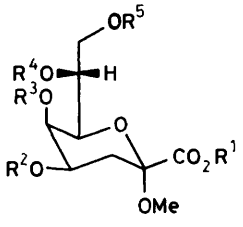
Phosphorylated Sugars. Part 24.[1] Methyl 3-Deoxy-α-D-*manno*-oct-2-ulopyranosidonic Acid 4-(Dihydrogen Phosphate): Synthesis, Stability in Acidic Medium, and Colorimetric Estimation

S. Robert Sarfati, Annick Le Dur, and Ladislas Szabó *
Equipe 'Endotoxines' (U.A.1116) du Centre National de la Recherche Scientifique, Institut de Biochimie, Université de Paris-Sud, 91405 Orsay, France

The synthesis of the title compound was accomplished in three steps starting from methyl (methyl 5-*O*-benzyl-3-deoxy-α-D-*manno*-oct-2-ulopyranosid)onate. When treated at 100 °C with acetate buffer of pH 4.5, the phosphorylated aldulosonic acid released inorganic phosphate more rapidly than upon exposure, at the same temperature, to 0.25M-HCl. The semicarbazide and thiobarbiturate assays could not be used to determine the amount of 3-deoxyoct-2-ulosonic acid present in the complex mixture of products formed in the latter conditions, despite an apparently univocal response in the former.

In connection with the structural analysis of the *Bordetella pertussis* endotoxin pursued in this laboratory, it was found necessary to examine, on the one hand, the behaviour of methyl 3-deoxy-α-D-*manno*-oct-2-ulopyranosidonic acid 4-phosphate (**4**) [as its diammonium salt] under such conditions of acidity as are employed for the cleavage of the hydrophilic and hydrophobic regions of lipopolysaccharides and, on the other hand, the evolution, during the acid treatment, of its response in the periodate–thiobarbiturate[2] and semicarbazide[3] assays which are widely used for the quantitative estimation of 3-deoxy-D-*manno*-oct-2-ulosonic acid in endotoxins.

The required phosphorylated methyl glycoside (**4**) was synthesized starting from methyl (methyl 5-*O*-benzyl-3-deoxy-α-D-*manno*-oct-2-ulopyranosid)onate[4] (**1**): the vicinal diol of this glycoside was transformed into the isopropylidene acetal (**2**) by treatment with 2,2-dimethoxypropane (DMP)–toluene-*p*-sulphonic acid (PTSA), whereafter the remaining hydroxy group was esterified with diphenyl phosphorochloridate in benzene solution in the presence of pyridine and 4-dimethylaminopyridine (DMAP). The resulting phosphotriester (**3**) was deprotected by hydrogenolysis; the benzyl group was removed first (Pd–C catalyst) followed by the phenyl groups (Adams' Pt catalyst). The acidity developed during the latter reaction was sufficient to remove the isopropylidene group, but did not affect the glycosidic bond. After saponification of the carboxylic ester with 0.1M-NaOH, exchange of Na^+ against NH_4^+, and purification of the product by column chromatography, first on silica gel and then on Sephadex G10, the diammonium salt of methyl 3-deoxy-α-D-*manno*-oct-2-ulopyranosidonic acid 4-phosphate (**4**) was isolated as a solid and characterized by its elemental analysis and its 400 MHz ^1H n.m.r. spectrum.



(**1**) R^1 = Me, R^2 = R^4 = R^5 = H, R^3 = CH_2Ph
(**2**) R^1 = Me, R^2 = H, R^3 = CH_2Ph, R^4R^5 = CMe_2
(**3**) R^1 = Me, R^2 = $P(O)(OPh)_2$, R^3 = CH_2Ph, R^4R^5 = CMe_2
(**4**) R^1 = R^3 = R^4 = R^5 = H, R^2 = PO_3H_2

Acidic solutions used for hydrolysis were M-HCl (cleavage of glycosidic bonds of neutral sugars for identification of glycoses), 0.25M-HCl (used for the release of Polysaccharide-2 from the *B. pertussis* endotoxin[5]), and 10mM-acetate buffer of pH 4.5 (specific cleavage of the glycosidic bond of 3-deoxyald-2-ulosonic acids[6]) all at 100 °C. As has been observed for other 3-deoxyald-2-ulosonic acid 4-phosphates,[7] the rate of phosphate-release from compound (**4**) was considerably slower at high acidities than at pH 4.5 (Figure 1); in the latter case the rate-determining step was very probably the hydrolysis of the glycosidic bond, and phosphate-release proceeded by elimination rather than by hydrolysis.[7] These results suggested that, if present, conservation of the structure of 3-deoxy-D-*manno*-oct-2-ulosonic acid 4-phosphate during acid-catalysed hydrolysis of endotoxins was more likely to occur at relatively low pH values. Accordingly, the phosphorylated glycoside (**4**) was treated with 0.25M-HCl at 100 °C and the kinetics of inorganic phosphate-release and the evolution of the glycoside's response in the semicarbazide[3] (to estimate the amount of 'reducing' 3-deoxyoct-2-ulosonic acid 4-phosphate present) and the thiobarbiturate[2] assays (to determine 'reducing' dephosphorylated 3-deoxyoct-2-ulosonic acid) were measured simultaneously

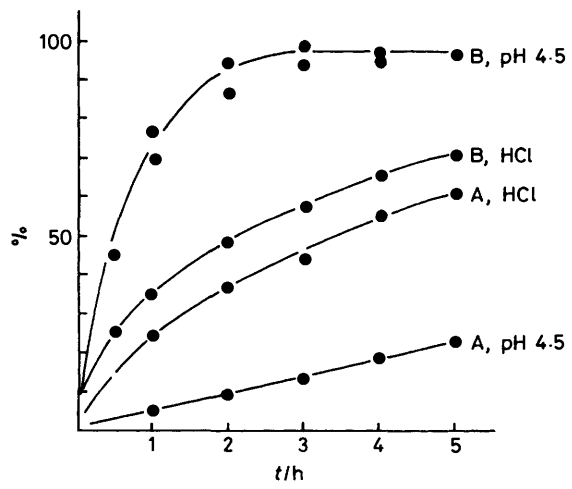


Figure 1. Kinetics of the appearance of inorganic phosphate upon treatment, at 100 °C, of 3-deoxy-D-*manno*-oct-2-ulosonic acid 5-(dihydrogen phosphate) (A), and of methyl 3-deoxy-α-D-*manno*-oct-2-ulopyranosidonic acid 4-(dihydrogen phosphate) (B) with M-HCl and 10mM-sodium acetate buffer (pH 4.5)

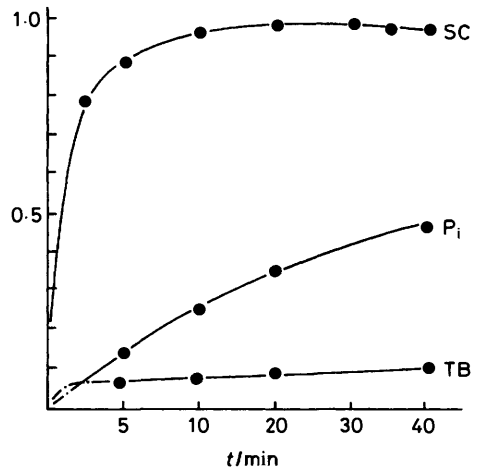


Figure 2. Kinetics of the appearance of compounds reacting in the thiobarbiturate (TB) and semicarbazide (SC) assays, and of inorganic phosphate (P_i) during treatment of methyl 3-deoxy-α-D-*manno*-oct-2-ulopyranosidonic acid 4-(dihydrogen phosphate) with 0.25M-HCl at 100 °C. Absorbances measured in the thiobarbiturate and semicarbazide tests are expressed as μmol of 3-deoxy-D-*manno*-oct-2-ulosonic acid (KDO)/μmol of methyl 3-deoxy-α-D-*manno*-oct-2-ulopyranosidonic acid 4-(dihydrogen phosphate) in the sample, the ammonium salt of KDO being used as reference. The amount of inorganic phosphate measured is expressed as μmol of inorganic phosphate found/μmol of total phosphate present in the sample

(Figure 2). An attempt to measure the kinetics of cleavage of the glycosidic bond of compound (**4**) by the semicarbazide reaction failed: indeed, despite its apparent simplicity (Figure 2, SC) suggesting that it was univocal and led, in a fast reaction, to the exclusive formation of an α-keto acid that was stable under the conditions employed, kinetics of phosphate-release revealed (Figure 2, P_i) that not only was only 20% of the total phosphate content released when cleavage of the glycosidic bond appeared to be quantitative (20 min), but also that formation of inorganic phosphate continued at about the same rate during the next 40 min, during which time the apparent α-keto acid content remained constant. Our conjecture that, under the conditions employed, hydrolysis of the glycosidic bond of the methyl aldulopyranosidonic acid (**4**) was followed by other reactions that were not revealed by the semicarbazide assay was confirmed by the evolution of the periodate–thiobarbiturate reaction. This test is based on the condensation of the fragment $OHCCH_2C(O)CO_2H$, produced from 3-deoxyald-2-ulosonic acids upon treatment with periodate, with 2-thiobarbituric acid to afford a red dye. 4-*O*-Substituted 3-deoxyald-2-ulosonic acids cannot yield this fragment directly when treated with periodate: the very weak response (3—5% of that given by an equivalent amount of unsubstituted 3-dexoyald-2-ulosonic acid) is due to ill-defined secondary reactions.[8] As seen from Figure 2, TB the intensity of the response of compound (**4**) in the thiobarbiturate test is negligible: were it due to free 3-deoxyoct-2-ulosonic acid, it would indicate that about 10—12% of the total amount of 3-deoxyald-2-ulosonic acid present in the sample was continuously present as such during the acid treatment. However, upon paper electrophoresis (pH 5, 200mM-pyridinium acetate; 50 V cm^{-1}) no free 3-deoxyoct-2-ulosonate could be detected by the periodate–thiobarbiturate spray[9] in the reaction mixture. Analysis by paper electrophoresis (under the aforementioned conditions) of the products formed after 15 min of hydrolysis, *i.e.* when, according to the semicarbazide test, the phosphorylated α-keto acid appeared to be the main product present in the reaction mixture, at least four negatively charged compounds could be detected by the periodate–silver nitrate reagent,[10] two of which were phosphate esters.[11]

These reactions have important consequences for the structural analysis of endotoxins. Indeed, a unit of 3-deoxy-D-*manno*-oct-2-ulosonic acid ('KDO'), substituted at O-5 by a heptose, is a common structural feature of most lipopolysaccharides analysed so far, and quite often, particularly in enterobacterial endotoxins, the sequence [sugar chain]–heptose–heptose–1,5-KDO is known to be present. It is also established that in some endotoxins a phosphorylated KDO is one of the constituents. If in the sequence [sugar chain]–heptose–KDO–KDO the KDO unit substituted by heptose at position 5 also carries a phosphate substituent on O-4, acid hydrolysis will transform this KDO unit; should the new structure remain attached to the sugar chain, it is likely to lead to confusing responses in the colorimetric assays widely used for the estimation of 3-deoxy-D-*manno*-oct-2-ulosonic acid. If, because of conditions of pH and temperature, it is detached from the sugar chain, a heptose will appear as the terminal unit of the latter. Finally, if the structure heptose–1,5-KDO–1,5-[KDO 4-phosphate] is present, the phosphorylated KDO will be transformed into a 5-substituted furoic acid and, being separated from the sugar chain, remain undiscovered.

Experimental

General methods were those used previously.[1]

Kinetics and Analytical Procedures.—Six samples (78.40 μg each) of compound (**4**) in 0.25M-HCl (200 μl) were placed in sealed tubes and kept at 100 °C for 0, 5, 10, 20, 30, and 50 min, respectively. Aliquots (50 μl) were used to estimate 3-deoxyoct-2-ulosonic acid by the periodate–thiobarbiturate reaction according to ref. 2. Inorganic phosphate was estimated[12] in aliquots of 45 μl; appropriate controls were run to establish that the amount of HCl present in the sample did not invalidate the results. For the estimation of α-keto acids, M-NaOH (15 μl) was added to aliquots (60 μl) of the hydrolysates, followed by water (125 μl) and by the semicarbazide reagent[3] (1 ml). The mixtures were incubated at 37 °C for 1 h and the absorbance was read at 250 nm.

Methyl (*Methyl* 5-O-*Benzyl*-3-*deoxy*-7,8-O-*isopropylidene*-α-D-manno-*oct*-2-*ulopyranosid*)*onate* (**2**).—Methyl (methyl 5-*O*-benzyl-3-deoxy-α-D-*manno*-oct-2-ulopyranosid)onate (**1**)[4] (1.5 g, 0.421 mmol), dried to constant weight *in vacuo*, was dissolved in a stirred mixture of dry acetonitrile (15 ml), dry *N*,*N*-dimethylformamide (2 ml), DMP (5 ml), and PTSA (50 mg) kept at 40 °C. After 30 min, t.l.c. [methanol–chloroform (1:9) or toluene–ethyl acetate–methanol (7:2:2)] showed the reaction to be complete. The cooled mixture was diluted with dichloromethane, neutralized with solid $NaHCO_3$, and filtered; the filtrate was washed sequentially with ice–water, cold, saturated aq. $NaHCO_3$, and ice–water, and dried (Na_2SO_4). After removal of the solvent, the residue was chromatographed on a column (2 × 100 cm) of silica gel [Merck 60; ethyl acetate–hexane (1:1)] to give the *title compound* as an oil (1.19 g, 72%) (Found: C, 58.4; H, 7.1. $C_{20}H_{26}O_8 \cdot H_2O$ requires C, 58.25; H, 6.80%); $[\alpha]_D^{20}$ +64.5° (*c* 1.25 in $CHCl_3$); δ_H(400 MHz; $CDCl_3$) 1.38 (3 H, s, CMe), 1.45 (3 H, s, CMe), 1.84 (1 H, t, $J_{3ax,3eq} = J_{3ax,4} = 12.5$ Hz, 3-H_{ax}), 2.10 (1 H, dd, $J_{3eq,3ax}$ 12.5, $J_{3eq,4}$ 5.2 Hz, 3-H_{eq}), 3.21 (3 H, s, CO_2Me), 3.75 (3 H, s, 2-OMe), 4.7 and 4.9 (together 2 H, 2 d, J_{gem} 12 Hz, CH_2Ph), and 7.33 (5 H, Ph); δ_C (20 MHz; $CDCl_3$) 25.1 and 26.7 (CMe_2), 35.6 (C-3), 50.9 and 52.2 (Me), 65.7 (C-4), 67.8 (C-8), 73.1 or 73.7 (C-6 or C-7), 74.8 (C-5 and $PhCH_2$), 99.1 (C-2), 109.0 (CMe_2), 127.6—128.3 (aromatic CH), 138.4 (quaternary aromatic C), and 168.2 (C-1).

Methyl (*Methyl* 5-O-*Benzyl*-3-*deoxy*-7,8-O-*isopropylidene*-α-D-manno-*oct*-2-*ulopyranosid*)*onate* 4-(*Diphenyl Phosphate*) (**3**).—Anhydrous pyridine (2 ml) and DMAP (75 mg) were added to a stirred solution of compound (**2**) (0.8 g, 2 mmol) in dry benzene (5 ml) containing diphenyl phosphorochloridate (1 g, 4 mmol) and the progress of the reaction was monitored by t.l.c. [ethyl acetate–hexane (1:1)]. When all of the starting material had disappeared (1 h), cold water (1 ml) was added to the cooled solution. After 1 h the reaction mixture was diluted with benzene (25 ml) and the solution was washed, first with ice-cold, saturated, aq. $NaHCO_3$ and then water; it was then dried (Na_2SO_4). Solvents were removed, and the residue was purified by column chromatography [2 × 10 cm; silica gel; ethyl acetate–hexane (1:1)] to give the oily *diphenyl phosphate* (**3**) (0.812 g, 67%) (Found: C, 61.0; H, 5.95. $C_{32}H_{37}O_{11}P$ requires C, 61.1; H, 5.9%); $[\alpha]_D^{20}$ +42.96° (*c* 1.16 in $CHCl_3$); δ_H(90 MHz; $CDCl_3$) 1.33 (3 H, s, CMe), 1.45 (3 H, s, CMe), 2.4 (2 H, d, *J* 8 Hz, 3-H_2), 3.23 (3 H, s, CO_2Me), 3.75 (3 H, s, 2-OMe), 4.65 (2 H, d, *J* 3 Hz, CH_2Ph), and 7.26—7.33 (15 H, Ph); δ_C (20 MHz; $CDCl_3$) 25.3 and 26.9 (CMe_2), 33.7 (C-3), 51.2 and 52.2 (Me), 67.4 (C-8), 99.1 (C-2), 109.3 (CMe_2), 120—120.3, 125.6, 138.6, 150.43—150.82 (aromatics), and 165.7 (C-1).

Diammonium (*Methyl* 3-*Deoxy*-α-D-manno-*oct*-2-*ulopyranosid*)*onate* 4-(*Dihydrogen Phosphate*) (**4**).—Pd–C (10% Pd; 0.1 g) catalyst was added to a stirred solution of compound (**3**) (0.566 g, 0.9 mol) in anhydrous ethanol (10 ml) and the suspension was treated with H_2 at atmospheric pressure and 22 °C; t.l.c. [ethyl acetate–hexane (1:1)] showed the reaction to be complete 1 h later. PtO_2 (Adams' catalyst, 0.1 g) was then added and hydrogenation was continued for 20 h; after addition of more PtO_2 (50 mg) and further hydrogenation (3 h), t.l.c. [ethyl acetate–ethanol–water (4:2:1)] and paper electrophoresis (0.2M-pyridinium acetate; pH 5; 40 V cm^{-1}; visualization by phosphate spray[11]) showed the reaction to be complete. After removal of the catalyst, the filtrate was neutralized with ammonia and the solvents were removed. No aromatic or isopropylidene methyl hydrogens were present in the ^1H n.m.r. spectrum of the resulting syrup (0.265 g, 78%), which was dissolved in 0.1M-NaOH in 50% aq. methanol (5 ml) and the mixture was stirred for 4 h at room temperature. Paper electrophoresis (conditions as above) then showed that the saponification was complete. The solution was passed through a small column of Amberlite IRN 77 (NH_4^+) resin, the eluate and aqueous washings were pooled, and the solvent was evaporated off. The residue was purified by chromatography on a silica gel column [2 × 10 cm; propan-1-ol–conc. ammonia (*d* 0.88)–water (11:4.5:4.5)]; pooled fractions were concentrated and passed through a column (1.5 × 60 cm) of Sephadex G10 gel in (1:1 000) ammonia–water. Fractions (4 ml) containing the phosphorylated glycoside (**4**) were pooled, the volume was reduced to *ca.* 1 ml, and acetone was added to precipitate the *title compound*, which was recovered by centrifugation (0.147 g, 42%) (Found: C, 28.1; H, 6.6; N, 7.3. $C_9H_{23}N_2O_{11}P{\cdot}H_2O$ requires C, 28.1; H, 6.5; N, 7.3%); $[\alpha]_D^{20}$ +29.5° (*c* 1.13 in water); δ_H(400 MHz; D_2O), 1.66 (1 H, dd, J_{gem} 13 Hz, 3-H_{ax}), 1.9 (1 H, dd, J_{gem} 13, $J_{3eq,4}$ 5 Hz, 3-H_{eq}), 2.9 (3 H, s, 2-OMe), 3.34—3.46 (2 H, m, 6-and 7-H), 3.68—3.77 (2 H, m, 8-H_2), 3.95 (1 H, d, *J* 2.75 Hz, 5-H), and 4.17—4.27 (1 H, m, 4-H).

References

1 Part 23, F. Trigalo and L. Szabó, *J. Chem. Soc., Perkin Trans. 1*, 1982, 1733.
2 Y. D. Kharkanis, J. Y. Zeltner, J. J. Jackson, and D. J. Carlo, *Anal. Biochem.*, 1978, **85**, 595.
3 J. MacGee and M. Doudoroff, *J. Biol. Chem.*, 1954, **210**, 617.
4 D. Charon and L. Szabó, *J. Chem. Soc., Perkin Trans. 1*, 1980, 1971.
5 A. Le Dur, M. Caroff, R. Chaby, and L. Szabó, *Eur. J. Biochem.*, 1978, **84**, 579.
6 M. R. Rosner, H. G. Khorana, and A. C. Satterthwait, *J. Biol. Chem.*, 1979, **254**, 5918.
7 A. Danan, M. Mondange, S. R. Sarfati, and P. Szabó, *J. Chem. Soc., Perkin Trans. 1*, 1982, 1275.
8 D. Charon and L. Szabó, *Carbohydr. Res.*, 1974, **34**, 271.
9 L. Warren, *Nature (London)*, 1960, **186**, 237.
10 W. E. Trevelyan, D. P. Proctor, and J. S. Harrison, *Nature (London)*, 1950, **166**, 444.
11 B. R. Bochner, D. M. Maron, and B. N. Ames, *Anal. Biochem.*, 1981, **117**, 81.
12 R. Parvin and R. A. Smith, *Anal. Biochem.*, 1969, **27**, 65.

Received 31*st March* 1987; *Paper* 7/566