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Preparation and structural determination of large oligosaccharides derived from acharan sulfate

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Abstract—The structures of a series of large oligosaccharides derived from acharan sulfate were characterized. Acharan sulfate is an unusual glycosaminoglycan isolated from the giant African snail, *Achatina fulica*. Oligosaccharides from decasaccharide to hexadecasaccharide were enzymatically prepared using heparin lyase II and purified. Capillary electrophoresis and gel electrophoresis confirmed the purity of these oligosaccharides. Their structures, determined by ESI-MS and NMR, were consistent with the major repeating sequence in acharan sulfate, →4)-α-D-GlcN$_p$Ac-(1→4)-α-L-IdoA$_p$2S-(1→, terminated by 4-linked α-D-GlcN$_p$Ac residue at the reducing end and by 4,5-unsaturated pyranosyluronic acid 2-sulfate at the non-reducing end.

Keywords: Acharan sulfate; Oligosaccharides; Structural determination

1. Introduction

Acharan sulfate is a glycosaminoglycan (GAG), isolated from the giant African snail, *Achatina fulica*.[1] It is polydisperse having an average molecular weight of 29,000[1] and a major repeating disaccharide structure of →4)-α-D-GlcN$_p$Ac-(1→4)-α-L-IdoA$_p$2S-(1→. It has been suggested that the structure of acharan sulfate is complicated by the presence of minor unsulfated disaccharide sequence of the structure →4)-α-D-GlcN$_p$Ac-(1→4)-α-L-IdoA$_p$-(1→.[2] It has been suggested that the heterogeneity of acharan sulfate may be related to its localization in the body of giant African snail.[3]

Acharan sulfate is the major glycoconjugate of snail mucus and it is proposed to have a number of important biological roles in snail physiology.[4] Purified acharan sulfate also shows various interesting biological activities. Inhibition of angiogenesis by acharan sulfate has been studied in experimental inflammation models. These studies show that acharan sulfate inhibits angiogenesis in the inflammatory granulation tissues probably due to the inhibition of capillary tube formation of vascular endothelial cells.[5] Acharan sulfate is also able to suppress tumor growth both in vivo and in vitro, an activity which is believed to be related to its inhibition of angiogenesis.[6] Acharan sulfate's activity against cultured Lewis lung carcinoma (LLC) cells is associated with its binding the protein receptor nucleolin found on the surface of the LLC cells.[7] Acharan sulfate derivatives also show a long duration anticoagulant activity and protective effects in vivo.[8]

The high molecular weight of GAGs usually limits their therapeutic applications. While oligosaccharides can be prepared by controlled chemical or enzymatic depolymerization of GAGs, specific sequences of appropriate size critical to the biological activities are often difficult to prepare. In the case of acharan sulfate, it is important to prepare relatively large, homogeneous

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oligosaccharides containing the repeating structure →4)-α-D-GlcN$_p$Ac-(1→4)-α-L-IdoA$_p$2S-(1→. These requirements make the separation and purification of such oligosaccharides challenging. Moreover, the presence of monotonous repeating sequence makes the structural determination of such oligosaccharides quite difficult. In previous studies, acharan sulfate-derived oligosaccharides of sizes as large as octasaccharide have been prepared and structurally determined.[2] The purification and structural analysis of larger acharan sulfate oligosaccharides, required for the therapeutic exploitation of this GAG are discussed in the current paper.

2. Results and discussion

Acharan sulfate was depolymerized through the controlled catalytic action of *Bacteroides stercoris* heparin lyase II.[9] At 40–60% reaction completion, a mixture containing larger oligosaccharides (between decasaccharide and hexadecasaccharide) was obtained as by analysis using capillary electrophoresis (CE) (Fig. 1). Size fractionation using gel permeation chromatography on a Bio-Gel P-10 followed by a strong anion exchange HPLC afforded oligosaccharides **1**–**4**.

The structure of smaller acharan sulfate-derived oligosaccharides (disaccharide through octasaccharide) had already been determined by our laboratory[2] so we turned to the structural determination of larger oligosaccharides required for biological evaluation. Acharan sulfate-derived oligosaccharides **1**–**4** were obtained in 86–92% purity as determined by CE analysis. Polyacrylamide gel electrophoresis (PAGE) is among the highest resolution methods for the analysis of GAGs and GAG-derived oligosaccharides.[10] All the four acharan sulfate-derived oligosaccharide samples showed bands prominent in the center of the PAGE gel visualized with alcian blue staining (Fig. 2A). Oligosaccharides **1** and **2** showed single bands suggesting these oligosaccharides were >90% pure, while oligosaccharides **3** and **4** showed major bands together with minor bands suggesting these oligosaccharides were >85% pure, consistent with the results of CE analysis. In comparison to the ladder of heparin oligosaccharide standards, **1** migrated on PAGE at a position close to that of a 12-sulfated heparin derived octasaccharide, of molecular weight 2308, which is close to a dodeca-saccharide derived from acharan sulfate. Oligosaccharides **2**–**4** differed in migration by an amount that corresponds to ~500 mass units suggesting that these

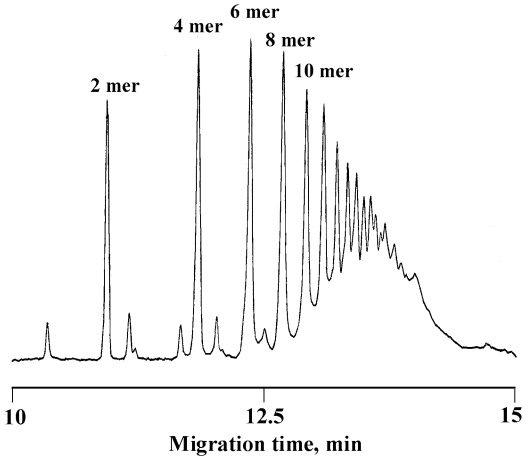

Figure 1. CE analysis of acharan sulfate after 40% digestion with heparin lyase II. Absorbance at 230 nm is plotted as a function of migration time. The peaks corresponding to the disaccharide (2-mer) through octasaccharide were confirmed using authentic standards that had been previously prepared and characterized in our laboratory.[2] The peaks decasaccharide (10-mer) and higher oligosaccharides (not labeled) initially deduced by peak counting, required purification and characterization.

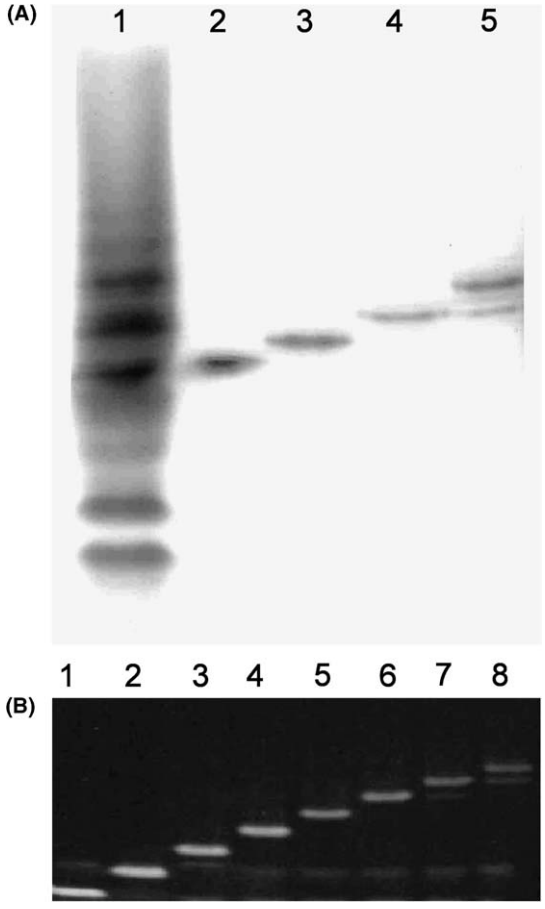

Figure 2. PAGE analysis of acharan sulfate-derived oligosaccharides. **A**. PAGE analysis of oligosaccharides visualized with alcian blue. Lane 1: Oligosaccharide standards derived from bovine lung heparin; Lane 2: Oligosaccharide **1**; Lane 3: Oligosaccharide **2**; Lane 4: Oligosaccharide **3**; Lane 5: Oligosaccharide **4**. **B**. PAGE analysis of fluorescently labeled oligosaccharides visualized with transillumination. Lane 1: 2-mer-ANTS; Lane 2: 4-mer-ANTS; Lane 3: 6-mer-ANTS; Lane 4: 8-mer-ANTS; Lane 5: Oligosaccharide **1**-ANTS; Lane 6: Oligosaccharide **2**-ANTS; Lane 7: Oligosaccharide **3**-ANTS; Lane 8: Oligosaccharide **4**-ANTS.

oligosaccharides corresponded to dodeca-, tetradeca-, and hexadeca-saccharides. Since the acharan sulfate-derived oligosaccharides are less highly sulfated than those derived from heparin, it appeared likely that **1**–**4** corresponded to a decasaccharide–hexadecasaccharide and they gave the same migration times as the peaks labeled 10-mer–16-mer in CE analysis (Fig. 1). PAGE analysis of these oligosaccharides, following fluorescent labeling with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and visualized by transillumination further establish the purity of these oligosaccharides (Fig. 2B).

Electrospray ionization mass spectrometry (ESI-MS) analysis was next used to determine the molecular weight of each acharan sulfate-derived oligosaccharide. Acharan sulfate disaccharide and tetrasaccharide, previously prepared in our laboratory[2] were used to optimize the ESI-MS parameters. Molecular ions were observed for acharan sulfate-derived disaccharide and tetrasaccharide in both positive- and negative-ion modes (Table 1). A doubly charged negative ion at *m*/*z* 480.2 was also observed for the tetrasaccharide. ESI mass spectral analysis has proved difficult for large oligosaccharides. However, for the large acharan sulfate-derived oligosaccharides prepared in the current study, it gave interpretable negative-ion mass spectra. All of the molecular ions for the oligosaccharides **1**–**4** were multiply charged, as is commonly observed in the ESI mass spectra of GAG-derived oligosaccharides due to their multiple sulfate group substitutions.[11] Surprisingly, no ions associated with fragmentation were observed in the mass spectra of oligosaccharides **1**–**4**, thus, the resulting spectra could be easily interpreted. The mass spectrum of oligosaccharide **4**, presented in Figure 3, is a representative example of the mass spectra obtained for **1**–**4** and the interpreted spectral data are presented in Table 1. Oligosaccharide **4** corresponds to one of the largest sulfated oligosaccharides yet analyzed using mass spectrometry.

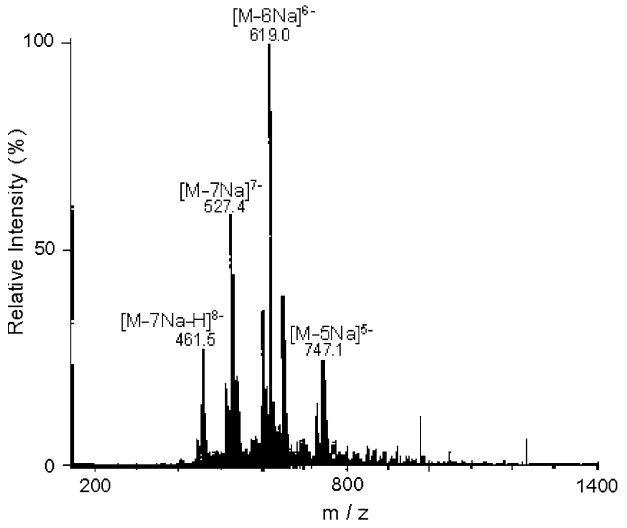

Figure 3. ESI-mass spectrum of Oligosaccharide **4**.

The ESI-MS analysis of **1**–**4**, confirmed the assignments made by PAGE analysis, showing they correspond to acharan sulfate-derived deca-, dodeca-, tetradeca-, and hexadeca-saccharides having 5, 6, 7, and 8 sulfate groups, respectively.

Next, the ^{1}H NMR spectra of **1**–**4** were obtained at 500 MHz using Shigemi tubes. These spectra were similar to those previously reported for smaller acharan sulfate-derived oligosaccharides.[2] Signals at 2.03, 2.06, and 2.07 ppm confirmed the presence of the *N*-acetyl groups of the glucosamine residue and the downfield shift of the peak corresponding to H-1 of the uronic acid (5.56 ppm) demonstrates that this residue is iduronic acid 2-sulfate (Fig. 4). ^{1}H–^{1}H COSY NMR spectra of **1** and **2** were recorded to facilitate the assignment of most of the proton resonances and the assignment of COSY NMR obtained for oligosaccharide **1** is shown in Figure 4. The COSY spectra obtained for oligosaccharides **1** and **2** were con-

Table 1. ESI-MS analysis of acharan sulfate oligosaccharides

Compd.	Size	Molecular formula	Molecular weight	Ion observed in positive mode	Ion observed in negative mode
	2-mer	$C_{14}H_{20}NNaO_{14}S$	481.36	504.1 = $[M+Na]^{+}$	458.2 = $[M-Na]^{-}$
	4-mer	$C_{28}H_{40}N_2Na_2O_{28}S_2$	962.73	1029.0 = $[M+3Na-2H]^{+}$	983.0 = $[M+Na-2H]^{-}$ 480.2 = $[M-2H]^{2-}$
1	10-mer	$C_{70}H_{100}N_5Na_5O_{70}S_5$	2406.81	nd	779.0 = $[M-3Na]^{3-}$ 578.9 = $[M-4Na]^{4-}$ 458.6 = $[M-5Na]^{5-}$
2	12-mer	$C_{84}H_{120}N_6Na_6O_{84}S_6$	2888.17	nd	698.9 = $[M-4Na]^{4-}$ 554.9 = $[M-5Na]^{5-}$
3	14-mer	$C_{98}H_{140}N_7Na_7O_{98}S_7$	3369.53	nd	650.9 = $[M-5Na]^{5-}$ 539.0 = $[M-6Na]^{6-}$
4	16-mer	$C_{112}H_{160}N_8Na_8O_{112}S_8$	3850.89	nd	747.1 = $[M-5Na]^{5-}$ 619.0 = $[M-6Na]^{6-}$ 527.4 = $[M-7Na]^{7-}$ 461.5 = $[M-7Na-H]^{8-}$

nd: not detected.

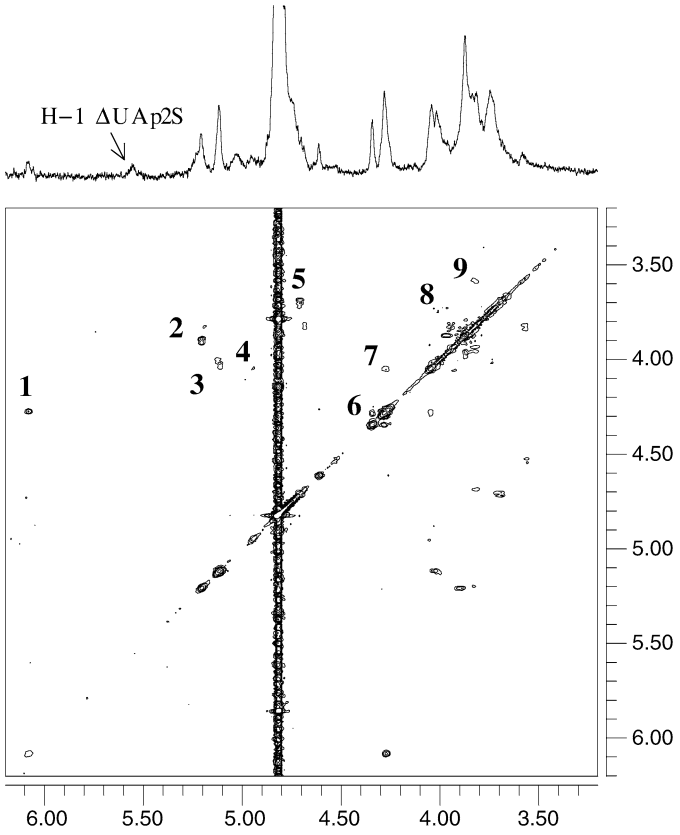

Figure 4. Partial ^{1}H–^{1}H COSY NMR spectrum (3.2–6.2 ppm) of oligosaccharide **1**. Selected cross-peaks have been labeled: (1) H-3/H-4 of ΔUA_p2S; (2) H-1/H-2 of $GlcN_pAc$ (α); (3) H-1/H-2 of $GlcN_pAc$ (internal); (4) H-4/H-5 of $IdoA_p2S$; (5) H-1/H-2 of $GlcN_pAc$ (β); (6) H-2/H-3 of $IdoA_p2S$; (7) H-3/H-4 of $IdoA_p2S$; (8) H-2/H-3 of $GlcN_pAc$ (internal); (9) H-5/H-6a of $GlcN_pAc$ (β).

sistent with the structures: ΔUA_p2S-(1→[4)-α-D-$GlcN_pAc$-(1→4)-α-L-$IdoA_p2S$-(1→]$_n$-4)-D-GlcNAcα,β $n = 4$ for **1** and $n = 5$ for **2** (Table 2). The 1D ^{1}H NMR spectra for oligosaccharides **3** and **4** were also consistent with their structure, but the 2D COSY spectra could not be obtained due to the limited amounts of these samples.

In conclusion, a series of large acharan sulfate-derived oligosaccharides were prepared, purified, and their structures were determined with MS and NMR analysis. These acharan sulfate-derived decasaccharide, dodecasaccharide, tetradecasaccharide, and hexadecasaccharide are among the largest sulfated oligosaccharides to be structurally determined thus far. These oligosaccharides will be invaluable in the study of the anti-cancer activities of acharan sulfate, and essential in developing a structure–activity relationship (SAR) for the activity of this unique GAG.

Table 2. Chemical shifts (ppm) of acharan sulfate-derived oligosaccharide **1**

	ΔUA_p2S	$GlcN_pAc$ (internal)	$IdoA_p2S$	$GlcN_pAc$	
				α	β
H-1	5.56	5.12	5.21	5.20	4.72
H-2	4.61	4.02	4.34	3.85	3.70
H-3	4.27	3.73	4.29	nd	nd
H-4	6.09	nd	4.04	nd	nd
H-5	—	nd	4.95	nd	3.58
H-6a	—	3.87	—	nd	3.83
H-6b	—	3.97	—	nd	3.94
N-Acetyl methyl	—	2.03–2.07	—	2.03–2.07	

nd = non determined.

3. Materials and methods

3.1. Materials

Acharan sulfate was prepared according to the previous procedure.[1] Heparin lyase II was purified from *Bacteroides stercoris* HJ-15 according to the method previously described.[9] Bio-Gel 10 gel (medium) was purchased from Bio-Rad (Hercules, CA). 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) was from Aldrich (Milwaukee, WI). All other reagents used were of analytical grade. HPLC system was equipped with AKTA^{TM} Purifier 10 controlled by Unicorn software 3.1 (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was a 5 μm Phenosphere strong-anion exchange (SAX) column from Phenomenex (Torrells, CA) of dimension 0.46×25 cm. UV spectrophotometry was performed

on a JASCO model V550 equipped with a thermostated cell (Tokyo, Japan).

3.2. Preparation of large acharan sulfate oligosaccharides

Acharan sulfate (500 mg, 10 mg/mL) in 50 mM sodium phosphate buffer, pH 7.0 was depolymerized using 500 μL of heparin lyase II purified from *Bacteroides stercoris* HJ-15 (20 mU/mL) at 37 °C. Aliquots were withdrawn at 1 h interval and immediately frozen and stored at −80 °C. At various time points, the absorbance at 232 nm was measured and digestion was continued until the absorbance was constant (complete digestion). The percent reaction completion at each time point was calculated by dividing the absorbance at 232 nm by the absorbance measured at reaction completion. The digestion mixture heated at 100 °C for 3 min when the absorbance at 232 nm indicated the digestion was 55% completion. After freeze-drying it was dissolved and centrifuged at 12,000*g* for 10 min to remove any particulates. The supernatant was applied to the Bio-Gel P-10 column (2.5 × 112 cm) equilibrated with 0.15 M NaCl at a flow rate 10 mL/h. The absorbance of each fraction was read at 232 nm. After collection and desalting, these oligosaccharides were further purified by SAX-HPLC to obtain purified acharan sulfate-derived oligosaccharide **1**–**4**.

3.3. Capillary electrophoresis

CE was performed using a system with advanced computer interface, equipped with high voltage power supply capable of constant or gradient voltage control using a fused silica capillary from GL Science (Tokyo). The composition of the acharan sulfate oligosaccharide mixture was confirmed using CE under normal polarity mode using a mixture of 40 mM disodium phosphate/40 mM sodium dodecylsulfate/10 mM tetraborate adjusted to pH 9.0 with 1.0 M hydrochloride. The fused silica capillary (75 μm I.D. × 375 μm O.D., 67 cm long) was automatically washed before use with 0.1 M sodium hydroxide, followed by nitrogen gas pressure injection (5 s) at a constant current 15 kV. The samples (1 mg/mL) were dissolved in water and loaded (7 nL) with nitrogen gas pressure injection.

3.4. Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed as previously described.[10] Briefly, a polyacrylamide mini-gel (8 × 6 cm, 22% total acrylamide) was prepared and 3 μg of each acharan sulfate-derived oligosaccharide was subjected to electrophoresis at 200 V for 90 min. Heparin oligosaccharide standards (15 μg), prepared from bovine lung heparin, were loaded on the same gel. Oligosaccharides were visualized by Alcian Blue staining. PAGE analysis was also conducted on fluorescently labeled oligosaccharides. Each oligosaccharide was resuspended in 5 μL of 0.15 M ANTS in 15% (v/v) acetic acid. Samples were incubated for 10–15 min at room temperature. Then 5 μL of 1.0 M sodium cyanoborohydride in dimethyl sulfoxide was added, and the suspension was mixed, briefly centrifuged to bring the reactants to the tip of the tube, and incubated for 16 h at 37 °C and then dried in a centrifugal vacuum evaporator. Each pellet was resuspended in 20 μL water and 5 μL glycerol. Separation of the labeled oligosaccharides was performed on polyacrylamide gel containing 30% acrylamide at a constant current of 25 mA at 4 °C for 90 min. Detection was performed on a transilluminator at 365 nm.

3.5. ESI-MS analysis

Both positive-ion and negative-ion modes of mass spectral analysis were performed on an Agilent MSD-Trap-SL mass spectrometer equipped with an electrospray ion source. Acharan sulfate-derived disaccharide and tetrasaccharide were used to optimize the acquisition parameters. Samples were dissolved in 1:1 water/methanol and introduced into ion source at a flow rate of 6 μL/min. Nebulizer pressure was set to 15 psi. Nitrogen gas was used as dry gas at a flow rate at 5 L/min and the dry temperature was at 325 °C.

3.6. NMR analysis

1D and 2D NMR spectra were recorded in a Varian Inova 500 MHz spectrometer. Before data acquisition, each sample was exchanged with 0.3 mL of ^2H_2O (99.9%) followed by lyophilization. The residue was redissolved in 75 μL of ^2H_2O (100%) and transferred to a Shigemi NMR microtube. For ^1H NMR, presaturation of the residual HO^2H peak was achieved during the relaxation delay. The spectra were recorded at 293 K with a flip angle of 90°, spectral width of 2806 Hz and a minimum of 64 accumulation pulses. One Hertz line broadening was applied in the data processing. ^1H–^1H COSY spectra were recorded at 293 K with a flip angle of 90°, spectral width of 2806 Hz and 512 × 2048 data points. A total of 32 scans and 600 increments was accumulated and a relaxation delay of 1 s was used.

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