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Structural characterisation of a new *O*-methylated heteroglycan, colleman, from the cyanolichen *Collema flaccidum*

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ABSTRACT

An alkali-extractable heteroglycan, colleman, was isolated from the cyanolichen *Collema flaccidum*, using ethanol fractionation and anion-exchange chromatography. The average molecular weight was estimated to be 360 kDa. Structural characterisation of the heteroglycan was performed by high-field NMR spectroscopy (1D proton, 2D-COSY, NOESY, 2D-TOCSY, ^{1}H ^{13}C-HSQC, HMBC, H2BC and HSQC-NOESY).

According to the data obtained, the structure of colleman is composed of repeating units of **A**, **B**, **C** and **D** in approximate molar ratio 5:5:2:1;

A: [3)2-OMe-β-Man*p*-(1→4)2-OMe-α-Ara*p*-(1→4)-β-Glc*p*A-(1→4)-β-Xyl*p*-(1→]n

B: [3)2-OMe-β-Man*p*-(1→4)-α-Ara*p*-(1→4)-β-Glc*p*A-(1→4)-β-Xyl*p*-(1→]n

(1→6)-β-Glc*p*

C: [3)2-OMe-β-Man*p*-(1→4)-α-Gal*p*-(1→4)-β-Xyl*p*-(1→4)-β-Xyl*p*-(1→]n

(1→6)-β-Glc*p*A

D: [3)2-OMe-β-Man*p*-(1→4)-α-Gal*p*-(1→4)-β-Xyl*p*-(1→4)-β-Xyl*p*-(1→]n

1. Introduction

Lichens are slow-growing symbiotic organisms consisting of a fungus and one or more photobionts that can either be algae or cyanobacteria (Nash, 1996; Rai & Bergman, 2002). Of the 13,500 lichen species growing worldwide (Hawksworth & Hill, 1984), less than 100 species have been investigated for their polysaccharide content (Olafsdottir & Ingolfsdottir, 2001). Lichenized ascomycota produce polysaccharides of three main structural types; linear or lightly substituted α- and β- glucans and branched galactomannans (Olafsdottir & Ingolfsdottir, 2001). In addition a few complex heteroglycans have been described (Iacomini, Zanin, Fontana, Hogge, & Gorin, 1987; Ingolfsdottir, Jurcic, Fischer, & Wagner, 1994; Olafsdottir et al., 1999a; Omarsdottir et al., 2006a; Prado, Gorin, Stuelp, Honda, & Iacomini, 1999; Sassaki et al., 2005). Lichen polysaccharides, which can be isolated in high yield, such as the glucans and galactomannans, are generally considered to be of fungal origin (Gorin, Baron, & Iacomini, 1988; Gorin, Baron, Silva, Teixeira, & Iacomini, 1993). This has been supported by comparing the polysaccharides isolated from the intact lichen with those produced by the aposymbiotically cultured mycobionts and photobionts (Cordeiro, de Oliveira, Buchi, & Lacomini, 2008; Cordeiro, Sassaki, & Iacomini, 2007; Cordeiro, Stocker-Worgotter, Gorin, & Iacomini, 2004; Ruthes et al., 2008; Takahashi, Takeda, & Shibata, 1979).

About 10% of all lichen species have cyanobacteria as the main or only photosynthetic partner (Nash, 1996; Rai & Bergman, 2002). In addition to photosynthesis, cyanolichens have the ability to fix atmospheric nitrogen for metabolic use (Carbonero, Tischer, Cosentino, Gorin, & Iacomini, 2003; Nash, 1996; Rai & Bergman, 2002).

Although for most lichen species the fungus forms the majority of the lichen biomass, the cyanolichens of the genera *Collema* are commonly called "jelly lichens" and contain cyanobacteria as the dominant partner of the symbiosis (Purvis, 2000; Rai & Bergman, 2002).

Collema flaccidum is a "jelly lichen" belonging to the family Collemataceae. Its symbiosis consists of ascomycota fungus and a

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Nostoc cyanobacterium (Honegger, 1991; Purvis, 2000). *C. flaccidum* is a robust creature which grows on cliffs and bare rocks and becomes gelatinous in the presence of water. Only one *Collema* species has been investigated for polysaccharide content before, i.e. *Collema leptosporum*, described as containing a galactoglucomannan and a (1 → 3) (1 → 6) branched β-glucan in addition to an uronic containing fraction which was not further specified (Prado et al., 1999). Structural characteristics of polysaccharides isolated from species of two other genera of cyanolichens have been described earlier; a galactomannan from *Leptogium azureum* (Carbonero et al., 2003), a highly branched galactomannan from *Peltigera canina* (Omarsdottir et al., 2006a) and a galactoglucomannan from *Peltigera aphthosa* (Gorin & Iacomini, 1985).

The aim of the present study is to characterise the structure of a new heteroglycan isolated from *Collema flaccidum* in detail, using high-field (800 MHz for ^{1}H) 1D and 2D nuclear magnetic resonance spectroscopy; COSY, NOESY, TOCSY, HSQC, HSQC-NOESY, HSQC-TOCSY, HMBC and H2BC and to provide useful NMR reference data for future studies on complex heteroglycans.

2. Experimental

2.1. Biological material

The lichen *Collema flaccidum* (Ach.) Ach. (Collemataceae) was collected in Akrafjall, in south-western Iceland in September 2004. The lichen was identified by S. Baldursdottir, lichenologist, Reykjavik, Iceland and by Dr. H. Kristinsson, lichenologist. A voucher specimen, Catalogue No. LA-31132 is deposited at the Herbarium of the Icelandic Institute of Natural History, Akureyri, Iceland.

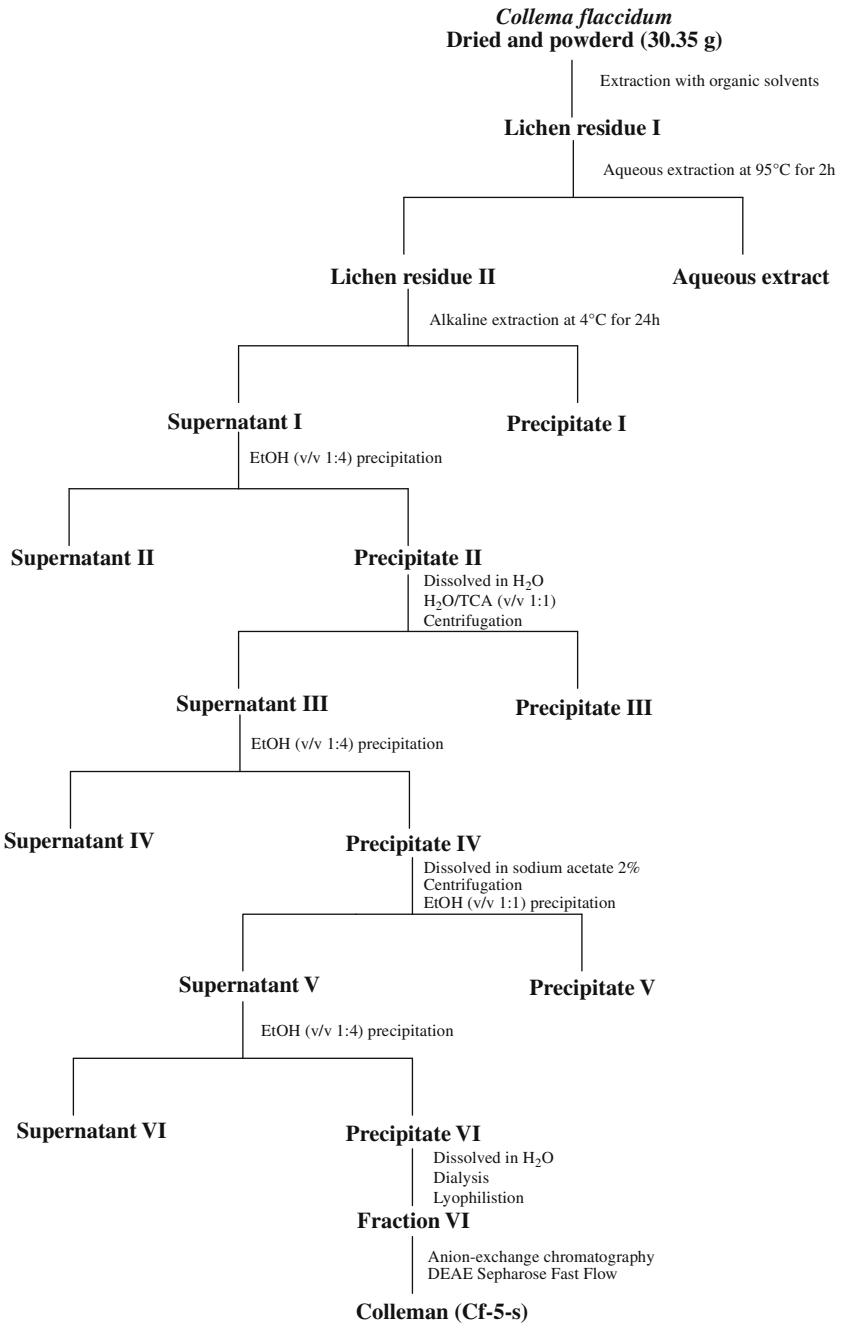

Fig. 1. Fractionation of the heteroglycan colleman (Cf-5-s), from the alkali extract of *C. flaccidum* by precipitation with ethanol, followed by anion-exchange chromatography.

2.2. Isolation and purification of the polysaccharide

The dried lichen material (30.35 g) was powdered and extracted in a Soxhlet apparatus with organic solvents (light petroleum, acetone and methanol), followed by extraction with hot (95 °C) distilled water for 2 h and hot filtration. The lichen residue from the water extraction was then extracted with 0.5 M aqueous NaOH according to Caldes (Caldes, Prescott, Thomas, & Baker, 1981) and kept at 4 °C overnight. The mixture was centrifuged and the supernatant processed further according to a previously described fractionation process (Fig. 1) (Caldes et al., 1981; Paulsen, Olafsdottir, & Ingolfsdottir, 2002) to give 604 mg of crude fraction named Cf-5. One hundred and fifty milligrams of this alkali-extractable, water-soluble fraction Cf-5 was then dissolved in 50 ml of water and purified further with anion-exchange chromatography on a 2.6 × 65 cm DEAE Sepharose Fast Flow column medium (Amersham, GE Healtcare), giving one fraction Cf-5-s using 0.25 M NaCl as the eluent. The fraction Cf-5-s was collected, dialysed (MWCO: 6–8 kDa) and lyophilized giving 73 mg (a yield of 0.24% from dry lichen material).

2.3. Determination of mean M_r and homogeneity

Homogeneity and mean M_r (relative molecular weight) of Cf-5-s was determined by HP-GPC on a Superose 6 HR 10/30 column (Amersham, GE Healthcare) eluted with 0.05 M sodium phosphate buffer pH 6.0, containing 0.15 M NaCl, with a flow rate of 0.5 ml/min, using refractive index detection (Hewlett Packard 1047A RI detector). The samples were applied in 1% solutions in the mobile phase, and the injected volume was 20 μl. For the M_r-estimation, calibration was performed using dextrans of known M_r (T10, T40, T70, T250, T500 and T2000, Amersham, GE Healthcare).

2.4. Enzymatic degradation

Cf-5-s (6.5 mg) was dissolved in 0.6 mL of D_2O and the pH adjusted to six using HCl. The solution was incubated with 40 μl of endo-1,4-xylanase from *Thermomonospora fusca* (Irwin, Jung, & Wilson, 1994) (Accession No. U01242 in GenBank, expressed in *Escherichia coli* and purified) and 40 μL of xylanase purified at Carlsberg Laboratorium from the fungus *Humicola insolens* expressing a synthetic gene encoding a 22-kDa xylanase in *Pichia pastoris* (Xyn prep 10, 22 # 33) at room temperature for several days.

Both enzymes were shown to be active when added to an α,β-1,4-xylan (6.5 mg) isolated from Birchwood dissolved in 0.6 mL of D_2O and incubated for 18 h at room temperature.

2.5. Nuclear magnetic resonance spectroscopy

The NMR chemical shifts were assigned from 2D homo- and heteronuclear experiments at 799.4 MHz for proton and

Table 1
^{1}H (800 MHz) and ^{13}C (201) MHz NMR chemical shifts (δ), NOE and HMBC correlations, for the heteroglycan Cf-5-s, isolated from the lichen *Collema flaccidum*.

		1	2	3	4	5a	5b/6a	6b	OMe	NOE/HMBC
A										
3)2-OMe-β-Man*p*	H	4.8	4.02	3.82	3.57	3.35	3.9	3.71	3.61	4.05
	C	103.5	78.6	80.6	66.5	77.3	62.2		62.4	79.5
4)2-OMe-α-Ara*p*	H	5.72	3.56	3.95	4.05	3.87	3.79		3.51	3.8
	C	97.2	78.9	68.4	79.5	63.8			58.8	76.5
4)β-Glc*p*A	H	4.56	3.34	3.72	3.8	3.86				3.81
	C	101.9	74.1	77.6	76.5	77.3	175.5			77.7
4)β-Xyl*p*	H	4.57	3.39	3.61	3.83	4.11	3.39			3.82
	C	101.6	73.7	75	77.7	64				80.6
B										
3)2-OMe-β-Man*p*	H	4.8	4.02	3.82	3.57	3.35	3.9	3.71	3.61	4.05
	C	103.5	78.6	80.6	66.5	77.3	62.2		62.4	79.4
4)α-Ara*p*	H	5.5	3.86	3.93	4.05	3.88	3.81			3.76
	C	99.9	69.7	69.6	79.4	64				77.4
4)β-Glc*p*A	H	4.56	3.35	3.75	3.76	3.8				3.81
	C	101.9	74	77.4	77.4	77.5	175.5			77.7
4)β-Xyl*p*	H	4.57	3.39	3.61	3.83	4.11	3.39			3.82
	C	101.6	73.7	75	77.7	64				80.6
C										
3)2-OMe-β-Man*p*	H	4.83	4.06	3.81	3.57	3.34	3.9	3.71		4.16
	C	103	78.5	80.6	66.6	77.3	62.2			77.9
β-Glc*p*(1–6)	H	4.54	3.31	3.51	3.39	3.47	3.93	3.73		3.85
	C	104	74	76.6	70.8	77.1	61.9			70
4.6)α-Gal*p*	H	5.18	3.9	3.99	4.16	3.83	4.2	3.85		3.67
	C	101.3	69.7	70.4	77.9	71.8	70			79
4)β-Xyl*p*	H	4.47	3.32	3.65	3.67	4.18	3.43			4.10/3.80
	C	102.7	73.7	75.6	79	65.3				77.7
4)β-Xyl*p*	H	4.57	3.39	3.61	3.8	4.11	3.39			3.81
	C	101.6	73.7	75	77.7	64				80.6
D										
3)2-OMe-β-Man*p*	H	4.83	4.06	3.81	3.57	3.34	3.9	3.71		4.16
	C	103	78.5	80.6	66.6	77.3	62.2			77.9
β-Glc*p*A(1–6)	H	4.52	3.35	3.52	3.54	3.79				3.86
	C	104.1	74	76.5	72.8	76.7	175.5			71
4.6)α-Gal*p*	H	5.18	3.9	3.98	4.16	3.83	4.2	3.86		3.67
	C	101.2	69.6	70.4	77.9	71.8	71			79
4)β-Xyl*p*	H	4.47	3.32	3.65	3.67	4.18	3.43			4.10/3.80
	C	102.6	73.7	75.6	79	65.3				77.7
4)β-Xyl*p*	H	4.57	3.39	3.61	3.8	4.11	3.39			3.81
	C	101.6	73.7	75	77.7	64				80.6

201.12 MHz for carbon, using acetone as reference for proton (δ2.22 ppm) and for carbon (δ30.9 ppm).

One mg of Cf-5-s was dissolved in 0.6 mL of D_2O and spectra were recorded on a BRUKER AVANCE 800 equipped with cryo probe in a 5 mm tube. All spectra were obtained with the temperature set to 33.5 °C.

In addition to 1D proton spectra a series of 2D spectra were obtained. The double quantum-filtered phase-sensitive COSY experiment were performed using the Bruker standard program DQFCOSY, with 0.3 s acquisition time and 4096 data points in the F2 dimension. The data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Similar, the nuclear Overhauser experiment was performed using the Bruker standard noesyphpr, with a mixing time of 300 ms. With the same amount of data points the TOCSY experiment was performed using standard Bruker program mlevphpr with a spinlock time of 80 ms.

In addition to 1D carbon spectra a series of heteronuclear experiments was performed using pulse field gradient programs HSQC, HMBC, HSQC-TOCSY, HSQC-NOESY and H2BC. All heteronuclear experiments were recorded with 2048 points in F2 and 1024 in F1 and zero-filled in both dimensions.

The spectra were assigned using the computer programs Topspin® and Pronto (Kjaer, Andersen, & Poulsen, 1994), which allows the simultaneous display of different two-dimensional spectra and the individual labelling of cross peaks.

3. Results and discussion

Cf-5-s was obtained from an alkali extract of *Collema flaccidum* followed by ethanol precipitation and anion-exchange chromatography. Cf-5-s was eluted as a single peak in HP-GPC and the mean M_r was determined to be 360 kDa by comparison to dextran standards.

The structure of Cf-5-s was established by ^1H and ^{13}C NMR spectroscopy and chemical shifts were assigned as shown in Table 1 using a series of 2D experiments. In addition, assignments of the chemical shifts of enzymatically hydrolysed polysaccharide fragments were used to support the assignment of the signals obtained from the intact heteroglycan.

The anomeric region of the COSY spectrum of Cf-5-s, can be divided into four major regions which showed well indicated anomeric signals (Fig. 2). The signals in the region at δ 5.50 and 5.72 ppm were assigned to anomeric protons of α-Ara*p* residues from the chemical shifts shown in Table 1, the 1H–1H three bond coupling constants ($J_{1,2} \sim 4$ Hz; $J_{2,3} \sim 10$ Hz; $J_{3,4} \sim 4$ Hz) and the characteristically small couplings (~1 to 3 Hz) between H-4 and H-5. The splitting of the anomeric α-Ara*p* signals into a major and a minor peak was not assignable, but probably originates from the order of the linking of different **A**, **B**, **C** and **D** units.

The signal at δ 5.18 ppm correlating to H2 at δ 3.90 were assigned to anomeric proton of α-Gal*p* residue, from the chemical shifts shown in Table 1, the 1H–1H three bond coupling constants ($J_{1,2} \sim 4$ Hz; $J_{2,3} \sim 10$ Hz; $J_{3,4} \sim 4$ Hz) and the characteristically small coupling (~1 Hz) between H-4 and H-5 (Bock, Pedersen, & Pedersen, 1984; Bock & Thøgersen, 1982; Duus, Gotfredsen, & Bock, 2000). Two signals at δ 4.83 and 4.80 ppm (Table 1) were assigned to the anomeric protons of β-Man*p* residues according to standard chemical shifts, the 1H–1H three bond coupling constants ($J_{1,2} \sim 1$ Hz; $J_{2,3} \sim 4$ Hz; $J_{3,4} \sim 10$ Hz). The small coupling constants $J_{1,2}$ and $J_{2,3}$ give rise to the characteristically very weak cross-peak in the phase-sensitive cosy (Bock et al., 1984; Bock & Thøgersen, 1982; Duus et al., 2000).

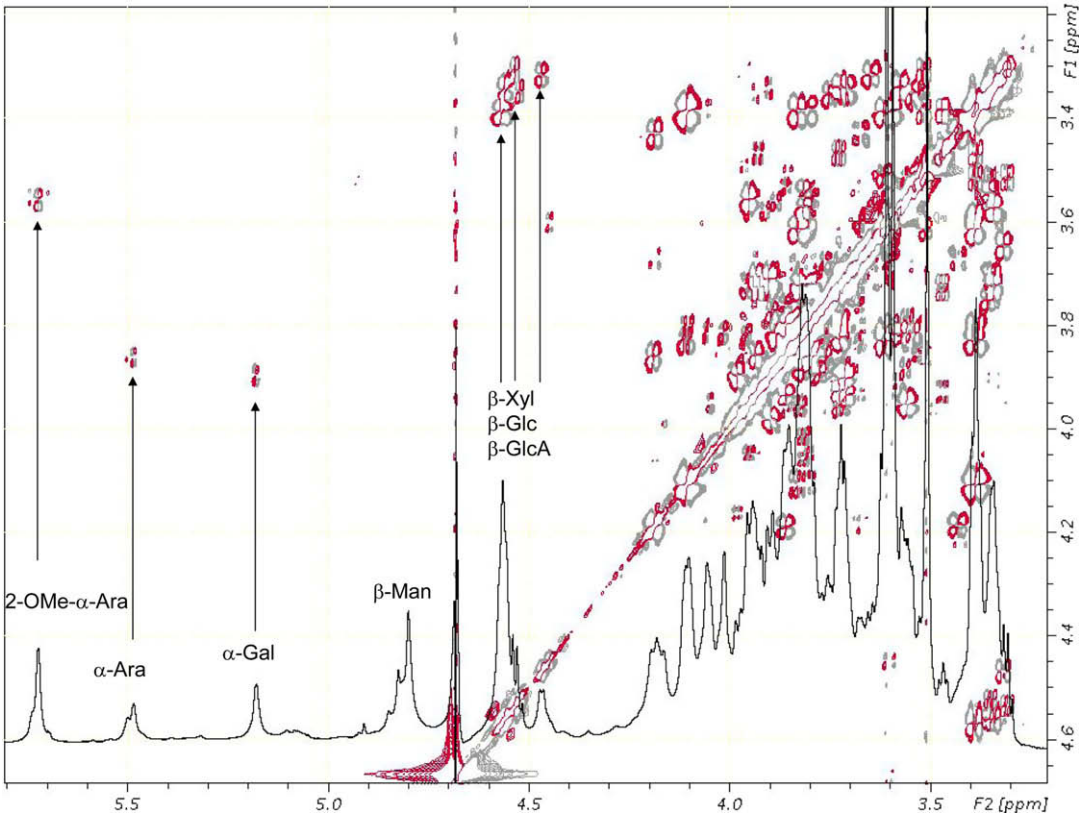

Fig. 2. 1D proton spectrum of colleman (Cf-5-s) with labelling of different anomeric signals overlaid with the DQFCOSY spectrum. Notice the weak anomeric signals in the COSY spectrum from β-Man*p* due to small coupling constants. The spectra shown represent the intact polysaccharide.

The remaining anomeric signals in the region δ 4.47–4.57 ppm were assigned to β-Xyl*p*, β-Glc*p*A and β-Glc*p* residues. The β-Xyl*p* residues were identified through upfield anomeric chemical shifts, coupling constants ($J_{1,2} \sim 8$ Hz; $J_{2,3} \sim 9$ Hz; $J_{3,4} \sim 9$ Hz) and the COSY correlations within their spin systems ended at H-5 with a well indicated cross-peak at δ 4.11/3.39 ppm and δ 4.18/3.43 ppm (Table 1) (Bock et al., 1984; Bock & Thøgersen, 1982; Duus et al., 2000).

The anomeric signals at δ 4.52 and 4.56 ppm were assigned to β-Glc*p*A residues from the chemical shifts (Table 1), the 1H–1H three bond coupling constants ($J_{1,2} \sim 8$ Hz; $J_{2,3} \sim 10$ Hz; $J_{3,4} \sim 10$ Hz), strong COSY and TOCSY cross-peaks between H1 and H-2,3,4, 5 and NOE correlations between H1–H3 and H1–H5, and a long range proton/carbon correlation between H5 and C6 carbonyl at δ 175.5 ppm. The anomeric signals at δ 4.54 ppm were assigned to β-Glc*p* residues from the chemical shifts (Table 1), the 1H–1H three bond coupling constants ($J_{1,2} \sim 8$ Hz; $J_{2,3} \sim 10$ Hz; $J_{3,4} \sim 10$ Hz), strong COSY and TOCSY cross-peaks between H1 and H-2,3,4,5, and 6a/6b and NOE correlations between H1–H3 and H1–H5 (Table 1) (Bock et al., 1984; Bock & Thøgersen, 1982; Duus et al., 2000).

Furthermore, inter-residual NOE and HMBC correlations in units **A** and **B** (δ 4.80/4.05; 79.5 ppm) were assigned between H-1 of β-Man*p* and H-4/C4 of α-Ara*p*, thus establishing a β-Man*p*(1 → 4)-α-Ara*p* linkages correlations (δ 5.72/3.80; 76.5 ppm in unit **A** and δ 5.50/3.76; 77.4 ppm in unit **B**) were inferred between the anomeric proton of the α-Ara*p* residues and H-4 and C-4 of residues β-Glc*p*A. Moreover, correlations (δ 4.56/3.83; 77.7 ppm) between H-1 of β-Glc*p*A and H-4 of β-Xyl*p* revealed that these residues are correspondingly (1 → 4)-linked and correlations (δ 4.57/3.82; 80.6 ppm) between H-1 of Xyl*p* and H-3 and C-3 of β-Man*p* shows that these residues are (1 → 3)-linked (Table 1).

In addition, NOE and HMBC correlations in units **C** and **D** (δ 4.83/4.16; 77.9 ppm) were assigned between H-1 of β-Man*p* and H-4/C4 of α-Gal*p*, thus establishing a β-Man*p*(1 → 4)-α-Gal*p* linkages and correlations (δ 4.54/4.20, 3.85; 70.0 ppm) between H-1 of β-Glc*p* and H-6 of β-Gal*p* (unit **C**) and (δ 4.52/4.20, 3.86; 71.0 ppm) between H-1 of β-Glc*p*A and H-6 of β-Gal*p* (unit **D**) revealed that terminal β-Glc*p* (unit **C**) or β-Glc*p*A (unit **D**) are (1 → 6) linked to α-Gal*p* residues. Furthermore, correlations (δ 5.18/3.67; 79.0 ppm) between H-1 of α-Gal*p* and H-4 of β-Xylp_a and (δ 4.47/3.80; 77.7 ppm) between H-1 of β-Xylp_a and H-4 of β-Xylp_b revealed that these residues are correspondingly (1 → 4) linked and correlations (δ 4.57/3.81; 80.6 ppm) between H-1 of Xyl*p* and H-3 and C-3 of β-Man*p* shows that these residues are (1 → 3)-linked.

The remaining ^{1}H and ^{13}C signals (Table 1) of the Cf-5-s polysaccharide were established through a combination of COSY, TOCSY, NOESY, HMBC, HSQC, H2BC HSQC-TOCSY and HSQC-NOESY experiments.

The anomeric carbon signals were identified in the region of the HSQC spectrum with resonances between δ 97.2 and 104.1 ppm (Table 1) (Fig. 3). All other carbon signals were assigned from proton/carbon correlations in the region between δ 60 and 82 ppm

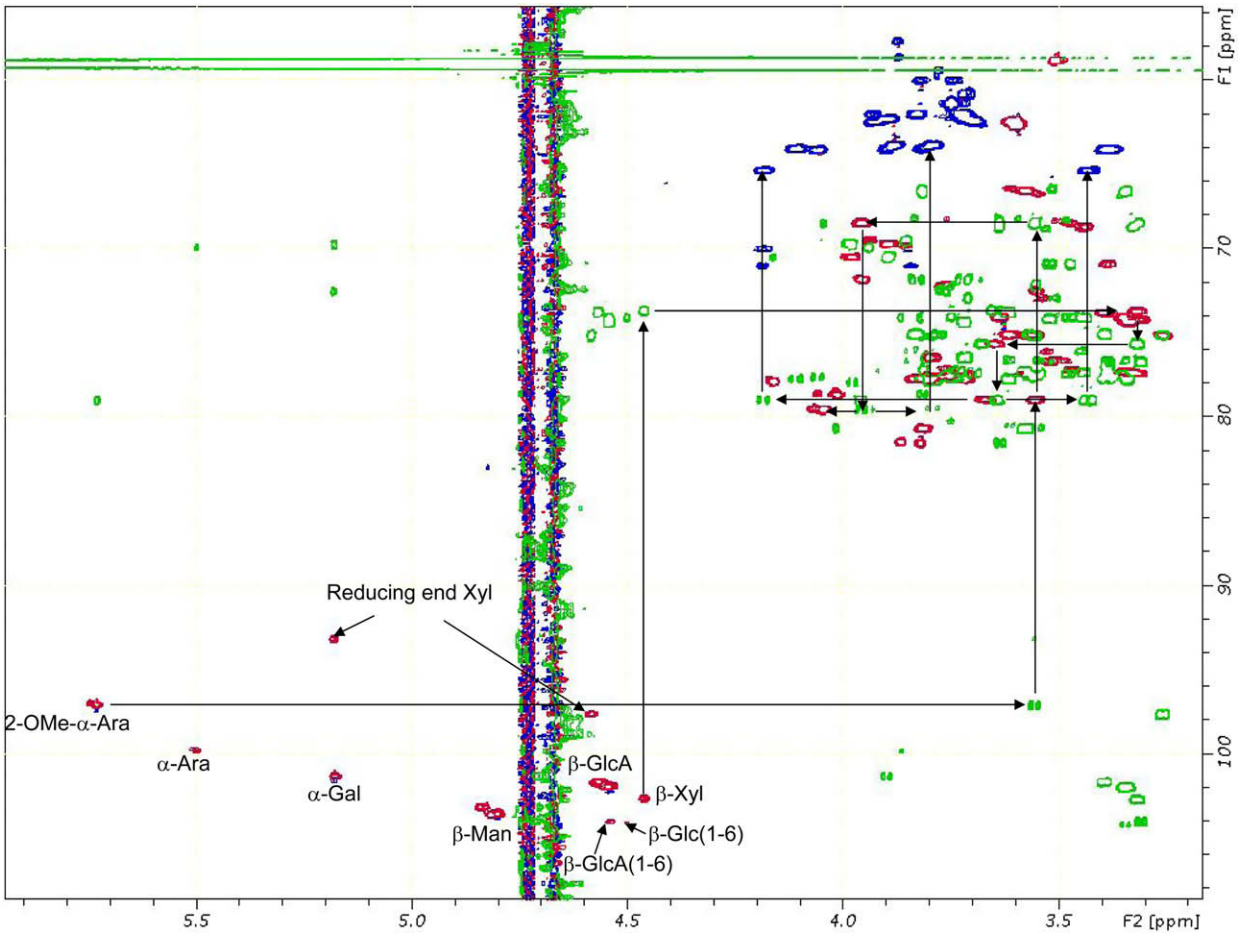

Fig. 3. HSQC (red: CH, blue: CH_2) spectrum of colleman (Cf-5-s) overlaid with the H2BC (green) spectrum. The intra-correlation of Xyl*p* C1–C2 and further onto C3, C4 and C5, is assigned by H2BC (showing H–C–C cross-peaks). Similar for the Ara*p* there are correlations from C1 to C2, C3, C4 and C5. The spectra shown represent the hydrolysed polysaccharide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(61.9 and 80.6). Downfield shifts for β-Man*p* C-3 at δ 80.6 ppm, β-Ara*p* C-4 at δ 79.4–79.5 ppm, β-Glc*p*A C-4 at δ 76.5 ppm, β-Xyl*p* C-4 at δ 77.7 and 79.0 ppm, α-Gal*p* C-4 at δ 77.9 ppm and C-6 at δ 70.0–71.0 ppm established the linkage positions for these residues (Table 1) (Bock et al., 1984). HSQC in combination with HMBC, HSQC-TOCSY and H2BC were used to assign C-2, C-3, C-4, C-5 and C-6 of the residues.

Three methoxy resonances (δ 3.61/62.4, 3.51/58.8 and 3.61/62.4 ppm) (Bock & Thøgersen, 1982) correlated to signals for H-2 of residues β-Man*p* (units **A** and **B**) and α-Ara*p* (unit **B**), establishing the 2-OMe-linkages. Strong carbonyl carbon signals in the HSQC spectrum at δ 175.5 ppm correlated through the HMBC to C-5/H-5 of residues β-Glc*p*A, (units **A**, **B** and **D**) (Table 1). In addition, the xylanase hydrolysed polysaccharide was assigned to consist of separated **A**, **B**, **C** and **D** units (larger fragments were present but they are not included in Table 1) with Xyl*p* at the reducing end, and the OMe-β-Man*p* unsubstituted at C3 (δ 3.63/73.6 ppm). The hydrolysed polysaccharide gave sharper and clearer spectra and the assignments are shown in Figs. 3 and 4 and Table 2.

Assignment of all ^{1}H and ^{13}C NMR signals for monosaccharide units of the polysaccharide Cf-5-s were accomplished and the complete analysis allowed revealing of the primary structure with approximate ratio of units **A**:**B**:**C**:**D**, 5:5:2:1. The authors suggest the trivial name colleman for the lichen polysaccharide Cf-5-s.

Generally free living cyanobacteria produce complex exopolysaccharides composed of 6–10 different monosaccharide residues both hexoses, deoxyhexoses and pentoses. These glycans are anionic in nature, which is due to the presence of acidic sugars like GlcA and GalA (De Philippis, Sili, Paperi, & Vincenzini, 2001; Otero & Vincenzini, 2003). In addition some have been shown to contain sulphated or amino sugars, and in rare cases methylated sugar units have been described, such as OMe Rha (Hu, Liu, Paulsen, Petersen, & Klaveness, 2003), 2-OMe Glc*p* (Brull et al., 2000) and 3-OMe Ara*f* (Volk, Venzke, & Blaschek, 2007). The photobiont of *Collema flaccidum* is a *Nostoc* cyanobacteria. Previously, exopolysaccharides of three species of unlichenised field growing *Nostoc* cyanobacetria, *Nostoc commune*, *Nostoc flagelliforme*, *Nostoc sphaeroides*, have been reported to consist of 1,4-linked Glc, Xyl and Gal residues, Man was also present and Ara was detected in a small amount in *N. flagelliforme* (Huang, Liu, Paulsen, & Klaveness, 1998). Further structural investigation of the *N. commune* polysaccharides revealed the presence of substantial amounts of Ara*f*, 2-OMe-Glc*p* and Glc*p*A in cultured samples (Brull et al., 2000). Structural investigation of an exopolysaccharide released by *Nostoc insulare* during photoautotrophic cultivation revealed the constituents: GlcA, Glc and Ara and for the first time 3-OMe-Ara*f* was described (Volk et al., 2007).

By comparing the results obtained in this study with earlier studies on lichens (Brull et al., 2000; Carbonero et al., 2005; Carbonero, Montai, Woranovicz-Barreira, Gorin, & Iacomini, 2002; Huang et al., 1998; Olafsdottir & Ingolfsdottir, 2001; Omarsdottir et al., 2006b; Pereyra, Prieto, Bernabe, & Leal, 2003; Prieto, Ahrazem, Bernabé, & Leal, 2004; Prieto, Leal, Bernabe, & Hawksworth, 2008; Reis, Tischer, Gorin, & Iacomini, 2002; Teixeira, Iacomini, & Gorin, 1995) the structure of colleman is different from the structure of all previously described lichen polysaccharides including the cyanolichen polysaccharides described from *Collema leptosporum* (Prado et al., 1999), *Leptogium azureum* (Carbonero et al., 2003) *P. canina*

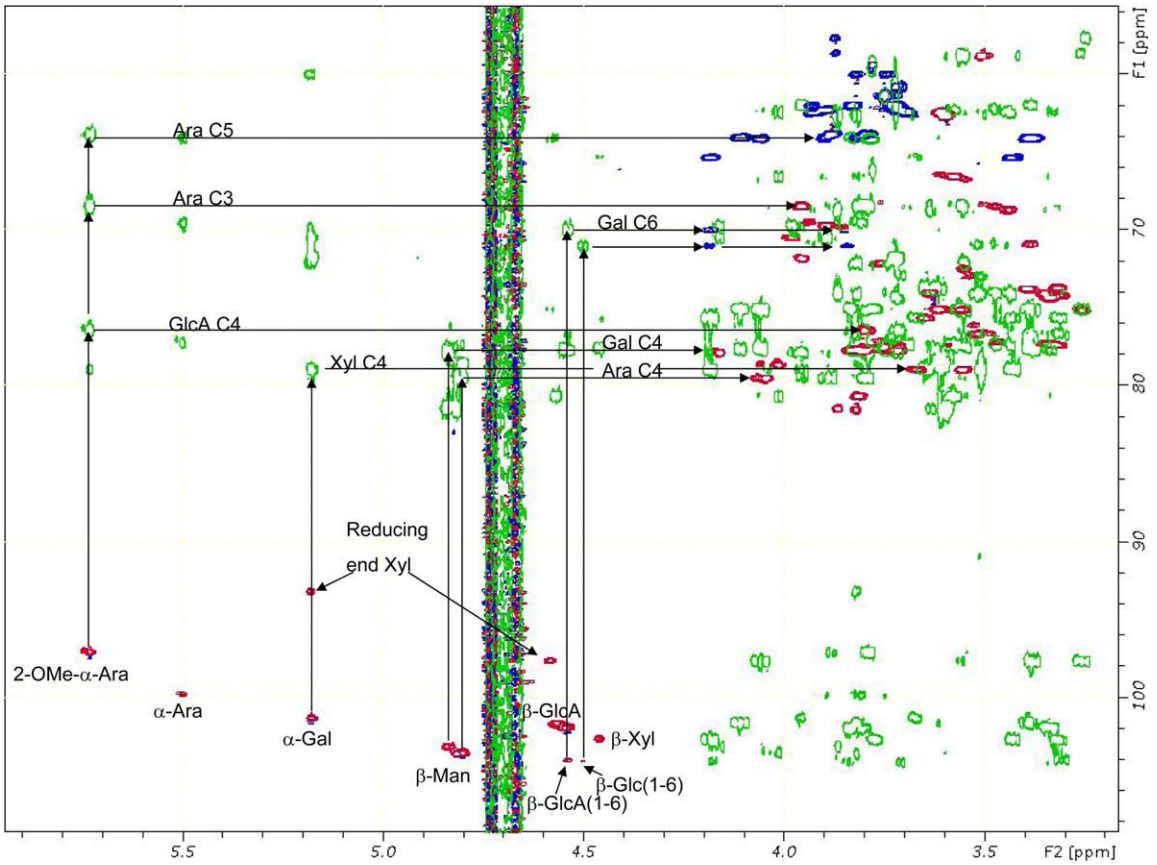

Fig. 4. The region shown is the same as shown in Fig. 3, except that here the HSQC (red: CH, blue: CH_2) spectrum is overlaid with the HMBC (green) spectrum. The assignments of intra-correlation H1–C3 and C5 of the Ara*p*, and inter-correlation from H1 of Ara*p* to C4 of Glc*p*A are shown. The assignment of the intra-correlation from the anomeric signals of the other units is also shown. The spectra shown represent the hydrolysed polysaccharide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
^{1}H (800 MHz) and ^{13}C (201) MHz NMR chemical shifts (δ), NOE and HMBC correlations, for the xylanase hydrolysed Cf-5-s.

		1	2	3	4	5a	5b/6a	6b	OMe	NOE/HMBC
A										
2-OMe-β-Man*p*	H	4.82	3.82	3.63	3.47	3.31	3.89	3.70	3.61	4.05
	C	103.1	81.1	73.6	68.0	76.9	61.9		62.0	79.1
4)2-OMe-α-Ara*p*	H	5.73	3.56	3.96	4.05	3.88	3.79			3.80
	C	96.7	78.5	68.0	79.1	63.5				76.1
4)β-Glc*p*A	H	4.55	3.35	3.75	3.80	3.81				3.80/3.77
	C	101.4	74.0	77.2	76.1	77.4	175.8			77.1/77.1
4)β-Xyl*p*	H	4.58	3.26	3.57	3.80	4.06	3.38			
	C	97.1	74.7	74.7	77.1	63.7				
4)α-Xyl*p*	H	5.18	3.55	3.75	3.77	3.82	3.75			
	C	92.7	72.1	71.7	77.1	59.6				
B										
2-OMe-β-Man*p*	H	4.82	3.82	3.63	3.47	3.31	3.89	3.70	3.61	4.05
	C	103.1	81.1	73.6	68.0	76.9	61.9		62.0	79.1
4)α-Ara*p*	H	5.50	3.86	3.94	4.05	3.90	3.82		3.51	3.80
	C	99.3	69.5	69.1	79.0	63.7			58.4	76.1
4)β-Glc*p*A	H	4.55	3.34	3.72	3.80	3.81				3.80/3.77
	C	101.4	74.0	77.2	76.1	77.4	175.8			77.1/77.1
4)β-Xyl*p*	H	4.58	3.26	3.57	3.80	4.06	3.38			
	C	97.1	74.7	74.7	77.1	63.7				
4)α-Xyl*p*	H	5.18	3.55	3.75	3.77	3.82	3.75			
	C	92.7	72.1	71.7	77.1	59.6				
C										
2-OMe-β-Man*p*	H	4.84	3.87	3.63	3.44	3.33	3.93	3.69	3.61	4.16
	C	102.6	81.0	73.6	68.3	76.9	62.0		62.0	77.5
β-Glc*p*(1–6)	H	4.54	3.31	3.52	3.39	3.47	3.94	3.73		4.18/3.85
	C	103.6	73.8	76.3	70.5	76.7	61.6			69.6
4.6)α-Gal*p*	H	5.18	3.90	3.98	4.16	3.96	4.18	3.85		3.67
	C	100.9	69.3	70.1	77.5	71.5	69.6			78.6
4)β-Xyl*p*	H	4.46	3.32	3.65	3.67	4.18	3.43			3.78/3.77
	C	102.3	73.3	75.2	78.6	64.9				77.1/77.1
4)β-Xyl*p*	H	4.58	3.26	3.55	3.78	4.06	3.38			
	C	97.1	74.7	74.7	77.1	63.7				
4)α-Xyl*p*	H	5.18	3.55	3.75	3.77	3.82	3.75			
	C	92.7	72.1	71.7	77.1	59.6				
D										
2-OMe-β-Man*p*	H	4.84	3.87	3.63	3.44	3.33	3.93	3.69	3.61	4.16
	C	102.6	81.0	73.6	68.3	76.9	62.0		62.0	77.5
β-Glc*p*A(1–6)	H	4.50	3.35	3.51	3.54	3.71				4.18/3.84
	C	103.7	74.0	76.2	72.6	77.1	176.4			70.6
4.6)α-Gal*p*	H	5.18	3.90	3.98	4.16	3.96	4.18	3.84		3.67
	C	100.9	69.3	70.1	77.5	71.5	70.6			78.6
4)β-Xyl*p*	H	4.46	3.32	3.65	3.67	4.18	3.43			3.78/3.77
	C	102.3	73.3	75.2	78.6	64.9				77.1/77.1
4)β-Xyl*p*	H	4.58	3.26	3.55	3.78	4.06	3.38			
	C	97.1	74.7	74.7	77.1	63.7				
4)α-Xyl*p*	H	5.18	3.55	3.75	3.77	3.82	3.75			
	C	92.7	72.1	71.7	77.1	59.6				

and *P. aphthosa* (Gorin & Iacomini, 1985; Omarsdottir et al., 2006a). The major differences are that colleman contains 2-OMe-β-Man*p*, and 2-OMe-α-Ara*p* residues not previously described from lichens or cyanobacteria. The 2-OMe-β-Man*p* is furthermore present in large amounts. Colleman also contains elevated amount of Xyl*p* and a considerable amount of Glc*p*A which is rare among the lichen polysaccharides although it is common amongst cyanobacteria. From the above it can be concluded that some structural features of colleman resembles those of polysaccharides isolated from the free living cyanobacterium *N. commune*. Therefore it is suggested that colleman is of cyanobacterial origin and similar polysaccharides can be expected to be found in other cyanolichens which have *Nostoc* cyanobacteria as the dominant symbiont.

It has been suggested that the presence of *O*-methylated sugar residues in heteroglycans isolated from cyanobacteria might be more common than previous studies have indicated, and the reason for this could be that the analytical methods used were not suitable for detecting these derivate sugars (De Philippis et al., 2001). The 2D NMR spectroscopy used in the present study for analysis of the structure of the cyanolichen heteroglycan, colleman, is a powerful method to reveal structural details including *O*-methylation of sugar residues and will undoubtedly be used to a larger extent in the future for structure elucidation of polysaccharides from cyanobacteria and cyanolichens.

Microbial polysaccharides are used commercially to some extent. The most common industrial use is as food coatings, emulsifying and gelling agents, bioflocculants and hydrating agents and they can be used to remove toxic metals from polluted waters (Otero & Vincenzini, 2003; Shah, Ray, Garg, & Madamwar, 2000). The lack of detailed structural information for the polysaccharides of cyanobacteria has limited the understanding of their role and possible industrial application (Volk et al., 2007, Pereira et al., 2009). In addition, a few *Nostoc* species have been used as food (Brull et al., 2000; Chu & Tsang, 1988) and as traditional medicine to treat cancer and gout (Hoppe, 1979) and furthermore various lichen polysaccharides have shown interesting effects on the immune system (Ingolfsdottir et al., 1994; Olafsdottir & Ingolfsdottir, 2001; Olafsdottir, Omarsdottir, Paulsen, Jurcic, &

Wagner, 1999b; Olafsdottir et al., 1999a; Olafsdottir, Omarsdottir, Paulsen, & Wagner, 2003; Omarsdottir, Freysdottir, Barsett, Paulsen, & Olafsdottir, 2005; Omarsdottir, Freysdottir, & Olafsdottir, 2007; Stuelp-Campelo et al., 2002) and it is of interest to investigate the industrial potential of colleman as well as its possible immunomodulating effects.

In conclusion, the complete NMR analysis of the lichen polysaccharide Cf-5-s or colleman, presented in this study revealed its primary structure. The detailed NMR assignments provide useful NMR reference data for future studies on colleman-like heteroglycans. According to the data obtained, colleman is a complex heteroglycan with a monosaccharide sequence of great variety, containing unusual 2-OMe Man*p* and 2-OMe-Ara*p* units and high levels of Xyl*p* and Glc*p*A. Colleman has some structural features resembling those of polysaccharides previously isolated from *Nostoc* cyanobacteria, and is therefore proposed to be of cyanobacterial rather than fungal origin.

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