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Synthesis of the carbohydrate moiety from the parasite *Echinococcus multilocularis* and their antigenicity against human sera

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ABSTRACT

Stereocontrolled syntheses of biotin-labeled oligosaccharide portions with a Galβ1-3GalNAc core of the Em2 glycoprotein antigen obtained from the parasite *Echinococcus multilocularis* have been accomplished. Trisaccharide Galβ1-3(GlcNAcβ1-6)GalNAcα1-R (**G**), tetrasaccharide Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα1-R (**J**) and pentasaccharide Galβ1-3(Galα1-4Galβ1-4GlcNAcβ1-6)GalNAcα1-R (**K**) (R = biotinylated probe) were synthesized by block synthesis by the use of 5-(methoxycarbonyl)pentyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-azido-4-*O*-benzyl-2-deoxy-α-D-galactopyranoside as a common glycosyl acceptor. Moreover, linear trisaccharide Galα1-4Galβ1-3GalNAcα1-R (**H**) and branched tetrasaccharide Galα1-4Galβ1-3(GlcNAcβ1-6)GalNAcα1-R (**I**) were synthesized by stepwise condensation. We examined the antigenicity of these five oligosaccharides by an enzyme linked immunosorbent assay (ELISA). Our results demonstrate that biotinylated oligosaccharides **H**, **I** and **K** show good serodiagnostic potential to detect infections caused by the parasite *E. multilocularis*. Among them the linear sequence Galα1-4Galβ1-3GalNAcα1-R in oligosaccharide (**H**) appears to show the highest sensitivity (95%). Moreover, our study clarified the dominant carbohydrate epitope of Em2 antigen.

1. Introduction

Alveolar echinococcosis (AE) in humans is a severe chronic helminthic disease caused by infection with the metacestode stage of *Echinococcus multilocularis*, a cestode of the genus *Echinococcus*, which is limited to the northern hemisphere such as Alps in Europe and Hokkaido in Japan. The adult parasites mainly reside in the intestines of foxes, wolves and dogs where they produce eggs. The eggs released in feces are usually ingested by rodents, the intermediate hosts of the parasite, or accidentally by humans [1,2]. Metacestodes are fluid-filled vesicles which are surrounded by an acellular, carbohydrate-rich surface structure that protects the parasite from immunological and physiological reactions of the host. In order to study the molecular features and selectivity of the immune response of metacestodes, an access to potential oligosaccharide epitopes expressed on the extracellular surface of *E. multilocularis* is required [3]. Moreover, there is demand for selective antigens that permit serological detection of echinococcosis in patients [4,5]. Persat et al. reported in 1991 that sera from AE patients recognized a neutral glycosphingolipid fraction extracted from metacestodes of *E. multilocularis* [6]. Structure determination of this glycosphingolipid fraction revealed that it belonged to the neogala-series (Galβ1-6Gal). In addition, it was further shown that neogala-based glycosphingolipids had significant serodiagnostic potential in differentiating alveolar from cystic echinococcosis (CE) [7]. Based on this information, we previously synthesized four kinds of glycosphingolipids, [Galβ1-6(Fucα1-3)Galβ1-6Galβ1-6] and its precursors [8], and reported their serodiagnostic potential [9]. In 1985, Gottstein isolated two protein fractions (Em1 and Em2) from an extract of *E. multilocularis* metacestode tissue and showed that Em2 was species specific for *E. multilocularis* [10].

Hülsmeier et al. reported that the structure of Em2 antigen was a novel mucin-type glycoprotein and studied the carbohydrate moieties in more detail [11]. However, they were unable to determine the exact linkage connectivity of the carbohydrate sequence in this novel mucin-type glycoprotein. Based on their estimated structures, we synthesized six oligosaccharides (**A**–**F**) with

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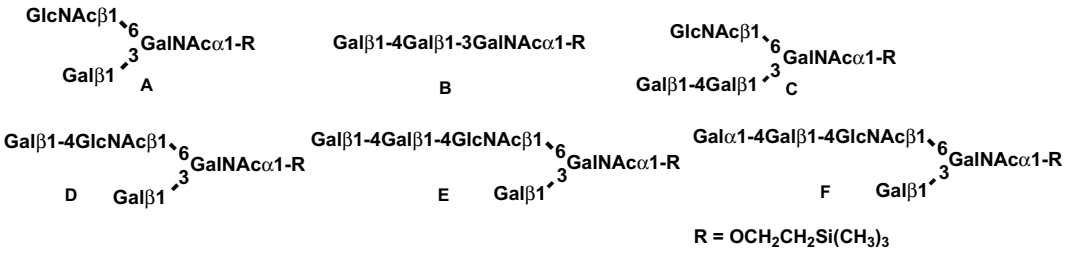


Fig. 1. Structures of the oligosaccharide derivatives, **A**–**F** from the parasite *Echinococcus multilocularis* synthesized in the previous report.

trimethylsilylethyl (TMSEt) group as an aglycon [12] (Fig. 1). However, these oligosaccharides did not react with sera of patients suffering from echinococcosis (data not shown) in an ELISA assay. Recently, Díaz et al. reported that the extracellular matrix of a related cestode *Echinococcus granulosus* contains a novel mucin-type O-glycan capping motif consisting of Gal*p*α1-4Gal linkages at the non-reducing end [13]. This information suggests that oligosaccharides containing Gal*p*β1-4Gal capping motifs, such as structures **B**, **C** and **E**, are not likely to be involved in the immune response. Moreover, in our previous investigation, we used TMSEt group as an aglycon, which seemed to have poor adherence ability to the microplate rendering from the use in ELISA assays. In order to enhance the adherence ability of the oligosaccharide probes to the plate, we decided to take advantage of the strong affinity of biotin to avidin. Biotinylated compounds have been widely used for investigating various biological responses [14–17]. We expected that biotinylated oligosaccharide probes may show improved adherence to streptavidin-coated microtitre plates that could find application as a potential serodiagnostic tool to detect AE infections in humans. We describe here the syntheses of biotinylated glycan portions of the glycoprotein antigen of *E. multilocularis*, **G**–**K** (Fig. 2), among which **H**, **I** and **K** contain the postulated novel capping motif Gal*p*α1-4Gal at the non-reducing end, and their antigenicity against sera of AE patients.

2. Chemistry

2.1. Syntheses of the target oligosaccharides *G*, *H*, *I*, *J* and *K*

As a temporary protecting group, 5-(methoxycarbonyl)pentyl group was chosen to ensure future conjugation to biotin for ELISA test for all target compounds. The synthetic routes for target compounds **G**–**K** are outlined in Schemes 2–6. Initially, disaccharide acceptor **6**, which serves as a common acceptor for the syntheses of compound **G**, **J** and **K**, was prepared (Scheme 1). Compound **2** was obtained by in situ conversion of glycosylnitrate **1** [18] into glycosyl iodide followed by coupling to methyl 6-hydroxyhexanoate using a combination of tetrabutylammonium iodide (TBAI) and iodine (I_2) as activator [12]. Although the preparation of 2-(trimethylsilyl)ethyl glycoside from glycosylnitrate **1** gave an anomeric ratio of 3:1 (α:β) in our previous paper [12], the anomeric ratio of 5-(methoxycarbonyl)pentyl glycoside **2** was 10:1 (α:β, from ^{1}H NMR). Zemplén deacetylation followed by protection of the 4- and 6-hydroxy groups as a benzylidine group provided **3**. It was possible to separate the major α-anomer from the minor β-glycoside at this stage. Glycosylation [19] of acceptor **3** with the trichloroacetimidate donor **4** [20] was carried out in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) and molecular sieves AW-300 in CH_2Cl_2 to afford desired β-glycoside **5** in 74% yield as a single anomer. Reductive ring opening of the benzylidene acetal **5** was achieved by treatment with dichlorophenylborane ($PhBCl_2$) and triethylsilane (Et_3SiH) in CH_2Cl_2 [21] to produce disaccharide acceptor **6** as a single regioisomer in 79% yield.

Glycosylation of imidate donor **7** [22] with acceptor **6** was achieved in the presence of TMSOTf to afford trisaccharide **8** in 81% yield (Scheme 2). Subsequently, reduction of the azido- and 2,2,2-trichloroethoxycarbonyl (Troc) groups of **8** with Zn-AcOH followed by debenzylation with catalytic hydrogenolysis over 10% Pd/C in THF and acetylation afforded **9** in 62% yield. After deacylation by Zemplén-based method, compound **10** was converted into an ethylenediamine monoamide [23] by exposure to ethylenediamine followed by conjugation to *N*-hydroxy succinimide ester of biotin (biotin-NHS) to afford target biotinylated trisaccharide **G** after column chromatographic purification on Sephadex LH-20 (Scheme 2).

The synthesis of tetrasaccharide **J** containing core-II type structure was achieved by glycosylation of disaccharide donor **11** with previously prepared acceptor **6** (Scheme 3). Thioglycoside donor **11** was prepared according to a previously established synthetic methodology [12]. Glycosylation of donor **11** with acceptor **6** in the presence of NIS, TfOH and MS AW-300 in CH_2Cl_2 afforded desired tetrasaccharide **12** in quantitative yield. The nature of the new glycosidic linkage was determined from the coupling constant of the anomeric proton (H-1 of GlcN,

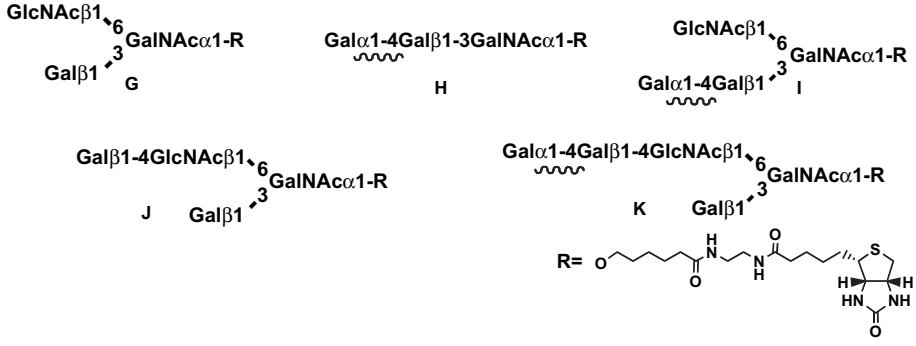
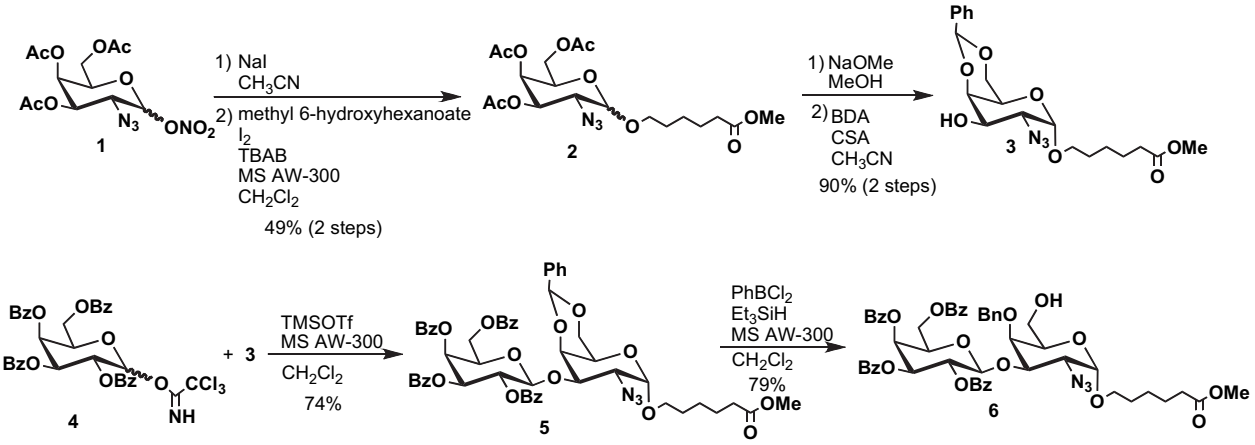


Fig. 2. Structures of the target oligosaccharide derivatives, **G**–**K**.

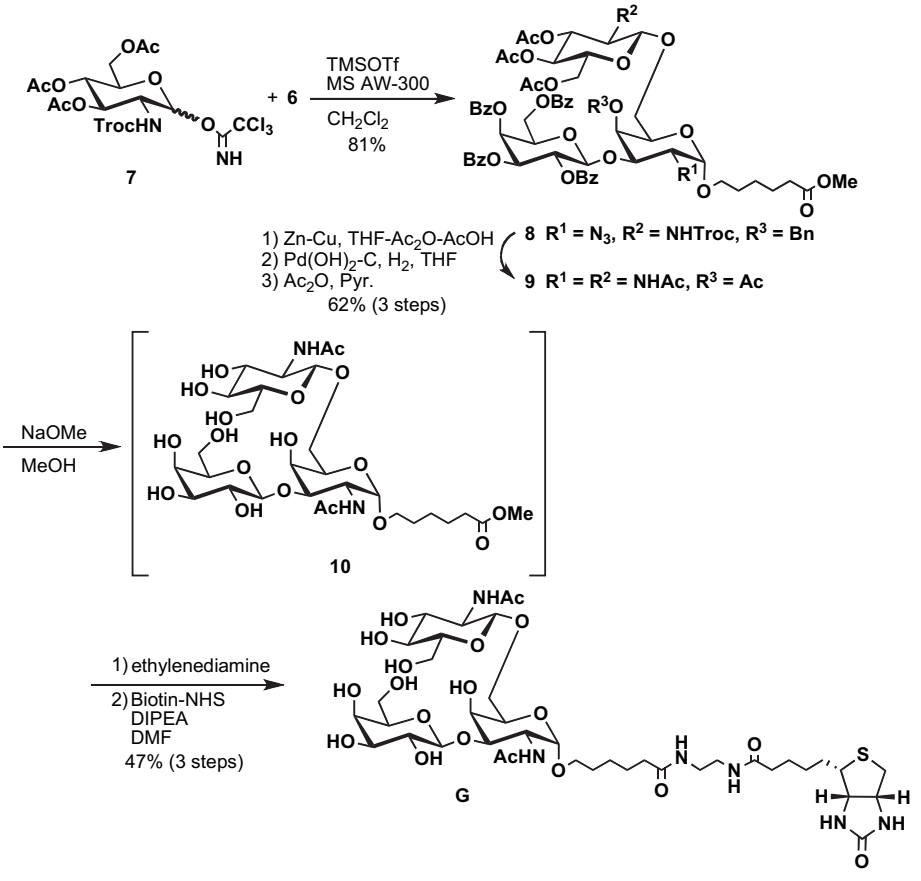


Scheme 1. Preparation of disaccharide acceptor.

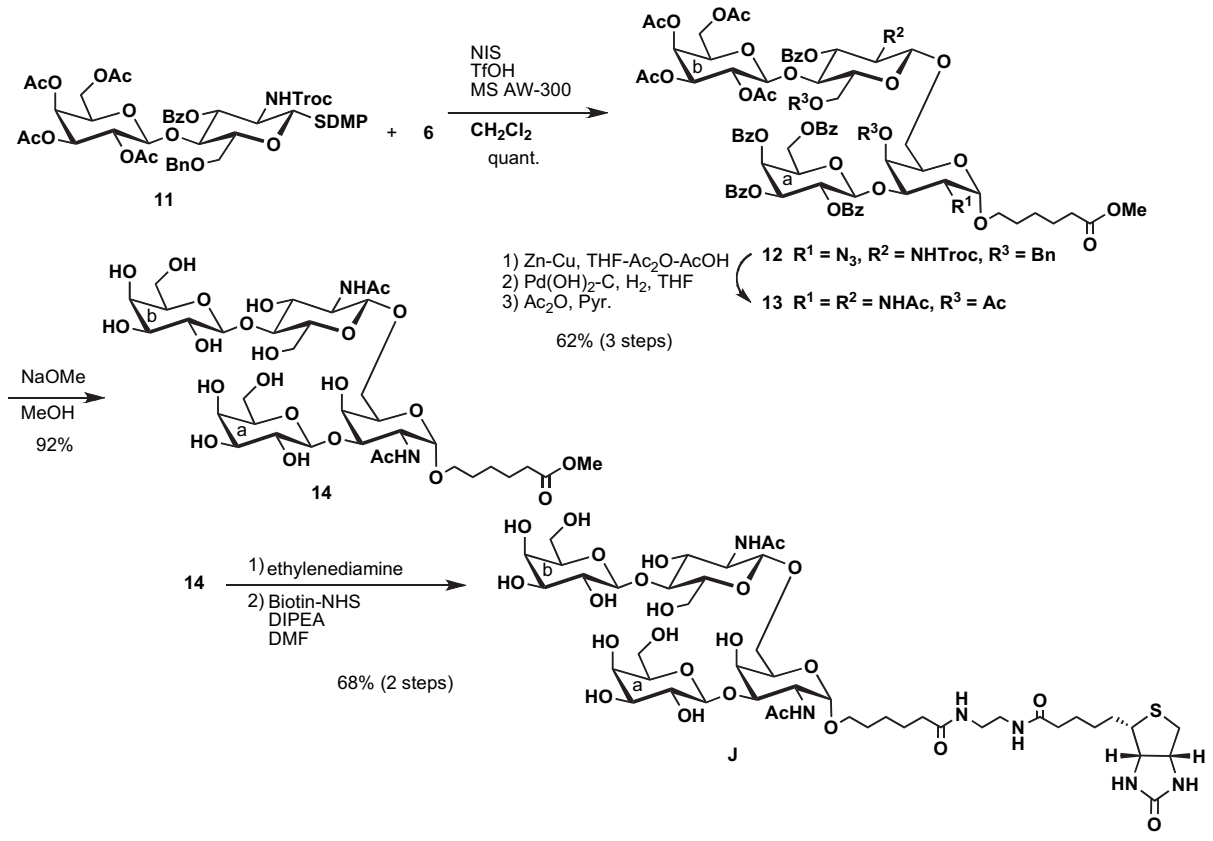
δ = 4.55 ppm, $J_{H1,\ H2}$ = 7.9 Hz). Removal of the Troc-protecting group using Zn–Cu in the presence of acetic anhydride and acetic acid in THF, followed by catalytic hydrogenation over 10% Pd–C in THF and subsequent *N*- and *O*-acetylation provided tetrasaccharide **13**. After deacetylation of **13**, 5-(methoxycarbonyl)pentyl glycoside **14** was converted into the ethylenediamine monoamide by exposure to ethylenediamine and conjugated to biotin to afford tetrasaccharide-biotin conjugate **J** in 68% yield (Scheme 3).

Branched pentasaccharide **K** was synthesized by glycosylation of thioglycoside donor **15** [12] with acceptor **6** (Scheme 4). NIS/TfOH-promoted activation of donor **15** and coupling to disaccharide acceptor **6** afforded desired β-glycoside **16** in 99% yield. Deprotection and biotinylation were performed as described for compound **J** to provide target pentasaccharide **K** (Scheme 4). Oligosaccharides **H** and **I** were prepared by stepwise condensation as described in our previous paper [12] (Schemes 5 and 6). Both oligosaccharides **H** and **I** are derived from a common trisaccharide **24**.

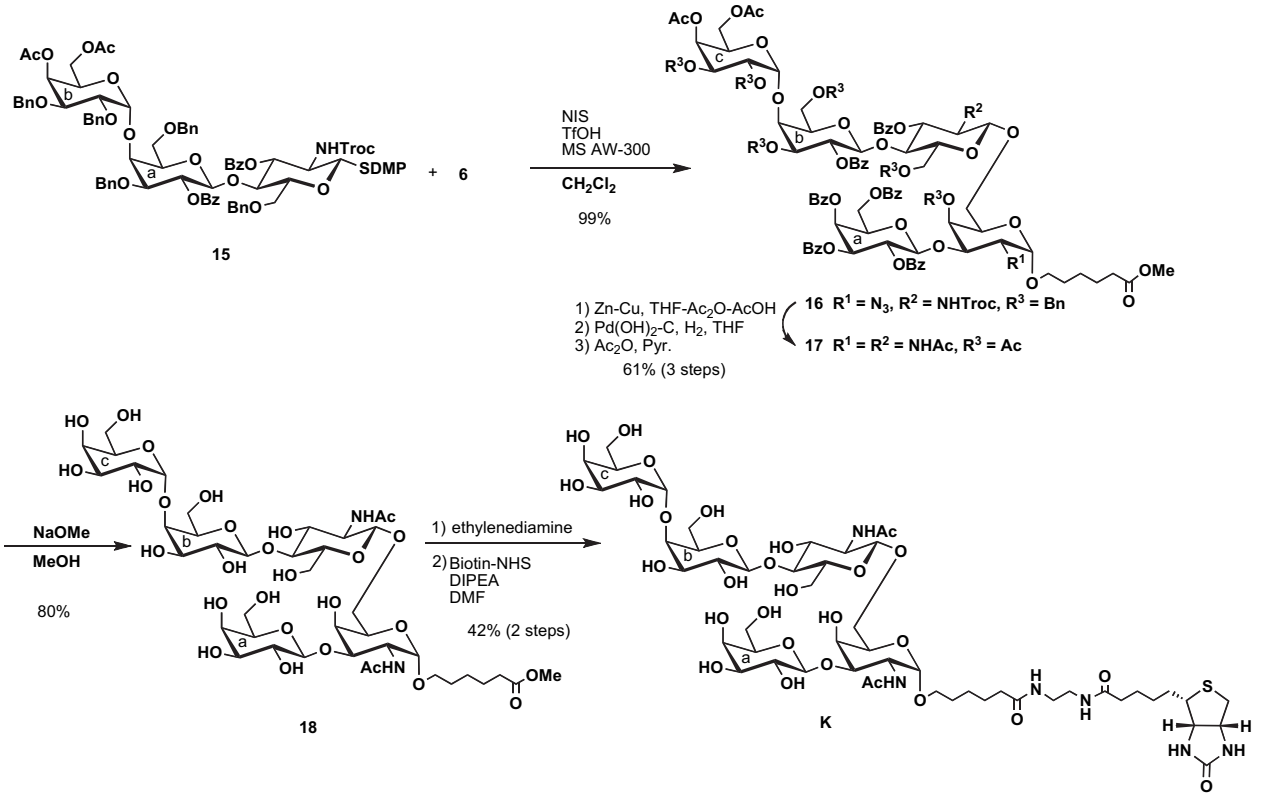
Common intermediate **24** was synthesized from the acceptor **3** and known thioglycoside donor **19** [12]. Glycosylation of acceptor **3** with **19** in the presence of NIS/TfOH provided disaccharide **20** in 75% yield. Selective removal of the chloroacetyl group in **20** with



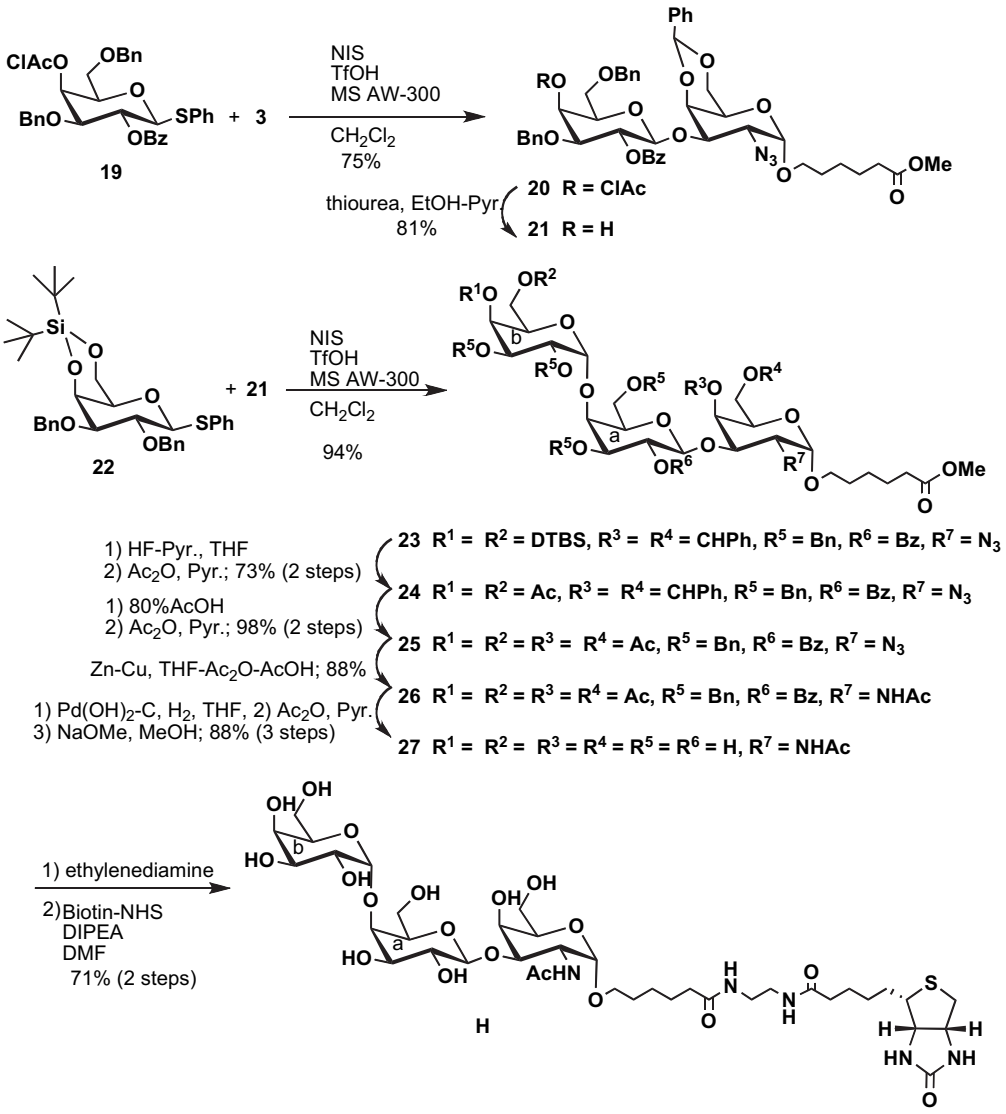
Scheme 2. Synthesis of trisaccharide **G**.



Scheme 3. Synthesis of tetrasaccharide **J**.



Scheme 4. Synthesis of pentasaccharide **K**.



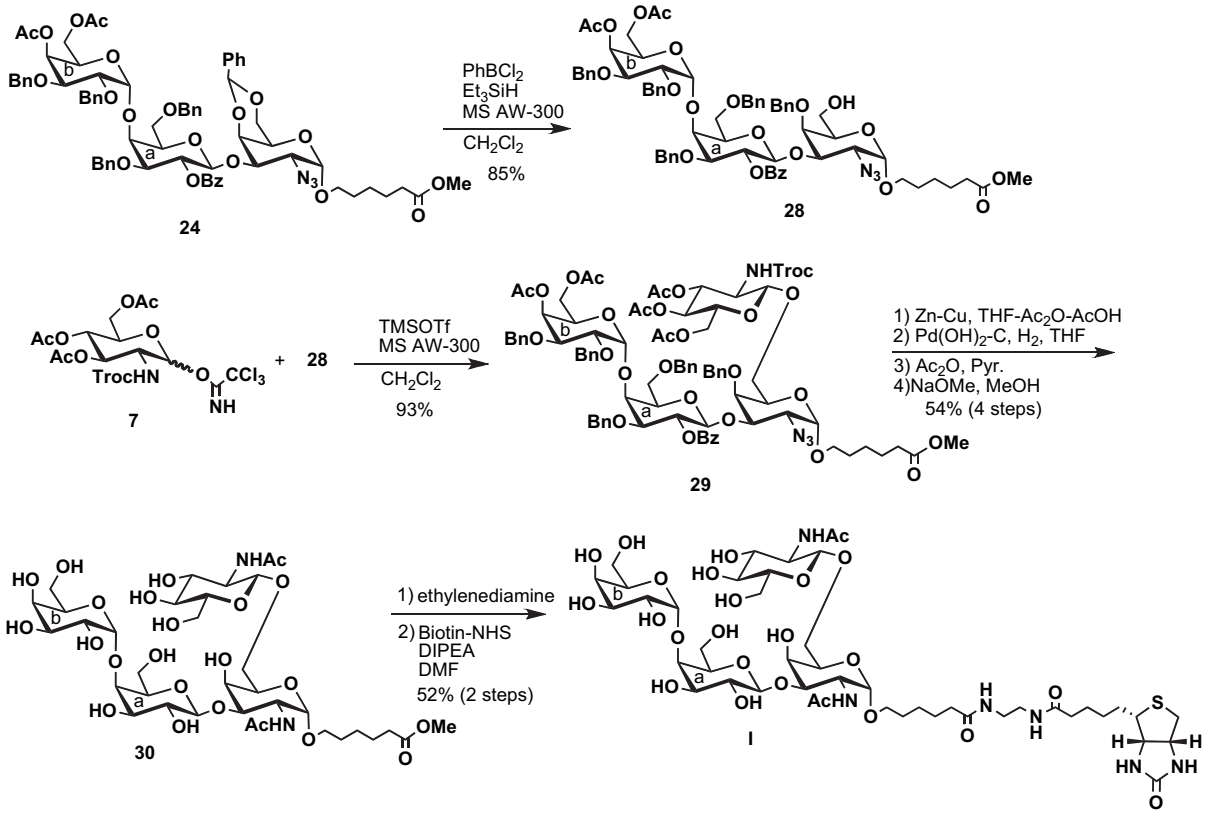
Scheme 5. Synthesis of trisaccharide **H**.

thiourea [24] produced disaccharide acceptor **21** which was used directly for the next glycosylation step. In order to prepare the Galα1-4Gal sequence with high α-stereoselectivity, we selected 4,6-*O*-di-*tert*-butylsilylene (DTBS)- protected galactose donor **22** [25]. Previous studies have indicated that DTBS-protected galactose donors induce high α-selectivity in glycosylation reactions [26]. NIS/TfOH-promoted activation of thioglycoside donor **22** and coupling to disaccharide acceptor **21** generated trisaccharide **23** in 94% yield. The newly formed α-glycosidic linkage was confirmed by ^{1}H NMR spectroscopy. The anomeric proton of the galactose moiety (b) in **23** appeared at 4.95 ppm as a doublet with a proton–proton coupling constant of 3.3 Hz (H-1 of Gal(b), δ = 4.95 ppm, $J_{H1b,H2b}$ = 3.3 Hz). Selective removal of the DTBS group in **23** was achieved with HF/pyridine followed by acetylation with acetic anhydride in pyridine to afford **24** in 73% yield. Global deprotection was performed by a combination of protection/deprotection steps. At first the benzylidene acetal was removed by acidic hydrolysis followed by *O*-acetylation using acetic anhydride in pyridine to afford **25** in 88% yield. Secondly, the azido-group was converted to an acetamido moiety by reduction with Zn/Cu AcOH in the presence of acetic anhydride. Finally, the obtained *N*-acylated product **26** was debenzylated by catalytic hydrogenolysis using Pearlman's catalyst followed by *O*-acetylation and deacetylation to produce deprotected target trisaccharide **27** in 88% yield. Compound **27** was then used for ligation to biotin using the common methodology to give target trisaccharide **H** (Scheme 5).

To prepare the branched tetrasaccharide **I**, it was necessary to introduce a (1→6)-linked *N*-acetyl glucosamine moiety into trisaccharide **24** (Scheme 6). In order to accomplish this, a reductive and regioselective ring opening of the benzylidene acetal group in **24** was necessary. This was achieved by exposure of **24** to Et_3SiH and $PhBCl_2$ in CH_2Cl_2 to afford **28**. Subsequently, trisaccharide acceptor **28** was glycosylated with glycosyl imidate **7** to generate tetrasaccharide **29** in 93% yield. The same deprotection and biotinylation reactions described for oligosaccharide **J** afforded target tetrasaccharide **I** in 52% yield (Scheme 6).

3. Antigenicity of oligosaccharides by ELISA

The reactivity of the five oligosaccharides **G**, **H**, **I**, **J** and **K** to alveolar echinococcosis (AE) patient sera was examined using microplates coated with streptavidin. The antibody response of the AE patient group differed from that of the normal healthy (NH) group (Fig. 3). AE patient group tended to show higher ELISA values



Scheme 6. Synthesis of tetrasaccharide **I**.

than NH group against oligosaccharide probes **H**, **I** and **K**, which have Galα1-4Gal sequence. On the contrary, very little response was observed to **G** and **J**, which lack the capping Galα1-4Gal sequence. Compound **H** showed the most strong response to the AE group resulting in clear differentiation between the AE and NH groups. The absorbance (A) values of AE group ($n = 60$) were significantly higher than those in the NH group ($n = 60$) ($P < 0.001$, Student's t-test). For the NH group shown in Fig. 3, the average A value against **H** was 0.24 $\pm$ 0.09; hence the cut off value that separated positive and negative was set to 0.42 calculated from the average A + (2 $\times$ standard deviation) for the 60 NH sera. Fifty-seven sera from the AE group showed A values above 0.42 (sensitivity: 95%); in contrast, all NH sera showed A values below 0.42 (specificity: 100%). This represents a higher sensitivity than the Em2 antigen (89.3%) previously reported by Gottstein [5]. Although the carbohydrate sequence of **K** and **F** are identical, the biotinylated probe **K** induced a higher response compared to **F** in the ELISA assay (results not shown). This is most likely the result of the significantly improved adherence of the biotinylated probe **K** to the streptavidin-coated microtitre plate leading to higher sensitivity and reproducibility in this serodiagnostic assay.

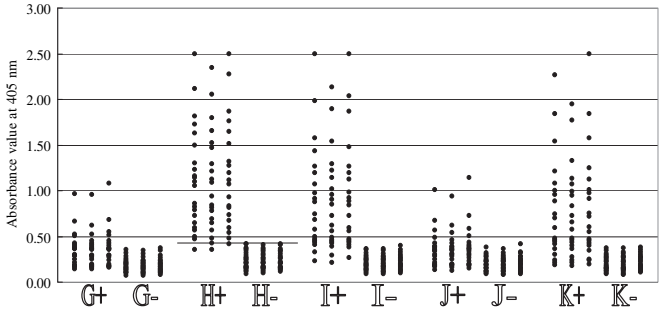


Fig. 3. ELISA reaction between human sera and the five oligosaccharides (**G**–**K**) used. (+): alveolar echinococcosis (AE) patient group (−):normal healthy (NH) group. The line of H show the cut off value (0.42).

4. Conclusions

We have prepared oligosaccharide-biotin conjugates **G**–**K** in order to study the antigenicity of putative carbohydrate sequences at the metacestode stage of the parasite *E. multilocularis*. Antigenicity of these compounds was examined by ELISA. The glycoconjugates **H**, **I** and **K** showed good serodiagnostic potential for alveolar echinococcosis. Among these compounds, the linear sequence Galα1-4Galβ1-3GalNAcα1-R in oligosaccharide (**H**) exhibited the highest sensitivity (95%) in the serodiagnostic assay. Moreover, we clarified the dominant epitope of Em2 antigen and confirmed that the unusual capping sequence Gal*p*α1-4Gal is an important contributor for selective antibody/antigen recognition. The synthetic biotinylated oligosaccharide probe **H**, will serve as a tool for diagnosis of AE patients by an ELISA assay.

5. Experimental section

5.1. General procedures

Optical rotations were measured with a Jasco P-1020 digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded with a Varian 500 FT NMR spectrometer. Me_4Si and acetone were used as internal standards for $CDCl_3$ and D_2O, respectively. MALDI-TOFMS was recorded on an AB Sciex Voyager RP mass spectrometer. High-resolution mass spectra were recorded on a JEOL JMS-700 under

FAB conditions. TLC was performed on Silica Gel 60 F254 (E. Merck) with detection by quenching of UV fluorescence and by charring with 10% H_2SO_4. Column chromatography was carried out on Silica Gel 60 (E. Merck).

5.2. 5-(Methoxycarbonyl)pentyl 3,4,6-tri-O-acetyl-2-azido-2-deoxy-D-galactopyranoside (**2**)

To a solution of **1** (870 mg, 2.31 mmol) in CH_3CN (10.0 mL) was added NaI (2.42 g, 18.6 mmol). The reaction mixture was stirred for 1 h at room temperature, then extracted with Et_2O, washed with aq. $Na_2S_2O_3$, dried ($MgSO_4$), and concentrated to give glycosyl iodide intermediate. To a solution of methyl 6-hydroxyhexanoate (1.01 g, 6.93 mmol), tetrabutylammonium iodide (TBAI) (1.71 g, 4.62 mmol) and MS AW300 (1.0 g) in CH_2Cl_2 (5.0 mL) were stirred under an atmosphere of argon for 2 h at room temperature, then solution of the glycosyl iodide in CH_2Cl_2 (5.0 mL) and iodine (I_2) (879 mg 3.47 mmol) were added and stirring was continued for 88 h. The reaction mixture was diluted with $CHCl_3$, washed with saturated aqueous $Na_2S_2O_3$, dried ($MgSO_4$), and concentrated. The product was purified by silica gel column chromatography (20:1 toluene–acetone) as eluent to give **2** (α:β ratio 10:1) (524 mg, 49%, two steps). MALDI-TOFMS: Calcd for $C_{19}H_{29}N_3O_{10}Na$: *m/z* 482.2 Found: *m/z* 482.7 $[M + Na]^+$.

5.3. 5-(Methoxycarbonyl)pentyl 2-azido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**3**)

To a solution of **2** (524 mg, 1.14 mmol) in MeOH (10 mL) was added NaOMe (31 mg, 0.51 mmol) and the mixture was stirred for 30 min, then neutralized with Amberlite IR 120 [H^+]. The mixture was filtered off and concentrated. To a solution of the residue (367 mg) in CH_3CN (10 mL) were added benzaldehyde dimethylacetal (0.34 mL, 2.28 mmol) and camphor-10-sulfonic acid (132 mg, 0.51 mmol) at room temperature. The reaction mixture was stirred for 3 h, then neutralized with Et_3N. After evaporation, column chromatography of the residue on silica gel (2:1 hexane-AcOEt) gave **3** (433 mg, 90%). $[\alpha]_D$ + 126 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ 7.50–7.37 (m, 5H, Ar), 5.58 (s, 1H, PhCH), 4.99 (d, 1H, $J_{1,2}$ = 3.4 Hz H-1), 4.31–4.26 (m, 2H, H-4, H-6a), 4.18 (dd., 1H, $J_{2,3}$ = 10.6 Hz, $J_{3,4}$ = 3.5 Hz, H-3), 4.11–4.08 (m, 1H, H-6b), 3.75–3.70 (m, 2H, OCH_2CH_2 a, H-5), 3.67 (s, 3H, OCH_3), 3.55–3.48 (m, 2H, H-2, OCH_2CH_2 b), 2.33 (t, 2H, *J* = 7.4 Hz), 1.69–1.60 (m, 4H,), 1.45–1.39 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.1, 137.3, 129.3, 128.3, 126.2, 101.3 (PhCH), 98.7 (C-1), 75.6 (C-4), 69.3 (C-6), 68.4 (OCH_2CH_2), 67.3 (C-3), 62.8 (C-5), 60.7 (C-2), 51.5 (OCH_3), 33.9, 29.1, 25.7, 24.6. HRFABMS: Calcd for $C_{20}H_{28}N_3O_7$: *m/z* 422.1927 Found: *m/z* 422.1974 $[M + H]^+$.

5.4. 5-(Methoxycarbonyl)pentyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**5**)

To a solution of **3** (138 mg, 0.33 mmol) and **4** (364 mg, 0.49 mmol) in dry CH_2Cl_2 (3.0 mL) was added MS AW-300 (500 mg), and the mixture was stirred for 2 h at room temperature, then cooled to −20 °C. TMSOTf (17.8 μL, 98.2 μmol) was added, and the mixture was stirred for 1 h at 0 °C, then neutralized with Et_3N. The precipitates were filtrated off and washed with $CHCl_3$. The combined filtrate and washings were successively washed with water, dried ($MgSO_4$), and concentrated. The product was purified by silica gel column chromatography (17:1 toluene-EtOAc) to give **5** (241 mg, 74%). $[\alpha]_D$ + 127 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ 8.09–7.16 (m, 25H, Ar), 6.00 (d, 1H, $J_{3',4'}$ = 3.4 Hz, H-4′), 5.92 (dd, 1H, $J_{1',2'}$ = 7.8 Hz, $J_{2',3'}$ = 10.4 Hz, H-2′), 5.60 (dd, 1H, $J_{2',3'}$ = 10.4 Hz, $J_{3',4'}$ = 3.4 Hz, H-3′), 5.49 (s, 1H, PhCH), 5.17 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.93 (d, 1H, $J_{1,2}$ = 3.3 Hz, H-1), 4.77 (dd, 1H, $J_{5',6'a}$ = 8.8 Hz, $J_{6'a,6'b}$ = 13.0 Hz, H-6′a), 4.47 (d, 1H, $J_{3,4}$ = 3.2 Hz, H-4) 4.44–4.40 (m, 2H, H-5′, H-6′b), 4.15–4.12 (m, 2H, H-3, H-6a), 3.76–3.74 (m, 2H, H-3, H-6b), 3.69–3.64 (m, 4H, OCH_2CH_2 a, OCH_3), 3.49 (s, 1H, H-5), 3.47–3.43 (m, 1H, OCH_2CH_2 b), 2.32 (t, 2H, *J* = 7.3 Hz), 1.69–1.59 (m, 4H), 1.42–1.36(m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ174.9, 166.0, 165.6, 165.2, 137.7, 133.6, 133.5, 133.3, 130.0, 129.8, 129.73, 129.72, 129.45, 129.44, 129.03, 128.97, 128.79, 128.71, 128.67, 128.57, 128.3, 128.2, 128.1, 126.1, 103.0 (C-1′), 100.6 (CHPh) 98.6 (C-1), 76.4 (C-3), 76.0 (C-4), 72.0 (C-3′), 71.5 (C-5′), 69.5 (C-2′), 69.0 (C-6), 68.3 (OCH_2CH_2), 68.2 (C-4′), 63.0 (C-5), 62.5 (C-6′), 58.5 (C-2), 51.5 (OCH_3), 33.9, 29.0, 25.7, 24.6.

HRFABMS: Calcd for $C_{54}H_{53}N_3O_{16}Na$: *m/z* 1022.3324 Found: *m/z* 1022.3276 $[M + Na]^+$.

5.5. 5-(Methoxycarbonyl)pentyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→3)-2-azido-4-O-benzyl-2-deoxy-α-D-galactopyranoside (**6**)

To a solution of **5** (207 mg, 0.21 mmol)) in dry CH_2Cl_2 (4.0 mL) was added MS AW-300 (200 mg), and the mixture was stirred for 2 h at room temperature, then cooled to −78 °C Et_3SiH (100 μL, 0.62 mmol) and $PhBCl_2$ (91.6 μL 0.70 mmol) were added, and the mixture was stirred for 5 min, then neutralized with Et_3N and added to MeOH. The precipitates were filtrated off and washed with $CHCl_3$. The combined filtrate and washings were successively washed with water, dried ($MgSO_4$), and concentrated. The product was purified by silica gel column chromatography (1:1 hexane-ethyl acetate) to give **6** (164 mg, 79%). $[\alpha]_D$ + 104 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$):δ 8.03–7.21 (m, 30H, Ar), 6.04 (d, 1H, $J_{3',4'}$ = 3.0 Hz, H-4′), 5.98 (dd, 1H, $J_{1',2'}$ = 7.8 Hz, $J_{2',3'}$ = 10.5 Hz, H-2′), 5.65 (dd, 1H, $J_{2',3'}$ = 10.3 Hz, $J_{3',4'}$ = 3.3 Hz, H-3′), 5.20 (d, 1H, $J_{1',2'}$ = 7.9 Hz H-1′), 5.19 and 4.75 (each d, 2H, J_{gem} = 11.2 Hz, benzyl methylene), 4.85 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 4.76 (d, 1H, $J_{6'a,6'b}$ = 11.4 Hz, H-6′a), 4.50 (dd, 1H, $J_{5',6'a}$ = 5.7 Hz, $J_{6'a,6'b}$ = 11.2 Hz, H-6′b), 4.45 (t, 1H, $J_{5',6'a}$ = 6.7 Hz, H-5′), 4.16–4.12 (m, 2H, H-4, H-3), 3.72–3.60 (m, 7H, H-2, H-5, H-6a, OCH_2CH_2 a, OCH_3), 3.46–3.37 (m, 2H, H-6a, OCH_2CH_2 a), 2.32 (t, 2H, *J* = 7.3 Hz), 1.67–1.56 (m, 4H), 1.41–1.35(m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ174.9, 166.0, 165.6, 165.34, 165.28, 138.2, 133.6, 133.5, 133.3, 133.2, 129.80, 129.78, 129.74, 129.70, 129.3, 129.2, 128.9, 128.8, 128.7, 128.6, 128.32, 128.28, 128.1, 103.0 (C-1′), 98.2 (C-1), 78.6 (C-3), 76.8 (C-4), 75.0 (CH_2Ph), 71.7 (C-3′), 71.5 (C-5′), 70.6 (C-5), 69.7 (C-2′), 68.1 (OCH_2CH_2, C-4′), 62.3 (C-6), 62.1 (C-6′), 59.4 (C-2), 51.5 (OCH_3), 33.9, 28.9, 25.7, 24.6. HRFABMS: Calcd for $C_{54}H_{55}N_3O_{16}Na$: *m/z* 1024.3480 Found: *m/z* 1024.3456 $[M + Na]^+$.

5.6. 5-(Methoxycarbonyl)pentyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→3)-[3,4,6-tri-O-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→6)]-2-azido-4-O-benzyl-2-deoxy-α-D-galactopyranoside (**8**)

To a solution of **6** (160 mg, 0.16 mmol) and **7** (150 mg, 0.24 mmol) in dry CH_2Cl_2 (2 mL) was added MS AW-300 (300 mg), and the mixture was stirred for 2 h at room temperature, then cooled to −20 °C. TMSOTf (8.7 μL, 48.0 μmol) was added, and the mixture was stirred for 1 h at −20 °C, then neutralized with Et_3N. The precipitates were filtrated off and washed with $CHCl_3$. The combined filtrate and washings were successively washed with water, dried ($MgSO_4$), and concentrated. The product was purified by silica gel column chromatography (5:1 toluene-EtOAc) to give **8** (190 mg, 81%). $[\alpha]_D$ + 65.0 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$):δ 5.20 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1 of Gal), 4.77 (d, 1H, $J_{1,2}$ = 3.4 Hz, H-1 of GalN), 4.76 (d, 1H, $J_{1,2}$ = 8.1 Hz, H-1 of GlcN). ^{13}C

NMR (125 MHz, $CDCl_3$): δ 102.3 (C-1 of Gal), 100.7 (C-1 of GlcN), 98.1 (C-1 of GalN). HRFABMS: Calcd for $C_{69}H_{74}Cl_3N_4O_{25}$: *m*/*z* 1463.3708 Found: *m*/*z* 1463.3666 *m*/*z* $[M + H]^+$.

5.7. 5-(Methoxycarbonyl)pentyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→3)- [2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-acetamido-4-O-acetyl-2-deoxy-α-D-galactopyranoside (**9**)

To a solution of **8** (190 mg, 0.13 mmol) in THF-AcOH-Ac_2O (3:2:1, 6.0 mL) was added Zn−Cu (760 mg). The mixture was stirred for 30 min at room temperature. After completion of the reaction, the solid was filtered off. The filtrate was concentrated and purified by silica gel column chromatography (3:2 toluene−acetone) to give an acetamido compound. To a solution of this compound (135 mg) in THF (2.0 mL) was hydrogenolysed in the presence of $Pd(OH)_2$/C (100 mg) for 8 h at room temperature. The mixture was filtered and concentrated, and the residue was acetylated with acetic anhydride (3.0 mL) in pyridine (5.0 mL). After the reaction was quenched with MeOH, toluene was added and co-evaporated several times. The product was purified by silica gel column chromatography (1:1 toluene−acetone) to give **9** (104 mg, 62%). $[\alpha]_D$ + 77.3 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$):δ 5.00 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal), 4.81 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1 of GalN), 4.62 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1 of GlcN). ^{13}C NMR (125 MHz, $CDCl_3$): δ 101.3 (C-1 of Gal), 101.0 (C-1 of GlcN), 97.0 (C-1 of GalN). HRFABMS: Calcd for $C_{65}H_{75}N_2O_{26}$: *m*/*z* 1299.4608 Found: *m*/*z* 1299.4567 $[M + H]^+$.

5.8. Biotinylated trisaccharide (G)

To a solution of **9** (100 mg, 77.0 μmol) in MeOH (5 mL) was added NaOMe (100 mg, 4.32 mmol) and the mixture was stirred at 50 °C for 19 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 H_2O to give **10**. The residue was dissolved in anhydrous ethylenediamine (10 mL) and heated at 70 °C for 44 h. The mixture was concentrated with toluene and the product was purified by Sephadex LH-20 column chromatography in H_2O to give an amine intermediate. The amine (42.3 mg, 56.9 μmol) was dissolved in DMF (6.0 mL), and the pH was adjusted to 8−9 using DIPEA. Biotine-NHS (23.3 mg, 56.9 μmol) was added and the reaction stirred for 12 h at room temperature. Toluene was added to and evaporated from the residue several times. The product was purified by Sephadex LH-20 column chromatography in H_2O to give **G** (35.0 mg, 47%). $[\alpha]_D$ + 56.7 (*c* 1.0, H_2O). ^1H NMR (500 MHz, D_2O):δ 4.67 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1 of GalN), 4.34 (d, 1H, $J_{1,2}$ = 8.4 Hz, H-1 of GlcN), 4.28 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal). ^{13}C NMR (125 MHz, D_2O): δ 104.2 (C-1 of Gal), 101.1 (C-1 of GlcN), 96.3 (C-1 of GalN). HRFABMS: Calcd for $C_{40}H_{69}N_6O_{19}SNa$: *m*/*z* 991.4158 Found: *m*/*z* 991.4184 $[M + Na]^+$.

5.9. 5-(Methoxycarbonyl)pentyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→3)-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-3-O-benzoyl-6-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→6)]-2-azido-4-O-benzyl-2-deoxy-α-D-galactopyranoside (**12**)

To a solution of **6** (100 mg, 99.8 μmol) and **11** (150 mg, 0.150 mmol) in dry CH_2Cl_2 (2 mL) was added powdered MS AW-300 (250 mg), and the mixture was stirred under Ar atmosphere for 2 h at room temperature, then cooled to −40 °C. NIS (67.5 mg, 0.30 mmol) and TfOH (2.7 μL, 30.0 μmol) were added to the mixture, which was stirred for 1 h at −50 °C, then neutralized with Et_3N. The precipitates were filtered off and washed with $CHCl_3$. The combined filtrate and washings were successively washed with saturated aqueous $Na_2S_2O_3$ and water, dried ($MgSO_4$), and concentrated. The product was purified by silica gel column chromatography (1:1 hexane-EtOAc) to give **12** (187 mg, quant.). $[\alpha]_D$ + 41.3 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$):δ 5.19 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1 of Gal a), 4.79 (d, 1H, $J_{1,2}$ = 3.3 Hz, H-1 of GalN), 4.65 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1 of GlcN), 4.45 (d, 1H, $J_{1,2}$ = 7.2 Hz, H-1 of Gal b). ^{13}C NMR (125 MHz, $CDCl_3$): δ 104.2 (C-1 of Gal a), 101.4 (C-1 of GlcN), 100.1 (C-1 of Gal b), 98.1 (C-1 of GalN). MALDI-TOFMS: Calcd for $C_{91}H_{95}Cl_3N_4O_{32}Na$: *m*/*z* 1883.5 Found: *m*/*z* 1883.6 $[M + Na]^+$.

5.10. 5-(Methoxycarbonyl)pentyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→3)-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-6-O-acetyl-3-O-benzoyl-2-deoxy]-β-D-glucopyranosyl-(1→6)]-2-acetamido-4-O-acetyl-2-deoxy-α-D-galactopyranoside (**13**)

Compound **13** was prepared from **12** (389 mg, 0.21 mmol) as described for preparation of **9**. The product was purified by silica gel column chromatography (2:1 toluene−acetone) to give **13** (214 mg, 62%). $[\alpha]_D$ + 59.1 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$):δ 5.01 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1 of Gal a), 4.81 (d, 1H, $J_{1,2}$ = 3.3 Hz, H-1 of GalN), 4.55 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1 of GlcN), 4.53 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1 of Gal b). ^{13}C NMR (125 MHz, $CDCl_3$): δ 101.31 (C-1 of Gal a), 101.25 (C-1 of GlcN), 100.9 (C-1 of Gal b), 97.1 (C-1 of GalN). MALDI-TOFMS: Calcd for $C_{82}H_{92}N_2O_{34}Na$: *m*/*z* 1671.5 Found: *m*/*z* 1671.5 $[M + Na]^+$.

5.11. 5-(Methoxycarbonyl)pentyl β-D-galactopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-α-D-galactopyranoside (**14**)

To a solution of **13** (210 mg, 77.0 μmol) in MeOH (15 mL) was added NaOMe (100 mg) at room temperature and the mixture was stirred at 50 °C for 12 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 H_2O to give **14** (102 mg, 92%). $[\alpha]_D$ + 41.2 (*c* 1.0, H_2O). ^1H NMR (500 MHz, D_2O):δ 4.67 (d, 1H, $J_{1,2}$ = 3.4 Hz, H-1 of GalN), 4.36 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1 of GlcN), 4.282 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal b), 4.276 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal a). ^{13}C NMR (125 MHz, D_2O): δ 104.3 (C-1 of Gal a), 102.5 (C-1 of Gal b), 101.0 (C-1 of GlcN), 96.4 (C-1 of GalN). HRFABMS: Calcd for $C_{35}H_{60}N_2O_{23}Na$: *m*/*z* 899.3485 Found: *m*/*z* 899.3520 $[M + Na]^+$.

5.12. Biotinylated tetrasaccharide (J)

Compound **14** (102 mg, 0.12 mmol) was dissolved in neat anhydrous ethylenediamine (10 mL) and heated at 70 °C for 48 h. The mixture was concentrated with toluene and the product was purified by Sephadex LH-20 column chromatography in H_2O to give an amine intermediate. The amine (84.5 mg, 93.4 μmol) was dissolved in DMF (10 mL), and the pH was adjusted to 8−9 using DIPEA. Biotine-NHS (38.3 mg 0.11 mmol) was added and the reaction stirred for 12 h at room temperature. Toluene was added to and evaporated from the residue several times. The product was purified by Sephadex LH-20 column chromatography in H_2O to give **J** (88.7 mg, 68%). $[\alpha]_D$ + 47.2 (*c* 1.0, H_2O). ^1H NMR (500 MHz, D_2O):δ 4.67 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1 of GalN), 4.36 (d, 1H, $J_{1,2}$ = 8.2 Hz, H-1 of GlcN), 4.284 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal b), 4.278 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal a). ^{13}C NMR (125 MHz, D_2O): δ 104.3 (C-1 of Gal a), 102.5 (C-1 of Gal b), 101.0 (C-1 of GlcN), 96.4 (C-1 of GalN). HRFABMS: Calcd for $C_{46}H_{73}N_6O_{24}SNa$: *m*/*z* 1153.4686 Found: *m*/*z* 1153.4659 $[M + Na]^+$.

*5.13. 5-(Methoxycarbonyl)pentyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→3)-[4,6-di-O-acetyl-2,3-di-O-benzyl-α-D-galactopyranosyl-(1→4)-2-O-benzoyl-3,6-di-O-benzyl-β-D-galactopyranosyl-(1→4)-3-O-benzoyl-6-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→6)]-2-azido-4-O-benzyl-2-deoxy-α-D-galactopyranoside (**16**)*

Compound **16** was prepared from **6** (60.0 mg, 59.9 μmol) and **15** (102 mg, 65.8 μmol) as described for preparation of **12**. The product was purified by silica gel column chromatography (16:9 hexane-ethyl acetate) to give **16** (143 mg, 99%). $[\alpha]_D$ + 71.1 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$):δ 5.15 (d, 1H, $J_{1,2}$ = 8.1 Hz, H-1 of Gal a), 4.97 (d, 1H, $J_{1,2}$ = 3.4 Hz, H-1 of Gal c), 4.75 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1 of GalN), 4.61 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal b), 4.52 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1 of GlcN),. ^{13}C NMR (125 MHz, $CDCl_3$): δ 102.9 (C-1 of Gal a), 101.4 (C-1 of GlcN), 101.1 (C-1 of Gal b), 100.4 (C-1 of Gal c), 98.0 (C-1 of GalN). MALDI-TOFMS: Calcd for $C_{128}H_{129}Cl_3N_4O_{36}Na$: *m*/*z* 2425.7 Found: *m*/*z* 2425.7 $[M + Na]^+$.

*5.14. 5-(Methoxycarbonyl)pentyl 2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl-(1→3)-[2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl-(1→4)-3,6-di-O-acetyl-2-O-benzoyl-β-D-galactopyranosyl-(1→4)-2-acetamido-6-O-acetyl-3-O-benzoyl-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-acetamido-4-O-acetyl-2-deoxy-α-D-galactopyranoside (**17**)*

Compound **17** was prepared from **16** (281 mg, 0.117 mmol) as described for preparation of **9**. The product was purified by silica gel column chromatography (3:2 toluene—acetone) to give **23** (142 mg, 61%). $[\alpha]_D$ + 95.1 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$):δ 4.97 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1 of Gal a), 4.92 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1 of Gal c), 4.78 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1 of GalN), 4.69 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1 of Gal b) 4.45 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of GlcN),. ^{13}C NMR (125 MHz, $CDCl_3$): δ 101.3 (C-1 of Gal b), 101.19 (C-1 of GlcN), 101.15 (C-1 of Gal a), 99.3 (C-1 of Gal c), 97.0 (C-1 of GalN). MALDI-TOFMS: Calcd for $C_{99}H_{110}N_2O_{42}Na$: *m*/*z* 2021.6 Found: *m*/*z* 2021.6 $[M + Na]^+$.

*5.15. 5-(Methoxycarbonyl)pentyl β-D-galactopyranosyl-(1→3)-[α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-α-D-galactopyranoside (**18**)*

Compound **18** was prepared from **17** (142 mg, 77 μmol) as described for preparation of **14**. The product was purified by Sephadex LH-20 column chromatography in H_2O to give **18** (59 mg, 80%). $[\alpha]_D$ + 61.8 (*c* 1.0, H_2O). ^1H NMR (500 MHz, D_2O):δ 4.76 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1 of Gal c), 4.67 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1 of GalN), 4.36 (d, 1H, $J_{1,2}$ = 8.7 Hz, H-1 of GlcN), 4.34 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal b), 4.28 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal a). ^{13}C NMR (125 MHz, D_2O): δ 104.3 (C-1 of Gal a), 102.8 (C-1 of Gal b), 101.0 (C-1 of GlcN), 99.8 (C-1 of Gal c), 96.4 (C-1 of GalN). HRFABMS: Calcd for $C_{41}H_{70}N_2O_{28}Na$: *m*/*z* 1061.4013 Found: *m*/*z* 1061.4044 $[M + Na]^+$.

5.16. Biotinylated pentasaccharide (K)

Compound **K** was prepared from **18** (102 mg, 0.116 mmol) as described for preparation of **J**, yielding 31 mg (42%). $[\alpha]_D$ + 68.7 (*c* 0.8, H_2O). ^1H NMR (500 MHz, D_2O):δ 4.76 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1 of Gal c), 4.67 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1 of GalN), 4.36 (d, 1H, $J_{1,2}$ = 8.7 Hz, H-1 of GlcN), 4.34 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal b), 4.28 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal a). ^{13}C NMR (125 MHz, D_2O): δ 104.3 (C-1 of Gal a), 102.8 (C-1 of Gal b), 101.0 (C-1 of GlcN), 99.8 (C-1 of Gal c), 96.4 (C-1 of GalN). HRFABMS: Calcd for $C_{52}H_{88}N_6O_{29}SNa$: *m*/*z* 1315.5214 Found: *m*/*z* 1315.5194 $[M + Na]^+$.

*5.17. 5-(Methoxycarbonyl)pentyl 2-O-benzoyl-3,6-di-O-benzyl-4-O-chloroacetyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**20**)*

Compound **20** was prepared from **3** (123 mg, 0.292 mmol) and **19** (222 mg, 0.350 mmol) as described for preparation of **12**. The product was purified by silica gel column chromatography (14:1 toluene-ethyl acetate) to give **20** (206 mg, 75%). $[\alpha]_D$ + 99.4 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ 8.01—7.06 (m, 20H, Ar), 5.68 (d, 1H, $J_{3',4'}$ = 3.3 Hz, H-4′), 5.46—5.42 (m, 2H, H-2′, PhCH), 4.91 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 4.82 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-1′), 4.65 and 4.42 (each d, 2H, J_{gem} = 12.7 Hz, benzyl methylene), 4.54 and 4.49 (each d, 2H, J_{gem} = 11.7 Hz, benzyl methylene), 4.37 (d, 1H, $J_{3,4}$ = 3.1 Hz, H-4), 4.19 and 4.11 (each d, 2H, J_{gem} = 15.2 Hz, $COCH_2Cl$), 4.16 (dd, 1H, $J_{5,6a}$ = 12.5 Hz, $J_{6a,6b}$ = 1.5 Hz, H-6a), 4.06 (dd, 1H, $J_{2,3}$ = 10.8 Hz, $J_{3,4}$ = 3.3 Hz, H-3), 3.90—3.87 (m, 2H, H-5, H-6b), 3.70—3.62 (m, 7H, H-2, H-3′, H-6′a, OCH_2CH_2 a, OCH_3), 3.59—3.56 (m, 2H, H-5′, H-6′b), 3.45—3.41 (m, 1H, OCH_2CH_2 b), 2.30 (t, 2H, *J* = 7.5 Hz), 1.66—1.57 (m, 4H), 1.40—1.33(m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ174.9, 167.0, 165.2, 137.7, 137.4, 136.9, 133.0, 129.90, 129.87, 129.0, 128.8, 128.6, 128.3, 128.2, 128.11, 128.06, 128.0, 127.8, 126.1, 102.5(C-1′), 100.5 (CHPh) 98.7 (C-1), 76.2 (C-3′), 75.8 (C-4), 75.4 (C-3), 73.8 (CH_2Ph), 72.1 (C-5), 71.1 (CH_2Ph), 70.7 (C-2′), 69.1 (C-6), 68.3 (C-4′), 68.1 (OCH_2CH_2), 67.9 (C-6′), 63.0 (C-5′), 58.5 (C-2), 51.5(OCH_3), 40.1 (CH_2Cl), 33.9, 29.0, 25.6, 24.6. HRFABMS: Calcd for $C_{49}H_{54}ClN_3O_{14}Na$: *m*/*z* 966.3192 Found: *m*/*z* 966.3234 $[M + Na]^+$.

*5.18. 5-(Methoxycarbonyl)pentyl 2-O-benzoyl-3,6-di-O-benzyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**21**)*

To a solution of **20** (125 mg 0.13 mmol) in EtOH-pyridine (3:1, 4 mL) was added thiourea (30 mg 0.40 mmol), and the mixture was stirred for 2 h at 80 °C. The mixture was diluted with $CHCl_3$, washed with saturated aqueous $NaHCO_3$ and water, dried ($MgSO_4$), and concentrated. The product was purified by silica gel column chromatography (1:1 hexane-ethyl acetate) to give **21** (93.4 mg, 81%). $[\alpha]_D$ + 89.2 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ 8.04—7.12 (m, 20H, Ar), 5.92 (dd, 1H, $J_{1',2'}$ = 8.0 Hz, $J_{2',3'}$ = 9.6 Hz, H-2′), 5.44 (s, 1H, PhCH), 4.91 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 4.80 (d, 1H, $J_{1',2'}$ = 8.0 Hz H-1′), 4.66 and 4.51 (each d, 2H, J_{gem} = 12.4 Hz, benzyl methylene), 4.61 and 4.56 (each d, 2H, J_{gem} = 11.7 Hz, benzyl methylene), 4.41 (d, 1H, $J_{3,4}$ = 3.1 Hz, H-4), 4.15—4.08 (m, 3H, H-3, H-6a, H-4′), 3.86—3.83 (m, 3H, H-6b, H-6′a, H-6′b), 4.16 (dd, 1H, $J_{5,6a}$ = 5.8 Hz, $J_{5,6b}$ = 6.8 Hz, H-5), 3.68—3.63 (m, 6H, H-2, H-3′, OCH_2CH_2 a, OCH_3), 3.57 (s, 1H, H-5′), 3.45—3.41 (m, 1H, OCH_2CH_2 b), 2.30 (t, 2H, *J* = 7.5 Hz), 1.66—1.57 (m, 4H), 1.40—1.35(m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ174.9, 165.3, 138.0, 137.7, 137.1, 129.8, 128.7, 128.5, 128.4, 128.2, 128.00, 127.96, 127.8, 127.7, 126.2, 102.5 (C-1′), 100.5 (CHPh) 98.7 (C-1), 78.4 (C-3′), 75.8 (C-4), 75.0 (C-3), 73.7 (CH_2Ph), 73.6 (C-5), 71.3 (CH_2Ph), 71.0 (C-2′), 69.4 (C-6′), 69.0 (C-6), 68.2 (OCH_2CH_2), 66.2 (C-4′), 63.1 (C-5′), 58.5 (C-2), 51.5 (OCH_3), 33.9, 29.0, 25.6, 24.6. HRFABMS: Calcd for $C_{47}H_{53}N_3O_{13}Na$: *m*/*z* 890.3476 Found: *m*/*z* 890.3494 $[M + Na]^+$.

*5.19. 5-(Methoxycarbonyl)pentyl 2,3-di-O-benzyl-4,6-O-di-tert-butylsilylene-α-D-galactopyranosyl-(1→4)-2-O-benzoyl-3,6-di-O-benzyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**23**)*

Compound **23** was prepared from **21** (90.0 mg, 0.10 mmol) and **22** (92.5 mg, 0.16 mmol) as described for preparation of **12**. The product was purified by silica gel column chromatography (14:1 hexane-ethyl acetate) to give **23** (132 mg, 94%). $[\alpha]_D$ + 114 (*c* 1.0, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$):δ 4.95 (d, 1H, $J_{1,2}$ = 3.3 Hz, H-1

of Gal b), 4.90 (d, 1H, $J_{1,2}$ = 3.0 Hz, H-1 of GalN), 4.79 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal a). ^{13}C NMR (125 MHz, $CDCl_3$): δ 102.7 (C-1 of Gal a), 101.0 (C-1 of Gal b), 98.7 (C-1 of GalN). HRFABMS: Calcd for $C_{75}H_{91}N_3O_{18}SiNa$: *m/z* 1372.5965 Found: *m/z* 1372.5992 $[M + Na]^+$.

5.20. 5-(Methoxycarbonyl)pentyl 4,6-di-O-acetyl-2,3-di-O-benzyl-α-D-galactopyranosyl-(1→4)-2-O-benzoyl-3,6-di-O-benzyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranoside (**24**)

A solution of **23** (746 mg 0.55 mmol) in THF (6 mL) was added HF-Pyr. (3.5 mL) at −10 °C and then was stirred for 4 h. The reaction mixture was added to water, extracted with ethyl acetate, and the organic layer was washed with saturated aqueous $NaHCO_3$ and water, dried ($MgSO_4$), and concentrated. The residue was treated with Ac_2O (6 mL) in pyridine (10 mL). The reaction mixture was poured into ice-water and extracted with $CHCl_3$. The extract was washed sequentially with 5% HCl, saturated aqueous $NaHCO_3$ and water, dried ($MgSO_4$), and concentrated. The product was purified by silica gel column chromatography (1:1 hexane-EtOAc) to give **24** (522 mg, 73%). $[\alpha]_D$ + 123 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$):δ 5.06 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1 of Gal b), 4.90 (d, 1H, $J_{1,2}$ = 3.8 Hz, H-1 of GalN), 4.80 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1 of Gal a). ^{13}C NMR (125 MHz, $CDCl_3$): δ 103.0 (C-1 of Gal a), 100.9 (C-1 of Gal b), 98.7 (C-1 of GalN). HRFABMS: Calcd for $C_{71}H_{79}N_3O_{20}Na$: *m/z* 1316.5155 Found: *m/z* 1316.5110 $[M + Na]^+$.

5.21. 5-(Methoxycarbonyl)pentyl 4,6-di-O-acetyl-2,3-di-O-benzyl-α-D-galactopyranosyl-(1→4)-2-O-benzoyl-3,6-di-O-benzyl-β-D-galactopyranosyl-(1→3)-4,6-di-O-acetyl-2-azido-2-deoxy-α-D-galactopyranoside (**25**)

A solution of **24** (213 mg 0.165 mmol) in 80% AcOH was stirred at 70 °C for 2 h, then was diluted with toluene and concentrated. The residue was acetylated with acetic anhydride (3.0 mL) in pyridine (5.0 mL). After the reaction was quenched with MeOH, toluene was added and co-evaporated several times. The product was purified by silica gel column chromatography (3:2 hexane-ethyl acetate) to give **25** (208 mg, 98%). $[\alpha]_D$ + 121 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$):δ 5.07 (d, 1H, $J_{1,2}$ = 3.4 Hz, H-1 of Gal b), 4.85 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1 of GalN), 4.70 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1 of Gal a). ^{13}C NMR (125 MHz, $CDCl_3$): δ 102.0 (C-1 of Gal a), 100.5 (C-1 of Gal b), 98.0 (C-1 of GalN). HRFABMS: Calcd for $C_{68}H_{79}N_3O_{22}Na$: *m/z* 1312.5053 Found: *m/z* 1312.5048 $[M + Na]^+$.

5.22. 5-(Methoxycarbonyl)pentyl 4,6-di-O-acetyl-2,3-di-O-benzyl-α-D-galactopyranosyl-(1→4)-2-O-benzoyl-3,6-di-O-benzyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-di-O-acetyl-2-deoxy-α-D-galactopyranoside (**26**)

To a solution of **25** (208 mg, 0.161 mmol) in THF-AcOH-Ac_2O (3:2:1, 10 mL) was added Zn−Cu (800 mg). The mixture was stirred for 30 min at room temperature. After completion of the reaction, the mixture was filtered through Celite. The filtrate was concentrated with toluene and purified by silica gel column chromatography (5:1 toluene−acetone) to give **25** (184 mg, 88%). $[\alpha]_D$ + 124 (*c* 1.0, $CHCl_3$)

^{1}H NMR (500 MHz, $CDCl_3$):δ 5.06 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1 of Gal b), 4.90 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1 of GalN), 4.56 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1 of Gal a). ^{13}C NMR (125 MHz, $CDCl_3$): δ 100.8 (C-1 of Gal a), 100.6 (C-1 of Gal b), 97.1 (C-1 of GalN). HRFABMS: Calcd for $C_{70}H_{83}NO_{23}Na$: *m/z* 1328.5254 Found: *m/z* 1328.5278 $[M + Na]^+$.

5.23. 5-(Methoxycarbonyl)pentyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (**27**)

Compound **26** (184 mg, 0.141 mmol) in THF (5.0 mL) was hydrogenolysed in the presence of $Pd(OH)_2$/C (150 mg) for 4 h at room temperature, and the mixture was filtered and concentrated. The residue was acetylated with acetic anhydride (3.0 mL) in pyridine (5.0 mL). The reaction mixture was poured into ice-water and extracted with $CHCl_3$. After the reaction was quenched with MeOH, toluene was added and co-evaporated several times. To a solution of the residue (153 mg) in MeOH (6 mL) was added NaOMe (50 mg) at room temperature and the mixture was stirred at 40 °C for 4 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 H_2O to give **27** (84.0 mg, 88%). $[\alpha]_D$ + 127 (*c* 1.0, H_2O). ^{1}H NMR (500 MHz, D_2O):δ 4.77 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1 of Gal b), 4.69 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1 of GalN), 4.36 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1 of Gal a). ^{13}C NMR (125 MHz, D_2O): δ 104.5 (C-1 of Gal a), 100.0 (C-1 of Gal b), 96.6 (C-1 of GalN). HRFABMS: Calcd for $C_{27}H_{47}NO_{18}Na$: *m/z* 696.2691 Found: *m/z* 696.2707 $[M + Na]^+$.

5.24. Biotinylated trisaccharide (H)

Compound **H** was prepared from **27** (84.0 mg, 0.125 mmol) as described for preparation of **J**, yielding 82.3 mg (71%). $[\alpha]_D$ + 119 (*c* 1.0, H_2O). ^{1}H NMR (500 MHz, D_2O):δ 4.77 (d, 1H, $J_{1,2}$ = 4.0 Hz, H-1 of Gal b), 4.69 (d, 1H, $J_{1,2}$ = 3.8 Hz, H-1 of GalN), 4.35 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1 of Gal a). ^{13}C NMR (125 MHz, D_2O): δ 104.5 (C-1 of Gal a), 100.0 (C-1 of Gal b), 96.6 (C-1 of GalN). HRFABMS: Calcd for $C_{38}H_{65}N_5O_{19}SNa$: *m/z* 950.3892 Found: *m/z* 950.3934 $[M + Na]^+$.

5.25. 5-(Methoxycarbonyl)pentyl 4,6-di-O-acetyl-2,3-di-O-benzyl-α-D-galactopyranosyl-(1→4)-2-O-benzoyl-3,6-di-O-benzyl-β-D-galactopyranosyl-(1→3)-2-azido-4-O-benzyl-2-deoxy-α-D-galactopyranoside (**28**)

Compound **28** was prepared from **24** (270 mg, 0.21 mmol) as described for preparation of **6**. The product was purified by silica gel column chromatography (1:1 hexane-ethyl acetate) to give **28** (229 mg, 85%). $[\alpha]_D$ + 91.3 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$):δ 5.10 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1 of Gal b), 4.82 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1 of Gal a), 4.80 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1 of GalN). ^{13}C NMR (125 MHz, $CDCl_3$): δ 103.1 (C-1 of Gal a), 100.8 (C-1 of Gal b), 98.3 (C-1 of GalN). HRFABMS: Calcd for $C_{71}H_{81}N_3O_{20}Na$: *m/z* 1318.5311 Found: *m/z* 1318.5349 $[M + Na]^+$.

5.26. 5-(Methoxycarbonyl)pentyl 4,6-di-O-acetyl-2,3-di-O-benzyl-α-D-galactopyranosyl-(1→4)-2-O-benzoyl-3,6-di-O-benzyl-β-D-galactopyranosyl-(1→3)-[3,4,6-tri-O-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→6)]-2-azido-4-O-benzyl-2-deoxy-α-D-galactopyranoside (**29**)

Compound **29** was prepared from **28** (110 mg, 84.8 μmol) and **7** (106 mg, 0.17 mmol) as described for preparation of **5**. The product was purified by silica gel column chromatography (3:2 hexane-ethyl acetate) to give **29** (139 mg, 93%). $[\alpha]_D$ + 61.2 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$):δ 5.05 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1 of Gal b), 4.81 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1 of Gal a), 4.74 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1 of GalN), 4.69 (d, 1H, $J_{1,2}$ = 8.6 Hz, H-1 of GlcN). ^{13}C NMR (125 MHz, $CDCl_3$): δ 103.2 (C-1 of Gal a), 101.0 (C-1 of GlcN), 100.7 (C-1 of Gal b), 98.2 (C-1 of GalN). MALDI-TOFMS: Calcd for $C_{86}H_{99}Cl_3N_4O_{29}Na$: *m/z* 1779.5 Found: *m/z* 1778.8 $[M + Na]^+$.

5.27. 5-(Methoxycarbonyl)pentyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)- [2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-α-D-galactopyranoside (**30**)

To a solution of **29** (267 mg, 0.152 mmol) in THF-AcOH-Ac_2O (3:2:1, 12 mL) was added Zn–Cu (1 g). The mixture was stirred for 30 min at room temperature. After completion of the reaction, the solid was filtered off. The filtrate was concentrated and purified by silica gel column chromatography (1:1 toluene: acetone) to give an acetamido intermediate. This compound (183 mg) in THF (5.0 mL) was hydrogenolysed in the presence of $Pd(OH)_2/C$ (150 mg) for 5 h at room temperature, then the mixture was filtered and concentrated. The residue was acetylated with acetic anhydride (3.0 mL) in pyridine (5.0 mL). After the reaction was quenched with MeOH, toluene was added and co-evaporated several times. To a solution of per-acylated compound (133 mg) in MeOH (5 mL) was added NaOMe (50 mg) and the mixture was stirred at 40 °C for 7 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 H_2O to give **30** (71.9 mg, 54%). $[\alpha]_D$ + 87.3 (*c* 1.0, H_2O). ^1H NMR (500 MHz, D_2O):δ 4.77 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1 of Gal b), 4.67 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1 of GalN), 4.35 (d, 1H, $J_{1,2}$ = 7.6 Hz, H-1 of Gal a), 4.34 (d, 1H, $J_{1,2}$ = 8.6 Hz, H-1 of GlcN). ^{13}C NMR (125 MHz, D_2O): δ 104.5 (C-1 of Gal a), 101.1 (C-1 of GlcN), 100.0 (C-1 of Gal b), 96.4 (C-1 of GalN). HRFABMS: Calcd for $C_{35}H_{60}N_2O_{23}Na$: *m*/*z* 899.3485 Found: *m*/*z* 899.3530 $[M + Na]^+$.

5.28. Biotinylated tetrasaccharide (**I**)

Compound **I** was prepared from **30** (71.9 mg, 82.0 μmol) as described for preparation of **J**, yielding 48.3 mg (52%). $[\alpha]_D$ + 73.1 (*c* 1.0, H_2O). ^1H NMR (500 MHz, D_2O):δ 4.76 (d, 1H, $J_{1,2}$ = 4.0 Hz, H-1 of Gal b), 4.66 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1 of GalN), 4.35 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1 of Gal a), 4.34 (d, 1H, $J_{1,2}$ = 8.4 Hz, H-1 of GlcN). ^{13}C NMR (125 MHz, D_2O): δ 104.5 (C-1 of Gal a), 101.1 (C-1 of GlcN), 100.0 (C-1 of Gal b), 96.4 (C-1 of GalN). HRFABMS: Calcd for $C_{46}H_{73}N_6O_{24}SNa$: *m*/*z* 1153.4686 Found: *m*/*z* 1153.4681 $[M + Na]^+$.

5.29. Serum samples

Serum samples examined by ELISA were obtained from 60 patients who were confirmed to have AE and 60 NH individuals.

5.30. ELISA protocol

ELISA was performed using as previously described [27,28] with some modifications. The oligosaccharides in H_2O (13 pmol per well) were added to the wells of flat-bottomed microplates (Streptavidin C96, No. 236001; Nunc, Roskilde, Denmark) coated with streptavidin and these plates were incubated for 1 h at 37 °C. After the coating solution was discarded, the microplates were washed with 0.05% Tween-PBS (250 μL per well). Serum samples diluted 1:250 with 0.05% Tween-PBS (200 μL per well) were then added to the wells and incubated overnight at 4 °C. After washing with 0.05% Tween-PBS, 200 μL of anti-human IgG/HRP (P0214; DakoCytomation, Denmark; 1:1000 in 0.05% Tween-PBS) was added, and the microplates were incubated for 1 h at 37 °C. After further washing, bound antibodies were detected by the addition of ABTS peroxidase substrate solution (KPL, Gaithersburg, MD, USA, 200 μL per well). After incubation period of 8 min at 37 °C, the reaction was stopped by the addition of 1% SDS, and the absorbance (A) values were read at 405 nm on a microplate reader (Model 680; BIORAD, Hercules, California, USA).

5.31. Data analysis

The cut off value that separated positive and negative was defined as the average A plus two standard deviations for the 60 NH sera. The significant difference between the two groups was analyzed by the Student's *t*-test.

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