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Note

Structural analysis of levans by use of ^{13}C-n.m.r. spectroscopy*

FRED R. SEYMOUR,

Fleming Department of Rehabilitation, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030 (U.S.A.)

ROGER D. KNAPP,

Division of Atherosclerosis and Lipoprotein Research, Baylor College of Medicine, and Methodist Hospital, Texas Medical Center, Houston, Texas 77030 (U.S.A.)

AND ALLENE JEANES

Northern Regional Research Center, Agricultural Research, Science and Education Administration, U. S. Department of Agriculture, Peoria, Illinois 61604 (U.S.A.)

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We have previously employed ^{13}C-n.m.r. spectroscopy to examine the structure of a series of dextrans[2–6], and have made the following observations. Firstly, the 6-mono-*O*-substituted α-D-glucopyranosyl residues of the dextran backbone produce major resonances that dominate the spectra of the dextrans and that have constant chemical-shifts (independent of the nature and degree of branching of the dextran); secondly, the various, branch-point α-D-glucopyranosyl residues have diagnostic resonances for each type of branch-point residue, and the chemical shifts of these diagnostic resonances are independent of the degree of branching; and, thirdly, the relative peak-heights of these diagnostic resonances (relative to the major resonances) are proportional to the degree of branching. High-temperature recording-conditions increase the resolution of the spectra and decrease the number of acquisitions necessary for a good signal-to-noise spectrum. In general, when they are present, diagnostic branching-resonances can be observed corresponding to about one residue in ten chain-extending, 6-mono-*O*-substituted α-D-glucopyranosyl residue resonances.

In addition, we have also employed ^{13}C-n.m.r. spectroscopy to examine compounds containing variously *O*-substituted β-D-fructofuranosyl residues[1]. It was observed that the β-D-fructofuranosyl residue has ^{13}C-n.m.r. resonances that differ completely from those associated with the α-D-glucopyranosyl residues. *O*-Substitution at different carbon atoms of the β-D-fructofuranosyl residue results in resonance displacements, but the resonance displacements of C-1, C-2, and C-6 of this residue

*Levans, Part II. For Part I, see ref. 1.

are not so great as those *O*-substitution resonance-displacements associated with similar positions for the α-D-glucopyranosyl residue. Several fractions obtained by acid hydrolysis of the native levan produced by *Streptococcus salivarius* ATCC 13419 (fractions F2, F14, and E6) were examined by ^{13}C-n.m.r. spectroscopy, and found to be essentially identical in terms of minor and major resonances. Correlation of the spectra of various, β-D-fructofuranosyl-containing compounds allowed assignment of ^{13}C-n.m.r. resonances to specific carbon atoms.

Levans from the NRRL collection that were obtained under conditions similar to those used for the dextrans previously studied have now been examined by ^{13}C-n.m.r. spectroscopy. Due to the need for greater resolution, a temperature of 90°

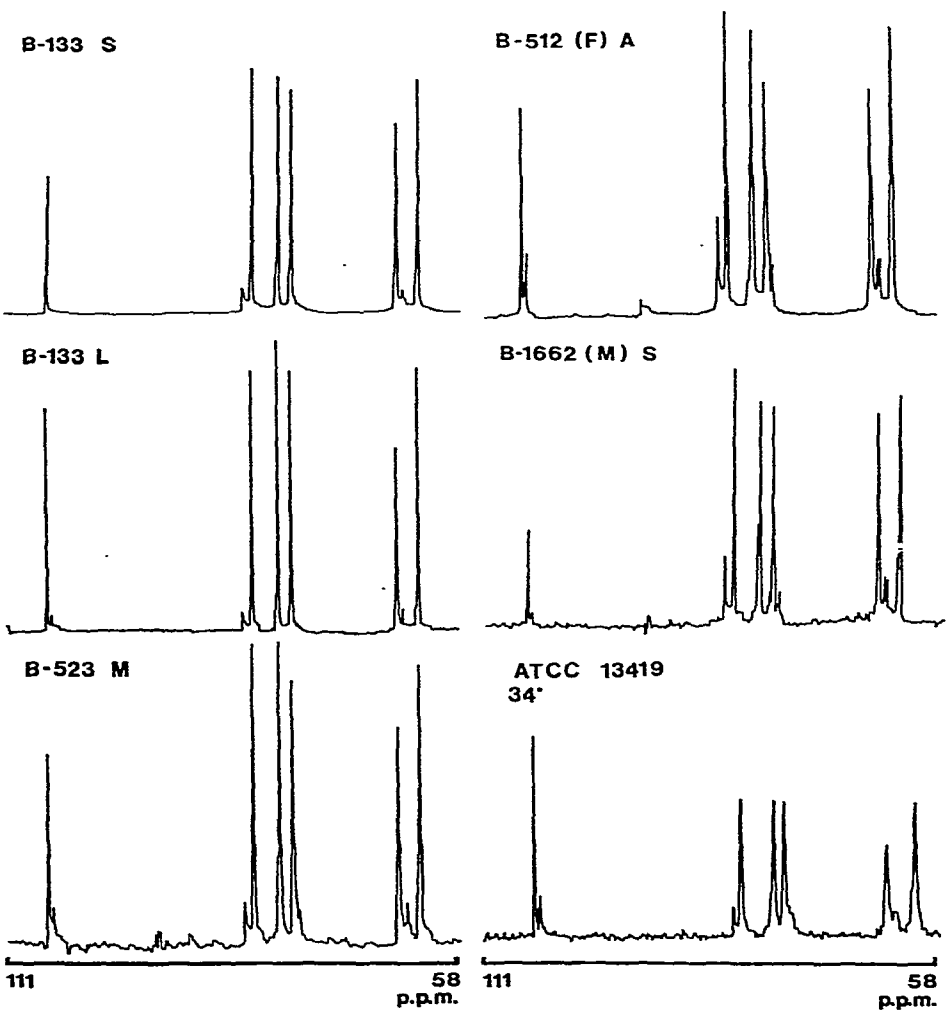

Fig. 1. ^{13}C-N.m.r. spectra at 90° (unless otherwise noted) for levan B-133 fractions S and L, levan B-523 fraction M, levan B-512(F) fraction A, levan B-1662(M) fraction S; and also, at 34°, for levan fraction E6 (low molecular weight) of native levan ATCC 13419.

was employed for recording the spectra of these levan fractions. To a first approximation, all levan fractions that have been studied have the same ^{13}C-n.m.r. spectrum, in contrast to the wide divergence in the ^{13}C-n.m.r. spectra of dextrans. However, certain minor resonances of these levans do differ, and these are discussed herein.

RESULTS AND DISCUSSION

The levan fractions studied are identified by the NRRL strain number of the producing organism and an identifying fraction-letter. These levans are: *Erwinia ananas* B-133 fractions S and L, *Leuconostoc mesenteroides* B-512(F) fraction A, *L. mesenteroides* B-523 fraction M, and *Bacillus* sp. B-1662(M) fraction S. The ^{13}C-n.m.r. spectra of these levan fractions were recorded at 90° (see Fig. 1 and Table I) and compared to those of previously studied levans (fractions F2, F14, and E6), which are products of the acid hydrolysis of the native levan produced by *S. salivarius* ATCC 13419.

The ^{13}C-n.m.r. spectra (34° and 90°) of levans B-512(F) fraction A and *S. salivarius* ATCC 13419 hydrolysis fractions were essentially identical. For comparison, the 34° spectrum of levan ATCC 13419, fraction F2, is included in Fig. 1, and, on

TABLE I

CHEMICAL SHIFTS FOR ^{13}C-N.M.R. SPECTRA OF LEVAN FRACTIONS

	Levan				
	B-133	*B-133*	*B-523*	*B-512(F)*	*B-1662(M)*
	Fraction				
	S	*L*	*M*	*A*	*S*
M[a]	105.68[b]	105.68	105.68	105.69	105.68
				105.27	105.28
	105.14	105.15	105.15	105.12	105.15
			93.31		
			93.00		
				91.82	
	82.87	82.88	82.87	82.86	82.87
M	81.80	81.82	81.82	81.82	81.82
	78.94 *s*[c]	78.98 *s*	78.96 *s*	78.96 *s*	78.97 *s*
M	78.74	78.78	78.76	78.76	78.76
M	77.26	77.27	77.30	77.27	77.27
	76.82	76.85		76.85	
			76.56	76.56	76.54
M	64.99	65.00	64.99	64.97	65.00
	64.23	64.23		64.23	64.24
			63.97	63.97	63.97
		62.79		62.77 *s*	62.76 *s*
M	62.45	62.48	62.48	62.48	62.48

[a]M indicates the six major resonances in the levan spectra. [b]The chemical shift in p.p.m., relative to tetramethylsilane, recorded at 90°. [c]The symbol *s* indicates a shoulder, or weak peak.

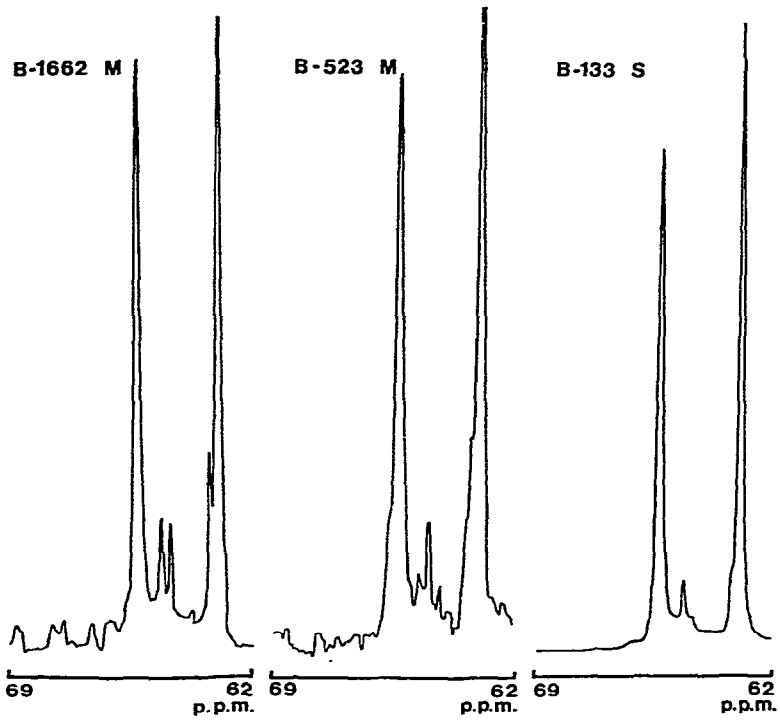

Fig. 2. ^{13}C-N.m.r. spectra of the expanded, 62–69-p.p.m. region of the data presented in Fig. 1, for levan B-1662(M) fraction S, levan B-523 fraction M, and levan B-133 fraction S.

raising the recording temperature to 90°, this spectrum became essentially identical to the 90° spectrum of levan B-512(F) fraction A shown in the same Figure.

Although the ^{13}C-n.m.r. spectra (90°) of the various levans, shown in Fig. 1 and Table I, are similar in the major resonances present, and generally similar in terms of the ratio of the peak heights of the major and minor resonances, an important difference was observed for the minor resonances in the 60–65-p.p.m. region. The spectra of levan B-512(F) fraction A, levan B-1662(M), and the acid-hydrolysis fractions of levan ATCC 13419 all contain two minor resonances, of equal intensity, at 63.97 and 64.23 p.p.m. However, the spectra of both the S and L fractions of levan B-133 contain only the 64.23-p.p.m. resonance, and, conversely, the levan B-523 fraction M spectrum contains only the 63.97-p.p.m. resonance (see Fig. 2). It is difficult to assess the precise meaning of the presence and absence of these resonances in the 60–65-p.p.m. region. It is possible that these minor resonances result from impurities, or from non-β-D-fructofuranosyl residues, in the various levan fractions. However, two effects suggest that these minor resonance-differences are not due to impurities. Firstly, levan is known to branch at approximately every seventh β-D-fructofuranosyl residue[7], and the ratio of the peak heights for the minor and major resonances is in general accord with this degree of branching. All minor resonances are of approximately the same intensity, and there are relatively few minor resonances

TABLE II

COMPARISON OF ^{13}C-N.M.R. RESONANCES TO VALUES OF SPECIFIC ROTATION FOR LEVAN FRACTIONS

NRRL strain	*Strain identification*	*Fraction*	$[\alpha]_D^{22}$ *(degrees[a])*	*N.m.r. peak 1 (64.23 p.p.m.)*	*N.m.r. peak 2 (63.97 p.p.m.)*
B-133	*Erwinia ananas*	S	−56.7	yes	
B-133		L	−54.7[b]	yes	
			−54.5		
B-523	*Leuconostoc mesenteroides*	M	−50.5		yes
B-512(F)		A	−55.2	yes	yes
B-1662(M)	*Bacillus* sp.	S	−49.4	yes	yes

[a]At c = 1.3, in water. [b]Duplicate determination. These rotational values are considered to be accurate to 1 part in 500, this being the difference in successive, one-second integrations. The weights of both the material and the water were known to better than 1 part in a thousand.

in the levan spectra to accommodate the (expected) additional resonances of the branch-point residues. These facts all suggest that the 63.97- and 64.23-p.p.m. resonances are associated with branching β-D-fructofuranosyl residues. Secondly, a dextran (α-D-glucan) would be the most probable impurity in these levans, and yet we have not observed dextran resonances near 63.97 or 64.23 p.p.m., and no other ^{13}C-n.m.r. spectral region suggests the presence of α-D-glucopyranosyl residues.

To check the possibility of different anomeric configurations of branch-point, D-fructofuranosyl residues, the specific rotations of the levan fractions were measured (see Table II). Were these resonance differences due to different anomeric configurations, ~10% of the β-D-fructofuranosyl residues, at branch-points, for the levan B-133 fractions S and L should be different from those of levan B-523 fraction M, with the remaining levan fractions exhibiting an intermediate value of the specific rotation. The data for specific rotations given in Table II show no direct correlation to the 60–65-p.p.m. region of the ^{13}C-n.m.r. spectra; these specific rotations were measured with sufficient precision that observation of differences in anomeric configuration at the 5–10% level could be anticipated. Therefore, it was concluded that only evidence that supports the β-D-fructofuranosyl-residue composition of these levan fractions had been obtained. Our rotational data are in reasonable agreement with the value $[\alpha]_D^{22} = -59.3°$, previously reported[7] for a levan elaborated by *Streptococcus salivarius* strain 51.

A remaining possibility for structural differences in levans is that the branching may occur at different carbon atoms, and not exclusively through 1,6-di-*O*-substituted β-D-fructofuranosyl residues, but information of branch-type nature is not currently available.

Employing levans from the NRRC collection, Allen and Kabat[8] demonstrated human antilevan production; the levan B-523 fraction M used by them was the same

as that reported in this article. These immunochemical studies were extended to mouse-myeloma proteins[9,10], the myeloma serum J606 being found to interact with all levans studied, but not with inulin. The J606 immunoglobulin was originally typed by Grey *et al.*[11], who used levans (their Table I) identical to the levan fractions of this report. Although some differences in precipitin data were observed, these differences were not so dramatic as those observed for the dextrans. Therefore, although the current immunochemical data on D-fructans clearly show that gross structural differences (*e.g.*, between levan and inulin) are detectable, the relationship of such immunochemical effects to more-subtle structural changes (*e.g.*, different branching residues for levan) remains in doubt.

EXPERIMENTAL

General. — The ^{13}C-n.m.r.-spectral conditions and methods for the preparation of samples have been described[2,3]. In general, a Varian XL-100-15 spectrometer equipped with a Nicolet TT-100 system was employed in the Fourier-transform mode. Samples (~200 mg) were dissolved in deuterium oxide (5 mL).

The specific rotations were measured with a Perkin–Elmer 241 polarimeter. Before being weighed, the samples were dried in high vacuum over phosphorus pentaoxide for 48 h, and then *in vacuo* over phosphorus pentaoxide for 18 h at 60°. Levan (~20 mg) was added to water (~1.5 mL), and the suspension was magnetically stirred for 10 min. The suspensions were then sonicated in a sonic bath for 10 min at room temperature, followed by sonication for 5 min at 60°. The second sonication was necessary in order to remove extensive opalescence from the solutions of levans B-512(F) fraction A and B-523 fraction M. Rotations were measured at 589 nm (22°), employing a 10-cm path-length. For all measurements, the light transmitted was equal to at least 85% of that for water-filled reference-cells. The rotational values were integrated over successive 1-sec intervals, and these values were stable for one part in 500.

Preparation of levans. — Levans from strains B-512(F) and B-523 were by-products isolated from experimental fermentations for dextran production on the pilot-plant scale. The media and conditions for culture were essentially the same as those previously detailed[12], with the initial fractionation and refractionations made by established procedures[13]. The crude levan fractions were precipitated by ethanol in the concentration range of 50–65%, and the re-fractionations were made in the range of 55–60% ethanol. The designation "M" for the B-523 levan accords with established terminology[13], and the designation "A" for the B-512(F) levan identifies the main product in a specific, refractionation sequence.

Erwinia ananas B-133 was cultured in a medium containing, in g per 100 mL of distilled water, sucrose, 10; Basamin Busch Yeast Hydrolyzate*, 0.10; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 0.25; and traces of mineral salts. Fermentation was conducted

*Basmin Busch Yeast Hydrolyzate is a product of Anheuser Busch, St. Louis, Missouri.

for 24 h in a completely filled, 11.35-L (3-gal) bottle, the initial and final pH values being, respectively, 7.4 and 5.6. The major products, fractions S and L, were separated from a spectrum of minor fractions by established procedures[13]. Fraction L was separated from fraction S by centrifugal sedimentation from dilute alcoholic and aqueous solutions. Both fractions were refractionated by use of aqueous ethanol in the concentration range of 55–60%; both were treated by the Sevag procedure as described by Staub[14] to lessen contamination by protein, and then both were dialyzed, in Visking-cellulose membranes, against distilled water. The retentates were filtered through sintered glass, and freeze-dried. Microanalyses showed: nitrogen <0.01, phosphorus <0.02, and ash <0.1%. Paper chromatography of acid hydrolyzates revealed no more than traces of any product other than D-fructose.

Bacillus sp. NRRL B-1662(M) was initially isolated, and designated strain N9, by Neill and co-workers[15], who studied the serological properties of its levan after synthesis by the action of cell-free extracts on sucrose solutions[15,16]. Fraction S was the major product of this strain when it was grown on a medium containing, in g per 100 mL of distilled water, sucrose, 10; Difco Bactopeptone, 1.0; Difco Yeast Extract, 0.5; and NaCl, 0.5. This levan fraction was separated[15] from another polysaccharide, composed of mannose, galactose, and glucose residues, that had a positive, specific optical rotation. Fraction S was treated by the Sevag procedure[14], dialyzed, and freeze-dried.

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