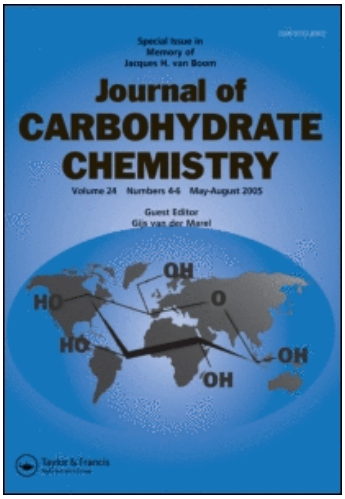

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RECENT DEVELOPMENTS IN TECHNOLOGY FOR GLYCOSYLATION WITH SIALIC ACID

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RECENT DEVELOPMENTS IN TECHNOLOGY FOR GLYCOSYLATION WITH SIALIC ACID*

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INTRODUCTION

Sialic acids are a class of nine-carbon monosaccharides found at the termini of oligosaccharides in many mammalian cellular systems.[1] This class is represented by the prototypical congener *N*-acetylneuraminic acid (NeuAc, **1**, Scheme 1). These unique sugars are ubiquitous, and they are present as components of both glycolipids and glycoproteins. Some examples of sialic acid containing oligosaccharides are shown in Scheme 2. Sialic acids are found in a variety of glycosidic linkages, some more common of which are α-2,3- or α-2,6-linkages to galactose residues. Additionally, they frequently exist as α-2,8-linked oligomers or polymers.

The lack of efficient technology to accomplish glycosylations with sialic acid is one of the long-standing deficiencies in carbohydrate chemistry.[2] Owing to the central role of sialic acids in carbohydrate recognition events, the development of high-yielding and operable methods to synthesize sialic acid glycosides has been the subject of considerable research. The development of such methods allows the construction of complex sialic acid containing glycoconjugates for the investigation of their roles in biochemical and cellular processes.

Most classical methods for synthesizing sialic acid glycosides are based on the reaction of an activated sialic acid such as **2** with an oligosaccharide glycosyl acceptor bearing a hydroxyl group nucleophile (Scheme 3).[2] The leaving group is typically a halogen, such as a chloride or bromide, and the activator is typically a heavy metal salt.[3,4] A regioselective union of the two reacting partners of course relies on appropriate protecting group patterns for both the glycosyl donor and acceptor com-

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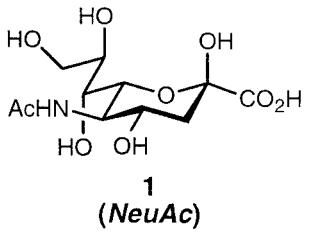


1
(*NeuAc*)

Scheme 1.

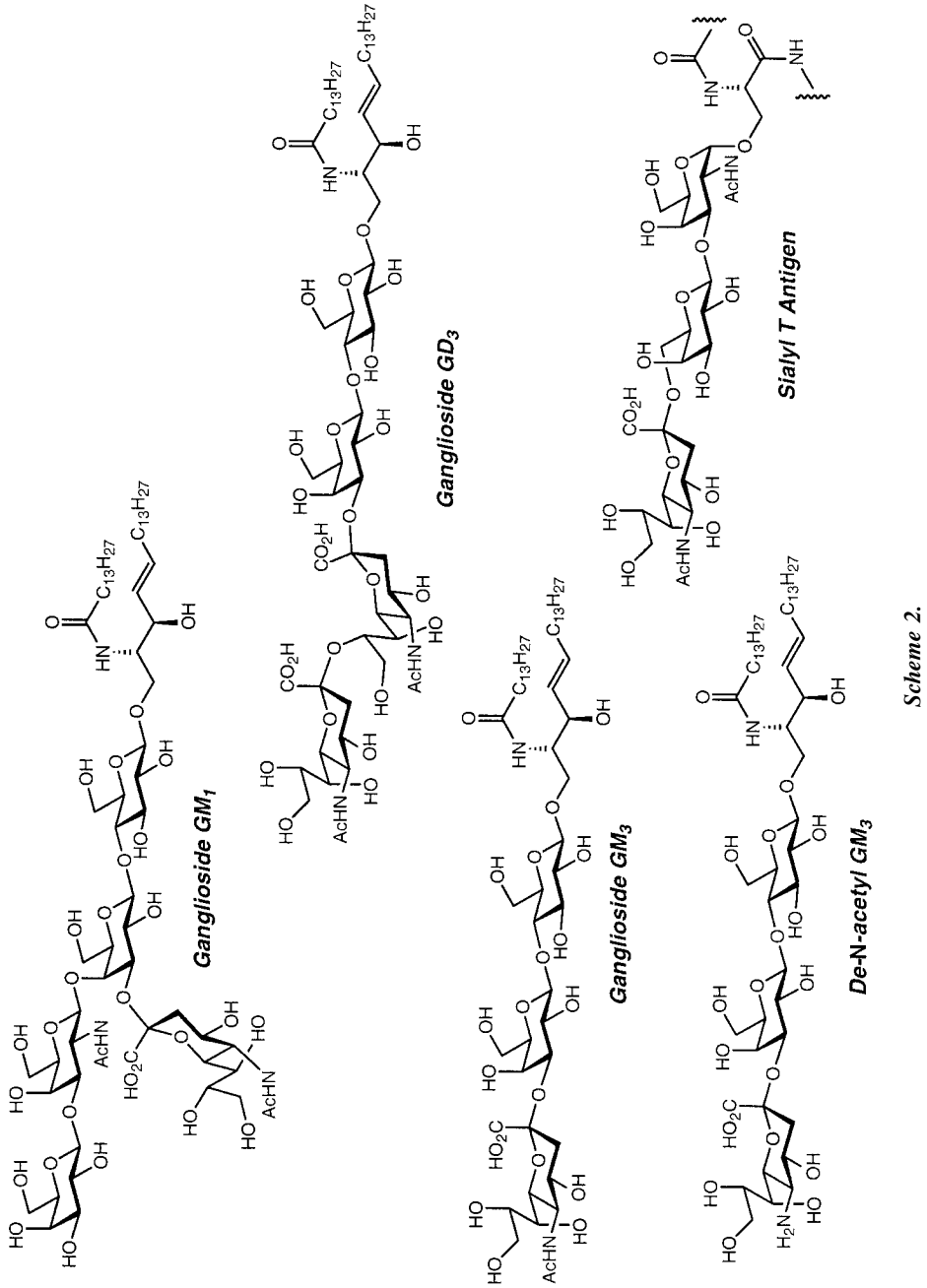
ponents. Many of the early methods are generally plagued with side reactions, low yields, and poor stereoselectivity. There are several reasons for these shortcomings. The electron-withdrawing carboxylate at the anomeric carbon disfavors the formation of the oxocarbenium ion **6** (**7**) en route to glycoside bond formation (Scheme 4). This oxocarbenium ion intermediate is also somewhat sterically hindered, so attack of hydroxyl nucleophiles can be slow. Therefore an elimination to provide **9** can be a significant competing reaction pathway. Additionally, since the reacting carbon is insulated from the nearest stereogenic center by either O6 or C3, there is very little steric biasing of one face of this oxocarbenium ion over the other. As a consequence, the stereoselectivity commonly observed is less than desirable. Complicating the issue further, the anomeric effect causes α-glycosides to be thermodynamically less stable than the corresponding β anomers. Heating a sialic acid ester in the presence of a primary alcohol and an acid catalyst results in exclusive formation of the β-glycoside (Scheme 5).[5] As a result of these factors, glycosylation reactions with sialyl donors often provide low yields of the desired sialic acid α-glycosides. Thus the synthesis challenge of efficient glycosylation with sialic acid is a daunting one indeed.

More recently, this long-standing problem has been revisited in a number of laboratories. This renewed focus was influenced significantly by the recent explosion of discoveries concerning the roles of oligosaccharides in biochemical processes of all types. Reviewed in this chapter are some selected recent advances in both chemical and enzymatic glycosylation with sialic acid.

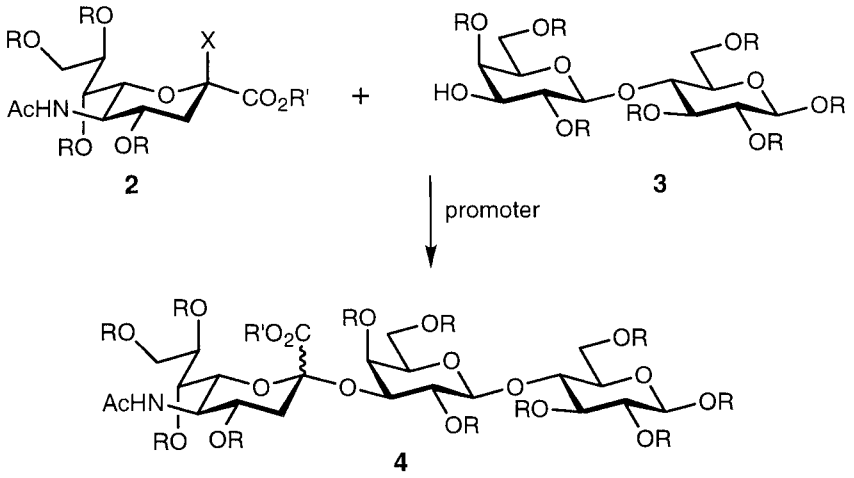
IMPROVED VARIATIONS OF THE CLASSICAL METHODS

Optimized Leaving Groups and Activation Methods

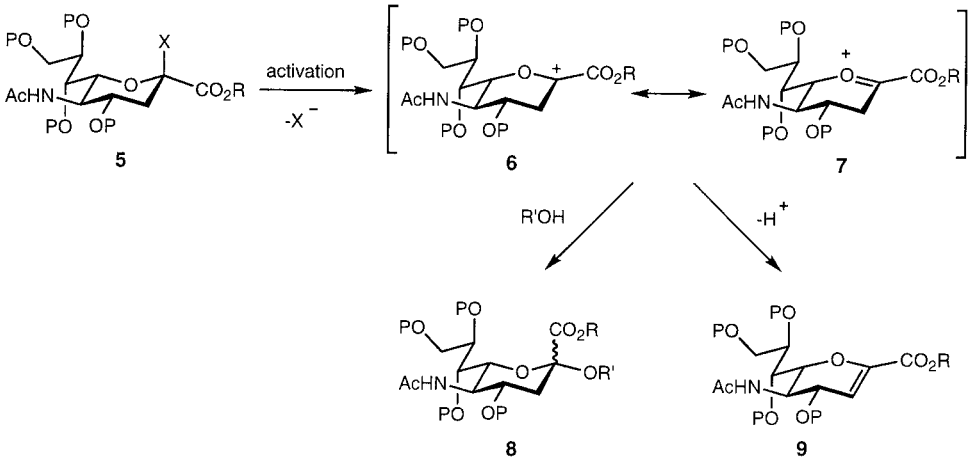
α-Glycosides of sialic acid are classically prepared by the Koenigs–Knorr[3] or Helferich[4] method, each of which involves the activation of a glycosyl halide by a metal salt and subsequent displacement by an alcohol (Scheme 6). Typically a metal salt such as silver(I) (Koenigs–Knorr) or mercury(II) (Helferich) complexes to a β-glycosyl halide **5** to form an activated intermediate **12**. This intermediate can then be directly displaced to give the product **8** or dissociate to form an ion pair **6**. It has been hypothesized that an ion pair may block the top face of the molecule and force bottom face attack to also provide the desired α-glycoside **8**. solvent polarity plays an important role in determining the mechanistic course of the reaction. One hypothesis is



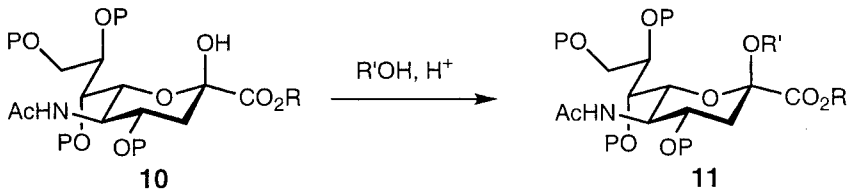
Scheme 2.



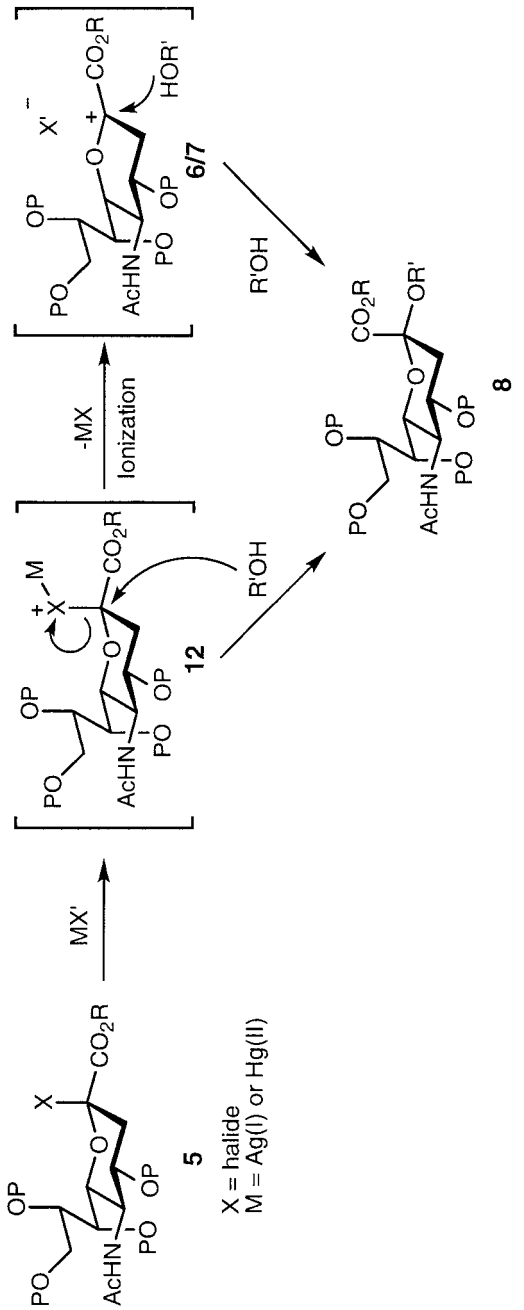
Scheme 3.



Scheme 4.



Scheme 5.

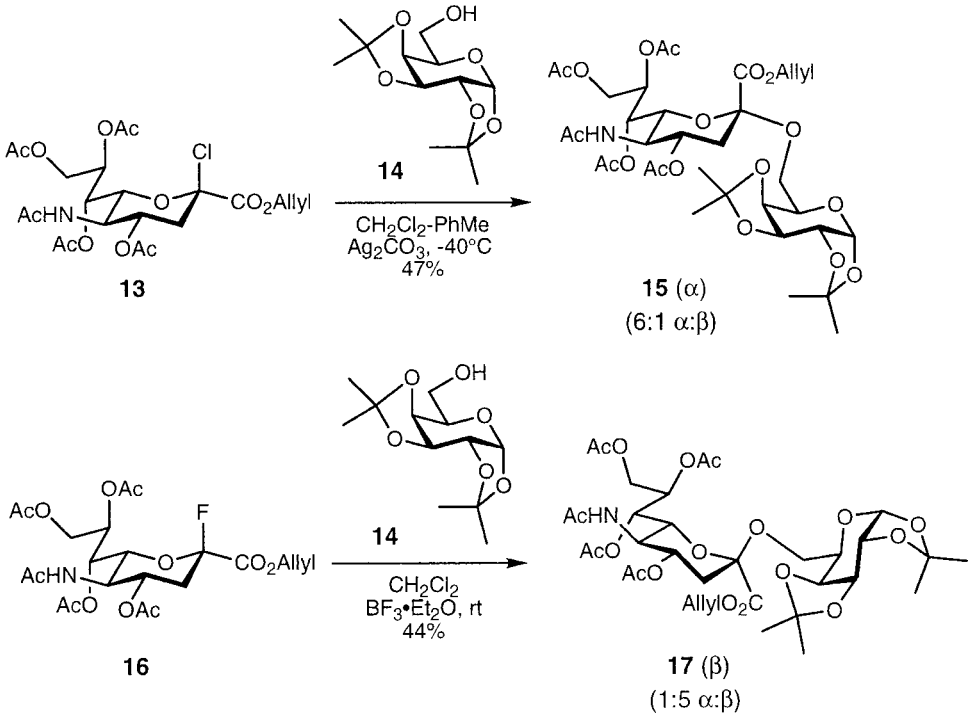


Scheme 6.

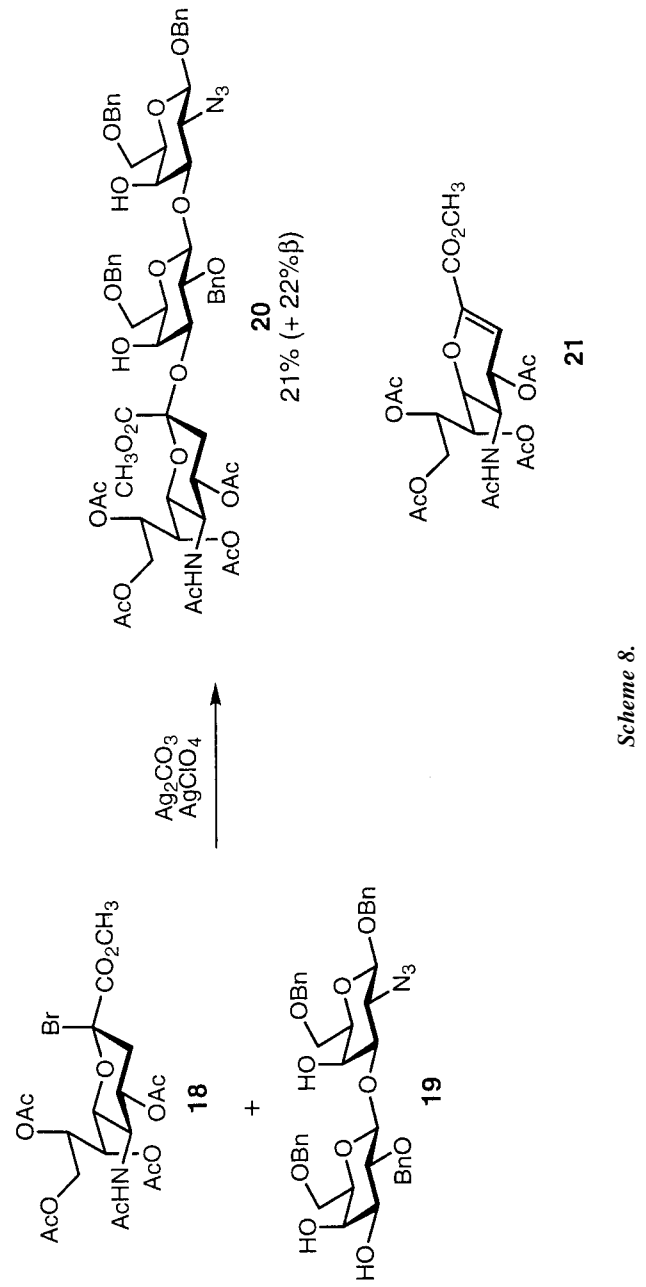
that more polar solvents favor the ion pair **6** because they can solvate the charged species, while the S_N2 pathway is preferred in nonpolar solvents.

Many variations of the metal promoters have been examined to probe the effect of the activator on the Koenigs–Knorr glycosylation yield and stereoselectivity. The promoters display a wide range of activity depending on the metal and counterion present. The order of reactivity among promoters with glycosyl halides was experimentally determined to be $AgOTf > Ag_2CO_3 >$ silver salicylate $> HgBr_2 > Hg(CN)_2$.[6] Generally, the more reactive silver promoters furnish higher stereoselectivities than the mercury salts, but give lower overall yields. In the majority of cases examined, the compound of choice was the most active promoter, AgOTf, or the least reactive, $Hg(CN)_2$.

Glycosyl halides are the typical sialyl donors in these processes. Sialyl chlorides are generally less reactive than bromides, but they are not as susceptible to decomposition and are sufficiently active under silver ion promotion to make them the most common halogenated glycosyl donor.[2] Sialyl fluorides are useful for glycosylation reactions but are usually activated under Lewis acidic conditions. An advantage of using fluoride glycosyl donors is their inherent preference to form β-glycosides. Under Koenigs–Knorr conditions, β-sialyl chloride **13** leads to predominantly the α-glycoside **15** in a glycosylation with primary alcohol **14**.[7a] However, when the β-fluoride **16** is activated with $BF_3{\cdot}OEt_2$, the β-glycoside **17** is afforded in a 5:1 β:α mixture of anomers (Scheme 7).



Scheme 7.



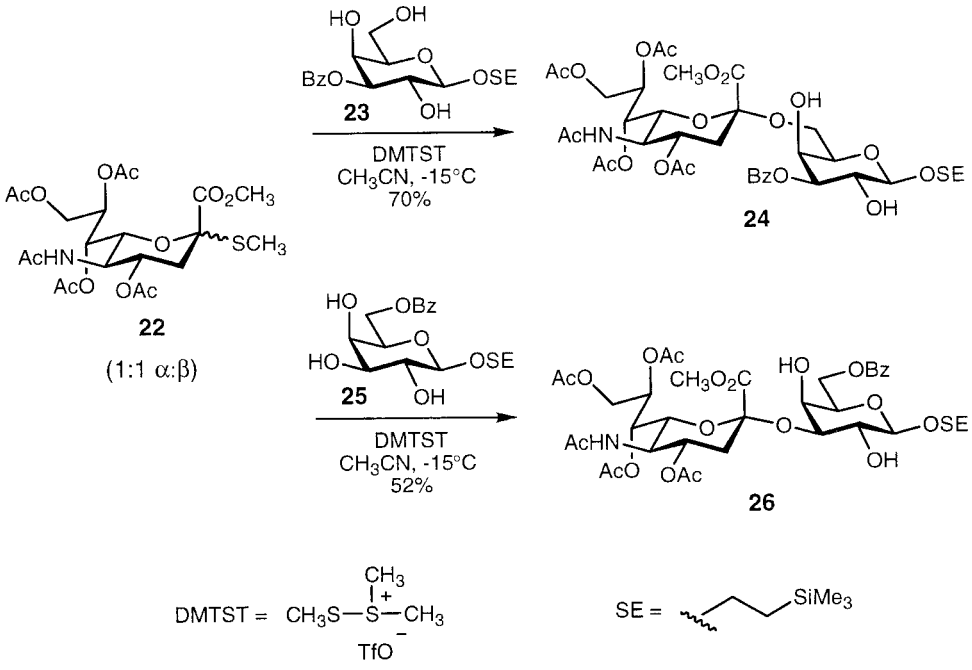
Scheme 8.

Methods derived from the Koenigs–Knorr approach are useful for glycosylation reactions involving reactive alcohols, such as primary alcohols. Unfortunately, when more hindered secondary alcohols are employed, the greater steric bulk of the nucleophile leads to lower stereoselectivities and yields. This is an important factor because many naturally occurring sialosides contain linkages to secondary alcohols. Sialyl bromides can be employed as more reactive donors in these instances.

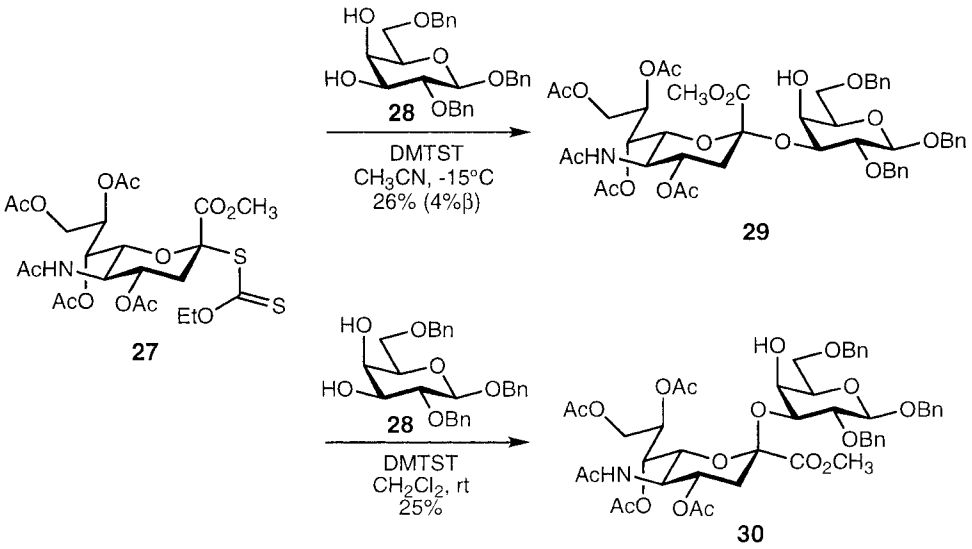
As a representative example, Paulsen and Von Reessen used a sialyl bromide to apply the Koenigs–Knorr method to the synthesis of an α-2,3-linked disaccharide (Scheme 8).[7b] Glycosylation of the galactose acceptor **19** with sialyl bromide **18** led to the desired disaccharide **20**, although with poor stereoselectivity (ca. 1:1 α:β). The low yields of Koenigs–Knorr glycosylations with secondary alcohols are partly due to elimination of the sialyl halide and formation of the 2,3-dehydro derivative **21**.

Many of the methods of activation using Koenigs–Knorr conditions require heterogeneous promoters and long reaction times at room temperature. In addition, the expense and toxicity of silver and mercury makes these methods impractical for large-scale syntheses. Consequently much research has focused on the development of glycosyl donors that could be activated at low temperature, under mild conditions, and without the need for heavy metal promoters.

Sialic acid thioglycosides, although initially synthesized for use as biological probes, have been found to be excellent glycosyl donors. Hasegawa and coworkers utilized thioglycosides in the synthesis of several α-2,3- and α-2,6-linked disaccharides. The methylsulfide donor **22** was activated by using the thiophile dimethyl(methylthio)sulfonium triflate (DMTST) at $-15°C$ in the presence of either



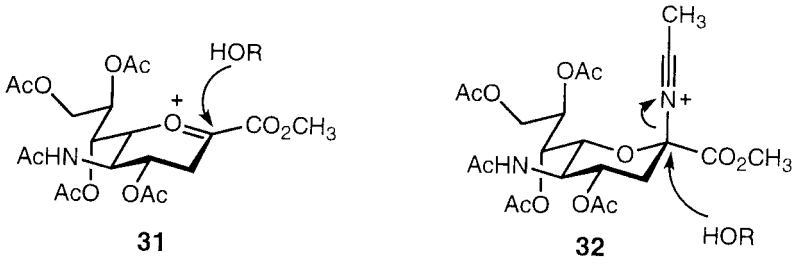
Scheme 9.



Scheme 10.

acceptor **23** or **25** to provide compounds **24** and **27**, respectively, as exclusively the α-glycosides (Scheme 9).[8] High stereoselectivities were observed for these glycosylations when the reactions were performed in acetonitrile, even though the thioglycoside starting material was a 1:1 mixture of anomers. This finding suggests that the stereoselectivity was not derived from the donor configuration as in Koenigs–Knorr glycosylations, but must depend on the reaction conditions.

In an attempt to determine the source of the stereocontrol, Sinäy and coworkers examined glycosylations of the xanthate donor **27** in polar and nonpolar solvent systems (Scheme 10).[9,10] Glycosylation of **28** in acetonitrile under DMTST activation afforded the α-2,3-linked disaccharide **29** in 26% yield as a 7:1 α:β mixture of diastereomers. However, the same reaction in CH_2Cl_2 gave exclusively the β-glycoside **30** in 25% yield (Scheme 10). The major by-product in both reactions was the 2,3-dehydro NeuAc derivative. Sinäy proposed that the glycosylation in CH_2Cl_2 probably involved an oxocarbenium ion **31** that underwent axial attack to form the β product (Scheme 11). However in acetonitrile, formation of a β-nitrilium intermediate **32**,



Scheme 11.

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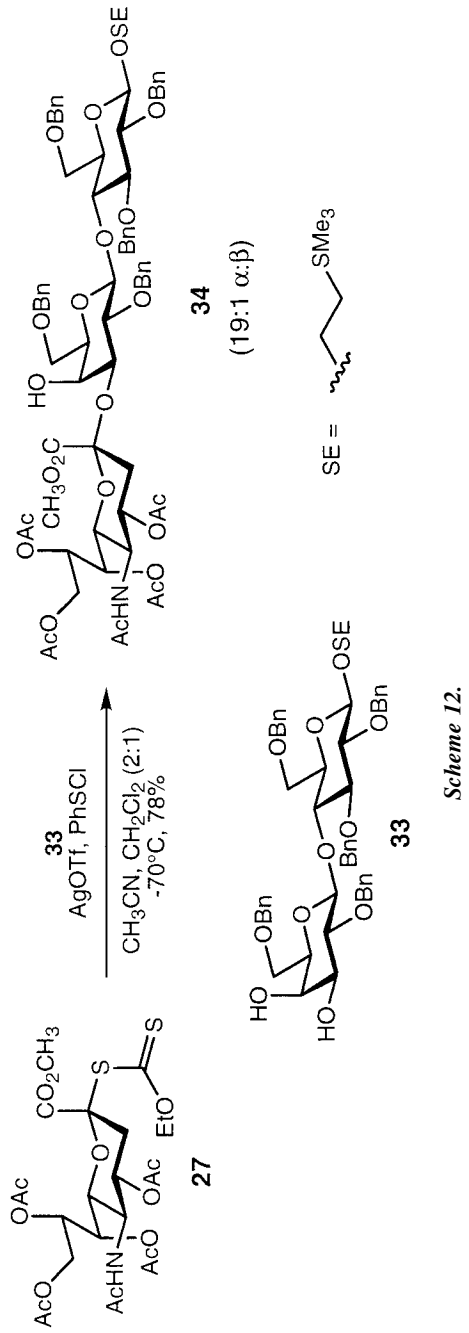
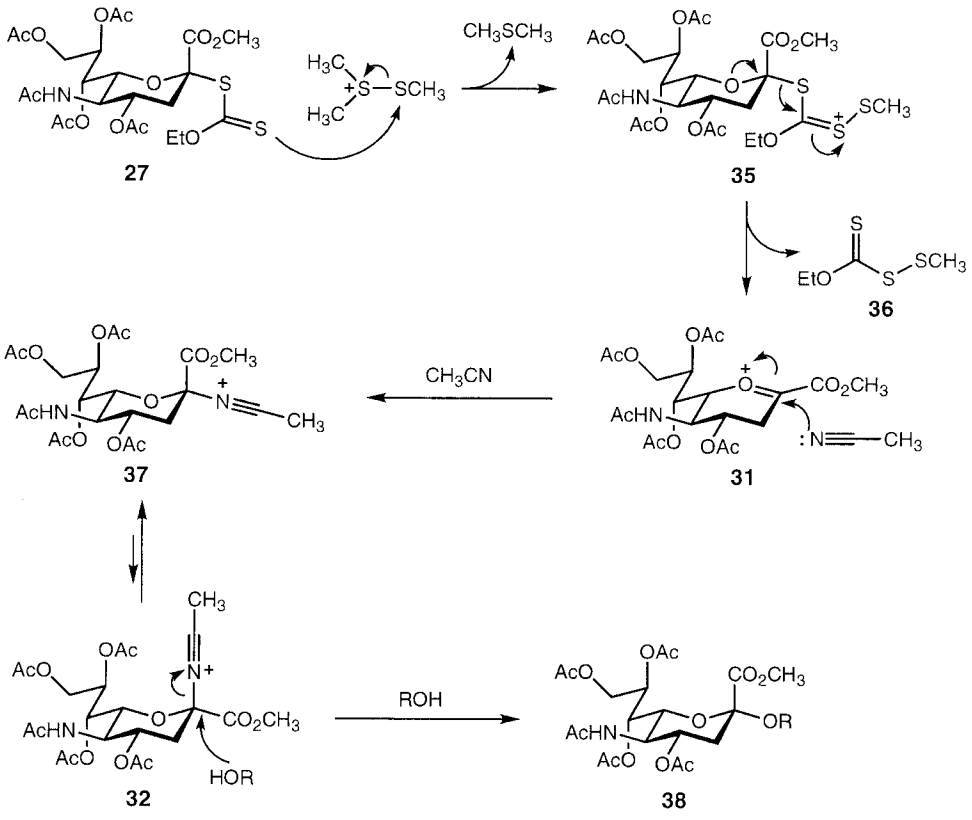


Scheme 12.

which undergoes S_N2 attack to afford the α-glycoside, may be the stereocontrolling element. The effect of acetonitrile has been studied in glucose and galactose systems, where the presence of axial nitrilium ions was supported by trapping and spectroscopic studies, both performed at low temperatures.[10]

The Whitesides group achieved a more efficient synthesis of α-sialosides from sialyl xanthates by modifying the reaction conditions of Sinäy's system.[11] Previously, Lönn and coworkers had demonstrated that lower temperatures and the use of milder promoter, methylsulfenyl triflate, increased the product yield and reaction stereoselectivity.[12] Whitesides and coworkers introduced an even milder promoter, phenylsulfenyl triflate (PST), which itself was prepared in situ from silver triflate and phenylsulfenyl chloride.[11] When sialylations of xanthate donor **27** were promoted by PST at $-70°C$ in a 2:1 ratio of CH_3CN to CH_2Cl_2, isolated yields of 60–80% and stereoselectivities of 19:1 $\alpha:\beta$ were observed. A representative example is shown in Scheme 12. Interestingly, when the reaction was conducted under high dilution (0.01 M in donor) the stereoselectivity improved to better than 99:1 $\alpha:\beta$, albeit at the expense of overall yield (52%).

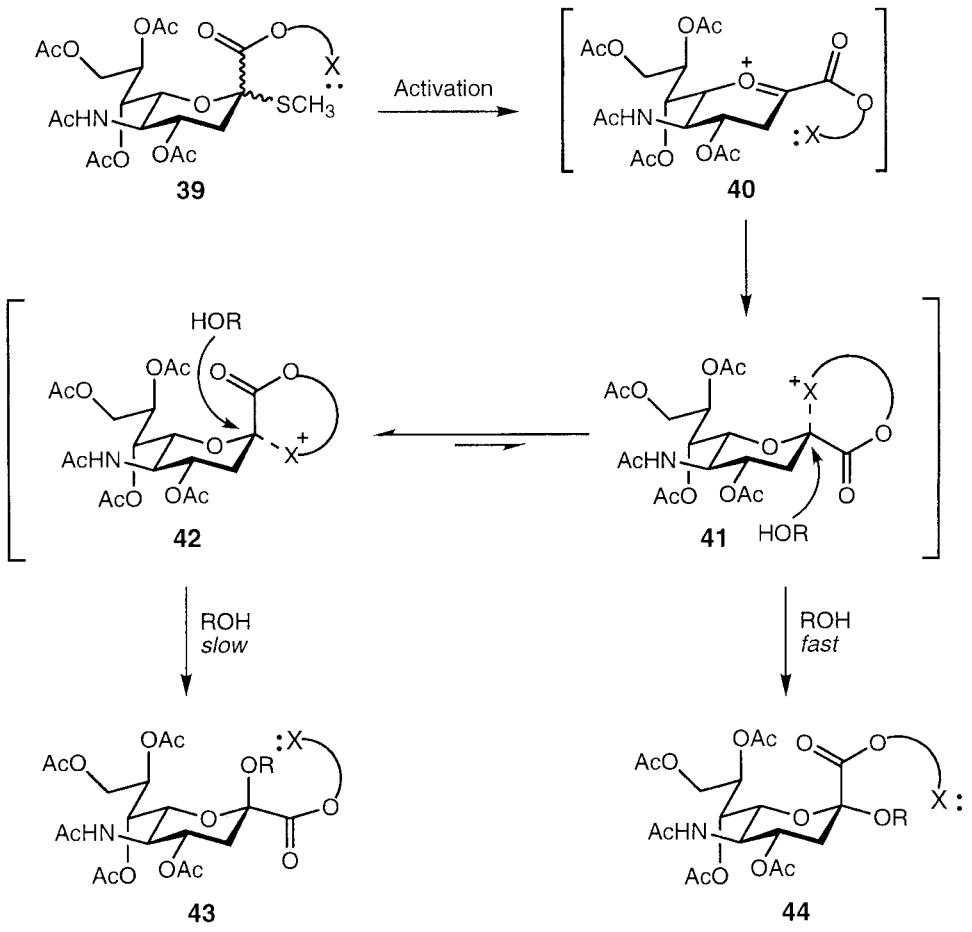
Regarding the reaction mechanism, Whitesides and coworkers also postulated that the presence of a β-nitrilium ion was the source of stereocontrol. In their pro-



Scheme 13.

posed mechanism, the xanthate **27** initially reacts with the PST promoter to form **35** (Scheme 13). Subsequent loss of **36** leads to the oxocarbenim intermediate **31**. Attack by acetonitrile is presumed to occur from the less hindered α face and leads to the presumably thermodynamically more favorable equatorial nitrilium ion **37** because of the reverse anomeric effect.[13,14] However the equatorial nitrilium ion is believed to be in equilibrium with the more reactive β species **32**. Acetonitrile is then displaced from this species by the glycosyl acceptor in an S_N2-like manner for form the α-glycosidic linkage in compound **38**.

The aforementioned experimental data do support this mechanism to some extent. First of all, the stereoselectivity is independent of the anomeric configuration of the starting material. A 1:1 mixture of xanthate anomers reacts to give predominantly the α-glycoside in acetonitrile, and the reaction by-product **36** was isolated and fully characterized. These data suggest that the xanthate is activated by the thiophilic promoter and glycosylation does not proceed through an S_N2-type mechanism. Finally,

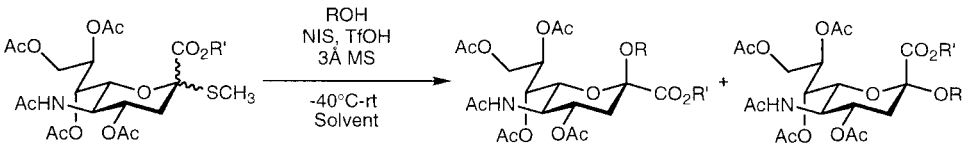


Scheme 14.

Whitesides proposes that the gain in stereoselectivity that was observed by performing the reaction dilute in acetonitrile corresponds to an increase in the [MeCN]/[acceptor] ratio. The authors believe that a competition exists between acetonitrile and the acceptor alcohol for the oxocarbenium ion intermediate. When the concentration of the glycosyl acceptor is high enough, the alcohol reacts directly with oxocarbenium ion **32** to form the β-glycoside. However, an increase in the amount of acetonitrile favors nitrilium ion formation over direct glycosylation, and since nitrilium ion formation is the stereocontrolling element, higher α:β ratios are observed.

Takahashi and coworkers examined a novel approach to stereoselective sialylations by incorporating an auxiliary into the ester.[15] An electron-donating group with an appropriate linker to the carboxylate carbon was anticipated to stabilize an oxocarbenium ion through long-range participation. Activation of compound **39**, where X is an electron-donating group, would produce oxocarbenium ion **40**. This intermediate could be stabilized by X to provide an equilibrating mixture of β and α ''onium'' isomers, **41** and **42**, respectively (Scheme 14). The authors predicted that the β-onium species **41** would preferentially undergo attack to afford the α-glycoside **44**.

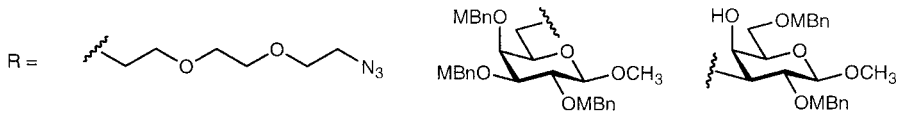
Preliminary experiments revealed that a sialyl donor containing a methyl sulfide and a 2-carbon spacer within the ester was the optimum substrate for these studies. This sialyl donor **48** was synthesized as an approximately 1:1 mixture of anomers and was activated with *N*-iodosuccinimide and trifluoromethanesulfonic acid. Glycosylations of compound **48** were compared with the methyl ester control **45** in various solvents to



45 R'=CH_3
48 R'=$CH_2CH_2SCH_3$

46 R'=Me
49 R'=$CH_2CH_2SCH_3$

47 R'=Me
50 R'=$CH_2CH_2SCH_3$



a **b** **c**

MBn=4-methylbenzyl

Entry	Donor (R')	Acceptor (R)	Solvent	Glycoside	Yield (%)
1	**45**	**a**	MeCN	**46:47** (62:38)	62
2	**45**	**a**	CH_2Cl_2	**46:47** (62:38)	70
3	**48**	**a**	MeCN	**49:50** (20:80)	50
4	**48**	**a**	CH_2Cl_2	**49:50** (38:62)	65
5	**48**	**a**	DME	**49:50** (05:95)	45
6	**48**	**b**	DME	**49:50** (16:84)	39
7	**48**	**c**	DME	**49:50** (09:91)	21

Scheme 15.

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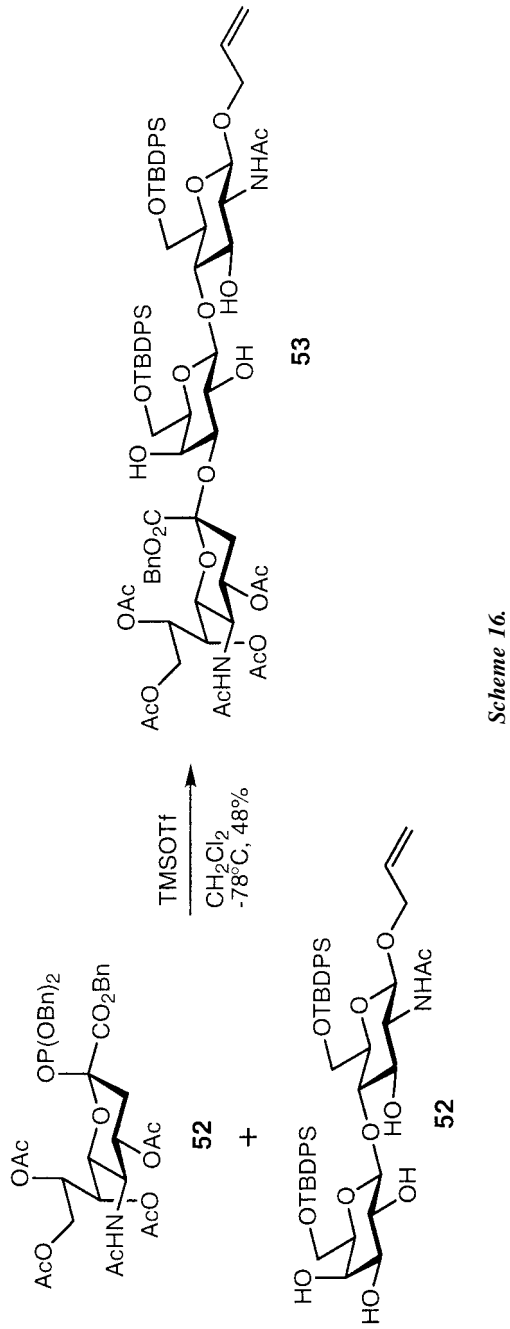
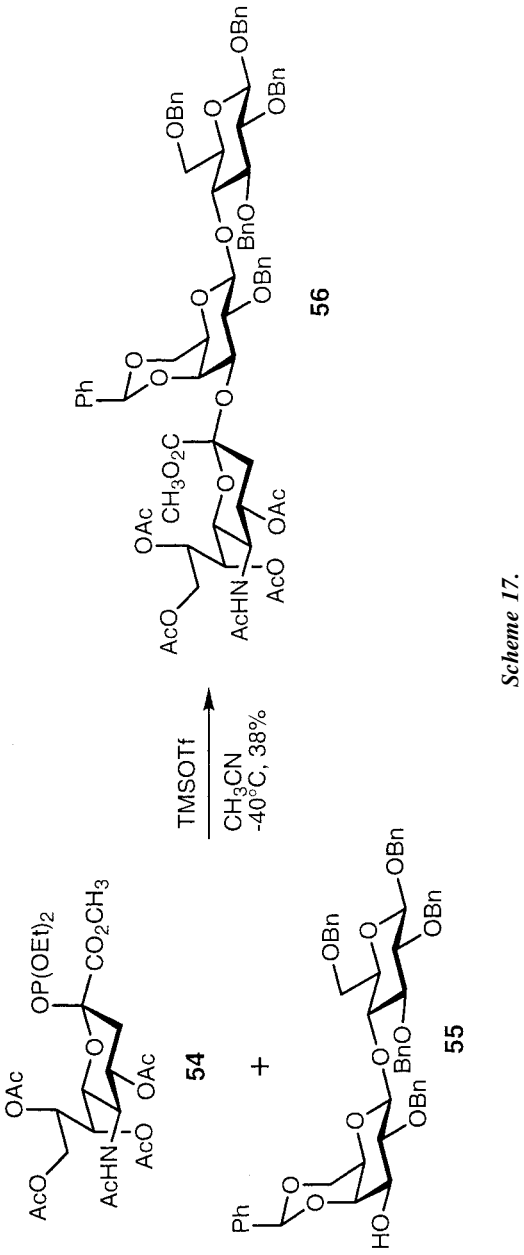


Scheme 16.



Scheme 17.

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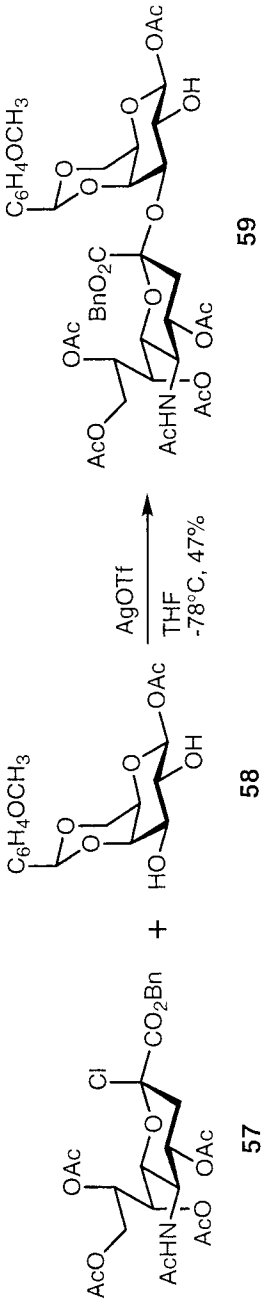


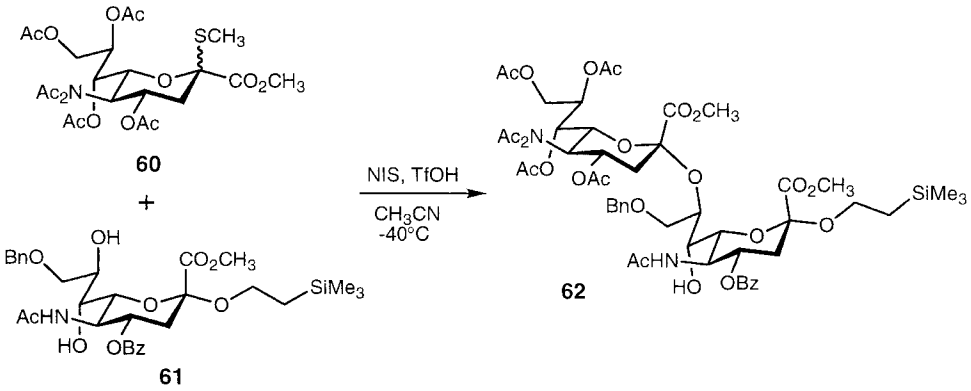
Scheme 18.

determine the extent of the enhancement of stereoselectivity (Scheme 15). Interestingly, glycosylations of 2-[2-(2-azidoethoxy)ethoxy]ethanol with donor **45** in acetonitrile or dichloromethane gave the same product ratios (entries 1 and 2), suggesting that for this system acetonitrile does not provide an increase in stereoselectivity. However, the tethered donor **48** predominantly favored formation of the α-glycosides (entries 3, 4, and 5), with the solvent dimethoxyethane (DME) providing the highest α:β ratios. Next, the sialyl donor **48** was utilized in the synthesis of α-2,6- and α-2,3-linked disaccharides under the optimal reaction conditions (entries 6 and 7). The selectivities observed were similar to those in the earlier examples, although increased formation of products derived from elimination caused the reaction yields to drop significantly.

A useful and powerful method that has been developed independently by Wong[16] and by Schmidt[17] and their colleagues utilizes sialyl phosphites as glycosyl donors. Phosphites are synthesized from the corresponding anomeric alcohols by reacting the latter with a phosphoramidite. This greatly shortens the reaction sequence needed to obtain the sialyl donors because many transformations that ordinarily would be needed to install a nonoxygen atom at the anomeric carbon are precluded, and several phosphoramidite reagents are readily available. An additional and significant advantage of these donors is that they are activated by many commonly used Lewis acids such as TMSOTf. Lewis acid activation is now a standard for glycoside synthesis, thus making these methods compatible with the most commonly used protecting group patterns and assembly strategies. Furthermore, sialyl phosphites are activated at low temperatures, and as a result generally provide products in high stereoselectivity. Some typical examples of the use of phosphites for sialylation are the Wong lab's synthesis **53**,[18] an intermediate en route to the sialyl Lexis X antigen (Scheme 16) and the Schmidt lab's synthesis of **56** (Scheme 17).[17a]

Protection Strategies to Optimize Yields and Regiocontrol

The course and outcome of glycosylation reactions with sialic acid can be greatly influenced in some instances by seemingly insignificant modifications of the protecting



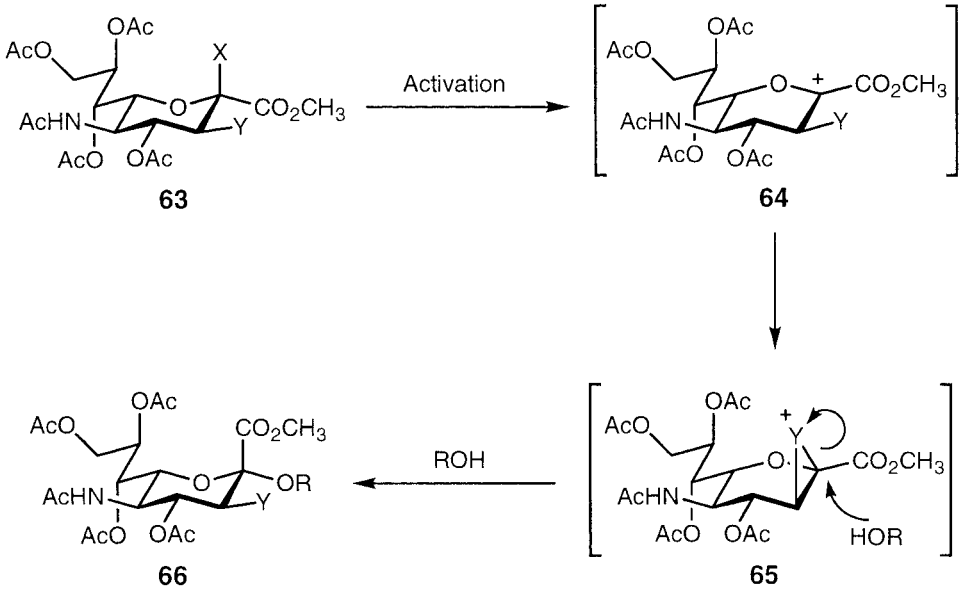
Scheme 19.

groups on the glycosyl donor or acceptor. For example, simply employing a benzyl ester rather than the more commonly used methyl ester to protect the carboxylic acid of NeuAc, in combination with a sialyl chloride as donor using Koenigs–Knorr-type conditions, has profound consequences on the stereoselectivity of the glycosylation.[19] This fact was recorded by Ratcliff and coworkers, and has been widely utilized.[19] A representative example is shown in Scheme 18.

The Boons laboratory has observed a dependence of the yield of glycosylation on the nature of the protecting group of the sialic acid C5 nitrogen.[20] When the nitrogen was engaged in an *N*,*N*-diacetylimide **60**, yields significantly increased relative to the simple acetamide, the latter bearing only one acetyl group. This observation was utilized to efficiently construct NeuAc-α-2,8-NeuAc linkages such as that in compound **62** (Scheme 19). The glycosylation of O8 of sialic acid by a sialyl donor is notoriously difficult to accomplish, and the donor **60** was nicely used to construct this linkage. A similar observation has been made by Hossain and Magnusson.[21]

USING DIRECTING GROUPS FOR STEREOCONTROL

Although several improved glycosyl donors have been developed to accomplish ''direct'' sialylations of acceptors, elimination reactions of the sialyl oxocarbenium ion intermediate often are significant competing nonproductive reaction pathways. Additionally, control of the stereochemistry at the NeuAc anomeric center is difficult and nontrivial. To alleviate these problems, several laboratories have investigated the use of



Scheme 20.

stereodirecting auxiliaries at C3 of NeuAc. The postulate is that this group Y in **63** (Scheme 20) could, after activation, assist in stabilizing the positive charge of the oxocarbenium intermediate **64**. In doing so, an intermediate such as **65** would be formed. Not only would this suppress elimination reactions, but one could imagine that the trajectory of attack of the glycosyl acceptor would be guided *anti* to the three-membered onium ion. As a result, the anomeric configuration might be assembled in a more predictable and α-selective fashion.

Goto and coworkers, who were early contributors, synthesized compounds of this nature from the 2,3-dehydro sialic acid **21**.[22] The Goto group initially investigated hydroxyl groups at C3 as directors and used the reliable Koenigs–Knorr method for glycosylation (Scheme 21).[22] The prototypical donor was the bromide **67**. As a means of examining the versatility of this sialyl donor, the α-2,9 and α-2,8 NeuAc dimers were chosen as the synthetic targets. Treatment of **67** with silver triflate and a selectively protected sialic acid glycal **68** afforded 42% of the α-2,9 dimer **69** along with a 21% of the β anomer. The secondary alcohol **70** was also glycosylated with **67** to give 26% of disaccharide **71**. Although the yield of the glycosylation was rather modest, the stereoselectivity was good (3:1 α:β). The Goto lab later found that bromides at C3 could also be used as directing groups.[23] This prompted a great deal of research into more convenient C3 substituents that would provide higher stereocontrol.

Ogawa and coworkers introduced C3 phenylselenyl and phenylthio substituents as stereocontrolling auxiliaries.[24,25] These directing groups had already been developed in the context of stereoselective syntheses of 2-deoxyglycosides by several groups.[26] Phenylthio and phenylselenyl neighboring groups were anticipated to provide better charge stabilization than a C3 hydroxyl substituent because of the increased polarizability of sulfur and selenium.[26] Initial glycosylation attempts with the phenylselenyl substituent proceeded with good stereoselectivity, although yields were low owing to elimination of a cationic selenium species to provide the corresponding 2,3-dehydro NeuAc derivative as the major product of the reaction.[24] The less polarizable phenylsulfide auxiliary was found to circumvent his problem.[25] Mercury-promoted glycosylation of the acceptor **73** with the donor **72** afforded the GM_3 precursor **74** in good yield with no trace of the β isomer (Scheme 22). An additional benefit of using a sulfur auxiliary over a hydroxyl was its relative ease of removal in one step by a radical reduction. Treatment of **74** with Bu_3SnH and IBN provided the trisaccharide **75** in 75% yield. The phenylsulfide auxiliary was also used to synthesize an α-2,8 NeuAc dimer.[25b] The Ogawa group immobilized these types of sialyl donor on solid support resins and demonstrated their ability to act as donors in solid phase glycosylations.[25c] Thioglycoside donors combined with C3 phenylthio directing groups have been effectively employed by the Magnusson laboratory.[27]

A disadvantage of auxiliary-directed glycosylation is that additional steps are required for the synthesis of the sialyl donor. To address this problem, the Whitesides group developed an expeditious route to a sialyl donor in two steps from the NeuAc glycal **21** (Scheme 23).[28] Treatment of **21** with 2,4-dimethylbenzenesulfenyl chloride afforded the crystalline intermediate **76** in 85% yield. This compound was quantitatively converted to the thioglycoside **77** by treatment with sodium methanethiolate.

Sialylations of **77** were achieved in good yield and stereoselectivity by using phenylsulfenyl triflate (PST) as a promoter and di-*tert*-butylpyridine (DTBP) as a

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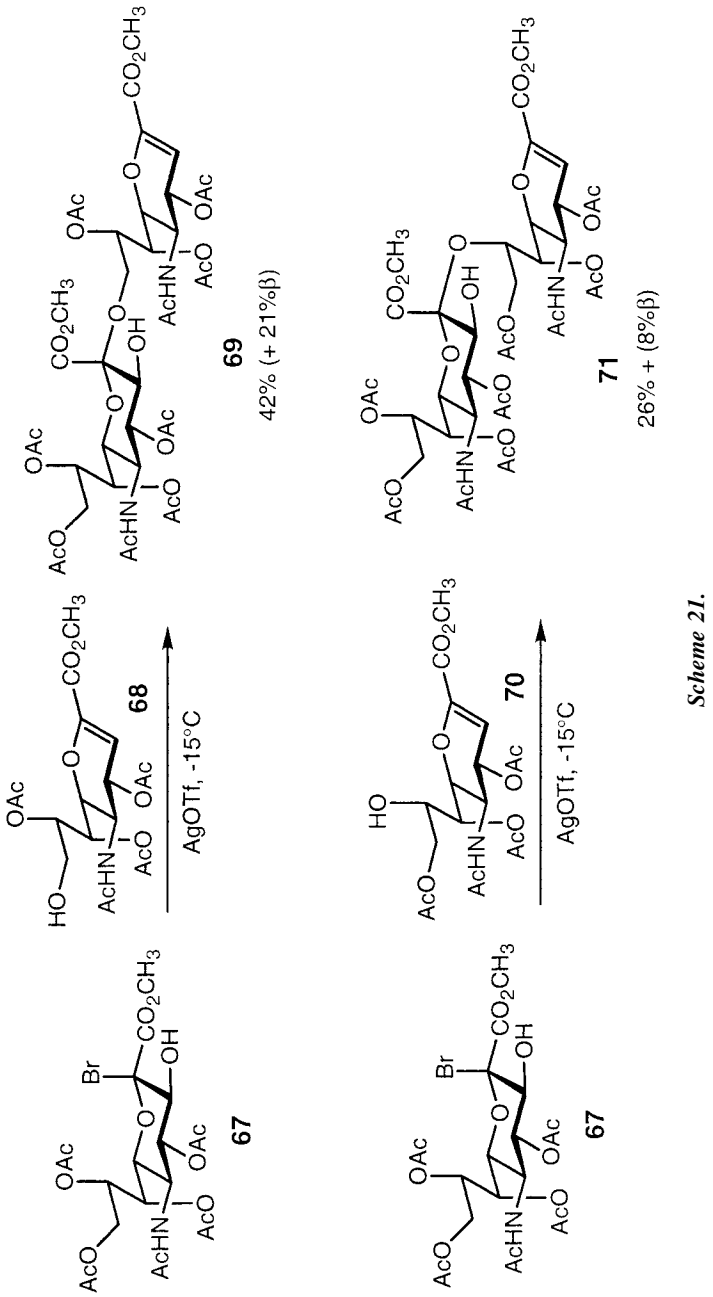
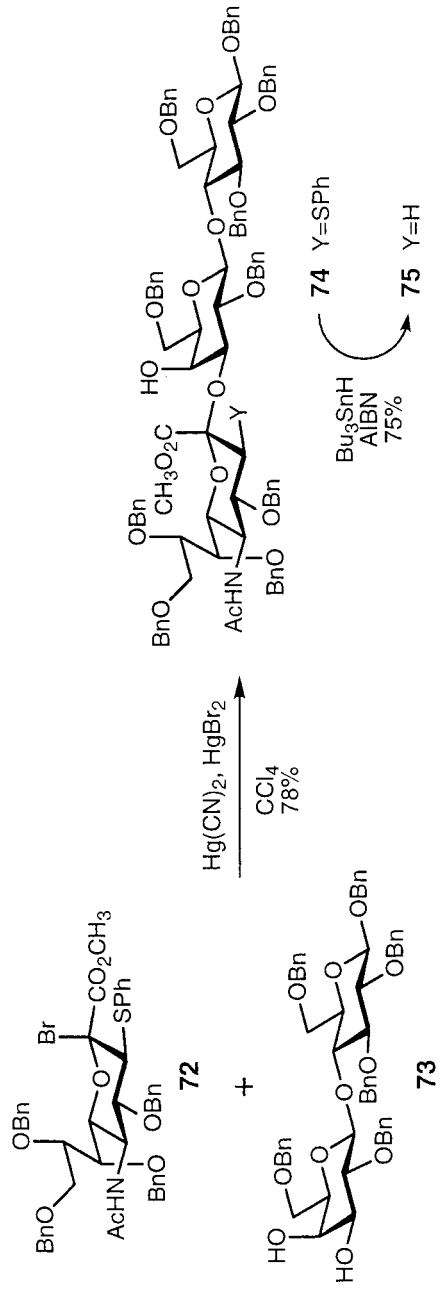
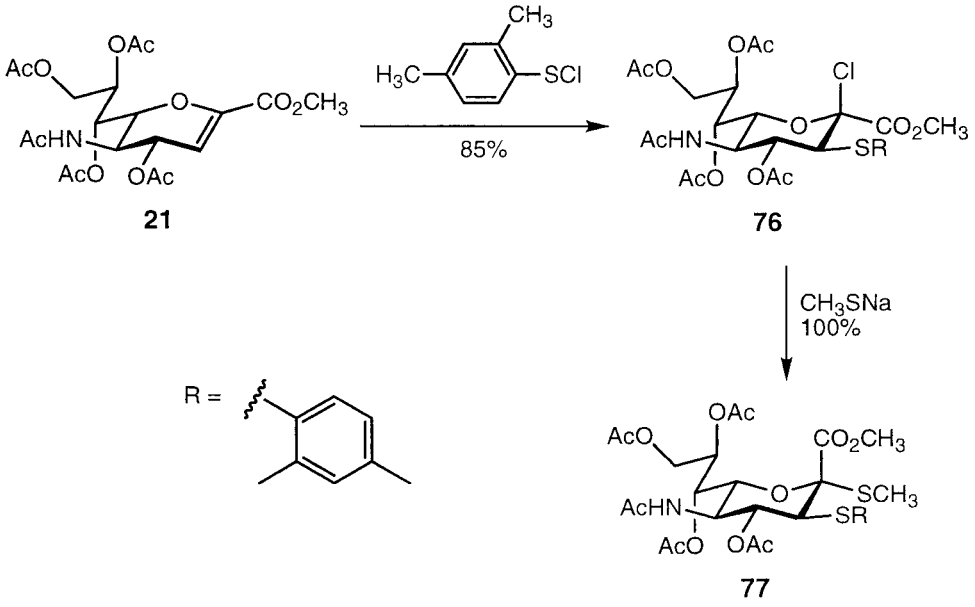


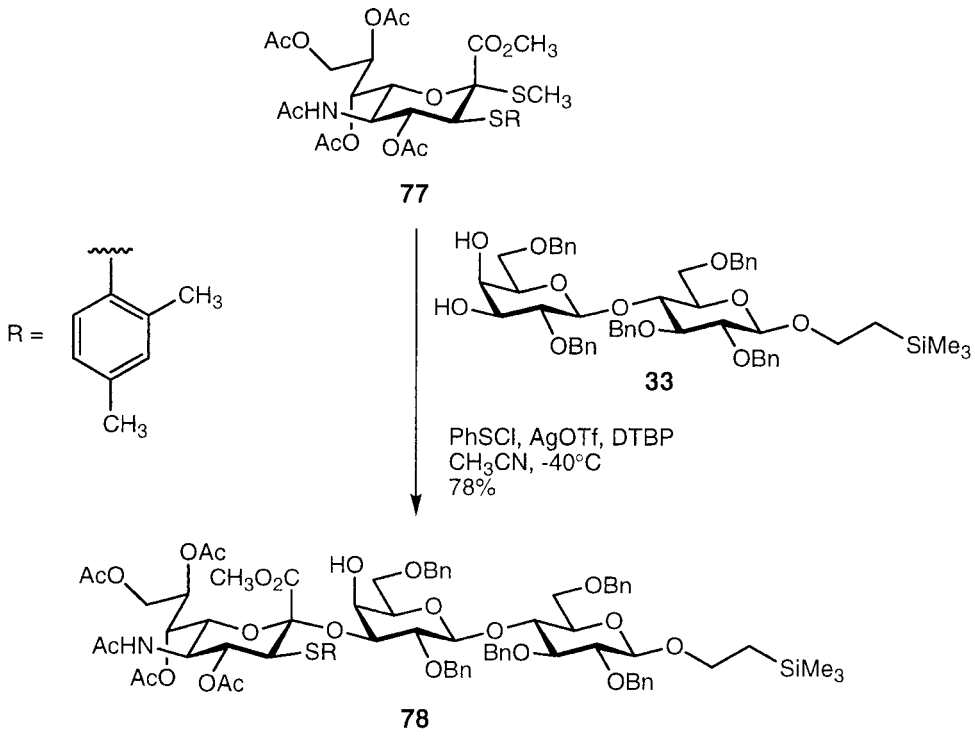
Scheme 21.



Scheme 22.



Scheme 23.

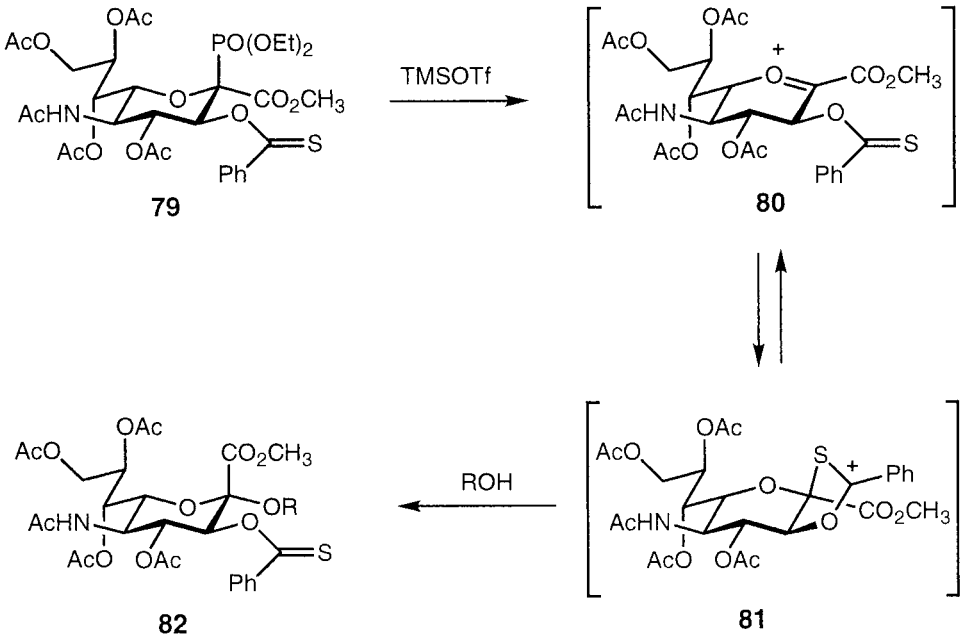


Scheme 24.

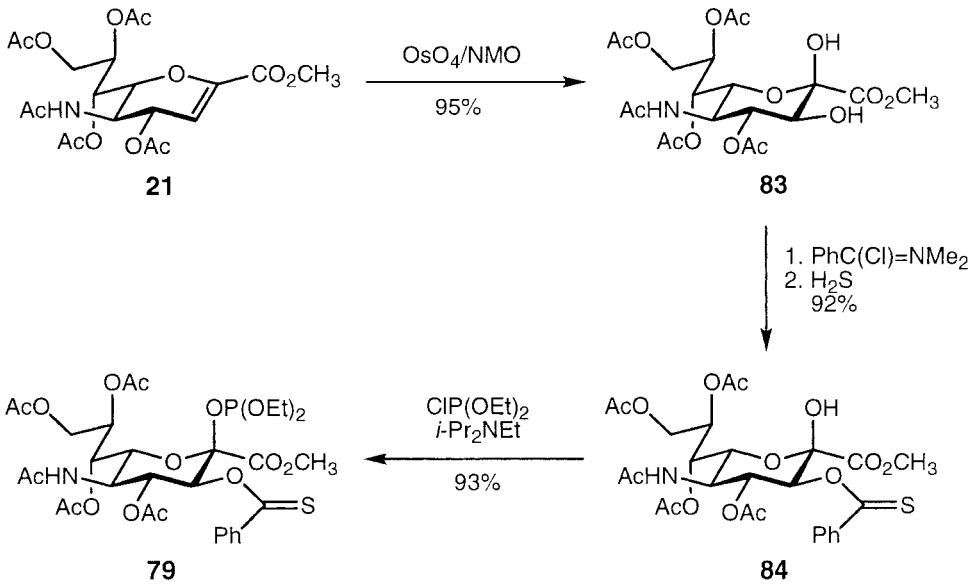
proton scavenger. The most impressive application of this donor was for the synthesis of the α-2,3-linked disaccharide **78**, which proceeded in 78% yield with no trace of the β isomer (Scheme 24). It was also demonstrated that the auxiliary could be efficiently removed by reduction with Bu_3SnH and AIBN in 94% yield.

In 1998 the Schmidt group looked to improve the efficiency and simplicity of sialyl glycosylations through the development of a phosphite donor containing a directing auxiliary at C3.[29] The primary objective was to develop an auxiliary that could be more easily introduced and removed and would more effectively direct the stereochemistry while minimizing side reactions. It was postulated that a better auxiliary might be one that could form a cyclic five-membered onium intermediate rather than a three-membered one. Compound **79** was found to meet the requirements. Acid-catalyzed activation of sialyl phosphite **79** should lead to oxocarbenium ion **80** without the need for stoichiometric promoters (Scheme 25). The intermediate oxocarbenium ion should readily undergo attack by the thionobenzoate auxiliary to form the intermediate **81**. Incorporation of the thionobenzoate auxiliary, which provides anchimeric assistance through a larger, more favorable five-membered ring, should provide higher stereocontrol than the three-membered episulfonium ions. Glycosylation should then proceed from the α face to provide the desired sialoside **82**. Following this reaction, the thionobenzoate auxiliary should be easily removed by treatment with Bu_3SnH/AIBN.

Many of the earlier syntheses of NeuAc derivatives containing auxiliaries were long and tedious because it was so difficult to obtain the correct stereochemistry at C3. However, Schmidt and coworkers discovered that the reaction of glycal **21** with OsO_4



Scheme 25.



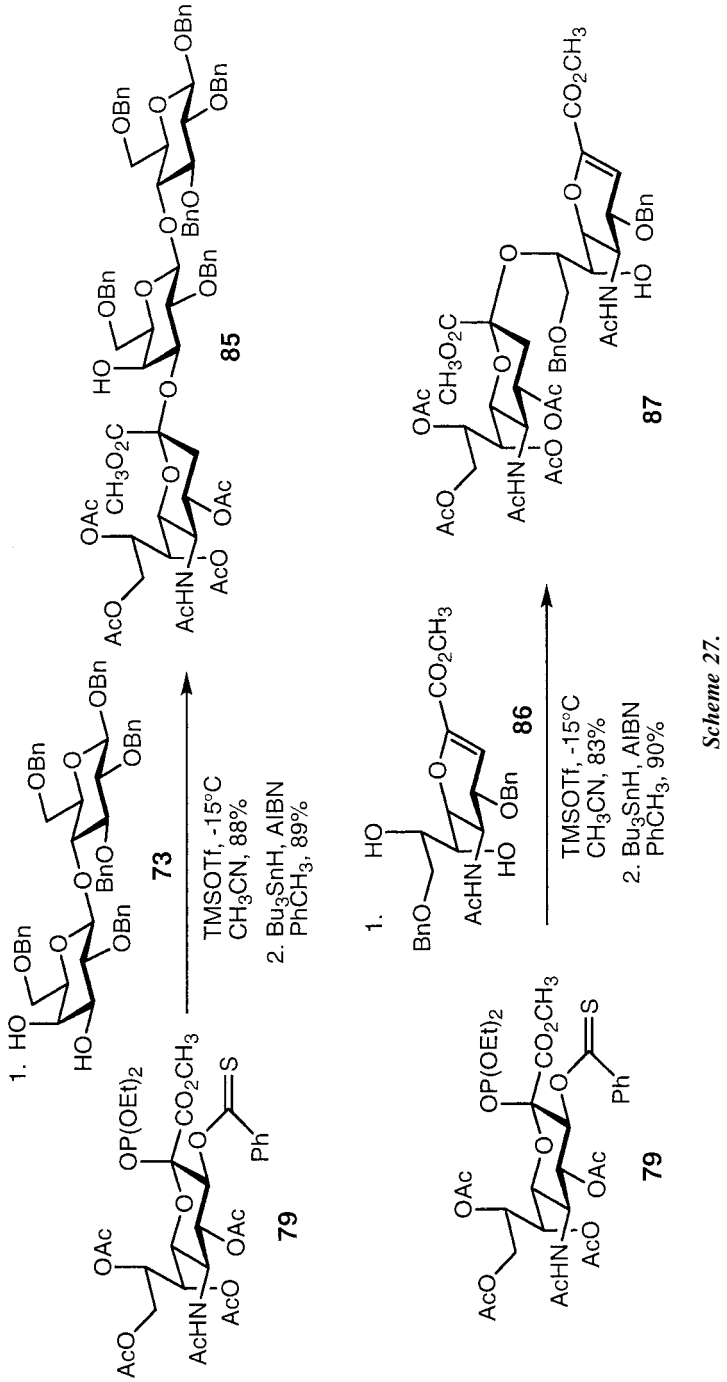
Scheme 26.

led to stereoselective dihydroxylation from the β face, providing diol **83** as the only product (Scheme 26). Subsequent regioselective introduction of the thiobenzoyl auxiliary was accomplished by treatment with *N,N*-dimethyl-α-chlorobenzimidium chloride followed by dihydrogen sulfide. Compound **84** was obtained as the product of this sequence in 92% yield. Finally, the alcohol was converted to the glycosyl phosphite **79** in a straightforward manner.

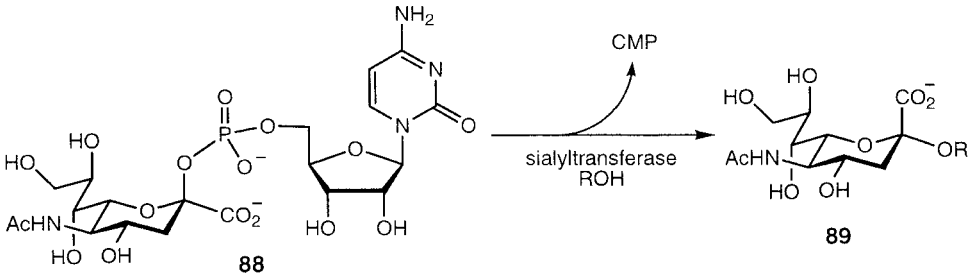
The utility of this donor was demonstrated in the synthesis of an α-2,3-disaccharide and an α-2,8-linked NeuAc dimer. Glycosylation of the protected lactose derivative **73** with donor **79** afforded an 88% yield of the protected GM_3 analog **85**, with no trace of the β-glycoside (Scheme 27). Likewise, glycosylation of glycal **86** provided an unprecedented 83% yield of the α-2,8 NeuAc dimer **87**, once again with no trace of the undesired β diastereomer. In both cases the auxiliary was removed with Bu_3SnH/AIBN in good yield.

USING SIALYLTRANSFERASES FOR THE SYNTHESIS OF SIALOSIDES

Enzyme-mediated glycosylations are powerful methods for the synthesis of complex carbohydrates, including those that contain sialic acid.[30,31] In mammalian systems, Leloir pathway glycosyltransferases are responsible for the biosynthesis of most glycoconjugates.[32] Sialyltransferases are a subset of the glycosyltransferases that transfer the NeuAc component of cytidinyl-5′-monophospho-β-*N*-acetylneuraminic acid (**88**, CMP-NeuAc) to acceptor hydroxyl groups with inversion of configuration at the anomeric center (Scheme 28).[30,31] Transferase-mediated sialylations are not burdened by some of the pitfalls of chemical glycosylations,



Scheme 27.



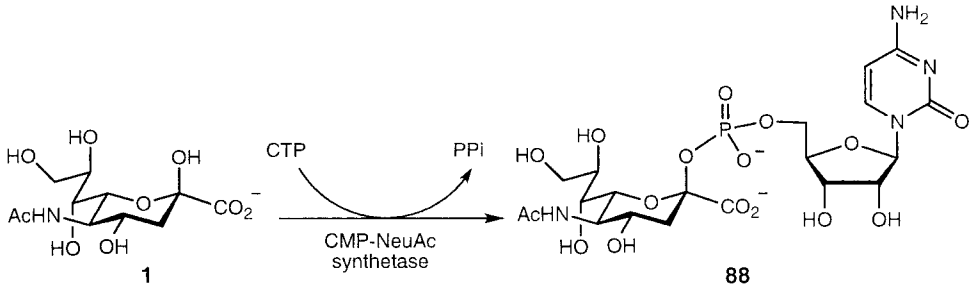
Scheme 28.

such as unwanted reaction pathways and the need for tedious protecting group schemes or stereocontrolling auxiliaries. In addition, sialyltransferases have been shown to tolerate several acceptor modifications,[33] although less is known about their donor specificity because the requisite CMP-NeuAc sugar donors are so difficult to synthesize.

Kinetic isotope effect studies with a particular transferase, namely, rat liver α-2,6-sialyltransferase, have shown that there is significant charge buildup at the anomeric center in the transition state.[34] This suggests that the glycosylation proceeds through an S_N1-like mechanism. CMP-NeuAc appears to be the common donor for all the sialyltransferases. However, each enzyme varies in regioselectivity and to some degree in acceptor specificity. Under the premise that an enzyme exists for the formation of each natural sialoside, there should be at least nine sialyltransferases responsible for the synthesis of the following disaccharides: NeuAc-α-2,6-Gal, NeuAc-α-2,3-Gal, NeuAc-α-2,6-GalNac, NeuAc-α-2,4-Gal, NeuAc-α-2,4-GlcNac, NeuAc-α-2,6-GlcNAc, NeuAc-α-2,6-Man, NeuAc-α-2,8-NeuAc, and NeuAc-α-2,9-NeuAc. However, only a small subset of the known and postulated sialyltransferases has been isolated and used for synthesis purposes.

The sialyltransferases are membrane-bound proteins located in the endoplasmic reticulum (ER) and in the Golgi apparatus. Information about their sequence homology is limited, but they do appear to share a common topography.[35] A catalytic domain resides at the C-terminus followed by an N-terminal segment that anchors the enzyme into the ER or Golgi membrane. Soluble, catalytically active sialyltransferases that lack the anchor segment have been isolated from milk, serum, and other body fluids, suggesting that this N-terminal anchor is not necessary for the enzyme to retain catalytic activity. However, the ability to obtain from natural sources quantities of most sialyltransferases that would be needed for synthesis applications is hampered by low tissue concentrations and difficult purifications.

The genes for members of some of the most common classes of sialyltransferases have been cloned and expressed.[33a] Expressing and isolating membrane-bound enzymes in high catalytic activity can be difficult; however, Paulson and coworkers replaced the anchor segment with a cleavable peptide in the expression of Gal-β-1,4-GlcNAc α-2,6-sialyltransferase.[35a,b] This allowed the enzymes to be secreted from the cell into the medium, thus simplifying the process of isolation. This technology has been used to express several sialyltransferases in quantities suitable for synthesis.

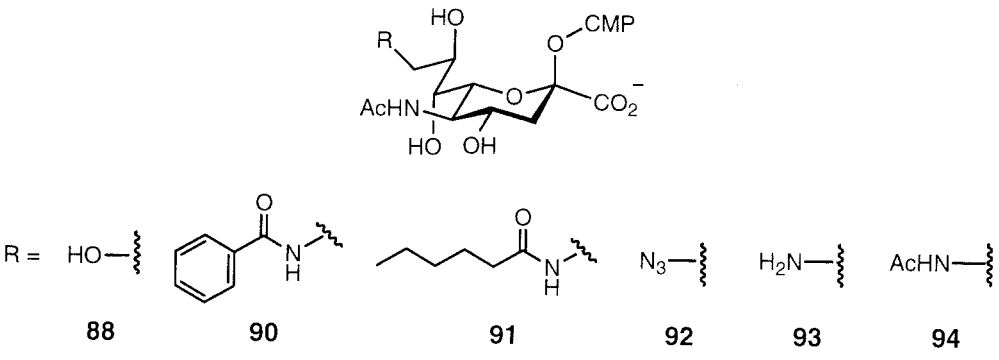


Scheme 29.

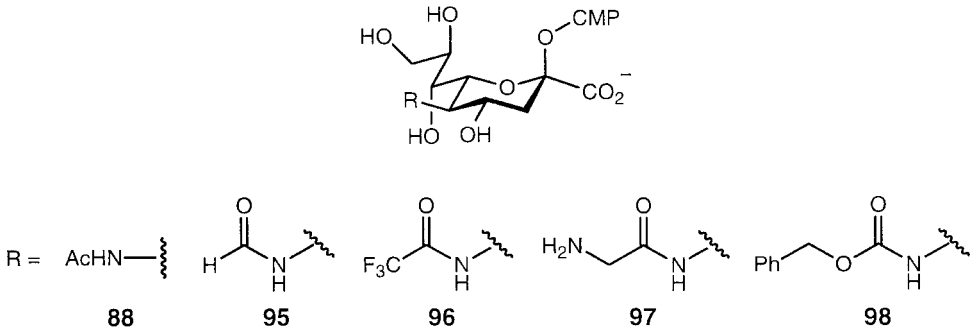
Gaining sialyltransferase accessibility is becoming a less serious problem as these enzymes become commercially available.

Synthesis of CMP-NeuAc and Related Derivatives

The primary method for the synthesis of CMP-NeuAc **88** is through the use of CMP-NeuAc synthetase, an enzyme that catalyzes the condensation of cytidine triphosphate (CTP) with sialic acid to produce CMP-NeuAc (Scheme 29).[30] CMP-NeuAc synthetase has been cloned from microbial sources and has been isolated from mammalian tissues.[36,37] The substrate specificity of each synthetase has been studied to some degree.[36,37] The mammalian version accepts C9 and some C8 modifications of NeuAc, as well as variations at the C5 position, such as replacement of the acetamide with OH (KDN) or hydroxylation of the acetamide (NeuAc). The microbial CMP-NeuAc synthetase has a high activity for C9-modified sialic acids, but does not tolerate alterations at the C5 position. Unfortunately, the microbial version is the more readily available enzyme, thus limiting the variety of analogs that can be prepared in this manner. Scheme 30 gives a representative sample of some important C9-derivatized CMP-NeuAc analogs.[37] Modifications at the C5 position are somewhat



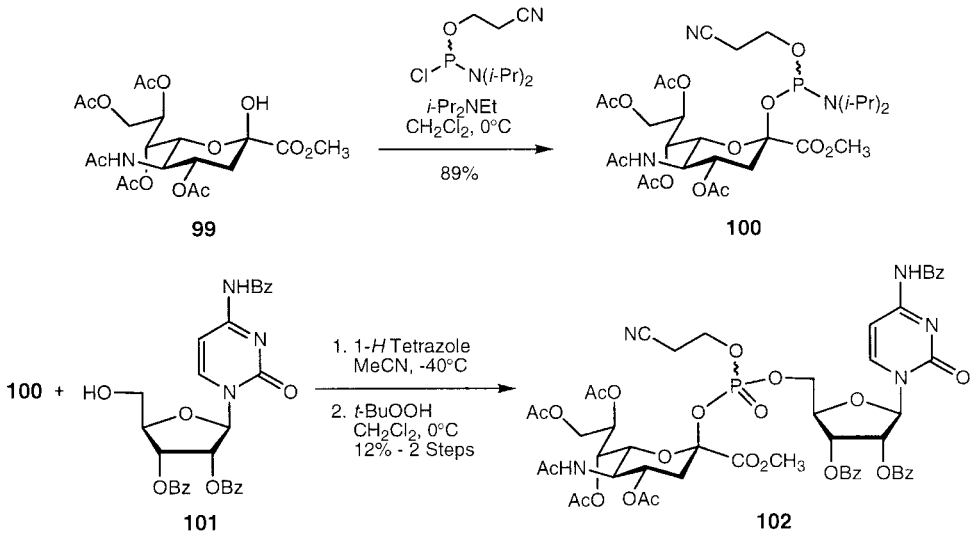
Scheme 30.



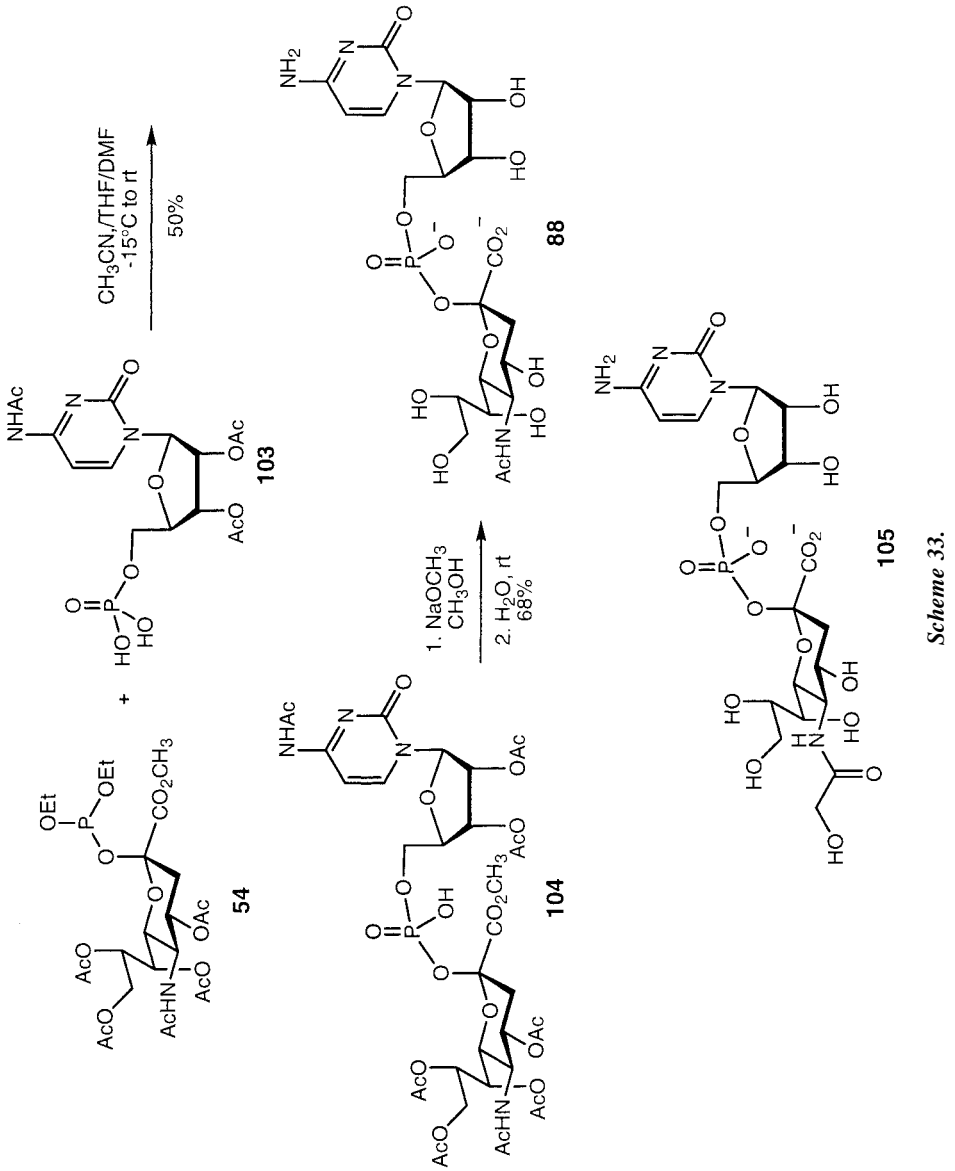
Scheme 31.

limited by the specificity of CMP-NeuAc synthetase for an amide at this position. Nevertheless, several analogs were prepared that incorporate sterically and electronically diverse substituents at C5 (Scheme 31).[37]

Wong and coworkers first attempted the nonenzymatic synthesis of CMP-NeuAc by employing phosphoramidite chemistry in a key step involving the ligation of a sialyl phosphoramidite and a selectively protected cytidine analog (Scheme 32).[38] Treatment of NeuAc derivative **99** with 2-cyanoethyl chlorophosphoramidite resulted in the formation of the β-sialyl phosphoramidite **100** in 89% yield. The sialyl donor was then coupled to the protected cytidine **101** under promotion by 1-*H* tetrazole to afford the intermediate phosphite, which was immediately oxidized with *tert*-butyl hydroperoxide to provide the protected CMP-NeuAc **102**. Deprotection of this intermediate was not reported; however, the Wong group demonstrated that the



Scheme 32.

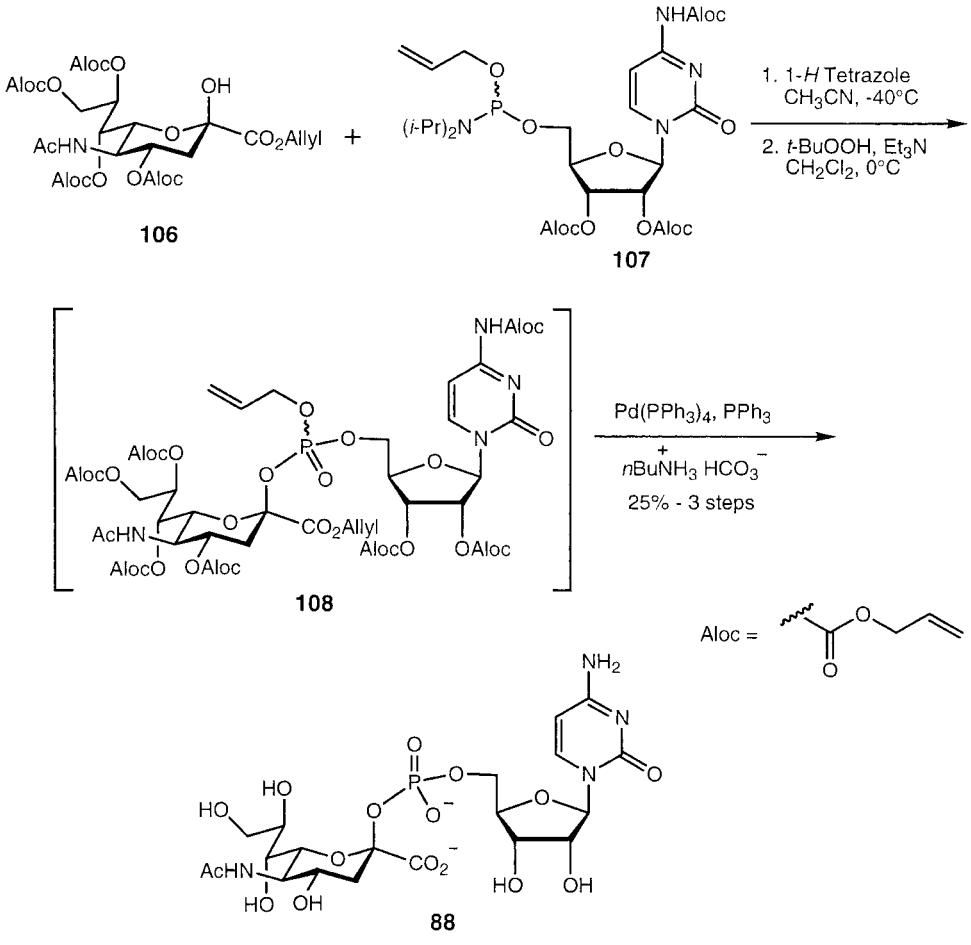


Scheme 33.

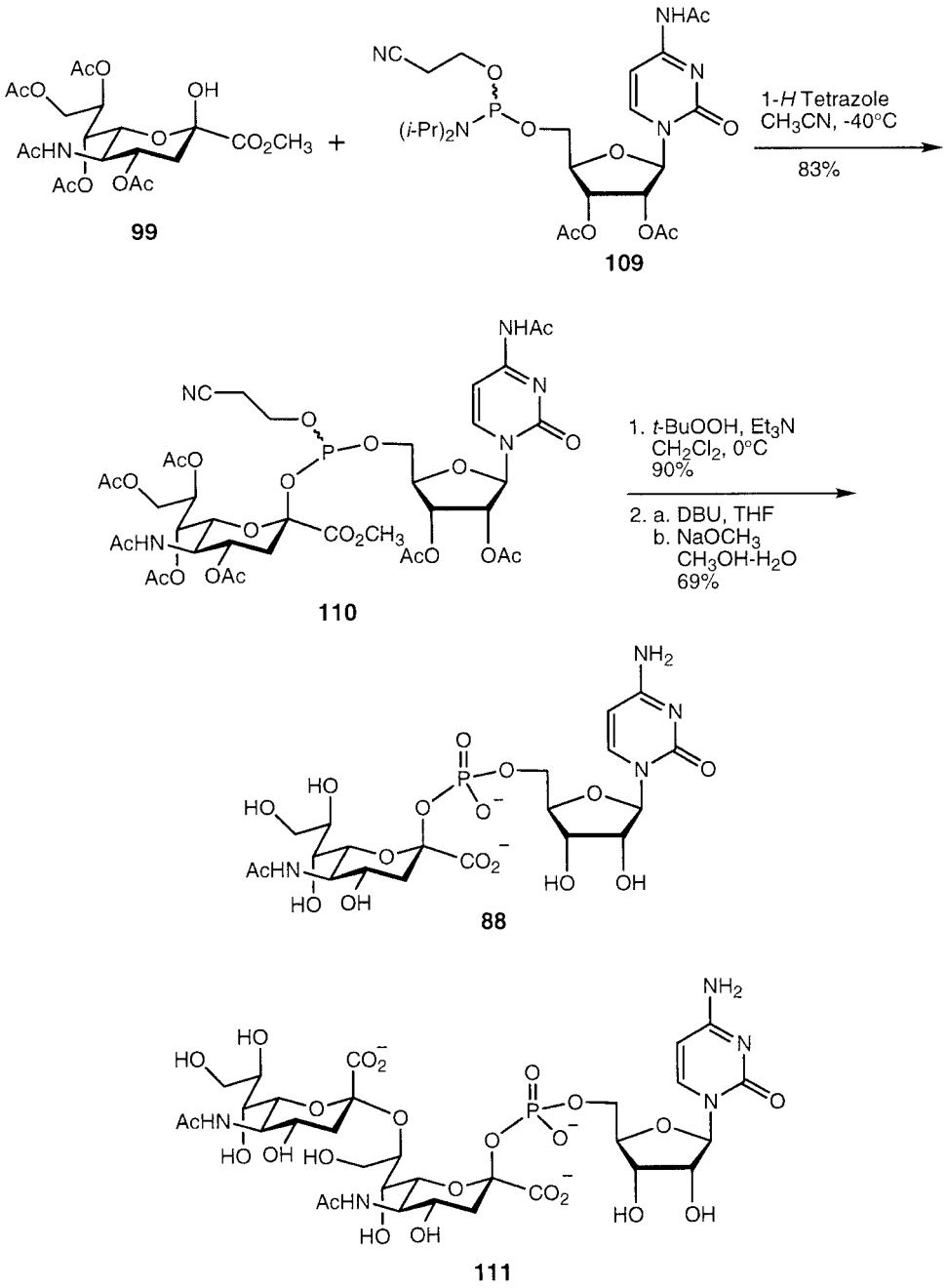
CMP-NeuAc core structure could be successfully synthesized utilizing phosphoramidite chemistry.

The Schmidt group utilized a sialyl phosphite in a very different synthesis strategy (Scheme 33).[39] Upon treatment of sialyl donor **54** with cytidine phosphoric acid **103**, a phosphite–phosphate exchange reaction occurred to give compound **104** exclusively as the β isomer. Deacylation by treatment with sodium methoxide followed by ester saponification through the addition of water provided CMP-NeuAc **88**. This method circumvented the need for an oxidation step or phosphorus deprotection. This method was also applied to the synthesis of another naturally occurring CMP-NeuAc derivative **105**.[40]

Hata and coworkers took a different approach to the ligation by utilizing cytidine phosphoramidite **107** and the tertiary anomeric alcohol of **106** as coupling partners (Scheme 34).[41] The coupling reaction successfully provided a phosphite intermediate, which was subsequently oxidized with *tert*-butyl hydroperoxide to provide the trialkyl



Scheme 34.



Scheme 35.

phosphate **108**. Attempts to purify the phosphotriester failed, but the production of **108** was supported by ^{31}P NMR spectroscopy of the crude reaction mixture. The protecting groups were then removed under mild conditions by treatment with tetrakis(triphenylphosphine)palladium(0) in the presence of the allyl scavenger *n*-butylammonium bicarbonate. Following purification by size exclusion chromatography, CMP-NeuAc **88** was obtained in acceptable overall yield (25% for three steps).

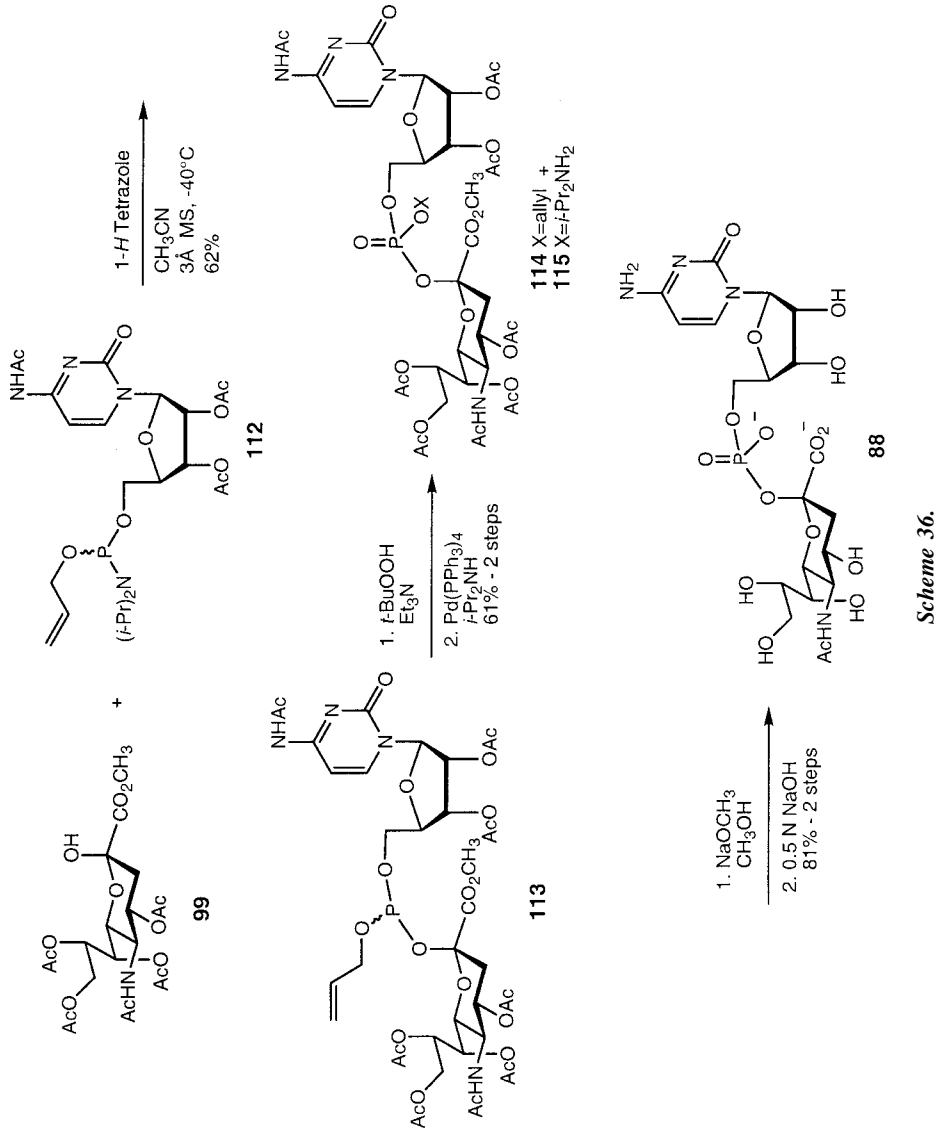
In a related synthesis of CMP-NeuAc, Kajihara and coworkers also used a cytidine phosphoramidite in the coupling reaction, although with different protecting group patterns on the coupling partners (Scheme 35).[42] The reaction of **99** with phosphoramidite **109** provided the phosphite **110** as a mixture of phosphorus diastereomers. Phosphite oxidation and subsequent treatment with methoxide led to decomposition. As an alternative, the cyanoethyl protecting group was first removed with DBU; then complete deprotection by treatment with sodium methoxide and sodium hydroxide afforded CMP-NeuAc **88** in good yield. This strategy was also applied to the synthesis of CMP-NeuAc-α-2,8-NeuAc **111**.[43]

Halcomb and Chappell developed a route to CMP-NeuAc **88** that promises to be general for the synthesis of virtually any derivative thereof.[44,45] The route (Scheme 36) utilizes a condensation of sialic acid derivative **99** with the phosphoramidite **112** to afford the phosphite **113** in 62% yield. Oxidation of the phosphite provided the phosphotriester **114**,[46] which was taken directly to the next transformation without purification (owing to its instability to chromatography). Deallylation of the phosphate gave compound **115** (61% for two steps), which was stable to silica gel chromatography. Compound **115** was deacylated with methoxide, and its methyl ester was subsequently saponified with NaOH to provide CMP-NeuAc **88**. The derivatives shown in Scheme 37 were synthesized according to this protocol and were investigated as substrates for sialyltransferases (see below).

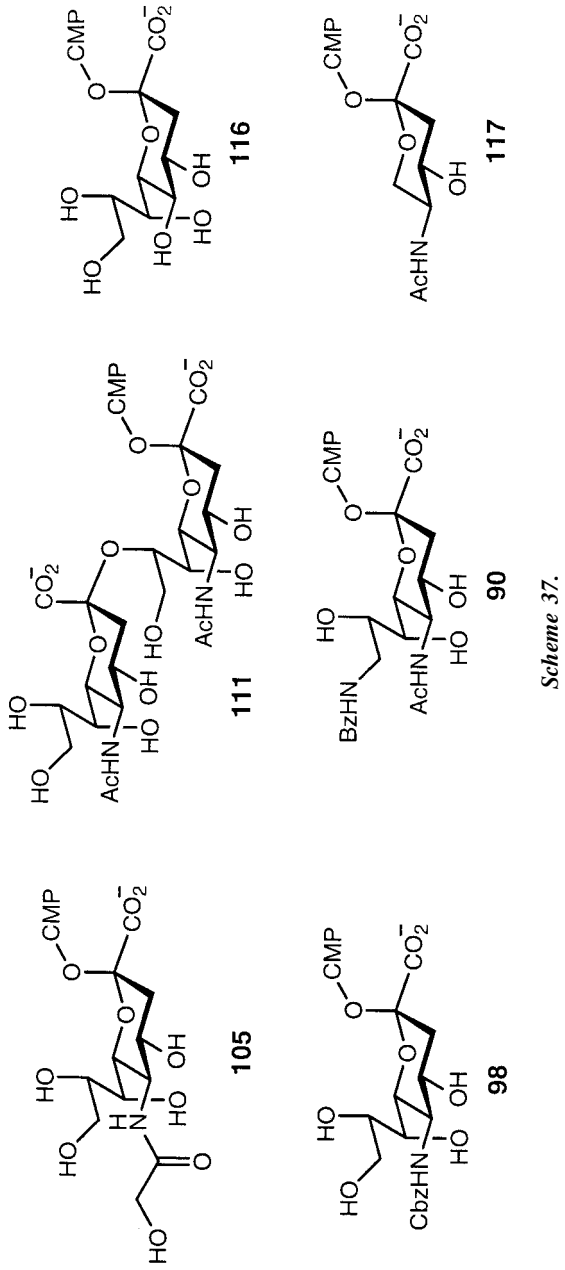
The synthesis of a CMP-NeuAc derivative that was bound to a solid support through the 9-position of the sialic acid has been reported by the Kajihara group.[47] This derivative is quite useful in that it can be utilized to immobilize glycoproteins onto a solid support by transferring the sialic acid to the terminus of the carbohydrate chain of the glycoprotein.

Synthesis of Wild-Type and Mutant Sialosides

The Brossmer and Paulson groups have studied the sialyltransferase donor specificity with a series of C5 and C9 conjugates, all of which were prepared through the CMP-NeuAc synthetase route.[37] The relative rates of these C9-modified CMP-NeuAc derivatives shown in Scheme 30 were compared against the natural donor CMP-NeuAc in sialyltransferase assays that utilized enzymes from different sources with their appropriate natural acceptors. The rat liver α-2,6-sialyltransferase tolerated a wide range of functional groups without significant decreases in the relative rates. Synthetically useful relative rates were observed for most of the CMP-NeuAc analogs with porcine sialyltransferase and rat liver α-2,3-sialyltransferase. The exception was the 9-amino analog, which was a poor substrate for both enzymes. Overall, these assays demonstrated that a wide variety of modifications at the C9 position are tolerated by these sialyltransferases.



Scheme 36.



Scheme 37.

The relative rates of sialylation for the C5-modified CMP-NeuAc derivatives shown in Scheme 31 were also determined.[37] All the C5-modified sialyl donors were accepted by the transferase enzymes, but the variants containing free amines were poor substrates for all the sialyltransferases examined. Generally, higher relative rates were observed for both α-2,6-sialyltransferases over the porcine α-2,3-sialyltransferase, although all the rates are high enough to be synthetically useful. This study has shown that numerous C5-modified CMP-NeuAc sialyl donors can be successfully utilized in the synthesis of modified glycoproteins.

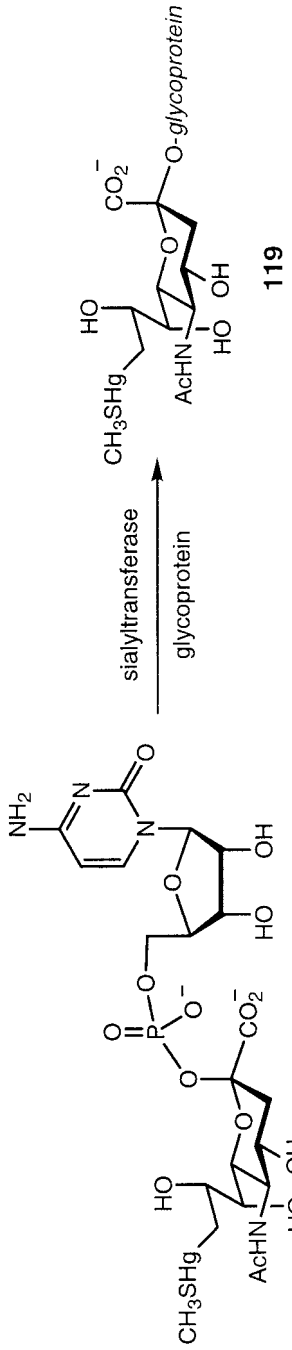
The sialyltransferases investigated were found to accept many variations at C5 and C9 of the sialic acid moiety. Halcomb and Chappell confirmed this fact with studies of sialyltransferases from different sources.[45] The C9- and C5-modified substrates in Scheme 37 were good substrates. However no activity was detected when compounds **111** and **117** were assayed. In addition to these examples, other CMP-NeuAc analogs that have been prepared and successfully transferred include C9 fluorescent compounds, C9 thioacetyl, C5 thioacetyl, and C4 deoxy.[37] A particularly useful modification introduced by the Wong lab incorporates a mercury atom at C9 (Scheme 38).[48] Transfer of this sialic acid to glycoproteins could greatly aid in X-ray crystallographic analysis of these biomolecules.

The field of solid phase synthesis has also benefited from enzyme technology. Several reports have described the use of sialyltransferases in the solid-supported synthesis of oligosaccharides that bear sialic acid.[49] Additionally, metabolic pathways have been harnessed by Bertozzi and coworkers to synthesize glycoconjugates on cell surfaces that bear modified sialic acids.[50]

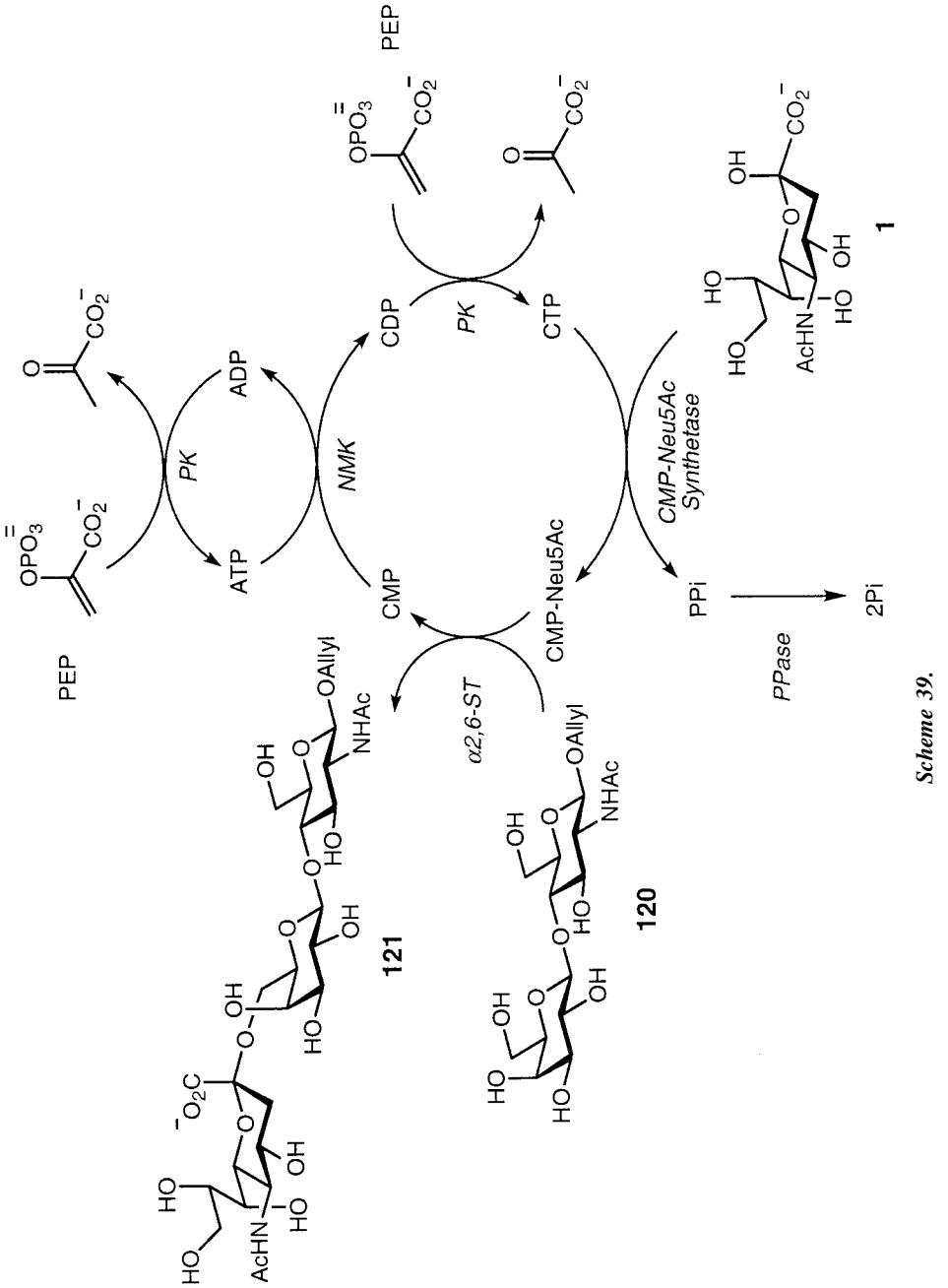
Cofactor Regeneration

Enzymatic methods are efficient for the assembly of complex oligosaccharides on a small scale, although the high cost of sugar nucleotides and problems with product inhibition detract from this approach in large-scale reactions. The synthesis of CMP-NeuAc by CMP-NeuAc synthetase requires stoichiometric amounts of expensive reagents, such as CTP, while the CMP by-product of the sialyltransferase reaction is an enzyme inhibitor. Ichikawa and Wong used the concept of in situ cofactor regeneration to alleviate these problems and increase the efficiency of sialylation (Scheme 39).[51]

The cascade begins with stoichiometric amounts of phosphoenolpyruvate (PEP), β-allyl-*N*-acetyl lactosamine **120**, NeuAc **1**, and catalytic quantities of ATP and CMP. Initially, CMP is converted to CDP by nucleoside monophosphate kinase (NMK) in the presence of ATP. The CDP produced reacts with PEP under pyruvate kinase (PK) catalysis to form CTP. Next, CMP-NeuAc synthetase catalyzes the in situ formation of the sialyl donor from NeuAc and CTP. The pyrophosphate by-product is decomposed to inorganic phosphate by inorganic pyrophosphatase (PPase). Subsequently, the α-2,6-sialyltransferase accomplishes the sialyation of the lactosamine acceptor **120** and produces the transferase inhibitor CMP as a by-product. The CMP concentrations are kept low by conversion to CDP, and in so doing the problem of product inhibition is minimized. The cycle afforded 21% of the sialylated trisaccharide **121**, which is remarkable considering the complexity of the system and number of synthetic steps that can be avoided.



Scheme 38.



Scheme 39.

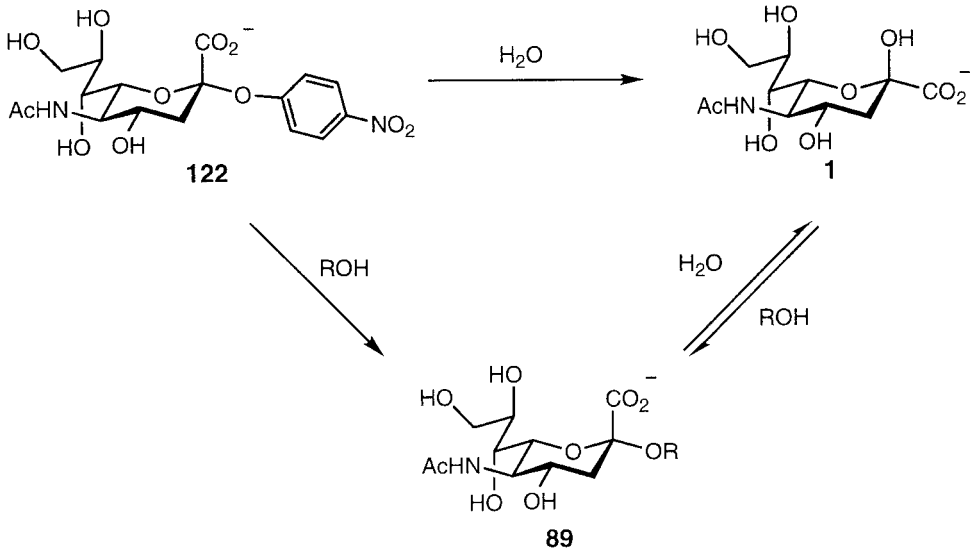
USING SIALIDASES FOR THE SYNTHESIS OF SIALOSIDES

Sialylhydrolases

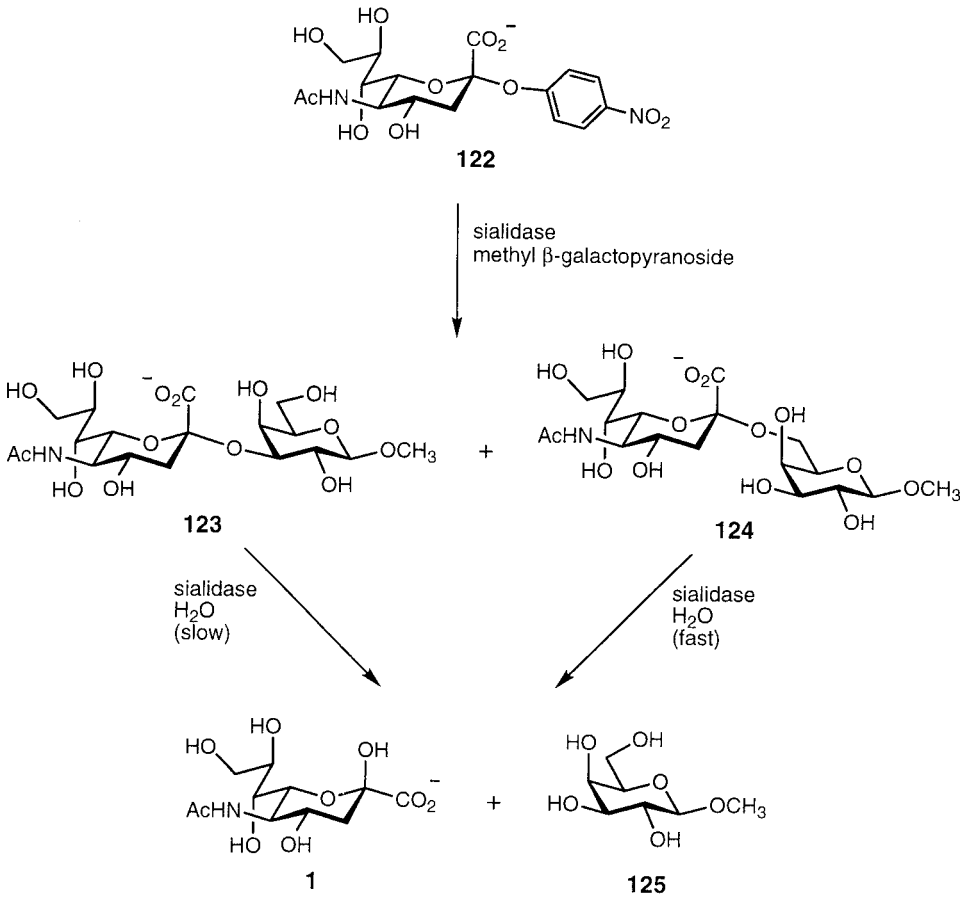
Sialidases are another class of enzymes that have been used in the synthesis of sialylated oligosaccharides. In addition to hydrolysis activity, sialidases are capable of sialoside transfer to alcohol donors. Using the 4-nitrophenylglycoside **122** as a sialyl donor, sialylidases can catalyze a transsialylation to an alcohol acceptor to generate the product **89**, or hydrolyze to NeuAc **1** (Scheme 40). To complicate matters further, the product **89** may also undergo sialidase-catalyzed hydrolysis to form NeuAc. So if any product is to be isolated, the rate of sialyl transfer must be much faster than either hydrolysis rate.

Thiem and Sauerbrei examined this concept to determine whether various sialidases could be used synthetically.[52] The rate of condensation, or reverse hydrolysis, was found to be negligible. However, the product hydrolysis rate was competitive with the rate of transsialylation to an alcohol acceptor. In an attempt to minimize product loss, reactions were stopped after 65–75% of the starting material had been consumed. Interestingly, a mixture of α-2,3 and α-2,6 regioisomers was obtained for reactions with an immobilized *Vibrio cholerae* sialidase (Scheme 41). In all cases, the α-2,6 isomer predominated, probably because of a combination of faster hydrolysis of the α-2,3 products (**123** → **1** + **125**) and lesser steric hinderance of the primary alcohol. Variations in the donor/acceptor ratio (1:7 optimized) had an effect on both reaction yield and regioselectivity, although most of the examples afforded only 2–3:1 α-2,6/α-2,3 product ratios in 14–20% overall yield.

Ajisaka et al. examined different sialidase sources and found that Newcastle disease virus (NDV) sialidase afforded predominantly the α-2,3 regioisomers, while



Scheme 40.

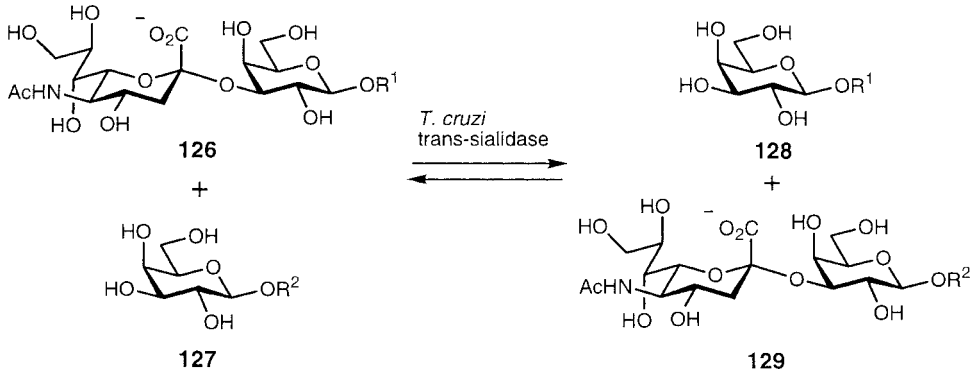


Scheme 41.

Arthrobacter ureafaciens and *Clostridium perfringens* sialidases, in addition to the *Vibrio cholerae* sialidase examined by Thiem, favored the α-2,6-linked products.[53] Unfortunately, the reaction yields did not improve for the new enzymes, varying from 0.8% to 3.6% isolated yield. In the case of NDV sialidase, the high selectivity for α-2, 3-sialosides stemmed from a large α-2,6/α-2,3 hydrolysis ratio. Hydrolysis of the α-2,6 products was found to be 28 times faster than the α-2,3 isomers. Interestingly, the α-2,6 preference of the other three enzymes was not correlated to product hydrolysis rates.

trans-Sialidases

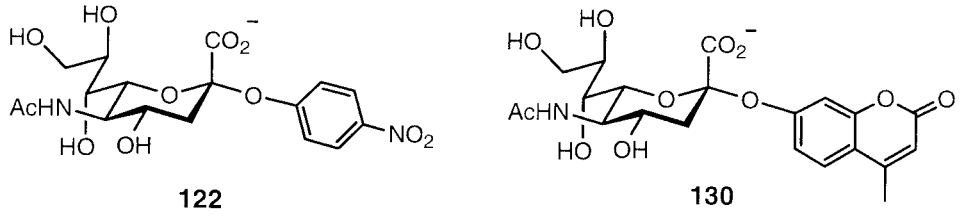
The *Trypanosama cruzi trans*-sialidase catalyzes the reversible transfer of NeuAc from a NeuAc-α-2,3-Gal-β-OR^1 sequence to an acceptor bearing the Gal-β-OR^2 motif (Scheme 42).[54] The enzyme is a particularly useful sialidase because it has very little hydrolytic activity and tends to almost exclusively catalyze transsialylation to a



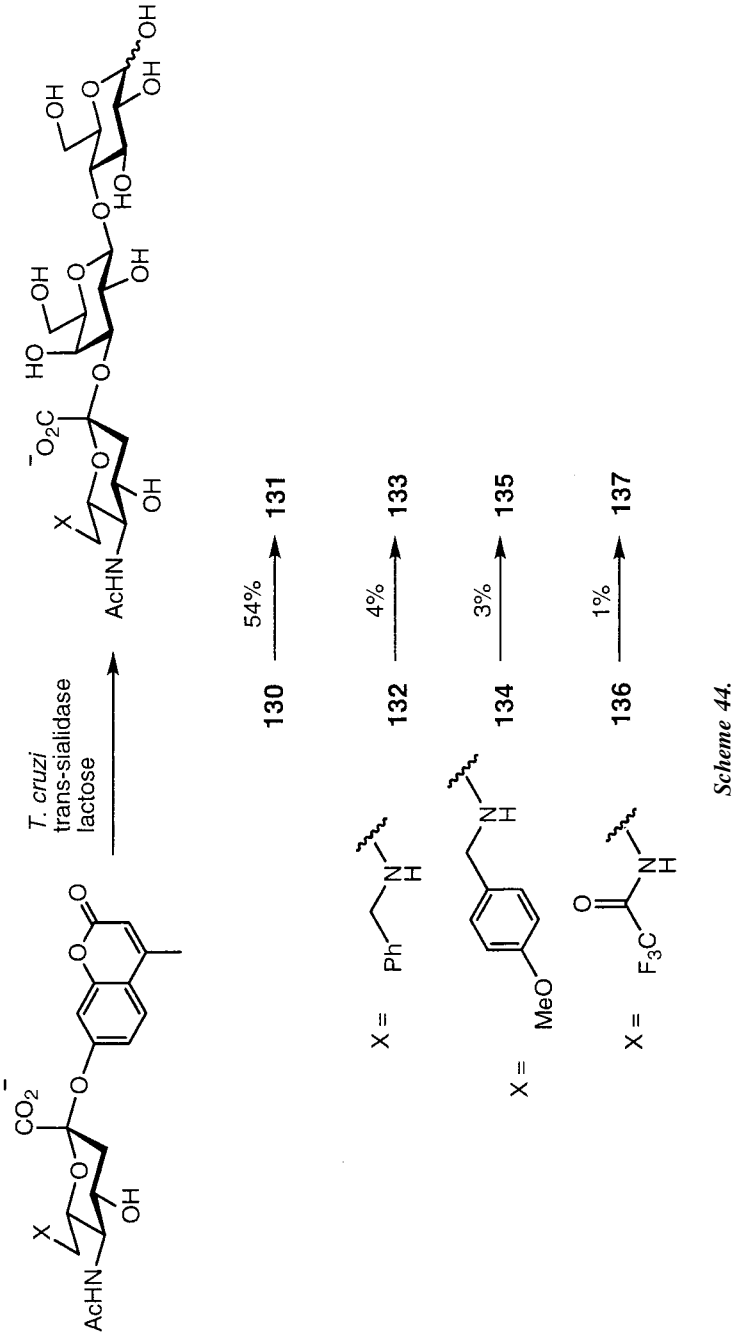
Scheme 42.

galactose. However a major drawback to this method is that to drive the glycosylation to completion, there is a need for large quantities of complex α-2,3-linked sialyl donors, which are generally difficult to obtain from natural sources. Other natural donors with α-2,6- or α-2,8-linked sialic acids have been examined but were discovered to be poor sialyl donors for α-2,3-sialylations catalyzed by *T. cruzi trans*-sialidase.[55] Simple aryl α-sialosides, such as the 4-nitrophenyl glycoside **122** and methylumbelliferone glycoside **130** (Scheme 43), have been found to be excellent substrates because of the irreversibility of the sialyl transfer, and these have become the most utilized sialyl donors for *trans*-sialidase-catalyzed glycosylations.

The donor specificity of *T. cruzi trans*-sialidase was examined with various side chain modified NeuAc glycosides. Vandekerckhove and coworkers found that methoxy or deoxy modifications to C9 of a NeuAc-α-2,3-Gal-β-OR donor did not affect *trans*-sialidase activity, although the same modifications at C4, C7, or C8 completely prevented transfer of the sialic acid. In addition, the C4- and C8-modified compounds were found to be mild inhibitors of the enzyme.[56] Lee and Lee extended these studies to more drastically modified NeuAc aryl glycosides.[57] The triol side chain of the methylumbelliferone-α-ketoside **130** was cleaved with periodate to afford the C7 aldehyde, which underwent reductive amination with different amines to provide several novel sialyl donors. Three of these derivatized sialic acids (those of **132**, **134**, and **136**) were successfully transferred to a lactose acceptor on analytical scales (50 nmol), albeit in much lower yield than NeuAc from aryl glycoside **130** (Scheme 44). NeuAc analogs



Scheme 43.



Scheme 44.

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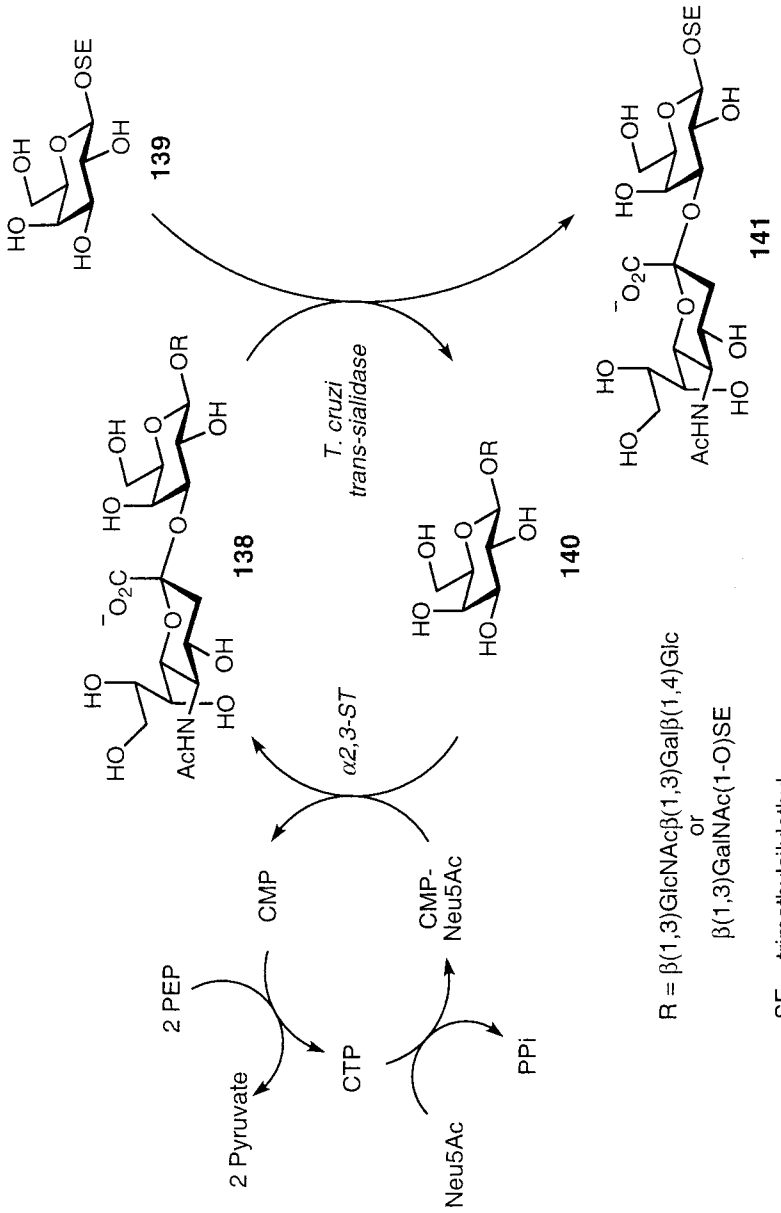

Scheme 45.

containing longer alkyl chain and terminal amine substituents were found not to be substrates for the *trans*-sialidase. Nonetheless, these studies helped to define the substrate requirements for *trans*-sialidase acceptance, and the latter work provides a novel method for the synthesis of chromophore labeled sialylated oligosaccharides.

Ito and Paulson designed a cofactor regeneration system that overcomes several limitations of *trans*-sialidase-catalyzed sialylations (Scheme 45).[58] The *trans*-sialidase is used in conjunction with α-2,3-sialyltransferase to effectively broaden the substrate specificity of the sialyltransferase. The β-trimethylsilyl galactoside **139** is known to be a poor substrate for α-2,3-sialyltransferase; however, it is readily accepted by *T. cruzi trans*-sialidase. The sialylated oligosaccharide **138** was synthesized by the CMP-NeuAc regeneration system developed by Ichikawa and Wong.[51] Then **139** underwent a transsialylation with **138** to provide the desired GM_4 precursor **141**. The regeneration of **138** from the by-product **140** by sialyltransferase drives the equilibrium toward the product **141**. This example demonstrates that *trans*-sialidases have some synthetic utility on their own but are much more general tools for oligosaccharide synthesis when coupled with sialyltransferases.

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