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Structural determination of an exocellular mannan from *Rhodotorula mucilaginosa* YR-2 using ab initio assignment of proton and carbon NMR spectra

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Abstract

The structure of the title mannan was determined exclusively by NMR. Because of the short relaxation time of the native mannan (100 kDa), a partially hydrolyzed mannan (10 kDa) was used for proton assignments by COSY, to correlate proton and carbon signals by HMQC, and to determine linkage positions between residues by HMBC. A further hydrolyzed mannan (oligomers of $\sim$1.5 kDa) was used to determine the anomeric configuration, using Wilker's quasi-3D method [Wilker, W.; Leibfritz, D. *Magn. Reson. Chem.* **1995**, *33*, 632–638]. The procedure presented here can be used to determine the structure of any polysaccharide.

Keywords: *Rhodotorula mucilaginosa*; Mannan; Structural determination; NMR; 2D method

1. Introduction

^{13}C NMR spectrometry is a very powerful method for determining the structures of polysaccharides. In particular, the ^{13}C chemical shifts of a glucan unequivocally[1,2] gives the positions of the inter-residue linkages and the anomeric configuration. Also, the anomeric proton splitting $^{3}J_{H-H}$, which depends on the dihedral angle between H-1 and H-2, shows the anomeric configuration. However, for mannans, having the 2-hydroxyl group axial, the carbon chemical shift gives no unequivocal information about the anomeric configuration,[2,3,4e] and the anomeric proton splitting has virtually the same values for both the α and β configurations. Although proton chemical shifts may suggest the anomeric configuration in a mannan, these shifts are affected by environmental circumstances, because the protons usually exist on the molecular surface, and it is inadvisable to use proton chemical shifts to assign molecular configuration. Previous reports have used anomeric $^{1}J_{C,H}$ values of 170 Hz for equatorial protons and 160 Hz for axial protons to assign the pyranose anomeric configuration,[4] but the variation in these constants depends on the mode of substitution, so there is still some ambiguity. In this report, we determine unequivocally the structure of a newly isolated *Rhodotorula* mannan, using NMR spectra. Before this work, Gorin et al. reported the structure of a linear *Rhodotorula glutinis* mannan (15 kDa) that has alternating

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Table 1
Proton and carbon chemical shifts and J_{H-H} constants of the 10 kDa mannan

Position	1	2	3	4	5	6	6′
Residue A							
Protons	4.73	4.25	3.98	3.70	3.48	3.75	3.89
	($J_{1,2}$ 1.7)	($J_{2,3}$ 1.8)	($J_{3,4}$ 10.9)	($J_{4,5}$ 8.2)	($J_{5,6}$ 8.2, $J_{5,6'}$ 2.4)	($J_{6,6'}$ −14.0)	
Carbons	100.9	68.3	79.7	65.9	76.9	61.8	
Residue B							
Protons	4.85	4.11	3.82	3.83	3.51	3.74	3.96
	($J_{1,2}$ 2.0)	($J_{2,3}$ 1.4)		($J_{4,5}$ 9.1)	($J_{5,6}$ 8.2, $J_{5,6'}$ 2.3)	($J_{6,6'}$ −12.9)	
Carbons	97.5	71.0	72.4	77.7	75.8	61.4	

β-(1→3) and β-(1→4) linkages. However, they determined the structure chemically and assigned its carbon spectrum afterwards.[5] Unfortunately, the 2D NMR method was not available in the mid 1970s, and therefore, there was some ambiguity in the signal assignments. Our assignment of the ^{13}C spectrum of the present mannan differs from that of Gorin et al. After our determination of the present mannan, Matsuo et al. showed that a *Leptospira* mannan has the same structure.[6] Our method is very useful, not only for determining the structures of mannans, but also for polysaccharides in general, without using any previous NMR data.

2. Results and discussion

The molecular weight of the mannan studied is 100 kDa, and we readily obtained its 1D proton and carbon spectra. However, the shorter relaxation time makes the FID signals in the 2D spectra much weaker.[7] The relaxation time of our mannan is relatively short, because of its molecular weight (100 kDa), making it difficult to obtain its 2D spectra. We compared the carbon spectrum of the original 100 kDa mannan with that of a partially hydrolyzed 10 kDa mannan, and obtained identical 1D carbon spectra for both mannans.[†] This suggests that these mannans have the same structure and different molecular weights. Therefore, we obtained several 2D spectra of the 10 kDa mannan.

First, we obtained the homonuclear correlation (COSY) spectrum of the 10 kDa mannan. This showed the connectivity between its vicinal protons, and its $^3J_{H-H}$ constants showed the ring conformation of each residue. As shown in Table 1 and Fig. 1, the two lowest-field signals are assigned to H-1, because there are two oxygen atoms on C-1. By conventional analysis of the 2D spectrum, the H-1 cross peak showed the absorption of H-2 as in Fig. 1 (shown with a straight arrow). We then identified the cross peak between H-2 and H-3 using the same method, and then determined the complete vicinal proton connectivity for the mannan. When several absorption peaks overlapped each other strongly, their correct connectivity could readily be solved by using the homonuclear Hartmann–Harn (HOHAHA) spectra of a target saccharide.[3] Fig. 1 clearly shows that the mannan has both types of residue, because it shows two types of anomeric proton and two complete connectivity sets. In addition, the ^{13}C spectrum (the ordinate in Fig. 2) of the mannan suggested that it contained two different residues, because we found 12 absorption peaks in the spectrum. Fig. 2 is the heteronuclear multiple quantum correlation spectrum with field-gradient pulses (HMQC-FG) of the 10 kDa mannan. This shows the connectivity between directly bound carbon and proton atoms. We assigned the C-6 and H-6 signals, because only C-6 had two protons directly bound to it. Moreover, the lowest two cross peaks between the lowest two proton peaks and the lowest

[†] The referee pointed out that the carbon 1D spectrum of the 10 kDa mannan should show the anomeric signals of the reducing-end residues. However, the reducing end residues constitute roughly 1/60 (1.7%) of the mannan. Furthermore, the anomeric equilibrium decreases their signal intensities, and thus these signals might be hidden by noise.

two carbon peaks were readily assigned to the two types of anomeric position. Thus, their carbon peaks could be assigned by cross peaks between carbon peaks and the assigned proton peaks.

The absorption peaks around 3.83 ppm in the proton spectrum (the abscissa in Fig. 2) had two proton peaks that overlapped markedly. These peaks were connected to two carbon atoms, C-3 and C-4, in the same residue. At this stage, we were able to assign all the proton peaks and all the carbon peaks as in Table 1.

The heteronuclear multiple bond correlation spectrum with field-gradient pulses (HMBC-FG) shown in Fig. 3 clearly shows the inter-residue linkage positions, with cross peaks between the lower-field H-1 peak on Residue B and the C-3 peak on Residue A, and between the higher field H-1 on Residue A and the C-4 peak on Residue B. Therefore, two different residues, which have $(1\rightarrow3)$ and $(1\rightarrow4)$ linkages, alternate in the mannan.

In the COSY spectrum in Fig. 1, we identified the splitting of each proton's absorption from the cross peaks, even though the 1D proton spectrum showed rather broad, minimally separated absorption peaks. Unfortunately, we could not determine the coupling constant between H-3 and H-4 on Residue B, for the same reason as described in the classic NMR textbook by Pople, Schneider, and Bernstein.[8] The results are shown in Table 1, and suggest that the two types of residue have the $^4C_1$ pyranose conformation.[9] There is no conflict between the assigned $^4C_1$ conformation and the $^3J_{H-H}$ constants shown in Table 1, because H-1–H-2 and H-2–H-3 are in gauche disposition, and the other vicinal ring protons are anti-disposed.

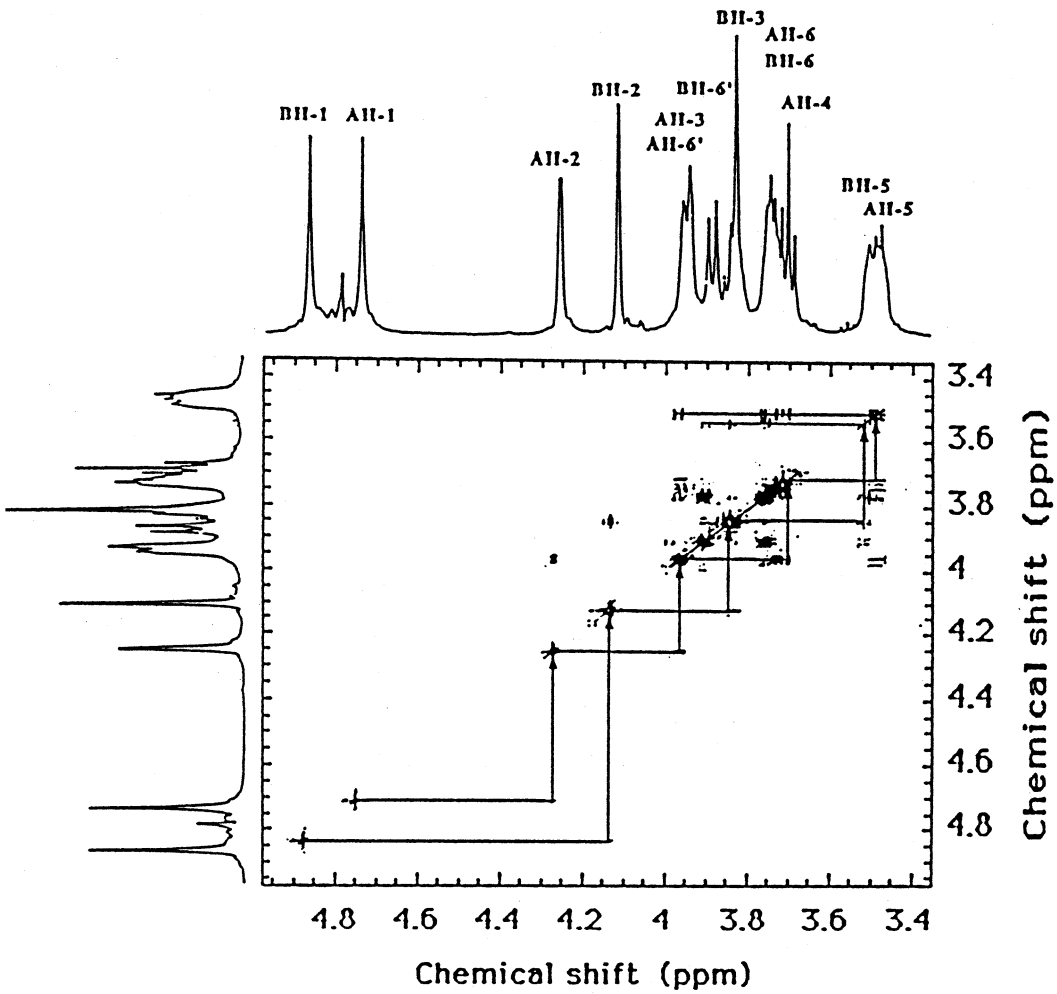

Fig. 1. The COSY spectrum of the 10 kDa mannan.

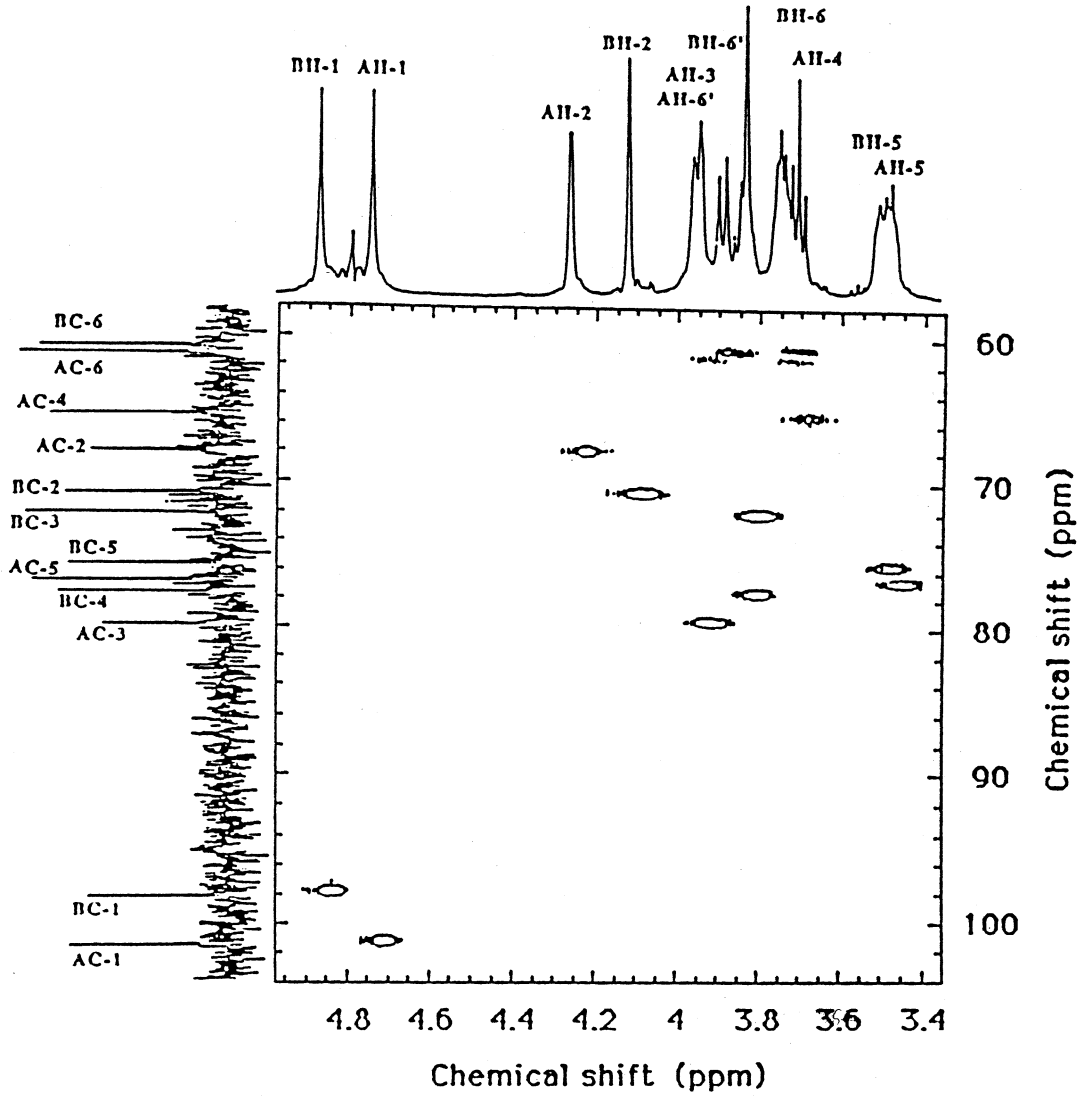

Fig. 2. The HMQC spectrum of the 10 kDa mannan.

Since the energetically lowest conformation of mannan is $^4C_1$, we cannot differentiate the α and β anomers from the coupling constant between H-1 and H-2, and the chemical shift of the anomeric carbon of the mannan shows no regular tendency,[2,3,4e] a situation very different for glucans.[1,2] Therefore, we tried to obtain the coupling constants between C-5 and H-1 for the methyl α- and β-mannopyranosides, using the method of Wilker and Leibfritz,[10] since we expected very different $^3J_{C-H}$ constants because of their different dihedral angles,[11] as shown before.[12] The results are shown in Table 2. We expected to assign the anomeric configuration of our mannan from the $^3J_{C-H}$ constant between C-5 and H-1. When we tried to obtain the $^3J_{C-H}$ value for the 10 kDa mannan, we were unsuccessful because of its relatively short relaxation time. We therefore used a further hydrolyzed mixture of mannan oligomers, smaller than the decamer. As shown in Table 2, we obtained small coupling constants for Residues A and B of the mannan, as expected for the β-anomeric configuration. This method can be applied to ketose-type saccharides, because we can use the $^3J_{C-H}$ constant in essentially the same manner as just described.

In conclusion, the mannan is shown to be a polysaccharide having alternating β-(1→3) and β-(1→4) linkages. We also showed that this method is applicable to the structural determination of other unknown polysaccharides, because it does not rely on previous

NMR spectral data for saccharides, or on any kind of chemical degradation other than depolymerization.

3. Experimental

Preparation of the mannan.—The inoculum was cultured in a 500 mL Erlenmeyer flask containing 100 mL YEDP medium (2% glucose, 1% polypeptone, and 1% yeast extract probably containing α-(1→6) and α-(1→3)-mannans) on a rotary shaker at 30 °C for 24 h. The culture medium was inoculated with 10^6 cells/mL. Batch fermentation was carried out in a laboratory fermentor TS-10 (Takasugi, Japan) in medium containing (g/L) sucrose 100.0, $(NH_4)_2SO_4$ 20.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, NaCl 0.1, $CaCl_2.2H_2O$ 0.1, and yeast extract 3.0, at pH 6.0. The fermentation was performed at 30 °C for 120 h, with agitation at 550 rpm, aeration at 1 vvm, and the pH was adjusted to 1.8 with aq ammonium.

The cell culture was centrifuged at 12,000*g* for 15 min to separate cells from the supernatant. The exocellular mannan in the supernatant was precipitated using 2 vols of 98% EtOH after ultrafiltration using Millipore PLGC 000 05 filters. The precipitate was recovered by centrifugation at 12,000*g* for 15 min, washed with EtOH, and dried.

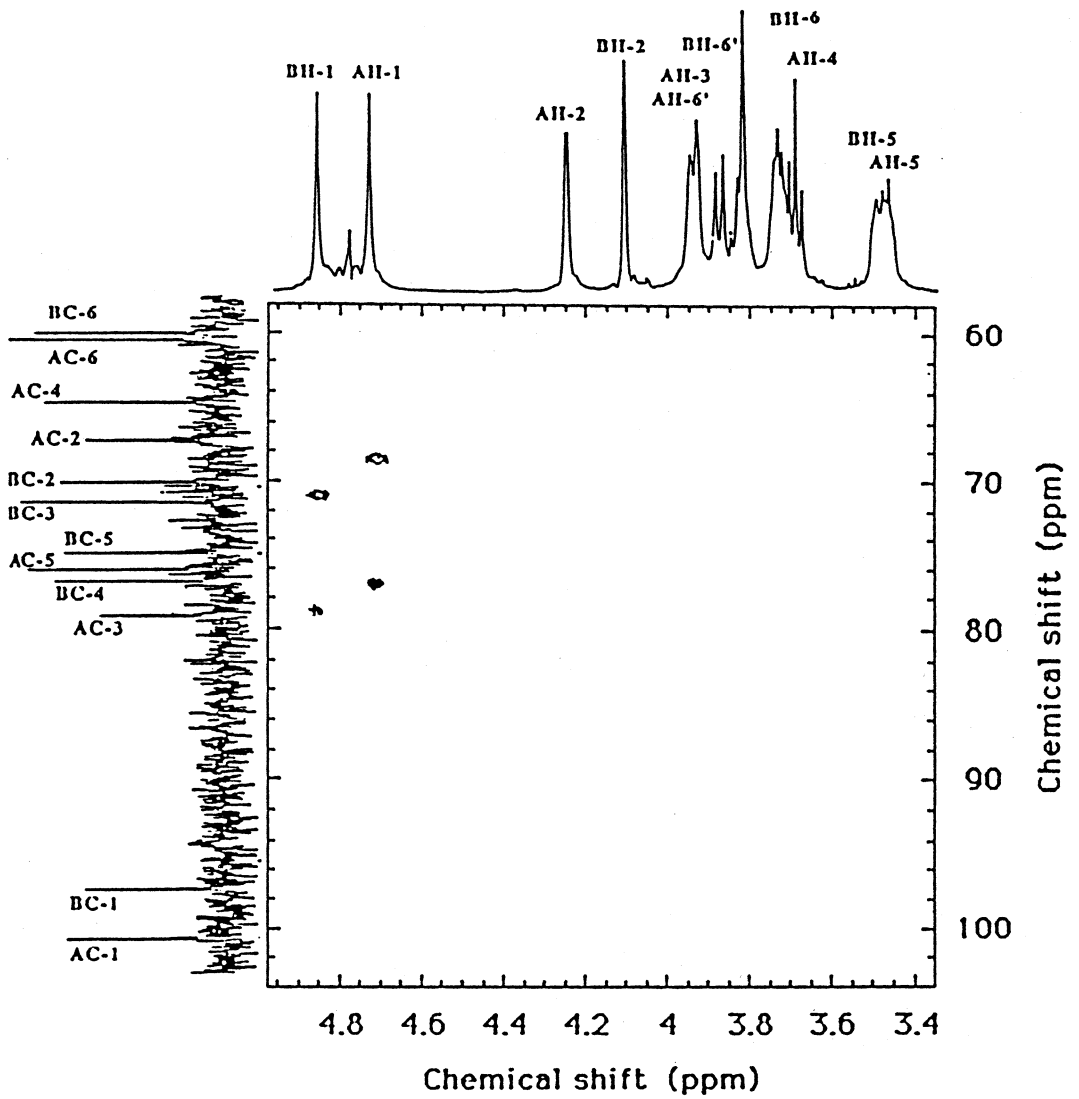

Fig. 3. The HMBC spectrum of the 10 kDa mannan.

Table 2
$^3J_{C\text{-}5\text{–}H\text{-}1}$ constants and dihedral angles between C-5 and H-1 of the 8kD oligomers

	J (Hz)	Dihedral angle (°)	
		Experimental [a]	Expected [b]
Methyl α-mannopyranoside	5.11	148	180
Methyl β-mannopyranoside	2.17	53	60
Residue A	2.72	46	
Residue B	1.12	68	

[a] The Karplus-type equation used in this table was $^3J_{C\text{–}H} = 5.5\cos^2\theta - 0.7\cos\theta + 0.6$.[11]

[b] Expected, since the typical chair form of cyclohexane is very similar to the $^4C_1$ form.

Preparation of the partially hydrolyzed mannan.—Crude exocellular mannan (7.8 g/L) was dissolved in distilled water and treated with Fehling's reagent. The sediment was recovered by centrifugation at 12,000*g* for 15 min. Copper was removed from the solution by a cation exchanger (Amberlite IR-120B). The purified mannan was precipitated with 2 vols of 98% EtOH. The precipitate was recovered by centrifugation at 12,000*g* for 15 min, washed with EtOH, and dried. The mannan was dissolved in 1 M HCl to give a concentration of 1 mg/mL. It was maintained at 50 °C for 5 h, made neutral with 1 M NaOH, and desalted using Amberlite IR-120 and IRA-410 ion-exchange resins. The fraction over 5 kDa was separated with a P-10 column (Pharmacia) and lyophilized.

Preparation of the manno-oligosaccharide.—The mannan was dissolved in 1 M HCl, as described for the partially hydrolyzed mannan. The fraction under 5 kDa was separated on a P-10 column, and the hepta-oligosaccharide (mean value) was separated by low-pressure liquid chromatography with Toyopearl HW-40, and lyophilized.

NMR methods.—Deuterium oxide was used as a solvent and sodium 4,4,4-trimethyl-4-sila-[$^2H_4$]pentanoate was selected as the internal standard. The NMR spectra were recorded using a JEOL Alpha 620 spectrometer at 620 MHz for protons and at 155 MHz for carbon. The concentration of the native mannan was saturated, and those of other partially hydrolyzed mannans were 20 mg/0.4 mL. The heteronuclear multi-bond correlation spectrum with field gradient pulses (HMBC-FG) was measured using the interval period between the first and second $\pi/2$ pulses of carbon fitted for $^3J_{C\text{–}H}$ 3 Hz. This interval time emphasizes a cross peak between C–H having a 3 Hz spin–spin coupling constant, and frequently the cross peaks between C–H with different coupling constants became weak or disappeared.[7] Three-bond C–H coupling constants were measured using the 2D version of the Wilker and Leibfritz method.[10] The τ_{max} was 200 ms with six samplings of τ between 40 and 180 ms, and the gradient- field pulse ratios used were $G_1{:}G_2{:}G_3 = 2{:}2{:}1$. The Karplus-type equation relating the three C–H bond-coupling constants and the dihedral angles in this paper is that derived experimentally by Mulloy et al.[11]

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