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ANALYSIS OF LINKAGE POSITIONS IN *Saccharomyces cerevisiae* D-MANNANS BY THE REDUCTIVE-CLEAVAGE METHOD*

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ABSTRACT

The positions of linkage in the D-mannans derived from *Saccharomyces cerevisiae* X2180 and its mutants, *mnn*1, *mnn*2, and *mnn*4, were established by perethylation and subsequent reductive cleavage with triethylsilane in the presence of boron trifluoride etherate ($BF_3 \cdot Et_2O$) or trimethylsilyl trifluoromethanesulfonate. With the latter as the catalyst, all glycosidic carbon–oxygen bonds were cleaved, to produce a mixture of ethylated 1,5-anhydro-D-mannitol derivatives. With $BF_3 \cdot Et_2O$ as the catalyst, 2-, 3-, and 6-linked residues were incompletely cleaved, and residues linked at both O-2 and O-6 were not cleaved at all. It was concluded that reductive cleavage is an attractive method for determination of the structure of polysaccharides.

INTRODUCTION

We recently described a new technique for determination of the structure of polysaccharides that, potentially, has significant advantages over standard methylation analysis[1]. This method is based upon methylation analysis, but departs from it significantly with regard to the types of fragments formed by cleavage of the fully methylated polysaccharide. In "standard" methylation analysis, the fully methylated polysaccharide is *hydrolyzed*, to produce partially methylated sugars that are subsequently characterized as their alditol acetates[2] or aldononitrile acetates[3] by combined gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.). This method suffers the disadvantage that it is incapable of distinguishing between 4-linked aldopyranosyl and 5-linked aldofuranosyl residues in a polysaccharide. The same deficiency applies to 5- and 6-linked 2-ketohexosyl residues. In the new technique that we reported[1], however, the linkage position(s) and ring form of each monosaccharide residue are established simultaneously. This method involves the ionic hydrogenation of all glycosidic carbon–oxygen bonds in the fully methyl-

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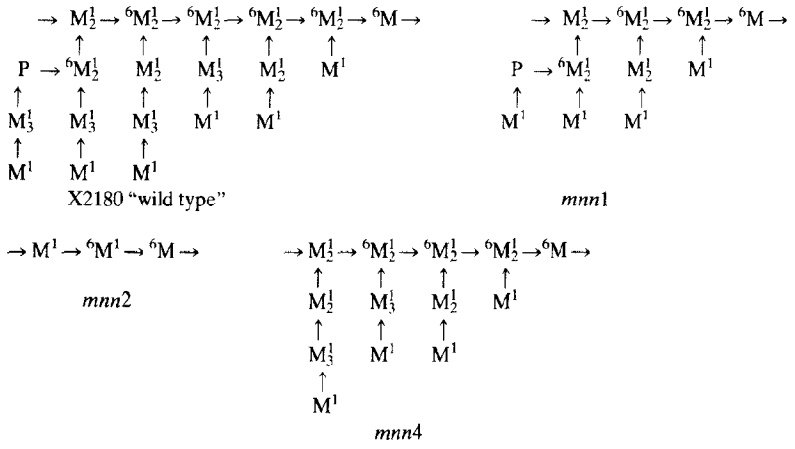


Fig. 1. Structures of the outer-chain D-mannans of *Saccharomyces cerevisiae* X2180 wild type and three mutants derived from it. [All D-mannosyl residues are in the pyranose ring-form (from ref. 5)].

ated polysaccharide with triethylsilane catalyzed by boron trifluoride etherate[1] or trimethylsilyl trifluoromethanesulfonate[4]. This *reductive cleavage* affords a series of partially methylated *anhydroalditols*, which are subsequently acetylated *in situ*, and analyzed by g.l.c.–m.s.

Studies with model glycosides[1,4] showed that reductive cleavage is regiospecific under these conditions; *i.e.*, selective cleavage of the glycosidic carbon–oxygen bond is observed *via* the formation and reduction of *cyclic* oxonium ions[4]. The regiospecificity of reduction is of critical importance if this reaction is to be generally applicable as a method for determination of the structure of polysaccharides, because any substantial degree of cleavage of the other acetal-oxygen bond (anomeric carbon atom–ring-oxygen atom) would lead to the formation of complex mixtures of *acyclic* oligomers that would be extremely difficult to fractionate and analyze. We have therefore begun a program to investigate the usefulness of this approach by using structurally well-characterized polysaccharides as models. *Saccharomyces cerevisiae* mannans are particularly useful in this regard, because of their content of branch points as well as several types of linkage, and because of the availability of mutant mannans of altered structure[5]. The mannans used in this study were the *S. cerevisiae* X2180 "wild type" and three mutants derived from it (*mnn*1, *mnn*2, and *mnn*4), whose generalized structures are given in Fig. 1.

RESULTS

Initial experiments were performed using fully methylated mannans. Although the expected results were obtained, the reductive-cleavage fragments were difficult to quantify accurately, because of the substantial volatility of the methylated 1,5-anhydromannitol derivatives. Better results were obtained by using perethylated mannans. The reductive cleavage of all four perethylated mannans

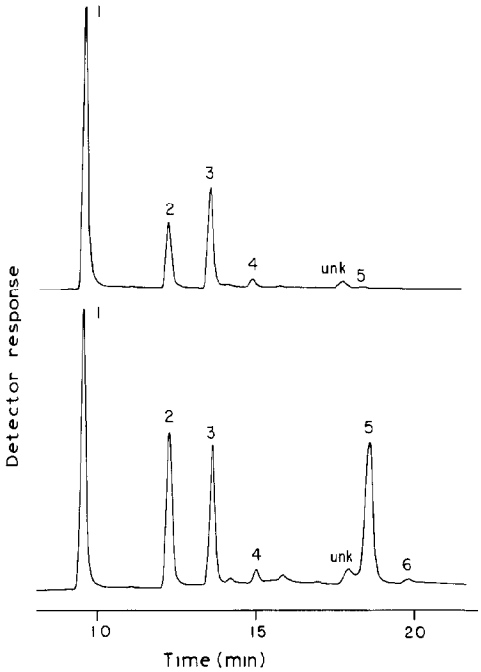
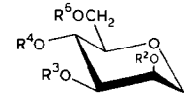


Fig. 2. Gas–liquid chromatograms on a column (3.18 mm × 3.66 m) of SP-2401, programmed from 170 to 250° at 6°/min, of the partially ethylated anhydroalditol acetates derived by reductive cleavage of perethylated *mnn*4 mannan with $BF_3 \cdot Et_2O$ as the catalyst (upper) and Me_3Si ester as the catalyst (lower). [The numbered peaks were identified as follows: (1) 1,5-anhydro-2,3,4,6-tetra-*O*-ethyl-D-mannitol, **1**; (2) 1,5-anhydro-2-*O*-acetyl-3,4,6-tri-*O*-ethyl-D-mannitol, **2**; (3) 1,5-anhydro-3-*O*-acetyl-2,4,6-tri-*O*-ethyl-D-mannitol, **3**; (4) 1,5-anhydro-6-*O*-acetyl-2,3,4-tri-*O*-ethyl-D-mannitol, **4**; (5) 1,5-anhydro-2,6-di-*O*-acetyl-3,4-di-*O*-ethyl-D-mannitol, **5**; (6) 1,5-anhydro-3,6-di-*O*-acetyl-2,4-di-*O*-ethyl-D-mannitol, **6**. Slight differences in retention times arise from the variability of the temperature program.]



1 $R^2 = R^3 = R^4 = R^6 = Et$
2 $R^2 = Ac, R^3 = R^4 = R^6 = Et$
3 $R^3 = Ac, R^2 = R^4 = R^6 = Et$
4 $R^6 = Ac, R^2 = R^3 = R^4 = Et$
5 $R^2 = R^6 = Ac, R^3 = R^4 = Et$
6 $R^3 = R^6 = Ac, R^2 = R^4 = Et$

was carried out with both boron trifluoride etherate ($BF_3 \cdot Et_2O$) and trimethylsilyl trifluoromethanesulfonate, separately, as the catalyst in the presence of triethylsilane (Et_3SiH). After workup, the products were acetylated, and the acetates analyzed by g.l.c. on SP-2401. Depending on the catalyst that was used, substantially different results were obtained. Shown in Fig. 2 are the chromatograms obtained for perethylated *mnn*4 mannan, which are representative.

The numbered peaks were identified through comparison to independently synthesized standards[6] by chemical-ionization (NH_3) mass spectrometry, electron-impact mass spectrometry, and g.l.c. retention-time. Peak 1 was identified as that of 1,5-anhydro-2,3,4,6-tetra-*O*-ethyl-D-mannitol (**1**), the expected product of non-reducing, terminal D-mannopyranosyl groups.

Peak 2 was identified as that of 1,5-anhydro-2-*O*-acetyl-3,4,6-tri-*O*-ethyl-D-mannitol (**2**), the product of 2-linked D-mannopyranosyl residues, and Peak 3, as that of 1,5-anhydro-3-*O*-acetyl-2,4,6-tri-*O*-ethyl-D-mannitol (**3**), the product of 3-linked D-mannopyranosyl residues. Unsubstituted D-mannopyranosyl residues in the backbone gave rise to Peak 4, *viz.*, 1,5-anhydro-6-*O*-acetyl-2,3,4-tri-*O*-ethyl-D-mannitol (**4**), whereas backbone D-mannopyranosyl residues substituted at O-2 produced 1,5-anhydro-2,6-di-*O*-acetyl-3,4-di-*O*-ethyl-D-mannitol (**5**, Peak 5). Peak 6 was identified as that of 1,5-anhydro-3,6-di-*O*-acetyl-2,4-di-*O*-ethyl-D-mannitol (**6**), arising from backbone D-mannopyranosyl residues substituted at O-3 (not shown in Fig. 1).

The integral values of all peaks were corrected for molar response by the effective carbon-response method[7]. This method was found to be applicable to anhydroalditols through a determination of the relative molar responses of fully ethylated, methylated, and acetylated 1,5-anhydro-D-mannitol; the determined values were in agreement with the calculated values ±3%. The response-corrected integrals were subsequently normalized to the integral obtained for nonreducing D-mannopyranosyl groups (**1**), in order to obtain the relative molar ratios of the derived anhydro-D-mannitol fragments (see Table I).

TABLE I

MOLAR RATIOS OF 1,5-ANHYDRO-D-MANNITOL DERIVATIVES (COMPOUNDS **1**–**6**) DERIVED BY REDUCTIVE CLEAVAGE OF *Saccharomyces cerevisiae* D-MANNANS

D-*Mannan*	*Catalyst*	*Molar ratio*[a]					
		1	**2**	**3**	**4**	**5**	**6**
X2180	ester[b]	**1.00**	0.53	0.59	0.06	0.78	0.03
	BF_3 Et_2O	**1.00**	0.23	0.46	0.08	0.01	—
*mnn*1	ester[b]	**1.00**	0.46	0.03	0.05	0.70	0.02
	$BF_3 \cdot Et_2O$	**1.00**	0.14	0.01	0.04	—	—
*mnn*2	ester[b]	**1.00**	0.46	0.18	6.85	0.13	0.17
	$BF_3 \cdot Et_2O$	**1.00**	0.26	0.14	4.90	—	—
*mnn*4	ester[b]	**1.00**	0.64	0.57	0.05	0.84	0.02
	$BF_3 \cdot Et_2O$	**1.00**	0.26	0.42	0.04	0.01	—

[a]Normalized to the basis of **1** as unity. [b]Trimethylsilyl trifluoromethanesulfonate.

DISCUSSION

The results obtained in reductive-cleavage reactions catalyzed by the Me_3Si ester (see Table I) are in full agreement with the published structures of *Sac-*

charomyces cerevisiae X2180 D-mannan and the D-mannans of its mutants. The only products detected in any of these reactions were partially ethylated derivatives of 1,5-anhydro-D-mannitol; g.l.c. analysis failed to reveal the presence of any higher-boiling oligomers that would have resulted from the formation and reduction of acyclic, oxonium-ion intermediates. In all of the D-mannans, the ratio of nonreducing terminal groups (**1**) to branch-point residues (**5** + **6**) is >1, but this is not unexpected, as nonreducing terminal groups also arise from the serine- and threonine-linked oligosaccharides of the protein core[8]. Branching occurs primarily at the O-2 atoms of the α-(1→6)-linked, backbone D-mannosyl residues of the X2180, *mnn*1, and *mnn*4 mannans, but branching also occurs to a small extent at the O-3 atoms of the backbone residues in these D-mannans, as evidenced by the detection of **6** in reductive-cleavage reactions. The occurrence of a small amount of branching at O-3 of the backbone residues was noted earlier, based on the results of standard methylation analysis[9]. In addition, a di-*O*-acetyl-monoanhydro-di-*O*-ethylhexitol of unknown structure was detected in traces in the reductive-cleavage products of the X2180 and *mnn*1 D-mannans, but in a more significant amount from *mnn*4 mannan. This unknown, which is eluted immediately prior to Peak 5 (see Fig. 2), may arise from another branched residue, but it is more likely to be the product of incomplete ethylation.

The reductive cleavage of *mnn*1 catalyzed by the Me_3Si ester produces very little of **3**, as expected, because of the absence of 3-linked D-mannopyranosyl residues in the outer core of the D-mannan. The small amount of **3** observed in this reaction arises from 3-linked residues in the inner core of the D-mannan, a structural region of the mannan not affected by the mutation[5] that produced *mnn*1. Similarly, the amounts of **1, 2, 3,** and **5** formed in the ester-catalyzed, reductive cleavage of perethylated *mnn*2 are attributable to the branched, inner core of the D-mannan, which is also not affected by the mutation that deletes all branching in the outer core[5].

A very interesting comparison may be made between the results obtained in the ester-catalyzed, reductive cleavages of perethylated X2180 and *mnn*4 D-mannans. These mannans differ structurally only by the absence of the D-mannobiosyl phosphoric diester unit from the *mnn*4 mannan (see Fig. 1). Because the phosphoric diester unit is attached to a 2-linked D-mannosyl residue in the X2180 mannan (see Fig. 1), the absence of this unit from the *mnn*4 mannan should result in the formation of a greater amount of **2** upon reductive cleavage. Indeed, the *mnn*4 mannan gives substantially more **2** than does the X2180 mannan, but the amounts of **3, 4, 5,** and **6** formed are virtually identical for the two mannans. Moreover, if the assumption is made that the difference in the amount of **2** formed from the *mnn*4 and X2180 mannans is attributable to the amount of 2-linked mannosyl residues in X2180 that contain a phosphoric diester unit, a mannose:phosphate ratio can be calculated for the X2180 mannan. The calculated mannose:phosphate ratio for the X2180 mannan is 28.2:1 [(1.00 + 0.64 + 0.59 + 0.06 + 0.78 + 0.03) ÷ 0.11]. The foregoing assumption is probably valid, because the mannose:phos-

phate ratio experimentally determined for the sample of X2180 mannan used in the experiment was 29:1. The fate of the phosphorylated D-mannosyl residue during the course of ethylation and reductive cleavage has not yet been established.

Substantially different results were obtained for all of the D-mannans when $BF_3 \cdot Et_2O$ was used as the catalyst in reductive depolymerization. The most notable result is the absence of **5** in $BF_3 \cdot Et_2O$-catalyzed reactions. The backbone D-mannosyl residues that are linked at both O-2 and O-6 are, therefore, not cleaved. Consequently, reductive cleavage under these conditions is nicely complementary to acetolysis, which selectively cleaves the 6-linked, backbone D-mannosyl residues [10]. Under the conditions used, backbone D-mannosyl residues linked only at O-6 were cleaved somewhat more slowly (compare the results obtained for *mnn*2), as were the 3-linked residues of the side-chains; 2-linked D-mannosyl residues were cleaved substantially more slowly, however. Although the Me_3Si ester is the more effective as a catalyst, the selectivity observed for $BF_3 \cdot Et_2O$ may make it useful in sequence studies.

From these results, it was concluded that our expectations based on initial studies with model glycosides[1,4,11] were justified, *i.e.*, reductive cleavage appears to be a valuable technique for polysaccharide structural characterization. This method has significant advantages over standard methylation analysis, in that (*a*) ring forms and linkage positions are established simultaneously, (*b*) it is less laborious, and (*c*) it may be useful for sequencing, because of kinetic differences for cleavage at different linkage positions.

EXPERIMENTAL

General. — *Saccharomyces cerevisiae* and its mutants were grown as previously described[12]. Isolation of mannans was accomplished as described by Kocourek and Ballou[13]. Perethylation of the mannans was performed by the procedure described by Sweet *et al.*[14], and the products were purified by gel-permeation chromatography in a column (2 × 80 cm) of Sephadex LH-20 with 1:2 (v/v) methanol–dichloromethane. Void-volume fractions were pooled, and evaporated to dryness under vacuum. Combined g.l.c.–m.s. was conducted in a Finnigan 4000 mass spectrometer equipped with a VG Multispec data-system. Column effluents were analyzed by chemical-ionization mass spectrometry, with ammonia as the reagent gas, wherein characteristic (M + 1) and (M + 18) ions were detected, and by electron-impact mass spectrometry in order to verify that the eluted components possessed mass spectra identical to those of independently synthesized standards[6]. All g.l.c. samples were "spiked" with authentic standards in order to verify their co-migration.

Reductive cleavage with $BF_3 \cdot Et_2O$ and Et_3SiH. — A 5-mL, round-bottomed flask containing a small, magnetic stirring-bar and 5 mg of perethylated polysaccharide (~0.02 mmol of acetal) was connected to a vacuum line, and the contents were dried overnight at room temperature. The flask was now cooled in an ice

bath, and dry CH_2Cl_2 (45 μL) was added. The solution was stirred until the perethylated polysaccharide dissolved, and Et_3SiH (30 μL; 0.19 mmol) and $BF_3 \cdot Et_2O$ (25 μL; 0.20 mmol) were sequentially added. The flask was sealed, and the solution was stirred for 24 h at room temperature. After quenching the reaction by the addition of methanol (1 mL), the mixture was passed through a bed (1 mL) of Dowex-AG 501 X-8-D resin contained in a small, glass pipet. The resin was eluted with additional methanol (5 mL), and the eluates were combined, and evaporated to dryness under vacuum at 20°.

Reductive cleavage with trimethylsilyl trifluoromethanesulfonate and Et_3SiH. — The procedure was the same as that just described, except that dry CH_2Cl_2 (200 μL), Et_3SiH (32 μL; 0.20 mmol), and the ester (20 μL; 0.1 mmol) were used.

Acetylation of ethylated anhydroalditols. — The products of the foregoing reactions were each dissolved in acetic anhydride (0.75 mL) and pyridine (0.75 mL), and heated for 1 h at 100° in a sealed ampoule. After being cooled to room temperature, the solution was added to ice (20 g) contained in a separatory funnel. Dichloromethane (30 mL) was added, and the organic layer washed successively with 30-mL portions of M H_2SO_4 (twice), saturated aqueous $NaHCO_3$ (twice), and water (twice), dried (anhyd. Na_2SO_4), and concentrated to ~1 mL under vacuum at room temperature. The concentrate was transferred to a small vial, and evaporated to dryness under a stream of dry nitrogen at room temperature.

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