ELSEVIER

Carbohydrate Research 270 (1995) 97–106

CARBOHYDRATE RESEARCH

Note

Identification of *N*-acetylglucosamine and 4-*O*-[1-carboxyethyl]mannose in the exopolysaccharide from *Cyanospira capsulata*

Domenico Garozzo [a], Giuseppe Impallomeni [a,*], Emanuela Spina [a], Luisa Sturiale [a], Attilio Cesàro [b], Paola Cescutti [1,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 Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, I-34127 Trieste, Italy*

Received 3 October 1994; accepted 5 December 1994

Keywords: LactylMan; Exopolysaccharide; *Cyanospira capsulata*; Cyanobacterium

Cyanobacteria, also called blue–green algae, are autotrophic prokaryotes; they are widespread in the environment and are capable of producing cell wall and extracellular polysaccharides. These materials possess industrially interesting solution properties [1] and their structures are relevant to our understanding of the microbial physiology and antigenicity. A new genus named *Cyanospira* has recently been isolated from the alkaline soda lake Magadi in Kenya [2,3]. Two species have been described: *Cyanospira rippkae* and *Cyanospira capsulata*. The latter takes its name from the gelatinous capsule that surrounds the microorganism cells that are arranged in colonies of helical thricomes. An exopolysaccharide has been isolated [2,3] from cultures of *Cyanospira capsulata* (CC-EPS), and this has been reported [3,4] to be composed of arabinose, fucose, glucose, mannose and galacturonic acid (GalA). Two other papers [5,6] have appeared dealing with the primary structure of CC-EPS. The structures proposed differ in the quantitative estimate of GalA. In Ref. [5], all five monosaccharides are reported to be present in equimolar ratio, whilst in Refs [4] and [6] GalA is reported to be present in a 2:1 ratio with respect to the other neutral sugars. This difference was explained by

* Corresponding author.

[1] Present address: USDA-ARS-ERRC, Philadelphia, PA 19118, USA

Elsevier Science Ltd.

SSDI 0008-6215(94)00007-7

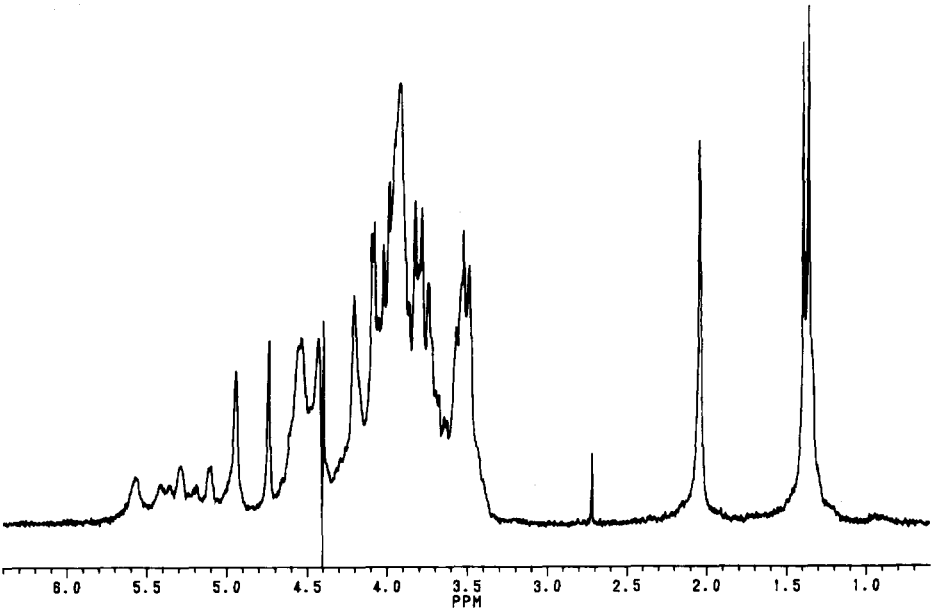


Fig. 1. ^{1}H NMR spectrum of the partially depolymerized exopolysaccharide from *Cyanospira capsulata*.

Cesàro et al. [6] as being due to the loss of uronic acids due to trifluoroacetic acid hydrolysis, whereas a more quantitative estimate of GalA is possible using 1 M HCl in anhydrous methanol.

In the present study we report the finding of two monosaccharides previously undetected in CC-EPS: *N*-acetylglucosamine (GlcNAc) and the rare acidic sugar 4-*O*-[1-carboxyethyl]mannose (4-lactylman).

The proton NMR spectrum of a sample of partially depolymerized CC-EPS is shown in Fig. 1. Although the NMR signals are quite broad, some information was obtained from the signals in the upfield region. The doublet at 1.38 ppm is characteristic of a $-CH_3$ group of a 6-deoxyhexose residue, thus confirming the presence of fucose, while the singlet at 2.05 ppm is typical of an acetyl group. Their integration gave a ratio of 2:1, respectively. The colorimetric assay [7] indicated the presence of hexosamine. Methanolysis followed by re-*N*-acetylation and derivatization of the methyl glycosides to TMS, and subsequent GC analysis identified this residue as GlcNAc. High performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) of the total acid hydrolysate confirmed the presence of GlcNAc (Fig. 2)

In order to isolate oligosaccharides from CC-EPS, we performed a partial hydrolysis and separated the hydrolysate by DEAE-Sephadex chromatography. The third peak eluted from the DEAE column had a very low response to the uronic acid assay, whereas its response was positive to the hexose assay. FABMS analysis of this fraction yielded a molecular mass of 252. The proton NMR spectrum of this compound is shown in Fig. 3. The doublets at 5.16 and 4.90 ppm ($J_{1,2}$ 1.5 and 1.0 Hz, respectively) are

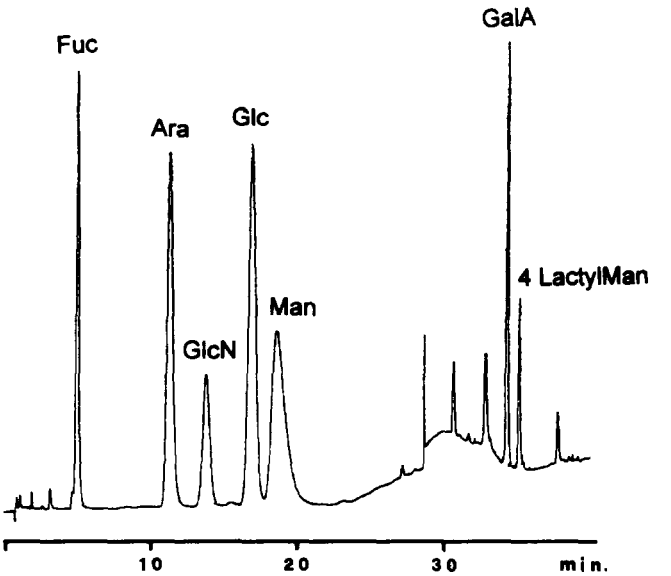


Fig. 2. High performance anion exchange chromatography of a total hydrolysate sample of the exopolysaccharide from *Cyanospira capsulata*.

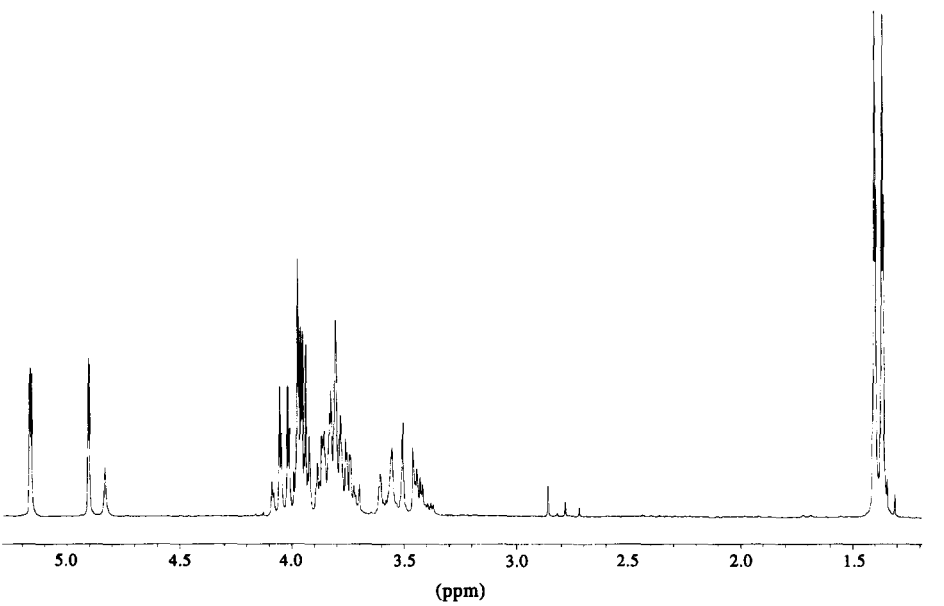


Fig. 3. ^{1}H NMR spectrum of the compound contained in the third peak from the Sephadex DEAE separation of a partial hydrolysate of the exopolysaccharide from *Cyanospira capsulata* desalted by BioGel P2 chromatography.

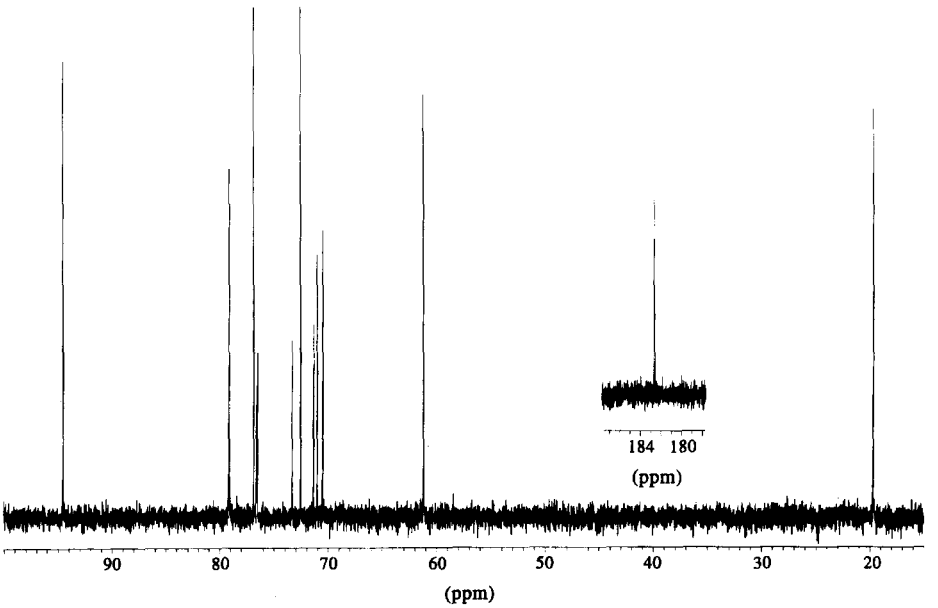


Fig. 4. ^{13}C NMR spectrum of the compound contained in the third peak from the Sephadex DEAE separation of a partial hydrolysate of the exopolysaccharide from *Cyanospira capsulata* desalted by BioGel P2 chromatography.

assigned to the anomeric proton and they result quite similar to those of mannose. The doublet at 1.38 ppm (^3J 7.1 Hz, plus a small splitting due to anomerization) corresponds to three protons and is therefore assigned to a methyl group. The quartet centred at 4.03 ppm shares the same coupling constant and anomerization splitting of the methyl group. It is therefore possible to hypothesize the presence of a CH_3–CH group having no scalar coupling with other protons. This was confirmed by a COSY 90 experiment that showed a strong correlation peak between the quartet at 4.03 and the doublet at 1.38 ppm, but no correlation between these two signals and all the other ring protons.

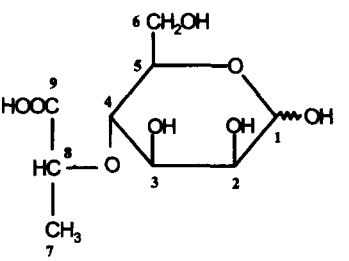
The ^{13}C NMR spectrum of the same sugar is shown in Fig. 4 and compared with mannose in Table 1: only one anomeric signal at 94.51 ppm is found, the 4α and 4β signals are shifted downfield at 76.86 and 76.58 ppm, respectively, and three more peaks are present at 19.69, 79.12 and 182.67 ppm in the sugar isolated from CC-EPS. DEPT NMR spectra showed that these peaks are due to a methyl, a methine and a carbon with no attached proton, respectively. The signal at 182.67 ppm is then assigned to a carboxylic group. The ^1H and ^{13}C NMR spectra suggest that the sugar under investigation is a mannose ring substituted at O-4, as indicated by the downfield shift of C-4, by a lactyl group (see Structure 1). This structure is consistent with the measured molecular mass of 252 and explains why an acidic sugar retained by an anion exchange column gives almost no response to the meta-hydroxybiphenyl assay for uronic acids. 4-Lactyl-man has been also identified in the total acid hydrolysate of CC-EPS (Fig. 2)

Table 1
Comparison between the ^{13}C NMR spectrum of the acidic monosaccharide isolated from the exopolysaccharide from *Cyanospira capsulata* (CC-EPS) and that of mannose. Δ is the chemical shift difference between the two sugars. For atom numbering see Structure 1

^{13}C atom	Monosaccharide		Δ
	CC-EPS (ppm)	Mannose (ppm)	
9 α,β	182.67	—	—
1 α,β	94.51	94.85, 94.48	0.34, 0.03
8 α,β	79.12	—	—
4 α	76.86	67.66	9.20
4 β	76.58	67.42	9.16
5 β	76.52	76.98	0.46
3 β	73.32	73.86	0.54
5 α	72.55	73.21	0.66
2 β	71.35	72.03	0.68
2 α	71.01	71.49	0.48
3 α	70.49	71.04	0.55
6 α,β	61.21	61.78	0.57
7 α,β	19.69	—	—

The heteronuclear $^{1}H-^{13}C$ chemical shift-correlated NMR spectrum (HETCOR) of 4-lactylman is shown in Fig. 5. This experiment proves unambiguously that the ^{13}C resonance at 94.51 ppm correlates with the two anomeric proton signals at 5.16 and 4.90 ppm, so that the presence of only one anomeric signal in the ^{13}C spectrum is explained by the fact that the α and β forms are isochronous. The HETCOR spectrum enabled us also to confirm the assignment (see Table 1) of the ^{13}C peak at 79.12 ppm to the methinic carbon of the lactyl group.

Reduction with metallic lithium in ethylenediamine to cleave the ether linkage of the lactyl group and reduce the aldehydic group of mannose [8] yielded mannitol, which was identified by its mass spectrum and by its retention time in GC-MS analysis. The position of the lactyl substituent in the mannose ring was further proved by GC-MS analysis of the carboxyl-reduced partially methylated alditol acetate. The mass spectrum shown in Fig. 6 clearly confirms that the mannitol derivative is substituted in position 4.



Structure 1

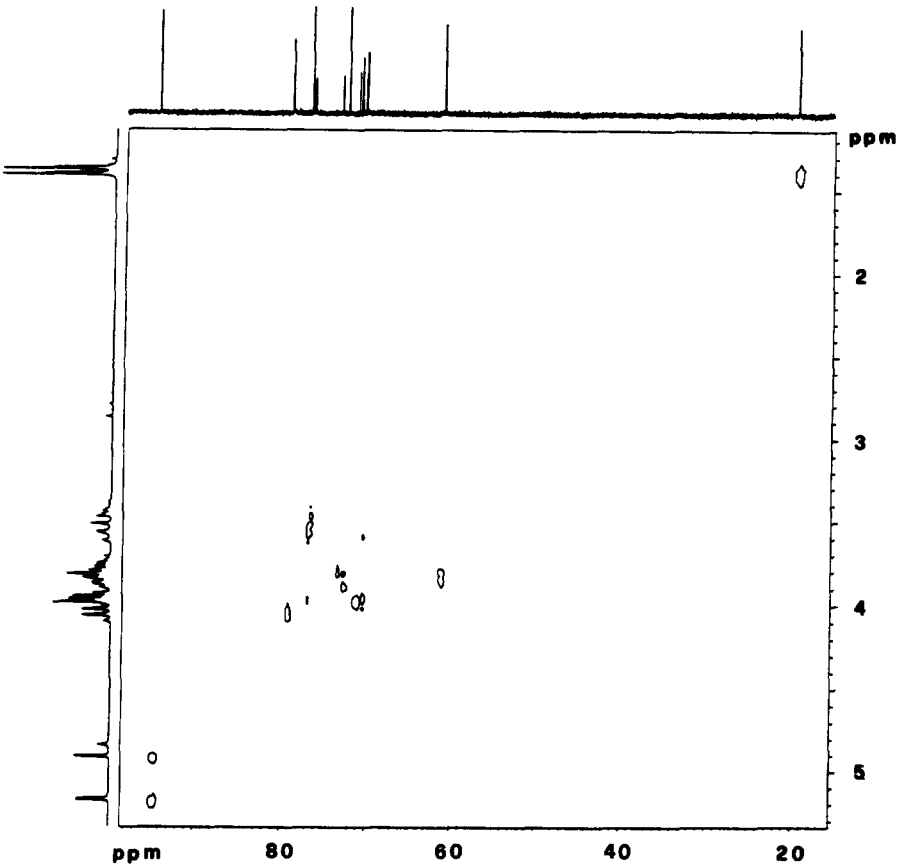


Fig. 5. $^{1}H-^{13}C$ chemical shift correlated NMR spectrum of the 4-lactylmannose sample isolated from the partial hydrolysate of the exopolysaccharide from *Cyanospira capsulata*.

4-Lactylmannose (or 4-*O*-[1-carboxyethyl]mannose) was first found in the extracellular polysaccharide from *Mycobacterium lacticolum* strain 121 by Kochetkov et al. [9]. These authors synthesised the 4-substituted mannose having the *S* and *R* forms of the lactyl group and reported the ^{13}C NMR spectra of the two diastereoisomers. Our monosaccharide from CC-EPS compares well with the ^{13}C NMR data of Kochetkov et al., but unambiguous determination of the absolute configuration of the lactyl group was not possible. Differences in the pH of the sample solution could explain the small differences in the measured chemical shifts between our data and that of Ref. [9].

Re-examining the ^{1}H NMR spectrum of the partially depolymerized CC-EPS (see Fig. 1), it is now apparent that the doublet at 1.38 ppm results from the overlapping of the methyl signals of fucose and 4-lactylman. In the anomeric region of the spectrum, two of the various signals are present at 4.94 and 4.73 ppm. These protons resonate at frequencies shifted slightly (0.1–0.2 ppm) upfield with respect to that of the reducing α

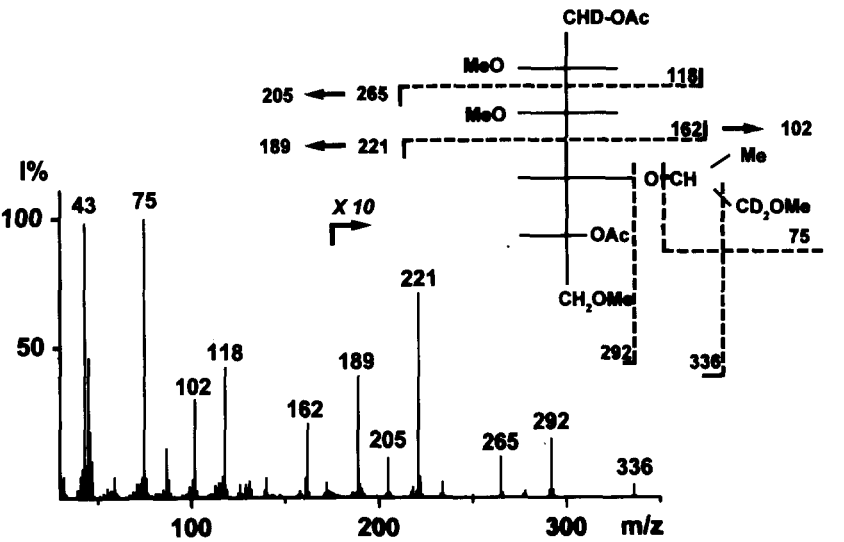


Fig. 6. Electron impact (70 eV) mass spectrum and relative fragmentation scheme of the carboxyl-reduced partially methylated alditol acetate of the 4-lactylmannose sample isolated from the partial hydrolysate of the exopolysaccharide from *Cyanospira capsulata*.

and β forms of mannose and 4-lactylman. This upfield shift is quite normal when going from the reducing to the glycosidic form of monosaccharides. The signals at 4.94 and 4.73 ppm can therefore be assigned to the anomeric protons of mannose and 4-lactylman, although it is not possible to distinguish between them.

Mannose is present in the CC-EPS in equimolar ratio with the other neutral sugars [6] and this must be true of 4-lactylman too, because of the equal intensity of the respective anomeric signals. The intensity ratio of 1:2 between the GlcNAc methyl at 2.05 ppm and those of fucose and 4-lactylman at 1.38 ppm indicates that the aminosugar is also present in equimolar ratio with 4-lactylman and the other neutral monosaccharides.

In conclusion, the exopolysaccharide from *Cyanospira capsulata* is composed of seven monosaccharides: fucose, arabinose, glucose, mannose, *N*-acetylglucosamine, 4-*O*-[1-carboxyethyl]mannose and galacturonic acid. All monosaccharide are present in equimolar ratio except GalA which has a molar ratio of 2:1 with respect to the other sugars.

Previous studies [5,6] and our evidence point towards a stereoregular structure with an octasaccharide repeating unit. Cesàro et al. [6] measured an equivalent weight of 490 for CC-EPS by potentiometric tritation. This value agrees with the theoretical equivalent weight of 464 calculated for an octasaccharide repeating unit with three carboxylic groups. In view of the interesting properties of the aqueous solutions of CC-EPS, it is worthwhile to define the structure of its repeating unit. Further work is now under way and will be reported shortly.

1. Experimental

Materials.—The sample of the exocellular polysaccharide from *Cyanospira capsulata* (CC-EPS) was kindly provided by Professor M. Vincenzini of the University of Firenze (Italy). CC-EPS was received as a crude precipitate from a culture broth of *Cyanospira capsulata* [2], grown in the conditions already described. Samples were isolated by precipitation with isopropyl alcohol from the cell-free supernatants of cultures of constant ages. 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. Partial depolymerization of the polymer was carried out on a solution of CC-EPS which was acidified to pH 3.5 by the addition of 0.1 M HCl and incubated at 100°C for 3–6 h. The sample was then neutralized, filtered and recovered by freeze-drying prior to NMR spectroscopic analysis

Monosaccharide analysis.—Analysis of monosaccharides by liquid chromatography was carried out by acid hydrolysis with 0.5 mL of 2 M trifluoroacetic acid (TFA) on 200 μg samples of CC-EPS at 120°C for 3 h. The TFA was then removed at 40°C with a stream of dry air and successive coevaporation with isopropyl alcohol. The high performance anion exchange (HPAE) apparatus consisted of a gradient pump equipped with a CarboPac PA-1 column (0.4 × 25 cm) and a pulsed amperometric detector (PAD) all from Dionex. Elution was 20 min with NaOH 16 mM, during which the neutral and basic sugars were eluted, and then by a NaOH and sodium acetate gradient from 16 to 200 mM and from 0 to 400 mM in 25 min, respectively. Sugar analysis by gas chromatography was performed using the trimethylsilyl ether derivatives, as described by York et al. [10], but in addition an acid treatment (TFA 2 M at 120°C for 3 h) was carried out before the methanolysis and the re-*N*-acetylation step (pyridine/acetic anhydride in methanol at room temperature for 25 min). 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) using helium as the carrier gas. Injections were made in the split mode with a 1 : 20 split ratio. A colorimetric assay for aminosugars was performed according to Johnson [7].

Partial acid hydrolysis of CC-EPS and isolation of 4-lactylmannose.—600 mg CC-EPS was partially hydrolysed with 400 ml 2 M TFA at 80°C for 2 h and then evaporated with isopropyl alcohol before applying it to a column (2.2 × 28.5 cm) of Sephadex DEAE A-25-120 (Sigma, St Louis, Missouri, equilibrated with a 50 mM buffer at pH 7.0) in 25 mL of phosphate buffer. HPAE-PAD analysis of the products eluted with an initial column wash with buffer showed that it is composed mainly of neutral monosaccharides. The retained material was eluted with a linear gradient from 0 to 350 mM of NaCl in 850 mL of phosphate buffer. Fractions (12 mL) were collected and assayed for hexose and uronic acid content using the anthrone and meta-hydroxybiphenyl assays respectively, as described in Ref. [9]. Six resolved peaks were obtained and the third peak, corresponding to 4-lactylman, was concentrated to 5 mL at 40°C under vacuum and injected into two consecutive columns (1.6 × 90 cm) of BioGel P2 (Biorad, Richmond, California) eluted with bidistilled water at room temperature at 10 mL h^{-1}. Fractions (3.2 mL) were collected and subjected to the anthrone assay. A single

broad peak was obtained and the BioGel P2 fractions were assayed by injecting them in the HPAE-PAD system. Essentially only one product was detected and the purest fractions were pooled together and analysed as described below.

Lithium reduction of 4-lactylman.—The reaction was conducted as described by Lau et al. [8]. About 250 μg of 4-lactylman was dissolved in 250 μL of anhydrous ethylenediamine in a screw-capped test tube with a magnetic stirring bar. Small pieces (2–3 mm) of lithium wire were added over a 1 h period in order to maintain the deep blue colour that develops when lithium is dissolved in ethylenediamine. Excess lithium was destroyed by slowly adding 4 mL of water to the ice cooled reaction which was then mixed with 20 mL of toluene and vacuum evaporated three times. The residue was then dissolved in 5 mL of bidistilled water, brought to pH 5 with AcOH and desalted by elution through a 10 mL column of Dowex 50 W × 8. The eluate was dried and treated with 300 μL of a 1:1 mixture of pyridine/acetic anhydride at 120°C for 20 min, partitioned between water and chloroform and then the organic phase was analysed by GC-MS.

Preparation of the carboxyl-reduced partially methylated alditol acetate of 4-lactylman.—About 100 μg of 4-lactylman was treated at 80°C for 1.5 h with 150 μL of 1 M HCl in anhydrous methanol. The reaction was then dried three times at 40°C with a stream of dry air after *t*-butyl alcohol was added. In order to reduce the methyl ester group, 300 μL of $Li(Et)_3BD$ 1 M in tetrahydrofuran (Super-Deuteride, Aldrich, Milwaukee, Wisconsin) was added and the reaction was kept at room temperature for 1 h, after which AcOH was added to the ice-cooled reaction till no further gas evolved. The carboxyl-reduced sugar was methylated and then deuterium reduced at C-1 by standard procedures [10,11].

NMR analysis.—Samples were exchanged three times with D_2O (99.9 atom% D, Aldrich) by evaporation at 40°C under a stream of dry air and finally dissolved in D_2O (99.96 atom% D, Aldrich). Spectra were run on an AC 200 F Bruker instrument (200 MHz for ^1H) interfaced with an Aspect 3000 computer using the Bruker DISR90 acquisition software. 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 room temperature for monosaccharides and at 60°C for the partially depolymerized CC-EPS. Chemical shifts are expressed in ppm from internal acetone (2.225 ppm for ^1H and 31.07 ppm for ^{13}C). The data of the COSY 90 experiment were acquired using the Bruker microprogram COSY.AU with a 415 Hz spectral width (SW) in 256 data points for F1 and 830 Hz SW in 1024 data points for F2. Data points were zero-filled in F1 and multiplied by an unshifted sinebell function in both dimensions before Fourier transformation. The $^1H-^{13}C$ chemical shift correlated experiment (HETCOR) data were acquired with the Bruker microprogram XHCORR.AU with a 421 Hz SW in 256 data points for F1 (^1H) and 4167 Hz in 4096 data points for F2 (^{13}C). Data points were zero-filled in F1 and multiplied by an unshifted sinebell function in F1 and by an exponential function in F2 before Fourier transformation.

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) with helium as the carrier gas and split injection with a 1:10 split ratio. The quadrupole analyser was set to scan from 30 to 400 m/z with 0.9 s scan time and 0.1 interscan delay. For FABMS a

Kratos MS 50 interfaced with an Eclipse computer (Data General) running under the DS90 Kratos software was used. Spectra of 4-lactylman were acquired in the negative and positive ion mode using glycerol as the FAB matrix.

Acknowledgments

The authors thank Dr M. Bosco, POLY-biòs Research Centre, Trieste, for recording the NMR spectrum of the polysaccharide. Work was carried out with grants from the Italian Ministry of University and Scientific Technological Research and from the Progetto Finalizzato Chimica Fine II, through CNR (Roma). One of the authors (L.S.) thanks the CNR for a fellowship.

References

[1] V. Crescenzi, *Trends Polym. Sci.*, 2(3) (1994) 104–109.
[2] G. Florenzano, C. Sili, E. Pelosi, and M. Vincenzini, *Arch. Microbiol.*, 140 (1985) 301–306.
[3] C. Sili, E. Pelosi, M. Vincenzini, R. Materassi, and G. Florenzano, *Proc. Eur. Congr. Biotechnol.*, Verlag Chemie, Weinheim, 1984, Vol. 1, pp. 91–96.
[4] A. Flaibani, Y. Olsen, and T.J. Painter, *Carbohydr. Res.*, 190 (1989) 235–248.
[5] M. Marra, A. Palmeri, A. Ballio, A. Segre, and M.E. Slodky, *Carbohydr. Res.*, 197 (1990) 338–344.
[6] A. Cesaro, G. Liut, C. Bertocchi, L. Navarini, and L. Urbani, *Int. J. Biol. Macromol.*, 12 (1990) 79–84.
[7] A.R. Johnson, *Anal. Biochem.*, 44 (1971) 628–635.
[8] J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 168 (1987) 219–243.
[9] N.K. Kochetkov, A.F. Sviridov, K.A. Arifkhodzhaev, O.S. Chizhov, and A.S. Shashkov, *Carbohydr. Res.*, 71 (1979) 193–203.
[10] W.S. York, A.G. Darvill, M. Mc Neil, T.T. Stevenson, and P. Albersheim, *Methods Enzymol.*, 118 (1985) 3–40.
[11] B.S. Valent, A.G. Darvill, M. McNeil, B.K. Robertsen, and P. Albersheim, *Carbohydr. Res.*, 79 (1980) 165–192.