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Note

A ^{13}C-n.m.r. analysis of linkages in lichen polysaccharides: an approach to chemical taxonomy of lichens

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It has been found that various kinds of water-soluble polysaccharides are contained in lichens in fairly high proportions; of these polysaccharides, lichenan[1], isolichenan[1], and pustulan[2] had been extensively studied to elucidate their chemical structures. The polysaccharides thus far known in lichens are the homo-D-glucans (1→3)-(1→4)-β-D-glucan (lichenan), (1→3)-(1→4)-α-D-glucan [isolichenan[1], PC-3 (ref. 3), EP-3 (ref. 4), and everniin[5]], (1→6)-β-D-glucan [pustulan and GE-3 (ref. 6)] and (1→3)-(1→4)-(1→6)-α-D-glucan (acroscyphan[4]).

In contrast to the lichen metabolites of lower molecular weight, the distribution of which is mostly species-specific[7], the lichen polysaccharides are rather characteristic of larger taxonomical groups[7, 8]. Hence, a wide survey of lichen polysaccharides was undertaken. It has also been demonstrated that some polysaccharides of lichens and higher fungi show host-mediated, antitumor activity against transplanted Sarcoma 180 in mice[4,6,9–11].

We now describe the isolation and ^{13}C-n.m.r. analysis of some lichen homo-D-glucans and their relative proportions in the lichens: the lichenan and isolichenan from *Cetraria richardsonii*, SJ-2-I from *Stereocaulon japonicum*[12] Th. Fr., PA-2 from *Pilophoron acicularis* (Svj.) Nyl., PC-3 from *Parmeria caperata* (L.) Ach., acroscyphan from *Acroscyphus sphaerophoroides* Lev., and SG-3 from *Sphaerophorus globosus* (Huds.) Vain.

EXPERIMENTAL

General. — Sugar analysis was performed with a JEOL JLC-6AH liquid-

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chromatographic autoanalyzer. Infrared spectra were recorded with a Jasco DS 402-G spectrophotometer. ^{13}C-N.m.r. spectra were recorded with a JEOL PFT-100/EC-100 spectrometer operating at 25.03 MHz, in the pulsed, Fourier-transform mode. Free-induction decays were accumulated with a 45° pulse and a repetition time of 1.2 sec. All spectra were recorded using 4K data-points and spectral width of 4 kHz. ^{13}C chemical shifts were expressed in p.p.m. downfield from external tetramethylsilane. All samples (80 mg/ml; pH 14) were contained in tubes (10 mm, o.d.).

Isolation and purification of SG-3 and PA-2. — *SG-3.* Thalli (42.6 g) of the lichen *Sphaerophorus globosus* were successively extracted with Me_2CO and 80% EtOH in order to remove soluble components. The residual thalli were further extracted with dist. H_2O on a boiling-water bath. The hot extract was concentrated *in vacuo*, and the concentrate was poured into EtOH (3 vol.) to afford a precipitate which was collected by centrifugation and dried, to give a pale-grey, water-soluble substance (polysaccharide fraction SG-1; yield 7.7 g, 18.1%). SG-1 was further separated into a cold-water-soluble fraction, SG-2 (yield 6.6%), and an insoluble fraction, SG-3 (1.9%), by the procedure of freezing and thawing. By determination with a sugar analyzer of the products of acid hydrolysis, it was proved that SG-2 contained mannose (major), galactose, and glucose, whereas SG-3 contained glucose only. Fraction SG-3 showed ν_{max}^{KBr} 844 cm^{-1}.

PA-2. — Thalli (292 g) of the lichen *Pilophoron acicularis* were extracted by the procedure just described. The resulting, pale-brown, water-soluble substance, PA-1 (yield 28.6 g, 9.8%), was separated into a cold-water-soluble fraction, PA-2 (yield 5.0%), and an insoluble fraction, PA-3 (yield 0.05%). Fractions PA-1, PA-2, and PA-3 contained glucose only, and showed ν_{max}^{KBr} 845, 849, and 850 cm^{-1}, respectively. Fraction PA-2 gave a single peak in gel filtration on Sephadex G-200.

Preparation of lichenan, isolichenan, PC-3, SJ-2-I, and acroscyphan. — The isolation procedures were essentially the same as those already described[3,4,12], and the physical and chemical data were identical with the values previously reported.

RESULTS AND DISCUSSION

Fig. 1 shows the ^{13}C-n.m.r. spectra of some lichen polysaccharides. The assignment of the ^{13}C signals of PC-3 [(1→3)-(1→4)-α-D-glucan containing each component in 1:1 ratio] was achieved from consideration of the relative peak-intensities (total 12), and the ^{13}C chemical shifts of (1→4)-α-D-glucans and (1→3)-α-D-glucans[13,14]. The assignment was confirmed by examination of the ^{13}C-n.m.r. spectra of other D-glucans, having different relative-intensities (see Table I). In a similar way, the ^{13}C-n.m.r. signals of lichenan were assigned on the basis of those given by (1→3)-β-D-glucans and cellobiose[14].

The ratio of (1→3)- to (1→4)-linkages was determined by comparing the integrated peak-intensity of C-3 [(1→3)-linkage] with that of C-4 [(1→4)-linkage] for both α- and β-D-linked D-glucans, as no overlaps of peaks appear in this region. The relative proportion of α-D-(1→6)-linkages was estimated in the region of the

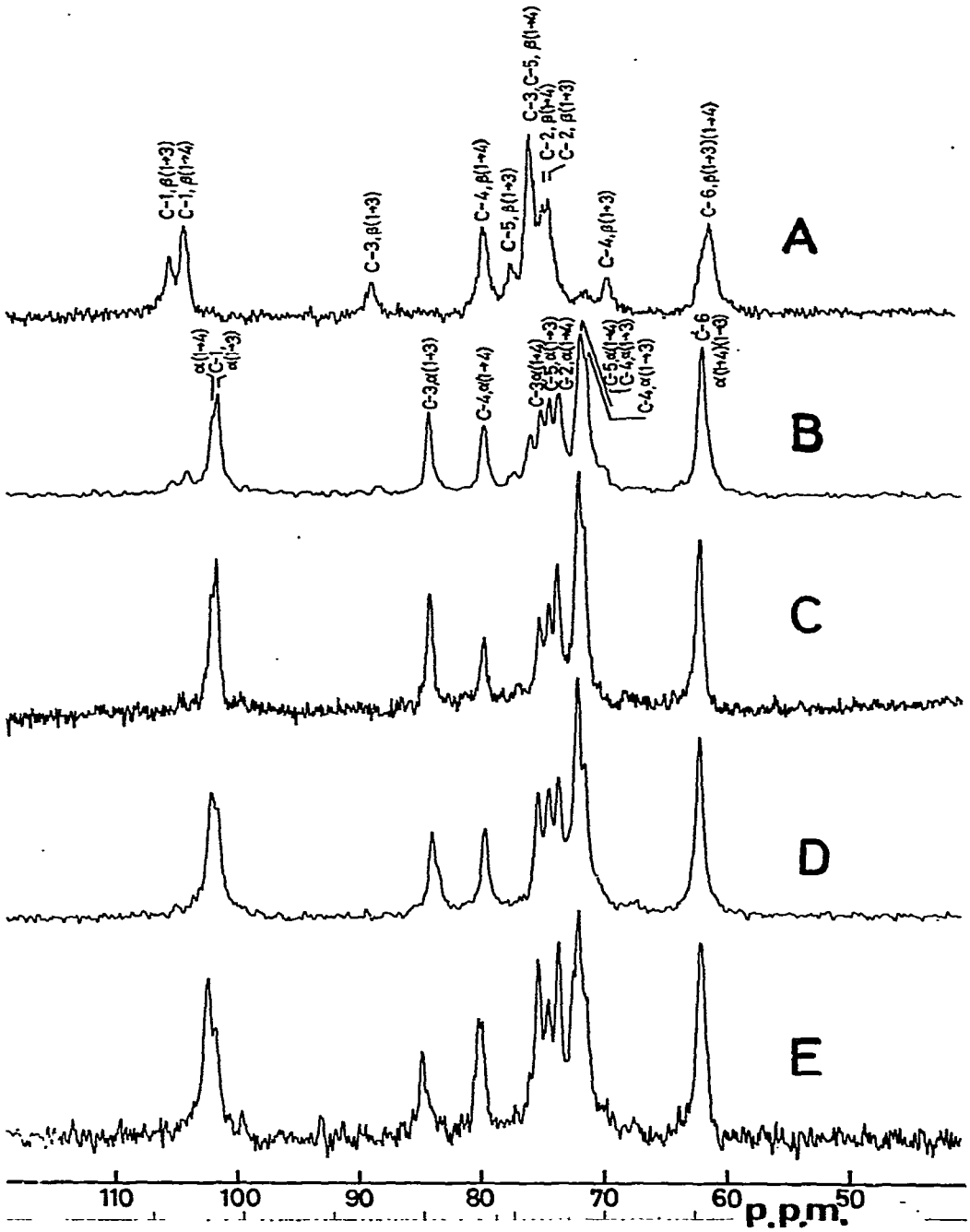

Fig. 1. ^{13}C-N.m.r. spectra of lichen polysaccharides in aqueous solution (80 mg/ml, pH 14). [A. Lichenan, 72,900 transients; B. Isolichenan, 75,120 transients; C. SJ-2-I, 13,000 transients, D. PC-3, 81,168 transients; and E. SG-3, 35,000 transients.]

TABLE I

^{13}C-N.M.R. SIGNALS, AND THEIR ASSIGNMENTS, FOR LICHEN POLYSACCHARIDES[a]

Lichen polysaccharides [α-(1→3)-(1→4)]							*D-Glucans having single D-glucosidic linkages*[b]		
PC-3	*Isolichenan*	*SJ-2-I*	*PA-2*	*SG-3*	*Acroscyphan*	*Assignment*	*α-(1→4)*	*α-(1→3)*	*α-(1→6)*
101.9(1)[c]	101.9	102.0	102.0	102.3	102.0	C-1 (1→4)	102.9		
101.3(1)	101.6	101.5	101.8	101.7	101.5	C-1 (1→3)		101.3	99.4 (C-1)
83.7(1)	84.3	84.1	84.7	84.7	83.5	C-3 (1→3)		83.2	
79.3(1)	79.8	79.6	79.9	80.1	79.6	C-4 (1→4)	80.6		
75.0(1)	75.1	75.1	75.1	75.2	75.1	C-3 (1→4)	75.4		75.4 (C-3)
74.2(1)	74.4	74.3	74.4	74.3	74.4	C-5 (1→3)		73.7	
73.3(1)	73.6	73.6	73.7	73.5	73.4	C-2 (1→4)	73.8		73.1 (C-2)
71.8(2)	71.9	71.9	71.9	71.9	71.9	C-5 (1→4)	72.6	72.2	71.8 (C-4)
						C-2 (1→3)			
71.2(1)	71.4	71.5	71.5	71.4	71.4	C-4 (1→3)		71.7	71.1 (C-5)
									66.8 (C-6)
61.9(2)	62.0	62.0	62.0	62.0	61.9	C-6 (1→3)	62.0	62.2	
						C-6 (1→4)			
(1:1)[d]	(3:2)[d]	(2:1)[d]	(2:1)[d]	(2:3)[d,e]	(2:3)[d,e]				
1:1[f]	3:2[f]	3:1[f]	—	—	—				

TABLE 1 *(continued)*

Lichen polysaccharide [*β-(1→3)-(1→4)*]	*Assignment*	D-*Glucans having single glucosidic linkages*[a]	
		β-(1→3)	*β-(1→4)*
105.6	C-1 (1→3)	104.7	
104.5	C-1 (1→4)		104.5
89.2	C-3 (1→3)	88.0	
79.9	C-4 (1→4)		71.4
76.2	C-5 (1→3)	77.8	
	C-3,5 (1→4)		78.2
75.0	C-2 (1→4)		74.9
74.6	C-2 (1→3)	74.9	
69.8	C-4 (1→3)	69.9	
62.0	C-6 (1→3)	62.5	
61.5	C-6 (1→4)		62.1
(3:7)[d]			
3:7[f]			

[a]P.p.m. from Me_4Si. [b]Data taken from ref. 13. [c]Relative peak-intensity. [d]Ratio of the integrated peak-intensity of C-3 [(1→3)-linkage] to that of C-4 [(1→4)-linkage]. [e]Peak for α-(1→6)-linkage less than 6%. [f]Data by chemical analysis. [g]Data for cellobiose, assignment based on ref. 14.

C-1 peak (99.4 p.p.m.). The composition thus obtained is in excellent agreement with the data given by chemical analysis (see Table I) for lichenan, isolichenan, and PC-3. The relative proportions of the peaks other than those for C-3 [(1→3)-linkage] and C-4 [(1→4)-linkage] are also consistent with the values for the polysaccharides shown in Fig. 1.

From Table I, it may be seen that the ratio of the α-D-(1→3)- to α-D-(1→4)-glucosidic linkages in acroscyphan, a polysaccharide from *Acroscyphus sphaerophoroides*, is 2:3, and that it contains, at most, 6% of α-D-(1→6)-linkages; the chemical evidence provided earlier had revealed the presence of α-D-(1→3)-, -(1→4)-, and -(1→6)-linkages in acroscyphan, but did not give their ratios. Polysaccharide SG-3 from *Sphaerophorus globosus* has been indicated by the ^{13}C analysis to have α-D-(1→3)-(1→4)-linkages in the ratio of 2:3, with not more than 6% of α-D-(1→6)-linkages. The results of the ^{13}C-n.m.r. study and the chemical evidence now strongly suggest that SG-3 is identical with acroscyphan; this would be reasonable, as the two lichens, *Acroscyphus sphaerophoroides* and *Sphaerophorus globosus*, belong to the same family, the *Sphaerophoraceae*. Furthermore, it is found that the ^{13}C-n.m.r. data for PA-2 from *Pilophoron acicularis* are identical with those for SJ-2-I, from *Stereocaulon japonicum*, in which the ratio of α-(1→3)- to α-(1→4)-linkages was determined as 3:1 from chemical analysis, and as 2:1 by ^{13}C-n.m.r. studies. As the genera of *Pilophoron* and *Stereocaulon* are also members of the *Stereocaulaceae*, the present result seems quite reasonable. Accordingly, it may be concluded that the chemical taxonomy of lichens on the basis of their polysaccharide content is readily achieved by examining the ^{13}C-n.m.r. spectra thereof.

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