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β-Galactosidase-catalyzed intramolecular transglycosylation

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Abstract—A novel transglycosylation strategy for efficient preparation of *N*-acetyllactosamine (Galβ1→4GlcNAc, LacNAc) and sialyl LacNAc was developed using β-galactosidase from *Bacillus circulans*. In order to minimize the competing hydrolysis by forcing the enzymatic transglycosylation to proceed in an intramolecular manner, a novel substrate **9** carrying the donor (galactose) and the acceptor (*N*-acetylglucosamine) components linked via a 2-hydroxy-5-nitro-benzylalcohol derived tether was prepared. Treatment with β-galactosidase from *B. circulans* afforded the transglycosylation product **11** in 26% yield. Furthermore, addition of sialyltransferase and CMP–sialic acid to this system gave sialyl LacNAc **18** in 39% yield.

N-Acetyllactosamine (Galβ1→4GlcNAc, LacNAc) is widespread as a constituent of glycoconjugates, including *N*- and *O*-linked glycoproteins,[1] glycolipids,[2] and glycosaminoglycans.[3]

It is also important as a precursor of ligands for cell adhesion molecules, for instance selectin[4] and CD22.[5]

Various approaches toward chemical synthesis of LacNAc have been investigated.[6–12] Most typically, it was prepared from lactose via lactal using Lemieux's azidonitration as the key transformation.[13,14] Enzymatic preparation of LacNAc has also been a subject of extensive investigation. For instance, galactosyltransferase-mediated glycosylation of *N*-acetylglucosamine (GlcNAc) with UDP–galactose provides a highly efficient access to LacNAc.[15] Although several types of glycosyltransferases are rapidly gaining practical use in preparative scale oligosaccharide synthesis,[16] limitation has been posed by the cost, availability and the stability of the enzymes and sugar nucleotides.

Alternatively, galactosidase derived from various sources, particularly the one derived from *Bacillus circulans*, have been used for the preparation of LacNAc, taking advantage of their transglycosylating activity.[17–20] A galactosidase based approach is attractive due to the following reasons: (1) inexpensive (lactose) or easily obtainable (aryl β-D-galactoside) galactosyl donor can be used, and (2) compared to glycosyltransferases, glycosidases are much more stable, easier to handle, and generally cheaper. However, in order to achieve efficient transglycosylation, a large excess of either glycosyl donor (i.e. lactose) or acceptor (GlcNAc) is usually required and isolation process thus tends to be complicated.

Since glycosidases are hydrolyzing enzymes in nature, it would be difficult to suppress the hydrolysis to an extent that transglycosylation using an equimolar amount of glycosyl donor/acceptor can be achieved in a synthetically useful efficiency. One potential tactic to alleviate this difficulty would be to make the glycosyl transfer entropically favorable by connecting the two components via a tether into a single molecule. On condition that donor and acceptor portions of the substrate fit simultaneously into the binding pocket of the enzyme, transglycosylation may be expected to proceed in an intramolecular manner and override the competing hydrolysis (Scheme 1).[21]

In order to examine this possibility, compounds **9** and **10** embracing two sugar residues with an *ortho* and *meta* relationship, respectively, were designed. It can be conceived that both of them are derivatives of *p*-nitrophenyl β-galactoside, an active and well-explored galactosyl donor. These compounds were prepared from commercially available 2-hydroxy-5-nitro- (**2**) (*ortho*) and 5-hydroxy-2-nitro- (**3**) benzyl alcohol (*meta*) as depicted in Scheme 2. Thus, Lewis acid-mediated reactions with glucosamine donor **1** afforded **4** and **5**. Subsequently, trichloroacetimidate **6**[22] was used as a

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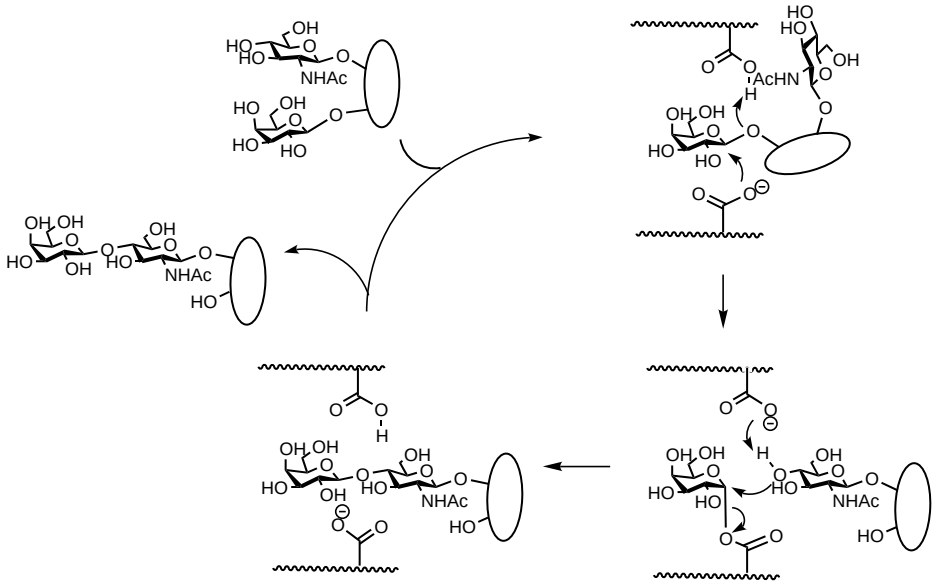
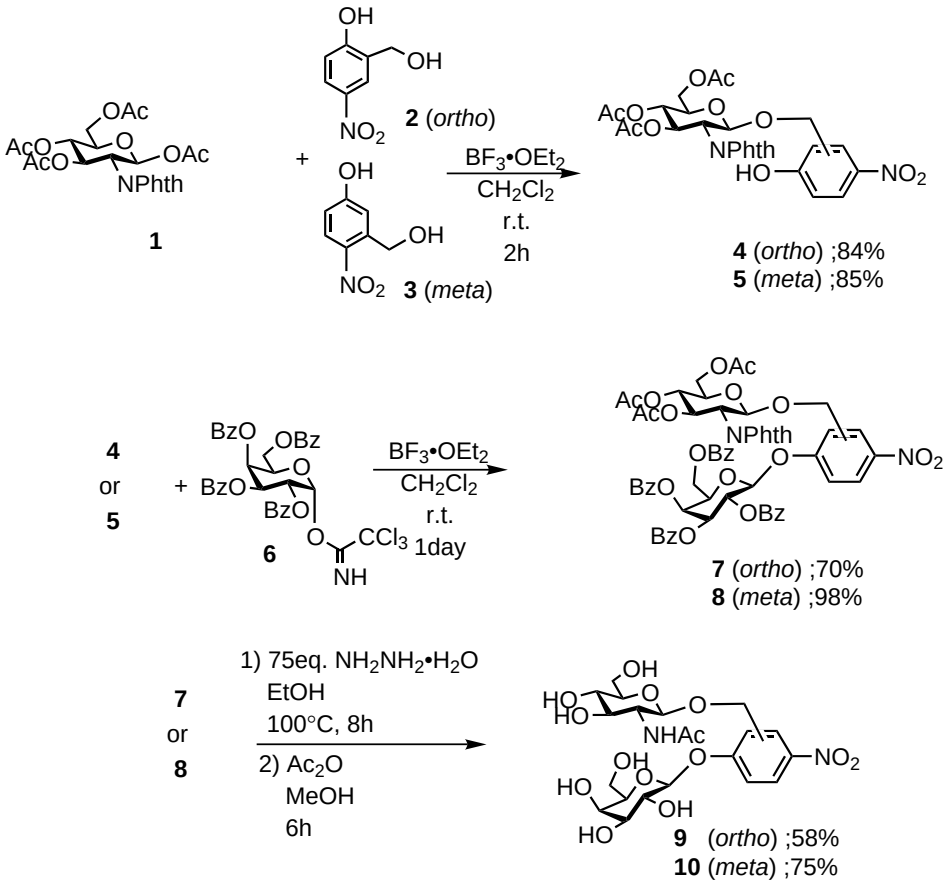


Scheme 1. β-Galactosidase-mediated intramolecular transglycosylation.



Scheme 2. Preparation of transglycosylation substrates.

galactosyl donor to glycosylate the phenolic hydroxyl group to give β-glycosides **7** and **8**. Somewhat unexpectedly, glycosylation of **4** was accompanied by the formation of a significant amount of corresponding α-isomer (α:β = 1:3.3). Finally, complete deprotection was followed by selective *N*-acetylation to give transglycosylation substrates **9** (*ortho*) and **10** (*meta*).

Enzymatic transglycosylation was examined using **9** or **10** (8 mM) in 50 mM sodium acetate buffer (pH 5.0) at

36°C in the presence of galactosidase from *B. circulans* (Biolacta, Daiwa Kasei Co., Ltd.) and the reactions were followed by analytical HPLC.[23] As shown in Fig. 1, profiles of two reactions are markedly different. From **9**, the formation of LacNAc derivative **11** (●) was dominant over **15** even at the early stage of incubation (i.e. [**9**]>[**13**]). Since rates of intermolecular glycosyl transfer likely to be similar between **9** and **13** ($k_1 \approx k_2$), this result indicates that the intramolecular pathway is functional ($k_3 > k_1$, k_2) (Scheme 3). On the other hand, the overall process from *meta*-oriented **10** was less efficient. Formation of LacNAc (**12**) lagged far behind

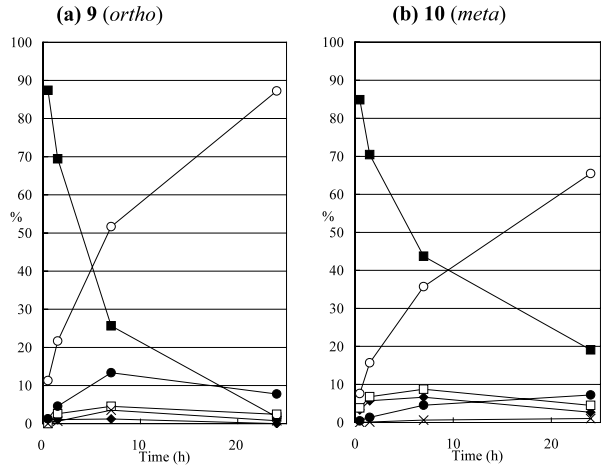
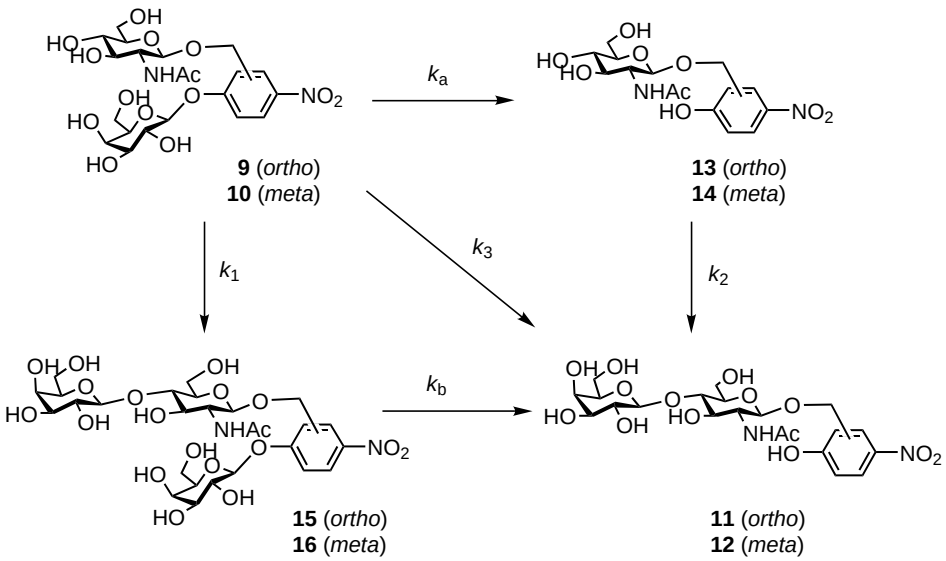


Figure 1. Time course of β-galactosidase-catalyzed reactions. (**a**) Compound **9** (8 mM) was incubated with 130 mU of β-galactosidase in 700 μl of acetate buffer (50 mM, pH 5.0) at 36°C. (**b**) Compound **10** (8 mM) was incubated with 390 mU of β-galactosidase under identical conditions as (**a**). Aliquots of the mixture was analyzed by HPLC. Peaks were assigned as follows based on MALDI-TOF MS analysis. ■ Starting material; (**9**/**10**); ○ GlcNAc (**13**/**14**); ● LacNAc (**11**/**12**); □ $Gal_2GlcNAc$ (**15**/**16**); × $Gal_2GlcNAc$ (regioisomer of **15**/**16**, structure not confirmed); ◆ $Gal_3GlcNAc$.

$Gal_2GlcNAc$ (**16**) and the direct pathway (**10**→**12**) seems insignificant. Gradual formation of **12** would most likely to be a result of intermolecular transglycosylation (**10**→**14**→**12** and/or **10**→**16**→**12**).

Using the preferred substrate **9**, effects of substrate concentration (entries 1–5), organic solvents (entries 6–12), and buffer pH (entry 13) were examined (Table 1). Contrary to several precedents on enzymatic transglycosylation,[24] beneficial effect of organic solvent was not observed. Preparative scale reaction was performed at 20 mM concentration (entry 4). Thus, using 50 mg (94 μmol) of compound **9** incubation with β-galactosidase (430 mU) for 3 h afforded 26% yield (13.1 mg) of **11**, the structure of which was rigorously confirmed by NMR analysis of corresponding acetate **17**.[25]

Since the product **11** should be susceptible to further digestion by β-galactosidase (to provide **13**), its yield does not necessarily reflect the genuine ratio of intramolecular transglycosylation and hydrolysis (i.e. k_3 versus k_a). As a matter of fact, the yield of **11** dropped to 18% after 4 h incubation (Table 1, entry 5). In order to minimize the extent of post-transglycosylation hydrolysis and to gain more precise estimation of the efficiency of transglycosylation, α-(2→6)-sialyltransferase (Rat, Recombinant, *Spodoptera frugiperda*; Calbiochem Co., Ltd.)[26,27] and CMP–sialic acid were added to the system to trap **11** as a galactosidase resistant product **18** (Scheme 4, Figs. 2 and 3). As expected, smooth formation of sialyl-α-(2→6)-LacNAc **18** (□) was observed and its amount did not decrease after prolonged incubation. In a preparative scale run, substrate **9** was used in 25 mg (47 μmol), that was incubated in 200 mM HEPES buffer (2.4 ml, pH 7.5) in the presence of β-galactosidase (100 mU), α-(2→6)-sialyltransferase (60 mU) and CMP–sialic acid (30 mg, 46 μmol). After 17 h, successive purification by Sephadex LH-20 and preparative HPLC afforded sialyl-α-(2→6)-LacNAc **18** in 39% yield (15.4 mg).[28,29]

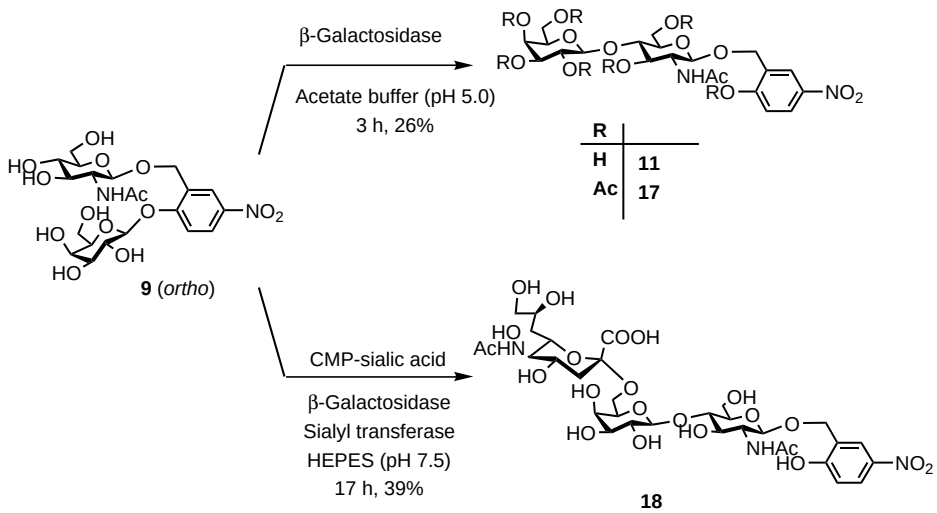


Scheme 3. Possible pathways leading to transglycosylation products.

Table 1. Effects of solvent and substrate concentration

Entry	**9** (mM)	Enzyme (mU)	Solvent (ml)[a]	Time (h)	Yield (%)
1	8	86	A (0.23)	8	15[b]
2	0.8	43	A (2.3)	48	4[b]
3	0.4	21.5	A (1.2)	144	3[b]
4	20	430	A (4.7)	3	26[c]
5	20	430	A (4.7)	4	18[c]
6	8	130	B (0.23)	24	7[b]
7	8	130	B (0.23)	72	14[b]
8	8	130	B (0.23)	96	14[b]
9	8	130	C (0.23)	24	2[b]
10	8	43	D (0.23)	8	10[b]
11	8	43	D (0.23)	24	13[b]
12	8	43	D (0.23)	72	1[b]
13	20	86	E (0.09)	4.5	15[b]

[a] A 50 mM acetate buffer pH 5.0; B 50% acetonitrile in A; C 60% triethyl phosphate in A; D 20% 2-ethoxyethyl ether in A; E 200 mM HEPES pH 7.5.
[b] Based on HPLC relative peak integrations, quantified at 245 nm.
[c] Isolated yield.



Scheme 4. Synthesis of LacNAc and sialyl LacNAc.

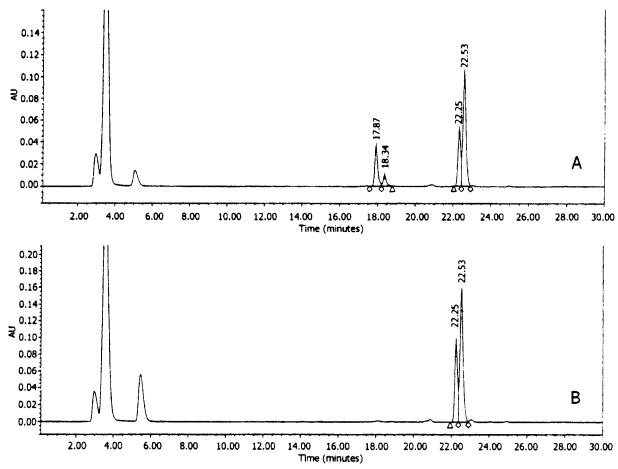


Figure 2. HPLC profiles of β-galactosidase/α-sialyltransferase reaction of **9**. A: 1.5 h, B: 6.5 h. Retention times of starting material (**9**), $Gal_2GlcNAc$ (**15**), NeuAcGalGlcNAc (**18**) and GlcNAc (**13**) are 17.87, 18.34, 22.25 and 22.53 min, respectively.

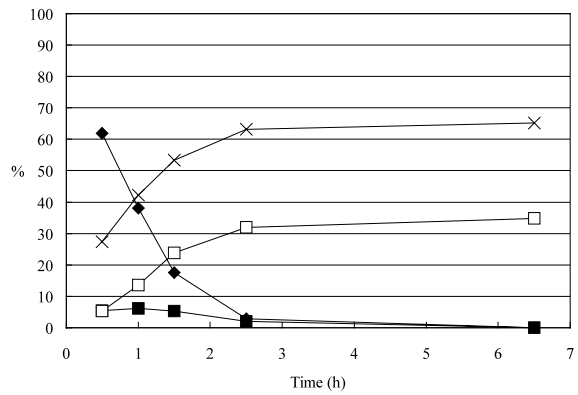


Figure 3. Time course of β-galactosidase/α-sialyltransferase reaction of **9**. Compound **9** (20 mM) was incubated with 86 mU of β-galactosidase and α-(2→6)-sialyltransferase (2.4 mU) in 95 μl of HEPES (200 mM, pH 7.5) 20 mM $MgCl_2$, 5.3 mM $MnCl_2$, 20 mM KCl, and CMP–sialic acid (20 mM) at 36°C. Peaks were assigned as follows based on MALDI-TOF MS analysis. ◆ Starting material (**9**); × GlcNAc (**13**); □ NeuAcGalGlcNAc (**18**); ■ $Gal_2GlcNAc$ (**15**).

In conclusion, a novel method for the preparation of LacNAc and sialyl LacNAc was developed using β-galactosidase-mediated intramolecular transglycosylation.

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25. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.35 (d, 1H, $J_{Ph4,Ph5}$ 2.7 Hz, Ph6), 8.23 (dd, 1H, $J_{Ph3,Ph4}$ 9.0 Hz, $J_{Ph4,Ph6}$ 2.7, Ph4), 5.64 (d, 1H, $J_{NH,2}$ 9.3 Hz, NH), 5.36 (d, 1H, $J_{3,4}$ 3.2 Hz, H-4Gal), 5.11 (dd, 1H, $J_{1,2}$ 8.1 Hz, $J_{2,3}$ 10.5, H-2Gal), 5.04 (t, 1H, $J_{2,3}=J_{3,4}$ 8.3 Hz, H-3GlcN), 4.98 (dd, 1H, $J_{2,3}$ 10.5 Hz, $J_{3,4}$ 3.4, H-3Gal), 4.83 (d, 1H, J_{gem} 13.4 Hz, PhCH), 4.65 (d, 1H, J_{gem} 13.4 Hz, PhCH′), 4.55 (dd, 1H, J_{gem} 12.2 Hz, $J_{5,6}$ 2.9, H-6Gal), 4.50 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1Gal), 4.41 (d, 1H, $J_{1,2}$ 7.6 Hz, H-1GlcN), 3.89 (t, 1H, $J_{5,6}=J_{5,6'}$ 7.1 Hz, H-5Gal), 3.81 (t, 1H, $J_{3,4}=J_{4,5}$ 8.5 Hz, H-4GlcN), 3.59 (m, 1H, H-5GlcN), 2.36, 2.15, 2.14, 2.09, 2.06, 2.05, 2.05, 1.97 (8s, 24H, 7Ac and NAc) ppm. HRMS (FAB) calcd for $C_{35}H_{44}N_2O_{21}Na$ (M+Na$^+$): 851.2334. Found: 851.2403.
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28. ^{1}H NMR (500 MHz, D_2O): δ 8.06 (d, 1H, $J_{Ph4,Ph5}$ 3.2 Hz, Ph6), 8.01 (dd, 1H, $J_{Ph3,Ph4}$ 9.2 Hz, $J_{Ph4,Ph6}$ 3.2, Ph4), 6.49 (d, 1H, $J_{Ph3,Ph4}$ 9.2 Hz, Ph3), 4.75 (d, 1H, J_{gem} 12.8 Hz, PhCH), 4.63 (d, 1H, J_{gem} 13.3 Hz, PhCH′), 4.57 (d, 1H, $J_{1,2}$ 8.2 Hz, H-1Gal), 4.40 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1GlcN), 2.64 (dd, 1H, $J_{3eq.,4}$ 4.1 Hz, J_{gem} 11.9, H-3eq. sialic acid), 2.00 and 1.95 (2s, 6H, 2NAc), 1.66 (dd, 1H, $J_{3ax.,4}=J_{gem}$ 11.9 Hz, H-3ax. sialic acid) ppm; ^{13}C NMR (150 MHz, D_2O, NaOD and MeOH): δ 176.4 (C_{Ph2}), 175.1 (C_{NAc}=O GlcN), 174.8 (C_{NAc}=O sialic acid), 173.7 (C-1 sialic acid), 133.1 (C_{Ph5}), 127.6 (C_{Ph4}-H), 127.2 (C_{Ph6}-H), 126.5 (C_{Ph1}), 119.6 (C_{Ph3}-H), 103.8 (C-1 Gal), 100.5 (C-1 GlcN), 100.4 (C-2 sialic acid), 80.2 (C-4 GlcN), 74.8 (C-5 GlcN), 74.0 (C-5 Gal), 73.1 (C-3 Gal), 72.7 (C-3 GlcN), 72.7 (C-6 sialic acid), 71.9 (C-8 sialic acid), 70.8 (C-2 Gal), 68.9 (C-4 Gal), 68.6 (C-7 sialic acid), 68.4 (C-4 sialic acid), 67.0 (C_{Bn}-H_2), 63.6 (C-6 Gal), 62.8 (C-9 sialic acid), 60.7 (C-6 GlcN), 55.4 (C-2 GlcN), 52.2 (C-5 sialic acid), 40.5 (C-3 sialic acid), 22.3 and 22.2 ($2C_{NAc}$-H_3) ppm.
29. Since compound **15** can be a substrate for the sialyltransferase, degalactosylation of the resultant trisaccharide might be functional as a minor pathway leading to **18**.