Microbial Glycosyltransferases for Carbohydrate Synthesis: α-2,3-Sialyltransferase from *Neisseria gonorrheae*

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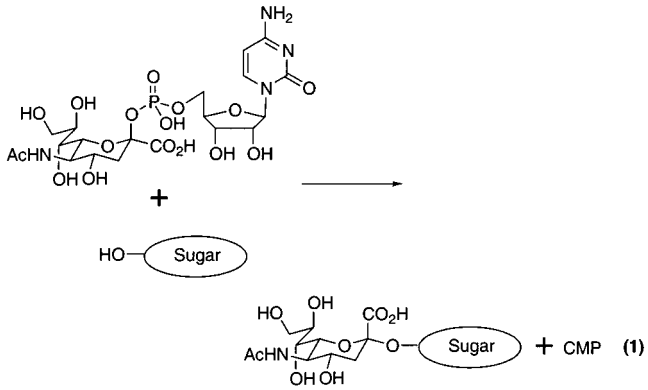
Abstract: The α-2,3-sialyltransferase from *Neisseria gonorrheae* was overproduced in *E. coli* for exploitation of its substrate specificity and synthetic utility. Several potential acceptor substrates were synthesized in this study, including mono- and oligosaccharides, glycolipids, and glycopeptides and their sulfate derivatives. Some CMP-sialic acid derivatives with modification at the C-5 position were also prepared for evaluation as donor substrates. It was found that the enzyme exhibits a broader acceptor substrate specificity when compared to other sialyltransferases, though the donor specificity is quite limited. Application of the enzyme to the preparative synthesis of representative sialyl glycoconjugates has been demonstrated. On the basis of this work and the work of others, this enzyme is the most versatile and synthetically useful among all sialyltransferases known to date, especially for the synthesis of sulfate-containing glycoconjugates.

Introduction

Many sugar nucleotide-dependent glycosyltransferases have been extensively used as glycosylation catalysts in oligosaccharide synthesis.[1] Enzymatic glycosylation is stereospecific, free of protecting group manipulation, and in general, more practical than the chemical glycosylation. Most of these enzymes are prepared from mammalian tissues by isolation or by gene cloning and expression in microbial or baculoviral systems.[2] Glycosyltransferases are, however, very specific for the sugar nucleotide substrate, though some can accept analogues with very small changes in structure.[1,3] Regarding the acceptor sugar specificity, most glycosyltransferases are specific for the terminal sugar moiety of accptor but are able to accommodate modifications at the group linked to the anomeric center [1,3] (i.e. the aglycon moiety). In addition to this relatively narrow substrate specificity, little is known with regard to the structures of glycosyltransferases complexed with substrates, making it very difficult to engineer the substrate specificity of these enzymes. Most of the synthetic applications of glycosyltransferases are therefore centered on the assembly of naturally occurring oligosaccharides found in mammalian systems. A recent study on TDP-sugar dependent microbial glycosyltransferases in vivo has shown that the enzymes accept a number of sugar nucleotide analogues as donor substrates,[4a] suggesting that some microbial glycosyltransferases may have more relaxed specificity and are thus useful as synthetic catalysts. Studies of these enzymes in vitro are, however, very limited due to the lack of TDP-sugars available for the experiment.[4b−d] As a part of our effort to exploit and expand the synthetic utility of glycosyltransferases, we report our study of the α-2,3-sialyltransferase from *Neisseria gonorrheae*. Sialyltransferases catalyze the transfer of sialic acid (*N*-acetylneuraminic acid, Neu5Ac) from the activated sugar nucleotide CMP-Neu5Ac to an acceptor sugar to form a sialoside (eq 1). Glycosidic linkages of sialic acid are predominantly



(1)

found in four forms, α-(2,3) or α(2,6) linkage to the C-3 or C-6 hydroxyl of galactose and α(2,8) or α(2,9) linkage to another sialic acid.[5] Sialyl glycosides are often found in glycoproteins, glycolipids, or polysaccharides and are associated with important biological functions.[5] Due to the hindered tertiary anomeric center and the lack of a participating auxiliary,

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chemical sialylation reaction often results in low yields and low stereoselectivity, and thus remains one of the most difficult glycosylation reactions.[6−12] Enzymatic sialylation using sialyltransferases therefore has been considered to be an attractive alternative.[1] The narrow substrate specificity of mammalian sialyltransferases, however, limits the usefulness of these enzymes in synthesis. The commercially available mammalian α-2,3-sialytransferase from rat liver, for example, only accepts *N*-acetyllactosamine as substrate; monosaccharides, sulfate-containing oligosaccharides, and glycopeptides are not acceptable.[1e] Bacterial sialyltransferases appear to have a broader substrate specificity. The α-2,6-sialyltransferase from *Photobacterium damse*, for example, has been shown to catalyze the transfer of sialic acid to a terminal galactose which is fucosylated or sialylated at the 2 or 3 position, respectively.[13] The α-2,3 sialytransferase from *Neisseria meningitidis* has been shown to sialylate the terminal galactose of *N*-acetyllactosamine, lactose, and α- or β-galactosides.[14a,b] A viral α-2,3-sialyltransferase was shown to sialylate fucosylated acceptors such as Lewis a and Lewis x.[14c] A bacterial α-2,8/2,9-sialyltransferase was shown to accept various oligosialic acids with alternate α-2,8 and α-2,9 linkages.[14d] Other microbial transferases have also been reported.[14e] However, the donor and acceptor specificities of all these microbial sialyltransferases, except the one from *N. meningitidis*, have not been well studied and their synthetic usefulness has not been well demonstrated. In this work, we exploit the substrate specificity of this microbial sialyltransferase using synthetic donor and acceptor analogues and derivatives, with particular emphasis on the development of enzymatic sialylation methods for the synthesis of important sialyl conjugates which are difficult or impractical to synthesize by other means.

Results and Discussion

Preparation of α-2,3-Sialyltransferase. The gene coding for the enzyme from *Neisseria gonorrheae* was initially cloned into pRSET vector (Invitrogen Co., Carlsbad, CA) for expression of the protein containing a hexahistidine tag at the N-terminus. The expressed protein was active, but was not able to bind to the Ni-affinity colunm. Using pET vector we are able to express the α-2,3-sialyltransferase with the histidine tag at the C-terminus. The expressed protein was more active and able to bind to the Ni-colunm. To purify the enzyme, the cells were passed through a french press followed by centrifugation. The supernatant was found to contain about 60% of the enzyme activity and the remaining activity was found in the cell pellet. The supernatant was passed through the Ni-colunm and eluted with 100 mM imidazole. The enzyme isolated through this simple procedure was shown to be quite homogeneous on SDS-page electrophoresis. It was thus used directly for determination of its specific activity and substrate specificity (Table 1).

Table 1. One-Step Purification of α-2,3-Sialyltransferase from 1 L of Culture by Affinity Column Chromatography

step	total protein (mg)	total activity (μmol/ h)[a]	specific activity (U/mg)	yield (%)
cell free ext.	3600	1587	0.44	100
pellet	12100	921	0.08	
after affinity column (from supernatant)	60.5	181	3.00	11.4

[a] The activity was measured based on the acceptor lactose (1 mM CMP-sialic acid, 1 mM acceptor, 10 mM $MgCl_2$, 50 mM Tris buffer, pH 7.5). One unit (U) of the enzyme activity is the formation of 1 μmol of product per hour.

Enzymatic Analysis. In general, the assay for acceptor specificity was carried out with CMP-[^{14}C]Neu5Ac as donor substrate. Sialylated products were separated from CMP-[^{14}C]-Neu5Ac by passing through Dowex 1×8 resin (phosphate form) and the isotope content was counted by scintillation counter. This method is, however, not effective for the analysis of acceptors containing negative charges or hydrophobic groups due to the problem of separation. Alternative methods were used for qualitative analysis to find synthetically acceptable substrates. Thus, for evaluation of acceptors carrying a hydrophobic aglycon, cold CMP-Neu5Ac was used and the product was isolated with a SepPak 18 solid-phase extraction cartridge and analyzed by electrospray mass spectrometry (ESI-MS). For evaluation of sulfate-containing oligosaccharides as acceptors, the reaction was analyzed by TLC with use of resorcinol to visualize the sialylated product. For compounds carrying a long aliphatic chain, the assay was carried out in the presence of 0.02% Triton X-100. To make sure that these qualitative methods are reliable, the sialylated product was further characterized by ESI-MS to ensure that sialylation does occur. The relative intensity of the sialylated product was then used to estimate the enzyme activity. Alternatively, the relative rates can be determined by using a pH indicator (e.g. phenol red, $\Delta\epsilon = 5.6 \times 10^4\ M^{-1}\ cm^{-1}$) in a minimal concentration of buffer (~2 mM). Several saccharides and derivatives were quickly surveyed by using one of these methods to determine their relative acceptor specificity. All numbers are based on lactose acceptor, the specific activity of which was determined to be 3 U/mg (1 U = 1 μmol product formed per hour).

Synthesis of Substrates. We are particularly interested in developing enzymatic methods for the sialylation of sulfate-containing oligosaccharides, glycopeptides, and glycolipids, as these sialosides exhibit important functions[1e,5] but are difficult to synthesize with traditional glycosylation methods. The phenolic sulfate group is too acid sensitive to be compatible with other protecting groups manipulations. The preparations of potential substrates shown in Table 2 were performed as follows. Allyl 6-*O*-sulfo-*N*-acetyllactosaminide (**7**) was synthesized as shown in Scheme 1. Glycosylation of allyl 6-*O*-

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Table 2. Substrate Specificiy of the α-2,3-Sialyltransferase

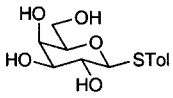
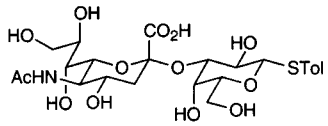
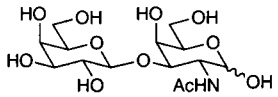
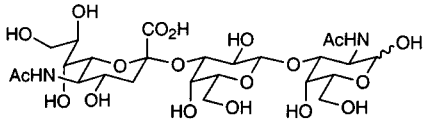
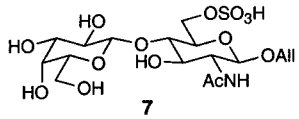
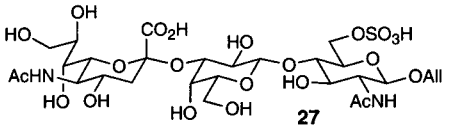
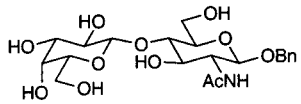
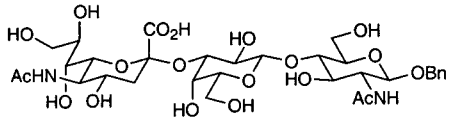
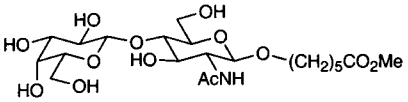
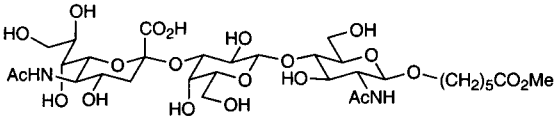
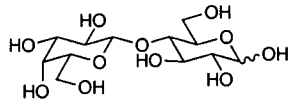
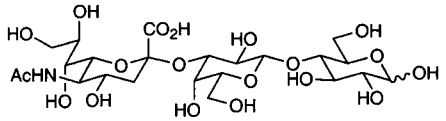
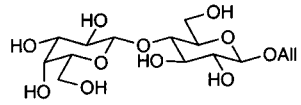
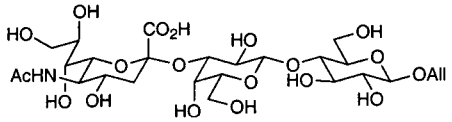
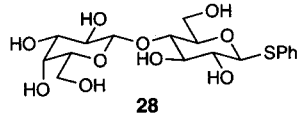
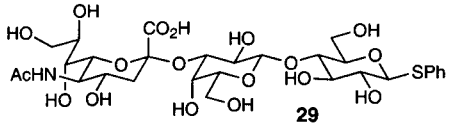
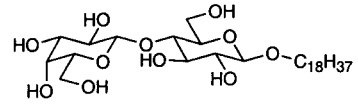
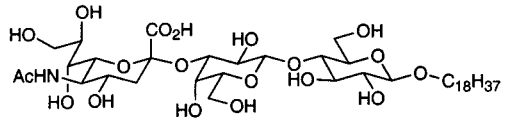
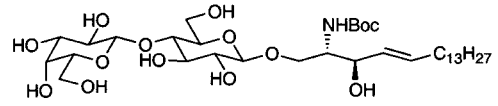
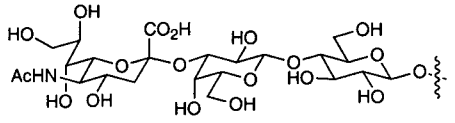
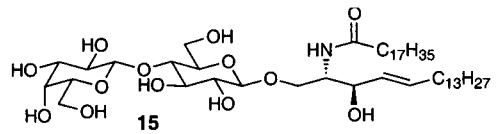
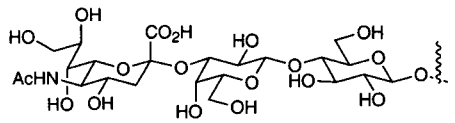
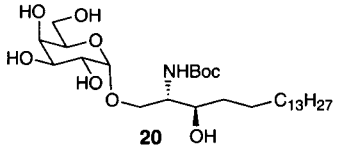
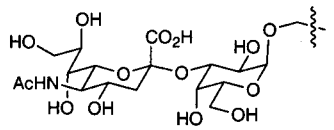
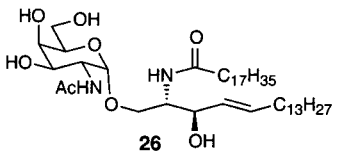
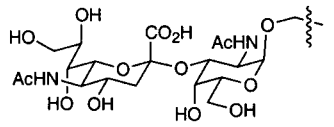
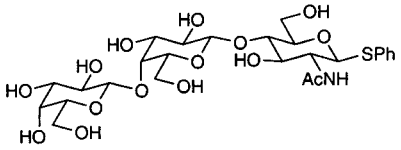
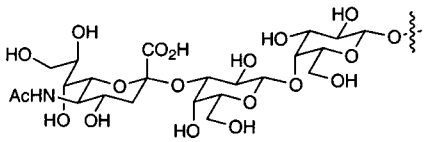
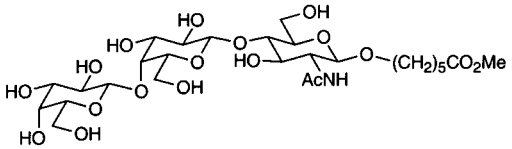
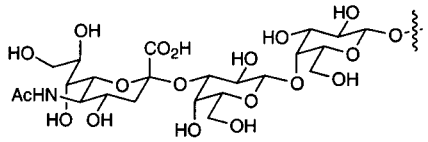
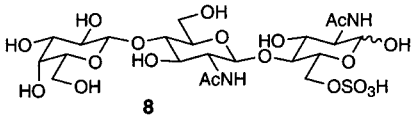
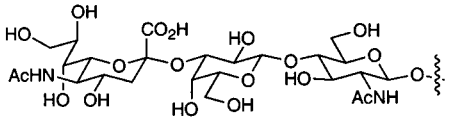
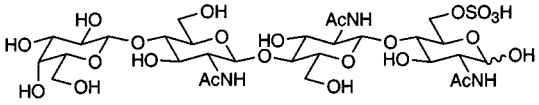
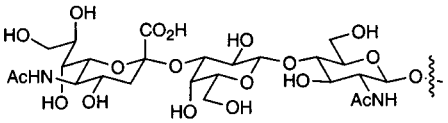
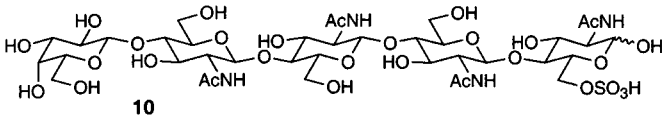
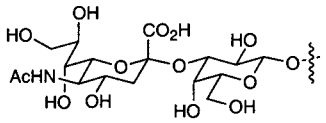
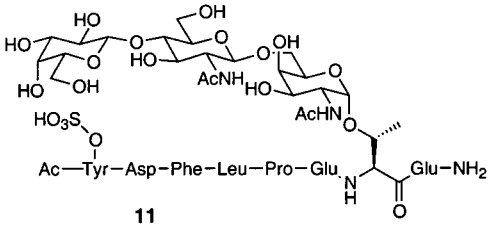
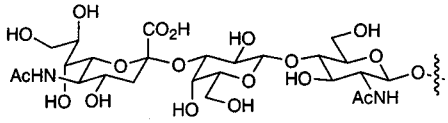
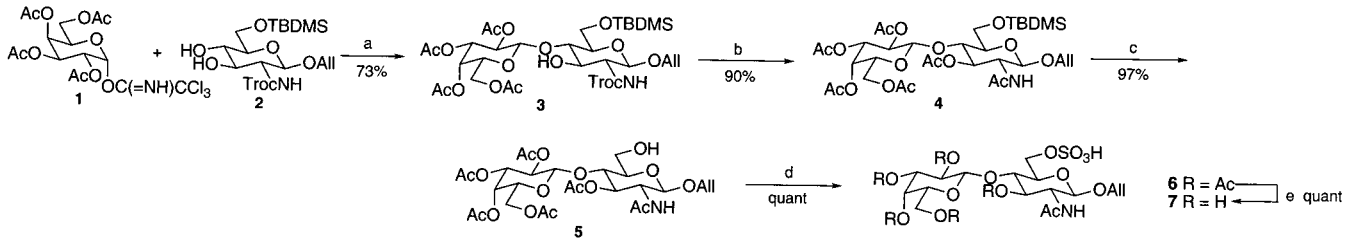
Acceptor	Product	relative rate[a]
		0.29
		0.26
 **7**	 **27**	1.40[b]
		1.35
		2.33
		1
		0.84
 **28**	 **29**	0.51
		0.26
 **14**		0.43
 **15**		0.28
 **20**		< 0.1[b]
 **26**		0.24

Table 2 (Continued)

Acceptor	Product	relative rate
		0.16
		0.24
 **8**		1.42[b]
 **9**		1.41[b]
 **10**		1.40[b]
 **11**		1.41[b]

[a] Based on the isotope assay as described in the Experimental Section. [b] Based on the TLC and/or ESI-MS analysis. Conditions for the assays are the same as that described in Table 1. All relative activities are based on lactose, the specific activity of which is 3 U/mg (1 U = 1 μmol product formed per hour). Compounds **7**−**11** and **28** were sialylated at the terminal galactose 3-position on a preparative scale.

Scheme 1[a]

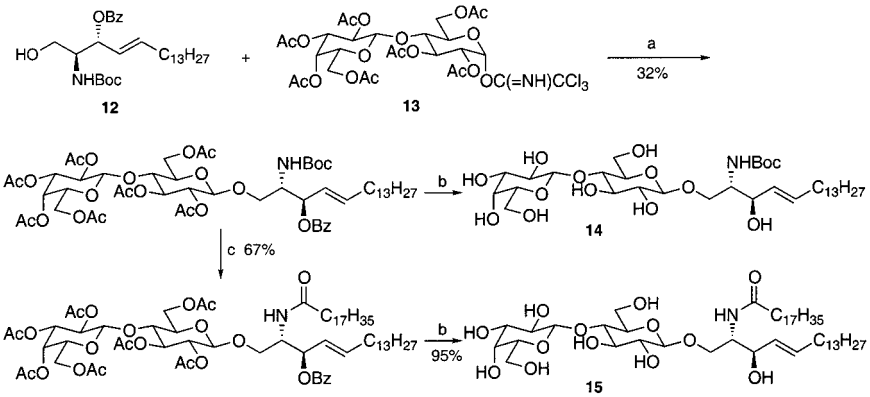


[a] Reagents and conditions: (a) TMSOTf/CH_2Cl_2/−48 °C; (b) Zn/AcOH then Ac_2O/Py; (c) 70% AcOH/70 °C; (d) SO_3·pyridine/THF; (e) NaOMe/MeOH.

TBDMS-*N*-Troc-β-D-glucosaminide (**2**) with 1.1 equiv of tetraacetylgalactosyl trichloroacetimidate **1** in CH_2Cl_2 at −48 °C in the presence of TMS triflate as an activator gave the $\beta(1 \rightarrow 4)$ linked disaccharide **3** regioselectively in 73% yield. The *N*-Troc group of **3** was removed with zinc in acetic acid, and the liberated amine was subsequently acetylated to give **4** in 90% yield. The TBDMS group was deprotected and the exposed hydroxyl group was converted to the sulfate group in nearly quantitative yield. Deacetylation with methanolic sodium methoxide gave **7**. Galactosyl chitooligosaccharides **8**−**10** containing a sulfate group at the reducing end 6-position were synthesized from chitooligosaccharides with use of Nod factor sulfotransferase coupled with regeneration of PAPS and β-1,4-galactosyltransferase as reported previously.[15a] The Tyr-*O*-sulfate-containing PSGL-1 glycopeptide **11** was prepared according to the procedure reported.[15b]

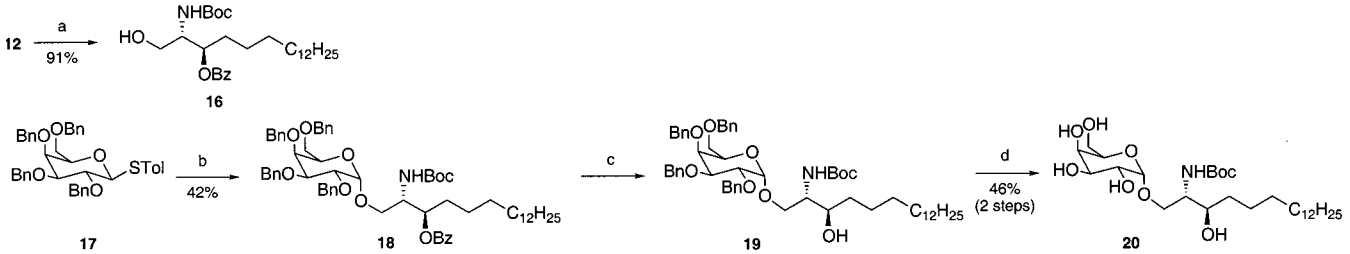
(15) (a) Burkart, M. O.; Izumi, M.; Chapman, E.; Lin, C.-H.; Wong, C.-H. *J. Org. Chem.* **2000**, *65*, 5565−5574. (b) Koeller, K. M.; Smith, M. E. B.; Huang, R.-F.; Wong, C.-H. *J. Am. Chem. Soc.* **2000**, *122*, 4241−4242.

Scheme 2[a]



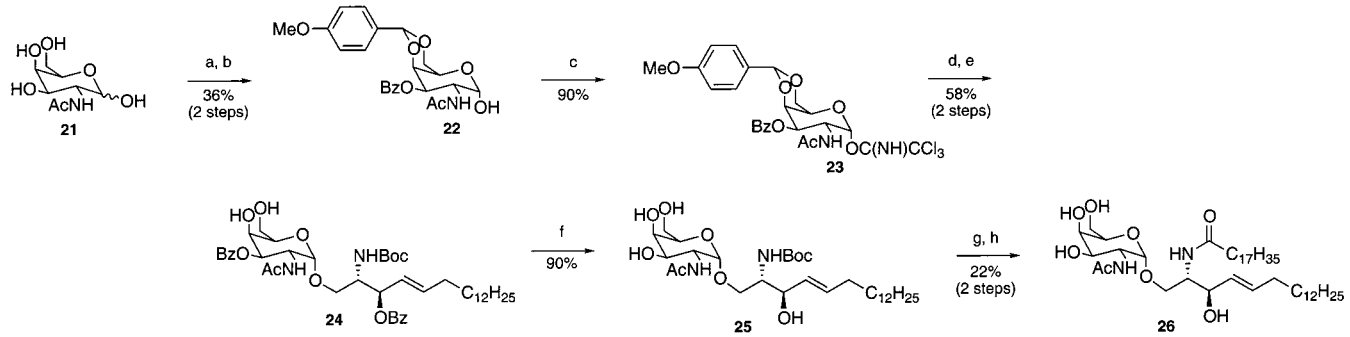
[a] Reagents and conditions: (a) $BF_3{\cdot}OEt_2$/MS 4 Å/CH_2Cl_2/−20 °C; (b) NaOMe/MeOH/CH_2Cl_2; (c) TFA/CH_2Cl_2 then $C_{17}H_{35}CO_2H$/EDC/CH_2Cl_2.

Scheme 3[a]



[a] Reagents and conditions: (a) H_2, Pd/C, EtOH; (b) **16**, NIS, TfOH, CH_2Cl_2, −50 °C; (c) NaOMe, MeOH; (d) H_2, Pd/C, EtOH.

Scheme 4[a]



[a] Reagents and conditions: (a) *p*-$MeOC_6H_4CH(OMe)_2$, TsOH; (b) BzCl, py, 0 °C; (c) CCl_3CN, DBU, CH_2Cl_2; (d) **12**, $BF_3{\cdot}OEt_2$, 1:1 Diox-THF, −20 °C, α/β = 8:1; (e) 80% AcOH; (f) NaOMe, MeOH; (g) 10%TFA in CH_2Cl_2; (h) polymer based acylation, NEt_3, CH_2Cl_2.

The synthesis of β-linked lactosyl sphingosines is illustrated in Scheme 2. Starting from the known compound 1,1-dimethyl [*R*-[*R**,*S**-(*E*)]]-2,2-dimethyl-4-(1-hydroxy-2-hexadecenyl)-3-oxazolidinecarboxylate,[16] we obtained *N*-(*tert*-butoxycarbonyl)-3-*O*-benzoyl-D-*erythro*-sphingosine (**12**) by benzoylation and subsequent mild hydrolysis of the isopropylidene acetal with acidic resin. Glycosylation of **12** with peracetylated lactosyl trichloroacetimidate (**13**) in CH_2Cl_2 at −20 °C in the presence of MS 4 Å and $BF_3{\cdot}OEt_2$ as an activator gave the β-lactoside which was deprotected to give **14**, or further transformed to **15**. The physical data of **14** and **15** are consistent with the reported values.[17]

Two α-linked sphingolipids (**20** and **26**) were also exploited as substrates, as these types of unusual linkages have been shown to activate killer T-cells through CD1 presentation.[18] The syntheses of **20** and **26** were shown in Schemes 3 and 4. It is noted that, even in the presence of a neighboring participating *N*-acetyl group, the BF_3 mediated glycosylation of **23** in dioxane gave mainly the α-product. A predominant β-product was obtained in other nonpolar solvents such as CH_2Cl_2. Perhaps the initial glycosyl cation generated formed an adduct with the lone-pair of the solvent through the neighboring group assistance, followed by a nucleophilic attack from the axial position to form the α-glycosidic linkage.

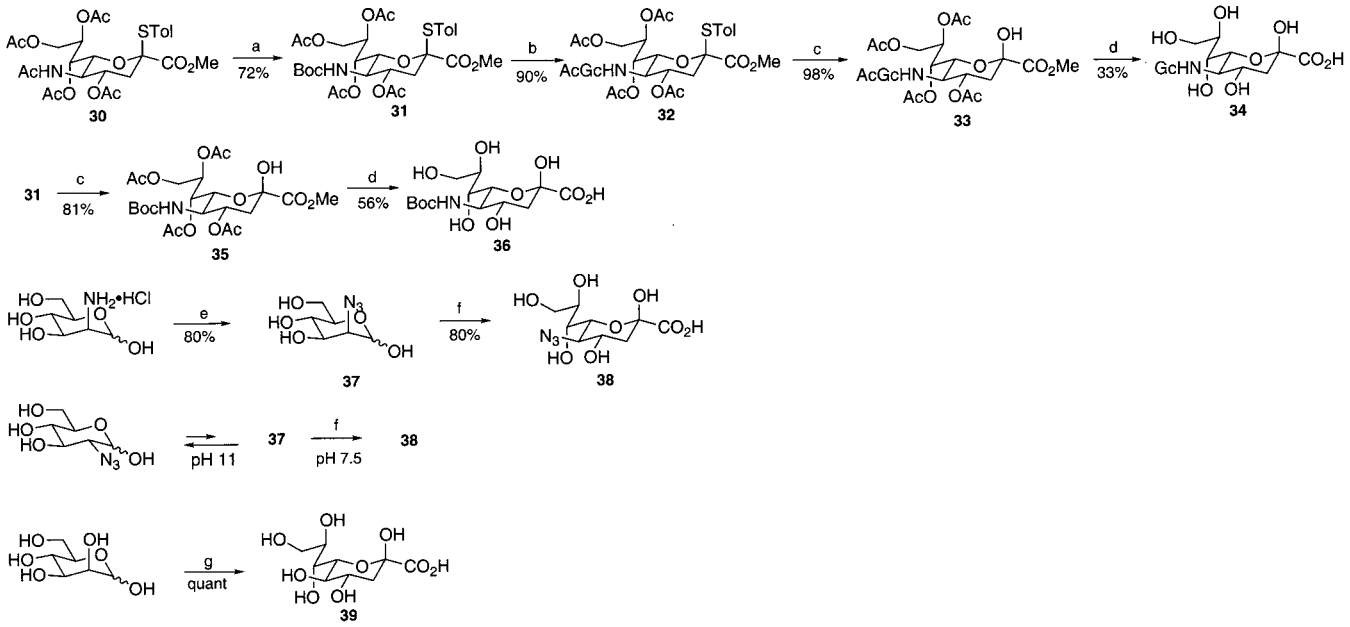
Acceptor Specificity and Representative Synthesis of Sialyl Conjugates. The synthetic acceptors and their relative activities tested as substrates for α-2,3-sialyltransferase are summarized in Table 2. The benzyl and methoxycarbonylpentyl glycosides of β-*N*-acetyllactosamine were excellent substrates, and addition of an extra β-galactosyl residue to the 4′-position was still

(16) (a) Koskinen, P. M.; Koskinen, A. M. P. *Synthesis* **1998**, 1075−1091. (b) Hoffman, R. V.; Tao, J. *Tetrahedron Lett.* **1998**, *39*, 3953−3956. (c) Herold, P. *Helv. Chim. Acta* **1988**, *71*, 354−361. (d) Hoffman R. V.; Tao, J. *J. Org. Chem.* **1998**, *63*, 3979−3985. (e) Koskinen, A.; Koskinen, P. United States Patent 5426228, 1995.

(17) Fiore, S.; Nicolaou, K. C.; Caufield, T.; Kataoka, H.; Serhan, C. N. *Biochem. J.* **1990**, *266*, 25−31.

(18) (a) Prigozy, T. I.; Naidenko, O.; Qasba, P.; Elewaut, D.; Brossay, L.; Khurana, A.; Natori, T.; Koezuka, Y.; Kulkarni, A.; Kronenberg, M. *Science* **2001**, *291*, 664−667. (b) Burdin, M.; Kronenberg, M. *Curr. Opin. Immunol.* **1999**, *11*, 326−331.

Scheme 5[a]



[a] Reagents and conditions: (a) (i) Boc_2O/DMAP/THF/50 °C, (ii) $NH_2NH_2 \cdot H_2O$/MeOH, (iii) Ac_2O/pyridine; (b) (i) PhOH/TMSCl/CH_2Cl_2, (ii) AcGcCl/DABCO/CH_2Cl_2; (c) NBS/aqueous acetone; (d) (i) NaOMe/MeOH, (ii) NaOH; (e) $TfN_3/CuSO_4/K_2CO_3$; (f) CH_3COCO_2Na/NeuAc aldolase (46 U/mmol); (g) CH_3COCO_2Na /NeuAc aldolase (9.2 U/mmol).

acceptable. Regarding sulfate-containing oligosaccharides, allyl 6-*O*-sulfo-*N*-acetyllactosaminide, galactosyl 6-*O*-sulfo-chitobiose and the corresponding chitotriose and chitotetraose, and the PSGL-1 glycopeptide carrying a sulfotyrosine residue were examined, and each gave the sialylated product in excellent yield. The commercially available rat α-2,3-sialyltransferase, however, can only accept galactosyl 6-*O*-sulfo-chitotetraose as a weak substrate.[15b]

The lactosides with allyl, phenylthio, octadecyl, *N*-Boc-sphingosine, or ceramide as an aglycon were good substrates. In addition, 4-methylphenylthio-*β*-galactoside, α-galactosyl and α-*N*-acetylgalactosaminyl *N*-Boc-sphingosine, lactose, allyl *β*-lactoside and phenylthio-*β*-lactoside, and lactosides with a long aliphatic chain were accepted as weak substrates. The mammalian sialyltransferase,[15b] however, cannot accept these monosaccharide derivatives, nor can the lactose derivatives. Among these, α-galactosyl sphingosine is the weakest substrate and is impractical for preparative synthetic application. The phenylthio sialyllactoside prepared here, however, can be used directly for the synthesis of such gangliosides or for condensation with another saccharide fragment since it can be easily protected and activated as a glycosyl donor in chemical glycosylation.

Additional preparative enzymatic syntheses were then carried out with use of allyl 6-*O*-sulfo-*N*-acetyllactosaminide (**7**) and phenylthio lactoside (**28**) as substrates, and 6-*O*-sulfo-sialyl-*N*-acetyllactosaminide (**27**) and phenylthio sialyllactoside (**29**) were isolated in quantitative and 63% yield, respectively. The chemical shift of δ 4.11 is characteristic of the H-3 of the galactosyl residue.[19] In addition, compounds **8**−**11** were also sialylated enzymatically on a preparative scale as shown in the previous communications.[15] It is noted that our attempts to sialylate compounds **7**−**11** and **28** with the mammalian sialyltransferase from rat liver failed.[15b]

Overall, as shown in Table 2, *Neisseria gonorrheae* α-2,3-sialyltransferase can sialylate *N*-acetyllactosamine, lactose, *β*-galactoside, and sulfated *N*-acetyllactosamine derivatives as well as their sphingolipid and sulfo-peptide conjugates. The compounds prepared in this study are biologically interesting and synthetically challenging.[1a,6] The sulfate-containing derivatives have not been prepared by any other sialyltransferase. With regard to other microbial sialyltransferases, the most studied is the one from *N. meningitidis* which has been shown to accept several galactooligosaccharides.[14a] Another potentially useful sialyltransferase is the one from myxoma virus,[14c] which accepts ∼6 galactosaccharides, including Lewis x, but monosaccharides are not substrates. Both of these sialyltransferases have not been shown to sialylate any glycolipids, sulfo-glycopeptides, and sulfo-oligosaccharides. Thus, compared to other known mammalian and microbial α-2,3-sialyltransferases, this enzyme appears to exhibit a broader acceptor specificity.

CMP-Neuraminic Acids as Donors. Cytidine-5′-monophospho-neuraminic acid (CMP-Neu5Ac) derivatives, especially those with a change of the NHAc group, were first examined as donor substrates since several 5-modified neuraminic acids exist in nature, i.e., *N*-glycolylneuraminic acid (Neu5Gc) and 2-keto-3-deoxy-nonulosonic acid (KDN), which carries an OH group instead of NHAc.[20] The unusual ganglioside *N*-glycolyl-GM3 was found in breast tumors and might be useful for immunological diagnosis and vaccine therapy.[21] Syntheses of these derivatives were exploited chemically and enzymatically by using CMP-neuraminic acid synthase. The synthesis of Neu5Gc and *N*-*tert*-butoxycarbonylamino-neuraminic acid (Neu5Boc) was first carried out from thioglycoside **30** (Scheme 5). We chose Neu5Boc as a candidate as it can be easily converted to other derivatives through exchange of the Boc group. To prepare this compound, the *N*-acetyl group of Neu5Ac has to be removed first. The procedures for the de-*N*-acetylation of Neu5Ac were fully documented in the literature,[22−30] and

(19) Sabesan, S.; Paulson, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 2068−2080.

(20) Varki, A. *Glycobiology* **1992**, *2*, 25−40.

(21) Marquina, G.; Waki, H.; Fernandez, L. E.; Kon, K.; Carr, A.; Valiente, O.; Perez, R.; Ando, S. *Cancer Res.* **1996**, *56*, 5165−5171.

(22) Roy, R.; Pon, R. A. *Glycoconj. J.* **1990**, *7*, 3.

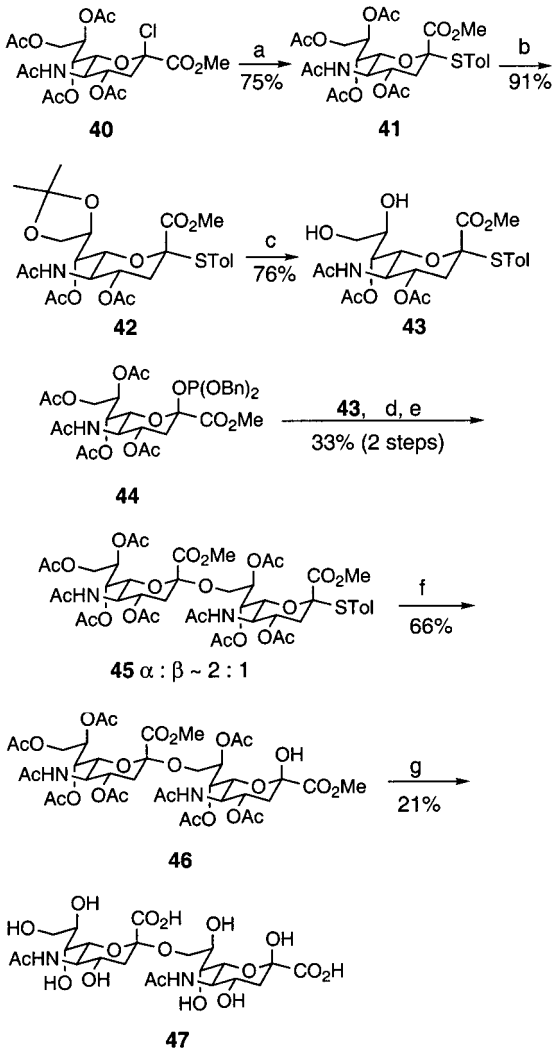
(23) Tanaka, M.; Kai, T.; Sun, X.-L.; Takayanagi, H.; Uda, Y.; Furuhata, K. *Chem. Pharm. Bull.* **1995**, *43*, 1844−1848.

among these conditions, the amide to carbamate conversion reported by Burk et al. seemed compatible with the acetyl and methyl ester protecting groups.[30] As shown in Scheme 5, introduction of the Boc group to **30** with di-*tert*-butyl dicarbonate (Boc_2O) and 4-(dimethylamino)pyridine (DMAP) in refluxing THF gave the *N*-acetyl-*N*-Boc derivative. The *N*-acetyl group was removed with hydrazine hydrate and MeOH at room temperature. Since partial de-*O*-acetylation was observed along with de-*N*-acetylation, the mixture of products was reacetylated to give the *N*-Boc derivative **31** in 72% yield. Deprotection of the Boc group was carried out by using TMSCl-PhOH in CH_2Cl_2[31] to give the free amine derivative with minimal acyl migration after short column chromatography to remove phenol. Acylation with acetoxyacetyl chloride and DABCO gave the fully protected Neu5Gc derivative **32** in 90% yield. Hydrolysis of the thioglycoside with NBS in aqueous acetone gave hemiacetal **33** in 98% yield. Deacetylation followed by saponification gave Neu5Gc (**34**) in 33% yield after purification by DEAE-sephadex A-25 chromatography. Neu5Boc was also prepared in the same way from thioglycoside **31**. Hydrolysis with NBS in aqueous acetone gave **35** in 81% yield. Another two-step deprotection gave Neu5Boc in 56% yield.

5-Azido-neuraminic acid ($Neu5N_3$)[32−34] and KDN were prepared with Neu5Ac aldolase. 2-Azido-mannose (**37**) was prepared from D-mannosamine hydrochloride via diazotransfer[35] in 80% yield. The Neu5Ac aldolase reaction with 4 equiv of sodium pyruvate without buffer[34] worked out very well to give $Neu5N_3$ (**38**) in 80% yield after purification by DEAE-sephadex A-25 chromatography. KDN (**39**) was prepared from D-mannose in quantitative yield with the same enzyme.[33] Alternatively, **37** was generated in situ from 2-azidoglucose at pH 11. Without isolation, the mixture was adjusted to pH 7.5 followed by addition of the aldolase and 4 equiv of pyruvate. This procedure is interesting as 2-azido-glucose is inexpensive and is more easily epimerized than *N*-acetyl-glucosamine.

The *N*-acetylneuraminic acid dimer with α(2→9) linkage [Neu5Acα(2→9)Neu5Ac (**47**)] was synthesized as shown in Scheme 6. Chloride **40** was converted to the α-thioglycoside with use of 4-methylthiophenol and benzyltriethylammonium chloride in 1 M NaOH and $CHCl_3$.[36] Vigorous stirring for 30 min under reflux gave the desired α-thioglycoside **41** in 75% yield. The acetyl groups were removed by treatment with methanolic sodium methoxide, and then the 8- and 9-OH groups were protected as an isopropylidene acetal.[37] The remaining free OH groups were acetylated, subsequently, to give the fully protected **42** in 91% yield. Acid hydrolysis of the 8,9-isopropylidene group in 90% acetic acid at 70 °C gave diol **43** in 76% yield. Sialylation of the diol **43** with sialyl phosphite **44** in acetonitrile[38] in the presence of molecular sieves AW-300 gave the 2→9 linked disaccharide as an anomeric mixture (α:β ∼ 2:1). After acetylation, the α-linked disaccharide **45** was isolated in 33% yield. Hydrolysis of the thioglycoside with NBS in aqueous acetone gave the hemiacetal **46** in 66% yield. Deacetylation and subsequent saponification gave Neu5Acα-(2→9)Neu5Ac (**47**) in 21% yield.

Scheme 6[a]



[a] Reagents and conditions: (a) TolSH/NaOH/ $BnEt_3NCl/CHCl_3$; (b) (i) NaOMe/MeOH, (ii) $Me_2C(OMe)_2$/CSA/acetone, (iii) Ac_2O/Py; (c) 90% AcOH/70 °C; (d) TMSOTf/MS AW-300/MeCN/−40 °C; (e) Ac_2O/Pyr; (f) NBS/ aqueous acetone; (g) (i) NaOMe/MeOH, (ii) NaOH.

Synthesis of CMP-Neu5Ac Derivatives. Enzymatic synthesis of CMP-Neu5Ac derivatives with the commercially available *E. coli* CMP-Neu5Ac synthetase[39,40] was first examined. The Neu derivative (6 mM) and CTP (10 mM) were dissolved in 100 mM Tris-HCl buffer (pH 9.0) in the presence of $MgCl_2$ (20 mM), and the solution was mixed with CMP-Neu5Ac synthetase and inorganic pyrophosphatase and gently stirred at 25 °C for 15 h. The product formation was analyzed by anion-

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(35) Alper, P. B.; Hung, S.-C.; Wong, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6029−6032.
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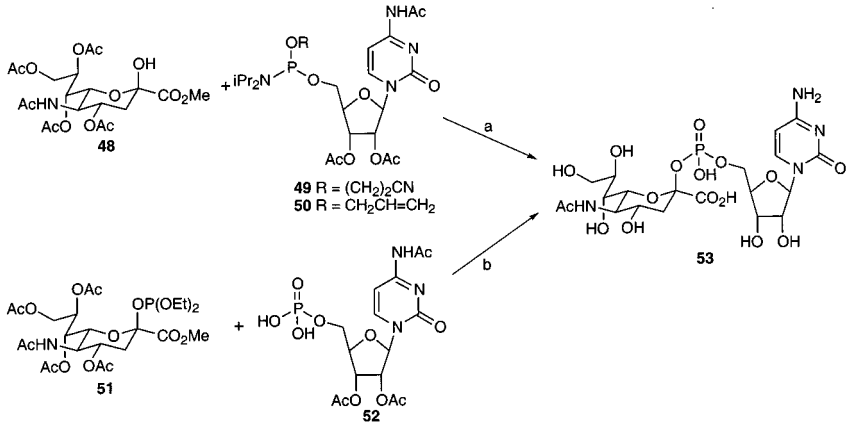
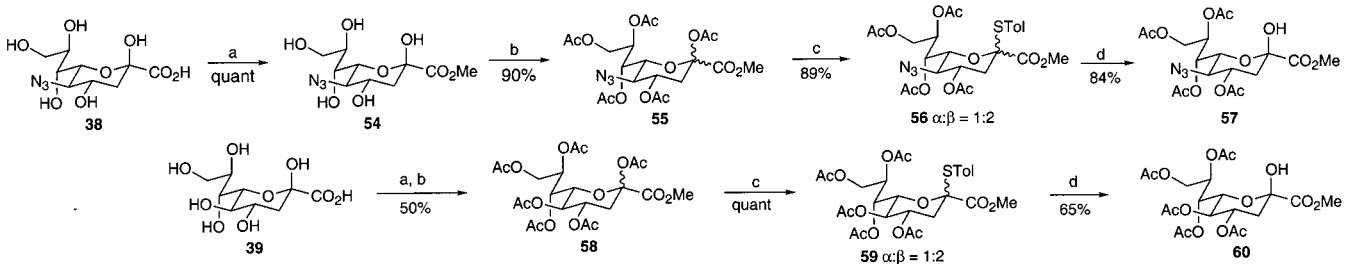
(40) Lin, J.; Shen, G.-J.; Ichikawa, Y.; Rutan, J. F.; Zapata, G.; Vann, W. F.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 3901−3910.

Scheme 7[a]



[a] Reagents and conditions: (a) (i) 1*H*-tetrazole/MeCN, (ii) tBuOOH/MeCN or DMD/CH_2Cl_2, (iii) DBU/THF or $(Ph_3P)_4Pd$/$^iPr_2NH$/MeCN, (iv) NaOMe/MeOH, (v) NaOH; (b) (i) DMF/MeCN, (ii) NaOMe/MeOH, (iii) NaOH.

Scheme 8[a]



[a] Reagents and conditions: (a) MeOH/Dowex (H^+); (b) Ac_2O/Py; (c) TolSH/BF_3·OEt_2/CH_2Cl_2; (d) NBS/aqueous acetone.

exchange HPLC with UV detection at 270 nm. To our surprise, Neu5Gc, Neu5Boc, $Neu5N_3$, KDN, and Neu5Acα(2→9)-Neu5Ac did not show any new product formation, with the exception of Neu5Ac, which gave CMP-Neu5Ac. Although this enzyme can accept Neu derivatives with modification at the 9-position with a large aromatic group,[41] it can neither accept the 9-phosphonate analogue of Neu5Ac[42] nor the disaccharide Neu5Acα(2→9)Neu5Ac.

Since enzymatic synthesis of CMP-Neu5Ac derivatives was unsuccessful, chemical synthesis of CMP-Neu5Ac and its analogues was carried out (Scheme 7). Coupling of a protected sialic acid hemiacetal (**48**) with a protected cytidine 5′-phosphoramidite (**49**, **50**) was first carried out with use of the 2-cyanoethyl phosphite[43] or the allyl phosphite method.[44] Another approach based on sialic acid phosphite (**51**) and protected cytidine 5′-monophoshoric acid (**52**)[45] was also examined. Both methods worked well for the preparation of CMP-sialic acid **53**. We then proceeded to prepare analogues.

Treatment of $Neu5N_3$ (**38**) with MeOH and an acidic resin gave methyl ester **54** in good yield (Scheme 8). Acetylation of **54** with acetic anhydride and perchloric acid,[46] however, gave a mixture of 2,3- and 3,4-ene compounds. Methyl ester **54** was then acetylated with acetic anhydride and pyridine to give peracetate **55**,[47] which was converted to the thioglycoside **56** with 4-methylthiophenol and BF_3·OEt_2 in 89% yield. Hydrolysis of thioglycoside **56** with NBS in aqueous acetone gave hemiacetal **57** in 84% yield. The hemiacetal of KDN (**60**)[48] was prepared from **59** in a similar manner.

As shown in Scheme 9, Neu5Ac, Neu5Gc, Neu5Boc, and $Neu5N_3$ react with the amidite to give their corresponding products in 25%, 27%, 26%, and 9% yield, respectively, but the KDN and Neu5Acα(2→9)Neu5Ac derivatives did not give the desired products. The hemiacetals of Neu5Ac, Neu5Boc, and KDN were then phosphitylated with diethyl chlorophosphite in the presence of DIEA to give the glycosyl phosphites in 93% and 51% yield, respectively. Phosphitylation of disaccharide **46** under the same conditions gave no reaction and only 2,3-elimination was observed with an increase of chlorophosphite and DIEA. On the other hand, phosphitylation of **46** with dibenzyl diethylphosphoramidite and 1*H*-tetrazole gave the desired disaccharide glycosyl phosphite **69** in good yield. Coupling of the phosphite of Neu5Ac, Neu5Boc, KDN, or Neu5Acα(2→9)Neu5Ac with triacetylcytidine 5′-phosphoric acid (**52**) gave the corresponding protected CMP-Neu5Ac derivatives in 65%, 59%, 39%, and 47%, respectively. Deprotection of each CMP-Neu5Ac derivative with methanolic sodium methoxide and subsequent saponification gave the desired CMP-Neu derivative after purification by gel permeation chromatography and lyophilization.

Donor Substrate Specificity. The synthetic CMP-Neu5Ac derivatives were tested as donor for the sialyltransferase reaction. A CMP-Neu5Ac derivative and phenylthio lactoside or methoxycarbonylpentyl *N*-acetyllactosaminide were incubated with the α-2,3-sialyltransferase in 0.1 M HEPES buffer (pH 7.5) in

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(44) Chappell, M. D.; Halcomb, R. L. *Tetrahedron* **1997**, *53*, 11109−11120.
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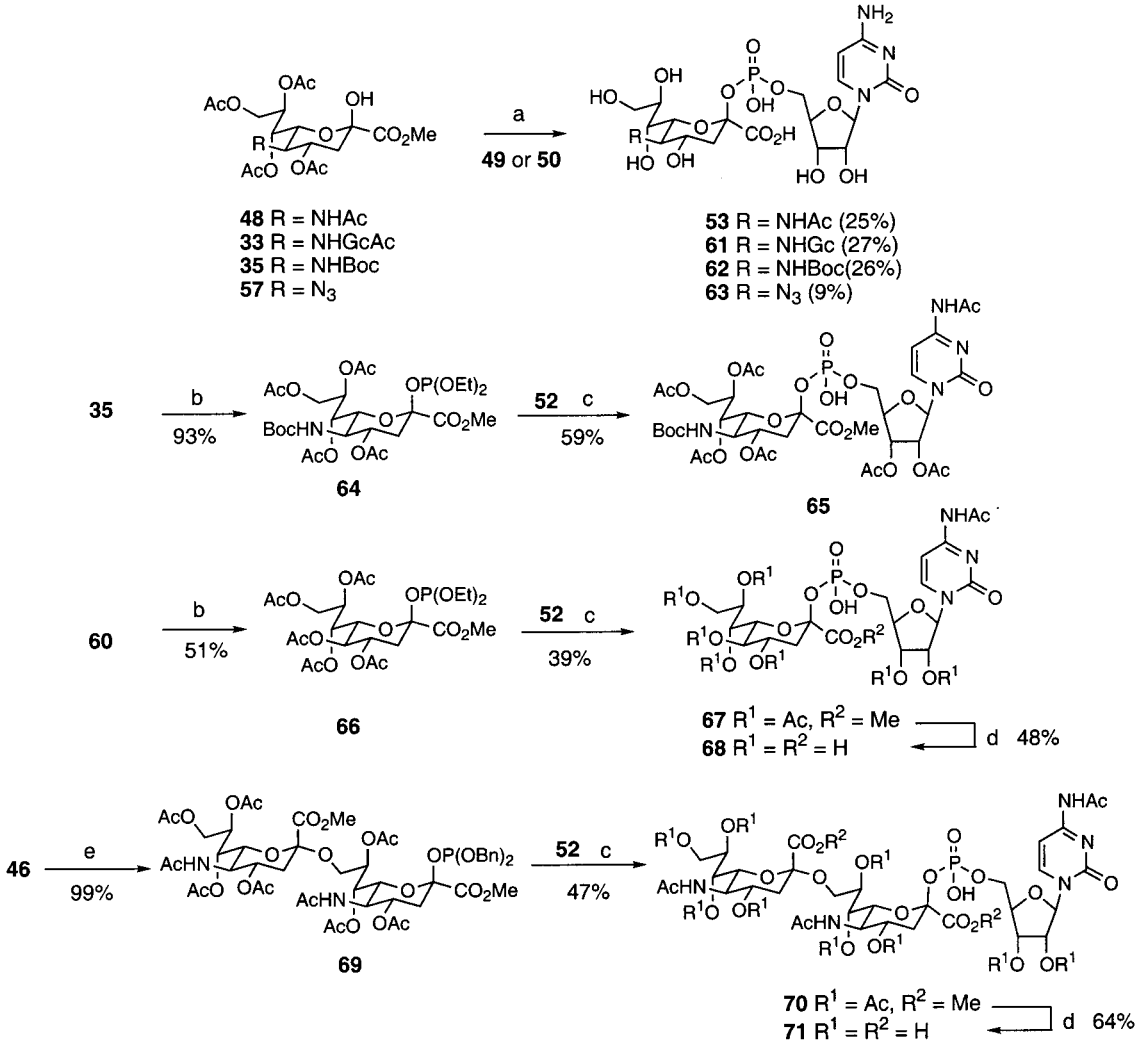
(48) Kajihara, Y.; Akai, S.; Nakagawa, T.; Sato, R.; Ebata, T.; Kodama, H.; Sato, K. *Carbohydr. Res.* **1999**, *315*, 137−141.

Scheme 9[a]



[a] Reagents and conditions: (a) (i) 1*H*-tetrazole/MeCN, (ii) tBuOOH/MeCN or DMD/CH_2Cl_2, (iii) DBU/ THF or $(Ph_3P)_4Pd$/$^iPr_2NH$/MeCN, (iv) NaOMe/MeOH, (v) NaOH; (b) $ClP(OEt)_2$/$^iPr_2NEt$/MeCN; (c) DMF/MeCN/−20 °C to room temperature; (d) (i) NaOMe/MeOH, (ii) NaOH; (e) $(BnO)_2PNEt_2$/1*H*-tetrazole/MeCN.

the presence of 10 mM $MgCl_2$, 0.2 mM DTT, and alkaline phosphatase at 25 °C for 2 days. The sialyloligosaccharide was isolated from the reaction mixture with use of a SepPak 18 solid-phase extraction cartridge and was examined by using anion-exchange HPLC and/or electrospray mass spectrometry (ESI-MS) in a negative mode. Under the assay conditions, CMP-Neu5Ac gave quantitatively the sialylated product. CMP-Neu5Gc, CMP-Neu5Boc, and CMP-KDN gave a detectable product by HPLC and ESI-MS, and most of the acceptor was unreacted. No detectable sialylated oligosaccharide was produced with CMP-$Neu5N_3$ and CMP-Neu5Acα(2→9)Neu5Ac. These results indicate that the sialyltransferase is quite specific for CMP-Neu5Ac as the donor substrate, similar to other known sialyltransferases.

In conclusion, the recombinant α-2,3-sialyltransferase from *Neisseria gonorrheae* was overexpressed in *E. coli* and purified by Ni-agarose affinity chromatography for the study of its synthetic utility. Chemo-enzymatic synthetic methods have also been developed to prepare potential substrates for the sialyltransferase. The enzyme was shown to catalyze the transfer of sialic acid to galactose and *N*-acetylgalactosamine with a variety of aglycon; to lactose, *N*-acetyllactosamine, and the glycolipid derivatives; to sulfated *N*-acetyllactosamine, 6-*O*-sulfo-chitotriose, and tetrose; and to tyrosine-sulfate containing glycopeptides and lactosides with a short aliphatic or aromatic aglycon. The α-linked galactosides with a long aliphatic aglycon are poor acceptors. Regarding the donor substrate specificity, the enzyme accepts Neu5Ac as an excellent substrate; Neu5Ac, Neu5Gc, Neu5Boc, and KDN are very weak substrates; $Neu5N_3$ and Neu5Acα(2→9)Neu5Ac are not substrates.

The sulfate-containing sialylated saccharides and conjugates prepared in this study are potentially useful as selectin blockers or as immunomodulators.[1a] These compounds are difficult or impractical to synthesize by other means, as the sulfate group is sensitive to acids and cannot sustain the condition of protecting group manipulation.[15b,49] In addition, enzymatic sialylation is stereospecific and free of protecting group manipulation and thus more practical than the chemical counterpart.[6] Compared with other sialyltransferases, this enzyme exihibits a broader substrate specificity and is the most versatile and synthetically useful sialyltransferase demonstrated to date. Work is in progress to use this expression system to prepare the enzyme for X-ray crystal structural study and to engineer the enzyme specificity.

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Experimental Section

Cloning of α-2,3-Sialytransferase from *Neisseria gonorrheae*. The DNA isolated from *N. gonorrheae* (ATCC 33084) was used for amplification of the gene coding for the α-2,3-sialyltansferase. The PCR was performed in a 100 μL reaction mixture containing 100 ng of DNA as template, 300 nmol of primers, 200 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and 1 U of Tag DNA polymerase. The forward primer (5-ATATTCATATGGGGTTGAAAAAAGTCTGTTT) containing an NdeI site and the reverse primer containg a HindIII site with the six His tag sequence (5-GGCGCCGAAGCTTAGTGGTGATGGTGGT-GAGCTTGTCATCGTCATCATTTTTATCGTCAAATGT) were used. The amplification was carried out in the following conditions: 94 °C, 1 min; 55 °C, 1 min; and 72 °C, 2 min for 35 cycles. The amplified fragment (1.2 Kb) was subcloned into pET23a vector (Novagen Co. Madison, WI). The ligation mixture was then transformed into the BL21(DE3) strain of *E. coli*. The insert was further confirmed by restriction enzyme digestion (NdeI and HindIII restricted cutting) and sequence analysis at the TSRI Core facility.

Preparation of α-2,3-Sialyltransferase. The transformed cells were inoculated in LB medium containing 30 μg/mL Kanamycin at 37 °C until OD600 reached 0.7−0.9 (about 2−3 h), then IPTG (1 mM) was added. The culture was then cultivated at lower temperature (25−30 °C) and incubation was continued overnight (18 h). The cells were harvested by centrifugation at 5000 × g for 5 min at 4 °C. The cell paste (3.2 g/L, wet cell) was suspended in a Tris-HCl buffer (20 mM, pH 7.8) containing 0.3 M NaCl, 1% Triton X-100, and 5 mM imidazole. Cells were then lysed three times by using a French press (Aminco Co., 1500 psi). The cell debris was removed by centrifugation at 27000 × g and the supernatant was collected and filtered through a 0.45 μm Millipore filter. The cleared lysate supernatant was loaded onto an equilibrated Ni-NTA agarose column and washed with a buffer containing 30 mM imidazole. The protein was eluted with 100 and 180 mM imidazole and was analyzed by 12% SDS-PAGE gel. The partially purified protein was dialyzed (overnight at 4 °C) against 4 L of cold Tris-HCl buffer (25 mM, pH 7.6) containing 0.2% Triton X-100, 10 mM $MgCl_2$ and 1 mM DTT.

Allyl *O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-2-acetamido-2-deoxy-6-*O*-sulfo-β-D-glucopyranoside (27). A solution of **7** (10 mg, 19 mmol) and CMP-Neu5Ac (21 mg, 23 mmol) in 100 mM HEPES buffer (pH 7.5, 3.8 mL) containing 20 mM $MgCl_2$, 0.2 mM DTT, 10 U alkaline phosphatase, and *Neisseria* α(2,3)-sialyltransferase was incubated for 2 days at 25 °C. TLC analysis (n-PrOH−H_2O−NH_4OH 7/2/1) showed that all donor was consumed. The solution was directly applied to a column of DEAE-sephadex (AcO^-), washed with water, and the product was eluted with 0.2 M NH_4OAc. Fractions containing the product were collected and passed through a column of Dowex HCR (H^+). The residue was concentrated and purified by size-exclusion chromatography (BioGel P4, 0.03 M NH_4HCO_3) and lyophilized to give **27** (18 mg, quant) as a white foam: ^{1}H NMR (600 MHz, D_2O) δ 5.92−5.85 (1H, m), 5.31−5.24 (2H, m), 4.60 (1H, d, J = 7.9 Hz), 4.58 (1H, d, J = 8.3 Hz), 4.40 (1H, d, J = 11.0 Hz), 4.34−4.31 (2H, m), 4.17−4.14 (1H, m), 4.11 (1H, dd, J = 3.1, 9.7 Hz), 3.95 (1H, d, J = 3.1 Hz), 3.92−3.58 (14H, m), 3.54 (1H, dd, J = 7.9, 9.7 Hz), 2.73 (1H, dd, J = 4.8, 12.3 Hz), 2.02 (6H, s, Ac×2), 1.80 (1H, t, J = 12.3 Hz); HR-MALDI−FTMS calcd for $C_{28}H_{44}N_2O_{22}SNa$ [M − 2H + Na]$^-$ 815.2004, found 815.1973.

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Supporting Information Available: Procedures for enzyme assay, synthesis, and characterization of compounds **2**−**7**, **12**−**47**, and **54**−**71**, and general procedures for the preparation of CMP-sialic acid derivatives (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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