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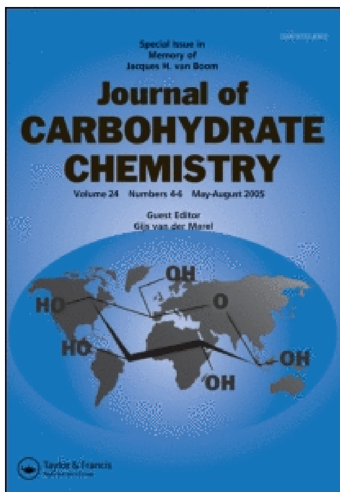

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
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Synthesis of β-GlcA-(1→3)-β-Gal and α-GalNAc-(1→4)-β-GleA-(1→3)-β-Gal as Biotinylated 2-Aminoethyl Glycoside and the Streptavidin Complex Formation

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To cite this Article Tamura, Jun-ichi , Miura, Yoshiaki and Freeze, Hudson H.(1999) 'Synthesis of β-GlcA-(1→3)-β-Gal and α-GalNAc-(1→4)-β-GleA-(1→3)-β-Gal as Biotinylated 2-Aminoethyl Glycoside and the Streptavidin Complex Formation', Journal of Carbohydrate Chemistry, 18: 1, 1 — 14

To link to this Article: DOI: 10.1080/07328309908543974

URL: http://dx.doi.org/10.1080/07328309908543974

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J. CARBOHYDRATE CHEMISTRY, 18(1), 1-14 (1999)

SYNTHESIS OF β-GlcA-(1→3)-β-Gal AND α-GalNAc-(1→4)-β-GlcA-(1→3)-β-Gal AS BIOTINYLATED 2-AMINOETHYL GLYCOSIDE AND THE STREPTAVIDIN COMPLEX FORMATION

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Received March 27, 1998 - Final Form October 6, 1998

ABSTRACT

Two oligosaccharides β-GlcA-(1→3)-β-Gal and α-GalNAc-(1→4)-β-GlcA-(1→3)-β-Gal related to the glycosaminoglycan linkage region were synthesized. Both oligosaccharides, having a 2-aminoethyl aglycon, were biotinylated and used as immunogens to prepare carbohydrate specific monoclonal antibodies. The biotin-conjugated trisaccharide was able to form a complex with streptavidin which could be used directly for immunization.

INTRODUCTION

The glycosaminoglycans are classified based on the types of hexosamine residues in the repeating disaccharide unit. Heparin and heparan sulfate have α-GlcNAc residues while chondroitin sulfate and dermatan sulfate have β-GalNAc in the repeating disaccharide unit, respectively. Both of these chains are linked to protein through a common tetrasaccharide: [β-GlcA-(1→3)-β-Gal-(1→3)-β-Gal-(1→4)-β-Xyl]. Many artificial β-

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xylosides can prime the biosynthesis as portions of this linkage region and of large chondroitin sulfate chains.[1] In 1995 Freeze's[2] group identified a pentasaccharide from melanoma cells with an α-GalNAc-(1→4) residue at the non-reducing end of the common tetrasaccharide, which did not fit into either of the categories. Subsequently, Sugahara's[3] group identified an α-*N*-acetylgalactosaminyltransferase (α-GalNAc transferase) in fetal bovine serum that was probably responsible for its formation. Although this novel sequence had not been previously found on any natural molecules, it was suggested to be an intermediate in the glycosaminoglycan biosynthesis or a stop signal[4] for chain elongation. Even though the α-GalNAc-(1→4)-β-GlcA sequence has not been found in glycoconjugates, the presence of an α-GalNAc transferase suggests the existence of a new biosynthetic pathway for the above disaccharide sequence. Since an α-GalNAc-(1→4)-β-GlcA sequence might be found in other glycoconjugates/glycosaminoglycans, this project had originally been started to synthesize an immunogenic oligosaccharide that could be used to generate a monoclonal antibody. This is necessary because lectins that recognize terminal α-GalNAc residues have been unsuccessful due to their low affinity. High affinity monoclonal antibodies against such carbohydrates can be prepared by linking the carbohydrates to biotin derivatives which are then coupled to streptavidin (StAv) for immunization. As shown in Scheme 1, the selected di- and trisaccharides (**1** and **2**) have a suitable spacer at the reducing end to allow conjugation with biotin for antigen formation. In the present paper, we describe a regio- and stereoselective synthesis of **1** and **2** as well as the respective biotinylamides **3** and **4**. Finally, complex formation of StAv and [^{3}H]-labeled **4** is also demonstrated.

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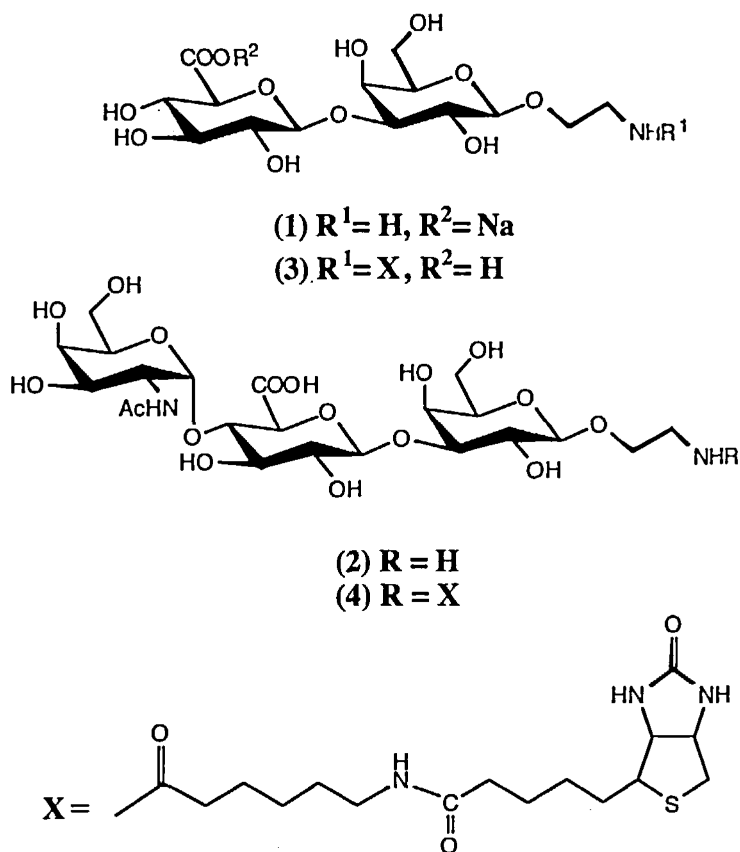
The β-GlcA-(1→3)-Gal sequence, which is a part of the glycosaminoglycan linkage region, has been synthesized during the last three decades. In 1967 Flowers[5] reported the first synthesis of the sequence by the use of (methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate) bromide according to Helferich conditions. Meyer's[6] and Nilsson's[7] groups independently improved the glycosylation using the same donor and suitably protected acceptors conducted under Koenigs-Knorr reaction conditions in 1991 and 1993, respectively. In 1993 and 1995, new synthetic methods employing glycosyl imidate or thioglycoside were introduced for this sequence by Jacquinet's[8] and Esko's[9] groups, respectively. Furthermore, the glycosidic portion of **2**, α-GalNAc-(1→4)-β-GlcA-(1→3)-Gal, was synthesized by Ogawa's[10] group as part of a glycosaminoglycan reducing end pentasaccharide.

RESULTS AND DISCUSSION

Both the planned oligosaccharides **1** and **2** are 2-aminoethyl glycosides. In a general procedure, a stepwise coupling to the aglycon was chosen. However, towards



Scheme 1

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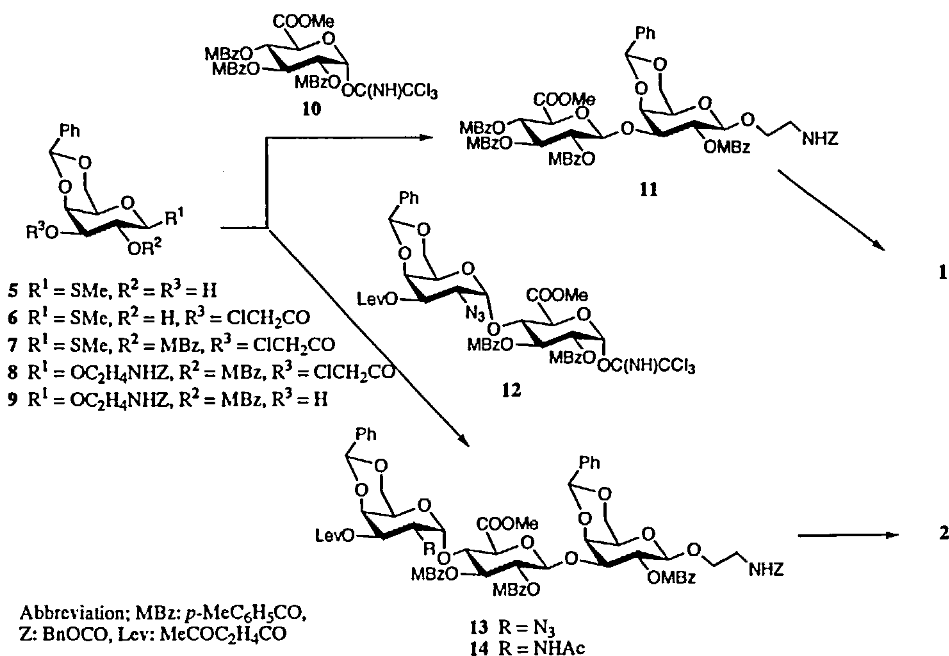
trisaccharide **2**, the final coupling was carried out by the use of disaccharide donor having an α-GalN3-(1→4)-β-GlcA sequence because of the low reactivity of GlcA 4-OH. Thus, the two known donors (**10**)[11] and (**12**)[10] could be effectively used. In order to distinguish the two amino groups on GalNAc and aglycon from each other, commercially available benzyl *N*-(2-hydroxyethyl)carbamate was adopted as an acceptor in the glycosylation with Gal donor (**7**). Oligosaccharides having similar aglycons were previously synthesized *via* azidoethyl[12] or azidopropyl glycoside,[13] an approach which is not suitable to this synthetic strategy because the disaccharide donor **12** has a 2-azido galactose moiety as a precursor of GalNAc. The synthetic strategies towards **1** and **2** are depicted in Scheme 2.

Gal donor **7** was first synthesized starting from the known methyl 4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (**5**).[14] Regioselective monochloroacetylation was



Scheme 2

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performed *via* dibutyltin oxide mediated 3-*O* activation[7] in 76% yield. The resultant 2-OH of **6** was then protected with a 4-methylbenzoyl group. The excess amount of pyridine in this acylation resulted in the removal of monochloroacetyl group during the reaction. When the base was limited to ~5 equiv compared to **6**, the desired fully protected **7** was obtained in 86% yield. The glycosylation of **7** with benzyl *N*-(2-hydroxyethyl)carbamate was successfully carried out by the action of *N*-iodo-succinimide-trifluoromethanesulfonic acid[15] to yield **8** in 94%. The monochloroacetyl group was removed with hydrazine acetate[16] and the common acceptor (**9**) having 3-OH on the Gal residue was obtained in 96% yield.

Stepwise glycosylation of **9** and the known GlcA donor **10** was carried out in the presence of $BF_3{\bullet}OEt_2$ promoter in toluene at -20 °C to give the β-linked disaccharide (**11**) stereoselectively in 39% yield. Having the desired disaccharide in hand, the deprotection procedures were performed. First, saponification with aq LiOH and successive aq NaOH media were carried out. Then, the resultant product, still having benzylidene acetal and benzyloxycarbonyl groups, was hydrogenolytically deprotected in the presence of a catalytic amount of Pd-C. Finally, the crude product was purified by use of

gel-permeation and the ion-exchange resin columns to give **1** as a sodium salt in 70% yield (three steps).

The trisaccharide **2** was synthesized as follows. Methyl (2-azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-levulinoyl-α-D-galactopyranosyl)-(1→4)-2,3-di-*O*-4-methylbenzoyl-1-*O*-trichloroacetimidoyl-α-D-glucopyranuronate (**12**) and the same acceptor **9** were coupled in the manner described above to give the desired trisaccharide (**13**) in 44% yield. When a similar disaccharide donor having acetamido instead of azido functionality was used in the same glycosylation procedure, the coupling yield was much lower (data not shown). Generation of the GalNAc moiety was tried by the reduction of azido group of **13**. As described previously,[10] we believed that Lindlar catalyst[17] would be superior to thiolacetic acid[18] for this purpose. Thus, the Lindlar catalyst was first applied for selective hydrogenolysis. However, we obtained somewhat unexpected result; the benzyloxycarbonyl group was removed during the reduction of azide. Consequently, thiolacetic acid was reexamined to avoid this unreliability and the desired GalNAc product was given in 87% yield without formation of the reducing end acetamide. The trisaccharide **14** was deprotected in the same manner as described above. In the final purification employing $(NH_4)_2CO_3$ buffer as the eluent, instead of distilled water, diffusion of the substrate on the gel-permeation column was averted. Thus, the trisaccharide **2** was successfully obtained in 72% yield after three deprotection steps. FABMS (positive as well as negative) supported the structure of **2** as a liberated uronic acid shown in Scheme 1.

Finally, saccharide and StAv complex formation was examined. Oligosaccharides **1** and **2** were coupled with biotin to give biotin conjugates **3** and **4**, respectively, formation of which was confirmed by MS measurement. A complex of **4** with StAv could not be detected by electrophoresis due to dissociation in the SDS sample buffer. We demonstrated complex formation by radiolabeling **3** with UDP-[^{3}H]GalNAc using the α-GalNAc transferase (Y. Miura and H. H. Freeze, unpublished data) to produce [^{3}H]-**4**. StAv and [^{3}H]-**4** were mixed in a conventional manner to give the trisaccharide-biotin-StAv complex. Complex formation was shown by gel-permeation chromatography using Sephadex G-50 as shown in Fig. 1. The uncomplexed molecule runs near the fully included volume, but after binding to StAv (60 kDa), it runs entirely in the void volume.

CONCLUSION

In this paper, we described the regio- and stereoselective syntheses of two glycosaminoglycan related oligosaccharides β-GlcA-(1→3)-β-Gal-(1→$OCH_2CH_2NH_2$ (**1**) and α-GalNAc-(1→4)-β-GlcA-(1→3)-β-Gal-(1→$OCH_2CH_2NH_2$ (**2**). Both the disaccharides **1** and **2** were biotinylated to give **3** and **4**, respectively, for use as

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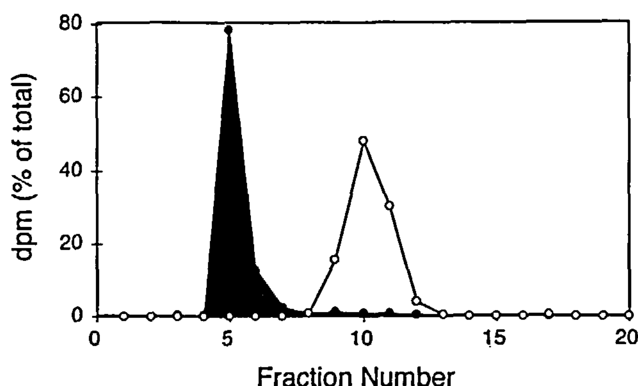

Fig. 1 Gel-permeation of [^{3}H]-4-StAv complex (●) and [^{3}H]-4 (O) by Sephadex G-50

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immunogens. The disaccharide-biotin conjugate **3** was enzymatically glycosylated with UDP-[^{3}H]-GalNAc to form the radiolabeled product which can be complexed with StAv. Biological studies with these substrates are under investigation.

EXPERIMENTAL

General methods. Melting points were determined with a Büchi melting point apparatus model 510 and were uncorrected. Optical rotations were measured at 22 ± 3°C with a JASCO DIP 310 or HORIBA SEPA-200 polarimeter in the solution of specified solvents. ^{1}H NMR spectra were measured with a JEOL EX 270 MHz spectrometer. Compounds **1** and **2** were measured with a JEOL 600 MHz spectrometer. Chemical shifts were expressed in ppm downfield from the signal for internal Me_4Si for $CDCl_3$ solutions. For solutions in D_2O, *tert*-BuOH served as the reference 1.23 and 31.1 ppm for ^{1}H and ^{13}C NMR, respectively. The FABMS for **1** and **2** were measured with a triple-stage quadrupole mass spectrometer (Finnigan MAT TSQ 700) equipped with a FAB ion source. The TOFMS for **3** and **4** were measured with MALDI I (Kratos Analytical). Radioactivity was counted with a Beckman LS 6000SC scintillation counter. Silica gel chromatography, analytical TLC and preparative TLC were done on a column of Wakogel® 200E and 300E (Wako) and glass plates coated with silica gel 60 F_{254} (E. Merck), respectively. Gels for size-exclusion chromatography (Sephadex LH-20, Biobeads S-X1) were purchased from Pharmacia and BIO•RAD, respectively. Molecular sieves were purchased from GL Science, Inc. and activated at 200 °C under vaccum prior

to use. Celite® 545 was purchased from Nacalai Tesque. All reactions in organic solvents were performed under an argon atmosphere.

Methyl 4,6-*O*-Benzylidene-3-*O*-chloroacetyl-1-thio-β-D-galactopyranoside (6). A solution of methyl 4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (5)[14] (203.4 mg, 79.3 μmol) and *n*-Bu_2SnO (261.5 mg, 1.05 mmol) in CH_2Cl_2 (6 mL) and MeOH (3 mL) was boiled under reflux for 2 h, cooled and concentrated under diminished pressure. The residue was diluted with CH_2Cl_2 (8 mL), a solution of $ClCH_2COCl$ (64 μL, 0.80 mmol) in CH_2Cl_2 (0.5 mL) was added and stirring continued for 15 min at rt. After work-up with ice, filtration on Celite and extraction with $CHCl_3$, the organic layer was washed with brine and saturated $NaHCO_3$, dried over anhyd $MgSO_4$, filtered and concentrated. The residue was subjected to silica gel column chromatography (toluene:ethyl acetate 7:1–4:1) to give **6** (199.0 mg, 76%): mp 168.0 °C (*n*-hexane–ethyl acetate, needle); $[\alpha]_D$ +81.1° (*c* 0.753, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 7.52–7.33 (m, 5H, aromatic H), 5.49 (s, 1H, PhC<u>H</u>), 4.96 (dd, 1H, $J_{2,3}$ = 9.57 Hz, $J_{3,4}$ = 3.63 Hz, H-3), 4.46 (dd, 1H, $J_{4,5}$ < 1.0 Hz, H-4), 4.35 (dd, 1H, $J_{5,6b}$ = 1.32 Hz, J_{gem} = 12.53 Hz, H-6b), 4.35 (d, 1 H, $J_{1,2}$ = 9.57 Hz, H-1), 4.16 (d, 2H, J = 2.64 Hz, ClC<u>H2</u>), 4.10 (ddd, 1H, $J_{2,OH}$ = 2.31 Hz, H-2), 4.02 (dd, 1H, $J_{5,6a}$ = 1.65 Hz, H-6a), 3.61 (bd, 1H, J < 1.0 Hz, H-5), 2.46 (d, 1H, OH-2), 2.26 (s, 3H, SMe).

Anal. Calcd for $C_{16}H_{19}ClSO_6$: C, 51.26; H, 5.12; Cl, 9.46; S, 8.55. Found: C, 51.17; H, 5.09; Cl, 9.69; S, 8.32.

Methyl 4,6-*O*-Benzylidene-3-*O*-chloroacetyl-2-*O*-(4-methylbenzoyl)-1-thio-β-D-galactopyranoside (7). To a solution of **6** (730.6 mg, 1.95 mmol) in CH_2Cl_2 (12 mL) was added pyridine (0.79 mL, 9.8 mmol) and *p*-toluyl chloride (1.0 mL, 7.6 mmol) at 0 °C. The reaction mixture was stirred at rt for 1 day and MeOH (6.0 mL) was added. The solution was concentrated under diminished pressure and the residue was subjected to silica gel column chromatography (*n*-hexane:ethyl acetate 8:1–7:1–5:1–4:1–3:1–2:1) to give **7** (823.0 mg, 86%): mp 163.5-164.0 °C (*n*-hexane–ethyl acetate, needles); $[\alpha]_D$ +75.5° (*c* 1.01, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 7.89 (d, 2H, J = 8.25 Hz, aromatic H), 7.51–7.23 (m, 7H, aromatic H), 5.77 (t, 1H, $J_{1,2}$ = $J_{2,3}$ = 9.90 Hz, H-2), 5.53 (s, 1H, PhC<u>H</u>), 5.25 (dd, 1H, $J_{3,4}$ = 3.63 Hz, H-3), 4.56 (d, 1H, H-1), 4.50 (bd, 1H, H-4), 4.40 (dd, 1H, $J_{5,6b}$ < 1.0 Hz, J_{gem} = 12.54 Hz, H-6b), 4.07 (dd, 1H, $J_{5,6a}$ < 1.0 Hz, H-6a), 4.02, 3.93 (ABq, 2H, J = 15.18 Hz, ClC<u>H2</u>), 3.69 (bs, 1H, H-5), 2.41 (s, 3H, Ph<u>Me</u>), 2.29 (s, 3H, SMe).

Anal. Calcd for $C_{24}H_{25}ClSO_7$: C, 58.46; H, 5.12; Cl, 7.19; S, 6.50. Found: C, 58.35; H, 5.06; Cl, 7.29; S, 6.49.

2-(*N*-Benzyloxycarbonyl)aminoethyl 4,6-*O*-Benzylidene-3-*O*-chloroacetyl-2-*O*-(4-methylbenzoyl)-β-D-galactopyranoside (8). A mixture of **7** (102.1 mg, 0.207

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mmol), benzyl *N*-(2-hydroxyethyl)carbamate (53.1 mg, 0.272 mmol) and powdered molecular sieves 4A (200 mg) in 1,2-dichloroethane (2 mL) was stirred at rt. After 1 h the solution was cooled to -20 °C, and then a solution of *N*-iodosuccinimide (50.5 mg, 0.224 mmol) and trifluoromethanesulfonic acid (2 µL, 0.02 mmol) in 1,2-dichloroethane (1 mL) and Et_2O (1 mL) was added. The reaction temp was raised gradually to -15 °C during 35 min. The reaction mixture was diluted with $CHCl_3$, quenched with saturated $NaHCO_3$, filtered on Celite, and extracted with $CHCl_3$. The organic layer was washed with saturated $NaHCO_3$, saturated $Na_2S_2O_3$, brine, dried over anhyd $MgSO_4$, filtered and concentrated under diminished pressure. The residue was purified by silica gel column chromatography (toluene: ethyl acetate 5:1–4:1) to give **8** (124.5 mg, 94%); mp 113.5-114.2 °C (*n*-hexane–ethyl acetate, white powder); $[\alpha]_D$ +64° (*c* 0.63, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 7.87 (d, 2H, J = 7.92 Hz, aromatic H), 7.55–7.14 (m, 12H, aromatic H), 5.64 (dd, 1H, $J_{1,2}$ = 7.92 Hz, $J_{2,3}$ = 10.56 Hz, H-2), 5.54 (s, 1H, PhC$\underline{H}$), 5.21 (dd, 1H, $J_{3,4}$ = 3.63 Hz, H-3), 5.18 (m, 1H, NH), 5.04, 4.92 (ABq, 2H, J = 12.54 Hz, PhC$\underline{H_2}$), 4.65 (d, 1H, H-1), 4.46 (d, 1H, H-4), 4.36 (d, 1H, J_{gem} = 12.54 Hz, H-6b), 4.09 (d, 1H, H-6a), 4.04, 3.95 (ABq, 2H, J = 15.18 Hz, ClC$\underline{H_2}$), 3.95, 3.68 (2m, 1Hx2, OCH_2), 3.59 (s, 1H, H-5), 3.34 (m, 2H, NCH_2), 2.36 (s, 3H, Ph$\underline{Me}$).

Anal. Calcd for $C_{33}H_{34}ClNO_{10}$: C, 61.91; H, 5.36; Cl, 5.54; N, 2.19. Found: C, 62.04; H, 5.34; Cl, 5.73; N, 2.19.

2-(*N*-Benzyloxycarbonyl)aminoethyl 4,6-*O*-Benzylidene-2-*O*-(4-methylbenzoyl)-β-D-galactopyranoside (9). To a solution of **8** (181.9 mg, 0.284 mmol) in 15 mL of EtOH-toluene (4:1) was added a hydrazine acetate (143.9 mg, 1.56 mmol) while stirring for 48 min prior to the removal of the solvents *in vacuo*. The residue was subjected to silica gel column chromatography (toluene: ethyl acetate 7:1–5:1–4:1–3:1–2:1) to give **9** (154.4 mg, 96%) as a syrup; $[\alpha]_D$ +10.0° (*c* 0.520, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 7.92 (d, 2H, J = 7.92 Hz, aromatic H), 7.54–7.13 (m, 12H, aromatic H), 5.59 (s, 1H, PhC$\underline{H}$), 5.33 (dd, 1H, $J_{1,2}$ = 7.92 Hz, $J_{2,3}$ = 9.90 Hz, H-2), 5.19 (m, 1H, NH), 5.05, 4.95 (ABq, 2H, J = 12.54 Hz, PhC$\underline{H_2}$), 4.58 (d, 1H, H-1), 4.36 (d, 1H, J_{gem} = 12.87 Hz, H-6b), 4.27 (d, 1H, $J_{3,4}$ = 3.96 Hz, H-4), 4.10 (dd, 1H, $J_{5,6a}$ < 1.0 Hz, H-6a), 3.92 (m, 2H, H-3, 1/2OCH_2), 3.69 (m, 1H, 1/2OCH_2), 3.54 (s, 1H, H-5), 3.36 (m, 2H, NCH_2), 2.62 (m, 1H, OH-3), 2.35 (s, 3H, Ph$\underline{Me}$).

Anal. Calcd for $C_{31}H_{33}NO_9$: C, 66.05; H, 5.91; N, 2.49. Found: C, 65.88; H, 5.89; N, 2.48.

2-(*N*-Benzyloxycarbonyl)aminoethyl [Methyl 2,3,4-tri-*O*-(4-methylbenzoyl)–β–D-glucopyranosyluronate]–(1→3)–4,6-*O*-benzylidene–2–*O*–(4-methylbenzoyl)-β-D-galactopyranoside (11). A mixture of **9** (97.5 mg, 0.173 mmol), methyl (2,3,4-tri-*O*-4-methylbenzoyl-α-D-glucopyranosyl trichloroacetimidate)uronate (**10**)[11] (194.4

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mg, 0.275 mmol) and powdered molecular sieves AW300 (540 mg) in toluene (11 mL) and CH_2Cl_2 (3 mL) was stirred at rt. After 35 min the solution was cooled to -20 °C, and then a solution of BF_3•OEt_2 (6 μL, 0.07 mmol) in CH_2Cl_2 (75 μL) was added. The same amount of promoter solution was added after 30 min. The reaction temp was raised gradually to rt overnight. The reaction mixture was diluted with ethyl acetate, quenched with saturated $NaHCO_3$, filtered on Celite and extracted with ethyl acetate. The crude product was roughly fractionized using gel-permeation chromatography (S-X1, toluene) and finally purified by silica gel column chromatography (toluene:ethyl acetate 10:1–5:1–2:1–1:1) to give **11** (73.9 mg, 39%) as a syrup; $[\alpha]_D$ +34.8° (*c* 0.620, $CHCl_3$). ^{1}H NMR data ($CDCl_3$): δ 7.78 (d, 2H, J = 8.25 Hz, aromatic H), 7.68 (m, 4H, aromatic H), 7.56–7.48 (m, 4H, aromatic H), 7.39–7.26 (m, 8H, aromatic H), 7.16 (d, 2H, J = 8.25 Hz, aromatic H), 7.02 (d, 4H, J = 8.25 Hz, aromatic H), 6.90 (d, 2H, J = 8.25 Hz, aromatic H), 5.75 (bt, 1H, J = 8.91 Hz, H-3'), 5.62 (bt, 1H, J = 9.57 Hz, H-4'), 5.56 (s, 1H, PhC<u>H</u>), 5.55 (dd, 1H, $J_{1,2}$ = 7.92 Hz, $J_{2,3}$ = 10.22 Hz, H-2), 5.46 (dd, 1H, $J_{1,2}$ = 7.26 Hz, $J_{2,3}$ = 8.58 Hz, H-2'), 5.18 (d, 1H, H-1'), 5.12 (m, 1H, NH), 5.01, 4.88 (ABq, 2H, J = 12.54 Hz, PhC<u>H</u>$_2$), 4.55 (d, 1H, H-1), 4.49 (d, 1H, $J_{3,4}$ = 3.30 Hz, H-4), 4.32 (d, 1H, J_{gem} = 11.87 Hz, H-6b), 4.30 (d, 1H, $J_{4,5}$ = 9.24 Hz, H-5'), 4.18 (dd, 1H, H-3), 4.09 (d, 1H, H-6a), 3.85, 3.59 (2m, 1Hx2, OCH_2), 3.59 (s, 3H, COOMe), 3.51 (s, 1H, H-5), 3.27 (m, 2H, NCH_2), 2.35, 2.34, 2.30, 2.27 (4s, 3Hx4, Ph<u>Me</u>).

Anal. Calcd for $C_{62}H_{61}NO_{18}$: C, 67.19; H, 5.56; N, 1.26. Found: C, 66.91; H, 5.64; N, 1.27.

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2-Aminoethyl (Sodium β-D-glucopyranosyluronate)-(1→3)-β-D-galactopyranoside (1). To a solution of **11** (40.9 mg, 36.9 μmol) in 6.2 mL of THF–H_2O (30:1) was added 1.25 N LiOH (0.5 mL) at 0 °C with stirring for 3.5 h. The mixture was concentrated under diminished pressure, taken up in 5 mL of MeOH–CH_2Cl_2 (4:1), and 0.5 N NaOH (1.8 mL) was added at 0 °C. The reaction temp was raised gradually to rt. After 19 h the reaction mixture was neutralized with 50% AcOH and the volatiles were removed under reduced pressure. The residue was then subjected to gel-permeation chromatography (G-15, H_2O), and the fractions containing the desired compound were freeze-dried. The residue was diluted with H_2O (5 mL) containing 5 drops of AcOH and stirred under H_2 atmosphere overnight in the presence of a catalytic amount of Pd-C. The mixture was filtered on Celite, the volatiles removed under reduced pressure, and the residue was purified by passing it through a gel-permeation column [LH-20, 0.1 M $(NH_4)_2CO_3$] and then on ion-exchange resin column [AG50W-X8 (Na^+)] to give 10.3 mg of powdered **1** (70%, 2 steps). $[\alpha]_D$ –21° (*c* 0.14, H_2O). ^{1}H NMR data (D_2O, 600MHz): δ 4.66 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1'), 4.49 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.19 (d, 1H, $J_{3,4}$ = 2.9 Hz, H-4), 4.11, 3.96 (2dt, 1Hx2, J_{vic} = 5.4 Hz, J_{gem} = 12.7 Hz, OCH_2),

3.80 (dd, 1H, $J_{2,3}$ = 9.8 Hz, H-3), 3.76 (d, 2H, J = 6.3 Hz, H-6), 3.70 (dd, 1H, H-2), 3.70 (m, 2H, H-5, 5'), 3.50 (m, 2H, H-3', 4'), 3.41 (dd, 1H, $J_{2,3}$ = 9.8 Hz, H-2'), 3.26 (t, 2H, NCH_2). ^{13}C NMR data (D_2O, 150MHz): δ 177.44 (C-6'), 105.08 (C-1'), 103.78 (C-1), 84.02 (C-3), 77.74 (C-5'), 76.84 (C-3'), 76.48 (C-5), 74.63 (C-2'), 73.25 (C-4'), 71.31 (C-2), 69.57 (C-4), 67.22 (OCH_2), 62.57 (C-6), 41.03 (NCH_2). FABMS (positive): *m/z* 444.2 $[M+2Na-H]^+$, 422.2 $[M+Na]^+$, (negative): *m/z* 398.1 $[M-H]^-$, 222.0 $[M-GlcA]^-$.

2-(*N*-Benzyloxycarbonyl)aminoethyl (2-Azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-levulinoyl-α-D-galactopyranosyl)-(1→4)-[methyl 2,3-di-*O*-(4-methylbenzoyl)-β-D-glucopyranosyluronate]-(1→3)-4,6-*O*-benzylidene-2-*O*-(4-methylbenzoyl)-β-D-galactopyranoside (13). A mixture of **9** (131.4 mg, 0.233 mmol), methyl {(2-azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-levulinoyl-α-D-galactopyranosyl)-(1→4)-[2,3-di-*O*-(4-methylbenzoyl)-α-D-glucopyranosyl trichloroacetimidate]}uronate **(12)**[10] (229.1 mg, 0.238 mmol) and powdered molecular sieves AW300 (1 g) in toluene (10 mL) and CH_2Cl_2 (3 mL) was stirred at rt. After 3 h the solution was cooled to -20 °C, and then a solution of $BF_3 \cdot OEt_2$ (27 μL, 0.24 mmol) in CH_2Cl_2 (0.67 mL) was added. The reaction temp was raised gradually to rt over 5.5 h. The reaction mixture was diluted with ethyl acetate, quenched with saturated $NaHCO_3$, filtered on Celite and extracted with ethyl acetate. The crude product was roughly fractionized using gel-permeation chromatography (S-X1, toluene) and finally purified by silica gel column chromatography (toluene:ethyl acetate 5:1–4:1–3:1–2:1–1:2–1:5) to give **13** (140.9 mg, 44%) as a syrup; $[\alpha]_D$ +112° (*c* 0.420, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 7.74–7.63 (m, 4H, aromatic H), 7.56–7.24 (m, 17H, aromatic H), 7.17-6.88 (m, 6H, aromatic H), 5.58 (bt, 1H, J = 9.07 Hz, H-3'), 5.51 (dd, 1H, $J_{1,2}$ = 7.92 Hz, $J_{2,3}$ = 10.56 Hz, H-2), 5.46, 5.45 (2s, 1Hx2, 2PhC<u>H</u>), 5.31 (bdd, 1H, J = 7.43, 9.07 Hz, H-2'), 5.12–5.09 (m, 3H, H-1', 1", NH), 5.09 (dd, 1H, $J_{3,4}$ = 3.30 Hz, H-3"), 5.01, 4.87 (ABq, 2H, J = 12.21 Hz, PhC<u>H2</u>), 4.51 (d, 1H, H-1), 4.37–4.29 (m, 4H, H-4, 6b or 6"b, 4', 4"), 4.15 (bd, 1H, J = 12.80 Hz, H-6"b or 6b), 4.12 (d, 1H, $J_{4,5}$ = 9.57 Hz, H-5'), 4.07 (dd, 1H, $J_{3,4}$ = 3.63 Hz, H-3), 4.04 (bd, 1H, J = 10.88 Hz, H-6a or 6"a), 3.96 (bd, 1H, J = 12.54 Hz, H-6"a or 6a), 3.88-3.81 (m, 1H, 1/2OCH_2), 3.77 (dd, 1H, $J_{1,2}$ = 3.30 Hz, $J_{2,3}$ = 11.22 Hz, H-2"), 3.68 (s, 3H, COOMe), 3.61 (s, 1H, H-5"), 3.61–3.53 (m, 1H, 1/2OCH_2), 3.47 (s, 1H, H-5), 3.26 (m, 2H, NCH_2), 2.74–2.53 (m, 4H, $2CH_2$), 2.34, 2.31, 2.29 (3s, 3Hx3, 3Ph<u>Me</u>), 2.08 (s, 3H, $COCH_3$).

Anal. Calcd for $C_{72}H_{74}N_4O_{23}$: C, 63.42; H, 5.48; N, 4.11. Found: C, 63.55; H, 5.59; N, 3.97.

2-(*N*-Benzyloxycarbonyl)aminoethyl (2-Acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-levulinoyl-α-D-galactopyranosyl)-(1→4)-[methyl 2,3-di-*O*-(4-methylbenz-

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oyl)–β–D–glucopyranosyluronate]–(1→3)–4,6–*O*–benzylidene–2-*O*-(4-methylbenzoyl)-β-D-galactopyranoside (14). To a solution of **13** (32.7 mg, 24.0 mmol) in pyridine (2 mL) was added AcSH (2 mL). The reaction mixture was stirred at 50 °C overnight and purified on a column of silica gel (toluene:ethyl acetate 2:1–1:1– ethyl acetate:MeOH 20:1–15:1) to give **14** (28.7 mg, 87%) as a syrup; $[\alpha]_D$ +142° (*c* 0.127, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 7.70–7.65 (m, 4H, aromatic H), 7.53–7.31 (m, 17H, aromatic H), 7.09 (d, 2H, J = 8.57 Hz, aromatic H), 7.02 (d, 2H, J = 7.92 Hz, aromatic H), 6.88 (d, 2H, J = 8.25 Hz, aromatic H), 5.57 (bt, 1H, J = 8.91 Hz, H-3'), 5.50 (dd, 1H, $J_{1,2}$ = 7.92 Hz, $J_{2,3}$ = 10.23 Hz, H-2), 5.46, 5.45 (2s, 1Hx2, 2PhC<u>H</u>), 5.33 (dd, 1H, $J_{1,2}$ = 7.26, $J_{2,3}$ = 9.24 Hz, H-2'), 5.10 (d, 1H, H-1'), 5.07 (d, 1H, $J_{1,2}$ = 3.63 Hz, H-1"), 5.02 (dd, 1H, $J_{2,3}$ = 11.55 Hz, $J_{3,4}$ = 3.30 Hz, H-3"), 5.01, 4.88 (ABq, 2H, J = 12.20 Hz, PhC<u>H</u>$_2$), 4.61 (m, 1H, H-2"), 4.52 (d, 1H, H-1), 4.40 (t, 1H, J $_{3,4}$ = $J_{4,5}$ = 9.24 Hz, H - 4'), 4.31 (m, 2H, H-4, 6b), 4.21 (bs, 1H, H-4"), 4.19 (bd, 1H, J $_{gem}$ = 11.55 Hz, H-6"b), 4.12 (d, 1H, H-5'), 4.05 (d, 1H, J = 11.54 Hz, H-6a), 3.97 (d, 1H, H-6"a), 3.84 (m, 1H, 1/2OCH_2), 3.69 (s, 3H, COOMe), 3.57 (m, 1H, 1/2OCH $_2$), 3.54 (s, 1H, H-5"), 3.48 (s, 1H, H-5), 3.25 (m, 2H, NCH $_2$), 2.8–2.5 (m, 4H, 2CH $_2$), 2.34, 2.30, 2.29 (3s, 3Hx3, 3Ph<u>Me</u>), 2.05 (s, 3H, COCH $_3$), 1.54 (s, 3H, NAc).

Anal. Calcd for $C_{74}H_{78}N_2O_{24}$: C, 64.42; H, 5.71; N, 2.03. Found: C, 64.42; H, 5.72; N, 2.12.

2-Aminoethyl (2-Acetamido-2-deoxy-α-D-galactopyranosyl)-(1→4)-(β-D-glucopyranosylonic acid)-(1→3)-β-D-galactopyranoside (2). To a solution of **14** (126.2 mg, 91.5 mmol) in THF (15 mL) was added 1.25 N LiOH (1.5 mL) at 0 °C, and the mixture was stirred for 2 h raising the temp gradually to rt. The mixture was concentrated under reduced pressure, taken up in 14.4 mL of MeOH–CH_2Cl_2 (5:1), and 0.5 N NaOH (7 mL) was added at 0 °C. The reaction temp was raised gradually to rt, additional 0.5 N NaOH (2.5 and 0.7 mL) were added after 1 and 4 days, respectively. The reaction mixture was neutralized with 50% AcOH after 4 days and the volatiles were removed under reduced pressure. The residue was diluted with H_2O (20 mL) containing AcOH (1 mL) and stirred under H_2 atmosphere overnight in the presence of a catalytic amount of Pd/C. The mixture was filtered on Celite and freeze-dried. The residue was finally purified by repeated gel-permeation column chromatography [LH-20, 0.1M $(NH_4)_2CO_3$] to give 39.9 mg of **2** (72%, 2 steps); $[\alpha]_D$ +102° (*c* 0.36, H_2O). ^1H NMR data (D_2O, 600MHz): δ 5.43 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1"), 4.64 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1'), 4.49 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.16 (1H, $J_{3,4}$ = 2.9 Hz, H-4), 4.15 (1H, H-2"), 4.11, 3.97 (1Hx2, OCH_2), 3.97 (1H, H-4"), 3.93 (1H, H-5"), 3.86 (1H, $J_{2,3}$ = 11.2 Hz, $J_{3,4}$ = 2.9 Hz, H-3"), 3.77 (1H, H-3), 3.76 (4H, H-6, 4', 5'), 3.71 (1H, H-5), 3.70 (3H, H-2, 6"), 3.68 (1H, H-3'), 3.41 (1H, H-2'), 3.25 (2H, NCH_2), 2.03 (3H, NAc). ^{13}C

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NMR data (D_2O, 150MHz): δ 177.06 (C-6'), 176.07 (<u>C</u>OCH3), 105.16 (C-1'), 103.75 (C-1), 98.28 (C-1"), 84.17 (C-3), 78.02 (C-5'), 77.87 (C-3'), 76.80 (C-4'), 76.46 (C-5), 75.14 (C-2'), 72.19 (C-5"), 71.22 (C-2), 69.65, 69.62 [C-4, 4"(changeable)], 68.96 (C-3"), 67.20 (OCH_2), 62.57 (C-6), 62.19 (C-6"), 51.17 (C-2"), 41.03 (NCH_2), 23.49 (CH_3). FABMS (positive): *m/z* 603.5 $[M+H]^+$, (negative): *m/z* 601.2 $[M-H]^-$.

2-(6-Biotinylamido)hexanamidoethyl (β-D-glucopyranosylonic acid)-(1→3)-β-D-galactopyranoside (3). To a solution of 50 μL of 0.1 M aq $NaHCO_3$ was added 0.72 μmol of **1**. A five-fold excess of NHS-LC-biotin (2 mg, Pierce) was added to the solution and then incubated at rt for one hour. Then 1 μL of AcOH and 200 μL of H_2O were added to the reaction mixture, and the solution was applied to a C18 reverse phase micro spin column, preactivated with MeOH and preequilibrated with H_2O. The column was washed four times with 200 μL of H_2O, and eluted twice with 200 μL of 50% MeOH. The eluate was dried, and the residues were dissolved in DMSO (10 μL), then diluted with CH_3CN (390 μL). The whole suspension was applied to a silica gel micro spin column, and washed with 400 μL of CH_3CN. The products were eluted with a stepwise gradient of H_2O in CH_3CN (5, 10, 15, 20, 25 and 50%). Each fraction was analyzed by TLC ($CHCl_3$:MeOH:H_2O 60:35:8) to locate the sugar derivatives. The eluates containing 25% and 50% H_2O in CH_3CN were combined, dried, and reconstituted in H_2O at 10 mM; the yield was quantitative. TOFMS (positive): *m/z* 737.4 $[M+H]^+$.

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2-(6-Biotinylamido)hexanamidoethyl (2-Acetamido-2-deoxy-α-D-galactopyranosyl)-(1→4)-(β-D-glucopyranosylonic acid)-(1→3)-β-D-galactopyranoside (4). The trisaccharide **2** was also biotinylated in the same way as above to give **4** quantitatively. TOFMS (positive): *m/z* 942.2 $[M+H]^+$.

Enzymatic addition of [^{3}H]GalNAc to 3. α-GalNAc transferase reaction was carried out in 50 μL of buffer containing 50 mM MES, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 40 μM UDP-[^{3}H]GalNAc (2.5 μCi) donor, 2 mM **3**, 50 μg of microsome fractions from mouse fetal brain, and 0.1% Triton X-100. After 16 h of incubation at rt, the mixture was heated at 100 °C for 5 min, and diluted with 350 μL of 20 mM $NaHCO_3$/5 mM EDTA. The solution was applied to a C18 reverse phase micro spin column and worked up as above. The dried residue from 50% MeOH elution was dissolved in 150 μL of buffer, 20 mM of Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, and applied to 1.5 mL *Helix pomatia* agglutinin (HPA) lectin affinity column (EY Laboratories, Inc.). The gel was incubated for 24 h at 4 °C and then washed with the buffer. Each 300 μL fraction was collected, and radioactivity was counted. The desired [^{3}H]-**4** was retarded on the column and separated from the parent biotinylated di-

saccharide **3**. Fractions containing radioactivity were combined and desalted using a C18 reverse phase micro spin column.

Binding of [^{3}H]-4 to StAv. StAv (5 μg, 21 munit, Sigma) in 50 μL of phosphate-buffered saline was mixed with approximately 20 pmol of [^{3}H]-**4** (50,000 dpm), and incubated at rt overnight. The mixture was applied to a 0.5 x 28 cm Sephadex G-50 column and eluted with 20 mM Tris-HCl, pH 7.4. One mL-fractions were collected and radioactivity was measured. A similar [^{3}H]-**4** was applied to a similar column and analyzed in the same way. The result is shown in Fig. 1.

ACKNOWLEDGMENTS

This work was financially supported by the CREST program of Japan Science and Technology Corporation (JST), the Kato Memorial Bioscience Foundation and by the American Cancer Society Grant BE-242. The authors are grateful to Drs. S. Manabe and H. Koshino (RIKEN) for NMR measurements and Dr. S. Kurono (RIKEN) for FABMS measurements. The authors also thank Ms. M. Yoshida and her staff (RIKEN) for elemental analyses.

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