Enzymatic and Chemical Approaches for the Synthesis of Sialyl Glycoconjugates[1]

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Due to their important role in the chemical and biological diversity of glycoconjugates, sialic acids mainly exist as terminal components of cell surface glycoproteins and glycolipids (1). These biomolecules are highly glycosylated by complex carbohydrate chains terminated by one or several sialic acid residues. Many significant biological events are associated with sialyl glycoconjugates. For instance, changes in either amount, type or linkage of sialic acids in tumor cell glycoconjugates can affect tumor growth and metastasis (2,3). Selectins, cell adhesion molecules implicated in the recruitment of leukocytes to lymphoid tissues and to sites of inflammation, require ligands possessing α2,3-sialic acids for proper recognition (4). The B cell-specific differentiation antigen CD_{22}, which is involved in cell activation, binds to cellular lactosamine sequences containing α2,6-sialic acids (5,6). Furthermore, striking differences have been found in the sialylation pattern of cells during development, activation, aging and oncogenesis (1). Consequently, tremendous efforts have been dedicated to understand the chemical and biological significance of sialic acid containing glycoconjugates, as well as to study their structures, metabolism and immunological activities. Since natural carbohydrates exist as diverse forms, it still remains a difficult challenge to obtain identical molecules in a reasonable amount for further investigations. Synthetic methods provide an efficient approach for the synthesis of sialylated glycoforms in terms of quantity and molecular variety.

Of all the reactions of glycosidic bond formation, addition of sialic acids is often considered to be the most laborious task due to problems of low reactivity, yield, and stereoselectivity (7,8). Here we present a review how the glycosidic linkages of sialic acids are generated by enzymatic and chemical methods.

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I. Enzymatic Approaches

Enzymatic methods to incorporate sialic acid residues into glycoconjugates require the utilization of cytidine-5'-monophosphate-*N*-acetylneuraminic acid (CMP-sialic acid or CMP-NeuAc) synthetase and a specific sialyltransferase (Figure 1). The former protein assists in the activation of sialic acid and catalyzes the reaction of sialic acid and cytidine-5'-triphosphate. The latter transfers the sialic acid moiety to the non-reducing end of an acceptor carbohydrate. Different types of sialyltransferases are required depending on various acceptors and glycosidic linkages of sialic acid. Therefore, how to obtain both enzymes is a prerequisite to execute the enzymatic sialylation.

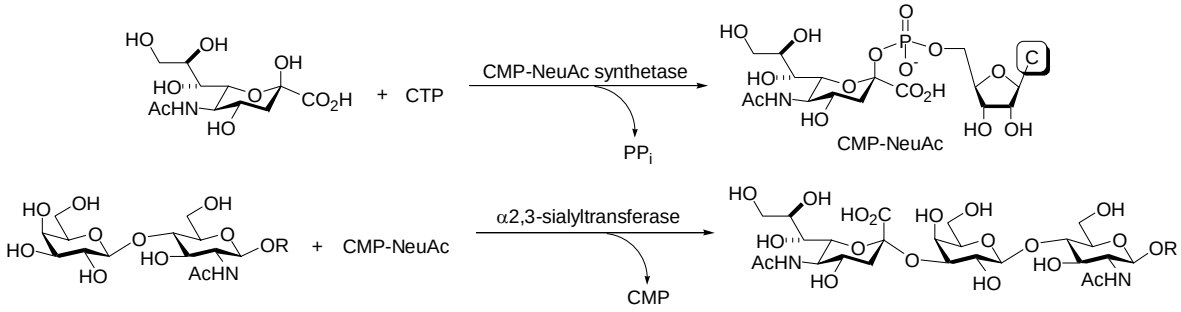


Figure 1. Reactions of CMP-NeuAc synthetase and sialyltransferase.

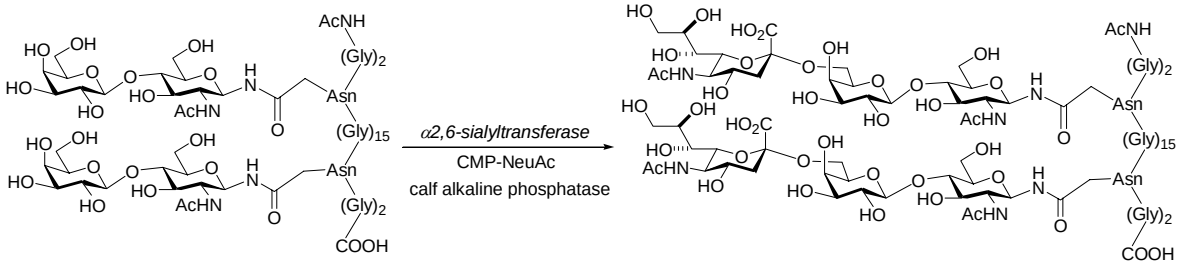
Although CMP-NeuAc is commercially available, its high cost (9) makes it advisable to use an efficient method of preparation. Several chemical syntheses of CMP-NeuAc have been reported (10-12). The number of reaction steps and tedious protection/deprotection procedures reflect the advantage of using CMP-NeuAc synthetase as a one-step enzymatic approach.

CMP-NeuAc synthetase, isolated from several mammalian tissues, has been used in the synthesis of CMP-NeuAc (13). The enzyme from *E. coli* was sequenced and cloned by Vann *et al.* (14-16) and further overexpressed by Shames *et al.* for large scale synthesis (17). Wong's group improved the overexpression and investigated the substrate specificity (18). The results indicated that CMP-NeuAc synthetase can tolerate the modification at the C-9 position of sialic acid without affecting the K_m value (18-21). Furthermore, sialic acid analogs, such as 9-deoxy, 7,9-dideoxy and 4,7,9-trideoxy-sialic acids were all accepted by the enzyme and converted to the corresponding CMP-NeuAc derivatives (22). However, neither the oxidation of hydroxyl group to a ketone introduced at the C-4, 7, or 8 position, nor their respective dimethylacetals were recognized as substrates by CMP-NeuAc synthetase (23).

Several α2,6- and α2,3-sialyltransferases have been used for carbohydrate

synthesis (24-26). These enzymes generally transfer *N*-acetylneuraminic acid to either the 6- or 3-position of terminal Gal or GalNAc residues. The α2,8-sialyltransferase exhibits specificity for the synthesis of α2,8-linked polysialic acids (27). Nearly twenty different sialyltransferases are known to synthesize all known sialyl-oligosaccharide structures (28). Since these enzymes share the same sugar donor (CMP-NeuAc) and recognize similar acceptor substrates, it was expected that they would exhibit some sequence homology. Surprisingly, there are no significant amino acid sequence similarities among the cloned sialyltransferases, with the exception of two short consensus sequences called the sialyl motifs (29,30). Several sialyltransferases have been successfully cloned on the basis of these sialyl motifs and PCR cloning strategy (31). However, these enzymes are limited to carbohydrate synthesis because they are membrane bound proteins and mainly expressed in specific mammalian cell lines (31) in insufficient amount (in terms of synthetic purpose). Since enzymatic processes are useful and convenient to provide sialyl glycoconjugates to meet research need in glycobiology, both expressed and isolated sialyltransferases have been used to perform enzymatic sialylation. We describe some representative examples below.

The pioneering work on sialyltransferases, including isolation, characterization, cloning and synthetic application, is due to Paulson *et al*. As early as in the middle of 1980's, his group reported the enzymatic synthesis of sialylated carbohydrates by using three purified mammalian sialyltransferases which accepted type 1 (Gal-β1,3-GlcNAc), type 2 (Gal-β1,4-GlcNAc), or type 3 (Gal-β1,3-GalNAc) oligosaccharides as substrates (24). In addition, linear and branched glycopeptides with multiple sialyl-*N*-acetyl lactosamine side chains (Scheme 1) were prepared using a combined chemical and enzymatic approach (32). After solid phase synthesis to incorporate β-GlcNAc-Asn and enzymatic galactosylation to add galactose, CMP-sialic acid and α2,6-sialyltransferase were employed to form the desired α2,6-sialyl linkage, as shown in Scheme 1 (32). Calf alkaline phosphatase destroyed the inhibiting side product CMP (32).

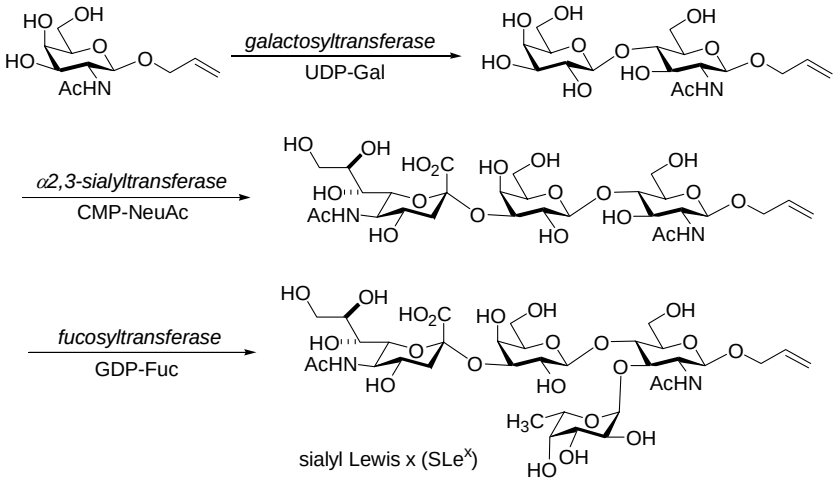


Scheme 1. Paulson's synthsis of sialyl glycopeptides using α2,6-sialytransferase

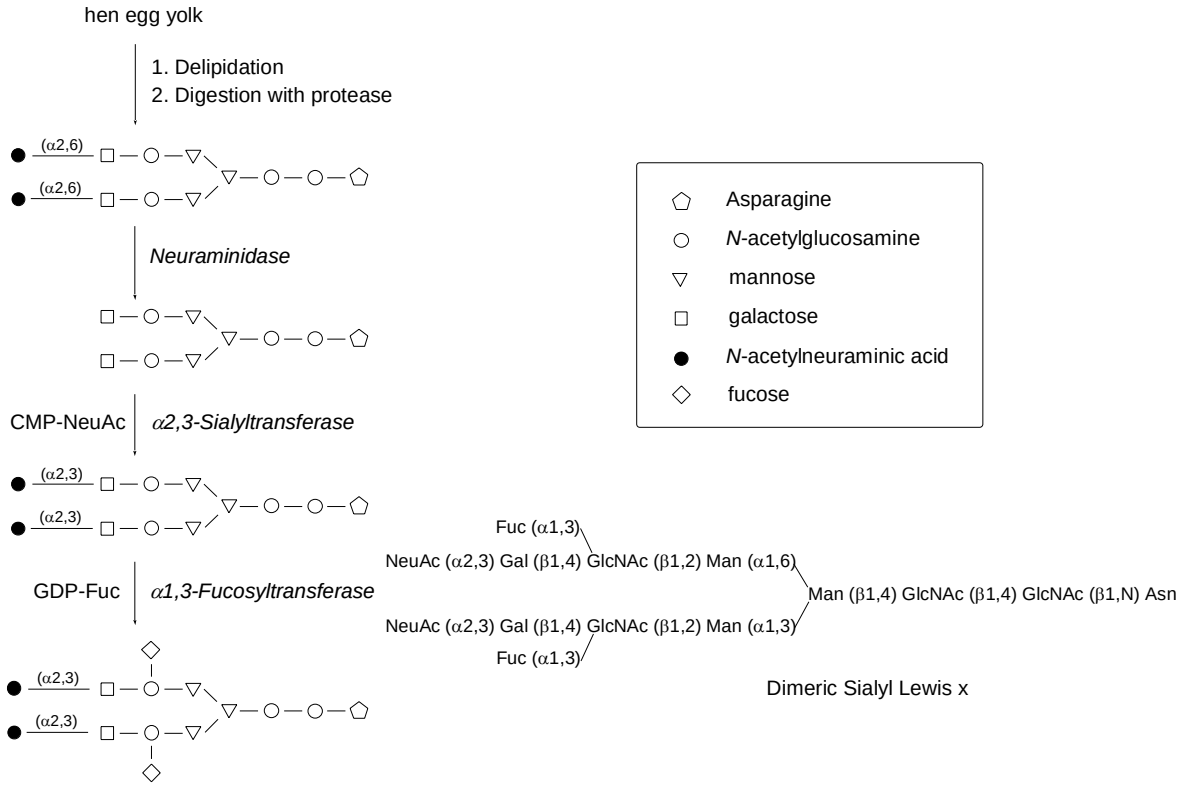
The tetrasaccharide sialyl Lewis x (SLe^x) is the carbohydrate epitope at the terminus of glycolipids displayed on the surface of neutrophils (33). SLe^x has been shown to be the

ligand recognized by E-selectins, which are expressed on the surface of endothelial cells during inflammation (33, 34). The cell adhesion process of neutrophils and endothelial cells occurs through the interaction of E-selectin and SLe^x (34). The SLe^x synthesis on a large scale (kg quantities) was carried out with glycosyltransferases by Wong *et al.* and was indeed a milestone in enzymatic catalysis (35). Scheme 2 shows the consecutive glycosylation steps using galactosyl-, sialyl-, fucosyl-transferases and the corresponding sugar nucleotides.

Furthermore, multivalent SLe^x can be prepared in an efficient way based on the enzymatic remodeling of naturally existing biomolecules (36,37). For example, biantennary α2,6-sialyl *N*-acetyllactosamine has been obtained after complete delipidation and digestion of proteases (36), as shown in Scheme 3. The subsequent treatment with neuraminidase and α2,3-sialyltransferase resulted in the desired α2,3-sialyl linkage. The succeeding fucosylation with α1,3-fucosyltransferase gave the divalent SLe^x containing thirteen sugar units (36).

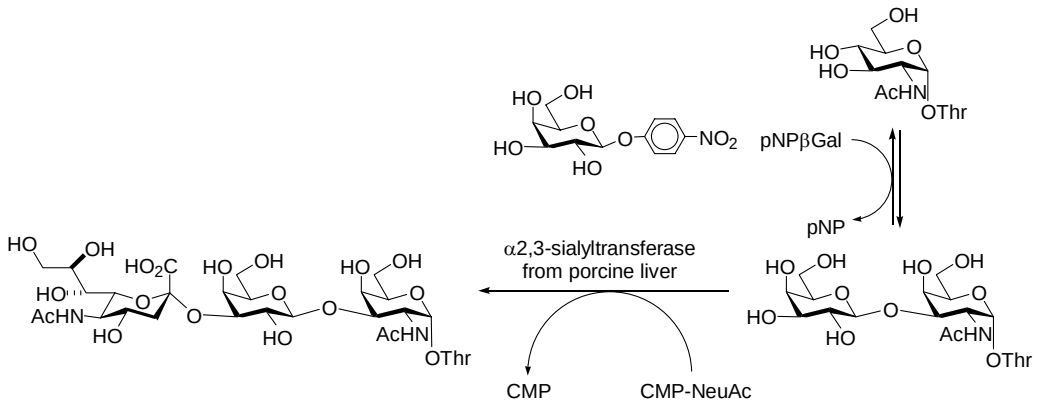


Scheme 2. Enzymatic synthesis of sialyl Lewis x developed by Wong *et al.*



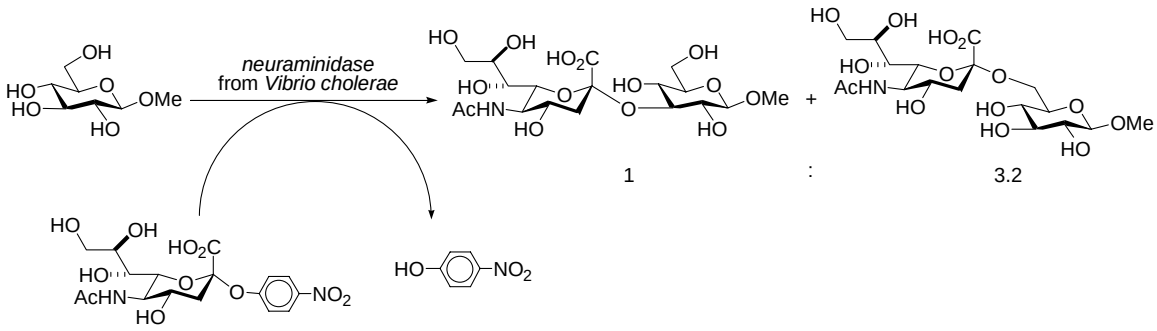
Scheme 3. Enzymatic synthsis of dimeric SLe^x glycopeptide

In addition of playing an important role in cell-cell recognition, sialic acid acts as molecular a mask or marker. The release of sialic acids from sialo-glycoproteins of the erythrocyte membrane causes exposure of the Thomsen-Friedenreich (TF) antigen (Galβ1,3-GalNAcα1-OThr)(38). Such consequence results in destruction of the red cell membrane followed by cell lysis (38). The sialylated TF antigen was recently synthesized by Thiem *et al* using a combination of β-galactosidase from bovine testes and α2,3-sialyltransferase from porcine liver (Scheme 4)(39). The former enzyme catalyzs the galactosylation in which *p*-nitrophenyl β-galactopyranoside (pNPβGal) functions as a donor. Despite the possibility of the reverse hydrolysis of the disaccharide intermediate, this reaction could be blocked by further sialylation leading to the final trisaccharide product.



Scheme 4. Enzymatic synthesis of the Thomsen-Friedenreich antigen

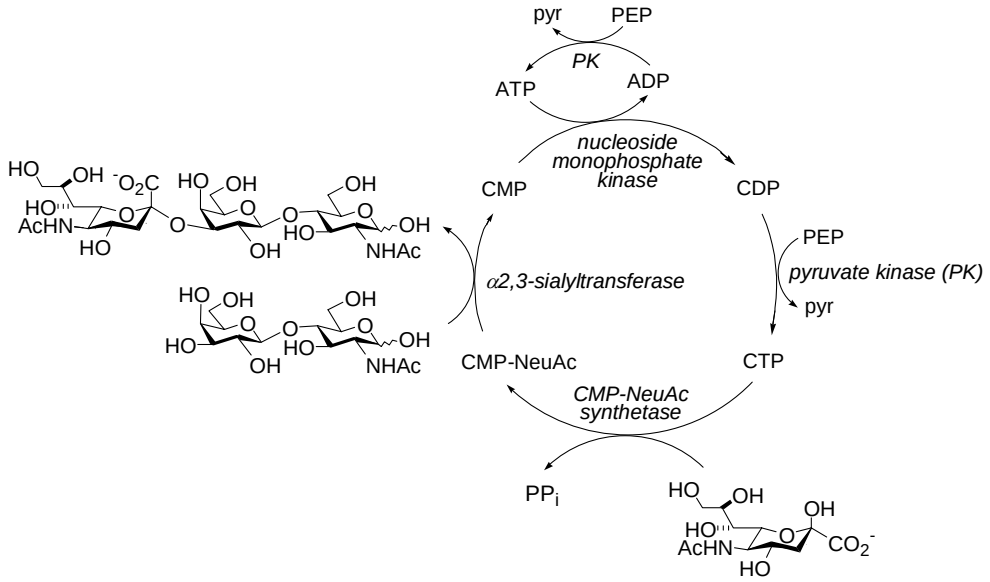
The reverse reaction of glycosidases can be considered as an alternative method of enzymatic glycosylation (40) because most glycosidases, unlike glycosyltransferases, are prevalently abundant in nature. This strategy has been introduced to the enzymatic sialylation. Thiem's group utilized *p*-nitrophenyl α-*N*-acetylneuraminic acid as the sialyl donor and neuraminidase from *Vibrio cholerae* to study trans-sialylation (Scheme 5)(41). Though various unusual di- and tri-saccharides can be prepared in this way and the rate of conversion can be measured based on the release of *p*-nitrophenol, an unsatisfactory low yield (14 to 20%) remained to be improved. Moreover, a mixture of α2,3- and α2,6-isomers was obtained even in the excessive presence of sialyl acceptor (41).



Scheme 5. Enzymatic sialylation using neuraminidase

Judging from the previous examples, silayltransferases are extremely powerful in terms of exclusive stereoselectivity and high efficiency (without protection and deprotection steps required in chemical synthesis). The enzyme reactions are, nevertheless, restricted to analytical- and small-scale synthesis due to the high cost of CMP-sialic acid (or CTP if CMP-NeuAc synthetase is used). The reactions also suffer from product inhibition caused by the released CMP. The regeneration of CMP-NeuAc *in situ* from CMP developed by Wong *et al.* is a simple solution to solve these two obstacles (42, 43). As shown in Scheme

6, nucleoside monophosphate kinase and pyruvate kinase are able to catalyze the transformation of CMP into CTP. In the regeneration system, the ultimate donor of phosphorylation comes from phospho(enol)pyruvate. As a matter of fact, Thiem's enzymatic synthesis of TF antigen was coupled with such a cofactor regeneration (39). Several advantages are offered in the *in situ* cofactor regeneration. First, the operating cost is reduced substantially since CMP-NeuAc or CTP is required in a catalytic amount. Second, product inhibition of CMP is minimized because of its low concentration. Finally, purification of the enzyme product is greatly improved without interfering CMP.



Scheme 6. *In situ* cofactor regeneration used in enzymatic sialylation

II. Chemical Approaches

Before the mid 80's, most chemical sialylations were carried out based on the Koenigs-Knorr reaction using the per-acetylated 2-halo-NeuAc **1** as the glycosyl donor. Unfortunately, for the sialylation occurring at the site of hindered alcohols (7), low α-selectivity and low yield were often observed while the elimination product **2** occurred as the major product. Recently, several new methods have been developed to obtain α-sialoglycosides as major isomers in high yields by the use of different sialyl donors, such as the 2-thioglycosides of sialic acid **3**, NeuAc phosphites **4**, S-glycosyl xanthates of NeuAc **5**, and 3-substituted NeuAc derivatives **6** (Figure 2). In the following, we discuss the sialylation reactions on the basis of glycosyl donors.

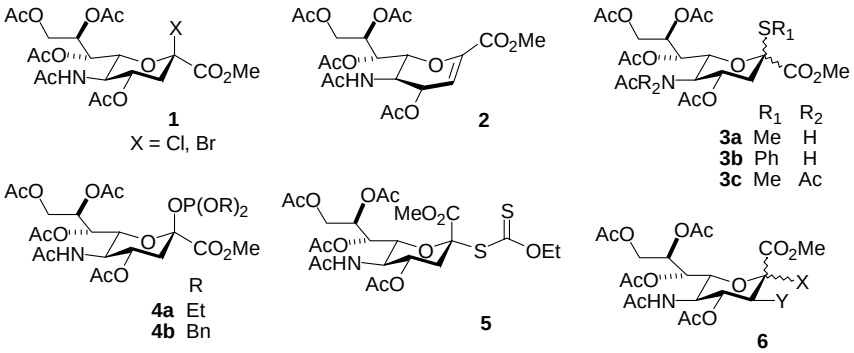


Figure 2. Various sialic acid donors

The first synthesis of thioglycosides was reported by Pivalova (44), and further investigated by Hasegawa *et al* (45-48). α-Glycosides were obtained predominantly in high yields using the anomeric mixture of either methyl (**3a**) or phenyl (**3b**) 2-thioglycoside as the sialyl donor, and DMTST (dimethyl(methylthio)sulfonium triflate) or NIS (*N*-iodosuccinimide)-T_fOH (*p*-trifluoromethanesulfonic acid) as the promoter. As shown in Figure 3 and Table 1 (45-50), various sugar acceptors were studied in acetonitrile at –40 ^oC. It should be noted that the secondary hydroxyls were sialylated to give exclusively the α-configuration (40-50%) in the presence of DMTST, while an anomeric mixture was obtained for the primary hydroxyls.

Table1. Glycosylation using the 2-thiolglycosides of NeuAc

Entry	Donor	Acceptor	Promoter	Yield (%) α	Yield (%) β	Reference
1	**3a**	**7**	DMTST	27	23	45
2	**3a**	**8**	DMTST	36	12	45
3	**3a**	**9**	DMTST	61	0	46
4	**3a**	**10**	DMTST	70	0	46
5	**3a**	**11**	DMTST	50	15	46
6	**3a**	**12**	NIS/T_fOH	70	0	47
7	**3a**	**13**	NIS/T_fOH	70	0	47
8	**3a**	**14**	DMTST	71	20	48
9	**3a**	**15**	DMTST	49	0	48
10	**3a**	**16**	DMTST	63	24	48
11	**3a**	**17**	DMTST	46	0	48
12	**3a**	**18**	NIS/T_fOH	59	10	46
13	**3a**	**19**	DMTST	40	0	47
14	**3a**	**20**	NIS/T_fOH	40	0	47
15	**3b**	**21**	NIS/T_fOH	65	0	49
16	**3c**	**9**	NIS/T_fOH	72	0	50
17	**3c**	**21**	NIS/T_fOH	71	0	50
18	**3c**	**22**	NIS/T_fOH	85	0	50

Moreover, under the condition of NIS/TfOH in acetonitrile, the reaction yield increased, but undesired β-glycosides were then obtained in some examples. Reducing the number of protecting groups in the acceptors improved yield and stereoselectivity. Interestingly, acceptors **10** and **11** containing different protecting groups at C-3 resulted in different

α-selectivity at the C-6 glycosylation site (entries 4 and 5). There are no systematic studies about the difference in activity between sialyl donors **3a** and **3b** (49). Recently, it was discovered by Boons *et al.* that the 5-*N*-diacetyl neuraminic acid derivative **3c** is significantly more reactive and does give higher yields in sialylation than the corresponding mono-*N*-acetylated derivative **3a** (entries 16-18) (50).

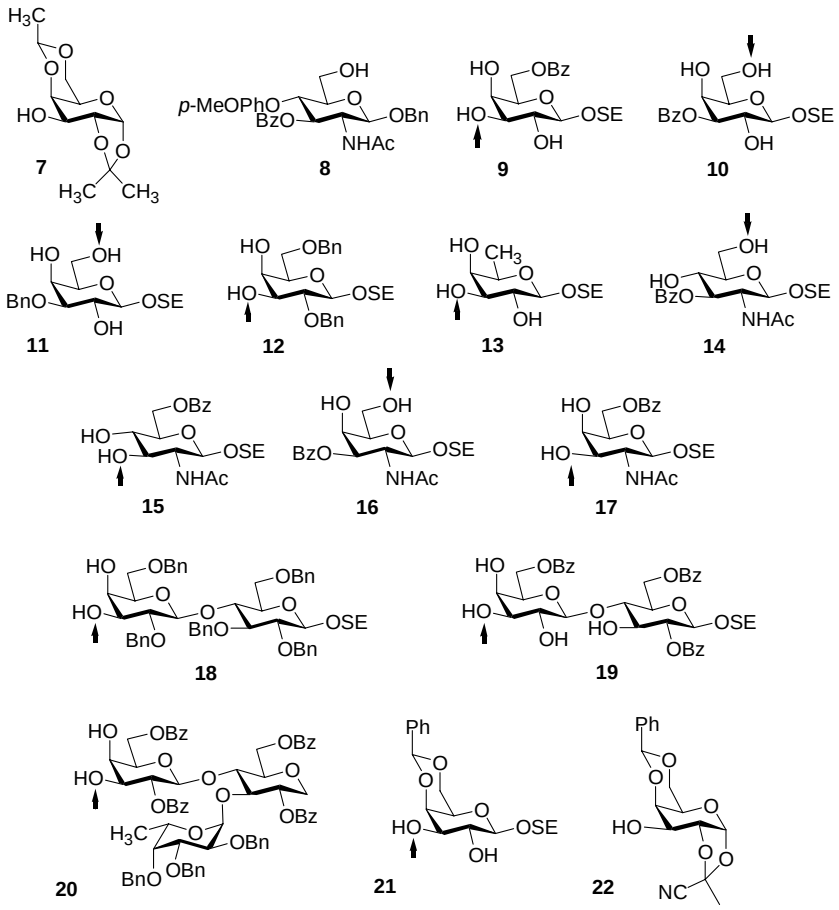


Figure 3. Structures of acceptors for glycosylation reactions using donors **3a-c**. Bold arrow indicates the glycosylation site. (SE: trimethylsilylethyl)

As shown in Figure 4, the 5-*N*-modified-2-thiosialosides (**23**, **24**) and deoxy sialic acid donors (**25, 26**) also show good α-selectivity (Table 2)(51-53). In addition, the S-sialyl xanthates **28** and **29**, first reported by Sinay *et al.* (54), showed comparable reactivity to that of the thiol analogs (**23, 24**) but with less α-selectivity (Table 3 and Figure 5). The reaction conditions of these two classes of sialyl donors are similar except the promoters are different (54-61). When benzoate was used to protect the hydroxyl groups of the acceptors, sialylation yield decreased (entries 8 and 9 in Table 3). The xanthate donors, however, are not as popular as the thiol ones.

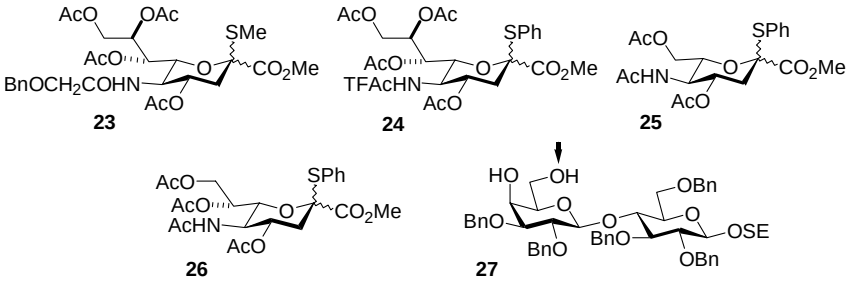


Figure 4. 2-Methyl thiol NeuAc analogues as sialic acid donors (compound **27** as the acceptor).

Table 2. Glycosylation using 2-thio NeuAc analogues

Entry	Donor	Acceptor	Promoter	Yield (%) α	Yield (%) β	Reference
1	**23**	**18**	NIS/T_fOH	55	0	51
2	**23**	**27**	NIS/T_fOH	50	13	51
3	**24**	**21**	NIS/T_fOH	61	0	52
4	**25**	**9**	NIS/T_fOH	45	0	53
5	**26**	**9**	NIS/T_fOH	45	0	53

Table 3. Glycosylation using the xanthates of NeuAc

Entry	Donor	Acceptor	Promoter	Yield (%) α	Yield (%) β	Reference
1	**28**	**30**	DMTST	48	16	54
2	**28**	**31**	DMTST	26	4	54
3	**28**	**32**	$AgOT_f$/MeSBr	71	4	55
4	**28**	**33**	$AgOT_f$/MeSBr	82	-	56
5	**28**	**34**	$AgOT_f$/MeSBr	56	-	57
6	**28**	**35**	$AgOT_f$/MeSBr	65	-	58
7	**28**	**36**	$AgOT_f$/PhSCl	58	-	59
8	**29**	**37**	$AgOT_f$/MeSBr	39	9	60
9	**29**	**38**	$AgOT_f$/MeSBr	15	5	60
10	**29**	**39**	$AgOT_f$/MeSBr	18	5	60
11	**29**	**40**	$AgOT_f$/MeSBr	41	18	61

Although the 2-thioglycosides of neuraminic acid have been applied for the addition of sialic acids, the requirement of at least two equivalents of thiophilic reagents is a disadvantage for this approach. Wong (12) and Schmidt (64) independently reported the utilization of sialyl phosphites as the donors of sialylations. As shown in Figure 6 and Table 4, these phosphite donors showed good α-selectivity for secondary hydroxyl acceptors. Nevertheless, as previously mentioned for most sialic acid donors, the obstacle still remains to obtain exclusive α-selectivity for sialylations occurring at primary hydroxyl groups. In order to solve this challenging problem, the anchimeric assistance by introducing an auxiliary group at C-3 has been proposed. As shown in Figures 7, 8, and Table 5 (67-73), the arylthio groups

present a good directing effect with excellent α-selectivity.

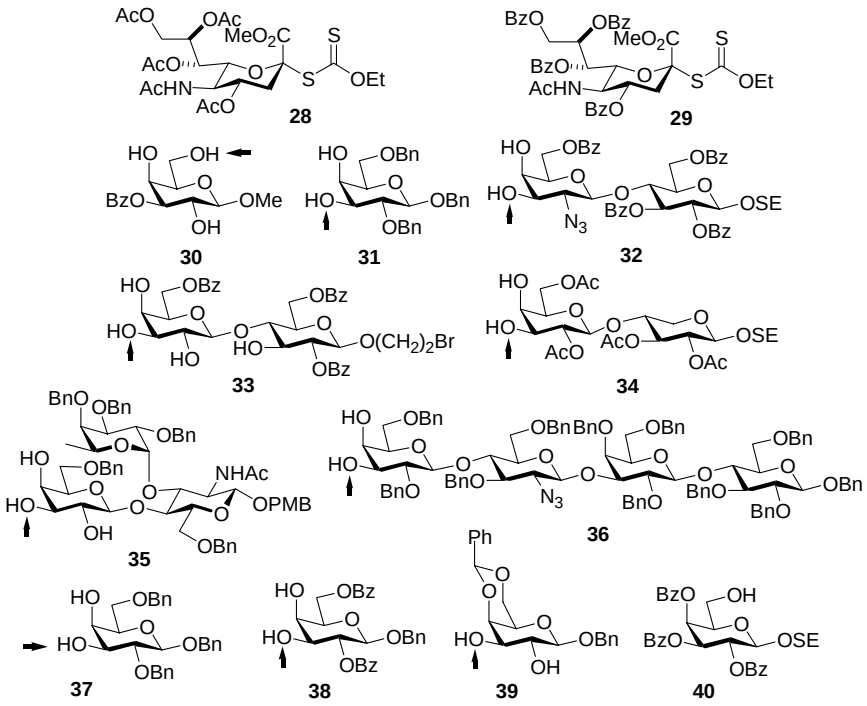


Figure 5. Structures of sialyl donors (xanthate type) and acceptors (Bold arrow indicates the glycosylation site).

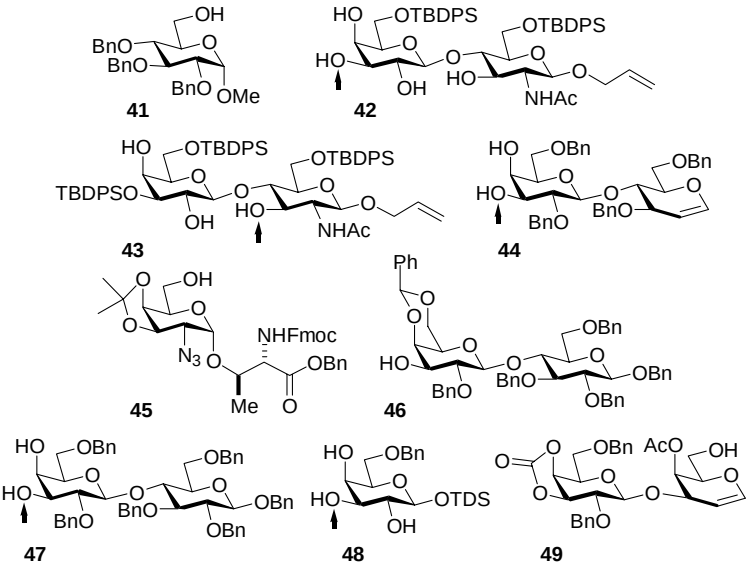


Figure 6. Structures of acceptors for the sialyl phosphite donors. (Bold arrow indicates the glycosylation site).

Table 4. Glycosylation using sialyl phosphites as donors

Entry	Donor	Acceptor	Promoter	Yield (%) α	Yield (%) β	Reference
1	**4a**	**41**	$TMSOT_f$	67	13	12
2	**4a**	**42**	$TMSOT_f$	67	11	12
3	**4a**	**43**	$TMSOT_f$	68	11	12
4	**4a**	**44**	$TMSOT_f$	75	0	62
5	**4a**	**18b**	$TMSOT_f$	77	0	62
6	**4a**	**45**	$TMSOT_f$	37	9	63
7	**4b**	**41**	$TMSOT_f$	56	14	64
8	**4b**	**46**	$TMSOT_f$	38	0	64
9	**4b**	**47**	$TMSOT_f$	55	0	65
10	**4b**	**48**	$TMSOT_f$	51	0	66
11	**4b**	**49**	$TMSOT_f$	67	17	63

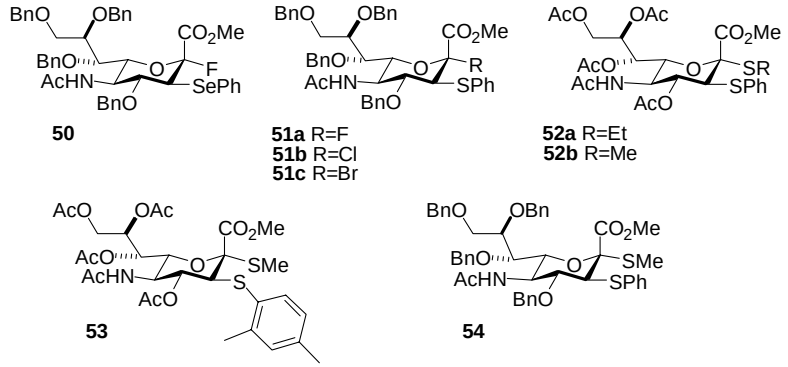


Figure 7. 3-Substituent NeuAc analogues as sialyl donors

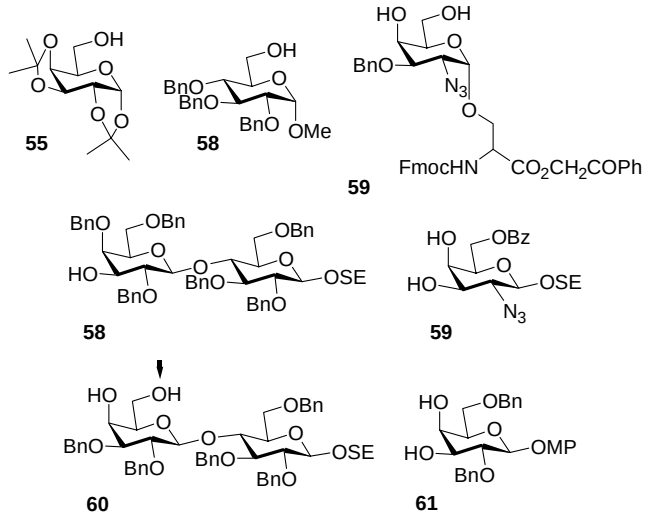


Figure 8. Structures of sialylation acceptors **55-61** for using 3-substituted NeuAc analogues (**50**-**54**) as sialyl donors

Table 5. Glycosylation using 3-substituted NeuAc analogues

Entry	Donor	Acceptor	Promoter	Yield (%) α	Yield (%) β	Reference
1	**50**	**55**	$AgOT_f$-$SnCl_2$	46	-	67
2	**50**	**56**	$AgOT_f$-$SnCl_2$	72	-	67
3	**50**	**47**	$AgOT_f$-$SnCl_2$	20	-	67
4	**51a**	**55**	$AgOT_f$-$SnCl_2$	68	4	68
5	**51b**	**56**	$Hg(CN)_2$-$HgBr_2$	71	0	68
6	**51b**	**47**	$Hg(CN)_2$-$HgBr_2$	64	-	68
7	**51c**	**57**	$Hg(CN)_2$-$HgBr_2$	85	-	69
8	**52a**	**58**	MSB/T_fOH	67	-	70
9	**52a**	**59**	MSB/T_fOH	77	-	70
10	**52a**	**18a**	MSB/T_fOH	71	0	70
11	**53**	**18a**	PhSCl/$AgOT_f$	78	0	71
12	**53**	**60**	PhSCl/$AgOT_f$	83	0	71
13	**52b**	**18a**	PhSCl/$AgOT_f$	83	0	72
14	**54**	**61**	NIS/T_fOH	85	0	73

The α-2,8-disialic acid, NeuAc-α(2-8)-NeuAc, is an essential component of some important glycoconjugates including gangliosides, oligo- and polysialic acids. These molecules play indispensable roles in numerous biological phenomena; e.g., tumor- associated antigens and bacterial toxins. As for the corresponding sialylations, the C-8 hydroxyl functionality as an acceptor exhibits low reactivity due to steric hindrance and intramolecular hydrogen bonding of 8-OH and 1-carboxylic ester (or 2-substituent)(74). On the other hand, attempts to prepare the α(2-8)-linkage using traditional sialyl donors, such as thioglycosides or phosphites, gave a very low yield and/or undesired β-linkage (7,45,74).

To solve such a problem, the auxiliary group at the C-3 position of sialyl donors was introduced for an efficient neighboring group participation. As shown in Table 6 and Figure 9 (74-80), dimeric sialic acid was formed as one isomer when the donor had the auxiliary group at C-3 (entries 7-9). The results appeared different from the examples without the C-3 auxiliary where α,β mixtures were obtained (entry 6). Additionally, the acceptors of 2,3-anhydroneuraminic acid derivatives, containing no hydrogen bonding as mentioned above, gave better yields of α, β mixtures when coupled with normal sialyl donors, e.g., **4b** and **3c**. Recently, Schmidt and coworkers reported that the phenoxythiocarbonyloxy group served as an excellent anchimeric group in terms of α-selectivity and yield (up to 83%) by using the phosphite as the leaving group.

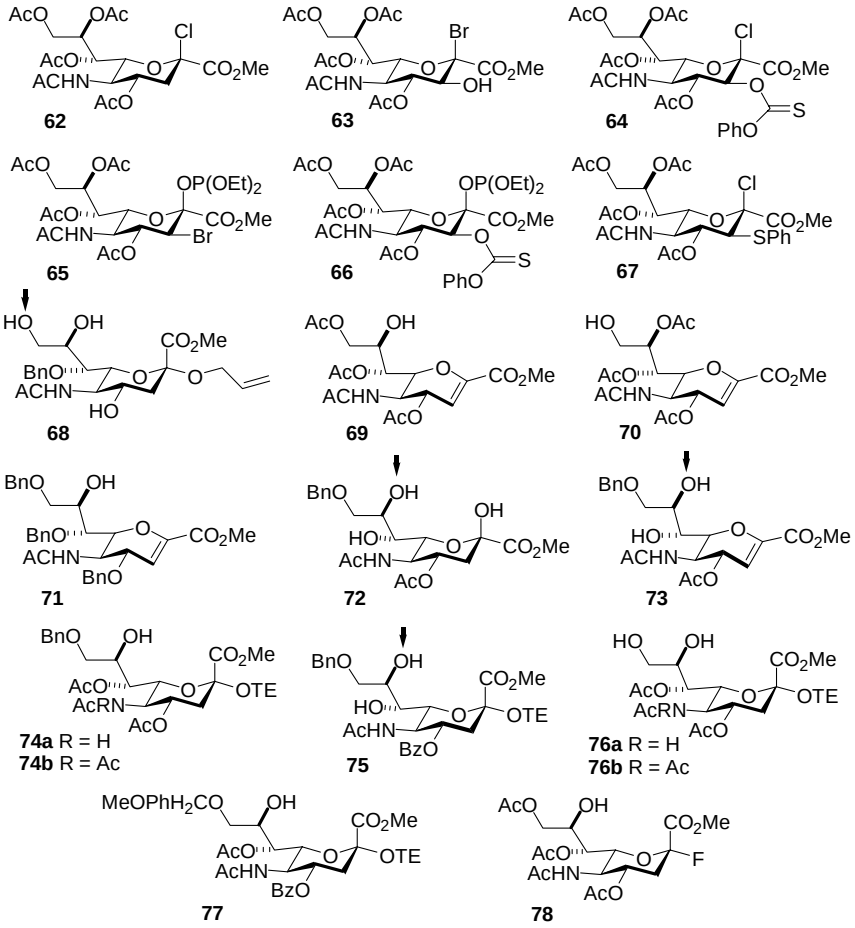


Figure 9. Sialic acid donors and acceptors for synthesis of NeuAcα(2-8)NeuAc (Bold arrow indicates the glycosylation site).

Being useful for establishing the NeuAc(2-3)Gal glycosidic linkage, the sialyl donor **3c** was also studied to prepare α-2,8 and α-2,9 sialic acid dimers. It demonstrated that the glycosyl acceptors of di-*N*-acetylated derivatives **74b** and **76b** generated significantly better results than those of mono-*N*-acetylated compounds **74a** and **76a** (entries 10, 11, 13, and 14 in Table 6). It indicated the possibility that the nucleophilicity of 8-OH could well be enhanced by removing the hydrogen bonding between 8-OH and 5-NAc.

Table 6. Synthesis of NeuAcα(2-8)NeuAc

Entry	Donor	Acceptor	Promoter	Yield (%) α	Yield (%) β	Reference
1	**62**	**68**	$Hg(CN)_2/HgBr_2$	55	28	75
2	**63**	**69**	$AgOT_f/Na_2HPO_4$	26	8	76
3	**63**	**70**	$AgOT_f/Na_2HPO_4$	42	21	76
4	**51c**	**71**	$Hg(CN)_2/HgBr_2$	64	-	77
5	**4b**	**72**	$TMSOT_f$	-	55	74
6	**4b**	**73**	$Sn(OT_f)_2$	14	54	74
7	**64**	**73**	$AgOT_f$/DTBP	68	-	74
8	**65**	**73**	$TMSOT_f$	-	58	78
9	**66**	**73**	$TMSOT_f$	83	-	78
10	**3c**	**74a**	NIS/T_fOH	8	8	79
11	**3c**	**74b**	NIS/T_fOH	32	18	79
12	**3c**	**75**	NIS/T_fOH	20	40	79
13	**3c**	**76a**	NIS/T_fOH	44	23	79
14	**3c**	**76b**	NIS/T_fOH	70	28	79
15	**52a**	**77**	$AgOT_f$/MSB	28	-	70
16	**67**	**78**	$AgOT_f/SnCl_2$	49	-	80

SUMMARY

In conclusion, either enzymatic or chemical approaches have their unique features and unavoidable disadvantages. Enzyme-catalyzed sialylations provide the desired sialo-glycosidic linkages in the two enzyme reactions (CMP-NeuAc synthetase and sialyltransferase) with exclusive stereoselectivity and high yield as long as the required sialyltransferase is available. High substrate specificity of the two enzymes is a limitation so that many unnatural glycoconjugates cannot be prepared enzymatically. As for chemical glycosylations of sialic acids, it is possible to introduce any modification in sialyl donor and acceptor, in addition to create special sugar linkages. Nevertheless, reducing the number of reaction steps (for preparing both donors and acceptors of glycosylation), and enhancing stereoselectivity, as well as reaction yield are still problems to be overcome.

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