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SYNTHETIC ANALOGUES OF SIALIC ACID AND THEIR BIOLOGICAL APPLICATION

by

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ABSTRACT

SYNTHETIC ANALOGUES OF SIALIC ACID AND THEIR BIOLOGICAL APPLICATION

by

BAO-JEN SHYONG

2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Neu5Ac2en) has been synthesized from N-acetylneuraminic acid (Neu5Ac) by a simple, quantitative, one-step conversion. Neu5Ac was dehydrated by peracetylation with trifluoroacetic anhydride (TFAA) in the presence of a catalytic amount of N,N-dimethyl-4-aminopyridine (DMAP). 2,3-Dehydro-2-deoxy-neuraminic acid (Neu2en) was also obtained when the ratio of TFAA/DMAP was changed to 4:3. Seven other sialic acid derivatives were prepared incorporating the half-chair conformation of Neu5Ac2en such as 2,3-epoxy-2-deoxy-N-acetylneuraminic acid, N-azidoacetyl neuraminic acid, and a substitution of N-acyl or 9-hydroxyl on Neu5Ac2en.

These derivatives were investigated for their ability to inhibit *Vibrio cholerae* sialidase using 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4MU-Neu5Ac) as the substrate. In a kinetic study, seven of the eight analogues of Neu5Ac2ene were shown to be competitive inhibitors with K_i values ranging from 10^{-3} M to 10^{-5} M. No inhibition was observed for the epoxide-Neu5Ac2ene compound .

An affinity chromatography method has been developed for separating *V. cholerae* neuraminidase from physiological protein mixtures. The affinity column was made by

coupling 2-deoxy-2,3-dehydro-N-deacetyl neuraminic acid (Neu2ene) to Bio-Rio Affi-Gel 10 activated agarose gel. Bound neuraminidase selectivly absorbed from a mixture of proteins was eluted from the column with 10% neuraminic acid in H_2O. The total activity of the recovered neuraminidase from the affinity column was evaluated using the 4MU-Neu5Ac assay, and the peroxidase-amplified assay. As judged by activity, 80-100% of the total activity applied to the column was recovered. The physical properties of the neuraminidase was examined by electrophoresis. Enzyme samples analyzed before and after the affinity chromatography appeared as 68 kDa and 16 kDa polypeptide bands while the molecular weight of *V. cholerae* neuraminidase has been previously reported as 24.3 kDa or 90 kDa polypeptides.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

A. INTRODUCTION

Sialic acids (neuraminic acids) are important components of the carbohydrate chains of glycoproteins and glycolipids, where they usually occur on the non-reducing termini of these molecules. The sialic acids are complex acidic sugars derived from neuraminic acid, a 5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid. The most common member of the sialic acid family is N-acetylneuraminic acid (Neu5Ac) (Fig. 1A). Because sialic acids occur on cell-surface glycoproteins and acidic glycosphingolipids (gangliosides) and contain a net negative charge, they have a pronounced effect on the surface charge and biological function of the cells in which they occur[1,2,3]. They are thought to be involved in the regulation of cell division by their incorporation into surface molecules by sialyltransferases or removal by sialidases (neuraminidases)[4,5].

Sialidase (neuraminidase, EC 3.2.1.18), designated as a N-acetylneuraminosyl-glycohydrolase, hydrolyzes the terminal α-ketosidic linkage of sialic acid residues from various sialylated glycoconjugates[1,2]. Sialidases have been detected in a variety of sources including human organs and microorganisms. Animals possess sialidases for the turnover of their sialoglycoconjugates[1,2]. The occurrence of these enzymes in microorganisms is often associated with pathogenicity[6,7,8]. The role of sialidases in viral infections has been the subject of many anti-infection studies[9]. The sialidase activity in

the cell culture medium of human foreskin fibroblasts is distinctly different between the sparse density and the confluent density cultures[4].

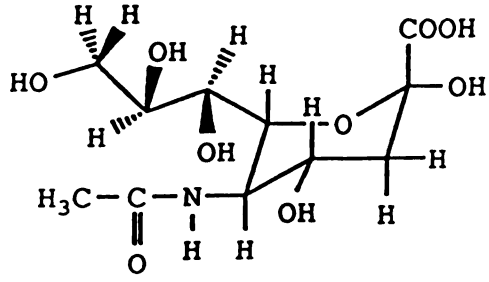
This study utilizes transition-state analogues of neuraminic acid to develop novel compounds for isolation and determination of sialidase from biological sources. One approach is to develop efficient inhibitors of these enzymes which requires that such molecules resemble the transition state for transformation of the substrates to products. A convenient transition state analogue for utilization in studies of sialidases is 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Neu5Ac2en) (Fig. 1B). This material is extremely expensive and difficult to synthesize from sialic acid [10,11,12]. These factors severely limit the application of Neu5Ac2en in the development of affinity matrices where considerable amounts of material are required. The most important step in this project was, therefore, to develop a simple, efficient chemical process for the conversion of sialic acid to Neu5Ac2en. The specific goals of this study were as follows:

(1)Develop a chemical process for converting sialic acid to Neu5Ac2en;

(2)Develop a method of coupling Neu5Ac2en to a solid support for use as an affinity matrix without affecting its inhibitory properties. This would make it possible to isolate sialidases from complex physiological mixtures such as spent cell-culture media;

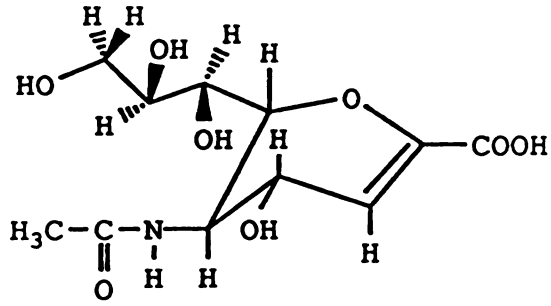
(3) Develop a photoactivatable sialidase inhibitor derived from Neu5Ac2en which can be prepared in a radiolabeled form for use as a photoaffinity probe for radiolabeling sialidases in complex systems[13,14,15]; and

(A)



Neu5Ac

(B)



Neu5Ac2ene

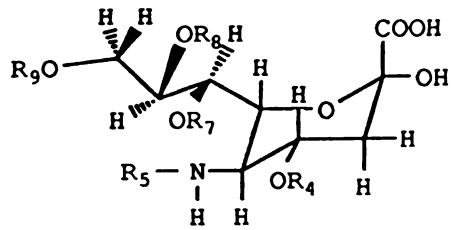
Figure 1: (A)The structure of free N-acetylneuraminic acid(Neu5Ac2en). .
(B)The structure of 2,3-dehydro-2-deoxyl-N-acetyl neuraminic acid (Neu5Ac2en).

(4) Validate the efficiency of the affinity matrix developed in (2) and the inhibitory power of the photoaffinity-labeled derivative developed in (3).

B. STRUCTURAL CHARACTERISTICS OF SIALIC ACIDS

Sialic acid is the trivial name for a series of neuraminic acid derivatives (IUPAC system name: 5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acids; Neu5Ac), which are acidic amino sugars. In a recent report[16], the number of sialic acids identified from natural sources was around 25. Neuraminic acid, with a carboxyl group at C-1, provides a negative charge on the carbohydrate components of glycolipids, glycoproteins, and other glycoconjugates. In addition to the carboxyl group, sialic acids have other functional groups such as N-acyl, C7-C8-C9 glycerol side chain, and multiple hydroxyl groups (Fig. 2, and Table 1). Two kinds of N-substituted sialic acids are widely encountered in nature, that is, N-acetyl (Neu5Ac) and N-glycolyl (Neu5Gc). In addition, the hydroxyl moieties at C-1, C-4, C-7, C-8, and C-9 can be methylated or esterified with acetyl, glycolyl, lactyl, methyl, sulphate, or phosphate groups[3]. In some glycoconjugates sialic acids with more than one substituent are found. Furthermore, unsaturated N-acetylneuraminic acid (Neu5Ac2en) has been found as a natural product in Kamerling *et. al.* study[17]. So far, no unsubstituted neuraminic acid has been isolated from natural sources.

In nature, sialic acids are usually α-ketosidically linked to other carbohydrates in glycoconjugates while free sialic acid exists almost completely as the β-anomer. The only β-ketosidic linkage involving sialic acid known to occur in nature is the activated



R_5	$R_{4,7,8,9}$
$-\overset{\overset{O}{\|}}{C}-CH_3$	$-H$
$-\overset{\overset{O}{\|}}{C}-CH_2OH$	$-\overset{\overset{O}{\|}}{C}-CH_3$
	$-\overset{\overset{O}{\|}}{C}-\underset{\underset{OH}{\|}}{CH}-CH_3$
	$-CH_3$
	$-SO_3H$
	$-PO_3H_2$

Figure 2: The structure of N-, O-substituents of sialic acids, which is adapted from Schauer,R. (1985).

Table 1: The influence in the activity of sialidase is contributed with N-, O-substitution (which is adapted from Schauer, R. and Corfield, 1981)

Moiety	Influence on Sialidase Activity	Literature
Aglycone or linkage to next monosaccharide	Aglycone specificity related to hydrophobicity. hydrolysis of 2-3, 2-6, and 2-8 linkage is enzyme specific and varies with the nature of linked monosaccharide.	Keilich, Ziegler, and Brossmer(1979). Drzeniek (1972, 1973).
Carboxyl function	It is essential for activity. Esterification will block the cleavage by sialidase	Gottschalk, and Drzeniek(1972).
Hydroxyl on the carbon 4 position	O-Acetylation results in abolition of or large reduction in cleavage rate. 4-O-methyl is less effective.	Schauer, and Failard (1968). Beau, and Schauer (1980)
Hydroxyl on the carbon 7, 8, or 9 position	O-Acetylation results in reduction of cleavage rate, but no abolition of activity.	Schauer, and Failard (1968).
N-acyl function activity.	Rate of cleavage for N-acetyl > N-glycolyl in natural substrates. Larger and smaller synthetic groups lead to reduced	Corfield et. al. (1980). Brossmer, and Nebelin (1969)
side chain from carbon 7 to 9	Shortening to C_8 and C_7 may influence the rate of cleavage $C_9 > C_8 > > C_7$.	Veh, and Schauer (1978). Suttajit and Winzler(1971)

nucleotide CMPβ(5-2)Neu5Ac (cytidine-5'-monophosphate-2-β-N-acetylneuraminic acid)[18,19]. Only a low level of free sialic acid has been detected in normal organisms[20,21,22].

Sialic acids occupy both terminal and internal positions in carbohydrate chains of glycoconjugates. Terminal sialic acids are usually linked to galactose (Gal), N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc), and glucose (Glc). The most common linkages found are α(2-3)Gal, α(2-6)Gal or α(2-6)GalNAc and less commonly α(2-4)Gal and α(2-4)GlcNAc[23,24]. The branched-terminal sialic acids found in colon mucus glycoproteins of rat were reported to have an α(2-6) linkage to Gal and GalNAc[25,26]. Ganglioside G_{D3} is an example of a glycoconjugate containing internal sialic acids, which are attached either to other sialic acids or to an oligosaccharide core[17]. The Neu5Acα(2-8)Neu5Ac-unit and Neu5Acα(2-3)-oligosaccharide core are common components in sialylpolysaccharides and oligosialylglycoconjugates[27,28,29]. The Neu5Acα(2-9)Neu5Ac, Neu5Acα(2-8)Neu5Ac, and/or Neu5Acα(2-6)Gal linked unit are present in bacterial polymers. For example, *E. coli* strain K92 (Bos 12) contains a mixture of α(2-9) and α(2-8) linkage; the polymers of *N. meningitis* Serotype C are made from the repeating unit α(2-9) linkage[30,31,32,33,34].

C. DISTRIBUTION AND BIOLOGICAL FUNCTIONS OF SIALIC ACIDS

The variety of substituents and linkage types of sialic acids provides structural diversity to oligosaccharides of glycoconjugates. Furthermore, the distribution of sialic acids also exhibits species- and tissue-specificity. For example, mammalian systems

contain N-acetyl, N-glycolyl, O-acyl, sulphate, and phosphate-substitued neuraminic acids while Hemichordata (*Dolichoglossus kowalevskii*) have only N-acetyl neuraminic acid[35,36]. Bovine breast muscle tissue has a higher Neu5Gc level (45%) than that of fetal bovine tissue (0%)[37]. Gangliosides, consisting of ceramide and sialyloligosaccharide moieties, are sialic acid-containing glycolipids. In the brain of horse, cow, pig, and sheep, the level of Neu5Gc-containing gangliosides was less than 2% of total sialic acids[38] while the proportion of that in the erythrocytes of horse and pig reach 95-100%[39,40]. No mammalian species has been found without sialic acid-containing glycoconjugates under normal conditions[41].

The distribution of N-acetyl, N-glycolyl, and O-acyl sialic acids in gangliosides was shown to correlate with developmental stages in various organisms[8,37,42]. Bovine fetal tissue was shown to have a higher level of sialic acids than adult tissues; however, adult tissues contained a higher percentage of Neu5Gc in the total sialic acids than fetal tissues. It was suggested that 9-O-lactyl-Neu5Ac was a differentiation marker in bovine tissues because it was only found in fetal brain tissues but not in the adult[37]. The percentage of Neu5Gc in gangliosides of rat intestine was shown to rapidly increase after the first 21 days of life. Similarly, the relative level of Neu5,9Ac_2 was shown to increase with maturation of erythrocytes in adult chickens and of colon in rat[43].

Evidence indicates that Neu5Ac is associated with changes in cell properties. The location of sialic acid-containing glycoconjugates on the plasma membrane of the cell was examined in order to correlate the specific functions on certain membrane areas[44]. The results indicated that the modification of protein-bound sialic acid occurred during the

mitosis of normal cells. It was also reported that the addition of extra Neu5Ac to growth media could stimulate DNA synthesis and increase cell density in a fibroblast cell line (GM03468A) *in vitro*[5]. This suggested that negatively charged Neu5Ac on glycoconjugates might be involved in cell surface regulation events such as cell recognition, masking of antigenic determinants, contact inhibition, and cell migration[45,46,47,48,49].

Total sialic acid (TSA) and lipid-bound sialic acid (LSA) content was measured for normal and transformed cell lines in tissue culture[50,51,52]. An increased level of sialic acid (0.6 μg) released per mg by sialidase was observed for a transformed baby hamster kidney cell line (BHK-21) as compared to normal cells[53]. The level of TSA in the serum of cancer patients with benign tumors was elevated from 1.74 mmol/L to 2.16-4.15 mmol/l[54]. A higher value of TSA was also observed in patients with malignant tumors[54,55]. The amount of Neu5Gc in total lipid-bound sialic acid of colon cancer patients is 1% while it is less than 0.01% in the healthy individuals[56].

It has been observed that the negative charge on sialic acids can influence the micro- and macro-structure of glycoproteins on the cell membrane. The conformation of some glycoproteins with sialic acid residues removed has been shown to change with a corresponding loss of activity[57,58,59]. Treatment of rat dermal fibroblasts with sialidase to expose the non-reducing-end saccharides of glycoproteins caused an increase in deformation of the cell membrane[60,61]. Intrinsic factor, a sialoglycoprotein, appeared to lose proteolytic resistance and binding ability to vitamin B_{12} after removal of the terminal sialic acids[62,63]. In pathological studies with *B. clostridium*, infection was shown to

occur after a bacterial sialidase cleaved sialic acid from cell surface glycoproteins on the host[64,65,66]. These tissues were then defenseless to clostridial proteases[67]. Desialylation of plasminogen (Pg) in human plasma by sialidase treatment alters its physiological functions, including an increase of amidolytic and fibrinolytic activity and a decrease in the binding ability to U937 cells[68]. However, Chinese hamster ovary (CHO) cells and mouse 3T3 cells treated with neuraminidase did not demonstrate a change in their binding ability on glass walls[69,70].

N- and O-acylated sialic acids in glycoconjugates have been described as antigenic determinants in various mammalian and microbial systems[56,71]. For example, Neu5Ac is the key sugar distinguishing the MN blood group substances[72,73,74]. Cholinergic-specific antigen (Chol-1_α) from rat brain was found to be a trisialoganglioside[75]. Cold agglutinins, which are antibodies raised against the terminal parts of N- or O-glycosidic oligosaccharide chains of glycoproteins in the membrane of erythrocytes, were able to bind Neu5Ac-containing antigens[72,73]. Waldenstrom macroglobulins also showed specific binding to Neu5Ac antigens[76,77]. N-glycolylneuraminic acid antigen was found in the blood group substances of East-Asian dogs. Hanganutziu-Deicher (HD) antibodies, discovered in sera of patients with several diseases, bound the Neu5Gc-antigen of gangliosides and glycoproteins[78,79]. Four antigenic gangliosides containing Neu5Gc were isolated from human colon cancer[80]. Evidence has also indicated that O-acetylation of sialic acids influences the immunogenicity of glycoconjugates in bacterial polysaccharides and human melanoma cells[81,82]. Colominic acid of *E. coli* K1, a homopolymer of Neu5Ac with O-acetylation at O-9 position, is more immunogenic than non-O-acetylated

homopolysaccharides[82]. Neu5,9Ac_2 found in the melanoma-assiciated ganglioside (G_{D3}), is considered to be a tumor-specific antigen[81,83].

D. SYNTHETIC SIALIC ACID DERIVATIVES

Studies of synthetic sialic acid derivatives provide a path to understanding the biological functions of sialic acids. They can be used as substrates and inhibitors of sialidases, sialyltransferases and other converting enzymes, and to study the mechanism of sialidase. The following discussion is limited to synthetic transition state analogues of sialidases. Synthetic sialo-oligosaccharide compounds have been reviewed by Van der Vleugel[84].

2-Deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en) has been known as a competitive inhibitor for various neuraminidases. The conformation of Neu5Ac2en is a half-chair form similar to the intermediate of hydrolysis reaction by sialidase. Neu5Ac2en has been found as a side product from the synthesis of α-linked sialodisaccharides[85,86,87]. Neu5Ac2en is synthesized from various halogenated neuraminic acids. For example, it is obtained by the treatment of 4,7,8,9-tetra-O-acetyl-2-chloro-2-deoxy-β-N-acetylneuraminic acid with an HCl-eliminating agent such as triethylamine (10 min, 20 °C) or silver carbonate (60-90 min, 80-90 °C), in dioxane or acetone, followed by O-deacetylation [12,71]. Another halogenated neuraminic acid, 4,7,8,9-tetra-O-*p*-nitrobenzoyl-2-bromo-2-deoxy-N-acetylneuraminic acid methyl ester, was converted quantitatively to Neu5Ac2en by using triethylamine or by treatment of molecular sieves 4A in dichloromethane. Neu5Ac2en was obtained in 73% yield by the full protection

bromo sugar after deacetylation[18]. In another scheme, Neu5Ac2en was prepared by prolonged heating (5hr, 90 °C) of 2,4,7,8,9-penta-O-acetyl-N-acetylneuraminic acid in dioxane, followed by O-deacetylation[12]. Other unsaturated sialic acid derivatives with different N-acyl groups are obtained from a similar process using different N-acylneuraminic acids as reactants[88].

The other common series of unsaturated sialic acids, 2-deoxy-2,3-dehydroneuraminic acid (Neu2en), are prepared from 2-deoxy-2,3-dehydro-N-benzyloxycarbonylneuraminic acid by hydrogenolytic cleavage with $Pd/BaSO_4/H_2$ [89]. The yield of this procedure has been reported to be around 73%. In addition, the benzhydryl ester of Neu2en is prepared with diphenyldiazomethane in methanol. A series of N-acyl neuraminic acid derivatives and their methyl esters are synthesized from Neu2en by various N-acyl transferring procedures[10,89]. Neu2en and its N-acyl derivatives allow us to study the effect of N-substituents on sialidase activity.

Synthetic analogues of sialic acid altered at the C-5 position were prepared on a microgram scale by Schreiner *et. al*[90]. 2-Deoxy-2,3-dehydro-N-trifluoroacetyl neuraminic acid (Neu5$CF_3$2en) was synthesized by hydrazinolysis of Neu5Ac2en in hydrazinium hydroxide (72hrs, 85 °C), followed by N-trifluoroacetylation of Neu2en[91]. This protocol provided a stereochemically defined procedure for the preparation of the N-substituted molecule compared with the traditional Kuhn-Baschang procedure[92] even though the yield of the final product (Neu5$CF_3$2en) was less than 25%. 2,3-Didehydro-2,3-dideoxy-D-glycero-D-galacto-2-nonulopyranosonic acid (Kdn2en), 5-azido-2,3-didehydro-2,3,5-trideoxy-D-glycero-D-galacto-2-nonulopyranosonic acid (5-azido-5-

deoxy-Kdn2en) were formed from the respective peracetylated methyl esters via dehydration and Zemphen saponification. These C-5 modified analogues of Neu5Ac2en were used to determine the interaction between the amino, carbonyl groups at C-5 position and the binding site of *V. cholerae* sialidase.

Synthetic analogues with alternations in the glycerol side chain of sialic acid have been investigated by Erich *et. al*[93]. 7-, 8-, 9-Deoxy-Neu5Ac2en was synthesized from the corresponding iodononulosonic acid methyl esters by xanthogenation, hydrolysis, peracetylation, and oxazolino-dehydration[94,95]. In this series of procedures, the 4,5-oxazolino derivative was a minor product when 1 M trimethylsilyl trifluoromethanesulfonate $CF_3SO_3Si(CH_3)_3$ in acetonitrile was used. The major products, peracetylated methyl esters of 9-deoxy-Neu5Ac2en derivatives, were prepared with only a catalytic amount of $CF_3SO_3Si(CH_3)_3$, followed by alkaline hydrolysis to give corresponding to deoxy-Neu5Ac2en compounds in 33-60% yield.

Synthetic analogues of sialic acid modified at C-4 were prepared to determine the structural requirements for sialidase activity. The C-4 hydroxyl group of sialic acids has been converted to azido, amino, N-formyl, N-acetyl, 4-epi-hydroxy, and 4-epi-azido substituents via the intermediate 4-epi-Neu5Ac2en [96,97,98]. The peracetylated methyl ester of Neu5Ac was treated with $CF_3SO_3Si(CH_3)_3$ in acetonitrile (2hrs, 50 °C) to give an 4,5-oxazoline compound, then with trifluoroacetic acid (TFA) in tetrahydrofuran/water to cleave the oxazolino ring, followed by deacetylation to form 4-epi-Neu5Ac2en. In another study, 4-epi-Neu5Ac2en was obtained by acetolysis in sulfuric acid and acetic anhydride at 40-50 °C for 7hrs.[99]. The azido substituents of Neu5Ac2en derivatives

were prepared from this epimeric derivative by reaction with triphenylphosphane/ammonia/diethylazodicarboxylate ($P(Ph)_3/NH_3$/DEAC). Other C-4 replacements were obtained by treatment of free 4-azido, or 4-epi-4-azido Neu5Ac with $P(Ph)_3/THF/H_2O$ and hydrolysis.

The above mentioned analogues were synthesized to further understand the structural requirements for the activity of sialidases *in vitro*. Another approach to obtain information about sialidases *in vivo* is to synthesize photoreactive analogues of sialic acids. The requirement of a photoactivable probe includes the specific binding to the active site of sialidase without azido interference. In Warner's studies[13,14,100], a series of photoreactable analogues were synthesized through preacetylated 9-O-tosyl-Neu5Ac2en methyl ester (Fig. 3). 9-Azido-Neu5Ac2en was obtained by treatment of the tosyl analogue with NaN_3 in dry dimethylsulfoxide under N_2 at 50 °C overnight. Next, 9-S-(4-azido-2-nitrophenyl)-5-acetamido-2,6-anhydro-2,3,5,9-tetradeoxy-9-thio-D-glycero-D-glacto-non-2-enonic acid (9-PANP-Neu5Ac2en) was prepared by introducing a photoactive compound, 4-fluoro-3-nitrophenylazide, to the C-9 position via a thio linkage. The 9-thioacetyl-Neu5Ac2en was formed by replacement of the tosyl group with potassium thioacetate in DMF (80 min, 60 °C). The photoactive product was obtained after 9-S-acetyl-Neu5Ac2en reacted with 4-fluoro-3-nitrophenylazide/ sodium methoxide in methanol, followed by peracetylation and base hydrolysis. Also, the radioactive photoprobe, 9-S-(4-azido-3,5-^{3}H-2-nitrophenyl)-5-acetamido-2,6-anhydro-2,3,5,9-tetradeoxy-9-thio-D-glycero-D-glacto-non-2-enonic acid (^{3}H-9-PANP-Neu5Ac2en) was prepared by using radioactive 4-fluoro-3-(2,6-^{3}H) nitrophenylazide as the starting material.

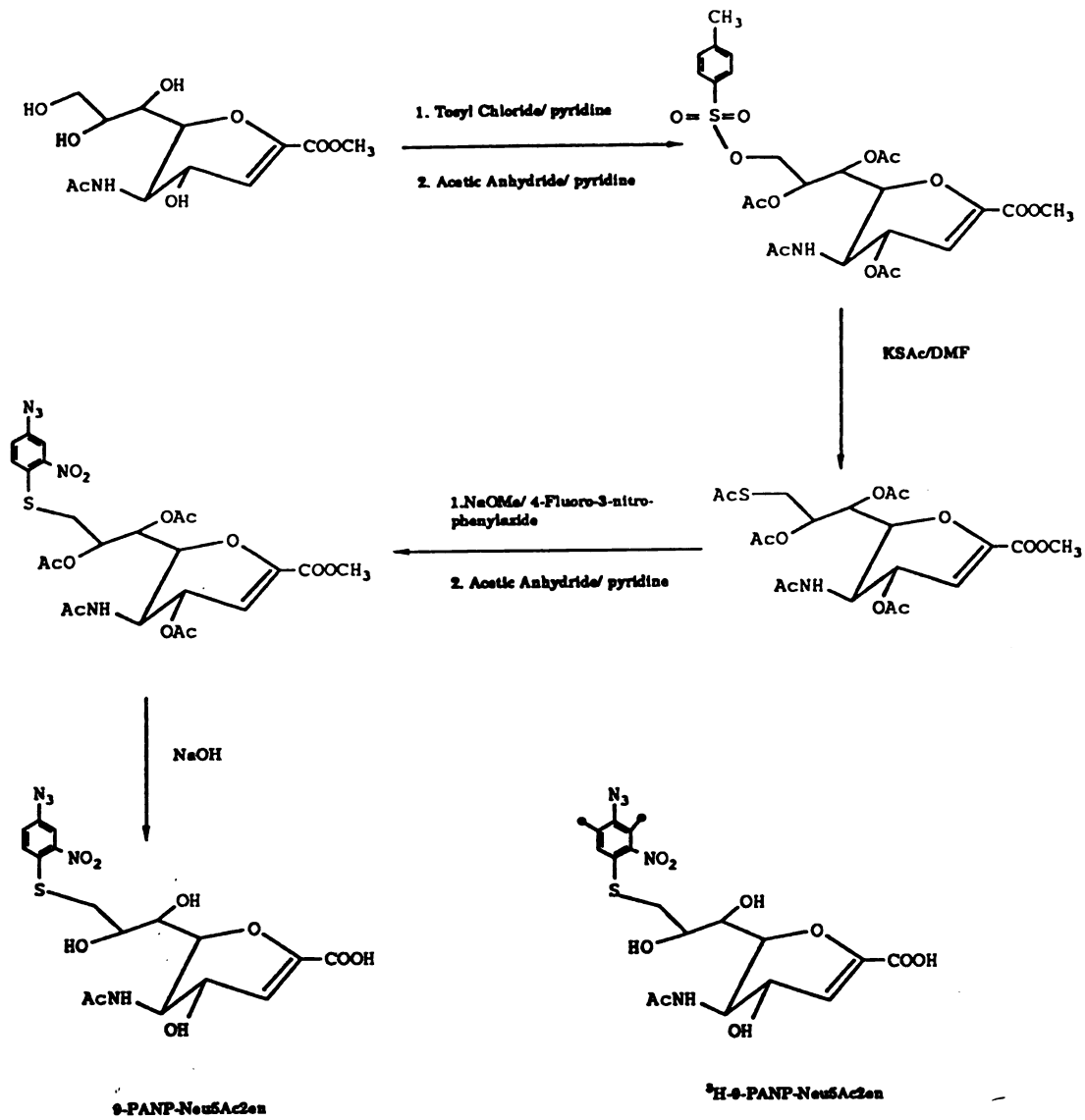


Figure 3: Reaction scheme for synthesis of ^{3}H-9-PANP-Neu5Ac2en, which is adapted from Warner, T. G.(1985).

These thio-Neu5Ac2en derivatives were shown to have K_i values (1.0×10^{-5} M) similar to Neu5Ac2en with lysosomal neuraminidases. In another study[15], a photoactivable heterobifunctional reagent, N-hydroxysuccinimide ester of 4-azidosalicylic acid (NHS-ASA), was incorporated into the C-9 position of 9-amino-Neu5Ac2en under slightly basic conditions (at pH>7, the half-life time was less than 2hrs.) (Fig. 4). Radioactive [^{125}I]ASA-Neu5Ac2en was also synthesized by coupling ^{125}I to the photoactive heterobifunctional reagent according to Hunter's method[101].

E. The CHARACTERISTIC OF SIALIDASE

Sialidases (neuraminidases, EC 3.2.1.18), designated as a number of N-acetylneuraminosyl-glycohydrolases, cleave the terminal α-ketosidic linkage of sialic acid residues from various sialylated glycoconjugates[102,103,104,105]. Sialidases have been isolated from a variety of sources including human organs and microorganisms. Sialidases found in bacterial systems are soluble exoenzymes while these enzymes in viruses compose a part of the envelope membrane to incorporate with hemagglutinin during infection[41]. In mammals, sialidase activity is detected in lysosomes, the Golgi apparatus, the plasma membrane and the cytosol by using gangliosides, sialylactoses, and 4-methylumbelliferyl-α-D-N-acetylneuraminic acid (4MU-NANA) as substrates [2,105-110].

The optimal pH of sialidase depends on substrate specificity and source. Sialidases found in bacteria and viruses have an optimal pH of 4.5 to 5.5, depending on the substrate[105,111,112,113,114]. In mammals, the optimum pH of lysosomal, Golgi, and plasma-membrane associated sialidases is 4.0 while that of soluble and extracellular

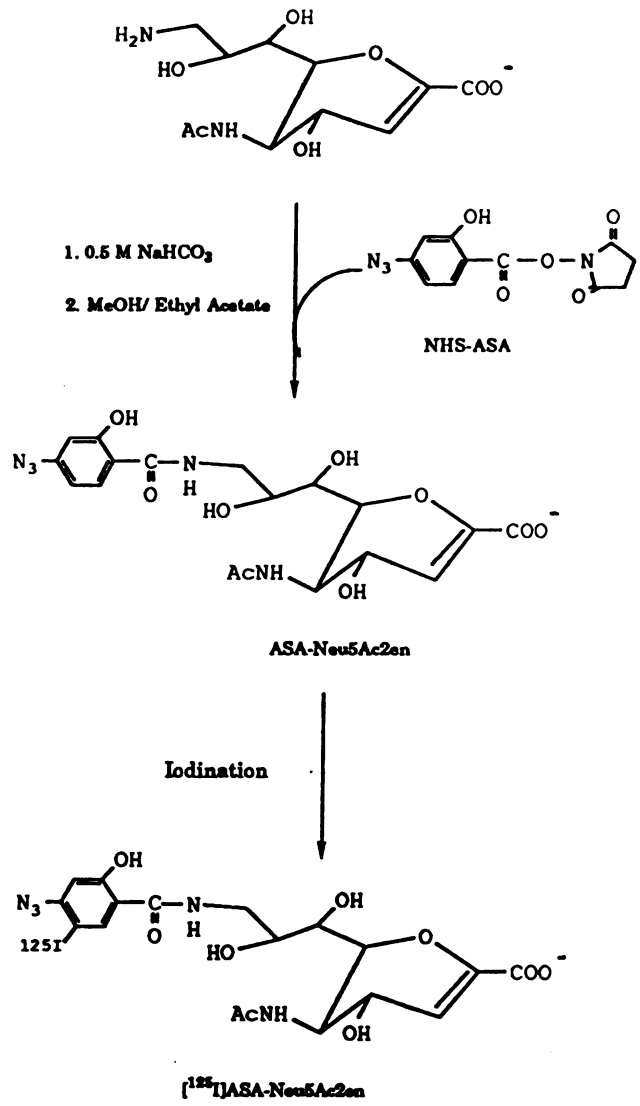


Figure 4: Reaction scheme for synthesis of ASA-Neu5Ac2en and [^{125}I]IASA-Nu5Ac2en, which is adapted from Horst *et. al.*(1990).

sialidases is reported at around pH 6-6.5[4,105,115].

A unique aspect of many types of sialidases is their wide substrate specificity[4,105,108,110,113]. The activity of various sialidases is studied by using (α(2-3)sialyllactose (α(2-3)II^3-Neu5AcLac) as a reference substrate. Bacterial and viral sialidases are shown to be relatively non-specific, attacking substrates such as gangliosides, sialyllactose, sialyloligosaccharides, and sialylglycoproteins. In addition, G_{M3}, α(2-3)II^3-Neu5AcLacCer, is a better substrate for *Vibrio cholerae* and *Clostridum perfringens* sialidase while α(2-6)sialyllactose (α(2-6)II^6 Neu5AcLac) is favored by *Arthrobacter ureafaciens* sialidase. Mammalian sialidases, on the other hand, are highly specific. Sialidases of rat liver Golgi hydrolyze the sialyllactoses, α(2-3)II^3 Neu5AcLac and α(2-6)II^{6}Neu5AcLac, but not gangliosides. Other factors, such as the N- and O-substitution, the length of side-chain, and linkage position in oligosaccharides, can also affect the activity of sialidases. Studies relating to these factors published before 1980 have been reviewed and summarized by Schauer[41].

More than one sialidase can occur in a given tissue. Using 4MU-Neu5Ac as a substrate, two different sialidases in human leucocytes have been isolated based on their concanavalin A (Con A) binding ability[116]. One is a Con A binding form coupled with β-galactosidase activity, primarily found in lymphocytes (>80%). The other is a non-Con A binding form which is mainly located in granulocytes. Both are classified as lysosomal sialidases. Two membrane-associated sialidases in rat brain are distinguished by a combination of biochemical and immunological methods[117]. Gangliosides are the substrates for one of the sialidases while the other had a wider range, including 4MU-

Neu5Ac, gangliosides, sialyloglycoproteins and sialyloligosaccharides. Additionally, antibodies raised against each sialidase did not cross react. Substrates for a cytosolic sialidase in rat liver included sialylated oligosaccharides, glycoproteins, glycopeptides, and gangliosides with an optimal pH of 6.0[118]. Lysosomal sialidases in rat liver only hydrolyzed low-molecular weight oligosaccharides and ganglosides under acidic pH conditions[117].

The activities of extracellular sialidases in the culture medium of human foreskin fibroblasts were detected by using [^{3}H]G_{M3} as substrate[4,119,120]. According to the pH profile, two forms of G_{M3} ganglioside sialidase were found that had maximal activities at pH near 4.5 and 6.5. The activity level of both sialidases in the culture medium was also highly dependent on the cell density. The level of sialidase activity at pH 4.5 from the culture medium increased from 4.1 pmol/hour/ml to 39 pmol/hour/ml while the cell density increases from sparse (1 x 10^3 cells/cm^2) to confluent density (6 x 10^4 cells/cm^2). In contrast, the activity of the extracellular sialidase with pH 6.5 maximum decreased from 9 pmol/hour/ml to a non-detectable level after the cell density reached confluence. The same experiment has also been performed on plasma-membrane bound sialidases[120]. No neutral sialidase activity was detected on the plasma-membrane bound fraction while the acidic sialidase activity was shown to be 10 times higher than that of culture medium. On a per cell basis, the acidic form of sialidase activity at confluency stage decreased to 40% of activity of sparse stage. Another study from Yogeeswaran[121] has demonstrated a similar event. Membrane-bound sialidase activity, which hydrolyzes gangliosides, decreased to around 50% at the touching stage (preconfluency density) of cell-cell contact

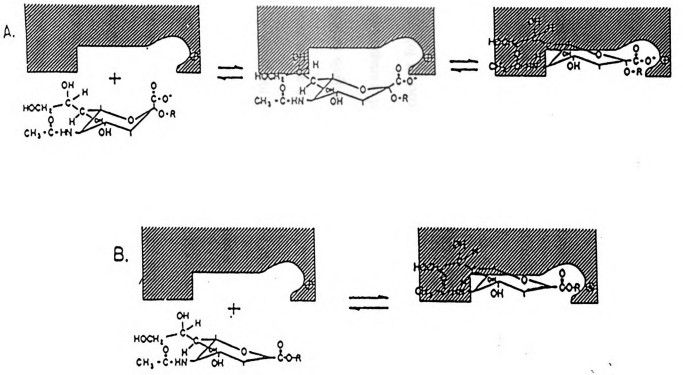
in mouse fibroblasts 3T3 cells. However, the sialidase activity of transformed cells showed no significant change at any stage of cell-contact.

A more sensitive assay of sialidase activity has been developed by Ogura *et al.* [115] to analyze low levels of extracellular sialidases in the culture medium. This assay employed ganglioside (G_{D1a}) as the substrate and measured the absorbance of the product (G_{M1}) coupled with cholera toxin B subunit-horse radish peroxidase at 490 nm. The detection limit of this assay for ganglioside sialidase was as low as 3 fmol/min. Using this assay, it has been reported that the culture medium of human fibroblast cells at pH 6.5 contained two sialidases. One of two sialidase activities (47-69 kDa) reached a maximum by addition of 1% Triton CF-54 while the other sialidase activity (16 kDa) was not affected by addition of the detergent. The relationship between these two forms of sialidases remains an open question.

F. The POSTULATED MECHANISM OF SIALIDASES

From studies on the hydrolysis of the glycosidic bond with egg-white lysozyme and other glycohydrolases, it has been suggested that the transition-state of substrates of sialidases were likely to be oxo-carbonium ions[122,123]. The mechanism of transition-state binding of sialidase from the soil bacterium *Arthrobacter Sialophilus* is postulated using Neu5Ac2en and other sialidase analogues as competitive inhibitors (Fig. 5)[124]. The K_i value of Neu5Ac2en and its methyl ester are 1.4×10^{-6} M and 4.8×10^{-5} M, respectively, while the K_m value of N-acetylneuraminlactose is 1.0×10^{-3} M. However, Neu5Ac-a-methyl ketoside methyl ester, Neu5Ac-β-methyl ketoside, and its methyl ester at 1.0 x

Figure 5: The postulated mechanism for the binding of substrates and transition-state analogues to *A. sailophiluse* sialidase (Miller, 1978). (A) Sialylglyconjugates was used as substrates of sialidases. (B) A transition-state analogue was used as a substrate of sialidases.



10^{-3} M are neither substrates nor inhibitors of this sialidase. This indicated that the free carboxyl group of sialic acid is essential for the substrate binding to sialidase but it is not necessary for transition-state analogue binding. The $^2C_5$ conformation of the sialic acid moiety on sialoglycoconjugates is converted to a half-chair conformation with a planar geometry at $O\text{-}C_2\text{-}C_3$ of the pyranose ring forming the oxo-carbonium cation ion intermediate. The half-chair conformation of Neu5Ac2en and its a-glycoside methyl ester fit into the active site of transition-stage of sialidases without any bond bending[124].

The protein sequence of neuraminidases from influenza A and B varies up to 70%; however, the active site of these sialidases has 24 amino acid residues which are highly conserved[25-132]. The mechanism of sialidase catalysis in influenza A/tokyo/3/67 (N2) strain has been studied by using site-directed mutagenesis[133]. Seven of 14 mutations were shown to possess no activity with the correct folding structure while two others were without the correct folding structure. One of two mutations, Asn-146 to Ser, is not located in the active site pocket. It has been known that Asn-146 is one anchor site for four N-linked carbohydrate side chain of salidases. While the activity of mutant Asn-146 vanished, it was suggested that the attachment of the carbohydrates could be important to form a proper folding structure and to remain active. Other mutations were located in the active site of sialidase. The catalytic mechanism of sialidase was deduced from these observations on the activity and the immunoprecipitation. In this model, His-274 is an indirect proton donor under the physiological pH, which causes a raise in the pK_a of Glu-276. Then the un-ionized carboxyl of Glu-276 breaks the glycosidic linkage by a protonation on the oxygen of the pyranose ring, the subsequent cation intermediate

might be stabilized by an acidic group (Glu-277), and a water molecule provides a proton to His-274 and a hydroxyl to the intermediate, leading to the release of the terminal sialic acid. Other conserved residues were involved in the substrate binding at the active site. Other strains of influenza A and B also displayed a similar catalytic model with a slight difference in the substrate binding residues[126,128,132]. A similar mechanism for lysozyme glycohydrolases and sialidases was postulated by Stryer[134]. The active site pocket involved two acidic amino acid residues (Glu-35 and Asp-52) located on the opposite side of the glycosidic bond cleaved by catalytic reaction.

To facilitate the characterization of the active site of sialidases, synthetic photoreactive radiolabelling probes provide a useful tool. For example, ^{125}I-ASA-Neu5Ac2en was used to label the active site of *C. perfringens* sialidase[15]. Photoaffinity-labeled sialidase (72 kDa) was cleaved with CNBr to generate 5 peptides with molecular weights of 27.2, 13.3, 11.4, 10.4 and 7.7 kDa. The 27.2 and 13.3 kDa peptides of 72 kDa sialidase are labelled with the radioionated molecule. This indicated that these two peptide chains should contain the active sites of *C. perfringens* sialidase. No detailed protein sequence was mentioned in this report. It is worth noting that the specificity of the photoprobe in mammalian sialidases was low. The active site of lysosomal neuraminidases in human placenta has been examined with a photolabelling transition-state analogue, ^{3}H-9-PANP-Neu5Ac2en[14]. After photolysis, the autoradiogram of the denatured enzyme complex exhibited two intense bands at 61 kDa and 46 kDa. The first 20 amino acids at the N-terminal of 46 kDa protein was shown to possess 85% identity with the cloned cDNA sequence of α-galactosidase B from human placenta and 68%

homology with the sequence of α-galactosidase A from human lung. It was suggested that the 46 kDa protein, associated with the photoprobe by non-specific hydrophobic interaction, was not a sialidase. The photoprobe was bound specifically with the 61 kDa protein since addition Neu5Ac2en decreased the intensity. However, the protein sequence of 61 kDa band could not be directly determined by automated Edman degradation due to an unknown blocking effect.

The lysosomal sialidase from human placenta has been reconstituted *in vitro* by Verheijen *et al.*[99,135]. They indicate that the full activity of lysosomal sialidase was present in a complex with β-galactosidase (64 kDa), and a protective protein (32 kDa). The inactive sialidase (66 kDa) isolated from human placenta includes a 38 kDa protein core with 7-14 kDa N-linked oligosaccharide chain. The active site of sialidase complex was located on this enzyme. The protective protein, however, was essential for full activity of the sialidase and the β-galactosidase[136]. The stoichiometry of this complex was not clear although the binding sites of β-galactosidase and sialidase on the protective protein were known to be on different domains. It was suggested that the protective protein is a processing enzyme because its protein sequence is highly homologous to yeast protease carboxypeptidase Y (CPY) and the yeast KEX1 gene product[137]. The activity and stability of sialidase significantly increased after addition of β-galactosidase. However, no effect occured after addition ofthe protective protein to inactive sialidase.

G. ACTIVATORS AND INHIBITORS OF SIALIDASE

An activator protein, β-glucosidase-stimulating protein, appears to stimulate the activity of oligosaccharide sialidases in fibroblasts from galactosialidosis and sialidosis

patients[138]. The activity of sialidase remained the same after desialylation of the activator. While all hydrolases are stimulated by β-glucosidase-stimulating protein, the mechanism of activation of the stimulator with sialidases is not yet clear.

It has been reported that some bacterial (*V. cholerae and D. pneumoniae*) and viral (some *influenza* virus strains) sialidases require divalent cation for activity, usually Ca^{+2} [1,41]. In *V. cholerae* sialidase, the Ca^{+2} ion is required for stability of the enzyme but not essential for the substrate binding[139,140]. It has been suggested that a salt bridge is formed between substrate and enzyme. In some mammalian sialidases, mono- and divalent cations are required for activity. A general effect of monovalent ions is increased ionic strength, which influences the pH value. A membrane-bound sialidase detected in Golgi apparatus from rat liver is stimulated by preincubating with 1 mM Ca^{+2} and Zn^{+2} [141]. The lysosomal sialidase of mouse liver is stabilized in the presence of phenylmethyl sulfonyl fluoride, Ca^{+2}, Mg^{+2}, Mn^{+2}, and Zn^{+2} [142]. It has been reported that one of membrane-bound ganglioside sialidases from human liver is activated by cholate[143]. The sialidase activity of human promyelocytic leukemia cell line (HL-60) is raised by incubation with 1 μM retinoic acid or 1.3% DMSO[144].

Inhibitors of sialidases have been classified into four groups according to source and character; they are naturally occurring compounds with high molecular weight and with low molecular weight, synthetic sialidase inhibitors, inorganic ions and other compounds. The influence of inhibitors *in vivo* is summarized in Table 2[145]. In general, the naturally occurring compounds with high molecular weight such as polyanionic polymers, are non-specific inhibitors due to the interaction between ions. The

Table 2: A summary of the inhibtion data, which is adapted from Schauer, R.(1982).

Inhibitor	Sialidase			Molecular weight	Type of inhibition	References
	viral	bacterial	mammalian			
Natural Compounds						
DNA, RNA, heparin	+++	+	ND	H	NC	Drzmiek, 1972, 1973
PSM	+++	++	ND	H	C	Drzeniek, 1972, 1973
ESM	++	++	ND	H	C	Sander and others, 1979
Neuraminin	++	-	ND	H	C	Zalan and others, 1975
Concanavalin A	++	-	ND	H	NC	Lin and others, 1977
II^3Neu5AcGgOse$_4$Cer micelles	ND	++	++	H	ND	Veh, Schauer, 1978
Neu5Ac	+	+	+	L	C	Drzeniek, 1972,1973,
Neu5Ac2en	+++	+++	++/-	L	C	Meindl and others, 1974
II^6Neu5AcLac	+	-	ND	L	C	Drzeniek, 1972
Siastatin A, B	-	++/-	++/-	L	C	Aoyagi and others, 1975
Panosialin	++	ND	ND	L	C	Aoyagi and others, 1971
Synthetic compounds						
Dextran sulphate	+++	+	ND	H	NC	Drzeniek, 1972, 1973
Neu5Acyl2en	+++	+++	++	L	C	Meindle and others, 1974
S ar d N-ketosides of Neu5Ac	+++	+++	ND	L	C	Khorlin and others, 1970
3-Aza-2,3,4-tri-deoxy-4-oxo-D-arabino-octonic acid & lactone	+++	+++	ND	L	C	Khorlin and others, 1970
N-substituted oxamic acids	+	++/-	++/-	L	C	Corfield and others,1981 Veh & Schauer, 1978
Ions						
Cu^{+2}	++	++	++	L	C	Rosenberg and others
Hg^{+2}	++	++	++	L	C	Drzeniek, 1972, 1973
Fe^{+3}	ND	ND	++	L	C	Corfield and others 1981

The strength of inhibition is indicated as weak (+) to very strong (+++) or no inhibition(-).
The type of inhibition is indicated as competitive (C), or non-competitive (NC). ND stands for no determination.
The molecular weight of inhibitors is indicated as high (H), and low (L).

effectiveness of inhibition of this group toward various sialidases is viruses > bacterial > mammalian. The next group of inhibitors with low molecular weight are shown to be more specific. The hydrolysis product of sialidase, Neu5Ac, at high concentration (up to 10-25 mM) inhibits the activity[146]. The inhibitor, Neu5Ac2en, was discovered in normal urine, serum and in saliva of human[22]. The level of Neu5Ac2en in sialuria patients is 10,000 times higher than that of normal[17,147]. The normal level of Neu5Ac2en in humans might cause strong inhibition of sialidases from microorganisms. Sialylα(2-6)lactose, a substrate of lysosomal sialidases in human, is a competitive inhibitor for viral sialidases from newcastle disease[139].

Investigations with synthetic sialidase inhibitors having structural similarity to sialic acids have been discussed. Other inhibitors with no connection to sialic acid structure were studied in the 1970's[148,149]. These results suggest that these compounds mainly inhibited the activity of bacterial and viral sialidases (K_i value was 4-5 x 10^{-1} mM). Inhibition by $HgCl_2$, $HgSO_4$, $Hg(NO_3)_2$, $Hg(OOCCH_3)_2$, and KIO_4 in *V. Cholerae* and *Cl. perfringens* sialidases *in vitro* was reported by Cabezas[150]. Other inorganic ions such as Cu^{+2}, Fe^{+3} also inhibit the activity of sialidases from all sources. It is suggested that the inhibition is non-specific and may be due to the interaction between ions and the thio group of enzyme.

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CHAPTER 2

SYNTHESIS OF SIALIC ACID ANALOGUES: A NOVEL, ONE-STEP CONVERSION OF N-ACETYLNEURAMINIC ACID AND THEIR DERIVATIVES

Running Title: Synthetic Analogues of Sialic Acid and Their Biological Application

ABSTRACT

2,3-Didehydro-2-deoxy-N-acetylneuraminic acid (Neu5Ac2en) has been synthesized from N-acetylneuraminic acid (Neu5Ac) by a simple, quantitative, one-step conversion. Neu5Ac was dehydrated by peracylation with trifluoroacetic anhydride (TFAA) in the presence of N,N-dimethyl-4-aminopyridine (DMAP) as base. 2,3-Didehydro-2-deoxy-neuraminic acid (Neu2en) was also obtained when the ratio of TFAA/DMAP was changed to 4:3. 2,3-Epoxy-2-deoxy-N-acetylneuraminic acid (2,3-epoxy-Neu5Ac) was prepared from Neu5Ac2en via epoxidation of the α,β-unsaturated acid with hydrogen peroxide-sodium tungstate. 8,9-Isopropylidene-2,3-didehydro-2-deoxy-N-acetylneuraminic acid was synthesized from Neu5Ac2en by using copper sulfate as a catalyst in acetone. A photoreactable compound, N-azidoacetyl neuraminic acid was obtained from Neu2en via a N-haloacetyl intermediate. Nine synthetic potential inhibitors of sialidase(s) have been prepared in this study; four of nine compounds have not been reported by other laboratories.

INTRODUCTION

5-N-Acetyl-5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid (N-acetylneuraminic acid, sialic acid, Neu5Ac) is a naturally-occurring acidic monosaccharide. Sialic acids are present in tissues from mammals, vertebrates, some bacteria, and the influenza virues[1]. The occurrence of sialic acids in nature has been reviewed[2,3,4,5,6]. The different substituents found on sialic acid in nature include N-acetyl, N-glycolyl, O-acetyl, O-methyl, O-phosphate, and O-sulphate[1,7,8]. Sialic acids

are a part of sialylosaccharide chains in glycoconjugates. They are located on terminal positons as well as internal positions in oligosaccharides via O-ketosidic α-2,3-, α-2,4-, α-2,6- and α-2,8- linkages. Free sialic acid has also been detected at low levels in human urine, serum, and saliva[9,10]. The variety of substituents and linkage types and locations of sialic acids affect the rate of catalysis of sialoglycoconjugate hydrolysis by sialidases[7,11,12,13,14]. Many biological functions have been attributed to sialic acid and its sialoglycoconjugates such as cell recognition and cell adhesion[15,16,17].

Sialidases (EC 3.2.1.18, N-acylneuraminosyl glycohydrolases) catalyze the hydrolytic cleavage of sialic acid residues from sialylglycoconjugates. It has been reported that sialidase participates in a number of physiological processes such as the regulation of cell proliferation[18], clearance of plasma proteins[19], the degradation of gangliosides and glycoproteins[20], and neurotransmission[21,22]. In pathogenic microorganisms, sialidases play an important role in the mechanism by which host cells are infected[23]. Extracellular sialidase activities have been detected at low levels in the culture medium of human fibroblasts[18,24]. Furthermore, it is known that several inherited metabolic diseases in humans result from sialidase deficiency[25,26]. The wide distribution of sialidases in cellular organelles and the diversity of biological functions has led to interest by several groups in the relationship between sialic acid structure and the activity of sialidases.

It has been reported that almost all mammalian, bacterial, and viral sialidases are inhibited by a transition state analogue, 5-N-acetyl-5-amino-2,3,5-trideoxy-D-glycero-D-galacto-nonulosonic acid (N-acetyl-2,3-dehydro-2-deoxyneuraminic acid,

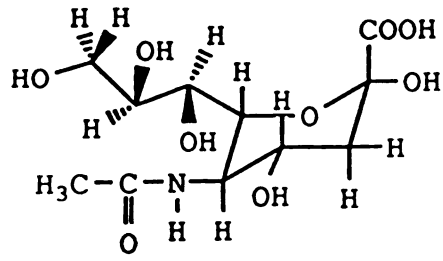
Neu5Ac2en)[27,28]. The conformation of the analogue is very similar to the oxonium ion intermediate formed during the hydrolysis reaction catalyzed by sialidases (Fig. 1)[29]. Thus, this analogue is an excellent probe to determine the active site(s) of sialidases. The synthesis of Neu5Ac2en in high yield has been a difficult problem due to its acid lability. Neu5Ac2en has been synthesized from 4,7,8,9-tetra-O-acetyl-2-deoxy-2-halo-neuraminic acid, by dehydrohalogenation with triethylamine or silver nitrate as catalyst in two laboratories[30,31]. One or more steps of protection and deprotection were usually required during the preparation of Neu5Ac2en and the yield of this procedure was less than 50 % due to degradation. It has been suggested that sialic acid derivatives with affinity labeling reagents could be useful for characterizing cellular sialidases and other sialic acid binding proteins[32,33,34]. Three photoactivatable transition state analogues of sialidase have been synthesized by introducing an aryl azide group in the C-9 position of Neu5Ac2en via a thio or an amide linkage[32,33,34]. In the present study, we provide a high-yield, one-step conversion to Neu5Ac2en by introducing and eliminating the trifluoracetyl group. Several other sialic acid derivatives were also prepared incorporating the half-chair conformation of Neu5Ac2en. Furthermore, a photoactivatable compound was obtained by incorporating an azide group at the N-5 position of Neu5Ac2en.

MATERIALS AND METHODS

A. Material

N-acetylneuraminic acid was a gift from the MECT Corporation, ToKyo. All

(A)



Neu5Ac

(B)

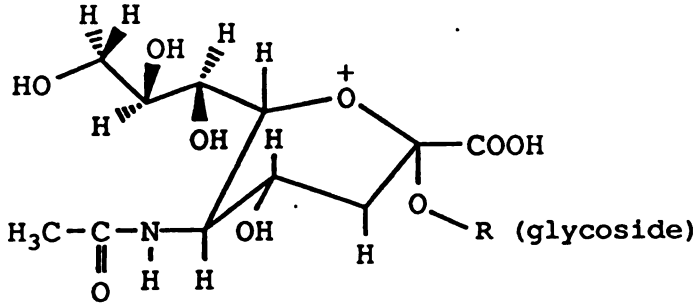


Figure 1: (a) The structure of Neu5Ac. (b) The half-chair conformation of sialyloglycoconjugates for the hydrolysis of sialidase.

other reagents were analytical grade and purchased from Sigma, J.T. Baker, or Aldrich. Thin-layer chromatography (TLC) was performed on pre-coated glass plates of silica gel 60 F_{254} (Merck Co.) with 1-propanol-water-30% ammonium hydroxide (6:3:1) as the developing solvent. Spots were detected by a carbohydrate-spray reagent containing 0.2% orcinol and 5% H_2SO_4 in methanol/water (3/1) and heating on a hot plate for 10 min. Orcinol reagent was used for colorimetric analysis of sialic acid derivatives within purification process[35]. HPLC was carried out using reversed-phase C_8 columns (Partisil 10 ODS-3, 22 x 250 mm, Whatman Co.) with a WATERS-600 instrument attached to Spectroflow 783 programmable UV detector at 232 nm and a WATERS-740 data processor. Compounds were eluted with a linear gradient of acetonitrile in water from 5% to 30% over 35 min at a flow rate of 10 ml/min. Size exclusion chromatography on a Bio-Rad P-2 (10 x 700 mm) column was performed with water as the mobile phase. IR spectra ($CaCl_2$ cell) were recorded with a Nicolet 710 FT-IR spectrometer. ^1H and proton-decoupled ^{13}C NMR spectra were measured in D_2O at 300 MHz by using a Varian VXR spectrometer. Negative FAB-MS spectra were obtained on a JEOL JMS HX-110 double focusing instrument. The samples were suspended in a matrix of triethanolamine.

Methods

Synthesis of 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid (Neu5Ac2en, B1)

N-acetyl-neuraminic acid (10 mg, 0.032 mmole) was added to a solution of N,N-dimethyl-aminopyridine (80 mg, 0.258 mmole) in dry acetonitrile (0.5 ml). The mixture

was cooled to -70 °C and then trifluoracetic anhydride (30 μl, 0.096 mmole) was added. The mixture was stirred for 1 hr. at 4 °C, and then heated in a teflon-lined screw capped vial for 6 hours at 70 °C. At the end of this period, the mixture was concentrated to dryness under a stream of nitrogen. The residue was dissolved in 1 ml H_2O, then the pH was adjusted to 11 with 30% ammonium hydroxide. The mixture was extracted with chloroform (x 3) to remove excess DMAP. The aqueous portion was applied to a Bio-Gel P-2 column for further purification. After solvent was removed by lyophilization, 8 mg (84%) of Neu5Ac2en was obtained. Large-scale synthesis of Neu5Ac2en has also been performed with 100 mg of starting material. Other reagents were scaled up with the same proportions as the small-scale. However, HPLC was required for further purificaton of Neu5Ac2en from the large-scale preparation. The recovery was 57% (54 mg). R_f: 0.643 (the R_f of Neu5Ac in this mobile phase was 0.473); ^{1}H-NMR (300 MHz, D_2O): δ 2.091 (s, 3H, $NCOCH_3$), 3.601 (dd, 1H, H-7), 3.646 (dd, 1H, H-9'), 3.885 (dd, 1H, H-9), 3.936 (ddd, 1H, H-8), 4.051 (dd, 1H,H-5), 4.213 (dd, 1H, H-6), 4.470 (dd, 1H, H-4), 5.692 (d, 1H, H-3); negative FAB-MS: m/z= 312$[M+Na-H]^-$, 290$[M-H]^-$, 272$[M-H_2O-H]^-$.

Synthesis of 2,3-dehydro-2-deoxy-neuraminic acid (Neu2en, B2)

Neu2en was prepared in the same manner as Neu5Ac2en except that the ratio of N,N-dimethyl-aminopyridime (40 mg, 0.128 mmole) to trifluoracetic anhydride (30 μl, 0.096 mmole) was changed to 4:3. Yield: 79% (6.4 mg). R_f: 0.707 ; ^{1}H-NMR (300 MHz, D_2O): δ 3.544 (dd, 1H, H-7), 3.602 (dd, 1H, H-9'), 3.855 (dd, 1H, H-9), 3.922 (ddd, 1H, H-8), 4.167 (dd, 1H,H-5), 4.345 (dd, 1H, H-6), 4.545 (dd, 1H, H-4), 5.681

(d, 1H, H-3); negative FAB-MS: m/z= 248[M-H]$^-$.

Synthesis of 5-acetamido-2,3-epoxide-3,5-dideoxy-β-glcero-D-galacto-nonulo-pyranosylonate (2,3-epoxide-Neu5Ac, B3)

The epoxidation of N-acetyl-2,3-dehydro-neuraminic acid was accomplished using a modification of the Kirshenbaum and Sharpless procedure[36,37]. Neu5Ac2en (5.59 mg, 0.019 mmole) and 0.1 equivalent of $Na_2WO_4 \cdot H_2O$ (6.7 mg, 0.0019 mmole) were added to 0.6 ml H_2O in a teflon-lined screw-capped vial. The mixture was stirred, and the pH of the solution was adjusted to around 6.2 with the addition of 0.1N NaOH and/or 1N H_2SO_4. After an additional 10 minutes stirring at room temperature, 1.2 equivalents of aqueous H_2O_2 (30% (w/v) solution, 8.84M, 6.7 μl) was added to the mixture. The pH of the reaction mixture was kept between 5.8 and 6.2. The progress of the reaction was followed by TLC. After 2 hours, the excess H_2O_2 was evaporated from the residue under a stream of nitrogen. The resulting mixture was purified by chromatography on a P-2 column. After the solvent was removed, the residue consisted of the 2,3-epoxy-Neu5Ac (R_f: 0.507) and the decomposed compound (2,3-diol; R_f: 0.489). According to NMR data, the conversion of epoxide compound from Neu5Ac2en was near 100% (6.01 mg). However, complete purification of the mixture was not successful. ^{1}H-NMR (300 MHz, D_2O): δ 2.004 (s, 3H, NCO$\underline{CH_3}$ of epoxide); 1.978 (s, 3H, NCO$\underline{CH_3}$ of diol), 3.444 (dd, 1H, H-7), 3.575 (dd, 1H, H-9'), 3.801 (dd, 1H, H-9), 3.930 (ddd, 1H, H-8), 3.978 (d, 1H, H-3), 4.052 (dd, 1H, H-4), 4.164 (dd, 1H, H-5), 4.274 (dd, 1H, H-6); negative FAB-MS: m/z= 306[M-H]$^-$ for epoxy-Neu5Ac and 324[M-H]$^-$ for 2,3-diol-Neu5Ac.

Synthesis of 2-deoxy-2,3-dehydro-N-chloracetyl neuraminic acid (Neu5ClAc2en, B4)

Aqueous triethylamine (20% (v/v)) was prepared with a pH around 11-12. Neu2en (5 mg, 0.0200 mmole) was added to 750 μl H_2O/CH_3CN solution (2/1 (v/v)). The pH of the aqueous solution was adjusted to 8 with 1 M $NaHCO_3$. Excess chloroacetic anhydride (35 mg, 0.20 mmole) was dissolved in 1 ml acetonitrile. The chloroacetic anhydride solution was added dropwise to the mixture with stirring at room temperature over 5 minutes. The pH of the reaction mixture was maintained in 7 to 8 range with 1 M $NaHCO_3$. The progress of the reaction was monitored by TLC. After 2 hours the reaction was complete, the resulting mixture was evaporated to dryness, resuspended in 1 ml H_2O, and passed through size exclusion column (Bio-Rad P-2 column) for further purification. The fractions containing the sialic acid derivatives were combinated and lyophilzed. Yield: 45%. R_f: 0.59; ^{1}H-NMR (300 MHz, D_2O): δ 3.985 (s, 2H, NCO$\underline{CH_2}$Cl), 3.491 (dd, 1H, H-7), 3.565 (dd, 1H, H-9'), 3.827 (dd, 1H, H-9), 3.904 (ddd, 1H, H-8), 4.173 (dd, 1H, H-5), 4.313 (dd, 1H, H-6), 4.496 (dd, 1H, H-4), 5.659 (d, 1H, H-3); nagative FAB-MS m/z= 324[M-H]$^-$, 326 [M-H]$^-$.

Synthesis of 2-deoxy-2,3-dehydro-N-bromoacetyl neuraminic acid (Neu5BrAc2en, B5)

2-Deoxy-2,3-dehydro-N-bromoacetyl neuraminic acid (Neu5BrAc2en) was obtained in a similar manner as N-ClAcNeu2en except that the bromoacetic anhydride was replaced with chloroacetic anhydride. Yield: 45%. R_f: 0.60; ^{1}H-NMR (300 MHz, D_2O): δ 3.689 (s, 2H, NCO$\underline{CH_2}$Br), 3.389 (dd, 1H, H-7), 3.526 (dd, 1H, H-9'), 3.777 (dd, 1H, H-9), 3.927 (ddd, 1H, H-8), 4.102 (dd, 1H, H-5), 4.248 (dd, 1H, H-6), 4.410 (dd, 1H, H-4), 5.578 (d, 1H, H-3); nagative FAB-MS m/z= 368[M-H]$^-$, 370 [M-H]$^-$.

Synthesis of 2-deoxy-2,3-dehydro-N-azidoacetyl neuraminic acid ($Neu5N_3Ac2en$, B6)

2-Deoxy-2,3-dehydro-N-azidoacetyl neuraminic acid was prepared from 2-deoxy-2,3-dehydro-N-aminoacetyl neuraminic acid with excess sodium azide (NaN_3) at 50 ^oC in methanol overnight. 2-Deoxy-2,3-dehydro-N-aminoacetyl neuraminic acid was obtained by the treatment of 2-deoxy-2,3-dehydro-N-chloracetyl neuraminic acid with 0.1 N aqueous NH_3 for 1 hour. Alternatively, N-azidoAcNeu2en could be directly prepared from N-haloacetyl Neu2en by addition of NaN_3 in methanol at 50 ^oC for 24 hours. A P-2 column was used for further purificaton. All operations with the azidoacetyl were carried out in darkness to prevent decomposition of the product. Recovery: 80%. IR (λ_{max}): 2114 cm^{-1} ; ^{1}H-NMR (300 MHz, D_2O): δ 3.814 (s, 2H, $NCOCH_2N_3$), 3.494 (dd, 1H, H-7), 3.529(dd, 1H, H-9'), 3.721 (dd, 1H, H-9), 3.908 (ddd, 1H, H-8), 3.916 (dd, 1H, H-5), 4.458 (dd, 1H, H-6), 4.581 (dd, 1H, H-4), 5.722 (d, 1H, H-3); negative FAB-MS: m/z= 331$[M-H]^-$, 303$[M-N_2-H]^-$.

Small-scale synthesis of 7,9-O-*m*-nitrobenzylidiene 2,3-dihydro-N-acetyl neuraminic acid (NB-Neu5Ac2en, B7)

Neu5Ac2en and *m*-nitrobenzylidiene dimethylacetatal were dried in a desiccator overnight. Neu5Ac2en (5 mg, 0.017 mmole) was dissolved in 200 μl of 0.01% TFA acetonitrile solution. Dimethylacetyl m-nitrobenzylidene (13.396 mg, 0.068 mmole) was added to the mixture and stirred overnight. The mixture was neutralized with triethylamine (1 drop) and reduced to dryness under N_2. The mixture was desalted by passing through a C_{18} SepPAK cartridge with the following sequence of solvents: H_2O (2ml), H_2O/MeOH (1/3; 2ml), MeOH (2ml). The product was eluted with the

H_2O/MeOH (1/3) fraction. R_f: 0.455; Yield: 78%; ^{1}H-NMR (300 MHz, D_2O): δ 1.978(s, 3H, NCO$\underline{CH_3}$), 3.209(dd, 1H, H-7), 3.559(dd, 1H, H-9'), 3.763(dd, 1H, H-9), 3.903(ddd, 1H, H-8), 4.182(dd, 1H, H-5), 4.344(dd, 1H, H-6), 4.505(dd, 1H, H-4), 5.617(d, 1H, H-3), 5.805(s, 1H, $OCOC_6H_4NO_2\underline{H}$); 5.927(s, 1H, $OCOC_6H_4NO_2\underline{H}$); 7.195-7.241(m, 1H, Ph), 7.527-7.594(m, 1H, Ph), 7.732-7.749(m, 1H, Ph), 8.08-8.209(m, 1H, Ph); negative FAB-MS: m/z 423[M-H]$^-$.

Synthesis of 9-O-*m*-aminobenzyl-2,3-dehydro-N-acetyl neuraminic acid (AB-Neu5Ac2en, B8)

9-O-*m*-Aminobenzyl-2,3-dehydro-N-acetylneuraminic acid was prepared by the reduction of 9-O-*m*-nitrobenzyl-2,3-dehydro-N-acetylneuraminic acid with palladium-poly (ethylenimine) (Pd-PEI) as a catalyst in H_2O/MeOH (5/1) in a sealed vial under a constant H_2 gas presure at room temperature overnight[38]. After the catalyst was removed, the filtrate was evaporated to dryness under N_2. Chromatography on a P-2 column was used for further purification. 9-O-*m*-Aminobenzyl-2,3-dehydro-N-acetylneuraminic acid was obtained by reducation of 7,9-*m*-nitrobenzylidene-2,3-dehydro-N-acetylneuraminic acid with sodium cyanoborohydride ($NaBH_3CN$) and hydrogen chloride gas (in ether) in THF for 2 hours. The R_f: 0.55; ^{1}H-NMR (300 MHz, D_2O): δ 1.978(s, 3H, NCO$\underline{CH_3}$), 3.209(dd, 1H, H-7), 3.559(dd, 1H, H-9'), 3.763(dd, 1H, H-9), 3.903 (ddd, 1H, H-8), 4.182(dd, 1H, H-5), 4.344(dd, 1H, H-6), 4.505(dd, 1H, H-4), 5.617(d, 1H, H-3), 5.851(d, 1H, $OCOC_6H_4NO_2\underline{H_2}$); 5.947(d, 1H, $OCOC_6H_4NO_2\underline{H_2}$), 7.523-7.631(m, 1H, Ph), 7.723-7.80(m, 1H, Ph), 8.138-8.289(m, 1H, Ph); negative FAB-MS: m/z=425[M-H]$^-$.

Large-scale synthesis of 9-O-*m*-aminobenzylidene-N-acetylneuraminic acid (AB-Neu5Ac, B9)

Neu5Ac and *m*-nitrobenzylidene dimethylacetal were dried in a desiccator overnight. Neu5Ac (100 mg, 0.3236 mmole) was dissloved in 1 ml freshly distilled N,N-dimethyl formamide. 1.2 equivilents of *m*-nitrobenzylidene dimethylacetal (76.50 mg) and a catalytic amount of toluenesulfonic acid were added to the mixture. The heterogeneous mixture was allowed to react in a water bath at 70-85 °C until the reactants were completely dissolved, and then allowed to stand for an additional 15 min. The mixture was neutralized with triethylamine (1 drop) and evaporated to dryness. The mixture was desalted by passing down a C_8 reverse phase column (1.5 x 7.0 cm). The following solvent system was employed; H_2O (20 ml), H_2O:MeOH(1:1; 20 ml), MeOH(20 ml), MeOH:$CHCl_3$(1:2; 20 ml), and $CHCl_3$(20 ml). The product, 7,9-*m*-nitrobenzylidene-N-acetylneuraminic acid (NB-Neu5Ac), was eluted with H_2O:MeOH(1:1). The reduction of NB-Neu5Ac was performed as that of NB-Neu5Ac2en. R_f: 0.455; Yield: 78%. ^{1}H-NMR (300 MHz, D_2O): δ 1.978(s, 3H, NCO$\underline{CH_3}$), 3.209(dd, 1H, H-7), 3.559(dd, 1H, H-9'), 3.763(dd, 1H, H-9), 3.903 (ddd, 1H, H-8), 4.182(dd, 1H, H-5), 4.344(dd, 1H, H-6), 4.505(dd, 1H, H-4), 5.617(d, 1H, H-3), 5.851(d, 1H, $OCOC_6H_4NO_2\underline{H_2}$); 5.947(d, 1H, $OCOC_6H_4NO_2\underline{H_2}$), 7.437-7.550(m, 1H, Ph), 7.662-7.760(m, 1H, Ph), 8.112-8.267(m, 1H, Ph); negative FAB-MS: m/z=441[M-H]$^-$, 171[$CH(OH)_2C_6H_4NO_2$]$^-$

Synthesis of 8,9-isopropylidene-2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid (IP-Neu5Ac2en, B10)

Neu5Ac2en (27.3 mg, 0.094 mmole) was dissolved in 6 ml of 0.01% TFA in acetone. A catalytic amount of copper sulfate was added to the mixture. The mixture was refluxed at 50 °C in an oil bath for 4 hours. The reaction was quenched by the addition of Na_2CO_3 (20 mg, 0.188 mmole) and the mixture was filtered to remove solids. The filtrate was evaporated under N_2, resuspended in 1 ml MeOH/H_2O (1/5), and applied to a cation exchange column (CM-Sephadex, 10 x 25 mm) to remove copper sulfate. H_2O was used as an eluent (2 ml). Yield: 100% (30 mg); R_f: 0.514; ^{1}H-NMR (300 MHz, D_2O): δ 1.329 (s, 6H, $\underline{CH_3}OCO\underline{CH_3}$), 2.023 (s, 3H, $NCO\underline{CH_3}$), 3.522 (dd, 1H, H-7), 3.590 (dd, 1H, H-9'), 3.851 (dd, 1H, H-9), 3.911 (ddd, 1H, H-8), 4.172 (dd, 1H, H-5), 4.328 (dd, 1H, H-6), 4.507 (dd, 1H, H-4), 5.664 (d, 1H, H-3); negative FAB-MS: m/z= 302[M-H]$^-$

Results and Discussions

The structures of the potential inhibitors of sialidases synthesized for this study are shown in Fig. 2. The structures of the Neu5Ac2en derivatives were determined by ^{1}H NMR spectroscopy (Table 1). In Neu5Ac2en, the signal of the vinyl proton at C-3 position appeared as a doublet at δ 5.692 ppm. While the H-3_{ax} and H-3_{eq} of Neu5Ac gave signals at δ 1.621 and 2.730 ppm, respectively, they vanished after the conversion was completed. The purity of the product was also shown on the ^{13}C NMR (D_2O, 20 °C) spectrum. The signal for C-2 and C-3 of Neu5Ac2en was present at δ 148.1 ppm

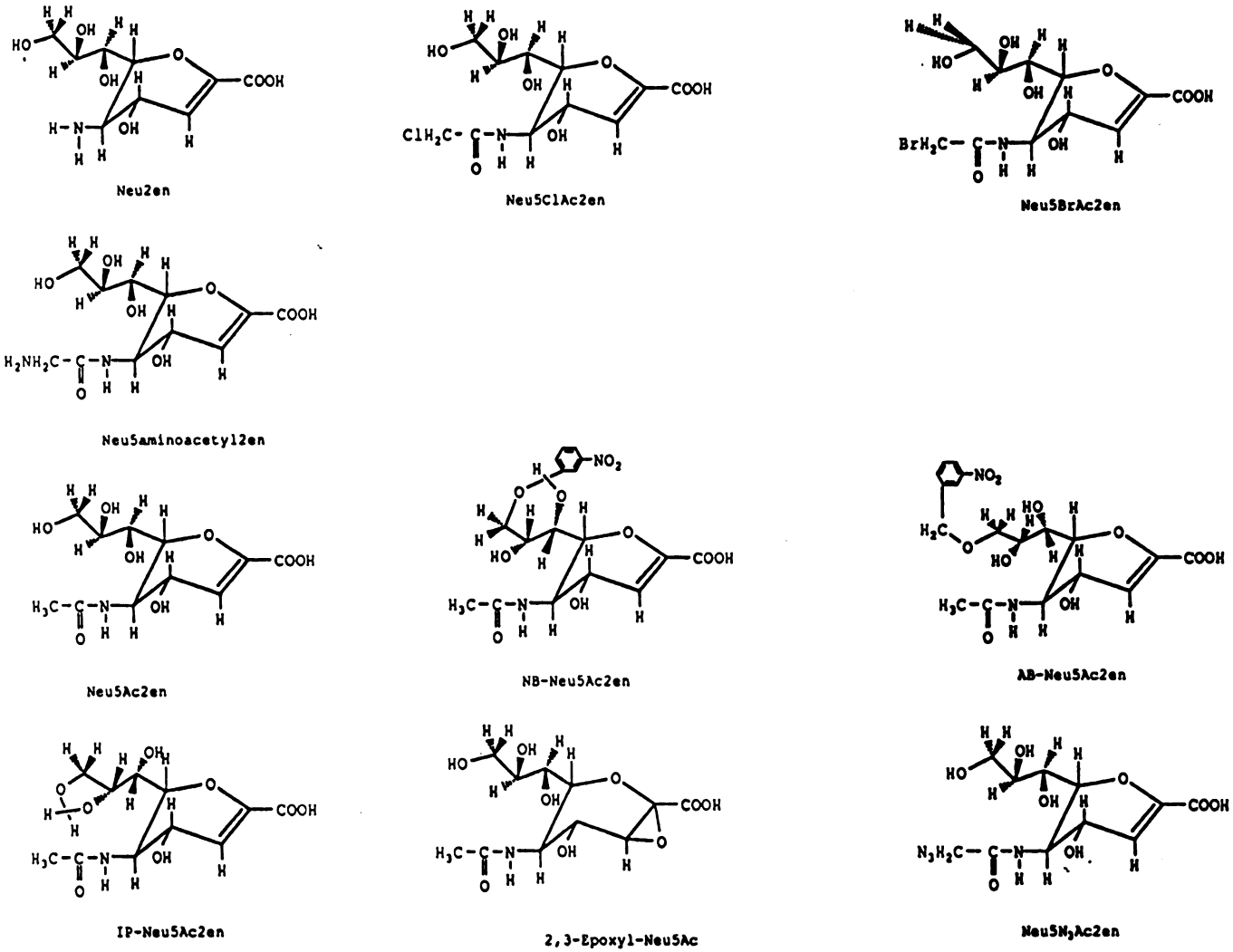


Figure 2: The potential inhibitors of sialidases have been synthesized for this project

Table 1: Relevant ^{1}H-NMR and TLC data for synthetic sialic acid derivatives in this study. Chemical shift value are obtained in ppm downfield from TMS for solution in D_2O at 20 °C.

Compound	Chemical shift (δ)												
	H-3_{ax}	H-3_{eq}	H-4	H-5	H-6	H-7	H-8	H-9	H-9'	5Ac	endo	exo	R_f
Neu5Ac*	1.639	2.484	4.066	3.921	4.141	3.456	3.92	3.90	3.622	2.054	-	-	0.473
Neu5Ac2en	-	5.672	4.470	4.051	4.213	3.601	3.936	3.885	3.646	2.091	-	-	0.643
Neu2en	-	5.681	4.545	4.167	4.345	3.544	3.922	3.855	3.602	-	-	-	0.707
2,3-epoxyl-Neu5Ac	3.978	-	4.052	4.164	4.274	3.444	3.930	3.801	3.575	2.004	-	-	0.507
Neu5ClAc2en	-	5.659	4.496	4.173	4.313	3.491	3.904	3.827	3.565	3.985	-	-	0.59
Neu5BrAc2en	-	5.578	4.410	4.102	4.248	3.389	3.927	3.777	3.526	3.689	-	-	0.610
Neu5N_3Ac2en	-	5.722	4.581	3.916	4.458	3.494	3.908	3.721	3.529	3.814	-	-	-
NB-Neu5Ac2en	-	5.617	4.505	4.182	4.344	3.209	3.903	3.763	3.559	1.978	5.805	5,927	0.455
AB-Neu5Ac2en	-	5.617	4.505	4.182	4.344	3.209	3.903	3.763	3.559	1.978	5.851	5.947	0.55
IP-Neu5Ac2en.	-	5.664	4.507	4.172	4.328	3.522	3.911	3.851	3.590	2.023	1.329+	-	0.514

* Kaverkamp , (1979a)

\+ The chemical shift of methyl proton of IP-Neu5Ac2en

and δ 107.8 ppm, respectively; those of C-2 and C-3 in Neu5Ac are δ 98.42 and 41.92 ppm, respectively. Other features of the spectra were essentially the same as those reported for the anticipated product. Thus, it is indicated that no epimerisations or further dehydrations occurred.

The spectral pattern of Neu2en was similar to that of Neu5Ac2en (Fig. 3, Table 1). However, the signal of acetamino proton at 2.091 ppm in Neu2en disappeared. The chemical shift for the vinyl proton at C-3 position of Neu2en was observed as a doublet at 5.832 ppm while the amino group was protonated. In 2,3-epoxy-Neu5Ac, the signal of the proton at the C-3 position was shifted from 5.692 ppm to 3.978 ppm. The β-effects for the H-4 of the epoxide group was an upfield shift of about 0.5 ppm. Chloroacetyl substitution at the N5 position moved the signal to 3.985 ppm. The long distance effects in H-4, H-5, and H-6 resulted in a downfield shift of about 0.1 ppm. The chemical shift for the endo and exo benzylidene proton on IPNB-Neu5Ac2en were present at 5.851 and 5.947 ppm, respectively. The β-effect of substitution on the H-7 and H-9 protons caused an upfield shift of about 0.2-0.4 ppm. The nitro reduction caused the chemical shift of aromatic protons to move downfield about 0.3 ppm. In IP-Neu5Ac2en, the chemical shift of methyl protons appeared at 1.329 ppm. A similar β-effect was also observed for H-8, H-9 protons.

The ^{1}HNMR spectral data of N-azidoacetyl Neu2en are presented in Fig. 4 and Table 1. The FT-IR spectra of the photoreactable compound are shown in Fig. 5 (see experimental for detail). The substitution of an azido group on the acetyl group (3.86 ppm) provided less deshielding than that of the more electronegative chloride atom on

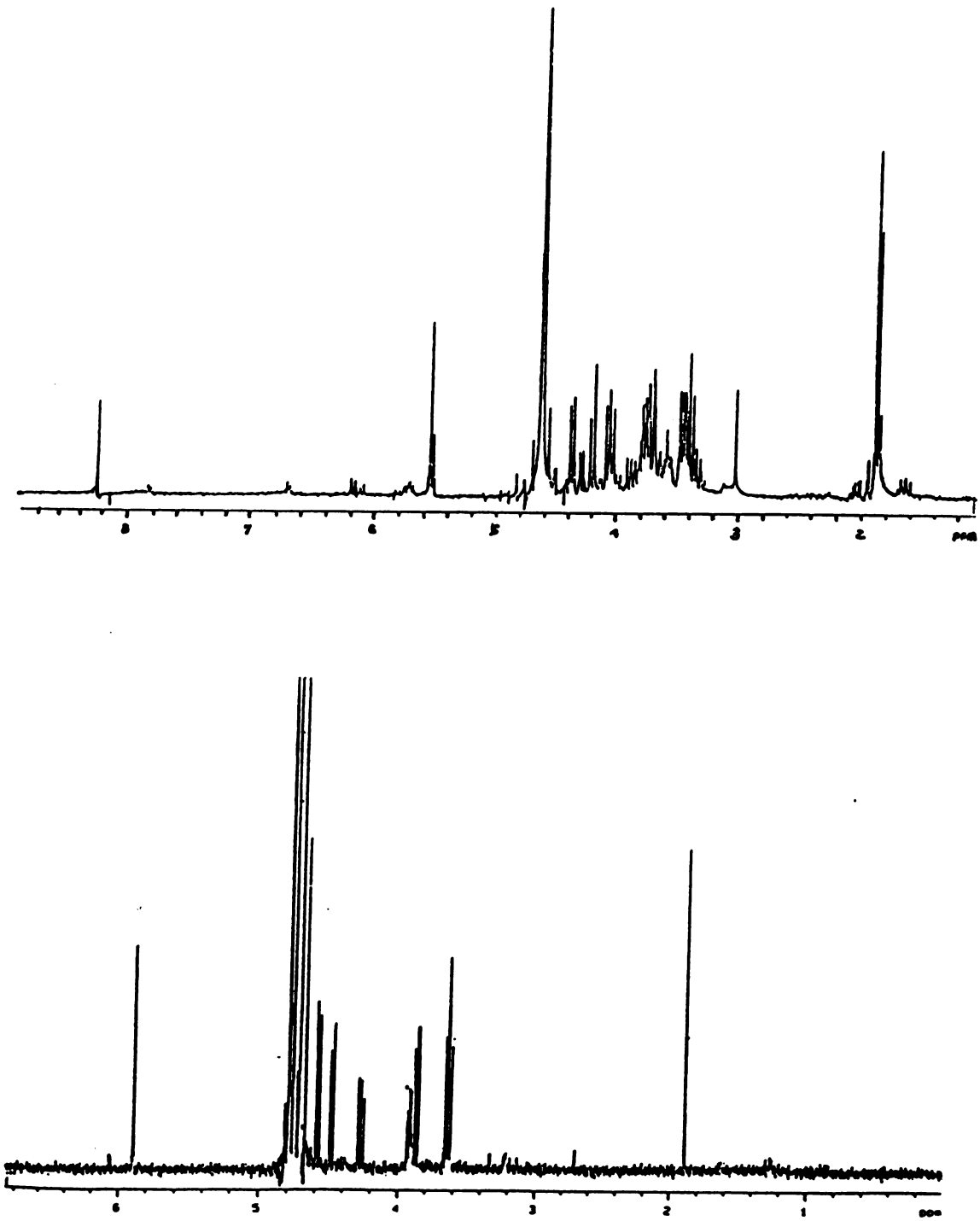


Figure 3: 300MHz ^{1}HNMR spectra of (A) Neu5Ac2en (B) Neu2en. Both samples were dissolved in D_2O and recorded at 20 °C.

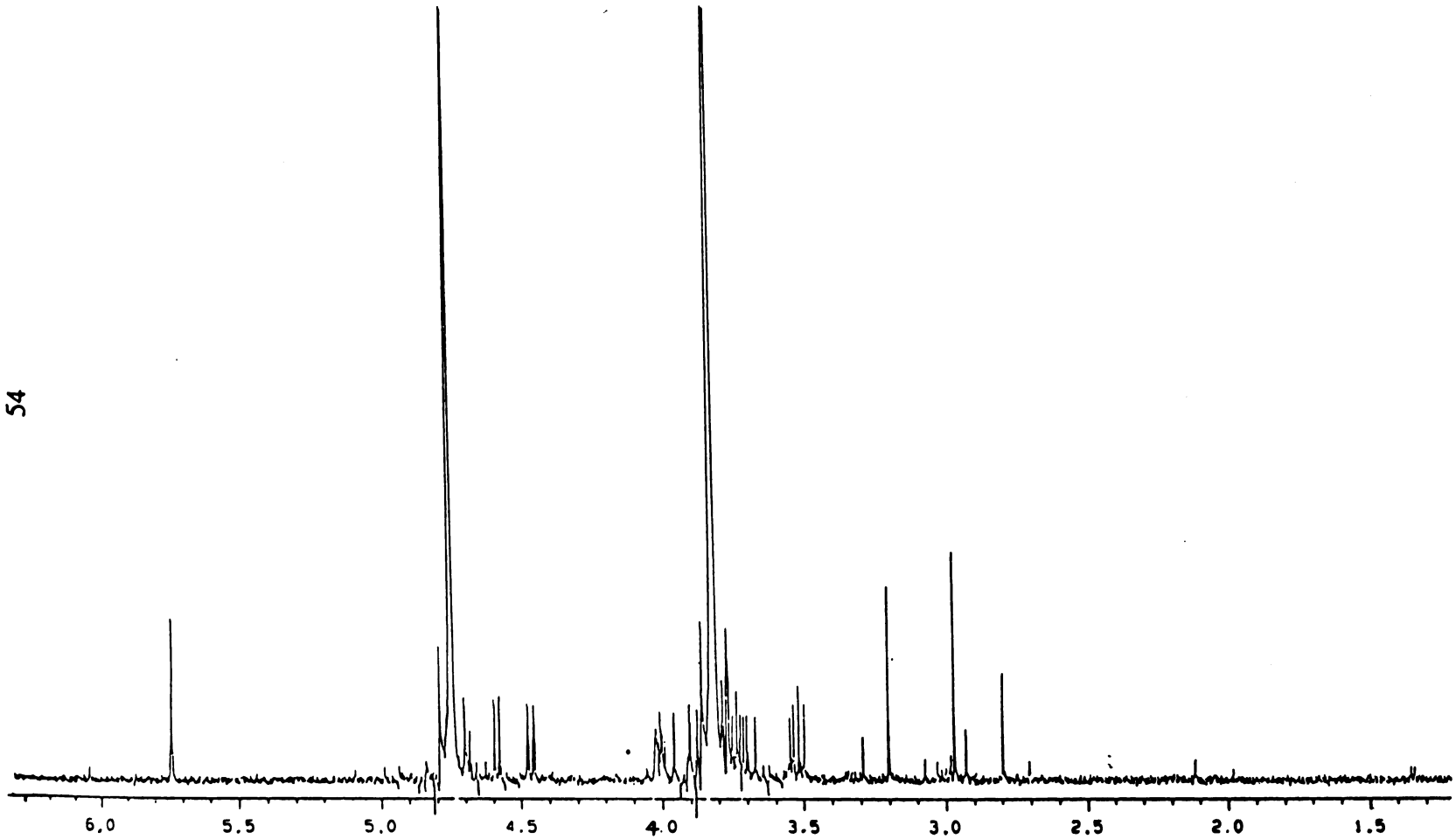


Figure 4: 300MHz ^{1}HNMR spectra of Neu5N_3Ac2en. Samples was dissolved in D_2O and recorded at 20 °C.

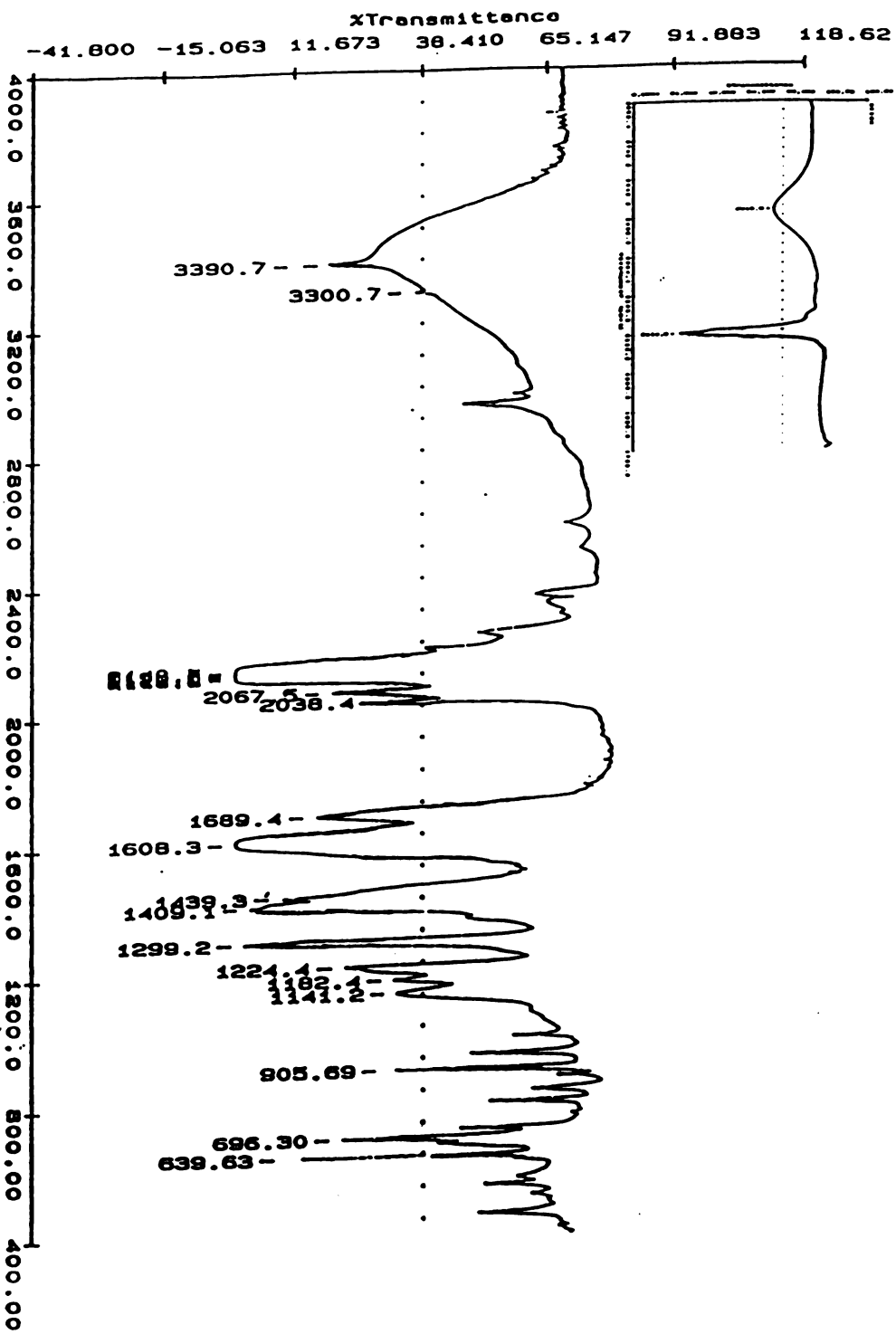


Figure 5: The whole range of infra-red absorbance spectrum of $Neu5N_3Ac2en$. The sample was prepared in D_2O and the spectrum was recorded by using $CaCl_2$ cell. Inset, the IR spectrum of $Neu5N_3Ac2en$ within the absorbance region of azido group.

the acetyl group present at 3.985 ppm. The results have demonstrated that the compound has been modified at N-5 position with an azidoacetyl although a photodecomposition experiment was not preformed to examine the photolysis products.

The conversion of Neu5Ac to Neu5Ac2en is a dehydration process. However, the acid lability of Neu5Ac caused rapid degradation during dehydration either at mild temperature or with mild acid. To overcome these disadvantages, a protecting group was required to activate the C-2 hydroxyl group of Neu5Ac. One or more deprotection steps are also required before elimination. In this work, we effected protection of the primary and secondary hydroxyl groups and activation of the C-2 tertiary hydroxyl group for elimination by preacylating with trifluoracetic anhydride in presence of N,N-dimethyl-4-aminopyridine. The trifluoroacetyl group not only provides a good leaving group to activate the hydroxy group at C-2 but also deactivates other hydroxyl groups during the elimination reaction. The trifluoracetates at other pyranose ring positions are able to withdraw electron density and to prevent the formation of the electron-deficient species during dehydration. The role of the N,N-dimethyl-4-aminopyridine in this reaction is critical in promoting the acylation of the sterically-hindered tertiary hydroxyl group under mild conditions.

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CHAPTER 3

INHIBITION STUDIES FOR SYNTHETIC SIALIC ACID ANALOGUES

Running Title:Synthetic Analogues of Sialic Acid and Their Biological Application

ABSTRACT

Eight derivatives of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en), described in the previous chapter, were investigated for their ability to inhibit *Vibrio cholerae* neuraminidase using 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4MU-Neu5Ac) as the substrate. In a kinetic study, seven of the eight analogues of Neu5Ac2en were shown to be competitive inhibitors with K_i values ranging from 10^{-4} M to 10^{-5} M. No inhibition was observed for the epoxide-Neu5Ac2en compound.

INTRODUCTION

Neuraminidase (sialidase) is a class of enzyme found in bacterial, viral and mammalian origin which selectively hydrolyzes the terminal sialic acid moieties from sialoglycoconjugates. Bacterial neuraminidases have been widely used to evaluate the neuraminidase inhibitors since large amounts of those enzymes were available. *Vibrio cholerae* neuraminidase was chosen for this study. This enzyme has maximum activity at pH 4.6, approximate K_m of 1.5 x 10^{-3} M with 4MU-Neu5Ac as the substrates, and was activated by 4 mM $CaCl_2$[1]. Inhibitors of neuraminidase have been divided into two groups according to whether their structures were related to sialic acid or not[2]. To specifically block the activity of bacterial neuraminidases, the inhibitors were required to have the structure related to the substrate or the product of the enzyme. 2-Deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en) and its methyl ester, transition state analogues of neuraminidase[3], have been shown to be competitive inhibitors of various neuraminidase with K_i values between $5x10^{-3}$ and $5x10^{-6}$ M depending on the enzyme

sources[4]. Additionally, it has been reported that naturally occurring substrates with various substitutions of Neu5Ac affect the cleavage rate of sialic acid by neuraminidase *in vivo* [5,6]. It has been concluded that Neu5Ac2en and its derivatives are good tools to study the enzyme properties because Neu5Ac2en is able to inhibit all neuraminidase(s) from viral, mammalian, and bacterial sources[7]. With regard to analogues of Neu5Ac2en, the degree of inhibition by compounds with different N-5 acyl groups has been reported by Meindle and Tuppy[4]. No inhibitory activity was observed for an acidic or basic substitution of N-acyl such as aminoacetyl or carboxyacetyl group. On the other hand, the N-acyl substitutents with a neutral group such as chloroacetyl or fluoroacetyl appeared to inhibit the enzyme to a certain degree[4]. Increasing the length of N-acyl substitution lowered the inhibitory effect in *V. Cholerae* neuraminidase. For example, the inhibitory effect for N-butyryl substituted Neu2en was 10 times less than that of Neu5Ac2en. When the N-acyl group was replaced with a benzoyl group, the K_i value was 3.0×10^{-2} M (Km=1×10^{-3} M for 4MU-Neu5Ac as substrate). The activity of bacterial and mammalian neuraminidases was completely blocked by acetylation of 4-hydroxyl group of Neu5Ac2en[8,9], while the inhibition constants of 4-epi-Neu5Ac2en was 2.7×10^{-4} M (comparison Km=5 mM for *V. cholerae* sialidase)[10]. The replacement of 9-hydroxyl with photolabelling compounds has been used to characterize *C. perfringens* neuraminidase[11,12]. These derivatives appeared to have K_i value similar to Neu5Ac2en (1.5×10^{-5} M). However, no report has been published about the inhibitory effect of 9-hydroxy in *V. cholerae* neuraminidase. The most potential synthetic inhibitor to date is 2-deoxy-2,3-dehydro-N-trifluoroacetyl neuraminic acid

(Neu5CF_3Ac2en) with a K_i value about $2.5x10^{-6}$ M[13].

In this paper, we describe and compare the inhibitory properties of Neu5Ac2en derivatives against *V Cholerae* neuraminidase *in vitro*. These Neu5Ac2en derivatives synthesized in this laboratory could be classified into two groups differing in the position of substitution N-acyl or 9-hydroxyl.

Materials and Methods

Materials. The following materials were purchased from commercial sources: 4MU-Neu5Ac, 4-methylumbelliferone (4MU), Neu5Ac, Neu5Ac2en and *V. Cholerae* neuraminidase (type IV, 0.7 U ml^{-1}) from Sigma Chemical Company (St. Louis, MO). The inhibitors were prepared as described in a previous chapter. Commercial Neu5Ac2en was used as the reference for the inhibition assay against *V. Cholerae* neuraminidase. All other chemicals and solvents were reagent grade or better. Aminco fluoro-colorimeter was obtained from Americaan Instrument Co. (Silver Spring, MD).

The preparation of enzyme assay. Neuraminidase activity was determined with a modification of the Potier et al. method[1]. The activity of *V. Cholerae* neuraminidase solutions purchased from commerical sources (Sigma) were diluted to 4 $\mu U\ \mu l^{-1}$ and used for these assayes. One unit (U) of neuraminidase activity was defined as 1 μmole 4MU-Neu5Ac hydrolysed per minute. Unless otherwise mentioned, a fluorogenic substrate (4MU-Neu5Ac) was used as the artificial substrate of neuraminidase. *V. Cholerae* neuraminidase activity was measured at 37 ^oC in a 0.2 ml total volume containing 0.1 M sodium acetate buffer (pH 5.0), 4 mM $CaCl_2$ and varied concentration of 4MU-

Neu5Ac and inhibitors for its optimum activity. For the blank sample, the acetate buffer was used to replace the enzyme. The hydrolysis reaction was terminated by the addition of 0.2 M glycine-sodium hydroxide solution (1 ml, pH 10.7) after 45-60 minutes of incubation. Free 4MU, the hydrolysis product, was measured by fluorescence emission at 450 nm and excitation at 365 nm with an Aminco fluoro-colorimeter. The fluorimeter was calibrated with 25 and 50 nM 4MU and referred as 50% and 100% of relative fluorescence intensity (RFI), respectively (Fig. 1). The relative fluorescence intensity of samples was determinated in an aliquot (200 μl) in a total volume 2.2 ml stopper solution containing 0.2 M glycine-sodium hydroxide (pH 10.7).

Experiments for Sialidase inhibition. For the determination of K_m values, 4MU-Neu5Ac was hydrolysed at the final concentration of 1, 0.5, 0.25, 0.1 mM under the experimental condition. The concentration of inhibitors are tested at 0.01 mM, 0.1 mM, and 1 mM in preliminary inhibition experiments. For the determination of Ki value, Neu5Ac2en and its analogues were investigated at 0.01, 0.05, and 0.1 mM using the substrate concentration described above. Two replications were prepared at each set of concentration. The kinetic models of these synthetic inhibitors were determined by the Lineweaver-Burk double reciproical plots. The constants (K_m and K_i values) were also calculated by fitting the data with a nonlinear regression program of Segal to the variants of the Michaelis-Menten equation for competitive, noncompetitive, uncompetitive and mixed inhibition[14].

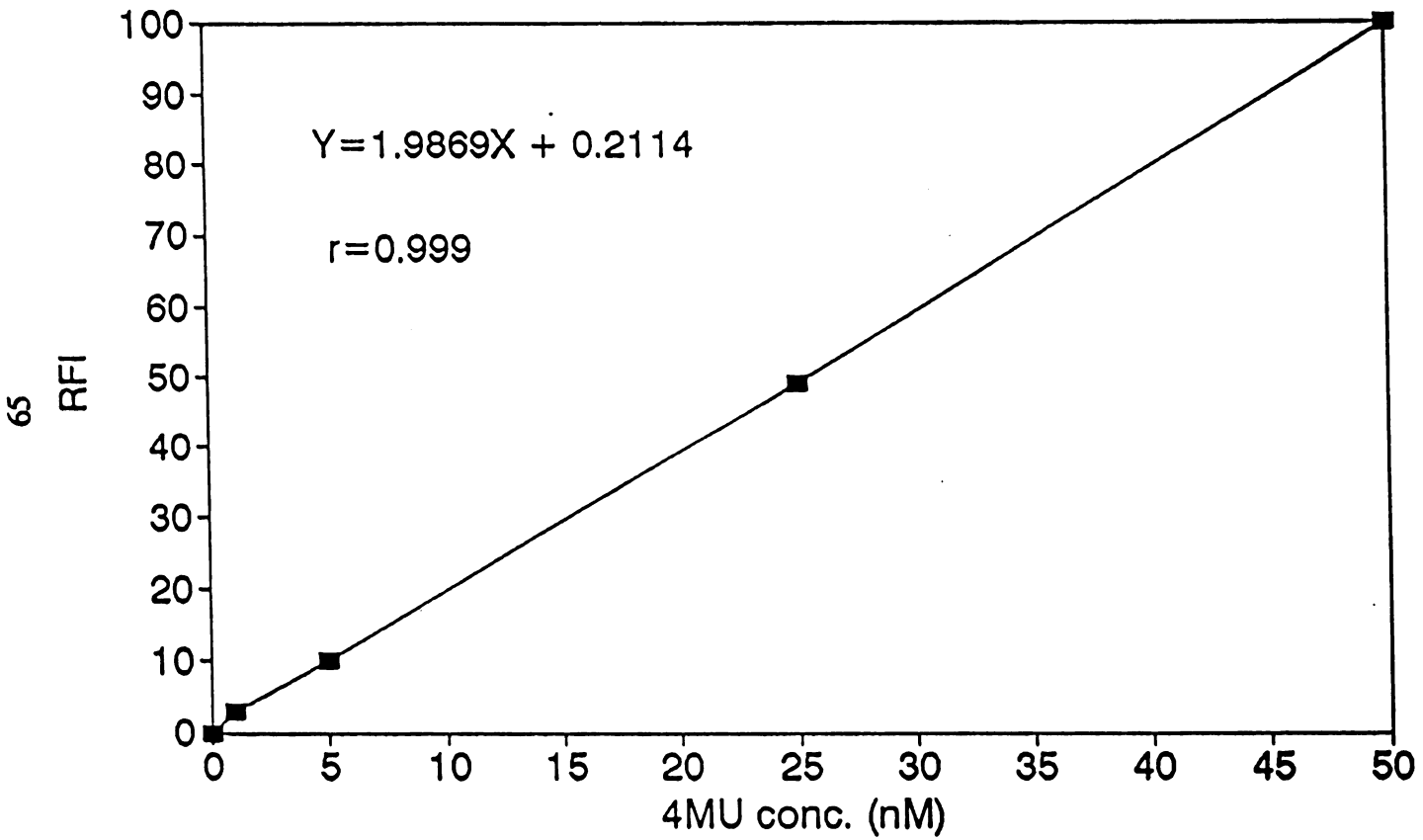

Figure 1: The standard curve for the relative fluoresence intensity (RFI) of 4MU from 0 nM to 50 nM.

Results and Discussions

The inhibition effect of these transition state analogues for *V. cholerae* neuraminidase activity has been evaluated by the inhibition assay with 1 mM substrate. The results summarized in Table 1 indicate that Neu5Ac2en and its derivatives varied at N-acyl and 9-hydroxyl positions are inhibitors of *V. cholerae* sialidase *in vitro*. The *V. cholerae* sialidase activity was inhibited from 90% to 20% varied with the concentration and substitution of inhibitor. For example, the remaining activity with the inhibitor (Neu5NClAc2en) at 1 mM, 0,1 mM, and 0.01 mM using 1 mM 4MU-Neu5Ac as substrate condition are 4%, 13%, and 24%, respectively, which has shown to be the strongest inhibitor in this study with a Ki value about 2.1×10^{-5} M. On the other hand, the remaining activity with AB-Neu5Ac2en at 1 mM, 0,1 mM, and 0.01 mM under 1 mM substrate condition are 23%, 58%, and 74%, respectively, which is the weakest inhibitor in this study with a Ki value about 1.1×10^{-4} M. Comparing the significance of inhibitory between commerical Neu5Ac2en (Neu5Ac2en(C)) and synthesized Neu5Ac2en (Neu5Ac2en(S)), the activity measured with 1 mM substrate is inhibited up to 82%, and 88% ($K_i = 3.5 \times 10^{-5}$ M), respectively. It suggested that no difference is observed in the inhibition effect for *V. cholerae* sialidase activity with these two Neu5Ac2en sources. The interesting analogue, epoxy-Neu5Ac, containing some degree of 2,3-diol-Neu5Ac has shown no inhibition effect for sialidase from *V. cholerae*.

The value of K_m determined from Lineweaver-Burk plot is about 3.86×10^{-3} M with 4MU-Neu5Ac as substrate for *V. cholerae* sialidase. Apparent K_m value similar to the result from Schreiner *et. al.* study (3.01×10^{-3} M)[13], but it has 2-3 times difference

Table 1: The remaining activity of *Vibrio cholerae* sialidase with synthesized Neu5Ac2en derivatives.
The activity was measureed in 0.1 M acetate buffer, pH 5.0 at 37 °C using 1 mM 4MU-Neu5Ac as substrate.

Inhibitor	Remaining Activity (%) with 1 mM substrate		
	1 mM	0.1 mM	0.01 mM
Neu5Ac methyl ester	110	106	99
Neu5Ac2en (C)	12	47	63
Neu5Ac2en (S)	18	43	67
Neu2en	16	54	74
Neu5ClAc2en	4	13	24
Neu5BrAc2en	16	49	89
$Neu5N_3Ac2en$	19	52	82
NB-Neu5Ac2en	10	52	79
AB-Neu5Ac2en	23	58	74
IP-Neu5Ac2en	14	45	71
Epoxyl-Neu5Ac*	78	106	117

comparing the reports from Potier *et. al.* ($1.5x10^{-3}$ M)[1]. Based on Lineweaver-Burk plots of the inhibition kinetics, these analogues substitued on the N-5 or 9-hydroxyl have been shown to be competitive inhibitors versus the substrate 4MU-Neu5Ac. The inhibition constant (K_i) of these compound are in the range $1.1x10^{-4}$ M to $2.1x10^{-5}$ M and summarized on Table 2. Inhibtion effect of *V. cholerae* sialidase by Neu5Ac2en(S) is present in Fig. 2 with a K_i of $3.5x10^{-5}$ M, which is 1.5 times higher than the previous report ($2.5x10^{-5}$ M)[15]. For Neu2en and $Neu5N_3Ac2en$, the inhibition efficacy are shown in Fig.3 and 4, respectively. The inhibition constant ($K_i=4.4x10^{-5}$ M) of Neu2en is found to be nearly equal to that of Neu5Ac2en ($3.5x10^{-5}$ M) in our study, which is three orders of magnitude higher than that of Zbiral *et. al.* study ($2.9x10^{-2}$M)[16]. This result could be explained by the neutral amine group on the C-5 position instead of a positive charge of the quaternary ammonium group. The inhibition by $Neu5N_3Ac2en$ is competitive with an apparent K_i ($6.2x10^{-5}$ M). This finding is encouraged and will allow the analogue to use for synthesis phtotoactivtable probe(s). The K_i value of other N-acyl substituted analogues such as Neu5NClAc2en, Neu5NBrAc2en, and N-aminoacetyl Neu2en are derived in the same manner as described in the methods (see Table 2), and are similar to the results from Meindl *et. al.* [4].

The kinetic results demonstrate that Neu5Ac2en derivatives with substitution on the 9-hydroxyl position are competitive inhibitors of sialidase from *V. cholerae*. The order of decreasing inhibition of this series compound is Neu5Ac2en, IP-Neu5Ac2en, NB-Neu5Ac2en, and AB-Neu5Ac2en. The inhibition constant of each inhibitor is shown in Table 2. Apparent K_i value of IP-Neu5Ac2en ($3.9x10^{-5}$ M), which formed an

Table 1: Inhibition of *Vibrio cholerae* neuraminidase by Neu5Ac2en and synthetic analogues

Inhibitor	Ki [mM]±SD
Neu5Ac2en (C)	$3.5x10^{-2}±6.7x10^{-3}$
Neu5Ac2en (S)	$3.5x10^{-2}±5.1x10^{-3}$
Neu2en	$4.4x10^{-2}±1.2x10^{-2}$
Neu5NClAc2en	$2.1x10^{-2}±9.4x10^{-3}$
Neu5NBrAc2en	$5.7x10^{-2}±1.4x10^{-2}$
$Neu5N_3Ac2en$	$6.2x10^{-2}±2.1x10^{-2}$
IP-Neu5Ac2en	$3.9x10^{-2}±1.7x10^{-2}$
NB-Neu5Ac2en	$7.9x10^{-2}±2.9x10^{-2}$
AB-Neu5Ac2en	$1.1x10^{-1}±5.1x10^{-2}$
Epoxyl-Neu5Ac	9.9±4
Control (4MU-Neu5Ac)	Km=3.9

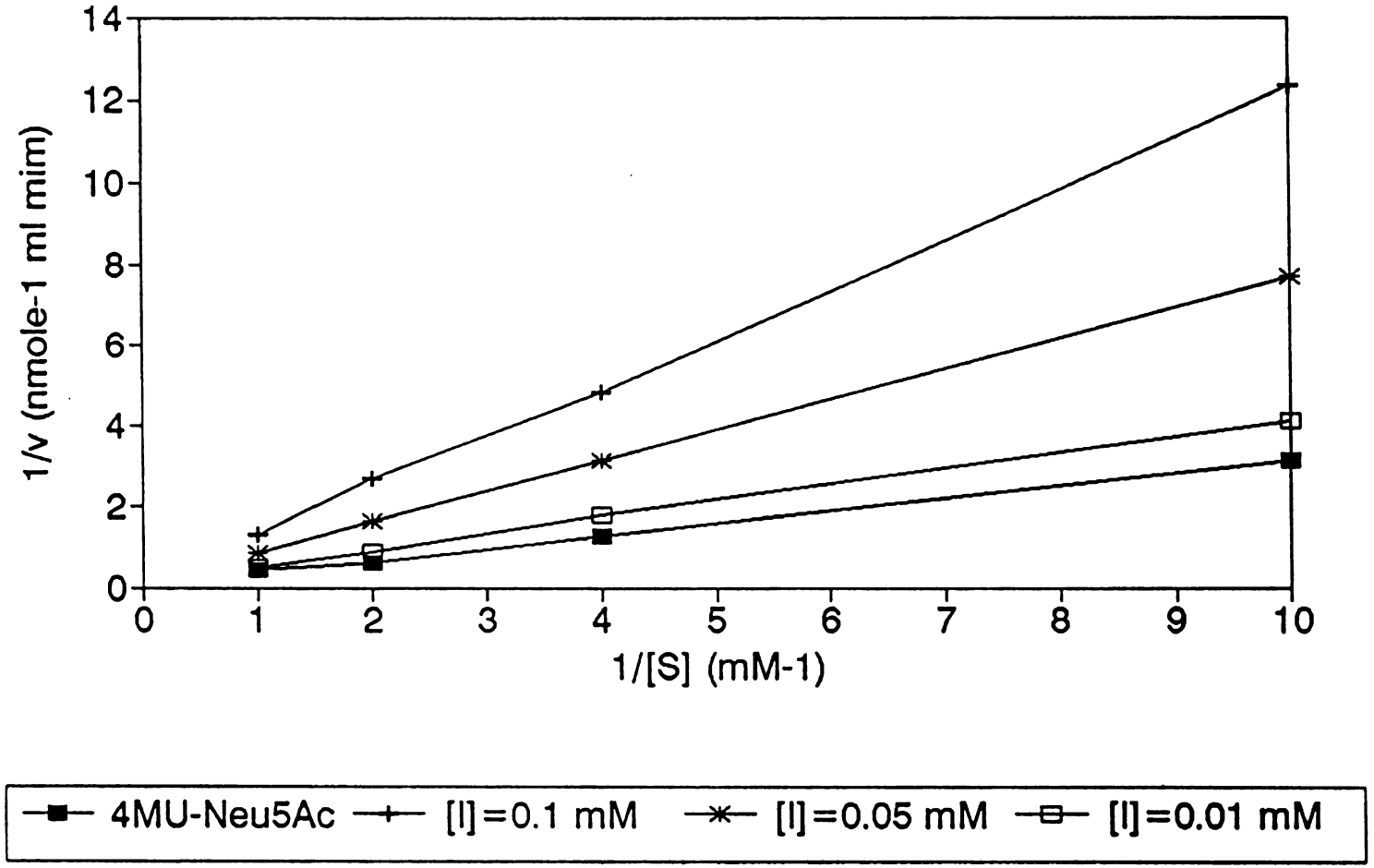

Figure 2: Inhibition kinetics of *V. cholerae* sialidase using 4MU-Neu5Ac as substrate in 0.1 M acetate buffer, pH 4.5 at 37 °C. The applied concentration of inhibitor (Neu5Ac2en(S)) is shown in figure legend.

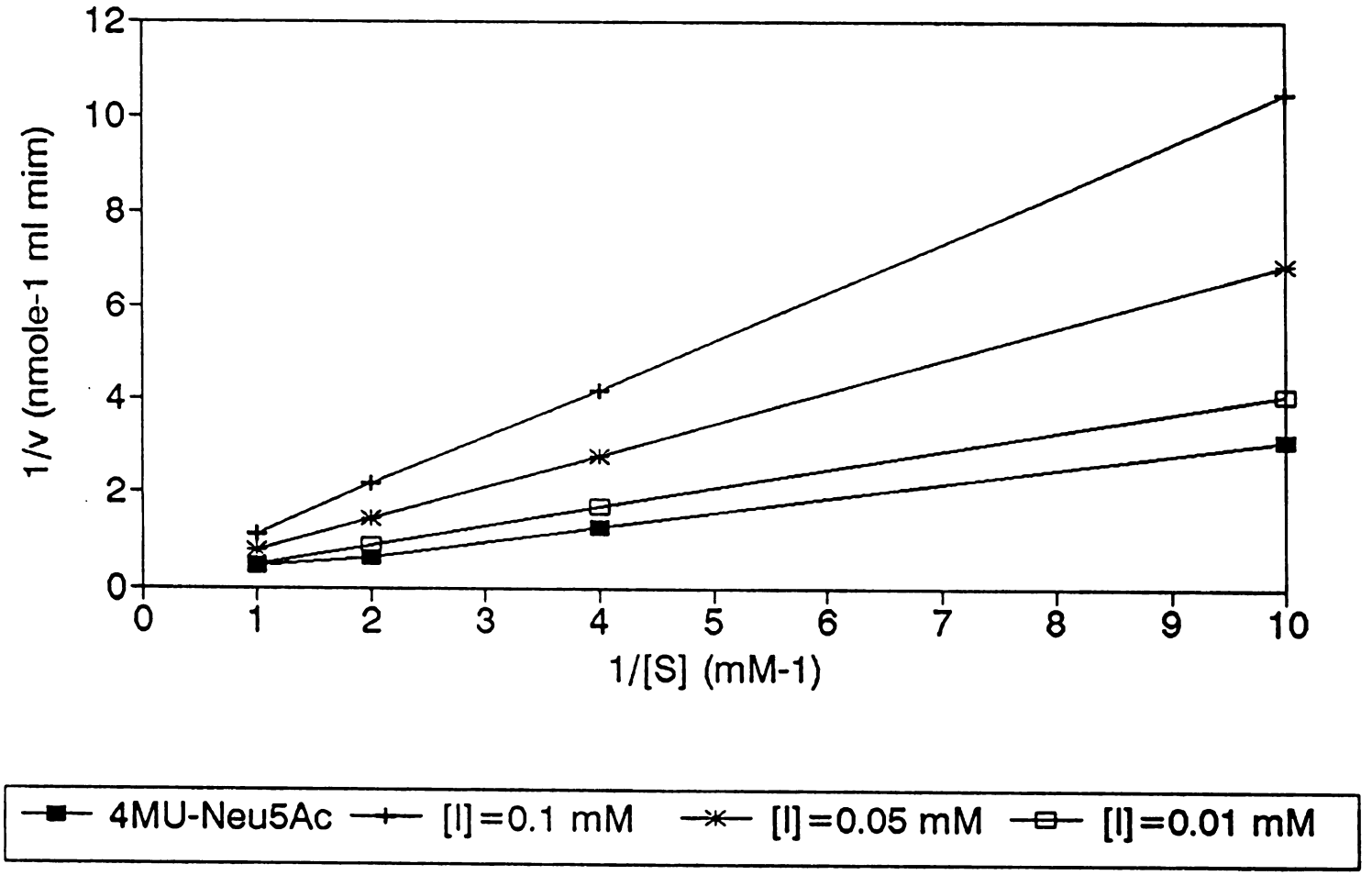

Figure 3: Inhibition kinetics of *V. cholerae* sialidase using 4MU-Neu5Ac as substrate in 0.1 M acetate buffer, pH 4.5 at 37 °C. The applied concentration of inhibitor (Neu2en) is shown in figure legend.

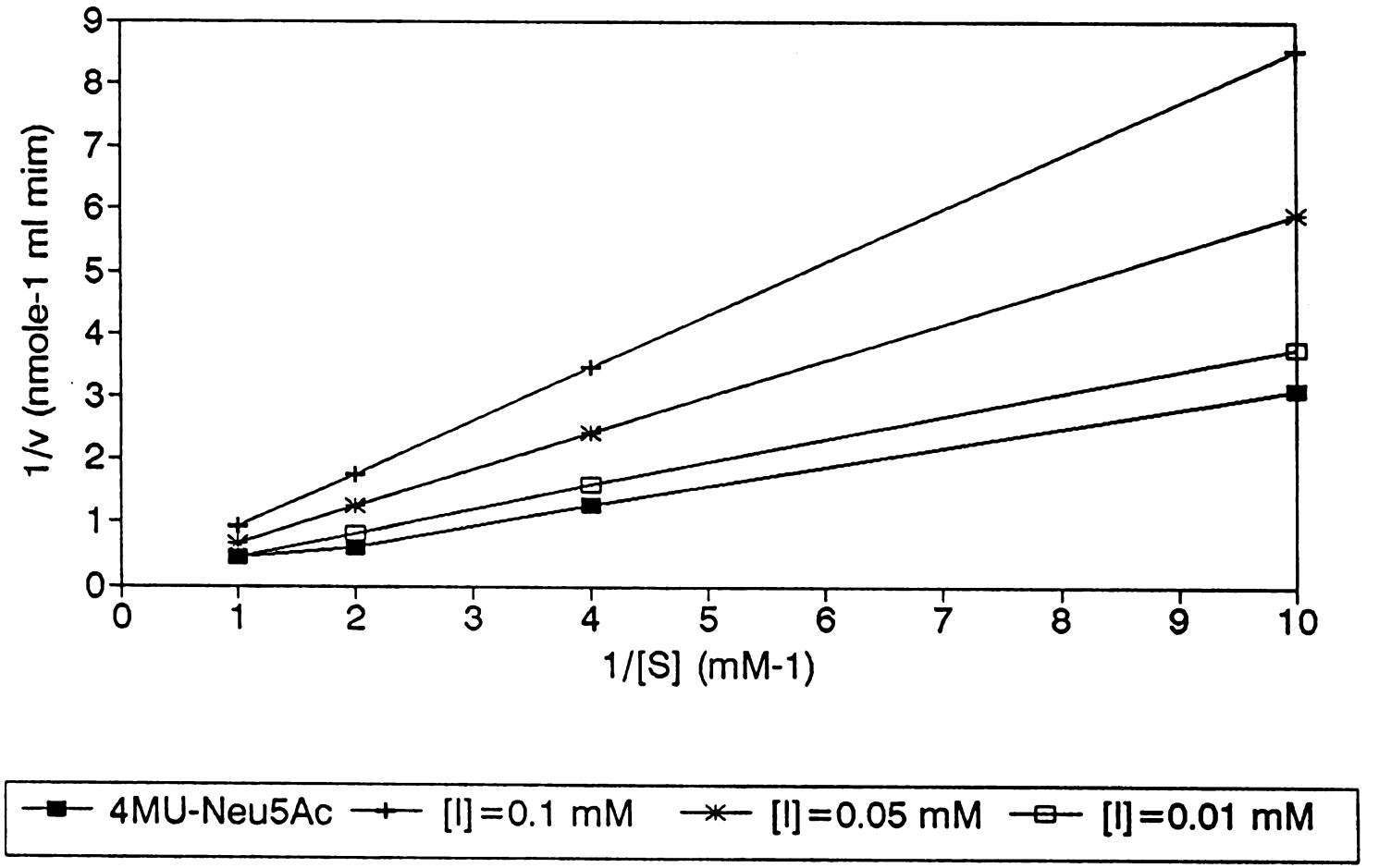

Figure 4: Inhibition kinetics of *V. cholerae* sialidase using 4MU-Neu5Ac as substrate in 0.1 M acetate buffer, pH 4.5 at 37 °C. The applied concentration of inhibitor ($Neu5N_3Ac2en$) is shown in figure legend.

isopropylidene linkage between the 8- and 9-hydroxyl, is likely the same as that of Neu5Ac2en ($3.5x10^{-5}$ M). A similar behavior is observed for the inhibition of NB-Neu5Ac2en (K_i $=7.9x10^{-5}$ M). The inhibitory effect by AB-Neu5Ac2en for *V, cholerae* sialidase ($K_i=1.09x10^{-4}$ M) is 20 fold less than that of Neu5Ac2en (Figure 5); this exception could not be a reasonably explained. Other inhibitors with the C-9 substituted Neu5Ac2en reported from Horst *et. al.* and Warner were shown to be like Neu5Ac2en[11,12]. The sialidase activity influence by deoxyl side chain of Neu5Ac2en analogues has been reported and the K_i range is from $1.1x10^{-5}$ M to $9x10^{-5}$ M[13,15]. The phenomenon indicates that the major force for the inhibition of sialidase causes from the half-chair configuration of sialidase analogue, and the N-acyl residue. The structure and conformation of the side chain of Neu5Ac2en derivatives has lesser influence for their inhibition on sialidase(s). However, a lone-pair electron element on the side chain of Neu5Ac2en congener(s) play a role in the recognition of sialidase.

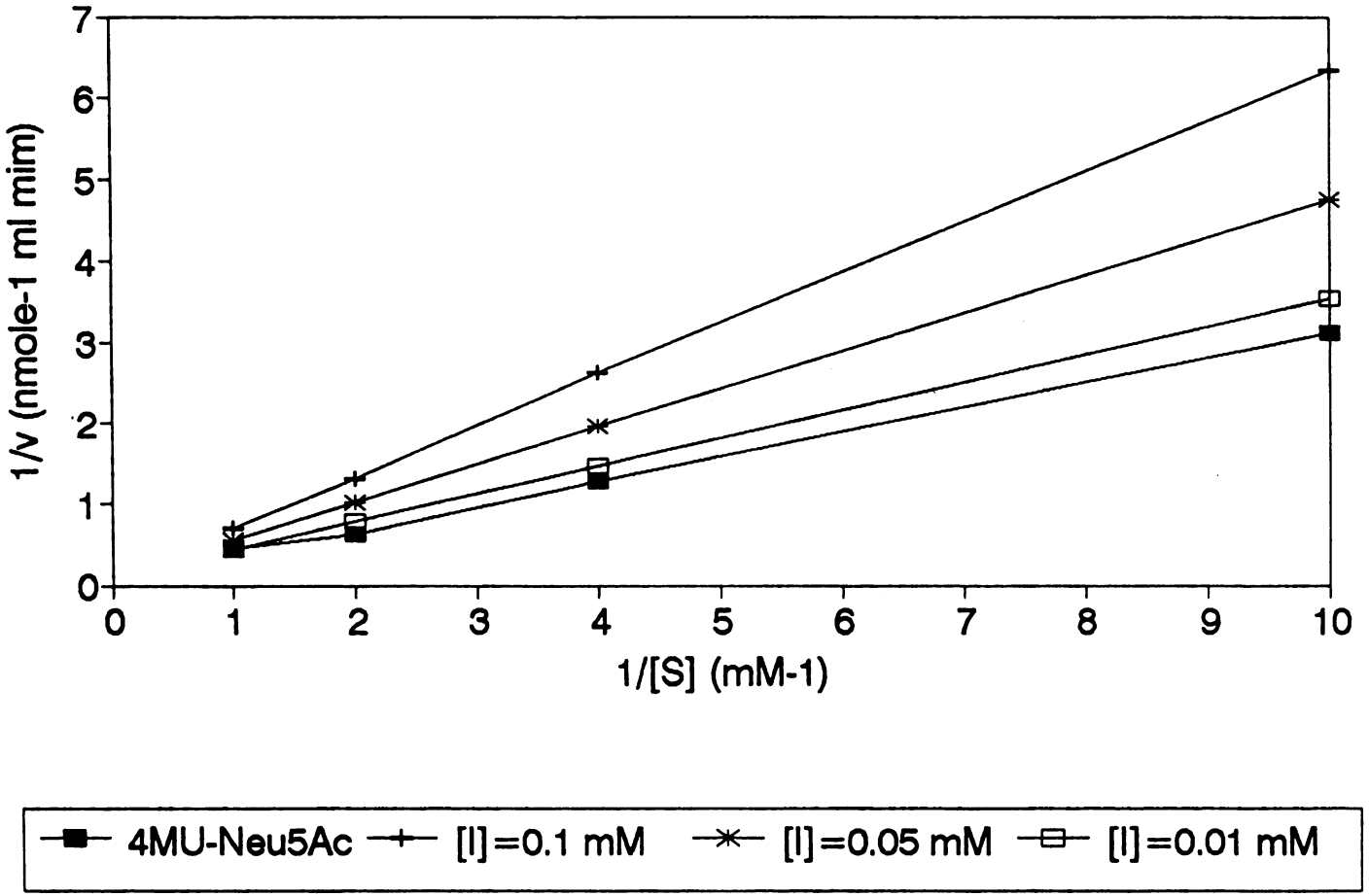

Figure 5: Inhibition kinetics of *V. cholerae* sialidase using 4MU-Neu5Ac as substrate in 0.1 M acetate buffer, pH 4.5 at 37 °C. The applied concentration of inhibitor (AB-NeuAc2en) is shown in figure legend.

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CHAPTER 4

AN AFFINITY CHROMATOGRAPHY METHOD - AN UTILIZATION OF SIALIC ACID DERIVATIVE

Running Title: Synthetic Sialic Acid Analogues and Their Biological Application

ABSTRACT

An affinity chromatography method has been developed for separating neuraminidases from physiological protein mixtures. The affinity column was made by coupling 2-deoxy-2,3-didehydro-N-deacetyl neuraminic acid (Neu2en) to Bio-Rad Affi-Gel 10 activated agarose gel. Bound neuraminidase was eluted from the column with 10% neuraminic acid in water. The totaal activity of the recovered neuraminidase from the affinity column was evaluated using the 4MU-Neu5Ac assay and the peroxidase-amplified assay[1,18]. As judged by activity, 80-100% of the total activity applied to the column was recovered. The physical properties of the recoved neuraminidase was examined by electrophoresis. Enzyme samples analyzed before and after affinity chromatography appeared as 68 kDa, and 16 kDa polypeptide bands while the molecular weight of *V. cholerae* neuraminidase has been previously reported as 24.3 kDa or 90 kDa polypeptides.

INTRODUCTION

Neuraminidase (N-acylneuraminosyl glycohydrolases; EC 3.2.1.18) has been found in a wide range of organisms from bacteria to humans. Procedures for purification of neuraminidase(s) with high specific activity from microorganisms have been well developed[2,3,4]. The activity of most animal neuraminidases has been reported but complete purification has been hampered by the fact that these enzymes are predominantly membrane bound having strong hydrophobic interaction[5,6,7]. In addition their lability, and low concentrations, as well as interference by other factors have added

to the difficulty of their isolation. Neuraminidase activity has also been detected in the cell-conditioned media of human fibroblast cell lines (FS-4 and GM03468A)[1,8,9]. The physical and biochemical properties of this neuraminidase differ from that of the membrane-bound neuraminidases[1,9,10].

The interaction of *Clostridium perfringens* neuraminidase with immobilized ligands such as sialic acid derivatives, and sialyl-glycoconjugates have been studied by Corfield *et. al.*[11]. In their study, adipic acid dihydrazido-Sepharose 4B (AD-Sepharose) and/or polymethylacrylic hydrazido-Sepharose 4B (PAH-Sepharose) were used as solid supports. Their results indicated that neuraminidase was strongly bound to the amino-linked adsorbents, could not be readily eluted with substrate or free sialic acid and recoveries were low (40%). On the other hand, binding of neuraminidase to non-substituted, blocked supports such as alkane chains from two carbons to ten carbons, or 4-nitrophenyloxamic acid were low. Neuraminidase adsorbed onto decyl-agarose support was recovered from the support in 80% yield and it was suggested that hydrophobic interactions between ligands and enzyme were involved. The same authors also reported a similar behavior for neuraminidases from *V. cholerae* and influenza virus strain 2A/Aichi/2/68.

A simple affinity chromatography method for the separation of neuraminidase(s) from other proteins would be a useful tool to extend neuraminidase research. Previous studies with immobilized ligands and neuraminidase(s) indicated that two factors should be considered in preparation of an affinity column. One is the specific binding of ligand with neuraminidase through a neuraminic acid analogue having a free C-1 carboxylic

group[12]. Next is the non-specific hydrophobic interactions between neuraminidase and the column support[13,14]. This paper reports the preparation of an affinity column which selectively absorbs neuraminidase using 2-deoxy-2,3-didehydro-N-deacetyl neuraminic acid (Neu2en) linked with agarose gel (Bio-Rad Affi G-10).

Methods and Materials

Materials: Bio-Rad Affi G-10 gel and poly-styrene EIA/RIA plates (96 wells) were obtained from Bio-Rad laboratories (Richmond, CA). The following substances were purchased from Sigma Chemical (ST Louis, MO): glucose oxidase type V, α-galactosidase, fetuin-agarose type III-A, *V. cholerae* neuraminidase type III (1 U/ml), 4MU-Neu5Ac, *o*-phenylenediamine tablet, bovine serum albumin (BSA), and G_{M1} and G_{D1a} gangliosides. *V. Cholerae* neuraminidase (500 U/ml) was obtained from Koch-Light laboratory (Colnbrook Bucks, England). Cholera toxin B subunit-horseradish peroxidase conjugate was purchased from LIST Biological Laboratories (Campbell, CA). AMICON centricon 10 filter was obtained from Amicon Division (Beverly, MA). Synthesis of Neu2en has been described in the previous chapter. Other solvents and reagents used were analytical grade or higher.

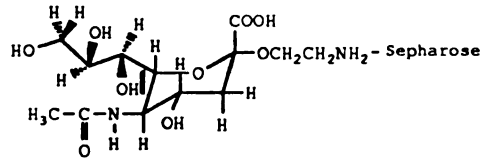
Instrument: An Aminco Fluoro-Colorimeter from American Instrument Co. (Siliver Spring, MD) was used to measure the relative fluorescence intensity (RFI) of free 4MU for 4MU-Neu5Ac assay at 365 nm for excitation and at 450 nm for emission, which was calibrated with 25 and 50 nM 4MU and their RFI was 50 and 100%, respectively. The sensitivity range of the instrument was fixed at 100 with a vernier control. A microtiter

plate reader from Bio-TEK Instruments Inc. (EIA Reader EL-307) was used to obtained the absorbance of the peroxidase-amplified assay at 490 nm.

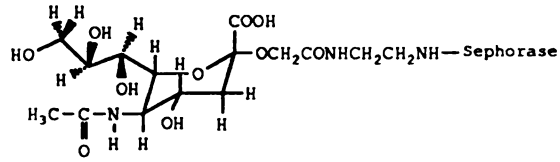
Preparation of immobilized sialic acid analogue on Bio-Rad Affi G-10 gel

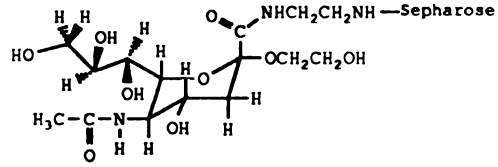
The activated affinity supports (Bio-Rad Affi G-10 gel) had a ten-carbon spacer arm. The immobilized ligand on Bio-Rad Affi G-10 gel was Neu2en (Figure 1C), which is a sialic acid derivative with a free amino group at the N-5 position. The coupling was performed in aqueous conditions following the product instructions[15], which was briefly described as the following. After the gel (1 ml) was washed with cold water (x 4 volumes) and drained through a Buchner funnel, the moist gel cake was transferred to a test tube (15 ml) and the cold ligand solution (20 mmoles/ml of gel) in 100 mM HEPES buffer (pH 9-10) was added. The gel slurry was agitated on a shaker at 4 ^{o}C overnight. Ethanolamine HCl (1 M, pH 8, 0.1 ml/per ml gel) was used to block any remaining active esters in the gel, and it was rocked at 4 ^{o}C for 1 hour. The gel was transferred to a poly-prep chromatography column and washed with water to remove the uncoupled ligand. Subsequently, the column was equilibrated with 100 mM sodium acetate buffer (pH 5.0). *V. Cholerae* neuraminidase (250 mU), glucose oxidase type V (1.25 U), and α-galactosidase (5 U) were mixed in 100 mM sodium acetate buffer (pH 5.0, 1 ml) and applied to the column. Glucose oxidase type V and α-galactosidase were eluted with 100 mM sodium acetate (pH 5.0, 40 ml), washed with H_2O (100 ml), and *V. cholerae* neuraminidase was subsequently eluted with 10% sialic acid (5 ml). The protein fractions were determined by the measurement of the UV absorbance at 280 nm and confirmed with Lowry's protein assay. Each protein peak was to concentrated to 0.5

(A)



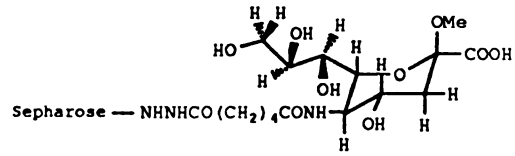
Neu5Ac via α-glycoside linkage



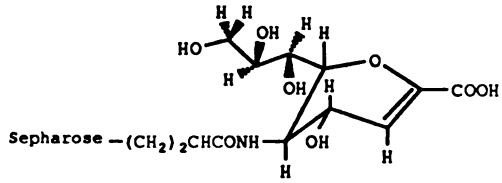


Neu5Ac via amide linkage

(B)

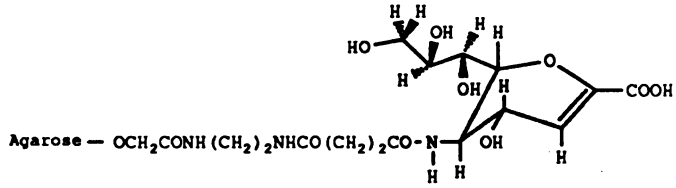


Neu-β-Me via amide linkage



Neu2en via amide linkage

(C)



Neu2ene Bio-Rad Affi G-10

Figure 1: The common applied affinity chromatography (A) Used sialic acid as ligand (B) Used sialic acid, its analogues, and sialylglycoproteins as ligands (C) Used Neu2en as ligand

ml in an AMICON centricon 10 cartridge before the activity assays and electrophoresis. The column was stored at 4 °C with 0.2% sodium azide.

Preparation of a fetuin-agarose affinity column chromatography

A control experiment was performed by preparing a fetuin-agarose column. Fetuin is a sialic acid containing glycoprotein, which should bind neuraminidase. Fetuin-agarose gel (200-300 mg/per ml, containing 2% free neuraminic acid) activated with cyanogen bromide was commercially available. The fetuin-agarose gel (1 ml) was transfered to a poly-prep chromatography column and washed with 100 mM sodium acetate buffer (pH 5.0) at 4 °C. The column was pre-saturated with 1% BSA solution to avoid non-specific binding of *V. cholerae* neuraminidase. Bound neuraminidase was eluted with 50 mM sodium phosphate buffer containing 1 M NaCl (pH 6.5, 10 ml)[11,13,16,17]. The column was stored at 4 °C with 0.2% sodium azide.

The activity assay

4MU-Neu5Ac assay

The assay of Potier *et. al.* was utilized with some modifications[18]. In this study, the activity of *V. Cholerae* neuraminidase was determined before and after affinity chromatography. The starting enzyme activity was 1 U/ml, which was used as the reference. Fractions from the affinity chromatography were diluted to 1 ml with buffer solution to compare with the reference. The assay mixtures contained the following components: 100 μl of 200 mM sodium acetate buffer containing 8 mM $CaCl_2$ (pH 5.0), 50 μl of 4 mM 4MU-Neu5Ac (the artificial substrate), and 50 μl of the enzyme solution. After 45 minutes incubation at 37 °C, the enzymatic hydrolysis was quenched by addition

of 1 ml of 0.2 M glycine-sodium hydroxide solution (pH 10.7). An aliquot (100 μl) of eluted neuraminidase was analyzed in a total volume 2.1 ml of 0.2 M glycine-NaOH solution (pH 10.7), and the RFI value for fluorescence emission at 450 nm was measured. Two sets of samples were prepared for each assay. Three readings were taken for each sample. Several batch dilutions of enzyme solution were required to obtain RFI readings in the liner range.

Peroxidase-amplified assay

V. Cholerae neuraminidase activity was assayed according to the method of Ogura et. al.[1]. A G_{M1} standard curve was linear up to 1 pmol (Figure 2). A 10 pmol sample of G_{D1a} was used as the substrate for the assay. No enzyme was added in the blank sample. The absorbance for hydrolysis of G_{M1} in the assay was subtracted from the blank to limit the background interference from G_{D1a}. The fractions assayed were treated in the same manner as they were for the 4MU-Neu5Ac assay. Two sets of samples were prepared for each assay. Five readings were taken for each sample.

SDS-Polyacrylamide Gel Electrophoresis

The physical properties of enzymes eluted from the affinity column were analyzed using the Bio-Rad Mini 1D Protean II Dual slab gel system. The concentrated column fractions were lyophilized, suspended in 100 μl SDS sample buffer of Laemmli[19], reduced with 25 mM 2-mercaptoethanol, and applied to SDS PAGE on 12% separating gels with a 8% stacking gel. After electrophoresis, the gel was pre-fixed in methyl alcohol containing 6.75 % acetic acid for 30 minutes, soaked in double distiation water overnight, and stained with silver nitrate as described by Giuliun *et. al.*[20,21]. A middle

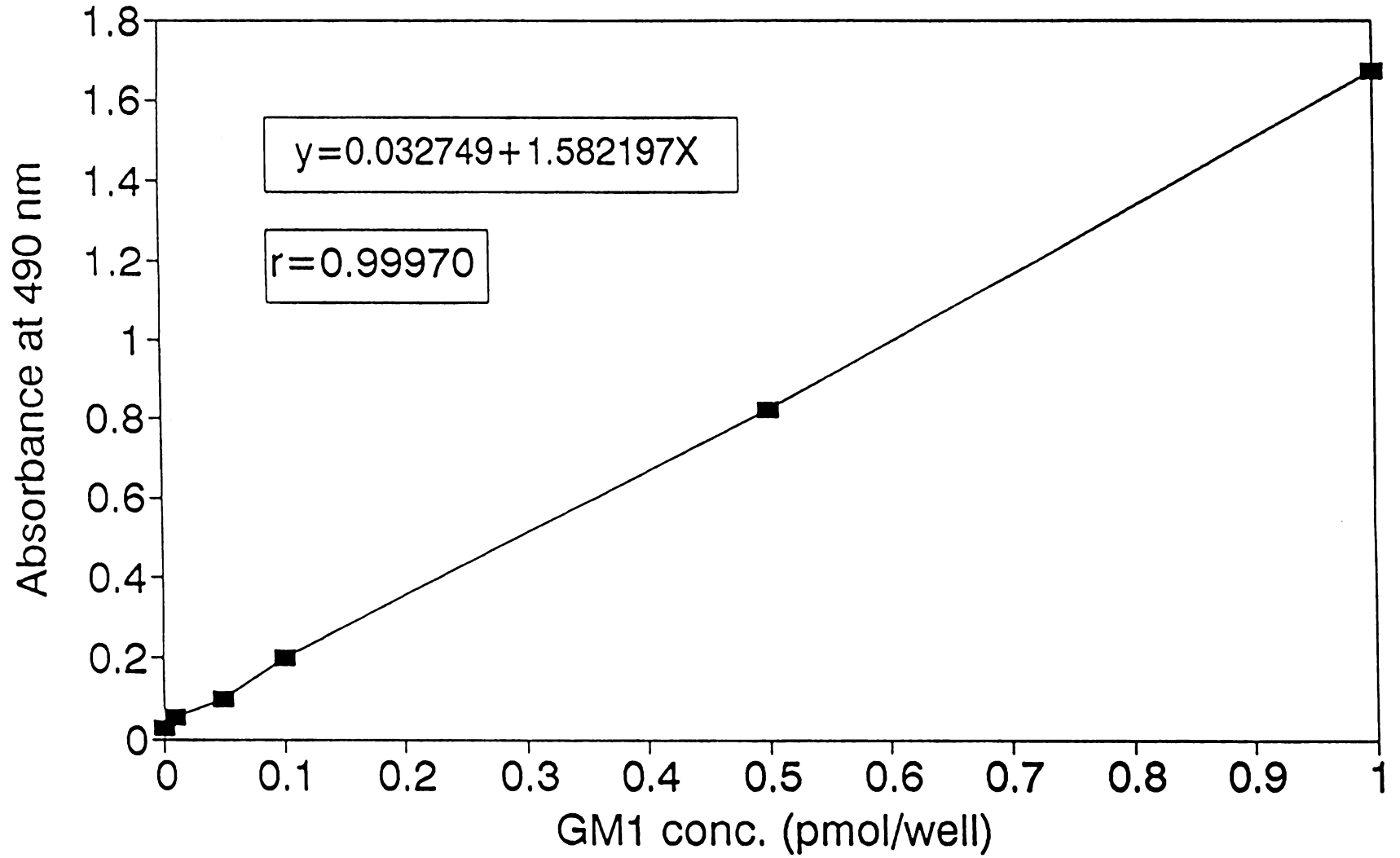


Figure 2: G_{M1} standard curve for sialidase activity by peroxidase-amplified assay

molecular-weight protein standard marker (Sigma Co.) was used to define the approximate molecular weights of bands on the gel.

Results

Affinity separation of *V. cholerae* neuraminidase from other enzymes

Conditions for optimal recovery of *V. cholerae* neuraminidase from an affinity column were determined by varying the sialic acid concentration in the eluent. Under the conditions of 4MU-Neu5Ac assay[18], a high concentration (Ki=25 mM) of sialic acid inhibited neuraminidase activity. The interference of sialic acid in peroxidase-amplified assay has not been previously reported. Therefore, both activity assays were performed after sialic acid was removed by size exclusion chromatography and with an ultrafilter PM-10.

The elution profile and electrophoresis pattern of fractions from the affinity Bio-Rad G-10 column are shown in Fig. 3,. *V. Cholerae* neuraminidase (1U, and 250 mU) was desorbed from the affinity column with 10% sialic acid (5 ml). The total activity of the recovered enzyme was examined using with both the 4MU-Neu5Ac and the peroxidase-amplified assay and approximated 1.1 U and 200 mU, respectively (Table 1). *V. Cholerae* neuraminidase isolated from the column migrated as a 68 kDa, and a 16 kDa polypeptides on a 12% SDS PAGE as did the starting material (Figure 4). The activity of the neuraminidase was not affected by the column according to the results of 4MU-Neu5Ac assay and peroxidase-amplified assay.

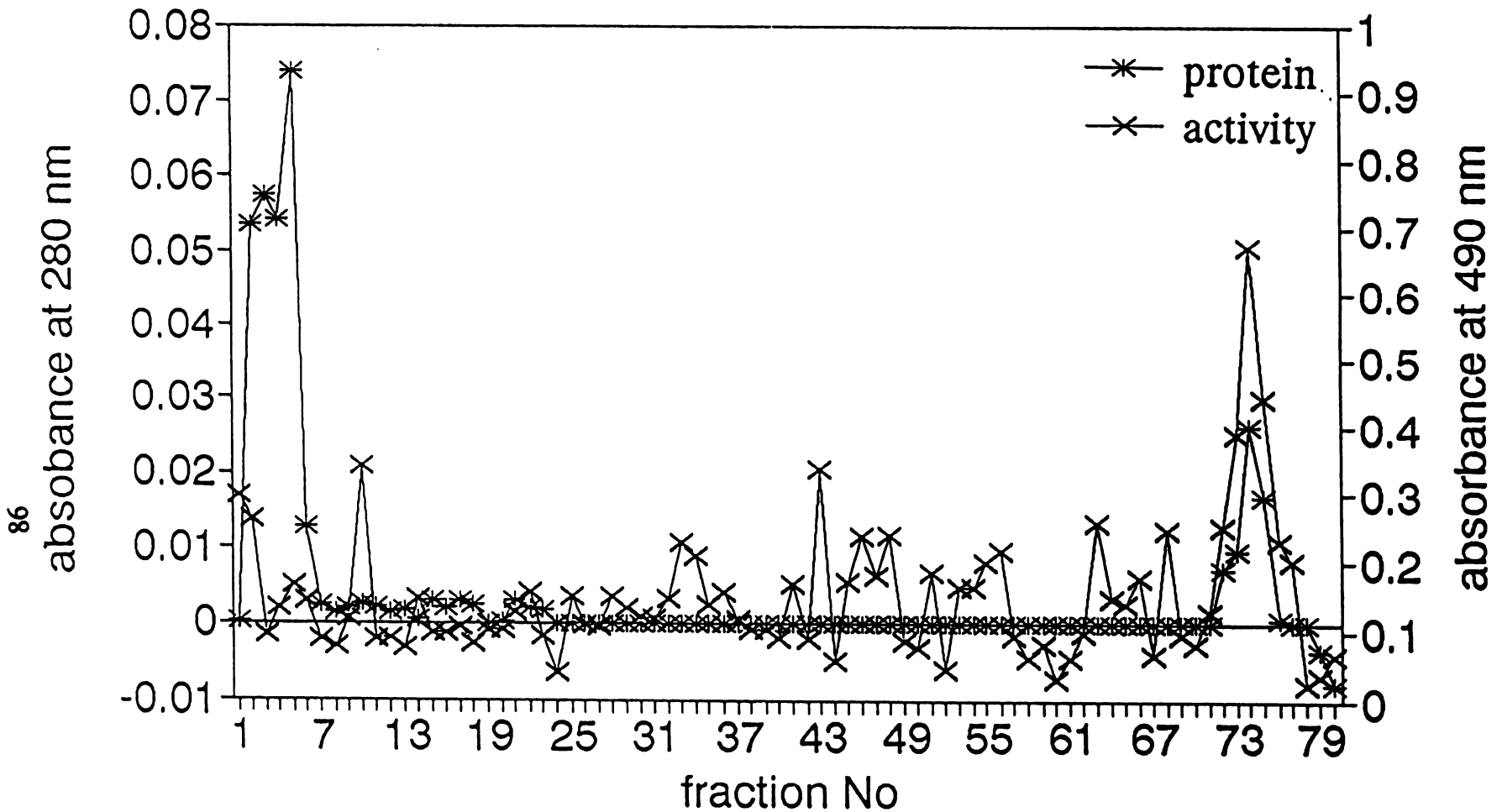


Figure 3: Elution profiles of Neu2en Bio-Rad Affi G-10 affinity chromatography. The protein mixture are a-galactosidase, glucose oxidase, and *Vibrio cholerae* sialidase. a-Galactosidase, and glucose oxidase was eluted with acetate buffer (100 mM, pH 5.0) from fraction 1 to 70. V.C. sialidases was eluted with 10% sialic acid in H_2O. The volume of each fraction was 2 ml.

Table 1: The recovered activity of *Vibrio cholerae* sialidase from Bio-Rad Affi-G10 affinity chromatogaphy

Immobilized ligand	Enzyme Applied (mU/mL)	Reccovered enzyme (mU/mL)	Percent enzyme activity recoverd
Control	4 mU/mL	4 mU/mL	100%
Neu2en	1000 mU/mL	1100 mU/mL*	110%
Neu2en	250 mU/mL	202.5 mU/mL**	81%
Fetuin	250 mU/mL	295 mU/mL***	118%

* The dilution factor of the enzyme solution is 250 .
** The dilution factor of the enzyme solution is 60.
*** The dilution factor of the enzyme solution is 60.

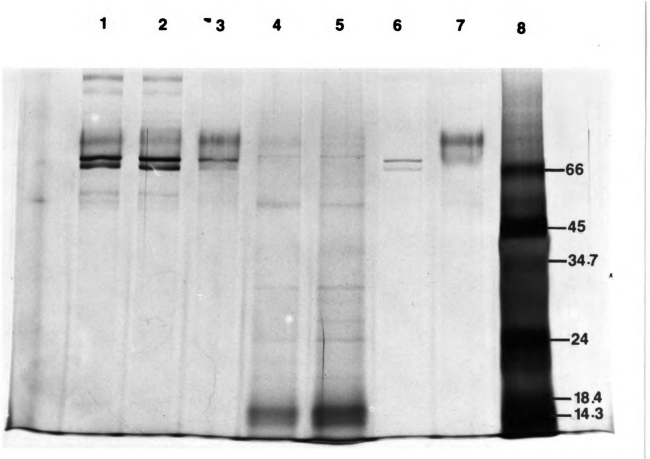


Figure 4: SDS-PAGE Analysis of *V. cholera* sialidase with an affinity column.

Lane 1: α-galactosidase, glucose oxidase, and *V. cholera* sialidase mixture. Lane 2: α-galactosidase, and glucose oxidase mixture. Lane 3: fractions pooled from the first affinity column peak. Lane 4: *V. cholera* sialidase standard. Lane 5: fractions pooled from the second affinity column peak. Lane 6: α-galactosidase standard. Lane 7: glucose oxidase standard. Lane 8: middle molecular weight marker from Sigma Co.

Neuraminidase bounded of fetuin-agarose column chromatography

Sialyl-glycoconjugates have commonly been employed as immobile ligands in column chromatography to adsorb neuraminidase from various biological sources[22,23,24,25]. The recovery of neuraminidase from an immobilized sialyl-glycoconjugate column is influenced by the nature of the immobilized sialyl-glycoconjugate and the amount of sialic acid in the sialyl-glycoconjugate. Fetuin is a plasma glycoprotein and a good substrate for neuraminidase, but is rapidly hydrolyzed by the enzyme[11]. Column degradation is a common problem in neuraminidase purification from all immobilized sialyl-glycoconjugate column[11,13,16,17]. After degradation, the resulting terminal saccharides of glycoconjugates may bind with other glycosidases and cause contamination during the process.

V. Cholerae neuraminidase (1 U) bound to a fetuin-agarose column is eluted with 50 mM sodium phosphate buffer containing 1 M NaCl (pH 6.0, 20 ml)[11]. The elution profile and electrophoresis pattern of the fetuin-agarose column is shown in Fig. 5. Under optimal conditions, the recovery of neuraminidase activity from the fetuin-agarose column was less than 10 %. To increase the yield of eluted neuraminidase, the salt concentration in the acetate buffer was raised to 2 M NaCl. However, no significant improvement in the recovery of neuraminidase was shown (Fig. 5). Bound neuramidase could be released from fetuin-agarose column while the column was placed at room temperature for 48 hrs.

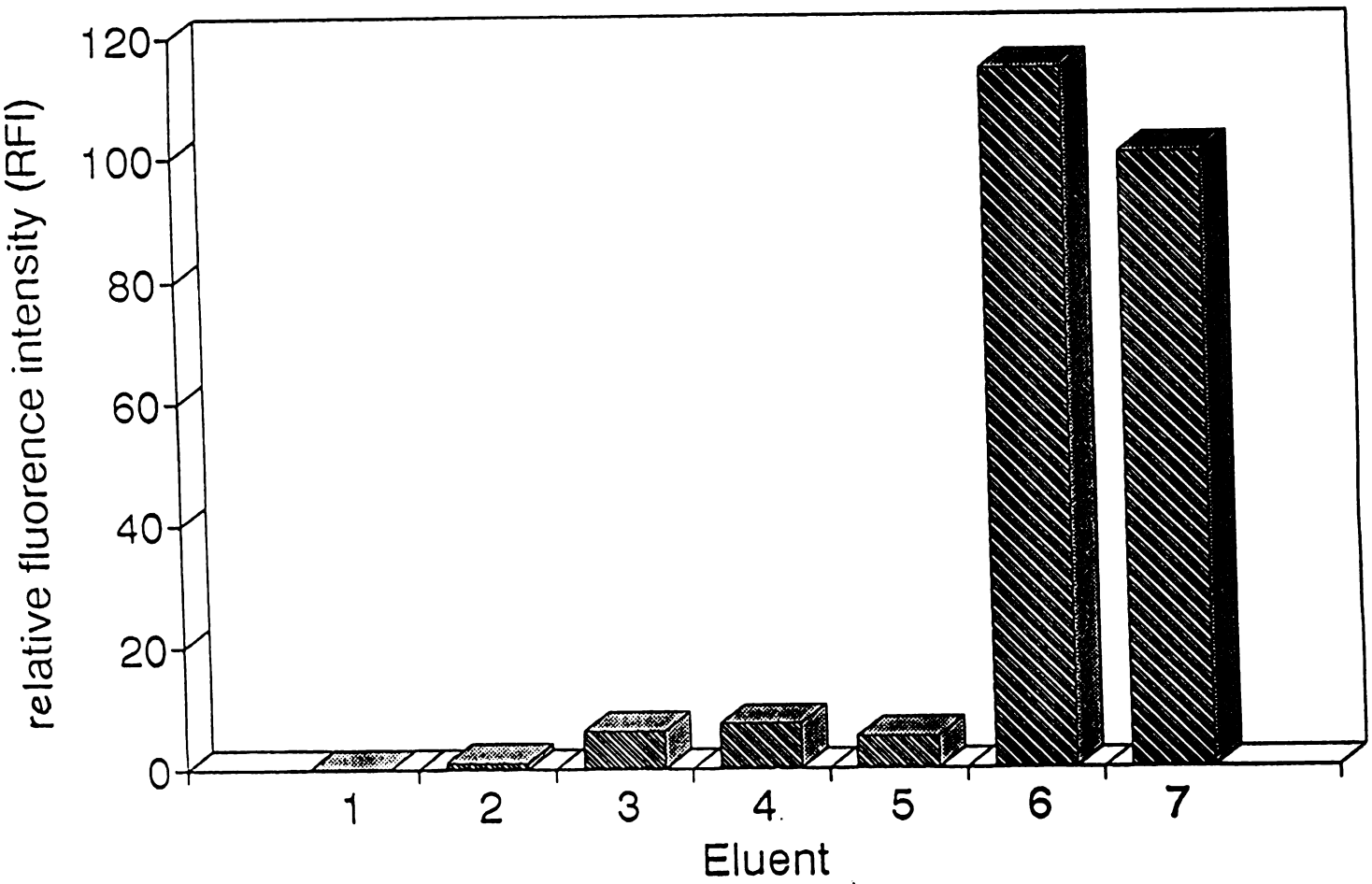


Figure 5: Elution profiles of fetuin-agarose affinity chromatography. Eluant 1: 5 ml Na-acetate buffer (100 mM, pH 5.0); eluant 2: 5 ml Na-acetate buffer (100 mM, pH 5.0); eluant 3: 10 ml NaCl (0.2 M)/phosphate buffer (50 mM, pH 6.0); eluant 4: 20 ml NaCl (1 M)/phosphate buffer (50 mM, pH 6.0); eluant 5: 20 ml NaCl (2 M)/phosphate buffer (50 mM, pH 6.0); eluant 6: 10 ml Na-acetate buffer (100 mM, pH 5.0) after the column was placed at room temperature for 48 hours; eluant 7: Control sample contains 250 mU V.C. sialidase in 5 ml acetate buffer (100 mM, pH 5.0).

Discussion

Sialic acid derivative(s) immobilized on a solid support through the amide (N-acetylneruaminamide) or the amino group of a-glycoside (N-acetylneuraminic acid) have been used for affinity chromoatography of *C perfringens* and *V. cholerae* neuraminidases (Fig. 1)[22,26,27]. These studies demonstrated that the N-acetyl neruaminamide-Sepharose did not significatly adsorb *C perfringens* and *V. cholerae* neuraminidases. These results were in agreement with the results of Miller *et. al.*[12], which suggested that the free carboxyl group of the sialic acid moiety on the substrates was essential for substrate binding to neuramidase within the catalytic stage.

The hydrophobic interactions between neuraminidase and various ligands have been studied using the enzyme from bacterial, viral and bovine kidney sources[13,28,29,30]. The binding of neuraminidases to alkyl agarose (range from C_2 to C_{10}) improved with increasing alkyl chain length. The neuraminidase could be desorbed from alkyl agarose with 1M NaCl or by shifting the pH from 4.5 to 6. This suggested that neuraminidase contained a hydrophobic site(s) which assist in the binding of neuraminidase to alkyl columns[11]. However, this interaction was shown to be a non-specific because a similar phenomenon was observed with other glycosidases treated in this manner[13]. The most selective binding of *C. perfringens* neuraminidase was observed on columns of Neu-β-Me and Neu2en linked to AD- or PAH-Sepharose via amide linkages. However, the enzyme was only recovered from these affinity columns after treatment with 0.05 M sodium acetate buffer (pH 5.3) at 20 °C and the recovery was low[11]. The bound neuraminidase on PAH-Sepharose failed to be eluted with

various substrates, products, or inhibitors such as sialyllactose (10 mM), Neu5Ac (100 mM), Neu-β-Me (100 mM), and Neu5Ac2en (10 mM)[2,11,27]. Further, hydrolysis of sialyllactose by neuraminidase bound to these columns demonstrated that the active site of the enzyme was not occupied by the bound ligand and was still available for enzymatic activity. This suggested that the spacer linkage from ligand to support was an important factor and that hydrophobic interactions might again be the main force for the quantitative binding of neuraminidase.

In our study, a transition state analogue (Neu2en) was coupled to the Bio-Gel Affi-10 affinity column. The results indicate that *V. cholerae* neuraminidase was not only adsorbed to the column specifically but was also easily eluted with 10% neuraminic acid (pH < 4.0). This is an improvement over the results of Corfield *et. al.*[11], in which the immobilization of Neu2en on PAH-Sepharose was shown to tightly bind two neuraminidases which could not be eluted with 100 mM Neu5Ac. The improvenment in recovery probably results from a reduction in hydrophobic interactions in the Neu2en Bio-Gel Affi-10 column by introducing a 10 carbon spacer arm with polar substituents rather than an alkyl spacer group as in the study of Corfield *et. al.*[11]. The specificity of neuraminidase binding to the Neu2en Affi-10 column was confirmed by the separation of *V. cholerae* neuraminidase from two other proteins in a mixture. The specificity probably arises from the interactions with the transition state analogue. The bound neuraminidase was successfully eluted with 10% neuraminic acid (pH < 4.0) illustrating that the Neu2en Affi-10 column is a true affinity chromatography.

The physical properties of *V. cholerae* neuraminidase have been determined with electrophoresis before and after affinity chromatography. Both samples appeared to be approximately 68 kDa, and 16 kDa polypeptides. However, the molecular weight of *V. cholerae* neuraminidase has been estimated by ultracentrifugation[32], and reported to be 10-20 kDa. The same enzyme was analyzed by gel electrophesis and reported to be 90 kDa[33] or to be 24-25 kDa[16]. The variation in the molecular weight reported for *V. cholerae* neuraminidase from different groups might arise from several different factors such as the ligand interactions, pH condition, glycolation migration, and partial degradation.

V. Cholerae neuraminidase was tightly bound to the fetuin-agaroses at 4 ^{0}C and barely desorbed from the column with high salt concentration in the buffer. Eventually, free sialic acid and bound neuraminidase were released from fetuin-agarose column after prolonged standing at the room temperature (48 hours). These observations are in partial agreement with the study by Corfield *et. al.*[11]. The large quantity (250 mU) of neuraminidase employed might cause an increase in the hydrophobic interactions and lead to poor recovery. Although fetuin is an affinity adsorbant for sialidase[13,16,17], it is not useful for chromatography because of the low recovery from the column and its degradation.

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CHAPTER 5

DICUSSIONS

I. SYNTHESIS OF NEURAMINIC ACID ANALOGUES

A. Develop a chemical process for converting sialic acid to Neu5Ac2en

During the last twenty years, several methods for the syntheses of Neu5Ac2en have been developed and are summarized on Fig. 1A. Usually, Neu5Ac2en is prepared from the fully protected 2-deoxy-2-halo sugar by dehydrohalogenation with base or silver carbonate[1,2,3]. The alternative process is to treat N-acetylneuraminic acid methyl ester with acetic anhydride in presence of a catalytic amount of sulfuric acid at 50 °C, followed by deacetylation with NaOMe/MeOH[4]. Under these conditions, a mixture containing Neu5Ac2en and the 4-epimer Neu5Ac2en was obtained. 2-Deoxy-2,3-dehydroneuraminic acid (Neu2en) was synthesized by hydrogenolytic cleavage of 2-deoxyl-2,3-dehydro-N-benzyloxycarbonyl-neuraminic acid, which was formed by reaction of sialic acid with diphenyldiazomethane[5].

In this work, a method for the synthesis of the transition state analogue of neuraminic acid (Neu5Ac2en and Neu2en) has been developed (Fig. 1B). This procedure has advantages over conventional methods in that it is a quantitative, simple one-step conversion from neuraminic acid to Neu5Ac2en, or Neu2en. In this reaction, epimerization at the C-4 position, which often accompanies the dehydration process, is prevented by using the base dimethylamino pyridine. A comparison between this method and the standard method for Neu2en synthesis, was made by varying ratios of

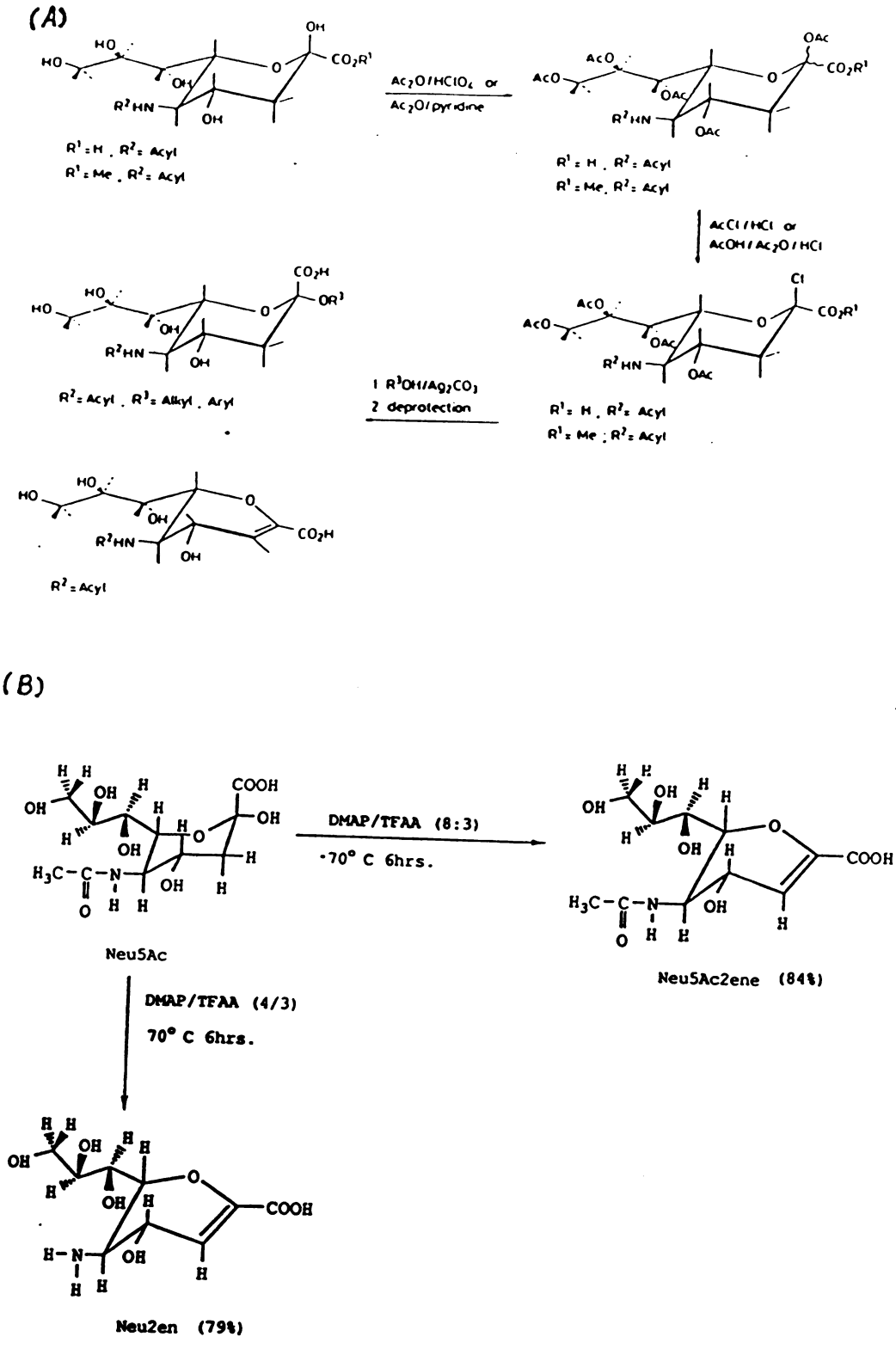

Figure 1: (A) The synthesis scheme of Neu5Ac2en was developed by Meindl and Tuppy[1,2,3]. (B) The synthesis scheme of Neu5Ac2en developed in this laboratory.

trifluoroacetic anhydride and dimethylamino pyridine. A series of derivatives at the N-5 position of Neu2en has been obtained using methods similar to previous studies[5]. These derivatives were assayed for their ability to competitively inhibit *V. cholerae* sialidase using 4MU-Neu5Ac as a substrate. This results were consistant with previous reports[6].

Three analogues of Neu5Ac2en with varying substituents at the 9-hydroxyl group were prepared in this study (Fig. 2A). The starting material, Neu5Ac2en, was selectively protected at the 7-hydroxyl and 9-hydroxy with *m*-nitrobenzylidene dimethylacetal and subsequently reduced with $NaBH_3CN$ and hydrogen chloride gas in THF. In a kinetic study, the 9-substituted analogues of Neu5Ac2en were shown to be competitive inhibitors with Ki values ranging from 110 to 80 μM, which is about 3 times less than that of Neu5Ac2en. These results are not in agreement with those obtained by Zibiral *et. al.*[7], who prepared 7-deoxy, 8-deoxy, or 9-deoxy-Neu5Ac2en from the corresponding peracetylated Neu5Ac methyl ester derivatives by treatment with trimethylsilyl trifluoromethanesulfonate ($CF_3SO_3Si(CH_3)_3$) (Fig. 2B). 9-Deoxy-Neu5Ac2en was shown to be a better inhibitor than Neu5Ac2en for sialidase from V. cholerae. To determine the contribution of the glycerol side-chain to inhibition, 1,2-O-*m*-nitrobenzenzlidene-3-hydroxyl glycerol was prepared and assayed in the same manner. No inhibition was observed. These results suggested that the binding site(s) of sialidase did not directly interact with by the side-chain of Neu5Ac2en. One drawback of the method for preparation of these 9-hydroxyl substituted compounds in this study is that the reduction regent ($NaBH_3CN$) is difficult to remove completely.

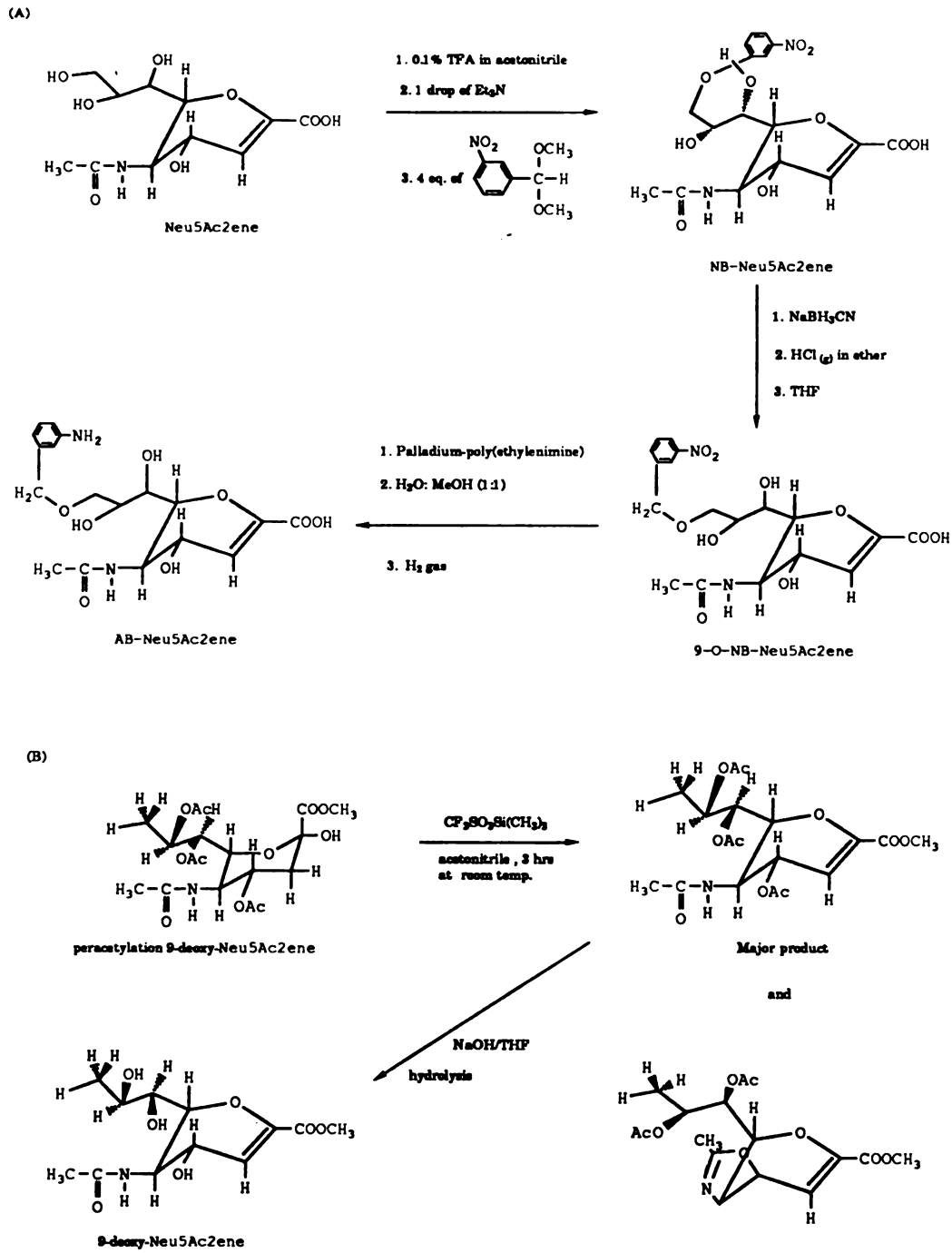

Figure 2: Reaction scheme for the synthesis of Neu5Ac2en derivatives with a modification on its glycerol side chain.(A) The synthesis scheme of AB-Neu5Ac2en developed in this laboratory.(B) 9-Deoxyl-Neu5Ac2en was prepared by Zbiral *et. al.*(7)

B. Develop a photo-activatable sialidase inhibitor

Two photoreactive, potential inhibitors of sialidase(s) have been prepared and analyzed by Warner and Horst *et. al.* (Fig. 3A)[8,9]. These photoactivatable groups were introduced into the C-9 position via a thio- or an amide- linkage. The starting material for both compounds was Neu5Ac2en methyl ester. Another photoreactive analogue of Neu5Ac2en, 5-azido-5-deoxy-Kdn2en, has been prepared by Schreiner *et. al.* by treatment of peracetylated 5-azide sialic acid methyl ester with $CF_3SO_3Si(CH_3)_3$ in presence of acetonitrile at 0 °C. However, this compound was not an inhibitor of sialidase from *V. cholerae* (Ki=2.5 mM)[10].

In this study, a photoactivatable compound, $Neu5N_3Ac2en$, was derived from Neu2en by incorporating an azide group to a haloacetyl group at the N-5 position (Fig. 3B). The photoreactable compound ($Neu5N_3Ac2en$) was shown to be a competitive inhibitor for *V. cholerae* sialidase (Ki= 6.1×10^{-2} mM), which could also be prepared in a radiolabeled form for use as a photoaffinity probe. To utilize a photoaffinity compound in physiological extracts, it is necessary to introduce a radiolabel photoreactable inhibitor without sacrificing its inhibition ability. We did not perform the radiolabeling synthesis because the procedure involved multiple enzymatic reactions to incorporate the ^{14}C into the pyranose ring of sialic acid. In accordance with the mechanism of catalysis of sialidase, Neu5Ac2en methyl ester has shown no influence on its inhibitory effectiveness. Therefore, it could be one candidate position to introduce a radiolabel. In a recent study, ^{14}C labeling at the C-2 position of Neu5Ac was prepared from N-acyl-2-amino-2-deoxyl-[1-^{14}C] glucose as precursors by a series of

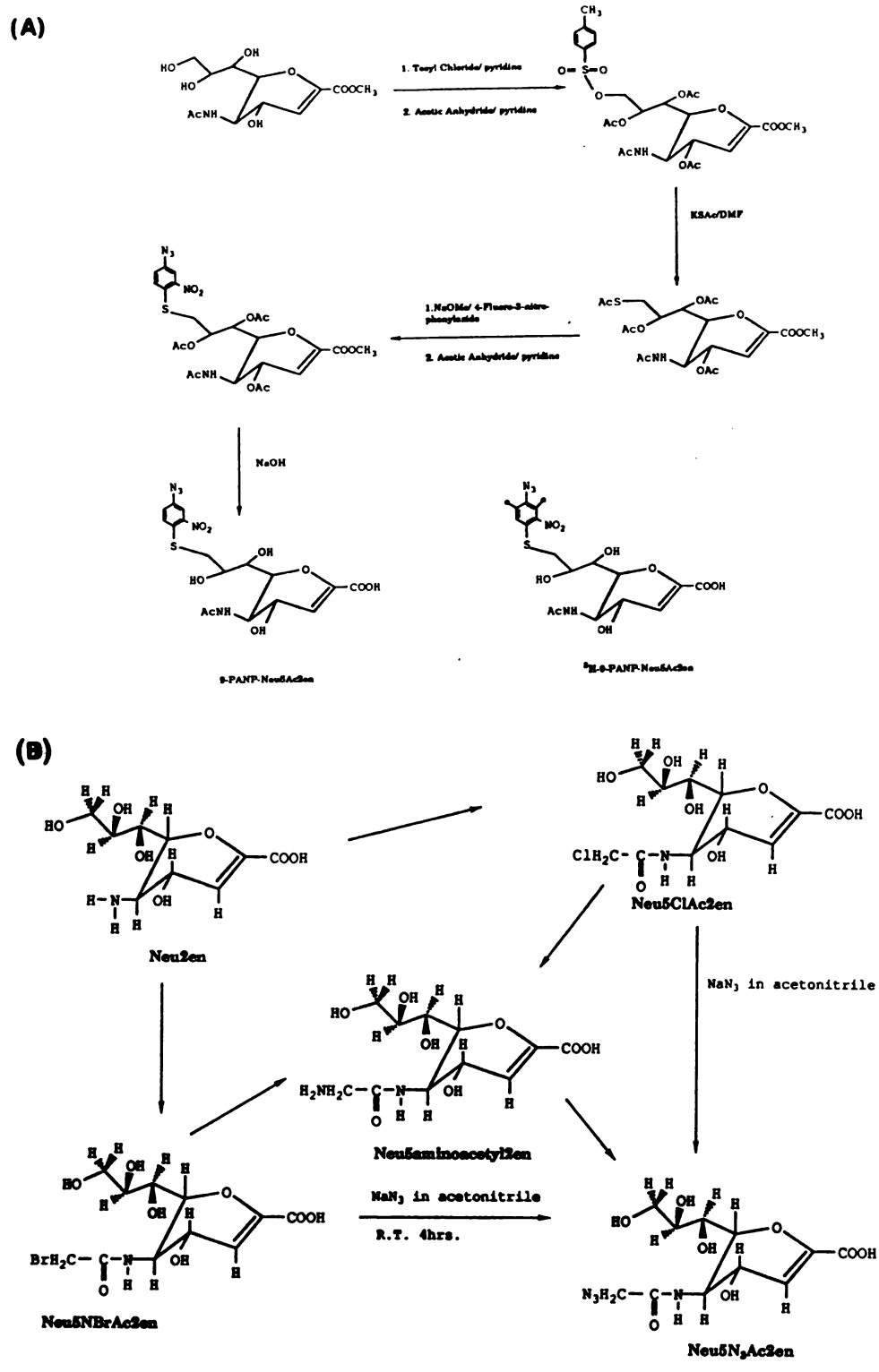

Figure 3: Reaction scheme for the synthesis of photoaffinity compound. (A) ^{3}H-9-PANP-Neu5Ac2en was prepared by Warner[8]; (B) Neu5N$_3$Ac2en was prepared in this laboratory.

enzymatic reactions[11].

II. AFFINITY CHROMATOGRAPHY FOR ISOLATION OF SIALIDASE FROM A PHYSIOLOGICAL COMPLEXES

It has been demonstrated that affinity chromatography is an efficient method to separate a particular enzyme from a phyiiological mixture[12,13,14,15]. Sialic acid derivatives immobilized on to various solid supports via α-glycosides have been employed in affinity chromatography systems for sialidase(s) purification[12,13,14]. It has also been reported that Neu2en and β-glycosides of sialic acid methyl ester (Neu-β-Me) were coupled to PAH-Sepharose matrix via the activation of acyl-hydrazido group with HNO_2[15, 16]. Coupling of sialic acid analogues having an aldehyde side-chain via reduction at the C-7 to soild supports was performed by reductive amination[12]. Sialoglycoconjugates have been immobilized on Sepharose and cellulose matrices[16,17]. Among those applications, the α-glycoside of Neu5Ac-Sepharose and related derivatives have been used to sucessfully purify sialidases from various sources because they can significantly adsorb sialidase in the presence of sodium acetate buffer (pH 5.5) and the enzyme can be desorbed from the column with the addition of the benzyl α-ketoside of Neu5Ac[12]. For the Neu2en-PAH-sepharose clomn, the binding of sialidase was strong, unfortunately there was no straightforward process to elute the bound sialidase. The main concern when using sialoglycoconjugates in affinity chromatography is that the column will be degraded by the enzyme.

In this study, Neu2en was immobilized on Bio-Rad Affi-Gel 10 via its free amino group. The following improvements over the Neu2en-PAH-sepharose column were

observed. First, the affinity column could selectively adsorb *V. cholerae* sialidase(s) from artificial protein mixtures (See details in chapter 4). The bound *V. cholerae* sialidase(s) could be quantitatively eluted with 10% aqueous sialic acid (Neu5Ac) at 4 °C. The activity of the recovered sialidase(s), as determined by the 4MU-Neu5Ac and the peroxidase-amplified assay, was retained. Overall, the Neu2en-Affi-10 column has advantages over α-glycoside of Neu5Ac-Sepharose and Neu2en-PAH-Sepharose chromatographies.

This study provided two useful tools for the handling of sialidase(s) in complex physiological mixtures. One is a series of sialic acid analogues with inhibitory characteristics and the other is an efficient affinity chromatography column. It will be interesting to examine the behavior of the affinity column when physiological extracts are applied. Problems might include the modification of the elution system, and the variety of the specificities within the families of sialidase(s).

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