Carbohydrate Research, 141 (1985) 283–288
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

CHARACTERIZATION OF 6-DEOXY-D-ALTRITOL IN THE CELL-WALL POLYSACCHARIDE OF *Nocardia asteroides* R 399

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(Received August 2nd, 1984; accepted for publication in revised form, December 28th, 1984)

ABSTRACT

A polyol, found in the cell-wall of *Nocardia asteroides* R 399 as a component of a neutral polysaccharide mainly composed of D-arabinose and D-galactose, was identified by mass spectrometry, paper chromatography, thin-layer chromatography, and gas chromatography as 6-deoxy-D-altritol.

INTRODUCTION

The value of cell-wall chemistry in the taxonomy of bacteria has been often presented. Cell walls of bacteria from the "CMN" group, *Corynebacteria, Mycobacteria,* and *Nocardia* consist of a basal structure of a peptidoglycan with disaccharide units linked to tetrapeptide units, long-chain α-branched β-hydroxylated fatty acids, and a neutral polysaccharide. The structure of mycobacterial polysaccharide has been found to be mainly composed of arabinose and galactose[1,2]. The chemical composition of the cell-wall polysaccharide of other bacteria of the "CMN" group has also been reported[3–5]. A polysaccharide having the following components was isolated from the cell-wall of *Nocardia asteroides* R 399: arabinose, galactose, glucose, and an unknown polyol. We report herein the isolation and characterization of this polyol.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer 241 photoelectric polarimeter. For the determination of carbohydrate components, samples were heated at 100° with M HCl for 15 h, or 2M HCl for 2 h, and analyzed as monosaccharide alditol acetates[6] by g.l.c. on a glass column (2 m × 1 mm) coated with 3% ECNSS-M on Gas Chrom Q (80–100 mesh) at 180°, or on capillary silica fused columns coated with SP 2100 (50 m), the temperature being raised from 180° to 250° (4°/min), or OV 101 (25 m) at a temperature increasing from 160° to 260° (4°/min).

Chromatographies. — Paper chromatography was performed with Whatman

0008-6215/85/$ 03.30

No. 1 or No. 3 MM paper by the descending method in the following solvent systems: (*A*) 6:4:3 (v/v) butanol–pyridine–water; (*B*) 10:10:2:1:1 (v/v) ethyl acetate–pyridine–water–acetic acid–propionic acid; and (*C*) 3:1:3 (v/v) ethyl acetate–pyridine–water. Chromatograms were revealed with alkaline[7] $AgNO_3$ after previous impregnation with an $NaIO_4$ solution.

T.l.c. was performed on Silica gel G plates (Merck) (0.25-mm thickness) in solvents *B*, (*D*) 2:1:2 (v/v) ethyl acetate–pyridine–water, or (*E*) 14:3:3 (v/v) ethyl acetate–2-propanol–methanol. The detection reagents were a modified Galanti mixture[8] [5:5:2 (v/v) 4% diphenylamine in ethanol–4% aniline in ethanol–H_3PO_4; plates were heated for a few min at 100°], 4% ethanolic H_2SO_4 (with heating at 100°), or 20% ethanolic *p*-toluenesulfonic acid (5 min at 120°).

G.l.c. analysis was performed with an Intersmat IGC 120 FL apparatus equipped with a flame-ionization detector, and g.l.c.–m.s. with a Hewlett-Packard 5710 A chromatograph in conjunction with a VG Micromass 305 mass spectrometer.

Gel permeation was performed on columns (1 m× 2.5 cm) of Sephadex G 50f + G 25f with 0.1M NaCl as eluting solvent.

Affinity chromatography was performed on Concanavalin A–Sepharose[9] 4B (Sigma) eluted first with 0.15M phosphate buffer, pH 7.0, containing 0.15M NaCl, and then with 0.01M borate buffer, pH 6.0, containing 0.5M NaCl. Fractions (2 mL) were collected at a flow rate of 21 mL · h^{-1} and analyzed for pentoses[10] and hexoses[11].

Cultures. — *N. asteroides* R 399 (ATCC 23824), defined as the reference strain of *Nocardia*, was grown on Sauton medium for three weeks at 37°. The cells were harvested by filtration, washed several times with water, and freeze-dried. They were defatted by successive extractions with 2:1 (v/v) chloroform–methanol.

Cell-wall preparation. — Defatted bacteria (15 g), suspended in water (30 mL), were disrupted by ultrasonic treatment with a 500 W MSE apparatus for 25 min, at time intervals of 5 min, at 0°. The suspension was centrifuged for 20 min at 5000*g*, EDTA slowly added to the supernatant solution to a 10mM final concentration, the mixture centrifuged for 1 h at 22 000*g*, and the pellet washed several times with de-ionized water. The crude cell-walls were suspended in water (20 mL) and treated with a 4% boiling $NaDodSO_4$ solution. The suspension was kept overnight at room temperature. After centrifugation at 22 000*g*, as just described, the cell-wall preparation was purified by treatment with pepsine, trypsine, and Pronase. The final preparations were found, by electron microscopy, free of unbroken cells or cytoplasmic contaminations.

Polysaccharide preparation. — Dried cell-walls (~2 g) were warmed at 70° with NaOH (30 mL) for 15 h under N_2 with continuous stirring. The insoluble material was removed by centrifugation at 5000*g* for 15 min. The supernatant solution was made neutral with acetic acid, centrifuged, and dialyzed for 3 days against distilled water. The undialyzable fraction was concentrated under reduced pressure and the polysaccharides were precipitated from the solution by the dropwise addi-

tion of cold ethanol. The cell-wall polysaccharide was obtained in the 80% ethanol fraction. This fraction was further purified by ethanol precipitation and column chromatography.

Preparation of 6-deoxyhexitols. — 6-Deoxy-D-galactitol, -D-glucitol, and -D-mannitol were prepared by reduction with $NaBH_4$ of commercially available L-fucose, D-quinovose, and L-rhamnose. 6-Deoxy-D-allose, -L-gulose, -L-idose, and -L-talose were generous gifts of Dr. T. Reichstein. The corresponding 6-deoxyhexitols were obtained by $NaBH_4$ (5 mg/mL of water) reduction for 15 h at room temperature.

6-Deoxy-D-altritol was synthesized from D-altrose as follows. Commercially available D-altrose (50 mg, 0.277 mmol, Sigma) was treated with 1.2M methanolic HCl at 100° for 24 h. To the methyl D-altroside solution in anhydrous pyridine (3 mL), were added tetraiodomethane (0.42 mmol, Fluka) and triphenylphosphine (0.84 mmol, Fluka). The mixture was kept for 18 h at 25°, added to methanol (0.5 mL), and evaporated. Methyl 6-deoxy-6-iodo-D-altroside was purified on a silica gel column with elution by chloroform and 20:1 (v/v) chloroform–methanol to give 20 mg (0.07 mmol). Catalytic reduction with H_2 (7 kPa at room temperature during 3 h) in the presence of Raney nickel in methanol gave methyl 6-deoxy-D-altroside. T.l.c. on Silica gel G with the upper phase of solvent *D* showed this compound (detected with ethanolic H_2SO_4) to be pure. Hydrolysis with M HCl at 100° overnight afforded 6-deoxy-D-altrose, which was found to be pure by t.l.c. on Silica gel G with solvent *E* and detection by spraying an ethanolic solution of *p*-toluenesulfonic acid, followed by heating for a few min at 120°. This was reduced with $NaBH_4$, as described earlier, to give 6-deoxy-D-altritol (5 mg).

Alditols were further acetylated with 1:1 (v/v) acetic anhydride–pyridine for 15 min at 100°; the 6-deoxy-D-hexitol acetates were dissolved in chloroform and analyzed by g.l.c.

RESULTS AND DISCUSSION

The extraction of cell walls of *Nocardia asteroides* R 399 by alkaline treatment gave an alkali-soluble polysaccharide. After neutralization, the polysaccharide was recovered by fractional precipitation with ethanol. The cytoplasmic polysaccharides were obtained in the fractions having 0 to 60% of ethanol, and the cell-wall polysaccharide was precipitated by the addition of ethanol to 80%. This last fraction was further purified by repeated fractional precipitation, by gel permeation chromatography on Sephadex G-50 and G-25, and by affinity chromatography on Concanavalin A–Sepharose 4B. The main fraction was eluted immediately from Concanavalin A–Sepharose.

Analysis of the purified polysaccharide showed the presence of arabinose, galactose, glucose, and, in small proportion, an unknown compound that had a retention time smaller than that of arabinose on g.l.c. and R_F values near those of erythritol on paper chromatography: R_{Glc} 1.2 (*A*) and 1.3 (*B*). It was detected on

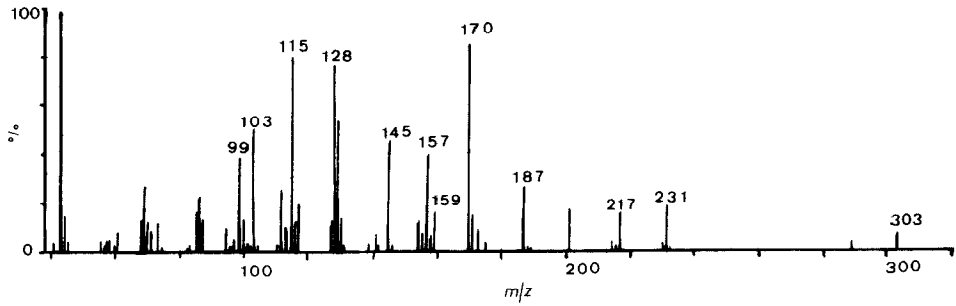
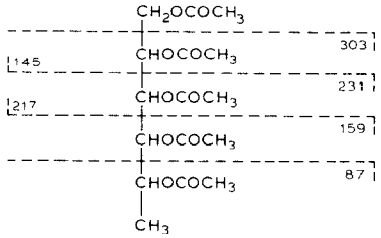


Fig. 1. Mass spectrum of the 1,2,3,4,5-penta-*O*-acetylhexitol isolated from nocardial polysaccharide.

paper by alkaline silver nitrate after sodium periodate oxidation. On t.l.c. on Silica gel G in solvents *B* and *C*, it moved faster than arabinose and was detected as a blue spot after the plate had been sprayed with the diphenylamine–aniline–phosphoric acid reagent. The unknown compound was prepared by chromatography on Whatman No. 1 paper in solvent *A*. It was reduced with sodium borodeuteride, acetylated, and analyzed by g.l.c.–m.s.; none of the fragments included a deuterium atom. When the compound was acetylated without reduction, its m.s. was identical with that of the foregoing one (Fig. 1). These results indicated the absence of an aldehyde group.

The mass spectrum of the acetylated derivative was identical with that of an authentic, acetylated 6-deoxyhexitol. Primary fragmentation peaks were observed at *m/z* 87, 145, 159, 217, and 231 (Scheme 1). Fragments at *m/z* 115, 129, 187, and



Scheme 1. Major m.s.-fragmentation pattern of 1,2,3,4,5-penta-*O*-acetyl-6-deoxyhexitols.

201 arose from ions at *m/z* 217, 231, 289, and 303, respectively, by loss of a ketene group and of an acetic acid molecule. The release of acetic acid from ions at *m/z* 217 and 159 gave fragments at *m/z* 157 and 99, respectively. Fragments having even mass number at *m/z* 128 and 170 are particularly important. Their presence in the spectra of fully acetylated 1-(or 6-)deoxyhexitols has been previously explained[12]. Thus, the structure of a 6-deoxyhexitol was assigned to the unknown compound. Its configuration was determined by comparison with authentic 6-deoxyalditols, after acetylation, by gas-liquid co-chromatography. None of the derivatives having the *galacto, gluco, manno, allo, gulo, ido,* and *talo* configuration had the same retention time as that of the fully acetylated unknown compound (Table I).

TABLE I

RETENTION TIMES IN G L C OF 1,2,3,4,5-PENTA-*O*-ACETYL-6-DEOXYHEXITOLS

Configuration	*Retention time (min)*[a]
allo	8.4
altro	6.4
galacto	9.8
gluco	12.2
gulo	12.4
ido	15.8
manno	8.6
talo	9.4
Unknown	6.4

[a]On a column of 3% ECNSS-M on Gas Chrom Q at a temperature of 180° isothermal.

6-Deoxy-D-altritol was prepared from commercially available D-altrose and acetylated. The retention time on g.l.c. of this derivative was identical with that of the acetylated unknown compound. Finally, the configuration of the polyol was determined by its optical rotation, $[\alpha]_D^{20}$ $-6 \pm 3°$ (*c* 0.02, water), the sign of the rotation being inverted in the presence of 5% ammonium molybdate. Similar results were obtained with synthetic 6-deoxy-D-altritol prepared from D-altrose. Thus, the unknown alditol was identified as 6-deoxy-D-altritol.

The cell-wall polysaccharide of *N. asteroides* R 399 is more complex than the arabinogalactan found in *Mycobacteria*. It contains, in addition, D-glucose and 6-deoxy-D-altritol. As far as we know, 6-deoxy-D-altritol had never been found in natural products up to date. The polyols found in bacteria are essentially glycerol and ribitol, which often are esterified with phosphate groups and involved in the structure of teichoic acids and in the linkage of peptidoglycan to teichoic acids. The only three 6-deoxyhexoses found in natural products belong to the L series: L-rhamnose, L-fucose, and 6-deoxy-L-talose; the latter compound is a component of some bacterial peptidoglycolipids, *i.e.*, mycosides C isolated from mycobacteria[13–17], such as *M. marianum, M. avium, M. smegmatis, M. scrofulaceum,* and *M. sp. 1217.* However, all the sugar components of the polysaccharide of *Nocardia* cell-walls have the D configuration.

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