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Carbohydrate Research 334 (2001) 39–47

CARBOHYDRATE RESEARCH

Isolation and NMR characterization of rosacelose, a novel sulfated polysaccharide from the sponge *Mixylla rosacea*

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Received 25 January 2001; accepted 14 May 2001

Abstract

Rosacelose, a new anti-HIV polysaccharide composed of glucose and fucose sulfate, has been isolated from an aqueous extract of the marine sponge *Mixylla rosacea*. Extensive use of ^{1}H and ^{13}C multidimensional NMR spectroscopy, combined with chemical analysis were used to establish a linear polysaccharide structure composed mainly of 4,6-disulfated 3-*O*-glycosylated α-D-glucopyranosyl and 2,4-disulfated 3-*O*-glycosylated α-L-fucopyranosyl residues (in a 3:1 molar ratio).

Keywords: *Mixylla rosacea*; Rosacelose; Sulfated polysaccharide; 2D-NMR; HIV; Fucoidan; Glucan; Marine sponge

1. Introduction

The role of sulfated polysaccharides in biological systems has attracted the attention of researchers in the last few decades. These compounds have been shown to be involved in several cellular processes, such as molecular recognition, cell development and differentiation, and cell–cell interaction.[1] Furthermore, a wide number of sulfated polysaccharides and other polyanionic substances are potent in vitro inhibitors of various viruses.[2–4] Sulfated dextran,[5] polysulfated pentosan,[6] galactan,[7] fucoidan[8] and carragenan[9,10] interfere with herpes simplex (HSV), vesicular stomatitis (VSV), type 2 poliovirus and replication of HIV-1 in vivo. Fucoidans, in particular, have also proved to have an anti-tumor activity.[11] The activity of these polysaccharides is strictly related to the presence of polyanionic charges. Nevertheless, the length of the sugar backbone and its structure can also affect the antiviral activity.[12–16]

In the marine environment, sulfated polysaccharides are common constituents of algae[17–19] and have also been isolated from tunicates.[20–24] Only few examples of a complete or partial identification of the structure of the sulfated units in sponge polysaccharides have been reported in the literature.[25,26] The sulfated polysaccharides play an important biological role in the sponges and, in particular, some sulfated polysaccharides (like glycosaminoglycans) have proved to have a specific role in sponge-cell aggregation.[1] These polysaccharides bind specific receptors and it is probable that the sugar rings act to spatially orientate the sulfates in a configuration that is

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0008-6215/01/$ - see front matter
PII: S0008-6215(01)00141-0

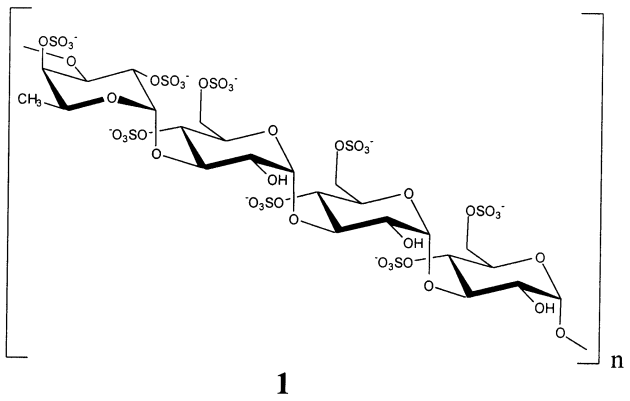

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Fig. 1. Structure of the preponderant repeating unit of the rosacelose from *M. rosacea* sp.

recognized by the receptors and it is this orientation of the sulfates that determines the specificity of the binding. Furthermore, the sulfate groups rather than the carbon ring of the sugars appear to play a key role in the binding. It is therefore important to determine the structure of the sulfated polysaccharides and their pattern of sulfation to clearly understand the biological mechanism of interaction in the marine sponges.

In this paper we report the results of the isolation and chemical characterization of a novel sulfated polysaccharide from the marine sponge *Mixylla rosacea* collected along the fiords of Galicia, Spain. This polysaccharide has been structurally characterized, as containing a linear arrangement of 4,6-disulfated 3-*O*-glycosylated α-D-glucopyranosyl (Fig. 1) and 2,4-disulfated 3-*O*-glycosylated α-L-fucopyranosyl residues in a 3:1 average molar ratio, by chemical degradation and extensive use of 2D NMR techniques (DQF-COSY,[27] HSQC,[28] HMBC,[29] TOCSY,[30] HSQC-TOCSY,[31] 2Q,[32] NOESY[33]).

2. Results and discussion

A polysaccharide fraction with high-molecular weight, containing a small amount of proteic impurities, was isolated from the aqueous extract of the sponge *M. rosacea* by ultrafiltration followed by precipitation from the concentrated aqueous solution with ethanol. A subsequent purification step, performed by gel-filtration on a column of Bio-gel A-1.5 m, gave a main polysaccharide fraction in the void volume, indicating that the molecular weight of its components was over 1.500 kDa. This fraction was in turn further purified by ion-exchange chromatography on a column of DEAE-cellulose (Fig. 2).

Fraction **A** was shown to contain proteic impurities while fraction **B** was totally composed of pure rosacelose (**1**). ^{1}H and 2D-COSY NMR spectra of the distinct sub-fractions of the chromatographic peak **B** were acquired to identify fraction **B** as a single component. The monosaccharide analysis of rosacelose (**1**) indicated that the polysaccharide contained D-glucose and L-fucose residues in a 3:1 molar ratio. The absolute configurations were determined, after hydrolysis, by HPLC chromatography with a chiral detector using convenient standards, while the anomeric configurations were obtained by

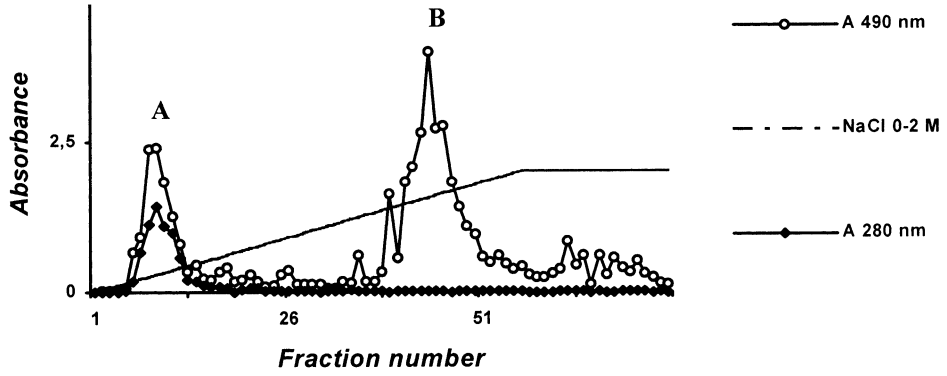

Fig. 2. Anion-exchange chromatography (DEAE-cellulose) of the sulfated polysaccharide from *M. rosacea* sp. The sulfated polysaccharidic fraction was applied on the column equilibrated with Tris–HCl 0.1 M pH 7.0 and eluted with a linear gradient of 0–2 M NaCl. Fractions were assayed by the phenol–H_2SO_4 reaction to estimate the sugar content (A 490 nm) and by UV-test (A 280 nm) to estimate the proteic one.

Table 1
^{1}H and ^{13}C chemical shift data for native and desulfated polysaccharide (rosacelose) and for α-methylglucoside and α-methylfucoside

	^{1}H chemical shifts (ppm)				^{13}C chemical shifts (ppm)			
	Rosacelose	Desulfated	α-D-Glc*p*-*O*-Me	α-L-Fuc*p*-*O*-Me	Rosacelose	Desulfated	α-D-Glc*p*-*O*-Me	α-L-Fuc*p*-*O*-Me
Glc								
1	5.31	5.31	5.10		99.3	99.5	100.0	
2	3.71	3.60	3.56		71.5	70.6	72.2	
3	4.06	3.83	3.72		79.3	80.2	74.1	
4	4.38	3.60	3.42		77.2	70.0	70.6	
5	4.32	3.97	3.77		68.8	71.7	72.5	
6	4.2, 4.37	3.7, 3.77	3.77, 3.87		67.6	60.5	61.6	
Fuc								
1	5.35	5.34		5.10	97.1	99.5		100.5
2	4.53	3.66		3.69	73.3	71.6		69.0
3	4.77	3.88		3.90	73.2	79.3		70.6
4	4.88	3.97		3.79	80.2	71.2		72.9
5	4.54	4.19		4.1–4.9	67.8	67.3		67.5
6	1.26	1.15		1.23	16.2	16.0		16.5

comparing ^{13}C anomeric chemical shifts with standard α and β methylglucoside and methylfucoside.

The sulfate content was determined as Na_2SO_4 by ion-exchange chromatography, after acid hydrolysis. The experimental value, 65% (Na_2SO_4–sulfated polysaccharide) of the dry weight of the polysaccharide, is in agreement with the hypothesis that, on average, every monosaccharide residue of this polysaccharide contained two sulfate groups and a minor portion of the glucose and fucose residues are characterized by a lower level of sulfation. In fact, the theoretical value for a polysaccharide composed of glucose and fucose in a 3:1 molar ratio, in which every monosaccharide residue contain two sulfate groups is 78% Na_2SO_4–sulfated polysaccharide.

The determination of proton and carbon chemical shifts (Table 1) of **1** from ^{1}H (Fig. 3) and ^{13}C NMR spectra was complicated by a general broadening and by a severe overlapping of the resonances. Moreover, several minor peaks were observable due to a small portion of fucose and glucose residues characterized by a different pattern of sulfation. However, the complete characterization of the spin systems and the identification of the glycosidation and sulfation positions were achieved through the use of 2D NMR techniques. Analysis of 2D-TOCSY spectra, usually applied in the determination of proton spin systems of oligo and polysaccharides, was complicated by a signal overcrowding in the region 3.5–4.5 ppm and by an overlap of the anomeric protons of fucose and glucose (5.35 and 5.31 ppm, respectively). Moreover, a broadening of the signals in the ^{1}H and ^{13}C spectra can be attributed to a random distribution of the disulfated glucose and fucose units of the polysaccharide.

In the HSQC-TOCSY spectrum (Fig. 4), the information about the proton–proton connectivities are filtered by the ^{13}C chemical shifts relative to the carbons connected to the protons from which the magnetization is generated. The better dispersion of the proton

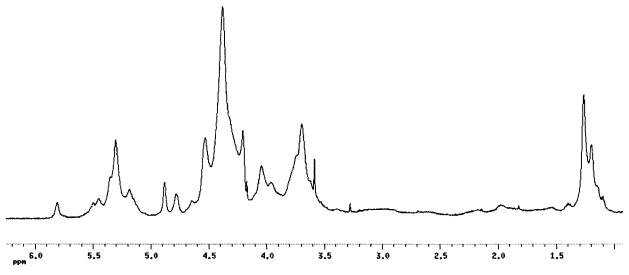

Fig. 3. ^{1}H spectrum (600 MHz) of the sulfated polysaccharide (**1**) was recorded for solution in D_2O at 70 °C.

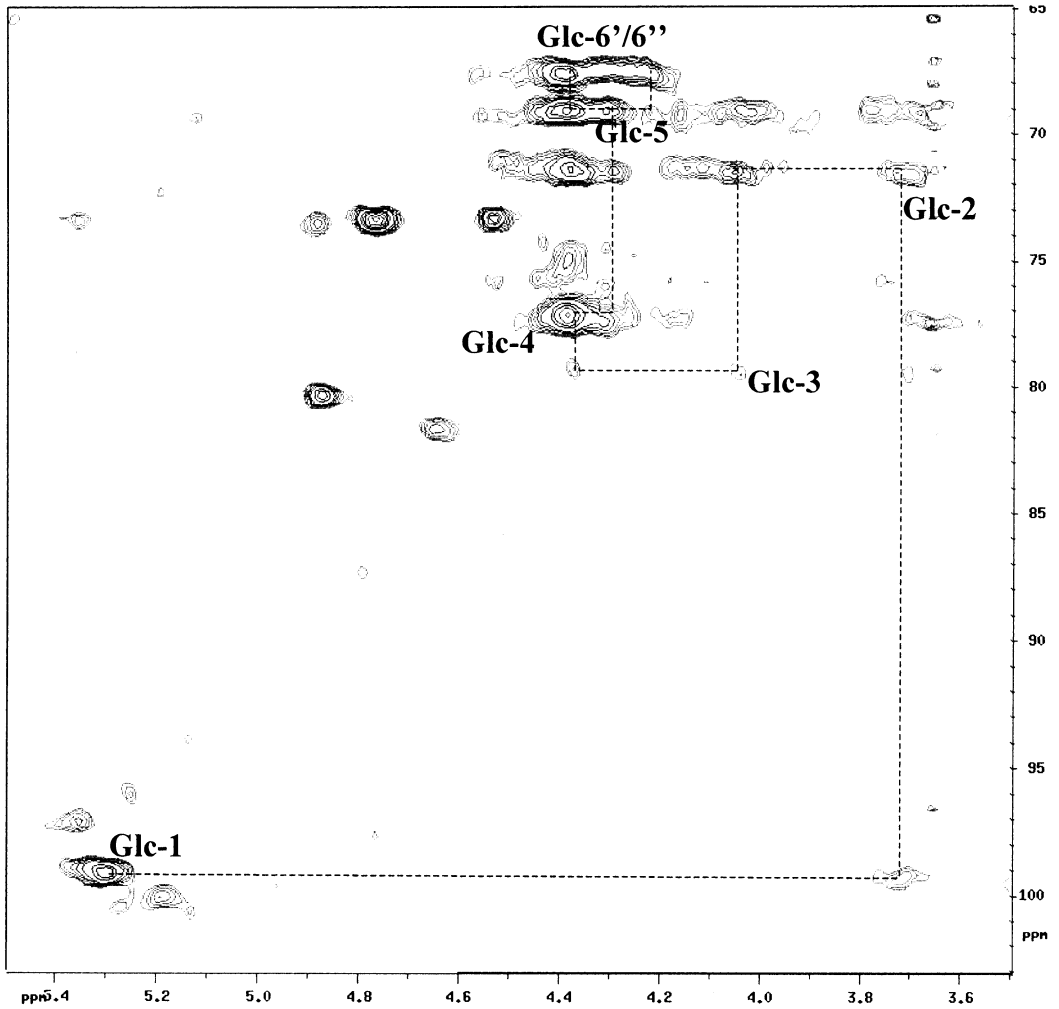

Fig. 4. Expansion of the HSQC-TOCSY spectrum (D_2O, 70 °C) of rosacelose (**1**). Dashed lines describe the correlations between the ^1H and ^{13}C resonances of glucose.

spin systems in the wider region of ^{13}C frequencies and the lack of diagonal peaks, present in homonuclear 2D-TOCSY spectrum, allowed us to obtain the ^1H and ^{13}C resonances of both the glucose and the fucose residues. In particular, the distinct anomeric carbons of the glucose and fucose units (99.3 and 97.1 ppm, directly correlated with 5.31 and 5.35 ppm) were especially suitable as starting points to obtain the proton and carbon chemical shifts of the two sugar units. It was possible to follow, starting from the anomeric resonances, the entire path of correlations relative to the ^1H and ^{13}C signals of each sugar spin system (dashed pathway in Fig. 4).

Analysis of the 2D-COSY (not shown) was used for the sequence specific assignment of the proton resonances obtained in the HSQC-TOCSY. To circumvent some of the inherent problems of the conventional COSY and DQF-COSY experiments, such as the presence of the diagonal peaks and the self-cancellation of antiphase components in broad signals, a 2Q experiment[32] (Fig. 5) was acquired in order to confirm and determine some crucial connectivities, i.e., H-4/H-5 (4.38 and 4.32 ppm) in the glucose and H-3/H-4 (4.77 and 4.88 ppm) in the fucose. In the 2Q spectrum, which has been used for the assignment of medium and large size proteins, it is possible to observe correlation peaks occurring at the sum of the chemical shifts of two coupled spins in ω_1 and at the chemical shift of each spin in ω_2 (Fig. 5) and that, for this reason, are spread over a much larger dimension with regard to conventional COSY, DQF-COSY and TOCSY techniques.

After the sequence-specific proton assignment for both the residues, the complete determination of the carbon resonances (Table 1) was pursued by the analysis of the HSQC spectrum. The chemical shift values in Table 1 have been compared with reported chemical shifts for standard α and β methylglucopyranosides and α and β methylfucopyranosides, indicating that rosacelose is basically constituted of 4,6-disulfated 3-*O*-glycosylated α-D-glucopyranosyl and 2,4-disulfated 3-*O*-glycosylated α-L-fucopyranosyl residues (in a 3:1 average molar ratio). In this linear structure, disulfated fucose units could occur randomly between the disulfated glucose residues, and **1** can be considered a predominant repeating unit proposed on the average ratio of the two residues. Indeed, lowfield chemical shift values for C-3, C-4 and C-6 (79.3, 77.2 and 67.6 ppm, respectively) and for H-3, H-4 and H-6 (4.06, 4.38 and 4.20/4.37 ppm) in the glucose residues suggest glycosylation or sulfation in these positions.[34–37] Analysis of homonuclear and heteronuclear 2D spectra of the desulfated derivative of **1** showed major upfield shifts for C-4 and C-6 (70.1 and 60.5 ppm) and for H-4 and H-6 (3.42 and 3.77/3.87 ppm), while a lowfield chemical shift was still observed for C-3 (80.2 ppm). These data established, for the glucose, the positions of the two sulfate groups at C-4 and C-6 and of the glycosidic linkage at C-3. In a similar way, we established the sulfation at C-2 and C-4 and the glycosylation at C-3, for the fucose residue. In fact, NMR data of **1** showed lowfield chemical shifts for C-2, C-3 and C-4 (73.3, 73.2 and 80.2 ppm) and for the corresponding protons H-2, H-3 and H-4 (4.53, 4.77 and 4.88 ppm). NMR analysis of desulfated derivative of **1** revealed upfield shifts for the resonances

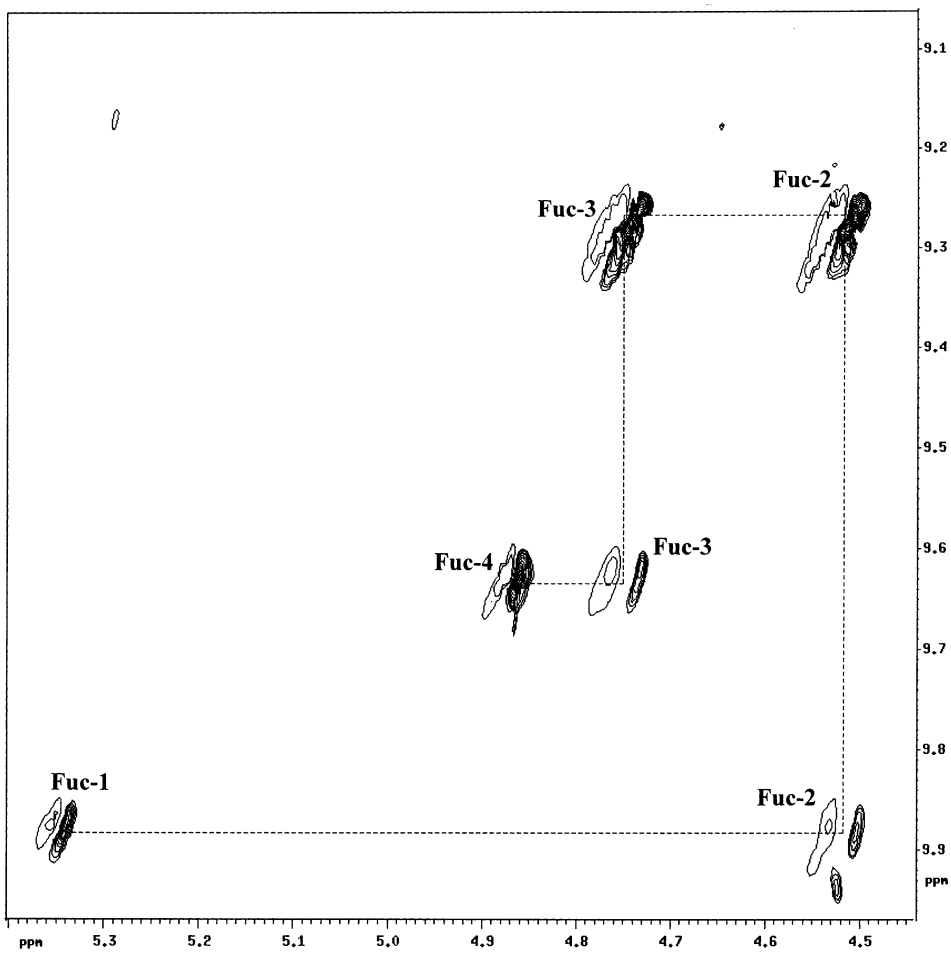

Fig. 5. Expansion of the 2Q spectrum (D_2O, 70 °C) of rosacelose (**1**). Dashed lines describe the correlations between the ^1H resonances of fucose.

C-2 and C-4 (71.6 and 71.2 ppm) and lowfield shift for C-3 (79.3 ppm). This clearly located glycosylation at C-3 and sulfation at C-2 and C-4, as supported also by upfield shifts for H-2 and H-4 (3.66 and 3.97 ppm). We observe also that H-3 is particularly downfield shifted in the sulfated polysaccharide, possibly due the presence of the two sulfations in the adjacent position 2-O and 4-O.

Further evidence for the (1→3)-glycosydic linkage was given by 2D-NOESY and HMBC spectra. Particularly diagnostic were two interresidue correlations observed in the NOESY spectrum; the first one between H-1 and H-3 of glucose and the second one between H-1 of fucose and H-3 of glucose. Indeed, intraresidue contact between H-1 and H-3 of glucose (1,3 equatorial–axial) can be ruled out on the basis of their 1,3 eq–ax relationship, indicating that the first correlation, observed in the set of NOESY spectra acquired with different mixing times (100, 50 and 25 ms) arises from spatial vicinity between a glucose anomeric proton and the H-3 of an adjacent glucose residue and cannot be interpreted as a spin diffusion effect.

Conclusive support to the (1→3)-glycosydic linkage was given by methylation analysis of the desulfated fraction, that revealed the expected 2,4,6-tri-*O*-methylglucose and 2,4-di-*O*-methylfucose and confirmed that the polysaccharide contains 3-linked glucose and 3-linked fucose residues.

The antiviral activity of rosacelose, predictable for its high degree of sulfation, has been confirmed by means of an anti HIV assay, in vitro, showing an IC_{50} of 5 μg/mL. Besides the antiviral activity, more recently, a biological role of sulfated polysaccharides regarding the structural integrity of the marine sponges has been reported, suggesting a similarity to the function played by proteoglycans in the connective tissues of vertebrates.[25]

In conclusion, in this paper we report the isolation, the purification, the structural characterization and the anti-HIV activity of rosacelose, a sulfated polysaccharide from the marine sponge *M. rosacea*. The knowledge of its structure can be relevant to understanding the molecular basis of the biological functions of sulfated polysaccharides in marine sponges, such as the antiviral and the aggregant activities.

3. Experimental

Biological material, extraction and purification.—The marine sponge *M. rosacea* was collected in the fiords of Galicia (Santiago de Compostela-Spain). The aqueous extract was lyophilized to give 9.0 g of a crude fraction. This fraction was dissolved in water (350 mL) in an ultrasonic bath and the insoluble portion was removed by filtration. The resulting viscous brown solution was filtered through an Amicon XM300 ultrafiltration membrane (300,000 MWCO) in an Amicon stirred cell (200 mL). The retentate, a brown, very viscous solution, was thoroughly washed with water and lyophilized to give 460 mg of a brown solid. The filtrate from the XM300 membrane filtered through membranes sequentially with decreasing MWCO (100.000–500) did not show any polysaccharidic material.

The polysaccharide extracted from the sponge was purified by gel-filtration chromatography on Bio-gel A1.5 m. In a typical chromatographic run, a portion of the crude polysaccharide (63.5 mg) was dissolved in the buffer $(NH_4)HCO_3$ (50mM, 3 mL), applied on the column (h: 108 cm, d: 1.5 cm) that had been equilibrated with the same buffer and eluted with the buffer 50 mM $(NH_4)HCO_3$ at a flow rate of 28 mL/h. Fractions (1.8 mL) were assayed by the phenol–H_2SO_4[38] reaction (490 nm) to estimate the sugar content and by UV-test at 280 nm to estimate the proteic one (UV-Beckman DU 640 spectrophotometer). The fractions containing the polysaccharide were pooled, desalted through an Amicon XM300 ultrafiltration membrane (300,000 MWCO) in an Amicon stirred cell (50 mL) and lyophilized. The sulfated polysaccharide was further purified by an anion-exchange chromatography on DEAE-cellulose (Matrex-500). In a typical run, the polysaccharidic fraction (60 mg) was dissolved in the buffer Tris–HCl (0.1 M, pH 7.0, 0.5 mL), applied on the column (h: 15 cm, d: 1 cm) equilibrated with Tris–HCl (0.1 M, pH 7.0) and eluted with a linear gradient of 0–2 M NaCl at a

flow rate of 0.4 mL/min. Fractions (1.1 mL) were assayed by the phenol–H_2SO_4 reaction to estimate the sugar content and by UV-test to estimate the proteic one. The fractions containing the polysaccharide were pooled, desalted through an Amicon XM300 ultrafiltration membrane (300,000 MWCO) in an Amicon stirred cell (50 mL) and lyophilized.

Chemical analysis.—The purified polysaccharide (6.3 mg) was hydrolyzed with 2 M TFA (300 μL) for 1 h at 120 °C. The TFA was removed with a dried stream of N_2. Monosaccharide analysis was obtained by HPLC using an APS2-450 × 4 mm I.D. column, and eluent 80:20 CH_3CN–water (v/v) with a flow rate of 1 mL/min at 35 °C and detector ELSD (a model Sedex-55 evaporative light scattering detector-S.E.D.E.R.E., France); temp: 80 °C; air flow: 6 L/min. The absolute configurations were determined by the same technique but with a different detector, a model OR-990 chiroptical rotation-monitor (Jasco Europe, Italy). Analytical liquid chromatographs were performed on a Waters chromatography equipped with Rheodyne model 7725i 20 μL injector and two model M510 solvent-delivery systems. Chromatographic data were collected and processed using 2010 CHROMATOGRAPHY MANAGER software (Waters Chromatography).

Methylation.—The desulfated fraction was methylated as follows. To a solution of the dry polysaccharide (1 mg, 60 °C overnight on P_2O_5) in methyl sulfoxide (1 mL) were added powdered NaOH (1 h) and methyl iodide (300 μL, overnight). The mixture was stirred at rt. The methylated polysaccharide was extracted with 1:1 $CHCl_3$–water three times and the organic phase was dried with N_2 stream. The methylated polysaccharide was hydrolyzed with 2 M TFA (200 μL) for 1 h at 120 °C, the solution was dried with an N_2 stream and the products were dissolved in EtOH (200 μL) and reduced with 2 mg $NaBD_4$ (overnight at rt). Excess $NaBD_4$ was destroyed by dropwise addition of HOAc acid and then the mixture was treated with 250 μL of a solution of 1:9 HOAC–MeOH and dried with an N_2 stream. This procedure was repeated four times. Successively, the mixture was repeatedly treated three times with MeOH. The resulting alditols were acetylated with Ac_2O (100 μL)–pyridine (200 μL) at 100 °C for 30 min, cooled and dried with an N_2 stream (with a few drops of isopropanol). The alditol acetates were extracted with $CHCl_3$–water (three times) and analyzed by GC–MS on a capillary column RTX-5 (Restek, 30 m × 0.25 i.d.) with a Hewlett–Packard 5890 instrument. The temperature program was: 90 °C for 1 min, 140 °C at 25 °C/min, 200 °C at 5 °C/min, 280 °C at 10 °C/min, and at 280 °C for 10 min. The carrier gas was He at flow rate 1 mL/min.

Sulfate determination.—The purified polysaccharide (3 mg) was hydrolyzed with 2 M HCl (4 mL) for 16 h at 100 °C. The acid solution was diluted at 10 mL with water and chromatographed using dried Na_2SO_4 as standard (pump system, GPM; column, IonPac AS4A-SC (4 × 25 mm) Dionex; precolumn, IonPac AS4A-SC (Guard 10–32) Dionex; eluent, 0.191 g Na_2CO_3 + 0.143 g $NaHCO_3$ in 1000 mL water; flow, 2 mL/min; suppressor, Anion Micromembrane Suppressor AMMS-II Dionex; loop, 25 μL Dionex; detector, PAD (conductibility)). Before the sulfate analysis of the polysaccharide, a calibration curve was achieved with Na_2SO_4 that was at 100 °C over night and then dissolved in water (1.042 mg and 2.780 mg in 100 mL).

Desulfation.—The polysaccharide (60 mg) was dissolved in water (HPLC, 15 mL), stirred for 20 min with 1.6 g of Dowex 50W-X8 resin (H^+ form, 200–400 mesh) and then the resin removed and the filtrate neutralized with pyridine and lyophilized. The pyridine salt of the rosacelose was dissolved in 15 mL of 9:1 Me_2SO–MeOH and heated for 3 h at 80 °C.[39] The reaction mixture was diluted with 300 mL of water and the desulfated derivative (15 mg) was recovered by ultrafiltration on an Amicon XM300 membrane and lyophilized.

NMR spectroscopy.—The spectra were recorded using a Bruker DRX600 spectrometer at $T = 343$ K. The polysaccharidic fractions were dissolved in 0.5 mL of 99.996% D_2O. The signal of HOD was used as standard (4.71 ppm). All spectra 2D-NMR were acquired in the phase-sensitive mode with the transmitter set at the solvent resonance and TPPI (time proportional phase increment) used to achieve frequency discrimination in

the ω_1 dimension.[40] The standard pulse sequence and phase cycling were used for 2Q, DQF-COSY, HSQC, HMBC and NOESY spectra. A total of 128 scans/t_1 value were acquired for the 2Q ($t_{1max} = 32$ ms) spectrum, 1 scan/t_1 value for the DQF-COSY ($t_{1max} = 30.4$ ms), 32 scans/t_1 value for the HSQC ($t_{1max} = 14.2$ ms) and 512 scans/t_1 value ($t_{1max} = 4.2$ ms) for the HMBC spectra. The TOCSY spectra were acquired using a DIPSI-2 sequence for spin locking with $t_{mix} = 70$ ms, 256 scans/t_1 value and $t_{1max} = 56.1$ ms. The HSQC-TOCSY spectra were acquired with 256 scans/t_1 value and $t_{1max} = 7.7$ ms. The NOESY spectra of D_2O solutions were recorded with saturation of the residual HOD resonance during the preparation and mixing periods and Hahn-echo to improve the quality of the baseline.[41,42] The NOESY spectra were acquired with mixing times of 25, 50 and 100 ms, 256 scans/t_1 value and $t_{1max} = 37.3$ ms. The NMR data were processed on a Silicon Graphic Indigo2 workstation using UXNMR software.

Anti HIV-activity.—Rosacelose (**1**) has been evaluated for its potential antiviral activity against HIV-1 at Campus Universitaire de Luminy, Marseille, France by Dr J.C. Chermann, by meaning the efficiency of the substrate to inhibit syncytia formation after HIV infection of MT4 cell line, as described previously.[43,44] The experimental IC_{50} value was 5 μg/mL.

Acknowledgements

The authors thank the Professor Eugenio Uriarte (Universidade de Santiago de Compostela, Departamento de Quimica Organica, Spain) for the collection of biological material; Dr J.C. Chermann (INSERM Unit U322 Campus Universitaire de Luminy, Marseille, France) for anti HIV assays; Professor Francesco Gasparrini (Università di Roma 'La Sapienza' Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Roma, Italia) and Dr Ernesto Vanini (Farmaceutici DAMOR, Napoli, Italia) for their kind support in the monosaccharide and sulfation analysis. Financial support was provided by MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica) PRIN 97, CNR (Consiglio Nazionale delle Ricerche) and Università di Salerno, Italy.

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