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Preliminary communication

Identification of 3-hydroxybutanoic acid as a component of the acidic extracellular polysaccharide of *Rhizobium trifolii* 0403

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The known non-carbohydrate components of the extracellular polysaccharide of *Rhizobium trifolii* are acetate[1,2], pyruvate[1–3], and succinate[4]. It has been shown that pyruvate substituents are strong immunodeterminants of rhizobial polysaccharides[1]. The acetate and pyruvate contents of the extracellular polysaccharide of some rhizobial polysaccharides are known to vary with the age of the culture[5]. Recently it has been reported that the levels of pyruvate and acetate in the capsular polysaccharide of *R. trifolii* 0403 vary with culture age[6]. During our study of the non-carbohydrate components of the acidic extracellular polysaccharide of *R. trifolii* 0403, we identified ether-linked 3-hydroxybutanoic acid as a substituent.

The extracellular polysaccharide was isolated from 5-day old, plate-grown cultures. A polysaccharide depolymerase[7] was used to generate oligosaccharide fragments from the parent polysaccharide[8]. These oligomers were purified by gel filtration through a column of Bio-Rad P-10 from which they were eluted as a single peak. They gave ^{1}H-n.m.r. spectra (250 MHz, D_2O) much more clearly resolved than those of the parent polymer. A 250-MHz Fourier-transform ^{1}H-n.m.r. analysis of these oligosaccharides (Fig. 1) revealed, by resonances (relative to external Me_4Si) between δ 1.23–1.43, the presence of acetal-linked pyruvate groups. Signals assigned to acetate groups appeared between δ 1.88 and 2.15. However, there were two other groups of resonance that could not be assigned to carbohydrate skeletal protons. One appeared between δ 1.03 and 1.17 (group I, Fig. 1) and the other between δ 2.36 and 2.58 (group II, Fig. 1). These signals were generally too complex to be interpreted directly.

The ^{1}H-n.m.r. spectrum was re-examined (Fig. 2) after mild hydrolysis of the oligosaccharides with base. This was accomplished by adding 10 μL of a 1% solution of deuterated sodium hydroxide to the sample (total volume 0.35 mL) and keeping it for 2 h at room temperature. After hydrolysis, the acetate-group signals in the spectrum collapsed to a sharp singlet at δ 1.80. The pyruvate group resonances now appeared as two sharp singlets at δ 1.35 and 1.38. The complex group of signals formerly between δ 2.36

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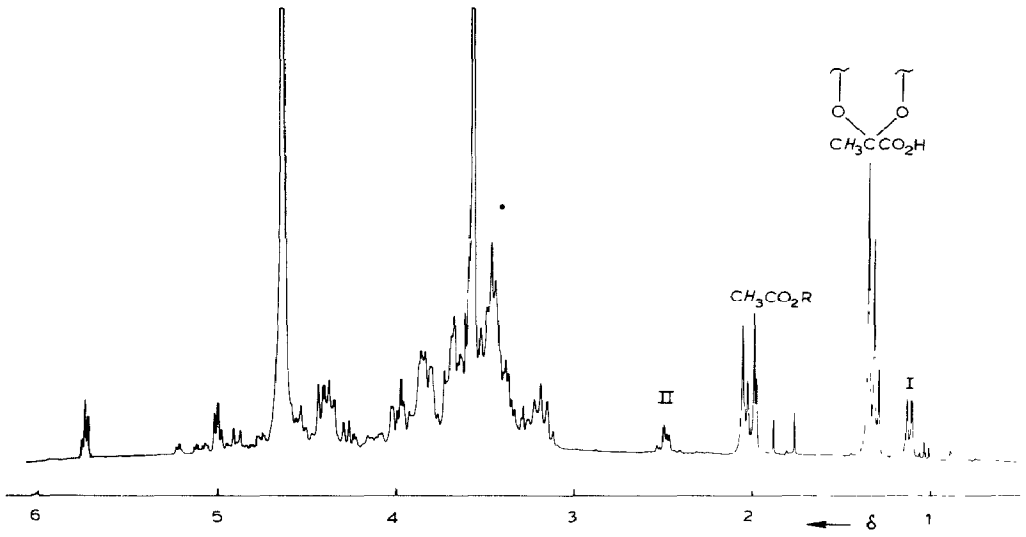

Fig. 1. 250-MHz Fourier-transform ^{1}H-n.m.r. spectrum of the oligosaccharide fragments generated by the action of a phage depolymerase on the extracellular polysaccharide of *Rhizobium trifolii* 0403.

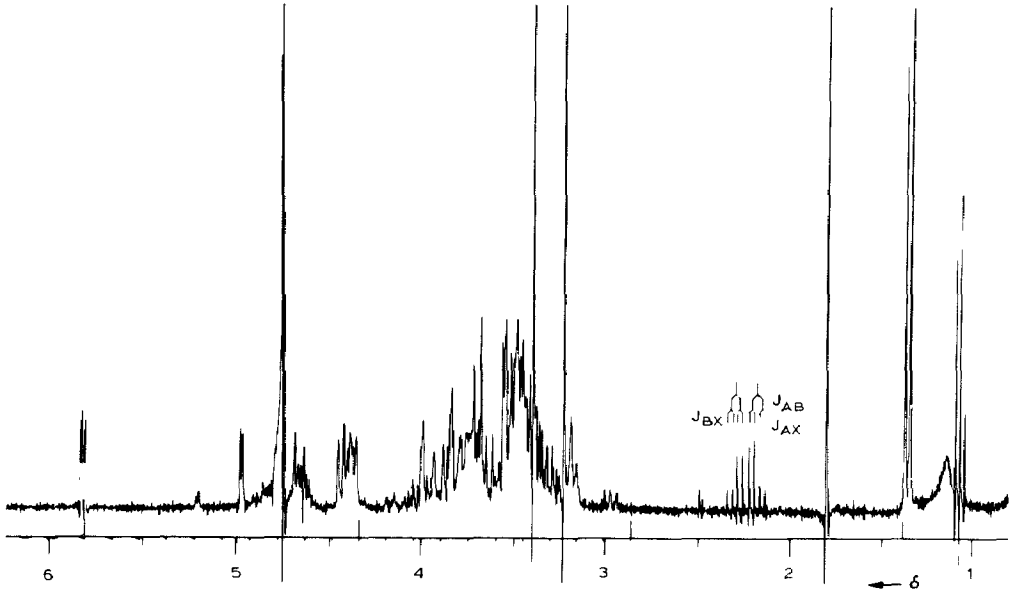

Fig. 2. Laurentz–Gauss resolution-enhanced 250-MHz ^{1}H-n.m.r. spectrum of the base hydrolyzed, enzymically produced oligosaccharides from the extracellular polysaccharide of *Rhizobium trifolii* 0403.

and 2.58 now appeared as a well defined set of resonances whose relative intensities and chemical shifts indicated them to be the two upfield components of an ABX spin-system. The A and B protons resonated at δ 2.18 and 2.30, respectively, and displayed a mutual

coupling (J_{AB}) of 14.7 Hz, a J_{AX} value of 6.4 Hz, and J_{BX} of 7.3 Hz. The large value of J_{AB} suggested geminal coupling, and indicated that the group of resonances was attributable to a methylene group adjacent to an asymmetric carbon atom bearing a single proton. The original chemical shift of the methylene protons and the upfield shift induced by addition of base indicated that this group was also adjacent to a carboxyl group. Upon treatment of the sample with base, the most upfield group of signals (group I, Fig. 1) became resolved into two intense peaks, together with another sharp, low-intensity peak.

Double-resonance experiments established the position of the third component of the ABX spin system (Fig. 3). Irradiation at the center of the AB components with enough power to decouple both protons from the X proton produced no immediately obvious changes in the other signals. However, subtraction of the decoupled spectrum from the undecoupled spectrum revealed a quartet (J = 7.8 Hz) at δ 4.02. Irradiation at this latter position collapsed the A and B signals to a pair of mutually coupled doublets (J = 14.7 Hz) and also collapsed the two large, upfield signals to a broad singlet (Fig. 3). This result indicated that the two upfield signals were due to a methyl group coupled to the proton that resonated at δ 4.02.

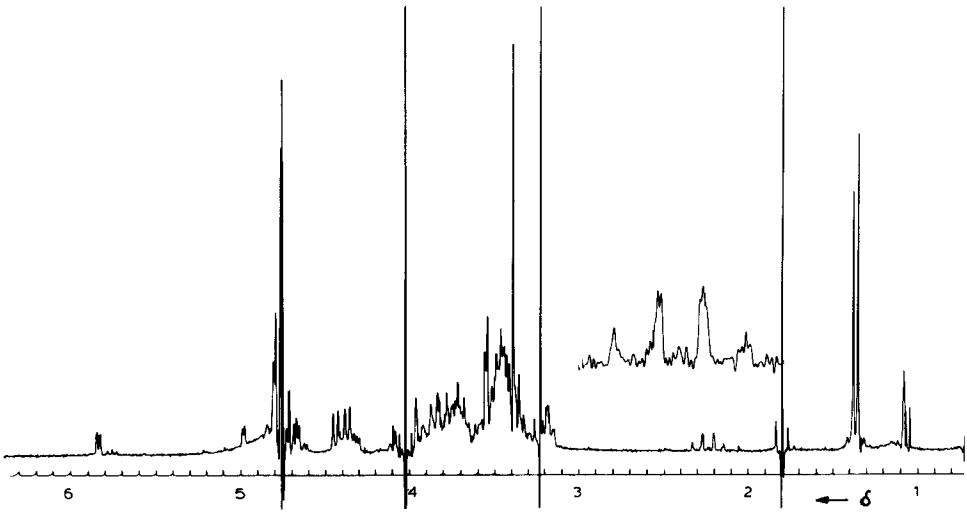

Fig. 3. 250-MHz ^{1}H-n.m.r. spectrum of the base-hydrolyzed oligosaccharides after irradiation of the signal at δ 4.02. Note the pair of mutually coupled doublets resulting from collapse of the pair of doublets of doublets assigned to the A and B components of the ABX system. This region of the spectrum is expanded in the inset.

The observed splittings, integrals, and chemical shifts of these groups of protons suggested a 3-hydroxybutanoyl moiety. The parameters measured in the experiment agreed with those observed from the spectrum of authentic sodium L-3-hydroxybutanoate (Fig. 4). The further splitting of the methylene signals of the oligosaccharide (Fig. 2) as compared with the splitting observed for the same group in the spectrum of the sodium salt of the free acid (Fig. 4) suggested that the 3-hydroxybutanoyl group might be attached to a sugar by an ether linkage. A bulky substituent on the 3-hydroxyl group of

3-hydroxybutanoic acid may lead to restricted rotation about the C-2—C-3 bond thus making the methylene protons (which are adjacent to a chiral center) magnetically non-equivalent. The fact that 3-hydroxybutanoic acid could not be detected by t.l.c. or l.c. analysis of the dialyzable fraction of the base hydrolyzate of both the oligosaccharide and polysaccharide also suggests an ether linkage. The spin multiplicities completely ruled out the possibility of the resonances being due to succinate or lactate residues. Succinate signals should collapse to a sharp singlet on hydrolysis of the ester groups by base.

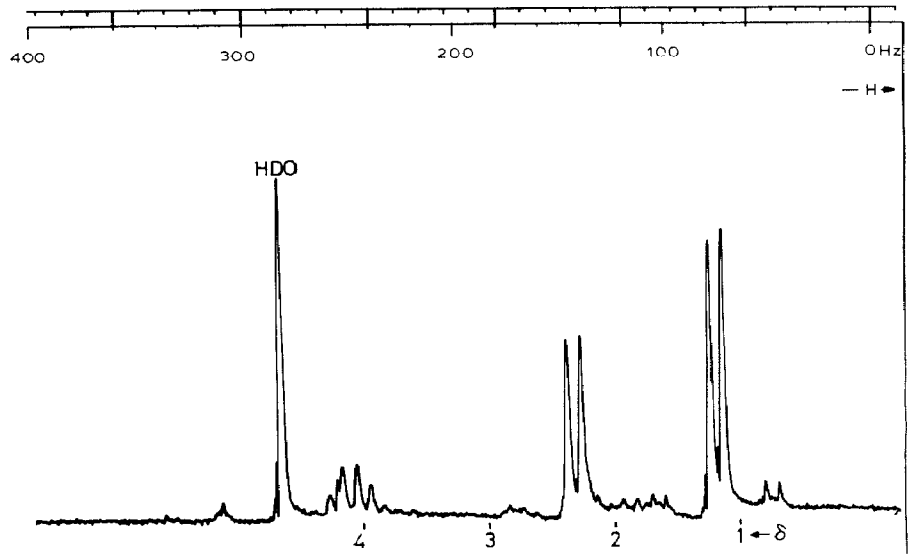

Fig. 4. 60-MHz ^{1}H-n.m.r. spectrum of the sodium salt of L-3-hydroxybutanoic acid in deuterium oxide.

The presence of 3-hydroxybutanoic acid in the extracellular polysaccharide was confirmed chemically. The oligosaccharide was digested in concentrated sulfuric acid for 15 min at 100°. This procedure was expected to yield *trans*-2-butenoic acid by elimination of the 3-alkoxy group from the butanoyl group. 2-Butenoic acid is stable under these conditions[9]. The presence of *trans*-2-butenoic acid in the processed mixture was indicated by l.c. (silica gel G with 5:3:1 dichloromethane—ethyl acetate—ethanol; acidic components located by spraying with 0.05% aqueous Bromthymol Purple adjusted to pH 8.0 with aqueous ammonia). Analysis by high performance liquid chromatography (l.c., detection at 235 nm, Bio-Rad Aminex HPX-87H column, 26 mM H_2SO_4 eluent, 55°) also indicated *trans*-2-butenoic acid to be present in the mixture. A compound having a retention time identical to that of an authentic sample of *trans*-2-butenoic acid (21.05 min) was collected in the l.c. effluent. Combined g.l.c.—m.s. (70eV, glass column of 15% SP1220 and 1% phosphoric acid on 100—120 mesh Chromosorb, temperature 100—160° at 5°/min, He carrier at 30 mL/min, detection by selective ion-monitoring at *m/z* 86) of this component gave a mass spectrum (Fig. 5) in which the base peak was found at *m/z* 86, the value of M^+ for 2-butenoic acid. Other diagnostic fragments were found at *m/z* 41 ($M - H - CO_2$) and 68 ($M - H_2O$). The mass spectrum and retention time (4.80 min) of the compound matched those of *trans*-2-butenoic acid. The u.v. spectrum of this

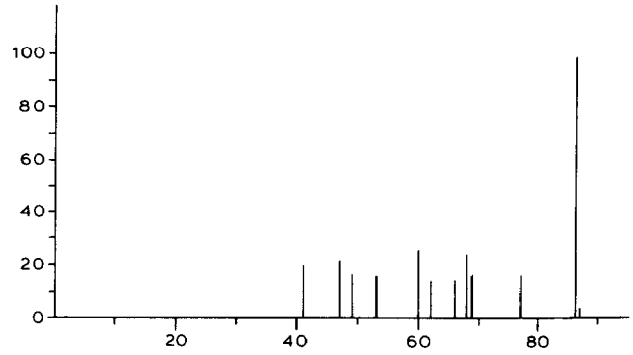

Fig. 5. Electron-impact mass spectrum of the component having the same l.c. retention-time as 2-butenoic acid.

compound in H_2SO_4 showed a maximum at the same position as that of *trans*-2-butenoic acid (235 nm in sulfuric acid solution). Essentially the same result was obtained when a sample of the parent polysaccharide was degraded and analyzed by the same method. Thus we conclude that ether-linked 3-hydroxybutanoic acid represents a novel non-carbohydrate substituent of the extracellular polysaccharide of *Rhizobium trifolii* 0403. Based on the ^{1}H-n.m.r. spectrum, the molar ratio of 3-hydroxybutanoate to pyruvate in the oligosaccharides was 1:4.0.

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REFERENCES

1 W. F. Dudman and W. F. Heidelberger, *Science*, 164 (1969) 954–955.
2 L. P. T. M. Zevenhuizen, *J. Gen. Microbiol.*, 68 (1971) 239–243.
3 B. K. Robertsen, P. Aman, A. G. Darvill, M. McNeil, and P. Albersheim, *Plant Physiol.*, 67 (1981) 389–400.
4 A. Amemura and T. Harada, *Carbohydr. Res.*, 112 (1983) 85–93.
5 M. C. Cadmus, K. A. Burton, and M. E. Slodki, *Appl. Environ. Microbiol.*, 44 (1982) 242–245.
6 J. Sherwood, G. Truchet, F. Dazzo, and J. Vasse, *North American Rhizobium Conf.*, Ithaca NY, 1983, abstr.
7 S. Higashi and M. Abe, *J. Gen. Appl. Microbiol.*, 24 (1978) 143–153.
8 A. Amemura, T. Harada, M. Abe, and S. Higashi, *Carbohydr. Res.*, 115 (1983) 165–174.
9 J. H. Law and R. A. Slepecky, *J. Bacteriol.*, 82 (1961) 33–36.
10 F. B. Dazzo, M. Urbano, and W. Brill, *Curr. Microbiol.*, 2 (1979) 15–20.