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Note

Synthesis of oligosaccharide phosphates: fragments of the biosynthetic intermediates of *Salmonella* O-specific polysaccharides

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Biosynthesis of many bacterial polysaccharides proceeds through the intermediate assembly of an oligosaccharide repeating-unit attached to a polyprenyl pyrophosphate acceptor followed by polymerisation. In structurally closely related O-specific polysaccharides of the *Salmonella* species which belong to serological groups A, B, D, and E, the formation of polyprenyl pyrophosphate derivatives of rhamnosyl-galactose and mannosyl-rhamnosyl-galactose has been demonstrated[1–4]. Unequivocal proof of the structure of the oligosaccharide chains of these intermediates was not presented, but results of structural analysis of the O-specific polysaccharides[5,6] allowed the structure 3-*O*-α-L-rhamnopyranosyl-α-D-galactopyranose (α anomer of **1**) to be assigned to the disaccharide intermediate. The trisaccharide intermediates should be of different structure for different strains of *Salmonella*, namely, 3-*O*-(4-*O*-α-D-mannopyranosyl-α-L-rhamnopyranosyl)-α-D-galactopyranose (α anomer of **4**) in serogroups A, B, or D_1, and the β-D-mannopyranosyl isomer (α anomer of **7**) in serogroups D_2 and E.

The disaccharide **1**[7,8], the trisaccharides **4**[8,9] and **7**[8,10–12], and the respective acetates **2**, **5**, and **8** have been synthesised as part of a study of bacterial antigenic polysaccharides. We now report the conversion of these derivatives into the corresponding α-phosphates **3**, **6**, and **9** which are fragments of the biosynthetic intermediates.

RESULTS AND DISCUSSION

Oligosaccharide acetates can be converted into the glycosyl phosphates by fusion with anhydrous phosphoric acid[13]. Although the method gives better results when AcO-1,2 are *trans*, reaction also occurs with the corresponding *cis* compounds. For glycosyl acetates having AcO-2 equatorial, the stereochemistry of the products depends on the duration of the fusion reaction, because the initially formed β-phosphates are converted into the more-stable α anomers. Consequently, more-

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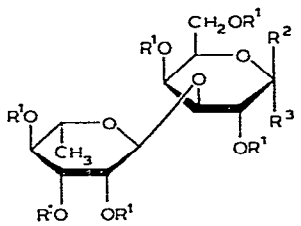
drastic conditions are necessary to obtain the α-phosphates, and some side-reactions may be expected.

The MacDonald reaction[13] has been used for preparation of *N*-acetylchondrobiosyl phosphate[14] and di-*N*-acetylchitobiosyl phosphate[15], the latter product unexpectedly being mainly the β anomer[16]. A facile synthesis of α-maltosyl and α-cellobiosyl phosphates has been described[17]. The stability of oligosaccharides which contain glycosidic bonds that are relatively labile towards acid hydrolysis (such as those containing α-L-rhamnosyl or β-D-mannopyranosyl residues) has not been investigated hitherto under the conditions of the MacDonald reaction.



1 $R^1 = H$, $R^2, R^3 = H,OH$

2a $R^1 = Ac$, $R^2 = H$, $R^3 = OAc$

2b $R^1 = Ac$, $R^2 = OAc$, $R^3 = H$

3 $R^1 = R^2 = H$, $R^3 = OPO_3H_2$

An equimolar mixture of the acetates **2a** and **2b**, prepared by treatment of **1** with acetic anhydride in pyridine[8], was used as the starting material for the synthesis of **3**. Treatment of **2ab** with 5 equiv of phosphoric acid under the standard conditions of the MacDonald procedure followed by deacetylation with lithium hydroxide gave, as shown by paper electrophoresis, mainly the disaccharide phosphate **3** and traces of a monosaccharide phosphate formed, presumably, by phosphorolysis of the rham-

TABLE I

DATA FOR DETERMINATION OF THE CONFIGURATION OF THE GLYCOSYL–PHOSPHATE LINKAGE IN OLIGOSACCHARIDE PHOSPHATES

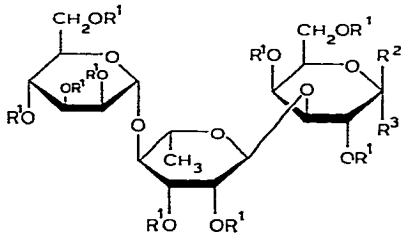
Glycosyl phosphate	$[M]_D$[a]	$[M']_D$[b]	$[M]_D - [M']_D$	$k^c \times 10^4$ *(sec^{-1})*
α-D-Galactopyranosyl phosphate	360[d]	144	216	9.2
β-D-Galactopyranosyl phosphate	100[d]	144	−44	12.3
3	208	18[e]	190	9.4
6	347	133[e]	214	8.2
9	175	−64[f]	239	9.6

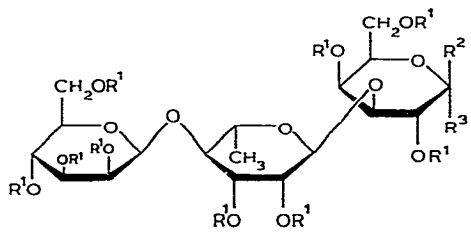
[a]Molar rotation of glycosyl phosphates in water. [b]Equilibrium molar rotation of the corresponding monosaccharides or oligosaccharides in water. [c]Observed first-order rate-constants for release of inorganic phosphate from glycosyl phosphates at 37° in 0.9M HCl with 1% of ammonium molybdate and 0.03% of Malachite Green. [d]Ref. 21. [e]Ref. 8. [f]Ref. 12.

nosyl-galactosyl linkage. The product **3** was purified by ion-exchange chromatography and isolated as the triethylammonium salt in a yield of 30%.

Analytical data are in accordance with the structure of **3**. Thus, all of the phosphate in **3** was acid-labile, and the ratios of phosphate, galactose, and rhamnose after acid hydrolysis were near-equimolar. Also, **3** consumed 2.03 mol of periodate per mol of phosphate and regenerated **1** on treatment with alkaline phosphomonoesterase. These data indicate the structure of **3**. The p.m.r. spectrum of **3** contained a signal at δ 5.39 with a splitting characteristic for α-D-galactopyranosyl phosphate, in addition to a signal for H-1 of the rhamnosyl residue. The high, positive optical rotation of **3** and the large positive difference between its $[M]_D$ value and that of equilibrated **1** (Table I) indicate the α-D configuration of the glycosyl-phosphate linkage.

A fast micro-method[18] for the identification of anomeric glycosyl phosphates is based on the determination of the rate of release of inorganic phosphate under conditions which allow continuous spectrophotometric monitoring of the reaction with a highly sensitive colorimetric reagent for inorganic phosphate[19,20]. Anomers of galactopyranosyl phosphate may be readily distinguished by the method (Table I), and **3** was hydrolysed at almost exactly the same rate as α-D-galactopyranosyl phosphate.





4 R^1 = H, R^2, R^3 = H, OH
5a R^1 = Ac, R^2 = H, R^3 = OAc
5b R^1 = Ac, R^2 = OAc, R^3 = H
6 R^1 = R^2 = H, R^3 = OPO_3H_2

7 R^1 = H, R^2, R^3 = H, OH
8a R^1 = Ac, R^2 = H, R^3 = OAc
8b R^1 = Ac R^2 = OAc, R^3 = H
9 R^1 = R^2 = H, R^3 = OPO_3H_2

A similar approach was used successfully for the preparation of trisaccharide phosphates **6** and **9**. The triethylammonium salt of **6** was isolated (40%) after fusion of **5ab**[9] with phosphoric acid followed by deacetylation and ion-exchange chromatography. In this way, **8ab**[12] was converted into the triethylammonium salt of **9** (43%).

The structures of **6** and **9** were established by the methods described above for **3**. Optical rotation data and the rate of release of inorganic phosphate in acid media (Table I) indicate the glycosyl-phosphate linkages in **6** and **9** to be α-D.

Thus, the MacDonald procedure is useful for the synthesis of the stable α anomers of glycosyl phosphates of oligosaccharides containing 6-deoxyhexosyl residues. The use of the oligosaccharide phosphates **3**, **6**, and **9** for the preparation of polyprenyl pyrophosphate derivatives will be described elsewhere.

EXPERIMENTAL

General methods. — Evaporations were performed *in vacuo* at <30° (bath). Optical rotations were determined with a Perkin–Elmer Model 141 polarimeter and $[M]_D$ values were calculated from the results of phosphorus determinations.

Paper chromatography (p.c.) and paper electrophoresis were performed on Filtrak FN-16 paper; phosphates were detected with the Hanes–Isherwood spray[22]. 0.05M Triethylammonium hydrogencarbonate (pH 8.5) was used for electrophoresis. P.c. was performed with *A*, 1-butanol–pyridine–water (6:4:3); *B*, 1-butanol–acetic acid–water (5:2:3); *C*, methanol–formic acid–water (16:3:1); and *D*, methanol–ammonia–water (6:1:3).

Monosaccharide composition of oligosaccharide phosphates was determined, after hydrolysis with 2M HCl for 2 h at 100°, by ion-exchange chromatography on a column (25 × 0.6 cm) of Dowex DA-X4 resin [elution with 0.5M sodium borate buffer (pH 8.5) at 55° and 60 ml/h, using a Technicon SC II system].

Periodate oxidation was performed in M acetate buffer (pH 4.0); the procedure of Avigad[23] was used for determination of periodate.

Phosphate was determined by a modification of the method of Hess and Derr[20]. The colour reagent was prepared by mixing 0.045% aqueous Malachite Green (60 ml) and ammonium molybdate solution (20 ml) prepared according to the original procedure (20 ml), but did not contain any detergent. For determination of acid-labile phosphate, a sample was treated with M sulphuric acid (0.2 ml) for 30 min at 100°, the colour reagent (2 ml) was added, and the absorbance at 660 nm was measured against a blank that contained water instead of the sample. For the determination of total phosphate, an aliquot of the solution was heated with 57% perchloric acid (0.2 ml) for 15 min at 200° and then treated as described above. The calibration curve was linear below 1.8 μg of phosphorus per sample. To measure the rate of release of inorganic phosphate from glycosyl phosphates, an aliquot (0.3–0.5 μg of phosphorus) was added to a thermostated (37°) spectrophotometric cell (1-cm path-length) which contained 2 ml of the colour reagent. The change of absorbance at 660 nm was measured automatically with a Unicam SP-8000 spectrophotometer and rate constants were calculated by plotting $\ln(A_\infty - A_t)$ *versus* time. For enzymic dephosphorylation, glycosyl phosphates were incubated overnight at room temperature with alkaline phosphatase (Serva) in 0.2M glycine buffer (pH 8.8) which contained traces of Mg^{2+}.

3-O-α-L-Rhamnopyranosyl-α-D-galactopyranosyl phosphate (**3**). — A solution of 3-*O*-α-L-rhamnopyranosyl-α,β-D-galactopyranose hexa-acetate (35 mg, prepared by treatment of **1** with acetic anhydride–pyridine) in dry benzene was freeze-dried. Anhydrous phosphoric acid (25 mg) was added to the residue, the mixture was kept *in vacuo* for 2 h at 56–60° and then cooled, cold M lithium hydroxide (7 ml) was added, and the resulting alkaline solution was left for 18 h at room temperature. The precipitate was collected, and washed with 0.1M lithium hydroxide. The combined filtrate and washings were treated with Dowex 50W-X8 (H^+) resin to pH 8, diluted with water to 100 ml, and applied to a column (1.2 × 25 cm) of Dowex 1-X8 (HCO_3^-)

resin. The column was washed with water (30 ml) and eluted (60 ml/h) with a linear gradient (0→0.3M) of triethylammonium hydrogencarbonate (300 ml). Fractions (10 ml) were assayed for acid-labile phosphate. Fractions 12–20 (centre of the peak eluted at 0.14M salt) were combined and concentrated, and the triethylammonium hydrogencarbonate was removed by repeated distillation of water and then ethanol from the residue. Chromatographically homogeneous triethylammonium salt of **3** was obtained (17 μmol, 30%), $[\alpha]_D$ +34° (*c* 0.12, water), $M_{Glc\text{-}1\text{-}P}$ 0.85 (electrophoresis), $R_{Glc\text{-}1\text{-}P}$ 0.70 (solvent *B*), 1.25 (solvent *C*), and 1.07 (solvent *D*). Treatment of **3** with alkaline phosphatase gave **1**, R_{Gal} 0.85 (solvent *A*) The ratios of acid-labile phosphate, galactose, and rhamnose were 1.00:1.07:1.15. Consumption of periodate (mol per mol of phosphate): 1.28 (10 min), 1.88 (30 min), 1.95 (1 h), and 2.03 (2 h). N.m.r. data (D_2O, internal 1,4-dioxane): δ 5.39 (dd, 1 H, $J_{1,2}$ 2.5, $J_{1,P}$ 7.5 Hz, H-1) and 4.95 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'). See also Table I.

3-O-(4-O-α-D-Mannopyranosyl-α-L-rhamnopyranosyl)-α-D-galactopyranosyl phosphate (**6**). — Acetylated 3-*O*-(4-*O*-α-D-mannopyranosyl-α-L-rhamnopyranosyl)-α,β-D-galactopyranose (**5ab**, 20 mg) and anhydrous phosphoric acid (30 mg) were treated as described above. A column (1.0 × 18 cm) of Dowex 1-X8 resin was used for purification, and elution with 0→0.33M triethylammonium hydrogencarbonate (400 ml) gave the triethylammonium salt of **6** (8 μmol, 40%). $[\alpha]_D$ +23° (*c* 0.15, water), $M_{Glc\text{-}1\text{-}P}$ 0.76, $R_{Glc\text{-}1\text{-}P}$ 0.22 (solvent *B*). The ratios of acid-labile phosphate, galactose, rhamnose, and mannose were 1.00:1.08:0.97:1.05. See also Table I.

3-O-(4-O-β-D-Mannopyranosyl-α-L-rhamnopyranosyl)-α-D-galactopyranosyl phosphate (**9**). — Acetylated 3-*O*-(4-*O*-β-D-mannopyranosyl-α-L-rhamnopyranosyl)-α,β-D-galactopyranose (**8ab**, 30 mg) and anhydrous phosphoric acid (50 mg) were treated as described above. In ion-exchange chromatography (performed as described for **6**), the peak eluted with 0.15M salt was the triethylammonium salt of **9** (12 μmol, 43%), $[\alpha]_D$ +45° (*c* 0.15, water), $M_{Glc\text{-}1\text{-}P}$ 0.78, $R_{Glc\text{-}1\text{-}P}$ 0.27 (solvent *B*). Treatment of **9** with alkaline phosphatase gave the trisaccharide **7**, R_{Gal} 0.44 (solvent *A*). The ratios of acid-labile phosphate, galactose, rhamnose, and mannose were 1.00:0.92:1.01:0.94. The consumption of periodate in 2 h was 3.05 mol per mol of phosphate. See also Table I.

REFERENCES

1 A. Wright, M. Dankert, and P W Robbins, *Proc. Natl. Acad Sci. U S A*, 54 (1965) 235–241.
2 M. Dankert, A. Wright, W. S. Kelley, and P W. Robbins, *Arch Biochem. Biophys.*, 116 (1966) 425–435.
3 M. J. Osborn, N. A. Cynkin, J. M. Gilbert, L. Muller, and M. Singh, *Methods Enzymol*, 28 (1972) 583–601
4 V N. Shibaev, T. N Druzhinina, A. N. Popova, N. K Kochetkov, S Sh Rozhnova, and V. A Kilesso, *Bioorg. Khim.*, 5 (1979) 1071–1082.
5 O. Lüderitz, O. Westphal, A. M. Staub, and H. Nikaido, in G Weinbaum, S. Kadis, and S. J. Aje (Eds.), *Microbial Toxins*, Vol. 4, Academic Press, New York, 1971, pp 145–233
6 H Kita and H. Nikaido, *J. Bacteriol.*, 113 (1973) 672–679.
7 B. A. Dmitriev, A. Ya. Chernyak, and N. E Bayramova, *Izv Akad. Nauk SSSR, Ser Khim*, (1975) 142–148.

8 V. I. BETANELI, M. V. OVCHINNIKOV, L. V. BACKINOWSKY, AND N. K. KOCHETKOV, *Carbohydr. Res.*, 84 (1980) 211–224.
9 N. K. KOCHETKOV, E. M. KLIMOV, AND V. I. TORGOV, *Izv. Akad Nauk SSSR, Ser Khim*, (1976) 165–167.
10 N. K. KOCHETKOV, B. A. DMITRIEV, O. S. CHIZHOV, E. M. KLIMOV, N. N. MALYSHEVA, V. I. TORGOV, A. YA. CHERNYAK, AND N. E. BAYRAMOVA, *Izv. Akad. Nauk SSSR, Ser. Khim*, (1974) 1386–1392.
11 N. K. KOCHETKOV, B. A. DMITRIEV, N. N. MALYSHEVA, A. YA. CHERNYAK, E. M. KLIMOV, N. E. BAYRAMOVA, AND V. I. TORGOV, *Carbohydr. Res.*, 45 (1975) 283–290.
12 N. K. KOCHETKOV, B. A. DMITRIEV, AND A. V. NIKOLAEV, *Izv. Akad. Nauk SSSR, Ser. Khim*, (1977) 2578–2581.
13 D. L. MACDONALD, *Methods Carbohydr. Chem.*, 6 (1972) 389–392
14 A. H OLAVESEN AND E. A. DAVIDSON, *J Biol. Chem*, 240 (1965) 992–996.
15 J. H. WEDGWOOD, C. D. WARREN, R. W. JEANLOZ, AND J. L. STROMINGER, *Proc. Natl. Acad. Sci. U.S A*, 71 (1974) 5022–5026.
16 C. D. WARREN, A HERSCOVICS, AND R. W. JEANLOZ, *Carbohydr. Res.*, 61 (1978) 181–196.
17 V. N. SHIBAEV, YU. YU. KUSOV, M. F. TROITZKY, AND N. K. KOCHETKOV, *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1974) 182–184
18 L. L. DANILOV, N S. UTKINA, AND V. N. SHIBAEV, *Bioorg. Khim*, 6 (1980) 780–782.
19 K. ITAYA AND M VI, *Clin. Chim. Acta*, 14 (1966) 361–366
20 H. H. HESS AND J. E. DERR, *Anal. Biochem.*, 63 (1975) 607–613.
21 E. W. PUTMAN AND W. Z. HASSID, *J. Am. Chem. Soc.*, 79 (1957) 5057–5060.
22 C. S HANES AND F. A. ISHERWOOD, *Nature (London)*, 164 (1949) 1107–1109
23 G. AVIGAD, *Carbohydr. Res.*, 11 (1969) 119–123.