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Sulfated and pyruvylated disaccharide alditols obtained from a red seaweed galactan: ESIMS and NMR approaches

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Abstract

The water-soluble acid agaran isolated from *Acanthophora spicifera* (Rhodophyta) was submitted to alkaline treatment for the complete cyclization of α-L-Gal*p* 6-sulfate to 3,6-An-α-L-Gal*p* units. The modified agaran was then partially depolymerized using partial reductive hydrolysis. The resulting oligosaccharide mixture was fractionated by adsorption and ion-exchange chromatography. Fractions were purified by gel-filtration chromatography and studied by ESIMS and NMR spectroscopy, including 1D ^{1}H, ^{13}C, DEPT and 2D ^{1}H, ^{1}H COSY, TOCSY and ^{1}H, ^{13}C HMQC procedures. The following neutral, pyruvylated, sulfated and sulfated/pyruvylated disaccharide alditols were obtained: β-D-Gal*p*-(1 → 4)-3,6-An-L-GalOH; 4,6-*O*-(1-carboxyethylidene)-β-D-Gal*p*-(1 → 4)-3,6-An-L-GalOH; β-D-Gal*p* 2-sulfate-(1 → 4)-3,6-An-L-GalOH and 4,6-*O*-(1-carboxyethylidene)-β-D-Galp 2-sulfate-(1 → 4)-3,6-An-L-GalOH. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The main polysaccharides found in red seaweeds are linear sulfated galactans with alternating 3-*O*-linked β-D-Gal*p* and 4-*O*-linked α-Gal*p* units and in many cases, the latter residues appear as 3,6-AnGal*p* units.[1,2] These galactans differ in the enantiomeric configuration of the α-linked units, which are L in agarans and D in carrageenans.[3] Many of these polysaccharides show complex patterns of substitution due to the presence of sulfate and/or methyl groups at different positions, and/or pyruvic acid acetals at O-4,6 of the β-D-galactopyranosyl units.[2–5]

The production of oligosaccharides from polysaccharides is an essential tool to determine the complex pattern of substitution shown by several of these galactans, as well as an aid in NMR assignments. For this purpose, partial methanolysis,[6,7] partial enzymic hydrolysis[8–11] and partial acid hydrolysis[12,13] have been used on galactans that contain 3,6-AnGal*p* units (acid-labile sugar). Partial acid hydrolysis, carried out in the presence of borane·4-methylmorpholine complex (4-MMB, an acid-stable reducing agent), has been used as an efficient approach to obtain oligosaccharides from these galactans. This procedure (partial reductive hydrolysis) allows a selective splitting of 3,6-anhydrogalactopyranosidic bonds, yielding oligosaccharides with 3,6-AnGalOH residues as terminal units (oligosaccharide alditols), while sulfate groups are substantially retained.[14–16]

Sulfated oligosaccharides and their modified derivatives have been shown to have important biological activities,[17] such as antiviral[18] and antiangiogenic effects.[19] Oligosaccharides containing pyruvic acid acetals are components of several bacterial antigenic factors, and are therefore potential targets for inmunological and synthetic vaccine studies.[20,21]

In spite of potential applications of oligosaccharides, in general, chromatographic techniques have been used to obtain only a few milligrams (<5 mg) of these compounds.[22] We now describe a preparative procedure for the isolation, fractionation and purification of pyru-

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vylated and sulfated disaccharide alditols from acidic agaran from *Acanthophora spicifera*.[23] A study based on 1D and 2D NMR spectroscopy and ESI-mass spectrometry of isolated, reduced oligosaccharides was then performed for structural elucidation and NMR assignments.

2. Experimental

Extraction of polysaccharides.—Specimens of *A. spicifera* (Ceramiales, Rhodophyta) were collected on the shore of Bombinhas, Santa Catarina State, on the southern coast of Brazil. Algae samples were air dried, milled and then extracted three times with water (1.5 g%) at 80 °C with mechanical stirring for 8 h. The three successive extracts were treated with ethanol (3 vol), and the precipitated polysaccharides were dissolved in water, reprecipitated with ethanol (3 vol), centrifuged, redissolved in water, dialyzed and freeze-dried yielding three fractions, AWI (14.0% yield), AWII (12.3%) and AWII (2.1%).[24]

Chemical analyses.—Total carbohydrates were determined by the phenol–sulfuric acid method using galactose as standard,[25] sulfates by the method of Dodgson and Price,[26] pyruvic acid by that of Koepsell and Sharpe[27] and protein by the procedure of Lowry.[28] The monosaccharide composition of polysaccharides was determined by the reductive hydrolysis method,[29] while reduced oligosaccharides were hydrolyzed with 45% formic acid at 100 °C for 15 h, followed by reduction with $NaBH_4$ overnight.[30] For both hydrolyzates, the acetylated alditols were analyzed by GC and GC–MS. GC analyses were carried with a HP-5890 gas chromatograph equipped with a flame-ionization detector (FID) using a fused silica capillary column (30 m × 0.25 mm i.d.) WCOT-coated with a 0.20 μm film of DB-225. Chromatography was run isothermically at 210 °C (injector and FID at 250 °C) with nitrogen as carrier gas at a flow rate of 1 mL/min. GC–MS analyses were performed using a Varian 3300 chromatograph coupled to a Finnigan Mat ITD 800 spectrometer. Injections were carried out at 50 °C, followed by a gradient of 40 °C/min up to 230 °C (then hold) with helium at 1 mL/min as carrier gas.

Alkaline treatment of polysaccharide.—The alkaline treatment was carried out as previously described.[31] Briefly, polysaccharide fraction AWI was reduced (10% $NaBH_4$, 5 °C, 8 h) and heated in M NaOH at 80 °C. Samples were taken at intervals, and the 3,6-anhydrogalactose content was determined by the resorcinol method.[32] Heating was continued until the 3,6-anhydrogalactose content remained constant or at least after 4 h.

Partial reductive hydrolysis.—The alkali-treated AWI polysaccharide (2.0 g) was dissolved in water (150 mL), the solution was heated to 60 °C and 13.5 g of acid-stable reducing agent (97% borane·4-methylmorpholine complex, Sigma-Aldrich) was then added, followed by 2 M CF_3COOH (50 mL). The mixture was incubated at 65 °C for 8 h, and the acid was then evaporated with the aid of added water.[14] The residue was chromatographed on a 1:1 charcoal–Celite[33] column (13 × 10.5 cm, 1 L/h) first eluting with water (4 L, for elimination of monosaccharides and residual reducing agent) and then eluting with 50% aq EtOH (6 L), which was evaporated, yielding an oligosaccharide mixture (55%).

Fractionation and purification of oligosaccharide alditols.—The oligosaccharide mixture was resuspended in water (5 mL) and then applied to a DEAE-Sephadex A-25 (Cl^-) column (5 × 22 cm, 40 mL/h) for anion-exchange chromatography. Successive elutions were carried out with water (Fraction A), 0.1 M NaCl (Fractions B and C) and 0.2 M NaCl (Fraction D). Salts were removed by gel-filtration chromatography on a BioGel P-2 column (1.5 × 100 cm, 20 mL/h), eluted with water. Fraction D was desalted using a 1:1 charcoal–Celite column, first eluting with water (for elimination of salt) and then with 50% aq EtOH to recover the oligosaccharide alditols. Elution profiles of adsorption, anion-exchange and gel-filtration chromatographies were determined by the phenol–sulfuric method for sugar detection[25] and the silver nitrate test for chloride.[34]

Nuclear magnetic resonance spectroscopy.—For NMR analysis, a portion of the dried samples (2–8 mg) was exchanged with deuterium by repeated evaporations in D_2O and then dissolved in 99.99% D_2O (0.4 mL). NMR spectra were determined at 30 °C (40 °C for Fractions C and D) with a Bruker Advance DRX 400 spectrometer equipped with a 5-mm inverse probe. 1D ^1H, ^{13}C, DEPT and 2D ^1H, ^1H COSY, TOCSY and ^1H, ^{13}C HMQC spectra were obtained at base frequency of 100.63 MHz for ^{13}C and 400 MHz for ^1H nuclei. Chemical shifts are reported relative to an internal acetone standard at 2.225 and 30.20 ppm for ^1H and ^{13}C NMR spectra, respectively. For ^{13}C NMR spectroscopy, the pulse sequence was with a delay (*D1*) and acquisition time (*AQ*) of 0.10 s and 0.59 s, respectively, using a spectral width of 31.0 kHz, 32 K data points, 90° pulse (7.1 μs), and 2000–10000 scans; Fraction B was also analyzed with a longer pulse sequence, $D1 = 0.60$ s and $AQ = 0.90$ s. ^{13}C NMR DEPT spectra were obtained at $\theta z = 135°$ where $\underline{C}H$ and $\underline{C}H_3$ signals appear in a positive phase with $\underline{C}H_2$ in a negative phase. For ^1H NMR spectra, all fractions was submitted to $D1 = 1.0$ s and $AQ = 2.0$ s, using a spectral width of 8.0 kHz, 32 K data points, 90° pulse (7.1 μs), and 32 scans. The $DO\underline{H}$ signal was suppressed by low-power irradiation during relaxation.

Electrospray-ionization mass spectrometry.—For ES-IMS analysis, samples diluted with water (1 mg/mL) were directly injected into an acetonitrile flow (Shimadzu LC pump) in a Micromass Quattro LC spectrometer. Analyses incorporated single quadrupole scanning, searching for a mass range of m/z 100–2000. The tuning parameters in the positive-ion mode were: capillary (4.99 kV), cone (40 V), and ion energy (1.3 V), which gave rise principally to pseudomolecular ions for neutral, charged (sodium salt) and double-charged disaccharides (disodium salt) in the sodium form $[M+Na]^+$. In the negative-ion mode, the parameters were 4.00 kV, 100 V, and 1.6 V, for capillary, cone and ion energy, respectively, giving rise to negative ions for neutral $[M-H]^-$ and charged $[M-Na]^-$ disaccharide alditols. For the doubly charged oligosaccharide, the negative ions $[M-2Na+H]^-$ and $[M-Na]^-$ were observed.

3. Results and discussion

Polysaccharide extraction and alkaline treatment.—The monosaccharide composition of the principal polysaccharide extract AWI is shown in Table 1. Galactose is the main constituent unit, with less 3,6-anhydrogalactose (3,6-AnGal). Xylose and 6-*O*-methyl galactose are also present. The polysaccharides are sulfated and pyruvylated, with ca. two sulfate groups for every three sugar residues and one pyruvate group for every 16 sugar residues.

Polysaccharide AWI was submitted to alkaline treatment where Gal*p* 6-sulfate units, with a free hydroxyl group at C-3, are transformed into 3,6-AnGal, via a selective desulfation at C-6.[35] After this treatment, 3,6-AnGal content increased (+ 24.7 mol%) with a concomitant decrease of galactose content (− 24.2 mol%) (Table 1). These results show that the native polysaccharide have a high content of 4-*O*-linked galactosyl 6-sulfate units.

Comparison of the ^{13}C NMR spectrum of the alkali-treated polysaccharide with that of the native one, showed an increase in signal intensity at 100.7 ppm (C-1 of β-D-Gal*p* 2-sulfate linked to 3,6-AnGal*p*) and 98.1 ppm (C-1 of 3,6-An-α-L-Gal*p*). Significant decreases in the intensity of C-1 signals at 101.4-100.9 ppm (β-D-Gal*p* 2-sulfate linked to α-L-Gal*p* 6-sulfate) were also observed (data not shown), in agreement with the reaction processed during the alkaline treatment, which rendered a repetitive backbone.[23]

Partial reductive hydrolysis.—The alkali-treated polysaccharide was submitted to partial reductive hydrolysis,[14] utilizing the susceptibility of 3,6-anhydrogalactosidic linkages to selective reductive hydrolysis. The ^{13}C NMR spectrum of hydrolysis products showed the presence of three main signals at 63.6, 53.0 and 43.0 ppm, corresponding to 4-methylmorpholine (4-MM sub-product of 4-MMB, the reducing agent extensively used in this technique). The first two were identified by DEPT spectroscopy as being due to methylene carbons, the signal at 63.6 ppm to C-2 and C-6, and that at 53.0 ppm to C-3 and C-5 of the morpholine ring. The last signal corresponds to the methyl carbon N-linked to the same structure. The large amount of 4-MM present in the hydrolyzate could make difficult the subsequent chromatographic steps, so we introduced adsorption chromatography on a charcoal–Celite column to eliminate this contaminant, principally because it enables the use of large amounts of sample. The charcoal–Celite column was first eluted with water (to eliminate 4-MM) and then with 50% aqueous ethanol for complete elution of the oligosaccharides. ^{13}C NMR analysis of the latter eluate showed a pronounced decrease of 4-MM signals relative to those of the oligosaccharides (Fig. 1). Although charcoal–Celite adsorption chromatography is commonly used for separation of monosaccharides and salts from neutral and uronic acid-containing oligosaccharides,[33] it has not been reported for pyruvylated/sulfated and/or 3,6-anhydrogalactitol-containing oligosaccharides. It is an efficient method for the isolation of acidic oligosaccharide alditols from hydrolysis byproducts.

The ^{13}C NMR spectrum of the purified hydrolyzate contained an intense signal at 62.8 ppm (C-1 of 3,6-An-L-GalOH), whereas no anomeric signal corresponding to 3,6-An-α-L-Gal (98–96 ppm) was detected, indicating, as expected, that the 3,6-anhydrogalactosidic linkages were selectively cleaved during partial reductive hydrolysis.[14,16] The upfield signal at 25.1 ppm, attributed to the methyl group of a pyruvic acid acetal,[36]

Table 1
Monosaccharide composition and chemical analyses of AWI (native) and AWI A.T. (alkali-treated) polysaccharides

Fraction	AnGal (mol%)	Xyl (mol%)	6-Me Gal (mol%)	Gal (mol%)	Carbohydrate (%)	Sulfate (%, $NaSO_3$)	Protein (%)	Pyruvic acid (%)
AWI	15.7	6.1	0.7	77.5	62.2	25.5	2.3	2.0
AWI A.T.	40.4	5.5	0.7	53.3	73.6	n.d.[a]	0.8	2.8

[a] n.d. = not determined.

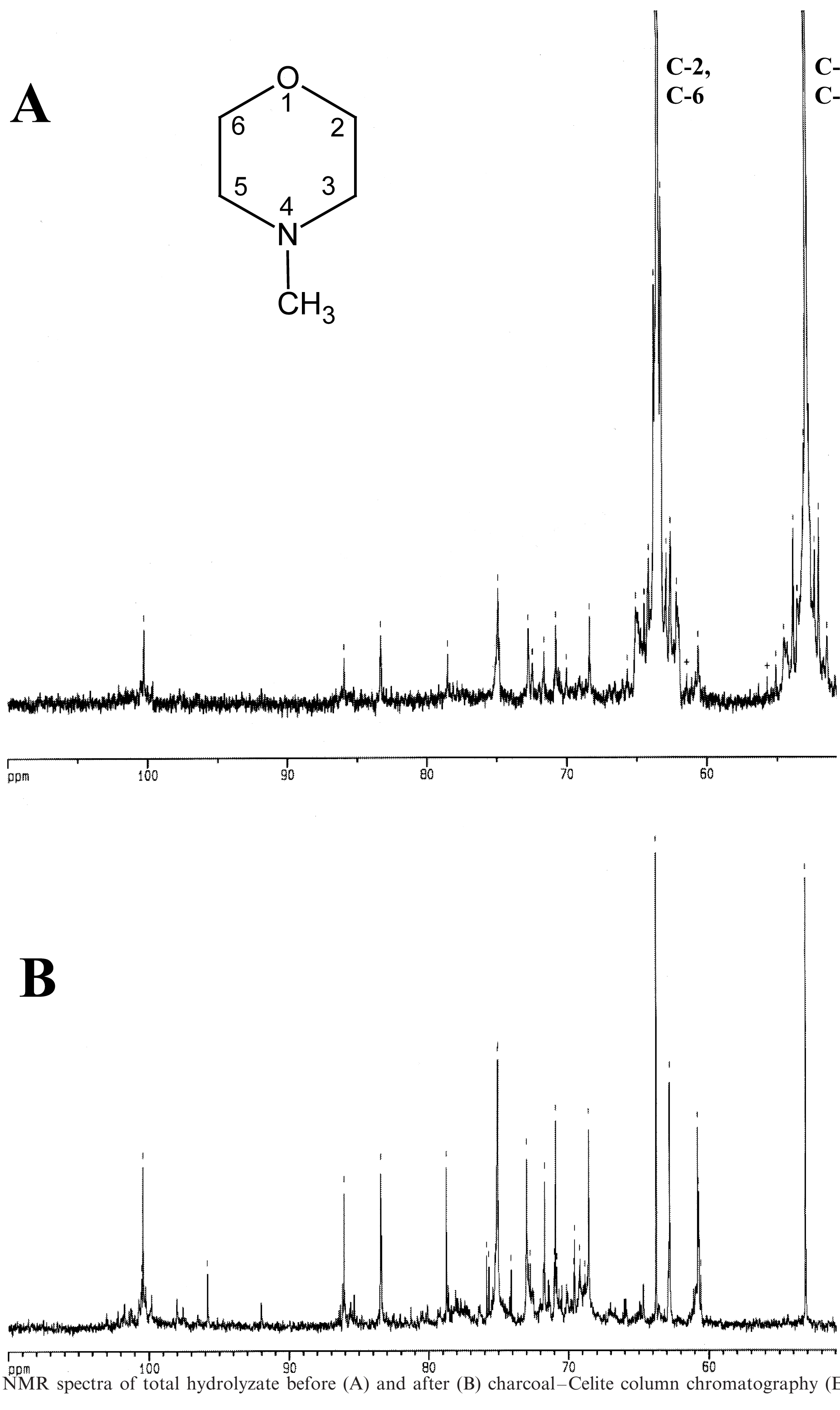

Fig. 1. ^{13}C NMR spectra of total hydrolyzate before (A) and after (B) charcoal–Celite column chromatography (EtOH eluate).

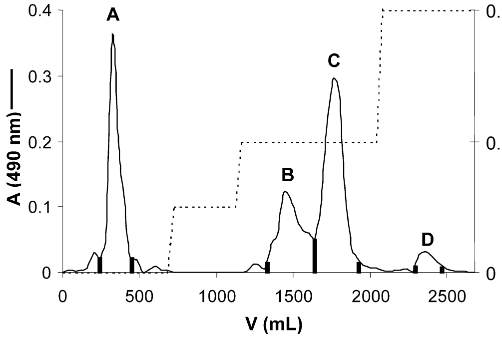

Fig. 2. Elution profile of ion-exchange chromatography on a DEAE-Sephadex A-25 column (5 × 22 cm × 40 mL/h). Elutions were carried out with water (Fraction A), 0.1 M NaCl (Fractions B and C) and 0.2 M NaCl (Fraction D), corresponding to neutral, pyruvylated, sulfated and sulfated pyruvylated oligosaccharide alditols, respectively.

indicates, that in addition to retention of sulfate groups,[14,16] the pyruvyl groups are also retained under hydrolysis conditions.

Fractionation and purification of oligosaccharide alditols.—Oligosaccharides were separated by anion-exchange chromatography on a DEAE-Sephadex A-25 (Cl^-) column, first eluted with water and then with NaCl solutions of increasing concentration. Aqueous elution yields fraction A, 0.1 M NaCl fractions B and C, and 0.2 M NaCl fraction D. On 0.05 M NaCl elution no sugar was detected (Fig. 2).

Fractions A, B and C were applied on to a low-pressure gel-filtration column (BioGel P-2) for desalting. For fraction A, this technique also eliminated residual 4-MM. Fraction D was desalted on a charcoal–Celite column because the low yield of carbohydrates and high content of salt of this sample did not enable the use of gel-filtration chromatography. Desalted fractions yielded oligosaccharide alditols **A** (73.5 mg, 3.7%), **B** (39.8 mg, 2.0%), **C** (110.0 mg, 5.5%) and **D** (2.2 mg, 0.1%).

*Oligosaccharide alditol **A**.*—^{13}C NMR analysis of **A** gave only one signal (102.2 ppm) in the anomeric region, which was assigned to C-1 of β-D-Gal*p*. Signals at 62.7, 72.9 and 60.9 ppm were DEPT inverted, and were attributed to C-1 and C-6 of 3,6-An-L-GalOH (reduced terminal unit) and C-6 of β-D-Gal*p*, respectively.[14,16] GC–MS analysis of **A**, after total hydrolysis, reduction and acetylation showed the presence of 3,6-AnGal and Gal (1:1 molar ratio), in agreement with NMR data. These results, together with its anion-exchange chromatography behavior (water elution), indicate that **A** is a neutral disaccharide alditol.

For complete ^{13}C and ^{1}H NMR assignments, **A** was also submitted to $^{1}H-^{13}C$ HMQC, and $^{1}H-^{1}H$ COSY and TOCSY examination. Considering the previously mentioned ^{13}C NMR assignments, their geminal protons could be assigned from the HMQC spectrum as H-1 (4.56 ppm), H-6 and H-6′ (3.76 ppm) of β-D-Gal*p* unit and H-1, H-1′ (3.65 ppm), H-6 (3.97 ppm) and H-6′ (3.87 ppm) of 3,6-An-L-GalOH. Having located H-1 of β-D-Gal*p*, H-2 (3.51 ppm) and H-3 (3.65 ppm), resonances were assigned from the COSY spectrum. For the reduced unit the approach was different, starting from the assigned H-6 and H-6′, H-5 (4.40 ppm), H-4 (4.33 ppm) and H-3 (3.91 ppm) were located using the same spectrum. The TOCSY experiment confirms the previous assignments and allowed identification of the H-4 (3.94 ppm) signal of β-D-Gal*p*. H-4 – H-5 coupling is very small in β-D-galactopyranoses,[4] so that no H-4/H-5 cross-peak was observed in the COSY and TOCSY spectra. The C-5 signal of the β-D-Gal*p* unit was assigned with the assistance of Me β-D-Gal*p* chemical shifts previously described.[37] These results show that the disaccharide alditol **A** is β-D-Gal*p*-(1 → 4)-3,6-An-L-GalOH (agarobiitol, see Fig. 6(A)). HMQC correlation data were employed again for complete ^{1}H and ^{13}C assignments (Table 2). Usov and Elashvili previously reported a complete assignment of the ^{13}C resonances of agarobiitol, isolated after partial reductive hydrolysis of agarans extracted from two species of the genus *Laurencia*.[14,16] Our ^{13}C NMR assignments agree with those of Usov and Elashvili considering an offset of (– 0.7 ppm), probably due to different calibrations of the spectra. Concerning ^{1}H NMR signals, our assignments are the same as those previously deduced, except for H-5 (4.40 ppm) of 3,6-An-L-GalOH that was assigned to signal at 5.00[14] and 4.00 ppm.[16] The use of 2D NMR techniques allowed us to assign H-5, H-6 and H-6′ of β-D-Gal*p* units that have not been previously identified.

Agarobiitol (M_n 326) was examined by ESIMS in the positive and negative-ion mode. The former gave a pseudomolecular ion (*m*/*z* 349), corresponding to the Na^+ form of agarobiitol $[M + Na]^+$ (Table 3, Fig. 6(A)) The latter gave the molecular ion of the deprotonated molecule (*m*/*z* 325) (Table 3). Usov and Elashvili[14,16] used secondary-ion mass spectrometry (SIMS) to analyze agarobiitol, also obtaining the pseudomolecular ion $[M + Na]^+$ (*m*/*z* 349).[14,16]

*Oligosaccharide alditol **B**.*—To characterize this fraction, we used the same approach as for fraction **A**, namely a combination of 1D and 2D NMR techniques. The ^{13}C NMR spectrum of **B** contained only one signal in the anomeric region at 102.0 ppm, assigned to β-D-Gal*p*. The signal at 62.9 ppm (DEPT inverted) was attributed to C-1 of 3,6-An-L-GalOH. These results, together with the chromatographic behavior of **B** (eluted with 0.1 M NaCl solution), indicate that it is an acidic disaccharide alditol. Its ^{13}C NMR spectrum contained a downfield signal at 25.0 ppm, also present in the native polysaccharide (data not shown), which was assigned to $\underline{C}H_3$ of a pyruvic acid acetal. The HMQC spectrum showed that this signal correlated with an intense resonance at 1.46 ppm, assigned to the methyl protons of the CH_3 group (Fig. 3). Garegg et al.[36] have shown, for 4,6-pyruvyl acetals on D-Gal*p*, that the signal of the methyl group, when the acetal carbon is in the R configuration, is characteristically at 24.5–25 ppm, while in the S form it is at 15–18 ppm; showing that pyruvyl group, in **B**, is in the R form.

As the acetal and carboxyl carbons of the pyruvate substituent were not detected in the ^{13}C NMR spectrum, another experiment was performed with a longer relaxation delay time. In this spectrum it was possible to identify the carboxyl carbon at 175.9 ppm, but not the acetal carbon signal, probably due to overlapping of signals with the C-1 of β-D-Gal*p*. Besides, the relatively long T_1 relaxation time of the acetal carbon makes difficult its detection by ^{13}C NMR spectroscopy.[5,38] For red algae galactans, the acetal carbon signal has only been reported in the ^{13}C NMR spectra of pyruvylated carrageenans isolated from *Callophycus* spp.[5] and oligosaccharides generated by enzymic hy-

Table 2
^{1}H and ^{13}C NMR assignments of **A**, **B**, **C** and **D** disaccharide alditols (values in ppm)

Unit	Carbon or proton	**A**		**B**		**C**		**D**	
		^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
β-D-Gal*p*	1	4.56	102.2	4.58	102.0	4.72	100.5	4.70	100.0
	2	3.51	70.6	3.64	70.2	4.25	78.8	4.26	78.7
	3	3.65	72.5	3.72	71.6	3.86	71.9	3.90	71.1
	4	3.94	68.5	4.17	70.8	4.00	68.6	4.23	70.4
	5	3.70	75.2	3.62	66.2	3.73	75.1	3.58	66.3
	6	3.76	60.9	4.06	64.8	3.79	60.8	4.02	64.9
	6′	3.76	–	3.94	–	3.79	–	3.91	–
Pyruvic acid acetal	CH_3	–	–	1.46	25.0	–	–	1.47	25.1
	COO^-	–	–	–	175.9	–	–	–	175.9
3,6-An-L-GalOH	1	3.65	62.7	3.71	62.9	3.67	62.9	3.71	63.0
	1′	3.65	–	3.71	–	3.67	–	3.71	–
	2	3.93	70.9	3.96	71.0	3.93	71.0	4.23	70.9
	3	3.91	83.5	3.96	83.5	3.93	83.5	3.96	83.6
	4	4.33	85.4	4.31	85.6	4.30	86.2	4.32	85.8
	5	4.40	75.2	4.40	75.2	4.44	75.2	4.43	75.2
	6	3.97	72.9	4.01	72.9	4.01	73.0	4.06	73.1
	6′	3.87	–	3.87	–	3.86	–	3.92	–

Table 3
Molecular and daughter ions formed in ESIMS spectra of disaccharide alditols **A**, **B**, **C** and **D** (X, Y and Z are fragments indicated in Fig. 6)

Ion mode	Ion	*m*/*z*			
		A	**B**	**C**	**D**
Positive	$[M+Na]^+$	349	–	–	–
	$[M+Na]^+$	–	441	451	–
	$[M+Na]^+$	–	–	–	543
Negative	$[M-H]^-$	325	–	–	–
	$[M-Na]^-$	–	395	405	–
	$[M-2Na+H]^-$	–	–	–	475
	$[M-Na]^-$	–	–	–	497
	$[X-Na]^-$	–	87	–	–
	$[HSO_4]^-$	–	–	97	–
	$[Y-Na-H]^-$	–	–	241	–
	$[M-2Na-H-SO_4]^-$	–	–	–	377
	$[Z-2Na]^{2-}$	–	–	–	156

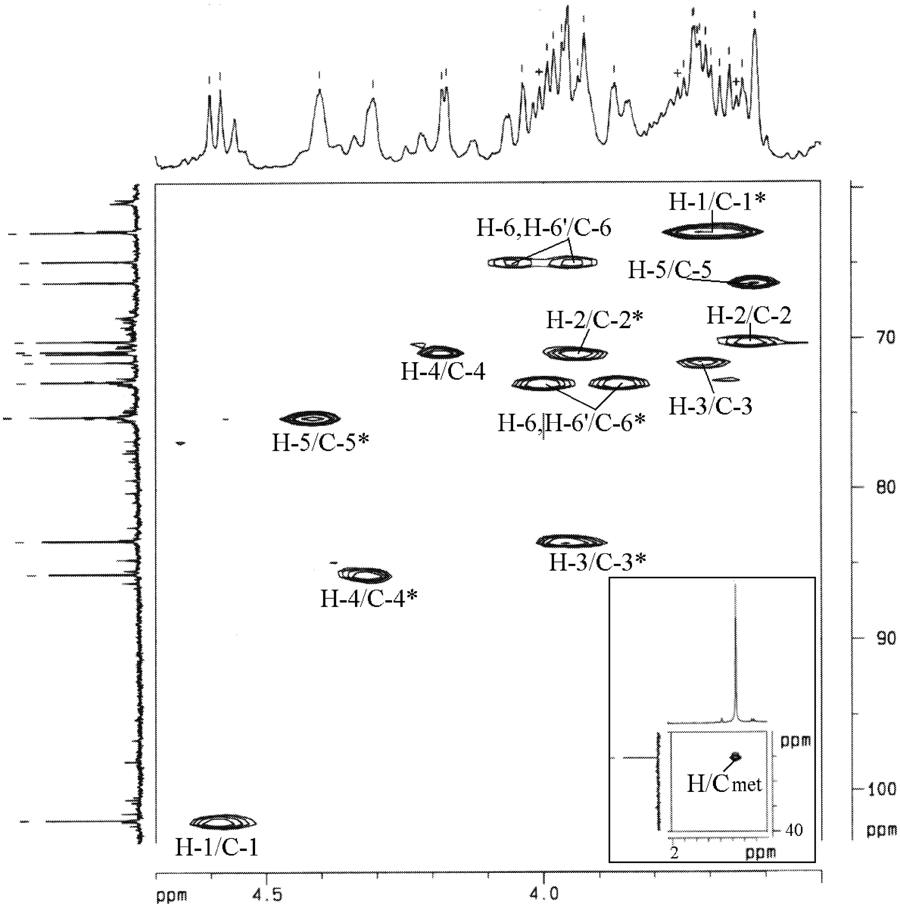

Fig. 3. 2D NMR HMQC spectrum of pyruvylated disaccharide alditol **B**. Inset: CH_3 of pyruvyl group cross-peak. Carbons with an asterisk correspond to the 3,6-An-L-GalOH unit.

drolysis from the agaran extracted from *Gracilaria compressa*[39] at 101.6 and 101.7 ppm, respectively.

The resonance at 66.2 ppm was assigned to C-5 of the pyruvylated β-D-Gal*p* units. Its position, relative to that of the corresponding signal for agarobiitol (75.2 ppm), was in agreement with the 9 ppm upfield shift of the C-5 signal of pyruvylated agarose. This intense shift is due to the β-effect of the two vicinal substituted carbons. The resonance at 64.8 ppm (DEPT inverted) was assigned to C-6 of the pyruvylated unit. The 3.9 ppm downfield shift of this signal, relative to that of agarobiitol, was consistent with the α-effect observed in pyruvylated agarose.[39,40]

With the help of 2D NMR experiments, complete ^1H and ^{13}C assignments of **B** were achieved (Fig. 3, Table 2), indicating that the structure of **B** was, 4,6-*O*-(1-carboxyethylidene)-β-D-Gal*p*-(1→4)-3,6-An-L-GalOH (pyruvylated agarobiitol, see Fig. 6(B)). NMR chemical shifts (^1H and ^{13}C) of 3,6-An-L-GalOH are nearly the same as those observed for agarobiitol, showing that the presence of the pyruvyl substituent only affects the substituted unit (β-D-Gal*p*).

ESIMS analysis of **B** in the positive-ion mode showed a pseudomolecular ion with m/z 441, attributed to the sodium salt of the pyruvylated agarobiitol (M_n 418) in the Na^+ form $[M+Na]^+$. The structure of **B** was confirmed by ESIMS analysis in the negative-ion mode, which gave the molecular ion (m/z 395) corresponding to $[M-Na]^-$, and an intense daughter ion (m/z 87) arising from pyruvate (Table 3, Fig. 6(B)).

*Oligosaccharide alditol **C***.—The ^1H NMR experiments with **A** and **B** were carried out at 30 °C. However, at this temperature the DO$\underline{H}$ signal (4.70 ppm) overlapped with the H-1 proton signal of **C**, so with this fraction experiments were performed at 40 °C where the DO$\underline{H}$ signal is shifted to 4.55 ppm and no overlapping was observed. ^{13}C NMR assignments showed an intense downfield displacement of the C-2 signal (+8.2 ppm) of the β-D-Gal*p* unit, relative to that of agarobiitol, which is consistent with α-sulfation. Upfield shifts of C-1 (−1.7 ppm) and C-3 (−0.6 ppm) signals of the same unit, when compared with those of agarobiitol, are in agreement with the β-effect of C-2 sulfation (Fig. 4). C-4, C-5 and C-6 assignments of β-D-Gal*p* unit are nearly the same as those of the unsubstituted disaccharide alditol **A**. The same behavior was observed for the resonances of 3,6-An-L-GalOH unit, with the exception of the C-4 signal that was shifted downfield by 0.8 ppm, probably due to sulfation at O-2 of the vicinal unit. For complete ^{13}C and ^{1}H assignments, **C** was also submitted to ^{1}H–^{13}C HMQC (Fig. 4), and ^{1}H–^{1}H COSY and TOCSY experiments (Table 2). These results showed that the structure of **C** was β-D-Gal*p* 2-sulfate-(1 → 4)-3,6-An-L-GalOH (agarobiitol 2-sulfate, see Fig. 6(C)).

Agarobiitol 2-sulfate has been previously obtained by partial reductive hydrolysis of the sulfated agaran isolated from the red seaweed *Laurencia nipponica*.[14] It was characterized by GLC analysis after complete hydrolysis and by ^{1}H and ^{13}C NMR spectroscopy. Our ^{13}C NMR assignments are in good agreement with those of Usov and Elashvili[14] with a constant shift (− 0.6 ppm) nearly the same as observed with agarobiitol. However, ^{1}H NMR assignments are the same as those deduced previously, except for H-2 of 3,6-An-L-GalOH assigned at 4.10 ppm, H-1 of the same unit and H-5, H-6 and H-6′ of β-D-Gal*p*, which have not been previously assigned.

The chemical structure of **C** was also studied by ESIMS. The positive-ion mode showed a pseudomolecular ion with *m*/*z* of 451, corresponding to the sodium salt of a monosulfated disaccharide alditols (M_n 428) in the Na$^+$ form [M + Na]$^+$ (Table 3). Usov and Elashvili[14] also obtained the pseudomolecular ion (*m*/*z* 451) of agarobiitol 2-sulfate by SIMS in the positive-ion mode. ESIMS analysis of **C** in the negative-ion mode gave the molecular ion with *m*/*z* 405 [M − Na]$^-$, accompanied by daughter ions corresponding to HSO_4^- (*m*/*z* 97) and sulfated hexopyranose (*m*/*z* 241) (Table 3, Fig. 6(C)).

Oligosaccharide alditol ***D***.—As with **C**, NMR experiments of **D** were carried out at 40 °C. Its ^{1}H and ^{13}C NMR spectra showed that the main component of this fraction was contaminated by the presence of other oligosaccharides in minor proportions. The presence of these contaminants did not affect NMR elucidation. In the ^{13}C NMR spectrum we observed that the C-1 signal (100.0 ppm) of the β-D-Gal*p* unit was shifted 0.5 ppm upfield when compared with C-1 of **C** (100.5 ppm), and the C-2 chemical shift (like **C** was + 8.1 ppm relative to agarobiitol) was in accordance with the presence of sulfate substitution. The other resonances of this unit (C-3–C-6) were shifted when compared with agarobiitol 2-sulfate suggesting the presence of another substituent. The most upfield ^{13}C signal (25.1 ppm), which correlates in HMQC with an intense ^{1}H singlet at 1.47 ppm, indicated that this substituent could be pyruvyl (data not shown). This was confirmed with the assignments of C-4 (70.4 ppm), C-5 (66.3 ppm) and C-6 (64.9 ppm) similar to the corresponding ones of the pyruvylated agarobiitol (**B**). C-3 resonance (71.1 ppm) showed a 0.5 and 0.6 ppm upfield shift relative to that of **B** and **C**,

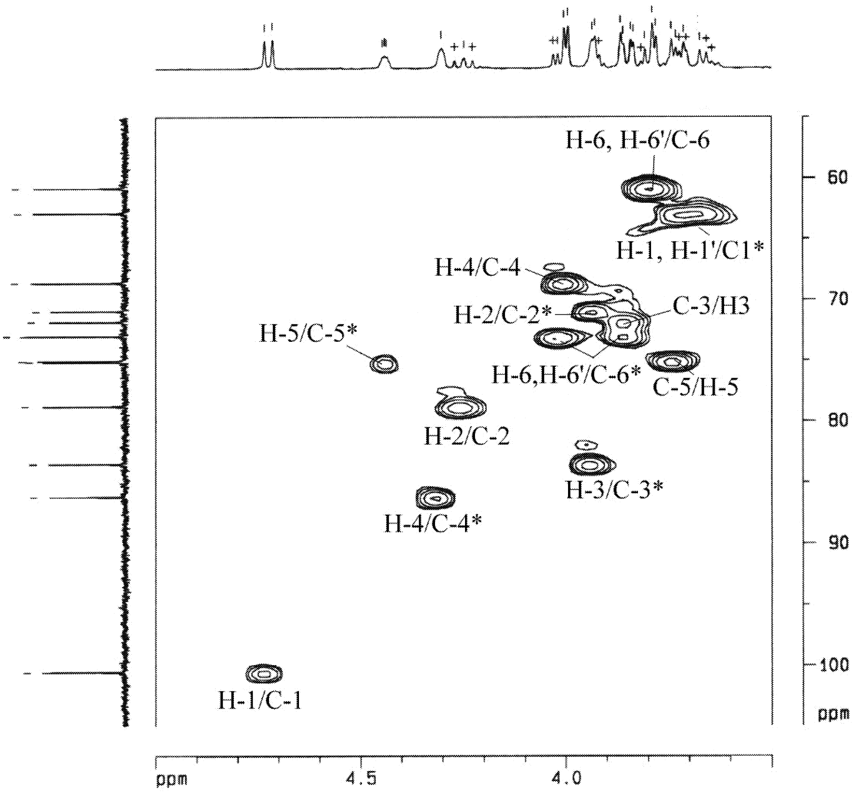

Fig. 4. 2D NMR HMQC spectrum with the complete assignment of sulfated disaccharide alditol **C**. Carbons with an asterisk correspond to the 3,6-An-L-GalOH unit.

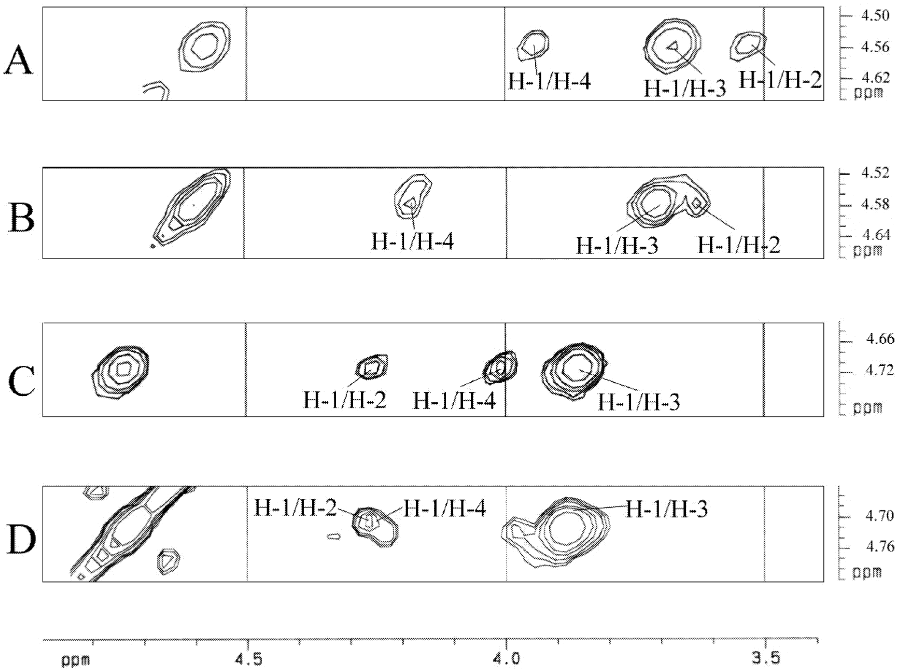

Fig. 5. Sections of 2D NMR TOCSY spectra of disaccharide alditols **A**, **B**, **C** and **D** (H-1 correlations region of β-D-Gal*p* unit).

respectively. This shift is in accordance with the simultaneous β-effects of sulfation (at C-2) and pyruvylation (at C-4) on the vicinal carbon C-3.

The ^{1}H and ^{13}C resonances of 3,6-An-L-GalOH unit of **D** are similar to the ones of agarobiitol (**A**) except for the 0.4 ppm downfield shift of C-4, intermediate between the shift observed for the corresponding signal of pyruvyl agarobiitol (**B** + 0.2 ppm) and agarobiitol 2-sulfate (**C** + 0.8 ppm).

In addition, the TOCSY spectra of **A**, **B** and **C** showed characteristically three correlations for H-1 of the β-D-Gal*p* unit (H-1/H-2, H-1/H3 and H-1/H-4), whereas **D** showed only two connectivities. This difference was due to overlapping of H-2 and H-4 signals as a consequence of the downfield shift of these protons. This overlapping would be expected from the analysis of the TOCSY spectra of **B** and **C** (Fig. 5).

ESIMS analysis of **D** in the positive-ion mode gave a pseudomolecular ion with m/z 543, corresponding to the disodium salt of sulfated and pyruvylated disaccharide alditols ($M_n = 520$) in the Na$^+$ form $[M + Na]^+$. This was confirmed by repeating the analysis in the negative-ion mode, which gave the pseudomolecular ions with m/z 497 and 475 attributed to $[M - Na]^-$ and $[M - 2Na + H]^-$, respectively. Also detected was the pseudomolecular ion with m/z 377, which corresponded to the above-mentioned disaccharide alditol lacking sulfate $[M - 2Na - H - SO_4]^-$ (Table 3, Fig. 6(D)).

The NMR and ESIMS results showed that the main component of **D** is the disaccharide alditol, 4,6-*O*-(1-carboxyethylidene)-β-D-Galp 2-sulfate-(1 → 4)-3,6-An-L-GalOH (pyruvyl agarobiitol 2-sulfate). As far as we know this is the first report of the preparation and characterization of a pyruvylated/sulfated disaccharide alditol from the galactans of red seaweeds.

The agarans biosynthesized by *A. spicifera* are highly sulfated on C-2 of the β-D-Gal*p* units, with some of these residues 4,6-pyruvylated. Only a low proportion of the β-D-units are non-sulfated and pyruvylated. The α-L-units are present as 3,6-anhydrogalactose or its biological precursor, galactopyranose 6-sulfate.[23] The alkaline treatment transformed Gal*p* 6-sulfate into 3,6-AnGal*p*, and in this way simplified the polysaccharide structure and increased the potential points of cleavage during partial hydrolysis. As a consequence of this modification, the principal products of the partial reductive hydrolysis were, as expected, disaccharide alditols.

The presence of agarobiitol 2-sulfate as the principal partial hydrolysis product is in agreement with the main repeating disaccharide structure of the alkali-treated polysaccharide. However, the relative high yield of agarobiitol and pyruvylated agarobiitol was not expected to occur, considering that nearly all the β-D-Gal*p* units of the native polysaccharide were C-2 sulfated. These results indicate that part of the sulfate was hydrolyzed under the partial reductive hydrolysis conditions, increasing the yield of the before mentioned oligosaccharides. The relative acid lability of the sulfate at C-2 of the β-D-Gal*p* units, when compared with those at other substitution positions, was also observed during autohydrolysis of *lambda*- and *theta*-carrageenans.[41] Under these conditions, the sulfate group was removed, whereas the C-2 sulfate groups of the α-units remained stable.

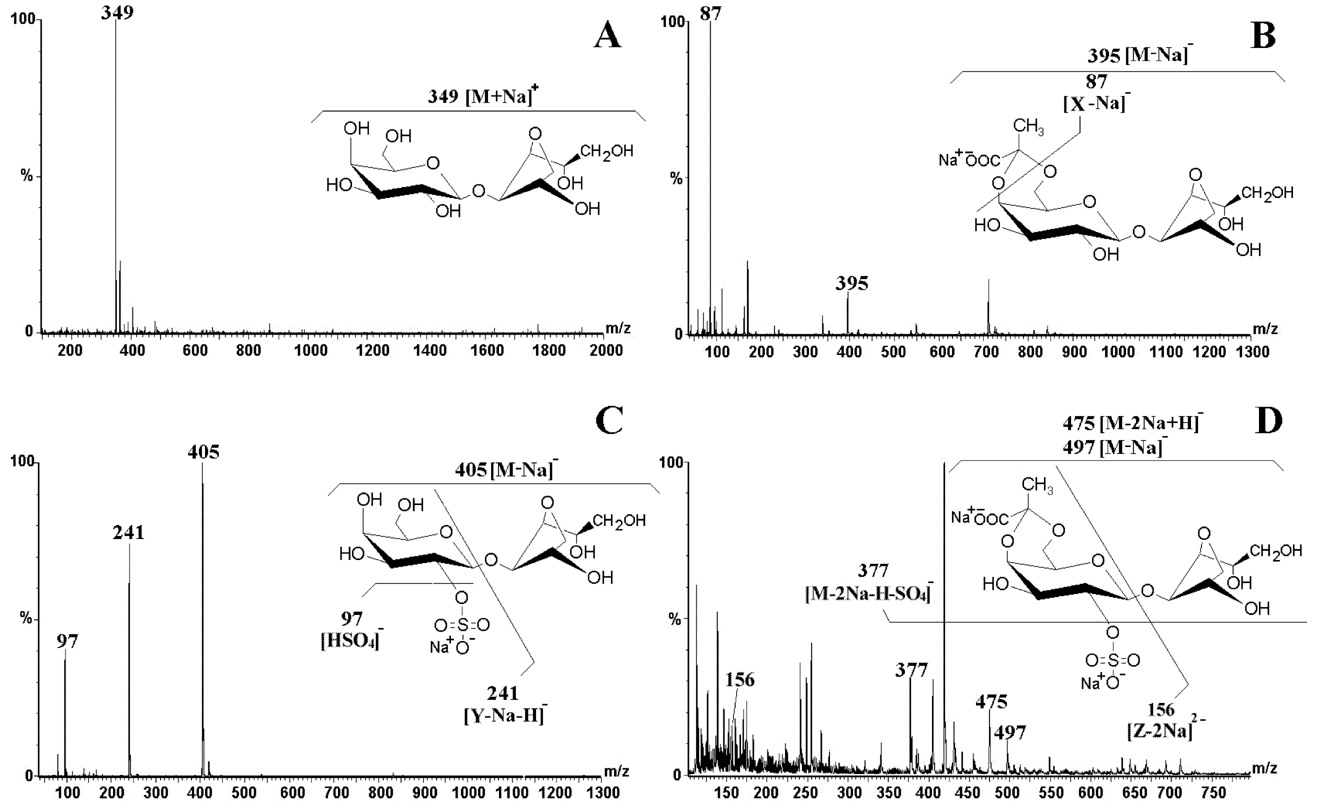

Fig. 6. ESIMS spectra of **A** (M_n 326), **B** (M_n 418 sodium salt), **C** (M_n 428 sodium salt) and **D** (M_n 520 disodium salt).

In conclusion, partial reductive hydrolysis was used to obtain oligosaccharide alditols from the alkali-treated agaran isolated from the red seaweed *A. spicifera*. This method allowed the preparation of neutral, pyruvylated, sulfated and sulfated/pyruvylated disaccharide alditols.

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