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The Study of Polysialic Acid Conjugates

Robert A. Pon

Thesis submitted to the School of Graduate Studies and Research in partial fulfillment for the degree of Master of Science in Chemistry.

University of Ottawa

Robert A. Pon, Ottawa, Canada, 1992

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List of Abbreviations

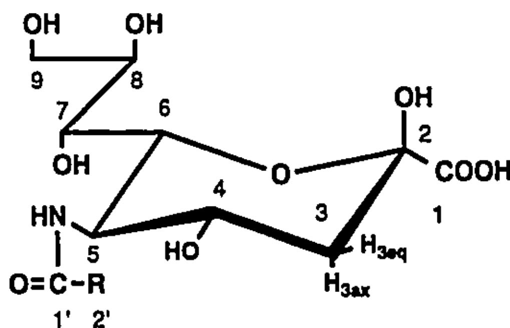
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
^{13}C NMR	carbon-13 nuclear magnetic resonance spectroscopy
Ab	antibody
Ag	antigen
BSA	bovine serum albumin
Cer	ceramide
CI-MS	chemical ionization mass spectrometry
colominic acid	*Escherichia coli* K1 capsular polysaccharide
CSA	chicken serum albumin
D_2O	deuterium oxide
DID	double immunodiffusion
DMSO-d_6	deuterated dimethyl sulfoxide
DP	degree of polymerization
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
ELISA	enzyme linked immunosorbent assay
Fab	single antibody binding site (fragment of an antibody)
FAB-MS	fast atom bombardment mass spectrometry
Gal	galactose
GBMP	*Neisseria meningitidis* serogroup B capsular polysaccharide
Glu	glucose

H-3_{ax}	the axial proton of sialic acid in the 3-position
H-3_{eq}	the equatorial proton of sialic acid in the 3-position
HPLC	high performance liquid chromatography
hr	hour
IgG	immunoglobulin (IgG isotype)
Im	immunogen
Kd	kilodalton
NCAM	neural cell adhesion molecule
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
PBS	phosphate buffered saline (pH 7.6, 0.1M PO_4^-, 0.15M NaCl)
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC-HPLC	size exclusion HPLC
TLC	thin layer chromatography
TT	tetanus toxoid
WGA	wheat germ agglutinin

Chapter 1- Introduction

1.1 Introduction

Sialic acid is a general name given to more than 30 different derivatives of neuraminic acid which is an acidic amino sugar comprised of nine carbons (fig. 1.1).



1-1a R = CH_3
1-1b R = CH_2OH

Figure 1.1- Neu5Ac and Neu5Gc structure

They are found extensively in nature, ranging from protozoa to man and have been comprehensively reviewed (Schauer, 1982). Due to the nature of this thesis, discussion will be limited to two types of sialic acid- namely N-acetylneuraminic acid (**1-1a**) and N-glycolylneuraminic acid (**1-1b**), and their relevance to mammalian species and in particular man.

N-Acetylneuraminic acid or 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (**1-a**) commonly known as Neu5Ac is the most ubiquitous form of sialic acid. N-Glycolylneuraminic acid or 3,5-dideoxy-5-hydroxyacetamido-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (**1-b**) referred to as

Neu5Gc is the second most prevalent sialic acid which is not normally found in humans (Schauer, 1982). These two sialic acids are present in generally one of two forms: (1) as part of complex oligosaccharides of glycoproteins and glycolipids, usually occupying the terminal or penultimate positions (Kitazume *et al.*, 1992) or (2) as internally polymerized forms.

1.2 Sialyloligosaccharides

Besides free sialyloligosaccharides found in urine or milk, sialyloligosaccharides are components of one of two general categories: (1) sialylglycoproteins and (2) sialylglycolipids. Indeed, no mammalian species has been found devoid of sialic acid. As glycoproteins, they can be found in serum, mucins, membranes, and brain tissues, while the sialylglycolipids are usually found as cell wall components, termed gangliosides, generally in nervous tissues (Schauer, 1982). The vast majority of sialic acid found in sialyloligosaccharides occurs in the monomeric form, always α-linked, and in terminal positions. There are some notable exceptions such as gangliosides with terminal disialyl residues, or brain and cell surface sialylglycoproteins (N-CAMs) both containing homopolymers of sialic acid. It is not surprising due to the widespread distribution and occurrence of sialic acid in terminal positions, that they are involved in a variety of biochemical and immunochemical events. Sialic acid oligosaccharides have been implicated in a number of biological roles such as (1) cell and tissue protection against invasive pathogens (Kitazume, 1992), (2) modulation of hormonal activity (Amano, 1989), (3) ion exchangers (Schauer, 1982), (4) plays a part

in cell differentiation (Feizi, 1985) and development (Hanai et *al.*, 1988; Goto et *al.*, 1982), and (5) plays a major role in nerve transmission (Schauer, 1982). Most of these processes are thought to be mediated by the negative charge of sialic acid and its density on cell-surfaces (Kitazume, 1992). The presence of sialyloligosaccharides is not always beneficial since they also act as cell surface receptor determinants for a number of pathogens such as human influenza virus (Paulson, 1985), mycoplasma pneumoniae (Loomes *et al.*, 1984), bacterial toxins (Schauer, 1982b) and lectins (Ravindranath *et al.*, 1985). They are also the immunodominant epitopes in certain forms of cancer (Higashi, 1990; Hakomori, 1984; 1985), Waldenstrom's macroglobulinemia (Tsai *et al.*, 1977) and act as human erythrocyte autoantigens resulting in chronic lymphocytic leukemia (Roelke, 1984). As such, they represent a much studied and important category of cell markers, particularly as tumour cell markers.

Two sialic acids relevant to this thesis are the Hanganutziu-Deicher (HD) antigen, in which a terminal α-linked Neu5Gc residue (**1-1b**) represents the immunodominant epitope (Higashi *et al.*, 1977), and an α-(2→8) linked disaccharide of Neu5Ac, which is found in a number of tumour related gangliosides (Paulson *et al.*, 1982; Hakomori, 1984).

Since the isolation of abundant quantities of pure sialylated gangliosides is next to impossible, synthetic versions provide the only means by which to study their participation in immunochemical processes. An array of monosialylated gangliosides have been prepared synthetically using a combination of classical and

enzymatic techniques (Sabesan & Paulson, 1986). From a survey of the synthetic gangliosides known to date, it is readily apparent that those gangliosides containing an α-(2$\rightarrow$8) linked Neu5Ac disaccharide are still lacking. This is due primarily to the synthetically difficult α linkage required. In fact, apart from the α-(2$\rightarrow$8) linked Neu5Ac disaccharide synthesized by Goto (Okamoto *et al.*, 1986, 1988), other synthetic attempts are still lacking. The disialyl Neu5Ac gangliosides (for a partial list see table 1-1) are extremely important since they are known tumour markers for certain forms of cancer. Monoclonal antibodies raised against these epitopes would be very useful for cancer diagnosis and treatment. Suitable macromolecular forms of these disialylated epitopes may also constitute potential anti-cancer vaccines. The HD antigen has recently been reviewed (Higashi, 1990) and its importance as a cancer determinant is clearly seen since it is not normally expressed in humans. The presence of glycolylneuraminic acid containing gangliosides in various human tissues and cells correlates with a number of cancers including colon cancer, melanoma, retinoblastoma, malignant lymphoma, seminoma, chondrosarcoma, and ovarian cancer. Neu5Gc containing glycoproteins have recently been implicated in a number of cancers such as gastric (Fukui *et al.*, 1989), and liver and breast cancers (Higashi, 1990), although work in this area is preliminary.

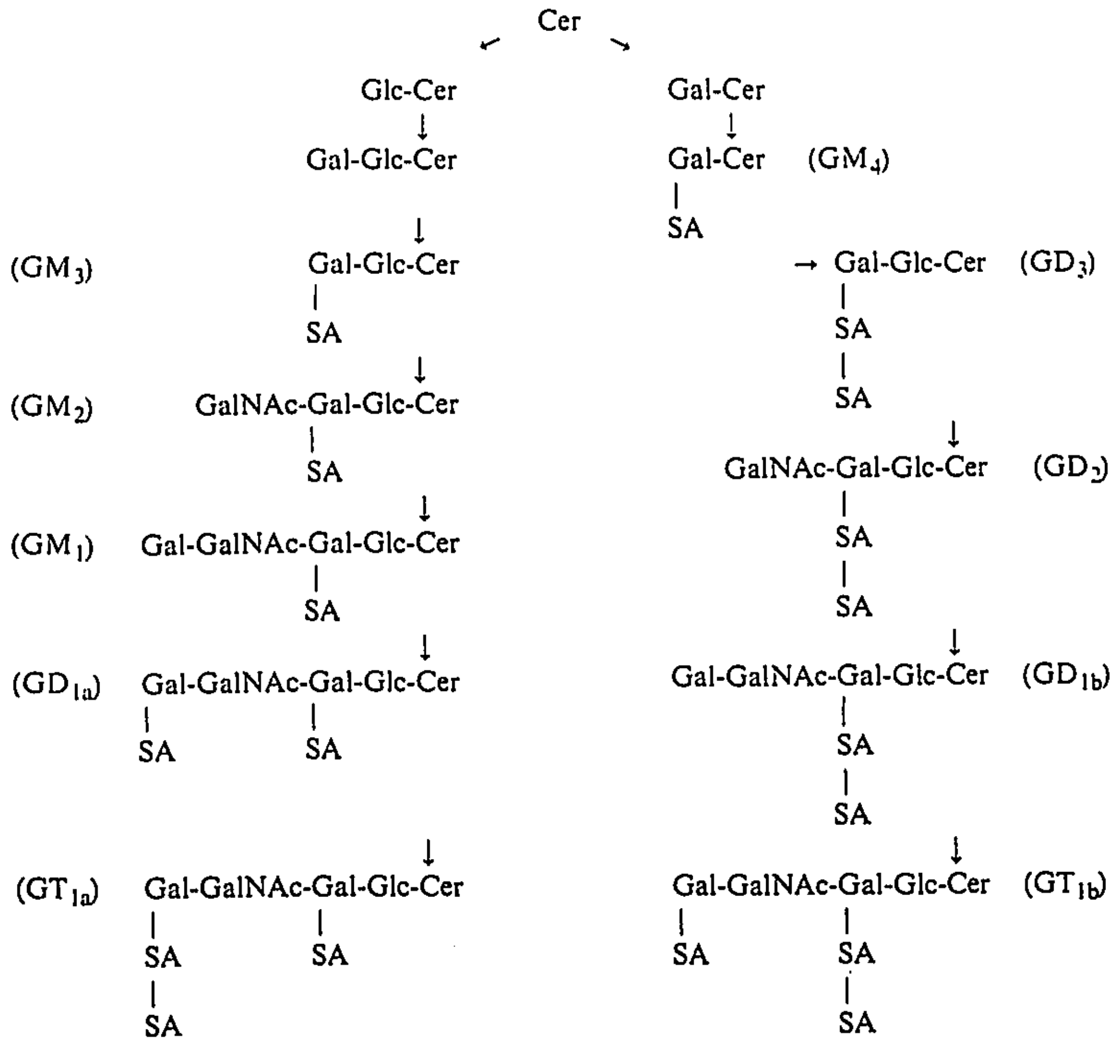


Table 1-1 Partial list of some disialylated gangliosides SA= either Neu5Ac or Neu5Gc; Glc= glucose; Gal= galactose; GalNac= N-acetylgalactosamine; Cer= ceramide (N-palmitylsphingosine).

TABLES NOT AVAILABLE

See article by Frederick Troy in the journal Glycobiology v.2., p5-23 1992

Little work has been done on gangliosides containing an α-(2→8) linked Neu5Gc disaccharide due to a lack of the naturally occurring gangliosides, however it has been suggested (Higashi, 1990) that a melanoma expresses a GD3 type ganglioside (see table 1-1) containing this diglycolyl residue. It is probably inevitable as our technology progresses and our abilities to detect specific sugars amid a large population of sugars, that this diglycolyl epitope will be found as a component of other cancerous tissues.

1.3 Polysialic acid

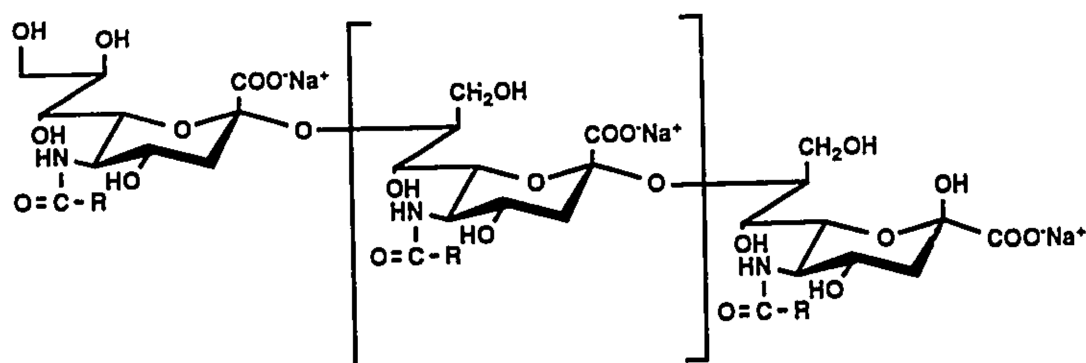
Polysialic acid is a term representing polymerized forms of sialic acid composed of either Neu5Ac (**1-1a**) or Neu5Gc (**1-1b**). Sialylpolymers are exclusively α-linked but can be found as α-(2→8), α-(2→9), or alternating α-(2→8) and α-(2→9) ketosidic linkages (table 1-2). Degrees of polymerization (DP) of the sialic acid residues can range from two (as described above) to well over 200 residues. Polysialic acids are major components of bacterial capsules, fish egg glycoproteins, neuronal glycoproteins in mammals, and are present as glycoproteins on some tumour cells (table 1-3) (Troy, 1992). They, like their sialyloligosaccharide counterparts, play in important biological processes as outlined in table 1-4. Among the more prominent roles of polysialic acid that is pertinent to this thesis, is its ability to render certain bacteria more resistant to immune processes allowing them to colonize and become disease causing.

The material presented in this thesis touches on the various aspects outlined above for both oligo- and polysialic acids, however

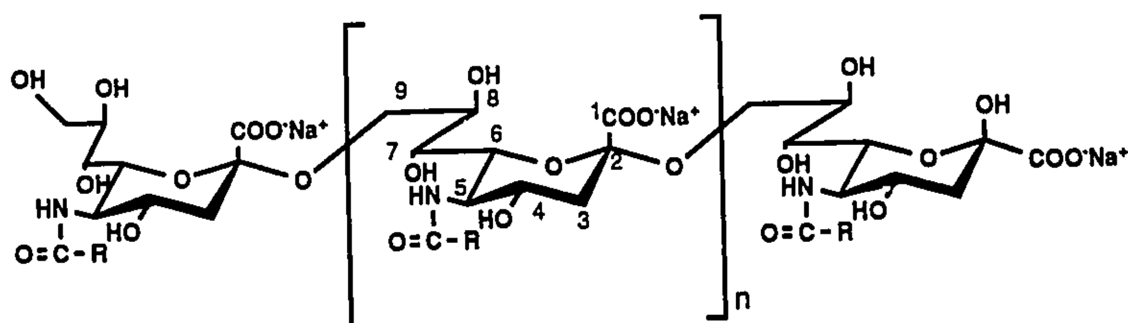
the main emphasis is directed towards the ongoing study of the interaction of the immune system with polysialic acid derived from the capsule of *Neisseria meningitidis* serogroup B.

1.3.1 Poly α-(2→8) linked N-acetyl neuraminic acid

Neisseria meningitidis is a gram negative bacteria which causes meningitis in humans. Pathogenic strains are invariably encapsulated which helps the bacteria evade normal bodily defences by masking important surface epitopes with their polyanionic shield. Serogrouping of the bacteria is based on the composition of the capsular polysaccharide which forms an integral part of the capsule. Two serogroups of *N. meningitidis* , namely serogroup B and C, are both homopolymers of sialic acid (see fig. 1-2). Structural analysis of these homopolymers revealed that both were composed of Neu5Ac as repeating unit with the residues linked α-(2→9) in serogroup C and linked α-(2→8) in serogroup B (Bhattacharjee *et al.*, 1975). The capsular polysaccharide of *Escherichia coli* K1 (known as colominic acid), also a leading cause of meningitis in humans, was found to be structurally (Liu *et al.*, 1971) and immunochemically identical (Kasper *et al.*, 1973) to the group B capsular polysaccharide of *N. meningitidis* (GBMP). The two differ only in their molecular weight and the presence of 1,2- diacylglycerophosphate glycosidically linked to the reducing residue of GBMP (Gotschlich *et al.*, 1981). These two organisms are responsible for the majority of bacterial meningitis which remains both in endemic and epidemic proportions throughout the world (Jennings, 1990).



R= CH_3 *Neisseria meningitidis* serogroup B capsular polysaccharide **(1-2)**
R= CH_3 *Escherichia coli* K1 capsular polysaccharide (colominic acid) **(1-3)**
R= CH_2OH N-Glycolyl colominic acid **(1-4)**



R= CH_3 *Neisseria meningitidis* serogroup C capsular polysaccharide **(1-5)**

Figure 1.2- Structures of the most common forms of polysialic acid.

The poor immunogenicity of GBMP or colominic acid is cited as the main reason why there does not exist a suitable vaccine against these two organisms. Strangely enough, group C meningococcal capsular polysaccharide (GCMP) consisting of the homopolymer of α-(2→9) linked Neu5Ac, is strongly immunogenic in persons over 5 years of age (Artenstein *et al.*, 1970). The demand for an effective vaccine against group B meningitidis and *E. coli* K1 has prompted a

tremendous amount of research into understanding the poor immunogenicity of the two polysaccharides. Some notable contributions from these studies include the recognition that an unusually large epitope of the polysaccharide (~10 residues) is required to bind to antibodies directed against the capsular polysaccharide of GBM (Jennings *et al.*, 1985; Finne & Makela, 1985). This contradicts the generally accepted value of 5-6 carbohydrate residues as being the maximum size that an antibody binding site can accommodate (Kabat, 1960). These findings have led to the proposal that the required epitope must be conformational in nature (Jennings *et al.*, 1984; Finne & Makela, 1985). Various attempts have been made to relate these immunological properties to the three-dimensional structure of the antigen. Lindon and co-workers (1984) observed by ^{1}H NMR studies that the B polysaccharide tumbles as a rigid species with internal rotation only at C-9 of the exocyclic side chain, compared to GCMP which shows segmental motion in the C-7 to C-9 side chains. Following this, it was found by high resolution NMR (Michon *et al.*, 1987), that oligosaccharides of GBMP show large linkage heterogeneity and that a pentasaccharide is the minimum required before a linkage conformation is generated which is similar to the conformation found in GBMP. They speculate that at least two residues are needed on either side of the actual epitope in order to stabilize the conformation of the internal residues. Providing a decasaccharide is the minimum size required for binding, elimination of the two exterior residues on both the reducing and non-reducing termini, leaves a conformational epitope composed of six residues consistent with Kabat's (1960) findings. A number of studies have

implicated the possibility that the conformation of the polysaccharide adopts helices in solution which is consistent for both the large epitope requirement and helps to explain the poor immunogenicity of the polysaccharide. A human monoclonal IgM (IgM^{NOV}) was found which exhibits cross reactive binding to both GBMP, polynucleotides and denatured DNA (Kabat *et al.*, 1986). Since the only common feature between the two polymers is the polyanionic charge distribution, this led the investigators to propose the possible existence of GBMP as a helix. Recent NMR and molecular modelling studies (Yamasaki & Bacon, 1991; Brisson *et al.*, 1992) have both suggested that the GBMP can adopt several helices some of which mimic the conformation of DNA.

The poor immunogenicity of the Group B polysaccharide can also be attributable to the findings that poly Neu5Ac has been found as constituents of developing human brain tissue (i.e. N-CAM sialylglycoproteins) (Finne & Makela, 1985) and expressed on developing rat kidney, heart and muscle tissue (Saukkonen *et al.*, 1986; Finne *et al.*, 1987; Hayrinen *et al.*, 1989). This has led to the concept of "antigen mimicry" where the bacteria have evolved capsules that are immunochemically identical to structures present on human tissues thus invoking immunological tolerance (the inability to produce antibodies that would react with one's own tissues). This generally accepted theory has led to controversy regarding the outcome of a vaccine that is based on the capsular polysaccharide due to its potential pathological repercussions (Rougon *et al.*, 1986). Advocates of a polyNeu5Ac specific vaccine claim that brain tissue is not accessible to circulating antibodies

(Moreno *et al.*, 1985; Lifely *et al.*, 1986) and extraneous polysialic acid is present only during the fetal stage. If however, polyNeu5Ac expression is found in extraneural tissues in humans as demonstrated in the rat, then the potential deleterious effects must be carefully considered. It is worthwhile noting that convalescent patients from *Neisseria meningitidis* serogroup B infection have high levels of anti-GBMP antibodies but do not show any ill effects (Lifely *et al.*, 1987)

1.4 Immunology of polysaccharides

The end goal of this thesis is to gain a better knowledge of the interaction of oligo-/polysialic acid with the immune system and its machinery, and ultimately, to use this understanding to generate useful vaccines or immunotherapies against invasive bacteria, viruses, or cancers. In order to devise new strategies and to develop new concepts, a sound immunological background is a prerequisite in order to critically evaluate these ideas, hence a brief overview of the immunology of polysaccharides is warranted.

An antigen (Ag) by definition is a species that reacts specifically with the combining site (the recognition portion) of an antibody (Ab). An immunogen (Im) however, is a species capable of eliciting an antibody response which will generate specific antibodies to the Im. It is important to understand that all Ag's are not necessarily immunogenic but all Im's are by definition also antigenic. An epitope is that part of an Ag that is specifically recognized by an antibody combining site. An Ag can have one or many hundreds of epitopes. In this work, we will be studying primarily antigenic

relationships while attempting to create new Im's in the process. Some polysaccharides are intrinsically immunogenic (i.e. Group C meningococcal polysaccharide) and are capable of generating an Ab response. These polysaccharides elicit for the most part pentameric IgM Ab's, which are considered as " first line" Ab's (fig. 1.3).

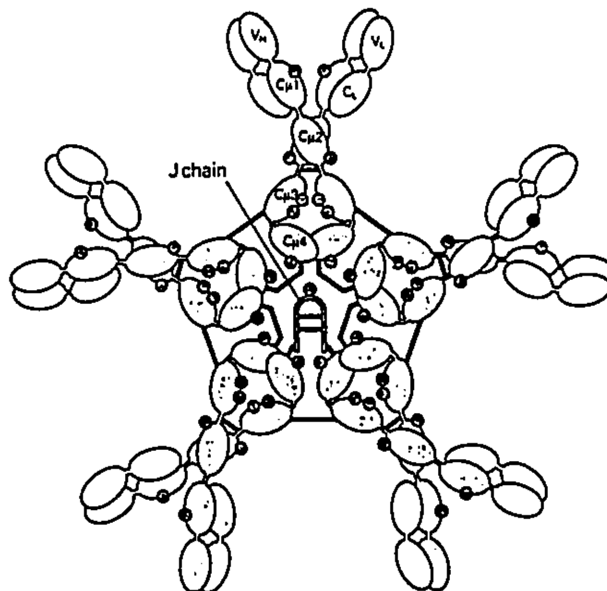


Figure 1.3- Pentameric IgM antibody. Diagram shows the 10 binding sites of the IgM antibody.

Ideally, a good vaccine is one that elicits a secondary response which generates a more effective IgG type Ab and produces a concomitant memory process. In other words, upon encountering the same stimuli, the immune response is quick to generate protective Ab's before that stimuli can do any damage. Polysaccharides do not produce IgG or secondary responses and are known as T-independent antigens (Kasper, 1986). Vaccines composed of polysaccharides are generally effective in adults since the IgM Ab's

produced can often be long lived (~5 years). They are not effective in children under the age of 2 due to an immature immune system and this is the principal reason why meningococcal meningitis is such a serious disease among children. Polysaccharides can be made into T-dependent Ag's (i.e. they are capable of raising IgG's and creating a memory response) by covalently coupling them to protein carriers. The protein acts like the trigger in the immune response and a wide spectrum of Ab's are produced against the polysaccharide and protein carrier alike. This process will be discussed in more detail in chapter 4. The B polysaccharide as we have seen, is weakly immunogenic even when coupled to a protein carrier producing primarily IgM type Ab (Skevakis *et al.*, 1984) for the various reasons already discussed. Interestingly, a modified GBMP- protein conjugate vaccine (Jennings & Roy, 1985) in which an additional methylene group has been added to the N-acyl sidechain (i.e. N-propionyl rather than N-acetyl), has succeeded in "tricking" the immune system. Antibodies generated are of the IgG isotype and have been found to be protective against *Neisseria meningitidis* serogroup B (Jennings *et al.*, 1989) and is currently in phase 1 clinical studies.

1.5 Thesis objectives

Ultimately, the objective of this thesis is to develop a semi-synthetic vaccine that would prove effective against the pathogens, *Neisseria meningitidis* serogroup B and *Escherichia coli* type K1. In work directed to this end goal, procedures will be worked out for the isolation of sialyloligosaccharides from natural sources, the synthesis of a bifunctional spacer and various structural analogs of the group B

meningococcal capsular polysaccharide, and novel methods of conjugation of oligo-/ polysialic acid to macromolecules such as proteins. Various studies will be conducted in order to gain a better understanding of the interaction of polysialic acid and the immune system including preliminary mapping of an antibody binding site of an antibody specific to *Niesseria meningitidis* serogroup B capsular polysaccharide and the determination of the fine specificities in structural requirements for this antibody.

Chapter 2 Preparation of Polysialic Acid Antigens

2.1 Introduction

The principal goal of this research project is to better understand the nature of the interaction between sialic acid or polysialic acid and the immune system and, with this greater comprehension, perhaps be able to manipulate these biological processes. The first step in trying to understand these complex and diverse processes is through a study of the immune system's army- namely the antibody molecule (Ab). Much information can be gained from studying the Ab combining site of an antibody directed against sialic or polysialic acid.

The group of Jennings and co-workers (1985) and Kabat and co-workers (1988) have both studied the common relationship between the number of sialic acid residues (DP) needed for maximum binding to two different Ab's specific to the capsular polysaccharide of group B meningococcus (GBMP). In both cases, it was found that the number of sialic acid residues was far greater than the expected 5-6 residues which was thought to be the limit to which the Ab binding site could accommodate (Kabat, 1960). This work has led to the valid conclusion that the epitope must be conformational in nature and is even presumed to be helical (Yamasaki & Bacon, 1991; Brisson *et al.*, 1992). Despite these conclusions, two important factors became evident. Both studies were carried out on pentameric (10 binding sites) horse IgM antibodies H.46 (Sarff *et al.*, 1975) and on a human monoclonal IgM^{NOV} antibody specific to GBMP (Kabat *et al.*, 1986). Results on the size

requirement for binding were achieved through competitive inhibition with discrete oligosaccharides in an ELISA assay. The significance of this can be seen when one compares the affinity vs. the avidity. Affinity is an intrinsic phenomena described by $K_a = \frac{[Ab\text{-}Ag]}{[Ab]\,[Ag]}$ where [Ab] is the concentration of a single binding site and [Ag] is the concentration of a single Ag epitope. Avidity on the other hand, describes the overall binding phenomena and is not an intrinsic value.

antibody	Fab	IgG	IgG	IgM
effective antibody valence	1	1	2	up to 10
antigen valence	1	1	n	n
equilibrium constant (L/M)	10^4	10^4	10^7	10^{11}
advantage of multivalence	—	—	10^3-fold	10^7-fold
definition of binding	affinity	affinity	avidity	avidity
	intrinsic affinity		functional affinity	

Fig. 2-1 Affinity and avidity. Multivalent binding between antibody and antigen (avidity or functional affinity) results in a considerable increase in stability as measured by the equilibrium constant, compared to simple monovalent binding (affinity or intrinsic affinity, here arbitrarily assigned a value of 10^4 L/M). This is sometimes referred to as the 'bonus effect' of multivalency. Thus there may be a 10^3-fold increase in the binding energy of IgG when both valencies (combining sites) are utilized, and a 10^7-fold increase when IgM binds antigen in a multivalent manner.

With a pentameric IgM (10 binding sites; cf. section 1.3.2) bound to a multivalent (many epitopes) polysaccharide antigen (as in the

competitive inhibition assay), avidity effects may dominate and dictate the behaviour of binding (fig. 2.1). Simply said, the possibility exists that a much larger oligosaccharide is needed in order to inhibit 50% binding to the native polysaccharide due to the need to offset the avidity effects.

In order to investigate further the relationship between the DP of Neu5Ac and H.46 IgM antibody, a study was initiated to elucidate as a first approximation, the direct binding of Neu5Ac oligosaccharides to the Ab, and eventually, to elucidate relative binding constants of the H.46 Ab to the oligosaccharides of increasing size. In order to achieve these ends, two criteria must be fulfilled-

(1) The need for a reasonable quantity of Neu5Ac oligosaccharides ranging from DP 1-15.

(2) The need for multivalent forms of the individual oligosaccharides.

Multivalent forms of the oligosaccharides are necessary for certain direct binding assays and can be achieved through the formation of protein glycoconjugates or acrylamide copolymers and will be discussed in a later section (c.f. chapter 4). A ready supply of pure oligosaccharides will allow entry into the determination of binding constants through classical techniques such as equilibrium dialysis (cf. chap.5), microcalorimetry (Sigurskold *et al.*, 1991), fluorescence studies (Glaudemans, 1991) or NMR techniques such as labelling experiments (Berman *et al.*, 1985; Kronis & Carver, 1985).

It has previously been mentioned that sialic acid oligosaccharides, in particular N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) oligosaccharides, are biologically

very important. They have been implicated in various forms of cancer, regulatory roles, as oncofetal and as tumour cell antigens, and in recognition phenomena. It is therefore also in our interest to obtain oligosaccharides of both Neu5Ac and Neu5Gc which will allow entry into the synthesis of complex gangliosides and neo-glycoproteins. These substrates have importance in serodiagnosis, modulation of cell-cell interactions, and as potential cancer vaccines.

The second step towards our study of the immunology of polysialic acid is by mapping the Ab combining site of an antibody specific to poly α-(2$\rightarrow$8) Neu5Ac. Most of the work studying the interaction of poly α-(2$\rightarrow$8) Neu5Ac with Ab over the last 20 years has been restricted to principally three different Ab's- a fact in itself which indicates the peculiar nature of poly α-(2$\rightarrow$8) Neu5Ac. The best known of the three Ab's is the H.46 horse IgM (Sarff *et al.*, 1975) specific to the capsular polysaccharide of *Neisseria meningitidis* serogroup B, although recently most of the research has been focused on the first and only monoclonal Ab of the IgG isotype specific to GBMP (Frosch *et al.*, 1985). The third Ab is a human monoclonal IgM Ab isolated from serum which is also specific to GBMP and oddly enough, to polynucleotides (Kabat *et al.*, 1986). The common feature with all three Ab's is that the polyanionic nature of the GBMP is essential for binding. Limited modifications of the N-acyl group are tolerated by either IgM but not by the IgG monoclonal (F. Michon, personal communication). Whether the carboxyl functionality is part of the Ab combining site or whether it functions to maintain the polysaccharide in a necessary spatial relationship, is still not completely clear (Michon *et al.*, 1987; Yamasaki & Bacon,

1991; Brisson *et al.*, 1992). Ongoing research such as X-ray crystallography of the Ab-Ag complex (D. Bundle, personal communication), or solution transferred NOE studies (J.Brisson, personal communication) are trying to gain a better insight into the conformational aspects of binding. It is thus our intention to synthesize a series of analogs of the GBMP in order to probe the fine specificity of the equine IgM Ab (H.46). We hope to gain valuable insight into the structural requirements of the Ab combining site and at the same time, it will afford us with a source of *de novo* substrates to create new and potentially useful artificial immunogens.

2.2 Results and Discussion

2.2.1 Part A- Sialyloligosaccharides

2.2.1.1 Thin layer chromatography of sialyloligosaccharides

Due to the acid lability of the glycosidic linkage of polysialic acid, mild acid hydrolysis was employed to generate oligosaccharides of both colominic acid (poly α-(2→8) Neu5Ac) (**2-1**) and glycolyl colominic acid (poly α-(2→8) Neu5Gc) (**2-2**) (fig. 2.2). Classical methods employ hydrolysis in 0.1M H_2SO_4 or HCl at 80 °C for 60-90 min. (Schauer & Corfield, 1982), however substantial amounts of higher oligosaccharides (DP>5) are not abundant. We deemed it worthwhile to undertake a comprehensive study to optimize hydrolytic conditions that will enable us to maximize the yield of any desired oligosaccharide (DP 1-20). Preliminary conditions used were those of Jennings *et al.* (1985) where colominic acid was hydrolyzed at pH 2 with HCl at 80 °C for 1 hr. It was in our interest to also set up TLC conditions that would enable us to visualize the individual oligosaccharides and to estimate the extent of hydrolysis. Various TLC conditions were tried (such as propanol:H_2O (Schauer & Granger, 1982) or butanol:propanol:0.1M HCl (Svennerholm, 1958), however the solvent system of propanol:H_2O:25% NH_4OH in the ratio of 6:2:0.5 was found to give the best resolution.

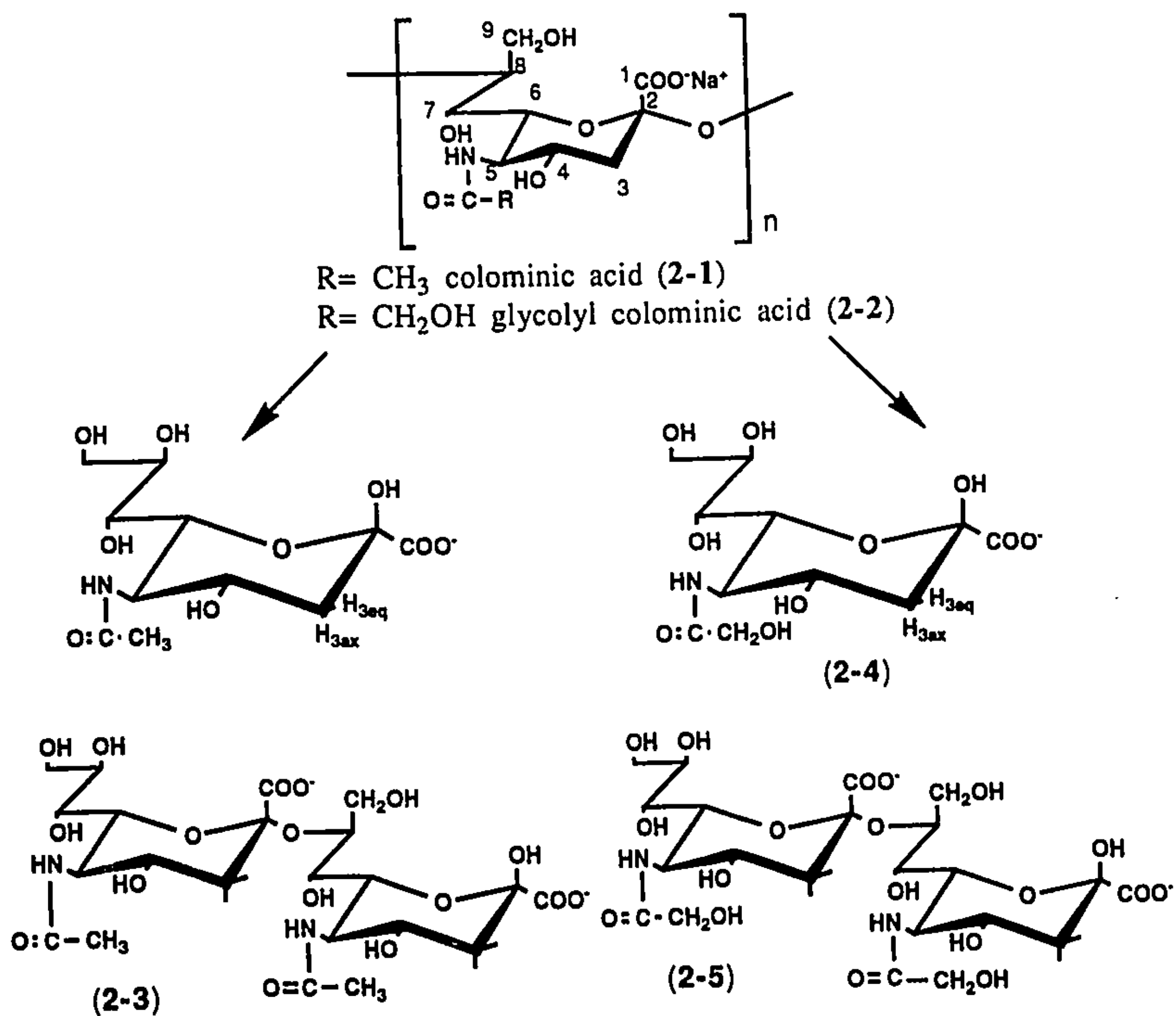


Figure 2.2- Important sialic acid mono and disaccharides.

Double elution of 20 cm plates and visualization with either resorcinol or phosphomolybdate spray, allowed detection of oligosaccharides up to DP 16 (fig.2.3).

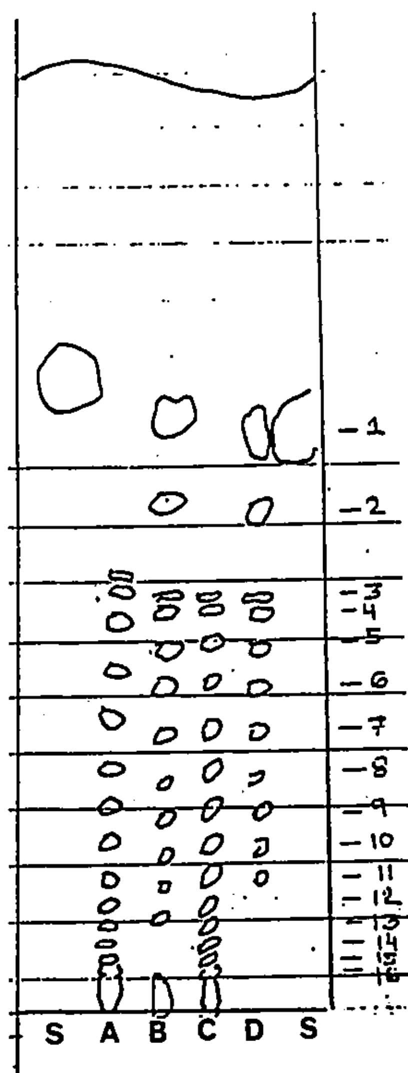


Figure 2.3- TLC of hydrolyzed colominic acid doubly eluted in n-propanol:H_2O:25% NH_4OH (6:2:0.5) and visualized with resorcinol reagent.

Spraying of the plates with water and preferential drying of the oligosaccharides allowed an estimate of the amount of each oligosaccharide relative to one another. It was realized after obtaining these results, that a TLC- direct binding study may be viable. In order to carry out a direct binding study, some manner of immobilizing the oligosaccharides to the silica plate must be performed. Successful examples employ the use of amino bonded silica gel HPTLC plates (Tang *et al.*, 1985), where the resolved

oligosaccharides were reductively aminated to the plate, followed by an overlay of an enzyme linked specific Ab. Alternatively, resolved oligosaccharides have been converted to neo-glycolipids (Stoll *et al.*, 1988) and due to their hydrophobic properties, are effectively immobilized onto silica gel during subsequent treatment with Ab's. Since we would like to see how many Neu5Ac residues are required to bind with H.46 IgM antibody, a TLC method such as those described may prove useful and warrants further investigation.

2.2.1.2 Colominic Acid- Hydrolysis Study

It was decided that the best approach to study the rate of hydrolysis of colominic acid was through a colourimetric comparison of the total to reducing sialic acid ratio. Although a number of colourimetric assays exist for the determination of total sialic acids (Schauer & Corfield, 1982), the method of choice is the resorcinol/ Cu^{2+} assay (Svennerholm, 1957) due to its simplicity, selectivity, and reasonable sensitivity. The mechanism for the chromophore production is complex and is still not well understood. The strong acid conditions promotes glycosidic cleavage, de-N-acetylation, and decarboxylation followed by rearrangements to yield a species capable of condensing with resorcinol (Aronson & Peters, 1976). Complexation with Cu^{2+} gives rise to a coloured chromophore (blue) with an absorption maximum at 580 nm. The assay is linear over a broad range and is sensitive to a limit of ~10 ug (32 nmol) Neu5Ac.

Reducing analysis is based on the reducing potential of the terminal ketal where Fe^{3+} is reduced to Fe^{2+} according to the method of Park and Johnson (1949). Complexation of Fe^{2+} with ferric

ammonium sulfate yields a chromophore (green) with a maximum absorption at 690 nm. The method is sensitive (5-30 ug or 16-100 nmol), however it is prone to variability and it is recommended that results be obtained in quadruplicate in order to get a reliable value.

The total/ reducing assay was applied successfully in the determination of the average molecular weight of two different sources of colominic acid. Colominic acid from Sigma Chemicals was found to have an average of 25 Neu5Ac residues per chain of polysaccharide or an average molecular weight of 8300 g/mol while colominic acid from Nacalai Tesque was significantly larger with an average molecular weight of 11500 g/mol or 35 Neu5Ac/ chain of polysaccharide. This result is significant when trying to optimize conditions for higher oligosaccharides. The total/ reducing ratio can also be used to verify the degree of polymerization of various oligosaccharides especially those where NMR techniques are unreliable (i.e. DP> 10).

Colominic acid (av. molecular weight 11.5 Kd) (**2-1**) was subjected to various hydrolytic conditions in which pH and temperature were varied. At various time intervals, aliquots were taken and subjected to an average chain length determination as described above. From this information, we were able to set up hydrolytic conditions optimized for any desired oligosaccharide from DP 1-25 (figure 2.4).

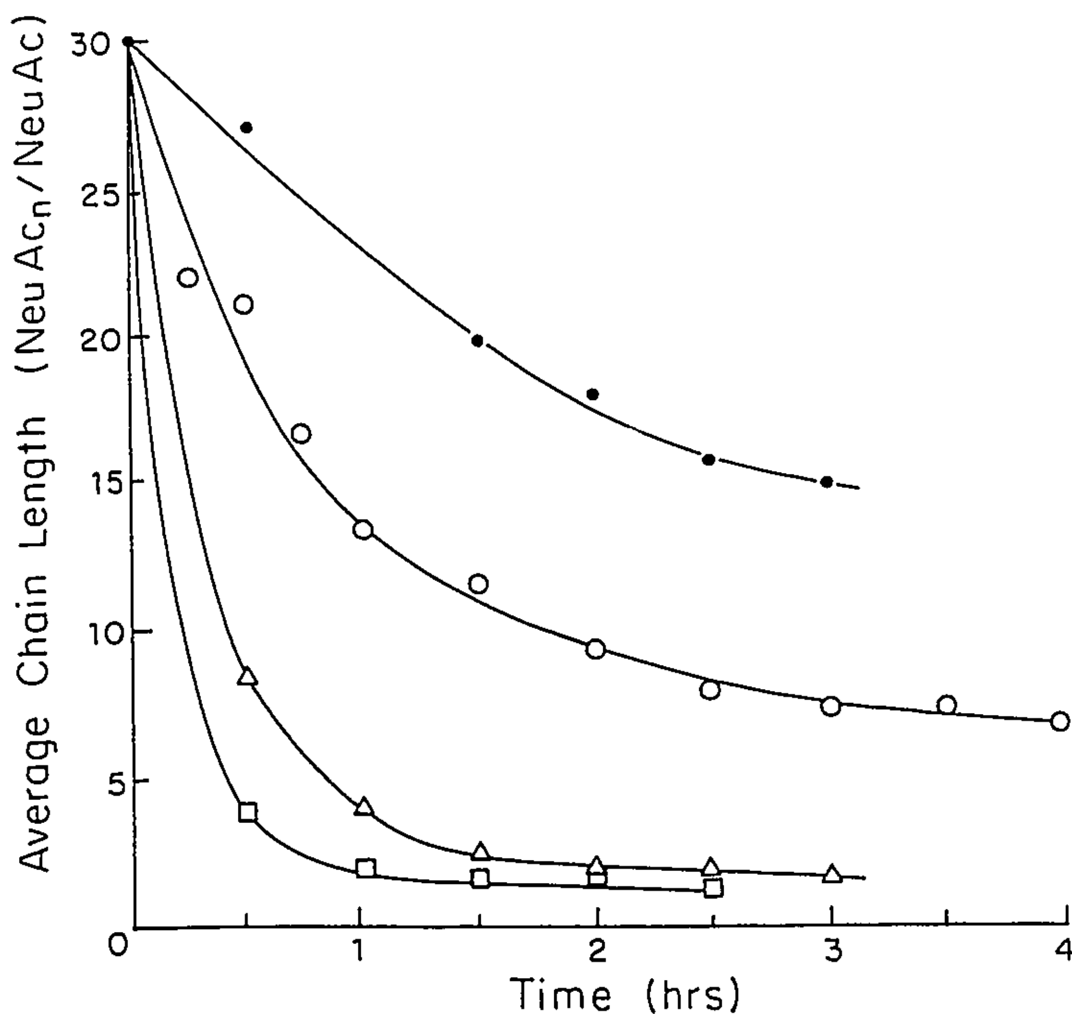


Figure 2.4- Rate of acid hydrolysis of colominic acid at: pH 7.0, 100 °C (•); pH 3, 50 °C (o); pH 3.0, 70 °C (Δ); pH 2.0, 80 °C (°)

A direct application of this study was the production of appreciable amounts of the α-(2→8) Neu5Ac disaccharide (**2-3b**) from an optimized hydrolysis (pH 3.0/ HCl, T=70 °C, T=3 hr) of the parent Neu5Ac homopolymer (colominic acid). The conditions were such that the disaccharide was isolated in 28% recovered yield after purification by gel permeation chromatography on a Biogel P-10 column in 0.03M NH_4HCO_3 (fig. 2.5).

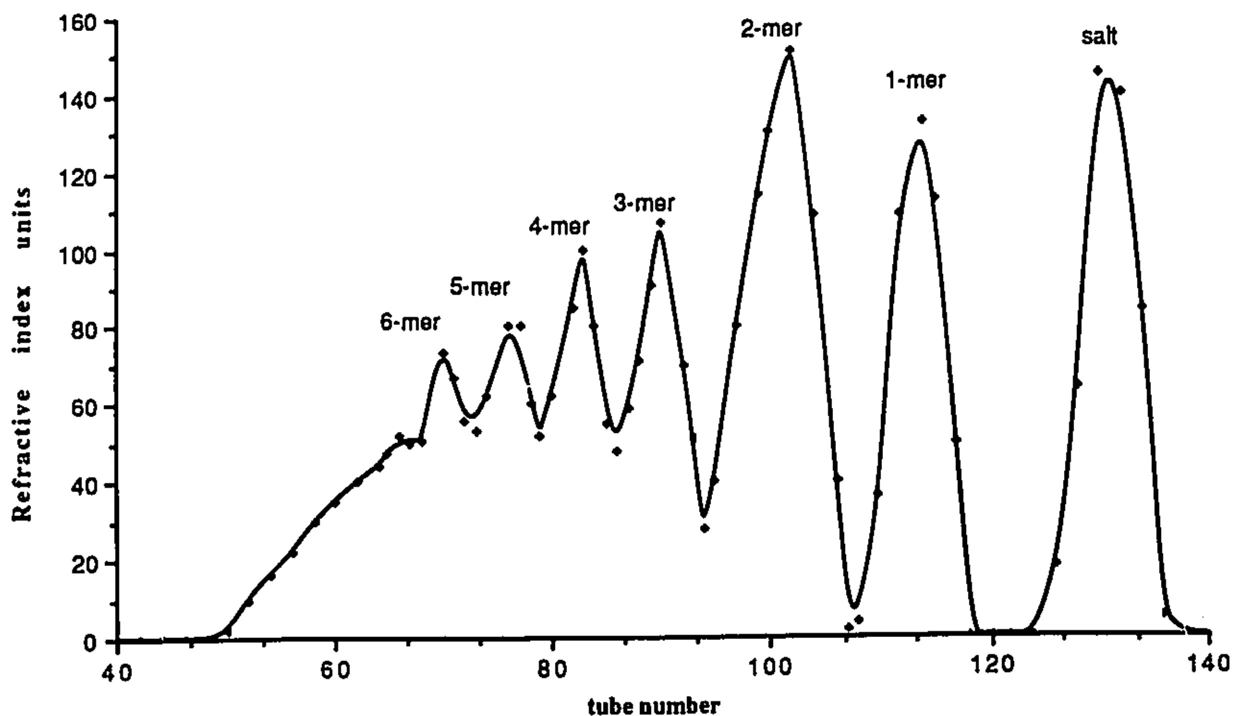


Figure 2.5- Fractionation of acid hydrozylate (pH 3, 70 °C, 3h) of colominic acid by size exclusion chromatography on a Biogel P-10 column in 0.03M NH_4HCO_3.

All spectroscopic analyses (FAB-MS, $[\alpha]_D$, TLC) proved unambiguously the presence of the pure Neu5Ac disaccharide (**2-3**) as demonstrated by the high resolution ^{1}H- and ^{13}C-NMR spectrums (fig.2.6 and tables 2-1 and 2-2).

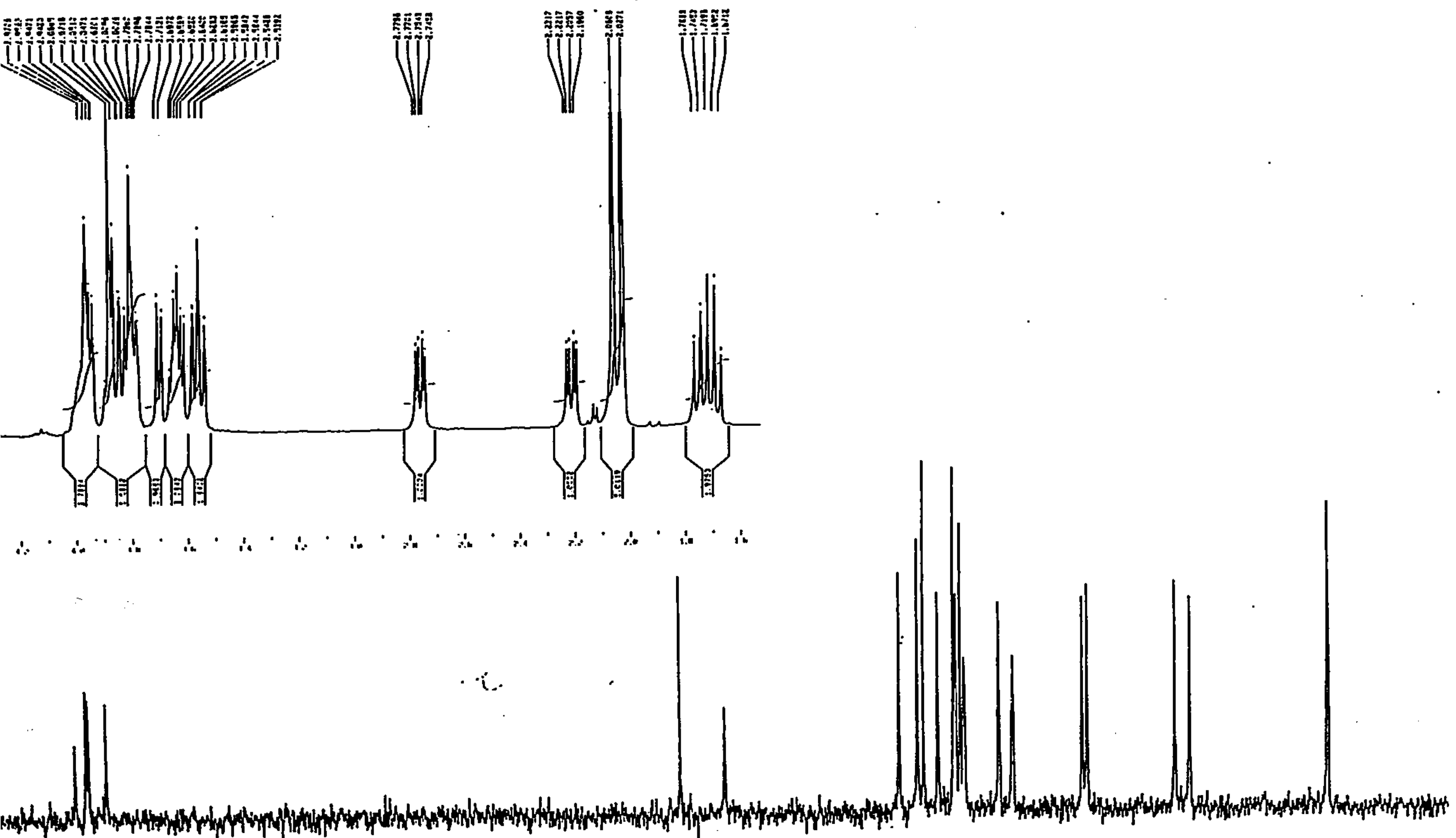


Figure 2.6- ^{1}H NMR and ^{13}C NMR spectra of $(Neu5Ac)_2$

Table 2-1

Partial ^1H NMR[a] chemical shifts of Neu5Ac and Neu5Gc mono & disaccharides in D_2O at 300 K

Neu5Ac[b] proton	non-reducing	reducing residue	Neu5Gc proton	monomer (a)	non-[c] reducing	reducing residue
$H\text{-}3_{ax}$	1.728 (m) 1H	1.728 (m) 1H	$H\text{-}3_{ax}$	1.852 (dd) 1H	1.697 (m) 1H	1.697 (m) 1H
$H\text{-}3_{eq}$	2.222 (dd) 1H	2.770 (dd) 1H	$H\text{-}3_{eq}$	2.233 (dd) 1H	2.720 (dd) 1H	2.210 (dd) 1H
CH_3	2.068 (s) 3H	2.035 (s) 3H	CH_2OH	4.143 (s) 2H	4.057 (s) 2H	4.098[d] (s) 1H 4.090[c] (s) 1H

a : referenced to HOD at 4.756 ppm

b : 500 MHz NMR shifts

c : 300 MHz NMR shifts

d : geminal protons

Table 2-2

^{13}C NMR (75.4 MHz) chemical shifts[a] of Neu5Ac and Neu5Gc mono & disaccharides in D_2O at 300 K

Neu5Ac carbon	non-reducing	reducing residue	Neu5Gc carbon	monomer	non-reducing	reducing residue
C-1	173.6	177.1	C-1	177.5	173.3	177.5
C-2	102.9	97.3	C-2	97.2	102.6	97.4
C-3	41.9	40.1	C-3	40.2	41.9	40.1
C-4	69.3	67.8	C-4	67.8	71.0	67.8
C-5	52.7	53.3	C-5	52.8	52.2	52.9
C-6	73.8	71.2	C-6	70.8	73.3	70.9
C-7	68.9	68.4	C-7	69.3	68.7	68.0
C-8	73.0	76.0	C-8	71.2	72.9	76.2
C-9	63.6	61.9	C-9	64.1	63.3	61.8
C=O	175.8[b]	175.5[b]	C=O	176.3	176.3[b]	176.2[b]
CH_3	23.1	23.1	CH_2OH	61.8	61.8	61.8

a : In ppm from internal acetone (31.07 ppm)

b : Tentative assignments.

Having a ready supply of this disaccharide is particularly relevant since the α-(2→8) linkage is extremely difficult to obtain synthetically. Apart from the elegant synthesis of Goto (Okamoto *et al.* ,1986, 1988), the α-(2→8) Neu5Ac disaccharide remains synthetically elusive. Recently, it was demonstrated that the Neu5Ac disaccharide obtained in this manner, could be efficiently transformed into a useful glycosyl donor (Abbas *et al.*, 1990) leading toward the synthesis of complex gangliosides (Diakur and Roy, 1991) and neo-glycoproteins. These may find potential applications in cancer diagnosis and treatment as either immunotherapeutics or *de novo* vaccines.

Besides N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) is the most ubiquitous of the sialic acids. Commercial natural sources of the very important monosaccharide are quite expensive (10 mg/ US$ 100.00) and the α-(2→8) linked disaccharide was not available at the time of this work. Both of these substrates are biologically very important as previously outlined in the introductory comments. We were able to take advantage of the α-(2→8) linkage of colominic acid (**2-1**) in the role of a blocked glycoside to convert it to the homopolymer of α-(2→8) Neu5Gc (**2-2**) (*vide infra* for a more complete discussion). Hydrolysis of the Neu5Gc homopolymer (N-glycolyl colominic acid) under the same conditions set forth for the optimized disaccharide hydrolysis of colominic acid met with a similar success. Purification of the oligosaccharides by gel filtration on a Biogel P-10 column in 0.03 M NH_4HCO_3 afforded the monosaccharide in 38% and the disaccharide in 32% overall yield.

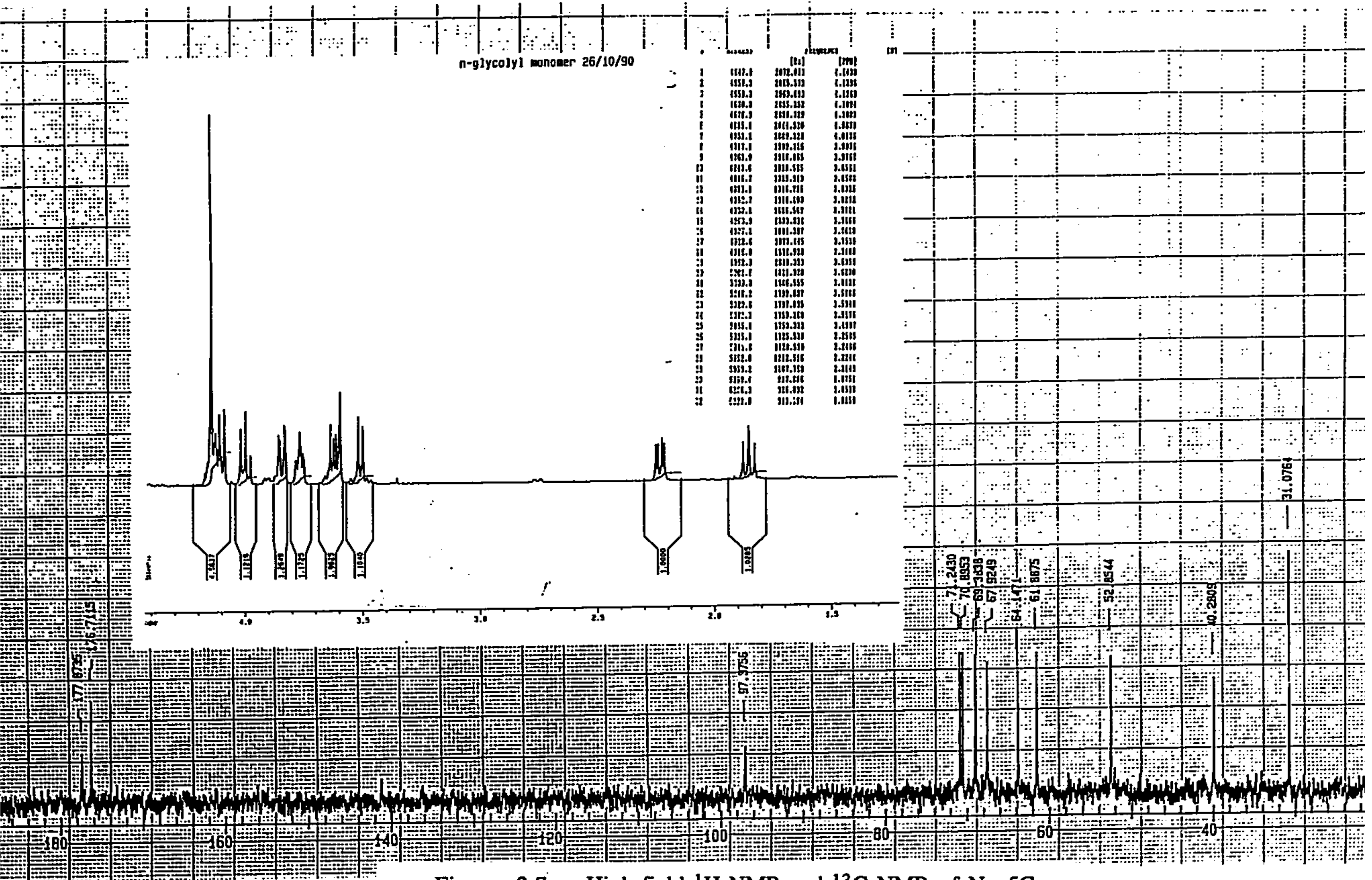


Figure 2.7- High field ^{1}H NMR and ^{13}C NMR of Neu5Gc monosaccharide

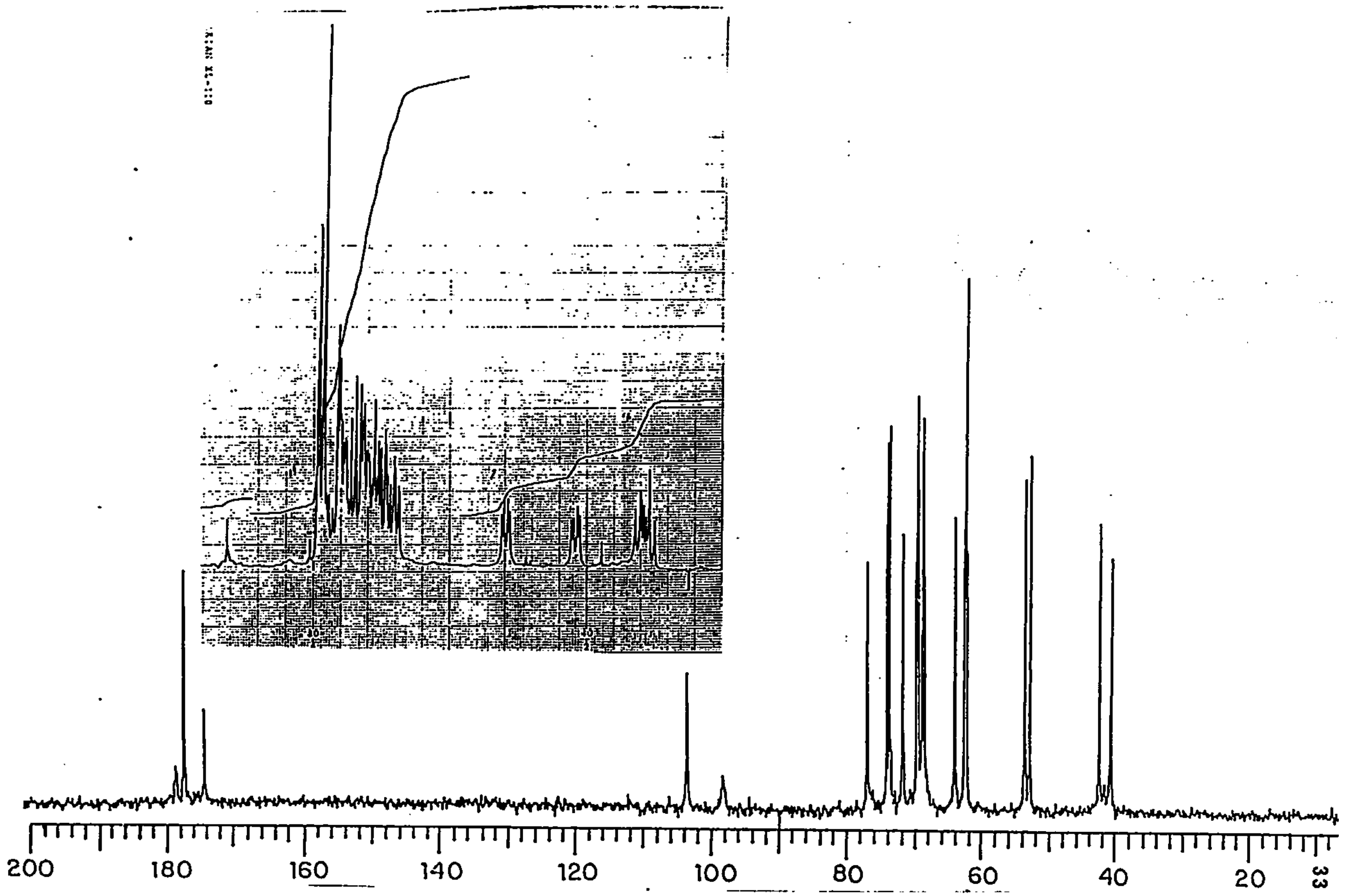


Figure 2.8- High field ^{1}H NMR and ^{13}C NMR of $(Neu5Gc)_2$.

Physical data for the monosaccharide (Neu5Gc) (**2-4**) were in complete agreement with a commercial sample (Sigma Chemicals) based on TLC, FAB-MS $[\alpha]_D$, ^{1}H- and ^{13}C NMR (Schauer, 1982). FAB-MS, and ^{1}H- and ^{13}C NMR data were also consistent for the isolation of the pure Neu5Gc dimer (**2-5**) and the purity of each oligosaccharide is demonstrated by their high field NMR spectrums (fig. 2.7 and 2.8 and tables 2-1 and 2-2). An alternate method of producing the monosaccharide from N-glycolyl colominic acid was developed (Roy & Boratynski, 1990) in which the polysaccharide was enzymatically hydrolyzed by an exo-neuraminidase in a membrane enclosed enzymatic catalysis (MEEC) system. The enzyme, which acts solely on the terminal non-reducing end, produces the dialyzable product which is isolated in pure form. The overall cost of producing Neu5Gc monosaccharide from either of the above two methods is approximately 50-100 times less than isolation from natural sources whereas the disaccharide's value is undefined due to its extreme rarity in mg amounts.

2.2.1.3 Isolation of sialic acid oligosaccharides

Isolation of sialic acid oligosaccharides was achieved in two general ways:

(1) Anion exchange chromatography

(2) Gel permeation chromatography

The degree of polymerization or size of the oligosaccharides needed dictated which method was to be employed. For oligosaccharides with DP> 5 anion exchange was the only possibility while gel permeation chromatography was the method of choice for smaller

oligosaccharides due to the ease of use and shorter column times required.

Mixtures of sialyloligosaccharides could be separated into individual components on a DEAE-Sephadex anion exchange (Cl^-) column according to the method of Nomonto *et al.*, (1982), with applications to polyNeu5Ac by Jennings and co-workers (1985). Elution of the column with an increasing NaCl gradient afforded oligosaccharides up to a DP of 16 (fig. 2-9).

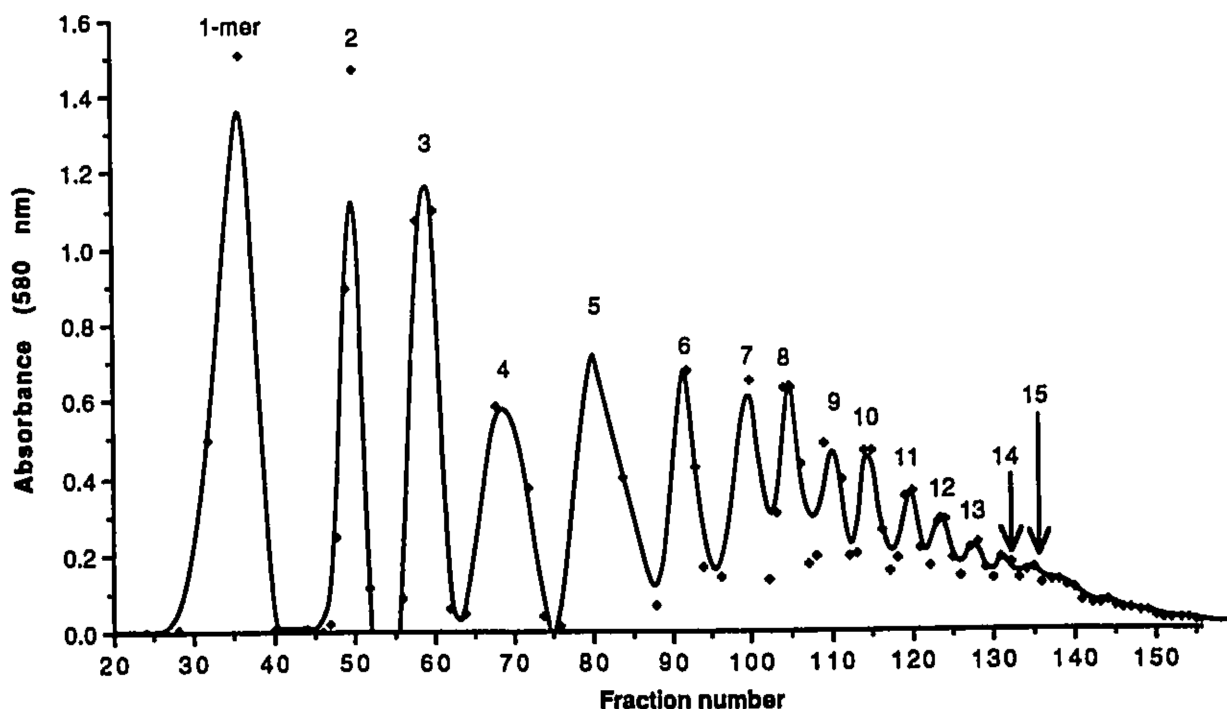


Figure 2.9- Acid hydrolysis (pH 2, 80 °C, 1hr) profile of colominic acid on a DEAE Sephadex A-25 column using a Tris(HCl)-NaCl gradient system.

The separation is time consuming- 3 days for the column elution followed by individual desalting and lyophilization of the

oligosaccharide fractions (~2 weeks overall). The advantages are the good resolution and reproducible results. The purification of sialyloligosaccharides could be improved by using a gradient of pyridinium acetate buffer as opposed to the Tris(HCl)-NaCl buffer system previously used (Jennings *et al.*, 1985). It was found that elution of the oligosaccharides with a linear gradient of 0.05M-1.85M pyridinium acetate (pH 5.4) yielded a resolution similar to the Tris(HCl)-NaCl method with the added advantage that the tedious desalting stage of the oligosaccharide fractions is omitted due to the volatility of the buffer (fig. 2.10).

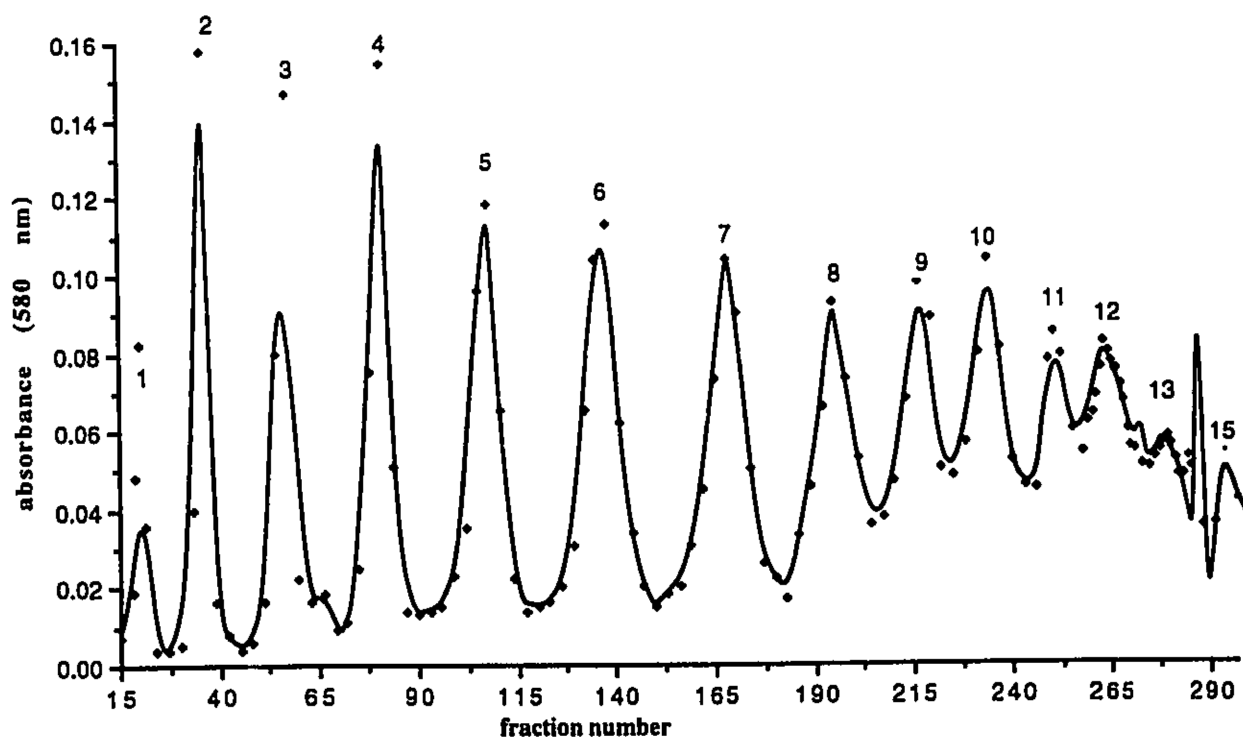


Figure 2.10- Acid hydrolysis (pH 7, 100 °C, 2hr) profile of colominic acid on a DEAE Sephadex A-25 column using a pyridinium acetate gradient system.

For long term storage of the oligosaccharides, it is advisable to change the counter ion to sodium by titration with NaOH since the pyridine salt tends to decompose over time leaving behind the acid form. Since the oligosaccharides elute sequentially, it is only necessary to verify the identity of one oligosaccharide in order to assign the others. This can be done either by ^{1}H NMR analysis of the N-acetyl heterogeneity at 2.07 ppm or integration of the reducing H-3 proton (2.22 ppm) to the remaining H-3 protons (2.6-2.8 ppm) or by colourimetric determination of the total sialic acid / reducing sialic acid ratio as previously described. The latter method enables the identification of oligosaccharides that are not readily determined by ^{1}H NMR spectroscopy.

For separation of a large amount of smaller sialyloligosaccharides (Dp< 5), the method of choice is gel extrusion chromatography. Three different matrices were tried each with a different molecular weight cutoff. It was found that overall resolution up to DP 5 was best achieved on a Biogel P-10 column (mol. wt. cutoff 100 KD) (fig.2.5). Again the use of a volatile buffer removes the need for a desalting stage and simplifies the purification. Interestingly, when one compares the molecular weights of the fractionated oligosaccharides, it is found that the apparent weight is much larger than that predicted by either neutral oligosaccharides or globular proteins. Indeed this is a general phenomenon encountered repetitively with pore diffusion techniques (dialysis, SDS-PAGE, etc.) and will be discussed in more detail (*vide infra*).

2.2.2 Part B- Analogs of colominic acid

As mentioned previously, the capsular polysaccharide of *Neisseria meningitidis* serogroup B (GBMP) and *Echerichia coli* K1 (colominic acid) are structurally (Bhattacharjee *et al.*, 1975; Liu *et al.*, 1971) and serologically (Grados & Ewing, 1970; Kasper *et al.*, 1973) identical both being homopolymers of α-(2$\rightarrow$8) Neu5Ac residues. Since colominic acid is commercially available, it is a convenient and appropriate starting material in which to make derivatives that represent analogs of GBMP.

The structure of the repeat unit of colominic acid logically leads to two areas that are well suited for derivatization (fig. 2-11):

(1) modifications at the carboxyl functionality.

(2) modifications of the N-acetamido functionality.

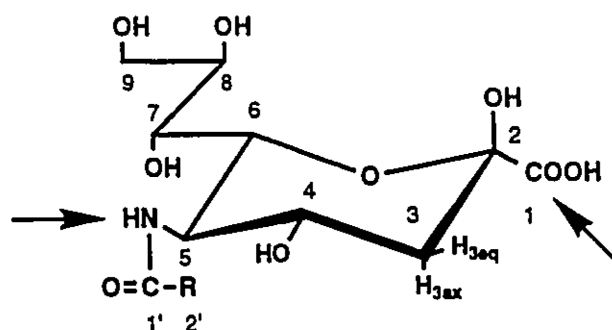


Figure 2.11- Derivatization points of sialic acid.

Modifications of the N-acetamido functionality

Analogs of the N-acetamido functionality were designed to satisfy two criteria:

(1) the effect of hydrophobic vs. hydrophilic substituents.

(2) the effect of increasing substituent size. With these goals in mind, the fully de-N-acetylated colominic acid (**2-6**) was considered as a useful precursor which would yield, in a divergent manner, an array of analogs (fig. 2-12).

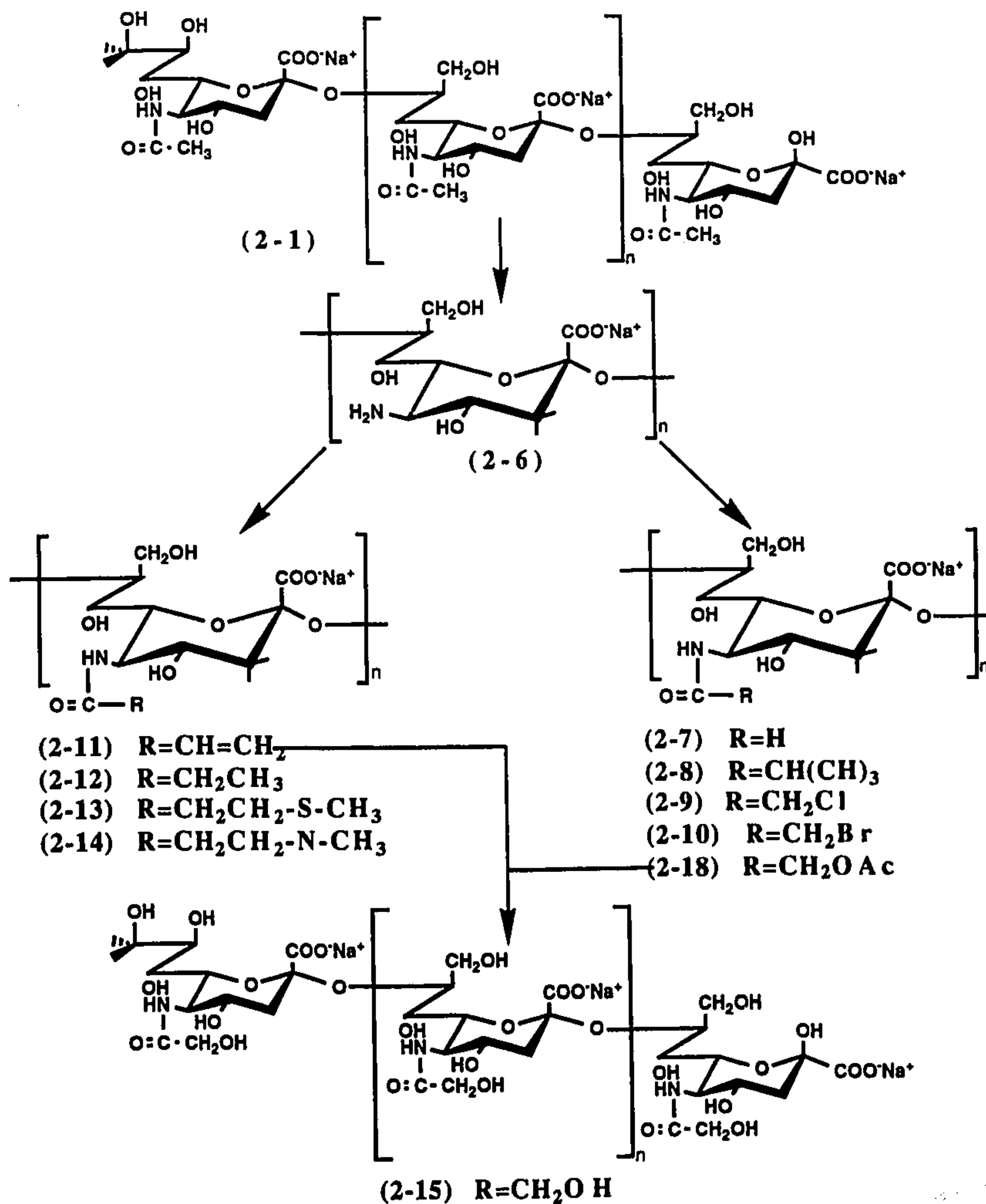


Figure 2.12- N-Acyl analogs of colominic acid.

Table 2-3

^{1}H NMR Chemical Shifts (ppm) in D_2O[1] at 300 K of Colominic Acid Analogs

Compound	H-3a	H-3e	H-4	H-5	H-6	H-7	H-8	H-9	H-9'
Colominic acid (2-1)[2]	1.74 (t)	2.67 (d)	3.60 (t)	3.82 (t)	3.63 (m)	3.90 (s)	4.10 (s)	4.19 (d)	3.66 (m)
De-N-Ac colominic acid (2-6)[3]	1.80 (t)	2.67 (d)	3.6 (bd)	2.98 (t)	3.56 (bd)	3.89 (s)	4.05 (s)	3.74 (d)	3.60 (bd)
N-Formyl colominic acid[3] (2-7)[4]	1.78 (bd)	2.67 (d)	3.80 (bd)	3.9 (d)	3.8 (bd)	3.95 (s)	4.16 (bd)	4.16 (bd)	3.8 (bd)

1: From internal acetone (2.225 ppm) or HOD (4.756 ppm) at 300 K

2: 500 MHz NMR shifts, N-Ac (2-1): singlet at 2.08 ppm.

3: 300 MHz NMR shifts

4 : N-CHO: (syn and anti); 8.03 (s) and 8.26 (s) ppm.

Table 2-3 (contn'd)

Compound	H-3a	H-3e	H-4	H-5	H-6	H-7	H-8	H-9	H-9'	N-Acyl group			
										RCH	RCH_2	RCH_3	RNH-NH_2
N-Isobutanoyl[1] colominic acid (2-8)	1.75 (bd)	2.66 (bd)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.55 (bdq)	-	1.1 (bd)	-
N-Chloroacetyl[2] colominic acid (2-9)	1.71 (bd)	2.68 (bd)	n.a.	n.a.	n.a.	n.a.	4.1 (bd)	4.1 (bd)	n.a.	-	4.23 (s)	-	-
N-Bromoacetyl[2] colominic acid (2-10)	1.74 (bd)	2.68 (bd)	n.a.	n.a.	n.a.	n.a.	4.1 (bd)	4.1 (bd)	n.a.	-	n.a.	-	-
N-Acryloyl colominic acid (2-11)[2]	1.74 (t)	2.61 (d)	~3.7 (bd)	~3.7 (bd)	~3.7 (bd)	3.83 (s)	~4.05 (bd)	~4.05 (bd)	~3.7 (m)	5.62 (d)	6.08 (m)	-	-
N-Propionyl[3] colominic acid (2-12)	1.74 (t)	2.65 (d)	~3.7 (bd)	3.82 (d)	~3.7 (bd)	3.90 (s)	~4.2 (bd)	~4.2	~3.7 (bd)	-	2.34 (q)	1.16 (t)	-

1: 200 MHz NMR shifts.

2: 300 MHz NMR shifts.

3: 500 MHz NMR shifts.

Table 2-3 contn'd.

Compound	H-3a	H-3e	H-4	H-5	H-6	H-7	H-8	H-9	H-9'	N-Acyl group			
										RCH	RCH_2	RCH_3	$RNH-NH_2$
S-Methyl propionyl[1] colominic acid (2-13)	1.7 (bd)	2.6 (bd)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	3.7 (bd) 2.6 (bd)	2.05 (s)	-
N-Methyl propionyl[1] colominic acid (2-14)	1.7 (bd)	2.6 (bd)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	3.27 (bdt) 2.8 (bd)	2.71 (s)	-
N-Glycolyl colominic[2] acid (2-15)	1.72 (t)	2.71 (d)	3.68 (bd)	3.87 (bd)	3.71 (bd)	3.86 (bd)	4.12 (s)	4.19 (s)	3.70 (bd)	-	-	4.10 4.21 (m)	-
O-Acetoxy-N-glycolyl colominic acid[1] (2-18)	1.71 (bd)	2.62 (bd)	3.7 (bd)	3.9 (bd)	3.7 (bd)	3.8 (bd)	4.1 (bd)	4.1 (bd)	3.8 (bd)	-	4.71 (s)	2.75 (s)	-

[1]: 200 MHz NMR shifts.

[2]: 500 MHz NMR shifts.

Table 2-3 contn'd.

Compound	H-3a	H-3e	H-4	H-5	H-6	H-7	H-8	H-9	H-9'	NAc	Carboxyl group		
											RCH_2	RCH_3	$RNH-NH_2$
Carboxyl reduced[1] colominic acid (2-21)	1.71 (t)	2.42 (d)	3.89 (m)	3.89 (m)	3.74 (bd)	3.74 (bd)	4.11 (s)	3.97 (d)	3.74 (bd)	2.06 (s)	3.81 3.81 (bds)	-	-
Colominic acid hydrazide[1] (2-22)	1.93 (t)	2.81 (d)	n.a.	3.88 (d)	3.74 (bd)	n.a.	n.a.	3.96 (d)	3.75 (bd)	2.05 (s)	-	2.05 (s)	2.90 (m)
Colominic acid[2] hydroxylamine (2-23)	1.92 (bd)	2.75 (bd)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.05 (s)	-	2.05 (s)	-

1: 500 MHz NMR shifts.

2: 300 MHz NMR shifts.

Table 2-4

^{13}C NMR Chemical Shifts (ppm) in D_2O[1] at 300 K of Colominic Acid Analogs

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	N-Acyl region[2]				
										C-1'	C-2'	C-3'	C-4'	C-5'
Colominic acid[3] (**2-1**)	173.9	101.8	40.7	69.2	53.3	74.0	70.0	78.7	62.1	175.7	23.4	-	-	-
De-N-Ac colominic acid[3] (**2-6**)	174.1	102.0	40.8	67.7	54.3	73.7	70.2	78.0	62.1	-	-	-	-	-
N-Isobutanoyl[4] colominic acid (**2-8**)	173.8	102.1	41.1	68.9	53.0	74.1	69.9	78.0	62.2	183.0	36.2	19.8	-	-
N-Chloroacetyl[4] colominic acid (**2-9**)	174.5	102.0	40.8	68.8	53.8	73.8	70.1	78.4	62.2	171.6	43.4	-	-	-
N-Bromoacetyl[4] colominic acid (**2-10**)	174.4	102.1	40.9	68.9	53.9	74.0	70.1	78.1	62.2	171.8	29.3	-	-	-

1: from internal acetone (31.07 ppm) or dioxane (67.4 ppm)

2: C-1' corresponds to the N-acyl carbonyl; C-2'-C-5' represent consecutive atoms from C-1'.

3: 75.4 MHz NMR shifts

4: 50.3 MHz NMR shifts

Table 2-4 contn'd.

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	N-Acyl region[1]				
										C-1'	C-2'	C-3'	C-4'	C-5'
N-Acryloyl colominic acid[2] (2-11)	174.1	102.0	40.3	69.3	53.3	74.1	70.5	78.0	62.2	170.0	130.6	129.0	-	-
N-Propionyl[2] colominic acid (2-12)	174.0	101.8	40.9	69.1	53.1	74.0	70.0	78.5	62.1	179.7	30.1	10.2	-	-
S-Methyl propionyl[3] colominic acid (2-13)	174.2	102.2	40.8	69.1	53.2	73.7	70.4	77.9	62.1	176.6	36.4	30.0	-	15.1
N-Glycolyl colominic acid[4] (2-15)	174.1	101.6	40.9	68.5	53.2	73.7	70.0	78.5	62.1	176.7	62.1	-	-	-

[1]: C-1' corresponds to the N-acyl carbonyl; C-2'-C-5' represent consecutive atoms from C-1'.

[2]: 75.4 MHz. NMR shifts.

[3]: 50.3 MHz NMR shifts.

[4]: 125.7 MHz NMR shifts

Table 2-2 contn'd.

										N-Acyl region[1]				
O-Acetoxy-N-glycolyl colominic acid[2] (2-18)	174.4	101.9	40.8	68.9	53.3	73.9	70.0	78.4	62.0	171.9	63.8	-	177.1	21.0
Carboxyl reduced[2] colominic acid (2-21)	60.9	102.4	38.4	68.4	53.5	73.2	69.0	73.9	62.1	175.6	23.1	-	-	-
Colominic acid[3] hydrazide (2-22)	167.4	102.8	40.8	66.9	52.7	75.1	67.1	75.8	60.9	175.6	22.9	-	-	-
Colominic acid[2] hydroxylamine (2-23)	166.6	102.5	40.6	68.7	52.5	74.0	67.1	75.5	61.6	175.6	22.9	-	-	-

1: C-1' corresponds to the N-acyl carbonyl; C-2'-C-5' represent consecutive atoms from C-1'.

2: 50.3 MHz NMR shifts.

3: 125.7 MHz NMR shifts.

2.2.2.1 De-N-acetylated colominic acid (2-6)

The low molecular weight colominic acid (**2-1**) (11 KD) was treated with 2M NaOH/ $NaBH_4$ at 110 °C for 7 hr in a sealed tube according to the procedure of Jennings & Roy (1985) for the GBM polysaccharide. The rate of hydrolysis of the N-acetyl group was followed as a function of time (fig. 2-13) and behaved in a similar manner to GBMP (Jennings *et al.*, 1986).

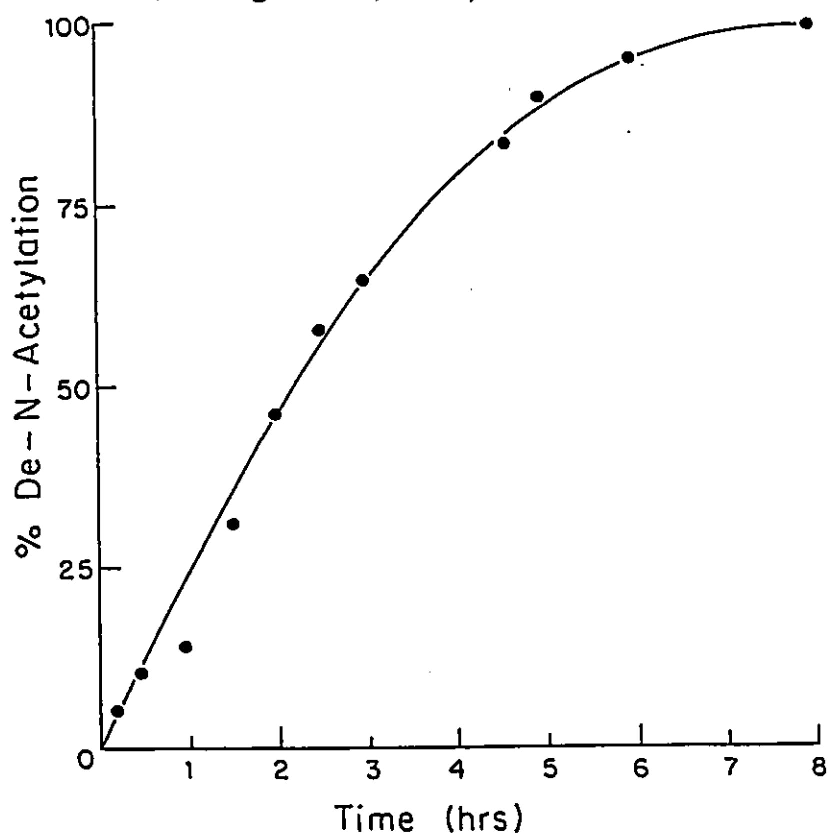


Figure 2.13- Rate of de-N-acetylation of colominic acid in 2M NaOH at 110 °C.

The rate of hydrolysis (de-N-acetylation) was determined by ^1H NMR spectroscopy (300 MHz, D_2O) upon integration of residual N-

acetamido protons (2.08 ppm) vs. either $H\text{-}3_{ax}$ (1.76 ppm) or $H\text{-}3_{eq}$ (2.64 ppm). As de-N-acetylation proceeded, the ^{1}H NMR spectrum (table 2-3) revealed the increasing appearance of an overlapping doublet of doublets at 2.98 ppm which is attributable to H-5 of (**2-6**) and represents an upfield shift of 0.84 ppm. Integration of this new peak vs. either of the H-3 protons provided a further check on the rate of hydrolysis vs. time. The product (**2-6**) after 7 hr showed complete disappearance of the N-acetamido protons and a new peak at 2.98 ppm integrating 1:1 with either H-3 proton. Complete de-N-acetylation was further characterized by the disappearance of both carbon resonances of the acetamido group in ^{13}C NMR (50.3 MHz; D_2O) (table 2-4). The C-5 chemical shift in (**2-6**) is shifted downfield (1.0 ppm) relative to (**2-1**) with an accompanying upfield shift of 1.5 ppm for C-4. This large upfield shift is not seen in C-6 and hence may represent a possible hydrogen bond between the acetamido carbonyl and the C-4 hydroxyl of (**2-1**)- not unlike a situation found in the monosaccharide (Czarnieki & Thornton, 1977). The poly amino derivative (**2-6**) was isolated in 82% yield† and represents an analog of GBMP that exhibits increased hydrophilicity as well as introduces a positively charged region into the molecule when protonated. We were now in a position to synthesize an array of N-acyl derivatives generally formed by reacting (**2-6**) with the desired acid chloride or acid anhydride.

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† : yields are calculated based on the molecular weights of the monomeric repeating unit.

2.2.2.2 N-Formyl colominic acid (2-7)

The de-N-acetylated polysaccharide (**2-6**) was reacted with a mixed anhydride of formic acid and acetic acid thereby taking advantage of the more electrophilic formyl carbonyl to give the pure poly N-formyl colominic acid (**2-7**) in 88% yield. The ^{1}H NMR spectrum of **2-7** (300 MHz; D_2O, table 2-3) revealed the characteristic formyl resonances at 8.03 and 8.26 ppm (Hirano & Yazi, 1980) in a 2:8 ratio integrating for 1 proton, reflecting the syn and anti conformation of the formyl proton. The H-5 proton of **2-7** shifted downfield by ~0.9 ppm with the concomitant disappearance of ninhydrin positive material, suggested the complete derivatization of **2-6**. This derivative is interesting since it possesses the carbonyl but does not have a hydrophilic or hydrophobic extension and binding studies will yield significant information on the importance of the carbonyl alone relative to the N-acetylated polysaccharide (**2-1**).

2.2.2.3 N-Isobutanoyl colominic acid (2-8)

The title compound (**2-8**) was synthesized in a straight-forward manner by treating (**2-6**) with an excess of isobutyric anhydride at 0 °C in an aqueous alkaline medium (NaOH). The extent of the reaction was estimated through the disappearance of ninhydrin positive material. The product was isolated in 85% yield (based on the monomer), and by ^{1}H NMR (300 MHz; D_2O) and ^{13}C NMR (50.3 MHz; D_2O) spectroscopy (table 2-3 and 2-4) proved unambiguously the formation of **2-8**. The methine proton at 2.55 ppm (broad quartet) and the two methyl groups at 1.1 ppm (broad

doublet) were indicative of the isobutanoyl group. These protons integrated in the proper proportions to either the H-3_{eq} (2.66 ppm) or H-3_{ax} (1.75 ppm) protons denoting 100% derivatization. The ^{13}C NMR (50.3 MHz; D_2O) spectrum corroborated the formation of **(2-8)** with peaks at 36.2 and 19.8 ppm for the methine and methyl carbons respectively. Interestingly, the isobutanoyl carbonyl as well as several ring or exocyclic carbons show modest shift differences relative to the parent polysaccharide **(2-1)**. It is postulated that the bulky group has steric interactions with the carbonyl group (C-1) and/ or the hydroxyl group at C-9 (Baumann *et al.*, unpublished results). In this respect, the synthesis of **(2-8)** well represents an analog of **(2-1)** that is sterically hindered in the N-acyl region.

2.2.2.4 Halogenated Analogs (2-9) & (2-10)

A series of halogenated derivatives were thought to present an interesting angle relevant to the antibody binding study. Acylation of the primary amine of **(2-6)** with trifluoroacetic anhydride in aqueous conditions was met with limited success due to the rapid decomposition of the anhydride to it's homologous acid. Much more success came from acylation of the amine with chloroacetyl chloride or bromoacetyl bromide which yielded derivatives **(2-9)** and **(2-10)** respectively where one acetamido proton is replaced by either a large chlorine or an even larger bromine atom. The acylation reactions proved to be tricky since elevated pH's led to heterogeneous products- resulting presumably from a substitution of the halogen by the hydroxide ions present. Evidence for this came from treatment of 2-bromoacetyl colominic acid **(2-10)** with either

NaOH or NH_4OH with the subsequent formation of two distinct products apart from the starting materials. The NaOH treated material gave a product whose ^{13}C NMR chemical shifts matched those of poly N-glycolyl colominic acid (**2-15**) (see below) albeit the product was heterogeneous. Treatment of **2-10** with NH_4OH yielded a product whose ^{1}H NMR spectrum (table 2-3) showed a new singlet at 3.27 ppm consistent for a O=C-CH_2-NH_2 group. It is also interesting to note that when employing an acetate buffer to maintain a given pH, treatment of **2-6** with either bromoacetyl bromide or chloroacetyl chloride produced mixed bromoacetyl/ acetyl (65:35) or chloroacetyl/ acetyl (55:45) derivatives. This result is obviously due to the formation of the mixed anhydride and the % N-acetylation reflects the reactivity of each acid halide. So under mild pH conditions (pH~ 8-8.5), the amino polysaccharide (**2-6**) was effectively acylated with either chloroacetyl chloride or bromoacetyl bromide to give the N-chloroacetyl derivative (**2-9**) in 84% yield or the N-bromoacetyl derivative (**2-10**) in 66% yield. The ^{1}H NMR spectrum (200 MHZ, D_2O) of **2-9** (table 2-3) was characterized by the appearance of a broad singlet at 4.23 ppm integrating for two protons relative to either H-3 protons. This is consistent for a methylene group located between an amide and a chlorine atom (Silverstein *et al.*, 1974). Similarly, ^{13}C NMR analysis of **2-9** (table 2-4) allowed the assignment of the resonance at 43.4 ppm to the methylene carbon α to the chlorine atom. Further evidence came from a ^{13}C-ADEPT spectrum analysis that showed conclusively that this peak was indeed a methylene. The ^{1}H NMR spectrum (200 MHz; D_2O) of the bromo derivative (**2-10**) was not very informative since

the new methylene resonance is expected to appear amongst the ring protons. Integration of the ring and exocyclic peaks relative to the H-3 protons did show however the presence of two additional protons due to the bromomethylene group. ^{13}C NMR and ADEPT analysis of **2-10** (table 2-4) was much more conclusive with the somewhat odd yet characteristic appearance of the methylene carbon α to a bromine at 29.3 ppm. This heavy halogen upfield shift is due to the "softness" or polarizabilty of the halogen and is most pronounced with Br relative to Cl (Abraham & Loftus, 1981). It is worth noting that comparison of the carbon resonances of **2-9** & **2-10** with the isobutanoyl derivative (**2-8**), shows some similar trends which may reflect once again the steric bulk at this position.

2.2.2.5 N-Acryloyl colominic acid (2-11)

The acryloyl derivative (**2-11**) proved to be one of the key analogs synthesized due to its ability to act as as precursor for a number of other analogs, its ability to be easily radiolabelled for use in radioimmunoassays (RIA) or ^{3}H NMR binding studies, or its intrinsic reactivity allowing for novel methods of synthesizing glycoconjugates (c.f. chap. 4). The derivative (**2-11**) was prepared by treating **2-6** with a slight excess of a dioxane solution of acryloyl chloride at 0 °C while maintaining the pH between 10-11 with NaOH. The extent of the reaction was monitored in the usual way through the disappearance of residual amine (ninhydrin). It was deemed necessary to let the reaction mixture sit at a high pH (~11) for a short period of time (30 min.) in order to remove O-esters that were detected with non- treated material (^{1}H NMR- heterogeneity of the

olefinic region and new peaks in the 4.5-5 ppm region). After workup by dialysis, lyophilization produced the desired product (**2-11**) in 94% yield. It was found convenient to combine the de-N-acetylation/ re-N-acryloylation steps in a one pot reaction sequence without the concomitant isolation of the intermediate (**2-6**). ^{1}H- and ^{13}C NMR (tables 2-3 and 2-4) were completely consistent for the formation of the title product (**2-11**). The ^{13}C NMR spectrum showed the characteristic signals of N-acryloyl residues. The carbonyl carbon was shifted upfield by ~5 ppm to 170 ppm consistent with the formation of an α,β unsaturated system, and the olefinic methine and methylene carbons at 130.6 and 129.0 respectively. ^{1}H NMR analysis showed resonances centered at 6.08 (broad multiplet; 2H's) and 5.62 ppm (broad doublet; 1H) consistent with the olefinic methylene and methine protons respectively. Integration of the olefinic protons vs. the H-3 ring protons clearly showed that the derivatization was complete.

2.2.2.6 N-Propionyl colominic acid (2-12)

The preparation of N-propionyl colominic acid (**2-12**) has been accomplished via acylation of the de-N-acetylated polysaccharide (**2-6**) with propionic anhydride (Jennings & Roy, 1985) and has been the subject of extensive studies (Kabat *et al.*, 1988; Jennings *et al.*, 1989; Lifely & Esdaile, 1991; Baumann *et al.*, unpublished results) due to its improved immunogenicity vs. the native colominic acid. In our hands, N-propionyl colominic acid (**2-12**) was prepared in quantitative yield via reduction of the double bond under atmospheric hydrogen over 10% palladium on charcoal as catalyst.

The ^{1}H- and ^{13}C NMR spectroscopic data (tables 2-3 and 2-4) were completely consistent with the literature values (Jennings *et al.*, 1986; Baumann *et al.*, unpublished results) and were characterized by the loss of the olefinic carbons (130.6 and 129.0 ppm) and olefinic protons (5.62- 6.08 ppm) with the appearance of two upfield carbon shifts at 30.1 and 10.2 ppm corresponding to the new methylene and methyl protons respectively. The synthesis of **2-12** in this way, represents a roundabout route relative to the direct acylation of **2-6**, however it demonstrates the applicability of introducing a radiolabel (i.e. $^{3}H_2$) at this position in high yields.

2.2.2.7 Michael-type adducts of N-acryloyl colominic acid

The presence of the conjugated double bond of the acryloyl derivative (**2-11**) led to the investigation of its use as a Michael-type acceptor. Two different nucleophiles of varying strength- namely the thio methoxide ion and a less powerful methylamine, were added to an aqueous alkaline solution of **2-11** (pH 9.5) and allowed to react overnight. Following dialysis and lyophilization, the 3-thiomethyl propionyl (**2-13**) and the 3-aminomethyl propionyl (**2-14**) derivatives of colominic acid were produced in 70% and 80% yield respectively. Addition in both cases took place exclusively at the β carbon, as judged by the appearance in the ^{1}H NMR spectrum of **2-13** (table 2-3) of two new peaks at 3.7 and 2.6 ppm integrating for two methylene protons each. These correspond to the methylene protons α to the carbonyl and the methylene protons α to the S-methyl group respectively resulting from the conjugate addition. Complete disappearance of the olefinic protons also substantiated the

conjugate addition. Integration of the S-methyl peak at 2.05 or either of the two new methylene groups vs. the H-3 protons clearly showed 100% modification of the N-acryloyl starting material. The ^{13}C NMR spectrum (table 2-4) corroborated the interpretation of the ^{1}H NMR spectrum with the appearance of two new methylene carbons from an ADEPT analysis at 36.4 and 30.0 ppm. The spectrum was also characterized by the S-methyl carbon appearing at 15.1 ppm. The pattern and interpretation of the NMR analysis for **(2-14)** paralleled that found for **(2-13)** with the two new methylene peaks appearing at 3.27 (2H) and 2.80 (2H) ppm and the N-methyl peak at 2.05 (3H) ppm. The good reactivity of the acryloyl functionality towards the strong sulfur nucleophile is not unexpected however, the quantitative addition of the weaker nitrogen nucleophile is pleasing since it allows entry into novel glycoconjugates of polysialic acid through the abundant ε-amine groups of the lysine residues in proteins (cf. chap.4).

2.2.2.8 N-Glycolyl colominic acid (2-15)

The most prevalent form of polysialic acid is the N-acetylneuraminic acid (Neu5Ac) homopolymer with either the α-(2→8) (GBMP; colominic acid) **(2-1)** or α-(2→9) (Group C meningococcal polysaccharide- GCMP) **(1-5)** linkages. The biologically relevant N-glycolylneuraminic acid (Neu5Gc) **(1-1b)** has until recently been found as a monomeric constituent of complex oligosaccharides usually in the terminal position (Higashi, 1990). Recently however, glycoproteins composed primarily of poly Neu5Gc **(2-2)** have been found in a number of *Salmonidae* fish eggs (Troy,

1992; Iwasaki *et al.*, 1990; Inoue *et al.*, 1986). The degree of polymerization of the poly Neu5Gc chains of these novel glycoproteins range from 4~25 residues. Although poly Neu5Gc has not as yet been found in human tissues, it is commonly known that the presence of Neu5Gc monosaccharides is generally related to cancerous tissues. A synthetic version of poly Neu5Gc (**2-15**) would be useful not only as an analog for binding studies, but would also provide a ready source of the potentially relevant polysaccharide from which a large supply of the biologically important oligosaccharides could be obtained. The synthesis of poly Neu5Gc (glycolyl colominic acid) (**2-15**) from colominic acid (**2-1**) was accomplished in two different manners of which the first method was dictated by the lack of suitable glycolylating reagents such as 1,3-dioxoan-2,4-dione or glycolic acid anhydride. Advantage was taken of the N-acryloyl colominic acid substrate (**2-11**), since reductive ozonolysis of the double bond should yield the desired product. The feasibility of this synthetic route in an aqueous medium was checked using a synthetic model compound, 1,6-di-N-acryloyl hexanediamine (**2-16**) (fig. 2-14).

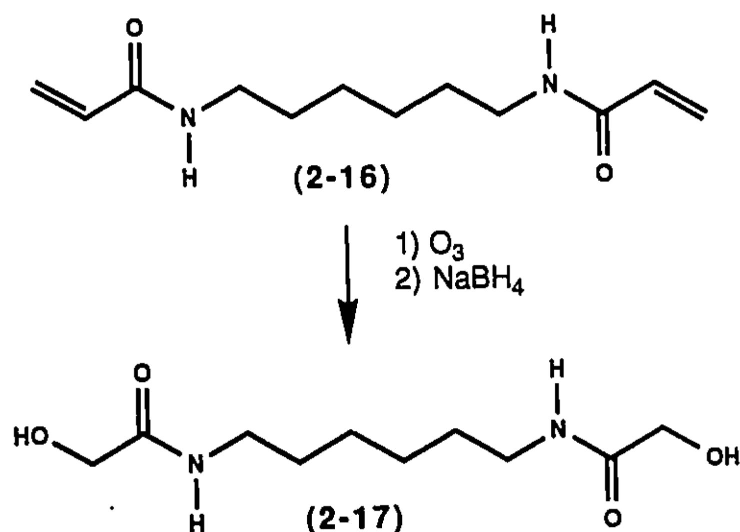


Figure 2.14- Model compound for reductive ozonolysis study.

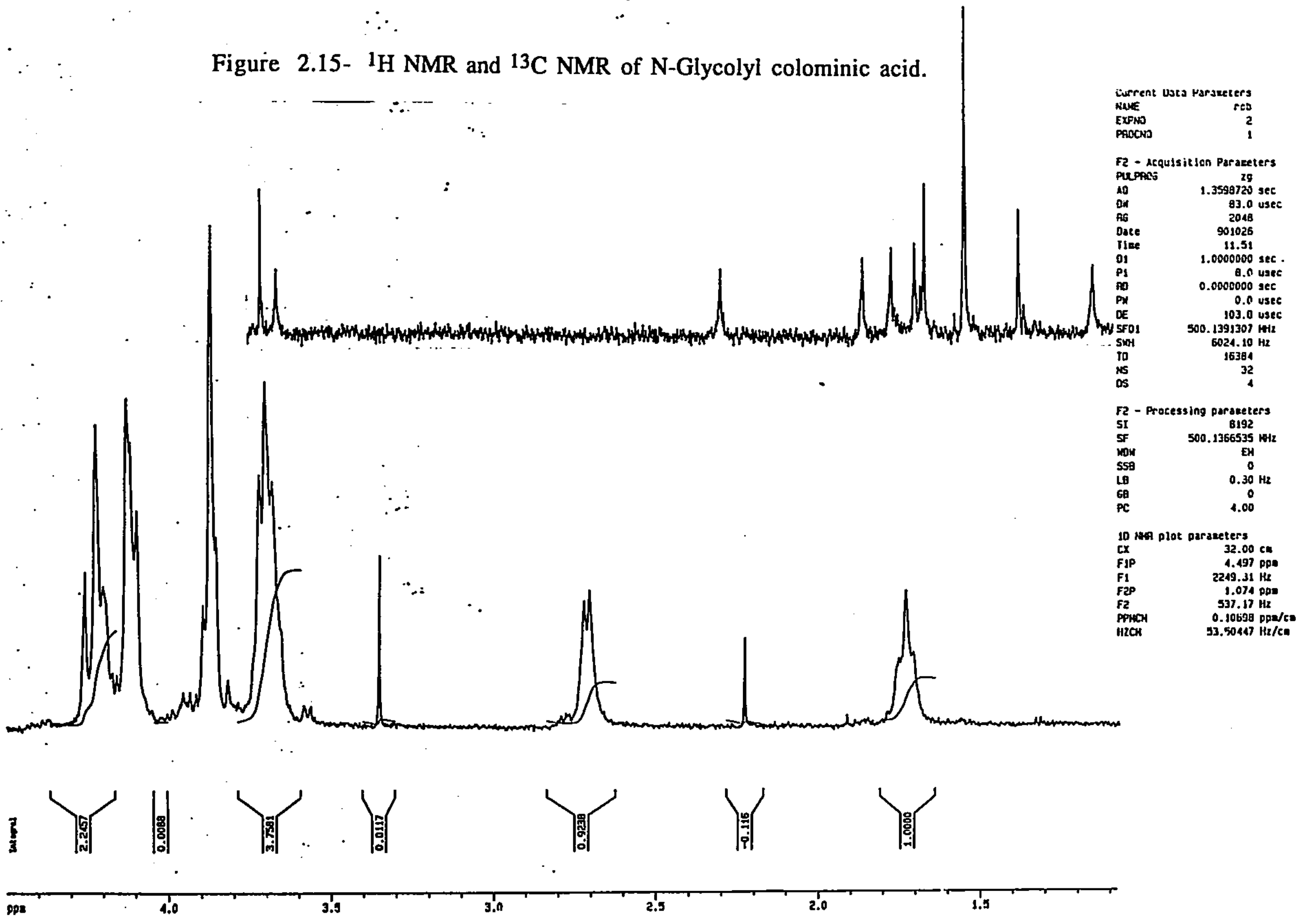
Indeed from ^{1}H NMR, ^{13}C NMR and FAB mass spectrometry, the desired product (**2-17**) was obtained in a straight forward manner and in high yield. Following the successful results obtained with the model compound (**2-16**), a water:methanol mixture (2:1, -20 °C) of the N-acryloylated polysaccharide (**2-11**) was treated with ozone for 20 minutes. The usual blue tinge indicating excess O_3 normally seen in methanol or other organic solvents, was not apparent in the water:MeOH mixture. The O_3 treatment time was estimated by trial and error by determining any residual N-acryloyl groups present after complete workup (^{1}H NMR spectroscopy). The ozonide formed was immediately reduced *in situ* with excess $NaBH_4$ and after suitable reaction time and purification, gave poly N-glycolyl colominic acid (**2-15**) in 78% yield. ^{1}H- and ^{13}C NMR spectroscopic features are identical to those described below in the alternate synthesis of **2-15**.

During the course of this study, the commercial availability of the reagent acetoxyacetyl chloride (Aldrich) allowed for a second and more direct approach to the synthesis of **2-15**, although the latter method was more flexible in that a radiolabel could be easily introduced (i.e. NaB^3H_4 reduction). Thus de-N-acetylated colominic acid (**2-6**) was treated under pH controlled conditions with an aqueous dioxane solution of acetoxyacetyl chloride (~50 eq) at 0 °C. The extent of reaction was estimated through the disappearance of amine positive material in the usual manner (ninhydrin). Maintaining the pH at 7-7.5 with NaOH at 0 °C and careful workup of the solution, yielded the C-2' O-acetylated glycolyl colominic acid (**2-18**) in 67% yield. This was characterized in its ^1H NMR spectrum (table 2-3) by the appearance of the O-acetyl signal at 2.15 ppm and the downfield appearance of a new peak at 4.71 ppm (2H's) corresponding to the C-2' methylene protons. The structure of **2-18** was further verified by ^{13}C NMR spectroscopy and ADEPT analysis, with the appearance of resonances arising from the new carbonyl carbon (C-4', 177.1 ppm), a new methylene carbon (C-2', 63.8 ppm) and the acetoxy methyl carbon (C-5', 21.0 ppm).

The N-glycolyl colominic acid derivative (**2-15**) was obtained by treatment of **2-18** either *in situ* or after purification with NaOH at a pH of ~12 for 2 hr in order to hydrolyze all the ester linkages. The derivative (**2-15**) was isolated in 89% overall yield from **2-6** after dialysis and lyophilization. Strong evidence for the successful synthesis of this biologically important polysaccharide is found in the ^1H NMR and ^{13}C NMR spectra of the polysaccharide (fig. 2.15). The ^1H NMR spectrum of **2-15** differed from that of **2-18** by the

disappearance of the peaks at 4.71 and 2.15 ppm consistent with the loss of the O-acetyl group. The ^{13}C NMR spectrum also showed the loss of the O-acetyl signal as judged by the disappearance of a carbonyl peak at 177.1 ppm and the acetoxy methyl carbon at 21.0 ppm. Coincidentally, the new hydroxymethyl carbon (C-2') appears at the same shift as the C-9 hydroxymethyl carbon at 62.1 ppm. Further structural proof for **2-15** came from its degradation into oligosaccharides and their subsequent characterizations (cf. section 2.2.1).

Figure 2.15- ^{1}H NMR and ^{13}C NMR of N-Glycolyl colominic acid.



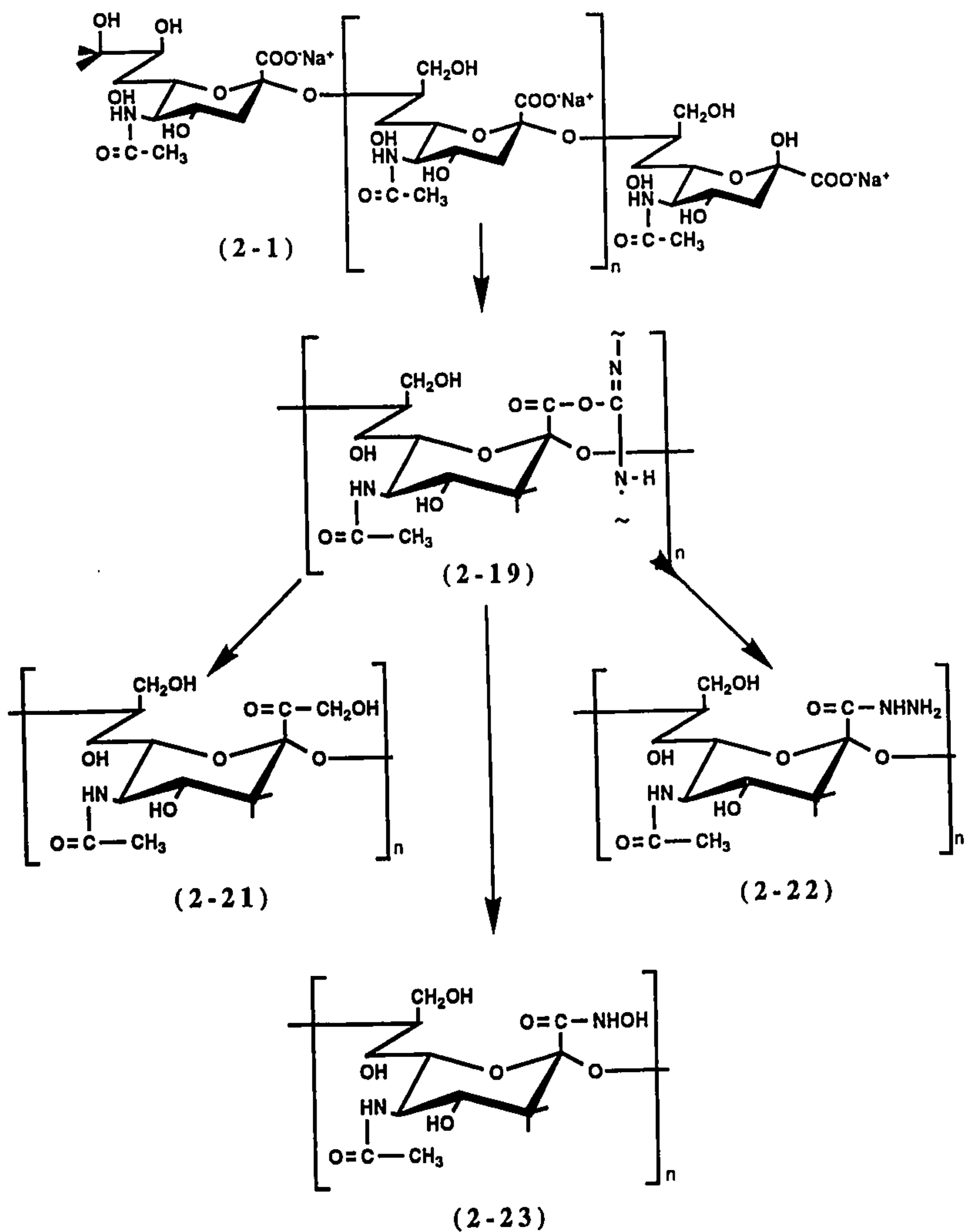


Figure 2.16- Analogs of carboxyl modified colominic acid.

Modifications of the carboxyl functionality

The carboxyl terminus of the GBMP and the *E. coli* K1 antigen (colominic acid) have been the subject of much research (Brisson *et al.*, 1992; Yamasaki *et al.*, 1991; Lifely & Esdaile, 1991; Kabat *et al.*, 1988; Michon *et al.*, 1987). From this work, the unique properties of the GBM or *E. coli* K1 capsular polysaccharides have all been attributed to the carboxyl functionality although the interpretations vary somewhat. It was in our interest to modify the carboxyl group of colominic acid in order to ascertain how pertinent its effect is upon binding with the horse IGM (H.46) antibody. The various carboxyl modified derivatives were all synthesized via activation of the carboxyl terminus (**2-19**) with the water soluble carbodiimide EDC (fig.2.16). The active ester intermediate was formed by titrating the polysaccharide at its pK_a (4.75) in the presence of the carbodiimide. Complete carboxyl activation was ascertained by a levelling off of the rising pH. Another phenomenon encountered was the precipitation of the activated colominic acid upon treatment with EDC. This can be accounted for by the formation of an internal lactone (**2-20**) between the C-9 hydroxyl and the carboxyl group of the adjacent sialic acid residue (fig. 2.17). The resulting lactone is insoluble in water and its properties have been extensively studied (Lifely *et al.*, 1981; 1984).

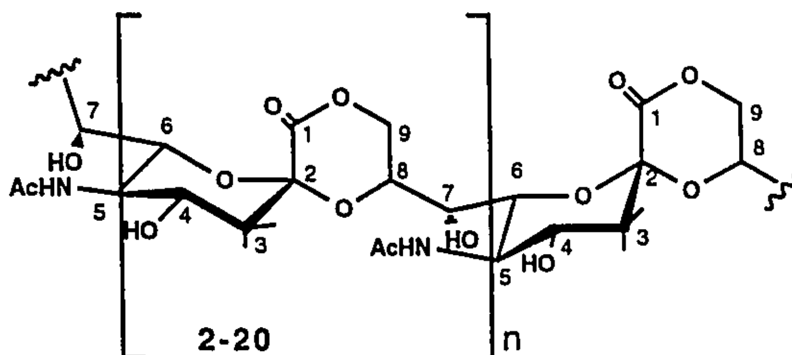


Figure 2.17- Colominic acid lactone.

2.2.2.9 Carboxyl reduced colominic acid (2-21)

The carboxylate groups of colominic acid were reduced into primary alcohols according to the method of Conrad & Taylor (1972) by reducing the EDC active ester (**2-19**) with $NaBH_4$. In order for complete reduction to occur it was found that a second treatment of the polysaccharide was generally necessary. The yield of the reduced material (**2-21**) was 85% based on the monomeric units. The reduced polymer was characterized primarily by the appearance of a new peak at 60.9 ppm consistent with a primary hydroxy methylene group. A study directed at elucidating the conformation of the reduced derivative (**2-21**) in relation to the native polysaccharide has allowed the complete assignment of the high field ^{1}H- and ^{13}C NMR spectrum (table 2-3 & 2-4) (Baumann *et al.*, unpublished results). The C-1 hydroxy methylene protons were found amongst the ring protons at 3.81 ppm. Preliminary evidence from this study has indicated that the reduced polymer lacks much of the ordered conformations of the native polysaccharide due to the removal of the charge.

2.2.2.10 Colominic acid hydrazide (2-22)

To compliment the synthesis of the neutral polysaccharide (**2-21**) described above, it was in our interest to synthesize other carboxyl modified analogs with varying properties. The synthesis of colominic acid hydrazide (**2-22**) was accomplished by treating the active ester of colominic acid (**2-19**) with hydrazine. Subsequent work-up furnished the hydrazide (**2-22**) in 73% yield. In the ^{1}H NMR spectrum (table 2-3), the H-3_{ax} proton was shifted downfield by 0.2 ppm to 1.93, and likewise the H-3_{eq} proton was shifted downfield by 0.14 ppm to 2.81 relative to the native polysaccharide. This shift is presumably due to an inductive effect of the hydrazide. A small broad peak at 2.90 ppm integrating for ~0.3 protons was tentatively assigned to the α-NH proton of the hydrazide group. The ^{13}C NMR spectrum (table 2-4) revealed a 6.5 ppm upfield shift of the C-1 carbonyl which is consistent for the formation of a hydrazide (Johnson & Jankowski, 1972). There were also large differences in the C-7, C-8, and C-9 shifts relative to colominic acid. These may be accounted for by a change in the conformation of the hydrazide derivative relative to the native colominic acid due perhaps to the partial positive charge that exists at physiological pH's (pK_a~7). Further evidence for the complete derivatization of (**2-1**) was the colourimetric determination of 0.93 moles of hydrazide (TNBS assay)/ mole of sialic acid (resorcinol assay).

2.2.2.11 Hydroxamic acid of colominic acid (2-23)

Treatment of (**2-19**) with hydroxylamine at pH 9.5 yielded the hydroxamic acid of colominic acid (**2-23**) in 46% yield. Complete derivatization was ascertained by the disappearance of the C-1

carboxyl carbonyl at 173.9 ppm in the ^{13}C NMR spectrum (table 2-4) with the concomitant appearance of a lower field (166.6 ppm) resonance corresponding to the hydroxamic acid carbonyl. Much of the same shift differences seen in the hydrazide derivative (**2-22**) were also seen in the hydroxamic acid derivative leading to the assumption that both polysaccharides should behave identically in serological testing. In the ^{1}H NMR, again the H-3_{ax} (1.92 ppm) and H-3_{eq} (2.75 ppm) were shifted downfield by 0.2 and 0.1 ppm respectively relative to the native colominic acid.

Interestingly, a number of other derivatives of the carboxyl terminus were attempted such as the treatment of the active ester (**2-19**) with NH_4OH, H_2N-CH_3, HN-$(CH_3)_2$ to give the primary, secondary, and tertiary amide respectively. In all cases, the results were little if any derivatizations.. A possible explanation is that the lactone (**2-20**) (fig. 2-17) is preferably formed and is reasonably difficult to derivatize. Only strong nucleophiles such hydrazine and hydroxylamine were able to open the lactone resulting in the hydrazide and hydroxamic acid respectively.

2.3 Summary

In summary, methods were established in which relatively large quantities of sialic acid oligosaccharides, whether Neu5Ac (**1-1a**) or Neu5Gc (**1-1b**), were obtained from natural sources or from a chemically modified natural source. These oligosaccharides will be used in mapping the size requirement of an antibody binding site (H.46 IgM) (c.f. chapter 5) as well as to provide a source for potential glycosyl donors which may be used in the synthesis of complex gangliosides and glycoproteins. These sialylated macromolecules may

find applications in potential cancer diagnosis and treatment as either vaccines or immunotherapies.

A number of well defined colominic acid analogs were also prepared with modifications taking place at two different regions of the sialic acid molecule. These analogs will be used to probe the binding specificity of an antibody directed against the capsular polysaccharide of *Neisseria meningitidis* serogroup B (c.f. chapter 5). From these studies, it is hoped to gain valuable information in the design of effective vaccines against both this and the *E. coli* K1 pathogen which are responsible for the majority of infant meningitis.

2.4 Experimental

General Methods

All solvents used were reagent grade and used without prior treatment. Distilled water was used in all cases except in selected dialysis purification steps. All reagents and chemicals unless otherwise noted, were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and used without prior purification. Colominic acid was purchased from either Sigma Chemical Co. (St. Louis, MO.) or Nacalai Tesque Inc. (Kyoto, Japan) and allyl Neu5Ac was obtained from Dr. R. Roy (Roy *et al.*, 1987). All buffer salts were reagent grade and used without any prior treatment. Tetanus toxoid was a gift of Dr. P. Rousseau (IAF, Laval, Canada), purified porcine IgG antibody was kindly donated by Dr. J. Boratynski of our lab, while bovine and chicken serum albumin (BSA and CSA) were purchased from Sigma Chemical Co. (St. Louis, MO.). The equine antibody H.46 was kindly donated by Dr J. Robbins (FDA, Bethesda, MD.) and the plant lectin WGA (*Triticum vulgaris*) was purchased from Sigma Chemical Co. (St. Louis, MO.). Peroxidase conjugated goat anti-horse IgM antibody, rabbit anti-pig IgG and IgM antibody, goat anti-bovine serum albumin antibody and tetramethyl benzidine (TMB) peroxidase substrate were obtained from Kirkegaard and Perry laboratories (Gaithersburg, MD.). Dialysis was performed with cellulose acetate tubing (Sigma Chemical Co., St. Louis, MO.) with molecular weight cutoff of 10 KD. UV analysis was performed using a Corning UV/Vis spectrophotometer (Fisher, Canada) and ELISA optical densities were read at 450 nm using a Titertek Multiscan M.C. (Flow Laboratories,

Meckenheim, F.R.G.) All melting points were recorded on a Gallenkamp apparatus and are uncorrected. Optical rotations were measured on a Perkin Elmer 241 polarimeter and were run at room temperature. Elemental analyses were conducted by Guelph Chemical Lab Ltd. (Guelph, Ont.).

SDS-polyacrylamide gel electrophoresis and immunoelectrophoresis were carried out on homemade systems operating between 160 and 200 V and 20-40 mA.

A variety of chromatographic methods were employed throughout this work. Column chromatography was performed in water or buffered solution as described, using the following matrices: Biogel P-2, Biogel P-6DG, Biogel A.5 (Bio-Rad, Richmond, CA.) and Sephadex G-10 and Sephadex G-100 (Pharmacia Fine Chemicals, Montreal, Canada). Peaks were detected using a Waters Associates R403 differential refractometer (Millford, MA, USA). High performance liquid chromatography (Superose-12, Pharmacia) was conducted on a Pharmacia (Montreal, Canada) dual pump (2148) system with UV detection using phosphate buffered saline (PBS) as eluent. Routine analytical thin layer chromatography (TLC) was performed using Merck pre-coated silica gel plates (60 F-254) and developed in the following solvent systems:

Solvent A: n-propanol: water 7:3

Solvent B: CH_3CN: 10% acetic acid 8:2

Solvent C: n-propanol: water 9:1 with 0.5% triethylamine

Solvent D: n-propanol: water: 25% NH_4OH 7:3:1

Solvent E: $CHCl_3$: methanol 1:1

Solvent F: n-propanol: water: 25% NH_4OH 6:2:0.5

The TLC plates were visualized using a molybdate reagent (2.5% wt/v ammonium molybdate/ 1% w/v ceric sulfate in 10% H_2SO_4) phosphomolybdate reagent (5% phosphomolybdic acid in EtOH), resorcinol reagent (appendix-A), $KMnO_4$, I_2, or UV.

Chemical ionization mass spectrometry was carried out on a VG 7070-E mass spectrometer using methane as the ionizing gas. FAB mass spectrometry in both the positive and negative mode was performed also on the VG 7070-E machine using glycerol as the matrix.

NMR data was collected on a number of machines. Routine ^1H NMR spectra were recorded on a Varian Gemini 200 (200 MHz), a Varian XL-300 (300 MHz) and high resolution ^1H NMR spectra, were recorded using a Bruker AMX-500 (500 MHZ) instrument. The same instruments were used to acquire respectively the 50.3 MHz, 75.4 MHz, and 125.7 MHz ^{13}C NMR spectra. DEPT analyses were performed either on the Gemini 200 or XL-300 instruments. NMR spectra were obtained at 25 °C or 300 K in either DMSO-d_6 or D_2O using as references either acetone (^1H NMR (δ=2.225 ppm); ^{13}C NMR (δ=31.07 ppm)), dioxane (^{13}C NMR (δ=67.4 ppm), or the HOD shift at 300 K (^1H NMR (δ=4.756 ppm)). The following designations are used: s=singlet, d= doublet, t= triplet, q= quartet, bd= broad, ov= overlapping, and m= multiplet.

Acid Hydrolysis Of Poly α-(2→8) N-Acetylneuraminic Acid (Colominic Acid) (2-1)

The following is a typical example of the hydrolysis of colominic acid (**2-1**):

Colominic acid (100 mg; 0.32 meq) was dissolved in 10 ml of distilled water (10 mg\ml) and the pH was lowered to 2 with 0.1M HCl. The solution was heated in an oil bath at 80 °C for 60 minutes. The solution was immediately cooled to room temperature and the pH was raised to between 7-8 with 0.1M NaOH. Lyophilization yielded the oligosaccharide mixture as their sodium salt which were either used as is or desalted on a Sephadex G-10 column (100 x 1.6 cm) equilibrated in distilled water and eluted by gravity at a flow rate of 30 ml\hr. Recovery of the oligosaccharides were typically >90% in terms of original polysaccharide amounts.

The extent of depolymerization could be visualized on TLC by double elution of a 20 x 5 cm plate in a n-propanol:H2O:25% NH_4OH (6:2:0.5) solvent system with detection by molybdate, phosphomolybdate, or resorcinol.

Separation Of Hydrolyzed Poly α-(2→8) N-Acetylneuraminic Acid Into It's Oligosaccharides

Anion exchange

(A) The above mixture of oligosaccharides was dissolved in 0.01M Tris(HCl) buffer (pH 7.6) and applied to a 1.5 X 50 cm column of DEAE Sephadex A-25 (Cl^-) equilibrated in the same buffer. The column was eluted with a linear gradient (2L) of 0.6M NaCl in 0.01M Tris(HCl) (pH 7.6) at a flow rate of 18 ml\hr maintained by a peristaltic pump. Fractions (7 ml) were collected over 3 days and the individual tube were screened for sialic acid by the resorcinol method (appendix A). Tubes corresponding to discrete oligosaccharides were pooled,

concentrated to dryness, and desalted on a Sephadex G-10 column (100 X 1.6 cm) equilibrated in water. The pure oligosaccharide- sodium salt was obtained from lyophilization of the desalted fraction.

(B) In a similar manner, an oligosaccharide mixture was applied to a DEAE Sephadex A-25 (OAc^-) column equilibrated in 50 mM pyridine acetate (pH 5.0). The column was eluted with an increasing linear gradient (2L) of 1.85M pyridine acetate at a flow rate of 17 ml\hr. Fractions were collected over a three day period and the individual tubes were screened for sialic acid by the resorcinol method (appendix A). Tubes corresponding to pure oligosaccharides were pooled, lyophilized, redissolved in water, and relyophilized to produce the oligosaccharide-pyridinium salt. For long term storage, the pure oligosaccharides were titrated to pH 7.5 with NaOH and relyophilized.

Gel filtration

(C) The mixture of oligosaccharides [$(Neu5Ac)_n$ or $(Neu5Gc)_n$] was dissolved in a small volume of a NH_4HCO_3 solution (0.03M; pH=7.6) and applied to a Biogel P-10 column (100x 1.6 cm) equilibrated in the same buffer. The column was eluted (18 ml/hr) and the column effluent was monitored by refractive index. In this manner, pure oligosaccharides (DP 1→5) were obtained after lyophilization as their ammonium salts.

N-Acetyl monomer (**2-3a**): recovered yield- 29%, FAB-MS for $C_{11}H_{19}O_9N$: m/z 308 (M-1), $[\alpha]_D$ = -32.4° (H_2O, c=1 mg/ml), see tables 2-1 & 2-2 for NMR data

N-Acetyl dimer (**2-3b**): recovered yield- 28%, FAB-MS for $C_{22}H_{36}O_{17}N_2$: m/z 599 (M-1), $[\alpha]_D$ =-11.2° (H_2O, c=5 mg/ml), see tables 2-1 & 2-2 for NMR data

N-Glycolyl monomer (**2-4**): recovered yield- 38%, FAB-MS for $C_{11}H_{19}O_{10}N$: m/z 324 (M-1), $[\alpha]_D$ = -21.8° (H_2O, c=5 mg/ml), see tables 2-1 & 2-2 for NMR data

N-Glycolyl dimer (**2-5**): recovered yield- 32%, FAB-MS for $C_{22}H_{36}O_{19}N_2$: m/z 631 (M-1), see tables 2-1 & 2-2 for NMR data

Poly α-(2→8)-De-N-Acetylated Neuraminic Acid (De-N-Ac colominic acid) (2-6)

Colominic acid (**2-1**) (100 mg; 0.32 meq) in 4 ml 2M NaOH containing 10 mg $NaBH_4$ was treated at 110 °C in a sealed tube for 7 hours. The slightly yellow solution was exhaustively dialyzed against 0.01M NH_4HCO_3 (pH=7.5) and lyophilized to give 70.5 mg (82%) of product as its ammonium salt.

The rate of de-N-acetylation was studied with 10 samples of colominic acid treated as above for various time periods (see fig. 2.13 for a typical run). After workup, ^{1}HNMR integration analysis revealed the extent of N-acetyl hydrolysis.

^1H NMR (300 MHz, D_2O): see table 2-3

^{13}C NMR (75.4 MHz, D_2O): see table 2-4

Poly α-(2→8)-N-Formyl Neuraminic Acid (N-Formyl colominic acid) (2-7)

De-N-Ac colominic acid (**2-6**) (10 mg; 0.39 meq) was dissolved in a 5:1 solution of formic acid/ acetic anhydride (500 ul) at O °C. The solution was allowed to warm to r.t. and left to sit for 24 hr. The mixture was poured cautiously into a saturated sodium bicarbonate solution containing ice, followed by dialysis against running water and lyophilization to afford 10.3 mg (88%; Na salt) of the title product.

^1H NMR (300 MHz, D_2O): see table 2-3

Poly α-(2→8)-N-Isobutanoyl Neuraminic Acid (N-Isobutyl colominic acid) (2-8)

To a cooled solution (5 ml; 0 °C) of de-N-Ac colominic acid (**2-6**) (40 mg; 0.15 meq) was added isobutyric anhydride (5x 20 ul) over a 60 min period while maintaining the pH between 8.5 and 11 with NaOH (0.5M). At the end of this period, the mixture tested negative by ninhydrin. The solution was chromatographed directly on a Biogel P6DG column (1.6X 100) in water and the void peak was collected and lyophilized to 42.6 mg (84.7%) of product (**2-8**) as its sodium salt.

^1H NMR (200 MHz, D_2O): see table 2-3

^{13}C NMR (50.3 MHz, D_2O): see table 2-4

Poly α-(2→8)-N-(Bromoacetyl) Neuraminic Acid (Bromoacetyl colominic acid) (2-10)

A solution (7 ml) of de-N-Ac colominic acid (**2-6**) (40 mg; 0.15 meq) was cooled to 0 °C and the pH was adjusted to 7.5. While maintaining the pH between 6-8.5 with NaOH (0.5M), neat bromoacetyl chloride (6X 50 ul) was added over 60 minutes. The reaction was monitored by ninhydrin for residual amine, however the results were not reliable due to the formation of coloured by-products.. The pH was raised to 8.5 and the solution was allowed to stand for 30 minutes, followed by exhaustive dialysis against water and lyophilization to yield 38 mg of product (66%; Na salt) (**2-10**).

^{1}H NMR (300 MHz, D_2O): see table 2-3

^{13}C NMR (50.3 MHz, D_2O): see table 2-4

Poly α-(2→8)-N-(Chloroacetyl) Neuraminic Acid (Chloroacetyl colominic acid) (2-9)

De-N-Ac colominic acid (**2-6**) (40 mg; 0.15 meq) was treated in a similar manner as described above (**2-10**) using chloroacetyl chloride yielding 44 mg (84%*) of the title product (**2-9**).

^{1}H NMR (300 MHz, D_2O): see table 2-3

^{13}C NMR (50.3 MHz, D_2O): see table 2-4

Poly α-(2→8)-N-Acryloyl Neuraminic Acid (N-Acryloyl colominic acid) (2-11)

The following is an example of a one pot, preparative scale reaction:

Colominic acid (**2-1**) (100 mg; 0.32 meq, 5 mg/ml) was de-N-acetylated as above followed by direct N-acryloylation in the following manner. The solution was diluted ten fold and the pH lowered to 10 with 1M HCl. A 1:1 solution of acryloyl chloride in dioxane was added in increments to the ice cold solution until complete amine derivatization occurred based on a negative ninhydrin test. The pH was maintained between 9 and 11 with periodic additions of NaOH (2M). The reaction was allowed to stand for an additional hour at pH 11 to ensure the complete hydrolysis of possible esters formed. The solution after dialysis (running water) was lyophilized to give **2-11** as a white powder (82.0 mg; 79%; Na salt).

^{1}H NMR (300 MHz, D_2O): see table 2-3

^{13}C NMR (75.4 MHz, D_2O): see table 2-4

Poly α-(2→8)-N-Propionyl Neuraminic Acid (N-Propionyl colominic acid) (2-12)

N-Acryloyl colominic acid (**2-11**) (50 mg; 0.15 meq) was dissolved in 20 ml water containing 10% Pd on charcoal (10 mg). Hydrogen gas was bubbled through the solution at room temperature for 24 hrs. The Pd\C was removed by filtration through celite and the material was lyophilized to give pure **2-12** (48.4 mg ,96%) as a fluffy off-white powder.

^{1}H NMR (300 MHz, D_2O): see table 2-3

^{13}C NMR (75.4 MHz, D_2O): see table 2-4

Poly α-(2→8)-N-(3-S-Methyl-Propionyl) Neuraminic Acid (S-Methyl propionyl colominic acid) (2-13)

Sodium thiomethoxide (50 mg; 0.71 mmol) was dissolved in an aqueous solution (3 ml) containing N-acryloyl colominic acid (**2-11**) (20 mg; ca. 0.062 meq). The pH was adjusted to 9.5 with NaOH (0.1M) and the solution was allowed to sit at r.t. overnight, followed by exhaustive dialysis against water and lyophilization to afford 16.1 mg (70%) of the title product (**2-13**) as its sodium salt.

^{1}H NMR (200 MHz, D_2O): see table 2-3

^{13}C NMR (50.3 MHz, D_2O): see table 2-4

Poly α-(2→8) N-(3-N-Methyl-Propionyl) Neuraminic Acid (N-Methyl propionyl colominic acid) (2-14)

N-Acryloyl colominic acid (**2-11**) (15 mg; 0.046 meq) was dissolved in 2M methylamine hydrochloride (2 ml) previously adjusted to pH 9.5 with NaOH (0.1M). The solution was left to sit at r.t. for 24 hr, dialyzed once against PBS, followed by exhaustive dialysis against water and lyophilization to give 13 mg (80%) of product as its sodium salt.

^{1}H NMR (200 MHz, D_2O): see table 2-3

Poly α-(2→8)-N-(Acetoxy-Acetyl) Neuraminic Acid (O-Acetoxy-N-glycolyl colominic acid) (2-18)

To an ice cold solution of de-N-Ac colominic acid (**2-6**) (100 mg; 0.37 meq) in 10 ml water at pH 7.5, was added single drop aliquots (~50ul) of a dioxane solution of acetoxyacetyl chloride (1:1). The pH was maintained below 8 at all times with NaOH (2M). The

acid chloride was added until complete disappearance of the amine function as determined by a negative ninhydrin test. The solution was applied to a Bio-Gel P6DG column (1.6x100) in water where the higher molecular weight fraction was collected and lyophilized to yield 91.2 mg (67%) of **2-18** as a white powder (sodium salt).

^{1}H NMR (200 MHz, D_2O): see table 2-3

^{13}C NMR (50.3 MHz, D_2O): see table 2-4

1,6-Di-N-Acryloyl Hexanediamine (2-16)

1,6-Hexanediamine (500 mg, 4.3 mmol) was dissolved in a 1:1 solution of methanol:water (10 ml). With vigorous stirring, a dioxane solution (1:1) of acryloyl chloride (1 ml, 1.5 eq) was added all at once. The pH immediately dropped from ~10 to ~2 due to the formation of HCl. The pH of the solution was brought back up to 10 with NaOH (4M) and the solution was treated a second time in the same manner with a further 1.5 eq of acid chloride. Cation resin (Amberlite IR-120 Na form) was added to the solution to remove any residual starting material, and the solution was filtered. TLC at this point revealed a UV positive product that was able to decolourize permanganate at a Rf of 0.66 (solvent B). Concentration of the solution by rotary evaporation under reduced pressure, followed by desalting on a 100 x 1.6 cm column of Sephadex G-10 in water, and lyophilization, yielded the pure title product **2-16** in 86% yield.

TLC (solvent B)- single spot (Rf= 0.66, UV positive, decolourizes $KMnO_4$)

CI-MS for $C_{12}H_{20}O_2N_2$: 225 m/z (M+1)

^1H NMR(300 MHz; DMSO-d_6): δ: 1.28 (overlapping t, 4H, RNH-CH_2-CH_2-$\mathbf{CH_2}$), 1.43 (apparent t, 4H, RNH-CH_2-$\mathbf{CH_2}$-CH_2), 3.11 (doublet of t, 4H, RNH-$\mathbf{CH_2}$-CH_2-CH_2), 5.58 (dd, 2H, H_a), 6.06 (dd, 4H, H_b), 6.21 (dd, 4H, H_c), 8.07 (t, 2H, R**NH**-CH_2-R)

^{13}C NMR(75.4 MHz; DMSO-d_6): δ: 26.3 (RNH-CH_2-CH_2-CH_2), 29.1 (RNH-CH_2-CH_2-CH_2), 38.6 (RNH-CH_2-CH_2-CH_2), 124.9 (R-CH=CH_2), 131.7 (R-CH=CH_2), 164.5 (R-CONR)

1,6-Di-N-Glycolyl Hexanediamine (2-17)

1,6-Di-N-acryloyl hexanediamine (**2-16**) (50 mg) was dissolved in a 1:1 solution of methanol-water (10 ml) and cooled to 0 °C. ozone was passed through the solution for 5 minutes without any observed indication of excess ozone present (i.e. without any blue colour formation). Solid $NaBH_4$ (200 mg) was added carefully to the ozonolyzed solution in order to minimize bubbling. The solution was allowed to warm to room temperature and sit for 1 hr. Cation resin (Amberlite IR-120 H^+ form was added until the pH dropped to ~2. The resin was filtered and the solvent was removed under reduced pressure. The solid residue was redissolved in MeOH and concentrated to dryness a number of times in order to remove methyl borate byproducts. TLC on the recuperated material (49.1 mg, 94.7% yield) revealed complete disappearance of the starting **2-16** and the presence of a single product **2-17** with Rf= 0.21 (solvent B).

TLC: (solvent B): single product (Rf=0.21, does not decolourize $KMnO_4$)

CI-MS for $C_{10}H_{20}N_2O_4$: 232 m/z (M+1)

^{1}H NMR (300 MHz, DMSO-d_6): δ: 1.23 (overlapping t, 4H, RNH-CH_2-CH_2-CH_2), 1.38 (overlapping t, 4H, RNH-CH_2-CH_2-CH_2), 3.07 (doublet of t, 4H, RNH-CH_2-CH_2-CH_2), 5.43 (s, 4H, CONH-CH_2-OH), 7.69 (t, 2H, RNH-CH_2-CH_2-CH_2).

^{13}C NMR (75.4 MHz, DMSO-d_6): δ: 26.2 (RNH-CH_2-CH_2-CH_2), 29.4 (RNH-CH_2-CH_2-CH_2), 38.1 (RNH-CH_2-CH_2-CH_2), 40.2 (CON-CH_2-OH), 171.5 (CON-CH_2OH).

Poly α-(2→8)-N-Glycolyl Neuraminic Acid (N-Glycolyl colominic acid) (2-15)

(A) De-O-Acetylation of poly α-(2→8) N-(acetoxy-acetyl) neuraminic acid (**2-18**) : The pH of a solution of O-acetoxy-N-glycolyl colominic acid (**2-18**) (50 mg; 0.13 meq) in water, was raised to 12-13 with NaOH (2M) and was maintained in this range for a period of 2 hours at room temperature. The solution was neutralized with 1M HCl, dialyzed against running water, and then lyophilized to yield 39.5 mg (89%) of title product as its sodium salt (**2-15**).

^{1}H NMR (500 MHz, D_2O): see table 2-3

^{13}C NMR (125.7 MHz, D_2O): see table 2-4

b) Reductive Ozonolysis of Poly α-(2→8) N-Acryloyl Neuraminic Acid (**2-11**): N-Acryloyl colominic acid (**2-11**) (100 mg; 0.31

meq) was dissolved in a 2:1 water-methanol mixture and was cooled to -20'C. Ozone was bubbled through the solution for 25 minutes, residual ozone was removed with nitrogen, and an excess of sodium borohydride (~20 mg) was immediately added. The solution was allowed to sit at room temperature for 2 hours, dialyzed against running water, and lyophilized to give the title product (**2-11**) (95.6 mg; 94%; Na salt).

^{1}H NMR (500 MHz, D_2O): see table 2-3

^{13}C NMR (125.7 MHz, D_2O): see table 2-4

Poly α-(2→8)-N-Acetyl Neuraminic Acid- EDC Active Ester (Colominic acid-EDC*) (2-19)

The following is a general method for the preparation *in situ* of carboxy-activated polysialic acid with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC): Colominic acid (**2-1**)(50 mg; 0.16 meq) was dissolved in 10 ml of water and the pH was adjusted to 4.75 with HCl (0.1M). In small increments, EDC (250 mg; 1.3 mmol) was added to the solution while keeping the pH at 4.75 with HCl (0.1M). The addition was done over a period of 2-3 hr until the pH stabilized. The solution was treated in manners described below.

Poly α-(2→8)-N-Acetyl Neuraminitol (Carboxy reduced colominic acid) (2-21)

To 50 mg of the colominic acid-EDC* (**2-19**) active ester was added sodium borohydride (200 mg) portionwise in order to limit bubbling. The pH was monitored and kept at ~8.5 with 0.5M HCl. The solution was allowed to sit overnight followed by exhaustive dialysis

against water and lyophilization to give the reduced product (**2-21**) in 85% yield (38 mg).

^1H NMR (500 MHz, D_2O): see table 2-3

^{13}C NMR (50.3 MHz, D_2O): see table 2-4

<u>Poly α-(2$\rightarrow$8) N-Acetyl Neuraminic Acid Hydrazide (Colominic acid hydrazide) (2-22)</u>

To 50 mg (0.16 mmol) of the colominic acid-EDC* (**2-19**) was added all at once 400 ul of NH_2-NH_2·H_2O (8.0 mmol) at room temperature. The solution was allowed to sit for 12 hr followed by dialysis against distilled water at 4 °C and lyophilization to provide **2.22** in 73% yield (38.2 mg).

^1H NMR (500 MHz, D_2O): see table 2-3

^{13}C NMR (125.3 MHz, D_2O): see table 2-4

<u>Poly α-(2$\rightarrow$8) N-Acetyl Neuraminic Acid Hydroxyl Amine (Colominic acid hydroxyl amine) (2-23)</u>

The preformed EDC active ester of colominic acid (**2-19**) was treated in the same manner as above with a 1M solution of hydroxylamine·HCl (8.0 ml; 8.1 mmol) previously adjusted to pH 10 with NaOH. Workup in the above manner yielded the title product (**2-23**) in 43% yield (22.8 mg).

^1H NMR (300 MHz, D_2O): see table 2-3

^{13}C NMR (50.3 MHz, D_2O): see table 2-4

Chapter 3- Heterobifunctional Spacer

3.1 Introduction

It is a well known phenomenon that extension of protein coupled small molecules (haptens) away from the protein by means of a spacer arm, can sometimes play a crucial role in terms of immunogenic responses to the haptens (Kimura *et al.*, 1990; Boullanger *et al.*, 1985). Although probably more complex, a simplified explanation is that the hapten is extended away from protein residues that may block or mask relevant immune cell processes required for expression of anti-hapten antibodies. Similarly in terms of antigenicity, the spacer arm extends the hapten so that it is able to reach the antibody binding pockets situated not on the surface but within the protein (cavity-type binding pockets) (Roy and Tropper, 1988a) as depicted schematically in figure 3-1a. In order to elicit optimal T and B cell response, the spatial requirements between hapten and its carrier support have been reported to be in the range of 7-70 Å (Porro, 1987; Alkan *et al.*, 1972). A serious limitation of using spacer technology is the possible production of neo-immunodominant determinants within either the hapten-spacer or the spacer-carrier linkage regions (Roy and Laferriere, 1988; Porro, 1987). Since haptens by definition, are small molecules containing only one epitope, the immunogenic potential of the linkage region is greater relative to a polysaccharide-spacer conjugate where there are many carbohydrate epitopes to one linkage region. Consequently, the need for a spacer in polysaccharide conjugates is generally dictated by the method of conjugation rather than any spatial requirements.

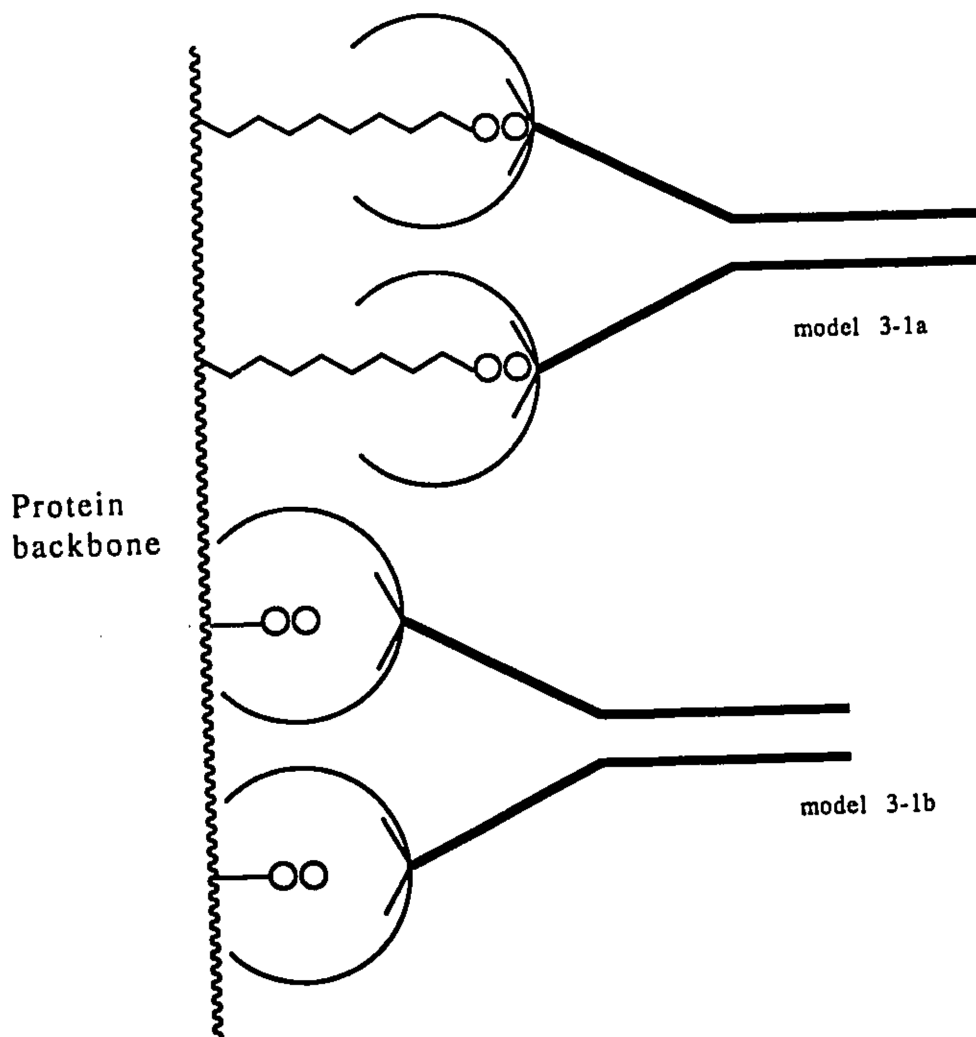


Figure 3.1- Schematic representation of the function of spacer molecules. Model 3.1a demonstrates binding of the antibody to the hapten due to its extension away from the protein. Model 3.1b shows the inability of the antibody combining site to reach the hapten due to steric interactions.

With these general principles in mind, our goal was to synthesize a spacer incorporating the following characteristics:

i) Heterobifunctionality	-Incorporation of two functional groups which allows selective coupling to either the protein or carbohydrate moiety.
ii) Unobtrusive	-As 'natural-like' as possible in order to minimize immunodominance.
iii) Chemistry	-Functional groups must be reactive so that coupling is fast and proceeds in a defined and predictable manner.
iv) Toxicity	-Should be devoid of potential toxic groups upon breakdown. Toxic reagents used in coupling should be minimized.
v) Versatility	-Should be non-limiting and able to be used in a variety of ways.
vi) Geometry	-Should be of optimal length.
vii) Hydrophilic	-should minimize hydrophobic interactions and be completely water soluble.

It is hoped that with a spacer of this nature, that oligo-/polysaccharide conjugates to either protein or water soluble acrylamide polymers will allow exploration of the interaction between antibodies and polysialic acids from both an immunogenic and antigenic point of view.

3.2 Results

3.2.1 N-Acryloyl glycine (3-2)

Starting from glycine ethyl ester hydrochloride (**3-1**) (fig. 3.2), the free acid (as its sodium salt) was generated *in situ* through saponification of the ester with NaOH (25% w/v). Complete hydrolysis was judged by TLC with the formation of a single product of lower R_f and disappearance of starting material. In a Schotten-Baumann type reaction (March, 1977), the amino group was acylated smoothly with neat acryloyl chloride while maintaining the pH of the solution at ~9 with solid NaOH. Complete derivatization of the amine was verified through a negative ninhydrin test as well as a new single product (R_f=0.24) which decolourized $KMnO_4$ and was UV positive. It was found that extraction of the acidic solution was best accomplished with multiple extractions of warm EtOAc since solubility of the product was low in other organic solvents such as $CHCl_3$, Et_2O, or even cold EtOAc. The product crystallized readily from EtOAc in 77% yield. The yield could be improved by exhaustive extraction of the acidic solution since residual product remained as judged by TLC. Melting point analysis of the crystalline product showed decomposition at 128.1 °C which was reasonable when compared to literature values which ranged from 110- 138 °C (Smith *et al.*, 1958; Kaczmar *et al.*, 1976; Heilmann and Smith, 1979). ^1H- ^{13}C NMR (300 MHz; DMSO-d_6) are consistent for the desired product with the appearance of the well characterized olefinic AMX spin system (Roy and Laferriere, 1990b; Kallin *et al.*, 1989). Integration of the olefinic protons vs. the glycine methylene protons gives the expected 3:2 ratio.

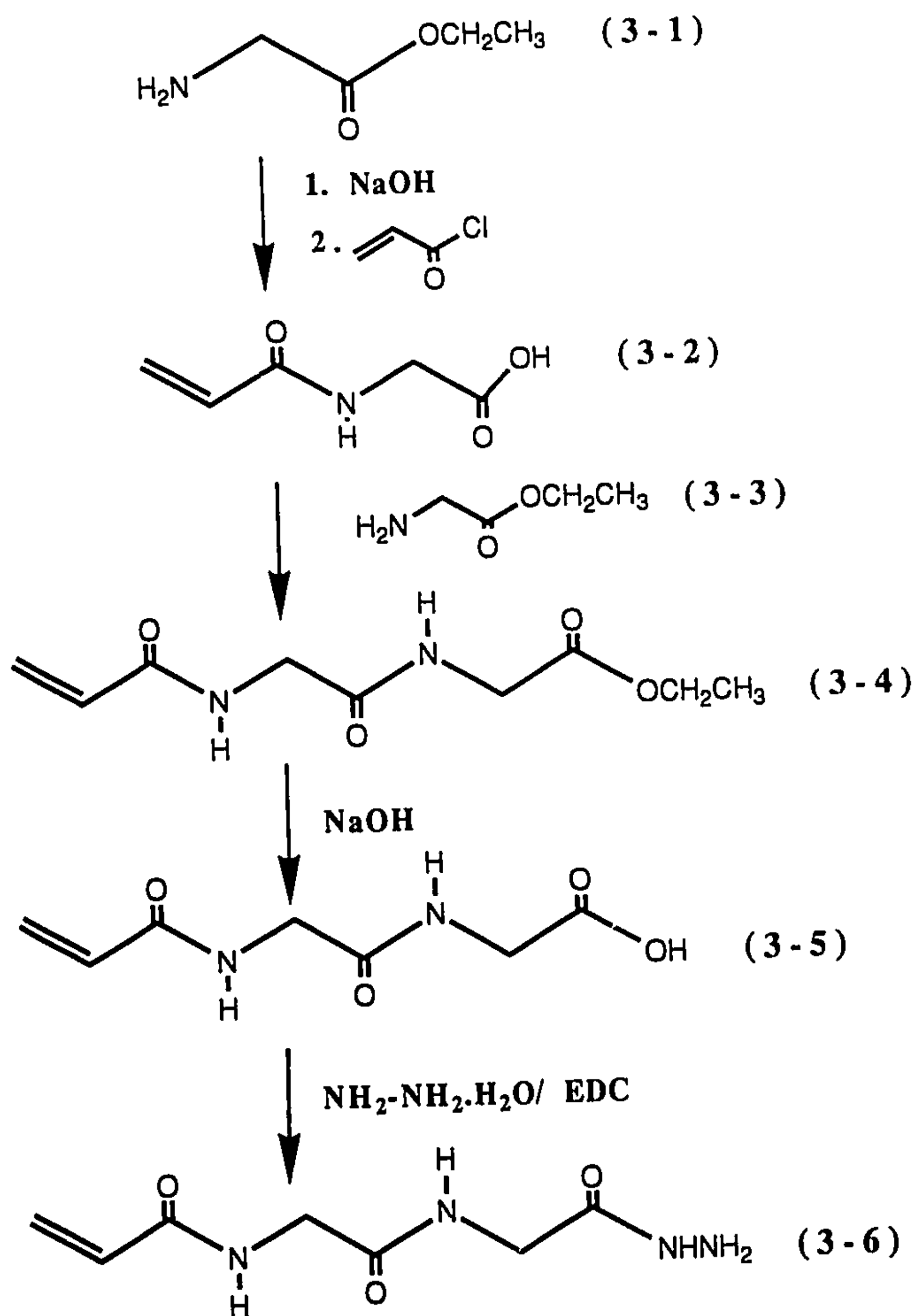
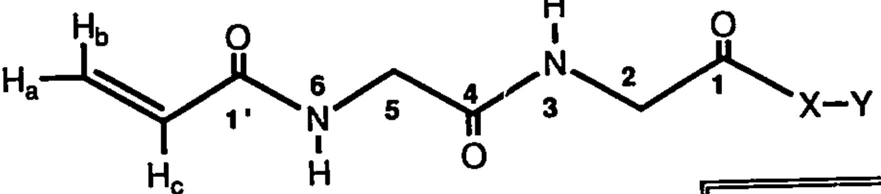


Figure 3.2- Synthetic scheme for the production of a heterobifunctional spacer.

Table 3-1 ^1H NMR (300 MHz) Chemical Shifts (ppm) And Coupling Constants (Hz) Of The Bifunctional Spacer In DMSO-d_6 At 25 °C

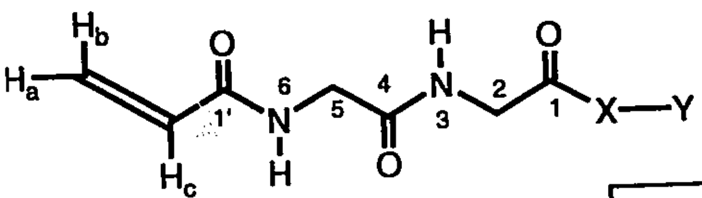


Comp.	H_2	H_3	H_5	H_6	H_a	H_b	H_c	Other: OCH_2	Other: CH_3	Other: NH	Other: NH_2
No.	J_{2-3}	J_{3-2}	J_{5-6}	J_{6-5}	J_{a-b} J_{a-c}	J_{b-a} J_{b-c}	J_{c-a} J_{c-b}	$J_{CH2-CH3}$	$J_{CH3-CH2}$		
3 - 2	- -	- -	3.77[1] (5.9)	8.41 (5.7)	5.60 (2.4) (9.9)	6.07 (2.4) (17.1)	6.28 (9.9) (17.2)	- -	- -	- -	- -
3 - 4	3.80 (2.2)	8.39 (2.1)	3.83 (2.2)	8.32 (2.1)	5.60 (2.3) (10.1)	6.08 (2.3) (17.1)	6.29 (10.1) (17.1)	4.07 (7.2)	1.18 (7.1)	- -	- -

---

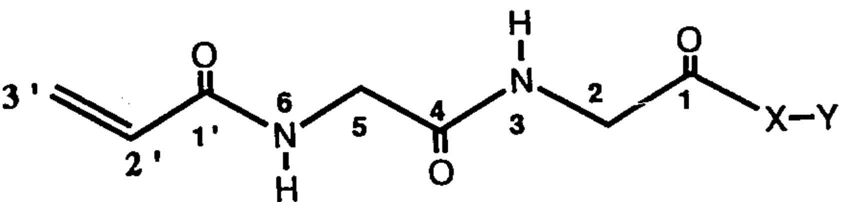
1: assumes position C-4 as the carboxylic acid carbon.

Table 3-1. ^{1}H NMR cont'd (300 MHz) Chemical Shifts (ppm) And Coupling Constants (Hz) Of The Bifunctional Spacer In DMSO-d_6 At 25 °C



Comp.	H_2	H_3	H_5	H_6	H_a	H_b	H_c	Other			
								OCH_2	CH_3	NH	NH_2
No.	J_{2-3}	J_{3-2}	J_{5-6}	J_{6-5}	J_{a-b} J_{a-c}	J_{b-a} J_{b-c}	J_{c-a} J_{c-b}	$J_{CH2-CH3}$	$J_{CH3-CH2}$		
3 - 5	3.76 (5.9)	8.24 (6.0)	3.81 (6.0)	8.39 (5.2)	5.61 (2.2) (10.1)	6.09 (2.2) (17.1)	6.30 (10.0) (17.1)	- -	- -	- -	- -
3 - 6	3.65 (5.9)	8.15 (5.8)	3.80 (5.8)	8.36 (5.8)	5.61 (2.2) (10.1)	6.08 (2.1) (17.1)	6.29 (10.1) (17.1)	- -	- -	8.98	4.22

Table 3-2. ^{13}C NMR (50.3 MHz) Chemical Shifts (ppm) In DMSO-d_6 At 25 °C Of The Bifunctional Spacer Synthesis



Comp.No	C_1	C_2	C_4	C_5	$C_{1'}$	$C_{2'}$	$C_{3'}$	Other	
								X	Y
3 - 2	--	--	171.3[1]	40.7	165.0	131.3	125.8	--	--
3 - 4	171.8	42.9	171.7	41.9	168.2	131.5	128.6	62.6	14.8
3 - 5	171.5	40.7	170.1	41.8	165.3	131.7	125.8	--	--
3 - 6	169.1	40.6	170.8	41.9	165.0	131.5	125.5	--	--

[1]: Assumes C-4 as the carboxylic acid carbonyl.

3.2.2 N-Acryloyl glycylglycine ethyl ester (3-4)

Preparation of the acryloylated dipeptide (**3-4**) was accomplished in a manner similar to Smith and Unruh (1957) using EDC to activate the carboxylate of acryloylated glycine (**3-2**) and displacement of the active ester with glycine ethyl ester (**3-3**). EDC was used rather than DCC due to the easier removal of the urea by-product of EDC. The high solubility of the EDC urea in EtOH combined with the low solubility of the product **3-4** was advantageous since simple filtration afforded the pure product. The yield of the product could be increased by concentrating the supernatant, recharging the acid (**3-2**) with additional EDC, and collection of the precipitate. Overall the acryloylated glycylglycine ester (**3-4**) was recovered in 96% yield as a single product by TLC (R_f=0.80, solvent E). ^{1}H NMR (300 MHz; DMSO-d_6) (appearance of an additional methylene at 3.83 ppm integrating 2:3 to the olefinic protons), ^{13}C NMR (3 carbonyl signals attributable to C1, C4, C'1 at 171.8, 171.7, and 168.2 ppm respectively as well as a new methylene carbon at 42.9 ppm), and CI-MS (M+1=215 m/z for $C_9H_{14}O_4N_2$) of the filtered product were identical to a crystallized (m.p. 148.9-149.1 °C, EtOH) sample and in subsequent reactions used as such. The literature melting point of 148-150 °C (Smith and Unruh, 1957) agreed well to the value found.

3.2.3 N-Acryloyl glycylglycine (3-5)

At this point in the synthesis, it was hoped that treatment of the ester (**3-4**) with hydrazine hydrate would afford the hydrazide (**3-6**) (fig. 3.3).

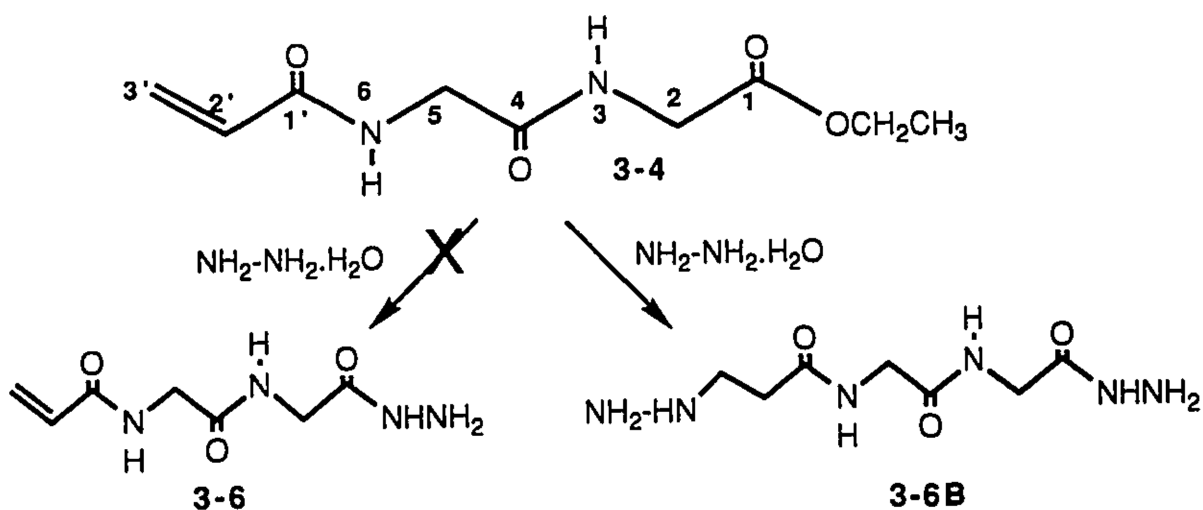


Figure 3.3- Bis addition of hydrazine.

However, under all reaction conditions tried, hydrazinolysis of the ester produced either no significant reaction or a crystalline product whose structure is tentatively assigned to (**3-6B**) based on TLC and ^{1}H NMR evidence. The material had a low R_f (baseline, solvent E), decolourized $KMnO_4$, stained positive for hydrazide (TNBS assay-appendix A), but was UV negative which led to the suspicion that it was not **3-6**. Subsequent ^{1}H NMR (300 MHz; DMSO-d_6) analysis revealed complete disappearance of the olefinic protons. The appearance of four N-H signals downfield and two triplets at 2.4 (H-3') and 2.79 (H-2') ppm integrating for 2 protons each, led to the proposed structure (**3-6B**). It was evident that the Michael adduct was formed first when a sample taken after a short period revealed the olefinic protons had disappeared but ~ 30% of the ethyl ester was still present. From these results, it was clear that the reactivity of the ethyl ester was not sufficient relative to the reactivity of the acryloyl

functionality. In order to increase the reactivity at this center, the ester (**3-4**) was saponified in alcoholic NaOH. That complete ester hydrolysis had occurred was determined by TLC (single spot at R_f=0.13, solvent E). After acidification with cation resin (H^+), simple filtration and solvent removal gave the pure N-acryloyl glycylglycine (**3-5**) in quantitative yield. The product was crystallized from hot EtOH (m.p. 133.1-134.3 °C) where all spectroscopic data (^1H NMR, ^{13}C NMR, and CI-MS) were consistent with the formation of **3-5**. Comparison of the non-crystalline product with the crystallized material showed no appreciable differences and was used as such in subsequent reactions.

3.2.4 N-Acryloyl glycylglycine hydrazide (3-6)

This step of the synthesis was by far the most difficult owing to the finicky solubility of both the product and the by-products. The strategy was again to activate the carboxylate with an active ester, followed by quantitative displacement with 1 eq. of NH_2-NH_2·H_2O. Reagents tried were EDC, DCC, and ethyl chloroformate. Each of the coupling reagents succeeded in that a new polar, hydrazide +'ve (TNBS), $KMnO_4$, and UV positive spot was seen by TLC (R_f=0.39, solvent E). The difficulty lay in the purification of the newly formed product. Extraction of **3-6** into organic solvents was also not successful. Removal of excess DCC urea (R_f=0.9, solvent E) would be expected to be a simple extraction into organic solvents leaving the product **3-6** behind. Residual DCC urea however, was always found in the water layer. While the breakdown products of ethyl chloroformate are simply CO_2 and EtOH, the problem arose due to the need for anhydrous conditions (Chen *et al.*,1987) combined with the

low solubility of the starting acid (**3-5**) in dry THF at relatively low temperatures (0-20 °C). Although this latter method gave reasonable yields, the DCC coupling procedure was opted for despite the pernicious solubility of the urea by-product. Reaction of the starting acid (**3-5**) with DCC and 1 equivalent of NH_2-NH_2·H_2O in t-butyl alcohol was followed by the subsequent co-precipitation of the suspected product **3-6** and DCC urea. After filtration, the bulk of the urea could be removed by extracting the solid with water. The water layer containing the suspected product was lyophilized to a fluffy product. In order to purify the product from residual urea, the product was absorbed onto 1-2 gm of silica gel in MeOH. Following removal of the solvent, a $CHCl_3$ slurry of the gel was applied to the top of a 25 ml flash chromatography column and was eluted with a 3:1 $CHCl_3$:MeOH solvent system. Interestingly, the urea came off with the void volume and could visibly be seen since it immediately crystallized in the tubes. Subsequent pooling and evaporation of the solvent yielded the product in 65% overall yield from the starting acid (**3-5**). Crystallization of the hydrazide (**3-6**) from EtOH afforded needles with a decomposition point of 172.9 °C. Spectroscopic and elemental evidence is strong for the successful isolation of **3-6**. The proton NMR spectrum (500 MHz; DMSO-d_6) (fig. 3.4) was characterized by the downfield appearance of a broad singlet at 8.9 ppm due to the α-hydrazide proton (O=C-NH-NH_2) and the 0.1 ppm upfield shift of the H-2 methylene protons (3.65 ppm). A broad, barely discernible peak integrating for 0.2 protons at 4.22 ppm was due to the NH_2 protons of the hydrazide. CI-MS, calculated for $C_7H_{12}N_4O_3$, showed the expected M+1 peak at 201 m/z.

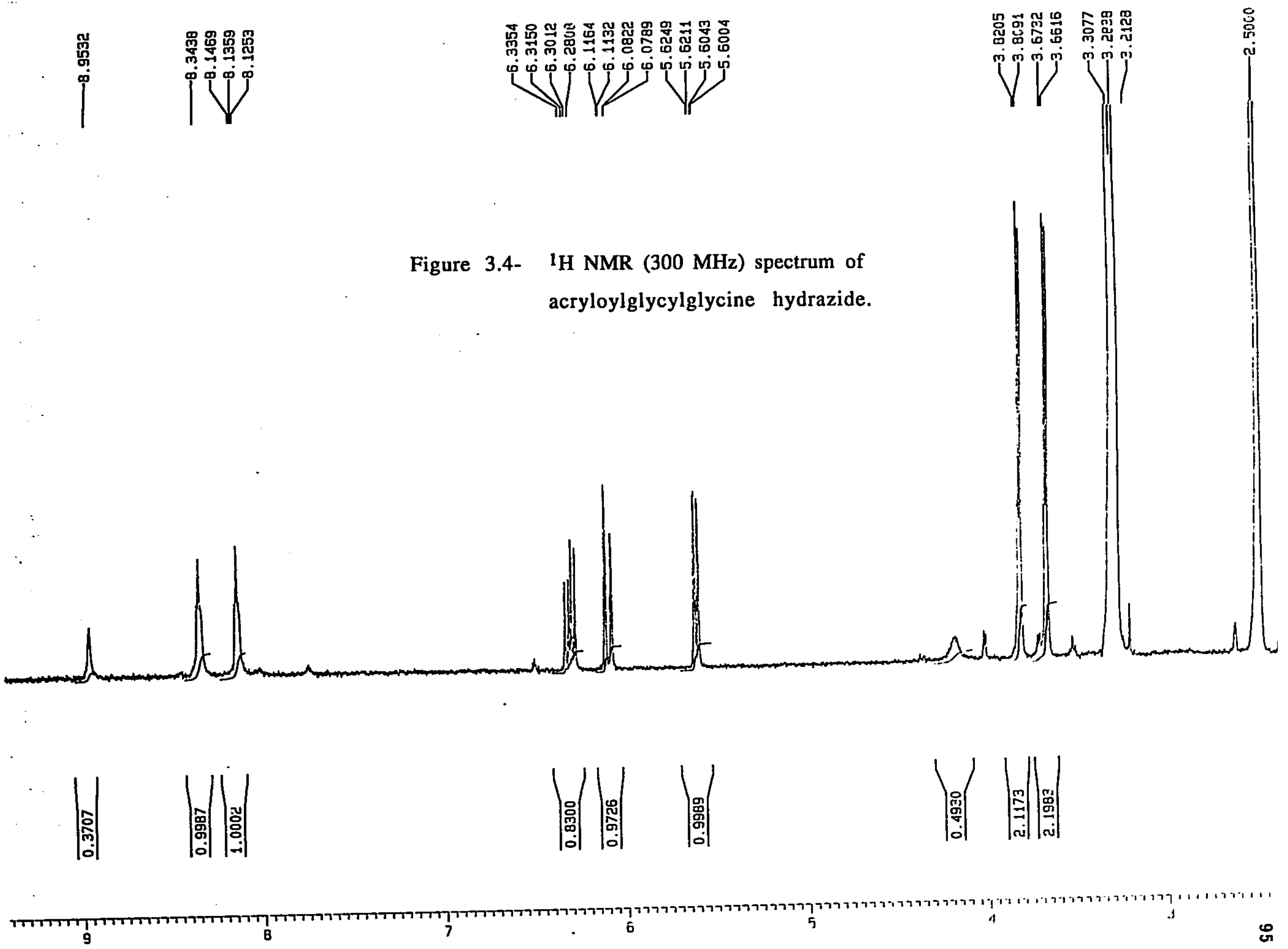


Figure 3.4- ^{1}H NMR (300 MHz) spectrum of acryloylglycylglycine hydrazide.

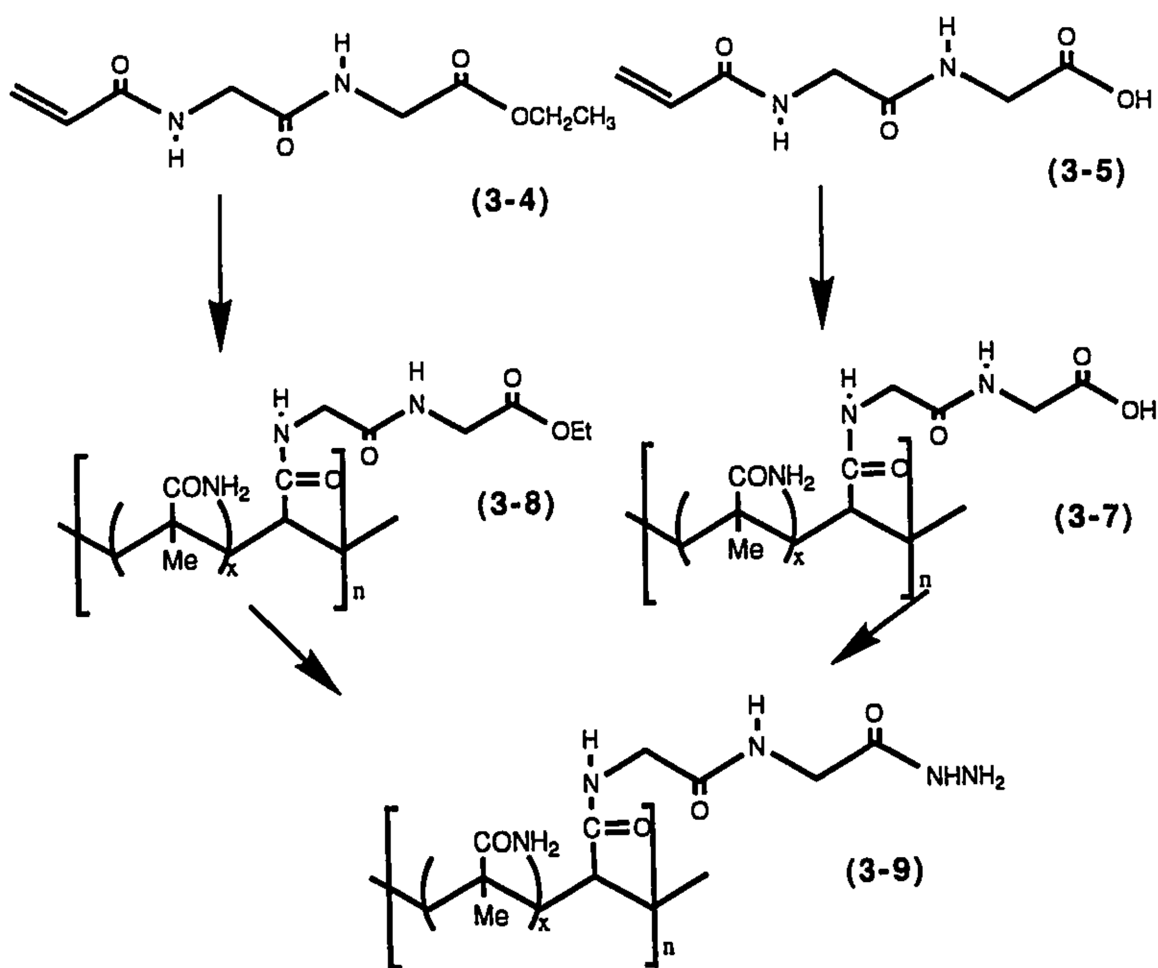


Figure 3.5- Methacrylamide copolymers.

3.2.5 Methacrylamide copolymers

Carbohydrate structures exposed on the cell surface as either glycolipids or glycoproteins serve as recognition structures in many biological processes (Paulson, 1985; Feizi, 1985, and Hakomori, 1984). In order to investigate these processes at both a molecular and cellular level, it is often fundamental that macromolecular multivalent forms of these surface oligosaccharides be available. Protein glycoconjugates are well documented versions of these macromolecular conjugates and will be discussed in a later chapter.

Soluble oligosaccharide-acrylamide copolymers offer an alternative to their protein counterparts and have recently been used to study a variety of immunochemical processes (Roy *et al.*, 1991; Roy and Laferriere, 1990a; Kallin *et al.*, 1989; Roy and Tropper, 1988a,b). There exists distinct advantages of these neo-glycopolymers compared to their protein counterparts such as being weakly immunoreactive in immunoassays. A direct application of this property is their use as screening antigens in monoclonal antibody production since only antibodies directed to the carbohydrate portion will be selected. Glycoprotein antigens are often short lived in terms of stability due primarily to the inherent instability of most proteins in terms of denaturation. Neo-glycopolymers on the other hand, are not prone to denaturation and provide very hardy and long lived sources of multivalent carbohydrate antigens. It is our goal to make use of the synthetic spacer with a terminal acryloyl group that is suitable for copolymerization with methacrylamide, to generate multivalent sialyloligosaccharide copolymers. As a first approach to the synthesis of these sialyloligosaccharide-methacrylamide copolymers, a water soluble copolymer composed of a methacrylamide backbone with pendant spacer hydrazides will be synthesized. The pendant hydrazides will then be amenable to attach suitable sialyloligosaccharides in the various manners outlined and discussed in chapter 4.

Copolymers of glycylglycine ethyl ester (**3-4**) and of glycylglycine (**3-5**) were prepared via radical copolymerization with methacrylamide in aqueous solutions under previously described conditions (Roy and Laferriere, 1988; Roy and Tropper, $1988_{a,b}$) (see

fig. 3.5). Polymerization was heat initiated at 100 °C in the presence of ammonium persulfate to give linear copolymers of broad molecular weight distributions. Typically, a 5:1 molar ratio of methacrylamide to the acryloyl substrate was used. From the ^1H NMR spectrum (300 MHz; D_2O) the integration of the methacrylamide CH_3 group to the ethoxy methylene protons of **3-8** indicated a 1:6 molar ratio of glycylglycine ester (**3-4**) to methacrylamide. Similarly, integration of the glycylglycine methylenes of **3-6** to the CH_3 methacrylamide protons in the copolymer **3-7**, led to a 1:8 molar ratio of glycylglycine (**3-5**) to methacrylamide. The spacer ester-methacrylamide copolymer (**3-8**) was converted to the spacer hydrazide-methacrylamide copolymer (**3-9**) by hydrazinolysis of the ester functionality resulting in 53% derivatization of the ester to hydrazide. This was calculated by determining the molar ratio of hydrazide (TNBS assay) to methacrylamide (9.0% mole/mole methacrylamide) and comparing to the theoretical value (17% mole/mole methacrylamide) found in **3-8** by the integration of the corresponding signals of the ^1H NMR spectrum. Glycylglycine-methacrylamide copolymer (**3-7**) was converted into the hydrazide copolymer (**3-9**) by coupling hydrazine to the EDC activated intermediate. Again from comparison of the moles of hydrazide formed (TNBS) vs. the theoretical number of moles of glycylglycine in **3-7**, the % of derivatization was 71% with 8.5 moles glycylglycine hydrazide/ 100 moles methacrylamide. It was found to be more practical to copolymerize either the spacer acid (**3-5**) or the spacer ester (**3-4**) first, followed by conversion to the hydrazide copolymer (**3-9**) rather than trying to copolymerize the spacer hydrazide (**3-6**)

directly. In this way, it eliminated the tedious purification after the DCC coupling of hydrazine. It also allowed direct hydrazinolysis of the spacer ester copolymer (**3-8**) which previously was not possible on the monomer (**3-4**) without getting double addition of hydrazine. The introduction of the powerful hydrazide nucleophile onto the copolymer will eventually allow the covalent attachment of sialyloligosaccharides by reductive amination (c.f. chapter 4) thus generating neo-sialylglycopolymers.

3.3 Discussion

A spacer composed of glycine- a fundamental amino acid, was chosen principally in order to minimize the "foreignness" of the substrate in terms of immunogenicity. In other words, by using a non-obtrusive species, we had hoped that protein conjugates linked through this spacer, would raise antibodies not to the linker region but mainly to the carbohydrate epitopes. Being of moderate polarity, hydrophobic effects of long commonly used alkyl chains (Lemieux *et al.*, 1975) are not encountered. The necessary spatial requirements (Porro, 1987; Alkan *et al.*, 1972) was fulfilled by using a glycine dipeptide and when appropriately functionalized, stretches to ~12Å in length. The significance of oligosaccharides derivatized with a spacer such as this becomes apparent when macromolecular conjugates are formed and are used in immunochemical assays. The extension of the oligosaccharide away from the surface of the macromolecule (i.e. protein, lipid, or polymer) can have a marked influence on the binding of antibodies and lectins (Roy and Tropper, 1988a).

The choice of a hydrazide functionality was two-fold. At physiological pH's, the hydrazide functionality is more reactive relative to its amino counterparts (Zon & Robbins,1983). This can be explained by the lower pK_a of the hydrazide (i.e. at moderate pH's, the degree of protonation is less relative to amines) and the α-effect, which contributes to an increased nucleophilicity of the hydrazide (Pratt and Bruice, 1972). Secondly, upon reaction with an aldehyde, the hydrazone formed has been reported to be relatively stable between pH 2-10 (Hofmann and O'Shannessy, 1988; Bayer *et al.*,

1988; Bailey & Butterfield, 1981). It was hoped that upon reaction with the hemiketal of sialic acids, the hydrazone like bond formed would also be stable. This would preclude the use of additional and sometimes toxic reagents such as $NaBH_3CN$ which in animal studies, should always be avoided if possible.

Incorporation of an acryloyl group allows entry into two different classes of compounds- namely Michael-type additions onto amines such as the ε-amine of protein lysine residues (Roy *et al.*, 1991; Roy and Laferriere, 1990a) and secondly, copolymerization in the presence of reactive monomers such as acrylamide to form linear polymers with pendant spacer residues. Prior derivatization of the acryloylated spacer with oligo- or polysaccharides will afford glycopolymers or pseudo polysaccharides upon copolymerization with acrylamide (Roy and Laferriere, 1990a; Roy and Tropper, 1988a; Kallin *et al.*, 1989; Weigel *et al.*, 1982).

The usefulness of the acryloylated glycylglycine hydrazide spacer (**3-6**) and its respective methacrylamide copolymer (**3-9**) is clearly demonstrated in the preparation of oligosaccharide-protein conjugates or multivalent oligosaccharide-methacrylamide copolymers which will be described in detail in subsequent chapters. Their use as possible immunogens as well as their importance as multivalent coating antigens in ELISA will also be discussed.

3.5 Experimental Methods

General Methods

For a complete description of general methods, please see the general method entry in chapter 2 (section 2.7). For a more complete description of the ^1H NMR and ^{13}C NMR chemical shift values, please see tables 3-1 and 3-2.

N-ACRYLOYL GLYCINE (3-2)

Glycine ethyl ester hydrochloride (**3-1**)(10.0 gm, 71.7 mmol) was added to a solution of 10 gm of NaOH in 40 ml water and stirred at room temperature for ten minutes. Complete saponification was verified by TLC (1:1 $CHCl_3$-MeOH, Rf=0.06).

To the ice cooled solution was added dropwise 8.73 ml (107 mmol, 1.5 eq.) of acryloyl chloride. The pH of the solution was maintained above 9 with the addition of solid NaOH. The reaction was monitored through the disappearance of the ninhydrin positive starting material and was deemed complete after 1.5 eq of acid chloride was added. This was verified by TLC (Rf=0.24, solvent E). The pH of the solution was lowered to 1.5 with concentrated HCl and the solution was multiply extracted with warm ethyl acetate. The combined organic extracts were dried with Na_2SO_4 and evaporated to a yellow solid. The solid was crystallized from EtOAc to yield 7.12 gm (77%) of the title product **3-2**.

TLC (Rf=0.24, solvent E)

m.p. (EtOAc, 128.1°C dec.)

Anal. Calc. for $C_5H_7O_3N$: C: 46.50, H: 5.46, N:10.85; found C: 46.55, H: 5.48, N: 10.64.

^1H NMR (300 MHz; DMSO-d_6): δ 3.77 (d, 2H, NH-CH_2), 5.60 (dd, 1H, H_a), 6.07 (dd, 2H, H_b), 6.28 (dd, 1H, H_c), 8.41 (t, 1H, NH-CH_2)

^{13}C NMR (50.3 MHz; DMSO-d_6): δ 40.7 (C_5), 125.8 ($C_{3'}$), 131.3 (C2'), 165.0 ($C_{1'}$), 171.3 (C_4)

N-ACRYLOYL GLYCYLGLYCINE ETHYL ESTER (3-4)

Glycine ethyl ester (**3-3**) (4.79 gm, 1.2 eq.) was dissolved in 40 ml of ethanol containing 7.44 gm EDC (38.8 mmol,1 eq.) and 5.00 gm (38.8 mmol) of N-acryloylated glycine (**3-2**) The precipitated mixture was stirred at room temperature for five hrs. and left to sit overnight. The precipitate was removed by filtration, washed with cold ethanol, and dried. TLC analysis (solvent E) of the supernatant revealed the presence of starting acid (**3-2**). After concentration, a further 250 mg (1.3 mmol) aliquot of EDC was added, left to stand, and the precipitate was recovered as already described. The combined precipitates yielded 7.96 gm (96%) of pure title compound **3-4**. Crystallization from hot EtOH afforded 5.25 gm of **3-4** in 66% yield.

TLC (Rf=0.80, solvent E)

m.p. (EtOH, 148.9-149.1 °C)

CI- M.S. for $C_9H_{14}O_4N_2$: m/z 215 (M+1)

Anal. Calc. for $C_9H_{14}O_4N_2$: C: 50.46, H: 6.59, N: 13.08 found C: 50.04, H: 6.31, N: 12.83.

^1H NMR: (300 MHz; DMSO-d_6): δ 1.18 (t, 3H, OCH_2-CH_3), 3.80 (d, 2H, H_2), 3.83 (d,2H, H_5), 4.07 (q, 2H, OCH_2-CH_3), 5.60 (dd, 1H, H_a), 6.08 (dd, 2H, H_b), 6.29 (dd, 1H, H_c), 8.32 (t, 1H, H_6), 8.39 (t, 1H, H_3)

^{13}C NMR (50.3 MHz; DMSO-d_6): δ 14.8 (OCH_2-CH_3), 41.9 (C_5), 42.9 (C_2), 62.6 (OCH_2-CH_3), 128.6 ($C_{3'}$), 131.5 ($C_{2'}$), 165.3 ($C_{1'}$), 170.1 (C_4), 171.5 (C_1)

N-ACRYLOYL GLYCYLGLYCINE (3-5)

Glycylglycine ethyl ester (**3-3**) (5.00 gm, 23.4 mmol) was dissolved in 70 ml of a 90:10 MeOH:H_2O solution. The pH of the solution was raised to 12 with 2N NaOH and maintained at this level until the complete disappearance of starting material as judged by TLC (1:1 $CHCl_3$: MeOH, Rf=0.13). Cation resin (Amberlite IR-120-H^+ form) was added to the solution until the pH dropped to ~3. The resin was removed by filtration and the methanol:water mixture was removed under vacuum. Recovery of the title compound **3-5** was quantitative and the product used without further need of purification.

TLC (Rf=0.13, solvent E)

m.p. (EtOH, 133.1-134.3°C)

CI-M.S. for $C_7H_{10}O_4N_2$: m/z 187 (M+1)

^1H NMR (300 MHz; DMSO-d_6): δ 3.76 (d, 2H, H_2), 3.81 (d,2H, H_5), 5.61 (dd, 1H, H_a), 6.09 (dd, 2H, H_b), 6.30 (dd, 1H, H_c), 8.39 (t, 1H, H_6), 8.24 (t, 1H, H_3)

^{13}C NMR (50.3 MHz; DMSO-d_6): δ 40.7 (C_2), 41.8 (C_5), 125.8 ($C_{3'}$), 131.7 ($C_{2'}$), 165.3 ($C_{1'}$), 170.1 (C_4), 171.5 (C_1)

N-ACRYLOYL GYLCYLGLYCINE HYDRAZIDE (3-6)

Glycylglycine (**3-5**) (1.0 gm, 5.38 mmol) was dissolved in 50 ml warm t-butyl alcohol to which 1.34 gm (6.5 mmol, 1.2 eq) of dicyclohexyl carbodiimide (DCC) was added. After ten minutes at room temperature, 10.8 ml (5.4 mmol, 1 eq) of a 0.5 M solution of hydrazine monohydrate in ethanol was added dropwise over a period of one hour. The mixture was stirred overnight, analyzed by TLC (1:1 $CHCl_3$:MeOH, Rf=0.39), and the solvent evaporated. The solid residue was extracted twice with water in order to remove the bulk of the DCC urea side-product, and the solution was lyophilized to a fluffy white powder. The crude material was purified by flash chromatography (3:1 $CHCl_3$:MeOH) yielding 700 mg (65%) of the title compound **3-6**.

TLC R_f=0.39, solvent E

m.p. (EtOH, decomposition pt. 179.3°C)

CI-M.S for $C_7H_{12}O_3N_4$: m/z 201 (M+1)

Anal. Calc. for $C_7H_{12}O_3N_4$: C:42.00, H: 6.04, N: 27.99; found C: 42.47, H: 6.10, N: 28.10.

^{1}H NMR (300 MHz; DMSO-d_6): δ 3.65 (d, 2H, H_2), 3.86 (d,2H, H_5), 4.22 (bd, .2H, NH-NH_2), 5.61 (dd, 1H, H_a), 6.08 (dd, 2H, H_b), 6.29 (dd, 1H, H_c), 8.15 (t, 1H, H_2), 8.36 (t, 1H, H_6), 8.98 (bd, 1H, NH-NH_2)

^{13}C NMR (50.3 MHz; DMSO-d_6): δ 40.6 (C_2), 41.9 (C_5), 125.5 ($C_{3'}$), 131.5 ($C_{2'}$), 165.0 ($C_{1'}$), 170.8 (C_4), 169.1 (C_1)

Glycylglycine Methacrylamide Copolymer (3-7)

To a solution containing 100 mg (0.538 mmol) of N-acryloylated glycylglycine (**3-5**) in 1.0 ml degassed and deionized water was added 228 mg (2.68 mmol; 5 eq) of methacrylamide. The polymerization was initiated by the addition of 3.0 mg of ammonium persulphate and heating the solution at 100°C for 15 minutes. The reaction was re-initiated with a further 3.0 mg of persulphate and another 15 minute heating period. The solution was exhaustively dialyzed against running water and lyophilized to provide 237 mg of polymer.

The proton NMR spectrum (300 MHz; D_2O) revealed that glycylglycine residues were present in a 1:8 molar ratio to methacrylamide (12% mole/mole methacrylamide) from the integration of the methylene residues of glycylglycine (3.72-3.87 ppm) to the methacrylamide methyl protons (1.09 ppm).

Glycylglycine Ethyl Ester-Methacrylamide Copolymer (3-8)

To a solution of 100 mg of N-acryloylated glycylglycine ethyl ester (3-4) (0.467 mmol) in 1.0 ml degassed and deionized water was added 200 mg (2.35 mmol; 5 eq) of methacrylamide. The reaction was initiated with 3.0 mg ammonium persulphate and heated at 100°C for 15 minutes. The reaction was re-initiated a second time in the same manner. The solution was exhaustively dialyzed against running water and lyophilized to provide 214.3 mg of the product.

The proton NMR spectrum (300 MHz; D_2O) of **3-5** revealed that the glycylglycine ethyl ester content was 17% (mole/mole methacrylamide) or present in a 1:6 molar ratio to methacrylamide from the integration of the O-CH_2 group (4.0 ppm) to the methacrylamide methyl group (1.08 ppm).

Glycylglycine Hydrazide-Methacrylamide Copolymer (3-9)

(A) Glycylglycine-methacrylamide copolymer (3-7) (25.0 mg) was dissolved in 15 ml water and the pH was adjusted to 4.8 with HCl. EDC (36 mg, 188 mmol) was added to the solution while maintaining the pH between 4.8-5.0. After pH stabilization,100 ul (0.1 mmol) of a 1M $NH_2NH_2.H_2O$ solution was added. The solution was stirred overnight, followed by exhaustive dialysis against running water, and lyophilization to yield 27.0 mg of product.

Quantitative TNBS (appendix A) analysis revealed a hydrazide incorporation of 16.5% (wt/wt polymer) or 8.5% (mole/mole methacrylamide).

(B) Glycylglycine ethyl ester-methacrylamide copolymer **(3-8)** (25.0 mg) was dissolved in 3.0 ml water to which 100 ul of a solution of hydrazine hydrate(1M, 0.1 mmol) was added. The reaction was allowed to stand for four days at room temperature, dialyzed against water, and lyophilized to yield 27.5 mg of product.

Hydrazide formation was verified by the disappearance of the ester peak in the ^{1}H NMR spectrum (300 MHz; D_2O) at 4.0 ppm. Colourimetric analysis (TNBS- appendix A) for the hydrazide group revealed its incorporation at 17.5% (wt/wt polymer) or 9.0% (mole/mole methacrylamide).

Chapter 4- Sialic Acid Containing Macromolecules

4.1 Introduction

Sialic acid mono-/ oligo-/ or polysaccharides coupled to a macromolecular support, such as proteins, polymers, or lipids modify substantially the way in which they react with the immune system (*in vivo*) or in immunochemical assays (*in vitro*). The most important effects are summarized as follows:

i) Non-immunogenic→immunogenic: Other than high molecular weight polysaccharides, typically carbohydrates are not immunogenic on their own (Paul *et al.*, 1971; Hurn & Chantler, 1980). It is a well established phenomenon that the coupling of a small non-immunogenic molecule (hapten) to a larger immunogenic protein results in the production of antibodies (Ab's) to the hapten (Landsteiner, 1917) and its application to carbohydrates is no exception (Avery & Goebel, 1931a,b). The same phenomenon has been found to occur with glycolipids in the form of micelles (Harwood, 1992; Fillet *et al.*, 1988)) and, although limited in study, glycopolymers hold promise for the future (Petrov *et al.*, 1985). Even though some high molecular weight polysaccharides are immunogenic and protective (i.e. *Neisseria meningitidis* serogroups A & C capsular polysaccharides) (Gotschlich *et al.*, 1969; Artenstein *et al.*, 1970), they are still poorly immunogenic in children under 2 years of age. Covalent coupling of these polysaccharides to protein macromolecules render them more immunogenic and become suitable infant vaccines (Anderson, 1988). The reason

for this increase in immunogenicity can largely be explained by the following entry.

ii) Conversion from a thymus-independent (TI) to a thymus-dependent (TD) antigen: As mentioned previously, polysaccharides are considered as TI antigens and smaller oligosaccharides are characterized as neither since on their own they do not elicit an Ab response. Polysaccharides have the ability to interact directly with Ab- forming cells to stimulate them to produce specific antibody, invariably of the IgM isotype. As the immune process matures however, there is no accompanying maturation of the quality of the Ab (i.e. increased affinity and isotype switching to IgG class Ab's) and no memory process is evolved. The covalent coupling of oligo-/ polysaccharides to TD proteins, invokes another process involving the participation of a subclass of lymphocytes known as T-helper cells (fig. 4.1). This response arises from the recognition of T-cell epitopes present on the carrier protein which are lacking on the oligo-/ polysaccharides. The result of this TD pathway is the production of high affinity and second generation IgG isotype antibodies, which is normally the desired response. Also, as the immune process matures there is the formation of memory cells which are long lived and are able to "switch on" instantaneously if ever the stimuli (i.e. oligo-/ polysaccharide from the bacteria) is encountered again. This is a most important consideration in the design of vaccines to bacterial polysaccharides.

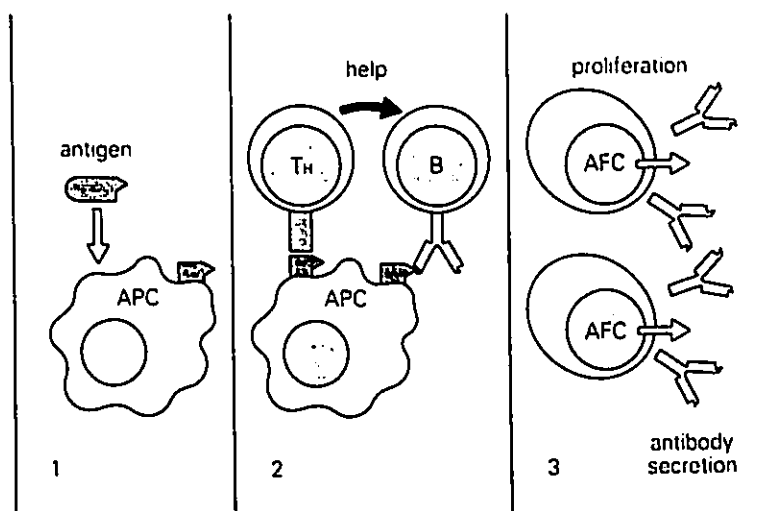


Fig. 4.1 Simplified overview of the immune response.
1. Antigen encountering the immune system is processed by antigen-presenting cells (APCs) which retain fragments of the antigen on their surfaces.
2. T-helper cells (TH) recognize the antigen via their surface receptors and provide help to B cells (B) which also recognize antigen by their surface receptors (immunoglobulin).
3. The B cells are stimulated to proliferate and divide into antibody-forming cells (AFCs) which secrete antibody.

iii) Multivalency: The third characteristic imparted by conjugating oligo-/ polysaccharides to proteins is the creation of multivalent antigens. Antibodies are at least divalent in terms of binding sites (e.g. IgG isotypes) and can be at most decavalent (e.g. IgM isotypes). The ability of an Ab to form crosslinked complexes with antigen to form large macromolecular lattices is fundamental to a variety of immunochemical assays and is the key mechanism in which appropriate signals are delivered to effector cells (*in vivo*) to promote phagocytosis, lysis, or release of cytotoxic factors. Immunochemists have often taken advantage of the

precipitating nature of these lattice complexes in order to gain insights into the structural requirements of the Ag or to determine useful kinetic and thermodynamic information as will be demonstrated in the following serological section. Polysaccharides typically are composed of a number of oligosaccharides which repeat themselves in a predictable fashion. For convenience, let a pentasaccharide repeat unit constitute one antigenic determinant for a particular Ab, then it is clear that a polysaccharide containing 50 repeat units, has the capability of having many Ab's bind to it at once. Hence, the polysaccharide is considered a multivalent antigen and can form cross-linked lattice structures with antibodies. The presence of 10 epitopes does not preclude the binding of 10 Ab's since other forces such as conformational changes, steric interactions, and ionic repulsions and attractions govern the microenvironment. Oligosaccharides on the other hand, may contain only one antigenic determinant and are thus unable to crosslink antibodies. Conjugation of oligosaccharides to protein, polymer, or lipid allows them to be presented to the Ab in a manner which allows the Ab to form extended networks.

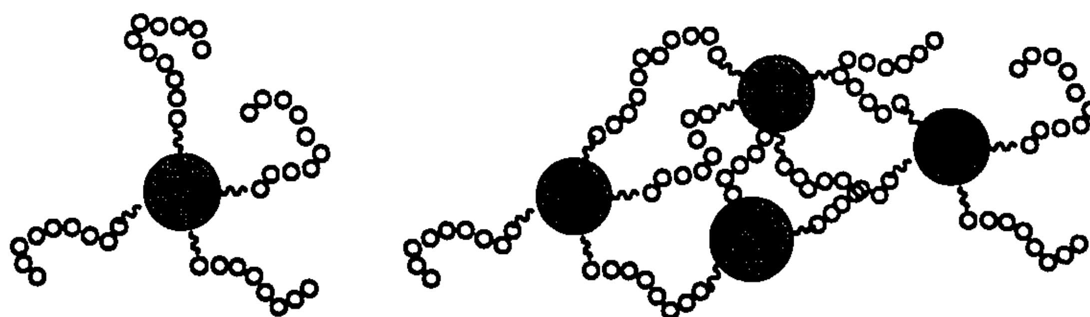
iv) Immobilization of antigen: Immobilization of an antigen on a solid surface is often necessary with immunochemical techniques due mainly to the need for repeated purification steps. Proteins, acrylamide based copolymers, and lipid glycoconjugates all bind strongly to hydrophobic surfaces such as polystyrene plates and effectively immobilizes the hydrophilic oligo-/ polysaccharide moiety of the

glycoconjugate. Purification between successive reagents is simply a matter of washing away the non-bound material leaving behind the oligo-/ polysaccharide bound to the surface.

The choice of carrier proteins is largely dictated by which applications the glycoconjugate will serve. As an immunogen, the choices are restricted to highly immunogenic proteins which can be purified to a well defined state. The most common proteins are derived from bacterial or mammalian sources with the obvious exclusion of human sources when considering a potential human vaccine. Bacterial derived carrier proteins like tetanus or diptheria toxoids, have the advantage of boosting pre-existing antibody levels to those pathogens as well as raising Ab's to the carbohydrate portion. A major disadvantage associated with using these toxins is that a detoxification stage is required. Detoxification reduces the number of functional groups available for conjugation purposes resulting in a reduced carbohydrate loading which is often crucial for an effective vaccine. Detoxification also reduces the number of T and B cell epitopes of the carrier protein rendering it less immunogenic. Despite these limitations, the toxins derived from bacteria are still the most ubiquitous form of carrier proteins. New generation carrier proteins (Pappenheimer *et al.*, 1972; Black *et al.*, 1988) are genetically engineered bacterial toxins that are non-toxic, not requiring a detoxification step, yet are immunochemically indistinct from the native protein. As an antigen, the choice of carrier is more flexible and is usually governed by availability, cost, stability, and a high number of desired functional groups in which to effect

conjugation. The best antigens are however those derived from synthetic acrylamide copolymers since they are much more stable than glycoproteins. They also represent "true" antigens since they are composed solely of acrylamide and carbohydrate, unlike glycoproteins which exhibit a large number of protein based antigens which may cause non-specific interactions with antibodies in immunochemical assays.

Effective glycoconjugates require a number of considerations in their design. Firstly, due to the low solubility of oligo-/ polysaccharides and proteins in organic solvents, all coupling chemistry must take place in aqueous media. The chemistry of the coupling reaction should be well defined and specific, forming stable non-reversible linkages without significantly modifying the structure of either the carbohydrate portion or the protein. The mechanism of the coupling should be quick and efficient relative to the stability of both the oligo-/ polysaccharide and the protein in the reaction medium, and require the minimum in chemical manipulations. The introduction of neo-antigens in the linkage region should be kept to a minimum which is an important consideration when dealing with linking spacers. Finally, consideration must be given into what type of glycoconjugate is desired. Reaction of more than one functional group of the polysaccharide can result in a macromolecular cross-linked product (fig. 4.2b).



Specific conjugation model 4-2a   Random conjugation model 4-2b

Figure 4.2- Conjugation models

Activation of the polysaccharide in a random manner produces epitopes of various sizes resulting in an ill- defined conjugate. The advantage lies in the presumed increase in immunogenicity of a high molecular weight cross-linked product, based on results obtained with protein vaccines. Monofunctionalized oligo-/ polysaccharides (fig. 4.2a) have the advantage of being well defined in terms of epitope size and the number of oligo-/ polysaccharide chains per protein molecule. These types of conjugates also reflect more realistically the capsular polysaccharides found on bacterial surfaces. Orientation is also an important factor. Many methods will present the oligo-/ polysaccharides in an orientation that is not naturally expressed by the bacteria (i.e. will link through a non- reducing terminal rather than through the natural reducing linkage).

The functional groups available in proteins are the ε-amino groups of lysine residues, the sulfhydryl group of cysteine, the carboxylic acids of aspartic and glutamic acid, and to a lesser extent,

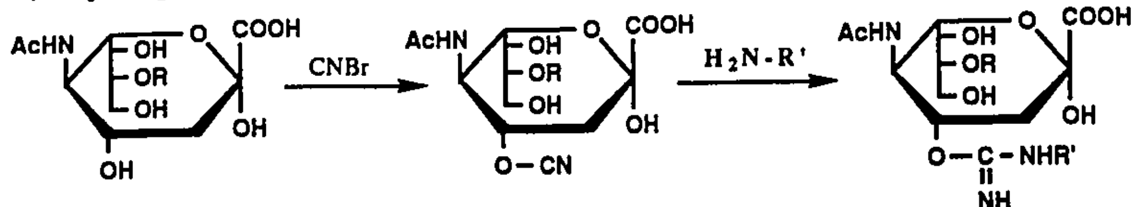
the phenolic hydroxyl group of tyrosine. Activation of carbohydrate residues is usually accomplished by activation of possible carboxylic acids, the random activation of some of the ring hydroxyls, introduction of terminal aldehydes, or modifications involving the introduction of a reactive linker. Conjugation can also take place via the hemiacctal or hemiketal functionality of reducing sugars. For practical purposes, direct coupling of carbohydrates to proteins without interposing any spacer groups are usually limited to the condensation of carboxyl or aldehyde groups with primary amines. Some of the more wide spread techniques are listed in table 4-1. It is important to note the distinction between specific coupling techniques (preferred due to a predictable outcome) or random coupling techniques. The trend is definitely toward the more defined coupling technique as various "random" vaccines are proving to be unreliable (Ward, 1991).

This chapter is devoted to the elucidation of methods in which to couple sialic acid oligo-/ polysaccharides to macromolecular supports for the various reasons mentioned above and at the same time, adhering to the outlined criteria and limitations of the coupling reaction. Currently, there are a number of methods using such techniques as described in table 4-1, however none of them are completely satisfactory due to one or more critical reasons.

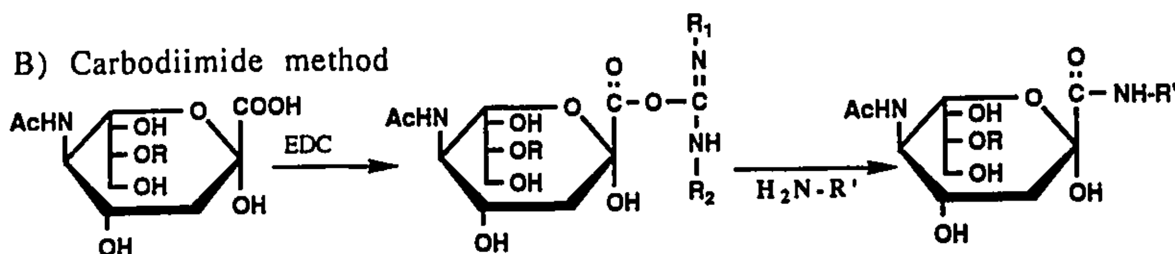
Reaction Type/ Substrate	Active Intermediate	Bond Type	Specificity
Aromatic Amine	diazonium salt	azo linkage	random
Carboxylic acids	mixed anhydrides	amidation	random
Carboxylic acids	carbodiimide ester	amidation	random
Oxidation	disulfide	disulfide bond	random
Substitution	bromo derivative	thioether bond	specific/random
Linker method	activated spacer	various	specific/random
Selective periodate oxidation	aldehyde	amination	specific
Reducing sugar	hemiacetal	amination	specific

Table 4-1- Partial list of some of the more prominent conjugation techniques.

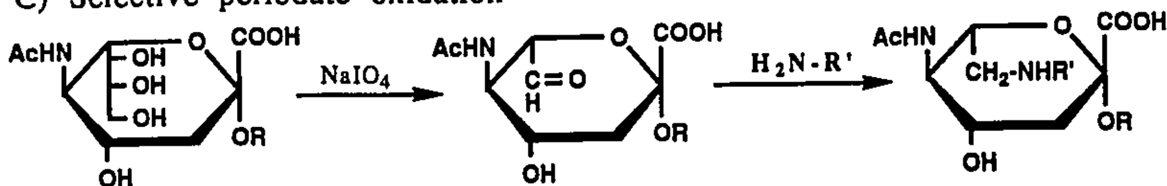
A) Cyanogen bromide method



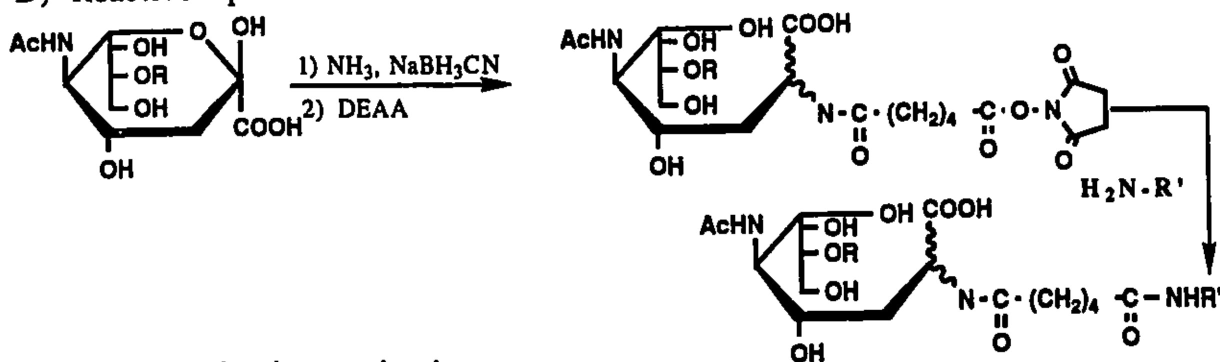
B) Carbodiimide method



C) Selective periodate oxidation



D) Reactive spacer method



E) Direct reductive amination

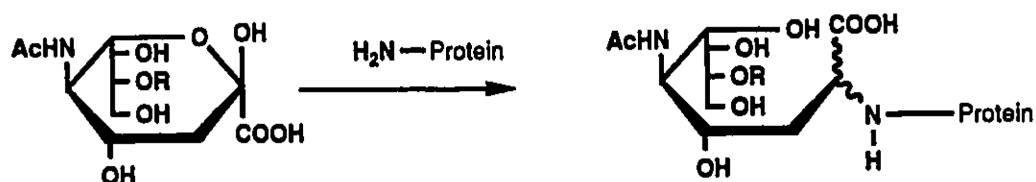


Figure 4.3- Sialic acid conjugation methods.

The existing methods (see fig. 4.3) can be categorized as those resulting from random activation of the sialic acid oligo-/ polysaccharide and consequentially results in the formation of crosslinked products, or those resulting from specific activation or derivatization of the sialic acid oligo-/ polysaccharide. Examples of the former case involve the cyanogen bromide activation of non-specific ring hydroxyls followed by condensation with amine (Kaijser *et al.*, 1983) (fig. 4.3-a), or the random activation of some of the carboxyl residues with the carbodiimide, EDC and condensation with amine (Beuvery *et al.*, 1983; 1986) (fig.4.3-b). Examples of the latter situation are the selective periodate oxidation of the vicinal diol of the exocyclic side chain of sialic acid (Jennings & Lugowski, 1981; Jennings & Roy, 1986) (fig. 4.3-c), or the introduction of a spacer selectively at the reducing terminus (Porro, 1987; Porro *et al.*, 1986; Porro *et al.*, 1983) (fig. 4.3-d). Direct reductive amination of the sialic oligo-/ polysaccharide (fig.4.3-e) has been attempted (Roy *et al.*, 1984a) but showed only limited success due to the poor reactivity of the reducing ketose. In this chapter, we will discuss the synthesis of various oligo-/ polysialic acid conjugates to either proteins or synthetic polymers based on a variety techniques including an improved method for direct reductive amination of amine onto the reducing ketose of oligo-/ polysialic acid and a *de novo* Michael-type method of conjugation. Evaluation of each technique with regard to it's advantages and disadvantages will also be discussed.

4.2 Results

Macromolecular sialic acid containing conjugates pertaining to this work consist of:

(1) protein based neo-glycoproteins

(2) polymer based neo-glycopolymers.

Emphasis will be placed on the synthesis of neo-glycoproteins since glycopolymers are usually generated from copolymerization of monomers as described in chapter 3, however alternate routes of synthesis of these glycopolymers will be discussed. Various sialyl mono-/ oligo-/ and polysaccharides were preliminarily modified in order to introduce a reactive group onto the carbohydrate segment subsequent to conjugation with proteins. A recurrent reaction sequence is the reductive amination of aldehydes, hemiacetals, or hemiketals with either ammonia, a primary amine, or a hydrazide. The reaction sequence is depicted in figure 4.4.

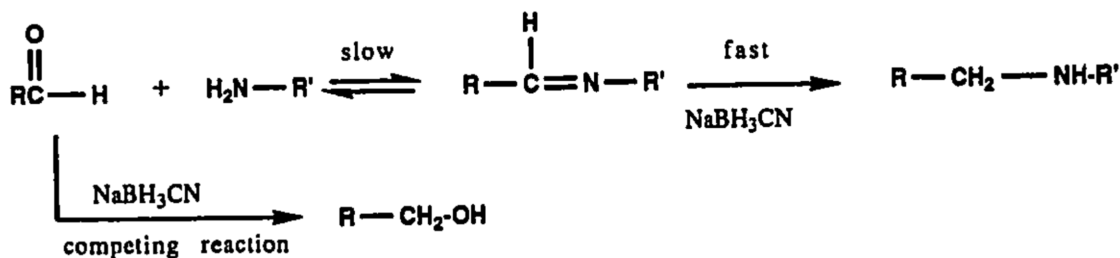


Figure 4.4 Reductive amination reaction scheme

The reversible Schiff base or hydrazone linkage is reduced to the stable C-N bond with $NaBH_3CN$. The major competing reaction is the slow reduction of the aldehyde or ketone to their respective alcohols

(fig. 4.4). Formation of the imine is rate determining and unfavourable in aqueous solutions. However, quick reduction of the imine is sufficient to drive the reaction forward. In the case of hemiacetal and hemiketal derivatizations, the terminal reducing sugar is opened to produce an acyclic alkylamine spacer (fig. 4.5).

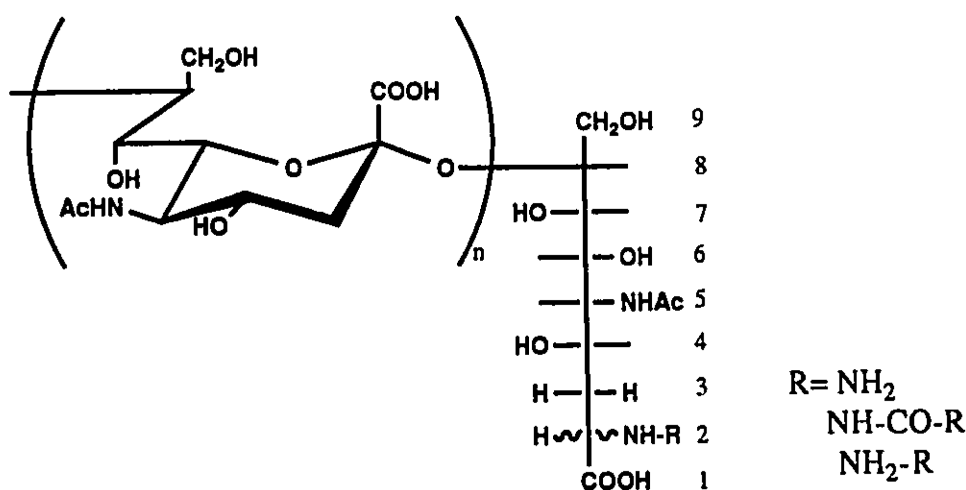


Figure 4.5- Acyclic alkylamine derived from reductive amination of $(Neu5Ac)_n$ with ammonia, hydrazide, or primary amine.

4.2.1 Functionalization of sialic acid substrates

4.2.1.1 Aldehydo derivatives

α-2-Oxoethyl Neu5Ac (**4-2**) (see fig. 4.6) was prepared from the α-allyl glycoside of Neu5Ac (**4-1**) by reductive ozonolysis according to the well established methods (Roy *et al.*, 1987; Roy & Laferriere, 1990b). Treatment of **4-1** in methanol with ozone at -78 °C allowed the efficient insertion of ozone into the double bond to

form the unstable ozonide. The extent of the reaction was estimated by the appearance of a blue colour signifying the presence of excess ozone. Reductive workup with dimethyl sulfide, followed by removal of solvents, produced the aldehydo compound (**4-2**) as an oil in quantitative yield.

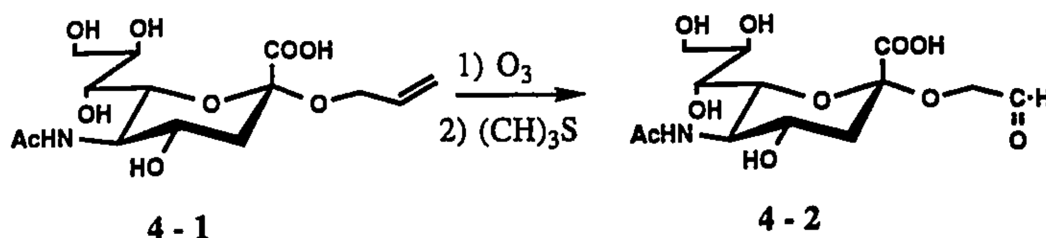


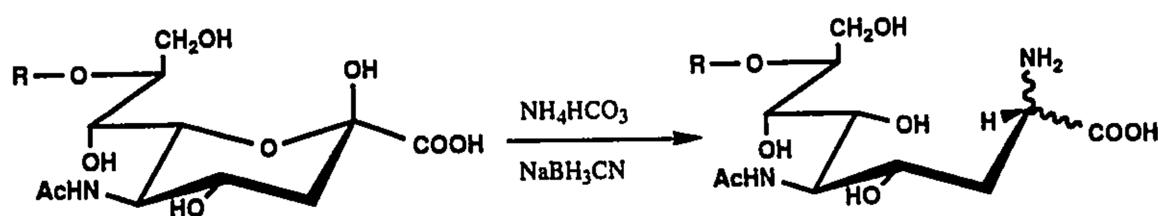
Figure 4.6- Synthesis of 2-oxoethyl Neu5Ac

The purity and identity of the product was verified by comparison to a known standard in TLC (single spot, Rf= 0.16 solvent B) and by negative FAB-MS which showed the characteristic (M-1) peak at 350 m/z.

4.2.1.2 Amino derivatives

A series of Neu5Ac oligosaccharides and colominic acid were reductively aminated with ammonia through the terminal reducing ketal functionality (fig. 4.7). The monosaccharide (**4-3**) was reductively aminated with NH_4HCO_3 in refluxing methanol since the solubility was good and the exclusion of water is known to increase the reaction rate (Stoll *et al.*, 1988). The reaction was followed by TLC and was stopped upon disappearance of the starting Neu5Ac and the appearance of a polar product at Rf 0.11 (solvent A). The product,

Neu5Ac-NH_2 (**4-7**) isolated in 76% yield, was reasonably complicated in its ^1H NMR (300 MHz; D_2O) spectrum due to the resultant diastereomeric mixture. Both of the resonances corresponding to the ring H-3_{ax} and H-3_{eq} protons at 1.83 and 2.21 ppm respectively, collapsed into a broad multiplet encompassing the N-acetamido protons between 1.8- 2.1 ppm and integrating for 5 protons overall.



R=H **4 - 3**

R= Neu5Ac **4 - 4**

R=$(Neu5Ac)_2$ **4-5**

R=$(Neu5Ac)_n$ **4-6**

R=H **4 - 7**

R= Neu5Ac **4 - 8**

R=$(Neu5Ac)_2$ **4-9**

R=$(Neu5Ac)_n$ **4-10**

Figure 4.7 Reductive amination of ammonia onto the reducing end of Neu5Ac.

Two peaks (apparent dd) appeared downfield at 4.43 and 4.48 ppm integrating for 0.5 H each and was attributed to the new H-2 proton of the two diastereomers. The ^{13}C NMR (50.3 MHz; D_2O) revealed a doublet in the C-3 region (34.8 ppm) corresponding to the two diastereomers and a lack of a C-2 anomeric carbon (97.6 ppm) (Schauer, 1982) consistent with the open form of the sugar. The open

form C-2 appeared as a doublet at 54.0 ppm consistent for a methine carbon α to both an amine and a carboxylic acid (Johnson & Jankowski, 1972). Neu5Ac disaccharide $(Neu5Ac)_2$ **(4-4)**, Neu5Ac trisaccharide $(Neu5Ac)_3$ **(4-5)** and colominic acid (Neu5Ac~30) **(4-6)** were reductively aminated with a saturated solution of NH_4HCO_3 in sodium borate buffer (0.2 M, pH 9.2) due to a lack of solubility in MeOH. The reaction time was substantially longer (5 days) than the corresponding monosaccharide due to the reversible nature of the Schiff base in aqueous media. The disaccharide derivative $(Neu5Ac)_2$-NH_2 **(4-8)** was recovered in 86% yield after gel extrusion chromatography and careful separation to remove residual $(Neu5Ac)_2$. Purification in this way is feasible since the amino derivative is retained slightly on a Sephadex matrix due to ionic interactions with the small number of carboxyl groups present on the gel. FAB-MS (for $C_{22}H_{37}O_{16}N_3.NH_4$: m/z 602 (M+1), m/z 600 (M-1)) and ^1H- and ^{13}C NMR spectroscopy were consistent for the reductively aminated dimer **(4-8)**. Collapse of the reducing terminal was observed by the disappearance of the H-3_{eq} and H-3_{ax} of the reducing end at 2.77 and 1.73 ppm respectively, and a lack of the reducing anomeric carbon at 97.3 ppm. The H-2 proton of each diastereomer was located downfield at 4.32 and 4.45 ppm, integrating for 1 proton overall. The C-2 carbon of the opened sugar was assigned to the resonance at 54.5 ppm (apparent doublet) as seen in the derivatized monosaccharide **(4-7)**. The ^1H- and ^{13}C NMR were also consistent for the presence of one intact α-2 linked Neu5Ac residue (Roy & Pon, 1990).

The reductively aminated trisaccharide $(Neu5Ac)_3$-NH_2 (**4-9**) was isolated in the same manner as above in 80% yield and all spectral analyses were consistent for the title product (**4-9**). FAB-MS showed a peak at 891 m/z (M-1) (negative mode) and a peak at 893 m/z (M+1) (positive mode) for $C_{33}H_{53}O_{24}N_4$. NH_4 salt. ^{1}H- and ^{13}C NMR (500 MHz and 50.3 MHz respectively, D_2O) analysis was essentially the same as described for the disaccharide derivative (**4-8**) showing the presence of the terminal alkyl amine with an intact Neu5Ac-α-(2→8)-Neu5Ac-α-(2→ moiety. The shifts of the H-3 protons established unequivocally the non-reducing residue from the interior α-(2→8) linked Neu5Ac residue with the H-3_{eq} of the non-reducing residue occurring at a lower field (2.77 ppm) relative to the interior H-3_{eq} at 2.70 ppm. Comparison of the ^{13}C NMR shifts of the corresponding $(Neu5Ac)_3$ (**4-5**) (Michon *et al.*, 1987), allowed the partial assignment of the complex ^{13}C NMR profile. In a similar manner as described for the disaccharide (**4-4**) and trisaccharide (**4-5**), colominic acid (**4-6**) with an average molecular weight of 12 KD (Neu5Ac~40)was reductively aminated with ammonia. Purification of the polysaccharide was achieved by removal of the small molecular weight components by dialysis through a semi-permeable membrane (mol. wt. cutoff= 10 KD). Recovery of the modified polysaccharide (**4-10**) was virtually quantitative (94%). ^{1}H- and ^{13}C NMR (500 MHz & 50 MHz respectively; D_2O) analyses were not very informative due to the small percentage of derivatized sialic acid relative to the intact sialic acid residues. In the ^{1}H NMR spectrum however, the presence of a small broad resonance in the 4.2- 4.4 ppm region was indicative of the reductively aminated reducing residue. The presence of

primary amine was confirmed by the positive ninhydrin result obtained with the polysaccharide after the ammonium counter ions were exchanged for sodium.

4.2.1.3 N-Acryloylated derivatives

A synthetic heterobifunctional spacer- Neu5Ac conjugate (**4-11**), terminating with a reactive N-acryloyl group, was prepared by reductively aminating 2-oxoethyl Neu5Ac (**4-2**) with N-acryloylglycylglycine hydrazide (**3-6**) (fig. 4.8). This was accomplished in refluxing methanol in the presence of $NaBH_3CN$ and followed by TLC. After the complete disappearance of the aldehydo Neu5Ac (**4-2**) and the concomitant formation of a new product which was UV and KMnO4 positive, one equivalent of $NaBH_4$ was added to ensure complete reduction of the hydrazone formed. This step was necessary since it was found that $NaBH_3CN$ did not completely reduce the hydrazone linkage and subsequent workup showed two products; the desired product (**4-11**) and residual Neu5Ac aldehyde (**4-2**). It is presumed that longer reaction times would overcome this observation, however the $NaBH_4$ treatment is a viable time saving option. One precaution must be taken with this last step and the reduction should be done at room temperature and with a limited amount of $NaBH_4$.

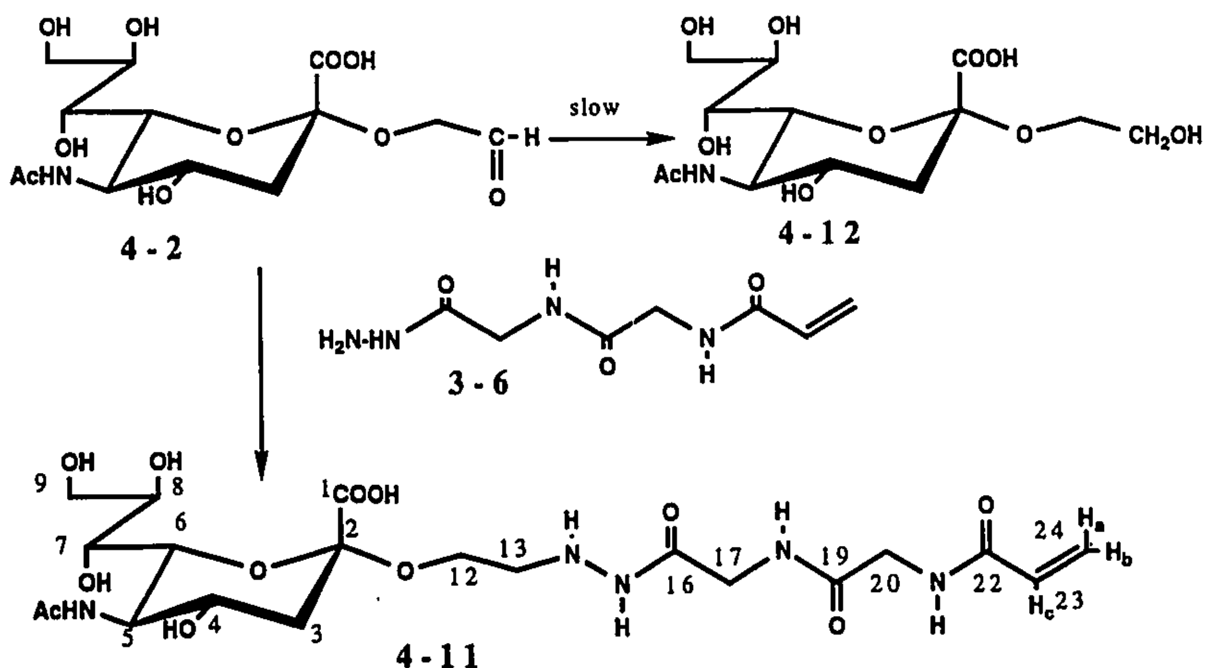


Figure 4.8- Neu5Ac-acryloylglycylglycine conjugate

Indeed it was found that treatment of **4-11** with a large excess of $NaBH_4$ caused partial reduction of the conjugated double bond of the N-acryloyl group which is a known phenomenon (Kadin, 1965). The sialic acid- spacer conjugate (**4-11**) was purified by preparative TLC (solvent E) in order to remove the principle by-product which is the reduced form of 2-oxoethyl Neu5Ac (**4-12**) and was isolated in 76% yield. The product was characterized by TLC (single product, UV, $KMnO_4$, and resorcinol positive; Rf= 0.28 solvent C), ^1H NMR (olefinic protons in 5.7-6.5 ppm range, the C-20 and C-17 methylenes at 4.05 and 3.93 ppm, H-3_{eq} at 2.75 ppm, H-3_{ax} at 1.72 ppm, and the N-acetamido protons at 2.05 ppm) and by ^{13}C NMR (two new methylene carbons as determined by a DEPT experiment at 68.3 and 71.4 ppm for the linkage region methylenes (C-13 and C-12

respectively), the characteristic olefinic shifts at 129.8 and 130.8 ppm, and the C-1 to C-9 carbons shifts of the Neu5Ac ring and side chain carbons).

The corresponding N-acryloylated dimer derivative (**4-13**), trimer derivative (**4-14**), and polymer derivative (**4-15**) were obtained by treating their respective amino derivatives (**4-8**), (**4-9**) and (**4-10**) with acryloyl chloride under basic aqueous conditions at 0 °C (fig. 4.9).

NaOH

R= Neu5Ac **4-8**
R=$(Neu5Ac)_2$ **4-9**
R=$(Neu5Ac)_3$ **4-10**

R= Neu5Ac **4-13**
R=$(Neu5Ac)_2$ **4-14**
R=$(Neu5Ac)_3$ **4-15**

Figure 4.9- N-Acryloylated Neu5Ac oligosaccharide series.

The HCl generated was neutralized by the addition of NaOH and the pH was maintained between 8.5-10 in order to minimize base hydrolysis of the acid chloride and to maximize the relative amount of free amine. Disappearance of ninhydrin positive starting material allowed the determination of the extent of the reaction. Workup of the products by gel extrusion chromatography on a Sephadex G-10 column in water revealed in the oligosaccharide cases, a molar ratio of the acryloyl group to single H-3 proton that was greater than 1

and significant heterogeneity in the N-acetyl region. This led to the conclusion that O-acrylates were also formed during the reaction and a saponification stage with NaOH was implemented prior to the workup of the derivatized oligo-/ polysaccharide. In this manner, N-acryloylated $(Neu5Ac)_2$ (**4-13**), N-acryloylated $(Neu5Ac)_3$ (**4-14**), and mono-N-acryloylated colominic acid (**4-15**) were isolated after gel extrusion purification in 92%, 85%, and 87% (recovered) yields respectively. Compounds **4-13** and **4-14** when analyzed by FAB-MS, gave molecular ions consistent with the desired products. High resolution ^{1}H NMR (500 MHz, D_2O) of the two oligosaccharides (table 4-2) both showed similar spectra with three distinct regions of overlapping dd's in the olefinic region, corresponding to the H_a (5.77 ppm), H_b (6.21 ppm), and H_c (6.43 ppm) N-acryloyl protons of the diastereomeric mixture. Interpretation of the coupling constants is prohibitive due to the complexity of the diastereomeric mixture. The olefinic protons integrated in a 3:1 ratio vs. the H-3eq proton in **4-13** (2.75 ppm) or vs. either of the H-3_{eq} protons in **4-14** (2.77 or 2.70 ppm). The two resonances from the H-2 proton in both cases shifted unexpectedly upfield by ~0.12 ppm perhaps owing to an anisotropic effect of the conjugated double bond. The remainder of the proton spectrum of **4-13** or **4-14** is consistent with the preservation of one terminal α 2-linked Neu5Ac residue in **4-13** and a terminal α-(2→8) linked disaccharide in **4-14**.

	4-13		4-14		
	residue A	residue B	residue A	residue B	residue C
H-2	-	4.33 (m, 0.5H) 4.25 (m, 0.5H)	-	-	4.24 (dd, 0.5H) 4.33 (dd, 0.5H)
H-3	ax. 1.78 (dd, 1H) eq. 2.75 (dd, 1H)	1.88 (m, 1H) 2.0-2.1 (m, 1H)	ax. 1.73 (dd, 1H) eq. 2.78 (m, 1H)	ax. 1.73 (dd, 1H) eq. 2.65 (m, 1H)	1.86 (m, 1H) 1.95 (m, 1H)
CH_3	2.03 (s, 3H)	2.07 (s, 3H)	2.03 (s, 3H)	2.07 (s, 3H)	2.07 (s, 3H)
H_a	-	5.77 (overlap dd,1H)	-	-	5.77 (overlap dd, 1H)
H_b	-	6.21 (m, 1H)	-	-	6.19 (m, 1H)
H_c	-	6.43 (m, 1H)	-	-	6.37 (m, 1H)

Table 4-2 ^{1}H NMR shifts (500 MHz, D_2O, 300 K) of **4-13** and **4-14** in ppm referenced to HOD at 4.756 ppm.

^{13}C NMR (50.3 MHz, D_2O) analysis of the two N-acryloylated oligosaccharides (table 4-3) revealed the characteristic olefinic carbon shifts at 128.1 and 130.6 ppm for the methylene and methine carbons respectively. Doublet peaks were observed for C-3, C-5, and C-9 of both **4-13** and **4-14** (table 4-3) signifying the presence of the two diastereomers. The C-2 resonance of the terminal N-acryloylated alkyl amine of both products was found in the 54 ppm region. An example of both the high field ^{1}H NMR and ^{13}C NMR spectra of the N-

acryloylated Neu5Ac disaccharide derivative (**4-14**) is given in figure 4.10 and 4.11.

	4-13		**4-14**		
	residue A	residue B	residue A	residue B	residue C
C-1	174.1	175.1	174.1	175.2	175.3
C-2	101.3	53.9	101.5	102.8	54.7 d
C-3	41.1	36.1 d	41.0	41.1	36.5 d
C-5	53.1	52.4	52.5	53.2	53.9
C-6	72.5	73.4	69.0	69.7	n.a.
C-8	74.4	78.5	72.5	73.4	78.6
C-9	63.3	61.7	63.4	63.4	62.0
C=O	175.6	175.6	175.7	175.7	174.1
CH_3	23.1	22.7	22.8	23.1	22.7
CH=C	-	130.6	-	-	130.8
$C=CH_2$	-	128.1	-	-	128.2
O=C-N	-	174.1	-	-	174.1

Table 4-3 ^{13}C NMR (125.7 MHz, D_2O, 300 K) shifts of **4-13** and **4-14** in ppm referenced from external acetone at 31.07 ppm.

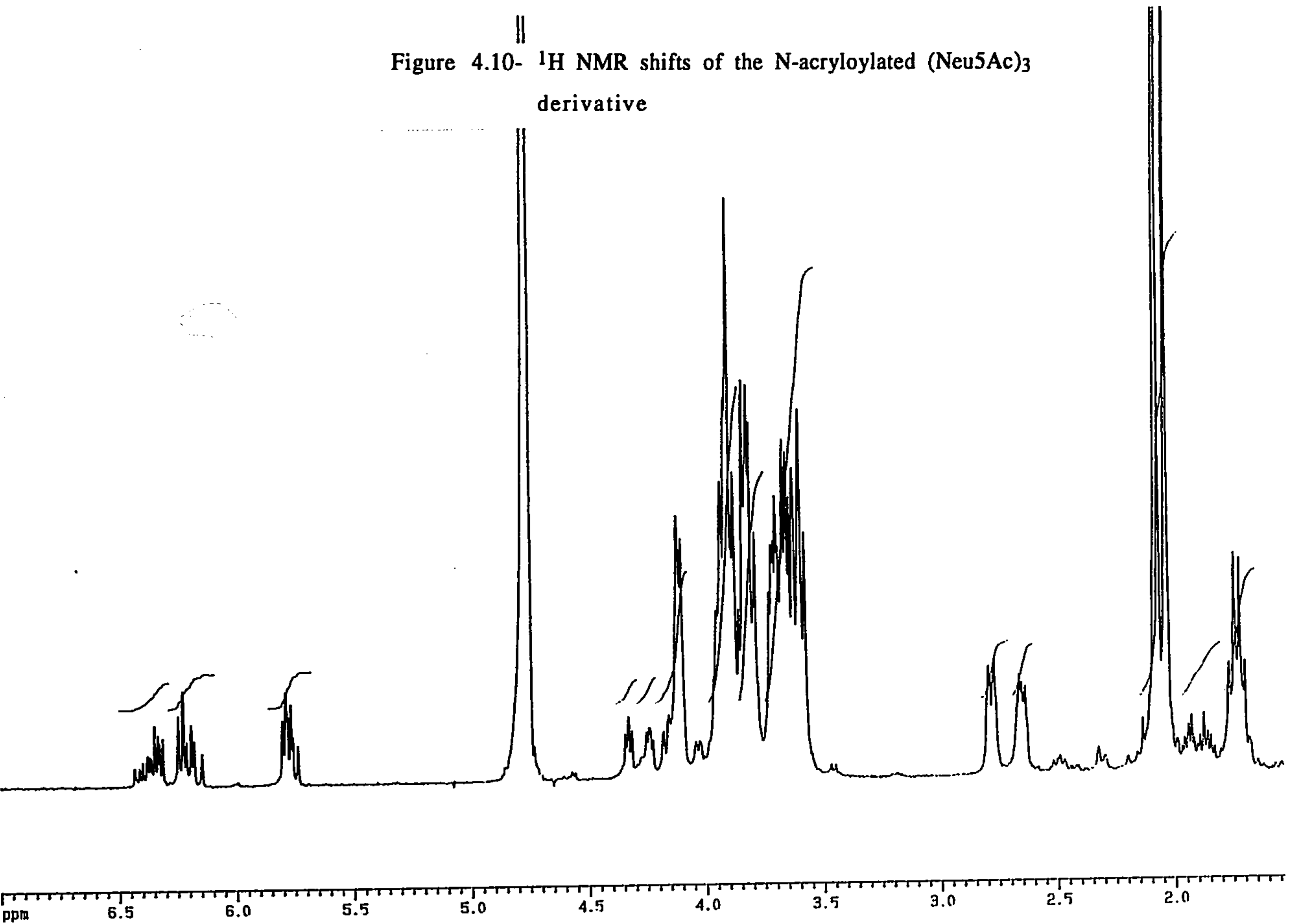
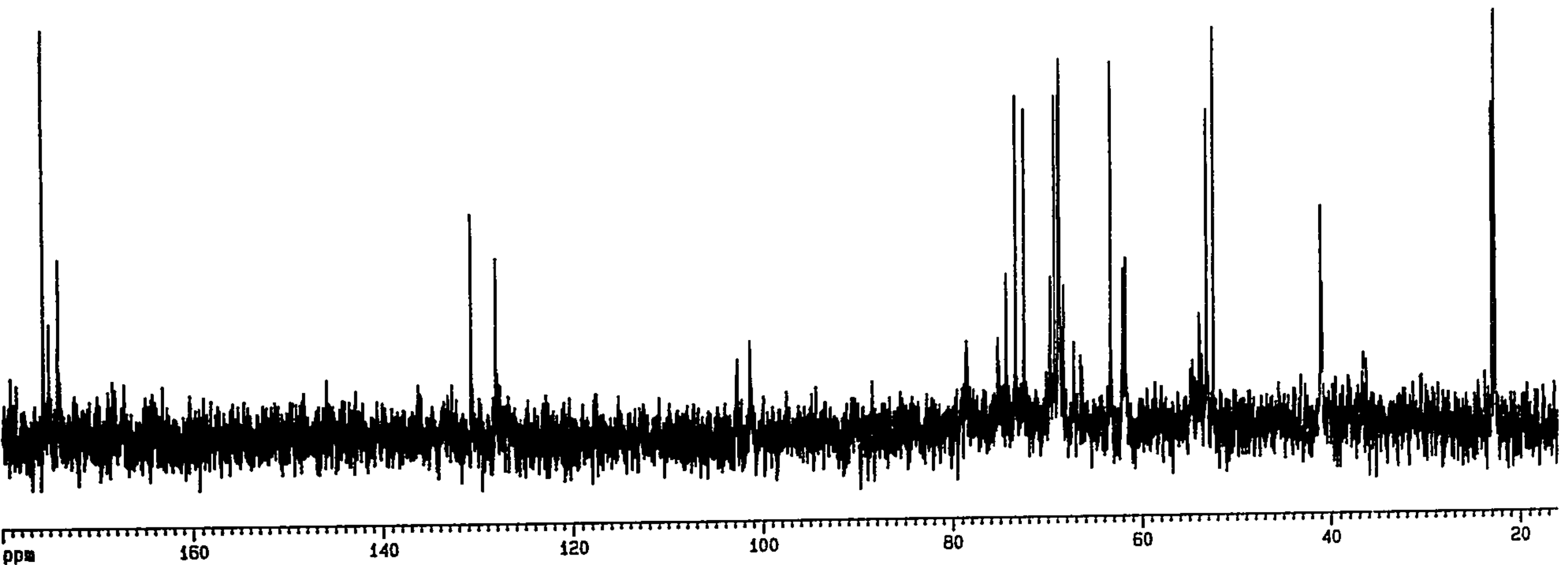


Figure 4.10- ^{1}H NMR shifts of the N-acryloylated $(Neu5Ac)_3$ derivative

Figure 4.11- ^{13}C NMR shifts of the N-acryloylated $(Neu5Ac)_3$ derivative



It is interesting at this point to make the comparison between the N-acryloylated $(Neu5Ac)_2$ derivative (**4-13**) and the N-acryloylated glycylglycine Neu5Ac conjugate (**4-11**). N-acryloylated $(Neu5Ac)_2$ (**4-13**) is characterized by the presence of one intact Neu5Ac residue α-linked to a hydrophilic open chain spacer, derived from the terminal Neu5Ac reducing residue, and terminating with a reactive acryloyl group (fig. 4.12).

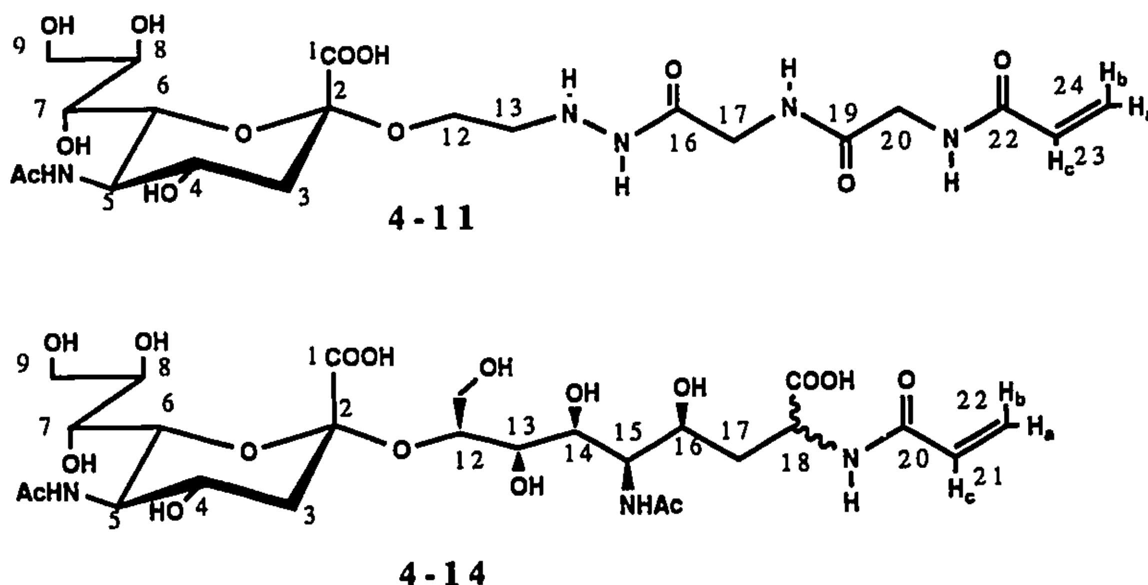


Figure 4.12- Comparison of two linker arms of Neu5Ac.

The N-acryloylated glycylglycine-Neu5Ac conjugate **(4-11)** resembles closely **(4-13)** in terms of functionalities present, spacer length, and hydrophilicity indicating that the acyclic spacer formed upon reductive amination has all the requirements normally associated with an ideal spacer (cf. chap. 3). The mono-N-acryloylated polysaccharide **(4-15)** was fractionated on a Biogel A.5

column equilibrated in PBS (pH=7.6), taking care that no sodium azide was used as column preservative. Four fractions of descending molecular weight were obtained in this manner. The average molecular weight of each fraction was estimated upon calibration of a gel extrusion column (Superose-12 HR 10/10) with purified oligosaccharides of known molecular weight. Comparison of the retention times on HPLC of the fractions to the standard curve yielded the average molecular weight of the fractions. The fraction corresponding to an average molecular weight of 3-4 KD (HPLC determined) was analyzed by ^{1}H NMR (500 MHz, D_2O). The characteristic N-acryloyl resonances (multiplets at 5.76 (H_a), 6.18 (H_b), and 6.36 (H_c) ppm) were integrated and compared to the integral for the H-3_{ax} proton at 1.75 ppm and the ratio was found to be one acryloyl residue to 11.8 Neu5Ac residues which corresponds to an average molecular weight of 3.85 KD in good agreement with the HPLC result.

The final N-acryloylated derivative synthesized differed from the others in that it was not introduced solely onto the reducing sugar. Colominic acid was de-N-acetylated under limiting conditions (see time course study, section 2.4.1) in order to afford a polysaccharide with randomly distributed free amino groups along the polysaccharide backbone (fig. 4.13).

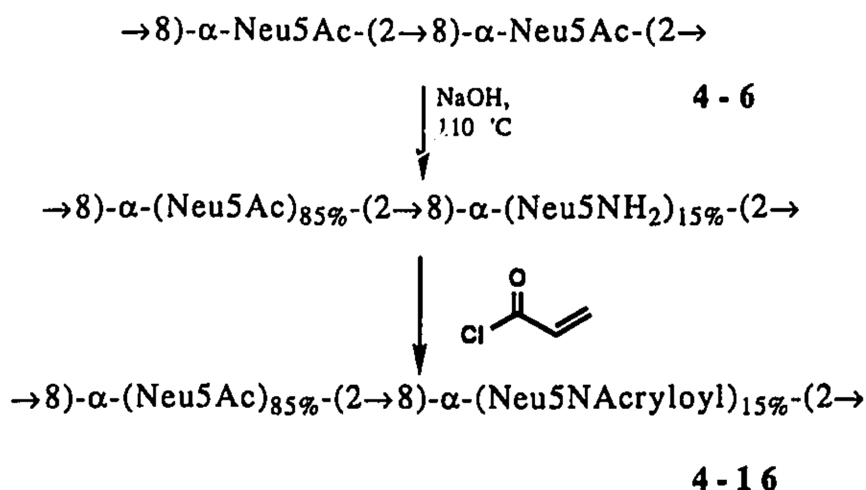


Figure 4.13- Synthetic scheme for the generation of 15% N-acryloylated colominic acid

Re-N-acryloylation was performed without any prior purification of the partially de-N-acetylated material in the same way as described for the synthesis of poly N-acryloyl colominic acid (**2-11**). The recovered yield of the partially derivatized colominic acid (**4-16**) was 84%. ^{1}H NMR (300 MHz, D_2O) analysis revealed the N-acryloyl group present at ~15% relative to the native Neu5Ac residues. It is presumed that de-N-acetylation occurs randomly, however recent results seem to indicate that the two termini are slightly more susceptible to base hydrolysis.

4.2.2 Modified protein substrates

Bovine serum albumin (**4-17**) (BSA; M_r 67,000) was modified according to the method of Robbins and Zon (1983) in order to provide an alternate form of the protein in which pendant spacers may allow increased accessibility for conjugation. Adipic acid

dihydrazide (AAD; **4-18**) was randomly incorporated onto aspartic or glutamic acid residues via their activation with the water soluble carbodiimide EDC (fig. 4.14).

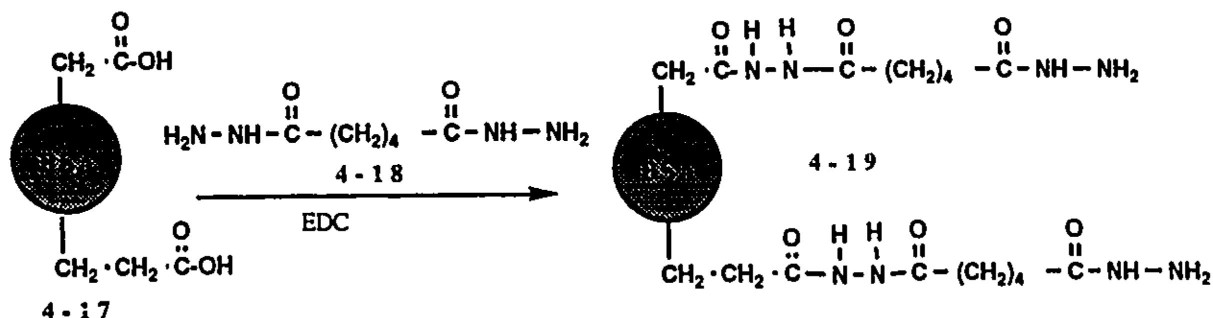


Figure 4.14- Synthesis of BSA- adipic acid hydrazide

The isolated modified protein produced a significant amount of precipitate upon redissolution presumably from protein crosslinking and was removed. The soluble fraction was isolated in 64% recovered yield. It was found that the lyophilized BSA-AAH (**4-19**) was not stable at 4 °C over long periods of time. Quantitative TNBS assay (appendix A) for hydrazide revealed a molar ratio of 10 adipic acid hydrazides (AAH) residues per mole BSA.

4.2.3 Protein glycoconjugates

4.2.3.1 A) Reductive amination via hemiacetal/ketals

The neutral disaccharide β-D-galactose-(1→4)-D-glucose (lactose) (**4-20**) was reductively aminated via the terminal reducing

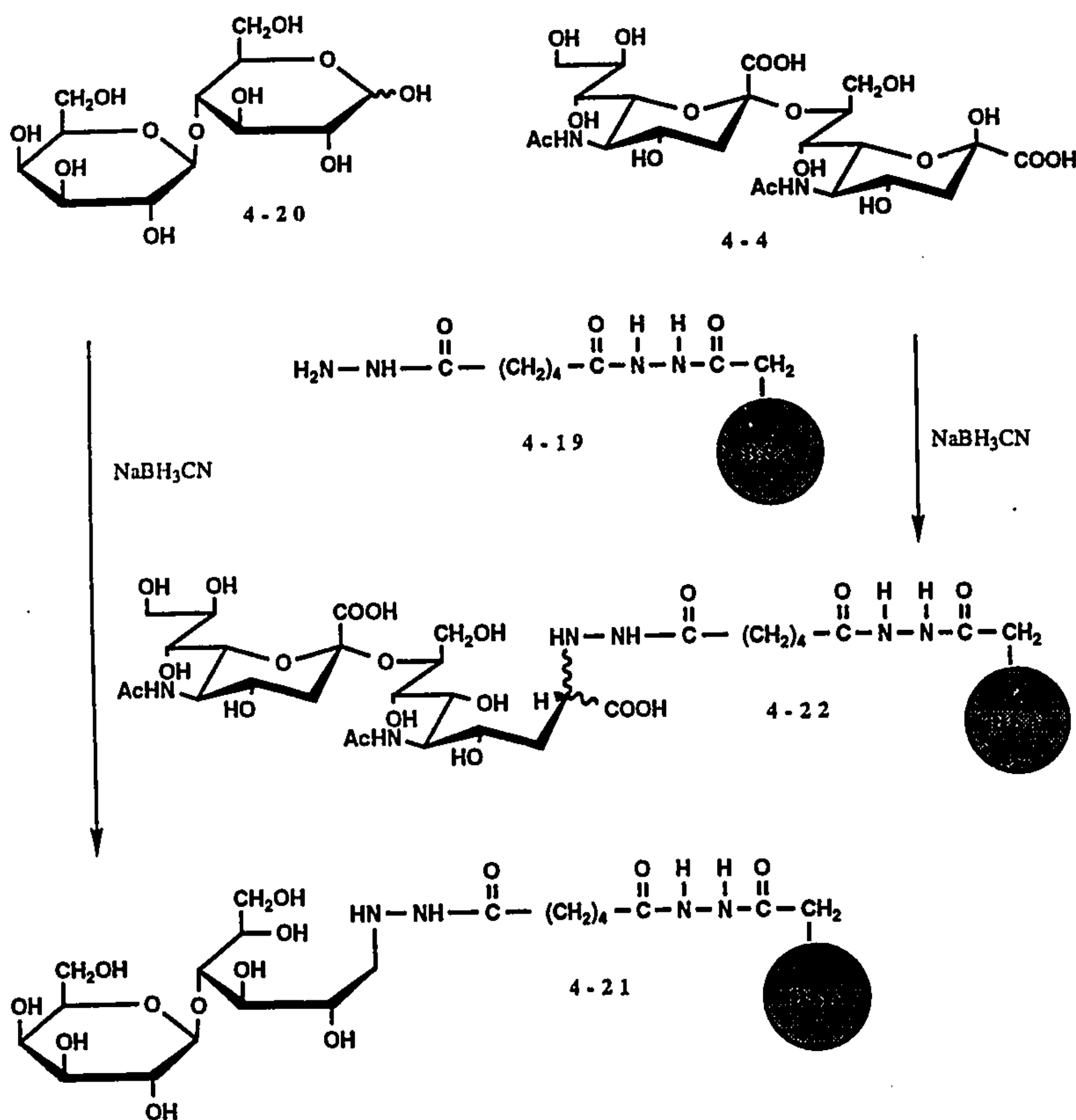


Figure 4.15- Reductive amination of two oligosaccharides to BSA-AAH.

hemiacetal of the glucose residue onto the modified BSA-AAH protein substrate (**4-19**) (fig.4.15). Coupling took place in borate

buffer (0.2M, pH 9.0) at 50 °C for 4 days in the presence of $NaBH_3CN$. Purification consisted of exhaustive dialysis followed by lyophilization to give the glycoconjugate (**4-21**). Quantitative analysis for galactose (Dubois method- appendix A) revealed a ratio of 27 moles of galactose per mole of BSA-AAH. The presence of residual hydrazide was negative (TNBS) indicating all of the incorporated AAH functionalities as well as some of the 59 lysine residues of BSA were modified.

Similarly, N-acetylneuraminic acid disaccharide $(Neu5Ac)_2$ (**4-4**) was reductively aminated onto BSA-AAH (**4-19**) via the hemiketal group of the end reducing sialic acid residue (fig. 4.15). Reactions conditions were slightly different to those described above with a longer reaction time (7 days) and a lower temperature (37 °C). Sialic acid content of the purified glycoconjugate (**4-22**) was determined by a quantitative resorcinol assay (appendix A) and found to be present in 3% (wt/wt) corresponding to 6.7 moles Neu5Ac per mole BSA-AAH. This material when analyzed by double immunodiffusion (DID) using the lectin WGA (cf. chapter 5 for further information and discussion), showed no precipitin bands indicating the absence or low incorporation of terminal Neu5Ac. After further studies with this lectin, it was found that this result is very dependant on the incorporation of terminal Neu5Ac, explaining the lack of serological evidence indicating the successful formation of a Neu5Ac containing conjugate (Roy *et al.*, 1991).

Two Neu5Ac oligosaccharides were successfully conjugated to BSA (**4-17**) via the direct reductive amination of the ε- amino group of lysine through the reducing hemiketal of sialic acid (fig. 4.16).

$(Neu5Ac)_3$ (**4-5**) was coupled in this way under forcing conditions (50 °C, 10 days, borate buffer 0.2M, pH 8.2) and the resulting conjugate **4-23** was isolated in 62% recovered yield. Colourimetric analysis on the conjugate **4-23** revealed the presence of 9.3 chains per protein. The term chains per protein represents in molar terms the number of moles of trisaccharide that has reacted with lysine. In terms of Neu5Ac however, there are only two residues per chain since reductive amination through the reducing end results in the open form of the sugar. Once again this conjugate was tested by DID with WGA as precipitating lectin and found to be negative. This negative result was interpreted in the same manner as discussed above.

NH_2 + $(Neu5Ac)_n$ $\xrightarrow{NaBH_3CN}$

R—O, CH_2OH, OH, OH, AcHN, HO, H, COOH, N

n=3 **4-5**

n=7 **4-24**

$R=(Neu5Ac)_2$ **4-23**

$R=(Neu5Ac)_6$ **4-25**

Figure 4.16- Direct reductive amination of two Neu5Ac oligosaccharides onto BSA.

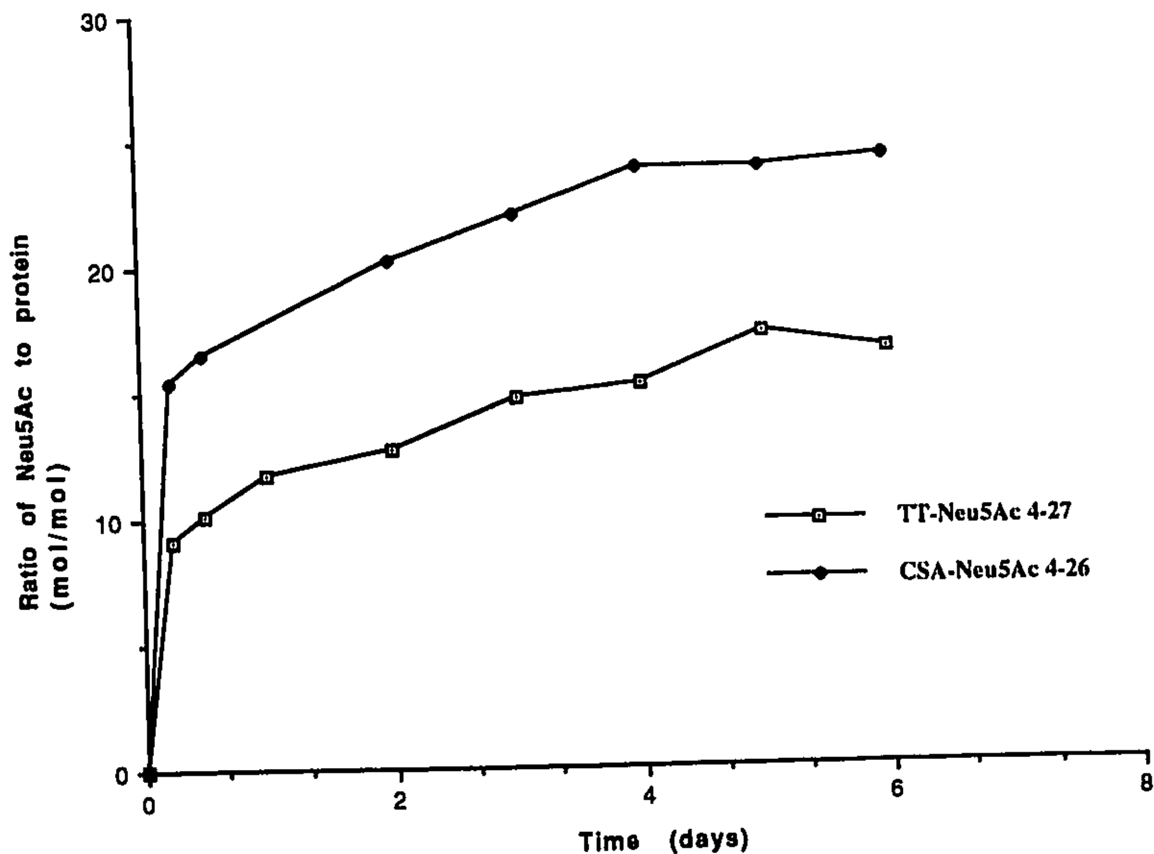
A Neu5Ac heptasaccharide $(Neu5Ac)_7$ (**4-24**) was reductively aminated to BSA (**4-17**) under even more forceful conditions

determined to be the optimal conditions for direct reductive amination through the hemiketal of sialic acid. Optimum conditions required the use of borate buffer (pH 9.2; 0.2M) to give a final protein concentration of 20 mg/ml. At least 30 equivalents of $NaBH_3CN$ to oligosaccharide are required. Incubation at 50 °C was found to give maximum conjugation in 5 days, while at 37 °C, the reaction required at least 7 days. This was determined by monitoring over time the increase in molecular weight of the protein as the sialic acid oligosaccharide is added, by size exclusion HPLC. Quantitative resorcinol (sialic acid) and BCA (protein) (appendix-A) of the resulting conjugate (**4-25**) determined sialic acid present at 16.7% (wt/wt) corresponding to 6.1 chains/BSA (i.e. 6.1 chains of 6 repeating α-(2$\rightarrow$8) linked Neu5Ac residues). This conjugate (**4-25**) was studied in an ELISA direct binding assay with anti-group B meningococcal horse antiserum H.46 and was found to bind with the H.46 antibody (cf. chapter 5).

4.2.3.2 Reductive amination via terminal aldehydes

A time course study for the direct coupling of 2–oxoethyl Neu5Ac (**4-2**) to the unmodified ε-amino group of the lysine residues on chicken serum albumin (**4-37**) (CSA; M_r: 67000) and tetanus toxoid (**4-35**) (TT; M_r: 150,000) is depicted in figure 4-17 and the results are tabulated in table 4-4. The couplings were carried out at 37 °C in borate buffer (0.2M; pH 8.2) with a protein concentration of 3 mg/ml. Aliquots were removed after discrete time periods and the material was analyzed for sialic acid using the resorcinol method (appendix A). A comparison of the two curves shows the greater

reactivity of the smaller molecular weight CSA due mainly to the large number (59) of accessible lysine residues. The lower incorporation of sialic acid onto TT can be accounted for by a decrease in accessible lysine residues after the detoxification process (formaldehyde treatment). Even though it is estimated that ~66 lysine groups are unmodified after detoxification (Bizzini *et al.*, 1970), it must be remembered that formalin treatment will modify the most accessible lysine residues first leaving the more "buried" ones behind.



Dialysis and lyophilization of each individual sample afforded the CSA-Neu5Ac conjugates (**4-26**) and the TT-Neu5Ac conjugates (**4-27**) with various Neu5Ac loadings. The TT-Neu5Ac conjugate (**4-27**) is particularly relevant as an immunogen since antibodies specific to terminal Neu5Ac have yet to be reported.

Time	TT-Neu5Ac		CSA-Neu5Ac	
(days)	% (wt/wt)	Neu5Ac/TT (mol/mol)	% (wt/wt)	Neu5Ac/CSA (mol/mol)
0.25	1.9	9.1	7.1	15.4
0.5	2.1	10.1	7.5	15.4
1.0	2.4	11.7	-	-
2.0	2.6	12.7	9.2	20.2
3.0	3.0	14.7	10.0	22.0
4.0	3.1	15.2	10.8	23.8
5.0	3.6	17.2	10.8	23.8
6.0	3.4	16.5	11.0	24.2

Table 4-4 Conjugation results of **4-26** and **4-27**.

As previously mentioned, terminal Neu5Ac residues on cell surfaces mediate a variety of biological processes including being receptors for viral adhesion. They can also be tumour cell markers when present in high densities and as such antibody immunotherapy may become a viable option.

Two polysaccharides, namely N-glycolylcolominic acid **(4-28)** and colominic acid **(4-6)**, were linked through their non-reducing termini according to the method of Jennings & Lugowski (1981) (fig. 4.18).

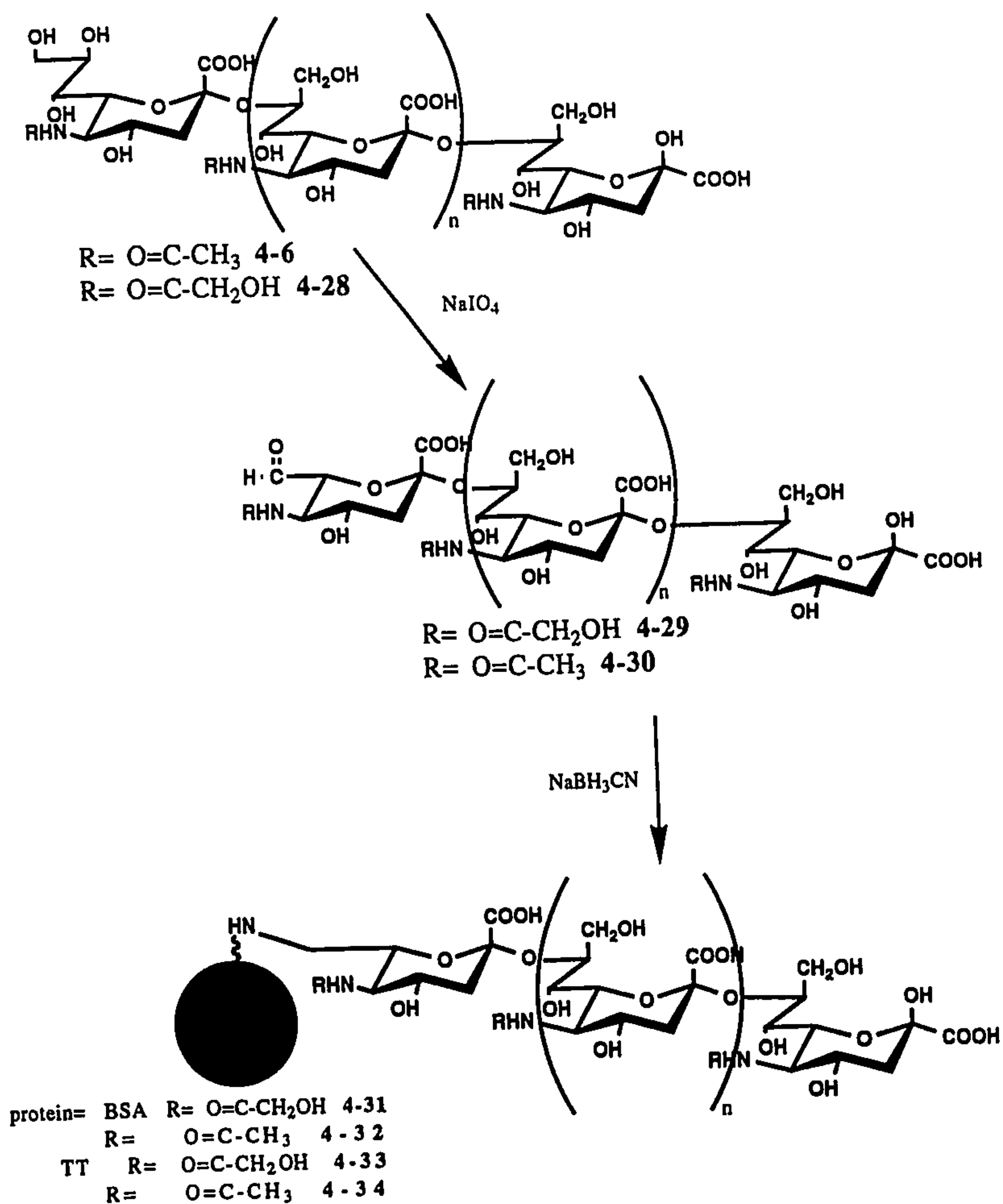


Figure 4.18- Sialylpolysaccharide conjugation to protein through the non-reducing terminus.

A discrete molecular weight fraction (~5-6 KD) of each polysaccharide was selectively oxidized in the exocyclic side chain of the non-reducing end, by oxidative cleavage between C-7 and C-8 with $NaIO_4$ to generate a free terminal aldehyde. The purified aldehydo polysaccharides ([ox] N-glycolylcolominic acid **4-29**; [ox] colominic acid **4-30**) were conjugated to both TT (**4-35**) and BSA (**4-17**) by reductive amination in phosphate buffer (0.75M; pH 8; 37 °C) for 5 days. The conjugates were purified by gel extrusion where the high molecular weight fractions corresponded to the newly formed conjugates. The % sialic acid was found to be higher with the BSA conjugates (BSA-glycolylcolominic acid (**4-31**), 19.5% wt/wt; BSA-colominic acid (**4-32**), 22% wt/wt) relative to the TT conjugates (TT-glycolylcolominic acid (**4-33**, 12% wt/wt; TT-colominic acid (**4-34**), 13.6% wt/wt), for the same reasons as outlined in the previous section. For both the TT and BSA conjugates, this represents ~4 chains per protein molecule.

4.2.3.3 Protein glycoconjugates via Michael-type additions

Based on the ready addition of sodium thiomethoxide and methylamine onto N-acryloyl colominic acid (**2-11**) (cf. chapter 2), it was hoped that similar Michael type additions, with lysine residues as nucleophiles, would constitute a novel method of conjugating sialyl oligo-/ polysaccharides to proteins.

The first model used to test the feasibility of conjugate addition of lysine was the coupling of 15% N-acryloylated colominic acid (**4-13**) to BSA (**4-17**) or to porcine IgG (**4-36**) (fig. 4-19). It was found that the most efficient conditions required a high concentration of Michael

acceptor to protein and the conjugation was best performed in borate buffer at pH 9.2. Alternate buffers such as phosphate (various pH's) and sodium hydroxide solutions (pH 10) were not satisfactory in terms of conjugation.

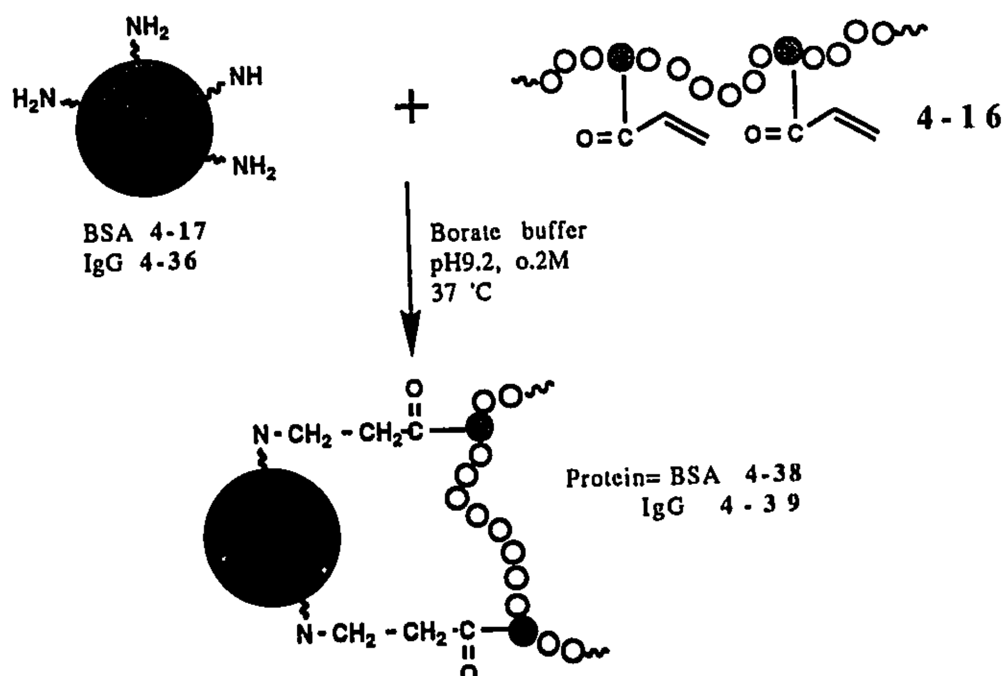


Figure 4.19- Protein conjugation with 15% N-acryloylated colominic acid via a Michael-type addition.

Purification of the high molecular weight BSA-15% N-acryloyl conjugate (**4-38**) by gel filtration yielded upon protein (Lowry method- appendix A) and sialic acid (resorcinol- appendix. A) analysis, a conjugate with 9.8% wt/wt sialic acid content. The kinetics of the conjugation could be visualized by monitoring the increase in molecular weight of the protein by gel extrusion HPLC (see fig. 4.20).

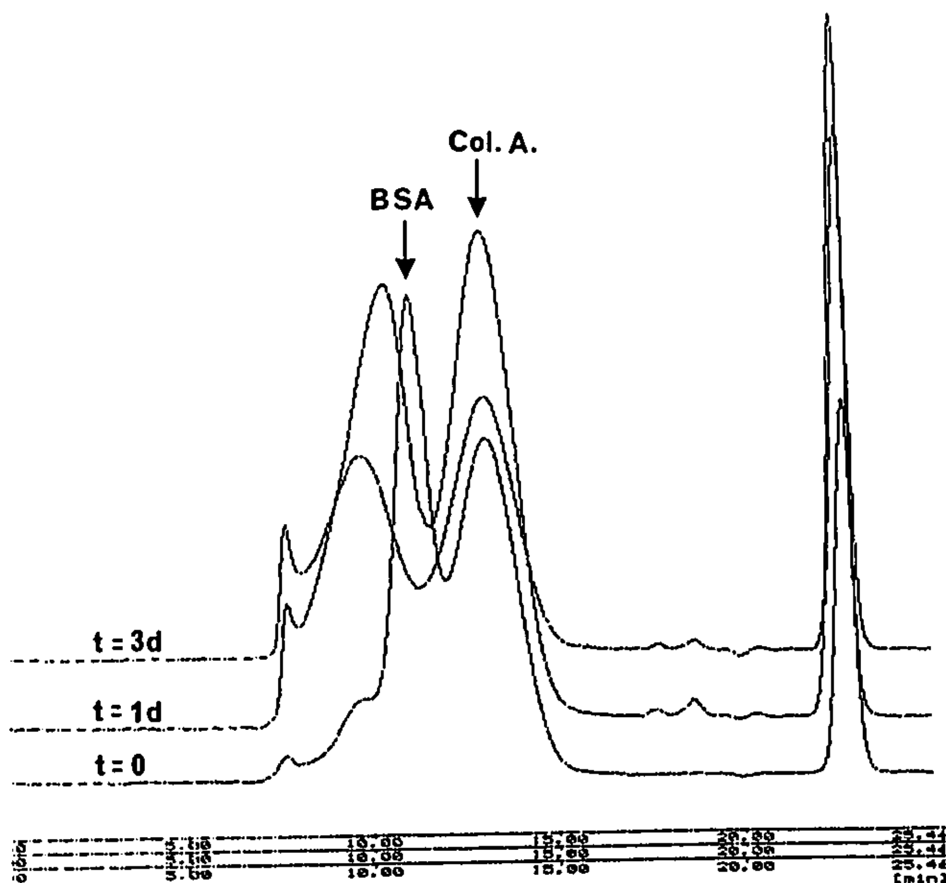


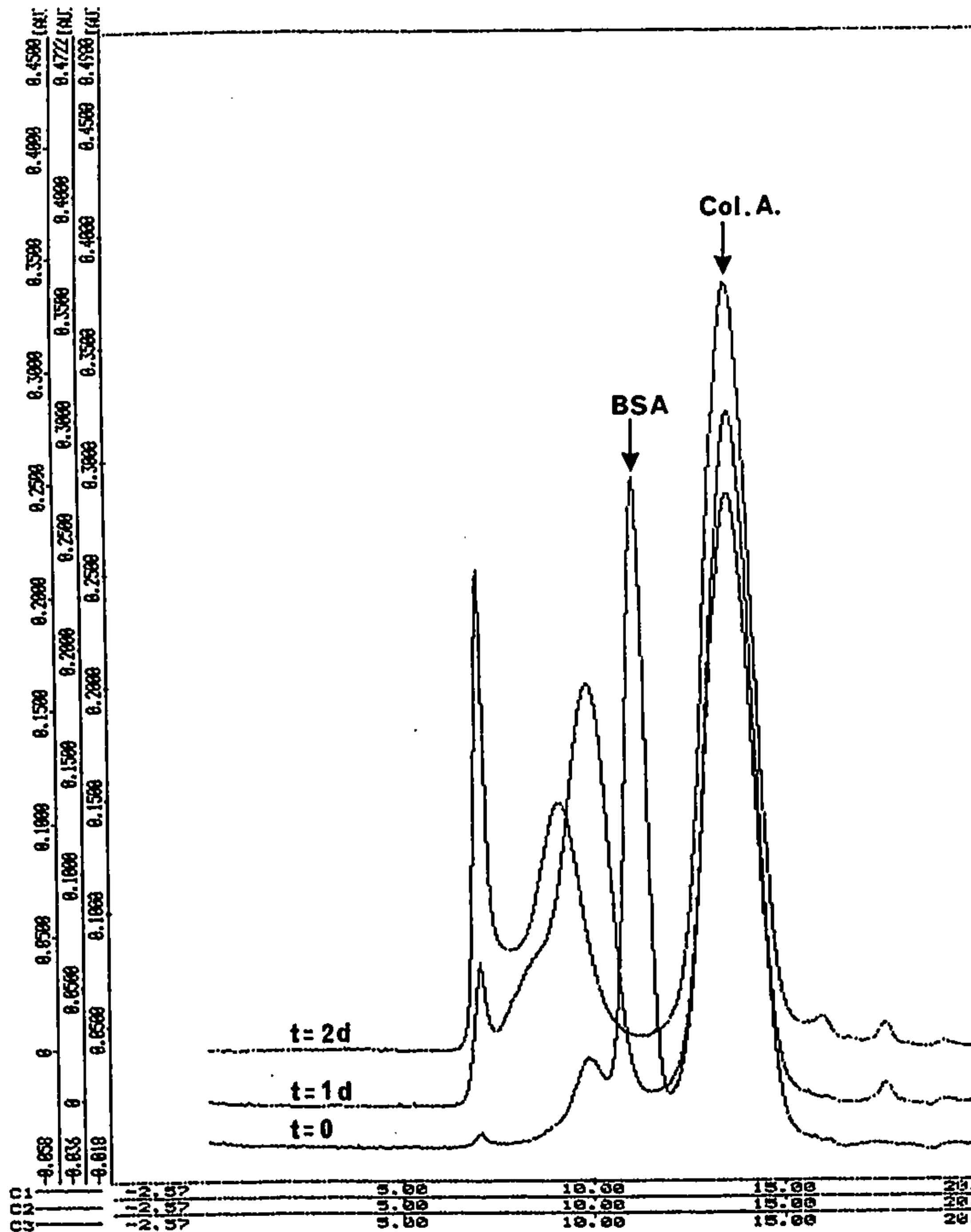
Figure 4.20- HPLC reaction profile of the coupling of 15% N-acryloyl colominic acid to TT

The reaction produced a significant amount of precipitate as time progressed and this was accounted for by the extensive cross linking due to the abundance of reactive Michael acceptors per polysaccharide chain. The low sialic acid content of the resulting conjugate, reflects this observation since only the proteins with a low sialic acid content remained soluble. The IgG-15% N-acryloylcolominic acid conjugate (**4-39**) was analyzed qualitatively only and was not isolated. Both conjugates were analyzed by DID, immunoelectrophoresis, and sodium dodecyl sulfate polyacrylamide

gel electrophoresis in order to determine whether the method resulted in the formation of true covalent conjugates. Based on the results of these assays (cf. chapter 5 for profiles and more details), it was concluded that the Michael addition of amine onto the acryloyl groups of **4-39** was an efficient and effective process.

With this information in hand, we turned towards the synthesis of more defined oligo-/ polysialic acid glycoconjugates of this kind. The preparation of the N-acryloylated disaccharide (**4-13**), trisaccharide (**4-14**), and polysaccharide (**4-15**) derivatives allowed entry into defined oligo-/ polysaccharide conjugates via Michael-type additions. Basically the N-acryloylated derivatives **4-13**, **4-14**, and **4-15** were conjugated to BSA (**4-17**) or TT (**4-31**) in essentially the same manner using high amounts of Micheal acceptor to protein (>100 eq) in concentrated solutions (20 mg protein/ml buffer). Incubation at 37 °C or 50 °C for 3-4 days yielded similar results. The sialic acid to protein content and the corresponding number of chains to protein of the BSA-(Neu5Ac) (**4-40**), BSA-$(Neu5Ac)_2$ (**4-41**), BSA-colominic acid (**4-42**), and TT-colominic acid (**4-43**) conjugates are summarized in table 4-5. As can be seen with the polysaccharide results, this method represents an efficient means of conjugating polysaccharides to protein. The HPLC (superose-12) reaction profile of the polysaccharide coupling is shown in fig. 4-21. The oligo-/ polysaccharide conjugates were analyzed in a direct ELISA binding study and will be discussed in detail in the following chapter.

Figure 4.21- HPLC profile of the conjugation of colominic acid to protein via Michael addition



Conjugate	% Neu5Ac/protein (wt/wt)	Chains/protein
BSA-(Neu5Ac) **4-40**	11.5	25.4
BSA-$(Neu5Ac)_2$ **4-41**	8.1	9.0
BSA-colominic acid **4-42**	93	14
TT-colominic acid **4-43**	15	4.8

Table 4-5 Neu5Ac content of various conjugates formed via a Michael-type addition onto protein.

4.2.4 Glycopolymers

In general, neo-glycopolymers are made from copolymerization of acrylamide or methacrylamide with a suitably derivatized oligosaccharide in manners outlined in chapter 3. Three novel sialyloligosaccharide glycopolymers were synthesized by either reductive amination of sialic acid onto preformed methacrylamide copolymers with pendant spacer hydrazide chains or the fortuitous copolymerization of the N-acryloylated $(Neu5Ac)_3$ derivative **(14)** with acrylic acid.

In an attempt at a one pot synthesis of the N-acryloylated $(Neu5Ac)_3$ derivative **(4-14)** from $(Neu5Ac)_3$ **(4-5)** by reductive

amination of ammonia followed by N-acryloylation, it was observed that rather than the expected product (**4-14**), a very polar product (TLC solvent D) was formed instead. Dialysis of the material to remove any low molecular weight components followed by lyophilization gave a product with a mass of almost twice the original amount of $(Neu5Ac)_3$. ^1H NMR analysis (300 MHz; D_2O) revealed the presence of intact Neu5Ac residues with the presence of 9 protons belonging to the acetamido groups, (2.05-2.1 ppm) and 2x $H\text{-}3_{eq}$ protons (2.6-2.8 ppm) as expected. However, two large, very broad peaks in the 1.72 and 2.38 ppm region seemed to indicate the presence of a polymeric backbone and indeed the shifts are consistent with the methylene and methine protons of a polyacrylic acid backbone. It would seem that during the course of acryloylating the terminal amine of (**4-9**), one of the extraneous species present initiated the copolymerization of the acryloylated trimer with the acrylic acid present in solution to give the copolymer **4-44** (fig. 4.22). Quantitative resorcinol analysis revealed a 16% wt/wt sialic acid content and ^1H NMR integration of the $H\text{-}3_{eq}$ protons to the polyacrylic acid methine proton, revealed incorporation of the N-acryloylated $(Neu5Ac)_3$ derivative (**4-14**) at 7.1% mole/mole. DID analysis on the copolymer with WGA as precipitating lectin revealed a positive precipitin reaction.

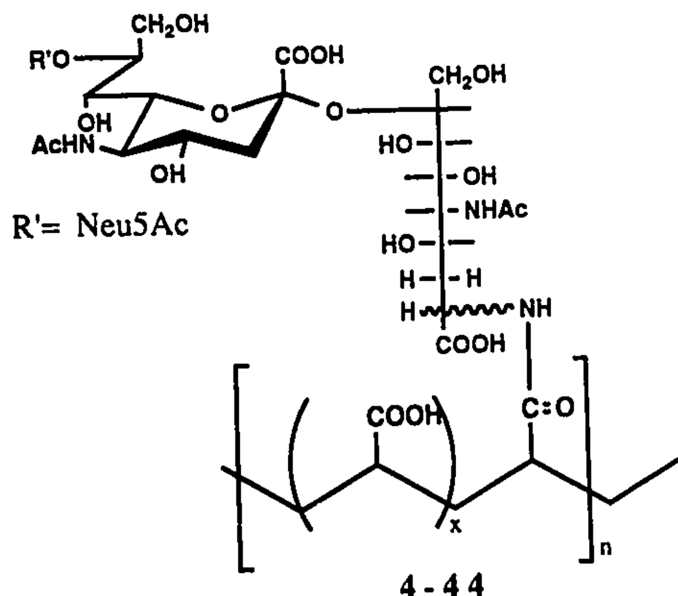


Figure 4.22- Neu5Ac disaccharide-acrylic acid copolymer

Alternatively, two neo-glycopolymers were synthesized using a preformed synthetic methacrylamide copolymer with pendant glycylglycine hydrazide sidechains (**3-9**). The 2-oxoethyl Neu5Ac derivative (**4-2**) possessing a terminal aldehyde in the aglycon region was mixed with the copolymer. Formation of the hydrazone-like bond between the pendant spacer of the copolymer (**3-9**) and the aldehydo Neu5Ac (**4-2**) was visualized by the disappearance of the starting aldehyde by TLC (solvent A) and the formation of a polar, resorcinol positive baseline product. The hydrazone was reduced with the addition of $NaBH_4$ and the material was purified by dialysis. Sialic acid analysis on the glycopolymer revealed a 5.6% wt/wt incorporation. ^1H NMR (300 MHz; D_2O) of the conjugate (**4-45**) (fig. 4.23) confirmed the presence of

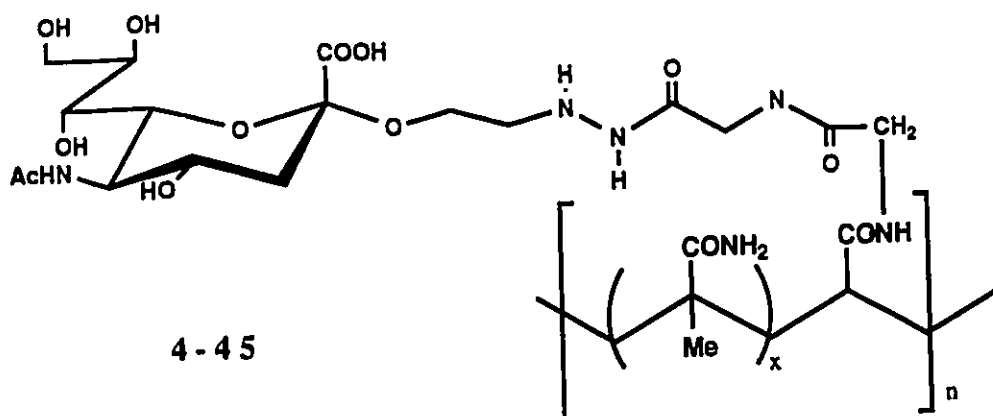


Figure 4.23- Neu5Ac-glycylglycine spacer-methacrylamide copolymer

a conjugate showing the distinct resonances of the ring protons (3.4-4.2 ppm) and the N-acetyl group at 2.01 ppm. Integration of the N-acetamido protons vs the methyl group of the methacrylamide backbone demonstrated the Neu5Ac present in 5.3 mole % indicating that 63% of the pendant spacer hydrazides of the copolymer were modified.

Along similar lines, $(Neu5Ac)_3$ (**4-5**) was directly reductively aminated through its reducing keto residue onto the spacer hydrazide-methacrylamide copolymer (**3-9**) in borate buffer (pH 9.2, 0.2M) at 50 °C for 5 days (fig. 4.24). Purification of the sialylated copolymer (**4-46**) by dialysis followed by sialic acid analysis, revealed the copolymer conjugate contained 8.6% sialic acid by weight. Double immunodiffusion analysis on the glycopolymer (**4-46**) with WGA did not result in the precipitation of the lectin for the same reasons as discussed above.

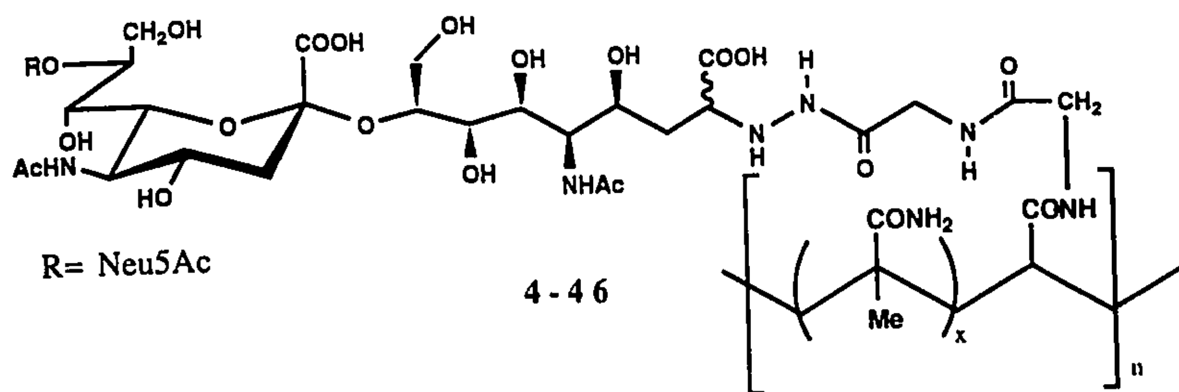


Figure 4.24- Direct reductive amination of $(Neu5Ac)_3$ onto a glycylglycine hydrazide- methacrylamide copolymer.

4.3 Discussion

One of the principal goals of this thesis was the development of efficient manners in which to conjugate sialic acid, sialyloligosaccharides, and polysialic acid to macromolecular supports such as proteins and synthetic polymers. The difficulty in understanding the complex biological functions of sialic and polysialic acid can in large part be attributable to the difficult chemistry inherent to this sugar. It has already been pointed out that there exists only one synthesis of the biologically important α-(2→8) linked disaccharide (Okamoto *et al.*, 1986; 1988), thereby supporting this claim. It is crucial that synthetic sialic acid containing probes be available in order to study such an array of biological interactions and it is for this reason, that so much emphasis was placed in obtaining macromolecular, multivalent forms of oligo-/ polysialic acid. In order to synthesize defined glycoconjugates of polysialic acid, all chemical transformations must take advantage of the unique character of the reducing terminal (not including the specific activation of the exocyclic side chain of the non-reducing end which will be discussed further on). Analysis of the reducing end structure reveals three distinct regions of potential derivatization: (1) the hemiketal, (2) the equatorial carboxylic acid (β-anomer), and (3) the anomeric hydroxyl. Most of our attempts centered on taking advantage of the hemiketal center due to the limited reaction paths available and a lack of success with the other two centers. In all cases, the hemiketal was exploited by the introduction of an amine function by reductive amination. The two strategies were to (i) directly link the carbohydrate moiety to the amine bearing groups of

either proteins or polymers or (2) to derivatize the amine in such a way as to introduce a new reactive center which would be available for conjugation to proteins or for copolymerization with acrylamides.

In theory, direct reductive amination of sialyloligosaccharides through their ketose reducing ends to proteins is the method of choice based on the simplicity of the reaction, it can be performed in aqueous conditions, it forms stable nonreversible covalent linkages, there is no prior need to derivatize either the carbohydrate or protein, and the reaction is predictable with no extraneous side reactions. In practice however, it is a difficult reaction and depending on the function of the conjugate, may not be a viable option. Reductive amination of proteins to reducing aldoses is a slow but useful method of conjugation and has been effectively used in the preparation of a number of conjugates (Anderson *et al.*, 1985; Cho *et al.*, 1984; Lin and Lee, 1982, Schwartz and Gray, 1977). In our hands, we also found that reductive amination of lactose onto BSA also proceeded with reasonably high incorporations of this disaccharide. Direct reductive amination of the terminal ketose of polysialic acid oligomers were for the most part unsuccessful or resulted in very low incorporations of the sialyloligosaccharides. These results were consistent with others (Roy *et al.*, 1984b; Jennings and Lugowski, 1981; Jennings *et al.*, 1983) who also determined that ketoses of sialic acid and KDO (3-deoxy-D-*manno* -octulosonic acid) were unreactive. The poor reactivity of the ketose can be explained by a number of factors: (1) the rate limiting step in reductive amination is the amount of the acyclic form of the sugar present in solution. Adjustment of pH, buffer and temperature have a pronounced effect

on the proportion of acyclic sugar with aldohexoses, but the effect seems to be much less pronounced with terminal ketoses (Roy *et al.*, 1984a). (2) the reactivity of an aldehydo carbonyl is known to be greater than that of a keto carbonyl (3) the presence of a carboxylate α-to the reactive center acts like an anionic shield which may exhibit repulsive forces to macromolecules bearing the reactive nucleophiles (4) the reversible nature of the imine formed in aqueous media compounds the effects of the previous factors. The poor reactivity of the hemiketal was not limited to macromolecular amines but as well to smaller substrates with reactive amino groups such as allylamine, hexanediamine and adipic acid dihydrazide. Despite the immediate lack of success encountered, the reductive amination of sialyloligosaccharides was still a worthwhile target and methods to improve the reactivity were investigated over the course of this work. One of the difficulties encountered in studying this reaction was the lack of an abundant source of model compound, such as the $(Neu5Ac)_2$ (**4-4**) which after coupling would possess, in addition to the alkylamine from the opened reducing end, one intact Neu5Ac residue that can be detected through colourimetric analysis. After investigating extensive combinations of nucleophiles, buffers, pH's, and concentrations, a set of conditions was found which resulted in modest incorporations of sialyloligosaccharides onto proteins and synthetic polymers. The key factor was determined to be the need for a highly concentrated solution of protein (typically 20 mg/ml or greater) and a high excess of reducing sugar (typically 100 equiv or greater relative to the protein). The ratio of $NaBH_3CN$ to reducing sugar was also critical (typically 30:1) since it must be present in

sufficient quantity to quickly reduce the transient imine formed yet limit the competing slow reduction of the ketone which is the major side product. Fortuitously, this reduction is slow at pH's above 6 however, small amounts of $NaBH_4$, which is present in impure $NaBH_3CN$, will seriously impede the conjugation reaction. The reaction also proceeds best in borate buffer (pH 9.2) at 50 °C, which probably enhances the relative amount of the acyclic reducing residue (Roy *et al.*, 1984a). Three noteworthy limitations of these new conditions are (1) not all proteins will be able to withstand these forcing conditions and this situation must be evaluated. (2) it may not be feasible to use such a large excess of the sialyloligosaccharide due to a lack of material (3) the reaction becomes increasingly more difficult with longer sialyloligosaccharides due mainly to the polyanionic nature of the polysaccharide.

Alternate chemistry at the hemiketal center is the introduction of a more reactive functional group. Based on the correlation between ligand size and concentration and the effective amination of the keto functionality, reductive amination of ammonia using very high excesses (saturated solutions) allowed for the efficient incorporation of a primary amine with the concomitant generation of a nine atom hydrophilic spacer. The efficiency of this reaction is directly attributable to the ease in which the small molecule can penetrate the polyanionic shield of the carboxylate residues and the overwhelming number of nucleophiles available for carbonyl attack relative to the few present on proteins. Derivatization of the primary amine with an acryloyl group allows entry into both a novel method of synthesizing glycoconjugates and the potential to form neo-

glycopolymers through copolymerization with acrylamides (Roy *et al.*, 1991). This type of substrate is particularly relevant when one exploits their ability to form experimental TD immunogens (i.e. protein conjugates), and then screen the antibodies produced with their polymer counterpart. Since the only structural element in common is the carbohydrate moiety, screening the antibody pool yields information specific to the carbohydrate residues only. Comparison of the protein incorporations obtained with N-acryloylated sialyloligosaccharides to their neutral counterparts (Roy *et al.*, 1991 ; Roy and Laferriere, 1990a), suggests a lower reactivity of the acryloyl group as a Michael acceptor. It is thought that once again the close proximity of the carboxyl group impedes the conjugate addition of lysine derived from protein. Incorporation of a spacer to distance the Michael acceptor from the carboxyl group will allow evaluation of this hypothesis. Protein or polymer conjugates synthesized in this manner are ideal since no chemical reagents are needed and the reaction is spontaneous and specific, performed under mild alkaline conditions and temperatures, with relatively short incubation times. The linkage however is not as stable as the secondary amine formed by reductive amination since the reaction is reversible in strongly basic solutions.

One other successful conjugation model was the generation of terminal free aldehydes by selective oxidation of an appropriate functional group. The generation of 2-oxoethyl Neu5Ac (**4-2**) bearing a free aldehyde in the aglycon portion, and its application to the formation of CSA-Neu5Ac (**4-26**) and TT-Neu5Ac (**4-27**) conjugates with various degrees of incorporation, demonstrates the good

reactivity of this substrate. The introduction of this glycosidic aldehyde is limited to small oligosaccharides due to the need for anhydrous conditions, necessitating the derivatization of the oligosaccharides, in order to introduce the allyl glycoside precursor. The group of Jennings *et al.*, (1981;1986) successfully introduced a free terminal aldehyde into the non-reducing terminus via selective periodate cleavage of the vicinal diol at C-7 and C-8 of the exocyclic sidechain (fig. 4.18). We were also able to prepare conjugates in this manner however, some important points must be considered when evaluating these conjugates as immunogens or more importantly, when using these multivalent antigens to probe antibodies specific to the capsular polysaccharide of *Neisseria meningitidis* serogroup B. By coupling the polysaccharide through the non-reducing end, the antigen is presented in an anti-sense to the natural presentation occurring on the surface of the bacteria. This has both implications from an immunogenic and antigenic point of view, where the importance falls off with increasing size of the polysaccharide. If one were to conduct binding studies with sialyloligosaccharide conjugates differing in their degrees of polymerization and linked through the non-reducing end with antibodies raised to the natural capsule of the bacteria, interpretation of the results may not reflect the actual situation. It is a well known phenomenon in enzyme chemistry, that enzymes are stereospecific recognizing and modifying only those substrates possessing the right stereochemistry. It is logical to assume that antibodies behave in a similar fashion recognizing only key parts of the antigenic determinant in a specific orientation. Reversing the presentation of the oligosaccharide on the carrier

protein, would force the antibody to flip in order to bind to its required orientation. The close proximity of the oligosaccharides to the protein surface may not sterically allow the Ab to flip and hence,binding is inhibited. Binding would occur only when the size of the oligosaccharide reached a critical value that would allow the Ab to reverse its orientation without the concomitant steric/ ionic interactions thus leading to a false interpretation of the actual DP needed for binding. Another important factor worthy of consideration is the loss of the penultimate sugar (i.e. the non-reducing residue) upon linking of the oligo-/polysaccharide through the non-reducing terminus. Kabat has classified antibody combining sites to polysaccharides into two classes (1) cavity type sites or endbinders (2) groove type sites (Kabat *et al.*, 1988). Although it is presumed that Ab interaction with polysialic acid fits primarily into groove-like combining sites, there may be a significant proportion of endbinder antibodies present in the antisera. The binding energy with this type of antibody combining site comes principally from its interaction with the terminal non-reducing sugar and to a lesser degree, with its immediate neighbouring residues (Cisar *et al.*, 1975). Obviously conjugation through this residue removes: (1) the potential to raise endbinder antibodies and, (2) the binding contribution of these Ab's in immunological assays which would not give a truly representative picture. Since the study of the binding interaction of sialic acid oligosaccharides with H.46 antibodies is one of the goals, this method of conjugation was not considered.

Two other conjugation models that did not meet with success will be briefly discussed. An alternate strategy to introduce a

reactive amine group into the reducing terminus of sialyloligosaccharides, was the attemnted formation of their respective glycosylamines (Likhosherstov *et al.*, 1986). The formation of the glycosylamine of Neu5Ac was studied by ^{13}C NMR spectroscopy. A saturated D_2O solution of NH_4Cl and Neu5Ac in a NMR tube was left at room temperature and the extent of reaction was monitored over time by ^{13}C NMR. After 5 days, a new compound estimated at less than 10% of the starting Neu5Ac appeared and was presumed to be the glycosylamine. After 4 additional days, the level of glycosylamine did not increase significantly however the dark colour of the solution suggested Neu5Ac decomposition. Recently, the group of Kallin *et al.*, (1989) by studying the formation of lactosylamine, did not meet with success when using a variety of ammonium based salts but did discover that good yields of the amine glycoside were obtained with NH_4HCO_3. They proposed that the formation of a stable N-glycosyl carbamate (fig. 4.25) intermediate was critical to the formation of the glycosylamine.

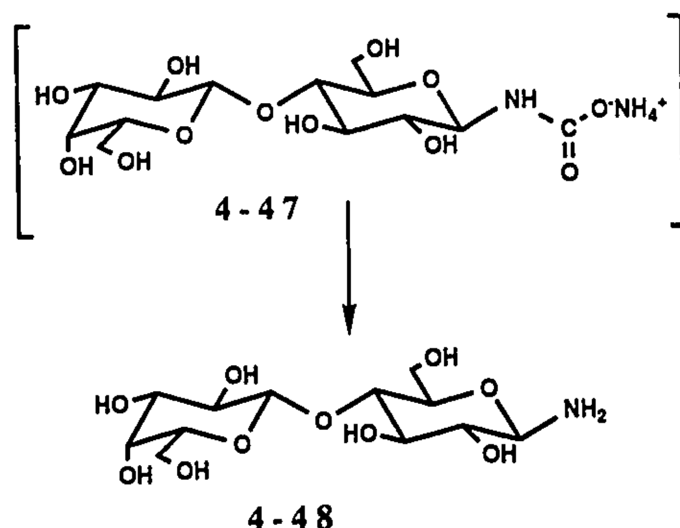


Figure 4.25- Lactosylamine via lactosylcarbamate intermediate.

These conditions have been applied successfully to the formation of a sialylactosylamine derivative (Roy and Laferriere,1990a; Roy *et al.*, 1991)). In light of these recent findings, a reinvestigation into Neu5Ac glycosylamine formation is warranted.

Finally, a strategy to take advantage of the spontaneous lactonization of colominic acid or GBMP (Lifely *et al.*, 1987) in acidic medium or in the presence of a carbodiimide was undertaken. The C-9 hydroxymethyl group is positioned in a favourable position to attack the C-1 carbonyl of its adjacent residue to form the internal lactone (fig. 4.26).

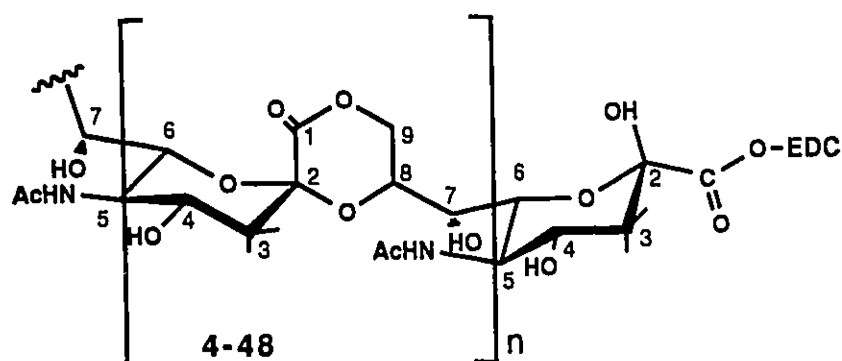


Figure 4.26- Lactone formation between two α-linked residues of Neu5Ac leaving the terminal β-linked reducing end as the EDC activated ester.

The β configuration of the reducing residue does not allow the formation of this internal ester so it was hypothesized that complete lactonization in the presence of EDC would leave the terminal carboxyl activated for selective nucleophilic attack. Attempts to modify the polysaccharide in this manner were not successful. In order to investigate further this reaction, the monosaccharide (β-Neu5Ac) was treated with EDC and various amines to give the resulting amides. This reaction was however also not successful due to the formation of a polar product (TLC) in all cases. At first this was thought to be the O-acylisourea (fig. 4.27) but treatment with base did not hydrolyze the suspected O-acylisourea back to the starting acid despite quite strong conditions. Hoare and Koshland (1967) have shown that a competing byproduct with carbodiimide activated carboxylic acids is the rearrangement to the N-acylurea (fig. 4.27) which may explain our results.

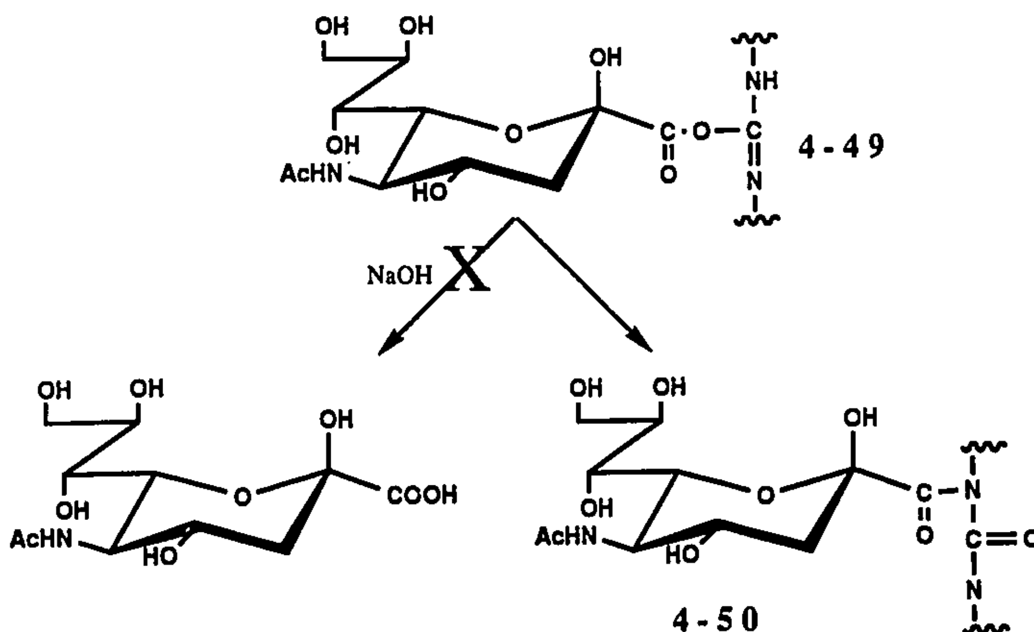


Figure 4.27- Possible rearrangement to the N-acylurea.

Throughout the course of this chapter, little mention has been made of the serological aspects of these synthetic conjugates and whether they are indeed useful. These considerations will be discussed in more detail in the following chapter along with all the immunochemical properties associated with them.

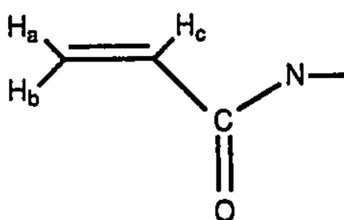
In summary, a great deal of effort has been devoted to the development of techniques for attaching sialyl oligo-/ polysaccharides to larger molecules such as proteins and polymers to give high molecular weight multivalent conjugates. Emphasis has been placed on the need to conjugate the carbohydrate specifically through its reducing end in aqueous conditions to yield highly defined, "natural looking" neo-glycoproteins and neo-glycopolymers with minimum chemical modifications. In this way, we were able to develop conditions that allow the direct reductive amination of terminal hemiketal residues with amines derived from proteins and

synthetic polymers alike. Studies in this area also allowed the development of a *de novo* coupling technique that makes use of the bifunctional acryloyl group to afford both protein conjugates via Michael-type reactions or synthetic polymers upon their copolymerization with acrylamides (Roy *et al.*, 1991). Conjugation of sialic acid substrates through these techniques has allowed the production of a number of immunogens, some of which are currently being evaluated in animal studies.

4.8 Experimental Methods

General Methods

For a complete description of general methods, please see the general method entry in chapter 2 (section 2.7). Immunochemical procedures are described in the experimental section of chapter 5. For a detailed description of the various colourimetric analyses, refer to appendix A. Protons of the acryloyl group are designated as H_a, H_b, and H_c as illustrated below.



Sialic Acid- Linkers

2-Oxoethyl 5-acetamido-3,5-dideoxy-D-*glycero* -α-D-*galacto* -2-nonulopyranosidonic acid (2-Oxoethyl Neu5Ac) (4-2)

Neu5Ac α-allyl glycoside (**4-1**) (25 mg; 72 umol) was dissolved in MeOH (20 ml) and cooled to -78 °C. Ozone was bubbled through the solution until a persistent blue colour (excess ozone) was observed. The solution was flushed with nitrogen followed by reduction with dimethyl sulfide (500 ul). The solution was allowed to warm to room temperature and sit for 2 hr. Excess DMS and MeOH was removed *in vacuo* followed by redissolution in water and lyophilization to yield the known title product (**4-2**) as an oil in quantitative yield.

TLC: Single product Rf= 0.16 solvent B

FAB-MS: for $C_{13}H_{20}O_{10}N$: 350 m/z (M-1)

5-Acetamido-2-amino-2,3,5-trideoxy-D-*glycero*-D-*galacto*-nononic acid (Neu5Ac-NH2) (4-7)

N-Acetylneuraminic acid (**4-3**) (100 mg; 0.32 mmol) was dissolved in a 0.5 M solution (30 ml) of NH_4OAc in MeOH containing 100 mg $NaBH_3CN$ (1.6 mmol) and heated to reflux. The reaction was stopped after 2 hr as judged by TLC (disappearance of Neu5Ac; solvent A) and the MeOH was removed. The sample was dissolved in water (5 ml) and the product was purified on a Sephadex G-10 column (100x 1.6 cm) in water. The sialic acid negative (resorcinol) and amino positive (ninhydrin) peak was pooled and lyophilized to 75 mg (76%) of title product (**4-7**).

TLC: Rf=0.11 solvent A- ninhydrin positive

^{1}H NMR:(300 MHz; D_2O) δ: disappearance of **H-3$_{ax}$** and **H-3$_{eq}$** protons, 1.90 (m, 1H, **H-3$_a$**), 2.05 (m, 1H, **H-3$_b$**), 2.07 (d, 3H, O=C-CH_3), 4.34 (dd, 0.5 H, **H-2**, $J_{H2',H3a}$=$J_{H2',H3b}$= 10.8 Hz), 4.48 (dd, 0.5 H, **H-2'**, $J_{H2,H3a}$≈$J_{H2,H3b}$=10.3 Hz),

^{13}C NMR:(50.3 MHz; D_2O) δ: disappearance of anomeric **C-2**, 22.6 (O=C-**CH_3**), 34.8 (d, **C-3**), 54.0 (d, **C-2**), 54.6 (d, **C-5**), 64.1 (**C-9**), 67.1 (**C-4**), 68.5 (**C-7**), 70.1 (**C-6**), 71.5 (**C-8**), 175.6[a] (-N-C=O), 175.5[a] (**C$_1$**)

8-O-(α-D-5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid)-5-acetamido-2-amino-2,3,5-trideoxy-D-*glycero*-D-*galacto*-nononic acid ($(Neu5Ac)_2$-NH_2) (4-8)

N-Acetylneuraminic acid dimer $(Neu5Ac)_2$ **(4-4)** (30 mg, .0.05 mmol) was dissolved in 750 ml of sodium borate buffer (pH 9.2, 0.2M) to which 200 mg of NH_4HCO_3 (2.53 mmol) was added. To this solution was added 15 mg of $NaBH_3CN$ (0.24 mmol) and the solution was incubated at 37 °C. After 72, an additional 5 mg of $NaBH_3CN$ was added and the solution was heated for an additional 48 hr The solution was diluted to 2ml and applied to a 100 x 1.6 cm column of Sephadex G-10 and eluted in water. The void volume and ninhydrin positive peak was pooled and lyophilized to 26.2 mg (86%) of **4-8** as a fluffy white material.

^1H NMR: (500 MHz; D_2O) in ppm.

Proton	residue A[b]	residue B[c]
H-2	-	4.32 (dd, 0.5H)
		4.45 (dd, 0.5H)
H-3	axial 1.78 (t, 1H)	$H-3_a$ 1.85 (m, 1H)
	equat. 2.75 (t, 1H)	$H-3_b$ 2.0-2.1 (m, 1H)
CH_3	2.04 (s, 3H)	2.07 (s, 1.5H)
		2.08 (s, 1.5H)

^{13}C NMR:(50.3 MHz; D_2O) in ppm,

res.	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C=O	CH_3
A[b]	174.3	102.6	41.0	69.0	52.4	73.5	68.7	72.8	63.4	175.7	22.7
B[c]	175.1	54.5	34.9	n.a.	54.8	n.a.	68.3	75.1	61.8	175.7	22.7

[a]: assignments may be reversed.

[b] : non-reducing residue.

[c] : reflects both diastereomers.

FAB-MS: for $C_{22}H_{37}O_{16}N_3.NH_4$: 602 m/z (M+1)
600 m/z (M-1)

α-D-Neu5Ac-(2→8)-α-D-Neu5Ac-(2→8)-D-Neu5Ac-NH_2 ((Neu5Ac)$_3$-NH_2) (4-9)

N-Acetylneuraminic acid trimer (Neu5Ac)$_3$ **(4-5)** (20 mg, 0.022 mmol) was dissolved in 500 μl of sodium borate buffer (0.2 M, pH 9.2) containing 200 mg of NH_4HCO_3 (2.5 mmol) and 10 mg of $NaBH_3CN$ (0.16 mmol). The solution was heated for 72 hr at 37°C whereupon an additional 5 mg of $NaBH_3CN$ was added. The solution was heated for a further 24 hr and then diluted to 2 ml and applied to a 100 x 1.6 cm column of Sephadex G-10 in water. The column was eluted and the corresponding ninhydrin positive peak was collected and lyophilized to afford **4-9** (16.0 mg; 80%).

^1H NMR (500 MHz, D_2O) in ppm.

Proton	residue A[b]	residue B	residue C[c]
H-2	-	–	4.28 (dd, 0.5H) 4.43 (dd, 0.5H)
H-3	axial 1.75 (t, 1H) equat. 2.77 (dd,	axial 1.75 (t, 1H) equat. 2.70 (dd,1H)	H-3_a 1.85 (m, 1H) H-3_b 2.0-2.1 (m,1H)
CH_3	2.04 (s, 3H)	2.08 (s, 3H)	2.07 (s, 1.5H) 2.08 (s, 1.5H)

^{13}C NMR: (50.3 MHz, D_2O) in ppm.

res.	C-1	C-2	C-3	C-5	C-8	C=O	CH_3
A[b]	173.8	101.6	40.4	52.4	72.5	175.6	23.0
B	173.7	102.5	41.1	53.0	78.1	175.6	23.0
C[c]	175.1	54.3	34.8 d	54.9 d	74.6	175.6	22.7

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[b] : non-reducing terminus.

[c] : reflects both diastereomers.

FAB-MS for $C_{33}H_{53}O_{24}N_4.NH_4$: 893 m/z (M+1)
891 m/z (M-1)

Reductive amination of ammonia to the reducing end of colominic acid (Colominic Acid-NH_2 (4-10)

Colominic acid (**4-6**) (200 mg; average mol. wt. 12,000) was dissolved in 2.0 ml of sodium borate buffer (0.2M; pH= 9.24)

containing 500 mg (6.3 mmol) of NH_4HCO_3 and 100 mg (1.6 mmol) of $NaBH_3CN$. The saturated solution was heated at 37° C for 3 days whereupon a further 100 mg (1.3 mmol) of NH_4HCO_3 was added and heating continued for 2 more days. The solution was dialyzed at 4 °C against PBS (pH= 7.3) followed by dialysis against distilled water and lyophilization to 188 mg of title product (**4-10**).

Ninhydrin analysis of the purified (**4-10**) as its sodium salt was positive for amine.

Reductive amination of N-acryloyl glycylglycine hydrazide (3-6) to 2-Oxoethyl Neu5Ac (4-2) - (4-11)

2-Oxoethyl Neu5Ac (**4-2**) (25 mg; 72 umol) was dissolved in MeOH (10 ml) to which 50 mg of N-acryloylated glycylglycine hydrazide (**3-6**) (250 umol) was added. $NaBH_3CN$ (10 mg; 160 umol) was added and the solution was heated to reflux. After complete disappearance of the starting aldehyde with the concomitant formation of a new U.V. and $KMnO_4$ positive product (TLC; solvent C), $NaBH_4$ (2.5 mg; 71 umol) was added to the solution at RT to ensure complete reduction. Excess $NaBH_4$ was destroyed by lowering the pH of the methanol solution to ~6 with HCl (0.1M). After MeOH removal, the sample was dissolved in water and applied to a Sephadex G-10 column (100X1.6 cm) equilibrated in water. After elution, the void peak (resorcinol and $KMnO_4$ positive) was collected and lyophilized. TLC analysis at this point revealed the presence of a small amount of material at Rf 0.37 (solvent C) corresponding to reduced **4-2**. The product (**4-11**) was purified by preparative TLC (solvent C) and afforded pure **4-11** after workup in 76% yield (29 mg).

TLC: Single product- Rf=0.25 solvent C

^1H NMR (200 MHz; D_2O): δ: 1.72 (t, 1H, **H-3**$_{ax}$, $J_{H3ax,H3eq}$=11.8 Hz), 2.05 (s, 3H, **N-acetyl**), 2.75 (dd, 1H, **H-3**$_{eq}$, $J_{H3eq,H3ax}$=12 Hz, $J_{H3eq,H4}$=5.0 Hz), 3.93 (s, 2H, **H-17**), 4.05 (s, 2H, **H-20**), 5.82 (dd, 1H, **H**$_a$, $J_{a,c}$=9.0 Hz, $J_{a,b}$=2.5 Hz), 6.26 (dd, 1H, **H**$_b$, $J_{b,c}$=17 Hz, $J_{b,a}$=2.5 Hz), 6.35 (m, 1H, **H**$_c$, $J_{c,b}$=16.7 Hz, $J_{c,a}$=9.0 Hz),

^{13}C NMR(50.3 MHz, D_2O): δ: 23.3 (N-Ac), 41.4 (**C-3**), 43.9 (**C-17 & C-20**), 53.3 (**C-5**), 64.1 (**C-9**), 68.3 (**C-13**), 69.6 (**C-4**), 69.7 (**C-7**), 71.4 (**C-12**), 73.1 (**C-8**), 74.0 (**C-6**), 101.3 (**C-2**), 129.8 (**C-24**), 130.8 (**C-23**)

N-Acryloylated (Neu5Ac)$_2$-NH_2 derivative (4-13)

To a solution (1 ml) of (Neu5Ac)$_2$-NH_2 (**4-8**) (25 mg) adjusted to pH 9 (0.1M NaOH) and cooled to 0 °C, was added in increments a 1:1 solution of acryloyl chloride in dioxane (6x 50 ul) over a period of 1 hr. The pH was maintained in the 9-11 range with 1 M NaOH. The pH was raised to 12 (NaOH) for 15 minutes while the solution warmed to R.T., followed by purification on a Sephadex G-10 column (1.6x 100 cm) equilibrated in water. The void peak was collected and lyophilized to 26.8 mg of title product (**4-13**) in 90% yield.

^1H NMR: see table 4-2.

^{13}C NMR: see table 4-3.

FAB-MS for $C_{25}H_{39}O_{17}N_3Na_2$: 701 m/z (M+1) +'ve mode

for $C_{25}H_{40}O_{17}N_3Na$: 676 m/z (M-1) -'ve mode

N-Acryloylated (Neu5Ac)$_3$-NH$_2$ derivative (4-14)

In an analogous manner as above, (Neu5Ac)$_3$-NH$_2$ (**4-9**) (16 mg) was treated with a dioxane solution of acryloyl chloride yielding after purification 15.4 mg of the title product (**4-14**) in 84% yield.

^1H NMR: see table 4-2.

^{13}C NMR: see table 4-3.

FAB-MS for $C_{36}H_{55}O_{25}N_4Na_3$: 1014 m/z (M+1) +'ve mode.

for $C_{36}H_{56}O_{25}N_4Na_2$: 991 m/z (M-1) -'ve mode

Mono-N-acryloylated colominic acid (4-15)

Colominic acid-NH$_2$ (**4-10**) (100 mg) was dissolved in 5.0 ml distilled water and cooled to 0° C. The material was treated with the slow addition of a 1:1 acryloyl chloride- dioxane solution while maintaining the pH between 9-10 with the addition of 0.5M NaOH. After complete addition of the acid chloride, a ninhydrin test showed negative amine. The material was exhaustively dialyzed against

distilled water at 4° C and lyophilized to afford 86.7 mg of title product (**4-15**). The product was fractionated on a 100 X 1.6 cm column of BioGel A.5 (200-400) in PBS (containing no azide) into four fractions of descending molecular weight (approx. 10,000 to 4000 D) where each fraction was dialyzed at 4°C against distilled water and lyophilized.

Mono-N-acryloylated colominic acid fraction I : 4.7 mg (~9-10 KD)
Mono-N-acryloylated colominic acid fraction II: 15.8 mg (~ 7-8 KD)
Mono-N-acryloylated colominic acid fraction III: 32.6 mg (~ 5-6 KD)
Mono-N-acryloylated colominic acid fraction IV: 19.8 mg (~ 3-4 KD)

15% N-Acryloyl colominic acid (4-16)

Colominic acid (**4-6**) (50 mg) was dissolved in 2M NaOH (2 ml) in a test tube containing 5 mg $NaBH_4$. The tube was sealed and heated to 105 °C for 15 minutes followed by immediate cooling. The solution was diluted to 10 ml with distilled water and the partially deacetylated product was reacylated with a 1:1 solution of acryloyl chloride/ dioxane (~250 ul) while maintaining the pH between 8.5-10 with NaOH (2N NaOH). Complete amine derivatization was confirmed by a negative ninhydrin test. The solution was exhaustively dialyzed against water and lyophilized to give 42 mg of product (**4-6**).

^{1}H NMR (300 MHz; D_2O): Ratio of residual N-acetyl (2.08 ppm) to H-3eq (2.67 ppm) revealed 14.8% de-N-acetylation.

Modified Protein Substrates

Bovine serum albumin- adipic acid hydrazide (BSA-AAH) (4-19)

Bovine serum albumin (**4-17**) (BSA) (50 mg, 0.74 umol) and adipic acid dihydrazide (**4-18**) (AAD) (75 mg; o.44 mmol) were dissolved in 10 ml water. To this solution was added in increments EDC (100 mg; 0.52 mmol) while titrating the solution's pH to 4.75 with HCL (0.1M). The solution was allowed to sit for an additional hour after the EDC addition followed by exhaustive dialysis against water and lyophilization to 46.4 mg of product (**4-19**). Upon redissolution, a significant amount of precipitate was observed hence the material was centrifuged (5 min. at 10,000 g) and the water soluble fraction was used in subsequent reactions (final recovered yield 32 mg).

From the ratio of hydrazide (determined by the TNBS assay) to protein, the conjugate contained 10 moles of hydrazide to 1 mole of BSA.

Protein Glycoconjugates

A) Reductive amination via hemiacetals or ketals:

Conjugation of lactose (β-D-Gal-(1→4)-D-Glu) (4-20) onto BSA-AAH (4-19)- (4-21)

BSA-AAH (**4-19**) (10 mg; 0.15 umol) and lactose (**4-20**) (10.5 mg; 31 umol) were dissolved in 3 ml borate buffer (0.2M; pH=9) containing $NaBH_3CN$ (1.3 mg; 20 umol). The capped solution was

heated at 52 °C for 4 days, followed by exhaustive dialysis against water and lyophilization to 7.3 mg of product (**4-21**).

Quantitative colorimetry for galactose (Dubois method) indicated 27 moles of lactose substituted per mole of protein. A subsequent TNBS assay for residual hydrazide was found to be negative.

Conjugation of $(Neu5Ac)_2$ (4-4) onto BSA-AAH (4-19)- (4-22)

A solution (1 ml) of $NaBH_3CN$ (20 mM in 0.2M phosphate buffer pH 8) was added to 10 mg $(Neu5Ac)_2$ (**4-4**) (16.7 umol) and 10 mg BSA-AAH (**4-19**) (0.15 umol) dissolved in 4 ml phosphate buffer (0.2M; pH=8). The solution was stirred for 7 days at 37 °C, followed by exhaustive dialysis against water and lyophilization to afford pure **4-22** (7.6 mg) as a fluffy material.

The sialic acid content of the conjugate was determined by a quantitative resorcinol assay and found to be 3% (wt/wt) or 6.7 mol (Neu5Ac) per mol protein. Double immunodiffusion analysis against WGA did not reveal any precipitation of the lectin.

Conjugation of $(Neu5Ac)_3$ (4-5) onto BSA (4-17) - (4-23)

To a solution of BSA (**4-17**) (5 mg; 0.074 umol) in borate buffer (0.2M; pH=8; 2 ml) was added $(Neu5Ac)_3$ (**4-5**) (10 mg; 11.2 umol) and $NaBH_3CN$ (20 mg; 0.32 mmol). The solution was stirred at 50 °C for 10 days followed by dialysis against tap water and freeze-drying to 3.1 mg of title product (**4-23**).

Sialic acid content (resorcinol) was found to be 8.5% (wt/wt) or 9.3 chains/protein. Double immunodiffusion analysis against WGA was negative.

Conjugation Of $(Neu5Ac)_7$ (4-24) To BSA(4-17) - (4-25)

In a similar manner as above, BSA (**4-17**) (10 mg; 0.15 umol), $(Neu5Ac)_7$ (**4-24**) (12 mg; 5.8 umol), and $NaBH_3CN$ (10 mg; 0.16 mmol) were all dissolved in 200 ul of borate buffer (0.2M; pH 9.2) and incubated at 50 °C for 11 days. Conjugation was followed by HPLC (Superose-12) with a higher molecular weight shift of the protein peak over time. The conjugate was purified by gel extrusion (Biogel A.5 in PBS), dialyzed, and lyophilized to 2.8 mg of title product (**4-25**).

Sialic acid content (resorcinol) to protein (BCA) was found to be 16.7% (wt/wt) or 6.1 moles $(Neu5Ac)_7$ to 1 mole BSA. The substrate was able to bind horse H.46 IgM antiserum in an ELISA assay.

B) Reductive amination via terminal aldehydes:

Conjugation of 2-Oxoethyl Neu5Ac (4-2) onto tetanus toxoid (TT) (4-35) - (4-27) or chicken serum albumin (CSA) (4-37) - (4-26) (ref MC-130)

To a solution of either TT (**4-35**) (30 mg; 0.2 umol) or CSA (**4-37**) (30 mg; 0.44 umol) in borate buffer (10 ml; 0.2M, pH=8.2) was added 2-Oxoethyl Neu5Ac (**4-2**) (52 mg; 0.15 mmol) and $NaBH_3CN$ (83 mg; 1.32 mmol). The solutions were heated at 37 °C with aliquots

(1 ml) removed over a period of 6 days. The pH of all fractions was lowered to ~5.4 with acetic acid, followed by exhaustive dialysis against running water, and lyophilization to give the purified TT-Neu5Ac series of conjugates (**4-27**) or the CSA-Neu5Ac series of conjugates (**4-26**).

The individual fractions were diluted to a known volume and analyzed for sialic acid content by the resorcinol method. For results, see table 4-4. The CSA conjugates with an incorporation of 20 Neu5Ac residues or more were able to precipitate WGA in DID analyses. The TT conjugates did not precipitate WGA in all cases.

Conjugation of colominic acid (4-6) or glycolylcolominic acid (4-28) onto BSA (4-17) or TT (4-35) via terminal aldehyde groups

(A) Activation Of Polysaccharides: Polysaccharides were fractionated into several molecular weight ranges on a BioGel A.5 column (1.6x100 cm) in PBS. The fraction corresponding to K_{av}=0.6 (mol. wt.~ 5-6 KD) of both colominic acid (**4-6**) and glycolylcolominic acid (**4-28**) was used in the conjugation to protein. Each sliced fraction was desalted on a Biogel P6DG column (1.6x100 cm) in water and lyophilized to a white fluffy material. The low molecular weight fraction of **4-6** (36 mg) and **4-28** (35 mg) were oxidized with a solution of sodium periodate in water (0.1M; 3.6 ml) in the dark for 15 minutes. Excess periodate was quenched with ethylene glycol (200 ul; 10 eq.) and the solutions were allowed to stand at R.T. for an

additional hour. The oxidized fragment of glycolylcolominic acid (**4-29**) and colominic acid (**4-30**) were purified on a Biogel P6DG column in water and concentrated to dryness.

(B) Conjugation To Protein: To either BSA (**4-17**) (10 mg; 0.15 umol) or TT (**4-35**) (5 mg; 0.033 umol) was added [o] glycolylcolominic acid (**4-29**) (10 mg) or [o] colominic acid (**4-30**) (10 mg) and $NaBH_3CN$ (10 mg; 0.16 mmol). The mixtures were dissolved in 3 ml of K_2HPO_4 (0.75M; pH=8) and heated at 37 °C for 5 days. Purification of the conjugates was accomplished by gel extrusion chromatography (Biogel A.5) where the high molecular weight material was pooled, dialyzed, and lyophilized.

BSA-glycolylcolominic acid (**4-31**): 12.2 mg, 21.9% sialic acid/BSA

BSA-colominic acid (**4-32**): 11.9 mg, 19.4% sialic acid/BSA

TT-glycolylcolominic acid (**4-33**): 5.68 mg, 13.6% sialic acid/TT

TT-colominic acid (**4-34**): 5.57 mg, 11.5% sialic acid/TT

C) Glycoconjugates via Michael addition:

Conjugation of 15% N-Acryloyl colominic acid(4-16) onto BSA (4-17) or IgG (4-36)

BSA (**4-17**) (5 mg) or IgG (**4-36**) (5 mg) was combined with 15% N-acryloylated colominic acid (**4-16**) (10 mg) and dissolved in 200 ul borate buffer (0.1M; pH 8.3). The solutions was incubated at

50 °C for 3 days followed by purification of the conjugate on a Sephadex G-150 column (1.6x100 cm) in 0.03M NH_4HCO_3. Lyophilization of the high molecular weight fraction yielded between 2-3 mg of either the BSA-15% acryloyl colominic acid conjugate (**4-38**) or the IgG-15% acryloyl colominic acid conjugate (**4-39**).

Sialic acid content (resorcinol) to BSA (Lowry) was estimated at 9.8% (wt/wt). SDS-PAGE electrophoresis of both (**4-38**) and (**4-39**) showed diffuse bands corresponding to higher molecular weight materials relative to their respective native protein. Immunoelectrophoresis of the two products demonstrated unequivocally the successful formation of conjugate.

Conjugation of N-acryloylated $(Neu5Ac)_2$ (4-13) onto BSA (4-17) - (4-40)

BSA (**4-17**) (3 mg; 0.044 umol) was combined with N-acryloylated $(Neu5Ac)_2$ (**4-13**) (10 mg; 0.015 mmol) and dissolved in 150 ul borate buffer (0.2M; pH=9.2). The solution was heated for 3 days at 37 °C, followed by purification by gel extrusion chromatography on a Biogel A.5 column (100x 1.6 cm) equilibrated in PBS. The high molecular weight fractions were pooled, dialyzed against distilled water, and lyophilized to 2.83 mg of product (**4-40**).

Sialic acid (resorcinol) to protein (BCA) analysis indicated sialic acid incorporation at 11.5% (wt/wt) or 25 moles Neu5Ac per mole BSA. The conjugate was a poor substrate in an ELISA binding assay against horse H.46 antiserum.

Conjugation of N-acryloylated $(Neu5Ac)_3$ (4-14) onto BSA (4-17) - (4-41)

In the same manner as above for **4-40**, the N-acryloylated $(Neu5Ac)_3$ derivative (**4-14**) (10 mg; 0.011 mmol) was reacted with BSA (**4-17**) (3 mg; 0.044 umol). After column purification and dialysis, lyophilization yielded 3.1 mg of product (**4-41**).

Colourimetric assays (resorcinol and BCA) indicated a 8.1% sialic acid to protein ratio (wt/wt) or 9 moles of $(Neu5Ac)_2$ to 1 mole of BSA. The conjugate was able to bind horse H.46 antiserum poorly.

Conjugation of mono-N-acryloylated colominic acid (4-15) Onto BSA (4-17) - (4-42) or TT (4-35) - (4-43)

The following is a typical example with a selected fragment of mono-N-acryloylated colominic acid of known size:

In a 1.5 ml Eppendorf centrifuge tube was added BSA (**4-17**) (3 mg; 0.044 umol) or TT (**4-35**) (3 mg; 0.02 umol) to a 150 ul solution of mono-N-acryloylated colominic acid (**4-15**) (15 mg; ~ 5-6 KD) in sodium borate buffer (0.2 M; pH 9.24). The mixture was heated at 37 °C for 72 hr and monitored for increase in molecular weight by gel extrusion HPLC (Superose-12). After one day, there was a complete disappearance of the relevant protein being replaced by a higher molecular weight product. At the end of the reaction, the material was purified by fractionation on a 100 X 2.5 cm column of BioGel A.5 (100 X 2.5 cm) in PBS, where the high molecular weight peak was collected, dialyzed at 4° C against dist. water, and lyophilized to give the respective conjugate (typically recoveries were ~ 80-100% of the original amount of protein).

Sialic acid (resorcinol) to protein (BCA) ratios were calculated and the BSA-colominic acid conjugate (**4-42**) was found to contain 93% Neu5Ac/ BSA and the TT-colominic acid conjugate (**4-43**) was found to contain 14.8% Neu5Ac/TT corresponding to 14 chains of colominic acid per BSA or 4.8 chains of colominic acid per TT molecule respectively, when assuming an average molecular weight of 4.5 KD for the polysaccharide fraction used. The two conjugates were able to bind horse H.46 antiserum in an ELISA assay.

Glycopolymers

$(Neu5Ac)_2$-sodium acrylate copolymer (4-44)

$(Neu5Ac)_3$ (**4-5**) (20 mg; 21.2 umol) was dissolved in MeOH (30 ml) containing NH_4OAc (130 mg; 1.70 mmol) and $NaBH_3CN$ (6.7 mg; 0.11 mmol). The methanolic solution was refluxed for 4 hr with periodic monitoring by TLC (solvent D). The disappearance of starting material and appearance of a ninhydrin positive spot indicated the reaction's end. The methanol was removed *in vacuo* and washed twice further with MeOH. The material was dissolved in water (5 ml) and the pH was raised to 10 with NaOH (0.5M). At 0 °C, the solution was treated with acryloyl chloride (2x 50 ul) while maintaining the pH at 10 with NaOH (2M). After a negative ninhydrin test, the solution was allowed to sit at R.T. at pH 10 for 20 minutes. The material was analyzed by TLC (solvent D) and the major product was baseline located. The solution was neutralized to pH 7.4 (0.2M HCl) and extensively dialyzed against distilled water. Lyophilization of the solution yielded 33 mg of product (**4-44**).

Sialic acid content (resorcinol) was found to be 15.9% (wt/wt). The polymer conjugate was able to precipitate WGA in a DID analysis.

TLC: Baseline product- solvent D

^{1}H NMR: δ: 1.72 (bd, 2H, -(CHCOONa-C$\underline{H}_2$)$_n$-), 2.05-2.1 (2x s, 9H, NAc), 2.38 (bd, 1H, -(C$\underline{H}$COONa-CH_2)$_n$-), 2.6-2.8 (2x m, 2H, 2x H_{3eq}), H_{3eq}:-(C$\underline{H}$COONa-CH_2)$_n$-= 7.1%

Conjugation of 2-Oxoethyl Neu5Ac (4-2) onto glycylglycine hydrazide-methacrylamide copolymer (3-9) - (4-45)

In 2 ml of water was dissolved 2-oxoethyl Neu5Ac (**4-2**) (10 mg; 0.027 mmol) and glycylglycine hydrazide-methacrylamide copolymer (**3-9**) (10 mg). Disappearance of the starting aldehyde (solvent A) indicated hydrazone formation and the material was reduced with $NaBH_4$ (20 mg; 0.53 mmol). After sitting for 2 hr at R.T., the solution was dialyzed and lyophilized.

Sialic acid analysis on the conjugate (resorcinol) revealed a 5.6% (wt/wt) incorporation.

^{1}H NMR: δ: 0.8-1.3 (bd, 57H, -(CH_2-CC$\underline{H}_3$$CONH_2$)$_n$-), 1.5-1.9 (bd, 38H, -(C$\underline{H}_2$-CCH_3CONH_2)$_n$-), 2.01 (s, 3H, N-Ac); N-Ac: -(CH_2-CC$\underline{H}_3$$CONH_2$)$_n$- = 5.3%

Conjugation of (Neu5Ac)$_3$ (4-5) onto glycylglycine hydrazide-methacrylamide copolymer (3-9) - (4-44)

A solution of borate buffer (600 ul; 0.07M; pH 9.2) containing 5.0 mg glycylglycine hydrazide-methacrylamide copolymer (**3-9**), 5.1 mg (5.72 umol) (Neu5Ac)$_3$ (**4-5**), and 18 mg (0.287 mmol)

$NaBH_3CN$ was heated at 50°C for five days. The solution was exhaustively dialyzed against distilled water and lyophilized to 4.6 mg of product (**4-44**).

The material was analyzed quantitatively for sialic acid by the method of Svennerholm (resorcinol) and found to contain 8.6% sialic acid by weight. The conjugate was not able to precipitate WGA in a double immunodiffusion study.

Chapter 5- Immunochemical Considerations

5.1 Introduction

Throughout the course of this thesis, a number of sialic or polysialic acid substrates have been synthesized with the end goal of studying their interaction with known equine IgM antibodies. This chapter will focus on the methods by which various glycoconjugates were characterized and preliminary serological results of the various studies will be presented. In order to determine the end effect of polysaccharide structural changes or to study aspects such as the minimum number of sialic acid residues needed for antibody binding, two key criteria are needed: 1) methods which allow us to "view" and evaluate macroscopic events such as antigen-antibody binding and 2) a source of antibodies that possess specificities to the substrate that is under investigation. Clearly this is critical when undertaking a structure-activity relationship. These antibodies also serve a dual role as probes in serological assays to detect and characterize a wide range of glycoconjugates.

It was mentioned in Chap.1 that antibodies to polysialic acid were rare owing to the low immunogenicity of the polysaccharide and were limited to three principal sources. The antibody available to us was the equine H.46 anti-Group B meningococcal IgM antibody (Sarff *et al.*, 1975) which was derived from a whole cell immunization. The horse antiserum is polyclonal (i.e.. contains many antibodies of different affinities and specificities) and is probably the most studied anti-GBMP antibody due to the large amounts that were made available for study. Ideally, the use of a monoclonal anti-GBMP

IgG antibody system (derived from a hybrid, single clone antibody producing cell-line that secretes antibody of the same isotype, specificity, and affinity) would be more appropriate, due to a decrease in complexity relative to working with a polyclonal serum.

Since one of the grey zones associated with the H.46 antiserum is whether or not it will bind small (less than 10 repeats) sialyloligosaccharides, it was not an ideal candidate as a detecting reagent for the characterization of novel sialylglycoconjugates. Unfortunately, antisera specific to small oligosaccharides or Neu5Ac itself are not available and the production of such antisera is currently in progress. The detection of these small sialylglycoconjugates was attempted using a plant lectin, *Triticum vulgaris* which is a wheat germ agglutinin (WGA) that is capable of binding terminal non-reducing Neu5Ac oligosaccharides amongst a number of other oligosaccharides (Goldstein and Poretz, 1986). The ability of this lectin to precipitate multivalent Neu5Ac containing macromolecules is not straightforward however, and this topic will be discussed in more detail (*vide infra*).

5.2 Immunochemical techniques

A brief description of the various immunochemical techniques relevant to this work will be outlined.

5.2.1 Double Immunodiffusion (Ouchterlony and Nilsson, 1978)

The technique of double immunodiffusion (DID) takes advantage of one of the best known properties of antibodies and that is their ability to precipitate multivalent antigens. This process

depends on the ability of the Ab to form a lattice of cross-linked Ab-Ag which eventually precipitates out of solution. If either the Ab or the Ag is monovalent (i.e. a Fab fragment of an antibody or a single antigenic determinant) then lattice formation is not possible and precipitation does not occur. The principle of DID is to let a solution of Ag and an Ab containing solution diffuse towards one another through a porous matrix like agarose. When the concentration of both the Ab and Ag reaches a critical level, precipitation occurs leaving behind a visible precipitin line at some point between the application points of the Ag and Ab. The shape and number of precipitation lines formed yield important information regarding the homology of epitope structures and of the constituent antiserum.

5.2.2 Immunoelectrophoresis (IEP) (Johnstone & Thorpe, 1982)

The technique of immunoelectrophoresis is essentially the same as described for DID with an added separation stage. A mixture of antigens, when applied to an agar/ agarose gel with an electric potential across its surface, will migrate towards either the anode (negatively charged antigens) or the cathode (positively charged antigens) where the distance migrated depends on the overall charge of the individual antigens. After separation by charge, an antiserum is applied to the gel and the immunodiffusion process takes over. The technique has the added advantage that impure glycoconjugates contaminated with the free carbohydrate can be separated and an accurate interpretation can be made. The process works equally well in the reverse sense by first electrophorizing the antibody mixture followed by antigen application.

5.2.3 Quantitative immunoprecipitation (Kabat & Schiffmann, 1962)

Relying on the same principles already described, quantitative immunoprecipitation allows one to isolate the Ab-Ag cross linked complex formed by the interaction of the antiserum with antigen in solution. Measuring the amount of protein in the Ab-Ag complex allows one to compare the relative binding strength of the antibody to other antigens. It is in this way that we can determine the relative effect of antigen structural modifications.

5.2.4 Enzyme linked immunosorbent assay (ELISA) (Engvall & Perlmann, 1971)

The enzyme linked immunosorbent assay (ELISA) has fast become the standard assay for detecting antibodies or antigens due to its versatility, sensitivity, safety, and because it is extremely economical in its use of reagents. Antigen is generally immobilized onto the surface of a polystyrene plate followed by incubation with increasing dilutions of the antiserum. After removal of non-bound antibody, a second species specific antibody (conjugate antibody) that is covalently coupled to an enzyme such as a peroxidase, is then incubated in the plate. Removal of excess conjugate antibody is followed by the addition of a colourless substrate which, after reaction with the bound enzyme, yields a coloured complex. The colour is directly proportional to the amount of Ab specific to the bound Ag. A number of variant assays can be performed using similar principles including competitive inhibition and sandwich ELISA's. The method is most powerful when screening for specific

antibodies in a polyclonal mixture or screening monoclonal fusion products since a high number of tests can be performed at once in a small amount of time.

5.2.5 Equilibrium dialysis (Berzotsky & Berkower, 1984)

The classical method used to determine binding information between Ag and Ab is through equilibrium dialysis. This method has many advantages such as solution kinetics, it is a true equilibrium process not requiring approximations, and the concentrations of ligand can be measured without disturbing the equilibrium. The principle is based on the diffusion of ligand across a semi-permeable membrane enclosing the antibody in question. Measurement of the free ligand and bound ligand at various concentrations allows the determination of K_A through a Scatchard plot. The method suffers from some notable drawbacks such as the equilibrium process involving transfer of Ag across the membrane. It is absolutely necessary that this state is reached which in some cases may require prohibitively long time periods (Cheng & Carlson, 1983). It also requires relatively large amounts of both Ag and Ab which may also be prohibitive. Interpretation of the results becomes increasingly more difficult when dealing with multivalent Ab's and Ag's where intrinsic values are influenced significantly by avidity effects.

5.2.6 Other methods to characterize glycoconjugates

Some notable biochemical techniques that are useful in the characterization of glycoconjugates are size exclusion HPLC (SEC-HPLC) and SDS polyacrylamide gel electrophoresis (SDS-PAGE). Both

HPLC) and SDS polyacrylamide gel electrophoresis (SDS-PAGE). Both techniques rely on fractionation of different molecular weight complexes through a matrix of defined pore size. In the case of SDS-PAGE, mobility of the conjugate is dictated by an applied electric potential gradient. SDS binds proteins to an equal extent giving the protein a uniform negative charge allowing for a separation based on molecular weight only. In this way, protein molecular weights can be estimated by the addition of standard proteins treated in a similar manner. This type of analysis cannot be applied to protein glycoconjugates for two reasons. (1) polysaccharides show very different mobilities through gels relative to globular proteins. (2) the binding of SDS to glycoconjugates is not well understood and may not be uniform with different conjugates leading to migration due to charge as well as by molecular weight. Only relative changes in molecular weight can be inferred from SEC-HPLC and SDS-PAGE.

5.3 Immunochemical characterization of sialylglycoconjugates

5.3.1 Double immunodiffusion studies with WGA

Initially, one of the most challenging aspects of this thesis was the characterization of the various sialylglycoconjugates synthesized through the various methods already discussed. Particularly challenging was the detection of sialylglycoconjugates derived from small sialyloligosaccharides due to the lack of an appropriate biological probe. For the most part, detection of these conjugates relied upon colourimetric analysis to quantitate any sialic acid present on the protein or polymer substrate. The limitation of this technique is the relatively low sensitivity of the colourimetric assays and hence, the large consumption of the often precious conjugates. It was our intention to make use of the binding properties of the plant lectin WGA (Goldstein & Poretz, 1986) to facilitate the detection of our novel sialylglycoconjugates. This was based on the lectin's ability to recognize terminal non-reducing Neu5Ac residues. Invariably, DID studies with this lectin showed no binding with the various sialyloligosaccharide protein conjugates despite colourimetric evidence that sialic acid was indeed present. From the accumulated evidence gathered from the repeated studies with sialyloligosaccharide- WGA interactions and the additional observations made from an intensive study of the WGA binding site towards Neu5Ac (Laferriere, PhD thesis 1990), it appears as if there is a minimum terminal Neu5Ac concentration threshold that must be surpassed in order to bind with WGA. As shown in table 5-1 with

BSA conjugates containing various levels of Neu5Ac, the conjugates with either 6 or 14 Neu5Ac residues per BSA did not precipitate WGA and precipitation was only observed with conjugates containing 23 or 36 Neu5Ac residues/BSA.

Table 5-1. Double immunodiffusion results of protein conjugates with various Neu5Ac contents with wheat germ agglutinin (WGA)

BSA[a]-(Neu5Ac)$_n$	Precipitin formation[b]	CSA-(Neu5Ac)$_n$	Precipitin formation	TT-(Neu5Ac)$_n$	Precipitin formation
n=6	–	n=15	–	n=9	–
n=14	–	n=17	–	n=12	–
n=23	++	n=20	+	n=15	–
n=36	++	n=22	+	n=17	–
		n=24	++		

[a] : donated by C. Laferriere.

[b] : visual observation.

DID studies with both the TT- and CSA-Neu5Ac conjugates (**4-27** & **4-26**) with different Neu5Ac incorporations, showed similar precipitating behaviours as the BSA-Neu5Ac conjugates. Precipitation occurred with CSA-Neu5Ac conjugates with greater than 20 Neu5Ac residues/protein, while TT-Neu5Ac conjugates with as many as 17 Neu5Ac residues/protein failed to precipitate WGA. This threshold phenomenon is most likely due to the inability of the lectin to form

extensive cross-linked lattices due to a low density of the antigen on the protein surface. The significance of this result can be readily seen when we compare a sialylmonosaccharide conjugate with a sialyltrisaccharide conjugate, both of which contain 10% Neu5Ac/protein (mole/mole). In the former case, the concentration of terminal Neu5Ac is ~22 Neu5Ac residues/protein. In the latter case, the concentration of terminal Neu5Ac is 3x's less and only ~7 Neu5Ac/protein and would yield a negative result using WGA as a detecting agent. Since this work focuses mainly on glycoconjugates with oligo-/ polysialic acids and incorporation of these substrates typically were found to be in the 5-20% Neu5Ac/protein range, WGA was found to be an unreliable probe in the detection of these sialylglycoconjugates.

The late acquisition of a number of sensitive instruments allowed a more complete characterization of synthetic sialylconjugates both from a physical and a serological standpoint. An example of each technique as applied to the characterization of a sialyl oligo-/ polysaccharide conjugate will be given.

Physical methods

5.3.2 SDS-PAGE

SDS-PAGE was performed on two protein (IgG and BSA) glycoconjugates formed by the Michael addition of 15% N-acryloyl colominic acid (**4-16**) as seen in figure 5.1.

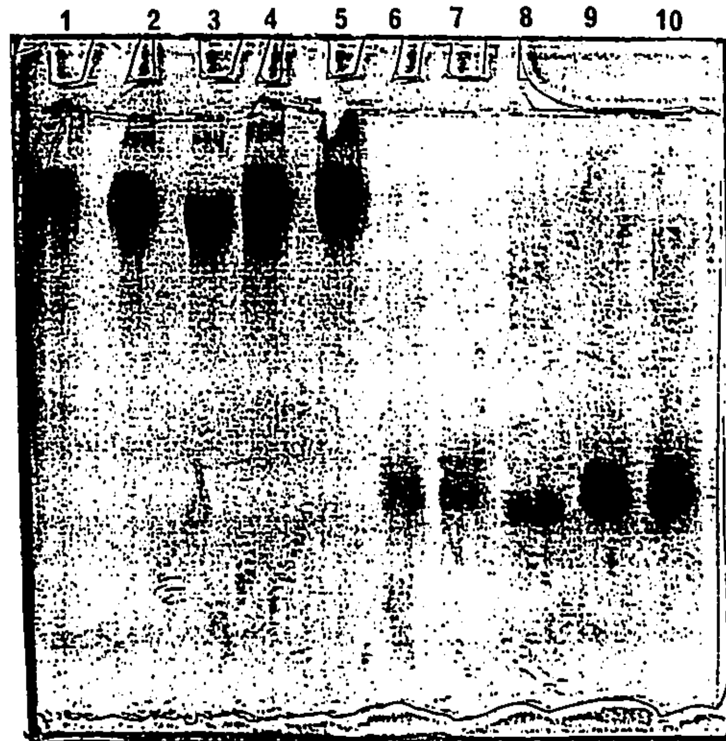


Figure 5.1- SDS-PAGE profile of two protein sialylglycoconjugates. lanes 1, 2, 4, and 5- IgG-colominic acid **4-39** (Michael adduct under various conditions, pH 8.3, 37°C; pH 10.2, 37°C; pH 8.2, 50°C; and pH 10.2, 50°C respectively), lane 3 and 8- porcine IgG and BSA respectively, and lanes 6, 7, 9, and 10- BSA-colominic acid **4-38** (Michael adduct under various conditions, pH 8.3, 37°C; pH 10.2, 37°C; pH 8.2, 50°C; and pH 10.2, 50°C respectively). Samples were run on a 7.5% gel at 16 V/cm for ~2hr at room temperature.

From the decreased mobility of the protein glycoconjugates relative to their native carrier protein, it is apparent that these conjugates represent higher molecular weight forms as would be expected by

the addition of polysaccharide chains. The bands produced are quite diffuse owing to the heterogeneity of the polysaccharide mixture and the previously mentioned poor understanding of how SDS binds to polysialic acid and whether there are differences with polysaccharides of differing lengths.

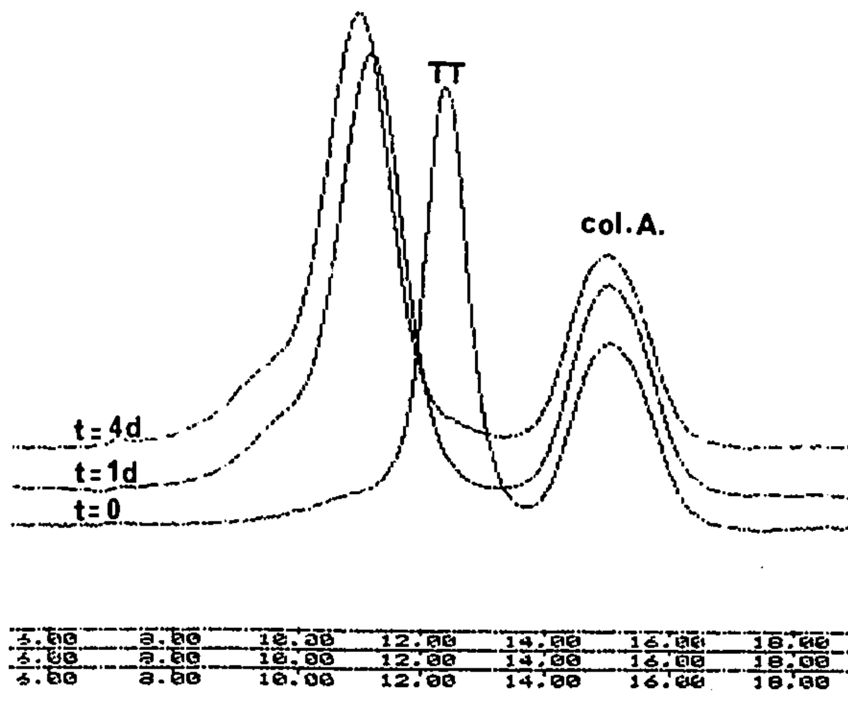


Figure 5.2- HPLC profile of the conjugation of oxidized colominic acid (**4-43**) to tetanus toxoid. (Superose-12, (Pharmacia) in PBS at 1 ml/min with UV detection at 214 nm).

5.3.3 Size exclusion HPLC (SEC-HPLC)

Perhaps the most useful of all the techniques used to characterize sialylglycoconjugates because it allows real time monitoring of the conjugation reaction without prior purification. It is

also useful to calibrate the average molecular weights of polysaccharide fractions based on retention times and allows semi-preparative purification of the reaction mixtures. As an example of its application, the time course coupling of a periodate generated colominic acid fraction (**4-34**, ~3 KD) to tetanus toxoid (**4-31**) is shown in fig 5.2. It can be seen that after one day at 37 °C, the peak corresponding to the native protein is shifted to a higher molecular weight due to the conjugation of the colominic acid fragment.

Serological methods

5.3.4 Immunoelectrophoresis (IEP)

Most proteins under electrophoretic conditions (pH>8) are negatively charged and will migrate towards the positive pole (anode). A notable exception are the immunoglobulins which are generally positively charged and migrate towards the negative pole (cathode). It can be expected then that sialylglycoprotein conjugates should behave differently from their respective native protein due to the additional negative charge associated with the polycarboxylates. An example which clearly shows the electrophoretic differences in both the type of carrier protein used (BSA or IgG) and the effect of conjugation can be seen in figure 5.3. This profile shows the conjugation of 15% N-acryloyl colominic acid to either BSA or IgG.

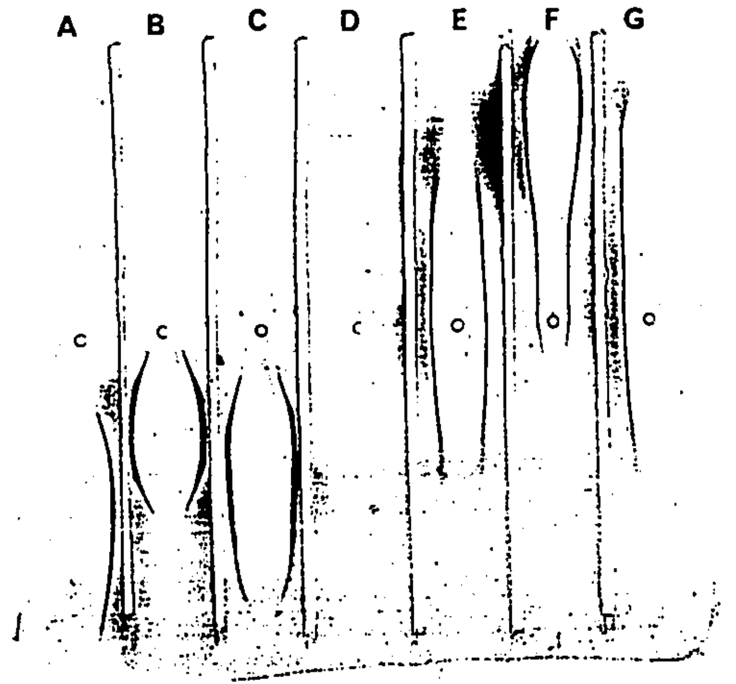


Figure 5.3- Detection of protein sialylglycoconjugates by immunoelectrophoresis. Bands were visualized with either rabbit anti-porcine IgG or goat anti-bovine serum albumin. Lane A- BSA-colominic acid (**4-38**, Michael adduct) , lane B- BSA, lane C- mix BSA+BSA-colominic acid (**4-38**), lane E- IgG-colominic acid (**4-39**, Michael adduct), lane F- porcine IgG, and lane G- mix IgG+IgG-colominic acid (**4-39**).

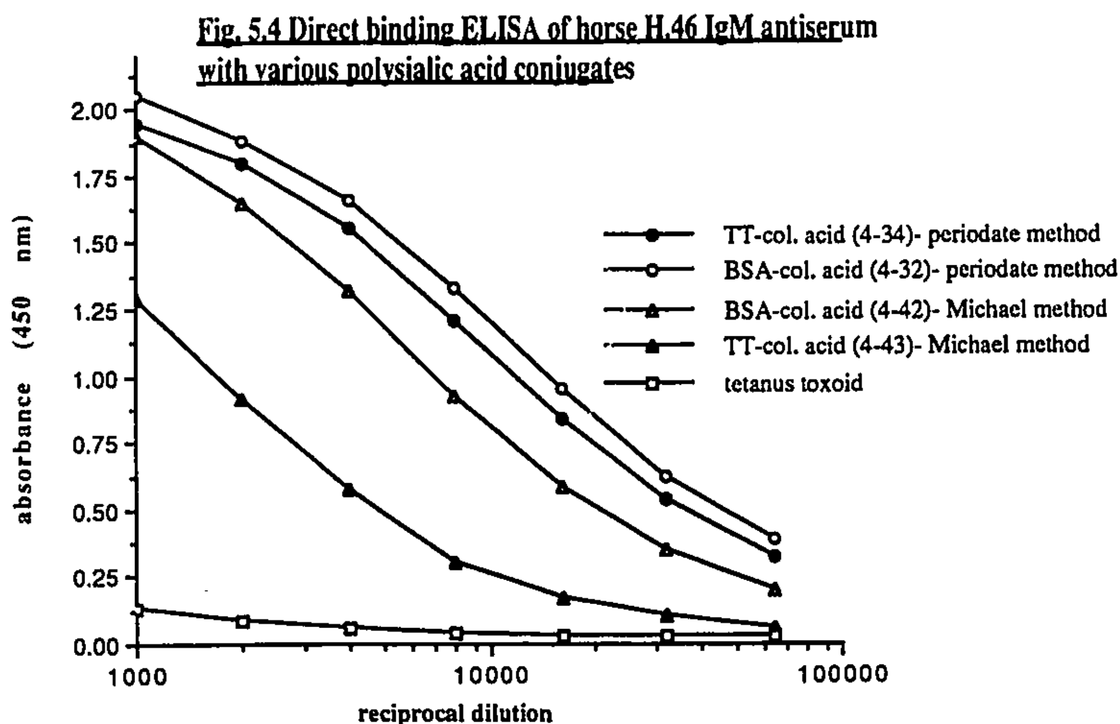
The differences in the electrophoretic mobility of the two conjugates can be clearly seen with the mixed fractions of conjugate and protein proving that they are indeed unique bands. The H.46 antiserum used in some of the previous assays had suffered from age and was found to contain few viable anti-GBMP antibodies which made detection of

the conjugates in the normal fashion (i.e. immunodiffusion) impossible. The conjugates were detected using a mouse anti-BSA or mouse anti-pig IgG antiserum which is why the native protein bands are visible. Proof that the bands corresponding to the conjugates indeed contained the conjugated polysaccharide came from overlaying the gel with the undiluted H.46 antiserum where after 24 hr at 4 °C, a band appeared which corresponded exactly to the band detected with the anti-BSA or anti-IgG antisera.

5.3.5 Enzyme linked immunosorbent assay (ELISA)

The last immunochemical assay used to detect the presence of newly formed sialylglycoconjugates was the ELISA system. The main advantages of ELISA are its simplicity, ability to screen many antigens at once, and its extreme sensitivity thus requiring very little amounts of conjugate. In a typical ELISA experiment only ~20 ug of conjugate is needed for characterization. ELISA was employed to verify the results of conjugation reactions where the protein or polymer conjugate was used as a coating antigen and immobilized to the wells of a polystyrene plate. An example of the type of result observed is given in fig. 5.4, which shows the screening of four potential conjugates using the H.46 antiserum to detect the presence of colominic acid on the conjugate. In this assay system, the colominic acid protein conjugate represented the coating anigen and the H.46 antiserum was the source of the detecting anibodies. The immune complex (Ab-Ag complex) was detected using a peroxidase labelled goat anti-horse IgM antibody and the appropriate enzyme substrate (TMB). The difference seen with the curve representing the TT-

colominic acid conjugate formed by Michael addition of mono-N-acryloylated colominic acid onto TT, probably reflects the poor incorporation of polysaccharide onto the protein rather than an inability of the H.46 antibodies to bind to this substrate.



With the advent of new instrumentation requiring very little substrate and time, the characterization of *de novo* sialylglycoconjugates has become a problem of the past. The importance of this technology as a time saving device cannot be emphasized enough since characterizations that would normally have required 1-2 weeks can now be accomplished in a matter of hours. They allow more highly defined and systematic production of potentially new immunogens that may well one day become a vaccine of the future.

5.4 Interaction of structurally modified polysaccharides with horse anti-Group B meningitidis IgM antibody (H.46)

Preliminary studies were undertaken to determine the fine specificities of a polyclonal IgM antibody (H.46, Sarff *et al.*, 1975) specific to the capsular polysaccharide of *Neisseria meningitidis* . As previously discussed (chapter 2), modification of the GBMP or the structurally homologous colominic acid was ideally located at either the carboxyl or N-acetyl functionalities. During a time course study on the rate of de-N-acetylation of colominic acid, a number of substrates were isolated with various degrees of residual N-acetyl group. Double immunodiffusion analysis (DID) of these substrates with H.46 antibody, revealed the effect of removing the N-acetyl functionality and at which point antigenicity of the polysaccharide was lost (see table 5-2). It was quite evident that antigenicity of the polysaccharide was lost after 55% of the polysaccharide was de-N-acetylated. If we assume de-N-acetylation was a random event, then it appears as if at least two consecutive Neu5Ac residues are needed in order for binding to occur. Loss of these two consecutive residues destroys the ability of the polysaccharide to bind with the H.46 antibody.

Table 5-2. Effect of de-N-acetylation on the antigenicity of colominic acid towards horse H.46 IgM antiserum by double immunodiffusion.

% de-N-acetylation[a]	precipitin formation[b]	% de-N-acetylation	precipitin formation
0	+++	57.7	+
5	+++	63.8	-
10.2	+++	83.3	-
13.9	+++	89.6	-
31.1	+++	95.1	-
45.6	++	100	-

[a] : determined by ^1H NMR analysis- see section 2.4.1

[b] : visual observation.

Table 5-3. Double immunodiffusion results of various colominic acid analogs towards horse H.46 IgM antibody.

Substrate	Precipitin formation[a]	Substrate	Precipitin formation
colominic acid (2-1)	++++	N-Acryloyl colominic acid (2-11)	++++
De-N-acetylated colominic acid (2-6)	-	N-Propionyl colominic acid (2-12)	++++
N-Formyl colominic acid (2-7)	-	N-Glycolyl colominic acid (2-15)	-
N-Isobutanoyl colominic acid (2-8)	+	Carboxy reduced colominic acid (2-19)	-
N-Chloroacetyl colominic acid (2-9)	++	Colominic acid hydrazide (2-22)	-
N-Bromoacetyl colominic acid (2-10)	+	Colominic acid hydroxylamine (2-23)	-

[a] : visual observations.

DID analyses were performed on all of the modified colominic acid analogs synthesized and described in chapter 2 and the results are summarized in table 5-3. From these results, it is clear that any modification of the carboxyl terminus of the polysaccharide is not tolerated by the antibody while varying results were obtained due to N-acyl modifications. N-propionyl and N-acryloyl colominic acid both gave strong precipitin bands with H.46 while the 3-chloropropionyl, 3-bromopropionyl , and isobutanoyl colominic acid showed weaker but detectable precipitin bands. The remaining polysaccharides did not give any visible precipitin lines with H.46 and are considered as poor substrates.

In order to quantitate the effect of polysaccharide structural changes on the binding ability to H.46, a quantitative immunoprecipitation study was undertaken. The preliminary results with a number of modified substrates are given in table 5-4 and depicted in figure 5.5.

Table 5-4 Immunoprecipitation results of various colominic acid analogs with horse H.46 IgM antiserum.

Substrate	Protein precipitated (ug) at 2x's Ab dilution	% Binding relative to colominic acid
colominic acid (**2-1**)	44.0	100
de-N-acetylated colominic acid (**2-6**)	-	-
N-glycolyl colominic acid (**2-15**)	-	-
N-propionyl colominic acid (**2-12**)	18.7	42.5
N-formyl colominic acid (**2-7**)	-	-
N-acryloyl colominic acid (**2-11**)	28.5	64.8

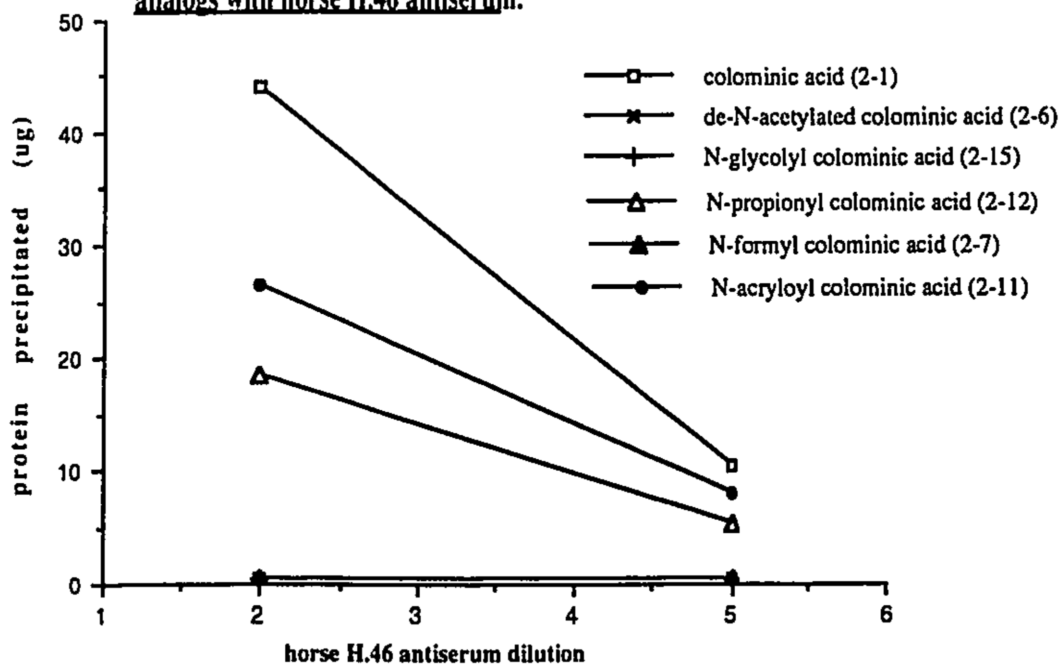


Fig. 5.5 Immunoprecipitation of various colominic acid analogs with horse H.46 antiserum.

A more comprehensive study involving the behaviour of antibody binding with various antigen dilutions was not performed due to a lack of available antibody. The preliminary results with the limited number of modified substrates tested showed the same trends as seen in the DID studies. Normalizing the amount of antibody precipitated with colominic acid, the relative precipitating power of the remaining analogs can be quantitated. N-acryloyl colominic acid was the best substrate tested being between 66-75% as effective as colominic acid followed by the N-propionyl derivative at 44-52% as effective. The remaining modified substrates were unable to precipitate any significant amount of H.46 antibody leading to their recognition as poor substrates.

From the above results two general conclusions can be made concerning the specificity of the H.46 antibody combining site:

(1) Maintenance of the polyanionic character of the polysaccharide is absolutely essential for binding to occur.

(2) There exists much more flexibility in the structural changes allowed in the N-acyl region.

The immunodominance of the polycarboxylate residues has been precedented by Lifely and coworkers (1981, 1984) who showed that the formation of internal ester linkages between the C-9 hydroxyl and the carbonyl functionality of the adjacent residue in GBMP (fig 4.25), abolished antigenicity with H.46 antibody. Jennings and coworkers (1986) reported that the formation of the methyl amide of GBMP as well as the carboxyl reduced derivative, also abolished precipitation of the H.46 antibody in immunodiffusion studies. Our results with similar derivatives of colominic acid showed the same tendencies both in DID and in immunoprecipitation studies. Furthermore, two additional derivatives (colominic acid hydrazide (**2-22**) and the hydroxamic acid of colominic acid (**2-23**)) both of which retain the carbonyl character and are capable of forming hydrogen bonds, did not restore antigenicity. The hydrazide with a pK_a~7 exists at physiological pH's with a significant proportion of positive charge as opposed to the almost exclusive anionic form of the acid (pK_a~3.7) which again does not appear to be significant. In light of these results, it would appear as if the anionic nature of the carboxyl is strongly involved in the binding site, probably

coordinated with a positively charged amino acid such as histidine, arginine, or lysine. Alternatively, the polyanionic charge may be responsible for maintaining a spatial distribution as suggested by Kabat *et al.*, (1986) based on results with a human monoclonal antibody IgM^{NOV} which cross reacts with both GBMP and polynucleotides such as poly (A) or poly (I). Since the only common element between the two is the polyanionic nature of the polymer, it follows that the antibody is recognizing the charge distribution. This argument does not hold up well with the H.46 antibody or a number of other antibodies specific to GBMP since they do not show any cross reaction towards these polynucleotides (M. Kulakowska, personal communication). The exact role of the carboxylic acid functionality is still the object of debate and current ongoing research into its exact role will be briefly discussed in the next section.

The H.46 antibody showed more tolerance for changes at the N-acetyl position of colominic acid and a number of conclusions can be drawn in regards to the fine specificity of the Ab combining site. Paralleling results found with the GBMP (Jennings *et al.*, 1986), complete removal of the acetyl groups abolished all antigenicity. Whether this is due to an associated change in conformation, introduction of positive charge, or the loss of the relatively hydrophobic group is not clear. Evidence that supports the latter situation comes from the recognition by H.46 of those modified substrates with a hydrophobic N-acyl functionality like a propionyl, an acryloyl, and to a small degree an isobutanoyl group. Other more hydrophobic substituents such as the N-butanoyl, N-pentanoyl, and N-hexanoyl versions of colominic acid have also been found to bind

with H.46 although the interaction varies with the chain length of the substituent (Lifely & Esdaile, 1991). Introduction of a hydrophilic substituent, namely the N-glycolyl derivative, totally abolished binding in both DID and immunoprecipitation studies. This would lend credence to the idea that the hydrophobic N-acyl substituents are key elements involved in the H.46 Ab combining site although they, unlike the carboxyl group, are not immunodominant. Further evidence that indicates the hydrophilicity of the N-glycolyl group causes a lack of binding with H.46 comes from the detailed high resolution NMR analysis of the polysaccharide which suggests that it adopts the same solution conformations as the native N-acetyl derivative (Baumann *et al.*, unpublished results). Interestingly the N-formyl derivative, lacking a methyl sidechain, does not bind with the H.46 antibody. There also appears to be a size limitation within the Ab combining site since it is clear that as the substituents become bulkier or more extended, antigenicity falls off. Whereas the propionyl derivative binds strongly to H.46, the isobutanoyl derivative does not. A similar situation arises with the 3-chloropropionyl and 3-bromopropionyl derivatives. It was apparent by DID, that the bulkier bromo derivative was not as good a substrate as the more compact chloro derivative.

In summary, from the preliminary mapping of the H.46 antibody combining site with a number of structurally defined polysaccharide derivatives, it is apparent that the carboxyl functionality is most probably involved in a direct interaction with basic amino acids within the binding pocket of the antibody. It is also a distinct possibility that this functional group may impose

conformational restrictions of the polysaccharide due either to a repulsive effect of the anionic charge and/or the ability to form key intramolecular hydrogen bonds. It also appears as if there is a more flexible region of the antibody pocket which interacts with the hydrophobic regions of the polysaccharide at the N-acetyl terminus. This interaction seems to be limited to small hydrophobic groups and antigenicity falls off as the substituent becomes bulkier or becomes more hydrophilic in nature.

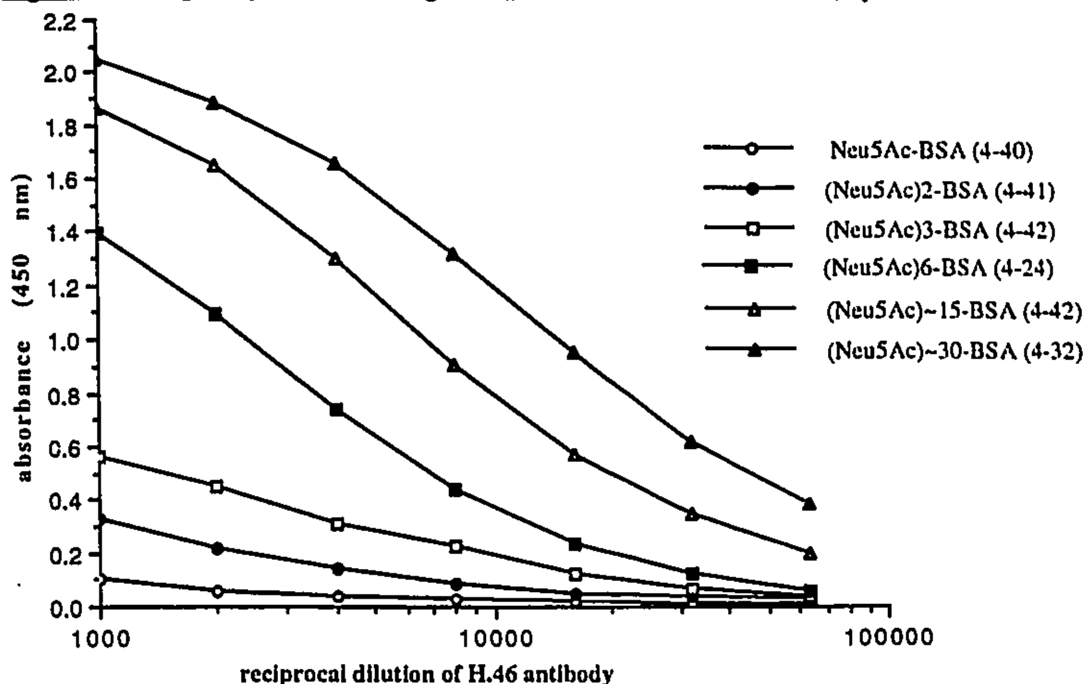
5.5 Preliminary direct binding study of sialyloligosaccharide- protein conjugates with horse anti-GBMP IgM antibody (H.46)

One of the initial goals of this thesis was to perform a direct binding study of Neu5Ac oligosaccharides with the anti-GBMP IgM antibody H.46, since at the time, this study had not been carried out. Our first approach was to use the classical method of equilibrium dialysis in order to gain binding information but quickly realized that this method, although theoretically sound, suffered from two major shortcomings critical to the success of the assay. Firstly, due to the lack of a microequilibrium dialysis system (Cheng & Carlson, 1983) and the need to determine bound and free antigen (sialyloligosaccharides) by colourimetry, both a large supply of Neu5Ac oligosaccharides from DP 1→15 and consequently, H.46 IgM antibody, would be needed. Obtaining large quantities of pure oligosaccharides while not impossible, is very time consuming and the horse antiserum was available to us only in short supply. More importantly, preliminary dialysis studies with Neu5Ac oligosaccharides revealed a critical limitation of this method. It was found that the time for Neu5Ac oligosaccharides to reach equilibrium was prohibitively long (i.e. monomer- 7 hr; dimer- 15 hr; trimer- 23 hr) and higher oligosaccharides such as the decamer did not reach equilibrium even after 4 days. Since the true binding constant reflects an equilibrium process, the method fails since in some cases equilibrium is never attained. The most reasonable explanation for this phenomenon is the hydrodynamic volume of the

sialyloligosaccharides makes them appear to be much larger in size than they physically are. This pseudo increase in molecular weight does not allow them to pass freely through the semi-permeable membrane (10 KD cutoff) - a process needed to reach equilibrium. This phenomenon is not limited to passage through semi-permeable membranes but has also been observed in gel extrusion chromatography. For example, the retention time of a 10 KD fraction of polysialic acid on a gel extrusion column represents a 65 KD globular protein or a 100 KD neutral polysaccharide (personal observation). That the charge is once again implicated in this effect comes as no surprise and reduction of the carboxyl group causes the polysaccharide to behave more like a neutral polysaccharide in gel extrusion chromatography.

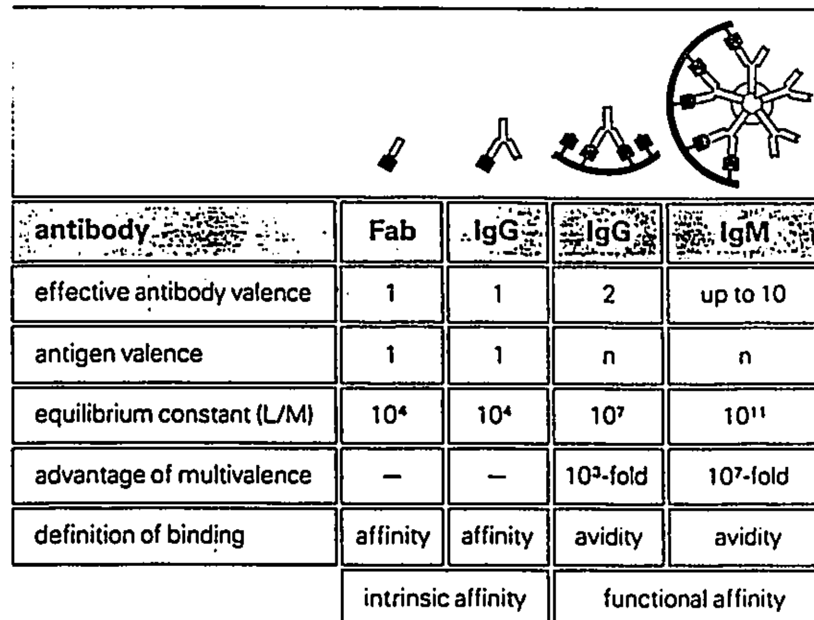
Some preliminary results were obtained however from a direct binding ELISA assay with sialyloligosaccharide- protein conjugates used as coating antigens. The results are shown in figure 5.6. The mono-, di-, and trisaccharide conjugates were made via Michael conjugation of the pure monofunctionalized N-acryloyl derivatives while the 15-mer conjugate was formed via Michael addition of a narrowly defined mixture of monofunctionalized N-acryloyl oligosaccharides with an average molecular weight of 4500 or 15 repeat units.

Fig. 5.6- Binding study of Neu5Ac oligosaccharides with horse H.46 antibody



The hexasaccharide conjugate was formed by the direct reductive amination of a Neu5Ac heptasaccharide onto BSA. The polysaccharide (~35 residues) conjugate was synthesized through the activation of the non-reducing terminus with periodate followed by reductive amination. In terms of the number of chains of oligosaccharide to protein, the various sialyloligosaccharide conjugates tested were not all at the same level. This however is not a significant factor since the coating antigen is present in the wells of the microtiter plate under saturating conditions (i.e. the surface is uniformly coated) and the antibody does not require the need for multivalent forms of the antigen in order to bind. From the binding profiles, it would appear as if the mono-, di-, and trisaccharide conjugates are relatively poor

ligands for the H.46 Ab, although there is a visible trend in that there is increased binding with an increase in DP. Comparison of the hexasaccharide conjugate, the 15-mer conjugate, and the native colominic acid conjugate reveals an increase in binding with an increase in chain length. This result is in contrast to the more classical carbohydrate binding studies (Kabat, 1960) where the maximum level of antibody binding occurs with oligosaccharides that saturate the Ab combining site (generally 6-7 residues in size) and the binding does not increase substantially with an increase in oligosaccharide size beyond this size. Clearly in our situation we do not see this type of levelling-off effect as in the classical case. Since it is inconceivable that the antibody combining site is larger than 6-7 sialic acid residues, it must be concluded that the antibody H.46 recognizes a conformational epitope of polysialic acid requiring an extended oligosaccharide for maximum binding. The results obtained in this direct binding study concur with the results obtained by Jennings and coworkers (1985) and Kabat and coworkers (1988) using inhibition studies. The possibility existed in these latter two cases, that their large oligosaccharide requirement result may have been influenced by the avidity effects of the IgM antibody (polyvalent) and the polysaccharide (also multivalent). In other words, their studies may have required a long sialyloligomer to inhibit the binding of the native polysaccharide to the H.46 or the IgM^{NOV} antibodies because shorter oligosaccharides may not have been sufficiently 'powerful' to displace the large polysaccharide which may have bound in a multipoint fashion (fig. 5.7).



antibody	Fab	IgG	IgG	IgM
effective antibody valence	1	1	2	up to 10
antigen valence	1	1	n	n
equilibrium constant (L/M)	10^4	10^4	10^7	10^{11}
advantage of multivalence	–	–	10^3-fold	10^7-fold
definition of binding	affinity	affinity	avidity	avidity
	intrinsic affinity		functional affinity	

Figure 5.7- Affinity vs. avidity effects.

Although avidity effects cannot be completely ruled out in our situation, it is much more unlikely to be a factor for the following reason. The true or intrinsic binding constant is determined by the interaction of one epitope of an antigen with one binding site of the antibody. Avidity effects come into play when more than one epitope or more than one binding site is present. In the case of an IgM, there is necessarily 10 binding sites. In our case however, the use of increasingly larger oligosaccharides in a direct binding assay necessitates that there is only one epitope of Neu5Ac present and

avidity cannot be a factor. The limitation with this type of analysis is that the real epitope of the polysaccharide is not known and as the oligosaccharide size increases, the potential that avidity will be a factor also increases. Based on the shape of the binding curves (fig. 5.6), it is reasonable to assume that a second epitope was not involved in the binding with H.46 Ab since the slope is continuous reflecting no change in the reaction order.

Although our direct binding results are limited, it appears as if they are consistent with a conformational epitope proposed by other groups. Since the inception of this work, a number of other studies regarding this binding phenomenon of polysialic acid have appeared and a current view of this unique polymer will be briefly outlined in the Recent and Future Prospects section.

5.6 Experimental

General Methods

For a complete description of general methods, please refer to the general methods entry in chapter 2 (section 2.7).

Double radial immunodiffusion techniques

Preparation of the agarose plates:

Agarose (200 mg, BDH) was added to phosphate buffered saline (PBS) (0.01M PO_4^{-3}, 0.15M NaCl, pH 7.3, 16 ml) and the mixture was heated (100 °C) until dissolution. Polyethylene glycol (PEG 8000) (4.0 ml of a 10% solution in PBS) was added to the hot solution to give an overall concentration of 1% agarose in PBS. The solution was distributed onto 5 cm Petri plates (2.7 ml per plate) and allowed to solidify on a level surface. The plates were stored at 4 °C under a moist atmosphere for a period not exceeding two days. For longer storage, the addition of sodium azide (0.02%) prevented bacterial growth.

Immunodiffusion experiment:

Just prior to use, a series of equally spaced wells were cut using a hole punch according to various templates. The gel plugs were removed from the plate by gentle aspiration. Typically, the antibody or lectin (WGA) was added to the center well and the antigen solutions were added to the surrounding wells. The normal concentrations of both the antigens and the lectin were either 1 or 2 mg/ml in PBS. The horse IgM (H.46) antibody was typically used as a

5 or 10 times concentrated solution of the original sample furnished to us. The antibody was applied (~50 ul) and allowed to diffuse followed by the addition of a second volume of PBS as wash over a 3 hr period. The antigen solutions were then added (~50 ul) and allowed to diffuse at room temperature (3 hr) followed by storage at 4 °C for 12-15 hr under a moist atmosphere. The bands were detected initially by the appearance of the white precipitin arc and stained if desired.

Staining of precipitin lines:

PBS (2-3 ml) was added to the developed plates and stored at 4 °C. The PBS solution was discarded every 3 hr and replaced with fresh PBS. This was continued for 3-4 changes of PBS. The precipitin lines were stained by covering the gel with a Coomasie blue solution (0.025% w/v Coomasie brilliant blue R in MeOH/H_2O/HOAc 50:45:5) until the bands became visible (30-60 min). The stain was removed at this point followed by the addition of destain (H_2O/HOAc/MeOH 87:8:5). The destain was replace every 2-3 hr until the background gel lost its colour. The gels were dried between filter paper at room temperature but became very fragile.

Immunoelectrophoresis (IEP) techniques

IEP was carried out on a homemade apparatus consisting of a prepared agar or agarose glass plate, platinum electrodes, buffer chambers, and a voltage supply. Typically, a 1.5% solution of agar or agarose in barbitol buffer (0.05M, pH 8.3) was prepared by heating the mixture at 100 °C until dissolved. The hot agar solution was distributed (20 ml) evenly over the surface of a 10 x 10 cm glass

plate. Wells were cut (1 mm) along the center line of the cooled gel and antigen solution (5 ul of typically 1 mg/ml solutions in PBS). The plate was electrophorized at 160 V and 20 mA for 2.5 hr by overlaying the two ends with wetted filter paper (Whatman #1) immersed in barbitol buffer. After electrophoresis, channels were cut between the wells and the gel removed by aspiration. Detecting antibody or lectin (1-2 mg/ml in PBS) was added to the channels and developed and stained as above for double immunodiffusion.

Immunoprecipitation Techniques

Antigen solutions (2 mg/ml) in PBS were prepared. The original horse IgM (H.46) solution was diluted 2x's and 5x's with PBS. In a series of micro-centrifuge tubes was added 100 ul of each antigen (20 ug) and 100 ul of either the 2x's or 5x's diluted antibody. The solutions were incubated at 4 °C for 4 days. Individual tubes freshly removed from the 4 °C environment were centrifuged (10,000 rpm) for 7 minutes followed by cooling at -15 °C for 5 minutes. The supernatants were discarded and the pellets (rarely visible) were washed with cold PBS (300 ul), recentrifuged, and cooled. This process was repeated 3x's with removal of the supernatant to leave the semi-dry pellets. The pellets were then assayed for protein according to the method of Lowry (appendix A) using immunoglobulin as standard.

Enzyme linked immunosorbent assay (ELISA)

The following is a standard method for measuring total antibody:

A solution of the oligo-/ polysaccharide protein conjugate (10 ug/ml in PBS) was used to coat the wells of a polystyrene microtiter plate (Corning). The antigen solution (100 ul) was pipeted into the wells (1 ug/ well) and allowed to incubate at 37 °C for 2 hr or room temperature overnight. The antigen solution was removed followed by washing the plate with PBS (300 ul). The wells were blocked with a 0.1% solution of BSA (300 ul, 1 hr at 37 °C), followed by removal and washing with PBS containing 0.05% Tween 20 (4x's). The appropriately diluted horse H.46 antiserum (10^{-3} to 64 x 10^{-3}) was added (100 ul) to each well and the plate was incubated for 2 hr at 37 °C. The plate was emptied and washed with PBS-Tween (5x's 300 ul). The peroxidase labelled goat anti horse IgM diluted 1:2000 with PBS-Tween, was added to each well (100 ul) and incubated for 30 minutes at r.t. The conjugate antibody was removed and the plate was washed with PBS-Tween (5x's 300 ul). The freshly prepared tetramethyl benzidine (TMB) peroxidase substrate was added to each well followed by gentle agitation. The progress of the reaction was stopped after 15 min by the addition of 50 ul of H_3PO_4 (50 ul/ well). The absorbances were read at 450 nm using a Titertek multiscan ELISA plate reader.

Sodium dodecylsulfate- polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a homemade apparatus consisting of two 15x15 cm glass plates, plastic spacers, a plastic bag, buffer chamber, and voltage supply. Gels were run at either 12.5% w/v (for BSA conjugates) or 7.5% w/v (for TT conjugates) final acrylamide

concentration according to the protein to be electrophorized. For example, a 7.5% w/v acrylamide gel was prepared by mixing 7.5 ml of an acrylamide- N,N'-methylene bisacrylamide stock solution (30:0.8) with Tris-HCl (3M, pH 8.8, 3.75 ml), 10% SDS in water (0.3 ml), 1.5% ammonium persulfate (1.5 ml), and water (16.95 ml). The solution was vacuumed degassed for five minutes followed by initiation with tetramethylethylenediamine (TEMED) (15 ul). The solution was injected between the two glass plates and allowed to polymerize. A stacking gel (3.75% w/v total acrylamide) was prepared by mixing the acrylamide-bisacrylamide stock solution (2.5 ml), Tris-HCl (0.05M, pH 6.8, 5.0 ml), 10% SDS (0.2 ml), 1.5% ammonium persulfate (1.0 ml), water (11.3 ml) and initiating the polymerization with TEMED (15 ul). This stacking solution was overlaid on the polymerized resolving gel and allowed to polymerize around the channel comb. After polymerization the channel comb was removed and the excess solution was removed by absorption with filter paper.

Samples were prepared by mixing in a 1:1 volume the protein conjugate (1 mg/ml in PBS) with a solution consisting of Tris-HCl (0.1M, pH 6.8), 2% SDS, 10% glycerol, and 0.001% bromophenol blue as tracking dye. The sample was heated at 100 °C for five minutes and applied to the gel channel (10-20 ul). The gel was electrophorized for ~2 hr or until the tracking dye reached the opposite pole at 160 V and 20 mA. The gel bands were visualized by staining the gel with Coomasie brilliant blue as described above for staining immunodiffusion bands.

6.1 Summary

In summary, the results presented in this work has provided useful information regarding the size and structural requirements for binding of poly α-(2$\rightarrow$8) Neu5Ac to the equine IgM antibody (H.46) specific to the capsular polysaccharide of *Neisseria meningitidis* serogroup B. It was found through a direct binding study that the binding of Neu5Ac oligosaccharides to the antibody increased progressively from DP 2$\rightarrow$15 without any observed plateau effect normally encountered with classical carbohydrate antibody interactions. Since this plateau phenomenon usually occurs at $\sim$6 residues in length, reflecting the maximum size of an antibody combining site, it was proposed that the epitope required by the H.46 antibody is indeed conformational in nature. This result is consistent with the results obtained through inhibition of binding studies with H.46 IgM antibody (Jennings *et al.*, 1986) and a human monoclonal antibody IgM^{NOV} (Kabat *et al.*, 1988). From the similarity of these results, it was concluded that the unusually large epitope required for binding was not distorted by any avidity effects that potentially may have dominated in the inhibition studies. The study of the specific interaction of the H.46 IgM antibody with poly α-(2$\rightarrow$8) Neu5Ac was extended to elucidate the structural requirements of poly α-(2$\rightarrow$8) Neu5Ac needed for antibody binding. This was accomplished through the synthesis of several analogs of poly α-(2$\rightarrow$8) Neu5Ac and studying their interaction with H.46 IgM. It was found that any modification affecting the negative carboxylate terminus completely abolished binding. In a similar manner, insertion of hydrophilicity at the N-terminus also destroyed the

polysaccharide. Hydrophobic modifications were tolerated at this terminus however, there also appears to be a size requirement involved since binding decreases with increasing substituent size. The results from this study indicates that the charge is necessary to either participate directly in the binding site and/ or to allow the polysaccharide to assume the proper conformation needed for binding. The results also indicate that there is a hydrophobic interaction between the N-acyl side chain and the antibody combining site which is restricted by the size of the substituent.

The two studies mentioned above also generated key derivatives that find applications in other areas. Methods were developed to produce the first ever synthetic version of the biologically important N-glycolyl colominic acid polysaccharide amongst a series of *de novo* polysaccharides that could potentially lead to interesting vaccines. Methods were also worked out to produce in high yields the biologically relevant α-(2$\rightarrow$8) N-acetylneuraminic acid disaccharide which is not available as of yet, in large quantities. The monosaccharide and α-(2$\rightarrow$8) linked disaccharide of N-glycolylneuraminic acid, both of which are biologically important and are not readily available, were also produced in high yields in a semi-synthetic manner.

An eleven atom heterobifunctional linker molecule was synthesized from the amino acid glycine as starting material, incorporating a reactive acryloyl and hydrazide terminus. Applications of the molecule included the reductive amination of the spacer onto various sialyloligosaccharides and the formation of

sialylglycopolymers through copolymerization of the spacer derivatives with methacrylamide.

Finally, an extensive study was carried out on new and improved ways to covalently link sialic acid oligo-/ polysaccharides to macromolecular supports such as proteins and polymers. Conditions were found that makes the direct reductive amination of sialyloligosaccharides to proteins or polymers, through the hemiketal functionality of the reducing terminal, a viable option. A *de novo* method of conjugation was also developed relying on the efficient addition of amine in a Michael-type reaction with suitably acryloylated sialyl oligo-/ polysaccharides (Michael acceptor). This method of conjugation was successfully applied to a number of acryloylated sialyl oligo-/ polysaccharides with various proteins to form new sialylglycoproteins.

7.1 Recent and Future Prospects

A lot has been learned from this and the large number of other studies, on the interaction of mono-/ oligo-/ and polysialic acid with biological systems. A complete understanding of these complex interactions however, is still far from being finished although not from lack of interest. Sialic and polysialic acid remains one of the hottest topics of carbohydrate research due principally to their ubiquitous presence in biological systems and their related associations with such diverse processes as cell development and differentiation, cell-cell communication, cell adhesion, bacterial pathogenesis, cancer metastastis, tumour oncodevelopmental antigens, and even HIV pathogenesis. It is not surprising based on these findings that there is even an international conference devoted solely to this molecule. With so much interest in this sugar, it is understandable that through the course of this work, a number of studies have appeared which have corroborated our findings and has extended these lines of research even further.

One of the main limitations of this and the previous binding studies (Jennings *et al.*, 1985; Kabat *et al.*, 1988)) was the use of pentameric IgM antibodies which have 10 binding sites. This automatically invokes the possibility that all antibody- antigen interactions may reflect not only the intrinsic affinity of the antibody but also added effects due to avidity. In our study, we have minimized this prospect by using a direct binding assay unlike the inhibition analyses where small oligosaccharides may not have the ability to displace a larger substrate that displays multipoint attachment. We have not however, eliminated the possibility that

avidity does not play a role. The work of Hayrinen *et al.*, (1989), unequivocally demonstrates that indeed an antibody specific to GBMP requires an unusually large epitope for binding, and binding increases as the size of the polymeric Neu5Ac chain increases. That this binding is not a measure of avidity but of true affinity is presupposed since they used a Fab fragment (a portion of an antibody with only 1 binding site) which by definition can only be related to an intrinsic interaction. Comparison of the Fab fragment and the parent antibody showed that each bound oligomeric Neu5Ac to the same extent indicating that the parent antibody was unable to bind the oligosaccharides in a multivalent manner. Recently, an elegant experiment was performed which studied the interaction of sialyloligosaccharides of increasing size with a Fab fragment, using titration microcalorimetry (B. Sigurskold, submitted for publication). Not only were they able to determine true binding constants (K_A) but they were able to measure directly enthalpic parameters leading to a complete description of the binding process. They found that indeed binding with the Fab fragment increased up to ~17 residues in length at which point the binding curve began to plateau. They estimate the minimum size of the epitope for 90% binding to be in the 10-12 residue range as compared to the more classical 5-6 residues found in other carbohydrate- antibody binding systems. One additional parameter found, interestingly enough, was that a polysaccharide of ~40 residues was needed before a second Fab fragment was found to bind. In other words, it appears as if a 40 residue homopolymer of Neu5Ac contains only two functional epitopes. Both of these studies support our direct binding results which seemed to suggest that

binding to the H.46 antibody increased with increasing size of the Neu5Ac repeat unit up to at least a 15-mer.

Since the inception of the conformational epitope theory, a number of studies have recently appeared which attempt to relate the three dimensional structure of the polysaccharide with its binding properties. The first comprehensive conformational study (Michon *et al.*, 1987) suggested that at least a decasaccharide was needed for binding, since the outer two residues at each end of the polymer were needed to stabilize the interior 6 residues which serves as the epitope. Yamasaki and Bacon (1991), based on a combination of quantitative 2D NOE and rigid molecular modeling analyses, have proposed that the polysaccharide exists as a helix in solution and attributes the lack of binding with smaller oligosaccharides with their inability to exist in this helical state. The most current interpretation of the unique conformation of the polysaccharide suggests that the rigid model approach used in the two prior studies is not valid. Brisson and coworkers (1992) have studied the conformation of the polysaccharide from a dynamic point of view and concluded that it exists primarily as random coils with the ability to form local extended helices. It is thought that antibodies raised to the polysaccharide are able to recognize only the extended helical conformers of the polysaccharide (n=9) due to clonal deletion of the genes which would normally encode for antibodies capable of binding to shorter conformers of poly Neu5Ac. This is explained by the fact that short Neu5Ac chains are present in normal tissue and antibodies to these structures would result in an autoimmune process. Obviously, these hypotheses are inferred from

data accumulated through NMR and theoretical calculations of the polysaccharide alone and are subject to a large degree of uncertainty. Studies are under way to try and 'visualize' the conformation of the polysaccharide while bound to the antibody. The ultimate goal would be the X-ray crystallography of the antibody- antigen complex. However, efforts to co-crystalize the Ab-Ag complex have not yet met with success despite the successful crystallization of the Fab fragment alone (D. Bundle, personal communication). An alternate study to determine the bound conformation of the Neu5Ac homopolymer is underway using the technique of transferred NOE's. This is accomplished by detecting specifically the carbohydrate as it dissociates from the antibody since, during this time frame, the carbohydrate reflects the conformation of its bound state.

A number of NMR conformational studies have since been performed on some of the colominic acid analogs outlined in chapter 2. Two interesting results stemmed from the carboxyl reduced version of colominic acid and N-glycolyl colominic acid. It appears from the conformational analysis of the reduced material, that the polysaccharide exists in a random coil state without much probability of forming extended ordered three dimensional structures. Although this result tentatively explains the lack of antibody binding to the reduced polymer, it does not rule out the possibility that the lack of binding is due to the loss of a key determinant involved in the combining site. The N-glycolyl polysaccharide however, behaves conformationally in a manner similar to the group B polysaccharide yet it does not bind to antibodies (H.46 IgM- Sarff *et al.*, 1975; IgM^{NOV}- Kabat *et al.*, 1986; Frosch *et al.*, 1985) specific to the B

polysaccharide. This certainly suggests that the hydrophilic nature of the N-acyl side chain is responsible for the lack of antibody binding due probably to a complementarity mismatch. These two group B meningitidis and *E. coli* K1 capsular polysaccharide analogs have been conjugated to a protein carrier rendering them immunogenic. Work is currently in progress to evaluate the serology of these two analogs. A comparative study is also underway to try to explain the differences in immunogenicity between the group B and C meningitidis capsular polysaccharides by NMR and molecular modeling.

It was previously mentioned that the N-propionyl derivative of group B meningitidis and *E. coli* K1 (colominic acid) capsular polysaccharide has circumvented the poor immunogenicity of the parent homopolymers raising protective antibodies against the *Neisseria meningitidis* bacterium (Jennings *et al.*, 1986; 1989). It has been found interestingly enough that these protective antibodies are directed against an unknown structure found on the bacteria and not towards the capsular polysaccharide alone since they only poorly cross react with the native polysaccharide. This vaccine is currently moving towards monkey trials and will potentially be evaluated in humans in 1993. Monoclonal antibodies, that are protective against the *Neisseria meningitidis* bacterium and are specific only to the propionyl derivative, are being evaluated for cross reaction to tissues antigens by histochemical staining.

The controversial nature surrounding the group B vaccine is being evaluated in a pregnant monkey study. Since polysialic acid expression is highest in developing fetuses, the effects of vaccinating

pregnant monkeys and evaluating any potential harmful effects to either the mother or its offspring should clarify somewhat this ongoing debate (H. Jennings, personal communication).

Work is also in progress to generate specific monoclonal antibodies to terminal α-linked N-acetylneuraminic acid which to date has not been successfully accomplished. This work is based on the formation of sialyloligosaccharide-TT conjugates using the novel Michael addition technique. Based on our experiences, a specific monoclonal antibody would be extremely useful as a diagnostic tool in both tissue screening and as a means for evaluating novel sialic acid containing glycoconjugates.

Appendix A

Resorcinol test for total sialic acid (Svennerholm, 1957)

Reagents:

1) 2% w/v resorcinol in water stored at 4 °C.
2) 0.1M copper (II) sulfate.
3) Resorcinol reagent: 10 ml of 2% resorcinol in water is mixed with 80 ml of concentrated HCl, and 0.25 ml of 0.1M $CuSO_4$ solution. The volume is made up to 100 ml with distilled water and stored at 4 °C.
4) Extraction solvent is composed of 85:15 v/v butyl acetate:n-butanol.

Standard:

O.1 mg/ml solution of N-acetylneuraminic acid in distilled water.

Unknown samples (containing 10-70 ug sialic acid) are placed in pyrex tubes and the volume made to 1 ml with water. Appropriate volumes of the standard solution (0-100 ug sialic acid) are made to a final volume of 1 ml with distilled water. Resorcinol reagent (1 ml) is added to each tube, followed by vortexing and heating at 100 °C for 15 minutes. After cooling, 2 ml of extraction solvent is added to each tube, vortexed for 30 seconds, and allowed to settle for 15 minutes. A sample of the organic phase is removed and the absorbance is read at 580 nm with blank correction.

Ninhydrin test for primary amines

Reagents:

1) 2% w/v ninhydrin in 4% aqueous pyridine. The reagent should be stored at 4 °C.

Standard:

1 uM/ml solution of N-acetylglucosamine in water.

Unknown samples (0.1-0.8 uM/ml) are made to a 1 ml final volume in pyrex tubes. Ninhydrin reagent (1 ml) is added to the unknown solutions and to a series of known standard concentrations. The tubes are heated at 100 °C for 15 minutes and the dark blue solutions are diluted with 5 ml of distilled water. The absorbance is read at 570 nm against an appropriate blank.

TNBS assay for hydrazides (Lee, 1978)

Reagents:

1) 0.2% w/v solution of 2,4,6-trinitrobenzenesulfonic acid (TNBS) in water.
2) 0.2M solution of sodium borate buffer (pH 8.7).

Standard:

1 uM/ml solution of butanoic hydrazide in water.

To 0.5 ml of a sample solution (0.02-0.1 umol of hydrazide) and the respective standard solutions, is added 0.5 ml of borate buffer and 0.5 ml of TNBS reagent. The solutions are allowed to sit at room temperature for 30 minutes while the orange coloured chromophore develops. The solution are diluted with 2 ml of water and the absorbances are read at 500 nm.

Dubois test for total reducing sugars (Dubois *et al.*, 1956)

Reagents:

1) 5% v/v solution of phenol in water.

Standard:

0.1 mg/ml solution of galactose in water.

Standard solutions of galactose (0-20 ug) are made to a final volume of 200 ul with water. Unknown solutions are also made to a final 200 ul volume. 5% phenol solution (200 ul) is added to each sample. While vortexing, concentrated H_2SO_4 (1 ml) is added quickly to the center of the tube. The absorbance of the orange chromophore is read at 490 nm.

Park-Johnson assay for reducing sugars (Park & Johnson, 1949)

Reagents:

1) 0.05% w/v solution of potassium ferricyanide ($K_3Fe(CN)_6$ in water.
2) 0.065% w/v KCN in 0.05M Na_2CO_3.
3) 1.5 gm ferric ammonium sulfate $(NH_4)Fe(SO_4)_2.nH_2O$ in a 0.1% w/v solution of SDS in 0.05N H_2SO_4.

Standard:

0.1% w/v solution of N-acetylneuraminic acid in water.

Unknown sample and standard solutions (0-30 ug Neu5Ac) are made to a volume of 1 ml. Reagent 1 (1 ml) and reagent 2 (1 ml) are added to the samples and heated at 100 °C for 15 minutes. After

cooling, reagent 3 (5 ml) is added and the samples vortexed gently and allowed to sit at room temperature for 15 minutes. The absorbance of the light green chromophore is read at 690 nm.

Lowry method for protein determination (Lowry *et al.*, 1951)

Reagents:

1) 2% w/v solution of Na_2CO_3 in 0.1M NaOH.
2) 0.5% w/v $CuSO_4.5H_2O$ in 1% sodium tartrate prepared fresh before use.
3) Mixture of reagent 1 (50 ml) with reagent 2 (1 ml).
4) Folin reagent (2N) diluted 1:1 with water.

Standard:

1 mg/ml solution of gamma globulin in 0.1M NaOH.

Reagent 3 (1 ml) is added to protein samples (10-80 ug) in 0.1M NaOH and vortexed. The mixtures are allowed to sit for 10 minutes at room temperature followed by the addition of reagent 4 (100 ul). The samples are vortexed and allowed to sit at room temperature for 30 minutes, followed by measurement of the absorbance at 750 nm.

Appendix B- Claims to Original Research

1. Improved method for the isolation of sialic acid oligosaccharides by anion exchange chromatography using a pyridine acetate gradient buffer system.
2. Optimization of hydrolytic conditions for the generation of sialic acid oligosaccharides (Neu5Ac and Neu5Gc) of desired degrees of polymerization.
3. The synthesis and characterization of novel colominic acid analogs outlined as follows:

   N-acryloyl colominic acid (**2-11**)

   N-propionyl colominic acid via reduction of **2-11** (**2-12**)

   N-(3-thiomethyl propionyl) colominic acid (**2-13**)

   N-(3-aminomethyl propionyl) colominic acid (**2-14**)

   N-formyl colominic acid (**2-7**)

   N-isobutanoyl colominic acid (**2-8**)

   N-chloroacetyl colominic acid (**2-9**)

   N-bromoacetyl colominic acid (**2-10**)

   O-acetoxy-N-glycolyl colominic acid (**2-18**)

   N-glycolyl colominic acid (**2-15**) via (1) acylation of **2-1** with acetoxyacetyl chloride followed by NaOH hydrolysis. (2) Reductive ozonolysis of **2-11**.

   Carboxyl reduced colominic acid (**2-21**)

   Colominic acid hydrazide (**2-22**)

   Colominic acid hydroxylamine (**2-23**)
4. Synthesis and characterization of a novel heterobifunctional spacer (**3-6**) and derived methacrylamide copolymers.

5. New conjugation methodology introduced includes:

   - An improved method for the direct reductive amination of terminal ketoses onto amino groups of proteins and methacrylamide copolymers.
   - Elucidation of a novel conjugation method involving a Michael-type addition onto amino functionalities of proteins.

6. The synthesis and characterization of a number of novel sialylglycoconjugates as follows:

   - N-glycolyl colominic acid- tetanus toxoid or bovine serum albumin conjugates.
   - Neu5Ac oligosaccharides and polysaccharide- tetanus toxoid , IgG, or BSA conjugates via Michael addition of the appropriately derivatized sialylated substrate.
   - A novel multi point protein conjugate of colominic acid via Micheal addition of amino groups onto 15% acryloylated colominic acid (**4-38**).

7. Preliminary studies investigating the direct binding of multivalent Neu5Ac oligosaccharides with horse H.46 IgM antibody show that binding increases with increasing degree of polymerization of the Neu5Ac oligosaccharide.

8. Preliminary studies mapping the antibody combining site of the H.46 antibody suggest a hydrophobic binding pocket in the antibody combining site which accommodates hydrophobic substituents of colominic acid at the N-acetyl position.

Appendix C- Publications Arising From This Research

Publications arising from this research

1) Roy, R., and Pon, R. (1990) "Efficient Synthesis of α-(2→8) Linked N-Acetyl and N-Glycolylneuraminic Acid Disaccharides from Colominic Acid", *Glycoconjugate J.* , 7, 3-12.

2) Abbas, s., Sugiyama, S., Diakur, J., Pon, R., and Roy, R. (1990) "Synthesis of α- and β-Methyl Neu5Ac α-(2→8) Neu5Ac Disaccharides", *J. Carbohydr. Chem.*, 9(6), 891-901.

3) Roy, R., Pon, R., Tropper, F., and Andersson, F. (1992) "Michael Addition of Poly-L-Lysine to N-Acryloylated Sialosides. Syntheses of Influenza A Virus Hemagglutinin Inhibitor and Group B Meningococcal Polysaccharide Vaccines", accepted for publication in *J. Chem. Soc., Chem. Commun.*

4) Roy, R., Pon, R., and Gamian, A., " Immunogenicities of Synthetic Sialic Acid Glycoconjugates", manuscript in preparation.

5) Roy, R., Laferriere, C., Pon, R., Boratynski, J., and Cousineau, L. (1990) "Synthetic Studies Related To Artificial Sialic Acid Conjugates" Proceedings from the XVI IUPAC International Carbohydrate Symposium, Yokohama, Japan.

References

Abbas. S., Sugiyama, S., Diakur, J., Pon, R., and Roy, R. (1990) *J. Carbohydr. Chem.*, **9**, 891-901.

Abraham, R., and Loftus, P. (1983) in *Proton and Carbon-13 NMR Spectroscopy* p. 25, John-Wiley & Sons, New York.

Alkan, S., Bush, M., Nitecki, D., and Goodman, J (1972) *J. Exp. Med.*, **136**, 387-391.

Amano, J., Sato, S., Nishimura, R., Mochizuki, M., and Kobata, A. (1989) *J. Biochem.*, **105**, 339.

Anderson, P. (1988) U.S. Patent 4,762,713.

Anderson, P., Pichichero, M., and Insel, R. (1985) *J. Clin. Invest.*, **76**, 52-59.

Aronson, L., and Peters, M. (1972) *Carbohydr. Res.*, **47**, 345-353.

Artenstein, M., Gold, R., Zimmerly, J., Wyle, F. Schneider, H., and Harkins, C. (1970) *N. Engl. J. Med.*, **282**, 417-420.

Avery, O., and Goebel, W. (1931a) *J. Exp. Med.*, **54**, 431-436.

Avery, O., and Goebel, W. (1931b) *J. Exp. Med.*, **54**, 437-447.

Bailey, K., and Butterfield, G. (1981) *Can. J. Chem.*, **59**, 641-646.

Bayer, E., Ben-Hur, H., and Wilchek, M. (1988) *Anal. Biochem.*, **170**, 271-281.

Berman, E., Brown, J., Lis, H., and Sharon, N. (1985) *Eur. J. Biochem.*, **152**, 447-451.

Berzofsky, J., and Berkower, I. (1984) in *Fundamental Immunology* (Paul, W., Ed.) p 595, Raven Press, New York.

Beuvery, E., Miedema, F, van Delft, R., and Haverkamp, J. (1983) *Infect. Immun.*, **40**, 39-45.

Beuvery, E., Roy, R., Kanhai, V., and Jennings, H. (1986) *Dev. Biol. Standard* , **65**, 197-204.

Bhattacharjee, A., Jennings, H., and Kenny, P., Martin, A., and Smith, I. (1975) *J. Biol. Chem.* , **250**, 1926.

Bizzini, B., Blass, J., Turpin, A., and Raynaud, M. (1970) *Eur. J. Biochem.* , **17**, 100-105.

Black, W., Munoz, J., Peacock, M., Schad, P., Cowell, J., Burchall, J., Lim, M., Kent, A., Steinman, L., and Falkow, S. (1988) *Science* , **240**, 656-659.

Boullanger, P., Andre, C., and Descotes, G. (1985) *J. Carbohydr. Chem.* , **4**, 279-291.

Brisson, J., Baumann, H., Imberty, A., Perez, S. and Jennings, H. (1992) *Biochemistry* , **31**, 4996-5004.

Chen, F., Lee, Y., Steinauer, R., and Benoiton, L. (1987) *Can. J. Chem.* , **65**, 619.

Cheng, A., and Carlson, G. (1983) *Anal. Biochem.* , **134**, 505-511.

Cho, S., Fuijiwara, T., Hunter, S., Rea, T., Gelber, R., and Brennan, P. (1984) *J. Infect. Dis.* , **150**, 311-322.

Cisar, J., Kabat, E., Dorner, M., and Liao, J. (1975) *J. Exp. Med.* , **142**, 435.

Conrad, H., and Taylor, R. (1972) *Biochemistry* , **11**, 1383.

Czarnieki, M., and Thorton, E. (1977) *J. Am. Chem. Soc.* , **99**, 8273-8282.

Diakur, J., and Roy, R. (1991) *J. Carbohydr. Chem.* , **10**, 947-957.

Dick, W., and Beurret, M. (1989) in *Contributions to Microbiology and Immunology* (Cruse, J., and Lewis, R., Ed.) vol. **10**, pp 48-114, Basel, Karger.

Dubois,H., Gilles, K., Rebers, P., and Smith, F. (1956) *Anal. Chem.*, **28**,350-356.

Engvall, E., and Perlmann, P. (1971) *Immunochemistry* , **8**, 871.

Feizi, T. (1985) *Nature* , **314**, 53.

Fillet, H., Blake, M., MacDonald, C., and McCarthy, M. (1988) *J. Exp. Med.* , **168**, 971.

Finne, J., and Makela, H. (1985) *J. Biol. Chem.* , **260**, 1265-1270.

Finne, J., Bitter-Suermann, D., Goridis, C., and Finne, U. (1987) *J. Immunol.*, **138**, 4402-4407.

Frosch, M., Gorgen, I., Boulnois, G., Timmis, N., and Bitter-Suermann, D. (1985) *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 1194-1198.

Fukui,Y., Maru, M., Ohkawara, K., Osada, Y., Wang, D., Ito, T., Higashi, H., Naiki, M., Wakamiya, N., and Kato, S. (1989) *Biochim. Biophys. Res. Commun.* , **160**, 1149-1154.

Glaudemans, C. (1991) *Chem. Rev.* , **91**, 25.

Goldstein, I., and Poretz, R. (1986) in *The Lectins, Properties, Functions, and Applications in Biology and Medicine* (Liener, I., Sharon, N., and Goldstein, I., Eds.) p 103, Academic Press, Florida.

Gotschlich, E., Fraser, B., Nishimura, O., Robbins, J., and Liu, T. (1981) *J. Biol. Chem.* , **256**, 8915.

Gotschlich, E., Goldschneider, I., and Artenstein, M. (1969) *J. Exp. Med.* , **129**, 1367-1384.

Grados, O. and Ewing, W. (1970) *J. Infect. Dis.* , **122**, 100-103.

Gray, G. (1974) *Arch. Biochem. Biophys.* , **163**, 426-428.

Hakomori, S. (1984) *Ann. Rev. Immunol.* , **2**, 103-126.

Hakomori, S. (1985) *Cancer Res.* , **45**, 2405-2414.

Hanai, N., Dohi, T., Nores, G., and Hakomori, S. (1988) *J. Biol. Chem.*, **263**, 6296-6301.

Harwood, J. (1992) *TIBS*, **17**, 203.

Hayrinen, J., Bitter-Suermann, D., and Finne, J. (1989) *Molec. Immun.*, **26**, 523-529.

Heilmann, S., and Smith, H. (1979) *J. Appl. Poly. Sci.*, **24**, 1551-1564.

Higashi, H. (1990) *Trends in Glycoscience & Glycotechnology*, **2**, 7-15.

Higashi, H., Naiki, M., Matuo, S., and Okouchi, K. (1977) *Biochim. Biophys. Res. Commun.*, **79**, 388-395.

Hiranano, S., and Yagi, Y. (1980) *Carbohydr. Res.*, **83**, 103-108.

Hoare, D., and Koshland, D, (1967) *Carbohydr. Res.*, **242**, 2447.

Hoffman, W., and O'Shannessy, D. (1988) *J. immunol. Meth.*, **112**, 113-120.

Hurn, B., and Chantler, S. (1980) *Meth. Enzymol.*, **70**, 104.

Inoue, S., and Inoue, Y. (1986) *J. Biol. Chem.*, **261**, 5256-5261.

Iwasaki, M., Inoue, S., and Troy, F. (1990) *J. Biol. Chem.*, **265**, 2596-2602.

Jennings, H. (1990) in *Current Topics in Microbiology and Immunology*, vol. **150**, pp 97-127, Springer-Verlag, Berlin.

Jennings, H., and Lugowski, C. (1985) *J. Immunol.*, **127**, 1011.

Jennings, H., and Roy, R. (1985) in *Pathogenic Neisseriae* (Schoolnik, G., Ed.) Proceedings of the Fourth International Symposium (American Society for Microbiology) pp 628-632

Jennings, H., Gamian, A., Michon, F., and Ashton, F. (1989) *J. Immunol.*, **142**, 3585-3591.

Jennings, H., Katzenellenbogen, E., Lugowski, C., Michon, F., Roy, R., and Kasper, D. (1984) *Pure Appl. Chem.*, **56**, 893-905.

Jennings, H., Lugowski, C., and Ashton, F. (1983) *Infect. Immun.*, **43**, 407-412.

Jennings, H., Roy, R., and Gamian, A (1986) *J. Immunol.* , **137**, 1708-1713.

Jennings, H., Roy, R., and Michon, F. (1985) *J. Immunol.* , **134**, 2651-2657.

Johnson, L., and Jankowski, W. (1972) in *Carbon-13 NMR Spectra* , Jonh-Wiley and Sons, New York.

Johnstone, A., and Thorpe, R. (1982) in *Immunochemistry in Practice* (Johnstone, A. and Thorpe, R., Eds.) Blackwell Scientific Publications, Oxford.

Kabat, E., and Schiffman, G. (1962) *J. Immunol.* , **88**, 782.

Kabat, E., Liao, J., Osserman, E., Gamian, A., Michon, F., and Jennings, H. (1988) *J. Exp. Med.* , **168**, 699-711.

Kabat, E., Nickerson, J., Liao, Grossbard, L., Osserman, E., Glickman, E., Chess, L., Robbins, J., Schneerson, R., and Yang, Y. (1986) *J. Exp. Med.* , **164**, 642.

Kabat. E. (1960) *J. Immunol.* , **84**, 82-85.

Kaczmar, U., and Traser, S. (1976) *Makromol. Chem.* , **177**, 1981-1989.

Kadin, S. (1965) *J. Org. Chem.*, **31**, 620.

Kaijser, B., Larsonn, P., Olling, P., and Schneerson, R. (1983) *Infect. Immun.* , **39**, 142-146.

Kallin, E., Lonn, H., Norberg, T., and Elofsson, M (1989) *J. Carbohydr. Chem.* , **8**, 597-611.

Kasper, D. (1986) *J. Infect. Dis.* , **153**, 407-415.

Kasper, D., Winkelhake, J., Zollinger, W., Brandt, B., and Artenstein, M. (1973) *J. Immunol.* , **110**, 262-268.

Kimura, K., Arata, Y., Yasuda, T., Kinosita, K., and Nakanishi, M. (1990) *Immunology* , **69**, 323-328.

Kitazume, S., Kitajima, K., Inoue, S., and Inoue, Y. (1992) *Anal. Biochem.* , **202**, 25-34.

Kronis, A., and Carver, J. (1985) *Biochemistry* , **24**, 834.

Laferriere, C. (1990) PhD Thesis.

Landsteiner, C. (1917) *J. Exp. Med.* , **59**, 751.

Lee, Y. C., (1978) *J. Carbohydr. Res.* , **67**, 509-514.

Lemieux, R., Bundle, D., and Baker, D. (1975) *J. Am. Chem. Soc.* , **97**, 4076.

Lifely, M., and Esdaile, J. (1991) *Immunology* , **74**, 490-496.

Lifely, M., Moreno, C., and Lindon, J. (1987) *Vaccine* , **5**, 11-26.

Lifely, R., and Moreno, C. (1986) *Lancet* , **2**, 214.

Lifely, R., Gilbert, A., and Moreno, C. (1981) *Carbohydr. Res.* , **94**, 193.

Lifely, R., Gilbert, A., and Moreno, C. (1984) *Carbohydr. Res.* , **134**, 229.

Lifely, R., Norwicka, U., Krambovitis, E., and Esdaile, J. (1987) in *Gonococci & Meningococci* (Poolman, J., Zanen, T., Meyer, f., Heckels, J., Makela, H., Smith, H., and Beuvery, E., Ed.) p 147, Kluwer Academic Publishers, Dobrecht.

Likhosherstov, L., Novikova, S., Derevitskaja, V., and Kochetkov, K. (1986) *Carbohydr. Res.* , **146**, C1-C5.

Lin, K., and Lee, C. (1982) *Immunology* , **46**, 333-342.

Lindon, C., Vinter, J., Lifely, R., and Moreno, C. (1984) *Carbohydr. Res.* , **133**, 59-74.

Liu, T., Gotschlich, E., Dunne, F., and Jonssen, E. (1971) *J. Biol. Chem.*, **246**, 4703-4712.

Loomes, M., Uemura, K., Childs, R., Paulson, J., Rogers, G., Scudder, P., Michalski, J., Hounsel, E., Taylor-Robinson, D., and Feizi, T. (1984) *Nature*, **307**, 560.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951) *J. Biol. Chem.*, **193**, 265-275.

March, J. (1977) in *Advanced Organic Chemistry, Reaction, Mechanism, and Structure* p. 382, 2nd ed., McGraw-Hill, New York.

Michon, F., Brisson, J., and Jennings, H. (1987) *Biochemistry*, **26**, 8399-8405.

Moreno, C., Lifely, R., and Esdaile, J. (1985) *Infect. Immun.*, **47**, 527.

Nomoto, H., Iwasaki, M., Endo, T., Inoue, S., Inoue, Y., and Matsumura, G. (1982) *Arch. Biochem. Biophys.*, **218**, 335-341.

Okamoto, K., Kondo, T., and Goto, T. (1986) *Tetrahedron Letters*, **27**, 5229-5232.

Okamoto, K., Kondo, T., and Goto, T. (1988) *Tetrahedron*, **4**, 1291-1298.

Ouchterlony, O., and Nilsson, L. (1978) in *Handbook of Experimental Immunology* (Weir, D., Ed.) ch. 19, Blackwell Scientific Publications, Oxford.

Pappenheimer, A., Uchida, T., and Harper, A. (1972) *Immunochemistry*, **9**, 891-906.

Park, J., and Johnson, M. (1949) *J. Biol. Chem.*, **181**, 149-151.

Paul, W., Katz, D., and Benacerraff, B. (1971) *J. Immunol.*, **107**, 685-688.

Paulson, J. (1985) in *The Receptors* (Conn, M., Ed.) vol. 2, pp 131-219, Academic Press, Orlando.

Petrov, R. (1985) *Vaccine* , 3, 391.

Porro, M. (1987) in *Towards Better Carbohydrate Vaccines* (Bell, R., and Torrigiani, G., Eds.) pp 279-305, Wiley, Chichester.

Porro, M., Constantino, P., Giovannoni, F., Pellegrini, V., Tagliaferri, L., Vannozzi, F., and Viti, S. (1986) *Molec. Immun.* , **23**, 385-391.

Porro, M., Constantino, P., Viti, S., and Saletti, M. (1983) *Med. Trop.* , **43**, 129-132.

Pratt, P., and Bruice, T. (1972) *J. Org. Chem.* , **37**, 3563.

Ravindranath, M., Higa, H., Cooper, E., and Paulson, J. (1985) *J. Biol. Chem.* , **260**, 8850.

Roelcke, D. (1984) *Funkt. Biol. Med.* , **3**, 106-127.

Rougon, G., Dubois, C., Buckley, N., Magnani, J., and Zollinger, W. (1986) *J. Cell Biol.* , **103**, 2429-2437.

Roy, R, Katzenellebogen, E., and Jennings, H. (1984a) *Can. J. Biochem. Cell Biol.*, **62**, 270-275.

Roy, R, Williams, R., and Jennings, H. (1984b) *Carbohydr. Res.* , **127**, 165.

Roy, R. and Laferriere, C. (1990a) *J. Chem. Soc. Chem. Commun.* , 1709-1711.

Roy, R., and Boratynski, J. (1990) *Biotech. Letters* , **12**, 347-352.

Roy, R., and Laferriere, C. (1988) *Carbohydr. Res.* , **177**, C1.

Roy, R., and Laferriere, C. (1990b) *Can. J. Chem.* , **68**, 2045-2054.

Roy, R., and Pon, R. (1990) *Glycoconjugate J.* , **7**, 3-12.

Roy, R., and Tropper, F. (1988a) *Glycoconjugate J.* , **5**, 203-206.

Roy, R., and Tropper, F. (1988b) *J. Chem. Soc. Chem. Commun.* , 1057-1060.

Roy, R., Laferriere, C., Gamian, A., and Jennings, H. (1987) *J. Carbohydr. Chem.*, **6**, 161-165.

Roy, R., Laferriere, C., Pon, R., Boratynski, J., and Cousineau, L. (1990) *Synthetic studies related to artificial sialic acid conjugates* , Proceedings from the XVI International Carbohydrate Symposium, Yokohama, Japan, Aug. 16-23 1990.

Roy, R., Tropper, F., Morrison, T., and Boratynski, J. (1991) *J. Chem. Soc. Chem. Commun.* , 536-538.

Roy, R., Tropper, F., Romanowska, A., Letellier, M., Cousineau, L., Meunier, S., and Boratynski, J. (1991) *Glycoconjugate J.* , **8**, 75-81.

Sabesan, S., and Paulson, J. (1986) *J. Am. Chem. Soc.* , **108**, 2068.

Sarff, L., McCracken, G., Schiffer, M., Glode, M., and Robbins, J. (1975) *Lancet*, **1**, 1099-1104.

Saukkonen, K., Haltia, M., Frosch, M., Bitter-Suermann, D., and Leinonen, M. (1986) *Microb. Pathogen.* , **1**, 101-105.

Schauer, R. (1982b) in *Advances in Carbohydrate Chemistry and Biochemistry* (Tipson, R. and Horton, D., Eds.) vol. **40**, pp 131-234, Academic Press, New York.

Schauer, R., and Corfield, A. (1982) in *Sialic Acids: Chemistry, Metabolism, and Function* (Schauer, R., Ed.) vol. **10**, p. 52, Springer-Verlag, Wien.

Schwartz, B., and Gray, G. (1977) *Arch. Biochem. Biophys.* , **181**, 542-549.

Sigurskjold, B., Altman, E., and Bundle, D. (1991) *Eur. J. Biochem.* , **197**, 239-246.

Silverstein, R., Bassler, C., and Morill, T., (1974) in *Spectrometric Identification of Organic Compounds* , 2ed., John-Wiley & Sons, New York.

Skivakis, L., Frasch, C., Zahradnik, J., and Dolin, R. (1984) *J. Infect. Dis.* , **149**, 387.

Smith, D., and Unruh, C. (1957) *J. Org. Chem.* , **23**, 301-303.

Stoll, M., Mizuochi, T., Childs, R., and Feizi, T. (1988) *Biochem. J.* , **256**, 661-664.

Svennerholm, L. (1957) *Biochim. Biophys. Acta.* , **24**, 604.

Svennerholm, L. (1958) *Nature,* **181**, 1154-1155.

Tang, P., Gooi, H., Hardy, M., Lee, Y., and Feizi, T. (1985) *Biochim. Biophys. Res. Commun.* ,**132**, 474-480.

Troy, F. (1992) *Glycobiology* , **2**, 5-23.

Tsai, C.M., Zopf, D., Yu, R., Wistar, R., and Ginsburg, V. (1977) *Proc. Natl. Acad. Sci.* U.S.A., **74**, 4591-4594.

Ward, J. (1991) *Vaccine* , **9**, S-17.

Weigel, P., Schnaar, R., Roseman, S., and Lee, Y. (1982) *Meth. Enzymol.* , **83**, 294.

Yamasaki, R., and Bacon, B. (1991) *Biochemistry* , **30**, 851-857.

Zon, G., and Robbins, J. (1983) *Carbohydr. Res.* , **114**, 103-121.

Abstract

This work describes the synthesis of a number of *Neisseria meningitidis* serogroup B or *E. coli* K1 (colominic acid) capsular polysaccharide analogs including the α-(2$\rightarrow$8) homopolymer of N-glycolylneuraminic acid. This represents the first ever synthesis of this biologically relevant polysaccharide. These analogs were used to probe the fine specificities of a polyclonal equine IgM (H.46) antiserum specific to the capsular polysaccharide of *Neisseria meningitidis* serogroup B by a combination of double immunodiffusion and immunoprecipitation analyses. It was determined that the carboxylic acid terminus of colominic acid is immunodominant and any structural changes at this functionality abolishes antigenicity completely. Structural changes of the N-acetyl group are tolerated by the H.46 antiserum providing the substituent is hydrophobic and sterically compact. These results suggest that the carboxylic acid terminus is either involved directly in the antibody combining site or functions to maintain the polysaccharide in a required conformation. They also suggest that the N-acetyl functionality is required in a hydrophobic interaction with complementary amino acids in the combining site of the H.46 antibodies.

This work also describes the optimization and isolation of both N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) oligosaccharides from their parent homopolymers. In this way, appreciable amounts of the rare and biologically important monosaccharide of N-glycolylneuraminic acid (Hanganutziu-Diecher antigen) and disaccharides of N-acetyl and N-glycolylneuraminic acid

were isolated. Macromolecular forms of a series of Neu5Ac oligosaccharides allowed the determination, through a direct ELISA binding study, that the H.46 antibody requires an unusually large epitope (> 15 Neu5Ac residues) for maximum binding. These results parallel results obtained through indirect methods (Jennings and coworkers, 1985; Kabat and coworkers, 1988) and suggests the required epitope is conformational in nature.

A novel heterobifunctional linker incorporating reactive hydrazide and N-acryloyl functionalities was synthesized in good overall yield. The usefulness of this linker was demonstrated by effecient reductive amination of sialyloligosaccharides with the hydrazide portion of the linker. The versatility of the N-acryloyl group was demonstrated by the radical copolymerization of the linker with methacrylamide to form random polymers with pendant hydrazide chains. Sialyloligosaccharides were covalently coupled to these pendant chains to form neo-sialylglycopolymers.

Finally, methods were improved or developed to efficiently couple sialic acid mono-, oligo-, and polysaccharides to macromolecules such as methacrylamide based copolymers or proteins. A novel method of conjugation involving a Michael-type addition of amines or hydrazides onto suitably derivatized N-acryloylated sialyloligo-/ polysaccharides is described. Improved methods for the direct reductive amination of the reducing ketose of sialyloligosaccharides to proteins or methacrylamide copolymers are also discussed.