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Dehydrogenation of the Phosphonate Analogue of Glucose 6-Phosphate by Glucose 6-Phosphate Dehydrogenase

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6,7-Dideoxy-α-D-*gluco*-heptose 7-phosphonic acid, the isosteric phosphonate analogue of glucose 6-phosphate, was synthesized in six steps from the readily available precursor benzyl 4,6-*O*-benzylidene-α-D-glucopyranoside. The analogue is a substrate for yeast glucose 6-phosphate dehydrogenase, showing Michaelis–Menten kinetics at pH7.5 and 8.0. At both pH values the K_m values of the analogue are 4–5-fold higher and the V values approx. 50% lower than those of the natural substrate. The product of enzymic dehydrogenation of the phosphonate analogue at pH8.5 is itself a substrate for gluconate 6-phosphate dehydrogenase.

Phosphonate analogues in which a C–CH_2–P moiety replaces the C–O–P grouping of a carbohydrate phosphate ester might be expected to act as substrates for certain enzymes that catalyse reactions of the corresponding phosphates. *S*-(+)-3,4-Dihydroxybutylphosphonic acid (I) is an isosteric analogue of *sn*-glycerol 3-phosphate and has been shown to be dehydrogenated by rabbit muscle glycerol 3-phosphate dehydrogenase, showing kinetic parameters very similar to those of the natural substrate (Adams *et al.*, 1974). The same enzyme also catalyses the reverse action, in which 4-hydroxy-3-oxobutylphosphonic acid (II) (Dixon & Sparkes, 1974), the analogue of dihydroxyacetone phosphate, is reduced to compound (I). In this case the rate of reaction is an order of magnitude lower than that of the natural substrate (Cheng *et al.*, 1974; Stribling, 1974). Further examples are the substrate activity of the 1-phosphonomethyl analogue of fructose 1,6-diphosphate for aldolase (Stribling, 1974), of the phosphonate analogue of 3-phospho-D-glycerate for phosphoglycerate kinase (Orr & Knowles, 1974) and of the product of the latter reaction for glyceraldehyde 3-phosphate dehydrogenase (Dixon & Sparkes, 1974).

Isosteric phosphonate analogues do not always retain the substrate activity of the parent phosphate; the dihydroxyacetone phosphate analogue (II), which is a substrate not only for glycerol 3-phosphate dehydrogenase but also for aldolase (Stribling, 1974), does not show substrate activity with triose phosphate isomerase (Dixon & Sparkes, 1974). Moreover it is unlikely that compounds containing the C–CH_2–P moiety will be split by enzymes catalysing phosphate cleavage, although it is possible that such analogues may act as inhibitors; the 1-phosphonomethyl isostere of fructose 1,6-diphosphate is a competitive inhibitor of fructose diphosphatase (Stribling, 1974).

The selective substrate and inhibitory activity of isosteric phosphonate analogues suggests that these compounds may have a use as metabolic inhibitors. In view of the central position occupied by glucose 6-phosphate (X) at the intersection of a number of metabolic pathways, it was decided to examine the activity of its phosphonate analogue with relevant enzymes. We now report the synthesis of this analogue, 6,7-dideoxy-α-D-*gluco*-heptose 7-phosphonic acid (IX), and the details of its dehydrogenation by yeast glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–$NADP^+$ oxidoreductase, EC 1.1.1.49).

Experimental

Materials

Unless otherwise stated laboratory reagents were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Column chromatography was carried out with silica gel; particle size 0.05–0.2mm (Merck, Darmstadt, W. Germany). Light petroleum refers to the fraction b.p. 60–80°C. Concentrations were carried out under diminished pressure. N.m.r. (nuclear-magnetic-resonance) spectra were measured with a JEOL JNM-4H-100 spectrometer at 100MHz.

Chemical syntheses

Benzyl 2,3-di-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside (IV). A mixture of benzyl 4,6-*O*-benzylidene-α-D-glucopyranoside (III) (36g) (Inch & Lewis, 1972) and NaH (8g) in dry dimethylformamide was stirred at 0–10°C for 30min. Benzyl bromide (33ml)

was added dropwise to the stirred mixture, which was then stirred overnight at 22°C. Excess of NaH was destroyed by the addition of methanol and the mixture was poured into water. The solid precipitate was filtered, and recrystallized from methanol to give *benzyl* 2,3-*di-O-benzyl*-4,6-*O-benzylidene*-α-D-*gluco*-*pyranoside* (IV) (30g, yield 55%), m.p. 132–133°C, $[\alpha]_D^{22}$ +26±0.5° (*c* 1.5 in chloroform) (Found: C, 75.7; H, 6.4; $C_{34}H_{34}O_6$ requires C, 75.8; H, 6.3%).

Benzyl 2,3,4-*tri-O-benzyl*-α-D-*glucopyranoside* (*V*). $LiAlH_4$ (10g) was added in portions to a solution of the benzylidene acetal (IV) (30g) in ether (300ml) and dichloromethane (300ml). The mixture was heated under reflux, a solution of $AlCl_3$ (30g) in diethyl ether (300ml) added, and the whole was boiled under reflux for 3h. Excess of $LaAlH_4$ in the cooled reaction mixture was decomposed by the addition of ethyl acetate and water, and the resulting precipitate was removed by filtration. The filtrate was concentrated, leaving a residue which was recrystallized from di-isopropyl ether, giving *benzyl* 2,3,4-*tri-O-benzyl*-α-D-*glucopyranoside* (V) (28g, yield 93%), m.p. 84–85°C, $[\alpha]_D^{22}$+67.0±1.0° (*c* 2.0 in chloroform) (Found: C, 75.3; H, 6.7; $C_{34}H_{36}O_6$ requires C, 75.5; H, 6.7%).

Benzyl 2,3,4-*tri-O-benzyl*-6,7-*dideoxy*-α-D-*gluco*-*hept*-6-*enopyranoside* 7-*phosphonic acid diphenyl ester* (*VII*). A mixture of the benzyl glycoside (V) (28g), pyridine (6ml), H_3PO_4 (3ml) and dicyclohexylcarbodi-imide (60g) in dimethyl sulphoxide (50ml) was stirred for 16h at room temperature and poured into a solution of oxalic acid (60g) in methanol (100ml). Sufficient diethyl ether was added to effect complete precipitation of salts, which were removed by filtration, and the filtrate was concentrated, poured into water and extracted with ether. The combined ether extracts were dried (over $MgSO_4$) and concentrated to give crude benzyl 2,3,4-tri-*O*-benzyl-α-D-*gluco*-hexodialdo-1,5-pyranoside (VI) (30g), which was not purified but treated immediately with diphenyl triphenylphosphoroanylidene-methylphosphonate (30g) (Jones *et al.*, 1968), in dimethyl sulphoxide (150ml) at 70–80°C for 8h. The cooled reaction mixture was poured into water, extracted with ether and the ether extracts were dried (over $MgSO_4$), concentrated and chromatographed on a column (30cm×4cm) of silica gel. Elution with light petroleum/ether (3:2, v/v) gave a product (R_F 0.3) which was recrystallized from di-isopropyl ether to give *benzyl* 2,3,4-*tri-O-benzyl*-6,7-*dideoxy*-α-D-*gluco*-*hept*-6-*enopyranoside* 7-*phosphonic acid diphenyl ester* (VII) (15.7g, yield 39%), m.p. 75–76°C, $[\alpha]_D^{20}$ +70.5±1.0° (*c* 2.0 in chloroform). N.m.r. data (C^2HCl_3): δ 6.19 (octet, 1 proton, $P-\underline{CH_a}=CH_b-\underline{CH_c}$, $J_{P,H_a}=24$Hz, $J_{H_a,H_b}=17$Hz, $J_{H_a,H_c}=2$Hz) (Found: C, 73.3; H, 5.9; $C_{47}H_{45}O_8P$ requires C, 73.5; H, 5.9%).

Benzyl 2,3,4-*tri-O-benzyl*-6,7-*dideoxy*-α-D-*gluco*-*hept*-6-*enopyranoside* 7-*phosphonic acid dibenzyl ester* (*VIII*). A solution of the diphenyl ester (VII) (2.2g) in dry dimethylformamide (30ml) was added rapidly to a suspension of NaH (0.34g) in benzyl alcohol (0.6ml) and dry dimethylformamide (1.2ml). After 15min the mixture was poured into water (60ml) containing acetic acid (0.9ml), extracted with ethyl acetate (3×50ml) and the combined ethyl acetate extracts were successively washed with aq. $NaHCO_3$ and water, dried (over $MgSO_4$) and concentrated. The residue was chromatographed on a column (30cm×2cm) of silica gel, when benzyl alcohol was eluted with light petroleum/ether (3:2, v/v) and the product with ether. Recrystallization of the product from di-isopropyl ether gave *benzyl* 2,3,4-*tri-O-benzyl*-6,7-*dideoxy*-α-D-*gluco*-*hept*-6-*enopyranoside* 7-*phosphonic acid dibenzyl ester* (VIII) (1.1g, yield 48%), m.p. 98–99°C, $[\alpha]_D^{22}$+63±1° (*c* 2.0 in chloroform). N.m.r. data (C^2HCl_3): δ 6.03 (octet, 1 proton, $P-\underline{CH_a}=CH_b-\underline{CH_c}$, $J_{P,H_a}=24$Hz, $J_{H_a,H_b}=17$Hz, $J_{H_a,H_c}=1.5$Hz); 6.86 (octet, 1 proton, $P-CH_a=\underline{CH_b}-CH_c$, $J_{H_a,H_b}=17$Hz, $J_{P,H_b}=6$Hz, $J_{H_b,H_c}=5$Hz) (Found: C, 73.7; H, 6.3. $C_{49}H_{51}O_8P$ requires C, 73.7; H, 6.4%).

6,7-*Dideoxy*-α-D-*gluco*-*heptose* 7-*phosphonic acid* (*IX*). A solution of the benzylated derivative (VIII) (2g) in ethanol (50ml) was hydrogenolysed over 10% palladium on charcoal at room temperature and atmospheric pressure until hydrogen uptake ceased (uptake 400ml, theoretical value 392ml). The solution was filtered and concentrated to give 6,7-*dideoxy*-α-D-*gluco*-*heptose* 7-*phosphonic acid* (IX) (600mg, yield 93%) as a glass-like solid. $[\alpha]_D^{20}$+61.9±1.0° (*c* 1.8 in ethanol). No aromatic protons were present in the n.m.r. spectrum.

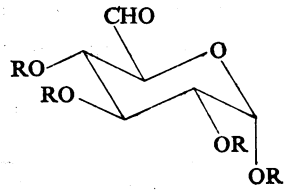
pK_a measurement

The secondary pK_a values of phosphonate (IX) and phosphate (X) were obtained by titration of a 1mM solution of the compounds with 50mM-NaOH at 25°C (ionic strength 0.101±0.001 mol/l) using a glass electrode.

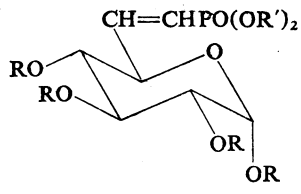
Kinetic determinations

Glucose 6-phosphate dehydrogenase (yeast), gluconate 6-phosphate dehydrogenase [6-phospho-D-gluconate–$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.44] (yeast), $NADP^+$ and glucose 6-phosphate (disodium salt) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.

Initial rates of glucose 6-phosphate dehydrogenase reactions were followed by determining formation of NADPH from $NADP^+$. The increase in E_{340} was measured in a Unicam SP.1800 spectrophotometer

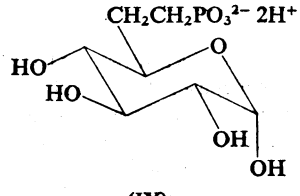


(VI) $R = CH_2C_6H_5$

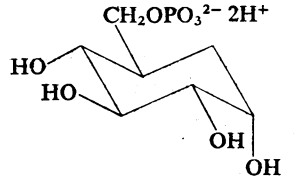


(VII) $R = CH_2C_6H_5$; $R' = C_6H_5$

(VIII) $R = R' = CH_2C_6H_5$



(IX)



(X)

at 25°C. Reaction mixtures contained 0.1M-Tris/HCl buffer adjusted to the required pH with 2M-NaOH and various concentrations of substrates in a 5cm-path-length cell (final vol. 5.005ml). Reactions were started by the addition of enzyme (0.05μg in 5μl of buffer containing 1% bovine serum albumin). A standard assay for glucose 6-phosphate dehydrogenase activity, with 0.05mM-$NADP^+$ and 0.1mM-glucose 6-phosphate, was performed at regular intervals during each experiment to check the stability of the enzyme. Initial rates were determined for five concentrations of one substrate at each of five different fixed concentrations of alternate substrate. Double-reciprocal plots were calculated from the unweighted data by the method of least squares and values of K_m and V were determined from plots of intercepts against concentration of alternate substrate as described by Florini & Vestling (1957).

Results and Discussion

6,7-Dideoxy-α-D-*gluco*-heptose 7-phosphonate (IX), the isosteric phosphonate analogue of glucose 6-phosphate (X), was synthesized in six steps from the readily available precursor benzyl 4,6-*O*-benzylidene-α-D-glucopyranoside (III). Benzylation of the benzylidene acetal (III) under standard conditions led to benzyl 2,3-*O*-benzyl-4,6-*O*-benzylidene-α-D-glucopyranoside (IV), the acetal ring of which was cleaved by using $LiAlH_4/AlCl_3$ (Liptak *et al.*, 1975) to give a high yield of crystalline benzyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranoside (V). Oxidation of the primary hydroxyl group of compound (V) by the Pfitzner–Moffatt procedure (Pfitzner & Moffatt, 1965; Zissis & Fletcher, 1970) gave the 6-aldehydo derivative (VI), which was not isolated but converted directly into the unsaturated phosphonate ester (VII) by treatment with the stable Wittig reagent diphenyl triphenylphosphoroanylidene-methylphosphonate (Jones *et al.*, 1968). Although diphenyl phosphonates may be hydrogenolysed over platinum oxide at high pressure (see, e.g., Adams *et al.*, 1974), the conversion of the diphenyl phosphonate (VII) into the free acid (IX) was achieved more readily via the dibenzyl ester (VIII). The transesterification of compound (VII) to compound (VIII) was effected by using the benzyl alcohol/NaH procedure of Jones & Moffatt (1968, 1971). Finally the dibenzyl ester (VIII) was hydrogenolysed over palladium/charcoal at atmospheric pressure and room temperature to give the free phosphonic acid (IX). The immediate precursors (VII and VIII) of the final product (IX) were well characterized, highly crystalline compounds with *trans* geometry about the double bond as shown (see the Experimental section) by the coupling constants of the olefinic protons (Griffin & Mitchell, 1965).

Alternative procedures involving protection of the primary hydroxyl group of benzyl α- or β-glucopyranoside by a triphenylmethyl (trityl) ether, followed by benzylation and detritylation to compound (V) (or its β-anomer), were found to give much poorer yields than the $LiAlH_4/AlCl_3$ ring-opening sequence described above.

6,7-Dideoxy-α-D-*gluco*-heptose 7-phosphonate (IX) was dehydrogenated by $NADP^+$ in the presence of glucose 6-phosphate dehydrogenase, showing Michaelis–Menten kinetics. The kinetic parameters for this reaction are compared with those for the

Table 1. *Comparison of kinetic parameters for glucose 6-phosphate and its phosphonate analogue*

Conditions are as described in the Experimental section. Values are ±S.E. (Bliss, 1967).

	(IX) or (X) K_m (μM)		$NADP^+$ K_m (μM)		V (relative to that of glucose 6-phosphate)	
Substrate	pH7.5	pH8.0	pH7.5	pH8.0	pH7.5	pH8.0
6,7-Dideoxy-α-D-*gluco*-heptose 7-phosphonate (IX)	227±6	192±9	6.2±0.7	6.5±0.3	0.58±0.06	0.46±0.07
Glucose 6-phosphate (X)	51±2	46±1	7.5±0.3	6.8±0.4	1.00	1.00

natural substrate, glucose 6-phosphate (X), in Table 1. The similarity of $K_m^{NADP^+}$ values for glucose 6-phosphate (X) and its phosphonate analogue (IX) suggests that the mechanism of the enzymic reaction does not differ greatly in the two cases. If K_m is regarded as a crude measure of affinity of the substrate for the enzyme then it is apparent that at both pH values examined the binding of the analogue (IX) is significantly weaker than that of glucose 6-phosphate (X). The phosphonate (IX) has secondary pK_a 7.46 and will contain 52 and 78% of the dianionic form at pH7.5 and 8.0 respectively, whereas glucose 6-phosphonate, whose secondary pK_a was found to be 6.21, will contain 95% dianionic form at both pH values. The value of K_m of the analogue (IX), however, is not greatly decreased on raising the pH of the assay from pH7.5 to 8.0 and it is accordingly unlikely that the ionic state of the analogue is a major factor in determining its higher K_m value compared with that of glucose 6-phosphate. The apparent decrease in affinity caused by replacing the C–O–P grouping of the natural substrate by C–CH_2–P could result either from specific interactions of the oxygen atoms or from minor geometric changes resulting from the substitution. These changes are reflected in the lower V values of the analogue compared with those of glucose 6-phosphate.

A reaction mixture containing 0.35mM-$NADP^+$ and 0.064mM phosphonate analogue (IX) was set up as for initial-rate studies (see the Experimental section) and allowed to proceed to completion as followed by NADPH production. Addition of gluconate 6-phosphate dehydrogenase (40μg in 20μl) caused a further steady reproducible increase in E_{340} of 0.003unit/min [the corresponding rate using glucose 6-phosphate (X) was 0.044unit/min]. It appears therefore not only that the phosphonate analogue (IX) is a substrate for glucose 6-phosphate dehydrogenase but also that its oxidation product at pH8.5, the corresponding analogue of gluconate 6-phosphate, is a substrate for gluconate 6-phosphate dehydrogenase. This result, which agrees with the findings of Webster and his co-workers (D. Webster, W. R. Jondorf & H. B. F. Dixon, unpublished work) suggests the possibility of enzymic conversion of the glucose 6-phosphate analogue (IX) into the phosphonate analogue of ribose 5-phosphate and of enzymic synthesis *in vitro* of the corresponding analogues of nucleotides and NAD^+.

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References

Adams, P. R., Harrison, R. & Inch, T. D. (1974) *Biochem. J.* **141**, 729–732

Bliss, C. I. (1967) *Statistics in Biology*, vol. 1, pp. 438–439, McGraw-Hill, New York

Cheng, P.-J., Hickey, R., Engel, R. & Tropp, B. E. (1974) *Biochim. Biophys. Acta* **341**, 85–92

Dixon, H. B. F. & Sparkes, M. J. (1974) *Biochem. J.* **141**, 715–719

Florini, J. R. & Vestling, C. S. (1957) *Biochim. Biophys. Acta* **25**, 575–578

Griffin, C. E. & Mitchell, T. D (1965) *J. Org. Chem.* **30**, 1935–1939

Inch, T. D. & Lewis, G. J. (1972) *Carbohydr. Res.* **22**, 91–101

Jones, G. H. & Moffatt, J. G. (1968) *J. Am. Chem. Soc.* **90**, 5337–5338

Jones, G. H. & Moffatt, J. G. (1971) *U.S. Patent* 3558595

Jones, G. H., Hamamura, E. & Moffatt, J. G. (1968) *Tetrahedron Lett.* **55**, 5731–5734

Liptak, A., Jodal, I. & Nanasi, P. (1975) *Carbohydr. Res.* **44**, 1–11

Orr, G. A. & Knowles, J. R. (1974) *Biochem. J.* **141**, 721–732

Pfitzner, K. E. & Moffatt, J. G. (1965) *J. Am. Chem. Soc.* **87**, 5661–5669

Stribling, D. (1974) *Biochem. J.* **141**, 725–728

Zissis, E. & Fletcher, H. G. (1970) *Carbohydr. Res.* **12**, 361–368