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Dehydrogenation of a Phosphonate Substrate Analogue by Glycerol 3-Phosphate Dehydrogenase

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S-(+)-3,4-Dihydroxybutylphosphonic acid, an isosteric analogue of *sn*-glycerol 3-phosphate, was synthesized stereospecifically and shown to be an effective substrate for rabbit muscle glycerol 3-phosphate dehydrogenase (*sn*-glycerol 3-phosphate–NAD^+ oxidoreductase, EC 1.1.1.8). Non-isosteric phosphonate analogues of *sn*-glycerol 3-phosphate showed neither substrate nor inhibitory activity with the enzyme.

Replacement of the C–O–P moiety in a phosphate ester by C–CH_2–P might be expected to result in little change in the overall geometry of the molecule. Analogues so derived from certain enzyme substrates could accordingly themselves show substrate activity. Although the stability of the C–CH_2–P grouping makes such analogues unlikely substrates for enzymes catalysing phosphate cleavage, they may well act as inhibitors. This possible selective substrate and inhibitory activity suggests that isosteric phosphonate analogues might be useful as metabolic inhibitors.

Although enzymic studies have been carried out with isosteres of phospholipids (Rosenthal & Pousada, 1968) and nucleotides (Hampton *et al.*, 1973), in which C–O–P of a natural substrate has been replaced by C–CH_2–P, no details of enzyme studies with corresponding analogues of simple carbohydrate phosphates have been reported. We have therefore synthesized *S*-(+)-3,4-dihydroxybutylphosphonic acid (compound V, Scheme 1), an isosteric analogue of *sn*-glycerol 3-phosphate (I) and examined its behaviour with glycerol 3-phosphate dehydrogenase of rabbit muscle. A synthesis of dilithium *RS*-3,4-dihydroxybutylphosphonate by a different route has been reported (Kabak *et al.*, 1972).

Experimental

Materials

Unless otherwise stated laboratory reagents were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. T.l.c. was performed by upward irrigation on microscope slides coated with silica gel G (E. Merck, Darmstadt, W. Germany), and the plates were developed with 50% (w/v) H_2SO_4 and/or iodine vapour. Column chromatography was carried out with silica gel (Merck, particle size 0.05–0.2mm). Light petroleum refers to the fraction b.p. 60–80°C. Concentrations were carried out under diminished pressure. N.m.r. spectra were measured with a JEOL JNM-4H-100 spectrometer at 100MHz.

Chemical syntheses

RS-2,3-Dihydroxypropylphosphonic acid [R form (II) and S form]. Barium *RS*-2,3-dihydroxypropylphosphonate was prepared from 1,2-*O*-isopropylidene-*rac*-glycerol (VIII) (Ralph N. Emanuel Ltd., Wembley, Middx., U.K.) by the procedure of Baer & Basu (1969). The free acid was generated by stirring the barium salt with the acid form of a sulphonated polystyrene resin (Amberlite IR-120) as described by Baer *et al.* (1969).

Diphenyl S-(+)-3,4-O-isopropylidene-but-1-enylphosphonate (IV). Diphenyl triphenylphosphoranylidene-methylphosphonate was obtained by quaternization of triphenylphosphine with diphenyl chloromethylphosphonate (McCall & McConnell, 1959) followed by neutralization with NaOH by the procedure of Jones *et al.* (1968); it had m.p. 149–150°C.

A solution of diphenyl triphenylphosphoranylidene-methylphosphonate (15.0g) and 2,3-*O*-isopropylidene-D-glyceraldehyde (III) (4.2g) [prepared by lead tetra-acetate oxidation of 1,2:5,6-di-*O*-isopropylidene-D-mannitol as described by Baer & Fischer (1939)] in dimethyl sulphoxide (100ml) was stirred for 5h at 60–80°C. The reaction was monitored by t.l.c. in ether–light petroleum (4:1, v/v), when the aldehyde (III) had R_F 0.3 and the product R_F 0.4. The reaction mixture was poured into water, extracted with ether and the ether extract dried (over $MgSO_4$), concentrated and chromatographed on a column of silica gel. Elution with ether–light petroleum (4:1, v/v) gave diphenyl *S*-(+)-3,4-*O*-isopropylidene-but-1-enylphosphonate (IV) (5.2g, 48%) as a chromatographically homogeneous syrup, $[\alpha]_D^{22}$ +1.7 ±0.5° (*c* 2.0 in chloroform). N.m.r. data (C^2HCl_3): δ 1.39 (singlet, 6 protons, isopropylidene); 3.58, 4.15 (triplets, 2 protons, H-4,4′); 4.70 (broad multiplet,

1 proton, H-2); 6.25 (octet, 1 proton, H-1, $J_{1,P}$ 22.5Hz, $J_{1,2}$ 17.5Hz, $J_{1,3}$ 1.5Hz); 6.85 (octet, 1 proton, H-3, $J_{3,P}$ 3.6Hz, $J_{2,3}$ 3.2Hz). The first-order coupling-constant data were consistent with those obtained from similar compounds (Paulsen *et al.*, 1971*a*).

S-(+)-3,4-*Dihydroxybutylphosphonic acid* (*V*). Hydrogenation of the unsaturated diphenylphosphonate (IV) was carried out under increased pressure of H_2, since attempted hydrogenation under atmospheric pressure was only partially successful. A solution of compound (IV) (4.6g) in methanol was shaken with Adams catalyst (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) under an atmosphere of H_2 (approx. 600kPa) at 22°C. Completion of the reaction was ascertained by t.l.c. in ether–light petroleum (4:1, v/v), when the diphenylphosphonate (IV) had R_F 0.4 and the product R_F 0.0. The reaction mixture was filtered and the filtrate concentrated to give *S*-(+)-3,4-dihydroxybutylphosphonic acid (V) (1.7g, 95%), $[\alpha]_D^{22}$ +18.5±1.0° (*c* 2.0 in ethanol). The n.m.r. spectrum (in dimethyl sulphoxide) showed neither aromatic nor isopropylidene protons. Shaking with ^2H_2O removed acidic and hydroxyl protons, leaving only two groups of protons (δ 1.0–2.0, 4 protons, and δ 3.0–3.6, 3 protons). The spectrum was thus entirely consistent with the assigned structure. The i.r. spectrum showed absorptions at 3247 (CO–H); 2632, 2273, 1667 [(P)O–H]; 1163 (P=O); and 980, 943 [P–O(H)]cm^{-1}.

Dibenzyl-(1*RS*,2*R*)-1,2,3-*trihydroxy*-2,3-*O*-*isopropylidene-propylphosphonate* (*VI*). A solution of dibenzyl hydrogen phosphite (12.0g) (prepared as described by Atherton *et al.*, 1945), 2,3-*O*-isopropylidene-D-glyceraldehyde (5.0g) and triethylamine (0.2g) in benzene (100ml) was heated at 60–80°C for 3h. The product crystallized from the cooled reaction mixture, was filtered and recrystallized from acetone to give dibenzyl-(1*RS*,2*R*)-1,2,3-trihydroxy-2,3-*O*-isopropylidene-propylphosphonate (VI) (7.0g, 46%) [R_F 0.55, benzene–ether–ethanol (5:5:1, by vol.)], m.p. 91–94°C, $[\alpha]_D^{22}$ +14.0±1.0° (*c* 1.3 in chloroform) (Found: C, 61.3; H, 6.4; $C_{20}H_{25}O_6P$ requires C, 61.3; H, 6.4%) as a mixture (60:40) of isomers as shown by n.m.r. of the benzylic protons at δ 5.11 (cf. Paulsen *et al.*, 1971*b*).

(1*RS*,2*R*)-1,2,3-*Trihydroxypropylphosphonic acid* (*VII*). The dibenzyl phosphonate (VI) (2.0g) in methanol was hydrogenolysed over palladium (10%) on charcoal (0.1g) at atmospheric pressure and room temperature. Filtration and concentration of the reaction mixture gave (1RS,2R)-1,2,3-trihydroxypropylphosphonic acid (VII) (0.8g, 91%) as a syrup [R_F 0.0 in benzene–ether–ethanol (5:5:1, by vol.)]. The n.m.r. spectrum of compound (VII) showed neither benzylic nor isopropylidene protons. The i.r. spectrum showed absorptions at 2632, 2273, 1667 [(P)O–H]; 1176 (P=O); 1020, 943 [P–O(H)]cm^{-1}.

Phenyl 1,2-*O*-*isopropylidene-rac-glycer*-3-*yl methylphosphonate* (*IX*). Phenyl methylphosphonochloridate was prepared by mixing equimolar quantities of phenol and methylphosphonic dichloride (Kinnear & Perren, 1952) in ether containing excess of triethylamine at room temperature. The reaction mixture was concentrated and the residual syrup distilled to give a product, b.p. 106°C/400Pa (3mmHg).

A solution of phenyl methylphosphonochloridate (2.2g) and 1,2-*O*-isopropylidene-*rac*-glycerol (VIII) (1.3g) in pyridine was stored at room temperature overnight. The reaction mixture was diluted with chloroform, washed successively with 1M-HCl, aqueous $NaHCO_3$ and water, dried (over $MgSO_4$) and concentrated under decreased pressure. Purification by column chromatography on silica gel afforded a chromatographically homogeneous syrup (yield 2.0g, 70%). N.m.r. data (in C^2HCl_3): δ 1.30, 1.37 (doublet, 6 protons, isopropylidene); 1.62 (doublet, 3 protons, P–CH_3, $J_{P,CH}$ 17.5Hz); 3.60–4.35 (multiplet, 5 protons, glycerol protons); 7.1–7.4 (multiplet, 5 protons, aromatic).

rac-Glycer-1-*yl hydrogen methylphosphonate* (*X*). The phenyl ester (IX) (2.0g) in methanol was hydrogenolysed over Adams' catalyst (0.5g) at atmospheric pressure and room temperature. The solution was filtered and concentrated to give the product as a syrup (yield 1.1g, 91%). N.m.r. data (C^2HCl_3+ ^2H_2O): δ 1.51 (doublet, 3 protons, P–CH_3, $J_{P,CH}$ 17Hz); 3.50–3.70 (multiplet, 4 protons, H-1,3); 3.75–4.10 (multiplet, 1 proton, H-2). There were no other signals in the n.m.r. spectrum, showing that hydrogenation had caused complete removal of the phenyl group and that hydrolysis of the isopropylidene group had occurred spontaneously. The i.r. spectrum showed absorptions at 3333 (CO–H); 1316, 909 (P–CH_3); 1198 (P=O); 1042 (P–O–C) and 990 [P–O(H)]cm^{-1}.

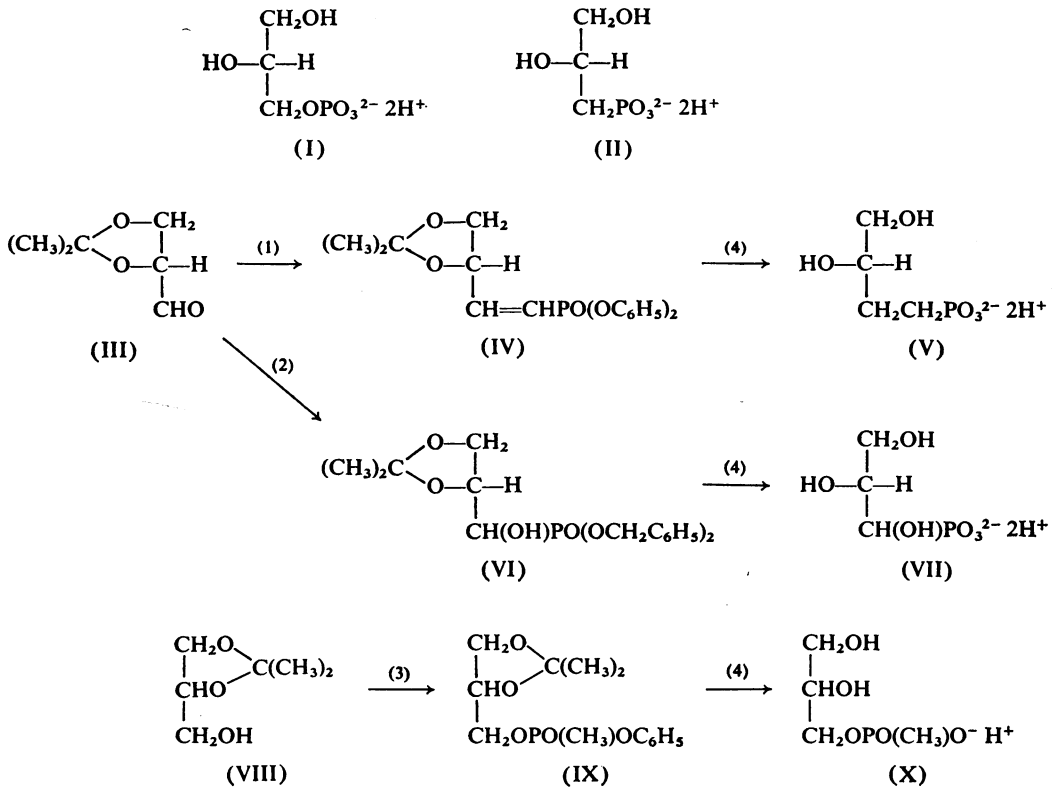
pK_a measurement

The secondary pK_a of phosphonate (V) was obtained by titration of a 40mM solution of it with 1M-NaOH at 20°C (ionic strength 0.12±0.01).

Kinetic determinations

Glycerol 3-phosphate dehydrogenase (rabbit muscle), NAD^+ and *sn*-glycerol 3-phosphate [di-(cyclohexylammonium) salt] were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. All other chemicals were obtained from BDH Chemicals Ltd. Di(cyclohexylammonium)*sn*-glycerol 3-phosphate was converted into its disodium salt, before use, by passage down a column of sulphonated polystyrene resin (Bio-Rad AG 50W-X8; Na^+ form) (Bio-Rad Laboratories Ltd., St. Albans, U.K.) followed by freeze-drying.

Initial rates of enzyme reactions were followed by determining formation of NADH from NAD^+. The increase in E_{340} was measured in a Unicam SP.1800



Scheme 1.

(1) $(C_6H_5)_3\overset{+}{P}-\bar{C}HPO(OC_6H_5)_2$ (3) $CH_3(C_6H_5O)POCl$

(2) $(C_6H_5CH_2O)_2PHO/(C_2H_5)_3N$ (4) Catalytic hydrogenation

spectrophotometer at 20°C. Reaction mixtures (final pH9.6) contained 0.1M-glycine and 0.1M-hydrazine (adjusted to pH9.6 with 1M-NaOH), 0.2μg of glycerol 3-phosphate dehydrogenase and various concentrations of substrates and inhibitors in a total volume of 1.005ml. Reactions were started by addition of enzyme. A standard assay for glycerol 3-phosphate dehydrogenase activity, with 2mM-NAD^+ and 2mM-*sn*-glycerol 3-phosphate as substrates, was performed before and after each series of experiments to check the stability of the enzyme. Values of K_m and V were obtained by extrapolation to infinite concentration of alternate substrate as described by Florini & Vestling (1957), by using intercepts from double-reciprocal plots computed by the method of least-squares.

Results and Discussion

Synthesis of each of the analogues (V), (VII) and (X) of *sn*-glycerol 3-phosphate (I) was accomplished in two steps from a readily available precursor (III or VIII) (Scheme 1). Purification of intermediates (IV) and (IX) was effected by column chromatography and of intermediate (VI) by recrystallization. As the final step in each synthetic route involved a simple catalytic hydrogenation of a chromatographically pure well-characterized intermediate, purification of the final product via a crystalline salt was unnecessary. In fact the products (V), (VII) and (X) were all shown to be pure by n.m.r. spectroscopy.

S-(+)-3,4-Dihydroxybutylphosphonate (V) behaved as a substrate of glycerol 3-phosphate dehydrogenase, showing Michaelis–Menten kinetics and kinetic parameters very similar to those obtained with the natural substrate, *sn*-glycerol 3-phosphate (I) (Table 1). As the phosphonate analogue (V) differs structurally from compound (I) only in containing CH_2–CH_2–P in place of the phosphate CH_2–O–P grouping, the similarity in kinetic parameters for compounds (I) and (V) suggests that the esterified C-3 oxygen of *sn*-glycerol 3-phosphate has no major direct interaction with the enzyme.

Table 1. *Comparison of kinetic parameters for sn-glycerol 3-phosphate and its phosphonate analogue*

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

Substrate	$K_m^{(I) \text{ or } (V)}$ (mM)	$K_m^{NAD^+}$ (mM)	V (relative to that of compound I)
sn-Glycerol 3-phosphate (I)	0.29±0.01	0.18±0.03	1.00
S-(+)-3,4-Dihydroxybutylphosphonate (V)	0.17±0.01	0.18±0.01	0.80±0.09

It is likely that the substrates bind to the enzyme in their dianionic forms, since the monoanionic *rac*-glycerol 1-methylphosphonate (X) behaved neither as a substrate nor as an inhibitor at concentrations up to 10mM. At the pH (9.5) of our enzyme assay more than 98% of both *sn*-glycerol 3-phosphate (I) (secondary pK_a 6.45) and *S*-(+)-3,4-dihydroxybutylphosphonate (V) (secondary pK_a 7.65) will exist as their dianions. However, it is possible that the phosphonate (V) will behave as a less effective substrate than compound (I) at physiological pH values when it will contain relatively less of the dianionic form.

R-(−)-2,3-Dihydroxypropylphosphonate (II) and (1*RS*,2*R*)-1,2,3-trihydroxypropylphosphonate (VII) are both analogues of *sn*-glycerol 3-phosphate (I) in which the CH_2–O–P moiety of compound (I) has been replaced by the C–P linkage. Baer *et al.* (1969) examined the behaviour of compound (II) and its racemate with rabbit muscle glycerol 3-phosphate dehydrogenase. They reported that neither modification showed substrate activity at a concentration of 0.08mM, nor did 0.15mM solutions of either form inhibit the dehydrogenation of 0.04mM-*sn*-glycerol 3-phosphate. We found no detectable substrate activity of 10mM-*rac*-2,3-dihydroxypropylphosphonate or of 10mM-(1*RS*,2*R*)-1,2,3-trihydroxypropylphosphonate (VII). Moreover, neither compound at a concentration of 10mM gave detectable inhibition of the dehydrogenation of 0.1mM-*sn*-glycerol 3-phosphate. It appears therefore that the spatial relationship of C-3 to P must approximate to that in the CH_2–O–P grouping for efficient binding to the enzyme.

The substrate activity of *S*-(+)-3,4-dihydroxybutylphosphonate (V) for glycerol 3-phosphate dehydrogenase is consistent with the finding of Stribling (1974) that 4-hydroxy-3-oxobutylphosphonic acid (Dixon & Sparkes, 1974), the corresponding analogue of dihydroxyacetone phosphate, is a substrate for the same enzyme acting in the reverse direction. Further demonstrations of substrate (Orr & Knowles, 1974; Stribling, 1974) and inhibitory (Stribling, 1974) activity of phosphonomethyl analogues with glycolytic enzymes indicate that such isosteric analogues may well find general application as metabolic probes.

Note Added in Proof (Received 21 May 1974)

Since submission of this paper, a report has been published (Cheng *et al.*, 1974) containing details of the substrate activity of *RS*-3,4-dihydroxybutylphosphonate with rabbit muscle glycerol 3-phosphate dehydrogenase.

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