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Note

Isolation of D-*threo*-pent-2-ulose from the lipopolysaccharide of *Pseudomonas diminuta* N.C.T.C. 8545

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Among the aerobic pseudomonads, the closely related species *Pseudomonas diminuta* and *Pseudomonas vesicularis* form an isolated subgroup, the inclusion of which in the genus *Pseudomonas* is increasingly suspect[1,2]. Chemotaxonomic grounds for the separate status of these species include the possession by their type strains of a unique range of polar lipids[3–5] and lipopolysaccharides in which lipid A is based on 2,3-diamino-2,3-dideoxy-D-glucose rather than 2-amino-2-deoxy-D-glucose[6]. The lipopolysaccharide from *P. diminuta* N.C.T.C. 8545 has other unusual features[7,8], to which can be added the presence of D-*threo*-pent-2-ulose, evidence for which is now presented.

As with most other bacterial lipopolysaccharides, mild hydrolysis of the lipopolysaccharide from *P. diminuta* with 1% acetic acid gives lipid A and water-soluble products which can be fractionated by chromatography on Sephadex. The polymeric fraction (D1) isolated by using Sephadex G-50 is the putative O-specific side-chain, and is a partially acetylated mannan having a disaccharide repeating-unit[8]. The products of lower molecular weight are not fully resolved on Sephadex G-50 or G-25, but include an oligomeric core-fraction (D2) containing D-glucose, an aldoheptose, and a 3-deoxy-2-octulosonic acid (but no hexosamine or phosphorus), and a monomeric fraction (D3) in which free 3-deoxy-2-octulosonic acid and inorganic orthophosphate have been identified[7].

During chromatography on Sephadex G-10 of the mixed D2 and D3 fractions from the last of five batches of lipopolysaccharide used for structural studies, separation of D3 into two subfractions was observed (Fig. 1*a*). Paper electrophoresis confirmed that the reactivity of subfraction D3a with phenol–sulphuric acid was attributable to free 3-deoxy-2-octulosonic acid, and also showed the presence of a little orthophosphate and traces of amino acids, but no neutral monosaccharides. Subfraction D3b gave a lemon-yellow colour (λ_{max} 477 nm) with phenol–sulphuric acid, and accounted for ~15% of the weight of the parent lipopolysaccharide. Paper electrophoresis of D3b at pH 5.3 showed the absence of phosphates and ninhydrin-

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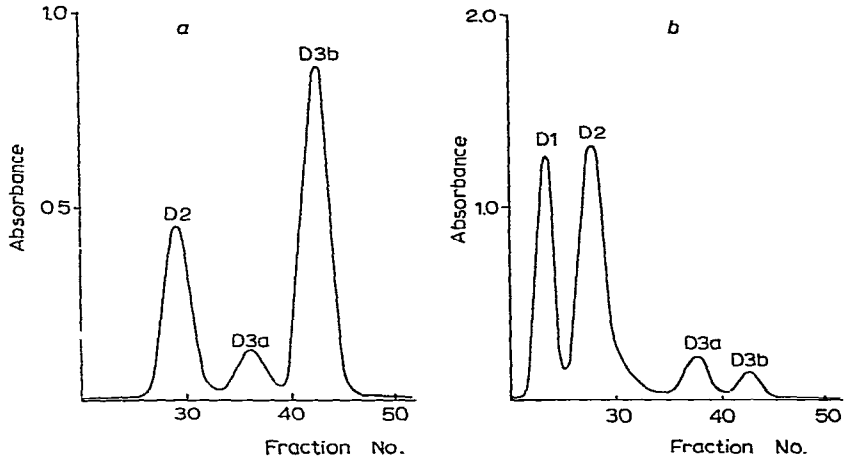


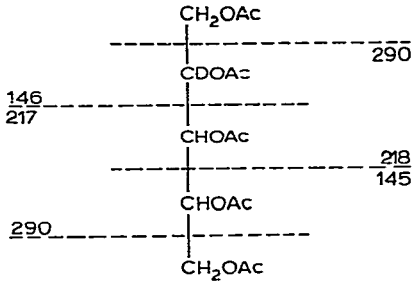
Fig. 1. Chromatography on Sephadex G-10 of water-soluble products obtained by mild hydrolysis of lipopolysaccharides from *P. diminuta* N.C.T.C. 8545 with 1% acetic acid at 100° for 2 h. Samples were applied to a column (82 × 1.5 cm) and eluted with aqueous pyridine–acetic acid (pH 5.4) at a flow rate of 5 ml.h^{-1}. Fractions (2 ml) were analysed for total carbohydrate by the phenol–sulphuric acid method, and then combined as indicated (D1, D2, D3a, and D3b). *a*, Products of low molecular weight from a pentulose-rich batch of lipopolysaccharide; *b*, total water-soluble products from a batch of lipopolysaccharide having a low content of pentulose.

reactive compounds, and the presence of a neutral component which strongly reduced alkaline silver nitrate and gave a reddish-brown colour with aniline hydrogenoxalate, but no reaction with the periodate–thiobarbituric acid reagent. The absence from D3b of the known neutral components of the lipopolysaccharide (glucose, mannose, and an aldoheptose) was confirmed by using the cysteine–sulphuric acid assay, g.l.c. for alditol acetates, and paper chromatography (p.c.). The results of the further studies described below showed that D3b was essentially pure D-*threo*-pent-2-ulose (D-xylulose).

In the diagnostic colour reactions with phenol–sulphuric acid, orcinol–hydrochloric acid–iron(III) chloride, and cysteine–carbazole–sulphuric acid, both D3b and the reference pentulose gave identical absorption spectra and (in the third reaction) an identical rate of colour production at room temperature. Identical mobilities and colour reactions were also found by p.c. (solvents *A*, *B*, and *C*), t.l.c. [solvents *F* (for silica layers impregnated with sodium dihydrogenphosphate), *I*, *J*, and *K* (all for layers of silica gel 60 F_{254})], and paper electrophoresis in borate buffer (pH 9.2). G.l.c. for alditol acetates from D3b gave two peaks having the retention times of the penta-acetates of arabinitol and xylitol and relative areas 3:4. Confirmatory identification of the pentitols was obtained by p.c. (solvents *A* and *D*), t.l.c. [solvents *E* (for cellulose sheets impregnated with tungstate at pH 6), *F* and *G* (for silica layers impregnated with sodium dihydrogenphosphate), and *H* (for layers of silica gel 60 F_{254})], paper electrophoresis in borate buffer (pH 9.2) and in calcium acetate–acetic acid, and h.p.l.c. of the *p*-nitrobenzoic esters.

The formation of both arabinitol and xylitol indicated that D3b contained a

threo-pent-2-ulose, and this inference was supported by g.l.c.–m.s. of the alditol acetates obtained after reduction with sodium borodeuteride. Deuterium labelling at C-2 was proved by the formation of major primary fragments with *m/z* 290, 218, 217, 146, and 145 (but not 289) (see formula **1**). Furthermore, the value of $[\alpha]_D$ for D3b was −28° (*c* 0.7, water; based on g.l.c. of the pentitol acetates), approaching that recorded (−33°) for D-*threo*-pent-2-ulose.



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The unexpected finding of the pentulose as a major component of this latest batch of lipopolysaccharide prompted a re-examination of three earlier batches. In previous studies of these batches, grades of Sephadex inappropriate for the resolution of subfractions of D3 had been used. Also, any pentulose would have been destroyed under the conditions of acid hydrolysis used to determine the monosaccharide compositions of the lipopolysaccharides and their fractions (this was confirmed by paper chromatography and g.l.c. analysis of the latest batch of lipopolysaccharide). The re-examination showed the pentulose to be present in all three batches of lipopolysaccharide, but in much smaller amounts. This is illustrated in Fig. 1*b*, which shows the elution profile for the total water-soluble products from a batch which gave relatively low yields of both D1 and D3b. Thus, D-*threo*-pent-2-ulose seems to be a consistent, but quantitatively variable, component of lipopolysaccharide preparations from *P. diminuta* N.C.T.C. 8545. It is unlikely to be an artefact of a retro-aldol condensation of the 3-deoxy-2-octulosonic acid also released on mild, acid hydrolysis: the pentulose and related pentoses have not been found in similar hydrolysates from other lipopolysaccharides containing a 3-deoxy-2-octulosonic acid.

Although the polymeric origin of the pentulose in *P. diminuta* is clear from the fact that it is present in material extracted from isolated cell-walls by aqueous phenol and is not lost during the dialysis stage of the preparation, it may not be an integral component of the lipopolysaccharide. Indeed, the observation that it may contribute at least 15% to the weight of the extract or be only a minor component indicates that it may form a separate polymer. However, it should be noted that at least 83% of the phenol–sulphuric acid reactivity of the pentulose-rich batch of lipopolysaccharide was associated with material excluded from Sephadex G-100 during chromatography in the presence of sodium dodecyl sulphate, and that the periodate–Schiff-reactive

material in this preparation migrated into the gel during electrophoresis in polyacrylamide in the presence of the surfactant[8]. Thus, it is possible that lipopolysaccharide preparations from *P. diminuta*, like those from some other bacteria[9], may contain species having chemically distinct side-chains. Further work is necessary to establish the architecture of the lipopolysaccharide and the significance of the isolated fractions.

Whatever the molecular origin of the pentulose in the cell envelope of *P. diminuta*, this appears to be the first time that the monosaccharide has been isolated from a bacterial polymer. On the other hand, 3-deoxy-D-*manno*-2-octulosonic acid is a "normal" component of bacterial lipopolysaccharides[9] and occurs in various other polysaccharides[10–13], while *N*-acetylneuraminic acid is encountered fairly commonly[14–16] and other glyculosonic acids occasionally[17]. Fructose appears to be the only neutral ketose previously isolated from bacterial polysaccharides. It is present in some capsular polysaccharides produced by strains of *Haemophilus influenzae* type e[18,19] and in the lipopolysaccharides of *Vibrio cholerae*[20–23]. Like the pentulose in the *P. diminuta* lipopolysaccharide, the fructose in *V. cholerae* lipopolysaccharides apparently occurs as acid-labile furanosyl residues. Although the fructose is not considered to be part of the O-specific chain[23–25], evidence for its location is inconclusive[22,26]. In contrast to the lipopolysaccharide of *P. diminuta*, the *Vibrio* products apparently lack a 3-deoxy-2-octulosonic acid.

EXPERIMENTAL

Isolation and fractionation of lipopolysaccharide. — Methods used for the extraction of lipopolysaccharide from cell walls of *P. diminuta* N.C.T.C. 8545, the degradation of the lipopolysaccharide with 1% acetic acid, and the fractionation of water-soluble products by chromatography on Sephadex G-50 and G-10 were those described previously[7,8].

Chromatographic and electrophoretic methods. — Paper chromatography and electrophoresis were performed using Whatman No. 1 paper. T.l.c. was performed with layers of silica gel 60 F_{254} (Merck), untreated or impregnated with sodium dihydrogenphosphate by immersion[27] or spraying[28], and with layers of cellulose (Merck) impregnated with sodium tungstate at pH 6 (ref. 29). The following solvents were used: *A*, the upper phase of ethyl acetate–pyridine–water (5:2:5); *B*, the upper phase of butan-1-ol–ethanol–water–0.88 ammonia (40:10:49:1); *C*, butan-1-ol–pyridine–water (10:3:3); *D*, butan-1-ol–0.75M boric acid[30] (85:10); *E*, butan-1-ol–ethanol–water[29] (40:11:19); *F*, propan-2-ol–acetone–0.1M lactic acid[27] (4:4:2); *G*, propan-2-ol–acetone–0.2M lactic acid[28] (6:3:1); *H*, ethanol–acetone–0.1M boric acid[31] (4:5:1); *I*, acetone–chloroform–water[32] (17:2:1); *J*, butan-1-ol–acetone–water[33] (4:5:1); *K*, propan-2-ol–ethyl acetate–water[33] (83:11:6). Buffer systems used for paper electrophoresis were aqueous pyridine–acetic acid[7] (pH 5.3), 0.05M sodium tetraborate[34] (pH 9.2), and 0.2M calcium acetate–0.2M acetic acid[35]. Detection was effected with ninhydrin, the Hanes/Isherwood reagent, the Warren reagents,

alkaline silver nitrate, aniline hydrogenoxalate, and the periodate–Schiff reagent (all described previously[7,8]), an aniline–diphenylamine reagent[32], the periodate–"tetrabase" reagents[31], naphthoresorcinol reagents[33,36], an anthrone reagent[36], and an orcinol reagent[36] followed by aniline hydrogenoxalate.

The *p*-nitrobenzoic esters of pentitols were prepared and analysed by h.p.l.c. as described by Schwarzenbach[37]. Alditol acetates were prepared by standard methods and were identified by g.l.c. at 190° on a column (1.6 m × 2 mm) packed with 3% of Silar 10C on Gas Chrom Q. G.l.c.–m.s. analysis was performed at the Physico-Chemical Measurements Unit, Harwell.

Other analytical methods. — The phenol–sulphuric acid reaction[38] was used to monitor chromatographic separations on Sephadex and for the comparison of fraction D3b with reference D-*threo*-pent-2-ulose (Sigma). The cysteine–carbazole reaction[39] and the orcinol reaction[40] were also used for the comparison of pentuloses, and the cysteine reaction[41] was used for the detection of aldoheptose. The specific rotation of the pentulose in fraction D3b was determined with a Bendix polarimeter (model 143A). For the detection of acid-stable monosaccharides, samples were hydrolysed for 2 h at 105° with 2M hydrochloric acid, and the hydrolysates were neutralised with Dowex 1 (HCO_3^-) resin.

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