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Chemoenzymatic synthesis of a 3^{IV}, 6^{III}-disulfated Lewis^x pentasaccharide, a candidate ligand for human L-selectin

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Abstract

The disulfated pentasaccharide 3-*O*-SO_3^--β-D-Gal*p*-(1 → 4)-[α-L-Fuc*p*-(1 → 3)]-6-*O*-SO_3^--β-D-Glc*p*NAc-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc*p* was prepared according to a chemoenzymatic approach, starting from 4-methoxybenzyl *O*-(4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1 → 4)-*O*-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, obtained in six steps from hepta-*O*-acetyl lactosyl bromide. Coupling of this lactose derivative with *O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl) trichloracetimidate afforded, after dephthaloylation and re-*N*-acetylation, 4-methoxybenzyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 3)-*O*-(2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1 → 4)-*O*-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside. Regioselective sulfation at the primary position of the glucosamine residue was then successfully achieved and the benzyl groups were removed. Enzymatic galactosylation of 4-methoxybenzyl *O*-(2-acetamido-2-deoxy-6-*O*-sulfo-β-D-glucopyranosyl)-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-glucopyranoside sodium salt, and subsequent regioselective sulfation at position 3 of the outer galactose residue through the stannylene procedure, led then to 4-methoxybenzyl *O*-(3-sulfo-β-D-galactopyranosyl)-(1 → 4)- *O*-(2-acetamido-2-deoxy-6-sulfo-β-D-glucopyranosyl)-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-glucopyranoside disodium salt, which was finally fucosylated using human milk α-(1 → 3/4)-fucosyltransferase affording, after anomeric deprotection, the target pentasaccharide. © 1998 Elsevier Science Ltd.

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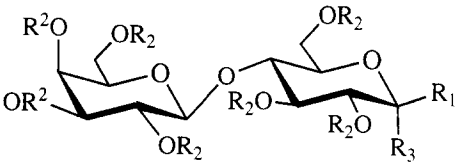
1. Introduction

Selectins, a family of adhesion molecules, mediate the interaction of circulating leukocytes with endothelial cells during the first step of recruitment to sites of inflammation. While P- and E-selectin, induced at the surface of endothelial cells in response to inflammatory signals, recognize carbohydrate ligands on leukocytes, L-selectin constitutively expressed on most leukocytes, interacts with carbohydrate ligands on endothelial cells during leukocyte migration at the site of tissue injury and also participates in the binding of these cells to the endothelial venules of peripheral lymph nodes in the normal process of recirculation of lymphocytes. Since the discovery of selectins, much work has been devoted to the characterization of carbohydrate ligands involved in the adhesion processes either in vivo or in vitro.

Three years ago, one of us reported the chemical synthesis of the 3^{IV}-sulfated Lewis^a pentasaccharide, the most powerful monovalent ligand for human E-selectin so far [1,2]. In continuation of this research program, we were interested in elucidating the specificity for L-selectin. It was already known for some years that neoglycolipids derived from an equimolar mixture of 3^{III}-sulfated Lewis^a and Lewis^x tetrasaccharides isolated from an ovarian cystadenoma were able to bind to L-selectin, along with E-selectin [3]. More recently, studies concerning the in-vivo specificity of L-selectin have led to partial characterization of the oligosaccharide structures of one physiological ligand, the GlyCAM-1 glycoprotein, occurring in mouse lymph nodes [4]: the 6-sulfated and 6′-sulfated sialyl Lewis^x sequences have been evidenced as the major capping groups of three O-linked chains of GlyCAM-1 [5,6]. However, the sulfation site involved in the interaction with L-selectin still remains controversial [7–9].

Whereas chemical synthesis of polysulfated lactose [10], 3^{II}, 6^{I}- and 3^{II}, 6^{II}-disulfated Lewis^x trisaccharide derivatives [7], 6^{II}-sulfated sialyl Lewis^x tetrasaccharide [11] and chemoenzymatic synthesis of 6^{II}-sulfated sialyl Lewis^x pentasaccharide [12] have been already reported, we now describe the synthesis of the 3^{IV}, 6^{III}-disulfated Lewis^x pentasaccharide **1** that was achieved according to a combined chemical and enzymatic approach, which shortened the number of steps as compared with the chemical synthesis alone.

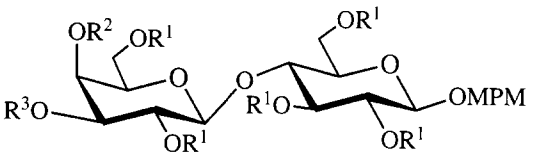
2. Results and discussion



2 $R^1 = H$, $R_2 = Ac$, $R_3 = Br$

3 $R^1 = OMPM$, $R^2 = Ac$, $R^3 = H$

4 $R^1 = OMPM$, $R^2 = R^3 = H$

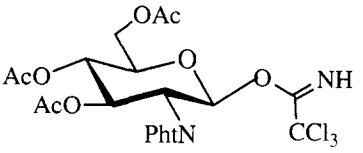


5 $R^1 = H$, $R^2, R^3 = C(CH_3)_2$

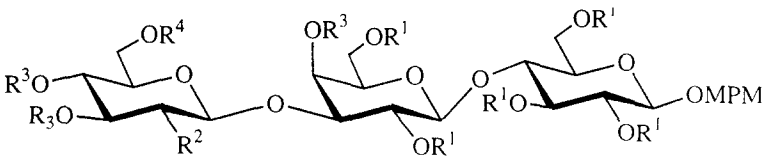
6 $R^1 = Bn$, $R^2, R^3 = C(CH_3)_2$

7 $R^1 = Bn$, $R^2 = R^3 = H$

8 $R^1 = Bn$, $R^2 = Ac$, $R^3 = H$



9

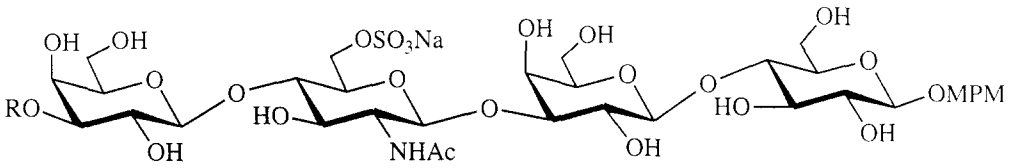


10 R^1 = Bn, R^2 = NPht, R^3 = R^4 = Ac

11 R^1 = Bn, R^2 = NHAc, R^3 = R^4 = H

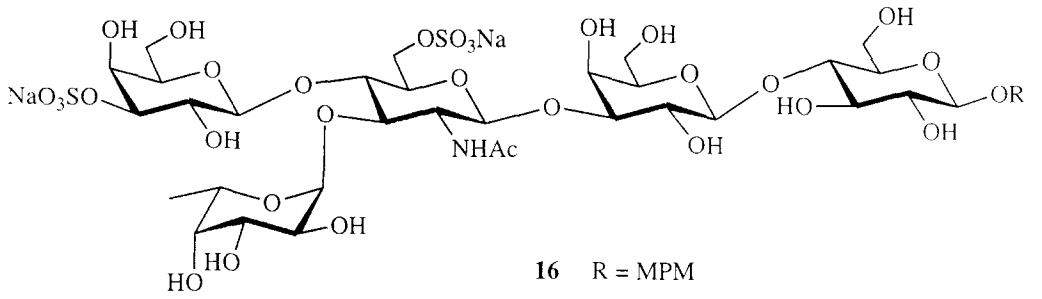
12 R^1 = Bn, R^2 = NHAc, R^3 = H, R^4 = SO_3Na

13 R^1 = R^3 = H, R^2 = NHAc, R^4 = SO_3Na



14 R = H

15 R = SO_3Na



16 R = MPM

1 R = H

The lactose derivative **8** was prepared in six steps from the common hepta-*O*-acetyl lactosyl bromide (**2**). The new 4-methoxybenzyl lactoside (**3**) was prepared from **2**, in the usual conditions, by using mercuric acetate in 4-methoxybenzyl alcohol. The 4-methoxybenzyl group was chosen because of its orthogonality with the benzyl protecting group [13]. Either it can be selectively removed to further extend the oligosaccharide chain, or retained during hydrogenolysis of a benzyl group by hydrogen transfer from cyclohexene using palladium hydroxide as a catalyst. The glycoside **3** obtained in 70% yield was de-*O*-acetylated with triethylamine in aqueous methanol giving **4** (95%); then, reaction of **4** with 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid monohydrate as described [14] afforded **5** (70%). Benzylation followed by acid hydrolysis of the isopropylidene group gave the diol **7** (87%) which was converted into the mono-acetate **8** in 86% yield by treatment with trimethylorthoacetate and subse-

quent acid catalyzed rearrangement of the intermediate orthoester [15]. Glycosylation of **8** with the imidate **9**, catalyzed by trimethylsilyl trifluoromethane sulfonate according to the Schmidt procedure [16], provided the trisaccharide **10** (77%). Conversion of the phthalimido group into the acetamido group under standard conditions afforded the trisaccharide **11** (54%) having four free hydroxyl groups which, treated with the sulfur trioxide–trimethylamine complex in anhydrous *N,N*-dimethylformamide, yielded the 6-monosulfated trisaccharide **12** (75%), as evidenced by the downfield shifted resonance for C-6^{III} at 65.87 ppm in its ^{13}C NMR spectrum. Selective removal of the benzyl groups in the presence of the anomeric 4-methoxybenzyl substituent could be achieved by treatment with palladium hydroxide on carbon and cyclohexene in methanol under reflux for 60 h, giving the monosulfated trisaccharide glycoside **13** in 70% yield. The downfield shifts for H-6^{III} and H-$6'^{III}$ at 4.33 and 4.21 ppm in the ^1H NMR spectrum of **13** confirmed the structure. Palladium hydroxide was preferred to metallic palladium which results in some desulfation. The trisaccharide **13** turned out to be a good substrate for the bovine milk β-(1 → 4)-galactosyltransferase [12]. Thus, the tetrasaccharide **14** was isolated in 84% yield after incubation of **13** with the enzyme, together with UDP-galactose epimerase, UDP-glucose and alkaline phosphatase [17]. The second sulfate group was then regioselectively introduced onto **14** having 11 free hydroxyl groups, by stannylene-directed sulfation with the sulfur trioxide–trimethylamine complex [18,19]. The 3^{IV},6^{III}-disulfated tetrasaccharide **15** was thus obtained in 60% yield after purification on DEAE-Sephadex and elution with triethylammonium hydrogen carbonate buffer. Natural and cloned fucosyltransferases have been already reported to be able to accept sulfated carbohydrates as substrates [12,20]. In our case, fucosylation of **15** was achieved using GDP-fucose and human milk α-[1 → 3/4] fucosyltransferase [21] in the presence of manganese chloride and alkaline phosphatase, affording **16** in 90% yield. The enzyme was used adsorbed on SP-Sephadex, the main advantage of this immobilization being concentration of the enzymatic activity. Finally, catalytic hydrogenation afforded the disulfated pentasaccharide target **1** in quantitative yield. Thus, from the chemically prepared trisaccharide **13**, a straightforward synthesis of **1** was achieved in 45% overall yield, in only four steps, two being enzymatic. The recent availability of cloned fucosyltransferases [1], allowing the scaling up of the syntheses, makes this strategy still more attractive. The 3^{IV}, 6^{III}-disulfated Lewis^x pentasaccharide **1** is now under biological evaluation towards human L-selectin.

3. Experimental

General methods.—All moisture-sensitive reactions were performed under a nitrogen atmosphere using dried glassware. All solvents were dried over standard drying agents and freshly distilled prior to use. Flash column chromatography was performed on Silica Gel 60A C.C. (6–35 μ) (S.D.S.). Reactions were monitored by TLC or HPTLC on Silica Gel $60F_{254}$ (E. Merck) with detection by charring with 10% H_2SO_4 in EtOH or 2% orcinol in 10% H_2SO_4 in EtOH. Melting points were determined with a capillary apparatus and are uncorrected. Optical rotations were measured with a Jasco digital micro polarimeter. IR Spectra were recorded on a Bruker IF S66. NMR Spectra were recorded at room temperature with Bruker AC 200, AC 250 or AM 400 spectrometers; chemical shifts are given relative to Me_4Si in $CDCl_3$; for NMR spectra in deuterium oxide, acetone (δ 2.22) was used as internal reference. The proton chemical shifts were assigned using ^1H homonuclear decoupling experiments. Elemental analyses were performed at the Service Central de Microanalyses du CNRS (Gif sur Yvette, France). Mass spectra were recorded on a Finigan MATT 95 apparatus coupled with electrospray. The following materials were purchased from the sources in parentheses: bovine milk D-GlcNAc-β-(1 → 4)-galactosyltransferase, UDP-glucose-4-epimerase, SP-Sephadex-C50-120 (Sigma); calf intestine alkaline phosphatase (Boehringer Mannheim); Bio-Beads SM-2 (100–200 mesh), AG 50W-X8 resin (100–200 mesh) (Bio-Rad), DEAE-Sephadex A-25 (Pharmacia); GDP-[^{14}C-fucose] (specific activity 285 mCi/mmol) (Amersham). GDP-fucose was synthesized according to published procedures [22,23].

Immobilization of human milk α-(1 → 3/4)-fucosyltransferase.—Radiochemical assay and partial purification of human milk α-(1 → 3/4)-fucosyltransferase were performed according to published procedure [21]. Thawed milk (0.14 U, 250 mL) was delipidated by centrifugation for 1.5 h at 5000 ×

[1] Through a french network (Réseau GTrec) devoted to production and study of recombinant glycosyltransferases, which is supported by the Ministère de l'Education Nationale, de la Recherche et de la Technologie.

g, mixed with dry SP-Sephadex C-50 (0.5 g) and gently stirred overnight. After adsorption, the resin was washed with water, equilibrated with 50 mM sodium cacodylate buffer (pH 7.5) and stored in the same buffer (0.13 U of fucosyltransferase adsorbed on SP-Sephadex C-50, 93% of loaded enzyme activity).

4-Methoxybenzyl O-*(2,3,4,6-tetra*-O-*acetyl-β*-D-*galactopyranosyl)-(1 → 4)-2,3,6-tri*-O-*acetyl-β*-D-*glucopyranoside* (**3**).—To a soln of hepta-*O*-acetyl lactosyl bromide (**2**, 8.5 g, 12.17 mmol) in 4-methoxybenzyl alcohol (16.8 g, 121.7 mmol) was added mercuric acetate (3.9 g, 12.17 mmol). The mixture was stirred for 2 h, then diluted with CH_2Cl_2 and filtered through Celite. The filtrate was washed with saturated aq $NaHCO_3$ soln, water, 20% potassium iodide solution and water again, dried (sodium sulfate) and concentrated. Flash chromatography (3:2 hexane–EtOAc) of the residue gave **3** (6.44 g, 70%); $[\alpha]_D^{25}$ −20° (*c* 2.0, CH_2Cl_2); R_f 0.21 (3:2 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.20 (d, 2 H, *J* 8.5 Hz, Ph), 6.88 (d, 2 H, Ph), 5.34 (d, 1 H, $J_{3,4}$ 3 Hz, H-4^{II}), 5.15 (t, 1 H, $J_{2,3} = J_{3,4}$ 9 Hz, H-3^{I}), 5.1 (dd, 1 H, $J_{1,2}$ 8.5, $J_{2,3}$ 9 Hz, H-2^{II}), 4.94 (dd, 1 H, H-2^{I}), 4.93 (dd, 1 H, H-3^{II}), 4.79 (d, 1 H, J_{gem} 12 Hz, Ph–CH), 4.53 (d, 1 H, Ph–CH′), 4.50 (d, 1 H, $J_{1,2}$ 8 Hz, H-1^{I}), 4.47 (d, 1 H, $J_{1,2}$ 8 Hz, H-1^{II}), 4.1 (m, 3 H, H-6^{I}, H-6′^{I}, H-6^{II}), 3.8 (s, 3 H, OCH_3), 3.60 (m, 1 H, H-5^{I}) and 2.23, 2.05, 2.02, 2.0, 1.96 (5 s, 21 H, 7 OAc).

4-Methoxybenzyl O-*β*-D-*galactopyranosyl)-(1 → 4)*-O-*β*-D-*glucopyranoside* (**4**).—A suspension of the peracetylated derivative **3** (3.6 g, 4.76 mmol) in 8:1:1 MeOH–water–triethylamine (100 mL) was stirred overnight at room temperature. The mixture was then concentrated to dryness and coevaporated several times with toluene. Recrystallization from water-EtOH gave **4** (2.1 g, 95%); mp 172–175 °C; $[\alpha]_D^{25}$ −17.5° (*c* 2.16, water); R_f 0.46 (3:5:1 2-propanol–EtOAc–water); ^{1}H NMR (deuterium oxide, 250 MHz); δ 7.4 (d, 2 H, *J* 8.5 Hz, Ph), 7.0 (d, 2 H, Ph), 4.82 (d, 1 H, J_{gem} 12 Hz, Ph–CH), 4.63 (d, 1 H, Ph–CH′), 4.48 (d, 1 H, $J_{1,2}$ 8 Hz, H-1^{I}), 4.41 (d, 1 H, $J_{1,2}$ 8 Hz, H-1^{II}), 3.96 (dd, 1 H, $J_{5,6}$ 2 Hz, $J_{6,6}$, 12 Hz, H-6^{I}), 3.6 (s, 3 H, OCH_3), 3.33 (t, $J_{2,3}$ 8 Hz, H-2^{I}); ^{13}C NMR (CD_3OD, 50 MHz): δ 131.5 (2 C–Ph), 114.70 (2 C–Ph), 104.80 (C-1^{II}), 102.65 (C-1^{I}), 80.32, 76.93, 76.36, 76.3, 74.53, 72.47, 71.7, 70.14 (C-2^{I}, C-3^{I}, C-4^{I}, C-5^{I}, C-2^{II}, C-3^{II}, C-4^{II}, C-5^{II}, CH_2), 62.40, 61.70 (C-6^{I}, C-6^{II}), 55.8 (OCH_3). Anal. Calcd for $C_{20}H_{30}O_{12} \cdot 0.5H_2O$: C, 50.95; H, 6.58; O, 42.46. Found: C, 51.09; H, 6.42; O, 42.60.

4-Methoxybenzyl O-*(3,4*-O-*isopropylidene-β*-D-*galactopyranosyl)-(1 → 4)*-O-*β*-D-*glucopyranoside* (**5**).—A soln of **4** (2.5 g, 5.4 mmol) in 2,2-dimethoxypropane (100 mL) containing *p*-toluenesulfonic acid monohydrate (100 mg) was heated at 70 °C for 2 h, then the mixture was neutralized with triethylamine, concentrated to dryness under reduced pressure and coevaporated with toluene to remove traces of triethylamine. The resultant residue was refluxed with 10:1 MeOH–water (60 mL) for 3 h. Then, the mixture was concentrated and coevaporated with toluene. Crystallization from boiling EtOH gave **5** (1.90 g, 70%); R_f 0.33 (9:1 CH_2Cl_2–EtOH); mp 200–203 °C; $[\alpha]_D^{25}$ −9° (*c* 2.04, CH_3OH); ^{1}H NMR (Me_2SO, 250 MHz): δ 7.3 (d, 2 H, *J* 8 Hz, Ph), 6.85 (d, 2 H, Ph), 5.5 (d, 1 H, *J* 5 Hz, OH), 5.23 (d, 1 H, *J* 5 Hz, OH), 4.9 (t, 1 H, *J* 5 Hz, OH), 4.68 (t, 1 H, *J* 5 Hz, OH), 4.76 (d, 1 H, J_{gem} 12 Hz, Ph–CH), 4.52 (d, 1 H, J_{gem} 12 Hz, Ph–CH′), 4.28 (d, 2 H, $J_{1,2}$ 8 Hz, H-1^{I}, H-1^{II}), 4.12 (dd, 1 H, $J_{3,4}$ 5, $J_{4,5}$ 1 Hz, H-4^{II}), 3.98 (dd, 1 H, $J_{2,3}$ 7 Hz, H-3^{II}), 3.76 (s, 3 H, OCH_3), and 1.42, 1.28 (2 s, 6 H, Me_2C); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 130.92 (2 C–Ph), 114.68 (2 C–Ph), 111.5 (CMe_2), 104.18, 102.82 (C-1^{I}, C-1^{II}), 81.04, 80.87, 76.45, 76.39, 75.35, 75.06, 74.87, 74.76, 71.54 (C-2^{I}, C-3^{I}, C-4^{I}, C-5^{I}, C-2^{II}, C-3^{II}, C-4^{II}, C-5^{II}, CH_2), 62.40, 61.94 (C-6^{I}, C-6^{II}), 55.67 (OCH_3), and 28.39, 26.48 (Me_2). Anal. Calcd for $C_{23}H_{34}O_{12}$: C, 54.97; H, 6.82; O, 38.21. Found: C, 54.80; H, 6.79; O, 37.93.

4-Methoxybenzyl O-*(2,6-di*-O-*benzyl-β*-D-*galactopyranosyl)-(1 → 4)*-O-*2,3,6-tri*-O-*benzyl-β*-D-*glucopyranoside* (**7**).—To **5** (1.6 g, 3.19 mmol) dissolved in Me_2NCHO (30 mL), were added benzyl bromide (2.3 mL, 19 mmol) and NaH (0.82 g, 20 mmol). The reaction mixture was stirred at 0 °C for 3 h, then diluted with CH_2Cl_2 and washed with water, aq $NaHCO_3$, water, dried and concentrated. Flash chromatography of the residue with (4:1 hexane–EtOAc containing 1% triethylamine) gave **6** (2.9 g, 94%). A soln of **6** (2.9 g, 3 mmol) in 70% acetic acid (50 mL) was kept for 3 h under reflux. The reaction mixture was then diluted with CH_2Cl_2 and the organic layer was washed with a saturated aq $NaHCO_3$ soln, water, dried and evaporated. Compound **7** crystallized from Et_2O–pentane (2.4 g, 87%); R_f 0.71 (3:2 hexane–EtOAc); mp 90 °C; $[\alpha]_D^{30}$ +3° (*c* 1, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.45–7.15 (m, 27 H, Ph), 6.85 (d, 2 H, *J* 8 Hz, Ph), 5.0–4.50 (m, 9 H, 9 Ph–CH), 4.45 (m, 5 H, H-1^{I}, H-1^{II}, 3 Ph–CH), 3.8 (s, 3 H, OCH_3), 2.5 (d, 1 H, *J* 4 Hz, OH) and 2.4 (d, 1 H, *J* 4 Hz, OH); ^{13}C NMR

($CDCl_3$, 50 MHz): δ 159.0 (C–Ph), 139.0–137.5 (C–Ph), 130.0 (2 C–Ph), 128.7–127.0 (C–Ph), 113.7 (2 C–Ph), 103.0, 102.17 (C-1^I, C-1^{II}), 83.0, 81.9, 79.9, 75.1, 75.0, 74.9, 73.46, 73.18, 73.0, 70.67, 68.63, 68.5, 67.0 (C-2^I, C-3^I, C-4^I, C-5^I, C-6^I, C-2^{II}, C-3^{II}, C-4^{II}, C-5^{II}, C-6^{II}, 6 CH_2) and 55.24 (OCH_3). Anal. Calcd for $C_{55}H_{60}O_{12}$: C, 72.35; H, 6.62; O, 21.03. Found: C, 72.37; H, 6.65; O, 21.23.

4-Methoxybenzyl O-*(4-O-acetyl-2,6-di-O-benzyl-β-*D*-galactopyranosyl)-(1 → 4)-O-2,3,6-tri-O-benzyl-β-*D*-glucopyranoside* (**8**).—To a soln of **7** (2.1 g, 2.3 mmol) in toluene (40 mL) were added trimethylorthoacetate (16 mL) and *p*-toluenesulfonic acid monohydrate (130 mg). The soln was stirred for 1.5 h at room temperature. The mixture was then diluted with CH_2Cl_2, neutralized with saturated aqueous $NaHCO_3$ soln, washed with water, dried and evaporated to dryness. The residue was dissolved in aq 80% acetic acid (40 mL) and the soln was stirred at room temperature for 1.5 h. Solvents were evaporated under diminished pressure, the last traces of acetic acid being removed by coevaporation with toluene. Purification by flash chromatography (2:1 hexane–EtOAc) gave **8** (1.4 g, 86%) as a syrup; $[\alpha]_D^{27}$ −40°(*c* 0.57, CH_2Cl_2); R_f 0.36 (2:1 hexane–EtOAc); ^1H NMR ($CDCl_3$, 250 MHz): δ 7.4–7.1 (m, 27 H, Ph), 6.85 (d, 2 H, *J* 8 Hz, Ph), 5.34 (d, 1 H, $J_{3,4}$ 3 Hz, H-4^{II}), 5.0–4.56 (m, 9 Ph–CH), 4.50–4.40 (m, 4 H, H-1^I, H-1^{II}, 2 Ph–CH), 4.23 (d, 1 H, J_{gem} 12 Hz, Ph–CH), 4.07 (t, 1 H, $J_{3,4} = J_{4,5}$ 10 Hz, H-4^I), 3.85 (s, 3 H, OCH_3), 2.5 (d, 1 H, OH) and 2.03 (s, 3 H, OAc); ^{13}C NMR ($CDCl_3$, 62.9 MHz): δ 159.25 (C–Ph), 138.99–137.89 (C–Ph), 129.62 (2 C–Ph), 129.46 (C–Ph), 128.45–127.32 (C–Ph), 113.75 (2 C–Ph), 102.5, 102.17 (C-1^I, C-1^{II}), 82.71, 81.69, 80.07, 76.36, 75.02, 74.94, 73.36, 73.20, 72.40, 70.67, 70.20, 69.52, 68.0, 67.13, 30.85, 60.77 (C-2^I, C-3^I, C-4^I, C-5^I, C-6^I, C-2^{II}, C-3^{II}, C-4^{II}, C-5^{II}, C-6^{II}, 6 CH_2), 55.23 (OCH_3) and 20.77 ($OCOCH_3$). Anal. Calcd for $C_{57}H_{62}O_{13}$: C, 71.68: H, 6.54; O, 21.78. Found: C, 71.89, H, 6.25; O, 21.54.

4-Methoxybenzyl O-*(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranosyl)-(1 → 3)-O-(4-O-acetyl-2,6-di-O-benzyl-β-*D*-galactopyranosyl)-(1 → 4)-O-2,3,6-tri-O-benzyl-β-*D*-glucopyranoside* (**10**).—Trimethylsilyl trifluoromethane sulfonate (28 μL, 92.4 mg, 0.146 mmol) in CH_2Cl_2 (280 μL) was added to a soln of **8** (1.4 g, 1.46 mmol) in CH_2Cl_2 (10 mL). A soln of the imidate **9** [16] (1 g, 1.76 mmol) in CH_2Cl_2 (5 mL) was then slowly added to the mixture cooled at −70 °C. After 0.5 h, the reaction was stopped by the addition of 10% triethylamine in CH_2Cl_2 and washed with water. The residue was chromatographed on silica gel (2:1 hexane–EtOAc) to give **10** (1.53 g, 77%); $[\alpha]_D^{25}$ +13°(*c* 1, CH_2Cl_2), R_f 0.28 (2:1 petroleum ether–EtOAc); ^1H NMR ($CDCl_3$, 250 MHz): δ 7.6–6.7 (m, 33 H, Ph), 5.8 (dd, 1 H, $J_{3,4}$ 9 Hz, $J_{2,3}$ 10 Hz, H-3^{III}), 5.53 (d, 1 H, $J_{1,2}$ 9 Hz, H-1^{III}), 5.4 (d, 1 H, $J_{3,4}$ 3 Hz, H-4^{II}), 5.17 (t, 1 H, $J_{4,5}$ 10 Hz, H-4^{III}), 4.90–4.76 (m, 3 H, 3 Ph–CH), 4.62 (d, 1-H, $J_{1,2}$ 8 Hz, H-1^{II}), 4.62 (d, 1 H, *J* 11 Hz, Ph–CH), 4.48 (d, 1 H, $J_{1,2}$ 8 Hz, H-1^I), 4.48 (d, 1 H, *J*11 Hz, Ph–CH), 3.8 (s, 3 H, OCH_3), 3.55 (dd, $J_{2,3}$ 8 Hz, H-3^{II}) and 2.05, 2.0, 1.9, 1.55 (4 s, 12 H, 4 OAc); ^{13}C NMR ($CDCl_3$, 62.9 MHz): δ 139.0–138.18 (C–Ph), 130.96 (2 C–Ph), 129.54–123.36 (C–Ph), 113.7 (2 C–Ph), 102.1, 101.8, 98.2 (C-1^I, C-1^{II}, C-1^{III}), 82.6, 81.6, 79.2, 78.8, 76.0, 75.09, 74.90, 74.69, 74.37, 73.50, 73.10, 72.54, 71.70, 70.6, 70.47, 69.64, 68.78, 68.23, 66.34, 61.5 (C-2^I, C-3^I, C-4^I, C-5^I, C-6^I, C-2^{II}, C-3^{II}, C-4^{II}, C-5^{II}, C-6^{II}, C-3^{III}, C-4^{III}, C-5^{III}, C-6^{III}, 6 CH_2), 55.22 (C-2^{III}), 54.86 (OCH_3), and 20.69, 20.40 (CH_3CO). Anal. Calcd for $C_{77}H_{81}O_{22}$: C, 67.39; H, 5.95; N, 1.02; O, 25.65. Found: C, 66.94; H, 5.96; N, 1.27; O, 25.54.

4-Methoxybenzyl O-*(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 3)-O-(2,6-di-O-benzyl-β-*D*-galactopyranosyl)-(1 → 4)-O-2,3,6-tri-O-benzyl-β-*D*-glucopyranoside* (**11**).—Hydrazine monohydrate (10 mL) was added to **10** (1.28 g, 0.93 mmol) in EtOH (90 mL). The mixture was refluxed for 24 h, then concentrated, and the remaining solvents evaporated with an excess of toluene. The residue was dissolved in MeOH (60 mL) and treated with Ac_2O (6 mL) at room temperature. After 24 h, the soln was concentrated and coevaporated with EtOH and toluene. Flash chromatography (95:5 CH_2Cl_2–EtOH) gave **11** (0.58 g, 54%) as a powder; $[\alpha]_D^{27}$ −4° (*c* 1.05, CH_2Cl_2); R_f 0.44 (9:1 CH_2Cl_2–EtOH); ^1H NMR ($CDCl_3$, 400 MHz): δ 7.5–7 (m, 27 H, Ph), 6.8 (d, 2 H, *J* 8 Hz, Ph), 6.15 (1 H, NH), 4.96 (d, *J* 11 Hz, Ph–CH), 4.82 (m, 3 H, 3 Ph–CH), 4.68–4.50 (m, 6 H, H-1^{III}, 5 Ph–CH), 4.43–4.26 (m, 5 H, H-1^{II}, H-1^I, 3 Ph–CH), 4.05 (d, 1 H, $J_{3,4}$ 3 Hz, H-4^{II}), 3.8 (s, 3 H, OCH_3), and 1.42 (s, 3 H, $NHCOCH_3$); ^{13}C NMR (CD_3OD, 62.9 MHz): δ 173.0 (CO), 139.5–138.5 (C–Ph), 129.98 (2 C–Ph), 128.59 (C–Ph), 128.36–127.16 (C–Ph), 112.98 (2 C–Ph), 102.95, 102.65, 102.31 (C-1^I, C-1^{II}, C-1^{III}). 83.0, 82.9, 81.89, 79.05, 76.67, 76.29, 75.08, 74.60, 73.35, 70.14, 68.56 (C-2^I, C-3^I, C-4^I, C-5^I, C-2^{II}, C-3^{II}, C-4^{II}, C-5^{II}, C-3^{III}, C-4^{III}, C-5^{III}), 75.51, 75.22, 74.88, 73.80, 73.37, 70.99, 68.98, 68.31 (6 CH_2, C-6^I, C-6^{II}), 61.28 (C-6^{III}),

56.41 (C-2^{III}), 55.41 (OCH_3) and 22.75 (CH_3CONH). Anal. Calcd for $C_{63}H_{75}NO_{18} \cdot H_2O$: C, 66.71; H, 6.66; N, 1.23. Found: C, 66.64; H, 6.64; N, 1.11.

4-Methoxybenzyl O-*(2-acetamido-2-deoxy-6-*O-*sulfo -β-*D-*glucopyranosyl)-(1 → 3)*-O-*(2,6-di-*O-*benzyl-β-*D-*galactopyranosyl)-(1 → 4)*-O-*2,3,6-tri-*O-*benzyl-β-*D-*glucopyranoside sodium salt* (**12**).—To a soln of **11** (200 mg, 0.18 mmol) in Me_2NCHO (5 mL) was added trimethylamine–sulfur trioxide (50 mg, 0.36 mmol). The reaction mixture was stirred for 24 h at 40 °C, then the reaction was stopped by the addition of MeOH (500 μL). Compound **12** was obtained by flash chromatography (4:1 CH_2Cl_2–MeOH) and converted into its sodium salt by passing over a column of AG 50W-X8 resin (Na^+ form) (164 mg, 75%); $[\alpha]_D^{27}$ −11.5° (*c* 2.17, CH_2Cl_2); R_f 0.58 (4:1 CH_2Cl_2–MeOH); ^{1}H NMR (4:1 $CDCl_3$–CD_3OD, 250 MHz): δ 7.4–7.1 (m, 27 H, Ph), 6.8 (d, 2 H, *J* 8 Hz, Ph), 4.93 (d, 1 H, *J* 11 Hz, Ph–CH), 4.84 (d, 2 H, *J* 11 Hz, 2 Ph–CH), 4.1 (d, $J_{3,4}$ 3 Hz, H-4^{II}), 3.95 (t, $J_{3,4} = J_{4,5}$ 9 Hz, H-4^{I}), 3.8 (s, 3 H, OCH_3), 3.2 (m, 1 H, H-5^{I}), 1.7 (s, 3 H, $NHCOCH_3$); ^{13}C NMR ($CDCl_3$–CD_3OD 4:1, 62.9 MHz): δ 172.94 (CO), 138.65–137.55 (C–Ph), 129.39 (2 C–Ph), 128.02–126.67 (C–Ph), 113.40 (2 C–Ph), 102.61, 101.97, 101.71 (C-1^{I}, C-1^{II}, C-1^{III}), 82.46, 82.40, 81.14, 78.2, 77.5, 75.98, 74.45, 73.84, 72.57, 68.93, 68.58 (C-2^{I}, C-3^{I}, C-4^{I}, C-5^{I}, C-2^{II}, C-3^{II}, C-4^{II}, C-5^{II}, C-3^{III}, C-4^{III}, C-5^{III}), 74.48, 74.4, 74.0, 73.2, 72.82, 70.40, 69.03, 67.67 (6 CH_2, C-6^{I}, C-6^{II}), 65.87 (C-6^{III}), 55.33 (C-2^{III}), 54.84 (OCH_3) and 22.22 (CH_3CONH). Anal. Calcd for $C_{63}H_{72}NO_{20}SNa \cdot H_2O$: C, 61.19; H, 6.03; N, 1.13. Found: C, 60.91; H, 6.04; N, 0.97.

4-Methoxybenzyl O-*(2-acetamido-2-deoxy-6-*O-*sulfo -β-*D-*glucopyranosyl)-(1 → 3)*-O-*β-*D-*galactopyranosyl)-(1 → 4)*-O-*β-*D-*glucopyranoside sodium salt* (**13**). —To a soln of **12** (100 mg, 0.09 mmol) in MeOH (4 mL) was added 20% palladium hydroxide/carbon (30 mg) and cyclohexene (2 mL). The mixture was refluxed for 60 h, filtered through Celite and evaporated in vacuo. The residue was loaded onto a column of Bio-Beads SM-2 (20 × 1 cm) in water and the product was eluted with (1:9) 2-propanol–water; after concentration, the eluate was passed through a small column of AG 50W-X8 ion-exchange resin (Na^+ form) to give **13** as its sodium salt (47.5 mg, 70%); $[\alpha]_D^{26}$ −5° (*c* 0.37, water); R_f 0.44 (3:5;1 2-propanol–EtOAc–water); IR (KBr): ν_{max} 3433 (OH), 1649 (C=O), 1251 (S=O) cm^{-1}; ^{1}H NMR (see Table 1). ^{13}C NMR (deuterium oxide, 62.9 MHz): 130.7 (2 C–Ph), 114.14 (2 C–Ph), 102.97, 100.79 (C-1^{I}, C-1^{II}, C-1^{III}), 82.31, 78.3, 75.11, 74.85, 74.5, 73.54, 72.81, 71.2, 69.95, 69.63, 68.39 (C-2^{I}, C-3^{I},

Table 1
Selected 400-MHz ^{1}H NMR data for **13–15** and **1**

Residue	Proton (*J*)	d (ppm), *J* (Hz)			
		13	**14**	**15**	**1**
Glc^{I}	H-1 ($J_{1,2}$)	4.52 (8.0)	4.50 (8.0)	4.51 (8.0)	α 5.2 (4.0)
					β 4.65 (8.0)
	H-2	3.32 (t)	3.30 (t)	3.30 (t)	3.27 (t)
β-D Gal^{II}	H-1 ($J_{1,2}$)	4.42 (8.0)	4.40 (8.0)	4.41 (7.0)	4.43 (8.0)
	H-4 ($J_{3,4}$)	4.17 (3.0)	4.16 (2.0)	4.17 (3.0)	4.18 (3.0)
β-D GlcNAc^{III}	H-1 ($J_{1,2}$)	4.77 (8.0)	4.69 (7.8)	4.69 (8.5)	4.72 (8.0)
	H-6′ ($J_{6,6'}$) ($J_{5,6'}$)	4.29 (11.0) (5.0)	4.29 (11.0)	4.29 (10.0) (5.0)	4.34
	H-6 ($J_{5,6}$)	4.32 (< 1)	4.37	4.39 (< 1)	4.38 (2.0)
	NAc	2.03	2.03	2.03	2.03
β-D Gal^{IV}	H-1 ($J_{1,2}$)		4.49 (8.0)	4.61 (8.0)	4.61 (8.0)
	H-3 ($J_{2,3}$)			4.33 (10.0)	4.32 (10.0)
	H-4 ($J_{3,4}$)		3.90 (3.0)	4.27 (3.0)	4.27 (3.0)
α-Fuc^{V}	H-1 ($J_{1,2}$)				5.12 (4.0)
	H-5 ($J_{5,6}$)				4.81 (6.0)
	CH_3				1.17
other	Ar	7.02 (9.0)	7.01 (9.0)	6.99 (9.0)	
		7.4 (9.0)	7.40 (9.0)	7.39 (9.0)	
	CH–Ar	4.87 (11.0)	4.86 (11.0)	4.85 (11.0)	
		4.69 (11.0)	4.67 (11.0)	4.68 (11.0)	
	OCH_3	3.84	3.83	3.83	

C-4^{I}, C-5^{I}, C-2^{II}, C-3^{II}, C-4^{II}, C-5^{II}, C-3^{III}, C-4^{III}, C-5^{III}, CH_2), 67.1 (C-6^{III}), 61.17, 60.10 (C-6^{I}, C-6^{II}); 55.61 (C-2^{III}), 55.09 (OCH_3), and 22.22 ($NHCOCH_3$). LRMS (positive mode): *m/z* 790.0 $[M + Na]^+$.

4-Methoxybenzyl O-*β*-D-*galactopyranosyl-(1 → 4)*-O-*(2-acetamido-2-deoxy-6-sulfo-β*-D-*glucopyranosyl)-(1 → 3)*-O-*β*-D-*galactopyranosyl-(1 → 4)*-O-*β*-D-*glucopyranoside sodium salt* (**14**).—The trisaccharide **13** (25 mg, 0.033 mmol), UDP-Glc (37 mg, 0.060 mmol), bovine milk D-GlcNAc *β*-(1 → 4)-galactosyltransferase (0.25 U), UDP-glucose-4-epimerase (1 U), calf intestine alkaline phosphatase (4 U) were incubated at 37 °C for 72 h in 50 mM sodium cacodylate buffer (1 mL, pH 7.4) containing 10 mM manganese chloride. Then, the mixture was applied to a column of Bio-Beads SM-2 (20 × 1 cm). After washing with water, the product was eluted with 5:95 2-propanol–water; the eluate was concd, passed through a small column of AG 50W-X8 ion exchange resin (Na^+ form), and then lyophilized to give **14** as its sodium salt (25 mg, 84%); $[\alpha]_D^{26}$ −1° (*c* 1.15, water); IR (KBr): ν_{max} 3420 (OH), 1653 (C=O), 1249 (S=O) cm^{-1}; ^1H NMR (see Table 1); ^{13}C NMR (deuterium oxide, 100 MHz): *δ* 174.67 (CO), 159.0 (C–Ph), 130.42 (2 C–Ph), 113.9 (2 C–Ph), 128.91 (C–Ph), 112.72, 102.55, 102.4, 100.56 (C-1^{I}, C-1^{II}, C-1^{III}, C-1^{IV}), 82.07, 78.27, 77.39, 75.14, 74.82, 74.57, 74.24, 72.56, 72.28, 72.20, 71.92, 70.92, 70.77, 69.68, 68.40, 68.09 (C-2^{I}, C-3^{I}, C-4^{I}, C-5^{I}, C-2^{II},C-3^{II}, C-4^{II}, C-5^{II}, C-3^{III}, C-4^{III}, C-5^{III}, C-2^{IV}, C-3^{IV}, C-4^{IV}, C-5^{IV}, CH_2), 66.31 (C-6^{III}), 60.88, 60.83, 59.93 (C-6^{I}, C-6^{II}, C-6^{IV}), 55.18 (C-2^{III}), 54.96 (OCH_3) and 21.98 ($NHCOCH_3$). LRMS (positive mode): *m/z* 951.9 $[M + Na]^+$.

4-Methoxybenzyl O-*(3-sulfo-β*-D-*galactopyranosyl-(1 → 4)*-O-*(2-acetamido-2-deoxy-6-sulfo-β*-D-*glucopyranosyl)-(1 → 3)*-O-*β*-D-*galactopyranosyl-(1 → 4)*-O-*β*-D-*glucopyranoside disodium salt* (**15**).—Compound **14** (25 mg, 0.027 mmol) and dibutyltinoxide (10 mg, 0.040 mmol)) were stirred in refluxing MeOH (1 mL) under nitrogen overnight. The solvent was removed in vacuo and the dry dibutylstannylene derivative was treated with Me_3N–sulfur trioxide (75 mg, 0.054 mmol) at room temperature overnight. The reaction mixture was then diluted with MeOH (3 mL) and evaporated under reduced pressure. The residue, dissolved in water, was loaded onto a DEAE-Sephadex A-25 column (25 × 2 cm), eluting with a linear gradient of 0 to 2 M triethylammonium hydrogen carbonate buffer (pH 8). Fractions containing the disulfated tetrasaccharide (eluting at 400 mM triethylammonium hydrogen carbonate) were pooled and twice lyophilized. The bis(triethylammonium) salt was converted to the disodium salt by passing through a column of AG 50W-X8 resin (Na^+ form) and the eluate was lyophilized affording **15** (16.5 mg, 60%); $[\alpha]_D^{26}$ −0.5° (*c* 0.56, water); R_f 0.073 (6:2:3 EtOAc–water–acetic acid); IR (KBr): ν_{max} 3429 (OH), 1653 (C=O), 1249 (S=O); ^1H NMR (see Table 1); ^{13}C NMR (deuterium oxide, 100 MHz): *δ* 130.4 (2 C–Ph), 113.85 (2 C–Ph), 102.7, 102.5, 101.9, 100.55 (C-1^{I}, C-1^{II}, C-1^{III}, C-1^{IV}), 79.8 (C-3^{IV}), 82.2, 78.35, 77.5, 74.85, 74.6, 74.45, 74.25, 72.7, 72.3, 72.05, 71.0, 69.7, 69.05, 68.15, 66.7, 66.4, (C-2^{I}, C-3^{I}, C-4^{I}, C-5^{I}, C-2^{II}, C-3^{II}, C-4^{II}, C-5^{II}, C-3^{III}, C-4^{III}, C-5^{III}, C-6^{III}, C-2^{IV}, C-4^{IV}, C-5^{IV}, CH_2), 61.0, 60.75, 60.0 (C-6^{I}, C-6^{II}, C-6^{IV}). 55.3 (C-2^{III}), 55.0 (OCH_3) and 21.5 ($NHCOCH_3$). LRMS (negative mode): *m/z* 492.6 $[M - 2\ Na]^{2-}$.

4-Methoxybenzyl O-*(3-sulfo-β*-D-*galactopyranosyl-(1 → 3)*-O-*[(α*-L-*fucopyranosyl)(1 → 4]]*-O-*(2-acetamido-2-deoxy-6*-O-*sulfo-β*-D-*glucopyranosyl)-(1 → 3)*-O-*β*-D-*galactopyranosyl-(1 → 4)-β*-D-*glucopyranoside disodium salt* (**16**).—The tetrasaccharide **15** (4 mg, 3.9 μmol)), GDP-fucose (6.2 mg, 7.8 μmol), human milk *α*-(1 → 3/4)-fucosyltransferase adsorbed on SP-C50 Sephadex (4 mU) and alkaline phosphatase (0.4 U) were incubated at 37 °C for 48 h in 50 mM sodium cacodylate buffer (2 mL, pH 7.5) containing 8 mM manganese chloride, 1.6 mM adenosine triphosphate and 1.6 mM NaN_3. The gel was filtered and washed with the same buffer. The filtrate and washings were combined and applied to a column of DEAE-Sephadex A-25 (HCO_3^- form). Elution with a gradient of 0 to 2 M triethylammonium hydrogen carbonate buffer (pH 8.0) gave **16** as its bis(triethylammonium) salt which was converted to the disodium salt by passing through a column of Bio-Rad AG 50W-X8 resin (Na^+ form). The freeze-dried eluate afforded **16** (4.0 mg, 90%); $[\alpha]_D^{26}$ −21° (*c* 0.5, water); R_f 0.65 (3:3:2 2-propanol–EtOAc–water); IR (KBr): ν_{max} 3429 (OH), 1652 (C=O), 1249 (S=O); ^1H NMR (deuterium oxide, 250 MHz): *δ* 7.35 (d, 2 H *J* 8 Hz, Ph), 6.98 (d, 2 H, Ph), 5.08 (d, 1 H, $J_{1,2}$ 4 Hz, H-1^{V}), 4.87 (q, 1 H, $J_{6,5}$ 6 Hz, H-5^{V}), 4.71 (d, 1 H, $J_{1,2}$ 8 Hz, H-1^{III}) 4.64 (d, 1 H, J_{gem} 8 Hz, Ph–CH), 4.57 (d, 1 H, $J_{1,2}$ 8 Hz, H-1^{IV}), 4.47 (d, 1 H, $J_{1,2}$ 8 Hz, H-1^{I}), 4.37 (d, 1 H, $J_{1,2}$ 8 Hz, H-1^{II}), 4.32 (m, 2 H, H-6^{III}, H-6′^{III}), 4.24 (dd, 1 H, $J_{3,4}$ 3 Hz, $J_{2,3}$ 10 Hz, H-3^{IV}), 4.22 (d, 1 H, H-4^{IV}), 4.13 (d, 1 H, $J_{3,4}$ 3 Hz, H-4^{II}), 3.82 (s, 3 H, OCH_3), 3.26 (dd, 1 H, H-2^{I}), 2.0 (s, 3 H, OAc)

and 1.15 (d, 3 H, CH_3); LRMS (negative mode): *m/z* 565.6 $[M - 2\ Na]^{2-}$.

O-(3-Sulfo-β-D-galactopyranosyl-(1 → 3)-O-[(α-L-fucopyranosyl)(1 → 4)]-O-(2-acetamido-2-deoxy-6-O-sulfo - β - D - glucopyranosyl) - (1 → 3) - O - β - D - galactopyranosyl-(1 → 4)-αβ-D-glucopyranose disodium salt (**1**).—A mixture of 4:1 MeOH–water (2 mL) was added to **16** (4 mg, 3.5 μmol) and the resulting mixture was stirred under hydrogen gas atmosphere in the presence of 10% palladium–carbon (4 mg). After one night, the mixture was filtered through Celite and evaporated. The residue was converted to its disodium salt by passing through a column of Bio-Rad AG 50W-X8 resin (Na^+ form); lyophilisation of the eluate afforded **1** (3.6 mg, 98%); $[\alpha]_D^{27}$ −3° (*c* 0.3, water); R_f 0.45 (3:3:2 2-propanol–EtOAc–water); IR (KBr): ν_{max} 3419 (OH), 1657 (C=O), 1261 (S=O) cm^{-1}; LRMS (negative mode): *m/z* 505.4 $[M - 2\ Na]^{2-}$.

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