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STRUCTURAL STUDIES OF VARIANOSE

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ABSTRACT

The complex carbohydrate varianose, elaborated by *Penicillium varians*, has been investigated, using methylation analysis, specific degradations, and n.m.r. studies. A main structural element in the glucogalactan portion of the molecule is a tetrasaccharide unit having the structure:

$$\begin{array}{l} \rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow \\ \qquad\qquad\quad 2 \\ \qquad\qquad\quad \uparrow \\ \qquad\qquad\quad 1 \\ \alpha\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Gal}f \end{array}$$

The possibility that this is a true repeating-unit, but that varianose has been enzymically modified during its production, is discussed. The presence of both α- and β-D-galactofuranosyl residues in varianose is an unusual feature.

INTRODUCTION

The extracellular polysaccharide, varianose, elaborated by *Penicillium varians*, G. Smith, was found[1] to contain D-galactose, D-glucose, and an unidentified sugar in the proportions 70:14:14. On methylation analysis, 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-galactose were obtained, indicating that varianose contains terminal D-glucopyranosyl groups linked to a D-galactan backbone. Because of the resistance of varianose to hydrolysis by 0.01M hydrochloric acid at 100°, it was assumed that the D-galactosyl residues were pyranosidic. The unidentified sugar was suspected to be either D-idose or L-altrose. We now report further structural studies of varianose.

RESULTS AND DISCUSSION

Varianose was isolated as previously described and further purified by dialysis. It had $[\alpha]_{578}$ +14°, in good agreement with the previously recorded[1] value, $[\alpha]_D$ +15°. An acid hydrolysate of the polymer contained D-galactose, D-glucose, and

D-mannose in the relative proportions 70:15:15. The product also contained nitrogen (N, 1%) and phosphorus (P, 0.2%). Attempts to fractionate the material by gel filtration were not successful. Treatment with 0.1M trifluoroacetic acid at 85° for 90 min, with fractionation of the product by gel filtration, yielded a polymeric material rich in mannose, a glucosylgalactose, and galactose as the main components.

In the peptidoglycan elaborated by *Penicillium charlesii*, which has been investigated by Gander *et al.*[2-4] and by Gorin and Mazurek[5], β-D-galactofuranosidic chains are linked to the rest of the molecule. It seems probable that varianose has a related structure, but with glucogalactan moieties. However, this has not been investigated, and the present study concerns only the glucogalactan part of the polymeric material.

In the ^{1}H-n.m.r. spectrum of varianose, the main signals in the region for anomeric protons were observed at δ 4.98, 5.12, 5.27, and 5.31. The presence of β-D-glucopyranosidic or β-D-galactopyranosidic residues, which give signals for their anomeric protons at higher fields, is therefore excluded. In the ^{13}C-n.m.r. spectrum, four strong signals were observed in the region for anomeric carbons, at 98.5, 100.1, 107.0, and 109.2 p.p.m. The two latter signals strongly indicate the presence of β-D-galactofuranosyl residues.

Methylation analysis of varianose (Table I) confirmed the previous identification[1] of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-galactose. In addition, four further galactose derivatives and three mannose derivatives were obtained; at the time of the previous work[1], chromatographic techniques were not available. The methylated sugars were analysed by g.l.c.–m.s. of their alditol acetates[6]. As the 2,3,4,6-tetra-*O*-methyl derivatives of glucitol and mannitol are not separated on the columns used, the ratio between the corresponding sugars was determined in a separate experiment, in which the mixture of methylated sugars was acetylated and analysed by g.l.c.[7]. However, the value (19%) for 2,3,4,6-tetra-*O*-methyl-D-

TABLE I

METHYLATION ANALYSIS OF VARIANOSE

Methylated sugar[a]	T^b *(OV-225)*	T^b *(SP-1000)*	*Mole %*
2,3,4,6-Glc	1	1	19
2,3,4,6-Man	1	1	2
2,3,5,6-Gal	1.10	1.06	5
3,4,6-Man	1.80	1.63	6
3,5,6-Gal	2.00	1.86	20
2,3,6-Gal	2.33	1.86	28
2,3,5-Gal	2.78	1.96	4
3,4-Man	4.35	3.34	2
3,5-Gal	4.92	4.06	14

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.* [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OV-225 column at 190° and on an SP-1000 glass-capillary column at 220°.

glucose must be too high, as it exceeds the percentage of D-glucose in the sugar analysis. As seen from Table I, most of the galactose derivatives must originate from furanosidic residues. That the alditol having the highest retention time derives from 3,5-di-*O*-methyl-D-galactose, and not from 2,4-di-*O*-methyl-D-galactose, was demonstrated by g.l.c.–m.s. of the alditol acetates obtained after reduction with sodium borodeuteride.

In the partial hydrolysis with acid discussed above, not even traces of oligosaccharides containing terminal D-galactopyranosyl groups could be observed. This indicates that the 2,3,6-tri-*O*-methyl-D-galactose derives from D-galactofuranosyl residues substituted at O-5 and not from D-galactopyranosyl residues substituted at O-4.

The glucosylgalactitol obtained after partial hydrolysis with acid had $[\alpha]_{578}$ +102°. In the ^{1}H-n.m.r. spectrum of the corresponding alditol, prepared by reduction with sodium borodeuteride, the signal for the anomeric proton appeared at δ 5.16 ($J_{1,2}$ 4 Hz). Hydrolysis of the permethylated alditol yielded a mixture of 2,3,4,6-tetra-*O*-methyl-D-glucose and 1,3,4,5,6-penta-*O*-methyl-D-galactitol-*1-d*. These results demonstrate that the disaccharide is 2-*O*-α-D-glucopyranosyl-D-galactose. In agreement with this, the permethylated disaccharide alditol gave the expected mass spectrum[8]. The partial structure **1** is accordingly established.

α-D-Glc*p*-(1→2)-D-Gal*f*-(1→

1

Varianose was subjected to a Smith degradation[9], that is, periodate oxidation, borohydride reduction, and hydrolysis with acid under mild conditions. Because of the furanosidic linkages in the polymer, the hydrolysis conditions are critical. It was found that less than 20% of the furanosidic linkages in varianose were cleaved on treatment with 0.2M trifluoroacetic acid at 22° for 40 h, and these conditions were chosen for the hydrolysis of the polyalcohol. Part of the product was methylated and investigated by g.l.c.–m.s. The main component (*T* 3.0 relative to fully methylated melibiitol on OV-1 at 195°) gave a mass spectrum expected for a pentofuranosyl-hexofuranosyl-tetritol[8,10]. Small amounts of a pentofuranosyl-tetritol and a hexofuranosyl-tetritol were also observed.

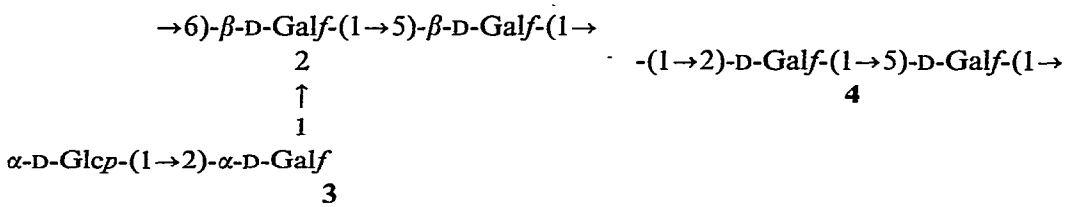
The main component, which was isolated by gel filtration, had $[\alpha]_{578}$ −1°, and yielded equimolecular amounts of threitol, arabinose, and galactose on acid hydrolysis. On methylation analysis, 2,3,5-tri-*O*-methyl-L-arabinose and 3,5,6-tri-*O*-methyl-D-galactose were obtained. The low value for the optical rotation indicated that the L-arabinofuranosyl and D-galactofuranosyl residues were both either α-linked or β-linked. In the ^{1}H-n.m.r. spectrum, the signals for anomeric protons appeared at δ 5.22 ($J_{1,2}$ small) and 5.12 ($J_{1,2}$ 4 Hz). These are the coupling constants expected for furanosides in which H-1 and H-2 are *trans* and *cis*, respectively[7]. In the ^{13}C-n.m.r. spectrum, signals for the anomeric carbons appeared at 107.3 and 102.6 p.p.m. These values are of the magnitudes expected for β-D-galactofuranosides (or α-L-arabinofuranosides) and α-D-galactofuranosides (or β-L-arabinofuranosides), re-

spectively[11]. Moreover, the former signal has been shifted 2 p.p.m. upfield because of substitution at O-2. Partial hydrolysis of the trisaccharide with acid gave a D-galactofuranosyl-threitol. Its anomeric carbon gave a signal at 108.5 p.p.m., demonstrating that it was a β-D-galactofuranoside. The trisaccharide obtained on Smith degradation consequently has the structure **2**. The position in which the D-threitol is substituted has not been proved, as the volatile threitol derivative was lost in the methylation analysis of the trisaccharide. However, the data from the methylation analysis of the polymer indicate that the D-threitol moiety must derive from D-galactofuranosyl residues, substituted at O-5.

β-L-Ara*f*-(1→2)-β-D-Gal*f*-(1→3)-D-Threitol
2

The four signals for anomeric carbons in the ^{13}C-n.m.r. spectrum of varianose can now be assigned as follows. Those at 109.2 and 107.0 p.p.m. are given by β-D-galactofuranosidic residues, with the signal at higher field reflecting substitution at O-2. The signal at 100.1 p.p.m. is most probably given by the α-D-glucopyranosyl groups, and that at 98.5 p.p.m. by the α-D-galactofuranosyl residues, the latter being shifted upfield because of substitution at O-2.

There is only one possible structure (**3**) that can give the trisaccharide **2** as the main product of Smith degradation.



3

Of the two minor products, the galactofuranosyl-threitol may be an artefact formed by partial hydrolysis of **2**. The arabinosyl-threitol, however, should derive from partial structures **4**, in which the (1→2)-linkage is broken as a result of the degradation; such structural elements are probably rather insignificant.

The tetrasaccharide unit **3** is a main structural feature in varianose. As discussed above, it is possible that varianose is a peptidoglycan that contains glucogalactan components, and that these components originally have a regular structure and are composed of the tetrasaccharide repeating-unit **3**. However, during the cultivation of the organism, which lasts for several weeks, the glucogalactan may be somewhat modified by the action of extracellular, hydrolytic enzymes produced by the mould. This could explain the less-regular structure indicated by the methylation analysis.

β-D-Galactofuranosyl residues are often part of natural carbohydrates, but α-D-galactofuranosyl residues are less common. To the best of our knowledge, varianose is the first natural carbohydrate in which both α- and β-D-galactofuranosyl residues have been observed.

EXPERIMENTAL

General methods. — Treatment with 0.5M trifluoroacetic acid at 100° overnight was used for complete hydrolysis of unmethylated and methylated products. Methylation analyses were performed as previously described[6]. Methylated oligosaccharides were separated by g.l.c. on 3% OV-1 columns at 170–190°. Sephadex G200 was used for attempted fractionation of the polymeric material. Paper chromatography was performed on Whatman No. 1 paper with ethyl acetate–acetic acid–water (3:1:1). T.l.c. was performed on silica gel with dichloromethane–acetic acid–methanol–water (10:5:3:2). N.m.r. spectra were recorded at 99.6 MHz (^{1}H) and 25.1 MHz (^{13}C) for solutions in deuterium oxide at 85°. Sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate was used as internal reference (^{1}H) and tetramethylsilane as external reference (^{13}C).

Varianose. — (*a*) *Isolation. Penicillium varians* was grown and varianose isolated from the culture medium as described by Haworth *et al.*[1]. The product was further subjected to dialysis and recovered by freeze-drying. Sugar analysis (g.l.c.–m.s. of the alditol acetates) showed the presence of D-mannose, D-galactose, and D-glucose in the proportions 15:70:15. The D configuration of the sugars was demonstrated by the method of Leontein *et al.*[12].

(*b*) *Partial, acid hydrolysis.* The polysaccharide (100 mg) was treated with 0.1M trifluoroacetic acid (10 ml) at 85° and the reaction was monitored by ^{1}H-n.m.r. spectroscopy. After 90 min, changes in the n.m.r. spectrum had become insignificant, indicating that most of the furanosidic linkages had been cleaved. The solution was then concentrated and the product was subjected to gel filtration. A fraction (17 mg) eluted with the void yielded mainly D-mannose on acid hydrolysis. Next followed an oligosaccharide fraction (8 mg), a disaccharide fraction (18 mg), and a monosaccharide fraction (40 mg). On methylation analysis of the oligosaccharide fraction, no 2,3,4,6-tetra-*O*-methyl-D-galactose was obtained. This fraction was not further investigated. The monosaccharide fraction contained D-galactose and D-glucose in the ratio 10:1.

The disaccharide fraction, $[\alpha]_{578}$ +102° (*c* 1.5, water), moved as a single spot in paper chromatography. On hydrolysis, it yielded equal amounts of D-galactose and D-glucose. The disaccharide alditol was prepared by reduction with sodium borodeuteride. The fully methylated alditol gave a single peak in g.l.c., and its mass spectrum contained, *inter alia*, peaks with *m/e* (relative intensities and designations in parenthesis): 88 (100, H_1), 89 (44), 101 (78, F_1), 172 (12, bA_3), 187 (22, aA_2), 204 (1, bA_2), 219 (3, aA_1), and 236 (11, bA_1).

(*c*) *Smith degradation.* Varianose (500 mg) was treated with 0.03M periodate in 0.1M sodium acetate buffer of pH 3.9 (100 ml) in the dark at 4° for 5 days. Excess of periodate was reduced by addition of ethylene glycol (1 ml), the solution was dialysed overnight and concentrated to 100 ml, and sodium borohydride (0.6 g) was added. After 4 h, the solution was neutralised with acetic acid, dialysed, and freeze-dried. The polyalcohol (350 mg), on acid hydrolysis, yielded equimolecular amounts of

arabinose and galactose. A solution of the polyalcohol (200 mg) in 0.2M trifluoroacetic acid (20 ml) was kept at 22° for 40 h and then concentrated. Part of the residue was methylated, and analysed by g.l.c.–m.s. The main component gave, *inter alia*, peaks at *m/e*: 88 (7, H_1), 89 (74), 101 (100, F_1), 111 (21 aA_3), 115 (63, cA_2), 143 (61, aA_2), 147 (8, cA_1), 175 (46, aA_1), 207 (23, bcJ_1), 287 (0.6, bcA_3), 315 (0.6, baA_3), 319 (0.6, bcA_2), 347 (0.4, baA_2), 379 (0.1, baA_1), and 411 (0.5, $abcJ_1$). On gel filtration of the degraded material, a main component (39 mg) was eluted in the trisaccharide region. It had $[\alpha]_{578}$ −1° (*c* 0.7, water) and yielded equimolecular amounts of threitol, arabinose, and galactose on acid hydrolysis.

Partial, acid hydrolysis of the arabinosyl-galactosyl-threitol. — A solution of the title compound (16 mg) in 0.05M trifluoroacetic acid (0.4 ml) was kept at 85°, the reaction being monitored by t.l.c. After 60 min, when two-thirds of the starting material had disappeared, the solution was concentrated and filtered through a column (1 × 4 cm) of Dowex-2 (HO^-) resin, in order to remove the reducing sugars from the mixture. The eluate was concentrated and fractionated by gel filtration. A product (2 mg), $[\alpha]_{578}$ −38° (*c* 0.14, water), was eluted in the disaccharide region; on hydrolysis, it yielded equimolecular amounts of threitol and galactose.

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