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Glycoconjugates: Design and Syntheses of Multivalent T-antigen Tumor Markers

In partial fulfillment of the requirements
for the degree of
Doctor of philosophy

In the Ottawa-Carleton Chemistry Institute
Department of Chemistry
University of Ottawa
Ottawa, Ontario
Canada

September 1997

Candidate

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Supervisor

René Roy

To my wife Hyunhee, my daughter Songe, and my son Jinwoo

Abstract

Cell surface carbohydrates, which are known front-line mediators in various cellular events, are involved in many recognition and adhesion processes with proteins from viruses, bacteria, toxin, and other cells. These carbohydrates are mainly composed of glycolipids and glycoproteins. The Thomsen-Friedenreich (TF or T) antigen has been known as a carbohydrate disaccharide, Gal-β-D-(1-3)-α-D-GalNAc α-linked to serine or threonine in glycoprotein and other mucins. It is widely distributed on common cancers of epithelial cells including breast cancers.

Due to difficulties in isolating them from natural sources, various synthetic methods were developed to achieve the synthesis of multivalent carbohydrate haptens in different forms varying in carbohydrate densities, conformations, and interglycosidic distances.

The synthesis of the T-antigen and its derivatives and modification of its aglycons having and give different terminal functional groups is described herein. Several glycosyl acceptors were synthesized and subsequent glycosylations were performed with glycosyl donors using TfOMe, TfOAg/2,4,6-collidine, NIS/TfOH, or $Hg(CN)_2$ as promoters to give β-(1-3) linked disaccharides. Especially, the Helferich methodology was quick, efficient, and provided the T-antigen in a simple one step reaction in excellent yields. The aglycon was modified to give sulfide bridged spacer arms containing end groups such as alcohol, amine, carboxylic acid, and thiol. Hydroformylation of allyl glycoside using rhodium catalyst afforded linear and branched aldehydes. In addition, ozonolysis of the allyl group also provided an aldehyde. The applications of these derivatives were extended to provide novel glycodendrimers, glycopolymers, and neoglycoproteins.

The synthesis of convergent dendrimers with valencies of between two and six T-antigen residues is described. The synthesis of dendritic cores was based on peptide coupling between N,N′-bis(acrylamido)acetic acid as a seed molecule and hexamethylene diamine or tris(2-aminoethyl)amine using HOBt/EDC strategy to give tetra- and hexa-valent structures. Conjugate addition of thiolated T-antigen to acrylamido functionality on the seed molecule gave divalent T-antigen in quantitative yield. Tetra- and hexa-valent

glycodendrimers bearing T-antigen were prepared by two different methods. First, thiolated T-antigen was added to the acrylamido functions of the dendritic cores. In addition, divalent T-antigen-bearing terminal carboxylic acid was coupled to polyamines using carbodiimide chemistry. The divalent T-antigen was also coupled to probes such as biotin and fluorescein to provide heterobifunctional dendrimers.

Hyperbranched L-lysine based dendritic cores bearing chloroacetylglycylglycine active ester were conjugated to thiolated T-antigen to give well defined di-, tetra-, and octa-valent glycodendrimers. A heterobifunctional dendrimer containing biotin was also prepared with tetravalent T-antigen dendrimer using TBTU coupling chemistry.

T-antigen containing dendrimers scaffolded on starburst^{TM} PAMAM dendrimers were prepared using peptide coupling strategy. PAMAM cores with generation G-0 to G-3 were conjugated to T-antigen bearing carboxylic acid to give "sugar balls" containing four to thirty-two T-antigen residues.

N-substituted glycosyl acrylamides were used as monomers to prepare neoglycoproteins and multivalent synthetic polyacrylamide based glycopolymers. Neoglycoproteins were synthesized by coupling N-acryloylated T-antigen residues onto proteins by conjugate addition. This methodology is simple, efficient and requires no additional reagents. Copolyacrylamide were prepared by two strategies. N-acryloylated carbohydrate and acrylamide were copolymerized using ammonium persulfate in aqueous conditions. Alternatively, preactivated poly(N-acryloxysuccinimide) and its analogues containing active esters were prepared. Graft conjugation of monomeric units bearing amino functional group such as carbohydrate hapten, NH_4OH, and alkyl amines was performed. In both methods, the carbohydrate density could be controlled at will through the choice of comonomer load ratio. The glycopolymers thus prepared had many advantages over the corresponding neoglycoproteins: high resistance to thermal, chemical, and biological degradation, and higher sensitivity during the bioassays.

Binding assays were performed using double radial immunodiffusion and turbidimetric analysis to confirm the ability of the glycoconjugates to bind to protein such as *Arachis Hypogaea* from peanut lectin. In addition, competitive enzyme linked

immunoassays were performed with monoclonal mouse IgG and peroxidase labeled goat anti-mouse IgG conjugates and all glycoconjugates showed enhanced inhibitory potentials.

Acknowledgment

Three and half years ago, I met Dr. René Roy. It was my best luck because I could join with his research group. I would like to express my gratitude to him. Dr. René Roy always opened his mind for discussions and help. His idea is creative in the field and makes me learn more. I really appreciate on the bottom of my heart. I will always remember you.

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I would like thank my wife Hyunhee for her love and encouragement, daughter, Songe, and son, JinWoo. Hyunhee sacrificed herself just for my study. I know she spent hard time more than I did. Thank you my love! I also thank to my father, N.K. Baek, mother, J.G. Yeon, father-in law, Y.S. Jeon, and B.Y. Kim for their encouragement and financial support.

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List of Schemes

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List of Abbreviation

Å	angstrong
Ac	acetyl
AcOH	acetic acid
AIBN	2,2'-azobisisobutyronitrile
β-Ala	beta-alanine
AgOTf	silver trifluoromethanesulfonate
ABTS	2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
Ac_2O	acetic anhydride
Bn	benzyl
bp	boiling point
BSA	bovine serum albumin
Bz	benzoyl
CI	chemical ionization
COSY	correlation spectroscopy
CPA	copolyacrylamide
CRD	carbohydrate recognition domain
cpm	count per minute
d	doublet
Da	dalton
DAST	diethylaminosulfur trifluoride
DCC	dicyclohexylcarbodiimide
DIPEA	diisopropylamine
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DMTST	dimethyl (methylthio) sulfonium triflate
DtBP	2,6-di-*tert*-butylpyridine
dt	doublet of triplet

EDC	1-(3-dimethylaminopropyl)-3-ethyl carbodiimide
ELLA	enzyme linked lectin assay
ELISA	enzyme linked immunosorbent assay
eq	equivalent
EtOAc	ethyl acetate
Et_2O	diethyl ether
Et_3N	triethyl amine
FAB-MS	fast atom bombardment ionization mass spectrometry
Gal	galactose
GalNAc	N-acetylgalactosamine
Glc	glucose
GlcNAc	N-acetylglucosamine
Gly	glycine
h	hour
HMQC	heteronuclear multiple quantum coherence
HOBt	1-hydroxybenzotriazole
HRP	horseradish peroxidase
Hz	hertz
IDCP	iodonium dicollidine perchlorate
IgG and IgM	immunoglobulins of class G and M
iPr	isopropyl
IR	infrared
KLH	keyhole limphets hemocyanin
Lys	lysine
M^+	parent molecular ion
MAb	monoclonal antibody
MAP	multiple antigen peptide
mCPBA	meta-chloroperoxybenzoic acid
Me	methyl
min	minute(s)

μL	microliter
mmol	millimolar
mol	mole
MS	mass spectrum
m/z	mass to charge ratio
M_v and M_n	viscosity and number average molecular weight
MW	molecular weight
NBS	N-bromosuccinimide
NHS	N-hydroxysuccinimide
NIS	N-iodosuccinimide
NMR	nuclear magnetic resonance
Nu	nucleophile
OD	optical density
PAMAM	poly (amidoamine)
PBS	phosphate buffered saline
Ph	phenyl
Phth	phthaloyl
Piv	pivaloyl
PNS	poly (N-acryloxysuccinimde)
ppm	parts per million
py	pyridine
p-TsOH	para-toluenesulfonic acid
rt	room temperature
RP	receptor protein
s	singlet
SD	standard deviation
t	triplet
tBu	*tert*-butyl
TBDMS-Cl	*tert*-butyldimethylsilyl chloride
TBDPS-Cl	*tert*-butyldiphenylslyl chloride

TBTU	O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
TFA	trifluoroacetic acid
TfOH	trifluoromethanesulfonic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMEDA	N,N,N',N'-tetramethylethylenediamine
TMS	trimethysilyl
TT	tetanus toxoid

Chapter 1. General Introduction

1.1. The role of cell surface carbohydrates

Glycobiology deals with the role of carbohydrates in biological events.[1] Carbohydrates are ubiquitous constituents of the cell surface and extracellular matrix serving as important source of energy and as major structural components of cell and tissues. Indeed, there are a number of biological phenomena which depend on carbohydrate-protein interactions. Carbohydrates are involved in key recognition events[2] with receptor proteins such as hormones, enzymes, toxins, lectins, antibodies, viruses and bacteria. They invoke numerous biological functions[1] such as cell growth, regulation and differentiation, cancer metastasis, inflammation, and microviral processes as shown in Fig. 1-1.

Cell surface carbohydrates are mainly composed of glycolipids, glycoproteins, proteoglycans, and capsular polysaccharides[3] and these glycoconjugates are externally expressed. This makes carbohydrates front-line mediators in intracellular events. The reason why carbohydrates are adaptable to work as such informative molecules comes from their structural complexity. This intrinsic nature can be easily envisaged when considering various structural combinations such as anomeric configuration, branching, linking position, and the possibility of bearing substituents (acetate, sulfate, phosphate, etc). Thus, carbohydrate residues have the potential to carry much more information than peptides of similar size. For example, four monosaccharides can produce more than 35,000 possible tetrasaccharide structures whereas only 24 tetrapeptide arrangements are possible with four amino acids.[4]

Sialic acids are well known as constituents of glycoproteins and glycolipids of cell membrane and are involved in a large variety of biological events.[5] Sialic acids terminate the carbohydrate groups of many cell surface glycoconjugates and contribute to the enormous diversity of natural oligosaccharide sequences from a limited number of

Fig. 1.1. Cell surface carbohydrates and their biological events.[2-b]

neutral core structures. These terminal substituents are ideally positioned to participate in protein-carbohydrate interactions mediating cell surface recognition phenomena. Cancer cells have been found to contain more sialic acid residues than normal cells and also specifically sialylated conjugates exhibit high concentration in various tumor cells.[6] Some examples are summarized in Table 1.1.

Table 1.1. Tumor associated sialic acid antigens.[6c, d]

Antigen	Structure	Cancer
Sialyl Le^a	Galβ(1-3)GlcNAcβ(1-3)Galβ(1-4)Glcβ1-1Cer 3 4 ↑ ↑ NeuAcα2 Fucα1	Gastrointestinal/ Pancreas (Cystic Fibrosis)
Sialyl Le^x	Galβ(1-4)GlcNAcβ(1-3)Galβ1-4Glcβ1-4Cer 3 3 ↑ ↑ NeuAcα2 Fucα1	Gastrointestinal/ Lung/Breast
GM_2	GalNAcβ(1-4)Galβ(1-4)Glcβ(1-1)Cer 3 ↑ NeuAcα2	Breast cancer Brain Tumor Human Melanomas
GD_3	NeuAcα(2-8)NeuAcα(2-3)Galβ1-4Glcβ(1-1)Cer	Human Melanomas
GM_3	NeuAcα(2-3)Galβ(1-4)Glcβ(1-1)Cer	Human Melanomas

Another function of sialyloligosaccharides is their participation in cellular recognition phenomena as well as their ability to serve as receptors for influenza virus. Therefore, carbohydrates can be used to intercept viral and bacterial adhesion before they bind host cells (Fig. 1.2).

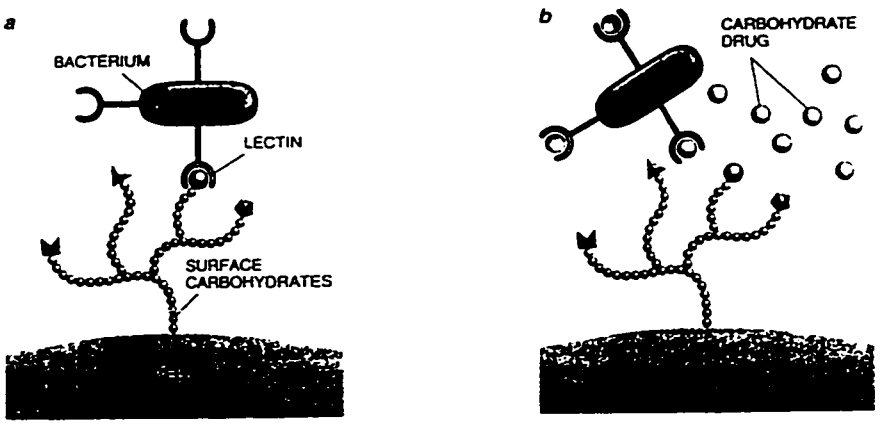


Fig. 1.2. Blocking bacterial attachment by carbohydrate drugs,[2-h] a) as a prelude to infection, bacterial surface proteins (lectin) attach to cell surface carbohydrates on susceptible cells; b) drug containing similar carbohydrates could prevent the attachment by competing binding to the lectins.

Thomson Friedenreich (T or TF) antigen is a cryptic O-linked disaccharide, β-D-Gal-(1-3)-α-D-GalNAc-O-serine/threonine found on human erythrocytes and epithelial cells which can be revealed by neuraminidase treatment. This antigen is important for the detection and immunotherapy of a number of common cancers including breast cancers (Fig. 1-3).[7]

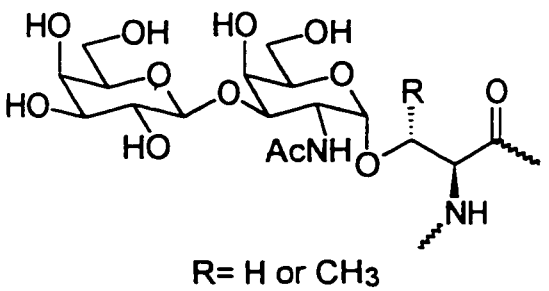


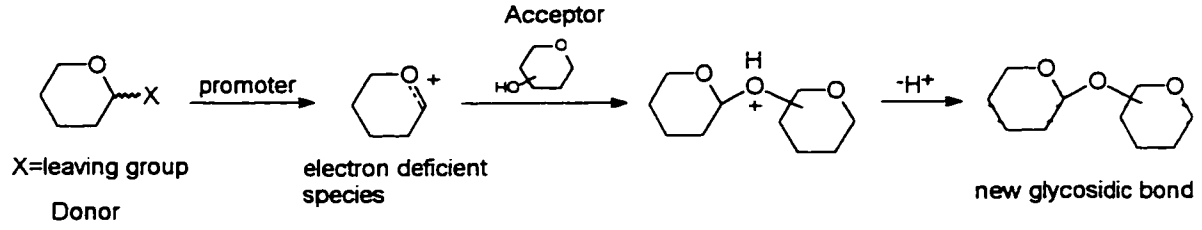
Fig. 1-3. Structure of T-antigen.

Springer *et al.*[9] have demonstrated the accumulation of T-antigen on cancers of epithelial origin and also showed that all normal donors have natural anti-T antibody levels. These antibody levels are often lower in patients with epithelial cancer during tumor progression. This suggested that, although these cancer cells are incapable of inducing an immune response against T-antigen, the low level of natural antibodies are able to recognize antigen on the tumor and to remove the infected cells by adsorption. Mice immunized with protein conjugated T-antigen have been reported to produce anti-T-antigen antibodies, to exhibit delayed type hypersensitivity (DTH) against natural T-antigen, and to demonstrate a significant level of protection against challenge with T-antigen-positive syngenetic tumor cells.[10] This conjugate was moderately immunogenic. Based on these facts, T-antigen is of considerable interest as a target for active immunotherapy against epithelial cancers. The T-antigen specificity has been investigated by agglutination techniques and radioimmunoassays using a number of derivatives of T-hapten as inhibitors.[7-b-e] T-antigen specific receptor proteins such as: peanut lectin from *Arachis Hypogaea*, *helix pomatia agglutinin*,[7-e] human hepatoma cell line, HepG2,[7-k] monoclonal antibody, 49H24,[7-c] and anti-KLH IgG antibody.[7-d]

1.2. Recent advances in glycosylation strategy

The role of cell surface carbohydrates in biological actions such as tumor progression and cell-cell recognition is significant. Furthermore, carbohydrates are capable of inducing protective antibody response and this immunogenic reaction is a major contributor to survival during infection.

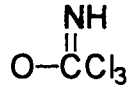
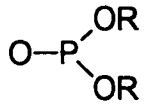
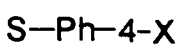
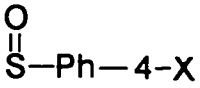
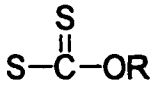
The chemical synthesis of oligosaccharides is usually complicated because the glycosidic linkages have to be introduced stereospecifically. To meet this demand, several synthetic strategies have been developed using a wide range of leaving groups and mild activation conditions.[11] Most glycosylations involve activation of glycosyl donors by an electrophile followed by the nucleophilic attack of glycosyl acceptors (Scheme 1-1).

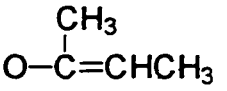
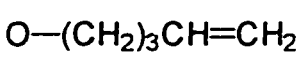
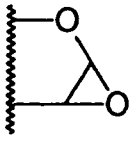


Scheme 1-1. Schematic representation of glycosylation reaction.

Reactive glycosyl donors for creating glycosidic bonds have been developed for all the sugars commonly found in nature and few examples are summarized in Table 1-2.[73-b]

Table 1-2. Recent development for the activation of glycosyl donors.

Leaving group (X)	Name	Typical promoters[73-c,d]
F	Fluorides	$SnCl_2/AgClO_4$ $HfCp_2Cl_2$ (AgOTf)
	Trichloroacetimidates	$BF_3{\cdot}OEt_2$, TMSOTf AgOTf
	Phosphites	$BF_3{\cdot}OEt_2$, TMSOTf
	Alkyl (aryl)thioglycosides	NIS/TfOH, DMTST, MeI
	p-X-phenylthioglycosides	NIS/TfOH, DMTST
	Sulfoxides	Tf_2O
	p-X-phenylsulfenylglycosides	TMSOTf
	Selenoglycosides	$AgOTf/K_2CO_3$
	Xanthates	MeSBr/AgOTf

	O-vinylglycosides	TMSOTf
	O-pentenylglycosides	NIS-TfOH
	Glycalepoxides	$ZnCl_2$

Inter-glycosidic bond formation in linear glycosylation is generally achieved by condensing a fully protected glycosyl donor bearing a leaving group at an anomeric center with suitably protected glycosyl acceptor containing one free hydroxyl group.[14, 73-d] In the classical Koenigs-Knorr method,[73-d] 1-bromo or chloro carbohydrate derivatives are used as glycosyl donors and high stereoselectivity can be obtained when a 2-acetoxy group is present. However, this method has some drawbacks for some oligosaccharide syntheses;

i) relatively harsh conditions are needed for the generation of glycosyl halides,

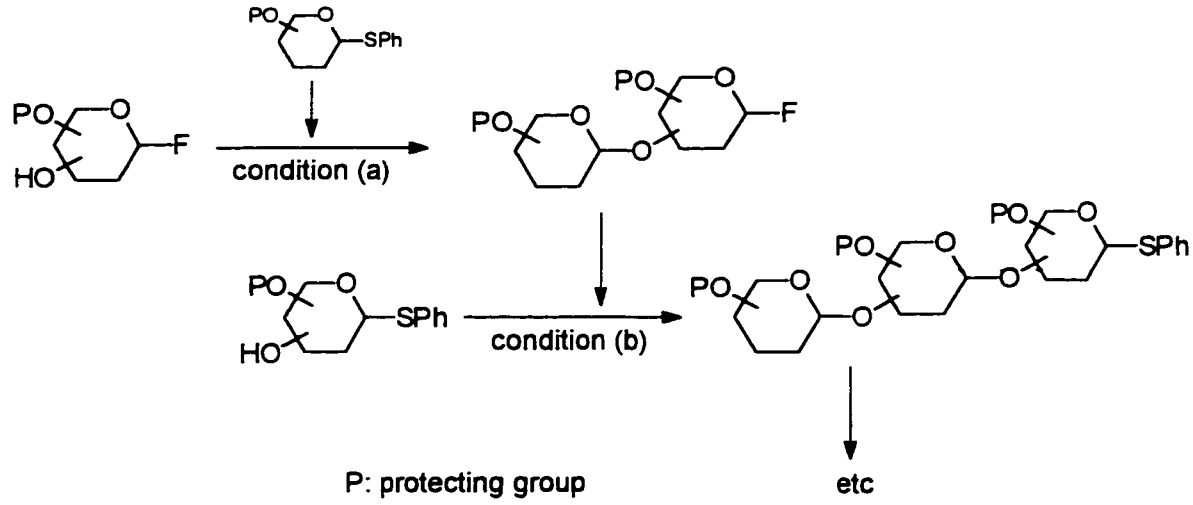
ii) glycosyl halides exhibit low thermal stability and can only be generated in situ at low temperature,

iii) these species is highly sensitive to hydrolysis,

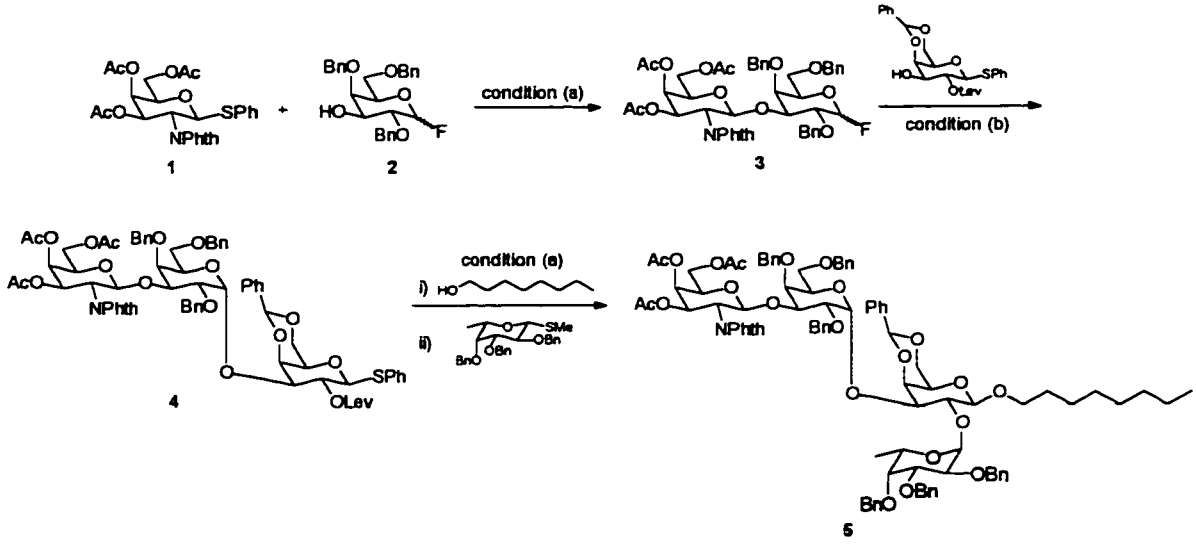
iv) heavy metal salts used as promoters are often dangerous (toxic and explosive).

To overcome these drawbacks, new glycosylation strategies were demanded to synthesize the desired oligosaccharides efficiently.

The synthesis of oligosaccharides consisting of as many as 20 monosaccharide units can be accomplished when a synthetic strategy is highly convergent.[13] In such glycosylation strategy, "orthogonal glycosylation strategy", most of the synthetic effort is directed toward the preparation of monomeric glycosyl donors and acceptors. The assembly of these units should involve minimum synthetic steps, high stereoselectivity and high yield. For example, execution of the synthesis used an orthogonal set of glycosylation reactions, namely activation of thioglycosides and glycosyl fluorides with several promoters (Scheme 1-2 and 1-3).

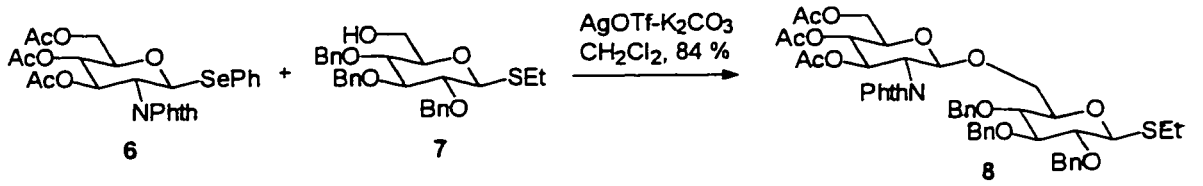


Scheme 1-2. Convergent orthogonal glycosylation methods in oligosaccharide synthesis; condition (a), NIS-AgOTf[14-a-c] or DMTST[14-d] and condition (b), Cp_2HfCl_2-$AgClO_4$.[14-e, f]



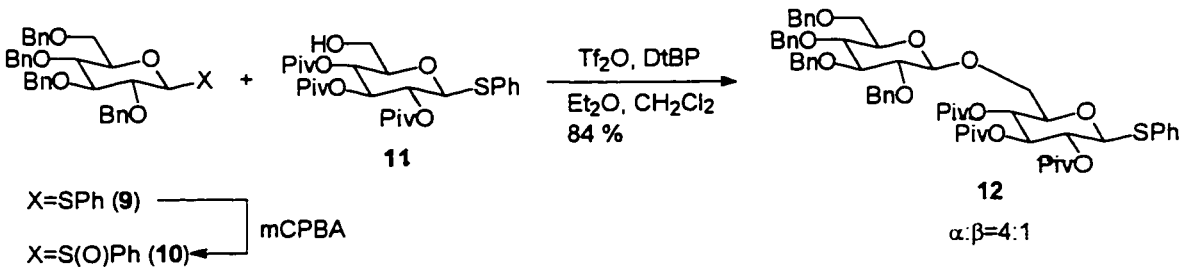
Scheme 1-3. Examples of orthogonal glycosylation for the synthesis of extended blood type B determinant.

Pinto *et al.*[15-a] have developed selective activation strategies in oligosaccharide synthesis. Instead of thioglycosides (**1**), highly reactive phenylseleno glycosides (**6**) were used for the glycosylation (Scheme 1-4). Phenylseleno glycosyl donors are activated by silver triflate and condensed with ethylthio glycosyl acceptors to give disaccharides (**8**).



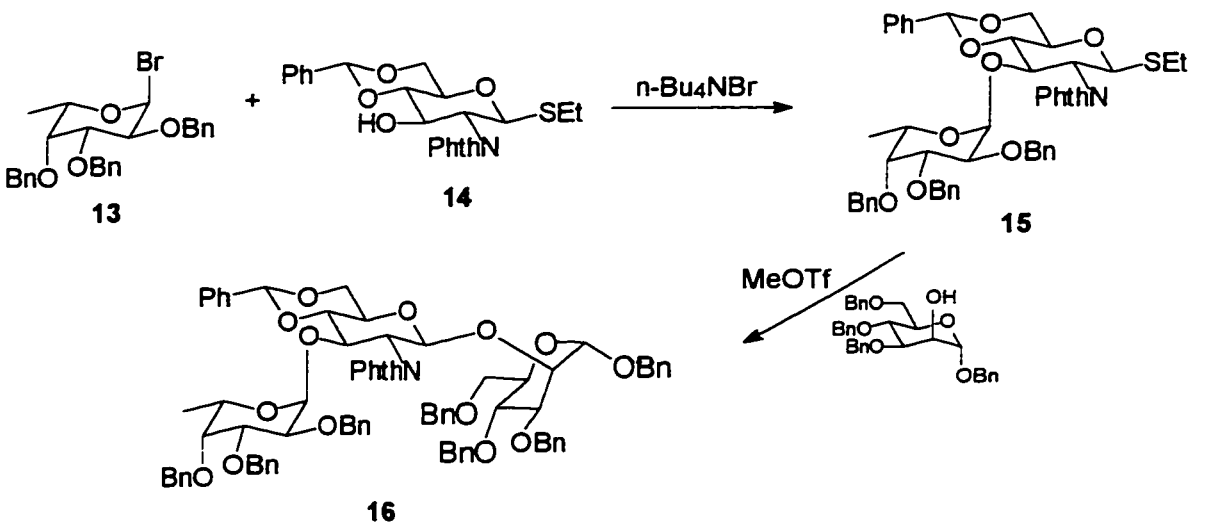
Scheme 1-4. Seleno- and thio-glycoside competitive glycosylation.

Oxidation of thioglycosides into sulfenylglycosides (**10**)[15-b] provides more active glycosyl donor than the precursors using appropriate promotors, hence, mild reaction conditions allow the use of acid sensitive protecting groups (Scheme 1-5). Another advantage is that thioglycosides used as glycosyl acceptors during the coupling reaction can be further oxidized to phenylsulfenyl glycosides which can then act as glycosyl donors for later glycosylation reactions.



Scheme 1-5. Glycosylation using phenylsulfenyl glycosides as a glycosyl donor.

When the anomeric substituent of an oligosaccharide is stable to withstand protecting group manipulations (act as a protecting group) and also have adequate reactivity to use as a glycosyl donor (act as a leaving group), selective two-stage activation is feasible (Scheme 1-6).[15-c] Glycosyl bromide (**13**) can be selectively activated in the presence of a thioglycoside (**14**) using n-Bu_4NBr to provide a disaccharide (**16**).

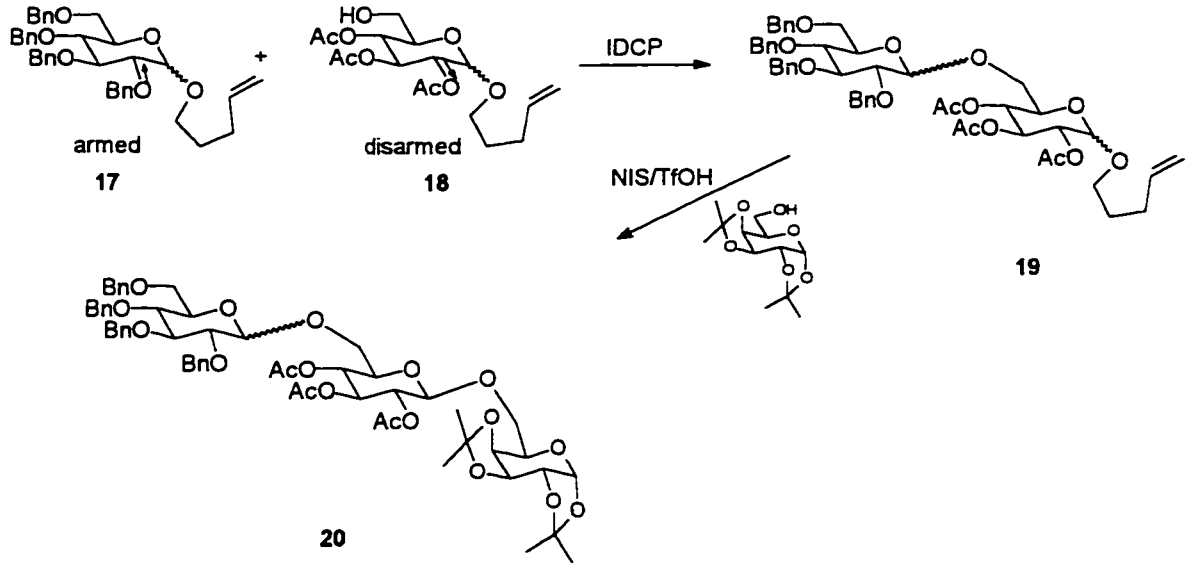


Scheme1-6. Trisaccharide synthesis using selective two-stage activation process.

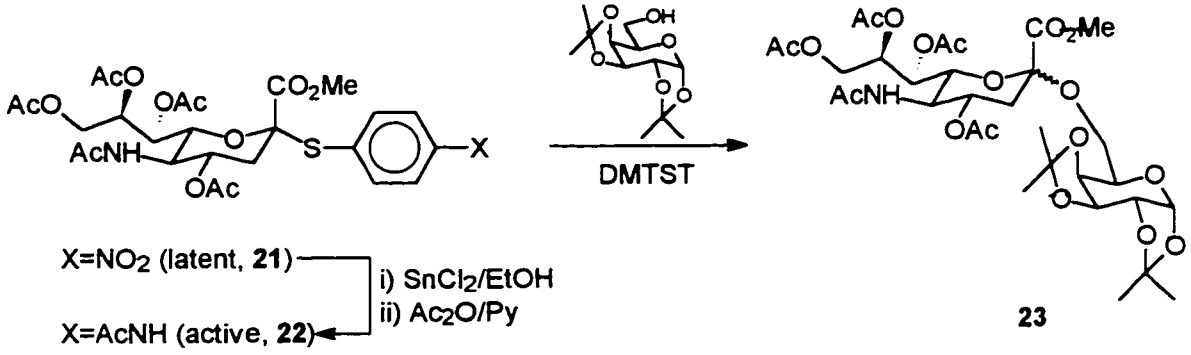
The disaccharide is used subsequently in the next glycosylation reaction as a donor and coupled with other acceptor in the presence of methyl triflate to give trisaccharide (**16**).

Fraser-Reid *et al.*[12] have introduced a chemoselective glycosylation strategy (armed-disarmed glycosylation strategy) in which a C-2 ether protected pentenyl glycoside (**17**) can be coupled chemoselectively to a acetylated pentenyl glycoside (**18**). The chemoselective glycosylation depends on the fact that C-2 ether activates (electron donating, armed) and C-2 ester deactivates (electron withdrawing, disarmed) the anomeric center (Scheme 1-7). Armed (-OBn) and disarmed (-OAc) monomers are coupled in the presence of iodonium di-collidine perchlorate (IDCP) and gives disaccharide (**19**) with anomeric mixture. The disarmed dimer is further glycosylated with another monomer (armed) using NIS/TfOH to afford trisaccharide (**20**).

A novel "active-latent" concept for the oligosaccharide synthesis was reported by our group.[12-g] This strategy relies on the selective activation of stable anomeric group (**21**) into a good leaving group (**22**) by a simple chemical interconversion. An elegant example is illustrated in Scheme 1-8 with synthesis of a sialoside.



Scheme 1-7. Chemoselective glycosylation (armed-disarmed) strategy.

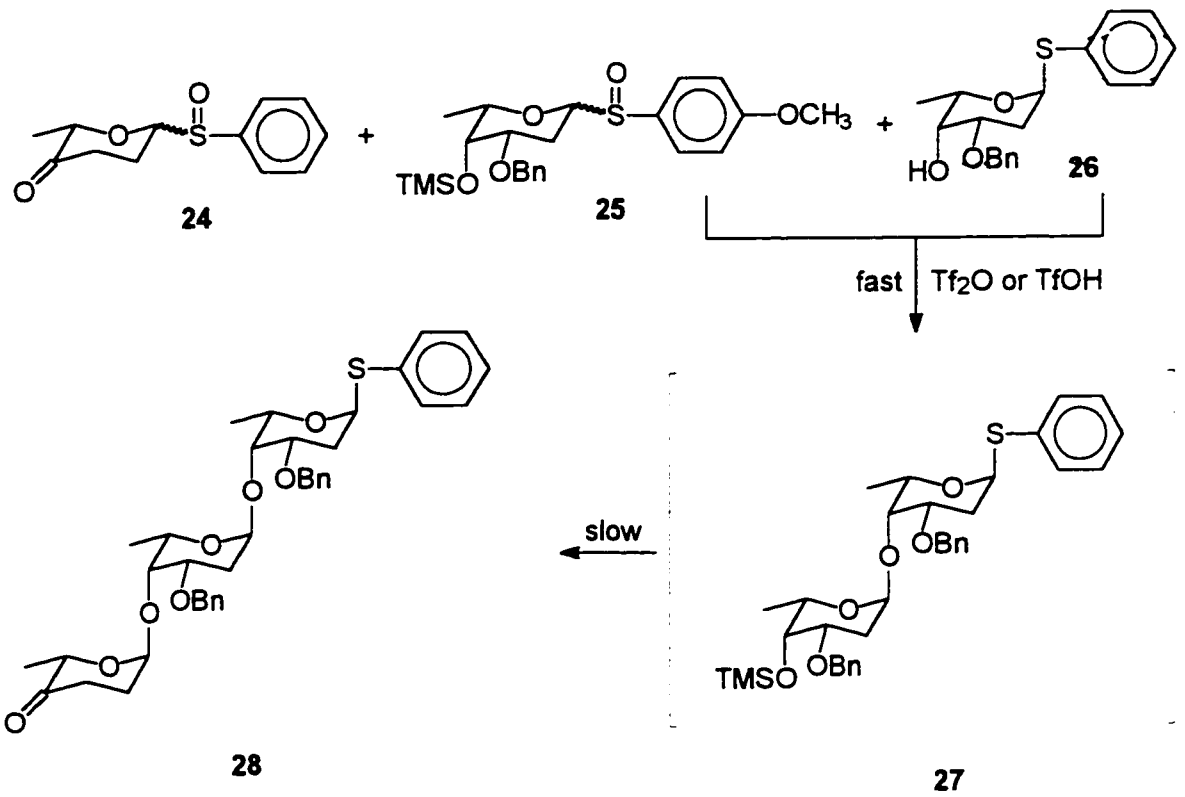


Scheme 1-8. Synthesis of sialosides using active-latent glycosylation strategy.

No glycosylation occured with the latent p-nitrophenylthio sialoside (**21**) in the presence of thiophilic promoter such as DMTST. Conversion of the nitro group into p-N-acetylphenyl sialoside (**22**) gave a α/β mixture of disaccharide under the same condition.

Another application of one-pot multi-step glycosylation strategy has been reported.[17] This method is based on the activation of anomeric center with appropriate promoters. For example, anomeric sulfoxides can be activated with Tf_2O or TfOH and

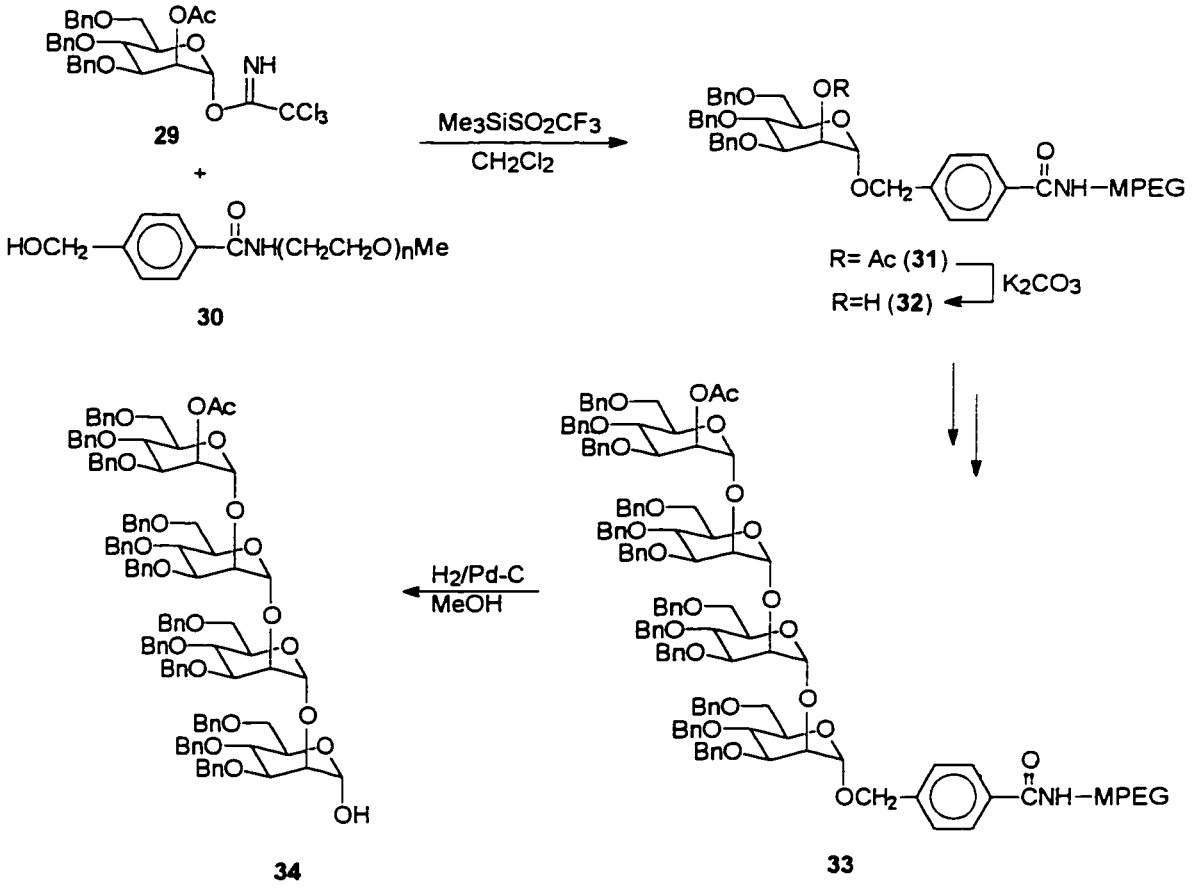
triflation of the sulfoxide acted as are rate limiting step (Scheme 1-9). Therefore, the reactivity of glycosyl donor depends on the substituents in the p-position of the phenyl ring and is established in the order: OMe>>H>NO_2. More reactive p-methoxyphenylthio glycosides act with acceptor selectively and subsequent glycosylation of phenylthio glycoside is normally followed to give trisaccharide (**28**).



Scheme 1-9. Synthesis of trisaccharide using one-pot glycosylation method.

Polymer supported solution synthesis of oligosaccharides has also been explored as an efficient oligosaccharide syntheses.[18] Solution phase synthesis offers many advantages over solid-phase synthesis because of the greater efficiency of the glycosylation step in solution. Selection of polymer support molecular weight was based on the consideration that the polymer bound oligosaccharides must remain soluble in the glycosylation step but can be precipitated readily from solution. The synthetic approach is illustrated in Scheme 1-10. Polyethyleneglycol-*co*-monomethyl ether (MPEG, M.W. 550) is glycosylated with

selectively protected benzyl and acetyl (C-2) mannose trichloroacetimidate (**29**). De-acetylation of the MPEG-monosaccharide can be accomplished under basic conditions. Repetition of glycosylation and hydrolysis provided the desired oligosaccharide (**33**). Hydrogenation of polymer supported oligosaccharide realesed pure oligosaccharide (**34**) from the polymer.



Scheme 1-10. Polymer supported solution synthesis of oligosaccharide.

1.3. Glycoside cluster effect

Fundamental understanding of carbohydrate-protein binding interactions at the molecular level has been of prime interest to glycobiologists. Generally, these interactions

are of low affinity when measured on a per-saccharide basis.[19] The dissociation constants (K_D) are usually in the ~μM to mM range. However, this problem can be overcomed by preparing glycoconjugates that provide multivalency above the level usually found available in nature. In addition, numerous protein receptors have several carbohydrate recognition domain (CRD) that bind to carbohydrate ligands. These multivalent binding opportunities from both carbohydrate ligands and protein receptors can enhance the overall binding avidity. Mammalian hepatic carbohydrate receptors which binds mono-, di- and trivalent galactose terminated oligosaccharides showed dramatic increase in affinities, up to 1:1,000:1,000,000, respectively.[20]

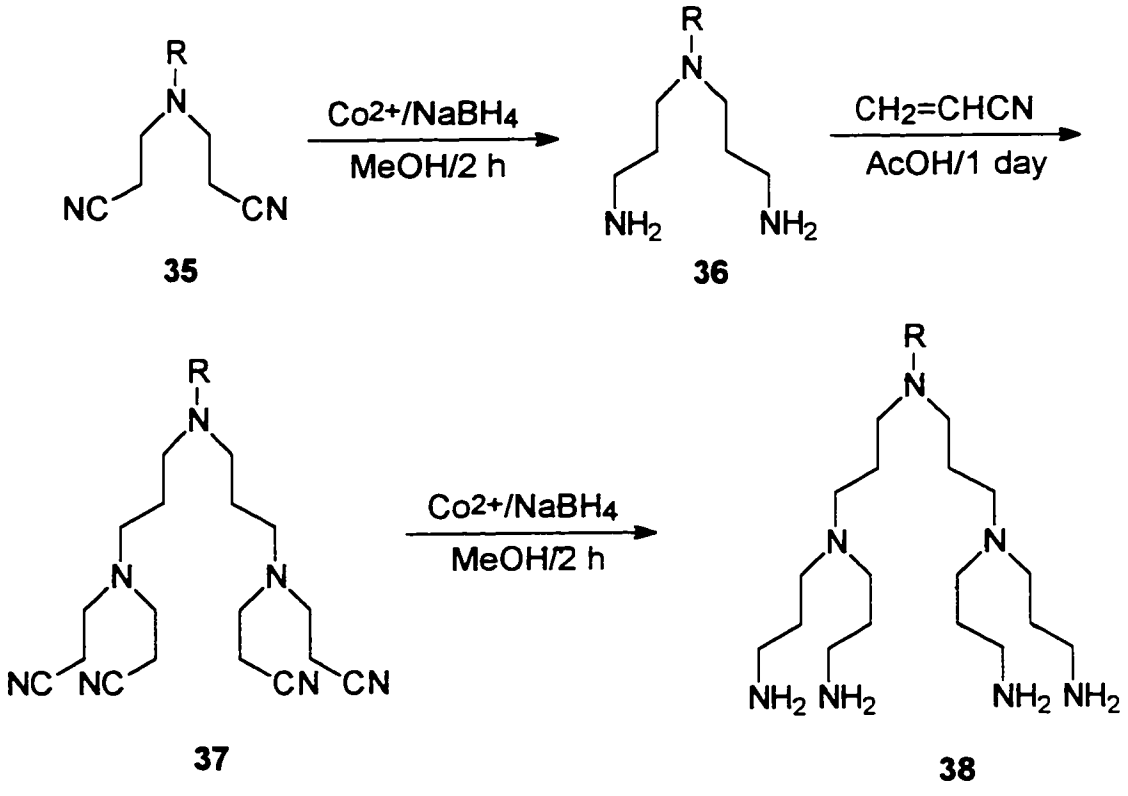
Because the spatial topography of terminal galactoside in the cluster is critical for maximum efficacy, chemically well designed glycoconjugates are necessary. Along with multivalent effect, the flexibility of their attachment can be another parameter that can control binding affinity.[21, 22] Therefore, in the following chapters, glycopolymers and neoglycoproteins containing T-antigen that mimic naturally occurring glycoconjugates will be discussed. To improve the content of size and carbohydrate heterogeneity, chemically well defined glycoconjugates with systematically arranged carbohydrate ligands will also be discussed for the understanding of carbohydrate receptor protein recognition.

1.4. Glycodendrimers

Dendrimers are tree like, three-dimensional polymers which posses three architectural components: a core, an interior, and a surface. These three regions are spatially defined by branching cells derived from connectors and branch junctures, all of which are tethered to the core residue by reiterative chemical bonds. Two methodologies for preparing hyperbranched dendritic polymers are known, a convergent[23~26, 185] and divergent[27, 28, 186, 210] strategies. These new monodisperse, high molecular weight polymers provide fundamental differences already recognized in the properties and behavior of classical polymer architectures such as those exhibited by linear, cross-linked, and branched classes. It has been received extensive attention in diverse areas: i) gene therapy,

biomimetics, drug delivery and immunoassays in life science, ii) molecular antennae and electroconductive dendrimers[29] in material science, iii) dendrimer catalyst and coatings in interfacial science, iv) molecular capsulation, lock-and -key complexes and v) self assembling dendrimers in supramolecular science.

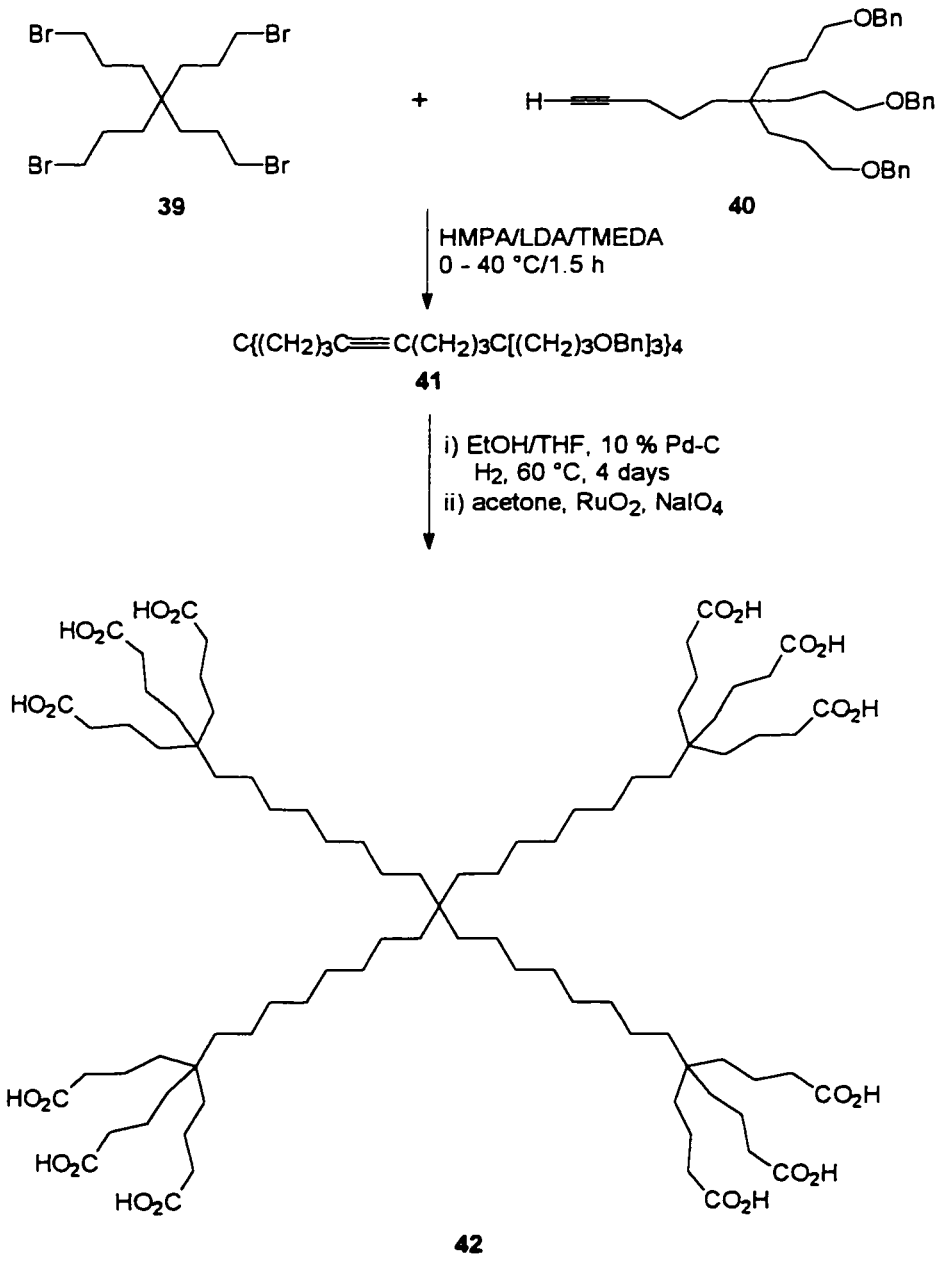
The preparation of "cascade" molecule was first reported by Vögtle *et al.*[30] in which Michael-type addition of an amine to acrylonitrile permited the attachment of the initial two arms (**36**). Subsequent reduction of the nitriles gave the diamines which can be subjected to the same synthetic sequence for the next generation (**38**) (Scheme 1-11).



Scheme 1-11. Preparation of cascade molecules.[30]

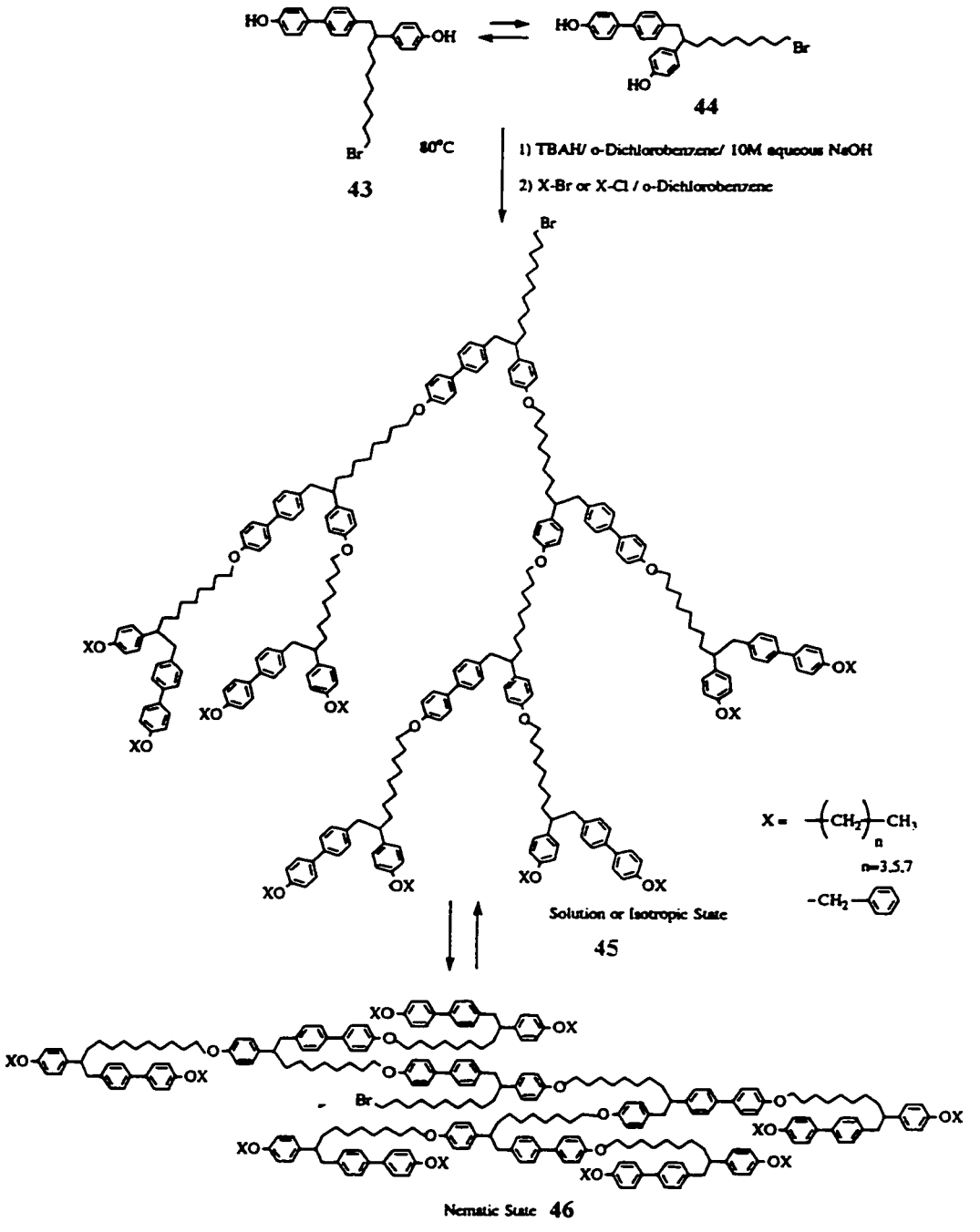
Newkome *et al.*[31-35] prepared unimolecular micelle-like dendrimers by treatment of tetrabromide central core and alkyne to generate dodecabenzyl ether as shown in Scheme 1-12. Concomitant reduction of triple bonds and hydrogenolysis of the benzyl ethers yielded 12-cascade: methane[4]:(nonylidyne):propanol which was transformed to the

corresponding bromide, then treatment with alkyne yielded dendrimer with 36-benzyl ether groups. Subsequent reduction and hydrogenolysis gave 36-cascade: methane[4]:(nonylidyne)^2:propanol. Oxidation of terminal alcohols by $RuO_2/NaIO_4$ afforded [8^2.3]micellanoic acid^{TM}, 36-cascade:methane[4]:(nonylidyne)^2:propanoic acid.



Scheme 1-12. Synthesis of dodecacarboxylic acids dendrimer.

Percec *et al.*[36, 37] reported the first thermotropic liquid crystalline dendrimer based on aromatic polyether (Scheme 1-13).



Scheme 1-13. Synthesis of liquid crystalline dendritic polyethers.

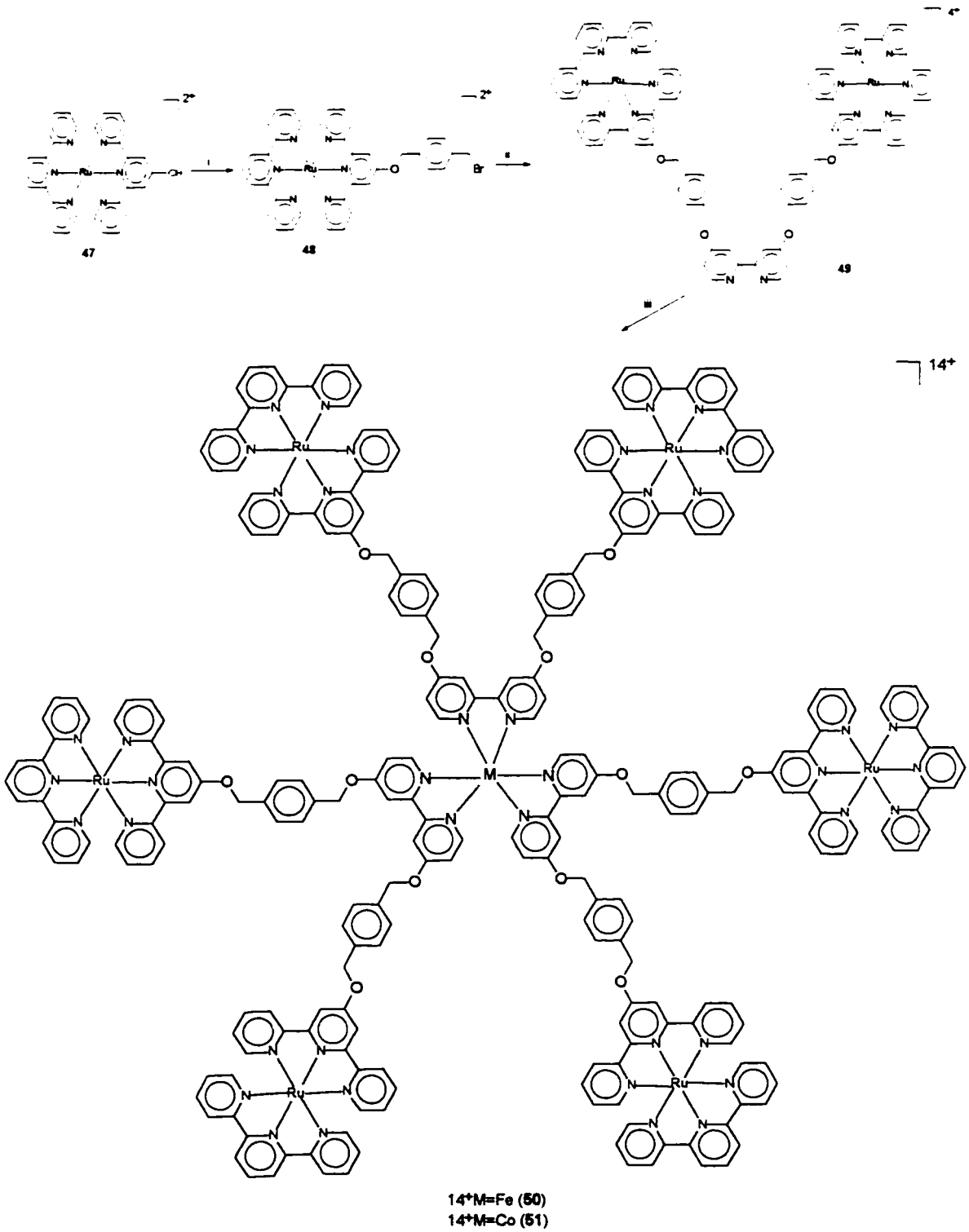
Due to the molecular flexibility, this dendrimer exhibited liquid crystalline properties.[38] The temperature for the transition from the nematic to the isotropic state for X=n-octyl, n-hexyl lied between 20~50°C. Conventional phase transfer catalyzed polyetherification between liquid-liquid two phase (organic-aqueous NaOH solution) and subsequent alkylation of unreacted phenolate chain ends with alkyl bromide or benzyl chloride were used for the preparation of dendritic polyethers.

Constable *et al.*[39] approached dendritic systems incorporating metal centers that are of interest as novel magnetic, electronic, or photooptical materials (Scheme 1-14).[40] This methodology relies on the initial synthesis of suitable bridged ligands, which are subsequently combined with the metal components to give metallodendrimers (**50** and **51**) and convergent approach allows the facile synthesis of species such as hexavalent dendrimers. The reaction of ruthenium complex with 2,2′-bipyridine-4 (1H), 4′ (1H)-dione (HO_2bpy) in the presence of K_2CO_3 gave divalent metallodendrimer. This dendritic compound is the building block for the heptanuclear species with $(NH_4)_2Fe(SO_4).6H_2O$ or $Co(O_2CMe)_2$ as core transition metals.

Majoral *et al.*[41] reported the synthesis of phosphorus containing dendrimers with chain end functionalities such as aldehyde, hydrazones, alcohols, α,β-unsaturated ketones or cinnamonitrile. Sinkai *et al.*[42] also reported new type of dendritic polymers, "crowned" arborols, using a convergent method.

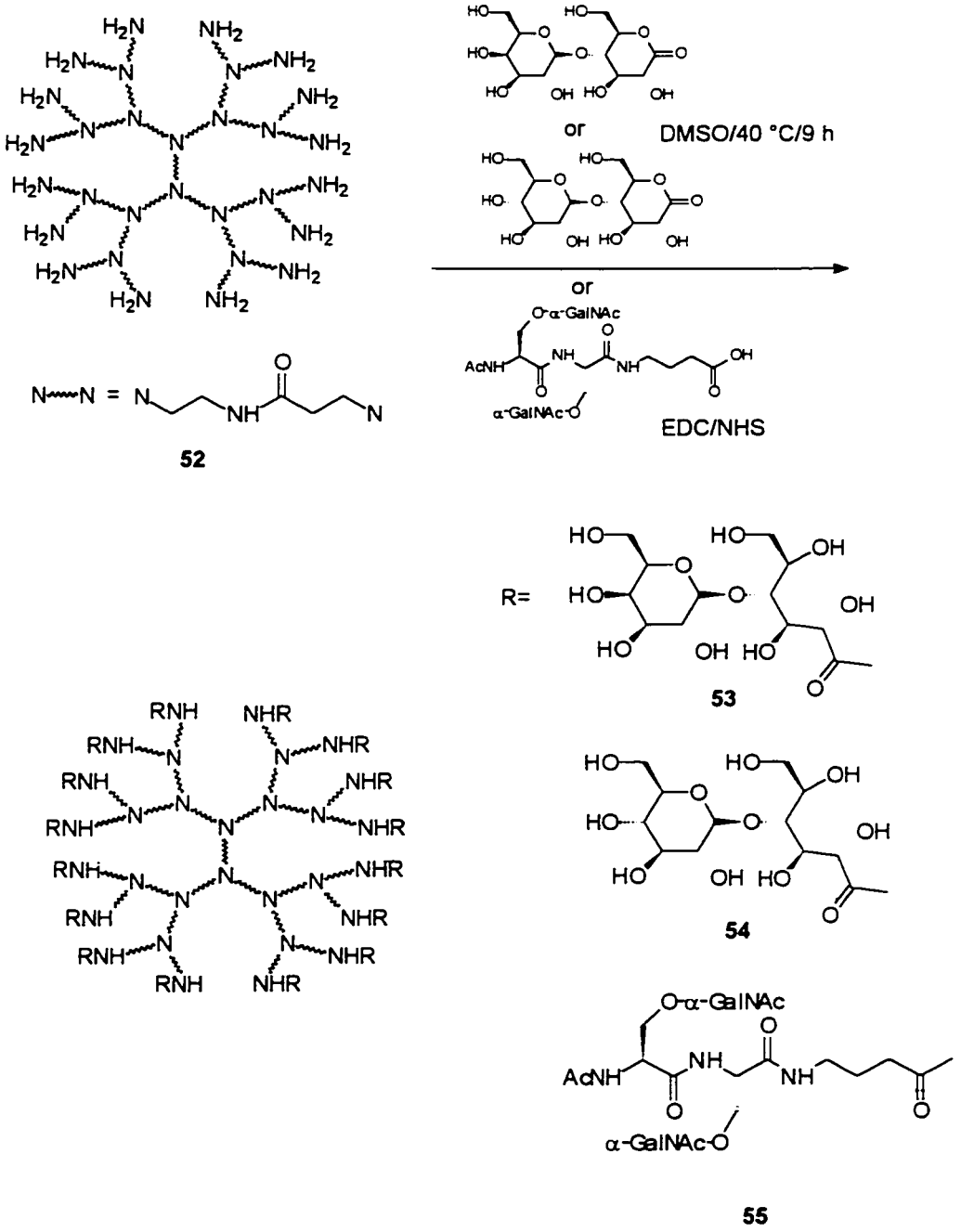
Kemmitt and Henderson [43] synthesized silatrane-containing dendritic wedge. This organosilicon dendritic wedge was the first example containing pentacoordinated silicon.

Only few carbohydrate-containing dendrimers have been prepared. Roy *et al.*[44] prepared L-lysine based multiple antigen peptide dendritic core (MAP) using solid phase peptide synthesis. At the end of dendrimers, sialic acid (Neu5NAc) residues were linked and di-, tetra-, octa-, and hexadecameric glycodendrimers were synthesized. For further extension of L-lysine based glycodendrimer, T-antigen was covalently attached to MAP core. This work will be presented in Chapter 5.

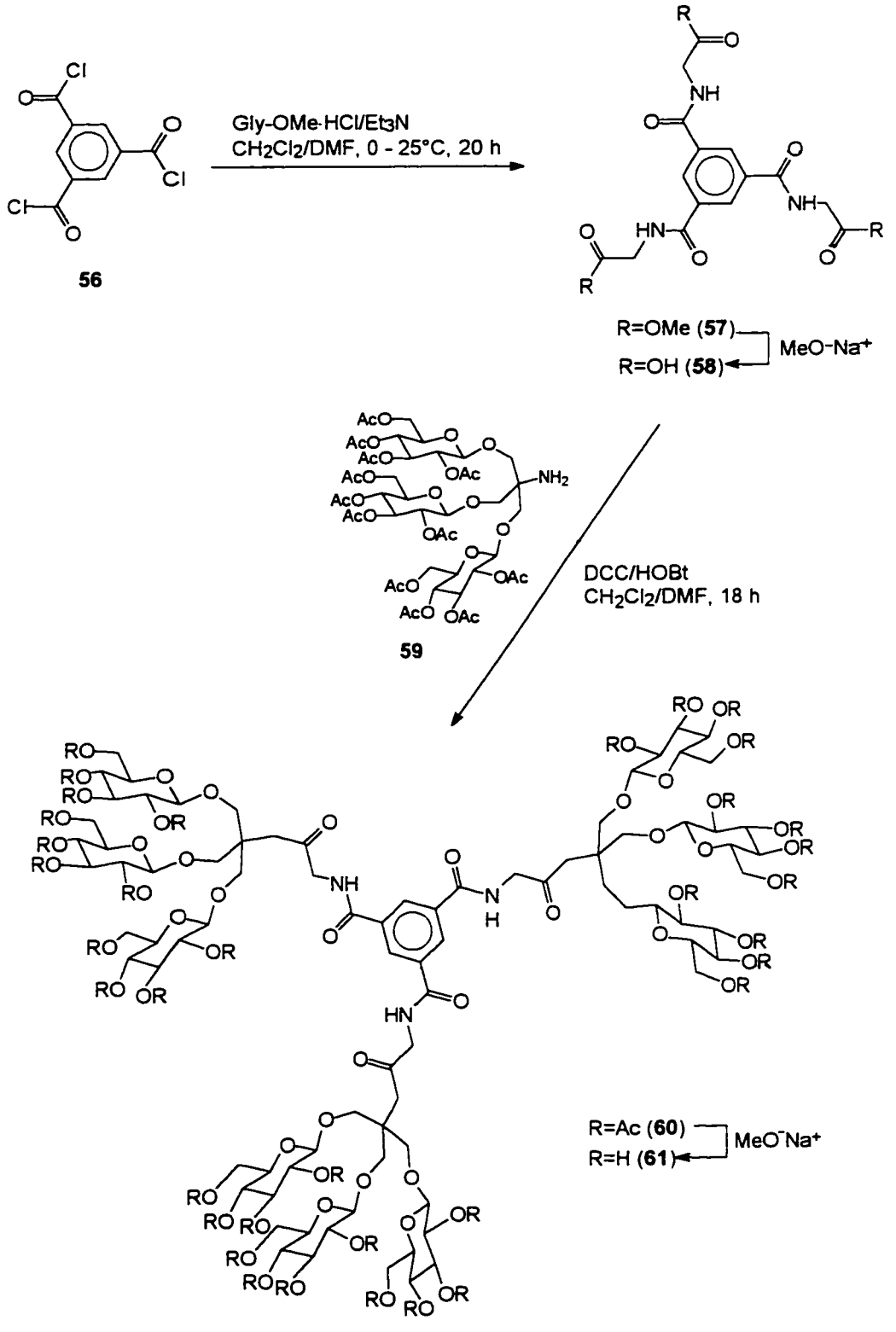


Scheme 1-14. Convergent synthesis of metallocentric metallodendrimer, i) 1,4-bis(bromomethyl)benzene, K_2CO_3, MeCN, ii) HO_2byp, K_2CO_3, MeCN, iii) $(NH_4)_2Fe(SO_4).6H_2O$ or $Co(O_2CMe)_2$, MeCN, H_2O.

Carbohydrate-persubstituted poly(amidoamine) (PAMAM) dendrimers (**53**~**55**) have also been prepared as mimics of naturally occurring multiantennary oligosaccharides[208, 212] (Scheme 1-15).



Scheme 1-15. Glycosylation of PAMAM dendrimers.[208, 212]



Scheme 1-16. Synthesis of glycodendrimer with nine residues.

Aoi *et al.*[208] prepared disaccharide lactone of lactose and maltose conjugated to terminal amines in PAMAM dendrimers. These conjugates showed binding interaction with Concanavalin A and Pea lectins. Similarly, Toyokuni and Singhal[212] have assembled dimeric T_N-antigen-serine peptide conjugate to 5th generation PAMAM *via* N-hydroxysuccinimide ester. Lindhost *et al.*[213] have coupled various glycoside to PAMAM using isothiocyanate method. Protected glycosyl isothiocyanates such as α-D-mannose, β-D-glucose, β-D-galactose, β-D-cellobiose and β-lactose have been employed for PAMAM glycosylation.

Ashton *et al.*[45] have achieved conjugation between glycosylated clusters and 1,3,5-benzenetricarboxylic acid to provide glycodendrimers (Scheme 1-16).

For a better understanding of carbohydrate-protein interactions in biological events, several glycodendrimers such as N,N′-bis(acrylamido)acetic acid, L-lysine and PAMAM based glycoconjugates with T-antigen have been prepared. Their binding abilities were also examined with receptor proteins (pytohemagglutinins) such as peanut lectin from *Arachis Hypogaea* and from mouse monoclonal antibodies (IgG and IgM).

1.5. Neoglycoproteins

In order to understand the various biological functions, carbohydrate haptens have to be covalently attached to carrier molecules like proteins (Fig. 1-4). The resulting neoglycoproteins have been used in screening, studying the binding interaction with antibodies, lectins and enzymes,[45, 46] synthetic vaccines[47], diagnostics, and targeted drug delivery systems.[48]

Preparation of neoglycoproteins should satisfy the following conditions;

i) compatible with an aqueous solution

ii) mild and effective under physiological conditions to prevent destruction or denaturation of the proteins

iii) compatible with multifunctional nature of the proteins

iv) versatile and applicable to various sugar residues

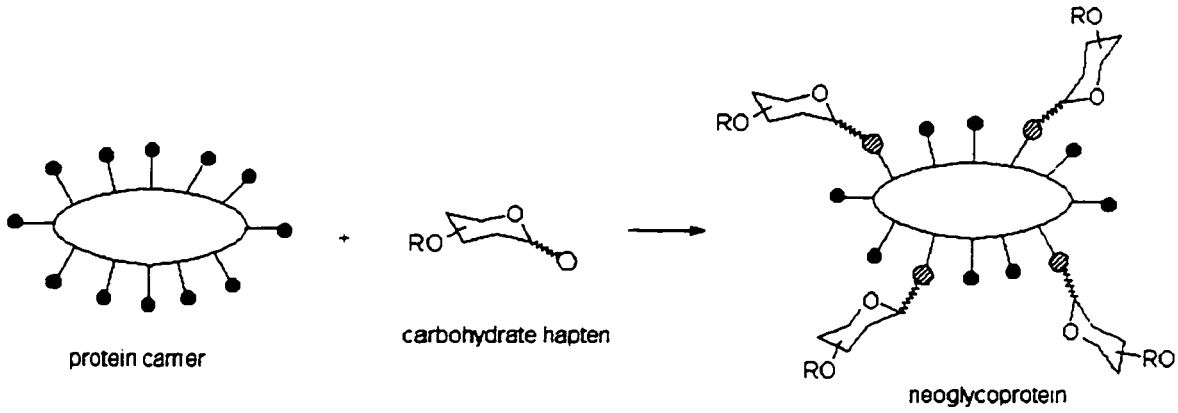
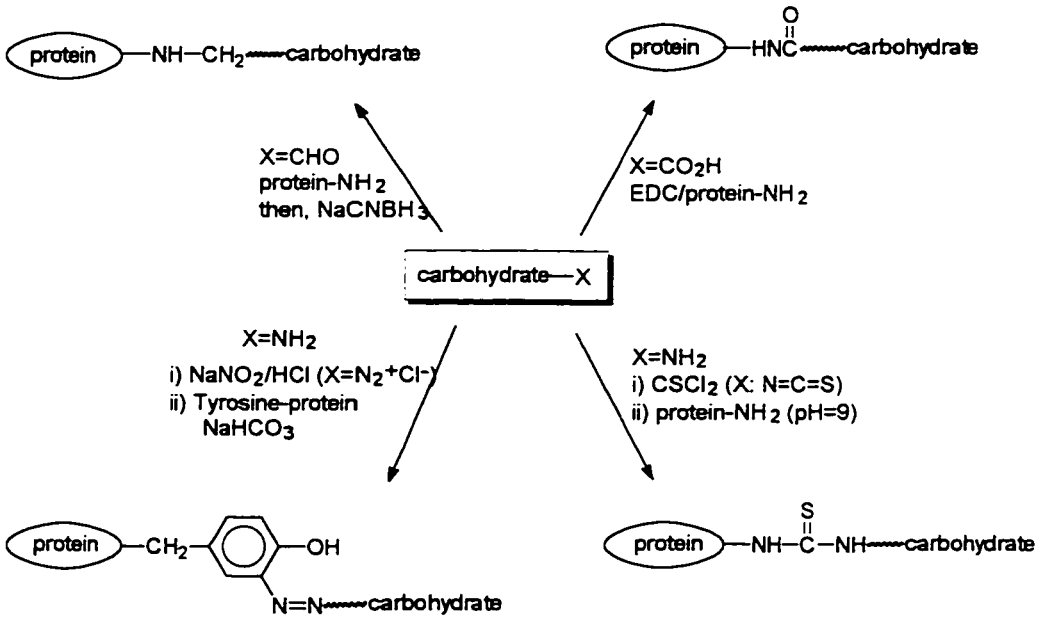


Fig. 1-4. Representation of neoglycoprotein synthesis.

v) easily manipulated and reproducible.

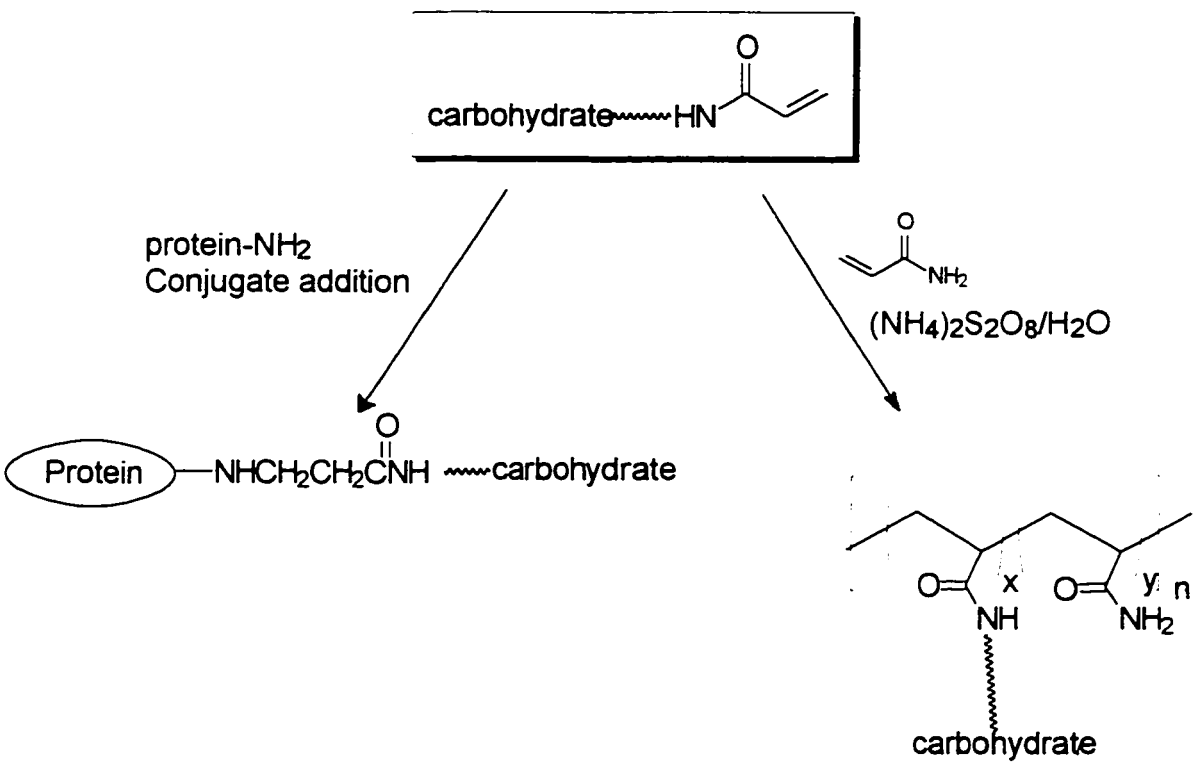
Roy *et al.*[49] have prepared glycoproteins with aldehyde terminated carbohydrate haptens and amine terminated poly L-lysine using sodium cyanoborohydride ($NaCNBH_3$). p-aminophenyl glycosides,[50] isothiocyanates[51] and alditol[52, 53] derivatives have been widely used (Scheme 1-17).



Scheme 1-17. Protein conjugation with carbohydrate haptens.

The diazotization of aromatic amines is not chemoselective and a large excess of diazophenyl glycoside is required for conjugation with the protein. The isothiocyanate method also requires the use of a large excess of toxic thiophosphagene to make the thiocarbamoyl linkage with amines in protein carriers. Amidation using carbodiimide chemistry is an alternative method to obtain neoglycoproteins (Scheme 1-17). A water soluble carbodiimide such as 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC) is used as a coupling reagent to link carbohydrate and protein (each part can be either in acid or amine form). However, very carefully controlled pH is required during the reaction to prevent the crosslinking of proteins. Thus each conjugation method to provide the desired neoglycoconjugates has its own drawbacks.

Roy *et al.*[55, 56] have reported a new methodology using a carbohydrate containing acrylamido function and a protein-carrier containing nucleophilic termini such as amine or thiol (Scheme 1-18).



Scheme 1-18. Synthesis of neoglycoproteins and neoglycopolymers using acrylamido functionalized carbohydrate haptens.

This methodology satisfies the required criteria mentioned earlier and no coupling reagents are needed. In addition, they can polymerize with/without comonomer like acrylamide.[57]

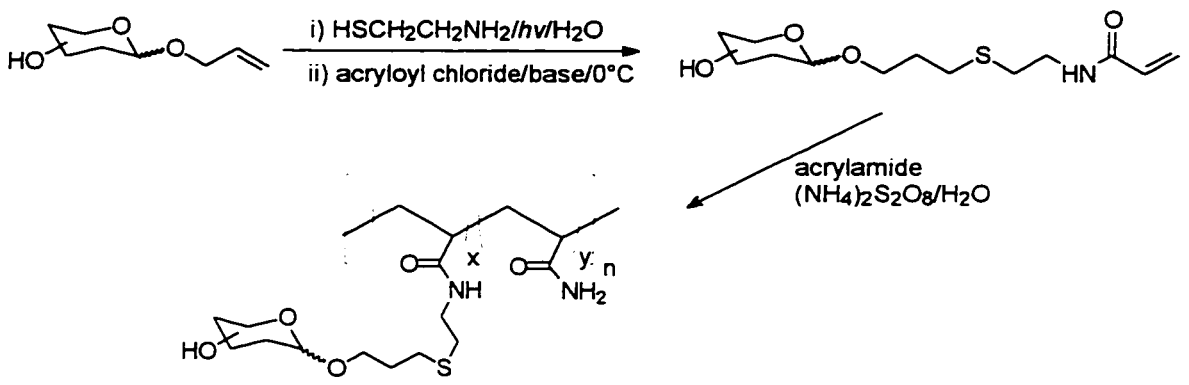
Even though neoglycoproteins are prepared successfully to serve biochemists and immunochemists for the investigation of carbohydrate-protein interactions, they still have some drawbacks. For example, because the protein carriers are composed of twenty different amino acids arranged in various sequences, structural characterization is difficult. Neoglycoproteins are also sensitive and biodegradable and, in some cases, have very short life-times under strictly controlled conditions as well as carbohydrate content in the conjugates can not be easily controlled. A critical drawback is that neoglycoconjugates give non-carbohydrate specific interactions caused by the protein carrier itself and can interfere with any recognition interactions during various immunochemical assays. These disadvantages have stimulated the chemist and biochemist to develop other types of glycoconjugates. As mentioned in Scheme 1-17, N-acrylamido functionalized carbohydrates are very feasible alternatives for the preparation of new glycoconjugates.

1.6. Glycopolymers

Since Horejsi[58, 224-a] reported the first glycopolymers involving allyl glycosides and acrylamide, water soluble antigenic glycopolymers have been synthesized with alkene terminated glycosides.[59, 60, 220-d] However, glycopolymers prepared from allyl glycosides suffer from a short distance between the polymer backbone and the carbohydrate moiety. As a result, the flexibility necessary for the carbohydrate to bind properly with the receptor protein[61] is limited due to the steric constraints imposed by the close proximity of the backbone and antigenic determinant. Nishimura *et al.*[62] prepared glycopolymers with elongated spacer arms such as n-pentenyl or n-undecylenyl chains. Unfortunately, the reactivities of the two monomers were too different and undesirable block copolymers were obtained as well long lipophilic chains resulted in solubility problem in water.[62-b]

To overcome these disadvantages, Roy *et al.*[61, 63, 220-b] suggested the use of N-acryloylated carbohydrates (Scheme 1-19). In this methodology, acrylamide was chosen as

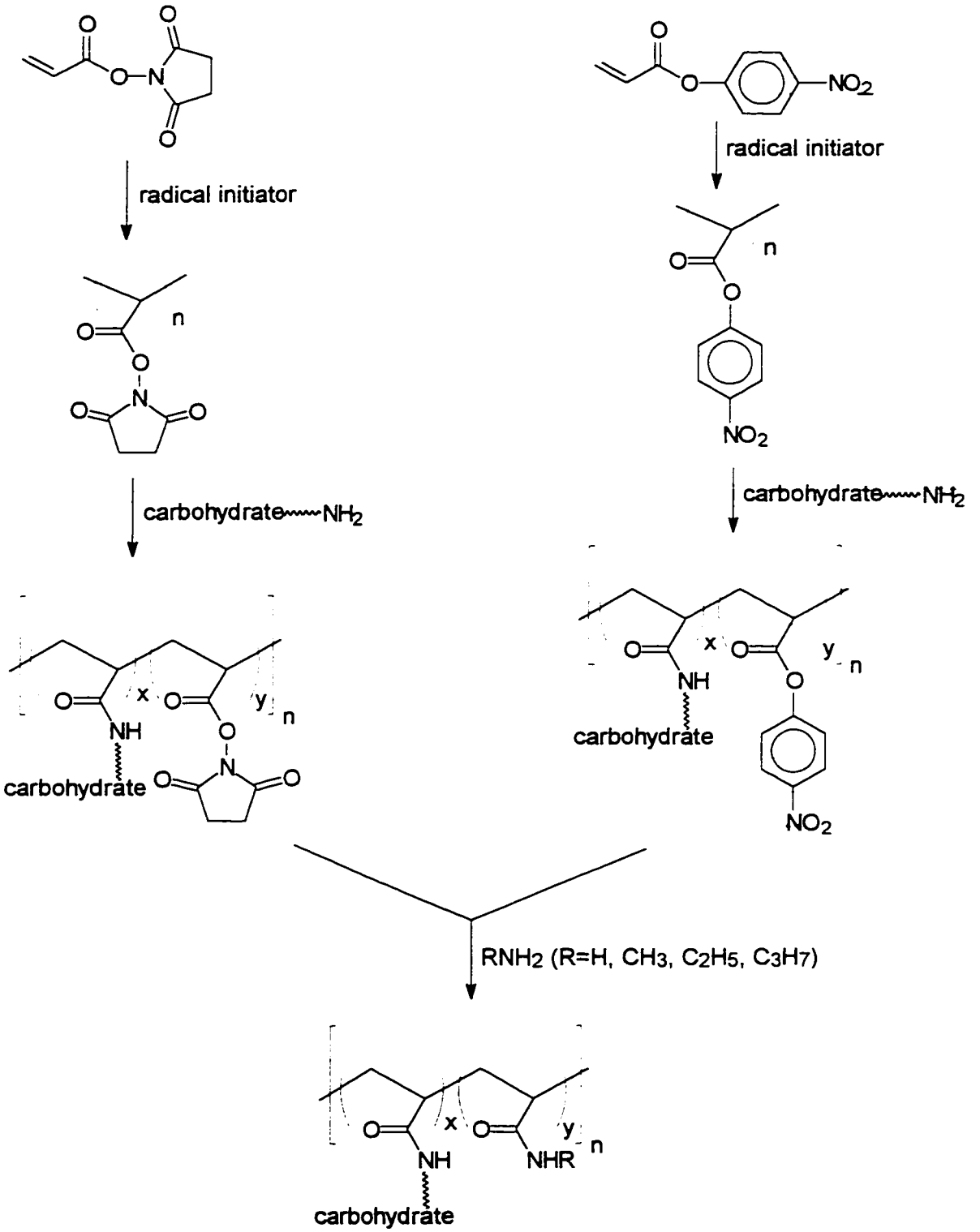
a comonomer due to its similar reactivity and equal chance to participate in polymerization.



Scheme 1-19. Preparation of copolyacrylamide containing carbohydrates from N-acryloylated carbohydrate hapten.

Furthermore, another N-acryloylated comonomer may be employed to prepare three components containing terpolymers.

Glycopolymers have some advantages over glycoproteins. They are cheap and easy to prepare and purify in either small or large quantities. They are thermally, chemically and biologically much more resistant than their neoglycoprotein counterpart. Their simple chemical structure allows for the characterization of their composition and structural features. Moreover, the artificial nature of the carrier does not lend itself to non-specific binding interaction between itself and proteins of interest so that it does not interfere with quantitative immunoassays or diagnostic applications. However, there is a drawback for these copolymerizations. In the randomly distributed copolymers, carbohydrate densities and molecular weight distributions may vary from batch to batch and if large quantities of glycopolymers are not prepared and used from the same batch for bioassays. To overcome this drawback, preactivated polymers such as poly(p-nitrophenylacrylate), poly(N-acryloxy succinimide) or poly(acrylamide-*co*-N-acryloxy succinimide) were synthesized.[218, 229, 230] These polymers can be prepared from the corresponding active esters namely N-acryloxy succinimide or 4-nitrophenyl acrylate by radical polymerization (Scheme 1-20).



Scheme 1-20. Synthesis of carbohydrate containing copolyacrylamide in two stages.

The molecular weight of the polymers can also be controlled through variation of the amount of radical initiator, temperature, solvent, concentration of solution. Once the desired molecular weight of polymers are obtained, graft conjugation can be performed with carbohydrate haptens which contain nucleophile such as amine to active ester functionalities to provide the glycosylated polymers. Subsequently, the remaining active ester is quenched by another nucleophile such as aqueous ammonia, methyl-, ethyl-, or propylamine to give the desired glycopolymers. Glycoside and effector content can be controlled by the amount of carbohydrate and effector used which are equipped with nucleophile. As long as preactivated ester polymers are used from the same batch, the physiological properties of glycopolymers will be identical and more reliable results can be obtained if these glycopolymers are used.

In this dissertation, the synthesis of several glycopolymers will be described. T-antigen monomers with different length of spacer arms are conjugated with acrylamide to give the glyco-*co*-polymers. At the same time, the same architecture of other glycopolymers is prepared from preactivated homo- and copoly acrylamide. Their effectiveness as antigens is also examined using double radial immunodiffusion assays, ELLA and ELISA with both lectin and antibodies. This thesis will touch upon these as well.

1.7. Immunochemical assays

1.7.1. Lectin and antibody

Lectins are non-enzymatic, carbohydrate-binding proteins of non-immunogenical origin that bind to specific carbohydrate structure.[64-69] They are a structurally diverse class of proteins, for example, legumes and cereals in plants and C- and S-type lectins in

animals. They have the intrinsic properties of specificity, molecular mass, number of binding sites (CRD) per subunit and the existance of disulfide bonds.

The functions and significance of lectins as cell surface carbohydrate receptors are now well known.[64-b] Lectins are relatively small proteins (usually ranging from 40 to 160kDa) made up of several subunits and exist as several isolectins (different isoelectric points). Some of them chelate to metals to complete the binding with carbohydrate haptens. They are also involved in the selectin-mediated cell-cell binding, the binding of sperm to sugar epitopes at the surface of the zona pellucida of egg (fertilization), cell growth, differentiation and migration process.[64-a]

The outstanding aspect of lectins is their specificity towards carbohydrates. Various lectins are well known to prove protein-carbohydrate interactions. A list of some lectins are summarized in Table 1-3.

Table 1-3. Lectins and characteristics.

Lectin	Origin	MAW (KDa)	Sugar specificity
Wheat Germ Agglutinin (WGA)	*Triticum Vulgaris*	43	GlcNAc NeuAc
Peanut lectin	*Arachis Hypogaea*	110	β-D-Gal β-D-Gal-(1-3)-α-D-GalNAc
RCA_{120}	*Ricimus Communis* (Castor bean)	120	β-D-Gal α-L-Rha
Maclura Pomifera	Osage orange	42	β-D-Gal
Sambucus Nigra	Elder stem and branches	140	Lactose
Galectin	animal cells	36	β-D-Gal

Peanut lectin is a tetrameric, carbohydrate free protein composed of four subunits where the subunit size is reported from 27 to 28 kDa. Peanut lectin contains one atom each of Ca^{++} and Mg^{++} per subunit. Extensive studies were performed for the carbohydrate binding specificity of peanut lectin by employing hapten-inhibition of peanut agglutinin, spectroscopic techniques and quantitative precipitation of glycoconjugates. Carbohydrate binding specificity is illustrated in Table 1-4. N-acetylgalactosamine and 2-deoxygalactose

Table 1-4. Inhibition of peanut agglutinin-blood group precursor substance precipitation by saccharides.[70]

Carbohydrate	Relative inhibitory potency[a]
Galactose	1.0
N-acetyl galactosamine	2.22
Methyl α-D-galactopyranoside	2.22
Methyl β-D-galactopyranoside	1.50
p-nitrophenyl α-D-galactopyranoside	1.50
β-D-Gal-(1-3)-α-D-GalNAc	54.5

a: Galactose is normalized to 1.0.

are inactive, suggesting the importance of the hydroxyl group at C-2 position, methyl α-galactose is somewhat better inhibitor than the anomeric β-galactoside. The importance of the hydroxyl group at the C-5 position is indicated by the failure of galactose-6-sulfate and L-arabinose to act as inhibitors. The disaccharide, β-D-Gal-(1-3)-α-D-GalNAc is the most complementary to the binding site being 55 times more active than galactose. The presence of aglycon does not affect the affinity of galactose indicating the absence of a hydrophobic region adjacent to the galactosyl binding site.

Antibodies or immunoglobulins are multivalent receptor proteins that constitute an important part of the immune system in higher living organisms. They are found in the blood serum and tissue of vertebrates and serve as a response against the appearance of antigens. These immune systems produce antibodies against various substances for regulatory functions including protection and eradication of infection.

For a better understanding of the immunogenic recognition of carbohydrate antigens, mouse monoclonal antibodies (IgM and IgG) are produced using the T-antigen conjugated BSA that we synthesized (Chapter 7).

On account of their specificities and multivalent nature, lectins and antibodies are used as good models for the formulation of carbohydrate-receptor protein complexes.

1.7.2. Double radial immunodiffusion

Double radial immunodiffusion assay[2-c] is a technique for testing qualitative agglutination. In this technique, glycoconjugate containing a buffer solution (PBS, pH=7.3) is placed into a well cut in an agar gel-coated glass plate. Lectin or antibody solution is placed in an adjacent well. The two solutions are then allowed to diffuse through the gel over time. If there is molecular recognition, a precipitate forms between the two wells. The precipitin band is visible and can be stained. This assay may be performed in an easy, quick manner without the need for any special instrumentation. Several photographs are shown in Chapter 7 indicating the formation of precipitin bands.

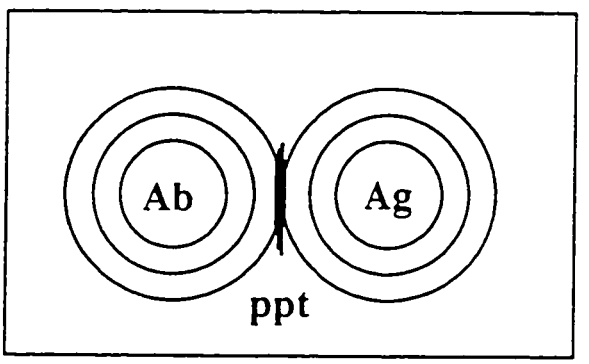


Fig. 1-5. Schematic representation of double immunodiffusion assay between antibody (Ab) and antigen (Ag).

1.7.3. Turbidimetric analysis

Turbidimetric analysis is also a simple qualitative test for the measurement of insoluble cross-linked carbohydrate-receptor protein lattice. The solution containing glycoconjugates is filled in a microtiter plate together with lectin or antibody. Optical density (O.D.) at certain wavelength is then measured as a function of time. Usually a large excess of glycoconjugates and receptors are used to induce precipitation from

solution, thus, a siginficant increase of O.D. values is expected. For the analysis of T-antigen containing conjugates, mostly 2 mg/mL of each solution (in PBS) is used.

1.7.4. Enzyme linked immunosorbent assays (ELISA)

ELISA is a quantitative inhibition assay which is much more sensitive than the previous two examples. It makes possible the determination of the concentration of a particular carbohydrate hapten to inhibit, i.e., IC_{50}. In competitive solid phase assay, a microtiter plate is coated with antigenic ligands such as naturally existing glycoproteins or synthetic glycopolymers (Fig. 1-6). The receptor and the glycoconjugate inhibitor with varying concentration are preincubated and added to the plates. An anti-receptor protein-enzyme conjugate is then added. If the glycoconjugate is an effective inhibitor, it will prevent the binding of the receptor protein to the coated antigen. Hence, the solid phase coated carbohydrate ligand competes with the free ligand in binding to the receptor protein. A multivalent glycoconjugate with strong inhibitory potential binds effectively to the receptor and thus reduces receptor-coated antigen binding interactions. The ability of inhibition is plotted as percent inhibition versus inhibitor concentration. These data provide an estimate of the concentration of glycoconjugate required for 50 % inhibition (IC_{50}).

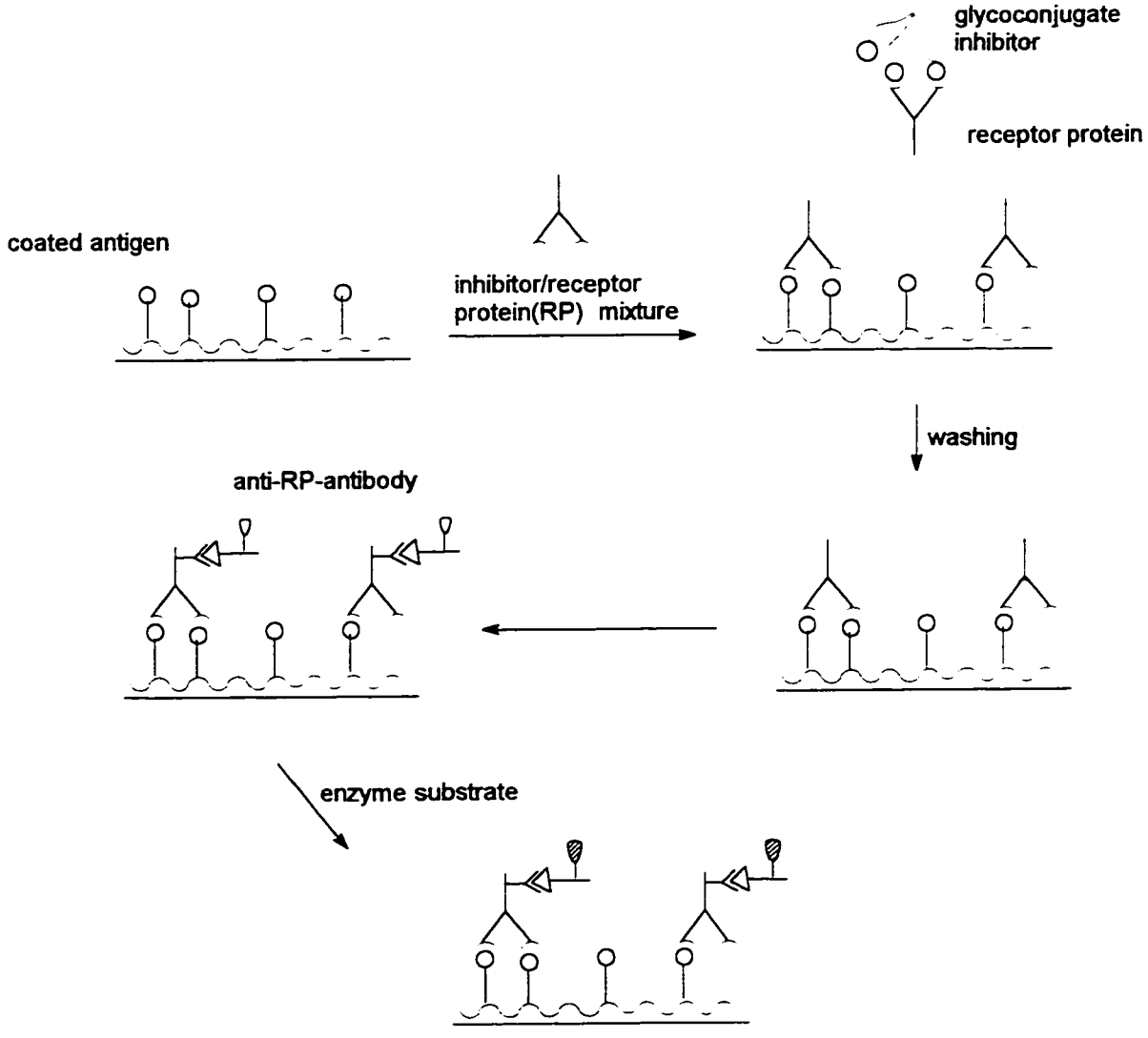
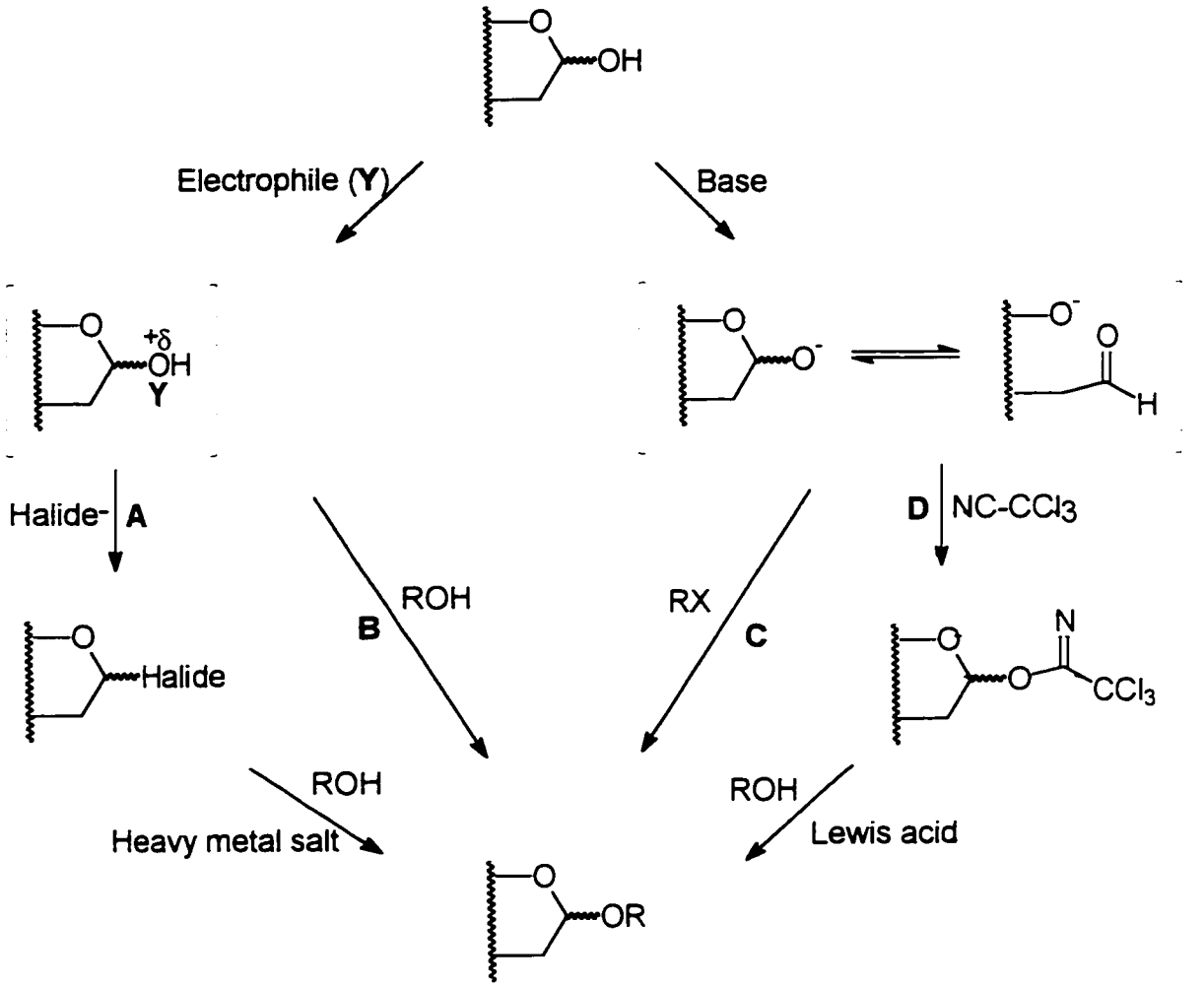


Fig.1.6. Schematic representation of competitive enzyme linked immunosorbent assay.

Chapter 2. Synthesis of Carbohydrate Haptens

2.1. Introduction

Recently, extensive biological studies of oligosaccharide containing naturally occurring products such as membranes, cell walls, and antibiotics have well established their key roles in many processes.[71, 72] Examples include fertilization, embryogenetics, hormone activities and cell proliferation and organization into specific tissues. To establish the biological role of oligosaccharides, a large number of well defined sugar moieties of different sizes which can be prepared by the efficient synthetic methodology is necessary. To meet this demand, many synthetic strategies have been developed to prepare oligosaccharides. In such a glycosylation, most of the synthetic effort is directed toward the preparation of glycosyl donors and acceptors.[72, 73] The use of glycosyl halide (X=Cl, Br) as an effective glycosyl donor in the glycosylation reaction was introduced by Koenigs and Knorr (**A**).[74] Scheme 2-1 derives the general principles of glycosylation. This method was useful for the synthesis of 1,2-trans glycosides which involved a two step reaction: first, introduction of a leaving group at the anomeric center by protonic or Lewis acid activation and subsequent nucleophilic substitution of the leaving group. Stereoselective α or β coupling can be achieved by exploitation of stereoelectronic effects (anomeric and inversed anomeric effect), neighboring participation and the choice of catalyst.[73-c] Method **B** proceeds glycosylation by a nucleophilic addition of glycosyl acceptor which possess a hydroxyl group. On the other hand, base activation can involve alkoxide at C-1 position (**C** and **D**). Ring-chain tautomerism and anomerization, unstable aldehyde and differentiated reactivities of the α- and β-alkoxide decrease stereoselectivity. Due to the low stability of glycosyl halides and toxicity of heavy metal salt promoters and the conversion difficulties of an intermediate oligosaccharide into such derivatives[76], new glycosyl donors having non-halogen leaving group at anomeric center have been developed.

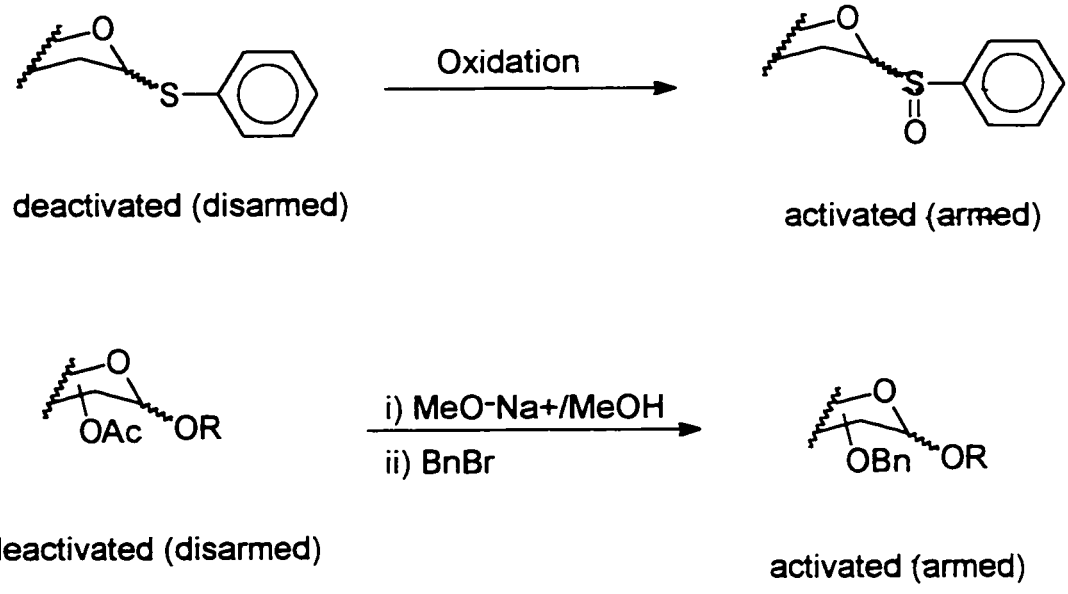


Scheme 2-1. General glycosylation concepts.[73-f]

Trichloroacetimidates[77], thioethers[78], fluoride[79], pent-4-enyl,[80] and phosphorus derivatives[81] are widely used donors. The development of armed and disarmed concept on glycosyl donor has provided a powerful tool to build up oligosaccharides efficiently.[82] This concept uses electron withdrawing protecting groups on a glycosyl donor for deactivation (disarmed) and electron donating groups for reactive (armed) donors.[80-d, 82] (Scheme 2.2) The category of armed and disarmed glycosyl donor can be applied to various donors such as thioglycoside,[83] glycals,[84] and 4-pentenyl glycosides.[85]

Even though there is no general explanation of reactivity, the choice of glycosyl acceptor to get the desired stereochemistry of the linkage is important as glycosyl donor

and promoter do.[86] Typically, acceptors are prepared with hydroxyl group but one derivatized with protecting groups. Glycal has been demonstrated to be efficient species



Scheme 2-2. Method of activated (armed) glycosyl donor.

for the synthesis of complex oligosaccharides[87] and selective protection of hydroxyl groups can be achieved using appropriate reagents.[91] The approach is also applicable to glycopeptides and oligosaccharides on the solid support.[88] In addition, glycal can be applied a different concept for glycosyl transfer or cycloaddition method with hetero atom containing diene.[89] Glycals, especially D-galactal and D-glucal, are intermediates for preparing selectively protected 2-azido-2-deoxy-D-galactopyranose and 2-azido-2-deoxy-D-glucopyranose building blocks.[90] The former glycoside is a precursor of N-acetyl-D-galactosamine which occurs in oligosaccharide antigenic determinants of a number of glycospingolipids[92] and extends to the T- and Tn antigen syntheses.[93]

The azidonitration of protected glycals disclosed by Lemieux *et al.*[90] is an attractive route to these derivatives and the 2-azido-1-nitrate adduct obtained can be transformed into various glycosyl donors by the substitution of a nitrate group.

Azido-phenylselenylation of double bond[94] is a useful reaction because it allows for the one-step introduction of two functionalities in a molecule by a electrophilic or radical

addition. For the selenoglycosides, the radically induced addition of glycals would afford 2-azido-2-deoxy-selenoglycoside which is a precursor of 2-amino-2-deoxy selenoglycosides.

Allyl glycosides have been used by carbohydrate chemists during oligosaccharide synthesis. The allyl group can be introduced at the anomeric center by standard Fischer (for α-glycosides) and Koenigs-Knorr (for β-glycosides) methods. By virtue of the alkene function, allyl glycosides can be functionalized to other groups. For instance, ozonolysis and subsequent reductive amination provides an alkylamine functional group. The detail of this method will be discussed in Chapter 3. For an anomeric protecting group, it has to be stable to several reaction conditions and can be removed by Pd/C in the presence of acid[95] or tris (triphenylphosphine) rhodium (I) chloride to isomerize the double bond followed by hydrolysis of the 1-propenylgroup with mercury (II) chloride in aqueous acetone.[96] Selective protection of hydroxyl group in allyl glycosides can also be achieved by the use of appropriate protecting groups such as t-butyldiphenylsilyl or benzylidene groups.

Despite the versatility of donors and acceptors, there is a still a hurdle to surmount in order to achieve successful glycosylation. It is significant that the choice of appropriate promoters is an important factor. The Koenigs-Knorr method used heavy metal (Hg, Ag) salt promoters involving glycosyl halide as a donor. Actually, this strategy gave an excellent glycosylation yield in our experience. New methods which involve non-halogen containing leaving groups have also been developed.[78-81] In these methods, richloroacetimidates are usually activated with trifluoroborane etherate ($BF_3 \cdot OEt_2$) or trimethylsilyl trifluoromethanesulfonate (TMSOTf).[97] Alkyl or aryl thioglycosides are activated by various thiophilic promoters[98] including NIS/cat. TfOH[99] and dimethyl (methylthio) sulfonium trifluoromethane sulfonate (DMTST).[100, 73-c,d] A variety of thiophilic reagent combinations have also been developed such as NBS/Bu_4NOTf[101], PhSePhth/TMSOTf,[102] and TfO_2.[103] These reagents react with sulfur atom to form cationic sulfonium ion ($R\text{-}S^{+}\text{-}L$) so that the glycosylation reactions can be accelerated in the presence of glycosyl acceptors.

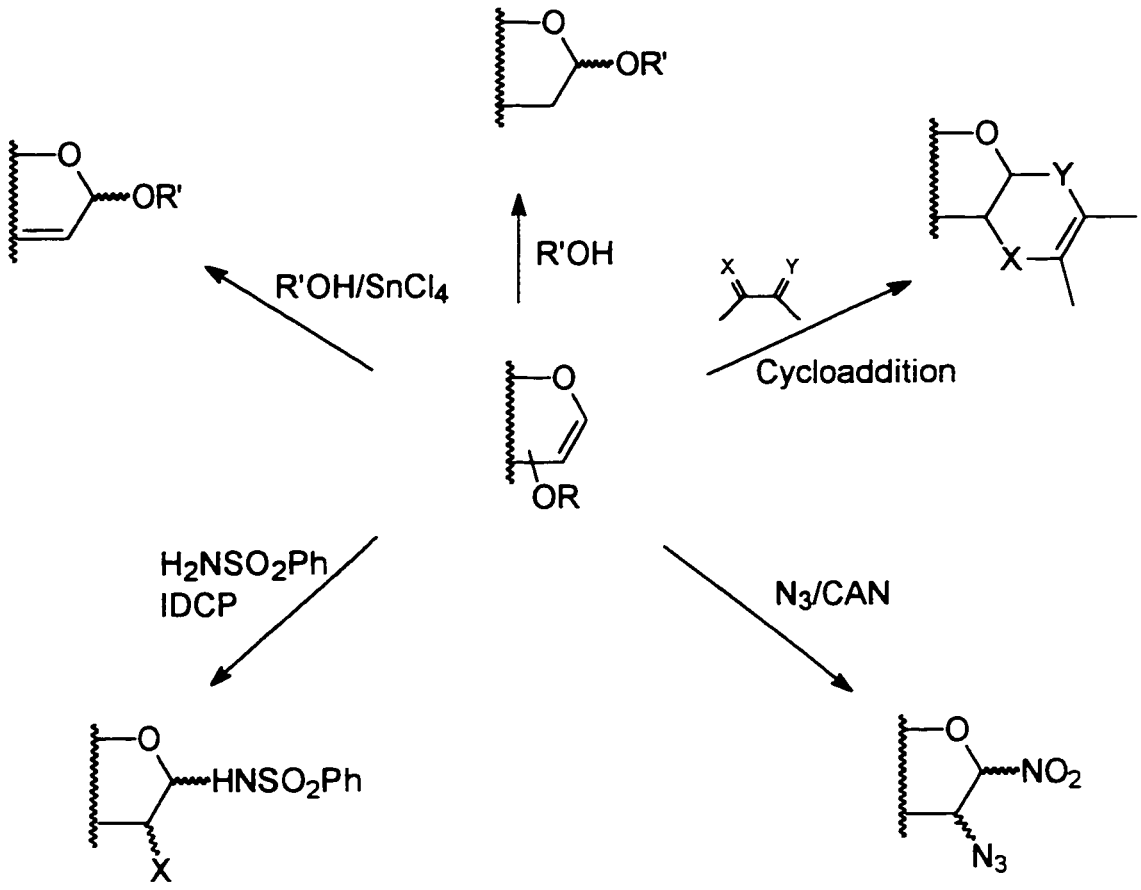
2-2. Glycosyl Acceptors

2-2-1. 1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol (Galactal)

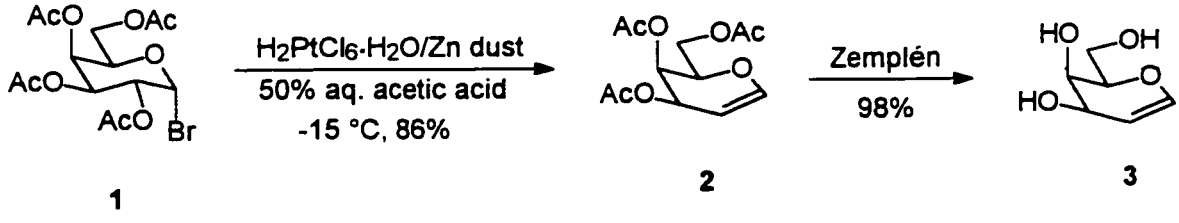
Since Lemieux *et al.*[104] have established the reaction of glycal and simple alcohol in the presence of I_2, Ag salt and base to give 2-deoxy-2-iodo glycosides, glycals have been important reaction intermediates especially in the synthesis of 2-deoxy-glycosides. Danishefsky *et al.*[105], Tatsuta *et al.*[106] and Thiem *et al.*[107] developed more practical promoters. The 2-deoxy-2-halo-α-glycosides obtained by these promoters can be converted into 2-deoxy-α-glycosides by reductive dehalogenation. This reaction methodology was efficiently applied to the synthesis of carbohydrate bearing natural products.[105, 108] Another application of glycals is the preparation of the 2-azido glycosides by azidonitration (ceric ammonium nitrate).[109] Furthermore, Danishefsky *et al.*[110] developed the sulfonamido glycosylation by the use of IDCP and benzenesulfonamide or N,N-dibromobenzenesulfonamide to prepare 2-deoxy-2-azido-β-glycosides. The glycosyl transformation of glycals is summarized in Scheme 2-3.

The synthesis of glycals is well established by Blackburne *et al.*[111]. They treated 2,3,4,6-tetra-O-benzoyl-1-thio-β-D-glucopyranose with lithium naphthalenide (2eq.) in THF at -78°C to give 3,4,6-tri-O-benzyl-O-glucal. William *et al.*[112] synthesized acetylated glycals from 2,3,4,6-tetra-O-acetyl glycosyl chloride in the presence of chromium diacetate dimer monohydrate and 1,2-diethanediamine followed by acetic anhydride.

Treatment of peracetylated galactosyl bromide with zinc dust/aqueous acetic acid and cat. H_2PtCl_6 at -15°C affords **2** in 86% yield after silica gel chromatography (Scheme 2-4).



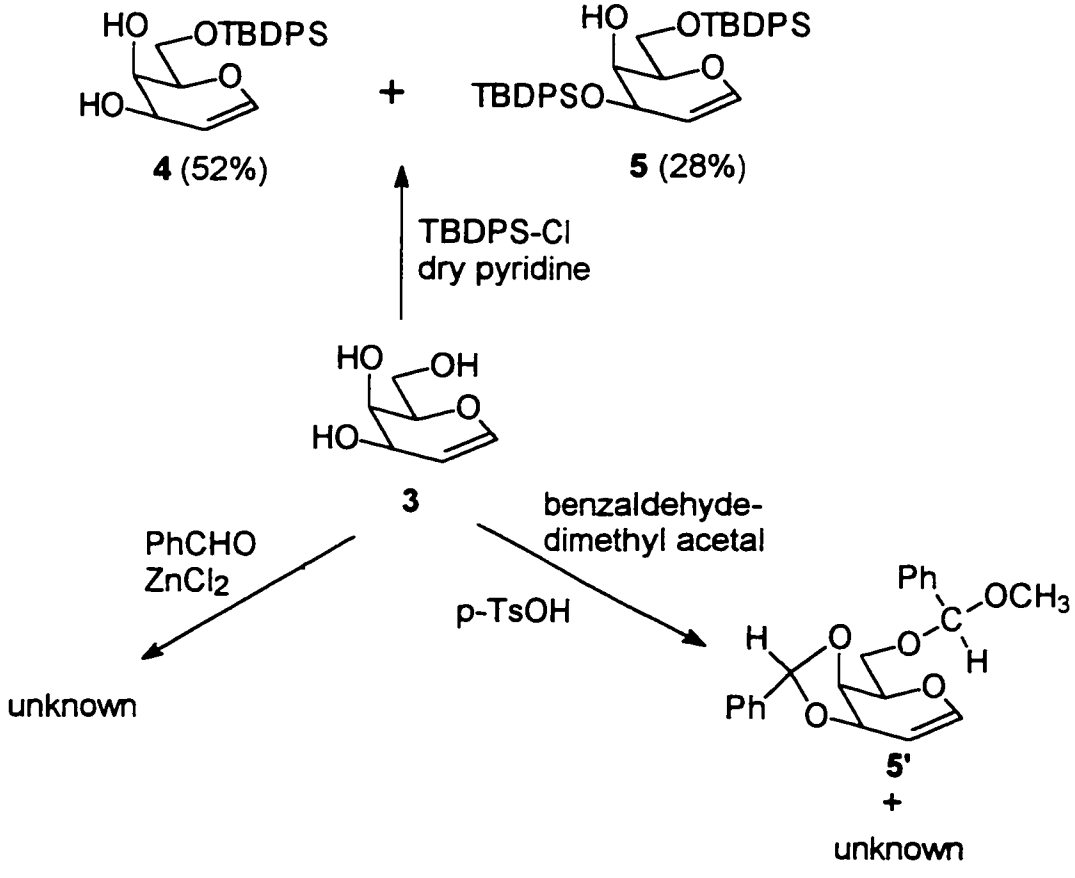
Scheme 2-3. The reactions of glycals.



Scheme 2-4. Preparation of galactal.

Deprotection of **2** was accomplished under Zemplén conditions (NaOMe, MeOH, pH~9). After neutralization with H^+ resin, the unprotected **3**, was obtained in 98% yield. The structure of **3** was determined according to the chemical shifts of the alkenyl protons and carbons (^{1}H-NMR, 6.49 ppm for H-1 and 4.80 ppm for H-2, ^{13}C-NMR, 145.4 ppm for C-1 and 104.1 ppm for C-2).

For the selective protection (Scheme 2-5), **3** was successfully treated with *t*-butyldiphenylsilyl chloride (1.3 eq.) in dry pyridine at room temperature. [91-c] The resulting mono- and disilyl derivatives **4** and **5** were isolated by silica gel chromatography to afford 52% and 28% yields, respectively. Compound **3** was also treated with benzaldehyde



Scheme 2-5. Selective protection of galactal.

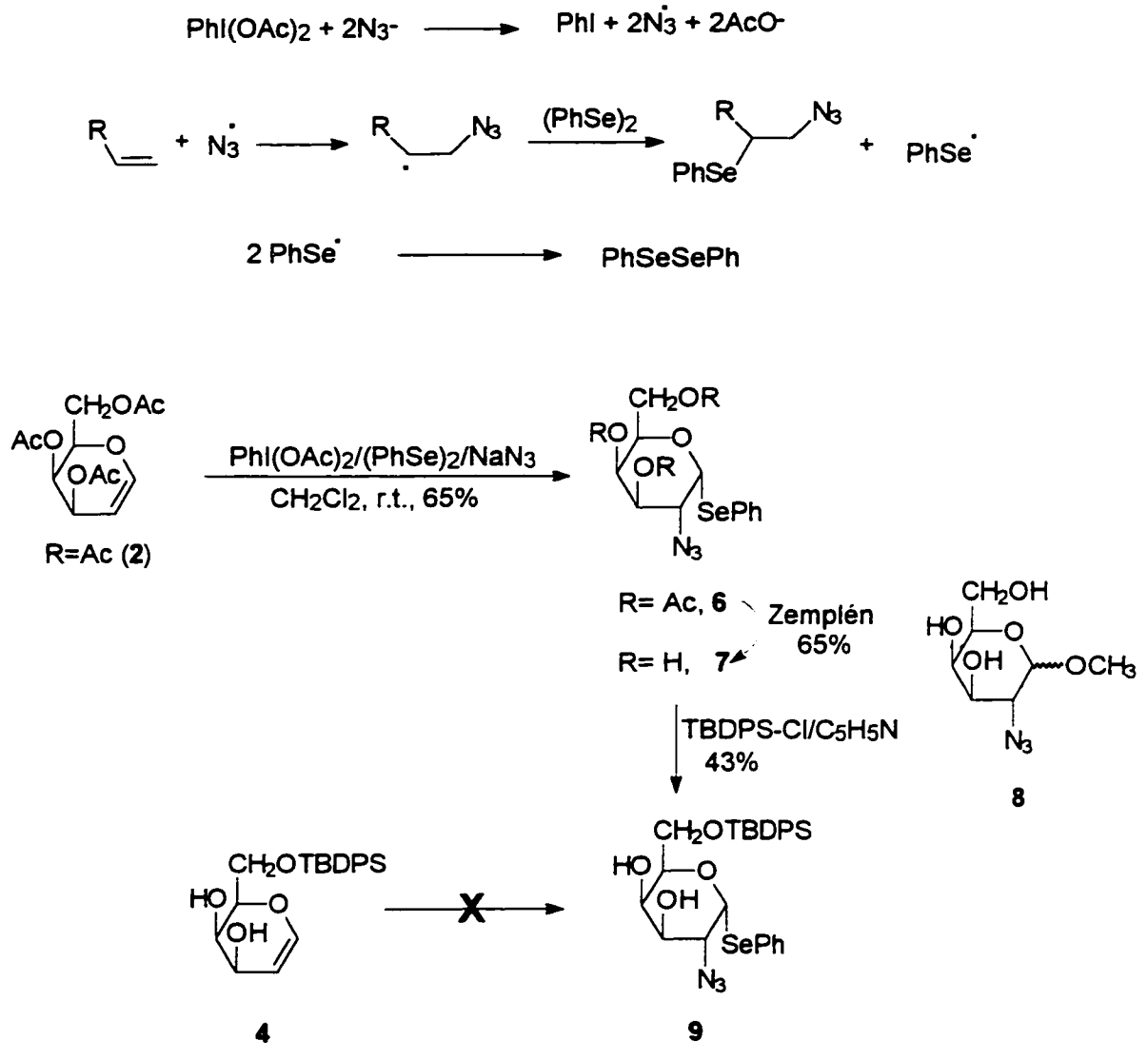
dimethylacetal in the presence of cat. *p*-toluenesulfonic acid monohydrate at room temperature. A plethora of products were obtained, one of which was recognizable from its ^{1}H-NMR spectrum as fully protected galactal **5'**. Treatment of **3** with benzaldehyde and anhydrous zinc chloride showed many unknown side products and the separation was difficult.

2.2.2. Azido Phenylselenylation of galactal

2-Amino-2-deoxy-glycosides are important compounds that are widely distributed in living organisms where they constitute building blocks of oligosaccharides.[115] They play important roles as ligands for protein molecules such as enzymes[116], antibodies[117] and lectins[118] and involve antigen-antibody recognitions.[119] The significant roles of these compounds have stimulated numerous synthetic methods for their preparations by simple and efficient procedures.[120, 115-c] 2-Azido-2-deoxy mono-and disaccharide derivatives are often intermediates in the synthesis of 2-amino-2-deoxy oligosaccharides. The azido function is nonparticipating group and does not cause steric hindrance for glycosylation. Therefore it can be used as an amino protecting group for the glycosyl donor.

The azido derivatives have been prepared by azidonitration of glycals.[109, 121] The 2-azido-1-nitrate adducts obtained could be transformed into various glycosyl donors by the displacement of nitrate to halide,[79] trichloroacetimidates[77] and S-xanthates.[121-a] However one-step synthesis of 2-azido-2-deoxy glycosides bearing a leaving group with good glycosylating properties is highly desirable. The azido-phenylselenylation of alkenes has been introduced by Pinto *et al.*[124], van Boom *et al.*[125] and Tingoli *et al.*.[94-b] With unsymmetrical olefins, the regioselectivity can be controlled by using electrophilic phenylselenium species (PhSeCl) and azide ions. In this case, Markovnikov adducts were obtained.[122-a, 123] Anti-Markovnikov addition to olefins can be accomplished with sodium azide and diphenyldiselenide in the presence of (diacetoxyiodo)benzene which oxidizes the azide ions into radical (Scheme 2-6).[94-b, 126-128] The free radical reaction of **2** with NaN_3 (11 eq.) and PhSeSePh (3.3 eq.) initiated by $PhI(OAc)_2$ was performed in dry CH_2Cl_2 at room temperature. After aqueous work-up and recrystallisation from Hexane/EtOAc (7/1)

enantiomerically pure **6** was obtained in 65% yield. It is a known compound and the physical data were in agreement with reported values.



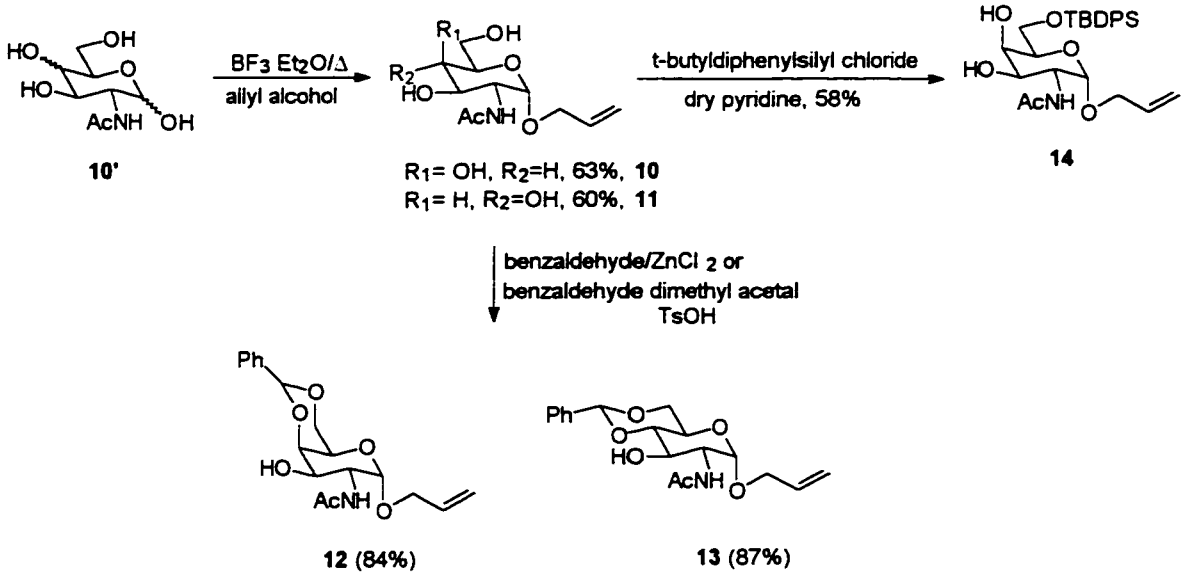
Scheme 2-6. Reaction mechanism of azido-selenylation of protected D-galactal.

Because only one face is hindered by acetyl group, equatorial azido addition at C-2 was obtained. Given this, the reaction gave a regio- and stereo-specific process.[94-c, 122] Deprotection of **6** was achieved under Zemplén condition to afford 65% yield of **7** as a yellowish powder. Unfortunately, a considerable amount of by-product resulted from the exchange of phenylselenyl by O-methyl group. Selective silylation at C-6 position was then performed and compound **9** was obtained in 43% yield. Another azidonitration reaction was tried from **3** but was not succesful. The structural assignment of phenyl 2-azido-2-deoxy-6-t-butyldiphenylsilyl galactosyl selenide (**9**) was established from ^{1}H-NMR, IR and MS spectroscopy. The signal for the anomeric proton at 5.86 ppm ($J_{1,2}$ = 5.3 Hz) indicated a 1,2-cis configuration. IR spectrum showed a typical azide absorption signal at 2110 cm^{-1}. (+) FAB-MS also showed a molecular ion peak at 584.18 (M+1, 7.1%).

2.2.3 Allyl Glycoside

By virtue of their versatile alkene function, allyl glycosides have been used for the synthesis of oligosaccharides and glycoconjugates.[129] This aglycon is also suitable to elongate spacer length and introduce other functionalities such as amino-, acrylamido-, hydroxy and carboxylic groups. These will be discussed in the next chapter.

Allyl α-D-GalNAc (**10**) and allyl α-D-GlcNAc (**11**) were prepared in 63% and 60% yields respectively using Fischer glycosidation with allyl alcohol as a reagent and solvent and $BF_3{\cdot}Et_2O$ as a catalyst (0.1 eq.) followed by recrystallization from absolute EtOH (Scheme 2-7). To be ready for the β-(1-3) linked glycosylation, **10** and **11** were partially protected with t-butyldimethylsilyl or benzylidene groups and purified by silica gel chromatography to afford **12**, **13** and **14** in 84%, 87% and 58% yields respectively. Compounds **12** and **13** were prepared as follows: first, **10** (or **11**) were exposed to benzaldehyde in the presence of zinc chloride at room temperature. Precipitation from the mixture of aqueous NH_4Cl and hexane and successive washing with hexane and water gave **12** (or **13**). The second method involved using benzaldehyde dimethyl acetal with cat. p-toluenesulfonic acid monohydrate at room temperature.



Scheme 2-7. Synthesis of selectively protected allyl α-D-glycosides.

The ^{1}H-NMR spectra of glycosides **10~14** showed $J_{1,2}$ values between 3.5 and 4.0 Hz and have α-configuration at anomeric center. As a protecting group was introduced, the J values **12~14** were increased by 0.2, 0.3 and 0.4 Hz with respect to the corresponding unprotected one. The coupling constant values did not change before and after the selective protection indicating that no major conformatonal changes occured. The two H-a protons (CH_2-CH=) are diastereotopic and thusshowed different chemical shifts with multiple splitting pattern because of the geminal coupling interactions with another H-a, H-b and the long range coupling with H-c and H-d. Compound **12** showed a ddt splitting pattern (J_{gem} = 13.0 Hz, J_{ab} = 6.2 Hz and ^4J = 1.3 Hz). All reaction products were completely analyzed and representative chemical shift values are given in Table 2-1 and 2-2. From the ^{13}C-NMR spectrum, the anomeric carbon of **11** was shifted downfield from 95.7 to 101.0 ppm upon 4,6-benzylidene protection and the other carbons revealed large downfield shifts. However, only slightly shifted values were obtained for **12** except C-5 (70.5 to 63.0 ppm) and C-6 (60.8 to 69.3 ppm). The chemical shifts of the aglycon protons were kept almost constant in the ^{1}H- and ^{13}C-NMR spectra.

Table 2-1. Results from allyl glycosides and derivatives.

compd	yield (%)[a]	m.p. (°C)	$[\alpha]_D$[b]
10	63	190~193	+194.8° (1.0, MeOH)
11	60	168~169	+148.8° (1.6, H_2O)
12	84	221~222	+132.0° (1.0, $CHCl_3$)
13	87	233~236	+96.0° (1.0, DMSO)
14	58	amorphous	+46.5° (2.1, $CHCl_3$)

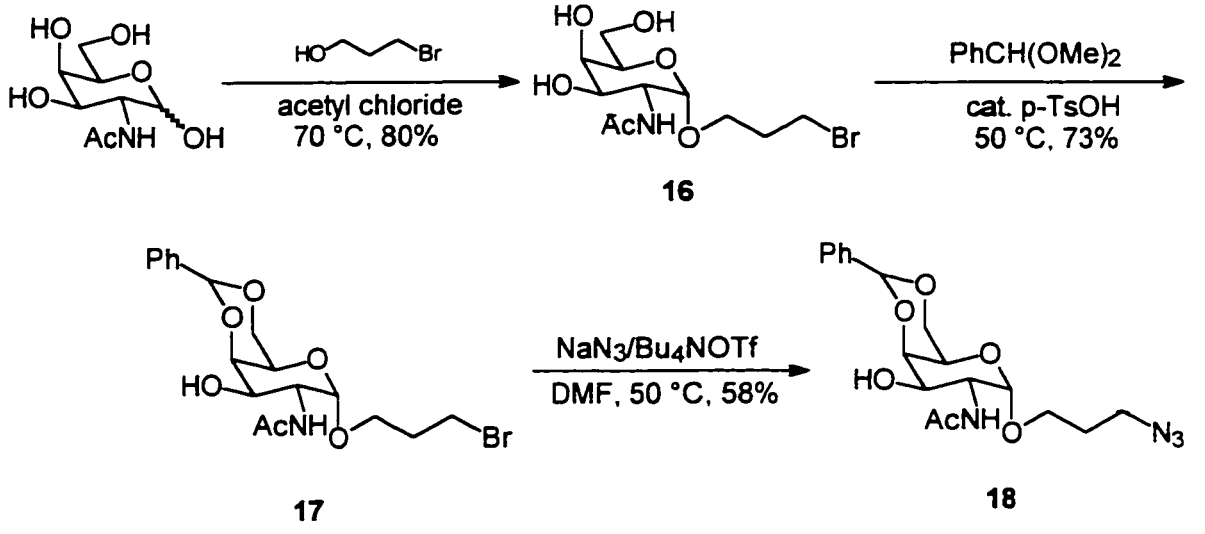
a: isolated yields.
b: the values in parenthesis are for concentration and solvent.

Table 2-2. ^{13}C-NMR Chemical shifts of O-allyl glycosides and derivatives.

compd	C-1	C-2	C-3	C-4	C-5	C-6	C-a	C-b	C-cd
10	95.8	49.4	67.2	68.5	70.5	60.8	68.0	133.2	117.4
11	95.7	53.2	71.5	69.6	70.6	60.1	68.0	133.2	117.5
12	97.5	50.3	69.0	75.5	63.0	69.3	68.4	137.4	117.8
13	101.0	54.3	97.0	67.4	82.1	67.7	68.1	134.5	117.0
14	94.3	53.9	71.7	68.6	70.1	63.6	63.6	hidden	117.9

2.2.4 3-Azidopropyl glycoside

The introduction of a non-participating azido group at the C-2 position takes advantage of the efficient synthesis of glycosamine derivatives. If the spacer having amino group like 3-aminopropyl exists at anomeric position, an alternative synthetic strategy is necessary. van Boeckel *et al.*[131] designed the glycosidation of galactosamine using the Fischer method.[130] This method is efficient when the glycosyl acceptor (alcohol) is used as solvent. Since 3-azidopropanol could not be used as a solvent, 3-bromo-1-propanol was chosen (Scheme 2-8).



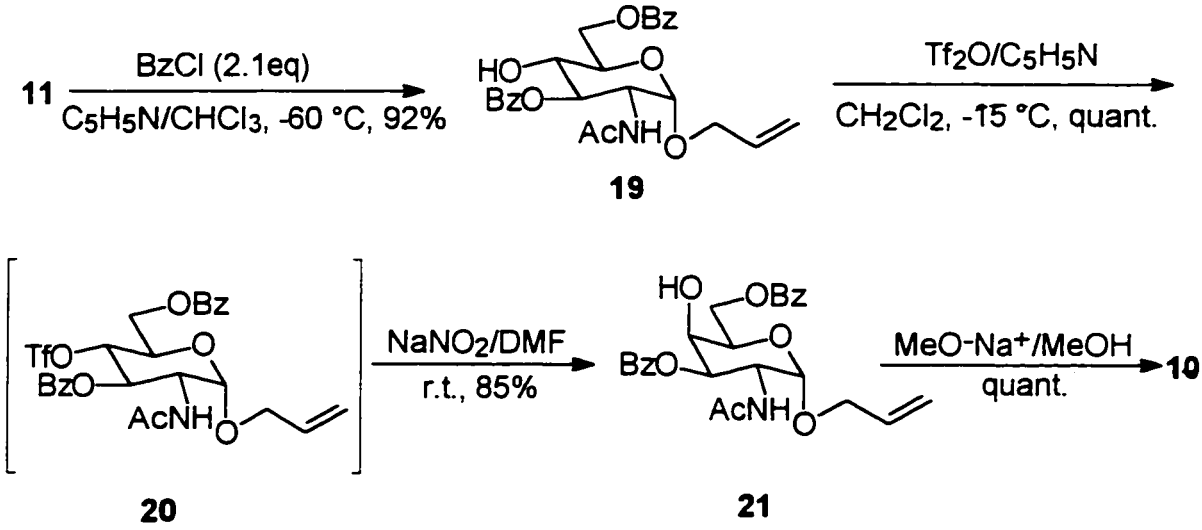
Scheme 2-8. Synthesis of 3-azidopropyl galactosamine, **18.**

The mixture of **15** and 3-bromo-1-propanol was heated under acidic conditions and **16** was isolated by silica gel chromatography in 80% yield. With an appropriate protection of compound **16** and compound **17** having a free OH at C-3 was obtained (73%) and azido group was introduced by treatment with NaN_3 and tetrabutylammonium triflate (Bu_4NOTf) in DMF to give **18** in 58% yield.

2.4. Epimerization of allyl α-D-N-acetyl glucosaminide to allyl α-D-N-acetyl galactosaminide

N-Acetyl galactosamine derivatives can be prepared from the corresponding N-Acetyl glucosamine epimer at C-4. Lattrel and Lohaus[132] isolated an alcohol with inverted configuration from the chiral secondary sulfonate with potassium nitrate. The compatibility of an ester group with this reaction condition was demonstrated in the synthesis of prostaglandins and steroids.[133] In view of these results, Dax *et al.*[134]

broadened the scope of this reaction to include the carbohydrate field and a D-glucose derivative was prepared. Trifluromethanesulfonate was used instead of 4-chlorobenzene sulfonate and toluene-*p*-sulfonate because of its better reactivity as a good leaving group in a nucleophilic substitution reaction.[135] Recently Lee *et al.*[136], Khan *et al.*[137] and Nashed *et al.*[138] prepared epimerized galactosamine derivatives in good yields. We extended this reaction to **11** for the epimerization at C-4 (Scheme 2-9).



Scheme 2-9. Epimerization of allyl α-D-GlcNAc to the corresponding allyl α-D-GalNAc derivative.

Selective 3,6-dibenzoylation of **11** with benzoyl chloride (2.1 eq.) in py/$CHCl_3$ (4/1, v/v) at -60 °C was accomplished to afford **19** in 92% yield along with a trace amount of tri-benzoylated **20**. Inversion of configuration at C-4 was carried out.[134] In brief, **19** was reacted with trifluoromethanesulfonic anhydride (Tf_2O) in C_5H_5N/CH_2Cl_2 to obtain the 4-triflyl intermediate (**20**) which was subsequently displaced with nitrite anions in DMF. During the aqueous work-up, the nitrite ester group was hydrolyzed to afford **21** in 85% yield. Deprotection under Zemplén conditions afforded **10** in quantitative yield. This compound was consistent with the other one prepared from Fischer glycosidation (Table 2-3).

Table 2-3. Physical properties of **10**, **19** and **21**.

compd	yield (%)	m.p. (°C)	$[\alpha]_D$	δ_{H-4}, ppm (J_{34})	δ_{C-4}, ppm
10	63	190 ~ 193	+194.8 (1.03, MeOH)	4.05 (3.3)	68.1
19	92	57 ~ 58	+81.9 (1.05, $CHCl_3$)	3.84 (9.2)	69.0
21	85	60 ~ 60	+80.6 (1.60, $CHCl_3$)	4.21 (2.9)	71.4

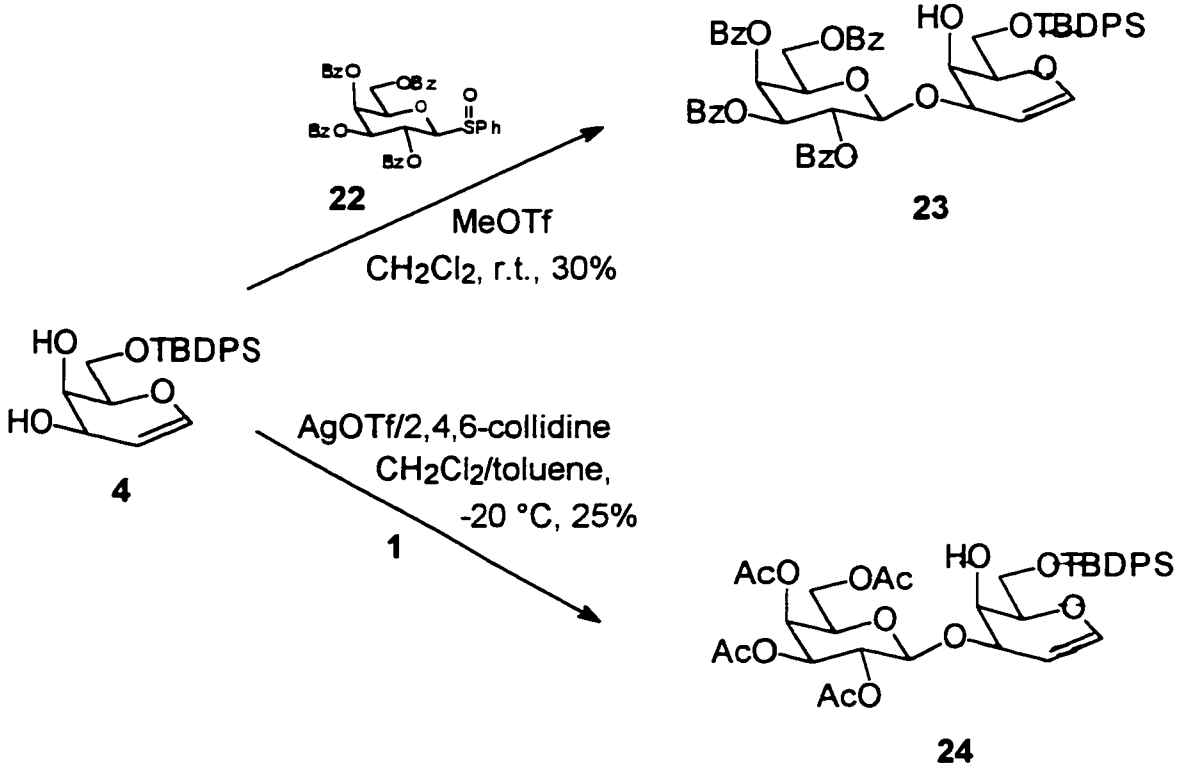
H-4 chemical shifts in **19** and **21** were 3.84 and 4.21 ppm, respectively and J_{34} values showed significant big differences. **19** showed a trans axial-axial coupling interaction between H-3 and H-4 with 9.2 Hz. However, **10** and **21** exhibited cis axial-equatorial interaction with 3.3 and 2.9 Hz. For ^{13}C-NMR, the chemical shifts for C-4 in **19** and **21** were obtained 69.0 and 71.4 ppm respectively. Accordingly, we judged that epimerization was performed successively. This reaction was very useful for providing a large amount of D-GalNAc from cheap and readily available D-GlcNAc using a straightforward procedure.

2.5. Glycosylation Strategy toward β-D-Gal-(1-3)-α-D-GalNAc (T-antigen)

2.5.1. The formation of β-(1-3) linkage using silyl glycoside

Given carbohydrate chemists' interest in the synthesis of glycals, glycosylation strategies where glycals are key building blocks for the synthesis of oligosaccharides have been investigated.[139, 140] It was already demonstrated that glycals have considerable advantages for complex carbohydrate synthesis.[141] Specifically, the selective exposure of a particular hydroxyl group on a glycal to serve as an acceptor is more straightforward than is the case in fully oxygenated pyranose derivatives. Furthermore, there are a number of mild and efficient methods for the activation of glycals as glycosyl donors. These strategies can be viable to provide various kinds of glycosidic linkages.[142]

Selectively protected hydroxyl group of partially protected galactal derivative **4** was used as a acceptor (Scheme 2-10). Glycosylation of sulfenylgalactoside **22** (1.2 eq.) with glycosyl acceptor **4** was performed in the presence of methyl triflate (MeOTf) in dry CH_2Cl_2 at room temperature to give the β-(1-3) linked disaccharide **23** in 30% isolated yield.



Scheme 2-10. Synthesis of β-(1-3) linked disaccharides.

Alternatively, **4** was glycosylated with peracetylated galactosyl bromide as a ligand and silver triflate (AgOTf)/2,4,6-collidine as a promoter (CH_2Cl_2/toluene at -20 °C) to afford **24** in 25% yield. In both reactions, molecular sieves (4 Å) were used to ensure strictly anhydrous conditions. The structural assignments of **23** and **24** were as follows. The β-linkage was readily assigned from the ^{1}H-NMR spectra which showed a doublet for H-1′ at 5.03 ppm ($J_{1,2'}$ = 7.6 Hz) for **23** and 4.62 ppm ($J_{1,2'}$ = 8.0 Hz) for **24**. The ^{13}C-NMR spectra also revealed the anomeric carbons of **23** and **24** at 100.8 and 100.3 ppm, respectively. The regioselectivity for the newly introduced glycosidic linkage was also

determined by ^{1}H-NMR spectroscopy. The H-3 signals for **23** and **24** showed a down field shift from 4.11 to 4.54 ppm and 4.56 ppm, respectively. In addition, the chemical shifts for C-3 were recorded from 67.1 ppm to 98.2 ppm for **23** and 98.1 ppm for **24**. The mass spectrum gave a molecular ion peak at 715.3 (M+1, 0.9%) for **24** but no spectrum was available for **23**.

Bay *et al.*[143] have accomplished the glycosylation of unprotected D-galactal using enzymatic method in 42% yield. On the other hand, Danishefsky *et al.*[140, 141, 145] recently applied this concept to the stereoselective glycosylation reaction of glycals in the presence of different promoters such as $AgClO^4/SnCl_2$,[141-b,c] $AgBF_4$,[141-b] AgOTf[144] and $ZnCl_2$[141-b, 145] depending on the glycosyl donors used.

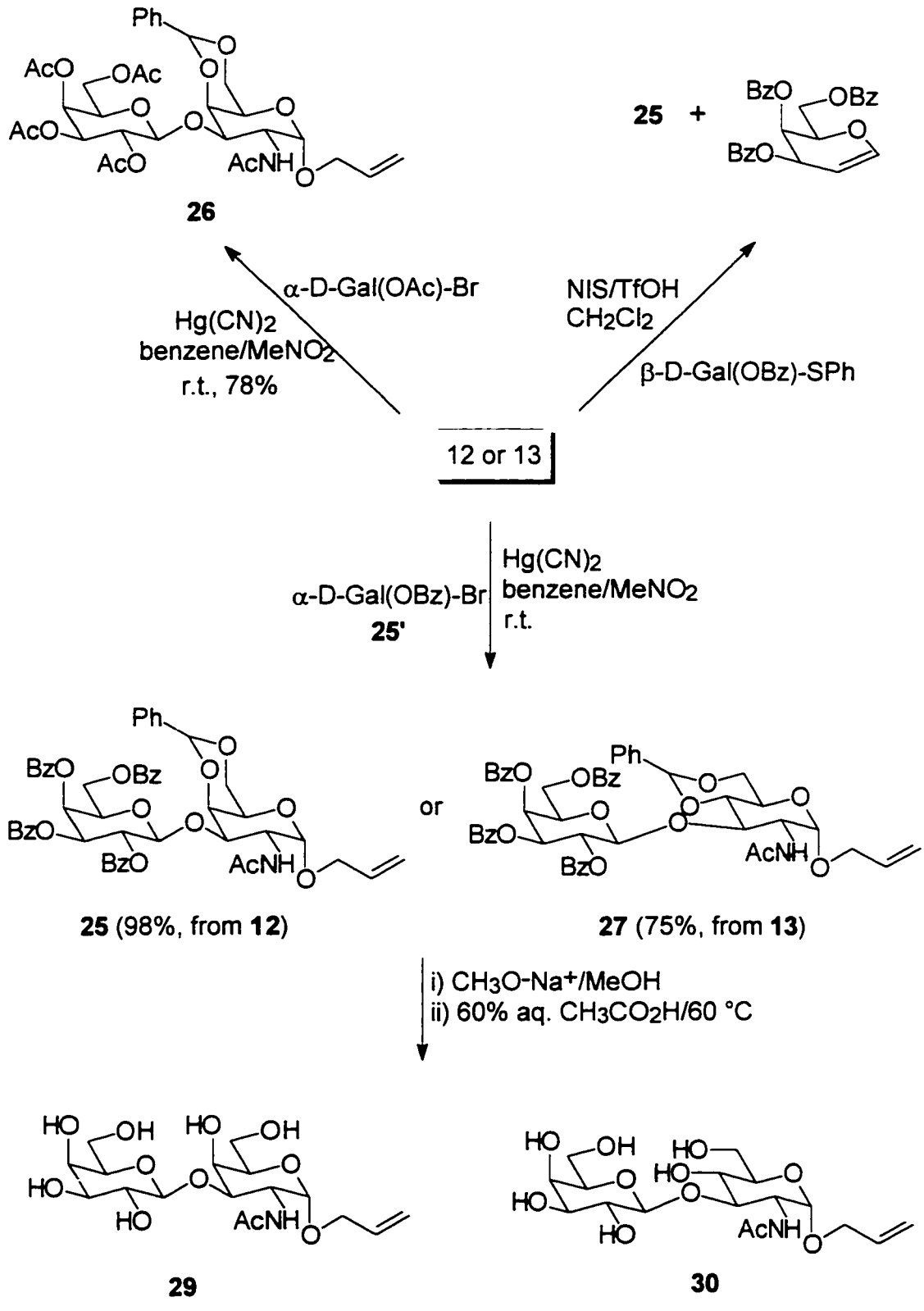
Compounds **23** and **24** were important intermediates to afford disaccharide, β-D-Gal-(1-3)-α-D-GalNAc (T-antigen) known to be a tumor-associated marker[146] and to synthesize more complex oligosaccharides or glycoconjugates. For instance, the double bond in galactal can be functionalized by azidonitration, glycosidation at the anomeric position and azide reduction to give a T-antigen derivative. Other glycosylation methods were attempted to improve the efficiency using phenyl 2-azido-2-deoxy-6-O-silyl-galactosyl selenide **9** as an acceptor with TfOH/NIS, AgOTF or MeOTf depending on the glycosyl donors (benzoylated thiophenyl galactoside or peracetylated galactosyl bromide). None of these were successful even under strictly controlled temperature from -76 °C to room temperature. Generally, silyl ethers such as t-butyldimethylsilyl and t-butyldiphenylsilyl groups can be eliminated using a variety of reagents, tetrabutylammonium fluoride,[147-a] $BF_3{\cdot}Et_2O$,[147-b] alkali metal tetrafluoroborate[147-c] and aqueous hydrofluoric acid.[147-d] The failure of coupling may be due to the poor stability of the silyl protecting group under the given conditions.

2.5.2. The formation of β-(1-3) linkage using allyl glycosides

The reactivity of glycosyl acceptors is approximate by in the order OH-6 > OH-3 > OH-2 > OH-4 for D-galacto and D-glucosides and is controlled by the protecting groups (steric and electronic effects). 4,6-Benzylidene protected **12** and **13** are satisfied for the β-

(1-3) linkage under the given reaction conditions. Having prepared the well-designed glycosyl acceptors, a series of coupling reactions of thioglycosides or protected glycosyl halides as donors with properly protected glycosyl acceptors **12** and **13** were performed using mercury salt at room temperature or NIS/TfOH at -30 °C (scheme 2-11). To create the anhydrous condition, **12** (or **13**) was dissolved in a mixture of benzene/nitromethane and coevaporated 3 times, once dried, twice the volume of solvent was used and reduced to less than half. Molecular sieves (4 Å) were then added. After addition of mercuric cyanide (1.5 eq.), the reaction was continued overnight at room temperature under heterogeneous conditions. The reaction was completed as judged by T.L.C. and aqueous work-up and purification by silica gel chromatography gave a white foamy solid in excellent yield (98%). The reaction was repeated with peracetylated galactosyl bromide to give **26** in 78% yield.[148] Since the glycosylation was successful, this strategy was applied to another disaccharide synthesis, i.e., β-D-Gal-(1-3)-α-D-GlcNAc derivative **27** to afford 75% yield.

One of the disadvantages in the Koenigs-Knorr method is that glycosyl halides exhibit low thermal stability and are highly susceptible to hydroysis. To overcome this drawback, thioglycosides were considered because of their good leaving groupability and high stability in many organic operations. Up to now, several different kinds of alkyl- and arylthio groups, including the heterocyclic thioglycosides groups, were developed[149] and thiophenyl derivative was chosen. The glycosylation reaction under the anhydrous condition was performed between benzoylated S-phenyl galactoside bromide and **12** using NIS/TfOH at-30°C. After a short time period, several side reactions were observed as judged by T.L.C. before the limiting agent (acceptor) was completely consumed. Two major adducts were isolated and assigned the structure **25** (14% yield) and 3,4,6-tribenzoyl galactal. On the basis of these results, the classical Helferich glycosylation method was excellent for the T-antigen synthesis with mild reaction condition and almost quantitative yield.



Scheme 2-11. Synthesis of β-D-Gal-(1-3)-α-D-GalNAc (**29**) and β-D-Gal-(1-3)-α-D-GlcNAc (**30**)

Table 2-3. Results of ^{1}H-NMR data of **25**,**27**, **29** and **30**.[a]

Residue	Proton	Chemical shift, ppm (coupling constant, Hz)[b]			
		25	**27**	**29**	**30**
GalNAc[c]	H-1	5.10 (3.4)	4.48 (3.7)	5.01 (3.8)	4.98 (3.6)
(GlcNAc)	H-2	4..61	4.29	4.41 (11.2)	4.18 (10.6)
	H-3	4.11	4.08	4.10	4.01
	H-4	4.41	3.88	4.29	3.67 (8.7)
	H-5	3.51	3.93 (11.1)	4.10	3.84 (12.1)
	H-6	4.11	4.38 (5.6)	3.68~3.78	3.91~3.76
		3.75 (12.4)	4.29		
Gal	H-1'[d]	5.16	5.10 (8.0)	4.53 (7.8)	4.51 (7.8)
	H-2'	5.72	5.67 (10.3)	3.59 (10.0)	3.59 (9.9)
	H-3'	5.60 (3.4)	5.48 (3.5)	3.69 (3.4)	3.72 (3.4)
	H-4'	5.98 (0.9)	5.88 (3.5)	3.98 (0.8)	4.03
	H-5'	4.41	3.88	3.72	3.91~3.76
	H-6'	4.68 (11.4)	4.29	3.86~3.78	3.94 (2.0)
		4.41	3.78 (10.2)		3.91~3.76

a: Recorded on a Brucker AMX-500 MHz spectrometer at ambient temperature and solvent.

b: Assignments are based on COSY and HMQC experiments. Chemical shifts are referred to internal $CHCl_3$ at 7.24 ppm for **25** and **27** and D_2O at 4.63 ppm for **29** and **30**. Spin-spin coupling constants appear in parenthesis.

c: GalNAc for **25** and **29** and GlcNAc for **27** and **30**.

d: The signal is partially hidden.

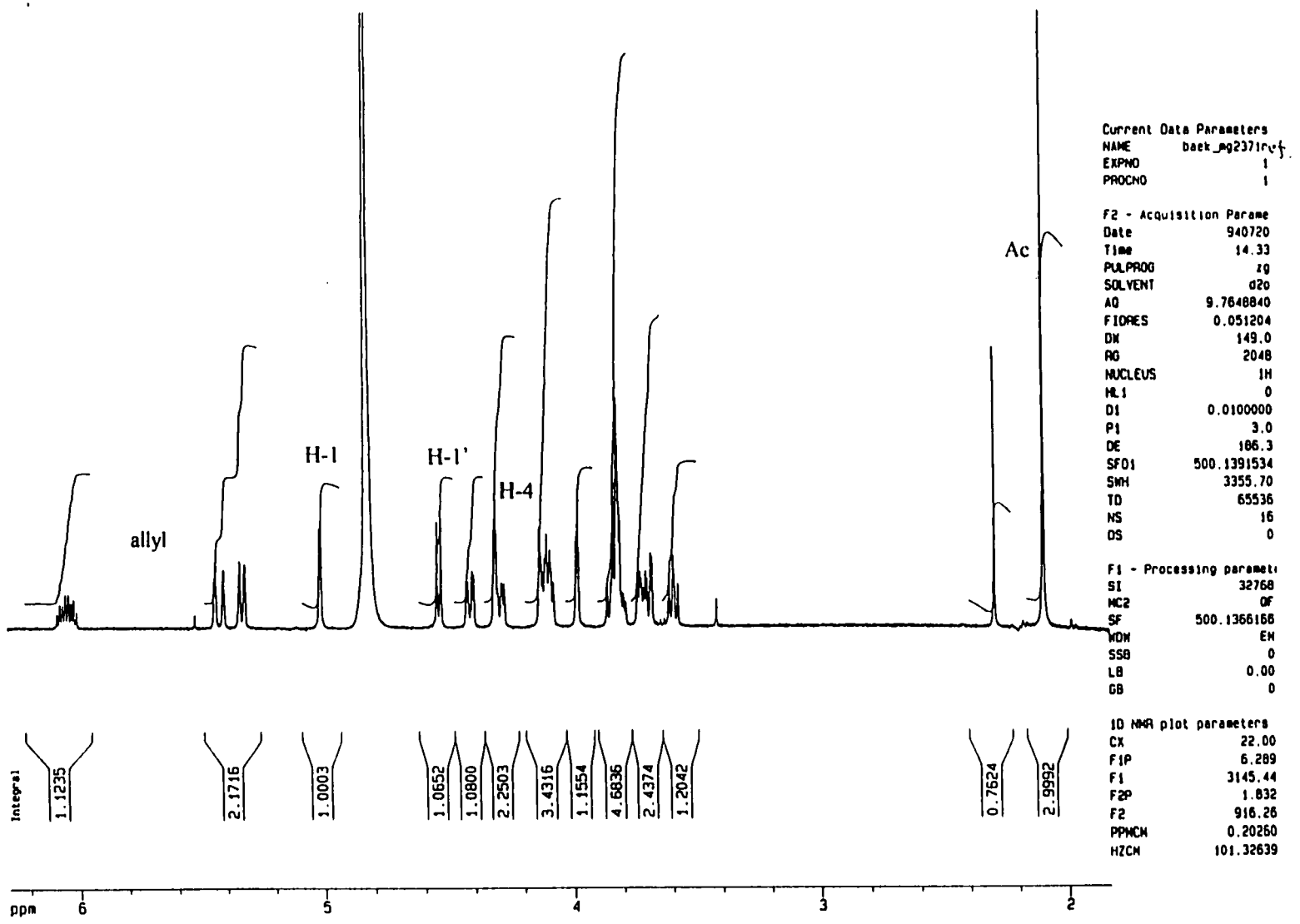


Fig. 2-1. ^{1}H-NMR Spectrum (500 MHz, D_2O) of disaccharide **29.**

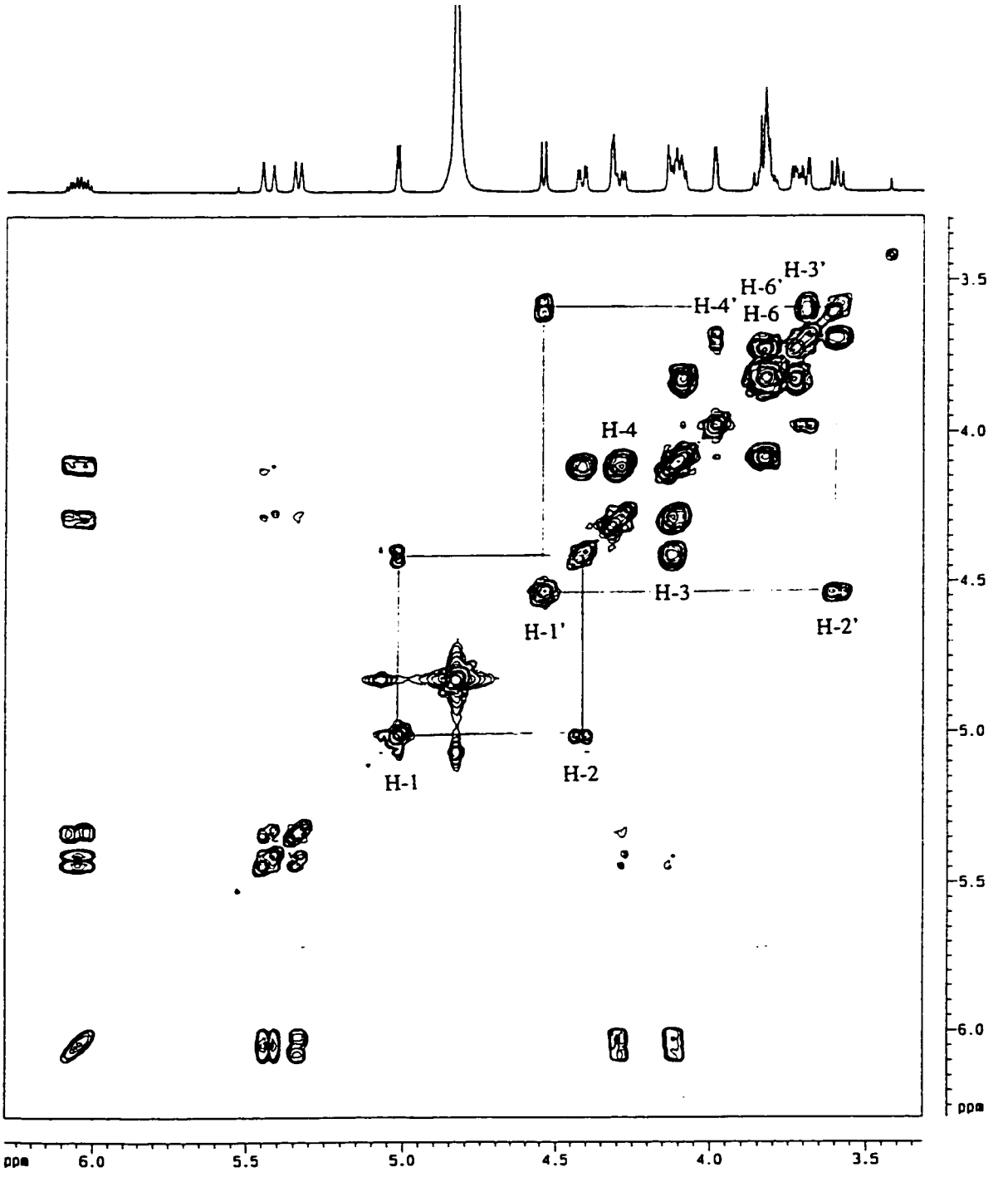


Fig. 2-2. ^{1}H-^{1}H COSY Spectrum (500 MHz, D_2O) of disaccharide **29.**

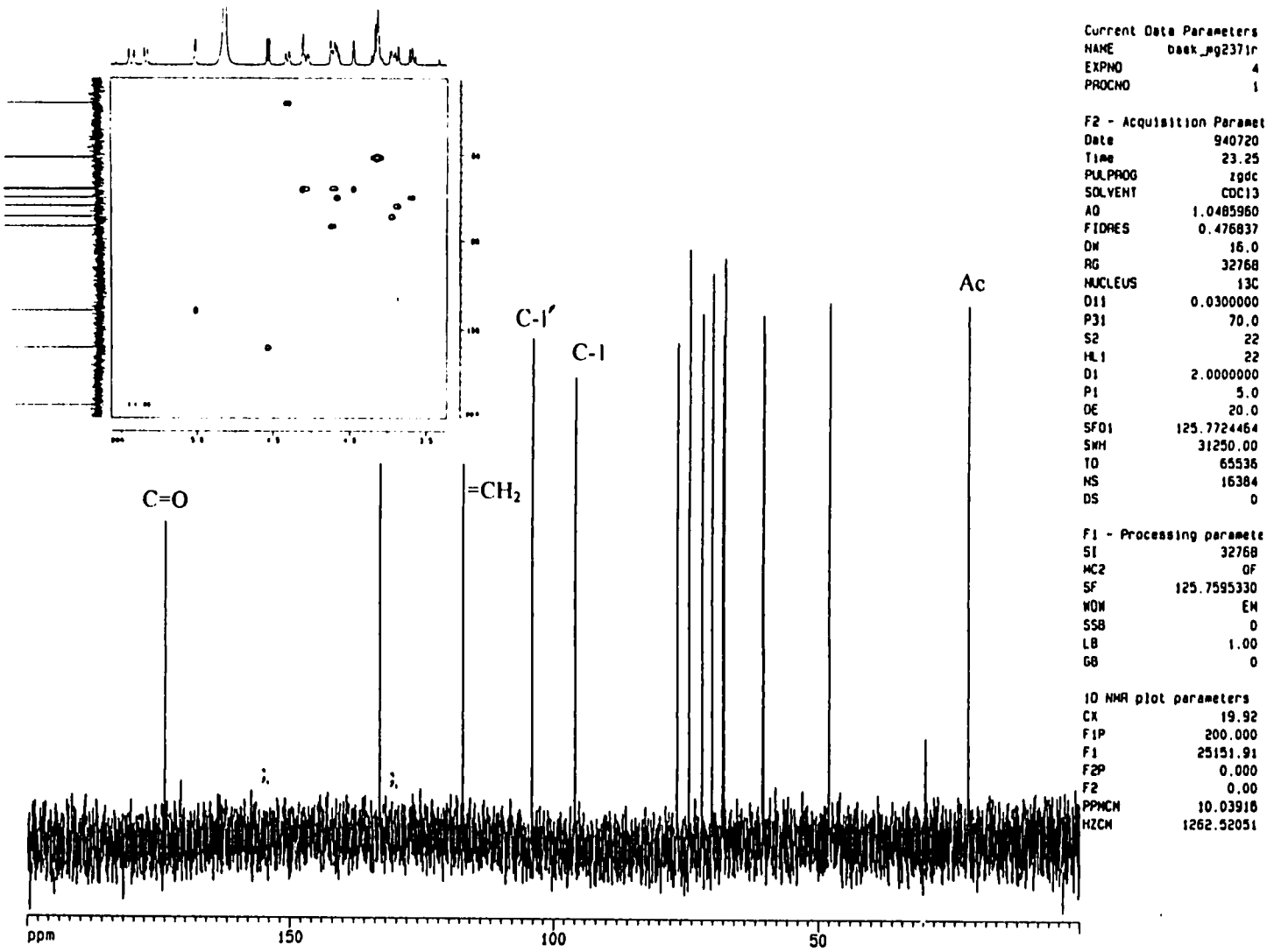


Fig. 2-3. ^{13}C-NMR and ^{1}H-^{13}C-HMQC (500 MHz, D_2O) of disaccharide **29.**

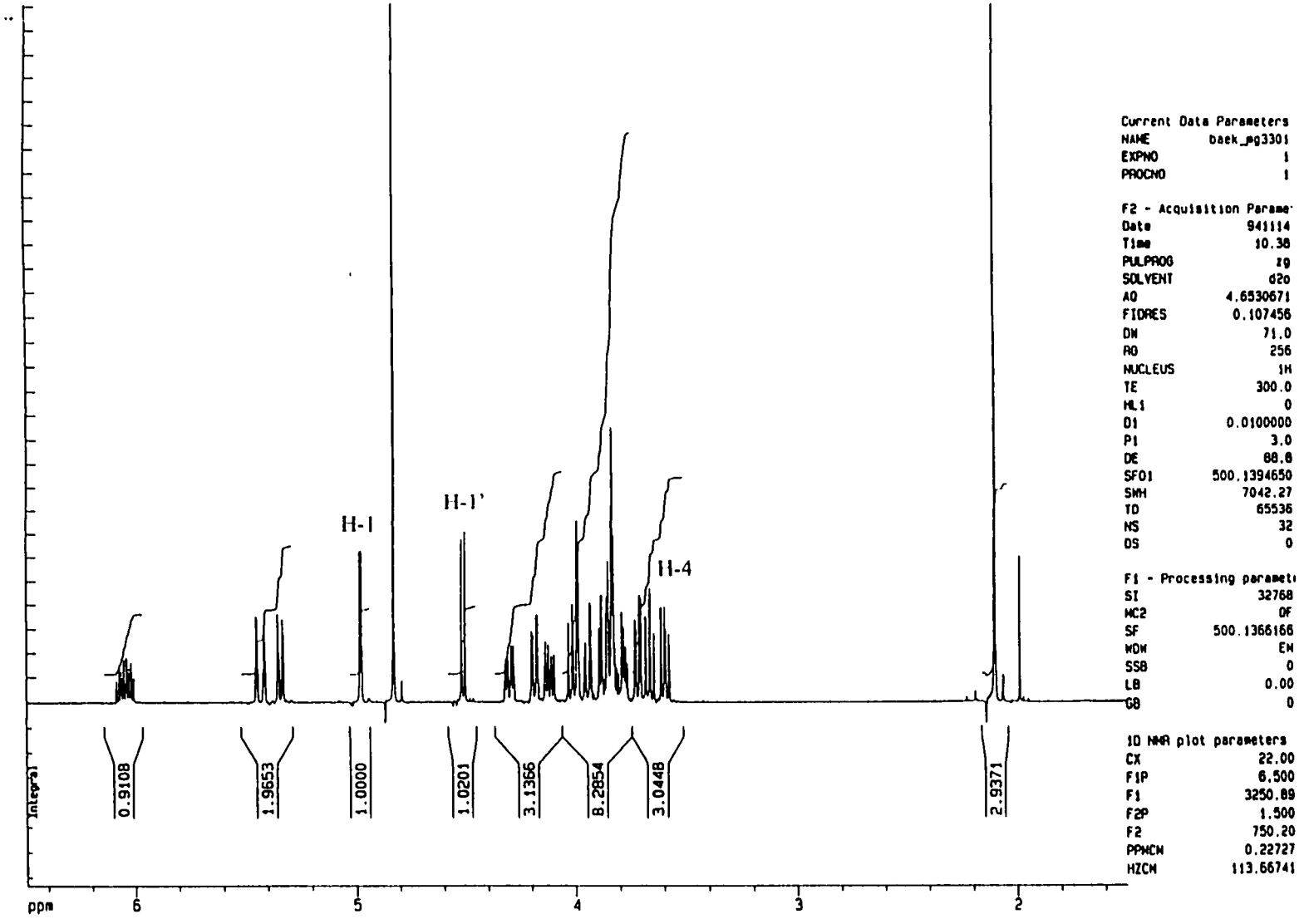


Fig. 2-4. ^{1}H-NMR Spectrum (500 MHz, D_2O) of disaccharide, **30.**

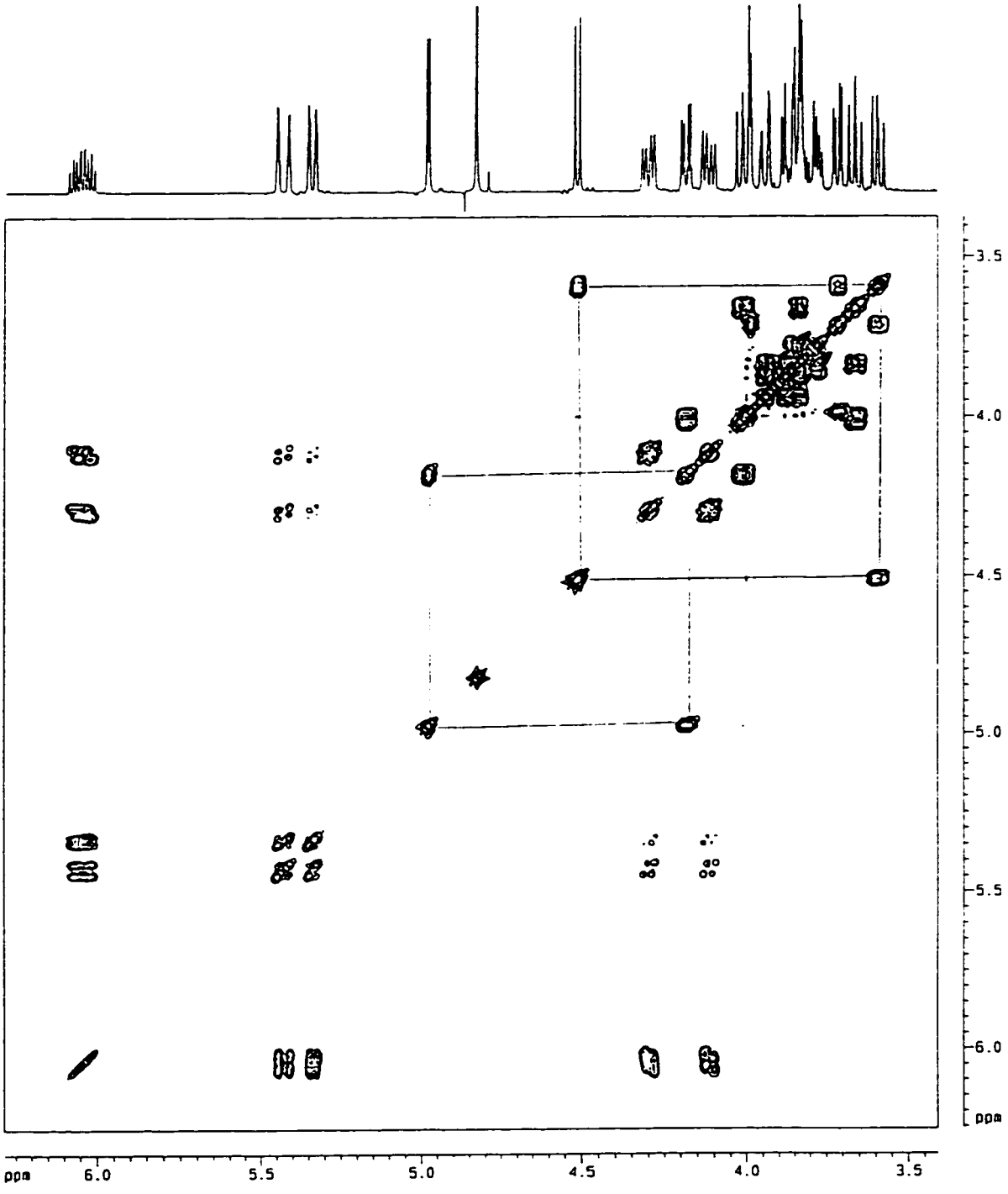


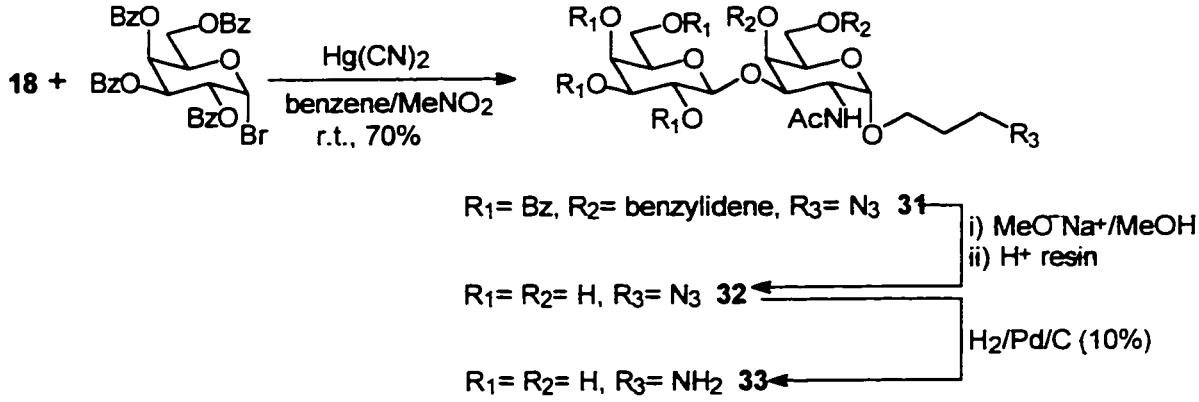
Fig. 2-5. ^{1}H-^{1}H COSY Spectrum (500 MHz, D_2O) of disaccharide **30.**

The manipulation of **25**, **26** and **27** was performed as follows: De-O-benzoylation or de-O-acetylation were accomplished under Zemplén condition to afford **28** in quantitative yield. **28** was treated with 60% aqueous acetic acid at 60 °C for 1.5 h. After silica gel chromatography and lyophilization, a white spongy solid **29** was obtained in 93% yield. The same procedure was applied for **27** to obtain **30**. The use of excess H^+ resin after Zemplén was chosen as a viable process to yield the same result. **29** and **30** can undergo many glycosidation reactions and these will be discussed in the next chapter. The ^{1}H-NMR chemical shifts and coupling constant values are summarized in Table 2-3. The structures were assigned in a similar manner as follows. Significantly, β-(1-3) linkage at C-1′ of **25** was readily assigned by J_{12}′ (8.0 Hz) at 5.16 ppm. **27** was also shown to have a similar coupling pattern at 5.10 ppm with the same J value. The α-linked anomeric proton at C-1 was assigned at 5.10 ppm (3.4 Hz) for **25** and 4.84 ppm (3.7 Hz) for **27**. Interestingly, two sets of geminal H-6 and H-6' protons in these compounds were detectable between 4.68 and 3.75 ppm and each proton was distinguishable from another. The J values of **25** were detectable such as 12.4 Hz for H-6 and 11.4 Hz for H-6′. As a benzylidene protecting group is introduced at C-4 and C-6, the molecule becomes more rigid so that the relative positions of the H-6 protons are fixed in different space. Therefore the stereochemistry of these protons is not equivalent any more. This explanation can be applied to H-6′ protons with the steric effect of the benzoyl substituents. For **29** and **30**, two different chemical shift patterns were shown when compared with the corresponding protected ones. First, The ^{1}H-chemical shift values do not change significantly, and even demonstrate the constant values. On the contrary, the galactosyl glycon has a downfield shift in the range of 0.7~2.2 ppm. Because the relatively weak electronic effect of the benzylidene group does not shield the protons in GalNAc or GlcNAc stay in the same positions. However, removal of the strong electron withdrawing benzoyl group which brings more deshielded environment reveals an upfield shift.

Peracetylation of **29** and **30** with excess of acetic anhydride in dry pyridine at room temperature gave 99% of **36** and **37**. The structual assignment was done based on ^{1}H- and ^{13}C-NMR and eight acetyl groups including N-acetyl group were observed.

2.5.3 The formation of β-(1-3) linkage using 3-azidopropyl glycoside

As we have azido-functionalized **18**, glycosylation was performed according to the Helferich method. For the synthesis of the β-interglycosidic bond in the required β-D-Gal-(1-3)-α-D-GalNAc-O$(CH_2)_3N_3$ disaccharide, benzoylated galactosyl bromide was used as a glycosyl donor to give **31** in 70% yield[131] (scheme 2-12).



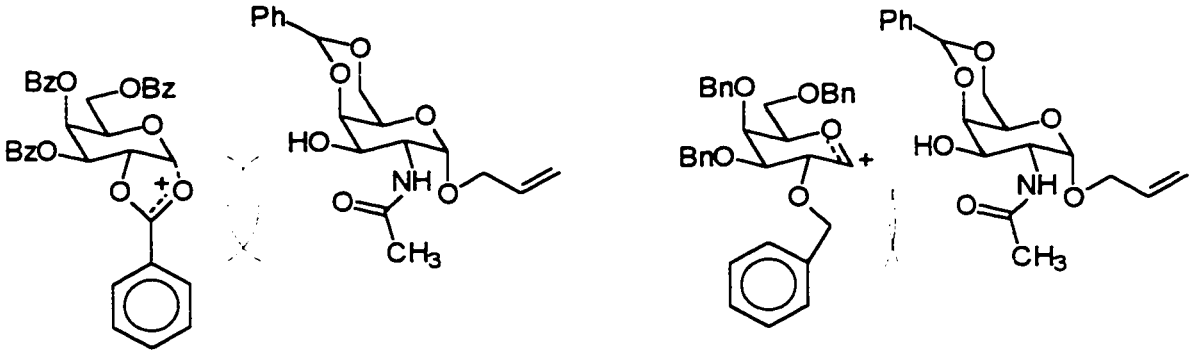
Scheme 2-12. Synthesis of β-D-Gal-(1-3)-α-D-GalNAc-O$(CH_2)_3N_3$ **33**.

Subsequent deprotection of **31** and lyophilization gave **32** in 90% yield. Reduction of the azido function in **32** in the presence of cat. Pd/C (10%) and H_2 bubbling afforded **33**. This process was straightforward with good yields and provided an amino functionalized T-antigen derivative. The structure of **31** was determined using ^{1}H- and ^{13}C-NMR spectra, particularly on the basis of the two anomeric protons. The signals of H-1 and H-1′ were shown at 5.14 ppm (7.6 Hz, β-configuration) and 5.10 ppm (3.3 Hz, α-configuration), respectively. Other results were consistent with the proposed structure (see experimental part). For the protons of -OCH_2 at C-1, H-6 and H-6′, two protons in each set were not chemically equivalent and had different chemical shifts. Interestingly, the methylene protons beside the azido group also showed the same tendency with signals at

3.30~3.27 ppm (dt, $J_{cc'}$ = 2.3 Hz, J_{bc} = 6.4 Hz). In ^{13}C-NMR, two anomeric carbons were readily detectable at 102.1 (C-1') and 98.0 (C-1) ppm. In the mass spectral analysis, all three compounds **31, 32** and **33** showed positive molecular ion peaks (M+1)+ at 985.4 (3.2%), 467.3 (17.3%) and 441.2 (3.7%), respectively.

2.5.4. The formation of β-(1-3) linkage using active donor

Glycosylation is affected by many factors such as solvent, neighboring group participation, promoters, and the nature of the leaving group at the glycosyl donor as was proven in the previous section. For example, the solvent directly affects the relative stabilities of the reactive intermediates and in some cases directly participates in the reaction.[115-c] In general, non-polar solvents favor the formation of α-glycosides and polar solvents favor β-glycosides.[150]



A (more hindered)

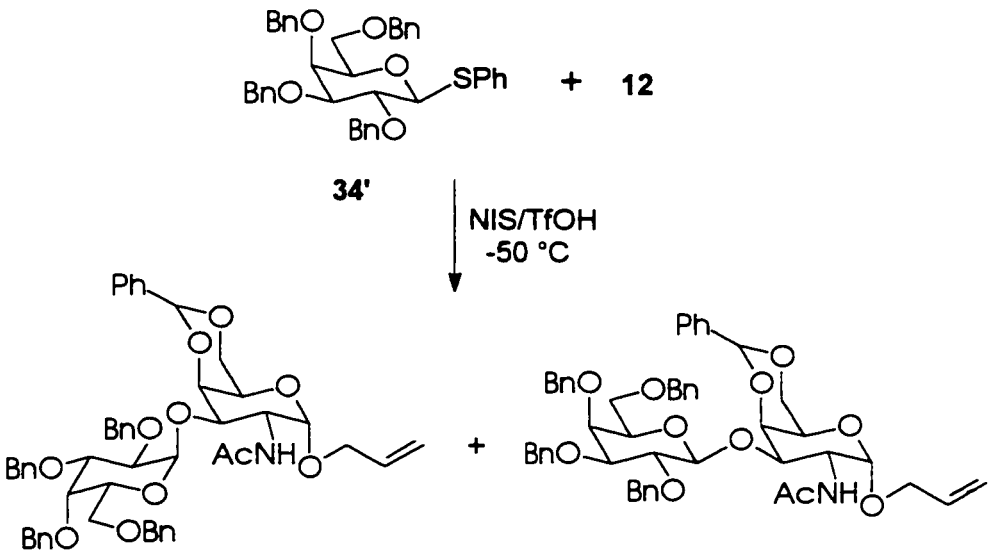
B (less hindered)

Fig.2-6. Steric hindrance in glycosylation.

When a bulky protecting group is involved in the glycosyl donor or acceptor or both, an important factor emerges namely the unfavorable steric interactions between protected donor and acceptor in the transition state.[151]. For instance, the benzoyl

protecting group at C-2 of the donor strongly participate in the formation of dioxolenium ion and prefers the formation of β-glycosides.

In our experience, although stereoselective β-linked disaccharide **25**, was obtained, one of the methods used to afford this product provided low yield (14%) possibly because of the proposed steric interaction between the dioxolenium ion of the donor and the N-acetyl group of the acceptor. To verify this postulate, phenyl 2,3,4,6-tetra-O-benzylated-1-thio-β-D-galactoside was chosen and the same reaction conditions employed in scheme 2-10 were applied in the glycosylation of **12**. The result gave 29% of α-(1-3)-linkedglycoside **34** and 21% of β-(1-3) glycoside **35** and the ratio of α/β was 1.33/1 (Scheme 2-13).



34 **35**

solvent	34 (α)	35 (β)	α/β ratio
CH_2Cl_2	29%	21%	1.33
CH_2Cl_2/toluene	26%	17%	1.53

Scheme 2-13. Glycosylation reaction using perbenzylated thioglycosyl donor.

The yield was considerably increased to an overall 50% yield. Here a rationale can be proposed. In the benzoylated glycosyl donor, dioxolenium state which blocks one side strictly leads to only β-configuration but there is also steric hindrance that prevents the approach of the acceptor for the coupling and a low yield resulted. However benzylated glycosyl donor opens both sides for the nucleophilic addition.. Therefore the unfavorable steric effect is considerably decreased thus making the approach of the counter part much easier. Because the anomeric center is opened on both sides, the α-and β-anomers were obtained. In brief, the solvent effect was briefly examined using toluene/CH_2Cl_2 (1/1) instead of CH_2Cl_2 itself and the same glycosylation reactions were performed. The mixture of α- (26%) and β-anomers (17%) was obtained and the ratio of α/β was 1.53/1. This result was consistent with the known solvent effect.[150, 151]

The structuresof **34** and **35** were assigned by ^{1}H- and ^{13}C-NMR spectra. Two anomeric protons were detected at 5.05 ppm ($J_{1,2'}$ = 3.8 Hz, H-1′) and at 5.03 ppm ($J_{1,2}$ = 3.5 Hz, H-1) for the α-anomer and at 5.13 ppm ($J_{1,2}$ = 3.5 Hz, H-1) and 4.54 ppm (H-1′) for the β-anomer. The chemical shifts of the anomeric carbons were also obtained at 97.4 ppm (C-1) and 96.6 ppm (C-1′) for **34** and 104.6 ppm (C-1′) and 97.5 ppm (C-1) for **35**. Other signals were properly assigned and well characterized (see experimental section). Mass spectra gave evidence of a common positive ion peak at m/z 827.4 (0.2 and 2.5% for **34** and **35** respectively) due to $(M+1)^+$.

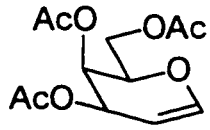
2.5. Conclusions

Several glycosylation strategies were successfully applied for the synthesis of β-D-Gal-(1-3)-α-D-GalNAc and β-D-Gal-(1-3)-α-D-GlcNAc derivatives. For the desired glycosylation, chemically well-defined acceptors were synthesized such as 1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol (D-galactal), azidoselenide, allyl glycosides and aminoalkyl glycosides and these were appropriately protected Epimerization of GlcNAc into a GalNAc derivative was performed. This procedure was useful to supply a large amount of GalNAc. β-Stereoselective glycosylation of the thio- or sulfenyl glycosides and glycosyl acceptors prepared was achieved using thiophilic promoters under modified reaction

conditions. By using glycosyl bromide (**25'**) and allyl 4,6-benzylidene glycosides (**12**)with mercury (II) salt as a promoter, disaccharides were synthesized in an excellent yield (98%). The other significant advantages of this strategy are that mild reaction conditions were applicable throughout the simple one-step reaction and only the β-anomer was formed selectively. To determine the participation effect of the neighboring group, differently protected thioglycosides (benzoyl or benzyl) were examined for their reactivity toward a common glycosyl acceptor. The benzoylated one revealed a serious steric hinderance due to the dioxolenium intermediate with the selective β-anomer. The opposite results were detected for the benzylated one with α/β mixtures dependent on the polarity of the solvent system used.

2.6. Experimental Methods

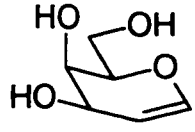
3,4,6-Tri-O-acetyl-1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol (2)



To a homogeneous solution of 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide (0.6 g, 1.46 mmol) in 50% aqueous acetic acid (30 mL) was added catalytic amount of $H_2PtCl_6 \cdot H_2O$ (3 drops) and zinc dust (0.6 g) at -15 °C. The reaction mixture was stirred vigorously for 2.5 h at the same temperature and then raised to room temperature. The reaction mixture was filtered through a celite pad and the filtrate was extracted with CH_2Cl_2 (100 mL x 3). The organic layer was combined, washed with water, dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with EtOAc/Hex (1/2) as an eluent to give 0.34 g of colorless viscous syrup (1.25 mmole, 86%). $[\alpha]_D$ = +70.0° (c = 0.8, $CHCl_3$); R_f = 0.29

(EtOAC/Hex, 1/2); CI-MS (ether): calcd. for $C_{12}H_{16}O_7$, 272.3, found, 272.9 (M+1, 2.3%); **^{1}H-NMR** δ ($CDCl_3$): 6.30 (dd, 1H, J_{12} = 6.3 Hz, J_{13} = 1.8 Hz, H_1), 5.41~5.38 (m, 1H, H_3), 5.29~5.26 (m, 1H, H_4), 4.60~4.54 (m, 1H, H_2), 4.18~4.06 (m, 3H, H_5, $2H_6$), 1.97~1.87 (3 singlets, 3Ac); **^{13}C-NMR** δ ($CDCl_3$): 171.0~170.6 (C=O), 145.94 (C_1), 99.4 (C_2), 73.3 (C_5), 64.4 & 64.3 (C_3 & C_4), 62.5 (C_6) 21.3~21.2 (C_{methyl}).

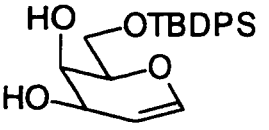
1,5-Anhydro-2-deoxy-D-lyxo-hex-1-enitol (D-galactal) (3)



To a solution of 3,4,6-tri-acetyl-1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol (**2**) 0.47 g, 1.73 mmol) in MeOH (30 mL) was added 1 M MeO^-Na^+/MeOH to adjust pH ~ 9 and the resulting solution was stirred for 40 min. at room temperature. The reaction mixture was neutralized by H^+-resin and filtered. The filtrate was treated with activated carbon for 15 min. followed by removal of the residue by filtration and evaporattion of the solvant. The product was dried under vacuum to give 0.25 g of colorless viscous syrup (1.70 mmol, 98 %). $[\alpha]_D$ = +78° (c = 1.6, MeOH); R_f = 0.6 ($MeOH/CH_2Cl_2$, 1/4); CI-MS (ether) (m/z): calcd. for $C_6H_{10}O_4$, 98.0, found, 99.0 (M+1, 3.4%); **^{1}H-NMR** δ (D_2O): 6.49 (ddd, 1H, J_{12} = 2.4 Hz, J_{13} = 2.0 Hz, J_{14} = 0.4 Hz, H_1), 4.81~4.79 (m, 1H, H_2), 4.57~4.55 (m, 1H, H_3), 4.15~4.12 (m, 1H, H_5), 4.03~4.02 (m, 1H, H_4), 3.94~3.84 (m, 2H, $2H_6$); **^{13}C-NMR** δ (D_2O): 145.4 (C_1), 104.1 (C_2), 78.5 (C_5), 66.1 (C_4), 65.4 (C_3), 62.8 (C_6).

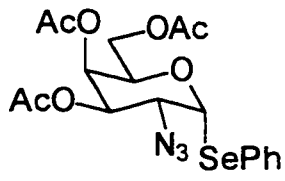
Anal. Calcd. for $C_{12}H_{16}O_7$ (272.3): C, 52.89; H, 5.94. Found: C, 51.67; H, 6.13.

6-(*Tert*-butyldiphenyl)silyl-1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol (4)



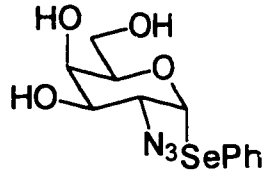
A solution of 1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol (**3**) (200 mg, 1.4 mmol) in dry pyridine (7 mL) was evaporated twice to remove moisture in the solution. Additional dry pyridine (15 mL) was added and evaporated again until the minimum amount of pyridine to dissolve the reactant (5 mL) remained, The solution was then saturated with N_2 and *tert*-butyldiphenylsilyl chloride (0.36 mL, 1.4 mmol) was added dropwise at room temperature and stirring was continued for 18 h. Additional silyl chloride (0.11 mL, 0.42 mmol) was added with stirring for 5 h at the same temperature. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and washed with water (3x). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with EtOAC/Hex (2/3) as an eluent to give 281 mg of monosilylated yellowish-viscous syrup at C-6 position (0.39 mmol, 52%) and disilylated by-product at C-4 and C-6 positions (0.39 mmol, 28%). $[\alpha]_D = 30.5°$ (c = 1.9, $CHCl_3$); R_f = 0.48 (EtOAc/Hex, 2/3); **^{1}H-NMR** δ ($CDCl_3$): 7.71~7.24 (m, 10H, 2Ph), 6.36 (dd, 1H, J_{12} = 5.8 Hz, J_{13} = 1.2 Hz, H_1), 4.73~4.68 (m, 1H, H_2), 4.37~4.31 (m, 1H, H_4), 4.15~4.08 (m, 1H, H_3), 3.99~3.86 (m, 3H, H_5, $2H_6$), 2.92 (d, 1H, $J_{OH\text{-}H3}$ = 5.3 Hz, OH at 4 position), 2.54 (d, 1H, $J_{OH\text{-}H4}$ = 10.1 Hz, OH at 5 position), 1.05 (s, 9H, *tert*-butyl); **^{13}C-NMR** δ ($CDCl_3$): 136.7~125.4 (2Ph), 144.8 (C_1), 103.3 (C_2), 76.7 (C_5), 67.1 (C_3), 64.8 (C_4), 64.0 (C_6), 27.3 (*tert*-butyl).

Phenylselenyl 3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranoside (6)



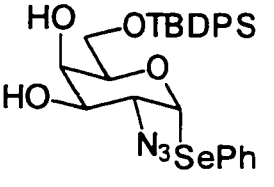
To a solution of 3,4,6-tri-O-acetyl-1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol (**2**) (600 mg, 2.21 mmol) in dry CH_2Cl_2 (40 mL) was added diphenyldiselenide (1.04 g, 3.32 mmol), sodium azide (0.72 g, 11.1 mmol) and (diacetoxyiodo)benzene (1.42 g, 4.42 mmol) and the reaction mixture was stirred for 7 days at room temperature. The solution was poured into saturated $NaHCO_3$ (30 mL) and extracted with CH_2Cl_2 (30 mL x 3). The organic layer was washed with water (3x) dried (Na_2SO_4) and condensed under reduced pressure. The residue was recrystallised with Hexane/EtOAc (7/1) to give 0.68 g of yellowish solid (1.44 mmol, 65%). IR (thin film, ν (cm^{-1}): 2113, azide); $[\alpha]_D$ = +165° (c = 1.0, CH_2Cl_2); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{18}H_{31}O_7N_3Se_1$, 471.09, found, 472.12 (M+1, 3.1%); **^{1}H-NMR** δ ($CDCl_3$): 7.63~7.22 (m, 5H, Ph), 5.98 (d, 1H, J_{12} = 5.1 Hz, H_1), 5.45 (dd, 1H, J_{34} = 3.9 Hz, J_{45} = 1.0 Hz, H_4), 5.10 (dd, 1H, J_{23} = 11.0 Hz, J_{34} = 3.8Hz, H_3), 4.68~4.62 (m, 1H, H_5), 4.23 (dd, 1H, J_{12} = 5.1 Hz, J_{23} = 10.9 Hz, H_2), 4.17~4.00 (m, 2H, $2H_6$), 2.13~1.96 (3 singlets, 3 Ac); **^{13}C-NMR** δ ($CDCl_3$): 170.5~169.9 (C=O), 135.4 (C_{ipso}), 129.5 (C_{para}), 128.1 (C_{meta}), 126.4 (C_{ortho}), 84.6 (C_1), 71.4 (C_3), 69.7 (C_4), 67.0 (C_5), 61.4 (C_6), 58.7 (C_2), 20.6 (C_{methyl}).

Phenylselenyl 2-azido-2-deoxy-α-D-galactopyranoside (7)



To a solution of phenylselenyl 3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranoside (**6**) (411 mg, 0.87 mmol) in MeOH (20 mL) was added MeO^-Na^+/MeOH (1 M) to adjust pH ~ 9 and stirring was continued for 1 h at room temperature. The reaction mixture was neutralized by acetic acid and was concentrated under reduced pressure. The residue was purified by silica gel column chromatography with EtOH/$CHCl_3$ (1/9) as an eluent to give 193 mg of yellowish powder (0.56 mmol, 65%). m.p.= 102.8~103.3 °C; $[\alpha]_D$ = +154.5° (c = 1.5, $CHCl_3$); R_f = 0.33 (EtOH/$CHCl_3$, 1/9), CI-MS (ether) (m/z): calcd. for $C_{12}H_{15}O_4N_3Se_1$, 345.02, found 346.01 (M+1, 1.5%); **^{1}H-NMR** δ ($CDCl_3$): 7.51~7.13 (m, 5H, Ph), 5.90 (d, 1H, J_{12} = 5.3 Hz, H_1), 4.15 (m, 1H, J_{34} = 4.5 Hz, J_{45} = 0.7 Hz, H_4), 4.12~3.99 (m, 2H, H_2, H_3), 3.88~3.70 (m, 3H, H_5, $2H_6$), 3.31, 2.62 & 1.98 (3 broad s, 3OH).

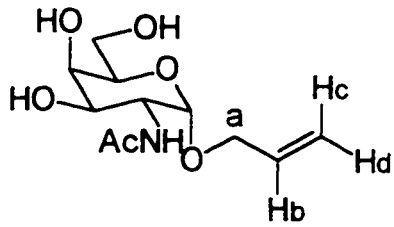
Phenylselenyl 2-azido-6-O-(*tert*-butyldiphenyl)silyl-2-deoxy-α-D-galactopyranoside (9)



To a solution of phenylselenyl-2-deoxy-2-azido-α-D-galactopyranoside (**7**) (240 mg, 0.7 mmol) and imidazole (238 mg, 3.5 mmol) in DMF (10 mL) was added silyl chloride (191 μL, 0.74 mmol). The resulting mixture was stirred overnight at room temperature. The solution was diluted with EtOAc (20 mL) and washed with water, brine, dried ($MgSO_4$) and filtered. The filtrate was condensed and purified by silica gel column chromatography with EtOAc/Hex (1/2) as an eluent to give 176 mg of white solid (0.3 mmol, 43%). m.p. = 71.3~72.5 °C; $[\alpha]_D$ = +109.6° (c = 1.4, $CHCl_3$); R_f = 0.58 (EtOAc/Hex, 2/3); IR (thin film, ν (Cm^{-1}), 2110, azide); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{28}H_{33}O_4N_3Si_1Se_1$, 583.14, found, 584.18 (M+1, 7.1%); **^{1}H-NMR** δ ($CDCl_3$): 7.63~7.03 (m, 15H, 3Ph), 5.86 (d, 1H, J_{12} = 5.3 Hz, H_1), 4.20~4.13 (m, 2H, H_3, H_4), 4.05

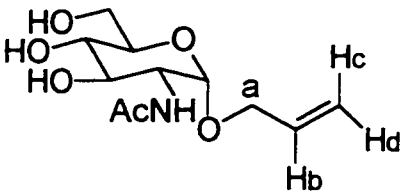
(dd, 1H, J_{12} = 5.3 Hz, J_{23} = 10.1 Hz, H_2), 3.78 (broad d, 2H, J_{56} = 4.5 Hz, $2H_6$), 3.70~3.64 (broad t, 1H, H_5), 3.50 (d, 1H, $J_{OH\text{-}H3}$ = 1.1 Hz, OH at 3 position), 2.53 (d, 1H, $J_{OH\text{-}H4}$ = 7.9 Hz, OH at 4 position), 0.95 (s, 9H, *tert*-butyl).

Allyl 2-acetamido-2-deoxy-α-D-galactopyranoside (10)



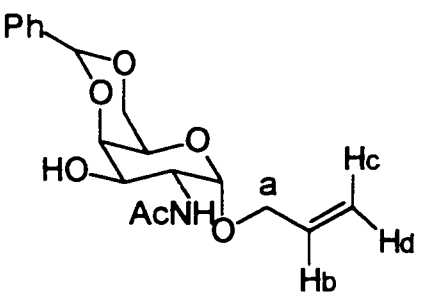
To a solution of dibenzoylated GalNAc derivative (**21**) (2 g, 4.26 mmol) in MeOH (20 mL) was added 2 or 3 drops of MeO^-Na^+ in MeOH (1 M) to adjust pH = 9~10. The mixture was stirred for 25 min. at room temperature. The sodium cations were eliminated using amberite IR-120, cationic (H^+) form. The solution was filtered and evaporated to dryness. The residue was dissolved in water (10 mL) and the immisible organic layer was removed. The aqueous layer was washed with $CHCl_3$ (3x) and lyophilized to afford 1.11 g (quantitative yield) of white solid. m.p. = 190.3~192.7 °C; $[\alpha]_D$ = +194.8° (c = 1.03, MeOH); R_f = 0.47 (EtOAc/iso-propyl alcohol/H_2O, 9/4/2); C.I.-M.S. (ether) (m/z): 262.12 (100%, M+1); **^{1}H-NMR**, δ (D_2O): 6.09~6.00 (m, 1H, H_b), 5.30 (dd, 1H, J_{gem} = 1.6 Hz, J_{trans} = 17.3 Hz, H_c), 5.32 (dd, 1H, J_{gem} = 1.5 Hz, J_{cis} = 10.3 Hz, H_d), 5.02 (d, 1H, J_{12} = 3.8 Hz, H_1), 4.28 (ddt, 1H, J_{ab} = 5.3 Hz, J_{gem} = 13.0 Hz, ^4J = 1.4 Hz, $1H_a$), 4.24 (dd, 1H, J_{12} = 3.8 Hz, J_{23} = 11.1 Hz, H_2), 4.10 (ddt, 1H, J_{ab} = 6.2 Hz, J_{gem} = 13.1 Hz, ^4J = 1.2 Hz, H_a'), 4.08~4.04 (m, 2H, H_4, H_5), 4.00 (dd, 1H, J_{23} = 11.1 Hz, H_3), 3.84~3.82 (m, 2H, $2H_6$), 2.09 (s, 3H's, Ac); **^{13}C-NMR**, δ (D_2O): 174.2 (C=O), 133.2 (C_b), 117.4 (C_{cd}), 95.8 (C_1), 70.5 (C_5), 68.1 & 68.0 (C_4, C_a), 67.2 (C_3), 60.8 (C_6), 49.4 (C_2), 21.5 (C_{methyl}).

Allyl 2-acetamido-2-deoxy-α-D-glucopyranoside (11)



To a solution of N-acetylglucopyranose (2 g, 9.0 mmol) in dry allyl alcohol was added a catalytic amount of $BF_3.Et_2O$. The mixture was refluxed for 3.5 h and stirred overnight at room temperature. The reaction mixture was neutralized with Et_3N, and co-evaporated with ethanol under reduced pressure. Crystallization from absolute ethanol afforded 1.53 g (65%) of white solid. m.p. = 166~168 °C; $[\alpha]_D = +145.6°$ (c = 1, MeOH); C.I.-M.S. (glycerol) (m/z): 262 (M+1, 100%); **^{1}H-NMR** δ (D_2O): 8.20 (d, 1H, $J_{2\text{-}NH} = 8.0$ Hz, NH), 6.03~5.90 (m, 1H, H_a) 5.30~5.19 (m, 2H's, H_{bc}), 4.93 (d, 1H, $J_{12} = 3.5$ Hz, H_1), 4.25 (dd, 1H, $J_{56} = 5.3$ Hz, $J_{66'} = 13.2$ Hz, H_6), 4.00 (dd, 1H, $J_{56'} = 6.1$ Hz, $J_{66'} = 13.3$ Hz, $H_{6'}$), 3.94~3.70 (m, 5H's, H_d, H_2, H_3, H_4), 3.50~3.44 (m, 1H, H_5), 2.01 (s, 3H's, AcNH); **^{13}C-NMR**, δ ($CDCl_3$): 174.1 ($C_{carbonyl}$), 133.1 (C_1), 117.5 (C_{bc}), 95.7,~69.6 (C_2, C_3, C_4, C_5), 68.0 (C_d), 61.1 (C_6), 53.2 (C_a).

Allyl 2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (12)



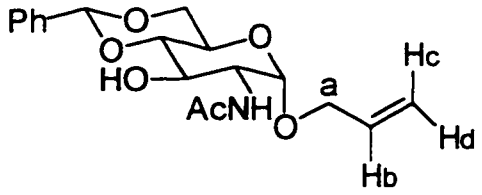
Method 1;

To a mixture of allyl GalNAc (**10**) (2.5 g, 9.6 mmol) in DMF (40 mL) and benzaldehyde dimethylacetal 5 mL (33.6 mmol, 5 eq.) was added a catalytic amount of p-toluenesulfonic acid monohydrate. The mixture was stirred for 5 h at room temperature, then poured into a mixture of saturated aqueous $NaHCO_3$ and $CHCl_3$. The organic layer was separated, washed with water, dried with Na_2SO_4 and concentrated to afford white powder (2.8 g, 8.03 mmol, 84%).

Method 2;

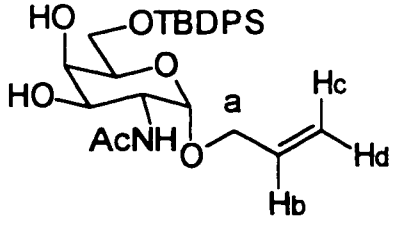
To a solution of allyl GalNAc (**10**) (1.8 g, 6.9 mmol), in benzaldehyde (30 mL) was added fused zinc chloride (1.9 g, 14 mmol, 2 eq.) and stirred under N_2 atmosphere overnight at room temperature. The mixture was poured into a cold mixture of 10% aqueous NH_4Cl (150 mL) and hexane (300 mL). The solution was mixed well and kept in ice bath for 3 h to induce solidification of the desired product. The product was filtered and washed successively with water, ether, and hexane to afford white powder (1.45 g, 4.0 mmol, 89%). m.p. = 220.7~222.0 °C; $[\alpha]_D$ = +132° (c = 1, $CHCl_3$); R_f = 0.44 (CH_2Cl_2/MeOH, 18/1); C.I.-M.S. (ether) (m/z): 350 (100%, M+1); **^{1}H-NMR**, δ ($CDCl_3$): 7.87~7.24 (m, 5H's, Ph), 5.92~5.83 (m, 1H, H_b), 5.74 (d, 1H, $J_{2\text{-}NH}$ = 9.0 Hz, NH), 5.55 (s, 1H, PhCH), 5.27 (dd, 1H, J_{gem} = 1.5 Hz, J_{trans} = 17.2 Hz, H_c), 5.21 (dd, 1H, J_{gem} = 1.3 Hz, J_{cis} = 10.4 Hz, H_d), 4.98 (d, 1H, J_{12} =3.5 Hz, H_1), 4.48~4.44 (ddd, 1H, J_{12} = 3.6 Hz, $J_{OH\text{-}H2}$ = 9.1 Hz, J_{23} = 10.9 Hz, H_2), 4.25 (dd, 1H, $J_{5\text{-}6a}$ = 1.5 Hz, $J_{6a\text{-}b}$ = 12.5 Hz, H_{6a}), 4.21~4.16 (m, 2H, H_4, 1H_a), 4.04 (dd, 1H, $J_{5\text{-}6b}$ = 1.6 Hz, J_{6ab} = 12.5 Hz, H_{6b}), 4.01 (ddt, 1H, J_{gem} = 13.0 Hz, $J_{a'b}$ = 6.2 Hz, J^4 = 1.3 Hz, 1$H_{a'}$), 3.85 (dd, 1H, J_{23} = 10.9 Hz, J_{34} = 3.5 Hz, H_3), 3.68 (broad s, 1H, H_5), 2.53 (broad s, 1H, OH), 2.03 (s, 3H, Ac); **^{13}C-NMR**, δ ($CDCl_3$): 171.2 (C=O), 137.5 (C_b), 133.4 (C_{ipso}), 129.1 (C_{para}), 128.2 (C_{ortho}), 126.3 (C_{meta}), 117.8 (C_{cd}), 101.3 (PhCH), 97.5 (C_1), 75.5 (C_4), 69.3 (C_6), 69.0 (C_3), 68.4 (C_a), 63.0 (C_5), 50.3 (C_2), 23.4 (C_{methyl}).

Allyl 2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside (13)



The reaction condition was the same as described in the synthesis of **12** and 2.9 g of white powder (8.35 mmol, 87%) was obtained. m.p. = 233~236 °C; R_f = 0.47 (CH_2Cl_2/MeOH, 18/1); $[\alpha]_D$ = +96.0° (c = 1, DMSO); CI-MS (ether) (m/z): calcd. for $C_{18}H_{23}O_6N_1$, 349.41, found, 350.40 (M+1, 100%); **^{1}H-NMR** δ ($CDCl_3$): 7.48~7.30 (m, 5H, Ph), 5.90~5.80 (m, 1H, H_b), 5.55 (s, 1H, CHPh), 5.30 (dd, 1H, J_{gem} = 1.6 Hz, J_{trans} = 17.0 Hz, H_c), 5.20 (dd, 1H, J_{cis} = 10.3 Hz, H_d), 4.85 (d, 1H, J_{12} = 3.8 Hz, H_1),4.28~3.56 (m, 8H, $2H_a$, H_2, H_3, H_4, H_5, $2H_6$), 2.93 (d, 1H, $J_{OH\text{-}3}$ = 3.1 Hz, OH), 2.04 (s, 3H, Ac); **^{13}C-NMR** δ ($CDCl_3$): 169.8 (C=O), 137.8 (C_{ipso}), 134.5 (C_b), 128.2 (C_{ortho}), 126.5 (C_{meta}), 117.0 (C_{cd}), 101.0 (C_1), 97.0 (C_3), 82.1 (C_5), 67.4 (C_4), 68.1 (C_a), 67.7 (C_6), 62.8 (CHPh), 54.3 (C_2), 22.7 (C_{methyl}).

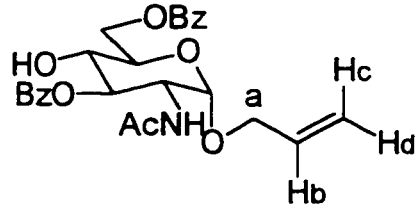
Allyl 2-acetamido-6-(*tert*-butyldiphenyl)silyl-2-deoxy-α-D-galactopyranoside (14)



To a solution of allyl 2-acetamido-2-deoxy-galactopyranoside (**10**) (0.8 g, 3.06 mmol) and imidazole (3.6 g, 52.0 mmol) in DMF (20 mL)was added (*tert*-butyldiphenyl)silyl chloride (836 μL, 3.21 mmol) and stirred for 1.5 h at room

temperature. The reaction mixture was poured into water (20 mL) and extracted with EtOAc (20 mL x 5). The organic layer was combined, and dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with EtOAc/EtOH (9/1) to give 0.89 g of yellowish sticky solid (1.78 mmole, 58%). $[\alpha]_D$ = +46.5° (c = 2.1, $CHCl_3$); R_f = 0.7 (EtOAc/EtOH, 9/1); **^{1}H-NMR** δ ($CDCl_3$): 7.68~7.20 (m, 10H, 2Ph), 5.90~5.82 (m, 2H, H_b, NH), 5.23 (dd, 1H, J_{gem} = 1.6 Hz, J_{trans} = 17.2 Hz, H_c), 5.19 (dd, 1H, J_{cis} = 10.4 Hz, H_d), 4.86 (d, 1H, J_{12} = 4.0 Hz, H_1), 4.32~4.27 (m, 1H, H_2), 4.17~4.12 (m, 1H, $1H_a$), 3.99 (broad s, 1H, H_4), 3.95~3.84 (m, 3H, $1H_a$, $2H_6$), 3.77 (broad t, 1H, H_5), 3.70 (broad d, 1H, J_{23} = 10.4 Hz, H_3), 2.04 (s, 3H, Ac), 1.04 (s, 9H, *tert*-butyl); **^{13}C-NMR** δ ($CDCl_3$): 172.5 (C=O), 135.6~129.8 (C_b, 2 Ph), 117.9 (C_{cd}), 96.3 (C_1), 71.7 (C_3), 70.1 (C_5), 68.6 (C_4), 68.0 (C_a), 63.6 (C_6), 5.9 (C_2), 26.8 (*tert*-butyl), 23.3 (C_{methyl}).

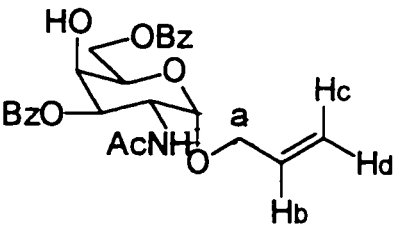
Allyl 2-acetamido-3,6-di-O-benzoyl-2-deoxy-α-D-glucopyranoside (19)



To a solution of allyl GlcNAc (**11**) (5.1 g, 19.5 mmol) in 60 mL of dry pyridine/chloroform (4/1, v/v) was added benzoyl chloride (4.98 mL, 42.9 mmol) in a dropwise fashion at -60 °C under N_2 atmosphere. The resultant white suspension was stirred for 2.5 h at the same temperature, then allowed to warm to room temperature. Upon addition of additional methanol (15 mL), the reaction mixture became a clear solution. The solvent was evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ (250 mL) and washed successively with 10% HCl, saturated aqueous $NaHCO_3$, and water, dried with Na_2SO_4, filtered and evaporated. The crude product was purified by silica gel column chromatography with $CHCl_3$/MeOH (25/1) as an eluent to afford 8.42 g (17.9 mmol, 92%) of desired product. m.p. = 57.3~57.8 °C; R_f = 0.64 ($CHCl_3$/MeOH,

20/1); $[\alpha]_D$ = +81.9° (c = 1.05, $CHCl_3$); C.I.-M.S. (ether) (m/z): calcd. for $C_{25}H_{27}N_1O_8$, 469.17, found, 470.12 (M+1, 23.5%); **^{1}H-NMR**, δ ($CDCl_3$): 8.04~7.34 (multi, 10H's, 2 phenyl's), 5.95 (d, 1H, $J_{2\text{-}NH}$ = 9.6 Hz, H_{NH}), 5.91~5.83 (m, 1H, H_b), 5.36 (dd, 1H, J_{23} = 10.8 Hz, J_{34} = 9.2 Hz, H_3), 5.26 (dd, 1H, J_{cd} = 1.5 Hz, J_{bc} = 17.2 Hz, H_c), 5.17 (dd, 1H, J_{cd} = 1.5 Hz, J_{bd} = 10.4 Hz, H_d), 4.89 (d, 1H, J_{12} = 3.6 Hz, H_1), 4.69 (dd, 1H, J_{56} = 4.6 Hz, $J_{66'}$ = 12.1 Hz, H_6), 4.54 (dd, $J_{56'}$ = 2.2 Hz, $J_{66'}$ = 12.1 Hz, $H_{6'}$), 4.45~4.40 (m, 1H, H_2), 4.20 (dd,1H, J_{ab} = 5.3 Hz, $J_{aa'}$ = 14.8 Hz, H_a), 4.04~3.98 (m, 2H's, H_5, $H_{a'}$), 3.86~3.81 (m, 1H, H_4), 3.68 (d, 1H, $J_{4\text{-}OH}$ = 5.0 Hz); **^{13}C-NMR**, δ ($CDCl_3$): 170.1 (C_b), 167.8 ~166.8 (C=O), 133.4~128.3 (2 phenyl's), 118.1 (C_{cd}), 96.6 (C_1), 74.7 (C_3), 70.5 (C_5), 69.0 (C_4), 68.5 (C_a), 63.5 (C_6), 51.6 (C_2), 23.0 (C_{methyl}).

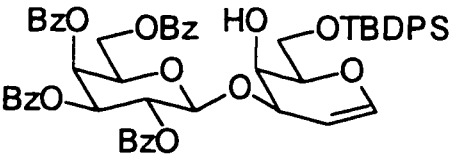
Allyl 2-acetamido-3,6-di-O-benzoyl-2-deoxy-α-D-galactopyranoside (21)



To a solution of triflic anhydride (1.3 mL, 7.68 mmol) in CH_2Cl_2 (30 mL) at -15 °C was added a solution of pyridine (1.24 mL, 16.0 mmol) in CH_2Cl_2 (5 mL). A white precipitate appeared during the initial phase of the reaction, but became clear after the complete addition of pyridine solution. A solution of allyl 3,6-dibenzoyl GlcNAc (**19**) (2.4 g, 5.12 mmol) in CH_2Cl_2 (10 mL) was added and the resulting mixture was stirred for 1.5 h at the same temperature under N_2 atmosphere. The reaction mixture was diluted with CH_2Cl_2 (100 mL) washed with 10% HCl, saturated aqueous $NaHCO_3$ and water, dried with Na_2CO_3 and filtered. The filtrate was evaporated to afford allyl 2-acetamido-2-deoxy-3,6-di-O-benzoyl-4-O-trifluoromethanesulfonyl-α-D-glucopyranose in quantitative yield as a yellow viscous syrup. This compound was used immediately in the next step without purification. The triflated compound was dissolved in DMF (20 mL), $NaNO_2$ (3.53 g, 51.2

mmol) was added and the mixture was stirred overnight at room temperature. The solution was diluted with CH_2Cl_2 (200 mL) and washed successively with brine and water, dried with Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography with $CHCl_3$/MeOH (30/1) as an eluent to afford 2.04 g (4.35 mmol, 85%) of white solid. m.p. = 59.7~60.3 °C; $[\alpha]_D$ = +80.6° (c = 1.6, $CHCl_3$); R_f = 0.33 ($CHCl_3$/MeOH, 30/1); C.I-M.S. (ether) (m/z): 470.15 (59.1%, M+1); **^{1}H-NMR**, δ ($CDCl_3$): 8.03~7.39 (m, 10H, 2 aromatics), 5.92~5.84 (m, 1H, H_b), 5.75 (d, 1H, $J_{2\text{-}NH}$ = 9.8 Hz, NH), 5.34 (dd, 1H, J_{23} = 11.2 Hz, J_{34} = 2.9 Hz, H_3), 5.24 (dd, 1H, J_{gem} = 1.5 Hz, J_{trans} = 17.2 Hz, H_c), 5.18 (dd, 1H, J_{gem} = 1.3 Hz, J_{cis} = 10.4 Hz, H_d), 4.97 (d, 1H, J_{12} = 3.7 Hz, H_1), 4.91~4.86 (m, 1H, H_2), 4.60 (dd, 1H, J_{56} = 5.8 Hz, $J_{66'}$ = 11.4 Hz, H_6), 4.52 (dd, 1H, $J_{56'}$ = 7.2 Hz, $J_{66'}$ = 11.3 Hz, $H_{6'}$), 4.27~4.19 (m, 3H, H_4, H_5, H_a), 4.04~4.00 (m, 1H, $H_{a'}$), 1.85 (s, 3H, Ac); **^{13}C-NMR**, δ ($CDCl_3$): 173.1, 169.5, 169.3(3 C=O), 136.2 (C_b), 136.5~131.4 (2 Ph), 100.1 (C_1), 75.0 (C_3), 71.6 (C_a), 71.4 (C_4), 70.5 (C_5), 66.5 (C_6), 50.3 (C_2), 26.3 (C_{methyl}).

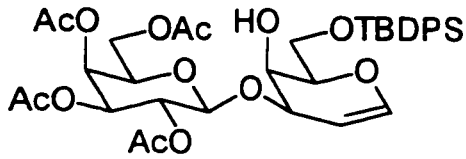
(2,3,4,6-Tetra-O-benzoyl-β-D-galactopyranosyl)-(1-3)-6-(*tert*-butyldiphenyl)silyl-1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol (23)



To a solution of phenylsulfenyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranoside (**22**) (1.04 g, 1.48 mmol) and 6-(tert-butyldiphenyl)silyl-D-galactal (**4**) (0.54 g, 1.40 mmol) in dry CH_2Cl_2 (30 mL) was added pulverized molecular sieves (300 mg, 4 Å) and stirred for 2 h at room temperature. Methyltrifluoromethanesulfonate (335 μL, 2.96 mmol) was added slowly under N_2 atmosphere and stirring was maintained for an additional 12 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and filtered through celite pad. The filtrate was washed with water and brine (30 mL) and dried ($MgSO_4$). The solution was then concentrated and the residue was purified by silica gel

column chromatography with EtOAc/Hex (1/3) as an eluent to give 0.12 g of white foam solid (0.42 mmol, 30 %). m.p. = 71~72 °C; R_f = 0.37 (EtOAc/Hex, 1/3); $[\alpha]_D$ = +52.0° (c = 1.5, $CHCl_3$); **^{1}H-NMR** δ ($CDCl_3$): 8.09~7.22 (30H, m, 6Ph), 6.28 (d, 1H, J_{12} = 4.7 Hz, H_1), 6.01 (d, 1H, J_{34}' = 3.4 Hz, J_{45}'≤ 1Hz, H_4'), 5.84 (dd, 1H, J_{12}' = 8.0 Hz, J_{23}' = 10.5 Hz, H_2'), 5.64 (dd, 1H, J_{34}' = 3.5 Hz, H_3'), 5.03 (d, 1H, J_{12}' = 8.0 Hz, H_1'), 4.59 (dd, 1H, J_{56}' = 6.6 Hz, J_{66}' = 11.3 Hz, $1H_6'$), 4.54~4.52 (m, 2H, H_2, H_3), 4.45 (dd, 1H, J_{56}' = 6.6 Hz, J_{66}' = 11.3 Hz, $1H_6'$), 4.18 (broad s, 1H, H_4), 3.96~3.92 (m, 3H, H_5, $2H_6$), 1.06 (s, 9H, t-butyl); **^{13}C-NMR** δ ($CDCl_3$): 165.9~165.2 (C=O), 145.6 (C_1), 135.6~127.7 (6 Ph), 100.8 (C_1'), 98.2 (C_3), 76.7 (C_5), 74.0 (C_2), 71.6 (C_3'), 71.5 (C_5'), 71.3 (C_2'), 69.8 (C_4'), 67.9 (C_4), 64.0 (C_6), 62.8 (C_6'), 26.8 (t-butyl), 19.2 (C_{methyl}).

(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-(1-3)-6-O-(*tert*-butyldiphenyl)silyl-1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol (24)

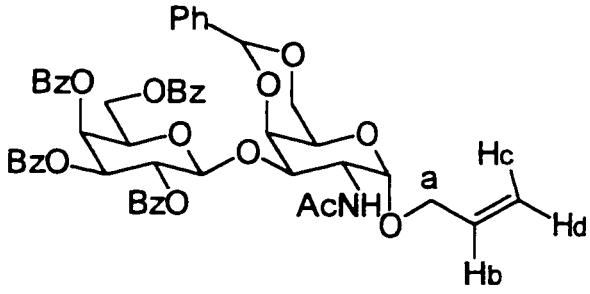


To a solution of 2,3,4,6-tetra-acetyl-α-D-galactopyranosyl bromide (**1**) (82.4 mg, 0.2 mmol), 6-(*tert*-butyldiphenyl)silyl-1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol (**4**) (69.2 mg, 0.18 mmol) in dry toluene 2 mL) was added pulverized molecular sieves (4 Å, 100 mg). The reaction mixture was saturated with N_2, cooled to -20 °C and stirred for 2 h. Silver trifluoromethanesulfonate (0.1 g, 0.4 mmol) and 2,4,6-collidine (27.8 μL, 0.21 mmol) in dry CH_2Cl_2/toluene (3/2) were then added under N_2 atmosphere. The reaction mixture was stirred for 40 min. at the same temperature and was then raised to room temperature. The solution was diluted with toluene (10 mL) and filtered through celite pad. The organic layer was washed with aqueous sodium thiosulfate (10 mL), water (10 mL) and dried (Na_2SO_4). The solution was condensed under reduced pressure and the residue was purified by silica gel column chromatography with EtOAc/Hex (1/3) as an eluent to give 32.1 mg of white foam solid (45 μmol, 25%). m.p. = 53.3~54.2 °C; $[\alpha]_D$ =

+25.0° (c = 1.7, $CHCl_3$); R_f = 0.24 (EtOAc/Hex, 1/2); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{36}H_{46}O_{13}Si_1$, 714.27, found, 715.32 (M+1, 0.9%); **^{1}H-NMR** δ ($CDCl_3$): 7.68~7.34 (m, 10H, 2Ph), 6.35 (dd, 1H, J_{12} = 6.3 Hz, J_{13} = 1.6 Hz, H_1), 5.37 (dd, 1H, J_{34}' = 3.4 Hz, J_{45}' = 0.9 Hz, H_4'), 5.21 (dd, 1H, J_{12}' = 7.9 Hz, J_{23}' = 10.5 Hz, H_2'), 5.02 (dd, 1H, J_{23}' = 10.5 Hz, J_{34}' = 3.4 Hz, H_3'), 4.62 (d, 1H, J_{12}' = 8.0 Hz, H_1'), 4.57~4.55 (m, 1H, H_3), 4.42~4.41 (m, 1H, H_2), 4.14 (broad s, 1H, H_4), 4.12~4.09 (m, 2H, $2H_6'$), 4.06~3.95 (m, 2H, H_5, $1H_6$), 3.93~3.90 (broad t, 1H, J_{45}' = 1.0 Hz, J_{56}' = 6.6 Hz, H_5'), 3.89~3.86 (m, 1H, $1H_6$), 2.14~1.96 (4 singlets, 12H, 4Ac), 1.05 (s, 9H, *tert*-butyl); **^{13}C-NMR** δ ($CDCl_3$): 170.2~169.3 (C=O), 145.5 (C_1), 135.6~127.7 (2Ph), 100.3 (C_1'), 98.17 (C_3), 76.7 (C_5), 73.3 (C_2), 71.0 (C_5'), 70.7 (C_3'), 68.8 (C_2'), 66.8 (C_4'), 63.8 (C_4), 62.4 (C_6), 61.1 (C_6'), 26.8 (*tert*-butyl), 20.7~20.5 (C_{methyl}).

Anal. Calcd. for $C_{36}H_{46}O_{13}Si_1$ (714.27): C, 60.54; H, 6.52. Found: C, 61.31; H, 6.75.

Allyl (2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-4,6-O-benzylidene-2-deoxy-α–D-galactopyranoside (25)



Method 1

To a solution of 2,3,4,6-tetra-O-benzoyl-1-thio-β-D-galactopyranose (211 mg, 0.31 mmol) and allyl 2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**12**) (60 mg, 0.17 mmol) in dry CH_2Cl_2 (2 mL) was added pulverized molecular sieves (4 Å, 100 mg).. The reaction mixture was saturated with N_2 and then stirred for 3 h at room

temperature and cooled to -30 °C. N-hydroxy succinimide (150 mg, 0.61 mmol) and trifluoromethanesulfonic acid (16.3 μL, 0.18 mmol) were added and stirring was proceeded for 2 h at the same temperature. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and filtered through a celite pad. The filtrate was washed successively with 10% $Na_2S_2O_4$ (10 mL), saturated $NaHCO_3$ (10 mL) and brine (10 mL). The solution was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with benzene/EtOAc (10/1) as an eluent to give 21 mg of white foamy solid (23 μmol, 14%).

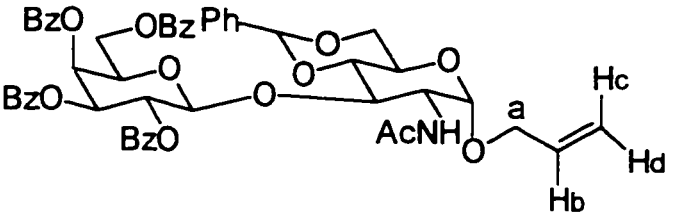
Method 2

Allyl 2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranose (**12**) (1.67 g, 4.78 mmol)was dissolved in 60 ml of 1:1 (v/v) anhydrous nitromethane-benzene containing 4 Å molecular sieves. To ensure dryness, the solution was concentrated (3x) by evaporation under reduced pressure. The same volume of solvent was added and then concentrated until 30 mL of solvent was remained. The temperature was adjusted to 25 °C under nitrogen and 1.80 g (7.13 mmol, 1.5 eq.) of mercuric cyanide and 4.70 g (7.13 mmol, 1.5 eq.) of 2,3,4,6-tetra-O-benzoyl-α-D-galactopyranosyl bromide were added. The solution was stirred for 18 h under the same condition. TLC (benzene/EtOAc, 2/1) indicated **12** was consumed completely. The solvent was evaporated under reduced pressure and the residue was dissolved in 40 mL of chloroform followed by filtration through a celite pad. The filtrate was successively washed with 10% aqueous potassium iodide, saturated sodium hydrogen carbonate and distilled water, then dried with magnesium sulfate. The solvent was evaporated to afford a white foamy solid. This crude product was purified by silica gel chromatography with benzene/ethyl acetate (15/1) as an eluent to give 4.31 g (4.67 mmol, 98%) of white foamy solid: m.p. = 109.7~111.0 °C; R_f= 0.59 (benzene/ethyl acetate, 1/2); $[\alpha]_D$ = +116.0° (c = 1.00, $CHCl_3$); (+) FAB-MS (glycerol) (m/z):928.3 (M+1, 3.0%), 1856 [2(M+1), 0.6%]; **^{1}H-NMR** δ ($CDCl_3$): 8.06~7.19 (m, 25H's, aromatic's), 5.98 (dd, 1H, $J_{34'}$ = 3.3 Hz, $J_{45'} \leq$ 1 Hz, H_4'), 5.85~5.78 (m, 2H's, H_b, H_2'), 5.60 (dd, 1H, $J_{23'}$ = 10.2 Hz, $J_{34'}$ = 3.4 Hz, H_3'), 5.48 (broad s, 1H, NH), 5.38 (s, 1H, PhC<u>H</u>), 5.23 (dd, 1H, J_{gem} = 1.5 Hz, J_{trans} = 17.2 Hz, H_c), 5.16 (m, 2H's,

J_{cis} = 8.0 Hz, H_d, H_1' is hidden by H_d), 5.10 (d, 1H, J_{12} = 3.4 Hz, H_1), 4.68 (dd, 1H, $J_{56'}$ = 6.9 Hz, $J_{6ab'}$ = 11.4 Hz, $H_{6a'}$), 4.63~4.58 (m, 1H, H_2), 4.46~4.36 (m, 3H's, H_4, H_5', $H_{6b'}$), 4.15~4.07 (m, 3H's, $1H_{6a}$, $1H_a$, H_3), 3.96 (dd, 1H, J_{gem} = 6.1 Hz, J_{ab} = 13.0 Hz, $1H_a$), 3.75 (dd, 1H, J_{56} = 1.4 Hz, J_{gem} = 12.4 Hz, $1H_{6b}$), 3.51 (m, 1H, H_5), 1.40 (s, 3H's, H_{methyl}); **^{13}C-NMR** δ ($CDCl_3$): 170.0~166.0 (five singlets, 5C=O's), 137.7~126.0 (multi, 30C's, 5aromatic's, C_b), 117.7 (C_{cd}), 102.0(C_1'), 100.9 (CPhCH), 97.4 (C_1), 75.8 (C_3), 75.4 (C_4), 71.8 & 71.8 (C_3' & C_5'), 70.2 (C_2'), 69.2 (C_6), 68.7 (C_a), 68.1 (C_4'), 63.0 (C_5), 62.7 (C_6'), 48.4 (C_2), 22.4 (C_{methyl}).

Anal. Calcd. for $C_{52}H_{49}O_{15}N_1$ (928.30): C, 67.20; H, 5.31; N, 1.53. Found: C, 66.84; H, 5.27; N, 1.48.

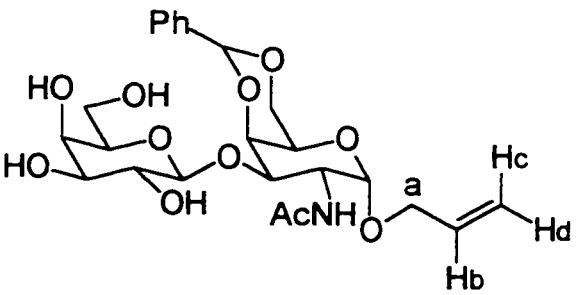
Allyl (2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside (27)



The reaction condition employed was similar to those described in allyl 2,3,4,6-tetra-O-benzoyl-β-D-glucopyranosyl-(1-3)-2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**13**) except that a longer reaction time (3 days) was required. 2.25 g (1.2 eq.) of donor and 1 g (2.86 mmol) of acceptor were used. Purification by silica gel column chromatography (benzene/EtOAc, 2/1) afforded 2 g (2.15 mmol, 75%) of white foamy solid. m.p. = 106.5~108.0 °C; R_f = 0.72 (benzene/EtOAc, 1/2); $[\alpha]_D$ = +100° (c = 1.0, CH_2Cl_2); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{52}H_{49}O_{15}N_1$, 927.31 found 928.32 (M+1, 2.8%); **^{1}H-NMR** δ ($CDCl_3$): 8.95~7.20 (m, 25H, 5aromatics), 5.88 (dd, 1H, $J_{34'}$ = 3.5 Hz, $J_{45'}$ = 1.1 Hz, H_4'), 5.77~5.69 (m, 1H,. H_b), 5.67 (dd, 1H,. $J_{12'}$ = 7.7 Hz, $J_{23'}$ = 10.3 Hz, H_2'), 5.59 (s, 1H, CHPh), 5.48 (dd, 1H, $J_{34'}$ = 3.5 Hz, H_3'), 5.23 (d, 1H, $J_{NH\text{-}2}$ = 8.7 Hz, NH), 5.16~5.12 (dd, 1H, J_{gem} = 1.,5 Hz, J_{trans} = 17.2 Hz, H_c), 5.14~5.12 (dd, 1H,

J_{cis} = 10.5 Hz, H_d), 5.10 (1H, J_{12}' = 8.0 Hz, H_1'), 4.84 (d, 1H, J_{12} = 3.7 Hz, H_1), 4.38 (dd, 1H, $J_{5\text{-}6a}$ = 11.1 Hz, $J_{6a\text{-}b}$ = 5.6 Hz, H_{6a}), 4.34~4.24 (m, 3H, H2, H_{6b}, H_{6a}'), 4.11~4.05 (m, 2H, $1H_a$, H_3), 3.93 (broad t, 1H, J_{56} = 11.1 Hz, H_5), 3.92~3.84 (m, 3H, $1H_a$, H_4, H_5'), 3.80~3.76 (broad t, 1H, $J_{6a\text{-}b}'$ = 10.2 Hz, H_{6b}'), 1.65 (s, 3H, Ac); **^{13}C-NMR** δ ($CDCl_3$): 170.0~165.1 (5 C=O), 137.1~125.9 (Cb, 5aromatics), 117.7 (C_{cd}), 101.7 (CHPh), 101.5 (C_1'), 97.2 (C_1), 81.8 (C_5'), 76.9 (C_3), 71.8 (C_3'), 71.4 & 71.4 (C_2', C_5), 69.0 (C_6'), 68.6 (C_a), 67.7 (C_4'), 62.9 (C_4), 61.3 (C_6), 52.3 (C_2), 22.7 (C_{methyl}).

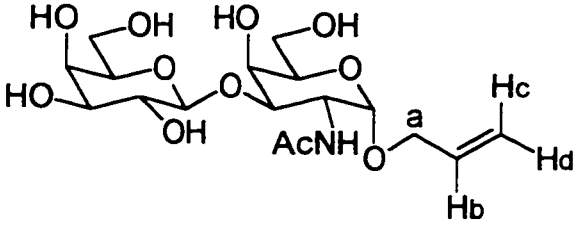
Allyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (28)



2.8 g (3.03 mmol) of **25** was dissolved in 20 mL of MeOH to which was added a catalytic amount of 1 M MeO^-Na^+ in MeOH to adjust pH = 9~10. The reaction mixture was stirred at room temperature for 30 min. with monitoring by TLC (benzene/EtOAc, 1/3). Sodium cations were removed by Amberite IR-120 cationic (H^+) form. The solution was filtered and evaporated to dryness. The crude product was dissolved in water and then the methylbenzoate layer was removed using a separatory funnel. The water layer was lyophilized to give **28** (1.57 g, quantitative yield) as a white solid:. m.p. = 117.5~118.8 °C; R_f = 0.9 ($CHCl_3$/MeOH/H_2O, 11/6/1); $[\alpha]_D$ = +118.6° (c = 1.00, H_2O); (+) FAB-MS (glycerol) (m/z):512.3 (M+1, 76.2%); **^{1}H-NMR** δ (D_2O):7.67~7.55 (m, 5H's, Ph), 6.10~6.02 (m, 1H, H_b), 5.85 (s, 1H, CHPh), 5.45 (dd, 1H, J_{gem} = 1.6 Hz, J_{trans} = 17.3 Hz, H_c), 5.35 (dd, 1H, J_{gem} = 1.7 Hz, J_{cis} = 10.4 Hz, H_d), 5.12 (d, 1H, J_{12} = 3.5 Hz, H_1), 4.73 (dd, J_{34} = 3.3 Hz, $J_{45}\leq$ 1Hz, H_4), 4.57 (dd, 1H, J_{12} = 3.5 Hz, J_{23} = 11.3 Hz, H_2),

4.55 (d, 1H, $J_{12'}$ = 7.8 Hz, H_1'), 4.35~4.25 (m, 4H's, H_3, $1H_a$, $2H_6$), 4.17 (ddd, 1H, J_{ad} = 1.3 Hz, J_{ab} = 5.0 Hz, J_{gem} = 12.9 Hz, $1H_a$), 4.08 (m, 1H, H_5), 3.97 (dd, 1H, $J_{45'}$ = 0.6 Hz, $J_{34'}$ = 3.4 Hz, H_4'), 3.88~3.81 (m, 2H's, $2H_6'$), 3.75~3.73 (m, 1H, H_5'), 3.68 (dd, 1H, $J_{34'}$ = 3.4 Hz, $J_{23'}$ = 9.9 Hz, H_3'), 3.53 (dd, 1H, $J_{12'}$ = 7.8 Hz, $J_{23'}$ = 10.0 Hz, H_2'), 2.10 (s, 3H's, H_{methyl}); **^{13}C-NMR** δ (D_2O): 174.1 (C=O), 136.3 (C_{ipso}), 133.2 (C_a), 129.5 (C_{para}), 128.2 (C_{meta}), 126.1 (C_{ortho}), 117.6 ($C_{bb'}$), 104.4 (C_1'), 100.8 (CHPh), 96.5 (C_1), 75.6 (C_4), 74.5 (C_3), 74.49 (C_5'), 72.0 (C_3'), 70.0 (C_2'), 68.5 (C_6), 68.3 (C_d), 68.1 (C_4'), 62.6 (C_5), 60.5 (C_6'), 48.1 (C_2), 21.5 (C_{methyl}).

Allyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-α-D-galactopyranoside (29)

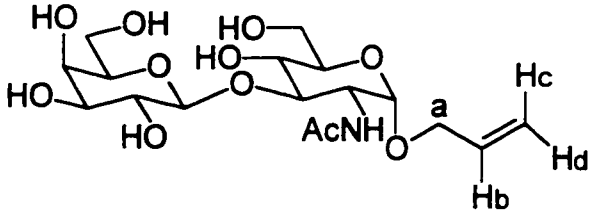


Compound **28** (1 g, 2.0 mmol) of was dissolved in 15 mL of 60% aqueous acetic acid and the resulting solution was stirred at 60 °C for 1.5 h. TLC ($CHCl_3$/MeOH/H_2O, 11/6/1) indicated the reactant which was U.V. detectable had completely disappeared and benzoic acid, a by-product was detected on the top of TLC plate. The crude product was purified by silica gel column chromatography with $CHCl_3$/MeOH/H_2O, 11/6/1 as an eluent. Evaporation and lyophilization gave 29 (0.79 g, 93%) as a white solid. m.p. = 230~232 °C; R_f = 0.53 ($CHCl_3$/MeOH/H_2O); $[\alpha]_D$ = +120.0° (c = 1.00, H_2O); (+) FAB-MS (glycerol) (m/z): 424.2 (M+1, 76.1%); **^{1}H-NMR** δ (D_2O): 6.08~6.01 (m, 1H, H_b), 5.42 (dd, 1H, J_{gem} = 1.6 Hz, J_{trans} = 17.3 Hz, H_c), 5.33 (dd, 1H, J_{gem} = 1.7 Hz, J_{cis} = 10.4 Hz, H_d), 5.01 (d, 1H, J_{12} = 3.8 Hz, H_1), 4.53 (d, 1H, $J_{12'}$ = 7.8 Hz, H_1'), 4.41 (dd, 1H, J_{12} = 3.7 Hz, J_{23} = 11.2 Hz, H_2), 4.31~4.27 (m, 2H's, H_4, $1H_a$), 4.13~4.07 (m, 3H's, H_3, H_5, $1H_a$), 3.98 (dd, 1H, $J_{34'}$ = 3.4 Hz, $J_{45'}$ = 0.8 Hz, H_4'), 3.86~3.78 (m, 4H's, $2H_6$, $2H_6'$),

3.74~3.71 (m, 1H, H_5'), 3.69 (dd, 1H, $J_{23'}$ = 10.0 Hz, $J_{34'}$ = 3.4 Hz, H_3'), 3.59 (dd, 1H, $J_{12'}$ = 7.7 Hz, $J_{23'}$ = 10.0 Hz, H_2'), 2.09 (s, 3H's, Ac); **^{13}C-NMR** δ (D_2O): 174.1 (C=O), 133.2 (C_b), 117.4 (C_{cd}), 104.2 (C_1'), 95.9 (C_1), 76.8 (C_3), 74.5 (C_5'), 72.1 (C_3'), 70.2 (C_5), 70.1 (C_2'), 68.3 (C_4), 68.1 (C_4'), 68.0 (C_a), 60.7 (C_6'), 60.5 (C_6), 48.1 (C_2), 21.5 (C_{methyl}).

Anal. Calcd. for $C_{16}H_{29}O_{10}N_1$ (424.20): C, 45.30; H, 6.92; N, 3.30. Found: C, 45.30; H, 6.93; N, 3.07.

Allyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-α-D-glucopyranoside (30)

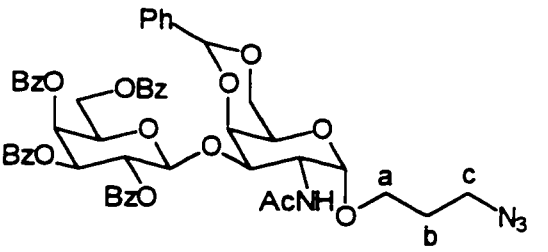


To a solution of allyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1-3)-2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside **27** (0.6 g, 0.65 mmol) in MeOH (20 mL) was added MeO^-Na^+ (1 M) to adjust pH ~ 9 and the solution was stirred for 30 min. at room temperature. Acetic acid was added to neutralize the reaction mixture and the solution was condensed under reduced pressure. Without purification, the residue was dissolved in 60% aq. acetic acid (20 mL) and stirred for 1.5 h at 60 °C. The reaction mixture was condensed by rotary evaporator and purified by silica gel column chromatography with $CHCl_3$/MeOH/H_2O (11/6/1) as an eluent to afford 0.23 g of white solid (0.54 mmol, 90%). m.p. = 189.5~191.0 °C; R_f = 0.51 (EtOAc/acetic acid/H_2O, 3/2/2); $[\alpha]_D$ = +81° (c = 1, H_2O); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{16}H_{29}O_{10}N_1$, 423.17, found, 424.26 (M+1, 36.3%); **^{1}H-NMR** δ (D_2O): 6.06~6.01 (m, 1H, H_b), 4.44 (dd, 1H, J_{gem} = 1.6 Hz, J_{trans} = 17.3 Hz, H_c), 5.34 (dd, 1H, J_{gem} = 1.6 Hz, J_{cis} = 10.5 Hz, H_d), 4.98 (d, 1H, J_{12} = 3.6 Hz, H_1), 4.51 (d, 1H, $J_{12'}$ = 7.8 Hz, H_1'), 4.31~4.28 (m, 1H, $1H_a$), 4.18 (dd, 1H, J_{12} = 3.6 Hz, J_{23} = 10.6 Hz, H_2), 4.13~4.09 (m, 1H, $1H_a$), 4.03~3.99 (m, 2H,

H_3, H_4'), 3.94 (dd, 1H, J_{gem} = 2.0 Hz, J_{56} = 12.1 Hz, H_{6a}'), 3.91~3.76 (m, 6H, H_5, H_5', $2H_6$, H_{6b}'), 3.72 (dd,. 1H, J_{23}' = 9.9 Hz, J_{34}' = 3.4 Hz, H_3'), 3.67 (broad t, 1H, J_{34} = 8.7 Hz, $J_{45} \leq$ 1 Hz, H_4), 3.59 (dd, J_{12}' = 7.8 Hz, J_{23}' = 9.9 Hz, H_2'), 2.10 (s, 3H, Ac); **^{13}C-NMR** δ (D_2O): 174.0 (C=O), 133.2 (C_b), 117.5 (C_{cd}), 103.0 (C_1'), 95.8 (C_1), 79.9 (C_3), 74.8 (C_5), 72.1 (C_3'), 71.2 (C_5'), 70.2 (C_2'), 68.2 (C_4), 68.1 (C_4') 68.0 (C_a), 60.5 & 60.1 (C_6 & C_6'), 52.0 (C_2), 21.5 (C_{methyl}).

Anal. Calcd. for $C_{16}H_{29}O_{10}N_1$ (424.20): C, 45.30; H, 6.91; N, 3.30. Found: C, 45.72; H, 6.56; N, 2.86.

3-Azidopropyl (2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (31)

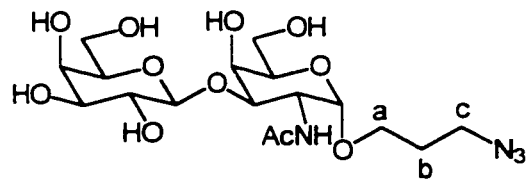


3-Aminopropyl 2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**18**) (0.54 g, 1.38 mmol) was dissolved in anhydrous nitromethane-benzene (1:1(v/v), 150 mL) containing 4 Å molecular sieves. To ensure dryness, the solution was concentrated (3x) by evaporation under reduced pressure. Twice the volume of solvent was added followed by concentration until 100 mL of solvent remained. The temperature was adjusted to 25 °C under nitrogen and 2,3,4,6-tetra-O-benzoyl-α-D-galactopyranosyl bromide **1** (1.35 g, 1.5 eq.) and mercuric cyanide (0.54 g, 1.5 eq.) were added. The solution was stirred overnight under the same condition. TLC (benzene/EtOAc, 1/2) indicated that the donor was consumed completely. The solvent was evaporated under reduced pressure and the residue was dissolved in $CHCl_3$ (100 mL) followed by filtration through a celite pad. The filtrate was successively washed with 10% aqueous potassium iodide, saturated sodium hydrogen carbonate and distilled water, then dried with sodium sulfate. The solution was condensed and the crude product was purified by silica gel

chromatography with benzene/ethyl acetate (1/2) as an eluent to give 0.9 g (1 mmol. 70%) of white solid. m.p. = 91.7~92.2 °C; R_f = 0.46 (benzene/ethyl acetate, 1/2); $[\alpha]_D$ = +120° (c = 1.3, $CHCl_3$); (+) FAB-MS (glycerol) (m/z): calcd for $C_{52}H_{50}O_{15}N_5$, 984.34, found, 985.39 (M+1, 3.2%); **^{1}H-NMR**, δ ($CDCl_3$): 8.06~7.19 (m, 25H, 5 aromatics), 5.97 (broad d, 1H, J_{34}'=3.27Hz, H_4'), 5.79 (dd, 1H, J_{12}' = 7.7 Hz, J_{23}' = 10.2 Hz, H_2'), 5.60 (dd, 1H, J_{23}' = 10.3 Hz, J_{34}' = 3.4 Hz, H_3'), 5.46 (d, 1H, $J_{2,NH}$ = 7.5 Hz, NH), 5.38 (s, 1H, -CHPh), 5.14 (d, 1H, J_{12}' = 7.6 Hz, H_1'), 5.10 (d, 1H, J_{12} = 3.3 Hz, H_1), 4.69 (dd, 1H, J_{56}' = 6.6 Hz, $J_{6a,6b}$ = 11.0 Hz, H_{6a}'), 4.59~4.54 (m, 1H, H_2), 4.44~4.35 (m, 3H, H_4, H_5', H_{6b}'), 4.14 (dd, 1H, $J_{5,6a}$ = 1.1 Hz, $J_{6a,6b}$ = 11.9 Hz, H_{6a}), 4.04 (dd, 1H, J_{12} = 11.3 Hz, J_{34} = 3.2 Hz, H_3), 3.75~3.72 (m, 2H, H_{6b}, H_a), 3.50 (broad s, 1H, H_5), 3.48~3.45 (m, 1H, H_a), 3.30~3.27 (dt, 2H, J_{cc}' = 2.3 Hz, J_{bc} = 6.4 Hz, H_c), 1.85~1.76 (m, 2H, H_b), 1.40 (s, 3H, H_{methyl}); **^{13}C-NMR**, δ ($CDCl_3$): 169.9~165.0 (C=O), 137.6~126.2 (aromatics), 102.1 (C_1'), 100.9 (-CHPh), 98.0 (C_1), 76.0 (C_3), 75.3 (C_4), 71.9 (C5'), 71.7 (C_3'), 70.4 (C_2'), 69.2 (C_6), 68.1 (C_4'), 65.3 (C_a), 63.1 (C_5), 62.6 (C_6) 48.6 (C_c), 48.5 (C_2), 28.6 (C_b), 22.5 (C_{methyl}).

Anal. Calcd. for $C_{52}H_{50}O_{15}N_5$ (984.34): C, 63.40; H, 5.10; N, 7.10. Found: C, 62.53; H, 5.12; N, 7.45.

3-Azidopropyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-α-D-galactopyranoside (32)

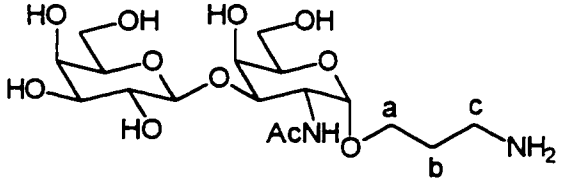


3-Azidopropyl (2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-(1-3)-2-acetamido -4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**31**) (0.7 g, 0.71 mmol) was dissolved in MeOH (50 mL) and a catalytic amount of 1 M MeO^-Na^+ was added to adjust pH = 9~10. The reaction mixture was stirred at room temperature for 30 min. with monitoring by TLC ($CHCl_3$/MeOH, 6/4). Excess anion was removed by Amberite IR-120 (cationic (H^+) form). More resin was added to remove the benzylidene protecting group

at 55 °C for 3 h. The solution was filtered and evaporated to dryness. The crude product was dissolved in water and $CHCl_3$ mixture and the organic layer was removed using a separatory funnel. The water layer was lyophilized to give white solid **32** (0.29 g, 0.63 mmol, 90%). m.p. = 156.8~157.6 °C; R_f = 0.42 ($CHCl_3$/MeOH, 6/4), $[\alpha]_D$ = +140° (c = 0.6, MeOH); CI-MS (ether): calcd for $C_{17}H_{30}O_{11}N_4$, 466.30, found, 467.30 (M+1, 17.3%); **^{1}H-NMR**, δ (D_2O): 4.96 (d, 1H, J_{12} = 3.8 Hz, H_1), 4.54 (d, 1H, J_{12}' = 7.8 Hz, H_1'), 4.40 (dd, 1H, J_{12} = 3.8 Hz, J_{23} = 11.1 Hz, H_2), 4.31 (broad d, 1H, J_{34} = 2.9 Hz, H_4), 4.10 (dd, 1H, J_{23} = 11.1 Hz, J_{34} = 3.1 Hz, H_3), 4.06 (broad t, 1H, J_{56} = 6.3 Hz, H_5), 3.98 (dd, 1H, J_{34}' = 2.8 Hz, J_{45}' = 0.6 Hz, H_4'), 3.90~3.79 (m, 5H, $2H_6$, $2H_6'$, $1H_a$), 3.74~3.71 (m, 1H, H_5'), 3.70 (dd, 1H, J_{23}' = 10.0 Hz, J_{34}' = 3.4 Hz, H_3'), 3.64~3.49 (m, 4H, H_2', $1H_a$, H_c), 2.10 (s, 3H, H_{methyl}), 2.08~1.96 (multi, 2H, H_b); **^{13}C-NMR**, δ (D_2O): 174.1 (C=O), 104.2 (C_1'), 96.8 (C_1), 76.7 (C_3), 74.5 (C_5'), 72.1 (C_3'), 70.2 (C_2', C_5), 68.3 (C_4), 68.1 (C_4'), 64.5 (C_a), 60.7 & 60.5 (C_6, C_6'), 48.2 (C_2), 47.8 (C_c), 27.5 (C_b), 21.5 (C_{methyl}).

Anal. Calcd. for $C_{17}H_{30}O_{11}N_4$ (466.33): C, 43.82; H, 6.54; N, 12.03. Found: C, 42.91; H, 6.61; N, 11.88.

3-Aminopropyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-α-D-galactopyranoside (33)



3-Azidopropyl-O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-α-D-galactopyranoside (**32**) (0.29 g, 0.63 mmol) was dissolved in MeOH (30 mL) and a catalytic amount of Pd/C (10%) was added. The resulting solution was bubbled with H_2 at room temperature for 45 min. The reaction was confirmed by a ninhydrin test. The solution was filtered through celite pad followed by evaporation to give a white solid (quantitative): m.p. = 89.6~90.0 °C; $[\alpha]_D$ = +84.0° (c = 1, MeOH); (+) FAB-MS (glycerol): 441.2 (M+1, 3.7%); **^{1}H-NMR**, δ (D_2O): 4.96 (d, 1H, J_{12} = 3.6 Hz, H_1), 4.54

(d, 1H, $J_{12'}$ = 7.7 Hz, $H_{1'}$), 4.39 (dd, 1H, J_{12} = 3.6 Hz, J_{23} = 11.0 Hz, H_2), 4.31 (broad d, 1H, J_{34} = 2.8 Hz, H_4), 4.09 (dd, 1H, J_{23} = 11.1 Hz, J_{34} = 2.8 Hz, H_3), 4.06 (broad t, 1H, J_{56} = 6.1 Hz, H_5), 3.98 (broad d, $J_{34'}$ = 3.1 Hz, $H_{4'}$), 3.68~3.79 (m, 5H, H_6, $H_{6'}$, H_a), 3.72~3.69 (m, 2H $H_{3'}$, $H_{5'}$), 3.63~3.58 (m, 2H, $H_{2'}$, H_a), 2.85 (broad t, 2H, J_{bc} = 6.9 Hz, H_c), 2.10 (s, 3H, H_{methyl}), 1.98~1.84 (m, 2H, H_b); **^{13}C-NMR**, δ (D_2O): 174.1 (C=O), 104.2 (C_1), 96.6 ($C_{1'}$), 76.8 (C_3), 74.5 ($C_{5'}$), 72.1 ($C_{3'}$), 70.2 ($C_{2'}$, C_5), 68.3 ($C_{4'}$), 68.2 (C_4), 65.4 (C_a), 60.8 & 60.5 (C_6, $C_{6'}$), 48.2 (C_2), 37.2 (C_c), 30.3 (C_b), 21.5 (C_{methyl}).

Anal. Calcd. for $C_{17}H_{32}O_{11}N_2$ (440.32): C, 46.33; H, 7.34; N, 6.36. Found: C, 45.78; H, 7.33; N, 5.39.

Allyl 2,3,4,6-tetra-O-benzyl-4,6-O-benzylidene-α/β-D-Gal-(1-3)-α-D-GalNAc

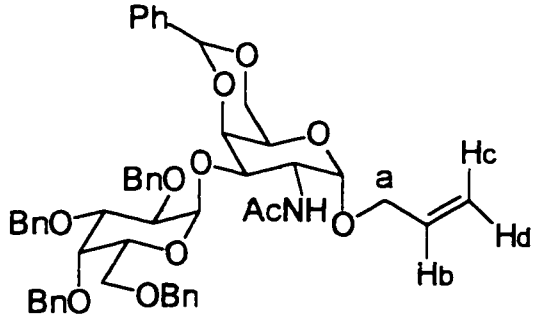
Method 1

To a solution of 2,3,4,6-tetra-O-benzoyl-1-thiophenyl galactopyranoside (106 mg, 0.17 mmol) and allyl 2-acetamido-4,6-O-benzylidene-2-deoxy-galactopyranoside (**12**) (50 mg, 0.14 mmol) in dry CH_2Cl_2 (2 mL) was added pulverized molecular sieves (4 Å, 120 mg) and the solution was stirred for 1 h under N_2 atmosphere. The reaction mixture was cooled to -50 °C and N-iodosuccinimide (76.5 mg, 0.34 mmol) and trifluoromethanesulfonic acid (9 μL, 0.1 mmol, 0.6 eq. for donor) were then added. The reaction was essentially completed in 30 min. as judged by TLC. The solution was diluted with CH_2Cl_2 (10 mL) and filtered through a celite pad. The filtrate was washed successively with 10% aq. $Na_2S_2O_3$ (10 mL), saturated aq. $NaHCO_3$ (10 mL), brine (10 mL) and dried (Na_2SO_4). The filtrate was condensed and the residue was purified by silica gel column chromatography with benzene/EtOAc (1/1). The products were dried in vacuo to give 33 mg of α-form (0.04 mmol, 29%) and 25.2 mg (0.03 mmol) of β-form. The overall yield was 50% and the ratio of α/β was 1.33/1.

Method 2

The reaction condition was the same as described in method 1 except the solvent used was CH_2Cl_2/toluene (1/1). The yield was 45.0 mg of α-form (0.052 mmol, 26%) and 29.6 mg of β-form (0.034 mmol, 17%). The overall yield was 43% and the ratio of α/β was 1.53/1.

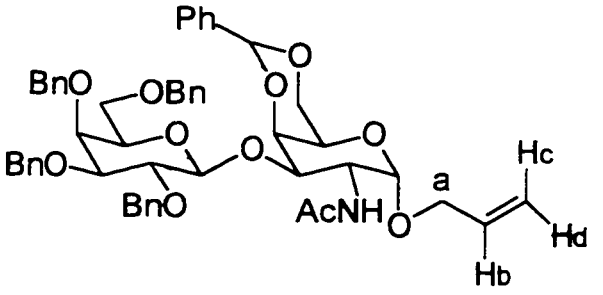
Allyl (2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl)-(1-3)-2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (34)



m.p.=44.3~45.1 °C; R_f = 0.73 (benzene/EtOAc, 1/2); $[\alpha]_D$ = +71.3° (c = 1.15, $CHCl_3$); (+) FAB-MS (glycerol) (m/z); cacld. for $C_{52}H_{57}O_{11}N_1$, 871.39, found, 872.40 (M+1, 0.2%); **^{1}H-NMR** δ ($CDCl_3$); 7.48~7.03 (m, 25H, 5Ar), 5.90 (d, 1H, $J_{2\text{-NH}}$ = 8.9 Hz, NH), 5.81~5.75 (m, 1H, H_b), 5.33 (s, 1H, CHPh), 5.24 (dd, 1H, J_{gem} = 1.6 Hz, J_{trans} = 17.2 Hz, H_c), 5.13 (dd, 1H, J_{cis} = 10.4 Hz, H_d), 5.05 (d, 1H, J_{12}' = 3.8 Hz, H_1'), 5.03 (d, 1H, J_{12} = 3.5 Hz, H_1), 4.93~4.79 (2 doublets, 2H, CH_2Ph), 4.71~4.67 (m, 3H, H_2, CH_2Ph), 4.61~4.38 (m, 4H, 2 CH_2Ph), 4.20 (m, 1H, H_5), 4.13~4.05 (m, 4H, h_2', H_5', $1H_6$, $1H_a$), 4.02~3.94 (m, 4H, H_3, H_3', $1H_6$, $1H_a$), 3.79 (d, 1H, J_{34}' = 1.9 Hz, H_4), 3.60 (broad s, 1H, H_4), 3.55 (dd, 1H, J_{56}' = 7.9 Hz, J_{gem}' = 9.9 Hz, $1H_{6a}'$), 3.31 (dd, 1H, J_{56}' = 3.9 Hz, J_{gem}' = 9,9 Hz, $1H_{6b}'$), 1.80 (s, 3H, Ac); **^{13}C-NMR** δ ($CDCl_3$); 170.1 (C=O), 138.8~126.1 (5 Ar), 117.5 (C_{cd}), 100.7 (CHPh), 97.4 (C_i), 96.6 (C_1'), 78.9 (C_3), 76.2 (C_2'), 75.4 (C_4'), 74.5~73.4 (3 CH_2Ph), 73.2 (C_3'), 72.8 (CH_2Ph), 70.7 (C_6'), 70.5 (C_5'), 69.4 (C_5, C_6), 68.5 (C_a), 63.1 (C_4), 48.2 (C_2), 23.0 (C_{methyl}).

Anal. Calcd. for $C_{52}H_{57}O_{11}N_1$ (871.39): C, 71.63; H, 6.63; N, 1.62. Found: C, 70.47; H, 6.43; N, 1.53.

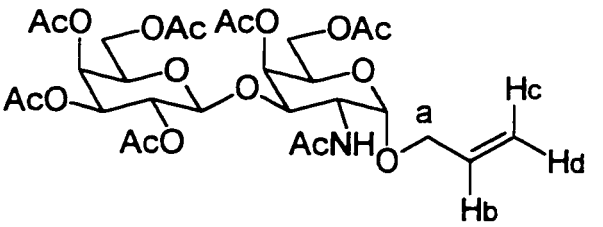
Allyl (2,3,4,6-tetra-O-benzyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (35)



m.p. = 31.6~32.5 °C; R_f = 0.82 (benzene/EtOAc, 1/2); $[\alpha]_D$ = +93.9° (c = 0.9, $CHCl_3$); (+) FAB-MS (glycerol) (m/z); cacld. for $C_{52}H_{57}O_{11}N_1$, 871.39, found, 872.40 (M+1, 2.5%); **^{1}H-NMR** δ ($CDCl_3$);7.54~7.19 (m, 25H, 5Ar), 5.85~5.81 (m, 1H, H_b), 5.72 (d, 1H, $J_{2\text{-}NH}$ = 8.1 Hz, NH), 5.46 (s, 1H, CHPh), 5.24 (dd, 1H, J_{gem} = 1.6 Hz, J_{trans} = 17.2 Hz, H_c), 5.17 (dd, 1H, J_{cis} = 10.4 Hz, H_d), 5.13 (d, 1H, J_{12} = 3.5 Hz, H_1), 4.92~4.73 (m, 4H, 2 CH_2Ph), 4.69~4.65 (m, 1H, H_2), 4.55~4.53 (m, 3H, H_1', CH_2Ph), 4.41 (2 dublets, CH_2Ph), 4.34 (d, 1H, J_{34} = 3.0 Hz, $J_{45} \leq$ 1Hz, H_4), 4.19~4.10 (m, 2H, 1H_6, 1H_a), 4.07 (dd, 1H, J_{23} = 11.3 Hz, J_{34} = 3.2 Hz, H_3), 4.00~3.96 (m, 1H, 1H_a), 3.89~3.81 (mi, 3H, H_2', H_4', 1H_6), 3.62~3.58 (m, 2H, H_5, 1H_6'), 3.53 (broad t, 1H, J_{56} = 6.1 Hz, H_5'), 3.50~3.44 (m, 2H, H_3', 1H_6'), 1.65 (s, 3H, Ac); **^{13}C-NMR** δ ($CDCl_3$); 170.0 (C=O), 138.8~133.9 (CHPh), 128.7~126.4 (4Bn), 117.4 (C_{cd}), 104.6 (C_1'), 100.8 (PhCH), 97.5 (C_1), 82.2 (C_3'), 78.8 (C_2'), 75.8 (C_4), 74.6 & 74.4 (2 CH_2Ph), 74.2 (C_3), 73.8 (C_5'), 73.7 (C_4), 73.6 & 73.1 (2 CHPh), 69.5 (C_6'), 69.3 (C_6), 68.6 (Ca), 63.2 (C_5), 49.0 (C_2), 23.0 (C_{methyl})

Anal. Calcd. for $C_{52}H_{57}O_{11}N_1$ (871.39): C, 71.63; H, 6.63; N, 1.62. Found: C, 70.39; H, 6.54; N, 1.57.

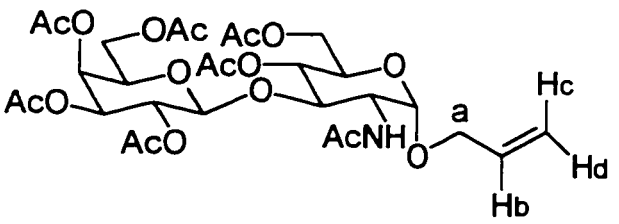
Allyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-galactotopyranoside (36)



To a solution of allyl Gal-(1-3)-GalNAc (**29**) (0.6 g, 1.42 mmol) in pyridine (30 mL) was added acetic anhydride (1.34 mL, 10 eq.) in a dropwise fashion at room temperature. The solution was stirred overnight at same temperature. The solvent was evaporated under reduced pressure to dryness. The residue was dissolved in CH_2Cl_2 (100 mL) and washed successively with saturated $NaHCO_3$ and distilled water. The solution was dried (Na_2SO_4), filtered and condensed. The residue was purified by silica gel chromatography with EtOAc/Hex/MeOH (12/6/1) as an eluent to give 0.93 g (1.4 mol, 98.6%) of white foamy solid: m.p. = 66.1~67.0 °C; R_f = 0.72 (EtOAc/Hex/MeOH, 12/4/1); $[\alpha]_D$= +47.0° (c = 1, $CHCl_3$); (+) FAB-MS (glycerol) (m/z); calcd. for $C_{29}H_{41}O_{17}N_1$, 675.24, found, 676.30 (M+1, 19.2%); **^{1}H-NMR** δ ($CDCl_3$): 5.92~5.84 (m, 1H, Hb), 5.62 (d, 1H, $J_{2\text{-NH}}$ = 8.8 Hz, NH), 5.35~5.33 (m, 2H, H_4, H_4'), 5.28~5.23 (dd, 1H, J_{gem} = 1.6 Hz, J_{trans} = 17.2 Hz, H_c), 5.23~5.21 (dd, 1H, J_{gem} = 1.4 Hz, J_{cis} = 10.4 Hz, H_d), 5.12 (dd, 1H, $J_{12'}$ = 7.9 Hz, $J_{23'}$ = 10.5 Hz, H_2'), 4.94 (d, 1H, J_{12} = 3.5 Hz, H_1), 4.93 (dd, 1H, $J_{34'}$ = 3.5 Hz, H_3' partially hidden), 4.57 (d, 1H, $J_{12'}$ = 7.9 Hz, H_1), 4.53~4.48 (m, 1H, H_2), 4.18~3.96 (m, 7H, $2H_6$, $2H_6'$, $2H_a$, H_5), 3.93 (dd, 1H, J_{23} = 10.9 Hz, J_{34} = 3.3 Hz, H_3), 3.86 (dt, 1H, $J_{45'}$ = 1.0 Hz, $J_{56'}$ = 6.8 Hz, H_5'), 2.14~1.95 (m, 21H, 7Ac); **^{13}C-NMR** δ ($CDCl_3$): 170.4~169.6 (C=O), 133.4 (C_b), 118.2 (C_{cd}), 100.6 (C_1'), 97.0 (C_1), 72.8 (C_3), 70.9 (C_5'), 70.8 (C_3'), 68.9~68.5 (C_2', C_4, C_a), 67.5 (C_5), 66.8 (C_4'), 62.6 & 61.1 (C_6 & C_6'), 48.8 (C_2), 23.4~20.5 ($7C_{methyl}$)

Anal. Calcd. for $C_{29}H_{41}O_{17}N_1$ (675.24): C, 51.50; H, 2.53; N, 2.10. Found: C, 50.07; H, 6.45; N, 1.90.

Allyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1-3)-4,6-2-acetamido-2-deoxy-di-O-acetyl-α-D-glucotopyranoside (37)



m.p. = 66.3~67.5 °C; R_f = 0.62 (EtOAc/Hex/MeOH, 12/4/1); $[\alpha]_D$ = +35.0° (c=1, $CHCl_3$); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{29}H_{41}O_{17}N_1$, 675.17, found, 676.3 (M+1, 12.0%); **^{1}H-NMR** δ ($CDCl_3$): 5.92~5.84 (m, 1H, H_b), 5.54 (d, 1H, J_{NH-2} = 10.1 Hz, NH), 5.32~5.30 (m, 2H, H_4', H_d), 5.27~5.22 (mi, 1H, H_c), 4.99 (dd, 1H, $J_{12'}$ = 7.6 Hz, $J_{23'}$ = 10.5 Hz, H_2'), 4.97~4.92 (m, 2H, H_3', H_4), 4.76 (d, 1H, J_{12} = 3.7 Hz, H_1), 4.53 (d, 1H, $J_{12'}$ = 7.6 Hz, H_1), 4.40~4.35 (m, J_{12} = 3.7 Hz, J_{23} = 10.3 Hz, H_2), 4.29~4.11 (m, 4H, $1H_6$, $2H_6'$, $1H_a$), 4.08 (dd, 1H, J_{56} = 10.6 Hz, J_{66} = 2.4 Hz, $1H_6$), 4.03~3.96 (m, 2H, $1H_6$, $1H_a$), 3.92~3.83 (m, 3H, H_3, H_5, H_5'), 2.11~1.93 (7 singlets, 21H, 7Ac); **^{13}C-NMR** δ ($CDCl_3$): 170.8~169.1 (7C=O), 133.2 (C_b), 118.7 (C_{cd}), 101.0 (C_1'), 96.8 (C_1), 76.2 (C_3), 70.9 (C_4), 70.3 (C_5), 69.0 (C_2'), 68.7 (C_a), 68.6 (C_3'), 68.1 (C_5), 66.8 (C_4'), 62.2 (C_6'), 60.9 (C_6), 52.2 (C_2), 23.4~20.5 (7 Ac).

Anal. Calcd. for $C_{29}H_{41}O_{17}N_1$ (675.24): C, 51.51; H, 6.13; N, 2.02. Found: C, 52.08; H, 6.36; N, 1.97.

Chapter 3. Functionalization of allyl glycosides

3.1. Introduction

The use of allyl glycosides in oligosaccharides is substantiated, when preparing glycoconjugates, by extending the length of aglycon through a thiol addition or functionalization to an aldehyde and carboxylic acid by ozonolysis[152] and/or transition metal catalyzed hydroformylation.[153] Primary alkylthiols bearing a different functional group at the opposite terminus, add to the double bond under mild, aqueous and U. V. light induced conditions. This reaction process allows for a stepwise lengthening of the spacer from one or two carbon elongation with allyl glycosides to a long unit. The resulting aglycon has a sulfide connection and this gives a spacer which is more hydrophilic than a hydrocarbon chain of similar length. The spacer modified carbohydrate haptens having different functional groups can be used in various conjugations.[177]

In addition, the acrylating procedure with acryloyl chloride can convert the terminal amine into acrylamidoalkylthio glycosides. Because these glycosides are basically N-substituted acrylamide, their reactivity during the polymerization is identical. These glycosides can also conjugate to other nucleophilic amine or thiol by the Michael addition to the acrylamide terminus. Once accomplished, the carbohydrate haptens can conjugate to proteins, polymers and dendrimers and the scope can be extended to other applications such as organometallic complexations.

Alternative routes to preparing functionalized carbohydrate haptens are via ozonolysis or hydroformylation of olefins to give the corresponding aldehydes with one carbon reduction or one carbon elongation. Transition metal complex catalyzed carbonylation of olefins,[153-a] acetylenes,[153-b] halides, alcohols,[153-c] nitro compounds,[153-b] etc have been extensively studied for insight into their reaction mechanisms as well as for applications of the reactions to organic syntheses. Recently, Alper *et al.*[153-a] found that a zwitterionic rhodium complex is an excellent catalyst for the hydroformylation of olefins with high regioselectivity observed for the branched or linear aldehyde depending on the

nature of the olefin. Rhodium complexes are also efficient catalyst for the selective formation of linear aldehydes in the presence of co-ligands.[154-156] Although many publications have been reported on these reactions, similar reactions involving carbohydrate moieties are rarely known. Therefore we applied this organometallic chemistry for the synthesis of hydroformylated carbohydrates.

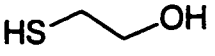
When olefins are treated with ozone at low temperature, they are converted to aldehydes. This reaction is very useful for introducing a carbonyl group to olefin as is achieved through hydroformylation. The results of the functionalization of allyl glycosides are described as follows.

3.2. Addition of thiol derivatives to allyl glycosides

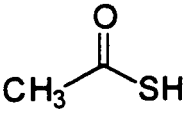
The lengthening of aglycon through an efficient thiol addition increases the applications of allyl glycosides. Bifunctional thiols bearing a different functional group at the opposing terminus add to olefins in aqueous medium.[157] Radical initiated thiol addition to allyl glycosides was used to prepare various 3-(alkylthio) propyl glycosides. Thiols used were shown below.

Cysteamine Hydrocloride, **38**

3-mercaptopropionic acid, **39**

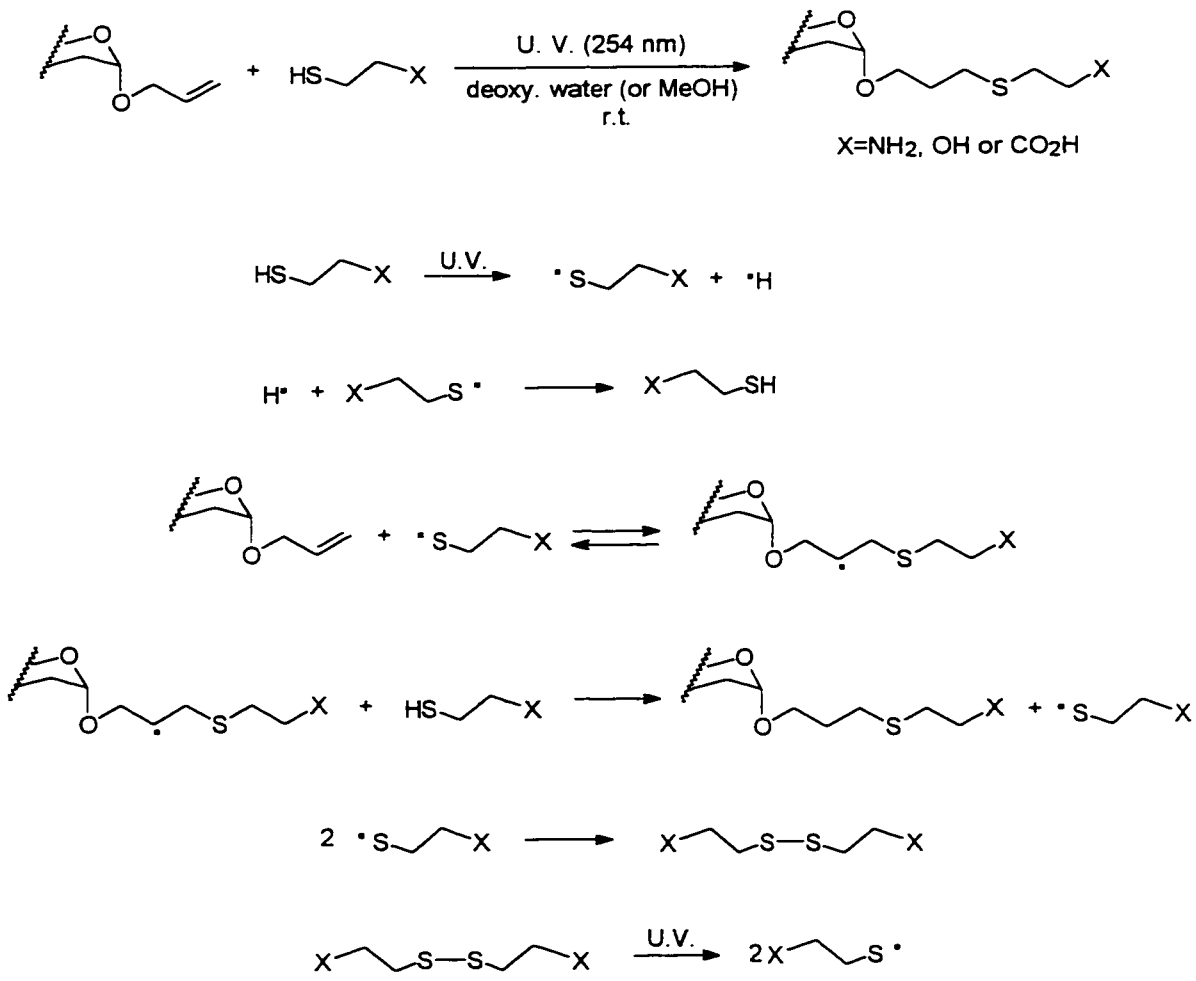


2-mercaptoethanol, **40**



Thioacetic acid, **41**

In the presence of radical initiator or U. V. light, thiols were added in an anti-Markovnikov fashion. Free radical mechanism were proposed in Scheme 3-1.



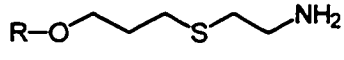
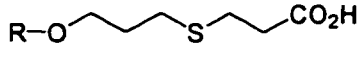
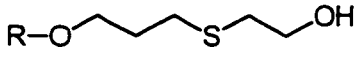
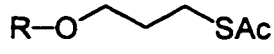
Scheme 3-1. Radical induced thiol addition to allyl glycosides.

The reaction is applicable to both protected and unprotected glycosides. A thiol is added in an appropriate solvent system depending on the glycoside, then the solution is irradiated intensively with a U.V. lamp.

The general procedure is described as follow. The appropriate thiol (**38**~**40**) and allyl glycoside (**29**, **30**, **36** or **37**) were dissolved in deoxygenated water (or MeOH). The solution was transferred to a quartz reactor and saturated with nitrogen then irradiated with a U.V. developing lamp (UVG-11 Mineralight) operating at 254 nm for 4~6 h. The desired product was purified with Dowex 50W (100 mesh) resin (NH_4^+ form) using a linear gradient in ammonium hydroxide for an unprotected sugar with X= NH_2. The

product (unprotected, X= CO_2H) was isolated with an anion exchange resin (Amberite IRA 400 OH^- form) column using an increasing acetic acid gradient. However an excess of disulfide remaining at the end of reaction often hampered purification. An excess of allyl glycoside was used to overcome this problem. Thioacetic acid was also reacted with allyl glycoside in the presence of AIBN in deoxygenated MeOH with refluxing to give thioester. Excess of thioacetic acid was used and regardless of whether they were protected or unprotected, the products could be separated easily from the disulfide formed by silica gel chromatography. 3-(alkylthio)propyl glycosides shown in Table 3-1 were isolated in good yields.

Table 3-1. Pysical properties of 3-(alkylthio) propyl glycosides.

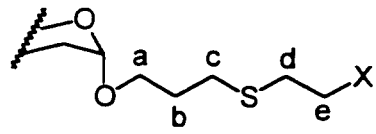
Glycoside	yield (%)	m.p. (°C)	$[\alpha]_D$
			
R= β-D-Gal-(1-3)-α-D-GalNAc (OH), **42**	63	108.5~109.6	+74.0° (1, H_2O)
β-D-Gal-(1-3)-α-D-GlcNAc (OH), **48**	90	113.0~115.0	+77.0° (1, H_2O)
			
R= β-D-Gal-(1-3)-α-D-GalNAc (OH), **43**	83	90.0~92.5	+76.0° (1, H_2O)
β-D-Gal-(1-3)-α-D-GalNAc (OAc), **44**	73	54.7~55.8	+55.8° (1, $CHCl_3$)
			
R= β-D-Gal-(1-3)-α-D-GalNAc (OAc), **45**	83	177.2~178.9	+75.9° (1.1, $CHCl_3$)
β-D-Gal-(1-3)-α-D-GlcNAc (OAc), **49**	90	136.5~137.8	+81.5° (0.7, $CHCl_3$)
			
R= β-D-Gal-(1-3)-α-D-GalNAc (OH), **46**	71	119.6~122.5	+84.0° (1, H_2O)
β-D-Gal-(1-3)-α-D-GalNAc (OAc), **47**	82	54.5~57.4	+69.6° (1.25, $CHCl_3$)
β-D-Gal-(1-3)-α-D-GlcNAc (OAc), **50**	79	55.2~59.2	+37.8° (1, $CHCl_3$)

These glycosides containing a sulfide-bridged aglycon have the following versatile applications for the conjugations. Amine terminus glycosides can undergo peptide

coupling to a carboxylic acid, N-acryloylation followed by polymerization or conjugate addition to another amine or thiol. Details were described in Chapter 7. Carboxylic acid terminus glycosides can also undergo the coupling reaction as does an amine terminus one. Thioacetates are deprotected to the corresponding thiols[159] under Zemplén condition then are coupled to an acryl group by conjugate addition. These reactions are straightforward and provide useful strategies to accomplish glycoconjugation. The structures were assigned based on ^{1}H- and ^{13}C-NMR and coincident with the proposed compounds (Table 3-2).

Table 3-2. ^{13}C-NMR Chemical Shifts of 3-(alkylthio)propyl glycosides.

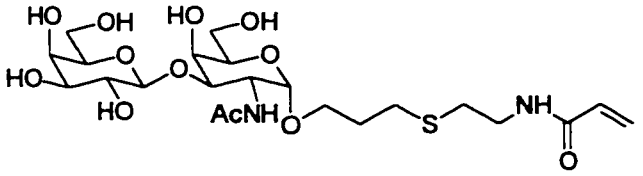
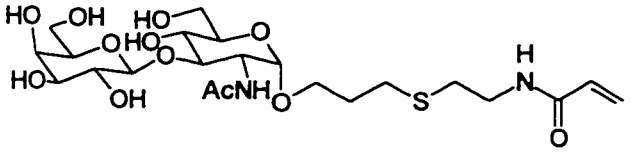
glycoside	C-1	C-1′	C-a	C-b	C-c	C-d	C-e	X
42[b]	96.7	104.2	66.0	27.9	27.2	30.8	38.6	-
43[b]	96.7	104.2	66.0	27.9	27.6	34.2	26.0	174.0 (CO_2H)
44[c]	97.0	100.6	65.4	28.0	28.0	34.3	26.1	173.7 (CO_2H)
45[c]	96.9	101.1	66.3	29.3	29.6	35.4	60.9	-
46[b]	96.7	104.2	65.7	27.8	25.2	-	-	-
47[c]	97.8	100.7	65.7	29.5	25.3	-	-	-
48[b]	96.6	102.9	65.9	27.9	27.2	30.7	38.6	-
49[c]	97.6	101.0	66.6	29.0	29.2	35.4	60.9	-
50[c]	97.6	101.1	65.5	29.5	25.2	-	-	-



a: Assignments were based on ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC experiments.
b: δ (ppm, D_2O)
c: δ (ppm, $CDCl_3$)

3-(2-N-acrylamidoethylthio)propyl glycosides **51** and **52**, of β-D-Gal-(1-3)-α-D-GalNAc and β-D-Gal-(1-3)-α-D-GlcNAc were prepared from precusor amines in quantitative yields. 3-(2-N-aminoethylthio)propyl glycosides **42** or **48**, were acrylated by a slow addition of acryloyl chloride in $CHCl_3$ to a methanolic solution at ice-water temperature. The use of anionic exchange resin (OH^- form) supports the reactivity of amines by HCl absorption. Once completed, the solution was filtered and the resin was washed with MeOH. Brief treatment with cationic resin (H^+ form) for neutrality followed by evaporation gave N-acryloylated glycosides. These compounds are susceptible to be polymerized by heat or light exposure. Therefore evaporation of solvent should be carried out at least lower than room temperature.

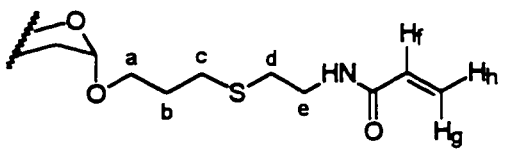
Table 3-3. Physical properties of **51** and **52.**

Glycosides		m.p. (°C)	$[\alpha]_D$
	**51**	N/A[a]	+92.2° (C = 1, H_2O)
	**52**	N/A[a]	+53.0° (C = 1, H_2O)

a: Thermal data could not be measured due to the polymerization.

Table 3-4. ^{13}C-NMR chemical shifts of **51** and **52.**[a]

glycoside	C-1	C-1′	C-a	C-b	C-c	C-d	C-e	C-f	C-gh
51	96.7	104.2	66.0	28.0	27.4	29.9	38.2	129.4	126.9
52	96.5	102.9	65.9	27.9	27.4	29.9	38.2	129.4	127.0



a: chemical shifts are in ppm for D_2O referenced to 4.63 ppm.

Because of stability problems **51** and **52** were prepared in situ just before the reaction to prevent undesired reactions. 3-(2-N-acrylamidoethylthio)propyl glycosides, **51** and **52** were analyzed and the physical properties are shown in Table 3-3 and 3-4.

3.3. Hydroformylation

3.3.1. Reaction conditions and mechanism

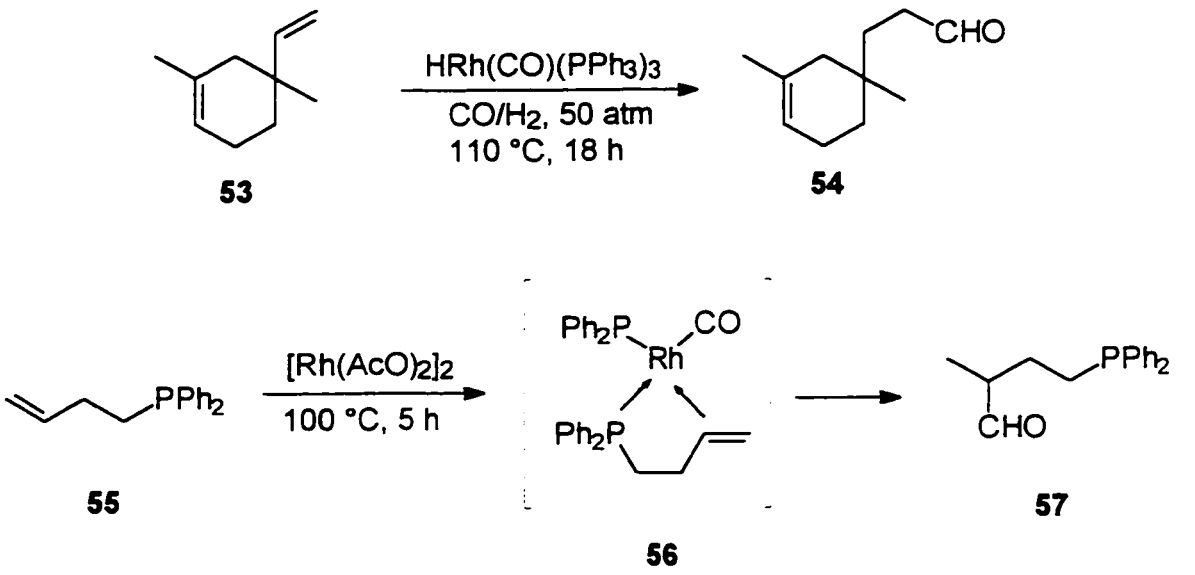
Several transition metal complexes have been employed as catalysts for the hydroformylation reaction. Cobalt,[160] rhodium,[157, 161] and ruthenium[162] based catalysts were more specifically overviewed. Cobalt complexes such as $Co_2(CO)_8$ and $Co_2(CO)_6(PPh_3)_2$ found to catalyze reactions of 1-hexene were reported and the modified catalyst with diphenylphosphine gave a high linear/branched aldehyde ratio at the beginning of the reaction.[163] The cluster $MeCCo_3(CO)_7$(DPM) where DPM is bis(diphenylphosphino)methane has been used as a catalyst to hydroformylate 1-pentene.[164] Ruthenium based complexes such as $Ru_2(O_2CR)_2(CO)_4L_2$[165] where R is Me, Ph, CF_3 or t-Bu and L is PPh_3 or $P(OMe)_3$ catalyze the hydroformylation of n-alkenes and further reduction leads to alcohol. However these transition metal-catalyzed reactions reveal low activities and a mixture of linear/branched usually results. The catalytic activity of rhodium complexes was found to be superior compared with cobalt or ruthenium complexes. As well, they were determined to be very effective catalysts for the regioselective hydroformylation of olefins. An investigation to find an effective catalytic system for improved regioselective aldehydes has been performed and the following factors were determined to contribute to regioselectivity;

i) steric effect of olefin

ii) ligand chelation effect to catalyst

For instance, 1,5-dimethyl-5-vinylcyclohexene, **53** gave the linear aldehyde, **54,** selectively (Scheme 3-2).[166] The regioselectivity is also affected by chelation of an alkene having heteroatoms like N, O or P as substituents. Hydroformylation of an alkenyl phosphine, **55**

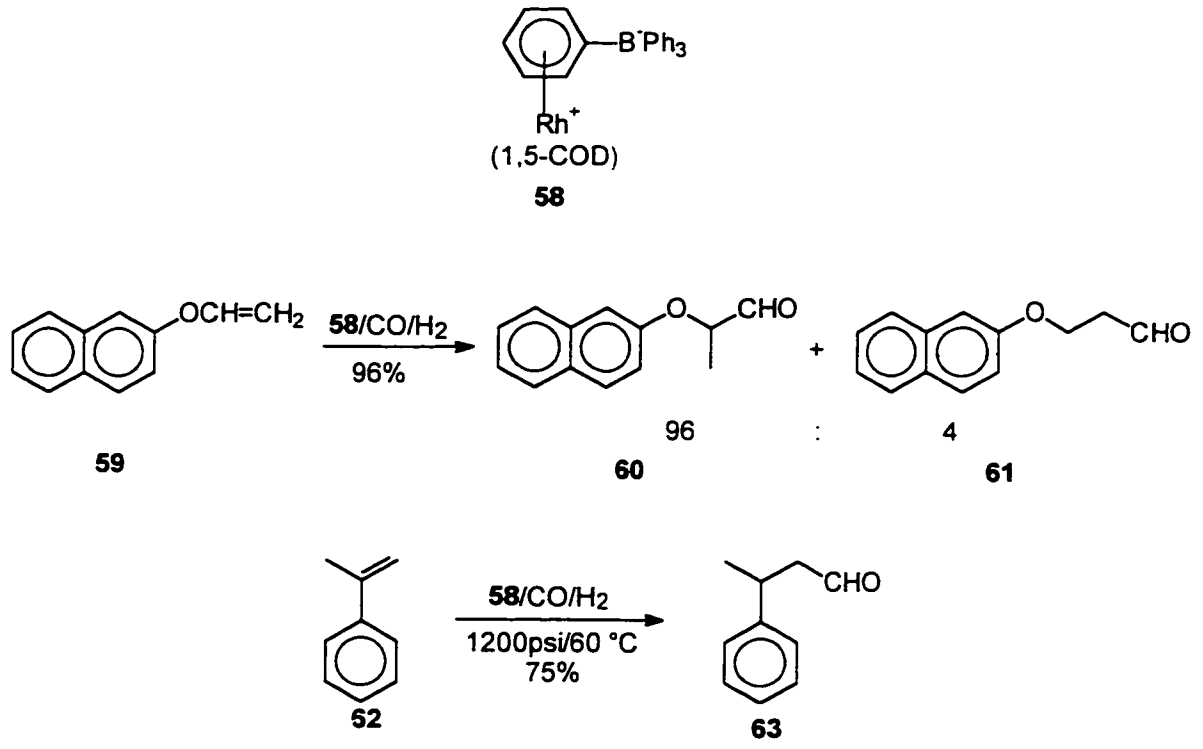
involves chelation of the phosphine to rhodium and the selective addition of the formyl group **57** results *via* five membered ring chelation **56**.[167]



Scheme 3-2. Rhodium catalyzed Hydroformylation of olefins.

The Rh-catalyzed hydroformylation of vinyl ethers, $ROCH{=}CH_2$ (R= alkyl, benzyl) gave a mixture of 2-alkoxy (or phenoxy)- and 3-alkoxy (or phenoxy)-propanal.[169] Addition of PPh_3 to the catalytic system increased β-isomer aldehyde formation. The photochemical hydroformylation reaction of 1-octene catalyzed by $RhCl_3$/norbornadiene proceeded at room temperature to give more than 90% selectivity for n-aldehydes.[168] Alper *et al.*[170] found the zwitterionic rhodium complex **58**, η^6-$C_6H_6BPh_3Rh(COD)$ was a very efficient and regioselective catalyst for the preparation of the branched aldehyde, **60**. When 1,1-disubstituted olefin **62** was used, the terminus aldehyde **63** was obtained selectively (Scheme 3-3).

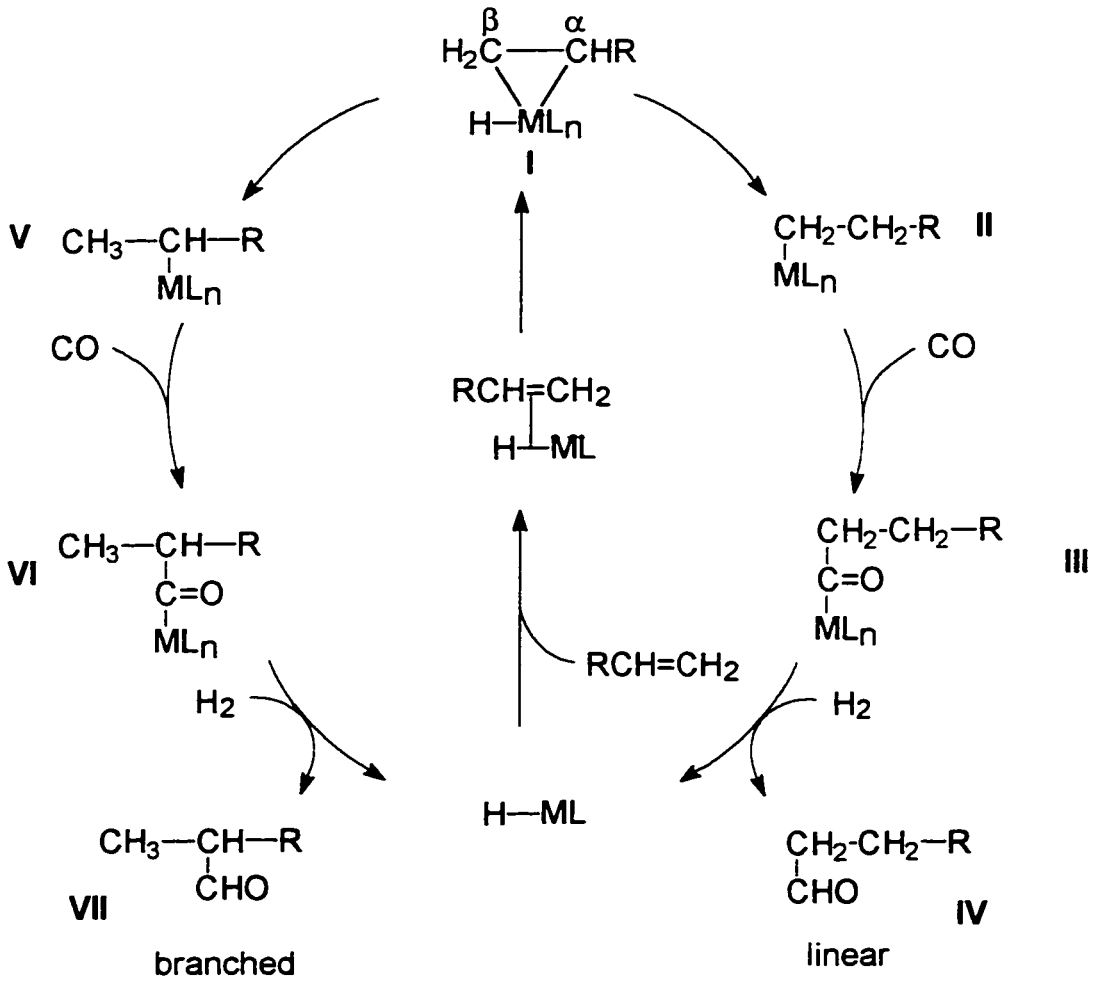
Generally simple aliphatic olefins are shown to have lack of regiocontrol while the presence of bulky substituents leads to less hindered aldehyde formation in a regioselective process. The remarkable dependency of regioselectivity on the catalyst metal species



Scheme 3-3. Zwitterion complex catalyzed Hydroformylation of olefins.

may well be accommodated by considering the stability and the isomerization ability of branched alkyl metal species and the relative rate of the migratory insertion of carbon monoxide into branched and linear alkyl-metal bonds. The regioselective hydroformylation of allyl acetate[171] was accomplished in the presence of **58** and 1,4-bis(diphenylphosphino) butane (dppb) (Rh/dppb, 1/2) with a ratio of linear to branched chain aldehyde of 95:5. A proposed mechanism is described in Scheme 3-4.

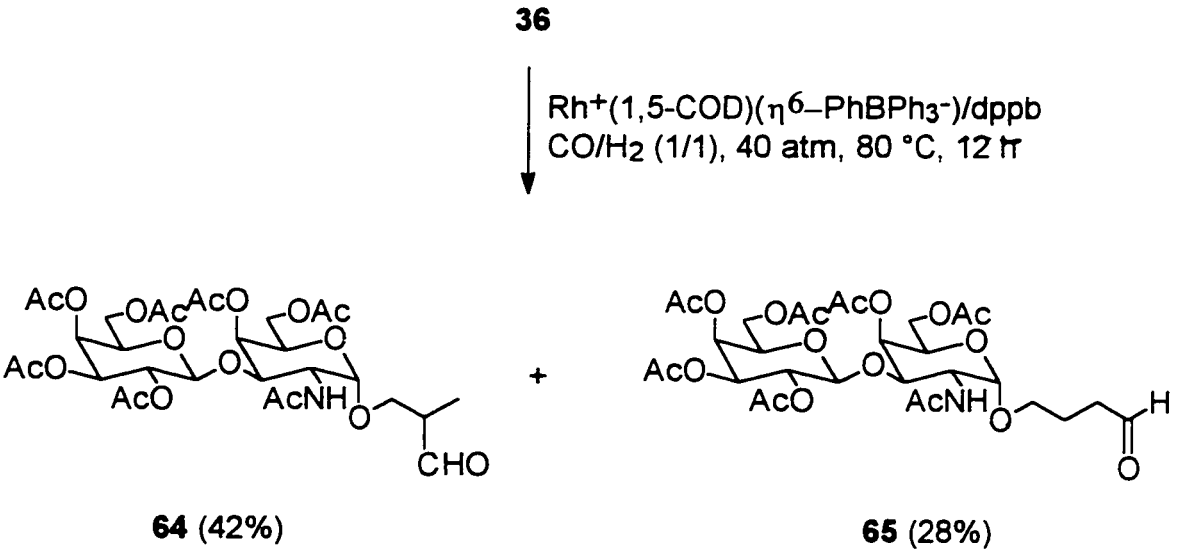
When an electron donating substituent is introduced into an olefin, the metal-C_α bond of an η^2-olefin-metal complex, **I** becomes stronger than the metal-C_β bond because of substantial stabilization of the formal negative charge developing on the C_α. Therefore the formation of the branched alkyl-metal species, **VI** should be more favorable than of the linear alkyl-metal species, **II**. Subsequent carbon monoxide insertion (**III** or **VI**) and hydrogenation forms linear- (**IV**) or branched- (**VII**) aldehyde.



Scheme 3-4 Proposed mechanism of the hydroformylation of olefins.

3.3.2 Results and Discussion

Because the Rh-complex **58** and 1,4-bis(diphenylphosphino)butane show good regioselectivity for simple olefins, this system was chosen for the hydroformylation of peracetylated allyl β-D-Gal-(1-3)-α-D-GalNAc **36** where the molar ratio of the Rh (I) catalyst **58** to dppb was 1/2. The solution was pressurized by syngas (CO/H_2, 1/1) to 40 atm. and stirred at 80 °C for 12 h. After evaporation of the solvent, the residue was purified by silica gel chromatography to give branched **64** and linear **65** aldehyde in 42% and 28% yield, respectively (Scheme 3-5).



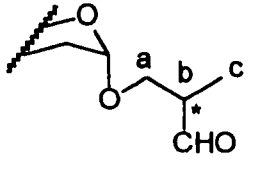
64 (42%) **65** (28%)

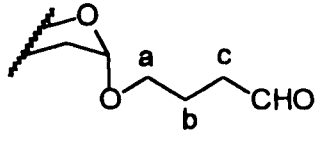
Scheme 3-5 Hydroformylation of peracetylated allyl β-D-Gal-(1-3)-α-D-GalNAc **36.**

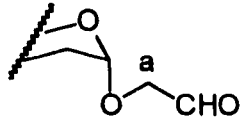
The structures of **64** and **65** were analyzed by ^{1}H- and ^{13}C-NMR spectroscopy and chemical shifts of the aglycons were very informative (Table 3-5). In the case of branched aldehyde **64**, H-a and H-b showed a strong deshielding effect due to electron withdrawing effect of the hydroformyl functional group. A $\Delta\delta$ = 0.3~0.4 ppm downfield shifts were observed for H-a compared with those of the linear aldehyde **65**. Proton H-b where the hydroformyl group is directly attached, showed a huge downfield shift when compared with H-b of **65** ($\Delta\delta$ = 0.8 ppm and the splitting pattern is much more complicated). Because **64** possesses a new chiral center at the C-b position, two different stereoisomers exist. As a result, two H-b signals are expected with different chemical shifts. However, the hydroformyl proton showed two doublets at 9.66, and 9.65 ppm.

Table 3-5. ^{1}H- and ^{13}C-NMR chemical shifts of **64**, **65** and **66**.[a]

Compd	^{1}H (δ, ppm)[b]					^{13}C (δ, ppm)[b]					
	H-a	H-b	H-c	NH	CHO	C-1	C-2	C-a	C-b	C-c	CHO
64	4.18~4.10	2.81~2.62	2.24~2.11	5.60[c]	9.66[d]	98.5	49.0	67.5	46.6	10.5	196.8
					9.65	97.8	49.0		46.3		
65	3.81~3.76	2.01~1.95	2.61~2.29	5.78	9.72	98.2	48.9	68.5	23.2~	41.8	202.9
	3.38~3.31								20.5[e]		
66	4.31	-	-	6.04	9.47	99.0	48.8	73.4	-	-	197.3

64: 

65: 

66: 

a: recorded on a bruker AMX-500 MHz spectrometer at ambient temperature.
b: assignment is based on COSY and HMQC experiments. Chemical shifts are referenced to internal $CDCl_3$ and are given in ppm.
c: the signal shows two set of doublets.
d: the C=O signal shows 2 doublets.
e: exact chemical shift of C-b is not available due to overlapping with other signals.

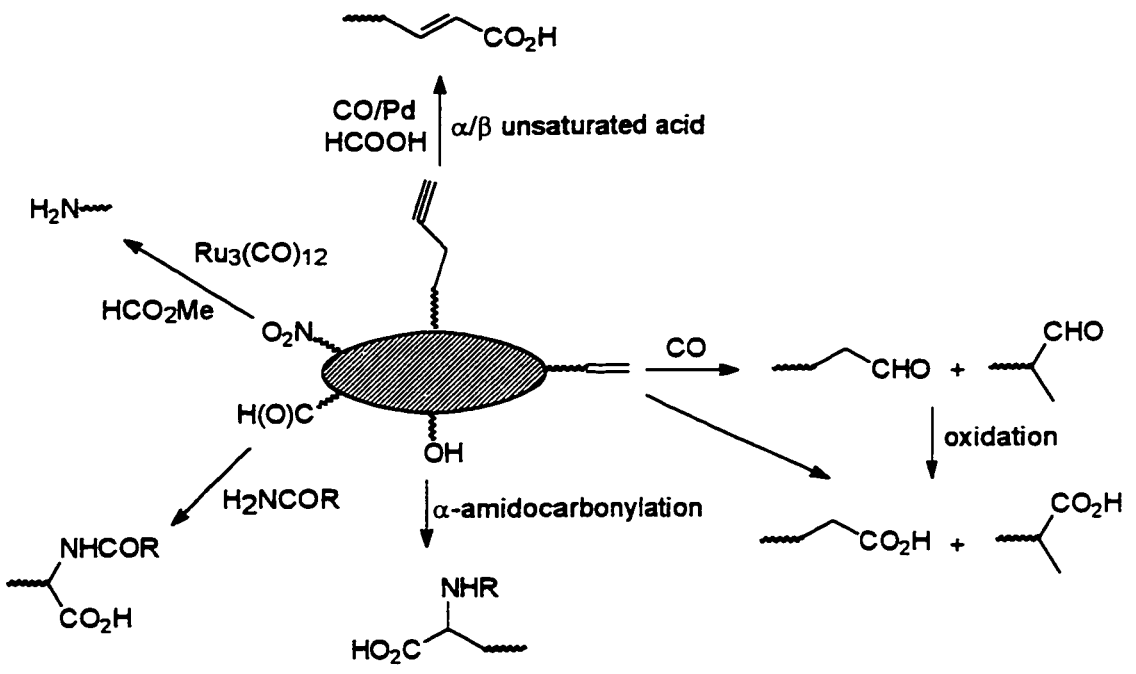
Actually the two protons at C-a are not chemically equivalent and this intrinsic property induces chirality and eventually affect the hydroformyl proton by long range coupling interaction. These two chiral centers induce two doublets. Overall integration of these signals demonstrates one proton. However, hydroformyl proton of **65** shows a singlet at 9.72 ppm. Another characteristic splitting pattern is observed for the amide proton of **64** at 5.60 ppm where two set of doublets are detected. As we saw in Table 3-5, H-c of **65** showed a downfield shift to 2.61~2.29 ppm compared with that of **64** (2.24~2.11 ppm) due to the neighboring hydroformyl deshielding effect. In the ^{13}C-NMR spectra, the chemical shifts of C-a of **64** and **65** show approximately the same values, 67.5 and 68.5 ppm, respectively. Due to the deshielding effect, significant downfield shifts of C-b of **64** and C-c of **65** are observed with $\Delta\delta$ = 23 and 31 ppm, respectively when compared with the corresponding isomers (Table 3-5). The carbonyl carbons in a hydroformyl group also show at 196.8 ppm for **64** and 202.9 ppm for **65**. From the existing chiral center at C-b and the other interaction (it could be the spatial interaction between the amide proton and the hydroformyl group which causes two doublets of the amide proton) in **64**, two carbonyl signals are detected at 46.6 and 46.3 ppm for C-b as well as 98.5 and 97.8 ppm for C-1 and 49.01 and 48.95 ppm for C-2. However, **65** shows normal ^{13}C-NMR pattern with their own values.

A lot of hydroformylation reactions are reported and, in many cases, poor regioselectivity was observed. As a result, a mixture was emerged which is a common drawback. We also face the same problem. More detailed research should be undertaken to overcome this problem. To solve this, the following variables should be investigated;

i) changing of the ratio of Rh^{+} complex/phosphine ligand

ii) other transition metal complexes such as cobalt, ruthenium or iridium as catalysts

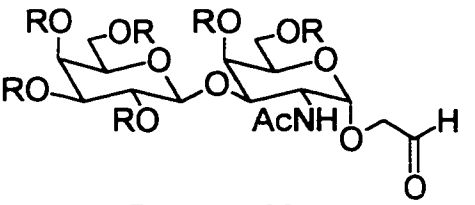
We should broaden our knowledge to other sugar moieties such as lactose, mannose, glucose, galactosamine or even oligosaccharides. Carbonylation of sugars bearing alcohols, amines, alkynes and nitro group is valuable challenge to extend the application of organometallic chemistry to carbohydrates (Scheme 3-6).



Scheme 3-6. Possible carbonylation of carbohydrate haptens.

3.4. Ozonolysis

Ozonolysis has been used for determining the structures of unsaturated compounds and it resulted in the degradation of large molecules into smaller fragments. For instance, ozonides are oxidized with peracids or H_2O_2 to afford carboxylic acids. They are reduced with $LiAlH_4$, $NaBH_4$, BH_3 or through catalytic hydrogenation with H_2 to afford alcohols.[175] Ozonides also can be reacted with ammonia to give the corresponding amine.[176] Allyl T-antigen **29** or **36** in $MeOH/CH_2Cl_2$ were treated with ozone at -78 °C and saturated with O_2. Ozone was then bubbled into the solution for 30 min.. The blue-colored solution indicated the formation of ozonide. The hydroformyl functional group was induced from ozonide by dimethyl sulfide as a reducing agent. Trimethyl phosphine[173] and thiourea[174] are viable subsittutes for dimethyl sulfide. The solution was kept in the cold room overnight. The solvent was evaporated to give a quantitative yield of aldehydes **66** and **67**. Along with carbonylation, ozonolysis is also an important synthetic strategy.



R= Ac **66**

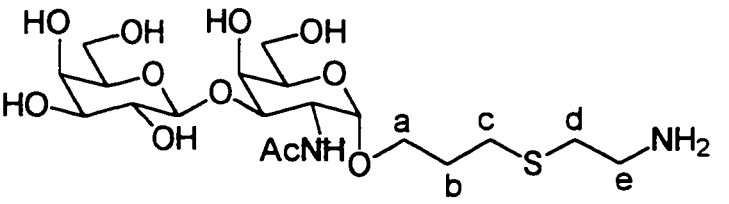
R= H **67**

3.5. Conclusions

Bifunctional thiol addition to allyl glycosides was performed successively through simple photoreaction. The elongated sulfide-bridged aglycons with novel functionalities at the opposite terminus have potentials to extend their applications. When hydroformylation of allyl glycoside was explored using a rhodium complex and a phosphine ligand, a mixture of linear and branched aldehydes was obtained in good yields. To improve the regioselectivity, an extensive study should be undertaken with various transition metal complexes as catalysts. Although the present study was limited to the hydroformylation of allyl T-antigen, our scheme should be equally applicable to different olefinic system and other sugars. The usefulness of the insertion of carbonyl groups was further substantiated by the ozonolysis of allyl glycosides.

3.6. Experimental methods

3-(2-Aminoethylthio)-propyl O-(β-D-galactopyranosyl)-(1-3)- 2-acetamido-2-deoxy - α-D-galactopyranoside (42)

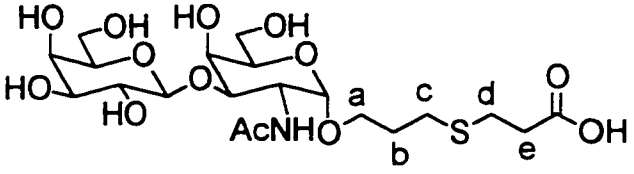


Allyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-α-D galactopyranose (**29**) (50 mg 0.118 mmol) and 12.5 mg (0.11 mmol) of cysteamine hydrochloride were dissolved in 1ml of deoxygenated water and irradiated (λ = 254 nm) under nitrogen atmosphere for 4.5 h. TLC (EtOAc/HOAc/H_2O, 3/2/2) indicated cysteamine was consumed completely (R_f = 0.44). The product was purified by cation exchange chromatography using Dowex 50W (100 mesh, NH_4^+ form). The product was applied to the column (27 x 0.9 cm) and eluted first with distilled water to give unreacted **29** followed by elution with a linear gradient (0 to 1.5 M, 1 L) in NH_4OH to give product. Product fractions were concentrated under reduced pressure to remove ammonia and the volume was reduced prior to lyophilization. Multiple lyophilization from water gave 36.9 mg (0.074 mmol, 63%) of **42** as a white sponge solid: m.p. = 108.5~109.6 °C; $[\alpha]_D$ = +74.0° (c = 1.00, H_2O); (+) FAB-MS (glycerol): calcd. for $C_{19}H_{36}N_2O_{11}S_1$, 500.20, found, 501.2 (M+1, 19.6%); **^{1}H-NMR** δ (D_2O): 4.97 (d, 1H, J_{12} = 3.8 Hz, H_1), 4.54 (d, 1H, J_{12}' = 7.8 Hz, H_1'), 4.40 (dd, 1H, J_{12} = 3.8 Hz, J_{23} = 11.1 Hz, H_2), 4.31 (d, 1H, J_{34} = 3.0 Hz, H_4), 4.10 (dd, 1H, J_{23} = 11.1 Hz, J_{34} = 3.0 Hz, H_3), 4.06 (m, 1H, H_5), 3.98 (d, 1H, J_{34}' = 3.4 Hz, H_4'), 3.90~3.79 (m, 5H's, $2H_6$, $2H_6'$, $1H_a$), 3.726 (m, 1H, H_5'), 3.69 (dd, 1H, J_{23}' = 9.9 Hz, J_{34}' = 3.4 Hz, H_3'), 3.64 (m, 1H, H_a), 3.59 (dd, 1H, J_{12}' = 7.8 Hz, J_{23}' = 9.9 Hz, H_2'), 3.05 (m, 2H's, H_c), 2.82 (t, 2H's, J_{de} = 6.6 Hz, H_d), 2.76 (t, 2H's, J_{bc} = 7.2 Hz, H_c), 2.10 (s, 3H's, H_{CH_3}), 1.98 (m, 2H's, H_b); **^{13}C-NMR** δ (D_2O): 174.0 (C=O), 104.2 (C_1'),

96.7 (C_1), 76.7 (C_3), 74.5 (C_5'), 72.0 (C_3'), 70.1 (C_2', C_5), 68.2 (C_4), 68.1 (C_4'), 66.0 (C_a), 60.7 (C_6'), 60.5 (C_6), 48.2 (C_2), 38.6 (C_e), 30.8 (C_d), 27.9 (C_b), 27.2 (C_c), 21.5 (C_{methyl}).

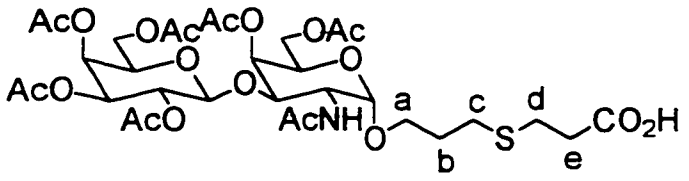
Anal. Calcd. for $C_{19}H_{36}O_{11}N_2S_1$ (500.20): C, 45.63; H, 7.31; N, 5.64. Found: C, 45.09; H, 6.95; N, 5.07.

3-(2-Carboxyethylthio)propyl β-D-galactopyranosyl-(1-3)-2-acetamido-2-deoxy-α-D-galactopyranoside (43)



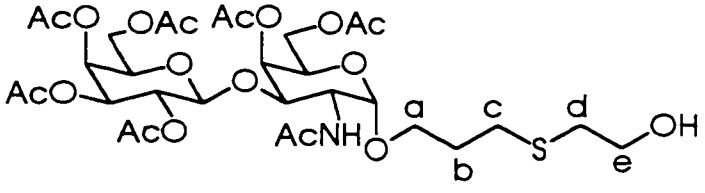
Allyl β-D-Gal-(1-3)-α-D-GalNAc (**29**) (100 mg, 0.24 mmol) was dissolved in deoxygenated distilled water (2.5 mL). 3- Mercaptopropionic acid (21 μL, 1 eq.) was added and the solution was saturated with N_2. The solution was then irradiated (254 nm) for 7 h. The reaction solution was loaded on an anion exchange resin (Amberite IRA 400 OH^-) column which was eluted with water followed by an increasing acetic acid gradient. The product-containing portion was pooled and concentrated under reduced pressure followed by multiple coevaporation with ethanol. A small amount of water was added to dissolve the product and lyophilized to afford 105.9 mg (2.0 mmol, 83%) of white spongy solid: m.p. = 90.0~92.5 °C; $[\alpha]_D$ = +76.0° (c = 1, H_2O); R_f = 0.33 ($CHCl_3$/MeOH/H_2O, 10/9/1); (+) FAB-MS (glycerol): 530.3 (M+1, 9.1%); **^{1}H-NMR**, δ (D_2O): 4.96 (d, 1H, J_{12} = 3.7 Hz, H_1), 4.54 (d, 1H, J_{12}' = 7.7 Hz, H_1'), 4.39 (dd, 1H, J_{12} = 3.7 Hz, J_{23} = 11.0 Hz, H_2), 4.31 (broad d, 1H, H_4), 4.10 (dd, 1H, J_{23} = 11.1 Hz, J_{34} = 3.1 Hz, H_3), 4.07 (broad s, 1H, H_5), 3.98 (broad d, 1H, J_{34}' = 3.5 Hz, H_4'), 3.89~3.79 (m, 5H, H_6, H_6', H_a), 3.74~3.72 (m, 1H, H_5'), 3.69 (dd, J_{23}' = 10.0 Hz, J_{34}' = 3.4 Hz, H_3'), 3.65~3.57 (m, 2H, H_2', H_a), 2.89 (t, 2H, J_{de} = 7.5 Hz, H_e), 2.80~2.73 (m, 4H, H_c, H_d), 2.10 (s, 3H, H_{methyl}), 2.01~1.96 (m, 2H, H_b); **^{13}C-NMR**, δ (D_2O): 174.0 (C=O), 104.2 (C_1'), 96.7 (C_1), 76.8 (C_3), 74.5 (C_5'), 72.0 (C_3'), 70.1 (C_2'), 70.1 (C_5), 68.2 (C_4), 68.1 (C_4'), 66.0 (C_a), 60.7 & 60.5 (C_6 & C_6'), 48.2 (C_2), 34.2 (C_d), 27.9 (C_b), 27.6 (C_c), 26.0 (C_e), 21.5 (C_{methyl}).

3-(2-Carboxyethylthio)propyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyronosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-galactopyranoside (44)



To a solution of peracetylated allyl Gal-(1-3)-GalNAc (**29**) (0.18 g, 0.26 mmol), mercaptopropionic acid (23 μL, 1 eq.) in deoxygenated MeOH (10 mL) was added a catalytic amount of AIBN and the solution was refluxed for 1 day under N_2 atmosphere. The solvent was then evaporated under reduced pressure. The residue was diluted with $CHCl_3$ (15 mL), washed with water, dried (Na_2SO_4) and condensed. The crude product was purified by silica gel column chromatography with EtOAc/AcOH (30/1) as an eluent and dried in vacuo to give 0.15 g (0.19 mmol, 73%) of white solid: m.p. = 54.7~55.8 °C; R_f = 0.38 (EtOAc/AcOH, 30/1); $[\alpha]_D$ = +55.8° (c = 1.2, $CHCl_3$); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{32}H_{47}O_{10}N_1S_1$, 781.25, found, 782.33 (M+1, 8.3%); **^{1}H-NMR** δ ($CDCl_3$); 2.78 (t, 2H, J_{de} = 7.4 Hz, H_e), 2.70~2.55 (m, 4H, H_c, H_d), 2.27~1.91 (m, 23H, 7Ac, H_b); **^{13}C-NMR** δ ($CDCl_3$); 65.4 (C_a), 34. 3 (C_d), 28.0 (C_b), 28.0 (C_c), 26.1 (C_e). The chemical shifts of other parts were the same as described for its precursor.

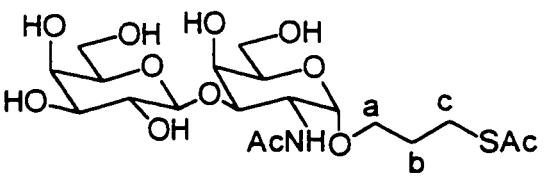
3-(2-Hydroxyethylthio)propyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyronosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-galactopyranoside (45)



A solution of peracetylated allyl β-D-Gal-(1-3)-α-D-GalNAc (**29**) (130 mg, 0.19 mmol) and 2-mercaptoethanol (14.9 μL, 1.1 eq.) in deoxygenated methanol (10 mL) was irradiated (λ = 254 nm) under N_2 atmosphere for 3.5 h. The solution was then evaporated to dryness. The residue was dissolved in $CHCl_3$ (30 mL), washed with water (3x) and dried (Na_2SO_4). The solution was condensed and purified by silica gel column chromatography with EtOAc/Hex/MeOH (6/3/1) as an eluent. The product was then dried in vacuo to give 122 mg of white solid (0.16 mmol, 83%): m.p. = 177.2~178.9 °C; R_f = 0.24 (EtOAc/Hex/MeOH, 12/4/1); $[\alpha]_D$ = +75.9° (c = 1.1, $CHCl_3$); (+) FAB-MS (glycerol) (m/z); calcd. for $C_{31}H_{47}O_{18}N_1S_1$, 753.25, found, 754.30 (M+1, 50%); **^{1}H-NMR**, δ ($CDCl_3$); 4.72 (d, 1H, J_{12} = 3.6 Hz, H_1), 4.56 (d,1H, J_{12}' = .6 Hz, H_1'), 4.00~3.76 (m, 3H, 1H_a, 2H_c), 3.53~3.49 (m, 1H, 1H_a), 2.95 (t, 1H, $J_{c\text{-}OH}$ = 5.8 Hz, OH), 2.96~2.69 (m, 4H, H_d, H_c), 2.12 (s, 3H, AcNH), 2.07~1.91 (m, 21H, 6Ac, 2H_b); **^{13}C-NMR** δ (D_2O); 101.1 (C_1'), 96.9 (C_1), 66.3 (C_a), 60.9 (C_c), 35.4 (C_d), 29.6 (C_c), 29.3 (C_b), 23.5~20.5 (7 C_{methyl})

Anal. Calcd. for $C_{31}H_{47}O_{18}N_1S_1$ (753.25): C, 49.40; H, 6.36; N, 1.93. Found: C, 48.77; H, 6.22; N, 1.80.

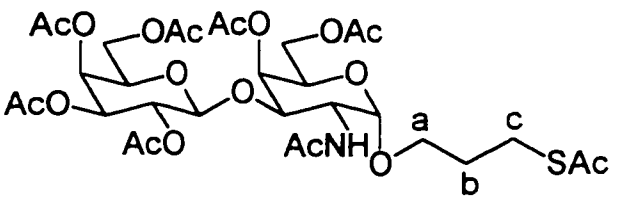
3-Thioacetyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-α-D-galactopyranoside (46)



Allyl β-Gal-(1-3)-α-GalNAc (**29**) (60 mg, 0.142 mmol) and 63 μL (0.85 mmol) of thioacetic acid was dissolved in 3 mL of deoxygenated methanol and refluxed for 1 day under nitrogen atmosphere. The solution was then evaporated under reduced pressure and redissolved with chloroform. This organic solution was washed with saturated $NaHCO_3$, distilled water (3x) and dried with sodium sulfate. The crude product was purified by silica gel column chromatography with $CHCl_3$/MeOH/H_2O, 7/4/0.8, and dried under vacuum to

afford 50 mg (71%) of ivory colored powder: m.p. = 119.6~122.5 °C; $[\alpha]_D$ = +83.0° (c = 1, H_2O); R_f = 0.69 ($CHCl_3$/MeOH/H_2O, 10/5/1); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{19}H_{33}O_{12}N_1S_1$, 499.17, found, 500.2 (M+1, 19.0%); **^{1}H-NMR**, δ (D_2O): 4.95 (d, 1H, J_{12} = 3.8 Hz, H_1), 4.55 (d, 1H, $J_{12'}$ = 7.8 Hz, $H_{1'}$), 4.39 (dd, 1H, J_{12} = 3.8 Hz, J_{23} = 11.1 Hz, H_2), 4.31 (d, 1H, J_{34} = 3.1 Hz, $J_{45} \leq$ 1 Hz, H_4), 4.10 (dd, 1H, J_{23} = 11.1 Hz, J_{34} = 3.1 Hz, H_3), 4.06~4.03 (m, 1H, H_5), 3.98 (d, 1H, $J_{34'}$ = 3.4 Hz, $H_{4'}$), 3.86~3.79 (m, 5H's, $2H_6$, $2H_{6'}$, $1H_a$), 3.74~3.72 (m, 1H, $H_{5'}$), 3.70 (dd,1H, $J_{23'}$ = 10.0 Hz, $J_{34'}$ = 3.3 Hz, $H_{3'}$), 3.59 (dd, 1H, $J_{12'}$ = 7.8 Hz, $J_{23'}$ = 9.9 Hz, $H_{2'}$), 3.60~3.56 (m, 1H, $1H_a$), 3.13~3.04 (m, 2H's, H_c), 2.46 (s, 3H's, -SAc), 2.10 (s, 3H's, AcNH-), 2.00~1.94 (m, 2H's, H_b); **^{13}C-NMR**, δ (D_2O): 201.4 (carbonyl carbon in thioacetyl group), 174.1 (carbonyl carbon in N-acetyl group), 104.2 ($C_{1'}$), 96.7 (C_1), 76.8 (C_3), 74.5 ($C_{5'}$), 72.0 ($C_{3'}$), 70.1~70.1 ($C_{2'}$, C_5), 68.3 (C_4), 68.1 ($C_{4'}$), 65.7 (C_a), 60.7 (C_6), 60.5 ($C_{6'}$), 48.2 (C_2), 29.5 (methyl carbon in thioacetyl group), 27.8 (C_b), 25.2 (C_c), 21.5 (methyl carbon in N-acetyl group).

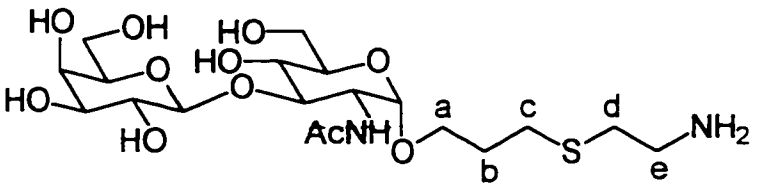
(3-Thioacetyl)propyl (2,3,4,6-tetra-O-acetyl β-D-galactopyronosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-galactopyranoside (47)



To a solution of allyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-galactopyranoside (**36**) (77.5 mg, 0.12 mmol) and thioacetic acid (50 μL, 6 eq.) in deoxygenated MeOH (3 mL) was added AIBN (3.8 mg, 0.1 eq.) and the reaction mixture was refluxed for 4 days under N_2 atmosphere. The resulting solution was condensed and dried in vacuo. The residue was dissolved in CH_2Cl_2 (10 mL) and washed successively with saturated sodium bicarbonate (5 mL) and water and dried with sodium sulfate. The solution was condensed under reduced pressure and the residue was purified by silica gel column chromatography with EtOAc/Hex/MeOH

(12/5/1) as an eluent to give 70.5 mg of white solid (94 nmol, 82%): m.p.= 54.5~57.4 °C; R_f = 0.39 (EtOAc/Hex/MeOH, 12/4/1); $[\alpha]_D$ = + 69.6° (c = 1.25, $CHCl_3$); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{31}H_{45}O_{18}N_1$, 751.2, found, 752.2 (M+1, 6.0%); **^{1}H-NMR** δ ($CDCl_3$): 6.05 (d,1H, J_{NH-2} = 9.1 Hz, NH), 5.31~5.30 (m, 2H, H_4, H_4'), 5.11~5.07 (dd, 1H, J_{12}' =7.9 Hz, J_{23}' = 10.5 Hz, H_2'), 4.93~4.91 (dd, J_{23}' = 10.5 Hz, J_{34}' = 3.5 Hz, H_3'), 4.81 (d, 1H, J_{12} = 3.6 Hz, H_1), 4.58 (d, 1H, J_{12}' = 7.9 Hz, H_1'), 4.52~4.48 (m, 1H, H_2), 4.15~3.82 (m, 7H, H_3, H_5, H_5', $2H_6$, $2H_6'$), 3.71~3.65 (m, 1H, $1H_a$), 3.46~3.38 (m, 1H, $1H_a$), 3.01~2.90 (m, 2H, H_c), 2.31~1.92 (8 singlets, 24H, 8Ac), 1.89~1.80 (m, 2H, H_b); **^{13}C-NMR** δ ($CDCl_3$): 170.5~169.7 (C=O), 100.7 (C_1'), 97.8 (C_1), 73.1 (C_3), 70.8(C_3', C_5), 68.9 (C_4'), 68.5 (C_2'), 67.5 (C_5'), 66.8 (C_4), 65.7 (C_a), 62.7 & 61.0 (C_6 & C_6'), 48.8 (C_2), 29.5 (C_b), 25.3 (C_c), 23.2~20.5 (Ac).

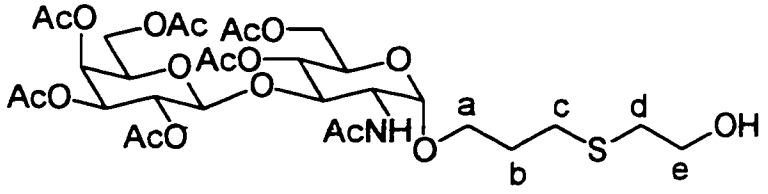
(2-Aminoethyl)thiopropyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-doxy-α-D-glucopyranoside (48)



The reaction conditions were the same as described for those of 3-(2-aminoethyl)thiopropyl β-D-galactopyranosyl-(1-3)-α-D-2-acetamido-2-deoxy galactopyranoside **42**. Sugar moiety (100 mg, 0.24 mmol) and 22.7 mg (0.20 mmol) of cysteamine were used. Purification with an ion exchange resin (NH_4^+ form) gave 88.6 mg (0.18 mmol, 90%) of white solid: m.p. = 113.0~115.0 °C; R_f = 0.21 (EtOAc/acetic acid/H_2O, 3/2/2); $[\alpha]_D$ = +77° (c = 1, H_2O); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{19}H_{36}O_{11}N_2S_1$, 500.20, found, 501.2 (M+1, 32.2%); **^{1}H-NMR** δ (D_2O): 4.92 (d, 1H, J_{12} = 3.6 Hz, H_1), 4.51 (d, 1H, J_{12}' = 7.8 Hz, H_1'), 4.17 (dd, 1H, J_{12} = 3.6 Hz, J_{23} = 10.6 Hz, H_2), 4.15~3.97 (m, 2H, H_3, H_4'), 3.96~3.80 (m, 5H, H_5, $2H_6$, $2H_6'$), 3.79~3.76 (m, 1H, H_5'), 3.71 (dd, J_{23}' = 10.0 Hz, J_{34}' = 3.4 Hz, H_3'), 3.67~3.63 (m, 2H, H_4, $1H_a$), 3.59 (dd,

1H, $J_{12'}$ = 7.8 Hz, $J_{23'}$ = 10.0 Hz, H_2'), 3.09 (t, 2H, J_{de} = 6.7 Hz, H_e), 2.83 (t, 2H, J_{de} = 6.6 Hz, H_d), 2.77 (t, 2H, J_{bc} = 6.9 Hz, H_c), 2.10 (s, 3H, Ac), 2.02~1.96 (m, 2H, H_b); **^{13}C-NMR** δ (D_2O): 173.9 (C=O), 102.9 (C_1'), 96.6 (C_1), 79.9 (C_3), 74.8 (C_5'), 72.1 (C_3'), 71.1 (C_5'), 70.2 (C_2'), 68.2 (C_4), 68.1 (C_4'), 66.0 (C_a), 60.5 & 60.1 (C_6 & C_6'), 52.0 (C_2), 38.6 (C_e), 30.7 (C_d), 27.9 (C_b), 27.2 (C_c), 21.5 (C_{methyl}).

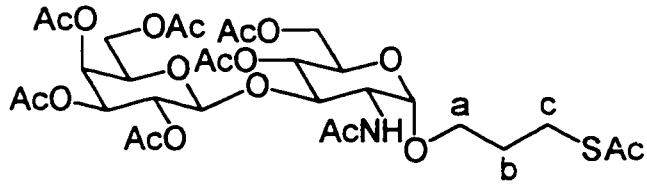
(2-Hydroxyethylthio)propyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyronosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-glucopyranoside (49)



Allyl β-D-Gal-(1-3)-α-D-GlcNAc (**29**) (290 mg, 0.43 mmol) and 2-mercaptoethanol (**40**) (31.7 μL, 1.1 eq) were used and the reaction procedure was the same as described above. The yield was 292 mg (0.39 mmol, 90%): m.p. = 136.5~137.8 °C; R_f = 0.21 (EtOAc/Hex/MeOH, 12/4/1); [α_D = +81.5° (c = 0.7, $CHCl_3$); (+) FAB-MS (glycerol) (m/z); calcd. for $C_{31}H_{47}O_{18}N_1S_1$, 753.25, found, 754.32 (M+1, 47.5%); **^{1}H-NMR** δ ($CDCl_3$); 4.72 (d, 1H, J_{12} = 3.7Hz, H_1), 4.56 (d, 1H, $J_{12'}$ = 7.5 Hz, H_1'), 3.82~3.75 (m, 3H, $2H_e$, $1H_a$), 3.53~3.49 (m, 1H, $1H_a$), 2.76~2.62 (m, 4H, $2H_c$, $2H_d$), 2.34 (t, 1H, $J_{e\text{-}OH}$ = 6.0 Hz, OH), 2.11 (s, 3H, AcNH), 2.06~1.99 (6 singlets, 18H, 3Ac), 1.98~1.89 (m, 2H, $2H_b$); **^{13}C-NMR** δ ($CDCl_3$); 101.0 (C_1'), 97.6 (C_1), 66.6 (C_a), 60.9 (C_e), 35.4 (C_d), 29.2 (C_c), 29.0 (C_b), 23.3~20.5 (7 C_{methyl}).

Anal. Calcd. for $C_{31}H_{47}O_{18}N_1S_1$ (753.25): C, 49.44; H, 6.30; N, 1.92. Found: C, 49.08; H, 6.23; N, 1.84.

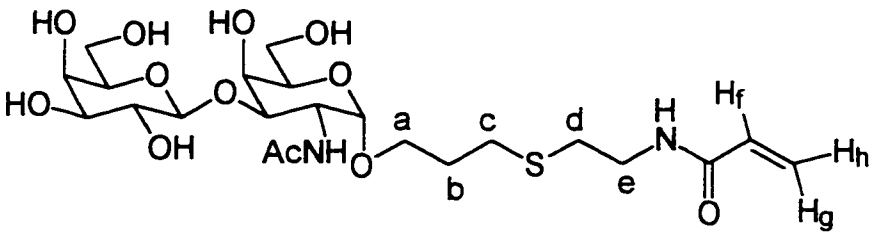
(3-Thioacetyl)propyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyronosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-glucopyranoside (50)



To a solution of allyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-glucopyranoside (**36**) (77.5 mg, 0.12 mmol) and thioacetic acid (50 μL, 6eq.) in deoxygenated MeOH (3 mL) was added AIBN (3.8 mg, 0.1 eq.) and the reaction mixture was refluxed for 3 days under N_2 atmosphere. The resulting solution was condensed and dried in vacuo. The residue was dissolved in CH_2Cl_2 (10 mL) and washed successively with saturated sodium bicarbonate (5 mL) and water and dried with sodium sulfate. The solution was condensed under reduced pressure and purified by silica gel column chromatography with EtOAc/Hex/MeOH (12/5/1) as an eluent to give 68.3 mg of white solid (91 nmol, 79%): m.p. = 55.2~59.2 °C; R_f = 0.40 (EtOAc/Hex/MeOH, 12/4/1); $[\alpha]_D$ = 37.8° (c = 1, $CHCl_3$); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{31}H_{45}O_{18}N_1S_1$, 751.2, found, 752.2 (M+1, 24.2%); **^{1}H-NMR** δ ($CDCl_3$): 6.10 (d,1H, $J_{NH\text{-}2}$ = 10.0 Hz, NH), 5.31 (dd, 1H, J_{34}' = 3.4 Hz, J_{45}' = 1.0 Hz, H_4'), 5.00 (dd, 1H, J_{12}' = 7.7 Hz, J_{23}' = 10.4 Hz, H_2'), 4.95~4.91 (m, 2H, H_3', H_4), 4.67 (d, 1H, J_{12} = 3.6 Hz, H_1), 4.56 (d, 1H, J_{12}' = 7.7 Hz, H_1'), 4.38~4.33 (dt, 1H, J_{12} = 3.6 Hz, J_{23} = 10.3 Hz, H_2), 4.21~3.98 (m, 4H, $2H_6$, $2H_6'$), 3.88~3.84 (m 3H, H_3, H_5, H_5'), 3.73~3.69 (m, 1H, $1H_a$), 3.43~3.38 (m, 1H, $1H_a'$), 3.08~3.05 (m, 1H, $1H_c$), 3.04~2.92 (m, 1H, $1H_c'$), 2.34 (s, 3H, Ac), 2.10~1.92 (7 singlets, 21H, 7Ac), 1.88~1.79 (m, 2H, H_b); **^{13}C-NMR** δ ($CDCl_3$): 170.7~169.1 (C=O), 101.1 (C_1'), 97.7 (C_1), 76.3 (C_3), 71.0 (C_4), 70.2 (C_5), 68.9 (C_2'), 68.7 (C_3'), 68.0 (C_5), 66.8 (C_4'), 65.5 (C_a), 62.3 & 60.7 (C_6, C_6'), 52.2 (C_2), 30.7 (AcNH), 29.5 (C_b), 25.2 (C_c), 23.2~20.5 (AcO).

Anal. Calcd. for $C_{31}H_{45}O_{18}N_2S_1$ (751.20): C, 49.50; H, 6.12; N, 3.74. Found: C, 49.84; H, 6.44; N, 3.75.

(2-N-acrylamidoethylthio)propyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy- α-D-galactopyranoside (51)

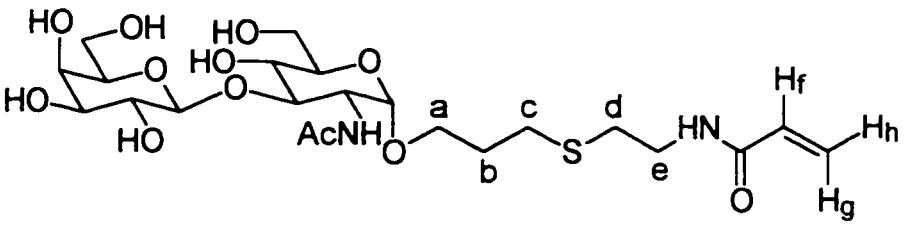


3-(2-Aminoethylthio)propyl glycoside (**42**) (33 mg, 66 nmol) was dissolved in 2 mL methanol. An excess of strong anion exchange resin (Amberite IRA-400, OH^-form), wet from prior washing with distilled water, was then added and the solution was cooled to 0 °C in an ice bath. 1.05 eq. of acryloyl chloride was added slowly in a dropwise fashion from a 5% solution in chloroform. TLC ($CHCl_3$/MeOH/H_2O, 6/4/1) indicated that **42** was completely consumed. The reaction was also monitored by the disappearance of amine by applying a small drop of the reaction solution to a piece of TLC plate then dipping it in a solution of 0.2% ninhydrin in 2% ethanolic pyridine. After heating, the absence of a red colored spot indicated consumption of amine. Once complete, the resin was filtered and washed with methanol. The filtrate was treated with cation exchange resin (Amberite IR-120, H^+ form). Filtration and evaporation of methanol under reduced pressure at ≤ 25 °C gave quantitative yield of **51**. M. P. could not be measured due to the polymerization: $[\alpha]_D$ = +92.2° (c = 1.00, H_2O); R_f = 0.52 (CH_3Cl/MeOH/H_2O, 6/4/1); (+) FAB-MS (glycerol): calcd. for $C_{22}H_{38}O_{12}N_2S_1$, 554.31, found, 555.4 (M+1, 48.6%); **^{1}H-NMR** δ (D_2O); 6.34 (dd, 1H, J_{cis} = 10.1 Hz, J_{trans} = 17.2 Hz, H_f), 6.27 (dd, 1H, J_{gem} = 1.5 Hz, J_{trans} = 17.1 Hz, H_g), 5.84 (dd, 1H, J_{gem} = 1.5 Hz, J_{cis} = 10.1 Hz, H_h), 4.95 (d, 1H, J_{12} = 3.7 Hz, H_1), 4.54 (d, 1H, J_{12}' = 7.8 Hz, H_1'), 4.39 (dd, 1H, J_{12} = 3.7 Hz, J_{23} = 11.1 Hz, H_2), 4.31 (d, 1H, J_{34} = 3.1 Hz, H_4), 4.10 (dd, 1H, J_{23} = 11.2 Hz, J_{34} = 3.1 Hz, H_3), 4.06 (m, 1H, H_5), 3.98 (dd, 1H, J_{34}' = 3.4 Hz, J_{45}' ≤ 1Hz, H_4'), 3.89~3.78 (m, 5H's, $2H_6$, $2H_6'$, $1H_a$), 3.72 (m, 1H, H_5'), 3.69 (dd, 1H, J_{23}' = 10.1 Hz, J_{34}' = 3.4 Hz, H_3'), 3.64~3.62 (m, 1H, H_a), 3.60 (dd,

1H, $J_{12'}$ = 7.8 Hz, $J_{23'}$ = 10.1 Hz, H_2'), 3.57 (t, 2H's, J_{de} = 6.6 Hz, H_e), 2.83 (t, 2Hs, J_{de} = 6.6 Hz, H_d), 2.78 (t, 2H's, J_{bc} = 7.1 Hz, H_c), 2.09 (s, 3H's, H_{CH_3}), 1.97 (m, 2H's, H_b); **^{13}C-NMR** δ (D_2O): 129.4 (C_f), 126.9 (C_{gh}), 104.2 (C_1'), 96.7 (C_1), 76.8 (C_3), 74.5 (C_5'), 72.0 (C_3'), 70.1 (C_2', C_5), 68.2 (C_4), 68.11 (C_4'), 66.0 (C_a), 60.7 (C_6), 60.5 (C_6'), 48.2 (C_2), 38.2 (C_e), 29.9 (C_d), 28.0 (C_b), 27.4 (C_c), 21.5 (C_{methyl}).

Anal. Calcd. for $C_{22}H_{38}O_{12}N_2S_1$ (554.31): C, 47.64; H, 6.91; N, 5.11. Found: C, 47.03; H, 7.14; N, 4.63.

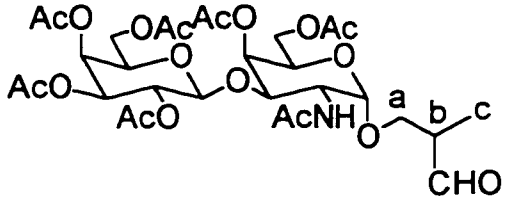
(2-N-acrylamidoethyl)thiopropyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-α-D-glucopyranoside (52)



The reaction conditions were the same as described in the synthesis of 3-(2-acrylamidoethyl) thiopropyl-β-D-galactopyranosyl-(1-3)-α-D-2-acetamido-2-deoxy-galactopyranoside (**51**). Sugar moiety (80.7 mg, 0.16 mmol) was used and 78.7 mg (0.14 mmol, 88%) of white spongy solid was obtained: m.p. = not available; R_f = 0.57 ($CHCl_3$/MeOH/H_2O, 6/4/1); $[\alpha]_D$ = +53.0° (c = 1, H_2O); (+) FAB-MS (glycerol) (m/z): calcd. for $C_{22}H_{38}O_{12}N_2S_1$, 554.60, found, 555.40 (M+1, 49%); The chemical shift values for the sugar part were same as those in the precursor.; **^{1}H-NMR** δ (D_2O): 6.35 (dd, 1H, J_{cis} = 10.1 Hz, J_{trans} = 17.1 Hz, H_f), 6.29 (dd, 1H, J_{gem} = 1.5 Hz, J_{trans} = 17.2 Hz, H_g), 5.84 (dd, 1H, J_{gem} = 1.5 Hz, J_{cis} = 10.1 Hz, H_h), 4.92 (d, 1H, J_{12} = 3.6 Hz, H_1), 4.51 (d, 1H, $J_{12'}$ = 7.8 Hz, H_1'), 3.59~3.55 (dt, 2H, J_{NH-e} = 2.4 Hz, J_{de} = 6.6 Hz, He), 2.83 (t, 2H, J_{de} = 6.6 Hz, H_d), 2.78 (t, 2H, J_{bc} = 7.1 Hz, H_c), 2.10 (s, 3H, Ac), 2.03~1.93 (m, 2H, H_b); **^{13}C-NMR** δ (D_2O): 173.9 & 168.1 (2 C=O), 129.4 (C_f), 127.0 (C_{gh}), 102.9 (C_1'), 96.5 (C_1), 65.9 (C_a), 38.2 (C_e), 29.9 (C_d), 27.9 (C_b), 27.4 (C_c), 21.5 (C_{methyl}).

Hydroformylation of allyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-galactopyranoside

To a solution of peracetylated allyl β-D-Gal-(1-3)-α-D-GalNAc (**36**) (0.3 g, 0.44 mmol) in CH_2Cl_2 (10 mL) was added 1/2 mole ratio of the Rh (I) catalyst, [Rh^+(1,5-COD)(η^6-$PhBPh_3^-$), 2.4 mg, 4.44 μmol] and bis(diphenylphosphino)butane (0.38 mg, 8.88 μmol). The solution was pressurized by syngas ($CO:H_2$=1/1) to 40 atm and stirred at 80 °C for 12 h. The solution was then evaporated under reduced pressure and the residue was purified by silica gel column chromatography with EtOAc/Hex/MeOH (12/4/1). The product was dried in vacuo to give 132 mg of 2-hydroformylpropyl isomer (0.19 mmol, 42%) and 87 mg of 3-hydroformylpropyl isomer (0.12 mmol, 28%). The overall yield was 70%.

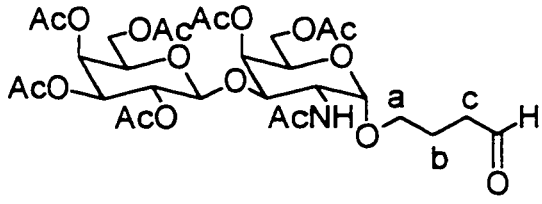


2-hydroformylpropyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-galactopyranoside (64)

m.p. = 67.3~69.0 °C; R_f = 0.51 (EtOAc/Hex/MeOH, 12/4/1); $[\alpha]_D$ = +38.2° (c = 1.1, EtOAc); (+) FAB-MS (glycerol) (m/z); calcd. for $C_{30}H_{43}O_{18}N_1$, 705.24, found, 706.19 (M+1, 3.6%); **^{1}H-NMR** δ ($CDCl_3$); 9.66 and 9.65 ppm (2 doublets, ahdehyde), 5.60 (2 set of doublet, 1H, $J_{2\text{-}NH}$ = 8.8 Hz, NH), 5.34~5.32 (m, 2H, H_4, H_4'), 5.12~5.09 (m, 1H, H_2'), 4.94 (d, 1H, J_{12} = 3.5 Hz, H_1), 4.92 (m, 1H, H_3'), 4.56 (d, 1H, $J_{12'}$ = 7.9 Hz, H_1'), 4.48~4.39 (m, 1H, H_2), 4.18~3.88 (m, 6H, H_5, H_6, H_6', H_a), 3.73~3.66 (dd, 1H, J_{12} = 3.5 Hz, J_{23} = 10.9 Hz, H_3), 3.66~3.59 (m, 1H, H_a), 2.81~2.62 (m, 1H, H_b), 2.24~2.11 (multi, 3H, H_c); **^{13}C-NMR**, δ ($CDCl_3$); 196.8 ($C_{aldehyde}$), 170.5~169.8 (C=O), 100.3 (C_1'),

98.5 & 97.8 ($C_{1\alpha}$, $C_{1\beta}$), 72.7 (C_3), 71.0 (C_3'), 70.8 (C_5'), 69.6 (C_4'), 68.5 (C_2'), 68.4 (C_5), 67.5 (C_a), 66.8 (C_4), 49.0 & 49.0 ($C_{2\alpha}$, $C_{2\beta}$), 46.6 & 46.3 ($C_{b\alpha}$, $C_{b\beta}$), 20.7~20.5 (C_{methyl}), 10.5 (C_c)

Anal. Calcd. for $C_{30}H_{43}O_{18}N_1$ (705.24): C, 51.05; H, 6.16; N, 2.00. Found: C, 50.80; H, 6.23; N, 1.90.

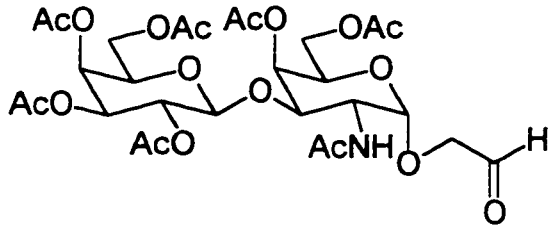


3-hydroformylpropyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl--α-D-galactopyranoside (65)

m.p. = 66.5~68.3 °C; R_f = 0.36 (EtOAc/Hex/MeOH, 12/4/1); $[\alpha]_D$ = +60.0° (c = 0.85, EtOAc); (+) FAB-MS (glycerol) (m/z); calcd. for $C_{30}H_{43}O_{18}N_1$, 705.24, found, 706.19 (M+1, 5.1%); **^{1}H-NMR** δ ($CDCl_3$); 9.72 (s, 1H, aldehyde), 5.78 (d, J_{NH-2} = 9.0 Hz, NH), 5.34 (dd, 1H, J_{34}' = 3.4 Hz, J_{45}' = 0.9 Hz, H_4'), 5.32 (broad d, 1H, J_{34} = 2.6 Hz, H_4), 5.11 (dd, 1H, J_{12}' = 7.9 Hz, J_{23}' = 10.4 Hz, H_2'), 4.95 (dd, 1H, J_{23}' = 10.5 Hz, J_{34}' = 3.5 Hz, H_3'), 4.83 (d, 1H, J_{12} = 3.6 Hz, H_1), 4.60 (d, 1H, J_{12}' = 7.9 Hz, H_1'), 4.18~4.10 & 3.98~3.87 (m, 4H, H_6, H_6'), 4.01 (m, 1H, H_5), 3.89 (dt, 1H, J_{45}' = 0.9 Hz, J_{56}' = 7.1 Hz, H_5'), 3.81~3.76 (m, 2H, H_3, H_a), 3.38~3.31 (m, 1H, H_a), 2.61~2.29 (m, 2H, H_c), 2.14~1.95 (m, 23H, 7Ac, H_b); **^{13}C-NMR** δ ($CDCl_3$); 202.9 (C=O, aldehyde), 170.5~169.7 (C=O), 100.7 (C_1'), 98.2 (C_1), 72.7 (C_3), 70.9 (C_3'), 70 9 (C_5'), 68.8 (C_4), 68.7 (C_2'), 68.5 (C_a), 67.5 (C_5), 66.8 (C_4'), 62.6 & 61.0 (C_6, C_6'), 48.9 (C_2), 41.8 (C_c), 23.2~20.5 (C_{methyl}, C_b).

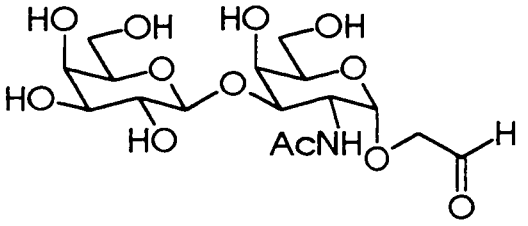
Anal. Calcd. for $C_{30}H_{43}O_{18}N_1$ (705.24): C, 51.05; H, 6.16; N, 2.00. Found: C, 50.67; H, 6.33; N, 1.83.

Hydroformylmethyl (2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-α-D-galactopyranoside (66)



A solution of peracetylated allyl T-antigen (**36**) (0.5 g, 0.74 mmol) in $MeOH/CH_2Cl_2$ (1/1, 20 mL) was cooled to -78 °C and saturated with O_2. Ozone was then bubbled for 30 min. to make ozonide. Once ozonide was formed, a blue-colored solution resulted. When the reaction was completed, dimethylsulfide was added and the solution was flushed with O_2. The solution was stirred for 3 h during which time the temperature was gradually increased to room temperature. The reaction mixture was then, kept in the refrigerator overnight. The solvent and excess dimethylsulfide was evaporated and dried under vacuum to give a white solid (0.5 g. quantitative): m.p. = 42.5~42.8 °C; R_f = 0.45 (EtOAc/Hex/MeOH, 12/4/1); $[\alpha]_D$ = +42.14° (c = 0.7, $CHCl_3$); (+) FAB-MS (glycerol) (m/z); calcd. for $C_{28}H_{39}O_{18}N_1$, 677.22, found, 678.30 (M+1, 7.5%); **^{1}H-NMR** δ ($CDCl_3$): 9.47 (s, 1H, C(O)H), 6.04 (d, 1H, $J_{NH\text{-}2}$ = 8.8 HZ, NH), 5.38 (broad d, 1H, J_{34} = 3.2 Hz, H_4), 5.35 (broad d, 1H, $J_{34'}$ = 3.5 Hz, H_4'), 5.10 (dd, 1H, $J_{12'}$ = 7.9 Hz, $J_{23'}$ = 10.6 Hz, H_2'), 4.95 (dd, 1H, $J_{34'}$ = 3.5 Hz, H_3'), 4.87 (d, 1H, J_{12} = 3.6 Hz, H_1), 4.60 (d, 1H, $J_{12'}$ = 7.9 Hz, H_1'), 4.42 (m, 1H, H_2), 4.31 (m, 2H, $H_{methylene}$), 4.17~4.06 (m, 4H, H_6, H_6'), 3.98~3.90 (m, 2H, H_3, H_5), 3.90 (broad t, 1H, $J_{56'}$ = 6.8 Hz, H_5')2.15~1.96 (m, 21H, H_{methyl}); **^{13}C-NMR** δ ($CDCl_3$); 197.3 ($C_{aldehyde}$), 170.5~165.8 (C=O), 100.6 (C_1'), 99.0 (C_1), 73.4 ($C_{methylene}$), 72.4 (C_3), 70.9 ($C_{5'}$), 70.8 (C_3'), 68.7 (C_4), 68.6 (C_2'), 66.8 (C_4'), 62.5 (C_5), 61.1 (C_6, C_6'), 48.8 (C_2), 23.3~20.5 (C_{methyl}).

Hydroformylmethyl O-(β-D-galactopyranosyl)-(1-3)-2-acetamido-2-deoxy-α-D-galactopyranoside (67)



Allyl β-D-Gal-(1-3)-α-D-GalNAc (**29**) (0.1 g, 0.24 mmol) was used and the reaction conditions were the same as those described above. 0.116 g of white spongy solid (quantitative) was obtained. The product was confirmed by a test with DNP (2,4-dinitrophenyl hydrazine). One drop of the sample solution in MeOH was applied to the TLC plate and dried. DNP power was applied on the surface of the TLC plate which was then left for a while. After the plate was cleaned, the spot was shown to have undergone reddish color change: m.p. = 186.3~187.2 °C; R_f = 0.46 (EtOAc/AcOH/H_2O, 3/2/2); (+) FAB-MS (glycerol) (m/z); calcd. for $C_{16}H_{27}O_{12}N_1$, 425.15, found, 426.26 (M+1, 2.4%); **^{1}H-NMR** δ (D_2O); 4.99 (d,1H, J_{12} = 3.8 Hz, H_1), 4.50 (d, 1H, J_{12}' = 7.8 Hz, H_1'), 9.32 (s, 1H, C(O)H), 4.08 (m, 2H, methylene); **^{13}C-NMR** δ (D_2O); 103.8 (C_1'), 96.1 (C_1), 70.7 (C(O)H), 70.6 ($C_{methylene}$).

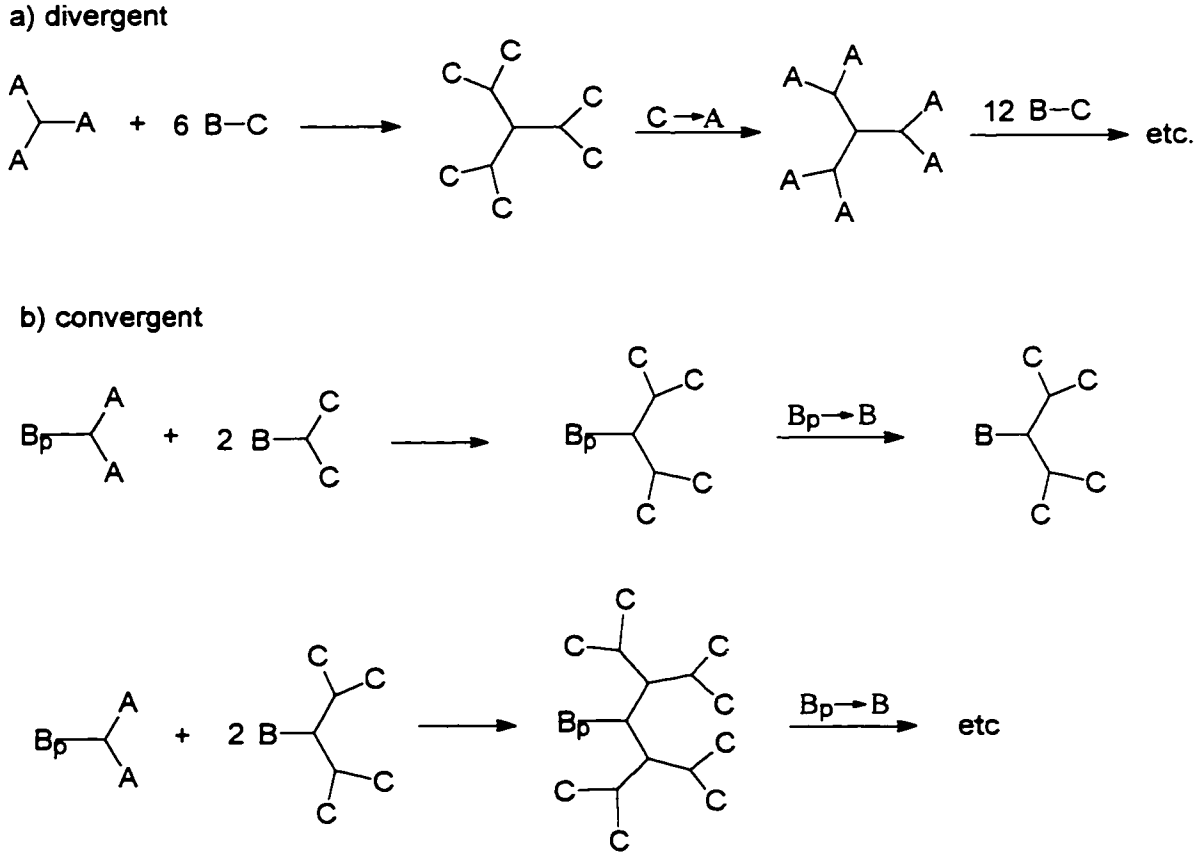
Chapter 4. Synthesis of N, N′-bis(acrylamido)acetic acid based glycodendrimers

4.1. Introduction

Cell surface carbohydrates found in the form of glycoproteins, glycolipids and proteoglycans are involved in a wide variety of important biological phenomena such as recognition of bacteria, viruses, hormones and other cells, cell growth, cancer cell metastasis and mounting of immune response, which depend on carbohydrate-protein interactions.[71, 178-180] In spite of their importance in specific recognition processes, individual interactions are of low affinity with dissociation constants (K_D's) in the micro~milimolar range.[181] It is known that multivalent carbohydrate-protein interactions cooperate in some recognition events to amplify binding affinity, thereby explaining the roles that these interactions play. These clusters or cooperative effects depend on the arrangement of multiple receptors in such a way as to bind to multiple carbohydrates having well organized architectures. It is also well known that various receptors have clustered carbohydrate recognition domains. Consequently, neoglycoproteins[183] and glycoproteins[184] have been used successfully to demonstrate amplified carbohydrate-protein binding interactions by factors as high as thousands. As a mimic of these multivalencies, chemically well defined biopolymers have been prepared in dendritic shapes.[180, 185, 186, 188] The species useful as inhibitors are anticipated to provide a complete understanding of these important recognition processes by virtue of their well characterized carbohydrate valency, shapes and orientation.

Dendrimers have been prepared by employing both convergent[185] and divergent strategies[186] (Scheme 4-1). The convergent method[185, 187] is a useful strategy to yield highly branched molecular trees. Trees prepared by the convergent method may be attached to a polyfunctional core, a technique called "double stage growth"[185] or branched-monomer approach.[188] In a similar way, combining the convergent and divergent

methods[189] with the same substrate using double protection were reported to grow trees rapidly.



B_p: protected form of a function B

Scheme 4-1. Systematic representation of synthetic strategies.

The divergent approach usually suffers from defects on the surface because of the higher number of branches as the number of generation increases. Excess reagent is needed and the elimination of this excess is necessary. One thing that the convergent approach suffers from is steric constraints when the dendrons are supposed to be attached to the core become too large. However, this drawback of the convergent method may be overcome if the dendron has a long string of length of spacers to eliminate this unfavorable interaction.

It is now well known that carbohydrate bearing thiols can conjugate to electrophiles such as α,β-unsaturated carbonyl compounds. When thiols are used as a nucleophilic periphery of the dendritic cores, the intra- and inter-molecular disulfide bridge formations are obtained and these also interfere with the designed nucleophilic thiol addition. Therefore partially glycosylated and partially oxidized disulfide containing dendrimers will be resulted. This makes the purification and characterization processes difficult. Nonetheless, the former approach eliminates the problems associated with disulfide formation. Since the carbohydrate bearing nucleophilic thiol conjugates to the electrophilic counterpart or oxidizes itself, the usage of excess of carbohydrate thiol can complete glycosidation of the dendrimers. Even dimeric carbohydrate disulfide can be recycled by simple disulfide cleavage.

As we discussed in Chapter 3, thiol functionalities are readily incorporated onto the carbohydrate moieties in the form of thioacetates. Thus deprotection of the thioacetyl group under Zemplén condition can generate a thiol which is subsequently used in nucleophilic addition reactions.

Based on such demands, we choose N, N′-bis(acrylamido)acetic acid (**68**) as a seed molecule which is a commercially available bifunctional compound.

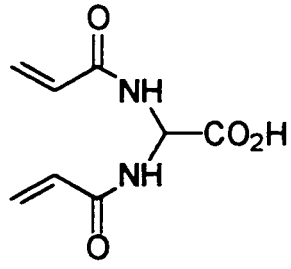


Figure 4-1. Structure of N, N′-bis(acrylamido)acetic acid **68.**

This molecule has some advantages. Firstly, two acrylamido functionalities are readily available for nucleophilic thiol addition under mild conditions. Secondly, the presence of a

carboxylic group at the opposite terminus allows scaffolding of the acrylamido functional group with a periphery of amines by condensation using HOBt/EDC method. Lastly, elongation of the spacer by the addition of a simple thiol such as cysteamine is easily accomplished with new terminus diamines and iterative conjugation with **68** can give multiple acryl amido groups on the surface at each generation (Scheme 4-2).

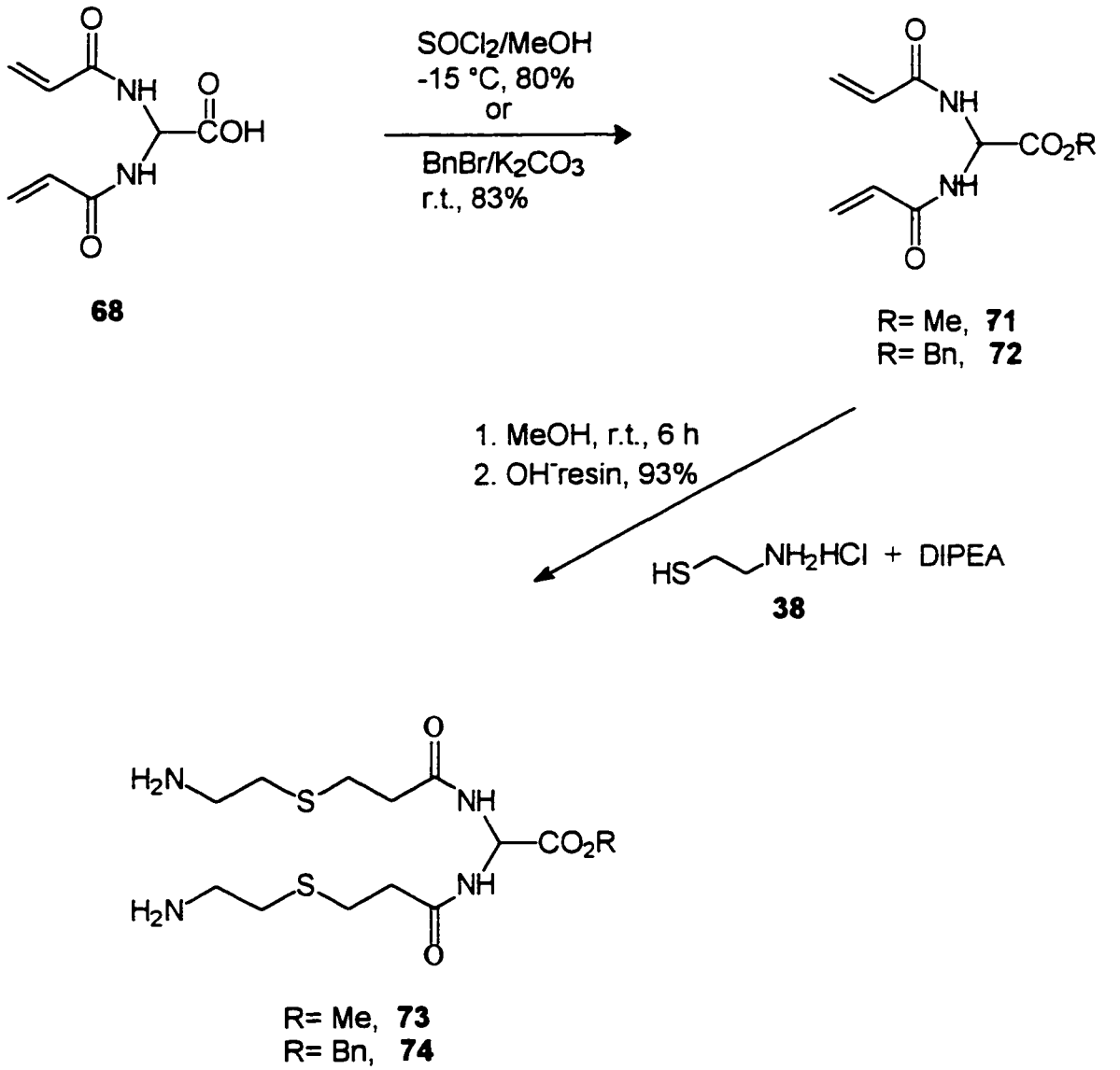
The other way to equip one molecule with multiamine functionalities results from acrylonitrile addition to amines and subsequent reduction of nitriles to give the corresponding amines. In this chapter, synthetic strategies of N, N′-bis(acrylamido)acetic acid based dendrimers and their glycosidation will be discussed.

4.2. Synthesis of N, N′-bis(acrylamido)acetyl cores

The following approach for the synthesis of dendritic core was initiated with N, N′-bis(acrylamido)acetic acid **68** as a seeding molecule and conjugated with cysteamine, **38**, hexamethylenediamine **69** and tris-(2-aminoethyl)amine **70** to give spherical dendrimers with between two and six valencies.

N, N′-bis(acrylamido)acetic acid **68** was esterified with MeOH *via* acyl chloride induced $SOCl_2$ to give the corresponding methyl ester **71** (MeOH, dropwise addition of $SOCl_2$, -15 °C, 80%) as shown in Scheme 4-2. Cysteamine **38** was chosen as a hydrophilic spacer with a sufficient length and amine functionalities to **68**. A slight excess of **38** for each acrylamido group was coupled to bis-acrylamido methyl ester **71** in a basic methanolic solution with DIPEA at room temperature and purified by silica gel chromatography with $CHCl_3$/MeOH/H_2O (10/6/1) and EtOAc/AcOH/H_2O (3/2/2) as eluents to afford bis-amino methyl ester **73** in 93% yield. The structure of **73** was confirmed by comparing the integration of the methyl group (3.95 ppm, s, 3H) with that of cysteamine methylene units (3.37 ppm, t, NH_2CH_2- and 3.01 ppm, t, -CH_2S-) and the ninhydrin (Kaiser) color test.[190] The reactions were quite straightforward and gave the dendritic precursor in high yield. Similarly, benzyl ester **74** prepared using benzyl

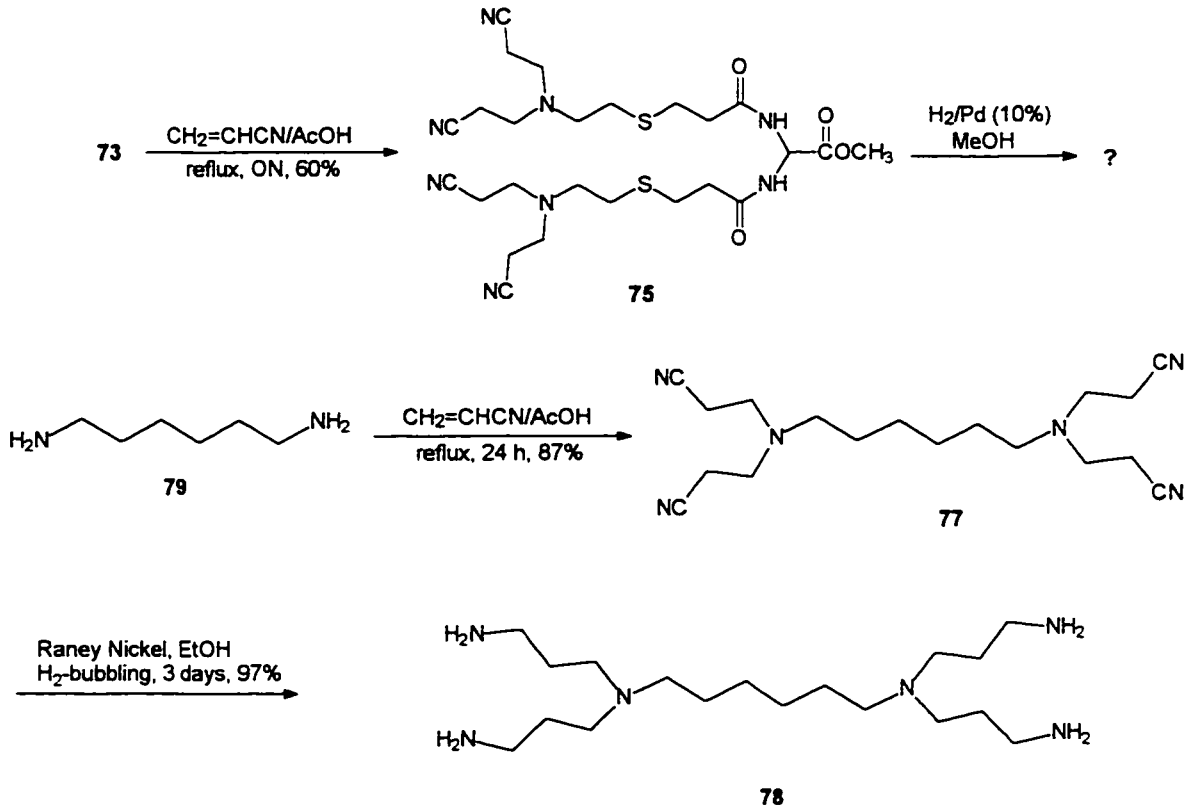
bromide under basic condition (BnBr, K_2CO_3, MeCN/DMSO=3/1, 83%), was subsequently conjugated with cysteamine to give bis-amino benzyl ester, **74**.



Scheme 4-2. Synthesis of bis-amino esters **73** and **74.**

Because the benzyl group disappeared after normal silica gel chromatography, the solution was simply condensed and dried under reduced pressure for the next reaction. Bis-amino esters **73** and **74** were suitable for the convergent growth of the dendritic architecture

because these extended spacers eliminate possible steric constraints for the completion of the generation. Tetravalent nitrile group containing dendrimers **75** and **77** were generated in a simple manner. Conjugate addition of acrylonitrile to amino groups in **73** was accomplished under reflux condition in the presence of acetic acid. **75** was isolated by silica gel chromatography to give 60% yield.

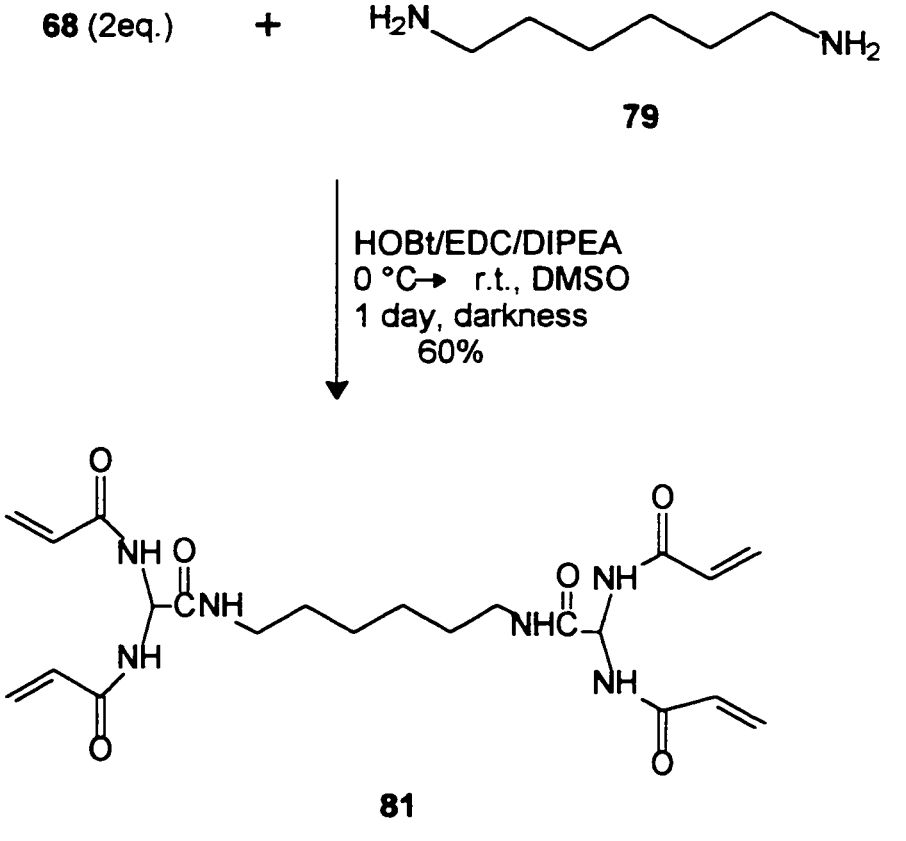


Scheme 4-3. Synthesis of tetravalent nitrile compounds **75** and **77**.

Reduction of **75** to the corresponding amine was not successful. During the reaction, **75** was completely consumed and a Kaiser test showed a positive result which indicated the presence of amine functionality. However, the ^{1}H- and ^{13}C-NMR spectra just showed complicated patterns and could not be used for structual determination. Another tethered tetravalent dendritic precursor **77**, composed of only a hydrocarbon chain, was prepared in the same manner (87% yield). Hydrogenolysis of was successfully performed with Raney

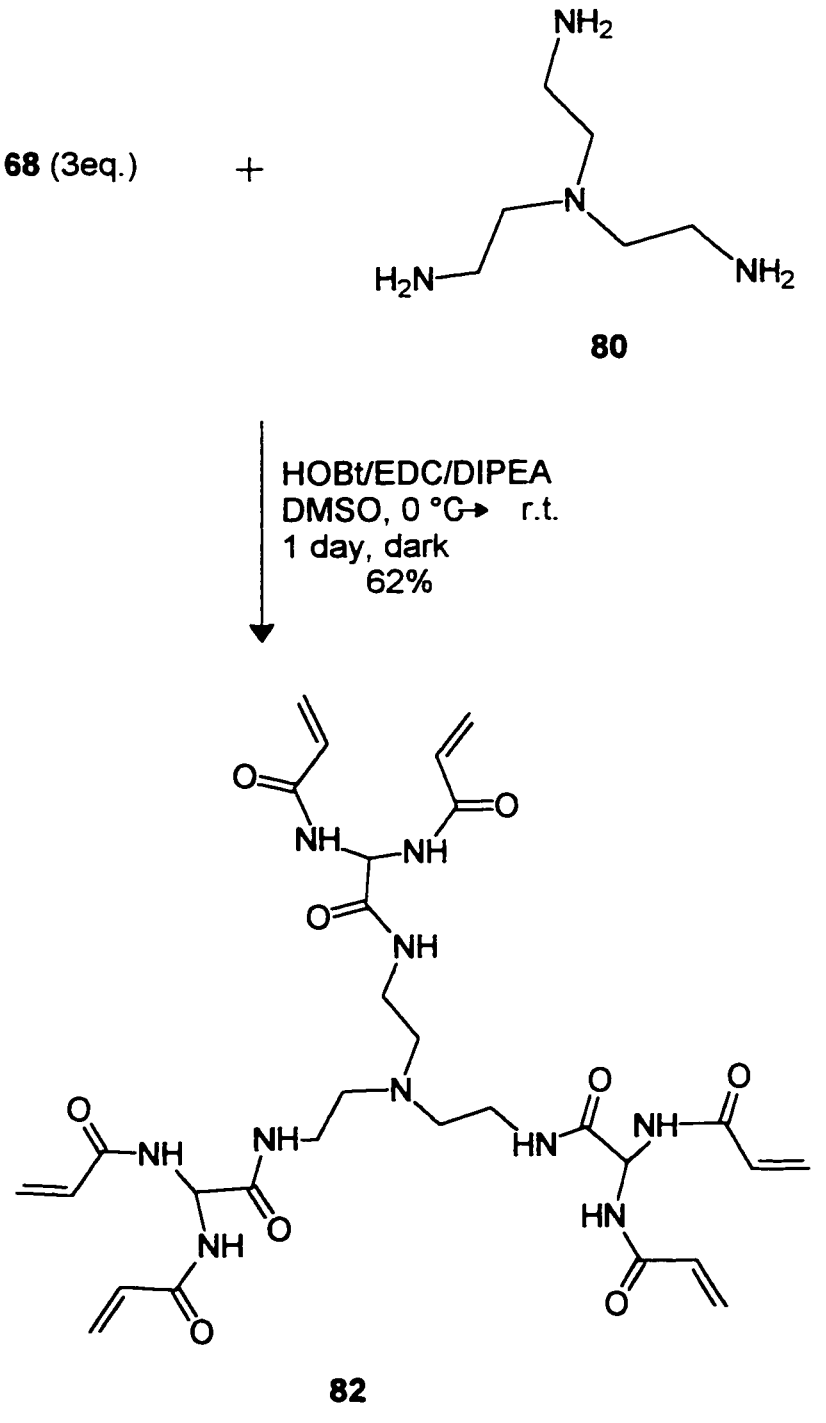
nickel in 97% yield. The structure was confirmed by ^{1}H-and ^{13}C-NMR spectral data. The mass spectrum showed a molecular ion peak at 345.30 (M+1, 1.6%).

Construction of other dendritic structures was accomplished by carbodiimide chemistry and hydroxy benztriazole (HOBt) activator strategies (EDC/HOBt/DIPEA, 25°C, darkness). N,N′-Bis(acrylamido)acetic acid **68** was coupled to hexamethylenediamine **79** or tris-(2-aminoethyl)amine **80** to give tetra- (**81**) and hexa- (**82**) valent dendrimers, respectively (Scheme 4-4 and 4-5).



81

Scheme 4-4. The tetravalent Bis[N, N′-bis(acrylamido) acetamido]hexane, **81.**



Scheme 4-5. The hexavalent Tris[N, N′-bis(acrylamido) acetamido]ethylamine, **82.**

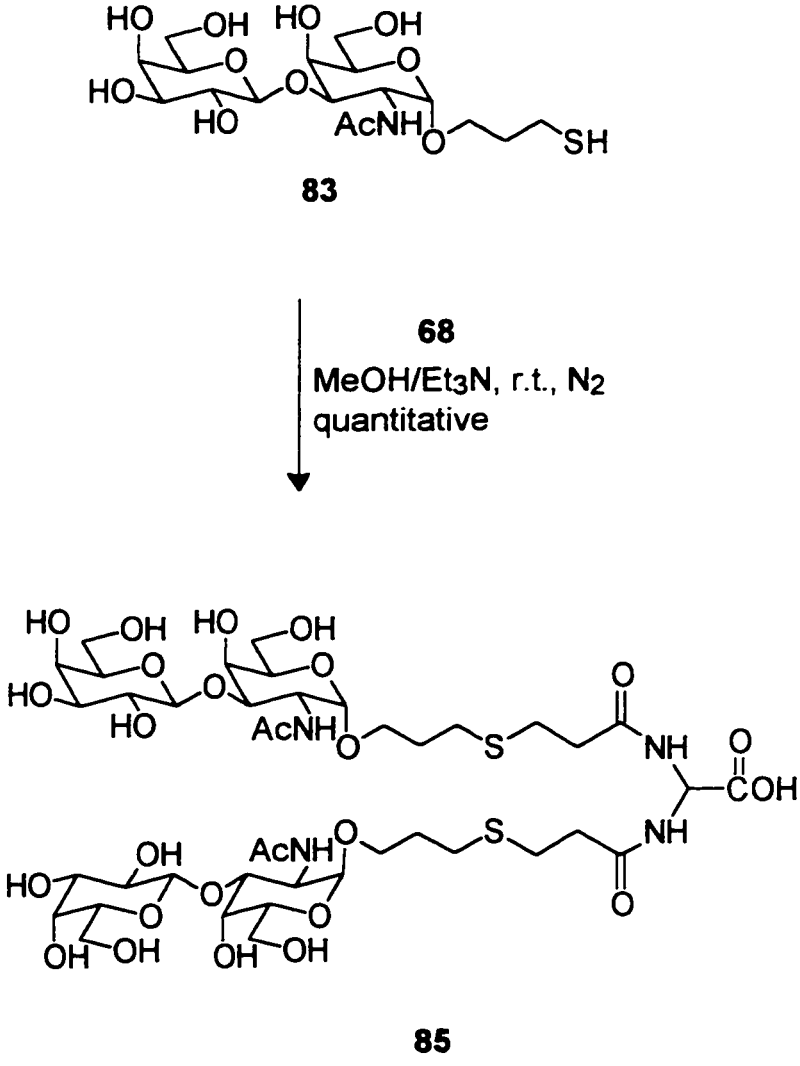
Multivalent dendrimers **81** and **82** were too polar to allow easy isolation *via* standard silica gel chromatographic techniques. Instead, the mixtures were subjected to precipitate in a solvent mixture of water and CH_2Cl_2 (1/1) followed by successive washings with water and CH_2Cl_2 to remove excess coupling reagents and DIPEA. Thus afforded **81** and **82** in 60 and 62% yields, respectively. Dendrimers **81** and **82** have simple structures due to C_2 and C_3 symmetry. Both display a typical splitting pattern for an acryloyl group between 5.6 and 6.1ppm in ^{1}H-NMR. Their integrations were compared with the methylene unit beside the amide bond and are consistent with the proposed structures.

4-3. Conjugation of 3-thiopropyl β-D-Gal-(1-3)-α-D-GalNAc and N, N′-bis(acrylamido)acetyl cores

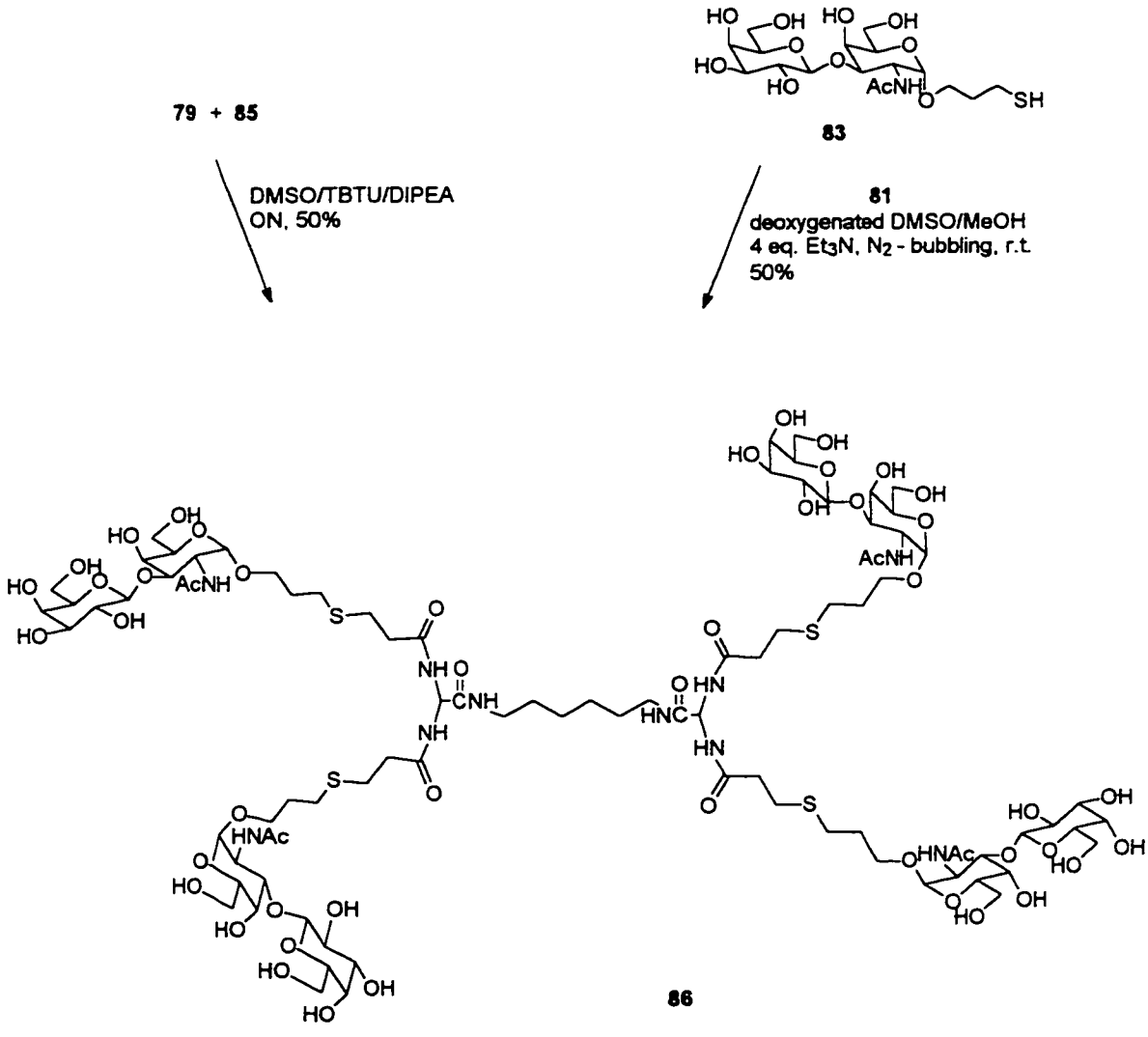
As demonstrated in Chapter 3, nucleophilic thiol addition to an electrophilic acryloyl functional group was very successful. In the same manner, carbohydrate thiols can also undergo conjugate addition at the periphery of the dendritic core. In order to prepare carbohydrate thiols, the precursors, thioacetates **46** and **47** were treated under Zemplén condition or with hydrazinium acetate in DMF[191], respectively. To accelerate de-S-acetylation of **47**, an excess of hydrazinium acetate was used and no de-O-acetylation was observed. The thio compound was purified by aqueous work-up and condensed under reduced pressure.

Dendritic cores **68**, **81** and **82** were treated with an excess of 3-thiopropyl β-D-Gal-(1-3)-α-D-GalNAc **83** or 3-thiopropyl-2′,3′,4′,6′-tetracetyl β-D-Gal-(1-3)-α-D-4,6-diacetyl-GalNAc **84** (1.2 eq. per acryloyl functional group, deoxygenated DMSO, N_2 - bubbling, overnight, 25 °C) to provide glycodendrimers **85**, **86**, **87** and **88**. DMSO was removed by lyophilization and the residue was redissolved in the minimum amount of water and the desired products were isolated by P-2 or P-4 size exclusion chromatography to afford **85**, **86** and **87** in moderate to excellent yields (quantitative, 50 and 32~45%), respectively. In each case, some disulfide by-product was formed during the reactions.

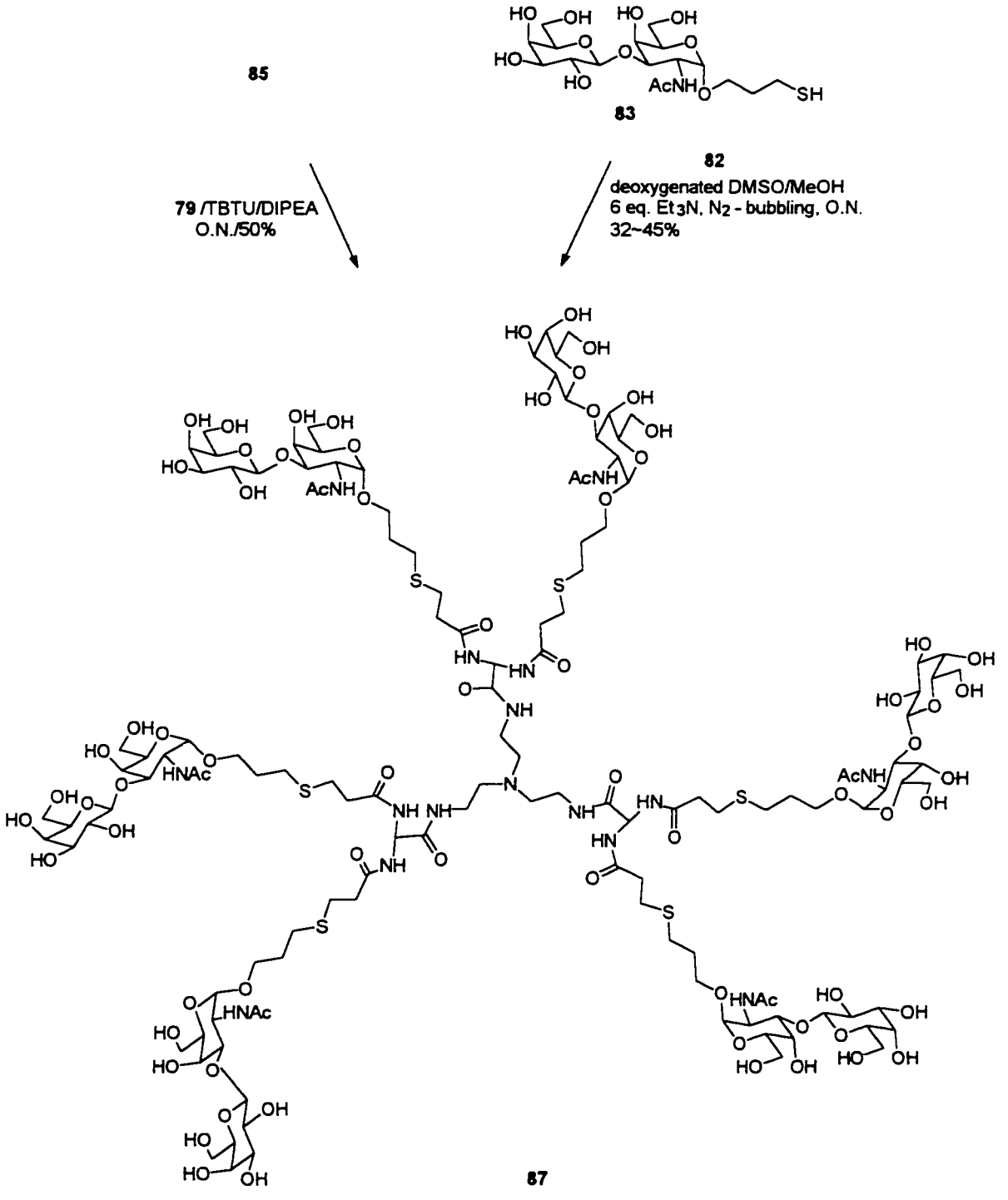
Alternatively, **87** could be prepared from **79** and **85** using TBTU (O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate) method to provide 50% purified yield.



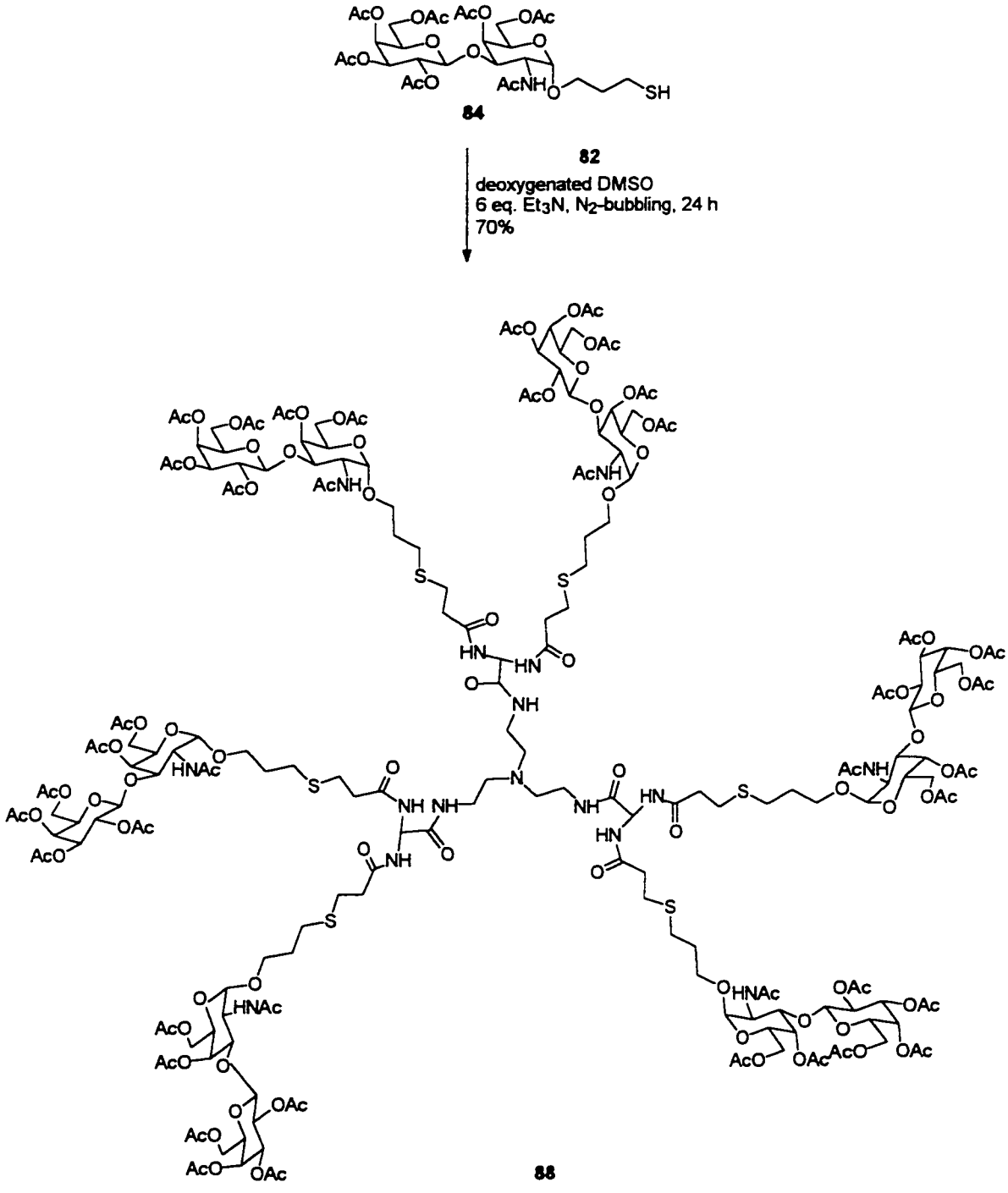
Scheme 4-6. Synthesis of divalent glycodendrimer **85.**



Scheme 4-7. Synthesis of tetravalent glycodendrimer **86.**

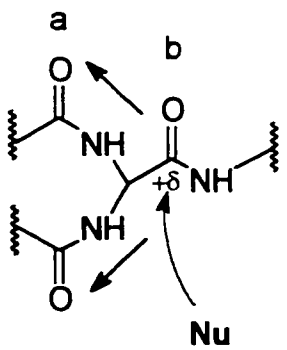


Scheme 4-8. Synthesis of hexavalent glycodendrimer **87**.



Scheme 4-9. Synthesis of fully protected hexavalent glycodendrimer **88.**

Complete conjugation was readily confirmed by ^{1}H-NMR spectral data which showed the absence of the well separated acryloyl signals between 5.6 and 6.1 ppm in D_2O and the emergence of new signals corresponding to the incorporated O-3-thiopropyl-β-D-Gal-(1-3)-α-D-GalNAc residues (H-1 at 4.96 ppm, H-1′ at 4.55 ppm and five methylene spacers at 3.80, 3.63, 2.90, 2.77, 2.67 and 2.00 ppm, $CDCl_3$) for dimer (**85**). Tetra- (**86**) and two hexa-(**87**, **88**) valent glycodendrimers were also clearly established the extent of the T-antigen residues. These signals were integrated relative to the anomeric signals at 4.55 ppm for H-1′ and 4.96 ppm for H-1 (Figure 4-2 ~ 4-5). In the case of **88**, the mixture was purified by silica gel chromatography with EtOAc/Hex/MeOH (12/5/1) as eluent to give 70% yield of hexavalent glycodendrimer. Interestingly, de-O-acetylation of **88** was not successful under Zemplén condition. After de-O-acetylation and P-4 size exclusion chromatographic purification (P-4 column), unknown compound which missed two methylene units in the core part was the sole product obtained. The ^{1}H-NMR of the unknown compound was very similar to that of dimeric dentrimer **85**. It might involved amide bond cleavage at the position (b). Because of the strong electron withdrawing effect of two neighboring amide groups (a), the carbonyl group (b) would be susceptible to nucleophilic attack to cleave the amide bond.



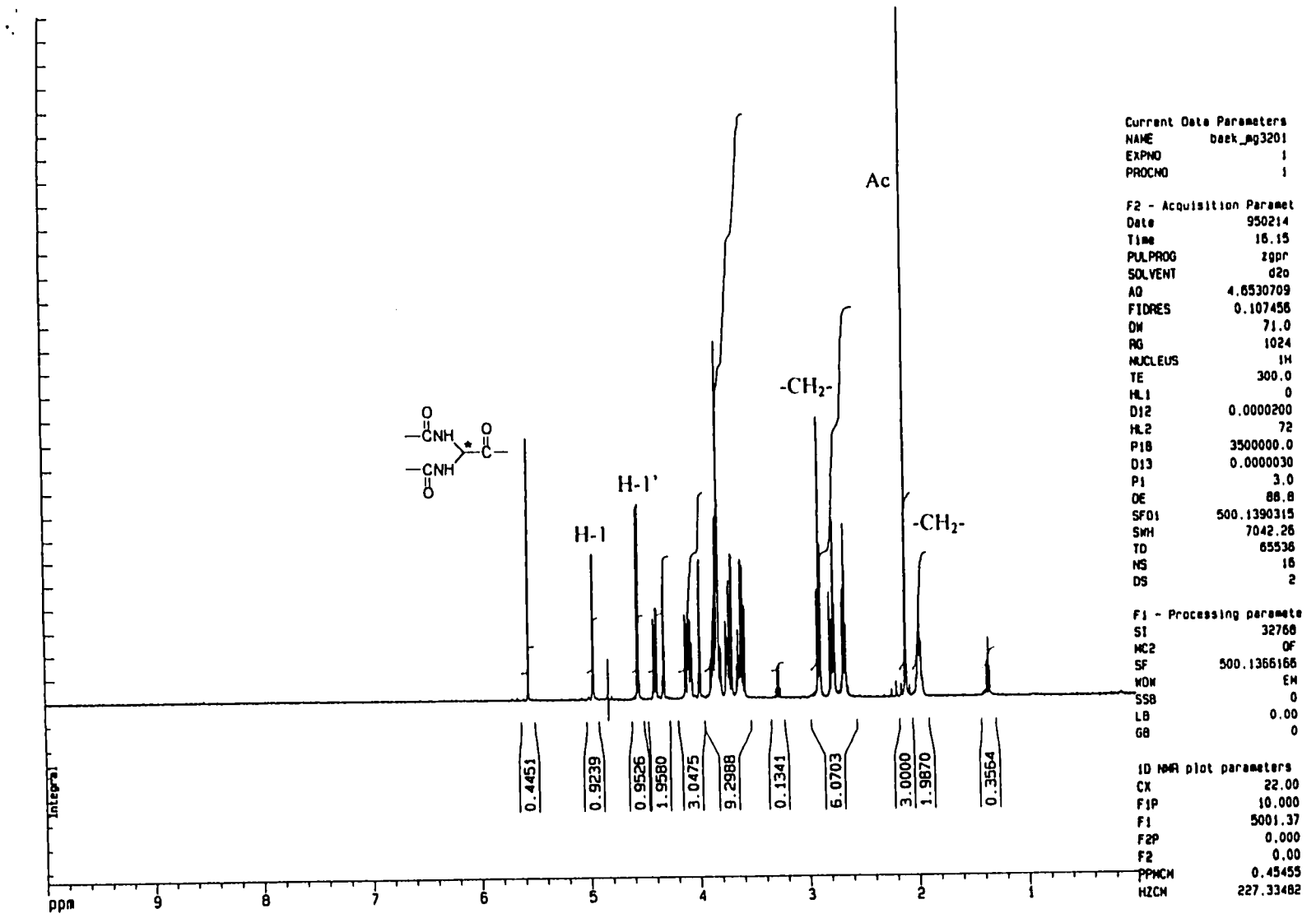


Fig. 4-2. ^{1}H-NMR (D_2O, 500 MHz) spectrum of divalent T-antigen dendrimer **85.**

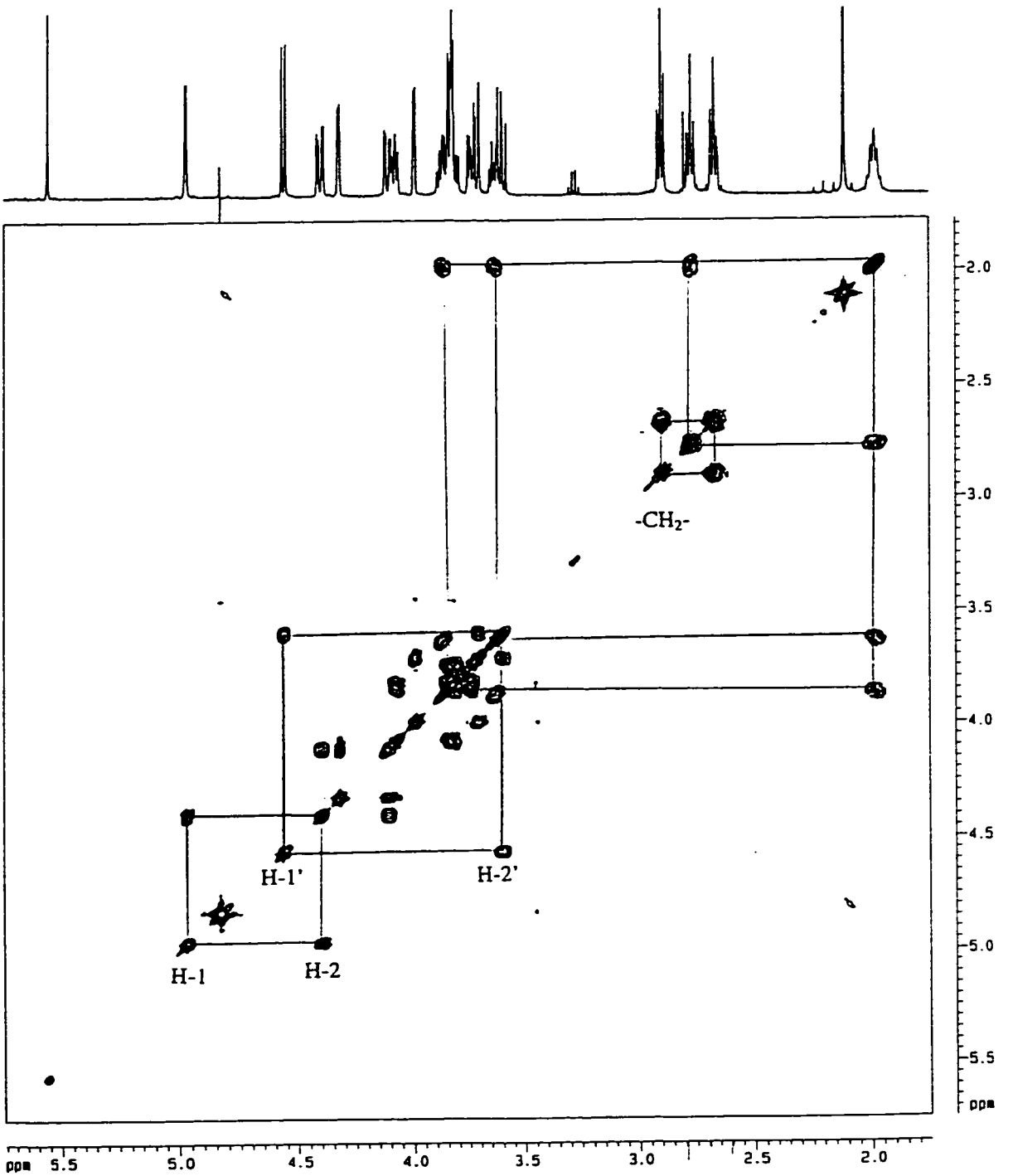


Fig. 4-3. ^{1}H-^{1}H COSY (D_2O, 500 MHz) spectrum of divalent T-antigen dendrimer **85**.

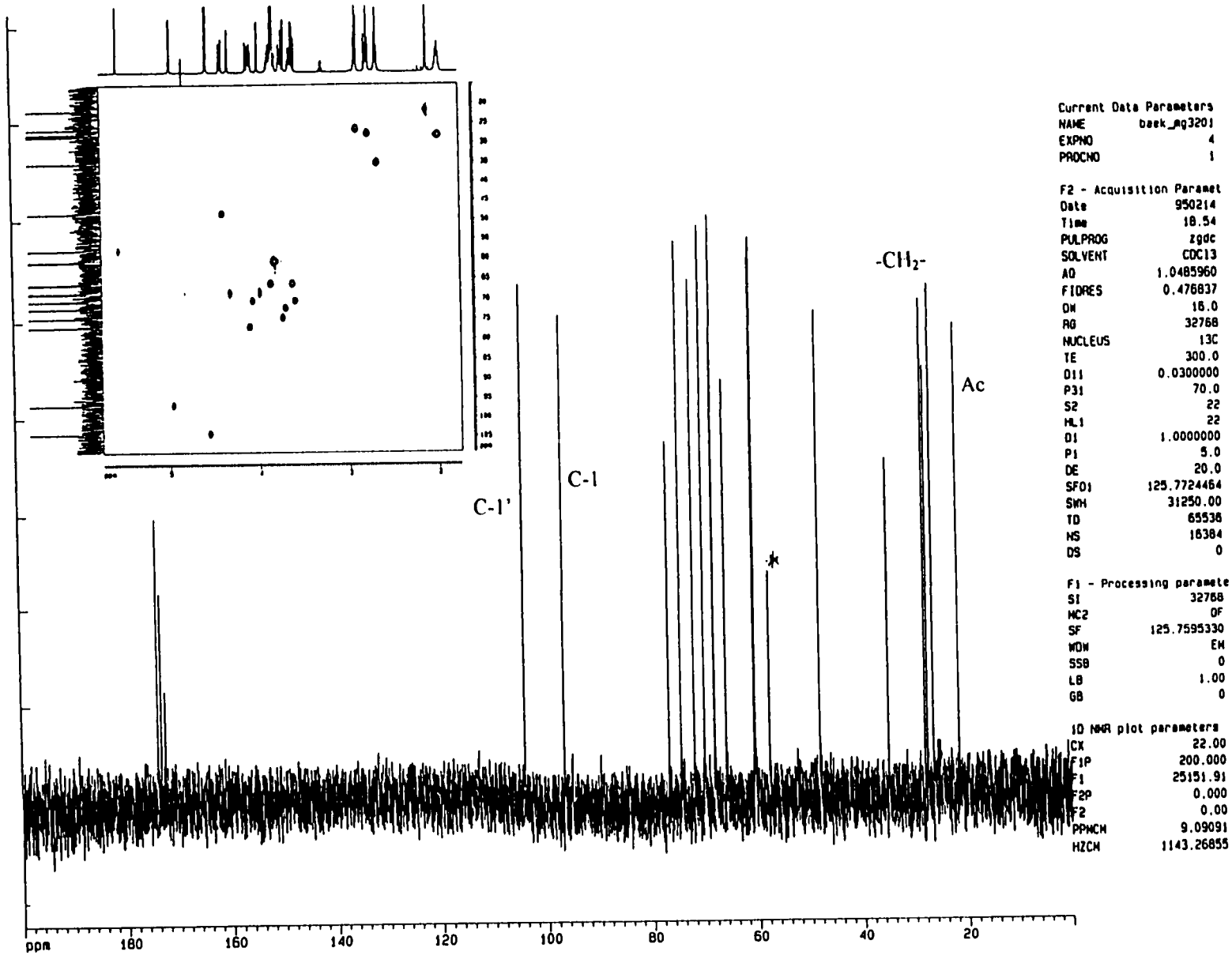


Fig. 4-4. ^{13}C-and ^{1}H-^{13}C HMQC (D_2O, 500 MHz) spectra of divalent T-antigen dendrimer **85.**

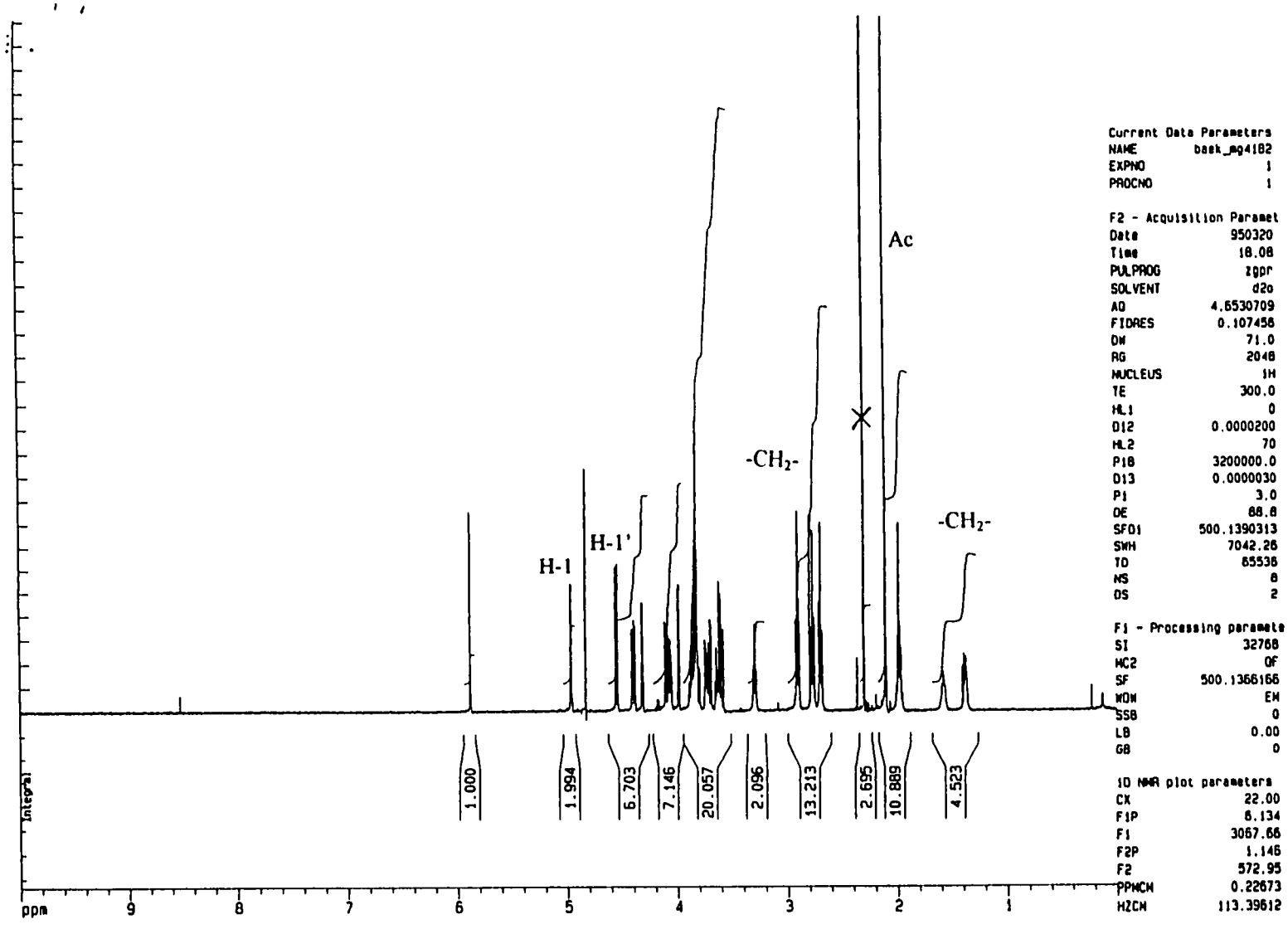
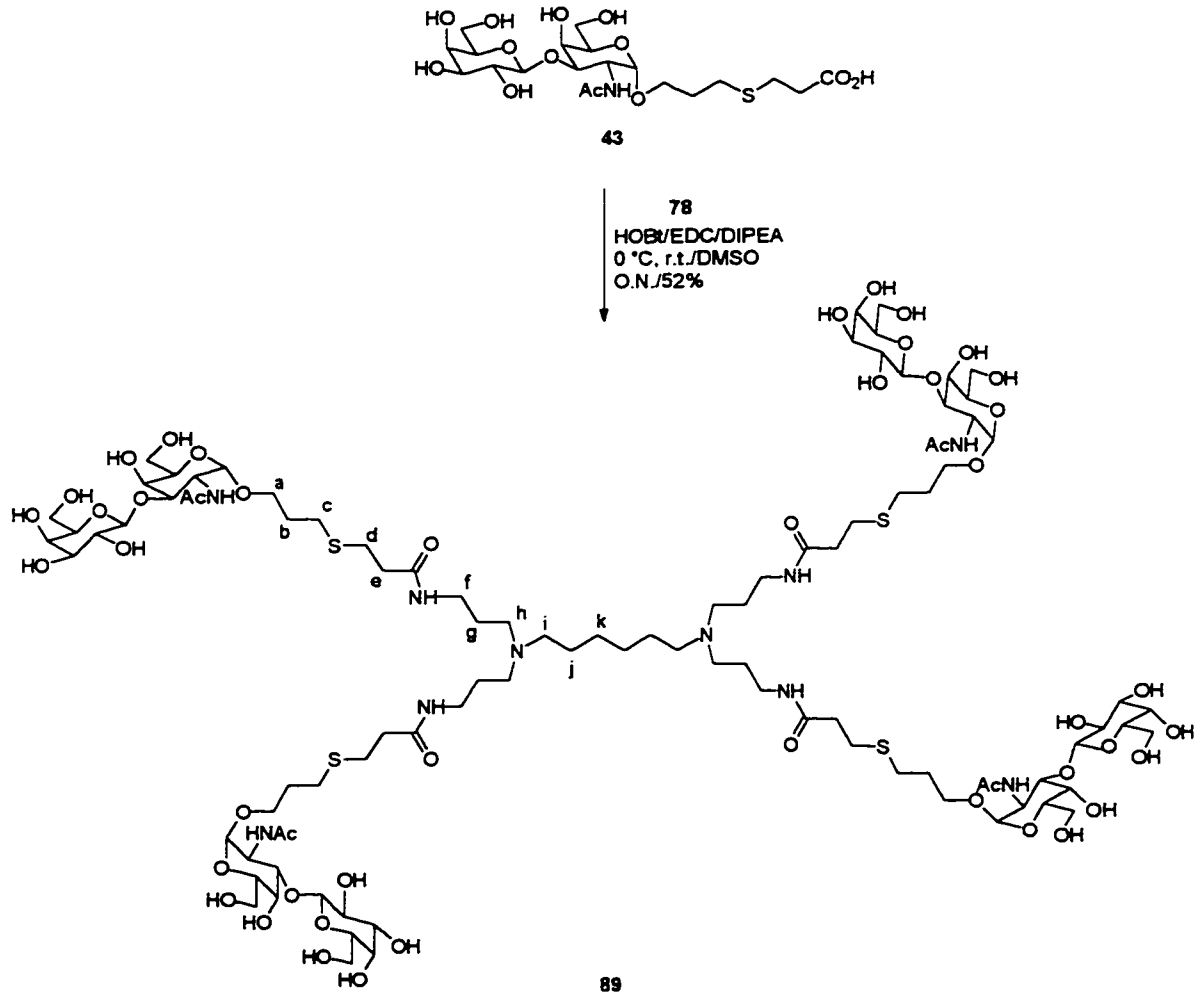


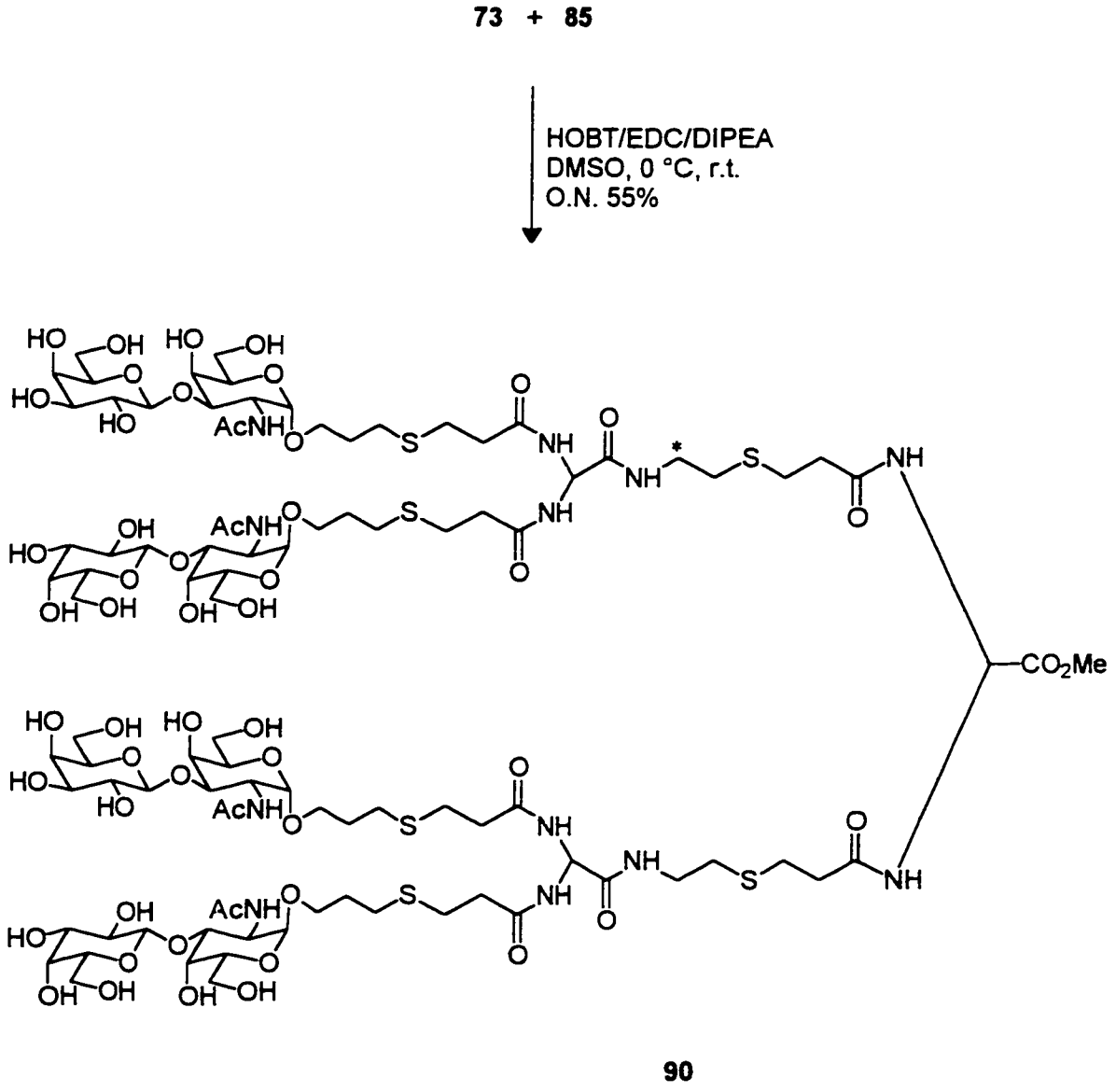
Fig. 4-5. ^{1}H-NMR (D_2O, 500 MHz) spectrum of tetravalent T-antigen dendrimer **86.**

4-4. Synthesis of other glycodendrimers based on amine terminated dendritic core

Multiamine equipped dendrimers **73**, **74** and **78** were coupled with a slight excess of 3-(2-aminoethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc **43** or T-antigen glycodendrimer **85** using HOBt/EDC strategies to provide a new type of T-antigen dendrimers **89** and **90** in moderate 52 and 65% yields, respectively (Scheme 4-10 and 4-11).



Scheme 4-10. Synthesis of tetravalent glycodendrimer **89**.



Scheme 4-11. Synthesis of tetravalent glycodendrimer **90.**

Since these glycodendrimers were too polar to isolate by normal silica gel chromatography, the P-4 size exclusion chromatographic technique was used with water as an eluent.

Complete coupling between the dendritic cores and carboxylic acid terminated carbohydrate haptens was confirmed by ^{1}H-NMR spectral data. For compound **89**, integration for the desired valency between anomeric proton. (H-1 at 4.95 ppm and H-1′ at 4.54 ppm, D_2O) and methylene unit (*) at 3.63 ppm (C(O)NH-$\mathbf{CH_2}$-) clearly showed the extent of T-antigen participation. For compound **90**, new anomeric proton signals were compared with a methylene unit (*) at 3.51 ppm and the desired valency was obtained (Fig. 4-6 ~ 4-8).

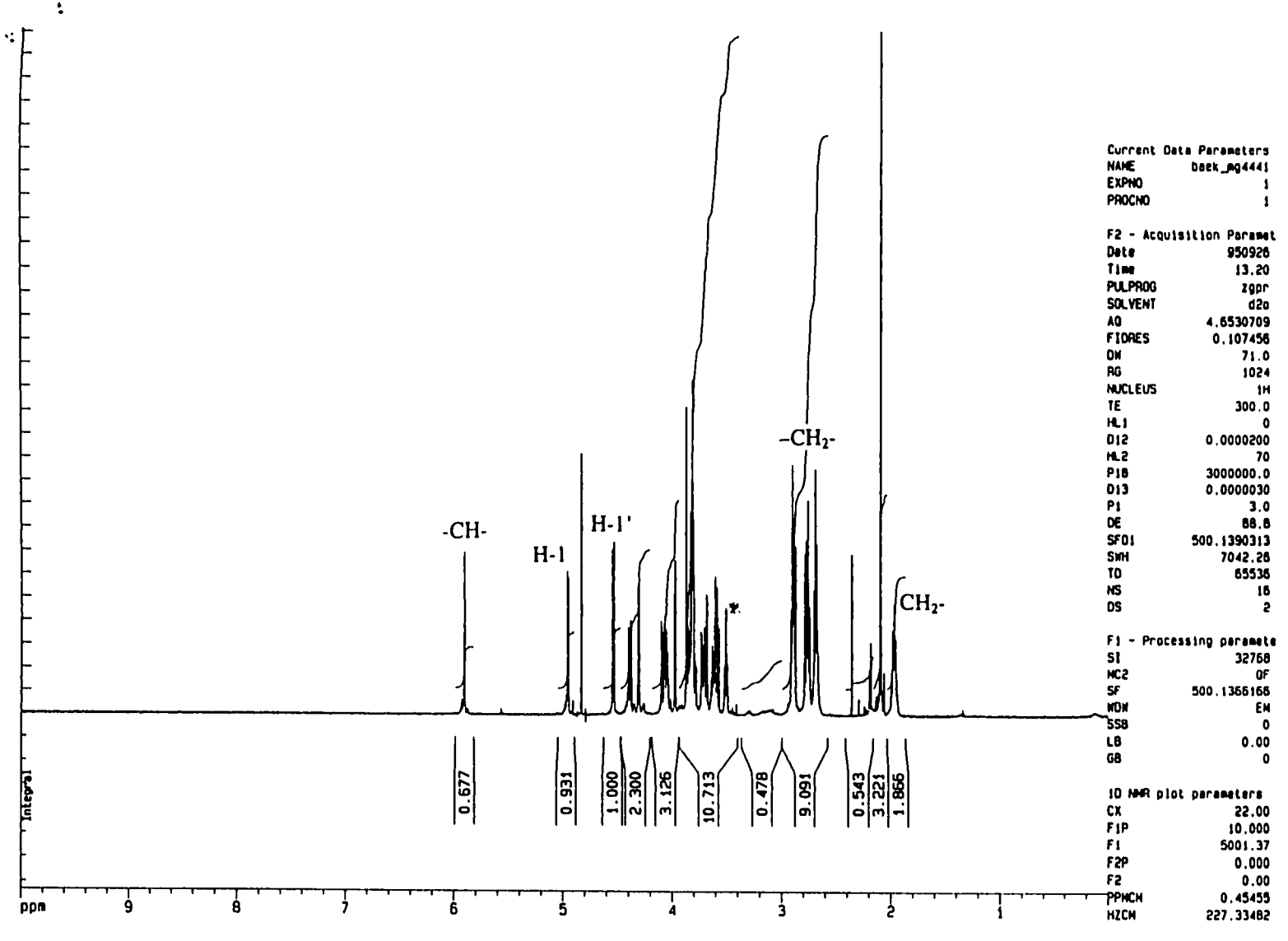


Fig. 4-6. ^{1}H-NMR (D_2O, 500 MHz) spectrum of tetravalent glycodendrimer **90**.

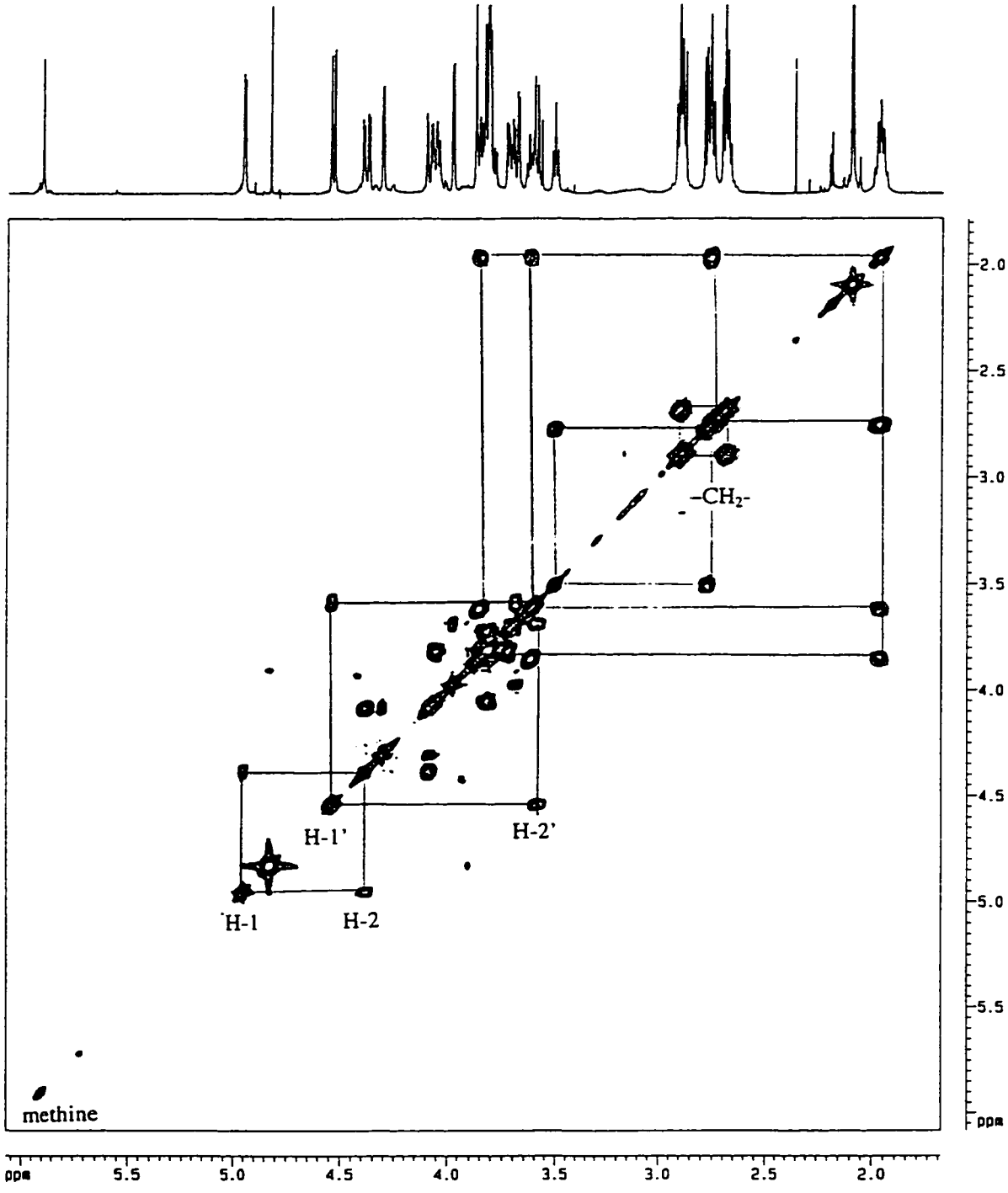
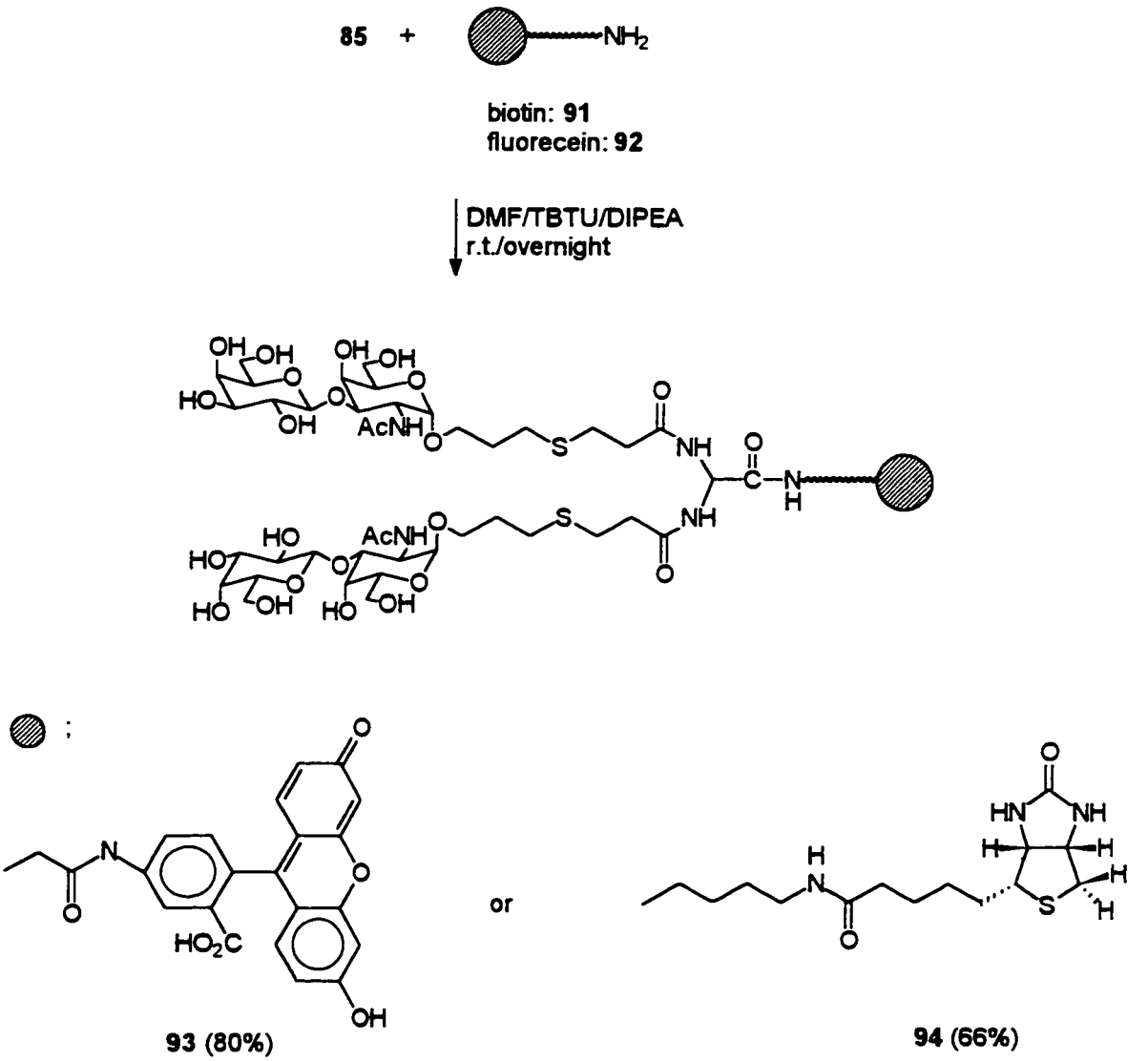


Fig. 4-7. ^{1}H-^{1}H COSY (D_2O, 500 MHz) spectrum of tetravalent glycodendrimer **90.**

4.5. Conjugation of divalent T-antigen and labeling agents

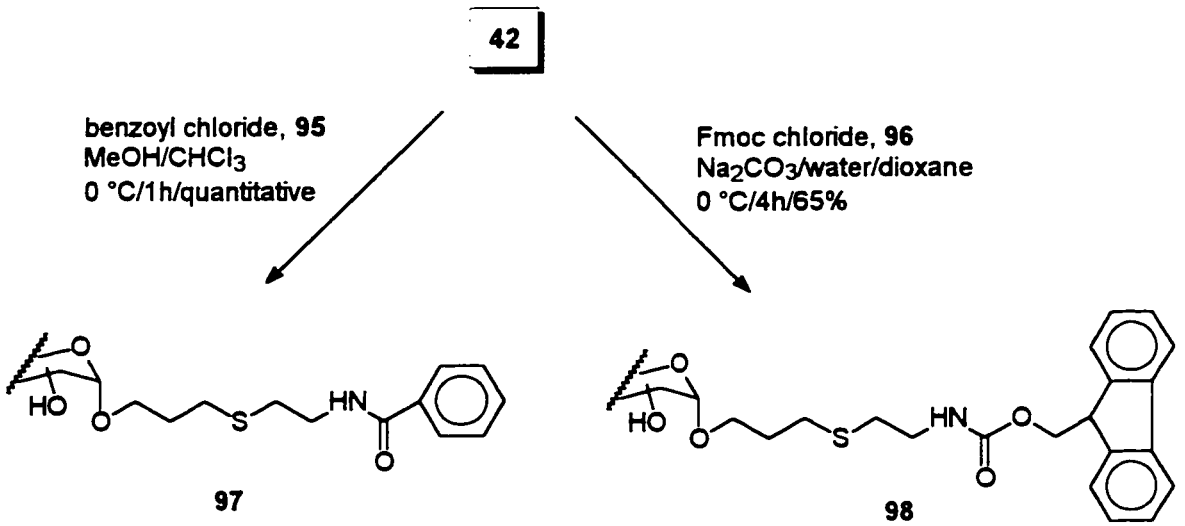
Since biotin labeled materials can be analyzed either by direct application of streptavidin (or avidin)-conjugated probe or sequential application of native (unconjugated) streptavidin followed by biotinylated probe, biotin labeled **93** was prepared. Because fluorescein is an excellent luminescent probe, labeled glycodendrimer **94** was also prepared (Scheme 4-12).



Scheme 4-12. Synthesis of labeled divalent T-antigen dendrimer **93** and **94.**

Divalent T-antigen **85** was coupled with primary amine containing biotin **91** or fluorescein **92** by TBTU/DIPEA strategies. The desired products were isolated by P-4 size exclusion chromatography in 80 and 60% yields, respectively.

Hydrophobic chromophores such as benzoyl-, **95** or 9-fluorenylmethyloxy- **96** labeled monomeric T-antigen **97** and **98** were also prepared in 60 and 65% yields, respectively (Scheme 4-13).



Scheme 4-13. Synthesis of aromatic chromophore labeled monovalent **97** and **98.**

4-6. Immunochemical assays

In order to determine the binding abilities of these glycodendrimers to the peanut lectin from *Arachis Hypogaea*, turbidimetric experiments were initially performed. The formation of precipitin complexes between peanut lectin and di- (**85**), tetra- (**86** and **89**), and hexa- (**87**) valent glycodendrimers was described in Figure 4-8 and 4-9. In most cases, maximum turbidity was obtained in 1 h.

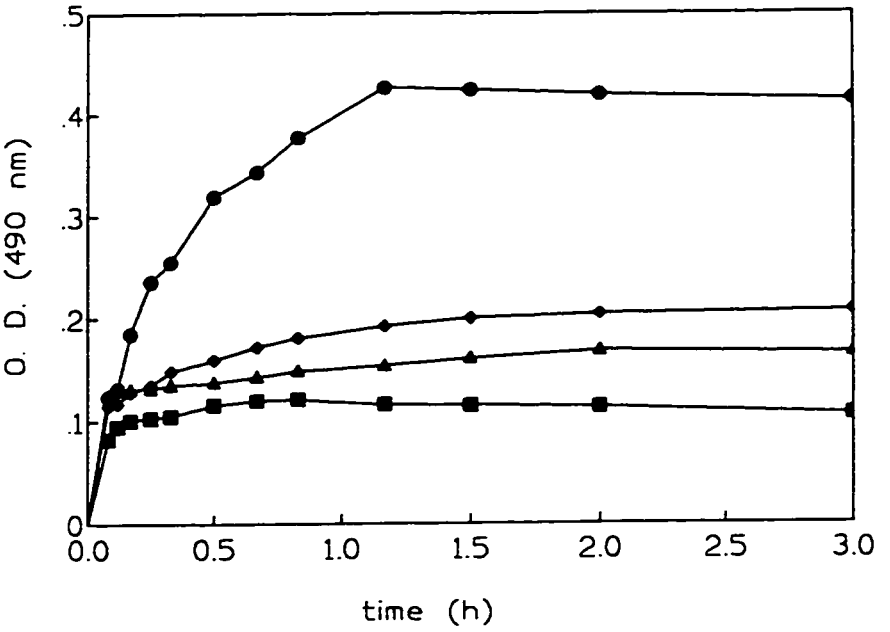


Fig. 4-8. Time course turbidimetric analysis of peanut lectin with T-antigen dendrimers **85** (■), **86** (▲), **87** (●), and **90** (◆).

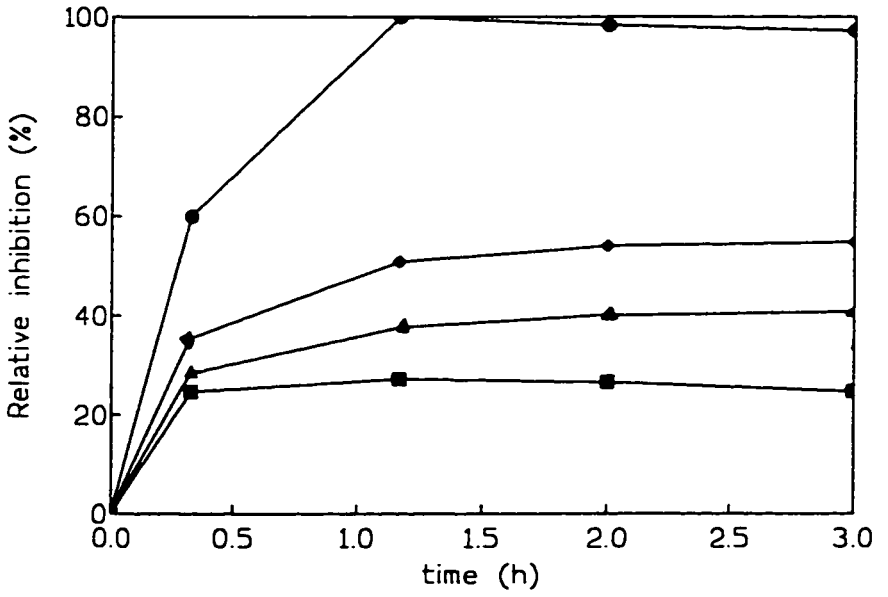


Fig. 4-9. Relative turbidities of T-antigen dendrimers **85** (■), **86** (▲), **87** (●), and **90** (◆) with respect to maximum value (O.D. = 0.43) of **87** at 1.3h.

These nano-quantitative experiments demonstrated that the hexavalent dendrimer **87** showed the most intensive precipitin (O. D. = 0.426). and this indicates a strong binding interaction between **87** and peanut lectin. Two tetramers **86** and **89** were showed moderate precipitin formation with optical density values of 0.18 and 0.13, respectively. Dimer **85** marked the lowest turbidity (O. D. = 0.1). The relative turbidities of di- and tetra valent dendrimers were only 50% when compared with that of hexavalent one. This implies that dimeric **85** exert the weakest binding and crosslinking interactions and readily soluble in the solution. From these results, multivalency effect was clearly demonstrated.

The inhibitory binding interactions of each dendrimer to mouse monoclonal IgG antibodies (mouse IgG MAb) were established by more quantitative and precise enzyme linked immunosorbent assays (ELISA). Mouse IgG MAb were generated using bovine serum albumin (BSA) conjugates derived from N-acrylamido T-antigen derivative **51**. T-antigen containing copolyacrylamide derived from **51** (1/10 mole ratio of repeating units) was used as a coating antigen. BSA conjugates and copolyacrylamide were prepared and the synthesis and physical properties will be described in Chapter 7. Before reaction with the coating antigen, mouse IgG MAb and glycodendrimers as inhibitors with varying concentrations were preincubated for the competitive inhibition of mouse IgG MAb. Goat-anti-mouse IgG MAb-enzyme conjugates were bound for the quantitative detection ofinhibitory properties of glycodendrimers. The results were shown in figure 4-10 and 4-11.

Table 4-1 Inhibitory potencies (IC_{50}'s) of the T-antigen-dendrimers to mouse IgG MAb.

compound	IC_{50} (nM)[a]	Relative potency[a]
29 (monomer)	2300	1
85 (dimer)	174 (347)	13.3 (6.6)
86 (tetramer1)	19 (76)	120.5 (30.1)
90 (tetramer2)	18 (72)	128.1 (32.2)
87 (hexamer)	48 (288)	47.8 (8.0)

a: values in parentheses are based on per T-antigen residue.

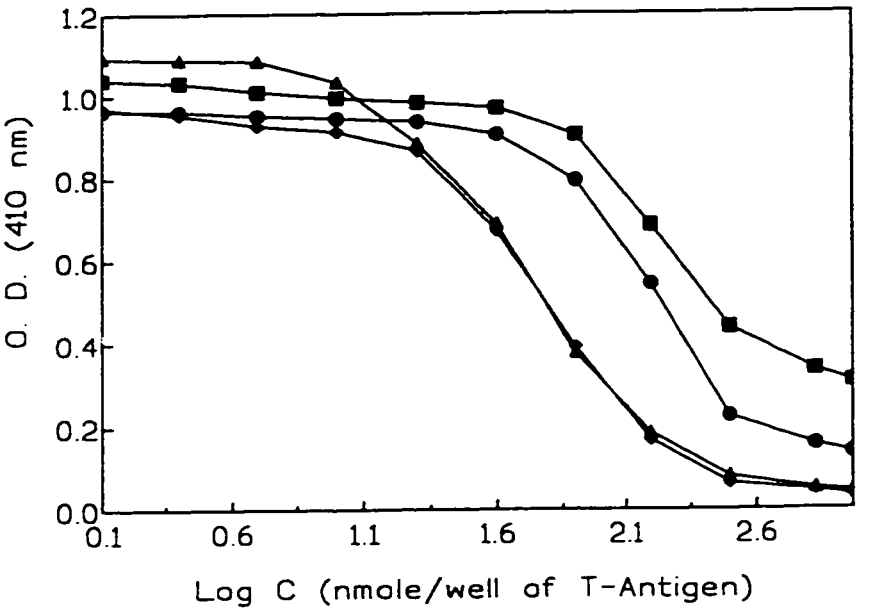


Fig. 4-10. Inhibition of binding of mouse IgG MAb to T-antigen-copolyacrylamide by dendrimers **85** (■), **86** (▲), **87** (●), and **90** (◆).

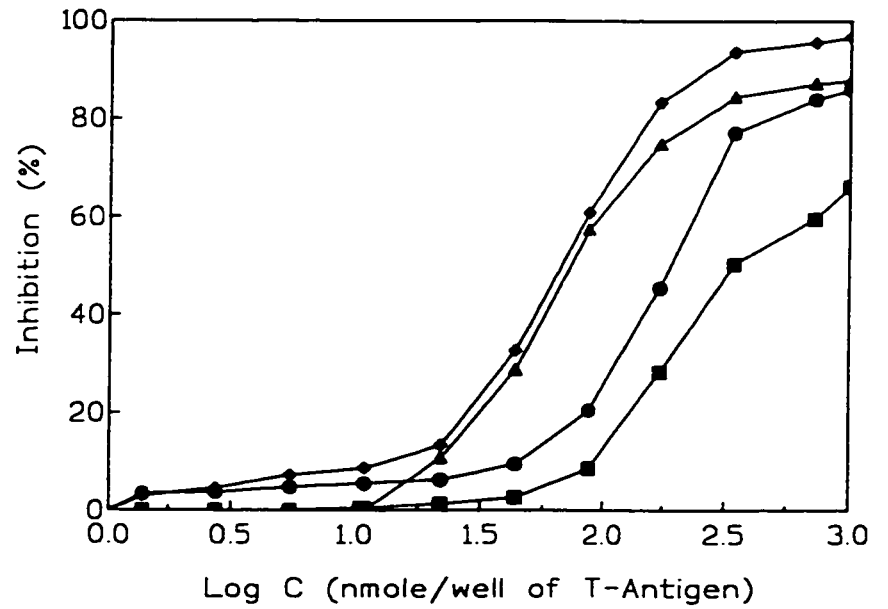


Fig. 4-11. Inhibition of binding of mouse IgG MAb to T-antigen-copolyacrylamide by dendrimers **85** (■), **86** (▲), **87** (●), and **90** (◆).

In the region of low concentration of inhibitors, no multivalency effect was observed. As the concentration of inhibitors increased, this effect was significant. Among these, tetramer **86** and **90** were shown to have the best inhibitory properties where they have IC_{50} values 19.09 and 17.96 nM, respectively (Table 4-1). Dimer **85** showed the lowest inhibition with an IC_{50} of 48.07 nM. On molecular level, the two tetramers were almost 120~130 times more potent than that of monomer **29**. Interestingly, for hexamer **87**, no further increase of inhibitory potency was observed with only 8 fold increase in inhibition potency on the level of T-antigen residues. These facts strongly indicate that not all T-antigen residues participate in the inhibition of mouse IgG MAb possibly due to inappropriate conformational environments such as insufficient length of spacers or lack of spatial freedom of each glycoside. As a result, unbound T-antigen residues reduce inhibitory potency. This phenomenon has been reported with sialoside containing polymers.[192]

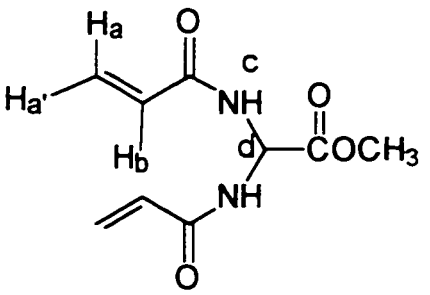
4.7. Conclusions

T-antigen containing dendrimers were prepared using the commercially available N, N′-bis(acrylamido)acetic acid as a seeding molecule. Divergently branched acrylamido group surfaced dendrimers and multiamine terminus obtained from the corresponding nitrile precursor were used as dendritic cores. Glycosidation of these cores was successfully performed using Michael addition of thiolated T-antigen to acryloyl functional group on the surface of the dendrimers or by means of HOBt/EDC coupling strategies. These synthetic glycodendrimers provided multiantennary glycan mimetics with excellent multivalency effects. The binding potential with peanut lectin to form stable cross-linked lattices was demonstrated by qualitative turbidimetric analysis. They were also tested in ELISA using T-antigen copolyacrylamide as a coating antigen, mouse monoclonal IgG, antibodies derived from BSA-T-antigen conjugates and goat-anti-mouse IgG MAb-enzyme conjugates as quantitative detector. Tetrameric glycodendrimers, **22**, **24** were the most potent inhibitors with 120~130 fold increase in inhibition with respect to the

monomer **29**. The quantitative results provide that multivalency is directly responsible for an increase of carbohydrate-receptor protein binding interactions.

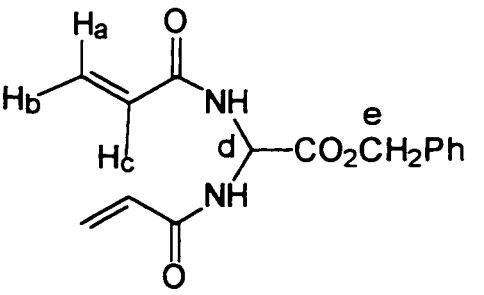
4.8 Experimental methods

Methyl N, N'-bis(acrylamido)acetate (71)



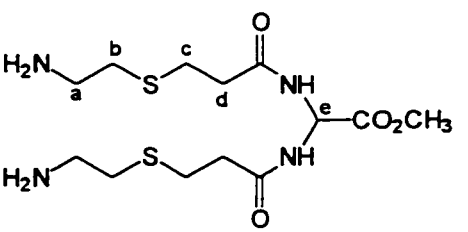
N, N'-Bis(acrylamido)acetic acid (2 g, 10.1 mmol) was dissolved in 120 mL of methanol and the solution was cooled to -15 °C. Thionyl chloride (884 μL, 12.12 mmol, 1.2 eq.) was added dropwise and the resulting mixture was stirred for 1 h. The temperature was allowed to rise to room temperature and the reaction was continued overnight. The solution was condensed to half volume, poured into a fresh silica gel column and separated with CH_3Cl/MeOH, 10/2 as an eluent to give 1.7 g (80%) of white powder: m.p. = 229~231 °C (soften temp.), 350 °C <; R_f = 0.73 (EtOAc/MeOH, 24/1); CI-MS (ether) (m/z): calcd. for $C_9H_{12}O_4N_2$, 212.08, found, 213.1 (M+1, 81.0%); **^{1}H-NMR**, δ (DMSO-d_6): 9.08 (d, 2H's, J_{cd} = 7.5 Hz, $2H_c$), 6.33 (dd, 2H's, J_{cis} = 10.2 Hz, J_{trans} = 17.1 Hz, $2H_b$), 6.14 (dd, 2H's, J_{gem} = 2.0 Hz, J_{trans} = 17.1 Hz, $2H_a$), 5.76 (t, 1H, J_{cd} = 7.6 Hz, H_d), 5.66 (dd, 2H's, J_{gem} = 2.0 Hz, J_{cis} = 10.2 Hz, $2H_{a'}$), 3.64 (s, 3H's, H_{methyl}); **^{13}C-NMR**, δ (DMSO-d_6): 168.7 (carbonyl carbon in ester group), 164.6 (carbonyl carbon in amide group), 130.7 (C_b), 126.9 ($C_{aa'}$), 56.1 (C_d), 52.5 (C_{methyl}).

Benzyl N, N′-bis (acrylamido) acetate (72)



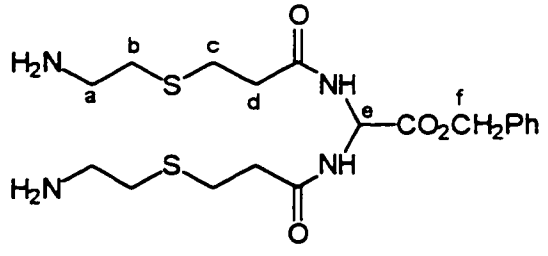
To a solution of N, N′-bis(acrylamido)acetic acid (1 g, 5.1 mmol) and benzyl bromide (0.67 mL, 1.1 eq.) in CH_3CN/DMSO (3/1, 160 mL) was added K_2CO_3 (0.71 g, 1.1 eq.) and the solution was stirred for 24 h. The solvent was evaporated under reduced pressure and lyophilization. The residue was dissolved in $CHCl_3$ (150 mL) and thoroughly washed with water and dried (Na_2SO_4). The solution was condensed, purified by fresh silica gel column chromatography with $CHCl_3$/MeOH (30/1) as an eluent and dried in vacuo to give 1.2 g (4.2 mmol, 82%) of white solid. m.p. = 190.2~191.1 °C; CI-MS (ether), (m/z); calcd. for $C_{15}H_{16}O_4N_2$, 288.16, found, 288.94 (M+1, 86.3%); **^{1}H-NMR** δ (DMSO-d_6): 9.12 (d, 2H, J_{NH-d} = 7.6 Hz, NH), 7.34~7.30 (m, 5H, aromaric), 6.35 (dd, 2H, J_{cis} = 10.2 Hz, J_{trans} = 17.1 Hz, H_c), 6.16 (dd, 2H, J_{gem} = 2.0 Hz, J_{trans} = 17.1 Hz, Ha), 5,83 (t, 1H, J_{NH-d} = 7.6 Hz, H_d), 5.67 (dd, 2H, J_{gem} = 2.0 Hz, H_b), 5.14 (s, 2H, H_e); **^{13}C-NMR** δ (DMSO-d_6): 168.7~164.6 (3C=O), 130.7 (C_c), 128.4~127.7 (aromatic), 126.9 (C_{ab}), 66.6 (C_e), 56.3 (C_d).

Methyl bis[N, N′-(2-(2-aminoethyl)thioethylamido)]acetate (73)



To a solution of 0.4 g (1.89 mmol) of methyl-N, N′-bis(acrylamido)acetate **71** in 30 mL of methanol was added a solution of 0.44 g (3.87 mmol) of cysteamine and 680 μL (3.87 mmol) of DIPEA in 3 mL of methanol. The solution was stirred for 6 h. at room temperature, condensed to minimum volume and purified by silica gel column chromatography with $CHCl_3$/MeOH/H_2O (10/6/1) and EtOAc/AcOH/H_2O (3/2/2) as eluents. The fractions containing product were collected and evaporated under reduced pressure, redissolved in 50 mL of distilled water and neutralized by OH^- resin(amberite IRA 400, OH^- form). The solution was filtered and the filtrate was lyophilized to give of hygroscopic amorphous solid (0.64 g, 93%): CI-MS (ether) (m/z): 367.2 (M+1, 4.6%); **^{1}H-NMR**, δ (D_2O): 6.00 (broad s, 1H, H_c), 3.95 (s, 3H′s, H_{methyl}), 3.37 (t, 4H′s, J_{ab} = 6.7 Hz, H_a), 3.01 (t, 4H′s, J_{ab} = 6.8 Hz, H_b), 2.99 (t, 4H′s, J_{cd} = 6.8 Hz, H_c), 2.77 (t, 4H′s, J_{cd} = 6.8 Hz, H_d); **^{13}C-NMR**, δ (D_2O): 173.8 (carbonyl carbon in ester group), 169.3 (carbonyl carbon in amide group), 56.1 (C_c), 53.2 (C_{methyl}), 37.9 (C_a), 34.4 (C_d), 27.9 (C_b), 25.8 (C_c).

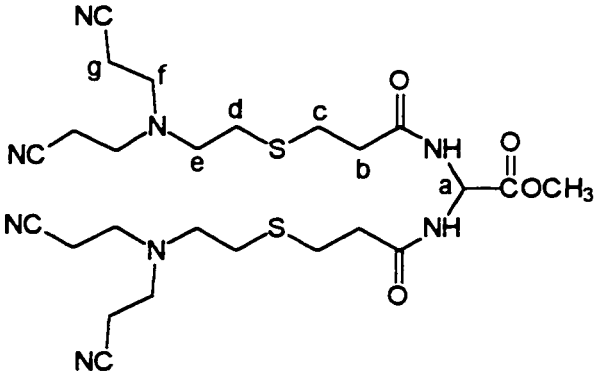
Synthesis of benzyl-bis[N,N′-(2-(2-aminoethyl)thioethylamido)]acetate (74)



To a solution of benzyl-bis (N, N′-acrylamido)acetate (0.2 g, 0.7 mmol), **72** in methanol (30 mL) was added a solution of cysteamine (0.16 g, 1.4 mmol) and DIPEA (0.24 mL, 1.4 mmol) in methanol (3 mL) and the solution was stirred overnight. Solvent was evaporated under reduced pressure. After silica gel column chromatography with EtOAc/water/AcOH, 3/2/2, as an eluent, the benzyl group was disappeared. Therefore, the residue was dried in vacuo without further purification to give **74** (0.32 g, quantitative) as an amorphous solid. This compound contained some DIPEA. **^{1}H-NMR** δ (D_2O):

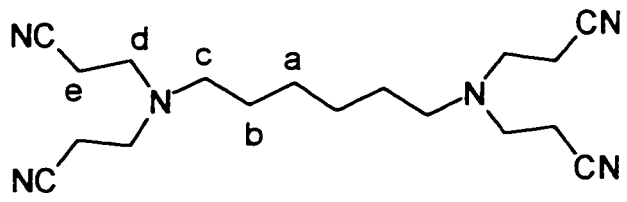
7.53~7.49 (m, 5H, aromatic), 5.57 (s, 1H, H_e), 4.83 (s, 2H, H_f), 3.43 (t, 4H, J_{ab} = 6.5 Hz, H_a), 3.09~3.06 (m, 8H, H_b, H_c), 2.94 (t, 4H, J_{cd} = 6.7 Hz, H_d); **^{13}C-NMR** δ (D_2O): 169.9~165.4 (3C=O), 124.6~123.3(aromatic), 59.7 (C_f), 54.1 (C_e), 38.4 (C_a), 34.2 (C_d), 29.9 (C_b), 24.6 (C_c).

Tetracyano methyl ester (75)



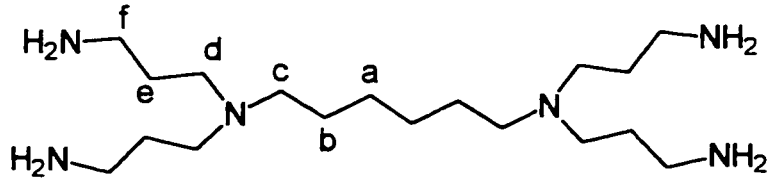
To a solution of bis-amino methyl ester (0.2 g, 0.5 mmol) in acrylonitrile (10 mL) was added acetic acid (0.16 mL, 2.5 eq. per amino group) and the solution was refluxed overnight. The excess of acrylonitrile was evaporated under reduced pressure. The residue was dissolved in ethyl acetate, washed with water (3x), dried (Na_2SO_4) and filtered. The filtrate was condensed and the residue was purified by silica gel column chromatography with EtOAc/MeOH (10/1) as an eluent. The product was dried in vacuo to give 0.11 g (0.33 mmole, 60%) of white solid: m.p. = 87.5~88.1 °C; **^{1}H-NMR** δ (DMSO-d_6): 8.81 (d, 2H, $J_{NH\text{-}a}$ = 7.6 Hz, NH), 5.62 (t, 1H, H_a), 3.62 (s, 3H, methyl), 2.79 (t, 8H, J_{fg} = 6.9 Hz, H_g), 2.69~2.65 (m, 8H, H_c, H_d), 2.58 (t, 8H, H_f), 2.55 (t, 4H, J_{de} = 6.6 Hz, H_e), 2.42 (t, 4H, J_{bc} = 7.4 Hz, H_b); **^{13}C-NMR** δ (DMSO-d_6): 170.8~168.9 (C=O), 119.9 (CN), 55.9 (C_a), 52.9 (C_c), 52.4 (C_{methyl}), 48.5 (C_g), 35.5 (C_b), 28.9 (C_f), 26.8 (C_d), 15.8 (C_e).

N, N, N′, N′-tetra-2-ethylcyano heaxmethylenediamine (77)



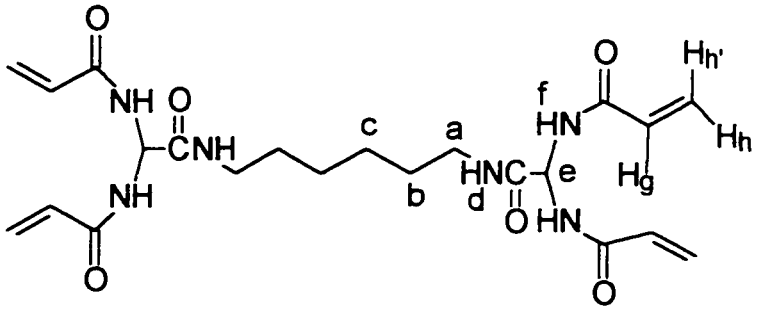
Hexanediamine (1 g, 8.61 mmole) was dissolved in 30 mL of acrylonitrile. 2.5 mL of acetic acid (~20 mmol per nitrile group) was added and the solution was refluxed (~125 °C) overnight. TLC (EtOAc/Hex, 3/1) and a ninhydrin test indicated that the starting material was completely consumed. The excess of acrylonitrile was distilled off under vacuum, the residue was extracted with $CHCl_3$ and added to 20 mL of concentrated ammonia solution. The organic phase was separated, washed with distilled water (3x) and dried with sodium sulfate. The crude product was purified by fresh silica gel column chromatography with ethylacetate as an eluent. The solution was condensed and dried under vacuum to afford 2.5 g (87%) of yellow colored viscous oil product: R_f = 0.63(EtOAc/Hex, 3/1); CI-MS (ether): 329.2(M+1, 1.9%); **^{1}H-NMR**, δ ($CDCl_3$): 2.81 (dd, 8H′s, $J_{cc'(gem)}$ = 1.8 Hz, J_{de} = 6.8 Hz, H_c), 2.50 (dd, 4H′s, $J_{cc'(gem)}$ = 1.7 Hz, J_{bc} = 7.1 Hz, H_c), 2.44 (t, 8H′s, J_{de} = 6.8 Hz, H_d), 1.45~1.42 (m, 4H′s, H_b), 1.29~1.34 (m, 4H′s, H_a); **^{13}C-NMR**, δ ($CDCl_3$): 118.7 (C≡N), 53.3 (C_c), 49.6 (C_e), 27.3 (C_b), 26.8 (C_a), 17.0 (C_d)

N, N, N′, N′-tetra(3-aminopropyl)hexamethylenediamine (78)



N, N′-Tetra(2-cyanoethyl)hexamethylenediamine 77 (1.2 g, 3.65 g) was dissolved in 50 mL of ethanol. 0.3 g of Raney Nickel was added and the solution was bubbled with hydrogen gas for 3 days. TLC ($CHCl_3$/MeOH/H_2O, 10/6/1) indicated that starting material was consumed completely. The solution was filtered and the filtrate was condensed under vacuum to afford 1.2 g (97%) of colorless oil product: CI-MS (ether) (m/z): calcd.for $C_{18}H_{44}N_6$, 344.36, found, 345.31 (M+1, 1.6%); **^{1}H-NMR**, δ (D_2O): 2.67 (t, 8H's, J_{ef} = 7.1 Hz, H_f), 2.58~2.52 (m, 8H's, H_c, H_d), 1.72~1.64 (m, 8H's, H_e), 1.53~1.52 (m, 4H's, H_b), 1.39~1.36 (broad t, 4H's, H_a); **^{13}C-NMR**, δ (D_2O): 52.6 (C_b), 50.2 (C_a), 38.6 (C_e), 27.7 (C_f), 26.3 (C_d), 24.5 (C_c).

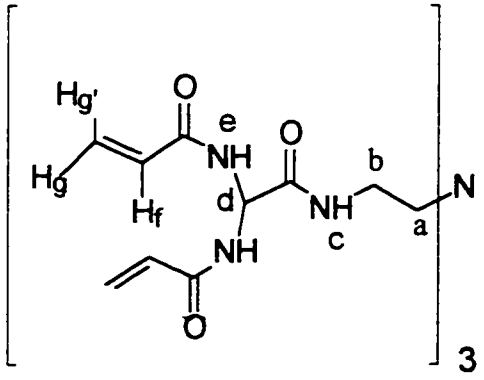
Tetravalent dendritic core (81)



To a solution of 0.44 g (2.22 mmol) of N, N′-bis(acrylamido)acetic acid in 40 mL of DMSO was added 0.45 g (3.33 mmol) of 1-hydroxybenzotriazole hydrate (HOBt), 0.12 g (1 mmole) of hexamethylenediamine and 87.1 μL (0.5 mmol) of DIPEA at 0 °C without light. The cloudy solution was stirred for 10 minutes after which 0.98 g (3.3 mmole) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was added. The suspension was allowed to rise in temperature to room temperature and was stirred for 1 day. TLC ($CHCl_3$/MeOH/H_2O, 10/3/0.5) indicated that the starting material was consumed completely. The solvent was removed by lyophilization and the yellow solid residue was mixed with CH_2Cl_2/H_2O (1:1 v/v ratio, 100 mL × 3) followed by suction filtration and vacuum drying to give 286 mg (60%) of insoluble ivory colored solid in both solvents: m.p. = 350 °C<; CI-MS: calcd. for $C_{13}H_{26}O_4N_4S_2$, 366.14, found, 367.20 (M+1,

4.6%); **^{1}H-NMR**, δ (DMSO-d_6): 8.64 (d,4H's, J_{ef} = 6.5 Hz, H_f), 8.02 (broad s, 2H's, H_d), 6.38~6.33 (m, 4H's, H_g), 6.10 (d, 4H's, J_{trans} = 17.1 Hz, J_{gem} = 2.0 Hz, $H_{h'}$), 5.84 (broad s, 2H's, H_e), 5.61 (d, 4H's, J_{cis} = 9.9 Hz, J_{gem} = 1.9 Hz, H_h), 3.04 (broad s, 4H's, H_a), 1.37 (broad s, 4H's, H_b), 1.21 (broad s, 4H's, H_c); **^{13}C-NMR**, δ (DMSO-d_6): 167.4 (C=O in amide group), 164.3 (C=O in acryloyl group), 131.2 (C_g), 126.2 ($C_{hh'}$), 57.1 (C_e), 38.8 (C_a), 28.8 (C_b).

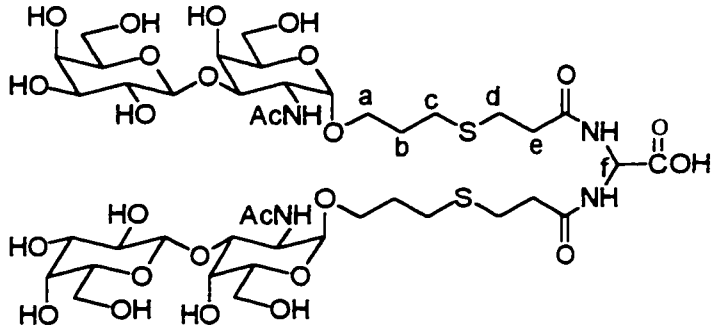
Hexavalent dendritic core (82)



To a solution of 0.48 g (2.42 mmol) of N, N'-bis(acrylamido)acetic acid in 50 mL of DMSO was added 0.49 g (3.62 mmol) of HOBt, 89.8 μL (0.6 mmole) of tris (2-aminoethyl) amine and DIPEA (73.2 μL, 94.2 mmol) at 0 °C without light. The cloudy suspension was stirred for 10 minutes after which 1.07 g (3.6 mmol) of EDC was added. The suspension was allowed to warm to room temperature and stirring was continued for 1 day. TLC ($CHCl_3$/MeOH/H_2O, 10/6/1) indicated complete consumption of the starting material. The solvent was eliminated by lyophilization and the yellow solid residue was mixed with CH_2Cl_2/H_2O (1:1 v/v ratio, 100 m L x 3) followed by suction filtration and vacuum drying to give 255 mg (62%) of product: m.p. = 350 °C <; (+) FAB-MS (glycerol) (m/z): cacld. for $C_{30}H_{42}O_9N_{10}$, 686.31, found, 687.4 (M+1, 16.3%); **^{1}H-NMR**, δ (DMSO-d_6): 8.71 (d, 6H's, J_{de} = 7.6 Hz, H_e), 7.97 (m, 3H's, H_c), 6.36 (dd, 6H's, J_{cis} = 10.2 Hz, J_{trans} = 17.1 Hz, H_f), 6.12 (dd, 6H's, J_{gem} = 2.0 Hz, J_{trans} = 17.1 Hz, H_g), 5.88 (d, 3H's, J_{de} = 7.7 Hz, H_d), 5.62 (dd, 6H's, J_{gem} = 1.9 Hz, J_{cis} = 10.3 Hz, $H_{g'}$), 3.11 (broad s,

6H's, H_b), 2.53 (screened by DMSO, 6H's, H_a); **^{13}C-NMR**, δ (DMSO-d_6); 167.6 (C=O in N-ethyl amide group), 164.5 (C=O in acryloyl group), 131.1 (C_f), 126.3 ($C_{gg'}$), 57.0 (C_d), 52.8 (C_a), 37.1 (C_b).

Divalent T-antigen dendrimer (85)

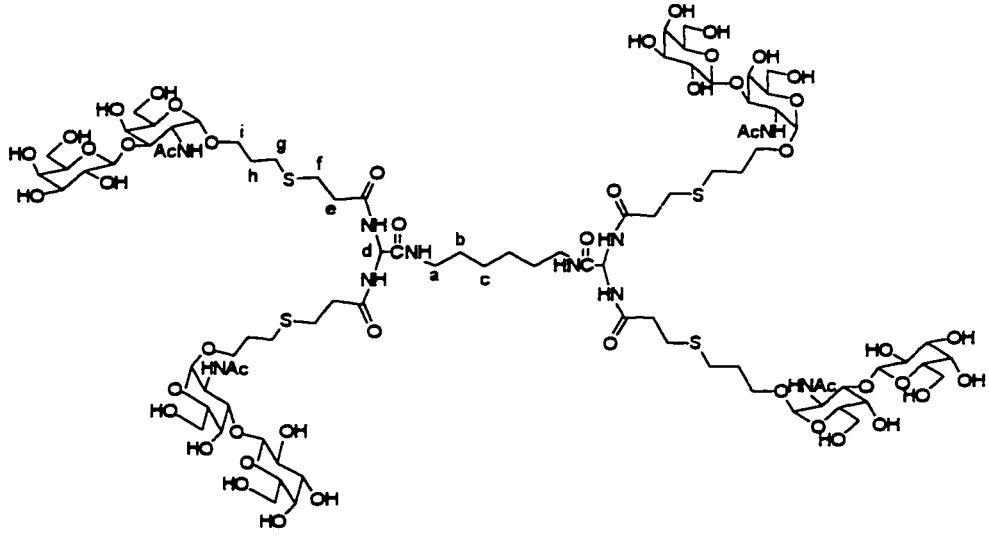


N, N'-Bis(acrylamido)acetic acid **68** (23.8 mg, 0.12 mmol) and 33.5 μL (0.24 mmol) of triethylamine was dissolved in 5ml of deoxygenated methanol and the mixture waskept under nitrogen atmosphere. 150 mg (0.3 mmol) of thioacetate **46** was dissolved in 1 mL of deoxygenated methanol and 1.10 mL of 0.23 M $CH_3O^-Na^+$/deoxygenated MeOH was added under nitrogen. After TLC indicated complete consumption of the starting material, this solution was transferred to the former solution by syringe and stirring was continued for 5 h. under nitrogen bubbling. The solution was condensed and purified by silica gel column chromatography with $CHCl_3$/MeOH/H_2O, 10/8/2 as an eluent to give **85** in quantitative yield: m.p. = 162.8~164.7 °C; $[\alpha]_D$ = +80.7° (c = 1, H_2O); R_f = 0.26 ($CHCl_3$/MeOH/H_2O, 10/8/2); **^{1}H-NMR**, δ (D_2O): 5.56 (s, 1H, H_f), 4.96 (d, 2H's, J_{12} = 3.8 Hz, H_1), 4.55 (d, 2H's, J_{12}' = 7.8 Hz, $H_{1'}$), 4.40 (dd, 2H's, J_{12} = 3.8 Hz, J_{23} = 11.1 Hz, H_2), 4.32 (d, 2H's, J_{34} = 2.9 Hz, $J_{45} \leq$ 1 Hz, H_4), 4.10 (dd, 2H's, J_{23} = 11.1 Hz, J_{34} = 3.1 Hz, H_3), 4.09~4.06 (broad t, 2H's, $J_{45} \leq$ 1 Hz, J_{56} = 6.4 Hz, H_5), 3.99 (d, 2H's, J_{34}' = 3.4 Hz, $J_{45}' \leq$ 1 Hz, $H_{4'}$), 3.89~3.79 (m, 10H's, $4H_6$, $4H_{6'}$, $2H_a$), 3.75~3.72 (m, 2H's, $H_{5'}$), 3.71 (dd, 2H's, J_{23}' = 10.0 Hz, J_{34}' = 3.4 Hz, $H_{3'}$), 3.65~3.60 (m, 2H's, H_a), 3.59 (dd, 2H's, J_{12}' = 7.8 Hz, J_{23}' = 10.0 Hz, $H_{2'}$), 2.90 (t, 4H's, J_{de} = 7.1 Hz, H_d), 2.77 (t, 4H's, J_{bc} =

7.4 Hz, H_c), 2.67 (t, 4H's, J_{dc} = 6.9 Hz, H_e), 2.04~1.95 (m, 4H's, H_b); **^{13}C-NMR**, δ (D_2O): 174.3 (carbonyl carbon in N-acetyl group), 173.4 (carbonyl carbon in N-methynyl amido group), 172.6 (carbonyl carbon in carboxylic group), 104.2 ($C_{1'}$), 96.7 (C_1), 76.8 (C_3), 74.5 ($C_{5'}$), 72.1 ($C_{3'}$), 70.2~70.1 ($C_{2'}$, C_5), 68.3 (C_4), 68.1 ($C_{4'}$), 66.1 (C_a), 60.7 (C_6), 60.5 ($C_{6'}$), 57.7 (C_f), 48.2 (C_2), 35.0 (C_e).

Anal. Calcd. for $C_{42}H_{71}O_{26}N_4S_2$ (1112.39): C, 45.31; H, 6.52; N, 5.03. Found: C, 46.04; H, 6.68; N, 4.29.

Tetravalent T-antigen dendrimer (86)

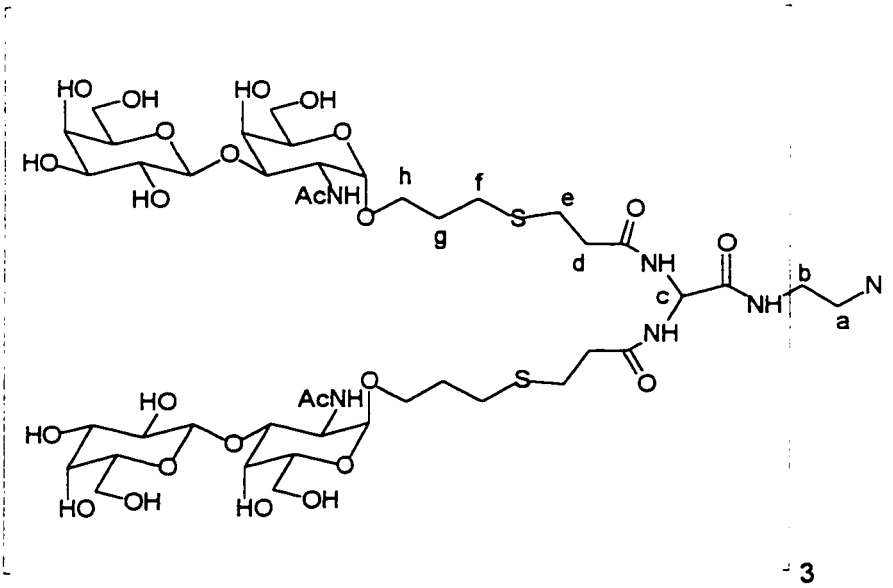


1,6-Bis[(N, N'-bis(acrylamido)acetamido]hexane **81** (5 mg, 10.5 μmol) and 7 μL (5 eq.) of triethylamine were dissolved in deoxygenated DMSO and the resulting solution was kept under nitrogen atmosphere. 25 mg (50 μmol) of **46** was dissolved in 1mL of deoxygenated methanol and 250 μL (50 μmol) of 0.2 M of $CH_3O^-Na^+$ in deoxygenated methanol was added at room temperature under nitrogen followed by neutralization using H^+ resin. The two solution were combined and stirred overnight at room temperature under nitrogen bubbling. TLC ($CHCl_3$/MeOH/H_2O, 10/8/2) indicated complete consumption of the thiol. The resulting solution was dried under vacuum and the crude product was purified by P-2 size exclusion chromatography with distilled water.

Lyophilization afforded 12 mg (50%) of white spongy solid and a trace amount of disulfide: m.p. = 149.2 ~153.5°C; $[\alpha]_D$ = +69.2° (c = 0.65, H_2O); **^{1}H-NMR**, δ (D_2O): 5.88 (s, 2H's, H_d), 4.97 (d, 4H's, J_{12} = 3.8 Hz, H_1), 4.55 (d, 4H's, $J_{12'}$ = 7.8 Hz, $H_{1'}$), 4.40 (dd, 4H's, J_{12} = 3.8 Hz, J_{23} = 11.1 Hz, H_2), 4.32 (d, 4H's, J_{34} = 2.9 Hz, $J_{45} \leq 1$ Hz, H_4), 4.10 (dd, 4H's, J_{23} = 11.1 Hz, J_{34} = 3.1 Hz, H_3), 4.06 (broad t, 4H's, J_{56} = 6.2 Hz, $J_{45} \leq 1$ Hz, H_5), 3.98 (d, 4H's, $J_{34'}$ = 3.2 Hz, $J_{45'} \leq 1$ Hz, $H_{4'}$), 3.89~3.79 (m, 20H's, $8H_6$, $8H_{6'}$, $4H_h$), 3.75~3.72 (m, 4H's, $H_{5'}$), 3.70 (dd, 4H's, $J_{23'}$ = 10.2 Hz, $J_{34'}$ = 3.4 Hz, $H_{3'}$), 3.65~3.61 (m, 4H's, $1H_i$), 3.60 (dd, 4H's, $J_{12'}$ = 7.8 Hz, $J_{23'}$ = 10.0 Hz, $H_{2'}$), 3.30 (t, 4H's, J_{ab} = 6.8Hz, H_a), 2.91 (t, J_{ef} = 6.9 Hz, H_f), 2.77 (t, 8H's, J_{gh} = 7.2 Hz, H_g), 2.69 (t, 8H's, J_{ef} = 6.9 Hz, H_e), 2.10 (s, 12H's, AcNH), 2.01~1.95 (m, 8H's, H_h), 1.58~1.56 (m, 4H's, H_b), 1.40~1.37 (m, 4H's, H_c); **^{13}C-NMR**, δ (D_2O): 173~168.1 (3 C=O's), 104.2 ($C_{1'}$), 96.7 (C_1), 76.8 (C_3), 74.5 ($C_{5'}$), 72.1 ($C_{3'}$), 70.2~70.1 ($C_{2'}$, C_5), 68.2 (C_4), 68.1 ($C_{4'}$), 66.0 (C_i), 60.7 (C_6), 60.5 ($C_{6'}$), 56.9 (C_d), 48.2 (C_2), 39.1 (C_a), 34.8 (C_e), 28.0 (C_h), 27.7 (C_b), 27.6 (C_g), 26.4 (C_f), 25.1 (C_c), 21.6 (C_{methyl}).

Anal. Calcd. for $C_{90}H_{156}O_{50}N_{10}S_4$ (2305.8): C, 46.80; H, 6.80; N, 6.10. Found: C, 45.87; H, 6.97; N, 5.45.

Hexavalent T-antigen dendrimer (87)



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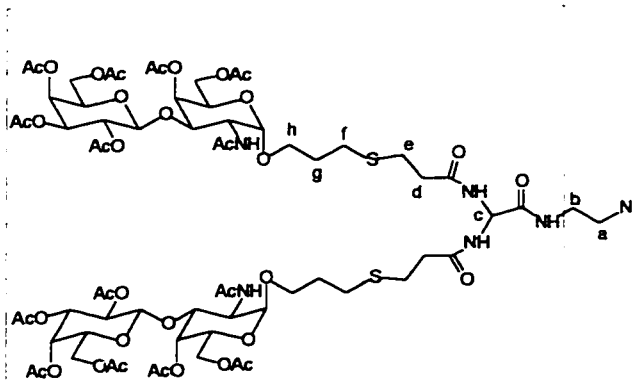
Compound **82** (5 mg, 7.3 μmol) and triethylamine (7.4 μL, 52.6 μmol) were dissolved in 1 mL of deoxygenated DMSO and the solution was kept under nitrogen atmosphere. 26.4 mg (52.6 μmol) of **46** was dissolved in deoxygenated methanol and 263 μL of 0.2 M $CH_3O^-Na^+$ in deoxygenated methanol was added under nitrogen atmosphere followed by neutralization using H^- resin. The two solutions were combined and stirred overnight at room temperature under nitrogen. TLC ($CHCl_3$/MeOH/H_2O) indicated complete consumption of thiol. The resulting solution was dried under vacuum and the crude product was purified by P-4 size exclusion chromatography with distilled water followed by lyophilization to afford 11.3 mg (3.2 μmol, 45%) of white spongy solid and 3.9 mg of disulfide: m.p. = 194~195 °C; $[\alpha]_D$ = +81.18° (c = 0.85, H_2O); **^{1}H-NMR**, δ (D_2O): 5.54 (s, 3H's, H_c), 4.96 (d, 6H's, J_{12} = 3.7 Hz, H_1), 4.54 (d, 6H's, J_{12}' = 7.7 Hz, $H_{1'}$), 4.39 (dd, 6H's, J_{12} = 3.7 Hz, J_{23} = 11.1 Hz, H_2), 4.31 (broad s, 6H's, H_4), 4.95 (dd, 6H's, J_{23} = 10.9 Hz, J_{34} = 3.4 Hz, H_3), 4.06 (t, 6H's, J_{56} = 5.6 Hz, $J_{45} \leq$ 1 Hz, H_5), 3.98 (d, 6H's, J_{34}' = 3.4 Hz, $J_{45}' \leq$ 1 Hz, $H_{4'}$), 3.86~3.78 (m, 30H's, 12H_6, 12$H_{6'}$, 6H_h), 3.73 (m, 6H's, $H_{5'}$), 3.70 (dd, 6H's, J_{23}' = 10.0 Hz, J_{34}' = 3.5 Hz, $H_{3'}$), 3.63~3.56 (m, 12H's, 6$H_{2'}$, 6H_h), 2.88 (t, 12H's, J_{de} = 6.9 Hz, H_e), 2.85 (t, 6H's, J_{ab} = 7.3 Hz, H_a), 2.75 (t, 12H's, J_{fg} = 7.1 Hz, H_f), 2.65 (t, 12H's, J_{de} = 6.8 Hz, H_d), 2.56 (t, 6H's, J_{ab} = 7.2 Hz, H_b), 2.09 (s, 18H's, AcNH); **^{13}C-NMR**, δ (D_2O): 174.1~173.6 (3 C=O's), 104,2 ($C_{1'}$), 96.7 (C_1), 76.8 (C_3), 74.5 ($C_{5'}$), 72.0 ($C_{3'}$), 70.2~70.1 ($C_{2'}$, C_5), 68.3 (C_4), 68.1 ($C_{4'}$), 66.0 (C_h), 60.7 (C_6), 60.5 ($C_{6'}$), 57.7 (C_c), 48.2 (C_2), 34.9 (C_b), 33.8 (C_d), 28.1 (C_g), 27.6 (C_f), 27.5 (C_a), 26.4 (C_e),, 21.7 (C_{methyl}).

Fully protected hexavalent T-antigen glycodendrimer (88)



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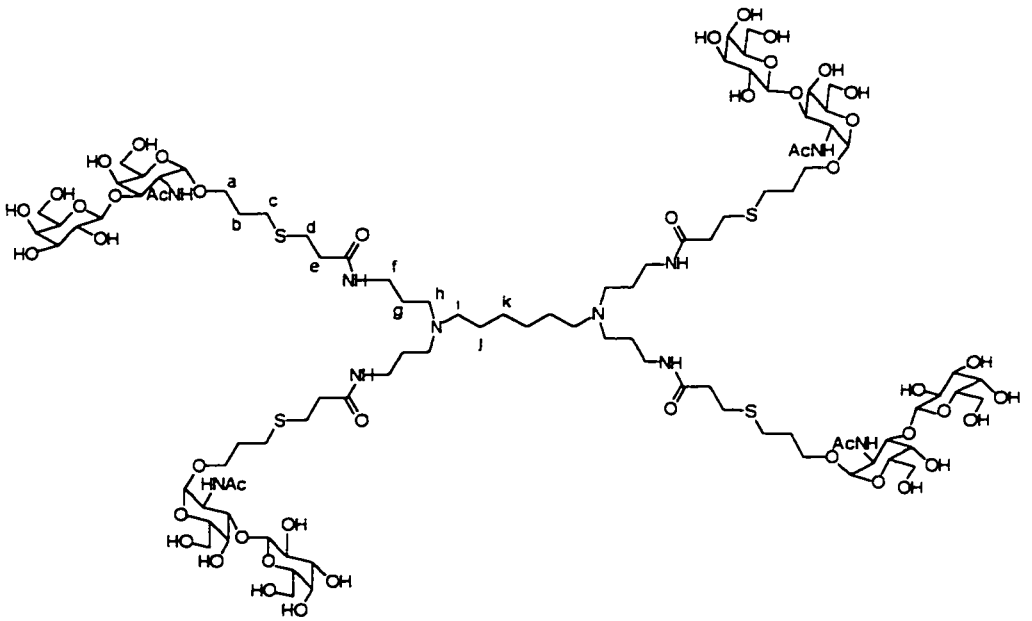
Dendritic core **82** (5 mg, 7.3 μmol) and 18 μL (0.129 μmol) of Et_3N were dissolved in 1mL of deoxygenated DMSO and kept under N_2 atmosphere. To a solution of **47** (60 mg, 80 μmol) dissolved in deoxygenated DMF (1 mL) was added 2.5 M hydrazinium acetate in DMF (100 μL, 3 eq.) and the solution was stirred for 30 minutes at room temperature. The reaction mixture was diluted with EtOAc (7 mL) and successively washed with 1 M HCl (4 mL), saturated $NaHCO_3$ (4 mL), water and saturated NaCl. The dried organic phase (Na_2SO_4) was briefly evaporated to dryness under reduced pressure. The residue was redissolved in deoxygenated DMSO (0.5 mL) to which was added to former solution. The reaction was continued overnight at room temperature under N_2 - bubbling. Completion of the reaction was judged by TLC using EtOAc/Hex/MeOH (12/5/1). DMSO was removed by lyophilization and the residue was purified by silica gel chromatography to give glycodendrimer **88** (25 mg, 5.1 μmol, 70%): **^{1}H-NMR** δ ($CDCl_3$): 5.73 (broad d, 9H, NH), 5.33 (broad s, 12H, H_4, H_4'), 5.12 (dd, 6H, $J_{12'}$ = 7.9 Hz, $J_{23'}$ = 10.4 Hz, H_2'), 4.96~4.91 (m, 12H, H_1, H_3'), 4.59 (d, 6H, $J_{12'}$ = 7.9 Hz, H_1'), 4.57~4.44 (m, 6H, H_2), 4.17~4.00 (m, 27H, H_6, H_6', H_f), 3.99~3.80 (m, 18H, H_5, H_5', H_3), 3.79~3.73 (m, 6H, H_a), 3.54~3.50 (m, 6H, H_a'), 2.82~2.65 (m, 12H, H_d), 2.64~2.57 (m, 18H, H_c, H_g), 2.18~1.86 (m, 150H, 48Ac, H_b, H_e), 1.42 (t, 6H, J_{gh} = 7.6 Hz, H_h); **^{13}C-NMR** δ ($CDCl_3$): 173.2~166.1 (C=O), 100.5 (C_1'), 97.8 (C_1), 72.8 (C_5), 70.9 (C_3), 70.8 (C_3'), 68.7 (C_4'), 68.6 (C_2'), 67.5 (C_c), 66.8 & 66.6 (C_h, C_4), 62.7~61.1 (C_5', C_6, C_6'),

49.0 (C_2), 35.7 (C_e), 34.3 (C_b), 33.0 (C_d), 28.9 (C_a), 28.7 (C_f), 21.5 (C_g), 28.7~20.5 (C_{methyl}).

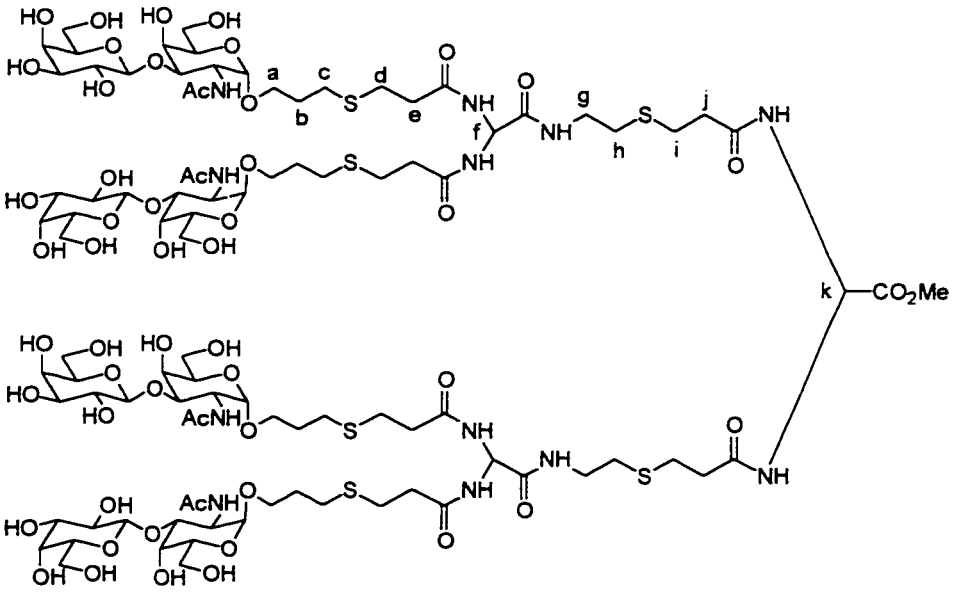
Tetravalent T-antigen glycodendrimer (89)



To a solution of 3-(2-carboxyethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc **43** (12.3 mg, 23.2 μmol) and N,N,N′,N′-tetra(3-aminopropyl)hexanediamine **78** (2 mg, 5.8 μmol) in DMSO (1 mL) was added EDC (6.3 mg, 46.6 μmol), HOBt (13.8 mg, 46.6 μmol) and DIPEA (20μL) at ice water temperature. The reaction mixture was stirred for 30 min. at the same temperature and then overnight at room temperature. The solution was dried by lyophilization and the residue was purified by size exclusion chromatography (P-4). The product containing fractions were collected and lyophilized to give 7.2 mg of white sponge solid (3.0 mmol, 52%): **^{1}H-NMR** δ (D_2O): 4.95 (d, 4H, J_{12} = 3.8 Hz, H_1), 4.54 (d, 4H, J_{12}' = 7.8 Hz, H_1'), 3.87~3.85 & 3.63~3.62 (m, 8H, H_a), 3.36 (t, 8H, J_{fg} = 6.3 Hz, H_f), 3.29~3.19 (m, 12H, H_h, H_i), 2.90 (t, 8H, J_{dc} = 6.9 Hz, H_c), 2.76 (t, 8H, J_{bc} = 7.1 Hz, H_c), 2.63 (t, 8H, J_{dc} = 6.9 Hz, H_d), 2.09 (s, 12H, Ac), 2.03~1.94 (m, 16H, H_b, H_g), 1.78 (broad s, 4H, H_j), 1.47 (broad t, 4H, H_k); **^{13}C-NMR** δ (D_2O): 104.2 (C_1'), 96.8 (C_1),

65.9 (C_a), 53.6 (C_i),52.3 (C_h), 28.9 (C_f), 28.6 (C_d), 28.0 (C_b), 27.5 (C_c), 26.7 (C_e), 25.0 (C_k), 23.0 (C_g), 22.7 (C_j), 21.6 (C_{methyl}).

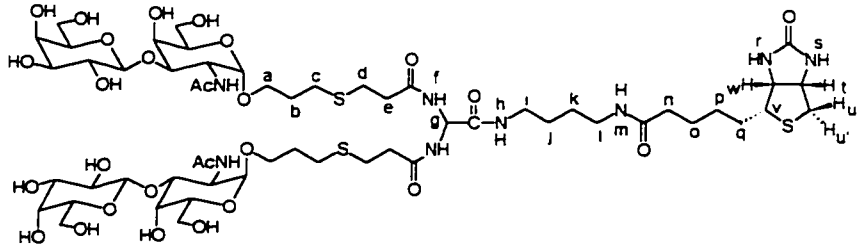
Tetravalent T-antigen dendrimer (90)



Compound **73** (13 mg, 11.7 μmol), **85** (2.1 mg, 5.8 μmol) DIPEA (100 μL) and HOBt (4 mg, 29.6 μmol) were dissolved in 1 mL of DMSO, cooled down to 0 °C and stirred for 10 minutes. 8.6 mg (29.0 μmol) of EDC was added and the reaction was continued. After 40 minutes, the temperature was allowed to rise to room temperature and stirring was continued overnight. The solution was dried by lyophilization, redissolved in minimum amount of water and purified by P-4 size exclusion chromatography followed by lyophilization to afford 9.2 mg (65%) of white spongy solid: m.p. = 127.0~131.0 °C; $[\alpha]_D$ = +57.5° (c = 0.8, H_2O); **^{1}H-NMR**, δ (D_2O): 5.90 (s, 3H's, H_f, H_k), 4.95 (d, 4H's, J_{12} = 3.7 Hz, H_1), 4.54 (d, 4H's, J_{12}' = 7.8 Hz, $H_{1'}$), 4.39 (dd, 4H's, J_{12} = 3.7 Hz, J_{23} = 11.1 Hz, H_2), 4.31 (d, 4H's, J_{34} = 3.0 Hz, $J_{45} \leq$ 1 Hz, H_4), 4.08 (dd, 4H's, J_{34} = 3.1 Hz, J_{23} = 11.1 Hz, H_3), 4.06 (t, 4H's, J_{56} = 6.2 Hz, H_5), 3.98 (d, 4H's, J_{34}' = 3.4 Hz, $H_{4'}$), 3.87 (s, 3H's, H_{methyl}), 3.87~3.78 (m, 20H's, 8H_6, 8$H_{6'}$, 4H_a), 3.73~3.71 (m, 4H's, $H_{5'}$), 3.69 (dd, 4H's, J_{34}' = 3.4 Hz, J_{23}' = 10.0 Hz, $H_{3'}$), 3.64~3.60 (m, 4H's, H_a), 3.61~3.57 (dd, J_{12}' = 7.8 Hz,

J_{23}' =10.0 Hz, $H_{2'}$), 3.51 (t, 4H's, J_{gh} = 6.6 Hz, H_g), 2.91~2.85 (m, 12H's, 8H_d, 4H_i), 2.79~2.74 (m, 12H's, 8H_c, 4H_h), 2.73~2.65 (m, 12H's, 8H_e, 4H_j), 2.09 (s, 12H's, ACNH), 2.00~1.94 (m, 8H's, H_b); **^{13}C-NMR**, δ (D_2O): 174.0, 173.9, 168.4 (3 C=O's), 104.2 (C_1'), 96.7 (C_1), 76.8 (C_3), 74.5 ($C_{5'}$), 72.2 (C_3'), 70.1, 70.1 (C_2', C_5), 68.2 (C_4), 68.1 (C_4'), 66.0 (C_a), 60.7 (C_6), 60.5 (C_6'), 56.8 (C_f, C_k), 53.2 (OCH_3), 48.2 (C_2), 38.5 (C_g), 34.8 (C_e), 34.7 (C_j), 29.9 (C_k), 28.0 (C_b), 27.6 (C_c), 26.4 (C_d), 26.1 (C_i), 21.6 (CH_3 in N-acetylgroup).

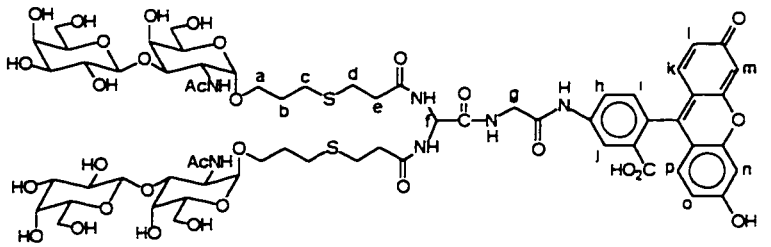
Biotin labeled divalent T-antigen dendrimer (93)



Divalent T-antigen **85** (16 mg, 14.4 μmol) and biotin (4.53 mg, 1 eq.) were dissolved in DMF (3 mL) and TBTU (5.6 mg, 1.2 eq.) was added. DIPEA was added to adjust pH = 8~9. The reaction mixture was stirred overnight at room temperature. The reaction was confirmed by a negative Kaiser test. DMF was removed by coevaporization with tert-buthanol under reduced pressure. The crude product was purified by size exclusion chromatography (P-4) with water as an eluent. The product portion was collected and lyophilized to afford 16.1 mg (11.43 μmol, 79%) of white spongy solid: m.p. = 170~172 °C; $[\alpha]_D$ = +22.2° (c = 1.4, HeOH); **^{1}H-NMR**, δ (D_2O): 4.95 (d,2H, J_{12} = 3.7 Hz, H_1), 4.67~4.65 (m, 1H, H_t), 4.54 (d, 2H, J_{12}' = 7.8 Hz, H_1'), 4.48~4.45 (m, 1H, H_w), 4.39 (dd, 2H, J_{12} = 3.7 Hz, J_{23} = 11.1Hz, H_2), 4.31 (broad d, 2H, H_4), 4.08 (dd, 2H, J_{23}= 11.1 Hz, J_{34} = 3.1 Hz, H_3), 4.05 (broad t, 2H, H_5), 3.97 (broad d, 2H, H_4'), 3.87~3.78 (m, 10H, H_6, H_6', H_a), 3.74~3.68 (m, 4H, H_3', H_5'), 3.63~3.57 (m, 3H, H_2', H_a), 3.39~3.35 (m, 1H, H_v), 3.31 (broad t, 2H, J_{ij} = 6.3 Hz, H_i), 3.24 (broad t, 2H, J_{kl} =6.5 Hz, H_l), 3.05 (dd, 1H, J_{tu} = 5.0 Hz, J_{gem} = 13.1 Hz, H_u), 2.93~2.81 (m, 5H, H_e, H_u'), 2.75 (t, 4H, J_{bc} = 7.1 Hz, H_c), 2.68 (t, 4H, $J_{d\,e}$= 6.8 Hz, H_d), 2.30 (t, 2H, J_{no} = 7.3 Hz, H_n), 2.10 (s,

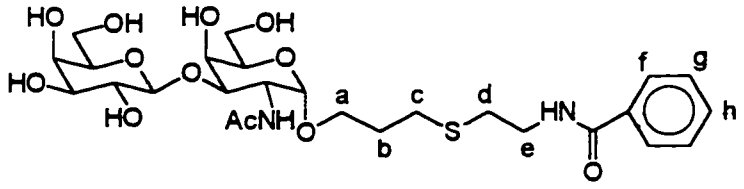
6H, H_{methyl}), 1.99~1.93 (m, 4H, H_b), 1.81~1.64 (m, 4H, H_o, H_q), 1.63~1.50 (broad s, 4H, H_j, H_k), 1.49~1.42 (m, 2H, H_p); **^{13}C-NMR**, δ (D_2O): 104.2 (C_1'), 96.8 (C_1), 76.9 (C_3), 74.5 (C_5'), 72.1 (C_3'), 70.2 & 70.2 (C_2', C_5), 68.3 (C_4'), 68.2 (C_4), 66.0 (C_a), 61.6 (C_w), 60.7 & 60.5 (C_6, C_6'), 59.8 (C_t), 57.0 (C_g), 54.9 (C_v), 48.3 (C_2), 39.3 (C_u), 38.9 (C_i), 38.4 (C_l), 35.1 (C_n), 34.8 (C_d), 28.0 (C_b), 27.6 (C_c), 27.4 (C_p), 26.4 (C_e), 25.3 (C_j, C_k), 24.7 (C_o, C_q), 21.6 (C_{methyl}).

Fluorescein labeled divalent T-antigen (94)



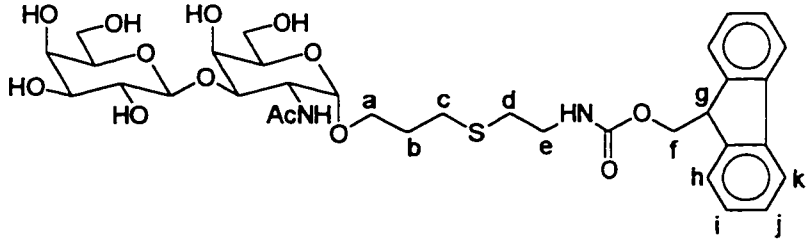
Divalent T-antigen **85** (10.07 mg, 9.05 μmol) and fluorescein (3.7 mg, 1 eq.) were used. The reaction conditions were same as those previously described. The yield was 9 mg of yellowish red spongy solid (6 μmol, 66%): m.p. = 129.5~132 °C; $[\alpha]_D$= +65.0° (c = 0.8, MeOH); **^{1}H-NMR** δ (D_2O); 8.05 (m, 1H, H_n), 7.92~7.86 (m, 2H, H_k, H_o), 7.75 (broad d, 1H, H_i), 7.50~7.45 (m, 2H, H_p, H_h), 7.34~7.26(m, 2H, H_l, H_j), 6.72 (broad s, 1H, H_m), 4.96 (d, 2H, J_{12} = 3.6 Hz, H_1), 4.55 (d, 2H, J_{12}' = 7.9 Hz), 2.90 (broad t, 4H, H_e), 2.76~2.64 (m, 8H, H_c, H_d), 2.07 (s, 6H, Ac), 1.97~1.88 (m, 4H, H_b). The chemical shifts for sugar part were same as in biotin labeled one.; **^{13}C-NMR** δ(D_2O); 179.6~158.2 (C=O), 137.3~124.6 (C_{ipso}), 131.1 (C_j, C_l), 124.6 (C_h, C_p), 122.4 (C_m), 121.7 & 117.3 (C_k, C_o), 121.0 (C_n), 110.9 (C_i), 104.2 (C_1'), 96.6 (C_1), 77.0 (C_3), 74.5 (C_5'), 72.2 (C_3'), 70.2 & 70.1 (C_2, C_5), 68.2 (C_4, C_4'), 66.0 (C_a), 60.7 & 60.5 (C_6, C_6'), 57.1 (C_f), 48.3 (C_2), 43.1 (C_g), 34.6 (C_d), 28.1 (C_b), 27.6 (C_c), 21.6 (C_{methyl}).

3-(2-benzamidoethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc (97)



To a solution of 3-(2-aminoethylthio)-propyl-β-D-Gal-(1-3)-α-D-GalNAc, **42** (25 mg, 49.9 μmol) in MeOH (2 mL) at 0 °C was slowly added bezoyl chloride, **95** (6.4 μL, 1.1 eq.) in $CHCl_3$ (1 mL). The solution was stirred for 1 h at the same temperature. The solvent was evaporated to dryness. The crude product was purified by fresh silica gel chromatography with $CHCl_3$/MeOH/H_2O (6/4/1) as an eluent to afford 36.2 mg (49.9 mmol, quantitative) of white solid: m.p. = 60.3~60.8 °C; $[\alpha]_D$ = +80.0° (c = 0.55, MeOH); **^{1}H-NMR**, δ (D_2O): 7.86 (d, 2H, J_{fg}=7.52Hz, H_f), 7.70 (m, 2H, H_g), 7.61 (m, 2H, H_h), 4.93 (d, 1H, J_{12} = 3.7 Hz, H_1), 4.52 (d, 1H, J_{12}' = 7.7 Hz, H_1'), 4.38 (dd, 1H, J_{12} = 3.6 Hz, J_{23} = 11.1 Hz, H_2), 4.28 (broad d, 1H, H_4), 4.08 (dd, 1H, J_{34} = 3.0 Hz, J_{23} = 11.0 Hz, H_3), 4.03 (m, 1H, H_5), 3.96 (broad d, J_{34}' = 3.2 Hz, $J_{45} \leq$ 1 Hz, H_4'), 3.93~3.57 (m, 11H, H_2', H_3', H_5', H_6, H_6', H_a, H_e), 2.92 (t, 2H, J_{de} = 6.6 Hz, H_d), 2.81 (t, 2H, J_{bc} = 7.1 Hz, H_c), 2.08 (s, 3H, H_{methyl}), 1.99 (multi, 2H, H_b); **^{13}C-NMR**, δ (D_2O): 174.0 & 170.4 (2C=O), 133.2 (C_{ipso}), 131.8 (C_f), 128.4 (C_g), 126.6 (C_h), 104.2 (C_1'), 96.7 (C_1), 76.8 (C_3), 74.5 (C_5'), 72.1 (C_3'), 70.2 (C_5), 70.1 (C_2'), 68.3 (C_4'), 68.1 (C_4), 66.0 (C_a), 60.7 & 60.5 (C_6, C_6'), 48.3 (C_2), 38.8 (C_e), 30.2 (C_d), 28.1 (C_b), 27.6 (C_c), 21.6 (C_{methyl}).

3-[2-(9-fluorenylmethyloxyamido)ethylthio]propyl β-D-Gal-(1-3)-α-D-GalNAc (98)



3-(2-Aminoethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc **42** (40 g, 79.9 μmol) was dissolved in 10% solution of Na_2CO_3 in water (1 mL). Dioxane (0.5 mL) was added and the mixture was stirred in an ice-water bath. 9-fluorenylmethyl chlorocarbonate, **96** (20.6 mg, 1 eq.) was added in small portion and the reaction was continued at ice-water bath temperature for 4hrs and then at room temperature for 8 h. The reaction mixture was dried by lyophilization and purified by silica gel chromatography with $CHCl_3$/MeOH (2/1) as an eluent to afford 40 mg (51.8 μmol, 65%) of white solid: m.p. = 57.5~58.1 °C; **^{1}H-NMR**, δ (DMSO): 7.87 (d, 2H, J_{hi} = 7.5 Hz, H_h), 7.67 (d, 2H, J_{jk} = 7.4 Hz, H_k), 7.53 (d,1H, $J_{2\text{-}NH}$ = 8.0 Hz, NH at C_2), 7.41~7.38 (m, 3H, H_i, -OC(O)NH), 4.74 (broad s, 1H, OH at C_3), 4.69 (d, 1H, J_{12} = 3.4 Hz, H_1), 4.60 (broad d, 1H, $J_{5\text{-}OH}$ = 4.9 Hz, OH at C_5), 4.46 (broad s, 2H, OH at C_6, C_6'), 4.40 (broad s, 1H, OH at C_4'), 4.35 (d, 1H, $J_{12'}$ = 7.6 Hz, H_1'), 4.29 (d, 2H, J_{fg} = 6.9 Hz, H_f), 4.25 (broad d, 1H, $J_{4\text{-}OH}$ = 3.6 Hz, OH at C_4), 4.20 (t, 1H, J_{fg} = 6.9 Hz, H_g), 4.16 (m, 1H, H_2), 3.94 (broad s, 1H, H_4), 3.73 (dd, 1H, J_{23} = 11.1 Hz, J_{34} = 2.7 Hz, H_3), 3.64~3.59 (m, 3H, H_4', H_5, H_a), 3.52~3.28 (m, 6H, H_2', H_6, H_6', H_a), 3.25 (broad t, 1H, H_3'), 3.15~3.12 (m, 2H, H_e), 2.58 (t, 2H, J_{bc}=7.02Hz, H_c), 2.53 (t, 2H, J_{de} = 7.2 Hz, H_d), 1.80 (s, 3H, H_{methyl}), 1.75 (m, 2H, H_b); **^{13}C-NMR**, δ (DMSO): 169.8 & 156.1 (2 C=O), 128.9 (C_i), 127.6~127.3 (C_l, C_m), 127.0 (C_j), 125.1 (C_k), 120.1 (C_h), 103.9 (C_1'), 97.1 (C_1), 76.2 (C_3), 75.3 (C_5'), 73.4 (C_3'), 71.2 (C_5), 70.8 (C_2'), 68.1 (C_4'), 67.3 (C_4), 65.6 (C_a),65.3 (C_f), 60.6 & 60.4 (C_6, C_6'), 48.6 (C_2), 46.7 (C_g), 40.2 (C_e), 30.6 (C_d), 29.1 (C_b), 27.6 (C_c), 22.7 (C_{methyl}).

Turbidimetric analysis between peanut lectin and di- (85), tetra- (86, 90) and hexa- (87) valent T-antigen dendrimers

Turbidimetric experiments were performed in Linbro (Titertek) microtitration plates where 50 μL/well of stock lectin solutions prepared from peanut lectin (2 mg/mL in PBS) were mixed with 50 μL of a stock solution of glycodendrimers, **85**, **86**, **87** and **90** (11 nmole/well of T-antigen for all dendrimers) and incubated at room temperature for 3 h. The turbidity of the solutions was monitered by reading the optical density (O. D.) at

490 nm at regular time intervals until no noticeable changes were observed. Each test was performed in triplicate.

Competitive double sandwich inhibition ELISA using mouse monoclonal IgG antibodies and T-antigen dendrimers, 85, 86, 87 and 90 as inhibitors

Linbro (titertek) microtiter plates were coated overnight at room temperature with T-antigen containing copolyacrylamide at 100 μL/well of a polymer stock solution, 10 μg/mL in 0.01 M phosphate buffer (pH = 7.3). Each well contained 1 μg/well of polymer and T-antigen content was 0.36 μg (0.85nmol) out of 1μg/well. The wells were then washed 3 times with 400 μL/well of 0.01 M phosphate buffer (pH= 7.3) containing 0.05% (v/v) Tween 20 (PBST). The wells were blocked with 150μL/well of 1% BSA in PBS for 1hr at 37 °C. At the same time, 50 μL/well of mouse monoclonal IgG antibodies solution in PBST (10 times dilution of a supernatant, 0.25 μmol/50 μL) and 50 μL of inhibitor solution in PBST with varying concentrations from 138 to 2.15 nmol/well of T-antigen by two fold serial dilution were mixed on Nunclon (Delta) microtiter plates and preincubated for 1hr at 37 °C. After washing with excess BSA with PBST, the wells were filled 100 μL/well of preincubated mouse IgG MAb/inhibitor solution and incubated again for 1hr at 37 °C. The wells were washed as described above with PBST and filled with 100 μL/well of goat-anti-mouse IgG MAb (receptor protein)-enzyme conjugates solution in PBST (1,000 times dilution of supernatant) followed by incubation for 1 h at 37 °C. The wells were washed and 50 μL/well of 2, 2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (1 mg/4 mL) in citrate-phosphate buffer (0.2 M, pH= 4.0 with 0.015% H_2O_2) was added. The reaction was stopped after 20 minutes by adding 50 μL/well of 1 M aqueous sulfuric acid solution and optical density was measured at 410 nm relative to 570 nm. Percent inhibitions were calculated as follows:

$$\% \text{ inhibition} = [A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})}/A_{(\text{no inhibitor})}] \times 100$$

IC_{50}'s were calculated as the concentration required for 50% inhibition of the coating antigen. All test were performed in triplicate.

Chapter 5 Synthesis of L-lysine based glycodendrimers

5.1. Introduction

Since virus-neutralizing antibodies[193] were prepared using the synthetic C-terminal hexapeptide of the protein of tobacco mosaic virus as antigenic determinants, great progress has been made in immunology at the molecular level regarding the recognition mechanism using synthetic peptides.[193-c] When synthetic peptides are considered as vaccine candidates, it may be necessary to avoid using a carrier protein which will be immunogenic itself. [200] The peptide antigen usually represents a minor fraction of the total molecular weight of the antigen-carrier conjugates. Thus conjugation of the peptide antigen to itself has been considered.[194] Based on such demands, Denkewalter *et al.*[195] prepared chemically well-defined monodisperse dendritic polypeptides using benzylhydrylamide and N^{α},N^{ε}-Boc-L-lysine p-nitrophenyl active ester as the branching residue (Fig. 5-1).

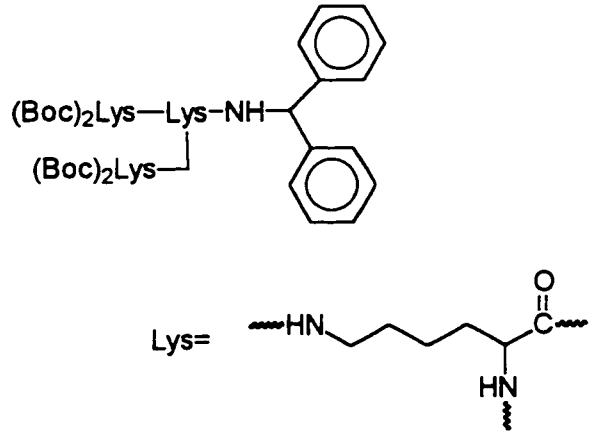
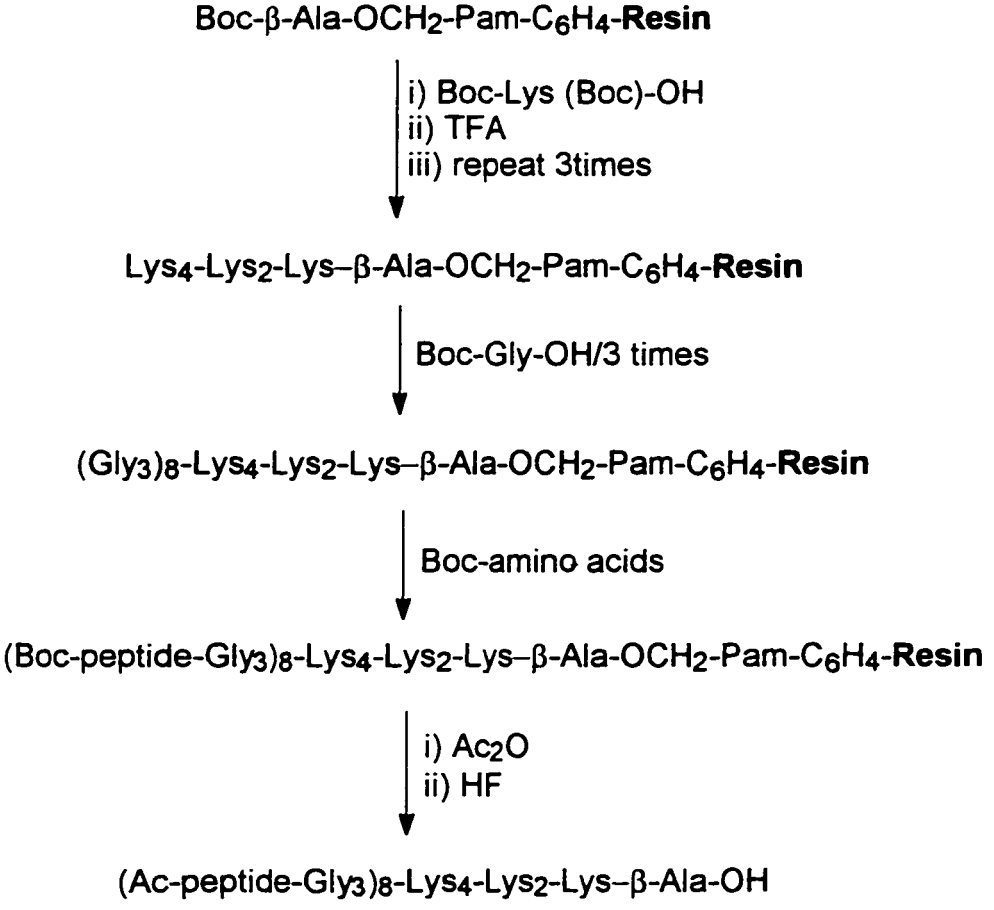


Fig. 5-1. Structure of L-lysine based dendritic core.

Tam *et al.*[196] have extensively studied the L-lysine based dendrimers using solid phase peptide synthetic strategies,[197, 198] for example, branching lysine core resulting an octavalent lysineyl scaffolding known as multiple antigen peptide (MAP) which consists of three levels of sequentially branched lysine, Lys_4-Lys_2-Lys-β-Ala.[199] (Scheme 5-1)



Scheme 5-1. Steps in synthesis of a MAP on the stepwise solid phase method.

Solid phase peptide synthesis is based on the iterative sequences such as the addition of N-protected amino acids to an insoluble polymer support, amino-protecting group cleavage,[202] and neutralization. Generally N-protecting groups are the acid labile t-

butoxycarbonyl (Boc) group or the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group.[203] The peptide coupling can be accomplished by carbodiimide chemistry with preactivated amino acid acids such as active esters.[198] This synthetic strategy has several advantages; Firstly, the synthesis is proceeded by a stepwise addition of sequential amino acids to afford high quality products. Secondly, the efficiency of the coupling is monitored by the disappearance of reacting amines using the Kaiser test. Thirdly, an excess of reagents can be removed by simple washing and filtration.

Roy *et al.*[201] have prepared hyperbranched L-lysine based dendrimers using β-Ala-p-benzyloxybenzyl alcohol (Wang) resin, N^{α},N^{ε}-di-Fmoc-L-lysine and pre-formed chloroacetylglycylglycine benzotriazoyl ester by solid phase peptide synthesis methods. The structures of these dendrimers are represented in Fig. 5-2 ~ 5-4.

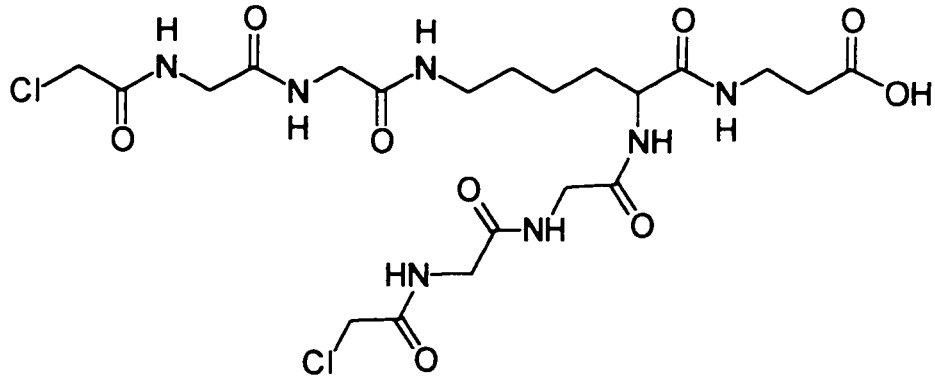


Fig. 5-2. Structure of divalent L-lysine dendrimer **99.**

One major characteristics of the MAP is that the core matrix is small and the bulk is formed by a high density of uniform peptide antigens layered around the core matrix. Therefore, dendrimers **99~101** represent ideal clusters to obtain the desired carbohydrate density, thus mimicking polyvalent glycoproteins.

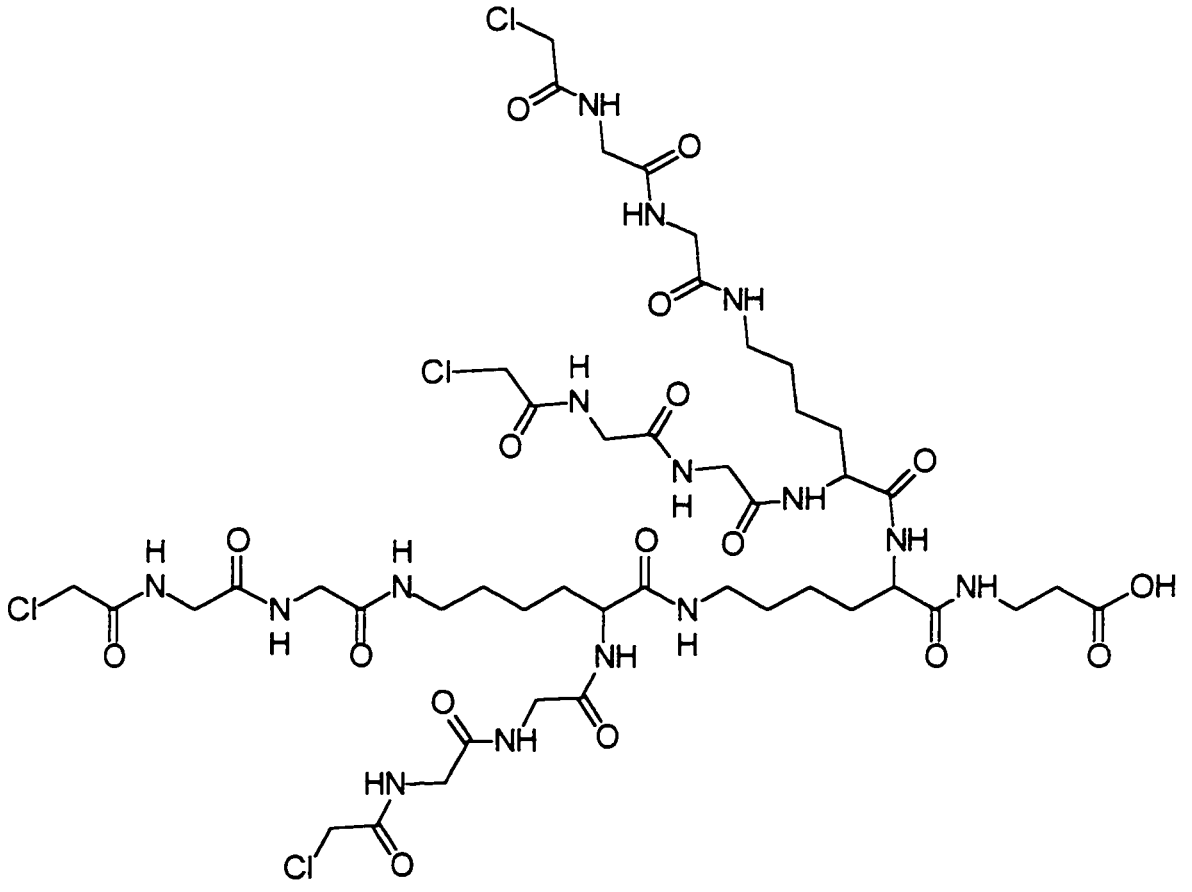


Fig. 5-3. Structure of tetravalent L-lysine dendrimer **100.**

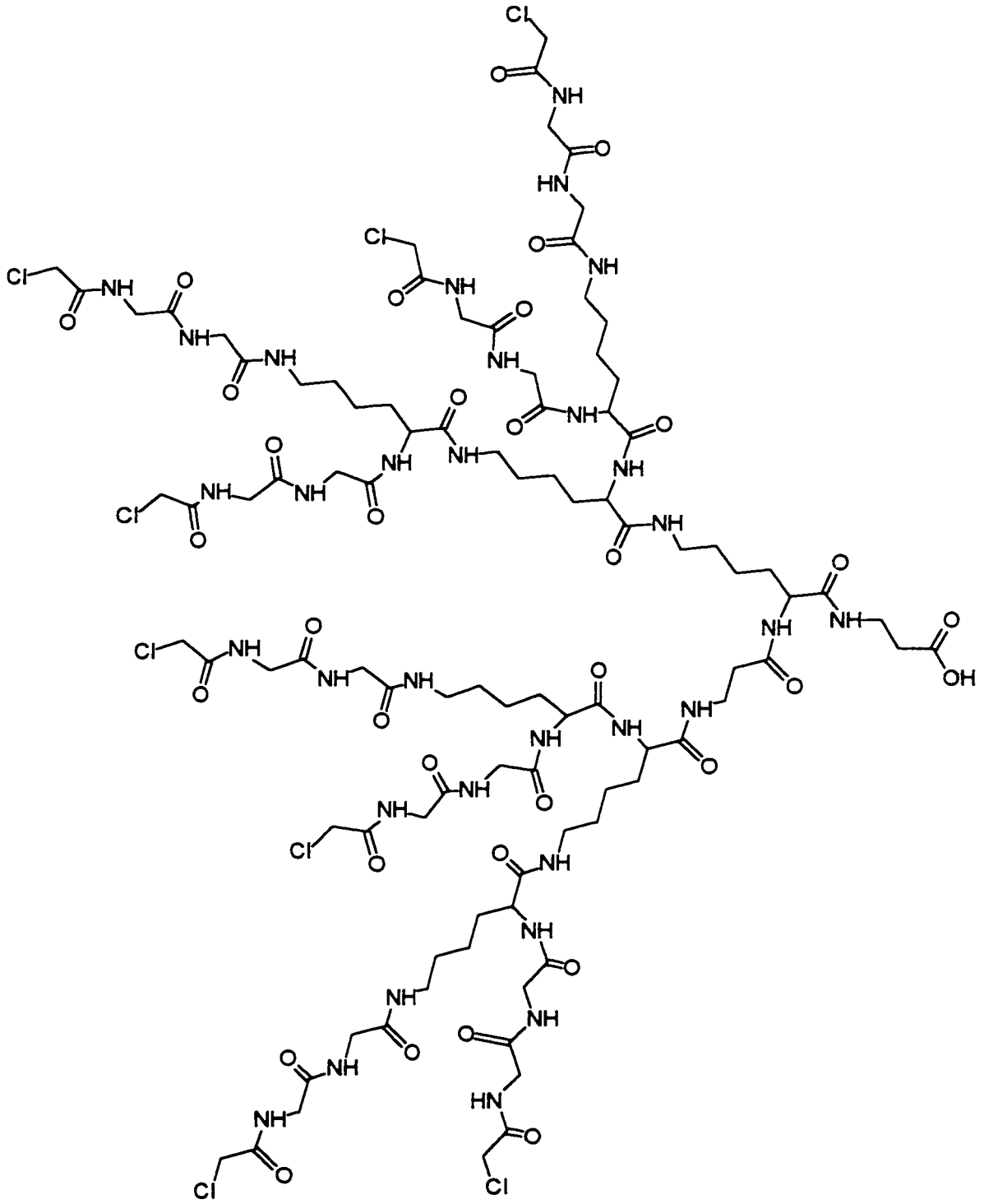
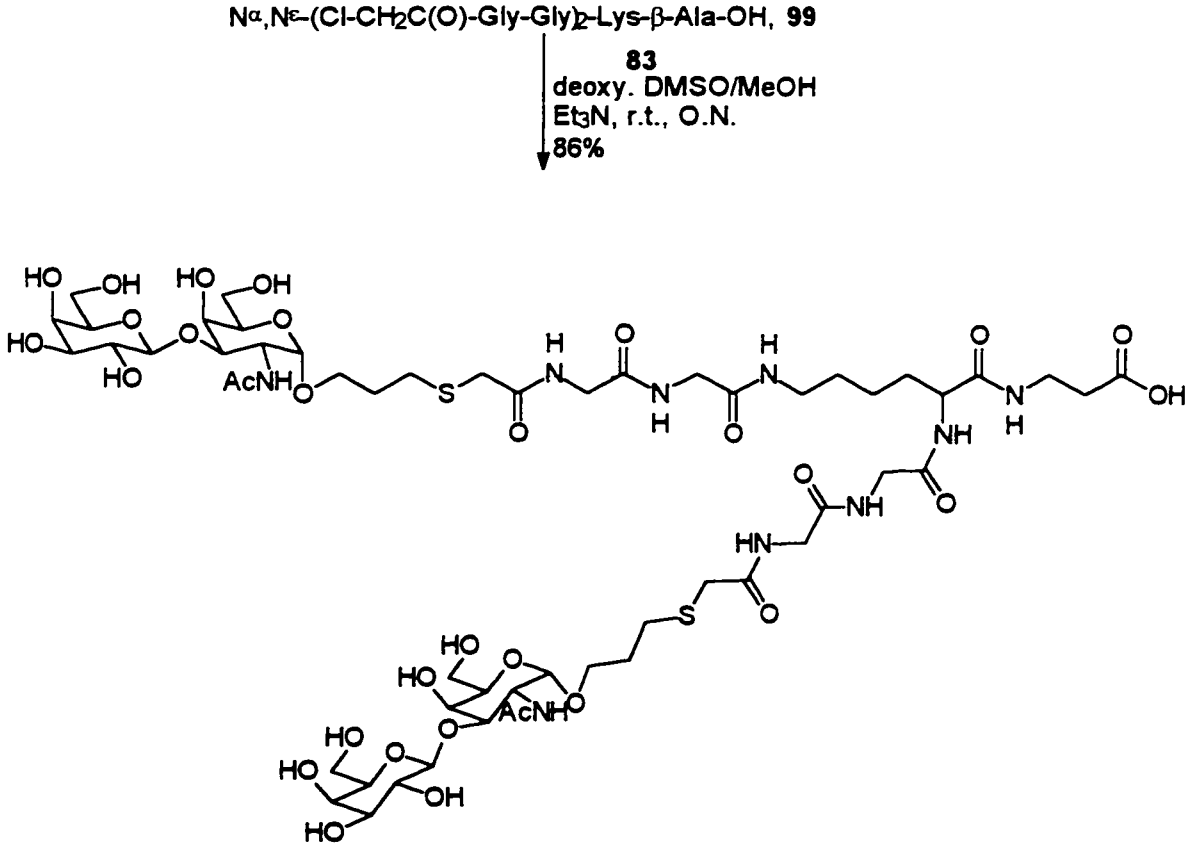


Fig. 5-4. Structure of octavalent L-lysine dendrimer **101.**

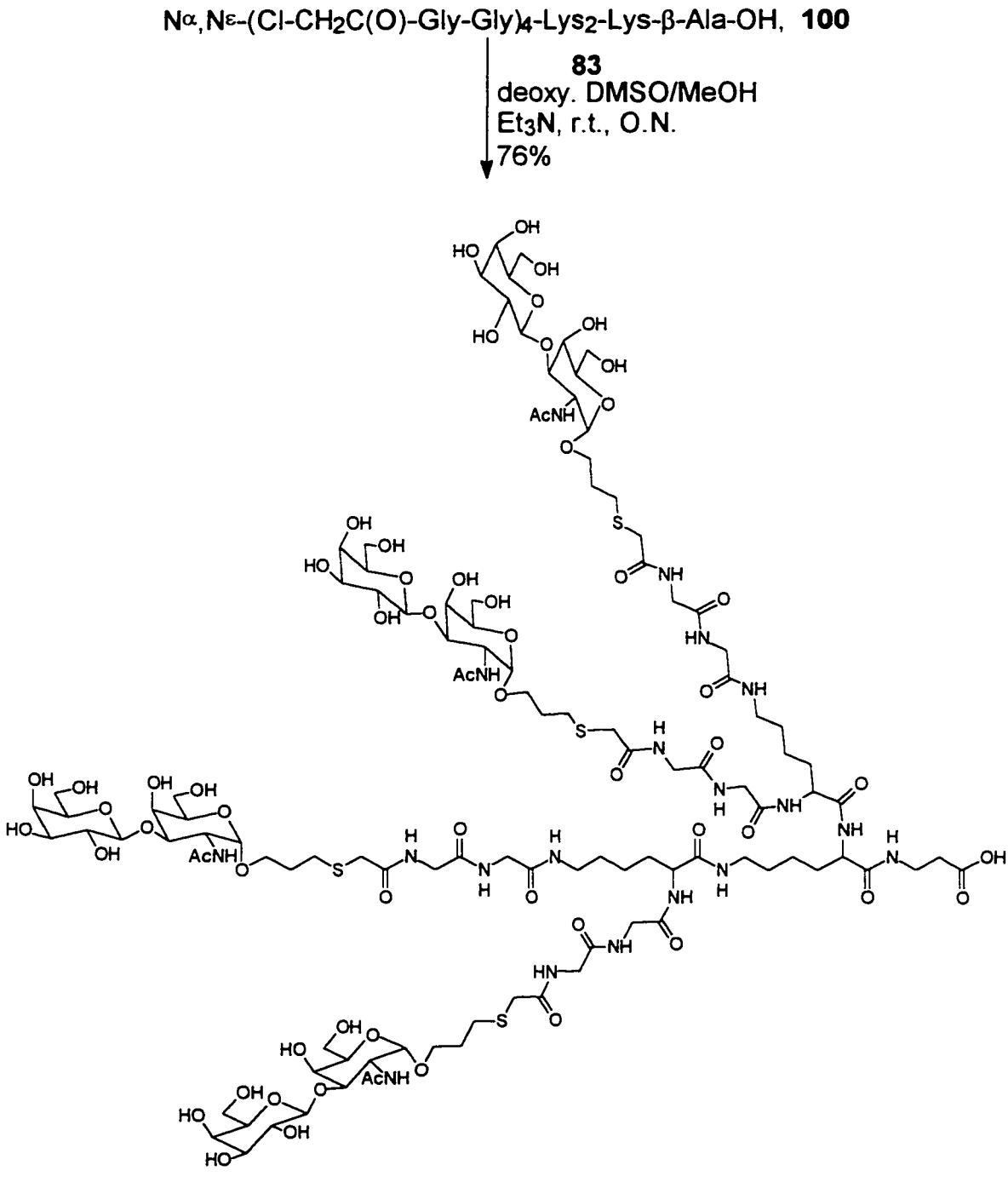
5.2. Glycosylation of L-lysine cores

As multivalency effects were proved in the previous chapters, L-lysine based T-antigen dendrimers were prepared to further demonstrate these effects and biochemical evaluations.

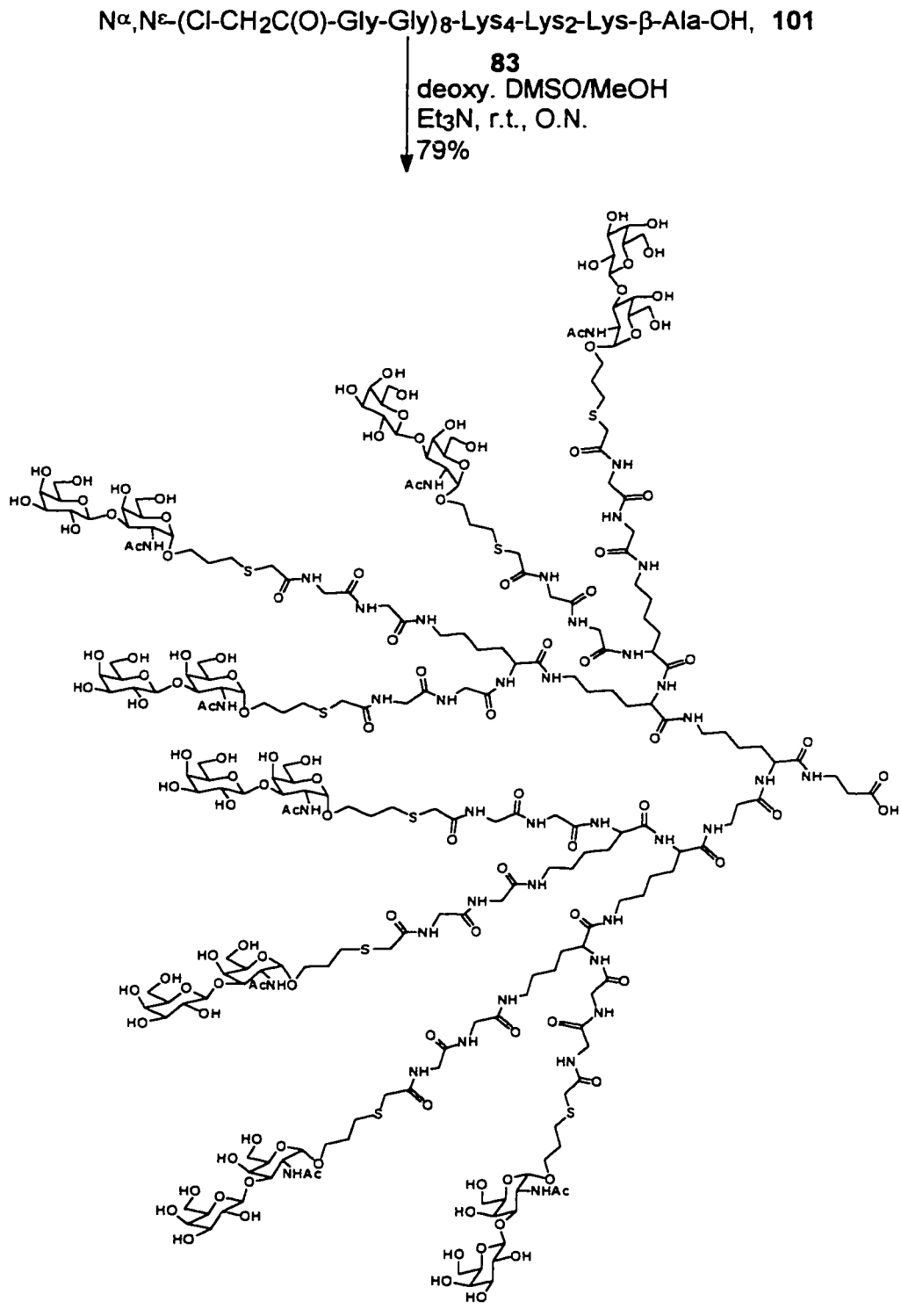
Conjugation of an excess of thiolated β-D-Gal-(1-3)-α-D-GalNAc **83** to each dendrimers **99**, **100** and **101** was accomplished by S_N2 chloride displacement (1.2 eq. of **83** per N-chloroacetyl group, deoxygenated DMSO/MeOH, Et_3N, 25 °C, overnight). All glycogendrimers were purified by size exclusion chromatography (P-2 or P-4) with water as an eluent followed by lyophilization to afford **102**, **103** and **104** in 76~86% yields.



Scheme 5-2. Synthesis of divalent L-lysine based T-antigen-dendrimer **102.**



Scheme 5-3. Synthesis of tetravalent L-lysine based T-antigen-dendrimer **103.**



Scheme 5-4. Synthesis of octavalent L-lysine based T-antigen-dendrimer **104.**

Completion of conjugation was confirmed by ^{1}H- and ^{13}C-NMR spectral data which revealed the presence of two anomeric centers as well as L-lysyl and β-alanyl residues. (Fig. 5-5~5-7) These signals were integrated relative to anomeric signals and established the extent of glycoside incorporation. The chemical shifts of well separated signals were summarized in Table 5-1 and showed constant chemical shifts regardless of the dendrimers.

Table 5-1. ^{1}H- and ^{13}C-chemical shifts of L-lysine based T-antigen dendrimers.

Compd	^{1}H (δ, ppm)				^{13}C (δ, ppm)			
	H-1	H-1′	Ala H-α	Lys H-δ	C-1	C-1′	Ala C-α	Lys C-δ
102	4.95	4.54	2.49	1.75	96.7	104.2	36.4	25.7
103	4.96	4.55	2.46	1.59	96.7	104.2	36.2	27.3
104	4.94	4.53	2.44	1.58	96.7	104.2	36.4	27.4

5-3. Conjugation of glycosylated L-lysine and biotin hydrazide

As mentioned for the importance of biotin labeled compounds (Chapter 4, section 4-5), the synthesis and biological application of biotinylated L-lysine based T-antigen dendrimers attest this fact. Biotin-tetravalent-T-antigen-L-lysine dendrimer **106** was prepared using **103** and biotin hydrazide **105** (TBTU, DIPEA, DMF, 25 °C, overnight, 92%). The solvent was removed under reduced pressure and the residue was purified by size exclusion chromatography (P-4) to afford the desired product **106** (Scheme 5-5). High resolution ^{1}H- and COSY NMR spectra (500 MHz, D_2O) revealed the key signals which represent the glycoside (4.96 ppm for H-1 and 4.55 ppm for H-1′), L-lysyl dendritic core (1.65~1.51 ppm for lysyl δ-CH_2) and biotin hydrazide (4.68 ppm for H-8 and 4.50 ppm for H-10, see the experimental methods for the counting of protons). The ratio of integration of these signals established each component in the biotin labeled product **106**, (Fig. 5-8 and 5-9).

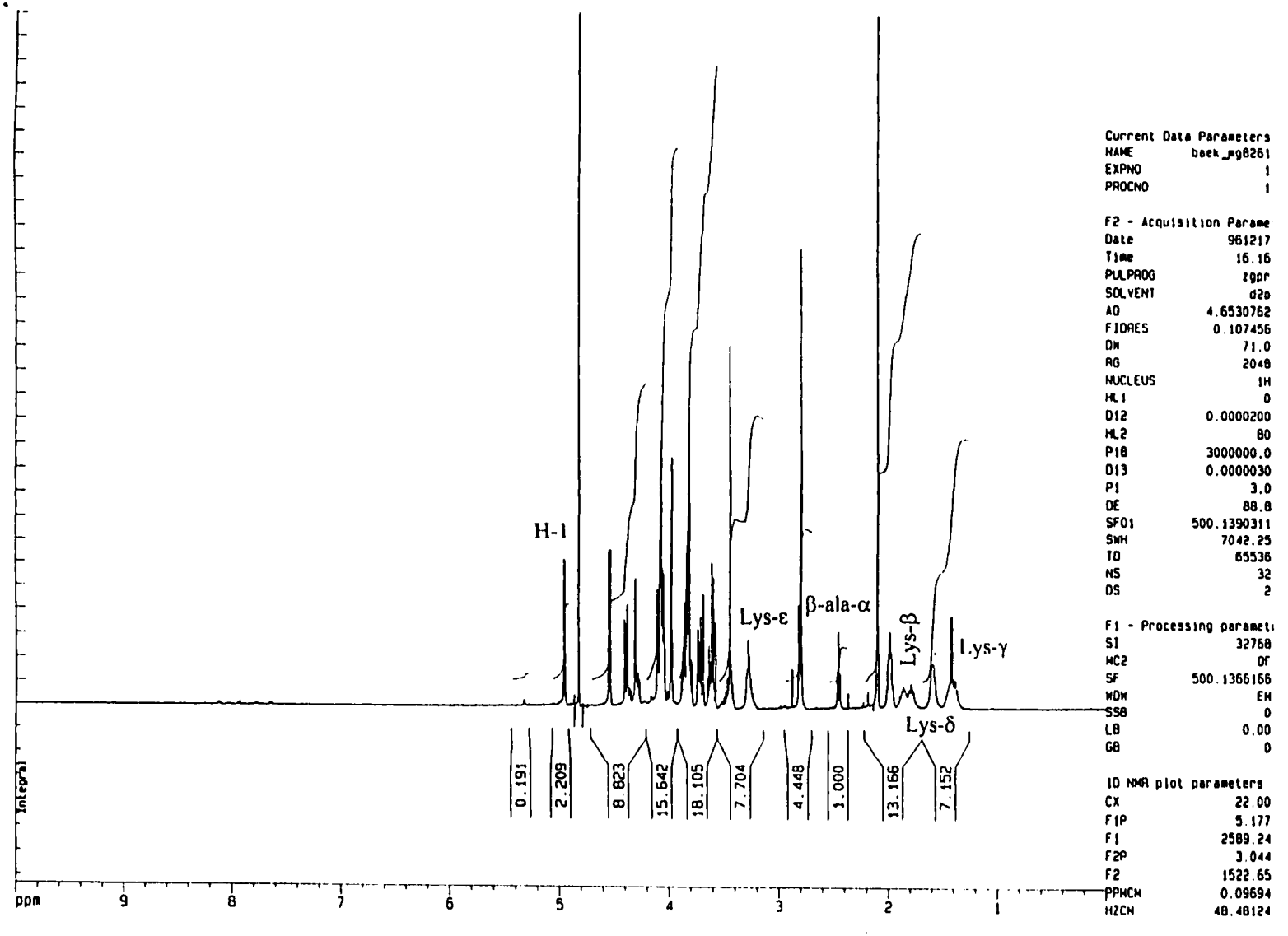


Fig. 5-5. ^{1}H-NMR spectrum (500 MHz, D_2O) of tetravalent T-antigen dendrimer **103.**

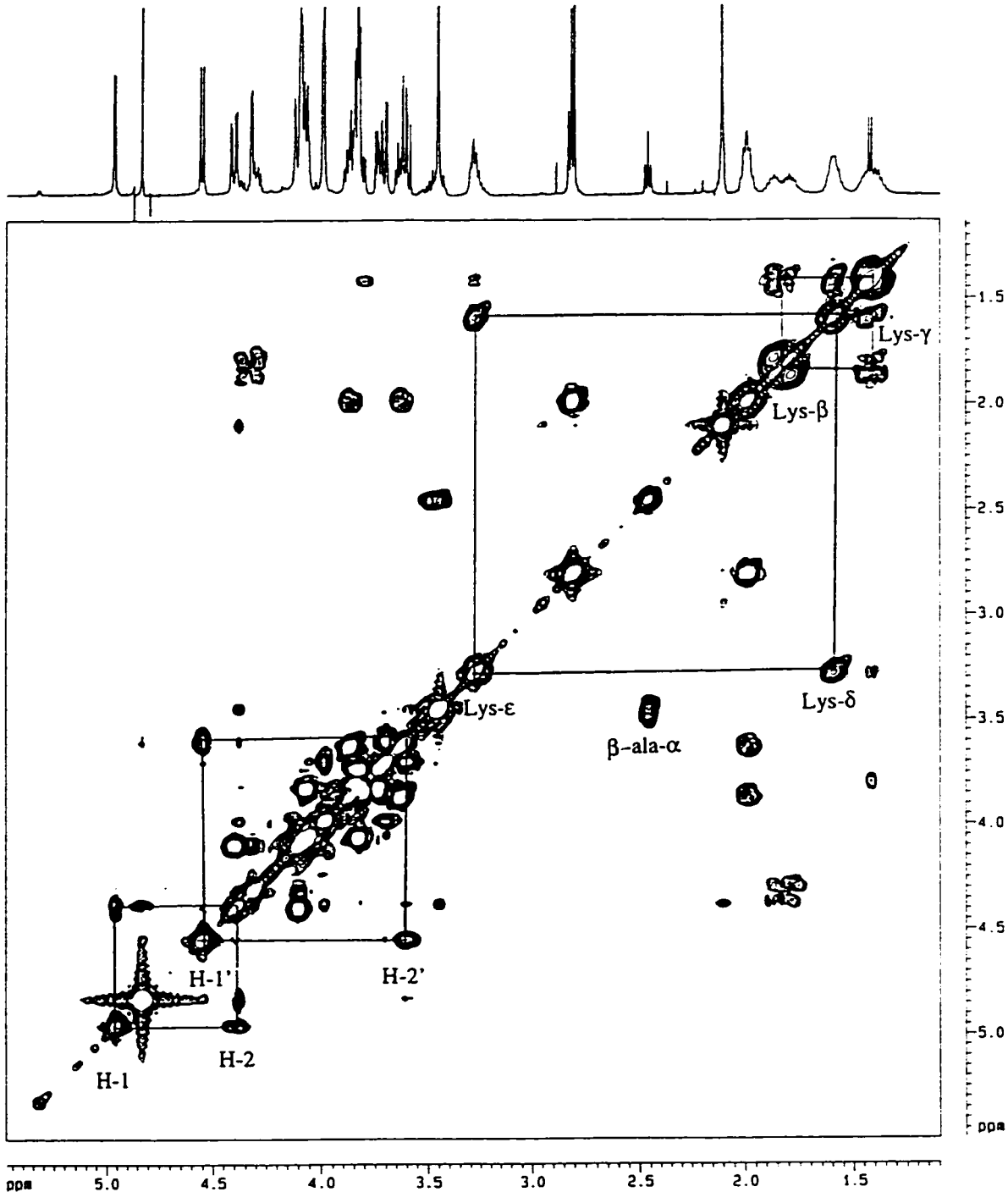


Fig. 5-6. ^{1}H-^{1}H COSY NMR spectrum (500 MHz, D_2O) of tetravalent T-antigen dendrimer **103.**

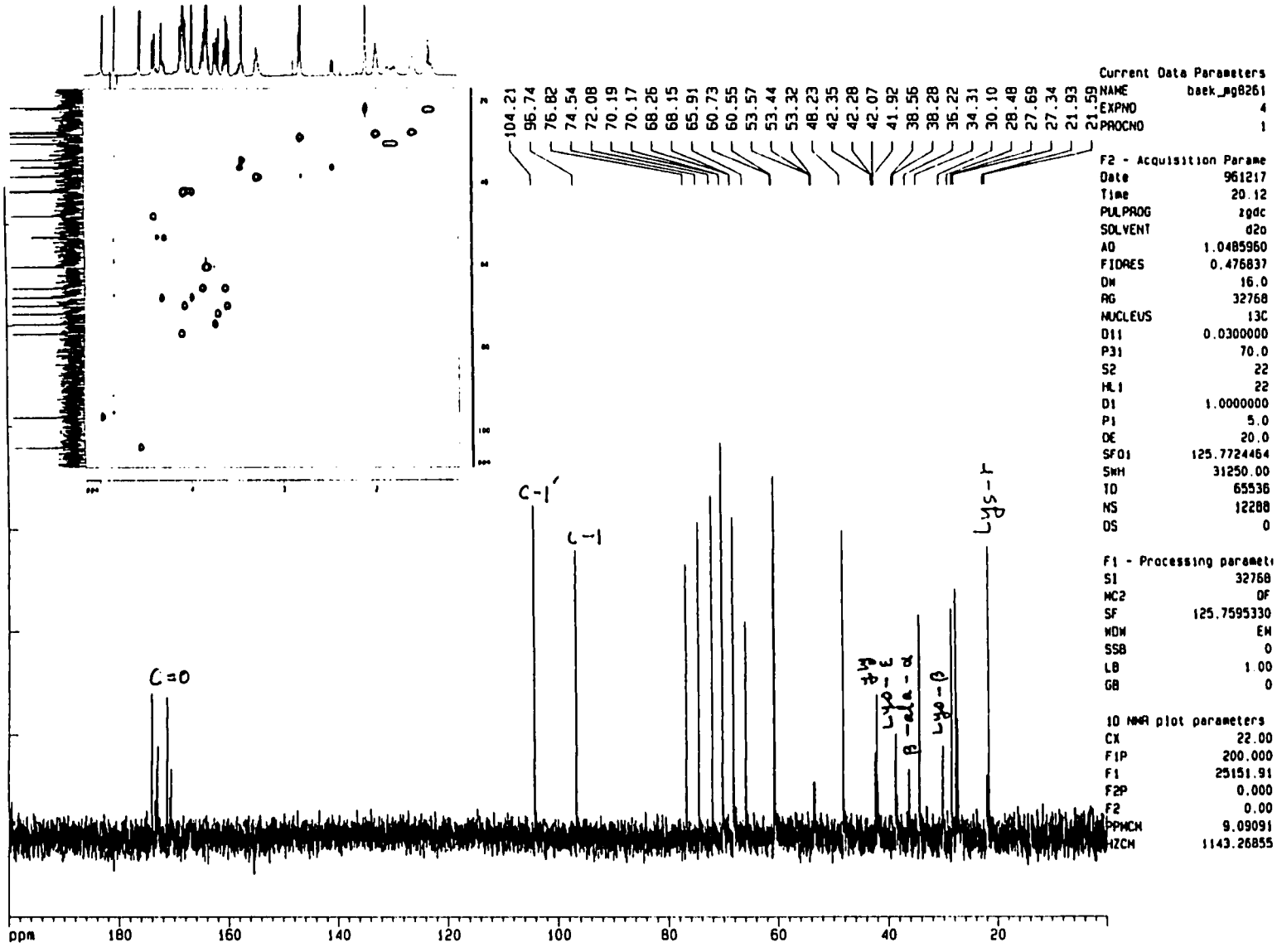
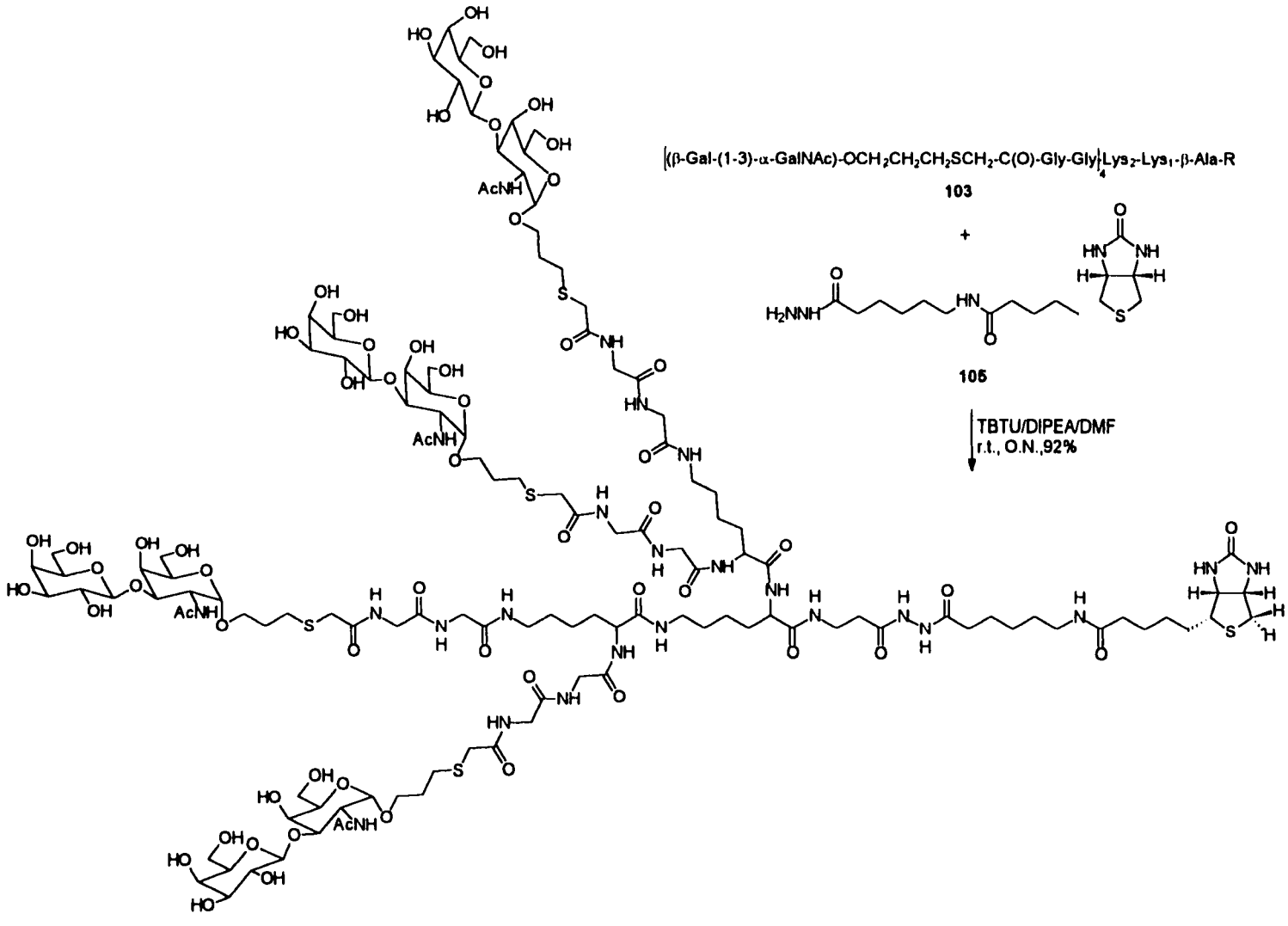


Fig. 5-7. ^{13}C- and ^{1}H-^{13}C HMQC NMR spectrum (500 MHz, D_2O) of tetravalent T-antigen dendrimer **103.**



Scheme 5-5. Synthesis of biotin labeled L-lysine based tetravalent T-antigen dendrimer **106**.

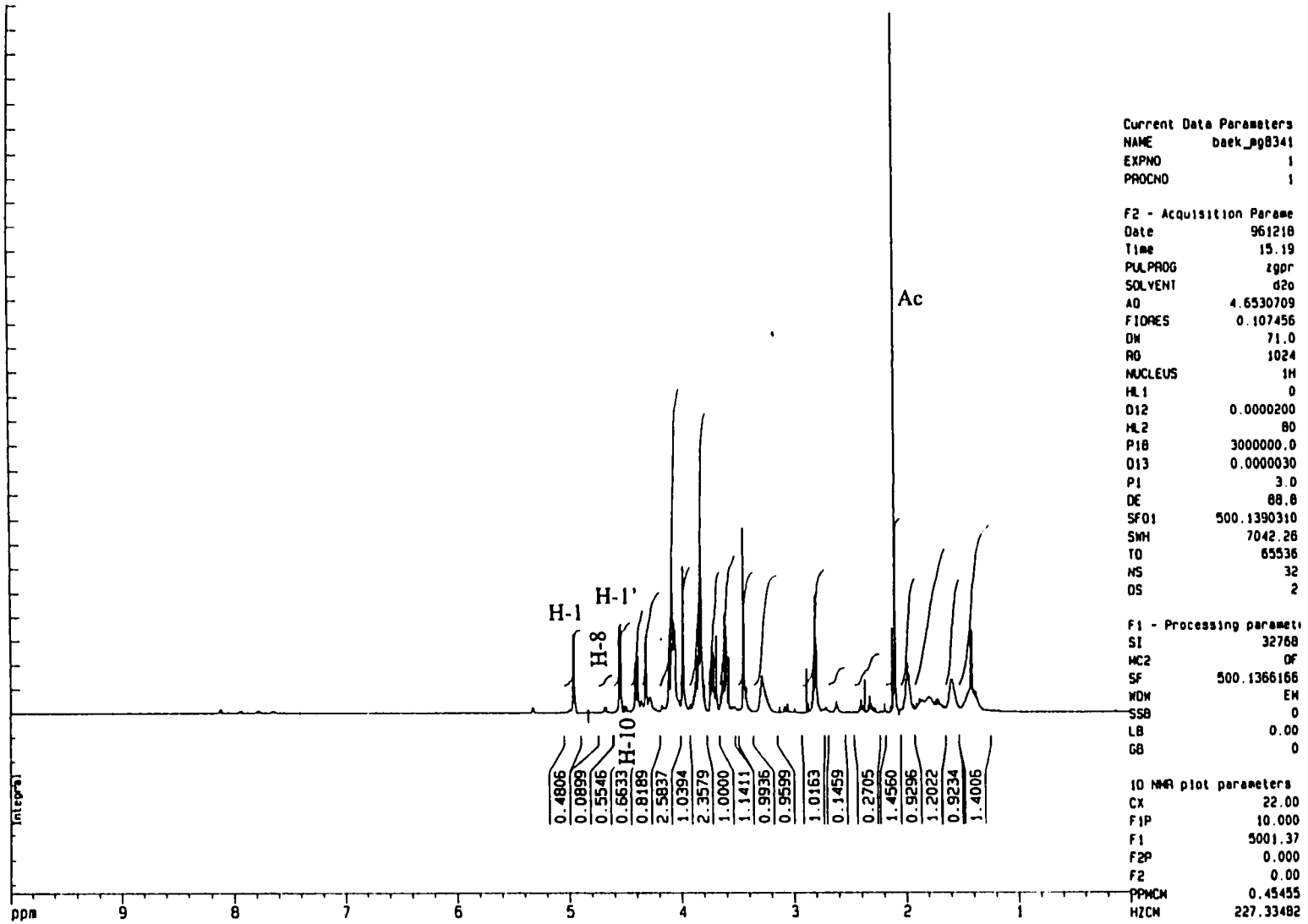


Fig. 5-8. ^{1}H-NMR spectrum (500 MHz, D_2O) of biotin labeled L-lysine based tetravalent T-antigen dendrimer **106.**

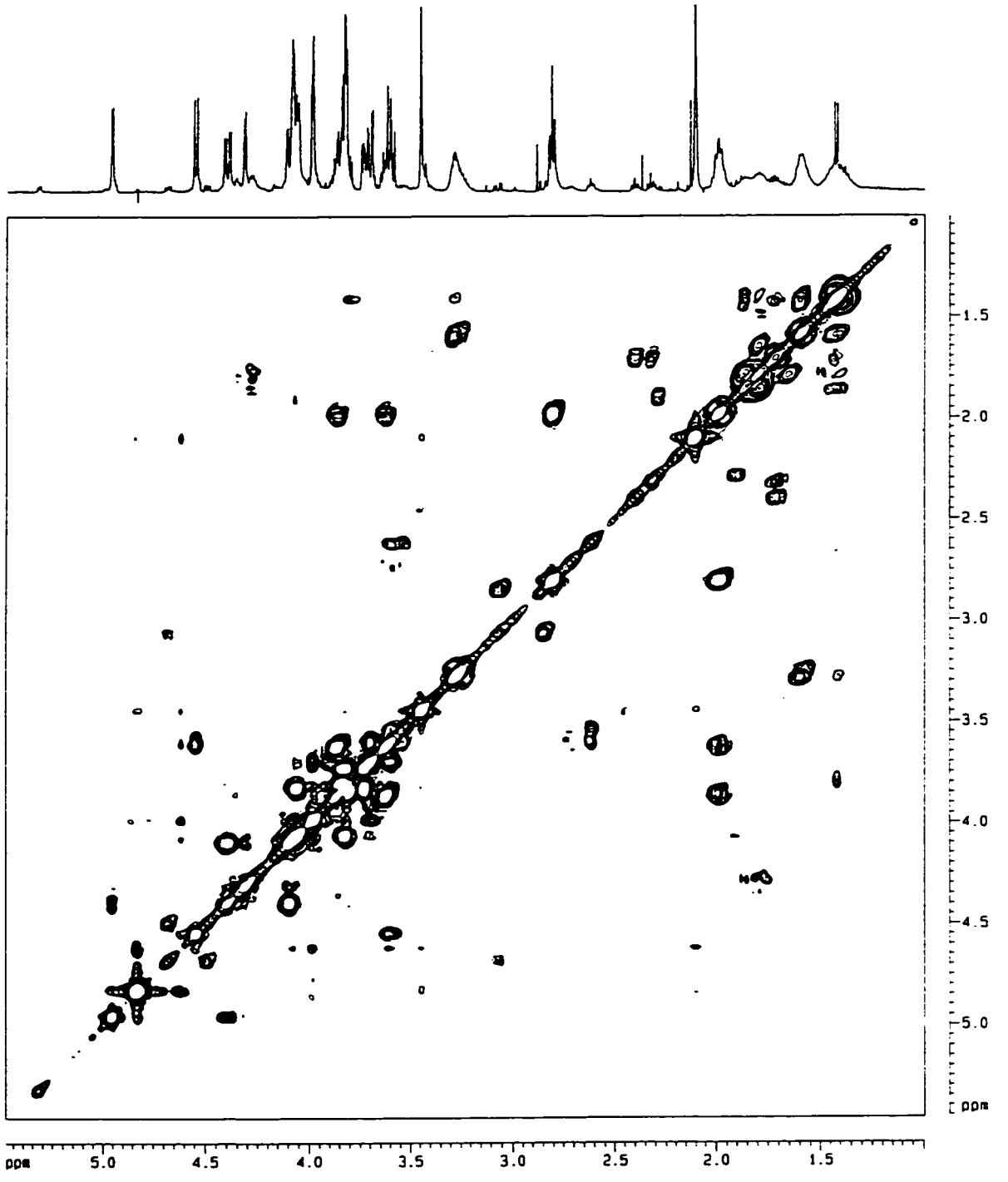


Fig. 5-9. ^{1}H-^{1}H COSY NMR spectrum (500 MHz, D_2O) of biotin labeled L-lysine based tetravalent T-antigen dendrimer **106.**

5-4. Immunochemical assays

Inhibitory binding interactions of biotin labeled L-lysine-tetravalent-T-antigen dendrimer **106** to mouse monoclonal IgG antibodies established the antigenic properties. The same mouse IgG MAb was used as mentioned in Chapter 4, section 4-6. Commercially available streptavidin was used as the coating material which specifically binds to biotin. After incubation at overnight 25°C, BSA blocking and washing , the wells were filled by biotin-T-antigen dendrimer solution, **106** in PBST with varying concentration by 10-fold dilution. Mouse IgG MAb were used to bind with the T-antigen in the dendrimers as the receptor protein (RP) and the assays were performed indirectly through goat anti mouse IgG MAb-enzyme conjugates. Residual enzymatic activity was then measured with 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)-H_2O_2

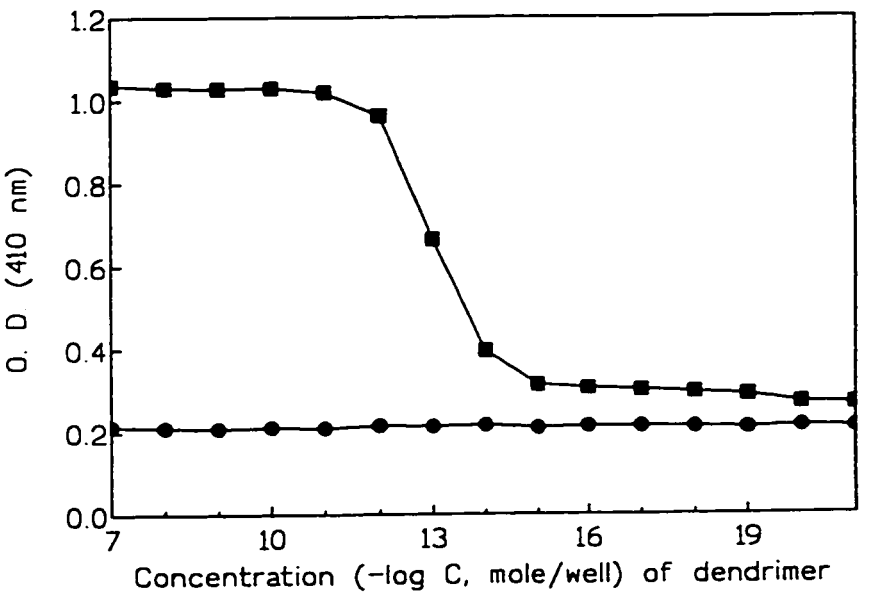


Fig. 5-10. ELISA using T-antigen dendrimer **103** (●) and biotin labeled T-antigen dendrimer **106** (■) and streptavidin as coating materials with mouse IgG-MAb, goat anti-mouse IgG-enzyme conjugates and ABTS-H_2O_2 as peroxidase substrates.

(0.015%) as peroxidase substrates. Another assays were performed with the L-lysyl T-antigen dendrimer **103** which does not possess biotin under the same conditions. The results are clearly shown in Fig. 5-10 and reveal the efficient binding interaction with streptavidin in proportion to the concentration of biotin-T-antigen dendrimer **106**. The optical density (O. D.) values were drastically increased at the concentration range between 1.528×10^{-10} and 1.528×10^{-12} mole/well and there was no further increase of O. D. values even at higher concentration of dendrimer. In contrast, T-antigen dendrimer **103** showed no binding interaction with coated streptavidin in the whole range of concentration. This means that the strong binding interaction between **106** and streptavidin exists and this interaction directly affects the content of T-antigen, thereby demonstrating the multivalency effect between T-antigen and receptor protein.

5-5 Conclusions

Hyperbranched L-lysine dendrimers containing N-chloroacetylglycylglycine spacers were conjugated with thiolated β-D-Gal-(1-3)-α-D-GalNAc by nucleophilic substitution of the N-chloroacetyl group. Each glycodendrimer was obtained in good yields and high purity. A biotinylated L-lysine based tetravalent dendrimer was successfully prepared using the TBTU strategy with an excellent yield. In immunological assays, biotinylated dendrimer was compared with the corresponding one which did not labeled. The former exhibited strong binding interactions with streptavidin which was used as the coating material and the concentration of dendrimers was in proportion to this interaction. This involved a high density of T-antigen, thus supported demonstration of the multivalency effect.

5-6 Experimental methods

General method

L-lysine dendritic core was dissolved in deoxygenated DMSO under N_2 bubbling condition. β-D-Gal-(1-3)-α-D-GalNAc-$OCH_2CH_2CH_2$-SAc (1.2 eq. per chloroacetyl group) was dissolved in deoxygenated methanol and $CH_3O^-Na^+$/deoxy. MeOH (0.3 M) was added dropwise until all of the -SAc was changed to -SH. Once the reaction was complete, IRA 120 (H^+ resin) was added to neutralize the excess of $CH_3^-Na^+$, then the mixture was filtrated. The filtrate was transferred to the former solution quickly. Triethylamine was added to adjust pH = 8~9 (This base was added more after 6 h). The solution was stirred overnight at room temperature under N_2 bubbling. After removal of solvent, the crude product was purified by size exclusion chromatography (P-2 or P-4) with distilled water as an eluent to afford white spongy solid in 76% ~ 86% yields.

[β-D-Gal-(1-3)-α-D-GalNAc-$OCH_2CH_2CH_2SCH_2C$(O)-Gly-Gly]$_2$-Lys-β-Ala-OH (102)

($ClCH_2C$(O)-Gly-Gly)$_2$-Lys-β-Ala-OH, **99** (20 mg, 32 μmol) and O-3-thiopropyl-β-D-Gal-(1-3)-α-D-GalNAc, **83** (35 mg, 1.2 eq. per chloroacetyl group) were used. After purification with size exclusion chromatography (P-2), 42.5 mg (27.6 μmol, 86%) of **102** was obtained. **^{1}H-NMR**, δ (D_2O): 4.95 (d, 2H, J_{12} = 3.7 Hz, H_1), 4.54 (d, 2H, J_{12} = 7.8 Hz, H_1'), 4.39 (dd, 2H, J_{12} = 3.7 Hz, J_{23} = 11.0 Hz, H_2), 4.42~4.31 (m, 4H, H_4, lysyl α-CH_2), 4.14~3.98 (m, 14H, H_3, H_4', H_5, glycyl CH_2), 3.89~3.79 (m, 10H, H_6, H_6', Ha), 3.74~3.70 (m, 4H, H_3', H_5'), 3.69~3.60 (m, 4H, H_2', H_a), 3.45 (broad s, 2H, β-alanyl β-CH_2), 3.07 (t, 2H, $J_{\delta\varepsilon}$ = 7.3 Hz, lysyl ε-CH_2), 2.81 (t, 4H, J_{bc} = 7.2 Hz, -CH_2S-), 2.49 (broad t, 2H, β-alanyl α-CH_2), 2.09 (s, 6H, CH_3), 2.00~1.96 (m, 4H, -OCH_2CH_2), 1.92~1.80 (m, 2H, lysyl β-CH_2), 1.78~1.71 (m, 2H, lysyl δ-CH_2), 1.51~1.42 (m, 2H, lysyl γ-CH_2); **^{13}C-NMR**, δ (D_2O): 174.0~170.8 (C=O), 104.2 (C_1'), 96.7 (C_1), 76.8 (C_3), 74.5 (C_5'), 72.1 (C_3'), 70.2 (C_2', C_5), 68.3 (C_4), 68.1 (C_4'), 65.9 (-OCH_2-), 60.7 & 60.5 (C_6 &

C_6'), 53.2 (lysyl C_α), 48.2 (C_2), 42.2~41.9 (glycyl CH_2), 38.7 (lysyl C_ε), 36.4 (β-alanyl C_α), 34.3 (β-alanyl C_β), 30.0 (lysyl C_β), 28.4 (-CH_2S-), 27.6 (-OCH_2**CH_2**-), 25.7 (lysyl C_δ), 21.6 (lysyl C_γ, CH_3).

[β-D-Gal-(1-3)-α-D-GalNAc-$OCH_2CH_2CH_2SCH_2C$(O)-Gly-Gly]$_4$-Lys_2-Lys_1-β-Ala-OH (103)

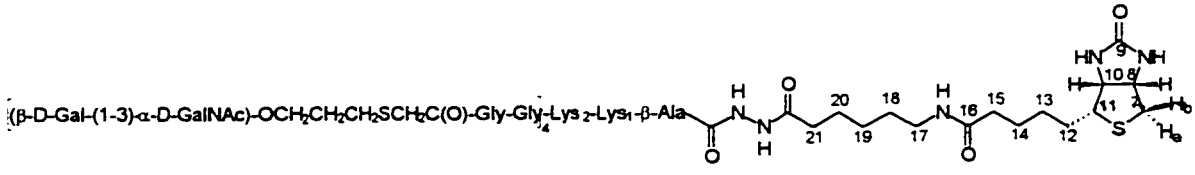
($ClCH_2C$(O)-Gly-Gly)$_4$-Lys_2-Lys_1-β-Ala-OH, **100** (16.7 mg, 13.5 μmol) and O-3-thiopropyl-β-D-Gal-(1-3)-α-DGalNAc, **83** (34 mg, 1.2 eq. per chloroacetyl group) were used. After purification with size exclusion chromatography (P-4), 30.1 mg (10.3 μmol, 76 %) of **103** was obtained. **^{1}H-NMR**, δ (D_2O): 4.96 (d, 4H, J_{12} = 3.8 Hz, H_1), 4.55 (d, 4H, J_{12}' =7.8 Hz, H_1'), 4.40 (dd, 4H, J_{12} = 3.8 Hz, J_{23} = 11.1 Hz, H_2), 4.32 (d, 4H, J_{34} = 3.0 Hz, $J_{45} \leq$ 1 Hz, H_4), 4.37~4.35 & 4.31~4.28 (m, 3H, lysyl α-CH_2), 4.11~4.05 (m, 20H, H_3, H_5, glycyl CH_2), 3.99 (m, 8H, H_4', glycyl CH_2), 3.89~3.79 (m, 20H, H_6, H_6', -OCH_2), 3.75~3.69 (m, 8H, H_3', H_5'), 3.65~3.58 (m, 8H, H_2', -OCH_2), 3.46~3.43 (m, 2H, β-alanyl β-CH_2), 3.29~3.23 (m, 6H, lysyl ε-CH_2), 2.83~2.77 (m, 8H, -SCH_2), 2.47~2.45 (t, 2H, $J_{\alpha\beta}$ = 7.0 Hz, β-alanyl α-CH_2), 2.01 (s, 12H, H_{methyl}), 2.02~1.95 (m, 8H, -OCH_2**CH_2**), 1.93~1.71 (m, 6H, lysyl β-CH_2), 1.60~1.57 (m, 6H, lysyl δ-CH_2), 1.44~1.37 (m, 6H, lysyl γ-CH_2); **^{13}C-NMR**, δ (D_2O): 174.2~170.5 (C=O), 104.2 (C_1'), 96.7 (C_1), 76.8 (C_3), 74.5 (C_5'), 72.1 (C_3'), 70.2 (C_5), 70.3 (C_2'), 68.3~ 68.2 (C_4, C_4'), 65.9 (-OCH_2), 60.7~60.6 (C_6, C_6'), 53.6~53.3 (lysyl C_α), 48.2 (C_2), 42.4~41.9 (glycyl CH_2), 38.6~38.3 (lysyl C_ε), 36.2 (β-alanyl C_α), 34.3 (β-alanyl C_β), 30.1 (lysyl C_β), 28.5 (-SCH_2), 27.7 (-OCH_2**CH_2**), 27.3 (lysyl C_δ), 21.9 (lysyl C_γ), 21.6 (C_{methyl}).

[β-D-Gal-(1-3)-α-D-GalNAc-$OCH_2CH_2CH_2SCH_2C$(O)-Gly-Gly]$_8$-Lys_4-Lys_2-Lys_1-β-Ala-OH (104)

($ClCH_2C$(O)-Gly-Gly)$_8$-Lys_4-Lys_2-Lys_1-β-Ala-OH, **104** (20 mg, 8 μmol) and O-3-thiopropyl-β-D-Gal-(1-3)-α-D-GalNAc, **83** (35 mg, 1.2 eq. per chloroacetyl group) were used. After purification with size exclusion chromatography (P-4), 39 mg (6.3 μmol, 79%)

of **104** was obtained. **^{1}H-NMR**, δ (D_2O): 4.94 (d, 8H, J_{12} = 3.7 Hz, H_1), 4.53 (d, 8H, J_{12}' = 7.8 Hz, H_1'), 4.38 (dd, 8H, J_{12} = 3.7 Hz, J_{23} = 11.1 Hz, H_2), 4.30 (d, 8H, J_{34} = 3.0Hz, J_{45} ≤ 1 Hz, H_4), 4.36~4.34 & 4.28~4.26 (m, 7H, lysyl α-CH_2), 4.10~4.03 (m, 32H, H_3, H_5, glycyl CH_2), 3.97 (m, 24H, H_4', glycyl CH_2), 3.87~3.80 (m, 40H, H_6, H_6', -OCH_2), 3.79~3.69 (m, 16H, H_3', H_5'), 3.68~3.56 (m, 16H, H_2', -OCH_2), 3.43 (broad s, 2H, β-alanyl β-CH_2), 3.27~3.25 (m, 14H, lysyl ε-CH_2), 2.80~2.79 (m, 16H, -SCH_2), 2.44 (t, 2H, $J_{\alpha\beta}$ = 7.0 Hz, β-alanyl α-CH_2), 2.08 (s, 24H, CH3), 1.98~1.96 (m, 16H, -$OCH_2\mathbf{CH_2}$), 1.85~1.78 (m, 14H, lysyl β-CH_2), 1.59~1.57 (m, 14H, lysyl δ-CH_2), 1.55~1.36 (mi, 14H, lysyl γ-CH_2); **^{13}C-NMR**, δ (D_2O): 174.0~170.5 (C=O), 104.2 (C_1'), 96.7 (C_1), 76.8 (C_3), 74.5 (C_5'), 72.1 (C_3'), 70.2 (C_2', C_5), 68.23 (C_4), 68.1 (C_4'), 65.9 (-OCH_2), 60.7 & 60.5 (C_6 & C_6'), 53.6~53.3 (lysyl C_α), 48.2 (C_2), 42.3~42.0 (glycyl CH_2), 38.6 (lysyl C_ε), 36.4 (β-alanyl C_α), 34.3 (β-alanyl C_β), 30.2 (lysyl C_β), 28.5 (-SCH_2), 27.7 (-$OCH_2\mathbf{CH_2}$), 27.4 (lysyl C_δ), 21.9 (lysyl C_γ), 21.6 (C_{methyl}).

[β-D-Gal-(1-3)-α-D-GalNAc-$OCH_2CH_2CH_2SCH_2C$(O)-Gly-Gly]$_4$-Lys_2-Lys_1-β-Ala-Biotin hydrazide (106)



T-antigen containing lysine based dendrimer (30 mg, 10.3 μmol), biotin hydrazide (3.8 mg, 10.3 μmol) and TBTU (4mg, 1.2 eq.) were dissolved in DMF (3 mL). DIPEA was added to adjust pH = 8~9. The solution was stirred overnight at room temperature. The solvent was removed to dryness under reduced pressure. The crude product was purified by size exclusion chromatography (P-4) to afford 31 mg (9.5 μmol, 92%) of white spongy solid. **^{1}H-NMR**, δ (D_2O): 4.96 (d, 4H, J_{12} = 3.8 Hz, H_1), 4.68 (dd, 1H, $J_{7a,8}$ = 4.5 Hz, $J_{8,10}$ = 8.0 Hz, H_8), 4.55 (d, 4H, J_{12}' = 7.8 Hz, H_1'), 4.50 (dd, 1H, $J_{8,10}$ = 8.0 Hz, $J_{10,11}$ = 4.5 Hz, H_{10}), 4.40 (dd, 4H, J_{12} = 3.7 Hz, J_{23} = 11.1 Hz, H_2), 4.31 (d, 4H, J_{34} = 2.9 Hz, J_{45} ≤ 1 Hz, H_4), 4.37~4.344 & 4.29~4.25 (m, 6H, lysyl α-CH_2), 4.11~4.05 (m, 20H,

H_3, H_5, glycyl CH_2), 3.99 (m, 8H, H_4', glycyl CH_2), 3.96~3.78 (m, 20H, H_6, H_6', -OCH_2), 3.74~3.67 (m, 8H, H_3', H_5'), 3.65~3.58 (m, 8H, H_2', -OCH_2), 3.57~3.49 (m, 2H, β-alanyl β-CH_2), 3.29~3.19 (m, 9H, H_{11}, H_{17}, lysyl ε-CH_2), 3.08~3.05 (dd, 1H, $J_{7a,8}$=5.02Hz, J_{gem} = 13.1 Hz, H_{7a}), 2.87~2.79 (m, 9H, -SCH_2, H_{7b}), 2.62 (t, 2H, $J_{\alpha\beta}$ = 6.7 Hz, β-alanyl α-CH_2), 2.40 (t, 2H, $J_{14,15}$ = 7.4 Hz, H_{15}), 2.32 (t, 2H, $J_{20,21}$ = 7.2 Hz, H_{21}), 2.10 (s, 12H, H_{methyl}), 2.00~1.97 (m, 8H, -OCH_2CH_2), 1.93~1.66 (m, 14H, H_{12}, H_{14}, H_{18}, H_{20}, lysyl β-CH_2), 1.65~1.51 (m, 8H, lysyl δ-CH_2), 1.47~1.36 (m, 12H, H_{13}, H_{19}, lysyl γ-CH_2); **^{13}C-NMR**, δ (D_2O): 174.0~170.5 (C=O), 104.2 (C_1'), 96.7 (C_1), 76.8 (C_3), 74.5 (C_5'), 72.1 (C_3'), 70.18 & 70.16 (C_5, C_2'), 68.2 & 68.1 (C_4, C_4'), 65.9 (-OCH_2), 61.6 (C_{10}), 60.7 & 60.5 (C_6, C_6'), 59.8 (C_8), 54.9 (C_{11}), 53.6~53.0 (lysyl C_α), 48.2 (C_2), 42.4~41.9 (glycyl CH_2), 39.5 (C_7), 38.6 (lysyl C_ε), 32.1 (C_{21}), 34.3 (alanyl C_β), 32.8 (C_{15}, alanyl C_α), 30.2~30.2 (lysyl C_β), 28.5 (-SCH_2), 27.7 (-OCH_2CH_2), 27.6 (lysyl C_δ), 28.5~27.6 (C_{12}, C_{13}, C_{18}), 24.9 (C_{19}), 24.7 (C_{14}, C_{20}), 22.0 (lysyl C_γ), 21.6 (C_{methyl}).

Double sandwich ELISA using streptavidin as coating materials, mouse monoclonal IgG antibodies and L-lysine based T-antigen dendrimers, 103 and 106.

Linbro (titertek) microtiter plates were coated overnight at room temperature with streptavidin at 100 μL/well of a polymer stock solution, 10 μg/mL in 0.01 M phosphate buffer (pH = 7.3). Each well contained 1μg/well of streptavidin. The wells were then washed 3 times with 400 μL/well of 0.01 M phosphate buffer (pH = 7.3) containing 0.05% (v/v) Tween 20 (PBST). The wells were blocked with 150 μL/well of 1% BSA in PBS for 1 h at 37 °C. After washing of excess BSA with PBST, the wells were filled 100 μL/well of dendrimer solutions with varying concentration by the serial 10-fold dilutions from 1.528 × 10-7 to 1.528 × 10-21 mole/well and incubated again for 1hr at 37 °C. The wells were washed as described with PBST and filled with 100 μL/well of mouse monoclonal IgG antibodies solution in PBST (10 times dilution of supernatant, 0.5 μmole/100μL). After incubation and washing, the wells were filled with 100 μL/well of goat-anti-mouse IgG MAb (receptor protein)-enzyme conjugates solution in PBST (1000 times dilution of supernatant) followed by incubation for 1 h at 37 °C. The wells were

washed and 50 μL/well of 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (1 mg/4mL) in citrate-phosphate buffer (0.2 M, pH = 4.0 with 0.015% H_2O_2) was added. The reaction was stopped after 20 minutes by adding 50 μL/well of 1 M aqueous sulfuric acid solution and optical density was measured at 410 nm relative to 570 nm. All tests were performed in triplicate.

Chapter 6. Synthesis of PAMAM based glycodendrimers

6.1. Introduction

The synthesis of carbohydrate containing polymeric materials is of considerable interest in biochemical and medicinal applications[204] because carbohydrate moieties play critical roles in cellular recognition,[205] transport,[206] and adhesion.[207] It is now well established that multiantennary oligosaccharides show a higher affinity for their receptors than simple monovalent-receptor binding. Okada *et al.*[208] have reported monodisperse glycopeptides[209-a] and sugar attached poly(2-oxazolines)[209-b] by utilizing living polymerization. Recently Roy *et al.*[201] have also reported multibranching glycodendrimers. However, geometric arrangement of terminal carbohydrates should be considered for their recognition.

Since Tomalia *et al.*[210] have synthesized poly(amido amine) (PAMAM) dendrimers by the divergent-growth procedure, the new concept has emerged for the macromolecular design inter-sugar space regulated carbohydrates exploiting a globular dendrimer skeleton. Starburst™ PAMAM dendrimers have been used as building blocks to attach various carbohydrate derivatives such D-glucose,[211] disaccharide lactones of lactose and maltose,[208] T_N-antigen attached peptide,[212] and α- and β-D-mannose, β-D-glucose, β-cellobiose and β-lactose isothiocyanate derivatives.[213]

T-antigen assembled PAMAM glycodendrimers have not been reporterd. To extend the concept of multivalency in comparision with other glycodendrimers in Chapter 4, fully T-antigen equipped globular PAMAM dendrimers "sugar balls" with highly ordered structures in which arranged carbohydrates on the peripheries of dendrimers were prepared. The synthesis of multiamine surfaced PAMAM dendrimers and their structures are depicted in Fig. 6-1~6-3.

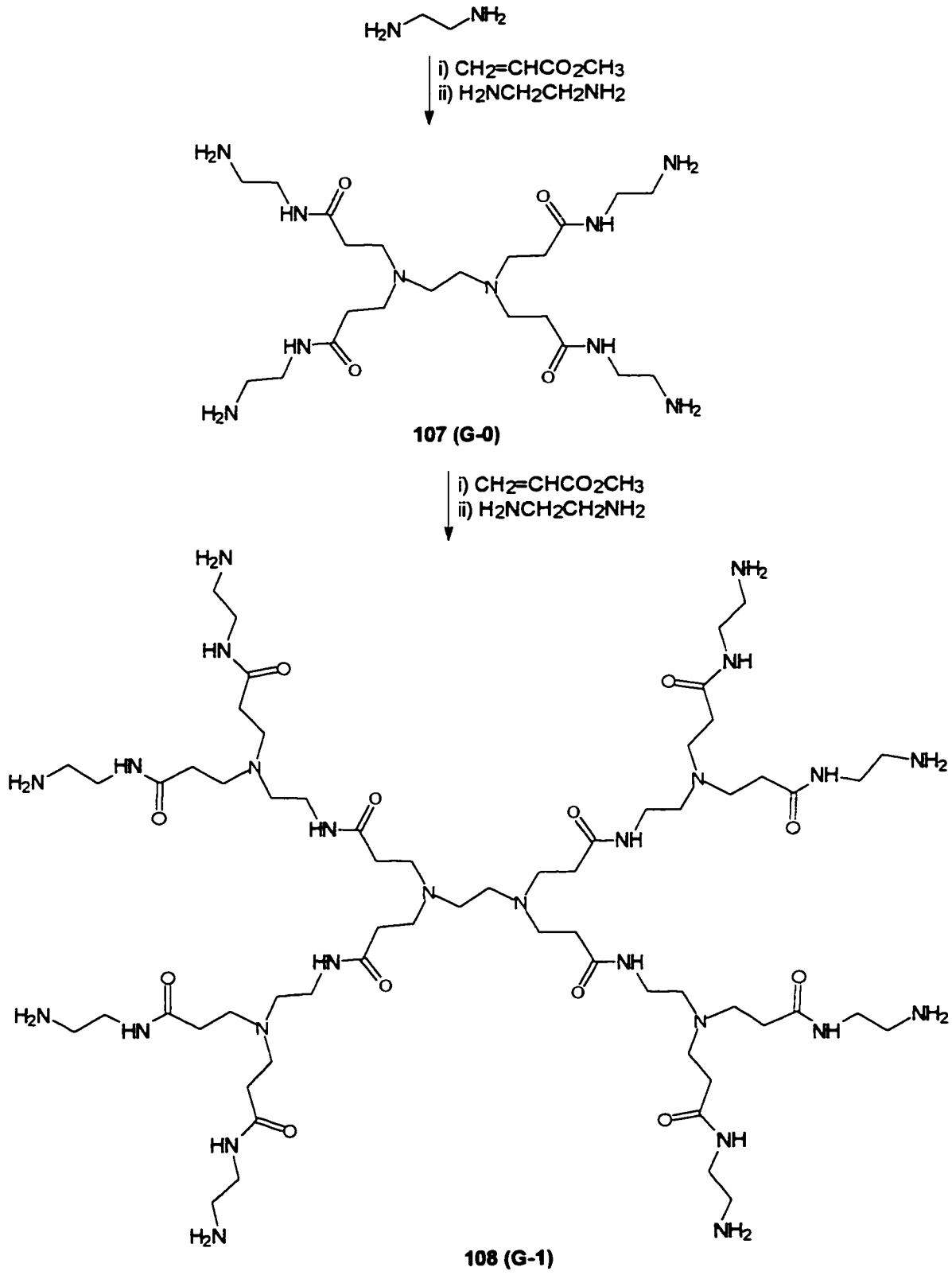


Fig. 6-1. Synthesis of starburst™ PAMAM dendrimers **107** and **108.**

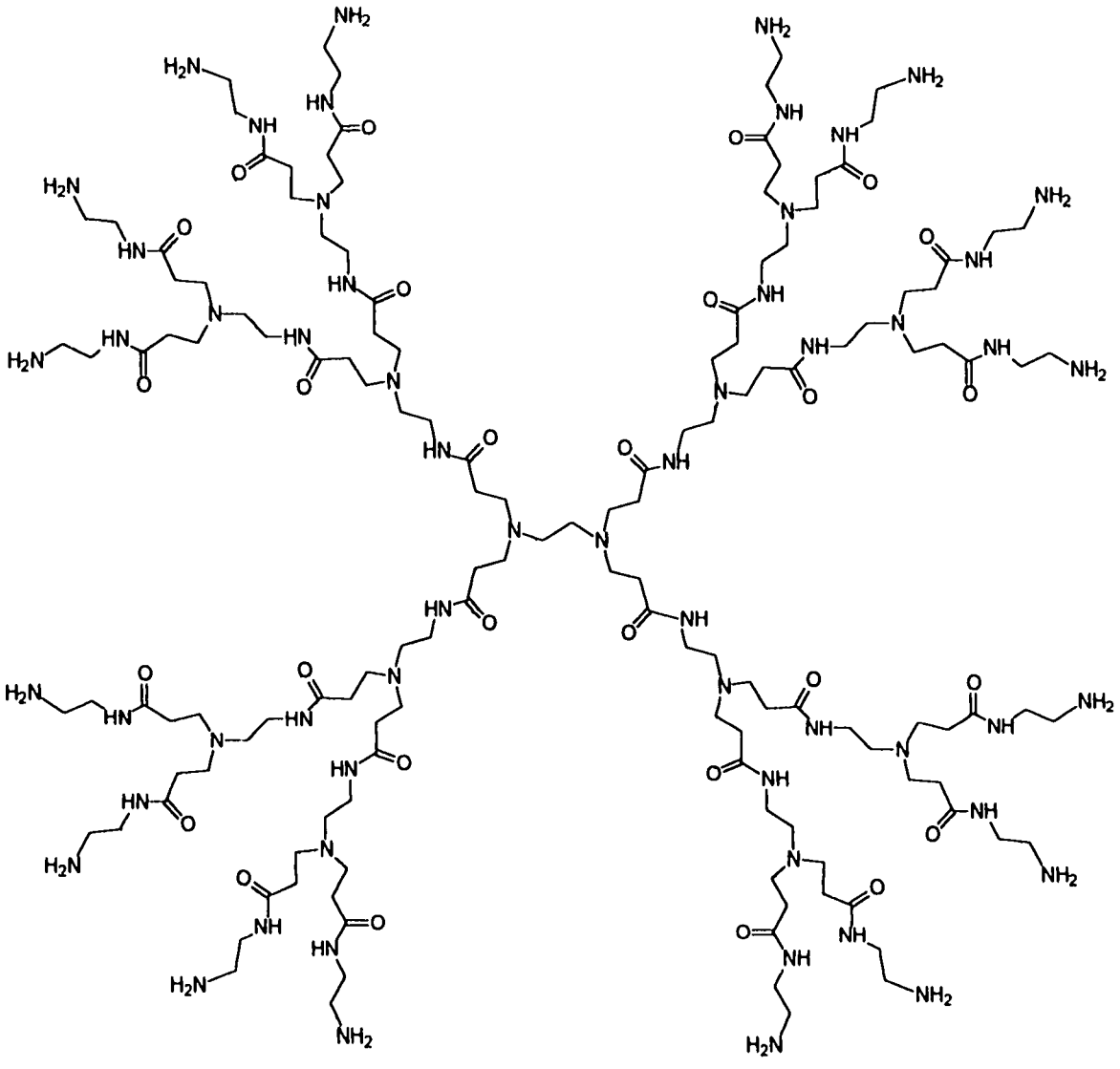


Fig. 6-2. 16 Amines surfaced starburst^{TM} PAMAM dendrimers **109 (G-2).**

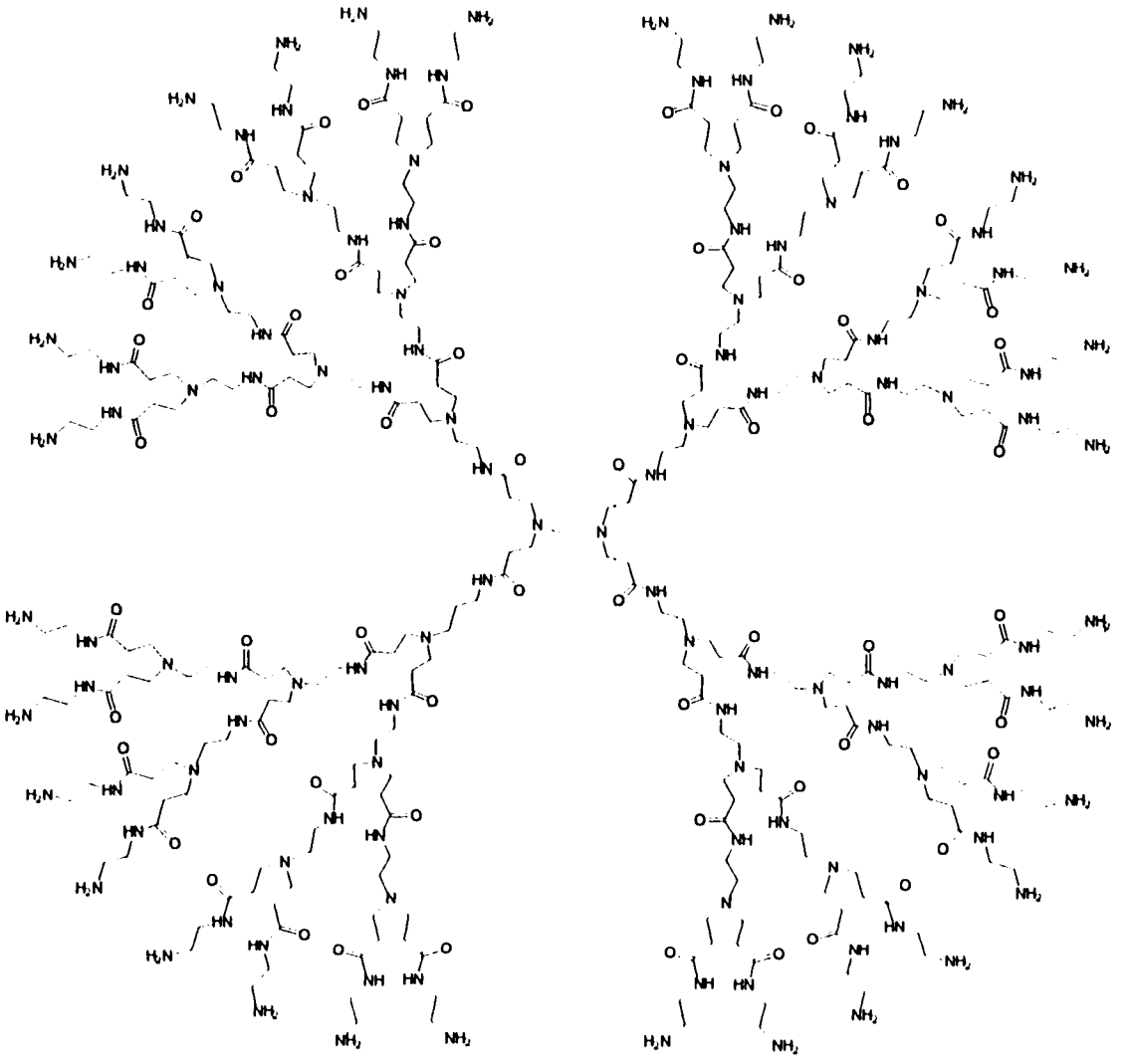
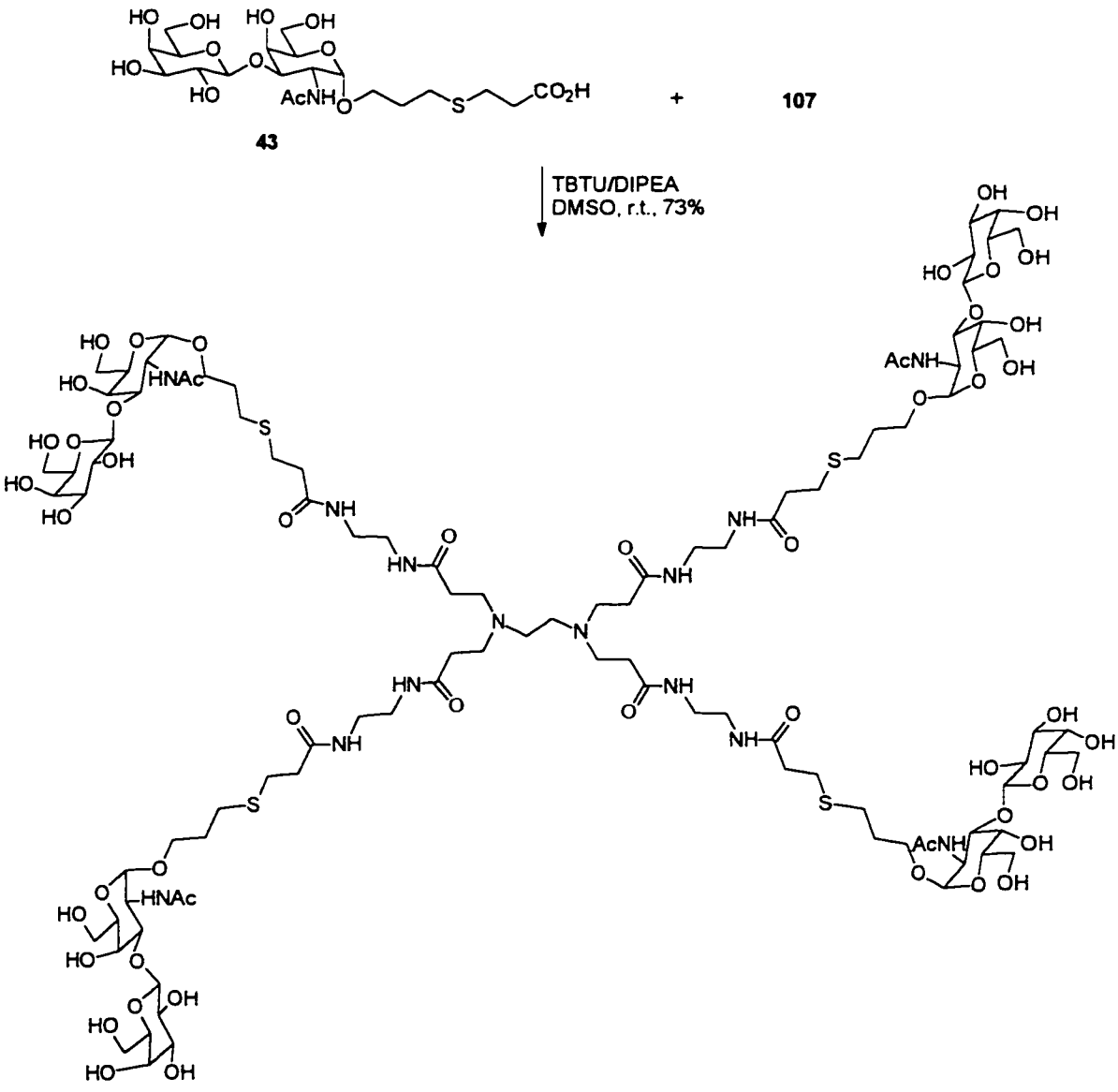


Fig. 6-3. 32 Amines surfaced starburst^{TM} PAMAM dendrimers **110 (G-3).**

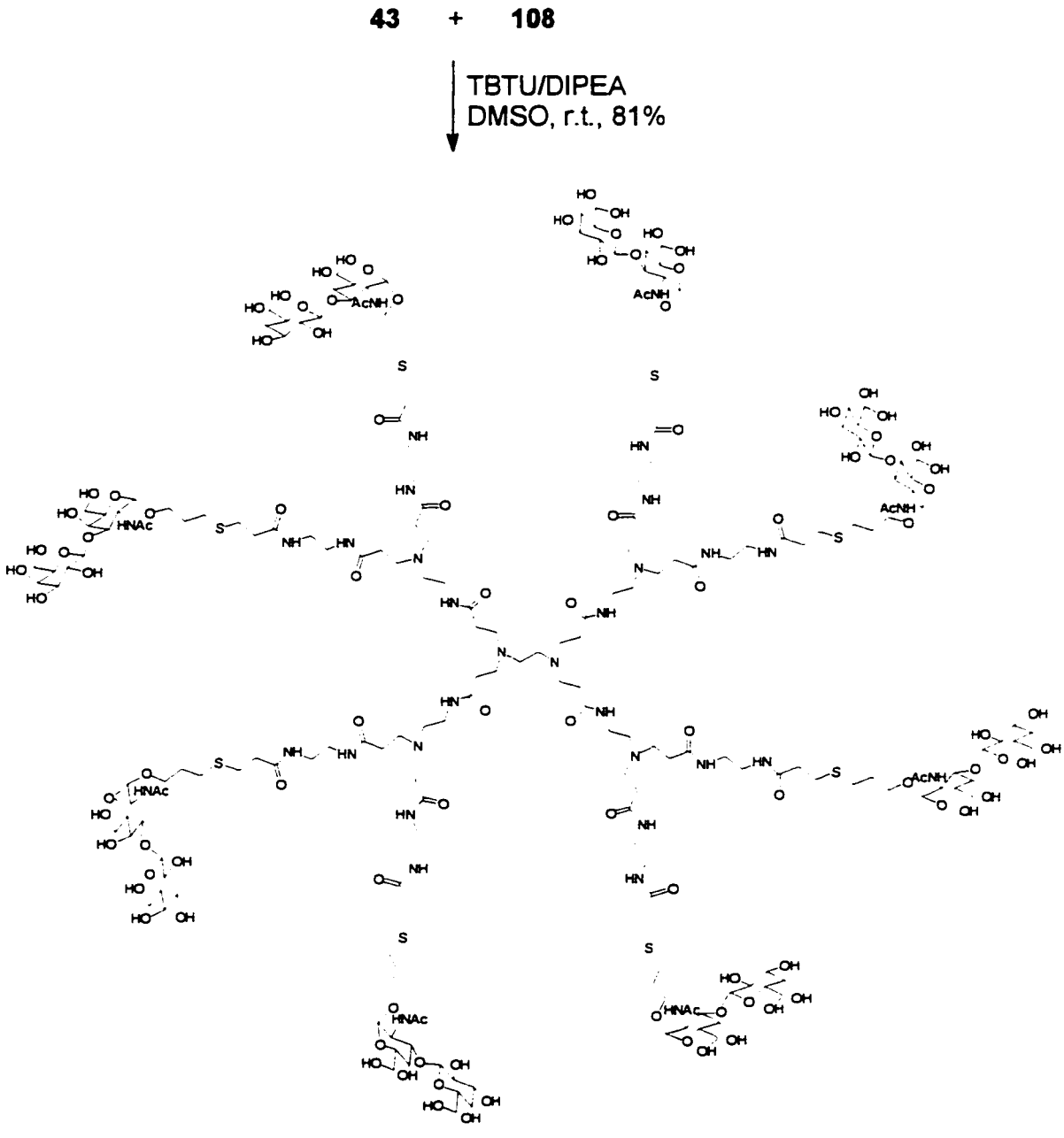
6-2. Conjugation of 3-(2-carboxyethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc to starburst™ PAMAM dendrimers

PAMAM dendrimers were conjugated with a slight excess of carboxylic acid terminated T-antigen, **43** by the TBTU strategy. Commercially available PAMAM dendrimers in MeOH, **107~ 110**, were dried under vacuum. The residue was dissolved in DMSO and the carbohydrate moiety, **43** (1.1 eq. per amine functionality) was added. TBTU (1.2 eq. per acid functionality) and DIPEA (pH = 8) were then added and the resulting solution was stirred overnight at room temperature (Scheme 6-1~6-4). The reaction mixture was lyophilized and the residue was purified by size exclusion chromatography (P-2 or P-4). After purification, PAMAM based T-antigen dendrimers, **111~114** with 4, 8, 16, and 32 T-antigen residues were obtained in excellent yields (73~99%).

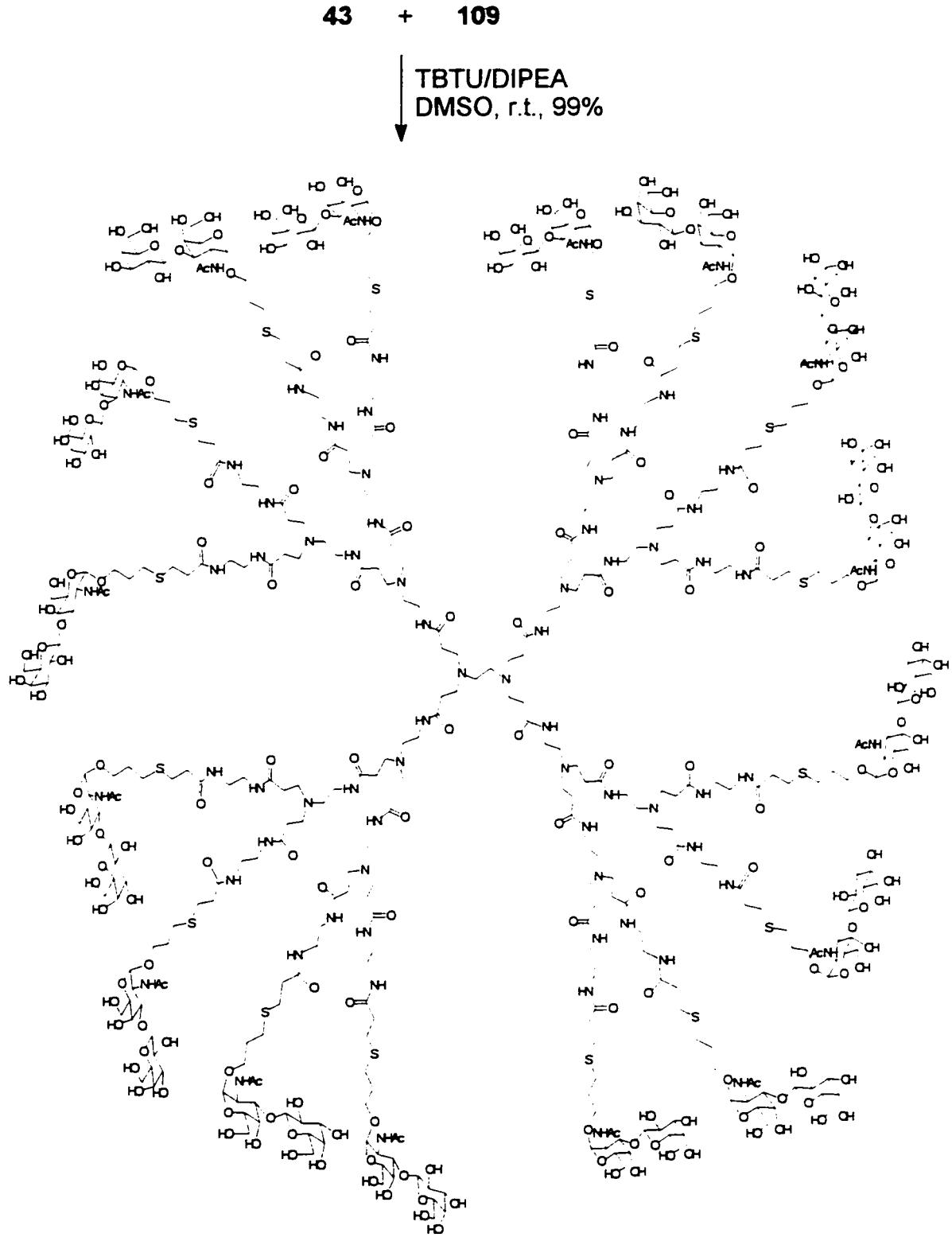
^{1}H-NMR spectra of these glycodendrimers clearly established total incorporation of the T-antigen residues by comparing the signals between the two methylene units of the ethanediamine of the dendritic core at 2.80 ppm for **111** and **112**, 2.75~2.79 ppm for **113** and 3.05 ppm for **114** and the two anomeric protons of T-antigen at 4.96 ppm for H-1 and 4.56 ppm for H-1′ in D_2O, respectively.



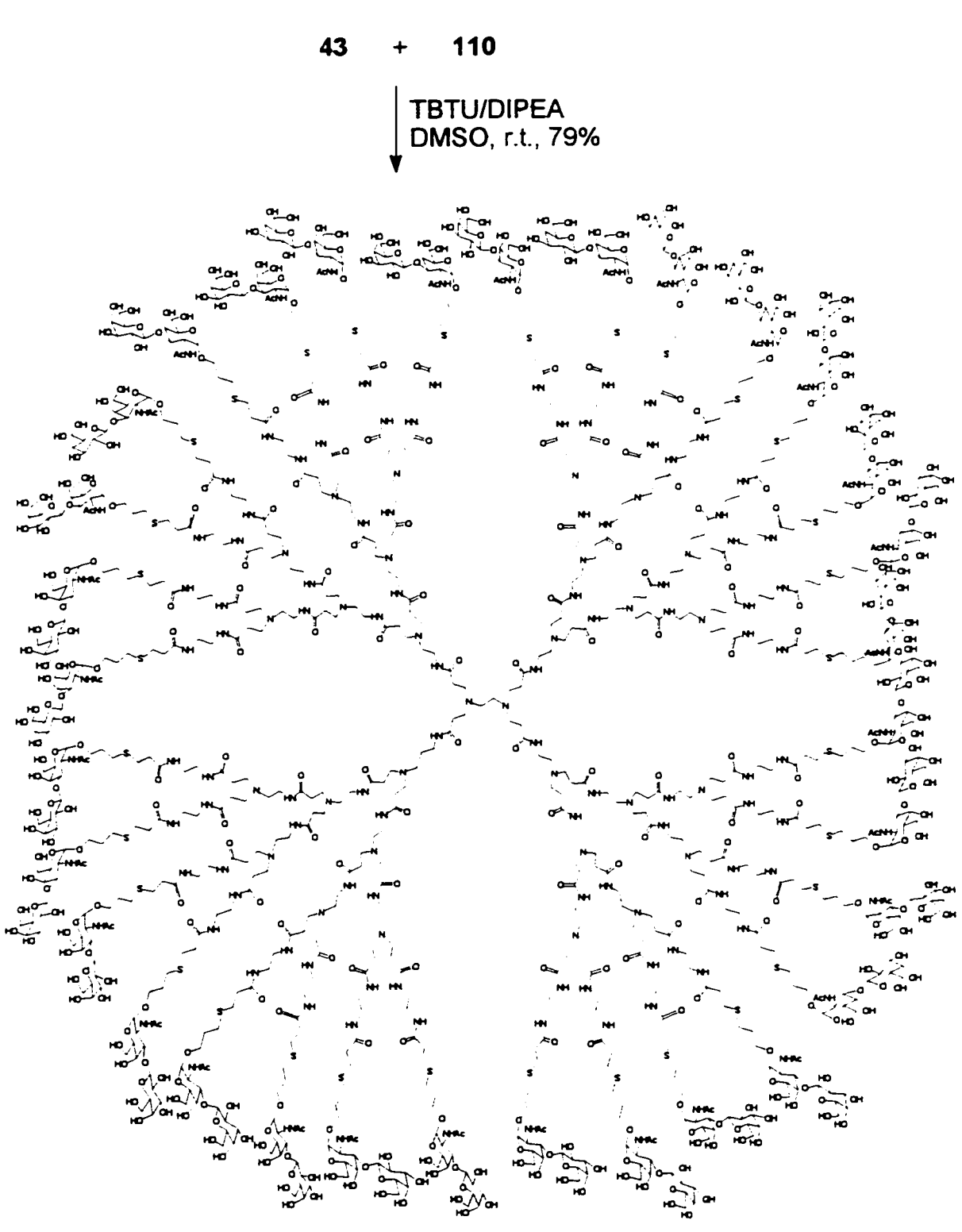
Scheme 6-1. Synthesis of tetravalent PAMAM based T-antigen dendrimer **111.**



Scheme 6-2. Synthesis of octavalent PAMAM based T-antigen dendrimer **112.**



Scheme 6-3. Synthesis of hexadecavalent PAMAM based T-antigen dendrimer **113**.



Scheme 6-4. Synthesis of 32-valent PAMAM based T-antigen dendrimer **114.**

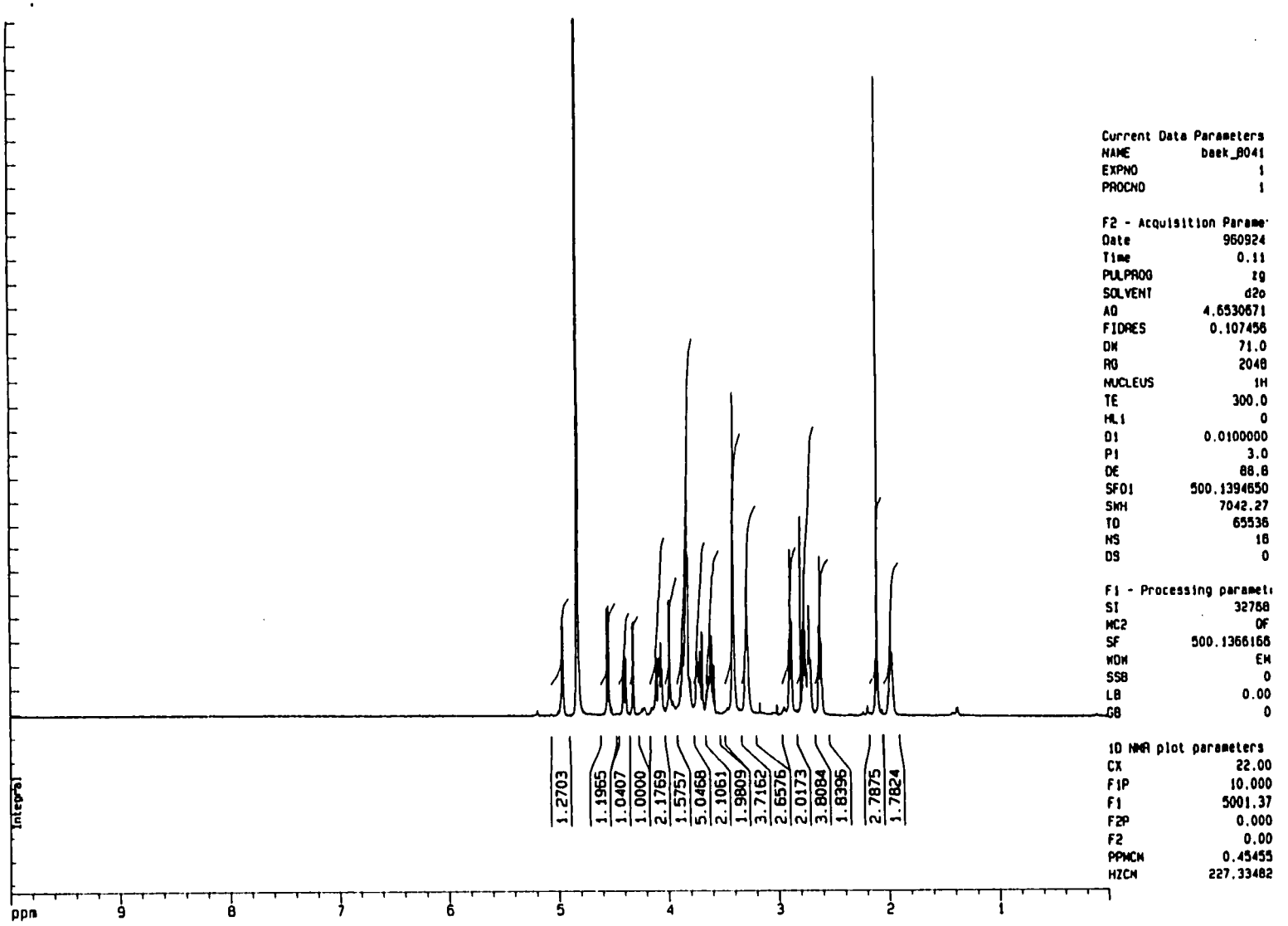


Fig. 6-4. ^{1}H-NMR spectrum (500 MHz, D_2O) of tetravalent PAMAM based T-antigen dendrimer **111.**

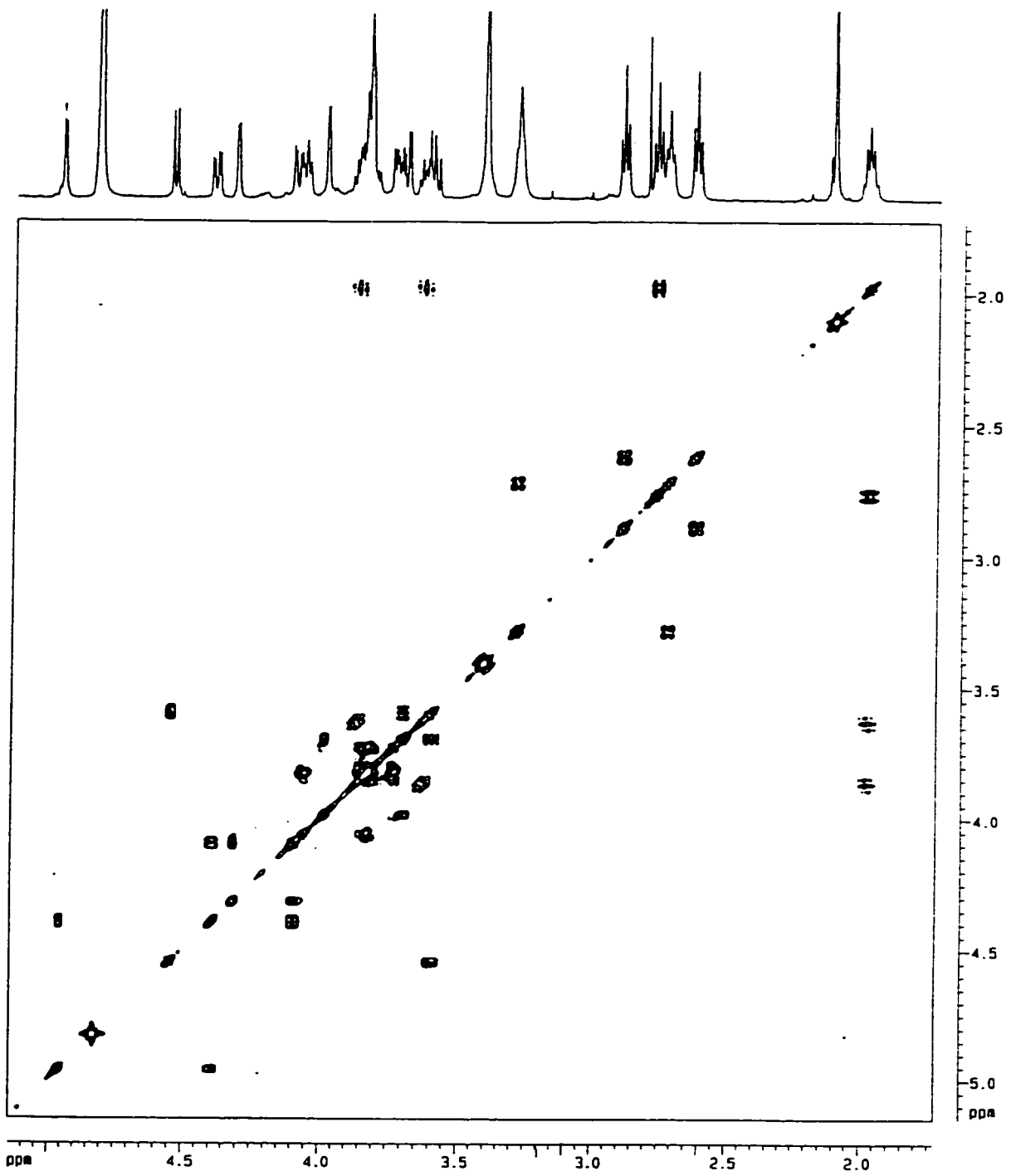


Fig. 6-5. ^{1}H-^{1}H COSY NMR spectrum (500 MHz, D_2O) of tetravlent PAMAM based T-antigen dendrimer **111.**

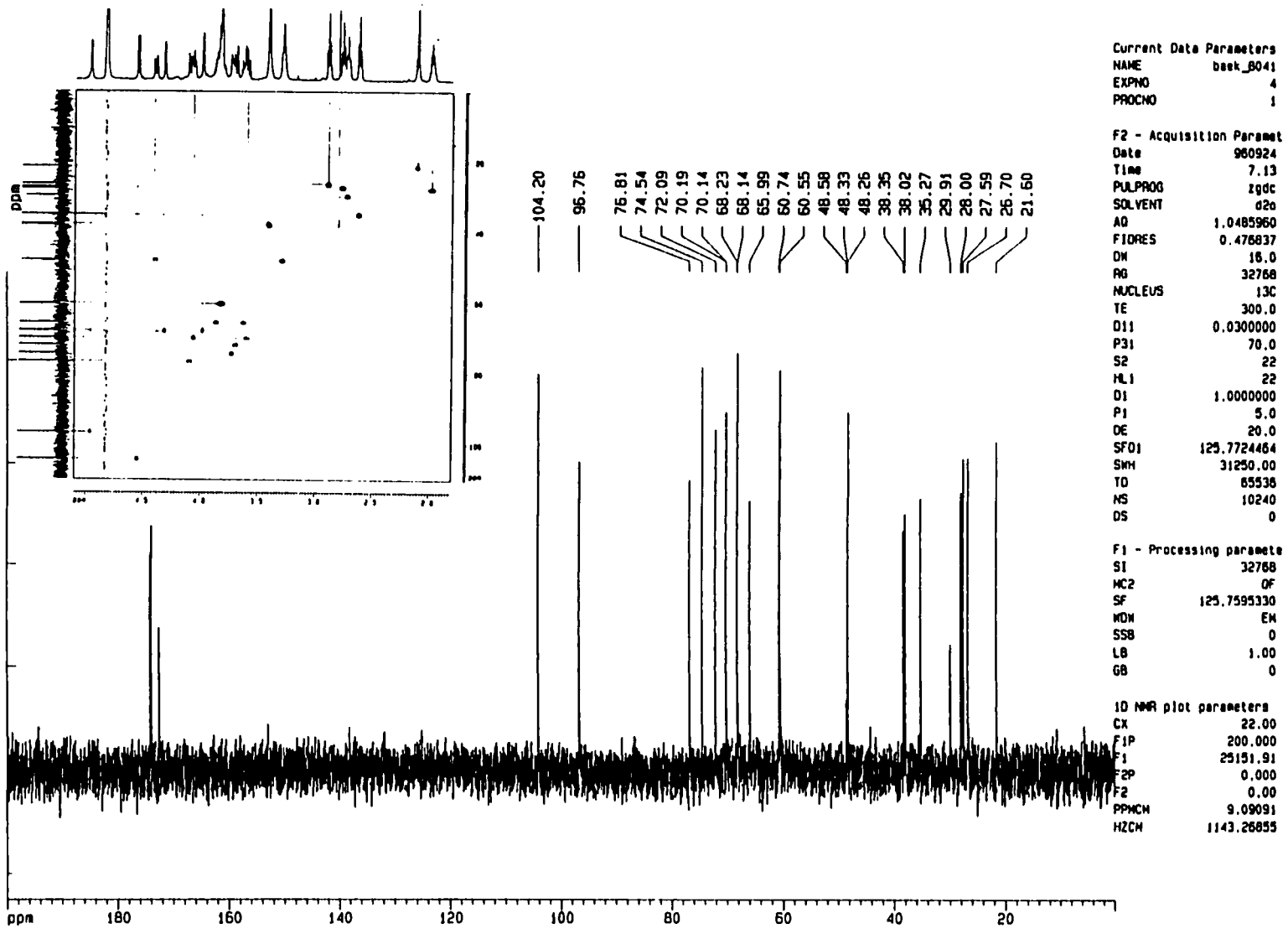


Fig. 6-6. ^{13}C-and ^{1}H-^{13}C HMQC NMR spectrum (500 MHz, D_2O) of tetravlent PAMAM based T-antigen dendrimer **111.**

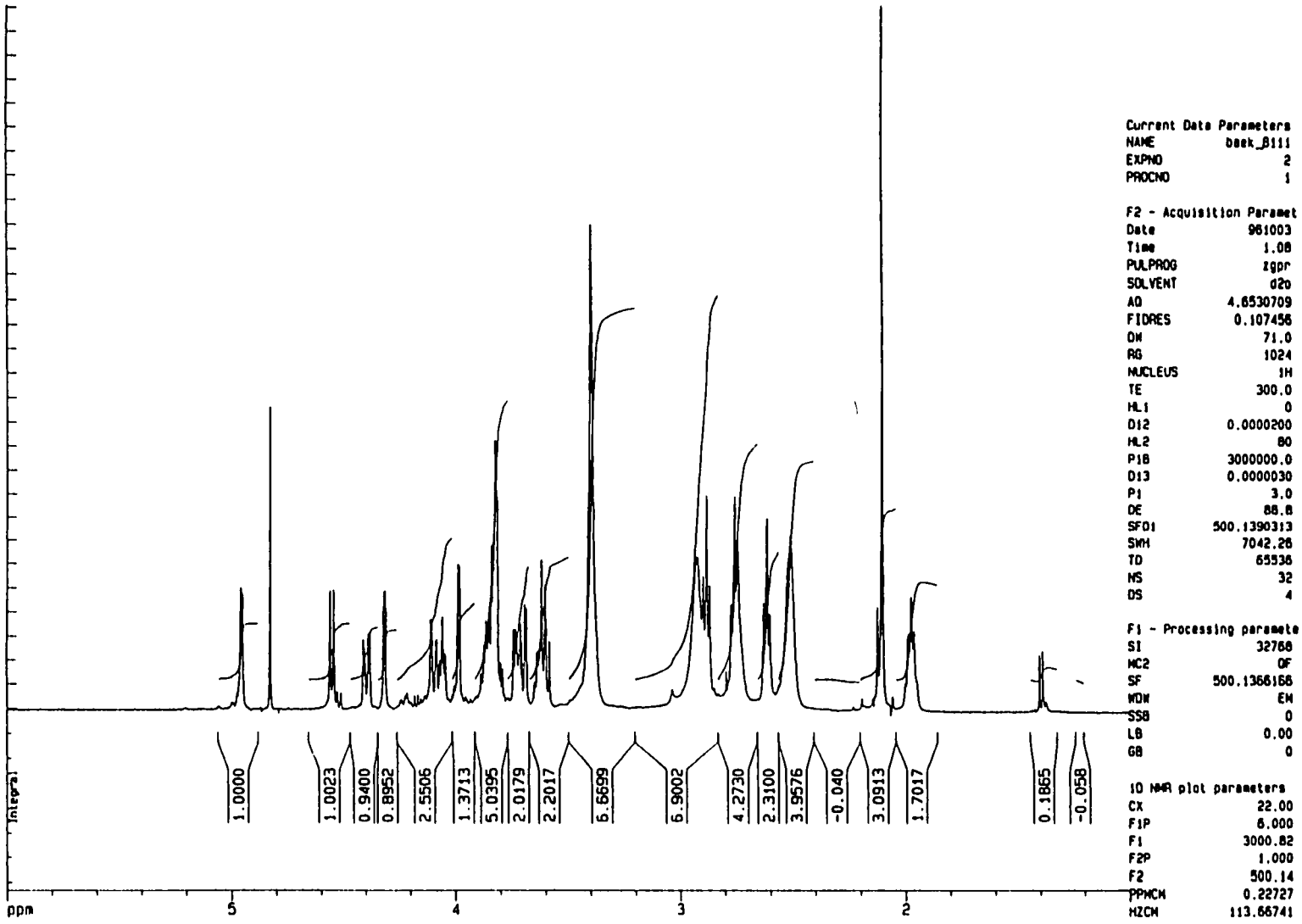


Fig. 6-7. ^{1}H-NMR spectrum (500 MHz, D_2O) of 32-valent PAMAM based T-antigen dendrimer **114.**

6-3. Immunochemical assays

Turbidimetric analysis and enzyme linked lectin assays (ELLA) were initially performed to determine the ability of these glycodendrimers to bind with peanut lectin. Time course formation of precipitin between glycodendrimers **111~114** and peanut lectin was shown in Fig. 6-8. Maximum turbidity which was in proportion to the number of T-antigen residues was obtained in 1h. These qualitative experiments demonstrated that the glycodendrimer containing 32 T-antigen residues **114** formed the most intense precipitin (O.D. = 0.41). All of the PAMAM based T-antigen dendrimers exhibited binding interaction with peanut lectin and also cleary showed the multivalency effect. The reactivity of the PAMAM glycodendrimers toward peanut lectin was further substantiated by ELLA as depicted in Fig. 6-9.

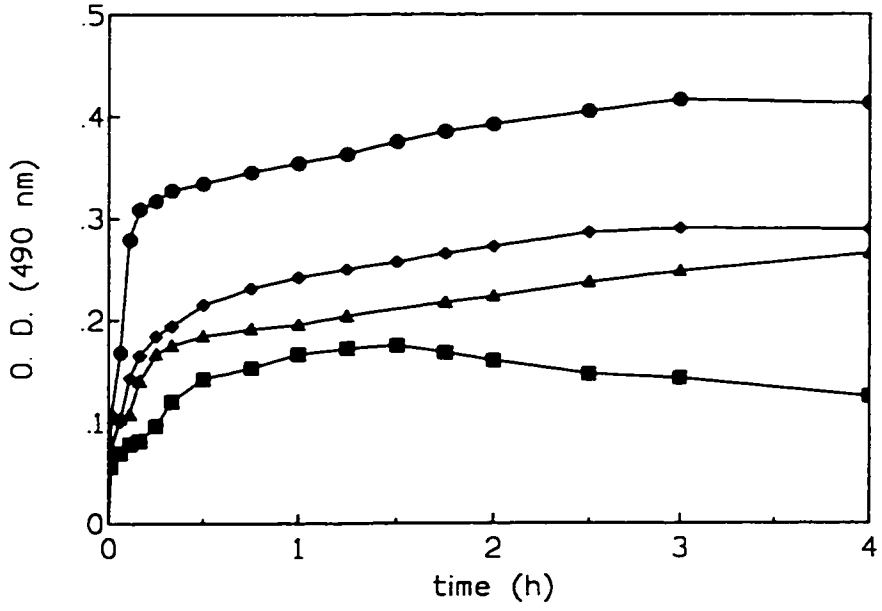


Fig. 6-8. Turbidimetric analysis of PAMAM based T-antigen dendrimers **111~114**, with peanut lectin from *Arachis Hypogaea.*

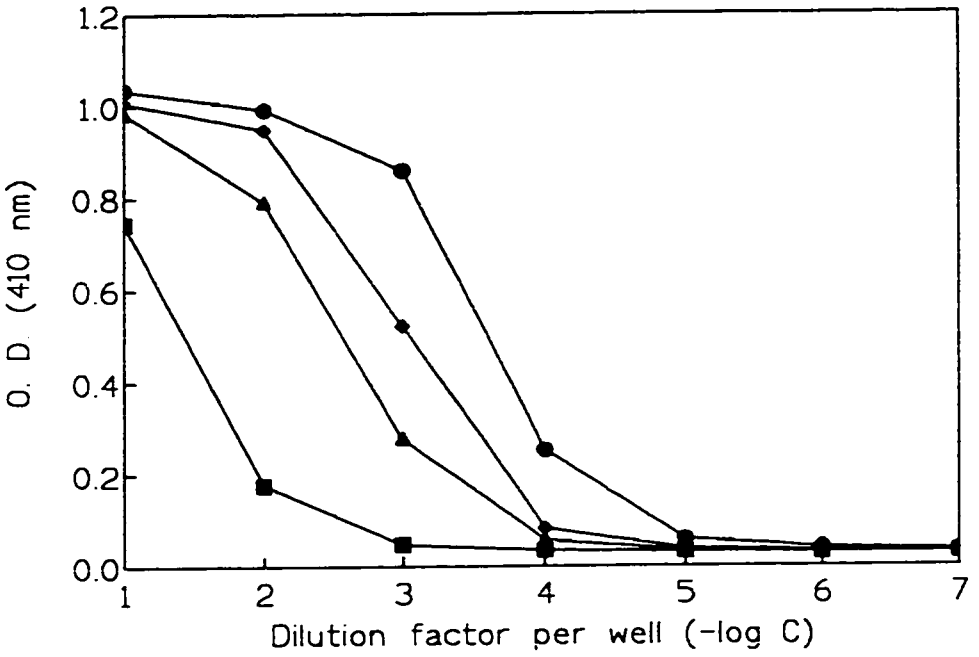


Fig. 6-9. Enzyme linked lectin assays (ELLA) of peanut lectin to PAMAM based T-antigen dendrimers **111**(■), **112** (▲), **113** (◆) and **114** (●) to immobilize *Arachis Hypogaea* horseradish peroxidase (HRP).

When optical density values were plotted as a function of the concentration of *Arachis Hypogaea*-HRP, thirty two valency **114** showed the highest O.D. values and vice versa for the tetravalency, **111**, in the whole range of concentration. From the terbidimetric analysis and ELLA, an increase of multivalency resulted in a steady increase of binding potential where **114** showed the most potent reactivity with peanut lectin. Similarly, Enzyme linked immunosorbent assays (ELISA) were performed with PAMAM glycodendrimers as coating antigens to immobilize mouse monoclonal IgG antibodies as receptor proteins. The results again confirmed the multivalency effect between T-antigen and receptor protein by the formation of strongly crosslinked carbohydrate-receptor protein complexes with high O. D. values (Fig. 6-10).

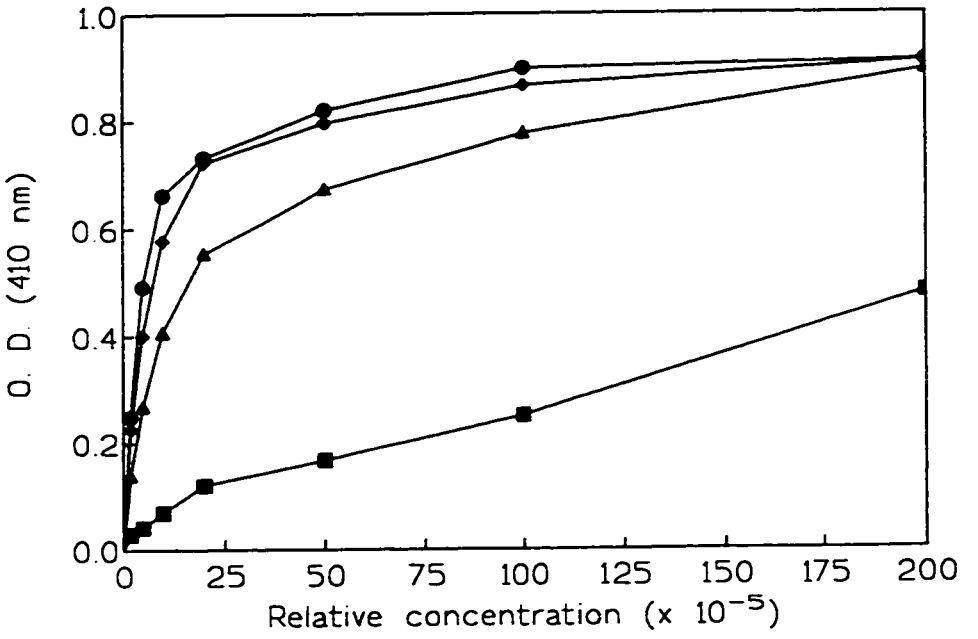


Fig. 6-10 Enzyme linked immunosorbent assays (ELISA) of PAMAM based T-antigen dendrimers **111**(■), **112** (▲), **113** (◆) and **114** (●) with mouse monoclonal IgG antibodies, goat anti-mouse IgG-enzyme conjugates and ABTS-H_2O_2 as peroxidase substrates.

On the basis of these preliminary assays, inhibitory potential was determined with T-antigen dendrimers **111~114** as inhibitors and T-antigen containing copolyacrylamide as coating antigens to immobilize mouse IgG MAb. Assays were performed indirectly through goat anti-mouse IgG MAb-enzyme conjugates (Fig. 6-11, 6-12). Before reaction with the coating antigen, the mouse IgG MAb and inhibitors with varying concentration were preincubated as described before.

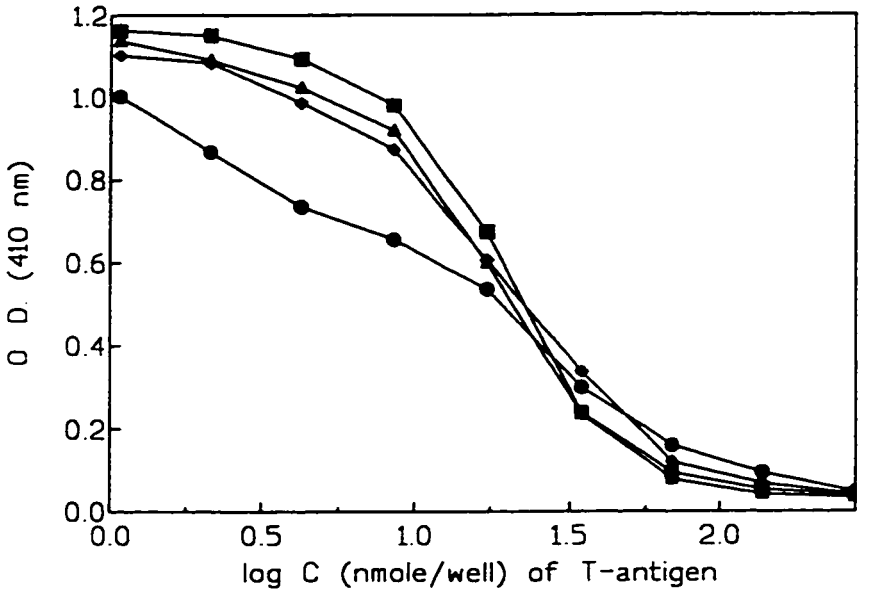


Fig. 6-11. Inhibition of binding of mouse IgG-MAb to coating antigen (T-antigen-copolyacrylamide) by **111**(■), **112** (▲), **113** (◆) and **114** (●).

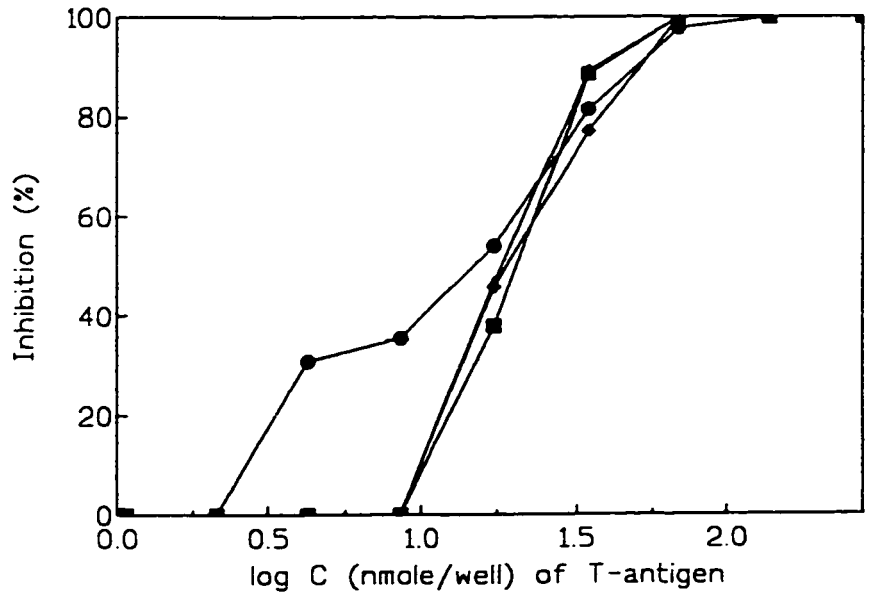


Fig. 6-12. Percent inhibition of binding of mouse IgG-MAb to coating antigen (T-antigen-copolyacrylamide) by **111**(■), **112** (▲), **113** (◆) and **114** (●).

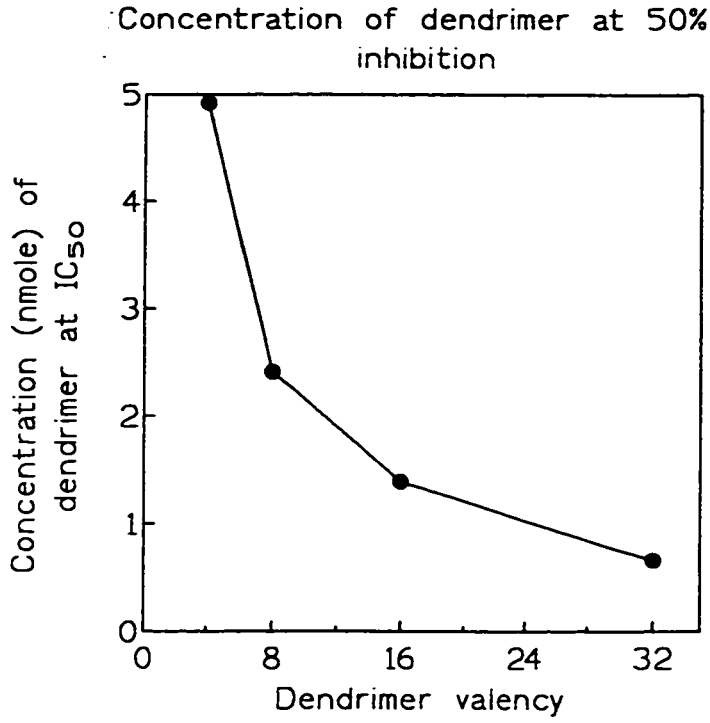


Fig. 6-13 Effect of dendrimer valency on the inhibition of mouse monoclonal IgG antibodies; PAMAM based glycodendrimers **111~114.**

The higher valency of the carbohydrate residues exhibited a stronger inhibitory potential. Dendrimers with valencies of four (**111**), eight (**112**), sixteen (**113**) and thiry two (**114**) showed IC_{50} values of 4.92, 2.41, 1.39 and 0.66 nmole, respectively (Table 6-1). When compared to the tetravalent N, N'-bis(acrylamido)acetic acid based glycodendrimer **90** the same valency of PAMAM T-antigen dendrimer **111** exhibited an inhibitory potential that was four times higher. This may have resulted from the accessibility of a number of T-antigen residues for the inhibition of receptor proteins due to readily available geometric arrangements as well as long enough spacers. Furthermore, in higher generations, these glycodendrimers showed up to twenty seven times better inhibitory properties than **90**.

Table 6-1. The amount of PAMAM T-antigen dendrimers to provide 50% inhibition of binding ofmouse IgG MAb to coating antigen (T-antigen-copolyacrylamide).

Compound	IC_{50} (nmole)[a]	Relative Potency[a]
90[b]	18 (72)	1
111	4.9 (20)	3.7 (4)
112	2.4 (19)	7.5 (3.7)
113	1.4 (22)	12.9 (3.2)
114	0.66 (21)	27.2 (3.4)

a: values in parentheses are based on T-antigen residues

b: N,N'-bis(acrylamido) acetic acid based glycodendrimer **90**, was chosen as a reference to compare inhibitory potency.

6.4. Conclusions

T-antigen containing globular glycodendrimers were synthesized with polyamine surfaced starburst™ PAMAM dendrimers using the TBTU strategy. The ability of these glycodendrimers to react with peanut lectin from *Arachis Hypogaea* and form insoluble carbohydrate-receptor protein complexes was confirmed by turbidimetric analysis. ELLA and ELISA with receptor proteins such as peanut lectin or mouse monoclonal IgG antibodies demonstrated the enhanced multivalency effect as the density of the carbohydrates increased. The tetravalent PAMAM based glycodendrimer exhibited better inhibitory potential than same valency of N, N'-bis(acrylamido) acetic acid based one. Furthermore, a higher generation of PAMAM glycodendrimers revealed incorporation of terminal T-antigen residues showing up to 27-fold increase in inhibition of the binding of mouse IgG MAb to coating antigens. These results confirmed the great feature of geometrically well designed PAMAM based glycodendrimers which possess molecular information and receptor protein markers.

6.5. Experimental methods

General Procedure

PAMAM solution in methanol was taken in a 25 mL round bottom flask and dried under vacuum. The residue was dissolved in DMSO and 1.1 eq of O-4-(2-carboxyethylthio) β-D-Gal-(1-3)-α-D-GalNAc, **43** and TBTU (1.2 eq per acid) was added followed by DIPEA to adjust pH = 8.5~9.0. The solution was stirred overnight at room temperature. The reaction was confirmed by a ninhydrin test. DMSO was eliminated by lyophilization. Without a further work-up procedure, the residue was purified by size exclusion chromatography (P-2, P-4) with distilled water as an eluent or dialysis with a 2,000 molecular weight cut-off tube against distilled water. The product portion was collected and lyopilized to afford white spongy solid with 73~99% yield.

Counting of the protons and carbons proceeded from the edge to the core of each glycodendrimer, **111~114**, by alphabetical order.

Tetravalent PAMAM based T-antigen dendrimers (111)

PAMAM (7.2 mg, 13.9 μmol) (**107**, G-0) dendrimer having 55.6 μmol of primary amine and 32.4 mg (61.16 μmol, 1.1 eq per amine) of carboxyacid terminated T-antigen were dissolved in 6 mL of DMSO. 23.6 mg (73.5 μmol, 1.2 eq per acid) of TBTU was added followed by DIPEA. The solution was stirred overnight. After purification, 26 mg (10.2 μmol, 73%) of product was obtained. **^{1}H-NMR**, δ (D_2O): 4.96 (d, 4H, J_{12} = 3.8 Hz, H_1), 4.55 (d, 4H, J_{12}' = 7.8 Hz, H_1'), 4.40 (dd, 4H, $J_{1\,2}$= 3.8 Hz, J_{23} = 11.1 Hz, H_2), 4.32 (broad d, 4H, J_{34} = 2.8 Hz, $J_{45} \leq 1$ Hz, H_4), 4.10 (dd, 4H, J_{23} = 11.1 Hz, J_{34} = 3.0 Hz, H_3), 4.07 (broad t, 4H, $J_{45} \leq 1$ Hz, J_{56} = 6.2 Hz, H_5), 3.99 (broad d, 4H, J_{34}' = 3.3 Hz, $J_{45}' \leq 1$ Hz, H_4'), 3.89~3.76 (m, 20H, $2H_6$, $2H_6'$, H_a), 3.75~3.72 (m, 4H, H_5'), 3.70 (dd, 4H, J_{23}' = 9.9 Hz, J_{34}' = 3.4 Hz, H_3'), 3.66~3.58 (m, 8H, H_a, H_2'), 3.41 (broad s, 16H, H_f, H_g), 3.29 (broad t, 8H, H_i), 2.89 (t, 8H, J_{de} = 6.9 2Hz, H_d), 2.80 (s, 4H, H_j), 2.77 (t, 8H, J_{bc} = 7.1

Hz, H_c), 2.72 (t, 8H, J_{hi} = 6.7 Hz, H_h), 2.62 (t, 8H, J_{de} = 6.9 Hz, H_e), 2.11 (s, 12H, methyl), 2.01~1.95 (m, 8H, H_b); **^{13}C-NMR**, δ (D_2O): 174.2~172.6 (C=O), 104.2 (C_1), 96.8 (C_1'), 76.8 (C_3), 74.5 (C_5'), 72.1 (C_3'), 70.2 (C_5), 70.1 (C_2'), 68.2 (C_4), 68.1 (C_4'), 66.0 (C_a), 60.7 & 60.6 (C_6 & C_6'), 48.6 (C_2), 48.3 (C_i), 38.4 ($C_{f,g}$), 38.0 (H_j), 35.3 (H_e), 29.9 (C_h), 28.0 (C_b), 27.6 (C_c), 26.7 (C_d), 21.6 (C_{methyl}).

Octavalent PAMAM based T-antigen dendrimers (112)

of PAMAM (8.37 mg, 5.85 μmol) (**108**, G-1) dendrimer having 46.8 μmol of primary amine and 27.3 mg (51.6 μmol, 1.1 eq per amine) of carboxyacid terminated T-antigen were dissolved in 5 mL of DMSO. 20 mg (62.3 μmol, 1.2 eq per acid) of TBTU was added followed by DIPEA. The solution was stirred overnight. After purification, 26.1 mg (4.7 μmol, 81%) of product was obtained. **^{1}H-NMR**, δ (D_2O): 4.96 (d, 8H, J_{12} = 3.7 Hz, H_1), 4.55 (d, 8H, $J_{12'}$ = 7.8 Hz, H_1'), 4.39 (dd, 8H, J_{12} = 3.7 Hz, J_{23} = 11.1 Hz, H_2), 4.32 (d, 8H, J_{34} = 2.7 Hz, $J_{45} \leq$ 1 Hz, H_4), 4.10 (dd, 8H, J_{23} = 11.1 Hz, J_{34} = 2.9 Hz, H_3), 4.06 (broad t, 8H, J_{56} = 6.6 Hz, H_5), 3.99 (d, 8H, $J_{34'}$ = 3.3 Hz, H_4'), 3.89~3.77 (m, 40H, H_6, H_6', H_a), 3.74~3.69 (m, 24H, H_5', H_3', H_k), 3.66~3.52 (m, 24H, H_2', H_a, H_l), 3.47 (broad t, 8H, H_j), 3.41 (broad s, 32H, H_f, H_g), 3.28 (broad t, 16H, H_h), 2.90~2.83 (m, 24H, H_e, H_m), 2.80 (s, 4H, H_n), 2.79~2.75 (m, 32H, H_c, H_i), 2.62 (t, 16H, J_{de} = 6.8 Hz, H_d), 2.10 (s, 24H, methyl), 1.99 (m, 16H, H_b); **^{13}C-NMR**, δ (D_2O): 174.2~171.4 (C=O), 104.2 (C_1'), 96.8 (C_1), 76.8 (C_3), 74.5 (C_5'), 72.1 (C_3'), 70.2 & 70.1 (C_5 & C_2'), 68.2 & 68.1 (C_4 & C_4'), 66.0 (C_a), 60.8 & 60.6 (C_6 & C_6'), 51.7 (C_j), 49.6 (C_l), 48.7 (C_2), 38.4~37.9 (C_f, C_g, C_n), 35.3 (C_d), 34.0 (C_k), 29.8 (C_i), 28.1 (C_b), 28.0 (C_m), 27.6 (C_c), 26.7 (C_e), 21.6 (C_{methyl}).

Hexadecavalent PAMAM based T-antigen dendrimer (113)

of PAMAM (11.5 mg, 3.52 μmol) (**109**, G-2) dendrimer having 56.32 μmol of primary amine and 32.8 mg (61.97 μmol, 1.1 eq per amine) of carboxyacid terminated T-

antigen were dissolved in 10 mL of DMSO. 24.0 mg (74.74 μmol, 1.2 eq per acid) of TBTU was added followed by DIPEA. The solution was stirred overnight. After purification, 40 mg (3.50 μmol, 99.0%) of product was obtained. **^{1}H-NMR**, δ (D_2O): 4.96 (d, 16H, J_{12} = 3.7 Hz, H_1), 4.56 (d, 16H, J_{12}' = 7.8 Hz, H_1'), 4.40 (dd, 16H, J_{12} = 3.7 Hz, J_{23} = 11.1 Hz, H_2), 4.32 (d, 16H, J_{34} = 3.0 Hz, $J_{45} \leq$ 1 Hz, H_4), 4.10 (16H, J_{23} = 11.1 Hz, J_{34} = 3.1 Hz, H_3), 4.06 (broad t, 16H, J_{56} = 6.4 Hz, H_5), 3.99 (d, 16H, J_{34}' = 3.3 Hz, H_4'), 3.89~3.76 (m, 80H, H_6, H_6', H_a), 3.75~3.69 (m, 32H, H_3', H_5'), 3.65~3.58 (m, 64H, H_2', H_a), 3.41~3.30 (m, 120H, H_f, H_g, H_k, H_o), 3.28~3.06 (m, 80H, H_i, H_j, H_m, H_n, H_p), 2.89 (t, 32H, J_{de} = 7.0 Hz, H_d), 2.79~2.75 (m, 92H, H_c, H_h, H_l, H_q, H_r), 2.62 (t, 32H, J_{de} = 7.0 Hz, H_e), 2.10 (s, 48H, methyl), 2.09~1.95 (m, 32H, H_b); **^{13}C-NMR**, δ (D_2O): 174.1~172.3 (C=O), 104.2 (C_1'), 102.8 (C_1), 76.9 (C_3), 74.7 (C_5'), 72.1 (C_3'), 70.4 (C_5), 70.2 (C_2'), 68.2 (C_4), 68.2 (C_4'), 66.0 (C_a), 60.8 & 60.6 (C_6 & C_6'), 53.9~48.3 (C_i, C_j, C_m, C_n, C_q), 49.3 & 43.0~38.0 (C_f, C_g, C_k, C_o), 48.8 (C_2), 35.3 (C_e), 31.5~28.1 (C_c, C_h, C_l, C_p, C_r), 27.6 (C_b), 26.7 (C_d), 21.7 (C_{methyl}).

Thirtytwo-valent PAMAM based T-antigen dendrimer (114)

PAMAM (12.4 mg, 1.80 μmol) (**110**, G-3) dendrimer having 57.6 μmol of primary amine and 33.5 mg (63.40 μmol, 1.1 eq per amine) of carboxyacid terminated T-antigen were dissolved in 8 mL of DMSO. 24.4 mg (76.0 μmol, 1.2 eq per acid) of TBTU was added followed by DIPEA. The solution was stirred overnight. After purification, 32.2 mg (1.38 μmol, 79%) of product was obtained. **^{1}H-NMR**, δ (D_2O): 4.96 (d, 32H, J_{12} = 3.7 Hz, H_1), 4.56 (d, 32H, J_{12}' = 7.7 Hz, H_1'), 4.40 (dd, 32H, J_{12} = 3.7 Hz, J_{23} = 1.1 Hz, H_2), 4.32 (broad d, 32H, J_{34} = 2.9 Hz, $J_{45} \leq$ 1 Hz, H_4), 4.10 (dd,32H, J_{23} = 11.1 Hz, J_{34} = 3.0 Hz, H_3), 4.06 (broad t, 32H, J_{56} = 6.1 Hz, H_5), 3.99 (broad d, 32H, J_{34}' = 3.4 Hz, H_4')3.95~3.79 (m, 160H, H_6, H_6', H_a), 3.78~3.69 (m, 64H, H_3', H_5'), 3.65~3.58 (m, 64H, H_2', H_a), 3.40 (broad s, 192H, H_f, H_g, H_h), 2.92 (broad s, 112H, H_k, H_l, H_o, H_p, H_s, H_t), 2.88 (t, 64H, J_{de} = 7.0 Hz, H_e), 2.77 (m, 128H, H_c, H_i), 2.62 (t, 64H, J_{de} = 7.0 Hz, H_d), 2.56~2.51 (broad s, 112H, H_j, H_m, H_n, H_q, H_r, H_u), 2.10 (s, 96H, methyl), 2.00~1.39 (m,

64H, H_b); **^{13}C-NMR**, δ (D_2O): 174.2~173.9 (six carbonyls), 104.2 (C_1'), 96.8 (C_1), 76.8 (C_3), 74.6 (C_5'), 72.1 (C_3'), 70.2 (C_2'), 70.1 (C_5), 68.2 (C_4), 68.1 (C_4'), 66.0 (C_a), 60.7 & 60.6 (C_6 & C_6'), 50.9 (C_i), 48.7 (C_k,C_l, C_o, C_p, C_s, C_t), 48.3 (C_2), 38.2~36.2 (C_f, C_g, C_h, C_v), 35.3 (C_d), 32.1 (C_j, C_m, C_n, C_q, C_r, C_u), 28.1 (C_b), 27.6 (C_c), 26.7 (C_e), 21.7 (C_{methyl}).

Anal. Calcd. for $C_{942}H_{1664}O_{444}N_{154}S_{32}$ (705.24): C, 50.8; H, 7.6; N, 9.7. Found: C, 49.85; H, 7.21; N, 8.79.

Turbidimetric analysis between peanut lectin from *Arachis Hypogaea* and PAMAM based T-antigen dendrimers, 111~114

Turbidimetric experiments were performed in Linbro (Titertek) microtitration plates where 50 μL/well of stock lectin solution prepared from peanut lectin (2 mg/mL in PBS) were mixed with 50 μL each of glycodendrimers **111~114** (2.1, 1, 0.54, 0.27 nmole/50 μL in PBS, respectively or 8.64 nmole of T-antigen residue each) and incubated at room temperature for 4 h. The turbidity of the solution was monitored by reading the optical density (O. D.) at 490 nm at regular time intervals until no noticeable changes could be observed. Each test was performed in triplicate.

Enzyme linked lectin assays (ELLA) between peanut lectin and PAMAM based T-antigen dendrimers, 111~114

Linbro (Titertek) microtitration plates were coated with 100 μL/well of PAMAM based T-antigen dendrimer solutions of stock solution of 10 μg/mL (1.4×10^{-9} mol/mL) in 0.01 M PBS (pH = 7.3) and incubated overnight at room temperature. After washing with PBST, blocking with BSA (1% PBS) for 1 h at 37 °C and washing again, the wells were filled with 100 μL/well of *Arachis Hypogaea*-peroxidase supernatant (0.1 mg/mL) with a serial dilution by a factor of 10 in PBST and incubated at 37 °C for 1h. The plates were washed and 50 μL/well of 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (1 mg/4 mL) in citrate-phosphate buffer (0.2 M, pH = 4.0 with 0.015% H_2O_2) was added. The reaction was stopped after 20 minutes by adding 50

μL/well of 1 M H_2SO_4 and optical density was measured at 410 nm relative 570 nm. Blank well contained citrate-phosphate buffer. Each test was performed in triplicate.

Enzyme linked immunosorbent assays (ELISA) between mouse IgG MAb and PAMAM based T-antigen dendrimers, 111~114

Linbro (Titertek) microtitration plates were coated with 100 μL/well of PAMAM based T-antigen dendrimer solution of a stock solution of 10 μg/mL in 0.01 M PBS (pH = 7.3) and incubated overnight at room temperature. After washing and blocking with BSA (1% in PBS) as described before, 100 μL/well of neat mouse IgG-MAb supernatant was added without dilution (5 μmol of antibodies/well) and incubated for 1 h at 37 °C. The plates were washed and 100 μL/well of goat anti-mouse IgG MAb-enzyme conjugates solution in PBST was added with varying concentration from 500 times to 50,000 times dilution by 2-fold dilution and incubated for 1 h. at 37 °C. After washing, 50 μL/well ABTS-H_2O_2 substrates (1 mg/4 mL) in citrate-phosphate buffer (0.2 M, pH = 4.0 with 0.015% H_2O_2) was added. The reaction was stopped after 20 minutes by adding 50 μL/well of 1 M H_2SO_4 and optical density was measured at 410 nm relative to 570 nm. A blank well contained citrate-phosphate buffer. Each test was performed in triplicate.

Competitive double sandwich inhibition ELISA using mouse monoclonal IgG antibodies and PAMAM based T-antigen dendrimers, 111~114 as inhibitors

Linbro (titertek) microtiter plates were coated overnight at room temperature with T-antigen containing copolyacrylamide at 100 μL/well of a polymer stock solution of 10 μg/mL in 0.01 M phosphate buffer (pH = 7.3). Each well contained 1 μg/well of polymer and the T-antigen content was 0.36 μg (0.85 nmol) out of 1 μg/well. The wells were then washed 3 times with 400 μL/well of 0.01 M phosphate buffer (pH = 7.3) containing 0.05% (v/v) Tween 20 (PBST). The wells were blocked with 150 μL/well of 1% BSA in PBS for 1 h at 37 °C. At the same time, 50 μL/well of mouse monoclonal IgG antibodies solution in PBST (10 times dilution of a supernatant, 0.25 μmol/50 μL) and 50 μL of inhibitor solution (PAMAM based T-antigen dendrimers) in PBST with varying

concentrations from 275 to 1.07 nmol/well of T-antigen by two fold serial dilution were mixed on Nunclon (Delta) microtiter plates and preincubated for 1 h at 37 °C. After washing of excess BSA with PBST, the wells were filled to 100 μL/well with preincubated mouse IgG MAb/inhibitor solution and incubated again for 1 h at 37 °C. The wells were washed as described above with PBST and filled with 100 μL/well of goat-anti-mouse IgG MAb (receptor protein)-enzyme conjugates solution in PBST (1,000 times dilution of supernatant) followed by incubation for 1 h at 37 °C. The wells were washed and 50 μL/well of 2, 2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (1 mg/4 mL) in citrate-phosphate buffer (0.2 M, pH = 4.0 with 0.015% H_2O_2) was added. The reaction was stopped after 20 minutes by adding 50 μL/well of 1 M aqueous sulfuric acid solution and the optical density was measured at 410 nm relative to 570 nm. Percent inhibitions were calculated as follows:

$$\% \text{ inhibition} = [A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})}/A_{(\text{no inhibitor})}] \times 100$$

IC_{50}'s were calculated as the concentration required for 50% inhibition of the coating antigen. All test were performed in triplicate.

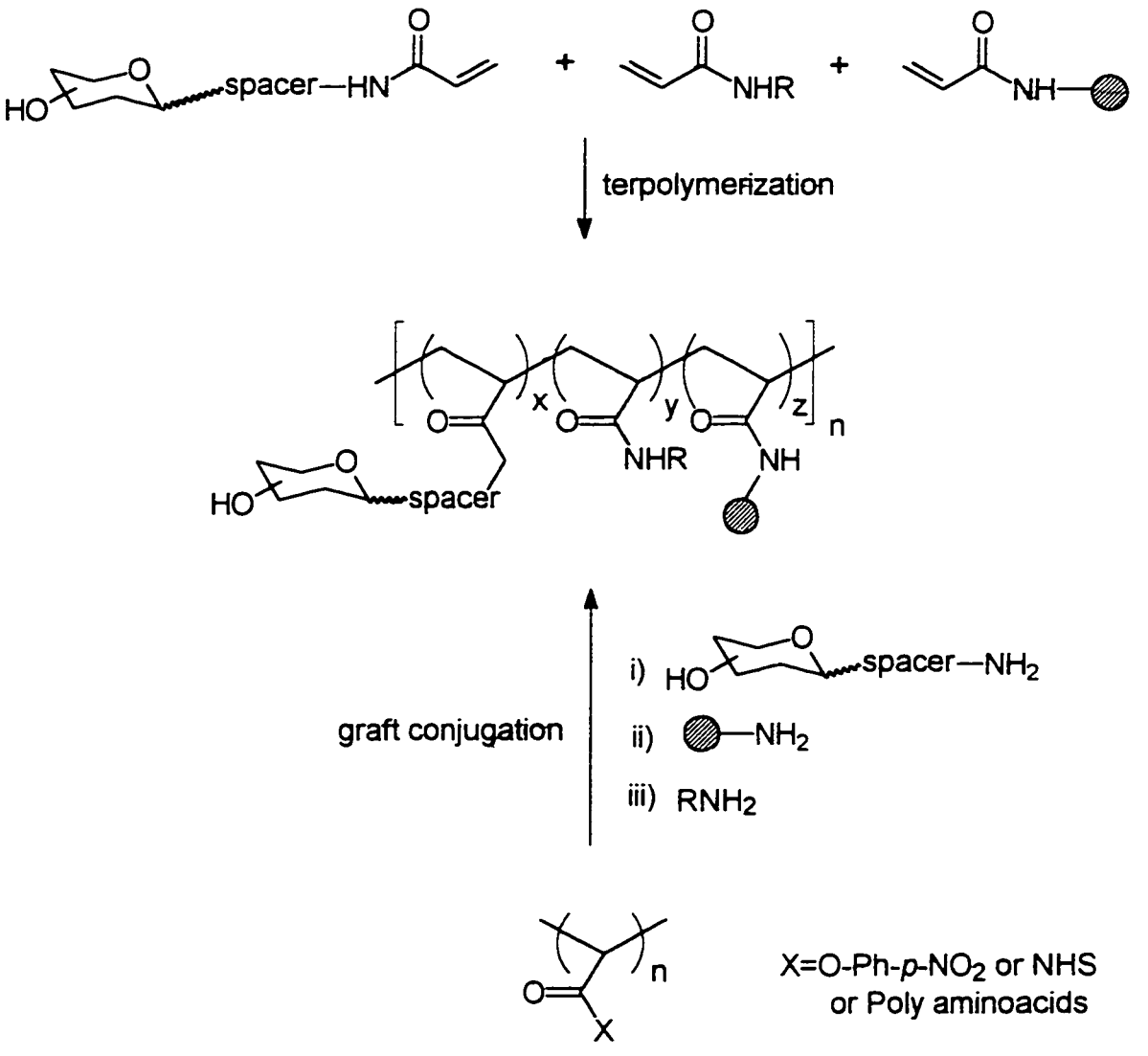
Chapter 7. Synthesis of custom designed glycopolymers bearing acrylamide

7.1. Introduction

As mentioned earlier, the multivalency effect of neoglycoproteins and glycopolymers enhances the binding avidity toward their receptors.[183-b] These synthetic macromolecules bearing carbohydrates have been used as models in vaccines,[213, 214] inhibitors of cell adhesion by virus, bacteria and toxins,[2-d] ligands in affinity chromatography[216] and drug carrier.[217] Furthermore fundamental insight into these carbohydrate-based interactions is dependent upon appropriate synthetic models that take into consideration the multivalent nature of cell surface carbohydrates.

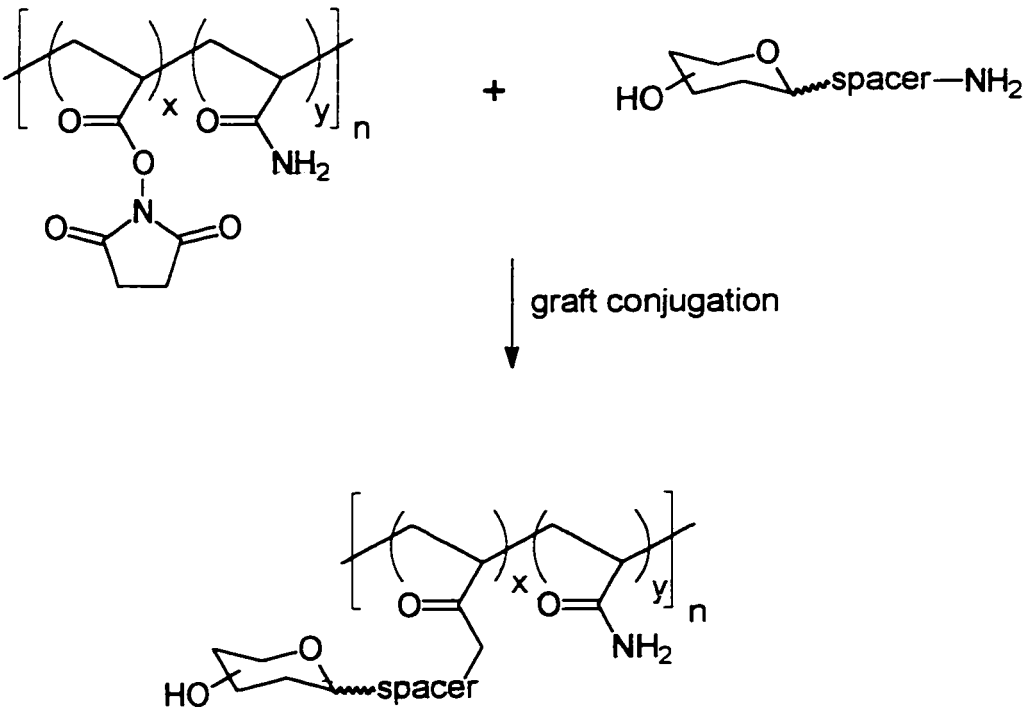
Multivalent carbohydrate containing macromolecules are well defined and easy to prepare in various shapes and orientations (Chapter 4, 5 and 6). Alternatively, the multivalency family of another type of neoglycoconjugate, the glycopolymers, have been reported.[218, 219] Even though these conjugates have their own drawbacks such as heterogeneity of their valency and random distribution of their carbohydrate ligand, they are still potent species as inhibitors or cell targeting agents.[219, 223] As well, they are straightforward to prepare and are useful in various immunoassays by virtue of their repeating units, biological activities and applications. Based on these facts, glycopolymers have been prepared in two ways (Scheme 7-1). In the first way, both carbohydrate monomer and backbone comonomer(s) were co-(ter-) polymerized in aqueous solutions using a radical initiator.[220] In the second way, suitably functionalized carbohydrate haptens were grafted onto preformed polymers bearing reactive functionalities such as poly(p-nitrophenylacrylate)[218, 221] and poly(N-acryloxysucciminde).[192-a, b, 222]

Allyl glycosides have been used as monomers[220-a, 224] to provide useful glycoconjugates. However, their short distance between the polymer backbone and the carbohydrate haptens have prevented strong binding with receptor proteins.



Scheme 7-1. Representation of synthetic strategies for glycopolymers.

To access deeper binding subsites for receptors, the allyl functions were transformed into longer, suitably functionalized aglycons by photo-induced thiol addition to the alkene function (Chapter 3). The monomers with an N-acrylamido function have been prepared and copolymerized with acrylamide to provide various glycopolymers in our laboratory.[219, 220, 223] To extend these concepts, T-antigen containing glycopolymers were synthesized by copolymerization of the monomers and/or graft conjugation of carbohydrate haptens onto preformed polymers (Scheme 7-1 and 7-2).



Scheme 7-2. Preparation of copolyacrylamide bearing carbohydrate hapten.

Furthermore, preactivated polymers based on N-acryloxysuccinimde were prepared in the desired molecular weight distribution and the density of the glycoside incorporated could be obtained either by control of the amount of glycosides or by control of the amount of active ester used as a comonomer component in the copolymers. Based on these results, it is expected that uniform glycosidic density through whole polymer chains is obtained and the discrete block copolymer formation is eliminated. Thus, chemically well defined glycopolymers can be obtained.

As acrylation of an amine functionality provides N-linked acryloyl function equipped with an carbohydrate haptens (Chapter 3), co- (ter-) polymerization of these leads to desirable randomly alternating copolymers where glycoside incorporation is controlled. In addition, these glycosides can be conjugated to proteins by the Michael

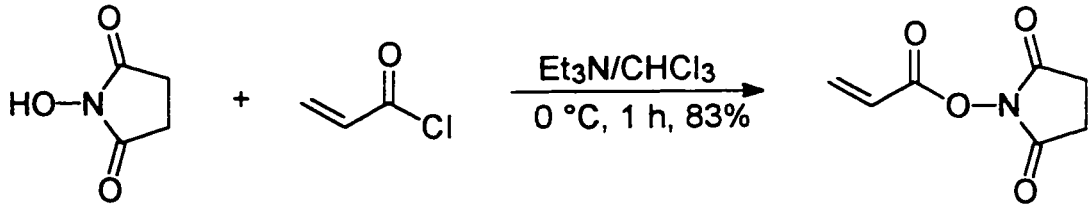
addition of nucleophilic amines to the acrylamide terminus. The labeling agents or effectors were chosen depending on the eventual use of the polymers. For example, biotin bearing glycoconjugates are used with streptavidin or avidin which specifically bind to bicyclic ring system in biotin in immunoassays.

In this chapter, preparation of the structurally modified glycopolymers and neoglycoproteins will be described and their biological activities will also be discussed.

7-2. Preparation of monomers

As described in Chapters 2 and 3, allyl glycosides **10**, **11**, **14**, **19**, **21**, **29**, **31**, and **37** were prepared by the conventional glycosylation methods in good or excellent yields. 3-(2-N-acrylamidoethylthio)propyl glycosides **51** and **52** were also prepared in a straightforward manner from the parent amines under the mild conditions.

The N-hydroxysuccinimide active ester group is readily introduced into the polymers[225] and has good chemical stability and physical properties, such as, it is an attractive monomer to use in the preparation of a water-soluble copolymer of acrylamide and N-acryloxysuccinimide. Scheme 7-3 shows the monomer preparation.



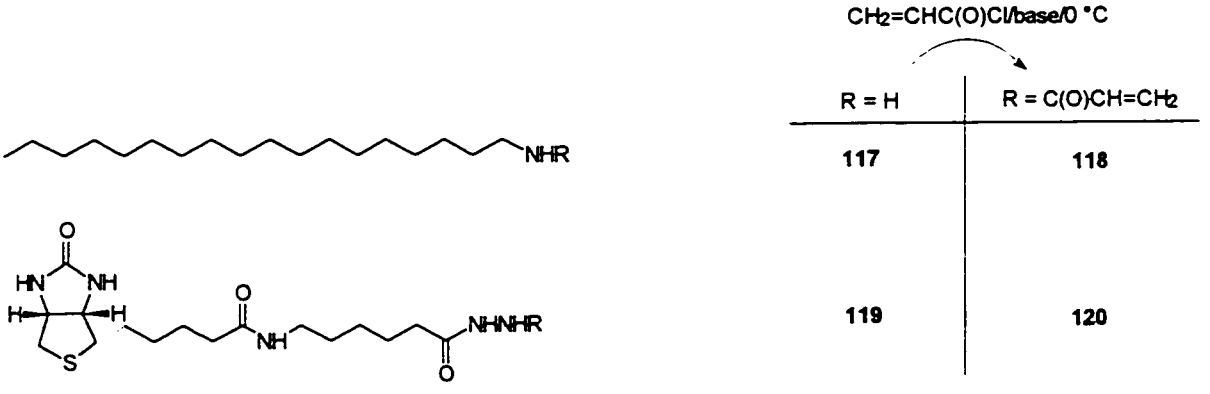
114 **116**

Scheme 7-3. Synthesis of N-acryloxysuccinimide **116.**

Acryloyl chloride was added to N-hydroxysuccinimide (NHS) and Et_3N in $CHCl_3$ at 0 °C and reacted for 1 h. After brief evaporation of the solvent in the presence of 2,6-di-*t*-butyl-4-methylphenol as a polymerization inhibitor, the remaining solution was mixed with

EtOAc/Hex (1/8) and crystallized in the refrigerator. Successive washing of the crystals formed with EtOAc/Hex (1/4 and 1/9) and hexane and drying under vacuum provided white crystals in 87% yield. No polymerization was detected and the typical chemical shift of the acryl functional group was observed between 6.80 and 6.25 ppm by ^{1}H-NMR.

The long hydrocarbon chain containing stearyl amine **117** was acryloylated in the same manner as for the preparation of **116** and was recrystallised from hot ethanol to give N-acryloylated stearyl amine device **118** in 96% yield. The effector **118** was incorporated in order to provide improved lipophilic properties which make glycopolymers as more effective coating antigens in bioassays. Biotin was chosen to make heterobifunctional glycopolymers since many useful streptavidin or avidin-labeled conjugates are commercially available and the affinity of these proteins to biotin is very strong ($K_D = 10^{-15}$).[226] Acryloylation of biotinamidocaproylhydrazide **119** was performed in the presence of anion (OH^-) exchange resin and gave **120** in 78% yield.



Scheme 7-4. N-acryloylation of effectors.

The N-hydroxysuccinimide active ester group showed both high reactivity and selectivity toward amine nucleophile. Alkylamine effectors **121~123** were chosen to enhance the hydrophobicity of the glycopolymers. Commercially available effector monomers, biocytin,

124 and fluorescein **125** with amine terminus were also chosen as biochemical probes (Fig. 7-1).

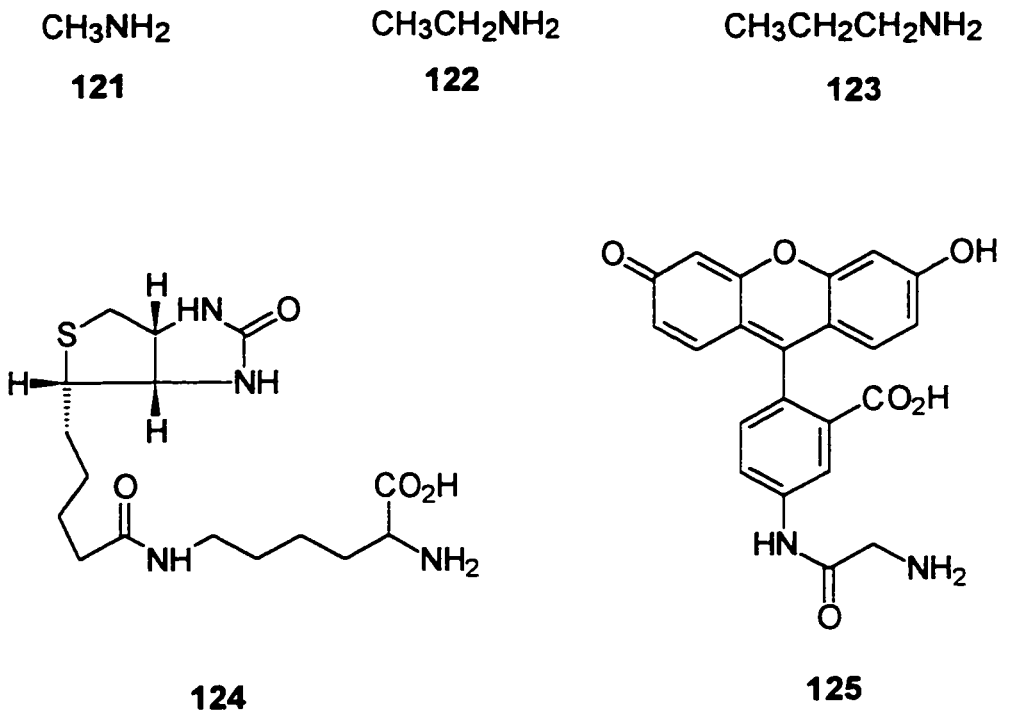


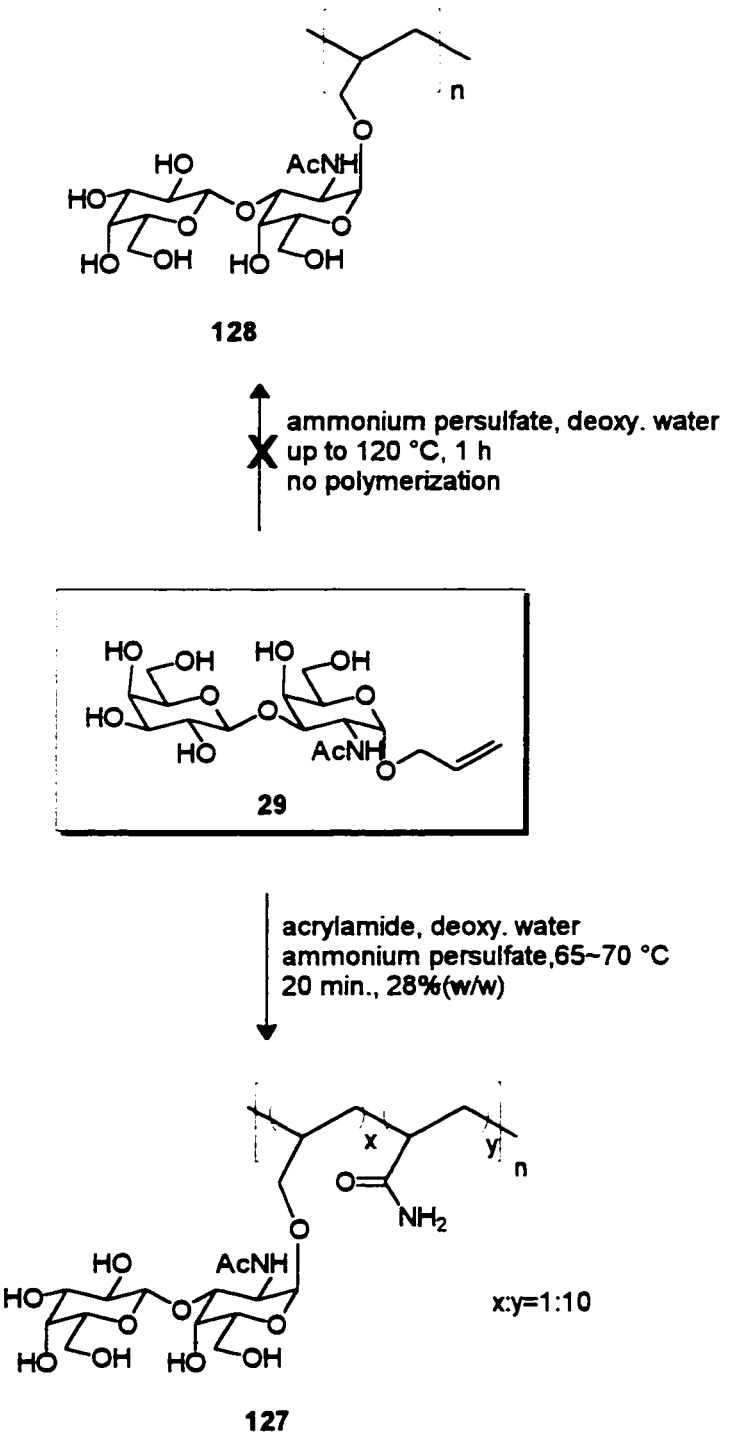
Fig. 7-1. Structures of effector monomers.

7.3. Glycopolymers by terpolymerization

7.3.1. Glycopolymers from N-acrylamido glycosides

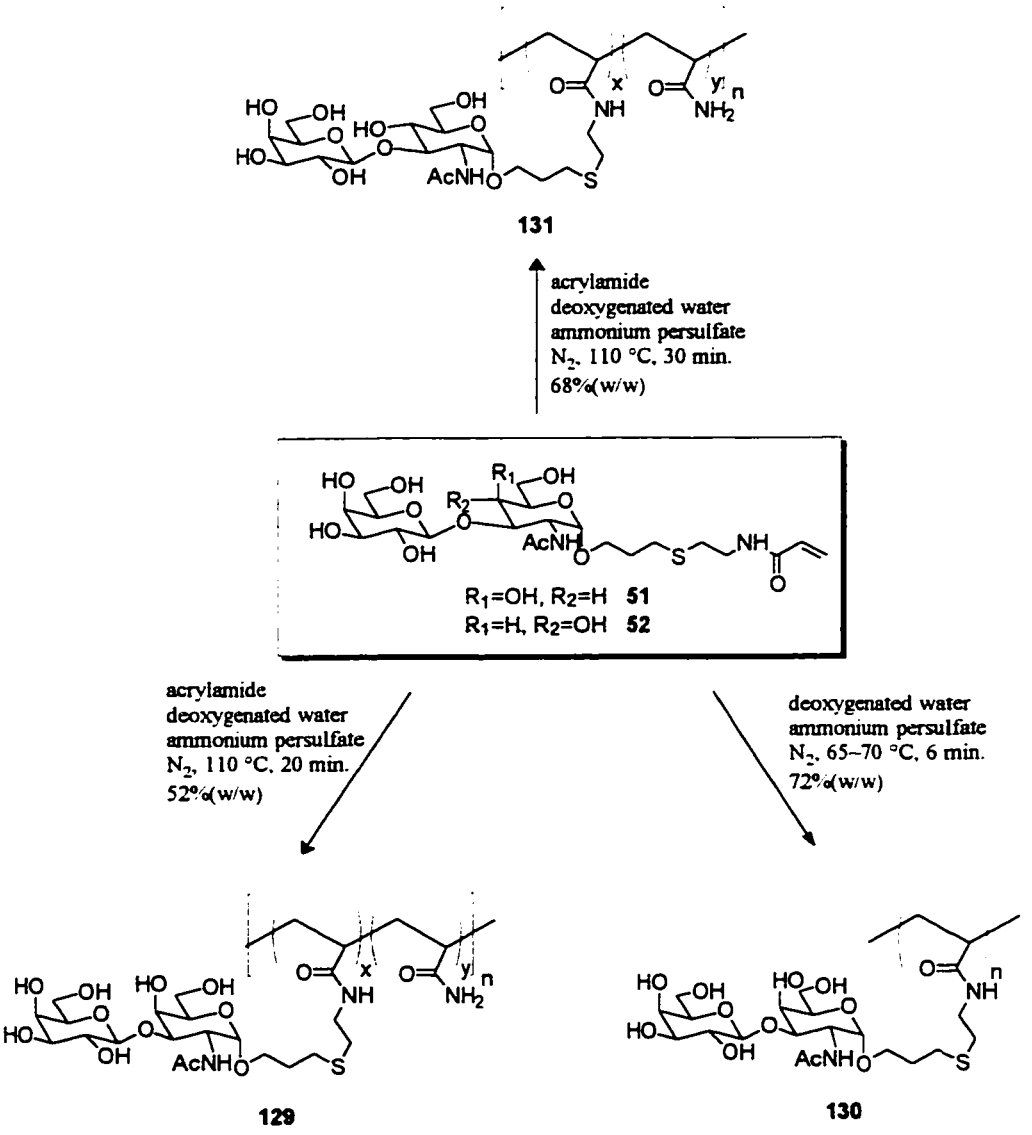
Allyl glycoside **29** was polymerized with acrylamide **126** as a comonomer (molar ratio of 1:10) in deoxygenated water in the presence of a catalytic amount of ammonium persulfate, $(NH_4)_2 S_2O_8$, [227] to afford glycopolymer **127** in 28% (w/w) after dialysis with a molecular weight cut-off of 12 kDa tubing against distilled water (Scheme 7-5). However, under the same reaction condition, homopolymerization of **29** to give **128** failed, even at longer reaction times and at higher temperature. The reason of the failure of **128** was that short spacers between the polymer backbone and the carbohydrate hapten can not compromise the steric hindrance between the monomer units and, as a result, subsequent monomer addition for the chain growth is difficult. Copolymerization with acrylamide facilitated the synthesis and gave **127** with 1/10 molar incorporation of **29/126** as judged by ^{1}H-NMR between anomeric signals at 4.98 ppm and the methine proton in the polymer backbone at 2.05~2.41 ppm. The copolymerization of **29** and **126** may lead to slightly blocked copolymer due to the lower reactivity of **29** thus leading to lower glycoside monomer incorporation. There was still a severe steric effect hampering the polymerization process, even in the case of the sterically less bulky comonomer, **126**. To eliminate such unfavorable interactions, allyl aglycon was elongated to 3-(2-N-acrylamidoethylthio)propyl residue. The elongated aglycon containing glycosides, **51** and **52**, were then polymerized in a similar manner (Scheme 7-6). Each polymer was dialyzed using molecular weight 12 kDa cut-off tubing against distilled water. The yields of the glycopolymers, **129**, **130** and **131** were 52, 72 and 68% (w/w), respectively. The carbohydrate to acrylamide incorporation ratios were 1/11 ~ 12 for **129** and 1/11 for **131**. Glycopolymers prepared from sulfide spacer bridged glycosyl acrylamides offer a considerable length of flexible spacer and homogeneously distributed copolymers rather than the block type ones. In addition, the monomer incorporation ratios within the glycopolymers were coincident with the monomers loaded in the polymerization vassel.

Thus, in both allyl and sulfide bridged spacer-glycosides, the sugar densities can be controlled at will.



Scheme 7-5. Polymerization of allyl T-antigen monomer **29.**

The molecular weight of the polymers **129** and **131** were estimated approximately by comparing the relative rate of diffusion of the polymers and peanut lectin (120 kDa) in a double radial immunodiffusion assay. Since polymers traveled a shorter distance than the lectin, the molecular weights of the polymers were thought to be higher than that of the lectin.



Scheme 7-6. Polymerization of spacer modified glycosides.

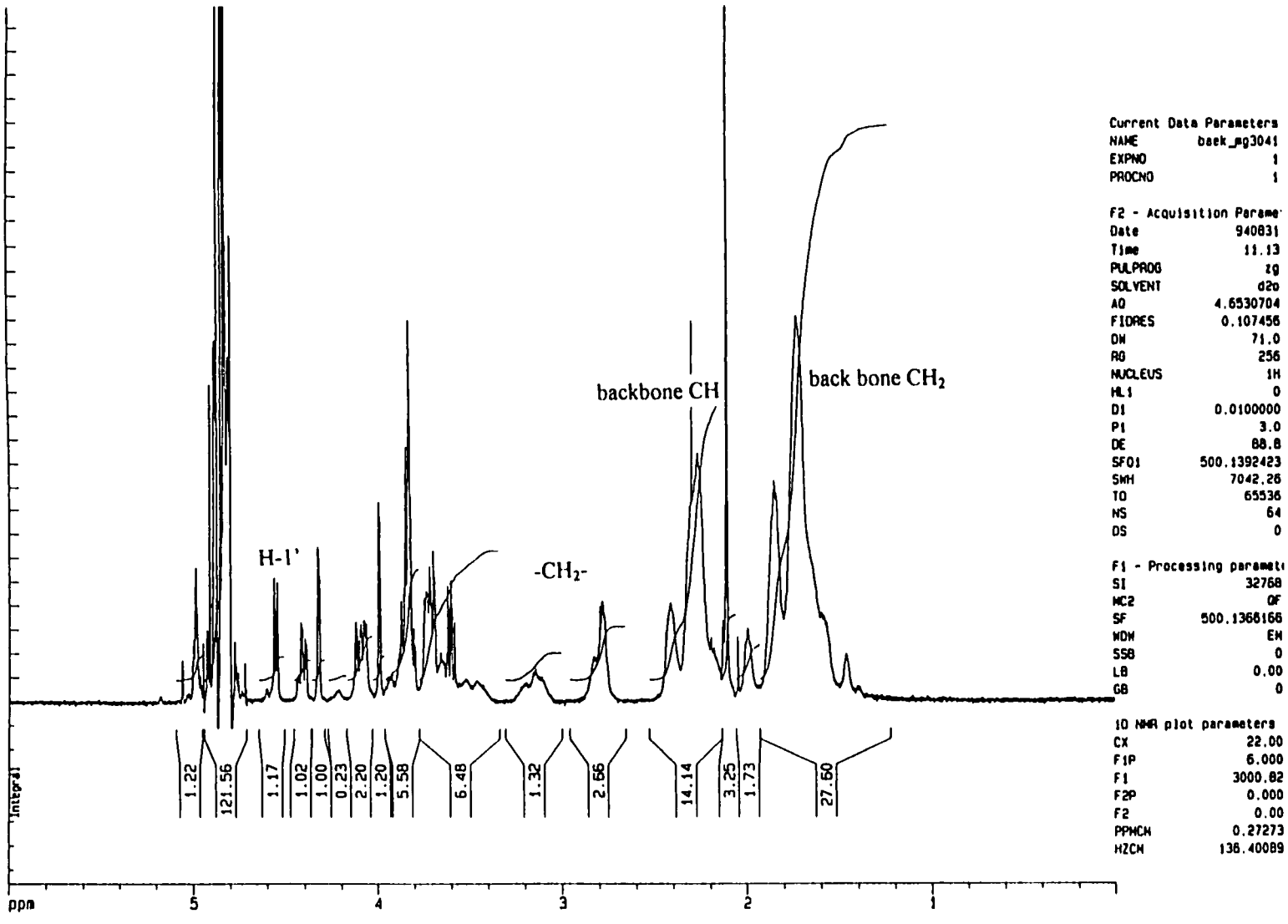
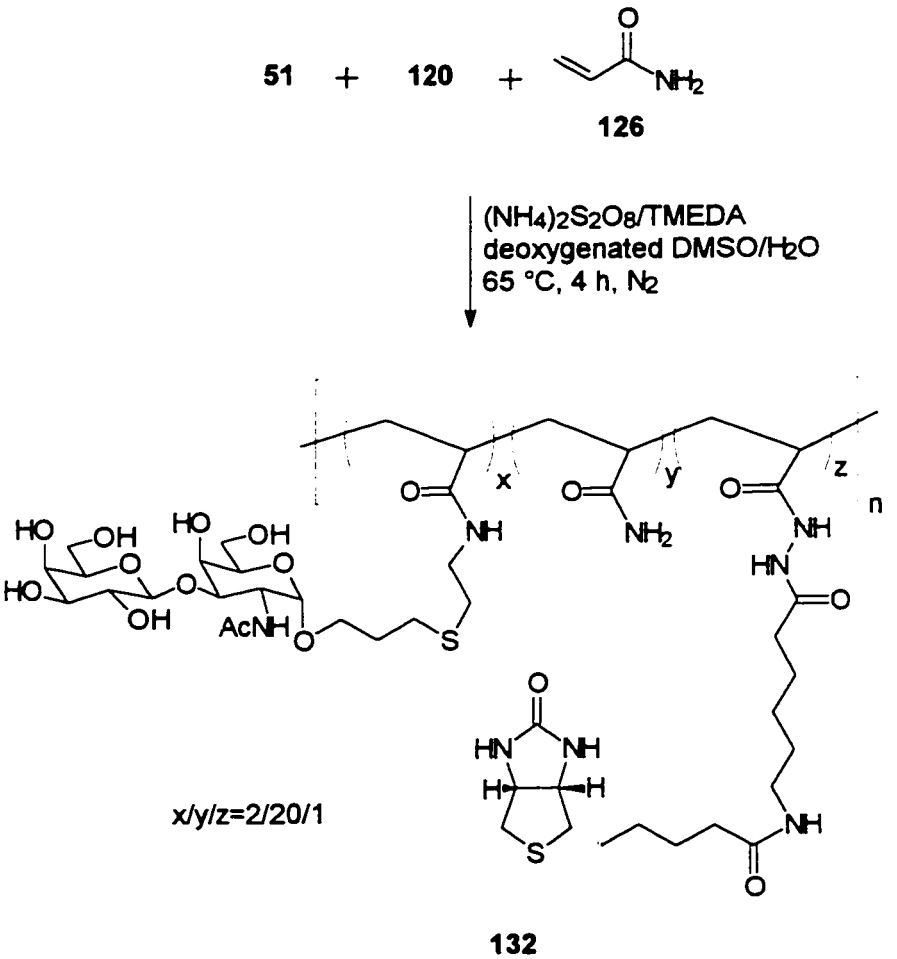


Fig. 7-2. ^{1}H-NMR spectrum (500 MHz, D_2O) of T-antigen-spacer CPA **129.**

7.3.2. Biotinylated glycopolymers

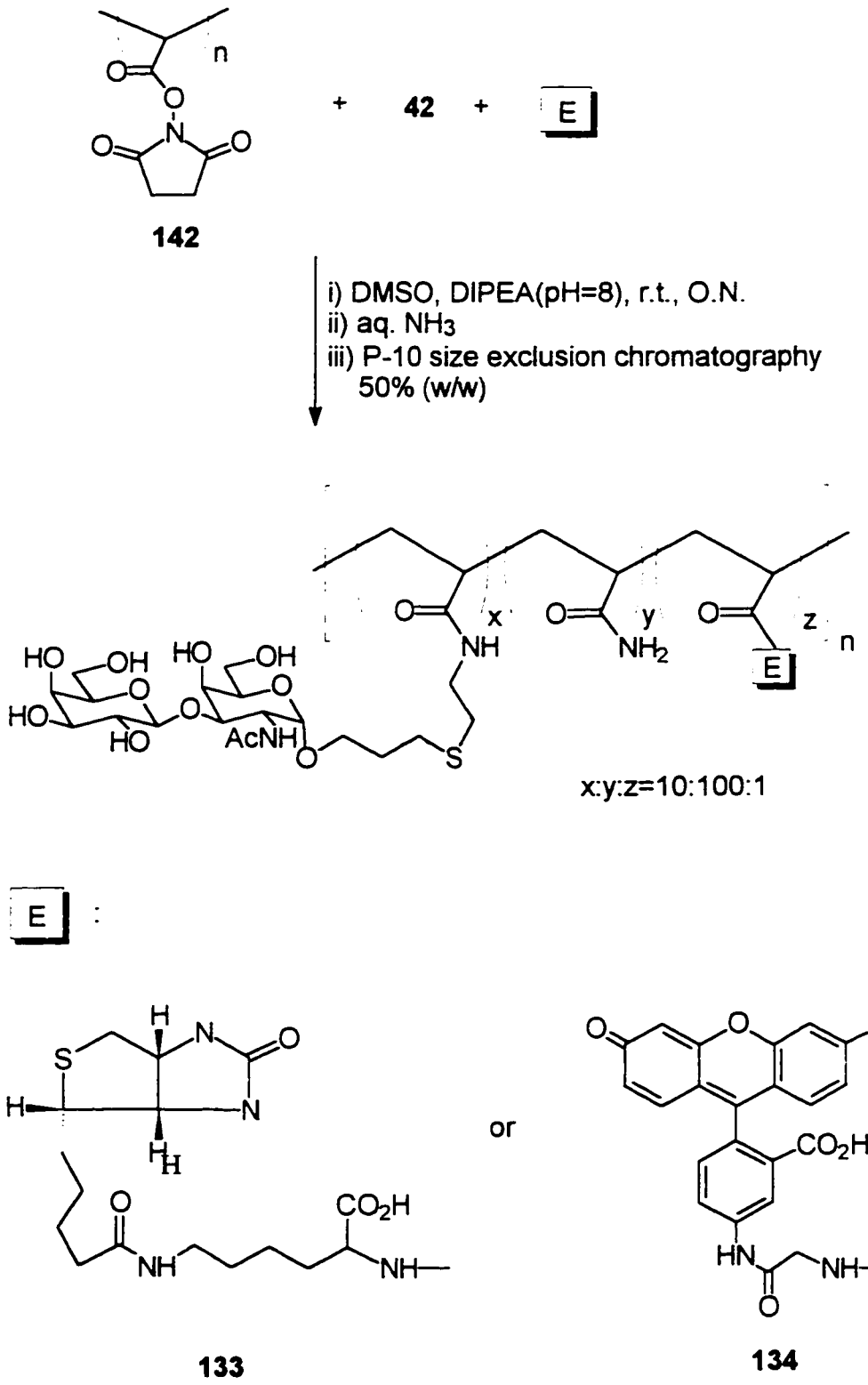
Glyco(ter)polymers containing biotin were synthesized with acryloylated spacer-T-antigen hapten (Scheme 7-7). For such a terpolymerization, the addition of DMSO was necessary to dissolve the biotin monomer, **120** and this did not affect the terpolymerization when performed at room temperature after initiation with ammonium persulfate and TMEDA.



Scheme 7-7. Synthesis of biotin/T-antigen-spacer-CPA **132** by terpolymerization.

T-antigen/biotin containing glyco(ter)polymer **132**, was prepared in 98% yield (molecular weight ≥ 12 kDa) by terpolymerizing acrylamide with 3-(2-N-acrylamidoethylthio)propyl-T-antigen **51**, and acryloylated biotin monomer **120**. The polymerization was performed in the mixed solvent (H_2O/DMSO, 2/1) for 4 h. Other effector labeled glycopolymers were also prepared with the preactivated poly(N-acryloxysuccinimide) (**140~142**), amine terminated T-antigen **42**, and effectors such as fluorescein, **134**. The synthesis of these homopolymers will be described in the following section. Poly(N-acryloxysuccinimide, PNS) was dissolved in DMSO and the solution containing **42** and biocytin, was combined and stirred for 2 days at room temperature in the presence of DIPEA. An excess of aqueous NH_3 was then added directly to quench the remaining active ester function. After the solution was lyophilized, the residue was dissolved in minimum amount of water and purified by size exclusion chromatography (P-10) or dialyzed with molecular weight 2,000 cut-off tubing against water for 3 days to afford **133** in 47% yield (w/w). The molar ratio of each component in **132** (1/2/20) and **133** (1/10/100) of biotin/T-antigen/acrylamide was confirmed by comparing the biotinyl proton at 4.7 ppm, the anomeric proton at 4.9 ppm and the methine or methylene proton at 1~2.3 ppm, all of which were identical to the feeding formulations. The presence of biotin in the polymer was confirmed by treatment with 0.1% 4-dimethylamino cinnamaldehyde in acidified ethanol. After brief heating, the presence of biotin was indicated by the appearance of a pink color. The analogue glycopolymer **129** was used as a negative control and did not develop any color change.

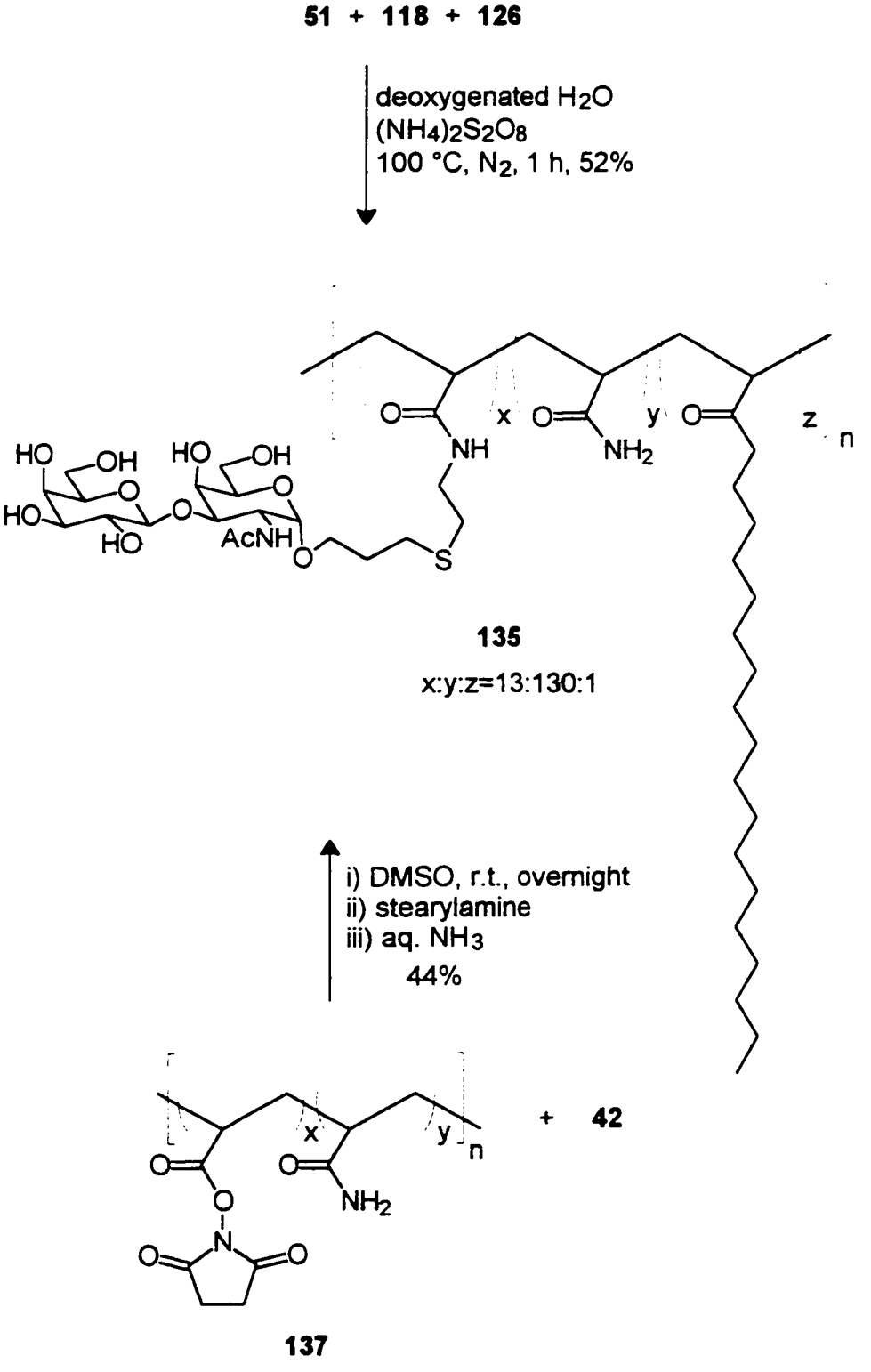
In a similar manner, fluorescein labeled terpolymer was also prepared to give a yellow spongy solid **134** in 56% yield (w/w). The ratio of acrylamide/T-antigen/fluorescein was determined by comparision with the methylene spacer of aglycon at 1.93 ppm, the distinct proton of fluorescein at 8.05 ppm and methylene or methine at 1~2.3 ppm and was approximately 100/10/1.



Scheme 7-8. Synthesis of effector containing terpolymers from PNS.

7.3.3. Stearylated glycopolymers

Copolyacrylamide glycopolymers have been proven to be excellent tools for coating antigens in solid phase ELISA/ELLA studies. As stated in the Chapter 6, the mechanism of adsorption of glycoconjugates to the wells of microtiter plates is by simple hydrophobic interaction with the well surface. However, a copolyacrylamide such as **129** is rather hydrophilic and exhibits excellent water solubility. Thus, an attempt was made to increase the lipophilicity of polymers by using the corresponding comonomer incorporated terpolymerization strategy. Stearyl amine **117** or N-acryloylated derivative **118** were chosen as effector monomers and used in two ways. (Scheme 7-9). First, N-acryloylated stearylated amine was terpolymerized with **51** and **126** to give the stearylated glyco(ter)polymer, staeryl/T-antigen-spacer-copolyacrylamide (CPA) **135** in 52% (w/w) yield. The reaction was performed at 100 °C for 1 h with ammonium persulfate as a radical initiator. Second, preformed poly(acrylamido-co-acryloxysuccinimide), mole ratio of 10/1) **137** was used as a polymeric support and conjugated to **42** and **117** to give the same glycopolymer **135** in 44% (w/w) yield. Grafting of monomeric units bearing a terminal amine was performed in DMSO. Preactivated copolymer **137** in DMSO was added to a solution of **42** in DMSO and the reaction mixture was stirred overnight at room temperature. **117** in *iso*-propanol was directly added and the reaction continued for another 6 h. The resulting solution was dialyzed with molecular weight 2,000 cut-off tubing against water for 3 days and lyophilized to give **135**. The ratio of components was determined in both cases by ^{1}H-NMR. A conservative estimate of 13/1 ratio of T-antigen/stearyl was made on the basis of the stearyl terminal methyl signal at 0.83 ppm as well as by comparing the integration of the backbone methylene signals near 1.7 ppm and assigning the increased integration area of the methylene signal to the stearyl effector which appears in this region. The ratio of acrylamide/T-antigen/stearyl was 130/13/1.

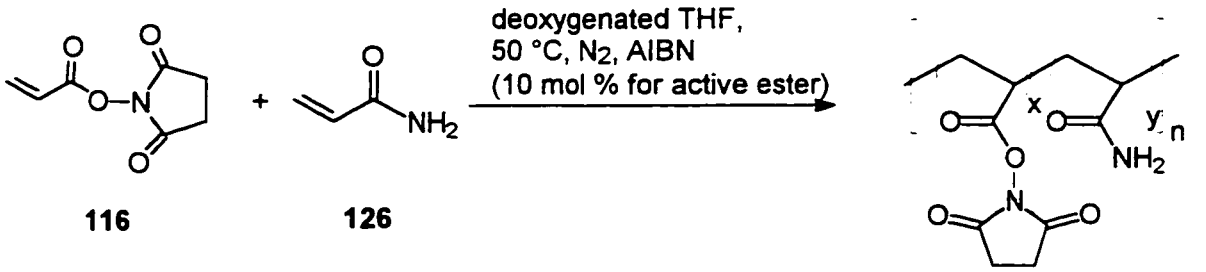


Scheme 7-9. Synthesis of stearyl/T-antigen-spacer-copolyacrylamide.

7.4. Preparation of poly(N-acryloxysuccinimide)

7.4.1. Synthesis of poly(acrylamide-co-N-acrylsuccinimide)

Preformed, water-soluble copolymers of acrylamide **126** and N-acryloxysuccinimide **116**, with varying monomer ratios were synthesized by free-radical polymerization in dry THF using thermal initiation with azo-bis(isobutyronitrile) (AIBN)[228, 229] (Scheme 7-10).



116 **126**

116 : 126 (mole ratio)	polymer	yield (%, w/w)
1 : 21	**136**	quantitative
1 : 11	**137**	quantitative
1 : 5.3	**138**	91
1 : 2.8	**139**	82

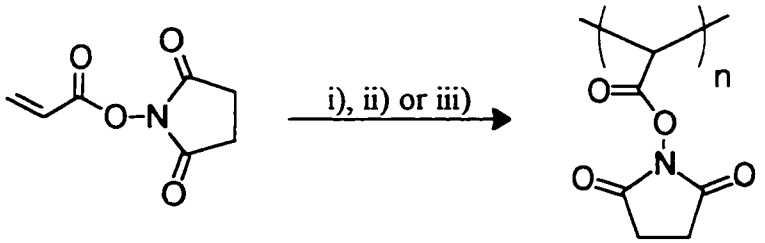
Scheme 7-10. Synthesis of poly(acrylamide-co-N-hydroxysuccinimide).

This method provided low molecular weight polymers which dissolved quickly and generated aqueous solutions of practical viscosities.

Polymers **116** and **126** with varying molar ratios, 1/20, 1/10, 1/5, 1/2.5, were dissolved in dry THF and degassed with N_2-bubbling for 30 minutes. Radical initiator was added and the solution was stirred overnight at 50 °C under N_2 atmosphere. The resulting white precipitate was filtered, washed three times with dry THF and dried under vacuum to afford white fluffy solid in quantitative~82% (w/w) yields for **136~139**. The ratio of the repeating units in each polymer was confirmed by comparision of the integration of ^{1}H-NMR signals between the two methylene units in the succinimide functional group at 2.8 ppm and the methylene unit in the polymer backbone at 1.5 ppm. Estimated ratios of the monomers were 1/20.6, 1/11. 1/5.3 and 1/2.8 for **136~139**, respectively. They showed stability at room temperature under dry conditions. When these polymers were exposed to the air, the active ester group was hydrolyzed as judged by TLC.

7.4.2. Synthesis of poly(N-acryloxysuccinimide) by homopolymerization

Poly(N-hydroxysuccinimide), a polymer preactivated by incorporation of active ester groups has been prepared in several molecular weight distributions and conjugated to sialic acid containing inhibitors of hemagglutination of influenza virus X-31.[230] On the basis of this facts, modified reaction conditions were developed to obtain homopolymer with molecular weight ranges of 28~45 kDa, **141** and **142** (Scheme 7-11). First, monomer **116** in dry THF (1 g in 100 mL) was degassed by N_2-bubbling and AIBN (10 mole%, 0.1 g) was added. The resulting solution was stirred at 55 °C for 1 day under N_2 atmosphere. After filtration and washing with dry THF, only 0.1 g (10 % (w/w) yield) of **140** resulted. Second, the concentration of the reaction solution was fixed to 1 M in benzene. After degassing, AIBN (3 %, w/w) was added and the resulting solution was stirred for 2 days at 70~75 °C under N_2 atmosphere. The precipitate was filtered, washed and dried to give **141** in 86% yield. Third, the reaction conditions employed were the same as those mentioned above except that a different AIBN content was used (1.5%, w/w). **142** was obtained in 85% (w/w) yield.



116

Scheme 7-11. Preparation of poly(N-acryloxysuccinimide) **140~142** i) 10 mol%, AIBN/degassed THF, N_2, 55 °C (**140**), ii) 3% w/w, AIBN/dry and degassed benzene, N_2, 70~75 °C (**141**), iii) 1.5% w/w, AIBN/dry and degassed benzene, N_2, 70~75 °C (**142**).

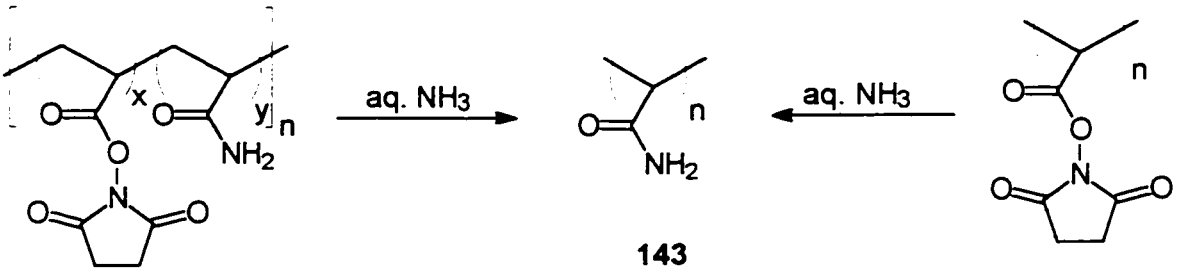
7.4.3. Molecular weight determination

A dilute polymer solution shows a considerably higher viscosity than pure solvent because of large differences in size between the polymer and the solvent molecules. The magnitude of the viscosity increase is related to the dimensions of the polymer molecules in solution. In other words, intrinsic viscosity, which represents the capacity of a polymer to enhance viscosity, increases with molecular weight. As such, viscosity measurements afford a measure of molecular weight. The quantities required and terminology used in dilute solution viscosity are summarized in Table 7-1.[231] Determination of molecular weight was performed using on Ostwald viscometer reading relative to water for series of poly(acrylamide-co-N-acryloxysuccinimide) derivatives **136~139** and poly(N-acryloxysuccinimide) **140~142**. Due to the poor stability of these polymers, they were manipulated to form polyacrylamide by quenching with excess of aqueous ammonia (Scheme 7-12). When aminolysis was completed, the resulting solution was lyophilized to dryness, then thoroughly washed with dry THF to remove N-hydroxysuccinimide (NHS) and to give pure polyacrylamide **143**.

Table 7-1. Quantities and terminology used in dilute solution viscosity.

Common name	IUPAC name	Symbols and definition[a]
Relative viscosity	Viscosity ratio	$\eta_r=\eta/h_o$
Specific viscosity	-	$\eta_{sp}=\eta_r-1$
Reduced viscosity	Viscosity number	$\eta_{red}=\eta_{sp}/C$
Inherent viscosity	Logarithmic viscosity number	$\eta_{inh}=\ln(\eta_r)/C$
Intrinsic viscosity	Limiting viscosity number	$[\eta]_{int} = \lim_{c\to 0}(\eta_{red})$

a: η_o: viscosity of solvent, η: viscosity of polymer solution of concentration C (mass per unit volume)



143

Scheme 7-12. Aminolysis of active ester polymers.

Polymer **143** was used to measure the viscosity. Molecular weight measurement was illustrated using **142** as a specimen. After measuring the flow time of the solution, intrinsic and reduced viscosities (equation 7-1 and 7-2) were calculated and extrapolated to infinite dilution to give the limiting viscosity number [η].

$$[\eta]_{int} = \lim_{c \to 0} (\eta_{red}) \qquad (7\text{-}1)$$

$$\eta_{red} = \eta_{sp}/C = \frac{\frac{\eta}{\eta_o} - 1}{C} \qquad (7\text{-}2)$$

$[\eta]$ of polymer is related to its viscosity-average molar mass, M_v, by the *Mark-Houwink* equation (7-3).

$$[\eta] = KM_v^a \qquad (7.3)$$

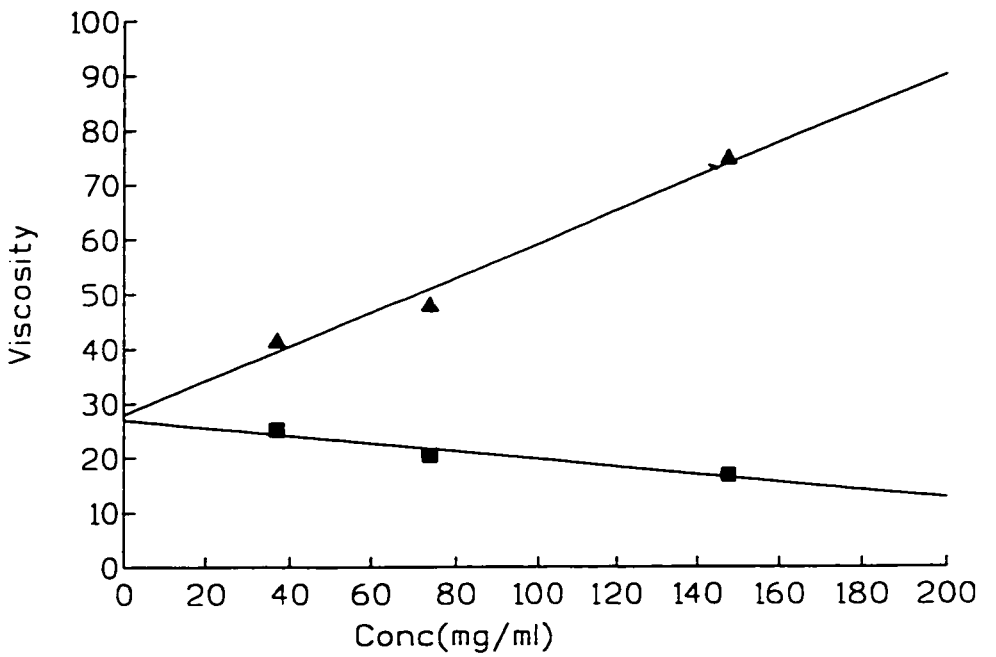


Fig. 7-3. Evaluation of $[\eta]$ for solution in water of polyacrylamide from **142** at 25°C using a dual *Huggins-Kraemer* plot.

where K and a are the characteristic constants for a given polymer/solvent/temperature system. For a simple polyacrylamide in a good solvent such as water, values of 5.6×10^{-3} and 0.8 were used for K and a, respectively as suggested by Molyneux.[232] From equation 7-3, the molecular weight (M_v) of **142** was determined to be 42.1 kDa. McNaughton *et al.*[233] proposed different values of K and a, namely 6.8×10^{-4} and 0.66 ± 0.05. Table 7-2 summarizes the molecular weights of the polymers.

Table 7-2. Determination of molecular weight of polymers.

polymer	Intrinsic ratio[a]	Limiting viscosity	M_v[b] (KDa)	M_n[c] (KDa)
136	1/20	11.54	13.9 (82)	2.5 (15)
137	1/11	8.44	9.4 (56)	1.5 (9)
138	1/5.3	6.85	7.2 (43)	1.1 (7)
139	1/2.8	N/A	-	-
140	-	N/A	-	-
141	-	20.40	28.3 (168)	5.6 (33)
142	-	28.03	42.1 (250)	9.2 (55)

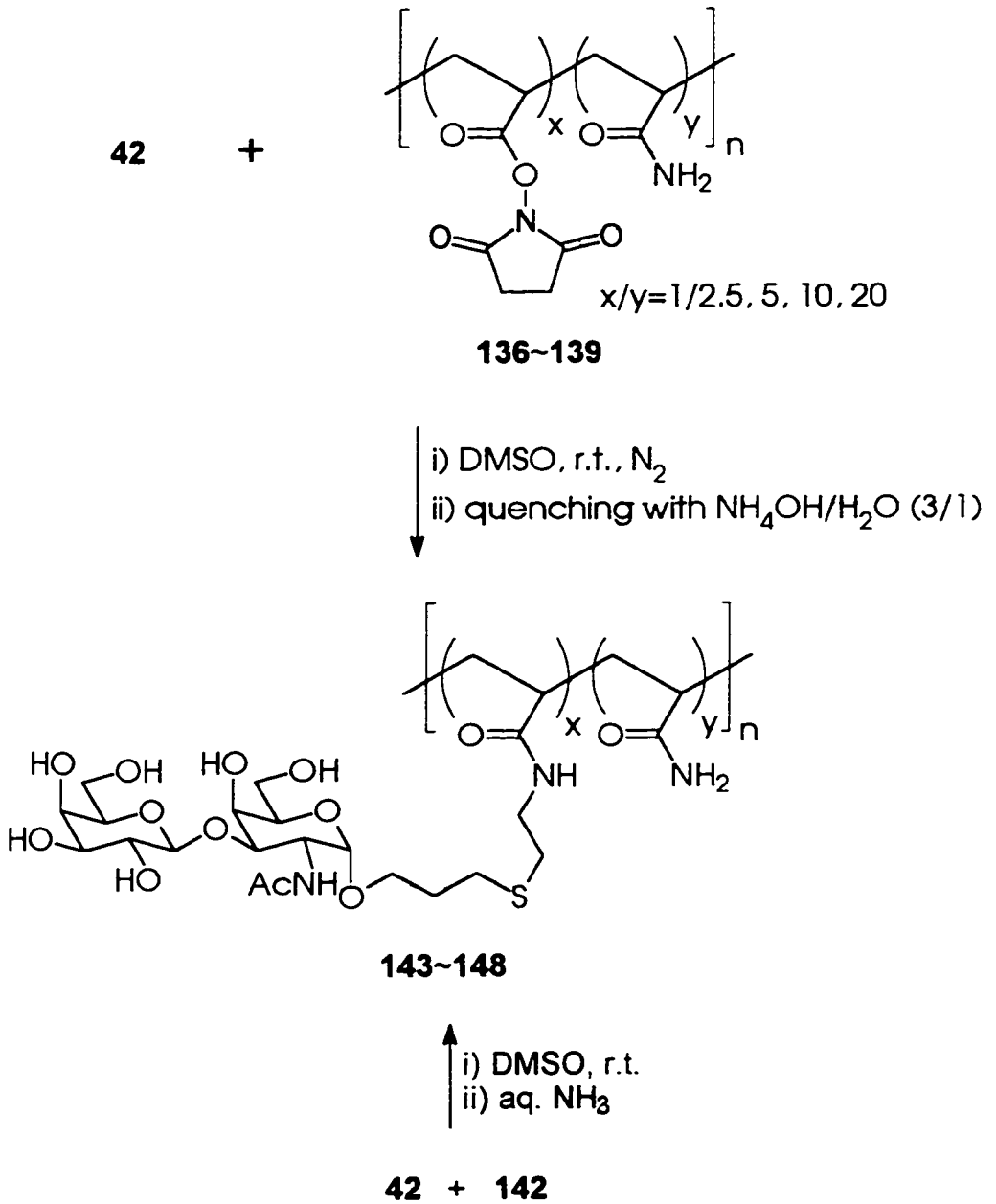
a: The monomer ratio was acrylamide vs active ester
b: Molineux relationship, $[\eta] = 5.6 \times 10\text{-}3 M_v^{0.8}$
c: McNaughton relationship, $[\eta] = 6.8 \times 10\text{-}4 M_n^{0.66}$
b,c: Parenthesis values refer to degree of polymerization.

7.5. Graft conjugation of carbohydrate hapten to poly(N-acryloxysuccinimide)

7.5.1. Conjugation of 3-(2-aminoethylthio)propyl T-antigen

The preparation of preactivated polymers is synthetically and mechanistically more attractive than copolymerization for several reasons. Firstly, The characteristics of the polymer backbone, molecular weight and polydispersity are kept constant because the same batch of polymers is used for every experiment. Secondly, homogeneous distributions of carbohydrate haptens along the polymer chain are obtained, thus carbohydrate density is constant for all of the polymers. Thirdly, preactivation is more convenient synthetically than copolymerization because the construction of many different polymers does not require synthesizing and characterizing different acrylamide monomers.

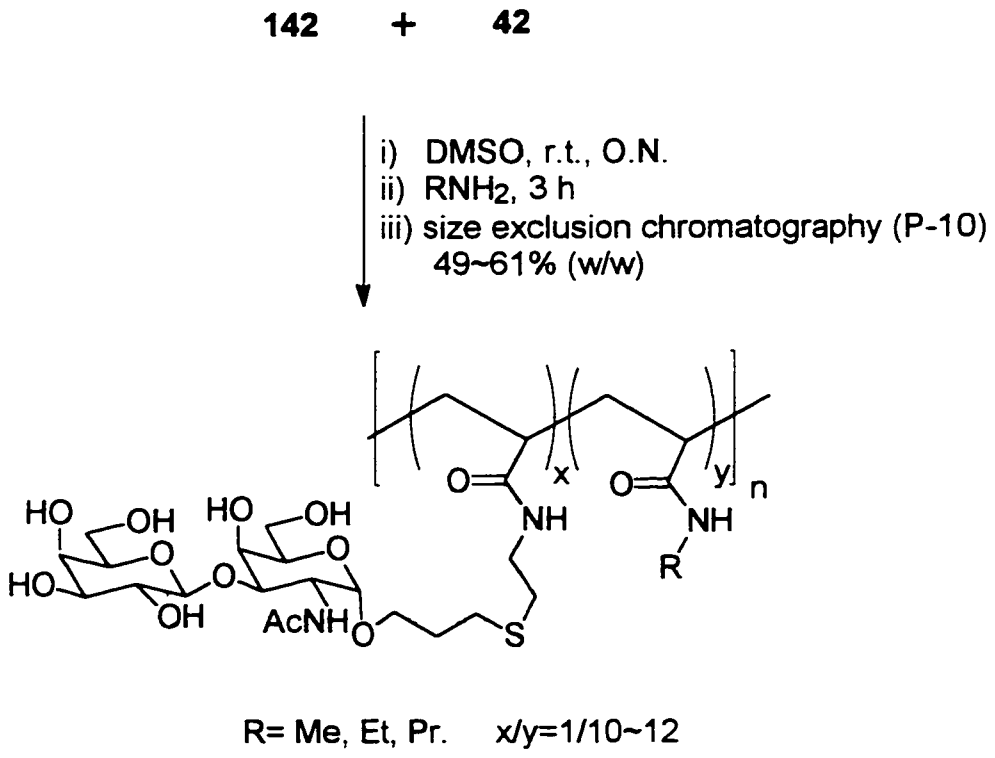
A solution of poly(acrylamide-co-N-acryloxysuccinimide) **136~139**, in DMSO was added to a solution of **42** and the resulting mixture was stirred overnight. Excess of active ester in the polymer backbone was quenched by ammonium hydroxide for 2 h, then lyophilized to dryness. After washing with dry THF, the residue was dried under vacuum to give ivory colored fluffy powder in 66, 97, 69 and 84% (w/w) for **143**, **144**, **145** and **146**, respectively. The carbohydrate content was confirmed by ^{1}H-NMR according to the anomeric proton at 4.97 ppm and the two methylene units of the succinimide group at 1.5 ppm. The incorporated ratios of T-antigen to acrylamide comonomer were 1/21.3, 1/12.6, 1/6.5, and 1/3.2 for **143**, **144**, **145** and **146**, respectively. These ratios were found to be approximately coincident with the ratio of compounds loaded into the reaction vessel before reaction. In a similar manner **147** and **148** were prepared from poly(N-acryloxysuccinimide) **142**, in 75 and 43% (w/w) yields, respectively. The ratio of carbohydrate unit relative to acrylamide was 1/11 for **147** and 1/11.8 for **148**.



Scheme 7-13. Synthesis of T-antigen-spacer-CPA from the active ester-polymers.

7.5.2. Preparation of water soluble glycopolymers quenched by alkylamine.

To increase the lipophilicity of the glycopolymers, another attempt was made by modifying the polymer backbone by increasing its lipophilic structure using methyl- (**121**), ethyl- (**122**) and propylamine (**123**) as quenching materials (Scheme 7-14).



Scheme 7-14. Preparation of lipophilic T-antigen glycopolymers, **149~151**

After substitution of the active ester with **42**, quenching with alkylamine and dialysis or size exclusion chromatography (P-10), alkyl amidated glycopolymers **149** (methylamine), **150** (ethylamine) and **151** (propylamine) were obtained in 49, 53 and 61% (w/w) yields, respectively. The carbohydrate content relative to the comonomer was 1/1~1/12. During the quenching process, an increase in the size of the alkyl amine employed resulted in an increased solution viscosity due to the formation of the high molecular weight polymer.

The binding ability of these polymers to the wells of the microtiter plates was examined by the amount of tritium (^{3}H) labeled Glucosamine (GlcNAc) bound. The test was performed as follows: The same amount of glycopolymers **127**, **133**, **148**, **149**, **150** and **151** in PBS were added and incubated. After washing the wells, tritium labeled GlcNAc was reacted with the coated polymers using UDP-GlcNAc transferase. After the same procedure, the degree of radioactive carbohydrate bound to the polymer was measured. The results are summarized in Table 7-3.

Table 7-3. Measurement of the amount of tritium (^{3}H) labeled GlcNAc bound to coated T-antigen polymers.

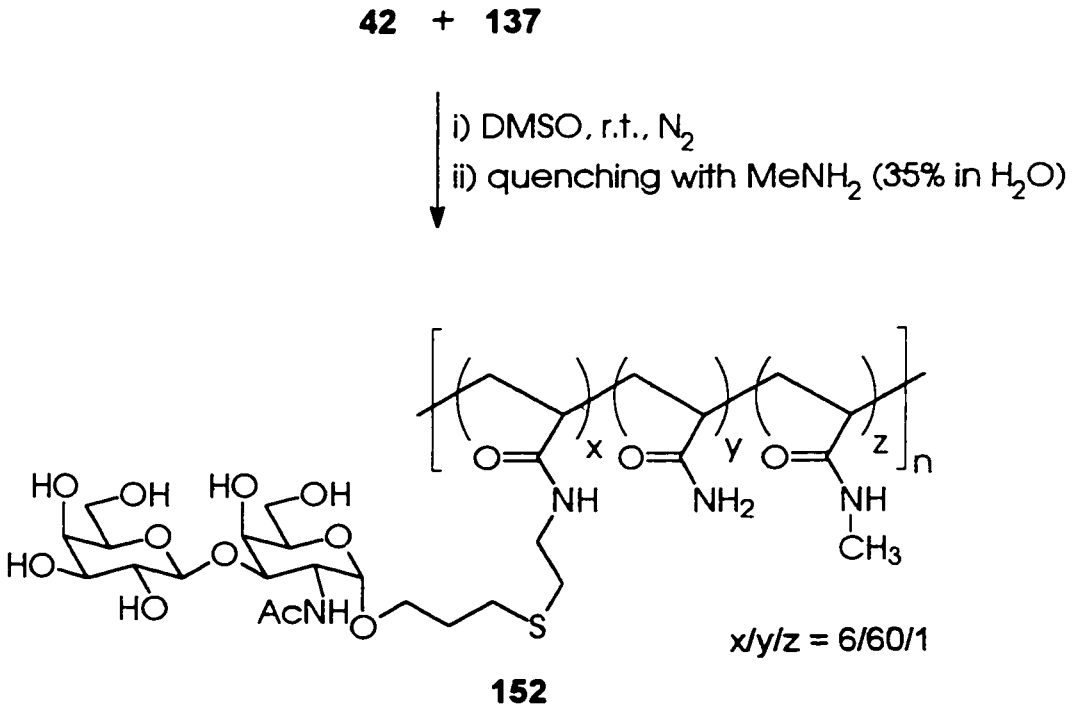
Compound	^{3}H-GlcNAc (cpm)[a]	SD (%)[b]
127	436	18
133	1531	11
148	2675	9
149	3707	6
150	4157	10
151	5275	9

a: cpm stands for count per minute.
b: SD stands for standard deviation.

As lipophilicity increases, the amount of ^{3}H-GlcNAc also increases two-fold from 2675 for **148** to 5275 for **151.** This result tells us that the more lipophilic the polymer, the better it sticks to the well of the plate and the more carbohydrate content results. Therefore, the possibility of ^{3}H-GlcNAc to bind with coated T-antigen is high. Glycopolymer **133** which has no spacer between the carbohydrate hapten and the backbone showed half content of ^{3}H-GlcNAc than that of **148** which has a spacer. Due to the lack of spacer in **133**, the sugar moiety may not readily available for the reaction and hence the low level of radio active GlcNAc participation is observed. Biotinylated terpolymer **127** showed the lowest value with the large standard deviation.

7.5.3. Preparation of terpolymers

Custom-designed glycoconjugates with the covalent addition of probes or effectors were synthesized from active ester polymers. In this way, glycopolymers bearing multiple functional components were introduced as useful tools in biological assays. As already illustrated in Schemes 7-8 and 7-9, biotin or fluorescein-marked terpolymers were successively prepared in addition to stearylated terpolymer. Lipophilicity controlled terpolymer was synthesized using poly(acrylamide-co-N-acryloxysuccinimide), methylamine **137** and **152** was afforded in 83% (w/w) yield. Methylamine was chosen as a quenching material (Scheme 7-15). The ratio of the repeating units, 1/6/60 (methylamido/T-antigen/acrylamide), was confirmed by ^{1}H-NMR according to the anomeric proton at 4.96 ppm, the methyl unit at 2.70 ppm and the methylene unit of the backbone at 1.70 ppm.



152

Scheme 7-15 Preparation of methyl/T-antigen-spacer-CPA **152**

7.6. Neoglycoproteins as vaccines from 3-(2-N-acrylamidoethyl) thiopropyl glycosides 51 and 52

In order to study biological processes, multivalent carbohydrate containing macromolecules have been reported.[218] It is also noteworthy the production of carbohydrate haptens which are covalently attached to carriers such as proteins. These neoglycoproteins have been used for the screening and studying of antibodies, lectins, and enzymes as well as immunogens as vaccine candidates.[15] Logically, vaccines where carbohydrate antigenic determinants are attached to immunogenic protein carriers can be synthesized.[25-28]

In considering the versatile applications of neoglycoproteins, a number of criteria must be addressed for the preparation of these glycoconjugates.

i) compatibility with an aqueous environment.

ii) mild reaction conditions to prevent destruction and denaturation of proteins.

iii) compatibility with the multi-functional nature of the proteins to prevent inter- and intra-protein crosslinking.

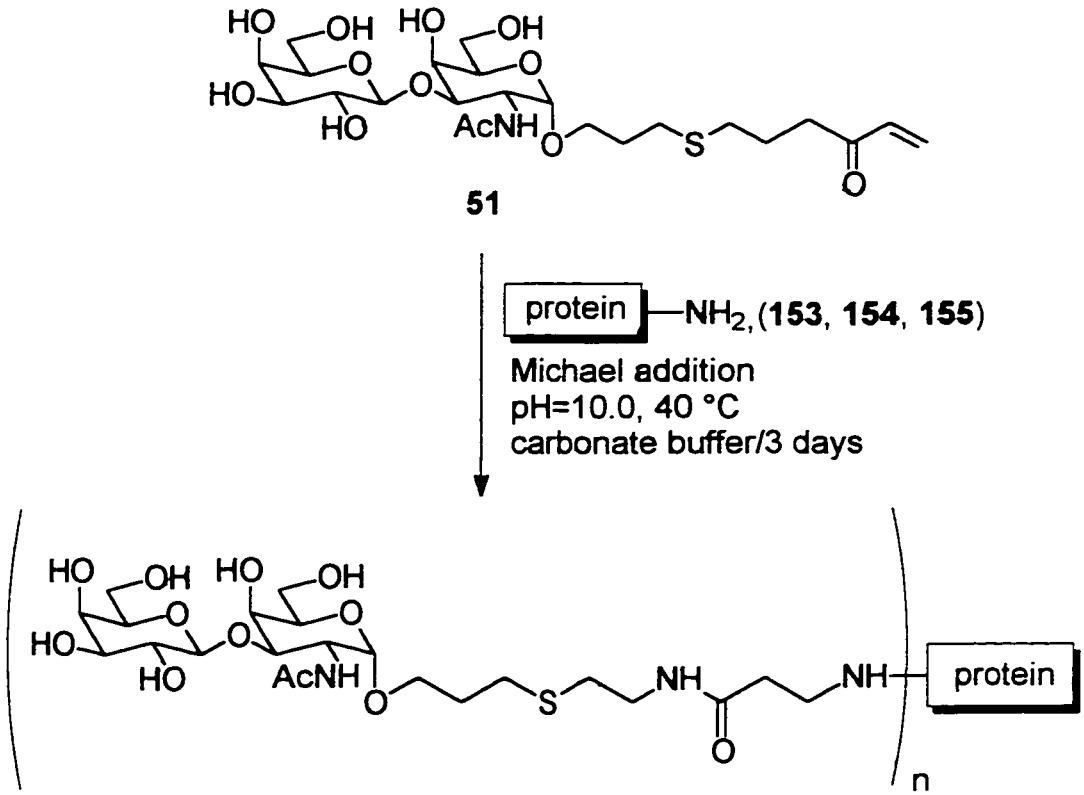
iv) no change in the overall charge of the proteins once modified.

v) versatile and applicable to various carbohydrate residues

vi) easy manipulation and reproducibility.

So far, several synthetic strategies have been developed such as diazotization of aromatic amines[30], isothiocyanate[31], amidation, N-hydroxysuccinimide esters[35] and reductive amidation.[36-39] However, these methodologies still have their own drawbacks in yielding the desired conjugates. Roy *et al.*[220-c] have developed a new glycoconjugation strategy based on the Michael addition reaction of nucleophiles such as amines, or thiols to N-linked acryl amido aglycon. This method satisfies the criteria mentioned for neoglycoprotein synthesis and does not require the use of additional reagents.

Accordingly, N-acrylamido functionalized carbohydrate **51**, was conjugated to carrier proteins (Scheme 7-16). Conjugation of **51** to bovine serum albumin (BSA, M.W. 66,000, **153**), tetanus toxoid (T.T., **154**) and hemocyanine from keyhole limpets



protein: Bovine serum albumine (BSA), **158**
Tetanus toxoid (T.T.), **159**
Keyhole limpets hemocyanin (KLH), **160**

Scheme 7-16 Synthesis of neoglycoproteins as vaccines.

(*megathura crenulata*) (KLH, **155**) were performed in carbonate buffer solution (pH = 10) at 40 °C for 3 days. Each solution was dialyzed exhaustively against distilled water and lyophilized to give the Michael adducts **158**, **159** and **160**. To afford a higher conjugation of T-antigen, the reaction temperature was changed to room temperature or 37 °C, and the BSA-conjugates **156** and **157** were obtained. The carbohydrate content of the conjugates was determined by the Dubois test[233] and the resulted are summarized in Table 7-4. In the same manner, β-D-Gal-(1-3)-α-D-GlcNAc-spacer-BSA conjugate **161**, was also prepared.

Table 7-4. Carbohydrate content of BSA-T-antigen conjugates.

Conjugate	Carbohydrate content (mol/1 mol BSA)
156	1.2
157	4.3
158	4.6

The glycoconjugates produced by conjugate addition of **51** have been evaluated in their respective biological assays. Thus, mouse monoclonal antibodies (IgG and IgM) were obtained by fusing normal antibody forming cells with B-cell tumor line (hybridomas) using the BSA conjugate **158**. These antibodies were already used for several bioassays in the previous chapter (see immunochemical section of Chapter 4, 5 and 6).

7.7 Immunochemical assays

The antigenicity of the glycopolymers and neoglycoproteins was qualitatively examined during the simple double radial diffusion assays using peanut lectin and/or rabbit serum. These receptor proteins are known to bind with T-antigen and T-antigen-bearing glycopolymers to form precipitin which are carbohydrate-receptor protein complexes. As shown in Fig. 7-4, both polymers **127** and **129**, formed precipitin with peanut lectin where **127** showed a more diffuse band than **129**. Since steric hindrance from the polymer backbone was the most importment factor to preventing a strong binding interaction between the carbohydrate hapten and the receptor protein, individual binding affinity was low. This unfavorable effect could be eliminated by the insertion of a flexible and hydrophilic spacer between them. Compound **129** showed a sharp and clear band which still existed after 7 days at room temperature, suggesting that multivalency and spacer arm effects were significant.

To demonstrate the binding specificity of T-antigen toward peanut lectin and to compare the affinity between glycopolymers and neoglycoproteins, another double

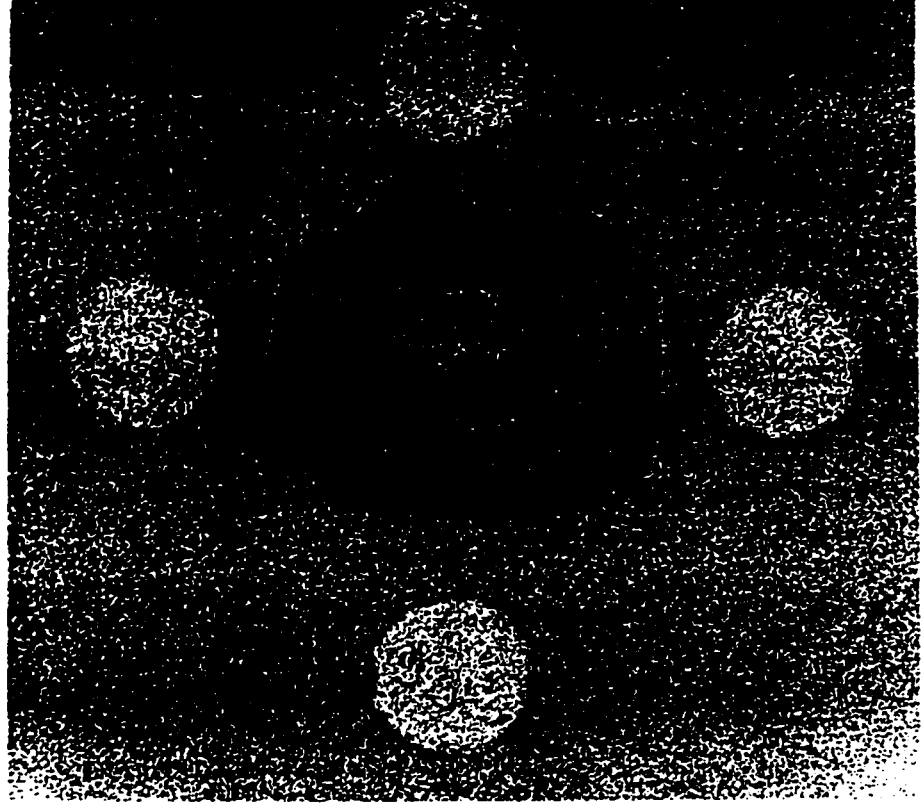


Fig. 7-4. Radial double immunodiffusion assay of peanut lectin (core) with **127** (no spacer, top and bottom) and **129** (with spacer, left and right).

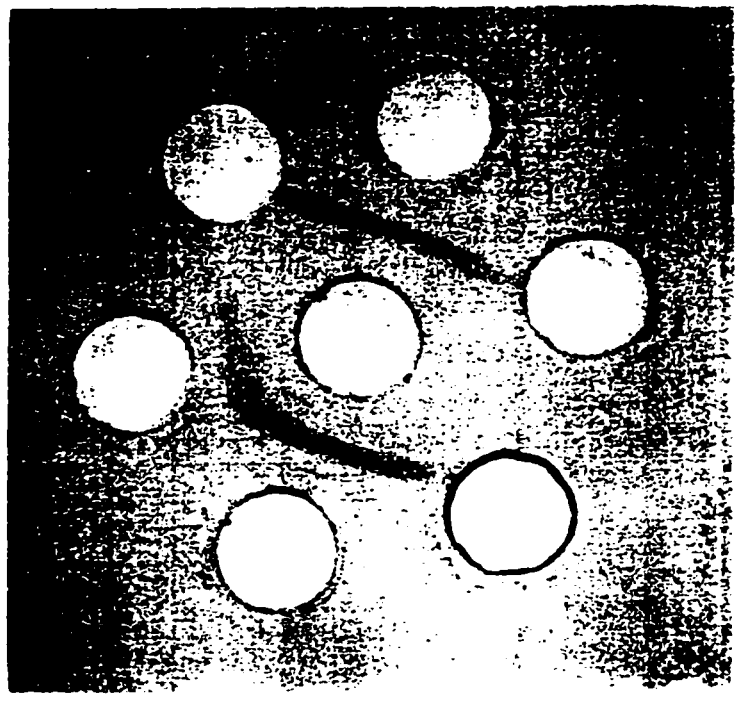


Fig. 7-5. Radial double immunodiffusion assay of peanut lectin (core) against **129**, **161**, negative control, **157**, **131** and negative control (clockwise from top).

immunodiffusion assay was performed with **129**, **131**, **157** and **161**. Polyacrylamide was chosen as a negative control. As expected, T-antigen conjugates, **129** and **157**, showed strong precipitin bands. The molecular weight of **129** could be assumed from the traveling distance toward the lectin well which was approximately the same as that of lectin. Compounds **131** and **161** showed a weak interaction and the precipitin disappeared after a few hours. From these facts, the axial hydroxyl group at the C-4 position is critical to recognizing the binding site of the receptor protein.

Carbohydrate-receptor protein interactions were demonstrated with the two kinds of glycoconjugates, two- (**85**), four- (**90**) and six- (**87**) valent glycodendrimers and BSA- (**158**), TT- (**159**) and KLH- (**160**)-T-antigen conjugates were chosen. Due to the relatively low molecular weights, they traveled very fast. The assays were performed in a time interval to load the conjugates.

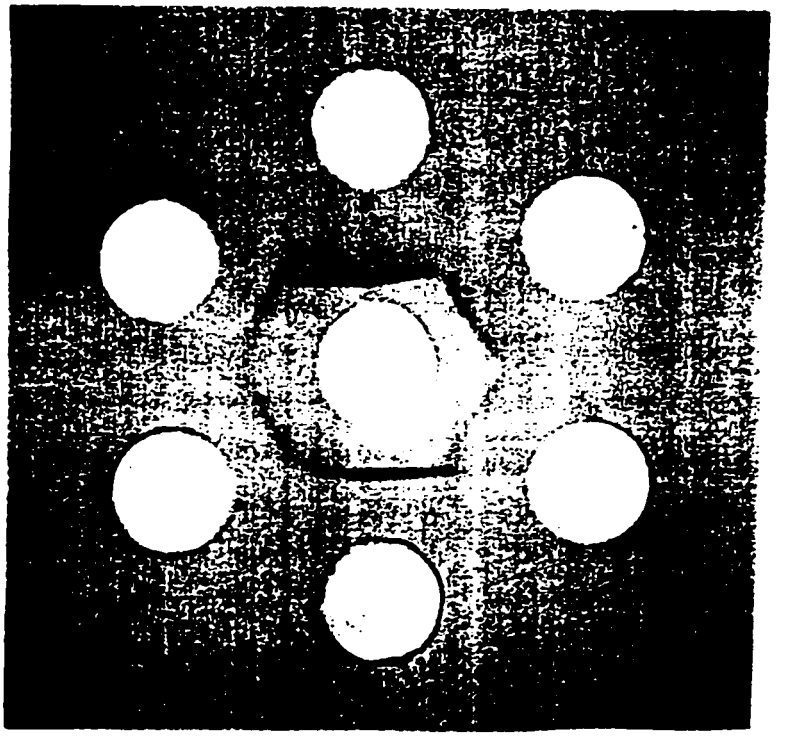


Fig. 7-6. Radial double immunodiffusion assays of peanut lectin (core) against glycodendrimer **85**, **87**, **90** and neoglycoprotein, **158**, **159**, **160** (clockwise from the top).

Once lectin, **158**, **159** and **160** were loaded into the wells, they were allowed to diffuse through agar gel for 18 h. Even though the precipitin was already formed, glycodendrimers were loaded into the wells and precipitin was observed to form in 6 h for all three compounds. Unlike the high molecular weight glycoproteins, the glycodendrimers showed fused precipitin bands.

A new type of double immunodiffusion assay was performed using biotin-labeled conjugate, **132** by dual streptavidin-biotin and T-antigen-peanut lectin interactions.

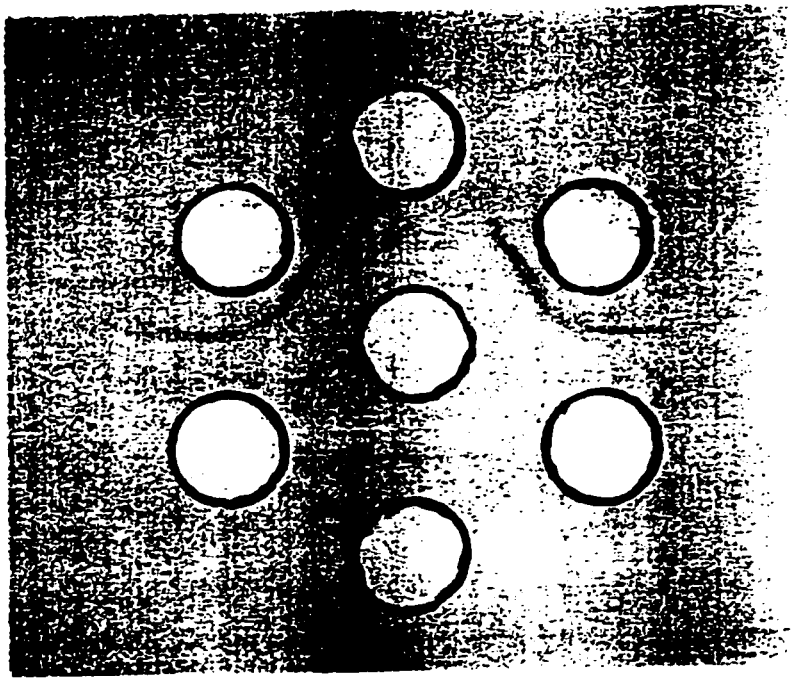


Fig. 7-7. Dual double immunodiffusion assays for streptavidin and/or peanut lectin against biotinylated glycopolymer **132** (core), negative control, **129** (lower left and right), streptavidin (top and bottom) and peanut lectin (upper left and right).

Biotinylated conjugate **132** showed two precipitin bands with streptavidin and peanut lectin. **129,** however, showed only one band with lectin. The same assay was performed using rabbit serum instead of peanut lectin. However, the interaction was too weak for complexes to be formed.

The advantages of lipophilicity of glycopolymers were estimated using **148**, **149**, **150** and **151** as coating antigens by ELISA (Fig. 7-9). When measured O.D. values were

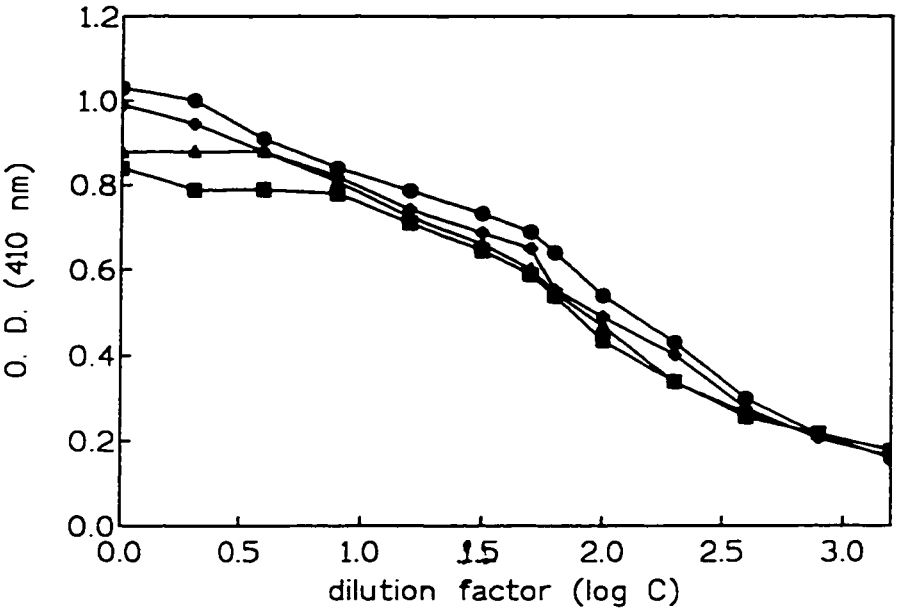


Fig. 7-8 ELISA of T-antigen-spacer-CPA **148** (-NH_2, ■), **149** (-$NHCH_3$, ▲), 150 (-$NHCH_2CH_3$, ◆), **151** (-$NHCH_2CH_2CH_3$, ●) with mouse IgG MAb and goat anti-receptor protein-enzyme conjugates.

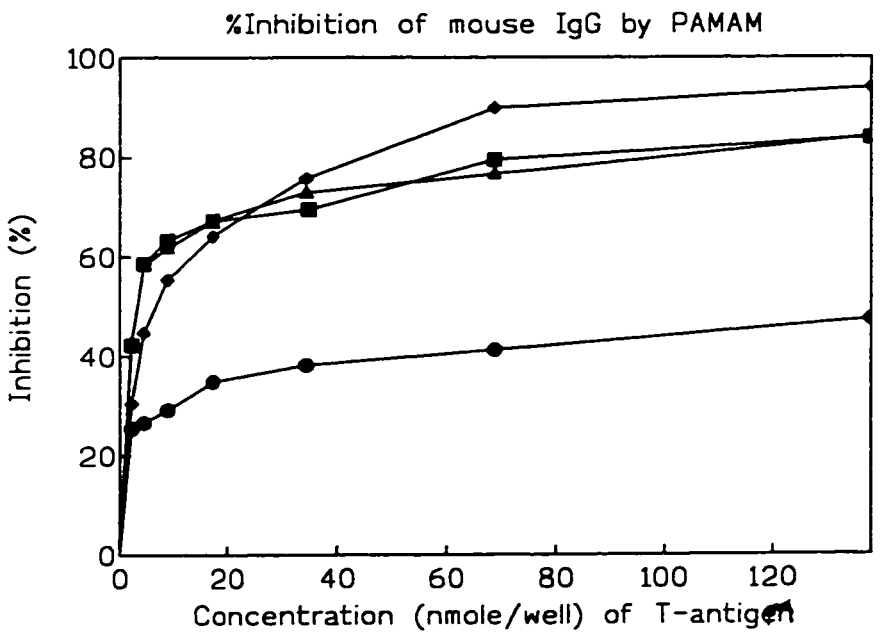


Fig. 7-9. Inhibition of binding of mouse IgG MAb to coating antigens, **148** (-NH_2, ■), **149** (-$NHCH_3$, ▲), **150** (-$NHCH_2CH_3$, ◆), **151** (-$NHCH_2CH_2CH_3$, ●), by PAMAM based glycodendrimer, **114**.

measured (410 nm) as a function of mouse IgG MAb concentration, the curves clearly indicated that the more lipophilic the glycopolymers, the better the coating ability on the microtiter plates.

IC_{50}'s for these glycopolymers **148**, **149**, **150** and **151**, by PAMAM glycodendrimer **114** were 3.7, 3.7, 3.9 and 146 nmol, respectively. As expected, the more lipophilic polymers bind tightly to the wells of microtiter plates and a high density of T-antigen was resulted. Therefore, the amount of inhibitor for 50% inhibition of the binding of antibodies must increase. Incidentally, polymer **151**, represented drastically increased inhibitor content at forty times higher than that of **148**.

As a result, the most lipophilic glycopolymer **151**, is the most powerful coating antigen to improve the sensitivity of the bioassays because only one fortieth the amount of **148** is needed.

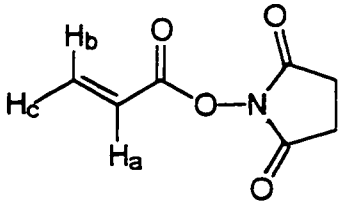
7-8. Conclusions

T-antigen containing glycopolymers were prepared in two ways. First, properly functionalized monomers were co- (ter-) polymerized using a radical initiator. Second, preactivated poly(N-acryloxysuccinimide) and its analogues were prepared under controlled molecular weight distributions. Amine-terminated glycosides and effectors were conjugated to the polymer backbone to afford the corresponding glycopolymers. As well as being synthetically more convenient than copolymerization, the latter method was better than the former method because the characteristics of the polymers such as chain length and polydispersity were kept constant (same batch of polymer was used for all experiments). Neoglycoproteins were also prepared as mimics of vaccine by conjugate addition of amine in protein to the acrylamido function in carbohydrate hapten. Double radial immunodiffusion assays for these glycoconjugates were extensively performed to evaluate their antigenicity with receptor proteins. All of the conjugates were active in terms of the formation of precipitin. A series of water soluble glycopolymers with increased lipophilicity were also prepared to be used as coating antigens with enhanced lipophilic interactions to the wells of microtiter plates in various bioassays. In an inhibition

assay, glycopolymer quenched by propylamine showed forty fold increased inhibitors for 50% inhibition than ammonia quenched polymer. This result improves effective binding of the more lipophilic polymer. Effectors such as biotin or fluorescein containing terpolymers were successfully synthesized in two methods as described.

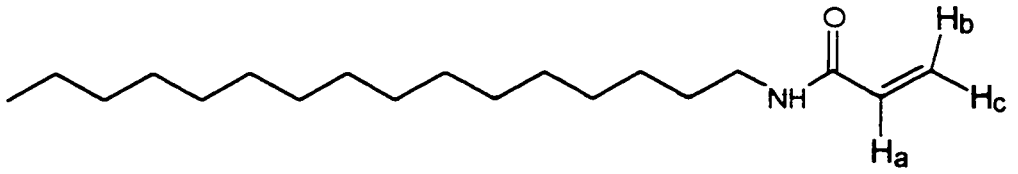
7.9. Experimental methods

N-acryloxysuccimide (116)



To a solution of N-hydroxysuccimide (10 g, 86.9 mmol), triethylamine (14.6 mL, 1.2 eq.) in $CHCl_3$ (150 mL) was added acryloyl chloride (8.5 mL, 1.2 eq.) dropwise over a 20 min. period at 0 °C. The solution was stirred an additional 30 min. at the same temperature, washed successively with, ice-water, brine, dried (Na_2SO_4) and filtered. The filtrate was added 2,6-di-*t*-butyl-4-methylphenol as a polymerization inhibitor and the solution aws condensed to a volume of 30 mL. Ethyl acetate (10 mL) and hexane (80 mL) were added slowly with stirring. The solution was kept in the refrigerator for 5 h. The crystals were filtered, washed successively with 60 mL of hexane/ethyl acetate (4/1), 60 mL of hexane/ethyl acetate (9/1) and 60 mL of hexane and dried in vacuo. The yield was 12.7 g (75.1 mmole, 86%): m.p. = 69.3~69.8 °C; R_f = 0.56 (ethyl acetate/hexane, 1/1); **^{1}H-NMR** δ ($CDCl_3$); 6.80 (dd, 1H, J_{trans} = 17.4 Hz, J_{gem} = 0.8 Hz, H_c), 6.43 (dd, 1H, J_{cis} = 10.9 Hz, H_d), 6.25 (dd, 1H, J_{cis} = 10.9 Hz, J_{trans} = 17.4 Hz), 3.01 (s, 4H, 2 methylene units)

N-Stearyl Acrylamide (118)



To a solution of stearyl amine (250 mg, 0.93 mmol) in CH_2Cl_2 (300 mL) containing triethylamine (400 μL, 2.9 mmol) was added a solution of acryloyl chloride (100 μL) in CH_2Cl_2 (12 mL) dropwise at 0 °C and stirring was continued for 30 minutes at the same temperature. The precipitate of triethylammonium chloride was filtered and washed with cold CH_2Cl_2. The filtrate was condensed and the resulting solid was dissolved in hot ethanol and recrystallized to afford 289.5 mg (96 %) of white crystalline powder: m.p. = 74.5~75.1 °C; R_f = 0.58 (EtOAc/Hex containg 0.5% i-PrOH) CI-MS (ether): 647 (2M+1, 29%), 234 (M+1, 100%); **^{1}H-NMR**, δ ($CDCl_3$); 6.25 (dd, 1H, J_{trans} = 17.0 Hz, J_{gem} = 1.6 Hz, H_b), 6.05 (dd, 1H, J_{cis} = 10.2 Hz, J_{trans} = 17.0 Hz, H_a), 5.60 (dd, 1H, J_{cis} = 10.2 Hz, J_{gem} = 1.6 Hz, H_c), 5.55 (broad, 1H,NH), 3.30 (dt, 2H, $J_{NH\text{-}methylene}$ = 5.9 Hz, $J_{methylene\text{-}methylene}$ = 7.1 Hz, N-CH_2), 1.51 (broad dt, 2H, J = 7.1 Hz, J = 5.9 Hz, CH_2), 1.17~1.27 (broad s, 30H, methylenes), 0.85 (t, 3H, $J_{methylene\text{-}methylene}$ = 6.8 Hz, CH_3); **^{13}-NMR**, δ ($CDCl_3$); 165.5 (C=O), 131.0 (C_a), 126.0 (C_{bc}), 40.0 (CH_2-NH), 29.5~26.7 (16 methylenes), 14.1 (CH_3).

β-D-Gal-(1-3)-α-D-GalNAc-CPA (127)

β-D-Gal-(1-3)-α-D-2-acetamido galactopyranoside, **29** (20 mg, 0.047 mmol) and acrylamide, compound **126** (33.5 mg, 0.47 mmol) were dissolved in 400 μL of deoxygenated water. The atmosphere within was evacuated by bubbling with nitrogen for 10 minutes. Solid ammonium persulfate (1 mg, 4.3 nmol) was added and the mixture was stirred vigorously at 65~70 °C for 20 minutes. TLC ($CHCl_3$/MeOH/H_2O, 10/9/1/)

indicated that some monomers remained unreacted. The polymerization was reinitiated as above and left at 100 °C for another 20 minutes. Dialysis and lyophilization gave 14.7 mg (27.5 w/w %) of **127**. ^{1}H-NMR (D_2O) integration gave a 1/10 by molar incorporation ratio of **29**/acrylamide.

β-D-Gal-(1-3)-α–D–GalNAc-Spacer-CPA (129)

3-(2-N-acrylamidoethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc **51** (18.5 mg, 0.033 mmol) and acrylamide (23.5 mg, 0.33 mmol) were dissolved in 300 μL of deoxygenated water. The reaction vessel was tightly fitted with a rubber septum. The atmosphere within was evacuated repeatedly (3X), then filled with nitrogen. 10 μL of 50 mg/mL solution (0.2 mole %) of amminium persulfate, $(NH_4)_2S_2O_8$ was injected and the solution was stirred vigorously at 110 °C for 20 minutes. TLC ($CHCl_3$/MeOH/H_2O, 10/6/1) indicated that the monomers were completely consumed. The resulting viscous solution was diluted with water and dialyzed exhaustively against distilled water. Lyophilization gave 22 mg (52% w/w) of white spongy solid. The ratio of acrylamide/sugar was determined by the anomeric proton (H_1') at 4.50 ppm and the polymer backbone (methylene or methine) at 1.5~2.3 ppm and was approximately 1/11.

β-D-Gal-(1-3)-α-D-GalNAc-spacer-homopolymer (130)

51 (5 mg, 0.01 mmol) was dissolved in 200 μL of deoxygenated water. The polymerization vessel was flushed with nitrogen and few granules of solid ammonium persulfate (< 1mg) were then added. The reaction mixture was stirred vigorously at 65~70 °C in a water bath for 6 minutes. TLC ($CHCl_3$/MeOH/H_2O, 10/9/1) indicated that the monomer was consumed completely. The viscous solution was diluted and dialyzed against water for 3 days. Lyophilization gave 3.6 mg (72 w/w %) of white spongy solid.

β-D-Gal-(1-3)-α-D-GlcNAc-Spacer-CPA (131)

3-(2-N-acrylamidoethyl)thiopropyl-β-D-Gal-(1-3)-α-D-GlcNAc **52** (25 mg, 0.045 mmol) and acrylamide **126** (32 mg, 0.45 mmol) were dissolved in 400 μL of deoxygenated water. The reaction vessel was tightly fitted with a rubber septum. The atmosphere within was evacuated repeatedly (3X), then filled with nitrogen. 10 μL of 50 mg/ml solution (0.2 mol %) of amminium persulfate, $(NH_4)_2S_2O_8$ was injected and the solution was stirred vigorously at 110 °C for 30 minutes. TLC ($CHCl_3$/MeOH/H_2O, 10/6/1) indicated that the monomers were completely consumed. The resulting viscous solution was diluted with water and dialyzed exhaustively against distilled water (2Lx3). Lyophilization gave 39mg (68.4% w/w) of white spongy solid. The ratio of acrylamide/sugar was determined using the anomeric proton (H_1') at 4.52 ppm and the polymer backbone (methylene or methine) at 1.5~2.3 ppm and was approximately 1/12.

Biotin/β-D-Gal-(1-3)-α-D-GalNAc-spacer-CPA (132)

Acrylamide **126** (18.41 mg, 259 μmol) and biotin monomer **120** (5.5 mg, 12.93 μmol) were dissolved in 500 μL of 1:1 ratio deoxygenated DMSO/H_2O mixed solvent. The other monomer **51** which was made *in situ* from 3-(2-aminoethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc **42** was dissolved in 500 μL of deoxygenated water. The two solutions were then combined and flushed with nitrogen. The resulting mixture was warmed to 65 °C and stirred vigorously to properly dissolve all the monomers. Once homogeneous, 7 μL of tetramethylethylenediamine (TMEDA) and 3 mg of solid ammonium persulfate were added and vigorous stirring at the room temperature proceeded for 4 h under nitrogen atmosphere. The clean viscous solution was diluted with water and dialyzed. A primary dialysis was performed in 2 L of 0.5 mM acetic acid followed by other dialysis against distilled water (2L x 2). Lyophilization gave 37.3 mg (98.2%, w/w) of white spongy solid **132**. Biotin incorporation was confirmed by the treatment with 0.1% 4-dimethylamino cinnamaldehyde in acidified ethanol. After brief heating, the presence of biotin was indicated by the appearance of a bright pink coloration of product. Biotinamido

caproylhydrazide as a positive control was colorized to dark pink (or red). The analogous β-D-Gal-(1-3)-α-D-GalNAc-spacer-CPA **129** which was used as a negative control didn't react with 4-methylamino cinnamaldehyde. ^{1}H-NMR (D_2O) conformed the presence of biotin from its characteristic signal found between 2.6~3.4 ppm. Two anomeric proton signals at 5.0 and 4.5 ppm indicated sugar content. From the integration of these signals, the ratio of monomers, acryl amide/biotin/Gal-GalNAc, in the polymer was approximately 20/1/2.

Terpolymer containing T-antigen and biocytin as a labeling agent (133)

To a solution of poly(N-acryloxysuccinimide) (67.7 mg, 345 μmol, M.W., 42 K) **142** and DIPEA (1~2 drops, pH ~ 8) in DMSO (3 mL), was added a solution of 3-(2-aminoethylthio)propyl-β-D-Gal-(1-3)-α-D-GalNAc **42**, (15 mg, 30 μmol) and biocytin **133** (1.1 mg, 3 μmol) in DMSO (1 mL). The solution was stirred for 2 days at room temperature. Once the reaction was completed, ammonium hydroxide (0.5 mL, neat) was added and stirring was continued for an additional 3 h. The solution was then lyophilized to dryness. The residue was dissolved in a minimum amount of water and purified by size exclusion chromatography (P-10). The fractions containing product were collected and lyophilized to give 39.6 mg (47%, w/w) (or dialyzed with 2,000 M.W. cut-off tube thoroughly against distilled water for 3 days). The resulting polymer was examined by acidified dimethylamino cinnamaldehyde and showed a positive color change to pink after brief heating. The ratio of acrylamide/sugar/biocytin was determined using the anomeric proton at 4.9 ppm, distinct protons at 4.7 ppm of biocytin and the polymer backbone (methylene or methine) at 1~2.3 ppm and was approximately 100/10/1.

Terpolymer containing T-antigen and fluorescein as a labeling agent (134)

Poly(N-acryloxysuccinimide) (67.7 mg, 345 mmol), T-antigen (15 mg, 30 mmol) and fluorescein (1.2 mg, 3 mmol) were used. The reaction conditions were the same as descried above except that the experiment was performed in the dark. 41.2 mg (56% w/w)

of yellowish spongy solid was obtained. The ratio of acrylamide/sugar/fluorescein was determined using the methylene spacer at 1.93 ppm, the distinct proton of fluorescein at 8.05 ppm and the polymer backbone at 1~2.3 ppm and was approximately 100/10/1.

Stearyl/β-D-Gal-(1-3)-α-D-GalNAc-spacer-CPA (135)

To a solution of poly(acrylamido-co-acryloxysuccinimide, 10/1 ratio, **137**) (30 mg, 34 nmol) in DMSO (1 mL) was added 3-(2-aminoethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc, **42**, (16 mg, 32 nmol) and the solution was stirred overnight at room temperature. Stearylamine (1 mg, 3.5 μmol) in *iso*-propanol (0.5 mL) was added and the solution was stirred another 6 h. Without purification, the solution was transferred to dialysis tubing (2,000 M.W. cut-off) and dialyzed thoroughly against water (2L x 3) for 3 days. The resulting solution was lyophilized to give 32.2 mg of white sponge solid (43.8%, w/w). The ratio of the repeating units was confirmed by ^{1}H-NMR according to the anomeric proton at 4.95 ppm, stearyl methyl at 0.83 ppm and the methylene units in the polymer backbone at 1.7 ppm and was 130/13/1 (acrylamide/sugar/stearyl).

Poly (acrylamide-co-N-acryloxysuccinimide)

General procedure

To a solution (1 M) of N-acryloxysuccinimide and acrylamide with varying molar ratio (1/20, 1/10, 1/5, 1/2.5) in dry THF degassed with N_2-bubbling for 30 minutes with vigorous stirring was added AIBN (0.1 eq. for N-acryloxysuccinimide). The solution was stirred overnight at 50 °C under N_2 atmosphere. Once the polymerization was initiated, the solution appeared foggy and eventually a white precipitate resulted. The solution was cooled to room temperature and filtered. The residue was washed with dry THF (3x) and dried in vacuo to give white fluffy solid with 81% ~ quantitative (w/w) yield. The ratio of the repeating units was confirmed by ^{1}H-NMR integration of the two methylene units in

the succinimide functional group at 2.8 ppm and the methylene units in the polymer backbone at 1.5 ppm.

Copolymer 136 (20/1 molar ratio)

N-acryloxysuccinimide (1 g, 5.9 mmol), acrylamide (8.4 g, 120 mmol) and AIBN (0.1 g, 0.59 mmol) were used and the yield was 9.4 g (quantitative). The ratio of the repeating units was 20.6/1 which was coincident with the number of moles of monomers loaded into the reaction vessel before the reaction.

Copolymer 137 (10/1 molar ratio)

N-acryloxysuccinimide (1 g, 5.9 mol), acrylamide (4.2 g, 59 mmol), and AIBN (0.1 g, 0.59 mmol) were used and the yield was 5.2 g (quantitative). The ratio of the repeating units was 11/1.

Copolymer 138 (5/1 molar ratio)

N-acryloxysuccinimide (1 g, 5.9 mol), acrylamide (2.1 g, 30 mmol), and AIBN (0.1 g, 0.59 mmol) were used and the yield was 2.8 g (91%, w/w). The ratio of the repeating units was 5.3/1.

Copolymer 139 (2.5/1 molar ratio)

N-acryloxysuccinimide (1 g, 5.9 mol), acrylamide (1.05 g, 15 mmol), and AIBN (0.1 g, 0.59 mmol) were used and the yield was 1.7 g (82%, w/w). The ratio of the repeating units was 2.8/1.

Poly(N-acryloxysuccinimide)

Method 1 (140)

To a solution of N-acryloxysuccinimide (1 g, 5.9 mmol) in dry THF (100 mL) degassed by N_2-bubbling for 30 min. was added AIBN (10 mole %, 0.1 g, 0.59 mmol). The solution was stirred at 55 °C for 1 day under N_2 atmosphere. Additional THF (100 mL) was added and stirring was continued for 10 min. The precipitate was separated by suction filtration and washed with dry THF(3x) and dried in vacuo to give 0.1 g (10%, w/w) of white solid

Method 2 (141)

To a solution (1 M) of N-acryloxysuccinimide (2 g, 11.8 mmol) in dry benzene was added AIBN (60 mg, 3%, w/w) and stirred for 2 days at 70~75 °C under N_2 atmosphere. After the solution was cooled to room temperature, the precipitate was filtered and washed with dry THF (5x). The residue was dried in vacuo to afford 1.64 g (86%, w/w) of white solid.

Method 3 (142)

The reaction conditions were the same as those employed in method 2 except that the amount of AIBN (30 mg, 1.5%, w/w). The residue was dried in vacuo to afford 1.55 g (85%, w/w) of white solid.

β-D-Gal-(1-3)-α-D-GalNAc-spacer-CPA from Poly (acrylamide-co-acryloxy succimide, 20, 10, 5, 2.5:1)

To a solution of poly(acrylamide-co-acryloxysuccimide) in DMSO was added a solution of 3-(2-aminoethylthio)propyl β-D-Gal-(1-3)-β-D-GalNAc **42**, in DMSO and the resulting mixture was stirred overnight. The solution was quenched by NH_4OH (0.3ml,

NH_4OH/H_2O=3/1) for 2hr and was then lyophilized to dryness. The residue was washed thoroughly with dry THF (10mL x 5) and dried in vacuo to give ivory colored fluffy powder in 65.6~97% (w/w) yield. The sugar content was confirmed by ^{1}H-NMR on the basis of the anomeric proton at 4.97 ppm and the methylene unit at 1.5 ppm.

1. β-D-Gal-(1-3)-α-D-GalNAc-spacer-CPA (143)

Polymer **136** (31.8 mg, 20 nmol), and 10 mg of **42** (20 nmol) were used. The yield was 27.4 mg (66%, w/w). The ratio of the repeating units was 21.3/1 (acrylamide/sugar moiety).

2. β-D-Gal-(1-3)-α-D-GalNAc-spacer-CPA (144)

Polymer **137** (31.7 mg, 36 nmol), and 18.2 mg of **42** (36 nmol) were used. The yield was 48.4 mg (97%, w/w). The ratio of the repeating units was 12.6/1.

3. β-D-Gal-(1-3)-α-D-GalNAc-spacer-CPA (145)

Polymer **138** (20.0 mg, 38 nmol), and 19.0 mg of **42** (38 nmol) were used. The yield was 26.9 mg (69%, w/w). The ratio of the repeating units was 6.5/1.

4. β-D-Gal-(1-3)-α-D-GalNAc-spacer-CPA (146)

Polymer **139** (16.0 mg, 46 nmole), and 23.1 mg of **42** (46 nmole) were used. The yield was 33.0 mg (84%, w/w). The ratio of the repeating units was 3.2/1.

Co-polyacrylamide containing T-antigen from Poly(N-acryloxysuccinimide) (147)

To a solution of poly (N-acryloxysuccinimide **142**) (20 mg, 120 μmol) in DMSO (3 mL) was added a solution of 3-(2-aminoethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc,

42 (30 mg, 60 μmol) in DMSO (1 mL) and stirred overnight at room temperature. After complete consumption of the sugar moiety, the solution was quenched by ammonium hydroxide (0.5 mL, neat) for 3 h. The solution was then lyophilized to remove the solvent and the residue was dissolved in a minimum amount of water followed by purification by size exclusion chromatography (P-10). The fraction containing the desired product was collected and lyophilized to give 30 mg of white sponge solid (75%, w/w). The ratio of the repeating unit, acrylamide/T-antigen, 1.2/1, was coincident with that of the monomers loaded before the reaction.

Co-polyacrylamide containing T-antigen from Poly (N-acryloxysuccinimide) (148)

To a solution of poly (N-acryloxysuccinimide **142**) (58.8 mg, 300 μmol) in DMSO (3 mL) was added a solution of 3-(2-aminoethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc, **42** (15.0 mg, 30 μmol) in DMSO (1 mL) and the mixture was stirred overnight at room temperature. After complete consumption of the sugar moiety, the solution was quenched with ammonium hydroxide (0.5 mL, neat) for 3 h. The solution was then lyophilized to remove the solvent and the residue was dissolved in a minimum amount of water followed by purification by size exclusion chromatography (P-10). The fraction containing the desired product was collected and lyophilized to give 32 mg of white sponge solid (43%, w/w). The ratio of the repeating unit, acrylamide/T-antigen, 11/1, was coincident with that of the monomers loaded before the reaction.

Preparation of co-polyacrylamide containing T-antigen from Poly(N-acryloxysuccinimide, 142) quenched by more hydrophobic materials (149~151)

More hydrophobic water-soluble copolymers containing T-antigen were prepared analogously by changing the quenching materials to increase hydrophobicity, i.e., methylamine, ethylamine and propylamine.. The reaction procedure was the same as that described above and the yields were 36.2 mg (49%, w/w) for **149**, 39.4 mg (53%, w/w) for **150** and 44.7 mg (61 %, w/w) for **151**. The ratios of the repeating units were determined using the anomeric protons in the sugar moiety at 4.95 ppm and the alkyl

protons in the quenching materials according to the ^{1}H-NMR and were 1/10 for **149**, 1/11.2 for **150** and 1/12 for **151**.

Methyl/β-D-Gal-(1-3)-α-D-GalNAc-spacer-CPA from Poly(acrylamide-co-acryl oxysuccinimide, 10/1 ratio) (152)

To a solution of poly (acrylamide-co-acryloxysuccinimide, **137**) (12.3 mg, 14 nmol) in DMSO (1 mL) was added 3-(2-aminoethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc **42** (6.0 mg, 12 nmol), in DMSO (0.5 mL) and the solution was stirred overnight. An excess of methyl amine (0.3 mL, 35% in H_2O) was then added and stirring was continued for another 3 h. The solution was then lyophilized to dryness. The residue was washed thoroughly with dry THF (10 mL x 5) and dried in vacuo to give a yellowish fluffy solid (15.2 mg, 83% w/w). This polymer was dialyzed (2,000 M.W. cut-off) against water (2L x 3) for 3 days to give a white spongy solid (7.0 mg, 38%). The ratio of the repeating units, 60/6/1 (acrylamide/sugar/methyl),was confirmed by ^{1}H-NMR on the basis of the anomeric proton at 4.96 ppm and the methyl proton at 2.7 ppm.

Conjugation of 3-(2-N-acrylamidoethylthio)propyl β-D-Gal-(1-3)-α-D-GalNAc, 51, to bovine serum albumin(BSA) by Michael addition (156~158)

Neoglycoconjugate (156)

To 1.0 mL volume of 0.2 M carbonate buffer of pH = 10 was added 66 mg of BSA (M.W.=66,000, 59 μmole lysine-NH_2, **153**) and 16.6 mg (30 μmole) of **51**. The solution was gently stirred at room temperature for 3 days in the dark. Even though TLC ($CHCl_3$/MeOH/H_2O, 6/4/1) indicated that unreacted **51** remained, the reaction was dialyzed exhaustively against distilled water and lyophilized to give 50 mg (61%, w/w) of white spongy solid. The measurement of the carbohydrate content showed 1.2 mole/mole BSA.

Neoglycoconjugate (157)

To 1.0 mL volume of 0.2 M carbonate buffer of pH = 10 was added 66 mg of BSA (59 μmol lysine-NH_2, **153**) and 18.5 mg (33 μmol) of **51**. The reaction conditions were the same as above except for a temperature change to 37 °C. The reaction solution was dialyzed and lyophilized to give 52 mg (62%, w/w) of white spongy solid. The carbohydrate content was 4.3 moles/mole BSA.

Neoglycoconjugate (158)

The same reaction was performed as described above except for changes pH and temperature, pH = 10.53 at 40 °C. The carbohydrate content was 4.6 moles/mole BSA.

Conjugation of 3-(2-N-acryl amidoethylthio)propyl-β-D-Gal-(1-3)-α-D-GalNAc, 51 to tetanus toxoid (TT) protein by Michael addition (159)

To compound **51** (5.33 mg, 9.61μmole) was added 12 mg of purified tetanus toxoid (4.8 μmol lysine-NH_2, **154**) in 1 mL of 0.2 M carbonate buffer, pH = 10.53. The suspension was homogenized by mixing on a vortex mixer and stirred gently at 40 °C for 3 days in the dark. The reaction mixture was diluted to 5 mL and then centrifuged at 14,000 g in a Fischer Microcentrifuge Model 235B for 5 minutes. The homogenous mixture was then dialyzed against distilled water and lyophilized to give 13.3 mg (79%, w/w) of protein conjugate, **159**.

Conjugation of 3-(2-N-acrylamidoethylthio)propyl-β-D-Gal-(1-3)-α-D-GalNAc, 51 to hemocyanine from keyhole limpets (*megathura crenulata*) (KLH) by Michael addition (160)

To compound **51** (35 mg, 70 μmol) was added 10 mg of KLH (M.W. several million, **155**) in 2 mL of 0.2 M carbonate buffer, pH = 10.53. The suspension was homogenized by mixing on a vortex mixer and stirred gently at 40 °C for 3 days. The

reaction mixture was dialyzed and lyophilized to give 8.5 mg of protein conjugate **160,** (19%, w/w).

Conjugation of 3-(2-N-acrylamidoethylthio)propyl-β-D-Gal-(1-3)-α-D-GlcNAc, 52, to bovine serum albumin(BSA) by Michael addition (161)

To 1 mL of 0.2 M carbonate buffer (pH = 10) solution of BSA (66 mg 59 μmol lysine-NH_2 **153**) was added 18.5 mg (33 μmol) of **52**. The reaction was performed as above except at a temperature of 40 °C. The reaction mixture was dialyzed and lyophilized to give 50 mg (61%, w/w) of white spongy solid.

Double immunodiffusion assays between T-antigen containing glycoconjugates and peanut lectin from *Arachis Pypogaea*

Agar gel diffusion experiments were performed using 1% agarose containing 2% poly(ethyleneglycol) (PEG, 8,000 molecular weight) in PBS. A glass plate was coated with hot 1% agarose containing 2% PEG in PBS and solidified for 20 minutes. A central well and four or six surrounding wells were cut in the gel.

1. Peanut lectin with 127 and 129

Peanut lectin (30 μL, 1 mg/mL PBS) was coperated into the core well and the outer wells were filled with **127** and **129** (30 μL, 1 mg/mL PBS). Precipitin bands between lectin and glycopolymers were allowed to form overnight at 4 °C.

2. Peanut lectin with 129, 131, 157 and 161

Peanut lectin (30 μL, 1 mg/mL PBS) was coperated into the core well and the outer wells were filled with **129**, **131**, **157**, **161** (30 μL, 1 mg/mL PBS) and polyacrylamide as a negative control (M.W., 100,000, 30 μL, 1 mg/mL PBS). Precipitin bands between lectin and glycopolymers and polyacrylamide were allowed to form overnight at 4 °C.

3. Peanut lectin with glycodendrimers 85, 87, 90 and neoglycoproteins 158, 159, 160

The core well was filled with peanut lectin (30 μL, 1 mg/mL PBS) and the outer wells were filled with **158**, **159** and **160** (30 μL, 1 mg/mL PBS). Precipitin bands between the lectin and the glycocojugates were allowed to form for 18 h at 4 °C. The rest of the wells were filled with **85**, **87** and **90** (30 μL, 1 mg/mL PBS) and were also allowed to form for 6 h at 4 °C.

Dual double immunodiffusion assays between 132 and streptavidin and/or rabbit serum

Biotinylated glycopolymer **132** (30 μL, 1 mg/mL PBS) was placed in the core well and the outer wells were filled with streptavidin, peanut lectin and glycopolymer without biotin **129**. Precipitin bands between **132** and streptavidin **129** ,**132** and peanut lectin were allowed to form overnight at 4 °C. The same assay was performed with rabbit serum instead of peanut lectin.

Enzyme linked immunosorbent assays (ELISA) between mouse IgG MAb and T-antigen containing glycopolymers 148~151

Linbro (Titertek) microtitration plates were coated with 100 μL/well of T-antigen containing glycopolymer solutions of a stock solution of 10 μg/mL for 148, 11.1 μg/mL for **149**, 12.1 μg/mL for **150** and 13.2 μg/mL for **151** (0.36 μg or 0.85 nmol/well of T-antigen) in 0.01 M PBS (pH = 7.3) and incubated overnight at room temperature. After washing and blocking with BSA (1% in PBS) as described before, 100 μL/well of mouse IgG-MAb supernatant was added with varying concentration from neat (no dilution, 5 μmole/well) to 1,600 times dilution (3.125 nmol/well) by two fold dilution and incubated for 1 h at 37 °C. The plates were washed and 100 μL/well of goat anti-mouse IgG MAb-enzyme conjugates solution in PBST (1,000 times dilution of supernatant) was added and incubated for 1 h. at 37 °C. After washing, 50 μL/well ABTS-H_2O_2 substrates (1

mg/4mL) in citrate-phosphate buffer (0.2 M, pH = 4.0 with 0.015% H_2O_2) was added. The reaction was stopped after 20 minutes by adding 50 μL/well of 1 M H_2SO_4 and the optical density was measured at 410 nm relative to 570 nm. A blank well contained citrate-phosphate buffer. Each test was performed in triplicate.

Competitive double sandwich inhibition ELISA using mouse monoclonal IgG antibodies and PAMAM based T-antigen dendrimer, 114 as a inhibitor

Linbro (Titertek) microtitration plates were coated with 100 μL/well of T-antigen containing glycopolymer solutions of a stock solution of 10 μg/mL for **148**, 11.1 μg/mL for **149**, 12.1 μg/mL for **150** and 13.2 μg/mL for **151** (0.36 μg or 0.85 nmol/well of T-antigen) in 0.01 M PBS (pH = 7.3) Each well contained 1 μg/well of polymer and the T-antigen content was 0.36 μg (0.85 nmole) out of 1 μg/well. The wells were then washed 3 times with 400 μL/well of 0.01 M phosphate buffer (pH = 7.3) containing 0.05% (v/v) Tween 20 (PBST). The wells were blocked with 150 μL/well of 1% BSA in PBS for 1 h at 37 °C. At the same time, 50 μL/well of mouse monoclonal IgG antibodies solution in PBST (10 times dilution of supernatant, 0.25 μmol/50μL) and 50μL of inhibitor solution (PAMAM based T-antigen dendrimers, **114**) in PBST with varying concentrations from 138 to 2.15 nmole/well of T-antigen by two fold serial dilution were mixed on Nunclon (Delta) microtiter plates and preincubated for 1hr at 37 °C. After washing the excess BSA with PBST, the wells were filled to 100 μL/well with preincubated mouse IgG MAb/inhibitor solution and incubated again for 1 h at 37 °C. The wells were washed as described above with PBST and filled with 100 μL/well of goat-anti-mouse IgG MAb (receptor protein)-enzyme conjugates solution in PBST (1000 times dilution of supernatant) followed by incubation for 1 h at 37 °C. The wells were washed and 50 μL/well of 2, 2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (1 mg/4 mL) in citrate-phosphate buffer (0.2 M, pH = 4.0 with 0.015% H_2O_2) was added. The reaction was stopped after 20 minutes by adding 50 μL/well of 1M aqueous sulfuric acid solution and the optical density was measured at 410 nm relative to 570 nm. Percent inhibitions were calculated as follows:

$$\% \text{ inhibition} = [A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})}/A_{(\text{no inhibitor})}] \times 100$$

IC_{50}'s were calculated as the concentration required for 50% inhibition of the coating antigen. All tests were performed in triplicate.

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Claims to Original Research

1. Several disaccharides were synthesized using TfOMe, TfOAg or $Hg(CN)_2$ as promoters: Allyl β-D-galactopyranosyl-(1-3)-α-D-2-acetamido-2-deoxy galactopyranoside, allyl β-D-galactopyranosyl-(1-3)-α-D-2-acetamido-2-deoxy glucopyranoside, 3-aminopropyl β-D-galactopyranosyl-(1-3)-α-D-2-acetamido-2-deoxy galactopyranoside, 2′,3′,4′,6′-tetra-O-benzoyl-β-D-galactopyranosyl-(1-3)-6-(tert-butylbiphenyl)silyl-1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol and 2′,3′,4′,6′-tetraacetyl-β-D-galactopyranosyl-(1-3)-6-(tert-butylbiphenyl)silyl-1,5-anhydro-2-deoxy-D-lyxo-hex-1-enitol. Especially, T - antigen was prepared in an excellent yield by one step reaction.
2. A strategy for preparing glycosides having extended sulfide bridged spacer arm was developed through an aqueous light-induced addition of functionalized thiol derivatives such as cysteamine, 2-mercaptoethanol, and 3-mercaptopropionic acid to allyl glycosides and these derivatives extended the applicatiions glycoconjugates syntheses.
3. Hydroformylation of allyl T-antigen was performed using rhodium catalyst to give linear and branched aldehyde. Ozonolysis of allyl aglycon also gave one carbon reduced hydroformyl aglycon. Hydroformylation of T-antigen opens new synthetic strategies for the functionalization of carbohydrates.
4. The N-acryloylation of terminal amino group of aglycon demonstrated as an effective method for preparing carbohydrate derivatives which can be polymerized with acrylamide comonomer or coupled to proteins by Michael addition.
5. The symmetrical convergent dendritic cores based on N,N′-bis(acrylamido)acetic acid were synthesized. The T-antigen containing glycodendrimers were prepared with valencies of between two and six using HOBt/EDC method or Michael addition of thiolated T-antigen to dendritic cores These dendritic cores were conjugated with carbohydrate hapten efficiently.
6. The hyperbranched L-lysine dendrimers were coupled with thiolated T-antigen to give another type of glycodendrimers through valencies of between two and eight.

7. T-antigen containing divalent conjugate based on N,N′-bis(acrylamido)acetic acid and tetravalent conjugate based on L-lysine were labeled with biotin and/or fluorescein. Biotin labeled ones were examined their biological activity using streptavidin. These bifunctional glycoconjugates have a potential to apply to bio-systems as sensors.
8. T-antigen containing spherical gylcodendrimers were prepared using peptide coupling strategy between T-antigen containing carboxylic acid functional group and amino group surfaced starburst™ PAMAM dendritic cores giving up to thirty-two valency.
9. N-acryloylated carbohydrate derivatives were employed as monomers in copolyacrylamide glycodendrimers and demonstrated as being superior to allyl glycosides. The incorporation of a spacer arm between the hapten and polymer backbone gives glycopolymer with enhanced binding affinities.
10. A new method for preparing glycopolymers were developed where the preactivated poly(N-acryloxysuccinimide) and its analogues involves. Graft conjugation of carbohydrate hapten and other comonomeric units bearing nucleophilic substituent, amino group, were performed to give the desired glycopolymers. This strategy has advantages when compared with the other one in terms of molecular weight distribution, carbohydrate density and biological activities.
11. To be used as a coating antigen for bioassays, the more lipophilic glycopolymers were prepared using alkyl amines such as methyl-, ethyl-, and propyl amine as quenching materials. The more liphophilic the glycopolymers, the highter liphophilic interaction is resulted.
12. Neoglycoproteins were prepared by Michael addition of amines on the proteins to N-acryloyl function as vaccine mimics
13. In immunochemical assays, all glycoconjugates exhibited enhanced inhibitory potentials. These results demonstrated the multivalency effect and their role in carbohydrate-protein interactions.

Publications

1. "Production and Structural Binding Requirements of Murine Monoclonal Antibodies to T-Antigen"
   M. G. Baek, R. Roy K. Rittenhouse-Diakun, Z. Xia and D. Pickhardt
   Immunological Investigations, submitted.

2 "Synthesis of T-Antigen Containing Glycodendrimers Based on PAMAM and Mouse Monoclonal IgG Antibody Binding Properties"
M. G. Baek and R. Roy
Angew. Chem. Int. Ed. Engl., Submitted

3 "Synthesis of N,N'-bis(acrylamido)acetic Acid Based T-Antigen Glycodendrimers and Carbohydrate-Protein Binding Properties"
M. G. Baek and R. Roy
JACS, submitted.

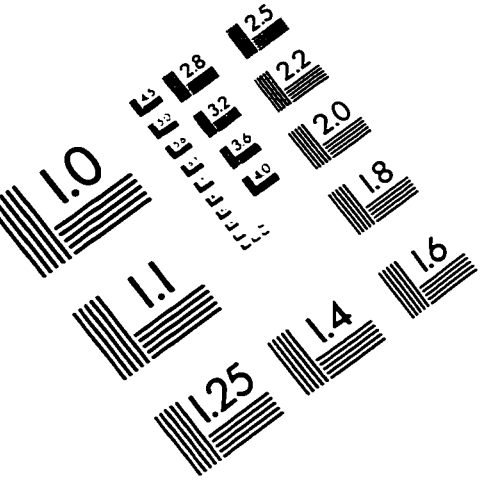
4 "Synthesis of L-Lysine Based Dendritic T-Antigen Tumor Markers and Mouse Monoclonal IgG Antibody Binding Properties"
M. G. Baek and R. Roy
Bioconjugate J., Submitted.

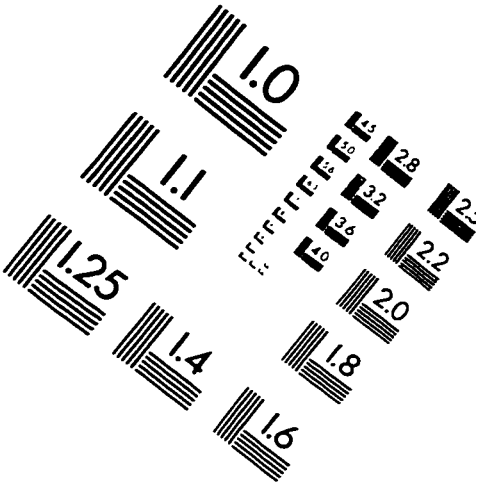
5 "Synthesis of T-Antigen Bearing PAMAM Based Glycoconjugates and Their Inhibitory Abilities toward Mouse Monoclonal Antibody"
M. G. Baek and R. Roy
Glycoconjugate J., Submitted.

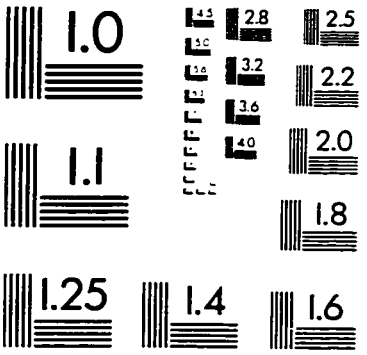
6 "Design and Synthesis of Water Soluble Glycopolymers with Enhanced Liphophilicity as Breast Cancer Markers"
M. G. Baek and R. Roy
JACS, Submitted.

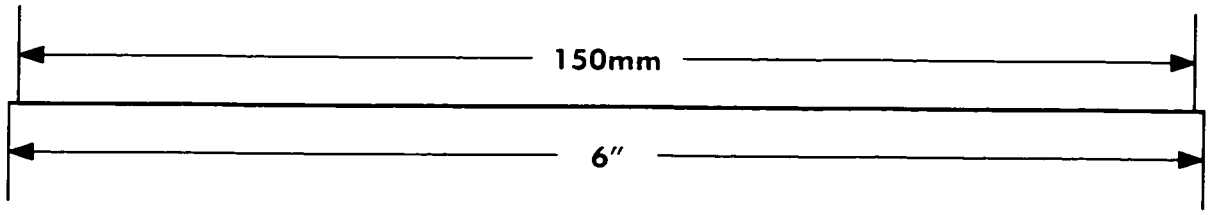
7 "Synthesis of Custom Designed T-Antigen Containing Glycopolymers: SN2 Substitution of Carbohydrate Amine to Poly(N-acryloxy succinimide) and Their Lectin Binding Properties"
M. G. Baek and R. Roy
Macromolecules, Submitted.

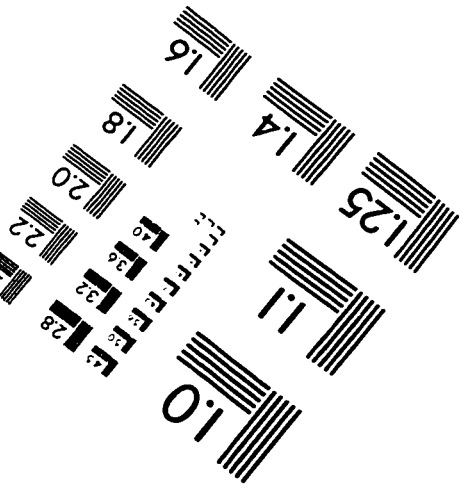
IMAGE EVALUATION TEST TARGET (QA-3)

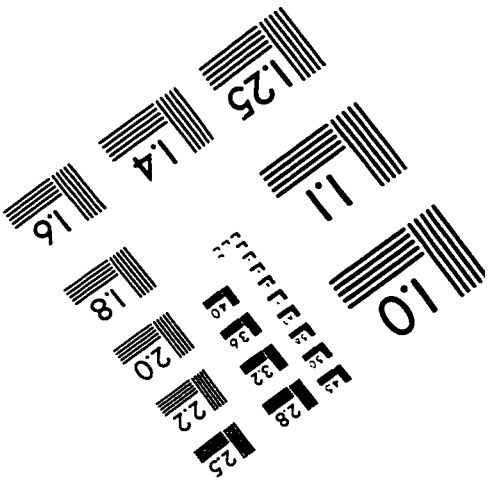












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