ELSEVIER

Carbohydrate Research 300 (1997) 315–321

CARBOHYDRATE RESEARCH

Chondroitin ABC lyase digestion of an ascidian dermatan sulfate. Occurrence of unusual 6-*O*-sulfo-2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-α-L-idopyranosyluronic acid)-β-D-galactose units

Paulo A.S. Mourão [a,*], Mauro S.G. Pavão [a], Barbara Mulloy [b], Robin Wait [c]

[a] *Departamento de Bioquímica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Caixa Postal 68041, Rio de Janeiro, RJ, 21941-590, Brazil*

[b] *National Institute for Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom*

[c] *Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom*

Received 12 October 1996; 18 January 1997

Abstract

A dermatan sulfate-like glycosaminoglycan was isolated from the body of the ascidian *Ascidia nigra* (*J. Biol. Chem.* 270: 31027–31036, 1995). ^{1}H NMR and fast atom bombardment mass spectrometry (FAB-MS) spectra of the tetra and disaccharides formed by chondroitin ABC lyase digestion support the proposed repeating disaccharide structure for this glycosaminoglycan, [→4)-α-L-IdoA(2SO_4)-(1→3)-β-D-GalNAc(6SO_4)-(1→], which differ from mammalian dermatan sulfate in its sulfation at both 2-position of the α-L-iduronic acid and the 6-position of the *N*-acetyl-β-D-galactosamine residue. © 1997 Elsevier Science Ltd.

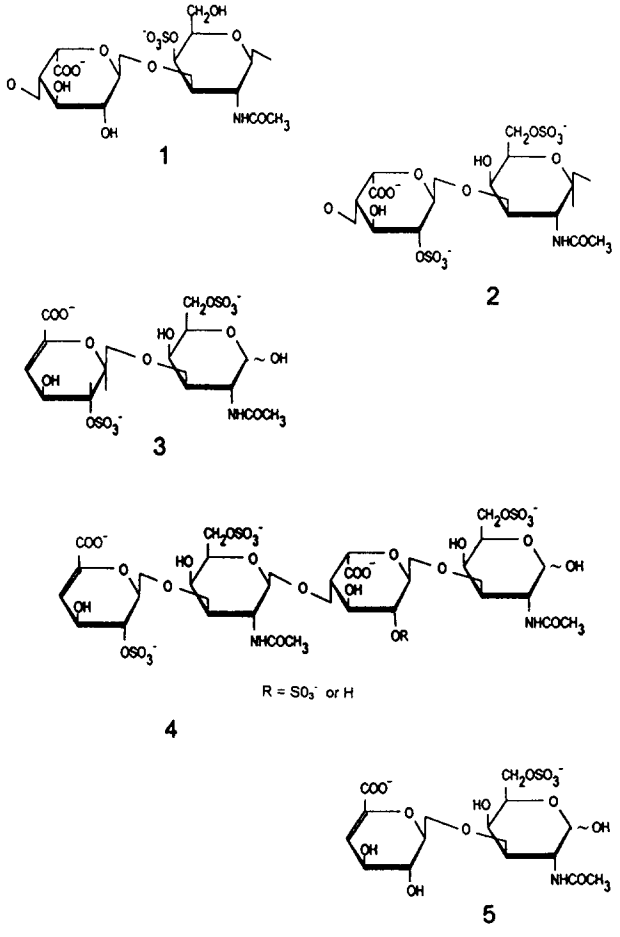
Keywords: Dermatan sulfate; Glycosaminoglycan; Tunicate

As part of our continuing studies of the structures of sulfated polysaccharides from vertebrates and invertebrates [1–7], we have isolated a unique dermatan sulfate from the viscera of the ascidian *Ascidia nigra* [8]. Mammalian dermatan sulfate has the structure shown in **1** in which the galactosamine residues are sulfated at the 4-position, and only a small proportion of iduronate residues are 2-*O*-sulfated. The repeating disaccharide of ascidian dermatan sulfate has the structure illustrated in **2** [8], which is sulfated at position 2 of the iduronate residue and position 6 of the *N*-acetyl-galactosamine residue. This polysaccharide proved to be important for eluci-

* Corresponding author. Fax: +55-21-2708647; e-mail: mourão@server.bioqmed.ufrj.br.

0008-6215/97/$17.00 © 1997 Elsevier Science Ltd. All rights reserved.
PII S0008-6215(97)00061-X

dating the structure which confers heparin cofactor II mediated anticoagulant activity, because, although it is more highly sulfated than mammalian dermatan sulfate, it has no measurable anticoagulant activity [8]. This anticoagulant activity is therefore not merely a function of 'charge density', but depends on the particular position of sulfation of *N*-acetyl–galactosamine residues of the repeating disaccharide unit.



The structure of this new dermatan sulfate was established by ^{1}H and ^{13}C NMR spectroscopy, and by the analysis of the products of chondroitin ABC lyase and 4,6-sulfatase digestions [8]. Because sulfatases capable of distinguishing between 2- and 3-sulfates are not available, the determination of the sites of sulfation of iduronate residues of this new glycosaminoglycan depends on NMR data on the whole polysaccharide and on comparison of the chromatographic migration of the products of chondroitin lyase digestion with standard compounds, and a standard 3-*O*-sulfated disaccharide is not available.

We have now separated the chondroitin ABC lyase degradation products by gel permeation chromatography and analysed them by NMR spectroscopy and FAB mass spectrometry. NMR data on the di- and tetrasaccharide fractions confirms that the iduronate residues are sulfated at the 2-position.

1. Results and discussion

The separation of the products of chondroitin ABC lyase digestion of the ascidian dermatan sulfate is illustrated in Fig. 1. Two oligosaccharide peaks with retention times characteristic of tetrasaccharide and disaccharide fragments were obtained, and a polymeric peak at the exclusion limit of the column (V_0). The polymeric peak had metachromatic activity and contained uronic acid, but this material was not susceptible to chondroitin ABC lyase digestion and was therefore thought to represent a non-galactosaminoglycan contaminant. Possibly tetrasaccharide is produced since the ascidian dermatan sulfate is degraded slowly by chondroitin ABC lyase, requiring longer incubation times and higher concentrations of enzyme than mammalian dermatan sulfate [8]. [1]

The ^{1}H NMR spectra of the di- and tetrasaccharide are shown in Fig. 2. The spectrum of the standard disaccharide $\Delta GlcA(2SO_4)$–$GalNAc(6SO_4)$ corresponding to the structure illustrated in **3** (Fig. 2A) was assigned by a 2D COSY experiment (data not shown), giving the data listed in Table 1 which are similar to those determined previously for the same compound, designated $\Delta Di\text{–}DS_D$ [9]. The ^{1}H NMR spectrum of the disaccharide fraction from ascidian dermatan sulfate is similar (Fig. 2B), and the assignments listed in Table 1 are based on this similarity. The large signal at 3.75 ppm arises from a contaminant, probably from the Tris buffer used in the chondroitin lyase digestion. A standard disaccharide $\Delta GlcA$–$GalNAc(6SO_4)$ ($\Delta Di6S$ [9]), sulfated only at the 6-position of the *N*-acetyl galactosamine residue (**5**), gave the spectrum illustrated in Fig. 2C. This

[1] We did not test directly if the tetrasaccharide releases disaccharide after a further incubation with chondroitin ABC lyase, since the small amount of tetrasaccharide obtained did not allow this experiment. However, we already showed, using paper chromatography, that increasing incubation time releases higher proportions of disaccharide [8]. Therefore, we believe that disaccharide could arise from tetrasaccharide after exhaustive digestion with chondroitin ABC lyase, although we have not demonstrated this experimentally.

Table 1
^{1}H chemical shifts of the constituent monosaccharides of the unsaturated disaccharides and tetrasaccharide derived from dermatan sulfate [a]

	Disaccharide from ascidian dermatan sulfate (**3**)		ΔGlcA(2SO$_4$)–GalNAc(6SO$_4$) (**3**)		ΔGlcA–GalNAc(6SO$_4$) (**5**)		Tetrasaccaride from ascidian dermatan sulfate (**4**)
	α	β	α	β	α	β	α / β
ΔUA(2SO$_4$)							
H1	5.57	5.54	5.58	5.55	5.24	5.20	5.52
H2	4.49	4.49	4.49	4.49	3.81	3.81	4.48
H3	4.21	4.21	4.21	4.21	4.13	4.13	4.21
H4	6.04	6.06	6.05	6.05	5.90	5.88	6.04
GalNAc(6SO$_4$)							
H1	5.23	n.d.	5.24	4.75	5.25	4.74	5.25, 5.21 [c]
H2	4.30	3.97	4.31	3.97	4.30	4.01	4.32
H3	n.d	4.04	4.14	4.02	4.14	3.96	n.d
H4	n.d	n.d	4.12	n.d	4.22	4.17	n.d.
H5	4.38	n.d	4.35	4.18 [b]	4.38	3.97	4.36
H6	n.d	n.d	4.21	4.2 [b]	4.20	4.22	n.d
H6′	n.d	n.d	4.13	4.2 [b]	4.14	4.16	n.d
CH$_3$	2.10	2.10	2.10	2.10	2.06	2.06	2.11, 2.07, 2.05
IdoA(2SO$_4$)							
H1							5.17 [c]
IdoA							
H1							4.95
H2							3.56

[a] Chemical shifts are referenced to internal trimethylsilylpropionic acid (TST) at 0 ppm; n.d., not determined.
[b] Approximate value due to overlapping resonances.
[c] Assignments may be interchanged.

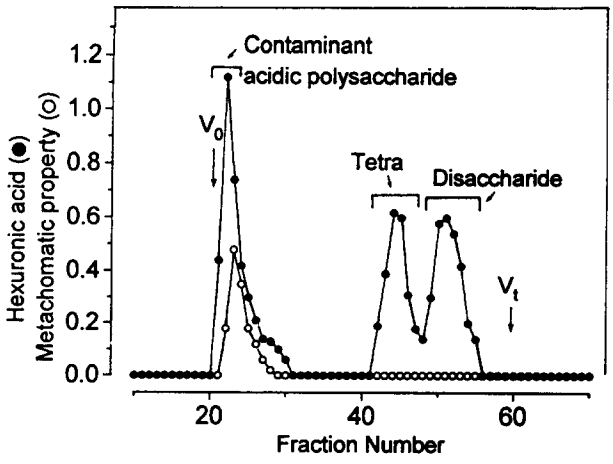


Fig. 1. Gel permeation chromatography on Sephadex G-50 of the products formed by chondroitin ABC lyase digestion of ascidian dermatan sulfate. Fractions of 1.5 mL were collected and assayed by metachromasia (○) and the carbazole reaction (●). Three groups of fractions were pooled separately, as indicated by the horizontal bars in the figure, and lyophilized.

spectrum is similar to that of the same compound described in Ref. [9], but the H2 resonance of the unsaturated uronic acid is 0.68 ppm upfield of that in the spectrum of ΔGlcA(2SO$_4$)–GalNAc(6SO$_4$) (Fig. 2A) and the disaccharide from ascidian dermatan sulfate (Fig. 2B), consistent with sulfation at that position in the latter two compounds. These data are not compatible with 3-*O*-sulfation of the uronic residue and establish the 2-*O*-sulfation of iduronate residues in the ascidian dermatan sulfate.

Comparison of the signal at ~5.90 ppm in the spectra in Fig. 2B and 2C suggests that the disaccharides obtained by chondroitin ABC lyase digestion of the ascidian dermatan sulfate contain small amounts (~10%) of non-sulfated iduronate (**5**) in addition to the predominant disaccharide sulfated at the 2-position of the uronic acid (**3**). This observation agrees with our previous interpretation of the ^{1}H NMR spectrum of the intact ascidian dermatan sulfate [8].

The ^{1}H NMR spectrum of the tetrasaccharide fraction (Fig. 2D) was assigned by comparison with the disaccharide spectra (Table 1). The prominent and well-resolved signal from H2 of the unsaturated uronic

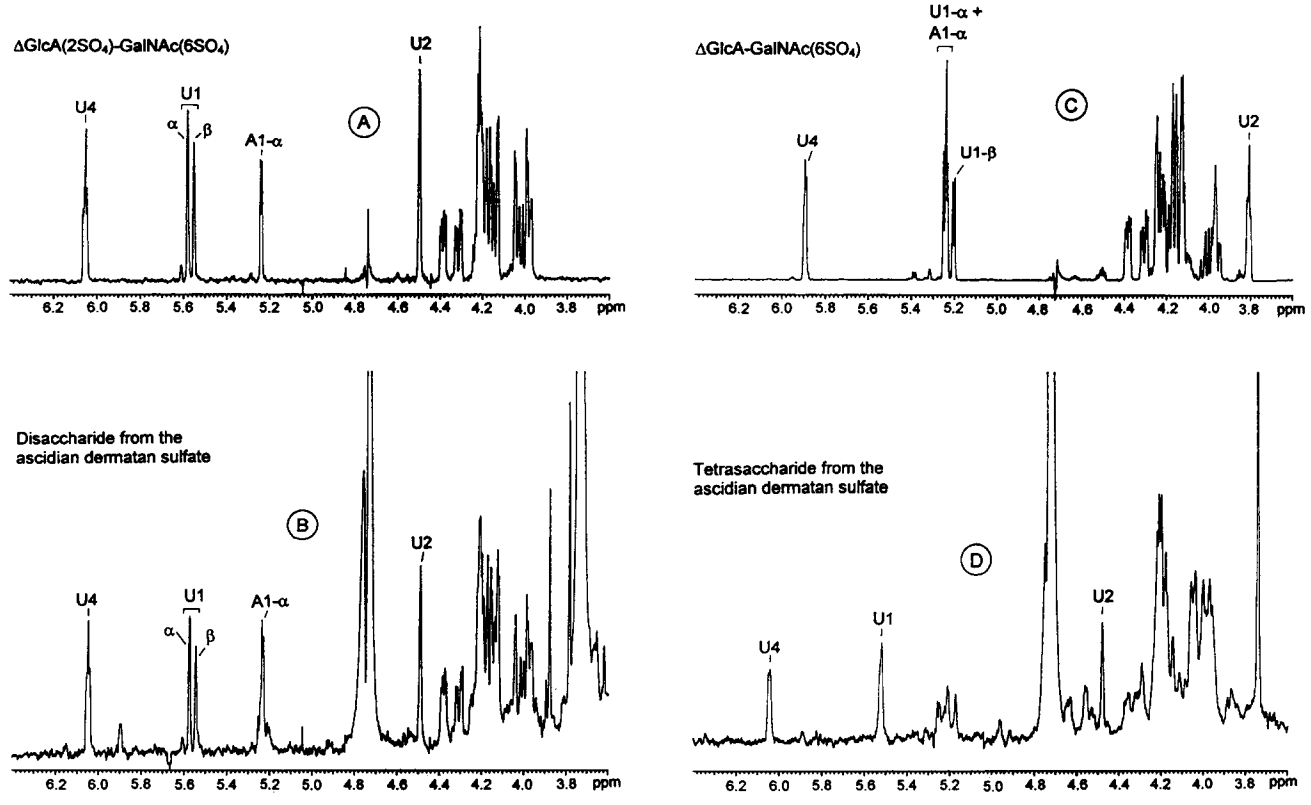


Fig. 2. ^{1}H NMR spectra at 500 MHz and 30 °C in D_2O of the standard disaccharides ΔGlcA(2SO$_4$)–GalNAc(6SO$_4$) (A) and ΔGlcA–GalNAc(6SO$_4$) (C), and of the disaccharide (B) and tetrasaccharide (D) fractions formed by chondroitin ABC lyase digestion of the ascidian dermatan sulfate (see Fig. 1). Signals designated by U refer to those produced by α-L-*threo*-hex-4-enopyranosyluronic acid, whereas those of *N*-acetyl-D-galactosamine are labeled A; α, α-anomer; and β, β-anomer.

acid at 4.48 ppm suggests that this residue is sulfated at the 2-position. Insufficient sample was available for 2D NMR spectroscopy, so assignments for the *N*-acetyl–galactosamine residue are not given; in the tetrasaccharide, *N*-acetyl–galactosamine is present both as a reducing terminal and as an internal residue, thus complicating the spectrum.

Signals at 5.25 ppm, 5.21 ppm, and 5.17 ppm were tentatively assigned to H1 of *N*-acetyl-α-D-galactosamine residues and to H1 of an internal, 2-*O*-sulfated α-L-iduronate residue (by comparison with values determined for internal IdoA residues in mammalian dermatan sulfate) [10]. A smaller signal at 4.96 ppm may be due to H1 of unsulfated iduronate [10]. The relative intensities of these signals indicate that most of the internal residues in the tetrasaccharide are

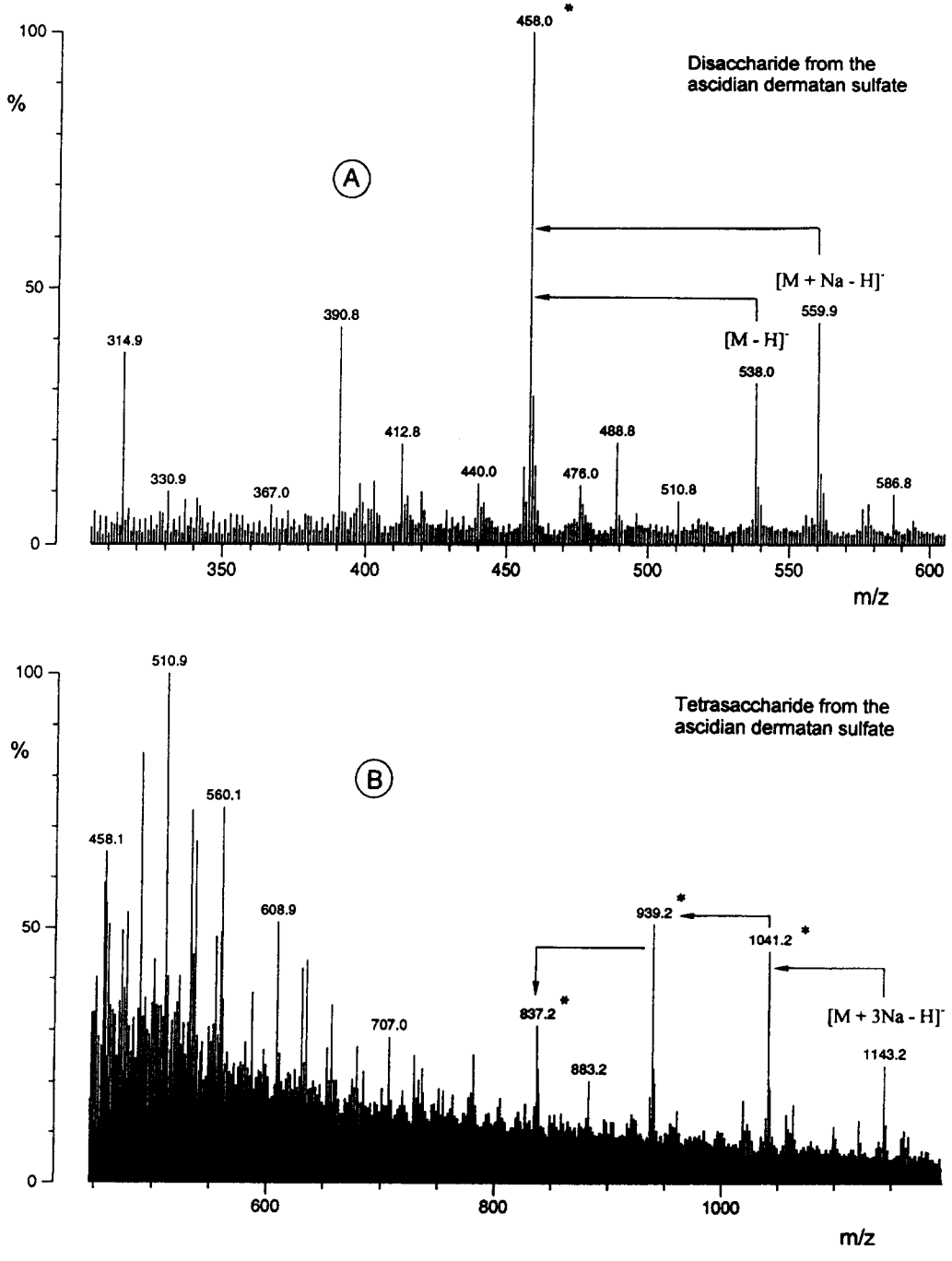


Fig. 3. Fast atom bombardment mass spectra of the disaccharide (A) and the tetrasaccharide (B) products of chondroitin ABC lyase digestion of ascidian dermatan sulfate. The signals marked by asterisks in both spectra result from the loss of sulfite from the molecular ions.

2-*O*-sulfated. The structure implied by these results is illustrated in **4**, and is consistent with that proposed for the repeating disaccharide of ascidian dermatan sulfate [8].

These compounds were further characterized by negative ion fast-atom bombardment mass spectrometry. A deprotonated molecule was observed at *m/z* 457.9 in the spectrum of the disaccharide ΔGlcA–GalNAc(6SO_4), which compares with a calculated value of 458.06 (empirical formula $C_{14}O_{14}$ $H_{16}NS$; **5**). Collision induced fragmentation of the deprotonated molecule resulted in a daughter ion spectrum characterized by an abundant decarboxylated fragment at *m/z* 414 and two weak glycosidic cleavage ions at 282 (Z_1) and 300 (Y_1) [11]. Loss of sulfate from the deprotonated molecule was not observed in either the conventional FAB spectrum or the collision spectrum.

Both the standard ΔGlcA(2SO_4)–GalNAc(6SO_4) disaccharide, and the disaccharide from enzymic digestion of ascidian dermatan sulfate (Fig. 3A), displayed deprotonated molecules at *m/z* 538 and *m/z* 560, consistent with $C_{14}O_{17}NS_2H_{18}$ (calculated value = 538.02) and $C_{14}O_{17}NS_2H_{17}Na$ (calculated value = 560.0), respectively. Facile loss of sulfite from the molecular ions is responsible for the abundant fragment at *m/z* 458. These data are is consistent with structure **3**, but data do not define the positions of sulfation.

A FAB–MS spectrum of the tetrasaccharide product of chondroitin ABC lyase digestion of ascidian dermatan sulfate (Fig. 3B) showed a deprotonated molecule at *m/z* 1143.1, consistent with the empirical formula $C_{28}O_{34}N_2S_4H_{38}Na_3$ (calculated value = 1142.99). Sequential losses of sodium sulfite (with hydrogen replacement) from this species resulted in the fragments at *m/z* 1041.2, 939.2 and 837.2. These data are consistent with structure **4** in which the internal iduronate residue is sulfated, so that the tetrasaccharide carries a total of four sulfates.

The mass spectrometric behavior of these compounds was consistent with that previously reported for glycosaminoglycan-derived oligosaccharides [12–15].

Overall, spectroscopic analysis of the tetra and disaccharides formed by chondroitin ABC lyase supported the proposed structure for the ascidian dermatan sulfate. This glycosaminoglycan has a distinctive structure, composed of repetitive disaccharide units of 6-*O*-sulfo-2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-α-L-idopyranosyluronic acid)-β-D-galactose.

2. Experimental

Isolation and purification of the ascidian dermatan sulfate.—Sulfated polysaccharides were extracted from the body of the ascidian *A. nigra* by papain digestion [5,7]. Dermatan sulfate was purified on a DEAE-cellulose column, developed by a linear gradient of NaCl, as described previously [8]. The purity of the dermatan sulfate preparation was confirmed by agarose gel electrophoresis, digestion with chondroitin AC and ABC lyases and by ^1H and ^{13}C NMR spectroscopy, as described previously [8].

Purification of the products formed by digestion of the ascidian dermatan sulfate with chondroitin ABC lyase.—Purified ascidian dermatan sulfate (~ 10 mg) was incubated with 1 unit of chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris* (Seikagaku American Inc., Rockville, MD, USA) in 1 mL of 50 mM Tris/HCl buffer (pH 8.0) containing 5 mM EDTA and 15 mM NaOAc. After incubation at 37 °C for 12 h, another 1 unit of enzyme was added to the reaction mixture, and the reaction was continued for an additional 12 h-period. The mixture was applied to a Sephadex G-50 column (88 × 1.5 cm) and eluted with 50 mM pyridine/acetate buffer (pH 6.0) at a flow rate of 6 mL/h. Fractions of 1.5 mL were collected and assayed by metachromasia using 1,9-dimethylmethylene blue [16] and the carbazole reaction [17]. The fractions containing the tetra- and disaccharides were pooled and lyophilized.

NMR spectroscopy.—^1H NMR spectra were recorded at 500 MHz using a Varian Unity 500 spectrometer in the FT mode. The tetra- and disaccharides samples were dissolved in ~ 0.7 mL of 99.8% D_2O (Goss Scientific, Ingatestone, United Kingdom) for NMR spectroscopy. The spectra were recorded at 60 °C, with suppression of the HOD signal by presaturation. Two-dimensional double-quantum filtered COSY spectra were recorded in the phase-sensitive mode using the pulse programs supplied by the manufacturer. All chemical shifts are relative to internal or external trimethylsilylpropionic acid.

Fast atom bombardment mass spectrometry.—Negative ion fast atom bombardment (FAB) mass spectra were obtained on a Kratos MS 80 RFA spectrometer, fitted with an Ion Tech FAB gun supplied with high-purity xenon gas. The matrices used were either triethanolamine or a 1:1 v/v mixture of glycerol with dithiothreitol and dithioerythritol (5:1 w/w). Aq solutions of the sample (1 μL containing

5–10 μg of sample) were mixed with a similar volume of matrix on the probe. The accelerating voltage was 4 kV and the instrument was scanned at 10 s per decade over the range 100–1500. The mass axes were calibrated with a mixture of sodium iodide and dithiothreitol. Collisional activation spectra were obtained by admitting helium into the collision cell in the field free region between the ion source and the electrostatic analyser, so as to attenuate the precursor beam by 50%. Daughter ion spectra were recorded by means of linked scans at a constant B/E ratio.

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq: FNDCT and PADCT), Financiadora de Estudos e Projetos (FINEP), Mizutani Foundation for Glycoscience (to PASM) and International Foundation for Science (to MSGP). We thank Adriana A. Eira for technical assistance.

References

[1] P.A.S. Mourão and A.S. Perlin, *Eur. J. Biochem.*, 166 (1987) 431–436.
[2] P.A.S. Mourão and I.G. Bastos, *Eur. J. Biochem.*, 166 (1987) 639–645.
[3] R.P. Vieira and P.A.S. Mourão, *J. Biol. Chem.*, 263 (1988) 18176–18183.
[4] M.S.G. Pavão, R.M. Albano, A.M. Lawson, and P.A.S. Mourão, *J. Biol. Chem.*, 264 (1989) 9972–9979.
[5] R.P. Vieira, B. Mulloy, and P.A.S. Mourão, *J. Biol. Chem.*, 266 (1991) 13530–13536.
[6] J.A. Santos, B. Mulloy, and P.A.S. Mourão, *Eur. J. Biochem.*, 204 (1992) 669–677.
[7] B. Mulloy, A.C. Ribeiro, A.P. Alves, R.P. Vieira, and P.A.S. Mourão, *J. Biol. Chem.*, 269 (1994) 22113–22123.
[8] M.S.G. Pavão, P.A.S. Mourão, B. Mulloy, and D.M. Tollefsen, *J. Biol. Chem.*, 270 (1995) 31027–31036.
[9] S. Yamada, K. Yoshida, M. Sugiura, and K. Sugahara, *J. Biochem.*, 112 (1992) 440–447.
[10] V. Bossennec, M. Petitou, and B. Perly, *Biochem. J.*, 267 (1990) 625–630.
[11] B. Domon and C.E. Costello, *Glycoconjugate J.*, 5 (1988) 397–409.
[12] V.N. Reinhold, S.A. Carr, B.N. Green, M. Petitou, J. Choay, and P. Sinay, *Carbohydr. Res.*, 161 (1987) 305–313.
[13] L.M. Mallis, H.M. Whang, D. Loganathan, and R.J. Linhardt, *Anal. Chem.*, 61 (1989) 1453–1458.
[14] T. Li, S. Okuda, T. Hirano, and M. Ohashi, *Glycoconjugate J.*, 11 (1994) 123–132.
[15] T. Li, M. Kubota, S. Okuda, T. Hirano, and M. Ohashi, *Glycoconjugate J.*, 12 (1995) 162–172.
[16] R.W. Farndale, D.J. Buttle, and A.J. Barret, *Biochim. Biophys. Acta*, 883 (1986) 173–177.
[17] T. Bitter and H.M. Muir, *Anal. Biochem.*, 4 (1962) 330–334.