Synthesis of bioactive sialosides

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Abstract: Sialosides of glycoprotein and glycolipid carbohydrate groups are
well documented to play important roles in biological recognition. This class of
oligosaccharides also poses unique challenges to the synthetic chemist.
Accordingly, the synthesis of natural sialosides and related analogs has been the
subject of intense investigations over the last decade. Advances in both chemical
and enzymatic approaches to the synthesis of sialosides have lead to increasingly
practical synthetic strategies. Recent progress is summarized in this report with
emphasis on the strategies for the use of sialyltransferases in enzymatic
sialylation.

Sialic acids terminate the carbohydrate groups of many cell surface glycoproteins and glycolipids
and contribute to the enormous diversity of natural oligosaccharide sequences generated from a
limited number of neutral core structures. As terminal substituents they are ideally suited to
participate in protein-carbohydrate interactions that mediate cell surface recognition phenomena.
Indeed, cell surface sialosides are known to serve as ligands for microbial toxins (Ref. 1), for
microbial adhesins which mediate attachment to host cells (Ref.1-3) and for animal lectins that
mediate cell-cell adhesion (Ref. 1,4-6; See Table 1).

TABLE 1.   CELL SURFACE SIALOSIDES RECOGNIZED AS LIGANDS BY
             CARBOHYDRATE BINDING PROTEINS

Carbohydrate binding protein	Cell Surface Sialoside Ligand[a]
Viral and bacterial carbohydrate binding proteins	
Influenza virus hemagglutinin (human)	NeuAcα2,6Galβ1,4GlcNAc-R
Influenza virus hemagglutinin (avian,equine)	NeuAcα2,3Galβ1,4GlcNAc-R
Sendai virus hemagglutinin	NeuAcα2,8NeuAcα2,3Gal-R
E. coli adhesin	NeuAcα2,3Galβ1,4Glcβ-ceramide (GM3)
Cholera toxin	Galβ1,3GalNAcβ1,4 　　　　　Galβ1,4Glcβ-ceramide (GM1) NeuAcα2,3
Mammalian cell adhesion receptors	
E-selectin and P-selectin	NeuAcα2,3Galβ1,4 　　　GlcNAc-R (SLe^x) Fucα1,3 NeuAcα2,3Galβ1,3 　　　GlcNAc-R (SLe^a) Fucα1,4
Bone marrow macrophage lectin	NeuAcα2,3Galβ1,3GalNAc-R

[a]R indicates the remaining core carbohydrate structure to which the sialoside sequence is attached.

Much attention has focused on the newly described selectin family of adhesion molecules which mediate recruitment of white blood cells to sites of inflammation and tissue injury in view of the potential for therapeutic relevance to treatment of these diseases. Two of these selectins, E-selectin and P-selectin are expressed on vascular endothelial cells (and on platelets for P-selectin) and mediate the initial attachment of leukocytes by binding sialyl-Lewis X containing glycoconjugates (Ref.4,5).

Advances in both the chemical and enzymatic approaches to synthesis of these compounds has provided systematic and reliable routes for the production of small quantities of many complex sialosides. The pace of progress suggests that practical and economic synthesis of targeted sialoside structures will be achieved in the foreseeable future. It is our expectation that the practical synthesis of many sialosides (and other oligosaccharides) will blend chemical and enzymatic steps to afford the most efficient synthetic routes. A summary of the status of sialoside synthesis with strategies for combining chemical and enzymatic methodologies is presented below. It should also be apparent that the principles described for the use of sialyltransferases in synthesis of sialosides applies generally to the use of glycosyltransferases for carbohydrate synthesis. For several excellent reviews, see Toone et al. (Ref.7), Ichikawa et al. (Ref.8) and David et al (Ref.9).

PROGRESS IN CHEMICAL SYNTHESIS OF SIALOSIDES

Formation of the glycosidic bond of the 2-keto sugar N-acetylneuraminic acid (sialic acid) is still considered one of the most fundamental problems in synthetic carbohydrate chemistry (Ref.10,11). Compared to formation of the typical hexopyranoside, the formation of sialic acid glycoside is hindered electronically as well as sterically by the presence of the geminal carboxylate group. Moreover, the lack of a functional group at the 3-carbon excludes the use of neighboring group participation for stereochemical control. In addition, the α configuration of the glycosidic linkage of natural sialoside is thermodynamically less favored with respect to the anomeric effect. For these reasons, early attempts utilizing peracetylated chloride **1a** or bromide **1b** were satisfactory only for reactive glycosyl acceptor substrates (Ref.12) and, once applied to relatively hindered alcohols to produce sialoside like **2** (Fig.1), this method resulted in low yields and little stereochemical preference for the desired α-isomer (Ref.13,14). Nonetheless, these sialic acid donors were successfully applied to the synthesis of various naturally occurring sialosides of gangliosides (Ref.15) and glycopeptides (Ref.16). The efficiency of this classical reaction was later refined by taking advantage of solvent effects (Ref.17) or using an insoluble catalyst (Ref.18).
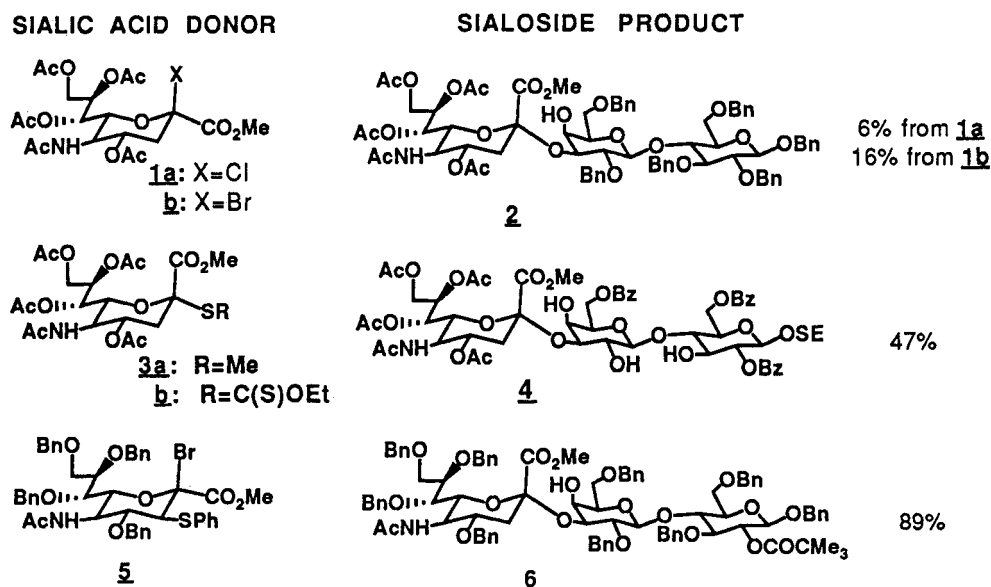

Figure 1. Key sialic acid donors and typical products. The products formed by chemical glycosylation can either be deblocked to yield the free oligosaccharides or used as glycosyl donors for the synthesis of more complex structures.

More dramatic improvements have come from examination of various leaving groups and activating agents. Based on developments of glycosylation using thioglycosides as donor substrates (Ref.19-23), corresponding sialic acid derivatives **3** were extensively investigated and shown to be superior to conventional 2-halo derivatives in both yield and stereoselectivity. Soft (thiophilic) nucleophiles, such as dimethylmethylthiosulfonium triflate (DMTST) (Ref.24-26), NIS-triflic acid (Ref.27), methanesulfenyl bromide-silver triflate (Ref.28), and benzeneselenenyl triflate (Ref.29,30) were shown to be effective. Certain dipolar solvents such as acetonitrile or THF are essential in order to get high stereoselectivity. This type of reaction currently constitutes the most practical method for synthesis of moderately complex molecules. Figure 1 demonstrates the typical example (Ref.24). By using 2 equivalents of the sialic acid donor **3a**, α-product **4** was obtained in 47% yield. Syntheses of various gangliosides have been achieved based on this method (Ref.31-35). Very recently, it was reported that similar trisaccharide can be synthesized in an even higher yield from **3b** (Ref.28). Even further improvement using this approach may come from investigation of other leaving groups such as fluoride (Ref.36) or imidate (Ref.37).

The concept of using a stereocontrolling auxiliary, similar to that developed for 2-deoxyglycoside synthesis (Ref.38), has recently been successfully applied to the synthesis of sialosides (Ref.39-42). Among various C-3 substituents tested, the phenylthio group was demonstrated to be the best (Ref.40,42).

TABLE 2.   EXAMPLES OF COMPLEX SIALOSIDES PRODUCED BY CHEMICAL SIALYLATION

Structure		Amount Produced[a]	Reference
NeuAcα2,3Galβ-ceramide (GM4)		A	32
NeuAcα2,3Galβ1,4Glcβ-ceramide (GM3)		A	33
		B	41
NeuAcα2,3Galβ1,3GalNAcβ1,4Galβ1,4Glcβ-ceramide (GM1b)		nd	15
NeuAcα2,3Galβ1,3GlcNAcβ1,3Galβ1,4Glcβ-ceramide		nd	34
NeuAcα2,6Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ-ceramide		B	35
NeuAcα2,3Galβ1,4 　　　　　　　＼ 　　　　　　　　GlcNAcβ1,3Galβ1,4Glcβ-ceramide 　　　　　　　／ 　Fucα1,3		nd	31
NeuAcα2,3(Galβ1,4 　　　　　　　＼ 　　　　　　　　GlcNAcβ1,3)₂Galβ1,4Glcβ-R 　　　　　　　／ 　Fucα1,3	R=O(CH₂)₆CO₂ᵗBu	nd	44
NeuAcα2,8NeuAcα2,3Galβ1,4Glcβ-ceramide (GD3)		nd	43
NeuAcα2,6 　　　　＼ 　　　　　GalNAcα-R 　　　　／ 　Galβ1,3	R=serine	C	16
	R=OH	B	17
(NeuAcα2,6GalNAcα-Ser)ₙ-Val	n=2,3	C	45
NeuAcα2,6Galβ1,4GlcNAcβ1,2Manα1,6 　　　　　　　　　　　　　　　＼ 　　　　　　　　　　　　　　　　Manβ1,4GlcNAcβ1,4GlcNAc 　　　　　　　　　　　　　　　／ NeuAcα2,6Galβ1,4GlcNAcβ1,2Manα1,3		nd	46

[a]A: >50 mg, B:10-50 mg, C: 1-10 mg, nd: not described

This reaction seems to be the most powerful with respect to yield and the selectivity for formation of the α glycoside bond. The trisaccharide 6 (Fig.1) was obtained in 89% yield based on the sialic acid donor 5 (Ref.41). Especially challenging molecules such as disialoganglioside GD3 (Ref.43), dimeric sialyl Lewis X (Ref.44) and multiple sialylated glycopeptide (Ref.45) have been synthesized by this method. However, the overall efficiency starting from sialic acid is not optimal because the preparation of the sialic acid donor 5 takes multiple steps and the C-3 substituent must be removed later. As described above, these basic sialic acid donors have been used in synthetic strategies for the synthesis of more complex sialosides. Examples of several complex sialosides that have been synthesized successfully are given in Table 2.

PROGRESS IN ENZYMATIC SYNTHESIS OF SIALOSIDES

Sialyltransferase family. Sialyltransferases carry out the addition of sialic acid to growing oligosaccharides during the natural biosynthesis of glycoproteins and glycolipids following the general reaction **I** with CMP-NeuAc as the activated glycosyl donor. These enzymes have high

$$\text{CMP-NeuAc} + \text{HO-Acceptor} ----------> \text{NeuAc-O-Acceptor} + \text{CMP} \quad \textbf{(I)}$$
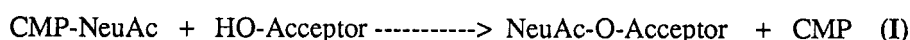
specificity for their acceptor substrate (typically a terminal disaccharide sequence is recognized) and transfer with strict stereochemical and linkage specificity. As a consequence of this specificity, 10-12 enzymes are believed to be required to synthesize all known natural sialosides (Ref.47). Four of the enzymes capable of forming several of the common linkages found in natural sialic acid containing glycoconjugates have been purified to homogeneity (Ref.47-49).

TABLE 3.   PURIFIED SIALYLTRANSFERASES AND PRODUCTS FORMED IN NATURAL SIALOSIDES.

Sialyltransferase (ST)	Product(s)
Galβ1,4GlcNAc α2,6 ST	NeuAcα2,6Galβ1,4GlcNAc-R
Galβ1,3/4GlcNAc α2,3 ST	NeuAcα2,3Galβ1,3GlcNAc-R NeuAcα2,3Galβ1,4GlcNAc-R
Galβ1,3GalNAc α2,3 ST	NeuAcα2,3Galβ1,3GalNAc-R
GalNAc α2,6 ST	NeuAcα2,6GalNAcαThr/Ser

	Galβ1,3 ⟍
	       ⟍ GalNAc αThr/Ser
	       ╱
	NeuAcα2,6 ╱

Combined chemical and enzymatic synthesis of sialosides. Sialyltransferases were first used to synthesize milligram amounts of sialosides for the purpose of characterizing the anomeric specificity of purified or partially purified enzymes following reaction with their preferred acceptor substrates (Ref.50,51). Once the difficulty in the chemical synthesis of sialosides was fully appreciated, the sialyltransferases began to be considered as enzymatic catalysts in an alternative approach combining both chemical and enzymatic steps (Ref.52,53). The basic strategy takes advantage of the fact that sialic acid occupies terminal positions on many carbohydrate groups. This allows a strategy starting with chemical synthesis of the neutral acceptor, deblocking to the

free oligosaccharide and enzymatic sialylation to finish the synthesis. Despite the limitation imposed by the difficulty in the purification of the sialyltransferases from tissues, the synthesis of a number of complex sialosides have been synthesized by this approach (Table 4).

TABLE 4.   SIALYLOLIGOSACCHARIDES   PREPARED   USING SIALYLTRANSFERASES

Sialyloligosaccharide		Amount Produced[a]	Reference
NeuAcα2,6GalβOMe		C	52
NeuAcα2,6GalβOPhNO$_2$		C	54
NeuAcα2,3GalβOPhNO$_2$		C	54
NeuAcα2,6Galβ1,4GlcOMe		C	52
NeuAcα2,3Galβ1,4GlcOMe		C	52
NeuAcα2,3Galβ1,3GalNAcOR;	R=Et, EtBr	B	55
NeuAcα2,3Galβ1,3GalNAcOR;	R=H, (CH$_2$)$_5$COOCH$_3$	C	52
NeuAcα2,6Galβ1,4GlcNAc OR;	R=Me, H	C	52,55-57
		A	58
NeuAcα2,3Galβ1,4GlcNAcOMe		C	52
NeuAcα2,6Galβ1,4GlcNAcβ-N-Asn		B	59
NeuAcα2,3Galβ1,3GlcNAcOR;	R=Me, Ph, (CH$_2$)$_5$COOMe	C	52
NeuAcα2,3Galβ1,3GlcNAcOMe		B	55
NeuAcα2,6Galβ1,4GlcNAcβOR		B	60
R=Aloc-Phe-Asn-Ser-Thr-Ile-OH or			
H-Gly-Gly-Asn-Gly-Gly-Gly-OH			
NeuAcα2,6Galβ1,4GlcNAcβ1,2ManαOMe		B	61
NeuAcα2,3Galβ1,3GlcNAcβ1,3GalβO(CH$_2$)$_8$COOMe		C	62
NeuAcα2,3Galβ1,3GlcNAcβ1,6GalβO(CH$_2$)$_8$COOMe		C	62
NeuAcα2,3Galβ1,3GlcNAcβ1,4Galβ1,4Glc, LSTa		C	52
NeuAcα2,6Galβ1,4GlcNAcβ1,4Galβ1,4Glc, LSTc		B	52
NeuAcα2,3Galβ1,4GlcNAcβ1,4Galβ1,4Glc, LSTd		C	52
NeuAcα2,6Galβ1,4GlcNAcβ1,4Galβ1,4Glcβ–sphingosine		B	b
NeuAcα2,3Galβ1,4GlcNAcβ1,4Galβ1,4Glcβ–sphingosine		B	b

NeuAcα2,3Galβ1,3 \
          \GalNAcOPh   C   63 \
   NeuAcα2,6/

3Me-O-Galβ1,4Glcβ1,6   C   64 \
          \GlcNAcβ1,3Galβ1,4Glcβ1,6 \
  NeuAcα2,3Galβ1,4/   \GlcNAcβMe \
           NeuAcα2,3Galβ1,4/

NeuAcα2,6Galβ1,4GlcNAcβ1,4 \
                  \Galβ-O(CH$_2$)$_5$COOMe   C   65 \
NeuAcα2,6Galβ1,4GlcNAcβ1,2/

NeuAcα2,6Galβ1,4GlcNAcβ1,6 \
                  \Galβ-O(CH$_2$)$_5$COOMe   C   65 \
NeuAcα2,6Galβ1,4GlcNAcβ1,3/

[a]A:>50 mg, B:10-50 mg, C: 1-10 mg.
[b]J.J. Gaudino and J.C. Paulson, In preparation.

Addressing the limitations in enzymatic glycosylation. Potential limitations in the use of sialyltransferases for practical large scale synthesis were immediately recognized; 1) limited availability of the enzymes purified from tissues, 2) the necessity of synthesizing the activated

donor substrate CMP-NeuAc and 3) the strict specificity of the enzymes restricting their versatility for synthesis of analogs of natural sialosides. Contributions from several laboratories have helped eliminate or minimize each of these limitations.

The problem of limiting enzymes has been attacked by efforts to clone the cDNAs of each sialyltransferase and express them as recombinant proteins. The cDNAs for three of the sialyltransferases listed in Table 3 (all but the GalNAc α2,6 sialyltransferase) have now been cloned (Ref.66-69). They share a common topology found in all glycosyltransferases cloned to date, consisting of an N-terminal signal-anchor sequence responsible for anchoring the enzyme in the membrane of the Golgi apparatus inside the cell and a large C-terminal catalytic domain (Ref.66). This topology has allowed a method of expressing soluble recombinant proteins, namely by replacing the signal anchor sequence with a cleavable signal-peptide allowing the synthesis of soluble enzymes secreted from the cell (Ref.66). Each of the sialyltransferases has been expressed as recombinant soluble enzymes in quantities suitable for large scale (>1g) reactions (Ref.58,68-70). By production of such recombinant sialyltransferases in high yield expression systems, a virtually unlimited supply of enzymes can be secured.

To address the need to use expensive CMP-NeuAc as a donor substrate, Ichikawa et al. (Ref.58) devised an enzymatic 'CMP-NeuAc cycle' for *in situ* regeneration of CMP-NeuAc during the course of the enzymatic sialylation. This cycle is based on the principle established earlier by Wong et al. (Ref.71) for *in situ* regeneration of UDP-Gal during a galactosyltransferase reaction. In the cycle, CMP-NeuAc is synthesized from a catalytic amount of CMP which is converted to CTP using two equivalents of phosphoenolpyruvate (PEP) and is then coupled to NeuAc by means of a recombinant CMP-sialic acid synthetase. As the CMP-NeuAc is consumed by the sialyltransferase the CMP released is recharged to CMP-NeuAc until the sialyltransferase reaction is finished. Remarkably, the cycle is also compatible with the *in situ* generation of NeuAc from ManNAc and pyruvate using N-acetylneuraminic acid aldolase, allowing synthesis of sialosides from inexpensive starting materials in a one pot reaction (Ref.72). This efficient regeneration system almost completely eliminates the need for prior synthesis of CMP-NeuAc for use in stoichiometric reactions.

The specificity of glycosyltransferases in general (Ref.8,73,74) and sialyltransferases in particular (Ref.8,52,74-76) have been examined by a number of laboratories to assess their adaptability for synthesis of analogs of the oligosaccharides they produce in nature. The central finding is that the enzymes typically have high selectivity for the spectrum of substrates found in nature, but exhibit surprising flexibility for unnatural analogs of their substrates. While key hydroxyl groups are essential for the substrates to be recognized by the enzymes, non-essential positions can be analogued and used as glycosyltransferase substrates to yield novel structures not found in nature. For the sialyltransferases this has been demonstrated for acceptor substrates (Ref.52,75) and for the donor substrate CMP-NeuAc (Ref.76,77). These studies demonstrate that sialyltransferases can be used to make analogs of natural sialosides within the limitations of their acceptor specificity. We have used this principle in exploring the use of sialyltransferases to prepare sialoside glycosyl donors for a general approach to the block synthesis of more complex sialosides.
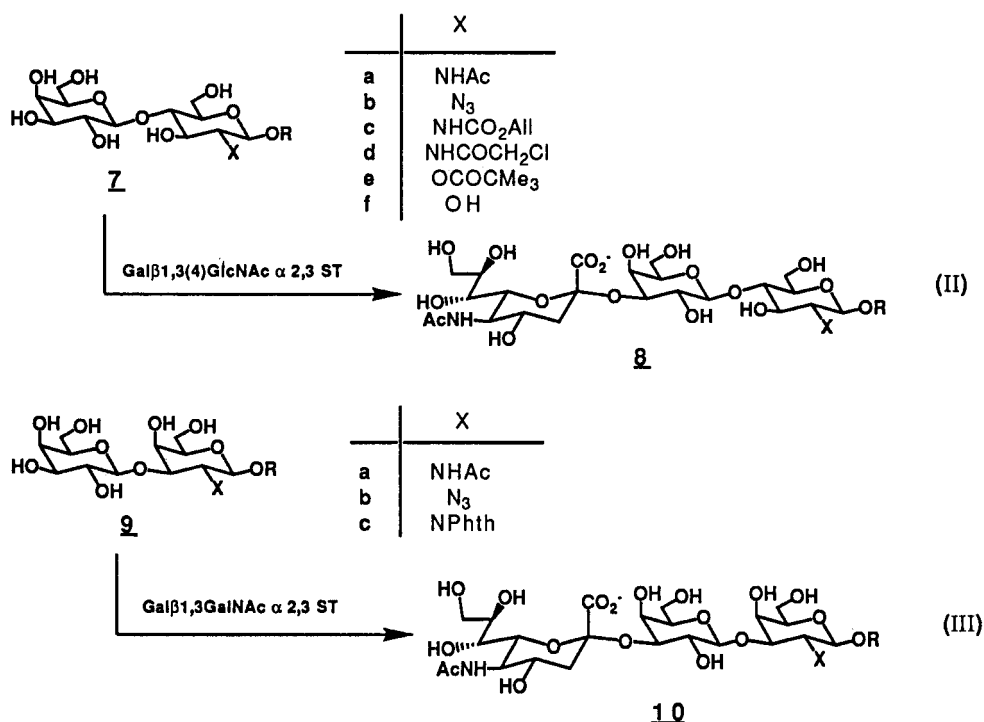
UTILITY OF SIALYLTRANSFERASES FOR PREPARATION OF OLIGOSACCHARIDE GLYCOSYL DONORS

For synthesis of complex oligosaccharides the generally accepted strategy is to use a retrosynthetic approach involving the assembly of suitable oligosaccharide glycosyl donors and acceptors which can be joined to make larger structures (Ref.78). This typically simplifies the problem of evolving independent synthetic strategies to synthesize a series of related compounds since the protected oligosaccharide blocks can often be used for synthesis of multiple compounds. Although synthesis of sialic acid containing glycosyl donors has become increasingly efficient, the overall yield starting from sialic acid is typically 15-20% due to the additional steps of protection of the

sialic acid and the excess of the sialic acid glycosyl donor required to achieve efficient glycosylation of the glycosyl acceptor. For this reason it was of interest to develop an efficient synthesis of sialoside glycosyl donors using a combined chemical and enzymatic approach.
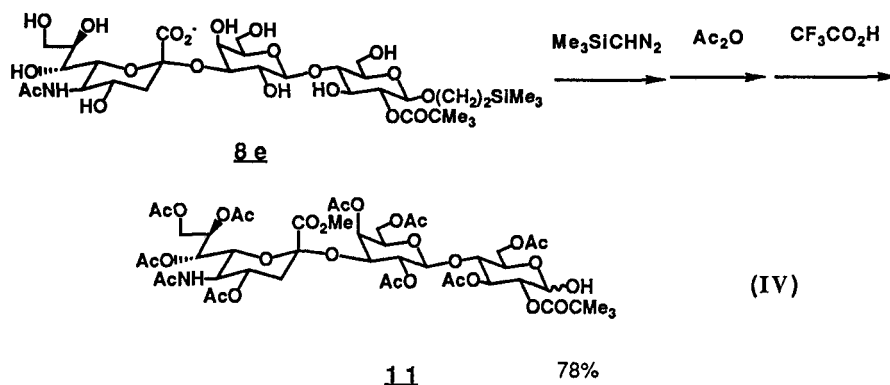
In order for a combined chemical and enzymatic synthesis strategy to be practical, several conditions must be met. Since the products can be considered 'bulk' intermediates, the enzymatic sialylation must be doable in a large scale with reasonable economy. This problem is now solved in principle, through developments of recombinant enzyme production and the CMP-NeuAc cycle (see above). It is also necessary that the specificity of the sialyltransferases are broad enough to tolerate some minor modifications of substrates so that the products can be efficiently converted to suitable glycosyl donors. Especially important is C-2 of the reducing terminal residue since the stereoselectivity and efficiency of chemical glycosylation is highly dependent on suitable substituents (or protecting groups) at this position. Finally, once enzymatic sialylation is complete, the product must be efficiently protected by blocking groups and converted to an activated glycosyl donor.

To address the question of the sialyltransferases utilizing appropriate acceptor substrates a variety of synthetic disaccharides were investigated as substrates for the Galβ1,3/4GlcNAc α2,3 sialyltransferase (Equation II) and the Galβ1,3GalNAc α2,3 sialyltransferase (Equation III). In addition to the natural substrates of these two enzymes (**7a** and **9a**, respectively), analogs with substitutions at the C-2 positions were all found to be effective substrates (**7b-e** and **9b,c**, respectively). The 2-O-pivaloyl derivative **7e** was especially interesting since it was actually a much better acceptor (lower Km and higher Vmax) than the parent compound lactose (**7f**). In addition, the pivaloyl substitution at C-2 is known to have particular value in improving the efficiency and stereochemical selectivity of glycosylation reactions.

	X
a	NHAc
b	N_3
c	$NHCO_2All$
d	$NHCOCH_2Cl$
e	$OCOCMe_3$
f	OH

Galβ1,3(4)GlcNAc α 2,3 ST (II)

8

	X
a	NHAc
b	N_3
c	NPhth

9

Galβ1,3GalNAc α 2,3 ST (III)

10

In fact all the substituents at C-2 were chosen for their potential value in synthesis of useful glycosyl donor substrates. For example, N-phthaloyl (Ref.79), N-allyloxycarbonyl (Ref.80) and O-acyl (Ref.78) groups are known to direct the stereochemical fidelity of glycosylation reactions with selectivity for a β (1,2-trans) product while a 2-azido derivative can be used for both α(1,2-cis) and β-glycoside synthesis depending on the nature of leaving groups and reaction conditions

(Ref.81, 82). Therefore, judicious use of the corresponding sialoside products is expected to give an access to various natural sialosides such as ganglioside GD1a and O-linked oligosaccharides of glycoproteins (e.g., NeuAcα2,3Galβ1,3GalNAcαThr/Ser).

$$\underset{\textbf{8 e}}{\text{(structure)}} \xrightarrow{\text{Me}_3\text{SiCHN}_2} \xrightarrow{\text{Ac}_2\text{O}} \xrightarrow{\text{CF}_3\text{CO}_2\text{H}} \underset{\textbf{11} \quad 78\%}{\text{(structure)}} \qquad \text{(IV)}$$

In order to utilize these sialyltransferase products as glycosyl donors, protection of hydroxyl groups followed by selective deblocking and activation of the anomeric position are required. Preliminary efforts suggest that this can be accomplished in an efficient manner as indicated in Equation **IV**. Thus, it is anticipated that a combined chemical and enzymatic synthesis of sialic acid containing oligosaccharide glycosyl donors may represent a viable strategy for synthesis of complex sialosides. Indeed, compound **11** has been successfully used in the synthesis of ganglioside GM3 (NeuAcα2,3Galβ1,4Glcβ-ceramide) with an overall yield of 40% starting from free NeuAc (Y. Ito and J. C. Paulson, in preparation).

SUMMARY AND PERSPECTIVE

As presented above there have been rapid advances in development of both chemical and enzymatic methods of glycosylation, with each method offering certain advantages while posing certain limitations. Increasing applications of a combined chemical and enzymatic approach to the synthesis of sialosides and other oligosaccharides offers the opportunity to explore the most efficient use of the diverse methodologies now available to the synthetic chemist. Through such pioneering efforts it is likely that highly practical large scale synthesis of complex carbohydrates will be achieved.

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