Carbohydrate Polymers **23** (1994) 5–12

Application of reductive cleavage in the structural investigation of the antigenic polysaccharides of *Aspergillus fumigatus* and *Penicillium digitatum* with respect to the determination of the ring size of the galactose moieties

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(Received 13 May 1993; revised version received 24 July 1993; accepted 16 August 1993)

The structures of the antigenic extracellular polysaccharides of *Aspergillus fumigatus* and *Penicillium digitatum* have been investigated with respect to the linkage positions and ring size of the galactose moieties by the reductive-cleavage technique and by standard methylation analysis. 6-*O*-Linked and 5,6-di-*O*-linked galactofuranose residues were identified as new structural features. While 4-*O*-linked galactopyranose is also present in the extracellular polysaccharide of *A. fumigatus*, no significant amounts could be determined in the extracellular polysaccharide of *P. digitatum*. Results were proved by a two-step degradation procedure involving selective reductive cleavage of the galactofuranosyl side chains using a mixture of trimethylsilyl trichloroacetate and borontrifluoride etherate (5:1) as mild Lewis acid, and triethylsilane followed by subsequent remethylation, complete degradation, and GLC–MS analysis.

INTRODUCTION

The structures of the antigenic extracellular polysaccharides (EPS) of mould species belonging to the genera *Penicillium* and *Aspergillus* (Ascomycetes) are of great interest, as immunoassays have been developed for the sensitive and specific detection of these moulds in food (Notermans & Heuvelman, 1985; Notermans & Kamphuis, 1990; De Ruiter *et al.*, 1993) and for recognition of invasive aspergillosis in humans (Ste-Marie *et al.*, 1990).

In several studies these EPS have been characterized as glycopeptides containing galactomannans as the main antigenic polysaccharides (Gander *et al.*, 1974; Bardalaye & Nordin, 1977; Barreto-Bergter *et al.*, 1980, 1981; Gomez-Miranda & Leal, 1981; Bennett *et al.*, 1985; Latgé *et al.*, 1991). The backbone of the EPS is constituted of α(1-2)-linked and α(1-6)-linked mannopyranosyl residues. Chains of β(1-5)-linked galactofuranosyl residues are linked to the mannan backbone. The additional presence of (1-4)-linked galactopyranosyl residues has also been demonstrated for *A. niger* (Bardalaye & Nordin, 1977) and *A. fumigatus* (Latgé *et al.*, 1991). The galactomannan can be associated with various amounts of glucans. Differences in the structure of these polysaccharides may be due to different conditions of growth and age of the cultures and to various isolation and purification procedures, e.g. extraction from the cell walls or precipitation from the culture fluids and may also be dependent on the strain.

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Carbohydrate Polymers 0144-8617/94/$07.00

Previous studies on the antigenic properties of these moulds pointed to an important role for β-5-*O*-linked D-galactofuranoses (Notermans *et al.*, 1988). However, the use of a purified exo-β-D-galactofuranosidase revealed that the galactose residues of the EPS could only be degraded partially (Van Bruggen-Van der Lugt *et al.*, 1992), while the antigenicity completely disappeared. Elucidation of the linkage positions by standard methylation analysis does not allow a discrimination between 4-*O*-linked galactopyranoses and 5-*O*-linked galactofuranoses. In most studies, only their considerable difference in susceptibility towards acid hydrolysis was used to reveal the occurrence of both linkages in the antigenic galactomannans (Miyazaki & Naoi, 1974; Bardalaye & Nordin, 1977; Kamphuis *et al.*, 1992). The method of Darvill *et al.* (1980) is based on the same principle and includes two-fold standard methylation analysis with partial hydrolysis, the second time with total hydrolysis. However, this allows only a qualitative discrimination between 4-*O*-linked pyranoses and 5-*O*-linked furanoses, but no quantification, if both types of residue are present. NMR-spectroscopy has also been used for this purpose (Unkefer & Gander, 1979), but was inappropriate to detect minor constituents. The reductive cleavage technique (Jun & Gray, 1987) in principle enables simultaneous determination of linkage positions and ring size of these galactose moieties (Gruber & Gray, 1990).

One of the disadvantages of the use of reductive cleavage is the possible occurrence of isomerization, which is influenced by the Lewis acid used. The addition of boron trifluoride etherate ($BF_3{\cdot}OEt_2$) to trimethylsilyl trifluoromethanesulphonate (TMS triflate), TMS trifluoroacetate or TMS trichloroacetate, is reported (Mischnick *et al.*, 1991) to suppress or at least to diminish ring-size isomerization, and additionally has a synergistic effect on the Lewis acid strength as described for TMS methanesulphonate (TMS mesylate) by Jun & Gray (1987). Isomerization also depends on the stereochemistry and on the residue attached to C-5 (e.g. H, CH_2OH, CH_2OCH_3, CH_2NH_2, $COOCH_3$) of the aldoglycosyl ring (Mischnick *et al.*, 1991). In the case of permethylated 1,4-glucans, isomerization has been suppressed (Jun & Gray, 1987). Unprotected hydroxyl groups favour rearrangement (Mischnick & Krebber, 1989), and 1,4-xylans are reported to yield about 35% of the ring contracted 1.4-anhydroxylitol with TMS mesylate/$BF_3{\cdot}OEt_2$ and even up to 72% with TMS triflate solely (Heims *et al.*, 1989). Gruber and Gray (1990) could suppress rearrangement of 5-*O*- and 3,5-di-*O*-linked arabinofuranosyl and of 5-*O*-linked galactofuranosyl residues during reductive cleavage with careful use of TMS mesylate/$BF_3{\cdot}OEt_2$. In addition, isomerization is always favoured by humidity (Bennek *et al.*, 1983).

In the present study, the possible presence of 4-*O*-linked galactopyranoses in addition to the 5-*O*-linked galactofuranoses in EPS of species of the genera Aspergillus and Penicillium is assessed by reductive cleavage and this method is compared with conventional methylation analysis. To get reliable results, the extent of isomerization of 4-*O*-linked galactoses was studied on a model compound. Furthermore, the applicability of a two-step degradation procedure was tested.

EXPERIMENTAL

General

Triethylsilane (Et_3SiH) was purchased from Merck (Darmstadt, FRG), TMS triflate, TMS mesylate, TMS trifluoroacetate, TMS trichloroacetate, and 1-methylimidazole were obtained from Fluka (Neu-Ulm, FRG) and $BF_3{\cdot}OEt_2$ was from Aldrich (Steinheim, FRG). Dry dimethylsulphoxide and dichloromethane were stored over 4-Å molecular sieves.

EPS preparations

Mould strains used in this study were *P. digitatum* Sacc. H-236 and *A. fumigatus* Fres. M51-1, which were grown at 23°C in shaking cultures with yeast nitrogen base (Difco Labs., Detroit, MI, USA) as the basal synthetic culture medium as described (De Ruiter *et al.*, 1991).

Permethylation

This was carried out by a modified Hakomori procedure (D'Ambra *et al.*, 1988). Crude products were purified by size-exclusion chromatography using an LH-20 columm.

GLC

GLC was carried out on a Carlo Erba Fractovap 4160 gas chromatograph, equipped with an on-column injection system, a DB 5 capillary column (40 m × 0·25 mm), a flame-ionization detector, and an integrator 3390A from Hewlett-Packard (Table 1, C). Two-dimensional GLC was performed on a Siemens Sichromat 2 instrument equipped with a CP Sil 5 CB column and a CP Sil 19 CB column (Chrompack, both 25 m × 0·25 mm), using the temperature programs listed in Table 1 (A + B). The molar ratios of the components were calculated from the peak areas of the GLC chromatograms using the molar response factors calculated by the ECR concept (Sweet *et al.*, 1975).

GLC–MS

Mass spectra were obtained with a Hewlett-Packard HP 5840-A/5985-A GLC–MS system and with a VG/70-250S instrument. For CI-mass spectra, ammonia was used as reactant gas.

Table 1. Relative retention times of the degradation products of the EPS of *Penicillium* and *Aspergillus* species obtained by reductive cleavage

Compound	Number[a]	Relative retention times[b]		
		A	A + B	C
1,4-Anhydro-D-galactitols				
2,3,5,6-tetra-*O*-methyl	**2**	0·819		0·814
5-*O*-acetyl-2,3,6-tri-*O*-methyl	**6**	0·989	1·000	0·992
6-*O*-acetyl-2,3,5-tri-*O*-methyl[c]	**8**	1·037	1·033	1·045
5,6-di-*O*-acetyl-2,3-di-*O*-methyl[d]	**13**	1·174	1·155	1·220
1,5-Anhydro-D-galactitols				
2,3,4,6-tetra-*O*-methyl[e]	**16**	0·819[f]		0·814
4-*O*-acetyl-2,3,6-tri-*O*-methyl	**4**	0·941	0·952	0·945
4,6-di-*O*-acetyl-2,3-di-*O*-methyl[d]	**12**	1·138		1·172
1,5-Anhydro-D-mannitols				
2,3,4,6-tetra-*O*-methyl	**3**	0·843		0·848
2-*O*-acetyl-3,4,6-tri-*O*-methyl	**5**	0·941	0·961	0·951
6-*O*-acetyl-2,3,4-tri-*O*-methyl	**10**	1·076		1·107
2,3-di-*O*-acetyl-4,6-di-*O*-methyl	**11**	1·126		1·167
2,6-di-*O*-acetyl-3,4-di-*O*-methyl	**14**	1·174	1·165	1·233
1,5-Anhydro-D-glucitols				
2,3,4,6-tetra-*O*-methyl	**1**	0·783		0·779
4-*O*-acetyl-2,3,6-tri-*O*-methyl	**7**	1·000	1·000	1·000
3-*O*-acetyl-2,4,6-tri-*O*-methyl	**9**	1·037	1·051	1·052
4,6-di-*O*-acetyl-2,3-di-*O*-methyl	**15**	1·199		1·249

[a]Compound numbers correspond to the peak numbers in Fig. 1.
[b]GLC conditions:
A: 25 m CPSil 5 CB, carrier gas He, 120°C, 5°C/min → 290°C.
B: 25 m CPSil19 CB, carrier gas He, 110°C (20 min.), 3°C/min → 270°C.
C: 40 m DB5, carrier gas: H_2, 70°C (1 min), 20°C/min → 130°C, 4°C/min → 290°C.
A + B: two-dimensional GLC.
[c]Not detected for the EPS from *A. fumigatus* previously described (Latgé *et al.* 1991).
[d]Only traces for the EPS of *A. fumigatus* (M51-1), not detected for the EPS of *A. fumigatus* previously described (Latgé *et al.*, 1991).
[e]Only formed in the two-step degradation procedure applied to the EPS of *A. fumigatus* (see text).
[f]Separated from **2** by two-dimensional GLC A + B[b] with 1°C/min instead of 3°C/min in the temperature program of B (relative retention time not determined).

Reductive cleavage

Reductive cleavage experiments were carried out according to the procedure described by Jun and Gray (1987). All reactions were performed in silylated glass vials and the permethylated samples (0·1–1 mg) were dissolved in dichloromethane (50–100 µl). Mixtures of TMS mesylate, TMS trifluoroacetate, and TMS trichloroacetate with $BF_3{\cdot}OEt_2$ (5:1) and TMS triflate alone were used as Lewis acids in a 10–25 fold excess per glycosidic bond together with equimolar amounts of triethylsilane. Reductive cleavage was allowed to proceed for 17 h at room temperature or for 2–4 h at room temperature and for additional 16 h at 4°C (partial reductive cleavage). The work-up procedure and the acetylation of the samples were carried out as described (Jun & Gray, 1987).

Remethylation

Remethylation of the partially degraded samples was performed with sodium hydroxide and methyl iodide in dimethylsulphoxide (Ciucanu & Kerek, 1984).

Standard methylation analysis

This was carried out as earlier described (Mischnick, 1989). Hydrolysis was performed for 1 or 2 h with 2 M trifluoroacetic acid at 120°C.

RESULTS AND DISCUSSION

Methylation analysis

The permethylated fungal polysaccharides were purified by size-exclusion chromatography and the polymeric part (about 90%) was further investigated. After reductive cleavage (Fig. 1) with TMS triflate or TMS mesylate/$BF_3{\cdot}OEt_2$ as the Lewis acid and triethylsilane followed by acetylation, up to six different anhydrogalactitols (4 major 1,4-anhydro- and two minor 1,5-anhydro derivatives), five 1,5-anhydromannitols and four 1,5-anhydroglucitols could be identified as listed in Table 1. The structures of all components were confirmed by standard methylation analysis (Fig. 2) with the restriction, that 4-*O*-linked pyranoses and 5-*O*-

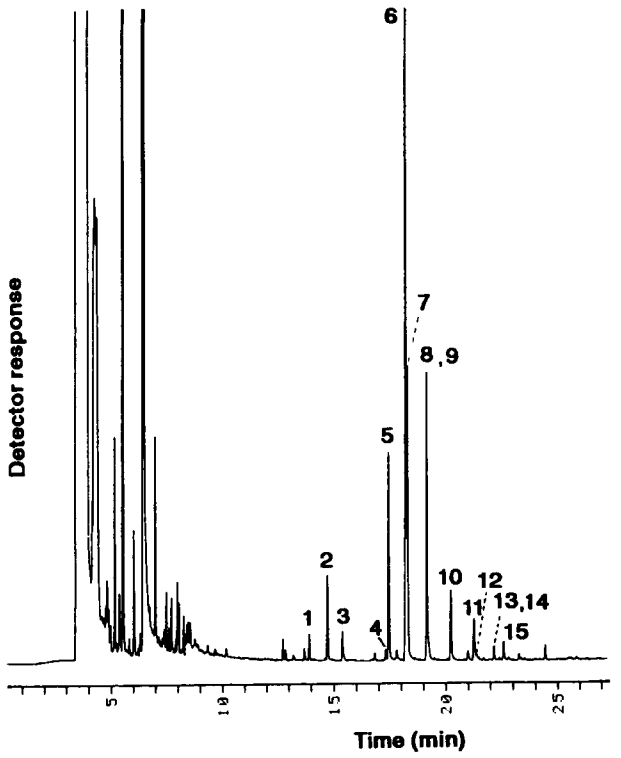

Fig. 1. Gas liquid chromatogram of the partially methylated anhydroalditol acetates derived by reductive cleavage of the permethylated EPS of *P. digitatum*. The peaks were numbered according to the compound numbers, as listed in Table 1. GLC conditions: see Table 1, column C.

linked furanoses yield identical partially methylated alditol acetates. Some components could only be separated and quantified by the use of two-dimensional GLC. The GLC-retention times of the different components relative to 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol (**7**) and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol (**7'**) were determined on three different stationary phases and listed in Table 1 (reductive cleavage) and Table 2 (standard methylation analysis), respectively. The identity of the degradation products was proved by their CI- and EI-mass spectra and by comparison of the relative retention times with authentic standards or values reported in the literature.

Glucose and mannose residues occur in the pyranose form solely. Besides terminal, 4- and 4,6-di-*O*-linked glucose residues, 3-*O*-linked moieties as known from the glucans of *A. niger* were also detected. As expected, terminal, 2-, 6-, and 2,6-di-*O*-linked mannopyranoses were present in all the EPS investigated. Significant amounts of 2,3-di-*O*-linked mannopyranosyl residues were only detected in the EPS of *P. digitatum* (Gander *et al.*, 1974). It can be expected that traces of this compound, which were found in the Aspergillus EPS, may be caused by slight undermethylation.

Terminal galactose residues exclusively occur in the furanose form, since only 1,4-anhydro-2,3,5,6-tetra-*O*-methyl-D-galactitol (**2**) and 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl-D-galactitol (**2'**), were found with regard to the non-terminal galactose residues. Only traces of, or no 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-galactitol (**4**) were obtained by reductive cleavage accomplished with TMS mesylate/$BF_3 \cdot OEt_2$ and Et_3SiH (Jun & Gray, 1987; Gruber & Gray, 1990) in addition to the expected compound 5-*O*-acetyl-1,4-anhydro-2,3,6-tri-*O*-methyl-D-galactitol (**6**) for the EPS of *P. digitatum*. Similar results were obtained with TMS trifluoroacetate/$BF_3 \cdot OEt_2$ as the Lewis acid mixture, with the restriction, that less mannosyl residues are found, which

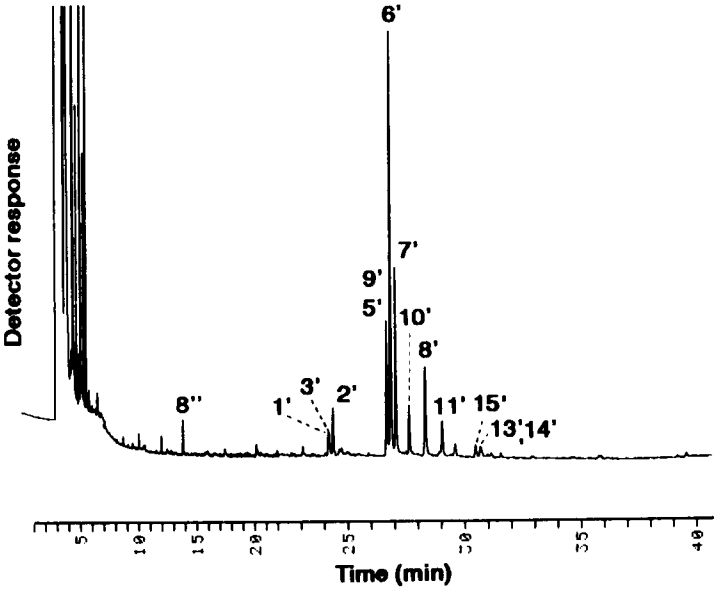

Fig. 2. Gas–liquid chromatogram of the partially methylated alditol acetates derived by standard methylation analysis of the EPS of *P. digitatum*. The peaks were numbered according to the compound numbers, as listed in Table 2. GLC conditions: 40 m DB5, carrier gas: H_2, temperature program: 70°C(1 min), 20°C/min → 130°C, 4°C → 220°C(10 min), 5°C → 290°C.

Table 2. Relative retention times of the degradation products of the EPS of *Penicillium* and *Aspergillus* species obtained by standard methylation analysis

Compound	Number[a]	Relative retention times[b]		
		A′	A′ + B′	C′
D-Galactitols				
1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl	**2′**	0·860		0·897
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl[e]	**16′**	0·884		0·915
1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl	**4′/6′**	0·990	0·995	0·993
1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methyl[c]	**8′**	1·054		1·046
1,4,5,6-tetra-*O*-acetyl-2,3,-di-*O*-methyl[d]	**12′/13′**	1·133	1·066	1·124
1,6-anhydro-2,3,5-tri-*O*-methylgalactofuranose	**8″**	0·42		
D-Mannitols				
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl	**3′**	0·854		0·890
1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl	**5′**	0·986	0·986	0·989
1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl	**10′**	1·029	1·023	
1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl	**11′**	1·077	1·071	
1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl	**14′**	1·133	1·064	1·124
D-Glucitols				
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl	**1′**	0·853		0·890
1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl	**7′**	1·000	1·000	1·000
1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl	**9′**	0·986	0·986	0·989
1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl[c]	**15′**	1·125	1·062	1·117

[a]Compound numbers correspond to the peak numbers in Fig. 2.
[b]GLC conditions:
A′: 25 m CPSil 5 CB, carrier gas: He, 120°, 5°C/min → 190°C, 1°C/min → 210°C, 5°C/min → 290°C.
B′: 25 m CPSil19 CB, carrier gas: He, 110°C (35 min), 3°C/min → 270°C.
C′: 40 m DB5, carrier gas: H_2, 70°C (1 min), 30°C/min → 180°C, 3°C/min → 290°C.
A′ + B′: two-dimensional GLC.
[c]See Table 1.
[d]See Table 1.
[e]See Table 1.

are known to need stronger degradation conditions (Bowie *et al.*, 1984). Therefore, it is most likely that no 4-*O*-linked galactopyranose is present in the EPS of *P. digitatum*. Opposed to this, significant amounts of compound **4** were obtained for the Aspergillus EPS in addition to **6**, which is the major component. Compound **4** could be formed by isomerization from 5-*O*-linked galactofuranose or may originate from 4-*O*-linked galactopyranose.

The presence of 6-*O*-acetyl-1,4-anhydro-2,3,5-tri-*O*-methyl-D-galactitol (**8**) and related compound **8′** indicated the presence of 6-*O*-linked galactofuranose residues. The EI-mass spectrum of **8** showed a characteristic fragment ion at *m/z* 117 (rel. abundance 100%) of the CH_2OAc-CHOMe (C6–C5) residue and the corresponding cyclic fragment of this primary cleavage at *m/z* 131 (5%), which after loss of methanol gave *m/z* 99 (9%). The fragment at *m/z* 101 (14%) may contain C4–C3–C2 (Heyns & Scharmann, 1966). Further minor fragments (rel. abundance <2%) are presumably caused by cleavage of C6 (*m/z* 175) and/or consecutive eliminations of methanol (*m/z* 143 and 111 or *m/z* 184). In addition signals at *m/z* 217 (M-31) and *m/z* 205 (M-43) are observed. However, the amount of **8′** was always lower than that of **8**. Finally, it was found that under the conditions of conventional methylation analysis up to 40% 1,6-anhydro-2,3,5-tri-*O*-methyl-D-galactofuranose (**8′**) was formed from 6-*O*-linked galactofuranosyl residues in addition to the reduced product **8′**. Its formation may be favoured by the lability of the 1-6 galactofuranosidic bond, making the galactosyl residue linked to the 6-position a good leaving group. The molecular mass of 204 for **8′** was determined by CI-MS. Its EI mass spectrum was identical with that reported by Heyns and Scharmann (1966) for 1,6-anhydro-2,3,5-tri-*O*-methyl-D-galactofuranose. This anhydro derivative can easily be overlooked due to its much higher volatility (see Table 2 and Fig. 2). 6-*O*-Linked galactofuranose has already been found in a cell-wall arabinogalactan derived from *Mycobacterium smegmatis* (Gruber & Gray, 1990), in EPS of *Sporothrix schenckii* and *Ceratocystis stenoceras* (Mendonça-Previato *et al.*, 1980), in the extracellular glucogalactan of *P. varians* (Jansson & Lindberg, 1980), in the cell walls of *Aureobasidium (Pullularia) pullulans* (Brown & Lindberg, 1967), and traces in the cell wall of the conidia of *A. fumigatus* (Barreto-Bergter *et al.*, 1981). While a considerable amount of the 6-*O*-linked galactofuranose was present in the EPS of *A. fumigatus* Fres. M51-1, it could not be detected in the galactomannan of another *A. fumigatus* strain as described previously by Latgé *et al.* (1991, results not shown).

Furthermore, a significant amount of 5,6-galactofuranose branching points has been found for *P. digitatum* (Van Bruggen–Van der Lugt *et al.*, 1992). 4,6-Di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-D-galactitol (**12**) was always detected beside the 1,4-anhydro isomer (**13**). No model studies were performed to study this phenomenon. However, since the ratio of **12** is higher with TMS triflate than with TMS mesylate/BF_3·OEt_2 and due to the higher tendency of isomerization for branched sugar units, it can be assumed, that only the 5,6-di-*O*-linked galactofuranose is originally present in the EPS.

Two-step degradation procedure

For successful use of the reductive-cleavage technique, rearrangement of the sugar ring must be excluded or at least controlled. Initial experiments with per-*O*-trimethylsilylated methyl-D-galactopyranoside with subsequent acetylation already showed that the pyranosyl form is very prone to ring contraction, yielding 2,3,5,6-tetra-*O*-acetyl-1,4-anhydro-D-galactitol as the main product. Reductive cleavage of permethylated methyl-4-*O*-(α-D-galactopyranosyl)-D-galactopyranoside with TMS-triflate/Et_3SiH yielded **6** and **4** in a ratio of 52:48 (besides the terminal moiety), while with TMS mesylate/BF_3·OEt_2 the ratio was 13:87. This confirmed the strong tendency of ring contraction for galactopyranose and demonstrated that isomerization during reductive cleavage is more favoured by TMS triflate. To prove whether the significant amount of **4** (4-5% with respect to all galactose moieties) found for the EPS of *A. fumigatus* (see above) is formed from 4-*O*-linked galactopyranose or resulted from rearrangement, a two-step degradation procedure was applied. First, the EPS was only partially degraded under catalysis of a very mild Lewis acid mixture (TMS trichloroacetate/BF_3·OEt_2, 5:1), similar to the method of Darvill *et al.* (1980). Under these conditions the galactofuranosyl moieties were nearly exclusively cleaved. A small portion of the anhydrogalactitols liberated were acetylated and quantified. The main part of the partially degraded sample was remethylated and further degraded by reductive-cleavage using TMS mesylate/BF_3·OEt_2 or by hydrolysis and subsequent reduction and acetylation. The occurrence of 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-galactitol (**16**) or 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol (**16'**) indicated that new terminal galactopyranoses originally linked in the chain had been formed by partial degradation (Fig. 3). By this procedure a total amount of about 7% 4-*O*-linked galactopyranose (with respect to all galactose moieties) was calculated for the EPS of *A. fumigatus*. Therefore, the presence of both 4-*O*-linked galactopyranose and 5-*O*-linked galactofuranose was proved in the EPS of *A. fumigatus*.

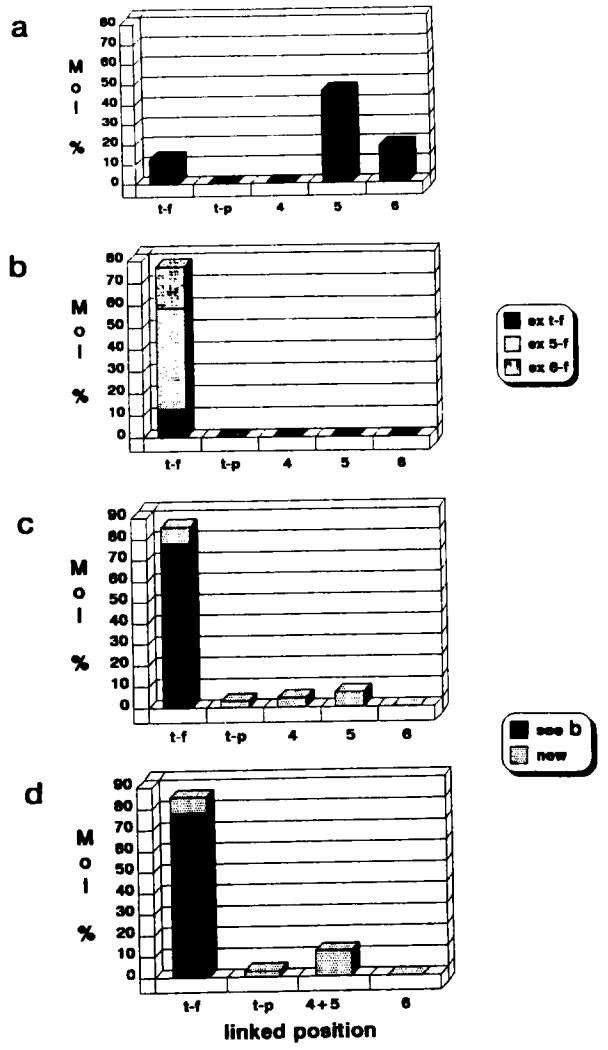

Fig. 3. Composition of the galactose moieties of the EPS of *A. fumigatus* after partial reductive cleavage (a), remethylation (b) and subsequent total reductive cleavage (c) or total hydrolysis, reduction and acetylation (d). t-f, Terminal furanoside; t-p, terminal pyranoside.

Comparison of reductive cleavage and standard methylation analysis

Both methods yielded the same qualitative results, when complete degradation was achieved. The quantitative results obtained by these two types of methylation analysis are shown in Table 3 and were similar for most of the constituents identified, especially when TMS triflate was used as the Lewis acid. With TMS mesylate/BF_3·OEt_2 the relative molar ratios of the anhydroalditols formed from 2-*O*-linked mannosyl residues **5**, **11** and **14** decreased in all cases. Residues of 2-, 3- and 6-*O*-linked mannopyranose and the corresponding di-*O*-linked residues are reported to be only partially or even not at all degraded with catalysis of BF_3·OEt_2, but can be cleaved completely with TMS triflate (Bowie *et al.*, 1984). For TMS mesylate solely, which is not able to cleave 4-*O*-linked glucopyranoses (Jun & Gray, 1987),

Table 3. Relative molar ratios of the constituents of the EPS from *P. digitatum* and *A. fumigatus* obtained by reductive cleavage using TMS mesylate/BF_3·OEt_2 (A) or standard methylation analysis (B)

Compound	(Number)	*P. digitatum*[a]		*A. Fumigatus*	
		A	B	A	B
Galactose moieties					
t-gal-*f*	(**2**/**2′**)	2·0	2·0	2·0	2·0
4-gal-*p*	(**4**)	—[b]		0·7 (1·0)[d]	
5-gal-*f*	(**6**/**6′**)	15·3	15·4	8·7	9·1
6-gal-*f*	(**8**/**8′** + **8″**)	7·2	6·5	2·6	2·2
4,6-gal-*p*	(**12**)[c]	0·2		0·1	
5,6-gal-*f*	(**13**/**13′**)	0·6	0·4	0·2	Traces
Mannose moieties					
t-man-*p*	(**3**/**3′**)	1·3	1·0	1·1	0·8
2-man-*p*	(**5**/**5′**)	4·7	5·0	1·8	2·1
6-man-*p*	(**10**/**10′**)	2·4	2·2	0·6	0·5
2,3-man-*p*	(**11**/**11′**)	1·2	1·6	0·2	0·4
2,6-man-*p*	(**14**/**14′**)	0·5	0·7	0·6	1·3

[a]Average of two different batches of *P. digitatum* EPS.
[b]Traces were summarized with **6**.
[c]Assumed to be formed via isomerization.
[d]Value in brackets calculated from the two-step degradation procedure (see text).

we observed a surprising selectivity for the 2-*O*-linked mannopyranoses. In contrast, the relative molar ratios of **8** and **13** were always higher than those of the corresponding galactitols **8′** and **13′**. 1,6-Anhydrogalactofuranose (**8″**) was identified as additional product of 6-O-linked galactofuranosyl residues. The combined amounts of **8′** and **8″** were in better agreement with the result of reductive cleavage. The remaining difference may be caused by losses of the very volatile compound **8″** during evaporation steps. The corresponding 5-*O*-acetyl-1,6-anhydro-2,3-di-*O*-methyl-D-galactofuranose (**13″**) was not detected, presumably due to the relative small amount of 5,6-di-*O*-linked galactofuranose present in the EPS.

CONCLUSIONS

Reductive cleavage provides a valuable method to elucidate the ring size and the linkage positions of the galactose residues in the EPS of *Penicillium* and *Aspergillus* species. The occurrence of 6-*O*- and 5,6-di-*O*-linked galactofuranosyl residues as new structural features in the EPS of *Penicillium digitatum* and *Aspergillus fumigatus* was demonstrated. The occurrence of minor amounts of galactopyranose residues in the EPS of *A. fumigatus* could be confirmed using a two-step reductive-cleavage procedure in which ring isomerization was suppressed. This procedure included the use of TMS trichloro-acetate/BF_3·OEt_2 as a very mild Lewis acid.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) and by the Netherlands' Foundation for Chemical Research (SON) with financial aid from the Netherlands' Technology Foundation (STW).

We gratefully acknowledge Dr J.-P. Latgé (Pasteur Institute, Paris, France) for critical reading of the manuscript.

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