INFECTION AND IMMUNITY, Nov. 1973, p. 685-693

Vol. 8, No. 5
Printed in U.S.A.

Comparison of the Rhamnomannans from the Human Pathogen *Sporothrix schenckii* with Those from the *Ceratocystis* Species

L. R. TRAVASSOS, P. A. J. GORIN, AND K. O. LLOYD

Departments of Dermatology and Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032, and Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan, Canada

Received for publication 28 June 1973

Rhamnomannans from several isolates of *Sporothrix schenckii* and *Ceratocystis* species were compared. No major differences emerged in the analysis of the carbohydrate composition of polysaccharides from either species. Methylation analysis for the identification of the glycosidic linkages present in the polysaccharides showed that they all were very similar with minor differences being observed among strains and between polysaccharides obtained at 25 and 37 C. Partially methylated derivatives were identified by gas-liquid chromatography and mass spectrometry. Their proportions suggest that polysaccharides from *S. schenckii* and *C. stenoceras* have a general structure similar to that of *C. ulmi*: a (1 → 6)-linked α-D-mannopyranosyl main chain substituted in the 3-positions by α-L-rhamnopyranosyl and in many cases by α-L-rhamnopyranosyl (1→2)-L-rhamnopyranosyl side chains. Differences among polysaccharides resided in the proportions of the dirhamnosyl side chains and of 4-*O*-substituted and 2,4-di-*O*-substituted mannose units. Increased proportions of dirhamnosyl side chains were observed in the polysaccharides from *S. schenckii* strains and from a pathogenic variant of *C. stenoceras* grown at 25 C. Proton magnetic resonance spectra (H-1 region) of the rhamnomannans showed that *S. schenckii* strains could be placed in two different groups similar but not identical to any *Ceratocystis* species. These spectra were very close to those of the polysaccharides from the *C. clavata* and *C. ambrosia* groups of *Ceratocystis* species.

Sporothrix schenckii is the agent of sporotrichosis which is usually a subacute or chronic subcutaneous infection in humans occasionally becoming systemic. Attempts to produce adequate antigens for immunological tests have led to preparations of varied complexity and activity. Concentrated culture filtrates (24), whole yeast cells and cell walls (20), and crude polysaccharide fractions (19) were some of the sources of these antigens. Earlier studies (3) showed that polysaccharides from an *S. schenckii* antigenic preparation contained mannose, rhamnose, and glucose. Ishizaki (13) obtained three serologically active polysaccharides after purification of the carbohydrate-containing fraction accumulated in the culture medium of *S. schenckii*. Sugars in these fractions were mainly mannose and rhamnose. Galactose and glucose which appeared as minor constituents were probably impurities of the rhamnomannan preparations. A homogeneous, serologically active peptido-rhamnomannan was isolated in this laboratory from the culture medium and from the yeast cells of *S. schenckii*. Besides rhamnose and mannose it contained only a trace of galactose and no detectable glucose (16).

Polysaccharides containing rhamnose as a major constituent can also be found in ascomycetes of the genus *Ceratocystis* (25) but are uncommon in other fungi (4, 5). Similarities in some morphological and physiological characteristics among saprophytic and pathogenic strains of *Ceratocystis* and *S. schenckii* were construed as evidence that *S. schenckii* might be the asexual form of one or more species of *Ceratocystis* (21, 26). Mutants from the wild type of a particular species of *Ceratocystis* (*Ceratocystis stenoceras*) were obtained showing a pathogenicity for hamsters and resembling *S. schenckii* in several aspects (17, 18). The relationship between *S. schenckii* and *Cerato-*

cystis species is further stressed by the immunological cross-reactivity of their culture filtrates (2).

The present investigation was conducted to compare the chemical structure of rhamnomannans from isolates of *S. schenckii* with those from strains of *C. stenoceras* including a pathogenic variant (18).

MATERIALS AND METHODS

Strains. Strains of fungi were isolated from both human and nonhuman sources. *S. schenckii* 1099.13 was originally isolated from a water well in Algier; it was received from the Pasteur Institute (no. 854), Paris. *S. schenckii* 1099.10, 1099.18, and CH-254 were isolated from patients with sporotrichosis. *C. stenoceras* 1099.11 is the wild culture originally isolated from a human scalp, and *C. stenoceras* 1099.12 is a pathogenic variant derived from the wild type after the successful infection of hamsters with the 1099.11 strain. Both strains were received from F. Mariat and correspond to cultures IP-1013-70 (wild type) and IP-1021-70 (pathogenic variant) of 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).

Medium and growth conditions. Fungi were grown in the following medium (g/liter): yeast nitrogen base (Difco) 6.7; Casamino Acids (Difco) 2.5; glucose, 50; and 1 ml of a vitamin mixture containing per 100 ml: thiamine-hydrochloride, 50 mg; riboflavin, 50 mg; Ca pantothenate, 50 mg; nicotinic acid, 50 mg; pyridoxine-hydrochloride, 10 mg; *p*-aminobenzoic acid, 10 mg; inositol, 10 mg; folic acid 1 mg; biotin, 0.4 mg. Glucose was autoclaved separately and added aseptically. Cells from 48-h cultures on brain heart infusion agar slants were suspended in saline and inoculated into 200 ml of medium in 1-liter flasks. These starter cultures were incubated with shaking at 25 or 37 C for 3 to 5 days. Some strains grew very poorly at 37 C and therefore their polysaccharides were only isolated from cultures at 25 C. Cells from the starter cultures were transferred to 3 liters of medium distributed in volumes of 1 liter in 2.8-liter Fernbach flasks or in volumes of 500 ml in 1-liter De Long flasks capped with stainless steel caps. Incubation was at 25 or 37 C with shaking (New Brunswick's gyratory shakers) for 5 to 7 days.

Extraction of polysaccharides. The procedure of Gorin and Spencer (10) was essentially followed. Modifications and other details on the methods used are indicated below. Fungal cultures (3 liters) were autoclaved at 15 psi for 30 min. Cells were harvested by centrifugation and washed once in saline. Supernatant fluids of cultures were pooled, 1 g% of Na-acetate was added, and the polysaccharides were precipitated with 3 volumes of ethanol. The precipitate was separated by decantation and finally by centrifugation. Ethanolic precipitates and the mass of cells were suspended in 2% aqueous KOH (300 to 400 ml) and extracted for 2 h at 100 C in a water bath. The brownish suspension was then neutralized with a few milliliters of glacial acetic acid, was centrifuged, and the supernatant fluid was evaporated down to 100 to 150 ml. The concentrated solution was again centrifuged to eliminate any insoluble material and was precipitated with 3 volumes of ethanol. Precipitate was separated by filtration through a glass filter (F), washed several times with ethanol, and dried. Polysaccharides were weighed at this stage, then dissolved in water (100 ml) at 100 C for 2 h in a water bath. Any insoluble residue was centrifuged off and the solution was precipitated with an equal volume of Fehling's solution. Precipitation was carried out at 4 C overnight. The insoluble copper complexes were then centrifuged, washed three times with 2% KOH, once with ethanol, and then isolated by filtration. The blue precipitate was ground to a fine powder, washed with ethanol, and refiltered. Copper complexes were shaken in a suspension with Amberlite IR-120 for 1 h at room temperature. Supernatant fluids were separated by decantation and the resin beads were washed twice with small volumes of distilled water. Solutions were then precipitated with 4 volumes of ethanol containing 2 to 3 drops of hydrochloric acid. Occasionally, the precipitates formed as a gummy mass sticking to the glass filters. In these cases the solids were redissolved in a reduced volume of distilled water and reprecipitated with 4 to 5 volumes of ethanol until a powder could be isolated by filtration. We cannot assert whether the rhamnomannans extracted by the present procedure were exclusively formed by the yeast or the mycelium (+ conidia) phase of the fungi because a mixture of these forms was always present at the time the 37 C cultures were harvested. Rhamnomannans accumulated in the medium were analysed jointly with the cell polysaccharides. The accumulation occurred during a 5-day growth period in which transitions between morphological forms could have taken place. At 37 C in the medium used there was a high percentage of yeast cells, whereas at 25 C the cultures were entirely in the mycelial phase. No attempts were made to separate the mycelia from yeasts in the cultures where the two forms were present. Filtration procedures to separate filaments would still have given a mixture of conidia and yeasts in the filtrate. Almost pure yeast forms could be obtained with the same strains in brain heart infusion media at temperatures higher than 28 C. Since, however, other studies on the polysaccharides from *Ceratocystis* (11, 25) employed the vitamin-enriched yeast-nitrogen base-Casamino Acids medium, this was used in the present investigation.

Analytical methods. Total carbohydrate, nitrogen, and phosphate were determined by the phenol-sulfuric acid method (8), by the ninhydrin method (23), and by the method of Ames (1), respectively. Methylpentoses were determined by the cysteine-sulfuric acid method (7) by using rhamnose as standard. Quantitative determination of monosaccharides in the polysaccharides was performed by gas-liquid chromatography of the corresponding alditol acetates (22) on stainless-steel columns (6 ft by ⅛ in.) containing 3% nitrile-polyester copolymer (ECNSS-M) or 2% neopentylglycolsuccinate (NPGS) at 185 or 195 C, respectively. A detailed description of the procedure used was given in a previous publication (15). Specific rotations were determined at room temperature by using a Perkin-Elmer 141 polarimeter.

Methylation-hydrolysis of polysaccharides. Methylation of polysaccharides was carried out by the dimethylsulfinyl anion and methyl iodide method (12) as described earlier (15) except that a second methylation with methyl iodide and silver oxide was not done. All polysaccharides studied dissolved readily in dimethyl sulfoxide. Methylated polysaccharides were hydrolysed in 80% formic acid at 100 C and then in 0.5 N H_2SO_4 at 100 C. The *O*-methyl sugars were identified by gas-liquid chromatography on 2% NPGS or 3% ECNSS-M columns as their methylglycosides, trimethylsilyl derivatives, and *O*-methylalditol acetates as described previously (15). Nonmethylated and monomethyl alditol acetates were not detected on 3% ECNSS-M columns at 185 C indicating that a satisfactory degree of methylation of the polysaccharides had been achieved. Retention times were calculated in relation to that of the corresponding derivative of 2,3,4,6-tetra-*O*-methylmannose. Peak areas of the *O*-methylalditol acetates run at 160 C on 3% ECNSS-M columns were measured to determine the approximate proportions of the methylated derivatives to 2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetylmannitol. No corrections were made for possible losses by evaporation of the more volatile 2,3,4-tri-*O*-methyl-1,5-di-*O*-acetylrhamnitol.

Pure methylated standards. Pure samples of dimethyl and trimethyl mannosides were received from C. E. Ballou. A tosyl derivative of 2,3-di-*O*-methylrhamnoside, kindly provided by G. O. P. Aspinall, was reduced with sodium amalgam according to Tipson (27) and the resulting tosyl-free rhamnoside was hydrolysed in 0.5 N H_2SO_4 at 100 C, reduced with $NaBH_4$ and acetylated to yield the corresponding 2,3-di-*O*-methyl-1,4,5-tri-*O*-acetylrhamnitol. From G. O. P. Aspinall we received also a sample of 3,4-di-*O*-methylrhamnose which was reduced and acetylated.

Mass spectra of methylated O-acetyl alditols. (Mass spectra were performed at the Institutionen for organisk kemi, Stockholms universitet, Stockholm, Sweden). The partially methylated *O*-acetyl alditols prepared from the polysaccharides of *S. schenckii* 1099.18 grown at 25 and 37 C were injected into a Perkin-Elmer 270 gas chromatograph-mass spectrometer, with a OV-225 S.C.O.T. column (50 ft by 0.02 in.) and a programmed temperature rise of 1 C/min from 175 to 195 C. Methylated *O*-acetyl alditols were eluted from the OV-225 column in the same order as from the ECNSS-M column. The mass spectra were recorded at an ionization potential of 70 eV, ionisation current of 75 μamp, and an ion-source temperature of 120 C.

Nuclear magnetic resonance spectroscopy. The NMR spectra of polysaccharides from strains 1099.11, 1099.12 (25 C and 37 C), 1099.13, 1099.18 (25 and 37 C), and CH-254 were obtained by using a 100 MHz Varian nuclear magnetic resonance spectrometer from 20% solutions in D_2O at 70 C with tetramethylsilane (τ = 10) as the external standard.

RESULTS

Extraction and analyses of the polysaccharides. Final yields of polysaccharides from 3-liter cultures of the different strains at 25 C varied generally from 1 to 2 g (dry weight) except for one strain (1099.11) which yielded 3.1 g. Mutant strain 1099.12 grew less abundantly at 37 C and the final yield in polysaccharides was half that at 25 C; the strains of *Sporothrix* isolated from human sources grew equally well at 25 or at 37 C and the polysaccharide yield at 37 C was only slightly less than at 25 C. In two instances the amounts of polysaccharides extracted from the culture medium (PM) and from the cells (PC) were compared. The dry-weight ratios PM/PC were 2.8 and 1.2. Analytical data for the polysaccharides are shown in Table 1. No major differences emerged in the analysis of the carbohydrate composition from *S. schenckii* and from *C. stenoceras*. The

TABLE 1. *Analyses of S. schenckii and C. stenoceras polysaccharides* (%)

Strains	Rhamnose		Mannose glc	Galactose glc	Total carbohydrate col[c]	N	P	$[\alpha]_D^{29}$ (deg.)
	col[a]	glc[b]						
C. stenoceras								
1099.11 (25 C)	46.9	47.4	48.6	5.2	109.5	0.8	tr[d]	−1.1
1099.12 (25 C)	62.5	59.5	42.4	tr	100.9	0.25	0.2	−8.4
1099.12 (37 C)	46.4	49.5	47.3	tr	92.3	0.7	0.38	+3.3
1099.40 (25 C)	57.2	57.4	46.5	0.0	104.2	0.3	0.2	−15.6
1099.41 (25 C)	43.3	45.0	53.1	0.0	98.3	0.2	0.18	−1.0
S. schenckii								
1099.13 (25 C)	47.8	51.0	48.1	4.7	103.5	0.25	0.2	+0.1
1099.18 (25 C)	52.1	48.0	48.3	tr	98.0	0.25	0.2	+0.8
1099.18 (37 C)	49.3	53.5	54.1	tr	108.0	0.37	0.37	+7.3
1099.10 (25 C)	48.9	47.5	46.2	0.0	100.2	0.5	0.2	+1.1
1099.10 (37 C)	49.3	47.3	51.7	tr	106.3	0.4	0.19	+1.1

[a] Colorimetric: cysteine-sulfuric acid method.
[b] glc, Gas-liquid chromatography.
[c] Colorimetric: phenol-sulfuric acid method.
[d] tr, Trace.

amounts of L-rhamnose and D-mannose were approximately the same in these polysaccharides except in two cases—strains 1099.12 at 25 C (but not at 37 C) and 1099.40—where rhamnose clearly predominated over mannose. The nature of the D-mannose and L-rhamnose enantiomers has been previously established in *S. schenckii* CH-254 polysaccharide by isolation of crystalline derivatives of the sugars (16). D-Mannose and L-rhamnose are also the monosaccharides of *C. ulmi* rhamnomannan (11). The small amount of galactose in two polysaccharides may be interpreted as impurities of the rhamnomannan preparations which also contained traces of nitrogen and phosphorus. Analytical data on the rhamnomannan of *S. schenckii* CH-254 have been given previously (16).

Methylation analysis. Identification of the partially methylated sugars was made by comparing the retention times of the three derivatives formed with those of appropriate standards. It became evident that all polysaccharides were quite similar with some minor differences being observed among strains and between polysaccharides obtained from cells grown at 25 or 37 C. The major peaks corresponded to the derivatives of 2,4-di-*O*-methylmannose and 2,3,4-tri-*O*-methylrhamnose suggesting an α-(1 $\rightarrow$ 6)-D-mannopyranosyl main-chain substituted in the 3-positions mainly by α-L-rhamnopyranosyl side chains as was found for *C. ulmi* (11). This structure is supported by methylation analysis of a peptido-rhamnomannan preparation from *S. schenckii* CH-254 previously hydrolysed at pH 1.1, 100 C for 5 h to partially remove the rhamnose end units (16). A 20-fold increase in the proportion of the 2,3,4-tri-*O*-methylmannose derivative was observed roughly corresponding to a simultaneous decrease in the proportion of the 2,4-di-*O*-methylmannose derivative. Varying amounts of di-*O*-methylrhamnose derivatives were also detected in all the chromatographs. Identification of the sugar as 3,4-di-*O*-methylrhamnose was made by showing that the tri-*O*-acetylrhamnitol derivative had a retention time identical to that of a pure sample of the 3,4 rather than the 2,3-di-*O*-methyl-rhamnose derivative. The identification was confirmed by examining the mass spectrum for the corresponding peak in the gas chromatograph, which revealed the formation of ions corresponding to the degradation pattern of the 3,4-di-*O*-methyl-rhamnose derivative. The results indicate that the *C. stenoceras-S. schenckii* group has dirhamnopyranosyl side chains as in *C. ulmi*. However, since the dirhamnopyranosyl side chain in *C. ulmi* had previously been shown to have a (1 $\rightarrow$ 4) linkage by a periodate oxidation method (11) the structure of *C. ulmi* was reexamined by methylation analysis. By using this method it has now been shown that *C. ulmi* polysaccharide also has the (1 $\rightarrow$ 2)-linked rhamnose residues. Patterns of the gas chromatographs of the *O*-acetyl derivatives of the partially methylated alcohols prepared from the polysaccharides of some strains of *S. schenckii*, *C. stenoceras*, and *C. ulmi* are shown in Fig. 1. Mass spectra of the *O*-acetyl derivatives from the polysaccharides of one strain of *S. schenckii* grown at 25 or 37 C confirmed their identity as 2,3,4-tri-*O*-methyl-1,5-di-*O*-acetylrhamnitol, 2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetylmannitol, 3,6-di-*O*-methyl-1,2,4,5-tetra-*O*-acetylmannitol, and 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylmannitol. Small amounts of the *O*-acetyl derivatives of 3,4,6-tri-*O*-methyl, 2,4,6-tri-*O*-methyl, and 2,3,4-tri-*O*-methyl-mannitol were also present. Table 2 indicates the prominent *m/e* values for the identified derivatives (6) as they were eluted in the gas chromatography. The presence of 2,3,4,6-tetra-*O*-methyl-mannose derivatives could only be detected as traces and in many cases not even so.

Approximate proportions of the partially methylated alditols. The proportions of the methylated derivatives for the different polysaccharides are shown in Table 3. Since the presence of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylmannitol was only detected as traces in most cases, the proportions of the *O*-acetyl derivatives of 2,3,4-tri-*O*-methylrhamnitol ought to be identical to those of 2,4 + 3,6-di-*O*-methylmannitol. In some cases the amounts of the *O*-acetyl derivatives of 2,3,4-tri-*O*-methylrhamnitol were less than expected. This was probably due to the partial evaporation of the very volatile rhamnitol derivative during the methylation-hydrolysis-acetylation technique. Small differences in the peak areas can also occur due to different detector responses to the methylated derivatives.

The linkages in the polysaccharides from the various isolates of *C. stenoceras* and *S. schenckii* are extremely similar except for variations in the proportions of the dirhamnosyl side chains. Usually, growth at 37 C led to the formation of polysaccharides with fewer dirhamnosyl groups as compared with the corresponding polysaccharides formed at 25 C. The proportions of the dirhamnosyl chains were high in two strains of *S. schenckii* and in the mutant strain of *C. stenoceras*. However, one strain of *S. schenckii* (1099.18) had proportions similar to those of the other strains of *C. stenoceras* even while growing at 25 C. Rise in growth tempera-

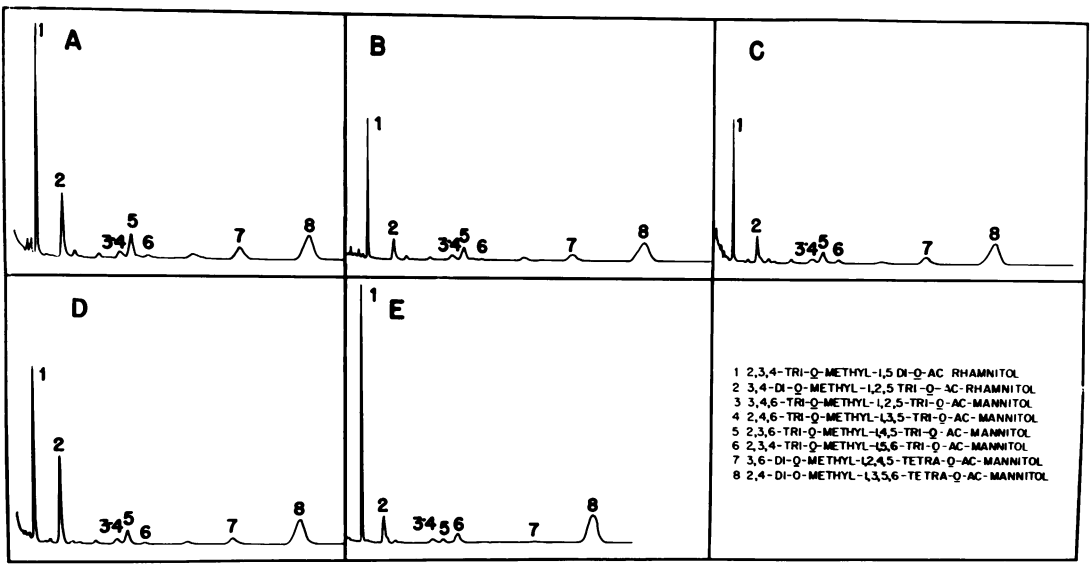


FIG. 1. *Gas chromatograms of the partially methylated alditol acetates from methylation analysis of polysaccharides from: A, S. schenckii 1099.10 (25 C); B, S. schenckii 1099.10 (37 C); C, C. stenoceras 1099.11 (25 C); D, C. stenoceras 1099.12 (25 C); E, C. ulmi (CBS 374.67).*

TABLE 2. *Prominent m/e values for the identified methylated derivatives from S. schenckii (1099-18) polysaccharide*

O-methyl-O-acetyl alcohol	m/e
I 2,3,4-tri-*O*-methyl-1,5-di-*O*-acetyl-rhamnitol	175, 161, 131, 117, 115, 101, 89, 43
II 3,4-di-*O*-methyl-1,2,5-tri-*O*-acetyl-rhamnitol	189, 173, 159, 131, 129, 89, 87, 71
III 3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetyl-mannitol	189, 161, 129, 101, 99, 87, 45
IV 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetyl-mannitol	201, 173, 161, 159, 129, 117, 101, 87, 45
V 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetyl-mannitol	233, 173, 161, 117, 113, 101, 99, 87, 45
VI 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetyl-mannitol	189, 173, 161, 129, 117, 101, 99, 87, 43
VII 3,6-di-*O*-methyl-1,2,4,5-tetra-*O*-acetyl-mannitol	233, 189, 129, 113, 99, 87, 45
VIII 2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetyl-mannitol	233, 201, 189, 173, 129, 117, 87

ture (37 C) is also accompanied by a decrease in the amounts of the 3,6-di-*O*-methylmannitol *O*-acetyl derivatives.

Nuclear magnetic resonance spectroscopy. The signals at the H-1 region were analyzed in order to compare the structures of the various polysaccharides. The patterns for eight polysaccharides (including one from *C. ulmi*) are shown in Fig. 2. Polysaccharide from *C. stenoceras* strain 1099.11 gave a proton magnetic resonance (PMR) spectrum identical to those described for the *C. clavata* group (25). The pathogenic mutant of this wild strain, *C. stenoceras* 1099.12, when grown at 25 C had a spectrum similar to the wild type except for the presence of an additional peak at τ 4.42 and the loss of peak at τ 4.64. When the mutant strain grew at 37 C, however, a profound change in the PMR spectrum (H-1 region) took place: the peak at τ 4.42 and the three peaks with signals at higher field than τ 4.51 could not be detected. Polysaccharide from the *S. schenckii* strain isolated from the water well (1099.13) gave a PMR spectrum similar to that of the rhamnomannan from the mutant strain of *C. stenoceras*. The peaks at τ 4.70 and τ 4.79 were minor but the

TABLE 3. *Relative peak areas of the methylated derivatives from the polysaccharides of S. schenckii and C. stenoceras strains*

O-acetyl derivatives[a]	*C. stenoceras*					*S. schenckii*					
	1099.11	1099.12		1099.40	1099.41	1099.13	1099.18		1099.10		CH-254[b]
	25 C	25 C	37 C	25 C	25 C	25 C	25 C	37 C	25 C	37 C	28 C
2,3,4-tri-*O*-methylrhamnitol	1.0	1.06	1.09	1.14	1.4	1.04	1.42	1.04	1.14	0.91	1.37
3,4-di-*O*-methylrhamnitol	0.3	0.86	0.15	0.37	0.48	0.88	0.41	0.14	0.64	0.26	0.14
2,4-di-*O*-methylmannitol	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
3,6-di-*O*-methylmannitol	0.26	0.28	0.16	tr	0.3	0.46	0.49	0.25	0.38	0.24	0.35
2,3,6-tri-*O*-methylmannitol	0.24	0.25	0.4	tr	0.39	0.5	0.4	0.47	0.41	0.29	0.54
3,4,6-tri-*O*-methylmannitol plus 2,4,6-tri-*O*-methylmannitol	0.15	0.12	0.22	tr	0.18	0.19	tr	0.1	0.13	0.15	0.14
2,3,4-tri-*O*-methylmannitol	0.1	tr	tr	0	0.19	tr	tr	tr	tr	tr	0.1
2,3,4,6-tetra-*O*-methylmannitol	tr	tr	tr	0	0	tr	tr	tr	0	0	0.14

[a] Values for the 2,3,4-tri-*O*-methylrhamnitol *O*-acetyl derivatives are not corrected for losses by evaporation; retention times for the 3,4,6-tri-*O*-methyl and 2,4,6-tri-*O*-methylmannitol *O*-acetyl derivatives did not allow a good separation of the peaks.

[b] Methylation analysis made on a rhamnomannan obtained from a peptido-rhamnomannan (16) after hydrolysis in KOH 2% for 2 h and ethanolic precipitation.

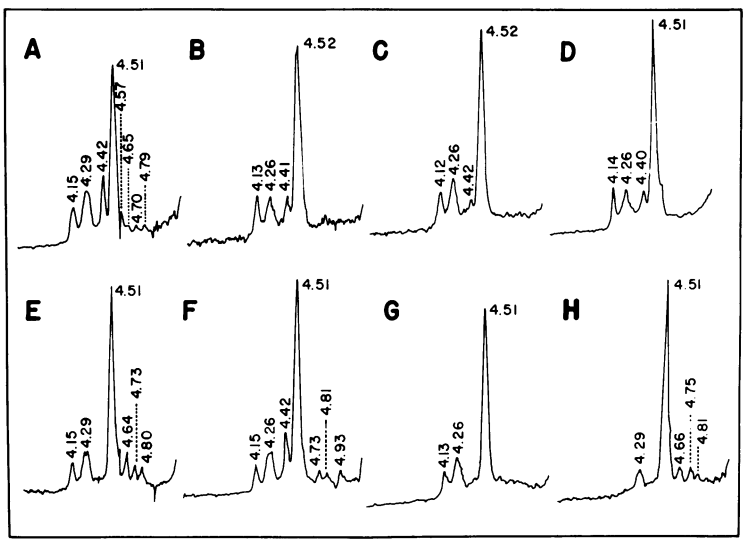


FIG. 2. *PMR (H-1 region) spectra of polysaccharides from: A, S. schenckii 1099.13 (25 C); B, S. schenckii 1099.18 (25 C); C, S. schenckii 1099.18 (37 C); D, S. schenckii CH-254 (28 C); E, C. stenoceras 1099.11 (25 C); F, C. stenoceras 1099.12 (25 C); G, C. stenoceras 1099.12 (37 C); H, C. ulmi (CBS 374.67).*

peak at τ 4.42 was as prominent as in the spectrum of the mutant rhamnomannan. The spectrum of the polysaccharide from one strain of *S. schenckii* (1099.18) resembled that for the mutant strain grown at 37 C rather than at 25 C: peaks at higher field than τ 4.52 were not observed. A small peak at τ 4.41 appeared in the spectrum of the polysaccharide from strain 1099.18 at 25 C but was reduced to a trace in the polysaccharide formed at 37 C. As noted before (11, 25) the spectrum of the rhamnomannan from *C. ulmi* differs from that of the rhamnomannans from the *C. clavata* group in lacking a peak at τ 4.12. It also does not contain the peak at τ 4.41 which appears in the PMR spectra of the *S. schenckii* polysaccharides. The spectrum of the polysaccharide from *S. schenckii* CH-254 originally isolated as a peptido-rhamnomannan (16) was identical to the spectrum of the rhamnomannan from another isolate of *S. schenckii* (1099.18) grown at 25 C.

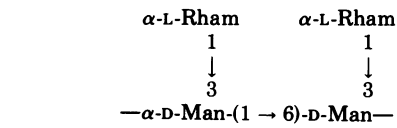
DISCUSSION

Fungal polysaccharides may contain hexoses, aminosugars, hexuronic acids, methylpentoses, and pentoses. Fucose is the methyl pentose of

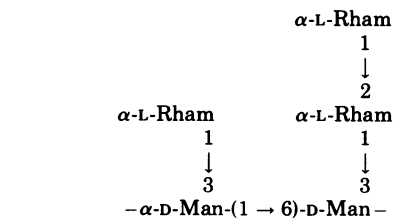
several mucorales and basidiomycetes (9), whereas rhamnose, which is generally absent in most fungal polysaccharides, is a major component of the alkali-extractable polysaccharides from several *Ceratocystis* species (25). This unique distribution of rhamnose is important in defining this particular group of higher fungi. The characterization of a peptido-rhamnomannan from *S. schenckii* (16) along with other provocative reports on morphological similarities among the conidial forms of *S. schenckii* and those of several *Ceratocystis* species (21, 26) raises the question of whether *S. schenckii* is the conidial state of a particular *Ceratocystis* species or a common denominator for the conidial states of several anascigerous *Ceratocystis* species. If the second alternative pertains, the denomination *S. schenckii* would not apply to a definite species but to a group of several morphologically related fungal species able to cause sporotrichosis.

Several reports by Mariat and collaborators (17–19, 28) tend to support the idea that one particular species of *Ceratocystis*, *C. stenoceras*, would be a likely candidate for the perfect form of *S. schenckii*. Pathogenic variants ("mutants") from *C. stenoceras* were apparently easy to obtain. A single passage in hamsters induced a stable pathogenic variant which does not form perithecia and resembles *S. schenckii* nonpigmented strains (21). It is probable that passage in hamsters selected preexisting pathogenic forms in the original culture of *C. stenoceras*.

The alkali-extractable polysaccharides from *C. stenoceras* and *S. schenckii* are very similar in their carbohydrate content and in their main glycosidic linkages. These linkages, which are also found in the *C. ulmi* rhamnomannan, can be summarized as follows:



and



The $[\alpha]_D$ values for all polysaccharides suggest that most of the constituent sugars are α-linked. Methylation-hydrolysis studies showed that most, if not all, nonreducing ends are occupied by L-rhamnopyranosyl residues. One strain of *S. schenckii* (CH-254) previously studied (16), however, had a small percentage (10 to 20%) of D-mannopyranosyl terminal residues which were responsible for the precipitation of the peptido-rhamnomannan by Concanavalin A. Methylation analysis on the polysaccharide from this strain after partial removal of the rhamnose end units by mild-acid hydrolysis showed the presence of an α-(1 → 6)-linked mannopyranosyl main chain as in the *C. ulmi* rhamnomannan. Methylation data also suggest that a certain number of 4-*O*-substituted and 2,4-di-*O*-substituted mannose units may exist but their location either in the main chain or in side-chains remains to be elucidated. Of particular interest is the variation in the proportions of the 3,4-di-*O*-methylrhamnose derivatives in the methylated polysaccharides. As a rule, the amounts of these dimethyl derivatives were much less when strains grew at 37 C. Under the conditions used for extraction of the polysaccharides we cannot assert at present if this alteration in structure is related to the transition mycelium → yeast. It is clear though that certain biochemical reactions related to the biosynthesis of the dirhamnosyl side chains or to the process of their addition to the (1 → 6)-linked α-D-mannopyranosyl main chain have been partially inhibited upon raising the temperature. Another derivative that seemed to be formed in less quantities at 37 C was that of 3,6-di-*O*-methylmannose. This result implies a lesser percentage at 37 C of the 2,4-di-*O*-substituted mannose units. Dimorphic transition in *S. schenckii* probably does not involve only alterations in the rhamnomannan structures. Profound alterations in other main components of the cell wall probably take place as is generally the case with other fungi (14). Increased proportions of the 3,4-di-*O*-methylrhamnose derivatives (up to 80% the amount of the 2,4-methyl-*O*-mannose derivatives) suggesting increased proportions of α-L-rhamnopyranosyl-(1 → 2)-L-rhamnopyranosyl side chains were observed in two strains of *S. schenckii* and in the pathogenic mutant of *C. stenoceras*. Dirhamnosyl side chains can represent up to 30% of the total end groups in polysaccharides from the *C. stenoceras* strains grown at 25 C. The rham-(1 → 2)-rham sequence in the *S. schenckii-C. stenoceras-C. ulmi* group parallel the *O*-α-L-rham-(1 → 2)-L-fuc sequence in the heteropolysaccharide of *Candida bogoriensis* (9).

In contrast to the relatively small differences in the proportions of certain linkages which could be observed in the rhamnomannans from *S. schenckii*, *C. stenoceras*, and *C. ulmi* by

methylation analysis, the PMR spectra of the polysaccharides showed definite differential patterns for some strains. The wild type of *C. stenoceras* (strain 1099.11) followed the same pattern of the so-called *C. clavata* group (25). The pathogenic mutant grown at 25 C, however, gained an additional peak at τ 4.42. Since this remarkable difference in structure appeared after the infection of susceptible animals with the wild type one should expect that the same could have happened with some isolates of *S. schenckii*. Peaks at τ 4.41 to 4.43 appear in the spectra of two polysaccharides from human-isolated strains of *S. schenckii*: the 1099.18 strain of the present investigation and the CH-254 strain studied previously (16). The spectra of these strains would be identical to those of the *C. ambrosia* group (25) were it not for the additional peak at τ 4.41 which is also a main difference between the spectra of the polysaccharides from the wild type and the mutant of *C. stenoceras*. It is tempting to suggest that these strains of *S. schenckii* may have been derived from any one of the *Ceratocystis* species of the *C. ambrosia* group. Among these species is *C. nigrocarpa* which was cited by Taylor (26) as having sympodulospores and yeast-like budding forms at 37 C not significantly different from those of typical strains of *S. schenckii*. The homogeneity of the *S. schenckii* group is, however, questioned by the PMR spectrum of the polysaccharide from the strain isolated from the water well. The spectrum of this strain was much closer to that of the mutant strain of *C. stenoceras* including the presence of peaks at τ 4.42 and the peaks at higher field than τ 4.51. Therefore, at least two groups of *S. schenckii* strains are evident as far as the structures of their rhamnomannans are concerned. These observations are based on the analysis of the PMR spectra of polysaccharides from strains grown at 25 C. Polysaccharides obtained at 37 C gave identical PMR spectra for both the mutant strain of *C. stenoceras* and a strain of *S. schenckii* (strain 1099.18).

A study of the PMR spectra (H-1 region) of rhamnomannans from several strains of *S. schenckii* isolated from human sources would indicate whether there was homogeneity within the group. The present data with a few strains suggest that *S. schenckii* rhamnomannans obtained at 25 C have structures different from any of the *Ceratocystis* groups as defined by PMR spectra (25). A similarity but not an identity could be found among the polysaccharides from *S. schenckii*, the *C. clavata* group (including *C. stenoceras*), and the *C. ambrosia* group.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-08478 from the National Institute of Allergy and Infectious Diseases and by the Brown-Hazen Fund of Research Corporation. K. O. L. is a Research Career Development Awardee of the Public Health Service. L. R. T. has been a fellow of the John Simon Guggenheim Memorial Foundation (1972) and of the Brazilian National Research Council (1973).

We are grateful to M. Silva-Hutner for her advice and encouragement and also to E. V. Passos for her technical assistance.

We are indebted to Sigfrid Svensson and his collaborators of the Institutionen for organisk kemi, Stockholms universitet, for the data on mass spectrometry.

LITERATURE CITED

1. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol. **8**:115-118.
2. Andrieu, S., J. Biguet, and S. Massamba. 1971. Etude immunologique comparée de *Sporothrix schenckii* et des souches saprophytes voisines. Sabouraudia **9**:206-209.
3. Aoki, Y., H. Nakayoshi, S. Asaga, and M. Ono. 1967. Studies on the immunologically active substances in fungi. Jap. J. Med. Mycol. **8**:284-291.
4. Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. **22**:87-108.
5. Bartnicki-Garcia, S. 1970. Cell wall composition and other biochemical markers in fungal phylogeny, p. 81-103. *In* J. B. Harborne, (ed.) Phytochemical phylogeny, Academic Press Inc., New York.
6. Bjorndal, H., B. Lindberg, and S. Svensson. 1967. Mass spectrometry of partially methylated alditol acetates. Carbohyd. Res. **5**:433-440.
7. Dische, Z., and L. B. Shettles. 1948. A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595-603.
8. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350-356.
9. Gorin, P. A. J., and J. F. T. Spencer. 1970. Structural chemistry of fungal polysaccharides. Advan. Carbohyd. Chem. **23**:367-417.
10. Gorin, P. A. J., and J. F. T. Spencer. 1968. Galactomannans of *Trichosporon fermentans* and other yeasts; proton magnetic resonance and chemical studies. Can. J. Chem. **46**:2299-2304.
11. Gorin, P. A. J., and J. F. T. Spencer. 1970. Structures of the L-rhamno-D-mannan from *Ceratocystis ulmi* and the D-gluco-D-mannan from *Ceratocystis brunnea*. Carbohyd. Res. **13**:339-349.
12. Hakomori, S. 1964. A rapid permethylation of glycolipid and polysaccharide catalysed by methylsulfinyl carbanion in dimethylsulfoxide. J. Biochem. (Tokyo) **55**:205-208.
13. Ishizaki, H. 1970. Some antigenic substances from culture filtrate of *Sporotrichum schenckii*. Jap. J. Dermatol. **80**:16-23.
14. Kanetsuna, F., L. M. Carbonell, I. Azuma, and Y. Yamamura. 1972. Biochemical studies on the thermal dimorphism of *Paracoccidioides brasiliensis*. J. Bacteriol. **110**:208-218.
15. Lloyd, K. O. 1970. Isolation, characterization and partial structure of peptido galactomannans from the yeast form of *Cladosporium werneckii*. Biochemistry **9**:3446-3453.

16. Lloyd, K. O., and M. A. Bitoon. 1971. Isolation and purification of a peptido-rhamnomannan from the yeast form of *Sporothrix schenckii*. Structural and immunochemical studies. J. Immunol. **107**:663–671.
17. Mariat, F. 1971. Adaptation de *Ceratocystis* a la vie parasitaire chez l'animal. Etude de l'aquisition d'un pouvoir pathogène comparable a celui de *Sporothrix schenckii*. Sabouraudia **9**:191–205.
18. Mariat, F. 1971. Adaptation de *Ceratocystis stenoceras* (Robak) C. Moreau a la vie parasitaire chez l'animal. Etude de la souche sauvage et des mutants pathogènes. Comparaison avec *Sporothrix schenckii* Hektoen et Perkins. Rev. Mycol. (Paris) **36**:3–24.
19. Mariat, F., R. V. Victoria, C. de Bièvre, and P. Lavalle. 1970. Sur le polyoside de *Sporothrix schenckii* antigene metabolique (sporotrichine) utilisé dans le diagnostique de la sporotrichose. Bull. Soc. Fran. Mycol. Med. **17**:5–6.
20. Nielsen, H. S., Jr. 1968. Biologic properties of skin test antigens of yeast form *Sporotrichum schenckii*. J. Infect. Dis. **118**:173–180.
21. Nicot, J., and F. Mariat. 1973. Caractères morphologiques et position systématique de *Sporothrix schenckii* agent de la sporotrichose humaine. Mycopathol. Mycol. Appl. **49**:53–65.
22. Sawardeker, J. S., J. H. Sloneker, and A. Jeanes. 1965. Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. Anal. Chem. **37**:1602–1604.
23. Schiffman, G., E. A. Kabat, and W. Thompson. 1964. Immunochemical studies on blood groups XXX. Cleavage of A, B, and H blood-group substances by alkali. Biochemistry **3**:113–120.
24. Schneidau, J. D., L. M. Lamar, and M. A. Hairston. 1964. Cutaneous hypersensitivity to sporotrichin in Louisiana. J. Amer. Med. Ass. **188**:371–373.
25. Spencer, J. F. T., and P. A. J. Gorin. 1971. Systematics of the genera *Ceratocystis* and *Graphium*. Proton magnetic resonance spectra of the mannose-containing polysaccharides as an aid in classification. Mycologia **63**:387–402.
26. Taylor, J. J. 1970. A comparison of some *Ceratocystis* species with *Sporothrix schenckii*. Mycopathol. Mycol. Appl. **42**:233–240.
27. Tipson, R. S. 1963. Reductive desulfonylation. p. 250–251. *In* R. L. Whistler, M. L. Wolfrom and J. N. BeMiller (ed.), Methods in carbohydrate chemistry, vol II, Academic Press Inc., New York.
28. Victoria, R. V., C. de Bièvre, and F. Mariat. 1970. Résultats préliminaires concernant la parenté chimique entre *Ceratocystis* sp G 118 et *Sporothrix schenckii* IP-29. Bull. Soc. Fran. Mycol. Med. **17**:7–8.