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Characterization of inhibitory activities and binding mode of synthetic 6′-modified methyl *N*-acetyl-β-lactosaminide toward rat liver CMP-D-Neu5Ac: D-galactoside-(2 → 6)-α-D-sialyltransferase

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ABSTRACT

6′-Deoxy (**12**), 6′-thio (**13**), and 6′-*O*-tetrahydropyranosyl (**14**) analogues of methyl *N*-acetyl-β-lactosaminide (**3**), were synthesized from lactose. NOE experiments proved that they adopt the same conformation as that of **3**. Inhibition studies using these synthetic analogues, including the disulfide dimer **15**, toward (2 → 6)-α-sialyltransferase (EC 2.4.99.1) revealed that the 6′-deoxy analogue **12** had remarkable inhibitory activity as the first acceptor-analogue inhibitor for this enzyme. It is noteworthy that the disulfide **15** also behaves as an inhibitor. The results indicated that chemical modification at the 6′-position of **3** did not cause much decrease in the binding affinity to the sialyltransferase. Further, a novel possibility that the acceptor and the acceptor-analogue inhibitor can bind simultaneously to the sialyltransferase was proposed based on the inhibition studies with **12** and CMP.

INTRODUCTION

Glycosyltransferases catalyze transfer of an activated glycosyl residue in a sugar nucleotide to an acceptor, and have strict specificities for both donor and acceptor. Although several glycosyltransferases have been cloned[1] and expressed in *E. coli*[2], their recognition domains of the substrates, that is, binding sites of the glycosyl donor as well as the glycosyl acceptor, have not been elucidated. On the other hand, studies on inhibitory activities of several acceptor analogues recently reported by Hindsgaul et al.[3] seem to give a clue for the recognition of the glycosyl acceptors. According to the inhibitory activities of their deoxygenated analogues in

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which the hydroxyl group to be glycosylated was removed, glycosyltransferases were divided into the following two groups. In the first group the hydroxyl group to be glycosylated is essential for the binding. In contrast to this, the second group does not require the hydroxyl function for binding.

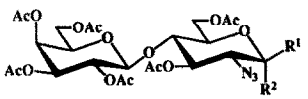
CMP-β-D-Neu5Ac: D-galactoside-(2 → 6)-α-D-sialyltransferase (EC 2.4.99.1) is responsible for the construction of the Neu5Acα2 → 6Gal sequence with strict acceptor specificity[4]. The best acceptor of this enzyme is the β-D-Gal-(1 → 4)-GlcNAc unit of the *N*-linked complex oligosaccharide. However, the same disaccharide unit of the *O*-linked oligosaccharide was shown to be a poor acceptor. In relation to the difference in acceptor specificity, we were interested in the biochemical behavior of 6′-modified β-D-Gal-(1 → 4)-GlcNAc analogues. The inhibitory activities of the substrate analogues are valuable in order to investigate the binding or recognition mechanism of the enzyme. The acceptor-analogue inhibitors of fucosyltransferases[3], glucosaminyltransferases[3], and galactosyltransferase[5] have been reported. Simple nucleotides such as CDP and CMP are known as inhibitors of sialyltransferases[6,7]. Further, a derivative of 5′-*O*-(*N*-acetyl-α-neuraminyl)-5-fluorouridine was reported to inhibit sialyltransferase activity of intact cells and a cell homogenate of splenic lymphocytes[8]. Although the actually effective form for the last compound is not clear, these are analogues of the donor, CMP-β-D-Neu5Ac. The 6′-modified acceptor analogues of (2 → 6)-α-sialyltransferase, 6′-deoxy (**12**), 6′-thio (**13**), and 6′-*O*-tetrahydropyranyl (THP, **14**) derivatives of *N*-acetyl-β-lactosaminide were synthesized. If (2 → 6)-α-sialyltransferase belongs to the above-described second group, the 6′-deoxy analogue **12** must be a good inhibitor. In contrast to this, if it belongs to the first group, the 6′-SH analogue is expected to behave as either an inhibitor or sialyl acceptor, because the thiol group has a tendency to donate a proton more easily than a hydroxyl group to form a hydrogen bond. The THP residue was introduced in order to elucidate the steric tolerance of the binding site.

Herein we report the synthesis of 6′-deoxy (**12**), 6′-thio (**13**), its disulfide (**15**), and 6′-*O*-THP (**14**) analogues of methyl *N*-acetyl-β-lactosaminide (**3**), and their inhibitory activities toward (2 → 6)-α-sialyltransferase. The binding mode of the most potent inhibitor **12** is also discussed.

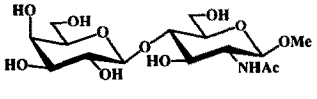
RESULTS AND DISCUSSION

Synthesis of 6′-modified methyl N-*acetyllactosaminides.*—Since the anomeric configuration at the reducing end of β-D-Gal-(1 → 4)-GlcNAc unit in complex oligosaccharides is fixed for the β configuration, we selected, as the starting material, methyl *O*-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (methyl *N*-acetyl-β-lactosaminide, **3**), which was prepared by a slightly modified Lemieux procedure[9]. The β-glycoside was obtained in an excellent yield by treatment of glycosyl chloride **1** with 4–5 equiv of sodium methoxide for a few min instead of the more generally used Koenigs–Knorr method[10].

For its isolation and purification, this methyl glycoside was peracetylated with acetic anhydride and pyridine in the presence of 4-dimethylaminopyridine to give **2** together with its α anomer ($\alpha:\beta$ ratio 1:35) in 89% overall yield. Methyl β-glycoside **2** was further transformed into **3** by reduction of the 2-azido group, followed by *N*-acetylation, and *O*-deacetylation, in 93% overall yield. It was proved that this glycosylation was convenient for the multigram-scale synthesis of **3** without the use of heavy metals or expensive silver salts.



1 R^1= H, R^2= Cl
2 R^1= OMe, R^2= H

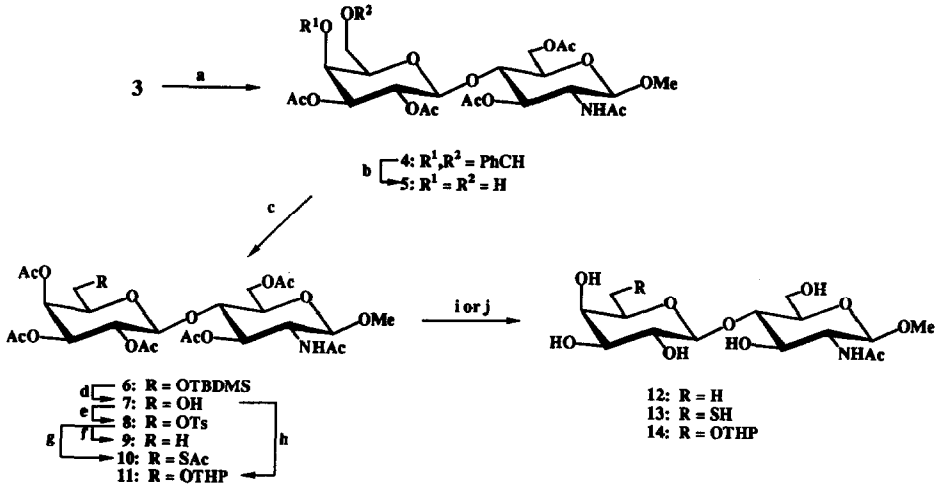


3

The synthesis of 6′-modified analogues of **3** is outlined in Scheme 1. Treatment of **3** with benzaldehyde dimethyl acetal and camphorsulfonic acid, followed by acetylation, gave the 4′,6′-*O*-benzylidene acetal **4** in 89% overall yield. Acetal **4** was hydrolyzed with 60% acetic acid to give a 4′,6′-diol **5** in 98% yield. Selective 6′-*O*-silylation of **5** with *tert*-butylchlorodimethylsilane, followed by 4′-*O*-acetylation, gave **6** in 78% yield. *O*-Desilylation with 60% acetic acid furnished the desired 6′-alcohol **7** and 4′-alcohol in 76 and 20% yields, respectively, the latter was formed by the migration of the 4′-*O*-acetyl group to the 6′-position.

The 6′-alcohol **7** was then smoothly converted to the 6′-tosylate **8** with *p*-toluenesulfonyl chloride in pyridine (77%). For the synthesis of the 6′-deoxy analogue **12**, the 6′-tosylate **8** was treated with sodium iodide, followed by homolytic reduction with tributyltin hydride, which led to the 6′-deoxy derivative **9** in 82% overall yield. Treatment of **7** with 2,3-dihydropyran and pyridinium *p*-toluenesulfonate gave the 6′-*O*-THP derivative **11** (1:1 diastereomeric mixture) in 74% yield. *O*-Deacetylation of **9** and **11** with sodium methoxide gave the desired 6′-deoxy **12** and 6′-*O*-THP analogue **14** in 93 and 86% yields, respectively.

The 6′-SH analogue **13** was also derived from the 6′-tosylate **8**. Treatment of **8** with potassium thioacetate gave **10** in 84% yield. However, *O*-deacetylation of **10** by use of sodium methoxide gave multiple compounds. The 6-thioacetate **10** was smoothly deacetylated with NH_4OH in the presence of DL-dithiothreitol as the reagent of sulfur antioxidation to give pure **13** in 45% yield. The 6′-SH analogue **13** was further transformed to the disulfide tetrasaccharide **15** in water for 72 h at 37°C (83%). This dimerization reaction could be monitored by the ^1H NMR (310 K, D_2O) signals of the 6′-methylene protons. The NMR spectra of pure **15** (top) and of the mixture of **13** and **15** (bottom) are shown in Fig. 1. (These spectra were measured at 303 K, because the HOD signal hid the signals of H-1 and H-1′ at 310 K.) At a concentration of 3 mM, **13** was changed by 20% to the disulfide **15** after 1 h. Under aerobic conditions, the 6′-SH analogue **13** may exist exclusively in the form of the disulfide **15**. Although **13** can be completely transformed into **15**, the lifetime of **13** was long enough for the short period of the sialyltransferase assay.

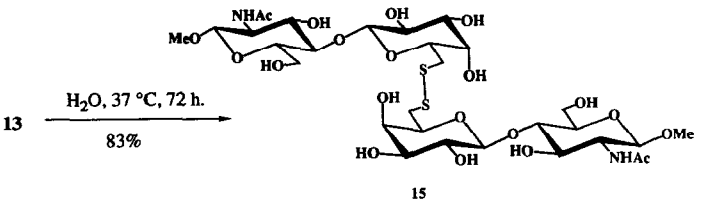


Reagents and conditions:
(a) (1) benzaldehyde dimethyl acetal, CSA, DMF, 50°C; (2) Ac_2O, pyridine, DMAP, 89% from **3**; (b) 60% AcOH, 90°C, 98%; (c) (1) Bu^tSiMe_2Cl, imidazole, DMF; (2) Ac_2O, pyridine, 78% from **5**; (d) 60% AcOH, 80°C, 76%; (e) TsCl, DMAP, pyridine, 50°C, 77%; (f) (1) NaI, dimethoxyethane, 80°C, (2) Bu_3SnH, AIBN, benzene, reflux, 82% from **8**; (g) KSAc, DMF, 80°C, 84%; (h) 2,3-dihydropyran, pyridinium *p*-toluenesulfonate, CH_2Cl_2, 74%; (i) NaOMe, MeOH, 93% (**12**), 86% (**14**); (j) 28% NH_4OH, MeOH, DL-dithiothreitol, 45%.

Scheme 1.

Conformational study of synthetic analogues by NMR spectroscopy.—It is essential to investigate the conformation of synthetic analogues for the evaluation of the relationship between chemical modification at the 6′-position and inhibitory activity. The ^1H NMR signals (500 MHz) of **3** and its analogues (**12**, **13**, and **15**) were assigned by extensive decoupling experiments (Tables I and II).

Lemieux and co-workers reported by HSEA calculation that glycosidic torsional angles of *O*-β-D-galactopyranosyl-(1 → 4)-2-deoxy-2-acetamido-D-glucopyranose were $\phi = 50°$, $\psi = 0°$ (refs. 11 and 12). This conformation was confirmed by ^1H nuclear Overhauser enhancement (NOE) experiments, since a 12% enhancement of the H-4 signal of *N*-acetylglucosamine was observed upon irradiation of the H-1 of the galactoside. The observed coupling constants around the pyranoside rings of compound **12**, **13**, and **15** indicated they adopt almost the same C-1 chair confor-



Scheme 2.

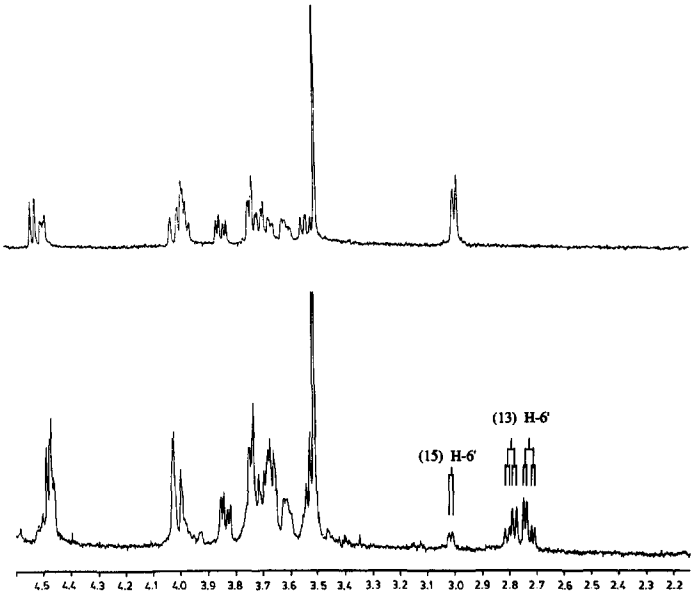


Fig. 1. 500-MHz ^{1}H NMR spectra of the disulfide dimer **15** and the partially dimerized 6′-thio analogue **13**. Top, **15**; bottom, during the dimerization reaction (1 h). Each spectrum was measured in D_2O at 303 K.

mations as the unmodified lactosaminide **3**. However, it was difficult to obtain the exact coupling constants of the 6′-*O*-THP analogue **14** due to the overlapping of diastereomeric ^{1}H NMR signals. In this case the conformation was evaluated only by NOE experiments. Thus NOE experiments were used to confirm the glycosidic

TABLE I

^{1}H NMR chemical shifts of compounds **3**, **12**, **13**, and **15** [a]

H-atom	**3**	**12**	**13**	**15**
1	4.46	4.46	4.46	4.46
2	[b]	3.73	[c]	[f]
3	[b]	3.68	[c]	[f]
4	[b]	3.63	[d]	3.66
5	3.59	3.60	3.60	3.61
6a	4.00	3.98	4.00	3.99
6b	3.83	3.82	3.82	3.83
1′	4.46	4.42	4.47	4.50
2′	3.53	3.49	3.52	3.54
3′	3.66	3.65	[d]	3.69
4′	3.92	3.75	4.01	3.96
5′	[b]	3.82	[e]	3.95
6′a	[b]	1.24	2.80	3.00
6′b	[b]		2.73	
OMe	3.50	3.50	3.50	3.50
NAc	2.03	2.03	2.03	2.03

[a] At 500 MHz, in ppm using TPS as an external standard in D_2O at 303 K. [b] 3.79–3.68, [c] 3.80–3.73, [d] 3.71–3.65, [e] 3.80–3.65, [f] 3.73–3.71.

TABLE II

^{1}H NMR spin–spin coupling constants (Hz) of compounds **3**, **12**, **13**, and **15** [a]

J_{H-H}	**3**	**12**	**13**	**15**
1–2	7.9	8.0	ND	ND
2–3	ND	8.0	ND	ND
3–4	ND	7.6	ND	ND
4–5	ND	7.8	ND	ND
5–6a	2.1	1.9	< 0.2	< 0.2
5–6b	5.2	6.3	5.2	5.5
6a–6b	12.2	12.2	12.7	12.6
1′–2′	8.0	8.0	7.7	8.2
2′–3′	9.9	9.8	8.7	10.2
3′–4′	3.4	3.6	ND	3.2
4′–5′	< 0.2	< 0.2	< 0.2	< 0.2
5′–6′a	ND	6.3	7.2	6.5
5′–6′b	ND		6.9	
6′a–6′b	ND		13.9	

[a] ND, Not detected.

torsional angle of our synthetic analogues, and the results are summarized in Table III.

The same magnitude of enhancement (9–16%) of the H-4 signals from the *N*-acetylglucosaminide moiety was observed for compounds **12**, **13**, and **14**. These data indicated that the torsional angle of three analogues are similar to **3**, namely, that the 6′-substitution had no significant effect on the conformation of the glycosidic linkage and pyranose rings. The glycosidic torsional angles of these three analogues may be around $\phi = 50°$ and $\psi = 0°$. In contrast to this, in the case of disulfide dimer **15**, NOE experiments were not successful. Thus the phase-sensitive 2D rotating frame nuclear Overhauser enhancement (ROESY) technique was used for this compound. Upon irradiation of the H-1 of the galactoside, the H-3 and H-5 signals of *N*-acetylglucosaminide were strongly enhanced, while H-4 showed weak enhancement. These ROESY data indicated that the glycosidic torsional angles of **15** were different from the other three analogues.

Inhibition studies of synthetic analogues.—The inhibitory activities of the synthetic acceptor analogues **12–15** toward (2 → 6)-α-sialyltransferase (rat liver) were

TABLE III

NOEs observed upon saturation of the H-1 resonance of compounds **3** and **12–14** [a]

Compound	Observed NOE (%) [b]		
	H-3′	H-5′	H-4
3	16	[c]	16
12	7	7	9
13	5	[c]	10
14	[c]	[c]	12

[a] NOE experiments were performed at 310 K at 500 MHz. [b] The observed NOEs for these signals were positive. [c] Not determined.

TABLE IV

Inhibition assay of methyl *N*-acetyl-β-lactosaminide analogues toward α-(2 → 6)-sialyltransferase

Compound		K_i (mM) [a] (inhibition mode)	Relative activity
6′-deoxy	**12**	0.76 (mixed) [b]	5.4
6′-SH	**13**	3.78 (mixed)	1.1
6′-*O*-THP	**14**	4.14 (mixed)	1.0
6′-S-S	**15**	2.00 (mixed)	2.0

[a] K_m = 0.90 mM for methyl *N*-acetyl-β-lactosaminide (**3**). [b] ±0.063.

evaluated according to the assay method of Paulson et al.[6]. In preliminary inhibition studies with the 6′-deoxy analogue **12** using methyl *N*-acetyl-β-lactosaminide **3** (0.5 mM) as acceptor, the fractional inhibition at 0.5 mM was 38%. In the cases of the 6′-SH (**13**) and 6′-*O*-THP (**14**) analogues, 40% inhibition was observed at 2 mM. The inhibition constants (K_i) of **12–15**, as well as the K_m value of **3**, were obtained as previously described[5]. The inhibitory activity of **12**, having the smallest K_i value (as shown in Table IV), is five times as high as those of **13** and **14**. For several known acceptor analogues which inhibit glycosyltransferases, the K_i values were larger than the K_m values[3] of the corresponding acceptors ($K_i/K_m > 1.8–7$)[13]. It is noteworthy that the remarkable inhibitory activity of **12** was characterized as the lowest K_i/K_m ratio (0.8).

Further, even **14** and **15**, which have bulky substituents at the C-6′ position, showed no remarkable decrease in activity. This fact indicates a rather large stereochemical latitude around the C-6′ position, or more exactly, around the galactose moiety in the acceptor-binding site of the (2 → 6)-α-sialyltransferase. This may explain the acceptor specificity, where the enzyme recognizes the β-D-Gal-(1 → 4)-GlcNAc residue, located not only at terminal, but also at internal positions of the oligosaccharide chain.

In addition to the relative ratios of K_i/K_m, the difference was also observed in the inhibition mode of the (2 → 6)-α-sialyltransferase. The known acceptor-analogue inhibitors toward (1 → 2)-α-L- and (1 → 4)-α-L-fucosyltransferases[3], two kinds of (1 → 6)-β-D-glucosaminyltransferases (V and mucin core-2)[3], and (2 → 4)-β-D-galactosyltransferase[5] showed competitive modes of action when the corresponding glycosyl acceptors were varied. Further, Paulson et al.[6] observed competitive inhibition for the (2 → 6)-α-sialyltransferase from bovine colostrum by varying the asialo-α_1-acid glycoprotein in the presence of *N*-acetyllactosamine. Considering the similarity[6] in the substrate specificity as well as the physicochemical behavior of two (2 → 6)-α-sialyltransferases from bovine colostrum and rat liver, the same inhibition mode can be expected. However, the observed inhibition mode of the sialyltransferase from rat liver was "mixed", when the concentration of **3** was varied in the presence of the acceptor analogues **13–15**, as shown in Fig. 2a for the case of **12**.

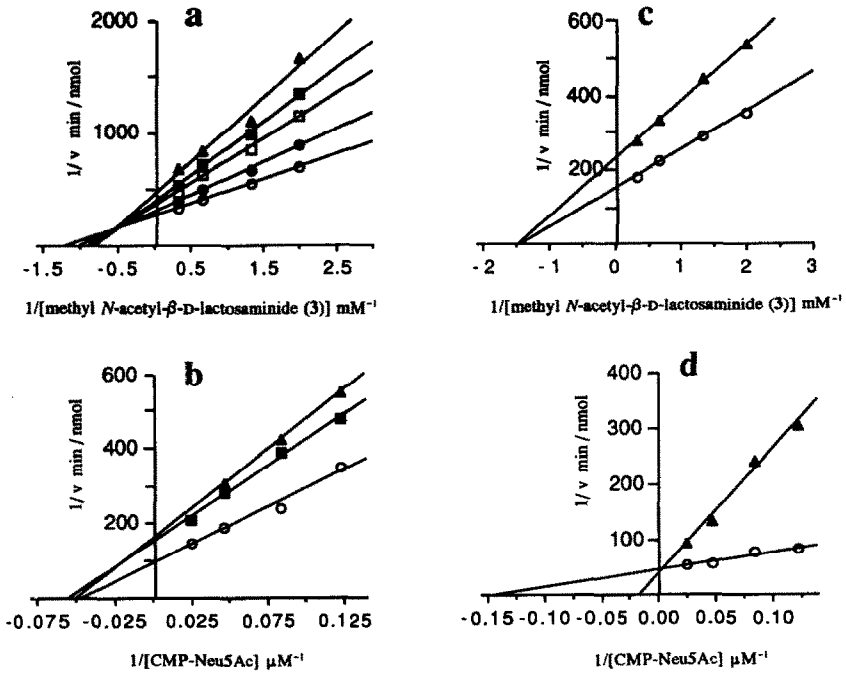


Fig. 2. Inhibition of sialyltransferase by the 6′-deoxy analogue **12** and CMP. (a) With **3** and **12** as varied substrate and inhibitor: ○, 0, ●, 250; □, 500; ■, 750; ▲, 1000 μM. CMP-Neu5Ac was used at a fixed concentration of 2.5 μM. Mixed inhibition with respect to **3**. (b) A fixed concentration of **3** (4.5 mM) was used with CMP-Neu5Ac and **12** as varied substrate and inhibitor: ○, 0; ■, 1; ▲, 2 mM. Noncompetative inhibition with respect to CMP-Neu5Ac. (c) With **3** and CMP as varied substrate and inhibitor: ○, 0; ▲, 6 μM. CMP-Neu5Ac was used at a fixed concentration of 2.5 μM. Noncompetative inhibition with respect **3**. (d) A fixed concentration of **3** (4.5 mM) was used with CMP-Neu5Ac and CMP as varied substrate and inhibitor: ○, 0; ▲, 60 μM. Competative inhibition with respect to CMP-Neu5Ac

An equilibrium random-order mechanism was proposed for the sialyltransferase from bovine colostrum[6]. In addition CMP was shown to inhibit the sialyl transfer noncompetitively when acceptor **3** was varied (Fig. 2b), indicating that the binding of CMP or CMP-Neu5Ac did not disturb that of **3**. An equilibrium random-order mechanism, in which an inhibitor competes with one substrate, is shown in Fig. 3 by the solid line[14a], but as a matter of course, this is not consistent with mixed inhibition[14b]. One possible explanation of the observed mixed inhibition is depicted with dotted lines in Fig. 3. These additional equilibria indicate that the accepter **3** and the inhibitor bind simultaneously to separate sites of the enzyme[14c]. In this model the equilibria are simplified assuming that the inhibitor has no effect on the binding of the donor and that EDAI is catalytically inactive.

Based on the velocity equation (eq *1*), the eqs *2* and *3* were derived[14c] for varied acceptor (lactosaminide) and varied donor (CMP-Neu5Ac), respectively. Equation *2*, where both the intercept and slope are linear with respect to [I] and α

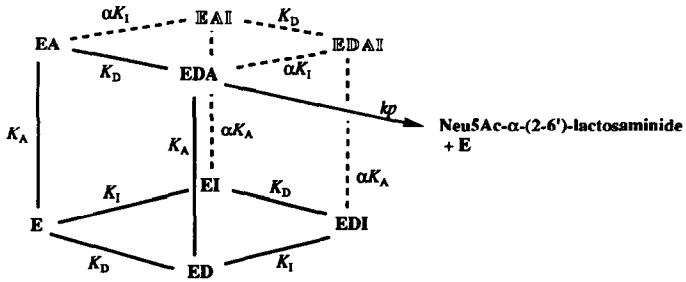


Fig. 3. Symbols used are defined as: E, enzyme; A, lactosaminide; D, CMP-Neu5Ac; I, inhibitor H-6 β-D-Gal-(1 → 4)-β-D-GlcNAc-(1-O)-Me; K, equilibrium constant.

$$v = \frac{\alpha K_I DIV_{max}}{\alpha K_I K_D K_A + \alpha K_I K_A D + \alpha K_D K_A I + \alpha K_A ID + \alpha K_I K_D A + \alpha K_I DA + K_D AI + DAI} \quad (1)$$

For lactosaminide

$$\frac{1}{v} = \frac{K_D + D}{DV_{max}} \left\{ \left(1 + \frac{I}{K_I} \right) \frac{K_A}{A} + \left(1 + \frac{I}{\alpha K_I} \right) \right\} \quad (2)$$

Mixed inhibition with respect to lactosaminide

$$\text{Slope} = \frac{K_D + D}{DV_{max}} \left(1 + \frac{I}{K_I} \right) K_A$$

$$\text{Intercept} = \frac{K_D + D}{DV_{max}} \left(1 + \frac{I}{\alpha K_I} \right)$$

For CMP-Neu5Ac

$$\frac{1}{v} = \frac{1}{V_{max}} \left[\left\{ \left(\frac{\alpha K_A + A}{\alpha K_I A} \right) I + \frac{K_A + A}{A} \right\} \frac{K_D}{D} + \left\{ \left(\frac{\alpha K_A + A}{\alpha K_I A} \right) I + \frac{K_A + A}{A} \right\} \right] \quad (3)$$

Noncompetitive inhibition with respect to lactosaminide

$$\text{Slope} = \frac{1}{V_{max}} \left\{ \left(\frac{\alpha K_A + A}{\alpha K_I A} \right) I + \frac{K_A + A}{A} \right\} K_D$$

$$\text{Intercept} = \frac{1}{V_{max}} \left\{ \left(\frac{\alpha K_A + A}{\alpha K_I A} \right) I + \frac{K_A + A}{A} \right\}$$

was estimated to be 2.3*, indicates a mixed inhibition system. The intercept and slope of eq *3* are linear with respect to [I], indicating a noncompetitive inhibition system. These four linear equations are consistent with the replots from Figs. 2a and 2b.

In conclusion, the 6′-modified *N*-acetyl-β-lactosaminide-analogues, i.e., 6′-deoxy (**12**), 6′-SH (**13**), 6′-*O*-THP (**14**), and dimeric disulfide (**15**) analogues have been designed and synthesized, and **12** was found to have a remarkable inhibitory activity toward the (2 → 6)-α-sialyltransferase from rat liver. The considerable

* The coefficient α (2.3) was estimated[14b] from the intersection ($-\alpha K_i$) of the [I]-axis in the intercept replot (intercept vs. [I]) and that ($-K_i$) in the slope replot (slope vs. [I]) of Fig. 2a.

activity shown by all of the synthesized analogues indicated that this transferase did not require the 6′-OH of the *N*-acetyllactosamine residue for its binding. Based on a mixed inhibition mode observed, a possibility of simultaneous binding of two *N*-acetyllactosaminides was proporsed.

Because the (2 → 6)-α-sialyltransferase has shown high substrate specificity for *N*-linked oligosaccharides having branched *N*-acetyllactosaminides, one would expect that if the (2 → 6)-α-sialyltransferase can simultaneously bind two branching *N*-acetyllactoasminides of the nonreducing end of *N*-linked oligosaccharides, these oligosaccharides may be potential sialyl acceptors compared to other oligosaccharides having no branched *N*-acetyllactosaminides. The biological meaning of this hypothesis is interesting and is expected to be clarified using more complex pseudooligosaccharides.

EXPERIMENTAL

General procedures.—NMR spectra were recorded with one of following instruments: JEOL EX-270, Bruker AC 300, and AM 500. The chemical shifts are presented in ppm and referenced to Me_4Si in $CDCl_3$ or sodium 3-(trimethylsilyl)propionate in D_2O as the internal or external standard, respectively, unless otherwise indicated. Optical rotations were measured with a JASCO DIP-4. High-resolution mass spectra were recorded on a JMS-SX102A instrument under FAB conditions. All reactions were monitored by TLC (Silica Gel 60-F_{254}, E. Merck) by charring after spraying with 5% H_2SO_4 in MeOH. Wako-Gel C-300 was used for flash column chromatography. (2 → 6)-α-Sialyltransferase (rat liver) was purchased from Boeringer-Mannheim. CMP-β-D-[U-^{14}C]-Neu5Ac was purchased from NEN Research Products.

Methyl O-*(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-azido-2-deoxy-β-*D*-glucopyranoside* (**2**).—A solution of **1** (4.9 g, 7.68 mmol) and NaOMe (32 mmol) in 1:3.5 CH_2Cl_2–MeOH (45 mL) was stirred at room temperature, neutralized with AcOH after 10 min, and concentrated. The residue was treated with pyridine–Ac_2O in the presence of 4-dimethylaminopyridine (DMAP). After being stirred for 12 h at room temperature, the mixture was concentrated. The residue was dissolved in $CHCl_3$, washed with brine, dried ($MgSO_4$), and concentrated to give **2** (ref. 10) (4.3 g, α : β ratio 1 : 35, 89%). ^{1}H NMR data: δ 5.35 (bd, 1 H, $J_{4',3'}$ 2.3 Hz, H-4′), 5.08 (dd, 1 H, $J_{2',1'}$ 7.6, $J_{2',3'}$ 10.2 Hz, H-2′), 5.00 (dd, 1 H, $J_{3,2}$ 10.2, $J_{3,4}$ 8.9 Hz, H-3), 4.95 (dd, 1 H, H-3′), 4.49 (dd, 1 H, $J_{6a,5}$ 1.6, $J_{6a,6b}$ 11.0 Hz, H-6a), 4.45 (d, 1 H, H-1′), 4.27 (d, 1 H, $J_{1,2}$ 7.9 Hz, H-1), 4.21–4.05 (m, 3 H, H-6′a,6′b,6b), 3.88 (bt, 1 H, H-5′), 3.72 (dd, 1 H, $J_{4,5}$ 8.9 Hz, H-4), 3.57 (s, 3 H, OMe), 3.38 (dd, 1 H, H-2), 2.15, 2.12, 2.11, 2.07, 2.03, 1.96 (each s, each 3 H, Ac).

Methyl O-*(β-*D*-galactopyranosyl)-(1 → 4)-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**3**).—To a solution of **2** (9.45 g, 14.9 mmol) in EtOAc (10 mL), was added a catalytic amount of Pd–C, and then H_2 was introduced. After being stirred for 12 h, the mixture was filtered through a Celite pad (washing with

$CHCl_3$), and the filtrate was concentrated. The residue was dissolved in pyridine–Ac_2O and stirred for 2 h. The residue obtained by concentration of the mixture was purified by flash column chromatography (EtOAc) to give methyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside (8.96 g). To a solution of this disaccharide (8.96 g) in dry CH_2Cl_2 (16 mL) was added dry MeOH (187 mL) and NaOMe (303 mg, 5.6 mmol), and the mixture was stirred for 2 h at room temperature. The mixture was neutralized with Dowex-50WX8 (H^+) and filtered through a pad of Celite. Concentration of the filtrate afforded **3** (ref. 10) (5.2 g, 93% overall yield). ^1H NMR data are presented in Tables I and II.

Methyl O-*(4,6-O-benzylidene-2,3,-di-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside* (**4**).—To a solution of **3** (580 mg, 1.46 mmol) in DMF (10 mL) at 0°C was added benzaldehyde dimethyl acetal (0.56 mL) and camphorsulfonic acid (238 mg), and the mixture was stirred for 2 h at 50°C. The mixture was quenched by the addition of Et_3N, then concentrated and dried. The residue was redissolved in pyridine, and 4-dimethylaminopyridine and Ac_2O was added, and the mixture was stirred overnight. After concentration, flash column chromatography (9:1 EtOAc–MeOH) gave **4** (845 mg, 89%): $[\alpha]_D^{25}$ + 27.2° (*c* 1.0, $CHCl_3$); mp 232–234°C; ^1H NMR data: δ 7.48–7.35 (m, 5 H, Ar), 5.89 (d, 1 H, $J_{NH,2}$ 9.6 Hz, NH), 5.48 (s, 1 H, PhC*H*), 5.27 (dd, 1 H, $J_{2',1'}$ 7.9, $J_{2',3'}$ 10.55 Hz, H-2′), 5.09 (dd, 1 H, $J_{3,4}$ 8.3, $J_{3,2}$ 9.9 Hz, H-3), 4.90 (dd, 1 H, $J_{3',4'}$ 3.6 Hz, H-3′), 4.54 (dd, 1 H, $J_{6a,6b}$ 11.9, $J_{6a,5}$ 2.6 Hz, H-6a), 4.49 (d, 1 H, H-1′), 4.36–4.28 (m, 3 H, H-1,4′,6′a), 4.17–4.03 (m, 3 H, H-2,6b,6′b), 3.80 (t, 1 H, $J_{4,5}$ 8.9 Hz, H-4), 3.63–3.57 (m, 1 H, H-5), 3.48 (bq, 1 H, H-5′), 3.45 (s, 3 H, OMe), 2.12, 2.07, 2.05, 2.04, 1.95 (each s, each 3 H, Ac). Anal. Calcd for $C_{30}H_{39}NO_{15}$: C, 55.13; H, 6.01; N, 2.14. Found: C, 54.64; H, 5.96; N, 2.10.

Methyl O-*(2,3,-di-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside* (**5**).—Compound **4** (464 mg, 0.71 mmol) was dissolved in 60% AcOH (9 mL) and heated at 90°C. After 2 h, the mixture was cooled to ambient temperature and concentrated. Flash column chromatography (4:1 EtOAc–MeOH) afforded **5** (394 mg, 98%): $[\alpha]_D^{23}$ + 6.7° (*c* 0.4, Me_2CO); mp 196–198°C; ^1H NMR data: δ 6.30 (d, 1 H, $J_{NH,2}$ 9.2 Hz, NH), 5.25 (dd, 1 H, $J_{2',1'}$ 7.9, $J_{2',3'}$ 10.6 Hz, H-2′), 5.09 (dd, 1 H, $J_{3,4}$ 9.2, $J_{3,2}$ 7.9 Hz, H-3), 4.91 (dd, 1 H, $J_{3',4'}$ 3.3 Hz, H-3′), 4.55 (d, 1 H, H-1′), 4.49 (dd, 1H, $J_{6a,6b}$ 11.9, $J_{6a,5}$ 3.0 Hz, H-6a), 4.41 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 4.16–3.51 (m, 8 H, H-2,4,5,6b,4′,5′,6′a,6′b), 3.45 (s, 3 H, OMe), 2.11, 2.10, 1.98 (each, s, each 3 H, Ac) 2.07 (s, 6 H, 2 Ac). Anal. Calcd for $C_{23}H_{35}NO_{15}$: C, 48.85; H, 6.24; N, 2.48. Found: C, 49.09; H, 6.21; N, 2.53.

Methyl O-*(2,3,4,-tri-*O*-acetyl-6-O-tert-butyldimethylsilyl-β-D-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside* (**6**).—To a stirred solution of **5** (229 mg, 0.41 mmol) in DMF (2 mL) at room temperature was added imidazole (183 mg, 2.43 mmol) and *tert*-butylchlorodimethylsilane (165 mg, 1.21 mmol). After 0.3 h, the mixture was quenched by the addition of excess MeOH and concentrated. The residue was dissolved in pyridine and Ac_2O was added. Con-

centration of the mixture and purification of the residue by flash column chromatography (EtOAc) afforded **6** (229 mg, 78%): $[\alpha]_D^{23}$ −30.6° (*c* 0.4, CH_2Cl_2); mp 103–106°C; ^{1}H NMR data: δ 5.86 (d, 1 H, $J_{NH,2}$ 9.2 Hz, NH), 5.47 (d, 1 H, $J_{4',3'}$ 3.3 Hz, H-4'), 5.11 (dd, 1 H, $J_{2',1'}$ 7.6, $J_{2',3'}$ 10.2 Hz, H-2'), 5.06 (dd, 1 H, $J_{3,4}$ 7.9, $J_{3,2}$ 9.2 Hz, H-3), 5.01 (dd, 1 H, H-3'), 4.36 (d, 1 H, $J_{1,2}$ 7.3 Hz, H-1), 4.14 (dd, 1 H, $J_{6b,5}$ 5.0 Hz, H-6b), 4.08 (m, 1 H, H-2), 3.81 (t, 1 H, $J_{4,5}$ 8.3 Hz, H-4), 3.73–3.51 (m, 4 H, H-6'a,6'b,5',5), 3.45 (s, 3 H, OMe), 2.13, 2.11, 2.07, 2.06, 1.98, 1.97 (each s, each 3 H, Ac), 0.86 (s, 9 H, Bu^t), 0.03, 0.01 (each s, each 3 H, SiMe). Anal. Calcd for $C_{31}H_{51}NO_{16}Si$: C, 51.58; H, 7.12; N, 1.94. Found: C, 51.28; H, 7.27; N, 2.05.

Methyl O-*(2,3,4,-tri-*O*-acetyl-β-*D*-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**7**).—Compound **6** (267 mg, 0.37 mmol) was dissolved in 60% AcOH (5.5 mL) and heated at 90°C. After 0.5 h, the mixture was cooled to ambient temperature and concentrated. Purification of the residue by flash column chromatography (19 : 1 EtOAc–MeOH) afforded **7** (171 mg, 76%): $[\alpha]_D^{25}$ −9.0° (*c* 1.0, $CHCl_3$); mp 116–118°C; ^{1}H NMR data: δ 5.96 (d, 1 H, $J_{NH,2}$ 9.6 Hz, NH), 5.37 (d, 1 H, $J_{4',3'}$ 3.0 Hz, H-4'), 5.14 (dd, 1 H, $J_{2',1'}$ 7.9, $J_{2',3'}$ 10.2 Hz, H-2'), 5.10 (t, 1 H, $J_{3,4} = J_{3,2} = 7.6$ Hz, H-3), 5.03 (dd, 1 H, H-3'), 4.57 (d, 1 H, H-1'), 4.51 (dd, 1 H, $J_{6a,6b}$ 11.6, $J_{6a,5}$ 3.3 Hz, H-6a), 4.40 (d, 1 H, $J_{1,2}$ 6.9 Hz, H-1), 4.18–4.06 (m, 2 H, H-2,6a), 3.84 (t, 1 H, $J_{4,5}$ 7.6 Hz, H-4) 3.45 (s, 3 H, OMe), 2.16, 2.11, 2.09, 2.07 (each s, each 3 H, Ac) 1.99 (s, 6 H, 2 Me). Anal. Calcd for $C_{25}H_{37}NO_{16}$: C, 49.42; H, 6.14; N, 2.31. Found: C, 49.31; H, 5.95; N, 2.27.

Methyl O-*(2,3,4,-tri-*O*-acetyl-6-*O*-p-toluenesulfonyl-β-*D*-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**8**).—To a stirred solution of **7** (55 mg, 91 μmol) in pyridine (0.5 mL) at 0°C was added 4-dimethylaminopyridine (3 mg, 27 μmol) and *p*-toluenesulfonyl chloride (173 mg, 0.91 mmol). The mixture was stirred for 2 h at 50°C. The mixture was cooled to ambient temperature, then quenched by the addition of excess of MeOH and concentrated. Purification of the residue by flash column chromatography (19 : 1 EtOAc–MeOH) afforded **8** (53 mg, 77%); $[\alpha]_D^{23}$ −14.1° (*c* 5.1, CH_2Cl_2); ^{1}H NMR data: δ 7.77, 7.38 (d, each 2 H, *J* 8.3 Hz, Ar), 5.80 (d, 1 H, $J_{NH,2}$ 9.2 Hz, NH), 5.35 (d, 1 H, $J_{4',3'}$ 3.6 Hz, H-4'), 5.11 (t, 1 H, $J_{3,2} = J_{3,4} = 8.3$ Hz, H-3), 5.08 (dd, 1 H, $J_{2',3'}$ 10.6, $J_{2',1'}$ 7.9 Hz, H-2'), 4.96 (dd, 1 H, H-3'), 4.52–4.46 (m, 2 H, H-1',6a), 4.38 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 4.17–3.93 (m, 6 H, H-2,5,6b,5',6'a,6'b), 3.81 (t, 1 H, $J_{4,5}$ 8.3 Hz, H-4), 3.69 (m, 1 H, H-5), 3.46 (s, 3 H, OMe), 2.47 (s, 3 H, *Me*-Ar), 2.11, 2.06, 2.05, 2.04, 1.98, 1.96 (each s, each 3 H, Ac). Anal. Calcd for $C_{32}H_{43}NO_{18}S$: C, 50.46; H, 5.68; N, 1.84. Found: C, 50.58; H, 5.75; N, 2.01.

Methyl O-*(2,3,4,-tri-*O*-acetyl-6-deoxy-β-*D*-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**9**).—To a solution of **8** (55 mg, 72 μmol) in dimethoxyethane (1 mL) was added NaI (108 mg, 0.72 mmol), and the mixture was heated at 100°C under Ar. After 12 h, the mixture was cooled to ambient temperature and concentrated. Purification of the residue by flash column chromatography (13 : 1 EtOAc–MeOH) gave methyl *O*-(2,3,4,-tri-*O*-acetyl-6-deoxy-6-iodo-β-D-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glu-

copyranoside. To a solution of this disaccharide and AIBN (5 mg) in dry dimethoxyethane (4 mL) was added tributyltin hydride (39 μL, 0.14 mmol), and the mixture was heated at 100°C under Ar. After 18 h, the mixture was cooled to ambient temperature and concentrated. The residue was triturated with 2:1 EtOAc–satd KF solution and the mixture was stirred for 2 h at room temperature. The organic layer was separated, washed with brine (×2), dried ($MgSO_4$), and evaporated. Purification of the residue by flash column chromatography (13:1 EtOAc–MeOH) afforded **9** (35 mg, 82%): $[\alpha]_D^{23}$ −10.7° (*c*, 1.1, CH_2Cl_2); ^1H NMR data: δ 5.64 (d, 1 H, $J_{NH,2}$ 9.2 Hz, NH), 5.21 (d, 1 H, $J_{4',3'}$ 2.3 Hz, H-4'), 5.11 (dd, 1 H, $J_{2',3'}$ 10.23, $J_{2',1'}$ 7.6 Hz, H-2'), 5.07 (dd, 1 H, $J_{3,2}$ 10.6, $J_{3,4}$ 7.6 Hz, H-3), 4.97 (dd, 1 H, H-3'), 4.50 (dd, 1 H, $J_{6a,5}$ 3.0, $J_{6a,6b}$ 11.9 Hz, H-6a), 4.47 (d, 1 H, H-1'), 4.34 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 4.14 (dd, 1 H, $J_{6b,5}$ 5.0 Hz, H-6b), 4.07 (m, 1 H, H-2), 3.79 (dd, 1 H, $J_{4,5}$ 8.6 Hz, H-4), 3.76 (bdd, 1 H, $J_{5',6'a} = J_{5',6'b} = 6.3$ Hz, H-5') 3.63 (m, 1 H, H-5), 3.46 (s, 3 H, OMe), 2.17, 2.11, 2.07, 2.06, 1.98, 1.97 (each s, each 3 H, Ac), 1.20 (d, 3 H, Me-6'). Anal. Calcd for $C_{25}H_{37}NO_{15}$: C, 50.76; H, 6.30; N, 2.37. Found: C, 50.77; H, 6.66; N, 1.90.

Methyl O-*(2,3,4,-tri-*O*-acetyl-6-S-acetyl-6-thio-β-*D*-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**10**).—To a solution of **8** (53 mg, 70 μmol) in DMF (1.7 mL) was added potassium thioacetate (79 mg, 0.69 mmol), and the mixture was heated at 80°C under Ar. After 12 h, the mixture was cooled to ambient temperature and evaporated. Purification of the residue by flash column chromatography (19:1 EtOAc–MeOH) afforded **10** (39 mg, 84%): $[\alpha]_D^{23}$ −6.0° (*c* 1.0, CH_2Cl_2); ^1H NMR data: δ 5.61 (d, 1 H, $J_{NH,2}$ 9.2 Hz, NH), 5.39 (d, 1 H, $J_{4',3'}$ 3.6 Hz, H-4'), 5.10 (dd, 1 H, $J_{2',3'}$ 10.6, $J_{2',1'}$ 7.9 Hz, H-2'), 5.08 (t, 1 H, $J_{3,4} = J_{3,2} = 7.9$ Hz, H-3), 4.95 (dd, 1 H, H-3'), 4.51 (dd, 1 H, $J_{6a,5}$ 2.6, $J_{6a,6b}$ 11.2 Hz, H-6a), 4.46 (d, 1 H, H-1'), 4.36 (d, 1 H, $J_{1,2}$ 7.3 Hz, H-1), 4.17–4.03 (m, 2 H, H-2,6b), 3.82 (t, 1 H, $J_{4,5}$ 8.3 Hz, H-4), 3.69–3.64 (m, 2 H, H-5,5'), 3.46 (s, 3 H, OMe), 3.04 (d, 2 H, $J_{6'a,5'} = J_{6'b,5'} = 7.3$ Hz, H-6'a,6'b), 2.35 (s, 3 H, SAc) 2.17, 2.12, 2.11, 2.05, 1.99, 1.97 (each s, each 3 H, Ac). Anal. Calcd for $C_{27}H_{39}NO_{16}S$: C, 48.72; H, 5.91; N, 2.10. Found: C, 48.87; H, 5.87; N, 2.15.

Methyl O-*(2,3,4,-tri-*O*-acetyl-6-*O*-tetrahydropyranyl-β-*D*-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**11**).—To a solution of **7** (55 mg, 91 μmol) in CH_2Cl_2 (1.0 mL) was added a catalytic amount of pyridinum *p*-toluenesulfonate and 2,3-dihydropyran (41 μL, 0.45 mmol). After 3 h, the mixture was diluted with $CHCl_3$, washed with satd aq $NaHCO_3$(×2), dried with $MgSO_4$, and evaporated. Purification of the residue by flash column chromatography (19:1 EtOAc–MeOH) afforded **11** (46 mg, 74%) as a 1:1 diastreomeric mixture. ^1H NMR data: δ 5.74, 5.73 (d, 0.5 H, $J_{NH,2}$ 9.6 Hz, NH), 5.47 (d, 0.5 H, $J_{4',3'}$ 3.3 Hz, H-4'), 5.44 (d, 0.5 H, $J_{4',3'}$ 3.0 Hz, H-4'), 5.15–4.97 (m, 3 H, 2 H-2',3'-3), 4.61 (bdd, 0.5 H, THP), 4.35 (d, 1 H, $J_{1',2'}$ 7.6 Hz, 2 H-1'), 3.45 (s, 3 H, 2 OMe), 2.14, 2.12, 2.07, 2.06 (each s, each 3 H, Ac) 1.98 (s, 6 H, Ac), 1.9–1.4 (m, THP). Anal. Calcd for $C_{30}H_{45}NO_{17}$: C, 52.09; H, 6.56; N, 2.03. Found: C, 51.79; H, 6.74; N, 1.92.

Methyl O-(6-deoxy-β-D-galactopyranosyl)-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**12**).—To a solution of **9** (10 mg, 17 μmol) in dry MeOH (0.5 mL) was added a catalytic amount of NaOMe, and the mixture was stirred at room temperature. After 3 h, the mixture was neutralized with Dowex-50WX8 (H^+) and filtered through a pad of Celite. The residue obtained by evaporation of the filtrate was purified by gel-permeation chromatography (Sephadex G-15, H_2O) to afford **12** (6 mg, 93%): $[\alpha]_D^{23}$ −17.2° (*c* 0.4, H_2O); ^1H NMR data are presented in Tables I and II. Calcd for $C_{15}H_{28}NO_{10}$: *m/z* 382.1713 (M + H); Found: *m/z* 382.1722.

Methyl O-(6-O-tetrahydropyranyl-β-D-galactopyranosyl)-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**14**).—To a solution of **11** (15 mg, 22 μmol) in dry MeOH (0.5 mL) was added a catalytic amount of NaOMe, and the mixture was stirred at room temperature. After 2 h, the mixture was carefully neutralized by dil AcOH in MeOH, and the solvents were evaporated. Purification of the residue by gel-permeation chromatography (Sephadex G-15, H_2O) afforded **14** (9 mg, 86%): $[\alpha]_D^{23}$ −42.7° (*c* 0.2, H_2O); ^1H NMR data: δ 4.72 (bs, 0.5 H, THP-anomer), 4.46 (d, 2 H, H-1,1′), 3.50 (s, 3 H, OMe), 2.01 (s, 3 H, Ac), 1.85–1.70, 1.65–1.45 (m, THP), Calcd for $C_{20}H_{36}NO_{12}$: *m/z* 482.2238 (M + H); Found: *m/z* 482.2238.

Methyl O-(6-thio-β-D-galactopyranosyl)-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**13**).—To a solution of **10** (32 mg, 48 μmol) in dry MeOH (2.5 mL) was added 28% NH_4OH (8 mL) and DL-dithiothreitol (10 equiv), and the mixture was stirred for 12 h at room temperature. The mixture was evaporated at 30–35°C. Purification of the residue by gel-permeation chromatography (Sephadex G-15, H_2O) afforded **13** (9 mg, 45%): $[\alpha]_D^{23}$ −12.5° (*c* 0.4, H_2O); ^1H NMR data are presented in Tables I and II. Calcd for $C_{15}H_{28}NO_{10}S$: *m/z* 414.1434 (M + H); Found: *m/z* 414.1420.

Methyl O-(6-deoxy-β-D-galactopyranosyl)-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside-6′-yl disulfide (**15**).—A solution of **13** (3.5 mg, 8.5 μmol) in H_2O (0.8 mL) was warmed at 37°C. After 72 h, the mixture was evaporated. Purification of the residue by gel-permeation chromatography (Sephadex G-15, H_2O) afforded **15** (3 mg, 83%): $[\alpha]_D^{23}$ +25.3° (*c* 0.5, H_2O); ^1H NMR data are presented in Tables I and II. Calcd for $C_{30}H_{53}N_2O_{20}S_2$: *m/z* 825.2634 (M + H); Found: *m/z* 825.2653.

Inhibition assay of the four synthetic analogues toward (2 → 6)-α-sialyltransferase.—Sialyltransferase activity was assayed according to the method of Paulson et al.[6] Inhibition assays were performed at 37°C for 15–60 min in 50 mM sodium cacodylate buffer solution (pH 6.0, 30 μL), which contained the following assay components: methyl *N*-acetyl-β-D-lactosaminide **1** (0.5–3 mM), analogue of **1**, bovine serum albumin (50 μg), Triton X-100 (0.5%), CMP-β-D-[U-^{14}C]Neu5Ac (2.5 μM, 12.1 GBq/mmol), and sialyltransferase (2.0×10^{-6} unit). The reaction was followed up to 15% consumption of CMP-Neu5Ac. The ^{14}C-labeled sialyllactosaminide was isolated by passage of the mixture diluted with 1 mL of 5 mM sodium phosphate (pH 6.8) through a Pasteur pipet column of Dowex-1X8 (PO_4^{2-}), and further eluted by 1 mL of 5 mM sodium phosphate. The ^{14}C-labeled sialyllac-

tosaminide was determined by scintillation counting. In general, the data points are the averages of at least duplicate values. The K_i as well as K_m values were obtained by the previously described method[5].

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