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FURTHER STUDIES ON THE RHAMNOMANNANS AND ACIDIC RHAMNOMANNANS OF *Sporothrix schenckii* AND *Ceratocystis stenoceras**†

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ABSTRACT

Studies were continued on the ^{13}C and proton nuclear magnetic resonance spectra of a series of mannose-containing polysaccharides formed by various strains of the closely related fungal species, *Sporothrix schenckii* and *Ceratocystis stenoceras*, and the criteria for their differentiation redefined. Exocellular filtrates of *C. stenoceras* 1099.40 contain mainly a galactan, with smaller proportions of a rhamnomannan having single-unit α-L-rhamnopyranosyl side-chains (**1**), an acidic rhamnomannan (**2**) containing glucuronic acid, and amylose. The mono-rhamnomannan was characterized by comparison of its ^{13}C spectrum with that of authentic polysaccharide. The acidic rhamnomannan was characterized partly with the aid of ^{13}C n.m.r. spectroscopy; in the process, fragments obtained by partial hydrolysis were used to assign signals of C-1 and other *O*-glycosylated ^{13}C nuclei. Other ^{13}C signals in spectra of polysaccharides from *C. stenoceras* and *S. schenckii* were assigned following tracer experiments that used media containing glucoses labeled with deuterium and ^{13}C, and by analogy with ^{13}C signal-displacement effects that are known to occur on *O*-glycosylation and *O*-methylation of hydroxyl groups. Evidence was obtained for the presence of structures **3**, **4**, and **5** in exocellular polysaccharide of *S. schenckii*. These structures give rise to C-1 signals that were assigned by reference to those of (1→4)-linked α-D-mannopyranose di- and tri-saccharides.

INTRODUCTION

Polysaccharides and peptido-polysaccharides have been frequently isolated from the outer layers of fungal cell-walls[1–3]. In yeasts, mannans are the main surface-

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antigens[4,5]. In human pathogens, mannans[6,7], galactomannans[8], phosphogalactomannans[9], glucomannans[10], and rhamnomannans[11] have been characterized.

Recently[12], we have shown that the human pathogen *Sporothrix schenckii* may synthesize different cell-wall surface polysaccharides depending on the growth conditions and cell morphology. As such polymers determine the antigenic specificity of cells, their transient or permanent exposure on the cell surface, or their excretion into the medium, as influenced by environmental stimuli, or by the life cycle of a particular organism, may be important in modulating the immune response in infected hosts.

In this human pathogen, rhamnomannans having single-unit α-L-rhamnopyranosyl and (1→2)-linked di-α-L-rhamnopyranosyl side-chains are formed at 37 and 25°, or in the yeast-like and mycelial phases respectively[11,12]. These groups are the main antigenic determinants in such molecules[13]. Changes in the molecular structure of polysaccharides from the same morphological type such as the incorporation of 4-*O*- and 2,4-di-*O*-substituted α-D-mannopyranose residues in the yeast rhamnomannan, are influenced by temperature. In young mycelium cultures of *S. schenckii*, a mannan is excreted into the medium together with increasing amounts of the rhamnomannan[12]. These polysaccharides could be separated on the basis of selective precipitation with concanavalin A.

In addition to the polysaccharides just referred to, the presence of an exocellular, galactose-containing polysaccharide in *S. schenckii* cultures has also been suggested[14]. As only traces of galactose were detected in our previous study on *S. schenckii* and *Ceratocystis stenoceras* polysaccharides after purification via precipitation with the Fehling reagent[11], it seemed possible that galactose was present as a galactan. Small amounts of a Fehling-precipitable galactomannan were isolated from the mycelium culture of one *S. schenckii* strain (1099.12) whose homogeneity, however, could not be fully ascertained. A similar galactomannan was not detected in another strain of *S. schenckii* (1099.18) which, nevertheless, synthesized rhamnomannans and a mannan[12].

S. schenckii and *C. stenoceras* are closely related fungi that coexist in their natural habitat of plants and their substrates. In a previous study[15], the polysaccharides from a number of strains of each species were examined by ^{13}C and proton n.m.r. spectroscopy and the spectra used as a means of differentiation between the two species. Six spectral types were obtained, and from these the following can be deduced: (*a*) only *S. schenckii* produces exocellular mannans in the early stages of growth showing ^{13}C signals at δ_c 103.3 (C-1 of the non-reducing end-groups) and δ_c 99.8 (C-1 of 2,6-di-*O*-substituted residues); (*b*) polysaccharides of *C. stenoceras* do not contain *O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranose side-chains showing ^{13}C signals at δ_c 103.7 (C-1 of terminal groups), at δ_c 96.8 (C-1 of 2-*O*-substituted residues), and a proton signal at τ 4.40 (origin unknown); (*c*) All strains of *C. stenoceras* give polysaccharides showing ^{13}C signals at δ_c 105.6, 81.6, and 80.3 (Fig. 1). The spectra of some *S. schenckii* polysaccharides also showed minor peaks

at δ_c 105.6. These signals were of particular interest, as they suggested the presence of chemical structures not previously elucidated[15].

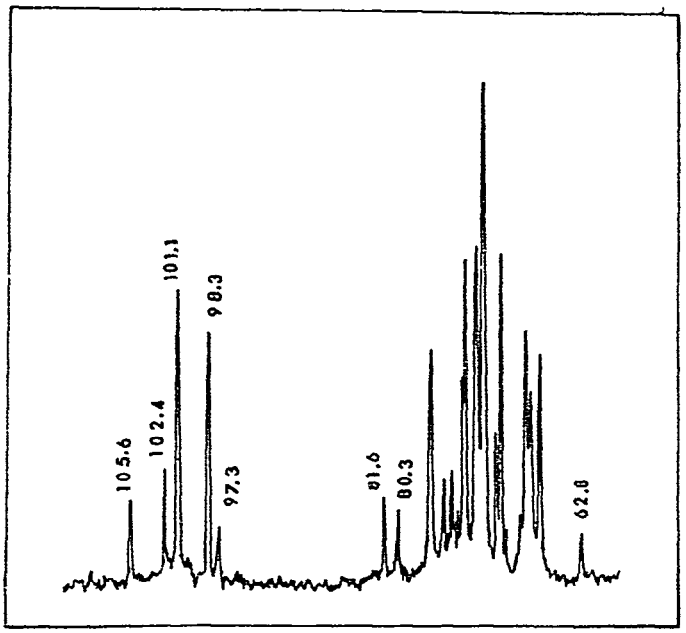


Fig. 1. ^{13}C n.m.r. spectrum of glucuronorhamnomannan of *Ceratocystis stenoceras* 1099.40.

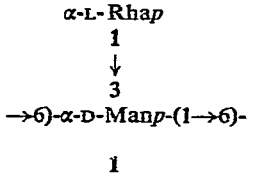
In the present paper, these three signals are attributed to the presence of rhamnomannans that also contain glucuronic acid. Individual signal-assignments could be made following determination of the chemical structure of the acidic rhamnomannan of *C. stenoceras* 1099.40. Other ^{13}C signals at δ_c 102.3 and 100.1 in spectra from *S. schenckii* polysaccharide were previously suggested to have arisen from the C-1 atoms of 4-*O*- and 2,4-di-*O*-substituted α-D-mannopyranose residues[12,15], and more-concrete evidence is now submitted as confirmation. Also, the assignments of other ^{13}C signals in the rhamnomannan spectra are made on the basis of ^{2}H and ^{13}C tracer-experiments with a strain of *S. schenckii*.

Chemical structures of C. stenoceras *polysaccharides* (**1** *and* **2**) *and* ^{13}C *signals of C-1 and* O*-glycosylated* ^{13}C *nuclei.* — The exocellular polysaccharide of *C. stenoceras* (1099.40) consists mainly of galactose, with smaller proportions of acidic rhamnomannan, rhamnomannan, and amylose (2%). Enrichment of rhamnomannans was effected through the insoluble copper-complex formed with Fehling solution, although the ^{13}C n.m.r. spectrum showed additional signals at δ_c 109.5, 85.0, 82.6, 78.6, and 64.5 that correspond to β-D-galactofuranose residues[16]. These residues, however, arise from a separate galactan, since fractionation on a column of DEAE-cellulose provided polysaccharides whose ^{13}C spectra corresponded to those of a (1→6)-

linked α-D-mannopyranose main-chain with each residue substituted in the 3-positions with α-L-rhamnopyranose groups[15,17], and the acidic rhamnomannan described next.

Similar fractionations were conducted with the polysaccharides obtained by alkaline extraction of cells of *C. stenoceras* 1099.40. As the extract contained a lower proportion of galactan than the exocellular material, it was possible by Fehling precipitation to obtain rhamnomannans free of galactan. This product also contained a mixture of a mono-rhamnomannan and an acidic rhamnomannan.

In order to determine the chemical structure of the acidic rhamnomannan, it was more convenient to examine the mixed *C. stenoceras* cell-wall rhamnomannan. The presence of glucuronic acid residues in the mixed rhamnomannan preparation was indicated by the presence of glucuronolactone, (detected on paper chromatograms) after hydrolysis, as well as by the low-field signal corresponding to CO_2H in its ^{13}C n.m.r. spectrum. D-Glucuronolactone was further characterized by conversion into D-glucose, which was attacked by D-glucose oxidase. Under weaker hydrolytic conditions, 2-*O*-β-D-glucopyranosyluronic acid-α,β-L-rhamnose was obtained and identified (see Experimental section). The proportion of glucuronic acid present in the mixed cell-wall rhamnomannans was reflected by its acid equivalent of 1662. A preponderance of structure **1** was indicated, as partial acetolysis of the mixed polysaccharides provided 3-*O*-α-L-rhamnopyranosyl-α,β-D-mannose in good yield via cleavage of the main chain. The latter was isolated following a Smith degradation incorporating mild hydrolytic conditions, and was assigned a (1→6)-linked, α-D-mannopyranose structure[18] on the basis of its ^{13}C n.m.r. spectrum.



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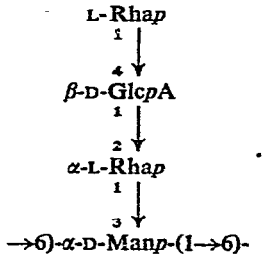

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In addition to giving a polysaccharide, the Smith degradation gave erythritol, erythronolactone, and possibly erythronic acid, as shown by paper chromatography. It is uncertain whether all of the erythritol arises from 4-*O*-substituted D-mannopyranose residues and/or starch, which is present in small amounts as amylose, as shown by an iodine test. The presence of erythronolactone is significant, as it indicates that the glucuronic acid residues are not non-reducing end-groups, but are 4-*O*-substituted. The pyranose form is the favored assignment, rather than a 5-*O*-linked furanose structure, as the glucosyluronic acid–rhamnose formed on partial acid hydrolysis shows considerable resistance to acid. The substitution at O-4 was confirmed by methylation of the polysaccharide followed by hydrolysis. From the mixed

O-methyl sugars, a spot on a paper chromatogram could be detected corresponding to 2,3-di-*O*-methylglucuronic acid. Treatment with methanolic hydrogen chloride, followed by reduction of the resulting methyl glycosides with lithium aluminum hydride gave a mixture that contained methyl 2,3-di-*O*-methyl-α,β-D-glucopyranoside, as shown by gas–liquid chromatography (g.l.c.).

Preferential removal of some of the side chains of the polysaccharides was effected with 0.185M sulfuric acid at 100°. The degraded polysaccharide had a ^{13}C n.m.r. spectrum lacking signals at δ_c 80.3 and 102.4 (Fig. 2), which may be interpreted as indication of removal of L-rhamnopyranosyl substituents from O-4 of glucopyranosyluronic acid residues. The signals arise, respectively, from C-4 of glucuronic

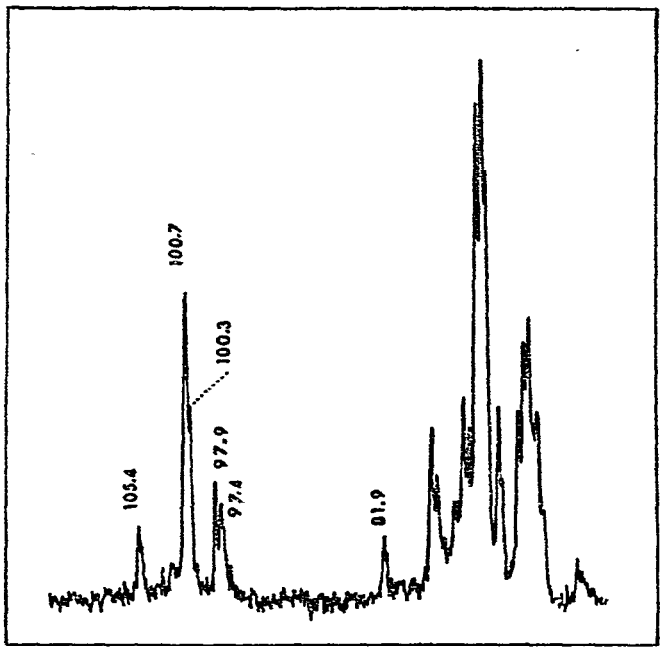


Fig. 2. ^{13}C n.m.r. spectrum of acid-degraded glucuronorhamnomannan of *Ceratocystis stenoceras* 1099.40.

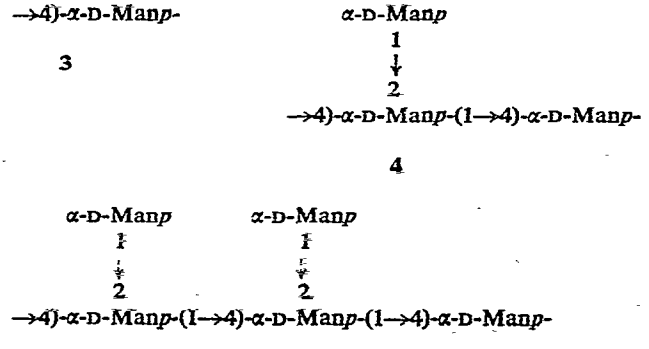
acid residues and C-1 of L-rhamnopyranosyl groups which are probably non-reducing end-groups (previous methylation data showed the absence of α-D-mannopyranosyl non-reducing end-groups[11]). The ^{13}C n.m.r. spectrum of the degraded polysaccharide also showed signals at δ_c 105.4, 97.4, and 81.9, which were interpreted as follows. The signals at δ_c 105.4 and 81.9 should arise from C-1 of β-D-glucopyranosyluronic acid and C-2 of 2-*O*-substituted L-rhamnopyranosyl residues, as their chemical shifts correspond to those of 2-*O*-β-D-glucopyranosyluronic acid-α-L-rhamnose. Also, the C-1 signal of methyl β-D-glucopyranosiduronic acid obtained at 33° in D_2O is observed at δ_c 104.3 (this value may be corrected to δ_c 104.9, as the present

determinations were performed[12] at 70°), and is close to the signal at δ_c 105.4. The signal at δ_c 97.4 arises from 2-*O*-substituted α-L-rhamnopyranosyl residues, upfield by 1.0 p.p.m. from that of C-1 of unsubstituted α-L-rhamnopyranosyl groups. Such an interpretation seems reasonable by analogy with the chemical shifts of C-1 atoms of unsubstituted and 2-*O*-substituted α-D-mannopyranose groups, which differ by 1.6 p.p.m.[19]. In the degraded polysaccharide, it appears that the glycosidic linkage attaching the 2-*O*-substituted L-rhamnopyranose residue to the main chain is rendered more acid-stable by the presence of the glucuronic acid substituent. The Smith degradation, ^{13}C spectra, and previous methylation data[11] indicates that this linkage is (1→3).

The data outlined here indicate that the cell-wall polysaccharides of *C. stenoceras* contain the predominant structure **1**, with smaller proportion of **2**.

Presence of structures **3**, **4**, *and* **5** *in* S. schenckii *polysaccharide and assignment of C-1 signals at* δ_c *100.1 and 102.3.* — Assignments of ^{13}C signals at δ_c 102.3 and 100.1, which appear in several spectra of polysaccharides from *S. schenckii* and *C. stenoceras*, were made as follows. Partial acid hydrolysis of the exocellular polysaccharide of *S. schenckii* 1099.12 dirhamnomannan gave 4-*O*-α-D-mannopyranosyl-α,β-D-mannose whose C-1 signal was at δ_c 102.8, thus indicating that the signal at δ_c 102.3 arises from 4-*O*-substituted α-D-mannopyranose residues. Similarly, the simultaneously formed *O*-α-D-mannopyranosyl-(1→4)-*O*-α-D-mannopyranosyl-(1→4) α,β-D-mannose gave rise to two C-1 signals, at δ_c 102.5 and 102.8.

As 2-*O*-substitution of an α-D-mannopyranosyl group by an α-D-mannopyranosyl group causes the C-1 signals to move upfield by 1.6 p.p.m.[19], it appears reasonable to propose that the upfield signal at δ_c 100.1 arises from 2,4-di-*O*-substitution of an α-D-mannopyranose residue. This type of substitution in the polysaccharide occurs, as shown by methylation data, and currently confirmatory evidence has been obtained from a Smith degradation under mild hydrolytic conditions. Erythritol, 2-*O*-α-D-mannopyranosyl-D-erythritol, and *O*-α-D-mannopyranosyl-(1→4)-*O*-α-D-mannopyranosyl-(1→2)-D-erythritol were found, and these could arise, respectively, from structures **3**, **4**, and **5**.



Side chains containing (1→2) linkages exist, as partial acetolysis gave 2-*O*-α-D-mannopyranosyl-α,β-D-mannose. The side chains of α-D-mannopyranose depicted in structures **4** and **5** should be substituted by α-L-rhamnopyranose groups in the parent polysaccharide, because methylation analysis gave only a trace of 2,3,4,6-tetra-*O*-methylmannose[11]. It appears likely that the single-unit side-chains are substituted at O-4 with rhamnopyranose groups since, if substitution occurred at O-2 or O-3, the glycosidic linkage would be stable to partial acetolysis. The products would then be the trisaccharides *O*-α-L-rhamnopyranosyl-(1→2)- or *O*-α-L-rhamnopyranosyl-(1→3)-α-D-mannopyranosyl-(1→2)-α,β-D-mannose, but these were not detected by paper chromatography.

Assignment of C-2–C-6 signals of mono-rhamnomannan of S. schenckii *and* C. stenoceras. — Other signals in the ^{13}C n.m.r. spectrum of *S. schenckii* rhamnomannans were of interest from the point of view of testing various techniques in signal assignment. The mono-rhamnomannan obtained[12] by growth of *S. schenckii* 1099.18 at 37° was of particular interest as its spectrum contained 12 signals arising from a (1→6)-linked α-D-mannopyranose main-chain, each residue being substituted in the 3-position by α-L-rhamnopyranosyl side-chains. The two C-1 signals have already been assigned[15], as has the CH_3 signal of the rhamnose residues, and so attention was paid to the 9 signals in the region δ_c 76.0 to 65.7 (Fig. 3)*. Most of the signals of the α-L-rhamnopyranosyl groups are readily assigned, as they should correspond closely to those of methyl α-L-rhamnopyranoside. However, the C-2 and C-3

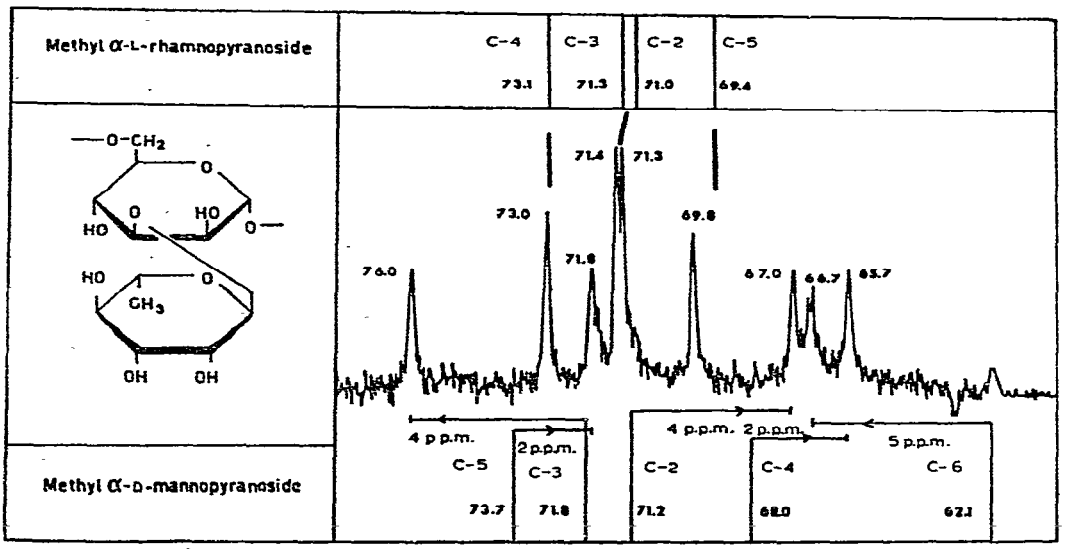


Fig. 3. Middle portion of the ^{13}C n.m.r. spectrum of the monorhamnomannan of *Sporothrix schenckii* 1099.18; assignments of signals and comparison of their chemical shifts with corresponding ones of methyl α-L-rhamnopyranoside and methyl α-D-mannopyranoside.

*All signals were obtained at 33° for the mono-rhamnomannan.

resonances cannot be distinguished with confidence as they are barely resolvable. Only two of the mannoside signals can be assigned with certainty, and this was done by comparison of their chemical shifts with those of methyl α-D-mannopyranoside (see Fig. 3). The polysaccharide signal at δ_c 76.0 must arise from C-3, which is *O*-substituted and should be located 5–10 p.p.m. downfield from the C-3 signal of methyl α-D-mannopyranoside[20], which lies at δ_c 71.8. The polymer signal at δ_c 71.8 should be from C-5 as it is slightly upfield from the C-5 signal[20] of methyl α-D-mannopyranoside (δ_c 73.7). Generally, 6-*O*-methylation of a mannopyranose residue results in such a C-5 upfield displacement[21]. The magnitude of the displacement on *O*-glycosylation can be gauged from the C-5 signal of a (1→6)-linked α-D-mannopyranose polymer[18], whose shift is close at δ_c 71.1 (Fig. 3).

In order to assign the C-2, C-5, and C-6 signals, which are closely distributed at δ_c 67.0, 66.7, and 64.7, a different approach was used. *S. schenckii* was grown on D-glucoses that were specifically isotopically labeled with the hope that the mannose and rhamnose moieties of the polysaccharide were formed without C–H bond cleavage or rearrangement of the carbon chain. When the D-glucose molecules are labeled with deuterium, the ^{13}C signals of the polysaccharide that are attached to deuterium would disappear[22]. Labeling of D-glucose molecules with ^{13}C should give rise to ^{13}C polysaccharide signals having enhanced intensities*.

Generally, C-6 signals of hexose monosaccharides may be assigned because, under partial decoupling conditions, a ^{13}C–^{1}H coupling takes place with two protons so that a triplet is observed, but the polysaccharide signals were too broad and poorly resolved for the recognition of a triplet. However, on growing *S. schenckii* on D-[6-$^{2}H_2$]glucose, the resulting polysaccharide does not give a signal at δ_c 66.7, thus characterizing the latter as the C-6 signal. This approach, however, was not applicable when using 3-^{2}H and 5-^{2}H derivatives of D-glucose, as the deuterium became randomized, and the spectra of the resulting polysaccharides were indistinguishable from that of unlabeled material.

When *S. schenckii* 1099.18 was grown on D-glucose containing double the natural abundance of ^{13}C in the 2-position (~2%), the spectrum of the resulting polysaccharide showed a signal of increased intensity at δ_c 67.0, which should thus correspond to C-2. Some increase was noted for the C-5 signal at δ_c 71.8, this indicating that, although most of the glucose chains remain intact, some fragmentation and rearrangement takes place during formation of the α-D-mannopyranose residues. In contrast, inspection of the ^{13}C signals of the α-L-rhamnopyranose residues shows no differences from the normal spectrum, suggesting that carbon chain-rearrangement is more extensive.

Another approach is to use D-glucose 90% labeled at C-1 with ^{13}C. The resulting polysaccharide should give a spectrum whose ^{13}C-2 signal should be removed from δ_c 67.0, as it would be split into a doublet because of ^{13}C–^{13}C

*As the yield of mono-rhamnomannan was low at 37°, the fungus was grown at 25°. Although the mono-rhamnomannan was mixed with other polysaccharides, its signals could be used in signal-assignment studies.

coupling with C-1 (J 45 Hz). Unfortunately, the C-6 signal at δ_c 66.7 increased to such an extent that it obscured other signals in this region of the spectrum.

EXPERIMENTAL

Preparation of ^{13}C n.m.r. spectra. — Carbon-13 magnetic resonance spectra were obtained by using a Varian XL-100-15 spectrometer with Fourier transform from D_2O solutions (0.85 ml) of compound (20–100 mg) contained in a coaxial glass cylinder fitting snugly within a 12-mm diameter × 20.3 cm tube maintained at 33°. Larger amounts of solute were dissolved in D_2O (2 ml) contained in the 12 mm × 20.3 cm tube similar to that just described, except that it was fitted with a Teflon vortex plug. To obtain a complete ^{13}C n.m.r. spectrum, the spectral width was 500 Hz, the acquisition time 0.4 sec, and the pulse width 50 μsec. In several experiments it was necessary to improve the resolution and the spectral width was narrowed to 500 Hz, with the acquisition time 4 sec and the pulse width 117 μsec. The chemical shifts are expressed as δ_c in p.p.m., relative to external Me_4Si, whose shift relative to D_2O (lock signal) was obtained in a separate experiment.

Chemical structure of rhamnomannans of C. stenoceras *1099.40.* — *C. stenoceras* was grown in 1-liter shake flasks, in 20 liters of medium, under the conditions described previously[11]. Following centrifugation and evaporation of the supernatant, polysaccharide (35 g) was precipitated by addition of an excess of ethanol. It contained mainly galactose, as shown by hydrolysis to aldoses and conversion into alditol acetates[23]. Also present was 1.3% of amylose, as shown by the amylase–amyloglucosidase dual enzyme-assay of Banks *et al.*[24].

Polysaccharides containing rhamnose and mannose and a trace of galactose were isolated from the crude mixture via their water-insoluble copper complexes formed with Fehling solution; yield 5.9 g; $[\alpha]_D^{25}$ −17° (c 0.3, water); acid equivalent 1,662. For ^{13}C n.m.r. spectrum see Fig. 1.

Polysaccharides (3.6 g) could also be obtained by extraction of the cells with 2% aqueous potassium hydroxide at 100°. This extract contained galactan, rhamnomannans, and a trace of amylose. Fractionation with Fehling solution gave a product that did not contain galactan, as the proportion of galactan in the crude, unfractionated polysaccharide was less than in the exocellular material.

Fractionation of the exocellular, purified polysaccharide (0.5 g) was performed on a DEAE-cellulose column in the acetate form. On washing with water, a mixture (106 mg) was obtained that appeared, by ^{13}C n.m.r. spectroscopy, to be a mixture of rhamnomannan and galactan. Passage of 5% acetic acid gave a fraction (70 mg) whose ^{13}C spectrum was identical to that of a polysaccharide having a main chain of (1→6)-linked α-D-mannopyranose residues, each residue being substituted at O-3 by an α-L-rhamnopyranosyl group[11]. An acidic polysaccharide (230 mg) was obtained on elution with 5% formic acid, and this gave ^{13}C signals at δ_c 80.2 and 81.4 of increased intensity.

Acid hydrolysis of C-stenoceras *1099.40 rhamnomannans.* — The mixed cell-wall

polysaccharides (1 g) obtained by precipitation with Fehling solution were hydrolyzed for 3 h at 100° in 2M sulfuric acid (10 ml). The solution was neutralized (barium carbonate) and treated with Amberlite IR-120 (H^+ form). The acidic components were absorbed on Dowex-1 (HCO_3^- form) and elution of the resin with dilute formic acid provided, after evaporation, a pink spot on a paper chromatogram having the R_F value of glucuronolactone [solvent: 40:11:19 (v/v/v) butyl alcohol–ethanol–water; spray: *p*-anisidine hydrochloride[25]]. The product was treated successively with refluxing, 3% methanolic hydrogen chloride, sodium borohydride in methanolic hydrogen chloride, and 0.5M sulfuric acid for 3 h at 100°. The resulting material corresponded to glucose on a paper chromatogram and was oxidized with D-glucose oxidase (Worthington), together with D-mannose.

Partial hydrolysis was conducted on the purified cell-wall polysaccharides (3 g) by using 0.5M sulfuric acid (40 ml) for 3 h at 100°. The mixture was processed in a similar way as already outlined, and the material (0.15 g) eluted from resin was fractionation on a cellulose column [eluant: 9:4:4 (v/v/v) ethyl acetate–acetic acid–water]. It had $[\alpha]_D^{25}$ −18° (*c* 0.5, water) and gave rhamnose and glucuronolactone (paper chromatogram) on hydrolysis with 2M sulfuric acid for 3 h at 100°. A 2-*O*-linkage was indicated, as the product was resistant to oxidation by lead tetraacetate[26], and the negative specific rotation is consistent with a β-glycosidic linkage. ^{13}C n.m.r. data (D_2O, 70°): 100.5 (Cα-1′), 104.8 (Cβ-1′), 94.5 (Cα-1), 94.2 (Cβ-1), 82.6 (Cα-2), and 82.1 (Cβ-2).

The *C. stenoceras* polysaccharide was partially hydrolyzed under the same conditions used to obtain 4-*O*-α-D-mannopyranosyl-α,β-D-mannose from the *S. schenckii* polysaccharide (see later). However, this disaccharide could not be detected on paper chromatograms with the 40:11:19 (v/v/v) butyl alcohol–ethanol–water solvent and *p*-anisidine hydrochloride as spray reagent.

The polysaccharide (1 g) was partially hydrolyzed in 0.185M sulfuric acid (10 ml) for 1 h at 100°, the solution was neutralized (barium carbonate), and evaporated to low volume. Degraded polysaccharide was obtained by precipitation with an excess of ethanol, yield 0.65 g, ^{13}C n.m.r. data: (D_2O, 70°) 173.0 (CO_2H), 105.4, 100.7, 100.3, 97.9, 97.4 (C-1 atoms), and 81.9 (C-2 of 2-*O*-substituted Rha*p*).

Smith degradation of C. stenoceras *1099.40 rhamnomannans.* — Hydrolysis of the polyalcohol, obtained by successive treatments[17] of the polysaccharide with sodium metaperiodate, at pH 2 for 1 h at 100° gave an ethanol-insoluble fraction whose ^{13}C n.m.r. spectrum corresponded to a (1→6)-linked α-D-mannopyranose polysaccharide[18]. ^{13}C n.m.r. data (D_2O, 70°): 101.1, 72.6 (large signal), 71.7, 68.6, and 67.6.

The ethanol-soluble fraction gave on a paper chromatogram [solvent: 40:11:19 (v/v/v) butyl alcohol–ethanol–water; spray: ammoniacal silver nitrate] spots corresponding to erythritol, R_{Gal} 0.3, and $R_{Glycerol}$ 1.0. The slowest spot corresponded to erythronic acid and the fastest one, apparently the 1,4-lactone, gave a brown rather than the black spot associated with glycerol. These fragments arise from 4-*O*-linked glucuronic acid residues.

Methylation of C. stenoceras *1099.40 rhamnomannans.* — Methylated polysaccharide was obtained as previously described[11], and was cleaved by refluxing overnight in 5% methanolic hydrogen chloride. The product was then converted into free *O*-methylaldoses with 3M sulfuric acid for 6 h at 100°, the solution was neutralized, and examined on a paper chromatogram [solvent: 40:11:19 (v/v/v) *n*-butyl alcohol–ethanol–water; spray: *p*-anisidine hydrochloride). A pink spot was detected having R_{Gal} 0.8, identical with that of 2,3-di-*O*-methyl-D-glucuronic acid[28]. However, as the other di-*O*-methylglucuronic acids were not available for comparison, it was reduced to a glucose derivative.

Accordingly, the original methyl glycosidic mixture was treated successively with lithium aluminum hydride in ether, 0.5M sulfuric acid for 18 h at 100°, and refluxing 3% methanolic hydrogen chloride for 3 h. G.l.c. on 10% neopentyl glycol sebacate on Chromosorb W at 160° gave 2 peaks having retention times of 24.3 and 35.2 min, which corresponded to methyl 2,3-di-*O*-methyl-α,β-glucopyranoside. Under similar conditions the 3,4 isomers had retention times of 18.9 and 22.0 min and the 2,4 isomers of 28.8 and 19.6 min.

Partial acetolysis of the rhamnomannans from C. stenoceras *1099.40.* — Under the same conditions as described next for the partial acetolysis of the *S. schenckii* polysaccharide, 3-*O*-α-L-rhamnopyranosyl-α,β-D-mannose[17] was obtained in 37% yield and identified by its ^{13}C n.m.r. spectrum.

Assignment of ^{13}C n.m.r. signals in the exocellular polysaccharides of S. schenkii *1099.12 and in the mono-rhamnomannan of* S. schenckii *1099.18.* — *A. Isolation of rhamnomannan. S. schenckii* was grown at 25° under the conditions previously described[11]. In a typical experiment, the culture medium was sterilized in an autoclave and centrifuged. Evaporation of the supernatant followed by precipitation with methanol and filtration provided mixed polysaccharides (8.2 g) that contained rhamnomannan and 5.9% of amylose[24]. Rhamnomannan was obtained in 10% yield, based on crude polysaccharide, via the copper complex formed with Fehling solution.

B. Partial acid hydrolysis of rhamnomannan. The rhamnomannan (0.6 g) was dissolved in 0.5M sulfuric acid (20 ml), which was maintained for 3 h at 75°. The solution was neutralized (barium carbonate), and evaporated to a syrup that was shown by paper chromatography [solvent: 40:11:19 (v/v/v) *n*-butyl alcohol–ethanol–water]; spray: *p*-anisidine hydrochloride to contain mainly rhamnose and a smaller proportion of mannose, galactose, and di- to penta-saccharides. Fractionation on a cellulose column provided rhamnose, mannose, and galactose (eluant: 7:1 (v/v) acetone–water], two disaccharides [7 mg and 6 mg, respectively with 4:1 (v/v) acetone–water] and a trisaccharide [8 mg; 3:1 (v/v) acetone–water].

The disaccharide migrating more rapidly on a paper chromatogram had $[\alpha]_D^{25}$ +23° (*c* 0.1, water); ^{13}C n.m.r. data (D_2O, 70°): δ_c 102.3 (C-1′).

Methylation by the method of Kuhn[29], followed by methanolysis in refluxing 5% methanolic hydrogen chloride, provided methyl 2,3,4,6-tetra-*O*-methyl- and methyl 2,3,6-tri-*O*-methyl-α-D-mannopyranoside, as shown by g.l.c.[30]. These

fragments, and the positive specific rotation, indicates that the disaccharide was 4-*O*-α-D-mannopyranosyl-α,β-D-mannose.

The slower-moving disaccharide was shown by a similar procedure to be 6-*O*-α-D-mannopyranosyl-α,β-D-mannose.

Likewise, the trisaccharide was found to be *O*-α-D-mannopyranosyl-(1→4)-*O*-α-D-mannopyranosyl-(1→4)-α β-D-mannose, $[\alpha]_D^{25}$ +39°; ^{13}C n.m.r. data (D_2O, 70°): δ_c 102.55, and 102.8 (substituted C-1 atoms).

C. Smith degradation of rhamnomannan. Rhamnomannan (0.5 g) was converted, by successive oxidation with sodium metaperiodate and reduction with sodium borohydride to a polyalcohol (0.28 g), which was partly hydrolyzed[27] in water for 1 h at pH 2 and 100°. Following deionization the residual polysaccharide (50 mg) was precipitated with ethanol and gave a ^{13}C n.m.r. spectrum identical with that of a (1→6)-linked α-D-mannopyranan[18]: ^{13}C n.m.r. data (D_2O, 70°): 101.1, 72.6 (large signal), 71.7, 68.6, and 67.6.

The ethanol-soluble fraction (102 mg) was fractionated on a column of cellulose [eluant: 7:1 (v/v) acetone–water], yielding erythritol (36 mg), characterized by g.l.c.[31]. Acetone–water (4:1,v/v) provided material (21 mg) whose R_F value on a paper chromatogram corresponded to that of a mannosylerythritol[32]. Another compound (12 mg) was eluted with 3:1 (v/v) acetone–water.

The second fraction had $[\alpha]_D^{25}$ +50° (*c* 0.2, water) and gave mannose and erythritol on acid hydrolysis. Methylation, followed by methanolysis, provided methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannopyranoside (g.l.c.), thus indicating a 2-*O*-α-D-mannopyranosyl-D-erythritol structure. 2-*O*-β-D-Mannopyranosyl-D-erythritol has $[\alpha]_D^{25}$ −36° (*c* 1.1, water)[32].

The last fraction gave mannose and erythritol on hydrolysis and had $[\alpha]_D^{25}$ +64° (*c* 0.2, water). Methylation, followed by methanolysis, provided methyl 2,3,4,6-tetra-*O*- and methyl 2,3,6-tri-*O*-methyl-α-D-mannopyranoside (g.l.c.)[30], corresponding to *O*-α-D-mannopyranosyl-(1→4)-*O*-α-D-mannopyranosyl-(1→2)-D-erythritol.

D. Partial acetolysis of rhamnomannan. Rhamnomannan (0.6 g) was partially acetolyzed under the conditions of Lee and Ballou[33], except that only 1-day's duration was used. Cellulose column-chromatography [eluant: 7:1 (v/v) acetone–water] provided 3-*O*-α-L-rhamnopyranosyl-α,β-D-mannose (50 mg) and *O*-α-L-rhamnopyranosyl-(1→2)-*O*-α-L-rhamnopyranosyl-(1→3)-α,β-D-mannose[13] (25 mg), which were identified by their ^{13}C n.m.r. spectra.

Extending the partial acetolysis for a further 4 days provided the 3-*O*-α-L-rhamnopyranosyl-α,β-D-mannose (66 mg) and a fraction (34 mg) containing 2-*O*-α-D-mannopyranosyl-α,β-D-mannose (identified by its p.m.r. spectrum[34]), contaminated by a little dirhamnosylmannose.

E. Preparation of D-*glucose labeled with* ^2H *and* ^{13}C. — The 3-^2H (ref. 35), 5-^2H (ref. 36) and 6-$^2H_2$ derivatives[37] of D-glucose were prepared by known routes, except that sodium borohydride was replaced by sodium borodeuteride in the reduction step.

D-[1-^{13}C]Glucose was prepared from D-arabinose by the cyanohydrin

synthesis[38] incorporating $K^{13}CN$ (90% enrichment). D-[2-^{13}C]Glucose was similarly prepared by using KCN and D-[1-^{13}C]arabinose, which was in turn prepared from D-erythrose[39].

Each of the 5 foregoing glucoses was converted to mono-rhamnomannan by *S. schenckii* 1099.18 via their copper complexes. The deuterated glucoses and D-[1-^{13}C]glucose were used as prepared, and D-[2-^{13}C]glucose was diluted with D-glucose so that its 2-position contained twice the natural abundance of ^{13}C.

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