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New Methods in Glycoconjugate Syntheses

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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

New Methods in Glycoconjugate Syntheses

Abstract

Glycoconjugates have become increasingly important in the study of immunological and cell surface recognition processes. A variety of new synthetic methods including new phase transfer catalysis methods have been developed to provide access to multifunctional carbohydrate haptens useful in a variety of different glycoconjugate and oligosaccharide syntheses. Methodology has been developed for the preparation of various *N*-substituted glycosyl acrylamides useful as single precursors towards both protein based immunoconjugates (neoglycoproteins) and multivalent synthetic polyacrylamide based glycoconjugates (glycopolymers). Neoglycoproteins have been synthesized by coupling *N*-substituted glycosyl acrylamides to nucleophilic sites on proteins by a new methodology based on Michael addition. This newly developed methodology is simple, quick, very efficient and requires no other chemicals. The polyacrylamide glycoconjugates have many desirable advantages over the protein based analogues. They are highly resistant to thermal, chemical and biological degradation, and appear to be much more sensitive during serological screening assays. They give no background interference during immunoprecipitation assays thus rendering these assays truly quantitative now. Their quick and effecient synthesis is very versatile in the sense that the incorporation of various appropriate comonomers can be effectively controlled to give these conjugates multiple specificities as well as enhanced solution and adsorption properties. These new methods have been used to produce a variety of *N*-acetylglucosamine conjugates. Together with the lectin from *Triticum vulgaris* they have been used as a model to investigate fundamental aspects of cell surface recognition interactions at the molecular level. The methodologies developed have been extended to a number of simple sugars and important oligosaccharide antigens to produce a variety of glycoconjugates useful as immunogens or

specialized screening antigens in diverse immunoassays.

As a second topic, phase transfer catalysis has been shown to be a very useful glycosylating methodology for the preparation of 1,2-*trans*-β-D-glycosides. Mild reaction conditions, using various peracetylated glycosyl halides (chlorides and bromides) as glycosyl donors, including 2-deoxy-, 2-acetamido-2-deoxy sugars and disaccharides, have been developed which allow quick and facile glycosylations of a wide range of nucleophiles. Aromatic alcohols, aryl and alkyl thiols, phenylselenol, carboxylic acids, dibenzyl phosphate, O-ethyl xanthate, azide, thiocyanate, *N*-hydroxy succinimide, a diimide (Barbital) and enolates have all been successfully glycosylated by a phase transfer catalysis strategy. Glycosylations were shown to be completely stereospecific , and proceed by an S_{N2} mechanism with inversion of configuration at the anomeric center. *p*-Nitro and *p*-formylphenyl glycosides, prepared by phase transfer catalysed glycosylation of substituted phenols, have been derivatized into useful neoglycoprotein and glycopolymer conjugates.

Acknowledgements

I have many people to thank for the help and encouragement I received from them over my years at the University of Ottawa.

First and foremost, I would like to thank my supervisor, Dr. René Roy, for his great interest, enthusiasm and guidance in this research work, as well as for allowing me the liberty to pursue my own ideas. The many, seemingly endless, discussions of ideas and concepts we had proved to be intellectually stimulating and were often the impetus for the development of many new ideas. It is unfortunate that we had more ideas than we had time and resources to allow for their development. I am sorry for not being able to report or bring to full fruition some of the many side projects worked on over the years.

I would like to express my gratitude to Dr. Anna Romanowska for her help in the electrophoresis and serological evaluation of many glycoconjugates. During the last two years, her close involvement and expertise have helped to provide a smooth progression in the developments of new glycoconjugation strategies.

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A special thanks is in order for Tony for his great devotion to the study of glycopolymer molecular dynamics by NMR, for obtaining high field NMR spectra from various government laboratories in the Ottawa region and for the solid state NMR work (I apologize, Tony, for not being able to present all of the work on which we collaborated. I hope we can publish some of this work when we're both a little less busy).

I'm greatly indebted to Micheline Tremblay for her help in the preparation of this document as well as for her support. To all my close friends and all the members of my family, thank you for your encouragement.

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Abbreviations

ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
Ac_2O	acetic anhydride
Ag	antigen
AHTL	*N*-acetylhomocysteinthiolactone
AIBN	azobisisobutyronitrile
AIDS	aquired immunodefiency syndrome
BBS	borate buffered saline
BSA	bovine serum albumin
BTEA	benzyltriethyl ammonium , $C_5H_6CH_2(Et)_3N^+$
b r	broad
°C	degree Celsius
CI-MS	chemical impact ionization mass spectrometry
COSY	shift correlation spectroscopy
CPA	copolyacrylamide
CPMA	copolymethacrylamide
d	doublet
Da	dalton
DEPT	distortionless enhancement by polarization transfer
DMF	dimethylformamide
DMSO	dimethylsulfoxide
$DMSO_{d6}$	hexadeuterated dimethylsulfoxide
EDC	1-(3-dimethylaminopropyl)-3-ethyl carbodiimide
EI-MS	electron impact ionization mass spectrometry
ELISA	enzyme linked immunosorbent assay
ELLA	enzyme linked lectin assay
equiv.	equivalent(s)
EtOAc	ethyl acetate
EtOH	ethanol
Et_2O	diethyl ether
FAB-MS	fast atom bombardment ionization mass spectrometry
Gal	galactose

GalNAc	*N*-acetylgalactosamine
Glc	glucose
GlcNAc	*N*-acetylglucosamine
HEMA	2-hydroxyethyl methacrylate
HETCOR	heteronuclear shift correlation spectroscopy
HIV	human immunodeficiency virus
HOAc	acetic acid
HPLC	high pressure liquid chromatography
hr(s)	hour(s)
HRP	horseradish peroxidase
IgG and IgM	immunoglobulins of class G and M
IR	infrared spectroscopy
Lac	lactose
lys	lysine
M	molar concentration, moles/litre
Malt	maltose
Man	mannose
ManNAc	*N*-acetylmannosamine
MeOH	methanol
min	minutes
mol	mole(s)
mp	melting point
mult	multiplet
Mv and Mn	viscosity and number average molecular weight
MW	molecular weight
NeuAc	*N*-acetylneuraminic acid
NMR	nuclear magnetic resonance
O.D.	optical density (absorbance)
n.O.e	nuclear Overhauser effect
Ø	phenyl
PBS	phosphate buffered saline
PCP	Pneumocystis carinii pneumonia
Pd/C	palladium on charcoal
PLL	poly-L-lysine
Pn23	type 23 streptococcus pneumoniae
PrOH	propanol

q	quartet
Rf	relative flow
Rha	rhamnose
RP	receptor protein
r t	room temperature
s	singlet
-S -	sulfide spacer, $O\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH_2\text{-}N$
sat.	saturated
SCS	substituent-induced chemical shift
SDS-PAGE	sodium dodecylsulfate - polyacrylamide gel electrophoresis
sec	second(s)
SMX	sulfamethoxazole
t	triplet
TBAH	tetrabutylammonium hydrogen sulfate , Bu_4NHSO_4
THF	tetrahydrofuran
TLC	thin layer chromatography
TMEDA	N,N,N',N'-tetramethylethylenediamine
TMP	trimethoprim
TT	tetanus toxoid
Tyr	tyrosine
UV	ultraviolet
WGA	wheat germ agglutinin
wt	weight

Common S.I. measurement units for mass, volume and distance have their usual symbols and significance.

Introduction

Carbohydrates are an essential class of compounds of all living organisms and are involved in a vast span of activities[1]. Carbohydrates are ubiquitous constituents of the cell surface and extracellular matrix serving as an important source of energy and as major structural components of cells and tissues. The more complex forms of carbohydrates are now understood to act as antigens[2] and cell surface receptors important in cell-cell recognition and adhesion processes[3]. Apart from their recognized roles in the physico-chemical properties of glycolipids and glycoproteins, carbohydrate residues can also serve as cell surface receptors for toxins[4], hormones[5], enzymes, lectins, antibodies, viruses and bacteria[6,7,8,9].

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1 J.F. Kennedy (ed.), *Carbohydrate Chemistry*. Clarendon Press, Oxford, England, 1988.

2 The term antigen is used throughout this dissertation as defined by: a)I. Roitt in *Essential Immunology*. 7th Edition, Blackwell Scientific Publications, Oxford, England. 1991, Chap 4 ; b) D. Catty in *Antibodies: A practical Approach*. IRL Press Ed. Oxford, England, Chap. 1, p.1 ; c) M.Z. Atassi, C.J. van Oss, D.R. Absolom (eds.) in *Molecular Immunology*. Marcel Dekker, New York, 1984. in particular Chap. 1, p.1 .
Antigen sensus stricto : a molecular hapten having the capacity of reacting with and binding to specific immunoglobulins and/or cellular receptors. M. Sela, *Science 66* , 1365 (1969). Therefore, in this context, antigens can react with antibodies and can thus produce immune complexes without necessarily inducing antibody formation as opposed to immunogens.

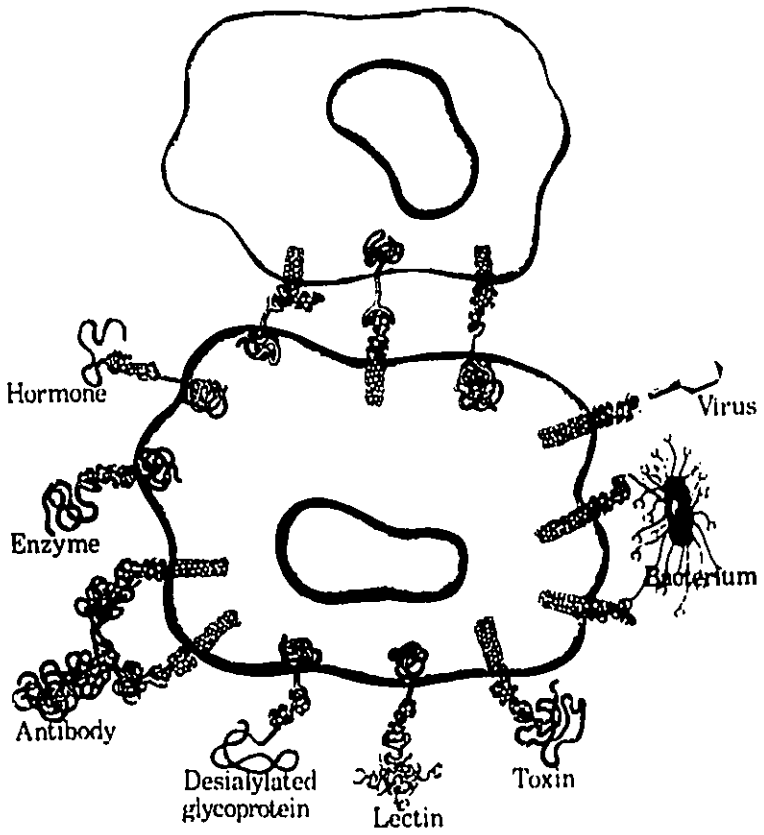
3 J. Montreuil, *Adv. Carbohydr. Chem. Biochem. 37* , 157 (1980); A. Gottschalk (ed.) in *Glycoproteins: Their Composition, Structure and Function*, Elsevier, Amsterdam, 2nd ed., 1972.

4 W. Reutter, E. Köttgen, C. Bauer and W. Gerok in *Sialic Acids. Chemistry, Metabolism and Function*. Cell Biology monograph series. Vol 10, R. Schauer (ed.), Springer-Verlag, Vienna, 1982.

5 H. Ankel, C. Krishnamurti, F. Besancon, S. Stefanos and E. Falcoff, *Proc. Natl. Acad. Sci. USA 77* , 2528 (1980).

6 I.J. Goldstein and R.D. Poretz in *The Lectins. Properties, Functions, and Applications in Biology and Medecine*. I.E. Liener, N. Sharon, I.J. Goldstein (eds.), Academic Press, Orlando, Florida. 1986.

7 J.C. Paulsen in *The Receptors*, vol 2, M. Conn (ed.), Academic Press, Orlando, Florida. 1985, p. 131-219.



A cell surface is comprised mainly of glycolipids, glycoproteins, proteoglycans and capsular polysaccharides[10]. This makes carbohydrates frontline mediators in intracellular events. What makes carbohydrates adaptable to work as such information molecules is in part due to their structural complexity. A small number of monosaccharide units can be assembled to provide a vast array of structures when we consider variations in their anomeric configuration, linkage position, branching and the possibility of bearing substituents. For this reason, a carbohydrate residue has the

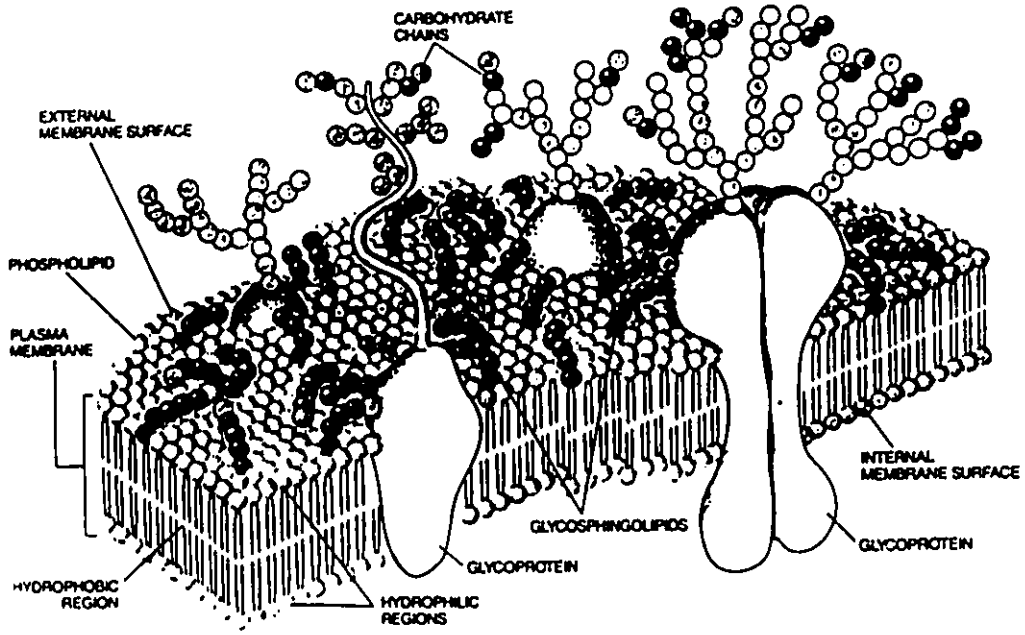
---

[8] A. Wright, M. McConnel and S. Kanegasaki in *Virus Receptors*, L.L. Randall and L. Philipson (eds.), *Receptors and Recognition*, series B, vol 7, Chapman and Hall, London, 1980.

[9] J.P. Arbuthnott and C.J. Smyth in *Adhesion of Microorganisms to Surfaces*, D.C. Elwood, J. Melling and P. Rutter (eds.), Academic Press, London (1975).

[10] N. Sharon in Complex Carbohydrates. *Their Chemistry, Biosynthesis and Functions*, Addison-Wesley, Reading MS, 1975.

potential to carry much more structural information than a peptide of similar size. For example four different monosaccharides can generate >35 000 tetrasaccharide structures by the action of relatively few enzymes with temporal or spatial regulation, but only 24 tetrapeptides can be assembled from four different amino acids[11].



Recognition and adhesion are basic cellular behavior required for many functions including cellular immune responses and pathogen infectivity. It also plays a critical role in biological development for processes such as inducing cell differentiation, effecting growth control, cell migration and initiating fertilization[12]. Carbohydrate-protein interactions are also implicated in tumor associated phenomena[13]. From the discussion above, it follows that glycoconjugates which are able to mimick or alter cell-cell and other cellular adhesion interactions have great potential in medical applications, and for the study of fundamental concepts underlying

---

[11] B.N.N. Rao, M. Moreland and B.K. Brandley, *Med. Chem. Res. 1* ,1 (1991).

[12] L. Glaser in *The Cell Surface: Mediator of Developmental Processes*, S. Subtelny and N.K. Wessels (eds.), Academic Press, New York, 1980.

[13] S. Hakomori, *Ann. Rev. Immunol.* 2 , 103 (1984).

protein-carbohydrate binding interactions such as affinity and avidity[14].

The obvious complexity of cell surfaces and natural carbohydrate based antigenic components has motivated immunochemists and molecular biologists to devise simpler, and more homogeneous synthetic glycoconjugate molecules as models for these components, as well as simple and effective methodologies for preparing them. Over the years, there have been many approaches for preparing multivalent carbohydrate ligands for these purposes. Artificial or synthetic carbohydrate protein conjugates, so called neoglycoproteins, were among the earliest materials used. Neoglycoproteins continue to attract the attention of researchers because of the large number of biochemical events that implicate glycoproteins and because there exists a variety of methods for their preparation[15]. Some neoglycoproteins bearing either viral or bacterial carbohydrate antigenic determinants can also be used very successfully as immunogens (vaccines) when the carbohydrate antigen alone is poorly immunogenic[16]. Other useful glycoconjugates include those of which carbohydrate antigens have been linked to

---

[14] In the literature, affinity and avidity have often been misused synonymously. Affinity generaly relates to a thermodynamic expression of the primary binding energy of a single binding site for an antigenic determinant. It may be considered as the sum of attractive and repulsive non-covalent intermolecular forces resulting from the interaction of a protein binding site and its homologous antigenic determinant. Experimentally, this term has its most precise application to monovalent hapten—anti-hapten systems. Avidity on the other hand, although dependent on affinity, also involves other contributing factors such as receptor valency, antigenic valency, and factors associated with binding but not directly concerned with the primary receptor—antigen interaction. (adapted from L.E. Glynn and M.W. Steward, Eds., in *Immunochemistry: An Advanced Textbook* , Wiley-Interscience, New York, 1977, p. 240)

[15] a) C.P. Stowel and Y.C. Lee, *Adv. Carbohydr. Chem. Biochem. 37* , 225 (1980); b) J.D. Aplin and J.C. Wriston Jr., *CRC Crit. Rev. Biochem. 10* , 259 (1981); c) C.P. Stowel and Y.C. Lee, *Methods Enzymol. 83* , 278 (1982); See also reference 1 p.526-528, and references cited within.

[16] J.F. Kennedy, Ed., *Carbohydrate Chemistry* . Clarendon Press, Oxford, England, 1988. p.537-552, and references cited within.

solid supports such as resin or latex beads[17], diatomaceous earth[18], silica[19], poly amino acids, polysaccharides such as starch[20] and agarose[21], and artificial polymer carriers such as polyacrylamide[22].

In part, this dissertation is focussed on the preparation of new types of glycoconjugates (glycopolymers and neoglycoproteins) and the development of related technologies useful in serological and immunochemical evaluation of carbohydrate-protein recognition and binding interactions as well as for immunogen development. The usefulness of these newly developed glycoconjugates and related methodologies used for their preparation has been evaluated with various antibodies and lectins. Some of this work has already been presented or published by this author with coworkers[23]. At first

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17 P.H. Iverius, *J. Biol. Chem. 247* , 2607 (1972); R. Barker, C.K. Chiang, I.P. Trayer and R.L. Hill, *Methods in Enzymol. 34* , 317 (1974);

18 R.U. Lemieux, D.A. Baker, W.H. Weinstein and C.M. Switzer, *Biochemistry 20* , 199 (1981); S. Shibata, I.J. Goldstein and D.A. Baker, *J. Biol. Chem. 257* , 9324 (1982).

19 W.I. Besinger, D.A. Baker, C.D. Buckner, R.A. Clift and E.D. Thomas, *New Engl. J. Med. 304* , 160 (1981).

20 I. Matsumoto and I. Osawa, *Biochem. Biophys. Res. Commun. 46* , 1810 (1972).

21 P. Vretblad, *Biochim. Biophys. Acta 434* , 169 (1976).

22 V. Horejsi, P. Smolek and J. Kocourek, *Biochim. Biophys. Acta 538* , 293 (1978); A. Ya. Chernyak, A.B. Levinski, B. Dmitriew and N.K. Kochetkov, *Carbohydr. Res. 128* , 269 (1984); R. Roy and C. Laferrière, *Carbohydr. Res. 177* , C1 (1988).

23 a) R. Roy and F.D. Tropper, *Glycoconjugate J. 5* , 203 (1988); b) R. Roy and F.D. Tropper, *J. Chem. Soc., Chem. Commun.* , 1058 (1988); c) R. Roy, F.D. Tropper, T. Morrison and J. Boratynski, *J. Chem. Soc., Chem. Commun.* , 536 (1991); d) R. Roy, F.D. Tropper, A. Romanowska, M. Letellier, L. Cousineau, S.J. Meunier and J. Boratynski, *Glycoconjugate J. 8* , 75 (1991); e) R. Roy, F.D. Tropper and A. Romanowska, *Bioconjugate J. 3* , 256 (1992); f) R. Roy, F. Tropper, A. Romanowska, R.K. Jain, C.F. Piskorz and K.L. Matta, *Bioorg. Med. Chem. Lett.*, (in press); g) R. Roy, F. Tropper and A. Romanowska, *J. Chem. Soc., Chem. Commun.* (in press); h) F.D. Tropper, M. Lamoureux and R. Roy, 57e Congrès de l'ACFAS, Montreal, Canada, May 1989; i) R. Roy and F.D. Tropper, 15th International Carbohydrate Symposium, Yokohama, Japan, August 1990; j) R. Roy, F.D. Tropper, F.O. Andersson, C. Laferrière and R.A. Pon, 59e Congrès de l'ACFAS, Québec, Canada, May 1991; k) F.D. Tropper and R. Roy, 74th Canadian Chemical Conference , Hamilton, Canada, August 1991; l) R. Roy, F.D. Tropper, J.R. Brisson and A.J. Williams, 16th International Carbohydrate Symposium, Paris, France, July 1992; m) A. Romanowska, F.D. Tropper and R. Roy, 16th International Carbohydrate Symposium, Paris, France, July 1992.

glance, this particular work has relevance and direct application to immunodiagnostics, immunology and microbiology.

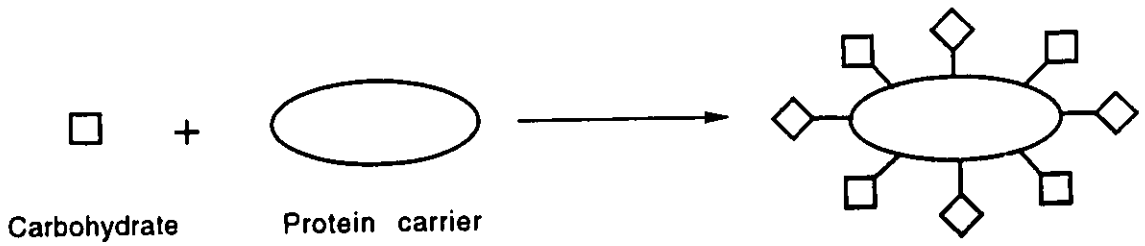
A second important aspect of this dissertation deals with phase transfer catalysed (PTC) glycosidation methodology. The work presented herein on this topic will demonstrate the value of PTC methodology for the 1,2-*trans* glycosylation of a very wide range of nucleophiles using various glycosyl halides. This general method was originally devised for preparing phenolic-glycoside inhibitors needed in binding studies with the lectin from *Triticum Vulgaris* (Wheat Germ Agglutinin, WGA). After mechanistic investigation of this reaction, the methodology was then successfully extended to prepare a wide range of useful glycosyl derivatives. The methodology has application to the preparation of synthetically and biologically relevant glycosyl derivatives such as nucleotides, glycophospholipids, enzyme substrates, amino- and thio-glycosides among others. Some of this work has also been described in the literature by this author and coworkers [23cdeg,24].

1.2 Neoglycoproteins

In order to study various adhesion and other biologically relevant processes, carbohydrate haptens have to be covalently attached to macromolecular carriers. So far, the most widely used carriers have been proteins. The resulting neoglycoproteins have been used *in vitro* for such things as screening and studying antibodies, lectins, enzymes and even whole microorganisms, and *in vivo* as immunogens. Their use, properties and preparation have been reviewed[15].

---

[24] a) R. Roy and F.D. Tropper, *Synth. Commun.* *20* , 2097 (1990); b) R. Roy and F.D. Tropper, *Can J. Chem.* , *69* , 817 (1991); c) F.D. Tropper, F.O. Andersson, C. Grand-Maître and R. Roy, *Synthesis*, *7* , 734 (1991); d) R. Roy, F.D. Tropper and C. Grand-Maître, *Can J. Chem.* , *69* , 1463 (1991); e) F.D. Tropper, F.O. Andersson, S. Braun and R. Roy, *Synthesis*, *7*, 618 (1992); f) F.D. Tropper, R. Roy, F.O. Andersson and C. Grand-Maître, *Carbohydr. Res.* , *229* ,149 (1992); g) F.D. Tropper, F.O. Andersson, S. Cao and R. Roy, *J. Carbohydr. Chem.* , *11* , 741 (1992).



When attached to immunogenic protein carriers, vaccines based on carbohydrate antigenic determinants can be prepared. Vaccines based on carbohydrate antigenic determinants, more often than not, must be produced this way as unconjugated oligo- and polysaccharides themselves are usually poorly immunogenic even in highly purified forms[25]. Although antibodies can be produced regularly against high molecular weight polysaccharide antigens such as dextran and levan[26] for example, polysaccharide antigens infrequently demonstrate delayed hypersensitivity (immunological memory) thus making them often unsuitable as vaccinating agents. Polysaccharides are considered as classical examples of T-cell independent antigens[26,27]. They can however be converted to T-helper cell dependent antigens when linked to immunogenic protein carriers[28]. T-cell dependence is usually necessary for immunological memory. Although vaccination with killed whole microorganisms or subcellular fractions thereof can be used to protect against infection, some serious drawbacks may be associated with such

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25 R.K. Cunningham in *Immunochemistry of Polysaccharide and Blood Group Antigens*, M.Z. Atassi, C.J. Van Oss and D.R. Absolom (eds.), *Molecular Immunology* , Marcel Dekker, New York, 1984.

26 E.A. Kabat, *Structural Concepts in Immunology and Immunochemistry* , Holt, Rinehart and Winston, New York, 1968.

27 a) W.E. Dick, Jr. and M. Beurret, *Glycoconjugates of Bacterial Carbohydrate Antigens in Conjugate Vaccines. Contri. Microbiol. Immumol.* J.M. Cruse and R.E. Lewis, Jr.(Eds). Basel, Karges, vol. 10, 48-114, (1989). b) R. Bell and G. Torrigiani, *Towards Better Carbohydrate Vaccines.* J. Wiley & Sons. Colchester, GB, (1987).

28 S.B. Svenson and A.A. Lindberg in *Progress in Allergy* , L.A. Hanson, P. Kallos and O. Westphal (eds.), Karger, Basel, 1983, vol 33, p. 120 ; R. Scheerson, J.B. Robbins, C. Chu, A. Sutton, G. Schiffman and W.F. Vann, *ibid* , p.144 ; H.J. Jennings and C. Lugowski, *J. Immunol. 127* , 104 (1984).

crude vaccines. They are often not as efficient as desired and can even be toxic to the recipient[25,29].

In view of the important uses of neoglycoproteins, effective methods and reagents for their preparation are of significant importance. A number of criteria must be considered for the synthetic development of these compounds. Synthetic methodologies for the preparation of neoglycoproteins must be mild and selective due to the relatively delicate nature of the multifunctional molecules involved. Ideally, preparative procedures, must be at the same time: (1) compatible with an aqueous environment, (2) be mild and effective under physiological conditions to prevent destruction and denaturation of the protein, (3) be chemoselective to simplify characterization, (4) be compatible with the multifunctional nature of the proteins to prevent inter- and intra-protein crosslinking, (5) lead to a linkage that is hydrolytically stable, (6) be high yielding due to the expensive nature of most important carbohydrate haptens and proteins, (7) be versatile and applicable to a wide variety of sugar residues, (8) be manipulatively easy and reproducible, (9) should not change the overall charge of the protein once modified. As much as possible, the use of relatively non-toxic reagents is also desired. Although many methods have been devised for conjugating haptens to proteins over the years, very few encompass all of these requirements. Diazonium, isothiocyanate, amidation and reductive amination couplings are the most widely used methods in generating neoglycoproteins and these will be discussed briefly here. The reader is directed to reference 15 for information on less commonly used methods.

Among the various existing methodologies, the use of *p*-aminophenyl glycosides[30] and alditol[31] derivatives still find widespread

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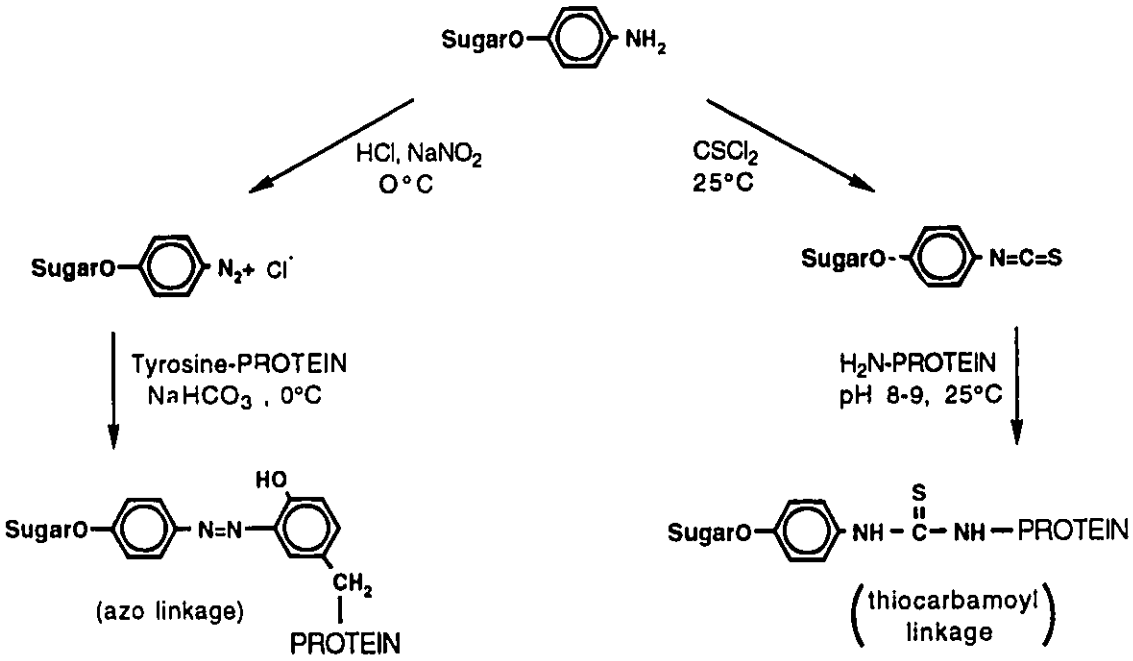
29 O. Westphal, K. Jann and K. Himmelspach in *Progress in Allergy* , L.A. Hanson, P. Kallos and O. Westphal (eds.), Karger, Basel, 1983, vol 33, p.9

30 O. Westphal and H. Feir, *Chem. Ber. 89* , 582 (1956).

31 D.A. Zopf, D.F. Smith, Z. Drzeniek, C.M. Tsai and V. Ginsberg, *Methods in Enzymol. 50* , 171 (1978); S.B. Svenson and A.A. Lindberg, *J. Immunol. Methods* 25 , 323 (1979).

application in spite of evident drawbacks associated with their use. For instance, conjugation by diazotization of the aromatic amine is not chemoselective and requires a large excess of the diazophenyl glycoside. As well, the conjugation is difficult to control in a reproducible manner and requires the use of noxious reagents. The isothiocyanate method also requires the use of labile and toxic thiophosgene reagent. A large excess of isothiocyanate reagent is also frequently required in this case.

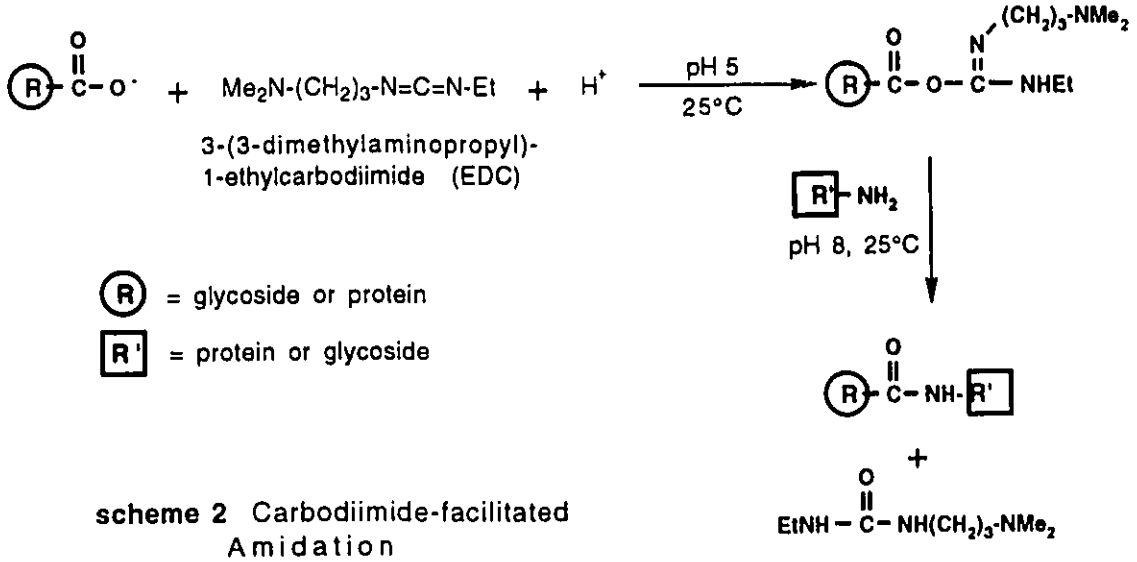


scheme 1 Protein conjugation with *p*-aminophenyl glycosides

Coupling by amidation with a water soluble carbodiimide is usually carried out as a one pot process. However, it requires careful control and variation of pH and results in at least partial crosslinking of the protein[32]. A general procedure is also difficult to apply with proteins of different pI. Protein amidation can also be realised with glycosides bearing acylating groups such as mixed anhydrides[33], acyl

[32] M-T.B. Davis and J.F. Preston, *J. Anal. Biochem. 116* , 402 (1981).
[33] R.R. King, F.P. Cooper and C.T. Bishop, *Carbohydr. Res. 55* , 83 (1977).

azides[34], nitrophenyl and *N*-hydroxysuccinimide esters[35]. These reagents can require several preparative steps and result in species sensitive to the alkaline aqueous milieu necessary for conjugation. Amidation procedures are not suitable for carbohydrates having free carboxyl and/or amine groups. One other important consideration in conjugation by amidation is the fact that it results in a change of the overall electronic charge on the protein which may result in denaturation and insolubility of the resulting neoglycoprotein.



scheme 2 Carbodiimide-facilitated Amidation

Reductive amination of free amine residues on proteins (ε-amine of lysine and the protein's N-terminus) using sodium cyanoborohydride ($NaCNBH_3$) with both reducing sugars[36] and aglycons[37,38,39] containing a terminal aldehyde group has proven to be a very effective method for preparing neoglycoprotein conjugates. Some advantages of this method include that it can be performed under extremely mild aqueous conditions with minimal chemical

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34 R.U. Lemieux, D.R. Bundle and D.A. Baker, *J. Am. Chem. Soc.* *97* , 4076 (1975); R.U. Lemieux, D.A. Baker and D.R. Bundle, *Can. J. Biochem.* *55* , 507 (1977).

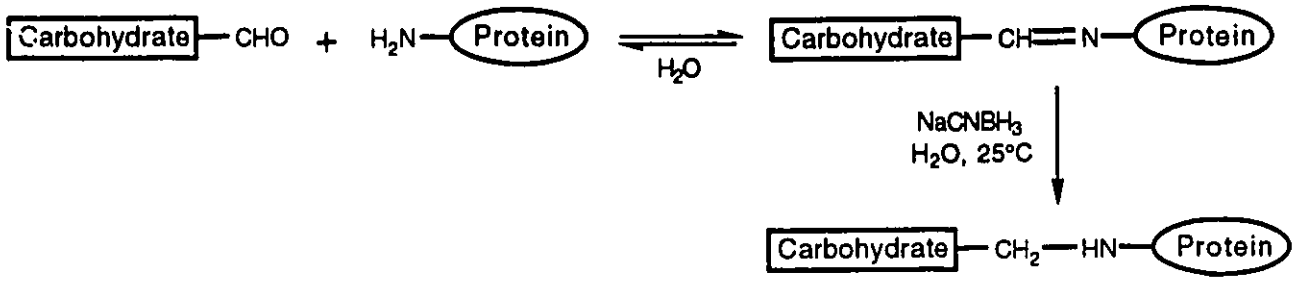
35 Y.S. Klausner and M. Bodansky, *Synthesis* , 453 (1972).

36 R. Roy, E. Katzenellenbogen and H.J. Jennings, *Can. J. Chem. Cell Biol.* *62* , 270 (1984).

37 R. Roy and C.A. Laferrière, *Can. J. Chem.* *68* , 2045 (1990).

38 B.J. Kamicker, B.A. Schwartz, R.M. Olson, D.C. Drinkwitz and G.R. Gray, *Arch. Biochem. Biophys.* *193* , 393 (1977).

manipulations being required and it does not change the overall charge of the protein. Reductive amination of oligosaccharides has certain drawbacks however. Besides being a slow reaction and requiring an excess of highly toxic $NaCNBH_3$, sacrificing the terminal reducing sugar can prove in some instances to be detrimental to the biological properties of the conjugate[35]. Reductive amination with suitably functionalized glycosides yields better results[39]. Reductive amination in conjunction with the description of a phase transfer catalysed procedure for introducing an *O*-linked *p*-hydroxy benzaldehyde aglycon will be discussed in section 5.4 for the preparation of *N*-acetyl glucosamine, glucose and lactose laden neoglycoproteins.

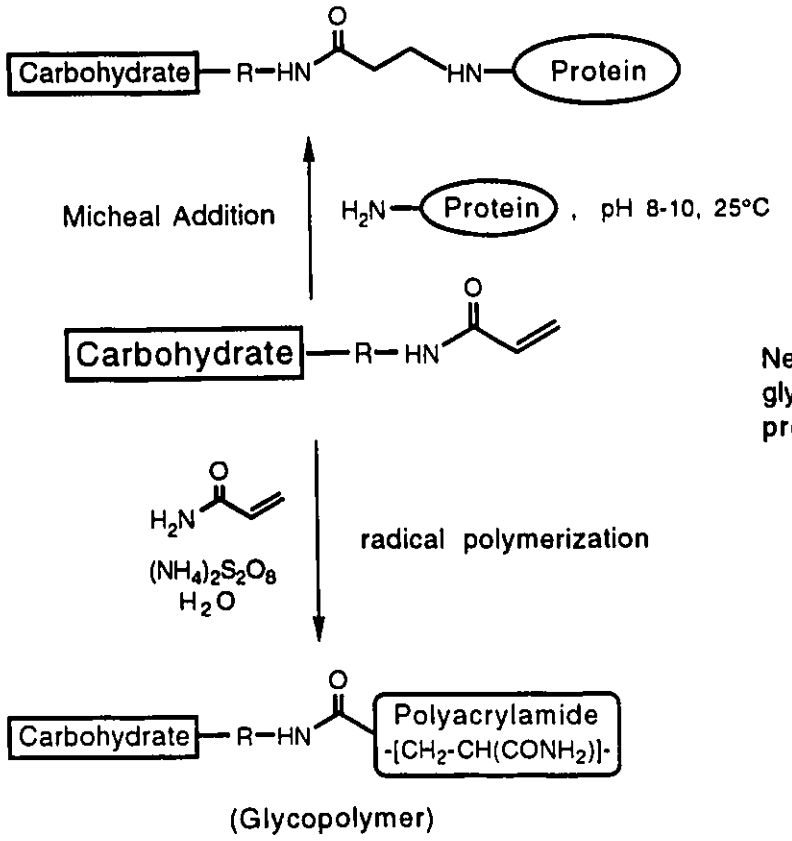


scheme 3 Protein conjugation by reductive amination

Investigations in the development of new glycoconjugation strategies has lead this research to a new approach in neoglycoprotein synthesis. This new method is based on the Michael addition reaction. In this method, carbohydrates having terminally *N*-linked acrylamide aglycons react with nucleophilic protein sites such as thiols (cysteine) and amines (lysine ε-terminus and protein *N*-terminus) under mildly alkaline conditions. This method satisfies all the desired criteria mentioned earlier for neoglycoprotein production, and requires no additional coupling reagents. Besides the ease of preparation, these acrylamide precursors have the added advantage that they can be used as comonomers for glycopolymer preparations (see next section). As will be discussed later, this

[39] R.T. Lee and Y.C. Lee, *Biochemistry* 19, 156-163 (1980).

method has been applied effectively for preparing potentially immunogenic neoglycoproteins of mono-, oligo- and poly-saccharides.



scheme 4

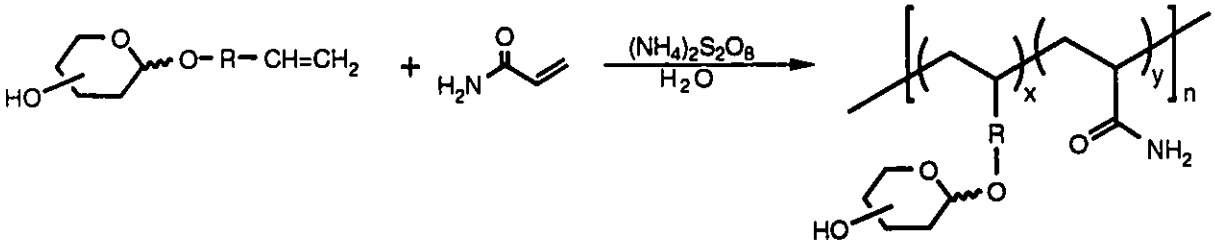
Neoglycoprotein and Glycopolymer glycoconjugates from acrylamide precursors

1.3 Glycopolymers

Neoglycoproteins have well served biochemists and immunochemists as tools to study carbohydrate-protein binding interaction thus far. However, these glycoconjugates are not without certain drawbacks. The very nature of the protein carrier, which is comprised of 20 different amino acids arranged in various sequences, makes the structural characterization of neoglycoproteins very difficult. Besides this, a variety of other drawbacks exist. Neoglycoproteins are sensitive and biodegradable compounds and so must be handled with care and often have relatively short shelf lives even under refrigerated conditions. Sugar densities on neoglycoproteins will

vary from batch to batch and cannot be easily controlled. They are also relatively costly to prepare and handle, often requiring special buffered solutions. One other very important consideration is that neoglycoproteins can give rise to non-carbohydrate specific interactions directed at the protein carrier which interferes with investigations during immunochemical assays.

Almost two decades have past since Horejsi and Kocourek[40] first described an entirely new approach to making useful artificial carbohydrate antigens which could alleviate those problems often associated with neoglycoproteins. In his method, simple allyl glycosides were copolymerized with acrylamide with a radical initiator in an aqueous environment to yield multivalent antigenic polymers bearing pendant carbohydrate residues. Originally referred to as pseudopolysaccharides, a term which can be misleading, these types of materials have since been named glycopolymers by analogy to glycolipids and glycoproteins[23e,41].



scheme 5 Glycopolymer synthesis

Glycopolymer

Apart from allyl glycosides[42], relatively few other alkene based glycosides have been used to prepare water soluble antigenic

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[40] V. Horejsi and J. Kocourek, *Biochim. Biophys. Acta* *297* , 346 (1973).

[41] R. Roy, F.D. Tropper, J.-R. Brisson and A.J. Williams, *Can. J. Chem.* , (submitted)

[42] a) V. Horejsi and J. Kocourek, *Methods in Enzymol. 34* , 361 (1973); K. Sutoh, L. Rosenfeld and Y.C. Lee, *Anal. Biochem.* *79* , 329 (1977); b) V. Horejsi, P. Smolcek and J. Kocourek, *Biochim. Biophys. Acta* *538* , 293 (1978); c) A.Y. Chernyak, A.B. Levinsky, B.A. Dmitriev and N.K. Kochetkov, *Carbohydr. Res. 110*, c16 (1982); A.Y. Chernyak, A.B. Levinsky, B.A. Dmitriev and N.K. Kochetkov, *Carbohydr. Res. 128*, 269 (1984); N. Kochetkov, *Pure and Appl. Chem. 56* , 923 (1984); A.Y. Chernyak, K. Antonov, N.K. Kochetkov, L.N. Padyukov and N.V. Tsetkova, *Carbohydr. Res. 141* , 199 (1985); P. Kosma, J.

glycopolymers. Improvements and variations have since been focussed mainly on increasing the aglycon length[23a,b,43,44] in order to provide a better spacer between the antigenic determinant and the polymer backbone, and by changing the polymerizable function on the glycoside aglycon from simple alkenes to more reactive acrylates[23a,b,c,45]. The choice of acrylamide as comonomer is well suited for glycopolymer synthesis. The resulting polymers are stable, non-ionic and extremely soluble in water. In addition, because polyacrylamide gels are extensively used by biochemists and immunochemists for electrophoresis work, experience as well as the materials required for preparing such glycopolymers is already present in laboratories where they are most needed.

There has also been attempts at preparing polystyrene glycopolymer systems as early as 1952 [46]. Polystyrene based glycopolymers prepared from vinylphenyl glycosides[47] and vinylbenzyl glyconamides[48] have provided antigenic materials in some instances. However, these materials were prepared as homopolymers and suffer from relatively poor solution properties in an aqueous environment. These materials may be more suited to the preparation of affinity chromatography resins or solid phase oligosaccharide synthesis supports once crosslinked [49].

Gass, G. Schultz, R. Christian and F.M. Unger, *Carbohydr. Res.* *167* , 39 (1987); J. Marino-Albernas, V. Verez-Bencomo, L. Gonzalez-Rodriguez, C.S. Perez-Martinez, E.G.A. Castell and A. Gonzalez-Segredo, *Carbohydr. Res.* *183* , 175 (1988).

43 S.I. Nishimura, K. Matsuoka and K. Kurita, *Macromolecules* *23* , 4182 (1990);

44 P. H. Weigel, R.L. Schnaar, S. Roseman and Y.C. Lee, *Methods Enzymol.* *83* , 294 (1982); R.T. Lee and Y.C. Lee, *Methods Enzymol.* *83* , 299 (1982)

45 a) E. Kallin, H. Lonn, T. Norberg and M. Elofsson, J. *Carbohydr. Chem.* *8* , 597 (1989); b) A.Y. Chernyak, A. Weintraub, T. Norberg and E. Kallin, *Glycoconjugate J.* *7* , 111 (1990); c) A. Spalstein and G. Whitesides, *J. Am. Chem. Soc.* , *113* , 487 (1991).

46 B. Helferich and H.J. Hoffmann, *Chem. Ber.* *85* , 175 (1952).

47 R.L. Whistler, H.P. Panzer and H.J. Roberts, *J. Org. Chem.* *26* , 1583 (1961); K. Kobayashi and H. Sumitomo, *Macromolecules* *13* , 234 (1980); Y. Koyama, A. Yoshida and K. Kurita, *Polym. J.* *6* , 479 (1986) and references cited within.

48 Y. Koyama, A. Yoshida and K. Kurita, *Polym. J.* *18* , 479 (1986) and references cited within; K. Kobayashi, H. Sumitomo, A. Kobayashi and T. Akaike, *J. Macromol. Sci. -Chem. A* *25* , 655 (1988) and references cited within.

49 J.M. Fréchet and C. Schuerch, *J. Am. Chem. Soc.* *93* , 492 (1971).

Methods of glycopolymer preparation have followed two strategies. The carbohydrate hapten is introduced into the glycopolymer either during the polymerization step by using a suitably functionalized carbohydrate monomer, or it is covalently attached to a preformed polymer matrix. The latter strategy has not been pursued to a great extent for the preparation of water soluble antigens but has been found useful in preparing crosslinked polyacrylamide based affinity chromatography[50]and cell immobilization media[51] . In those examples, glycosides with an amino group on the aglycon react with N-hydroxysuccinimide active esters incorporated in the polymer gels to form stable amide linkages.

Grafting strategies for preparing water soluble antigens include the reaction of epoxypropyl galactoside with polyvinyl alcohol[52], and the formation of malto-oligosaccharide hydrazones with polyacrylic acid hydrazide[53] . It is necessary to produce homogeneous and structuraly well defined antigenic material to study complex protein-carbohydrate interactions properly and for use in diagnostic applications. The functionalization of existing polymeric material is recognized as being synthetically more demanding, and may lead to species of ill defined structures which may be difficult to quantitate and/or lead to unwanted secondary effects.

Glycopolymers offer many advantages over neoglycoproteins, which have yet to be fully recognized. They are cheap and easy to prepare and purify in either small or large quantities (milligram to multigram preparations have been achieved with ease). They are thermally, chemically and biologically much more resistant than

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[50]R.L. Schnaar and Y.C. Lee, *Biochemistry 14* , 1535 (1975); R.L. Schnaar, P.H. Weigel, S. Roseman and Y.C. Lee, *Methods Enzymol. 83* , 306 (1982).

[51] P.H. Weigel, E. Schmell, Y.C. Lee and S. Roseman, *J. Biol. Chem. 253* , 330 (1978); R.L. Schnaar, P.H. Weigel, M.S. Kuhlenschmidt, Y.C. Lee and S. Roseman, *J. Biol. Chem. 253* , 7940 (1978); P.H. Weigel, R.L. Schnaar, M.S. Kuhlenschmidt, E. Schmell, R.T. Lee, Y.C. Lee and S. Roseman, *J. Biol. Chem. 254* , 10830 (1979).

[52] B. Kraska and L. Mester, *Tetrahedron Lett. 46* , 4587 (1978).

[53] H. Andresz, G.C. Richter and B. Pfannemuller, *Makromol. Chem. 179* , 301 (1978).

their neoglycoprotein counterparts. Their simple chemical structure allows for easier and more accurate composition and structural characterization with methods such as gel permeation chromatography, viscometry, NMR, chemical assays and elemental analysis[23e,38]. Moreover, the artificial nature of the carrier does not lend itself to non-specific binding interaction between it and the binding proteins of interests whether these proteins are in a purified form or come from crude animal sera. Unlike neoglycoproteins, the artificial nature of the acrylamide backbone also does not interfere with quantitative immunochemical assays such as immunoprecipitation which measure protein content in immunecomplexes. This renders these types of assays truly and unambiguously quantitative for the first time. Work with ordinary polyacrylamide standards has failed to show any kind of unspecific chemical or immunological response in all assays tried thus far. The hydrophilic, nonionic, strong and chemically resistant nature of polyacrylamide[54] and its high resistance to precipitation by electrolytes, seems to make it very well suited for its use in many immunochemical or diagnostic applications.

For glycopolymer preparation, one can take advantage of allyl glycosides which are commonly prepared during oligosaccharide syntheses (the allyl group is an effective protecting group in carbohydrate chemistry[55] and a precursor to reductive amination strategies to proteins[56]). However, glycopolymers prepared from allyl glycosides suffer from a very short spacer between the polymer carrier backbone and the carbohydrate hapten. This makes them suitable for work with lectins which have shallow binding sites, but unable to properly access the deeper binding sites encountered in antibodies[23a,b] due to steric constraints imposed by the close proximity of the backbone to the antigenic determinant.

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54 P. Molyneux (ed.), *Water-Soluble Synthetic Polymers: Properties and Behavior. Vol I*, CRC Press, Boca Raton, Florida, 1983. p. 84-92

55 R.W. Binkley (ed.), *Modern Carbohydrate Chemistry* , Marcel Dekker, New York, 1988. p.121-122, 154-155.

56 M.A. Bernstein and L.D. Hall, *Carbohydr. Res. 78* , C1 (1980).

However, some good results have been obtained with versions bearing extended oligosaccharide structures[39]. Even if the spacer arm is elongated, coacrylamide glycopolymers prepared with simple alkenyl type glycosides, such as n-pentenyl or n-undecenyl glycosides[40], suffer from the fact that they are formed from two distinctively different monomer types which have very different reactivity ratios[57]. This leads to the formation of a block type polymer and a glycoside incorporation that does not directly reflect the monomer loading prior to polymerization. This makes the carbohydrate density in the resulting glycopolymer difficult to predict and control. This problem can be resolved by using carbohydrate monomers wich contain a terminal acrylamide function. Using such comonomers, a more desirable randomly "alternating" copolymer is produced. Furthermore,the incorporated monomer ratio in the glycopolymer is identical to the ratio of monomers loaded in the reaction vessel. As mentioned in the previous section, the preparation of acrylamide type glycomonomers also offers the advantage that these materials may also be used for the preparation of neoglycoproteins by Michael addition (Scheme 4). Having the carrier as the only variable, a strategy is created by which antibodies directed solely at the carbohydrate hapten can be targetted with the glycopolymer after being elicited *in vivo* against the analogous neoglycoprotein.

This thesis will touch upon the preparation of glycopolymers from both allyl glycosides, and *N*-acrylamide-substituted-glycoside comonomers and their effectiveness as antigens with both lectins and antibodies. Water soluble glycopolymer antigens have direct application to immunodiagnostics. Two new types of *N*-acrylamide-substituted-glycoside comonomers have been developed, each of which take advantage of commonly used substrates, allyl and nitrophenyl glycosides. Allyl glycosides **1** can be effectively converted to 3-(2-(2-propenamido)ethylthio)propyl glycosides **2** by the light catalysed addition of 2-aminoethanethiol followed by *N*-

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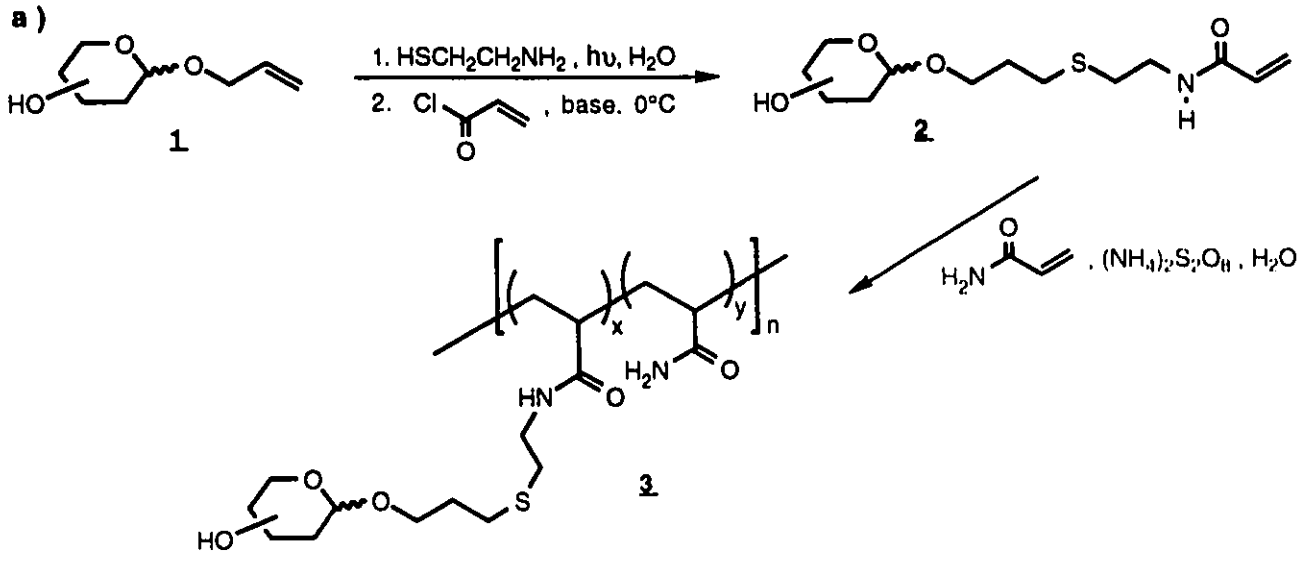
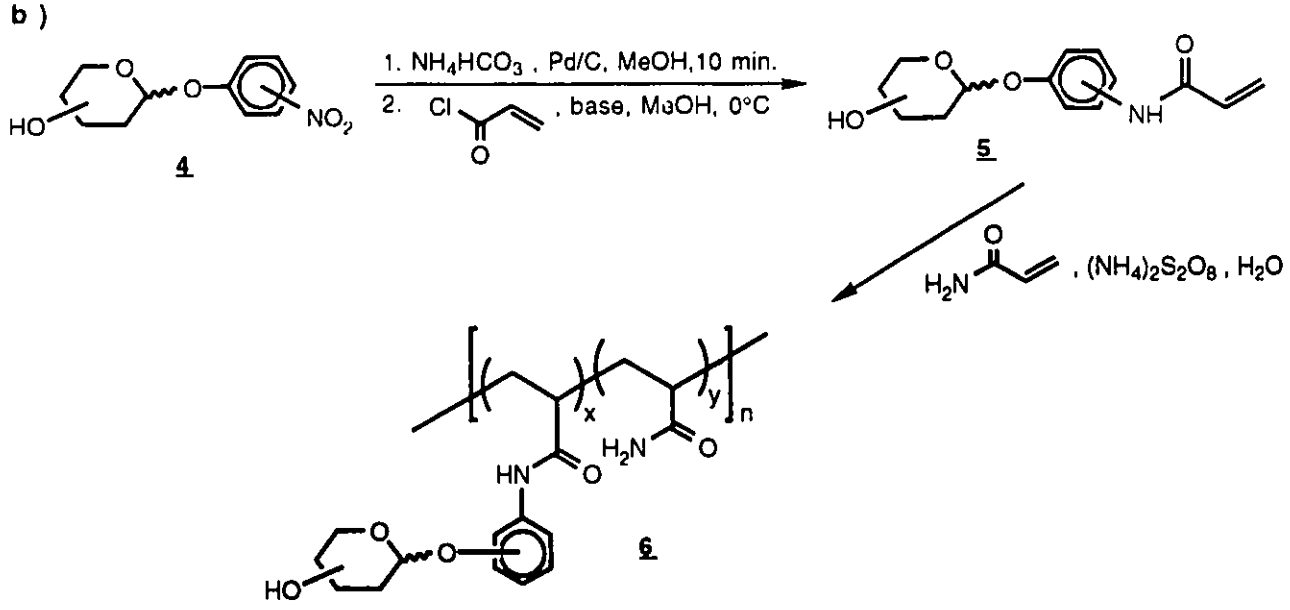
[57] J. Bradup and E.H. Immergut (eds.), *Polymer Handbook* , Wiley interscience, New York, 1975

acryloylation. On the other hand, propenamidophenyl glycosides **5** can be prepared quickly and efficiently from nitrophenyl glycosides **4** which are commonly used as enzyme substrates or precursors to aminophenyl glycosides used to prepare neoglycoproteins. This method is attractive in view of the fact that many nitrophenyl glycosides are already commercially available. It requires the reduction of the nitro group to the corresponding amine followed by *N*-acryloylation.

scheme 6 Preparation of coacrylamide glycopolymers from acrylamide-glycosides derived from **a)** allyl-, or **b)** nitrophenyl-glycosides.



b)



1.4 Lectins

Lectins were recognized as a class of proteins in the mid-1950's [58] with the first example surfacing as early as 1888 [59], and have since been defined as multivalent, carbohydrate binding proteins or glycoproteins of non-immune origin that can agglutinate cells or precipitate other materials such as polysaccharides and glycoconjugates having more than one saccharide of appropriate complementarity[60]. Their function and importance is now recognized as playing a major role in cellular adhesion processes mediated by cell surface carbohydrate receptors[61].

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[58] W.C. Boyd in *The Proteins* , H. Neurath and K. Bailey (eds.), Vol 2, Part 2, p. 756-844, Academic Press, New York, 1954.

[59] H. Stillmark, Ph.D. Thesis, University of Dorpat, Dorpat (Tartu).

[60] H.B.F. Dixon, *Nature 292* , 192 (1981).

[61] An excellent and well referenced text dedicated to lectins is ref. 6, *The Lectins. Properties, Functions and Application in Biology and Medecine.* , I.E. Liener, N. Sharon, I.J. Goldstein (eds.), Academic Press, Orlando, Florida. 1986. A second useful text is *Receptor-Specific Proteins. Plant and Animal Lectins* by E.R. Gold and P. Balding, American Elsevier, New York, 1975. A good general article can be found in *Molecular Immunology*, M.Z. Atassi,

Lectins are widely distributed among living organisms and have been isolated from a broad range of species in virtually every phyllum. They have been found in sources ranging from viruses to bacteria, to plants and fungi, to invertebrates such as snails, crabs and sponges, to vertebrates like fish, chickens and humans. Lectins are relatively small proteins (usually 40-160 kDa) made up of several subunits and usually exist as several isolectins (different isoelectric points). They have different patterns of glycosylation and many have metal chelates.

Lectins have been linked to a wide range of biochemical and biophysical events. They are used very successfully as biochemical research tools and probes, as well as in medical applications. Because of their carbohydrate specificities, they have been employed as highly discriminating agents in many types of work (see Table 1).

C.J. van Oss and D.R. Absolom (eds.), *Lectins* , M.E.A. Pereira , Marcel Dekker, New York, 1984.

Table 1 Major uses of lectins[62]

1	Blood typing; Identification of new blood types; Structural studies of blood types.
2	Cell separation; Study of cellular surfaces and cell recognition and adhesion phenomena.
3	Investigation of complex carbohydrate structures on surfaces of animal cells, bacteria and viruses and of subcellular particles. Identification of microorganisms.
4	Isolation, purification and structural studies of carbohydrates and glycoconjugates.
5	Study of cell surface structure variation during malignant transformation.
6	Mitogenesis of lymphocytes; studies of events occurring upon initiation of cell division.
7	Cell targetted drug carriers.
8	Clinical diagnostics
9	Toxins
10	Isolation of lymphocytes and marrow stem cells for transplantation.
11	Chromosomal probes.
12	Good multivalent models for antibodies.

Because virtually all cells are coated with carbohydrates, it is not suprising that lectins bind readily to cells and are involved in functions including pathogen infectivity, fertilization, cell-growth, -differentiation, and -migration. They are directly involved in many important cellular recognition and adhesion processes. "Lectinology" has thus begun to grow into an important field of the biological sciences. The development of simple and effective glycoconjugate tools for the study and isolation of lectins is therefore of considerable value.

The glycoconjugates developed during this research have been designed to probe three important factors involved in carbohydrate-

[62] Adapted in part from *Applications of Lectins* , H. Lis and N. Sharon, p. 293-370, ref. 6

lectin interactions: (1) valency of the ligand, (2) spatial disposition of sugar residues, and (3) chemical modification of interacting groups involved in binding. The last point relates to a better understanding of binding site requirements and limitations with respect to the arrangement of functinal groups present on the carbohydrate ligand. The first two points are related to the avidity phenomenon, that is, the dramatic increase in binding affinity observed with multivalent antigens.

General methods were developed for the preparation of antigenic glycoconjugates. Glycoconjugates have been produced with various glycosides, and with varying degrees of laden sugar densities. To investigate their effectiveness as antigens, various lectins were used to probe protein-carbohydrate interactions. A list of lectins used with some of their characteristics are outlined in Table 2.

Table 2 Lectins and characteristics[6]

Lectin	Origin	Molecular Weight (kDa)	Sugar specificity	Number of binding sites/molecule
Wheat Germ Agglutinin (WGA)	*Triticum Vulgaris*	43	GlcNAc NeuAc	4 2
Peanut Lectin	*Arachis Hypogea*	110	β-D-Gal	4
RCA_{120}	*Ricinus Communis* (Castor Bean)	120	β-D-Gal α-L-Rha	4 -
Maclura Pomifera	Osage Orange	42	β-D-Gal	-
Sambucus Nigra	Elder stems and branches	140	Lactose	4

1.5 Antibodies

Antibodies or immunoglobulins are multivalent receptor proteins that constitute an important part of the immune system in higher living organisms[Y] . They are found in the blood serum and tissues of vertebrates as a response against the appearance of antigens. This immune response is the basis of the whole field of immunology. Immune systems produce antibodies against various substances for various regulatory functions including protection and eradication of infection. A large number of antibodies are carbohydrate receptors. Protection from infection by pathogens, including viruses, bacteria and other microorganisms, through vaccination is an important scientific endeavour beneficial to society. As most ceils are covered with carbohydrate appendages, producing and understanding immunogenic recognition of carbohydrate antigens is of significant importance. Thus, monoclonal antibody research, diagnostics and other biomedical applications require good purification, diagnostic and immunogenic agents. With most work being related to peptide antigens, antibody recognition of carbohydrates is a relatively new area in immunology. Strategies and methods for the preparation of carbohydrate antigens and immunogens useful to these ends, are thus of important value. Investigations of lectin interactions are also important in our general understanding of carbohydrate recognition. Due to their specificities and multivalent nature, lectins can serve as good models for carbohydrate-binding antibodies.

The development of the glycoconjugates described in this work ultimately contributes to the field of immunology. The strategy of using acrylamide-glycosides for the simultaneous preparation of neoglycoproteins and glycopolymers (Schemes 4 and 6b), in addition to concepts and observations made from lectin models, has allowed

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[Y] The immune system is a very complex physiological system, as is the diversity in type and function of immunoglobulin proteins. References to advanced immunology or immunochemistry textbooks are suggested for a more in depth description of the immune system and its components. For example, see references 2 and 14.

the preparation of important carbohydrate-immunogens in addition to complimentory diagnostic agents for the antibodies raised specifically against the carbohydrate residues. Immunogens were prepared as tetanus toxoid conjugates for the more relevant oligosaccharide antigens. These are outlined in Table 3.

Table 3 Glycoconjugates of important carbohydrate antigens

	Antigen#	Significance	Immunogenic* Carrier	Diagnostic Antigen
1	Galβ(1–4)Glcβ1–	Gal/GlcNAc-hepatocyte receptors	BSA	Copolyacrylamide glycopolymer (with and without additional co-effectors)
2	Galβ(1–3) GalNAcα1–	"T-Antigen", is a tumor associated cell marker	TT	Copolyacrylamide -glycopolymer
3	Galβ(1–4) GlcNAcβ(1–6) GalNAcα1–	ABH type 2 glycoprotein determinant of human blood group activities	TT	Copolyacrylamide -glycopolymer
4	Galβ(1–3) GlcNAcβ(1–3) Galβ(1–3) GlcNAcβ1–	ABH type 1 glycoprotein determinant of human blood group activities	TT	Copolyacrylamide -glycopolymer
5	Galβ(1–3) GlcNAcβ(1–3) Galβ(1–4)Glcβ1–	"lacto-N-tetraose" meconium glycolipid determinant	TT	Copolyacrylamide -glycopolymer (with and without additional biotin co-effector)
6	$(NeuAc\alpha 2\text{–}8)_{15}$	menningococcal antigenic determinant	TT ¥	poly-L-Lysine - glycopolymer (with additional biotin co-effector)

monosaccharide residues quoted are pyranosides having the D-configuration
* BSA = bovine serum albumin, TT= tetanus toxoid protein
¥ prepared by collaborators (R. Roy and R. Pon)

Two different carrier proteins, namely bovine serum albumin (BSA) and tetanus toxoid (TT) have been used to prepare potentially useful immunogens. These two proteins were chosen as they are known to exhibit a large difference in immunogenicities when injected in model animals. The tetanus toxoid protein (TT) has been found to be very immunogenic in mammals. It's use as carrier increases the chances of producing a better quality immune response including immunological memory[63] . BSA conjugates are better suited for use as model carbohydrate-immunogens. BSA's use is popularized by it's low cost, ruggedness and acceptable responses in experimental animals. It's use has the drawbacks that responses against BSA protein sequences generate antibodies that interfere with many existing albumin type molecules (*in vitro* and *in vivo*)[63,64]. The use of albumin molecules as serological blocking agents or as co-diagnostic agents therefore becomes unsuitable when working with antibody sera raised against BSA conjugates.

In this work, some water soluble diagnostic glycopolymer antigens have been prepared in ways that incorporate secondary effector probes. The purpose for doing this is to extend the usefulness of the glycopolymers. In a first investigation, the physical properties of glycopolymer antigens were specifically altered by introducing methyl and stearyl alkyl groups into the glycopolymer structure for the purpose of providing more lipophilic antigens. In a second investigation, immunochemical probes have been introduced in order to provide more speciallized diagnostic antigens. Two useful immunochemical probes have been investigated, biotin and tyramine. Biotin is a very versatile probe. It's strong binding to avidin and streptavidin protein conjugates makes it an effective serological probe as well as a useful tool in immuno-fluorescence and -electron

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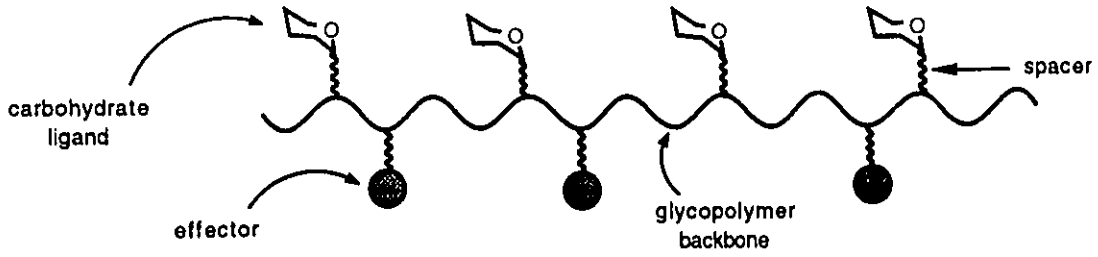
[63] M.Z. Atassi, *Immune Recognition of Proteins* in *Molecular Immunology*, M.Z. Atassi, C.J. van Oss and D.R. Absolom (eds.), Marcel Dekker, New York, 1984.

[64] J.L. Turk and D. Parker, *Interaction of Haptens with Proteins and their Immunogenicity* in *Immunochemistry: An Advanced Textbook* , L.E. Glynn and M.W. Steward,(Eds).Wiley-Interscience, New York, 1977, p.449

microscopy[65]. Tyramine is an analogue of the amino acid tyrosine. Its phenolic structure lends itself to simple electrophilic radioiodination labelling, thereby making conjugates bearing this effector also useful in radioimmunoassay type experiments[66]. A third immunochemical probe, in the form of a secondary carbohydrate, has also been introduced to give a bispecific antigen which is simultaneously recognized by diverse lectins and antibodies.



The acrylamide strategy has also been successfully applied with a non-carbohydrate hapten (sulfamethoxazole, **7**) to provide antigens useful in AIDS related immunological research. High doses of sulfamethoxazole are prescribed to AIDS patients afflicted by the lethal opportunistic infection *Pneumocystis carinii* pneumonia. However, patients can be or become very sensitive to this treatment as serious immunologically based allergic responses to the drug develop with continued therapy [67]. Various sulfamethoxazole conjugates were prepared to help trace the immunological

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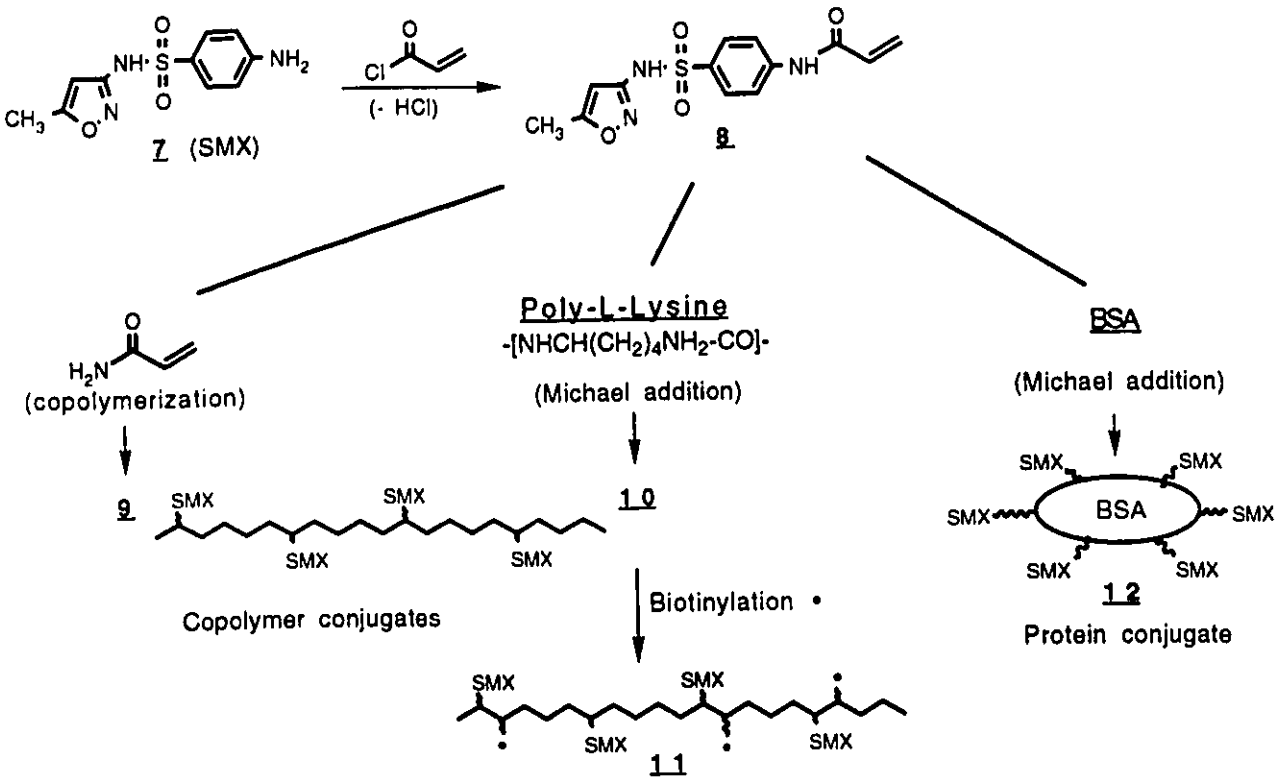
65 G.R. Bullock and P. Petrusz (eds.), *Techniques in Immunocytochemistry* , Vol 2 and Vol 3, Academic Press, San Diego, California, 1983 and 1985; D. Brada and J. Roth, *Anal. Biochem. 142* , 79 (1984); R.E. Morris and C.B. Saelinger, *J. Hystochem. Cytochem. 32* , 124 (1984).

66 D.J. McCormick and H. E. Schmitz, *Radioimmunoassay* in *Molecular Immunology*, M.Z. Atassi, C.J. van Oss and D.R. Absolom (eds.), Marcel Dekker, New York, 1984. p 402 - 425.

67 A.J.A. van Der Ven, P.P. Koopmans, TB. Vree and J.W.M. van der Meer, *The Lancet, 338* , 431 (1991).

development of the severe side effects. A very successful diagnostic conjugate **10** was developed to screen patients who can or should not be treated with the drug sulfamethoxazole. These conjugates are presently being used and investigated by colleagues in the Departments of Microbiology and Immunology, and the Faculty of Medicine, University of Ottawa.



Scheme 7 Sulfamethoxazole (SMX) conjugates

1.6 Serological assays

Various serological assays were performed as an integral part of the research presented herein. These assays give a relative or quantitative measure in binding affinity between complementary antigen and receptor. The serological assays were either of agglutinin/precipitin nature, as in quantitative precipitation and double immunodiffusion through agarose, or were performed at solid-liquid interfaces with enzyme-linked assays. These three types of assays were used to investigate the antigenicity of the glycoconjugates developed against lectins, antibodies and other receptor proteins. A brief description of the immunochemical techniques to be most encountered is appropriate and follows. More detailed descriptions can be found elsewhere[2c]. Experimental protocols will be described in their appropriate sections.

Agglutination is one of oldests and simplest tests used for demonstrating immunoadhesive type reactions *in vitro* [68] . Precipitation is a reversible process resulting from the agglutination of antigenic particles by crosslinking with multivalent receptor proteins directed at the antigenic determinants. Precipitation is an uncommonly sensitive assay, with direct visual observation being detectable with only a few micrograms per millilitre. In the method named immunoprecipitation, one measures the degree to which soluble antigens agglutinate soluble antibodies or lectins. The precipitated antigen-receptor protein complex is then washed of unbound materials and quantitatively assayed colorimetrically for its protein content. A optimal antigen/receptor protein (Ag/RP) ratio for the couple is thus calculated. The use of neoglycoproteins instead of glycopolymers as antigens results in an assay that is very difficult to evaluate because the precipitin complex is a non-stoichiometric association of two different proteins having dissimilar sensitivities to the developing reagents.

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[68] C.J. van Oss, *Agglutination and Precipitation* in *Molecular Immunology*, M.Z. Atassi, C.J. van Oss and D.R. Absolom (eds.), Marcel Dekker, New York, 1984. p 361 - 379

Glycopolymers on the other hand do not interefere with the chemical reactions necessary to evaluate protein content. The assay is performed by adding increasing amounts of antigen to solutions of constant concentration in binding protein, and determining the point of maximum agglutination (minimum Ag/RP ratio) by analyzing the washed precipitate that results. Assays were usually performed in 300 µl volumes with 1-100 µg of materials.

A relatively simple and effective way of evaluating agglutination is by the method of double radial diffusion in agar gel. This is more of a qualitative method that is not as sensitive as other assays but requires little material and attention. Because we are interested in developing very antigenic substances, radial diffusion has proven to be a very effective means of quickly screening and assessing the antigenicity of developed materials. In the method developed by Ouchterlony[69] almost a quarter century ago, small volumes (10-20 µl) of Ag and RP solutions (1 mg/mL) are deposited in separate wells punched in small agarose gel slabs. The Ag and RP diffuse toward each other. At their place of encounter, a sharp precipitin band is formed if there is molecular recognition resulting in Ag-RP complex formation. The sometimes curved precipitin lines can be visualized directly, and permanently stained or photographed. Results are usually observable within 24 hours. Direct permanent photographic records of the many radial diffusion assays performed throughout this research has not always been possible. Many of the immunodiffusion results will be described in text or through schematic representation based on detailed notes and dried gel specimens.

Heterogeneous enzyme immunoassay[70] such as ELISA (enzyme-linked immunosorbent assay) developed only twenty years ago has proven to

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69 O. Ouchterlony in *Handbook of Immunodiffusion and Immunoelectrophoresis* , Ann Arbor Science Publishers, Ann Arbor, Michigan, 1968.

70 For a good general review see K.W. Walls, *Enzyme Immunoassays i Molecular Immunology*. M.Z. Atassi, C.J. van Oss and D.R. Absolor Marcel Dekker, New York, 1984. p 427 - 445

be a very sensitive method for measuring antigen-receptor affinity[71]. In the classic ELISA, an antigen is adsorbed or fixed to a solid surface, then exposed to RP containing serum and washed of any unbound material. Detection of any immune-complex formation is then made with an enzyme conjugated anti-globulin directed to the RP. Washing and exposure to the required enzyme substrate then gives a photometrically measurable response indicative of the amount of bound RP. The assays are performed in small wells (300 µl × 96) moulded in polystyrene or polyvinyl chloride plates where the antigen is adsorbed from solution and coats the well suface by hydrophobic interactions. The assays are performed with serial dilutions of both serum and antigen, as well as inhibitor during competitive binding inhibition assays. Assays can be performed in a variety of ways either directly using avalable RP-enzyme conjugates, or indirectly through anti-RP-enzyme conjugates or other labeled reagent able to specifically interact with the immune-complex. ELLA, or enzyme linked lectin assay, is a particular example of a direct enzyme immunoassay method that will often be encountered throughout this work.

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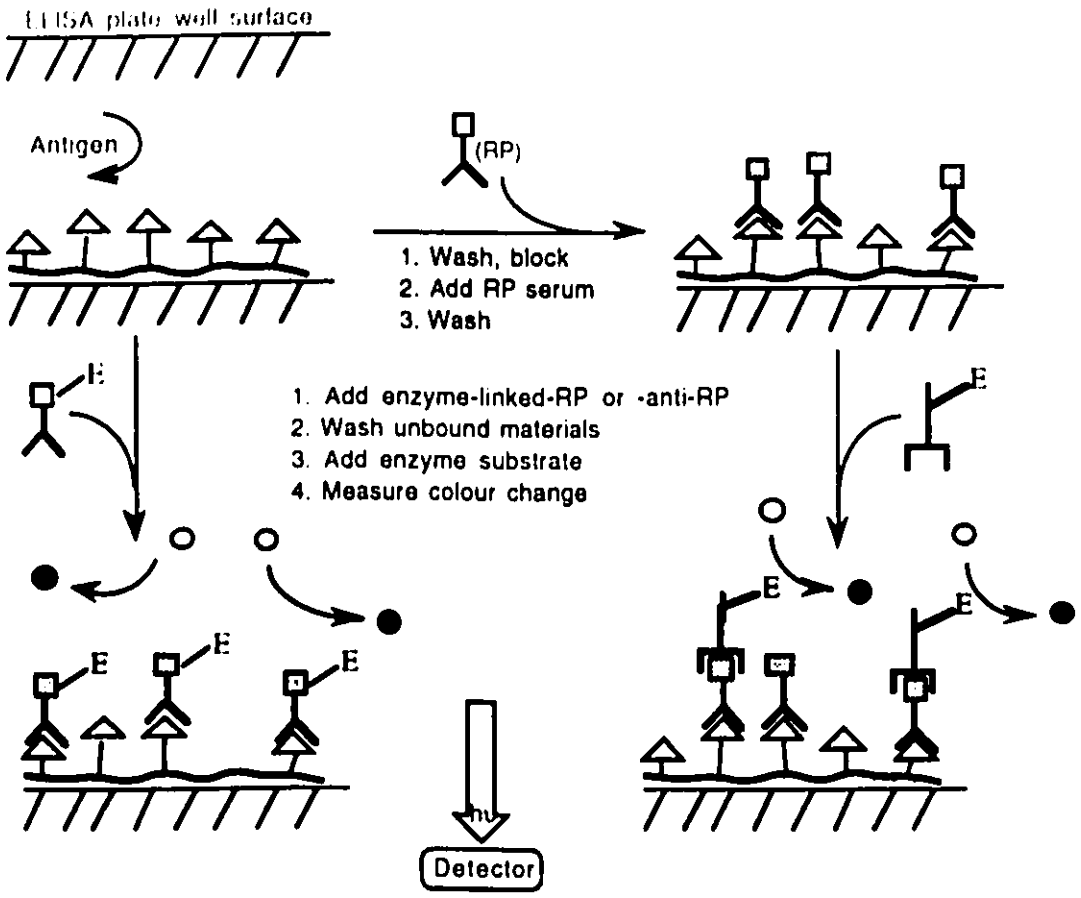
71 B.K. Van Weeman and A.H.W.M. Schuurs, *FEBS Lett.* 15, 232 (1971); E. Engvall and P. Perlman, *Immunochemistry* 8, 871 (1971).



Scheme 8 Representation of direct and indirect (sandwich type) heterogeneous enzyme immunoassays.

A large part of the serological evaluations performed during this research were made possible by the direct involvement of a colleague in our group, Dr. Anna Romanowska. Her contributions through the meticulous performance of many serological assays helped provide valuable information needed to properly characterize the glycoconjugates. Her close contact and informative discussions resulted in a sound team effort which helped smooth the glycoconjugate development.

2. *O*-Allyl glycosides as precursors to glycoconjugates

O-Allyl glycosides have long served carbohydrate chemists during oligosaccharide syntheses. The allyl group is easily introduced at the anomeric centre by standard Fischer (for α-glycosides) or Koenigs-Knorr (for β-glycosides) type methods. As an anomeric protecting group, it is stable to a variety of reaction conditions and can be removed in a mild two-step sequence using tris(triphenylphosphine) rhodium (I) chloride to isomerize the double bond followed by hydrolysis of the 1-propenyl group with mercury (II) chloride and oxide (or zinc (II) chloride and oxide) in aqueous acetone[72]. Removal can also be achieved by double bond isomerization with potassium *t*-butoxide in dimethyl sulfoxide followed by mild hydrolysis[73].

Allyl glycosides, by virtue of a versatile alkene function, can also be employed to provide useful glycoconjugate. For example, reductive ozonolysis, followed by reductive amination to a protein carrier, gives neoglycoproteins[54]. The alkene function can also be copolymerized with acrylamide for example to give antigenic glycopolymers[48]. Such glycopolymers have very short spacer lengths (a simple methylene bridge) and are suitable for studying receptor proteins such as lectins which contain relatively shallow binding sites. To access deeper binding subsites of lectins or antibodies, the allyl function can also be easily transformed into a longer, suitably functionalized aglycons by light catalysed thiol addition to the alkene (see section 2.3.2 and chapters 3 and 4). Various allyl glycosides were prepared as precursors to glycoconjugates having long and short spacer lengths (Scheme 9).

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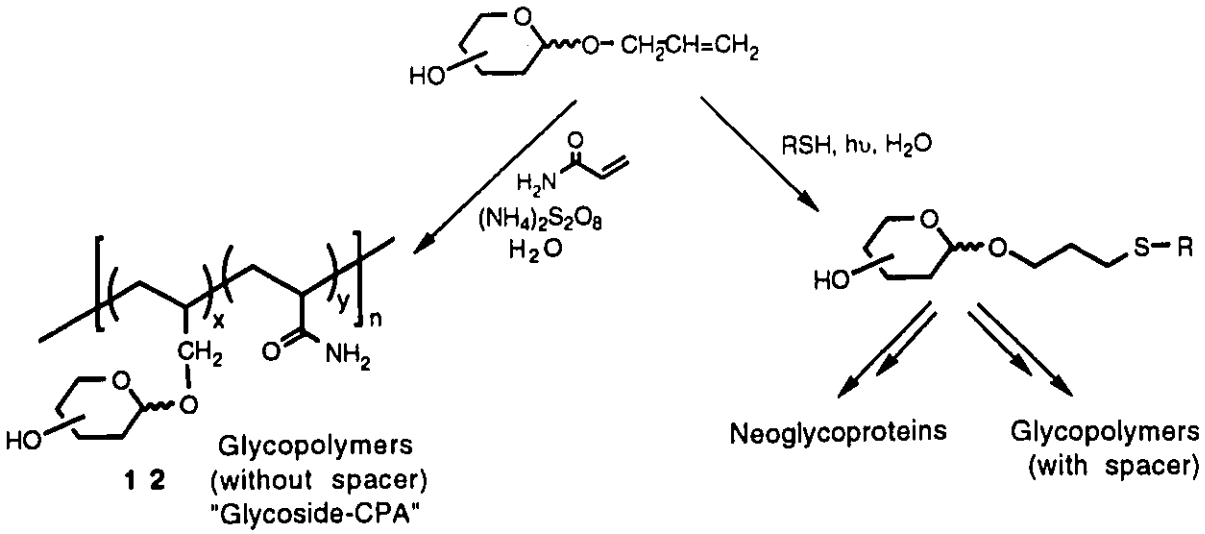
72 H.A. El-Shanawy and C. Schuerch, *Carbohydr. Res.* *131* , 239 (1984).

73 R.Giggs, S. Payne and R. Conant, *J. Carbohydr. Chem.* *2* , 207 (1983).



Scheme 9 Aliyl glycosides as precursors to various glycoconjugates

2.1 Preparation

As mentioned, 1-*O*-allyl-glycosides can be prepared by conventional glycosylation methodologies. Various allyl-glycosides to be used as precursors to the glycoconjugates were prepared by slight modifications to published procedures[74,75,76]. The allyl glycosides prepared are shown in Table 4.

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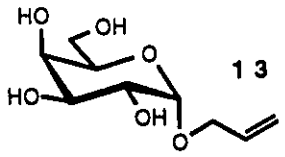
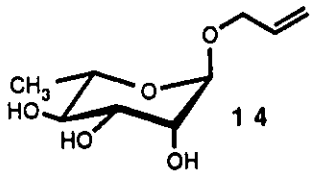
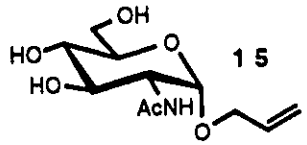
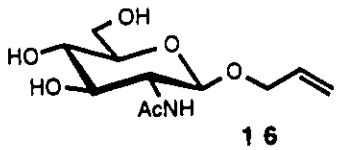
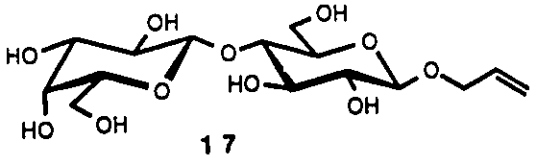
74 Y.C. Lee and R.T. Lee, *Carbohydr. Res. 37* , 193 (1974).

75 G. Luckacs, *Tetrahedron Lett. 13* , 1153 (1979).

76 V. Horejsi and J. Kocourek, *Biochim. Biophys. Acta 336* , 338 (1974).

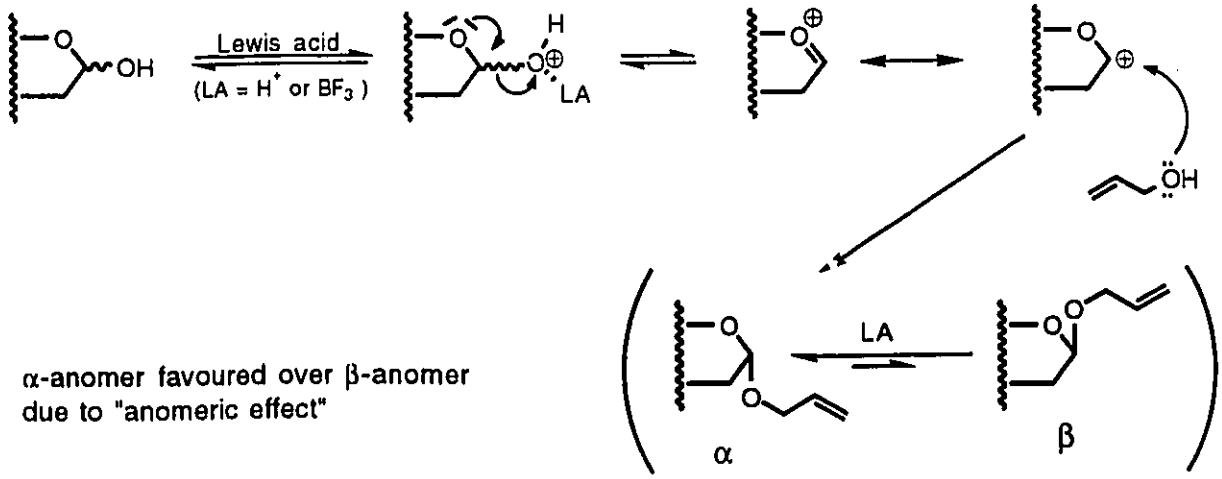
Table 4 Allyl glycosides

Glycoside	mp (°C)	$[\alpha]_D$ (H_2O)	references to (reported) values
 **13** Allyl α-D-Gal	144.6 - 146.1 (145 - 146)	+ 184.5 ° (+ 185 °)	74
 **14** Allyl α-L-Rha	oil	- 51.8 ° (- 56 °)	75
 **15** Allyl α-D-GlcNAc	171.3 - 173.2 (172 - 174)	+ 148.3 ° (+ 148.8 °)	74
 **16** Allyl β-D-GlcNAc	171.5 - 173.3 (171 - 172)	- 34.7 ° (- 33.9 °)	74
 **17** Allyl β-D-Lac	172.0 - 173.9 (171 - 172)	+ 17.2 ° (+ 16 °)	76

Allyl α-D-Gal **13** and α–L-Rha **14** were obtained in satisfactory yield and purity by Fischer glycosylation with strong, cation exchange resin as the catalyst, with allyl alchohol acting as both solvent and reagent. A weaker acid catalyst, boron trifluoride etherate ($BF_3 \cdot Et_2O$), was used in the case of allyl α-D-GlcNAc **15**.

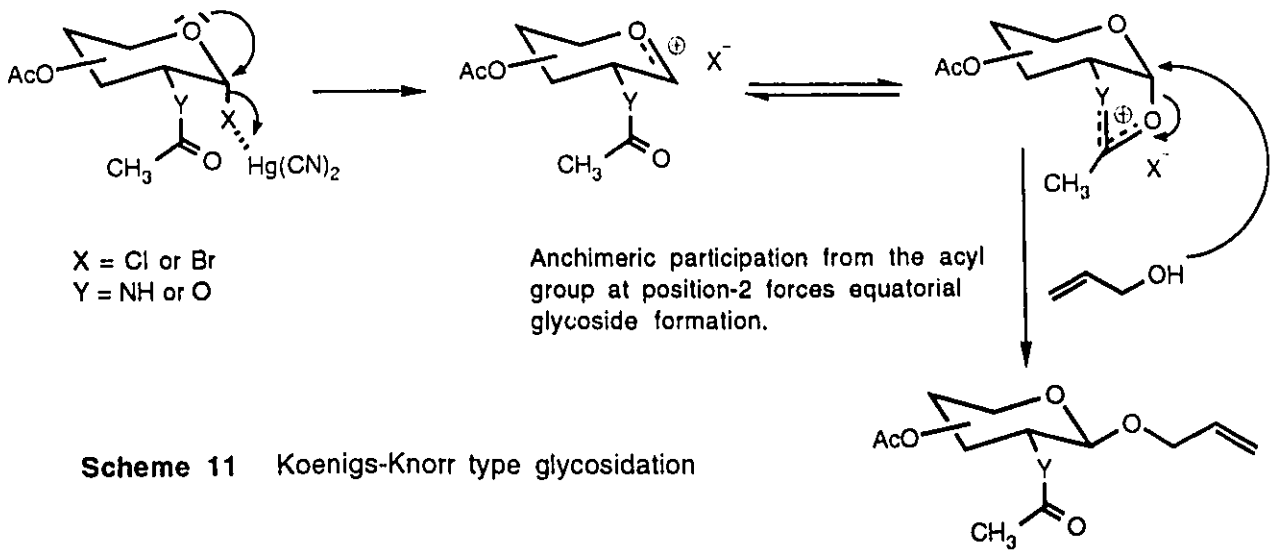
It was prepared in twice the yield (70%) reported in the literature[74] by simply doubling the amount of catalyst ($BF_3 \cdot Et_2O$) used. This result might be explained if one considers that, under these conditions, more catalyst might be free to react in glycoside formation rather than being complexed to the *N*-acetyl group.

Scheme 10 Fischer glycosidation



α-anomer favoured over β-anomer due to "anomeric effect"

Allyl glycosides with β anomeric configuration were prepared from the corresponding peracetylated glycosyl halides under Helferich modified Koenigs-Knorr conditions using mercuric cyanide $Hg(CN)_2$ according to known procedures. Here, 1,2 trans-glycosidation is favoured due to anchimeric participation by (Scheme 11) the acetate group at position 2 which forces nucleophilic attack to occur equatorially. Zemplén de-*O*-acetylation (cat.$CH_3O^-Na^+/CH_3OH$) afforded the desired free glycosides in a quick and smooth manner.

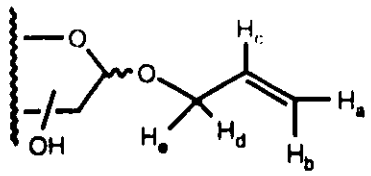


Scheme 11 Koenigs-Knorr type glycosidation

^{1}H-NMR spectra of these free glycosides except for the anomeric signal are relatively uninformative with respect to hydrogens contained in the sugar ring. The signals for these protons are amassed together in a small region between 3 and 4 ppm and appear collectively as large complex multiplets. Features which are discernable in the ^{1}H-NMR spectra are those signals belonging to the anomeric protons, the allyl groups, and to the lone methyl groups in the rhamnoside and N-acetyl glucosamine series. The appearance of the allyl group signals in the proton spectrum remained almost constant for all the glycosides prepared. Multiplicities of the dddd type were observed for each of the protons in the allyl group with little or no variation in chemical shift position or coupling constants from one glycoside to another. Representative values are given below and are consistent with values already reported for such systems[77] on a 500 MHz instrument.

[77] S.H. Tahir and O. Hindsgaul, *Can. J. Chem.* *64*, 1771 (1986).

Table 5 ^{1}H-NMR data for the allyl group in allyl glycosides 13 - 17.



Average δ (ppm, DMSO)	HH coupling constants
H_a = 5.14 ± 0.02 H_b = 5.29 ± 0.04 H_c = 5.89 ± 0.03 H_d = 4.03 ± 0.03 H_e = 3.88 ± 0.04	$J_{ab} \approx J_{ad} \approx J_{ae} \approx J_{bd} \approx J_{be}$ = 1.5 ± 0.3 Hz J_{ac} = 10.5 ± 0.2 Hz J_{bc} = 17.0 ± 0.2 Hz $J_{cd} \approx J_{ce}$ = 5.5 ± 0.2 Hz J_{de} = 13.5 ± 0.2 Hz

^{13}C-NMR data for the allyl glycosides synthesized are tabulated in Table 6.

Table 6 ^{13}C-NMR chemical shifts* of allyl glycosides

	Glycoside	C1	C2	C3	C4	C5	C6	O-CH_2-C	-CH=	$=CH_2$
13	Allyl α-D-Gal	98.2	70.2	70.0	71.7	69.2	61.9	68.9	134.2	118.8
14	Allyl α-L-Rha	99.6	70.8	71.0	72.7	69.3	17.5	68.8	133.9	119.0
15	Allyl α-D-GlcNAc	97.0	50.7	69.5	69.3	71.8	62.1	69.3	134.5	118.7
16	Allyl β-D-GlcNAc	100.9	56.3	74.7	70.7	76.7	56.3	71.2	134.2	118.9
17	Allyl β-D-Lac									
	(Glc)	101.8	75.2	76.1	71.8	75.6	61.8	71.5	134.1	119.5
	(Gal)	103.7	79.2	73.3	69.3	73.6	60.9			

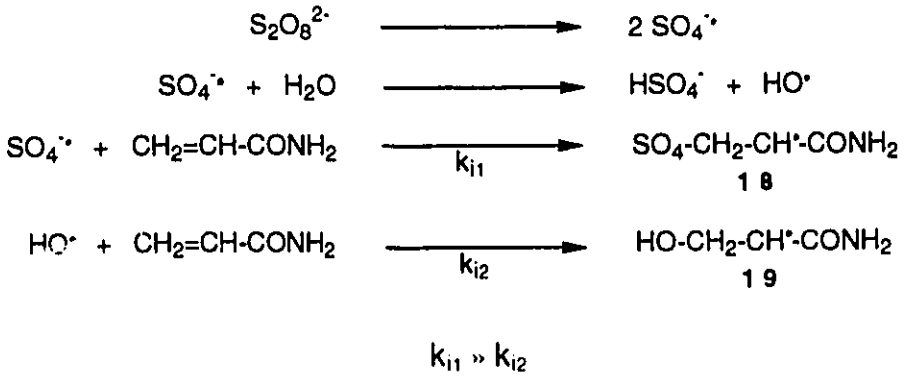
* 75.4 MHz, D_2O, 25 C, referenced to internal acetone (δCH_3 = 31.07ppm).

2.2 Copolymerization with acrylamide

Allyl glycosides were copolymerized with acrylamide in water under radical initiated conditions to give antigenic glycopolymers (**12**) of moderate molecular weight (50-100 kDa) as proposed by Horejsi[42b] and described in schemes 5 and 9. Ammonium persulfate ($(NH_4)_2S_2O_8$) was chosen as initiator because of its good solubility

in water and its high rate of dissociation to give the radical initiating species. The high rate at which $(NH_4)_2S_2O_8$ produces radical initiating species in boiling water allows for a relatively smooth and narrow molecular weight distribution of the resulting copolymer because most polymer chains are initiated simultaneously[78] .

The initiation mechanism is rather complex, and has been investigated thoroughly by others[78]. The main steps are summarized below.



The free radical species **18** and **19** then propagate the polymerization by a chain process after the initial electron transfer initiation step. Other initiation procedures for the polymerization of acrylamide in an aqueous environment have been reviewed[54]. Termination occurs by chain transfer to soivent and chain-chain coupling as the monomer concentration decreases.

The copolymerization of these two distinctively different monomer types (conjugated alkene in acrylamide versus a non-conjugated system in allyl glycosides) leads to a block type copolymer as suggested by their different reactivity ratio[57]. The lower reactivity of the allyl unit also results in a lower glycoside monomer incorporation than what is introduced in the polymerization vessel (Table 7). Approximately, a four fold mole percentage decrease in

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[78] J.P Riggs and F.J. Rodriges, *J. Polym. Sci. A1* , 5 , 3167 (1967).

glycoside incorporation with respect to monomer loading was observed under conditions used. The variation in monomer incorporation between similar trials is probably due to slight differences in reaction time and variation in stirring rates.

Table 7 Copoly (acrylamide/allyl-glycoside) glycopolymers

Polymer	Carbohydrate Unit	Monomer ratio: allyl glycoside/acrylamide (by mole)		Yield (% mass conversion)	$[\alpha]_D$ (c=1, H_2O)
		Reaction Load	* Polymer incorporation		
18	α-D-Gal	1:1.4	1:5.2	48	+51.7°
19	α-L-Rha	1:1.6	1:6.3	40	-1.8°
20	α-D-GlcNAc	1:1.8	1:5.1	67	+61.1°
21	β-D-GlcNAc	1:1.8	1:5.4	46	-
22	β-D-GlcNAc	1:1.8	1:7.7	41	-11.6°
23	β-D-GlcNAc	1:2.0	1:8.3	47	-
24	β-D-Lac	1:2.7	1:9.0	49	+1.3°

* Determined by ^{1}H-NMR and/or elemental analysis. See section 2.2.1

The glycopolymers introduced here were prepared with relative ease by boiling a concentrated, deoxygenated aqueous solution of the monomers ([monomers] ≥ IM) in the presence of the initiator $(NH_4)_2S_2O_8$ (0.4 mol %) for 10 to 15 min.. Under these conditions, mass conversion yields were on the order of 50%. Longer reaction times do increase yields, at the expense however of creating polymers of increasingly higher molecular weight and greater polydispersity. Molecular weights similar to those of lectins were desired to facilitate double radial diffusion assays.

Purification and isolation of the glycopolymers was achieved by simple dialysis against water followed by freeze-drying. Isolated polymers were white, spongy solids of very low density and were often statically charged.

2.2.1 Characterization of glycopolymers

Proper characterization of glycoconjugates is essential for a clear understanding of the fundamental aspects involved in binding interactions between receptor-proteins and carbohydrate laden surfaces or macromolecules. A clear idea of the glycopolymer structures is required to draw valid conclusions about their physiochemical and biophysical behaviour. Characteristics such as molecular mass, overall size and shape of the glycopolymers as well as hapten content are of primary importance. Investigation of these properties have been addressed through various methods and are discussed below.

A) Carbohydrate content.

The most important feature of any glycoconjugate is probably the carbohydrate density. Multivalency is an important factor in adhesion phenomena. Various methods have been utilized in the course of this work to quantitate the carbohydrate content in the glycopolymers produced. Carbohydrate monomer incorporation has been determined by colorimetric chemical essays, by elemental analysis and from ^{1}H-NMR spectrum integrations.

Colorimetric chemical assays such as the phenol/sulfuric acid assay devised by Dubois[79] or those of Elson and Morgan[80] test for amino sugars for example, are most often used in determining carbohydrate content of neoglycoproteins and can also be used with glycopolymers. These kinds of assays are actually slightly better suited to glycopolymer systems as the polymer backbone does not impart any background interference during assays in contrast to relatively more complex protein carriers. Although background

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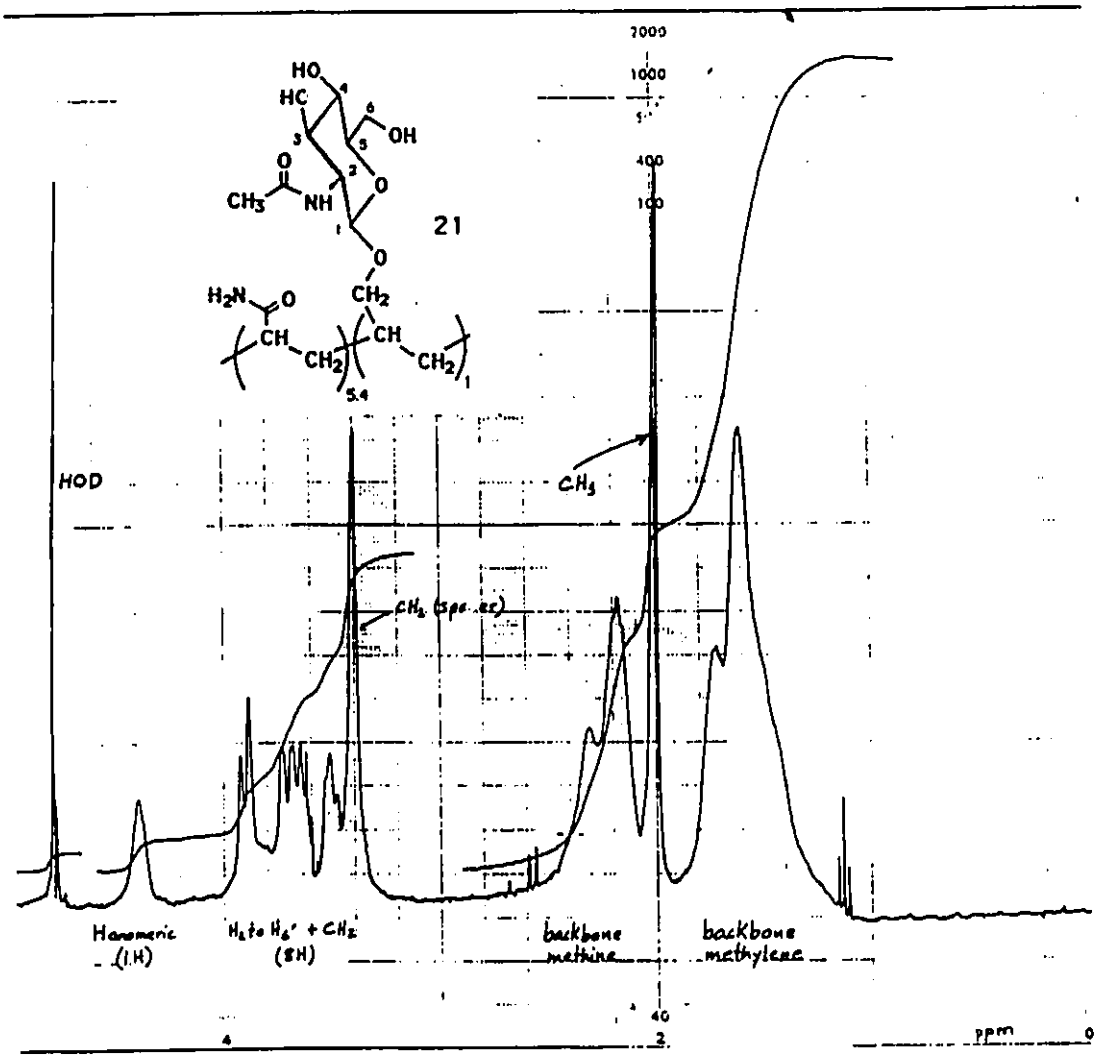
[79] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, *28*, 350 (1956).

[80] L.A. Elson and W.J.S. Morgan, *Biochem. J.*, *27*, 1824 (1933).

interference from any glycoconjugate carrier can be eliminated through the use of proper controls and blanks, other drawbacks to this kind of method still exist. Colorimetric assays are relatively tedious as they require the construction of calibration curves. The calibration curves as well as the samples submitted for assay must also be performed in triplicate. A more important consideration is the fact that both proteins and glycopolymers can retain water to as much as 10 to 15% by weight of isolated glycoconjugate which make a true and accurate determination very difficult. The presence of salts in many protein preparations also affects these determinations by masking the true weight of the glycoconjugate in a sample. A second recourse available for accurately determining carbohydrate content in neoglycoproteins is by amino acid analysis. However, this method requires specialized equipment which is not always available, and only gives the amount of modified amino acids in the free neoglycoproteins. Because some amino acids can be modified by more than one carbohydrate hapten with conjugation procedures such as reductive amination or Michael addition, true carbohydrate content is again difficult to quantitate properly.

^{1}H-NMR has proven to be a very simple, quick and effective tool for accurately determining the carbohydrate content in acrylate based glycopolymers. In the ^{1}H-NMR spectra of glycopolymers, resonances belonging to the sugar residues are found in the same chemical shift range as in the glycosides. Their position between 3.4 and 5 ppm are well removed from the residues arising from the polymer backbone found between 1 and 2.5 ppm. By comparing the relative integration ratios of these areas, an unambiguous value for the average carbohydrate density can be determined once the backbone resonance contribution from the glycoside comonomer has been taken into account. A typical ^{1}H-NMR spectra of a copoly (acrylamide/allyl-glycoside), glycopolymer is shown below along with spectra assignments and a detailed calculation of the carbohydrate content.

Figure 1 ^{1}H-NMR spectrum of copoly(acrylamide/allyl β-D-*N*-acetyl glucopyranoside) **21**



From the three glycoside resonance areas assigned in the spectrum of **21**, we find that one proton has an average integration value of 8.3 units. The total methylene and methine signals from all monomer units in the polymer backbone integrate for a total of 109 + 54 = 163 units. Subtracting the 3 proton contribution of the glycoside monomer unit (3 × 8.3 ≡ 25 units) leaves the total acrylamide

contribution ($x \times 3 \times 8.3$) at 138 units. Thus, **x** = 5.5 for the structure depicted for **21**.

One other method of accurately describing the molecular structure of glycopolymers synthesized is through elemental analysis. The relative monomer incorporation in **21** for example can be established using $(C_3H_5NO)_x(C_{11}H_{19}NO_6)_y \cdot nH_2O$ as the general structure of the copolymer obtained after freeze-drying. Water is incorporated in the molecular formula since polyacrylamides are known to strongly retain some water of hydration[54]. By setting up three equations for %C, %H and %N in addition to x+y+n=1, four simultaneous equations with three unknowns can be solved and verified algebraically:

$$\% \text{ Element} = \frac{am_{element} \times \sum_{x,y,n} E_{mu} \times M_{mu}}{\text{M.W. of the average repeating unit}} \qquad (1)$$

where : $am_{element}$ = atomic mass of the element
E_{mu} = number of particular elements per monomer type
M_{mu} = molar fraction of each monomer unit (x,y,n)

example: for **21**, $\%C = \frac{12.0115\ (3x + 11y)}{71.0794x + 261.2772y + 18.0152n}$

For example, elemental analysis of **21** gave C:45.41%, H:6.98% and N:12.38% which corresponds to an average monomer incorporation ratio of 5.3 acrylamide unit per allyl glycoside unit with a retained water content of 10.3% by weight. The average polymer repeating unit formula being $(C_3H_5NO)_{5.3}(C_{11}H_{19}NO_6)_1 \cdot 4.1H_2O$. This result varies by only 4% from the result obtained earlier by ^{1}H-NMR for the same glycopolymer. It is noteworthy that this method has the distinct advantage that the water content in the isolated glycopolymer, which is variable from batch to batch, can be calculated and taken into consideration during serological assays where exact amounts of glycoconjugates used is required.

B) Molecular weight of glycopolymers.

The allyl-glycoside glycopolymers discussed in this chapter were prepared according to the method outlined by Horejsi[42a] where molecular weights were determined by ultracentrifugation and shown to range from 40 to 100 kDa. The glycopolymers discussed in this chapter should also have molecular weights in this range as they were prepared in the same fashion. This assumption is consistent with the observed diffusion rate of the glycopolymers relative to the diffusion ratio of lectins of known molecular weight through agarose gels during double radial diffusion experiments.

An accurate measurement of molecular weight was performed for one of the allyl-glycoside glycopolymers (**21**) by viscometry, as described by Flory[81] using an Ostwald viscometer. Viscometry is a manipulatively facile method of determining molecular weights. However, this method usually requires up to 500mg of sample (recoverable). In this method, flow times of decreasing concentration of **21** in water are measured through an Ostwald viscometer, relative to pure water. Intrinsic and specific viscosities (equations 1 and 2 respectively) are then calculated and extrapolated to infinite dilution to give the "limiting viscosity number" $[\eta]$. $[\eta]$ is then related to a viscosity average molecular weight (M_V) through the Mark-Houinik expression (equ. 3) where K and **a** are empirical constants that depend on the solvent used and the polymer itself[81,82].

$$\lim_{c \to 0} \frac{(\eta/\eta_0) - 1}{c} = [\eta_{int}] \qquad (2)$$

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81 P.J. Flory in *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, New York 1953. p. 308-314.

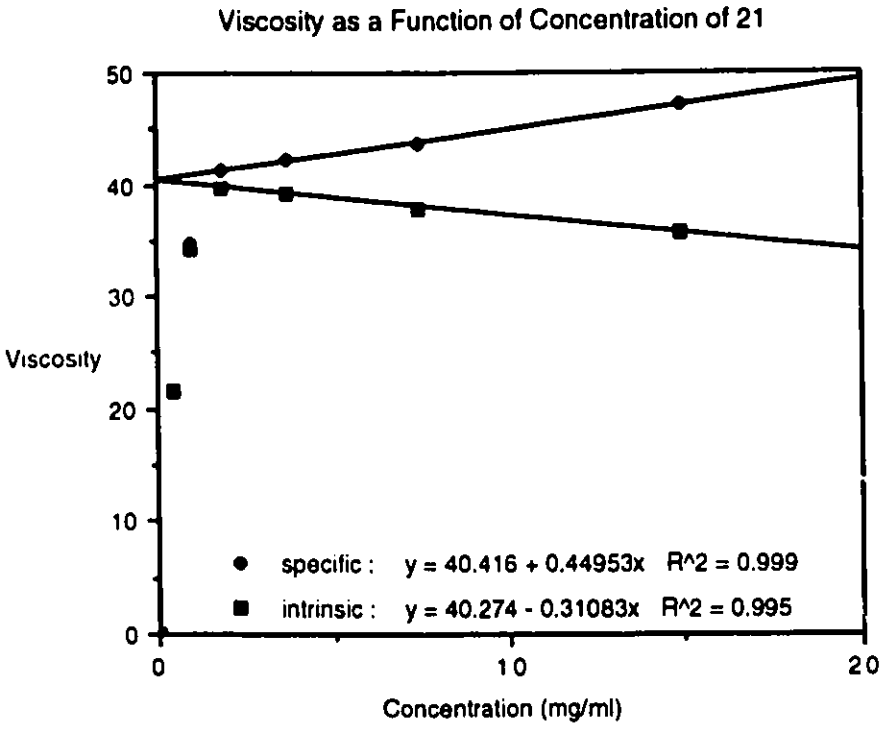
82 P.F. Onyon in *Techniques of Polymer Characterization*, P.W. Allen(Ed), Academic Press, New York, 1959; H. Tompa , *Polymer Solutions*, Academic Press, New York, 1956; C. Tanford , *Physical Chemistry of Macromolecules*, John Wiley & Sons, New York, 1961.

$$\lim_{c \to 0} \left(\frac{\eta/\eta_0 - 1}{c}\right) = [\eta_{sp}] \qquad (3)$$

$$[\eta] = K \cdot M_v^{a} \qquad (4)$$

Limiting viscosity numbers of 40.3 and 40.4 were obtained from intrinsic and specific viscosities respectively. Assuming a similar behaviour for the glycopolymer **21** and simple polyacrylamide in a "good" solvent such as water, values of 5.6×10^{-3} and 0.80 were used for K and **a** respectively as suggested by Molyneux[54]. This method gave a molecular weight (M_V) of 66.4 kDa for polymer **21**.

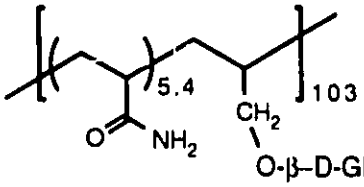
Figure 2



The method of molecular weight determination by viscometry for these glycopolymers was substantiated by gel permeation chromatography (GPC) results with secondary polyacrylamide standards and TSK G4000 SWXL column (see experimental section).

Thus, molecular weight determinations by GPC justify the **a** and K values used in the Mark-Houinik equation (3) and demonstrate that the physicochemical properties of the glycopolymers are closely related to that of simple polyacrylamide, in aqueous solution.

Thus, the average polymer chain for **21** is completely characterized by the molecular formula:



• 422 H_2O **21'**

C) Molecular size and shape.

The limiting viscosity number [η] derived from intrinsic viscosity (eg. 1) can also be used to deduce the size and shape of the glycopolymers in solution[83]. This was first described by Einstein[84]. The limiting number [η] = 40.3 found for **21** show that these glycopolymers exist in aqueous solution as random coils[83]. Simple polyacrylamide also exists as a random coil in water[85, 54]. A partial specific volume (υ_{eff}) of 16,1 $cm^3\ g^{-1}$ can be deduced from [η] according to equation 4 if the random coil is assumed to be sperical (μ = 2.5)[83].

$$\lim_{c \to 0} \frac{(\eta/\eta_0) - 1}{c} = \mu \cdot \upsilon_{eff} \qquad (5)$$

From the derived formula of **21**, it is possible to calculate the theoretical volume occupied by an average polymer chain in the aqueous solution at 25°C[86]. The root-mean-square length $\sqrt{\langle l^2 \rangle}$ of a

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[83] G.M. Barrow, *Physical Chemistry*, 4th Ed., McGraw-Hill, New York, 1979. Chap. 22, p. 755.

[84] A. Einstein, *Ann. Physik, 19*, 259 (1906) and *34*, 591 (1911).

[85] E.A. Bekturov and Z.K. Bakuova in *Synthetic Water Soluble Polymers in Solution*, Huthig and Wepf Verlag Basel, Heidelberg, New York, 1986. p. 127-135.

[86] K.J. Laidler and J.H. Meiser, *Physical Chemistry*, Benjamin-Cummings,

randomly coiled polymer chain is approximated by equation 5, where b is the bond length between atoms in the chain (b=1.12 Å for C-C)[87] and N the number bonds in the chain (for **21'**, N = [5.4 × 2 +1 × 2] × 103 = 1318).

$$\sqrt{\{l^2\}} = b\sqrt{N} \qquad (6)$$

$$V = 4/3\ \pi\ r^3 = 1/6\ \pi\ (\{l^2\})^{3/2} \qquad (7)$$

Thus, the estimated diameter ($\sqrt{\{l^2\}}$) of **21'** is 40.7Å, and its volume $3.52 \times 10^{-26}\ m^3$. These values have been calculated without considering any contribution from the carbohydrate ring, and therefore more likely represent only a lower limit.

D) Molecular dynamics of glycopolymers **20** and **21**.

An in-depth study of the molecular dynamics of the N-acetyl glucosamine glycopolymers **20** (α anomer) and **21** (β anomer) has been performed and presented elsewhere[41]. The details of these experiments and results are quite lenghty and complex, and are more suited to a physical chemistry forum. Therefore, only a brief abstract of this work is given.

^{13}C spin-lattice and spin-spin relaxation as well as nuclear Overhauser enhancements have been used to examine the molecular dynamics of both α and β anomeric forms of allyl-D-*N*-acetyl glucosamine/acrylamide glycopolymers. A comparative study of **20** and **21**, which have similar sugar densities was performed. ^{13}C-NMR spectra were obtained at four different magnetic field strengths corresponding to resononance frequencies of 50.3, 75.4, 100.6 and 125.7 MHz where the fast inversion-recovery technique (FIRFT)[88]

Menlo Park, California, 1982. p. 867-871.

[87] D.C. McCain and J.L. Markley, *J. Am. Chem. Soc. 108*, 4259 (1986).

[88] R.L. Vold, J.S. Waugh, M.P. Klein and D.E. Phelps, *J. Chem. Phys. 48*, 3831 (1968).

was used to determine T_1 relaxation rates of all carbon atoms for each field. The time scale of motions were determined by using various forms of the "model-free" approach[89]. This method has been used in the past to study the molecular dynamics of biologically related systems but never for glycopolymers[90].

These experiments have shown that the motions of the C-H vectors of the glycopolymer backbone may be described by a Lorentzian spectral density function, giving rise to an effective correlation time (1.15 ns) for overall tumbling of an average glycopolymer molecule. The overall motion of the glycopolymer molecules was shown to be anisotropic in nature by including spin-spin relaxation data in the analysis. The *N*-acetyl methyl and sugar hydroxymethyl group (C_6) exhibit independent internal motions and the activation energies associated with these motions were determined. Small differences in relaxation rates between the α- and β-anomeric forms suggest that the motions of the sugar ring are different for the two systems. This conclusion is supported by MM2 potential energy contour map calculations which indicate that the sugar residue in **21** (β-anomer) has almost twice the conformational flexibility of the sugar in **20** (α-anomer).

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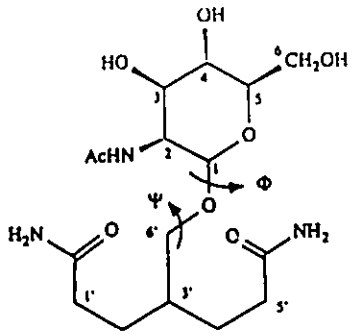
89 G. Lipari and A. Szabo, *J. Am. Chem. Soc. 104*, 4546 and 4559 (1982).

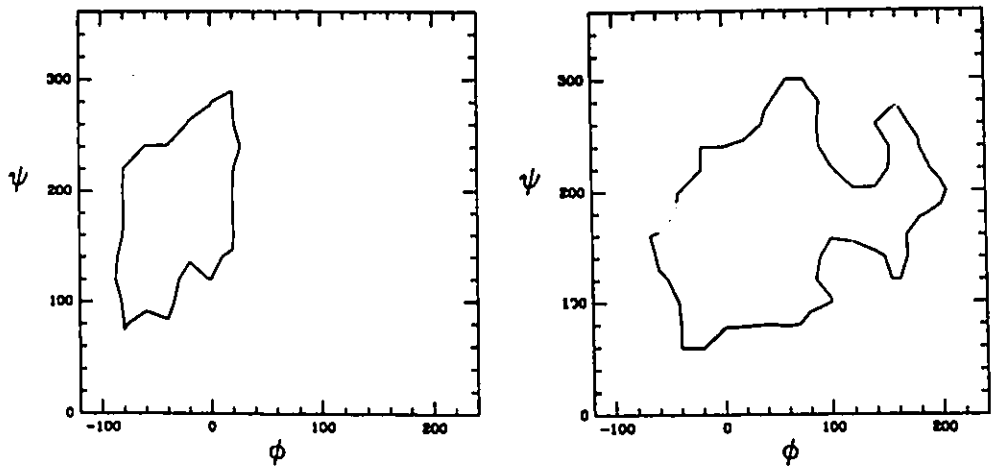
90 P.G. Schmidt, H. Sierzputowska-Gracz and P.F. Agris, *Biochemistry 26*, 8529 (1987) ; G.D. Henry, J.H. Weiner and B.D. Sykes, *Biochemistry 23*, 590 (1986) ; L.T. Hughes, J.S. Cohen, A. Szabo, C. Niu and S. Matsuura, *Biochemistry 23*, 4390 (1984) ; J.L. Dimiroli, H. Lam-Tanh, F. Toma and S. Fermandjian, *Biochemistry 23*, 3173 (1984).

Figure 3 Structures used for the potential energy calculations.



Figures 4 and 5 Potential energy contour map for the α-D-GlcNAc glycopolymer **20** and the β-D-GlcNAc glycopolymer **21**. The 10 kcal/mol levels with respect to the global minimum are shown.

20 **21**



E) ^{13}C-NMR of allyl-glycoside glycopolymers

Because of the high molecular weight of the glycopolymers and their relatively high viscosity in D_2O, the ^{1}H-NMR spectra of these materials are devoid of any fine structure, even at elevated temperatures. These materials, however, do give highly resolved ^{13}C-NMR spectra. The ^{13}C-NMR spectra of these materials appear almost as superimposed spectra of polyacrylamide and the respective carbohydrate. Typical features in their spectra are the

carbonyl resonances at 180 ppm with a small downfield shoulder peak, and broadened signals at 41 and 36 ppm belonging to the backbone methine and methylene carbons, respectively. The methylene spacer signal appears at 38 ppm as a shoulder to the backbone methylene signal. The anomeric carbons are found in their usual positions, however, their signals are considerably broad compared to the remaining sugar resonances. This may be attributed to more restricted motion of this particular centre relative to the backbone[41] or due to the effect of different stereochemical microenvironments in the polymer backbone (N.B. each alternating methine centre in the backbone may be of R or S absolute configuration and some tail to tail or head to head monomer links may be present in the chain). The ^{13}C-NMR chemical shifts of the allyl-glycoside copolyacrylamide glycopolymers have been tabulated below and a typical spectrum is shown also.

Table 8 ^{13}C-NMR chemical shifts of carbohydrate resonances in allyl-glycoside copolyacrylamide glycopolymers.

Glycopolymer	Sugar	C1	C2	C3	C4	C5	C6	NHAc
1 8	α-D-Gal	96.2	69.3	70.0	70.4	72.4	61.8	-
1 9	α- L-Rha	100.5	70.9	71.1	72.8	69.4	17.6	-
2 0	α-D-GlcNAc	97.9	54.6	71.6	70.8	72.7	61.4	175.8(C=O)
								22.8 (CH_3)
2 1	β-D-GlcNAc	102.3	56.3	74.4	70.7	76.6	61.5	175.0(C=O)
								23.0 (CH_3)
2 4	β-Lac							
	(Glc)	103.3	75.1	76.1	71.7	75.5	61.8	-
	(Gal)	103.6	79.2	73.3	69.3	73.6	60.9	-

* Measured at 75.4 MHz in D_2O at ambient temperature. Chemical shifts are given in ppm relative to TMS using acetone as internal standard (δCH_3 = 31.07 ppm).

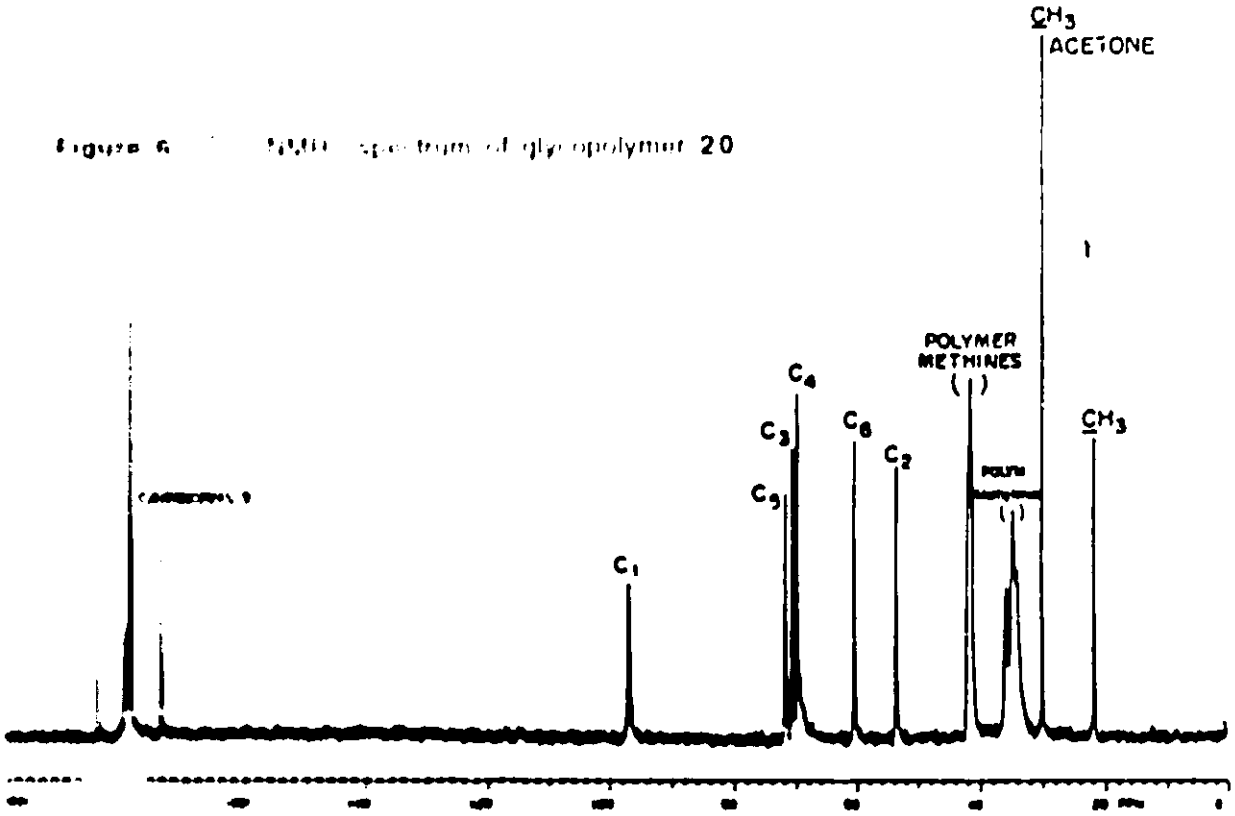


Figure 6 [illegible] spectrum of glycopolymer 20

2 2 2 Evaluation of antigenicity

The antigenicity of glycopolymers 18 to 24 was demonstrated during double radial diffusion assays with lectins Quantitative precipitation assays were also performed to quantitate the antigenicity of the N-acetyl glucosamine glycopolymers (both α and β anomers 20 and 21) towards the Wheat Germ Agglutinin lectin During double radial diffusion assays all glycopolymers reacted specifically only with those lectins which contain binding sites complementary to the respective carbohydrate residues bound to each polymer The results of these binding assays are tabulated in a quantitative fashion below

Table 9 Double radial diffusion assay for lectins and copoly(allyl-glycoside /acrylamide) glycopolymers.

Antigen		Lectin *				
hapten	glycopolymer	*Maclura Pomifera*	*Sambucus Nigra*	*Wheat Germ Agglutinin*	*Ricinus Communis*	*Arachis Hypogea*
α-D-Gal	18	+	-	-	+	+
α-L-Rha	19	-	-	-	+	-
α-D-GlcNAc	20	-	-	+	-	-
β-D-GlcNAc	21,22,23	-	-	+	-	-
β-Lac	24	+	+	-	+	+

* Other lectins, *Concanavalin A* (specific for α-D-Man and α-D-Glc) and *Dolichus Biflorus* (specific for α-D-GalNAc) did not prepicipitate any of the above glycopolymers but did precipitate glycopolymers of α-D-Man, α-D-Glc and α-D-GalNAc as expected[11].

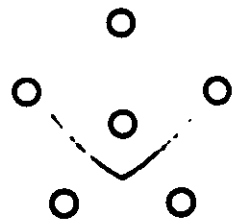


Figure 7
Radial double diffusion assay of WGA (center well) against glycopolymers 18 19 20 21 24 (clockwise from top)

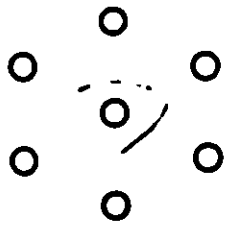
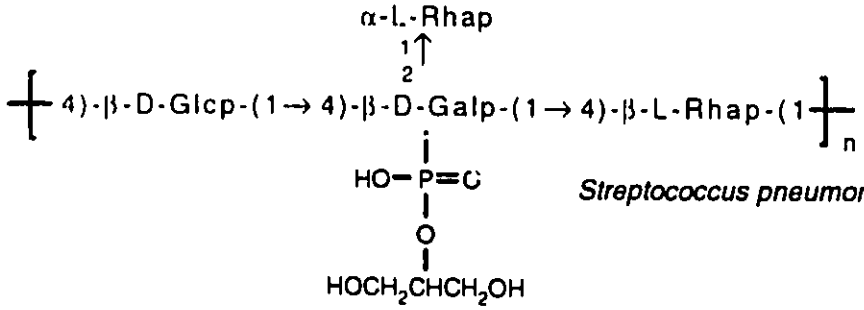


Figure 8
Radial double diffusion assay of *Maclura pomifera* (center well) against glycopolymers 18, 19, 24, 20, 21, 23 (clockwise from top).

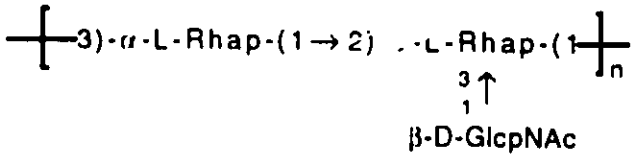
These simple binding assays demonstrate that lectins, unlike antibodies, possess binding sites which must be on the proteins outer surface since steric hindrance from the glycopolymer backbone is separated from the carbohydrate hapten by a methylene bridge

only. Agar gel diffusion assays with the α-L-Rha glycopolymer **19** showed a weak interaction with rabbit antisera raised against the capsular polysaccharide of *Streptococcus pneumoniae* type 23 (Pn 23; Statens Serum Institut, Denmark) possessing branched α-L-rhamnosyl residues but failed to precipitate rabbit raised Rhamnan antibodies. These antibodies did, however, give positive results with spacer modified α-L-Rha glycoconjugates (see later) On the other hand, GlcNAc glycopolymers **21**, **22** and **23** also failed to precipitate affinity purified IgG antibodies raised against the capsular polysaccharide (CPS) of the β-hemolytic Group A streptococci which possess branched β-D-GlcNAc residues. However, these antibodies were precipitated by spacer modified β-D-GlcNAc glycoconjugates (see later). This illustrates some of the drawbacks that these over-simplified glycopolymers have.

Bacterial antigen structures



Streptococcus pneumoniae Type 23 CPS



β-Hemolytic Group A *streptococci* CPS

Quantitative precipitation assays were performed on both α and β anomeric GlcNAc glycopolymers **20** and **21** with the wheat germ agglutinin (WGA) lectin. Prepared in the same way, **20** and **21** have similar densities and, assumably, molecular weight. Results of these

assays are given below (Fig. 9) and demonstrate the sensitivity of these simple glycopolymer antigens.

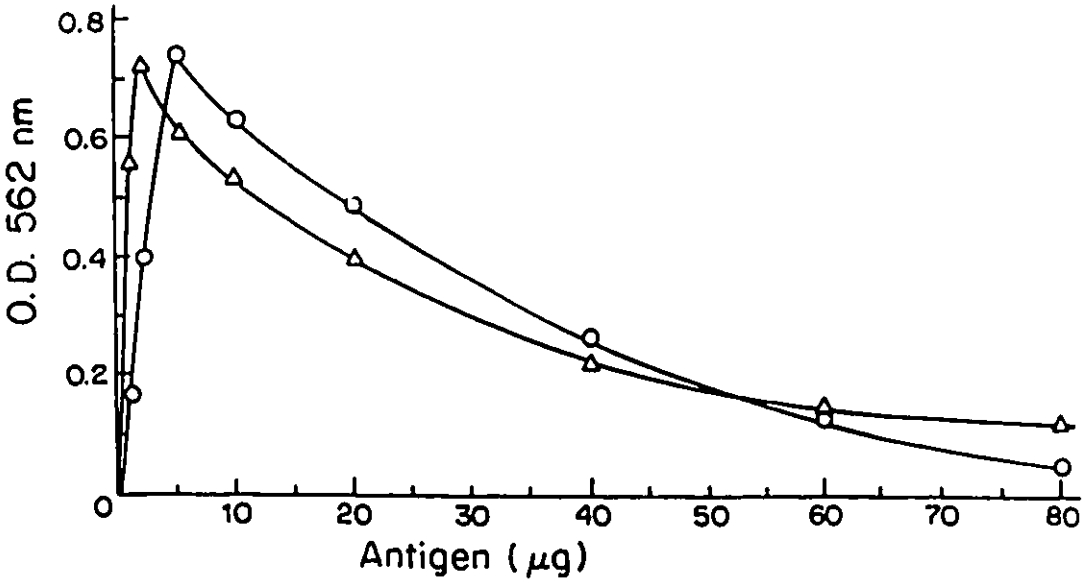


Figure 9 Quantitative precipitation curves of WGA by α-(Δ) and β-(O)-D-GlcNAc glycopolymers **20** and **21** respectively.

Maximum agglutination for 20μg of lectin was achieved with only 2μg of the α-glycopolymer, which bound strongest. The β-glycopolymer bound at a ratio of 5μg Ag /20μg WGA. Although WGA exhibits very little anomeric preference with methyl N-acetyl D-glucosaminides, a slight preference for the β-anomer has been demonstrated in the past[92]. The molecular dynamics data obtained for the α- and β-glycopolymers[41] suggests that, in this case, the better binding properties of the α-glycopolymer **20**, despite the lower overall mobility and perhaps accessibility of the sugar moiety over the β-glycopolymer **21**, might be associated with other geometrical constraints.

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[92] I.J. Goldstein, S. Hammarström and G. Sunblad, *Biochim. Biophys. Acta 405*, 63 (1975).

2.3 Experimental methods

General methods.

All solvents and reagents used were of reagent grade and, when required, further purifications were accomplished following published procedures[93a]. Evaporations were performed under reduced pressure provided from a water aspirator, at 20-40°C, with a Büchi rotary evaporator. Melting points were recorded with a Gallenkamp apparatus and are uncorrected. Optical rotations ($[\alpha]_D$)were recorded with a Perkin Elmer 241 polarimeter and were run at 23 ± 1°C.

Thin-layer chromatography (TLC) was performed using Silica Gel 60-F254 plates and column chromatography on Silica Gel 60 (230-400 Mesh, E. Merck No. 9385). The developed plates were routinely dipped in or sprayed with a solution of ceric sulfate (1%) and ammonium molybdate (2.5%) in 10% aqueous sulfuric acid and heated at ≈ 150°C. Other developers include 0.2% isatin in acidified ethanol, iodine, dilute aqueous potassium permanganate, 2% ninhydrin in 4 % aqueous pyridine (for amines), 0.2 % 4-dimethylamino cinnamaldehyde in acidified ethanol (for biotin) and ultraviolet light (for chromophores).

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL 300 or Gemini 200 spectrometer at 300 MHz or 200 MHz for protons and 75.4 or 50.3 MHz for carbon respectively, under internal lock condition unless indicated otherwise. Proton chemical shifts (δ) are given relative to internal chloroform (7.24 ppm) for $CDCl_3$ solutions, to internal acetone (2.216 ppm) for D_2O solutions and to DMSO (2.50 ppm) for DMSO-d_6 solutions unless stated otherwise. To simplify ^{1}H-NMR spectra, labile hydrogens in unprotected glycosides and glycopolymers were exchanged for deuterium by repeated

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[93] a) D.D. Perrin, W.C. Armarego and D.R. Perrin, *Purification of Laboratory Compounds*, 2nd Ed. Permagon, London, 1980; b) S.P. Colowick and N.O. Kaplan, *Methods Enzymol.*, *1*, 141 (1955).

lyophilization from D_2O solutions. Carbon chemical shifts are given relative to deuterochloroform (77.00 ppm), to internal acetone (31.07 ppm) for D_2O solutions and to DMSO-d_6 (39.50 ppm) unless stated otherwise. Analysis were done as first order approximations. Assignments are based on ^1H-^1H COSY and ^1H-^{13}C HETCOR experiments, and distortionless enhancement by polarization transfer (DEPT) experiments. Mass spectra were recorded with a VG 7070-E spectrometer (EI,CI-ether) or Kratos II H instrument (FAB-glycerol, CI-ether). Xenon was used as the neutral fast atom in FAB-MS experiments.

Infrared spectra were recorded on a Bomem Michelson series FT-IR instrument with computer workstation for solutions in chloroform. NaCl plates were used. UV-vis spectra were recorded on a Gilford instrument using quartz cuvettes.

Preparative scale size exclusion or ion exchange chromatographies were eluted with the help of a Pharmacia Peristaltic Pump P3. Chromatogram detection was performed with a Waters Differential Refractometer R401 or R403 and recorded on a Linear 1200 or 2000 chart recorder. Fractions were collected automatically using LKB 2112 Redirac or Pharmacia model 5051 fraction collectors

The pH of aqueous solutions were measured with a Fischer Scientific model 805 NP instrument fitted with a Fischer Scientific E-N5 pencil electrode at room temperature (23 ± 2 °C). Proper two point instrument calibration was routinely performed using commercial standard buffer solutions (pH 4.00, 7.00 and 10.00) from Fischer. Crude pH measurements were made with Hydrion test paper or S & S Panpeha pH indicator strips. When used for non-aqueous solutions such as methanol, pH test papers were always premoistened with a mist of distilled water.

All buffers used were prepared according to standard methods previously described[93h] and were always filtered before use. For synthetic purposes, Amberlite IRA-400 anion exchange resin or Amberlite IR-120 cation exchange resin was used unless indicated

otherwise.

Glycoconjugates were exhaustively dialysed against distilled water using cellulose tubing (Sigma, molecular weight cut-off of 12 kDa) unless indicated otherwise. Lyophilizations were performed using a VIRTIS-24 freeze drier.

Allyl α-D-galactopyranoside **13**

D-galactose (18.05 g, 0.103 mol, Sigma), Rexin 101 (H^+),10g resin (100 mesh) and allyl alcohol, 200 mL (Fluka) were refluxed for 90 min. in a 500 mL flask. The solution was allowed to cool for 45 min. after which the resin was filtered and then evaporated to yield a yellow syrup. The syrup was suspended in 30 mL of absolute ethanol and the product left to crystallize overnight at 4°C. The resulting crystals were filtered and washed with with cold ethanol. Repeated crystallition (2x) of the mother liquors yielded a total of 9.81 g (0.045 mol, 44%) of product, pure by t.l.c. (Rf = 0.43, 9/4/2 ethyl acetate/isopropanol/water) and NMR. Additional ^{1}H-NMR δ ($DMSOd_6$) : 4.58 (d, 1H, J_{12}=1.7 Hz, H_1). See Table 4 for mp and $[\alpha]_D$ values for this and following allyl glycosides.

Allyl α-L-rhamnopyranoside **14**

This product was prepared as described for **13**. However, the product was isolated by silica gel chromatography using chloroform/ methanol (8/1) as eluent. **14** (Rf = 0.32) was isolated in 61% yield as a clear oil. Additional ^{1}H-NMR δ ($DMSOd_6$): 5.15 (d,1H, J_{12} = 1.7 Hz, H_1), 1.15 (d, 3H, $J_{5,6}$ = 6.2 Hz, CH_3).

Allyl 2-acetamido-2-deoxy-α-D-glucopyranoside **15**

N-acetyl D-glucosamine (24.5 g , 0.111 mol) in 350 mL allyl alcohol containing 3.5 mL of boron trifluoride etherate was refluxed for 2.5 hrs. The resulting clear solution was evaporated and coevaporated several times with ethanol to yield a white crystalline mass.

Multiple crystallization from abs. ethanol gave 19.3 g (0.074 mol, 70%) of fluffy, white crystals pure by TLC (Rf = 0.63, 3/2/1, EtOAc/CH_3COOH/H_2O) and NMR. Additional ^{1}H-NMR δ ($DMSOd_6$) : 7.77 (d, 1H, $J_{NH,2}$ = 8.4 Hz, NH), 5.01 (d, 1H, J_{12} = 5.2 Hz, H_1), 1.84 (s, 3H, CH_3).

Allyl 2-acetamido-2-deoxy-β-D-glucopyranoside **16**

2-Acetamido-3,4,6-tri-*O* -acetyl-2-deoxy-α-D-glucopyranosyl chloride (40.80g, 0.112 mol), prepared according to Horton[94] and 30.3 g (0.12 mol) Hg $(CN)_2$ were stirred in 400 mL of dry allyl alcohol under an anhydrous atmosphere for 24 hrs. at room temperature. Evaporation of the allyl alcohol gave a solid mass, most of which dissolved on shaking with 400 mL $CHCl_3$. After filtration, the chloroform solution was washed extensively and successively with 10% aqueous KI, 10% aqueous $Na_2S_2O_3$ and water. It was then dried with $MgSO_4$ and evaporated to a crystalline mass. Repeated (4x) crystallizations of all liquors from abs. ethanol gave 25.55 g (0.066 mol, 60%) of acetylated **16**. mp = 162.9 - 164.9°C (lit.[95] mp = 160°C. ^{1}H-NMR δ ($CDCl_3$): 5.83 (H_c), 5.44 (d, 1H, $J_{NH,2}$ = 8.8 Hz, NH), 5.28 (dd, 1H, J_{34} = 9.6 Hz, H_3), 5.24 (H_b), 5.18 (H_a), 5.06 (dd, 1H, J_{45} = 10.0 Hz, H_4), 4.69 (d, 1H, J_{12} = 8.4 Hz, H_1), 4.32 (H_d), 4.24 (dd, 1H, J_{56} = 4.8 Hz, $J_{66'}$ = 12.2 Hz, H_6), 4.11 (dd, 1H, $J_{56'}$ = 2.4 Hz, $H_{6'}$), 4.07 (H_e), 3.85 (ddd, 1H, J_{23} = 10.6 Hz, H_2), 3.67 (ddd, 1H, J_{45} = 9.9 Hz, H_5), 2.07, 2.01, 2.00, 1.93 (4 x s, 4 x $COCH_3$). ^{13}C-NMR δ ($CHCl_3$): 170.7, 170.5, 170.0, 169.2 (4 x C=O), 133.4 (-CH=), 117.7 (=CH_2), 99.6 (C_1), 72.4 (C_5), 71.8 (C_3), 69.9 (O-CH_2-C=), 68.7 (C_4), 62.2 (C_6), 54.8 (C_2), 23.4 (CH_3, NHAc, 20.8, 20.75, 20.7 (3 x CH_3, OAc)).

Zemplén de-*O*-acetylation with sodium methoxide in methanol gave a quantitative yield of **16** after neutralization with cationic resin (Amberlite IR-129 (H)) and evaporation. Recrystallization of the product was achieved in 95% EtOH. Additional ^{1}H-NMR δ ($DMSO-d_6$) for **17**: 4.29 (d, 1H, J_{12} = 8.4 Hz, H_1), 1.80 (S, 3H, CH_3).

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[94] D. Horton, *Methods Carbohydr. Chem.*, *6* , 282 (1972).

[95] E. Thomas, *Carbohydr. Res.*, *13* , 225 (1970).

Allyl β-D-lactoside **17**

Compound **17** was prepared from 2,2',3,3',4',6,6' hepta-O-acetyl-β-D-lactosyl bromide by following the procedures outlined for **16**, in a 54% overall yield. Physical constants of acetylated **17** were in good agreement with those previously reported[96], see Table 4. Additional ^{1}H-NMR δ (D_2O) : 4.53 (d, 1H, J_{12} = 7.9 Hz,$H_{1'}$), 4.44 (d, 1H, J_{12} = 7.8 Hz, H_1).

Copoly(acrylamide/allyl-glycoside) glycopolymer preparation

Copolymers were prepared according to Horejsi *et al* [97] . Reactions were initiated with $(NH_4)_2S_2O_8$ and carried out at 100°C under a nitrogen atmosphere for 10 to 15 min. after which they were diluted with cold water, dialysed and lyophilized. Water used as polymerization solvent was deoxygenated effectively by simply boiling it vigorously over a propane flame for 10 to 15 min. followed by bubbling a steady stream of nitrogen through it via a fritted glass filter rod as it cooled. Deoxygenation by multiple freeze-thaw cycles or by bubbling helium or argon did not offer any advantages and was more time consuming.

Copoly(acrylamide/allyl α-D-galactopyranoside) **18**

Acrylamide (0.50 g, 7.0 mmol) and 0.99 g (4.5 mmol) of **13** were dissolved in 10.0 mL of deoxygenated water. The solution was immersed in a boiling water bath and after a few minnutes, solid ammonium persulfate ($(NH_4)_2S_2O_8$) (10 mg, 44 μmol) was added. The solution was stirred vigorously for 10 min. after which it was diluted with water, dialysed and lyophilized. 0.71 g (48%) of the corresponding glycopolymer was obtained.

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[96] V. Horejsi and J. Kocourek, *Methods Enzymol.* , *34* , 361 (1974).

[97] V. Horejsi, P. Smolcek and J. Kocourek, *Biochim. Biophys. Acta.*, *538* , 293 (1978).

Copoly(acrylamide/allyl α-L-rhamnopyranoside) **19**

Using the same procedure as for **18**, 440 mg of **19** was prepared by polymerizing 740 mg (3.63 mmol) of **14** and 369 mg (5.20 mmol) of acrylamide in 7.2 mL of deoxygenated water. Ammonium persulfate (7.2 mg, 32 µmol) was used to initiate the polymerization.

Copoly(acrylamide/allyl α-D-N-acetylglucosamine) **20**

As described above, 1.00 g (67%) of **20** was prepared using 0.50 g (7.03 mmol) of acrylamide and 1.00 g (3.82 mmol) of **15** in 10.0 mL of deoxygenated water. Ammonium persulfate (10.0 mg, 4.4 µmol) was used to initiate the polymerization.

Copoly(acrylamide/allyl β-D-N-acetylglucosamine)

a) compound **21**: 0.78 g (2.98 mmol) of **16**, 0.39 g (5.48 mmol) of acrylamide and 8 mg (35 µmol) $(NH_4)_2S_2O_8$ were stirred for 15 min. at 100°C in 8.0 mL of deoxygenated water. The usual processing gave 0.54 g (46%) of **21**.
b) compound **22**: 0.50 g (1.91 mmol) of **16**, 0.25 g (3.52 mmol) of acrylamide and 5 mg (22 µmol) $(NH_4)_2S_2O_8$ were dissolved in 5.0 mL of deoxygenated water and vigorously stirred at 100°C for 11 min.. 0.36 g (47%) of **22** were obtained after the usual processing.
c) compound **23**: 0.28 g (1.08 mmol) of **16** and 0.15 g (2.14 mmol) of acrylamide were dissolved in 3.0 mL deoxygenated water. 4 mg (17.5 µmol) of $(NH_4)_2S_2O_8$ were added and the solution heated at 100°C for 15 min.. The usual processing gave 175 mg (41%) of **23**.

Copoly(acrylamide/allyl β-D-lactoside) **24**

Allyl β-D-lactoside **17** (1.00 g, 2.62 mmol), 0.50 g (7.0 mmol) of acrylamide and 10 mg (44 µmol) of ammonium persulfate were dissolved in 10.0 mL of deoxygenated water. While stirring, this solution was immersed in a boiling water bath for 10 min.. The usual processing gave 0.73 g (49%) of **24**.

Molecular weight determination of glycopolymers by viscometry and gel permeation chromatography (GPC)

The viscosity average molecular weight of **21** was calculated from the measured intrinsic and specific viscosity in H_2O at 25.0°C with an Ostwald viscometer as described by Flory[81]. The viscometer was suspended in a thermostatted water bath which controlled the temperature to ± 0.1°C. The temperature was measured using a Cr/Al thermocouple. Flow times were measured twice, to within ± 0.1s for each trial. Flow times for10.00 mL samples were measured for distilled water and for seven two fold dilutions of a stock polymer solution originally prepared by dissolving 332.0 mg of lyophilized polymer (containing 10.3% of H_2O by weight - see section 2.2.1 a)) in 20.00 mL H_2O. Extrapolation to infinite dilution gave limiting viscosity values of 40.3 and 40.4 for intrinsic and specific viscosities respectively (correlation coeffecients > 0.995). The limiting viscosity number is related to the viscosity average molecular weight through the Mark-Houinik expression[81, 82] $\eta=KM_V^a$. Assuming similar behaviour of **21** and simple polyacrylamide in a "good" solvent such as water, values of 5.6×10^3 and 0.80 were used for K and **a** respectively[54]. This method gave a viscosity molecular weight average of 66.4 kDa.

Gel permeation chromatography was performed through a TSK G4000 SWXL column (7.5 x 30 cm) in 0.1M phosphate buffer (pH 7.0) at a flow rate of 1.0 mL/min. A Waters Model 991/625LC HPLC system equiped with a multiple diode array detector was used. Molecular weight calibrations were performed with both protein (Boehringer Mannheim, cat. no. 1213-776) and polyacrylamide standards (Polysciences). Absorbances were measured at 205 nm (polyacrylamides) or 280 nm (proteins). Both glycopolymers **20** and **21** had retention times between 7 and 12 min., centered at 10.0 min.. The 50 and 90 kDa polyacrylamide standards had retention times centered at 11.2 and 8.4 min. respectively, thus confirming the above viscometry measurements and method.

Viscometry and GPC results confirmed estimated molecular weights made from observing relative mobilities of glycopolymers and lectins of known molecular weights through agarose gels during double radial diffusion experiments.

Agar gel double radial diffusion assays

Double radial diffusion assays were performed on 1% agarose (BDH) containing 2% polyethylene glycol (PEG, MW8000, Sigma) in 0.1M phosphate-buffered saline (PBS) pH 7.2, according to Ouchterlony and Nilsson[98]. Both glycopolymers and lectins were used at a concentration of 1 mg/mL PBS unless indicated otherwise. Aliquots (20μL) of respective antigens and lectins were used to fill wells perforated in the agarose gel slabs (≈ 1 mm thickness). Precipitin bands were allowed to form overnight at 4°C.

Quantitative Precipitation Assays

Aliquots (100μL) of lectin solutions (200μg/mL PBS) or Pn23 CPS rabbit antibody serum were added to 1.5 mL Eppendorf microcentrifuge tubes (Brinkmann Instruments Co., Westbury, N.Y.). Aliquots of 1.0 to 200μL of glycopolymer stock solutions (1.0 mg/mL PBS) were then added, and the final volumes of all tubes were then adjusted to 300μL. Precipitation reactions were all run in triplicate. The solutions were then agitated with a vortex mixer and allowed to equilibrate for 2 hrs at 37°C and then for 4 days at 4°C. The tubes were then centrifuged for 15 min. at 14000g in a Fischer Microcentrifuge Model 235B. The precipitin pellets were washed three times with 300 μL of cold PBS with centrifugation between washes. The protein content of the pellets was determined using the bicinchoninic acid[99] (BCA) protein assay reagents of Pierce (Il., USA)

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[98] Ö. Ouchterlony and L.A. Nilsson, *Immunodiffusion and Immunoelectrophoresis* in *Handbook of Experimental Immunology* , D.M. Weir (ed.), chapter 19, Blackwell Scientific Publications, Oxford, 1978.

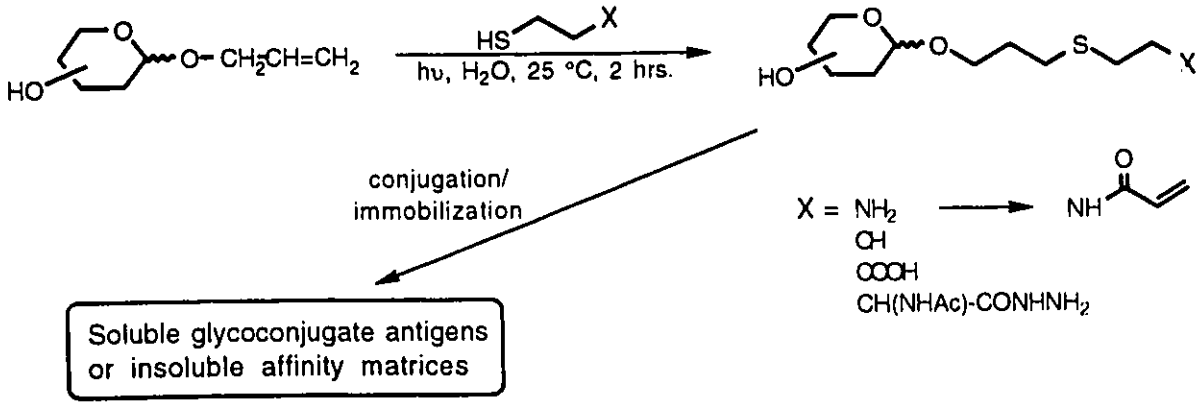
[99] P.K. Smith, R.I. Krohn, G.I. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Prorenzano, E.K. Fukimoto, N.M. Goeke, B.J. Olson and D.C. Klenck, *Anal.*

following the enhanced protocol described in the instruction manual. The absorbances were measured at 562 nm.

Biochem. *150*, 76 (1985).

3. Addition of thiols to allyl glycosides

The use of allyl glycosides in oligosaccharide synthesis is further substantiated, when preparing glycoconjugates, by extending the aglycon length through an efficient thiol addition reaction. Primary alkyl thiols bearing a different functional group at the opposing terminus, add smoothly to the alkene function under mild, aqueous, light promoted conditions. This method can be used to produce spacer modified carbohydrate haptens having different functional groups useful in various conjugation or immobilization procedures.



Scheme 12 Allyl-glycosides as precursors to spacer modified glycoconjugates

This strategy has various subtle advantages over existing ways of introducing spacers. Besides employing prevalent allyl glycosides, it is mild, performed in water, and compatible with a variety of functional groups. It permits a stepwise lengthening of the spacer, from one or two C-C bonds with allyl glycosides (polymerization or ozonolysis and reductive amination), to a considerably longer unit. The resulting aglycon contains a sulfide bridge midway through its length. This gives a spacer which is more hydrophilic than often used

[illegible]

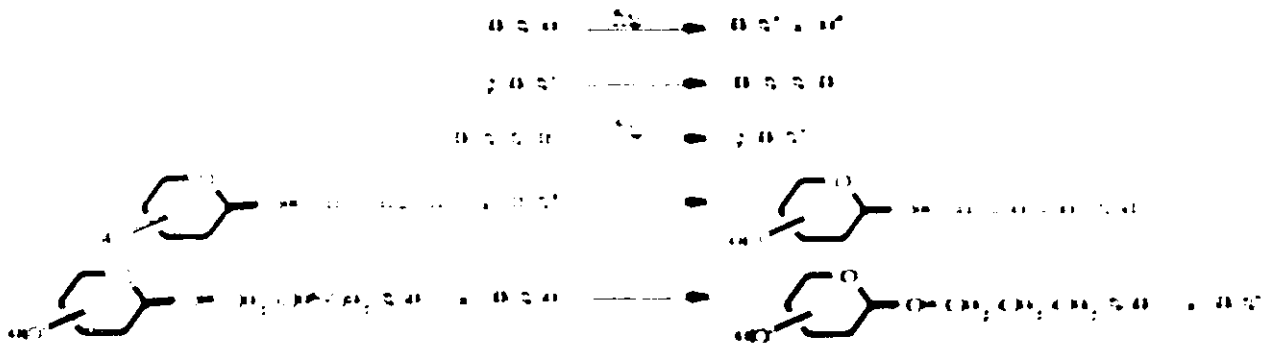
3.1 Reaction conditions evaluation and mechanism

[illegible] an acid by electrophilic nucleophile or free radical mechanisms. In the presence of a free radical initiator mercaptans add to double bonds by a free radical mechanism in an anti Markovnikov orientation. It is this last mechanism which was exploited to prepare the title compounds.

Sulfide elongation by thiol addition to allyl glycosides was first suggested from work done by Lee[1] in that work the methodology was investigated using as a thiol addition of 2-mercaptoethylamine hydrochloride to allyl glycosides and was performed in only moderate yields by refluxing a large excess of thiol to allyl glycosides in water at room temperature for long periods of time thus suggesting the potential for a radical coupling of these reagents. Subsequent studies[2] [illegible] aqueous solutions of allyl glycosides and 2-mercaptoethylamine hydrochlorides were exposed to an ultraviolet light source under deoxygenated conditions. The upper [illegible] of the reaction mixture revealed a quick and clean consumption of the allyl glycosides to the desired 3-(2-aminoethylthio)propyl glycosides. Completion was achieved in about one hour. Since dimerization of 2-mercaptoethylamine through disulfide

[illegible]

formation was observed. NMR analysis of the products indicated by the absence of new methyl peaks that thiol addition to the alkene proceeded exclusively in an anti-Markovnikov fashion consistent with the light promoted radical mechanism proposed below (Scheme 1).



Scheme 1. Proposed mechanism for thiol addition to allylglycosides

The reaction seems feasible only when unprotected allylglycosides and a thiol are dissolved in water then irradiated with ultraviolet light. Reactions were performed in an open beaker exposed to direct irradiation from a common ultraviolet developing lamp (UVG) or Rayonet light operating at 254 nm. The best results were obtained with deoxygenated water with the whole apparatus contained under a nitrogen atmosphere. The reaction does proceed in the presence of oxygen albeit at a seemingly slower pace as judged by TLC.

Various reaction conditions were tested using cysteamine hydrochloride as the thiol and allyl β-D-glucoside (**1**) or allyl α-D-glucoside (**2**) with water as solvent. These were performed to examine and further understand the reaction of thiol from an aqueous stock solution equally concentrated (1.25 M in cysteamine hydrochloride and **1**). Identical solutions were subjected to different lighting conditions. Scenes A, C, similarly equal volumes of an aqueous stock solution equally concentrated (1.25 M in the cysteamine hydrochloride disulfide dimer and **1**). Scenes B were subjected to

the same lighting conditions as above. Trials were performed simultaneously for a total of 3 hours in a) darkness, b) ambient laboratory light, c) 200W incandescent and d) concentrated ultraviolet light (with and without sodium bisulfite ($NaHSO_3$) as radical inhibitor) for both series. Reactions were monitored periodically by TLC (3/2/1/1 EtOAc/AcOH/EtOH/H_2O and 5/3/2 MeCN/acetone/5% $AcOH_{(aq)}$).

These experiments revealed no product formation for any reaction performed in the dark or in ambient light after 6 hours. For cysteamine as thiol source (Series A), a 200W incandescent light source provided only minimal product formation after 1 hour while the reaction was complete under concentrated ultraviolet irradiation. Slight product formation was detected when the disulfide was irradiated with concentrated ultraviolet light only. The presence of sodium bisulfite completely inhibited any product formation under conditions of ultraviolet irradiation. Combined, these observations are consistent with a radical mechanism.

Attempts at using methanol as solvent (13, cysteamine, U.V.) resulted in a sluggish reaction and difficult control of volume due to rapid evaporation. Attempts at adding cysteamine to allyl-glycosides under different radical initiating conditions also proved fruitless. The disulfide was the sole new product with H_2O_2 under aqueous thermal conditions (25 and 100 C). In a different attempt, acetylated 16 cysteamine and AIBN (azobisisobutyronitrile) were refluxed in tetrahydrofuran (THF) and quickly produced the disulfide dimer only.

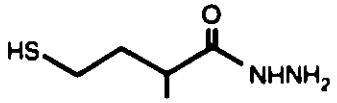
3.2 Preparation of various 3-(alkylthio) propyl glycosides

Ultraviolet light catalysed thiol addition to allyl-glycosides under aqueous conditions was used to prepare a variety of 3-(alkylthio) propyl glycosides. Various bifunctional mercaptans were used to give general access to spacer modified carbohydrate haptens useful in glycoconjugate syntheses. Thiols used are shown below.

25 cysteamine

27 3-mercaptopropionic acid

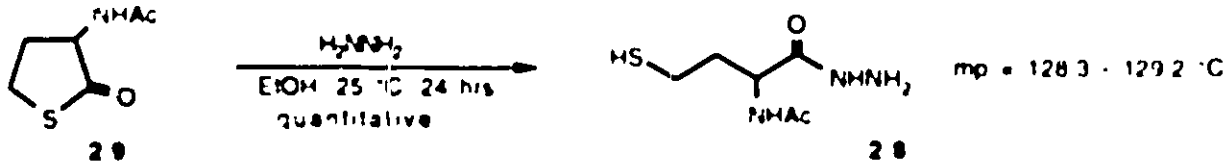
26 2-mercaptoethanol



28

DL-2-N-acetyl-4-mercaptobutanoylhydrazide

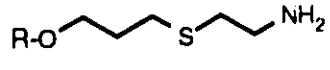
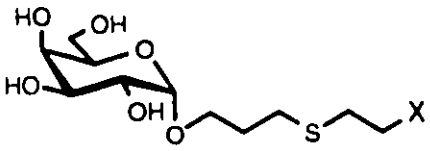
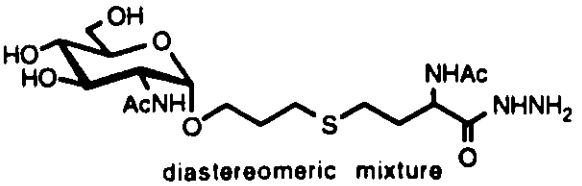
Thiols **25**, **26** and **27** were obtained from commercial sources while **28** was synthesized from DL-*N*-acetyl homocysteine thiolactone **29** and hydrazine in ethanol by a transacylation process.



29 **28**

New glycosides were prepared from a variety of allyl-glycosides isolated yields and physical data for each glycoside shown below are given in Tables 10 and 11

Table 10 3-(alkylthio)propyl-glycosides

Glycoside			Yield ¥ (%)	mp (°C)	$[\alpha]_D$ §	Possible conjugation method *
	R = α-L-Rha	**30**	98	oil	- 36.3 °	A, B, C, D
	α-D-GlcNAc	**31**	95	147.8 -148.2	+121.1 °	A, B, C, D
	β-D-GlcNAc	**32**	97	175.8 -177.4	- 13.6 °	A, B, C, D
	X = NH_2	**33**	91	93.1 - 94.0	+132.9 °	A, B, C, D
	COOH	**34**	81	decomp. 110	+ 88.9 °	E, F
	OH	**35**	94	oil	+123.8 °	-
 diastereomeric mixture		**36**	48	amorphous solid	+ 69.0 °	A, B, C, G

¥ Based on thiol as limiting reagent

§ C=1, H_2O for **30**, **31**, **32**, **33**; C=1, DMSO for **34**, **35**; C=1, CH_3OH for **35**.

* Possible conjugation methods include but are not limited to:

A) Carbodiimide coupling to COOH; B) Glutaraldehyde coupling to NH_2 ;
C) Acryloylation, then polymerization or Michael addition to NH_2 or SH;
D) Addition to cyanogen bromide or epoxide activated supports;
E) Carbodiimide coupling to NH_2 ; F) conversion to hydrazide followed byABCD;
G) Conversion to acyl azide (HNO_3), followed by addition to NH_2 .

Except for the hydrazide **36**, the 3-(alkylthio) propyl glycosides shown in Table 10 were isolated in high yields. The lowered yield for **36** has been assessed by TLC as being due to a reversion of the corresponding thiol **28** to the cyclic *N*-acetyl homocysteine thiolactone **29** during the addition reaction.

The presence of unreacted thiol or disulfide did often complicate isolation procedures. This is particularly true for those bearing a terminal amine or carboxylic acid groups, where ion exchange chromatography was used to isolate the products. It was found that using an excess of allyl-glyco[illegible] during the synthesis allowed easier isolation of the desired product. In this way, consumption of

the thiol and corresponding disulfide, which is either produced as a side reaction or is present in the commercial reagent, could be driven to completion. Thus the only products remaining at the end of the reaction would be the neutral unreacted allyl glycoside and the new sulfide which were easily separable based on their different ionic properties or mobilities on silica gel. The excess allyl glycoside was always completely recovered in a pure form with relative ease.

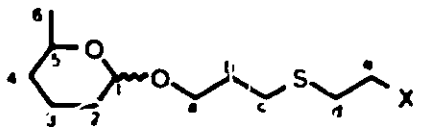
Table 11 ^{13}C-NMR data* for 3-(alkylthio) propyl glycosides

Carbon §	**30**[a]	**31**[a]	**32**[a]	**33**[b]	**34**[a]	**35**[a]	**36**[b]
C_1	99.5	96.7	101.1	99.0	98.8	98.2	97.7
C_2	70.4	53.9	55.3	70.1	69.5	69.5	54.6
C_3	70.6	70.7	74.0	70.0	68.7	68.7	71.8
C_4	71.8	70.5	70.5	71.7	71.1	71.2	70.9
C_5	68.4	72.8	76.9	69.0	68.3	68.3	72.3
C_6	18.0	60.8	60.9	61.9	60.5	60.4	61.4
C_a	64.8	65.4	66.9	67.1	65.6	65.6	67.2
C_b	29.3	29.3	29.5	29.3	29.4	29.6	29.2
C_c	27.7	27.7	27.4	28.3	27.9	28.2	28.8
C_d	33.1	34.5	33.2	39.5	35.0	33.8	31.8
C_e	40.7	41.2	40.5	41.6	26.6	60.9	28.0
C=O(Ac)	-	169.3	168.9	-	-	-	175.1
CH_3(Ac)	-	22.7	23.1	-	-	-	22.7
X	-	-	-	-	173.3 (COOH)	-	170.4(C=O) 168.0(C=O) 22.4 (CH_3)

a) δ (ppm, DMSOd_6); b) δ (ppm, D_2O)

* Assignments are based on 2 D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HECTOR experiments.

§ numbering system



3.3 3-(2-aminoethylthio)propyl glycosides

Of the types of 3-(alkylthio)propyl glycosides described in Table 10, only those with a terminal amine function (**30**, **31**, **32** and **33**) were used to prepare glycoconjugates. 3-(2-aminoethylthio)propyl glycosides have proven to be versatile precursors to different types of glycoconjugates. The terminal amine's nucleophilicity allows conjugation to a macromolecular carrier or support through a wide range of existing methods (reference 5 and Table 10), while the extended sulfide aglycon provides a spacer which is long, flexible and has the capacity to be modified further by oxidation of the sulfide. This methodology offers advantages over commonly used δ-(methoxycarbonyl)octyl aglycon and acyl azide methodology[101] as it is compatible with carbohydrates having sialic acid, Kdo or uronic acid residues[105] during conjugation procedures.

Because relatively polar reagents and products are encountered in this reaction, isolation and purification of 3-(2-aminoethylthio) propyl glycosides can be troublesome. Careful cation exchange chromatography can be used however to separate neutral unreacted allyl glycosides from unreacted cysteamine, its disulfide and the new glycoside on a relatively large scale. Multigram preparations have been purified on Dowex 50W (100 Mesh) resin (NH_4^+ form) by using a linear gradient in ammonium hydroxide. By this technique, all the above components could often be separated effectively. However, excess disulfide present at the end of the reaction often hampered purification by eluting partially with the desired product. As discussed earlier, using an excess of allyl glycoside eliminates this problem. A representative chromatogram is given below for an incomplete coupling reaction and shows the sequential elution of allyl α-D-galactose, cysteamine, cysteamine disulfide and the desired 3-(2-aminoethylthio)propyl α-D-galactopyranoside. The chromatographic separation was followed by monitoring the change

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[105] a) R. Roy and C.A. Laferrière, *Carbohydr. Res.* *177*, C1 (1988); b) H. Paulsen and F.C. Holtgen, *Tetrahedron Lett.* *32*, 2747 (1991); c)H. Paulsen and P. Matschulat, *Liebigs Ann.*, 487 (1991)

in refractive index and the fractions identified by TLC. A schematic representation of the ion exchange chromatography apparatus used is given below.

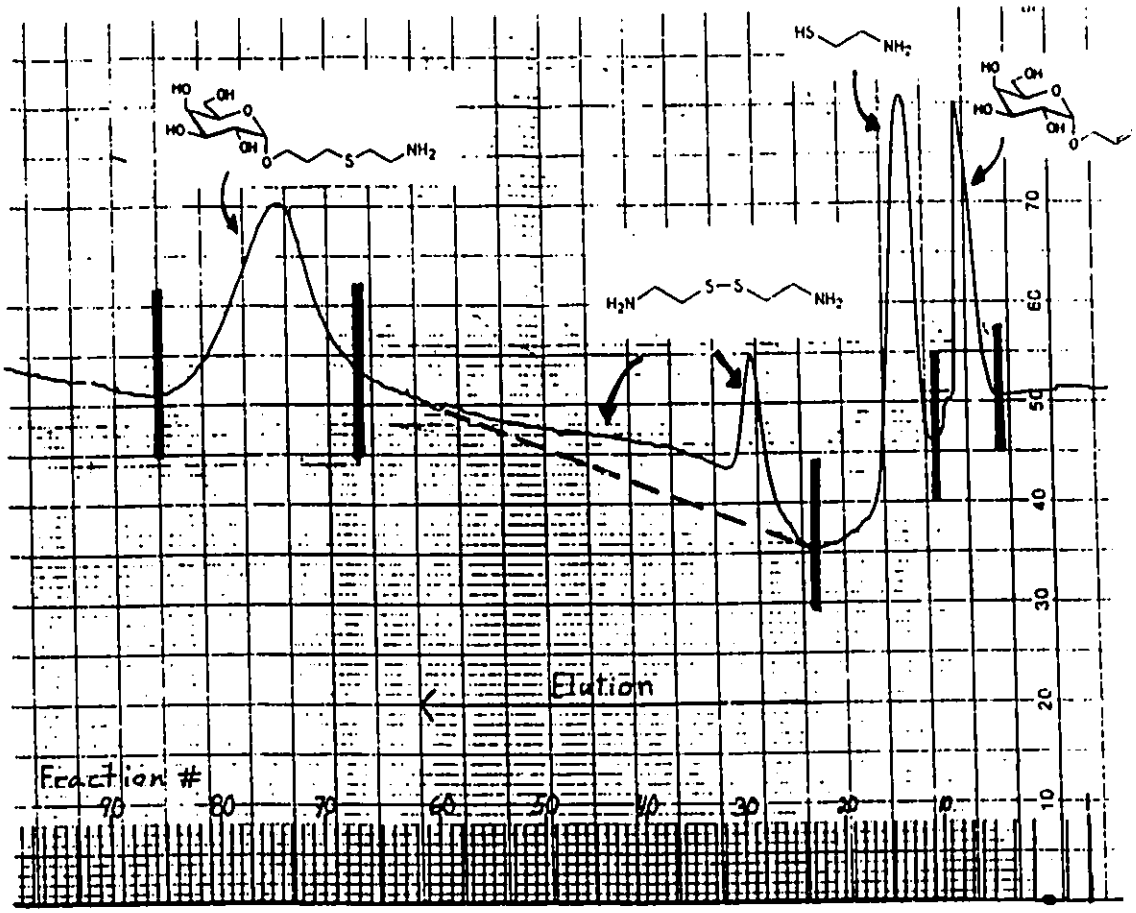


Figure 10 Cation (NH_4^+) exchange chromatogram for a crude product mixture from a cysteamine addition reaction to allyl α-D-Gal. 0.68g of product mixture was eluted from a 2.5 x 60 cm column of Dowex 50w (100 mesh) with an increasing linear gradient of NH_4OH. Products were identified by TLC alongside authentic samples.

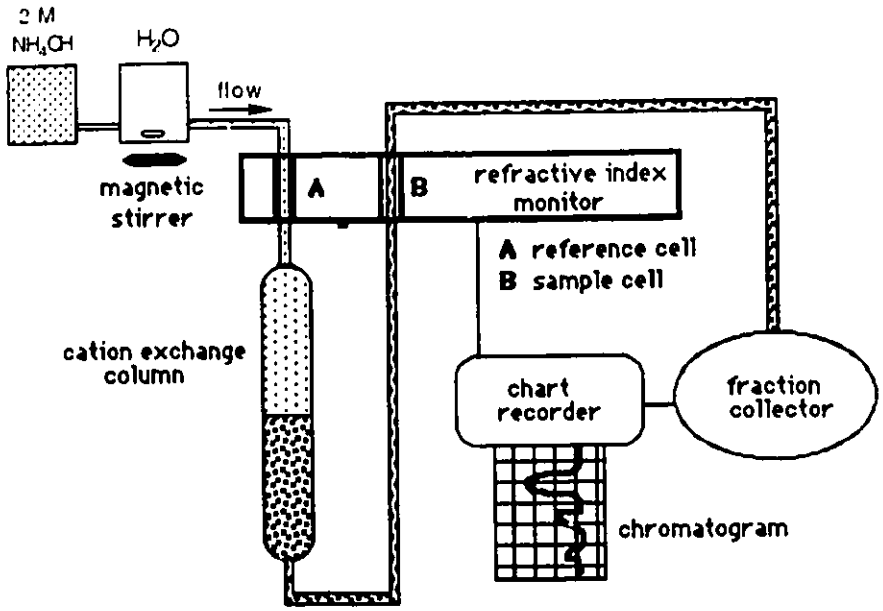


figure 11 Schematic representation of cation exchange chromatography apparatus for 3-(2-aminoethylthio)propyl glycoside purification.

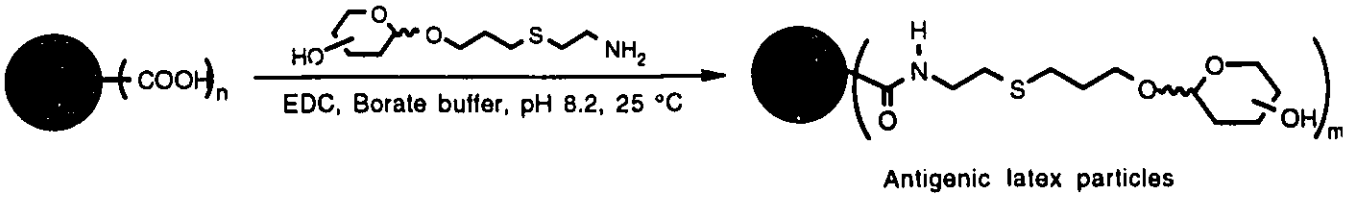
These carbohydrate derivatives were synthesized with the primary objective of preparing N-acryloylated analogues as comonomers for a new glycopolymer synthesis strategy (Chapter 4). To further substantiate the potential usefulness of 3-(2-aminoethylthio) propyl glycosides, water soluble carbodiimide coupling to carboxylated latex particles and synthetic polymers was performed.

a) Antigenic agglutination beads.

An important and commonly used immunodiagnostic assay methodology involves the agglutination of antigen laden microspheres by complementary antibodies present in body fluids. Although not as sensitive as other methods such as ELISA, this type of assay has the advantage that results are obtained very quickly and are detectable by simple visual observation. Many assays employing this technique are distributed commercially.

Antigenic latex beads were prepared for three different carbohydrate haptens and tested against various lectins and

antibodies. Glycosides **30**, **31** and **32** were coupled to carboxylated latex beads (Estapor, Rhône-Poulenc, 0.667 μm) in borate buffer pH 8.2 using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC). Sugar incorporation was determined at 2.8% by weight by the phenol-sulfuric acid method of Dubois[79] for the rhamnosylated beads and are assumed to be similar for the *N*-acetylglucosamine laden particles which were simultaneously prepared by the same procedure.



Antigenic latex particles

Scheme 14 Antigenic aglutination particles by heterogeneous carbodiimide facilitated amide formatior

Rhamnosylated beads agglutinated the Ricinus communis lectin and rabbit antibodies raised against pneumococcal type 23 (Pn23) capsular polysaccharides[106] but not WGA or affinity purified rabbit antibodies to the group-specific carbohydrate antigen of the β-hemolytic Group A streptococcal pyogenes[107]. Group A streptococcal antibodies did agglutinate β-D-*N*-acetylglucosamine conjugated beads while beads with the α anomer agglutinated WGA. Agglutination of WGA with β-D-*N*-acetylglucosamine covered beads was not attempted but should work in theory if one considers the glycopolymer immunodiffusion results described in Table 9. As little as 2 μg of lectin and antibody were sufficient to agglutinate 50 μg samples of antigenic beads in all trials performed.

In principle, 3-(2-aminoethyithio)propyl glycosides could be

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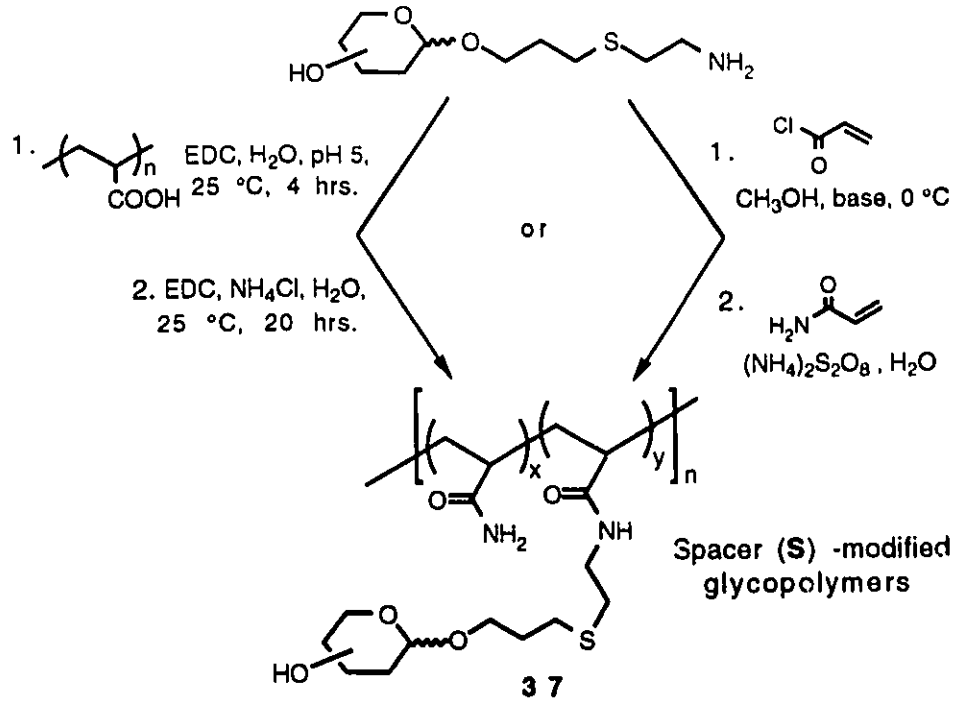
106 C. Jones, *Carbohydr. Res. 139* , 75 (1985); M. Heidelberger and W. Nimmich, *Immunochem. 13* , 67 (1976).

107 K.M. Knigge, J.L. Bahh, J.R. Sirca, K. Ancell, T.G. Bloomster and B.A. Marchlewicz, *J. Clin. Microbiol. 20* , 735 (1984).

immobilised to Eupergit C or a great variety of other commercially available activated supports for uses in affinity chromatography. This type of derivatization was not attempted in this work, however. The recognition of these possibilities only demonstrate the potential usefulness of 3-(2-aminoethylthio)propyl glycosides.

b) Antigenic, water-soluble glycopolymers.

3-(2-aminoethylthio)propyl glycosides were also used to prepare synthetic water-soluble glycopolymer antigens. These glycopolymers differ from the first glycopolymers introduced in that a long (≈ 11Å) spacer now separates the carbohydrate hapten from the polymer carrier. Two different synthetic approaches were investigated. In the first method, 3-(2-aminoethylthio)propyl glycosides were covalently attached to commercial polyacrylic acid polymers of known different molecular weights, through EDC activated amide formation[32]. In the second approach, the terminal amine of the glycosides in question were acylated with acryloyl chloride to give *N*-substituted glycoside acrylamides which could then be copolymerized to give glycopolymers identical to those of the first approach. This second method will be discussed in detail in the following chapter.



3-(2-N-acrylamidoethylthio)propyl glycoside copolyacrylamides

"glycoside- S -CPA"

Scheme 15 Synthetic strategies towards spacer-modified polyacrylamide glycopolymers

The transformation of polyacrylic acid to polyacrylamide glycopolymers was performed to further substantiate the usefulness of 3-(2-aminoethylthio)propyl glycosides and to give access to glycopolymers of different known molecular weights. The glycopolymers so produced could then be used as calibration standards in molecular weight determination and to investigate the effect of molecular weight on antigenicity of glycopolymers in general. With the 3-(2-aminoethylthio)propyl α-L-rhamnopyranoside **30**, water soluble acrylamide glycopolymers were prepared by EDC mediated amide formation with secondary polyacrylic acids of 50, 90, 150 and 200 kDa molecular weight averages. These polyacrylic acid standards were converted to polyacrylamide glycopolymers having a 10% by mole hapten load. The transformation was carried out in a two step sequence. Polyacrylic acids were first activated with 15 mole% of EDC then mixed with 10 mole% of **30** for an

appropriate length of time. In the second step, an excess of EDC followed by an excess of ammonium chloride was added to transform the remaining 90 mole % of carboxyl groups to amides, thus completing the transformation to polyacrylamides. For the 200 kDa standard, a commercial copoly (acrylamide/acrylic acid) polymer having 10% by mole of free carboxyl was used and so did not require treatment with ammonium chloride. All reactions were performed simultaneously in an identical manner with the pH controlled at or near pH 5. The reactions were followed by monitoring changes in pH during conjugation. Approximately 4 hours at room temperature were required to conjugate the glycosides. Capping the remaining carboxylates with ammonium chloride was performed overnight to ensure completeness. The final glycopolymers were isolated as spongy, white solids after exhaustive dialysis and freeze-drying. The results of these conjugations are given in Table 12.

Table 12 Polyacrylamide glycopolymer standards

Glycopolymer	Polyacrylic acid molecular weight (kDa)	mol % of rhamnose incorporation *	% recovered glypolymer mass †
38	50[a]	9.8	111
39	90[a]	11.4	106
40	150[a]	9.8	117
41	200[b]	3.4	56

* average value (± 0.5%) from ^{1}H-NMR and elemental analysis for $(C_3H_5NO)_x(C_{14}H_{25}NOS)_y \cdot nH_2O$. Values were also corroborated by phenol/H_2SO_4 analysis[79].
† Greater than 100% recovered mass yields (based on x=9 and y=1, see scheme 15) are due to retained water of hydration after lyophilization.
a) Purchased as 25% by weight aqueous solution.
b) Purchased as a solid with 10% free carboxyls.

Glycopolymers prepared from 50, 90 and 150 kDa polyacrylic acid standards were shown to have the desired 10% by mole hapten incorporation. However, only 3.4% by mole of the rhamnoside **30**

could be incorporated into the 200 kDa polymer characterized by the supplier as containing 10% free carboxyls. A second trial performed with this polymer and methyl amine as the amine to be coupled gave similar incorporation and isolation yield results. This suggests that low molecular weight species were present in the commercial sample and that this fraction may have contained most of the carboxyl containing residues making up the difference in carboxyl content claimed by the supplier. The slight deviations from the expected 10% hapten incorporation found in the glycopolymers **38**, **39** and **40** are probably due to inexactitudes in the commercial sample concentrations. The polyacrylic acid samples used to prepare **38**, **39** and **40** were purchased as 25% by weight aqueous solutions.

The α-L-Rha-**S**-CPA glycopolymers prepared above were assayed for antigenicity against Pn23 antibody serum by double radial immunodiffusion. Each glycopolymer, including **41**, with only a 3.4 mole% rhamnose content, were able to precipitate an antibody population in the serum. Glycopolymer **41** seemed to give weaker precipitin bands than the other three glycopolymers having a 10% by mole content of rhamnosyl residues however.

The immunodiffusion results with these and glycopolymer **19** indicate that a considerable antibody population in the sera was directed solely against simple α-L-rhamnosyl residues and demonstrates the importance of the immunodominant rhamnose side-chains within the Pn23 cell surface polysaccharide[108]. The glycopolymers were simultaneously assayed in one experiment. As expected, decreasing diffusion rates through the agarose gel was observed, as judged by the intermediate distances of precipitin bands formed, for glycopolymers increasing in average molecular weight. A representation, drawn to scale, has been reproduced below to show these observations. A photographic record of the experiment was not possible at the time, and interference from unprecipitated

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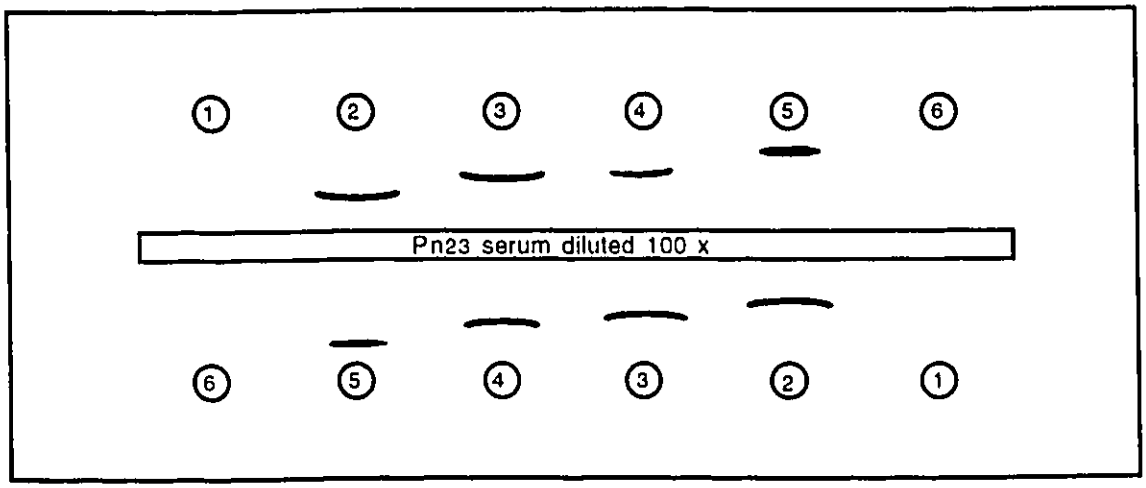
[108] For a review on cross-reacting rhamnose containing Pneumococci and Klebsella see: M. Heidelberger and W. Nimmich, *Immunochem.*, *13* , 67 (1976).

protein material which could not be washed away made permanent staining of the preciptin bands unsuitable.



Respective wells filled with [Ag]=2mg/ml
1: allyl α-L-Rha copolyacrylamide **19**
2: 50 kDa α-L-Rha- **S** -CPA **38**
3: 90 kDa α-L-Rha- **S** -CPA **39**
4: 150 kDa α-L-Rha- **S** -CPA **40**
5: 200 kDa α-L-Rha- **S** -CPA **41**
6: β-D-GlcNAc- **S** -CPA (negative control)

Figure 12 Representation of agarose gel immunodiffusion assay of Pn23 antibody serum against α-L-rhamnose glycopolymers.

3.5 Experimental

DL-2-N-Acetyl-4-mercaptobutanoylhydrazide **28**

DL-*N*-acetyl homocysteine thiolactone (5.80 g, 36.3 mmol, Aldrich) and 1.95 mL (40.0 mmol) of hydrazine monohydrate (Aldrich) were stirred overnight in 50 mL EtOH at 25°C. Evaporation under reduced pressure gave a quantitative yield of **28** pure by TLC (Rf = 0.42, 4/1 $CHCl_3$/MeOH). Recrystallization from ethanol gave a white crystalline powder mp = 128.3 - 129.2 °C. CI-MS (ether) gave m/e (ion, relative intensity): 383 (2M + 1, 11) 192 (M + 1, 100), 160 (M +

1 - N_2H_4, 41). ^{1}H-NMR δ ($DMSOd_6$): 9.11 (S, 1H, NH-N), 7.97 (d, 1H, J=7.5 Hz, NHAc), 4.28 (mult, 1H, CHN), 4.20 (s, 2H, N-NH_2), 2.37 (mult, 3H, SCH_2 + SH), 1.80 (s, 3H, CH_3), 1.78 (mult, 2H, C-CH_2-C). ^{13}C-NMR δ ($DMSOd_6$): 170.3, 169.1 (carbonyls), 50.2 (C-2), 36.7 (C-3), 22.6 (CH_3), 20.5 (C-4). This product appears stable for extended periods only when stored refrigerated.

3-(-2-Aminoethylthio)propyl α-L-rhamnopyranoside **30**

Compound**14** (3.00 g, 14.7 mmol) and 1.42 g (12.5 mmol) of cysteamine hydrochloride were dissolved in 20 mL of deoxygenated water and irradiated (λ = 254 nm) under a nitrogen atmosphere for 3 hrs. TLC (3/2/1, EtOAc/HOAc/H_2O) showed a clean and complete consumption of the thiol to the desired product (Rf = 0.39). No disulfide was observed. The product was isolated by cation exchange chromatography using Dowex 50W (100 mesh, NH_4^+ form). The product was applied to the column (65 x 2.5 cm) and eluted first with distilled water to give unreacted **14**, followed by elution with a linear gradient (0 to 1 M, 2.5L) in NH_4OH to give the desired product. Product fractions were pooled and concentrated under reduced pressure to remove ammonia and diminish the volume prior to lyophilization. Multiple lyophilizations from water gave 3.45 g (98%) of **30** as a sticky white amorphous solid, pure by TLC, ^{1}H and ^{13}C-NMR. The material behaved as though it was hygroscopic turning into a clear, glassy solid upon exposure to air and resisted crystallization from a variety of solvent systems.
$[\alpha]_D$ = -36.3° (c = 1.07, H_2O) CI-MS (ether) gave m/e (ion, relative intensity): 282 (M+1, 100), 147 (M - aglycon, 13) 136 ($HO(CH_2)_3S(CH_2)_2NH_3^+$, 50). ^{1}H-NMR δ ($DMSOd_6$): 4.48 (d, 1H, J_{12} = 1.5 Hz, H_1), 3.56 (mult, 2H), 3.34 (mult., 3H), 3.12 (t, 1H, J=9.5 Hz), 2.68 (mult,2H, H_e), 2.49 (mult, 4H, H_c + H_d), 1.70 (mult, 2H, H_b), 1.08 (d, 3H, $J_{5,6}$ = 1.5 Hz, CH_3). Other physical data can be found in tables 9 and 10.

3-(2-Aminoethylthio)propyl 2-acetamido-2-deoxy-α-D-glucopyranoside **31**

As a typical reaction, 1.20 g (4.60 mmol) of **15** and 0.44 g (3.87 mmol) of cysteamine hydrochloride were dissolved in 9.0 mL of deoxygenated water and irradiated as above for 1.75 hrs. TLC (3/2/1 EtOAc/HOAc/H_2O) showed a clean and complete consumption of the thiol to the desired glycoside. Cation exchange chromatography and solvent removal as above gave 1.30 g (95% based on the thiol) of pure **31** as a white powder which could be crystallized from EtOH/Et_2O. mp = 147.8 - 148.2°C, $[\alpha]_D$ = +121.1° (c = 1.01 H_2O) {lit.[74] mp = 149 - 151°C, $[\alpha]_D$ = +126.0°} Analytical data: % calculated for $C_{13}H_{26}N_2O_6S{\cdot}0.75H_2O$: C, 44.37; H, 7.68; N, 7.96; S, 9.11. Found: C, 44.47; H, 7.42; N, 8.02; S, 9.48. CI-MS (ether) gave m/e (ion, relative intensity): 339 (M+1 - O-aglycon, 30), 136 ($HO(CH_2)_2S(CH_2)_2NH_3^+$, 9). ^{1}H-NMR δ (DMSO$d_6$): 4.63 (d, 1H, J_{12}= 3.6 Hz), 3.60 (mult, 3H), 3.37 (mult, 7H), 3.12 (dd, 1H, $J_{a,b}$=8.7, $J_{a,a'}$=9.6 Hz, $H_{a'}$), 2.73 (t, 2H, $J_{d,e}$=7.2 Hz, H_d), 2.55 (mult, 4H, H_c+H_e), 1.83 (s, 3H, CH_3), 1.74 (mult, 2H, H_b). ^{13}C-NMR data are given in Table 10.

3-(2-Aminoethylthio)propyl 2-acetamido-2-deoxy-β-D-glucopyranoside **32**

Compound **16** (1.77 g, 6.78 mmol) and 0.76 g (6.70 mmol) of cysteamine hydrochloride were dissolved in 15 mL of deoxygenated water, then processed as above. 2.20 g of white, solid **32**, pure by TLC (Rf = 0.34, 3/2/1 EtOAc/HOAc/H_2O) and NMR was obtained. Compound **32** could be crystallized from abs. EtOH. mp = 175.8 - 177.4 °C, $[\alpha]_D$ = -13.6° (c = 0.83 H_2O) {lit.[74] mp = 175 - 177°C, $[\alpha]_D$ = -10.8°}. Analytical data: % calculated for $C_{13}H_{26}N_2O_6S{\cdot}1.5H_2O$: C, 42.70; H, 7.17; N, 7.67; S, 8.77. Found: C, 42.58; H, 7.50; N, 7.45; S, 9.00. CI-MS (ether) gave a spectrum similar to **31**. ^{1}H-NMR δ (D_2O): 4.31 (d, 1H, $J_{1,2}$= 8.4 Hz, H_1), 3.76 (mult, 2H), 3.52 (mult, 4H), 3.35 (mult, 1H), 3.26 (mult, 2H), 2.65 (t, 2H, $J_{d,e}$=7.0Hz, H_d), 2.48 (t, 2H, $J_{b,c}$=6.5Hz, H_c), 2.41 (mult, 2H, H_e), 1.86 (s, 3H, CH_3), 1.65 (mult, H_b). ^{13}C-NMR data are given in Table 10.

3-(2-Aminoethylthio)propyl α-D-galactopyranoside **33**

Compound **13** (3.82 g, 17.4 mmol) and 1.86 (16.4 mmol) of cysteamine hydrochloride were dissolved in 50 mol of deoxygenated water. The reaction was processed as above. 4.30 g (90%) of pure **33** was obtained after lyophilization. The product could be crystallized from $EtOH/Et_2O$. mp = 93.1-94.0°C, $[\alpha]_D$ = +132.9° (c = 2.5 H_2O) {lit.[74] mp = 88-89°C, $[\alpha]_D$ = +136.6°}, Rf = 0.24 (3/2/1 EtOAc/HOAc/H_2O). ^{1}H-NMR δ (D_2O): 4.67 (d, 1H, $J_{1,2}$= 2.8 Hz, H_1), 3.69 (mult, 2H), 3.55 (mult, 3H), 3.46 (mult, 2H), 3.32 (mult,1H), 2.82 (t, 2H, $J_{d,e}$=6.3 Hz, H_d), 2.53 (t, 2H, $J_{b,c}$=6.6 Hz, H_c), 2.42 (t, 2H, $J_{d,e}$=6.3 Hz, H_e), 1.65 (mult, 2H, H_b). ^{13}C-NMR data are given in Table 10.

3-(2-Carboxyethylthio)propyl α-D-galactopyranoside **34**

Compound **13** (0.41 g, 1.86 mmol) was dissolved in 40 mL of deoxygenated water. 3-Mercaptopropionic acid (160 µL, 1.86 mmol, Aldrich) was then added, and the solution irradiated as above for a total of 6 hrs. after which TLC showed complete consumption of **13** and the thiol to form **34** (Rf=0.40, 1/1 acetone/water). The reaction solution was then loaded on an anion exchange resin (Amberlite IRA400 (HO^-)) column (2 x 38 cm) which was eluted with water followed by an increasing acetic acid gradient. Fractions containing the desired product were pooled and concentrated under reduced pressure. Multiple coevaporation with ethanol and subsequent drying under vaccum gave **34** as a white solid pure by TLC and NMR. mp=decomposes at or near 110°C, $[\alpha]_D$ = +88.9° (c=1.10 DMSO). ^{1}H-NMR δ ($DMSOd_6$): 4.62 (d, 1H, $J_{1,2}$=3.1 Hz, H_1), 3.63 - 3.27 (mult, 8H), 2.65 (t, 2H, $J_{d,e}$=7.3 Hz, H_d), 2.57 (t, 2H, $J_{b,c}$=7.4 Hz, H_c), 2.45 (t, 2H, $J_{d,e}$=7.3 Hz, H_e), 1.75 (mult, 2H, H_b). ^{13}C-NMR data are given in Table 10.

3-(2-Hydroxyethylthio)propyl α-D-galactopyranoside **35**

Compound **13** (0.40 g, 1.82 mmol) and 130µl (1 equiv.) of 2-mercaptoethanol were dissolved in 6.0 mL of deoxygenated water and irradiated as above, for 1.5 hrs. The resulting product solution

was coevaporated with ethanol to an oil, then purified by silica gel chromatography with 4/1/1 $CHCl_3$/acetone/MeOH to remove traces of unreacted **13** and disulfide. Evaporation of the pooled, pure product fractions yielded 0.46 g (85%) of **35** as an oil which failed all crystallization attempts. $[\alpha]_D$ = +123.8° (c = 1.45, MeOH). CI-MS (ether) gave m/e (ion, relative intensity): 299 (M+1, 5), 281 (M+1 - H_2O, 2.5), 137 ($HO(CH_2)_3S(CH_2)_2OH$ + H, 50) 119 ($HO(CH_2)_3SCH_2CH_2^+$, 15). ^{1}H-NMR δ ($DMSOd_6$): 4.63 (d,1H, $J_{1,2}$=4.2 Hz, H_1), 3.70 (dd,1H, $J_{3,4}$=3, $J_{4,5}$<1 Hz, H_4), 3.64 (dt,1H, $J_{a,b}$=6.4, $J_{a,a'}$=10.0 Hz, diastereotopic H_a), 3.35-3.68 (mult, 5H), 3.51 (t, 2H, $J_{d,e}$=7.0 Hz, H_e), 2.59 (t, 2H, $J_{b,c}$=7.4 Hz, H_c), 2.56 (t, 2H, $J_{d,e}$=7.0 Hz, H_d), 1.77 (mult, 2H, H_b). ^{13}C-NMR data are given in Table 10.

3-[((R/S)3-N-Acetyl-3-hydrazinocarbonyl)-propylthio]propyl 2-N-acetamido-2-deoxy α-D-glucopyranoside **36**

Compound **15** (0.35 g, 1.34 mmol) and 0.23 g (1.20 mmol) of **28** were dissolved in 3.5 mL of deoxygenated water and irradiated for 24 hrs. The product solution was coevaporated to dryness with EtOH, dissolved in a minimum of MeOH, then applied to a silica gel column. Elution with 4/3/2 acetone/$CHCl_3$/MeOH) gave 0.28 g (48%) of solid **36**, pure by TLC (Rf = 0.10 4/1 $CHCl_3$/MeOH, Rf = 0.42, 2/1/1 acetone/$CHCl_3$/MeOH). This material failed all crystallization attempts. $[\alpha]_D$ = +69.0° (c = 1.21 DMSO). CI-MS (ether) gave m/e (ion, relative intensity): 451 (M - 1, 25), 409 (M-NHAc, 8), 393 (M-$CONHNH_2$, 6), 262 (GlcNAc-O-$CH_2CH_2^+$, 42), 204 (M-O-aglycon, 95). ^{1}H-NMR δ ($DMSOd_6$): 5.01 (d,1H, $J_{1,2}$=4.4 Hz, H_1), 3.62 (mult, 3H), 3.48 (mult, 3H), 3.35 (mult, 3H), 3.13 (mult,1H, H_5), 2.57 (t, 2H, $J_{b,c}$=7.1 Hz, H_c), 2.45 (t, 2H, $J_{d,e}$=7.7 Hz, H_d), 1.92 (s, 3H, CH_3), 1.85 (s, 3H, CH_3), 1.84 (mult, 2H, H_e), 1.74 (mult, 2H, H_b). ^{13}C-NMR data are given in Table 10.

Antigenic latex agglutination bead preparation

Carboxylated latex particles (Estapor, Rhône-Poulenc, Paris, 0.667μm,10% by weight solution in water) were covalently coated

with 3-(2-aminoethylthio) propyl glycosides **30**, **31** and **32** following the general procedure outlined. The latex beads were sonicated for 30 minutes prior to use. 200µl suspended particle solution was mixed with 200µl of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide solution 15 mg/mL for 30 minutes (pH 5.2). This suspension was then diluted to 4.0 mL with a borate buffered saline solution (0.14 M NaCl, 0.01 M borate, pH 8.2) and 20 µmol of hapten were then added. The solution was stirred for 2.5 hrs. at room temperature. Unreacted glycosides were removed by centrifugation and washing with 4 x1mL volumes of 1% BSA in PBS (pH 7.2) with resuspension of the beads by vigorous agitation from a high speed vortex mixer during each wash. The beads were stored in 1 mL volumes of 1% BSA in PBS to give a stock bead solution of 20 mg/mL.

Sugar content of agglutination beads

Incorporated sugar densities were determined from the rhamnosylated bead preparation. 200µl of a sonicated rhamnosylated bead suspension were centrifuged and washed twice with distilled water with resuspension and recentrifugation between washings. The beads were diluted to 1.00mL with water. Aliquots (200µl, 800 µg of beads) were diluted with 200µl of 5% aqueous phenol, agitated and finally treated with 1.00 mL of concentrated H_2SO_4 while vortexing. Absorbance of the resulting ye!lowish-brown solution was measured at 490 nm after centrifugation of the beads. Unconjugated beads were treated similarily and used as blank. A calibration curve was constructed with L-rhamnose. The sugar density was determined as 22 µg of Rhamnose per 800 µg of beads (2.8% by weight).

Agglutination assay with glycosylated latex particles

Stepwise volumes (1 to 25 µl) of lectins (WGA, *Ricinus communis*, 1 mg/mL PBS) and affinity purified rabbit antibodies to the group-specific carbohydrate antigen of the β -hemolytic Group A streptococcal pyogenes (Biomega, Laval, Quebec; 1 mg/mL PBS) were

added to 100 μl aliquots of glycosylated agglutination beads (1 and 2 mg/mL 1% BSA in PBS) and mixed. Volumes (20 μl) of10 and 25 fold dilutions of rabbit antisera raised against pneumococcal type 23 (Pn23) capsular polysaccharide (Statens Serum Institut, Denmark) were also assayed in the same manner as above. Assays were performed both in polystyrene ELISA plates (Linbro, Titertek, VA, USA) and flat glass plates. Unconjugated latex beads were used as secondary controls. Agglutination was determined visually in a qualitative fashion. Hapten specific agglutination was observed in all assays at all concentrations investigated. WGA was agglutinated by α-D-GlcNAc laden beads but not with rhamnosylated or unconjugated beads. Both the *Ricinus communis* lectin and Pn23 antibody sera were agglutinated by the rhamnosylated beads only. However, agglutination with Pn23 antibody sera appeared to be less intense. Group A streptococcal antibodies were agglutinated only by β-D-GlcNAc laden beads.

Acrylamide glycopolymers prepared from polyacrylic acid standards **38, 39, 40, 41**

Glycopolymers **38, 39, 40:** Secondary polyacrylic acid standards having average molecular weights of 50, 90 and 150 kDa (Polysciences) were purchased as solutions containing 25% solids in water. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.0 mL of a 20 mg/mL solution, 21 mmol,15 mol%, EDC, Aldrich) was added to 0.40g of each aqueous polyacrylic acid sample (1.39 mmol COOH). After stirring for 30 minutes, 1.0 mL of a 40mg/mL solution of the rhamnoside **30** (0.14 mmol,10 mol%) was added and the pH adjusted to 4.75 . The pH was kept between 4.8 and 5.0 with periodic additions of 0.3 M HCl for 4 hours after which the pH stopped rising. EDC (360 mg, 1.88 mmol, 1.5 equiv.) was then added and left to react for 30 minutes at pH 4.75 after which 5g (75 mol excess) of NH_4Cl were added and left to react overnight. The glycopolymers were then dialysed exhaustively against distilled water and freeze dried to give white, spongy solids.

Glycopolymer **41** was prepared using a copoly(acrylamide/acrylic acid) polymer of 200 kDa containing 10% COOH (Polysciences). 100 mg of this polymer and 40 mg of EDC were dissolved in 5.0 mL of water and stirred for 30 minutes. Compound **30** (50 mg) was then added and the pH adjusted to 4.8. This solution was left to react overnight after which it was dialysed and lyophilized as above.

All glycopolymers were found to be antigenic toward Pn23 antibody sera by double radial immunodiffusion. Glycoside incorporation for each glycopolymer was determined by ^{1}H-NMR, elemental analysis as well as by phenol/sulfuric acid assay of Dubois *et al.* [79]. All assays were consistent with each other. Results are given in Table 12.

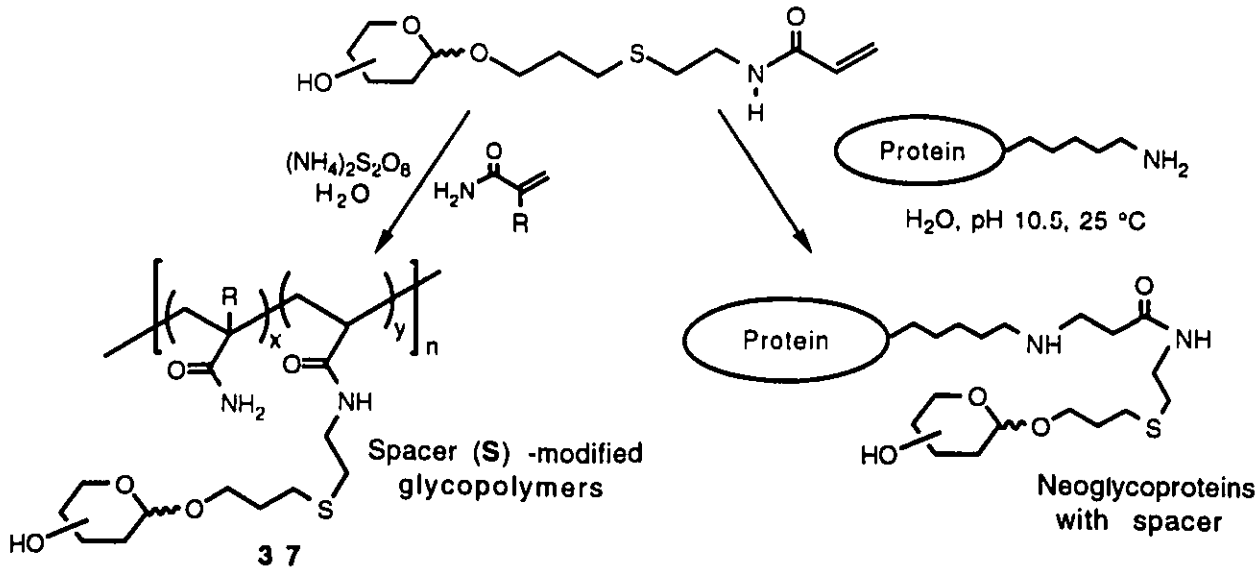
4. 3-(2-*N*-Acrylamidoethylthio)propyl glycosides

Synthetic glycopolymer antigens have proven to be useful tools in the isolation and study of carbohydrate binding proteins. As discussed previously, their increasing popularity since their recent introduction is due to advantages they have over more traditional glycoconjugates. The largest body of reported work with glycopolymers has been related to the use of those derived from allyl glycosides and acrylamide. However, such systems do have some shortcomings such as a very short spacer separating the haptens and the carrier, and the fact that block type copolymers are produced in which a monomer incorporation is difficult to control. For these reasons, a new strategy was developed to simultaneously remedy both of these problems while taking advantage of 3-(2-*N*-acrylamidoethylthio)propyl glycosides produced easily from allyl glycosides.

Through a quick and mild acylating procedure with acryloyl chloride, these last amines can be effectively converted to 3-(2-*N*-acrylamidoethylthio)propyl glycosides. Because these glycosides are basically *N*-substituted acrylamides, their reactivity during polymerization is essentially identical to simple acrylamide. Thus, copolymerization of these leads to more desirable, randomly alternating copolymers in which glycoside incorporation can be controlled at will.

In addition to being superior comonomers for glycopolymer preparations, these glycosides can be conjugated to proteins by the Michael addition of nucleophilic amine or thiol groups to the acrylamide terminus. Thus, spacer modified glycopolymers and neoglycoproteins can now be accessed with ease from the same glycoside derivatives thus greatly simplifying the conjugation methodology.

Scheme 16 Spacer-modified glycoconjugates from 3-(2-*N*-acrylamidoethylthio)propyl glycosides

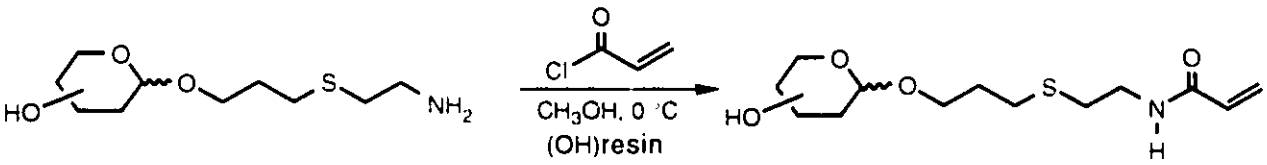


R = H : 3-(2-N-acrylamidoethylthio)propyl glycoside copolyacrylamides
"glycoside- **S** -CPA"

R = CH_3 : 3-(2-N-acrylamidoethylthio)propyl glycoside copolymethacrylamides
"glycoside- **S** -CPMA"

4.1 Preparation and characterization

3-(2-*N*-Acrylamidoethylthio)propyl glycosides of α- and β–D-*N*-acetylglucosamines, α-D-galactose and α-L-rhamnose were synthesized in a straightforward manner from the parent amines in high yields under mild conditions. The terminal amines group of the 3-(2-aminoethylthio) propyl glycosides were selectively acylated by a slow dropwise addition of acryloyl chloride to cooled (0°C) methanolic solutions of each amine, in quantitative yields. The use of anion exchange resin ($^-$OH form) proved to be effective as both a base and a chloride and acrylate ion sponge. Thus, filtration of the resin once the reaction is complete leaves a salt and acid free solution. Brief treatment with (H^+) resin to ensure neutrality followed by evaporation gave essentially pure *N*-acryloylated glycosides.



Scheme 17 *N*-Acryloylation of 3-(2-aminoethylthio)propyl glycosides

The presence of moisture in the reaction solution does not hamper the reaction but seems to help facilitate the ion exchange process. This last point is noteworthy as the amine precursors to the title compounds are isolated from aqueous solutions and may thus contain residual water. In fact the best results have been achieved when resin, still wet from washing, was employed.

The title compounds are quite stable when pure and stored refrigerated. Heat and light may initiate polymerization, especially when impurities are present. As a precautionary measure, solvent removal should be caried at ≤ 25°C and the pH of the evaporating solution checked when substantially concentrated. Traces of improperly removed acrylic acid formed during the reaction can be removed at this point by simple filtration through a mixed bed of ion exchange resin ($^-OH/H^+$) if necessary.

3-(2-*N*-Acrylamidoethylthio)propyl glycosides synthesized along with physical characteristics are shown below in tables 13 and 14.

Table 13 3-(2-aminoethylthio)propyl glycosides

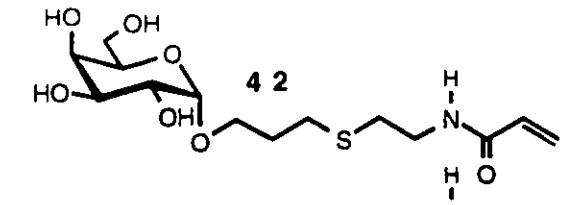
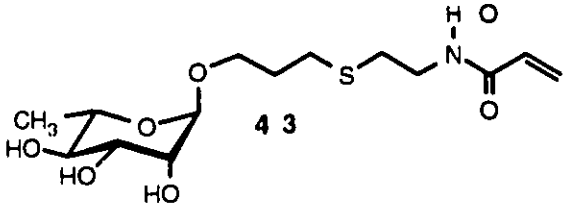
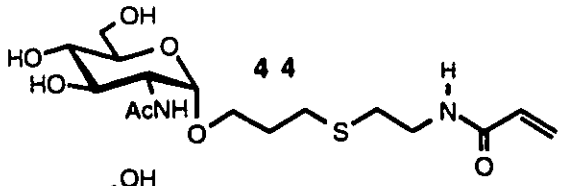
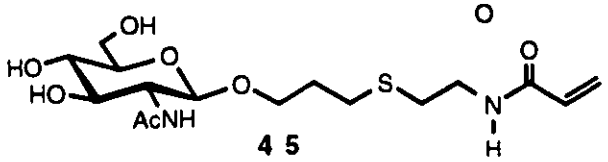
	mp (°C)	$[\alpha]_D$
 **4 2**	104.1 - 106.3 ($EtOH/Et_2O$)	+ 110.0 ° (C=1.2, DMSO)
 **4 3**	Oil	- 27.9 ° (C=1.7, DMSO)
 **4 4**	145.9 - 147.7 (CH_3CN)	+ 98.3 ° (C=1.2, DMSO) + 105.5 ° (C=1.2, H_2O)
 **4 5**	135.5 - 137.1 (CH_3CN)	-17.2 ° (C=0.92, DMSO) - 9.9 ° (C=1.2, H_2O)

Table 14 ^{13}C-NMR chemical shift* data for 3-(2-*N*-acrylamidoethylthio)propyl glycosides

Carbon†	42	43	44	45
C_1	98.8	99.9	96.7	101.1
C_2	68.4	70.5	54.1	55.5
C_3	68.8	70.8	70.6	74.1
C_4	69.6	72.0	70.4	70.6
C_5	71.2	68.5	72.8	76.9
C_6	60.6	18.0	60.8	61.0
C_a	65.5	64.9	65.5	67.0
C_b	29.4	29.2	29.2	29.4
C_c	30.5	30.5	30.5	30.6
C_d	27.8	27.8	27.7	27.5
C_e	38.7	38.7	38.4	38.7
COOH=	164.5	164.6	164.7	164.6
-CH=	131.5	131.5	131.5	131.5
$=CH_2$	125.1	125.2	125.3	125.3
C=O(Ac)	-	-	169.6	169.2
CH_3(Ac)	-	-	22.7	23.1

*Chemical shifts are in ppm for $DMSOd_6$ solutions with $DMSOd_6$ referenced to 39.50 ppm.
†Numbering system used is identical to that described for Table 10.

4.2 Copolymerization with acrylamide

3-(2-*N*-Acrylamidoethylthio)propyl glycosides were prepared for use as monomers in glycopolymer syntheses. This strategy was developed to remedy shortcomings found with glycopolymers prepared directly from allyl glycosides. Glycopolymers prepared with sulfide spacer modified glycosyl acrylamides offer a relatively non-hydrophobic flexible spacer of considerable length (≈11Å), and give randomly alternating copolymers rather than the block type copolymers produced by copolymerizing allyl glycosides. Thus, these glycopolymers are more uniform with respect to overall hapten density and are better suited for evaluating the important effects of interhapten distances during binding interactions with multivalent

receptors[11]. Randomly alternating, atactic copolymers are produced during copolymerization with acrylate comonomers because of the essentially identical reactivity ratios these possess[106]. This also leads to a monomer incorporation ratio within the glycopolymer which is identical to that loaded in the polymerization flask. Thus, unlike allyl glycoside glycopolymers, the sugar densities in the new glycopolymers can be controlled at will. Since our first report[23a,b] of this new methodology for preparing spacer modified glycopolymers, Paulsen and Höffgen[105b] have successfully applied this method to prepare antigenic glycopolymers of the gram-negative bacteria lipid A core region trisaccharide unit L-α-D-Hep(1→3)-L-α-D-Hep(1→5)-α-Kdo.

In addition to acrylamide, other acrylate type comonomers can be used to produce glycopolymers with different properties. The introduction of an effectors as a third comonomer species can also impart desirable physico- or imuno-chemical properties to the glycopolymer. Some of these aspects will be touched upon in later chapters.

Preparing spacer modified glycopolymers by radical copolymerization has several advantages over the functionalization of polyacrylic acid by EDC. By direct copolymerization, the glycopolymer backbone structure can be varied by using different acrylate comonomers to give glycoconjugates having different solution properties. A crosslinking agent such as *N,N'* methylene bisacrylamide can be added during the copolymerization to give antigenic gels useful in affinity chromatography or electrophoresis. The radical polymerizations are much quicker, being performed in minutes compared to a full day for polyacrylic acid amidation. Besides this, the EDC mediated process must be controlled carefully and may give polymers with some ill-defined appendages arising from secondary reactions associated with EDC activated esters[109].

The sulfide spacer modified glycopolymers discussed in this section

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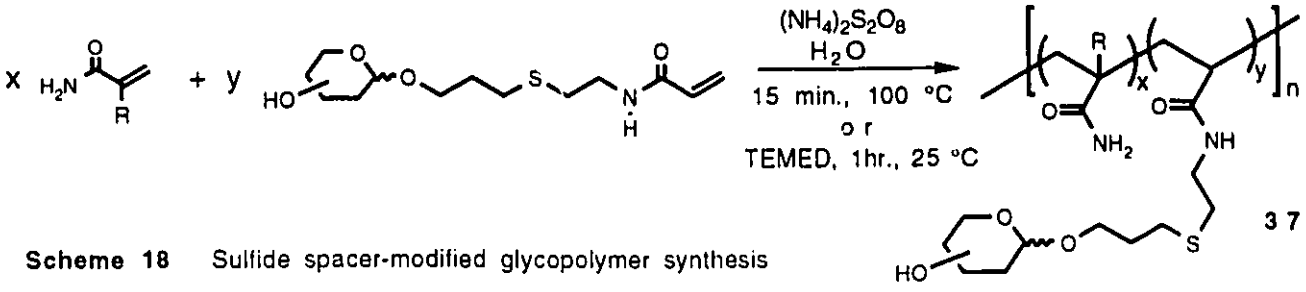
[109] Y. Inoue and K. Nagasawa, *Carbohydr. Res.*, *111*, 113 (1982).

have been prepared in the same fashion as the glycopolymers described earlier. Ammonium persulfate $(NH_4)_2S_2O_8$ was again used as initiator. However, two different approaches at catalyzing the initiation were used. Radical initiating species were generated from $(NH_4)_2S_2O_8$ by heat (100°C) or by catalysis with *N,N,N',N'*-tetramethylethylenediamine (TEMED)[110] at room temperature with identical success. TEMED promoted polymerization are milder but require at least one hour to yield sufficiently high molecular weight polymers. Also, dialysis of the glycopolymers must be performed in a mildly acidic medium (1 mM acetic acid) to completely remove the TEMED which otherwise seems to complex to the glycopolymer.



Scheme 18 Sulfide spacer-modified glycopolymer synthesis

37a R = H : 3-(2-*N*-acrylamidoethylthio)propyl glycoside copolyacrylamides "glycoside- **S** -CPA"

37b R = CH_3 : 3-(2-*N*-acrylamidoethylthio)propyl glycoside copolymethacrylamides "glycoside- **S** -CPMA"

Sulfide spacer-modified copolyacrylamide (glycoside-**S**-CPA) **37a** and copolymethacrylamide (glycoside-**S**-CPMA) **37b** glycopolymers produced by radical polymerization are described in Table 14. Copolymethacrylamide glycopolymers **37b** were prepared in order to provide more hydrophobic antigens which could prove to be superior as coating antigens in ELISA type assays. The increased hydrophobicity of the copolymer backbone should allow for stronger

[110] G. Odian, *Principles of Polymerization* , 2nd Ed. , Wiley Interscience Publications, New York, 1981, p. 327

adsorption of these glycopolymers to polystyrene plates. Moreover, the decreased hydrophilicity of such glycopolymers might force a quicker precipitation of precipitin complexes with antibodies or lectins. Furthermore, a nearly 20% increase in glycopolymer mass can be achieved by using methacrylamide over simple acrylamide without disturbing the hapten loading or the interhapten distances. This advantage could be considered when glycopolymers must be prepared from a limited amount of available antigen monomer.

The glycopolymers were prepared at various times during the course of this work and were prepared following a generalized procedure. Deoxygenated aqueous solutions, concentrated at 1M in total polymerizable acrylate groups were treated with 0.1 to 0.5 mol% of $(NH_4)_2S_2O_8$ then heated for 12 to 15 minutes at 100°C, or left to react at room temperature for one or more hours in the presence of 0.5 mol% of TEMED. The glycopolymers were then dialyzed exhaustively against distilled water and lyophilized to give white spongy solids.

The glycopolymers were characterized by ^{1}H-NMR spectroscopy and elemental analysis for hapten incorporation as well as by phenol/sulfuric acid assay for the rhamnose glycopolymers. Results from each method were consistent with each other. To note, corrections for the signal arising from the methylene unit (H_b) in the centre of the spacer's propyl chain must be made when monomer ratios are determined by ^{1}H-NMR. This signal appears at 1.7 ppm, under the backbone methylene signal (HECTOR). Other than this, ^{1}H-NMR spectra appear as those for the allyl glycoside glycopolymers except for additional signals arising from the spacer chain. As an example, the ^{1}H-NMR spectrum of **52** is given in Figure 13. ^{13}C-NMR spectra of the glycopolymers are relatively uninformative as in the case of allyl glycoside glycopolymers. Except for the backbone methine and methylene signals, all other signals appear as sharp lines at the same positions (± 1 ppm) as in their respective 3-(2-aminoethylthio)propyl glycosides. An example is given in Figure 14. Molecular weights were assessed in a qualitative fashion from their

relative mobilities through agarose gel and are estimated as being between 60 and 120 kDa.

Table 15 Sulfide spacer-modified glycopolymers

Glycopolymer#		Yield* (%)	Monomer Ratio (acrylamide/glycoside)	
			Reaction loading	Polymer incorporation¥
α-L-Rha-**S**-CPA	**46**	51	2.5	2.7
α-L-Rha-**S**-CPMA	**47**	48	2.6	2.8
α-D-GlcNAc-**S**-CPA	**48**	60	4.0	3.9
	49	40	homopolymer	homopolymer
	50	53	3.9	3.9
	51	80	5.0	5.1
β-D-GlcNAc-**S**-CPA	**52**	85	7.0	7.0
	53	26	9.0	9.0
	54	57	25	25.1
	55	55	40	40.3
β-D-GlcNAc-**S**-CPMA	**56**	40	10.0	10.1

CPA = copolyacrylamide, CPMA = copolymethacrylamide.
* Yield after lyophilization for material greater than 12 kDa.
¥ Determined by elemental analysis and/or ^{1}H-NMR. Compounds **45** and **46** have also been assayed by the phenol sulfuric acid method which gave similar results.

Figure 13 ^{1}H-NMR spectrum of β-D-GlcNAc-S-CPA 52

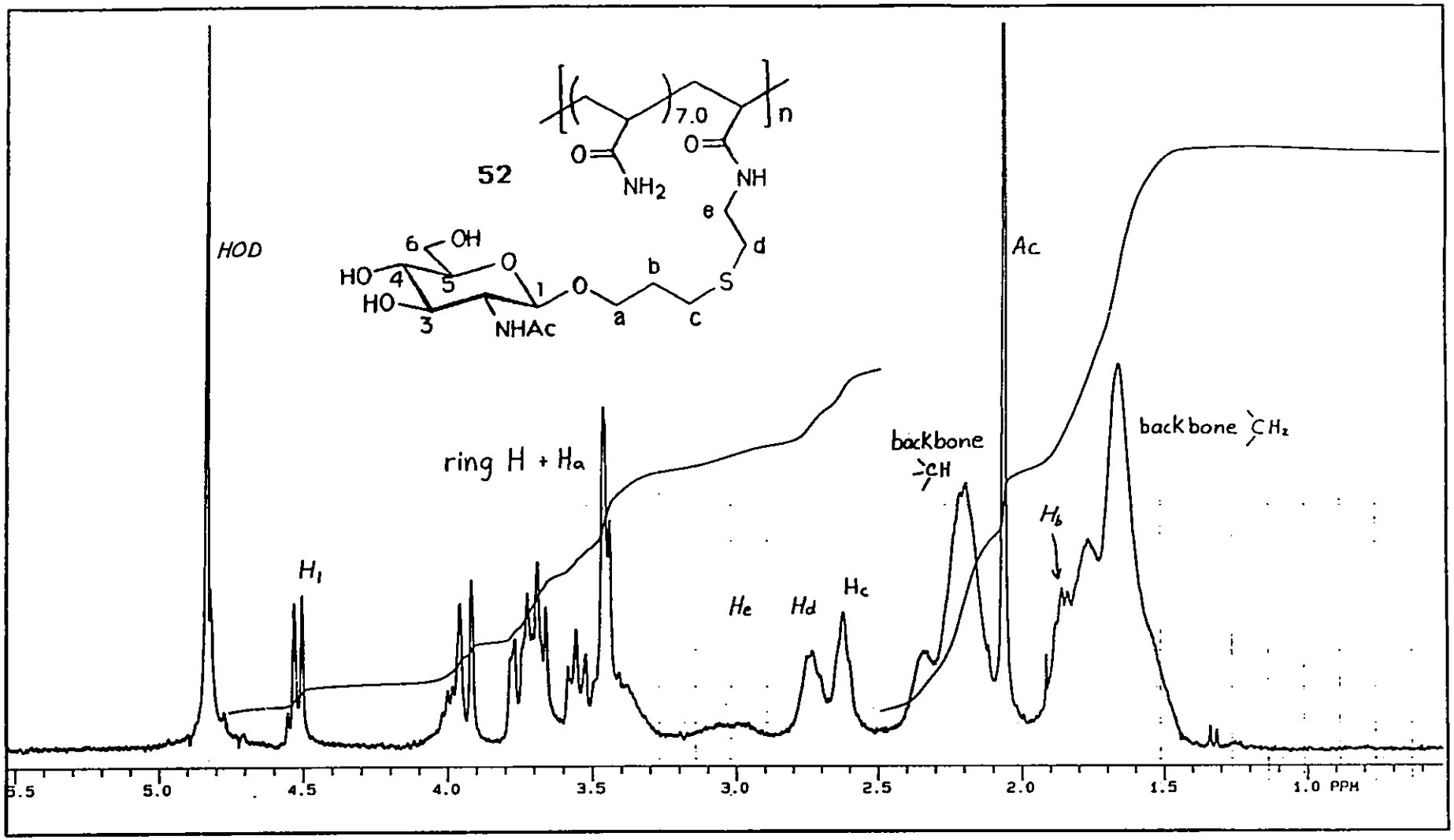
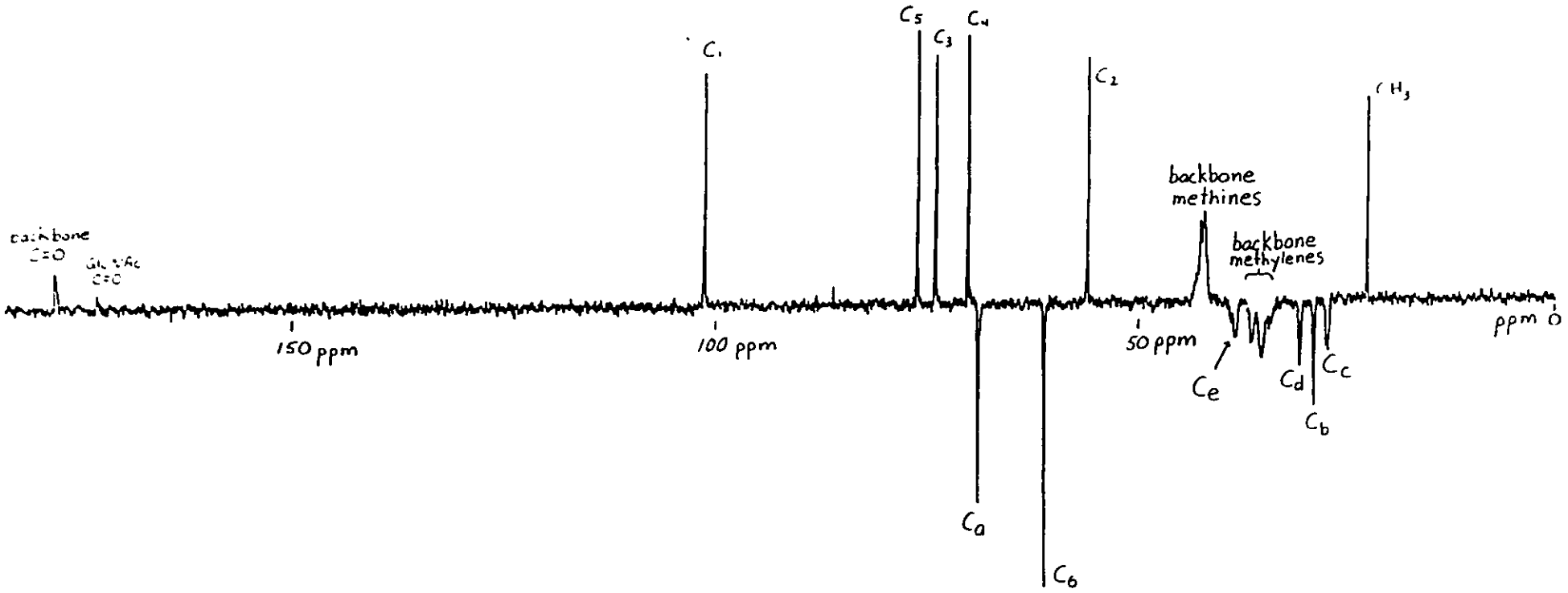


Figure 14 ^{13}C-NMR attached proton test (APT) spectrum of β-D-GlcNAc-S-CPA **52**



4.2.1 Serological evaluation of sulfide spacer modified glycopolymers

The antigenicity of the sulfide spacer modified glycopolymers were investigated by double radial diffusion, quantitative precipitation, and enzyme-linked assays. All glycopolymers described in Table 15 were shown to be antigenic in a specific manner towards the various lectins and antibodies presented earlier (section 2.2.2). These spacer modified glycopolymers were all found to be more potent antigens than their analogous glycopolymers prepared from allyl glycosides.

The rhamnosylated glycopolymers **46** and **47** were found to bind strongly to the *Ricinus Communis* lectin (RCA_{120}) and Pn23 CPS rabbit antiserum in double radial diffusion experiments. The value of the rhamnosylated copolyacrylamide glycopolymer **46** was also exemplified in quantitative precipitation experiments with Pn23 rabbit IgG antibodies. A quantitative curve for this couple is shown in figure 15.

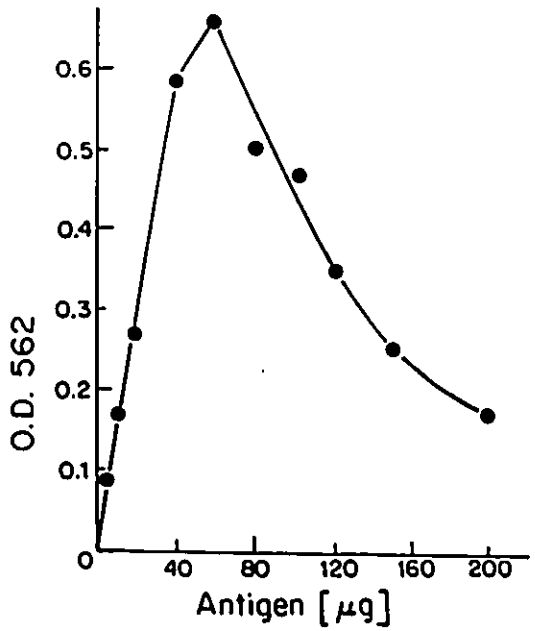


Figure 15 Quantitative precipitin curve of the α-L-Rha-S-CPA **46** with pneumococcus type 23 rabbit antiserum (100 μl).

Sensitization of ELISA plates with **46** (2 μg/well) was also useful in detecting Pn23 antibodies using either peroxidase labeled goat anti-

rabbit IgG or protein A [111], in the sandwich mode (see scheme 8, right). These assays were performed in a qualitative fashion by visual observation as the necessary equipment for quantification was not available at the time. Nonetheless, α-L-rhamnose antibodies were clearly detectable from 50 µl aliquots of 100 fold diluted Pn23 antiserum using 5 µg of horseradish peroxidase labeled goat anti-rabbit IgG or protein A. A negative control with the β-D-GlcNac-**S**-CPA **50** showed no binding to the Pn23 antibodies.

N-Acetylglucosamine containing glycopolymers were first tested by double radial immunodiffusion against Group A streptococcal CPS antibodies. Glycopolymers **48** and **50**, which have identical carbohydrate content but differ in anomeric configuration, were assayed simultaneously. Precipitin band formation was observed only for the β-D-GlcNAc-**S**-CPA **50** (Figure 16). This mirrors observations described earlier for the agglutination of antigenic latex particles by the same antibodies. This result demonstrates the importance of incorporating a spacer in glycopolymers in order to access the deeper binding sites found in certain antibodies which generally differ from their lectin counterparts (general observations made during this work). The analogous glycopolymer prepared from allyl β-D-GlcNAc could not agglutinate these antibodies (section 2.2.2).

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[111] Protein A is a polypeptide isolated from *Staphylococcus aureus* that binds immunoglobulin G molecules in the Fc region. See A. Surolia et al., *Trends. Biochem. Sci.*, 7 , 74 (1981); E. Ishikawa and K. Kato, *Scand. J. Immunol. Suppl.* 7 , 43 (1978).

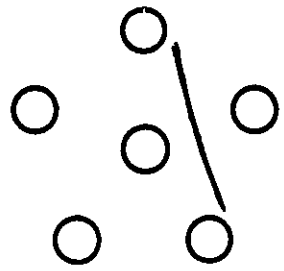

Figure 16 Immunodiffusion of anti-Group A Strep (center well) vs glycopolymers with and without spacers **48, 50, 46, 21, 20** (clockwise from top).

Both anomeric configuration of D-GlcNAc copolymers were also comparatively assessed with the WGA lectin. As observed for the analogous glycopolymers without spacers, the glycopolymer **48** with α-D-GlcNAc residues was found to bind slightly better than its comparable anomer **49** by quantitative precipitation assays (figure 17).

The effect of sugar density on glycopolymers antigenicity was assessed with the β-D-GlcNAc-**S**-CPAs **49** to **55**. Double radial diffusion assays against WGA revealed sharp precipitin bands with graded intensities which reflected hapten content in the glycopolymers. Even glycopolymer **55** with only one β-D-GlcNAc residue for every 40 acrylamide units clearly agglutinated WGA in these assays.

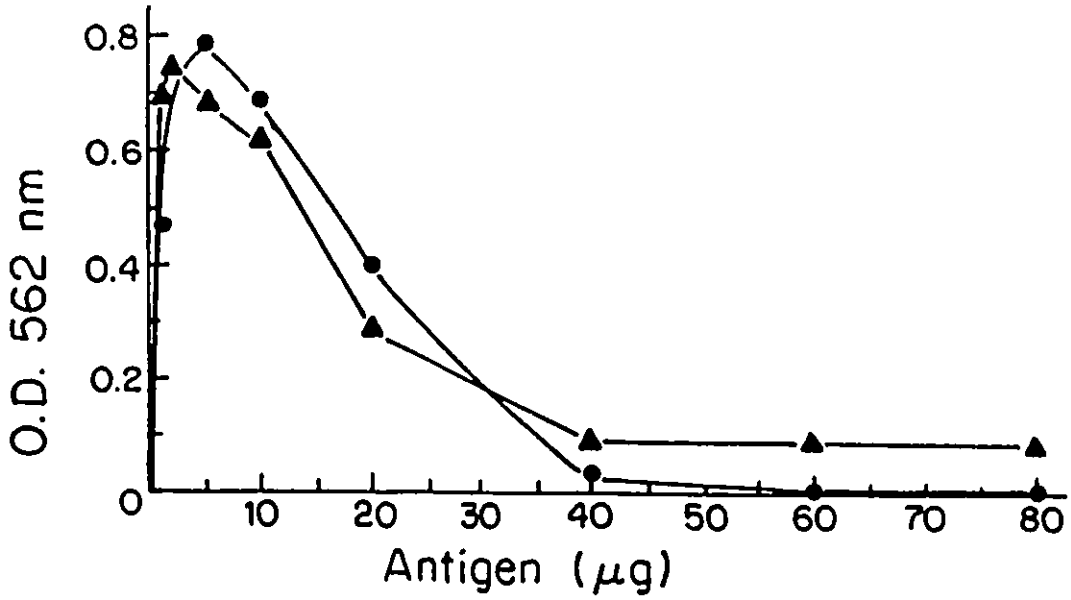

Figure 17 Quantitative precipitation curves of WGA by α-(▲) and β-(•) D-GlcNAc-S-CPA **48** and **49** respectively.

To quantitate the effect of hapten density on antigenicity, glycopolymers **49**, **50**, **53**, **54** and **55** were assayed for relative binding capacity by solid phase enzyme-linked lectin assay (ELLA) with horseradish peroxidase (HRP) labeled WGA. 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was used as HRP substrate. The results shown in figure 18 indicate that the homopolymer **49** and the copolymer **50** having a 1/4 ratio of β-D-GlcNAc to acrylamide residues were essentially equivalent in their ability to bind WGA and were slightly more sensitive than **53** (1/9) and **54** (1/25). **53** and **54** were of similar ability and far more sensitive (on a weight basis) than the copolymer **55** (1/40). Overall, the homopolymer as only marginally more sensitive as ELLA coating antigens (on a weight basis) than other glycopolymers with as low as one sugar unit per 25 acrylamide units.

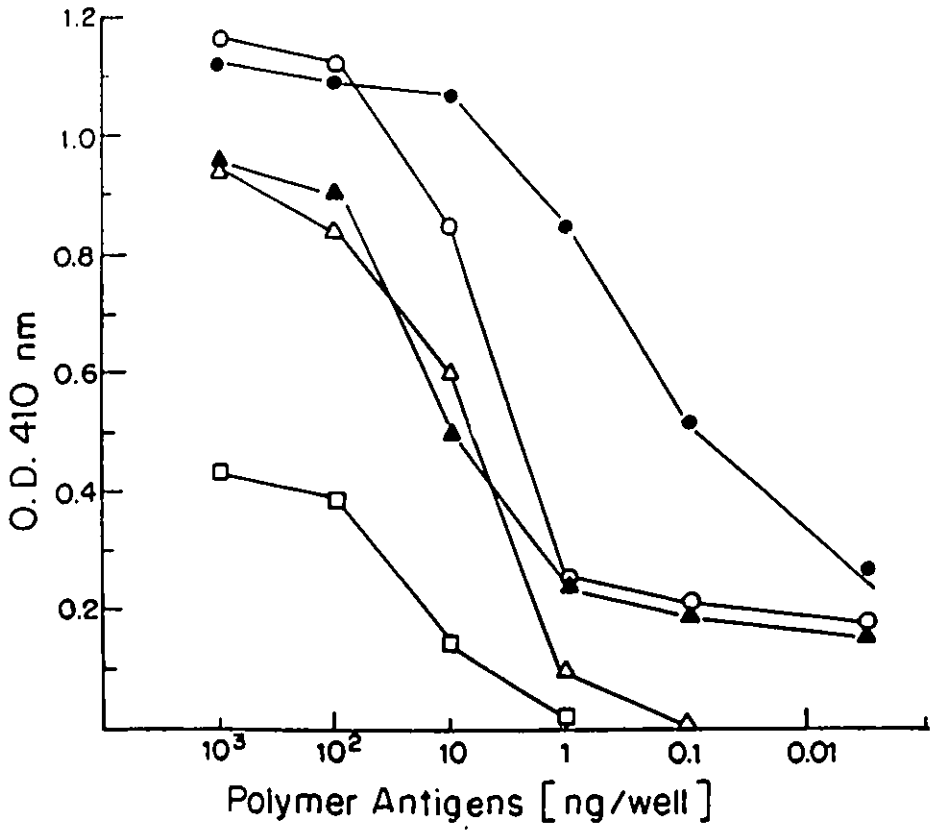

Figure 18 ELLA with β-D-GlcNAc-**S**-CPA glycopolymers. Glycopolymers **49** (O), **50** (•), **53** (Δ), **54** (▲) and **55** (□) were used as coating antigens to immobilize HRP labeled WGA. ABTS was used as HRP substrate.

To demonstrate the strength and specificity of the binding interaction between WGA and β-D-GlcNAc-**S**-CPA glycopolymers, quantitative hapten inhibition assays were performed by solid phase immunoassays using the ELLA technique. Glycopolymer **54** having only a 1:25 molar incorporation ratio of sugar to acrylamide was used to coat ELISA plates (100 ng/well). Various monosaccharides were then used to inhibit binding of HRP-WGA (50 ng/well). The results are given in figure 19 and Table 15 and demonstrate the relative inhibitory power of various monosaccharides.

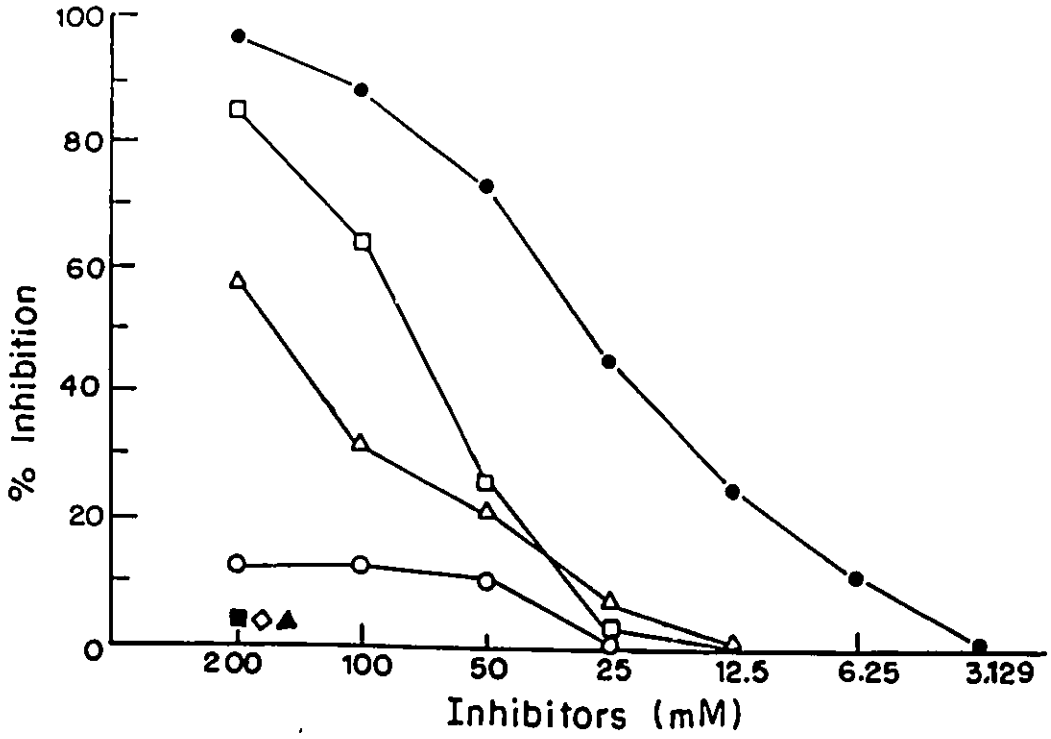

Figure 19 Quantitative hapten inhibition of HRP-WGA binding to β-D-GlcNAc-**S**-CPA **54** (1:25) by monosaccharides: GlcNAc (•), NeuAc(□), GalNAc (Δ), $GlcNH_2$ (O), ManNAc (Δ), Glc(◊) and Gal(▫). 100ng of **54** were used to coat wells. 50 ng/well of HRP-WGA were used.

Table 16 Inhibition of binding (ELLA) of HRP-WGA (50 ng) to β-D-GlcNAc-S-CPA **54** (1:25 incorporation; 100 ng) by monosaccharides.

Sugar inhibitor *	Concentration (mM) for 50% inhibition	Relative inhibitory strength
D-GlcNAc	23.75	1
Neu5Ac	40.6	0.58
D-GalNAc	175	0.14
D-ManNAc	>200	-
D-$GlcNH_2$	>200	-
D-Glc	>200	-
D-Gal	>200	-

* abbreviations have their usual meaning

The result trends were as expected [112]. Inhibitory potency was in the decreasing order: GlcNAc > Neu5Ac » GalNAc > ManNAc, $GlcNH_2$, Glc, Gal. However, the very high amount of D-*N*-acetylglucosamine required for inhibition was somewhat surprising. Goldstein and coworkers[113] reported that only 5.9 μM of *N*-acetylglucosamine were required for 50% binding inhibition of WGA by *p*-azophenyl β-D-*N*-acetylglucosaminide-BSA conjugates. Thus, the glycopolymer **54** is more than 4000 times more effective at binding WGA than the aryl-β-D-glucosaminide-BSA conjugates which should bind very strongly to WGA due to the aromatic aglycon (see later). This clearly demonstrates the superiority of the glycopolymers over neoglycoproteins as diagnostic antigens.

The importance of antigen multivalency in binding multivalent receptor proteins can be demonstrated by a quick calculation showing the ratio of free versus bound *N*-acetyl glucosamine residues required for inhibition. 100 ng of **54** were used to coat the ELISA plate wells. Taking into account that **54** contained 10% by weight of retained water and that this glycopolymer contained a sugar to acrylamide ratio of 1:25, only 41.5 pmol of immobilized *N*-acetylglucosamine (GlcNAc) could be present in the well assuming all of the 90 ng of **54** bound to the well surface. 100 μl of free GlcNAc at a concentration of 23.75 mM were required to inhibit 50% binding with WGA. This corresponds to a lower limit of more than a $5{,}7 \times 10^4$ mole excess of free GlcNAc to immobilized GlcNAc required for only 50% inhibition of binding. Approximately, a 6×10^5 mole excess is required for total inhibition. Thus, the importance of avidity in binding interactions between multivalent receptor proteins and glycopolymer antigens is clearly demonstrated.

In order to quantitate the effects of anomeric configuration, sugar density on the glycopolymer, and the effect of incorporating a spacer

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[112] Reference 6, p. 107

[113] I.J. Goldstein, S. Hammarström and G. Sundblad, *Biochim. Biophys. Acta*, *405*, 63 (1975).

in glycopolymers, comparative binding inhibition assays were performed using GlcNAc-glycopolymers designed to address these questions. The results of this comparative experiment are given in Table 16. The experiment involves glycopolymers with and without a spacer, and having both anomeric configurations. A group of β-D-GlcNAc-**S**-CPA having a range of sugar densities was used to evaluate the effect of sugar density on binding to WGA.

Table 17 a) Inhibition of binding (ELLA) of HRP-WGA to various D-GlcNAc-glycopolymers by D-GlcNAc.

	Glycopolymer†	Spacer	Acrylamide/ glycoside molar ratio	[GlcNAc] required for 50% inhibition (mM)	*Molar ratio of free: immobilized GlcNAc
20	α-D-GlcNAc-CPA	$-CH_2-$	5.1	20.0	1.27×10^4
21	β-D-GlcNAc-CPA	$-CH_2-$	5.4	5.6	3.61×10^3
23⁝	β-D-GlcNAc-CPA	$-CH_2-$	7.4	11.0	1.01×10^3
48	α-D-GlcNAc-**S**-CPA	$C_6H_{11}NOS$	3.9	170	1.14×10^5
49	β-D-GlcNAc-**S**-CPA	$C_6H_{11}NOS$	homopolymer	110	4.33×10^4
50	"	$C_6H_{11}NOS$	3.9	46.0	3.09×10^4
53	"	$C_6H_{11}NOS$	9.0	36.0	3.71×10^4
54	"	$C_6H_{11}NOS$	25	23.75	5.15×10^4
55⁝	"	$C_6H_{11}NOS$	40	14.0	4.53×10^3

† Coated with 100 ng/well.
⁝ Coated with 1 µg/well
* Assuming glycopolymers have similar water contents (10%) after lyophilization.

b) Relative binding affinities of various D-GlcNAc-glycopolymers

Glycopolymer†		Acrylamide/ glycoside (molar ratio)	Relative binding affinities	
			by mole of immobilized GlcNAc	by weight of glycopolymer
20	α-D-GlcNAc-CPA	5.1	0.1*	0.12
21	β-D-GlcNAc-CPA	5.4	0.032	0.033
23‡	β-D-GlcNAc-CPA	7.4	0.0089	0.0065
48	α-D-GlcNAc-**S**-CPA	3.9	1	1
49	β-D-GlcNAc-**S**-CPA	homopolymer	0.38	0.65
50	"	3.9	0.27	0.27
53	"	9.0	0.33	0.21
54	"	25	0.45	0.14
55‡	"	40	0.040	0.0082

† Coated with 100 ng/well.
‡ Coated with 1 µg/well
* Assuming glycopolymers have similar water contents (10%) after lyophilization.

These experiments confirm observations discussed earlier and clearly indicate that WGA binds stronger to α-D-GlcNAc than to its β-anomer. This preference is exhibited for glycopolymers equipped with and without a spacer. Based both on weight and moles of immobilized hapten, glycopolymers of comparable sugar densities show that the α anomer binds 3 times stronger the β-anomer in the case of glycopolymers which do not possess a spacer (**20** vs **21**) and 3.7 times stronger in the case of glycopolymers possessing a spacer (**48** vs **50**). The presence of a spacer between GlcNAc residues and the polymer carrier clearly enhances the overall binding affinity of glycopolymers towards WGA. On a mole basis of immobilized hapten, the incorporation of a spacer in glycopolymers increases binding affinity by a one order of magnitude for both anomeric configurations (**20** vs **48**; **21** vs **50** and **53**). The last five entries of Table 17 demonstrate the influence of sugar density on binding affinity. On a mole basis of immobilized β-D-GlcNAc, glycopolymers possessing from 0 to 25 acrylamide residues per glycoside unit are equally effective antigens (**49**, **50**, **53** and **54**). A decrease in

binding strength is observed when the sugar density is decreased to a 1:40 ratio with acrylamide (**55**). This suggests an optimal threshold monomer ratio between 25 and 40 acrylamides per glycoside unit in the final glycopolymer antigen for the strongest possible binding to WGA by a β-D-GlcNAc-**S**-CPA glycopolymer antigen.

A similar inhibition study (Table 18), this time using *p*-nitrophenyl-β-D-GlcNAc as inhibitor gave the same conclusions as discussed above. These experiments were performed simultaneously with those described above in Table 17. An interesting difference between the two sets of experiments is the observation that *p*-nitrophenyl β-D-GlcNAC is approximately 20 times more effective at inhibiting binding of WGA than free β-D-*N*-acetylglucosamine (compare values in Tables 17 and 18). Investigation of this effect is discussed in chapter 5.

Table 18 a) Inhibition of binding (ELLA) of HRP-WGA to various D-GlcNAc glycopolymers by *p*-nitrophenyl β-D-GlcNAc.

	Glycopolymer†	Spacer	Acrylamide/ glycoside molar ratio	[inhibitor] for 50% inhibition (mM)	*Molar ratio of free inhibitor to immobilized GlcNAc
20	α-D-GlcNAc-CPA	$-CH_2-$	5.1	1.0	695
21	β-D-GlcNAc-CPA	$-CH_2-$	5.4	0.13	93
23:	β-D-GlcNAc-CPA	$-CH_2-$	7.4	0.60	52
48	α-D-GlcNAc-**S**-CPA	$C_6H_{11}NOS$	3.9	>10.0	>7470
49	β-D-GlcNAc-**S**-CPA	$C_6H_{11}NOS$	homopolymer	>10.0	>4364
50	"	$C_6H_{11}NOS$	3.9	4.4	3271
53	"	$C_6H_{11}NOS$	9.0	2.0	2294
54	"	$C_6H_{11}NOS$	25	1.8	4343
55:	"	$C_6H_{11}NOS$	40	1.5	539

† Coated with 100 ng/well.
: Coated with 1 µg/well
* Assuming glycopolymers have similar water contents (10%) after lyophilization.

b) Relative binding affinities of various D-GlcNAc-glycopolymers

Glycopolymer†		Acrylamide/ glycoside (molar ratio)	Relative binding affinities	
			by mole of immobilized GlcNAc	by weight of glycopolymer
20	α-D-GlcNAc-CPA	5.1	< 0.093	< 0.10
21	β-D-GlcNAc-CPA	5.4	< 0.012	< 0.013
23‡	β-D-GlcNAc-CPA	7.4	< 0.007	< 0.006
48	α-D-GlcNAc-S-CPA	3.9	1	1
49	β-D-GlcNAc-S-CPA	homopolymer	< 0.58	<1
50	"	3.9	< 0.44	< 0.44
53	"	9.0	< 0.31	< 0.20
54	"	25	< 0.58	< 0.18
55‡	"	40	< 0.072	< 0.015

† Coated with 100 ng/well.
‡ Coated with 1 µg/well
* Assuming glycopolymers have similar water contents (10%) after lyophilization.

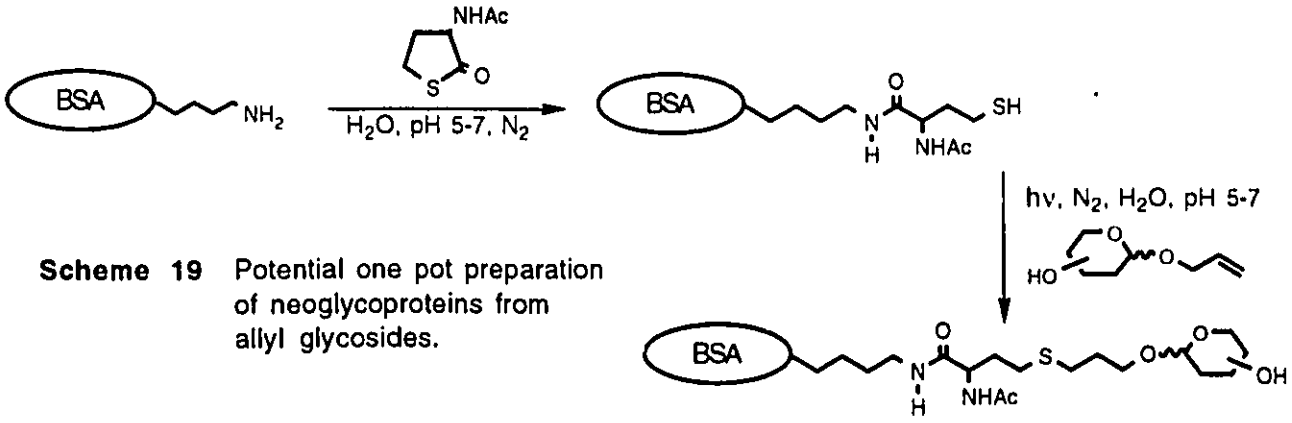
The effect of using methacrylamide over acrylamide as comonomer in providing potentially better glycopolymer antigens was investigated along with other structurally varied glycopolymers. The results of these experiments will be addressed in Chapter 10.

4.3 Neoglycoprotein preparation by Michael addition to acrylamides

As discussed earlier (section 1.3), various methods exist for conjugating carbohydrate haptens to proteins to produce useful neoglycoprotein conjugates. Because of drawbacks associated with these existing methodologies, interest was generated in developing new methodologies for neoglycoprotein preparation. A new method involving the Michael addition of nucleophilic protein sites to appropriate acrylamide containing haptens has been developed and will be discussed here.

4.3.1 Thiolated proteins

At first, results obtained with light promoted thiol addition to allyl glycoside prompted attempts to use this technique to covalently link allyl glycosides to thiolated proteins. A one pot, three component coupling was envisaged in which a spacer could also be incorporated between the carbohydrate hapten and the carrier protein. The reasoning, which is represented in scheme 19, was as follows: proteins could be efficiently thiolated by a known[114] ring opening trans-acylation of *N*-acetylhomocysteine thiolactone (AHTL) to free ε-amine groups of lysine residues under very mild conditions. The freed thiol groups could then react with allyl glycosides under the influence of ultraviolet light to provide a stable sulfide bridge.



Scheme 19 Potential one pot preparation of neoglycoproteins from allyl glycosides.

However, this idea proved better on paper than in practice. A variety of reaction conditions were tried with only very limited success. To a considerable extent, the thiolated proteins would denature or crosslink by disulfide bridge formation before addition to the alkene could take place. Keeping the pH mildly acidic and excluding oxygen did not help. The best conjugation results ever achieved was on the order of 3 glycoside residues per protein unit with most of the protein being precipitated.

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[114] J.F. Kennedy and A. Zamir, *Carbohydr. Res.*, *41*, 227 (1975).

The last result prompted the hypothesis that alkenes of higher reactivity such as the conjugated alkene group 3-(2-*N*-acrylamidoethylthio)propyl glycosides might provide a better alternative to allyl glycosides for a radical thiol addition. However, it was also recognized at this point that sulfide addition could possibly occur by an ionic Michael addition process. Recognizing that thiolated proteins could be crosslinked by disulfide formation as a result of ultraviolet irradiation, as was demonstrated earlier with cysteamine and other thiols in an aqueous environment, a conjugation attempt was performed in the absence of light at an alkaline pH. The results of these conjugation attempts under Michael addition conditions proved fruitful. Large amounts of glycosides could be coupled to thiolated BSA, with little protein precipitation occuring under certain reaction conditions.

Thus, BSA samples were thiolated with varying amounts of AHTL for 4 hrs. at 4°C in carbonate buffer (0.5M, pH 9.5, deoxygenated water) and under a nitrogen atmosphere to minimize disulfide formation. A five molar excess of the acrylamide galactoside **42** to AHTL was then added to each thiolated BSA stocks which were then divided and left to react at 25, 37 and 50 °C. Aliquots from each trial reaction were removed periodically, dialysed then assayed for carbohydrate content by a combination of a Lowry protein and a phenol/H_2SO_4 assay for Gal. All reactions performed at 50 °C began to precipitate protein after 8 hrs., while reactions of only heavily thiolated proteins precipitated after 16 hrs. when performed at 37°C. All remaining trials showed no signs of precipitation when stopped after 46 hrs. However, all aliquots which were removed periodically to be assayed for carbohydrate incorporation showed signs of precipitation after dialysis. Nonetheless, these solutions were each treated with NaOH to a final concentration of 0.1M in NaOH in order to solubilize the precipitated conjugates. Aliquots of each solution were then assayed for protein content by a modified[115] Lowry[116]

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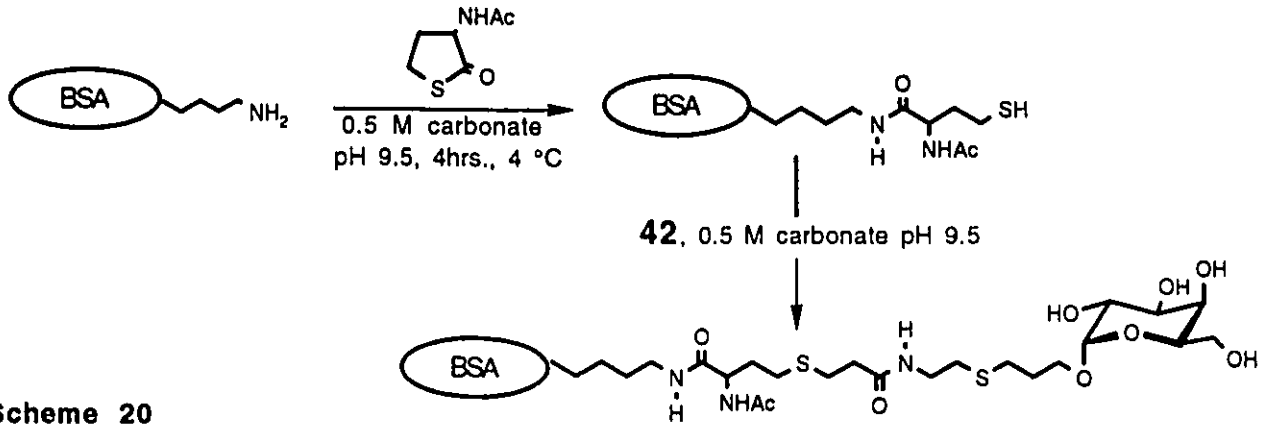
[115] E. Larson, B. Howlett and A. Jagendorf, *Anal. Biochem.*, *155* , 343 (1986).
[116] O.H. Lowry, *J. Biol. Chem.* , *193* , 265 (1951).

assay as well as for carbohydrate content by the phenol/sulfuric acid method. All assays were performed in triplicate with good agreement between individual values. However, the resulting Gal/BSA ratios determined appeared inconsistent within each reaction condition trial. Gal/BSA ratios were found to fluctuate over time within each reaction trial. The inconsistencies are assumed to be due to a high degree of inter- and intra-protein disulfide crosslinking which could affect the Lowry protein assay[115]. A trend could be indentified however, in which increased sugar incorporation reflected increases in concentration of both **42** and the thiolating reagent AHTL, reaction temperature and elapsed time of reaction.



Scheme 20

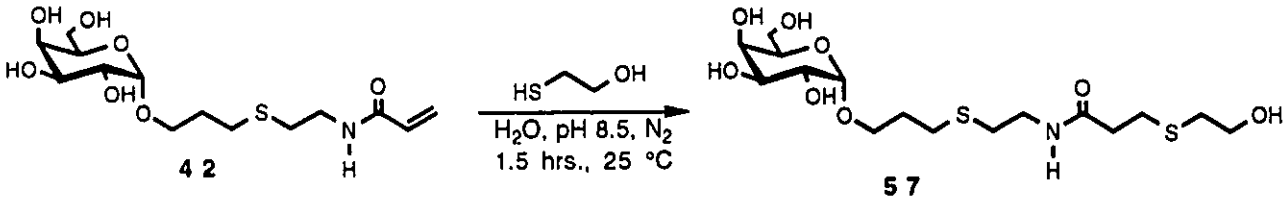
Neoglycoprotein preparation by Michael addition of thiolated proteins to N-glycoside substituted acrylamides

A double radial diffusion experiment with the α-D-galactose specific lectin *Maclura Pomifera* showed that conjugates prepared after only one hour at 25°C from a 1/0.5/2.5 molar ratio of lysine/AHTL/**42** ([BSA] = 5 mg/mL, 0.5M carbonate pH 9.5) were antigenic. Although the Michael addition of thiolated protein carriers to 3-(2-*N*-acrylamidoethylthio)propyl glycosides appeared as a potentially viable approach to preparing neoglycoproteins, the problems of precipitation associated with thiolated proteins made this approach unattractive. Further experiments with the aim of

optimizing conditions were not pursued. Efforts were directed towards a more simplified procedure instead. This procedure involves the use of unmodified proteins and is discussed in the next section.

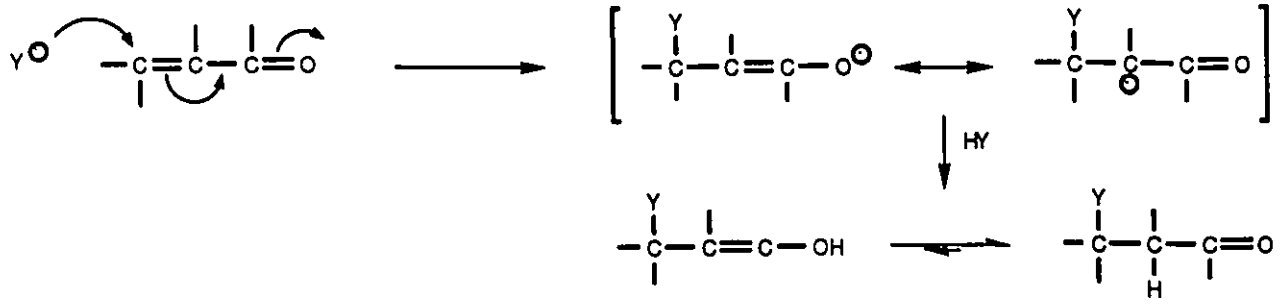
To demonstrate that *N*-acrylamide-substituted-glycosides were being covalently attached to the thiol groups by Michael type addition, a model reaction was carried out. As described in scheme 21, **42** was left to react with 2-mercaptoethanol under conditions similar to that used during protein conjugation. The product **57** was isolated as an oil in high yield (93%) and fully characterized (elemental analysis, FAB-MS, ^{1}H-and ^{13}C-NMR, 2D ^{1}H-^{1}H COSY, ^{1}H-^{13}C HETCOR, $[\alpha]_D$) as supporting evidence for the Michael addition process during protein conjugation.

Scheme 21 a) Michael addition of a thiol to an acrylamide glycoside.



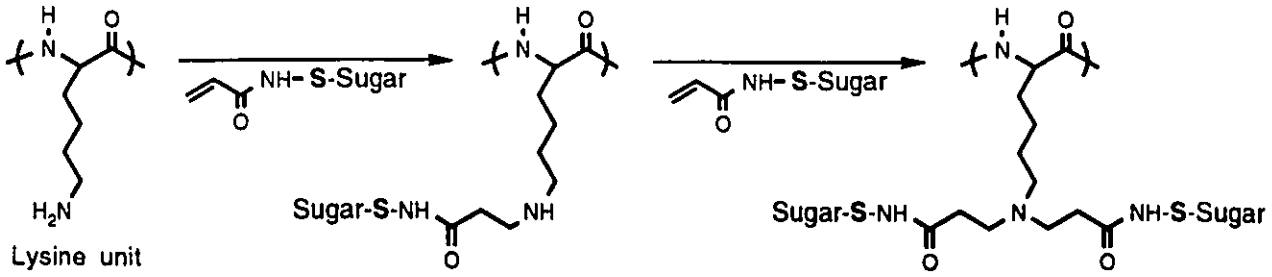
$C_{16}H_{31}NO_8S_2$, oil
m/e = 430 (M+1)
$[\alpha]_D$ = 79.2 ° (H_2O)

b) Generalized mechanism for a 1,4-conjugate (Michael type) addition.



4.3.2 Natural proteins

The results obtained with thiolated proteins encouraged us to believe that potential existed for a mild and effective way of preparing neoglycoproteins via a 1,4 conjugate addition (Michael type addition) of nucleophilic sites on proteins to *N*-acrylamide-substituted-glycosides. Our attention was focussed on amine groups which contribute to the natural make-up of proteins. The amino acid L-lysine appeared as a potential site to which could be added not just one but two acrylamide units. L-lysine provides a relatively accessible amine group at the end of a 4-carbon aliphatic chain and is usually found in considerable amounts on most proteins[117] . An important advantage to this method is that successful conjugation would provide a neoglycoprotein in which the ionic character of the protein would not be altered as the primary amines would simply be converted to secondary and/or tertiary amines.



Scheme 22 Protein conjugation by Michael addition of lysine residues to *N*-glycoside substituted acrylamides

There were concerns however, that unlike thiols, amines might not prove to be as nucleophilic under reaction condition parameters that would not destroy or denature proteins. These concerns later proved unwarranted as proteins conjugation was shown to proceed smoothly at room temperature under relatively mild alkaline conditions.

A trial experiment was performed in which BSA (59 lysine residues) was mixed with the 3-(2-N-acrylamidoethylthio)propyl α-D-

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[117] A.L. Lehninger, *Biochemistry* , Worth Publishers, New York, 1970. Chap. 5

galactoside **42** (1.05/1 **42** lysine) in a borate buffered saline solution (BBS, 0.01 M borate, 0.14 M NaCl, pH 9.2, [BSA] = 2 mg/ml) at room temperature. Aliquots were removed after 36 hours and tested by double radial diffusion against the lectin from *Maclura Pomifera* . Weak but clearly visible precipitin bands were formed after overnight incubation at 4°C. Because the aliquots were not dialyzed prior to the assay, a blank with the same ratio of free **42** to BSA was also tested but proved negative. Thus, protein conjugation by Michael addition appeared to be successful.

To evaluate the effectiveness of this new conjugation method, trials were performed under different sets of conditions. Trials were performed at 25°C using various reagent ratios in two different buffer systems (0.5 M carbonate or 0.05 M BBS) at pH 10.5. This pH level was chosen as it is the limiting level before BSA begins to denature at 25°C. Reagents were mixed and left to react for 4 days. After dialysis, aliquots of each trial were assayed for protein and for carbohydrate contents. The results, shown in Table 19, indicate that conjugation is more effective in a carbonate buffer than in a borate buffer. These results mirror observations made with p-*N*-acrylamidophenyl glycosides (see section 8.2). No protein denaturation was observed in any of the conjugation trials by SDS-PAGE electrophoresis. Lyophilization of these neoglycoproteins gave white, spongy solids which readily redissolved in aqueous solutions.

Table 19 BSA conjugation with **42** by Michael type addition (4 days, 25°C).

Reaction loading **42** / BSA	D-Gal : BSA found *	
	0.5 M Carbonate	0.05 M BBS
5	4	3
10	7.2	5.9
20	17.7	13.7
40	30	23
60	61	43

*Determined by Lowry protein assay and phenol/sulfuric acid assay for D-Gal.

These conjugates were all tested by double radial diffusion against the *Maclura Pomifera* lectin. Only the conjugates bearing more than 10 galactose units were found to be antigenic enough to show visible precipitin bands with the lectin. Precipitin bands of graded intensities reflecting sugar content in the glycoconjugates were observed.

To demonstrate that *N*-acrylamide-substituted-glycosides could be covalently conjugated to ε-amine groups on lysine residues, conjugations were performed using poly-L-lysine under reaction conditions similar to those above. Results of these experiments are discussed in later chapters. In addition, Michael addition products were isolated when simple amines were conjugated to *N*-acrylamide-substituted-glycosides under similar circumstances. Results of these experiments are discussed later in Chapter 9.

4.4 Experimental methods

General Methods :

General methods are as described in Chapter 2.

*3-(2-*N*-Acrylamidoethylthio)propyl glycosides.*

The title glycosides were all prepared with relative ease by the same general procedure outlined below. Reactions were performed equally well on scales of 50 mg to 4 g to give quantitative yields of product.

Pure 3-(2-acrylamidoethylthio)propyl glycosides were dissolved in methanol. An excess of strong anion exchange resin (Amberlite IRA-400 (HO^-) form) wet from prior washing with distilled water was then added and the solutions cooled to 0°C with an ice bath. While stirring at a low rate, 1.05 equivalents of acryloyl chloride (Aldrich)

were slowly added in a dropwise fashion from 5% solutions in either chloroform or 1,4-dioxane. Although acrylyolation of amines is essentially instantaneous, a rapid addition of acryloyl chloride leads to an incomplete consumption of the amine and the formation of unknown secondary products. *A slow addition of acryloyl chloride is essential for a clean quantitative transformation to the desired products* . Reactions were monitored by the disapearance of the amine by applying a small drop of the reaction solution on a piece of TLC plate then dipping it in a solution of 0.2% ninhydrin (Aldrich) in 2% ethanolic pyridine. After mild heating, the absence of a purple coloured spot indicated complete consumption of the amine. Reaction completeness and product purity was confirmed by TLC using 5/1 $CHCl_3$/MeOH as eluent. The acrylamides produced could be visualized by U.V.. All products and amine precursors could be visualized by treatment with either I_2, 1.2% isatin in ethanol or aqueous molybdic acid.

Once complete, the anion exchange resin is filtered and washed with methanol. The filtrate is then treated a moment with cation exchange resin (Amberlite IR-120 or Dowex 50W in (H) form). Removal of this resin followed by evaporation of methanol under reduced pressure at ≤ 25°C gave essentially quantitative yields of the desired glycosides, pure by TLC and NMR.

Additional data to that found in tables 12 and 13 for each 3-(2-acrylamidoethylthio) propyl glycoside prepared is given below.

*3-(2-*N*-Acrylamidoethylthio)propyl α-D-galactopyranoside* **42**

Compound **42** was prepared as outlined in the general method on a 4 g scale. The product was crystallized from EtOH/Et_2O. (+) FAB-MS (glycerol) gave (M+1, 20), 281 (M-$NHCOCHCH_2$, 8), 119 ($HO(CH_2)_3SCH_2CH_2^+$, 79). ^{1}H-NMR δ (DMSOd_6): 6.21 (dd, 1H, J_{cis} = 9.9, J_{trans} = 17.1 Hz, COCH=), 6.07 (dd, 1H, J_{gem} = 2.5 Hz, $=CH_2$ trans), 5.60 (dd, 1H, $=CH_2$ cis), 4.63 (d, 1H, J_{12} = 3.2 Hz, H_1), 3.35 - 3.69 (mult,

8H,H_2,H_3,H_4,H_5,H_6,$H_{6'}$,H_a). 3.29 (t, 2H, H_e), 2.60 (mult, 4H, H_c + H_d), 1.77 (mult, 2H, H_b).

*3-(2-*N*-Acrylamidoethylthio)propyl α-L-rhamnopyranoside* **43**

Compound **43** was prepared on several occasions, as outlined in the genaral procedure, without difficulty. CI-MS (ether) gave m/e (ion, relative intensity): 190 ($HO(CH_2)_3S(CH_2)_2NHCOCHCH_2$ + 1, 100), 147 (M - *O*-aglycon, 119 ($HO(CH_2)_3SCH_2CH_2^+$, 41). ^{1}H-NMR δ ($DMSOd_6$): 8.36 (t, 1H, $J_{e,NH}$ = 5.6Hz, NH), 6.12 (dd, 1H, J_{cis} = 10.0, J_{trans} = 17.1 Hz, -CH=), 5.95 (dd, 1H, J_{gem} = 2.2 Hz, $=CH_2$ trans), 5.46 (dd, 1H, $=CH_2$ cis), 4,41(d, 1H, $J_{1,2}$ = 1.2 Hz, H_1), 3.03 - 3.59 (mult, 10H, H_2,H_3,H_4,H_5,H_6,$H_{6'}$,H_a,H_e), 2.49 (mult, 4H, H_c + H_d), 1.69 (mult, 2H, H_b), 1.16 (d, 3H, J = 6.1 Hz, CH_3).

*3-(2-*N*-Acrylamidoethylthio)propyl 2-acetamido-2-deoxy-α-D-glucopyranoside* **44**

This material could be crystallized from either CH_3CN or EtOH/Ether. Analytical data: % calculated for $C_{16}H_{28}N_2O_2S \cdot 1.5\ H_2O$: C, 45.81; H, 6.73; N, 6.68; S, 7.64. Found: C, 45.54%; H, 6.46; N, 6.84; S, 7.41. (+) FAB-MS gave m/e (ion, relative intensity): 393 (M+1, 13), 264 (M-(NHCOCHCH$_2$ + NAAc), 20), 204 (M-O-aglycon, 100), 144 ($^+CH_2S(CH_2)_2NHCOCHCH_2$, 56), 119 ($HO(CH_2)_3SCH_2CH_2^+$, 80) CI-MS: 190 (HO-aglycon + 1, 100), 204 (M-O-aglycon, 27). ^{1}H-NMR δ ($DMSOd_6$): 6.21(dd, 1H, J_{cis} = 9.8, J_{trans} = 17.1 Hz, -CH=), 6.08 (dd, 1H, J_{gem} = 2.5 Hz, $=CH_2$ trans), 5.59 (dd, 1H, $=CH_2$ cis), 4.62 (d, 1H, $J_{1,2}$ = 3.6 Hz, H_1), 3.60 (mult, 4H), 3,47 (mult, 2H), 3.34 (mult, 3H), 3.13 dt, 1H, $J_{a',b}$ = 4.4, $J_{a,a'}$ = 9.0 Hz, $H_{a'}$), 2.61 (t, 2H, $J_{b,c}$ = 7.3 Hz, H_c), 2.57 (t, 2H, $J_{d,e}$ = 7.2 Hz, H_d), 1.83 (s, 3H, CH_3), 1.75 (mult, 2H, H_b).

*3-(2-*N*-Acrylamidoethylthio)propyl 2-acetamido-2-deoxy-β-D-glucopyranoside* **45**

This compound could be crystallized from warm CH_3CN. Analytical data: % calculated for $C_{16}H_{28}N_2O_2S \cdot 1.5\ H_2O$: C, 46.82; H, 7.37; N, 6.82; S, 7.81. Found: C, 46.66; H, 7.54; N, 6.75; S, 8.02. (+) FAB and CI-MS gave spectra similar to **44**. ^{1}H-NMR δ ($DMSOd_6$): 6.21 (dd, 1H, J_{cis} =

9.9, J_{trans} = 17.1 Hz, -CH=), 6.08 (dd, 1H, J_{gem} = 2.4 Hz, $=CH_2$ trans), 5.59 (dd, 1H, $=CH_2$ cis), 4.25 (d, 1H, $J_{1,2}$ = 8.0 Hz, H_1), 3.76 (mult, 1H), 3.67 (mult, 1H), 3.45 (mult, 2H), 3.31 (mult, 4H), 3.06 (mult, 2H), 2.56 (t, 2H, $J_{b,c}$ = 7.3 Hz, H_c), 2.50 (t, 2H, $J_{d,e}$ = 7.2 Hz, H_d), 1.80 (s, 3H, CH_3), 1.70 (mult, 2H, H_b).

Sulfide spacer-modified glycopolymers

α-L-Rha-S-CPA **46**: 0.30 g (4.2 mmol) of acrylamide and 0.60 g (1.79 mmol) of **43** were dissolved in 6.0 mL of deoxygenated water. Solid ammonium persulfate $(NH_4)_2S_2O_8$ (4 mg, 0.017 mmol) was then added and the solution stirred at high speed while in a boiling water bath for 10 minutes. The resulting viscous solution was diluted with water and dialyzed exhaustively against distilled water. Lyophilization gave 446 mg (51%) of white, spongy solid. ^{1}H-NMR (D_2O) integration gave a 1/2.9 by mol incorporation ratio of **43**/acrylamide. Phenol/H_2SO_4 assay gave a 1:3.2 incorporation. Elemental analysis of **46** gave C: 46.35%, H: 7.25%, N: 9.20%, S: 5.78% which corresponds to a 1/2.6 ratio based on moles S/(moles N - moles S).

α-L-Rha-S-CPMA **47**: 30.0 mg (0.352 mmol) of methacrylamide (BDH) and 46.0 mg (0.137 mmol) of **43** were dissolved in 500 µL of deoxygenated water in a small test tube containing a small magnetic stir bar. The polymerization vessel was flushed with nitrogen and 1µL (6.6 µmol) of TEMED (Aldrich) followed by 4 µL of a 50 mg/mL solution of $(NH_4)_2S_2O_8$ (0.88 µmol, 0.18% by mol) were then added. The reaction was left stirring overnight after which it was dialyzed and freeze-dried to yield 36.5 mg (48%) of spongy, white solid. ^{1}H-NMR (D_2O) integration gave a 1/2.8 molar incorporation ratio of **43** to methacrylamide. Phenol/H_2SO_4 assay gave a 1/3.0 ratio.

α-D-GlcNAc-S-CPA **48**: 27.7 mg (0.071 mmol) of **44** and 20.0 mg (0.28 mmol) of acrylamide were dissolved in 350 µL of deoxygenated water and initiated with 3 µL of a 100 mg/ml solution of $(NH_4)_2S_2O_8$ (0.4 mol %). The reaction was kept at 100°C for 12 minutes while stirring. A TLC (30/20/1, $CHCl_3$/MeOH/H_2O) revealed that most of

the monomers were unreacted. Thus, the initiation and polymerization was repeated as above. Dialysis and lyophilization gave 28.9 mg (60% of **48** as a white, spongy solid. $[\alpha]_D$= +34.0° (c= 1.03 H_2O). ^{1}H-NMR (D_2O) integration gave a 1/3.9 molar ratio of **44**/acrylamide incorporated in the glycopolymer. Elemental analysis gave for C: 43.67%, H: 6.78%, N: 11.03%, S: 4.18% which corresponds to a 1/4.05 ratio and 12.1% of retained water by the method outlined in Chapter 2.

β-D-GlcNAc-**S**-CPA **49**: 20.0 mg of **45** were deposited into a small (0.6 x 5 cm) test tube which was then tightly fitted with a rubber septum. The atmosphere within was repeatedly (3x) evacuated then filled with nitrogen. Deoxygenated water (60 µL) and 5 µL of 10 mg/mL solution of $(NH_4)_2S_2O_8$ (0.4 mol %) were then injected and the reaction immersed in a boiling water bath for 16 minutes. Stirring was performed by brief and brisk agitation from a vortex mixer on several occasions during polymerization. The glycopolymer was dialyzed and freeze-dried to give 8.0 mg (40%) of spongy, white solid.

β-D-GlcNAc-**S**-CPA **50**: 100 mg (0.255 mmol) of and 70 mg (0.99 mmol) of acrylamide were dissolved in 1.3 mL of water, sealed under a nitrogen atmosphere, then injected with 14 µL of a 100 mg/mL solution of $(NH_4)_2S_2O_8$ (0.5 mol %). The reaction was stirred rapidly at 100°C for 12 minutes TLC (30/10/1, $CHCl_3$/MeOH/H_2O) indicated that most of the monomers remained unreacted. The polymerization was reinitiated as above and left at 100°C for 12 minutes a second time. Dialysis and freeze-drying gave 91.2 mg (53%) of **50**. $[\alpha]_D$= -9.8° (c= 0.83, H_2O). A 1/3.9 **45**/acrylamide incorporation ratio was deduced from ^{1}H-NMR integrations. Elemental analysis gave (in %) C: 39.94, H: 6.02, N: 10.47 and S: 4.32 which corresponds to a 1/3.6 molar ratio of monomers and 13.1% of retained water by weight using the method and equation (1) described in Chapter 2.

β-D-GlcNAc-**S**-CPA **51**: 60 mg (0.153 mmol) of **45** and 55 mg (0.775 mmol) of acrylamide were polymerized for 15 minutes at 200°C in 1.5 mL of deoxygenated water under a nitrogen atmosphere. A 50 µL

volume of $(NH_4)_2S_2O_8$ (100 mg/mL, 2.4 mol %) was used to initiate polymerization. The solution was then dialyzed and freeze-dried to 92.6 mg (81%) of **51** as a white, spongy solid. A 1/5.1 molar ration of **45**/acrylamide was incorporated in the polymer as judged by ^{1}H-NMR integrations.

β-D-GlcNAc-**S**-CPA **52**: 39.2 mg (0.10 mmol) of **45**, 50.0 mg (0.70 mmol) of acrylamide and 5 μL of TEMED were dissolved in 950 μL of deoxygenated water while under a nitrogen atmosphere. The solution was then warmed slightly and injected with 50 μL of a 100 mg/ml solution of $(NH_4)_2S_2O_8$ (2.7 mol %). The polymerization was left to proceed at room temperature for one hour. The solution was then dialyzed against 5 l of 1 mM HOAc followed by multiple 5 L volumes of distilled water. Lyophilization gave 78.0 mg (85%) of **52** as a white, spongy solid. ^{1}H-NMR (D_2O) analysis gave a 1/7.0 molar ratio of **45**/acrylamide incorporation in the glycopolymer.

β-D-GlcNAc-**S**-CPA **53**: 39.3 mg (0.100 mmol) and 64.0 mg (0.900 mmol) of acrylamide were dissolved in 100 μL of deoxygenated water and put under a nitrogen atmosphere. The solution was injected with 10 μL of a 100 mg/mL solution of $(NH_4)_2S_2O_8$ (0.4 mol %) and then stirred vigorously at 100°C for 16 minutes. Dialysis and lyophilization gave 26.9 mg (26%) of **53** as a white, spongy solid. ^{1}H-NMR (D_2O) gave a 1/9.0 molar incorporation ratio of **45**/acrylamide.

β-D-GlcNAc-**S**-CPA **54**: 39.2 mg (0.100 mmol) of **45** and 177.8 mg (2.50 mmol) of acrylamide were dissolved in 2.5 ml of deoxygenated water while under a nitrogen atmosphere. A 25 μL volume of a 100 mg/mL solution of $(NH_4)_2S_2O_8$ (0.4 mol %) was then injected and the solution stirred vigorously at 100°C for 16 minutes. The resulting viscous solution was diluted, dialyzed and lyophilized to give 124.3 mg (57%) of **54**. ^{1}H-NMR (D_2O) analysis gave a 1/25.1 molar incorporation ratio of **45**/acrylamide. Elemental analysis gave (in %): C: 43.97, H: 6.35 N: 15.45, S: 1.34 which corresponds to a 1/24.9 ratio of monomer and 11.0% by weight of retained water.

β-D-GlcNAc-**S**-CPA **55**: 9.6 mg (24.5 μmol) of **45** and 69.4 mg (0.976

mmol) of acrylamide were dissolved in 1000 µL of deoxygenated water under a nitrogen atmosphere. A 10 µL volume of a 100 mg/mL solution of $(NH_4)_2S_2O_8$ (0.4 mol %) was then injected and the solution stirred vigorously at 100°C for 15 minutes. The resulting viscous solution was diluted, dialyzed and lyophilized. Compound **55** (43.7 mg, 55%) was obtained as a white, spongy solid. ^{1}H-NMR (D_2O) analysis gave a 1/40 molar incorporation ratio of **45**/acrylamide.

β-D-GlcNAc-**S**-CPMA **56**: 22.1 mg (56.3 µmol) of **45** and 48.0 mg (0.564 mmol) of methacrylamide were dissolved in 600 µL of deoxygenated water. A 25 µL volume of a 50 mg/mL solution of $(NH_4)_2S_2O_8$ (0.9 mol %) was then injected and the solution stirred vigorously at 100°C for 12 minutes. Dialysis and lyophilization gave 21 mg (30%) of **56** as a white, spongy solid. ^{1}H-NMR (D_2O) analysis gave a 1/40 molar incorporation ratio of **45**/acrylamide.

Quantitative immunoprecipitation

Quantitative immunoprecipitations were performed with the same protocols as described in the experimental section of Chapter 2.

Solid phase enzyme-linked assays

a) Detection of Pn23 CPS rabbit antibodies with α-L-Rha- **S***-CPA* **46** *by ELISA*

The wells of Linbro EIA microtiter plates (Flow Laboratories, Mississauga, ON, Canada) were coated at 4 °C overnight by using 200 µL of **46** (16 µg/mL). The plates were then blocked with 200 µL of 1% BSA in PBS at room temperature for 30 minutes. After washing once with 200 µL of 0.02% Tween 20 (Aldrich) in PBS, the wells were filled with 50 µL of Pn23 CPS rabbit antibody serum (Staten Serum Institut, Denmark) diluted gradually from 10 to 100 times with PBS. Plates were then washed five consecutive times with 200 µL of 0.02% Tween 20 in PBS. Detection of bound antibodies was performed by incubating for 1 hour at room temperature 50 µL volumes of either: a) goat anti-rabbit IgG-HRP concentrate (Sigma) diluted gradually from 50 to 1000 times in PBS, or b) Protein A-HRP

(Sigma) in gradual concentrations ranging from 0.1 to 1.0% in PBS. Plates were then washed five times with 200 µL of 0.02% Tween 20 in PBS. 50 µL of enzyme substrate (1:1 volumes of 0.003% H_2O_2 and 500mg/mL 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) each in 0.1 M phosphate-citrate buffer, pH 4.0) were then added to each well. The gradual green colouration was observed visually over a 15 minute period. All wells treated with Pn23-CPS antibody serum developped into positives assays. Wells not treated with Pn23-CPS antibody remained uncoloured. For assays detected with goat anti-rabbit IgG-HRP, colouration decreased with concentration of enzyme while changes in the PN23-CPS antibody serum showed no effect, thus suggesting that this method could detect lower than 100 x diluted Pn23-CPS antibody serum. For assays performed with Protein A-HRP, colouration decreased as a function of both the Pn23-CPS antibody serum and the enzyme.

b) ELLA with WGA and GlcNAc- **S***-CPAs* (Figure 16)

The wells of Linbro EIA microtiter plates were coated at room temperature overnight by using 100 µL of 10-fold dilutions of glycopolymers **49**, **50**, **53**, **54** and **55** (10 µg/mL). Plates were blocked with 300 µL of 0.2% BSA in PBS for 1 hour at room temperature. After washing (four times with 300 µL PBS), the wells were filled with 100 µL of 300x diluted (PBS) horseradish peroxidase-labeled Wheat Germ Agglutinin lectin (WGA-HRP, Sigma No. L3892, 1 mg/mL). The plates were incubated at room temperature for 3 hours and then washed five times with PBS. A 50 µL volume of enzyme substrate (ABTS, 250 µg/mL in citrate-phosphate buffer, 0.2 M, pH 4.0 with 0.01% H_2O_2) was then added. After 15 minutes, the optical density was measured at 410 nm on a Dynatech MR 600 spectrophotometer.

c) Inhibition of ELLA by monosaccharides (Figure 17 and Table 17)

The general protocol described above was followed. Plates were coated with glycopolymers (10 ng/well), blocked and washed. Plates were then incubated for 3 hours at room temperature after adding 50

μL of 2-fold serial dilution of various monosaccharides (0.400 M) and 50 μL of 150x diluted WGA-HRP (1 mg/mL), with gentle stirring provided from a low speed vortex mixer fitted with a microtiter plate holder. After washing, ABTS was added and the optical density was measured as described above.

Protein quantification by a modified Lowry [113] procedure

The method is essentially as described by others[112]. To 100 μL samples 1.00 mL of solution A is added. The solution is vortexed at a high speed for a moment then left for 10 minutes. A 100 μL volume of solution B is then added while the sample is vortexed at high speed. After 3 minutes, 100 μL of solution C is added and the samples mixed vigorously. After a 30 minute incubation period, optical densities are measured at 750 nm.

Solution A: 50 mL of 2% Na_2CO_3 in 0.1 M NaOH mixed with 1.0 mL of an aqueous solution 0.5% in $CuSO_4 \cdot 5H_2O$ and 1% in sodium tartrate.

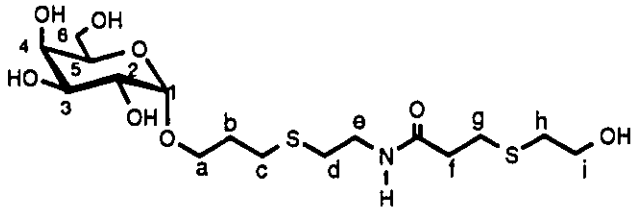
Solution B: 1N Folin and Ciocalteu's phenol reagent (Sigma).

Solution C: 20 mM dithiothreitol (Aldrich) in H_2O.

*3-([3-(2-Hydroxyethyl)thiopropanamido]-2-*N*-ethylthio)propyl-α-D-galactopyranoside* **57**

Compound **42** (342 mg, 0.973 mmol) and 90 μL (1.28 mmol,1.3 equiv.) of 2-mercaptoethanol were dissolved in 15 mL of deoxygenated water. The pH was adjusted to 8.5 with NaOH and the solution stirred at room temperature under a nitrogen atmosphere. The reaction was deemed complete by TLC (5/3/1 MeCN/acetone/10% aqueous HOAc and 4/1 $CHCl_3$/MeOH) after 1.5 hours without any secondary product formation detectable. The solution was then neutralized with acetic acid and the solvent removed by multiple coevaporations with ethanol. The resulting mass was dissolved in a minimum of methanol and filtered through silica gel. Elution, first with 7/1 followed by

4/1 $CHCl_3$/MeOH gave 388 mg (93%) of **57** as a clear oil after complete solvent evaporation. The product was homogeneous by TLC (Rf = 0.20 in 4/1 $CHCl_3$/MeOH; Rf = 0.48 in 5/3/1 MeCN/acetone/10% aqueous HOAc) and NMR. $[\alpha]_D$ = 79.2° (c = 1.50, H_2O).



(+)FAB-MS gave m/e (ion, relative intensity): 881 (2M + Na^+, 2), 859 (2M+H, 2), 452 (M + Na^+, 65), 430 (M + H^+, 51), 268 (HO-aglycon + H^+, 76). ^{1}H-NMR δ (DMSOd_6): 4.63 (d, 1H, $J_{1,2}$ = 3.2 Hz, H_1), 3.70 (dd, 1H, $J_{3,4}$ = 2.8, J_{45}< 1 Hz, H_4), 3.36-3.69 (mult, 9H,H_2,H_3,H_5,H_6,$H_{6'}$,H_a,H_i), 3.21 (t, 2H, $J_{d,e}$ = 6.3 Hz, H_e), 2.69 (t, 2H, $J_{h,i}$ = 7.3 Hz, H_h), 2.59 (t, 2H, $J_{h,i}$ = 7.6 Hz, H_h), 2.56 (t, 2H, $J_{b,c}$ = 7.0 Hz, H_c), 2.54 (t, 2H, $J_{d,e}$ = 6.3 Hz, H_d), 2.34 (t, 2H, $J_{f,g}$ = 7.3 Hz, H_g), 1.77 (mult, 2H, H_b). ^{13}C-NMR δ (DMSOd_6): 170.5 (C = 0), 98.9 (C_1), 71.2 (C_5), 69.5 (C_4), 68,7 (C_3), 68.3 (C_2), 65.6 (C_a), 60.7 (C_i), 60.5 (C_6), 38.6 (C_e), 36.0 (C_g), 33.8 (C_c), 30.5 (C_d), 29.4 (C_b), 27.8 (C_h), 27.4 (C_f).

5. *O*-Aryl 2-acetamido-2-deoxy-β-D-glucopyranosides

5.1 Wheat Germ Agglutinin binding inhibitors

The purpose of the work undertaken and described in this dissertation was to develop novel glycoconjugates which could be useful as tools to probe carbohydrate-protein interactions. As efforts began to direct themselves toward studying fundamental aspects of interactions with glycopolymers, attention was focussed on using WGA as a model carbohydrate binding protein. During the study of well-defined GlcNAC glycopolymers (Table 17), it was observed that *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside was a much better binding inhibitor of WGA than the corresponding free monosaccharide (Table 16). Enhanced binding to aryl glycosides has also been observed with other lectins[6].

The X-ray of WGA[118] (Figure 20) has shown the presence of tyrosyl residues in the carbohydrate binding sites. In particular, tyr 64 appears to be properly positioned to undergo aromatic ring stacking with a phenolic aglycon once the *N*-acetyl D-glucopyranoside ring is bound in its proper position, subsite A. To investigate the possibility of electron donor-acceptor (EDA)[119] complexation between the electron-rich tyrosine residue and the electron-poor aromatic ring of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, a series of aryl glycosides having a range of electronic contributions was required. Therefore, a series of aryl 2-acetamido-2-deoxy-β-D-glucopyranosides was synthesized to probe these potential electronic contributions and to evaluate their contributions to the

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[118] C.S. Wright, *J. Mol. Biol., 215* , 635 (1990) and references cited within.

[119] The term charge-transfer complexes has also been used. No actual transfer of charge is expected or proven to be responsible for bonding here. The more neutral name EDA has been adopted as suggested by J. March, *Advanced Organic Chemistry. Reactions, Mechanisms and Structure*, Wiley-Interscience, New York, 1985. p. 74-77; Good leading references are found in this reference.

relative binding energies with the lectin.

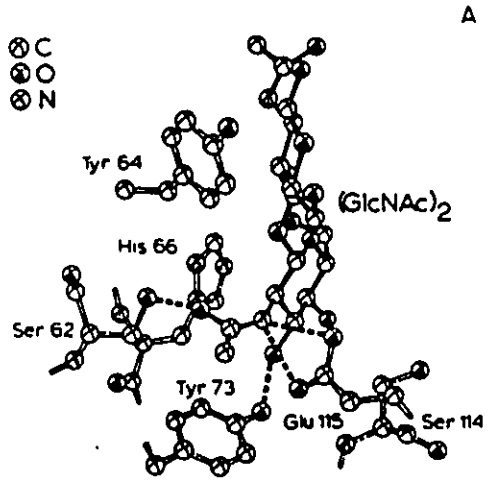

Figure 20 : X-ray of WGA binding subsite A complexed to dichitosan.

5.2 Phase transfer catalysed synthesis of aryl β-D-*N*-acetylglucosaminides

Aryl glycosides are widely distributed in nature and many of these compounds possess medicinal properties. Furthermore, they are known to undergo facile rearrangement into *C*-glycosides[120]. Other useful applications of aryl glycosides reside in their use as chromogenic substrates in enzymology[121] and as inhibitors in sugar-lectin interactions[6].

Few syntheses of aryl glycosides have appeared in the past[122]. Efficient glycosylation of reactive phenolic derivatives has shown

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[120] T. Kametani, H. Kondo and Y. Fugimori, *Synthesis* , 1005 (1988); T. Matsumoto, M. Katsuki and K. Suzuki, *Tetrahedron Lett.*, *29* , 6935 (1988).

[121] J.A. Cabezas, A. Reglero and P. Calvo, *Int. J. Biochem.*, *15* , 243 (1983).

[122] K. Igarashi, *Adv. Carbohydr. Chem. Biochem.*, *34* , 243 (1977) and references cited within.

to present particular difficulties[123]. The most general methods used to prepare these compounds was based on reactions of sodium phenoxides in aqueous acetone with yields ranging from 26-35% [124]. More recently, the less convenient solvent dimethyl formamide has been substituted for acetone to give yields in the 45-80% range[125]. In our view, these methods were unattractive in terms of yield or experimental ease. We thus attempted phase transfer catalysis (PTC) as a preparative method for the desired glycosides.

PTC using peracetylated glycosyl halides has recently been applied to the synthesis of aryl glycosides[126]. However, there has been no reported application of this procedure for the preparation of *O*-aryl 2-acetamido-2-deoxy-β-D-glucopyranosides until this work[127]. The most probable reason for not using PTC in past aryl 2-acetamido-2-deoxy-β-D-glucopyranosides **59** syntheses might be due to anticipated irreversible formation of 2-methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-d]-oxazoline **60** following a halide ion catalysed double inversion mechanism[128] on the substrate 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride **58**.

We hypothesized that formation of the oxazoline **60** could be prevented if nucleophilic attack by phenoxides at the anomeric centre could occur at a faster rate than the attack by the catalyst counteranions. Nucleophilic attack by counteranions such as chloride

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123 S. Koto, N. Morishima, M. Akan, T. Tsuchiya and S. Zen, *Bull. Chem. Soc. Jpn.*, *5* , 1895 (1981).

124 a) D.H. Leeback, *Biochem. Prep.*, *10* , 118 (1963); b) F. Shafizadeh, M.H. Meshrecki and R.A. Sussot, *J. Org. Chem.*, *38* , 1190 (1973).

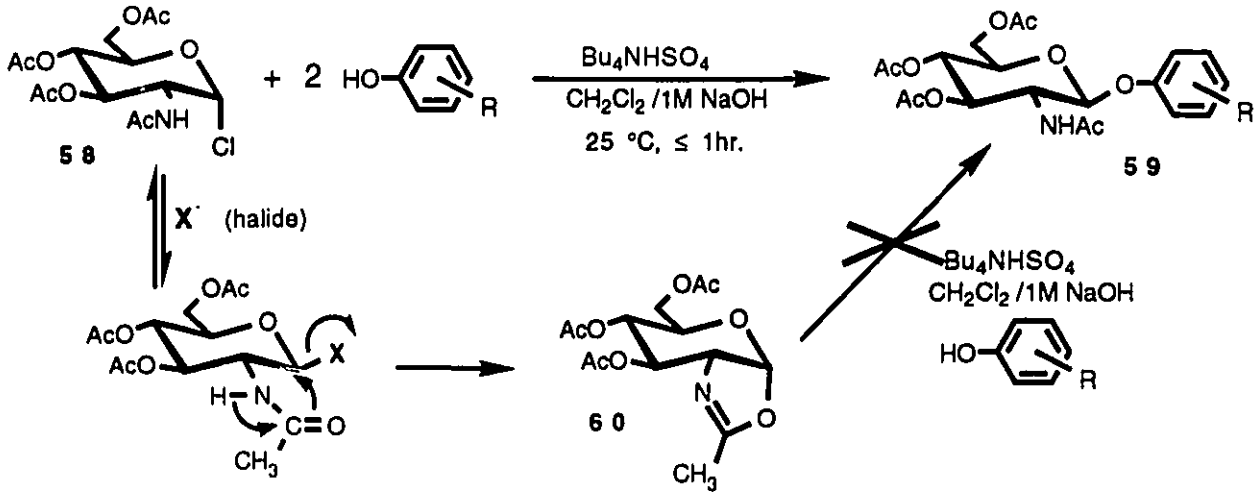
125 M.G. Vatina, V. Motodtsov and L.I. Fedoreeva, *Carbohydr. Res.*, *44* , 142 (1985).

126 a) K. Brewster, J.M. Harrison and T.D. Inch, *Tetrahedron Lett.*, 5051 (1979); b) D. Dess, H.P. Klein, D.V. Weinberg, R.J. Kaufman and R.H. Sidhu, *Synthesis*, 883 (1981); c) J. Bogusiak and W. Szeja, Pol. J. Chem., 59 , 293 (1985); d) J. Rothermel and J. Faillard, *Carbohydr. Res.*, *196* , 29 (1990).

127 Some of the material here has already been published by this author. See references 25a and 25b as a well as R. Roy, F.D. Tropper and A.J. Williams , *J. Magn. Reson. Chem.*, *29* , 852 (1991).

128 R.U. Lemieux and H. Driguez, *J. Am. Chem. Soc.*, *97* , 4063 (1972).

or bromide would lead to a reactive β-glycosyl halide followed by anchimeric displacement by the neighbouring acetamido group to give the oxazoline **60**. With this in mind, it became obvious that a non-nucleophilic and hydrophilic anion such as sulfate or hydrogen sulfate would solve part of the problem. However, it should be noted that for each nucleophilic substitution by a phenoxide there is one chloride anion released. To minimize this effect, two equivalents of phenoxide were used throughout the synthesis.



Scheme 23 Synthesis of aryl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosides by phase transfer catalysis.

Over 20 different aryl 2-acetamido-2-deoxy-β-D-glucopyranosides were prepared in a stereospecific manner using tetrabutylammonium hydrogen sulphate ($(n\text{-}C_4H_9)_4N^+ HSO_4^-$, TBAHS) as phase transfer catalyst in 1M NaOH and methylene chloride at room temperature. Glycosidation was effected in about one hour with yields ranging from 66-93%. Zemplén de-*O*-acetylation neatly afforded the desired unprotected glycosides. The effect of various catalysts and reaction conditions were evaluated and are discussed. In addition, several functional group manipulations were effected to widen the number and nature of aryl substituents.

5.2.1 Mechanism evaluation and reaction conditions optimization

As suspected, formation of the oxazoline **60** could be achieved under PTC conditions. In the first set of experiments, the phenoxide nucleophile was omitted and the reaction carried out with tetrabutylammonium bromide as catalyst in CH_2Cl_2 and NaOH. A good yield (69%) of the expected oxazoline **60** was obtained. This catalytic two phase system is conceptually similar to the reaction conditions previously used by Lemieux and Driguez[128] for the synthesis of **60** in acetonitrile alone. Formation of **60** was usually detectable (TLC) in most PTC glycosidations performed with **58** and a phenolic nucleophile. In view of the possibility of forming the aryl glycosides **59** from an intermediate oxazoline **60**, the former was used as substrate under the optimized set of conditions described above in Scheme 23. Treatment of **60** with *p*-hydroxybenzaldehyde and TBAHS (1 equiv.) and 1M NaOH in methylene chloride at room temperature gave no glycoside, thus illustrating that the oxazoline **60** is not an intermediate in the glycoside syntheses.

The efficiency of a number of phase transfer agents were compared to evaluate the best catalyst. Not surprisingly[129], the more lipophilic tetrabutylammonium (TBA) cation was slightly more effective (~2 times faster) than the corresponding benzyltriethylammonium (BTEA) chloride or bromide in methylene chloride. With 0.2 molar equivalents (limiting conditions) of TBA cation catalyst over **58** and phenol, the iodide counteranions gave more oxazoline than bromide or chloride. These differences seemed to disappear, however, under non-limiting conditions (1 equiv.) during quantitative experiments using chloride, bromide, iodide, hydroxide, or hydrogen sulfate counteranions and TBA cations in methylene chloride and 1M NaOH with *p*-iodo and *p*-chloro phenols (2 equiv.) as nucleophiles.

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[129] E.V. Dehmlow, *Angew. Chem. Int. Ed. Engl.*, *16*, 493 (1977).

It was also observed that the reaction with phosphonium salts[130] such as ethyltriphenylphosphonium bromide as catalyst, was faster than with TBA bromide. However, irreversible formation of by-products such as ethyldiphenylphosphine oxide[131] or ylide, consumed the catalyst and complicated the purification steps, thus rendering these catalysts inappropriate for the actual goals.

The nature of the organic phase was also investigated. In the past[126c], benzene or toluene have often been used under refluxing conditions with BTEA chloride as catalyst to effect glycosidations[132]. However, reactions were completed much faster with methylene chloride or 1,2-dichloroethane at room temperature than with benzene, even under refluxing conditions. These observations held true with either TBA bromide or BTEA chloride as catalysts. Thus, methylene chloride was used for all *O*-aryl glycoside syntheses.

The effect of the catalyst concentration was also evaluated. With TBA bromide, the reactions were faster with increasing concentrations (0.1 - 1 equiv.). Glycosidation did not proceed in the absence of a catalyst. At high concentration, fewer by-products were observed (TLC). The consumption of the chloride **58** was also faster under refluxing conditions but at the expense of forming more side products. Besides the oxazoline **60**, other products isolated from PTC glycosidation of **58** were those having a hydrolyzed anomeric centre and partially de-*O*-acetylated aryl glycosides. Transesterification by the phenoxides is postulated as a reaction pathway since minor quantities of acetylated phenols[133] were isolated in a few instances. As well, products[132] arising from nucleophilic attack of the solvent, methylene chloride, could also be identified after extended reaction periods.

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[130] A.W. Herriot and D. Picker, *J. Am. Chem. Soc.*, *97* , 2345 (1975).

[131] Identified by MS and ^{1}H-NMR spectroscopy, m.p.=122.6 - 123.8 °C.

[132] a) F. Chétien, P. DiCesare and B. Gross, J. *Chem. Soc. Perkin Trans.1* , 3297 (1988); b) J. Bogusiak and W. Szeja, *Carbohydr. Res.*, *141* , 165 (1985).

[133] Identified by MS and ^{1}H-NMR spectroscopy.

The basicity of the aqueous phase was also examined. Molar sodium hydroxide gave faster reactions and therefore fewer by-products (oxazoline **60** and anomeric hydrolysis) than 0.5 M (or less concentrated) NaOH, saturated sodium bicarbonate or 1M sodium carbonate. At a concentration of 2M NaOH, the reaction was even faster for most phenoxides but seemed to produce more de-*O*-acetylated glycosides (TLC).

5.2.2 Preparation, isolation and characterization

Following the observations made from above, a generalized procedure (described in Scheme 23) was used to prepare the desired aryl 2-acetamido-2-deoxy-β-D-glucopyranosides in good yields. No α-glycosides could be detected in the ^{1}H-NMR spectra of the crude reaction products, or by TLC. The glycosyl chloride **58** was dissolved in CH_2Cl_2 (100mg/ml) with one equivalent of TBAHS and 2 equivalents of phenoxide. An equal volume of 1M NaOH was then added, and the reaction stirred vigorously at room temperature until consumption of **58** was complete (30-60 minutes, TLC). After extractive work up, the desired products could be purified by silica gel chromatography or by direct crystallization (EtOH) from the evaporated organic extracts. All the anomeric protons of the per-*O*-acetylated aryl glycosides appeared between 5.03 and 5.55 ppm with vicinal coupling constants in accord with 1,2-trans β-D-linkages ($J_{1,2} \approx 8$Hz). The anomeric carbon signals appeared between 97.1 and 100.2 ppm with $^1J_{13C\text{-}1,1H\text{-}1}$ of 160 Hz, also consistent with a β-linkage (axial H-1)[134]. Finally, Zemplén de-*O*-acetylation of the per-*O*-acetylated glycosides with a catalytic amount of sodium methoxide in methanol gave quantitative yields of aryl 2-acetamido-2-deoxy-β-D-glucopyranosides.

A list of monosubstituted phenyl glycosides synthesized by PTC is given in Tables 20 and 21 along with physical properties of each.

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[134] K. Block and V. Pederson, *J. Chem. Soc. Perkin Trans. 2* , 293 (1974).

Complete NMR spectroscopic data are given in Tables 23 through 28 at the end of the following section.

Table 20 Physical properties and selected NMR spectroscopic data of aryl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosides prepared by phase transfer catalysis.

						Elemental Analysis¥					
						Calculated (%)			Found (%)		
#	Product X	Yield (%)	Melting Point (°C)	$[\alpha]_D$ ($CHCl_3$)	Formula	C	H	N	C	H	N
61	H	93	203.5-204.6	-12.4	$C_{20}H_{25}NO_9$	56.73	5.95	3.31	56.54	6.07	3.65
62	p-Me	84	197.4-197.8	-10.9	$C_{21}H_{27}NO_9$	57.66	6.22	3.20	57.46	6.38	3.12
63	m-Me	86	195.2-196.0	-10.8	$C_{21}H_{27}NO_9$	57.66	6.22	3.20	57.41	6.12	3.54
64	p-*t*-Bu	73	177.1-177.9	-9.2	$C_{24}H_{33}NO_9$	60.11	6.94	2.92	60.10	7.05	2.87
65	p-PH	89	242.2-242.6	-7.8	$C_{26}H_{29}NO_9$	62.52	5.85	2.80	62.47	5.94	2.93
66	p-OMe	85	195.8-196.4	-11.9	$C_{21}H_{27}NO_{10}$	55.63	6.00	3.09	55.69	6.09	3.34
67	p-F	77	197.7-198.4	-12.7	$C_{20}H_{24}NO_9F$	54.42	5.48	3.17	53.88	5.51	3.44
68	p-Cl	66	121.6-213.5	-9.6	$C_{20}H_{24}NO_9Cl$	52.47	5.28	3.06	52.24	5.49	3.23
69	p-Br	88	228.3-228.8	-8.6	$C_{20}H_{24}NO_9Br$	47.82	4.82	2.79	47.65	4.73	2.79
70	p-I	83	252.3-252.9	-4.9	$C_{20}H_{24}NO_9I$	43.73	4.40	2.55	43.55	4.74	2.43
71	p-NO_2	73	237.4-238.4	-9.6	$C_{20}H_{24}N_2O_4$	51.28	5.16	5.98	51.15	5.11	6.05
72	p-CHO	67	226.8-227.6	-16.9	$C_{21}H_{25}NO_{10}$	55.87	5.58	3.10	55.99	5.51	2.96
73	p-CN	80	243.1-244.0	-29.4	$C_{21}H_{24}N_2O_9$	56.25	5.39	6.25	55.84	5.51	6.34

¥ Satisfactory microanalyses (within ± 0.2%)were also obtained for all the halogens.

Table 21 Physical properties of aryl 2-acetamido-2-deoxy-β-D-glucopyranosides

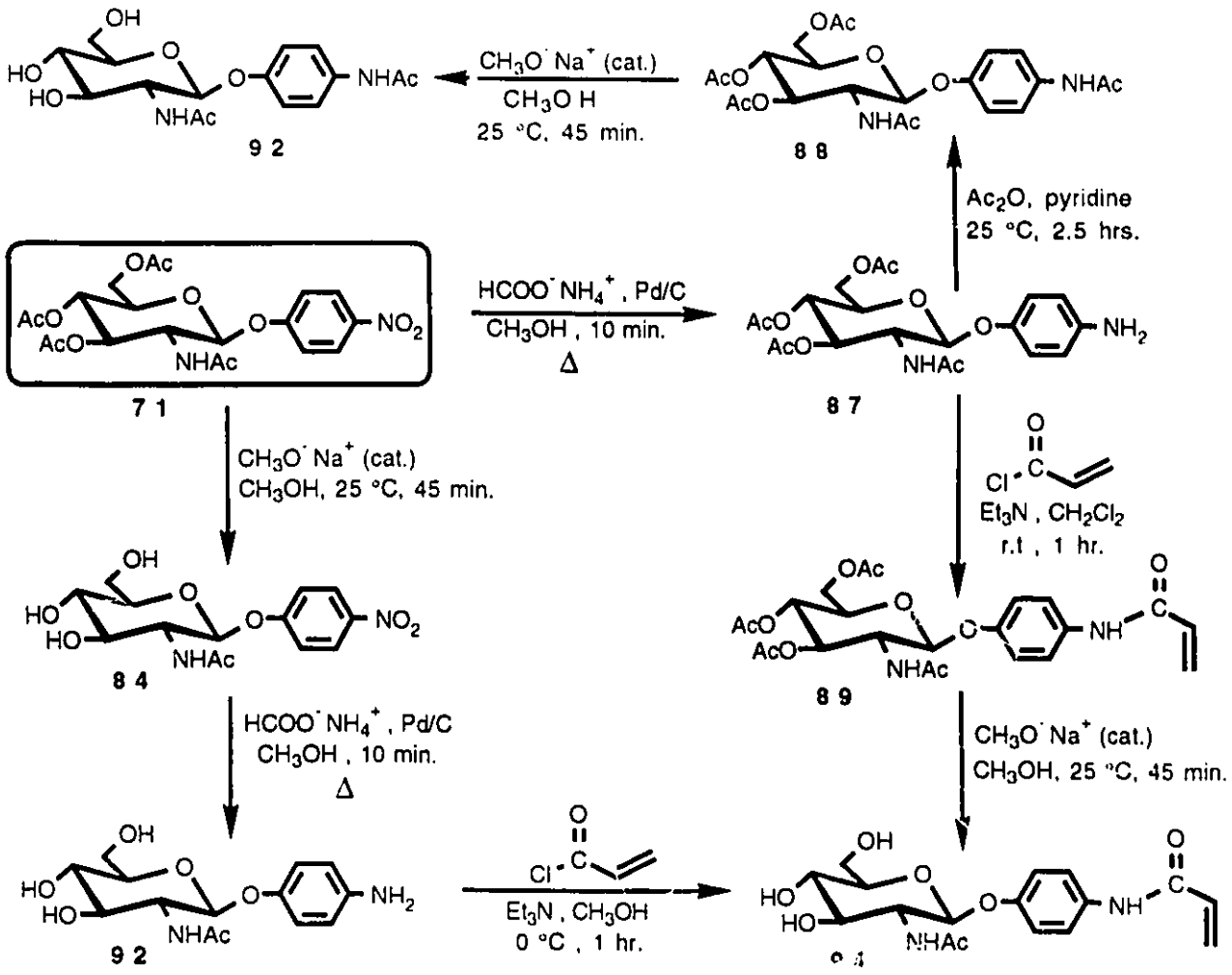
#	Product X	Melting Point (°C)	$[\alpha]_D$ ($CHCl_3$)	Formula	Elemental Analysis¥					
					Calculated (%)			Found (%)		
					C	H	N	C	H	N
74	H	240.9-241.3	-12.3	$C_{14}H_{19}NO_6$	56.78	6.68	4.73	56.56	6.44	4.71
75	p-Me	236.4-236.7	-5.3	$C_{15}H_{21}NO_6$	57.87	6.80	4.50	57.84	6.77	4.41
76	m-Me	225.5-226.3	-9.4	$C_{15}H_{21}NO_6$	57.87	6.80	4.50	58.00	6.81	4.53
77	p-*t*-Bu	205.7-206.3	-1.7	$C_{18}H_{27}NO_6$	61.17	7.70	3.96	61.18	7.51	4.04
78	p-PH	261.7-262.0	+13.7	$C_{20}H_{23}NO_6$	64.33	6.21	3.75	63.94	6.27	3.65
79	p-OMe	245.4-245.7	-6.2	$C_{15}H_{21}NO_7$	55.04	6.47	4.26	55.01	6.45	4.28
80	p-F	242.8-243.2	-14.8	$C_{14}H_{18}NO_6F$	51.16	5.72	4.23	53.33	5.75	4.44
81	p-Cl	249.5-250.1	-3.2	$C_{14}H_{18}NO_6Cl$	50.64	5.47	4.22	50.50	5.50	4.03
82	p-Br	243.9-244.5	+2.7	$C_{14}H_{18}NO_6Br$	44.64	4.68	3.78	44.70	4.82	3.72
83	p-I	249.2-249.6	+9.0	$C_{14}H_{18}NO_6I$	39.92	4.34	3.32	39.73	4.29	3.31
84	p-NO_2	206.2-206.7	-13.2	$C_{14}H_{18}N_2O_8$	49.11	5.17	8.10	49.12	5.30	8.18
85	p-CHO	195.0-195.9	-3.0	$C_{15}H_{19}NO_7$	55.50	5.81	4.45	55.38	5.89	4.31
86	p-CN	211.2-211.5	-4.3	$C_{15}H_{18}N_2O_6$	55.53	5.74	8.76	55.90	5.63	8.69

¥ Satisfactory microanalyses (within ± 0.2%) were also obtained for all the halogens.

To widen the range and nature of aryl substituents several different functional group transformations were performed starting from *p*-nitro- and *p*-formyl-phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides (Scheme 24 and 25). Thus, reduction of the *p*-nitro derivatives **71** or **84** by catalytic transfer hydrogenation[135] with 10% Pd/C and ammonium formate in methanol afforded the corresponding *p*-amino derivatives **87** and **92** in essentially quantitative yields. The amine derivatives could then be acetylated to the corresponding p-*N*-acetyl and p-*N*-acryloyl derivatives. Usual

[135] S. Ram and E. Ehrenkaufer, *Synthesis* , 91 (1988).

N-acetylation, (pyridine, acetic anhydride) of **87** afforded **88**, in a near quantitative yield. Zemplén de-*O*-acetylation of **88** proceeded smoothly to give the deprotected glycoside **93**. *N*-acryloylation of **87** with acryloyl chloride and triethylamine at 0 °C in methylene chloride or in methanol for **92** gave, in 95 ± 2% yields, **89** and **94** respectively. In the latter case, by-products had to be eliminated by treatment with ion-exchange resin. However, the scheme using the fully acetylated glycoside offered practical advantages since extraction of the product in an organic solvent was effective and straightfoward. Compound **94** could then be obtained by Zemplén de-*O*-acetylation with ease in quantitative yield. The p-*N*-acrylamidophenyl glycoside **94** was synthesized to access glycopolymer and neoglycoprotein conjugates by polymerization and Michael addition methodologies, and will be elaborated on further in Chapter 8.



Scheme 24 Synthetic transformations of p-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides

Oxidation of the per-*O*-acetylated *p*-formylphenyl glycoside **72** with bromine in basic methanol solution according to Lichtenthaler *et al.* [136] gave directly the *p*-carbomethoxyphenyl glycoside **90** in 55% yield which was eventually converted to the free acid **96** without difficulty. All attempts at increasing the yield for the bromine oxidation failed. Reduction of the aldehyde **72** with sodium borohydride gave the *p*-(hydroxymethyl) phenyl glycoside **91** in quantitative yield.

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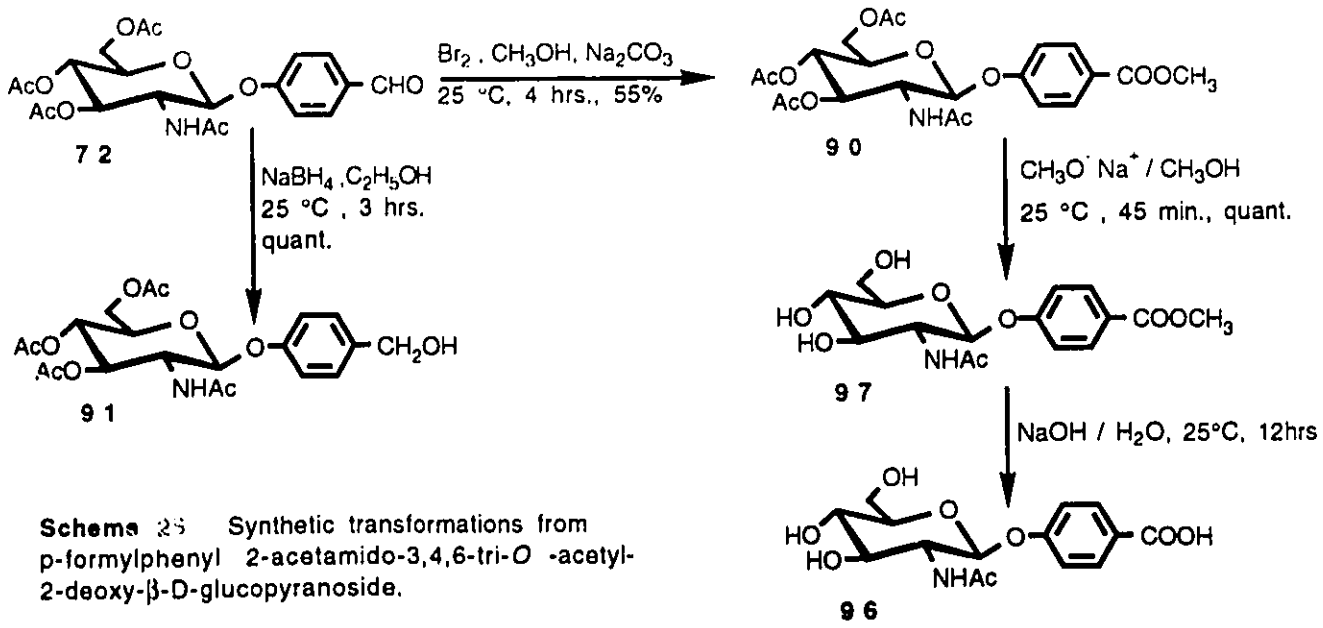
[136] F.W. Lichtenthaler, P. Jarglis and K. Lorenz, *Synthesis*, 790 (1988).



Scheme 25 Synthetic transformations from p-formylphenyl 2-acetamido-3,4,6-tri-*O* -acetyl-2-deoxy-β-D-glucopyranoside.

The new glycosides are listed in Tables 22 and 23 along with some of their physical properties. Complete NMR data can be found in tables 23 through 28.

Table 22 Physical properties and selected NMR spectroscopy data of aryl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosides.

						Elemental Analysis					
						Calculated (%)			Found (%)		
#	Product X	Yield (%)	Melting Point (°C)	$[\alpha]_D$ ($CHCl_3$)	Formula	C	H	N	C	H	N
87	p-NH_2	98	174.2-175.4	-8.6	$C_{20}H_{26}N_2O_9$	54.79	5.98	6.39	54.72	6.22	6.20
88	p-NHAc	96	252.1-253.0	-13.2	$C_{22}H_{26}N_2O_{10}$	55.00	5.87	5.83	55.14	5.81	5.97
89	p-NHC(O)-$CH=CH_2$	96	257.5-258.3	-2.6 (DMSO)	$C_{28}H_{28}N_2O_{10}$	56.09	5.73	5.69	56.12	5.83	5.74
90	p-CO_2Me	55	213.6-214.1	-13.5	$C_{22}H_{27}NO_{11}$	54.89	5.65	2.91	55.00	5.74	3.01
91	p-CH_2OH	98	195.8-196.8	-9.0	$C_{21}H_{27}NO_{10}$	55.63	6.00	3.09	55.60	6.05	3.15

Table 23 Physical properties and selected NMR spectroscopy data of aryl 2-acetamido-2-deoxy-β-D-glucopyranosides.

					Elemental Analysis					
					Calculated (%)			Found (%)		
#	Product X	Melting Point (°C)	$[\alpha]_D$ (DMSO)	Formula	C	H	N	C	H	N
92	p-NH_2	227.5-228.6	+3.7	$C_{14}H_{20}NO_6$	53.84	6.45	8.97	52.75	6.65	8.75
93	p-NHAc	244.9-245.4	+2.2	$C_{16}H_{21}N_2O_7$	54.41	6.02	8.17	54.39	5.99	7.93
94	p-NHC(O)-$CH=CH_2$	248.3-249.0	+8.4	$C_{17}H_{22}N_2O_7$	55.73	6.05	7.65	55.84	6.11	7.43
96	p-CO_2H	amorph.*	-5.7	$C_{15}H_{19}NO_8$	52.79	5.32	4.10	–	–	–
97	p-CO_2Me	230.2-230.6	-5.5	$C_{16}H_{21}NO_8$	54.17	6.13	4.02	54.08	5.96	3.94
98	p-CH_2OH	196.9-198.2	+2.4 (MeOH)	$C_{16}H_{21}NO_7$	55.16	6.47	4.15	55.04	6.47	4.28

* amorphous; lyophilized

Figure 20 Atom labelling scheme for aryl 2-acetamido-2-deoxy-β-D-glucopyranosides and ^1H-^1H COSY spectrum of *p*-chlorophenyl 3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside.

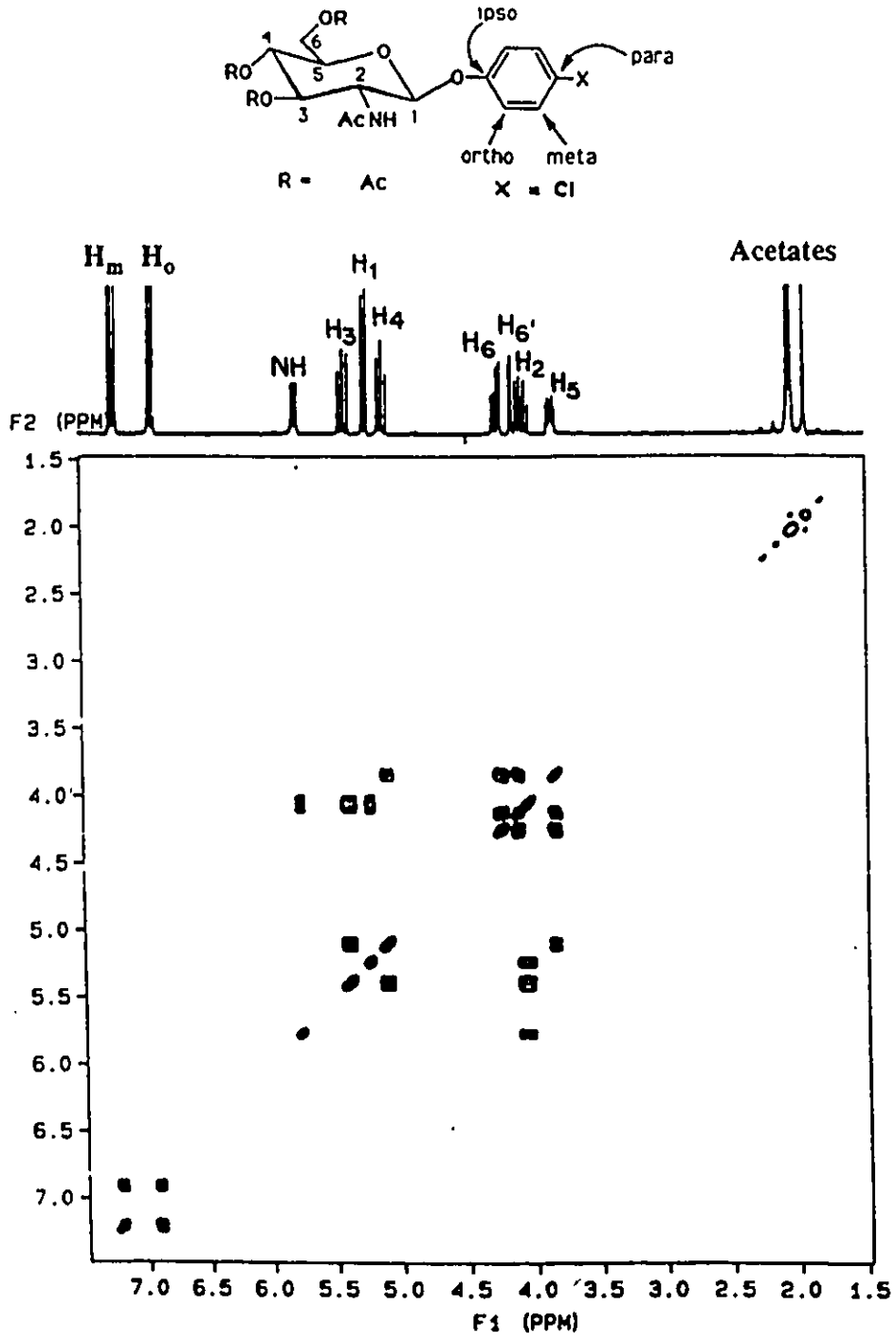


Table 24 ^{1}H-NMR chemical shifts (ppm) of non-acetylated glycosides

Compound[a] X	H-1 (d)	H-2-H-6	NH	H-*meta* (d)	H-*ortho* (d)	X	NAc (s)
H	4.95	3.67–3.77 (2H) 3.17–3.54 4(H)	[b]	7.26 m	7.02–6.90 m		1.82
CH_3	4.86	3.62–3.71 (2H) 3.12–3.51 (4H)	[b]	7.06	6.94	2.18 s	1.81
t-Bu	4.89	3.63–3.73 (2H) 3.18–3.52 (4H)	[b]	7.26	6.85	1.18 s	1.81
Ph	4.99	3.67–3.78 (2H) 3.17–3.55 (4H)	[b]		7.54 m (4H) 7.41 m (2H) 7.30 m (1H) 7.13 d (2H)		1.83
F	4.85	3.66–3.75 (2H) 3.16–3.54 (4H)	[b]	6.91–7.10 m (4H)		—	1.83
Cl	4.91	3.66–3.75 (2H) 3.19–3.53 (4H)	[b]	7.28	6.95	—	1.82
Br	4.92	3.63–3.77 (2H) 3.17–3.51 (4H)	[b]	7.42	6.91	—	1.80
I	4.91	3.65–3.74 (2H) 3.13–3.51 (4H)	[b]	7.57	6.78	—	1.80
OCH_3	4.92	3.23–3.74 (6H)	[b]	6.85 m (4H)		3.63 s	1.84
NH_2	4.68	3.14–3.70 (6H)	[b]	6.69	6.52	[b]	1.82
NHA_c	4.47	3.13–3.75 (6H)	7.81 d	7.46	6.89	9.85 s (NH) 2.00 s (CH_3)	1.81
$NHCOCHCH_2$	4.10	3.15–3.75 (6H)	7.81 d	7.57	6.93	—CH= 6.91 dd *trans* 6.22 dd *cis* 5.72 dd	1.81
NO_2	4.91	3.65–3.74 (2H) 3.14–3.52 (4H)	[b]	7.57	6.78 d	—	1.81
$Me_3N^+I^-$ [c]	5.30	3.94–4.07 (2H) 3.52–3.84 (4H)	[b]	7.80	7.25	3.64 s	2.03
CN	5.07	3.67–3.78 (2H) 3.15–3.52 (4H)	[b]	7.72	7.09	—	1.80
CHO	5.14	3.20–3.74 (6H)	[b]	7.87	7.14	9.89 s	1.79
$COOCH_3$	5.09	3.63–3.76 (2H) 3.17–3.55 (4H)	7.82 d	7.90	7.05	3.81 s	1.79
COOH[c]	5.22	3.93–4.06 (2H) 3.52–3.84 (4H)	[b]	7.78	7.08	[b]	2.02
CH_2OH	5.16	3.91–4.02 (2H) 3.50–3.82 (4H)	[b]	7.36	7.07	4.58 s	2.02
m-CH_3	4.90	3.64–3.73 (2H) 3.18–3.52 (4H)	[b]		7.13 m (1H) 6.76 m (3H)	2.21 s	1.81

[a] *para*-Substituents unless stated otherwise.
[b] Exchanged protons, ND.
[c] Spectrum obtained in D_2O with acetone as internal standard.

Table 25 ^{13}C-NMR chemical shifts (ppm) of non-acetylated glycosides

Compound[a] X	C-1	C-2	C-3	C-4	C-5	C-6	*ipso*	*ortho*	*meta*	*para*	X	NHCOCH$_3$	
H	99.37	55.55	74.15	70.41	77.31	60.80	157.86	116.56	129.65	122.20	—	169.60	23.02
CH_3	99.73	55.59	74.13	70.43	77.26	60.82	155.80	116.58	129.91	131.03	20.06	169.54	23.00
t-Bu	99.55	55.57	74.14	70.45	77.28	60.83	155.66	116.09	126.18	144.52	C 33.78 CH_3 31.23	169.53	22.97
Ph	99.39	55.56	74.10	69.39	77.35	60.80	157.43	116.99	134.3	139.91	*ipso* 134.30 *ortho* 126.45 *meta* 127.90 *para* 127.06	169.60	22.99
F	99.89	55.46	74.02	70.29	77.24	60.72	153.80 d (2.0)[b]	115.80 d (23.1)[b]	117.95 d (8.2)[b]	157.36 d (237.4)[b]	—	169.29	23.05
Cl	99.44	55.46	74.03	70.31	77.32	60.73	156.55	118.38	129.41	125.97	—	169.58	22.96
Br	99.32	55.03	74.00	70.28	77.31	60.70	156.98	118.83	132.31	113.67	—	169.52	22.95
I	99.16	55.41	73.98	70.26	77.29	60.68	157.59	119.23	138.17	85.00	—	169.48	22.94
OCH_3	99.52	55.63	74.16	70.48	77.26	60.88	154.78	114.66	118.04	151.91	56.43	169.61	23.47
NH_2	101.12	55.69	74.19	70.54	77.14	60.93	149.22	114.65	118.16	144.02	—	169.47	23.02
NHAc	99.82	55.53	74.13	70.42	77.25	60.79	153.50	116.79	120.45	134.04	23.70 168.1	169.52	22.98
$NHCOCHCH_2$	99.73	55.44	74.04	70.26	77.29	60.70	153.90	116.94	120.73	126.83	C=O 163.04 —CH= 132.09 CH_2= 133.67	169.57	23.08
NO_2	98.56	55.27	73.89	70.10	77.45	60.55	162.52	116.73	125.90	142.02	—	169.64	22.94
$Me_3N^+I^-$ [c]	99.78	56.07	74.10	70.38	76.89	61.20	158.15	118.59	122.46	142.14	57.98	175.74	22.92
CN	98.52	55.31	73.95	70.18	77.41	60.62	160.89	117.23	134.37	119.03	104.44	169.66	22.95
CHO	98.71	55.61	74.21	70.50	77.42	60.94	162.54	116.94	131.05	132.18	192.22	170.55	23.16
$COOCH_3$	98.29	55.26	73.86	70.06	77.23	60.50	160.85	115.92	130.95	122.98	165.52 51.86	169.05	23.05
COOH[c]	100.04	56.33	74.43	70.57	77.06	61.38	160.12	116.84	132.07	131.67	175.73	176.04	22.90
CH_2OH	99.35	55.48	73.95	70.25	77.13	62.41	156.26	115.99	127.57	135.98	70.67	168.95	23.09
m-CH_3	99.39	55.58	74.16	70.42	77.31	60.80	157.90	113.49 (C-2') 117.32 (C-6')	139.17 (C-3') 122.90 (C-5')	129.38	21.00	169.58	23.04

[a] *para* Substituents unless stated otherwise.
[b] Coupling constants for ^{13}C–^{19}F couplings (Hz).
[c] Spectrum obtained in D_2O with acetone as internal standard.

Table 26 ^{1}H-NMR chemical shifts (ppm) of acetylated glycosides

Compound[a] X	H-1 (d)	H-2 (ddd)	H-3 (dd)	H-4 (dd)	H-5 (ddd)	H-6 (dd)	H-6' (dd)	NH (d)	H-*meta* (d)	H-*ortho* (d)	X	Ac (s)
H	5.25	4.10	5.39	5.13	3.85	4.28	4.13	5.54	7.27 m		Combined m centred at 7.01	1.94, 2.03 2.04, 2.05
CH_3	5.18	4.07	5.37	5.13	3.68	4.27	4.12	5.58	7.05	6.86	2.27 s	1.93, 2.02 2.04, 2.05
t-Bu	5.21	4.09	5.38	5.12	3.83	4.27	4.13	5.54	7.27	6.90	1.26 s	1.93, 2.02 2.04, 2.05
Ph	5.30	4.14	5.41	5.13	3.88	4.29	4.15	5.77	7.48	7.04	7.40 broad m	1.95, 2.03 2.04, 2.06
F	5.18	4.05	5.38	5.11	3.82	4.27	4.13	5.65		6.94 (2H)[b]	—	1.94, 2.02 2.04, 2.05
Cl	5.23	4.06	5.39	5.10	3.84	4.26	4.12	5.77	7.20	6.90	—	1.92, 2.02 2.03, 2.04
Br	5.23	4.06	5.39	5.10	3.84	4.25	4.12	5.77	7.35	6.85	—	1.92, 2.02 2.03, 2.04
I	5.23	4.06	5.39	5.10	3.84	4.26	4.12	5.66	7.54	6.74	—	1.92, 2.02 2.04, 2.05
OCH_3	5.13	4.05	5.37	5.11	3.79	4.27	4.12	5.59	6.92	6.78	3.74 s	1.95, 2.01 2.03, 2.06
NH_2	5.03	4.05	5.34	5.09	3.75	4.25	4.11	5.81	6.79	6.56	NH_2 3.52 s (broad)	1.93, 2.00 2.02, 2.04
NHAc	5.05	4.05	5.22	4.96	3.71	4.15	3.99	7.21	7.32 d	6.81 d	8.75 s, NH 1.78 Ac	1.90, 1.90 1.94, 1.99
NO_2	5.55	ND[c]	5.23	4.96	ND[c]	ND[c]	ND[c]	8.13	8.23	7.24	—	1.77, 1.95 2 × 2.00
CN	5.43	4.05	5.44	5.11	3.92	4.26	4.12	5.92	7.55	7.03	—	1.90, 2.02 2.03, 2.04
CHO	5.42	4.11	5.43	5.12	3.92	4.27	4.14	5.75	7.81	7.07	9.89 s	1.93, 2.03 2.04, 2.05
$COOCH_3$	5.42	4.10	5.45	5.09	3.93	4.24	4.12	6.41	7.89	6.95	3.83 s	1.85, 2.00 2.01, 2.02
CH_2OH	5.24	4.08	5.39	5.12	3.84	4.27	4.13	5.56	7.27	6.96	4.62 d[d]	1.94, 2.03 2.04, 2.06
$NHCOCHCH_2$	5.16	4.12	5.35	5.10	3.81	4.26	4.11	5.97	7.47	6.92	COCH= 6.23 dd H-*trans* 5.73 dd H-*cis* 6.41 dd	1.94, 2.02 2.03, 2.05
m-CH_3	5.22	4.06	5.38	5.11	3.85	4.26	4.12	5.57	7.14	6.79 s (H-2') 6.77, 6.84 (H-4',6')	2.29 s	1.93, 2.02 2.04, 2.05

[a] *para* Substituents unless stated otherwise.
[b] Appears as AB system: one doublet, J = 6.3 Hz.
[c] Overlapping signals.
[d] Appears as AB system: one doublet, J = 3.6 Hz.

Table 27 ^{13}C-NMR chemical shifts (ppm) of acetylated glycosides

Compound[a] X	C-1	C-2	C-3	C-4	C-5	C-6	*ipso*	*ortho*	*meta*	*para*	X	C=O(Ac)	CH_3(NHAc)	CH_3(OAc)
H	98.92	54.66	71.83	68.56	72.03	62.10	157.17	116.96	129.60	123.20	—	169.61, 171.02 170.64, 170.83	23.13	20.47 2 × 20.55
CH_3	99.28	54.50	71.61	68.81	72.16	62.16	155.12	117.02	129.87	132.50	20.41	169.54, 170.70 2 × 170.80	22.89	2 × 20.32 20.41
t-Bu	98.88	54.42	71.51	68.69	72.09	62.08	154.83	116.39	126.22	145.82	C 33.95 CH_3 31.21	169.58, 2 × 170.79 170.87	22.95	20.38 2 × 20.47
Ph[a]	98.13	53.17	70.99	68.36	72.35	61.65	156.58	116.78	139.84, 134.90, 2 × 128.83, 2 × 127.84 126.98, 2 × 126.44			169.29, 169.72 169.81, 170.00 169.55	22.53	20.21 20.25 20.33
F	99.67	54.65	71.83	68.70	72.00	62.11	161.21	115.85 d (13.4)[c]	118.69 d (8.1)[c]	154.84 d (156.5)[c]	—	169.55, 170.69 170.79, 170.85	22.98	20.37 2 × 20.44
Cl	98.79	53.88	71.47	68.48	71.98	61.88	155.62	118.25	129.14	127.69	—	169.40, 170.48 2 × 170.79	22.69	20.22 2 × 20.30
Br	98.70	54.49	71.74	68.57	71.83	62.03	156.16	118.79	132.37	115.51	—	169.55, 170.70 170.83, 170.92	22.97	20.39 2 × 20.49
I	98.54	53.49	71.24	68.36	71.98	61.72	156.83	119.08	137.93	147.37	—	169.17, 170.12 170.19, 170.39	22.51	20.05 2 × 20.11
OCH_3	100.07	54.49	71.68	68.59	72.06	62.07	155.66	114.47	118.63	151.58	55.03	169.60, 170.71 170.83, 170.97	23.08	20.43 2 × 20.53
NH_2[a]	100.16	56.65	71.06	68.53	72.14	61.78	149.58	115.28	118.45	142.41	—	169.20, 170.09 170.25, 170.34	22.47	3 × 20.06
NHAc[a]	99.19	53.31	71.05	68.33	72.06	61.63	152.98	116.94	120.84	133.80	23.34	168.51, 169.09 169.97, 170.11 170.30	22.32	3 × 19.93
NO_2[a]	97.06	52.96	71.16	68.19	72.25	61.50	161.71	116.83	126.03	142.48	—	169.65, 169.95 170.03, 170.35	22.62	20.31 20.41 20.47
CN	97.54	53.43	71.47	68.21	71.71	61.58	159.88	116.98	133.48	147.6	105.57	169.09, 170.12 170.45, 170.61	22.43	3 × 20.01
CHO	97.72	53.86	71.72	68.50	71.95	61.88	161.69	116.67	131.52	131.31	190.77	169.23, 2 × 171.41 170.73	22.67	20.18 2 × 20.24
$COOCH_3$	97.79	54.48	71.80	68.48	71.87	62.00	160.57	116.08	131.50	124.65	166.66 51.92	169.57, 170.72 2 × 170.87	23.00	20.41 2 × 20.51
CH_2OH	99.05	54.35	71.67	68.59	72.03	62.09	156.52	116.93	128.37	135.90	64.30	169.70, 170.91 2 × 170.99	22.96	20.45 2 × 20.52
$NHCOCHCH_2$[a]	98.50	53.39	71.08	68.61	72.09	61.85	153.21	117.21	121.09	134.32	132.14 (—CH—) 127.21 (CH_2=) 163.48 (C=O) CH_3 21.25	169.93, 2 × 170.29 170.61	22.79	20.55
m-CH_3	98.87	54.68	71.??	68.69	72.11	62.22	157.20	113.75 (C-2') 117.73 (C-6')	139.7 (C-3') 123.92 (C-5')	129.27		169.58, 170.60 170.77, 170.97	23.08	20.42 20.47 2 × 20.50

[a] *para* Substituents unless stated otherwise
[b] Spectrum obtained in DMSO-d_6
[c] Coupling constants are for ^{13}C-^{19}F couplings
[d] Spectrum obtained in 10% DMSO-d_6 in $CDCl_3$ solution

Table 28 ^1H-^1H coupling constants (Hz) of non-acetylated glycosides [a]

Compound[a,b] X	J(12)	J(H-2, NH)	J(om)	J(X)
H	8.4	[c]	[d]	[d]
CH_3	8.4	[c]	8.7	—
t-Bu	8.5	[c]	8.9	—
Ph	8.4	[c]	[d]	[d]
F	8.4	[c]	[d]	—
Cl	8.4	[c]	9.0	—
Br	8.5	[c]	9.0	—
I	8.4	[c]	8.9	—
OCH_3	8.4	[c]	[d]	—
NH_2	8.3	[c]	8.8	[c]
NHAc	8.4	8.9	10.0	—
$NHCOCHCH_2$	8.4	9.2	9.0	J_{trans} = 17.1 J_{cis} = 9.8 J_{gem} = 2.6
NO_2	8.1	[c]	8.9	—
	8.4	[c]	9.4	—
CN	8.4	[c]	8.9	—
CHO	8.4	[c]	8.8	—
$COOCH_3$	8.5	9.0	8.9	—
COOH	8.5	[c]	8.9	—
CH_2OH	8.4	[c]	8.7	—
m-CH_3	8.5	[c]	[d]	—

[a] The proton signals from H-2 to H-6,6' give either two multiplets or a single multiplet as indicated in Table 1.
[b] *para* Substituents unless stated otherwise.
[c] Exchanged.
[d] Unresolved.

Table 29 ^1H-^1H coupling constants (Hz) of acetylated glycosides

Compound[a] X	J(12)	J(23)	J(34)	J(45)	J(56)	J(56')	J(66')	J(H-2, NH)	J(om)
H	8.0	10.2	9.4	9.5	5.2	2.6	12.3	8.9	[b]
CH_3	8.2	10.5	9.3	9.3	5.4	2.5	12.2	8.9	8.4
t-Bu	8.1	10.4	9.3	9.4	5.2	2.5	12.3	8.9	8.7
Ph	8.3	10.5	9.3	9.5	5.3	2.5	12.2	8.9	8.8
F	8.3	10.5	9.3	9.8	5.3	2.6	12.2	8.6	[c]
Cl	8.2	10.6	9.3	9.5	5.4	2.5	12.2	8.8	9.0
Br	8.1	10.5	9.4	9.5	5.4	2.4	12.2	8.7	8.6
I	8.3	10.4	9.3	9.5	5.4	2.4	12.2	8.3	8.9
OCH_3	7.5	10.0	9.3	9.6	5.2	2.5	12.3	8.3	8.9
NH_2	8.4	10.5	9.3	9.6	5.2	2.5	12.2	8.8	8.9
NHA_c	8.4	10.3	9.6	9.7	5.3	2.5	12.3	8.2	9.0
NO_2	8.5	10.3	9.4	9.6	[b]	[b]	[b]	11.0	9.2
$NHCOCHCH_2$	8.1	10.3	9.5	9.3	5.7	2.4	11.4	8.4	8.8
CN	8.1	10.2	9.6	9.5	5.6	2.3	12.3	8.6	8.6
CHO	8.1	10.4	9.2	9.4	5.5	2.5	12.2	8.4	8.7
$COOCH_3$	8.4	10.5	9.5	9.7	5.7	2.4	12.3	8.6	9.0
CH_2OH	8.3	10.3	9.4	9.7	5.4	2.6	12.2	9.0	8.7
m-CH_3	8.2	10.0	9.2	9.4	5.4	2.4	12.3	8.9	[d]

[a] *para* Substituents unless stated otherwise.
[b] Unresolved
[c] Appears as AB system: doublet, J = 6.3 Hz.
[d] $J(op)$ or $J(mp)$ = 7.6 Hz; $J(o'o)$, $J(op)$ and $J(o'p)$ = 0 Hz.

5.3 NMR chemical shift correlations

Linear free energy relationships have found increasing utility in the study of substituent effects in NMR spectroscopy[137,138]. In general, the contributions to chemical shift changes induced by substituents are attributable to a combination of resonance and inductive or field effects and depend on the type of system under investigation[137]. With a series of *p*-substituted phenyl β-D-*N*-acetylglucopyranosides at hand, the substituent-induced chemical shifts (SCS) of a series of aryl β-D-*N*-acetyl glucopyranosides was investigated. With these aryl glycosides, SCS have been shown to extend through the aglycon oxygen to both the anomeric carbon and proton as well as C-2. Since the chemical shifts of nuclei are intimately related to electron density, a correlation between Hammett σ-constants and the chemical shifts of H-1, C-1 and C-2 would be expected. Such a correlation has been observed with substituent constants designed to describe resonance contributions towards electronic density. This suggests that electronic effects of the *para*-substituents, felt by the anomeric centre, are mediated through the aglycon oxygen connecting the sugar and phenyl fragments via p-π orbital overlap.

Good correlations have also been observed between calculated and measured chemical shift values of some aromatic carbon nuclei in the aglycon.

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[137] For an excellent review on correlation of NMR chemical shifts with Hammett σ values see D.F. Ewing in *Correlation Analysis Chemistry: Recent Advances* , Chap. 8, N.B. Chapman and J. Shorter (Eds.), Plenum Press, New York, 1978.

[138] a) S. Ehrenson, R.T.C. Brownlee and R.W. Taft, *Prog. Phys. Org. Chem.*, *10* , 1 (1973); J. Bromilow, R.T.C. Brownlee, R.D. Topsom and R.W. Taft, *J. Am. Chem. Soc.*, *98* , 2020 (1976); O. Exner and M. Budesinsky, *Magn. Reson. Chem.*, *27* , 585 (1989)

General theory on Hammett type linear free-energy relationships

In 1937 Hammett[139] recognized that electronic influence of a substituent could be assessed from the reactivities of substituted benzenoid derivatives (k) with respect to unsubstituted derivatives (k_o) by the equation:

$$\log (k/k_o) = \rho\sigma \qquad (8)$$

or in terms of free energy:

$$\Delta\Delta G_o^\circ = -2.3\ RT\rho\sigma \qquad (9)$$

where σ is a Hammett constant for the substituent in question and ρ a proportionality constant which relates σ values to different reactions, mechanisms and conditions. R and T are the gas constant and temperature respectively. Since the inception of this theory, Hammett type linear free energy relationships have been used to successfully predict and correlate a wide range of chemical and physical properties for a variety of compounds and reactions. Much of this success is due to refinements of the Hammett equation and σ values[140]. Refinements to σ values have been directed in many directions. For example, σ values corresponding to different substitution patterns such as σ_{para} and σ_{meta} have met with great success. As well, σ values have been separated in terms of σ_R and σ_I parameters to quantitate subsituent influences in terms of resonance and inductive effects respectively (equations 10 and

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[139] L.P. Hammett, *J. Am. Chem. Soc.*, *59* , 96 (1937).

[140] There has been a variety of approaches to refining Hammett type correlations and σ parameters. A detailed discussion on the theory of linear free energy relationships and the Hammett equation is beyond the scope of this dissertation. The reader is directed to reviews on the subject, in particular: a) T.H. Lowry and K.S. Richardson, *Mechanism and Theory in Organic Chemistry* , 2nd Ed., Harper and Row, New york, 1981. p. 130-142; b) C. Hansch, A. Leo and R.W. Taft, *Chem. Rev.*, *91* , 165 (1991); c) O. Exner, *Correlation Analysis of Chemical Data* , Plenum Press, New York, 1988; d) N.B. Chapman and J. Shorter (Eds.), *Correlation Analysis in Chemistry: Recent Advances* , Plenum Press, New York, 1978.

11)[141].

$$\sigma = \sigma_R + \sigma_I \qquad (10)$$

$$\rho\sigma = \rho_R\sigma_R + \rho_I\sigma_I \qquad (11)$$

A particularly important refinement to Hammett σ values has been the introduction of the dual coefficients σ_p^+ and σ_p^-. These express enhanced mesomeric interaction and are to be used when the reaction site is directly conjugated with the substituents. σ_p^+ values are defined for electron-donor substituents and σ_p^- for acceptors. The σ coefficients introduced briefly here are among the most basic ones used in correlation analyses and have proven to be sufficient and well suited for the correlations analyses in the section as well as in section 5.4.

The initial idea to this work, born out of simple curiosity, was to see if electron donating/withdrawing effects of *para* substituents in aryl β-D-*N*-acetylglucosaminides **59** were carried through to the anomeric proton or carbon. If such was the case, correlation between NMR chemical shifts (δ) and Hammett-type parameters (σ) should be observed. With the initial NMR data obtained routinely after each synthesis (Tables 23, 24, 25 and 26), trends were clearly observed between both anomeric carbon and proton chemical shifts ($\delta_{C\text{-}1}$ and $\delta_{H\text{-}1}$) and established σ_p and σ_R values in both series of acetylated and non-*O*-acetylated β-D-*N*-acetylglucopyranosides. No trends could be observed for the inductive parameter σ_I. Having noted these trends, controlled experiments were performed to determine whether or not a strong Hammett-type correlation truly existed between the variables of interest.

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[141] There have been various treatments for quantifying resonance and field or inductive effects. The reader is directed to reference # 140 for more detailed descriptions.

Because the original ^1H and ^{13}C-NMR spectra for the title compounds were recorded for samples at arbitrary concentrations, an investigation of concentration dependence on δ_{H-1} and δ_{C-1} was performed. Using *p*-chlorophenyl-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside **68** in $CDCl_3$, ^{13}C-NMR and ^1H-NMR spectra were recorded for various fixed concentrations. The results shown in Table 21 and plotted in Figures 22 and 23 demonstrate that a strong concentration dependence in the form of a quadratic expression exists for δ_{H-1} and δ_{C-1}. This dependence has been correlated with excellent accuracy (correlation coefficient Irl = 1.000 for δ_{C-1} and r=0.998 for δ_{H-1}). Also, the concentration dependence for δ_{H-1} and δ_{C-1} are in the reverse sense. δ_{H-1} is directly proportional to sample concentration while δ_{C-1} is inversely proportional.

Table 30 C-1 and H-1 NMR chemical shifts of **68** as a function of sample concentration in $CDCl_3$.

Concentration		NMR chemical shifts (ppm)*	
mg/600µl	mM	δ_{H-1}[a]	δ_{C-1}[b]
2.0	0.728	5.2230	-
4.0	1.46	5.2255	-
6.0	2.18	5.2270	-
10.0	3.64	5.2305	99.018
15.0	5.64	5.2370	98.999
25.0	10.9	5.2470	98.960
50.0	18.2	5.2650	98.892
75.0	27.3	-	98.846

* determined with a Varian XL-300 spectrometer using a "double precision acquisition" technique

a) determined at 299.943 MHz.

b) determined at 75.429 MHz.

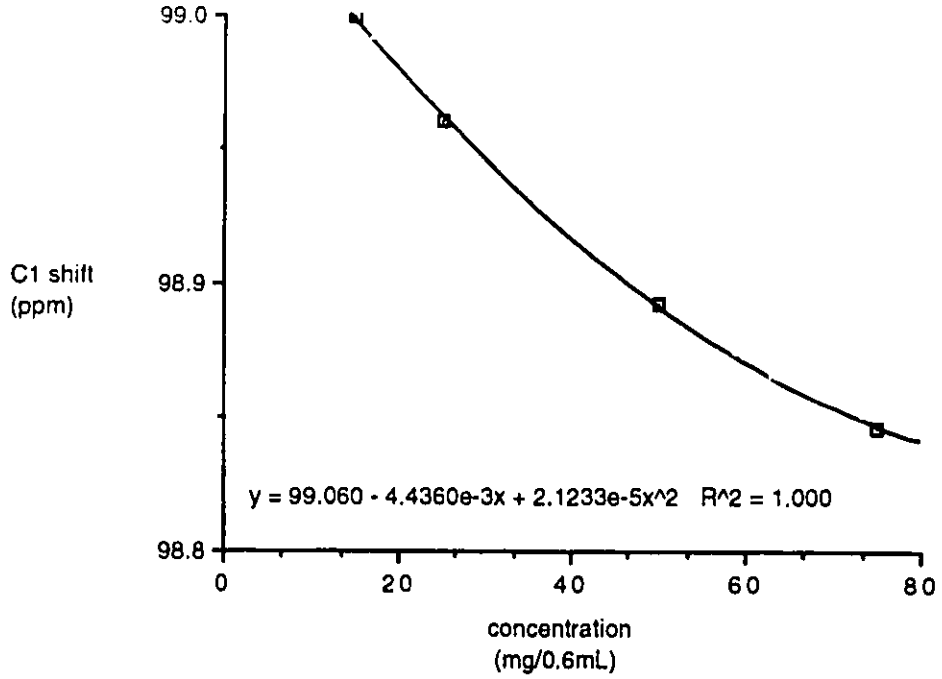

Figure 21 C1 chemical shift (δ_{C-1}) of **68** as a function of sample concentration in $CDCl_3$.

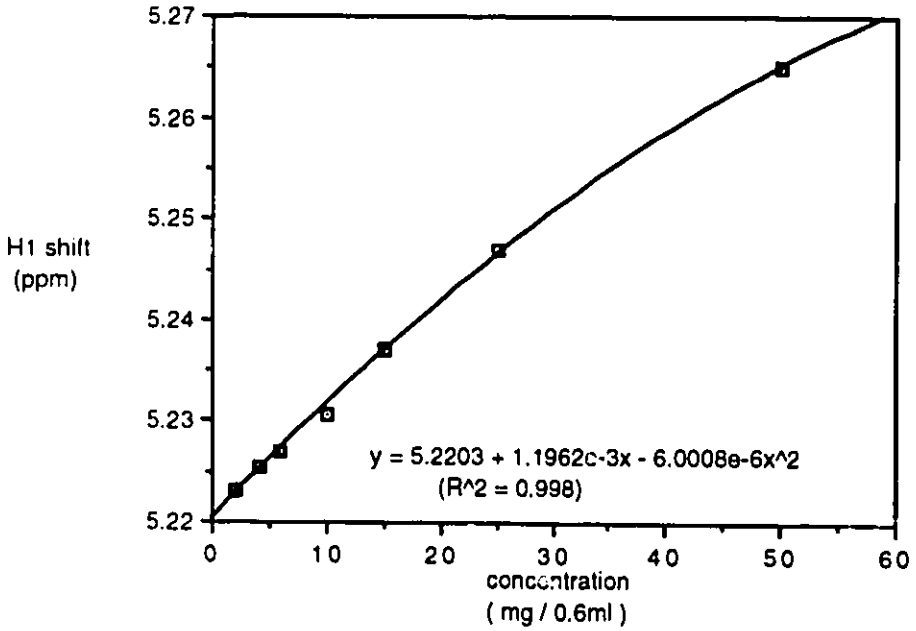

Figure 22 H1 chemical shift (δ_{H-1}) of **68** as a function of sample concentration in $CDCl_3$.

Having noted a strong concentration dependence on the chemical shifts of interest, standard conditions were set for measuring NMR spectra for a set of 9 different *p*-substituted phenyl β-D-*N*-acetylglucopyranosides. Proton chemical shifts were measured for all compounds at a concentration of 6.7 mg/ml of solvent while chemical shifts carbon resonances were measured at a concentration of 5.43×10^{-3} M in the appropriate solvent. Spectra for acetylated glycosides (OAc) were measured for solutions in $CDCl_3$ while non-acetylated glycosides (OH) were measured for solutions in $DMSOd_6$. All NMR measurements for each series were performed at 23 ± 1 °C during one sitting to minimize drifts due to external influences. Spectra were recorded in triplicate for each compound to within ±0.002 ppm for proton spectra and to within ±0.01 ppm for carbon spectra.

Table 31 Selected chemical shifts (ppm) for *para*-substituted phenyl β-D-*N*-acetylglucopyranosides.†

Substituent	$\delta_{H\text{-}1(OAc)}$	$\delta_{H\text{-}1(OH)}$	$\delta_{C\text{-}1(OAc)}$	$\delta_{C\text{-}1(OH)}$	$\delta_{C\text{-}2(OAc)}$	$\delta_{C\text{-}2(OH)}$
NH_2	5.051	4.673	100.42	101.00	54.75	60.88
OCH_3	5.122	4.798	100.10	100.39	54.81	60.79
CH_3	5.178	4.874	99.34	99.58	54.77	60.76
H	5.245	4.943	98.91	99.21	54.74	60.73
F	5.182	4.874	99.64	99.90	54.84	60.74
Cl	5.225	4.931	99.00	99.28	54.78	60.67
CHO	5.426	5.127	97.75	99.36	54.70	60.59
CN	5.403	5.095	97.73	98.37	54.71	60.56
NO_2	5.446	5.160	*	98.42	*	60.52

* *p*-nitrophenyl-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside was not soluble at the required concentration for ^{13}C-NMR spectra.

† refer to text for experimental conditions and chemical shift designations.

Table 31 lists measured chemical shifts of interest as a function of the corresponding *para*-substituent. These chemical shifts values were then plotted as a function of various Hammett-type σ

substituent constants. In particular, correlations were investigated with σ_p, σ_R, σ_I and the enhanced mesomeric contants $\sigma_p^{+/-}$[142]. A linear regression analysis was performed on all data sets and the resulting correlation coefficients (r) are shown in Table 32.

Table 32 Correlation coefficients as a function of σ parameters for each data set described in Table 22.

Chemical shift set	σ_p	σ_R	σ_I	$\sigma_p^{+/-}$
$\delta_{H\text{-}1(OAc)}$	0.946	0.963	NC	0.987
$\delta_{H\text{-}1(OH)}$	0.975	0.958	NC	0.994
$\delta_{C\text{-}1(OAc)}$	0.916	0.981	NC	0.974
$\delta_{C\text{-}1(OH)}$	0.933	0.946	NC	0.975
$\delta_{C\text{-}2(OAc)}$	NC	NC	NC	NC
$\delta_{C\text{-}2(OH)}$	0.986	0.903	NC	0.986

NC = no correlation

Linear regression analysis of the data sets show a definite trend in chemical shifts with respect to the Hammett constant σ_p (r = 0.916 to 0.986) for anomeric carbons and protons in both the acetylated (OAc) and non-acetylated (OH) glycosides. A good correlation is also observed for the C-2 chemical shifts of non-acetylated glycosides (r = 0.986) but no correlation observed for C-2 acetylated derivatives. The correlations improve for anomeric carbons and protons when inductive contributions of each substituent are removed and chemical shifts are compared to resonance contributions only (σ_R). This is justified as no correlation is observed between chemical shifts and the inductive parameter σ_I. Thus, the chemical shifts studied here appear to be influenced mainly by resonance effects. Recent literature reports[143,137,138c,d] have shown good correlations between chemical shifts and enhanced mesomeric coefficients $\sigma^{+/-}$, and discuss the better suitability of these parameters in correlating chemical shifts in aromatic systems. When anomeric carbon and

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[142] Values used for each σ constant are those suggested by Exner, Chap. 10 ref. 139d and are equivalent to those listed by Hansch, Leo and Taft (ref. 139b).

[143] B.M. Lynch, *Org. Magn. Reson.*, *6*, 190 (1974) and references cited within.

proton chemical shifts of acetylated and non-acetylated glycosides are correlated to σ^+/σ^- values, correlations become very good to excellent (r = 0.974 to 0.994) and are improved further when outlying data for the fluorinated derivatives are omitted (r = 0.987 to 0.997). To support any serious claims that there is a "precise" parallel between δ and σ, the linear correlation must be extremely good, i.e., significant to at least the 0.1% level (r = 0.925 or better for n=8)[136]. Such a good correlation has been shown here to exist through a total of 6 and 7 bonds away from the aryl substituents.

There is no doubt that the correlations described above are valid. However, some interesting curiosities are observed when the data are plotted. First, trends for carbon and proton chemical shifts are inversed with respect to σ parameters. As expected on a purely empirical basis, $\delta_{H\text{-}1}$is directly proportional to σ constants and thus inversely proportional to electron density in the aromatic ring. However, $\delta_{C\text{-}1}$ and $\delta_{C\text{-}2}$ values are directly proportional to electron density in the aromatic ring ($\delta_C \propto 1/\sigma$). This demonstates that two different mechanisms exist for the transfer of electron density around the carbon and hydrogen nuclei and that the notion of simple substituent electronegativity is insufficient to describe substituent-induced chemical shifts at the anomeric centre. The rationalization of this effect in terms of the relative importance of various nuclear shielding contributions to δ [137] are purely of a physical chemistry nature and far beyond the scope and main topic of this dissertation. A more in-depth analysis for the rationalization of these results will have to wait for a possible future collaboration with an expert in the field.

A second curiosity in the analysis has been observed when comparing differences in chemical shifts ($\Delta\delta_{C\text{-}1}$ and $\Delta\delta_{H\text{-}1}$) of substituted derivatives with respect to unsubstituted phenyl β-D-*N*-acetylglucopyranosides as is done in σ scales. Here, chemical shift differences are almost identical between acetylated and non-acetylated glycosides, i.e., $\Delta\delta_{C\text{-}1(OAc)} = \Delta\delta_{C\text{-}1(OH)}$ and $\Delta\delta_{H\text{-}1(OAc)} = \Delta\delta_{H\text{-}1(OH)}$. In other words, the substituent's effect on

anomeric carbon and proton chemical shifts are structure (acetylated versus non-acetylated glycosides), and importantly, solvent independent ($CDCl_3$ versus $DMSOd_6$). This confirms that no substituent field effects contribute to δ_{C-1} or δ_{H-1} and that substituent induced chemical shifts in aryl glycosides are mediated purely through resonance interactions. The observations discussed above are described in Figures 23 and 24.

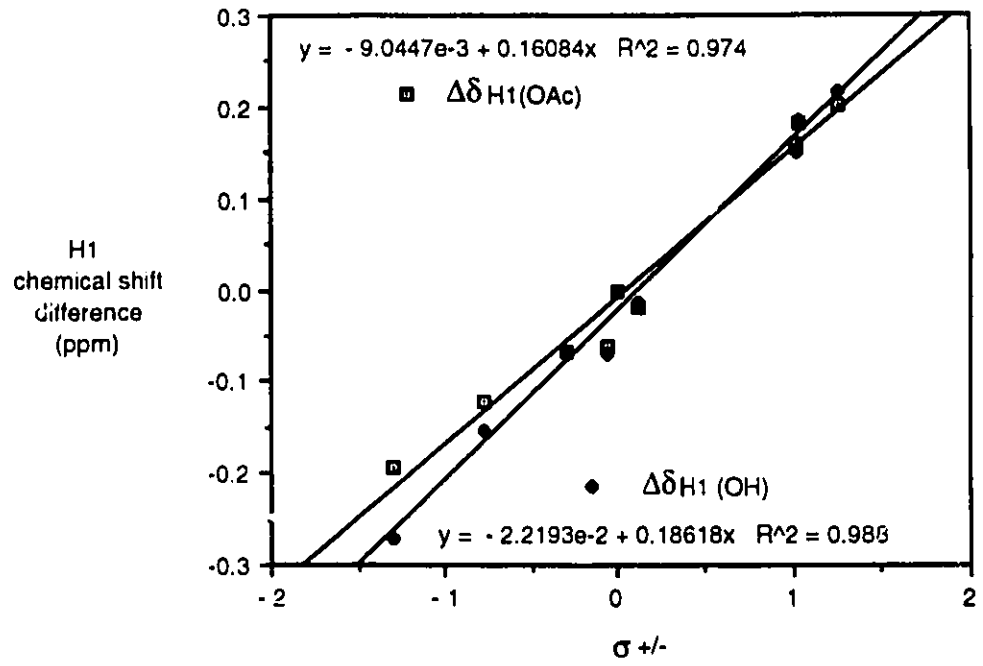

Figure 23 $\Delta\delta_{H-1}$ for *p*-substituted phenyl β-D-*N*-acetylglucopyranosides as a function of $\sigma^{+/-}$ values.

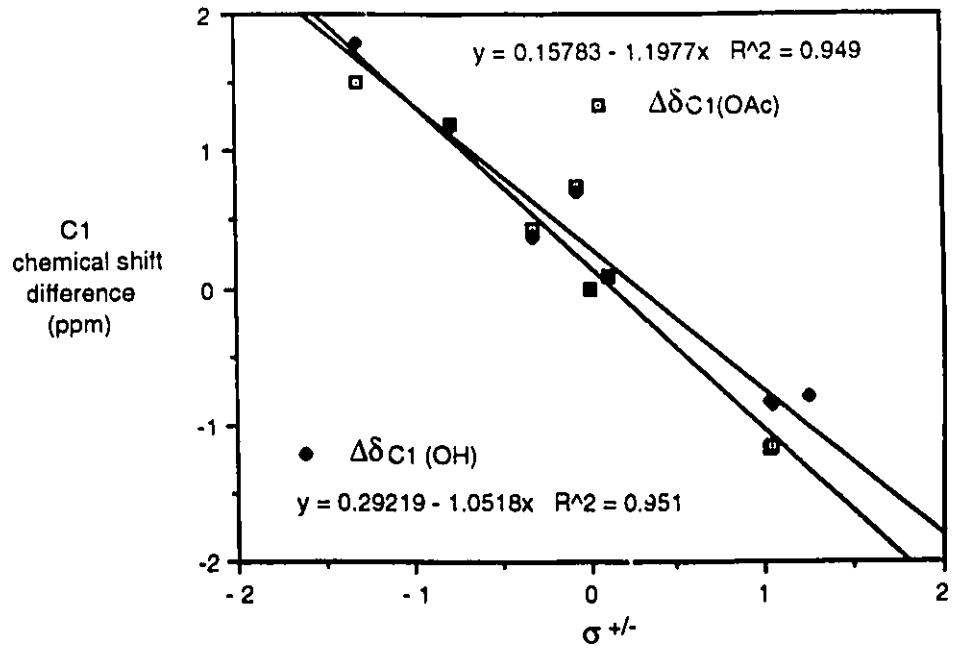

Figure 24 $\Delta\delta_{C\text{-}1}$ for *p*-substituted phenyl β-D-*N*-acetylglucopyranosides as a function of $\sigma^{+/-}$ values.

In light of the observations discussed above, correlation analysis of substituent-induced chemical shifts was performed on *p*-substituted anisoles (*p*-X-C_6H_4-OCH_3), which are conceptually similar in structure to the aryl glycosides studied above, to see if the same effects were also manifested in the anisole system. The results obtained confirmed a previous analysis of anisoles[144] in which subsituent induced chemical shifts could not be correlated to a simple Hammett parameter. For these compounds, only a Dual Substituent Parameter (DSP) approach involving σ_R and σ_I as described in equation 11 could provide good correlations. Thus, although anisoles and the aryl glycosides studied are similar in structure with the anomeric centre resembling the methoxy group of anisoles, two different mechanisms are involved in electron density distribution to the proton and carbon nuclei of interest. In anisoles, inductive field effects appear to be important in affecting chemical

[144] J. Bromilow, R.T.C. Brownlee and D.J. Craik, *Aust. J. Chem.*, *30*, 351 (1977).

shifts while they are unimportant in the aryl glycosides. This result might suggest that the sugar ring oxygen may play a role in the case of substituent-induced chemical shifts at the anomeric centre of aryl glycosides.

5.4 Binding inhibition of wheat germ agglutinin with aryl 2-acetamido-2-deoxy-β-D-glucopyranosides

The importance of protein binding to various organic molecules in terms of struture-activity relationships has long been of interest to biochemists and pharmacologists[145]. The binding of aryl glycosides to lectins, in particular, has also been investigated in the case of Concanavalin A (Con A, *Canavalia ensiformis*) and shown to correlate to various substituent parameters[146]. Binding affinity was reported to be highly dependant on electronic properties of the substituent. As stated at the beginning of this chapter, various *p*-substituted phenyl β-D-*N*-acetylglucopyranosides were synthesized to probe the carbohydrate binding site of WGA. Because the X-ray[118] of this lectin has revealed the presence of an electron-rich tyrosine residue (tyr 64) in close proximity to the anomeric centre of a complexed *N*-acetyl-D-glucopyranoside unit, the hypothesis of electron donor-acceptor (EDA) complexation was put forward to possibly explain the enhanced inhibitory potency of *p*-nitrophenyl β-D-N-acetylglucopyranoside **84** over the corresponding free monosaccharide (Tables 17 and 18). Thus, binding inhibition of WGA was performed by ELLA with various β-D-*N*-acetylglucopyranosides bearing *para* substituents of various electron donating or withdrawing capacities to investigate the possibility of EDA complexation within the WGA binding site. The reasoning behind this

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145 For an excellent review on correlation of stucture-activity relationships for biochemical systems see C. Hansch in *Correlation Analysis Chemistry: Recent Advances*, Chap. 9, N.B. Chapman and J. Shorter (Eds.), Plenum Press, New York, 1978.

146 F.G. Loontens, J.P. Van Wauve, R. De Gussem and C.K. De Bruyne, *Carbohydrate Research ,30* , 51 (1973) and references cited within.

experiment was that if EDA complexation was occuring, inhibition potency and hence binding of WGA to the aryl glycosides would increase as substituents became more electron-withdrawing. A good indication of electron donating or withdrawing potential for aromatic substituents is Taft's inductive parameter σ_I [142]. The inhibitory strength of each inhibitor was evaluated and correlated to σ_I values with the contention that if EDA complexation was occuring within WGA's binding site, a linear correlation between inhibition potency and corresponding σ_I values should be observed. In fact, such a linear correlation was demonstrated and thus suggests that enhanced binding of WGA to *p*-nitrophenyl *N*-acetyl-β-D-glucopyranoside is not due to a simple increase in hydrophobic interaction by the aglycon. An additional electronic contribution from the substituent is also important. The inhibition data gathered shows no correlation with any other substituent parameters such as σ_p, σ_R, $\sigma^{+/-}$, or the hydrophobicity constants π [144]. Hydrophobicity contributions from substituents in binding to Con A by *p*-substituted glycosides also proved to be insignificant compared to electronic effects[146]. Multiple parameter analyses using combinations of the above constants were not performed to improve the regression fits because of the complex nature of such statistical analyses, and correlations with σ_I alone are very good.

Table 34 Inhibitor of binding (ELLA) of WGA-HRP to β-D-GlcNAc-**S**-CPA (**54**) by *p*-substituted phenyl β-D-*N*-acetylglucopyranosides.

Inhibitor, substituent		[inhibitor] for 50% inhibition (nM)	Relative inhibitory potency	log M_{50}	σ_I *
80,	F	1.0	1.0	-9.00	0.52
84,	NO_2	1.8	0.56	-8.74	0.57
85,	CHO	1.7	0.59	-8.77	0.25
79,	OCH_3	2.6	0.38	-8.57	0.25
92,	NH_2	3.75	0.27	-8.43	0.01
74,	H	4.0	0.25	-8.40	0
75,	CH_3	4.3	0.23	-8.37	-0.05

* Values taken from reference 143

As seen in Table 33, binding inhibition is increasingly more effective as the substituent in *p*-subsituted phenyl β-D-*N*-acetylglucopyranosides increases in electron withdrawing potential. This effect can be correlated to σ_I values with excellent agreement (r=0.999) when values for the fluorine and formyl substituents are omitted and inhibitor potency is calculated relative to the *p*-nitro derivative (Fig. 25). Similarly, correlation to σ_I using the logarithm of the molar concentration required for 50% inhibition (log M_{50}) as a relative indicator of inhibitory strength[146] is also very good (|r|=0.994; Fig. 26). This correlation is significant to just above the 99.9% confidence level (r=0.991 for n=5)[137], and does apply to a range of substituents having different electronic properties. Because of the multicomponent nature of ELLA, the molar concentration required for 50% inhibition of ELLA (M_{50}) or the relative inhibitory strengths derived from M_{50} have no simple relation with K, the equilibrium constant of the interaction[70,147]. A linear free-energy energy relation does apply however as the experimental values are reproducible within constant experimental conditions, and inhibition strength is expressed as relative values[146,148]. A similar regression analysis performed on Con A required a dual substituent parameter approach involving both inductive and resonace contributions of the substituents[146]. In this study, inductive contributions of the substituents appear to be sufficient in correlating the inhibition data.

Data for the formyl and fluoro derivatives have been omitted from the final simple component (σ_I) correlation analysis as this data deviates strongly from the remaining data which is well behaved. Omission of the *p*-formyl data in the correlation analysis is justified since the aldehyde can undergo Schiff base[149] formation

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[147] C.J. van Oss, D.R. Absolom, *Nature and Thermodynamics of Antibody-Interactions* in *Molecular Immunology* , Chap 16 M.Z. Atassi, C.J. van Oss, D.R. Absolom (eds.), Marcel Dekker, New York, 1984.

[148] J.L. Webb, *Enzyme and Metabolic Inhibitors* , Vol. 1, Academic Press, New York, 1963.

[149] R.L. Reeves, *The Chemistry of the Carbohydrate Group* , Vol 1, S. Patai (Ed.),

with the many free amine groups on the proteins. Such glycosylated WGA-HRP conjugates could result in inter WGA complexation that would be stronger than complexation to the free inhibitor due to the multivalent nature of such interaction. As observed, this would artificially enhance the *p*-formyl inhibitor's potency. In a similar study[146] involving the lectin Con A, it has been suggested that hydrogen bonding of the formyl group might lead to an artificial increase in binding strength to the lectin. In our study however, the *p*-methoxy and *p*-amino (which is extremely susceptible to protonation) derivatives fit very well in the correlations which suggests that hydrogen bonding to the aglycon has no influence in *p*-subsituted phenyl β-D-*N*-acetylglucopyranosides binding to WGA . Omission of the *p*-fluorine value seems less justified and is based solely on previously documented observations that halides, and fluorine in particular, often do not fit in many Hammett-type correlations and are often treated as a different sub-class[142cd,146].

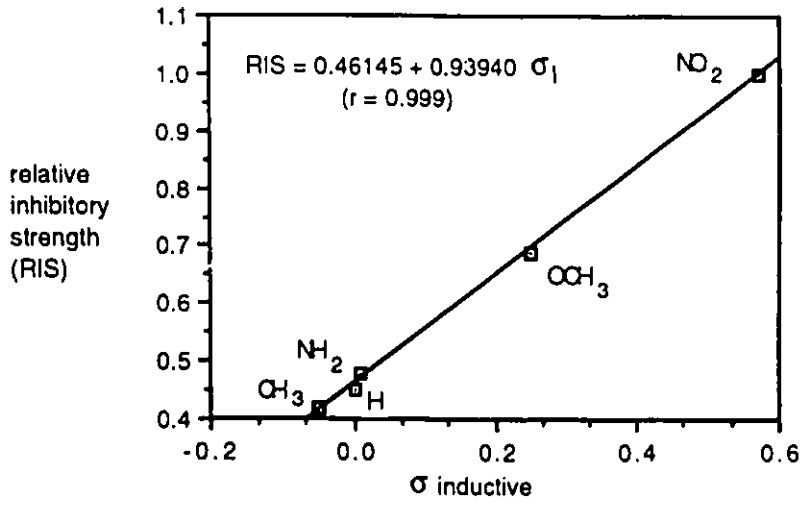

Figure 25 Correlation of relative inhibitory potency of *p*-subsituted phenyl β-D-*N*-acetylglucopyranosides to corresponding σ_I values.

Wiley, London, 1966. pp.567.

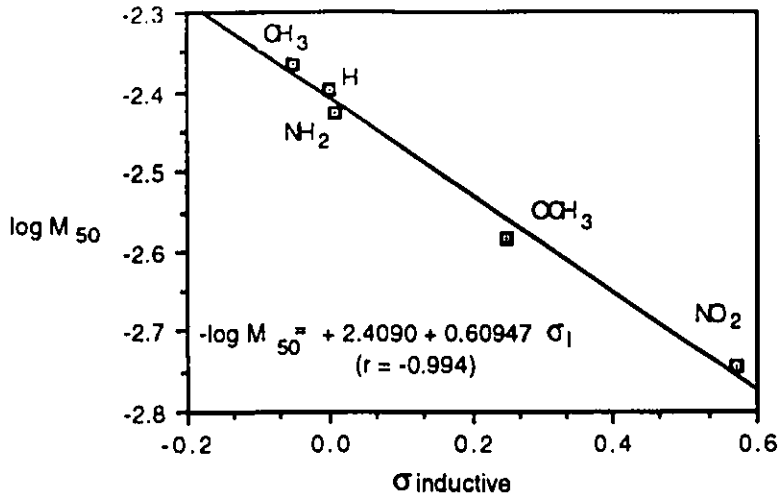

Figure 26 Correlation of inhibitory potency of *p*-subsituted phenyl β-D-*N*-acetylglucopyranosides, as a function of log M_{50} , to corresponding σ_I values.

Because a good correlation appears to exist between inhibition potency and electronic density in the aromatic ring of inhibitors, as quantitated by σ_I values, the hypothesis of EDA complexation within WGA's binding site appears quite plausible. Unlike the work done with Con A[146] , the Hammett reaction constant ρ in this study is positive, indicating that the *p*-substituted phenyl β-D-*N*-acetyl glucopyranosides interact favorably with an electron-donating site on WGA and that binding is enhanced by electron-withdrawing substituents. Supporting evidence for this conclusion could not be obtained by uv-spectroscopy due to interference of the many aromatic amino acid residues within WGA which could not be subtracted from the binding complex spectrum due to a lack of necessary equipment. Previously, a UV spectral study of aryl glycosides binding to Con A failed to show π–π charge complex behaviour[150]. The change in chromophore spectrum in that study was interpreted as resulting from a change in dielectric constant on binding with the lectin and not EDA complexation. Fluorescence spectroscopic studies were performed by our colleague, Dr. J.

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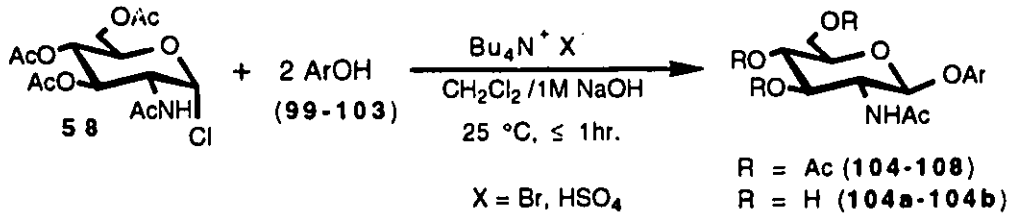
[150] I.S. Hassing and I.J. Goldstein, *Eur. J. Biochem.* , *16* , 549 (1970).

Boratynski, on this system but proved inconclusive due to tryptophan absorption and emissions which partially or totally masked the emission bands of interest. A more detailed study of EDA complexation within WGA's binding site will have to wait until later. One such study proposed is to identify a tight ring stacking between Tyr 64 and complexed aryl-β-D-*N*-acetylglucosaminide haptens by X-ray crystallography.

5.5 Phase transfer catalysed glycosylation of more complex aromatic alcohols

As described earlier, PTC has proven to be a useful method for preparing substituted-phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides. Besides using substituted phenols as nucleophiles, the same PTC conditions described earlier (Scheme 26) could be used effectively with a variety of more complex aromatic alcohols to produce the corresponding β-glycosides. Coloured indicators were used as nucleophiles to produce useful *N*-acetylglucosaminidase[151] enzyme substrates, and fluorimetric probes in lectin studies[152]. A steroid (Estrone) was also glycosylated as an entry to the synthesis of pro-drug type compounds. The aromatic alcohols used with their structure is given below.

Scheme 26 PTC synthesis of more complex aryl alcohols



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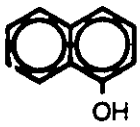
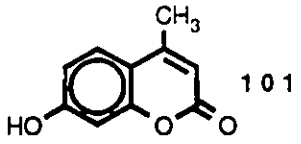
151 a) D. Pugh and N.G. Walker, *J. Histochem. Cytochem.*, *9* , 242 (1961); b) M.I. Shulman, V.A. Kulshin and A.Y. Khorlin, *Anal. Biocem.*, *101* , 342 (1980); c) T. Izumi and K. Suzuki, *J. Biol. Chem.*, *258* , 6991 (1983).

152 a) H. DeBock, H. Lis, H. van Tilbeurgh, N. Sharron and F.G. Loontiens, *J. Biol. Chem.*, *259* ,7067 (1984); b) M. Decastel, M. Vincent, K.L. Matta and J.P. Frémoy, *Arch. Biochem. Biophys.*, *232* , 640 (1984).



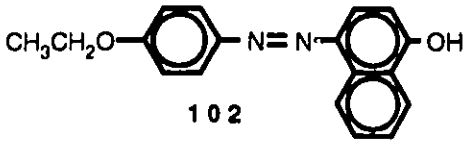
α-naphthol
99

β-naphthol
100



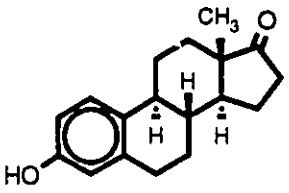
101

7-hydroxy 3-methyl coumarin



102

4-(p-ethoxyphenyl)azo naphth-1-ol



Estrone
103

Glycosylations were effected in a smooth manner, as described in Scheme 26, with yields generally ranging from 65 to 87% after chromatographic separation. Reaction with the alcohol **102** gave the expected glycosides in only 30% yield, however. The lowered yield in this case is attributed to the poor quality (TLC) of **102** which was purchased (Aldrich) as an indicator under the commercial name Fat Brown B. Zemplén de-*O*-acetylation gave the corresponding free glycosides (**104a** - **106a**) without difficulty. The glycosides produced, along with physical properties are given in Table 30. These experiments demonstrate the usefulness of PTC methodology in preparing glycosides of complex aryl alcohols that are often found in natural products.

Table 34 PTC synthesis of aryl β-D-*N*-acetylglucopyranosides from structurally complex aromatic alcohols.

					Elemental Analysis						
						Calculated (%)			Found (%)		
Nu*	Product	Yield (%)[a]	Melting Point[b] (°C)	$[\alpha]_D$[c]	Formula	C	H	N	C	H	N
99	**104**	80	212.1-212.6	-66.0°	$C_{24}H_{27}NO_9$	60.88	5.75	2.96	60.60	5.83	2.84
	104a		244.4-244.7	-85.0°	$C_{18}H_{21}NO_6$	62.24	6.09	4.03	62.26	6.10	4.07
100	**105**	70	220.1-220.5	-65.8°	$C_{24}H_{27}NO_9$	60.88	5.75	2.96	60.94	5.66	2.89
	105a		239.8-240.3	+15.8°	$C_{18}H_{21}NO_6$	62.24	6.09	4.03	62.00	5.77	4.05
101	**106**	87	255.3-255.6	-0.0°	$C_{24}H_{27}NO_{11}$	57.03	5.38	2.77	56.80	5.44	2.71
	106a		215.0-215.6	+3.5°	$C_{18}H_{21}NO_8$	56.99	5.58	3 69	56.84	5.74	3.60
102	**107**	30	274.2-275.2	+22.5°	$C_{32}H_{35}N_3O_{10}$	61.83	5.67	6.76	61.51	6.03	6.71
103	**108**	65	124 sinters	+54.6°	$C_{32}H_{41}NO_{10}$	64.09	6.89	2.34	63.97	6.86	2.34

* Nucleophile

a) Yields after silica gel chromatography. Zemplén de-*O*-acetylation gave quantitative yields of **104a - 106a**.

b) Crystallized from ethanol.

c) At 23 °C; C=1, $CHCl_3$ for **104 - 108**; C=1, DMSO for **104a - 106a**.

5.6 Experimental methods

General methods.

General methods are as described in section 2.3.

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl chloride **58**

The chloride **58** was prepared according to Horton[153] by stirring *N*-acetyl-D-glucosamine in acetyl chloride overnight under an anhydrous atmosphere. The product was always contaminated and thus purified by flash chromatography with a gradient of dry ethyl/acetate/hexane (1:1) to ethyl/acetate. On a large scale, 7 g could easily be purified, stored cold and dessicated for 8 months without any decomposition.

Aryl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosides **61 - 73**

Typical Procedure.

Chloride **58** (0.28 mmol), tetrabutylammonium hydrogen sulfate or bromide (1 equiv.) and the phenols (2 equiv.) were vigourously stirred at room temperature in methane chloride (1 mL) and 1M sodium hydroxide (1 mL). The reactions were monitored by TLC ($CHCl_3$/EtOAc/*n*-prOH:25/5/2, v/v). After complete consumption of the chloride **58** (30-45 min.), the reaction mixtures were worked up by adding ethyl acetate (10 mL). The resulting organic phases were successively washed with 1M NaOH (3 x 10 mL) followed by water (2 x 10 mL) and finely with saturated brine (10 mL). The dried organic

[153] D. Horton, *Methods Carbohydr. Chem.*, *6*, 282 (1972).

phases (Na_2SO_4) were evaporated under reduced pressure. The resulting residues were purified by preparative TLC using the above eluent. The aryl glycosides were extracted from the silica gel using ethyl acetate and crystallized from ethanol to give **61-73** in 66-93% yields.

The above procedure could be effectively prepared on large scale by direct crystallization of the glycosides obtained after the liquid-liquid extractions and evaporation of ethyl acetate. In the case of **71** (*p*-NO_2), the phenoxide was not entirely soluble in the two phase mixture, gentle warming rendered it soluble. All the products obtained (OAc and free OH) had physical constants (m.p. and $[\alpha]_D$) which generally agreed with those of the literature[154] where applicable.

Zemplén de-O-acetylation of aryl 2-acetamido-2-deoxy-β-D-glucopyranosides

Two general methods were employed. These were based on the solubility properties of the acetylated glycosides. Method A: for glycosides soluble or moderately soluble in methanol. These were de-*O*-acetylated in MeOH under standard conditions. The glycosides were dissolved in MeOH to which was added catalytic amount of 1M NaOMe in MeOH. The reaction mixtures were stirred for 1-2 hours at room temperature with monitoring by TLC ($CHCl_3$/MeOH 4/1). The sodium cations were removed with Amberlite IR-120 cationic (H^+). The solutions were filtered and evaporated to dryness. All the glycosides were obtained in almost quantitative yields by crystallization from ethanol or iso-propanol. Method B: for glycosides which were not very soluble in MeOH, an equal volume of benzene was added as co-solvent. The solutions were then treated with 1M NaOMe as above. The de-esterified glycosides progressively

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154 a) D.H. Leeback, *Biochemical Preparations*, *10* , 118 (1963); b) F. Shafizadeh, M.H. Meshreki and R.A. Susott, *J. Org. Chem.*, *38* , 1190 (1973); c) G.S. Jones and D.J. Kosman, *J. Biol. Chem.*, *255* , 11861 (1980).

precipitated out of the solutions. Complete precipitation was ensured by adding an equal volume of hexane or ether. The solutions were cooled and the products filtered. They were always of analytical purities but were usually recrystallized from ethanol.

Reduction of **71** *to the acetylated p-aminophenylglycoside* **87**.

Compound **71** (176 mg, 0.376 mmol), 10% palladium on charcoal (15 mg) and ammonium formate (90 mg, 4 equiv.) were suspended in MeOH (20 mL). The solution was brought to a full boil for two minutes in a stoppered round bottom flask after which time TLC ($CHCl_3$/MeOH, 4/1) indicated complete reaction. The solution was filtered through Celite which was rinsed with MeOH and evaporated to a white solid. Extraction of this residue with ethyl acetate and washing with saturated sodium hydrogen carbonate (2 x 20 mL) and water (2 x 20 mL) gave pure **87** (160 mg, 97%) after usual processing of organic phase. An analytical sample of **87** was obtained by recrystallization from warm (not boiling) ethanol, mp=172.2-175.4 °C .

Reduction of **84** *to the free p-aminophenyl glycoside* **92**

Compound **84** (50 mg, 0.146 mmol) was refluxed for 10 minutes in MeOH (2.5 mL) containing ammonium formate (50 mg, 5.3 equiv.) and 10% palladium on charcoal (6 mg). Some ammonium formate sublimed in the condenser. The reaction was stopped when TLC (CH_3CN/acetone/10% aq. HOAc, 5/3/1) indicated complete conversion of **84** (R_f 0.56)to **92** (R_f 0.35). Isolation by filtration and evaporation afforded pure **92** (44 mg, 98%).

p-N-*Acetamidophenyl 2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-β-D-glucopyranoside* **88**.

The *p*-aminopenyl glycoside **87** (0.52 g, 1.164 mmol) was treated

with pyridine (20 mL) and acetic anhydride (10 mL) for 1 hour at room temperature. Methanol (10 mL) was then added to destroy the excess anhydride and after 45 minutes the solvents were evaporated to dryness with a few co-evaporation from toluene. Crystallization of the residue from ethanol gave **88** in 96% yield (2 crops, 531 mg).

p-Carbomethoxyphenyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside **90**.

To a solution of p-formylphenylglycoside **72** (350 mg, 0.776 mmol) and sodium hydrogen carbonate (800 mg) in MeOH (15 mL) was added bromine (300 μL). The reaction mixture was stirred at room temperature for 90 minutes, after which time TLC still indicated some starting aldehyde. Subjecting the reaction mixture to refluxing conditions or addition of excess bromine did not improve the situation. Ethyl acetate (40 mL) was added and the resulting organic phase was repeatedly washed with distilled water until the solution became clear. Usual processing of the organic phase (Na_2SO_4) and evaporation afforded a white solid. Purification by flash chromatography with a gradient of ethyl acetate/hexane (1/1, v/v) and 0.5% methanol to 2/1 (v/v) gave pure **90** (206 mg, 55%). Crystallization from ethanol gave an analytical sample of **90**.

p-Hydroxymethylphenyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside **91**.

The *p*-formylphenylglycoside **72** (240 mg, 0.532 mmol) and sodium borohydride (15 mg, 0.395 mmol) were stirred at room temperature for 3 hours in a mixture of ethanol (4 mL) and methylene chloride (2 mL). TLC in chloroform-ethyl acetate-n-propanol (25/5/2) showed complete reaction: R_f 0.39 (**72**), R_f 0.20 (**91**). Chloroform (20 mL) and 0.5M HCl (20 mL) were added to the reaction mixture. The organic phase was washed with saturated sodium hydrogen carbonate (20 mL) followed by water (20 mL). Usual processing afforded **91** (240

mg, quant.) as an homogeneous white solid. An analytical sample was obtained from ethyl acetate-hexane.

p-Carboxyphenyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside **96**.

Compound **96** (58 mg) was prepared by aqueous saponification of **97** in 0.1M NaOH overnight at room temperature. After complete reaction (TLC), an excess of cation exchange resin (Dowex 50W, H^+ form) was added to neutralize the solution (2 hours, 25 °C). Filtration of the resin and lyophilization gave **96** in 90% recovered yield. All attempts at crystallization failed. The product was shown to be pure by TLC and NMR spectroscopy.

α-Naphthyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside **104**.

Compound **58** (407.2 mg, 1.114 mmol), 332.0 mg (2.233 mmol) of α-Naphthol (Aldrich) and 359.3 mg (1.114 mmol) of tetrabutyl ammonium bromide were stirred vigourously in a two phase system consisting of 4.0 mL of CHCl and 3.0 mL of 1M NaOH, at room temperature for approximately 30 minutes after which time TLC (25/5/2, $CHCl_3$/EtOAc/n-prOH) indicated complete consumption of the chloride **58**. The reaction was worked up by adding 50 mL of ethyl acetate and successively washing the organic phase with 40 mL of 1M NaOH and 40 mL of water (2x). The organic phase was then dried with Na_2SO_4, filtered and evaporated to a brownish mass. Compound **104** was recovered in 80% yield (422 mg) after three crystallization crops from iso-propanol. ^{1}H-NMR δ ($CDCl_3$): 8.15 (mult, 1H, aromatic H), 7.52 (d, 1H, J = 8.4 Hz, aromatic H), 7.47 (mult, 2H, aromatic H), 7.33 (dd, 1H, J = 7.4 Hz, J = 8.2 Hz, aromatic H), 7.02 (dd, 1H, J = 0.9, J = 8.7 Hz, aromatic H), 5.59 (d, 1H, $J_{2,NH}$ = 9.2 Hz NH), 5.30 (dd, 1H, $J_{2,3}$ = 10.4, $J_{3,4}$ = 9.3 Hz, H_3), 5.22 (d, 1H, $J_{1,2}$ = 8.3 Hz, H_1), 5.20 (dd, 1H, $J_{4,5}$ = 9.6 Hz, H_4), 4.51 (mult, 1H, H_2),

4.31 (dd, 1H, $J_{5,6}$ = 5.4 Hz, $J_{6,6'}$ = 12.3 Hz, H_6), 4,20 (dd, 1H, $J_{5,6'}$ = 2.6 Hz, $H_{6'}$), 3.91 (ddd, 1H, H_5), 2.05 to 2.08 (4s, CH_3). ^{13}C-NMR δ ($CDCl_3$): 169.3 to 170.8 (4 C=0), 153.1 (O-$C_{aromatic}$), 134.1, 127.1, 126.2, 125.5 (2C), 125.3, 122.3, 121.7, 108.8 (9 $C_{aromatic}$), 99.7 (C_1), 72.1 (C_5), 71.4 (C_3), 68.6 (C_4), 61.9 (C_6), 53.4 (C_2), 22.5 (CH_3, NHAc), 20.1 (3 x CH_3, OAc). CI-MS (ether) gave m/e (ion, relative intensity): 474 (M+1, 34), 330 (M-aglycon, 100), 270 (330-HOAc, 22), 210 (270-HOAc, 28), 150 (210-HOAc, 33).

β-Naphthyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside **105.**

The chloride **58** (430 mg, 1.18 mmol) was stirred with one equivalent of TBA bromide and 2 equivalents of β-naphthol in the same solvent system and under identical conditions as for **104**. Identical work up and crystallization procedure gave 369 mg (70%) of **105**. ^{1}H-NMR δ ($CDCl_3$): 7.67 to 7.78 (mult, 3H, aromatic H), 7.13 to 7.47 (mult, 4H, aromatic H), 5.75 (d, 1H, $J_{2,NH}$ = 8.8 Hz, NH), 5.42 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 9.3 Hz, H_3), 5.38 (d, 1H, $J_{1,2}$ = 8.2 Hz, H_1), 5.14 (dd, 1H, $J_{4,5}$ = 9.8 Hz, H_4), 4.28 (dd, 1H, $J_{5,6}$ = 5.5 Hz, $J_{6,6'}$ = 12.2 Hz, H_6), 4.16 (dd, 1H, $J_{5,6'}$ = 2.6 Hz, $H_{6'}$), 3.92 (ddd, 1H, H_5), 1.93 to 2.05 (4s, CH_3). ^{13}C-NMR δ ($CDCl_3$): 169.6 to 171.0 (4 C=0), 155.0 (O-$C_{aromatic}$), 134.2, 130.1, 129.6, 127.8, 127.1, 126.6, 124.7, 119.0, 111.5 (9 $C_{aromatic}$), 99.0 (C_1), 72.1 (C_5), 71.9 (C_3), 68.8 (C_4), 62.2 (C_6), 54.7 (C_2), 23.0 (CH_3, NHAc), 20.4 to 20.5 (3 x CH_3, OAc). CI-MS (ether) gave a similar spectrum to that described for the α-naphthyl analogue **104**.

7-O-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl) 4-methyl coumarin **106.**

The chloride **58** (505 mg, 1.38 mmol), 467 mg (1.38 mmol) of TBAHS and 485 mg (2.75 mmol) of 7-hydroxy-4-methyl coumarin were stirred vigourously in a combination of 5 mL of CH_2Cl_2 and 5 mL of

1M NaOH at room temperature for 30 minutes. The reaction was then worked up by adding 50 mL of ethyl acetate and successively washing the organic phase with 50 mL 1M NaOH (2x) and 50 mL H_2O (3x). During the extraction procedure, material began to crystallize out of organic phase and settle at the interface. The aqueous phase was removed, and the organic phase evaporated to dryness to yield a white crystalline mass. Recrystallization from $CHCl_3$/EtOH gave, in three crops, a total of 604 mg (87%) of **106**, pure by TLC and NMR. ^{1}H-NMR δ ($CDCl_3$): 7.45 (d, 1H, J = 8.5 Hz, aromatic H), 6.12 (d, 1H, $J_{allylic}$ = 1.2 Hz, C(O)-C<u>H</u>=), 6.00 (d, 1H, $J_{2,NH}$ = 8.9 Hz, NH), 5.41 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 9.3 Hz, H_3), 5.37 (d, 1H, $J_{1,2}$ = 8.2 Hz, H_1), 5.14 (dd, 1H, $J_{4,5}$ = 9.9 Hz, H_4), 4.27 (dd, 1H, $J_{5,6}$ = 5.5 Hz, $J_{6,6'}$ = 12.2 Hz, H_6), 4.18 (mult, 1H, H_2), 4,14 (dd, 1H, $J_{5,6'}$ = 2.6 Hz, $H_{6'}$), 3.92 (ddd, 1H, H_5), 2.35 (d, 3H, $J_{allylic}$ = 1.2 Hz, coumarin CH_3), 1.94 to 2.08 (4s, 4 acetate CH_3). ^{13}C-NMR δ ($CDCl_3$): 169.7 to 170.3 (4 C=0, Ac), 160.3 (C=O, coumarin), 159.5 (C_7, coumarin), 154.6 (C_9, coumarin), 153.5 (C_4, coumarin), 126.8 (C_5, coumarin), 114.9 (*C* -Me), 113.8 (C_8, coumarin), 112.3 (C_6, coumarin), 103.5 (C(O)-<u>C</u>H=) 97.5 (C_1), 72.4 (C_5), 71.2 (C_3), 68.5 (C_4), 61.7 (C_6), 53.1 (C_2), 22.6 (CH_3, NHAc), 20.3 to 20.4 ($3CH_3$, OAc) 18.0 (CH_3, coumarin). CI-MS (ether) gave m/e (ion, relative intensity): 506 (M+1,3), 330 (M-aglycon, 100), 177 (coumarin + 1, 100).

[4-(p-Ethoxyphenyl)azonaphth-1-yl] 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside **107**.

The chloride **58** (80.0 mg, 0.219 mmol), 74.0 mg (0.218 mmol) of TBAHS and 66.0 mg (0.226 mmol) of 4-(*p*-ethoxyphenyl)azonaphth-1-ol (Fat Brown B indicator, Aldrich) were stirred vigourously for 45 minutes in 1.0 mL of CH_2Cl_2 and 1.0 mL of 1M NaOH, at room temperature. After the usual extractive procedure, evaporation gave a yellow crystalline mass. The product was purified by silica gel chromatography (1:1 $CHCl_3$:EtOAc). Compound **107** was obtained in 30% yield (40.6 mg) after evaporation of solvent. The whole mass was recrystallized from 20 mL EtOH to give floculant yellow

needles. FAB (glycerol)- and CI (ether)-MS procedures failed to desorb the sample and thus could not provide an adequate spectrum. ^{1}H-NMR δ ($CDCl_3$): 8.90 (dd, 1H, J = 0.6, J = 7.7 Hz, aromatic H), 8.21 (dd, 1H, J = 0.8, J = 8.4 Hz, aromatic H), 7.99 (d, 2H, $J_{o,p}$ = 9.1 Hz, H meta to glycoside), 7.75 (d, 1H, J = 8.4 Hz, aromatic H), 7.59 (symmetrical mult (i4 lines), 2H, aromatic H), 7.06 (d, 1H, J = 8.5 Hz, aromatic H), 7.01 (d, 2H, $J_{o,p}$ = 9.1 Hz, H ortho to glycoside), 5.58 (d, 1H, $J_{2,NH}$ = 9.1 Hz, NH), 5.30 (dd, 1H, $J_{2,3}$ = 10.4 Hz, $J_{3,4}$ = 9.4 Hz, H_3), 5.25 (d, 1H, $J_{1,2}$ = 7.6 Hz, H_1), 5.21 (dd, 1H, $J_{4,5}$ = 9.4 Hz, H_4), 4.55 (ddd, 1H, H_2), 4.31 (dd, 1H, $J_{5,6}$ = 5.5 Hz, $J_{6,6'}$ = 12.3 Hz, H_6), 4.20 (dd, 1H, $J_{5,6'}$ = 2.5 Hz, $H_{6'}$), 4.12 (q, 2H, J = 7.0 Hz, CH_2), 3.94 (ddd, 1H, H_5), 1.94 to 2.09 (4s, CH_3(OAc)) 1.46 (t, 3H, J = 7.0 Hz, CH_3). ^{13}C-NMR δ ($CDCl_3$): 169.3 (C=O), 170.3 (C=0), 170.6 (C=0),170.1 (C=0), 161.3 ($C_{aromatic}$, ipso to glycoside),155.0 (quat. $C_{aromatic}$), 147.5 (quat. $C_{aromatic}$), 143.4 (quat. $C_{aromatic}$), 132.2 (quat. $C_{aromatic}$), 127.4 ($CH_{aromatic}$), 126.4 ($CH_{aromatic}$), 125.8 (quat. $C_{aromatic}$), 124.9 C meta to glycoside) 123.2 ($CH_{aromatic}$), 122.0 ($CH_{aromatic}$), 114.7 (C ortho to glycoside), 111.8 ($CH_{aromatic}$), 108.3 ($CH_{aromatic}$), 99.7 (C_1), 72.2(C_5), 72.1 (C_3), 68.3 (C_4), 63.8 (CH_2), 62.1 (C_6), 23.3 (CH_3, NHAc), 20.6 to 20.7 ($3CH_3$, OAc) 14.8 (CH_3 of aglycon).

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl estrone **108**.

The chloride **58** (350 mg, 0.957 mmol), 324.9 mg (.0957mmol) of TBAHS and 517.4 mg (1.914 mmol, 2 equiv.) of estrone (Aldrich) were stirred vigourously at room temperature in a two phase system ccmprised of 3.5 mL CH_2Cl_2 and 3.5 mL 0.1M NaOH for 30 minutes after which time TLC (2/1 EtOAc/hexane) indicated a complete consumption of **58**. After the usual extractive work up, the product was purified by radial silica gel chromatography (2/1 EtOAc/hexane). 369.6 mg (65%) of **108**, pure by TLC and NMR, was recovered after solvent evaporation. The product could be recrystallized slowly from ether/hexane. ^{1}H-NMR δ ($CDCl_3$): 7.17 (dd, 1H, J < 1, J=8.7 Hz, H meta to glycoside), 6.75 (dd, 1H, J = 1.7, J = 8.7

Hz, H_{ortho} next to H_{meta}), 6.71 (d, 1H, J = 1.7 Hz, H ortho to glycoside and steroid ring), 5.57 (d, 1H, $J_{2,NH}$ = 8.8 Hz, NH), 5.38 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 9.3 Hz, H_3), 5.16 (d, 1H, $J_{1,2}$ = 8.3 Hz, H_1), 5.11 (dd, 1H, $J_{4,5}$ = 9.8 Hz, H_4), 4.26 (dd, 1H, $J_{5,6}$ = 5.4, $J_{6,6'}$ = 12.2 Hz, H_6), 4.14 (dd, 1H, $J_{5,6'}$ = 2.4 Hz, $H_{6'}$), 4.08 (ddd, 1H, H_2), 3.84 (ddd, 1H, H_5), 2.85 (mult, 2H, steroid), 2.52 (d, 1H, J = 8.7 Hz, steroid), 2,46 (d, 1H, J = 7.8 Hz, steroid), 1.94 to 2.07 (4s, CH_3(OAc)), 0.88 (s, 3H, CH_3 steroid), remaining peaks between 1.23 and 2.37 ppm, belonging to the steroid aglycon integrate for 11H. ^{13}C-NMR δ ($CDCl_3$): 220.7 (C=O steroid), 170.7 (C=0, Ac), 170.5 (C=0, Ac),170.3 (C=0, Ac), 169.3 (C=0, Ac), 154.8 (O-$C_{aromatic}$), 137.8 (quat. $C_{aromatic}$), 126.2 (C_{meta} to glycoside), 116.9 (C_{ortho} to glycoside), 114.1 (C_{ortho} to glycoside), 98.9 (C_1), 72.1 (C_5), 71.9 (C_3), 68.6 (C_4), 50.4 (CH steroid), 48.0 (quat. steroid), 44.0 (CH steroid), 38.2 (CH steroid), 35.9 (CH steroid), 31.6 (CH_2 steroid), 29.7 (CH_2 steroid), 26.5 (CH_2 steroid), 25.9 (CH_2 steroid), 23.4 (CH_3, NHAc), 21.6 (CH_2 steroid), 20.7 to 20.8 ($3CH_3$, OAc), 13.9 (CH_3 steroid). FAB-MS (glycerol) gave m/e (ion, relative intensity): 1199 (2M+1, 2.4), 600 (M+1, 17), 330 (M-aglycon, 84), 269 (Estrone + 1, 58).

α-Naphthyl 2-acetamido-2-deoxy-β-D-glucopyranoside **104a**.

Zemplén de-*O*-acetylation of **104** (280 mg, 0.591 mmol) with a catalytic amount of sodium methoxide in methanol (20 mL). 2 mL of benzene were added as cosolvent to help solubilize **104**. After 3 hours at room temperature, the reaction was deemed complete by TLC (Rf **104a** = 0.59, 4/1 CH_2Cl_2/MeOH) with no secondary product formation observed. Some product had precipitated out of solution. Thus, methanol was added until resolubilization. The homogeneous solution was then treated with cation exchange resin (H^+) to remove Na^+ cations and neutralize the reaction. Filtration of the resin followed by evaporation of the solvent gave 205 mg (quant.) of **104a** as a white crystalline mass. The product was recrystallized from hot iso-propanol to give small white needles. CI-MS (ether) gave m/e (ion, relative intensity): 348 (M+1, 20), 204 (M-aglycon, 100, 145

(α-Naphthol + 1, 33). ^{1}H-NMR δ ($DMSOd_6$): 8.05 (mult, 1H, aromatic), 7.85 (mult, 1H, aromatic), 7.35 - 7.65 (mult, 4H, aromatic), 7.14 (d, 1H, J = 7.5 Hz, aromatic), 5.00 (d, 1H, $J_{1,2}$ = 8.4 Hz, H_1), 3.2 - 4.0 (mult, 6H, sugar ring H), 1.80 (s, 3H, CH_3). ^{13}C-NMR δ ($DMSOd_6$): 169.9 (C=O), 153.7 (O-$C_{aromatic}$), 134.1, 127.5, 126.6, 126.3, 125.5, 125.4, 121.8, 121.6, 108.9 (remaining $C_{aromatic}$), 100.7 (C_1), 77.5 (C_5), 74.0 (C_3), 70.5 (C_4), 60.9 (C_6), 55.3 (C_2), 23.1 (CH_3).

β-Naphthyl 2-acetamido-2-deoxy-β-D-glucopyranoside **105a**.

Compound**105a** was prepared by Zemplén de-*O*-acetylation of **105** with the procedure outlined for **104a**, in a quantitative yield. The product was crystallized from abs. EtOH. CI-MS (ether) gave a spectrum almost identical to **104a**. ^{1}H-NMR δ ($DMSOd_6$): 7.78 (mult, 3H, aromatic), 7.38 (mult, 3H, aromatic), 7.14 (d, 1H, J = 2.2, J = 8.9 Hz, aromatic), 5.10 (d, 1H, $J_{1,2}$ = 8.5 Hz, H_1), 3.2 - 3.9 (6H, sugar ring H), 1.83 (s, 3H, CH_3). ^{13}C-NMR δ ($DMSOd_6$): 169.7 (C=O), 155.5 (O-$C_{aromatic}$), 134.2, 129.5, 129.3, 127.7, 127.1, 126.6, 124.3, 118.9, 110.9 (remaining $C_{aromatic}$), 99.5 (C_1), 77.4 (C_5), 74.2 (C_3), 70.4 (C_4), 60.8(C_6), 55.6 (C_2), 23.1 (CH_3).

7-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl) 4-methyl coumarin **106a**.

A quantitative yield of **106a** was obtained by Zemplén de-*O*-acetylation of 320 mg of **106** under the same conditions specified for **104**. The product was recrystallized form EtOH 95%. FAB-MS (glycerol) gave m/e (ion, relative intensity): 380 (M+1, 7), 204 (M - aglycon, 45), 177 (aglycon + 1, 28). ^{1}H-NMR δ (D_2O): 7.55 (dd, 1H, $J_{5,8}$ = 1.7, $J_{5,6}$ = 8.9 Hz, H_5 coumarin), 6.96 (dd, 1H, $J_{6,8}$ = 2.3, $J_{5,6}$ = 8.9 Hz, H_6 coumarin), 6.87 (dd, 1H, $J_{5,8}$ = 1.7, $J_{6,8}$ = 2.3 Hz, H_8 coumarin), 6.10 (sharp mult, 1H, C(O)-CH=), 5.22 (d, 1H, $J_{1,2}$ = 8.3 Hz, $H_{anomeric}$), 3.5 - 4.1 (mult, 6H, sugar ring H), 2,32 (d, 3H, $J_{allylic}$ = 1.4 Hz, CH_3 coumarin), 2.07 (s, 3H, CH_3 NHAc). ^{13}C-NMR δ ($DMSOd_6$): 170.2 (C=O,

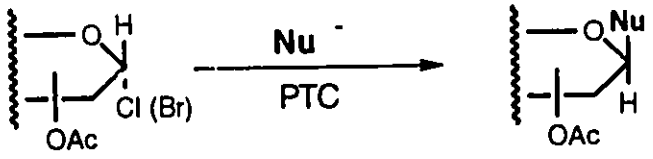
NHAc), 160.6 (C=O, coumarin) 160.5 (C_3, coumarin), 154.7 (C_7, coumarin), 153.7 (C_9, coumarin), 126.8 (C_5, coumarin), 114.6 (C_4, coumarin), 113.9 (C_8, coumarin), 112.1 (C_6, coumarin), 103.5 (C_2, coumarin), 99.1 (C_1), 77.5 (C_5), 74.1 (C_3), 70.5 (C_4), 60.9 (C_6), 55.5 (C_2), 23.1 (CH_3, NHAc), 18.2 (CH_3, coumarin).

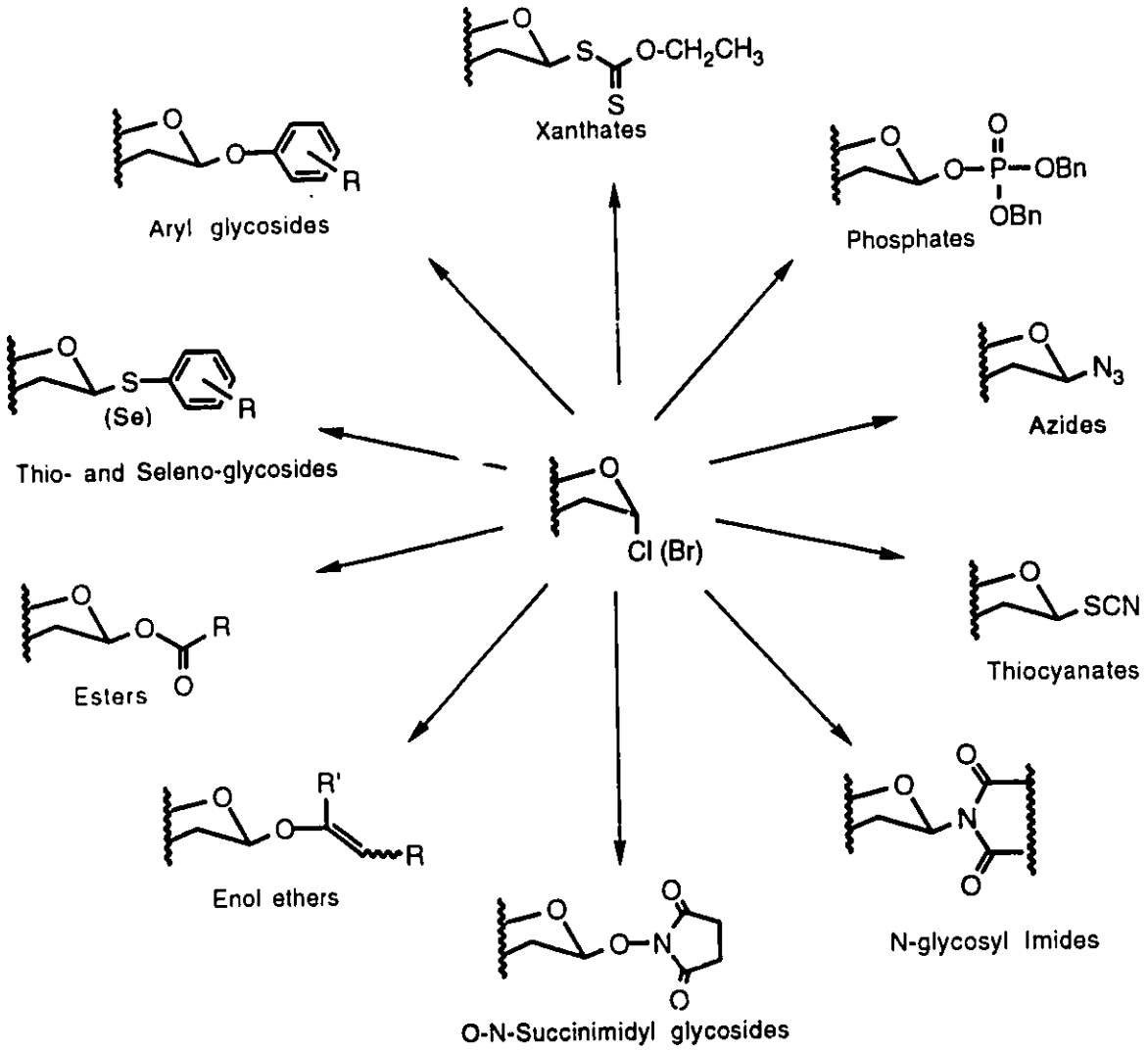
Accurate NMR chemical shift determination for Hammett correlations

^{1}H- and ^{13}C-NMR spectra were obtained under identical conditions for all samples. ^{1}H-NMR spectra were acquired at 299.943 MHz using a dedicated 5 mm ^{1}H probe. A 4000.0 Hz spectral window was excited with 33 °C pulse and the FID acquired over 2.997 seconds using 24000 points. The data was zero-filled to 32K before Fourier transformation to allow accurate chemical shift measurement. ^{13}C-NMR were acquired using a 5 mm broadband probe at a resonance frequency of 75.429 MHz. A 16502 Hz spectral window was excited with a 38 °C pulse and acquired over 0.909 seconds using 30016 points, zero-filled to 32K before Fourier transformation. Waltz-16 decoupling was used for all ^{13}C spectra. All spectra were recorded at ambient temperature (23 ± 1 °C) using a Varian XL-300 spectrometer. Samples were prepared by dissolving 4.0 mg samples for ^{1}H studies, and a weight equal to 5.46 x 10^{-5} moles for ^{13}C studies, in 600 µL of the appropriate solvent (see text). Spectra were recorded in triplicate for each compound to within 0.002 ppm for ^{1}H spectra and to within 0.01 ppm for ^{13}C spectra.

6. General synthesis of glycosyl derivatives by phase transfer catalysis

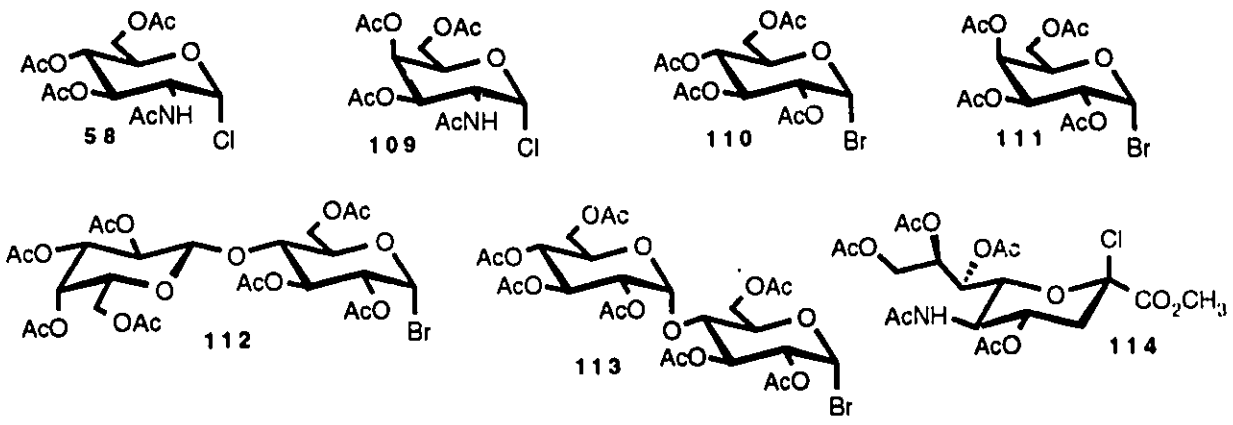
The PTC strategy used for the effective preparation of aryl 2-acetamido-2-deoxy β-D-glucopyranosides[24a,b] has been successfully extended to a wide range of glycosyl halides and a large variety of different types of nucleophiles[24c-g]. PTC glycosidation has proven to be an effective general method for preparing β-glycosides of glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, maltose and lactose as well as α-glycosides of *N*-acetylneuraminic acid. Coworkers have also successfully employed the PTC methodology to other glycosides and glycobiosides. Not only can phenolate type nucleophiles be glycosylated under PTC conditions but so can aryl and alkyl thiolates, aryl selenates, xanthates, phosphates, azide, thiocyanate, *N*-hydroxysuccinimide, amides, carboxylates and enolates. Glycosylations are always stereospecific with complete inversion of configuration at the anomeric centre. Thus, PTC methodology has proven to be very general indeed. PTC methodology has been used with success for various combinations of glycosyl halides and nucleophiles described above, to produce the desired glycosyl derivatives in a quick, mild, high yielding and stereospecific manner. The evaluation of the generality of this methodology is demonstrated for each different nucleophile type in the sections that follow.





Carbohydrate derivatives accessed from glycosyl halides by phase transfer catalysis

Glycosyl halides used in PTC glycosylations were prepared according to standard methods and are shown below.



6.1 Phenolates

As demonstrated in the last chapter, phenolic alcohols can be glycosylated effectively with 2-acetamido-2-deoxy-α-D-glucopyranosyl chloride **58**. The same PTC conditions (Scheme 23) have also been used with success for the galactosyl analogue **109**. As an example, the 7-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl) 3-methyl coumarin **115** was prepared in a first attempt in 27% overall yield from N-acetyl D-galactosamine in only two steps. Higher yields, resembling what was achieved for the corresponding gluco analogue **101**, should be achieved by optimizing the conditions for the preparation of 2-acetamido-2-deoxy-α-D-galactopyranosyl chloride **109** (see experimental). Nonetheless, a recent report by Lemieux and coworkers[155] described the first synthesis of this compound from 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl chloride in 7% overall yield only. **115** is an important agent for the detection of *N*-acetyl-β-D-

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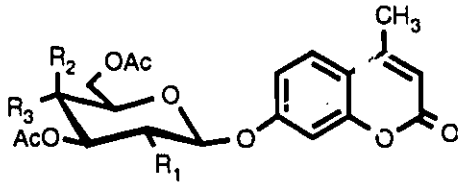
[155] R. Szweda, U. Spohr, R.U. Lemieux, D. Schindler, D.F. Bishop and R.J. Desnick, *Can. J. Chem.*, *67* , 1388 (1989).

galactosaminidase in tissues[151bc, 156] and a fluorimetric probe for lectin binding studies[152ab]. The β-D-glucoside analogue **116** was also prepared by PTC from the halide **110** in 48% yield.



	R_1	R_2	R_3
115	NHAc	OAc	H
116	OAc	H	OAc
116a	OH	H	OH

The same PTC reaction conditions also allowed the preparation of the *p*-formyl and *p*-nitrophenyl β-D-glucopyranoside derivatives **117** and **118** in 45% and 48% yields respectively. In all three cases (**116-118**), the glycal **119** was formed as the major secondary product in yields almost identical to that of the desired glycosides. This side product results from a competing dehydrohalogenation reaction by abstraction of the H-2 proton by base or the incoming nucleophile (Scheme 27). Only very little dehydrobromination could be observed (TLC) in the absence of the phase transfer agent when the halide was stirred in the usual two phase system overnight. Efforts to improve the yields using α-D-glucopyranosyl chloride instead of the bromide **110** failed. Only slow reactions (> 2 days) without any increase in yield was observed at room temperature, and almost exclusive formation of the 2-acetoxy-D-glucal **119** was obtained under refluxing conditions. Although considerable dehydrohalogenation occured in the gluco series, PTC using TBAHS in a CH_2Cl_2/1M NaOH two phase system appeared to be a convenient method for preparing aryl β-D-glucopyranosides. Thus, PTC glycosylation of aromatic alcohols is not limited to 2-acetamido-2-deoxy sugars only. Zemplén de-*O*-acetylation of **117** and **118** gave the corresponding deprotected glycosides **117a** and **118a** in

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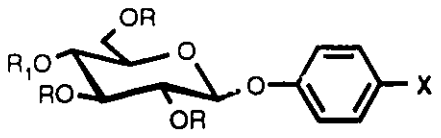
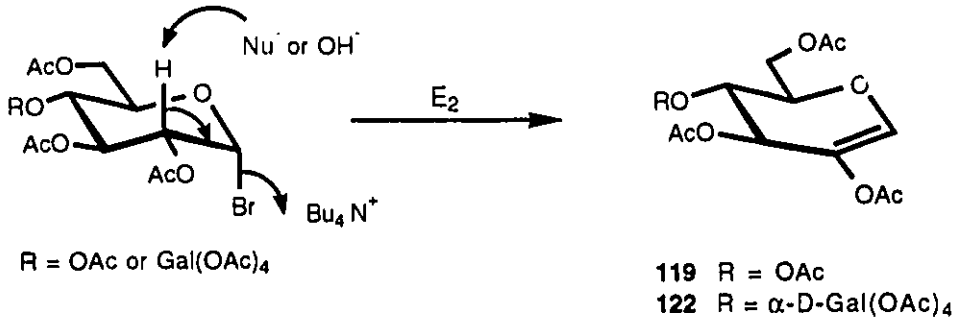
[156] B.G. Winchester, *Biochem. J.*, *124*, 929 (1971).

quantitative yields without any difficulty.



	R_1	R	X
117	Ac	Ac	CHO
117a	H	H	CHO
118	Ac	Ac	NO_2
120	α-D-Gal(OAc)$_4$	Ac	CHO
120a	α-D-Gal	H	CHO
121	α-D-Gal(OAc)$_4$	Ac	NO_2
121a	α-D-Gal	H	NO_2

PTC glycosylation of aromatic alcohols is not limited to monosaccharides. For example, with the per-acetylated lactosyl bromide **112**, the *p*-formyl and *p*-nitrophenyl β-D-lactosides **120** and **121** could be prepared in similar yields (45 and 50%) to the gluco series using the same reaction conditions as usual (TBAHS, CH_2Cl_2/1M NaOH, 25 °C, 1 hour). As in the case of the gluco series, the 2-acetoxy lactal **122** was a major side product in these syntheses also.



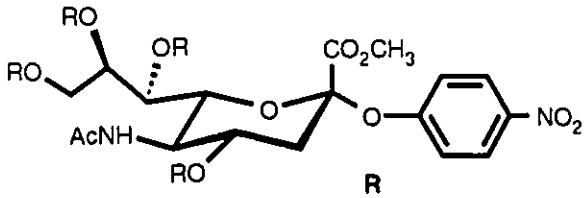
R = OAc or Gal(OAc)$_4$

119 R = OAc
122 R = α-D-Gal(OAc)$_4$

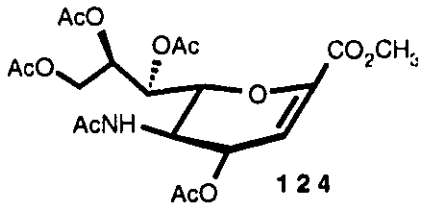
Scheme 27 Glycal formation by base assisted trans axial dehydrobromination by an E_2 type mechanism (shown for halides **110** and **112**).

The glycosyl chloride **114** was used to efficiently prepare the corresponding methyl (*p*-nitrophenyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate **123** under the same mild conditions as the other aryl glycosides in 64% yield. Again, the synthesis was stereospecific with ^{1}H-NMR analysis of the crude product mixture showing only the equatorial α-anomer being formed as judged by the presence of only one doublet

characteristic of the equatorial H_3 of NeuAc derivatives in the region δ: 2-3 ppm (2.60 ppm)[157]. Shortly after this preparation, others[158] published a similar PTC synthesis of **123**. However, the inferior (see section 5.2.1) BTEA chloride was used as catalyst. The reaction required refluxing conditions with benzene and gave an inferior yield. Again, dehydrohalogenation of the chloride **114** provided the major by-product **124** [159] which was identified by ^1H-NMR after chromatographic purification of the crude product mixture. Compound**123** is an important sialidase substrate[160]. The PTC methodology developed here allows for its preparation in the highest yielding and most convenient fashion reported so far[161]. This entry into PTC glycosidation of 2-deoxy sugars lacking a potential participating group next to the anomeric centre further substantiates the earlier claims (section 5.2) that neighbouring group participation is not involved in the formation of aryl 1,2-*trans* β-D-glycosides by PTC.



123 Ac
123a H



124

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157 R. Roy, C.A. Laferriere, A. Gamian and H.J. Jennings, *J. Carbohydr. Chem.*, *6* , 161 (1987) and references 8 and 9 within.

158 J. Rothermal and H. Faillard, *Carbohydr. Res.*, *196* , 29 (1990).

159 K. Okamoto, T. Konodo, T. Goto, *Bull. Chem. Soc. Jpn.*, *60* , 631 (1987).

160 V. Eschenfelder and R. Brossmer, *Carbohydr. Res.*, *162* , 294 (1987).

161 See also C. Laferriere, *Synthesis of Sialic Acid Antigens* , Ph.D. thesis, University of Ottawa, Ottawa, Canada, 1991.

Table 35 Physical properties of per-*O*-acetylated aryl 1,2-*trans* β-D-glycosides

Product	Yield (%)	Melting Point (°C) *	$[\alpha]_D$ # (CHCl3)	Formula	Elemental Analysis					
					Calculated (%)			Found (%)		
					C	H	N	C	H	N
115	27	219.3-220.2	-5.5°	$C_{24}H_{27}NO_{11}$	57.03	5.38	2.77	57.19	5.38	2.61
116	48	143.6-144.3	-36.0°	$C_{24}H_{26}O_{12}$	56.92	5.17	-	56.81	5.10	-
117	38	139.8-141.2	-16.6°	$C_{21}H_{24}O_{11}$	55.75	5.35	-	55.90	5.47	-
118	46	178.8-179.8	-40.5°	$C_{20}H_{23}NO_{12}$	51.18	4.94	2.98	51.14	4.99	3.06
120	45	89.7-91.8	-28.5°	$C_{33}H_{40}O_{19}$	53.51	5.44	-	53.39	5.47	-
121	57	133.3-135.4	-21.1°	$C_{32}H_{39}NO_{20}$	50.73	5.19	1.85	50.87	5.25	1.85
123	64	89.5-90.6 (sinters) 100.8-101.2 (melts)	+52.3°	$C_{26}H_{32}N_2O_{15}$	50.98	5.27	4.57	50.69	5.37	4.34

* crystallized from ethanol
# c=1

Table 36 Physical properties of non-acetylated aryl β-D-glycosides

Product	Melting Point (°C)*	$[\alpha]_D$ # (DMSO)	Formula	Elemental Analysis					
				Calculated (%)			Found (%)		
				C	H	N	C	H	N
116a	209.4-209.9	-93.2° (CH_3OH)	$C_{16}H_{18}O_8$	56.80	5.36	-	56.65	5.10	-
117a	157.6-159.4	-48.3°	$C_{13}H_{16}O_7$	54.95	5.67	-	54.71	5.69	-
120a	244.2-245.9	-11.7°	$C_{19}H_{26}O_{12}$	51.12	5.87	-	50.89	6.10	-
121a	249.0-250.2	+39.1°	$C_{18}H_{25}NO_{13}$	46.66	5.44	3.02	46.67	5.43	2.99

* crystallized from ethanol
# c=1

6.2 Thiolates

Thioglycosides make up an important family of carbohydrate derivatives. They have long been recognized as inhibitors for a number of glycohydrolase enzymes[162], and as such, have been used extensively as ligands for affinity chromatography[163]. Recently, they have gained interest because they represent stable latent glycosyl donors, useful in block-oligosaccharide synthesis, which resist most standard functional group manipulations[164]. More recently, long chain alkyl 1-thio-D-glucopyranosides have demonstrated liquid-crystal behaviour[165].

A wide variety of methods exist for the preparation of thioglycosides[166]. The most widely used procedures for the synthesis of 1,2-*trans*-thioglycosides are those based on Lewis acid catalyzed reactions between per-*O*-acetylated sugars and thiols[160a], or those using glycosyl halides with thiophenoxides[167]. However, both these procedures suffer from some drawbacks. The one phase thiophenoxide method, as with phenolates, yields products that are extensively contaminated with de-*O*-acetylation, while the Lewis acid catalyzed reaction gives mixtures of α, β-anomers and dithioacetals.

The success encountered in preparing 1,2-*trans*-*O*-aryl β-D-glycosides by PTC prompted the attempt in preparing thioglycoside analogues by the same method with the contention that the softer

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162 E. Jr. Steers, P. Cuatrecasas and H.B. Pollard, *J. Biol. Chem.*, *246* , 196 (1971).

163 J.H. Pazur, *Adv. Carbohydr. Chem. Biochem.*, *39* , 405 (1981).

164 F. Andersson, W. Birberg, P. Fügedi, P.J. Garegg, M. Nashed and A. Pilotti, *ACS Symp. Ser.*, *398* , 117 (1989); P. Fügedi, P.J. Garegg, H. Lönn and T. Norberg, *Glycoconj. J.*, *4* , 97 (1987).

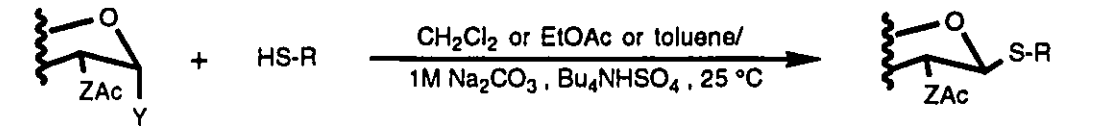
165 H.A. van Doren, R. van der Geest, R.M. Kellog and H. Wynberg, *Carbohydr. Res.*, *194* , 71 (1989).

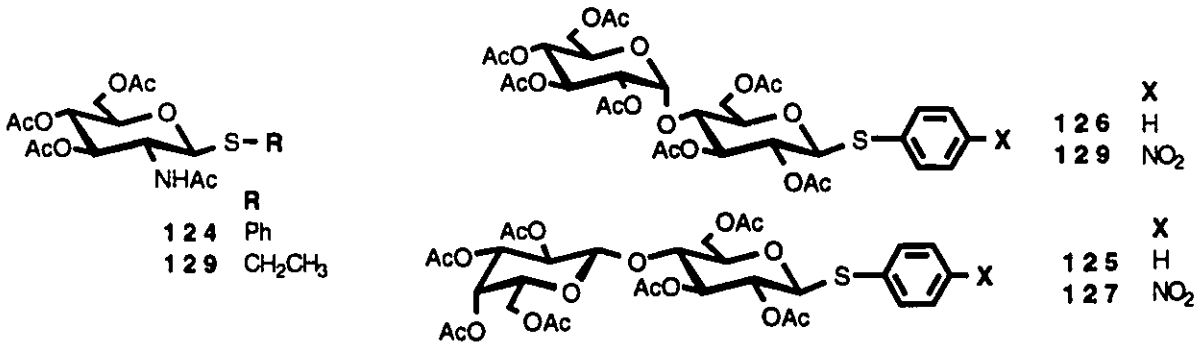
166 a) R.J. Ferrier and R.H. Furneaux, *Methods Carbohydr. Chem.*, *8* , 251 (1980); b) S. Hanessian and Y. Guindon *Carbohydr. Res.*, *86* , C3 (1980); c) V. Pozsgay and H.J. Jennings, *Tetrahedron Lett.*, *28* , 1375 (1987); and references cited therein.

167 C.B. Purves, *J. Am. Chem. Soc.*, *51* , 3619, 3631 (1929).

and more nucleophilic thiols should behave as well if not better than phenoxides. This assumption was justified as the glycosyl halides **58**, **112** and **113** quickly (≤ 30 minutes, and usually within 10 min.) gave the corresponding phenyl 1-thio-β-D-glycosides **124**, **125**, **126** in high yields (Table 37) when reacted with thiophenol under PTC conditions similar to that used for the phenolates (TBAHS, 25°C, CH_2Cl_2/1M NaOH for **124**, or 1M Na_2CO_3 for **125** and **126**). In this case, 1M Na_2CO_3 was found to be sufficient as the alkaline aqueous phase due to the increased acidity and nucleophilicity of benzenethiol. The disaccharides are of interest since they constitute integral components of a number of polysaccharide repeating units which can be adequately synthesized using widespread thioglycosyl donor technology[158].

Scheme 28 PTC synthesis of thio-glycosides





The reaction occured with complete stereocontrol to give 1,2-*trans*-β-D-thio-glycosides ($J_{1,2} \approx$ 10 Hz, C_1, δ = 85-86 ppm) by nucleophilic displacement (S_{N2}) of halide with inversion of configuration as judged by NMR spectroscopy of the crude product mixtures. In

contrast to the *O*-aryl glycoside syntheses, no substantial dehydrohalogenation products could be detected during these PTC syntheses. However, oxidation of the nucleophile to the corresponding disulfide and nucleophilic attack of the solvent to produce bis(phenylthio) methane and chloromethylphenyl sulfide were competing reactions[168]. These side reactions in some cases consumed the nucleophile (2 equiv.) before complete consumption of the glycosyl halides. Successive partial additions of the thiol or adding more than 2 equivalents of nucleophile could drive the reaction to completion, however. The use of either 1,2-dichloroethane, toluene, or ethyl acetate as the organic phase eliminated the problem without any decrease in isolated yield of the products[25c] or increase in reaction time.

The versatile transformations of the *p*-nitrophenyl group into latent thioglycosyl donor groups[169] and the usefulness of the *p*-acrylamidophenyl groups for the synthesis of protein and polymer glycoconjugates prompted the synthesis of the disaccharides **127** and **128** by the same PTC strategy as above. Although high yields of **127** and **128** could be achieved, much longer reaction times (up to 6 hours) were required. Excessive amounts of *p*-nitrothiophenol (>2 equiv.) were required as oxidation of the nucleophile to its disulfide and nucleophilic attack to the solvent (CH_2Cl_2) occurred readily. The poor solubility of the *p*-nitrophenyl thiolates in CH_2Cl_2/1M Na_2CO_3 could have been the source of these problems. Using ethyl acetate as the organic phase did, however, improve the reaction in terms of solubility and decrease in the amount of nucleophile required but had no major effect on yield or reaction time[24f].

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168 These products were isolated and characterized by MS, ^1H- ^{13}C-NMR spectroscopy.

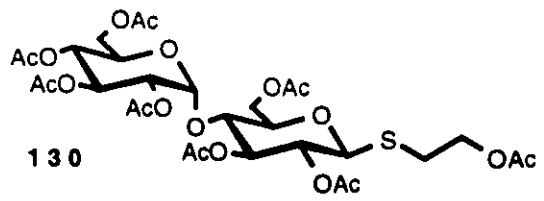
169 *p*-Nitrophenylthio glycosides are poor glycosyl donors because of the electron withdrawing nature of the nitro group which diminishes the nucleophilicity of the sulfur atom towards usual promoters. Reduction of the nitro group followed by acylation converts the aryl substituent into an electron donating group which can then promote glycosylation. R. Roy, F.O. Andersson, M. Letellier, *Tetrahedron Lett.*, in press.

Table 37 Synthesis and physical properties of peracetylated thioglycosides **124 - 130.**

Product	Yield (%)	Melting Point (°C)*	$[\alpha]_D$ ($CHCl_3$)	Formula	Elemental Analysis							
					Calculated (%)				Found (%)			
					C	H	N	S	C	H	N	S
124	95	204.9-205.6	-23.6°	$C_{20}H_{25}NO_8S$	56.92	5.17	-	7.30	54.51	5.70	3.58	7.45
125	92	165.1-166.7	-12.8°	$C_{32}H_{40}O_{17}S$	55.75	5.35	-	4.40	52.79	5.60	-	4.19
126	89	82 (amorph.)	+47.4°	$C_{32}H_{40}O_{17}S$	51.18	4.94	2.98	4.40	52.76	5.51	-	4.17
127	91	122.3-124.1	-26.2°	$C_{32}H_{39}NO_{19}S$	53.51	5.44	-	4.14	49.47	5.18	1.72	4.33
128	71	164.9-166.3	+42.1°	$C_{32}H_{39}NO_{19}S$	50.73	5.19	1.85	4.14	49.75	5.13	1.77	4.21
129	42	180.8-181.9	-40.7°	$C_{16}H_{24}NO_8S$	50.98	5.27	4.57	8.21	49.10	6.58	3.60	7.87
130	42	129-131	+57.7°	$C_{30}H_{42}O_{19}S$	48.78	5.73	-	4.34	48.68	5.86	-	4.13

* Crystallized from ethanol except for **126** (CH_2Cl_2) and **130** (ether/diisopropyl ether).

Besides aryl thiols, alkyl thiols can also be glycosylated under PTC conditions. The per-*O*-acetylated ethylthio β-D-*N*-acetylglucopyranoside **129** was prepared in a modest 42% yield, in a catalytic two phase system using CH_2Cl_2 and 1M NaOH. De-*O*-acetylation by the thiolate seems to compete with nucleophilic substitution at the anomeric centre. Considerable oxazoline **60** formation was also observed during the reaction. Comparatively, only very small quantities (about 10%) of methylthio β-D-*N*-acetylglucosamine were obtained when the smaller sodium methylthiolate was used as nucleophile. Similarily, the per-*O*-acetylated 2-acetoxyethylthio β-D-maltoside **130** was prepared in 42% overall yield after PTC glycosylation of 2-mercaptoethanol with acetobromomaltose **113** (CH_2Cl_2/1M NaOH, 3hrs., 25°C), followed by acetylation of the crude product mixture. Substitution of toluene for methylene chloride as the organic phase gave no reaction in this case.



130

These results confirm earlier observations by Bogusiak and Szeja[170] who showed that similar reactions with glycosyl and galactosyl bromides could be conducted at room temperature in benzene. Their results mirror those presented here as glycoside yields increase with increasing size of the thiol, and are almost identical to those obtained here for the corresponding glycosides (SCH_3, SCH_2CH_3 and SC_6H_5)

[170] J. Bogusiak and W. Szeja, *Pol. J. Chem.*, *59* , 293 (1985); *Chem. Abst.*, *104* , 186748 (1986).

Table 38 ^{13}C-NMR chemical shift data* for the per-*O*-acetylated thioglycosides

	C-1 C-1'	C-2 C-2'	C-3 C-3'	C-4 C-4'	C-5 C-5'	C-6 C-6'	C=0	CH_3	Aglycon			
									C_{ipso}	C_{meta}	C_{ortho}	C_{para}
124	86.4	53.1	73.6	69.5	75.5	62.3	169.5 170.4 170.7 171.0	20.3 20.4 20.5 23.0 (NHAc)	132.7	132.3	128.9	128.0
125	85.5 101.1	70.2 69.0	76.1 70.9	76.6 66.6	73.8 70.7	62.1 60.8	169.0 to 170.3 (7C)	20.5 to 20.8 (7C)	131.7	133.0	128.9	128.7
126	85.6 95.5	70.6 69.9	76.4 69.2	72.9 67.9	76.0 68.5	62.7 61.4	169.3 to 170.4 (7C)	20.5 to 20.8 (7C)	131.2	128.4	133.3	128.4
127	83.8 100.9	69.6 68.8	75.7 70.6	76.7 66.4	73.3 70.4	61.8 60.7	169.0 to 170.2 (7C)	20.1 to 20.4 (7C)	142.0	130.6	123.7	146.8
128	83.9 95.7	70.4 69.9	76.3 69.2	72.4 67.9	76.0 68.6	62.8 61.5	169.4 to 170.5 (7C)	20.8 to 20.4 (7C)	141.4	131.2	123.8	147.0
129	84.3	53.3	73.9	68.5	75.8	62.4	169.1 169.9 170.5 170.9	20.7 to 20.8 (3C) 23.3 (NHAc)	24.2 (CH_2)		14.8 (CH_3)	
130	83.2 95.6	70.6 70.0	76.2 69.3	72.6 68.0	76.3 68.6	62.8 61.5	169.4 to 170.5 (7C)	20.6 to 20.9 (4C)	63.6 ($-CH_2OAc$)		28.7 ($-CH_2-S-$)	

* In ppm for solutions in $CDCl_3$ at 75.4 MHz. Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments

Table 39 ^{1}H-NMR data* (δ and J) for peracetylated thioglycosides

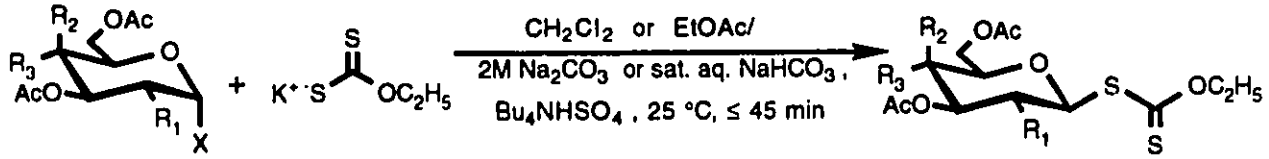
	H-1 d ($J_{1,2}$)	H-2 dd ($J_{2,3}$)	H-3 dd ($J_{3,4}$)	H-4 dd ($J_{4,5}$)	H-5 ddd ($J_{5,6a}$)	H-6a dd ($J_{6a,6b}$)	H-6b dd ($J_{5,6b}$)	CH_3 s	Aglycon
124	4.82 (10.4)	3.98 ddd (10.0)	5.20 (9.4)	5.03 (9.7)	3.70 mult	4.16 mult		1.96-2.05 4 s	7.47, mult, H_m 7.29, mult, H_o+H_p
125 Glc	4.65 (10.1)	4.88 (9.2)	5.19 (9.1)	3.72 (9.9)	3.62 (2.0)	4.56 (11.9)	4.08 (5.6)	1.94-2.12 7 s	7.45, mult, H_m 7.29, mult, H_o+H_p
Gal	4.44 (7.9)	5.08 (10.5)	4.92 (3.4)	5.32 (1.0)	3.64 (6.7)	4.10 (10.2)	4.04 (7.2)		
126 Glc	5.18 4 line AB_{mult} (9.3)		5.24 (9.1)	3.89 (9.6)	3.67 (2.5)	4.49 (12.0)	4.23 (4.8)	1.94-2.08 7 s	7.50, mult, H_m 7.27, mult, H_o+H_p
Glc'	5.34 (3.9)	4.79 (10.5)	4.92 (9.6)	4.99 (10.2)	3.89 (2.2)	4.17 (12.4)	3.99 (8.7)		
127 Glc	4.81 (10.1)	4.95 (9.0)	5.24 (8.8)	3.74 6 line AB_{mult}	(1.7)	4.53 (12.0)	4.10 (4.4)	1.95-2.13 7 s	7.54, d,, H_o 8.13, d, H_m $J_{o,m}$ = 9.0
Gal	4.46 (7.8)	5.09 (10.4)	4.94 (3.4)	5.33 (1.0)	3.85 (6.3)	4.12 (11.0)	4.04 (7.3)		
128 Glc	4.85 5 line AB_{mult} (8.9)		5.31 (8.8)	3.96 (9.8)	3.84 (2.5)	4.52 (12.3)	4.22 (5.1)	1.98-2.13 7 s	7.55, d, H_o 8.14, d, H_m $J_{o,m}$ = 9.0
Glc'	5.38 (4.0)	4.83 (10.5)	5.33 (9.7)	5.03 (10.0)	3.93 (2.5)	4.22 (12.5)	4.04 (2.5)		
129	4.56 (10.4)	4.04 mult	7 line AB mult	(9.7)	3.66 (4.9)	4.22 (12.4)	4.10 (2.5)	1.94-2.05 4 s	1.24, t, J=7.5, CH_3 2.70, dt, J=1.8, CH_2
130 Glc	4.57 (10.1)	4.85 (9.2)	5.27 (10.1)	3.97 (9.6)	3.68 (2.6)	4.49 (12.1)	4.18 (4.4)	2.98-2.12 8 s	2.93, ddd (1H, -CH_2-S) 2.76, ddd (1H, -CH_2-S)
Glc'	5.39 (4.0)	4.83 (10.6)	5.34 (9.6)	5.03 (10.1)	3.93 mult	mult at 4.21 overlap with -CH_2-OAc			J_{gem} = 14.0 J = 7.2 J = 6.7

* 300 MHz, $CDCl_3$. δ are in ppm, J are in Hz. Assignments are based on 2D ^{1}H-^{1}H COSY experiments.

6.3 *O*-Ethyl xanthate

S-glycosyl xanthates[171] are versatile synthetic carbohydrate derivatives[172]. This family of compounds have recently gained interest because of their use as efficient and stereoselective glycosyl donors in cases where the usual glycosyl halides and thioglycosides gave modest results[173]. *S*-glycosyl xanthates are also useful precursors to reducing sugars, 1-deoxy- and 1-thio-sugars, as well as 1-thioglycosides[165a, 166d].

Scheme 29 PTC synthesis of *O* -ethyl β-D-glycosyl dithiocarbonates



	X	R_1	R_2	R_3
58	Cl	NHAc	OAc	H
110	Br	OAc	H	OAc
111	Br	OAc	OAc	H
112	Br	OAc	H	α-D-Gal(OAc)$_4$

	R_1	R_2	R_3
131	NHAc	OAc	H
132	OAc	H	OAc
133	OAc	OAc	H
134	OAc	H	α-D-Gal(OAc)$_4$

Although previous syntheses of *S*-glycosyl xanthates have been relatively efficient, the success of PTC methodology encountered so far prompted the attempt at extending its use as an entry to this class of compounds[24g]. It was hypothesized that the soft nature of a xanthate nucleophile could provide good yields of the desired glycosides under mild conditions. This was truly justified, as a

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171 a) Work previously presented in: F.D. Tropper, F.O. Andersson, S. Cao and R. Roy, *J. Carbohydr. Chem.* , *11* , 741 (1992). See also: b) M. Sakata, M. Haga and S. Tejima, *Carbohydr. Res.*, *13* , 379 (1970); c) H. Paulsen, R. Rauwald and U. Weichert, *Liebigs Ann. Chem.*, 75 (1988); d) A. Marra, L.K. Shi Shun, F. Giuffeny and P. Sinay, *Synlett.*, 445 (1990).

172 a) A. Marra and P. Sinay, *Carbohydr. Res.*, *187* , 35 (1989); b) A. Marra and P. Sinay, *Carbohydr. Res.*, *195* , 303 (1990); c) W. Birberg and H. Lönn, *Tetrahedron Lett.*, *33* , 115 (1992) and references within; d) D. Horton and D.H. Hutson, *Adv. Carbohydr. Chem.*, *18* ,123 (1963).

173 K. Okamoto and T. Goto, *Tetrahedron*, *46* , 5835 (1990).

variety of *O*-ethyl β-D-glycosyl dithiocarbonates (**131 - 134**) were prepared in high yields (> 90%) under mild conditions using *O*-ethyl xanthic acid potassium salt (Scheme 29). Again, the reactions were highly stereoselective with only 1,2-trans β-D-*S*-glycosyl xanthates being detected. No mixture of anomers or glycosidic by-products could be detected from the crude reaction mixture by TLC, ^{1}H- and ^{13}C-NMR spectroscopy. No competitive dehydrohalogenation was detected as in the case of nucleophiles of high basicities seen earlier. The reactions were slightly faster in ethyl acetate than in methylene chloride. Control experiments revealed that the *O*-ethyl *S*-potassium dithiocarbonate was consumed by reacting with methylene chloride when used as the organic phase. When the reaction was conducted in the absence of a glycosyl halide in CH_2Cl_2, methylene bis-(*O*-ethyl xanthate) $(EtO\text{-}C(S)S)_2CH_2$ was obtained[174]. Product yields were virtually unchanged when using either ethyl acetate or methylene chloride as solvent. However, at least 2 equivalents of the nucleophile were necessary for complete consumption of the glycosyl halide in CH_2Cl_2 while 1.2 equivalents was sufficient for transformations performed in ethyl acetate. Most of the *S*-glycosyl xanthates prepared were previously known compounds and their physical data (m.p., $[\alpha]_D$) are in good agreement with reported values. Physical properties are given in Table 40. The fully assigned (2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR) ^{1}H- and ^{13}C-NMR spectroscopic data are given in Tables 40 and 41.

In conclusion, a mild, highly stereospecific and high yielding entry into useful *S*-glycosyl xanthates has been achieved with PTC methodology. Readily available reagents were used. The reactions occurred with complete inversion of configuration at the anomeric centres. This method has also proven successful in preparing an analogous sialic acid derivative[24g] and has potential use for other glycosyl halides.

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[174] Identified by CI-MS, ^{1}H- and ^{13}C-NMR spectroscopy.

Table 40 Physical properties of *S*-glycosyl xanthates **131-134**.

Product	Yield (%)	Melting Point (°C)	$[\alpha]_D$ ($CHCl_3$)	Formula	Elemental Analysis							
					Calculated (%)				Found (%)			
					C	H	N	S	C	H	N	S
131	91	139.0-140.2[a]	+33.3°	$C_{17}H_{25}NO_9S_2$	45.22	5.58	3.10	14.20	45.02	5.68	3.04	14.12
132	98	64.8-66.9[b]	+30.2°	$C_{17}H_{24}O_{10}S_2$	45.13	5.35	–	14.17	45.16	5.34	–	14.29
133	91	79.5-80.3[c]	+49.7°	$C_{17}H_{24}O_{10}S_2$	45.13	5.35	–	14.17	44.88	5.12	–	14.40
134	96	amorphous	+2.3°	$C_{29}H_{40}O_{18}S_2$	47.07	5.44	-	8.66	*	*	-	*

a) ether/diisopropyl/ether; b)crystallized on standing without solvent; c) CH_2Cl_2/ether/hexane
* Although pure by TLC and NMR, **133**, twice failed to give an acceptable analysis. CI-MS (ether) gave m/e: 740.8 (1,1%, M+1), 680.9 (4.4%, M-COS+1), 619.1 (46.3%, M-$EtOCS_2$·).

Table 41 ^{1}H-NMR data of *S*-glycosyl xanthates **131-134** ($CDCl_3$, 300 MHz)[a].

Compound[b]		H-1 d ($J_{1,2}$)	H-2 dd ($J_{2,3}$)	H-3 dd ($J_{3,4}$)	H-4 dd ($J_{4,5}$)	H-5 ddd ($J_{5,6\,a}$)	H-6a dd ($J_{6a,6b}$)	H-6b dd ($J_{5,6b}$)
131[d]		5.42 (10.9)	4.36 -	5.06 - 5.22 * (- , 9.4)		3.78 (4.9)	4.22 (12.5)	4.09 (2.3)
132		5.40 (10.4)	5.11 (9.0)	5.27 (9.3)	5.04 (9.9)	3.78 (4.9)	4.19 (12.5)	4.05 (2.2)
133		5.43 (9.7)	5.33 (9.2)	5.12 (3.4)	5.38 (3.3)	3.96 - 4.07 mult		
134	Glc	5.41 (10.6)	5.07 (9.1)	5.28 (8.4)	3.80 (10.0)	3.72 (1.9)	4.43 (11.8)	4.03-4.14
	Gal	4.44 (7.9)	5.09 (10.4)	4.92 (3.5)	5.33 (1.2)	3.85 (6.8)	4.03 - 4.14 (- , 6.8)	

a) Assignments are based on HETCOR and COSY experiments. Chemical shifts are referenced to internal $CHCl_3$ at 7.24 ppm and are given in ppm relative to TMS. Coupling constants (J) are given in Hz.
b) OAc, NAc: 1.85 - 2.13 ppm; singlets.
c) Ethyl: CH_2 : 4.57 - 4.62 (q, J = 7.1 Hz), CH_3 : 1.35-1.40 (t, J=7.1 Hz).
d) NH: 5.69 (d, $J_{2,NH}$ = 9.4 Hz).
* AB multiplet

Table 42 ^{13}C-NMR data[a] of compounds **130-133** ($CDCl_3$, 75.4 MHz)

Compound[b]		C-1	C-2	C-3	C-4	C-5	C-6	CH_2	CH_3
131		87.5	51.5	73.9	67.9	76.5	61.8	70.7	13.5
132		85.3	67.8	73.5	68.2	76.1	61.4	70.5	13.2
133		86.0	65.8	71.8	67.2	75.0	61.3	70.6	13.6
134	Glc	85.6	68.7	73.8	75.9	77.2	62.0	70.7	13.6
	Gal	101.0	69.0	70.9	66.6	70.7	60.8		

a) Assignments based on HETCOR and COSY experiments. Chemical shifts (ppm) are referenced to internal $CDCl_3$ at 77.0 ppm.
b) C=S: 209.8 - 210.5 ppm; C=O (Ac): 169.1 - 170.3 ppm; CH_3 (OAc): 20.1 - 21.1 ppm; CH_3(NAc): 23.0.

6.4 Dibenzyl phosphate

Carbohydrate phosphates are important intermediates in a wide variety of biosynthetic processes[175]. They constitute integral compounds of the core and lipid A region of lipopolysaccharides, bacterial polysaccharides, teichoic acids, sugar nucleotides, glycophospholipids and phosphoinositides. The occurrence and methods for their preparation have been reviewed[176]. Previous methods for the synthesis of glycosyl 1-phosphates are based essentially on two concepts. In the first one, phosphoric acid, dialkyl phosphates or their salts were used as nucleophiles on glycose peracetates, 1,2-orthoesters, halogenoses, 1,2-oxazolines or trichloroacetimidates. In a second approach, anomeric hydroxyl groups were activated as lithium or thalium salts and reacted with electrophilic dialkyl phosphorochloridates.

From the previous observations that soft nucleophiles, generated

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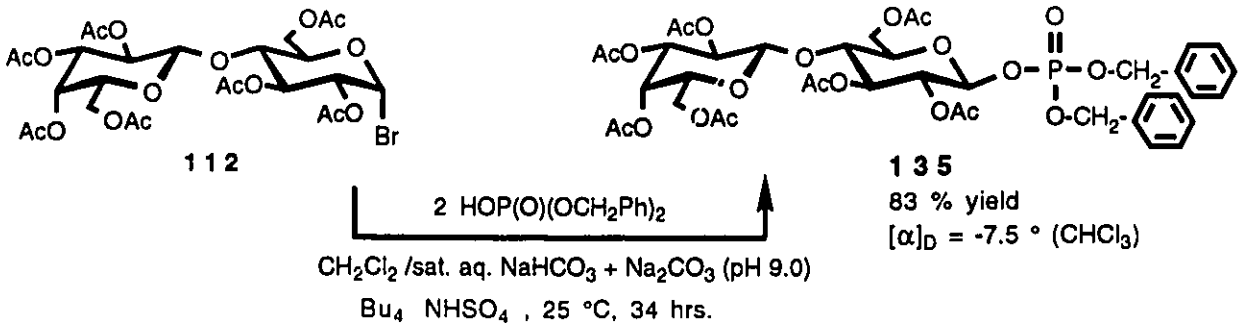
175 H. Kikaido and W.Z. Hassid, *Adv. Carbohydr. Chem. Biochem.*, *26* , 351 (1971).
176 J. Thiem and M. Franzkowiak, *J. Carbohydr. Chem.*, *8* , 1 (1989).

from weak acids (pKa ≤ 8), appear to be well suited for nucleophilic displacement of halide in glycosyl halides, an attempt at preparing glycosyl phosphates by PTC was tried. If successful, this procedure would give access to carbohydrate phosphotriesters from readily available reagents. In fact, such a procedure was found to give high yields of glycosyl- and glycobiosyl-dibenzyl phosphotriesters from various peracetylated glycosyl bromides, and has been published[24d].

Scheme 30 1,2-*trans* -β-D-Lactosyl phosphate synthesis by PTC



Treatment of acetobromo α-D-lactose **112** in methylene chloride and saturated sodium bicarbonate with dibenzyl phosphate (2 equiv.) and TBAHS (1 equiv.) at room temperature for 1.5 days, gave the phosphate **135** in 83% yield after chromatographic separation. By-products included the lactal **122** (4%) and products of anomeric hydrolysis (C_1-OH, ≈10%).

Obviously, the reaction was much slower than those using phenoxides, thiophenoxides or *O*-ethyl xanthate. Increasing the basicity of the aqueous phase with either 1M or 2M Na_2CO_3 or 1M NaOH did increase the rate of consumption for the lactosyl halide **112** but at the expense of lower product yield and higher yields of anomeric hydrolysis (TLC). The use of different organic solvents (EtOAc or toluene) or phase transfer catalyst resulted in slower reactions and lower yields.

Unlike the previous and later glycosylations, 2-acetamido-3,4,6-tri-

O-acetyl-2-deoxy-α-D-glucopyanosyl chloride **58** was incapable of producing the analogous β-D-glycosyl phosphate. The oxazoline **60** was the major product of all attempts and was formed at a higher rate (TLC) in the presence of dibenzyl phosphate than without. Thus, it is believed that the nucleophilic displacement of the α-chloride by dibenzyl phosphate might have occurred, followed by rapid intramolecular displacement of the phosphate aglycon by the amide function to provide the oxazoline **60**. This could be rationalized by a favoured anchimeric participation from the acetamido group in **58** over the acetyl group in the 2-position of **112**.

The β-D-lactosyl phosphate **135** was fully characterized by high field ^{1}H- and ^{13}C-NMR spectroscopy with the help of 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments. The anomeric carbon of **134** appeared at 96.2 ppm as a doublet ($^{2}J_{C,P}$ = 4.9 Hz) as well as the C-2 signal found at 71.5 ppm ($^{3}J_{C,P}$ = 9.0 Hz). The anomeric proton appeared as a doublet of doublets at 5.30 ppm ($^{3}J_{1,2}$ = 7.5, $^{3}J_{H,P}$ = 7.5 Hz). A fully assigned ^{1}H-NMR spectrum and 2D ^{1}H-^{1}H COSY spectrum of **134** is shown in Figures 27 and 28.

Figure 27 Resolution-enhanced 500 MHz ^{1}H-NMR spectrum of dibenzyl (per-*O*-acetylated β-D-lactosyl) phosphate **134**

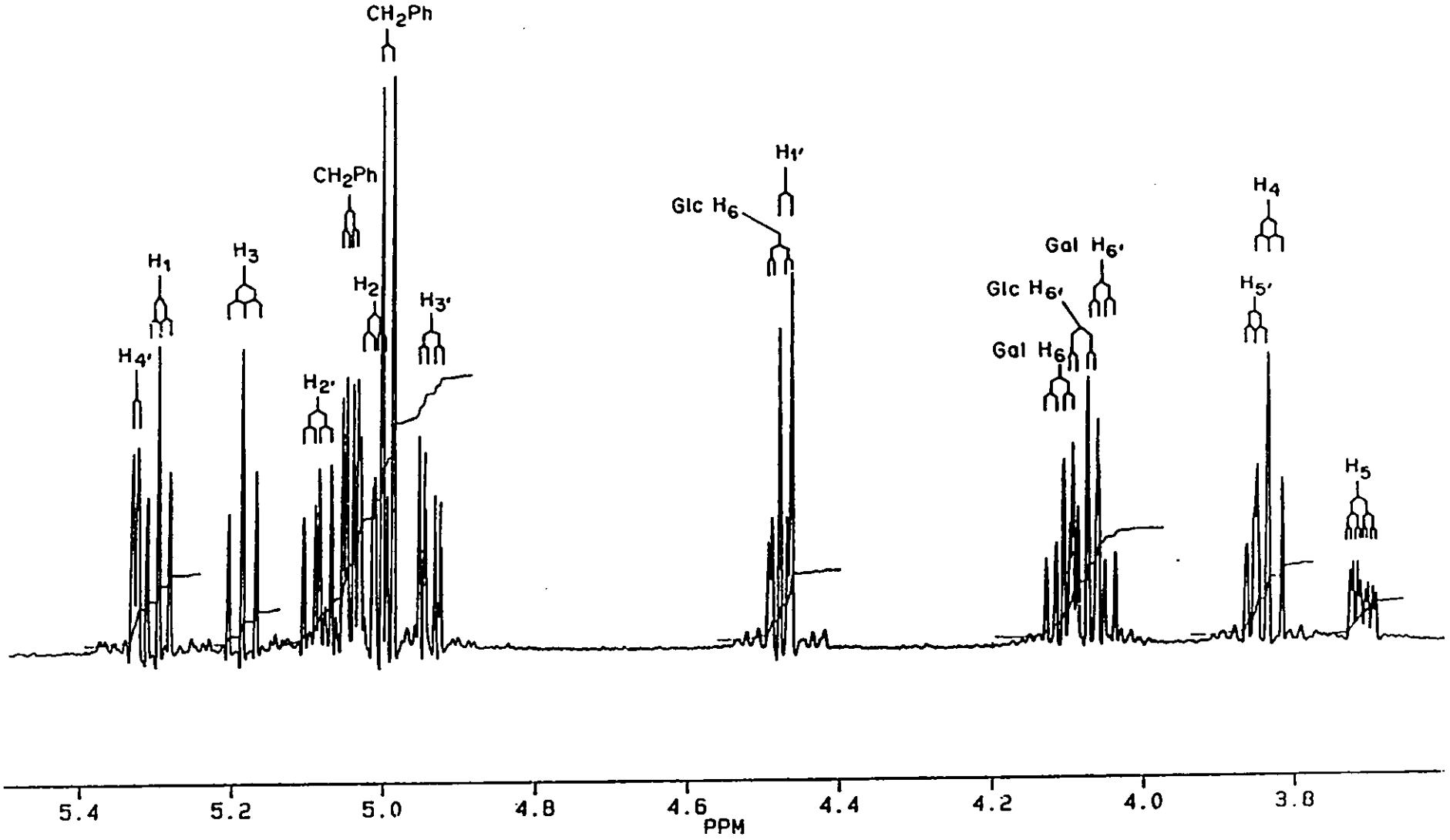
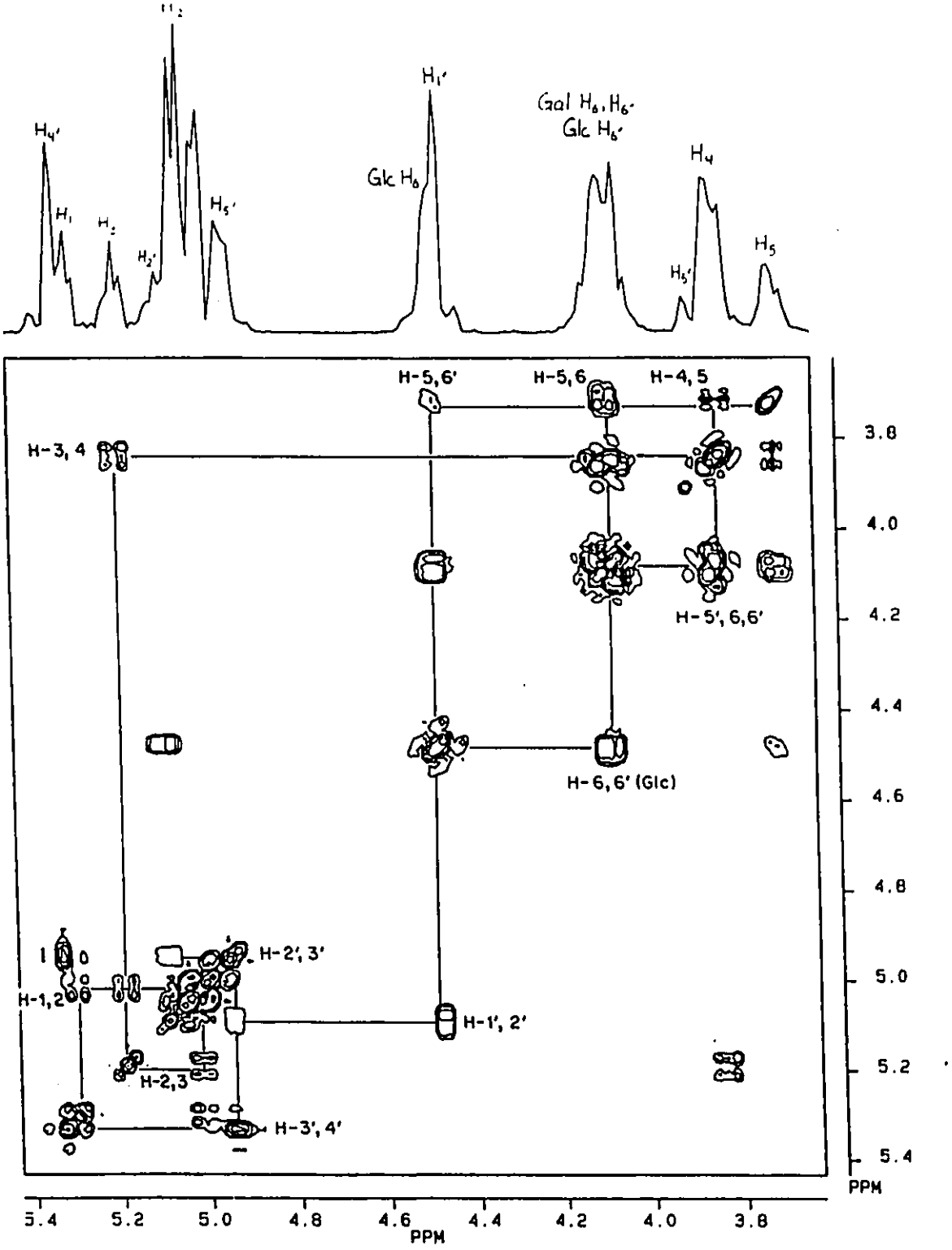


Figure 28 Expanded 2D ^{1}H-^{1}H COSY contour plot of the sugar ring proton region of **134**.



In conclusion, 1,2-*trans* β-D-glycosyl phosphotriesters can be synthesized in good yield with complete stereocontrol by PTC methodology[24d]. PTC presents clear advantages over previous methodologies because all reagents are stable, nontoxic and readily available. In addition, β-glycosyl phosphates can give access to α-glycosyl phosphates by Lewis acid catalyzed anomerization[177], and have been shown to act as useful glycosyl donors[178].

6.5 Azide

Glycosyl azides make up an important family of carbohydrate derivatives[179]. They have been widely used as precursors of glycopeptides[180] and heterocyclic *N*-glycosides[181]. The corresponding glycosylamines, which can be obtained by reduction of the azide group[182], can be used as monomeric precursors to glycopolymers after suitable *N*-acryloylation[45a,183]. Recent applications of solid-phase peptide synthesis methodologies to glycopeptide preparations[184] have reactivated interest in glycosyl azides[185]. This is particularily true in light of their use as protected glycosyl acceptors[186].

Previous syntheses of glycosyl azides from glycosyl halides were

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177 a) R.R. Schmidt, M. Stumpp and J. Michel, *Tetrahedron Lett.*, *23* , 405 (1982); b) R.R. Schmidt and M. Stumpp, *Liebigs Ann. Chem.* , 680 (1984).

178 a) S.I. Hashimoto, T. Honda and S. Ikegame, *J. Chem. Soc. Chem. Commun.* , 685 (1989); b) M. Michalska, *Heterocycles*, *28* , 1244 (1989); c) R.R. Schmidt, H. Gaden and H. Jatzke, *Tetrahedron Lett.*, *31* , 327 (1990);d) R.R. Schmidt, M. Behrendt and A. Toepfer, *Synlett.* , 694 (1990).

179 F. Micheel and A. Klemer, *Adv. Carbohydr. Chem. Biochem.*, *16* , 85 (1961).

180 H.G. Garg and R.W. Jeanloz, *Adv. Carbohydr. Chem. Biochem.*, *43* , 135 (1985).

181 G. Baum and F. Micheel, *Chem. Ber.*, *90* , 1595 (1957).

182 R. Roy and W.K.C. Park unpublished results.

183 R. Roy and C.A. Laferriere, *J. Chem. Soc. Chem. Commun.* , 1709 (1990).

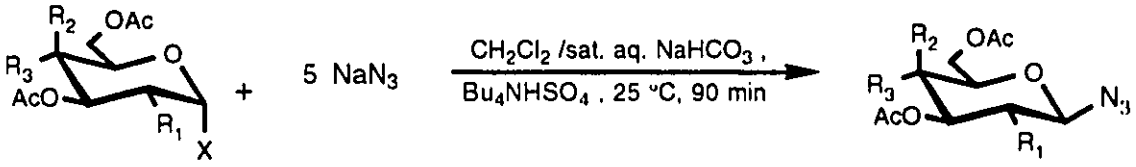
184 a) H. Kunz and B. Dombo, *Angew. Chem. Int Ed. Engl.*, *27* , 711 (1988);b) M. Hollósi, E. Kollát, I. Laczkó, K.F. Medzihradsky, J. Thurin and L. Otrös, *Tetrahedron Lett.*, *32* , 1531 (1991).

185 S.T. Anfiel and P.T. Jr. Lansbury, *J. Org. Chem.*, *55* , 5560 (1990).

186 H. Kunz, *Angew. Chem. Int Ed. Engl.*, *26*, 294 (1987).

accomplished under homogeneous one-phase conditions with silver or sodium azides in chloroform or formamide respectively[173,187]. More recently, they have been prepared from 1-*O*-acyl glycosides with trimethylsilyl azide and a Lewis acid catalyst[188]. Reports by Kunz and coworkers[189], then by Thiem and Wiemann[190], on the use of PTC for the synthesis of **138** or its corresponding chitobiosyl analogue with Aliquot 336 or TBAHS in chloroform prompted the application of the PTC conditions described earlier to the synthesis of a range of glycosyl azides to demonstrate the generality of this methodology. Under the very mild conditions reported here and already published with coworkers[24c], glycosyl azides **136-138** and disaccharides **139, 140** as well as an analogous sialic acid derivative (not shown) have been prepared in excellent yields (93-98%, Table 43).

Scheme 31 1,2-*trans* β-D-glycosyl azides by PTC



	X	R_1	R_2	R_3
58	Cl	NHAc	H	OAc
110	Br	OAc	H	OAc
111	Br	OAc	OAc	H
112	Br	OAc	H	β-D-Gal(OAc)$_4$
113	Br	OAc	H	α-D-Gal(OAc)$_4$

	R_1	R_2	R_3
136	NHAc	H	OAc
137	OAc	H	OAc
138	OAc	OAc	H
139	OAc	H	β-D-Gal(OAc)$_4$
140	OAc	H	α-D-Gal(OAc)$_4$

Thus, treatment of 1,2-cis-peracetylated α-D-glycosyl- and

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187 A. Bertho and H. Nüssel, *Ber., 63* , 836 (1930)

188 a) H. Kunz, W. Sager, D. Schanzenbach and M. Decker, *Liebigs ann. Chem.* , 649 (1991);b) H. Paulsen, Z. Györgydeak and M. Friedman, *Chem. Ber., 107* , 1568 (1974).

189 H. Kunz, H. Waldmann and J. März, *Liebigs Ann. Chem.* , 45 (1989).

190 J. Thiem and T. Wiemann, *Angew. Chem. Int. Ed. Engl., 29* , 80 (1990).

glycobiosyl halides (**58**, **110-113**) with TBAHS (1 equiv.) and sodium azide (3-5 equiv.) in a liquid two phase system (CH_2Cl_2/sat. $NaHCO_3$) for 2 hours at room temperature gave almost quantitative yields of the corresponding 1,2-*trans* β-D-glycosyl azides (**136-140**). All reactions occurred with complete anomeric inversions. No mixture of anomers or elimination by-products could be detected from the crude reaction mixtures by ^1H- and ^{13}C-NMR spectroscopy. No adverse solvent effects were noticed. Using ethyl acetate instead of methylene chloride gave identical results. Changing the aqueous phase from saturated $NaHCO_3$ to 1M or 2M Na_2CO_3 had no adverse effect either. A slight increase in reaction rate was observed, however.

Table 43 PTC synthesis of peracetylated 1,2-*trans* β-D-glycosyl azides and their physical constants[191].

					Elemental Analysis					
					Calculated (%)			Found (%)		
Product	Yield (%)	Melting Point (°C) #	$[\alpha]_D$ *	Formula	C	H	N	C	H	N
136	93	138.2-139.0	-31.0°	$C_{14}H_{19}N_3O_9$	45.04	5.13	11.26	44.93	5.32	11.21
137	96	103.3-104.0	-14.0°	$C_{14}H_{19}N_3O_9$	45.04	5.13	11.26	45.19	5.22	11.43
138	98	166-167 (dec)	-47.7°	$C_{14}H_{20}N_4O_8$	45.16	5.41	15.05	45.34	5.44	14.99
139	98	amorphous	-20.4°	$C_{26}H_{35}N_3O_{17}$	47.20	5.33	6.35	46.93	5.31	6.07
140	97	105.6-106.6	+51.8°	$C_{26}H_{35}N_3O_{17}$	47.20	5.33	6.35	47.15	5.40	6.28

crystallized from ethanol; * C=1, $CHCl_3$

Most of the above glycosyl azides were known compounds[173] and the physical data (m.p., $[\alpha]_D$) obtained are in good agreement with reported values (see experimental section). The fully assigned

[191] Physical constants were in good agreement with values reviewed in ref. #173.

(HETCOR, COSY) ^{1}H- and ^{13}C-NMR spectroscopic data (Tables 43 and 44) of **136-140** confirmed the 1,2-trans-β-D-anomeric configuration ($J_{1,2}$ = 8.7 ± 0.2 Hz; C-1, δ = 87.4-88.3 ppm). The infrared spectra of **136-140** gave $\nu_{N\equiv N}$ = 2120 ± 1 cm^{-1} for solutions in $CHCl_3$. Mass spectra of all glycosides CI (ether) or (+)FAB (glycerol) all gave clear molecular ion peaks (M + 1) with concomitant losses of N_3 or N_2 followed by HCN followed by sequential losses of HOAc. A representative spectrum is given for the peracetylated 2-acetamido-2-deoxy-β-D-glucopyranosyl azide **138** in Figure 29.

Table 44 ^{1}H-NMR data* of peracetylated glycosyl azides **136-140.**

Compound		H-1 d ($J_{1,2}$)	H-2 dd ($J_{2,3}$)	H-3 dd ($J_{3,4}$)	H-4 dd ($J_{4,5}$)	H-5 ddd ($J_{5,6a}$)	H-6a dd ($J_{6a,6b}$)	H-6b dd ($J_{5,6b}$)
136		4.65	4.96	5.22	5.11	3.79	4.28	4.17
		(8.9)	(9.3)	(9.5)	(9.9)	(4.7)	(12.5)	(2.4)
137		4.58	5.15	5.01	5.40	3.99	4.13 - 4.17 AB	
		(8.5)	(10.4)	(3.3)	(1.1)	(5.9)	(-)	(7.1)
138 #		4.75	3.89	5.23	5.07	3.77	4.25	4.13
		(9.3)	(10.3)	(9.5)	(9.7)	(4.7)	(12.5)	(2.4)
139	Glc	4.61	4.85	5.19	3.79	3.68	4.49	4.02-4.14
		(8.8)	(9.5)	(9.0)	(10.0)	(4.9)	(12.5)	(2.1)
	Gal	4.46	5.09	4.93	5.33	3.85	4.02 - 4.14	
		(7.9)	(10.4)	(3.4)	(1.1)	(7.2)	(-)	(-)
140	Glc	4.69	4.77	5.24	4.00	3.76	4.49	4.02
		(8.7)	(9.0)	(8.9)	(9.6)	(4.5)	(12.3)	(2.6)
	Gal	5.39	4.84	5.33	5.03	3.92	4.19 - 4.26 AB	
		(4.1)	(10.5)	(9.5)	(10.2)	(3.6)	(-)	(2.2)

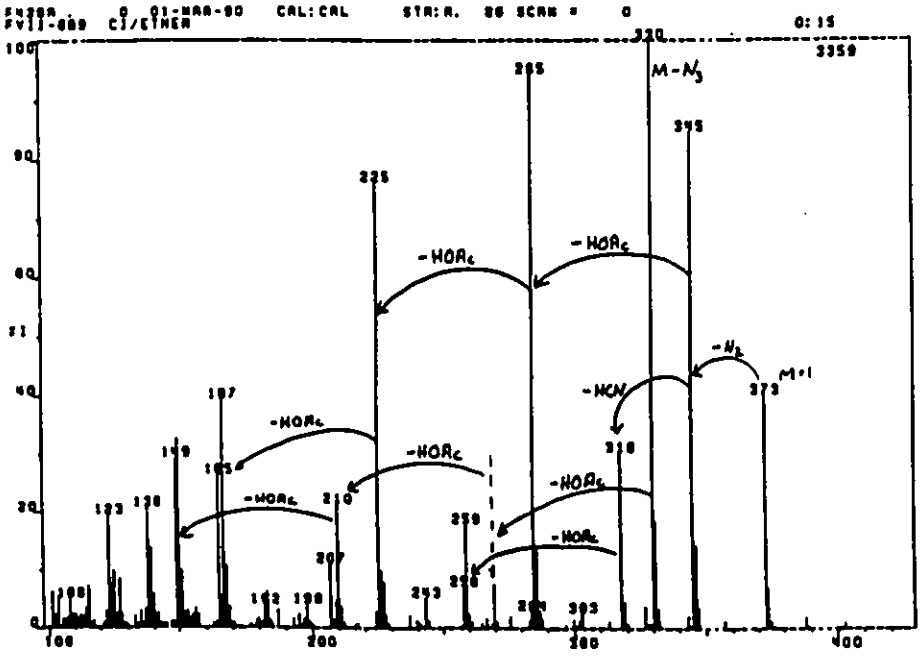
* Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments. Chemical shifts are in ppm relative to TMS and coupling constants (J) are in Hz for solutions in $CDCl_3$ (300 MHz); OAc and NAc CH_3 signals all appear as singlets 1.95 - 2.14 ppm.
# NH: 5.79 ($J_{2,NH}$ = 9.2 Hz).

Table 45 ^{13}C-NMR data[a] of peracetylated glycosyl azides **136-140** ($CDCl_3$, 75.4 MHz).

Compound		C-1	C-2	C-3	C-4	C-5	C-6	C=O	Ac(Me)
136		87.9	70.6	72.6	67.8	74.0	61.6	169.2-170.6 (4s)	20.5 20.7 (3s)
137		88.2	68.0	70.7	66.8	72.8	61.2	169.3-170.3 (4s)	20.5, 20.6 20.7 (2s)
138		88.3	53.9	72.0	68.1	73.8	61.8	169.5-170.3 (4s)	20.3, 20.4, 20.5, 23.0*
139	Gal	87.7	70.9	72.5	75.8	74.8	61.7	169.0-170.3	20.5, 20.6 (3s)
	Glc	101.1	69.0	71.0	66.5	70.7	60.8	(7s)	20.7 (2s), 20.8
140	Glc	87.4	71.5	75.1	72.3	74.2	62.5	169.4-170.5	20.5, 20.6 (3s)
	Glc'	95.7	69.9	69.2	67.9	68.6	61.4	(7s)	20.7 (2s), 20.8

a) Assignments are based on 2D ^1H-^1H COSY and ^1H-^{13}C HETCOR experiments. Chemical shifts (δ) are given in ppm relative to TMS.
* NHAc

Figure 29 CI-MS (ether) spectrum of **138**.



6.6 Thiocyanate

The successful application of PTC methodology to the preparation of 1,2-trans-β-D-glycosyl and -glycobiosyl halides from respective α-D-halogenoses with sodium azide prompted the attempt at preparing 1,2-trans-β-D-glycosyl thiocyanates using the same strategy. Glycosyl thiocyanates have recently been shown to act as good glycosyl donors in oligosaccharide synthesis[192] in a catalytic process involving triphenylmethylium cations. Very few glycosyl thiocyanates have been reported in the literature[186,193]. Applications other than as glycosyl donors have not yet been elaborated upon. They do have potential as hydrolysis-resistant ligands in glycoconjugate preparations. Reduction of the nitrile function (with $LiAlH_4$ for example) should give a thioglycoside with a short spacer terminated by an amine. As discussed throughout this dissertation, the amine can then be used for conjugation to various supports or carriers by a variety of methods .

Kotchetkov and coworkers[186] were able to prepare five different glycosyl thiocyanates in yields of 55 to 70% from 1,2-cis bromides in dry acetone, with potassium thiocyanate in the presence of 18-crown-6 ether. Glycosyl thiocyanates were isolated as kinetic products since, in solution, they can isomerize into isothiocyanates by an intramolecular rearrangement with inversion of anomeric configuration[186].

By applying a PTC strategy towards the synthesis of β-D-glucosyl thiocyanate, similar results to Kotchetkov and coworkers were obtained. Mixing acetobromoglucose **110** with an excess of potassium thiocyanate in a liquid two-phase system comprised of ethyl acetate and 1M Na_2CO_3 with TBAHS at room temperature for one hour gave an 82% yield of a 10:1 mixture (^{1}H-NMR) of β-D-glucosyl thiocyanate **141** and isothiocyanate isomer **142**.

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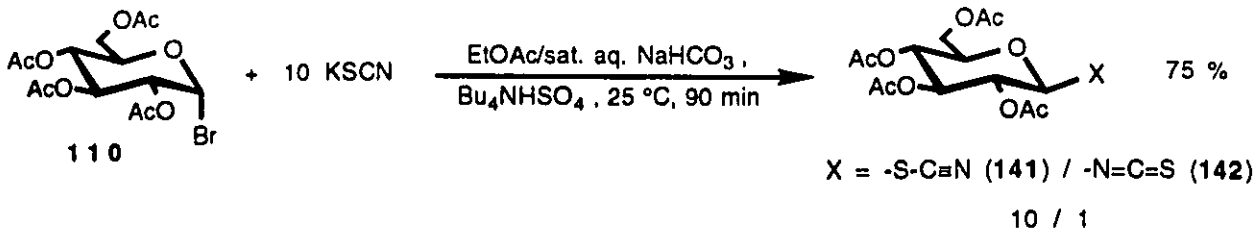
192 N.K. Kotchetkov, E.M. Klimov, N.N. Malysaeva and A.V. Demchenko, *Carbohydr. Res.*, *212* , 77 (1991).

193 a) Z. Witzak, *Adv. Carbohydr. Chem. Biochem.*, *44* , 91 (1986);b) A. Muller and A. Wilhelms, *Ber.*, *74* , 698 (1941).

Scheme 32 PTC glycosylation of thiocyanate anion



The reaction did not proceed in CH_2Cl_2, and gave poor results when the alkalinity of the aqueous phase was decreased. A large excess of KSCN (10 equiv) was required to effect the transformation cleanly and in reasonable time. The products were isolated by silica gel chromatography. The nature of the products were confirmed by IR spectroscopy. The thiocyanate **141** had a sharp $\nu_{S\text{-}C\equiv N}$ absorption at 2167 cm^{-1}, while the isothiocyanate **142** had a relatively strong and broad $\nu_{N=C=S}$ absorption at 2025 cm^{-1} [186].

Table 46 PTC synthesis of **141** and **142** with some physical constants

Product	Yield (%)[a]	Melting Point (°C)[b]	$[\alpha]_D$ *	Formula	Elemental Analysis							
					Calculated (%)				Found (%)			
					C	H	N	S	C	H	N	S
141[c]	68	114.5-114.8	- 18.5°	$C_{15}H_{19}O_9S$	46.27	4.92	3.60	8.23	46.33	5.02	3.49	8.39
142	7	113.6-115.7	+ 4.7°	$C_{15}H_{19}O_9S$	46.27	4.92	3.60	8.23	46.32	5.03	3.56	8.37

a) Isolated by silica gel chromatography.
b) Crystallized from ethyl acetate/hexane.
c) Lit.[186] yield 62%, $[\alpha]_D$ = -21° ($CHCl_3$), mp = 132-133.5°C (benzene/hexane).
* C=1, $CHCl_3$

Unlike Kotchetkov's synthesis, the isothiocyanate **142** produced by PTC had a 1,2-*trans* β-D configuration ($J_{1,2}$= 8.5 Hz). Thus, it must have been produced by nucleophilic displacement of bromide by isothiocyanate, which exists as a resonance form of thiocyanate

($^{-}$S-C≡N ↔ S=C=N^{-}), and not by a rearrangement process as previously determined.

Table 47 ^{1}H-NMR data* (300 MHz, $CDCl_3$) for glycosides **141** and **142**.

Product	H-1 d ($J_{1,2}$)	H-2 dd ($J_{2,3}$)	H-3 dd ($J_{3,4}$)	H-4 dd ($J_{4,5}$)	H-5 ddd ($J_{5,6a}$)	H-6a dd ($J_{6a,6b}$)	H-6b dd ($J_{5,6b}$)
141	4.87 (9.6)	5.12 (9.3)	5.25 (9.2)	5.13 (10.0)	3.81 (4.8)	4.26 (12.6)	4.17 (2.4)
142	5.00 (8.5)	5.09 (9.5)	5.19 (9.2)	5.12 (10.0)	3.73 (4.7)	4.22 (12.5)	4.12 (2.3)

* Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments. Chemical shifts are in ppm relative to TMS and coupling constants (J) are in Hz for solutions in $CDCl_3$ (300 MHz); OAc group CH_3 signals all appear as singlets 1.95 - 2.10 ppm.

Table 48 ^{13}C-NMR chemical shifts data* ($CDCl_3$, 75.4 MHz) for **141** and **142**.

Product	C-1	C-2	C-3	C-4	C-5	C-6	C=0	CH_3	Aglycon
141	83.4	70.6	73.0	67.4	77.0	61.4	169.1 169.2 169.9 170.5	20.4 20.5 (2C) 20.7	107.8 -S-C≡N
142	83.5	71.8	72.5	67.6	74.0	61.5	169.0 169.2 170.1 170.5	20.5 (3C) 20.7	144.2 -N=C=S

* Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments. Chemical shifts (δ) are given in ppm relative to TMS.

6.7 Carboxylates

From the observation that glycosyl phosphates[24d] and xanthates[24g] can be produced very efficiently and in a completely stereospecific manner by PTC from dialkyl phosphates or *O*-ethyl xanthic acid salts and glycosyl halides[24d], an analogous strategy was attempted to produce 1,2-*trans* β-D-glycosyl esters form carboxylic acid nucleophiles. Glycosyl esters are often encountered in carbohydrate chemistry as synthetic intermediates. They are also found in natural products in such compounds as the antitumor agent (+)-Phyllanthoside[194] for example. Apart from being used as glycosyl donors for the synthesis of glycosyl azides[183], glycosides [195], thioglycosides[106a] and nucleosides[196], some β-D-glycosyl esters have recently been shown to be useful glycosyl donors in oligosaccharide synthesis[197]. The α-D-glycosyl esters appear to be less suitable as glycosyl donors than the β-anomers. Glycosyl esters are usually obtained as anomeric mixtures by acylation of anomerically unprotected sugars or by acidolysis of simple glycosides[198]. Recently[191b], a method involving the addition of carboxylic acid cesium salts to glycosyl halides in dimethyl formamide stereospecifically gave β-1-*O*-acyl glycosides in moderate to good yields (66-85%).

The application of PTC to the preparation of β-1-*O*-acyl glycosides has proven to be an effective synthetic method. Yields are high and conditions are mild with the few examples investigated. S_{N2}

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[194] A.B. Smith III and R.A. Rivero, *J. Am. Chem Soc., 109* , 1272 (1987) and references cited within.

[195] a) T. Ogawa, K. Beppa and S. Nakabayashi, *Carbohydr. Res., 93* , C6 (1981);b) L.F. Tietze, R. Fisher, *Angew. Chem., 95* , 902 (1983).

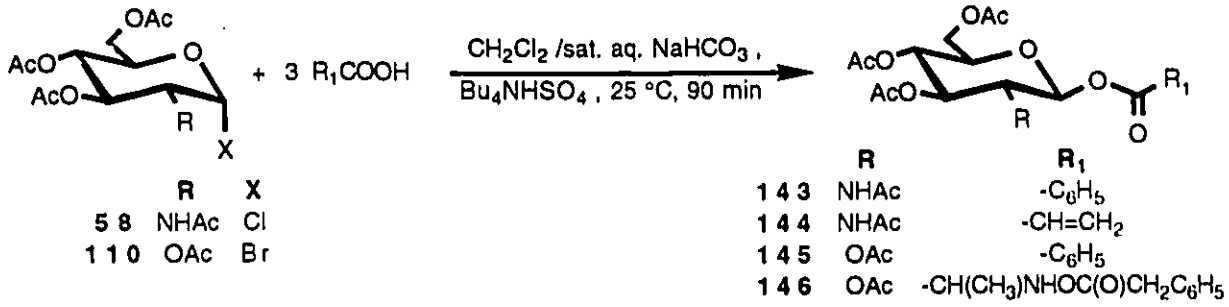
[196] a) H. Vorbrüggen, K. Krolikiewicz and B. Bhunuf, *Chem. Ber., 114* , 1234(1981);b) U. Niedballa and H. Vorbrüggen, *J. Org. Chem., 41* , 2084 (1976).

[197] a) F. Nicotra, L. Panza, A. Romano and G. Russo, *J. Carbohydr. Chem., 11* , 397 (1992); b) H. Kunz, R. Kullman, P. Wernig and J. Zimmer, *Tetrahedron Lett., 33* , 1969 (1992) and references cited within.

[198] a) B. Coxon and A.G. Fletcher, *J. Org. Chem., 26* , 2892 (1961);b) K. Takeo, *Carbohydr. Res., 77* , 245 (1979).

be performed in 60 minutes using a combination of CH_2Cl_2 and1M Na_2CO_3 as the reaction medium and TBAHS as phase transfer agent.

Scheme 33 Preparation of 1,2-*trans* β-D-glycosyl esters by PTC



	R	X
58	NHAc	Cl
110	OAc	Br

	R	R_1
143	NHAc	$-C_6H_5$
144	NHAc	$-CH=CH_2$
145	OAc	$-C_6H_5$
146	OAc	$-CH(CH_3)NHOC(O)CH_2C_6H_5$

Best results were achieved with an excess of carboxylic acid at mildly alkaline pH. It is suggested that the acidic phase transfer catalyst (Bu_4NHSO_4, TBAHS) and the carboxylic acid be carefully premixed in 1M Na_2CO_3, and then added to the glycosyl halide dissolved in CH_2Cl_2. In this way, possible effervescence due to CO_2 evolution can be controlled without any losses of reagents. In this fashion, 1,2-*trans*-β-D-glycosyl esters were synthesized with acrylic and benzoic acids as well as a carbobenzyloxy (CBZ) protected amino acid, *N*-CBZ-L-alanine. Transformations were carried out with acetobromoglucose **110** and acetochloro *N*-acetylglucosamine **58** (Scheme 33). Yields and physical constants are given in Table 49.

Table 49 Synthesis and physical properties of 1,2-*trans* β-D-glycosyl esters.

Product	Yield (%)	Melting Point (°C, EtOH)	$[\alpha]_D$ ($CHCl_3$)	Formula	Elemental Analysis					
					Calculated (%)			Found (%)		
					C	H	N	C	H	N
143	94	159.5-160.4	-68.3°	$C_{21}H_{25}NO_{10}$	55.87	5.58	3.10	55.92	5.61	3.00
144	76	145.8-147.4	-3.8°	$C_{17}H_{23}NO_8$	50.87	5.78	3.49	50.79	5.81	3.53
145	92	146.7-147.2	-25.6°	$C_{21}H_{24}O_{11}$	55.75	5.35	-	55.82	5.51	-
146	84	140.8-141.6	-4.1°	$C_{25}H_{31}NO_{13}$	54.25	5.65	2.53	54.29	5.70	2.59

Reaction with *N*-CBZ-L-alanine was performed as a model entry to glycoconjugate of amino acids containing a reactive anomeric ester bond[199]. Kunz and coworkers produced the allyloxycarbonyl protected analogue of **146** along with other similar β-D-glucosyl amino acids esters for this purpose[191b]. The method described here, however, is faster, much simpler and does not require impractical DMF as solvent. This method is also compatible with both 2-*O*-acyl and 2-deoxy-*N*-acetyl sugars.

6.8 Nitrogen nucleophiles

N-glycosides constitute an important class of carbohydrate derivatives. For example, nucleosides and many glycopeptides are *N*-linked glycosides. A PTC synthesis of *N*-linked glycosides was envisaged to further elaborate the usefulness of PTC glycosylations and potentially give access to these types of derivatives. The choice of nucleophiles was restricted to compounds of relatively acidic N-H bonds.

At first, attention was directed to imidazole (**144**, pKa = 6.9) as a nucleophile for a model entry into purine nucleoside synthesis.

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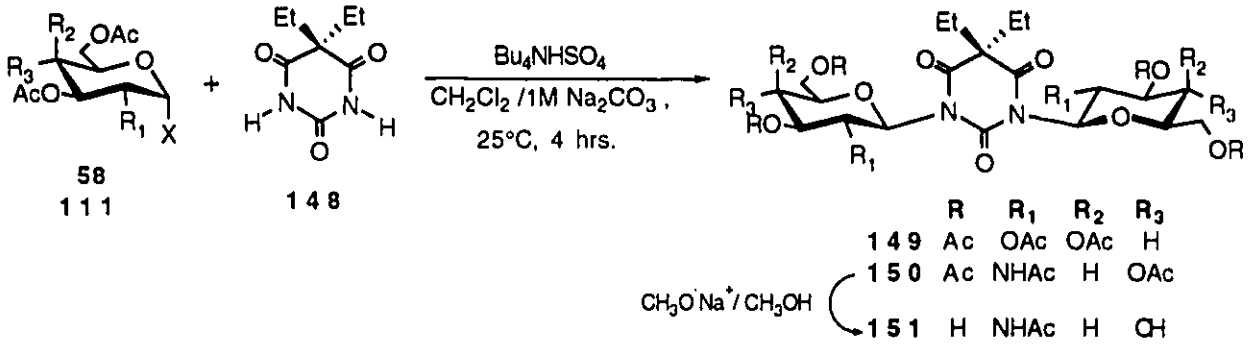
[199] a) D. Keglevic and S. Valentekovic, *Carbohydr. Res.*, *38* , 133 (1974)b) S. Horvat and D. Keglevic, *Carbohydr. Res.*, *108* , 89 (1982).

Attempts at glycosylating imidazole with various peracetylated glycosyl halides under PTC conditions failed to give synthetically useful results. With 1M NaOH as the aqueous base, no consumption of the halides could be observed with either ethyl acetate or benzene as the organic phase, even after 45 hours. Obviously, the imidazole anion/tetrabutylammonium couple is not very soluble in these organic solvents. Increasing the alkalinity with 2M NaOH had no effect. Conversely, when glycosylations were attempted with CH_2Cl_2 as the organic phase, the glycosyl halide was slowly consumed to give an excessive amount of unknown products (by TLC). Decreasing the alkalinity of the aqueous phase did not remedy the problem. Thus, these reactions were deemed synthetically useless and the strategy discontinued for the time being.



	R	R_1	R_2	R_3
149	Ac	OAc	OAc	H
150	Ac	NHAc	H	OAc
151	H	NHAc	H	OH

Scheme 34 PTC synthesis of bis *N*-glycosylated Barbital

The PTC synthesis of an *N*-glycoside was successful, however, when barbital (5,5-diethyl barbituric acid, **148**, pKa = 7.4) was used as a nucleophile and serves as a model entry into pyrimidine base-like aglycons. However, glycosylation of barbital could not be limited to one site only. Bisglycosylation of barbital was observed in all reactions (scheme 34). Monoglycosylation products could be observed by TLC when an excess (1.5 equiv.) of barbital was used but seemed to disappear over the extended reaction time (~4 hours) required for complete consumption of the glycosyl halide. Presumably, monoglycosylated barbital derivatives are soluble in the aqueous

phase since removal of a second acidic proton is required to form bis glycosylated products. While the remaining glycosyl halide becomes depleted, de-*O*-acetylation of the monoglycosylated product in the aqueous phase (1M Na_2CO_3) becomes important and causes the disappearance of this product from the organic phase (CH_2Cl_2). The only product remaining being the bis glycosylated compound which cannot be de-*O*-acetylated easily in the organic phase. Good yields (54-69%, purified by chromatography) of the bis glycosylated barbitals could be easily achieved when a 2.5 molar ratio of glycosyl halide (**58**, **111**) to barbital was used with TBAHS in a CH_2Cl_2/2M Na_2CO_3 solvent couple (scheme 34).

Table 50 PTC synthesis of bis *N*-glycosylated Barbital derivatives.

					Elemental Analysis					
					Calculated (%)			Found (%)		
Product	Yield (%)	Melting Point (°C)	$[\alpha]_D$ (C,solvent)	Formula	C	H	N	C	H	N
149	54	182.4-183.7 (CH_2Cl_2/Et_2O)	-21.1° (1.1,$CHCl_3$)	$C_{36}H_{48}N_2O_{21}$	51.19	5.73	3.32	51.20	5.96	3.41
150	69	190.1-191.4 (CH_2Cl_2/Et_2O)	-32.5° (1.2,$CHCl_3$)	$C_{36}H_{50}N_4O_{19}$	51.31	5.98	6.65	51.36	6.02	6.44
151	quant.	219.0-219.6 (i-prOH)	-37.3° (1.7, H_2O)	$C_{24}H_{38}N_4O_{13}$	48.81	6.49	9.49	48.85	6.64	9.21

Bis glycosylation was shown by ^{1}H-NMR, mass spectrometry as well as elemental analysis. The nature of the *N*-glycoside linkage was confirmed by ^{13}C-NMR spectroscopy ($\delta_{C\text{-}1}$ = 80.3 - 81.4 ppm). 1,2-*trans* diaxal coupling constants ($J_{1,2}$ = 10.0 ± 1 Hz) in the ^{1}H-NMR spectra also confirmed the β-D-anomeric configuration of each sugar. The two glycosyl substituents are not equivalent by NMR, however. Two distinct sets of equally intense sugar resonances are observed in the ^{1}H- and ^{13}C-NMR spectra of **149** and **150** for solutions in either $CDCl_3$ or $DMSOd_6$. Spectra become more complicated in the case of the de-*O*-acetylated version, **151**.

Figure 30 a) ^{1}H- and b) ^{13}C-NMR spectra of **150**

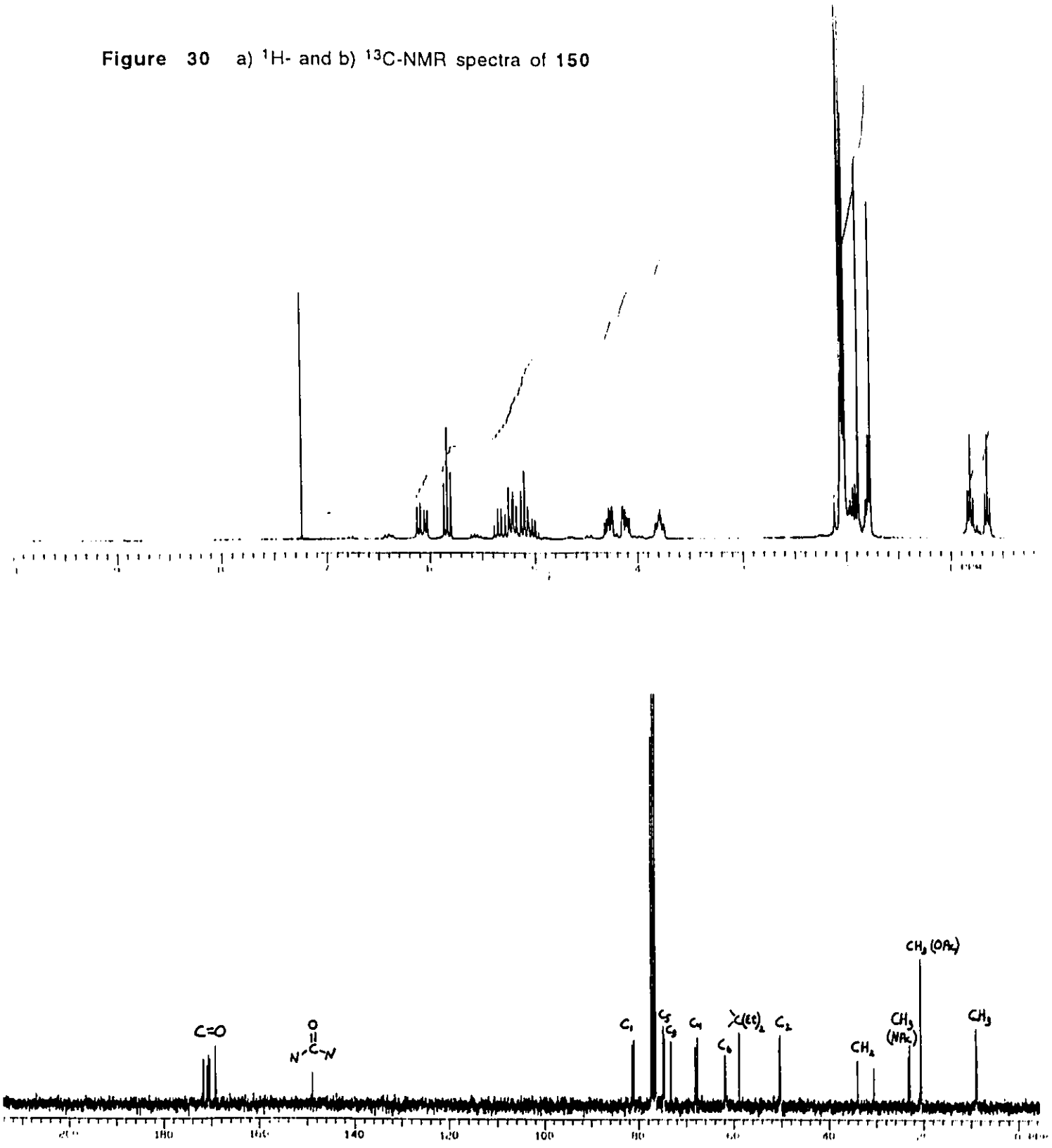
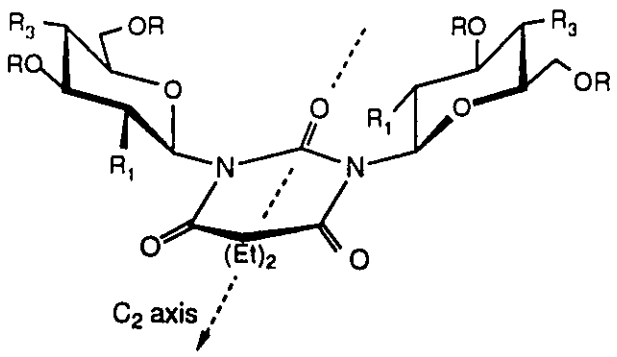


Figure 31



	R	R_1	R_2	R_3
149	Ac	OAc	OAc	H
150	Ac	NHAc	H	OAc
151	H	NHAc	H	OH

In the case of the per-*O*-acetylated glycosides **149** and **151**, there is a doubling of sugar resonances observed in the NMR spectra taken at 23 and 60 °C. This observation is difficult to explain considering generally accepted notions. Considering that the barbital exists as a planar and symmetric heterocyclic ring, by extension, derivatives **149-151** should also possess C_2 group symmetry (Figure 31). If free rotation about the C_1-N bond is permitted, chemical equivalence is expected for twin nuclei. A single set of resonances is expected. However, the existence of rotamers due to possible restricted rotation about the C_1-N bond could result in three possible isomers. But in this case, more than two sets of peaks would be expected. The suggestion that strong internal hydrogen bonding between the NHAc group and either of the two carbonyls of the barbital ring in **150** could contribute to the existence of inequivalent nuclei is made invalid by considering the identical NMR behaviour observed with the analogue **149**. The glucosyl analogue **149** cannot undergo such hydrogen bonding as it lacks a hydrogen bond donating group at C-2.

The adoption of a non-planar barbital ring conformation for barbital derivatives appears to be unlikely. However, if repulsive steric or electronic forces did exist in these derivatives such that the barbital ring was forced to adopt a puckered conformation, two different environments could be envisaged for the two halves of these derivatives as symmetry would be lost (Figure 32). With the considerations outlined above, a satisfactory explanation for the NMR behaviour of these derivatives cannot be provided at this time.

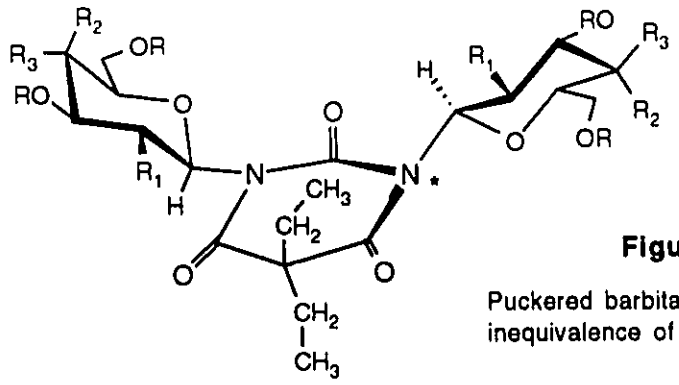


Figure 32

Puckered barbital ring conformation leading to the inequivalence of the two carbohydrate rings and twin nuclei

In the case of the de-*O*- acetylated bis glycosylated barbital **151**, the NMR spectra revealed multiple sets of resonances. In D_2O, three sets of resonances were observed for each carbon of the glycosyl residues in both the ^1H- and ^{13}C-NMR spectra . Multiple sets of peaks were also observed for the ethyl substituents. In addition, two of the three sets were of equal and low intensity while the third is of much higher intensity. When recorded at 70°C (D_2O), some of the multiple peak sets collapsed to one signal. When the NMR spectra of **151** was recorded in $DMSOd_6$ at ambient temperature, three identically intense sets of resonances were observed in both the ^{13}C- and ^1H-NMR spectra. When recorded at 150°C in $DMSOd_6$, resonances in the proton spectrum collapsed to give a single set of sharp resonances. However, multiple sets of resonances were still observed in the ^{13}C-NMR spectrum at this elevated temperature. These resonances were no longer of equal intensity, and chemical shift clusters appeared to be more spread out. These observations suggests that, unlike the peracetylated derivative **150**, the de-*O*-acetylated derivative **151** exists in solution as a more complex combination of different conformers. In addition, the possible existence of charge separated resonance structures such as those depicted in Figure 33 might be important contributors within the polar medium (D_2O, DMSO) in which they were observed.

The existence of multiple resonances in the NMR spectra are not due to impurities. Resonance intensities and patterns are different between D_2O and $DMSOd_6$ solutions. Compound **151** was a

recrystallized product shown to be pure by TLC and elemental analysis. ^{1}H-NMR integrations confirm the bis glycosylated nature of **151**. A truly satisfactory explanation for the NMR behaviour of **151** cannot be provided at this time.

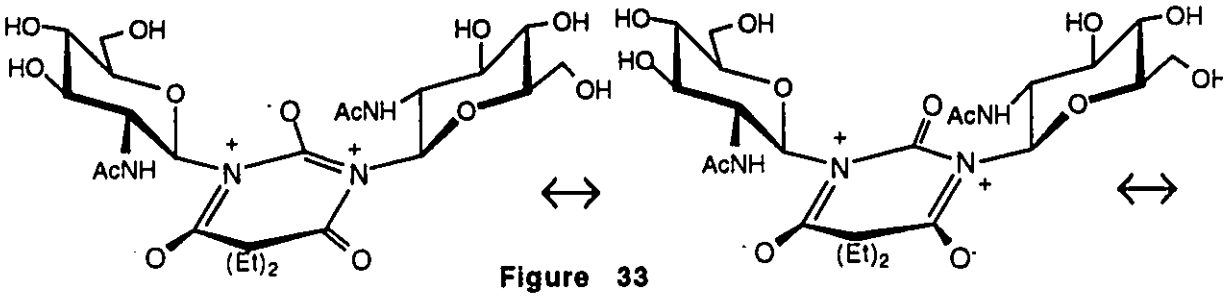
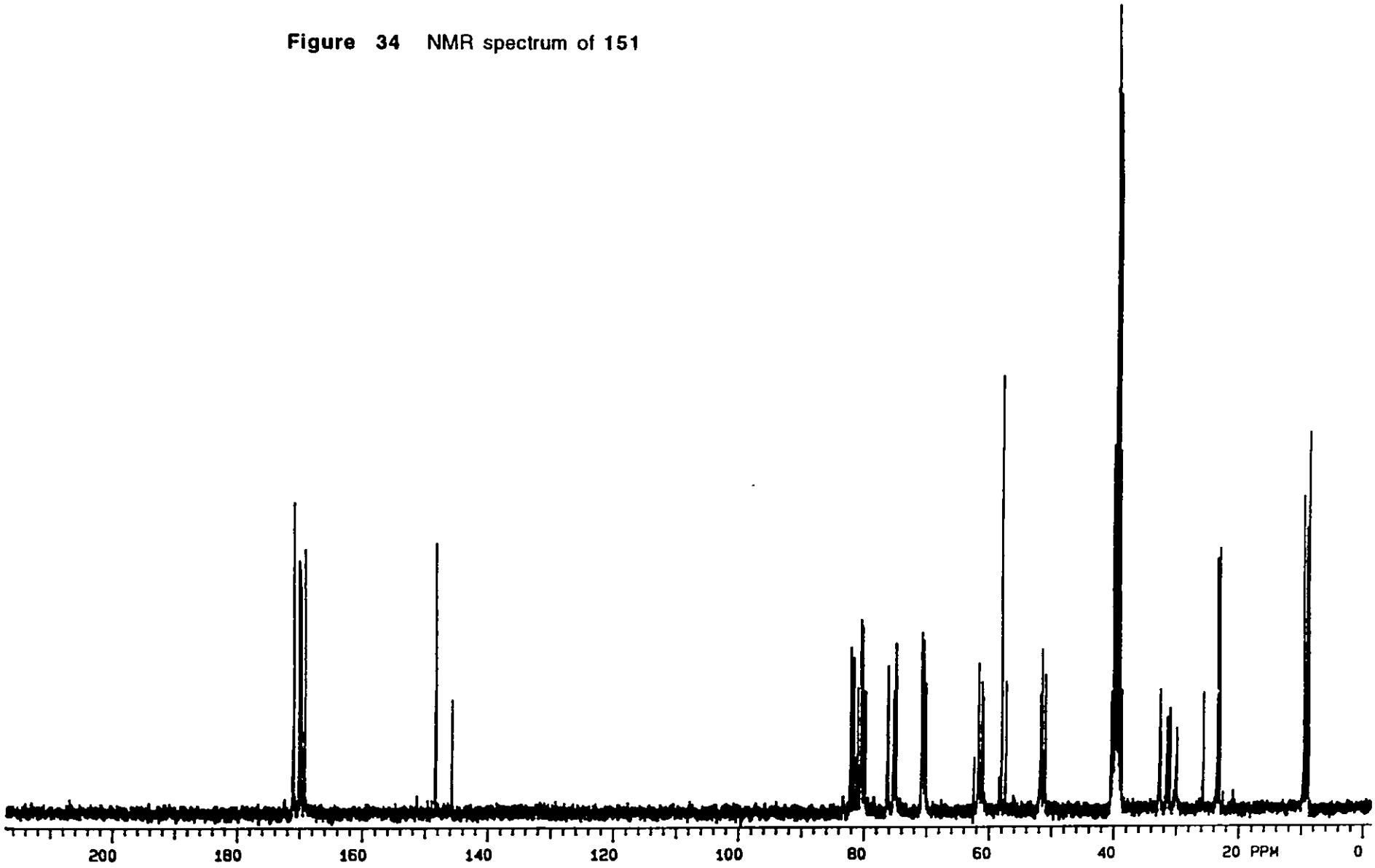


Figure 33

In this author's opinion, the existence of multiple resonances in the NMR spectra of these bis glycosylated barbital derivatives 149-151 cannot yet be satisfactorily explained. A complete rationalization of these NMR observations is beyond the scope of the dissertation and will have to wait for a later date when more elaborate experiments, including X-ray crystallography and molecular mechanics calculations, can be performed.

Figure 34 NMR spectrum of **151**



6.9 Enolates and other carbon nucleophiles

In recent years, there has been considerable attention paid to the synthesis of *C*-glycosides[201]. The interest in such compounds stems from the fact that, unlike *O*-, *S*- and *N*-glycosides, *C*-glycosides have a non-hydrolysable glycosidic linkage. They are highly resistant to chemical and enzymatic degradation[202]. *C*-glycosides also occur as subunits of a variety of important natural products[203]. Usual synthetic approaches to *C*-glycosides either involve carbanion or radical coupling methodologies. It was anticipated that, with the choice of a sufficiently strong carbon acid, PTC could also be used to provide a very simple access to 1,2-*trans* β-D-*C*-glycosides. In the first attempts, peracetylated glycosyl halides were treated with the following simple nucleophiles: KCN, CH_3NO_2, and CH_3CN. Reactions were attempted under a variety of different reaction conditions but all proved to be synthetically useless. A considerable amount of de-*O*-acetylated and other degradation products were obtained (TLC) when TBAHS in 1M NaOH and various organic solvents were tried. For such small and hard nucleophiles, perbenzylated glycosyl halides may prove to be more suitable. Softer nucleophiles such as the conjugate bases of $CH_2(NO_2)_2$ or $CH_2(CN)_2$ should be considered for future attempts.

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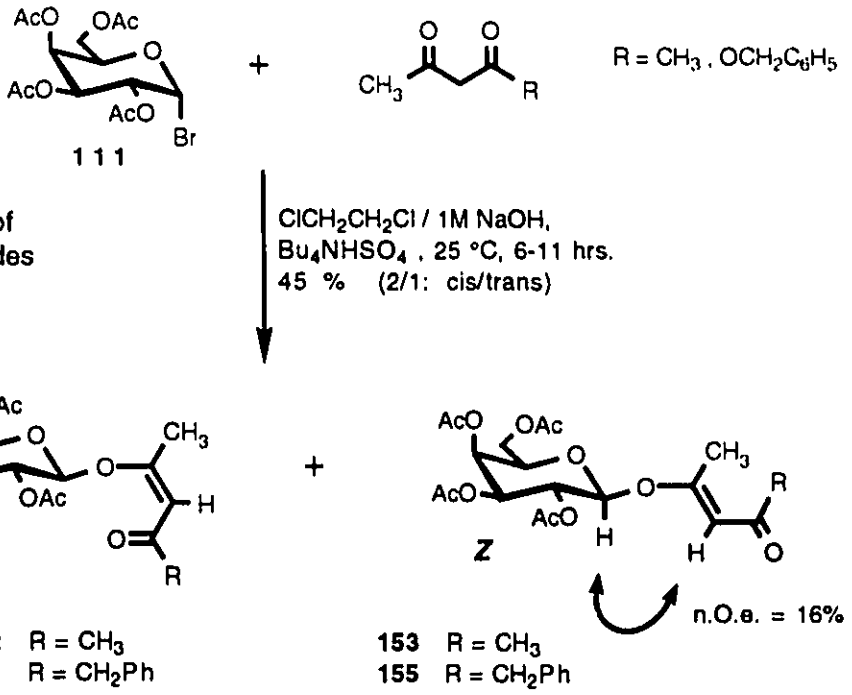
201 a) J.O. Nagy and M.D. Bednarsky, *Tetrahedron Lett.*, *32* , 3953 (1991) and references cited therein; b) D. Crich and L.B. Lim, *Tetrahedron Lett.*, *31* , 1897 (1990) and refences cited therein; c) G. Stork, H.S. Suh and C. Kim, J. *Am. Chem. Soc.*, *113* , 7054 (1991) and references cited therein; d) F. Nicotra, L. Panza and G. Russo, *J. Org. Chem.*, *52* , 5627 (1987); A. Hosomi, Y. Sakata and H. Sakurai, *Tetrahedron Lett.*, *25* , 2174 (1987); e) M.D. Lewis, J.K. Cha and Y. Kishi, *J. Am. Chem. Soc.*, *104* , 4976 (1982).

202 a) M.L. Shulman, S.D. Shiyan ans A.Y. Khorlin, *Carbohydr. Res.*, *33* , 229 (1974); b) M. Chmielewski, J.N. BeMiller and D.P. Cerretti, *Carbohydr. Res.*, *97* , C1 (1981); c) D.P. Cerretti, *Carbohydr. Res.*, *94* , C10 (1981).

203 a) F.J. MacDonald, D.C. Campbell, D.J. Vanderah, D.M. Washecheck, J.E. Burks and D. van der Helm, *J. Org. Chem.*, *40* , 665 (1975); b) D. T. Connor, R.C. Greenough and M. von Strandtman, *J. Org. Chem.*, *42* , 3664 (1977) and *43* , 5027 (1978).



Scheme 35
PTC synthesis of
O -enol glycosides

152 R = CH_3
154 R = CH_2Ph

153 R = CH_3
155 R = CH_2Ph

After the failed attempts mentioned above, softer, enolate type carbanions were envisaged as potentially more appropriate carbon nucleophiles for the goal of preparing *C*-glycosides from peracetylated glycosyl halides. However, no detectable *C*-glycosides could be produced with enolizable carbon acids. Instead, these nucleophiles gave modest yields (45%) of *O*-glycosylation of the generated enolates, wih poor *Z/E* selectivity in the resulting aglycon (scheme 35). These glycosylations were best performed at ambient temperature using TBAHS as phase transfer catalyst in a heterogeneous blend of 1,2-dichloroethane and 1M NaOH. Under these conditions complete consumption of the halide was effected in 6 to 11 hours. Much slower reaction rates were observed when toluene was used as the organic phase. As examples, PTC glycosylation of 2,4-pentanedione with acetobromogalactose **111** gave 47% *O*-alkylation product (**152/153** *Z/E* = 2/1) while benzyl 3-keto propanoate gave 45% *O*-alkylation (**154/155** *Z/E* = 2/1) after silica gel chromatography. The nature of the 1,2-*trans* β-D-anomeric configuration for all the *O*-enol-glycosides was confirmed from the

anomeric proton coupling constants ($J_{1,2}$ = 7.9 ± 0.1 Hz). Anomeric carbon resonances were found at 96.3-100.4 ppm.

The alkene stereochemistry in the aglycons was determined from nuclear Overhauser enhancement (n.O.e.) experiments. Thus, irradiation of the olefinic proton in the aglycon gave an n.O.e. of 16% for the anomeric proton in both *Z*-*O*-enol glycosides (**153** and **155**) while no such n.O.e. could be observed in the *E*-*O*-enol glycosides (**152** and **154**). Crystals suitable for X-ray crystallographic analysis could not be obtained to confirm the determined stereochemistries. Only very delicate twin needles could be obtained for the four different *O*-enol glycosides presented here.

Although the PTC methodology used thus far for preparing various 1,2-*trans* β-D-glycosides has so far not proven to be useful in preparing *C*-glycosides, it can be used to prepare *O*-enol glycosides in modest yields. Such compounds may have potential application in the future for assymetric synthesis with or without enzymatic catalysis. One can also envisage the preparation of disaccharides by Diels-Alder type chemistry performed on the aglycon. Clearly more experiments should be conducted to improve the yields and investigate the potential usefulness of such *O*-enol glycosides.

Table 51 Physical data for peracetylated *O*-enol β-D-galactopyranosides.

Product	Yield (%)	Melting Point (°C)	$[\alpha]_D$ ($CHCl_3$)	Elemental Analysis				
				Formula	Calculated (%)		Found (%)	
					C	H	C	H
152	32	125.4-126.6	-3.2°	$C_{19}H_{26}O_{11}$	53.02	6.09	53.23	6.12
153	15	125.6-126.3	-7.3°	$C_{19}H_{26}O_{11}$	53.02	6.09	53.25	6.13
154	30	oil	+11.4°	$C_{25}H_{30}O_{12}$	•	•	•	•
155	15	oil	-16.1°	$C_{25}H_{30}O_{12}$	•	•	•	•

• not performed.

Table 52 ^{1}H-NMR data* for *O*- enol β-D-galactopyranosides **152-155**.

Product	H_1 d ($J_{1,2}$)	H_2 dd ($J_{2,3}$)	H_3 dd ($J_{3,4}$)	H_4 dd ($J_{4,5}$)	H_5 ddd ($J_{5,6a}$)	H_{6a}/H_{6b} m ($J_{5,6b}$)	=CH- s #	CH_3 s #	R
152	5.08 (7.9)	5.40 (10.3)	5.06 (3.5)	5.41 (1.0)	3.98 (5.6)	4.13 (7.2)	5.20	2.07	2.29 (S,CH_3)
153	4.97 (8.0)	5.37 (10.5)	5.05 (3.4)	5.41 (1.0)	4.03 (5.5)	4.14 (7.3)	5.69	2.24	2.15 (S,CH_3)
154	5.10 (7.8)	5.43 (10.5)	5.03 (3.4)	5.39 (1.0)	3.94 (5.5)	4.11 (7.2)	5.09	2.13	5.03 (S,CH_2) 7.29 (m,C_6H_5)
155	4.91 (7.9)	5.32 (10.5)	5.02 (3.6)	5.39 (1.0)	3.97 (5.6)	4.11 (7.3)	5.30	2.25	5.09 (S,CH_2) 7.32 (m,C_6H_5)

* Chemical shifts are given in ppm for solutions in $CDCl_3$ (300 MHz). Coupling constants are given in Hertz. Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments. Resonances for the acetate methyls appear as singlets 1.97-2.14 ppm.

\# Signals can be resolved into doublets ($J_{allylic} \leq 0.5$ Hz)under high resolution conditions.

Table 53 ^{13}C-NMR chemical shift data* for peracetylated *O*-enol β-D-galactopyranosides **152 - 155**.

Product	C_1	C_2	C_3	C_4	C_5	C_6	=CH-	O-C=	C=O	CH_3	C=O(Ac)	CH_3(Ac)
152	96.3	68.1	70.5	66.6	71.2	61.2	112.6	161.4	197.7	18.3	168.9	20.4
											169.3	20.5
											170.0	(2C)
											170.1	20.6
153	96.7	67.9	70.4	66.7	71.4	61.5	103.8	168.7	197.0	18.5	169.1	20.5
											169.9	20.6
											170.0	(3C)
											170.2	
154 [a]	100.4	68.1	70.6	66.8	71.1	61.3	97.7	163.5	163.7	19.9	169.2	20.4
											170.1	20.5
											170.2	20.6
											170.3	20.7
155 [b]	97.0	68.0	70.5	66.8	71.5	61.5	95.7	166.8	169.1	18.3	169.9	20.3
											170.0	20.4
											170.1	20.5
											170.4	20.6

* Chemical shift values are in ppm and are for solutions in $CDCl_3$ (75.4 MHz). Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments.

a) C_{ipso}: 136.6; C_{ortho}: 128.3; C_{meta}: 128.0; C_{para}: 127.8; CH_2: 65.2.

b) C_{ipso}: 136.0; C_{ortho}: 128.5; C_{meta}: 128.4; C_{para}: 128.1; CH_2: 65.7.

6.10 *N*-Hydroxysuccinimide

O-amino glycosides are relatively rare in nature. They have so far only been discovered in calicheamicin[204] and esperamicin[205] antibiotics. Thus, no formal synthesis of *O*-oxamino glycosides has yet been reported in the literature. Very recently, Anderson and coworkers[206] reported the synthesis of two glycosides of *N*-hydroxysuccinimide from peracetylated sugars with Lewis acid catalysts in 75% yields. Other methods of preparing *N-O* linked

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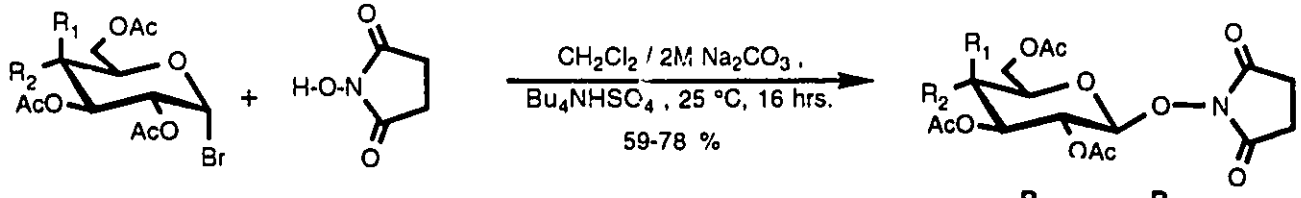
[204] D. Yang, S.H Kim and D. Kahne, *J. Am. Chem. Soc., 113* , 4715 (1991) and references cited within.

[205] R.L. Halcomb, M.D. Wittman, S.H. Olson and S.J. Danishefsky, *J. Am. Chem. Soc., 113* , 5080 (1991) and references cited within.

[206] M. Andersson and S. Oscarson, *16th International Carbohydrate Conference*, Paris, July 1992, Abstract # A083; M. Anderson and S. Oscarson, *Glycoconjugate J.* , 9, 122 (1992).

urethane glycosides include the addition of protected hydroxylamines to 2-deoxy glycals with triphenylphosphine hydrobromide as promoter, and glycosylation of *N*-hydroxylphthalimide by Mitsunobu reaction. These methods were employed in calicheamicin[204] and esperamicin[205] syntheses but suffer from poor yields and stereoselectivity.

The fair acidity (pKa = 6)[207] of *N*-hydroxysuccinimide prompted the attempt at preparing glycosides thereof by PTC in order to investigate the generality of this glycosylation methodology, and describe a formal synthesis of *O*-oxamino glycosides with a 1,2,-*trans* β-D-anomeric configuration. *N*-oxysuccinimidyl glycosides glycosides can be easily transformed by mild hydrazinolysis into *O*-oxamino glycosides useful in antibiotic syntheses, or converted into glycosyl hydroxamic esters with a terminally functionalized aglycon spacer suitable for glycoconjugate preparations. The latter derivatives could prove useful in determining the unknown effects of anomeric *O*-oxamino linkages in biological systems.



	R_1	R_2
156	H	OAc
157	OAc	H
158	H	α-D-Glc(OAc)$_4$

Scheme 36 PTC glycosylation of N-hydroxysuccinimide

1,2-*trans* β-D-glycosides and -glycobiosides of *N*-hydroxy succinimide were prepared with relative ease using mild PTC conditions (Scheme 36). Glycosylations were performed easily at room temperature using 2 equivalents of *N*-hydroxysuccinimide and TBAHS as catalyst. The best results were obtained with methylene chloride as the organic phase (1 mL/50-100 mg of glycosyl halide)

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[207] D.E. Ames and T.F. Grey, *J. Chem. Soc.*, 631 (1955).

and 2M Na_2CO_3 (1 mL/1 mL CH_2Cl_2) as the aqueous phase. Under these conditions, good yields (59 - 78%, isolated) of *N*-oxysuccinimidyl glycosides couls be obtained in 2 to 4 hours from peracetylated α-D-glycosyl bromides. The analogous glycosylation reaction could not be performed effectively with acetochloro *N*-acetylglucosamine **58**. With this glycosyl halide, the oxazoline **60** was the major product obtained (≥ 50%). The use of ethyl acetate instead of methylene chloride greatly reduced the reaction rate. Reactions could only be completed in two days when ethyl acetate was used. Decreasing the alkalinity of the aqueous phase by using 1M Na_2CO_3 or saturated $NaHCO_3$ also reduced the reaction rate. In this case, however, reactions could be effectively completed by stirring overnight. The only major side reaction in the PTC synthesis of *N*-oxysuccinimidyl glycosides was the dehydrobromination of the glycosyl halides. The extent of dehydrobromination was, however, less severe than with phenoxide nucleophiles (section 6.1).

Glycosylations were again judged to be stereospecific. Only 1,2-*trans*-β-D-glycosides could be detected by TLC and NMR spectra of the crude products. Glycosides **156 - 158** had anomeric proton resonances from 4.84 to 5.16 ppm with $J_{1,2}$ values of 6.3 Hz for the glucosyl and maltosyl derivatives **156** and **158**, and a $J_{1,2}$ value of 8.1 Hz for the galactosyl derivative **157**. Anomeric carbon chemical shifts were found between 102.3 and 103.7 ppm. Yields and physical data for these *N*-oxysuccinimidyl glycosides are given in Table 54. Complete NMR chemical shift data are given in tables 55 and 56.

Table 54 Isolated yields and physical data of peracetylated *N*-oxysuccinimidyl β-D-glycosides produced by PTC.

Product	Yield (%)	mp (°C) (EtOAc/hex)	$[\alpha]_D$ ($CHCl_3$)	Formula	Elemental Analysis					
					Calculated (%)			Found (%)		
					C	H	N	C	H	N
156	59	182.4-183.7	-43.0°	$C_{18}H_{23}NO_{12}$	48.54	5.21	3.14	48.51	5.26	3.09
157	78	190.1-191.4	-52.1°	$C_{18}H_{23}NO_{12}$	48.54	5.21	3.14	48.53	5.41	3.16
158	60	219.0-219.6	+36.5°	$C_{30}H_{39}NO_{20}$	49.12	5.36	1.91	49.36	5.50	1.86

Table 55 ^{1}H-NMR data* for peracetylated *N*-oxysuccinimidyl β-D-glycosides **156-158**.

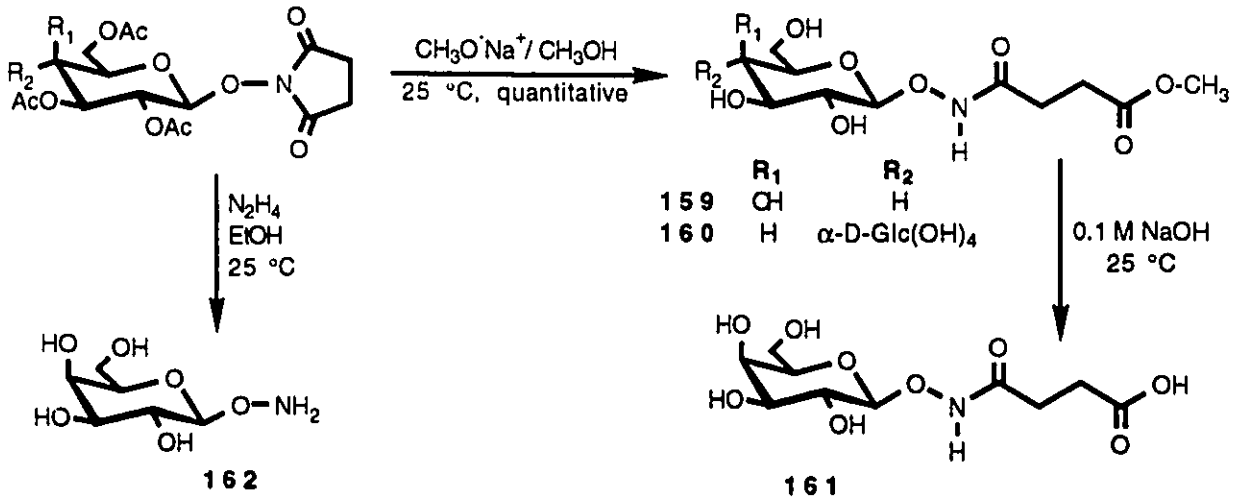
Product		H-1 d ($J_{1,2}$)	H-2 dd ($J_{2,3}$)	H-3 dd ($J_{3,4}$)	H-4 dd ($J_{4,5}$)	H-5 ddd ($J_{5,6a}$)	H-6a dd ($J_{6a,6b}$)	H-6b dd ($J_{5,6b}$)	CH_2 S
156		5.04 (6.3)	5.18	- (mult)	5.27	3.73 (4.9)	4.27 (12.5)	4.13 (2.5)	2.72
157		4.84 (8.2)	5.38 (10.4)	5.04 (3.4)	5.36 (1.0)	3.88 (6.2)	4.22 (11.3)	4.11 (7.4)	2.72
158	Glc	5.16 (6.3)	5.08 (7.1)	5.19 (8.3)	4.34 (9.6)	3.80 (3.5)	4.43 (12.2)	4.28 (4.6)	2.72
	Glc'	5.41 (3.9)	4.82 (10.5)	5.35 (9.5)	5.03 (10.0)	3.97 (4.3)	4.26 (12.4)	4.06 (2.0)	

* Chemical shifts are in ppm relative to TMS by referencing to internal $CHCl_3$ (7.24)ppm for solutions in $CDCl_3$ (300 MHz). Coupling constants (J) are in Hertz. Acetate methyl resonances appeared as singlets 1.92 - 2.14 ppm. Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments.

Table 56 ^{13}C-NMR chemical shifts data* for peracetylated *N*-oxysuccinimidyl β-D-glycosides **156 - 158**.

Product		C-1	C-2	C-3	C-4	C-5	C-6	CH_2
156		103.7	69.6	72.2	68.1	72.2	61.7	25.3
157		105.2	66.8	70.4	66.1	71.2	60.6	25.3
158	Glc	102.3	70.4	74.9	72.3	72.5	62.6	25.4
	Glc'	95.5	70.1	69.2	68.0	68.5	61.6	

* Chemical shifts are in ppm relative to TMS by referencing to internal $CDCl_3$ (77.0 ppm) for solutions in $CDCl_3$ (75.4 MHz). Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments. Acetate methyl resonances were always found in the 20.5 to 20.8 ppm region. All carbonyl resonances could be resolved in the 169.2 to 170.6 ppm region.



Scheme 37 Synthetic transformations of N-oxysuccinimidyl β-D-glycosides

In addition to de-*O*-acetylation, treatment of peracetylated *N*-oxysuccinimidyl β-D-glycosides **157** and **158** under Zemplén de-*O*-acetylation conditions with sodium methoxide in methanol resulted in the ring opening of the succinimidyl aglycon to give the

in the ring opening of the succinimidyl aglycon to give the corresponding β-D-glycosyl hydroxamic esters **159** and **160** bearing a terminal methyl ester function, in quantitative yields (Scheme 37). Although only the galactosyl and maltosyl derivatives **159** and **160** were isolated and fully characterized, the formation of the glucosyl derivative was verified by fast atom bombardment mass spectrometry (m/e: 928.2 (2.1%), 3M+1; 619.1 (10.0%), 2M+1; 310.1 (97%), M+1 for $C_{11}H_{19}NO_9$) and ^{13}C-NMR, however. In addition, saponification of **159** with 0.1 M NaOH gave the corresponding terminal carboxylic acid **161** in very good yield. Physical data for the above compounds are given in Table 57. The corresponding NMR data can be found in Tables 58 and 59.

Table 57 Physical data for *O*-β-D-glycosyl hydroxamic esters **159 - 161**.

Product	Yield	$[\alpha]_D$ (MeOH)	Formula	(+) FAB - MS data		
				m/e	% *	ion
159	quant.	-41.8°	$C_{11}H_{19}NO_9$	1237.2	0.6	4M+1
				928.2	2.1	3M+1
				619.1	10.6	2M+1
				310.1	98.8	M+1
				148.1	10.0	aglycon + 2H
				115.0	58.1	aglycon - OCH_3
160	quant.	+42.6°	$C_{17}H_{29}NO_{14}$	1415.6	1.8	3M+1
				994.1	10.1	2M+1
				472.3	70.1	M+1
				148.1	74.8	aglycon + 2H
				115.0	64.8	aglycon - OCH_3
161	quant.	-35.3°	$C_{10}H_{17}NO_9$	1181.4	0.5	4M+1
				886.3	1.1	3M+1
				591.2	8.2	2M+1
				296.1	83.7	M+1
				134.1	94.5	aglycon + 2H

* Intensity relaltive to base peak in spectrum (glycerol + 1).
\# All products were obtained as amorphous solids which resisted all attempts at crystallization

Table 58 ^{1}H-NMR data* for *O*-β-D-glycosyl hydroxamic esters **159-161**.

Product	H_1 d ($J_{1,2}$)	H_2 dd ($J_{2,3}$)	H_3 dd ($J_{3,4}$)	H_4 dd ($J_{4,5}$)	H_5	H_{6a}	H_{6b}	CH_2CO_2 t (J_{CH_2})	CH_2CON t (J_{CH_2})	CH_3 s
					(mult)					
159	4.66 (7.3)	3.62 - (mult)	3.86	3.94 (2.4)	3.62 - (mult)		3.86	2.73 (6.6)	2.52 (6.6)	3.71
160 Glc	4.73 (8.1)	3.39		to	3.97			2.72 (6.6)	2.50 (6.6)	3.71
Glc'	5.42 (3.9)			(mult)						
161	4.67 (7.3)	3.63 - (mult)	3.85	3.94 (2.4)	3.63 - (mult)		3.85	2.71 (6.6)	2.50 (6.7)	-

* Chemical shifts are given in ppm for solutions in D_2O (300 MHz). Coupling constants are given in Hertz. Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments.

Table 59 ^{13}C-NMR data* for *O*-β-D-glycosyl hydroxamic esters **159 - 161**.

Product	C_1	C_2	C_3	C_4	C_5	C_6	CH_2	CH_3	C=O
159	108.3	70.7	74.9	71.3	77.9	63.3	29.5	54.7	173.9
							31.1		177.5
160 Glc	107.5	74.0	78.2	77.2	78.6	62.8	29.5	54.7	174.0
Glc'	102.1	71.6	75.0	73.5	75.2	62.8	31.1		177.5
161	108.2	70.7	74.8	71.2	77.8	63.3	29.5	-	174.1
							31.1		178.8

* Chemical shift values are in ppm and are for solutions in D_2O (300 MHz) with acetone as internal reference (δ_{CH3} = 31.07 ppm). Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments.

The de-*O*-acetylation with concomitant ring opening of the succinimidyl aglycon under Zemplén conditions is conceptually similar to reactions performed very recently by Andersson and coworkers[206] where *N*-oxysuccinimidyl glycosides were treated with hydrazine to give the corresponding glycosyl hydroxamic esters

bearing a terminal hydrazide function. In this study, hydrazinolysis of *N*-oxysuccinimidyl glycosides with an excess of hydrazine was performed to determine if *O*-oxamino glycosides, useful in antibiotic synthesis, could be accessed from the succinimidyl glycosides (Scheme 37). Such glycosides also have potential in glycoconjugate synthesis after acryloylation of the amine. Indeed, the anticipated *O*-oxamino glycosides were produced smoothly with an excess (7 equiv.) of hydrazine in ethanol at room temperature (TLC). However, the polar and and hygroscopic nature of these *O*-oxamino glycosides in addition to the production of acetyl hydrazide and butanedioic dihydrazide[208] complicated the purification steps on large scales. Due to time constraints, this part of the study was left incomplete. The formation of *O*-oxamino β-D-galactopyranoside **162** was identified by mass spectrometry, ^{1}H- and ^{13}C-NMR spectroscopy only[209].

To conclude, PTC is a mild, simple and effective strategy for preparing novel *N*-*O*-linked glycosides having a 1,2-*trans*-β-D configuration. In addition, glycosides of *N*-hydroxysuccinimide are versatile reagents which can easily be derivatized to give potential precursors to glycoconjugates[206] or calicheamicin[204] and esperamicin[205] synthesis.

6.11 Benzeneselenol

Selenyl glycosides are non-natural compounds which have just recently attracted interest. Their synthesis and use as glycosyl donors in oligosaccharide synthesis was introduced shortly after this investigation into their synthesis by PTC. In comparison to phenol and thiophenol which have been demonstrated to be good

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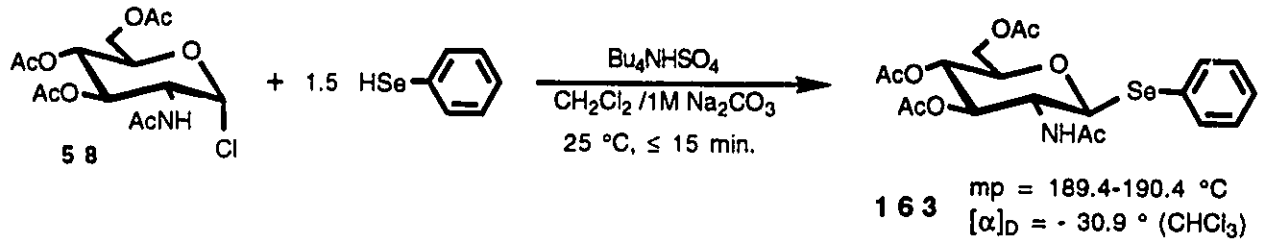
[208] Identified by mass spectrometry, ^{1}H- and ^{13}C-NMR. Butanedioic hydrazide crystallizes from ethanol and can be removed by filtration.

[209] CI-MS for $C_6H_{13}NO_6$ gave m/e (relative intensity, ion): 391.1 (8%, 2M+1), 196.0 (58%, M+1). ^{13}C-NMR δ (ppm, D_2O): 105.2 (C_1), 74.1 (C_5), 72.4 (C_3), 69.0 (C_4), 68.2 (C_2), 60.6 (C_6).

nucleophiles in PTC glycosylation strategies, benzeneselenol was also identified as a potentially very good nucleophile for S_{N2} displacement of anomeric halides. Its acidity is comparable to thiophenol. Selenium's position below sulfur in column VI A of the periodic table also suggested that benzeneselenol would behave as a good, soft nucleophile. The hypothesis that benzeneselenol would serve as a good nucleophile for PTC glycosylation was justified. Indeed, benzeneselenol did prove to be an excellent nucleophile during PTC glycosylation with acetochloro *N*-acetylglucosamine **58**. Phenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-seleno-β-D-glucopyranoside**163** was prepared in a 95% isolated yield in less than 10 minutes when 1.5 equivalents of benzeneselenol was stirred at room temperature with chloride **58** in the presence of TBAHS (1 equiv.) in a CH_2Cl_2/1M Na_2CO_3 solvent couple (Scheme 38). By-products of the reaction were identified as being bis(phenylseleno)methane from nucleophilic attack of the solvent, and oxidation of the benzeneselenol to the corresponding diselenide. These secondary reactions pathways mirror the observations described earlier for the thiophenol nucleophiles.



Scheme 38 PTC glycosylation of benzeneselenol

S_{N2} nucleophilic displacement of chloride by the benzeneselenide anion occurred with complete stereochemical inversion as with all nucleophiles investigated so far. The 1,2-*trans* β-D-configuration of **163** was confirmed by the anomeric proton resonance which appeared as a doublet at 4.97 ppm ($CDCl_3$) with a 1,2-*trans* diaxal coupling constant of 10.6 Hz. The anomeric carbon was found, shifted

considerably upfield, at 82.7 ppm ($CDCl_3$).

Glycosylation of benzeneselenol by PTC was also observed to be compatible with acetobromoglucose **110** and acetobromogalactose. TLC analysis of benzeneselenol glycosylation with these glycosyl halides proceeded with the same effectiveness as did the *N*-acetylglucosamine analogue **163**. These glycosides were not fully characterized, however. Their formation was confirmed by mass spectrometry only. The same PTC procedure described above also proved successful for colleagues[210] when performed with acetochloroneuraminic acid methyl ester **114**. This method should be applicable to other glycosyl halides as demonstrated with other nucleophiles.

Phase transfer catalysis has been shown to be a very useful glycosylating methodology for the preparation of 1,2-*trans* β-D-glycosides. Mild reaction conditions, using various peracetylated glycosyl halides (chlorides and bromides) as glycosyl donors, including 2-deoxy-, 2-acetamido-2-deoxy sugars and disaccharides, have been developed which allow quick and facile glycosylations of a wide range of nucleophiles. Aromatic alcohols, aryl and alkyl thiols, phenylselenol, carboxylic acids, dibenzyl phosphate, *O*-ethyl xanthate, azide, thiocyanate, *N*-hydroxysuccinimide, a diimide (Barbital) and enolates have all been successfully glycosylated by a phase transfer catalysis strategy. Glycosylations were shown to be completely stereospecific , and proceeded by an S_{N2} mechanism with inversion of configuration at the anomeric center. Derivatization of many glycosides has been performed to extend the usefulness of many PTC products. In particular, *p*-nitro and *p*-formylphenyl glycosides, prepared by phase transfer catalysed glycosylation of substituted phenols, have been derivatized into useful neoglycoprotein and glycopolymer conjugates and are discussed in the following chapters.

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[210] S. Meunier and R.Roy, unpublished results.

6.12 Experimental methods

General methods

General methods are as described in previous chapters.

Glycosyl halides

Glycosyl halides used were synthesized according to known standard procedures. Preparation of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy glucopyranosyl chloride was described earlier in Chapter 5.

Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycerol*-β-D-*galacto*-2-nonulopyranosyl)onate chloride **114** (acetochloroneuraminic acid methyl ester)[36] and 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl chloride **109** were prepared by stirring sealed, anhydrous solutions of the free sugars in acetyl chloride for 24-48 hours at room temperature. Evaporation of the acetyl chloride followed by several coevaporations with toluene gave **114** and **119** as glassy solids which were used without further purification. 2,3,4,6-Tetra-*O*-acetyl α-D-glucopyranosyl bromide **110** (acetobromoglucose) and 2,3,4,6-tetra-*O*-acetyl-α-D-galactosyl bromide **111** (acetobromogalactose) were prepared by the usual HBr/HOAc (35% w/w) treatment of the peracetates[211] and purified by recrystallization with EtOAc/hexane or ether/hexane. 2,2',3,3',4',6,6'-Hepta-*O*-acetyl α-D-lactosyl and maltosyl bromides (acetobromo-lactose,**112** and -maltose, **113**) were also prepared by treatment of the corresponding octaacetates with 35% HBr/HOAc (w/w)(1 hour, room temperature) and purified by flash chromatography using a hexane/EtOAc gradient. All peracetylated glycosyl bromides were of excellent purity when used in PTC

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[211] R.W. Jeanloz and P.J. Stoffyn, *Methods Carbohydr. Chem.*, *1*, 221 (1962).

glycosylations. 2,3,4,6-Tetra-*O*-acetyl α-D-glucopyranosyl chloride was prepared according to Lemieux[212] by $TiCl_4$ treatment of β-D-glucose pentaacetate in chloroform. Crystallization from ether/hexane gave the chloride as a very pure microcrystalline solid.

7-O-(2-Acetamido-2-deoxy-β-D-galactopyranosyl) 3-methyl coumarin **115**

N-acetyl D-galactosamine (100.1 mg , 0.453 mmol, Sigma) was dissolved in 1.0 ml of freshly distilled acetyl chloride at 0°C. After stirring for one hour at 0°C, the solution was allowed to react at room temperature overnight. Evaporation of the acetyl chloride under reduced pressure followed by multiple coevaporations with toluene gave the impure chloride **109** (80% by TLC) as a clear oil. After adding 153.6 mg (0.453 mmol) of TBAHS, 159.5 mg (0.905 mmol) of 7-hydroxy 4-methyl coumarin **101**, 2 mL of CH_2Cl_2 and 2 mL of 1M NaOH the resulting solution was stirred at room temperature for 50 min.. The reaction was then worked up by adding 20 mL EtOAc and successively washing the organic phase with 20 ml 1M NaOH, 20 mL H_20 (2X) and 10 ml sat. brine. The organic phase was then dried with Na_2SO_4, filtered and evaporated to a clear oil. Purification by preparative TLC (25/5/3 $CHCl_3$/EtOAc/n-prOH) gave, after overnight extraction of the silica in 40 mL 2/1 EtOAc/CH_2Cl_2, filtration and evaporation, 61.0 mg (27%) of **115**.

Compound **115** could be crystallized from ether/hexane to give white needles mp=219.3-220.2 °C. $[\alpha]_D$ = -5.5° (c= 0.73, $CHCl_3$) {lit.[149] amorphous, $[\alpha]_D$ = -3.0° (c= 0.4, $CHCl_3$)}. CI-MS (ether) gave (ion, relative intensity): 506 (M + 1, 3), 330 (M - aglycon, 100), 177 (7-hydroxy 4-methyl coumarin + 1, 100). ^{1}H-NMR δ ($CDCl_3$): 7.48 (dd, $J_{5,8}$ = 1.7, $J_{5,6}$ = 7.6 Hz, H_5 coumarin), 6.49 (mult, 2H, remaining aromatic H), 6.15 (d, 1H, $J_{allylic}$ = 1.2 Hz, C(O) - CH=), 5.64 (d, 1H $J_{2,\ NH}$ = 8.7 Hz, NH), 5.38 - 5.45 (AB mult, 2H, H_3 + H_4), 5.39 (d, 1H, $J_{1,2}$ =

212 R.U. Lemieux, *Methods in Carbohydr. Chem.*, *II* , 223 (1971).

8.3 Hz, H_1), 4.28 (ddd, 1H, $J_{2,3}$ = 10.5 Hz, H_2), 4.11 - 4.18 (mult, 3H, H_5+ H_6 + $H_{6'}$), 2.38 (d, 3H, $J_{allylic}$ = 1.2 Hz, CH_3 coumarin), 2.16 (s, 3H, CH_3, Ac), 2.09 (s, 3H, CH_3, Ac), 2.03 (s, 3H, CH_3, Ac), 1.96 (s, 3H, CH_3, Ac). ^{13}C-NMR δ ($CDCl_3$): 170.2 - 170.6 (4 C=0, Ac), 160.9 (C=O, coumarin), 159.6 (C_7, coumarin), 154.7 (C_9, coumarin), 152.4 (C_4, coumarin), 125.6 (C_5, coumarin), 115.2 (C_3, coumarin), 114.0 (C_8, coumarin), 112.9 (C_6, coumarin), 103.9 (C(O)-CH=), 98.5 (C_1), 71.4 (C_5), 69.7 (C_3), 66.6 (C_4), 61.6 (C_6), 51.2 (C_2), 23.4 (CH_3, NHAc), 20.6 (3 x CH_3, OAc), 18.6 (CH_3, coumarin).

7-O-(β-D-Glucopyranosyl) 3-methyl coumarin **116**

7-Hydroxy 4-methyl coumarin **101** (260 mg, 1.48 mmol), 260 mg (0.766 mmol) of TBAHS and acetobromo α-D-glucose **110** were stirred in 1.5 mL of CH_2Cl_2 and 1.5 mL of 1M NaOH at room temperature for 40 min., until TLC (1/1 EtOAc/hexane +1% n-propanol) indicated complete consumption of the bromide. The reaction was worked up as described for **115**. Purification by preparative TLC gave 60.0 mg (49%) of the known 2-acetoxy glucal **119** (Rf = 0.52) and 88.0 mg (48%) of **116** (Rf = 0.26). The glycoside could be crystallized from hot ether and a few drops of hexane. See table 35 for physical data.

CI-MS (ether) gave (ion, relative intensity): 507 (M + 1, 74), 331 (M - aglycon), 271 (331 - HOAc, 18), 177 (7-hydroxy 4-methyl coumarin + 1, 52), 169 (177 - H_20, 100). ^{1}H-NMR δ ($CDCl_3$): 7.47 (d, 1H, J = 8.6 Hz, H_5 coumarin),6.88 (mult, 2H, remaining aromatic H), 6.13 (d, 1H, $J_{allylic}$ = 1.1 Hz, C(O) - CH=), 5.07 - 5.34 (mult, 4H, H_1 + H_2 + H_3 + H_4), 4.25 (dd, $J_{5,6}$ = 5.6, $J_{6,6'}$ = 12.4 Hz, H_6), 4.12 (dd, 1H, $J_{5,6'}$ = 2.3 Hz, $H_{6'}$), 3.89 (ddd, $J_{4,5}$ = 10.5 Hz, H_5), 2.35 (d, 3H, $J_{allylic}$ = 0.8 Hz, CH_3 coumarin), 1.99 - 206 (4s, CH_3, Ac). ^{13}C-NMR δ ($CDCl_3$): 170.7, 170.3, 169.5, 169.4 (C=0, Ac), 160.8 (C=O, coumarin), 159.2 (C_7, coumarin), 154.9 (C_9, coumarin), 152.3 (C_4, coumarin), 125.8 (C_5, coumarin), 115.5 (C_3, coumarin), 113.9 (C_8, coumarin), 113.1 (C_6, coumarin), 103.9 (C(O)-CH=), 98.2 (C_1), 72.4 (C_5), 72.2 (C_3), 70.8 (C_2), 67.9 (C_4),

61.6 (C_6), 20.3 - 20.5 ($4CH_3$, Ac), 18.4 (CH_3, coumarin).

7-O-(β-D-Glucopyranosyl) 3-methyl coumarin **116a**

Compound **116** was deprotected by Zemplén de-*O*-acetylation under conditions already described to a quantitative yield of **116a**. The product was recrystallized from EtOH/ether. See table 36 for physical data.

CI-MS (ether) gave (ion, relative intensity): 339 (M + 1, 47),177 (7-hydroxy 4-methyl coumarin + 1, 100). ^{1}H-NMR δ (D_2O): 7.57 (d, 1H, $J_{5,6}$= 8.8 Hz, H_5 coumarin), 7.04 (dd, 1H, $J_{6,8}$= 2.5 Hz, H_6 coumarin), 6.93 (d,1H, H_8 coumarin), 6.10 (d, 1H, $J_{allylic}$ = 1 Hz, C(O) - CH=), 5.17 (d, 1H, $J_{1,2'}$ = 7.2 Hz, H_1), 3.50 - 4.02 (mult, 6H, remaining glucose H),5.6, 2.32 (d, 1H, $J_{allylic} \leq 1$ Hz, CH_3). ^{13}C-NMR δ ($DMSOd_6$): 160.4 (C=0, Ac),159.7 (C_7, coumarin), 153.6 (C_9, coumarin), 126.6 (C_5, coumarin), 114.3 (C_3 + C_4 coumarin), 113.6 (C_8, coumarin), 111.9 (C_6, coumarin), 103.4 (C_2 coumarin), 100.2 (C_1), 77.3 (C_5), 76.6 (C_3), 73.3 (C_2), 67.7 (C_4), 60.7 (C_6),18.1 (CH_3).

p-*Formylphenyl 2,3,4,6-tetra*-O-*acetyl-β-D-glucopyranoside* **117**

Acetobromoglucose **110** (250 mg, 0.61 mmol), 150 mg (2 equiv.) of *p*-hydroxy benzaldehyde (Aldrich) and 204 mg (1 equiv.) of TBAHS were stirred vigourously in methylene chloride (2.5 mL) and 1M NaOH (2.5 mL). After 40 min., the reaction mixture was processed as usual (see **115**). The residue obtained after evaporation of the organic phase was further purified by column chromatography using EtOH/hexane (1/1 by vol) as eluent to give pure **117** (104 mg, 38%) eluted after the product of elimination, 2,3,4,6-tetra-*O*-acetyl-D-glucal **119** (90.4 mg, 45%): mp = 64.9 - 66.2 °C, $[\alpha]_D$ = -33° (c=1, $CHCl_3$) {lit[213] mp 65 - 66 °C, $[\alpha]_D$ = -32°}. Compound **117** crystallized

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213 R.J. Ferrier and G.H. Sankey, *J. Chem. Soc. C.* , 2339 (1966).

from ethanol to give white needles. See table 35 for physical data.

^{1}H-NMR δ ($CDCl_3$): 9.90 (s, 1H CHO), 7.83 (d, 2H, $J_{m,o}$ = 8.7 Hz, H_{meta}), 7.07 (d, 2H, $J_{m,o}$ = 8.7 Hz, H_{ortho}), 5.10 to 5.31 (mult, 4H, H_1 to H_4), 4.27 (dd, 1H, $J_{5,6}$ = 5.3 Hz, H_6), 4.15 (dd, 1H, $J_{5,6'}$ = 2.5 Hz, H_6, $J_{6,6'}$ = 12.4 Hz, $H_{6'}$), 3.90 (ddd, $J_{4,5}$ = 9.6 Hz, H_5), 2.02 - 2.04 (4s, CH_3). ^{13}C-NMR δ ($CDCl_3$): 190.9 (CHO), 170.6, 170.3, 169.5, 169.4 (C=O, Ac), 161.3 (C_{ipso}), 131.9 (C_{meta} and C_{para}), 116.8 (C_{ortho}), 97.9 (C_1), 72.4 (C_3), 72.2 (C_5), 70.9 (C_2), 68.0 (C_4), 61.7 (C_6), 20.4 - 20.5 (4 CH_3).

p-*Formyphenyl β-D-glupyranoside* **117a**

De-*O*-acetylation of **117** under the usual Zemplén conditions, neutralization with H^+ resin, filtration and evaporation always gave a quantitative yield of **117a**. The product was crystallized from MeOH/ether. See table 36 for physical data.

^{1}H-NMR δ ($DMSOd_6$): 9.89 (s, 1H, CHO), 7.87 (d, 2H, $J_{m,o}$ = 8.8 Hz, H_{meta}), 7.20 (d, 2H, H_{ortho}), 5.05 (d, 1H, $J_{1,2}$ = 7.5 Hz, H_1), 3.17 - 3.70 (mult, 6H, remaining sugar H). ^{13}C-NMR δ ($DMSOd_6$): 191.2 (CHO),161.9 (C_{ipso}), 131.5 (C_{meta}), 130.3 (C_{para}), 116.3 (C_{ortho}), 99.6 (C_1), 77.1 (C_3), 76.4 (C_5), 73.1 (C_2), 69.5 (C_4), 60.5 (C_6).

p-*Nitrophenyl 2,3,4,6-tetra*-O-*acetyl-β-D-glucopyranoside* **118**

Acetobromoglucose **110** (10.0 g, 24.3 mmol), 6.7 g (2 equiv.) of *p*-nitrophenol and 8.2 g (1 equiv.) of TBAHS were vigourously stirred in a combination of 150 mL CH_2Cl_2 and 150 mL 1M NaOH. The phenoxide precipitated from solution ans was redissolved with gentle warming. After complete consumption of the bromide, the aqueous phase was separated. After exhaustive washing with 1M NaOH, sat. brine, the organic phase was dried with Na_2SO_4, filtered and evaporated to a yellow oil which crystallized upon the addition of ethanol. Recrystallization from ethanol gave pure **118** (4.65 g, 41%).

An additional 550 mg was obtained after silica gel chromatography of the mother liquor (total yield = 5.20 g, 46%). mp = 178.8-179.8°C, $[\alpha]_D$ = -40.5° (C=1.16, $CHCl_3$) {lit.[214] mp = 172-174 °C, $[\alpha]_D$ = -49.3°}.

CI-MS (ether) gave m/e (ion, relative intensity): 440 (M + 1 - NO, 24), 331 (M - aglycon, 100), 271 (331 - HOAc). ^{1}H-NMR δ ($CDCl_3$): 8.20 (d, 2H, $J_{m,o}$ = 9.3 Hz, H_{meta}), 7.05 (d, 2H, H_{ortho}), 5.11 to 5.32 (mult, 4H, H_1 to H_4), 4.27 (dd, 1H, $J_{5,6}$ = 5.4 Hz, H_6), 4.15 (dd, 1H, $J_{6,6'}$ = 12.4 Hz, $H_{6'}$), 3.91 (ddd, $J_{5,6'}$ = 9.6 Hz, H_5), 2.06, 2.05, 2.04, 2.03 (4, CH_3). ^{13}C-NMR δ ($CDCl_3$): 170.5, 170.2, 169.5, 169.3 (4 C=O), 161.2 (C_{ipso}), 143.2 (C_{para}), 125.8 (C_{meta}), 116.6 (C_{ortho}), 97.9 (C_1), 72.3 (C_5 + C_3), 70.7 (C_2), 67.8 (C_4), 61.6 (C_6), 20.3 (3 CH_3).

p-*Formylphenyl 2,2',3,3',4,4',6,6'-hepta*-O-*acetyl-β-D-lactoside* **120**

Acetobromolactose **112** (274 mg, 0.39 mmol), 96 mg (0.79 mg, 2 equiv.) of *p*-hydroxy benzaldhyde and 134 mg (0.40 mmol, 1 equiv.) of TBAHS were stirred vigourously in 2 mL CH_2Cl_2 and 2 mL 1M NaOH. After 45 min. at room temperature, the reaction was worked up as usual. Purification of the crude residue using EtOAc/hexane (1/1, v/v) containing 0.5% isopropyl alcohol gave 131 mg (45%) of pure **120** followed by the peracetylated-2-acetoxy-D-lactal (118 mg, 49%): mp 152.7 - 153.7 °C (EtOH), $[\alpha]_D$ = -19.7° (c=1, $CHCl_3$) {lit.[3] mp = 168 - 170 °C, $[\alpha]_D$ = -19 }. The pure solid **120** which failed recrystallization had mp = 89.7 - 91.8 °C (slow evaporation of CH_2Cl_2), $[\alpha]_D$ = -28.5° (c=1.12, $CHCl_3$).

^{1}H-NMR δ ($CDCl_3$): 9.90 (s, CHO), 7.82 (d, 2H, $J_{m,o}$ = 8.9 Hz, H_{meta}), 7.06 (d, 2H, H_{ortho}), 5.34 (dd, 1H, $J_{3',4'} = J_{4',5'}$ = 1.1 Hz, $H_{4'}$), 5.28 (ddd, 1H, $H_{5'}$), 5.11 (dd, 1H, $J_{1',2'}$ = 7.8 Hz, $H_{2'}$), 4.95 (dd, 1H, $J_{2',3'}$ = 10.4 Hz, $H_{3'}$), 4.49 (d, $H_{1'}$), 3.84 - 4.15 (mult, 9H, remaining H), 1.58 - 2.14 (7s, CH_3). ^{13}C-NMR δ ($CDCl_3$): 190.5 (CHO), 170.2, 170.0, 169.9, 169.8, 169.3, 168.9 (C=O), 161.0 (C_{ipso}), 131.7 (C_{para} and C_{meta}), 116.6

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[214] R.S. Sidhu, *Synthesis* , 883 (1981).

(C_{ortho}), 101.1 ($C_{1'}$), 97.6 (C_1), 76.0 (C_4), 73.0 (C_3), 72.6 (C_5), 71.2 ($C_{3'}$), 70.9 ($C_{5'}$), 70.7 (C_2), 69.1 ($C_{2'}$), 66.6 ($C_{4'}$), 61.9 (C_6), 60.8 ($C_{6'}$).

p-Formylphenyl β-D-lactoside **120a**

The heptaacetate **120** (187 mg, 0.25 mmol) was suspended in methanol (15 mL). Methanolic sodium methoxide (1M, 20 μL) was then added and the mixture was stirred overnight at room temperature followed by reflux for 10 min. to ensure complete de-*O*- acetylation. Ether (15 mL) was added to the cooled solution. After 40 min. at 0°C, the product was filtered and dried to give pure **120a** (111 mg, quant.) mp = 244.2 - 245.9 °C (decomp.), $[\alpha]_D$ = -11.7° (C = 0.98, DMSO).

Analytical data: calculated for $C_{19}H_{26}O_{12}$: C, 51.12; H, 5.87. Found: C, 50.89; H, 6.10. ^{1}H-NMR δ (D_2O): 9.83 (s, CHO), 7.97 (d, 2H, $J_{m,o}$ = 8.9 Hz, H_{meta}), 7.28 (d, 2H, H_{ortho}), 5.32 (d, 1H, $J_{1',2'}$ = 7.7 Hz, $H_{1'}$), 4.49 (d, 1H, $J_{1,2}$ = 7.7 Hz, H_1), 3.55 - 4.03 (mult, remaining). ^{13}C-NMR δ ($CDCl_3$): 191..2 (CHO), 161.8 (C_{ipso}), 131.5 (C_{meta}), 130.4 (C_{para}), 116.3 (C_{ortho}), 103.7 ($C_{1'}$), 99.1 (C_1), 79.9, 75.5, 75.0, 74.7, 73.2, 72.8, 70.5, 68.1 (ring-C), 60.0, 60.4 ($C_{6'}$,C_6).

p-Nitrophenyl 2,2',3,3',4',6,6'-hepta-O-acetyl-β-D-lactoside **121**

Acetobromolactose **112** (1.50 g, 2.14 mmol), 0.72 g (1 equiv.) of TBAHS and 0.60 g (2 equiv.) of *p*-nitrophenol were stirred vigourously in 10 mL CH_2Cl_2 and 10 mL 1M NaOH at room temperature for one hour. The reaction was worked up as usual. Recrystallizations of the crude residue from ethanol gave 0.79 g of **121** as white urchins of analytical quality. Silica gel chromatography of the remaining mother liquor gave an additional 134 mg of pure material. Total yield, 57%. (+) FAB-MS (glycerol) gave m/e (ion, realtive intensity): 727 (1.4, M - NO), 711 (1.3, M - NO_2), 619 (5.3, M-OPhNO_2), 559 (16, 619-HOAc), 331 (72, M - O-

$Glc(OAc)_4$-O-$PhNO_2$). NMR data can be found in Table 63 in Chapter 8.

p-*Nitrophenyl β-D-lactoside* **121a**

Typical procedure: 1.21 g (1.60 mmol) of pure **121** was dissolved in 25 mL of warm methanol. 40 µL of 1M $CH_3O^-Na^+/CH_3OH$ was then added and the reaction brought to a gentle reflux and left overnight. After adding 10 mL of ether and cooling, 733 mg (99%) of **121a** were filtered as a white microcrystalline powder of analytical purity. FAB-MS (glycerol) gave m/e (ion, relative intensity): 556 (1.0, M+Gly+1). 464 (1.4, M+1), 325 (6, M-O$PhNO_2$). NMR data can be found in Table 63 in Chapter 8.

*Methyl (*p*-nitrophenyl 5-acetamido-4,7,8,9-tetra*-O*-acetyl-3,5-dideoxy-D*-glycero-*α*-D*-galacto-*2-nonulopyranosid)onate* **123**

Crude acetochloroneuraminic acid methyl ester **114** (79.4 mg, 0.155 mmol) (95% pure by TLC), 33.0 mg (0.1 mmol) of TBAHS and 41 mg (0.3 mmol) of *p* -nitrophenyl were stirred in 0.6 mL of CH_2Cl_2 and 0.3 mL of 1.5M NaOH at room temperature for 20 min.. After the usual extractive work up, the product was isolated by preparative TLC using $CHCl_3$/EtOAc/n-prOH (25/5/1, v/v/v) as eluent and EtOAc/i-prOH (4/1, v/v) to extract the product from the scrapped silica. Filtration and evaporation gave 61.4 mg (64%) of **123** of analytical purity. The product was recrystallized from ether/pentane and had physical constants which agreed with those already published[154].

*Generalized PTC procedure for preparing peracetylated aryl 1,2-*trans*-1-thio-β-D-glycosides and glycobiosides* **124-128**

To solutions of the appropriate glycosyl halides **58**, **112** or **113** (1 equiv.) and TBAHS (1 equiv.) in CH_2Cl_2 or EtOAc (1mL/100 mg of the halide) is added 1M aq Na_2CO_3 (1 mL/100 mg of the halide) and

thiophenol or *p*-nitrophenol (3 equiv.). The two phase reaction mixture was stirred vigourously at room temperature and monitored by TLC (25/5/1 $CHCl_3$/EtOAc/n-prOH for **124**: Rf = 0.35; 1/1 hex/EtOAc including 0.5% i-prOH for **125**: Rf = 0.40,, **126**: Rf = 0.42, **127**: Rf = 0.37, **128**: Rf = 0.42). Reactions were complete within 30 min. for **124 - 126** and within 5.5 hours for **127** and **128**. After complete consumption of the halide, EtOAc was added and the organic phase washed successively with 2 portions of 1M NaOH, water and sat. NaCl. The organic extracts were then dried (Na_2SO_4), filtered and concentrated under reduced pressure the afford the crude glycosides. **124**, **125**, **127** and **128** were purified by crystallization from warm ethanol while **126** resisted all crystallization attempts and had to be purified by column chromotography on silica gel using a gradient of CH_2Cl_2/EtOAc (1/0, 1/1). Physical constants are given in Table 37 and agreed with those from the literature where applicable[158, 161,215]. NMR data given in Tables 38 and 39.

Ethyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-1-thio-β-D-glucopyranoside **129**

The glycosyl chloride **58** (100 mg, 0.273 mmol) and 93 mg (1 equiv,) of TBAHS were dissolved in 1 mL of CH_2Cl_2. 500 µl of a 10% (v/v) solution of ethanethiol (2.2 equiv.) in 2M NaOH was injected and the reaction stirred vigourously at room temperature for 25 min.. The reaction was then diluted with 10 mL EtOAc and washed with three 10 mL portions of water and 5 mL of sat. NaCl. The organic phase was dried (Na_2SO_4), filtered and concentrated under reduced pressure to give 67 mg of a white solid. Crystallization from ethanol gave 47 mg (44%) of pure **129**. See Tables 37, 38 and 39 for physical constants and NMR data.

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215 G. Wagner and R. Metzner, *Pharmazie*, *24* , 245 (1969); *Chem. Abst.*, *72* , 55827s (1970).

O-Ethyl-S-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl) dithiocarbonate **132**

The following represents a typical general procedure. To a solution of the appropriate glycosyl halide **58, 110 - 112** (1 equiv.), TBAHS (1 equiv.) and *O*-ethyl xanthic acid potassium salt (Aldrich, 1.2 or 2 equiv.) in methylene chloride (2 equiv.; 1 mL/100 mg halide) or ethyl acetate (1.2 equiv.; 1mL/100 mg halide) was added 2M sodium carbonate (1 mL/100 mg halide) (or sat. sodium hydrogen carbonate). The two phase reaction was mixed vigourously stirred at room temperature until TLC showed disappearance of the starting halide (< 45 min.). Ethyl acetate (10 times the reaction volume) or methylene chloride was then added. The organic phase was separated and successively washed with saturated sodium hydrogen carbonate, water and brine. The combined organic extracts were dried (Na_2SO_4), filtered and evaporated under reduced pressure to afford pure S-glycosyl xanthates **131 - 135**. Compound **132** crystallized in the flask on standing at 4 °C and was obtained in 98% yield; mp = 64.8 - 66.9 °C (prisms); $[\alpha]_D$ = +30.8° (c=1.0, $CHCl_3$); {lit.[216] mp = 88 - 89 °C (needles); $[\alpha]_D$ = +30.8° (c=3.20, $CHCl_3$).

O-Ethyl- S-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl) dithiocarbonate **133**

The residue obtained after evaporation of the organic extracts was chromatographed on a silica gel column using ethyl acetate/hexane (3/5, v/v) as eluent. The homogeneous fractions were evaporated to dryness to give pure **133** which crystallized on standing, 91% yield; mp = 79.5 - 80.4 °C (CH_2Cl_2, ether/hexanes); $[\alpha]_D$ = +49.7° (c=0.95, $CHCl_3$); {lit.[217] 79 - 80 °C; $[\alpha]_D$ = +51.6° (c=1.0, $CHCl_3$).

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[216] W. Schneider, R. Gille and K. Eisfeld, *Ber.*, *61* , 528 (1928); *Chem. Abst.*, *22* , 4107 (1928).

[217] M. Sakata, M. Haga and S. Tejima, *Carbohydr. Res.*, *13* , 379 (1970).

O-Ethyl S-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl) dithiocarbonate **131**

The oily residue obtained upon evaporation of the organic extracts crystallized from ether-diisopropyl ether to give **131** in 91% yield; mp = 139.0 - 140.2 °C; $[\alpha]_D$ = +33.3° (c=1.1, $CHCl_3$).

O-Ethyl S-(2,2',3,3',4',6,6'-hepta-O-acetyl-β-D-lactosyl) dithiocarbonate **134**

The oily residue obtained upon evaporation of the organic extracts was purified on a silica gel column eluted with CH_2Cl_2/EtOAc (6/1, v/v) to provide pure **134** in 96% yield. Compound **134** was obtained as a white foam and failed all crystallization attempts.

Dibenzyl (2,2',3,3',4',6,6'-hepta-O-acetyl-β-D-lactosyl)phosphate **135**

Acetobromolactose **112** (50 mg, 0.36 mmol), dibenzyl phosphate (149 mg, 0.54 mmol), 1.5 equiv.) and TBAHS (120 mg, 0.35 mmol), 1 equiv.) were dissolved in methylene chloride (2 mL). Saturated sodium hydrogen carbonate (2 mL) containing sodium carbonate (70 mg, pH 9.0) was then added to the organic solution. The two phase reaction mixture was vigourously stirred at room temperature for 34 hours until TLC (1/1, v/v, 0.1% Et_3N in hexane/ethylacetate) indicated complete conversion of **112** (Rf = 0.38) into the glycosyl phosphate **135** (Rf = 0.27). Addition of ethyl acetate (20 mL), washing the organic phase with sat. $NaHCO_3$, water and sat. NaCl, followed by drying (Na_2SO_4), filtration and evaporation of the organic phase furnished a glassy solid. Purification on silica gel column chromatography using chloroform/isopropanol as eluent (40/1, v/v) afforded 12 mg of the 2-acetoxylactal **122** resulting from α dehydrobromination side-reaction, followed by **135** (266 mg, 83%) as a glassy solid; $[\alpha]_D$ = -7.5° (c = 0.97, $CHCl_3$). Anal. calc. for

$C_{40}H_{49}O_{21}P$: C 53.57, H 5.51, P 3.45; found: C 53.45, H 5.62, P 3.71.

(-)FAB-MS m/e 809 (M^--benzyl, 13%). ^{1}H-NMR (500 MHz) δ ($CDCl_3$): 7.20 - 7.36 (mult, 10H, Ph), 5.33 (dd, 1H, $J_{3,4}$ = 3.5, $J_{4,5}$ < 1 Hz, $H_{4'}$), 5.30 (dd, 1H, $J_{1,2}$ = 7.5, $^3J_{1,P}$ = 7.5 Hz, H_1), 5.19 (dd, 1H, $J_{3,4}$ = 9.2, $J_{2,3}$ = 9.3 Hz, H_3), 5.09 (dd, 1H, $J_{1,2}$ = 7.8, $J_{2,3}$ = 10.4 Hz, $H_{2'}$), 5.01 (dd, 1H, H_2), 4.99 (app.d, 2H, $^2J_{C,P}$ = 7.3, J_{gem} <1Hz, CH_2Ph), 4.94 (dd, 1H, $H_{3'}$), 5.03 (dd, 2H, $^2J_{C,P}$ = 7.3, J_{gem} = 2.8 Hz, CH_2Ph), 4.48 (dd, 1H, $J_{5,6}$ = 2.1, $J_{6,6'}$ = 12.2 Hz, H_6Glc), 4.47 (d, 1H, $J_{1,2}$ = 7.8 Hz, $H_{1'}$), 4.11 (dd, 1H, $J_{5,6}$ = 6.9, $J_{6,6'}$ = 11.2 Hz, H_6Gal), 4.06 (dd, 1H, H_6Glc), 4.02 (dd, 1H, $J_{5,6'}$ = 5.1 Hz, H_6Glc), 3.85 (app.d, 1H, $J_{5,6'}$ = 6.3 Hz, $H_{5'}$), 3.84 (dd, 1H, $J_{4,5}$ = 10.0 Hz, H_4), 3.71 (ddd, 1H, H_5), 2.13, 2.02, 2.02 (2x), 2.00, 1.94, 1.88 (7s, 21H, OAc). ^{13}C-NMR δ ($CDCl_3$): 170.3, 170.1 (2x), 170.0,169.5, 169.0 (OAc), 135.3 (d, $^3J_{C,P}$ = 23.3 Hz, C_{ipso} Ph), 128.6 (C_{para}), 128.6, 128.5 (C_{meta}), 127.8, 127.7 (C_{ortho}), 101.1 ($C_{1'}$), 96.1 (d, $^2J_{C,P}$ = 4.9 Hz, C_1), 75.6 (C_4), 73.4 (C_5), 72.3 (C_3), 71.5 (d, $^3J_{C,P}$ = 9.0 Hz, C_2), 70.9 ($C_{3'}$), 70.7 ($C_{5'}$), 69.6 (d, $^2J_{C,P}$ = 6.2 Hz, CH_2Ph), 69.5 (d, $^2J_{C,P}$ = 6.3 Hz, CH_2Ph), 69.0 ($C_{2'}$), 66.5 ($C_{4'}$), 61.5 (C_6), 20.7 (2x), 20.6 (3x), 20.5, 20.4 (OAc).

PTC synthesis of glycosyl azides **136 - 140**

General procedure: To a solution of the appropriate glycosyl halides **58, 110 - 113** (1 equiv.), TBAHS (1 equiv.) and sodium azide (3-5 equiv.) in methylene chloride (1mL/100mg of the halides) was added saturated aqueous $NaHCO_3$ (1 mL/100 mg of the halides). The two phase reaction mixtures were vigourously stirred at room temperature for 1-2 hours, after which time TLC indicated complete transformation of the halides. EtOAc (10 times the volume of CH_2Cl_2) was added, the organic phase separated and successively washed with sat. $NaHCO_3$, water (2x) and brine. The combined extracts were dried (Na_2SO_4), filtered and evaporated under reduced pressure to afford pure glycosyl azides **136 - 140**. The dried azides

136 - 140 were homogeneous by TLC, ^{1}H- and ^{13}C-NMR spectroscopy. They were, however, crystallized for combustion analyses, mp and $[\alpha]_D$ measurements (see Table 43). The yields of **136 - 140** varied from 93-98% (Table 43).

PTC synthesis with thiocyanate as nucleophile: 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl thiocyanate **141** *and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate* **142**

Acetobromoglucose **110** (600 mg), 405 mg (1 equiv.) of TBAHS and 1.42 g (10 equiv.) of KSCN were stirred vigourously for one hour at room temperature in 3 mL ETOAc and 3 mL 1M Na_2CO_3. The reaction was worked up by adding 30 mL of EtOAc and washing the organic phase with sat. $NaHCO_3$, water (3x) and sat. brine. The organic phase was dried (Na_2SO_4), filtered and evaporated to an oil that crystallized upon standing. ^{1}H-NMR of the crude product mixture indicated a 10:1 ration of **141**:**142**. Silica gel chromatography with 2/1 hex/EtOAc containing 0.5% i-prOH gave 27 mg (7%) of the isothiocyanate **142** ($\nu_{N=C=S}$ = 2025 cm^{-1}) followed by 386 mg (68%) of the desired thiocyanate **141** ($\nu_{S-C\equiv N}$ = 2167 cm^{-1}). Both products were crystallized from EtOAc/hexane. Physical properties and NMR data are given in Tables 46 - 48.

2-Acetamido-3,4,6-tri-O-acetyl-1-benzoyl-2-deoxy-β-D-glucopyranose **143**

Acetochloro *N*-acetylglucosamine **58** (153.7 mg, 0.421 mmol), 154.0 mg (3 equiv.) of benzoic acid and 140.2 mg (1 equiv.) of TBAHS were stirred vigourously at room temperature in 2 mL CH_2Cl_2 and 2 mL sat. $NaHCO_3$. After 5 min., the pH was adjusted to 7.5 with 2M Na_2CO_3. The reaction was shown to be complete adter a total of 40 min. TLC (25/5/3, $CHCl_3$/EtOAc/n-propOH) indicated a complete and clean conversion of the halide **58** (Rf = 0.51) to the new product **143** (Rf = 0.47). Thus, the reaction was worked up by adding 20 mL of

EtOAc and washing the organic phase with 5 portions of 15-20 mL of sat. $NaHCO_3$, water and sat. brine. After drying (Na_2SO_4), filtration and evaporation to a solid, a crude yield >100% was obtained. TLC analysis indicated an incomplete removal of excess benzoic acid. The crude material was redissolved in CH_2Cl_2 and washed once with concentrated (>2M) Na_2CO_3, water, and sat. NaCl. Drying (Na_2SO_4), filtration and evaporation gave a white crystalline mass (183.7 mg, 97%) of essentially pure **143** (TLC, NMR). The product was recrystallized from EtOH/ether for elemental analysis, mp and $[\alpha]_D$ measurements (see Table 49).

^{1}H-NMR δ ($CDCl_3$): 8.03 (mult, 2H, H_{ortho}), 7.56 (mult, 1H, H_{para}), 7.41 (mult, 2H, H_{meta}), 5.97 (d, 1H, $J_{2,NH}$ = 9.4 Hz, NH), 5.86 (d, 1H, $J_{1,2}$ = 8.8 Hz, H_1), 5.12 - 5.26 (AB mult, 2H, H_3 and H_4), 4.54 (ddd, $J_{2,3}$ = 9.6 Hz), 4.28 (ddd, $J_{5,6a}$ = 4.5 Hz, H_{6a}), 4.11 (dd, $J_{5,6b}$ = 12.4 Hz, H_{6b}), 3.90 (ddd, $J_{5,6}$ = 2.3 Hz, H_5), 2.03 (s, 3H, CH_3), 1.83 (s, 3H, CH_3). ^{13}C-NMR δ ($CDCl_3$): 171.5, 170.9, 170.3 and 169.5 (4 C=O, Ac), 165.3 (C=O, benzoyl), 134.0 (C_{para}), 130.3 (C_{ortho}), 128.7 (C_{meta}), 128.5 (C_{ipso}), 93.2 (C_1), 72.8 (C_5), 72.6 (C_3), 67.9 (C_4), 61.6 (C_6), 52.3 (C_2), 23.0 (CH_3, NAc), 20.5 ($2CH_3$, OAc), 20.4 (CH_3, OAc).

2-Acetamido-3.4.6-tri-O-acetyl-2-deoxy-1-(prop-2-enoyl)-β-D-glucopyranose **144**

Acetochloro *N*-acetylglucosamine **58** (138.1 mg, 0.378 mmol),127.3 mg (1 equiv.) of TBAHS, and 60 µl (68 mg, 2.5 equiv.) of acrylic acid were vigourously stirred at room temperature in 1.5 mL CH_2Cl_2 and 1.5 mL sat. $NaCO_3$ was added to keep the pH slightly above 7. The reaction was allowed to proceed for 70 min. after which time it was diluted with 20 mL EtOAc. The organic phase was washed twice with 1M NaOH (15 mL), sat. $NaHCO_3$, water and sat. NaCl. Drying (Na_2SO_4), filtration and evaporation of the organic phase gave 126.7 mg of crude semi-solid material, impure by TLC (25/5/3, $CHCl_3$/EtOAc/n-prOH). Silica gel chromatography with a gradient of hexane/EtOAc (1/1, 2/3, 1/2) gave 115.1 mg (76%) of pure **144**. The product could

be crystallized slowly from EtOH/ether and a drop of hexane to give small, white cubes. Physical data are given in table 49.

^{1}H-NMR δ ($CDCl_3$): 6.50 (dd, 1H, J_{trans} = 17.0 Hz, J_{gem} = 1.6 Hz, $=CH_2$ trans), 6.10 (dd, 1H, J_{cis} = 10.4 Hz, -CH=), 5.93 dd, 1H, $=CH_2$ cis), 5.72 (d, 1H, $J_{1,2}$ = 8.8 Hz, H_1), 5.59 (d, 1H, $J_{2,NH}$ = 9.7 Hz, NH), 4.37 (ddd, 1H, $J_{2,3}$ = 9.9 Hz, H_2), 4.26 (dd,1H, $J_{5,6a}$ = 4.5 Hz, H_{6a}), 4.10 (dd,1H, $J_{6a,6b}$ = 12.4 Hz, H_{6b}), 3.82 (ddd, $J_{5,6b}$ = 2.3 Hz, H_5), 2.07, 2.03, 2.02 and1.88 (4s, CH_3).

2,3,4,6-Tetra-O-acetyl-1-benzoyl-β-D-glucopyranose **145**

Acetobromoglucose **110** (150 mg, 0.365 mmol),125 mg (1 equiv.) of TBAHS and 130 mg (3 equiv.) of benzoic acid were stirred overnight at room temperature in 2 mL CH_2Cl_2 and 3 mL of sat. $NaHCO_3$. TLC (1/1, hexane/EtOAc + 0.5% i-prOH) showed a complete consumption of the halide (Rf = 0.63) to cleanly give **145** (Rf = 0.56). Work up as above gave 160 mg (97%) of essentially pure **145**. Recrystallization was performed easily from warm ethanol. Physical data are given in table 49.

^{1}H-NMR δ ($CDCl_3$): 8.03 (mult, 2H, H_{ortho}), 7.59 (mult, 1H, H_{para}), 7.43 (mult, 2H, H_{meta}), 5.90 (mult, 1H, H_1, $J_{1,2}$ = 8.1 Hz, with virtual couplings from H_2/H_3 AB signal), 5.33 (mult, 2H, H_3/H_4 AB system), 5.18 (mult, 1H, H_4, $J_{4,5}$ = 10.0 Hz and virtual couplings from H_2/H_3 AB signal), 4.31 (dd, $J_{5,6a}$ = 4.5 Hz, H_{6a}), 4.12 (dd, 1H, $J_{6a,6b}$ = 12.5 Hz, H_{6b}), 3.92 (ddd, 1H, $J_{5,6b}$ = 2.3 Hz, H_5), 2.06, 2.04, 2.03, 1.97 (4s, CH_3). ^{13}C-NMR δ ($CDCl_3$): 170.3, 169.8, 169.2 and 169.1 (4 C=O, Ac), 164.2 (C=O, benzoyl), 133.8 (C_{para}), 129.9 (C_{ortho}), 128.4 (C_{meta}), 128.2 (C_{ipso}), 92.1 (C_1), 72.5 (C_5), 72.4 (C_3), 69.9 (C_4), 67.7 (C_2), 61.2 (C_6), 20.5 (CH_3), 20.4 ($2CH_3$), 20.3 (CH_3).

2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl N-carbobenzyloxy-L-alanine ester **146**

Acetobromo α-D-glucose **110** (411 mg, 1.00 mmol), 513 mg (2.3 equiv.) of *N*-CZB-L-alanine (Sigma) and 340 mg (1 equiv.) of TBAHS were stirred at room temperature in 4 mL CH_2Cl_2 and 4 mL of 1M Na_2CO_3 for 5 hours. TLC (1/1 EtOAc/hexane + 0.5% i-prOH) showed a complet and clean conversion of the halide **110** (Rf = 0.68) to the ester **146** (Rf = 0.62). Work up as above gave 464.5 mg (84%) of essentially pure **146** (TLC, ^{1}H- and ^{13}C-NMR). The product was recrystallized easily from abs. ethanol. Physical data are given in table 49.

^{1}H-NMR δ ($CDCl_3$): 7.33 (mult, 5H, aromatic), 5.72 (d, 1H, $J_{CH,NH}$ = 8.1 Hz, NH), 5.08 - 5.27 (mult, 4H, H_1, H_2, H_4), 5.08 (s, 2H, CH_2 Ala), 4.32 (dq, 1H, $J_{CH,\ CH3}$ = 7.4 Hz CH Ala), 4.26 (dd, $J_{5,6a}$ = 4.5 Hz, H_{6a}), 4.08 (dd, 1H, $J_{6a,6b}$ = 12.5 Hz, H_{6b}), 3.82 (ddd, 1H, $J_{5,6b}$ = 2.2 Hz, H_{6b}), 2.06, 2.01, 2.00 (3s, 12H, 4CH_3 OAc). ^{13}C-NMR δ ($CDCl_3$): 171.1, 170.4, 169.8, 169.2 and 169.1 (5 C=O), 155.4 (C=O), 136.0 (C_{ipso}), 128.3 (C_{ortho}), 128.0 (C_{para}), 127.8 (C_{meta}), 92.1 (C_1), 72.5 (C_5), 72.3 (C_3), 69.8 (C_4), 67.6 (C_2), 66.7 (CH_2 Ala), 61.2 (C_6), 49.4 (CH, Ala), 20.4 (CH_3, OAc), 20.3 (3CH_3, OAc), 17.4 (CH_3, Ala).

1,3-N,N'-Bis(2,3,4,6-tetra-O-acetyl-β-D-galactosyl) 5,5-diethyl barbiturate **149**

Acetobromogalactose **111** (1.07 g, 2.60 mmol, 2.5 equiv.), 0.19 g (1.04 mmol, 1 equiv.) of barbital **148** and 0.35 g (1.56 mmol, 1.5 equiv.) of TBAHS were vigourously stirred at room temperature overnight in a combination of 15 mL CH_2Cl_2 and 15 mL 2M Na_2CO_3. TLC (1/1 EtOAc/$CHCl_3$) showed a complete consumption of the bromide **111** (Rf = 0.79) to give **149** (Rf = 0.47) as the major product. The reaction was worked up by adding EtOAc, removing the aqueous phase and then washing the organic phase successively with sat. $NaHCO_3$ (2x), water (2x) and sat. NaCl. After drying (Na_2SO_4),

filtration and evaporation, a yellow, oily residue was obtained which resisted crystallization. Purification of **149** was performed by column silica gel chromatography with a gradient of $CHCl_3$/EtOAc (5/1, 4/1, 3/1). 472 mg (54%) of pure **149** was obtained after pooling and evaporation of the product fractions. **149** was crystallized from CH_2Cl_2/ether. Pnysical data are given in table 50.

CI-MS (ether) gave m/e (ion, relative intensity): 845 (M+1, 50), 515 (M+2-Gal(OAc)$_4$, 67), 331 (Gal(OAc)$_4^+$,100). ^{1}H-NMR δ ($CDCl_3$): 6.36 (dd, 1H, $J_{2,3}$ = 9.9 Hz, H_2), 6.07 (dd, 1H, $J_{2,3}$ = 9.9 Hz, H_2), 5.96 (d, 1H, $J_{1,2}$ = 9.4 Hz, H_1), 5.95 (d, 1H, $J_{1,2}$ = 9.4 Hz, H_1), 5.46 (dd, 1H, $J_{4,5}$ = 1.0 Hz, H_4), 5.44 (dd, 1H, $J_{4,5}$ = 1.0 Hz, H_4), 5.12 (dd, 1H, $J_{3,4}$ = 3.5 Hz, H_3), 5.08 (dd, 1H, $J_{3,4}$ = 3.5 Hz, H_3), 3.99 - 4.19 (mult, 6H, $2H_5$ + $4H_6$), 2.33, 2.20, 2.07, 2.02, 2.01, 1.99, 1.98, 1.90 (8s, 24H, CH_3 OAc), 2.0 (mult, 4H, CH_2 barbital obscured by acetate resonances), 0.87 (t, 2H, J = 7.5 Hz, CH_2 barbital), 0.81 (t, 2H, J = 7.5 Hz, CH_3 barbital). ^{13}C-NMR δ ($CDCl_3$): 171.6, 170.5, 170.3 (2C), 170.2, 170.0 (2C), 169.9, 169.7, 169.5 (10 C=O), 148.7 (N_2 C=O), 81.4, 80.3 ($2C_1$), 73.4, 72.9 ($2C_5$), 72.4, 71.8 ($2C_3$), 66.7, 66.6, 66.4, 66.2 ($2C_4$ and $2C_2$), 61.0 ($2C_6$), 58.9 ($C(Et)_2$), 35.1, 31.1, ($2CH_2$ barbital), 20.4 - 20.8 (CH_3,Ac), 9.0, 8.7 ($2CH_3$ barbital).

1,3-N,N'-*Bis(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-β-D-glucopyranosyl) 5,5-diethyl barbiturate* **150**

Acetochloro *N*-acetylglucosamine **58** (2.00 g, 5.47 mmol, 2.5 equiv.), 0.41 g (2.23 mmol, 1 equiv.) of barbital **148**, 1.13 g (3.33 mmol, 1.5 equiv.) of TBAHS were stirred vigourously overnight at room temperature in a blend of 25 mL CH_2Cl_2 and 25 mL 2M Na_2CO_3. TLC (EtOAc/$CHCl_3$, 2/1, v/v) showed a complete consumption of the halide **58** (Rf = 0.56) to give **150** (Rf = 0.18) and secondary products. Crystallization from CH_2Cl_2/ether gave 1.12 g of pure **150**. Silica gel chromatography (2/1, EtOAc/$CHCl_3$) of the mother liquor gave an additional 185 mg of product. Total yield, 1.50 g (69%). Physical data are given in table 50.

(+)FAB-MS (glycerol) gave m/e (ion, relative intensity): 843.3 (M+1, 5), 513.9 (M+2 - $Gal(OAc)_4$, 17), 330 ($Gal(OAc)_4^+$,30). ^{1}H-NMR δ ($CDCl_3$): 6.00 (d, 1H, $J_{2,NH}$ = 9.0 Hz, NH), 5.87 (d, 1H, $J_{2,NH}$ = 7.9 Hz, NH'), 5.82 (d, 1H, $J_{1,2}$ = 9.5 Hz, $H_{1'}$), 5.81 (d, 1H, $J_{1,2}$ = 9.1 Hz, H_1), 5.34 (ddd, 1H, $J_{2,3}$ = 9.0 Hz, H_2), 5.26 (dd, 1H, $J_{4,5}$ = 9.8 Hz, H_4), 5.22 (dd, 1H, $J_{3,4}$ = 9.3 Hz, $H_{3'}$), 5.09 (dd, 1H, $J_{3,4}$ = 9.7 Hz, H_3), 5.08 (dd, 1H, $J_{4,5}$ = 9.8 Hz, $H_{4'}$), 5.02 (ddd, 1H, $J_{2,3}$ = 10.1 Hz, $H_{2'}$), 4.29 (dd, 1H, $J_{5,6a}$ = 5.4, $J_{6a,6b}$ = 12.5 Hz, H_{6a}), 4.27 (dd, 1H, $J_{5,6a}$ = 2.6, $J_{6a,6b}$ = 12.2 Hz, H_{6a}), 4.13 (dd, 1H, $J_{5,6b}$ = 2.2, $J_{6a,6b}$ = 12.5 Hz, H_{6b}), 4.11 (dd, 1H, $J_{5,6b}$ = 4.5, $J_{6a,6b}$ = 12.2 Hz, H_{6b}), 3.74 - 3.80 (mult, 2H, $2H_5$, $H_{5'}$), 2.01 - 2.06 (6s, CH_3 OAc), 1.88 - 1.99 (mult obscured by OAc signals, 4H, CH_2 barbital), 0.82 (t, 3H, J = 7.4 Hz, CH_3 barbital), 0.65 (t, 3H, J = 7.2 Hz, CH_3 barbital). ^{13}C-NMR δ ($CDCl_3$): 171.8, 171.7, 171.0, 170.7, 170.6, 170.5, 170.3, 169.3, 169.2, 169.1 (C=O), 149.0 (N_2 C=O), 81.5, 81.1 ($2C_1$), 75.0, 74.9, 74.7, 73.3 ($2C_5$, $2C_3$), 68.1, 67.7 ($2C_4$), 62.0, 61.8 ($2C_6$), 58.9 ($C(Et)_2$), 50.3, 50.2 ($2C_2$), 34.0, 30.6 ($2CH_2$, barbital), 23.3, 23.0 (CH_3, NHAc), 20.5 - 20.7 (CH_3, OAc), 9.1, 8.8 ($2CH_3$, barbital).

1,3-N,N'-*Bis(2-acetamido-2-deoxy-β-D-glucopyranosyl) 5,5-diethyl barbiturate* **151**

Compound**150** (850 mg, 1.01 mmol) was dissolved in 45 mL of warm methanol. 1M CH_3ONa was added to pH 9-10. The solution was left stirring at room temperature for a few hours. TLC (30/10/1. $CHCl_3$/MeOH/H_2O, v/v/v) showed one homogeneous spot (Rf = 0.13) when charred after spraying with ethanolic H_2SO_4 (5%). The solution was neutralized with (H^+) resin, filtered and evaporated to a constant weight of 597 mg (quant.). A white microcrystalline powder was obtained after recrystallization from isopropanol (first crop, 545 mg). Physical data are given in table 50.

CI-MS (ether) gave m/e (ion, relative intensity): 590.9 (M+1, 2), 387.9 (M+2- GlcNAc, 90), 359.9 (338 - CO, 21), 262.0 (338 - $(Et)_2C(CO)_2$, 25), 204 (GlcNAc - OH, 100), 186 (204 - H_2O, 100), 168

(186 - H_2O, 94), 150 (168 - H_2O, 100). ^{1}H-NMR δ ($DMSO_{d6}$, 150 °C): 7.12 (broad s, 2H, NH), 5.72 (d, 2H, J_{12}= 9.8 Hz, H_1), 4.64 (mult, 2H, H_2), 3.75 (dd, 2H, $J_{5,6a}$ = 2.4, $J_{6a,6b}$ = 11.7 Hz, H_{6a}), 3.56 (dd, 2H, $J_{5,6b}$ = 5.2 Hz, H_{6b}), 3.55 (mult obscured by H_{6b}, 2H, H_4), 3.33 (mult, 4H, H_3 and H_5), 1.88 (q, 2H, J = 7.3 Hz, CH_2), 1.87 (q, 2H, J = 7.3 Hz, CH_2), 1.81 (s, 6H, CH_3 ,NAc), 0.78 (t, 6H, J = 7.3 Hz, CH_3). ^{13}C-NMR δ ($DMSO_{d6}$, 23 °C): 171.1, 170.9, 170.2, 169.9, 169.8, 169.3 (N-C=O), 148.3 (2 N_2C=O), 145.7 (N_2C=O), 82.0, 81.6, 81.0, 80.4, 80.3, 79.8 ($3C_1$ and $3C_5$), 76.1, 75.2, 74.8 ($3C_3$), 70.7, 70.4, 70.1 ($3C_4$), 61.6, 61.1, 60.9 ($3C_6$), 57.9, 57.3 (2 $C(Et)_2$), 51.7, 51.5, 50.9 ($3C_2$), 32.5, 31.3, 30.9, 29.8 (4 CH_2, barbital), 23.3, 23.0, 22.9 (3 CH_3, NAc), 9.4, 8.9, 8.6 ($3CH_3$, barbital).

PTC galactosylation of 2,4-pentanedione. Preparation of enolate glycosides **152** *and* **153**

Acetobromogalactose **111** (2.00 g, 4.86 mmol) and 1.65 g (1 equiv.) of TBAHS were dissolved in 20 mL of 1,2-dichloroethane. 20 mL of 1M NaOH were then added followed by 750 µl (1.5 equiv.) of 2,4-pentanedione. The reaction was stirred for a total of 11 hours at room temperature, after which TLC (1/1 EtOAc/hexane + 0.5% i-prOH) showed complete consumption of the halide (Rf = 0.17, *E*-*O*-enol-β-D-galactopyranoside **152**; Rf = 0.30, *Z*-*O*-enol-β-D-galactopyranoside **153**). The reaction was worked up by separating the aqueous phase, diluting the organic phase with EtOAc then successively washing it with sat. $NaHCO_3$, water (2x), and sat. brine. Drying (Na_2SO_4), filtration and evaporation under reduced pressure gave an oil. After dissolving in a minimum of CH_2Cl_2, the crude material was applied to silica gel column then eluted with a gradient of EtOAc/hexane (3/4, 1/1, 4/3; v/v) including 0.5% i-prOH. 0.31 g (15%0 of *Z*-enol galactoside **153** was recovered, followed by 0.67g (32%) of the *E*-enol galactoside **153**. Physical data for **152** and **153** are tabulated in section 6.10.

PTC galactosylation of benzyl 3-keto-butanoate. Preparation of enolate glycosides **154** *and* **155.**

Acetobromogalactose **111** (200 mg, 0.486 mmol), 165 mg (1 equiv.), and 140 µL of benzyl 3-keta butanoate (acetobenzyl acetate, Aldrich) were stirred vigourously at room temperature in 2.5 mL of 1,2-dichloroethane and 25 mL of 2M Na_2CO_3. After 6 hours, TLC (1/1, EtOAc/hexane + 0.5% i-prOH) showed a complete consumption of the halide **111**, to give two major products (Rf = 0.25, *E* -*O*-enol-β-D-galactopyranoside **154**; Rf = 0.39, *Z* -*O*-enol-β-D-galactopyranoside **155**). The reaction was worked up as described for **152/153**. The products were simplified by preparative TLC using $CHCl_3$/MeOH (30/1, v/v) as eluent. Extraction of the u.v. active bands with EtOAc gave 73 mg (30%) of **154** and 38 mg (15%) of **155**. All attempts at crystallization failed for both compounds. Physical data for **154** and **155** are tabulated in section 6.10.

N-*Oxysuccinimidyl 2,3,4,6-tetra*-O-*acetyl-β-D-glucopyranoside* **156**

Acetobromoglucose **110** (200 mg, 0.487 mmol), 170 mg (3 equiv.) of *N*-hydroxy succinimide (Aldrich) and 165 mg (1 equiv.) of TBAHS were stirred vigourously at room temperature in 3 mL of CH_2Cl_2 and 3 mLof 2M Na_2CO_3. After 2 hours, TLC (35/1, $CHCl_3$/i-prOH, v/v) showed a complete consumption of the bromide **110** (Rf = 0.64) to give **156** (Rf = 0.26) as the major product. The reaction was worked up by adding ethyl acetate (20 mL) and washing with sat. $NaHCO_3$ (2x), followed by distilled water and sat. NaCl. The organic extracts were dried (Na_2SO_4), filtered and evaporated under pressure to give a clear oil. The product**156** crystallized as a white, microcrystalline powder, pure by TLC, NMR and elemental analysis, upon addition of CH_2Cl_2 (85.4 mg). Crystallization of the mother liquor from CH_2Cl_2/ether gave an addtitional 42.4 mg of material. Total combined yield was thus 127.8 mg (59%). NMR and physical properties including elemental analysis is given in Tables 54, 55 and

56.

N-*Oxysuccinimidyl 2,3,4,6-tetra*-O-*acetyl*-β-*D-galactopyranoside* **157**

Acetobromogalactose **111** (1.00 g, 2.43 mmol), 0.56 g (2 equiv.) of *N*-hydrosuccinimide and 0,80 g (1 equiv.) of TBAHS wre stirred vigourously at room temperature in a combination of of 15 mL of CH_2Cl_2 and 15 mL of 2M Na_2CO_3. After 3 hours, the reaction was worked up in the same fashion as **156**. The crude **157** was then purified by silica gel chromatography using a gradient of EtOAc/hexane (1/1, 2/1, 3/1, v/v) containing 0.5% i-prOH. Pooled product fractions were evaporated under reduced pressure to give an oil which crystallized overnight upon standing under high vaccum. The product **157** was obtained in a yield of 846 mg (78%), pure by TLC (35/1 $CHCl_3$/MeOH, Rf = 0.36) and NMR. A small quantity of material was recrystallized from $CHCl_3$/ether/hexane to give small, white needles. NMR and physical data are given in Tables 54, 55 and 56.

N-*Oxysuccinimidyl 2,2',3,3',4',6,6'-hepta*-O-*acetyl*-β-*D-maltoside* **158**

Acetobromomaltose **113** (620 mg, 0.886 mmol), 300 mg (1 equiv.) of TBAHS and 204 mg (2 equiv.) of *N*-hydroxysuccinimide were vigourously stirred at room temperature overnight in 7 mL CH_2Cl_2 and 7 mL of sat. $NaHCO_3$. After a similar work up to that described for **156**, the crude maltoside **158** was purified by silica gel chromatography using EtOAc/hex (1/1, v/v) containing 0.5% i-prOH. A 394 mg (60%) yield of pure **158** was obtained after complete solvent removal. A portion of the product was crystallized from ethanol. NMR and physical data can be found in Table 54, 55 and 56.

Zemplén de-O-acetylation of peractylated N-oxysuccinimidyl β-D-glycosides. Preparation of 1-O-β-D-glycosyl 3-carboxymethyl propylhydroxamic esters **159** *and* **160**.

Glycosides **159** and **160** were both prepared by the same general procedure, conducted at room temperature. The *N*-oxysuccinimidyl glycosides **157** and **158** were dissolved in methanol (20 mg/mL). Solutions were treated with sodium methoxide by a dropwise addition of a solution of 1M CH_3ONa in CH_3OH until pH 8.5 - 9. Over time, the pH gradually decreased to near neutrality. Additional sodium methoxide was added until pH 8.5 - 9. After repeated additions of sodium methoxide, the pH of the solution stayed constant, and reaction products collapsed to one single homogeneous spot on TLC ($CHCl_3/MeOH/H_2O$, 30/10/1, v/v/v; Rf = 0.2 for **159** and **160**). Transformations were carried out slowly over a few days. After complete reaction, the solutions were then neutralized with (H^+) resin (Amberlite IR 120) filtered and evaporated to complete dryness under vacuum. Both glycosides **159** and **160** were obtained as glassy solids in quantitative yields and were pure by TLC and NMR. Both compounds failed all attempts at crystallization from a variety of solvents and solvent blends. NMR and physical data for **159** and **160** are given in Tables 57, 58 and 59.

β-D-Galactosyl 3-carboxypropylhydroxamic ester **161**

The methyl ester **159** (120 mg, 0.407 mmol), was stirred in 10 mL of 0.05M NaOH at room temperature overnight. The solution was then neutralized with strong (H^+) resin (Dowex 50W), filtered and lyophilized to give **161** as a white amorphous solid. A quantitative yield of pure **161** was obtained after drying the lyophilized product over P_2O_5 under vacuum for 3 days. All attempts at crystallization failed. NMR and physical data are given in Tables 57, 58 and 59.

Phenyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-1-seleno-β-D-glucopyranoside **163**

Acetochloro *N*-acetylglycosamine **58** (202.0 mg, 0.553 mmol), and 187.3 mg (1 equiv.) of TBAHS were stirred in 2 mL CH_2Cl_2 and 2 mL 1M Na_2CO_3 at room temperature for a moment. 100 µL (1.5 equiv.) of benzeneselenol (Aldrich) was then added to the mixture by syringe. A TLC analysis (25/5/1, $CHCl_3$/EtOAc/n-prOH) of the reaction mixture showed a complete and clean conversion of the halide **58** (Rf = 0.45) had occured within 10 min. The reaction was worked up by diluting with EtOAc (20 mL) and washing the organic phase with 0.5M NaOH, water and sat. NaCl. After drying (Na_2SO_4), filtration and evaporation of the organic extracts a yellow oil was obtained. The product **162** was purified by filtration through a short bed of silica gel. Elution with CH_2Cl_2 retained the product and liberated bis(phenylseleno) methane and diphenyl selenide. The product **162** was then eluted from the silica bed with EtOAc. A 258 mg (95%) yield of pure **162** was obtained after solvent removal. The amorphous white solid was then crystallized form warm ethanol. Physical data: mp = 189.4 - 190.4 °C, $[\alpha]_D$ = -30.9° (c=1.18, $CHCl_3$).

CI-MS (ether) for $C_{20}H_{25}NO_8Se$ gave m/e (ion, relative intensity): 490.9 (M+5, 2.8), 489.8 (M+4, 12.8), 488.8 (M+3, 13.1), 486.6 (M+1, 59.4), 330 (M - SeC_6H_5, 100), 270 (330 - HOAc, 11), 210 (270 - HOAc, 16), 150 (210 - HOAc, 50). ^{1}H-NMR δ ($CDCl_3$): 7.59 (mult, 2H, H_{meta}), 7.29 (mult, 3H, H_{ortho} + H_{para}), 5.57 (d, 1H, $J_{2,NH}$ = 9.3 Hz, NH), 5.12 (dd, 1H, $J_{3,4}$ = 9.4 Hz, H_4), 5.03 (dd, 1H, $J_{3,4}$ = 9.4 Hz, H_3), 4.97 (d, 1H, $J_{1,2}$ = 10.6 Hz, H_1), 4.06 - 4.21 (mult, 3H, H_2 + H_{6a} +H_{6b}), 3.64 (ddd, 1H, $J_{5,6a}$ = 3.0, $J_{5,6b}$ = 4.9 Hz, H_5), 2.05, 2.00, 1.99 and 1.95 (4s, CH_3, acetates). ^{13}C-NMR δ ($CDCl_3$): 171.2, 170.6, 170.0 and 169.3 (4 C=O), 134.7 (C_{meta}), 129.0 (C_{ortho}), 128.3 (C_{para}), 127.9 (C_{ipso}), 82.7, (C_1), 76.9 (C_5), 73.6 (C_3), 68.3 (C_4), 62.4 (C_6), 54.1 (C_2), 23.3 (CH_3, NAc), 20.6 - 20.7 (4 CH_3, OAc).

7. *p*-Formylphenyl glycosides as precursors to neoglycoproteins[218]

It is well established that artificial carbohydrate protein conjugates constitute useful antigens and immunogens from which immunodiagnostic reagents as well as animal and human vaccines can be derived[219]. They are also important tools for receptor studies, for the inhibition of adherence of bacteria and viruses, and for cell targetted drug delivery[15]. Existing methods for neoglycoprotein syntheses have been discussed earlier (Chapters 1 and 4). Of the existing methodologies, reductive amination of free amine residues on proteins (L-lysine ε-amino groups in particular) with suitable aldehyde containing carbohydrates offers certain advantages. These advantages include ease and mildness of the conjugation procedure, as well as an unchanged ionic character of the conjugate with respect to the native protein carrier. Protein conjugation by reductive amination, including the coupling mechanism has been discussed earlier (Chapter 1).

The interest in evaluating the threshold sugar densities on glycoproteins for the expression of antigenicity (avidity effect), coupled with the success of phase transfer catalysis (PTC) in preparing subsituted aryl glycosides, prompted the attempt at preparing neoglycoproteins with defined sugar densities by reductive amination of various *p*-formyphenyl glycosides as a general strategy (Scheme 39). There have been no prior reports of the direct incorporation of an aldehyde functionality by

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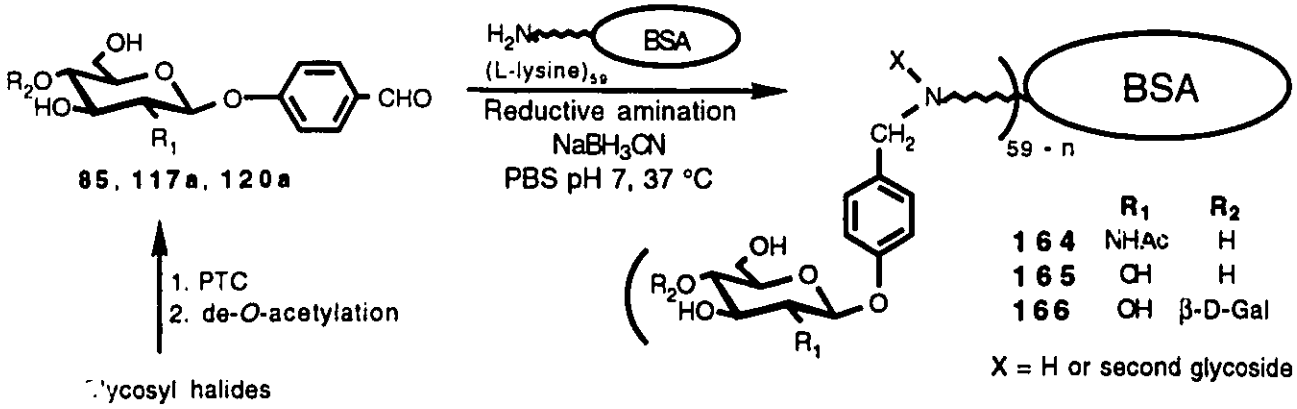
218 Previously described in part at the 15th International Carbohydrate symposium, Yokohama, Japan, August 12-17, 1990; and within R. Roy, F.D. Tropper, A. Romanowska, M. Letellier, L. Cousineau, S.J. Meunier and J. Boratynski, *Glycoconj. J.*, *8* , 75 (1991).

219 R. Bell and G. Torrigiani in *Towards Better Carbohydrate Vaccines* , R. Bell and G. Torrigiani (eds), John Wiley, London, 1987; W.E. Dick and M. Beurret in *Contributions to Microbiology and Immunology, Conjugate Vaccines* , Karger, Basel, vol. 10.

glycosylation of *p*-hydroxybenzaldehyde. The use of this aromatic aglycon has the advantage that the unprotected aldehyde function is compatible with a quick and mild PTC glycosylation procedure yet very reactive to amines to form reducible Schiff bases. Also, many carbohydrate binding proteins show enhanced binding affinities for aryl glycosides over non-aromatic analogues (Chapter 4).



Scheme 39 Neoglycoprotein synthesis by reductive amination with p-formylphenyl glycosides

Protein conjugations were performed at 37°C in PBS[220] with sodium cyanoborohydride ($NaBH_3CN$) as the reducing agent. *p*-Formylphenyl β-D-glycosides of *N*-acetylglucosamine (**85**), glucose (**117a**) and lactose (**120a**) were described earlier (Chapters 5 and 6). With these glycosides in hand, reductive amination of bovine serum albumin (BSA) was performed under different sets of molar reactant ratios (RCHO: NH_2: $NaBH_3CN$). Conjugation reactions were monitored as a function of time and aliquots were withdrawn at time intervals in order to obtain a series of neoglycoproteins having a wide distribution of sugar contents with different levels of antigenic expression. Carbohydrate contents were determined by the colorimetric phenol/sulfuric acid assay of Dubois and coworkers[221]

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[220] PBS is used as abreviation for 0.2M Phosphate Buffered Saline pH 7.0 as described earlier.

[221] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, 28, 350 (1956)

for the glucose and lactose conjugates **165** and **166**. In the case of the *N*-acetylglucosamine conjugates **164**, carbohydrate content was inferred from the number of modified L-lysine residues determined by amino acid analysis (Figure 35) BSA contains 59 L-lysine residues. It should be noted that the number of glucosamines coupled to BSA might be higher than the number of modified lysine groups since two aldehydes can react on each primary amine of the lysines. No other amino acids were modified by the conjugation procedure, and no protein denaturation was observed in the isolated conjugates.

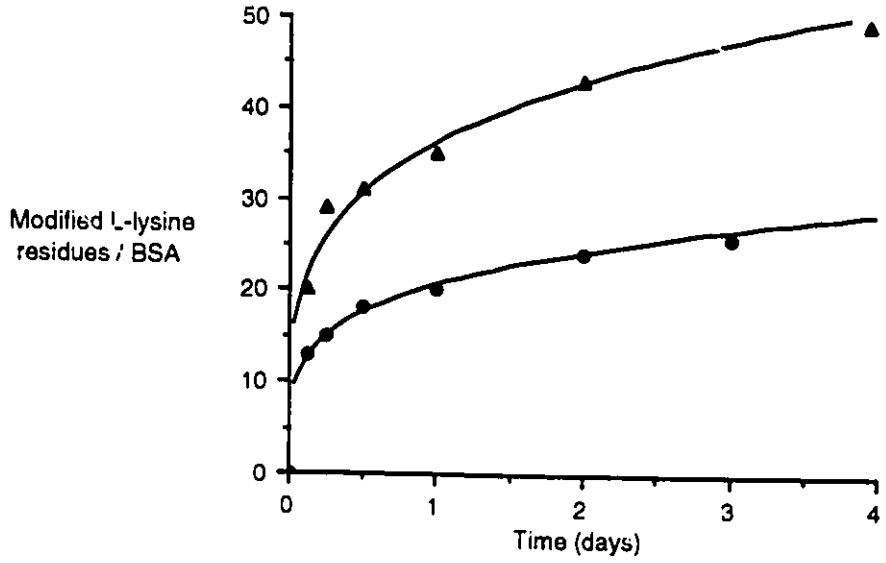

Figure 35 Time course of reductive amination of **85** with BSA in 0.2M PBS pH 7.0, 37°C. Ratio of **85**: L-lysine: $NaBH_3CN$ at 5:1:6 (▲) and at 2:1:2 (•).

Initial observations of the glucosaminide-BSA conjugates **164** clearly illustrate the effect of sugar content on the antigenicity of the neoglycoprotein conjugates. This was demonstrated by the graded intensities of the precipitin bands obtained with **164** and wheat germ agglutinin (WGA) by double radial diffusion in agarose gel (Figure 36). Conjugates obtained after 20 min., 1,2,4 and 6 hours, although known to contain some conjugated *N*-acetylglucosamine

residues (GlcNAc/ BSA ≤ 10), were not antigenic in these assays. At a concentration of 1 mg/mL, the GlcNAc-BSA conjugate **164** obtained after 12 hours (**58**: L-lysine : $NaBH_3CN$, 2:1:2, 18 GlcNAc/BSA) showed a weak precipitin band. However, the bands were very distinct at a GlcNAc/BSA ratio ≥ 20 (≥ 1 day). Interestingly, ELLA (not shown) appeared to be more sensitive in these assays since conjugates having low GlcNAc/BSA ratios (8 to12) still showed the capacity to bind to WGA. Further studies are in progress to evaluate the serological properties and extent of antigenicities of the conjugates by colleagues (Dr. A. Romanowska, Dr. R. Roy).

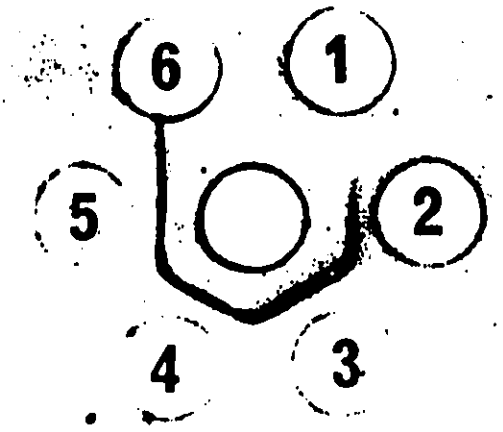

Figure 36 Agarose double radial diffusion of **164**. Middle wel: WGA; wells 1 to 5: conjugates (GlcNAc/BSA) obtained after 6 hrs. (15), 12 hrs. (18), 1 day (20), 2 days (24) and 3 days (26) respectively; well 6: BSA control.

In the case of the glucosyl and lactosyl conjugates **165** and **166**, the conjugation reaction was not monitored over time for hapten incorporation. In each of these two cases, two sets of different reactant ratios were used and the conjugates assayed for carbohydrate content after 90 hours at 37 °C. Molar ratios of 5:1:2 and 1:1:2 (glycoside : lysine : $NaBH_3CN$) were used to prepare conjugates **165** and **166** in PBS (10 mg BSA/mL PBS) at pH 7.0. The assayed carbohydrate hapten incorporations are given in Table 60. Hapten incorporations for trials performed under identical reaction conditions gave similar results for two different carbohydrate

derivatives. These results suggest that the general method and conditions described here should give similar results for other carbohydrate derivatives.

Table 60 Glycoside incorporation by reductive amination in BSA conjugates.

Reaction conditions*	Conjugates#	
glycoside : lysine : $NaBH_3CN$ (molar ratio)	**165** Glc/BSA	**166** Lac/BSA
5:1:2	47	41
1:1:2	15	6

* 90 hrs., 37 °C, 10 mg BSA/mL PBS pH 7.0.
\# Sugar/BSA ratios were determined colorimetrically by phenol/H_2SO_4 assay of Dubois *et al*[21].

The β-D-lactoside conjugates **166** showed similar behaviour to the GlcNAc-BSA conjugates during double diffusions assays performed against the *Ricinus Communis* and *Arachis Hypogea* lectins. A low amount of lactosyl residues (6) per BSA carrier was insufficient to agglutinate these lectins, when lectins and conjugates were used at concentrations of 1 and 2 mg/mL PBS in agar gel diffusion assays. Only the highly laden (Lac/BSA = 41) lactosyl conjugate **166** could agglutinate the lectins when BSA, a negative control, and an aryl lactoside-copolyacrylamide glycopolymer (**185a**), a positive control, acted accordingly in the assays (Figure 37).

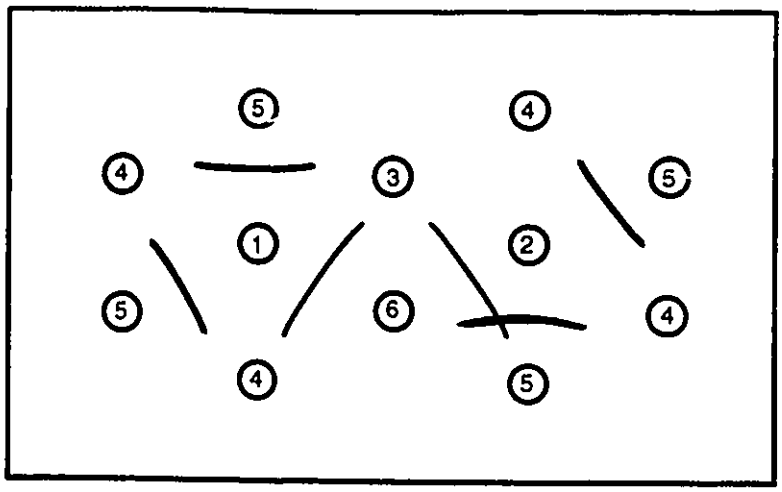

Figure 37 Scaled representation of a double radial diffusion assay for lactosyl conjugates **166**. Well 1: *Ricinus Communis* ; well 2: *Arachis Hypogea* ; well 3: BSA, (-) control; well 4: **166**, Lac/BSA = 6; well 5: **166**, Lac/BSA = 41; well 6: β-D-Lac-Ø-CPA **185**, (+) control.

The glucosyl conjugates **165** were not tested by immunodiffusion. Their preparation serve mainly to demonstrate the generality of the glycoconjugation strategy by reductive amination of *p*-formylphenyl glycosides prepared by PTC. The synthesis of **165** helped identify reaction condition limits for the reductive amination step. Thus, PTC represents an effective route toward the synthesis of *p*-formylphenyl glycosides which can then be efficiently coupled to proteins by reductive amination under neutral conditions. The strategy allows for the quick and easy preparation of neoglycoproteins which are antigenic toward lectins.

7.1 Experimental methods

General methods

General methods including protocols for double radial diffusion assays are as described earlier in previous chapters.

Direct conjugation of p-*formylphenyl glycosides to BSA to reductive amination*

Preparation of **164**

In two sets of experiments, bovine serum albumin (BSA, Sigma, Fraction V, 214 mg), the aldehyde **85** (300 mg) and sodium cyanoborohydride (66 mg) were homogenized and incubated at 37 °C in 0.2M sodium phosphate saline (PBS) pH 7.0 (10 mL). The molar ration of **85**:lysine-NH_2:$NaBH_3CN$ was therefore 5:1:6. Aliquots (1 mL) were withdrawn at time intervals and the reaction mixtures exhaustively dialyzed against distilled water, then freeze dried. Analysis of modified lysine residues was performed by amino acid analysis using the phenylisothiocyanate method (Mrs. C. Boucher, Bio-Mega Inc. Laval, Quebec). The reaction was repeated with a 2:1:2 molar ratio of **85**:lysine-NH_2:$NaBH_3CN$ at a BSA concentration of 10 mg/mL. In this case, GlcNAc/BSA ratios in the conjugates **164** were determined from UV absorbances measured at 271 nm and 280 nm, and standardized against analogous sialic acid conjugates characterized by the colorimetric resorcinol method[222].

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222 L. Svennehlom, *Biochim. Biophys. Acta.*, *24* , 601 (1957). See reference 218 for a complete description of the sialic acid neoglycoprotein conjugates prepared by the same method.

Preparation of **165** *and* **166**

In each of these two cases, two different reactant ratios were used, aliquots were however not taken at time intervals. For the conjugation of **117a**, the first molar ratio of **117a**:NH_2:$NaBH_3CN$ was 5:1:2 corresponding to 23 mg of **117a**, BSA (25 mg) and $NaBH_3CN$ (11.2 mg) in PBS (2.5 mL). In the second experiment, this ratio was1:1:2 (4.5 mg **117a**, 25 mg BSA, 11.2 mg $NaBH_3CN$). The conjugates were processed as before after 90 hours at 37 °C. The sugar content of **117a** in the first conjugate was 47 as measured by the phenol/sulphuric acid method, as described earlier. The second conjugate has 15 residues of **117a** per mole of BSA.

For the lactoside **120a** at a similar ratio of 5:1:2 and 1:1:2, the reaction mixtures contained 35.5 mg of **120a**, 25 mg BSA and 11.2 mg $NaBH_3CN$, and 7.1 mg of **120a**, 25 mg BSA and 11.2 mg $NaBH_3CN$ respectively in PBS (2.5 mL) at pH 7.0. The content of **120a** in the first conjugate was 41/BSA while it was 6/BSA in the second as determined by the colorimetric method described above.

8. Nitrophenyl glycosides as precursors to glycoconjugates[223]

Nitrophenyl glycosides have long been known to be useful chromogenic glycohydrolase substrates. Here, the value of nitrophenyl glycosides has been extended to their use as precursors for the synthesis of neoglycoprotein and glycopolymer conjugates. The strategy developed is of considerable value since many nitrophenyl glycosides are commercially availaole or accessed easily by phase transfer catalysed (PTC) glycosylation using peracetylated glycosyl halides. The strategy (Scheme 40) involves conversion of the aromatic nitro group into an acrylamide function followed either by copolymerization with suitable comonomers to give glycopolymers **167**, or conjugation to proteins and synthetic polypeptides by Michael addition of free amine residues to the acrylamide function to give neoglycoproteins **168** and poly-L-lysine polypeptide glycopolymers (Chapter 9). This strategy was used with great success to prepare a wide range of useful glycoconjugates ranging from mono- to tetra-saccharide derivatives with the corresponding nitrophenyl glycosides as precursors. Thus, immunogens as well as diagnostic antigens can be accessed from one compound, in this case, an acrylamidophenyl glycoside. The acrylamide to glycoconjugates strategy allows for glycopolymer mediated diagnosis and potential isolation of antibodies raised by the analogous immunogen. The implied vaccination/diagnosis strategy allows one to focus attention on antibodies which are directed solely against the carbohydrate hapten.

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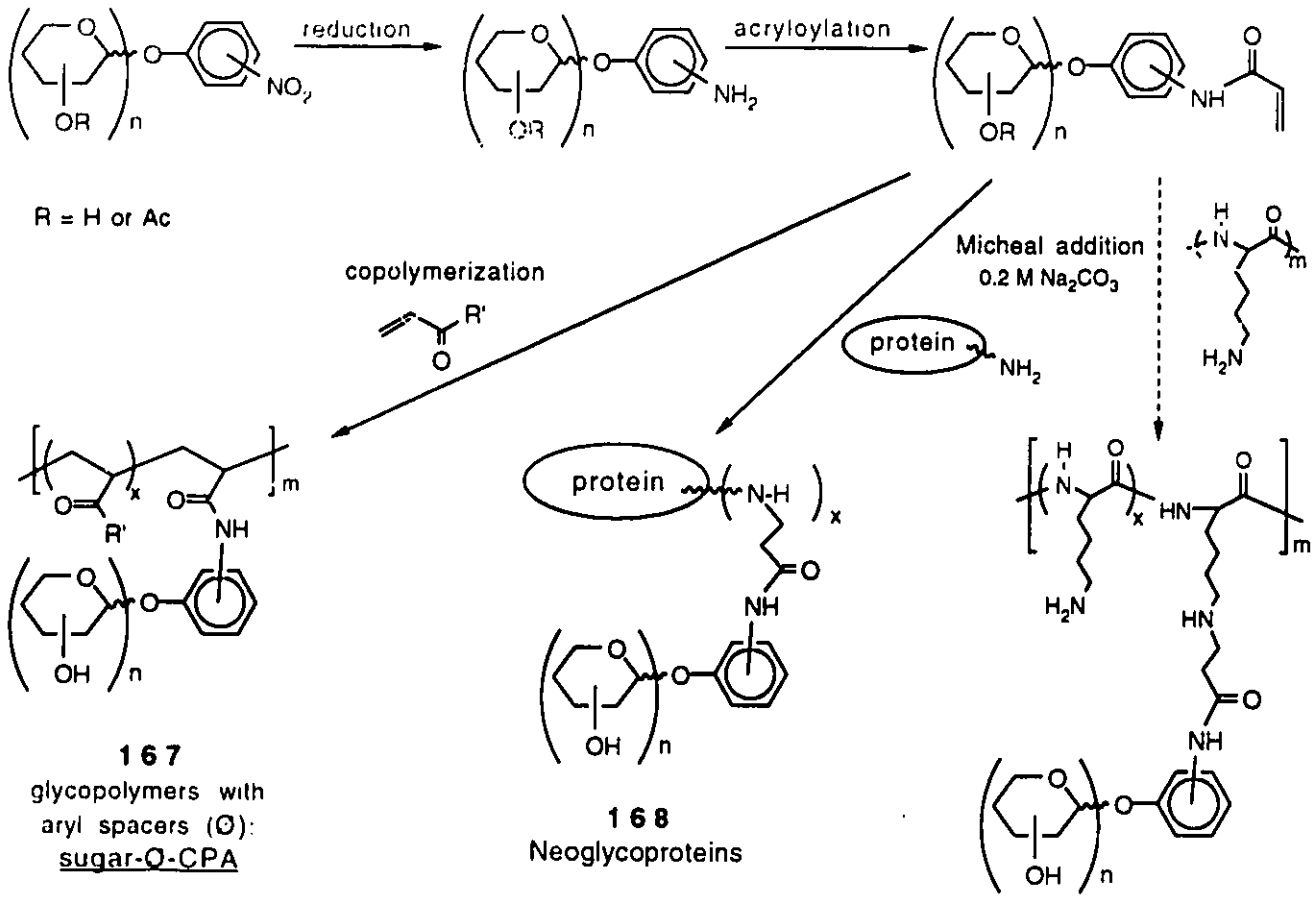
223 Previously introduced in part in a) R. Roy, F.D. Tropper, T. Morrison and J. Boratynski, *J. Chem. Soc. Chem. Commun.* , 536 (1991); b) R. Roy, F.D. Tropper and A. Romanowska, *Bioconjugate J.*, *3* , 256 (1992); c) R. Roy, F.D. Tropper, A. Romanowska, R.K. Jain, C.F. Piskorz and K.L. Matta, *Bioorg. Med. Chem. Lett.* (in press); d) R. Roy and F.D. Tropper, 15th International Carbohydrate Symposium, Yokohama, Japan, August 1992; e) F.D. Tropper and R. Roy, 74th Canadian Chemical Conference, Hamilton, Canada, August 1991.

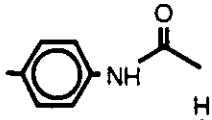
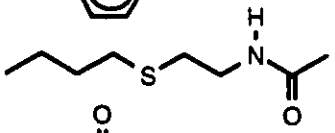
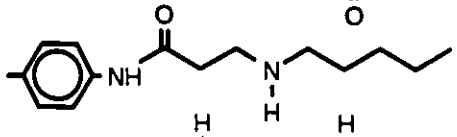
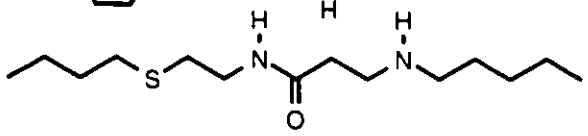


Scheme 40 General scheme for preparing diagnostic and immunogenic glycoconjugates from nitrophenyl-glycosides

Various different acrylate-type comonomers have proven to be compatible with *N*-acrylamidophenyl glycoside monomers for preparing aryl glycoside glycopolymers (abreviated hereon as sugar-Ø-CPA). The incorporation of a phenolic aglycon in this strategy has given access to glycopolymers with a spacer of intermediate length between those prepared earlier from allyl- and 3-(2-*N*-

acrylamidoethylthio) propyl-glycosides (Chapters 2 and 4). Thus, glycopolymers of varying spacer lengths, useful in studying binding site depths in carbohydrate receptor proteins, have been made available from popular carbohydrate precursors (Table 61).

Table 61 Glycopolymer spacers and associated extended lengths

*	Spacer	Lenghth (Å)
A		2.4
B		6.4
C		11
D		23
E		28

* Spacers A-C are associated with copolyacrylamide glycopolymers encountered thus far. Spacers D and E result in glycopolymers prepared by Michael addition of ε-amine groups of poly-L-lysine to the appropriate N-acrylamide-substituted-glycosides.

Neoglycoprotein conjugates prepared by Michael addition methodology (previously discussed in Chapter 4) have been prepared from various natural as well as synthetic protein carriers. Bovine serum albumin (BSA) and tetanus toxoid (TT) proteins have been used to prepare potentially useful immunogens. These two natural proteins were chosen as they are known to exhibit a large difference in immunogenic properties when injected in model animals. The properties and advantages of these immunogenic carriers have already been discussed in the first chapter (Section 1.5). Synthetic poly-L-lysine has also been employed as hapten carrier to provide a different type of glycopolymer with respect to the copolyacrylamide

versions. Poly-L-lysine conjugates and their properties will be discussed later in a separate chapter (Chapter 9).

The first part of this chapter will introduce the strategy as a general method with glycosyl, *N*-acetylglucosamine and lactosyl derivatives as models. The first section (8.1) will deal with the synthetic transformation of all nitrophenyl glycosides to acrylamidophenyl glycosides with specific emphasis on model carbohydrates. Following a description of the glycoconjugation work performed with model carbohydrates (section 8.2), work done with more complex derivatives will be discussed (section 8.3).

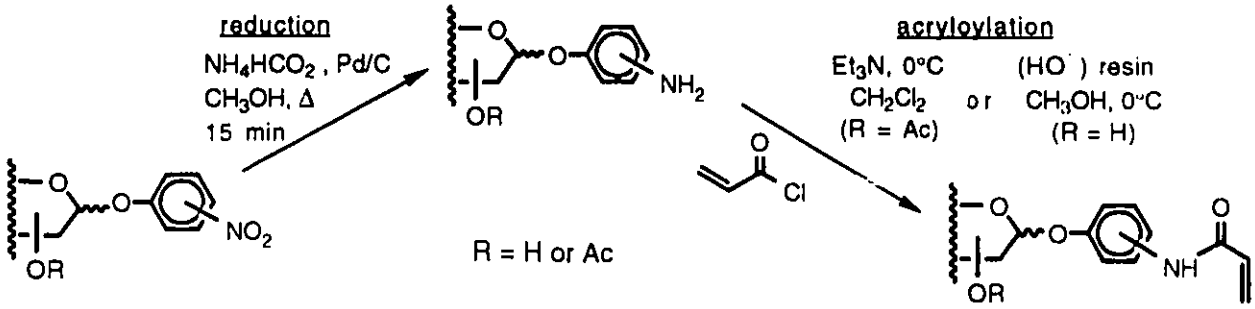
8.1 General conversion of nitrophenyl glycosides to corresponding acrylamides

The conversion of nitrophenyl glycosides into acrylamidophenyl glycosides has already been discussed for β-D-*N*-acetylglucosamine derivatives (Section 5.2.2). The procedure has been extended here to more carbohydrate derivatives to demonstrate the generality of the method.

The aromatic nitro group of aryl glycosides can be reduced quickly and easily to an amino group by mild catalytic transfer hydrogenation[224] in methanol using 5 or 10% palladium on charcoal (Pd/C), and ammonium formate as the hydrogen transfer agent. Reductions were usually complete within 10 minutes after mild heating. Conversions were usually quantitative by TLC, and high yields (94-98%) of pure isolated products were recovered. Complications, presumably in the form of incomplete reduction to give nitroso or hydroxylamine derivatives, have been observed in some trials and may be due to catalyst poisoning or an increase in pH

[224] S. Ram and R.E. Ehrenkaufer, *Synthesis* , 91 (1988).

due to liberated ammonia. Using a generous amount of Pd/C and an excess (5 equiv.) ammonium formate or the addition of 0.05% formic acid was shown to alleviate any complications. Once complete, filtration and evaporation of the reaction solution are the only necessary steps to obtain pure aminophenyl glycosides. Residual ammonium formate, if present, could be removed either by treatment with anion (HO^-) exchange resin to remove formate ions while ammonia is removed under vacuum, or by performing multiple freeze drying cycles from aqueous solutions of the non-acetylated products. A simple liquid/liquid extraction could be performed with acetylated versions. The reduction procedure was effective for both acetylated or unprotected glycosides and was successful on scales of a few milligrams to more than a gram.



Scheme 41 General synthetic scheme for preparing *N*-acrylamidophenyl glycosides from nitrophenyl glycosides.

Ortho- and *para-* nitrophenyl glycosides were reduced by this transfer hydrogenation procedure with the same ease. The reductions could be monitored effectively by TLC. All unprotected mono- and oligo-saccharide derivatives could be resolved effectively by TLC with $CHCl_3$/MeOH/H_2O (30/10/1, v/v/v) or MeCN/acetone/5% HOAc (5/3/2) which are very polar yet relatively rapid systems. Acetylated derivatives could be resolved with blends of ethyl acetate and hexane. In all cases, the amines were of lower Rf and became brightly coloured almost instantly after being wetted with

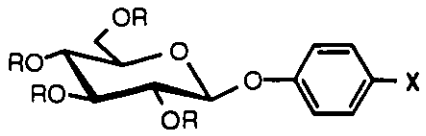
acidic cerium molybdate[225] when used as TLC developing reagent. This developing agent was found to be very sensitive to aminophenyl glycosides. *Ortho*-substituted derivatives turned bright orange while *para*-substituted produced a bright reddish-purple colour. In some trials, the glycosylated anilines were found to be sensitive to impurities and oxydation. When working on small scales or with complex oligosaccharides, it is suggested that the amines be acryloylated soon after they are prepared in order to minimize any possible losses.

N-Acryloylation of aminophenyl glycosides was performed with acryloyl chloride in the presence of a base at 0°C. Near quantitative yields of acrylamidophenyl glycosides were isolated when *N*-acryloylation of peracetylated derivatives were performed in CH_2Cl_2 with triethylamine as base, or in methanol with anion exchange resin (HO^-, Amberlite IRA-400) for unprotected glycosides. A slow, dropwise addition of acryloyl chloride is necessary to ensure a clean transformation. In this way, only one equivalent of acylating agent was necessary and no side products were obtained. These results mirror those observed during the synthesis of the analogous *N*-acetylglucosamine derivatives discussed previously (Section 5.2.2).

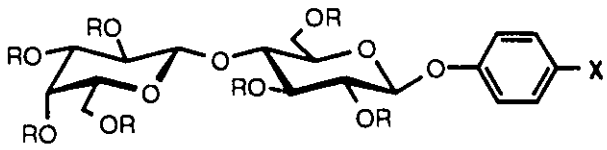
The tranformation of nitro- to *N*-acrylamido-phenyl glycosides using peracetylated glycosides offered practical advantages over the unprotected counterparts. Simple liquid-liquid extractions using volatile organic solvents could be used during work up steps while ion exchange processes had to be used in the case of non-acetylated derivatives. The first procedure proved to be manipulatively more facile for obtaining very pure compounds. Zemplén de-*O*-acetylation always gave the corresponding deprotected glycosides in quantitative yields.

---

[225] J.G. Kirchner, *Thin Layer Chromatography* , Interscience, NY, 1976, p. 151. 0.02M $(NH_4)_6Mo_7O_{24}$, 0.01 M $(NH_4)Ce(SO_4)_3$ in 10% H_2SO_4. Used routinely as TLC developing agent during synthesis. See experimental section.



	R	X
118	Ac	NO_2
170	Ac	NH_2
171	Ac	$NHCOCH{=}CH_2$
172	H	$NHCOCH{=}CH_2$



	R	X
121	Ac	NO_2
173	Ac	NH_2
174	Ac	$NHCOCH{=}CH_2$
121a	H	NO_2
175	H	NH_2
176	H	$NHCOCH{=}CH_2$

In the case of the lactosyl series, as in the case of *N*-acetylglucosaminides described earlier (5.2.), derivatizations were performed on both the acetylated and unprotected glycoside derivatives. High yields were obtained through both series (≥ 92%, Table 62). For the glucosides, the key conjugate precursor, p-*N*-acrylamidophenyl β-D-glucopyranoside **171**, was obtained via the peracetylated series due to the overall greater manipulative ease which protected derivatives offer. Yields, physical and NMR data (assigned by ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments) for the new glucosyl and lactosyl derivatives are given in Tables 62 through 65. As an example, 2-dimensional ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR spectra of **173** are also given in Figure 38.

Table 62 Synthesis and physical data for *p*-amino and *p*-N-acrylamido-phenyl β-D-glucosides and -lactosides **170 - 175**

Product	Yield (%)	Melting Point (°C)	$[\alpha]_D$ *	Formula	Elemental Analysis Calculated (%) C	H	N	Found (%) C	H	N
acetylated glycosides										
170	97	135.4-136.1 (EtOH)	-19.5° ($CHCl_3$)	$C_{20}H_{25}NO_{10}$	54.67	5.73	3.19	54.74	5.83	3.05
171	92	165.5-166.2 (EtOH)	-18.5° ($CHCl_3$)	$C_{23}H_{27}NO_{11}$	55.98	5.52	2.84	56.06	5.65	2.68
172	95	242.9-244.2 (EtOH)	-30.2° (DMSO)	$C_{15}H_{19}NO_7$	55.38	5.89	4.31	55.29	5.87	4.10
173	98	183.2-185.2 (EtOH/MeOH)	-15.0 °C ($CHCl_3$)	$C_{32}H_{41}NO_{18}$	52.82	5.68	1.92	52.73	5.50	1.75
174	95	117.8-118.7 (CH_2Cl_2)	-18.8 °C ($CHCl_3$)	$C_{32}H_{43}NO_{14}$	53.78	5.54	1.79	53.64	5.58	1.68
unprotected glycosides										
175	99	237.0-238.5 (EtOH) 270.2-271.5 (H_2O)	-24.2° (DMSO)	$C_{18}H_{27}NO_{11}$	FAB MS:			M + Na + Gly 1.2% M + Na 10.2% M + H 5.3%		
176	94	252.6-254.8 (MeOH)	-26.7° (DMSO)	$C_{21}H_{29}N_{12}$	51.74	6.00	2.87	51.97	5.99	2.76

* measured for 1% solutions

Table 63 ^{1}H-NMR data* of peracetylated *p*-amino and *p*-acrylamidophenyl glucosides and lactosides

	H-1 d ($J_{1,2}$)	H-2 dd ($J_{2,3}$)	H-3 dd ($J_{3,4}$)	H-4 dd ($J_{4,5}$)	H-5 ddd ($J_{5,6a}$)	H-6a dd ($J_{6a,6b}$)	H-6b dd ($J_{5,6b}$)	H-ortho d ($J_{o,m}$)	H-meta	X
170	4.88 (7.3)	5.29 - 5.31 (mult)		(9.6)	3.76 (4.9)	4.26 (12.3)	4.12 (2.4)	6.58 (8.6)	6.81	3.51 (br s, 2H, NH_2)
171	5.00 (7.7)	5.08 - 5.30 (mult)		(9.6)	3.81 (5.3)	4.26 (12.3)	4.13 (2.5)	6.93 (9.0)	7.48	7.3 (s, 1H, NH) 6.41(dd, $=CH_2$ trans) 6.20 (dd, -CH=) 5.74 (dd, $=CH_2$ cis) J_{cis} =10.0, J_{gem} =1.7 J_{trans} =16.8
173 Glc	4.85 (7.8)	5.10 (9.4)	5.23 (9.4)	3.85 (9.1)	3.68 (2.0)	4.47 (11.9)	4.07 (5.4)	6.57 (8.8)	6.79	3.51 (br, s, 2H, NH_2)
Gal	4.48 (7.8)	5.10 (10.4)	4.93 (3.4)	5.33 (1.0)	3.86 (mult. -)	4.01 - 4.14 (AB mult)				
174 Glc	4.98 (7.7)	5.14 (9.1)	5.25 (8.7)	3.86 (9.8)	3.75 (2.1)	4.47 (12.0)	4.07 (5.9)	6.93 (9.1)	7.48	7.20 (br, s, 1H, NH) 6.41 (dd, $=CH_2$ trans) 6.20 (dd, -CH=) 5.75 (dd, $=CH_2$ cis) J_{trans} =16.8, J_{cis} =10.2 J_{gem} =1.3 Hz
Gal	4.48 (7.8)	5.11 (10.4)	4.94 (3.4)	5.34 (1.0)	3.86 (mult. -)	4.01 - 4.14 (AB mult)				

*Chemical shifts δ are in ppm, coupling constants (J) are in Hz for solutions in $CDCl_3$ (300 MHz). Assignments are based on 2D ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments. Acetate methyl signals appear as singlets δ: 1.95 - 2.06 ppm.

Table 64 H-NMR data* for *p*-amino and *p*-acrylamido-phenyl β-D-glucosides and β-D-lactosides.

Product		H_1 d ($J_{1,2}$)	H_2 - H_{6b} (mult)	H_{ortho} d ($J_{o,m}$)	H_{meta} d	-CH= dd (J_{trans})	$=CH_2$ trans dd (J_{gem})	$=CH_2$ cis dd (J_{cis})
172		5.13 (7.5)	3.51 - 3.98	7.17 (9.0)	7.46	6.44 (17.1)	6.33 (1.7)	5.88 (9.8)
175	Glc	4.99 (7.9)	3.53 - 4.00	6.81 (9.0)	7.00	-	-	-
	Gal	4.47 (7.7)						
176	Glc	5.14 (7.8)	3.52 - 4.01	7.15 (9.1)	7.45	6.52 (17.0)	6.31 (1.7)	5.86 (9.1)
	Gal	4.48 (7.8)						

* Chemical shifts δ are given in ppm relative to TMS and coupling constants (J) are given in Hertz for solutions in D_2O (referenced to internal acetone δ = 2.216 ppm).

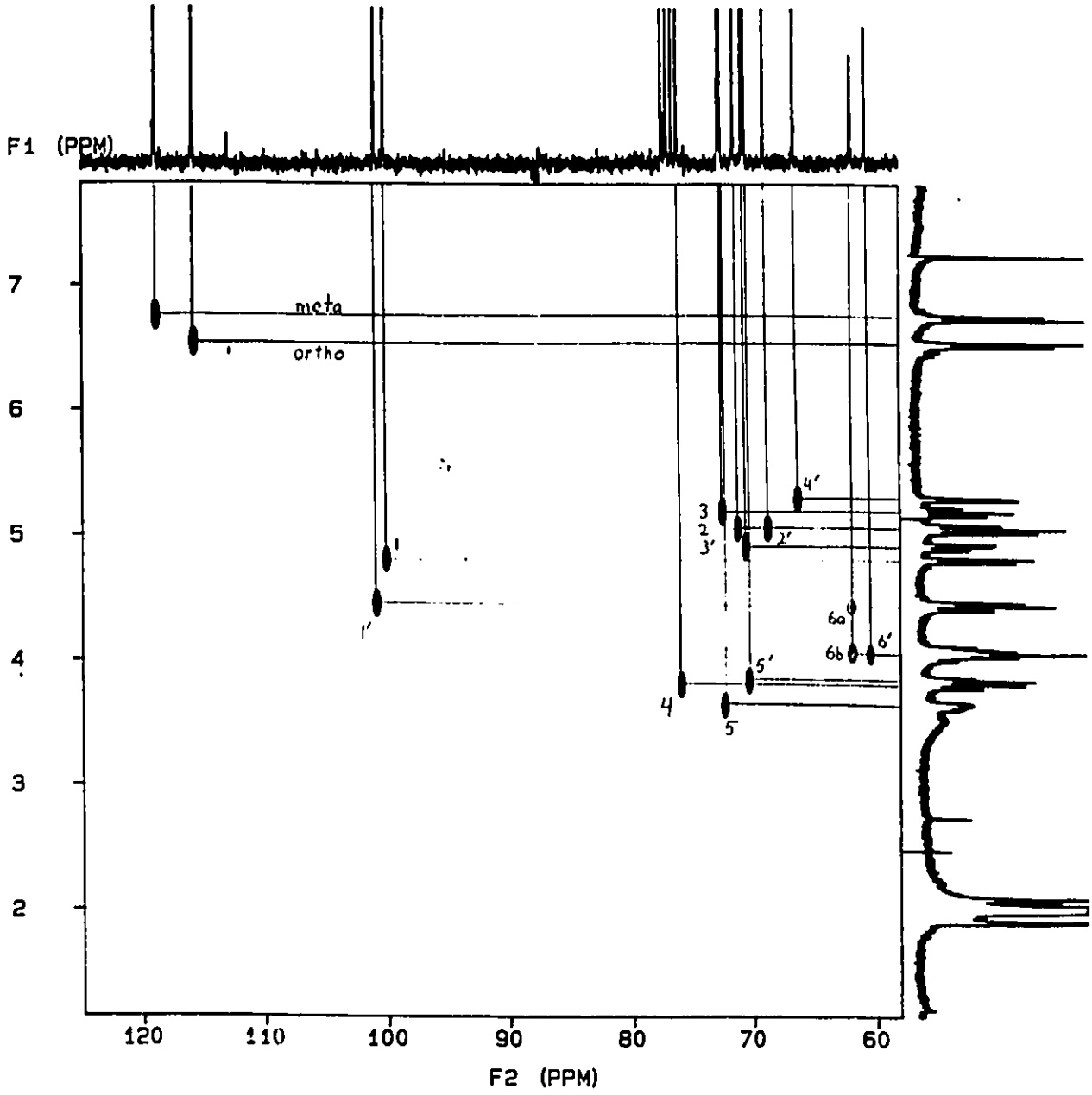
Table 65 ^{13}C-NMR data* for *p*-amino and *p*-acrylamido-phenyl β-D-glucosides and -lactosides.

Product		C_1	C_2	C_3	C_4	C_5	C_6	C_{ipso}	C_{ortho}	C_{meta}	C_{para}	X
Acetylated glycosides δ $CDCl_3$												
170		100.4	71.0	71.6	68.1	72.5	61.7	149.6	115.7	118.8	142.7	-
171		99.4	71.0	71.9	68.1	72.6	61.8	153.6	117.6	121.6	133.6	163.8 C=O 131.1 -CH= 127.8 $=CH_2$
173	Glc	100.2	71.6	72.8	76.2	72.7	62.0	149.6	115.7	118.8	142.4	-
	Gal	101.0	69.1	70.9	66.6	70.7	60.8					
174	Glc	99.1	71.4	72.8	76.2	72.7	62.0	153.4	117.5	121.3	133.2	163.2 C=O
	Gal	101.0	69.1	70.9	67.0	70.7	60.8					130.9 -CH= 127.8 $=CH_2$
Free glycosides δ $DMSOd_6$												
172		100.7	73.2	76.6	69.7	76.9	60.7	153.3	116.4	120.4	133.1	162.5 C=O 131.9 -CH= 127.8 $=CH_2$
175	Glc	101.6	74.8	75.6	80.4	74.9	60.3	148.6	114.5	117.7	142.5	-
	Gal	103.8	70.6	73.3	68.2	73.1	60.5					
176	Glc	100.2	74.8	75.5	80.1	74.8	60.4	153.2	116.4	120.4	133.2	162.6 C=O
	Gal	103.7	70.5	73.2	68.1	72.9	60.1					131.8 -CH= 126.3 $=CH_2$

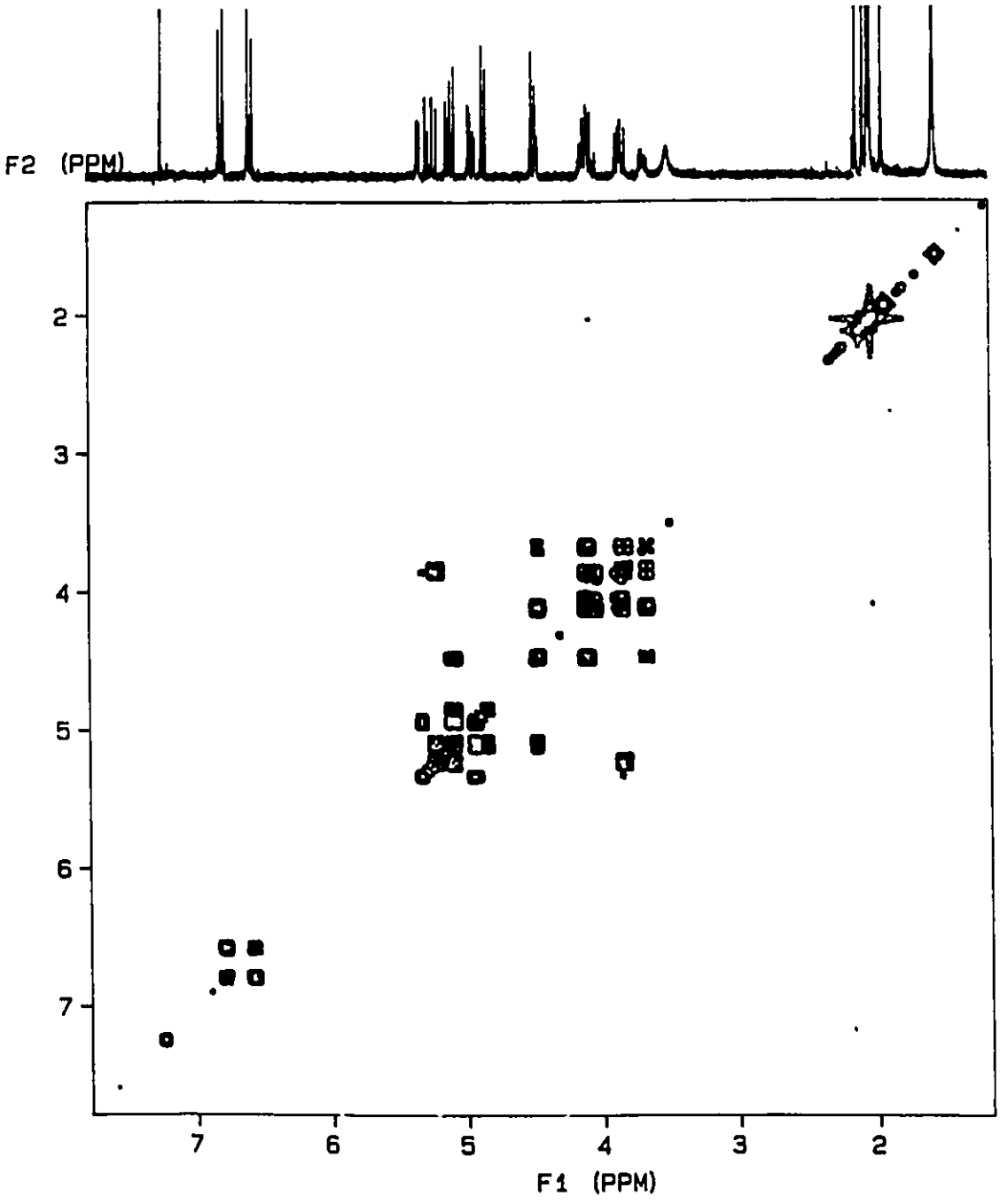
* Chemical shifts are in ppm relative to TMS and were recorded at 75.4 MHz for solutions in $CDCl_3$ (ref.77.00 ppm) for **170 - 174** : C=O @ 168.9 - 170.7 ppm, CH_3 @ 20.2 - 20.5 ppm; or for solutions in $DMSOd_6$ (ref. 39.50 ppm) for **172, 175** and **176.**

Figure 38 2-Dimensional correlation spectra of **173** performed for $CDCl_3$ solutions (Varian XL-300). **a)** ^{1}H-^{13}C HETCOR **b)** full scale ^{1}H-^{1}H COSY **c)** COSY expansion (3.5-5.5 ppm region).

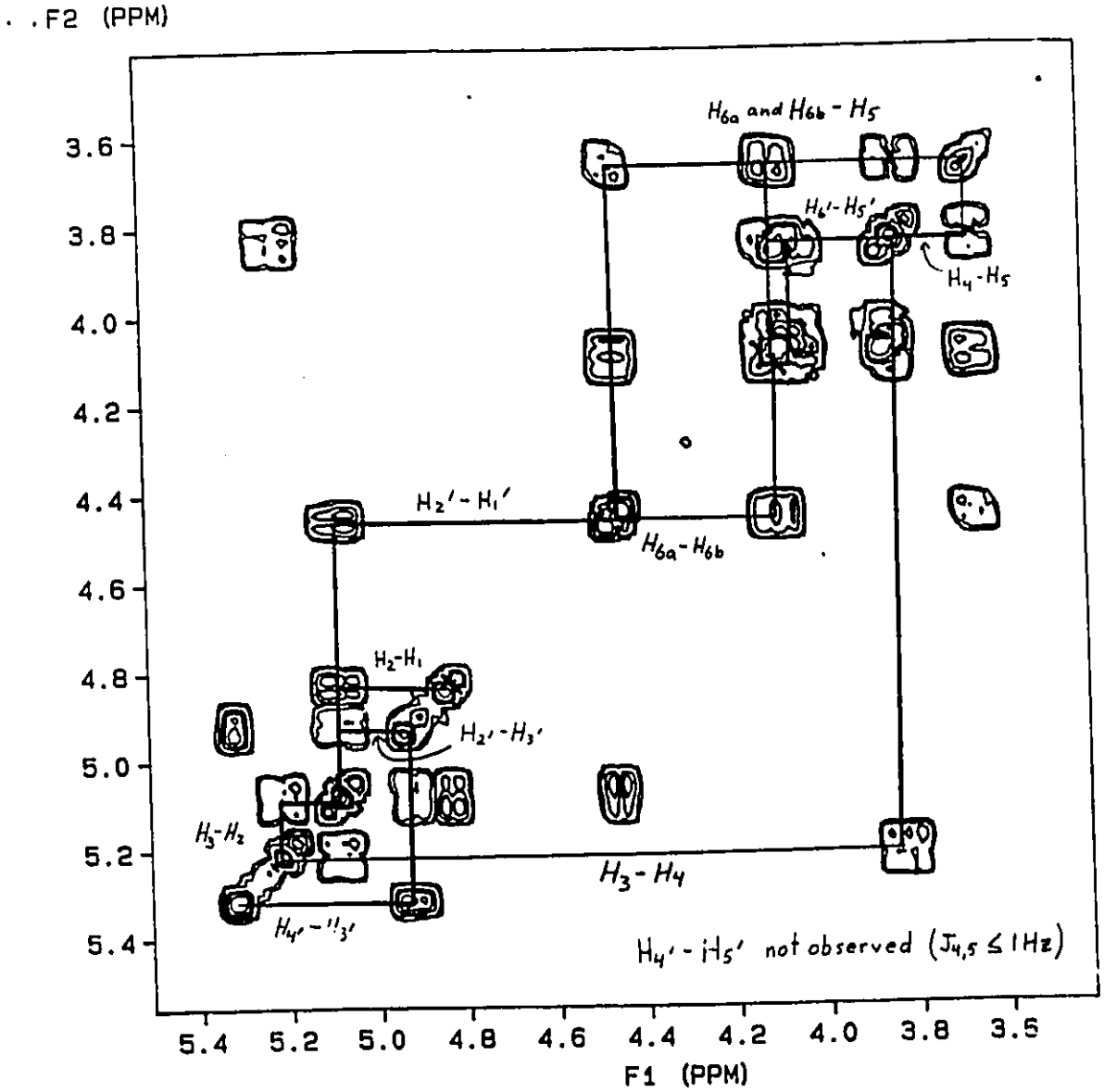
A)



B)



C)


. . F2 (PPM)
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4
H6a and H6b - H5
H6'-H5'
H4-H5
H2'-H1'
H6a-H6b
H2-H1
H2'-H3'
H3-H2
H3-H4
H4'-H3'
H4'-H5' not observed (J4,5 ≤ 1Hz)
5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.5
F1 (PPM)

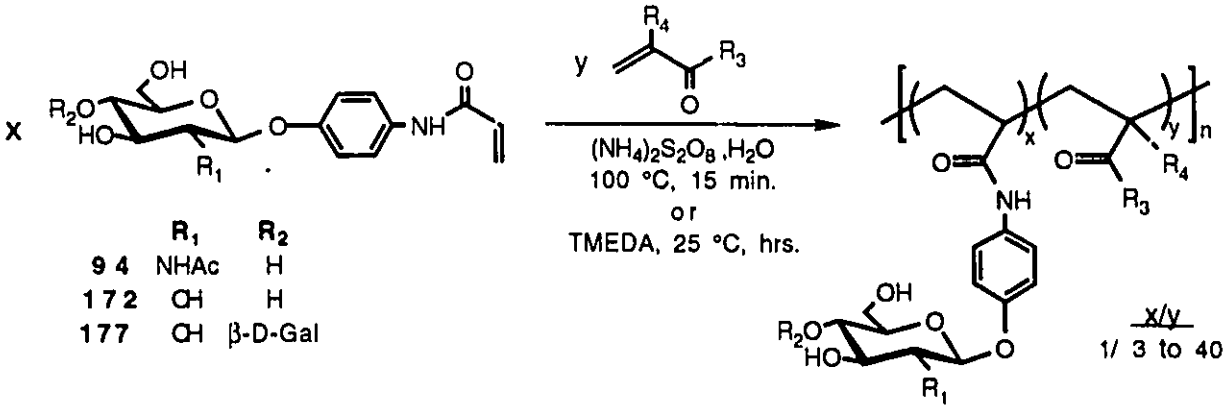
8.2 General glycosylation strategies employing *N*-acrylamidophenyl glycosides

The use of *N*-acryloylated carbohydrates as precursors to glycoconjugates has distinct advantages over previously reviewed methodologies since the conjugated acrylamide function offers two synthetic possibilities as originally proposed by this author and coworkers[222]. First, they can be efficiently copolymerized with acrylate-type monomers to give randomly alternating water-soluble glycopolymers and second, amine groups of proteins or functionalized polymers can be used as nucleophiles to give glycoconjugates by Michael addition (Scheme 40). A general description of glycopolymer and neoglycoprotein conjugate synthesis will be discussed separately with emphasis on work done with p-*N*-acrylamidophenyl β-D-glucoside, -*N*-acetylglucosaminide and lactoside models.

8.2.1 Glycopolymers from *N*-acrylamidophenyl glycosides. β-D-glucose, -*N*-acetylglucosamine and -lactose models.

The copolymerization of *N*-acrylamidophenyl glycosides with acrylate-type comonomers occurs with the same level of effectiveness as described earlier for 3-(2-*N*-acrylamidoethylthio) propyl glycosides (Chapter 4). Good yields (50-95%) of aryl-glycoside glycopolymers were obtained with a minimal investment of time and effort. As before, the polyvalent antigens produced here were purified and isolated easily by dialysis and freeze drying to give white, spongy solids containing typically between 9 and 15% of retained water. The randomly alternating glycopolymers were shown to have molecular weights of the same order as those described earlier (~100 kDa) when measured by gel permeation chromatography or by diffusion rates through agarose gels relative to known protein

and glycopolymer standards. Radical initiated polymerizations were effected using ammonium persulfate ($(NH_4)_2S_2O_8$) with heat (100°C, 15 min.) or *N,N,N',N'*-tetramethylethylenediamine (TMEDA) at room temperature for a few hours. In both cases, polymerizations proceeded smoothly and gave identical monomer incorporation ratios in the isolated glycopolymers. To properly remove TMEDA from glycopolymers, an initial dialysis in 5 mM acetic acid was required.



	R_1	R_2
94	NHAc	H
172	OH	H
177	OH	β-D-Gal

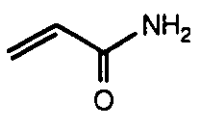
		R_2	R_1	R_3	R_4
β-D-GlcNAc-Ø-CPA	**181**	NHAc	H	NH_2	H
β-D-GlcNAc-Ø-CPMA	**182**	NHAc	H	NH_2	CH_3
β-D-GlcNAc-Ø-CPHEMA	**183**	NHAc	H	OCH_2CH_2OH	CH_3
β-D-Glc-Ø-CPA	**184**	OH	H	NH_2	H
β-D-Lac-Ø-CPA	**185**	OH	β-D-Gal	NH_2	H

Scheme 42 Synthesis of aryl glycoside glycopolymers

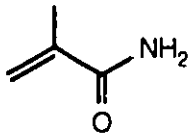
As described earlier for other glycopolymers generated from *N*-acryloylated carbohydrate derivatives, the monomer ratios incorporated within the glycopolymers were found to be almost identical to the load ratio. In fact, glycoside incorporation was sometimes found to be slightly superior than expected when lower amounts of co-acrylate monomers were used (Table 66; glycoside/co-acrylate:1/5, 1/10). This could suggest that *N*-aryl acrylamides might be slightly more reactive than the other, simpler

non-aromatic comonomers. Glycopolymer compositions were determined by elemental analysis and/or ^{1}H-NMR spectroscopy as described earlier. In these aryl glycoside glycopolymers however, the aromatic proton resonances in the ^{1}H-NMR spectra (D_2O) provide a second reporter group removed downfield (7.0 ppm, br, H_{ortho}; 7.5 ppm, br, H_{meta}) from the sugar resonances (~5 ppm, anomeric; 3.4 - 4.0 ppm, ring H) and the backbone signal (1.4 - 2.4 ppm). *N*-Acetyl groups were again found as relatively sharp singlets (2.0 ppm) while much of the remaining ^{1}H-NMR spectrum was composed of broadened signals similar to those encountered earlier with glycopolymers prepared from allyl glycosides (Chapter 2). To demonstrate the generality of the glycopolymerization strategy beginning from popular nitrophenyl glycosides, p-*N*-acrylamidophenyl glycosides of β-D-glucose **172**, β-D-*N*-acetylglucosamine **94** and β-D-lactose **176** were used to generate a wide range of glycopolymers (Scheme 42, Table 66).

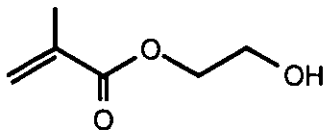
Glycopolymers with varying sugar densities were prepared with various acrylate-type comonomers to study the serological properties of these new glycoconjugates. The ability to synthesize antigenic glycopolymers from different acrylate comonomers could potentially prove useful in the custom design of glycopolymers having certain desired properties predetermined by the comonomer chosen. The comonomers chosen for this study are given below.



177

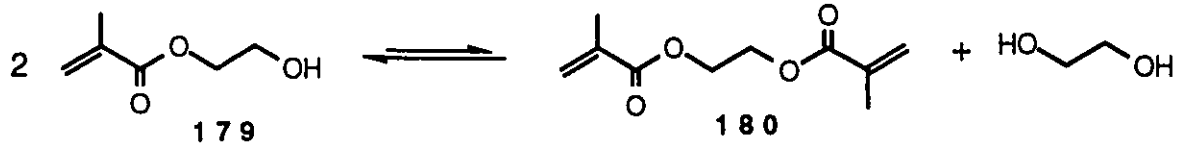


178



179

Copolyacrylate glycopolymers of β-D-*N*-acetylglucosamine (β-D-GlcNAc-Ø-copolyacrylate **181-183**) were synthesized using acrylamide **177** (β-D-GlcNAc-Ø-CPA, **181**), methacrylamide **178** (β-D-GlcNAc-Ø-CPMA, **182**), and hydroxyethyl methacrylate, HEMA, **179** (β-D-GlcNAc-Ø-CPHEMA, **183**). HEMA was chosen as a model comonomer because it gives water-soluble polymers, and has been approved to prepare human compatible surfaces for use in dentistry, opthamology and implants[226]. The copolyacrylamide (CPA) and copolymethacrylamide (CPMA) glycopolymers were produced without any difficulty. In the case of CPHEMA glycopolymers however, insoluble, crosslinked material was produced in addition to soluble β-D-GlcNAc-Ø-CPHEMA. This crosslinking is most likely due to the presence of bifunctional 2-methacryloxy ethyl methacrylate **180** present in the commercial sample (Aldrich). This by-product is formed by transesterification and can be difficult to remove properly prior to use. Soluble CPHEMA glycopolymer**183** was separated from insoluble material by simple filtration of the aqueous solution obtained after dialysis of the crude glycopolymers.



[226] a) M. Stol, *J. Bioact. Compat. Polym.* *6* , 308 (1991).;b) G.W. Shaw, D.J. Claremont and J.C. Pickup, *Biosens. Bioelectron.*, *6* , 401 (1991); c) J.M. Anderson and Q.H. Zhao, *MRS Bull.*, *16* , 75 (1991); and references cited within each.

Table 65 Aryl glycoside copolyacrylate glycopolymers.

Product	Glycopolymer name	% Yield (lyophilized)#	Molar ratio Sugar / co-acrylate		H_2O content (% by wt)†
			Load	Found *	
181a	β-D-GlcNAc-Ø-CPA	6	1:5	1:3	-
181b		25	1:10	1:6.2	14.0
181c		20	1:10	1:7.2	14.9
181d		97	1:20	1:20	11.9
181e		54	1:20	1:18	9.8
181f		81	1:40	1:38	8.6
182a	β-D-GlcNAc-Ø-CPMA	58	1:10	1:9	19.5
182b		54	1:20	1:20	10.2
183a	β-D-GlcNAc-Ø-CPHEMA	70	1:10	1:9.3	~0
183b		41	1:20	1:17.6	~0
184	β-D-Glc-Ø-CPA	76	1:10	1:8	2.9
185a	β-D-Lac-Ø-CPA	65	1:10	1:10	8.6
185b		65#	1:10	1:10	-

* Determined by elemental analysis and/or ^{1}H-NMR.
† Determined by elemental analysis.
# Polymerized in H_20 with $(NH_4)_2S_20_8$ as initiator at 100°C for 15-20 min. except for **185b** which was performed at 25°C in the presence of TMEDA. Total monomer concentration used was always 0.8M.

The polymerization conditions used produced glycopolymers with molecular weights comparable to those of lectins when considering their relative mobilities through agarose gel during immunodiffusion assays. Gel permeation chromatography (GPC) was performed on β-D-Lac-Ø-CPA **185a** to establish the true molecular weight and polydipersity of an average aryl glycoside glycopolymer. An HPLC chromatogram of **185a** performed on a TSK G4000 SWXL column (Figure 39) showed a rather narrow molecular weight distribution centered at around 120 kDa.

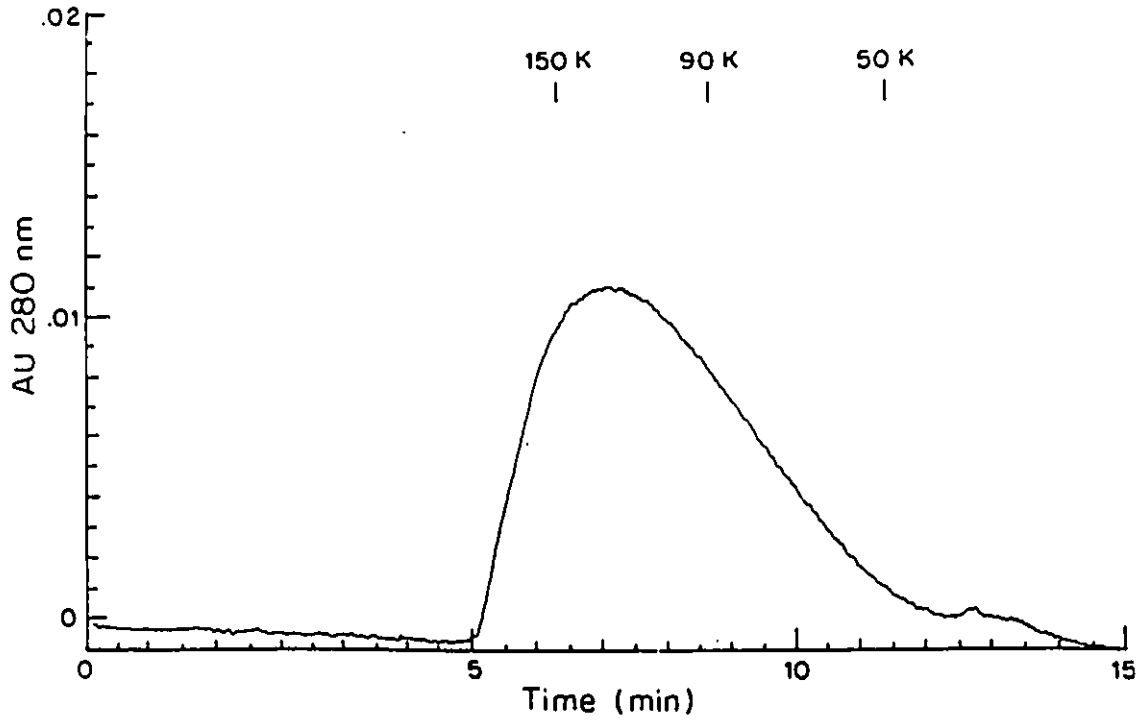


Figure 39 HPLC profile of **185a** on a TSK G4000 SWXL column in 0.1M phosphate buffer, pH 7.0. Bars indicate the molecular weight of polyacrylamide standards.

The homogeneity of the glycopolymer structures was established by ^{13}C-NMR spectroscopy. Glycopolymer spectra showed a single set of relatively sharp resonances for most of the carbohydrate hapten residues. The aromatic ortho carbon as well as the anomeric carbon attached to the phenyl ring were sharp but slightly broadened when compared to the other carbohydrate resonances. The carbohydrate hapten resonances all appeared at positions similar to those found in the analogous *p*-aminophenyl glycosides. The backbone methine and methylene resonances appeared in their usual positions as multiple broad peaks near 43 and 35 ppm., respectively. As an example, the ^{13}C-NMR spectrum of β-D-Lac-Ø-CPA **185a** is given in Figure 40.

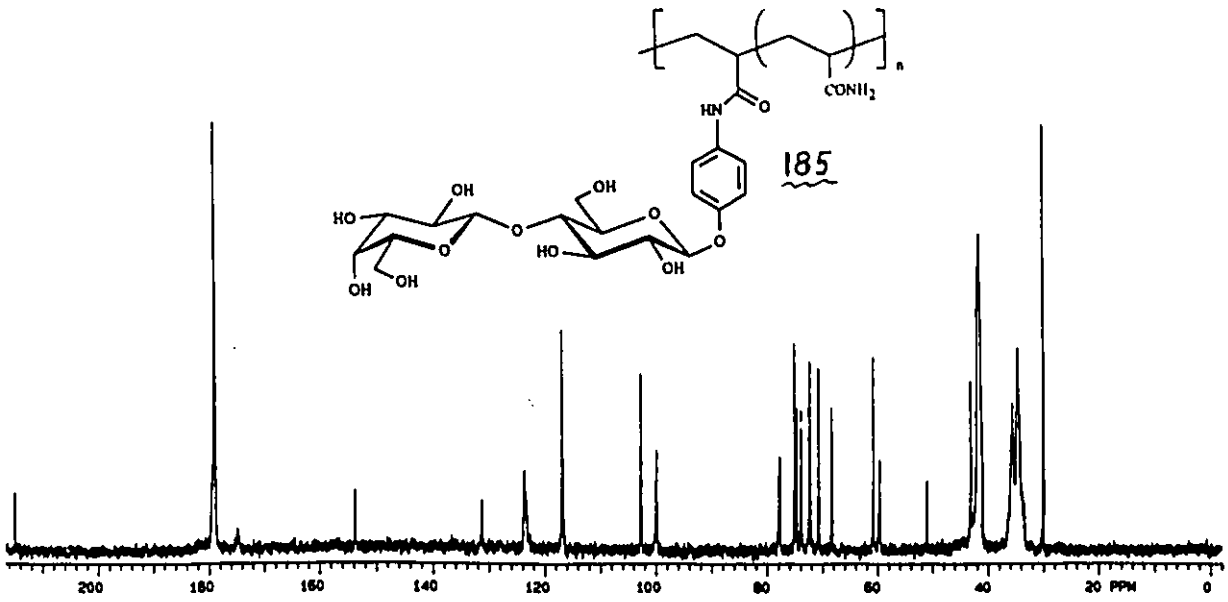


Figure 40 ^{13}C-NMR spectrum (75.4 MHz) of β-D-Lac-Ø-CPA **185a** in D_2O at 37°C. Resonances for internal acetone standard appear at 31.1 ppm (CH_3) and 217 ppm (C =O).

Double radial diffusion assays through agarose gel were performed with lectins to test the antigenicity of the new glycopolymers derived from aryl glycosides. Except for the glucose glycopolymers (**184**) which could not be tested for lack of an appropriate lectin, all glycopolymers proved to be antigenic towards specific lectins. For the β-D-*N*-acetylglucosamine glycopolymers **181-183**, sharp and intense precipitin bands were formed when assayed against Wheat Germ Agglutinin. Bands of graded intensities reflecting sugar content on the glycopolymers were clearly observed when assays were performed using sample concentrations of 0.5 and 1 mg/mL (Figure 41). No difference was perceptible by radial diffusion between similar laden glycopolymers made from different comonomers (β-D-GlcNAc-Ø-CPA, -CPMA, -CPHEMA). A photograph of

this assay is given in Figure 42.

These glycopolymers were also found to be good coating antigens during ELLA experiments with WGA. The β-D-GlcNAc-Ø-CPA glycopolymers were found to be as sensitive as the analagous β-D-GlcNAc-**S**-CPA glycopolymers introduced earlier (**50-53**). These results are discussed later in Chapter 11. A complete evaluation of all aryl β-D-*N*-acetylglucosamine glycosides is in progress[227] and results will be presented elsewhere in due course.

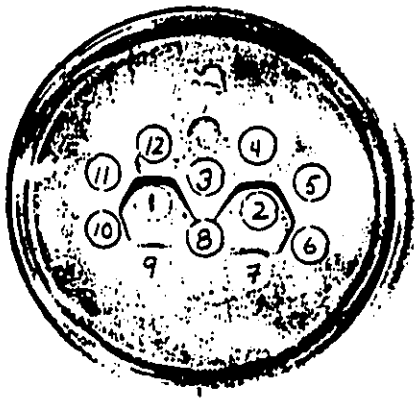


Figure 41 Agar gel diffusion assay of WGA (wells 1 and 2) against β-D-GlcNAc-Ø-CPA glycopolymers **181**. Well 3: β-D-GlcNAc-**S**-CPA **52**, (+) control; wells 4 and 12: **181a** (1:3); wells 5 and 11: **181c** (1:7); wells 6 and 10: **181e**; wells 7 and 9:**181f** (1:38); well 8: β-D-Lac-Ø-CPA **185a**, (-) control. Wells 2-8 (right): 1 mg;mL, wells 1, 9-12 (left): 0.5 mg/mL.

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[227] Dr. A. Romanowska and Dr. R. Roy, Department of Chemistry, University of Ottawa.

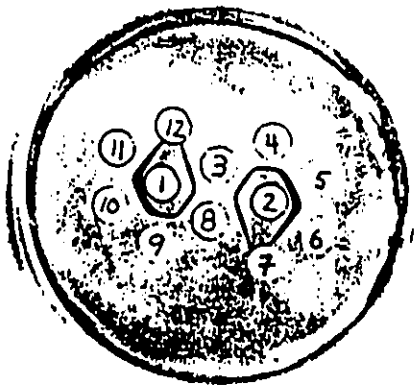

Figure 42 Agar gel diffusion assay of WGA (wells 1 and 2) against β-D-GlcNAc-Ø-glycopolymers **181-183**. β-D-GlcNAc-Ø-CPHEMA: well 3: **183a** (1:10); well 8: **183b** (1:20); β-D-GlcNAc-Ø-CPMA: well 5: **182a** (1:10); well 10: **182b** (1:20); β-D-GlcNAc-Ø-CPA: well 6: **181c** (1:7); well 11: **181e** (1:18). Wells 4 and 9: (+) control, wells 7 and 12 : (-) controls (see Figure 41).

The β-D-Lac-Ø-CPA glycopolymers (**185**) behaved as expected during double diffusion assays with lectins. Glycopolymers **185a** and **b** showed intense precipitin bands with the galactose specific lectin from *Ricinus Communis* (castor bean) and the lactose-specific lectin from *Arachis Hypogaea* (peanut) while no binding interaction could be observed with WGA which is specific for sialic acid and *N*-acetylglucosamine. The strong affinity of β-D-Lac-Ø-CPA for the peanut lectin was demonstrated in a quantitiative precipitation experiment (Figure 43). In this assay, close to 30 μg of **185b** was sufficient for maximum agglutination of only 20 μg of lectin from a solution volume of 300 μL (see experimental).

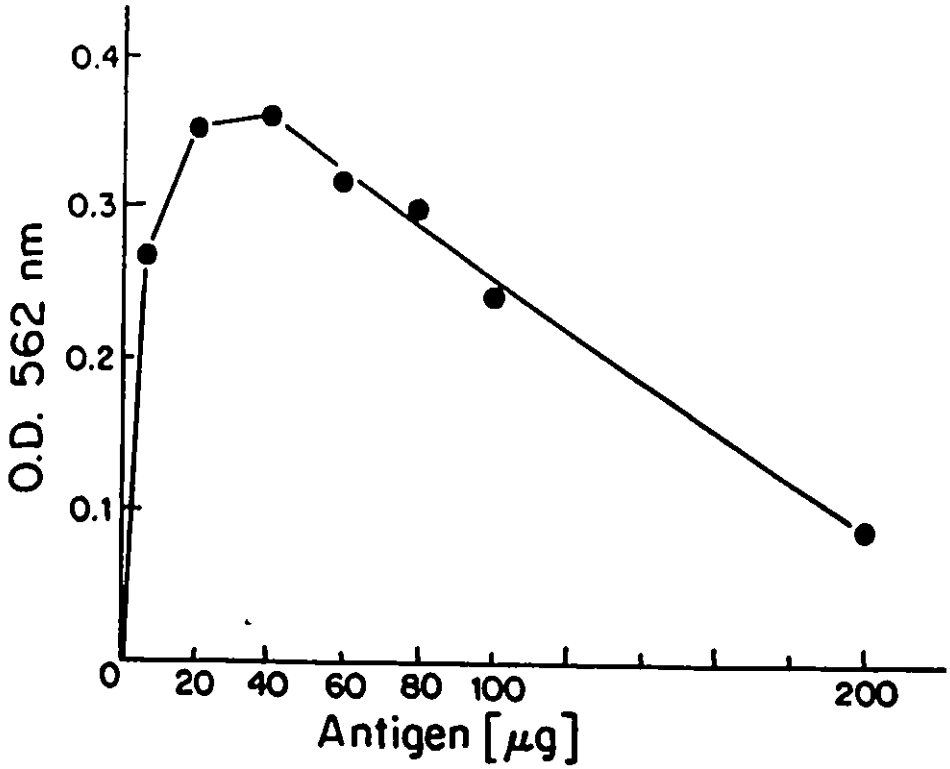

Figure 43 Quantitative precipitin curve of β-D-Lac-Ø-CPA **185b** with the *Arachis Hypogaea* lectin 20 μg in 300 μl PBS, pH 7.0.

The ability of aryl glycoside glycopolymers to serve as coating antigens in solid phase enzyme binding assays was also investigated for β-D-Lac-Ø-CPA **185b**. Two-fold serial dilutions of **185b** in PBS were used to coat the wells of microtiter plates. After standard manipulations, direct binding of horseradish peroxidase (HRP) - labeled peanut lectin was demonstrated using ABTS/H_2O_2 as enzyme substrates as described earlier (Chapters 4 and 5). With a pre-established lectin concentration, the optical density (O.D,) of the oxidized enzyme substrate at 410 nm could be directly correlated with antigen-polymer concentrations. The β-D-Lac-Ø-CPA glycopolymer **185b** at1 μg/well in the above ELLA assay was shown to be as sensitive as protein conjugates could be in similar assays. The high affinity of the multivalent glycopolymer **186b** towards peanut lectin was demonstrated by requiring as little as 12 ng/well for detection (O.D. = 0.4) of the peanut lectin-HRP conjugate (Figure 44).

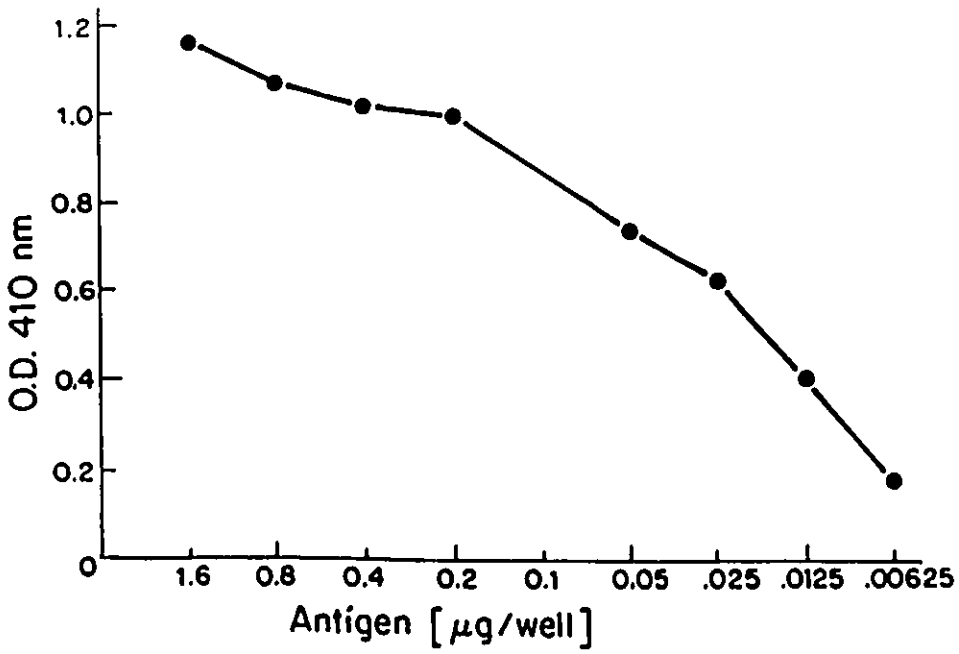

Figure 44 Enzyme linked lectin assay (ELLA) of β-D-Ø-CPA **185b** with HRP-labeled *Arachis Hypogaea* using ABTS as enzyme substrate.

Lactose was chosen as a good ligand model because it is an important hepatocyte[228] and human colorectal carcinoma lectin[229] receptors. The glucose and *N*-acetylglucosamine glycopolymers have primary uses in lectin binding studies. In conclusion, water-soluble aryl glycoside glycopolymers have shown to be very selective antigens in lectin model studies. The method is general and begins from popular or commercially available nitrophenyl glycoside precursors. The application of this strategy has been applied to other biologically relevent carbohydrates and will be presented later in this chapter.

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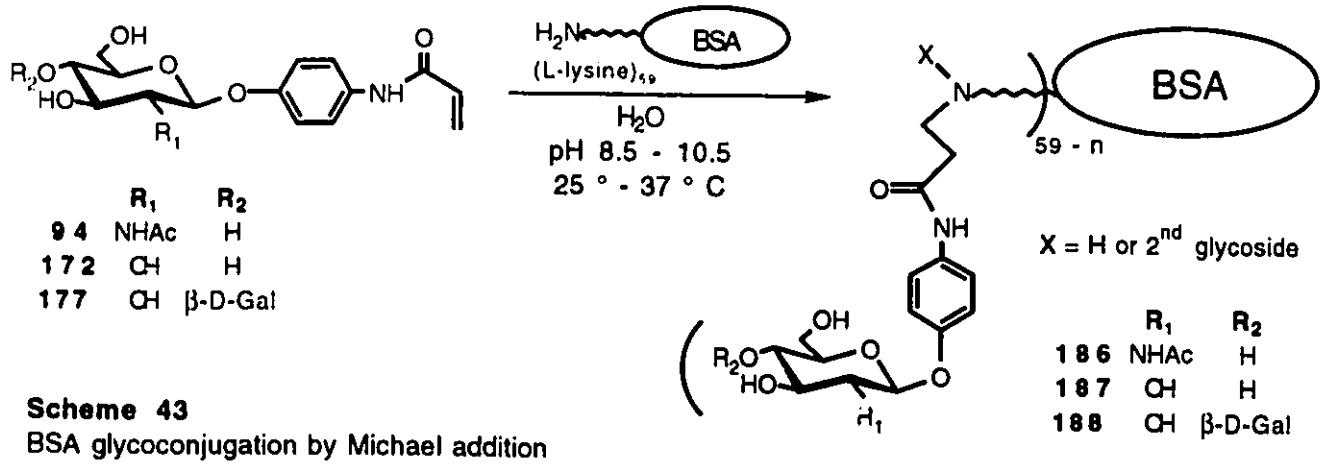
[228] a) R.L. Hudgin, W.E. Pricer Jr. and G. Ashwell, *J. Biol. Chem.*, *2497* , 5336 (1974); b) R.T. Lee and Y.C. Lee, *Glycoconjugate J.*, *4* , 317 (1987).

[229] R. Lotan, Y. Matsushita, D. Ohannesian, D. Carralero, D.M. Ota, K.R. Cleary, G.L. Nicolson and T. Imura, *Carbohydr. Res.* , *213* , 47 (1991).

8.2.2 Neoglycoproteins from *N*-acrylamidophenyl glycosides. β-D-Glucose, -*N*-acetylglucosamine and -lactose models.

N-acrylamidophenyl glycosides have been shown to be effective precursors to neoglycoproteins through coupling by Michael addition of protein-amine functions (ε-lysine group in particular) to the conjugated acryloyl function. The method has been used to attach a variety of glycosides to various proteins. The method has many advantages over existing methologies. As described earlier (Chapter 4), the method of Michael addition of protein-amine groups to acryloylated carbohydrate derivatives is mild, selective and efficient. The acryloylated carbohydrate precursors are stable reagents and require no additional reagents for coupling.



	R_1	R_2
94	NHAc	H
172	OH	H
177	OH	β-D-Gal

	R_1	R_2
186	NHAc	H
187	OH	H
188	OH	β-D-Gal

Scheme 43
BSA glycoconjugation by Michael addition (1,4-conjugate addition, see scheme 21 for general mechanism).

The covalent attachment of p-*N*-acrylamidophenyl β-D-glucoside **172**, -*N*-acetylglucosaminide **94** and -lactoside **175** to BSA was tested under various reaction conditions to optimize the reaction. Coupling to BSA (10 mg/mL) by the lactosyl derivative **176** was

tested under a reactant molar ratio of 2:1 sugar:amine in different buffers (phosphate, borate and carbonate), pH (8.5, 9.5 and 10.5) as well as temperatures (30 and 37°C). Reactions were followed over time by analyzing dialyzed aliquots of each reaction for lactose by the colorimetric phenol/H_2SO_4 assay. The results shown below in Figures 46 and 47 demonstrate clearly that conjugations are more rapid in a 0.2M carbonate buffer followed by 0.2M borate and phosphate buffers. As expected, the rate of lactose incorporation also increased with pH and temperature. The best results were achieved in 0.2M carbonate buffer, pH 10.5 at 30°C. At 37°C, protein degradation seemed to be occuring. No protein degradation could be observed for the plotted reaction conditions when the lactose-BSA conjugates were analysed by SDS-PAGE electrophoresis (figure 45). As shown in figure 45, mobilities during electrophoresis were clearly dependant on hapten density.

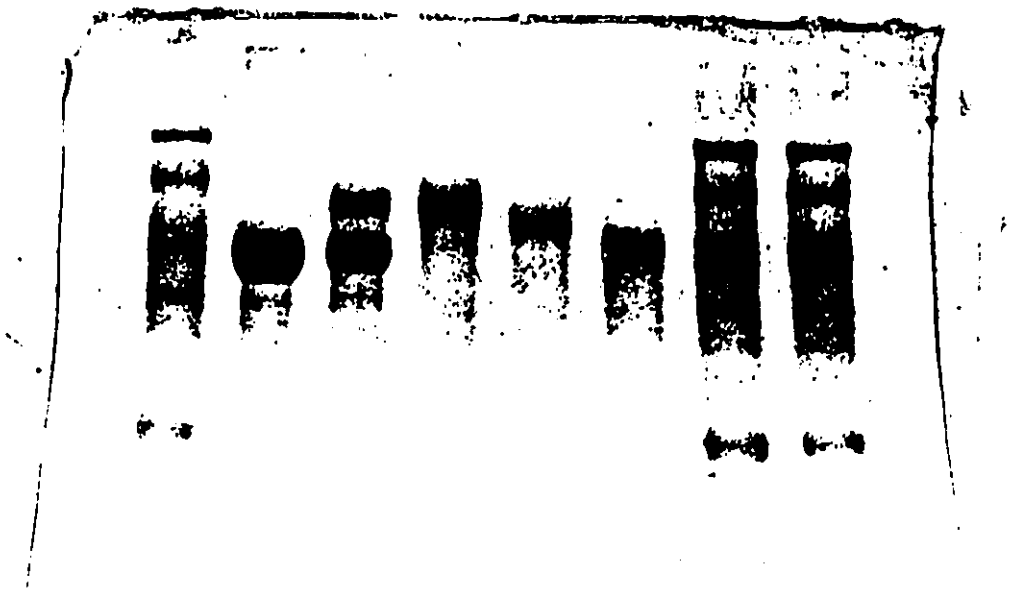


Figure 45 SDS-PAGE electrophoresis of Lac-BSA conjugates prepared by Michael addition. Lanes 1,7 and 8 (standards): myosine, 200 kDa; β-galactosidase, 111 kDa; phosphorylase 6, 97.4 kDa; BSA, 67 kDa; ovalbumin, 42.7 kDa. Lane 2: BSA; lane 3: BSA + Lac-BSA (45/1); lane 4: Lac-BSA (45/1); lane 5: Lac-BSA (23/1); lane 6: Lac-BSA (6/1).

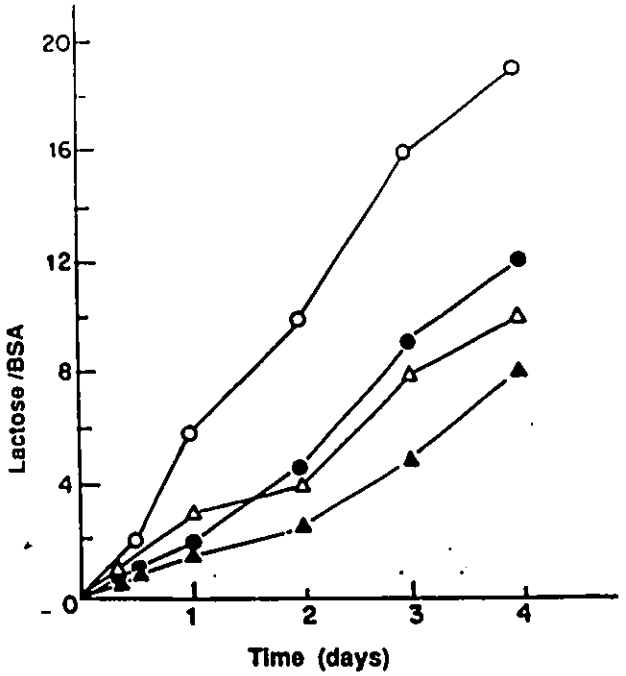


Figure 46 Time course of Michael addition of BSA to p-*N*-acrylamidophenyl β-D-lactoside **176** in 0.2M phosphate buffer, pH 8.5 (▲), pH 9.5(•) and borate buffer pH 8.5 (Δ), pH 9.5 (O).

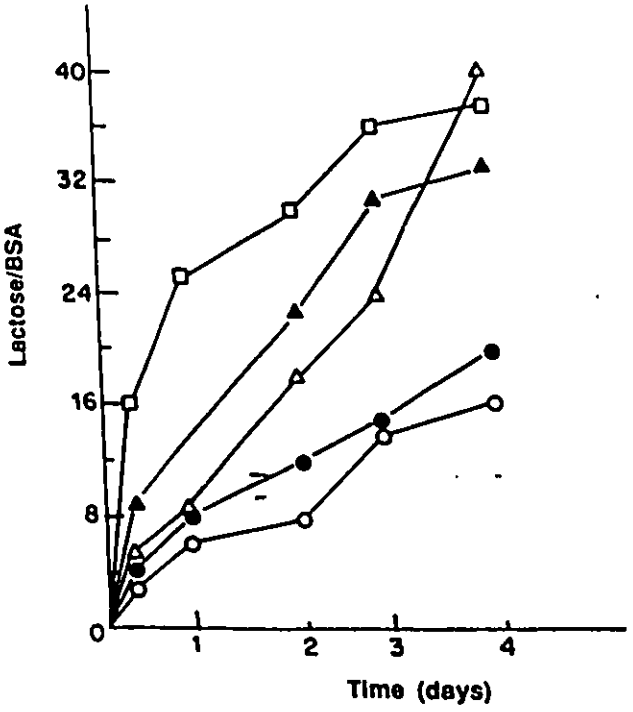


Figure 47 Time course of Michael addition of BSA to p-*N*-acrylamidophenyl β-D-lactoside **176** in 0.2M carbonate buffer, pH 8.5 , 30°C(O), 37°C (•); pH 9.5, 30°C (Δ), 37°C (▲); pH 10.5, 30°C (□).

Similar experiments were carried out with glucosyl and *N*-acetylglucosaminyl derivatives **172** and **94** and confirmed the results demonstrated for lactoside. When tested under different sets of reactant molar ratios, (2 or 4 :1, sugar : amine; BSA at 2 mg/mL), buffers (phosphate, borate and carbonate) and pH (8.0 and 9.0) for 3 days at 37°C, Michael addition did not furnish appreciable conjugation in the phosphate buffers while conjugations proceeded smoothly in both borate and carbonate buffers. The carbonate buffer was found to be more efficient than borate, and reactions were more effective at pH 9.0. In this case, the levels of incorporation were measured by comparing absorbances at 250 nm and 280 nm using HPLC purified conjugates and p-*N*-acetamidophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside **93** as a standard. The HLPC separations were performed on a TSK G4000 SWXL column using NaCl (0.07M) and phosphate buffer (0.02M, pH 6.7) as the mobile phase while absorbances at 250 and 280 nm were measured simultaneously with a diode-array detector. The sugar contents of the conjugates varied from 6 to 10 (borate, pH 9.0) to 30 to 60 (carbonate, pH 9.0).

As a general procedure, *N*-acrylamidophenyl glycosides are mixed with a protein (5-10 mg/mL) using a 2:1 molar ratio of sugar to lysine residues in 0.2M carbonate and left at room temperature for three days. These conditions give neoglycoproteins with high sugar incorporations with BSA (≥ 40 sugars/BSA). When performed with poly-L-lysine under the same conditions with a 6 :1 lysine : sugar ratio (poly-L-lysine at 6 mg/mL), p-*N*-acrylamidophenyl β-D-lactoside could be quantitatively coupled to the carrier (see Chapter 12). Glycoconjugates prepared this way were always very antigenic when assayed by agar gel diffusion with the appropriate lectins.

8.3 Glycoconjugates of biologically relevant carbohydrate haptens

Nitrophenyl glycosides of four important immunodominant carbohydrate haptens have been efficiently converted to *N*-acrylamidophenyl glycosides by acryloylation of aminophenyl glycosides produced by catalytic transfer hydrogenation of the parent nitro derivatives. All synthetic transformations were carried out on unprotected derivatives in methanol as described in Section 8.1. The acrylamidophenyl glycosides were subsequently used to prepare glycopolymers by radical initiated copolymerization with acrylamide and neoglycoprotein immunogens by Michael addtion to TT proteins as described in Section 8.2. The glycoconjugates were prepared as part of an immunization/diagnosis strategy to raise and isolate carbohydrate specific antibodies raised in rabbits to be used in cancer research. The derivatives described were prepared as part of a collaboration with researchers headed by Dr. K.L. Matta in the Department of Gynecologic Oncology at the Roswell Park Cancer Institute (Buffalo, N.Y.).

Some carbohydrate sequences[230] and the cryptic Thomsen-Friedenreich (T) antigen in particular (β-D-Gal-(1-3)α-D-GalNac-O-serine/threonine)[231] have been well established as tumor-associated markers. Advances in mapping these carbohydrate determinants have been made through the synthesis of well defined conjugates. Although there have been numerous syntheses of the T-antigen containing a wide variety of aglycons[232], there has been no

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230 a) T. Feizi, *Nature (London), 53* , 314 (1985); b) S. Hakomori, *Ann. Rev. Immuno., 2* , 103 (1984).

231 G.F. Springer, *Science 224* , 1198 (1984).

232 a) H.M. Flowers and D. Shapiro, *J. Org. Chem., 30* , 2041 (1965); b) K.L. Matta and J.J. Barlow, *Carbohydr. Res., 48* , 65 (1976); c) R. Kaifu and T. Osawa, *Carbohydr. Res., 69* , 79 (1979); d) J-C. Jaquinet and H. Paulsen, *Tetrahedron Lett.* , 1387 (1981); e) R.M. Ratcliffe, D.A. Baker and R.U. Lemieux, *Carbohydr. Res., 93* , 35 (1981); f) H. Paulson and M. Paal, *Carbohydr. Res., 113* , 203 (1983); g) H. Kunz, S. Birnbach and P. Wernig, *Carbohydr. Res., 202* , 207 (1990).

systematic study on the involvement of the aglycon regions (Serine/threonine) in the antibody combining sites. It is possible that the epitope density, more than the exact nature of the peptide sequences anchoring the T-antigen (and others) might be responsible for the high antigenic expression. Therefore, efficient access to immunogenic neoglycoproteins for which antibodies can be raised, coupled with the synthesis of antigenic glycopolymers useful for screening anti-carbohydrate antibodies are still valuable goals for this and other immunodominant epitopes.

Table 67 Immunodominant carbohydrate antigens and their associated significance.

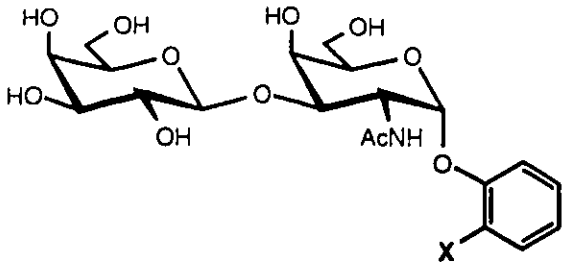
Product	Antigen	Significance	References
189	Galβ(1-3)GalNAcα1-	"T-antigen" tumor associated cell marker.	229, 233
190	Galβ(1-4)GlcNAcβ (1-6)GalNAcα1-	ABH type 2 blood group antigen.	234
191	Galβ(1-3)GlcNAcβ (1-3)Galβ(1-3) GlcNAcβ1-	- segment of ABH type 1 blood group antigen involved in the expression of Lewis blood group activity - found on ovarian cyst Le^a glycoproteins.	235
192	Galβ(1-3)GlcNAcβ (1-3)Galβ(1-4) Glcβ1-	"Lactose-N-tetraose" found on Le^a glycosphingolipids present in human tumors , meconium, and erythrocytes.	236

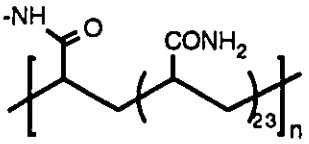
233 a) T.A. Beyer and R.C. Hill, *J. Biol. Chem.*, *255* , 5373 (1980).

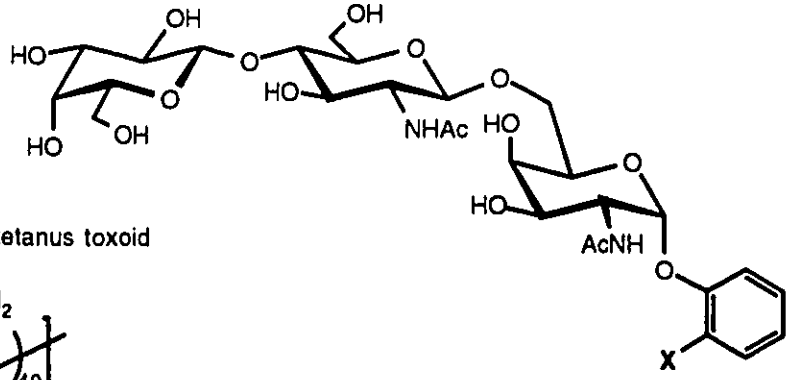
234 R.U Lemieux and M.H. Burzynska, *Can. J. Chem.*, *60* , 76 (1982).

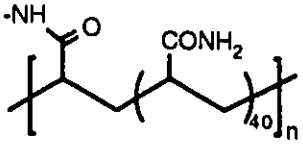
235 a) L. Rovis, B. Anderson, A. Kabat, F. Gruezo and J. Liaa, *Biochemistry 12* , 5340 (1973); b) W.M. Watkins, *Glycoproteins* , A. Gottschalk (ed.), Elsevier 1972, p. 830-891; c) T.B. Kuhlenschmidt and Y.C. Lee, *Biochemistry 23* , 3569 (1984).

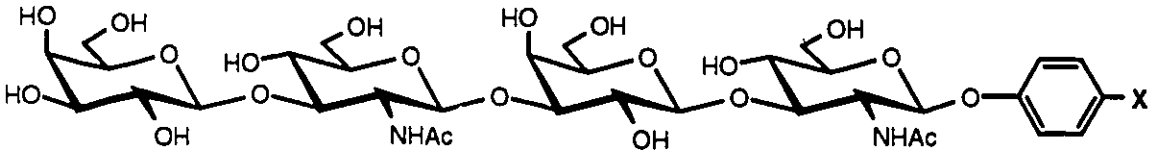
236 S. Sato, Y. Ito and T. Ogawa *Carbohydr. Res.*, 155 , C1 (1986).

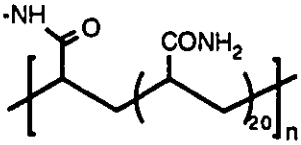


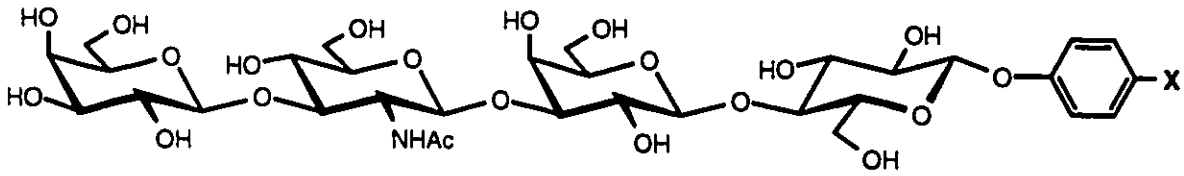
	X
193	NO_2
194	NH_2
195	$NHC(O)CH=CH_2$
196	$NHC(O)CH_2CH_2\text{-})_n$-tetanus toxoid
197	



	X
198	NO_2
199	NH_2
200	$NHC(O)CH=CH_2$
201	$NHC(O)CH_2CH_2\text{-})_n$-tetanus toxoid
202	



	X
203	NO_2
204	NH_2
205	$NHC(O)CH=CH_2$
206	$NHC(O)CH_2CH_2\text{-})_n$-tetanus toxoid
207	



	X
208	NO_2
209	NH_2
210	$NHC(O)CH=CH_2$
211	$NHC(O)CH_2CH_2\text{-})_n$-tetanus toxoid

Scheme 44
Aryl glycosides and glycoconjugates of immunodominant oligosaccharide antigens.

The T-antigen (**189**) and the D-lactosamine-trisaccharide (**190**) were supplied as their *ortho*-nitrophenyl glycosides **193** and **198** respectively, while the ABH type 1 determinant (**191**) and lacto-N-tetraose were supplied as *para*-nitrophenyl glycosides **203** and **208** respectively. Reduction of the aromatic nitro groups by catalytic transfer hydrogenation, using 5 or 10% Pd/C and ammonium formate as the transfer agent, proceeded smoothly to give the amines **194**, **199**, **204** and **209** in essentially quantitative yields. The reductions were carried out in methanol contaning 0.05% formic acid, by gently warming the reagents in a stoppered flask for a few minutes (< 5 min.). Filtration through Celite and treatment with anion (HO^-) exchange resin to neutralize formic acid, followed by evaporation gave the aniline derivatives. Except for **209**, the amine intermediates were never completely characterized for fear of decomposition. They were immediately converted to the corresponding *N*-acrylamidophenyl derivatives. The case of the *p*-aminophenyl glycoside **209** is noteworthy as it appears to have been isolated as its formate salt. The ^{1}H-NMR spetrum (D_2O, Figure 48) of **209** revealed a sharp singlet integrating for one proton at 8.45 ppm (HCO_2^-). Conversely, the ^{13}C-NMR spectum (D_2O, Figure 49) of the same sample revealed a superfluous carbonyl resonance at 171.1 ppm. Isolation of the formate salts of aniline derivatives were never observed for the analogous model glucose, *N*-acetylglucosamine and lactose derivatives discussed earlier.

Figure 48 ^{13}C-NMR (D_2O, 75.4 MHz) spectrum of **209**.

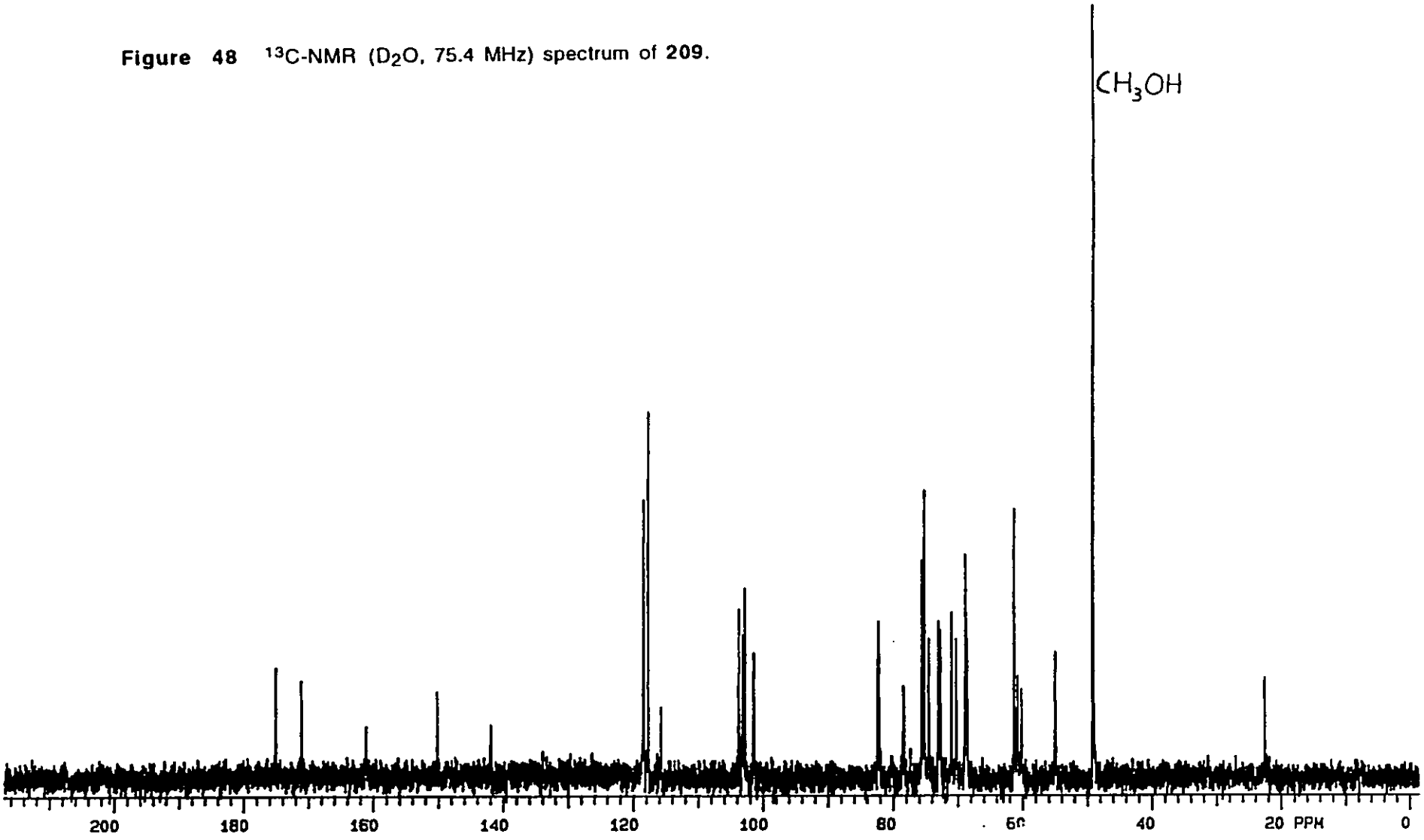
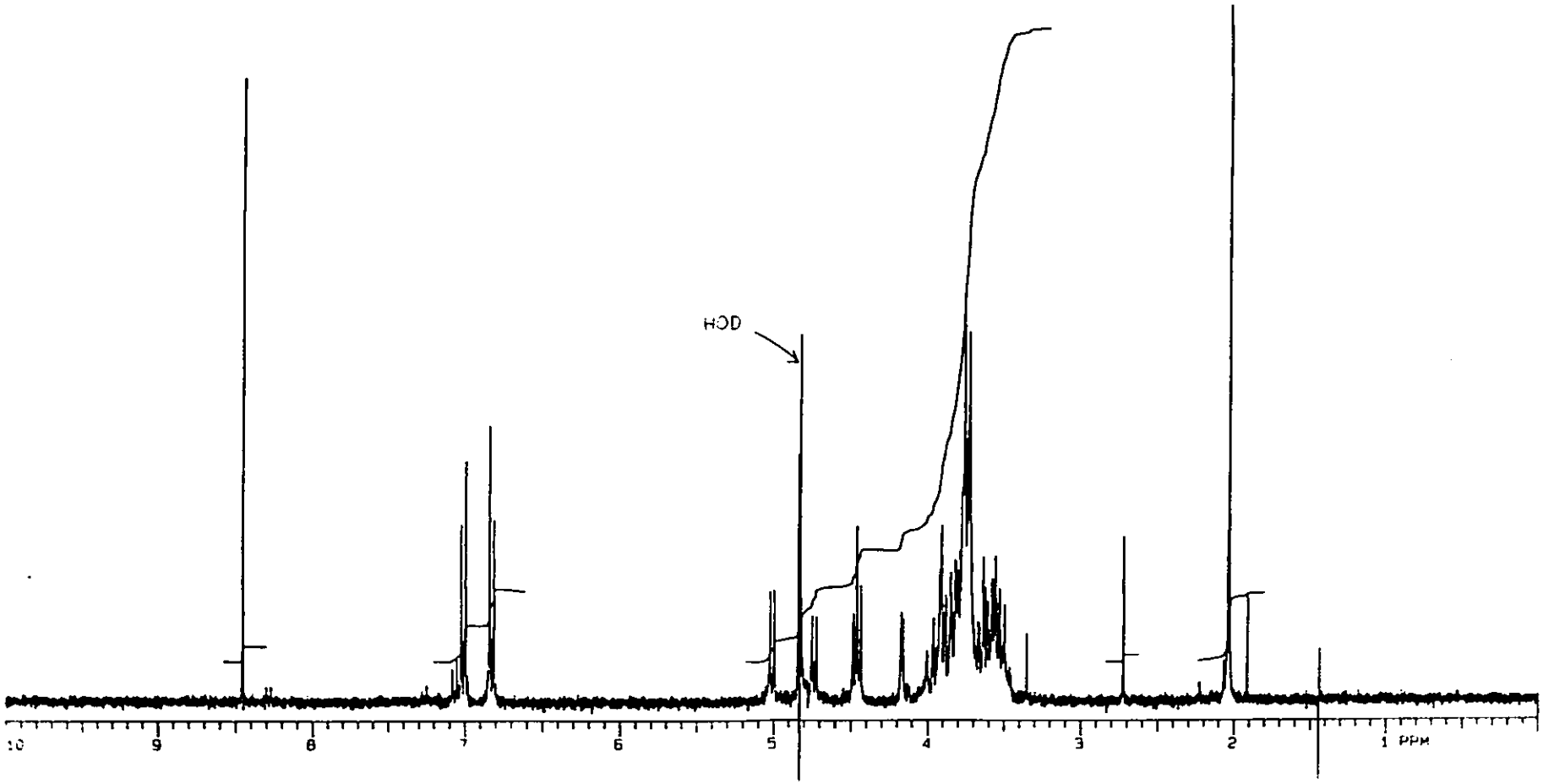
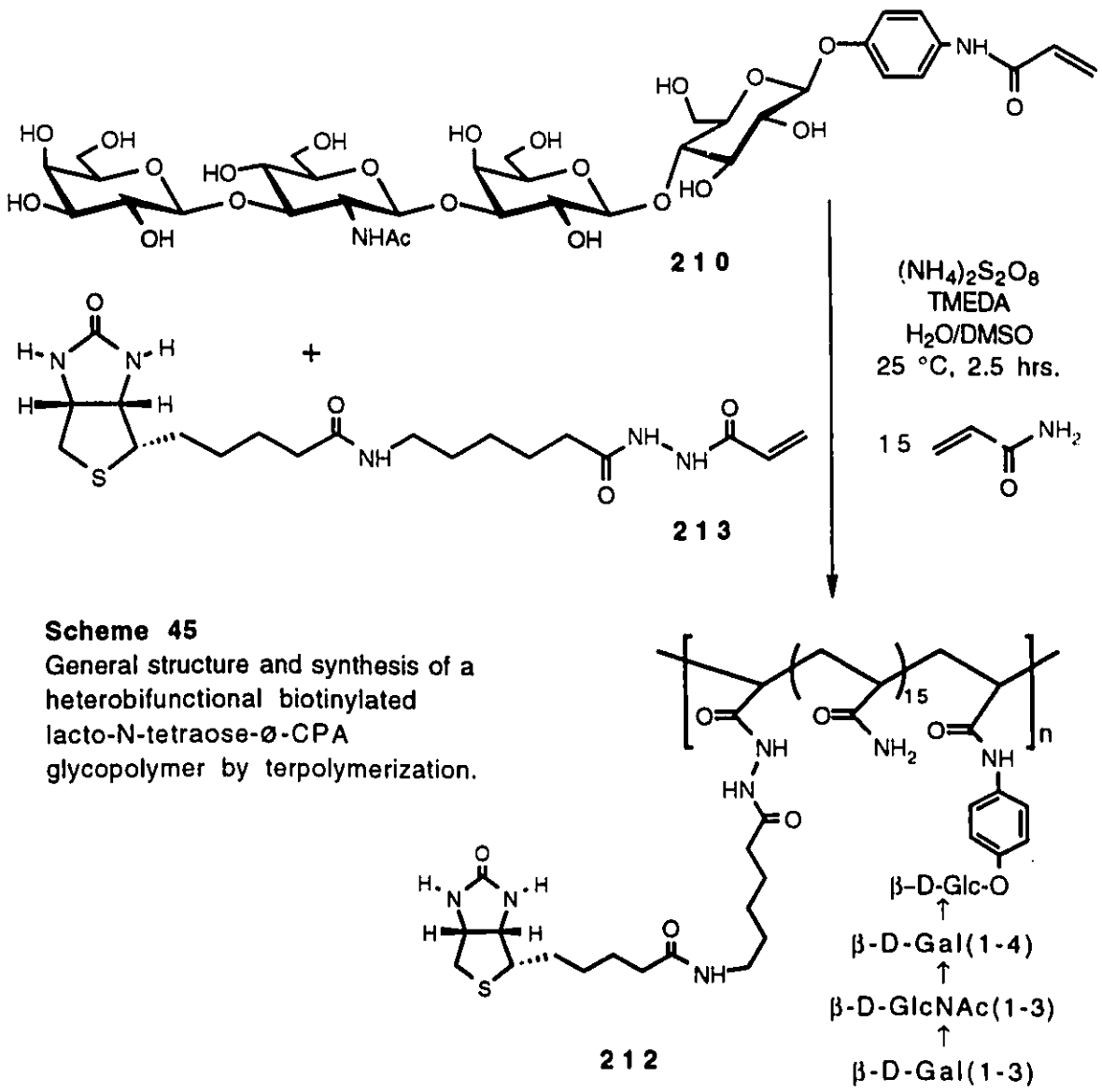


Figure 49 ^{1}H-NMR (D_2O, 300 MHz) spectrum of **209**.



N-Acryloylations were performed as discussed previously. Cooled (0°C) methanolic solutions of the amines **194**, **199**, **204** and **209** were each treated with anion (HO^-) exchange resin to which was added in a slow, dropwise fashion a slight excess of acryloyl chloride as dilute chloroform or 1,4-dioxane solutions. Products **195**, **200**, **205** and **210** were then isolated after filtration and evaporation. The acrylamide **200** was obtained pure in 98% overall yield from the parent nitro derivative **198** without any need for chromatography. The acrylamides **195** and **205** were slightly contaminated with acrylamide and an unknown acrylate containing compound, respectively. They were purified by size exclusion chromatography on a Sephadex LH-20 column using aqueous methanol as eluent. Compounds **195** and **205** were isolated in 95% and 55% overall yields, respectively. In the case of **205**, the lower yield is unexplainable except through unnoticed losses from excessive manipulations, as the parent amine **204** was isolated in quantitative yield and TLC analysis of the acryloylation reaction did not reveal any unexpected behaviour. In the case of the acrylamide **210**, which was produced quantitatively by TLC, a manipulative accident caused the loss and heavy contamination of the final product. Purification of recuperated product extracts on Sephadex LH-20 resulted in only a 10% overall yield isolation of **210** form the parent nitro derivative **208**.

All *N*-acrylamidophenyl glycosides **195**, **199**, **205** and **210** were used to prepare glycopolymer and neoglycoprotein conjugates by the methods elaborated upon earlier in this chapter. Copolyacrylamide glycopolymers **197**, **202**, **207** and **212** were obtained by radical initiated copolymerization of the respective *N*-acrylamidophenyl glycosides with acrylamide in deoxygenated water using ammonium persulfate and TMEDA at room temperature overnight.



Scheme 45
General structure and synthesis of a heterobifunctional biotinylated lacto-N-tetraose-ø-CPA glycopolymer by terpolymerization.

In the case of the lacto-N-tetraose antigen, the glycopolymer conjugate was prepared by terpolymerization of acrylamide, **210**, and *N*-acryloylated biotinamidocaproylhydrazide **213** (see later). This heterobifunctional glycopolymer was prepared as useful diagnostic antigen for the study of lacto-N-tetraose specific antibodies. The presence of a biotin effector allows for additional monitoring of the binding interaction between carbohydrate specific

antibodies and the glycopolymer by any number of existing avidin or streptavidin mediated labelling techniques such as immunogold staining for direct visualization by electron microscopy for example[237]. All glycopolymers were isolated in high yields (> 80%) by dialysis and freeze drying. ^{1}H-NMR spectroscopy was used to determine the monomer composition of each glycopolymer. Glycopolymers **197**, **202**, and **207** were shown to contain hapten to acrylamide molar ratios of 1:23 (21% by wt), 1:40 (18% by wt) and 1:20 (63% by wt) respectively. Glycopolymer **212** was shown to have a biotin to carbohydrate hapten to acrylamide molar ratio of 1:1:15.

Tetanus toxoid (TT) neoglycoprotein conjugates were prepared from each *N*-acryloylated carbohydrate hapten precursors by Michael addition, as described earlier. The immunogens **196**, **201**, **206** and **211** were prepared by simply mixing the corresponding *N*-acrylamidophenyl glycosides with purified tetanus toxoid protein[238] (5 mg/mL) in 0.2M carbonate buffer pH 10.0 at room temperature for three days. A molar ratio of acrylamide to TT L-lysine residues of 2:1 was used in each case. The neoglycoproteins so produced were purified and isolated by centrifugation, dialysis and freeze drying. The homogeneous reaction mixtures did not show any substantial protein degradation when analyzed by SDS-PAGE gel electrophoresis (not shown). Because of the highly valuable nature of the TT neoglycoprotein conjugates and the relatively small scale of the preparations (5-10 mg), the hapten content of each glycoconjugate was not directly evaluated. Previous work with model systems (Section 8.2.2), and the lactosyl analogues in particular, have shown that the conditions used here result in high incorporations of carbohydrate hapten. In the case of conjugate **206**, half of the acrylamide precursor **205** used during the conjugation procedure was recovered after dialysis of the crude neoglycoprotein.

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[237] a) G.R. Bullock and P. Petrusz (eds.), *Techniques in Immunocytochemistry Vol 2 and Vol 3* , Academic Press, San Diego, CA, 1982 and 1985; b) D. Brada and J. Roth, *Anal. Biochem.*, *42* , 79 (1984); c) R.E. Morris and C.B. Saelinger, *J. Hystochem. Cytochem.*, *32* , 124 (1984); d) J. E. Beesley, *Proc. Roy. Microscop. Soc.*, *20* , 187 (1985).

[238] MW = 150 kDa, ~60 free L-lysines per protein.

This suggests that as many as 60 carbohydrate haptens were conjugated to TT. In other words, all of the free L-lysine residues on TT had been modified.

The antigenicity of the neoglycoproteins produced was demonstrated with the T-antigen neoglycoprotein **196** as an example. Agar gel diffusion of **196** against the peanut lectin from *Arachis Hypogaea* produced an intense precipitin band while no interaction was observed with non-galactose-specific lectins such as WGA when used as controls (not shown). The antigenicity of **196** was also quantitated by enzyme linked lectin assays (ELLA) with horseradish peroxidase labeled peanut lectin (100 µg/well of two-fold serial dilutions from 1 mg/mL). Figure 50 shows a typical binding assay using **196** as coating antigen in microtiter plates (1 and 0.1 µg/well). Enzyme activity in the wells containing adsorbed lectin-antigen complexes was measured with ABTS as chromogenic enzyme substrate with optical densities measured at 410 nm after 15 minutes.

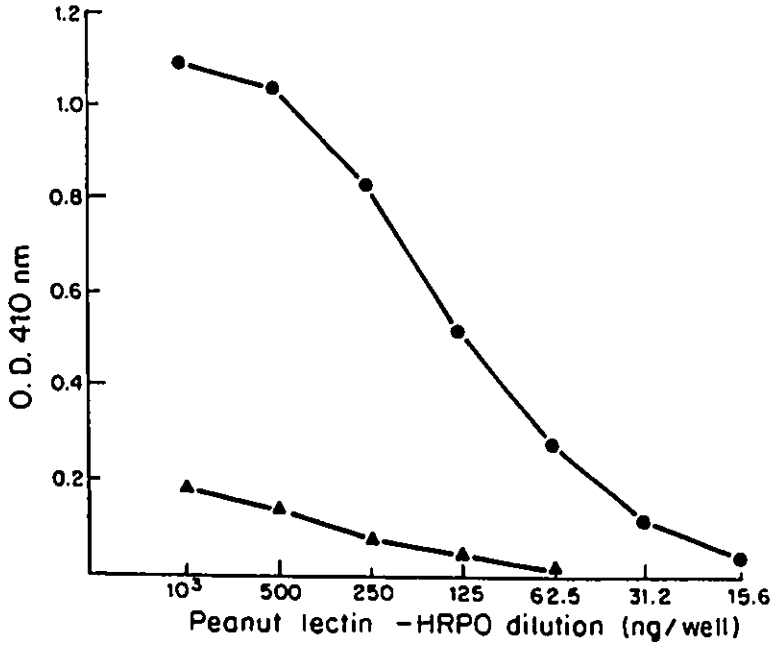

Figure 50 Enzyme linked lectin assay (ELLA) of neoglycoprotein **196** as coating antigen at 1 µg (•) and 0.1 µg (▲) per well using horseradish peroxidase labeled peanut lectin.

All glycopolymer and TT neoglycoprotein conjugates have been forwarded to collaborators at the Roswell Park Cancer Institute (Buffalo, USA) for immunological study. The immunogenicities of the neoglycoprotein conjugates **196**, **201**, **206** and **211** are presently being evaluated in rabbits. Results of this collaborative research effort will be presented in due course.

8.4 Experimental methods

General methods

General methods are as described in the previous chapters.

p-*Aminophenyl 2,3,4,6-tetra*-O-*acetyl β-D-glucopyranoside* **170**

p-Nitrophenyl 2,3,4,6-tetra-*O*-acetyl β-D-glucopyranoside **118** (224 mg, 0.476 mmol), 150 mg (2.4 mmol, 5 equiv.) of ammonium formate and 50 mg of 10% palladium on charcoal (Pd/C, Aldrich) were warmed in 25 mL of CH_3OH for two minutes and left to cool to room temperature. A TLC analysis (1/1, $CHCl_3$/EtOAc, v/v) showed that conversion of **118** (Rf = 0.60) to the amine **170** (Rf = 0.35) was clean and complete after the first minute of heating. The reaction was filtered through Celite with warm methanol used for rinsing. Evaporation (25 °C) of the methanol under reduced pressure gave a white solid mass which was taken up in CH_2Cl_2, and washed with sat. $NaHCO_3$ followed by water. The organic phase was dried (Na_2SO_4), filtered and evaporated to a white crystalline solid, pure by TLC and NMR. The product **170** was obtained in 210.7 mg (quant.) yield. The product was crystallized from ethanol to give fine, white needles. Physical and NMR data for this compound can be found in

Tables 62, 63 and 65 of this chapter. This compound could also be prepared effectively on a 1 g scale under the same conditions.

p-N-*Acrylamidophenyl 2,3,4,6-tetra*-O-*acetyl β-D-glucopyranoside* **171**

The amine **170** (200 mg, 0.454 mmol) and 500 µL of triethylamine were dissolved in 30 mL CH_2Cl_2 and then cooled in an ice bath. A slow, dropwise addition of acryloyl chloride (43 µL in 5 mL CH_2Cl_2, 1.1 equiv.) was then performed over 45 minutes. TLC (1/1, EtOAc/$CHCl_3$) showed a clean and complete conversion of the amine **170** (Rf = 0.35) to the acrylamide **171** (Rf = 0.42). The reaction was worked by successively washing the mixture with 0.5M HCl (2x), sat. $NaHCO_3$ (2x), water and sat. NaCl. The combined organic extracts were dried (Na_2SO_4), filtered and evaporated under reduced pressure to give a yellow oil. Filtration through a short silica gel plug using $CHCl_3$/MeOH (15/1, v/v) gave 211 mg (94% yield) of pure **171** after solvent removal. The product was crystallized from warm ethanol to give white needles. NMR and physical data are given in Tables 62, 63 and 65.

p-N-*Acrylamidophenyl β-D-glucopyranoside* **172**

Zemplén de-*O*-acetylation of **171** (310 mg) was performed in methanol (25 mL) at room temperature using a catalytic amount of 1M CH_3ONa/CH_3OH. A quantitative yield of **172** was obtained after neutralization with (H^+) resin, filtration and evaporation. The product could be crystallized from ethanol for physical data measurements. NMR and physical data are given in Tables 62, 63 and 65.

p-Aminophenyl 2,2',3,3',4',6,6'-hepta-O-acetyl β-D-lactoside **173**

A solution of *p*-nitrophenyl 2,2',3,3',4',6,6'-hepta-*O*-acetyl β-D-lactoside **121** (483 mg, 0.638 mmol) and ammonium formate (200 mg, 5 equiv) in 80 mL of methanol containing 100 mg 10% Pd/C was gently warmed for 5 minutes in a stoppered flask. After cooling, the reaction mixture was filtered through Celite which was rinsed with methanol. The methanol was evaporated and the crude product taken up in CH_2Cl_2. The organic phase was washed successively with sat. $NaHCO_3$ and water. After drying (Na_2SO_4), evaporation and complete solvent removal, 456 mg (98%) of pure **173** was obtained. The product could be crystallized from ethanol/methanol to give small needles suitable for elemental analysis and other physical measurements. NMR and physical data for this compound are given in Tables 62, 63 and 65.

p-N-*Acrylamidophenyl 2,2',3,3',4',6,6'-hepta*-O-*acetyl β-D-lactoside* **174**

A solution of the amine **173** (419 mg, 0.576 mmol) and triethylamine (350 µL) in 40 mL of CH_2Cl_2 was cooled to 0 °C. A solution of 60 µL (1.25 equiv.) acryloyl chloride in 15 mL CH_2Cl_2 was slowly added dropwise until complete consumption of the amine was inferred from the disappearance of a purple colouration when a drop of the reaction solution on a piece of TLC plate was wetted with the acidic cerium/molybdate TLC developing solution (see text). The reaction mixture was then allowed to reach room temperature while stirring for 30 minutes. The organic phase was then successively washed with 0.5M HCl (2x), sat. $NaHCO_3$, water and brine. The combined organic extracts were dried (Na_2SO_4), filtered and evaporated under reduced pressure (< 30 °C). The residue was purified by silica gel column chromatography with $CHCl_3$/EtOAc (1/1, v/v). The product **174** was thus obtained in a 426 mg (95%) yield as a white solid after complete removal of the solvent, and

was pure by TLC, NMR and elemental analysis (Tables 62, 63 and 65). The product failed all crystallization attempts with decomposition being the result when solvents such as ethanol were warmed. However, crystals were obtained from slow evaporation of CH_2Cl_2, mp = 117.8-118.7°C.

p-*Aminophenyl β-D-lactoside* **175**

The catalytic transfer hydrogenation method described for **173** was applied to *p*-nitrophenyl β-D-lactoside **121a**. Compound **121a** (178 mg, 0.384 mmol), ammonium formate (120 mg, 5 equiv.) and 25 mg of 10% Pd/C were warmed in 25 mL of CH_3OH for a few minutes inside a stoppered flask. TLC in $CHCl_3$/MeOH/H_2O (30/10/1) indicated complete conversion within 15 minutes of initial warming. The reaction mixture was filtered through Celite and rinsed with methanol. The filtrate was evaporated to dryness to give **175** still containing ammonium formate. The salt was removed by repeated freezedrying from water. The homogeneous product (quantitative) was crystallized from warm (not hot) ethanol (mp = 237.0-238.5 °C) or water (mp = 270.2 - 271.5 °C) and had physical properties consistent with those previously published[239]. The amine **175** could also be obtained in a quantitative yield by Zemplén de-*O*-acetylation of the peracetylated analogue **173** without difficulty. Physical and NMR data are given in Tables 62, 63 and 65.

p-*N-Acrylamidophenyl β-D-lactoside* **176** , *Method A:*

The peracetylated *N*-acryloyl derivative **174** (350 mg, 0.448 mmol) was treated with methanolic sodium methoxide as described above for **172**. Analytically pure **176** was obtained in 441 mg (98%) yield after crystallizations from ethanol. NMR and physical data are given in Tables 62, 63 and 65.

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[239] F.H. Babers and W.F. Goebel, *J. Biol. Chem.*, *105* , 473 (1934).

Method B: N-*Acryloylation of the unprotected lactoside* **176**

Compound **173** (141 mg, 0.326 mmol) was treated with triethyl amine (200 µL) in cold methanol (20 mL, 0°C). Acryloylation was performed by a slow, dropwise addition of 35 µL acryloyl chloride in 4 mL $CHCl_3$. After complete addition, the reaction mixture was stirred at room temperature for an additional hour. Ions were removed from solution by successive treatments with anionic resin (OH^-) followed by cationic resin (H^+) until the solution reached neutrality. Compound **176** was obtained in 94% yield after freeze drying the methanolic residue. The product was homogeneous by TLC in $CHCl_3$/MeOH/H_2O (30/10/1) and had physical constants consistent with the product obtained from method A.

*Copolymerization of p-*N*-acrylamidophenyl β-D-glycosides* (**94**, **172** and **176**) *with various acrylate-type comonomers* (**177-179**)

General preparation : Glycopolymers **181a - 185a** were prepared by heating solutions of the appropriate monomers in deoxygenated water under a nitrogen atmosphere. Polymerizations were carried out at 100 °C by submerging reaction flasks in a boiling water bath. Solutions were vigourously stirred during polymerizations. Total monomer concentrations were kept constant at 0.8M. Polymers were initiated with 0.4% $(NH_4)_2S_2O_8$ by injection of an appropriate volume of a 50 mg/mL solution in deoxygenated water. Polymerizations were carried out over 12-15 minutes at 100 °C. Monomer uptake was monitored by TLC ($CHCl_3$/MeOH/water, 30/10/1 or MeCN/acetone/5% aqueous HOAc, 5/3/1). In some cases, initiation appeared (TLC) to be unsuccessful after the first 12 minutes. In these cases, initiation was repeated and reactions heated an additional 12 minutes. Polymers were processed by exhaustive dialysis (10 kDa MW cutoff) in distilled water followed by freezedrying. They were all obtained as white, spongy solids and were usually produced on scales of 30 - 100 mg. Monomer incorporations in the glycopolymers were determined by ^{1}H-NMR (D_2O) by comparing integrations from the

aromatic protons (7 - 7.5 ppm) and sugar ring protons (3.5 - 5 ppm) to the backbone methine and methylene signals (1.4 - 2.4 ppm). The N-acetyl methyl signal at 2.0 ppm for the GlcNAc polymers was also used as reporter group. In most cases, the composition, including water content, of the glycopolymers was also verified by elemental analysis according to equation 1 and the steps outlined in Chapter 2. Values derived from ^{1}H-NMR and elemental analysis were always consistent, well within 10% of each other. See Table 66 for products, yields and associated calculated glycopolymer compositions including retained water content. Results of elemental analyses for most of the aryl glycoside glycopolymers are given below in a tabular fashion (Table 68).

Table 68 Elemental analysis of lyophilized aryl glycoside glycopolymers.

Glycopolymer	%C	%H	%N
181b	45.62	5.95	12.12
181c	45.15	6.01	12.01
181d	45.52	6.36	15.36
181e	46.97	6.51	14.92
181f	46.89	6.59	16.70
182a	45.27	6.28	11.46
182b	50.59	6.39	13.41
183a	51.63	6.75	0.69
183b	54.08	7.16	1.20
184	49.63	7.05	13.15
185a	46.71	6.19	11.78

Molecular weight determination of **185b**

Molecular weight determination was performed on a Waters Model 991/625LC HPLC system equiped with a multiple diode-array detector. Calibration was done with protein standards (Boehringer Mannheim, cat. no. 1213-776) and polyacrylamide standards (Polysciences) in 0.1M phosphate buffer (pH 7.0) on a TSK G4000

SWLX column (7.5 mm x 3.0 cm) at a flow rate of 1 mL/min. Absorbances were measured at 205 nm (polyacrylamides) or 280 nm (proteins and glycopolymer).

Agar gel diffusion assays

Assays were performed as described earlier in Chapter 2 experimental section (2.3) with lectins purchased from Sigma.

Quantitative precipitation with lectin

The lectin from *Arachis Hypogaea* (Peanut, Sigma No. L-0811) was diluted to 200 μg/mL in PBS and 100 μL solutions were added to 1.5 mL Eppendorf microcentrifuge tubes (Brinkmann Instruments Co., Westbury, NY). Aliquots of 1.0 to 200 μL of a stock solution of the polymer **185b** (1.0 mg/mL) in PBS were added to the tubes. The final volume of all the tubes were then adjusted to 300 μL. The reactions were run in triplicate. The solutions were gently agitated and allowed to incubate for 2 hours at 37 °C, then 4 days at 4 °C. The tubes were centrifuged for 15 minutes at 14,000 g in a Fisher Microcentrifuge model 235 B. The precipitin pellets were washed three times with 100 μL of cold PBS with centrifugation between washes. The protein content of the pellets was determined using the bicinchoninic acid (BCA) protein assay reagents of Pierce (Il, USA) following the enhanced protocol described in the instruction manual. The absorbances were measured at 562 nm on a Gilford instrument.

Enzyme linked lectin assay (ELLA)

The wells of Limbro EIA microtiter plates (Flow Laboratories, Mississauga, ON, Canada) were coated at room temperature overnight by using 100 μL of two-fold dilutions of polymer **185b** or the glycoprotein **196** (16 μg/mL) in PBS. The plates were blocked with

300 µL of 0.2% BSA in PBS for 1 hour at room temperature. After washing (4x) with PBS, the wells were filled with 100 µL of 300 x diluted (PBS) horseradish peroxidase labeled peanut lectin (1 mg/mL, Sigma No. L7759). The plates were incubated at room temperature for 3 hours and then washed 5 times with PBS. The enzyme substrate 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 250 µg/mL, 50 µL/well) in citrate-phosphate buffer (0.2M, pH 4.0 with 0.01% H_2O_2) was added. After 15 minutes the optical density was measured at 410 nm on a Dynatech MR 600 spectrophotometer.

*Conjugation of p-*N*-acrylamidophenyl lactoside* (**176**) *to BSA by Michael addition* (Figures 44 and 45).

To 5.0 mL volumes of 0.2M phosphate and borate buffers of pH 8.5 and 9.5 were added 50 mg of BSA (45 µmol lysine-NH_2) and 44 mg (90 µmol) of **176**. The four separate solutions were homogenized on a Vortex mixer then incubated in a thermostated water bath at 37°C. To 10.0 mL volumes of 0.2M carbonate buffers of pH 8.5, 9.5 and 10.5 were added 100 mg of BSA (90 µmol lysine-NH_2) and 88 mg (180 µmol) of **176**. Each of these solutions were homogenized on a Vortex mixer then separated into two 5 mL portions. One set of solutions was incubated at 30 °C while the other was incubated at 37°C. After 6, 12, 24, 48, 72 and 96 hours, 500 µL aliquots of each solution were removed from each reaction and dialyzed exhaustively against distilled water. The dialyzed samples were then diluted to 5.0 mL and analyzed spectrophotometrically for protein and carbohydrate content. Protein content was determined at 280 nm while carbohydrate content was assayed by the phenol/H_2SO_4 micro method of Fox and Robyt[240] in wells of microtiter plates using a Dynatech MR 600 spectrophotometer. All analyses were performed in triplicate.

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[240] J.D. Fox and J.F. Robyt, *Anal. Biochem.*, *195* , 93 (1991).

ortho-N-*Acrylamidophenyl* O-(β-D-*galactopyranosyl*)-(1-3)-2-*acetamido*-2-*deoxy*-α-D-*galactopyranoside* **195**

The nitrophenyl derivative **193** (7.1 mg, 14.1 μmol), 5 mg (5 equiv.) of ammonium formate and 5 mg of 5% Pd/c were mixed in 2 mL MeOH containing 0.05% formic acid, in a small round-bottom flask. The flask was stoppered and warmed briefly (1-2 min.) over a heat gun. While cooling to room temperature TLC (MeCN/acetone/10% HOAc in water, 5/3/1) showed only a partial reduction of **193** (Rf = 0.32) to the amine **194** (Rf = 0.23) which turned bright orange on contact with the acidic cerium molybdate developing solution. The reaction was warmed briefly and allowed to cool a second time. TLC then showed that a clean and complete conversion to the amine had been achieved. The product solution was filtered through a small bed of Celite using warm methanol for rinsing. After evaporation (≤ 30 °C), the resulting clear oil was dissolved in water and repeatedly lyophilized to remove any remaining ammonium formate or formic acid. The lyophilized amine **194** was then dissolved in 2 mL MeOH. Wet anion (HO^-) exchange resin (Amberlite IRA-400) was added and the solution cooled to 0°C. A dilute solution of acryloyl chloride in 1,4-dioxane (≤ 1%) was slowly added dropwise to the cool, stirring solution until TLC indicated a complete and clean conversion of the amine to the acrylamide **195** (Rf = 0.33). After warming to room temperature, the resin was filtered, rinsed with warm methanol and evaporated to a clear oil. The oil was dissolved in water and lyophilized to a white, fluffy solid (8.5 mg). An ^{1}H-NMR spectrum of this sample revealed the presence of a 2.5 molar excess of acrylamide to the product **195**. The acrylamide contaminant, produced most likely from acryloylation of incompletely removed ammonium formate in the previous stage, was removed by size exclusion chromatography on a column of Sephadex LH20 (1.5 x 85 cm) using MeOH/water (9/1, v/v) as eluent (flow rate = 32 mL/hr). The separation was followed with a Waters 700 series refractive index monitor, and recorded. An excellent separation was achieved. The product fractions (eluted first) were pooled and evaporated. The

product **195** was recovered in 95% overall yield (7.1 mg) as a white, lyophilized powder.

^{1}H-NMR data for **195**, δ (D_2O, referenced to HOD at 4.828 ppm): 7.15 - 7.44 (mult, 4H, aromatic), 7.57 (dd, 1H, J_{cis} = 10.2, J_{trans} = 17.0 Hz, C(O)CH=), 6.40 (dd, 1H J_{gem} = 1.2 Hz, C=CH_2 trans), 5.96 (dd, 1H, C=CH_2 cis), 5.71 (d, 1H, $J_{1,2}$ = 3.5 Hz, H_1), 4.53 (d, 1H, $J_{2,3}$ = 11.1 Hz, H_2), 4.43 (d, 1H, $J_{1,2}$ = 7.5 Hz, $H_{1'}$), 4.30 (d, 1H, $J_{3,4}$ = 2.4 Hz, $H_{4'}$), 4.03 - 4.11 (mult, 2H), 3.92 (dd, 1H, $J_{3,4}$ = 3.0, $J_{4,5}$ = ≤ 1 Hz, $H_{4'}$), 3.77 - 3.51 (mult, 7H), 2.05 (s, 3H, NAc).

ortho-N-*Acrylamidophenyl* O-*(β-D-galactopyranosyl)-(1-3)*-O-*(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1-6)-2-acetamido-2-deoxy-α-D-galactopyranoside* **200**

The nitro derivative **198** (6.2 mg, 8.8 μmol), 3 mg (5 equiv.) of ammonium formate and 10 mg of 10% Pd/C were mixed and briefly warmed in 2 mL MeOH containing 0.05% HCOOH in a stoppered flask. TLC using MeCN/acetone/10% HOAc in water (5/3/1) indicated a clean and complete conversion of **198** (Rf = 0.15) to the amine **199** (Rf = 0.09) which turned bright orange on contact with the acidic cerium molybdate developing solution. After processing as above, 6.0 mg (quant.) of the amine **199** was obtained as a white solid. The amine was dissolved in 2 mL MeOH, treated with anion (HO^-) exchange resin, and cooled to 0°C. Acryloyl chloride was slowly added dropwise as a 1% solution in $CHCl_3$ until a complete and clean conversion to the desired acrylamide **200** (Rf = 0.12) was achieved. After warming to room temperature, the resin was filtered and rinsed with warm methanol. Complete solvent removal gave an almost quantitative yield (6.3 mg) of the desired product **200** as a white solid, pure by TLC and ^{1}H-NMR.

^{1}H-NMR data for **200**, δ (D_2O): 7.16 - 7.47 (mult, 4H, aromatic), 6.55 (dd, 1H, J_{cis} = 10.1, J_{trans} = 16.8 Hz, C(O)CH=), 6.37 (dd, 1H J_{gem} = 1 Hz, C=CH_2 trans), 5.94 (dd, 1H, C=CH_2 cis), 5.69 (d, 1H, $J_{1,2}$ = 3.6 Hz,

H_1), 4.47 (d, 1H, $J_{1,2}$ = 7.4 Hz, $H_{1'}$), 4.43 (d, 1H, $J_{1,2}$ = 7.6 Hz, $H_{1''}$), 3.5 - 4.3 (mult, 18H), 2.04, 1.84 (2s, 2 x 3H, NAc).

para-N-*Acrylamidophenyl* O-*(β-D-galactopyranosyl)-(1-3)*-O-*(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1-3)*-O-*(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-β-D-glucopyranoside* **205**

The nitrophenyl derivative **203** (35.0 mg, 40.2 μmol), 12.5 mg (5 equiv.) of ammonium formate and 20 mg of 10% Pd/C, in 20 mL MeOH containing 0.05% HCO_2H, were gently warmed over a heat gun in a stoppered flask until dissolution of the glycoside **203** was deemed complete. The reaction mixture was then left to cool to room temperature while a TLC analysis (MeCN/acetone/5% HOAc in water, 5/3/2) indicated a clean and complete conversion of **203** (Rf = 0.17) to the desired amine **204** (Rf = 0.10). The amine produced a bright, reddish-purple colouration on contact with the acidic cerium molybdate developing solution. The product solution was filtered through Celite, using wet methanol for rinsing, and collected in a flask containing anion (HO^-) exchange resin to neutralize the formic acid. This solution was stirred at 30°C while being bubbled with nitrogen to remove any remaining amonia. The resin was filtered and rinsed with wet methanol. The solution volume was reduced to 10 mL. New anion (HO^-) exchange resin was added, and the solution cooled to 0°C. In a slow, dropwise fashion from a syringe was added 90 μL of 5% acryloyl chloride in 1,4-dioxane over the course of 3 hours. TLC then indicated a clean and complete conversion of the amine **204** to the desired acrylamide **205** (Rf = 0.14). The product solution was filtered and rinsed with warm, wet (5%) methanol. Evaporation of the methanol and lyophilization of the crude oil from water gave a white, fluffy solid. During weighing preparations, the flask containing **205** was dropped and shattered on the floor and bench. A benchtop extraction with water was performed. The lyophilized extract was then purified on Sephadex LH20 as described for **200** using MeOH/water (4/1) as eluent. Only 3.9 mg (10%) of pure

205 could be recovered after solvent removal.

^{1}H-NMR data for **205**, δ (D_2O, referenced to HOD at 4.828 ppm): 7.44 (d, 2H, $J_{o,M}$ = 9.0 Hz, H_{meta}), 7.11 (d, 2H, H_{ortho}), 6.42 (dd, 1H, J_{cis} = 9.8, J_{trans} = 17.1 Hz, C(O)CH=), 6.32 (dd, 1H J_{gem} = 1.5 Hz, C=CH_2 trans), 5.87 (dd, 1H, C=CH_2 cis), 5.24 (d, 1H, $J_{1,2}$ = 8.5 Hz, anomeric), 5.01 (d, 1H, J = 8.0, J = 9.7 Hz), 4.75 (d, 1H, $J_{1,2}$ = 7.7 Hz, anomeric), 3.4 - 4.2 (mult, 24H), 2.02, 2.03 (2s, 2 x 3H, NAc).

para-N-*Acrylamidophenyl* O-*(β-D-galactopyranosyl)-(1-3)*-O-*(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1-3)-β-D-galactopyranosyl)-(1-4)-β-D-glucopyranoside* **210**

The nitrophenyl derivative **208** (38.0 mg, 45.9 μmol), 40 mg (5 equiv.) of ammonium formate and 40 mg of 10% Pd/c were mixed in 20 mL MeOH containing 10 μL HCO_2H. This solution was then warmed gently in a stoppered flask over a heat gun until **208** seemed to be completely dissolved. The solution was left to cool to room temperature while TLC using MeCN/acetone/5% HOAc in water (5/3/2) indicated a clean and complete conversion of **208** (Rf = 0.50) to the desired amine **209** (Rf = 0.31). The amine turned bright purple on contact with the acidic cerium molybdate developing solution. The product solution was filtered through Celite, using wet (5%) methanol for rinsing, into a flask containing anion (HO^-) exchange resin. After 30 minutes at room temperature and 90 minutes at 4°C, the resin was filtered and the methanol evaporated. The resulting wet product was lyophilized to give **209** as a white powder (38.9 mg), pure by TLC. ^{1}H- and ^{13}C-NMR analysis of the product confirmed the high purity of the amine but suggested that it was isolated as its formate salt (see NMR data below). Nonetheless, this product was dissolved in wet (6%) methanol and then cooled to 0°C in the presence of anion (HO^-) exchange resin. A 60 μL volume of10% acryloyl chloride in 1,4-dioxane was added portionwise to the stirring solution over a 30 minute period. After stirring for a total of one hour at 0°C, the solution was warmed to room

temperature after which the resin was filtered and the methanol evaporated. The resulting wet product was lyophilized to a white powder which appeared to be homogenous by TLC. However, an ^{1}H-NMR spectrum of the crude product indicated the presence of a second unknown acryloylated compound. The crude product was therefore purified on Sephadex LH20 resin as described above, using MeOH/water (2/1) as eluent. The desired compound **210** was obtained in only 21.5 mg (55%) overall yield after complete evaporation of the solvents. This product was homogeneous by NMR and TLC (Rf = 0.40) and had $[\alpha]_D$ = -7.3° (C = 0.6, H_2O).

NMR data for the amine **209** : ^{1}H-NMR data δ (D_2O, referenced to HOD at 4.828 ppm): 8.45 (s, 1H, HCO_2^-), 7.01 (d, 2H, $J_{o,M}$ = 9.0 Hz, H_{meta}), 6.83 (d, 2H, H_{ortho}), 5.01 (d, 1H, $J_{1,2}$ = 7.8 Hz, anomeric), 4.74 (d, 1H, $J_{1,2}$ = 7.7 Hz, anomeric), 4.47 (d, 1H, $J_{1,2}$ = 7.3 Hz, anomeric), 4.45 (d, 1H, $J_{1,2}$ = 7.6 Hz, anomeric), 4.17 (dd, 1H, $J_{3,4}$ = 3.0, $J_{4,5} \leq 1$ Hz, H_4 Gal), 3.5 - 4.0 (mult, 23H), 2.03 (s, 3H, NAc). ^{13}C-NMR δ (D_2O, referenced to MeOH at 48.97 ppm): 175.1 (C=O, NHAc), 171.1 (C=O, HCO_2^-), 161.1 (C_{ipso}), 142.0 (C_{para}), 118.3 (C_{meta}), 117.6 (C_{ortho}), 103.6, 103.1, 102.7 and 101.3 (4 C_1), 82.2, 78.3, 75.4, 74.3, 75.0 (2C), 74.4, 72.8, 72.6, 70.8, 70.2, 68.6, 68.5, 61.1 (2C), 60.7, 60.6 and 60.0 (4 C_6 including one at 61.1), 54.8 (C_2 GlcNAc), 22.4 (NAc).

NMR data for the acrylamide **210**: ^{1}H-NMR δ (D_2O): 7.45 (d, 2H, $J_{o,m}$ = 9.1 Hz, H_{meta}), 7.15 (d, 2H, H_{ortho}), 6.43 (dd, 1H, J_{cis} = 9.7, J_{trans} = 17.0 Hz, C(O)CH=), 6.33 (dd, 1H, J_{gem} = 1.7 Hz, C=CH_2 trans), 5.88 (dd, 1H, C=CH_2 cis), 5.15 (d, 1H, $J_{1,2}$ = 8.0 Hz, anomeric), 4.74 (d, 1H, $J_{1,2}$ = 8.3 Hz, anomeric), 4.48 (d, 1H, $J_{1,2}$ = 8.3 Hz, anomeric), 4.45 (d, 1H, $J_{1,2}$ = 7.8 Hz, anomeric), 4.17 (dd, 1H, $J_{3,4}$ = 3.0, $J_{4,5} \leq 1$ Hz, H_4 Gal), 3.4 - 4.0 (mult, 23H), 2.04 (s, 3H, NAc). ^{13}C-NMR δ (D_2O): 175.9 (C=O, NAc), 167.8 (C=O, acrylamide), 154.9 (C_{ipso}), 131.1 (C_{para}), 129.2 (-CH=), 127.3 (=CH_2), 124.6 (C_{meta}), 118.0 (C_{ortho}), 104.3, 103.8, 103.4 and 101.1 (4 C_1), 82.9 (2C), 78.9, 76.1 (2C), 75.8 (2C), 75.1, 73.4 (2C), 71.6, 70.9, 64.4, 69.3, 69.2, 61.9 (2C), 61.4, 60.8 (4 C_6), 55.6 (C_2 GlcNAc), 23.1 (CH_3, NHAc).

Glycopolymer conjugates **197**, **202** *and* **207**

T-antigen glycopolymer **197**: The *N*-acryloylated precursor **195** (5.0 mg, 9.5 μmol) and acrylamide (10.4 mg, 146 μmol) were dissolved in 200 μL of deoxygenated water under a nitrogen atmosphere. TMEDA (2 μL) followed by 5 μL of ammonium persulfate (50mg/mL) were then added and the reaction mixture was left to stir overnight at room temperature. Dilution, and dialysis, first from 0.5mM to remove TMEDA followed by distilled water, gave 3.0 mg (19%) of **197** after lyophilization. ^{1}H-NMR spectroscopy indicated a 1:23 molar ratio of hapten to acrylamide in the isolated glycopolymer (see text).

ABH type 2 antigen glycopolymer **202**: Following the identical procedure as described for **197**, 3.4 mg (4.65 μmol) of **200** and 6.5 mg (91.5 μmol) of acrylamide gave 9.9 mg (quant.) of lyophilized **202**. Hapten content was assayed by ^{1}H-NMR spectroscopy and indicated a 1:40 molar incorporation ratio of **200** to acrylamide (see text).

ABH type 1 antigen glycopolymer **207**: Following the identical procedure as above, 2.0 mg (2.2 μmol) of **205** and 3.9 mg (55 μmol) of acrylamide were used to generate 5.1 mg (86%) of **207**. ^{1}H-NMR analysis indicated a 1:20 molar incorporation ratio of **205** to acrylamide (see text).

biotin /Lacto-N-*tetraose-ø-CPA glycopolymer* **212**: The Lacto- N-tetraose tetrasaccharide derivative **210** (12.4, mg, 14.5 μmol) and 15.5 mg (220 μmol) of acrylamide were partially dissolved in 250 μL of deoxygenated water. The biotin monomer **213** was then added as 125 μL volume of a 24.5 mg/mL solution in DMSO. To the now

homogeneous solution was added 4 μL of TMEDA followed by 5 μL of ammonium persulfate (60 mg/mL). The terpolymerization reaction was left stirring for 2.5 hours after which it was processed as above. The glycopolymer **212** was obtained in 26.1 mg yield (94%). ^{1}H-NMR (D_2O) analysis of the glycopolymer indicated a 1:1:15 molar incorporation ratio of biotin:Lacto- N-tetraose:acrylamide incorporation in **212**.

Tetanus Toxoid (TT) protein purification

A 4.0 mL aliquot of a prepurified tetanus toxoid preparation of unknown concentration (IAF-Biovac, lot AT-18) was diluted to 10 mL with distilled water and dialyzed twice against 5L volumes of distilled water overnight. After the second dialysis, the precipitated material 27 mg (lyophilized) was removed by centrifugation. The combined supernatants were lyophilized and gave 46 mg of soluble TT. The soluble TT fraction was assayed by SDS gel electrophoresis and was shown to contain mainly monomeric TT (150kDa) with almost no higher weight aggregates present. It was kept as a lyophilized solid at 4 °C prior to conjugations. This material was always soluble in water and did not denature or aggregate even after 4 days in 0.2M carbonate buffer pH 10.0 at room temperature.

TT protein conjugates **196, 201, 206, 211.**

T-antigen-TT conjugate **196** : To 2.0 mg (4 μmol) of **193** was added 5.1 mg of purified tetanus toxoid (2 μmol L-lysine) in 500 μL of 0.2M carbonate buffer pH 10.0. The suspension was homogenized by mixing on a Vortex mixer and left at room temperature for 72 hours. The reaction mixture was diluted to 2.5 mL and then centrifuged at 14,000 g in a Fischer Microcentrifuge Model 235B for 5 minutes. The homogeneous mixture was then dialysed against distilled water (4 x 1L) and lyophilized to give 5.8 mg of protein conjugate **196**.

ABH type 2 antigen-TT conjugate **201**: Conjugate **201** was prepared as described above for **196**. In this case however, 5.1 mg of purified TT (2μmol L-lysine) and 2.9 mg (4 μmol) of **200**, homogenized in 100 μL of buffer, was used. Lyophilization gave 7.9 mg of conjugate **201**.

ABH type 1 antigen-TT conjugate **206** : Conjugate **206** was prepared using the same procedure as above. In this case, 6.0 mg of purified TT (2.4 μmol L-lysine) was treated with 3.9 mg (4.4 μmol) of **205** in 750 μL of buffer for 96 hours. Processing as above gave 8.3 mg of **206**. Lyophilization of a primary dialysate (50 mL, 10 hours), after treatment with an excess of Amberlite mixed bed resin (H^+/OH^-; 30 min, 25 °C) to remove salts, gave 2.0 mg of recuperated **205**, pure by TLC.

Lacto- N*-tetraose-TT conjugate* **207**: Conjugate **207** was prepared as described above for the other TT conjugates. In this case, 3.1 mg (3.6 μmol) of **210** and 7.6 mg of purified TT (3.1 μmol L-lysine) homogenized in 750 μL of buffer was used. After 72 hours, the conjugate was processed as usual. Lyophilization gave 10.3 mg of **207**.

9. Poly-L-lysine as a macromolecular carrier. Lactose and colominic acid glycoconjugates by Michael addition.

Until this point, the synthesis of new water-soluble glycoconjugates has focussed on the use of natural proteins (neoglycoproteins) and synthetic polymers (glycopolymers) as carriers for carbohydrate haptens of interest. This chapter presents the use of semisynthetic poly-L-lysine as a water-soluble, macromolecular carrier. As a carrier, poly-L-lysine has properties intermediate to those of natural proteins and synthetic polymers. Unlike natural proteins which are comprised of various combinations of up to 20 different amino acids, poly-L-lysine is a homopolymer. The homogeneous composition of poly-L-lysine allows for the preparation of glycoconjugates for which structural compositions can be determined with relative ease with methods such as NMR spectroscopy. Yet, unlike synthetic copolyacrylamide glycopolymers, poly-L-lysine is easily metabolized by humans[241]. It is also poorly immunogenic. Thus, glycopolymers of poly-L-lysine have potential use as targetted drug delivery vehicles if one were to attach a drug to such a glycopolymer. Poly-L-lysine has already proven effective as a drug carrier to combat tumor cells simply because it is more quickly absorbed by these cells through endocytocis[242]. An additional advantage of using poly-L-lysine as carrier molecule is its commercial availability in a wide range of molecular weight fractions ranging from 1 kDa to more than 300 kDa (Sigma). This allows for the preparation of glycoconjugates of known and predetermined molecular weights to suit desired needs[243]. Another

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241 I.G. Donamura and O. Vogl, *Polymeric Drugs, Academic Press* , New York, 1978.

242 a) H. J-P. and W.C. Shen *Proc. Natl. Acad. Sci. USA, 75* , 3867 (1978); *78* , 7589 (1981); b) B.C.F. Chu and S.B. Howell, *Biochem. Pharmacol., 30* , 2545 (1981), *31* , 3513 (1982); c) A. Rorowsky, R.A. Forsch, J. Galivan, S.S. Susten and J.H. Freicheim, *Mol. Pharmacol., 27* , 141 (1985).

243 In this study, the poly-L-lysine used was purchased (Sigma) as a hydrobromide salt preparation of low polydispersity (Mw/Mn = 1.1) and of intermediate molecular weight (Mw = 70 kDa, degree of polymerization = 330)

advantage poly-L-Lysine may have over natural proteins, but which was not evaluated, is the potential for performing conjugations by Michael addition or even reductive amination (Chapter 7) at more elevated temperatures than commonly used for proteins, in order to increase reaction rates. Unlike proteins, poly-L-lysine does not possess a folded structure which can be denatured with heat.

Poly-L-lysine (**214**) is well suited for the preparation of glycopolymers. It has, as reactive sites, primary amine groups which are found, unhindered, at the end of the carbon chain (ε-amino groups). Thus, poly-L-lysine already contains within its structure a spacer of moderate length which separates any attached hapten from the polymer backbone. In this study, two different poly-L-lysine glycopolymers have been prepared by Michael addition of lysine terminal ε-amino groups to acrylamide containing carbohydrate and biotin derivatives. In addition to providing a new type of glycopolymer system, this work brings aditional evidence that acrylamide containing carbohydrates are conjugated to natural proteins such as BSA or TT in a covalent manner by a 1,4-conjugate addition (Michael addition) of lysine ε-amino groups to the acrylamide function when mixed under alkaline conditions.

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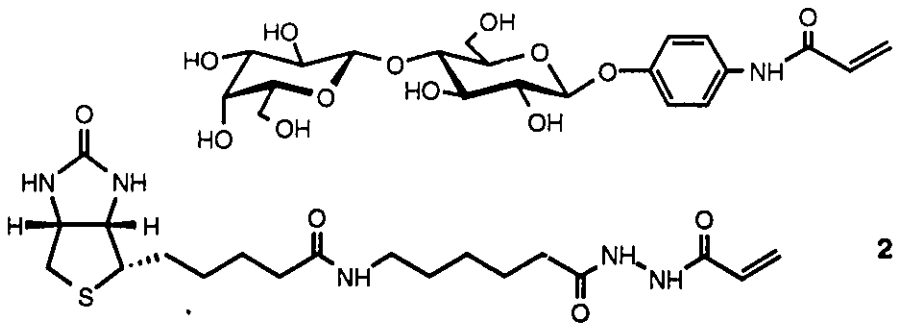
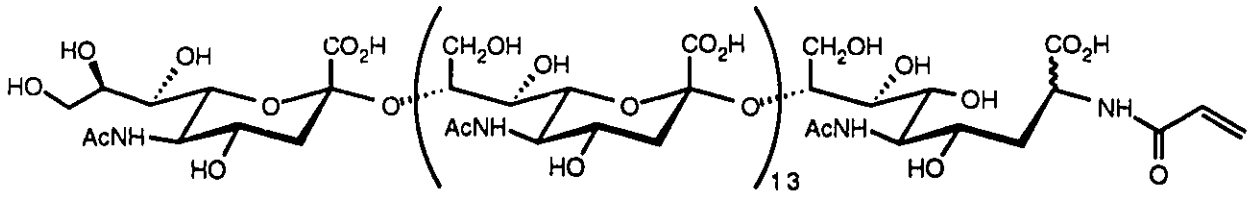
so that the new glycoconjugates could resemble the glycopolymers and BSA neoglycoprotein conjugates prepared so far.



176

213

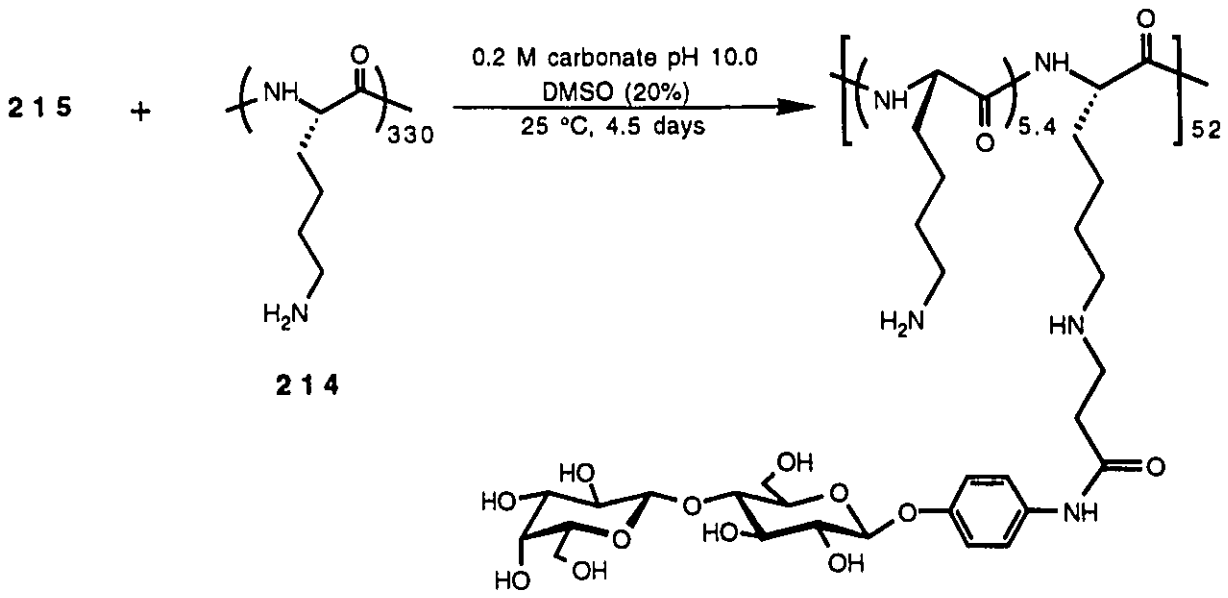


215

The ground work for developing poly-L-lysine based glycopolymers has been performed with the *p*-*N*-acrylamidophenyl β-D-lactoside precursor **176**, as well as a suitable pentadecamer of α-(2-8)-linked sialic acid (Neu5Ac) **215**. Conjugates were prepared easily by mixing *N*-acryloylated derivatives (**176**, **213** and **215**) with poly-L-lysine at room temperature in 0.2M carbonate buffer at pH 10.0. The presence of DMSO (20%) as cosolvent for **176** and **213** had no adverse effect on the conjugation reactions. With simple haptens such as lactose, purification and isolation of poly-L-lysine conjugates can be performed with ease by simple dialysis and freeze drying.

The Lac-Ø-PLL glycopolymer **216** was synthesized as a general entry to the use of poly-L-lysine as glycoconjugate carrier. The $(Neu5Ac)_{15}$/biotin-PLL glycoconjugate **218** was synthesized as part of a collaborative effort for work directed towards the development of a Group B menningoccocal vaccine. The biotinylated glycopolymer **218** was prepared as a diagnostic research tool for the identification and study of antibodies (IgG as well as IgM) that have been successfully raised for the first time against the colominic

acid oligomer which represents the cell surface capsular polysaccharide of the pathogenic *Escherichia coli* (K1 antigen) and group B *Neissera meningitidis* [244].



Scheme 46
Synthesis of an aryl β-lactose-poly-L-lysine glycopolymer by Michael addition. (average structures shown)

In preparing the Lac-Ø-PLL conjugate (Scheme 46), it was the intention to modify one of every six existing lysine residues on poly-L-lysine by mixing appropriate amounts of **176** and poly-L-lysine hydrobromide and assuming a quantitative conjugation of the added lactoside **176**. A quantitative uptake of **176** seemed feasible considering the results obtained earlier with folded, heterogeneous proteins such as BSA, the large excess of amine groups present during conjugation and the use of an extended reaction time (4.5 days) in a concentrated reaction medium (50 mg PLL/mL 0.2M carbonate pH 10.0 with 20% DMSO). Under these conditions however,

[244] H.J. Jennings, *Adv. Cahohydr. Chem. Biochem.* *41*, 155 (1983).

the isolated product revealed that one out of every 5.4 lysine residues was capped with the lactoside **176**, a sugar density higher than predicted possible. The lactoside incorporation was determined by ^{1}H-NMR spectroscopy by comparing the relative integration intensities of the lactoside's aromatic signals (6.9 - 7.5 ppm) and sugar ring resonanaces (3.5 - 4.0 ppm, H_2 - $H_{6'}$) to peaks belonging to poly-L-lysine (4.3, 2.9 and 1.2 - 2.0 ppm). The hapten's new propanamide signals were found at 2.65 (CH_2-N) and 2.0 ppm (CH_2-C(O)N). The two anomeric protons remain unchanged in their positions with respect to the precursor **176** and are found at 5.15 ppm (H_1) and 4.48 ppm ($H_{1'}$). The ^{1}H-NMR (D_2O) spectrum of the Lac-Ø-PLL conjugate **216** is shown below in Figure 51.

Assuming a quantitative uptake of **176** during conjugation (no unreacted **176** was recovered after lyophilization of the crude conjugate's dialysate), the higher than predicted lactose content in **216** suggests that commercial poly-L-lysine hydrobromide is supplied as a moderately hydrated preparation. This is not specified by the supplier. This material also appears to be hygroscopic. Thus, care should be taken in the future when similar conjugates of specific hapten densities are required. The Lac-Ø-PLL conjugate **216** was found to be very antigenic by a double diffusion assay in agarose gel against both *Ricinus Communis* and *Arachis Hypogaea* lectins while unmodified poly-L-lysine was not (not shown).

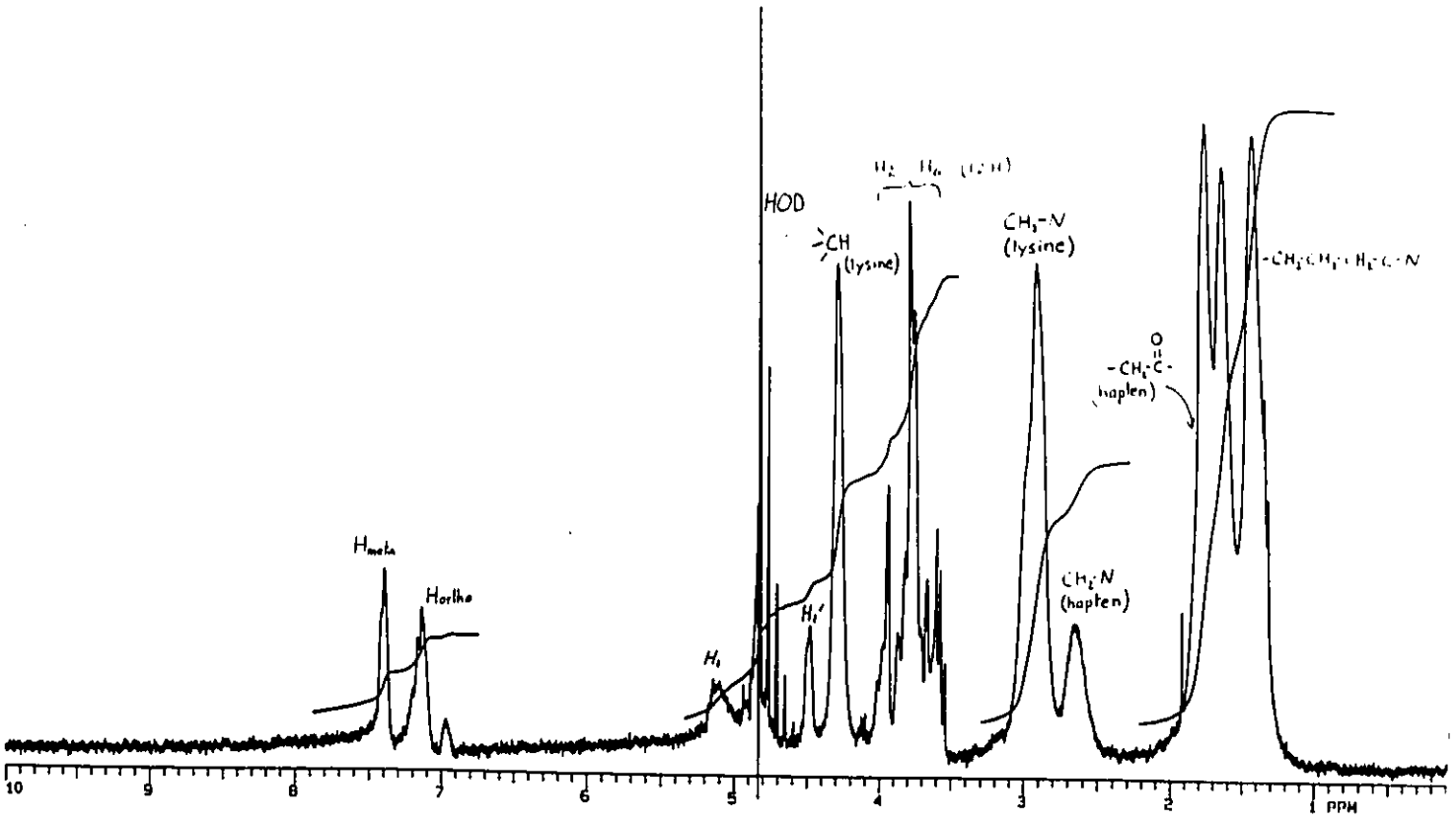


Figure 51 ^{1}H-NMR spectrum (D_2O, 300 MHz) of Lac-Ø-PLL **216**.

The $(Neu5Ac)_{15}$/biotin-PLL conjugate **218** was prepared in two steps (Scheme 47). In the first step, the reductively aminated then acryloylated reducing end[245] of the sialic acid pentadecamer **215** [246] from a GPC fractionated acid digest of colominic acid[247] was conjugated to poly-L-lysine in the hope of modifying 1 of every 50 lysine residues, assuming a quantitative uptake of the acrylamide **215** to give the $(Neu5Ac)_{15}$-PLL conjugate **217**. A 1:50 ratio of

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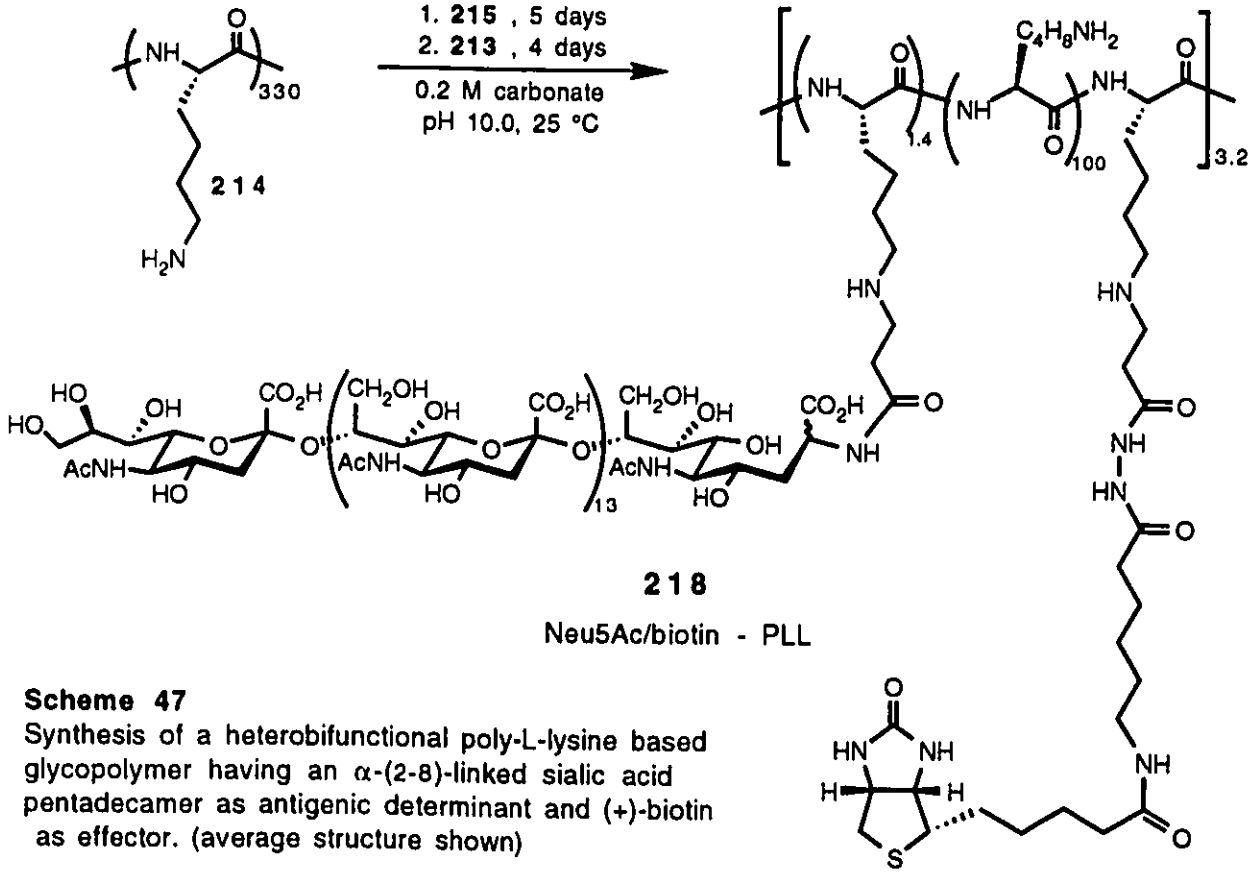
245 R. Roy, E. Katzenellenbogen and H.J. Jennings, *Can. J. Chem. Cell Biol. 62* , 270 (1984).

246 R. Roy and R. Pon, unpublished results. R. Pon, M.Sc. Thesis, Department of Chemistry, University of Ottawa, 1992.

247 R. Roy and R. Pon, *Glycoconjugate J.*, *7* , 3 (1990).

modified:unmodified lysine residues was envisaged for several reasons. First, the conjugate was to be used with purified IgG and IgM antibodies which have much higher binding affinities for oligosaccharide haptens than do lectins (typically ≥ 10^3 times stronger). Second, only a limited amount (5 mg, ~1 μmol) of **215** was available to produce a maximum amount of conjugate. Finally, because of possible anionic repulsion of the highly charged hapten residues, a high hapten loading may not be possible anyway. This last reason is based on previous observations that, unlike neutral sugars, high density sialic acid glycoconjugates are difficult to prepare[248].



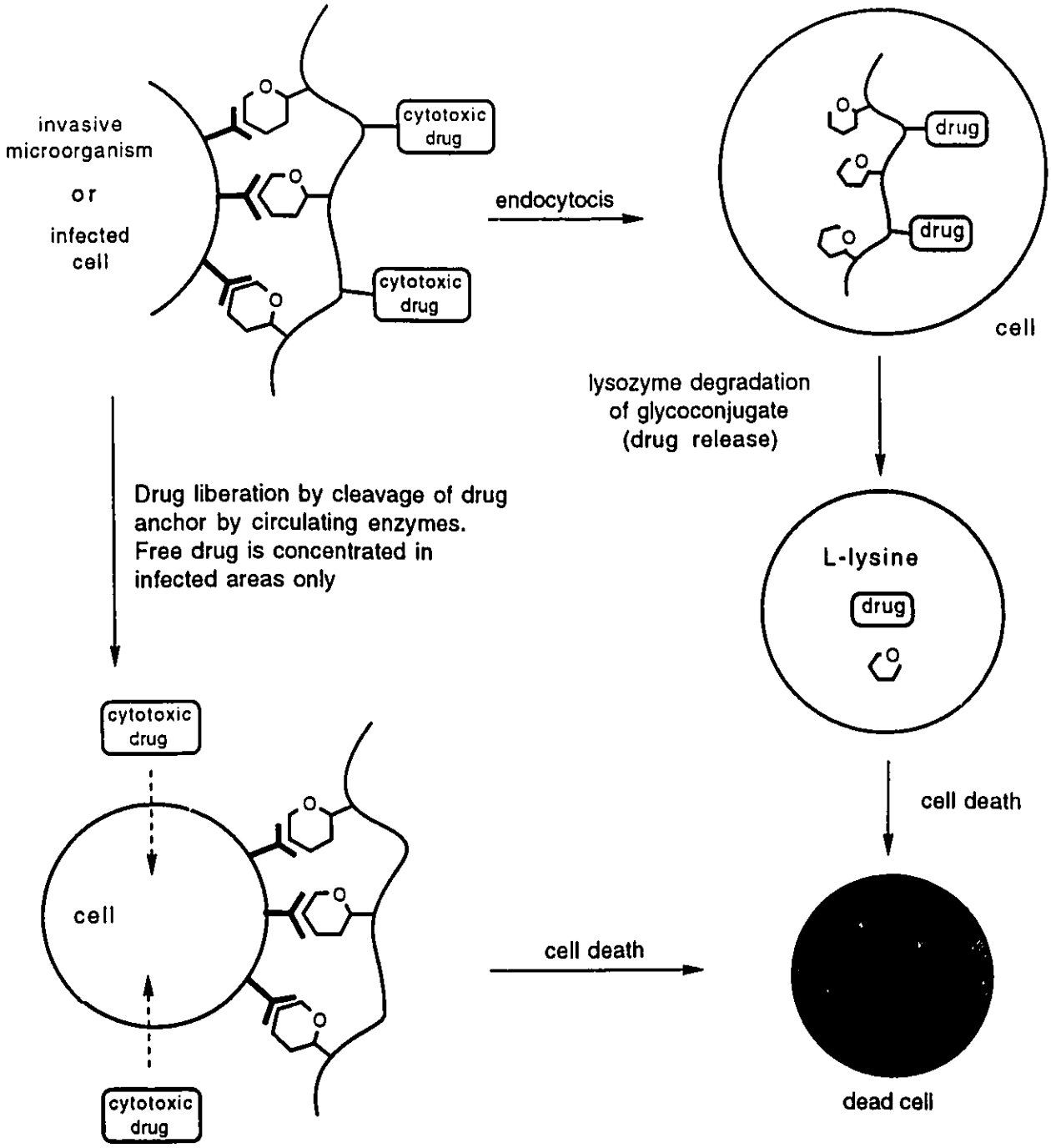
Scheme 47
Synthesis of a heterobifunctional poly-L-lysine based glycopolymer having an α-(2-8)-linked sialic acid pentadecamer as antigenic determinant and (+)-biotin as effector. (average structure shown)

[248] a) R. Roy and C. Laferriere, *Can. J. Chem.*, *68* , 2045 (1990); b) R. Roy and C. Laferriere, *J. Chem.Soc., Chem. Commun.*, 1709 (1990).

In fact, only one of every 70 lysine residues could be modified after 9 days of reaction time when the final heterofunctional $(Neu5Ac)_{15}$/biotin-PLL conjugate **218** was analyzed by ^{1}H-NMR spectroscopy. In this case, the loading density was determined by comparing the *N*-acetyl methyl resonance (2.10 ppm, 15 NAc) to the ε-methylene group of lysine (3.0 ppm) after removing unconjugated **215** from **218** by ion exchange chromatography on Sephadex DEAE-A25 (acetate form) with 0.01M NaCl as eluent. The purification was monitored by HPLC on TSK 4000 SWXL column (7.5 mm x 30 cm) using PBS (0.02M, pH 7.0) containing 0.02% NaN_3 as the mobile phase (1.0 mL/min). Under these conditions, **215** had a retention time of 11.0 min. while the desired conjugate was eluted after 12.0 min. The biotin content on the final conjugate **218** could not be assayed by ^{1}H-NMR spectroscopy due to the low amount of **213** used during preparation (biotin:lysine = 1:100) and the absence of any reporter groups unobstructed by the remaining glycopolymer resonances. Biotin conjugation was performed for 4 days, which has previously been shown to be sufficient for a high conjugation yield. Any unconjugated biotin, possibly present in the crude **218**, was removed by dialysis prior to the anion exchange chromatography purification. The presence of conjugated biotin was confirmed by ELISA using peroxidase labeled avidin (not shown, performed by collaborators) and by the appearance of a pink colouration when a sample was treated with 0.2% acidified ethanolic dimethylamino cinnamaldehyde[253]. The heterobifunctional $(Neu5Ac)_{15}$/biotin-PLL conjugate **218** has been forwarded to colleagues for further investigations. Results of further studies will be presented in due course.

In conclusion, poly-L-lysine has been used to prepare glycopolymers which have potential use in diagnostic applications and as useful non-immunogenic, biocompatible glycoconjugates. Poly-L-lysine glycopolymers have the added advantage over copolyacrylamide-type glycopolymers that secondary effectors, such as biotin for example, can be grafted onto the glycoconjugates after they have been

prepared and have undergone preliminary testing for antigenicity. Poly-L-lysine glycoconjugates could have future potential as cell directed drug delivery vehicles by using carbohydrate haptens specific for existing cell surface receptors and therapeutic drug effectors. The multivalent nature of such conjugates should provide a high affinity toward the targetted cells. Such a strategy could be applied to invasive microorganisms or affected cells in the body (Scheme 48).



Scheme 48 Representation of proposed possible use of biodegradable poly-L-lysine glycoconjugates

Experimental methods

General Methods

The general methods used were as described in previous chapters.

Lac-Ø-PLL conjugate **216**

To a small vial, containing a micro stir bar was added 24.8 mg (118 μmol NH_2) of poly-L-lysine hydrobromide (Sigma, P-2636, lot 81H5541, $Mw_{(vis)}$ = 69,000, $DP_{(vis)}$ = 333, $Mw/Mn_{(sec\text{-}LALLS)}$ = 1.1), 9.7 mg (20 μmol) of p-*N*-acrylamidophenyl β-D-lactoside **176** and 2000 μL of 0.2M carbonate buffer pH 10.0. After stirring for 15 minutes, the lactoside **176** could not be properly dissolved. The solution quickly became homogeneous after adding 500 μL of DMSO. The reaction was left gently stirring for 4.5 days after which a primary dialysis in 25 mL of distilled water was performed. Treatment of this dialysate with mixed bed resin (H^+/HO^-) followed by lyophilization did not provide any detectable, unconjugated **176**. The crude glycopolymer **216** was then exhaustively dialyzed against two 5L volumes of distilled water, and freeze dried. The glycopolymer **216** was isolated as a white, spongy solid in 25.0 mg yield. ^{1}H-NMR (D_2O) analysis (see text and Figure 47) of **216** revealed that glycopolymer **216** contained a 1:5.4 ratio of lactose to unmodified lysine residues.

^{13}C-NMR data δ D_2O (referenced to internal CH_3OH at 48.97 ppm): 173.8 (C=O), 169.9 (C_{ipso}), 132.1 (C_{para}), 123.7 (C_{meta}), 117.2 (C_{ortho}), 103.2 (C_1 Glc), 100.4 (C_1 Gal), 78.3, 75.6, 75.1, 74.4, 72.8 (2C), 71.1, 68.7 (C_2 to C_5 Glc and Gal), 61.2, 60.1 ($2C_6$), 53.6 (C_α lysine), 41.2 (-CH_2-N lactoside), 39.4 (C_ε lysine), 30.7 (CH_2 lysine), 27.3 (CH_2-C(O)N lactoside), 26.8 and 22.5 ($2CH_2$ lysine).

$(Neu5Ac)_{15}$ /biotin-PLL conjugate **218**

In a small vial was dissolved 5.0 mg (1μmol) of **214** (Mw = 5 kDa)[246] and 11.8 mg (56 μmol) of poly-L-lysine hydrobromide (Sigma, P-2636) in a combination of 50 μL 2M NaOH and 150 μL 0.2M carbonate buffer pH 10.0. This solution was stirred gently for 5.5 days at room temperature, then diluted with 3 mL of water, dialyzed and lyophilized to give crude **217** as a white, spongy solid. At this point a decision was made to incorporate a biotin effector into the glycoconjugate. Thus, **217** was redissolved in 50 μL 2M NaOH and 150 μL 0.2M carbonate buffer pH 10.0. A 30 μL volume of *N*-acryloylated biotinamidocaproyl hydrazide **213** (see next chapter) in DMSO (8.2 mg/mL, 0.58 μmol) was then added, and the solution (pH 10-10.5) gently stirred for 4.0 days. After dilution, dialysis and freeze drying, crude **218** was obtained as a white, spongy solid. HPLC analysis of the crude conjugate **218** on a TSK 4000 SWXL column (7.5 mm x 30 cm) using PBS (0.2M, pH 7.0) containing 0.02% NaN_3 as the mobile phase (1.0 mL/min.) revealed two peaks at 11.0 and 12.0 min. The peak centered at 11.0 min. was identified as unreacted poly sialic acid acrylamide **214** by comparison to an authentic sample. The crude conjugate **218** was therefore purified on Sephadex DEAE-A25 resin (acetate form) on a small column (0.5 x 10 cm). The purified $(Neu5Ac)_{15}$/biotin-PLL conjugate **218** was obtained after eluting with 5 mM NaCl, pH 7.2. The purity of **218** was assessed by the complete disappearance of the peak at 11.0 min. in the HPLC profile. Salt free **218** (10.1 mg) was obtained after dialysis and freeze drying.

The poly α-(2-8)-Neu5Ac content in **218** was assayed by ^{1}H-NMR spectroscopy in D_2O by comparing integration intensities for the *N*-acetyl methyl resonance of Neu5Ac at 2.10 ppm and the lysine's unobstructed ε methylene resonance found at 3.00 ppm. The biotin content could not be assayed by ^{1}H-NMR spectroscopy due to the low amount of **213** used (1% by mol) and the absence of a clearly resolved reporter group signal. Biotin conjugation was however demonstrated by ELISA using peroxidase labeled avidin (not shown,

performed by Dr. Anna Romanowska) as well by reacting a small aqueous sample of **218** with 0.2% ethanolic dimethylamino cinnamaldehyde[253]. This last test proved positive by giving a pink colour when a small aqueous sample of **218** was adsorbed to a piece of TLC plate, sprayed with the above reagent then heated. Poly-L-lysine or **214** failed to give any reaction under the same conditions.

10. Custom designed glycopolymer synthesis by terpolymerization[249]

The strategy of copolymerizing acrylamides with various acryloylated carbohydrate derivatives to produce water-soluble glycopolymers has been extended to the production of heterobifunctional glycopolymers by the addition of acryloylated effector molecules as tertiary monomers during polymerization. Such a three component copolymerization (terpolymerization) strategy has been successfully used for the first time to produce various glyco(ter)polymers possessing a variety of interesting and useful properties (Figure 52). Different tertiary effector monomers have been synthesized and copolymerized to confer diverse and desirable immuno- and physico-chemical properties to synthetic polymer glycoconjugates. In particular, carbohydrate and biotin effectors have been added as biochemical probes, while a phenolated effector has been added as a radioiodinatable tracer probe. The addition of a long chain aliphatic hydrocarbon effector has provided a glycopolymer with lipophilic character which has enhanced properties as a coating antigen during solid phase enzyme assays.

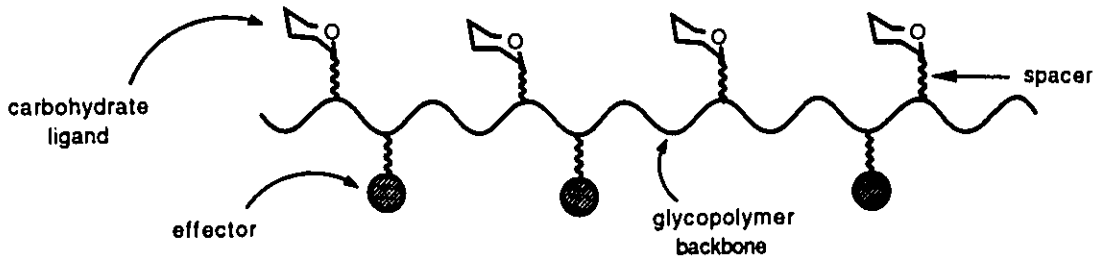


Figure 52 Schematic representation of heterobifunctional glycopolymers.

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[249] Presented in part at the 16th International Carbohydrate Symposium, July 5 - 10, 1992, Paris, France and in R. Roy, F. Tropper and A. Romanowska, *J. Chem. Soc. Chem. Commun.*, (in press).

10.1 Effector comonomer preparation

The first example of a terpolymerized heterobifunctional glycopolymer was demonstrated in section 8.3 when (+)-biotin was incorporated into a lacto-N-tetraose copolyacrylamide glycopolymer (**212**). An analogous heterobifunctional poly-L-lysine glycopolymer $(Neu5Ac)_{15}$/biotin-PLL (**218**), produced by Michael addition, was also discussed in Chapter 9. Other biotinylated glycopolymers have also been synthesized and are discussed in this chapter. Biotin was chosen as an obvious candidate for making heterobifunctional glycopolymers since many useful avidin or streptavidin-labeled conjugates are commercially available[250]. The affinity of these proteins to biotin are among the highest known ($K_D=10^{-15}$)[251]. Therefore, these couples represent powerful diagnostic potential. Other effectors such as *N*-acryloylated stearylamine and *N*-acryloylated tyramine have been synthesized and incorporated into glycopolymers. The *N*-acryloylated stearylamine device **222** was incorporated in order to provide improved lipophilic properties which make glycopolymers more effective as coating antigens in enzyme linked immunosorbent assays (ELISA or ELLA). The *N*-acryloylated tyramine monomer **223** was introduced to provide glycopolymers on which radioactive iodine labeling can be accomplished under usual conditions[66] by mimicking tyrosine residues of proteins. A glyco(ter)polymer has also been synthesized in which the effector is a second carbohydrate hapten.

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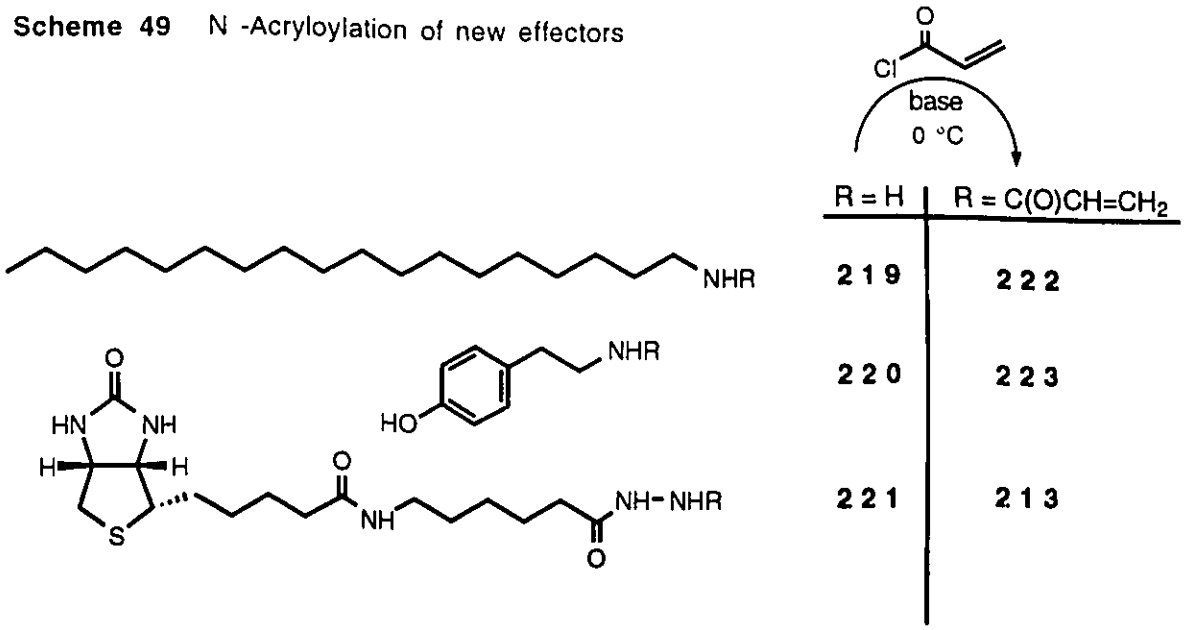
[250] M. Wilchek and E.A. Bayer, *Anal. Biochem.*, *171* , 1 (1988).
[251] J. Green, *Advan. Protein Chem.* , *29* , 85 (1975).

Scheme 49 N -Acryloylation of new effectors

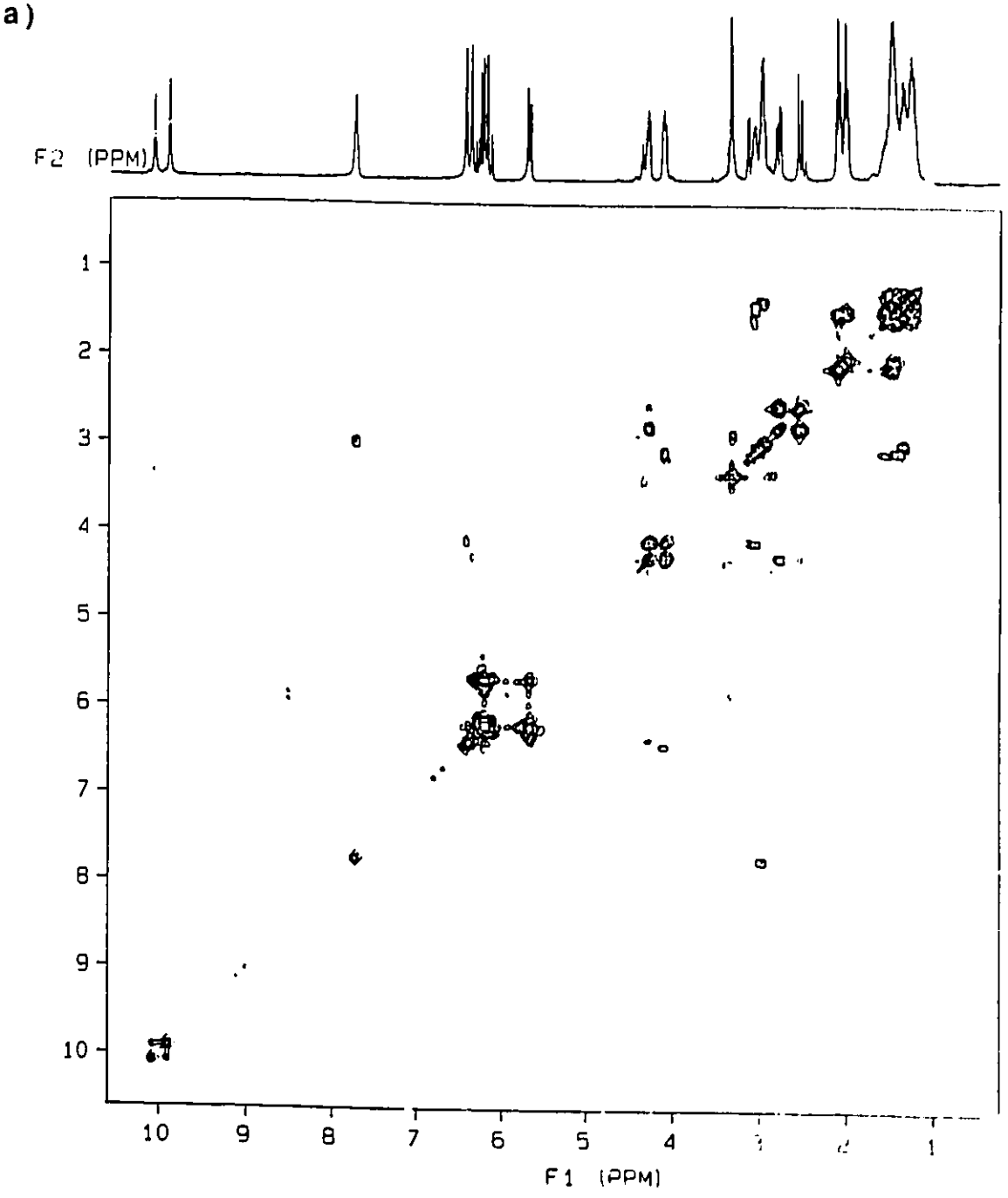


The syntheis of new copolymerizable effector monomers was straightforward. Suitable amine (**219**, **220**) or hydrazide (**221**) derivatives were *N*-acryloylated with acryloyl chloride as acylating agent in the presence of a base (Scheme 49). *N*-acryloylated effectors were chosen since they would have similar polymerization reactivities to acrylamides already used in glycopolymer syntheses and thereby permit greater flexibility in generating glycopolymers of desired compositions simply through the choice of a monomer load formulation. The *N*-stearyl acrylamide derivative **222** was produced in essentially quantitative yield without need for chromatographic purification by performing the acryloylation in methylene chloride in the presence of triethylamine, at 0°C, The *N*-2-(*p*-hydroxyphenyl)ethyl acrylamide derivative **223** was isolated in only 64% yield after acryloylation of tyramine (**220**) in methanol at 0°C. Obvious side reactions such as *O*-acylation and/or ring acylation were the most likely sources of product loss, in this case. Acryloylation of the commercially available biotinamidocaproylhydrazide **221** was performed in methanol at 0°C in the presence of anion (HO^-) exchange resin and gave **213** in a total

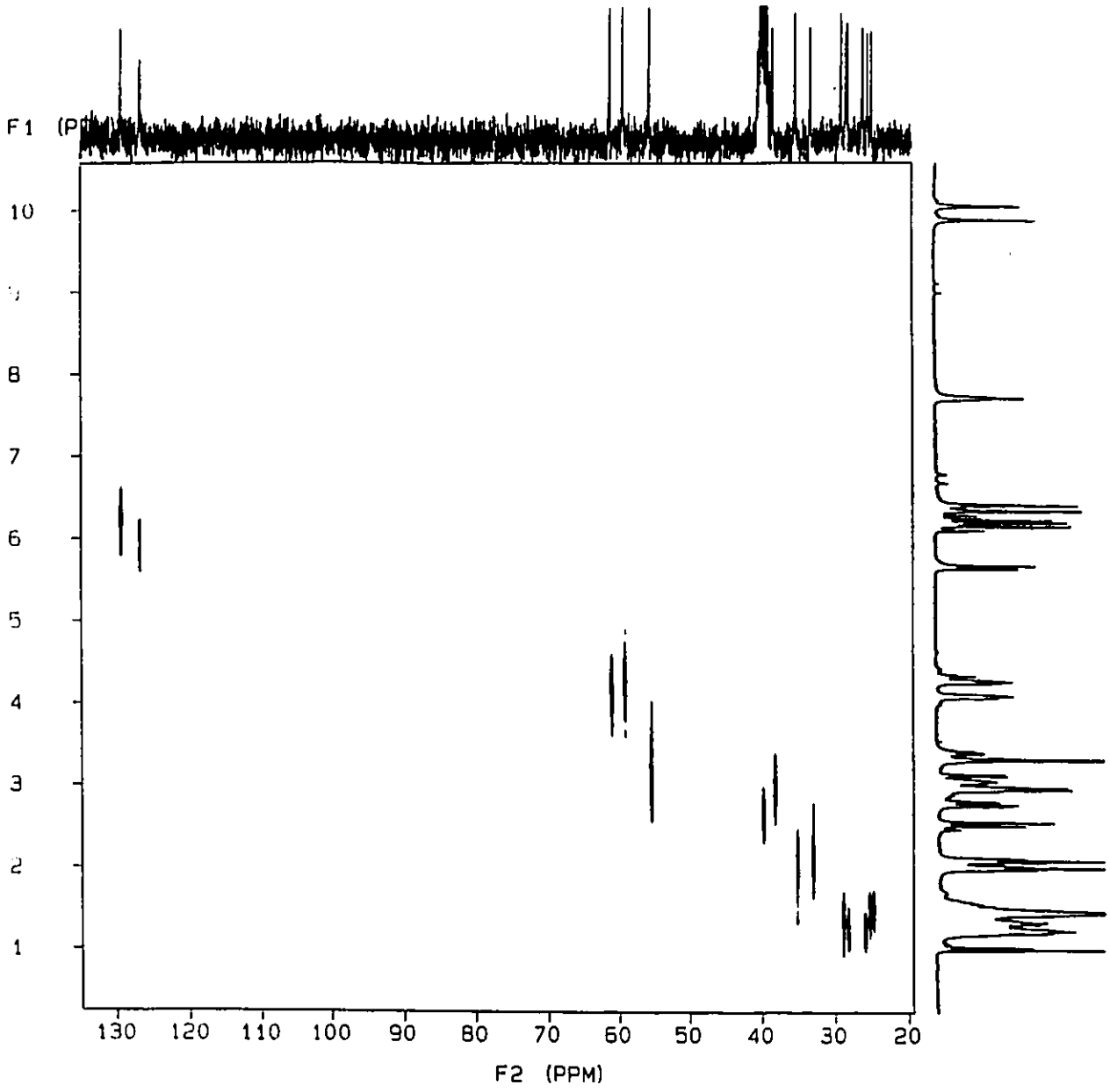
yield of 78% after partial crystallization and preparative TLC purification. In this case, the impure hydrazide (only 90-95% pure by TLC) and the poor solubility of **221** and **213** in common solvents[252] could be blamed for lowering the yield. The *N*-acryloylated biotin monomer **213** was fully characterized by NMR spectroscopy. Two-dimensional ^{1}H-^{1}H COSY as well as 1-, 2- and 3-bond ^{1}H-^{13}C HETCOR experiments were used to fully assign all resonances (see experimental section). A COSY and partial 1-bond ^{1}H-^{13}C HETCOR spectrum of **213** is shown below in Figure 53.

[252]Biotin has poor solubility properties in most solvents at 25 °C (22mg/100mL water, 80mg/100mL alcohol). M. Windholz (ed.), The Merck Index, 10th Edition, Merck & CO., Rahway, N.J., 1983.

Figure 53 a) ^{1}H-^{1}H COSY spectrum of **213** (connectivities have been omitted for clarity).
b) 1-bond ^{1}H-^{13}C HETCOR spectrum of **213**.
(performed on a Varian XL-300 spectrometer for solutions in $DMSOd_6$).



b)

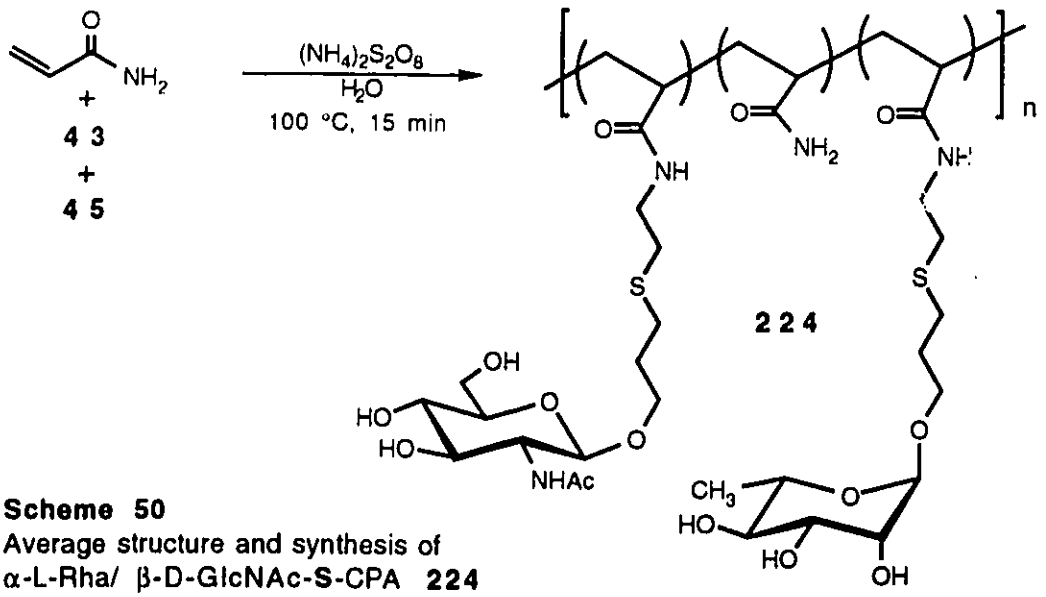


10.2 Glyco(ter)polymer preparation.

Glyco(ter)polymers were prepared in the same fashion as simple glycopolymers discussed previously. Selected monomer ratios of the three components: acrylamide, effector and carbohydrate reagents were subjected to radical initiated polymerizations in deoxygenated water using ammonium persulfate at 100 °C or at room temperature with added TMEDA. Except for the stearylated glycoconjugate, the composition of the isolated glyco(ter)polymers were always found to be identical to the monomer load ratios used when analyzed by ^{1}H-NMR spectroscopy. As with previous conjugates, simple dialysis against water followed by freeze drying gave glyco(ter)polymers as spongy, white solids.

10.2.1 Dual carbohydrate glycopolymer.

With a wide range of *N*-acryloylated carbohydrate derivatives in hand, a glyco(ter)polymer containing two different carbohydrate haptens was an obvious choice for the first attempt at preparing bifunctional glycopolymers. Thus, the bispecific α-L-Rha/β-D-GlcNAc-**S**-CPA glyco(ter)polymer **224** was synthesized from 3-(2-*N*-acrylamidoethylthio)propyl glycoside precursors **43** (α-L-Rha) and **45** (β-D-GlcNAc) in 59% yield by heat (100 °C) catalyzed initiation with ammonium persulfate in water. A monomer incorporation of 1:1.8:5.2 of **43**:**45**:acrlyamide was shown by ^{1}H-NMR to make up the new glycoconjugate's structural composition. This determination was easily carried out by comparing integration intensities of methyl reporter groups belonging to each hapten (2.0ppm, s, NAc, GlcNAc and 1.3 ppm, d, $J_{5,6}$ = 6 Hz, CH_3 Rha) to the backbone methylene and methine signals (1.6 and 2.2 ppm, broad). A correction for the obscured spacer methylene (H_b) found within the backbone methylene resonances was made, as described previously in Chapter 4.



Scheme 50
Average structure and synthesis of α-L-Rha/ β-D-GlcNAc-**S**-CPA **224** by terpolymerization

The bispecificity of the dual α-L-Rha/β-D-GlcNAc-**S**-CPA glyco(ter)polymer **224** was demonstrated with various lectins and antibodies, each specific for either of the two carbohydrate haptens during double radial diffusion assays in agarose gel. The behaviour of **224** towards the lectins of *Triticum Vulgaris* (WGA) and *Ricinus communis* , or rabbit-raised IgG against the capsular polysaccharide of the β-hemolytic Group A streptococci and the serotype 23 capsular polysaccharide of *Streptococcus pneumoniae* (Pn23) was as described earlier for the individual α-L-Rha-**S**-CPA (**46**) and β-D-GlcNAc-**S**-CPA (**52**) glycopolymers (Chapter 4). The new bifunctional glyco(ter)polymer **224** gave sharp and intense precipitin bands with all these carbohydrate binding proteins while showing no recognition by the *N*-acetylgalactosamine specific lectin from *Dolichus biflorus* (assays not shown). The affinity of **224** for WGA and the Pn23 antibodies was quantitated by immunoprecipitation experiments (Figures 54 and 55). The results show that the presence of a secondary carbohydrate hapten on the glycopolymer does not adversely affect the binding interaction of the other. Thus, only 10 μg of **224** was required to effectively precipitate 20 μg of WGA in

300 μL of PBS while only 20 μg of **224** were required for maximum agglutination of 100 μg of Pn23 rabbit antibody serum diluted to 300 μL with PBS. These values are very similar to those found earlier for the individual and analogous α-L-Rha-**S**-CPA and β-D-GlcNAc-**S**-CPA glycopolymers (see Figures 15 and 17, Chapter 4).

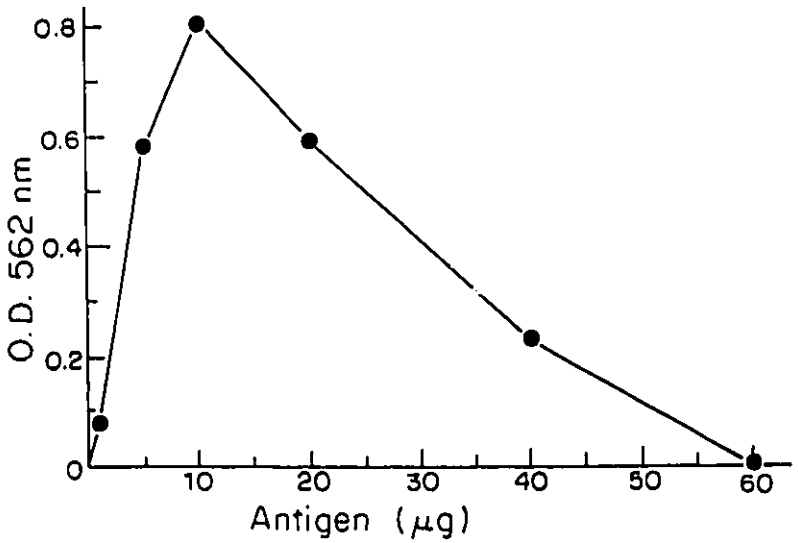


Figure 54 Quantitative precipitation of WGA (20 μg/300 μL) PBS by **224**.

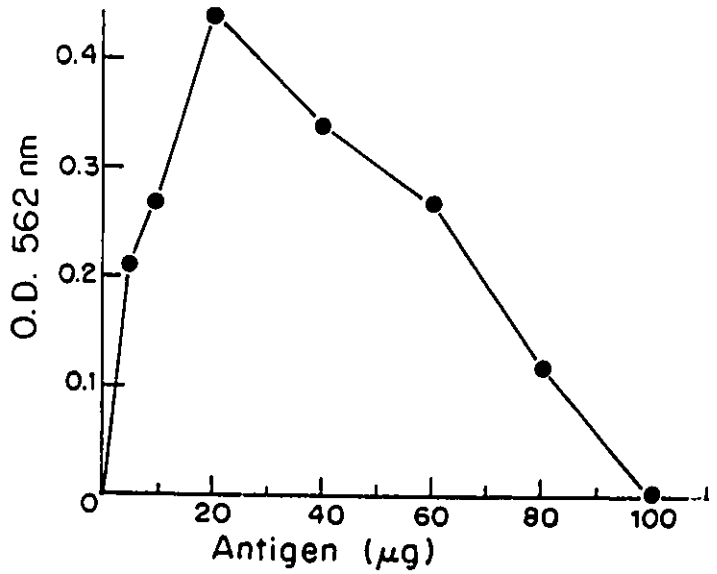
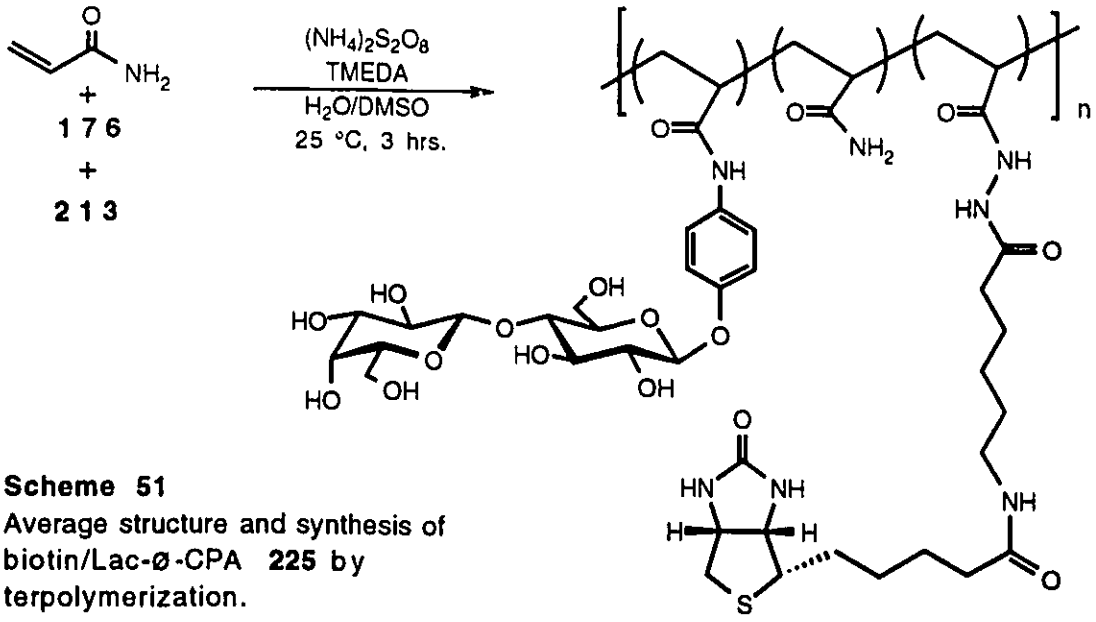


Figure 55 Quantitative precipitation of rhamnose specific antibodies from 100 μL of Pn23 rabbit-raised IgG antibody serum in 300 μL in PBS.

The strategy of incorporating two different carbohydrate haptens within a single glycopolymer by terpolymerization (or by Michael addition to poly-L-lysine, Chapter 9) has proven successful in producing a model biospecific antigen. The strategy described here has potential as a method for preparing carbohydrate based multivalent, bispecific immunoadhesins. As in this case, one carbohydrate ligand can be directed to lectins on a cell surface while the other carbohydrate hapten can be recognized by an antibody.

10.2.2 Biotinylated glyco(ter)polymers.

Glycopolymers contining an additional (+)-biotin ligand have been discussed previously in the case of a lacto-N-tetraose glycopolymer antigen **212**. As model studies previous to the synthesis of **212**, glyco(ter)polymers containing (+) biotin were synthesized with β-D-lactoside and β-D-*N*-acetylglucosaminide haptens. For such glyco(ter)polymers, the addition of DMSO to the polymerization solution was required to properly dissolve the biotin monomer **213**. The presence of DMSO did not adversely affect the outcome of the polymerization when performed at room temperature after initiation with ammonium persulfate and TMEDA. In all cases where **213** was used as comonomer in terpolymerization, the relative amount of **213** to the other monomers within the isolated glycoconjugate was always identical to what was loaded into the reactions. Thus, *N*-acryloylation of hydrazides provides monomers having polymerization reactivities very similar or identical to acrylamides.



Scheme 51
Average structure and synthesis of biotin/Lac-Ø-CPA **225** by terpolymerization.

A dual β-D-lactose/biotin containing glyco(ter)polymer, biotin/Lac-Ø-CPA **225** was synthesized in 57% yield (Mw ≥ 12 kDa) by terpolymerizing acrylamide with the acryloylated biotin monomer **213** and the p-*N*-acrylamidophenyl β-D-lactoside **176** (Scheme 51). The polymerization was carried out in a 2/1 blend of deoxygenated water/DMSO for 3 hours. ^{1}H-NMR spectroscopy was used to evaluate the glyco(ter)polymer's structural composition. Analysis of the proton spectrum, given in Figure 53, indicated a 1:2:10 molar incorporation ratio of biotin:lactose:acrylamide monomers. The glyco(ter)polymer's composition was identical to the reaction formulation. This determination was carried out by comparing characteristic resonances of **213** found unobstructed between 2.6 and 3.4 ppm (see experimental for **213**) to the aromatic resonances of the lactoside hapten (7.0 - 7.5 ppm) as well as the anomeric signals found at 5.14 ppm (br, H-1 Glc) and 4.50 (d, $J_{1,2}$ = 7.5 Hz, H-1 Gal) to the glycopolymer backbone resonances near 2.3 and 1.7 ppm (methines and methylenes). Corrections were made for 8 biotin methylene resonances found hidden under the backbone signal. The presence of biotin in this and similar terpolymers can also be

confirmed quickly by reacting a small sample of purified glycopolymer, either as an aqueous drop on a piece of TLC plate or in solution in a test tube, with 0.1-0.2% dimethylamino cinnamaldehyde in acidified ethanol. After heating briefly, the presence of biotin is indicated by the appearance of a bright pink colouration. Glycopolymers deficient in biotin do not react with this commercial reagent.

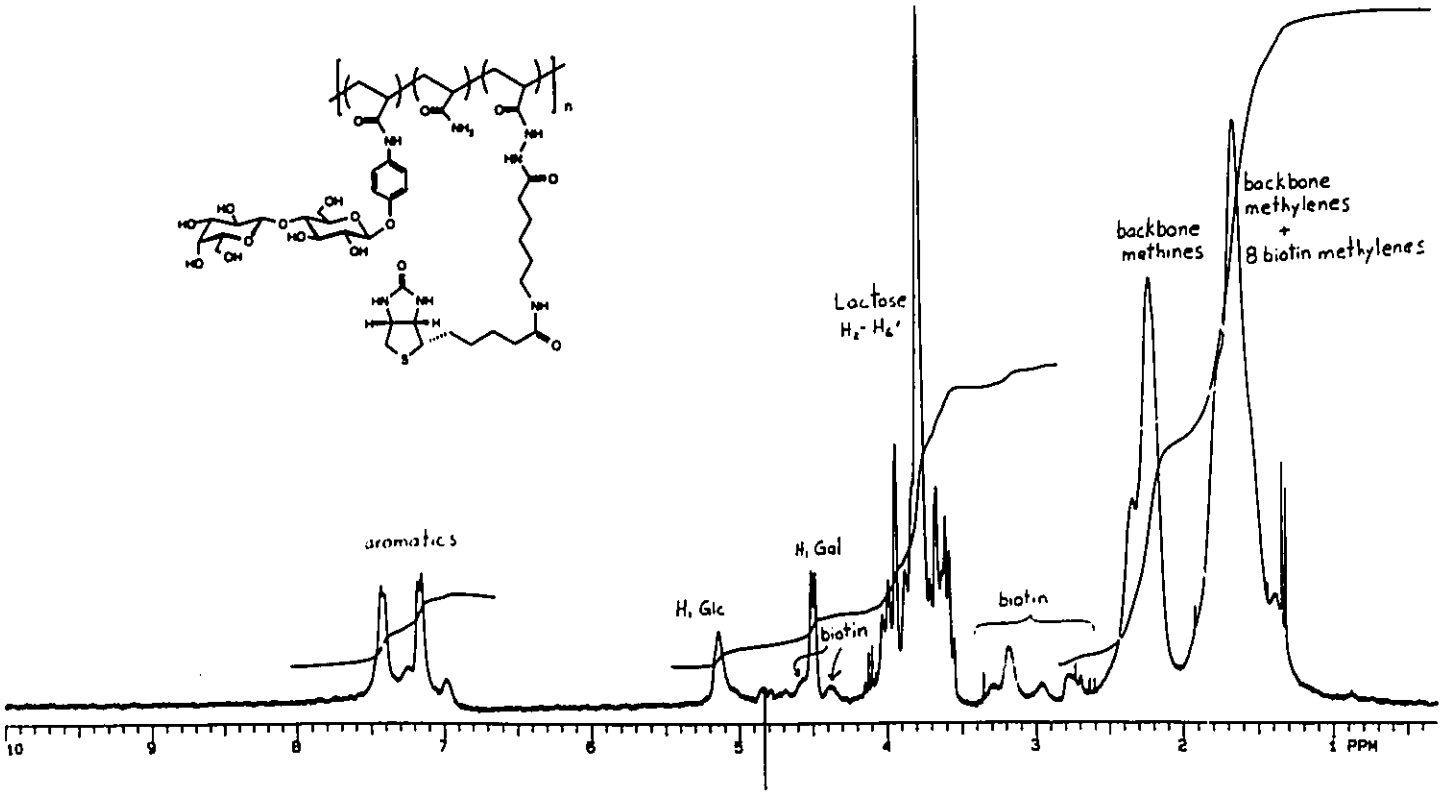


Figure 56 ^{1}H-NMR spectrum (D_2O, 300 MHz) of the biotin/Lac-Ø-CPA glyco(ter)polymer **225**

The biotin/Lac-Ø-CPA glyco(ter)polymer **225** was assayed for dual antigenicity by radial diffusion through agarose gel against streptavidin (specific for (+)-biotin) and peanut lectin (specific for lactose). Conjugate **225** showed sharp precipitin bands with both proteins. The use of **225** as immunoadhesin between the above binding protein failed to give conclusive results as it appears that streptavidin is a glycoprotein which is also recognized by the peanut lectin from *Arachis Hypogaea* . This was confirmed by an agarose gel diffusion assay between both proteins. A scaled representation of a radial diffusion assay demonstrating all the above interactions is shown in Figure 57.

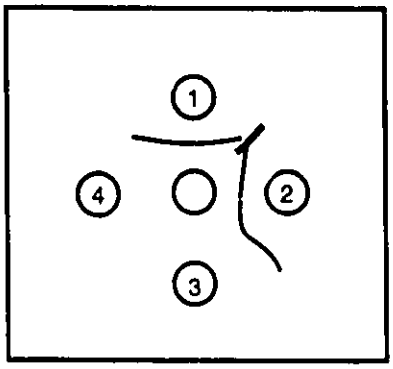
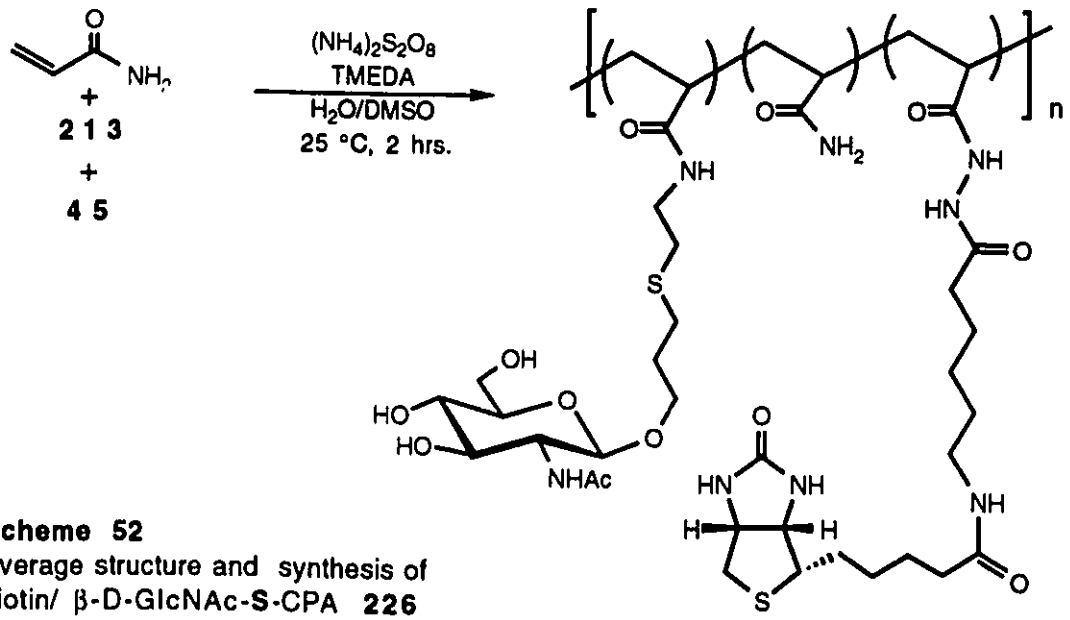


Figure 57 Scaled representation of an agarose gel diffusion assay of **225** (center well) against streptavidin (well 1, and peanut lectin (well 2). Well 3 and 4 contain β-D-Lac-Ø-CPA **185b**. All materials were assayed at 1 mg/mL in PBS.

In addition to **225**, a biotin containing glyco(ter)polymer was also prepared with the β-D-*N*-acetylglucosaminide monomer **45** (Scheme 52). In this case, the biotin/β-D-GlcNAc-**S**-CPA **226** was prepared in 96% yield. The terpolymerization was carried out and DMSO with a total monomer concentration of 1M. After initiation with 0.2% (by mol) of ammonium persulfate, the polymerization gave clear and very viscous solution after only 1.5 hours. ^{1}H-NMR analysis of the purified biotin/β-D-GlcNAc-**S**-CPA (**226**) conjugate revealed a 1:5:50 molar incorporation ratio of biotin:β-D-GlcNAc:acrylamide

monomers, an identical ratio to the reaction load. This determination was performed by comparing relative integration intensities from the sharp *N*-acetyl methyl signal (2.03 ppm) of the sugar resonances as described for the analogous biotin deficient β-D-GlcNAc-**S**-CPA **52** (Figure 13, Chapter 4). Biotin content relative to the sugar was determined in the anomeric proton region of the ^{1}H-NMR spectrum where the biotin's bridgehead proton signals are also visible.

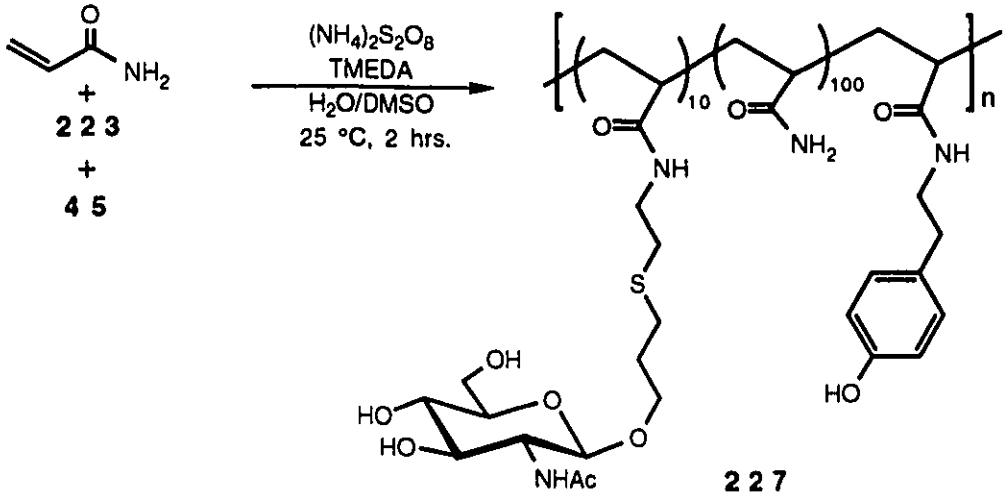


Scheme 52
Average structure and synthesis of biotin/ β-D-GlcNAc-**S**-CPA **226** by terpolymerization.

The antigenicity of **226** was tested by a double radial diffusion assay in agarose gel against streptavidin (for biotin) and WGA (for GlcNAc). The glyco(ter)polymer **226** produced sharp precipitin bands with both proteins when diffused at original concentrations of 1 mg/mL. The simple β-D-GlcNAc-**S**-CPA **53** did not agglutinate streptavidin. The serological properties of the biotinylated glyco(ter)polymers are still being evaluated and results will be presented by colleagues in due course.

10.2.3 Phenolated glycopolymers

Radioiodinated protein conjugates have long been used in immunochemical studies[66]. Radioactive labeling techniques are popular because of their great sensitivity. To demonstrate that suitably functionalized glycopolymer conjugates can also be prepared for potential use in radioimmunoassays[66], a phenolated glycopolymer **227** was synthesized (Scheme 53).



Scheme 53
Average structure and synthesis of N-tyramide/ β-D-GlcNAc-S-CPA **227** by terpolymerization.

Thus, the glucosaminide **45** and the *N*-acryloylated tyramine **223** were terpolymerized with acrylamide to give an *N*-tyramide containing glycopolymer **227**. The *N*-tyramide/β-D-GlcNAc-**S**-CPA glyco(ter)polymer **223** was prepared with a 1:10:100 molar ratio of **223**:**45**:acrylamide monomers. It was isolated in 53% yield as a white and somewhat brittle solid, and was shown to have the same composition as the formulation described for its synthesis. The structural composition of **227** was determined by ^{1}H-NMR spectroscopy. The *N*-tyramide:GlcNAc ratio was judged from the two

aromatic signals appearing as doublets ($J_{o,m} \equiv 8$ Hz) at 6.87 and 7.20 ppm, and the anomeric proton signal at 4.53 ppm. Because of the small amount of *N*-acryloylated tyramine **223** used in the synthesis, the spectrum intensity must be elevated for this step. The GlcNAc:acrylamide ratio was determined in the usual fashion for any β-D-GlcNAC-**S**-CPA (see for example Chapter 4, Figure 13). A GlcNAc:acrylamide value of 1:10 was obtained by comparing integration intensities of signals from the anomeric proton, the remaining sugar ring resonances and the spacer's methylene groups (3.3 - 4.2 ppm, 8H; 2.6, 2.7 and 3.0 ppm, 2H each), as well as the *N*-acetyl methyl singlet at 2..05 ppm, to the typical polymer backbone methine and methylene resonances (2.3 and 1.7 ppm).

The antigenicity of the *N*-tyramide/β-D-GlcNAc-S-CPA glyco(ter)polymer **227** was verified by an agarose gel diffusion assay. WGA gave a sharp precipitin band when diffused toward **227**, just as it did earlier for the β-D-GlcNAc-**S**-CPA **53**. The new phenolated glycopolymer **227** was also shown to behave as a good coating antigen during ELLA with horseradish peroxidase labeled WGA (HRP-WGA) and ABTS/H_2O_2 as enzyme substrate (Figure 58). During ELLA, the use of 1 ng of **227** as coating antigen was sufficient to detect ($O.D._{410\ nm}$ = 0.6) 1 ng of HRP-WGA in solution. With similar sugar densities, the behaviour of **227** during ELLA was the same as the analogous, *N*-tyramide deficient β-D-GlcNAc-**S**-CPA (**53**) (compare Figures 59 and 60).

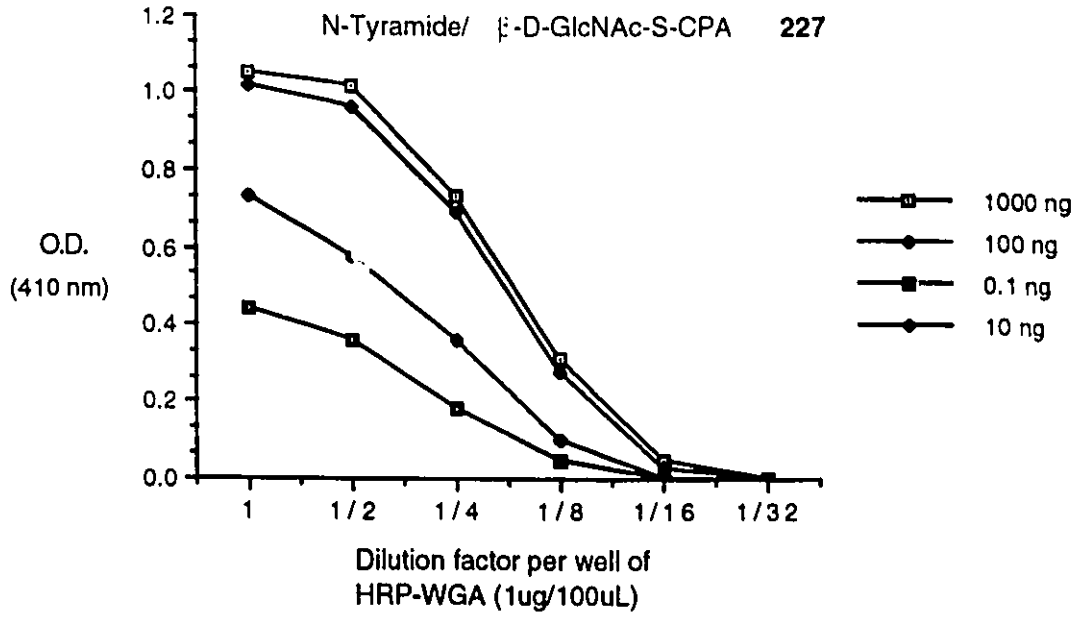
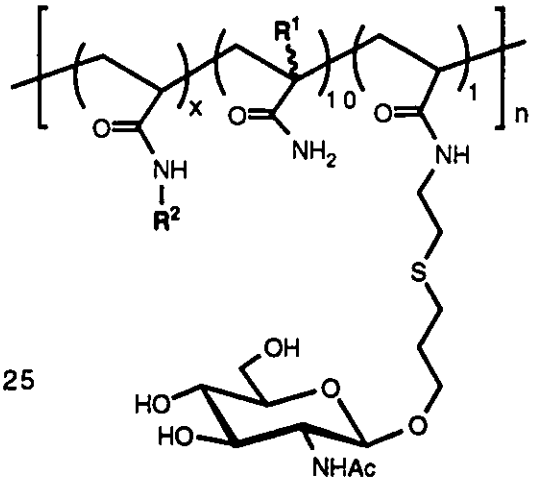


Figure 58 Enzyme linked lectin assay (ELLA) of **227** used as coating antigen with HRP-WGA and ABTS/H_2O_2 as enzyme substrate.

The use of **227** as a radioiodinated glycoconjugate probe could not be tested due to limited access to the radioassay facilities required for such a study. However, there is no reason to believe that the *N* - tyramide probe could not be radioiodinated using standard techniques[66]. The phenolic nature of the *N*-tyramide probe is structurally very similar to tyrosine amino acid residues found on proteins which are labeled readily. Therefore, the synthesis of **277** by terpolymerization serves more as a demonstration for the concept of preparing potentially useful heterobifunctional synthetic glycoconjugates by terpolymerization of suitable acrylamide monomers.

10.2.4 Stearylated glycopolymers

Copolyacrylate and copolyacrylamide glycopolymers in particular have proven to be excellent glycoconjugates useful in diagnostic applications. They have proven to be very well suited as coating antigens in solid phase ELISA and ELLA studies. As described previously (Section 1.6), the mechanism by which glycoconjugates are adsorbed to wells of microtiter plates is by simple hydrophobic interaction with the plastic surface. The polyacrylamide backbone of such glycopolymers has a rather poor lipophilic character. The polyamide structure of copolyacrylamides such as **53** is actually rather hydrophilic and gives the glycopolymer its excellent water solubility. Thus, attempts were made at modifying the carrier backbone by increasing its lipophilic structure to possibly improve its adsorption to lipophilic surfaces.

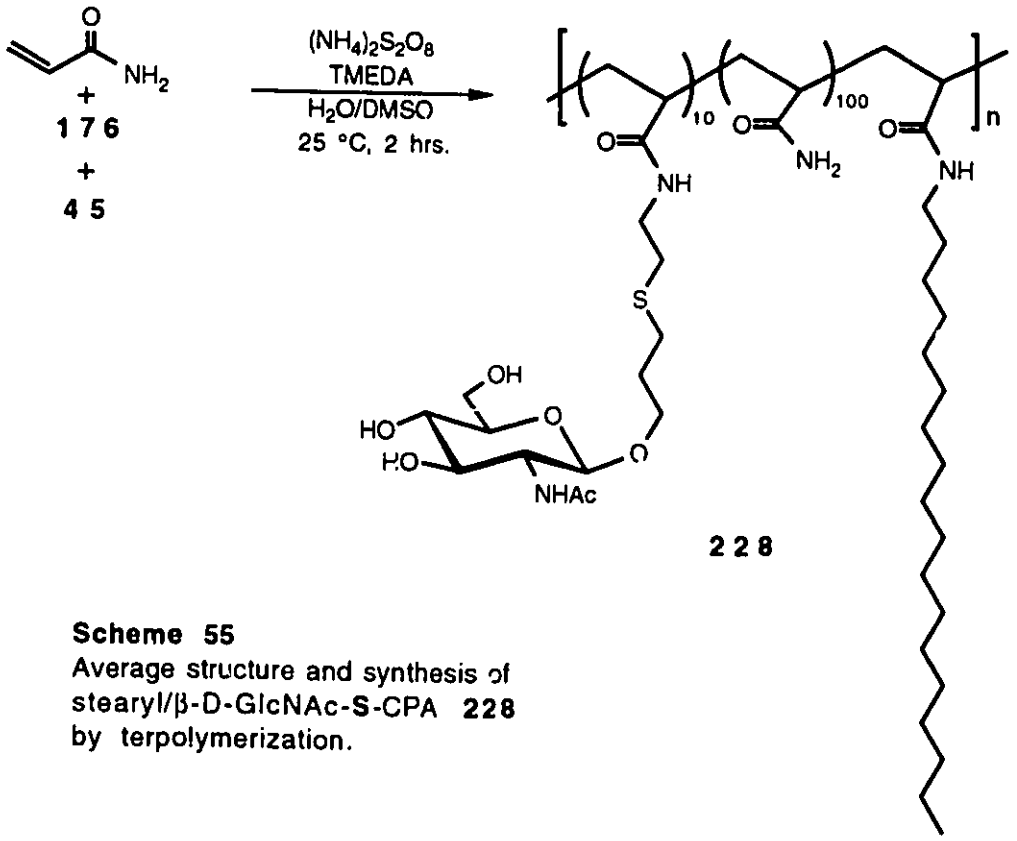


	R^1	R^2	x
53	H	H	0
56	CH_3	H	0
227	H	$CH_2CH_2C_6H_4OH$	0.1
228	H	n-$C_{17}H_{36}$	0.025

Scheme 54 Alkylated polyacrylamide glycopolymers

An initial attempt to demonstrate the concept was made earlier (Sections 4.2 and 7.2.1) by preparing glycopolymers with a copolymethacrylamide backbone such as β-D-GlcNAc-S-CPMA **56**, for example. Copolymethacrylamide glycopolymers appeared to exhibit a more lipophilic character than copolyacrylamide analogues by requiring longer to solubilize properly and by giving seemingly

more viscous aqueous solutions. Their adsorbtion properties during ELLA with WGA appeared to be slightly improved over analogous copolyacrylamide antigens (Figures 59 and 60). In a second attempt at incorporating lipophilic sites on glycopolymers, a terpolymerization strategy was attempted for the incorporation of a long, lipophilic effector in the form of a long hydrocarbon side chain (*N*-octadecyl, $C_{18}H_{37}$). Thus, stearyl amine was *N*-acryloylated and terpolymerized in water with **45** and acrylamide to give the stearylated copolyacrylamide glyco(ter)polymer stearyl/β-D GlcNAc-**S**-CPA **228** in a 59% yield (Scheme 55). Terpolymerization to give **228** was performed under standard conditions at 100°C for 15 minutes using ammonium persulfate as the radical initiator.



Scheme 55
Average structure and synthesis of stearyl/β-D-GlcNAc-**S**-CPA **228** by terpolymerization.

The stearyl/β-D-GlcNAc-**S**-CPA conjugate **228** was formulated to give a 1:10:100 ratio of stearyl:GlcNAc:acrylamide composition. The desired 1:10 incorporation ratio of GlcNAc: acrylamide in the

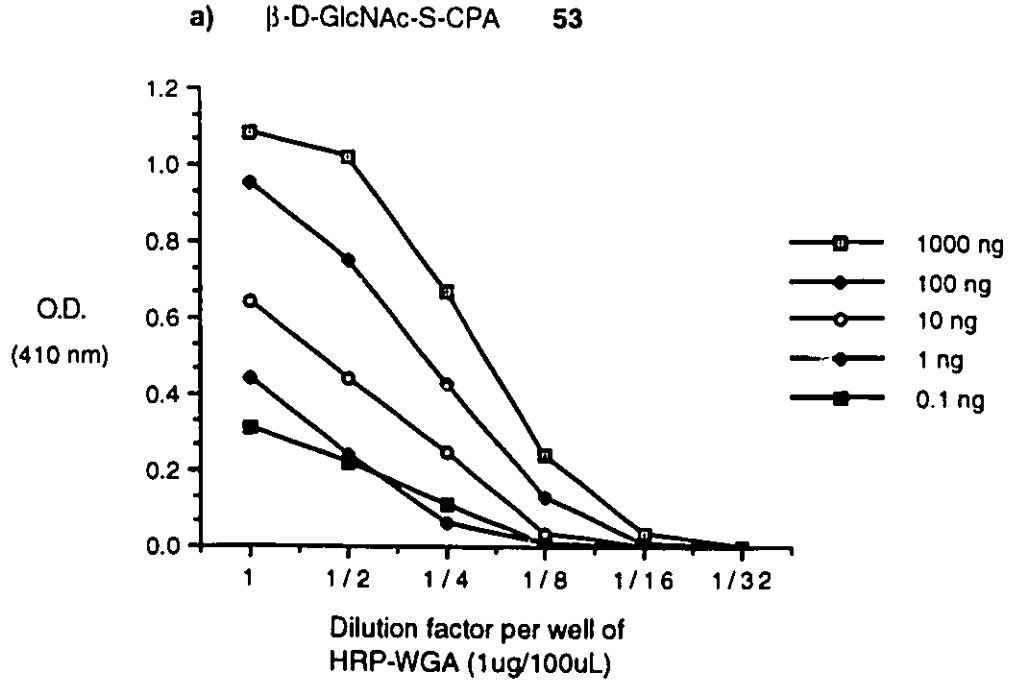
isolated glyco(ter)polymer was clearly observed by ^{1}H-NMR spectroscopy. However, the stearyl effector content proved difficult to accurately assess. A conservative estimate of a 1:23 ratio of stearyl:GlcNAc was made from what appears to be the stearyl group's terminal methyl signal at 0.80 ppm (t, J≈7Hz), and by comparing the integration of the backbone methine and methylene signals near 2.2 and 1.7 ppm and assigning the increased integration area in the large methylene signal the methylenes belonging to the stearyl effector (15 CH_2) which appear in this region.

The lower than desired amount of *N*-stearyl acrylamide (**222**) incorporation in the glyco(ter)polymer **228** was not totally unexpected based on the poor solubility of **222** in water. It is for this reason that the terpolymerization was performed at 100°C with vigourous agitation. Under these conditions, **222** (mp = 78°C), if not fully dissolved, could at least be present as a melted suspension during the polymerization and thus have a better chance of reacting. No organic cosolvent was found to be suitable for solubilizing **222** under the polymerization conditions used. The incorporation of stearyl side chains to the glycopolymer was revealed by the cloudy appearance of the aqueous solutions of the new glyco(ter)polymer **228**. Unlike simple copolyacrylamide glycopolymers which give clear solutions when dissolved in water, the new stearylated glycopolymer **228** appears as a mildly foggy solution which may be caused by the increased hydrophobicity of the glycoconjugate. The cloudy appearance of **228** in solution was not due to suspended insoluble particulates since centrifugation at 14,000 g did not yield any precipitate or change in the solution's appearance.

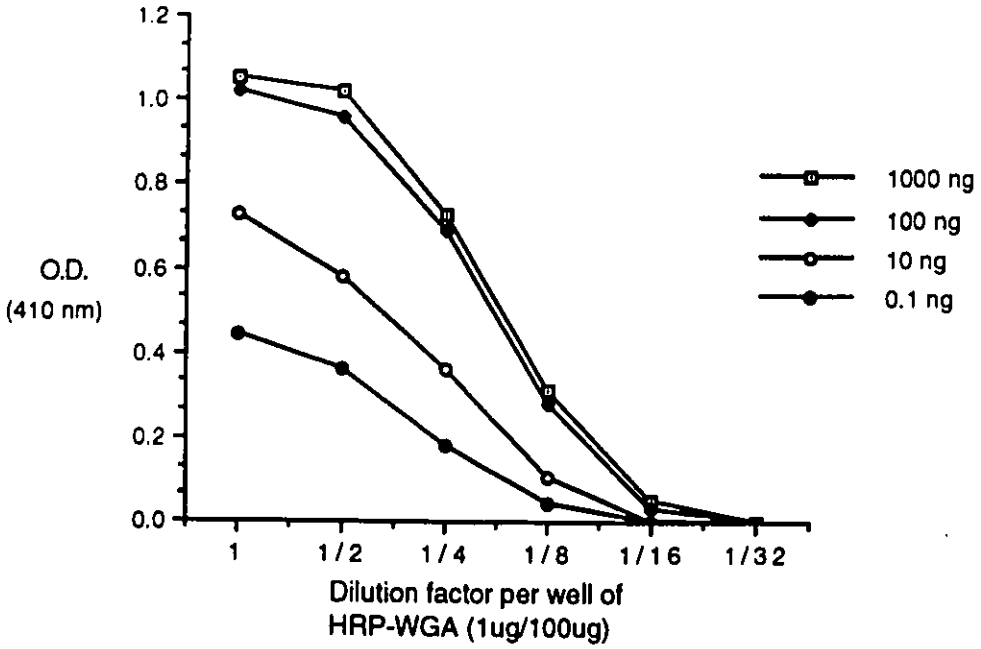
The effect of incorporating lipophilic groups in copolyacrylamide GlcNAc-glycopolymers on the capacity of being better adsorbed to the surface of plastic microtiter well surfaces was assayed by ELLA using HRP-WGA and ABTS/H_2O_2 as enzyme substrates. The stearylated copolyacrylamide glycopolymer **228** did not appear to be much better than the β-D-GlcNAc-**S**-CPMA glycopolymer **56** as a coating antigen when assayed at 10 ng/100 μL/well. However, both

228 and **56** appeared to be better then a simple β-D-GlcNAc-**S**-CPA **53** (Figure 59) at an HRP-WGA working concentration of 1 μg/100 μL/well. A clear distinction between these glycopolymers and the *N*-tyramide/β-D-GlcNAc-**S**-CPA **227** could be observed when used at a coating concentration of 10 ng/100 μL/well (Figure 60). Under these conditions, the immobilization and detection of 0.5 μg of HRP-WGA by glycopolymers of similar hapten densities was found to be in the decreasing order : stearylated CPA > CPMA > tyraminylated CPA > regular CPA glycopolymers. The stearylated glycopolymer **228** was twice as efficent as the version without the stearyl chain effector (**53**).

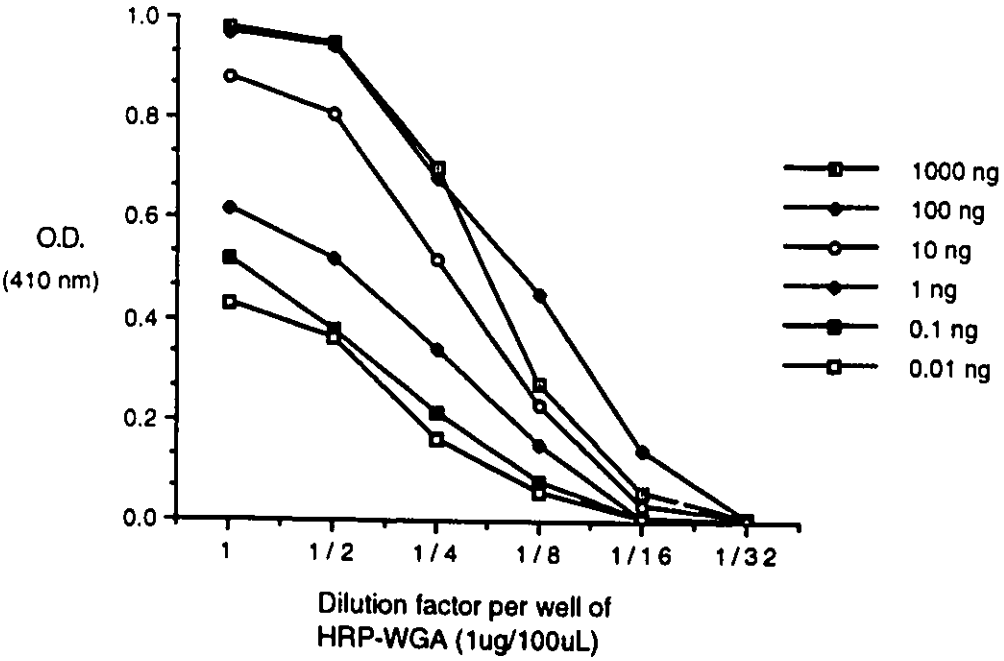
Figure 59 (Below) ELLA of glycopolymers having similar hapten densities (10% by mol) but different lipophilic character as coating antigens with HRP-WGA using ABTS/H_2O_2 as enzyme substrate.
Coating antigens: **a)** β-D-GlcNAc-**S**-CPA **53**
b) β-D-GlcNAc-**S**-CPMA **56**
c) stearyl/β-D-GlcNAc-**S**-CPA **228**



b) β-D-GlcNAc-S-CPMA 56



c) stearyl/ β -D-GlcNAc-S-CPA **228**



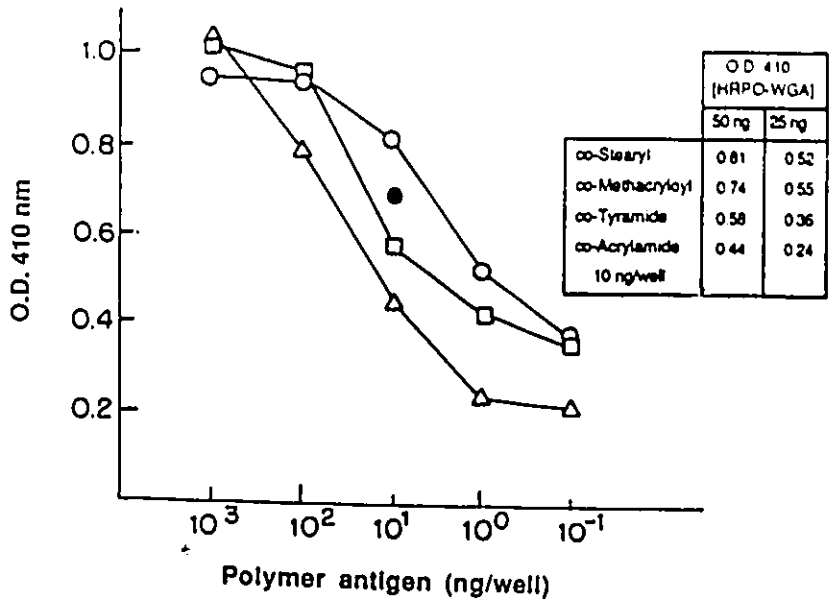


Figure 60 (Below) Comparative ELLA of glycopolymer coating antigens **53** (Δ), **56** (•, one point), **227** (□), and **228** (O) with HRP-WGA (0.5 mg/100 mL/well) using ABTS/H_2O_2 as enzyme substrate.

In conclusion, custom designed glycopolymers can be prepared by terpolymerization of acrylamide monomers to improve or impart desired bio- or physico-chemical properties to glycopolymer antigens. The incorporation of secondary carbohydrate or biotin ligands gives novel multivalent heterobifunctional glycoconjugates with potential use as immunoadhesins or specialized diagnostic agents. By incorporating a phenolated effector, glycopolymers can be made to serve in radioimmunoassays while increasing the lipophilic character with hydrophobic effectors can give rise to more effective coating antigens for use in solid phase enzyme linked assays. The work discussed in this chapter describes more the feasibility and intrinsic properties of the concept rather then provide an exhaustive evaluation of each glycopolymer's potential application. In closing, it must be noted that the strategy of incorporating specialized effectors or probes in glycopolymer antigens is not limited to the terpolymerization of acrylamides. The strategy is also adaptable to poly-L-lysine conjugates as described in the previous chapter with the biotin/$(Neu5Ac)_{15}$-PLL conjugate **218**.

10.3 Experimental methods

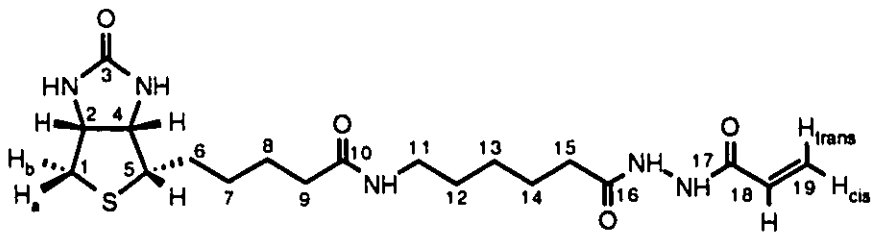
General methods

General methods were as described in earlier chapters.

Acryloylation of biotinamidocaproylhydrazide , **213**

Biotinamidocaproylhydrazide **221** (45.0 mg, 0.121 mmol, Sigma, ~95% pure by TLC) was dissolved in warm methanol (85 mL)[251]. Anion (HO^-) exchange resin was added and the solution placed in an ice bath. To this stirring solution was added, in a slow dropwise fashion, a solution of 11 µL (0.135 mmol) acryloyl chloride in 500 µL of 1,4-dioxane. The reaction was monitored by TLC ($CHCl_3$/MeOH, 4/1) and showed a complete and clean conversion of **221** (Rf = 0.20) to **213** (Rf = 0.40) after 3 hours. The impurity (Rf = 0.50, presumably biotinamidocaproyl methyl ester) in the starting material was not affected. The reaction was then warmed to near 50°C, the resin was filtered and washed with warm methanol. Evaporation of the methanol gave a white solid which could not be redissolved very well in most solvents. It was redissolved in a minimum of hot methanol (~20 mL), allowed to cool at room temperature then stored in the freezer compartment of a refrigerator (-15°C). After two weeks, a white, cottony mass was filtered, washed with cold ethanol then dried under vaccum to yield 22 mg (43%) of **213**, pure by TLC and NMR. The product had mp = 185.7-187.1°C and $[\alpha]_D$ = +35.4° (c=0.82, DMSO). The mother liquor was concentrated and 200 µL of DMSO was added to completely solubilize the remaining crude material. This solution was applied to a half-size preperatory TLC plate. The resulting wide and doubled application band was concentrated to single thin band by multiple short elutions with pure ethanol. The TLC plate was then eluted with $CHCl_3$/MeOH (4/1, v/v). The band corresponding to the desired product **213** was scraped

from the glass and then extracted with EtOAc/EtOH (4/1). The silica was filtered and the solvent evaporated. An additional 18 mg (35%) of pure **213** was thus obtained, giving a total yield of 78% for **213**.



213

(-) FAB-MS for $C_{19}H_{31}N_5O_4S$ gave m/e (ion, relative intensity): 424.2 (M-1, 50), 199.0 (M - $C(O)NH(CH_2)_5C(O)NHNHC(O)CH=CH_2$, 57). ^{1}H-NMR δ (DMSO$d_6$): 10.02 and 9.84 (2s, 2x1H, hydrazide NH), 7.68 (t, J = 5.6 Hz, amide NH), 6.38 and 6.32 (2s, 2x1H, urea NH), 6.27 (dd, 1H, J_{cis} = 9.9, J_{trans} = 17.1 Hz, H_{18}), 6.19 (dd, 1H, J_{gem} = 2.5, J_{trans} = 17.1 Hz, H_{19} trans), 5.68 (dd, 1H, J_{cis} = 9.9, J_{gem} = 2.5 Hz, H_{19} cis), 4.30 (dd, $J_{2,4}$ = 7.7, $J_{1a,2}$ = 5.0 Hz, H_2), 4.12 (dd, $J_{4,5}$ = 5.0 Hz, H_4), 3.09 (mult, 6 lines, 1H, H_5), 3.00 (dt, 2H, $J_{11,12}$ = 6.4, $J_{11,NH}$ = 5.6 Hz, H_{11}), 2.81 (dd, 1H, J_{gem} = 12.4, $J_{1a,2}$ = 5.0 Hz, H_{1a}), 2.57 (d, 1H, H_{1b}), 2.13 (t, 2H, $J_{8,9}$ = 7.3 Hz, H_9), 2.04 (t, 2H, $J_{14,15}$ = 7.3 Hz, H_{15}), 1.43 - 1.62 (mult, 6H, methylenes 6, 8 and 14), 1.37 (mult, 6 lines, 2H, H_{12}), 1.21 - 1.34 (mult, 4H, methylenes 7 and 13).

^{1}H-NMR δ (D_2O/DMSOd_6, 5/1, referenced to HOD at 4.828 ppm): 6.33, 6.34, 6.35 (3 line AB mult, intensity 2:1:1, J = 4.0 and 3.6 Hz, 2H, -CH=C and $=CH_2$ trans), 5.92 (dd, 1H, J = 4.0 and J = 7.6 Hz, $=CH_2$ cis), 4.61 (dd, 1H, $J_{2,4}$ = 8.0, $J_{1a,2}$ = 4.2 and $J_{1b,2}$ = 0 Hz, H_2), 4.42 (dd, 1H, $J_{4,5}$ = 4.4 Hz, H_4), 3.34 (symetrical mult, 1H, H_5), 3.20 (t, 2H, $J_{11,12}$ = 6.8 Hz, H_{11}), 2.99 (dd, 1H, $J_{1a,2}$ = 4.9, $J_{1a,1b}$ = 13.0 Hz, H_{1a}), 2.78 (d, 1H, $J_{1a,1b}$ = 13.0 Hz, H_{1b}), 2.36 (t, 2H, $J_{8,9}$ = 7.3 Hz, H_9), 2.26 (t, 2H, $J_{14,15}$ = 7.3 Hz, H_{15}), 1.50 - 1.78 (mult, 8H, methylenes 6, 8, 12 and 14), 1.35 - 1.50 (mult, 4H, methylenes 7 and 13).

^{13}C-NMR δ (DMSOd_6): 171.8 (C_{16}), 170.8 (C_{17}), 163.2 (C_3), 162.7 (C_{10}), 129.3 (C_{18}), 126.6 (C_{19}), 61.0 (C_4), 59.2 (C_2), 55.4 (C_5), 40.0 (C_1), 38.3 (C_{11}), 35.2 (C_{15}), 33.1 (C_9), 28.9 (C_{12}), 28.2 (C_6), 28.0 (C_7), 25.9

(C_{13}), 25.3 (C_{14}), 24.8 (C_8).

N-Stearyl acrylamide **222**

Stearyl amine (250 mg, 0.928 mmol) was dissolved in 25 mL of CH_2Cl_2 containing 400 µL of triethyl amine. The stirring solution was cooled to 0°C and a solution of acryloyl chloride 100 µL in CH_2Cl_2 (8 mL) was added dropwise over a 40 min. period. The precipitated triethylammonium chloride was filtered and washed with ice cold CH_2Cl_2. The CH_2Cl_2 was evaporated and the resulting solid redissolved in hot ethanol. the product (Rf = 0.58 in EtOAc/Hex containing 0.5% i-prOH) was left to crystallize at room temperature. Three crops gave 289.5 mg (96%) of **222** as a white microcrystalline powder which had mp = 74.5-75.1°C.

Elemental analysis for $C_{21}H_{41}NO$ gave: % calc'd C, 77.95; H, 12.77; N, 4.33; % found: C, 77.74; H, 12.56; N, 4.36. CI-MS (ether) gave m/e (ion, relative intensity): 647 (2M+1, 29), 324 (M+1, 100). ^{1}H-NMR δ ($CDCl_3$): 6.25 (dd, 1H, J_{trans} = 17.0, J_{gem} = 1.6 Hz, $=CH_2$ trans), 6.05 (dd, 1H, J_{cis} = 10.2, J_{cis} = 10.2 Hz, C(O)CH=C), 5.60 (dd, 1H, J_{cis} = 10.2, J_{gem} = 1.6 Hz, $=CH_2$ cis), 5.55 (broad, 1H, NH), 3.30 (dt, 2H, J_{NH,CH_2} = 5.9, J_{CH_2,CH_2} = 7.1 Hz, N-CH_2),1.51 (app dt, 2H, J = 7.1, J = 5.9 Hz, CH_2), 1.17 - 1.27 (br. s, 30H, methylenes), 0.85 (t, 3H, J_{CH_2,CH_2} = 6.8 Hz, CH_3). ^{13}C-NMR δ ($CDCl_3$): 165.5 (C=O), 131.0 (-CH=), 126.0 ($=CH_2$), 40.0 (-CH_2-N), 31.9, 29.7 (7C), 29.6 (3C), 29.5, 29.4, 29.3, 26.9, 26.7 (16 methylenes), 14.1 (CH_3).

N-*2-(p-Hydroxyphenyl)ethyl acrylamide* **223**

Tyramine hydrochloride (0.32 g, 1.84 mmol, Aldrich) was dissolved in 10 mL of methanol and 1 mL of triethylamine. The solution was cooled to 0 °C and a slow, dropwise addition of acryloyl chloride (200µL in 5 mL 1,4-dioxane) was made until TLC ($CHCl_3$/MeOH, 10/1) indicated a complete consumption of tyramine (Rf = 0) to give **223**

as the major product (Rf = 0.52). The reaction was worked up by evaporating to a crystalline mass and redissolving the crude product mixture in 30 mL of EtOAc. The organic phase was washed with 0.5M HCl to remove excess triethylamine followed by sat. $NaHCO_3$, water and brine. The organic extracts were dried (Na_2SO_4), filtered and evaporated to an oil. Attempts at crystallizing the product from various solvent systems proved fruitless. The product **223** was isolated by silica gel chromatography using $CHCl_3$/MeOH (15/1) as eluent. The desired product was isolated in a 225 mg (64%) yield as a glassy solid which could not be crystallized.

Physical data for **223**: CI-MS (ether) gave m/e (ion, relative intensity): 382.9 (2M + 1, 9), 191.9 (M + 1, 100). ^{1}H-NMR δ ($CDCl_3$): 7.00 (d, 2H, $J_{o,m}$ = 8.3 Hz, H_{ortho}), 6.78 (d, 2H, H_{meta}), 6.40 (br, 1H, OH), 6.26 (dd, 1H, J_{trans} = 17.0, J_{gem} = 1.5 Hz, $=CH_2$ trans), 6.02 (dd, 1H, J_{cis} = 10.1, J_{trans} = 17.0 Hz, C(O)CH=), 5.75 (br, 1H, NH), 5.61 (dd, 1H, J_{cis} = 10.1, J_{gem} = 1.5 Hz, $=CH_2$ cis), 3.54 (dt, 2H, J_{NH,CH_2} = 6.2, J_{CH_2,CH_2} = 7.0 Hz, $-CH_2$-N), 2.75 (t, 2H, J_{CH_2,CH_2} = 7.0 Hz, $-CH_2$-Ph). ^{13}C-NMR δ ($DMSOd_6$): 156.3 (C_{para}), 132.4 (-CH=), 130.6 (C_{ipso}), 130.1 (C_{ortho}), 124.9 ($=CH_2$), 115.7 (C_{meta}), 41.5 ($-CH_2$-N), 35.2 ($-CH_2$-Ph).

α-L-Rha/β-D-GlcNAc-S-CPA **224**

Acrylamide (110 mg, 1.55 mmol), the α-L-rhamnosyl monomer **43** (174 mg, 0.537 mmol) and the β-D-*N*-acetylglucosaminide **45** (117 mg, 0.298 mmol) were dissolved in 8.0 mL of deoxygenated water in a flask filled with nitrogen. The flask was immersed in a boiling water bath and after a few minutes, initiated with 40 µL of $(NH_4)_2S_2O_8$ (100 mg/mL, 0.4% by mol). The polymerization reaction was stirred vigourously at 100 °C for 15 minutes before being diluted and exhaustively dialyzed against distilled water. Lyophilization gave 401 mg (59%) of **224** as a white, spongy solid. ^{1}H-NMR analysis of **224** revealed a monomer incorporation ratio identical to the reaction load ratio (α-L-Rha:β-D-GlcNAc:acrylamide = 1:1:6).

^{1}H-NMR data for **224** . δ (D_2O referenced to HOD at 4.84 ppm): 4.52 (d, 1H, $J_{1,2}$ = 8.4 Hz, H_1 GlcNAc), 4.97 (d, 1H, $J_{1,2}$ = 1.6 Hz, H_1 Rha), 3.2 - 3.8 (mults, br, 15H), 2.6 (br, 8H, CH_2-S-CH_2 spacer), 2.2 (br, 12H, backbone methines), 2.0 ppm (s, 3H, CH_3 GlcNAc), 1.6 (br, 28H, backbone methylenes and H_b spacer), 1.33 (d, 3H, $J_{5,6}$ = 6.0 Hz, CH_3 Rha).

Biotin/β-D-Lac-Ø-CPA **225**

Acrylamide (27.4 mg, 385 μmol), 8.2 mg (19.3 μmol) of the biotin monomer **213** and 18.8 mg (36.8 μmol) of the lactoside **176** were dissolved in 400 μL of deoxygenated water and 200 μL of DMSO in a small stoppered test tube, under a nitrogen atmosphere. The solution was warmed to 65°C and stirred vigourously to properly dissolve all the monomers. Once homogeneous, 5 μL of TMEDA followed by 50 μL of ammonium persulfate (50 mg/mL) were injected. After stirring at room temperature for 3 hours, the clear, viscous solution was diluted with water, and dialyzed. A primary dialysis was performed in 5L of 0.5mM HOAc followed by two other dialyses in 5L of distilled water. Lyophilization gave 31.0 mg (57%) of **225** as a white, spongy solid. Biotin incorporation was verified by treating a small sample of **225** with 0.2% 4-dimethylamino cinnamaldehyde in acidified ethanol (Sigma, a reagent specific for detecting biotin[253]) and by observing the generation of a pink to red colouration. The analogous β-D-Lac-Ø-CPA **185** did not react with 4-dimethylamino cinnamaldehyde. ^{1}H-NMR (D_2O) analysis of **225** confirmed the presence of biotin from the characteristic resonance of **213** found unobstructed between 2.6 and 3.4 ppm. The aromatic resonances of the lactoside hapten (7.0 - 7.5 ppm) as well as the anomeric signals found at 5.14 (br, Glc) and 4.50 (d, $J_{1,2}$ = 7.5 Hz, Gal) were clear indications of lactose content in **225**. Comparison of intensities for various reporter groups of the biotin and lactoside monomers to the

[253] P. György, *The Vitamins* , vol. VII, P. György and W.N. Pearson (eds.), Academic Press, New York, 1967, p. 303-313.

polymer backbone's characteristic methine and methylene resonances at 2.3 and 1.7 ppm indicated a 1:2:10 molar incorporation ratio of biotin:lactose:acrylamide in **225**. Corrections for the 8 methylene resonances of biotin, found under the backbone methylene signal, were made for this determination.

*Biotin/β-D-GlcNAc-***S***-CPA* **226**

Acrylamide (71.1 mg, 1.00 mmol), 39.0 mg (0.10 mmol) of the 3-(2-*N*-acrylamidoethylthio)propyl β-D-*N*-acetylglucosaminide **45**, and a small magnetic stir bar were placed in a test tube. The test tube was sealed with a septum, evacuated and filled with a nitrogen atmosphere. Deoxygenated water (800μL) and DMSO (300μL) were then added using a syringe followed by 15 μL of TMEDA. The solution was homogenized at 65°C for a few moments, then injected with 50 μL of ammonium persulfate (100 mg/mL). The polymerization was allowed to stir vigourously for 2 hours at room temperature. The resulting clear viscous solution was diluted with 10 mL of hot water then dialyzed once against 5 L of 0.5mM HOAc followed by two 5L volumes of distilled water. Lyophilization gave 118.8 mg (97%) of **226** as a white, spongy solid. ^{1}H-NMR (D_2O) indicated a 1:5:50 molar incorporation ratio of **213**:**45**:acrylamide. Biotin content in the glyco(ter)polymer was confirmed by treating a small sample of **226** with 0.2% ethanolic dimethylamino cinnamaldehyde which resulted in a pink colouration. The analogous but biotin deficient β-D-GlcNAc-**S**-CPA glycopolymer **53**, as expected, failed to give a positive result for biotin when treated in the same manner.

The ^{1}H-NMR spectrum of **226** appeared, as did the spectrum of the β-D-GlcNAc-**S**-CPA glycopolymer **52** (see Figure 13, Section 4.2), with most biotin resonances being obscured by resonances from the two more dominant monomers. Partially obstructed by the anomeric proton doublet at 4.53 ppm, signals from H_2 and H_4 of biotin could be measured relative to the anomeric proton by expanding and intensifying this region.

N-*Tyramide/β-D-GlcNAc*-**S**-*CPA* **227**

2-*N-p* -Hydroxyphenylethyl acrylamide **223** (100 μL of 20 mg/mL 0.1M NaOH, 0.01 mmol), acrylamide (71.1 mg, 1.00 mmol) and the β-D-acetylglucosaminide **45** (39.2 mg, 0.1 mmol) were boiled in 1.0 mL of deoxygenated water under a nitrogen atmosphere for a few moments before 3.5 mg (1.4 mol%) of solid ammonium persulfate were added. After 15 min. of vigourous stirring at 100°C, the clear viscous solution was diluted with 12 mL of water and dialyzed against three 5L volumes of distilled water. Lyophilization gave 59.4 mg (53%) of dry, white solid. ^{1}H-NMR confirmed the 1:10:100 **222**:**43**:acrylamide composition of **227**.

^{1}H-NMR δ (D_2O): 7.20 (d, $J_{O,M} \approx 8$ Hz, H_{ortho}), 6.88 (d, $J_{o,m} \approx 8$ Hz, H_{meta}), 4.53 (app t, J = 7.9 Hz, $H_{anomeric}$), 3.3 - 4.2 (mult, H_2 - H_6 and H_a spacer, GlcNAc), 3.0 (br, H_e spacer), 2.75 (H_c spacer), 2.65 (H_d spacer), 2.1 - 2.4 (br, backbone methines), 2.06 (s, CH_3 NAc), 1.4 - 2.4 (br, backbone methylenes and H_b spacer).

Stearyl/β-D-GlcNAc-**S**-*CPA* **228**

N-Stearyl acrylamide **222** (3.2 mg, 0.01 mmol), acrylamide (71.1 mg, 1.00 mmol), and the *N*-acryloylated β-D-*N*-acetylglucosaminide **45** (39.2 mg, 1 mmol) were vigourously stirred in 1.1 mL of deoxygenated water at 100 °C, under a nitrogen atmosphere. After 10 min., 5 mg (2% by mol) of solid ammonium persulfate was added, the test tube was refilled with a nitrogen atmosphere and fitted with a septum. The polymerization was left stirring at high speed for 12 min. at 100°C. The resulting viscous solution was diluted with 12 mL of water and dialyzed against 5 volumes of water (5L) over the course of 3 days. The slightly foggy solution was centrifuged at 14, 000 g for 15 min. No sedimentation or change in the the solution's appearance was observed. The dialyzed solution was then lyophilized to yield 66.5 mg (59%) of **228** as a white,

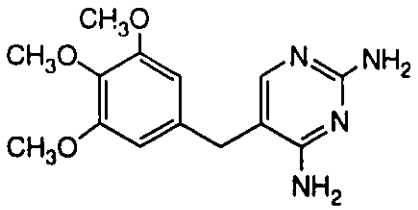
spongy solid. ^{1}H-NMR (D_2O) analysis (see text of Section 10.2.4) of **228**'s structural composition indicated a 1:10 ratio of GlcNAc:acrylamide, while a 1:23 estimate of stearyl:GlcNAc ratio was made (see text).

Serological assays

The serological assays performed were as described in earlier chapters. Avidin, streptavidin and peroxidase labeled streptavidin were purchased from Sigma (A9390, S4762 and S5512). HRP-WGA and affinity purified antibodies to Pn23 and Group A Streptoccoci capsular polysaccharides have been described in earlier chapters. Procedures for double radial diffusion assays were as described in earlier chapters as well as for the enzyme linked assays.

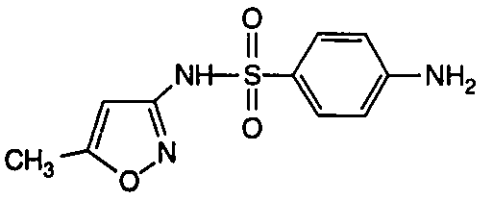
11. Design and synthesis of drug conjugates for diagnosis and study of immune hypersensitivity toward sulfamethoxazole in persons with AIDS.

People with acquired immune deficiency syndrome (AIDS) usually succumb to opportunistic infections common in the the late stages of HIV (human immunodeficiency virus) infection. Pneumocystis carinii pneumonia (PCP) is a lethal bacterial infection which is one of the most frequent opportunistic infections in HIV-seropositive patients[254]. Cotrimoxazole, a combination of trimethoprim (TMP) and sulfamethoxazole (SMX) is widely used for treating a variety of bacterial and parasitic infections including PCP. In HIV-seropositive patients, 40-80% may show or develop adverse reactions to cotrimoxazole whereas only 1-3% of the general population show any adverse reaction[255].



trimethoprim (TMP)

5-[(3,4,5-trimethoxyphenyl)methyl]
-2,4-pyrimidinediamine



sulfamethoxazole (SMX)

4-amino-N-(5-methyl-3-isoxazolyl)
benzenesulfonamide

It has been shown that SMX is the cause of most of the idiosyncratic reactions towards cotrimoxazole[256]. The high frequency of adverse reactions to SMX in HIV-seropositive patients may be due to several

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254 J.A. Levy, *AIDS Pathogenesis and Treatment* , Dekker, New York, 1989.

255 A.J.A. van Der Ven, P.P. Koopmans, TB. Vree and J.W.M. van der Meer, *The Lancet, 338* , 431 (1991).

256 F.M. Gordin, G.L. Simon, C.B. Wofsy and J. Mills, *Ann. Int. Med., 100* , 495 (1984).

factors[256,257]. In particular, it was hypothesized that adverse reactions could result from the immune dysfunction seen in HIV-seropositive patients. The polyclonal B cell activation, polyclonal gammopathy, and decreased T-supressor cell functions seen in these individuals could lead to an exagerated immune response to SMX or its metabolites[1]. Thus, a collaborative study was undertaken to analyse the antibody response to SMX in HIV-seropositive patients using a rational enzyme immunoassay strategy[258].

This chapter will focus mainly on the development of SMX drug immunoconjugates prepared for the study, and some of the assay results obtained. All serological assays were performed by Mr. P. Daftarian[258] and are presented here for clarity. The SMX drug conjugates developed for this study have so far helped to demonstrate increased levels of IgM, IgG_1 and IgG_3 antibodies to SMX in HIV-seropositive patients. A diagnostic test capable of identifying patients at risk of developing adverse drug reaction to SMX by detecting the presence of specific anti-SMX antibodies could be a potential outcome of this work.

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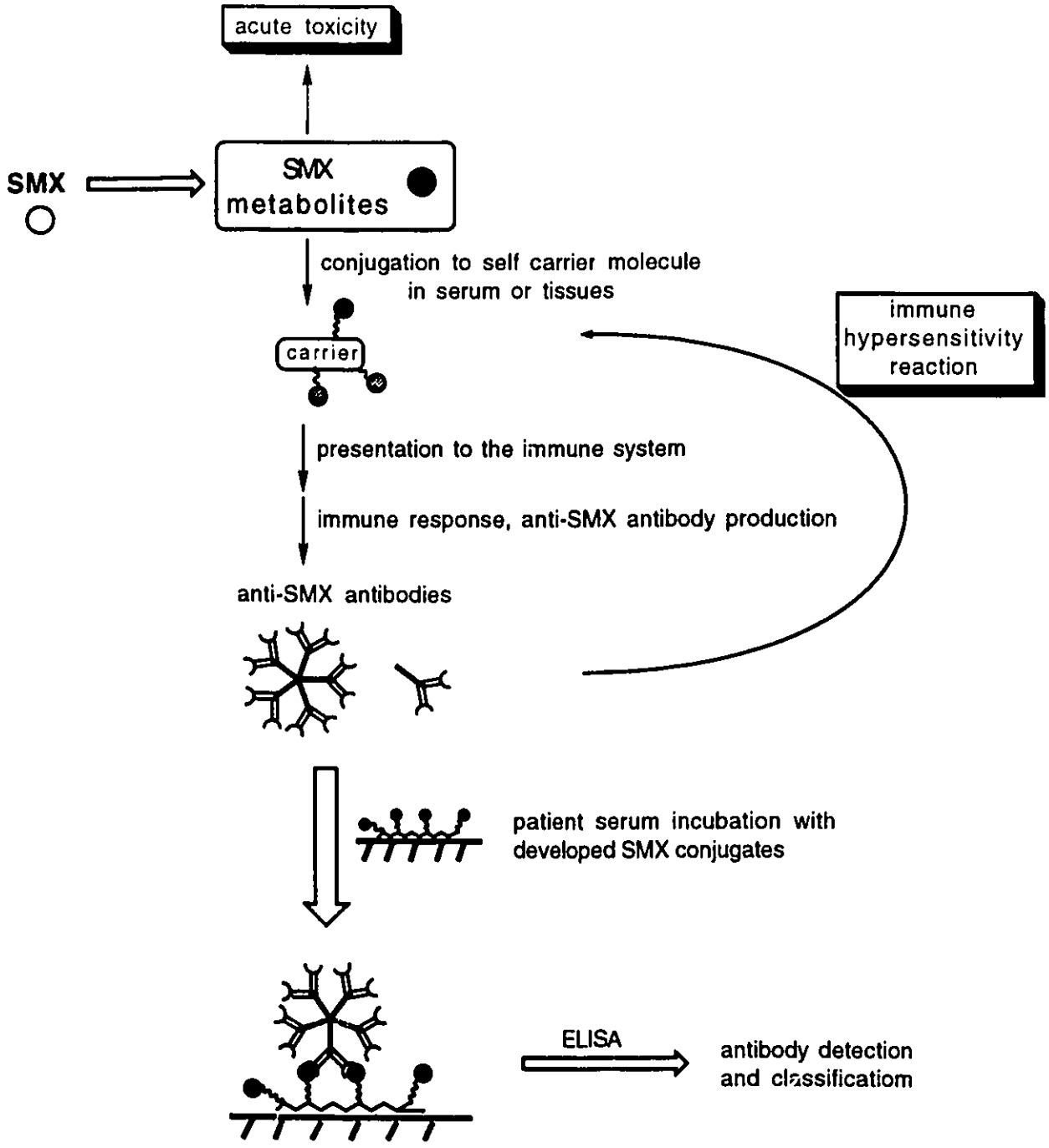
[257] a) J. Uetrecht, *Reviews in Toxicology*, *20* , 213 (1990); b) B.K. Park, J.W. Coleman and D. Kitteringham, *Biochem. Pharmacol.*, *36* , 581 (1978).

[258] This work was presented in part at the Annual Meeting of the Association of Medical Laboratory Immunologists, August, 1991, Chicago. The overall study was approved by the Human Experimentation Committee at the Ottawa General Hospital. Members of the collaborative research effort include Mr. Pirouz Daftarian, Prof. Lionel Filion from the Departments of Microbiology, Immunology and Chemistry at the University of Ottawa, as well as Dr. F. Diaz-Mitoma (M.D.) and Dr. Brian Conway from the Division of Virology, Children's Hospital of Eastern Ontario (Ottawa, Canada).



Scheme 56 Proposed pathways for idiosyncratic drug reactions to sulfamethoxazole (SMX) in AIDS patients and study goal.

11.1 Drug conjugate development

Sulfamethoxazole (SMX) drug conjugates were developed following the same strategies outlined earlier with carbohydrates. The aromatic amino group of SMX was an obvious choice for an *N*-acryloylation and conjugation strategy as a first attempt. The choice of using the amine terminus as the linkage position to the conjugates was, in part, due to the obvious ease of applying an acrylamide conjugation while providing generally unaltered structure in which presentation from any conjugate would be the least hindered. This strategy had proven successful with carbohydrate derivatives. Also, when SMX is metabolized, the only terminal reactive site in SMX, the amine group is converted to reactive nitrogen species such as nitroso and hydroxylamine derivatives which can then be conjugated to carrier proteins[260,261,259]. *N*-Acetylation is also a known metabolic pathway for arylamines but leads to unreactive species[257a]. Reactivity of the amine group *in vivo* suggests that altering the potential charge of this centre by acylation might not effect binding to SMX specific antibodies particularily if presentation of SMX to the immune system by attachement to a carrier was also provided this way.

N-Acryloylation of SMX to provide the acrylamide precursor to SMX-immunoconjugates was performed with acryloyl chloride in methanol using triethylamine as base (Scheme 57). The acrylamide **229** was isolated in 76% yield in a first attempt. Reactivity of the sulfonamide nitrogen and poor solubility of SMX derivatives appeared to be contributing factors to the less than quantitative yield of this transformation.

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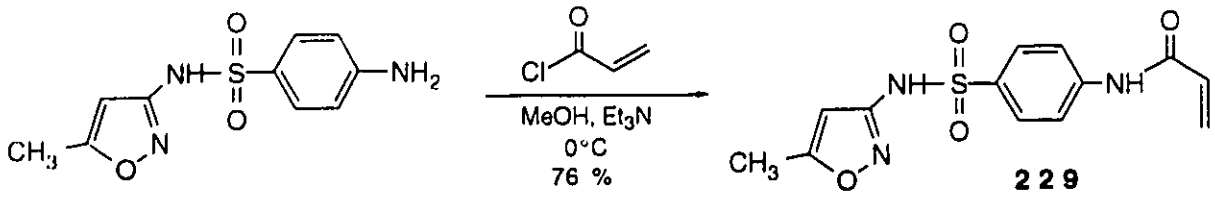
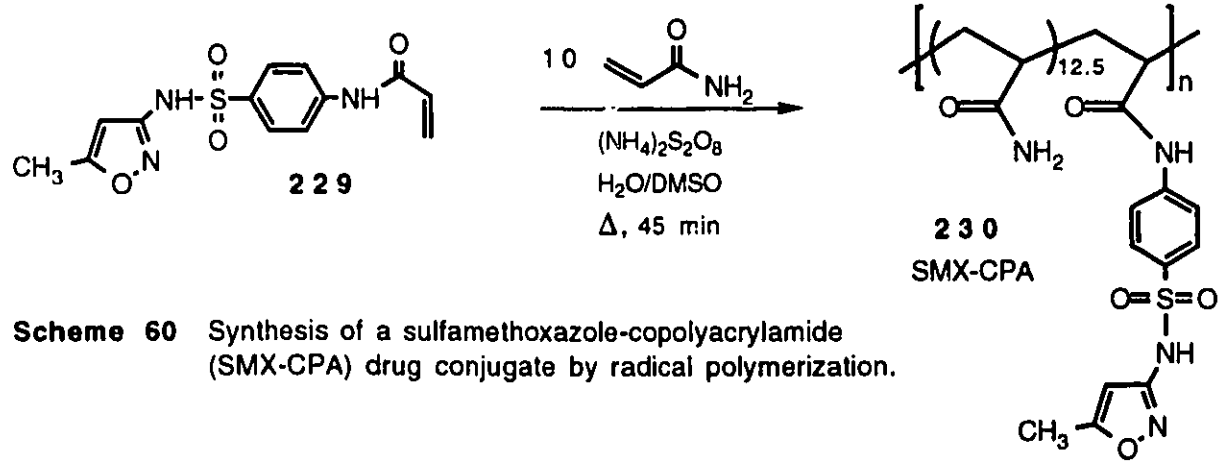
[259]A.E. Cribb, M. Miller, J.S. Leeder, J. Hill and S.P. Spielberg, *Drug Metab. Dispos.* *19*, 900 (1990).



Scheme 57 *N*-Acryloylation of sulfamethoxazole (SMX)

The first SMX drug conjugate was prepared by the copolymerization of acrylamide and the *N*-acryloylated SMX derivative **229** (Scheme 58). Acrylamide (10 equiv.) and **229** were refluxed under a nitrogen atmosphere for 45 min. in a blend of deoxygenated water and DMSO (4/1, v/v). These conditions were used to compensate for the poor solubility of **229** in water. The polymerization was initiated with ammonium persulfate (0.3% by mol) and gave a 90% yield of lyophilized sulfamethoxazole-copolyacrylamide conjugate **230** (SMX-CPA). Purification prior to final isolation was performed by dialysis (10 kDa MW cutoff).

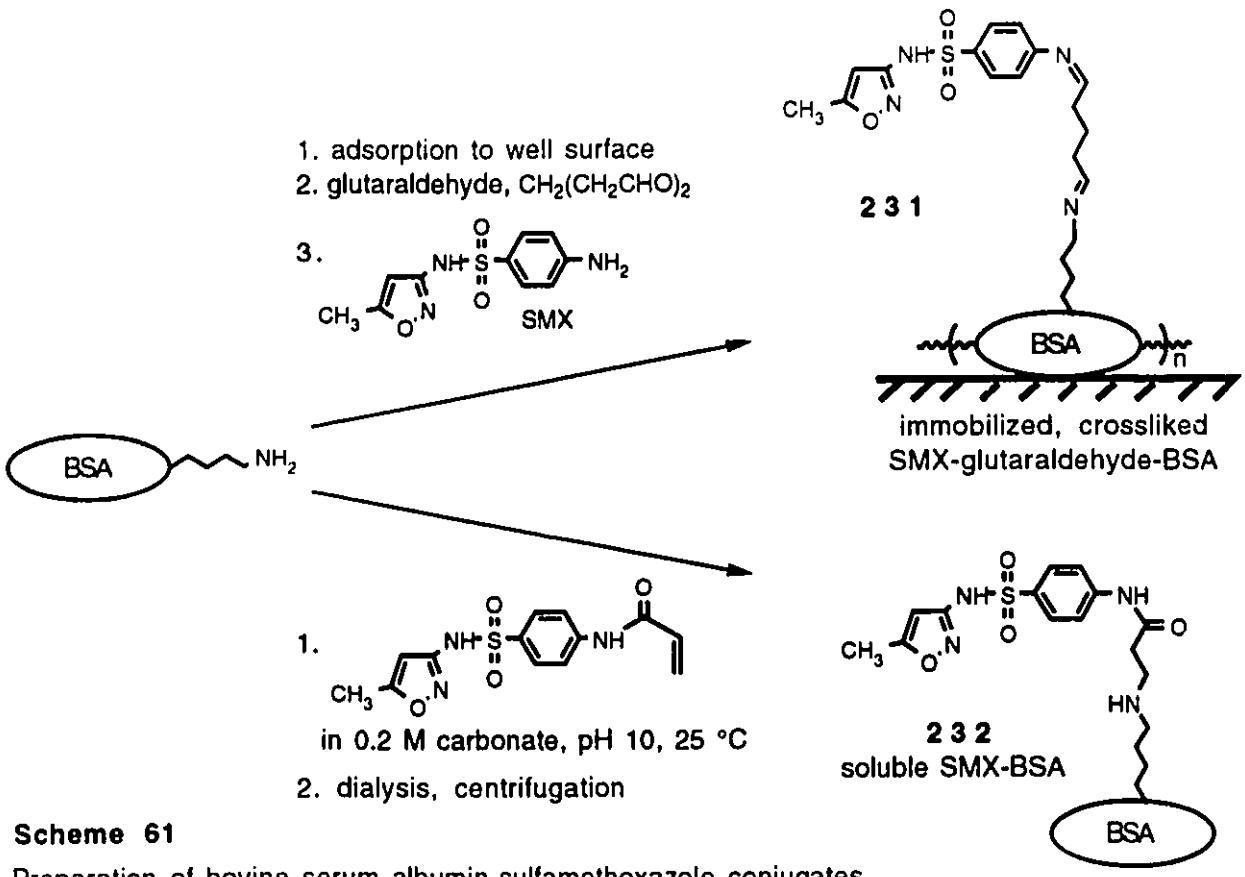


Scheme 60 Synthesis of a sulfamethoxazole-copolyacrylamide (SMX-CPA) drug conjugate by radical polymerization.

The water soluble SMX-CPA **230** was shown to contain a 1:12.5 molar ratio of **229**:acrylamide by ^{1}H-NMR spectroscopy. The slightly

lower than anticipated incorporation of SMX in the isolated polymer conjugate may have been due to the poor solubility of the sulfamethoxazole monomer **229**. The composition analysis of SMX-CPA **230** was performed by comparing integration intensities of SMX aromatic protons (br, 7.4 - 7.9 ppm) and the oxazole ring proton (br, 5.8 ppm) to the polymer backbone methine and methylene signals (br, 2.0 - 2.5 ppm and 1.4 - 2.0 ppm). Corrections for the SMX methyl signal also appearing as part of the methine signal at 2.0 - 2.5 ppm was performed. Elemental analysis of the lyophilized SMX-CPA conjugate indicated a 1:12.4 composition ratio of **229**:acrylamide as well as a 13.6% (by wt) water content. Thus a true polymer mass conversion yield of 78% was achieved for SMX-CPA **230**. The ^{13}C-NMR spectrum (D_2O) of SMX-CPA **230** confirmed the homogeneous nature of the polymer. Single sharp resonances were observed for SMX carbon nuclei while the backbone methine and methylene resonances gave the usual broadened signals near 43 and 36 ppm, respectively.

Serological evaluation of the SMX-CPA drug conjugate with serum of AIDS patients diagnosed with SMX hypersensitivity showed only a very modest ability to detect anti-SMX antibodies during solid phase ELISA when compared to an SMX-BSA conjugate prepared by Michael addition. The lack of any spacer between the SMX hapten and the copolyacrylamide backbone in the SMX-CPA conjugate appears to be the most obvious reason for the poor recognition of antibodies. As discussed previously with carbohydrate systems, antibodies have relatively deep binding sites within their structure which may not be accessible without a spacer. However, the presence of anti-SMX antibodies in the serum of AIDS patients was clearly observed by ELISA when different sulfamethoxazole-bovine serum albumin conjugates were employed as coating antigens. This confirmed the need for a spacer to properly present the SMX hapten to the antibody binding site, as well as reinforcing the belief that linking SMX to a carrier by the amine terminus was a good method for the presentation of SMX haptens .



Scheme 61

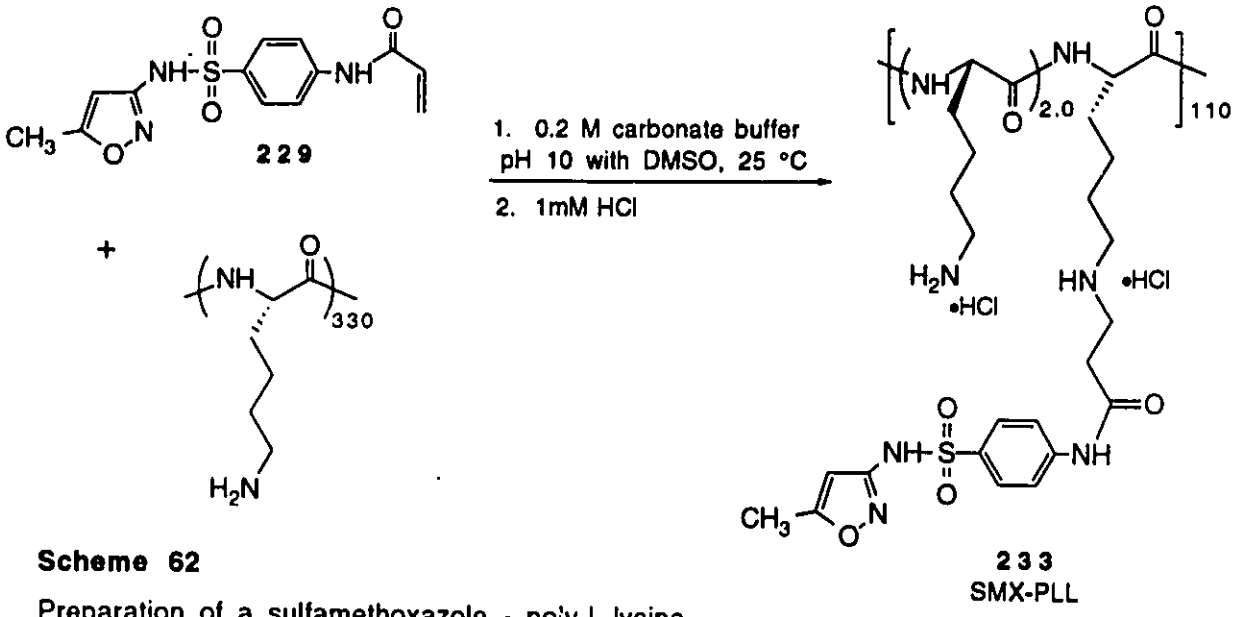
Preparation of bovine serum albumin-sulfamethoxazole conjugates by glutaraldehyde condensation and Michael addition.

BSA conjugates of SMX were prepared by a glutaraldehyde condensation strategy as well as by Michael addition of **229** to L-lysine ε-amino groups, and thus have a spacer arm inherent in their structure. SMX-glutaraldehyde-BSA antigens **231** were prepared in ELISA microtiter plates as an integral part of the immunoassay. The variable and ill-defined nature of **231** produced a high background signal during ELISA. This interference could have been due to the reaction of immunoglobulins with uncapped aldehyde groups to form Schiff base linkages, or by non-SMX-specific interaction with the BSA carrier. The synthesis of a SMX-BSA conjugate **232** by Michael addition with **229** was performed to provide better defined protein conjugates free of any possible chemically reactive sites. Mixing a 2 mole ratio of the *N*-acryloylated SMX derivative **229** to BSA lysine

residues in 0.2M carbonate buffer (pH 10.0, [BSA] = 10 mg/mL) for 5 days at room temperature gave the SMX-BSA conjugate **232** as a material which precipitated partially upon dialysis. Centrifugation and lyophilization of the supernatant gave a 50% yield of soluble conjugate. The precipitated material was identified as proteins having high amounts of SMX incorporation. Presumably, SMX imparts hydrophobic character to BSA which then precipitates beyond a threshold level of SMX incorporation. The soluble fraction described above was used in enzyme assays for the reasoning that such a preparation contained a maximum amount of SMX which did not precipitate BSA. Decreasing reagent concentrations and the reaction time to 3 days did not substantially improve the yield of soluble conjugate. Insoluble fractions could be rendered soluble by adding HCl to increase the ionic character of the protein. However, such conjugates were not considered to be suitable for our assay.

Using soluble SMX-BSA **232** in ELISA did improve background interference slightly when compared to the SMX-glutaraldehyde-BSA conjugate **231**. However, the ill-defined nature of BSA conjugates in general and possible problems of non-SMX-specific interaction associated to using natural proteins as hapten carriers in immunochemical experiments, prompted the synthesis of sulfamethoxazole-poly-L-lysine conjugates (SMX-PLL). The homogeneous and non-natural character of poly-L-lysine (PLL) reduces the chance of any non-SMX-specific antibody detection as well as provide other advantages. Unlike BSA conjugates, an accurate determination of immobilized SMX could be made with relative ease using a non-destructive technique such as ^{1}H-NMR spectroscopy. Accurate SMX content determinations for the SMX-BSA conjugate **232** would require more involved techniques such as amino acid analysis, for example. Although SMX and the analogous N^4-acetylated derivative **233** (not shown; prepared by acetylation of SMX with Ac_2O/pyridine) has a UV absorption band at 260nm, the intensity is highly dependant on pH (hyperchromic effect with increasing pH) and is obscured by BSA absorptions appearing in the same region. The homogeneous polyamine structure of PLL and PLL

conjugates prepared by Michael addition allows for a more random and greater incorporation of SMX while retaining water solubility. Increases in hydrophobicity from conjugated SMX haptens can be offset by lowering the pH to increase protonation of the amino groups. Increasing the ionic character of SMX-PLL this way ensures water solubility. In one trial, a SMX-PLL conjugate was prepared where 42% of available lysine residues had been modified with an SMX ligand, and was easily solubilized by titration with a small amount of HCl.



Scheme 62

Preparation of a sulfamethoxazole - poly-L-lysine conjugate (SMX-PLL) **233** by Michael addition.

An SMX-PLL conjugate **233** was prepared with a high SMX hapten content (33% by mol) for the ELISA studies of AIDS patient serum. High amounts of immobilized SMX ligands were desired in order to maximize the sensitivity of any ELISA experiment through avidity effects. The SMX-PLL conjugate **233** was prepared by a Michael addition methodology by mixing the *N*-acryloylated SMX derivative **229** and poly-L-lysine (MW = 69 kDa) in a blend of 0.2M carbonate buffer pH 10.0 and DMSO (9/1, v/v) at room temperature for 4.5 days. The addition of DMSO was necessary to ensure a good solubility of

229 and did not adversely affect the reaction. In conjugation trials, *N,N* -dimethylformamide (DMF) instead of DMSO was also used without adverse effects. DMSO was prefered over DMF as a general cosolvent because amines are commonly found in commercial DMF samples[93a] and could compete for acryloylated derivatives. Dialysis of the crude SMX-PLL conjugate **233** in distilled water resulted in precipitation of the polymer conjugate. After dialysis, the SMX-PLL conjugate was easily resolubilized by slow titration with a small quantity of dilute HCl. To provide a more general method, and to give better defined and reproducible conjugates, dialysis was performed in 1 mM HCl which inhibited any precipitation. Lyophilization then gave **233** as a white, spongy solid which could be completely redissolved in water without any difficulty (≥ 40 mg/mL). A 33% by mol incorporation of SMX hapten in the conjugate **233** was determined by ^{1}H-NMR (D_2O) spectroscopy (Figure 61) while only a 31% by mol equivalent of *N*-acryloylated SMX (**229**) to lysine was provided for the reaction. The slightly higher than quantitative uptake of SMX in the new SMX-PLL conjugate **233** is explained again by the presence of water in the commercial poly-L-lysine sample used. The 1:2 SMX:free lysine ratio determination was made by comparing integration intensities of the aromatic and oxazole ring protons found at 7.4 - 7.7 ppm (br, 4H) and 5.7 ppm (br, 1H) respectively to the lysine resonances described earlier. The backbone methine resonance appearing as a relatively sharp signal at 4.35 ppm (H_α) serves as a good reporter for total lysines (conjugated and unconjugated). The remaining signals at 3.35 and 3.15 ppm (-CH_2-N-CH_2-, SMX-lys), 3.00 (H_ε), 2.0 (br, CH_2-C(O), SMX-lys), 1.70 (H_β + H_δ), 1.45 ($H\gamma$ + CH_3 of SMX) can also be used in the SMX content determination and gave the same result as above. The ^{13}C-NMR spectrum (D_2O) of SMX-PLL **233** confirmed the homogeneity of the polymer drug conjugate by providing a single set of relatively sharp resonances for poly-L-lysine and somewhat broadened lines for many of the SMX ligand nuclei.

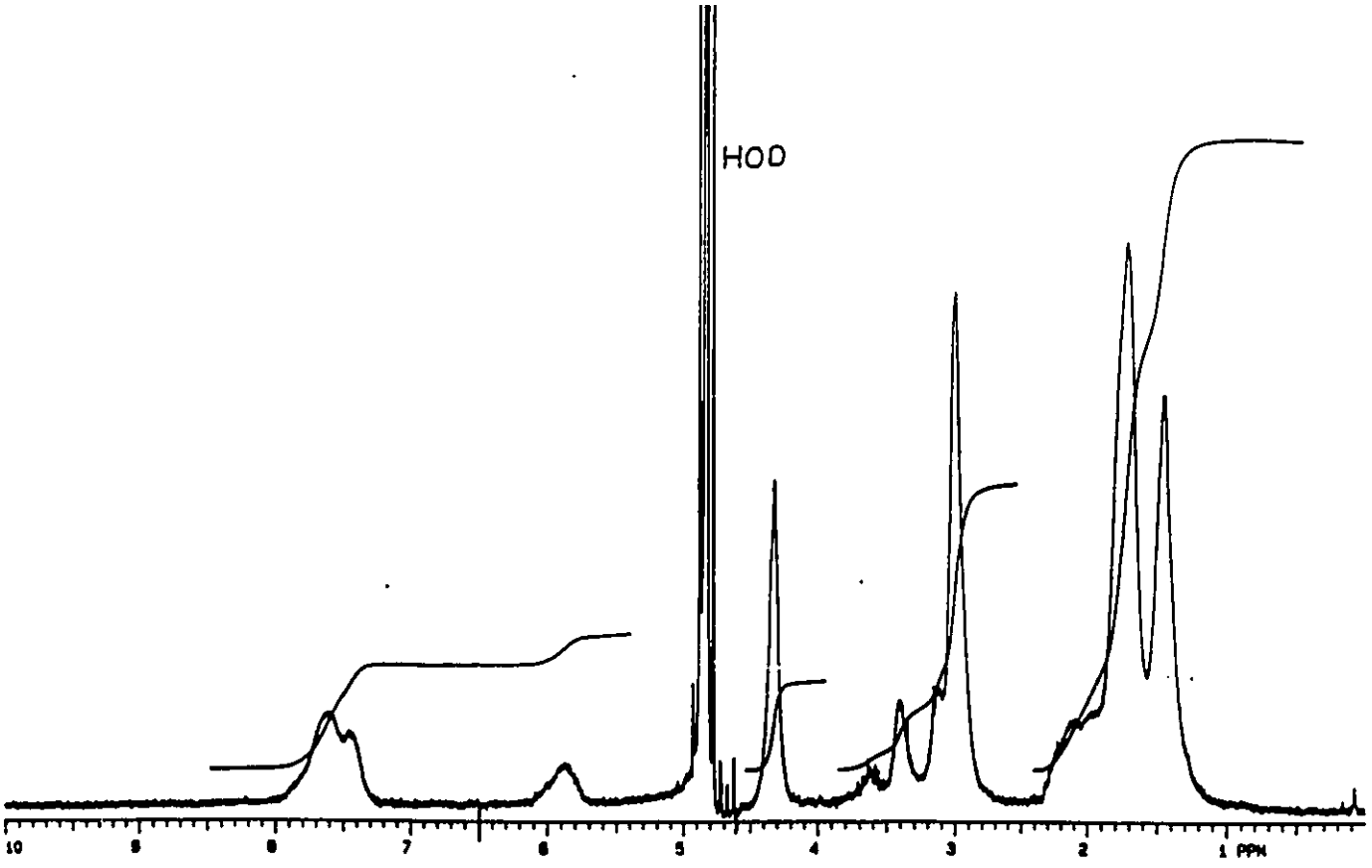


Figure 61: ^{1}H-NMR (300 MHz, D_2O) of SMX-PLL **233**

As suspected, the SMX-PLL conjugate **233** proved to be a superior antigen in enzyme linked immunoassays. It was found to be more sensitive than any BSA conjugate while providing very little background interference during ELISA experiments. Increased sensitivity is most likely due to the higher density of immobilized SMX hapten found in the poly-L-lysine conjugate over BSA conjugates. Increased background interference observed with BSA conjugates may be due to the presence of anti-albumin type antibodies present in blood serum which recognize the BSA carrier. The non-immunogenic nature of poly-L-lysine reduces the likelihood of antibody recognition of the poly-L-lysine carrier.

11.2 Assays and results

For the study, ELISA experiments were performed by coating microtiter plate wells with SMX conjugates followed by an incubation period with blood serum from various patients. Single serum samples were obtained from 40 HIV-seropositive individuals, 20 with a history of adverse reactions following 5-10 days of therapy with cotrimoxazole, and 20 individuals who had taken sulfa drugs without adverse reactions. A control population of 20 infants (age 9 ± 3 months), and 18 healthy adults with no history of adverse reactions to cotrimoxazole was used in the study. Detection of any anti-SMX antibodies bound to SMX conjugates was made using peroxidase labeled anti-IgM, anti-IgG, anti-IgE, anti-IgG_1, anti-IgG_2, anti-IgG_3 and anti-IgG_4 antibodies using *o*-phenylenediamine (OPD) as enzyme substrate. Colour development was measured at 450 nm after 40 min with an automated microtiter plate reader. Optical density (O.D) values more than two standard deviations above the mean O.D. measurement of infant and adult volunteers was used as criteria to define the presence of SMX directed antibodies in patient's sera. Infants were chosen as negative controls as they were less likely to have received cotrimoxazole and were less likely to have detectable levels of maternal antibodies against SMX.

Table 68 Detection of SMX specific antibodies in AIDS patients having adverse reactions to cotrimoxazole treatments by ELISA using BSA-gluteraldehyde-SMX **231** as coating antigen.

Immunoglobulin classes	Mean optical densities with standard deviations	
	Patients[a] (n=9)	Controls[b] (n=8)
IgG	0.86 ± 0.12	0.25 ± 0.22
IgM	1.46 ± 0.39	0.32 ± 0.23

a) HIV-seropositive patients with a history of adverse reactions to cotrimoxazole.
b) HIV-seronegative patients with no history of adverse reaction to cotrimoxazole.

Initial results with SMX-glutaraldehyde-BSA conjugates **231** (Table 68) showed increased levels of anti-SMX IgG and IgM antibodies in HIV-seropositive patients (8) having a previous reaction to cotrimoxazole when compared to HIV-seronegative patients (9). In addition, antibody levels were shown to be higher in HIV-seropositive patients with adverse reaction to cotrimoxazole than in HIV-seropositive patients not expressing any adverse effects to the drug when the SMX-PLL conjugate **233** was used in the assays (Table 69). Significantly increased levels of anti-SMX IgM antibodies over other antibody classes was observed in all HIV-seropositive patients. However, levels were higher in patients showing adverse reactions to cotrimoxazole (Figure 62). Although 85% of HIV-seropositive patients had detectable anti-SMX IgG antibodies in circulation, the levels were again higher in those patients experiencing hypersensitivity to cotrimoxazole. Further study demonstrated that the anti-SMX IgG antibodies were exclusively of the IgG_2 and IgG_3 subclasses (Table 70, Figure 63). No IgE antibodies directed against SMX could be detected in any patient.

Figure 62 Measured (ELISA) optical densities (O.D.) representing IgM and total IgG anti-SMX antibodiy levels in patients treated with SMX. No significant levels of IgE antibodies were detected.

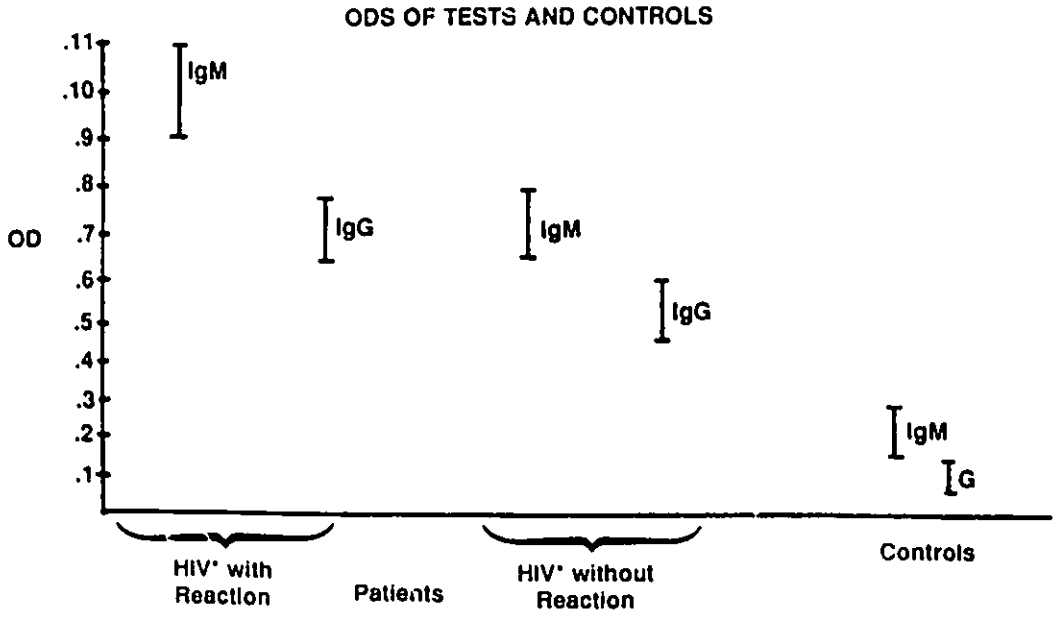


Table 69 Frequency and mean O.D. values# representing anti-SMX antibodies in blood sera of normal adults, infants and HIV-seropositive patients measured by ELISA using SMX-PLL **233** as coating antigen (1 μg).

Subject	Sample size	IgM		IgG	
		O.D.	frequency	O.D.	frequency
HIV(-) adults	10	0.17 (±0.05)	0	0.11 (±0.02)	0
HIV(+) with reaction	20	1.05 (±0.24)	20	0.72 (±0.07)	19
HIV(+) with no reaction	20	0.71 (±0.05)	9	0.52 (±0.08)	17
infants	20	0.09 (±0.02)	0	0.09 (±0.03)	0

Standard deviations are given in parentheses.

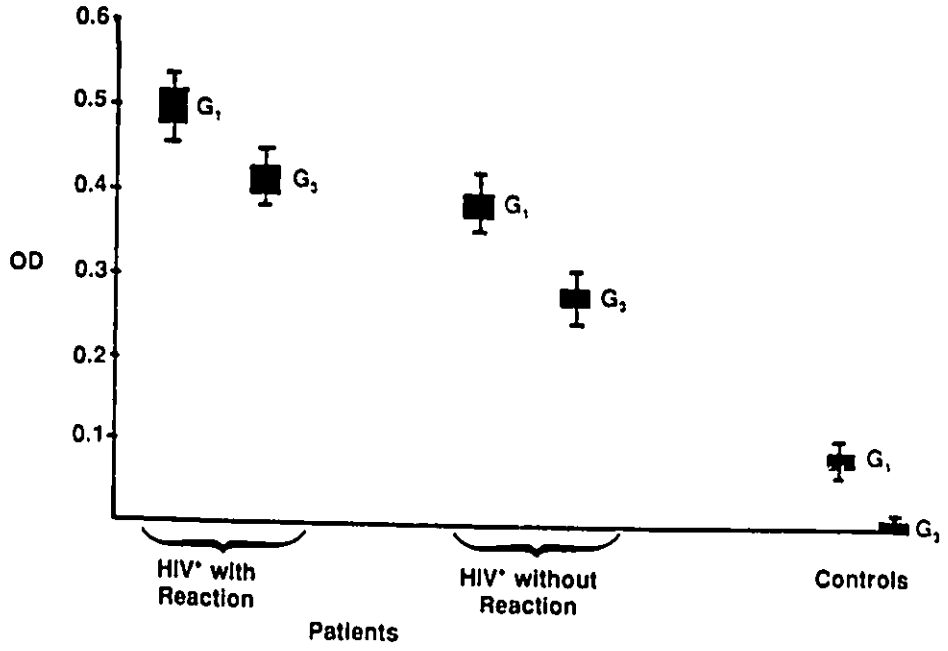


Figure 63 Measured (ELISA) optical densities (O.D.) representing levels of IgG_1 and IgG_3 subclasses in patients treated with SMX. No significant levels of IgG_2 or IgG_4 could be detected.

Table 70 Frequency (F) and mean O.D. values representing anti-SMX IgG subclasses in blood sera of infants, normal adults and HIV-seropositive patients with and without adverse reactions to cotrimoxazole using SMX-PLL **233** as coating antigen in ELISA.

Subject	Sample size	IgG_1		IgG_2		IgG_3		IgG_4	
		O.D.	F	O.D.	F	O.D.	F	O.D.	F
HIV(-) adults	10	0.10 (±0.03)	0	0.10 (±0.02)	0	0.0	0	0.0	0
HIV(+) with reaction	20	0.50 (±0.08)	19	0.10 (±0.01)	0	0.41 (±0.03)	19	0.0	0
HIV(+) with no reaction	20	0.40 (±0.07)	17	0.10 (±0.02)	0	0.26 (±0.03)	17	0.0	0
infants	20	0.00	0	0.02 (±0.02)	0	0.0	0	0.0	0

The SMX-PLL conjugate **233** was also useful in studying the potential development of an immune response against SMX in HIV infected individuals To monitor the development of anti-SMX antibodies prospectively, 3 additional patients (2 HIV-seropositive, and 1 HIV-seronegative) were studied with serum samples taken prior, during and after cotrimoxazole therapy. The two HIV-seropositive patients received cotrimoxazole intravenously (100 mg SMX and 20 mg TMP per kg/day) for the treatment of Pneumocystis carinii pneumonia. Cotrimoxazole treatment had to be discontinued in both patients at days 8 and 10 of therapy due to adverse reaction (rash and fever). The HIV-seronegative patient was treated with oral cotrimoxazole (1600 mg SMX and 320 mg TMP per day) for a respiratory infection. No anti-SMX antibodies could be detected in any of the subjects prior to treatment, or at any time for the HIV-seronegative patient. In contrast, anti-SMX IgG and IgM were present after treatment began in the 2 HIV-seropositive subjects, and remained elevated 3-4 weeks after therapy was discontinued.

Table 71 Detection of anti-SMX IgM in blood sera of patients undergoing cotrimoxazole therapy. Optical densities measured by ELISA using SMX-PLL **233** as coating antigen.

Time (in weeks)	Patient A HIV (-)	Patient B HIV (+)	Patient C HIV (+)
0	0.31	0.43	0.54
2	0.36	1.05	1.29
5	0.36	0.93	1.03

Treatment in patients B and C was discontinued after 8 and 10 days respectively due to hypersensitivity symptoms.

The ELISA strategy using BSA and, in particular, PLL conjugates of SMX has helped to demonstrate the evolution of an immune response to SMX in HIV infected individuals. The results obtained show that anti-SMX antibodies are present in the majority of HIV-seropositive patients treated with SMX whether they have experienced side effects to SMX or not. However, higher antibody levels were observed in patients who could not tolerate cotrimoxazole therapy than in those who did. A humoral response to SMX was characterized by the detection of increased levels of SMX specific IgM, IgG_1 and IgG_3 antibodies. The high presence of IgG_3 antibodies, the most efficient subclasses to initiate complement cascade[260], suggest that complement activation could be involved in hypersensitivity to SMX. Additional evidence for the role of humoral immunity to SMX in AIDS patients comes from patients studied before, during and after SMX treatment (Table 71). An immune response to SMX treatment was only detected in HIV-seropositive patients .

With both the bovine serum albumin (BSA) and poly-L-lysine (PLL) sulfamethoxazole (SMX) immunoconjugates, the detection of anti-SMX IgM antibodies identified patients with adverse reaction to SMX better than IgG antibodies. Antibodies against SMX have previously been observed[260,261] but not fully studied in patients with adverse

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[260] C. Ressler, M.D. Greaney, G. J. Tatake, L.R. Brettman and M. Ballow, *J. Allergy Clin. Immunol.*, *85* , 156 (1990).

[261] a) T. J. Sullivan, R. Davidson, R. Budens and S. Nightingale, *Clin. Pharmacol.*, 9 , 136 (1990). b) C. Ressler, M. Knapp, G. J. Tatake, M. Ballow and P.

reactions to this drug, in both HIV-seronegative and seropositive populations. In one case[262], a less effective poly-L-lysine conjugate and conjugation procedure to that described here was used. In the study described here, many individuals with detectable anti-SMX antibodies had undergone treatment without adverse side effects. This suggests that a low-level of antibodies to SMX can be tolerated without overt symptoms, and that there might be a critical level of antibodies to SMX necessary to develop a reaction (rash and fever). The mechanism for the development of side effects to SMX treatment is likely complex, involving toxic and immunologic components. A more indepth rationalization of SMX toxicology in infected individuals is beyond the scope of this dissertation and this author's direct involvement. A more detailed look at the development of immune hypersensitivity to sulfamethoxazole based on observations described here and elsewhere[263], has already been presented[258]. A full description of the study is forthcoming[264].

11.3 Inhibition studies

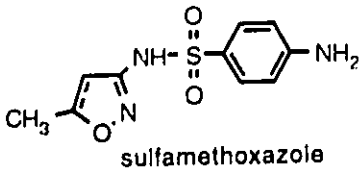
Binding inhibition studies were performed to confirm the specificity of our assays as well as to evaluate structural features required for the antigenic determinant of anti-SMX antibodies. Sulfamethoxazole (SMX) and other structurally related compounds were used in the inhibition assays. The structures of the inhibitors are shown below and include popular sulfa drugs.

Rapacz, *J. Allergy Clin. Immunol.*, 79 , 198 (1987).

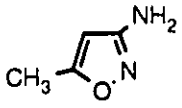
262 J.G. Tatke, M. Knapp and C. Ressler, *Bioconjugate Chem.*, 2 , 124 (1991).

263 P. Daftarian, Ph.D. thesis, Department of Immunology, University of Ottawa, Ottawa, Canada, submitted September 1st, 1992.

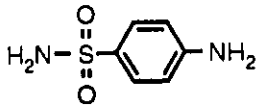
264 a) P.Daftarian, B. Conway, F.D. Tropper, R. Roy, L. Filion and F. Diaz-Mitoma. Classes and Subclasses of Antibodies to Sulfamethoxazole in AIDS patients. Manuscript in final stage of preparation. To be submitted to *J. Allergy Clin. Immunol.* ; b) F.D. Tropper, P. Daftarian, B. Conway, L. Filion, F. Diaz-Mitoma, R. Roy. Design and Synthesis of Drug Conjugates for the Diagnosis and Study of Immune Hypersensitivity towards Sulfamethoxazole. Manuscript in preparation, to be submitted to *Med. Chem. Lett.*



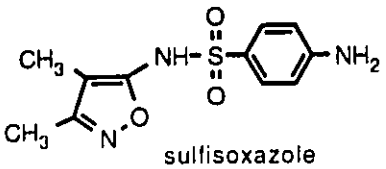
sulfamethoxazole
(SMX)



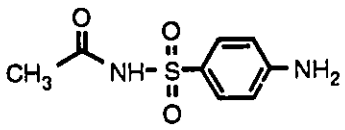
3-amino 5-methyl isoxazole
(AMI)



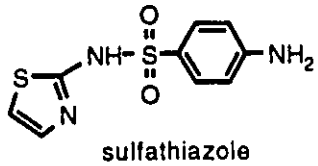
sulfanilamide
(SM)



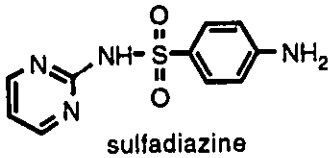
sulfisoxazole
(SXZ)



N^1-acetyl sulfanilamide
(ASM)



sulfathiazole
(STZ)



sulfadiazine
(SDZ)

The specificity of our assay for detecting anti-SMX antibodies from the blood serum of HIV infected individuals treated with cotrimoxazole was confirmed during ELISA inhibition studies. The blood serum of 4 HIV-seropositive patients having high levels of anti-SMX IgM antibodies was used for the study. The serum was diluted 80 fold with PBS and then incubated for one hour at 37 °C with varying amounts of different inhibitors before being assayed. The BSA-gluteraldehyde-SMX conjugate **231** was used as coating antigen for the assay of 100 µL volumes of preincubated sera samples.

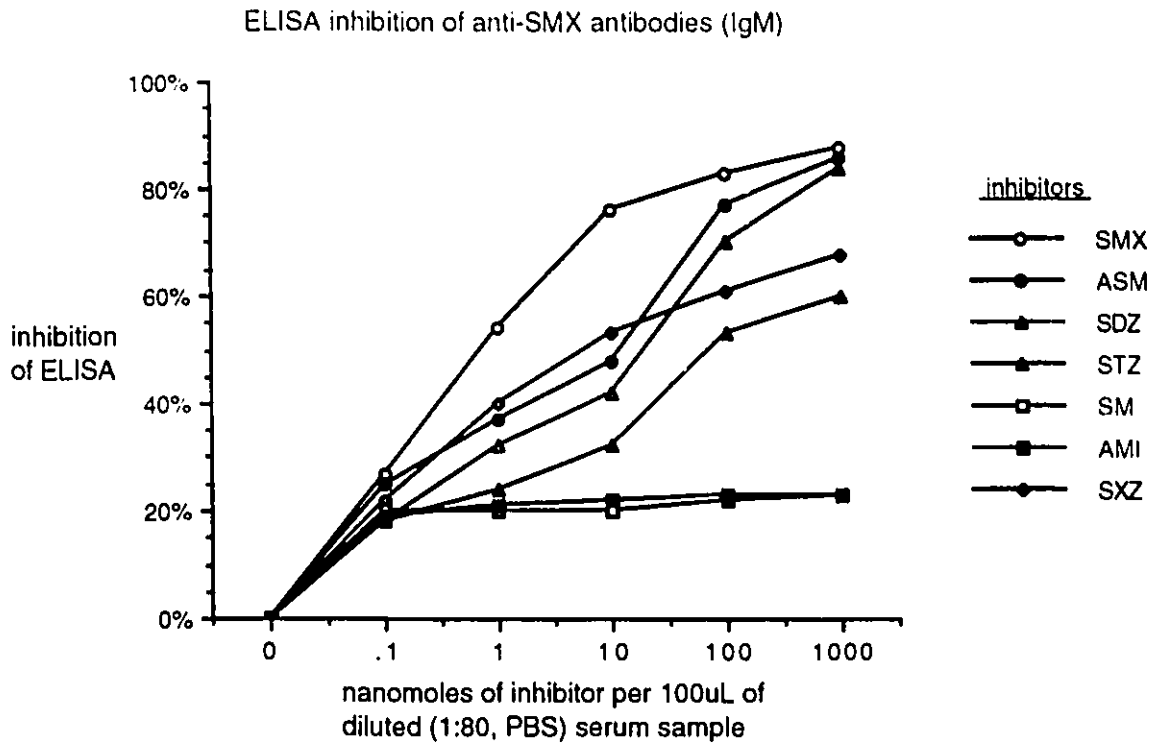


Figure 64 Binding inhibition curves for the anti-SMX ELISA with selected inhibitors.

The results (Figure 64) clearly indicate that SMX is the best inhibitor for the assay and demonstrates the specificity of the assay. Trimethoprim did not inhibit the ELISA, nor did the two half-components of SMX, 3-amino 5-methyl isoxazole (AMI) or sulfanilamide (SM). The requirement of a full two ring structure of SMX was also demonstrated with the SMX analogue sulfasoxazole (SXZ) which was only 10 times less effective as inhibitor. Sulfadiazine (SDZ) and N^1-acetyl sulfanilamide (ASM) could also inhibit binding but to a much lesser extent. The ability of ASM to inhibit binding and not simple sulfanilamide suggests that an acidic sulfonamide N-H group is required, and that an ionic interaction at this site may be involved. Inihibition data from, and structural differences in, sulfisoxazole (SFZ) and sulfathiazole (STZ) when compared to SMX suggest that the presence of heteroatoms (N,O) in the isoxazole ring of SMX are necessary and may be involved in a

polar interaction such as hydrogen bonding within the binding site. This is also supported by inhibition data of sulfadiazine (SDZ) which contains a nitrogen centre in the same relative position as SMX.

More work is required and is presently underway to better characterize the antigenic determinant responsible. Results will be presented in due course. The inhibition results presented here suggest that sulfa drugs not recognized by the anti-SMX antibodies could be alternative drugs for the therapy of Pneumocystis carinii pneumonia (PCP) for individuals who exhibit hypersensitivity towards sulfamethoxazole.

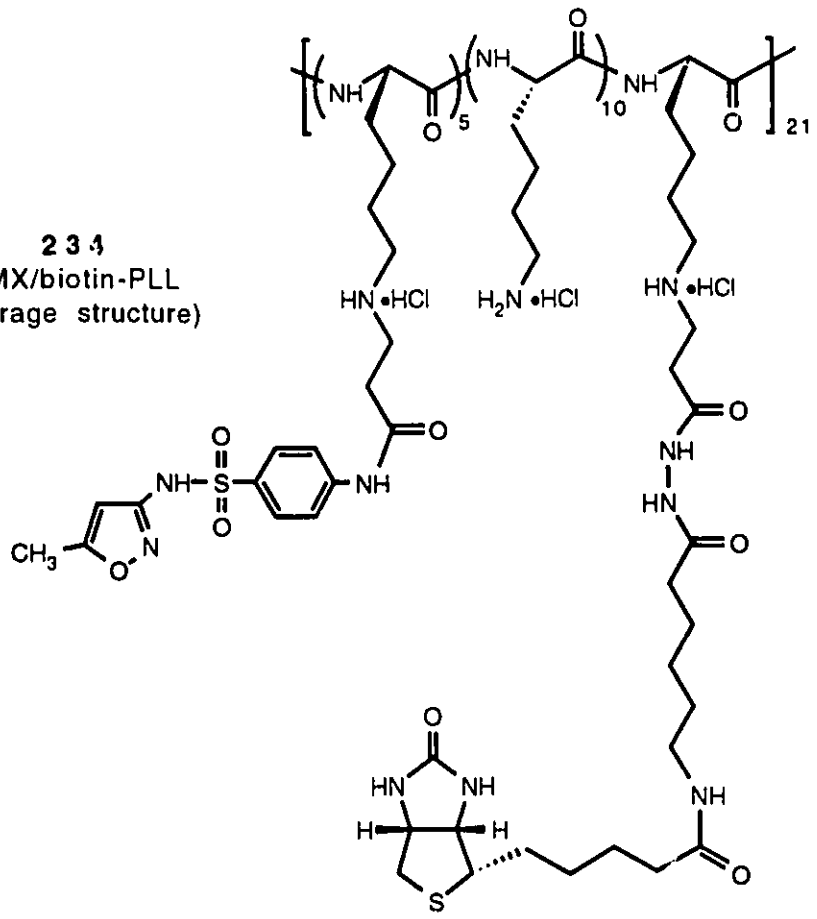
11.4 Outlook to future studies

The effect of HIV infection on the human immune system is a complex chain of events which is not clearly understood. Individuals afflicted with AIDS suffer a viral invasion and subsequent breakdown of the immune system which leaves them incapable of effectively combatting infectious agents. It has been demonstrated that sulfamethoxazole stimulates an immune response in HIV-seropositive patients which often results in hypersensitivity reactions. For this reason, reaction to SMX may be a good model to study immunoregulation in AIDS patients.

Preliminary work by collaborators on this project has recently shown a high incorporation of SMX-PLL conjugate **233** into T-cells of HIV-seropositive treated with SMX. As a tool for more elaborate research of this and other immunoregulation processes, a biotinylated SMX-PLL conjugate (**234**) was prepared. (+)-Biotin haptens were incorporated as immunochemical probes to monitor the uptake of poly-L-lysine-sulfamethoxazole conjugates within immune processes. Biotinylation of SMX-PLL **233** was accomplished by the same procedure described previously for the $(Neu5Ac)_{15}$/biotin-PLL conjugate **218**. Briefly, a sample of the SMX-PLL conjugate **233** and the *N*-acryloylated biotin monomer **213** were

stirred in 0.2M carbonate buffer (pH 10.0) containing 15% DMSO for 4.5 days to give the biotinylated conjugate SMX/biotin-PLL **234**.

234
SMX/biotin-PLL
(average structure)



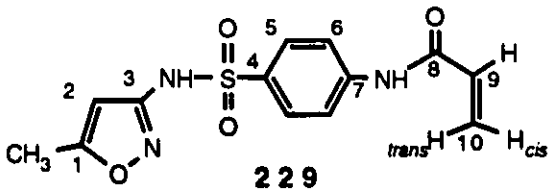
Incorporation of biotin by Michael addition was performed in a way designed to give a 1:5 molar ratio of biotin:SMX. Biotin incorporation in **234** could not be assayed accurately by ^{1}H-NMR spectroscopy due to interference from the poly-L-lysine signals. Observations from all other Michael addition conjugation reactions performed under identical conditions suggest that a quantitative or near quantitative uptake of **213** should have been realized in this case also. However, the presence of covalently bound biotin in **234** was verified with 0.2% cinnamaldehyde in acidified ethanol. Treating a small sample of **234** with this reagent gave a bright pink colouration when heated briefly, while the biotin deficient conjugate **233** did not react. The SMX/biotin-PLL conjugate **234** has been forwarded to collaborators

for further investigation of the immune response to SMX in AIDS patients. Results of these investigations will be presented in due course.

11.5 Experimental methods

Acryloylation of sulfamethoxazole **229**

Sulfamethoxazole (402.1 mg, 1.59 mmol, Aldrich) was dissolved in 250 mL of methanol containing 1.1 mL of triethylamine. After cooling to 0 °C, a 4% solution of acryloyl chloride in CH_2Cl_2 was added in a very slow, dropwise fashion until TLC (16/1/1, $CHCl_3/CH_3OH/CH_3CN$) showed a complete consumption of the amine (Rf = 0.35) to give the acrylamide **229** (Rf = 0.55) as the major product. After evaporation of the methanol, the residue was taken up in EtOAc and 0.1M HCl. The aqueous phase was quickly separated and the organic phase washed with sat. $NaHCO_3$. During this extractive work up, while floculent crystals appeared in the aqueous phase. These were filtered and washed with copious amounts of water. This material (260 mg, dried under vaccum over P_2O_5) was identified by ^{1}H-NMR and CI-MS to be the desired pure product (TLC and elemental analysis). Evaporation of the ethyl acetate phase, after washing with water then drying over Na_2SO_4, gave a slightly yellow, crystalline mass which contained more of the desired product (TLC). This product was crystallized from acetone/water after being chromatographically purified through a short silica gel plug using $CHCl_3/CH_3OH/CH_3CN$ (14/1/1, v/v/v) as eluent. An additional 110 mg of **229** was recovered for a total yield of 370 mg (76%). The melting point of both isolates were identical (mp= 258.8-261.4 °C).



229

CI-MS (ether) gave only the molecular ion (M+1) at m/e = 308 (100%). ^{1}H-NMR δ (DMSOd_6): 7.78 - 7.85 (symmetrical mult, 4H, H_5 and H_6), 6.43 (dd, 1H, J_{cis} = 9.9, J_{trans} = 17.0 Hz, H_9), 6.29 (dd, 1H, J_{gem} = 2.1 Hz, H_{10}, trans), 6.12 (d, 1H, $J_{allylic}$ = 0.9 Hz, H_2), 5.81 (dd, 1H, H_{10}, cis), 2.28 (d, 3H, $J_{allylic}$ = 0.9 Hz, CH_3). ^{13}C-NMR δ (DMSOd_6): 170.3 (C_8), 163.7 (C_1), 157.6 (C_3), 143.3 and 133.4 (C_4 and C_7), 131.3 (C_9), 128.1 (C_5 and C_{10}), 119.2 (C_6), 95.4 (C_2), 12.1 (CH_3). λ_{max} = 293 nm (CH_3CN) or 278.5 nm (0.01M NaOH).

SMX-CPA conjugate **230**

The *N*-acryloylated sulfamethoxazole derivative **229** (50.0 mg, 0.163 mmol) and acrylamide (115.1 mg, 1.62 mmol) were refluxed in 2.5 mL of water and DMSO (4/1, v/v) under a nitrogen atmosphere. After a few minutes (<5), the polymerization was initiated by injecting 25 μL of ammonium persulfate (50 mg/mL). The initially turbid solution turned to a clear viscous one after continued reflux for 45 min. The resulting solution was diltuted with water then dialyzed exhaustively against distilled water. Lyophilization gave 148.3 mg (90%) of white spongy SMX-CPA **230** polymer which had λ_{max} = 263.5 nm (H_2O). Elemental analysis of this material gave C:44.97%, H:6.22%, N:15.80% and S:2.42% which corresponds to a 1:12.4 molar incorporation ratio of **229**:acrylamide and a retained water content of 13.6% (by weight) according to the procedure outlined in Chapter 2 (equation 1).

^{1}H-NMR δ (D_2O): 7.4 - 7.9 (broad mult, 4H, phenyl), 5.8 (broad, 1H, oxazole =CH-), 2.0 - 2.5 (broad, 13.5H, backbone methine + 3H from the SMX methyl), 1.4 - 2.0 (broad, 27H, backbone methylene). Integration ratios indicated a 1:12.5 molar ratio of **229**:acrylamide, consistent with the elemental analysis results. ^{13}C-NMR δ (D_2O): 180.3 (C=O), 176.3 (C_1, SMX), 172.2 (C_3, SMX), 141.5 and 137.1 (C_1 and C_7, SMX), 128.8 (C_5, SMX), 42.5 (broad, backbone methine), 36.6 and 35.8 (broad, backbone methylene), 12.6 (CH_3, SMX).

SMX-glutaraldehyde BSA conjugate **231**

The conjugate **231** was prepared as described by Saunders[265] in polystyrene flat-bottom 96-well microtiter plates (NUNC, Gibco, Montreal, Canada). Briefly, 100 µL of BSA dissolved in distilled water (30 µg/mL) was deposited in each well. The plates were alowed to dry for 1 hour at 37 °C after which 100 µL of 0.2% glutaraldehyde, pH 7.2 (JBS Supplies, EM Services, Toronto, Canada) was added. The plates were incubated at 37°C for 30 min. then washed five times with distilled water (300 µL). Sulfamethoxazole (100 µL of a 10 µg/mL solution in PBS pH 8.0) was added to each well, and the plates incubated for 2 hours at room temperature. Any remaining unreacted aldehyde groups were capped with 200 µL of 1M glycine (Sigma) in PBS pH 7.2 with a 1 hour incubation period at 37 °C. Finally, the plates were washed 5 times with PBS pH 7.2 containing 0.5% Tween 20 (Sigma) and blocked with 1% gelatin (Bio Rad, Montreal, Canada) in PBS pH 7.2 for 2 hours at room temperature. Plates were then stored at 4 °C for later use.

SMX-BSA conjugate **232**

BSA (20.0 mg, 18 µmol lysines) and **229** (10.8 mg, 35 µmol) were homogenized in 2 mL of 0.2M carbonate buffer pH 10.0 using a Vortex mixer. After 5 days at room temperature, the clear solution was dialyzed against three 5 L volumes of distilled water. During dialysis, a precipitate was formed. The precipitate was sedimented by centrifugation at 14, 000 g. The supernatant was lyophilized and gave 9.9 mg (50%) of SMX-BSA **232** which redissolved readily in water. The precipitate protein could be redissolved by titration with dilute HCl or NaOH. The sulfamethoxazole content could not be assayed by UV spectroscopy due to overlapping tyrosine and

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[265] G.C. Saunders, *The art of solid phase enzyme immunoassay including selected protocols.* in Immunoassay in the Clinical Laboratory, R.M. Nakamura, W.R. Dito and E.S. Tucker (eds.), Alan R. Liss, New York, 1979, p. 100 - 118.

tryptophane bands in the 260-270 nm region containing the sulfamethoxazole absorption. This SMX-BSA **232** conjugate was found to be only slightly better as coating antigen than **231** prepared by glutaraldehyde condensation.

SMX-PLL conjugate **233**

Poly-L-lysine hydrobromide (110 mg, 0.523 mmol of lysine) and **229** (50 mg, 0.163 mmol) was dissolved in 9 mL of 0.2M carbonate buffer pH 10.0 and 1 mL of DMSO. The reaction was stirred gently at room for 4.5 days. After 1 day, 1000 µL of this solution was removed and treated with **213** to give the SMX/biotin-PLL conjugates (see next). The solution was dialyzed against distilled water (5 L) which caused the poly-L-lysine conjugate to precipitate after roughly 10 min. Dialysis was then carried out against 1mM HCl (5 L) which caused the conjugate to resolubilize. Lyophilization gave 125.1 mg of **233** as a white, spongy solid. ^{1}H-NMR spectroscopy analysis indicated that on average, one of every three lysine residues had been modified (33% by mol). The determination was carried out by comparing integration intensities of the oxazole ring proton signal to the backbone methine signal.

^{1}H-NMR (D_2O): all peaks are broad with no fine structure. Centered positions are reported. d: 7.7 (2H, H_5 SMX), 7.4 (2H, H_6 SMX), 5.7 (1H, =CH-, oxazole), 4.35 (3H, backbone methine), 3.35 (H, $C(O)CH_2$-SMX), 3.8 - 3.2 (8H, CH_2-N SMX and H_ε lys), 1.7 (11H, H_β + H_δ lys and CH_3 SMX), 1.45 (6H, H_γ lys). ^{13}C-NMR δ (D_2O, referenced to internal methanol at 48.97 ppm): 173.3 (C=O lys), 170.1 (C=O SMX), 161.5 (C_1 SMX), 158.3 (C_3 SMX), 141.0 and 135.5 (C_4 and C_7 SMX), 127.5 (C_5 SMX), 119.8 (C_6 SMX), 95.8 (C_2 SMX), 53.2 (C_α lys), 42.7 (CH_2-N SMX), 39.0 (C_ε lys), 31.7 ($CH_2C(O)$ SMX), 30.3 (CH_2 lys), 26.1 (CH_2 lys), 21.9 (CH_2 lys), 11.4 (CH_3 SMX),

SMX/biotin-PLL conjugate **234**

The 1000 µL aliquot (52.3 µmol lysine and 16.3 µmol **229**) was

treated with 180 µL of **213** (8.2 mg/mL DMSO 3.5 µmol) and left to stir at room temperature for 4.5 days. Dialysis in 1mM HCl (5 L) followed by lyophilization gave 12.5 mg of **234** as a white, spongy solid. The ^{1}H-NMR spectrum appeared as described for **233** with no truly discernible biotin resonances unobstructed lysine signals. Biotin content was demonstrated by reacting a small sample of **234** (~100 µg) with 0.2% cinnamaldehyde in acidified ethanol (Sigma). Gentle warming resulted in a pink colour generation while the biotin deficient conjugate **233** did not react under identical conditions.

Enzyme linked immunoassays.

SMX coating anitgens.

When SMX-PLL **233** or SMX-BSA **232** were used as coating antigens (1 µg/well), 100 µL of each antigen (10 µg/mL) were deposited in wells of flat-bottom polystyrene microtiter plates (NUNC) which were then allowed to dry at 17°C (1hr.). Plates were then blocked with 1% gelatin in PBS pH 7.2 (200µL, 2 hrs. rt) and washed (5x) with PBS pH 7.2 containing 0.5% Tween 20.

Evaluation of study samples

Subject sera (100 µL, diluted 1:40 in PBS pH 7.2) were added to each well. After incubation (1hr. at 37°C) the plates were washed with PBS pH 7.2 containing 0.5% Tween 20 (3x 200 µL). Antibody classes and subclasses were evaluated with 100 µL of appropriately diluted peroxidase labeled anti-IgM (1:4000), anti-IgG (1:2000), anti-IgE (1:45000), anti-IgG_1 (1:160), anti-IgG_2 (1:160), anti-IgG_3 (1:160), anti-IgG_4 (1:160). Peroxidase labeled anti-IgE and anti-IgG subclasses were purchased from Active Site (Birmingham, England). Peroxidase labeled anti-IgM and anti-IgG were purchased from Taco Inc. (Burlingame, CA, USA). After a 1 hr. incubation period at 37°C, the plates were washed (3x 200µL) with PBS pH 7.2 containing 0.5%

Tween 20. Plates were developed by adding the peroxidase substrate ortho-phenyldiamine (100 µL, 2 mg/mL), and after 40 min., measuring the colour development at 450 nm with an automated plate reader (Dynatech MR 600).

Hapten inhibition assays

For this analysis, 50 µL of serum from 4 subjects having high anti-SMX IgM levels were diluted 1:40 in PBS pH 7.2 and preincubated with 50 µL of inhibitors (purchased from Sigma and Aldrich) of appropriate concentrations (1nM to 1 µM) for one hour at 37 °C. All samples were processed for ELISA testing as described above using plates coated with SMX-glutaraldehyde BSA **231**. The reduction in measured antibody levels was taken as an indication of inhibition using the following equation:

$$\% \text{ inhibition} = (1 - O.D._{inhibited} / O.D._{uninhibited}) \times 100$$

Conclusion

The general object of the research undertaken for this Ph.D. dissertation was the development of new glycoconjugates useful as tools for studying carbohydrate-protein recognition and binding interactions. The primary focus was directed towards a rational strategy for developing well-defined and superior water-soluble polymers bearing pendant carbohydrate residues. These so-called "glycopolymers" were designed to act as multivalent antigens specific for selected lectins and antibodies. This goal in mind, coupled with particular interest in wheat germ agglutinin binding site requirements, has led to a better understanding of both systems. During research, different new avenues were opened and capitalized upon to provide maximum product diversity and usefulness from each step. Born out of this research has been the development of a wide variety of improved glycoconjugation strategies as well as an important and general phase transfer catalyzed glycosylation methodology. All new methods and strategies were developed and shown to be general, then applied to more complex or important examples.

A variety of glycopolymer systems, having different structures, functions and properties were created. A general copolyacrylate-type (copolyacrylamide in particular) glycopolymer design and synthesis strategy has provided multivalent carbohydrate antigens having many superior or desirable properties. As antigens, glycopolymers have many desirable properties and are superior in every way to popular neoglycoprotein conjugates. Unlike neoglycoproteins however, copolyacrylamide glycopolymers are non-immunogenic and resistant to metabolism. Of the many desirable properties they possess, they are inexpensive to prepare and isolate in excellent purity on a wide scale range (10 mg - grams). They are chemically and enzymatically very resistant and can be stored at room temperature for seemingly indefinite periods without any

degradation or loss in activity[1]. They are well-defined, homogeneous macromolecules which can be fully characterized by simple methods such as NMR. The sugar content and molecular weight can be controlled at will by a simple choice of reagent concentrations to provide glycopolymers which exist in water as dynamic random coils with randomly alternating monomer compositions. Although not requiring the use of any buffers, solubility is unaffected by even highly ionic solutions. Because of their synthetic nature and poor immunogenicity, they do not provoke any non-hapten-specific binding and do not interfere in quantitative protein assays. A simple extension to a terpolymerization strategy provides heterobifunctional glyco(ter)polymers with specialized features.

The conversion of carbohydrates and terpolymerizable effectors to the necessary *N*-substituted acrylamide monomers, is accomplished by mild acryloylation of a terminal amino or hydrazide group. Such amine groups are easily introduced to carbohydrate haptens by mild reduction of popular nitrophenyl glycosides or by a light catalyzed cysteamine addition to allyl glycosides. In this way, both aromatic and aliphatic spacer arms are provided in high yields. In addition to being better monomers for aqueous radical copolymerization with acrylate-type comonomers such as acrylamide, the acrylamide function also serves very well in a new glycoprotein synthesis methodology. Michael addition of amino (or thiol) groups of proteins (ε-amines of L-lysine in particular) is a simple, mild and effective way of covalently attaching carbohydrates to proteins. The dual reactivity of the acrylamide function allows for the simultaneous access to both glycopolymers and neoglycoprotein conjugates. Thus, immunogens and antigens can be produced which share only the features of the hapten of interest. The new Michael addition procedure for preparing neoglycoproteins is superior in other ways such as that it requires no additional reagents beyond the desired protein carrier and the *N*-acryloylated hapten. High levels of hapten incorporation can be achieved without altering the overall net ionic

[1] Some glycopolymers are now as old as five years.

charge of the protein or degrading it.

Poly-L-lysine based glycopolymers have also been produced which bridge the gap between natural protein carriers and synthetic copolyacrylate-type glycopolymers. The natural composition of poly-L-lysine allows its use *in vivo* while being poorly immunogenic. It also provides well-defined homogeneous multivalent antigens with excellent serological properties (immunodiffusion, quantitative precipitation and ELISA). As a carrier, poly-L-lysine provides a water-soluble structure having built-in aliphatic spacer arms terminated by reactive amino groups to which carbohydrates, effectors or other haptens may be grafted to by various methods.

The strategies developed for preparing glycoconjugates as tools for protein receptor studies has been extended to the preparation of therapeutic drug conjugates. Immunodiagnostic sulfamethoxazole conjugates have been prepared to study and diagnose immune hypersensitivity to sulfamethoxazole in HIV seropositive patients. From this, it follows that the same strategies could be performed for peptide antigens after *N*-acryloylation of the *N*-terminus or the *C*-terminus if this one is converted to a hydrazide group, for example.

Beyond the antigenic and immunogenic conjugate design strategies, a general phase transfer catalyzed glycosylation methodology has also been developed. Born out of observations made during synthesis of aryl glycoside inhibitors, phase tranfer catalysis has proven very useful as a method for preparing a wide range of glycosyl derivatives. It is a simple and effective procedure which is completely stereospecific and applicable to relatively soft and acidic nucleophiles. Using peracetylated glycosyl halides, glycosylation occurs by S_{N2} displacement of axially disposed halides to give equatorially disposed aglycons. The method is very general. It has been successfully applied to a wide range of nucleophiles and carbohydrates. Monosaccharides ranging from glucose and galactose to their corresponding 2-acetamido-2-deoxy analogues, to sialic acid have proven to be compatible with this methodology, as well as

numerous disaccharides.

Claims to original research

The work described in this dissertation includes new ideas, strategies and methodologies which have contributed to advancements in the field of carbohydrate chemistry and immunochemistry. Some of the new strategies and methodologies that have already been published have generated interest in the scientific community in the form of collaborations and adaptations of this work by well-respected, international research labarotories.

For example, Hans Paulsen (Hamburg, Germany) and coworkers have been successfully applying the strategy of radical addition of cysteamine to allyl glycosides folowed by *N*-acryloylation of the amine and copolymerization with acrylamide to produce water-soluble glycopolymers of complex and biologically relevant oligosaccharide antigens[105bc]. The strategy of preparing glycopolymer and neoglycoprotein conjugates from nitrophenyl glycoside precursors attracted the attention of Kushi L. Matta (Buffalo, NY) which resulted in a collaboration to produce conjugates of biologically relevant oligosaccharide antigens for use in cancer research (Section 8.3). Expertise acquired in preparing glycoconjugates from *N*-acryloylated carbohydrate derivatives sparked an interdisciplinary collaboration with physicians and researchers from the Virology Division of the Children's Hospital of Eastern Ontario and the Departments of Immunology and Microbiology at the University of Ottawa to develop sulfamethoxazole conjugates for the study and diagnosis of immune hypersensitivity in HIV seropositive patients. Glycopolymers of *N*-acetylglucosamine have been requested by Dr. Kenneth Lloyd of the Sloan Kettering Cancer Institute in New York City for testing in cancer research.

To summarize the major contributions brought forth through the work presented in this dissertation, a list of strategies, methodologies and results is described below in a general manner.

New compounds are not listed.

1. A method for preparing glycosides having an extended flexible spacer arm bearing reactive terminal groups has been provided through an aqueous light-catalyzed addition of functionalized thiols to allyl glycosides. The method has allowed for the preparation of carbohydrate derivatives which can then be coupled to proteins, polymers or solid supports. For example, with cysteamine as the thiol, 3-(2-aminoethylthio)propyl glycosides were produced as precursors to glycopolymers through various strategies.

2. Methods and strategies have been discussed for the coupling of 3-(2-aminoethylthio)propyl glycosides to carboxylated particles (polymers, latex particles) for the preparation of defined research and diagnostic agents.

3. The *N*-acrylyolation of terminal amino groups of aglycon spacer arms has been demonstrated as an effective method for preparing carbohydrate derivatives which can be copolymerized with acrylate type comonomers or coupled to proteins by a Michael addition process.

4. The use of *N*-acryloylated carbohydrate derivatives as monomers in copolyacrylamide glycopolymer syntheses has been demonstrated as being superior to allyl glycosides previously employed. The use of an all-acrylamide monomer based strategy for preparing glycopolymers allows for the preparation of materials in which exact carbohydrate content can be controlled at will through the choice of comonomer load ratio, while the incorporation of a spacer arm between the hapten and polymer backbone has given glycopolymers with enhanced binding affinities.

5. The use of different acrylate type comonomers to prepare glycopolymers has shown to be compatible with the glycopolymer strategy and provides materials having slightly different physico-chemical properties to simple acrylamide based systems.

6. Glycopolymers have been shown to be very effective and useful antigens in simple serological assays such as immunodiffusion, quantitative precipitation and solid phase enzyme linked assays. These glycoconjugates appear to be more sensitive than neoglycoprotein conjugates. The use of synthetic copolyacrylamide glycopolymers allows, for the precise determination of agglutinated lectin or antibody from solution during quantitative precipitation experiments. Glycopolymers have been effective as coating antigens during enzyme linked assays when used in amounts as low as 0.1 ng/well.

7. A terpolymerization of acrylamide monomers strategy has given access to heterobifunctional glycopolymers which, depending on the effector chosen, can be useful as immoadhesins or specialized glycoconjugate probes.

8. A new method for preparing neoglycoproteins has been developed which involves the 1,4-conjugate addition (Michael addition) of lysine ε amines to *N*-acryloylated carbohydrates. This method is very efficient and mild. It has the advantage of using the same carbohydrate derivatives used to prepare glycopolymers.

9. The use of poly-L-lysine as a biodegradable glycopolymer backbone has been introduced. Poly-L-lysine glycopolymers and drug conjugates have also been shown to be excellent diagnostic agents in serological assays.

10. Sulfamethoxazole protein and copolymer conjugates have been developed to study the immune response against the drug sulfamethoxazole in HIV seropositive patients. The conjugates produced have proved useful in diagnosing HIV seropositive patients susceptible to adverse reaction to sulfamethoxazole and in identifying immunoglobulin classes and subclasses directed against sulfamethoxazole .

11. Phase tranfer catalysis has been demonstrated as being a simple and efficient glycosylation methodology adaptable to a wide range of

nucleophiles. The method has the advantage of requiring relatively benign reagents under mild conditions. It is adaptable to a variety of different sugars and is completely stereospecific. It provides the first entry into the hydroxylamino glycoside family found in esperamycin and calicheamycin antibiotics.

12. The hypothesis of electron donor-acceptor complexe formation between aryl 2-acetamido-2-deoxy-β-D-glucopyranosides and tyrosine residues within wheat germ agglutinin's binding sites has been given support through a linear free energy relationship study.

13. A strong correlation has been shown to exist between chemical shifts of anomeric carbon and hydrogen nuclei in *p*-substituted phenyl β-D-glycosides of *N*-acetylglucosamine to Hammett substituent constants. Correlations were found to be structure and solvent independent.

Publications arising from work described in this thesis

1. François D. Tropper, Fredrik O. Andersson, SuoDing Cao and René Roy; Stereospecific Synthesis of Thioglycosyl Xanthates by Phase Transfer Catalysis. *J. Carbohydr. Chem., 11* , 741 - 750 (1992).

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3. François D. Tropper, Fredrik O. Andersson, Chantal Grand-Maître and René Roy; Phase Transfer Catalyzed Synthesis of *para* - Nitrophenyl-1-thio-β-D-Glycobiosides. *Carbohydr. Res., 229* , 149-154 (1992).

4. René Roy, François D. Tropper, Anna Romanowska, Rakesh K. Jain, C.F. Piskorz and Kushi L. Matta; Direct access to Neoglycoprotein and Glycopolymers from Single Precursors. Synthesis of T-antigen and *N*-Acetyl-Lactosamine-β-D-(1→6)-α-D-GlcNAc Conjugates. *Bioorg. Med. Chem. Lett.* (in press).

5. René Roy, François D. Tropper and Anna Romanowska; Custom Designed Glycopolymer Syntheses by Terpolymerization. *J. Chem. Soc., Chem. Commun.* (in press).

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10. René Roy and François D. Tropper. Carbohydrate Protein Interactions. Syntheses of Agglutinin Inhibitors of Wheat Germ Agglutinin by Phase Transfer Catalysis. *Can. J. Chem., 69* , 817-824 (1991).

11. René Roy, François D. Tropper, Tara Morrison and Janusz Boratynski; Syntheses and Transformations of Glycohydrolase Substrates into Protein Conjugates based on Michael Additions. *J. Chem. Soc. Chem. Commun.* , 536-538 (1991).

12. René Roy, François D. Tropper, Anna Romanowska, Marie Letellier, Luc Cousineau and Serge Meunier; Expedient Syntheses of Neoglycoproteins Using Phase Transfer Catalysis and Reductive Amination as Keys Reactions. *Glycoconjugate J., 8* , 75-81 (1991).

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14. René Roy and François D. Tropper; Synthesis of Copolymer Antigens Containing 2-Acetamido-2-deoxy-α or β-D-Glucopyranosides. *Glycoconjugate J., 5* , 203-206 (1988).

15. René Roy and François D. Tropper; Synthesis of Antigenic Carbohydrate Polymers Recognized by Lectins and Antibodies. *J. Chem. Soc., Chem. Commun., 15* , 1058-1060 (1988).

Manuscripts which are in preparation or have been submitted for publication.

1. René Roy, François D. Tropper, Jean-Robert Brisson and Antony Williams; Global and Molecular Dynamics of Poly(glycosyl-acrylamide) Glycopolymers from ^{13}C-NMR Relaxation Studies. Submitted to *Can. J. Chem.*

2. René Roy, Robert A. Pon, François D. Tropper and Fredrik O. Andersson; Michael Addition of Poly-L-Lysine to *N*-Acryloylated Sialosides. Synthesis of Influenza A Virus Hemagglutinin Inhibitor and Group B Meningococcal Polysaccharide Vaccines. Submitted to *J. Chem. Soc., Chem. Commun.*

3. René Roy, François D. Tropper, Anna Romanowska, Rakesh K. Jain, C.F. Piskorz and Kushi L. Matta. Neoglycprotein and Glycopolymer Syntheses from Single *N*-Acryloylated Carbohydrate Precursors. Manuscript in preparation. Article requested by Y.C. Lee, editor of *Methods in Enzymology* .

4. François D. Tropper, Anna Romanowska, Fredrik O. Andersson and René Roy. Tailor-made Glycopolymer Syntheses. Manuscript in preparation. Article requested by Y.C. Lee, editor of *Methods in Enzymology* .

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6. Pirouz Daftarian, Brian Conway, René Roy, François D. Tropper, Lionel Filion and Francisco J. Diaz-Mitoma. Classes and Subclasses of Antibodies to Sulfamethoxazole in Persons with AIDS. Submitted to *J. Allergy Clin. Immunol.*

7. François D. Tropper, Pirouz Daftarian, Brian Conway, Lionel Filion,

Francisco J. Diaz-Mitoma and René Roy. Design and Synthesis of Sulfamethoxazole Drug Conjugates for Diagnosing and Studying Immune Hypersensitivity Toward Sulfamethoxazole in Patients with AIDS. Manuscript in preparation - to be submitted to *Med. Chem. Res.*

Conference Presentations

1. René Roy, François D. Tropper, Jean-Robert Brisson, Antony J. Wiliams; Global and Internal Molecular Dynamics of Poly(acrylamide-co-allyl 2-acetamido-2-deoxy-D-glucopyranosides) Glycopolymers from ^{13}C-NMR Relaxation Studies. 16th International Carbohydrate Symposium, July 5-10, 1992, Paris, France.

2. Anna Romanowska, François D. Tropper and René Roy. Custom Designed Glycopolymer Syntheses by Terpolymerization. 16th International Carbohydrate Symposium, July 5-10, 1992, Paris, France.

3. Antony Williams, François D. Tropper, René Roy and Stéphane Braun; Conformational Isomerization and Internal Motions in the Side Chains of β-Gluco and β-Thioglucopyranosides. 37th Canadian Spectroscopy Conference, Ottawa, Canada, August, 1991.

4. François D. Tropper and René Roy; Newer Aspects of Carbohydrate Protein Interactions. 74th Canadian Chemical Conference, Hamilton, Canada, June 1991.

5. René Roy, François D. Tropper, Fredrik O. Andersson and Chantal Grand-Maître; Carbohydrates and Interdisciplinary Research. 10th Annual Ottawa-Carleton Organic Chemistry Symposium, May 1991.

6. René Roy, François D. Tropper, Fredrik O. Andersson, Craig Laferrière and Robert A. Pon; Synthèses et propriétés antigéniques de glycoconjugués. 59e Congrès de l'ACFAS, Québec, Canada, May 1991.

7. René Roy and François D. Tropper; Expedient Synthesis of Glycoconjugates Based on Phase Transfer Catalysis. 15th International Carbohydrate Symposium. Yokohama, Japan, August 1990.

8. François D. Tropper, Marc Lamoureux and René Roy; Synthèse et sérologie de copolymères antigéniques possédant les résidus 2-acetamido-α ou β-D-glucopyranosides. 57e Congrès de l'ACFAS. Montréal, Canada, May 1989.