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Note

Capillary electrophoresis for monitoring chemical reactions: sulfation and synthetic manipulation of sulfated carbohydrates

Robert J. Kerns, Ioncho R. Vlahov, Robert J. Linhardt *

Division of Medicinal and Natural Products, Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52242 USA

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The lack of a simple and rapid method for the analysis of structurally similar, highly charged, carbohydrates has made monitoring their chemical modification much more difficult than in traditional synthetic reactions where thin-layer chromatography (TLC) is used. High-performance liquid chromatography (HPLC), frequently used in the analysis of reaction mixtures, requires substantial sample preparation and long analysis times. Capillary electrophoresis (CE) allows rapid, repetitive, high-resolution analysis with little, if any, sample preparation. The use of CE as an analytical tool for a large variety of chemical compounds has seen a rapid increase in recent years [1]. CE separates analytes in a narrow fused silica capillary (50–100 μm $\times$ 0.5–1 m) under high voltage (5–25 kV) [2]. Under normal polarity separations, a small amount of concentrated sample in a basic buffer is applied at the anode and detected at the cathode. Negative analytes, such as sulfated sugars, are prevented from migrating under electrophoresis toward the anode by the bulk flow of solvent towards the cathode. This electroosmotic flow is caused by the applied voltage across the charged silinol residues (resulting from the basic buffer) of the capillary column [1,2]. Applications for carbohydrates have included analysis and separation of highly charged glycosaminoglycans and their derivatives, sialic acid containing oligosaccharides, and carbohydrates derivatized with charged groups [2]. The utility of CE to follow chemical reactions of highly charged carbohydrates of very similar structure has not yet been reported.

Chemical synthesis of biologically active, sulfated oligosaccharides and polysaccharides (i.e., heparin, chondroitin sulfate, heparan sulfate, sulfated cyclodextrins) typically leaves

* Corresponding author.

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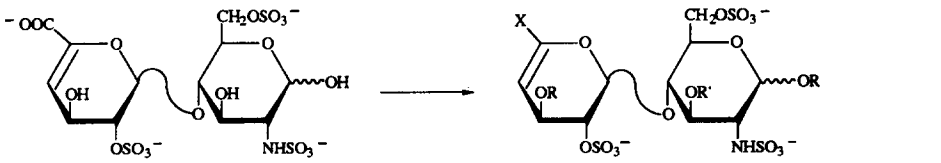


1

2 R = R' = $COCH_3$, X = CO_2^- 4 R = $CO(CH_3)_3$, R' = H, X = CO_2^-, β
3 R = $COCH_3$, R' = H, X = CO_2^- 5 R = $CO(CH_3)_3$, R' = H, X = CO_2^-, α
6 R = R' = H, X = CO_2Bn

Scheme 1.

the addition of the sulfate moiety until as late in the synthetic scheme as possible. In the final synthetic step, only high-yielding deprotection reactions remain. Only the disappearance of unsulfated starting material, in a high-yielding sulfation step, or the complete deprotection of sulfated product is usually monitored. TLC on cellulose [3], or silica gel plates [4] has been useful for analyzing mono- or di-anionic species or molecules with a significant lipophilicity. In our laboratory, we have recently found it necessary to follow the chemical modification of highly sulfated carbohydrates, where structural alterations occur without a change in molecular charge. Information on reaction completion and composition of product mixtures (containing components having three or more negative charges) is needed. Based on this requirement, we anticipated that CE would provide a rapid and sensitive method to follow both chemical reactions and product purification. Extensive studies in our laboratory had failed to successfully apply TLC to the analysis of these highly charged compounds. The use of CE is described here as an analytical tool for monitoring chemical reactions of sulfated sugars. This methodology should aid in developing new methods for the chemical synthesis and modification of sulfated organic compounds.

Trisulfated disaccharide **1** (Scheme 1) was obtained by enzymatic depolymerization of heparin as previously described by our group [5]. Acylation of hydroxyl groups on heparin and heparin fragments has been shown using acid anhydrides and ion-pairing agents [6]. However, no information on the regioselectivity of acylation or the degree of desulfation of unrecovered material (if present) is known. As part of our continuing effort to investigate the biological activity of derivatized heparins, we have begun to examine the chemical synthesis and derivatization of heparin oligosaccharides. Introduction of hydroxyl protecting groups on **1** were required for subsequent synthetic manipulations. Acetylation, pivaloylation, and benzylation reactions of **1** were followed by CE using absorbance detection at 232 nm. Detection at this wavelength is based on the presence of the chromophoric unsaturated uronic acid residue. These analyses result in a time–course distribution of reaction products. Samples for analysis (2–5 μL) were removed from the mixture (using a 10-μL syringe through the rubber septa) and diluted (quenching the reaction) using 10–20 μL of deionized, distilled water at 0°C. These diluted aliquots were then analyzed directly on CE (Fig. 1). Purified products were co-injected with reaction samples or compared by similarity in migration time to verify peak identity.

CE analysis of the acetylation of **1** is shown in Fig. 1A. Disaccharide **1** having a migration time of 25 min is observed only at time 0 (electropherogram a). At 1 h, a cluster of peaks having a major component **3** are detected between 20 and 23 min (electropherogram b). The peaks converged into a single peak **2** (electropherogram c), while peak **1** disappeared

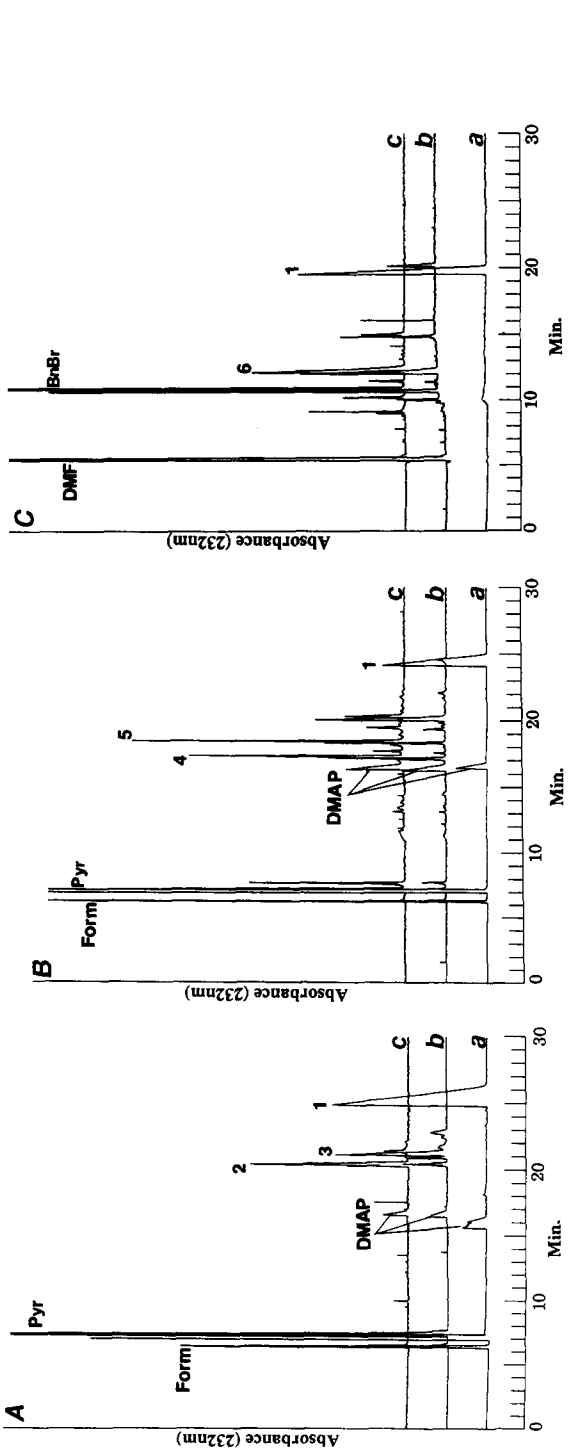


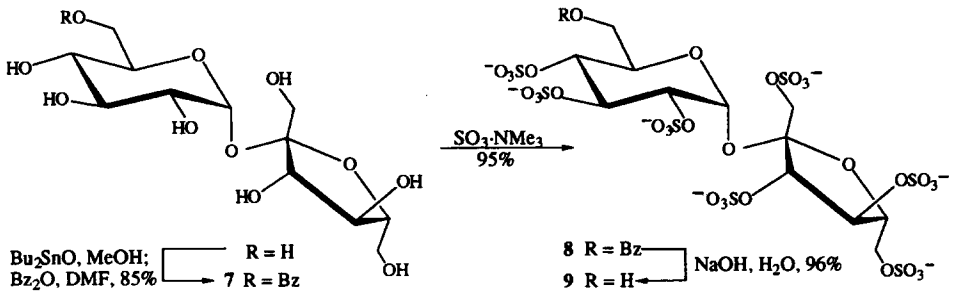
Fig. 1. Electropherograms of reactions of **1** at selected times. A, Ac_2O–DMAP–Pyr–H_2NCHO (a, 0 h; b, 1 h; c, 9 h). B, Piv_2O–DMAP–Pyr–H_2NCHO (a, 0 h; b, 5.5 h; c, 24 h). C, NaH–BnBr–Me_2NCHO (a, 0 h; b, 4 h; c, 23 h).

and peak **3** greatly diminished. Formamide (Form) and pyridine (Pyr) (6–8 min) and 4-(dimethylamino)pyridine (DMAP) (16-17 min) are observed in all three electropherograms. HPLC purification of the major products, followed by spectral characterization, showed **2** to be peracetylated while **3** was only diacetylated. This reaction can be driven to completion providing high yields of **2**. These electropherograms show the disappearance of starting material, the appearance and subsequent disappearance of partially acetylated intermediates, and the concomitant increase in peracetylated product **2**. This is the same type of information that TLC would traditionally provide when used to monitor a reaction. If the acetylation reaction proceeds for a prolonged time (electropherogram not shown), desulfated products become prominent (8-15 min). These desulfated products appear to arise from acetylative desulfation of the glucosamine residue [7].

Selective protection of **1** could be achieved, leaving the C-3 glucosamine hydroxyl group unprotected, by using the bulky pivaloyl group. The pivaloylation of **1** (Fig. 1B) again shows a decrease of starting material over time to afford two products **4** and **5**. The ratio of these two products did not change, even following a prolonged reaction time (5 days) and in the presence of a large excess of acylation reagent (data not shown). Again, pyridine and formamide are observed in the electropherograms migrating between 6 and 7 min. Isolation and characterization of **4** and **5** demonstrated that they were the β and α forms, respectively, of a single dipivaloylated derivative. This demonstrates that the bulky pivaloyl group does not readily react at the hindered C-3 of the glucosamine and leaves this center open for subsequent derivatization. It is important to note that sulfation of this unprotected hydroxyl group would give rise to the 3-sulfated glucosamine residue found in the antithrombin III binding site of heparin [8]. The ability of CE to separate the α and β anomers of these dipivaloyl products demonstrates the high level of resolution that can be achieved by this technique. The peak observed at 8 min (Fig. 1B) was not isolated and may be either a small amount of perpivaloylated or desulfation product.

Benzylation of the tetramethylammonium salt of **1** gave an unexpected result. The electropherograms of this reaction (Fig. 1C) again show the disappearance of starting material **1** with the rapid appearance of a single major product **6**. Little or no change in subsequent electropherograms was observed throughout an extended reaction time, and no other products were isolated. Benzyl bromide (10.5 min) and DMF (5 min) are observed in the upper two electropherograms. Isolation of **6** and its characterization showed it to be the benzyl ester derivative of **1**. None of the benzyl ether containing products, expected under the reaction conditions used, were observed.

The second synthetic scheme (Scheme 2) involves the preparation of sulfated sucrose derivatives. These derivatives are based on sucrose octasulfate (SOS), a compound that is recognized to act in biological systems as a heparin mimetic [9]. Our laboratory set out to prepare selectively modified sulfated sucrose derivatives for biological testing. Sucrose was first selectively benzoylated at the C-6 position to provide **7** [10]. The sulfation of **7** affords **8** and the subsequent selective hydrolysis of the benzoyl ester in the presence of the sulfate esters affords sucrose heptasulfate, **9**. Both reactions were followed by CE as described for the heparin disaccharide derivatives, except detection was at 206 nm and the reaction aliquots (10–20 μL) were quenched with CE buffer (100–200 μL) prior to their analysis by CE.



Scheme 2.

Electropherograms of the sulfation of **7** (Fig. 2A) show its rapid and complete conversion to two main products within 1 h. The peak at 23.5 min corresponds to the fully sulfated product **8**. The second peak, migrating slower than **8** at 26 min (electropherogram b), surprisingly appears to correspond to an undersulfated intermediate that gradually disappears by 15 h. Under normal polarity CE, molecules with higher negative charge generally have longer migration times. On a shorter reaction time (0.5 h, electropherogram not shown), undersulfated intermediates appear as expected at earlier migration times (<19 min). The peak corresponding to **8** is rather broad, resulting from its high level of sulfation [11] and overloading of the column necessitated by the low detection sensitivity of the benzoyl group. Co-injection of **8** with the 1 h reaction sample confirmed that the product migrating at 23.5 min was **8** and the product migrating at 26 min (Fig. 2A, electropherogram b) was an intermediate. DMF migrated at 5 min in the electropherogram. Debenzoylation of **8** (Fig. 2B) was followed by observing an increase in sodium benzoate (Fig. 2B, 8–10 min) as well as the concomitant decrease in starting material **8**. Product **9**, containing no chromophore, is not observed in these electropherograms. The presence of **9** was confirmed by its isolation from the mixture in high yield. Electropherogram a (Fig. 2B) is the pure

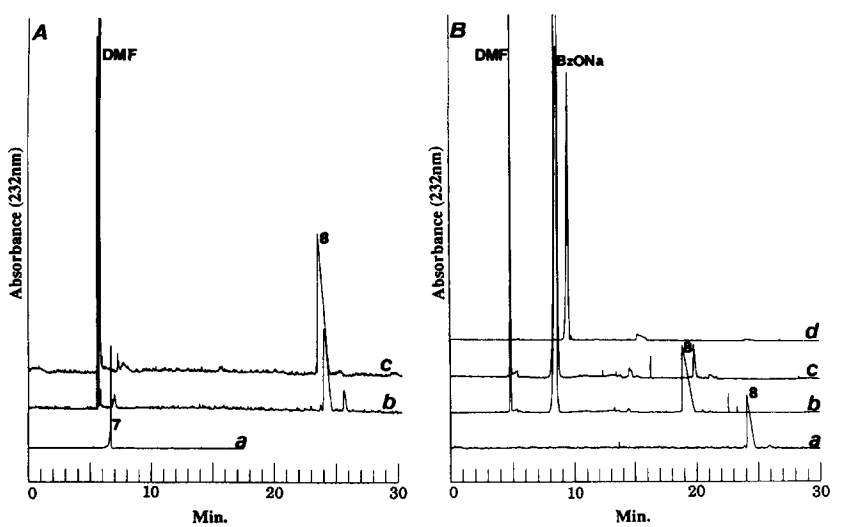


Fig. 2. Electropherograms of: A, sulfation of 6-*O*-benzoyl sucrose **7**, $SO_3 \cdot NMe_3$, Me_2NCHO, 50°C (a, 0 h; b, 0.5 h; c, 1 h); B, debenzoylation of **8**, NaOH, Me_2NCHO, 4°C, (a, purified **8**; b, 1 h; c, 9 h; d, 18 h).

starting material **8** while electropherograms b–d contain a trace of DMF impurity from the bulk material **8** used in the reaction. A significant difference in migration times was observed for **8** (Fig. 2B, electropherograms a and b). The presence of sodium benzoate appears to affect migration times. Thus, the pure starting material **8**, was co-injected with each reaction sample to confirm the assignment of peaks. The minor peak at 14–15 min together, with the peak corresponding to benzoate, disappear upon chloroform extraction of the acidified aqueous mixture allowing pure product to be isolated in excellent yield. CE demonstrates that debenzoylation occurs at a rate significantly faster than desulfation, as benzoate is the only new peak observed in electropherograms of the mixture.

The ability of CE to monitor the disappearance of reactants, the presence of intermediates, and the appearance of products has been demonstrated using relatively simple reactions. These highly charged compounds preclude the use of TLC for monitoring reactions. In addition, TLC would not be expected to give the high level of sensitivity and information provided using CE. The high sensitivity of CE means that only a small amount of sample is required, making CE ideal for monitoring microscale reactions. The high resolution of CE permits the separation of complex mixtures that might be impossible to separate by a low-resolution technique such as TLC. The time required for CE sampling of the reaction is minimal and can be shortened further by reducing the capillary length or using a different buffer [12]. Extension of this methodology to other types of reactants and reactions is possible. Use of surfactants, as well as the addition of up to 10% water-miscible organic solvent (i.e., methanol or acetonitrile), permits the analysis of less polar compounds [12]. CE can also separate uncharged molecules because of the presence of electroosmotic flow [12], suggesting the utility of this method for following derivatization of small amounts of neutral carbohydrates. Advances in the use of indirect detection for CE [11] may also extend these methods to compounds where no chromophore is present in either reactant or product molecules.

1. Experimental

General methods.—All reactions were monitored using a fused silica (externally coated except where the tube passed through the detector) capillary tube (75 μm i.d., 375 μm o.d., and 75 cm long) from Dionex. The capillary tube was washed extensively with 0.1 M phosphoric acid, 0.5 M NaOH, and deionized, distilled water before use. The capillary was rinsed with operating buffer [10 mM sodium borate, 50 mM sodium dodecylsulfate (SDS) at pH 8.8] in the instrument. Before each reaction, standards were run to confirm operating consistency. The sample was injected by gravity injection (45 mm), the injection time was 25 s (corresponding to 15 nL). CE was performed under normal polarity at a constant voltage of ca. 20 kV. Detection was by ultraviolet absorbance at 232 or 206 nm as described in the text. ^{1}H NMR spectra were recorded at 25°C on a Bruker WM 360, AMX 600, or Varian Unity 500 spectrometer. All NMR samples were repeatedly lyophilized from ^2H_2O before analysis and contained TSP as internal standard.

Mass spectra were obtained using a VG ZAB-HF instrument in the fast-atom bombardment (FAB) ionization mode. The FAB ion source used was a standard VG Analytical, Inc. system equipped with a saddle field atom gun. Xenon was used for the bombarding

fast-atom beam; typical operating conditions were beam energies of 8 keV and neutral beam currents equivalent to 1.5 mA supplied by an ION TECH (Model B 50) current and voltage regulator/meter. Negative-ion FAB spectra were obtained by signal adding four or eight scans with the use of the multichannel analysis (MCA) software of the VG 11-250J data system. Triethanolamine was used as the matrix. Molecular ion and fragment ions are defined as previously described [13].

Strong anion-exchange high performance liquid chromatography (SAX-HPLC) used dual, face programmable, Shimadzu (Kyoto, Japan) LC-7A titanium-based pumps. The system was equipped with a Rheodyne (Cotati, CA) no. 7125 titanium injector and a Pharmacia LKB (Piscataway, NJ) 2141 variable wavelength UV detector with a Shimadzu Chromatopac C-R2A integrating recorder. Preparative separations relied on a 2 × 25 cm, 5-μm particle size, Spherisorb SAX-HPLC column from Phase Separations, Norwalk, CT.

*3-O-Acetyl-4-deoxy-2-O-sulfo-α-*L-threo-*hex-4-enopyranosyluronic acid-(1 → 4)-1,3-di-O-acetyl-2-deoxy-2-sulfamido-6-O-sulfo-α-*D-*glucopyranose, tetrasodium salt* (**2**) *and 3-O-acetyl-4-deoxy-2-O-sulfo-α-*L-threo-*hex-4-enopyranosyluronic acid-(1 → 4)-1-O-acetyl-2-deoxy-2-sulfamido-6-O-sulfo-α-*D-*glucopyranose, tetrasodium salt* (**3**).—Disaccharide **1** (tetrasodium salt) (46.8 mg, 70.4 μmol) was dissolved in 1 mL of dry formamide under N_2. To this solution, 1 mL of dry pyridine containing DMAP catalyst was injected. At time zero the CE sample was taken (Fig. 1A shows CE sampling data). At 0°C with stirring, Ac_2O (120 μL, 1.26 mmol) was injected. After 30 min the ice bath was removed and the mixture warmed to room temperature. After 9 h the reaction flask was placed in an ice bath and quenched with 2 mL of water and stirred at room temperature for 2 h. The crude solution was evaporated under high vacuum, followed by addition of water, to give 2 mL. The mixture was purified using SAX-HPLC [5]. Fractions containing **2** and **3** were verified by CE. Desalting on Bio-Gel P-2 with water eluent, concentration under high vacuum, passing through Dowex 50 (Na^+), and lyophilization gave amorphous solids.

Compound **2**: 25 mg, 45%; migration time (CE) 20.5 min; FABMS: *m/z* 790, 768, 746 $[M-5H+4Na]^-$, $[M-4H+3Na]^-$, $[M-3H+2Na]^-$, 688, 666, 644 $[M-4H+4Na-NaSO_3]^-$, $[M-3H+3Na-NaSO_3]^-$, $[M-2H+2Na-NaSO_3]^-$; ^1H NMR (360 MHz, ^2H_2O): δ 2.11, 2.13, 2.22 (3 s, 9 H, CO_2CH_3), 3.70 (dd, 1 H, $J_{2,3}$ 11 Hz, H-2), 4.17–4.31 (m, 4 H, H-4,5,6A,6B) (confirmed by decoupling experiments), 4.62 (dd, 1 H, H-2′), 5.19 (dd, 1 H, H-3), 5.33 (dd, 1 H, $J_{3',4'}$, 5.1 Hz, H-3′), 5.53 (d, 1 H, $J_{1',2'}$, 1.7 Hz, H-1′), 5.93 (d, 1 H, H-4′), 6.25 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1).

Compound **3**: 3 mg; migration time (CE) 21.5 min; FABMS: *m/z* 748, 726, 704, 682 $[M-5H+4Na]^-$, $[M-4H+3Na]^-$, $[M-3H+2Na]^-$, $[M-2H+Na]^-$, 624, 602 $[M-3H+3Na-NaSO_3]^-$, $[M-2H+2Na-NaSO_3]^-$; ^1H NMR (500 MHz, ^2H_2O): δ 2.14, 2.20 (2 s, 6 H, CO_2CH_3), 3.40–3.89 (m, 2 H, $J_{4,5}$ 9.0 Hz, H-3,4), 3.48 (dd, 1 H, $J_{2,3}$ 10 Hz, H-2), 4.1 (m, 1 H, H-5), 4.21 (d, 1 H, ABX, $J_{A,B}$ 10, $J_{AX} \approx 0$ Hz, H-6A), 4.34 (dd, 1 H, J_{BX} 4.5 Hz, H-6B), 4.67 (m, 1 H, H-2′), 5.49 (dd, 1 H, $J_{3',4'}$, 4.0 Hz, H-3′), 5.61 (d, 1 H, $J_{1',2'}$, 2.5 Hz, H-1′), 5.91 (d, 1 H, H-4′), 6.31 (d, 1 H, $J_{1,2}$ 3.0 Hz, H-1).

*4-Deoxy-3-O-pivaloyl-2-O-sulfo-α-*L-threo-*hex-4-enopyranosyluronic acid-(1 → 4)-2-deoxy-1-O-pivaloyl-2-sulfamido-6-O-sulfo-β-*D-*glucopyranose, tetrasodium salt* (**4**) *and 4-deoxy-3-O-pivaloyl-2-O-sulfo-α-*L-threo-*hex-4-enopyranosyluronic acid-(1 → 4)-2-deoxy-1-O-pivaloyl-2-sulfamido-6-O-sulfo-α-*D-*glucopyranose, tetrasodium salt* (**5**).—To **1** (tetrasodium salt) (22.8 mg, 34.3 μmol) in 0.5 mL of dry formamide maintained under

N_2, 0.5 mL of dry pyridine containing DMAP catalyst was injected. At time zero the CE sample was taken (Fig. 1B shows CE sampling data). At 0°C with stirring, pivalic anhydride (150 μL, 741 μmol) was injected, the ice bath was removed, and the mixture was allowed to warm to room temperature. After 6 h, 150 μL of pivalic anhydride was injected. After stirring for 5 days (electropherograms at 24 h and 5 days were nearly identical), the mixture was cooled to 0°C, 2 mL of H_2O was added, and it was stirred for 2 h. The crude mixture was dried under high vacuum, water was added to give 2 mL, and the products were fractionated using SAX-HPLC [5]. Fractions containing **4** and **5** were verified using CE. Desalting on Bio-Gel P-2 with water eluent, concentration under high vacuum, passing through Dowex 50 (Na^+), and lyophilization gave white amorphous solids.

Compound **4**: 8 mg, 28%; migration time (CE) 17.1 min; FABMS: *m/z* 832, 810, 788, 766 $[M-5H+4Na]^-$, $[M-4H+3Na]^-$, $[M-3H+2Na]^-$, $[M-2H+Na]^-$, 708, 686, 664 $[M-3H+3Na-NaSO_3]^-$, $[M-2H+2Na-NaSO_3]^-$, $[M-H+Na-NaSO_3]^-$, 465 $[M-2H+2Na-(\Delta UA\text{-}3\text{-}O\text{-}piv)]^-$; ^1H NMR (500 MHz, ^2H_2O): δ 1.22, 1.25 [2 s, 18 H, $CO_2(CH_3)_3$], 3.33 (dd, 1 H, $J_{2,3}$ 9.0 Hz, H-2), 3.82–3.92 (m, 3 H, H-3,4,5, confirmed by decoupling), 4.27 (dd, 1 H, J_{BX} 1.5 Hz, H-6B), 4.31–4.35 (dd, 1 H, $J_{A,B}$ 11 Hz, J_{AX} 4.0 Hz, H-6A), 4.67 (m, 1 H, $J_{2',3'}$ 1.5 Hz, H-2'), 5.4 (dd, 1 H, $J_{3',4'}$ 4.0 Hz, H-3'), 5.55 (d, 1 H, $J_{1',2'}$ 3.0 Hz, H-1'), 5.67 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 5.93 (d, 1 H, H-4').

Compound **5**: 6 mg, 21%; migration time (CE) 18.5 min; FABMS: *m/z* 832, 810, 788, 766 $[M-5H+4Na]^-$, $[M-4H+3Na]^-$, $[M-3H+2Na]^-$, $[M-2H+Na]^-$, 708, 686, 664 $[M-3H+3Na-NaSO_3]^-$, $[M-2H+2Na-NaSO_3]^-$, $[M-H+Na-NaSO_3]^-$; ^1H NMR (500 MHz, ^2H_2O): δ 1.20, 1.24 [2 s, 18 H, $CO_2(CH_3)_3$], 3.5 (dd, 1 H, $J_{2,3}$ 11 Hz, H-2), 3.74 (m, 1 H, H-5), 3.90–3.95 (m, 2 H, H-3,4), 4.23–4.29 (m, 2 H, 2×H-6), 4.66 (m, 1 H, H-2'), 5.36 (m, 1 H, $J_{3',4'}$ 4.0 Hz, H-3'), 5.55 (d, 1 H, H-1', <1 Hz), 5.95 (d, 1 H, H-4'), 6.3 (d, 1 H, $J_{1,2}$ 3.0 Hz, H-1).

*Benzyl 4-deoxy-2-*O*-sulfo-α-*L*-*threo*-hex-4-enopyranosyluronate-(1→4)-2-deoxy-2-sulfamido-6-*O*-sulfo-*D*-glucopyranose, trisodium salt* (**6**).—Disaccharide **1** [tetramethylammonium salt, prepared by passing the sodium salt through Dowex 50 (H^+) resin and titrating with tetramethylammonium hydroxide] (50 mg, 57.5 μmol) was dissolved in dry Me_2NCHO (1.5 mL) under N_2. Benzyl bromide (0.1 mL, 841 μmol) and NaH (10 mg, 417 μmol) were added under N_2 flow. Fig. 1C shows CE sampling data. After 23 h the mixture was poured over ice (5 mL) and extracted with $CHCl_3$ (3×20 mL). The aqueous layer was evaporated under high vacuum and dried by forming an azeotrope with EtOH. The dry white solid was repeatedly washed with EtOH to give the crude product as a glass or as a white solid upon lyophilization (50 mg, 93%). The product was dissolved in 1:1 water–MeOH, passed through Sephadex LH-20 with the same solvent to give **6** in 99+% purity by CE. Migration time (CE) 12.5 min; FABMS: *m/z* 732, 710, 688, 666 $[M-4H+3Na]^-$, $[M-3H+2Na]^-$, $[M-2H+Na]^-$, $[M-H]^-$, 608, 586 $[M-2H+2Na-NaSO_3]^-$, $[M-H+Na-NaSO_3]^-$, 642, 620 $[M-3H+3Na-Bn]^-$, $[M-2H+2Na-Bn]^-$, 360 [13] $[M-H+Na-\Delta UA]^-$; ^1H NMR (500 MHz, ^2H_2O): δ 3.30 (dd, 1 H, $J_{2,3}$ 9.5 Hz, H-2), 3.72–3.84 (m, 2 H, H-3,4), 4.15–4.17 (m, 2 H, 2×H-6), 4.24–4.30 (m, 1 H, H-5), 4.35–4.37 (m, 1 H, $J_{3',4'}$ 4.5 Hz, H-3'), 4.62 (m, 1 H, H-2'), 4.81–5.33 (dd, 2 H, OCH_2-phenyl), 5.44 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 5.59 (d, 1 H, $J_{1',2'}$ 3.0 Hz, H-1'), 6.35 (d, 1 H, H-4'), 7.43–7.52 (m, 5 H, aromatic).

β-D-Fructofuranosyl 6-O-*benzoyl-α-D-glucopyranoside* (**7**).—Sucrose (5 g, 14.6 mmol) and dibutyltin oxide (4 g, 16.1 mmol) were refluxed for 1 h in anhyd MeOH (75 mL). The clear solution was evaporated to dryness, followed by coevaporation with toluene (3×50 mL). The white solid was dissolved in dry Me_2NCHO (25 mL), cooled in an ice bath, and benzoic anhydride (3.63 g, 16 mmol) was added. The mixture was stirred for 4 h at 4°C, then for 48 h at ambient temperature. Water (75 mL) was added, and the solution was washed with EtOAc (3×50 mL). The aqueous layer was evaporated to dryness with toluene (3×50 mL). The syrup was crystallized from 1:2 MeOH–Et_2O to give **7** in 85% yield; R_f 0.47 (7:2:1 2-propanol–water–ammonia); ^1H NMR (600 MHz, ^2H_2O): δ 3.53–4.24 (m, 11 H), 4.57, 4.66 (2 H, ABX, J_{AB} 12, J_{AX} 5.1, J_{BX} 2.1 Hz, 2×H-6), 5.42 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 7.5–8.2 (5 H, aromatic).

1′,3′,4′,6′-*Tetra*-O-*sulfo-β-D-fructofuranosyl* 6-O-*benzoyl*-2,3,4-*tri*-O-*sulfo-α-D-glucopyranoside, heptasodium salt* (**8**).—Disaccharide **7** (0.9 g, 2 mmol) and trimethylamine–sulfur trioxide complex (2.44 g, 175 mmol) were stirred under N_2 in 12 mL of dry Me_2NCHO at 50°C for 5 h (Fig. 2A shows CE sampling data). An additional portion of Me_2NCHO (20 mL) and trimethylamine–sulfur trioxide complex (1.4 g) was added, and the suspension was kept at 50°C overnight. 1:1 Methanol–water was added to the mixture, and the suspension was layered on a column of Sephadex LH-20 and eluted with the same solvent system. The combined product was passed through Dowex 50 (Na^+) resin. After evaporation, the colorless glass was dissolved in water and lyophilized to give **8** as a white powder in 95% yield. Migration time (CE) 23–24 min; ^1H NMR (600 MHz, ^2H_2O): δ 4.24 (dd, 1 H, J_{AB} 11, $J_{5',6'A}$ 8.0 Hz, H-6′A), 4.25 (d,1 H, J_{AB} 11 Hz, H-1′A), 4.35 (d, 1 H, H-1′B), 4.42 (ddd, 1 H, $J_{4',5'}$ 8.0, $J_{5',6'B}$ 2.3 Hz, H-5′), 4.48 (dd, 1 H, $J_{1,2}$ 3.7, $J_{2,3}$ 10 Hz, H-2), 4.51 (dd, 1 H, H-6′B), 4.57 (ddd, 1 H, $J_{4,5}$ 10, $J_{5,6B}$ 1.0 Hz, H-5), 4.64 (dd, 1 H, J_{AB} 12 Hz, H-6A), 4.68 (dd, 1 H, $J_{3,4}=J_{4,5}=9.8$ Hz, H-4), 4.75 (dd, 1 H, H-6B), 4.78 (dd, 1 H, H-3), 4.86 (dd, 1 H, $J_{3',4'}$ 8.0 Hz, H-4′), 5.15 (d, 1 H, H-3′), 5.87 (d, 1 H, H-1), 7.54–8.12 (5 H, aromatic).

1′,3′,4′,6′-*Tetra*-O-*sulfo-β-D-fructofuranosyl* 2,3,4-*tri*-O-*sulfo-α-D-glucopyranoside, heptasodium salt* (**9**).—Disaccharide **8** (120 mg) in 2.5 mL of N NaOH was stirred overnight at 4°C (Fig. 2B shows CE sampling data). The reaction was quenched with 3 mL of N HCl and extracted with $CHCl_3$ (3×5 mL). The aqueous layer was passed through Dowex 50 (Na^+) resin, evaporated under high vacuum, redisolved in water, and lyophilized to give a white powder (**9**) (96% yield). The NMR (^2H_2O) data was identical with that of commercially available material: ^1H NMR (500 MHz): δ 3.96 (2 H, ABX, J_{AB} 12, J_{AX} 1.0, J_{BX} 1.8 Hz, 2×H-6), 4.18 (ddd, 1 H, $J_{4,5}$ 10 Hz, H-5X), 4.22, 4.33 (dd, 2 H, J_{AB} 12 Hz, 2×H-6′), 4.34–4.41 (m, 3 H, H-2,1′A,5′), 4.46 (dd, 1 H, $J_{3,4}$ 10 Hz, H-4), 4.54 (dd, 1 H, J_{AB} 12, J_{BX} 0.7 Hz, H-1′B), 4.72 (dd, 1 H, $J_{2,3}$ 10 Hz, H-3), 4.88 (dd, 1 H, $J_{3',4'}$ 8.0 Hz, H-4′), 5.13 (d, 1 H, H-3′), 5.83 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1).

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