ELSEVIER

Carbohydrate Research 307 (1998) 113–124

CARBOHYDRATE RESEARCH

The structure of the exocellular polysaccharide from the cyanobacterium *Cyanospira capsulata*

Domenico Garozzo [a], Giuseppe Impallomeni [a,*], Emanuela Spina [a], Luisa Sturiale [b]

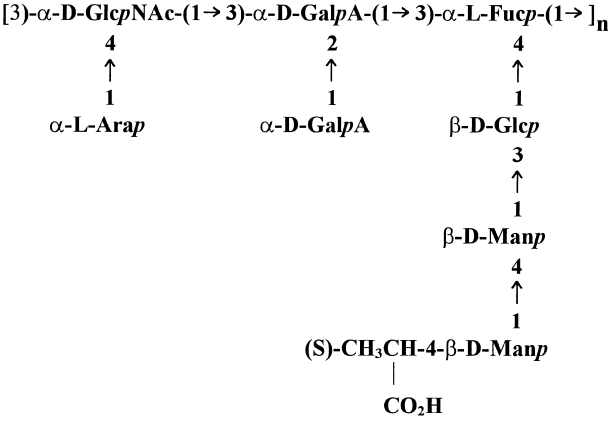
[a] *Istituto per la Chimica e la Tecnologia dei Materiali Polimerici, Consiglio Nazionale delle Ricerche, Viale A. Doria 6, 95125 Catania, Italy*

[b] *Dipartimento di Scienze Chimiche, Università di Catania, Viale A. Doria 6, 95125 Catania, Italy*

Received 10 October 1997; accepted 19 January 1998

Abstract

The exocellular polysaccharide produced by the cyanobacterium *Cyanospira capsulata* has been subjected to partial acid hydrolysis and *N*-deacetylation-nitrous acid deamination. The oligosaccharides released have been isolated by weak anion exchange and aqueous size exclusion chromatography, and characterized by a combination of 1D and 2D nuclear magnetic resonance spectroscopy, mass spectrometry, sugar compostion and linkage analyses. The polysaccharide has an octasaccharide repeating unit with the following structure:



Keywords: *Cyanospira capsulata*; Cyanobacterium; Blue-green algae; Exopolysaccharide, structure; 4-*O*-(1-carboxyethyl)mannose

* Corresponding author. Tel: +39-95-339926; Fax: +39-95-221541; e-mail: gimpa@dipchi.unict.it

0008-6215/98/$19.00
PII S0008-6215(98)00036-6

1. Introduction

Cyanobacteria, also called blue-green algae, are autotrophic prokaryotes widespread in the environment and capable of producing storage, cell envelope and exocellular polysaccharides. These materials possess industrially interesting solution properties [1,2] and their structures are relevant to our understanding of their rheological and physicochemical behavior as well as of the microbial physiology and antigenicity.

A new genus named *Cyanospira* has recently been isolated from the alkaline soda lake Magadi in Kenya [3,4]. Two species have been described: *Cyanospira rippkae* and *Cyanospira capsulata*. The latter takes its name from the gelatinous capsule that surrounds the micro-organism cells that are arranged in colonies of helical thricomes. During its diazotrophic growth *C. capsulata* releases in the culture medium an acidic exopolysaccharide (hereafter referred to as CC-EPS) whose aqueous solution properties have been investigated [5–8]. It has been found that CC-EPS displays a somewhat unusual behavior. In particular, the shear dependent properties are similar to those of xanthan gum, which is known to adopt a conformationally ordered structure with "stiff" polymer chains, but the time dependent properties are more similar to those of the plant gum guar [5,8], which is instead known to adopt in solution a flexible random coil structure. Because of these properties CC-EPS may be regarded as a material for which industrial exploitation is to be considered. Relevant to this point is the stability of molecular and rheological properties of CC-EPS under different growth conditions [9] and the stability of the cell line, which has been shown to remain viable after desiccation for as long as 7 years [10].

Despite the scientific interest demonstrated by these studies and the fact that some papers have appeared dealing with the primary structure of the polysaccharide [6,11–13], this fundamental information is still missing. We have recently shown [14] that this polysaccharide is composed of seven monosaccharides: Fuc, Ara, Glc, Man, GlcNAc, GalA, and the rare acidic sugar 4-*O*-(1-carboxyethyl)-mannose (4LacMan). The last monosaccharide was first found in the extracellular polysaccharide from *Mycobacterium lacticolum* strain 121 [15], and lactic acid has been reported to occur as an ether-linked substituent in a number of bacterial polysaccharides [16], besides the well known case of *N*-acetylmuramic acid (2-acetamido-3-*O*-(1-carboxyethyl)-2-deoxyglucose) found in bacterial peptidoglycan. This is however the first instance among polysaccharides from cyanobacteria. All monosaccharides are present in equimolar amount but the GalA which has a molar ratio of 2:1 with respect to each of the other sugars. NMR analysis of CC-EPS in D_2O is severely hampered by the high viscosity of its solutions, even at relatively low concentration and high temperatures. Partial depolymerization improves the quality of the ^1H NMR spectrum [14], but the signals are still quite broad and little information can be extracted concerning the composition and structure of CC-EPS.

In this study we have resorted to the degradation of CC-EPS to oligosaccharides by partial acid hydrolysis with trifluoroacetic acid and, alternatively, by *N*-deacetylation-nitrous acid deamination. Partial acid hydrolysis gives a complex mixture of oligosaccharides, whilst HONO deamination produces an oligosaccharide which originates from the polysaccharide repeating unit. Isolation of these oligosaccharides allowed us to completely map the primary structure of the polysaccharide. The detailed structural analysis of these products of CC-EPS degradation is here reported.

2. Experimental

Materials.—The sample of exocellular polysaccharide from *Cyanospira capsulata* (CC-EPS) was kindly provided by Professor A. Cesàro of the University of Trieste, Italy. CC-EPS samples were isolated by precipitation in isopropyl alcohol from cell-free supernatants of cultures of *C. capsulata* of constant ages, grown in the conditions already described [3]. The crude polymer, a white fibrous material, is soluble in water and forms very viscous solutions. Aqueous solutions of the crude CC-EPS are opalescent and have a pH value of about 9.5, the same as that of the culture medium from which the polysaccharide was extracted. Solvents and reactants were all from Aldrich, unless otherwise stated.

Monosaccharide and linkage analyses.—Absolute configuration of monosaccharides was determined according to Gerwig et al. [17]. In the case of 4LacMan the lactyl group was removed by action of BBr_3 in CH_2Cl_2 on the peracetylated sugar [18] and the absolute configuration of the freed Man determined. The lactyl group was established to be in the S configuration by observing the

NOE contacts in the ^{1}H NMR spectrum of the acetylated lactone derivative [19]. GC analyses were run on a Dani 6800 gas chromatograph equipped with a flame ionization detector and a DB1 column (30 m×0.32 mm i.d., J & W Scientific); injections were in the split mode with a 1:20 split ratio. Linkage analyses were carried out by the Hakomori method as described by York et al. [20] on 1 mg CC-EPS samples reduced at the carboxyl groups of GalA and 4LacMan with $NaBD_4$ after condensation with water soluble carbodiimide [21]. Oligosaccharides (100–200 μg) were first permethylated and methyl carboxylate groups reduced with 250 μL of $Li(Et)_3BD$ 1 M in THF for 1 h at RT, the reducing agent destroyed with AcOH and removed as borate methyl ester. The sample was again methylated repeating twice the addition of base and CH_3I and purified by solid phase extraction on SepPak cartridges (Waters). Permethylated samples were hydrolyzed (0.5 mL CF_3COOH 2 M at 120 °C for 3 h), the resulting monosaccharides deuterium reduced at C-1 ($NaBD_4$ 10 mg/mL in NH_4OH 1 M, 1 h at RT) and finally, after removal of borate, acetylated with 250 μL pyridine/acetic anhydride 1:1 at 120 °C for 20 min. The acylation mixture was carefully evaporated at RT after addition of toluene and dissolved in chloroform for analysis by GC and GC-MS.

Partial acid hydrolysis of CC-EPS and isolation of oligosaccharides.—600 mg CC-EPS was partially hydrolyzed with 400 mL CF_3COOH 2 M at 80 °C for 2 h and the acid was removed in a rotavapor after addition of isopropyl alcohol. The hydrolysis mixture was dissolved in 25 mL of 50 mM phosphate buffer at pH 7.0 and applied to a column (2.2×28.5 cm) of Sephadex–DEAE A-25-120 (Sigma) equilibrated in the same buffer. The column was first washed with a two-column volume of buffer to elute unretained material, that was shown by high performance anion exchange chromatography (HPAEC) to be composed mainly of neutral monosaccharides. The retained material was eluted with a 0 to 350 mM linear gradient of NaCl in 850 mL of buffer at pH 7.0. Fractions (12 mL) were collected and assayed for hexose and uronic acid content using the anthrone and meta-hydroxybiphenyl assays as described in ref. [20]. Five major resolved peaks were obtained and the fractions were pooled together accordingly. Each pool was concentrated to 5 mL at 40 °C under vacuum and injected in two BioGel P2 (Biorad) columns (1.6×90 cm) in series eluted with bidistilled water at 10 mL/h. Fractions (3.2 mL) were collected and assayed for neutral and acidic sugars and by conductimetry. Carbohydrate positive fractions were analyzed by HPAEC. The HPAEC apparatus consisted of a gradient pump equipped with a Rheodyne injection valve, a CarboPac PA-1 column (0.4×25 cm) and a pulsed amperometric detector (PAD), all from Dionex; analyses were performed with a 40 min linear gradient 0–400 mM NaOAc in NaOH 200 mM at 1 mL/min. BioGel P2 fractions containing the same oligosaccharide were pooled together.

Nitrous acid degradation of CC-EPS.—CC-EPS (100 mg) was deacetylated with NaOH 2 M at 100 °C for 3 h, after which the reaction was neutralized with AcOH and dialyzed (membrane cut off = 1000) against bidistilled water. ^{1}H NMR analysis showed that GlcNAc deacetylation was almost complete. The polysaccharide was treated with 100 mg $NaNO_2$ in 4 mL AcOH 0.7 M; the resulting solution had a pH = 4 and was stood at RT for 90 min, after which 0.5 mL 25% NH_4OH was added to stop the reaction and 50 mg $NaBH_4$ added. After 1 h at RT excess reducing agent was destroyed by addition of AcOH and borate removed as methyl esters. The product was dissolved in 4 mL 50 mM AcOH and injected in a BioGel P4 (Biorad) column (1.6×90 cm) eluted with 50 mM AcOH at 10 mL/h. 3 mL fractions were collected and assayed for hexoses. Carbohydrate-positive fractions were analyzed by HPAEC-PAD as above described.

Mass spectrometry.—GC-MS analyses were run on a Trio 1 GC-MS system (Fisons) equipped with a SP2330 column (30 m×0.32 mm i.d., Supelco) or a DB1 column (30 m×0.32 mm i.d., J&W Scientific) with helium as the carrier gas and split injection with a 1:10 split ratio. The quadrupole analyzer was set to scan from 30 to 400 m/z with 0.9 s scan time and 0.1 interscan delay. FABMS spectra of oligosaccharides were acquired with a Kratos MS 50 interfaced with an Eclipse S/120 computer (Data General) running under the DS90 Kratos software. Spectra were acquired in negative and positive ion mode using glycerol or glycerol/thioglycerol 1:1 as the FAB matrix. Reduced and peracetylated fractions were prepared according to procedures described above or with trifluoroacetic anhydride/AcOH [22].

NMR spectroscopy.—Samples were exchanged three times with D_2O (99.9 atom% D) by evaporation or lyophilization and finally dissolved in

D_2O (99.96 atom% D). Spectra of the reducing fractions were run on an AC 200 F Bruker instrument interfaced with an Aspect 3000 computer. 1D spectra were acquired with a 0.22 Hz/point digital resolution and 4.1 s pulse repetition time for ^1H and 0.61 Hz/point and 1.5 s for ^{13}C, at RT. Chemical shifts are expressed in ppm from internal acetone (2.225 ppm for ^1H and 31.07 ppm for ^{13}C). High field 1D and 2D experiments were run on AMX 500 or DRX 600 Bruker spectrometers. Double quantum filtered correlation spectroscopy (DQF-COSY) spectra were acquired using standard Bruker pulse sequence with TPPI [23] in F1. Data matrices (1024×512 or 2048×512 points) were multiplied by a shifted sine bell function in both dimensions before zero-filling and FT. 2D total correlation spectroscopy (TOCSY) [24] spectra used a MLEV-17 pulse train for isotropic mixing, flanked by two 2.5 ms trim pulses. Three experiments (80, 160 and 240 ms mixing times) were acquired for each sample. Phase sensitive (TPPI) data matrices were filtered as done for DQF-COSY before FT. 1D TOCSY spectra used DANTE [25] pulses for selective excitation; spin-lock times were tailored to each case to solve ambiguities or resolution problems intervened in the assignment process of 2D experiments. ^1H-^{13}C correlation spectra were carried out by inverse detection using heteronuclear single quantum coherence (HSQC) [26]. A BIRD pulse [27] was used to select ^{13}C-bound protons and decoupling of ^{13}C was attained by the GARP sequence [28]. 4K×512 data matrices were filtered with gaussian function in F2 and shifted squared sine function in F1 (phase sensitive by TPPI) and zero-filled in F1 before FT. Heteronuclear multiple bond correlation (HMBC) [29] experiments were acquired with pulsed field gradients coherence selection. The delay for evolution of long range heteronuclear couplings was set to 60 ms. Data matrices were treated as described for HSQC experiments before FT. Experimental data were processed using the software FELIX (Biosym) running on a Silicon Graphics IRIS workstation or using the Bruker WINNMR program running on an IBM compatible PC.

3. Results and discussion

Composition and linkage analyses of CC-EPS.—We have already shown [14] that CC-EPS is composed of Fuc, Ara, Glc, Man, GlcNAc, 4LacMan and GalA in a molar ratio of 1:1:1:1:1:1:2. Linkage analysis of the carboxyl reduced polysaccharide confirmed the results obtained by Marra et al. [12] for Fuc (3,4-linked), Ara (terminal), Glc (3-linked), Man (4-linked) and GalA (2,3-linked and terminal in equal amounts). GlcNAc and 4LacMan, that were previously not identified in CC-EPS, are present as 3,4-linked and terminal sugars, respectively. GC analysis of monosaccharides as the TMS derivatives of glycosides of optically pure 2-butanol [17] allowed us to assign Fuc and Ara to the L and Glc, Man, GlcNAc , GalA and Man of 4LacMan to the D absolute configuration. The chiral carbon of the carboxyethyl group of 4LacMan is in the S absolute configuration (see Experimental section).

Partial acid hydrolysis of CC-EPS and isolation of pure oligosaccharides.—600 mg of the polysaccharide was hydrolyzed with CF_3COOH 2M at 80 °C for 2 h. The Sephadex–DEAE separation of the hydrolysis mixture is shown in Fig. 1. Five resolved intense peaks are present, as revealed by the anthrone assay for hexoses and by the m-hydroxybiphenyl assay for uronic acids. As reported previously [14], peak 3 has been shown to consist solely of the rare acidic monosaccharide 4-*O*-(1-carboxyethyl)-mannose, which explains the null response to the uronic acid assay and the fact that this fraction is nonetheless retained by the weak anion exchange stationary phase.

All peaks of the Sephadex–DEAE separation were analyzed for heterogeneity by HPAEC-PAD. Peaks 2 and 3 were essentially pure compounds, whilst peaks 1, 4 and 5 appeared to be complex mixtures of oligosaccharides. BioGel P2 size exclusion chromatography was used to isolate pure oligosaccharides (>90% as estimated by HPAEC-PAD and NMR) by analyzing by HPAEC-PAD every carbohydrate-positive fraction of the BioGel P2 chromatographic runs. In this way it was possible to isolate two pure oligosaccharides from peak 1 (compounds **1a** and **1b**), four from peak 4 (compounds **4a**, **4b**, **4c** and **4d**), and two from peak 5 (compounds **5a** and **5b**). Peak 2, which already contained only one compound, was simply purified by this procedure and labeled as **2a**.

Structural analysis of oligosaccharides from CC-EPS partial hydrolyzate.—All oligosaccharides were subjected to composition and linkage analyses. FABMS analysis in positive and negative ion mode of the reducing underivatized oligosaccharides gave the molecular mass of each fraction. FABMS was also applied on the reduced and peracetylated

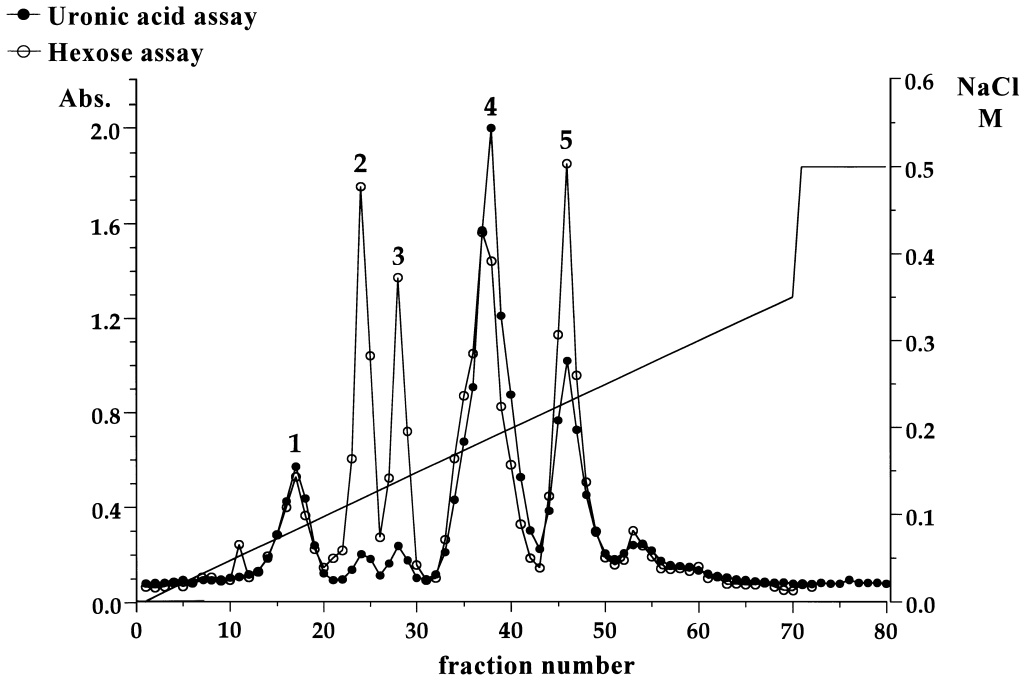


Fig. 1. Sephadex–DEAE separation of CC-EPS partial hydrolyzate.

samples of some of the oligosaccharides in order to get information on the sequence. The spectra contained besides the molecular ions also some structurally informative fragment ions, which are reported in the discussion of the single oligosaccharides when appropriate. All fractions were also analyzed by ^{1}H (200 MHz) and ^{13}C (50 MHz) NMR spectroscopy in D_2O; proton spectra are shown in Fig. 2.

Compound **2a**.—FAB mass spectra of **2a** indicated a molecular weight of 414 ($(M-H)^-$ at *m/z* 413 in negative, $(M+Na)^+$ at *m/z* 437 and $(M-H+2Na)^+$ at *m/z* 459 in positive ion mode). Given the monosaccharide composition of CC-EPS, this value can be accounted for by a structure $(4LacMan)_1(Hex)_1$, Hex being Glc or Man (the molecular mass of 4LacMan is 252). Composition and linkage analyses showed that fraction **2a** is a disaccharide composed of a terminal 4LacMan linked to position 4 of a reducing Man. In Fig. 2 the ^{1}H NMR spectrum is shown: the signals at 5.17 ($J_{1-2}=1.4$ Hz) and 4.91 ($J_{1-2}=1.0$ Hz) ppm are assigned to the α and β anomeric protons of Man. Reduction with $NaBH_4$ causes these signals to disappear. The resonance at 4.74 ppm ($J_{1-2}=1$ Hz, measured by Lorentz to Gauss resolution enhancement), with intensity equal to the sum of the two anomeric signals of Man, is assigned to the β glycosidic ^{1}H of the 4LacMan residue (the large singlet at 4.82 ppm present in all spectra is due to residual HOD). The β configuration was deduced from the ^{1}H-1 chemical shift and coupling constant and from the ^{13}C-1 chemical shift (100.94 ppm), and was confirmed by the value of 161 Hz measured for the one bond ^{13}C–^{1}H coupling constant for the anomeric nuclei in the proton-coupled DEPT spectrum of the reduced disaccharide. In Fig. 2 the ^{1}H NMR spectrum of 4LacMan is also reported for comparison. The structure of fraction **2a** is shown in Scheme 1.

Compound **4d**.—Composition and linkage analyses showed that **4d** is composed of terminal Glc, GlcNAc and GalA, 2,3-linked GalA and 3,4-linked reducing Fuc. This is consistent with the molecular mass of 881 determined by FABMS. In the negative ion FAB mass spectrum besides pseudomolecular ions at *m/z* 880 $(M-H)^-$, 902 $(M-2H+Na)^-$ and 924 $(M-3H+2Na)^-$, fragments at *m/z* 740 and 594 are found. The ion at *m/z* 740 confirms the presence of a terminal hexose (loss of 162 a.m.u. from the most abundant pseudomolecular ion at *m/z* 902); *m/z* 594 corresponds to a loss of Glc and Fuc (902−162−146) suggesting that Glc is linked to Fuc. Therefore, the fragment at *m/z* 594 is formed by a branched GalA bearing a GlcNAc and another GalA (Scheme 1). The ^{1}H NMR (Fig. 2) gave five anomeric signals between 5.6 and 4.6 ppm. The α and β form of reducing Fuc were found at 5.25 ($J_{1-2}=3.6$ Hz) and 4.67 ($J_{1-2}=7.9$ Hz) ppm. The sum of their intensities accounts for one

proton, compared to signals at 5.58, 5.35 and 5.21 ppm, assigned to three α glycosidic linkages. The additional anomeric resonance is probably a β linkage at about 4.55 ppm, which overlaps with other signals. At 2.04 and 1.32 ppm two signals are present, each integrating for three protons. The first is a singlet assigned to the methyl group of GlcNAc, the second is a doublet due to the methyl of Fuc partially split by anomerization.

^{1}H NMR was repeated on the reduced sample, **4dr** (Fig. 3, Table 1). Having completely assigned the proton spectrum, the ^{13}C resonances were identified through inspection of a proton detected ^{1}H–^{13}C correlation experiment (HSQC, data reported in Table 1). The HMBC experiment gave all correlations between the anomeric proton and carbon of one sugar unit and the carbon and proton, respectively, of the monosaccharide to which it is linked, allowing us to assign to **4d** the structure depicted in Scheme 1.

Compound **5a**.—**5a** has a molecular mass of 1409, which is the highest among the oligosaccharides obtained from CC-EPS. Composition and linkage analyses give identical results to those of the original polysaccharide, except for Fuc, which is a reducing sugar in **5a**, and for GlcNAc, which is found as 4-linked monosaccharide instead of 3,4-linked as in CC-EPS. The presence of a reducing Fuc is also revealed by the ^{1}H NMR spectrum shown in Fig. 2, where the α anomeric resonance of Fuc is readily identifiable because of its intensity (lower than that of the other anomeric protons)

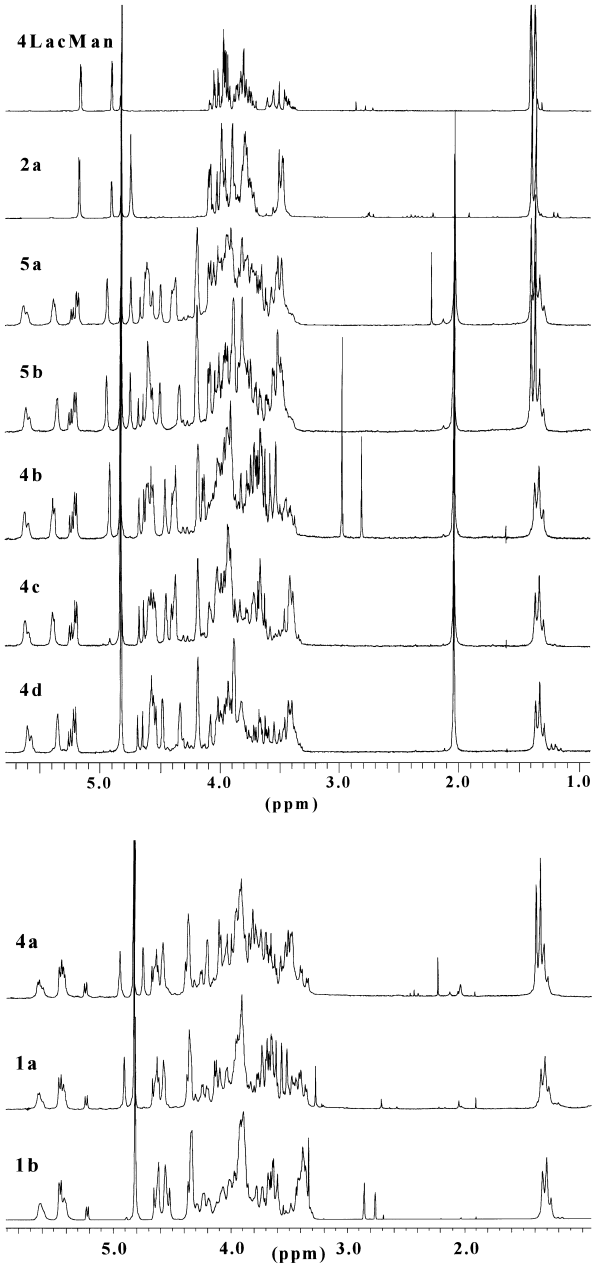


Fig. 2. 200 MHz ^{1}H NMR spectra in D_2O of CC-EPS oligosaccharides isolated from CF_3COOH partial hydrolyzate. Structures are shown in Scheme 1.

2a

β-4LacMan-(1→4)-Man

4d

α-GlcNAc-(1→3)-α-GalA-(1→3)-Fuc
2 4
↑ ↑
1 1
α-GalA β-Glc

5a

α-GlcNAc-(1→3)-α-GalA-(1→3)-Fuc
4 2 4
↑ ↑ ↑
1 1 1
α-Ara α-GalA β-Glc
3
↑
1
β-Man
4
↑
1
β-4LacMan

4c

α-GlcNAc-(1→3)-α-GalA-(1→3)-Fuc
4 2 4
↑ ↑ ↑
1 1 1
α-Ara α-GalA β-Glc

4b

α-GlcNAc-(1→3)-α-GalA-(1→3)-Fuc
4 2 4
↑ ↑ ↑
1 1 1
α-Ara α-GalA β-Glc
3
↑
1
β-Man

5b

α-GlcNAc-(1→3)-α-GalA-(1→3)-Fuc
2 4
↑ ↑
1 1
α-GalA β-Glc
3
↑
1
β-Man
4
↑
1
β-4LacMan

4a

α-GlcN-(1→3)-α-GalA-(1→3)-Fuc
4 2 4
↑ ↑ ↑
1 1 1
α-Ara α-GalA β-Glc
3
↑
1
β-Man
4
↑
1
β-4LacMan

1a

α-GlcN-(1→3)-α-GalA-(1→3)-Fuc
4 2 4
↑ ↑ ↑
1 1 1
α-Ara α-GalA β-Glc
3
↑
1
β-Man

1b

α-GlcN-(1→3)-α-GalA-(1→3)-Fuc
4 2 4
↑ ↑ ↑
1 1 1
α-Ara α-GalA β-Glc

Scheme 1.

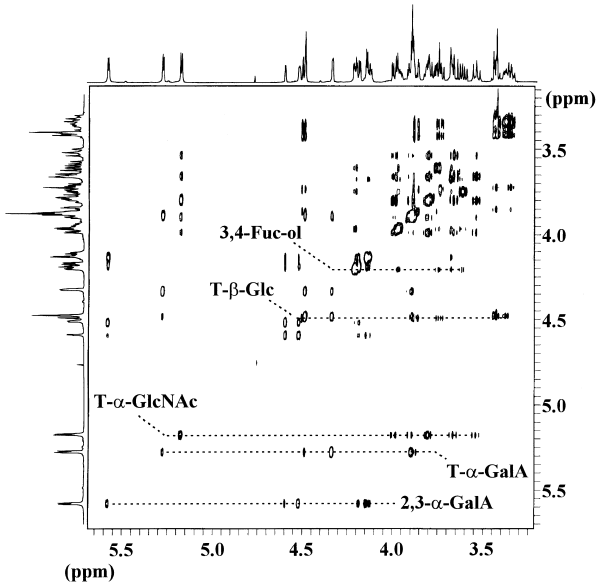


Fig. 3. Partial 500 MHz TOCSY NMR spectrum of fraction **4dr** acquired with 240 ms spin–lock time. For assignments see Table 1.

and its chemical shift and coupling constant. The anomeric region between 5.6 and 5.2 ppm is very similar for **4d** and **5a**, i.e. probably 2,3-linked GalA, terminal GalA and GlcNAc are in the α configuration also in **5a**. At 4.94 and 4.75 ppm two new anomeric signals are present. The latter is the same of that found in fraction **2a** (Fig. 2) and may therefore be attributed to β-^{1}H-1 of 4LacMan. The occurrence of this monosaccharide in **5a** is also evidenced by the doublet due to the lactyl methyl group at 1.38 ppm, superimposed to the methyl signal of Fuc at slightly higher field. From the data given so far fraction **5a** may be thought of as the repeating unit of CC-EPS. ^{1}H NMR was repeated on the reduced sample, **5ar** (Table 2). Anomeric chemical shifts and coupling constants of Man and 4LacMan did not allow unambiguous determination of their configuration, therefore proton coupled DEPT spectra were acquired and the ^{1}H-1–^{13}C-1 coupling constant measured: in both cases a value of 162 Hz established that these sugars are β linked. ^{13}C signals were assigned by recording at 500 MHz a HSQC spectrum and are reported in Table 2. Finally, a gradient enhanced HMBC spectrum established the sequence of **5a** which is shown in Scheme 1. Partial sections of the HMBC experiment with the relevant assignments are shown in Fig. 4a and b.

Compounds **4b**, **4c** *and* **5b**.—The monosaccharides constituent **4b**, **4c** and **5b** and their linkages are shown in Scheme 1. These data are in

Table 1
^{1}H and ^{13}C NMR data[a] of CC-EPS fraction **4dr** in D_2O

	1	2	3	4	5	6a	6b
2,3-α-Gal*p*A							
^{1}H ppm	5.582	4.193	4.131	4.519	4.600		
(J_{H-H})	(3.4)	(10.5)	(3.1)	(1.6)			
^{13}C ppm	96.25	70.43	71.80	66.80	72.94	176.10	
T-α-Gal*p*A							
^{1}H ppm	5.277	3.906	3.878	4.334	4.486		
(J_{H-H})	(3.3)	(10.5)	(3.0)	(1.4)			
^{13}C ppm	96.38	68.68	70.61	71.31	72.86	176.30	
T-α-Glc*p*NAc							
^{1}H ppm	5.176	3.987	3.658	3.536	3.808	3.898	3.79
(J_{H-H})	(3.8)	(10.5)	(8.9)	(9.8)			
^{13}C ppm	93.10	54.17	72.19	70.63	73.14	61.48	
Acetyl-CH_3	^{1}H 2.037	^{13}C 22.89					
Acetyl-CO-		^{13}C 175.65					
T-β-Glc*p*							
^{1}H ppm	4.491	3.340	3.429	3.421	3.370	3.870	3.736
(J_{H-H})	(7.8)	(9.6)				(2.2, 12.3)	(5.7)
^{13}C ppm	105.74	74.42	76.48	70.42	76.59	61.74	
3,4-Fuc-ol							
^{1}H ppm	3.748	3.963	4.208	3.673	4.135	1.267	
(J_{H-H})	(3.9, 12.0)	(7.6)	(2.4)	(3.2)		(6.6)	
^{1}H ppm	3.606						
(J_{H-H})	(6.3, 12.0)						
^{13}C ppm	63.78	73.32	80.53	83.35	68.13	19.72	

[a] Chemical shifts are expressed in ppm from internal acetone (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C).

Table 2
^{1}H and ^{13}C NMR data of CC-EPS fraction **5ar** in D_2O

	1	2	3	4	5	6a	6b
2,3-α-Gal*p*A (a) [b]							
^{1}H ppm	5.620	4.180	4.136	4.527	4.637		
(J_{H-H})	(3.2)	(10.6)	(3.0)	(1.6)			
^{13}C ppm	95.92	69.42	71.14	66.54	72.78	175.73	
T-α-Gal*p*A (b) [b]							
^{1}H ppm	5.299	3.899	3.930	4.373	4.463		
(J_{H-H})	(3.3)		(3.1)	(1.4)			
^{13}C ppm	95.74	68.63	70.63	71.12	72.72	176.00	
T-α-Glc*p*NAc (c) [b]							
^{1}H ppm	5.175	4.009	3.810	3.704	4.016	3.967	3.870
(J_{H-H})	(3.8)	(10.6)	(8.2)	(10.5)			
^{13}C ppm	92.51	53.64	70.82	79.29	71.40	60.66	
Acetyl-CH_3	^{1}H 2.03 ppm	^{13}C 19.70 ppm					
Acetyl-CO-	175.57 ppm						
4-β-Man*p* (d) [b]							
^{1}H ppm	4.917	4.197	3.818	3.842	3.517	3.900	3.747
(J_{H-H})	(1.0)	(2.8)	(8.8)				
^{13}C ppm	101.36	70.76	72.26	77.45	75.73	61.29	
T-β-4LacMan*p*(e) [b]							
^{1}H ppm	4.741	4.086	3.768	3.500	3.485	3.927	3.767
(J_{H-H})	(1.0)	(3.1)	(8.4)				
^{13}C ppm	100.96	70.673	73.09	76.62	76.77	61.15	
Lactyl-CH_3	^{1}H 1.374 ppm	^{13}C 19.70 ppm					
Lactyl-CH-	^{1}H 4.018 ppm	^{13}C 79.20 ppm					
Lactyl-COOH		^{13}C 182.48 ppm					
T-β-Glc*p*(f) [b]							
^{1}H ppm	4.526	3.473	3.697	3.535	3.405	3.876	3.750
(J_{H-H})	(8.0)	(9.3)	(8.7)	(9.8)		(2.3; 12.3)	(5.7)
^{13}C ppm	105.45	74.10	84.98	68.92	76.05	61.72	
T-α-Ara*p*(g) [b]							
^{1}H ppm	4.375	3.618	3.686	3.952	3.960		
(J_{H-H})	(7.6)	(9.8)	(2.8)				
^{1}H ppm					3.675		
^{13}C ppm	104.38	71.74	73.06	69.20	67.07		
3,4-Fuc-ol(h) [b]							
^{1}H ppm	3.745	3.963	4.225	3.683	4.134	1.266	
(J_{H-H})		(7.6)	(2.3)			(6.6)	
^{1}H ppm	3.601						
(J_{H-H})	(11.9; 6.2)						
^{13}C ppm	63.72	73.34	80.44	83.28	68.17	19.62	

[a] Chemical shifts are expressed in ppm from internal acetone (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C).
[b] Letters in parentheses refer to sugar units as symbolized in Fig. 4a and b.

accord with the molar masses as determined by positive and negative ion FABMS. The ^{1}H NMR spectra are shown in Fig. 2. The region between 5.6 and 5.1 ppm for these oligosaccharides is identical to that of **4d** and **5a** and the anomeric signals may therefore be assigned as for those two CC-EPS fragments, namely, starting from the leftmost: branched α-GalA, terminal α-GalA, reducing α-Fuc and α-GlcNAc. The last monosaccharide is terminal in **4d**, but 4-linked in **4c**. This fact and the presence of terminal Ara is what distinguishes **4c** from **4d**. L-Ara is found terminal in the polysaccharide and in **5a**, where it is linked through an α linkage (for **5ar** $\delta = 4.375$ ppm and $J_{1-2} = 7.6$ Hz, Table 2). In **4c** L-Ara must be also α linked because the anomeric signals not assigned yet all lie at chemical shifts incompatible with a β linkage. The α-L-Ara anomeric resonance at about 4.39 ppm is partially overlapped with another signal. FABMS of **4c** after reduction with $NaBH_4$ and peracetylation gave the correct molar mass (nominal 1687 a.m.u.) and some fragment ions, which may

be interpreted as oxonium ions produced by glycosidic cleavage with charge retained on the non reducing end (A1-type cleavage [22]), at *m/z* 259, 303, 331 and 546. These correspond to terminal Ara, GalA, Glc and Ara-GlcNAc, respectively. From these data the structure depicted in Scheme 1 may be deduced for **4c**. The same considerations regarding Ara apply when comparing the ^{1}H NMR spectrum of **4d** with that of **4b**. In the case of **4b** a new anomeric signal appears at 4.92 ppm (J_{1-2} < 1 Hz). This is the same signal found in the spectrum of **5a** and assigned through NMR analysis of **5ar** to β-Man. In fact Man is present in **4b** as terminal sugar, whilst Glc, terminal in the smaller oligosaccharides **4d** and **4c**, is now 3-linked. Also in this case FABMS of the reduced and peracetylated compound furnished a molar mass (nominal 1975 a.m.u.) consistent with composition data and informative fragment ions at *m/z* 259, 303, 331, 546 and 619. These, with the exception of *m/z* 619, are the same as those obtained for reduced and peracetylated **4c** and are interpreted accordingly. Fragment at *m/z* 619 is readily assigned to an oxonium ion formed by two hexoses, i.e. Man and Glc. It is therefore possible to draw for **4b** the structure shown in Scheme 1. The anomeric region of the ^{1}H NMR spectrum of **5b** (Fig. 2) is very similar to that of **5a**, the major difference occurring around 4.39 ppm, where the terminal α-L-Ara anomeric signal present in **5a** is no longer found in the case of **5b**. Composition and linkage analyses and molar mass (nominal 1277 a.m.u.) substantiate the information drawn from 1D NMR. In particular, Ara is not among the sugars constituent **5b** and GlcNAc, which is 4-linked in **5a**, is now terminal. The presence of terminal β-4LacMan is evident, besides composition data, from the anomeric resonance at 4.75 ppm and the methyl doublet at 1.38 ppm, partially overlapped with the Fuc methyl signal at slightly higher field.

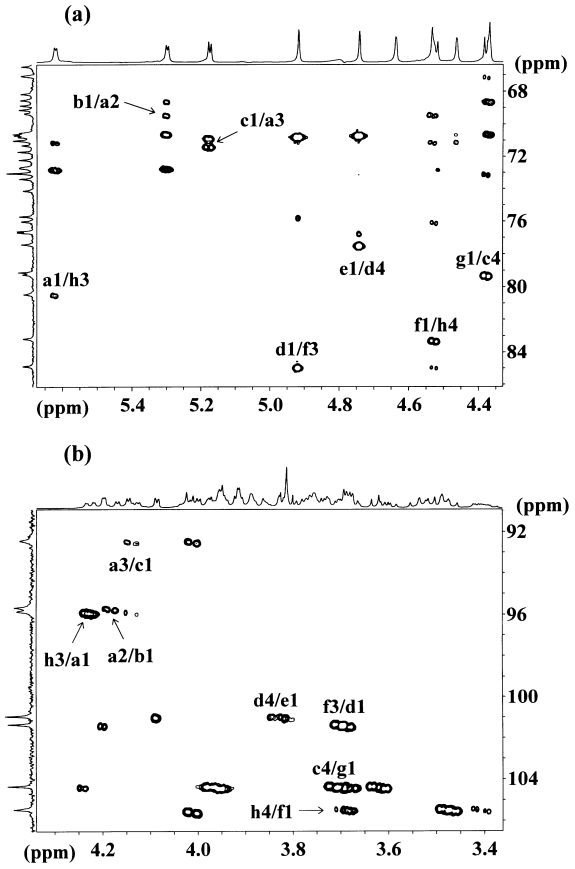
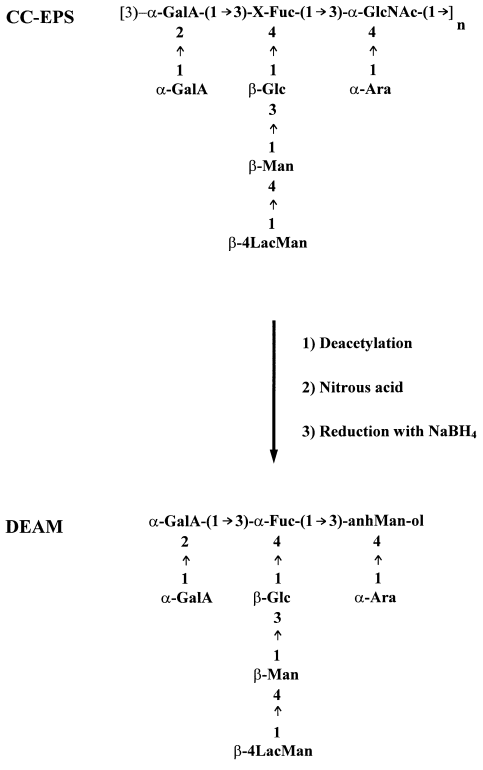


Fig. 4. Partial ^{1}H–^{13}C long range gradient enhanced correlation spectrum recorded at 600 MHz of fraction **5ar**. Labels on cross peaks identify correlations establishing the sequence: letters symbolize monosaccharides as reported in Table 2. For instance, b1/a2 identify the long range correlation between ^{1}H-1 of unit b (T-α-GalA) and ^{13}C-2 of unit a (2,3-α-GalA).

Compounds **4a**, **1a** *and* **1b**.—The structures proposed for **4a**, **1a** and **1b** are shown in Scheme 1. These oligosaccharides give on composition and linkage analyses the same results of already characterized compounds **5a**, **4b** and **4c**, respectively. The molar masses are however not the same, there being a 42 a.m.u. difference between **5a** and **4a**, **4b**



Scheme 2.

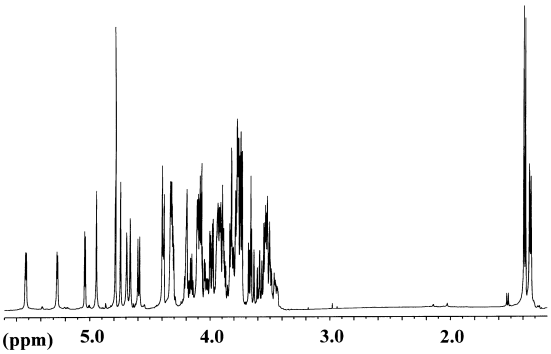


Fig. 5. 500 MHz ^{1}H NMR spectrum in D_2O of fraction DEAM (Scheme 2) obtained by nitrous acid deamination of CC-EPS.

and **1a**, **4c** and **1b**. This may be explained by supposing that in **4a**, **1a** and **1b** GlcNAc has lost the acetyl group during the partial acid hydrolysis used to degrade CC-EPS. This hypothesis is clearly proved by ^{1}H NMR spectra of **4a**, **1a** and **1b** in Fig. 2, where the α-GlcNAc anomeric proton resonating at 5.20 ppm in all CC-EPS oligosaccharide examined so far (except **2a**) is missing. A new signal is in its stead present at 5.45 ppm ($J_{1-2} = 3.5$ Hz), partially overlapped with ^{1}H-1 of terminal GalA at slightly higher field. This new resonance may be assigned to ^{1}H-1 of α-2-amino-2-deoxy-glucose (α-GlcN). Finally, even more evident

Table 3
^{1}H and ^{13}C NMR data of CC-EPS fraction DEAM in D_2O

	1	2	3	4	5	6a	6b
2,3-α-Gal*p*A (a) [b]							
^{1}H ppm	5.524	3.988	4.077	4.393	4.665		
(J_{H-H})	(3.8)	(10.4)	(3.4)	(1.4)			
^{13}C ppm	97.70	72.68	68.16	71.42	72.01	174.64	
T-α-Gal*p*A (b) [b]							
^{1}H ppm	5.265	3.883	3.988	4.327	4.694		
(J_{H-H})	(3.9)	(10.3)	(3.5)	(1.4)			
^{13}C ppm	96.37	68.53	69.92	71.09	72.07	174.98	
3,4-α-Fuc*p*(c) [b]							
^{1}H ppm	5.036	4.034	4.097	4.103	4.303	1.339	
(J_{H-H})	(3.8)	(10.3)				(6.7)	
^{13}C ppm	98.83	68.61	74.05	79.42	68.57	15.99	
4-β-Man*p* (d) [b]							
^{1}H ppm	4.943	4.195	3.822	3.830	3.530	3.909	3.754
(J_{H-H})	(0.9)						
^{13}C ppm	101.15	70.68	72.18	77.36	75.65	61.23 [c]	
T-β-LacMan*p* (e) [b]							
^{1}H ppm	4.743	4.074	3.775	3.520	3.518	3.934	3.747
(J_{H-H})	(1.0)						
^{13}C ppm	100.83	70.72	73.12	76.42	74.19	61.18	
Lactyl-CH_3	^{1}H 1.385 ppm	^{13}C 19.45 ppm					
Lactyl-CH-	^{1}H 4.093 ppm	^{13}C 78.62 ppm					
Lactyl-COOH		^{13}C 181.42 ppm					
3-β-Glc*p*(f) [b]							
^{1}H ppm	4.592	3.526	3.748	3.586	3.448	3.923	3.798
(J_{H-H})	(7.9)	(9.3)	(8.8)	(10.0)			
^{13}C ppm	103.60	76.52	84.75	68.61	76.17	61.34 [c]	
T-α-Ara*p*(g) [b]							
^{1}H ppm	4.390	3.535	3.665	3.934	3.896		
(J_{H-H})	(7.6)	(9.7)	(3.5)	(1.4)			
^{1}H ppm					3.645		
(J_{H-H})					(1.2; 12.9)		
^{13}C ppm	104.56	71.44	72.85	68.90	67.05		
3,4-anhMan*f*(h) [b]							
^{1}H ppm	3.737	4.197	4.310	4.325	4.156	3.767	
(J_{H-H})	(5.8)	(9.3)		(9.3)	(5.8)		
^{13}C ppm	61.75	83.37	83.13	85.48	83.40	61.34 [c]	

[a] Chemical shifts are expressed in ppm from internal acetone (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C).
[b] Letters in parentheses refer to sugar units as symbolized in Fig. 6a and b.
[c] Assignments may be interchanged.

is the absence in the spectra of these three oligosaccharides of the singlet at about 2.03 ppm due to the methyl group of the acetyl substituent.

Nitrous acid degradation of CC-EPS.—CC-EPS was deacetylated and then treated with nitrous acid and the reaction products reduced giving 2,5-anhydromannitol (anhMan-ol) in place of GlcNAc (Scheme 2). The reaction mixture was worked up to remove borate and injected in a BioGel P4 column eluted with AcOH 50 mM. Carbohydrate positive fractions (anthrone assay) were analyzed by HPAEC-PAD and by FABMS. The results are in accord with the structure illustrated in Scheme 2. Fig. 5 shows the ^{1}H NMR spectrum at 500 MHz of DEAM. Signals at 5.524 and 5.265 ppm are due to branched α-GalA and terminal α-GalA. The doublet at 5.036 ppm ($J_{1-2}=3.8$ Hz) was assigned to ^{1}H-1 of an α-Fuc unit. 2D and 1D selective TOCSY spectra acquired with a set of different spin–lock times and a DQF-COSY spectrum were sufficient to assign all the proton resonances. Through the proton–carbon correlation spectrum (HSQC) all the $_{13}$C resonances were assigned. Table 3 reports these data. The gradient enhanced HMBC spectrum reported partially in Fig. 6a and b allowed to trace all long range correlations through glycosidic linkages. These data confirm all the structural details gained in the study of the partial hydrolyzate of CC-EPS and the α configuration of Fuc determined from the 1D ^{1}H NMR of DEAM. The HMBC experiment also established what had been deduced by comparing the linkage analysis results for GlcNAc in the polysaccharide and in the oligosaccharides, i.e. that Fuc is linked to position 3 of GlcNAc.

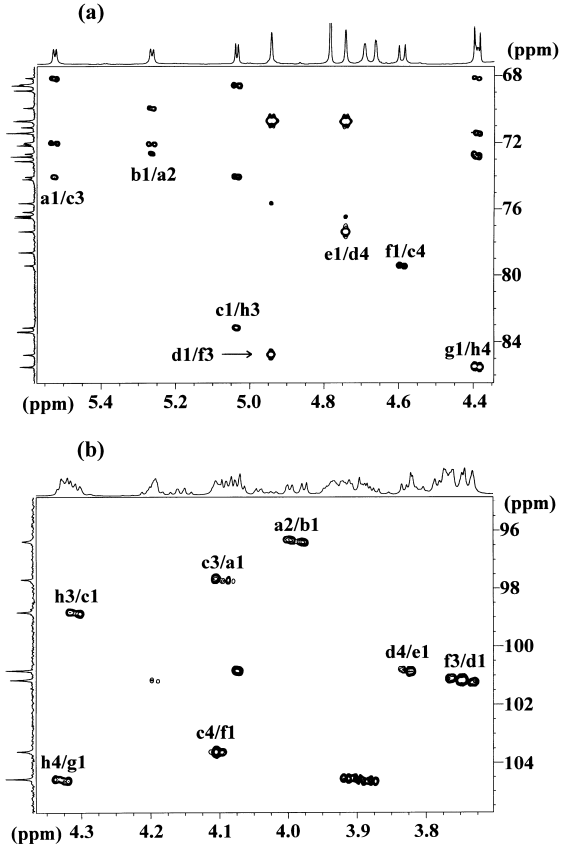


Fig. 6. Partial ^{1}H–^{13}C long range gradient enhanced correlation spectrum recorded at 600 MHz of fraction DEAM (Scheme 2). Labels on cross peaks identify correlations establishing the sequence: letters symbolize monosaccharides as reported in Table 3. For instance, a1/c3 identify the long range correlation between ^{1}H-1 of unit a (2,3-α-GalA) and ^{13}C-3 of unit c (3,4-α-Fuc).

4. Conclusions

Although NMR analysis of the original polysaccharide was not possible because of severe line broadening, isolation of several oligosaccharides from degradation reactions allowed us to completely map CC-EPS structure. Previous investigations by other authors [9,13] showed that the cyanobacterium *Cyanospira capsulata* produces an exocellular polysaccharide with a sugar composition fairly constant with respect to culture age and conditions. All published data [6,9,11–14] agree on a stoichiometric ratio between constituent monosaccharides. The amounts of pure oligosaccharides isolated from CF_3COOH partial hydrolyzate in this study account for a high percent value (about 60%) of the material obtained by DEAE chromatography, indicating that the structures characterized are representative of the polysaccharide. Formation of the octasaccharide DEAM (Scheme 2) by a selective degradation reaction, foreseen thanks to the characterization of partial hydrolysis products, constitutes a good confirmation of the structure proposed. These data allow to conclude that CC-EPS is a regular polysaccharide with the repeating unit illustrated in the Abstract section.

Acknowledgements

We thank Professor Attilio Cesàro for kindly providing us with CC-EPS. Many thanks are due to Professor Herman van Halbeek for hosting one of the authors (G.I.) in his lab at the Complex Carbohydrate Research Center of the University of

Georgia (Athens, GA, USA) during part of this study. We are deeply indebted to Dr. John Glushka, manager of NMR facilities at the CCRC, for patient and friendly help in setting up and processing of high field NMR experiments. Partial financial support from the Italian Ministry for University and Scientific and Technological Research (MURST) and from the National Research Council (CNR) is gratefully acknowledged.

References

[1] V. Crescenzi, *Trends Polym. Sci.*, 2 (3) (1994) 104–109.
[2] C. Bertocchi, L. Navarini, A. Cesàro, and M. Anastasio, *Carbohydr. Polym.*, 12 (1990) 127–153.
[3] G. Florenzano, C. Sili, E. Pelosi, and M. Vincenzini, *Arch. Microbiol.*, 140 (1985) 301–306.
[4] C. Sili, E. Pelosi, M. Vincenzini, R. Materassi, and G. Florenzano, *Proc. Eur. Congr. Biotechnol.*, 1 (1984) 91–96.
[5] L. Navarini, C. Bertocchi, A. Cesàro, R. Lapasin, and V. Crescenzi, *Carbohydr. Polym.*, 12 (1990) 169–187.
[6] A. Cesàro, G. Liut, C. Bertocchi, L. Navarini, and R. Urbani, *Int. J. Biol. Macromol.*, 12 (1990) 79–84.
[7] R. Lapasin, S. Pricl, C. Bertocchi, L. Navarini, A. Cesàro, and R. De Philippis, *Carbohydr. Polym.*, 17 (1992) 1–10.
[8] L. Navarini, A. Cesàro, and S.B. Ross-Murphy, *Carbohydr. Polym.*, 18 (1992) 265–272.
[9] M. Vincenzini, R. De Philippis, C. Sili, and R. Materassi, *J. Appl. Phycol.*, 5 (1993) 539–541.
[10] C. Sili, A. Ena, R. Materassi, and M. Vincenzini, *Arch. Microbiol.*, 162 (1994) 20–25.
[11] A. Flaibani, Y. Olsen, and T.J. Painter, *Carbohydr. Res.*, 190 (1989) 235–248.
[12] M. Marra, A. Palmeri, A. Ballio, A. Segre, and M.E. Slodky, *Carbohydr. Res.*, 197 (1990) 338–344.
[13] M. Vincenzini, R. De Philippis, C. Sili, and R. Materassi, *Appl. Microbiol. Biotechnol.*, 34 (1990) 392–396.
[14] D. Garozzo, G. Impallomeni, E. Spina, L. Sturiale, A. Cesàro, and P. Cescutti, *Carbohydr. Res.*, 270 (1995) 97–106.
[15] N.K. Kochetkov, A.F. Sviridof, K.A. Arifkhodzhaev, O.S. Chizhov, and A.S. Shashkov, *Carbohydr. Res.*, 71 (1979) 193–203.
[16] L. Kenne and B. Lindberg, *Bacterial Polysaccharides*, in G.O. Aspinall (Ed.), *The Polysaccharides*, Vol. 2, Academic Press, Orlando, FL, 1983, pp 287–363.
[17] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 77 (1979) 1–7.
[18] G.R. Gray, *Methods Enzymol.*, 83 (1982) 248–260.
[19] W.B. Severn and J.C. Richards, *J. Am. Chem. Soc.*, 115 (1993) 1114–1120.
[20] W.S. York, A.G. Darvill, M. McNeil, T.T. Stevenson, and P. Albersheim, *Methods Enzymol.*, 118 (1985) 3–40.
[21] R.L. Taylor and H.E. Conrad, *Biochemistry*, 11 (1972) 1383–1388.
[22] A. Dell, *Methods Enzymol.*, 193 (1990) 647–660.
[23] D. Marion and K. Wuthrich, *Biochem. Biophys. Res. Commun.*, 113 (1983) 967–974.
[24] A. Bax and D.G. Davis, *J. Magn. Reson.*, 65 (1985) 355–360.
[25] G.A. Morris and R. Freeman, *J. Magn. Reson.*, 29 (1978) 433–462.
[26] G. Bodenhausen and D.J. Ruben, *Chem. Phys. Lett.*, 69 (1980) 185–188.
[27] J.R. Garbow, D.P. Weitekamp, and A. Pines, *Chem. Phys. Lett.*, 93 (1982) 504–508.
[28] A.J. Shaka, P.B. Barker, and R. Freeman, *J. Magn. Reson.*, 64 (1985) 547–552.
[29] A. Bax and D. Marion, *J. Magn. Reson.*, 78 (1988) 186–191.