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Structural characterisation of a highly branched galactomannan from the lichen *Peltigera canina* by methylation analysis and NMR-spectroscopy

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Abstract

An alkali-extractable water-soluble polysaccharide, Pc-4, was isolated from the lichen *Peltigera canina* using ethanol fractionation and size exclusion chromatography. The average molecular weight of Pc-4 was estimated to be 53 kDa. Structural characterisation of Pc-4 was performed by methanolysis, methylation analysis and NMR-spectroscopy (1D proton, COSY, NOESY, TOCSY and HSQC).

According to data obtained, Pc-4 is a highly branched galactomannan, with (1→6)-linked α-mannopyranosyl units in the main chain, which are mainly disubstituted at O2 and O4 by single unsubstituted units of α-Man*p* and β-Gal*p*, respectively. Pc-4 resembles the lichen galactomannans described previously; however, Pc-4 has higher degree of branching.

Keywords: Lichens; *Peltigera canina*; Galactomannan; Methylation analysis; NMR-spectroscopy

1. Introduction

The foliose lichen *Peltigera canina* (L.) Willd belonging to the family of Peltigeraceae (Kristinsson, 1964), is a lichen species that grows circumpolar in the northern hemisphere, arctic to temperature but is scattered and rare in the southern hemisphere (Vitikainen, 1994). Glucans, galactomannans and complex heteroglycans from lichens have been shown to have various biological effects, including immunomodulating activities (Olafsdottir & Ingólfsdóttir, 2001). In a recently published study, four heteroglycans, Pc-1, Pc-2, Pc-3 and Pc-4 were isolated from the aqueous and alkali extracts of *P. canina* and tested for in vitro immunomodulating activities and shown to influence cells both from the innate and adaptive immune systems. The structures of these heteroglycans were not determined apart from their monosaccharide composition. Methanolysis of the alkali-extractable water-soluble polysaccharide, Pc-4, resulted in a molar ratio mannose:galactose:glucose of 67:30:3 (Omarsdottir, Freysdottir, Barsett, Paulsen, & Olafsdottir, 2005). Apart from *P. canina* only one species of the genus *Peltigera* has been investigated for polysaccharide content, i.e. *Peltigera aphthosa,* described as containing a galactoglucomannan with a molar ratio of 38:11:44 (Gorin & Iacomini, 1985).

Galactomannans have been isolated from several lichen species and shown to consist of (1→6)-α-D-mannopyranosyl backbone, but differ in the substitution pattern at O2 and O4. The units of the backbone can be unsubstituted, monosubstituted at O2 by α-Man*p* or α-D-Gal*p*, monosubstituted at O4 by β-Gal*p*, or disubstituted at O2 and O4 by α-D-Gal*p* and β-D-Gal*p* or α-D-Man*p* and β-D-Gal*p*, respectively (Baron, Iacomini, Fanta, & Gorin, 1991; Carbonero, Tischer, Cosentino, Gorin, & Iacomini, 2003; Iacomini, Schneider, & Gorin, 1985; Olafsdottir & Ingólfsdóttir, 2001; Prado, Gorin, Stuelp, Honda, & Iacomini, 1999; Woranovicz, Gorin, Marcelli, Torri, & Iacomini, 1997). The methods that have been used to determine the structural features of these lichen

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galactomannans are mostly methylation analysis and simple 1D ^{13}C and ^{1}H (anomeric region) NMR-spectroscopy, with a couple of exceptions were 2D NMR spectroscopy is also applied (Carbonero et al., 2003; Prado et al., 1999).

The aim of the present study is to determine the structural features of Pc-4, using methylation analysis and high field (800 MHz for ^{1}H) 1D and 2D nuclear magnetic resonance spectroscopy; COSY, NOESY, TOCSY and HSQC and to provide useful NMR reference data for future studies on lichen galactomannans.

2. Experimental

2.1. Biological material

The lichen *P. canina* (L.) Willd (Peltigeraceae) was collected in 2002 in Fljotshlid in south-western Iceland. The lichen was identified by S. Baldursdottir, lichenologist, Prokaria Ltd, Reykjavik, Iceland and by Dr Hördur Kristinsson, Director of the Icelandic Institute of Natural History, Akureyri, Iceland. A voucher specimen (catalogue nr. AMNH L-28941) is deposited at the Icelandic Institute of Natural History, Akureyri, Iceland.

2.2. Isolation and purification of the polysaccharide

The dried lichen material (197 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 (Scheme 1) (Caldes et al., 1981; Paulsen, Olafsdottir, & Ingólfsdóttir, 2002) to give fraction VI. Of this alkali-extractable, water-soluble fraction VI, 150 mg were dissolved in 3 ml of 0.2 M aqueous NaOH and purified further on a 1.6×85 cm Sephacryl S-400 column (Amersham Biosciences), by elution with the same solvent. The eluted fractions were tested using the phenol/sulphuric acid test (Dubois, Gilles, Rebers, & Smith, 1956). The positive fractions (100–200 ml) were combined, dialysed through a membrane with 6–8 kDa M_r-cut-off and lyophilised to give 101 mg of Pc-4.

2.3. Determination of mean M_r and homogeneity

Homogeneity and mean M_r of Pc-4 was determined by HP-GPC on a Superose 6 HR 10/30 column (Amersham Biosciences) 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 (HP 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 Biosciences).

2.4. Monosaccharide composition

The monosaccharide composition of the purified water-soluble polysaccharides was determined by GC of the trimethylsilylated derivatives of the methyl glycosides obtained by methanolysis of 1 mg of polysaccharide in 1 ml of 4 M HCl in methanol with mannitol as an internal standard (Barsett & Paulsen, 1992; Reinhold, 1972).

2.5. Methylation analysis

One milligram of Pc-4 in dimethyl sulphoxide was methylated using NaOH and methyl iodide according to Ciucanu and Kerek (1984) as described by Kim and Carpita (1992). After hydrolysis with 2.5 M trifluoroacetic acid, the methylated sugar residues were converted to partially methylated alditol acetates by reduction with $NaBD_4$ in 2 M NH_4OH followed by acetylation with acetic anhydride. The derivatised sugar residues were extracted into dichloromethane and evaporated to dryness, dissolved again in 100 µl methanol and analysed by GC-MS (Samuelsen, Paulsen, Wold, Otsuka, Yamada and Espevik, 1995).

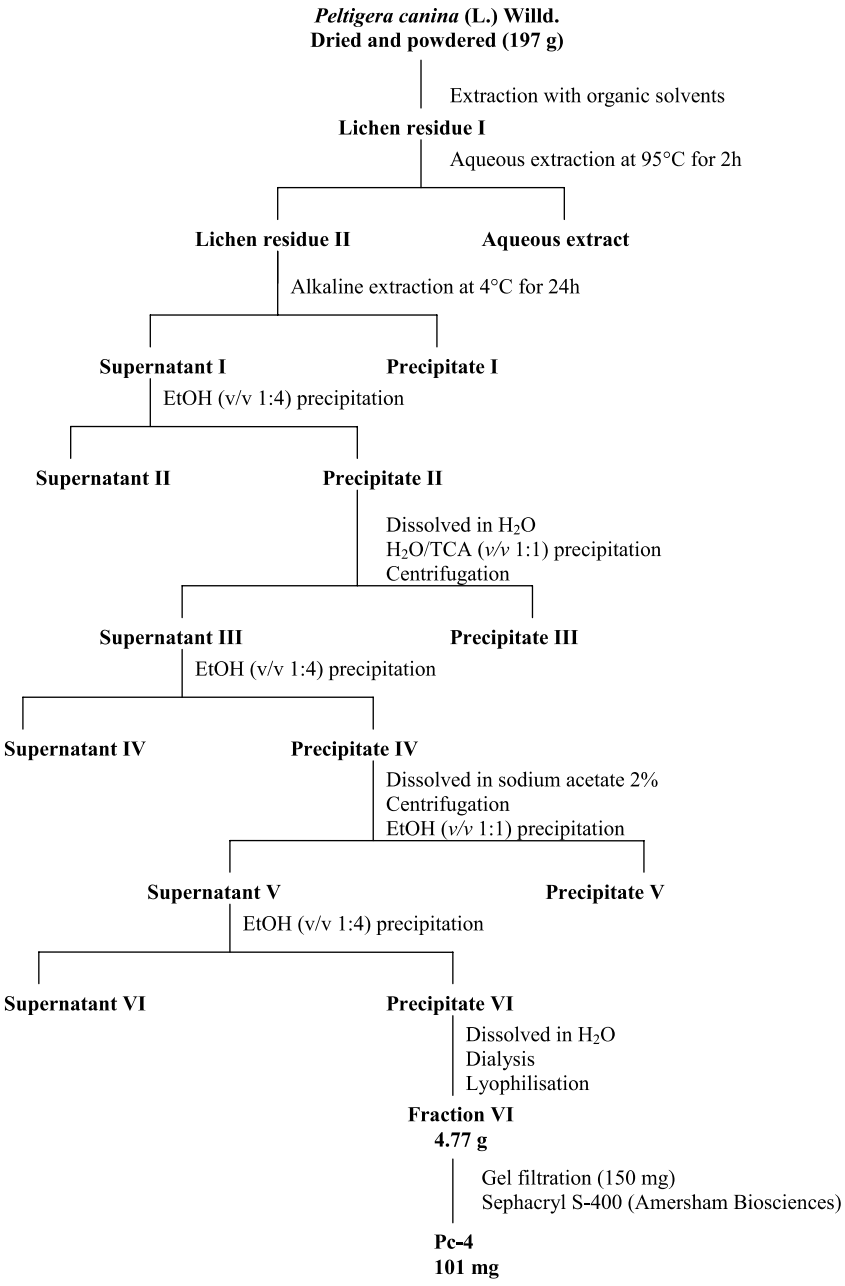
2.6. Nuclear magnetic resonance spectroscopy

The NMR chemical shifts were assigned from 1D and 2D homo- and heteronuclear experiments recorded at 799.96 MHz for proton and 201.12 MHz for carbon, using acetone as reference for proton (δ 2.225 ppm) and 1,4-dioxane for carbon (δ 67.4 ppm).

Pc-4 (2 mg) was dissolved in 0.7 mL of D_2O and spectra were recorded at 25 °C on a Varian UNITY INOVA 800 in a 5 mm tube.

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 Varian standard program tndqcosy, with 0.37 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 Varian standard tnnoesy, with a mixing time of 75 ms. The TOCSY experiment was performed using standard Varian program tntocsy with a spinlock time of 80 ms. A heteronuclear experiment was performed using the pulse field gradient program gHSQC.

The spectra were assigned using the computer program Pronto (Kjaer, Andersen, & Poulsen, 1994), which allows



Scheme 1. Fractionation of polysaccharides from the alkali extract of *P. canina* by precipitation with ethanol, followed by gel filtration. The plant material was previously extracted with organic solvents and water.

the simultaneous display of different two-dimensional spectra and the individual labelling of cross peaks.

3. Results and discussion

Precipitate VI from the alkali extraction of *P. canina* L. was dissolved in 50 ml of water, dialysed and lyophilised to give 4.77 g of fraction VI (2.4%, yield). One hundred and fifty milligram of the fraction was further purified with gel filtration (size exclusion chromatography) to give 101 mg of the galactomannan Pc-4 (a yield of 1.6% from dry plant material). Pc-4 was eluted as a single peak in HP-GPC and the mean M_r was determined to be 53 kDa by comparison to dextran standards. Methanolysis, followed by GC of the trimethylsilyl derivatives, showed that Pc-4 contained mannose:galactose:glucose in a molar ratio of 69:30:1. This is in agreement with the previously published ratio (Omarsdottir, Freysdottir, Barsett, Paulsen & Olafsdottir, 2005), apart from the amount of glucose which is down to 1% instead of 3%. The absolute configuration of mannose and galactose was presumed to be the D-configuration based on previous studies of lichen galactomannans (Gorin & Iacomini, 1984).

Table 1
GC-MS analysis of partially *O*-methylated alditol acetates obtained from the methylated galactomannan Pc-4

O-Me-alditol acetates	Molar ratio (%)[a]	Linkage type[b]
2,3,4,6-Me_4-Man	34.6	Manp-(1→
2,3,4,6-Me_4-Gal	27.3	Galp-(1→
2,3,4-Me_3-Man	4.7	6)-Manp-(1→
3,4-Me_2-Man	7.2	2,6)-Manp-(1→
2,3-Me_2-Man	6.1	4,6)-Manp-(1→
3-Me-Man	16.5	2,4,6)-Manp-(1→

[a] Molar response determined according to (Sweet, Shapiro, & Albersheim, 1975), *O*-Me-alditol acetates <2% are not included.
[b] Based on derived *O*-methylated acetates.

Partially methylated alditol acetates analysed by GC-MS revealed a highly branched polysaccharide, containing non-reducing terminal units of Man*p* (34.6%) and Gal*p* (27.3%) as well as 6-*O*-linked Man*p* (4.7%), 2,6-di-*O*-linked Man*p* (6.1%), 4,6-di-*O*-linked Man*p* (7.2%) and 2,4,6-tri-*O*-linked Man*p* (16.5%) units (Table 1). The results of the methylation analysis are in agreement with a galactomannan structure with a (1→6)-α-D-mannopyranosyl main-chain, linked at O2 and O4 by Man*p* and Gal*p*. However, the number of terminal units are considerably higher than the number of branching points as discussed below.

The structure of Pc-4 (Fig. 1) was further analysed by NMR-spectroscopy using a series of one- and two-dimensional spectra (1D proton, DQF-COSY, NOESY, TOCSY and HSQC). The anomeric region of the proton spectra could be divided into two regions (Fig. 2 and Table 2). The signals in the region from δ 4.33–4.36 ppm were assigned to anomeric protons of terminal β-Gal*p* (residue B) from the chemical shifts (Bock, Pedersen, & Pedersen, 1984), the ^1H–^1H three bond coupling constants ($J_{1,2}\sim 7$ Hz; $J_{2,3}\sim 10$ Hz; $J_{3,4}\sim 3$ Hz) and NOE correlations between H1–H3 and H1–H5 (Duus, Gotfredsen, & Bock, 2000). Furthermore, NOE correlation between the anomeric proton of B and H4 of C, e.g. between δ 4.36 and 3.96 ppm, showed that residue B was linked to O4 of residue C. This is corroborated by the carbon shift of the α-Man*p* C4 at δ 76.9 ppm compared to the chemical shift of C4 for the non-substituted α-Man*p* at δ 71.5 ppm (Vinogradov, Petersen, & Duus, 2000). The remaining anomeric signals in the region δ

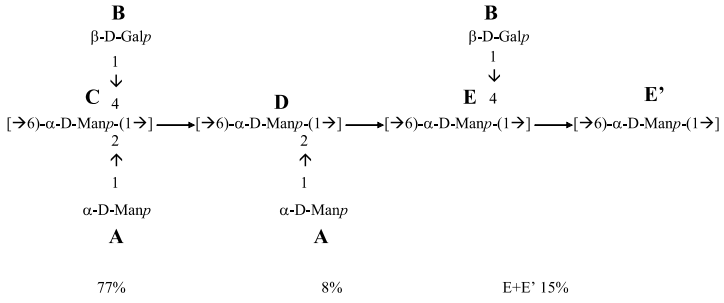


Fig. 1. Repeating units of the galactomannan Pc-4. The approximate percentage of each unit is estimated according to the integrals from ^1H NMR data.

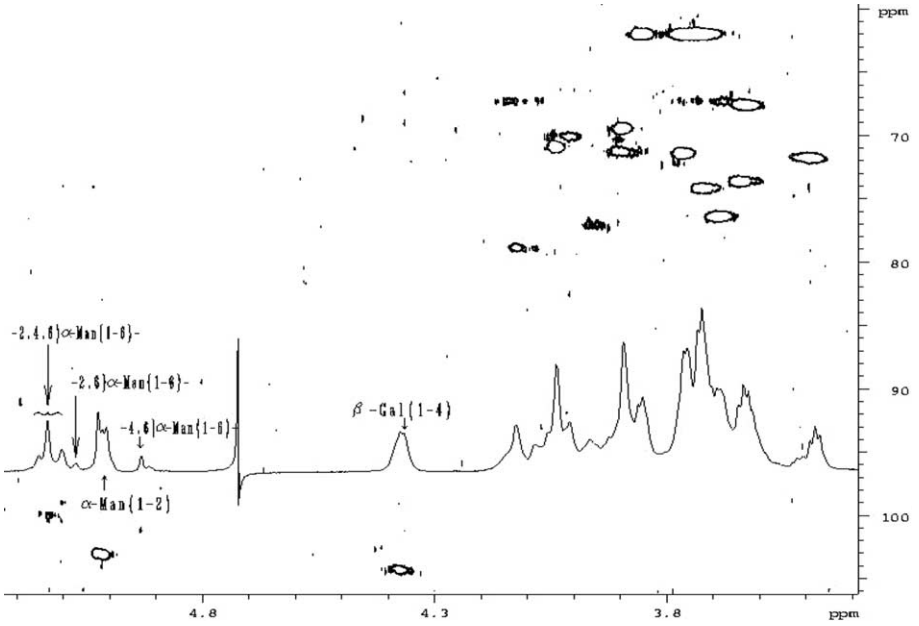


Fig. 2. The anomeric region of ^1H–^{13}C HSQC spectrum of the galactomannan Pc-4.

Table 2
^{1}H and ^{13}C NMR chemical shifts (δ), NOE correlations and relative integrals of the anomeric signals, for the highly branched galactomannan, Pc-4 isolated from the lichen *Peltigera canina*

	Unit		1	2	3	4	5	6a	6b	NOE	Integral
t-α-Man(1→2)	A	H	5.00	4.01	3.74	3.60	3.70	3.83	3.69	4.10	1.0
		H	4.99	4.01	3.74	3.59	3.69	3.83	3.69	4.02/4.06	
		H	4.98	4.01	3.73	3.59	3.68	3.83	3.69	4.09	
		C	102.9	70.9	71.4	67.3	74.1	61.9			
t-β-Gal(1→4)	B	H	4.36	3.47	3.66	3.87	3.65	3.73	3.70	3.89	1.0
		H	4.34	3.45	3.64	3.86	3.65	3.73	3.70	3.93	
		H	4.34	3.44	3.60	3.86	3.65	3.73	3.70	3.93	
		H	4.33	3.49	3.65	3.86	3.65	3.73	3.70	3.86	
		C	104.2	71.7	73.5	69.4	76.3	61.9			
2,4,6)-α-Man(1→6)	C	H	5.11	4.10	3.99	3.96	3.86	4.12	3.65	4.12/3.65	1.0
		H	5.13	4.09	4.01					4.13/3.64	
		H	5.12	4.09	4.01					4.13/3.64	
		C	99.8	78.7	70.0	76.9	71.2	67.3			
		H	5.07	4.02	4.00					4.13/3.64	
		H	5.08	4.06	3.98	3.86				4.13/3.64	
		C	98.8	78.7	70.0	76.9					
2,?)-α-Man(1→?)	D	H	5.04	3.99	3.96						0.1
		C	99.1	79.1	70.0						
4,?)-α-Man(1→6)[a]	E	H	4.90	4.03	3.88	3.92				4.04/3.79	0.2
		C	101.1	70.0	70.2	76.9					
Ref.: Vinogradov, Petersen, & Duus, 2000											
t-α-Man(1→2)		C	102.3	70.2	70.2	66.9	73.3	61.1			
2,6)α-Man(1→6)		C	98.5	78.8	70.5	67.1	72.6	61.0			
Ref.: Bock, Pedersen, & Pedersen, 1984											
t-β-Gal(1→4)		C	104.9	72.9	74.0	69.8	76.4	62.1			

? is not assignable but is probably 6. D and E are likely to be single branched versions of C and to induce the variation of A, B and C.
[a] Having the same anomeric chemical shift, →6)-α-Man(1(6) is likely to contribute to the integral of 0.2.

4.90–5.20 ppm were assigned to α-Man*p*, according to chemical shifts and ^{1}H–^{1}H three bond coupling constants ($J_{1,2} \sim 1$ Hz; $J_{2,3} \sim 3$ Hz; $J_{3,4} \sim 10$ Hz) (Duus, Gotfredsen & Bock, 2000; Vinogradov, Petersen & Duus, 2000). The anomeric signal of α-Man*p* at δ 4.98–5.00 ppm was assigned to a terminal α-Man*p*-unit linked to O2 (residue A) of the α-Man*p* backbone (residue C) by NOE correlation between H1 of residue A and H2 of residue C, e.g. between δ 5.00 and 4.10 ppm. Also NOE correlation between H1 of A and H1 of C, e.g. δ 5.00 and 5.11 ppm were observed, as previously described for α-(1→2) linked mannans (Vinogradov et al., 2000). The anomeric signals in the region of δ 5.07–5.13 ppm were assigned to originate from the →6)-α-Man*p*-(1→ backbone by NOE correlation between, e.g. H1 at δ 5.11 ppm and a set of H6 protons at δ 4.12/3.65 ppm. This is confirmed by the C6 carbon chemical shift at δ 67.3 ppm, compared to unsubstituted C6 chemical shift at δ 61.9 ppm (Vinogradov et al., 2000). Residue C is substituted at O2 and O4 by the above discussed α-Man*p* and β-Gal*p*, respectively. The assignment of the α-Man*p* anomeric signals at δ 5.04 and 4.90 ppm was difficult due to overlapping of signals. However, referring to the methylation analysis and the assigned shift, it is suggested that these units are a part of the →6)-α-Man*p*-(1→ backbone, which is substituted as →2,6)-α-Man*p*(1→6 (residue D) and →4,6)-α-Man*p*-(1→6 (residue E), respectively. It cannot be determined by NMR if →6)-α-Man*p*-(1→6 residue signals are contributing to the integral of the chemical shifts assigned to E, →4,6)-α-Man(1→6 residue. Judging from the methylation data it seems likely that this signal includes a reasonable amount of the unsubstituted backbone units →6)-α-Man(1→6 as well (E′ in Fig. 1).

The molar ratio of 1:1:1:0.1:0.2 for A/B/C/D/E+E′ (Table 2), based on the relative integrals of the anomeric signals in the ^{1}H NMR are in agreement with the monosaccharide composition given by the methanolysis. This is in accordance with an unusually compact lichen-galactomannan structure were the main chain is dominated by (1→6)-α-D-mannopyranosyl units branched at O2 and O4, with terminal α-Man*p* and β-Gal*p*, respectively. According to the integrals of the ^{1}H NMR anomeric signals of the backbone residues C, D and E/E′, the percentages were estimated to be 77, 8 and 15%, respectively (Fig. 1). The NMR integration can contain some uncertainty from differences in relaxation, but results point to a highly branched structure. The low proportion of branching points relative to terminal units in Pc-4, as determined by the methylation analysis, disagrees with the proportions determined by NMR integration, and could theoretically be explained by under-methylation of residue C of the highly branched backbone, due to steric hindrance. However, in a case of under-methylation of residue C, non-methylated mannose units, present after GC-MS of the products obtained after the complete methylation procedure, should be found as hexa-acetyl-mannitol. As this is not observed, under-methylation of residue C cannot be expected to explain this relatively high degree of non-reducing terminal units compared to branching points. Other plausible explanations could be chemical degradation of the polymer or incomplete reduction of the O3-methylated derivative of residue C. It is also possible that the O-methylated mannan core of this highly branched polymer, might be incompletely hydrolysed, resulting in too low proportion of branched units compared to end units in the methylation results. The use of stronger acidic conditions and/or longer reaction times for the hydrolysis of galactomannans is described in the literature (Ahrazem, Leal, Prieto, Barbero, & Bernabé, 2001; Carbonero, Cordeiro, Mellinger, Sassaki, Stocker-Wörgötten & Gorin, 2005; Prado, Gorin, Stuelp, Honda & Iacomini, 1999). Methylation analysis of polysaccharides has the advantage of providing detailed information on linkage types and observes units present in small amounts, however it can be expected that uncertainties resulting from chemical reactions and quantification of the chromatographic data may result in errors in the molar ratio of more than 10% (Vincent, 2003).

The advantage of using NMR spectroscopy for structure elucidation of polysaccharides is that it does not involve uncertainties resulting from chemical degradation, and produces data for the intact polymer. The enhanced resolution capacity of high field NMR is definitely an advantage when analysing large and rigid polysaccharides where broad signals and overlapping can be a problem.

In conclusion, Pc-4 galactomannan resembles some of the lichen galactomannans described previously, consisting of (1→6)-α-D-mannopyranosyl backbone, branched at O2 and O4 with α-Man*p* and β-Gal*p*, respectively, however Pc-4 has a higher degree of branching. In addition, the detailed NMR data for Pc-4, provided in this study, can certainly be valuable for future characterisation of lichen galactomannans.

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