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Chemoenzymatic preparation of dermatan sulfate oligosaccharides as arylsulfatase B and α-L-iduronidase substrates

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Dermatan sulfate was partially depolymerized with chondroitin ABC lyase to obtain an oligosaccharide mixture from which an unsaturated disulfated tetrasaccharide was purified and characterized using nuclear magnetic resonance spectroscopy and electrospray ionization mass spectrometry. Chemical removal of the unsaturated uronate residue with mercuric acetate, followed by de-4-*O*-sulfation with arylsulfatase B (*N*-acetylgalactosamine 4-sulfatase) and *N*-acetylhexosaminidase catalyzed removal of the 2-acetamido-2-deoxy-D-galactospyranosyl residue at the non-reducing end afforded a monosulfated disaccharide of the structure α-L-idopyranosyluronic acid (1 → 3)-α,β-D-2-acetamido-2-deoxy-4-*O*-sulfo galactopyranose. This monosulfated disaccharide serves as a substrate for mammalian α-L-iduronidase as demonstrated using fluorophore assisted carbohydrate electrophoresis.

Keywords: **dermatan sulfate, oligosaccharide, substrates, iduronidase, arylsulfatase, *N*-acetylgalactosamine 4-sulfatase, nuclear magnetic resonance spectroscopy, electrospray ionization mass spectrometry**

Introduction

Enzymes are indispensable tools for the analysis and sequencing of newly isolated glycan structures [1–6]. In the glycosaminoglycan field such enzymes include the well-established bacterial polysaccharide lyases [7–9] as well as recently introduced mammalian exoglycosidases, involved in glycosaminoglycan catabolism [10] and the sequencing of heparan sulfate [11]. New enzymes are also being developed as therapeutics. These applications require small, specific, oligosaccharide substrates for use in routine bioassays, quality control and to better understand substrate specificity. Two newly available commercial enzymes, MPS-I iduronidase [12–14] and MPS VI, arylsulfatase B (*N*-acetylgalactosamine 4-sulfatase) [15–17] are such enzymes that require substrates of structures α-L-idopyranosyluronic acid (1 → 3)-α,β-D-2-acetamido-2-deoxy-4-*O*-sulfo-galactopyranose (or α-L-Ido*p*A (1 → 3)-α, β-D-Gal*p*NAc4S) and β-D-Gal*p*NAc4S-(1 → 4)-α-L-Ido*p*A-(1 → 3)-α,β-D-Gal*p*NAc4S, respectively. Since *de novo* chemical synthesis of such oligosaccharides would require many synthetic steps, we undertook the chemoenzymatic preparation of these substrates. This paper reports the chemoenzymatic synthesis and utilization of these oligosaccharide substrates.

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Materials and methods

Preparation of dermatan sulfate tetrasaccharide

Dermatan sulfate-derived tetrasaccharide was prepared as previously described [18]. Briefly, dermatan sulfate from porcine intestinal mucosa (Celsus Laboratories, Cincinnati, OH), with a homogenous repeating structure [→ 4)Ido*p*A (1 → 3)Gal*p*NAc 4S (1 →]$_n$, was prepared in 50 mM Tris-HCl-sodium acetate, pH 8.0 buffer (50 ml, 20 mg/ml). Chondroitin ABC lyase (2 U, EC 4.2.2.4, Sigma Chem., St. Louis, MO) treatment, at 37°C until the reaction was 50% complete, afforded an oligosaccharide mixture. Concentration to 10 ml by rotary evaporation at 40°C and fractionation on a Bio-Gel P6 superfine (Bio-Rad, Richmond, CA) column (4.8 × 100 cm) in 100 mM sodium chloride, afforded sized oligosaccharide fractions. The tetrasaccharide fraction (the next to last peak eluting from this column detected by absorbance at 232 nm) was desalted on a Bio-Gel P2 column (4.8 × 70 cm) eluted with distilled water and freeze-dried. The tetrasaccharide

(100 mg) was purified by strong anion exchange (SAX) high pressure liquid chromatography (HPLC) on a Spherisorb 2.0 × 25 cm column (Waters, Miliford, MA) eluted with a 0–2 M sodium chloride gradient. The major peak was collected, desalted on Bio-Gel P2 and freeze-dried. The resulting product was > 99% pure by analytical SAX-HPLC and > 96% pure by capillary electrophoresis (CE) analyses [18]. The oligosaccharide was dissolved in D_2O (99.0 atom %) filtered through a 0.45 µm syringe filter and freeze-dried to remove exchangeable protons. After exchanging three times, the sample was dissolved in D_2O (99.96 atom %). 1D, ^{1}H-NMR experiments were performed on a Varian VXR-500 spectrometer equipped with a 5 mm triple resonance tunable probe with standard Varian software at 298°K on 700 µl samples at 0.1–0.5 mM. The HOD signal set as a reference at 4.75 ppm was suppressed by presaturation for 3 s. The tetrasaccharide **1** was assigned the structure ΔU*p*A (1 → 3)-*β*-D-Gal*p*NAc4S (1 → 4)-α-L-Ido*p*A (1 → 3) α,*β*-D-Gal*p*NAc4S (where ΔU*p*A is 4-deoxy-α-L-*threo*-hex-enopyranosyluronic acid) based on ^{1}H-NMR. ^{1}H-NMR (500 MHz, D_2O)ΔU*p*A; H1, *δ* 5.233; H2, 3.810; H3, 4.002; H4, 5.925. *β*-D-Gal*p*NAc4S; H1, 4.680; H2, 4.039; H3, 4.133; H4, 4.602; H5, 3.866; H6, 3.76; NAc, 2.090. α-L-Ido*p*A; H1, 4.9; H2, 3.512; H3, 3.810; H4, 4.091; H5, 4.6663. α,*β*;-D-Gal*p*NAc4S H1α, 5.184, *β* 4.732; H2α, 4.328, H3α 4.149, *β* 4.002; H4α 4.681; *β* 4.602; H5α 4.241; *β* 3.86, H6α 3.76, *β* 3.7; NAcα,*β* 2.029. Electrospray ionization-mass spectrometry (ESI-MS) (negative ion) was performed using a Micromass, Inc., (England) Autospec equipped with electrospray interface. Nitrogen gas was used as bath (250 l/h) and nebulizer (12 l/h). The electrospray ion source was at 80°C and the spray needle was at 7.7 kV. Tetraethylammonium iodide in acetonitrile calibrant was used to analyze oligosaccharide sample in 1:1 water: acetonitrile containing 0.05% ammonium hydroxide (also used as mobile phase). Spectra were obtained on 20 µl of sample in 30–40 scans and processed using OPUS software. The ESI-MS showed a parent ion $[M\text{-}3H]^{3-}$, at m/z 305, calcd. mass 918 amu.

Chemical removal of terminal unsaturated uronic acid residue [18]

Tetrasaccharide was dissolved at a concentration of 1 mg/ml in water. Mercuric acetate reagent (35 mM) was prepared by dissolving 113 mg of $Hg(OAc)_2$ in 10 ml of distilled water adjusted to pH 5 with a few drops of acetic acid. In a typical experiment, 1 ml of oligosaccharide solution (1 mg/ml) was treated with 1 ml of mercuric acetate reagent, stirred for 10 min at room temperature. The reaction mixture was passed over a pre-washed Dowex 50W-X8H^+ column (1 × 5 cm), and then washed with 5 column volumes of distilled water. The total eluent was adjusted to pH 7 using sodium bicarbonate solution, and freeze-dried. The resulting oligosaccharide was redissolved in 0.5 ml of water and then was applied to a Sephadex G-25 column (2.5 × 50 cm) and elution was monitored by absorbance at 210 nm. The first peak eluting was collected and desalted on a Bio-Gel P2 column (1 cm × 25 cm) and freeze-dried. The trisaccharide **2** was assigned the structure *β*-D-Gal*p*NAc4S(1 → 4)-α-L-Ido*p*A (1 → 3)-α,*β*-D- Gal*p*NAc4S based on ^{1}H-NMR and ESI-MS analysis(as described for **1**). ^{1}H-NMR (500 MHz, D_2O) *β*-D-Gal*p*NAc4S; H1 *δ* 4.577; H2, 4.004; H3 3.98; H4, 4.672; H5, 3.83; H6, 3.78; NAc, 2.042. α-L-Ido*p*A; H1, 4.9; H2, 3.504; H3, 3.84; H4, 4.085; H5, 4.69. α,*β*-D-Gal*p*NAc4S H1α, 5.180, *β*, 4.748; H2α, 4.325, *β*, 4.020; H3α, 4.168, *β*, 3.98; H4α, 4.722, *β*, 4.71; H5α, 4.286, *β*, 3.84; H6aα, 3.79, *β*, 3.78; H6bα, 3.71, *β* 3.78; NAcα, *β*, 2.025. ESI-MS (negative ion) parent ion $[M\text{-}2H]^{2-}$, at m/z 379, calcd. mass 760 amu.

Preparation of oxidized dermatan sulfate

Sodium periodate (1.46 g) was dissolved in citrate buffer (50 mM citric acid, pH 3.0) and cooled to 4°C. Dermatan sulfate (1.0 g) was dissolved in the same citrate buffer (50 ml) and transferred in a slow stream into the reaction flask containing the well stirred periodate solution maintained at 4°C. More buffer (50 ml) was used to rinse the flask that contained DS solution and the washings were transferred into the reaction flask. After 16 h of reaction at 4°C, a solution (10 ml) of ethylene glycol in water (30% v/v) was added and reaction continued for 48 h. The reaction mixture, a clear solution, was dialyzed against deionized water using cellulose acetate dialyzing tubes (MWCO 6000–8000) and then freeze-dried to give the periodate oxidized dermatan sulfate (PIODS).

Preparation of PIODS column

Hydrazide activated Sepharose (10 ml, BioRad) was transferred into a conical flask (50 ml) and washed successively with deionized water (2 × 50 ml) and 0.1 M acetate buffer (pH 4.8) containing 0.5 M NaCl (2 × 50 ml). PIODS (70 mg) was dissolved in minimum buffer (~ 20 ml) and added to the resin. The flask was shaken at 4°C for 30 h at the end of which *p*-anisaldehyde (500 µl) was added and reaction continued for another 24 h at 4°C. Finally, sodium cyanoborohydride (100 mg) was added into the reaction mixture and stirring continued at 4°C (16 h). The reaction mixture was acidified (pH 3–4) with 1 M acetic acid, the slurry transferred into a column fitted with fritted glass filter and the solid washed thoroughly with deionized water (1 L), 25% ethanol in water (1 L) and finally with 20% ethanol in water (500 ml).

Purification of arylsulfatase B

Glacial acetic acid (1 µl/ml of lysate) was added to arylsulfatase B lysate (BioMarin Pilot plant) to adjust its pH to 4.5 and the turbid solution was centrifuged at 14 000 rpm for 3 min to pellet the particulates. Chromatography was done using GradiFrac (Pharmacia). The supernatant (6 ml) was loaded onto 1 ml of resin prepared as described above using 25 mM acetate buffer (pH 4.5), eluted with the same buffer until no more absorbance was detected and then with 25 mM

Tris acetate buffer (pH 6.0) which produced a broad peak with weak activity (tested against 4-methylumbelliferryl (4-MU)-sulfate, John Hopwood, personal communication). Elution with 25 mM Tris acetate buffer (pH 6.0), containing 1 M NaCl in a steep gradient (0–100%, 10 ml) gave a sharp peak and the corresponding eluant showed high activity. Single peak fractions were collected from 3 runs on the same resin and concentrated to about 3 ml and salt exchanged (Centricon 10 filter) using about 15 ml of 25 mM acetate buffer (pH 6.0). Desalting was repeated two more times. Arylsulfatase B solution (1.6 ml) thus obtained was diluted to 2 ml and used for desulfation.

Enzymatic desulfation of trisaccharide

Trisaccharide **1** (8.26 mg) was dissolved in 1 ml water to give a 10 mM stock solution. To 10 µl of this trisaccharide stock solution, 10 µl of 0.5 M acetate buffer (pH 5.6), 10 µl of distilled water and 10 µl of purified arylsulfatase B (1.2 U/ml) was added and incubated at 37°C for 3 days. More (10 µl) arylsulfatase B (2.4 U/ml) was added and the incubation was continued for another 3 days. The reaction was monitored for completion by periodically removing 1 µl aliquots and fluorescently labeling for analysis by gel electrophoresis.

Conversion of monosulfated trisaccharide to monosulfated disaccharide and GalpNAc

The reaction mixture (70 µl) obtained by enzymatic desulfation was acidified with 100 mM phosphoric acid (35 µl). Protein and inorganic phosphate were precipitated by the addition of 3 volumes of ethanol and removed by centrifugation. The ethanol solution of monosulfated trisaccharide product was dried by Speedvac and the residue reconstituted with 30 µl of water and 10 µl of 0.5 M sodium phosphate buffer (pH 4.0). Next, 10 µl of *N*-acetylhexosaminidase (162 U/ml, from Jack bean, supplier: V-Labs, Covington, LA; alternatively, Hexase II, 34 U/ml, supplier: Glyko, Novato, CA) was added and the mixture was incubated at 37°C for 4 days after which an additional 10 µl of enzyme was added. The reaction was monitored for completion by periodically removing, and fluorescently labeling 1 µl aliquots for analysis by gel electrophoresis. After 12 weeks, additional enzyme (10 µl) was added and digestion continued for two more weeks before the reaction was complete.

Iduronidase treatment of monosulfated disaccharide

The *N*-acetyl-hexosaminidase treated reaction mixture (2 µl) containing monosulfated disaccharide (**5**) was mixed with 0.5 M sodium acetate buffer (pH 5.6) and 1 µl of distilled water followed by 5 µl of α-L-iduronidase (132 KU/ml, BioMarin). The mixture was incubated overnight at 37°C and the product, after evaporation, was investigated by gel electrophoresis following usual fluorescent labeling.

Fluorescent labeling and analysis of products by electrophoresis

To each oligosaccharide containing aliquot, 0.5 µl of 100 mM phosphoric acid was added, followed by 2.5 µl of 300 mM 2-aminoacridone (AMAC) fluorescent label, 2.5 µl of 30% acetic acid, and 5 µl of 1 M $NaCNBH_3$ in dimethyl sulfoxide. The labeling reaction was incubated at 37°C overnight and then evaporated to dryness on a Speedvac. The labeled sample was reconstituted in 10 µl of DMSO and 10 µl of 25% glycerol in water and 4 µL was analyzed on an *N*-linked profiling gel (Glyko, Novato, CA) run at 30 mA constant current for 45 min at 6°C using OLIGO running buffer (Glyko). The gel was imaged using a Glyko SE 2000 imager.

Results and discussion

Dermatan sulfate, a glycosaminoglycan containing a major repeating structure of $\rightarrow 4)\alpha$-L-Ido*p*A$(1 \rightarrow 3)$-β-D-Gal*p*NAc4S$(1 \rightarrow$, is currently under evaluation as a clinical antithrombotic [18] and is thus, available in large quantities. Partial enzymatic depolymerization of dermatan sulfate with chondroitin ABC lyase afforded a mixture of oligosaccharides [18]. Fractionation of this oligosaccharide mixture by size and charge resulted in the preparation of multimilligram quantities of tetrasaccharide. The purity of this tetrasaccharide was confirmed by analytical SAX-HPLC and CE and its structure was established to be, ΔU*p*A $(1 \rightarrow 3)$-β-D-Gal*p*NAc4S $(1 \rightarrow 4)$-α-L-Ido*p*A $(1 \rightarrow 3)$-α,β,-D-Gal*p*NAc4S **1** (Figure 1) by ^{1}H-NMR spectroscopy and ESI mass spectrometry. Mercuric acetate treatment was used to remove the unnatural unsaturated uronate residue (ΔU*p*A) afforded through the eliminase action of the chondroitin ABC lyase at the non-reducing terminus [20]. The product of this reaction was purified by gel permeation chromatography (Figure 2). The disulfated trisaccharide **2** was fluorescently labeled by reductive amination (Figure 1) and analyzed by gel electrophoresis. It showed a single, rapidly migrating band low in the gel (a small amount of impurity in AMAC appeared as a band in all runs) indicating the high purity of this sample (Figure 3). The structure of the resulting disulfated trisaccharide **2** was confirmed by ^{1}H-NMR spectroscopy and ESI-MS as β-D-Gal*p*NAc4S$(1 \rightarrow 4)$-α-L-Ido*p*A $(1 \rightarrow 3)$-α,β-D-Gal*p*NAc4S (Figure 1).

The sulfate group at the 4-position of the non-reducing terminal Gal*p*NAc residue could be removed on exhaustive treatment with arylsulfatase B. Fluorescent labeling followed by gel electrophoresis analysis confirmed that de-4-*O*-sulfation had taken place, as indicated by the appearance of a new slower migrating band with a decreased net charge. Treatment of this monosulfated trisaccharide **3** with *N*-acetylhexosaminidase afforded two saccharide products, D-Gal*p*NAc **4** and α-L-Ido*p*A $(1 \rightarrow 3)$-α, β-D-Gal*p*NAc4S **5**. Fluorescent labeling of the product mixture and analysis by gel electrophoresis showed the disappearance of the band corresponding to the

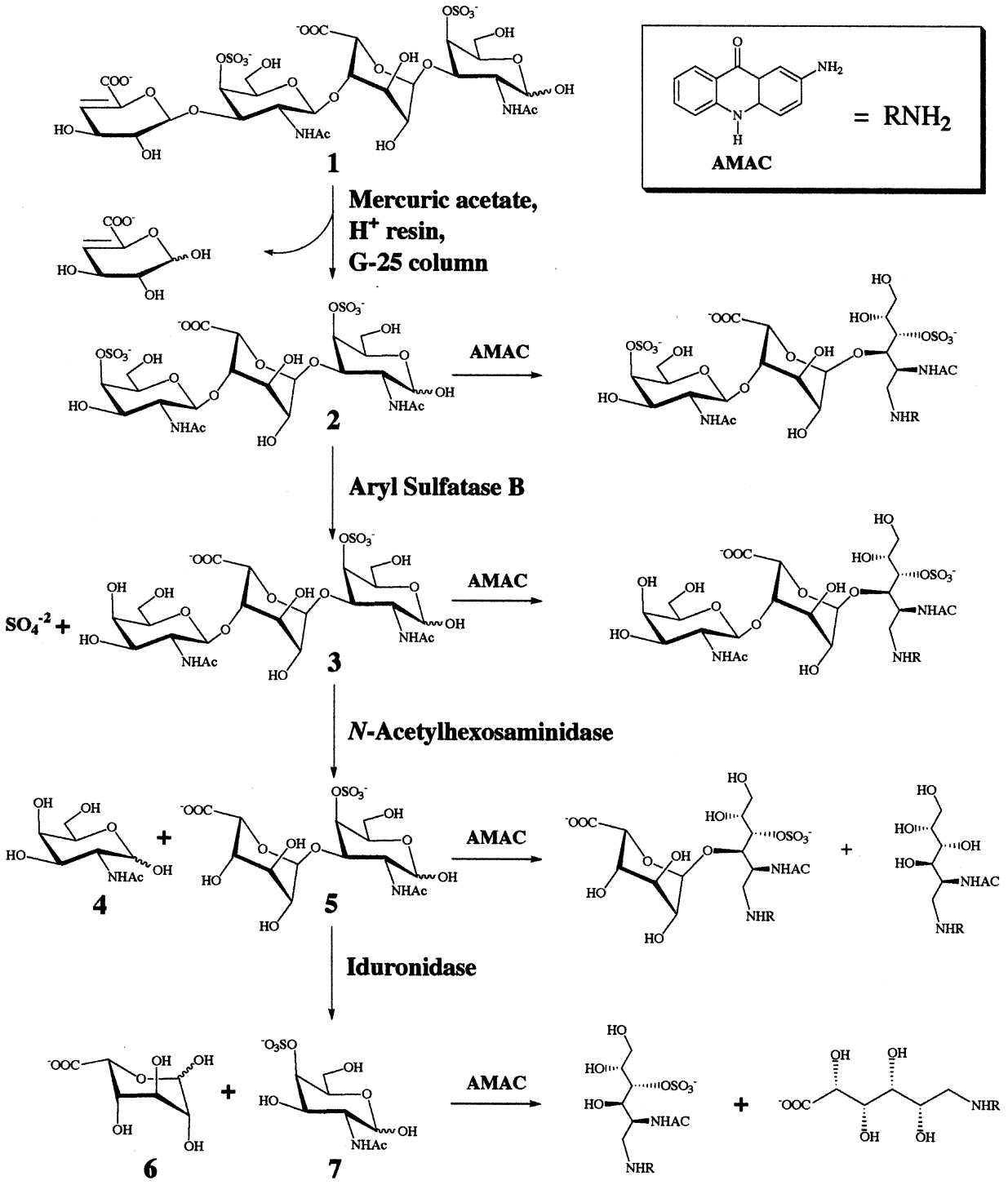

Figure 1. Structure of the oligosaccharides prepared in this study and their fluorescently labeled derivatives.

monosulfated trisaccharide **3** and the appearance of two new bands. One slowly migrating band observed at the top of the gel co-migrated with the fluorescently labeled D-Gal*p*NAc standard and a second rapidly migrating band at the bottom of the gel corresponded to the small, highly charged fluorescently labeled monosulfated disaccharide **5**. Treatment of this reaction mixture with α-L-iduronidase followed by fluorescent labeling and gel electrophoresis showed the disappearance of the band corresponding to the fluorescently labeled monosulfated disaccharide and the appearance of two new bands

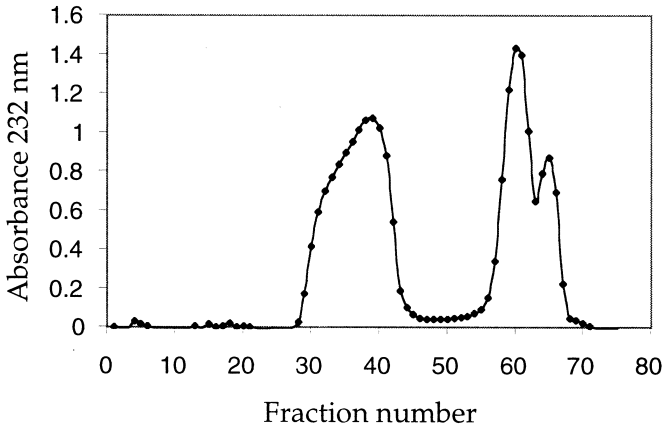

Figure 2. Purification of disulfated trisaccharide following mercuric acetate treatment on Sephadex G-25 column. Each 3.2 ml fraction was collected in a period of 4 min. The peak eluting first (fractions 28–42) contains the disulfated trisaccharide product.

assignable to fluorescently labeled L-Ido*p*A **6** (runs midway between GalA and GlcA, unpublished data) and fluorescently labeled D-Glc*p*NAc4S **7**.

In conclusion, this study demonstrated that the chemoenzymatic preparation of a monosulfated disaccharide as an α-L-iduronidase substrate was possible starting from readily available dermatan sulfate. Furthermore, the structure of a disulfated trisaccharide determined by ^{1}H-NMR and ESI-MS, was confirmed using microanalytical fluorophore assisted carbohydrate electrophoresis based sequencing method.

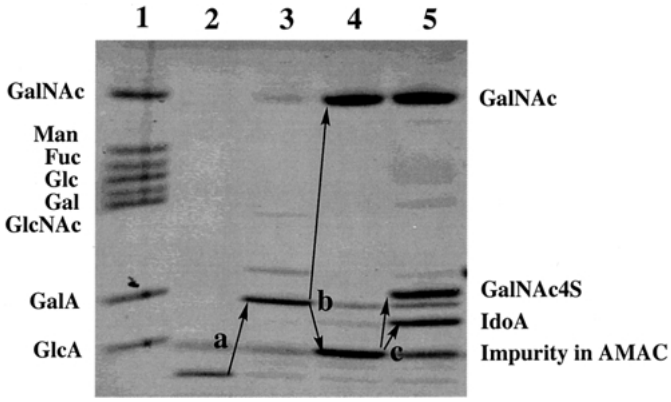

Figure 3. Polyacrylamide gel electrophoresis of oligosaccharides fluorescently labeled with AMAC. Lane 1 contains a mixture of monosaccharide standards; Lane 2 contains disulfated trisaccharide; Lane 3 contains disulfated trisaccharide treated with (**a**) arylsulfatase B (the monosulfated trisaccharide product ran just below the GalA standard); Lane 4 contains disulfated trisaccharide treated with (**a**) arylsulfatase B and (**b**) *N*-acetyl hexosaminidase (the monosulfated disaccharide product runs at the same position as a contaminant present in the AMAC label seen in lanes 2–5); and Lane 5 contains disulfated trisaccharide treated with (**a**) arylsulfatase B, (**b**) *N*-acetylhexosaminidase and (**c**) α-L-iduronidase.

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