O-SULFATION OF 4-*O*-SUBSTITUTED DERIVATIVES OF D-GLUCURONIC ACID

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Abstract

The sulfation of heparan sulfate, 2-sulfation of D-glucuronic acid, is regioselective as has been reported. 4-O-substituted D-glucuronic acid derivatives were synthetized in this work to study the sulfation of monosacharide models. No selectivity between hydroxyls 2- and 3- was obtained.

Resumen

La sulfatación de heparan sulfato es regioselectiva en la posición 2 de las unidades de ácido D-glucurónico, según se observó previamente. Se sintetizaron derivados 4-O-sustituidos de ácido D-glucurónico a fin de estudiar la sulfatación en monosacáridos modelo. No se obtuvo selectividad entre los hidroxilos 2 y 3 de los derivados preparados.

Keywords: Sulfation; Heparan sulfate; D-Glucuronic acid

Introduction

Sulfated glycosaminoglycans (GAGs) are complex polysaccharides composed of alternating units of hexuronic acid and hexosamine. Both heparin (HEP) and heparan sulfates (HS) have 1→ 4 linkages and 2-amino-2-deoxy-D-glucose, but while 2-sulfated L-iduronic acid is the major uronic acid in HEP, D-glucuronic acid is more abundant in HS.

Sulfate groups are disposed allowing the polysaccharide to bind the plasma proteins such as antithrombin III (AT III), heparin cofactor II (HCII), lipoprotein lipase (LPL) and low-density lipoproteins (LDL), and therefore the sulfation pattern is closely related to the biological activity of the polymer [1].

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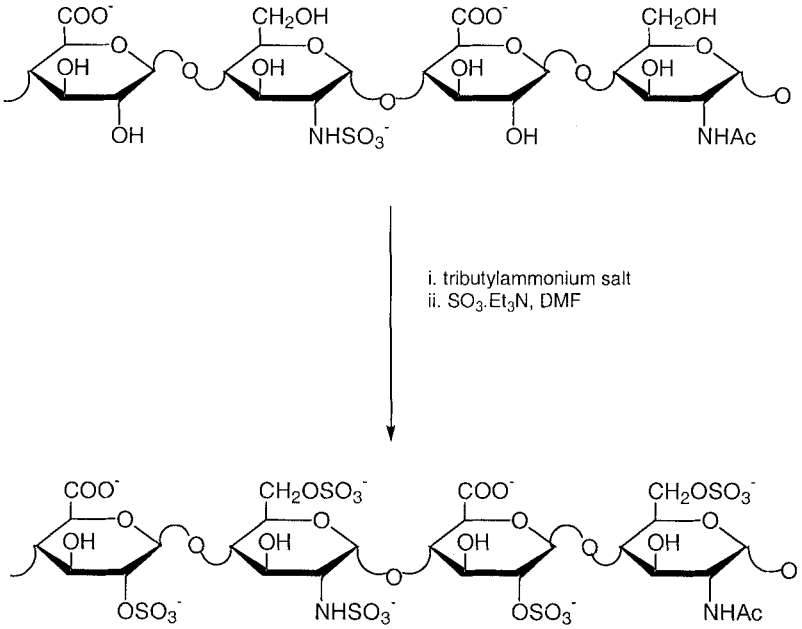
An increasing charge density of GAGs is usually associated with stronger protein binding [1]. Consequently, a number of studies have been carried out in order to modify the sulfate content of HEP and HS [2,3].

The natural abundance and wide distribution of HS make this polysaccharide a suitable starting material for the synthesis of modified GAGs. Our previous studies on chemical modification of GAGs include *N*-salicylate derivatives of HEP [4], carboxymethyl derivatives of HS [5], sulfated derivatives of previously periodate-oxidized HS [6] and selective 2-sulfation of D-glucuronic acid units in HS [7] .

Sulfation of HS has been reported under conditions that minimized *N*-desulfation (sulfur trioxide - triethylamine complex in dry *N*,*N*-dimethylformamide (DMF), Scheme 1). The ^{13}C NMR analysis of the sulfated product indicated that only sulfation at C-2 of D-glucuronic acid units occurred. This result was further confirmed by methylation and GLC-MS analysis of the corresponding alditol acetates [7].



Scheme 1

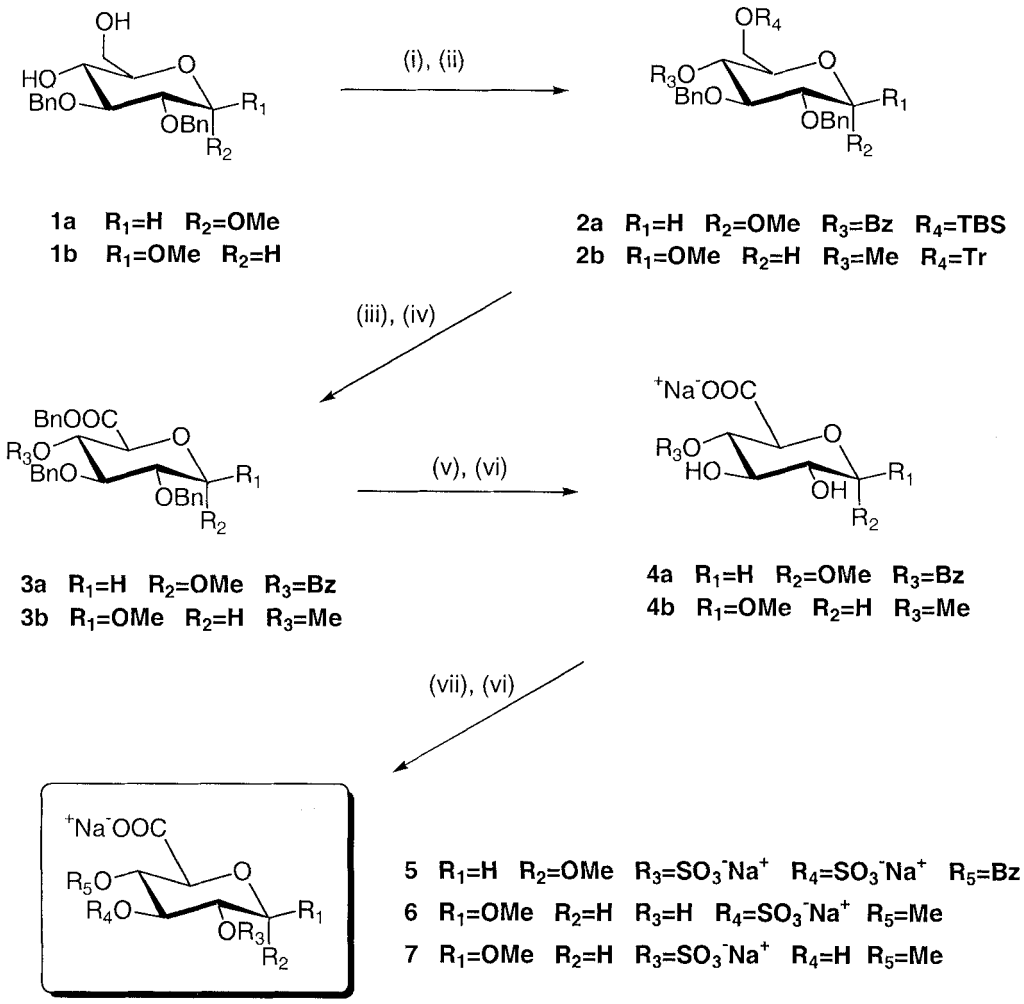
The selectivity observed in the sulfation of the D-glucuronic acid moieties of HS [7] led us to study monosaccharide models in the same experimental conditions. In this paper we report on the sulfation of 4-*O*-protected glucuronic acid derivatives.

Results and Discussion

The literature is more abundant in acylation reactions than in the preparation of inorganic esters of monosaccharides. Sulfation of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside with an excess of pyridine-sulfur trioxide complex in pyridine has been

reported to yield substitution at 2- as the major product, while the β anomer lead to a 3:2 ratio of 2- and 3-sulfated products [8]. We performed [9] the sulfation of methyl 4,6-*O*-benzylidene-β-D-glucopyranoside in identical experimental conditions as those used for HS to study the influence of reaction conditions. A 3:2 mixture of 2- and 3-sulfated products (70 % yield), in agreement with the previous report [8], was obtained, along with a small proportion (12 %) of the 2,3-disulfated product.

We turned our attention to suitably protected D-glucuronic acid derivatives, as monosaccharide models of the units present in HS chains.



Scheme 2

The synthetic sequence of the monosaccharide models is shown in Scheme 2. Methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside **1a** [10] was selective silylated at *O*-6 and benzoylated at *O*-4 to give **2a** in 85 % yield. Jones oxidation lead to the uronic acid which was directly transformed ($KHCO_3$, Bu_4NI, BnBr, DMF) to the benzyl ester **3a** (64 % yield). Hydrogenolysis of benzyl groups followed by ion-exchange workup afforded methyl 4-

O-benzoyl-α-D-glucopyranosiduronic acid **4a** as its sodium salt in 90 % yield. Sulfation of **4a** using 1.0 to 2.0 equivalents of $SO_3{\cdot}Et_3N$ left starting material unchanged. This lack of reactivity has been previously reported for benzylidene derivatives [8]. When a large excess of the complex (5-fold) was used, as in the case of HS [7], the major product obtained was the 2,3-disulfated product. In the ^1H NMR spectrum of the main product, the shift observed for H-1 was +0.21 (from δ 5.02 in **4a** to 5.23), for H-2 it was +0.54 (from δ 3.89 to 4.43), for H-3 it was +0.66 (from δ 4.16 to 4.82). These results are in accordance with the presence of two sulfate groups at C-2 and C-3 that allowed us to assign the major sulfated product as methyl 4-*O*-benzoyl-2,3-di-*O*-sulfo-α-D-glucopyranosiduronate, trisodium salt **5**.

Since no selectivity was observed, a monosaccharide model more structurally related to heparan sulfate was synthesized. Compound **1b** was tritylated at *O*-6 and then methylated to give **2b** in 75 % yield. As above, Jones oxidation lead to the uronic acid which was directly converted to the benzyl ester **3b** (64 % yield). Hydrogenolysis of benzyl groups followed by ion-exchange workup afforded **4b** in 85 % yield.

Sulfation of **4b** with an excess of freshly prepared sulfur trioxide-triethylamine complex was performed until complete disappearance (TLC) of the starting material. By TLC, one spot of a product more polar than **4b** was detected. NMR allowed to determine that the product was in fact a mixture of both monosulfated derivatives of **4b**. In the ^{13}C NMR spectrum of the mixture, the resonance assigned to C-1 of **4b** (δ 102.3) was not observed, and two new resonances at δ 102.0 and 100.5 appeared, corresponding to the 3-*O*- and 2-*O*- sulfated glucuronates **6** and **7**, respectively. From their relative intensity, it was possible to estimate a similar proportion of the two products. Again, the sulfation showed no selectivity between the 2- and 3-hydroxyls.

The presence of a sulfate group at glucuronic acid residues in HS have biological relevance. 2-Sulfated D-glucuronic acid units in HS are major components in the naturally highly sulfated HS from rat liver tissues [11] that do not exhibit anticoagulant properties in spite of their high degree of sulfation [12]. On the other hand, it has been suggested [13,14] that HS oligosaccharides with a high content of sulfated GlcA units are accumulated in the nuclei of cultured rat hepatocytes and may be involved in cell growth proliferation. Selective chemical sulfation of HS [7] may allow to modulate the biological activity of the polysaccharide and could be explained on the basis of the enhanced reactivity of HO-2 (or the lack of reactivity of HO-3).

Since no regioselectivity was observed in our monosaccharide models, the regioselective 2-sulfation of D-glucuronic acid units in the polymer [7] should be related to the polysaccharide conformation as a consequence of the structure of the glycosidic chain. In HS, the regular sequence is composed by a β-D-glucuronic acid unit linked to HO-4 of *N*-acetyl or *N*-sulfate-D-glucosamine residue. The linking of the glucosamine to the next glucuronate is α, giving an alternating sequence. This anomeric configuration would allow the formation of hydrogen bonds between both residues preventing the sulfation of HO-3 or increasing the reactivity of HO-2. Another possibility involves a conformational arrangement of the polymeric chain that would expose preferentially one of two available hydroxyls. Our results do not allow to support one of these hypothesis.

In conclusion, on sulfation of monosaccharide derivatives of D-glucuronic acid, no selectivity between hydroxyls 2- and 3- was observed. Sulfation of oligosaccharide models, molecular modelling and cristallographic studies would be complementary approaches to understand the selectivity observed in polysaccharide chains in future investigations.

Experimental

General.— Optical rotations were measured at 20 ± 2 °C with a Perkin Elmer Model 241 digital polarimeter, using a 10 cm 1 mL cell. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker ACE 200 instrument. FAB MS were obtained on a VG-ZAB SEQ4F mass spectrometer on a glycerol matrix. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60 F_{254} (layer thickness, 0.2 mm; E. Merck, Darmstadt, Germany), detection by charring with sulfuric acid. Flash column chromatography was performed on silica gel 60 (230-400 mesh, E. Merck).

Materials.— Triethylamine-sulfur trioxide complex was prepared according to Nair and Bernstein [15].

Methyl 2,3-di-*O*-benzyl-4-*O*-benzoyl-6-*O*-tert-butyldimethylsilyl-α-D-glucopyranoside (**2a**).

To a solution of methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside **1a** [10] (989.8 mg, 2.64 mmol) in CH_2Cl_2 (9 mL), triethylamine (0.625 mL, 4.5 mmol, 1.7 equiv.), 4-dimethylaminopyridine (1.8 mg, 0.04 equiv.) and *tert*-butyldimethylsilylchloride (616.8 mg, 4.1 mmol, 1.55 equiv.) were added under nitrogen, the mixture was stirred overnight at room temperature. Then, more triethylamine (0.624 mL, 1.77 equiv.) and benzoyl chloride (0.48 mL, 1.55 equiv.) were added. After 16 h, the mixture was diluted with CH_2Cl_2, washed with water, and concd. Flash chromatography on silica gel (95:5 hexane-EtOAc) yielded pure **2a** (1.33 g, 85% yield), as a colourless oil: $[\alpha]_D$ -10.1º (*c* 1.0, $CHCl_3$), ^{1}H NMR ($CDCl_3$): δ 8.19-7.27 (15 H, Ph), 5.33 (dd, 1 H, $J_{3,4}$ 9.4 Hz, $J_{4,5}$ 9.4 Hz, H-4), 4.94-4.75 (m, 5 H, $J_{1,2}$ 3.5 Hz, CH_2Ph, H-1), 4.24 (dd, 1 H, $J_{2,3}$ 9.4 Hz, H-3), 4.13 (m, 1 H, H-5), 3.85-3.77 (m, 3 H, H-2, H-6, H-6'), 3.62 (s, 3 H, OCH_3), 1.01 (s, 9 H, t-BuSi), 0.15 and 0.13 (two s, 6 H, CH_3Si). ^{13}C NMR: δ 165.27 (CO), 138.16-127.36 (Ar.), 97.94 (C-1), 79.80, 79.31 (CH_2Ph), 75.35, 73.40, 71.16, 70.67 (C-2, C-3, C-4, C-5), 62.85 (C-6), 55.10 (OCH_3), 25.79 (*t*-BuSi), 18.21 (CH_3Si).

Anal. Calc. for $C_{34}H_{44}O_7Si$: C, 68.89; H, 7.48. Found: C, 68.54; H, 7.23.

Benzyl (methyl 2,3-di-*O*-benzyl-4-*O*-benzoyl-α-D-glucopyranosid) uronate (**3a**).

To a solution of **2a** (1.16 g, 1.6 mmol) in acetone (38.5 mL), chromium trioxide (509.3 mg, 5.09 mmol, 2.6 equiv.) in 3.5 M H_2SO_4 (2.24 mL) was slowly added at 0 °C. After 4 h the mixture was poured into iced water and extracted with CH_2Cl_2. The organic layer was dried and the solvent evaporated. The residue was dissolved in DMF (55 mL). $KHCO_3$ (1.23 g), tetrabutylammonium iodide (3.62 g) and benzyl bromide (0.46 mL, 3.92 mmol) were added. After 20 h at room temperature, the solvent was evaporated. CH_2Cl_2 and water were added, the organic layer was separated, dried and concentrated under re-

duced pressure. Flash chromatography on silica gel gave **3a** (724 mg, 64% yield), as a syrup: $[\alpha]_D$ + 4.2º (*c* 1.06, $CHCl_3$), ^{1}H NMR ($CDCl_3$): δ 7.92-7.02 (20 H, Ph), 5.37 (dd, 1 H, $J_{3,4}$ 9.5 Hz, $J_{4,5}$ 10.1 Hz, H-4), 5.13 (d, 1 H, J_{gem} 12.1 Hz, CHPh), 4.91-4.61 (m, 6 H, CH_2Ph, H-1), 4.36 (d, 1 H, H-5), 4.10 (dd, 1 H, $J_{2,3}$ 10.1 Hz, H-3), 3.69 (dd, 1 H, $J_{1,2}$ 3.6 Hz, H-2), 3.43 (s, 3 H, OCH_3). ^{13}C NMR: δ 168.29 (C-6), 165.19 (CO), 137.94-127.48 (Ar.), 98.70 (C-1), 78.80, 78.49 (CH_2Ph), 75.46, 73.69, 71.67, 68.69, 67.64 (C-2, C-3, C-4, C-5, CH_2Ph), 56.00 (OCH_3).

Anal. Calc.for $C_{35}H_{34}O_8$: C, 72.15; H, 5.88. Found: C, 71.81; H, 6.02.

Methyl 4-*O*-benzoyl-α-D-glucopyranosiduronic acid, sodium salt (**4a**).

A solution of **3a** (724 mg, 1.24 mmol) in MeOH (50 mL) was hydrogenated in the presence of 5% Pd/C at 60 psi. After 8 h at room temperature the mixture was diluted with water and filtered. The filtrate was stirred with ion-exchange resin (Na^+ form), then filtered and concentrated, giving pure (by TLC) **4a** (372 mg, 90% yield): $[\alpha]_D$ + 82.5º (*c* 1.01, H_2O) . ^{1}H NMR (D_2O): δ 8.10-8.05 and 7.71-7.52 (5 H, Ph), 5.14 (dd, 1 H, $J_{3,4}$ 10.1 Hz, $J_{4,5}$ 10.0 Hz, H-4), 4.92 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 4.18 (d, 1 H, H-5), 4.06 (dd, 1 H, $J_{2,3}$ 10.0 Hz, H-3), 3.79 (dd, 1 H, H-2), 3.47 (s, 3 H, OCH_3). ^{13}C NMR: δ 175.67 (C-6), 168.38 (CO), 134.80-129.55 (Ar.), 100.12 (C-1), 74.11, 71.94, 70.79 (C-2, C-3, C-4, C-5), 56.15 (OCH_3).

FAB MS: 311.0765 (M-Na^+). $C_{14}H_{15}O_8$ requires 311.0767.

Methyl 4-*O*-benzoyl-2,3-di-*O*-sulfo-α-D-glucopyranosiduronic acid, trisodium salt (**5**).

To a solution of **4a** (114.0 mg, 0.34 mmol) in dry DMF (4 mL), SO_3.Et_3N (62.0 mg, 0.34 mmol) was added. After 24 h at room temperature, TLC showed only starting material. Additional SO_3·Et_3N (124.0 mg, 0.68 mmol) was added. After 24 h, a product was detected by TLC, although most of starting material remained unreacted. After a further addition of SO_3·Et_3N (217.0 mg, 1.20 mmol) the reaction was left 48 h at room temperature, when most of the starting material was consumed as shown by TLC. After neutralization, the mixture was concentrated under vacuum. Water (20 mL) was added and the soln was stirred with ion-exchange resin (Na^+ form), then filtered and concd. Flash chromatography on silica gel (8:2 CH_2Cl_2-MeOH) yielded 80 mg (46%) of **5**: $[\alpha]_D$ + 16.1º (*c* 1.0, H_2O). ^{1}H NMR (D_2O): δ 8.08-8.05 and 7.72-7.50 (5 H, Ph), 5.39 (dd, 1 H, $J_{3,4}$ 10.1 Hz, $J_{4,5}$ 10.0 Hz, H-4), 5.27 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 4.87 (dd, 1 H, $J_{2,3}$ 10.0 Hz, H-3), 4.49 (dd, 1 H, H-2), 4.26 (d, 1 H, H-5), 3.52 (s, 3 H, OCH_3). ^{13}C NMR: δ 175.10 (C-6), 168.13 (CO), 134.62-129.17 (Ar.), 98.13 (C-1), 77.13, 75.86, 72.22, 70.70 (C-2, C-3, C-4, C-5) 56.42 (OCH_3). FAB MS: 514.9543 (M-Na^+). $C_{14}H_{13}O_{14}Na_2S_2$ requires 514.9542.

Methyl 2,3-di-*O*-benzyl-4-*O*-methyl-6-*O*-trityl-β-D-glucopyranoside (**2b**).

To a solution of methyl 2,3-di-*O*-benzyl-β-D-glucopyranoside [16] (**1b**, 4.43 g, 7.2 mmol) in CH_2Cl_2, triethylamine (1.8 mL, 12.96 mmol), trityl chloride (2.20 g, 7.92 mmol) and dimethylaminopyridine (50 mg) were added at 0°C. After 24 h at room temperature, a satd. aq. solution of NH_4Cl was added, and the mixture was extracted with CH_2Cl_2. The organic layer was dried ($MgSO_4$) and concentrated to a syrup. The tritylated product was

eluted from a short column of silica gel (10:1 cyclohexane-ethyl acetate) and dissolved in dry DMF (30 mL). Iodomethane (0.52 mL, 8.64 mmol) and sodium hydride (55 % in mineral oil, 0.2 g, 8.64 mmol) were added at 0°C. After stirring overnight at r.t., methanol was added and the mixture extracted with dichloromethane. The organic layer was washed with water and brine, dried ($MgSO_4$) and concentrated. Flash chromatography on silica gel (15:1 cyclohexane-ethyl acetate) gave pure **2b** (3.4 g, 75 % yield): $[\alpha]_D$ +1.6 (c 1.1, $CHCl_3$), ^{1}H-NMR (200 MHz): δ 7.54-7.17 (m, 25 H, Ph), 4.90–4.71 (m, 4 H, CH_2Ph), 4.34 (d, 1 H, $J_{1,2}$ = 7.26 Hz, H-1), 3.63 (s, 3 H, OMe), 3.52-3.46 (m, 4H, H-2, H-3, H-4, H-6a), 3.30-3.20 (m, 4H, H-5, OMe), 3.12 (dd, 1H, $J_{6a,6b}$ = 10.0, $J_{5,6b}$ = 3.64 Hz, H-6b). ^{13}C-NMR (50 MHz): δ 144.04 and 128.82-128.01 (Ph), 104.59 (C-1), 86.3 (CPh_3), 84.64, 82.44, 79.90, 75.78, 74.79, 74.58 (C-2,C-3,C-4,C-5, 2 CH_2Ph), 62.36 (C-6), 60.56 (OMe), 56.56 (OMe).

Benzyl (methyl 2,3-di-*O*-benzyl-4-*O*-methyl-β-D-glucopyranosid)uronate (**3b**).

To a solution of **2b** (3.4 g, 5.4 mmol) in acetone, a solution of CrO_3 (1.3 g) in 3.5 M H_2SO_4 (5.9 mL), was added at 0 °C. After 3 h, the mixture was poured into iced water and extracted with CH_2Cl_2. The organic layer was dried ($MgSO_4$), the solvent evaporated and the residue was dried overnight over P_2O_5. The crude acid was then dissolved in anhydrous DMF (120 mL). $KHCO_3$ (4 g, 41.4 mmol), tetrabutylammonium iodide (5.8 g), and benzyl bromide (1.35 mL, 13.23 mmol) were added. After 20 h, the solvent was evaporated, and the residue partitioned between water and CH_2Cl_2. The organic layer was dried ($MgSO_4$), filtered and evaporated to a syrup. Flash chromatography on silica gel (20:1 cyclohexane-ethyl acetate) gave **3b** (1.65 g, 64 % yield): $[\alpha]_D$ +4.8 (c 1.2, $CHCl_3$), ^{1}H-NMR (200 MHz): δ 7.6-7.05 (m, 15 H, Ph), 5.28 and 5.23 (two d, 2H, J_{gem} = 11.2 Hz, CH_2Ph), 4.88-4.65 (m, 4 H, CH_2Ph), 4.34 (d, 1 H, $J_{1,2}$ = 7.6 Hz, H-1), 3.82 (d, 1 H, $J_{4,5}$ = 9.1 Hz, H-5), 3.53 (m, 5 H, H-3, H-4, OMe), 3.41 (t, 1 H, $J_{2,3}$ = 8 Hz, H-2), 3.35 (s, 3 H, OMe). ^{13}C-NMR (50 MHz): δ 168.51 (C-6), 138.41, 135.23, 128.56, 127.62 (Ph), 104.94 (C-1), 83.71 (C-3), 81.6 (C-2), 81.11(C-4), 75.55, 74.75, 74.47, 67.19 (CH_2Ph, C-5), 60.52 (OMe, C-4), 57.31 (OMe).

HR CIMS: Calcd. for $C_{29}H_{31}O_7$ $(M-1)^+$: m/z 491.2070. Found: 491.2065

Calcd. for $C_{29}H_{36}O_7N$ $(M+NH_4^+)$: m/z 510.2492. Found: 510.2492

Methyl 4-*O*-methyl-β-D-glucopyranosiduronic acid, sodium salt (**4b**).

Compound **3b** (150.0 mg, 0.39 mmol) in 1:1 ethyl acetate-methanol, was hydrogenated at 4 atm over Pd/C 10 % for 6 h. The catalyst was filtered and the solvent evaporated. The free acid was converted to the sodium salt by ion-exchange chromatography (Amberlite IRA 120, Na^+ form), giving **4b** (72.5 mg, 85 % yield): $[\alpha]_D$ -17.6 (c 0.95, H_2O), ^{1}H-NMR (D_2O, 400 MHz): δ 4.33 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1), 3.73 (d, 1 H, $J_{4,5}$ = 9.8 Hz, H-5), 3.55-3.48 (m, 4 H, H-3, OMe), 3.45 (s, 3 H, OMe), 3.28 (dd, 1 H, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 9.4 Hz, H-2), 3.25 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.8 Hz, H-4). ^{13}C-NMR (50 MHz): δ 172.44 (C-6), 102.25, (C-1), 80.64, 74.03, 73.09, 71.85 (C-2, C-3, C-4, C-5), 59.13 (OMe), 56.43 (OMe).

HR FABMS (Neg.): Calcd. for $C_8H_{12}O_7Na$ $(M-H)^-$: m/z 243.0481. Found: 243.1105

Methyl 4-*O*-methyl-3-*O*-sulfo-β-D-glucopyranosiduronic acid, disodium salt (6), and methyl 4-*O*-methyl-2-*O*-sulfo-β-D-glucopyranosiduronic acid, disodium salt (7).

To a solution of **4b** (72.7 mg, 0.30 mmol) in dry DMF (5 mL), $SO_3.Et_3N$ (45.8 mg, 0.30 mmol) was added. The mixture was stirred at r.t. and more $SO_3.Et_3N$ was added until all the starting material was consumed and a new product more polar appeared as shown by TLC. After neutralization, the mixture was concentrated under vacuum. Water (20 mL) was added and the soln. was stirred with ion-exchange resin (Na^+ form), then filtered and concentrated. ^{1}H-NMR (500 MHz, D_2O): δ 4.50 (t, 1H, J = 7.8 Hz, H-2 of **7**), 4.48 (d, 1H, J = 7.8 Hz, H-1 of **7**), 4.40 (d, 1 H, J = 8.0 Hz, H-1 of **6**), 4.33 (t, 1 H, J = 9.1, H-3 of **6**), 3.92 (d, 1 H, J = 8.5 Hz, H-5 of **6**), 3.81 (d, 1 H, J = 10.0 Hz, H-5 of **7**). ^{13}C-NMR (50 MHz): δ 172.45, 172.30 (C-6), 102.00, (C-1 of **6**), 100.50 (C-1 of **7**), 59.15, 59.09 (OMe), 56.50, 56.45 (OMe).

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