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Discrimination Between *Sporothrix schenckii* and *Ceratocystis stenoceras* Rhamnomannans by Proton and Carbon-13 Magnetic Resonance Spectroscopy

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The proton and ^{13}C magnetic resonance (CMR) spectra of 10 *Sporothrix schenckii* and three *Ceratocystis stenoceras* rhamnomannans were studied. On the basis of their CMR spectra, the rhamnomannans from *S. schenckii* strains could be classified into two major types, I and II, readily distinguishable from the polysaccharides formed at the same temperature (25 C) by *C. stenoceras*. Rhamnomannans from *S. schenckii* synthesized at 37 C gave CMR spectra (type III) differing from those of polysaccharides of types I and II and from those of *C. stenoceras* polysaccharides. The contention that *C. stenoceras* might be the perfect form of *S. schenckii* is not supported by the present data. A presumptive pathogenic mutant of *C. stenoceras* synthesized a type I rhamnomannan characteristic of *S. schenckii*, clearly differing from the rhamnomannans from *C. stenoceras* synthesized in the same conditions. Nuclear magnetic resonance spectroscopy revealed differences in the structures of the polysaccharides from these species not previously recognized by methylation analysis.

Proton magnetic resonance (PMR) spectra of yeast mannans have been used as a method of fingerprinting different polysaccharides (10, 11). Several polysaccharides from filamentous fungi of the genus *Ceratocystis* have also been studied by this method (12). These polysaccharides were classified into eight groups according to the similarity of their spectra.

Recently, ^{13}C magnetic resonance (CMR) spectra have been advantageously employed as a fingerprinting method of fungal polysaccharides because of their greater number of well-resolved signals as compared with those in the PMR spectra. The assignments of several signals to particular structures present in oligosaccharides and in yeast polysaccharides have been established (3, 5).

The rhamnomannans from the human pathogen *Sporothrix schenckii* have structures closely related to those of some *Ceratocystis* polysaccharides (15). Although no major differences could be recognized between polysaccharides from *S. schenckii* and *Ceratocystis stenoceras* by methylation analysis, the PMR spectra of these polysaccharides showed definite patterns allowing the recognition of different structures. The differences were mostly evident when the polysaccharides were isolated from cultures grown at 25 C and much less with cultures grown at 35 to 37 C (14, 15).

In the present work the rhamnomannan structures from several strains of *S. schenckii* were examined by PMR and CMR spectroscopy in an attempt to determine the homogeneity of this species. In addition, we compared these spectra with those for the polysaccharides from *C. stenoceras*, a species that has been proposed as the perfect stage of *S. schenckii* (7, 8).

MATERIALS AND METHODS

Microorganisms. All cultures used in this work belong to the Mycology Laboratory, Department of Dermatology, College of Physicians and Surgeons of Columbia University. Strains of *S. schenckii* no. 1099.10, 1099.15, 1099.16, 1099.18, 1099.23, 1099.24, 1099.26, and 1099.27 were isolated from human patients with sporotrichosis. *S. schenckii* 1099.13 was isolated from a water well in Algiers; it was received from the Pasteur Institute, Paris. *C. stenoceras* 1099.40 and 1099.41 were received from the Centraalbureau voor Schimmelcultures (CBS 237.32 and CBS 360.71). *C. stenoceras* 1099.11 and 1099.12 were received from F. Mariat and correspond to cultures IP-1013-70 (wild type) and IP-1021-70 (pathogenic mutant) of the Pasteur Institute. Due to several of its characteristics, among which are the guanine/cytosine content of its deoxyribonucleic acid (DNA) and

the high percentage of hybridization with an *S. schenckii* DNA (submitted for publication), strain 1099.12 was reclassified by us as *S. schenckii* 1099.12.

Polysaccharides. Polysaccharides were isolated from cells and from the supernatant fluid of cultures obtained at 25 or 37 C according to the procedure used before (15). Polysaccharides formed at 37 C were only obtained from a few cultures of *S. schenckii* that grew well at this temperature.

Nuclear magnetic resonance spectroscopy. PMR spectra of *S. schenckii* and *C. stenoceras* polysaccharides were obtained in a 100 MHz Varian nuclear magnetic resonance (NMR) spectrometer from 20% solutions in D_2O at 70 C with tetramethylsilane (TMS) (τ = 10) as the external standard. Results are expressed in τ values to ease their comparison with spectra already published using this unit (12, 15). Only the H1 region of the spectra was examined.

^{13}CMR spectra were obtained in a Varian XL 100-15 NMR spectrometer with Fourier transform on D_2O solutions (5 ml) of polysaccharide containing natural abundances of ^{13}C at 70 C in a tube 12 mm in diameter. The number of transients used varied from 5,000 for a 20% solution to 40,000 for a 3% solution. The sweep width was 5,000 Hz (198.4 ppm), the acquisition time 0.4 s, and the pulse width 50 μs. The maximal decoupling power (10 W) was used centered on 500 Hz downfield from the ^1H resonance of TMS. Chemical shifts are expressed in parts per million, the term δ_c referring to the downfield difference of the particular signal and that of the external standard, TMS. The signal of an internal standard, dioxane, was 68.0 ppm.

RESULTS

CMR spectra. Tables 1 and 2 show the values for the CMR signals of *S. schenckii* polysaccharides that were classified into types I, II, and III on the basis of these spectra. Minor differences were observed in the spectra of the various type I polysaccharides that are probably due to the poor resolution of the peaks rather than to different polysaccharide structures. Peaks at δ_c 105.3 to 105.4 were absent or ill defined within type I spectra.

Type II *S. schenckii* polysaccharides that can be compared with type I polysaccharides because both types were obtained at the same temperature (25 C) gave different CMR spectra: the peak at δ_c 80.2 to 80.4 was absent. More noticeable, however, was the absence of the signal at δ_c 96.6 to 96.7 that was a common feature among spectra of type I polysaccharides. A very small peak at δ_c 97.0 appeared in the spectra of two polysaccharides from the three classified as type II.

Spectra of polysaccharides of *S. schenckii* obtained at 37 C (type III) were quite different from those of types I and II. Peaks at δ_c 80.0 and 80.2 were lacking and, in the C1 region, the peaks at δ_c 96.5, 103.5, and 105.4 were absent. Within this group, the polysaccharide of strain

TABLE 1. *^{13}C magnetic resonance signals of S. schenckii polysaccharides of type I*[a]

^{13}C magnetic resonance signals						
1099.10	1099.18	1099.23	1099.26	1099.12	1099.13	1099.27
18.4	18.3	18.3	18.2	18.3	18.3	18.3
62.7	62.7	62.6	62.6	62.6	62.6	62.5
66.5	66.4	66.4	66.4	66.4	66.3	66.2
67.3	67.3	67.2	67.2	67.3	67.0	67.0
67.9	67.7	67.7	67.6	67.7	67.6	67.5
70.3	70.3	70.2	70.2			
70.6	70.6	70.5	70.4	70.5	70.5	70.5
71.9	71.8	71.8	71.8	71.7	71.6	71.7
72.6	72.4	72.4	72.4	72.4	72.3	72.3
73.8	73.7	73.6	73.6	73.7	73.6	73.5
75.9	75.7	75.6	75.6	75.6	75.6	75.6
76.9	76.9	76.9	76.7	76.8	76.7	76.7
80.1	80.0	80.0	80.1	80.1		79.9
80.4	80.2	80.2	80.3	80.3	80.3	80.2
96.8	96.5	96.6	96.6	96.7	96.6	96.5
98.1	98.1	98.0	98.0	98.0	97.9	97.9
99.8	99.8	99.7	99.8	99.8		
100.1	100.1	100.0	100.0	100.1	100.0	99.9
101.1	100.9	100.9	100.9	100.9	100.9	100.9
102.3	102.3	102.3	102.3	102.1	102.1	102.1
103.7	103.5	103.5	103.7	103.7	103.7	103.5
			105.4 (?)	105.3	105.4	105.3

[a] δ_c values in parts per million, relative to external TMS.

TABLE 2. *^{13}C magnetic resonance signals of S. schenckii polysaccharides of types II and III*[a]

^{13}C magnetic resonance signals					
Type II strains			Type III strains		
1099.15	1099.16	1099.24	1099.10	1099.12	1099.18
18.2	18.2	18.2	18.4	18.3	18.2
62.6	62.6	62.6	62.7	62.6	62.6
66.4	66.4	66.4	66.5	66.4	66.4
67.3	67.3	67.3	67.4	67.2	67.3
67.6	67.7	67.7	67.9	67.7	67.6
70.2	70.2	70.2	70.3	70.2	70.2
70.4	70.5	70.5	70.6	70.5	70.5
71.8	71.8	71.8	72.0	71.8	71.8
72.4	72.4	72.4	72.6	72.4	72.4
73.6	73.6	73.6	73.7	73.6	73.6
74.9	74.9	74.6		75.0	
75.6	75.7	75.6	75.7	75.6	75.7
76.7	76.7	76.7	76.9	76.7	76.9
80.0	80.0	80.1			
97.0	97.0				
97.9	98.0	98.0	98.1	98.0	98.0
99.8		99.7	99.9	99.9	99.8
100.0	100.0	100.0	100.1		100.0
100.9	100.9	100.9	101.1	100.9	100.9
102.3	102.3	102.3	102.3	102.3	102.3
103.3	103.3	103.5			
105.4	105.4	105.4			

[a] Polysaccharides of type III were isolated from cultures grown at 37 C. δ_c values are in parts per million.

1099.12 was a little different from the others (Table 2).

C. stenoceras polysaccharides obtained at 25 C gave spectra of a very particular type readily distinguishable from any *S. schenckii* polysaccharide. Prominent differences were the absence of peaks at δ_c 103.5 to 103.7, 99.7 to 99.8, and 96.5 to 96.6 in the C1 region. Additional peaks at δ_c 75.0 to 75.2, 81.4, and at 97.0 and the presence of an important peak at δ_c 105.4 to 105.5 were differential features of the spectra of *C. stenoceras* polysaccharides (Table 3, Fig. 1). In accordance with the simpler structure of *C. stenoceras* 1099.40 polysaccharide, signals at δ_c 100.0 and 62.7 were virtually absent in its CMR spectrum. Figure 1 depicts the partial spectra of polysaccharides of the different types indicating their main differences. The upper-field region with the signal of the ^{13}C nuclei of the methyl group of rhamnose (δ_c 18.3) is not shown in Fig. 1.

PMR spectra. Spectra at the H1 region for both *S. schenckii* and *Ceratocystis* (*C. stenoceras* and *C. ulmi*) polysaccharides showed a few minor peaks at higher field than τ 4.51 (12, 15). These peaks were small and sometimes ill defined in the spectra of *S. schenckii* polysaccharides and will not be considered in the classification of the different polysaccharides.

Seven strains of *S. schenckii* formed polysaccharides at 25 C having spectra (H1 region) identical to A and B in Fig. 2. Polysaccharides from these strains had been classified as type I by CMR spectroscopy. Three strains of *S. schenckii* had polysaccharides obtained at 25 C giving spectra identical to C in Fig. 2. These strains were classified as type II by CMR spectroscopy. PMR spectra of *S. schenckii* polysaccharides of type II were similar to the spectra of *S. schenckii* polysaccharides obtained at 37 C (type III) and to those of *C. stenoceras* polysaccharides (spectra D and E, respectively, in Fig. 2). With the three *C. stenoceras* polysaccharides there was no trace of a peak at τ 4.41 to 4.42, which seems characteristic of *S. schenckii* polysaccharides' spectra of type I. *S. schenckii* type II polysaccharides and those obtained at 37 C (type III) usually showed a minor peak at τ 4.41 or a trace of it. The rhamnomannan of strain 1099.40 of *C. stenoceras*, which has a simpler structure than those of other *C. stenoceras* strains as it contains only traces of 2,4-di-*O*-substituted and 4-*O*-substituted D-mannopyranose units (15), gave a PMR spectrum with only a very minor peak at τ 4.14 (F in Fig. 2).

TABLE 3. *^{13}C magnetic resonance signals of C. stenoceras polysaccharides*[a]

^{13}C magnetic resonance signals		
1099.11	1099.40	1099.41
18.3	18.3	18.3
62.6	62.7[b]	62.7
66.4	66.4	66.4
67.2	67.3	67.4
67.7	67.7	67.8
70.2	70.2	70.3
70.6	70.6	70.6
71.8	71.8	71.9
72.5	72.5	72.6
73.6	73.6	73.7
75.2	75.0	75.1
75.7	75.7	75.8
76.7	76.7	76.9
80.1	80.2	80.2
81.4	81.4	81.4
97.0	97.0	96.8
98.0	98.0	98.1
100.0	100.0[b]	100.2
100.9	100.9	101.1
102.3	102.3	102.3
105.4	105.4	105.5

[a] δ_c values are in parts per million.
[b] Very small peaks.

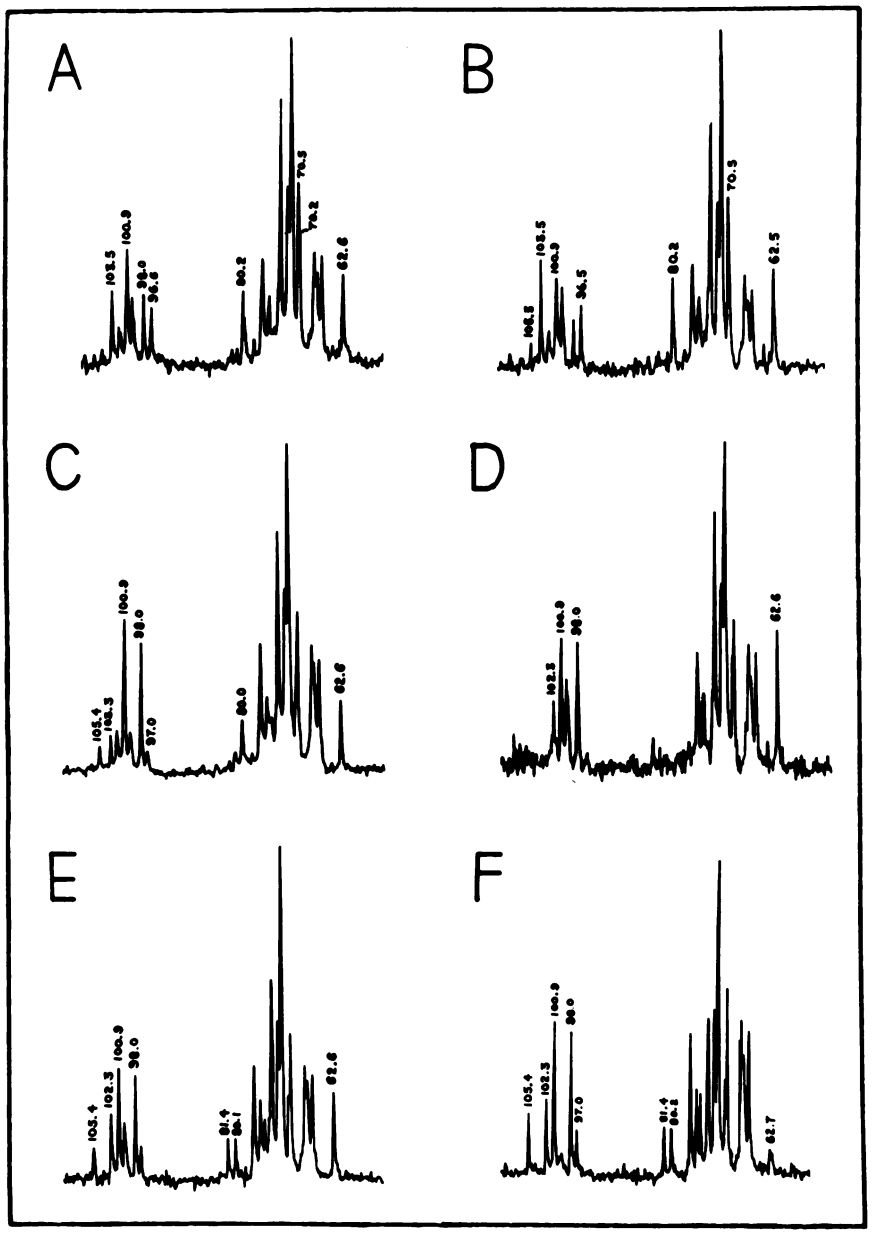

FIG. 1. *Partial CMR spectra of polysaccharides from (A) S. schenckii 1099.23 (type I); (B) S. schenckii 1099.27 (type I); (C) S. schenckii 1099.16 (type II); (D) S. schenckii 1099.18 (grown at 37 C, type III); (E) C. stenoceras 1099.11; (F) C. stenoceras 1099.40.*

DISCUSSION

On the basis of their NMR spectra, the rhamnomannans from *S. schenckii* strains grown at 25 C could be classified in two major types (I and II). Ten rhamnomannans from 10 different strains isolated from different sources and diverse geographic regions were studied in the present work. It is probable then that the results are truly representative of the species *S. schenckii*.

It is interesting to ask whether one type of polysaccharide arose by mutations in cells originally producing the other type, or whether both types were concurrently selected in nature after emerging independently. In the latter case the

species *S. schenckii* could include morphologically similar microorganisms that may, however, have derived from different species of *Ceratocystis*. Several suggestions of such relationships between *S. schenckii* and species of *Ceratocystis* have been pointed out (13). Similarities between the conidial and yeast-budding forms of *S. schenckii* and those of a few species of *Ceratocystis* were studied. Other reports suggest that a particular species of *Ceratocystis*—*C. stenoceras*—might be the perfect stage of *S. schenckii* inasmuch as their morphological similarities and the possible isolation of pathogenic mutants of wild-type *C. stenoceras* were considered (7, 8). However, the wild-type culture of *C. stenoceras* from which the pathogenic mutants were isolated might have consisted of a mixture of *C. stenoceras* and

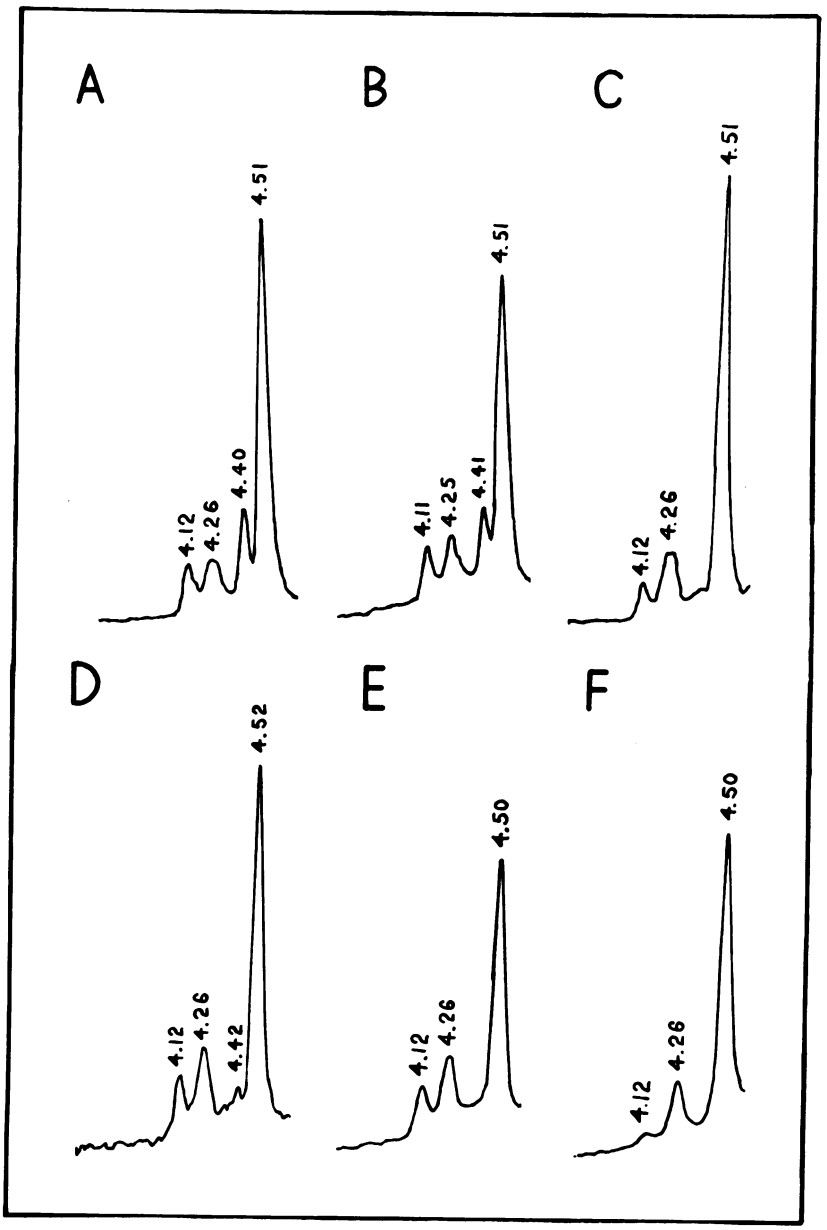

FIG. 2. *PMR spectra (H1 region) of polysaccharides from (A) S. schenckii 1099.23; (B) S. schenckii 1099.10; (C) S. schenckii 1099.16; (D) S. schenckii 1099.18 (grown at 37 C); (E) C. stenoceras 1099.41; (F) C. stenoceras 1099.40.*

S. schenckii with morphologically indistinguishable conidial and yeast forms: inoculation into animals might have selected the pre-existing pathogenic forms. The possible heterogeneity of the original culture of wild-type *C. stenoceras* apparently was not excluded. One of the presumptive pathogenic variants of *C. stenoceras* (1099.12) has been included in our studies. Its polysaccharide synthesized at 25 C was typical of *S. schenckii* and clearly different from those of *C. stenoceras*. Both the PMR and CMR spectra placed this polysaccharide in group I of *S. schenckii* polysaccharides. The spectra of three polysaccharides from typical strains of *C. stenoceras* were identical and readily distinguishable from those of *S. schenckii*. On this basis it seems that type I-polysaccharide-producing strains of *S. schenckii* have an as yet unknown perfect stage among the *Ceratocystis* species. PMR spectra (H1 region) of type II polysaccharides were very similar to those of *C. stenoceras* polysaccharides. The signal at τ 4.41 to 4.42 characteristic of type I polysaccharides was virtually absent in type II. However, the CMR spectra clearly showed that these polysaccharides were quite different from those of *C. stenoceras*.

The suitability of the choice of this phenotype in assessing the relationships among species may be questioned in light of the considerable change in structure observed in polysaccharides formed at 37 C as compared with those from the same strains growing at 25 C. PMR spectra for polysaccharides formed at 37 C (type III) resembled those of type II and *C. stenoceras* polysaccharides. In some cases, however, a minor signal at τ 4.40 was present, although much reduced in size when compared with those appearing in the spectrum of the corresponding polysaccharide formed at 25 C. We did not compare, however, the CMR spectra of polysaccharides from *S. schenckii* and *C. stenoceras* formed at 37 C. The strains of *C. stenoceras* grew very poorly at this temperature as did one strain of *S. schenckii* isolated from a nonhuman source (1099.13). The presence of peaks at τ 4.40 to 4.42 in the PMR spectra was also evident with *S. schenckii* polysaccharides formed at 35 C on prolonged incubation. Toriello et al. (14) observed that a peak at τ 4.40 was present in the spectra of polysaccharides isolated from the culture filtrates without alkali treatment and not in those of polysaccharides extracted from cells with 2% KOH. The CMR spectra of *S. schenckii* polysaccharides obtained at 37 C were much simpler than those of types I and II or of *C. stenoceras* polysaccharides since they lacked signals at δ_c 105.4, 103.5, 96.6 in the C1 region, and at δ_c 80.0 and 80.2.

PMR and CMR spectra proved to be very helpful in fingerprinting *S. schenckii* and *Ceratocystis* polysaccharides. Being closely related, differences in the structures of these polysaccharides could hardly be detected by methylation analysis (15). By using this technique, it was shown that all the rhamnomannans had the structures shown in Fig. 3. The polysaccharides from the various species grown at 25 and 37 C had varying amounts of the mono- and dirhamnosyl side chains as well as small amounts of 2,4-di-*O*-substituted and 4-*O*-substituted α-D-mannopyranose and traces of other linkages. The possibility cannot be excluded that the samples obtained at 25 and 37 C consist of different ratios of two different polysaccharides with high and low amounts, respectively, of the dirhamnosyl side chains.

Specific structural assignments to the various CMR signals cannot be done accurately at present, since there are no previous studies on spectra of oligosaccharides containing both rhamnose and mannose. A few tentative assignments, however, may be suggested. (i) The signal at δ_c 62.6 to 62.7 probably represents the resonance of the C6 from α-D-mannopyranose units unsubstituted at O6. This assignment is made by comparison with the C6 signal of methyl-D-mannopyranoside (2, 9) and the C6 signals of nonreducing end units in mannose-containing oligosaccharides (3). (ii) The δ_c 96.5 to 96.7 peak can be assigned to C1 of 2-*O*-substituted α-L-rhamnopyranosyl units by analogy with structures containing 2-*O*-substituted D-mannose units (3). This signal is absent from spectrum D, which arises from a polysaccharide with few 2-*O*-linked units (15). (iii) The signal at δ_c 97.9 to 98.1 corresponds to C1 resonances of α-L-rhamnopyranosyl nonreducing end units. This assignment is made by comparison with

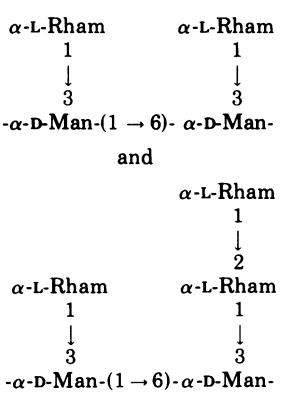

FIG. 3. *Rhamnomannan structures detected by methylation analysis. Abbreviations: Rham, rhamnose; Man, mannose.*

the spectrum of a disaccharide, 3-*O*-α-L-rhamnopyranosyl-α,β-D-mannose, isolated by partial acetolysis of the rhamnomannan from *C. ulmi* (4). This spectrum (unpublished data) has signals at δ_c 94.9 and 97.4 that correspond to C1 of the reducing unit and C1 of the nonreducing unit, respectively. (iv) The δ_c 100.0 to 100.1 signal seems to arise from C1 of 2,4-di-*O*-substituted α-D-mannopyranose units since it appears in the spectra of all polysaccharides containing these structures. It is reduced to a trace in the polysaccharide from strain 1099.40 that contains only traces of 2,4-di-*O*-substituted and 4-*O*-substituted α-D-mannopyranose units. (iv) The signal at δ_c 100.9 can be assigned to C1 of 3,6-di-*O*-substituted α-D-mannopyranosyl units. This is a major signal in all the spectra as would be excepted from the structure of these polymers (15). A similar signal (δ_c 100.5) is found in the spectrum of a rhamnomannan from *Hyalodendron pyrium*. This polymer also contains a (1 → 6)-linked α-D-mannopyranosyl main chain substituted in the number 3 positions by α-L-rhamnopyranosyl units (unpublished data). The hyphomycete *Hyalodendron* has been reported as the conidial state of certain *Ceratocystis* species (1).

The polysaccharide of *C. stenoceras* 1099.40 with a simplified structure that contains only traces of 2,4-di-*O*-substituted and 4-*O*-substituted mannopyranose units still gave a CMR spectrum with five signals at the C1 region. Since according to the methylation analysis (15) only three signals were expected—C1 of the α-L-rhamnopyranosyl nonreducing end unit, C1 of the 2-*O*-substituted α-L-rhamnopyranosyl internal unit in the side chain, and the C1 of the 3,6-di-*O*-substituted α-D-mannopyranosyl units in the main chain—this finding implies a hitherto unrecognized feature of the structure of the polysaccharide of *C. stenoceras*. These additional signals in the spectrum could be due to β-linked rhamnopyranosyl units. Yeast mannans containing both β- and α-linked D-mannopyranose units have already been described (6).

Further assignments of the CMR signals to particular structures and confirmation of the present assignments will be possible upon studying the complete structure of selected polysaccharides of *S. schenckii* and *C. stenoceras* and the NMR spectra of their fragments obtained by partial acetolysis.

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