Synthesis and Antigenicity against Human Sera of a Biotin-Labeled Oligosaccharide Portion of a Glycosphingolipid from the Parasite Echinococcus multilocularis

Noriyasu Hada, a Ayaka Kitamura, a Kimiaki Yamano, b Frank Schweizer, c and Fumiyuki Kiuchi* a

a Faculty of Pharmacy, Keio University; 1–5–3 Shibaokoen, Minato-ku, Tokyo 105–8512, Japan; b Hokkaido Institute of Public Health; Kita-19, Nishi-12, Kita-ku, Sapporo 060–0819, Japan; and c Departments of Chemistry and Medical Microbiology, University of Manitoba; Winnipeg, Manitoba R3T 2N2, Canada.

Received February 29, 2016; accepted March 14, 2016

Synthesis of a biotinylated analog of the carbohydrate portion of a glycosphingolipid from the parasite Echinococcus multilocularis has been achieved. We synthesized β-D-Galp-(1→6)-β-D-Galp-(1→6)-α-L-Fucp-(1→3)-β-D-Galp-(1→R; biotin probe) (1) and compared the antigenicity by an enzyme linked immunosorbent assay (ELISA) with biotinylated trisaccharide α-D-Galp-(1→4)-β-D-Galp-(1→3)-α-D-Galp-(1→R; biotin probe) (F), which has been shown to have significant antigenicity. Both of the oligosaccharides reacted with sera of alveolar echinococcosis (AE) patients, but showed different reactivity. Among the 60 sera of AE patients, more sera reacted with the linear sequence Galα1→4Galβ1→3GalNAcβ1→R of oligosaccharide (F) than for branched compound 1. Some sera showed high specificity to one of the compound, indicating that the antibodies in the sera of AE patients differ in their specificity to recognize carbohydrate sequences of glycosphingolipids. Our results demonstrate that both of the biotinylated oligosaccharides 1 and F have good serodiagnostic potential and are complementary to detect infections caused by the parasite Echinococcus multilocularis.

Key words glycosphingolipid; Echinococcus multilocularis; biotin probe; chemical synthesis

In our continuing studies to elucidate the biological function of carbohydrate moieties of glycosphingolipids (GSLs) and glycoproteins (GPs) from invertebrates, we have synthesized oligosaccharides found in various Protostomia phyla.1–16 Our interests have been focused on the unique saccharide chain structure of GSLs and GPs found in several parasites, Echinococcus multilocularis, Schistosoma mansoni,13,14 Ascaris suum15 and Toxocara canis.16 Among them, we are strongly interested in the structures of GSLs and GPs from E. multilocularis. E. multilocularis is a parasite, which belongs to the class Cestoda of the phylum Platyhelminthes and causes alveolar echinococcosis (AE), a severe parasitic zoonosis that can be fatal without appropriate treatment. Therefore, a simple and reliable diagnosis method is important. Diagnosis of AE is based on immunological response, detected by enzyme-linked immunosorbent assay (ELISA) for primary screening and by the Western blotting method for secondary confirmation tests, of the serum of patients to the crude antigen extracted from E. multilocularis cysts.17–19 However, no perfect antigen exists that sera from AE patients recognized a neutral glycosphingolipid fraction from E. multilocularis and determined the structures of some of the glycosphingolipids isolated from this fraction. Furthermore, Hülsmeier et al. reported20 that Em2, an antigen extracted from E. multilocularis, is a mucin-type glycoprotein. Based on these information, we synthesized four glycosphingolipids20 and five carbohydrate structures of glycoproteins6,20 of E. multilocularis, and examined antigenicity of the pure compounds by ELISA for their serodiagnostic potential.5,6,23 Among the synthesized compounds (A–I, a glycosphingolipid, Galβ1→6(Fucα1→3)Galβ1→6Galβ1→Cer (D), and a biotinylated carbohydrate, Galα1→4Galβ1→3GalNAcβ1→R-biotin-probe (F), showed good serodiagnostic potential for AE (Fig. 1). However, the potential of the two compounds cannot be compared directly because of the difference of the reducing end structure, a ceramide and a biotin probe. In this paper, we describe the synthesis of the biotinylated analog of D (I) and compare the antigenicity against sera of AE patients with compound F.

Results and Discussion

Chemistry The synthetic strategy for oligosaccharide 1 is shown in Chart 1. Suitably protected monosaccharide derivatives (2–5) were chosen as building blocks. As a temporary protecting group of the reducing end for the target compound, 5-(methoxy carbonyl)pentyl group was chosen to ensure future conjugation to biotin for ELISA. The synthetic route for the target compound 1 is shown in Charts 2–4. Initially, reducing end monosaccharide acceptor 2 was prepared from commercially available 1,2,3,4,6-pentaacetyl-β-D-galactose in a five-step process (Chart 2). At first, coupling of 1,2,3,4,6-pentaacetyl-β-D-galactose with methyl 6-hydroxyhexanoate in the presence of SnCl2 in dry CH2Cl2 for 16h at room temperature gave compound 6 (47%). Com-
pound 6 was converted to the glycosyl acceptor 2 by a series of reactions involving deacetylation, protection of primary alcohol with tert-butyldiphenylsilyl (TBDPS) chloride and benzoylation (7) (57%, 3 steps), followed by removal of the TBDPS group (94%). Acceptor 3 was prepared from known 2-(trimethylsilyl)ethyl 3-O-(2-naphthylmethyl)-β-D-galactopyranoside (8)24) by the following five-step procedure. Protection of primary alcohol with tert-butyldimethylsilyl (TBDMS) chloride, followed by benzoylation gave compound 9 (79%, 2 steps). Selective removal of the 3-O-naphthylmethyl (Nap) group from 9 by 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) followed by chloroacetylation produced compound 10 (86%, 2 steps), and finally deprotection of the TBDMS group in 10 under an acidic condition by TsOH afforded the acceptor 3 in 81% yield (Chart 3).

Synthesis of the oligosaccharide 1 was initiated by coupling of glycosyl donor 425) to galactose-based acceptor 3 in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf)26) to produce disaccharide 13 in 90% yield. The β-glycosidic linkage in 13 was confirmed by 1H-NMR spectroscopy. The anomeric proton of the nonreducing end galactose moiety appeared as a doublet with a homonuclear coupling constant of 7.8 Hz. Selective removal of 2-(trimethylsilyl)ethyl (TMS-ethyl) group in 13 with trifluoroacetic acid (TFA) in CH2Cl2, followed by treatment with CCl3CN in the presence of 1,8-diazabicyclo[5,4,0]-7-undecene (DBU)27) provided

---

Fig. 1. Structures of the Compounds A–I, Derivatives of the Oligosaccharides from the Parasite Echinococcus multilocularis, Synthesized in the Previous Report

Chart 1. Synthetic Strategy of Compound 1
α-trichloroacetimidate 14. Glycosylation of disaccharide donor 14 with acceptor 2 in the presence of TMSOTf and MS4Å in CH2Cl2 produced desired trisaccharide 15 in 90% yield. Removal of the 3-O-chloroacetyl group in 15 was achieved with thiourea yielding trisaccharide acceptor 16 in 93% yield. Glycosylation of the acceptor 16 with the donor 5 in the presence of N-iodosuccinimide (NIS), trifluoromethanesulfonic acid and MS 4 Å in CH2Cl2 afforded desired disaccharide 17 in 99% yield, as evidenced by 1H-NMR spectroscopy (H-1 of Fuc δ 5.06 ppm, J = 3.6 Hz). Deprotection of the benzyl group in 17 under neutral condition by hydrogenation over 10% Pd–C, and removal of the benzoyl groups under the Zemplén condition, followed by purification by column chromatography on Sephadex LH-20, furnished desired precursor 18. Conversion of 18 into its ethylenediamine monoamide by exposure to ethylenediamine followed by conjugation with N-hydroxysuccinimide ester of biotin (biotin-NHS) afforded the target biotinylated tetrasaccharide after column chromatographic purification on Sephadex LH-20 (Chart 4). The structure and purity of 1 was demonstrated by its 1H-NMR and high resolution-electrospray ionization (HR-ESI)-MS data.

Antigenicity of the Oligosaccharides  Reactivity of the oligosaccharides 1 and 5 to sera of AE patients was examined by ELISA using microplates coated with streptavidin (Fig. 2). As reported previously, compound 5 showed strong response to the AE group. On the contrary, although some of the patients’ sera reacted strongly and the average of absorbance values of AE group (n=60) was significantly higher than that of the NH group (n=60) (p<0.001, Student’s t-test), the average reactivity of compound 1 was not strong compared with that of 5. However, comparison of the reactivity of individual serum against 1 and 5 revealed that each serum showed different reactivity to the oligosaccharides (Fig. 3). Most of the sera which reacted strongly (absorbance >0.5) with 5 showed weak reactivity (absorbance <0.5) with 1. On the contrary, the sera which reacted strongly (absorbance >0.5) with 1 showed less reactivity to 5 except for 2 sera. Ten of the sera reacted to 5 more than 10 fold (maximum 25 fold) compared with 1 and one serum showed specificity to 1 (12 fold compared with 5). These results indicate that the specificity of the antibodies in the sera is not uniform, and the two oligosaccharides are complementary as the antigen for serological diagnosis of AE. The average reactivity of 5 was stronger than that of 1, suggesting that the linear trisaccharide structure of 5 is important as a core structure for the antigenicity. However, further studies will be necessary to elucidate structure–antigenicity relationship of oligosaccharides found in E. multilocularis.

Conclusion  We prepared oligosaccharide-biotin conjugate 1 and compared the antigenicity with the previously synthesized oligosaccharide derivative 5 by ELISA for their serodiagnostic potential. Sixty sera of AE patients showed different reactivity to the two oligosaccharides, which indicated that the antibodies in the sera of AE patients recognize a different glycosphingolipid sequences. As the antigenicity of the two oligosaccharides was complementary, a mixture of the two compounds will show superior serodiagnostic potential for diagnosis of AE than a single compound.

Experimental  General Optical rotations were measured with a Jasco P-1020 digital polarimeter. 1H- and 13C-NMR spectra were recorded with a Varian 500 FT NMR spectrometer. Me4Si
and acetone were used as internal standards for CDCl₃ and D₂O, respectively. ESI-HR-MS was recorded on a JEOL MS T-100 mass spectrometer. TLC was performed on Silica Gel 60 F254 (E. Merck) with detection by quenching of UV fluorescence and by charring with 10% H₂SO₄. Column chromatography was carried out on Silica Gel 60 (E. Merck). 2,3,4,6-Tri-O-benzoyl-α-D-galactopyranosyl trichloroacetimide (4), 2-(trimethylsilyl)ethyl 3-O-(2-naphthylmethyl)-β-D-galactopyranoside (8), phenyl 2,3,4-tri-O-benzyl-L-thio-α-L-fucopyranoside (5) were prepared as reported. 1,2,3,4,6-Pentaacetyl-β-D-galactose was purchased from Tokyo Chemical Industry Co.

5-(Methoxycarbonyl)pentyl 2,3,4,6-Tetra-O-acetyl-β-D-galactopyranoside (6) To a solution of 1,2,3,4,6-penta-O-acetyl-β-D-galactopyranose (500 mg, 1.28 mmol) in CH₂Cl₂ (5.0 mL) cooled at 0°C were added methyl 6-hydroxyhexanoate (277 mg, 1.92 mmol), and 4Å powdered molecular sieves (250 mg) and SnCl₄ (150 µL, 1.28 mmol). The mixture was stirred for 17 h at room temperature. Et₃N (0.1 mL) was added, and the mixture was filtered, washed with cold water, dried (MgSO₄), and concentrated. The residue was purified by silica gel column chromatography using 2:1 hexane-EtOAc as eluent to give 6 (288 mg, 47%). [α]D²⁵ −14.9° (c=0.1, CHCl₃). 1H-NMR (CDCl₃) δ: 5.39 (1H, d, J₃,4 = 3.1 Hz, H-4), 5.20 (1H, dd, J₂,3 = 10.3 Hz, J₁,2 = 7.9 Hz, H-2), 5.02 (1H, dd, H-3), 4.46 (1H, d, H-1), 4.20–4.11 (2H, m, H-6a, b), 3.92–3.87 (2H, m, H-5, –OCH₂CH₂CH₂CH₂CH₂–), 3.67 (3H, s, –OC₃H₃), 3.50–3.46 (1H, m, –OC₃H₂CH₂CH₂CH₂–), 2.15–1.99 (12H, m, Ac×4). 13C-NMR (CDCl₃) δ: 173.9, 170.3, 170.2, 170.1, 169.2, 101.2 (C-1), 70.8 (C-3), 70.5 (C-5), 69.7

Char 4. Synthesis of Tetrasaccharide 1

![Synthesis of Tetrasaccharide 1](image-url)
(–OCH2CH2CH2CH2CH2–), 68.8 (C-2), 67.0 (C-4), 61.2 (C-6), 51.4 (CH3), 33.8, 29.0, 25.3, 24.5, 20.61, 20.55, 20.5. HR-ESI-MS: Calcd for C12H32O12Na: m/z 499.1791. Found: 499.1793 [M+Na]+.

5-(Methoxycarbonyl)pentyl 2,3,4-Tri-O-benzoyl-6-O-tert-butylidiphenylsilyl-β-D-galactopyranoside (7) To a solution of 6 (270 mg, 0.57 mmol) in MeOH (5.0 mL) was added NaOMe (10 mg) and stirred for 30 min at room temperature, then neutralized with Amberlite IR 120 [H+]. The mixture was filtered and concentrated to give a residue. To a solution of this residue in N,N-dimethylformamide (DMF) (2.5 mL) was added imidazole (73.5 mg, 1.08 mmol) and tert-butylidiphenylsilyl-chloride (169 µL, 0.65 mmol) at 0°C, and the reaction mixture was stirred for 3 h at 0°C. The mixture was diluted with CHCl3, washed with 5% HCl, aq NaHCO3 and water, dried (MgSO4), and concentrated. The product was purified by silica gel column chromatography using 2:1 toluene–acetone as eluent to give 5-(methoxycarbonyl)pentyl 6-O-tert-butylidiphenylsilyl-β-D-galactopyranoside. To a solution of this compound in pyridine (2 mL) was added benzoyl chloride (198 µL, 1.70 mmol). The mixture was stirred for 18 h at 0°C, then diluted with CHCl3, washed with 5% H Cl, aq NaHCO3 and water, dried (MgSO4), and concentrated. The product was purified by silica gel column chromatography using 20:1 toluene-acetone as eluent to give compound 7 (279 mg, 57% 3 steps); [α]D25 +80.6° (c=1.1, CHCl3). 1H-NMR (CDCl3) δ: 8.04–7.09 (25H, m, 5×Ph), 6.04 (1H, d, J3,4=3.4 Hz, H-4), 5.70 (1H, d, J1,2=7.6 Hz, J2,3=10.4 Hz, H-2), 5.62 (1H, dd, J3,4), 4.72 (1H, d, J4,5), 4.06 (1H, m, H-5), 3.91 (1H, dt, –OC2H5), 3.49 (1H, dt, –OC2H5), 3.60 (3H, s, –OC3H5), 3.49 (1H, dt, –OC2H5), 3.49 (1H, dt, –OC2H5), 3.49 (1H, dt, –OC2H5). 13C-NMR (CDCl3) δ: 173.9, 165.6, 165.4, 165.2, 135.5, 135.4, 133.2, 133.1, 132.9, 132.5, 130.0, 129.8, 129.7, 129.6, 129.53, 129.48, 129.45, 129.0, 128.5, 128.3, 128.2, 127.7, 127.6, 101.6 (C-1), 73.8 (C-5), 71.9 (C-3), 70.1 (C-2), 69.8 (–OCH2CH2CH2CH2CH2–), 67.9 (C-4), 61.3 (C-6), 51.4 (CH3), 33.7, 29.0, 26.6, 25.3, 24.4, 19.0. HR-ESI-MS: Calcd for C50H54O11NaSi: m/z 881.3332. Found: 881.3322 [M+Na]+.

Fig. 2. ELISA Reaction of the Oligosaccharides I and F with Human Sera


(–OCH2CH2CH2CH2CH2–), 68.8 (C-2), 67.0 (C-4), 61.2 (C-6), 51.4 (CH3), 33.8, 29.0, 25.3, 24.5, 20.61, 20.55, 20.5. HR-ESI-MS: Calcd for C12H32O12Na: m/z 499.1791. Found: 499.1793 [M+Na]+.

5-(Methoxycarbonyl)pentyl 2,3,4-Tri-O-benzoyl-6-O-tert-butylidiphenylsilyl-β-D-galactopyranoside (7) To a solution of 6 (270 mg, 0.57 mmol) in MeOH (5.0 mL) was added NaOMe (10 mg) and stirred for 30 min at room temperature, then neutralized with Amberlite IR 120 [H+]. The mixture was filtered and concentrated to give a residue. To a solution of this residue in N,N-dimethylformamide (DMF) (2.5 mL) was added imidazole (73.5 mg, 1.08 mmol) and tert-butylidiphenylsilyl-chloride (169 µL, 0.65 mmol) at 0°C, and the reaction mixture was stirred for 3 h at 0°C. The mixture was diluted with CHCl3, washed with 5% HCl, aq NaHCO3 and water, dried (MgSO4), and concentrated. The product was purified by silica gel column chromatography using 2:1 toluene–acetone as eluent to give 5-(methoxycarbonyl)pentyl 6-O-tert-butylidiphenylsilyl-β-D-galactopyranoside. To a solution of this compound in pyridine (2 mL) was added benzoyl chloride (198 µL, 1.70 mmol). The mixture was stirred for 18 h at 0°C, then diluted with CHCl3, washed with 5% HCl, aq NaHCO3 and water, dried (MgSO4), and concentrated. The product was purified by silica gel column chromatography using 20:1 toluene-acetone as eluent to give compound 7 (279 mg, 57% 3 steps); [α]D25 +80.6° (c=1.1, CHCl3). 1H-NMR (CDCl3) δ: 8.04–7.09 (25H, m, 5×Ph), 6.04 (1H, d, J3,4=3.4 Hz, H-4), 5.70 (1H, d, J1,2=7.6 Hz, J2,3=10.4 Hz, H-2), 5.62 (1H, dd, H-3), 4.72 (1H, d, J4,5), 4.06 (1H, m, H-5), 3.91 (1H, dt, –OC2H5), 3.49 (1H, dt, –OC2H5), 3.60 (3H, s, –OC3H5), 3.49 (1H, dt, –OC2H5). 13C-NMR (CDCl3) δ: 173.9, 165.6, 165.4, 165.2, 135.5, 135.4, 133.2, 133.1, 132.9, 132.5, 130.0, 129.8, 129.7, 129.6, 129.53, 129.48, 129.45, 129.0, 128.5, 128.3, 128.2, 127.7, 127.6, 101.6 (C-1), 73.8 (C-5), 71.9 (C-3), 70.1 (C-2), 69.8 (–OCH2CH2CH2CH2CH2–), 67.9 (C-4), 61.3 (C-6), 51.4 (CH3), 33.7, 29.0, 26.6, 25.3, 24.4, 19.0. HR-ESI-MS: Calcd for C50H54O11NaSi: m/z 881.3332. Found: 881.3324 [M+Na]+.

5-(Methoxycarbonyl)pentyl 2,3,4-Tri-O-benzoyl-β-D-galactopyranoside (2) A solution of 7 (279 mg, 0.33 mmol) and acetic acid (92 µL, 1.63 mmol) in tetrahydrofuran (THF) (1.6 mL) was added 1 M tetrabutylammonium fluoride (TBAF) in THF (640 µL, 0.65 mmol) at 0°C and then stirred for 18 h. After concentration of the mixture, the residue was
dissolved in water, extracted with CHCl₃, washed with aq NaHCO₃ and water, dried (MgSO₄), and concentrated. The product was purified by silica gel column chromatography using 27:7 toluene–EtOAc as eluent to give compound 2 (192 mg, 79%). 

A solution of 9 (208 mg, 0.28 mmol) in CH₂Cl₂–H₂O (19:1, 2.0 mL) was added DDQ (127 mg, 0.56 mmol) at room temperature and then stirred for 17 h. The precipitates were filtrated off and washed with CHCl₃. After concentration, the residue was dissolved in water, extracted with CHCl₃, washed with saturated aqueous NaHCO₃, dried (MgSO₄), and concentrated. The product was purified by silica gel column chromatography (6:1 hexane–AcOEt) as eluent to give 6-hydroxyl compound (148 mg). To a solution of this compound in CH₂Cl₂ (2.4 mL) was added CH₃COCl (39 µL, 0.50 mmol) and pyridine (0.4 mL). The reaction mixture was stirred for 45 min. at 0°C, then diluted with CHCl₃, washed with 5% HCl, aq NaHCO₃ and water, dried (MgSO₄), and concentrated. The product was purified by silica gel column chromatography using 15:1 hexane–EtOAc as eluent to give compound 10 (164 mg, 86% 2 steps). δ° 0.5 +58.1° (c=0.5, CHCl₃). ¹H-NMR (CDCl₃) δ: 8.06–7.21 (10H, m, 2 × Ph), 5.71 (1H, d, J₃,4=3.4Hz, H-4), 5.48 (1H, d, J₄,5=7.9Hz, J₃,4=10.6Hz, H-2), 5.33 (1H, dd, H-3), 4.65 (1H, d, H-1), 3.96 (1H, dt, –OC₂H₅–), 3.87 (1H, br t, H-5), 3.85 and 3.78 (2H, each d, J=14.7Hz, –OC₂H₅–), 3.77–3.65 (2H, m, H-6a, b), 3.52 (1H, dd, H-2, –OC₂H₅–), 2.76–0.74 (2H, m, –OC₂H₅–), –0.10 and –0.16 (15H, m, –OSi(CH₃)₂–), –CH₃, –0.14 (9H, m, Si(CH₃)₃). ¹³C-NMR (CDCl₃) δ: 206.9, 166.8, 165.8, 165.0, 133.4, 133.2, 129.9, 129.7, 128.5, 128.4, 100.9 (C-1), 73.6 (C-5), 73.3 (C-3), 69.7 (C-2), 67.6 (–OC₂H₅–), 67.5 (C-4), 60.6 (C-6), 40.5 (Si(CH₃)₃). 30.8, 25.6 (–OC₂H₅–), –1.6, –5.7, –5.8. HR-ESI-MS: Calcd for C₃₆H₄₇ClO₉NaSi: m/z 765.3255. Found: 765.3291 [M+Na]⁺.

**Fig. 3. Correlation of the Reactivity of Each Serum to Oligosaccharides A and F**
galactopyranoside (15) Compound 15 was prepared from 14 (441 mg, 0.37 mmol) and 2 (192 mg, 0.31 mmol) as described for preparation of 13. The product was purified by silica gel column chromatography (3:2 hexane–EtOAc) to give 15 (460 mg, 90%). \([\alpha]_D^{25} +76.2^\circ\) (c=1.1, CHCl3). 1H-NMR (CDCl3) \(\delta = 8.11–7.21\) (45H, m, 9\(\times\)Ph), 5.87–5.85 (2H, m, H-1 of Gal c, Gal a), 5.79 (1H, d, \(J_{1,2} = 3.3\) Hz, H-4 of Gal b), 5.69–5.62 (2H, m, H-2 of Gal c, Gal a), 5.54–5.48 (3H, m, H-2 of Gal b, H-3 of Gal c, Gal a), 5.34 (1H, dd, \(J_{1,2} = 10.51\) Hz, H-3 of Gal b), 4.68 (1H, d, \(J_{1,2} = 7.9\) Hz, H-1 of Gal a). 13C-NMR (CDCl3) \(\delta = 173.9, 166.6, 165.7, 165.5, 165.4, 165.3, 165.2, 165.0, 164.9, 133.6, 133.3, 133.2, 131.0, 130.3, 130.5, 129.79, 129.70, 129.6, 129.41, 129.31, 129.26, 129.24, 129.17, 129.94, 128.9, 128.7, 128.6, 128.5, 128.49, 128.47, 128.44, 128.43, 128.3, 128.22, 128.20, 101.4 (C-1 of Gal a), 100.4 (C-1 of Gal c), 76.7, 72.8, 72.7, 71.9, 71.62, 71.57, 71.0, 69.83, 69.77, 69.6, 69.5, 68.6, 67.8, 67.7, 67.3, 65.5, 61.3, 51.4, 40.5, 33.6, 28.9, 25.3, 24.4, MALDI-TOF-MS: Caled for C_{34}H_{53}O_{28}Na: m/z 1676.4501. Found: 1676 [M+Na]^+. 5-(Methoxycarbonyl)pentyl 2,3,4,6-Tetra-O-benzyl-β-D-galactopyranosyl-(1→6)-2,3,4-di-O-benzoyl-β-D-galactopyranosyl-(1→6)-2,3,4,6-Tetra-O-benzyl-β-D-galactopyranosyl-(1→6) 2,4-di-O-benzyl-β-D-galactopyranosyl Trichloroacetimide (14) To a solution of 13 (653 mg, 0.57 mmol) in CH_{2}Cl_{2} (2.5 mL) cooled to 0°C was added CF_{3}CO_{2}H (5.0 mL), and the mixture was stirred for 30 min at room temperature and concentrated. EtOAc–toluene (1:2) were added and the mixture was concentrated to give the reducing sugar. To a solution of the residue in CH_{2}Cl_{2} (3.0 mL) cooled at 0°C were added DBU (25 µL, 171 µL) and CCl_{4}CN (575 µL, 5.71 mmol). The mixture was stirred for 2h at 0°C. The mixture was concentrated and the residue was purified by silica gel column chromatography using 2:1 hexane–EtOAc as eluent to give 14 (680 mg, quant.). \([\alpha]_D^{25} +75.0^\circ\) (c=0.8, CHCl3). 1H-NMR (CDCl3) \(\delta = 8.36\) (1H, s, NH), 8.10–7.20 (30H, m, 6\(\times\)Ph), 6.73 (1H, d, \(J_{1,2} = 3.5\) Hz, H-1), 5.96 (1H, d, \(J_{1,2} = 3.7\) Hz, H-4), 5.90 (1H, d, \(J_{1,2} = 3.7\) Hz, H-4), 5.84 (1H, dd, \(J_{1,2} = 3.3\) Hz, \(J_{2,3} = 10.7\) Hz, H-3), 5.74–5.67 (2H, m, H-2, H-2'), 5.54 (1H, dd, \(J_{1,2} = 3.5\) Hz, \(J_{2,3} = 10.3\) Hz, H-3'), 4.87 (1H, d, \(J_{1,2} = 7.9\) Hz, H-1), 4.61 (1H, t, \(J_{2,3} = 6.4\) Hz, H-5), 4.26–4.09 (4H, m, H-5', 6a, 6a', 6b', 6b), 3.98–3.85 (8H, m, H-6b, CICH_{2}CO–). 13C-NMR (CDCl3) \(\delta = 166.5, 166.8, 165.7, 165.4, 165.0, 164.0, 133.7, 133.6, 133.2, 131.7, 130.1, 129.9, 129.80, 129.75, 129.72, 129.68, 129.32, 129.30, 129.24, 129.84, 128.89, 128.7, 128.6, 128.53, 128.48, 128.4, 128.3, 128.2, 100.6 (C-1'), 93.4 (C-1), 90.5 (C-3Cl), 76.7, 71.7 (C-3'), 71.2 (C-5'), 70.7 (C-5), 69.6 (C-2'), 69.5 (C-3), 68.2 (C-4'), 67.8 (C-4'), 67.6 (C-2), 66.6 (C-6), 61.5 (C-6'), 45.0 (CICH_{2}CO–). 5-(Methoxycarbonyl)pentyl 2,3,4,6-Tetra-O-benzyl-β-D-galactopyranosyl-(1→6)-2,3,4-di-O-benzyl-3-O-chloroacetyl-β-D-galactopyranosyl-(1→6)-2,3,4-tri-O-benzoyl-β-D-galactopyranoside (17) To a solution of 16 (341 mg, 0.25 mmol) and 5 (210 mg, 0.40 mmol) in dry CH_{2}Cl_{2} (3.0 mL) was added powdered MS 4 Å (300 mg), and the mixture was stirred under Ar atmosphere for 2h at room temperature, then cooled to –60°C. NIS (90 mg, 400 µmol) and TIOH (3.5 µL, 40 µmol) were added to the mixture and the mixture was stirred for 30min. at –60°C then neutralized with Et_{3}N. The precipitates were filtered off and washed with CHCl_{3}. The combined filtrate and washings were successively washed with saturated aqueous Na_{2}SO_{4} and water, dried (MgSO_{4}), and concentrated. The product was purified by silica gel column chromatography (14:1 toluene–EtOAc) to give 17 (393 mg, 99%). \([\alpha]_D^{25} +58.1^\circ\) (c=2.0, CHCl3).
1H-NMR (CDCl₃) δ: 5.06 (1H, d, J₁₂=3.6 Hz, H-1 of Fuc), 4.73 (1H, d, J₁₂=7.7 Hz, H-1 of Gal c), 4.65–4.53 (2H, m, H-1 of Gal a, Gal b). 13C-NMR (CDCl₃) δ: 101.4 (C-1 of Gal a), 101.3 (C-1 of Gal b), 100.8 (C-1 of Gal c), 98.6 (C-1 of Fuc). MALDI-TOF-MS: Calcd for C₃₁H₅₄O₂₂Na: m/z 2007.6772. Found: 2007 [M+Na]⁺.

5-(Methoxycarbonyl)pentyl β-D-Galactopyranosyl-(1→6)-α-L-fucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside (18) Compound 17 (150 mg, 75.5 µmol) in 1:1 THF–MeOH (6.0 mL) was hydrogenolysed for 2 h at room temperature. The mixture was filtered and concentrated. To a solution of 1,2-dioxygen-MeOH (4.0 mL) was added NaOMe (50 mg) at room temperature and the mixture was stirred at 40°C for 16 h, then neutralized with Amberlite IR 120[H⁺]. The mixture was filtered off and concentrated. The product was purified by Sephadex LH-20 column chromatography in MeOH to give 18 (28 mg, 48%). [α]D²⁵ = −58.1° (c=0.4, MeOH). 1H-NMR (CD₂OD) δ: 5.12 (1H, d, J₁₂=3.9 Hz, H-1 of Fuc), 4.40 (1H, d, J₁₂=7.6 Hz, H-1 of Gal c), 4.31 (1H, d, J₁₂=7.5 Hz, H-1 of Gal b), 4.22 (1H, d, J₁₂=7.3 Hz, H-1 of Gal a). 13C-NMR (CDCl₃) δ: 176.9, 106.2, 105.9, 105.7, 103.6, 77.6, 76.0, 75.9, 75.8, 75.7, 75.6, 74.5, 73.4, 73.3, 72.6, 72.5, 71.4, 71.1, 70.83, 70.75, 70.6, 69.0, 63.4, 52.9, 50.7, 50.4, 49.4, 35.6, 31.3, 27.5, 26.6, 17.7. HR-ESI-MS: Calcd for C₃₁H₅₄O₂₂Na: m/z 801.3004. Found: 801.3030 [M+Na]⁺.

Biotinylated Tetrasaccharide (1) Compound 18 (28 mg, 36.0 µmol) was dissolved in neat anhydrous ethylene diamine (3 mL) and heated at 70°C for 48 h. The mixture was concentrated by azeotropic distillation with toluene and the product was purified by Sephadex LH-20 column chromatography in H₂O to give 19 (29 mg, 78%). [α]D²⁵ = −51.5° (c=0.4, H₂O). 1H-NMR (D₂O) δ: 5.16 (1H, d, J₁₂=4.1 Hz, H-1 of Fuc), 4.53 (1H, d, J₁₂=7.6 Hz, H-1 of Gal a), 4.45 (1H, d, J₁₂=7.6 Hz, H-1 of Gal c), 4.40 (1H, d, J₁₂=7.9 Hz, H-1 of Gal b). 13C-NMR (D₂O) δ: 104.0 (C-1 of Gal b), 103.7 (C-1 of Gal a), 103.4 (C-1 of Gal c), 101.6 (C-1 of Fuc). HR-ESI-MS: Calcd for C₂₉H₄₃N₂O₂₅Sn: m/z 1055.4206. Found: 1055.4276 [M+Na]⁺.

Serum Samples Serum samples of 60 patients, who were confirmed to have AE, and those of 60 healthy individuals, which are kept in Hokkaido Institute of Public Health, were used for ELISA assay under approval of the institute.

ELISA Protocol ELISA was performed using as previously described¹, with some modifications. The oligosaccharides in H₂O (13 pmol per well) were placed in the wells of flat-bottomed microplates (Streptavidin C96, No. 236001; Nunc, Roskilde, Denmark) coated with streptavidin and incubated for 1 h at 37°C. After removal of the solution, the wells were washed with 0.05% Tween-PBS (250 µl/well). Serum samples diluted 1:250 with 0.05% Tween-PBS (200 µl/well) were then added to the wells and incubated overnight at 4°C. After removal of the serum and washing with 0.05% Tween-PBS, 0.2 µl of anti-human immunoglobulin G (IgG)/horseradish peroxidase (HRP) (P0214; DakoCytomation, Denmark; 1:1000 in 0.05% Tween-PBS) was added, and the microplate was incubated for 1 h at 37°C. After washing of the wells, bound antibodies were detected by the addition of 2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) peroxidase substrate solution (KPL, Gaithersburg, MD, U.S.A, 200 µl per well). After incubation period of 8 min at 37°C, the reaction was stopped by the addition of 1% sodium dodecyl sulfate (SDS), and the absorbance (A) values were read at 405 nm on a microplate reader (Model 680; BIORAD, Hercules, California, U.S.A.).

Data Analysis The significant difference between the two groups was analyzed by the Student’s t-test.

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research (No. 25460131) and by Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and MEXT-supported program for the strategic research foundation at private universities (centers of excellence for research) in “molecular nanotechnology for green innovation,” FY 2012–2016.

Conflict of Interest The authors declare no conflict of interest.

References