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Vitamin B_6—Phosphonic Acids*

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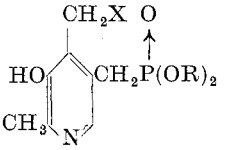
It is generally assumed that pyridoxal and pyridoxamine, in acting as coenzymes in various systems, are attached to the respective apoenzymes by means of a 5-phosphoric acid group. The four nuclear substituents of the three forms of vitamin B_6 can be varied within narrow limits to yield compounds which exhibit some measure of vitamin B_6-activity in several *in vitro* and *in vivo* systems. These variations have been summarized by Shive.[1] The same author has also listed those structural variants which cause the resulting analogues to act as vitamin B_6 antagonists (reference 1, page 660). The most significant alterations of the 5-hydroxymethyl group in both the protagonists and antagonists concerned compounds containing methyl, acetoxymethyl, aminomethyl, and bromomethyl groups in position 5, or a 4,5-epoxydimethyl group. No structural variations of phosphorylated analogues of vitamin B_6 involving the 5-methyl phosphoric acid group have been reported. In the framework of our studies on the effect of replacing phosphoric acid groups by phosphonate groups in natural metabolites,[2–5] we have now synthesized and tested several phosphonate analogues of pyridoxamine, pyridoxine, desoxypyridoxine and methoxypyridoxine.

The general formula of these compounds may be represented as I where R is *iso*propyl or hydrogen, and X is H, OH, OCH_3, or NH_2.



I

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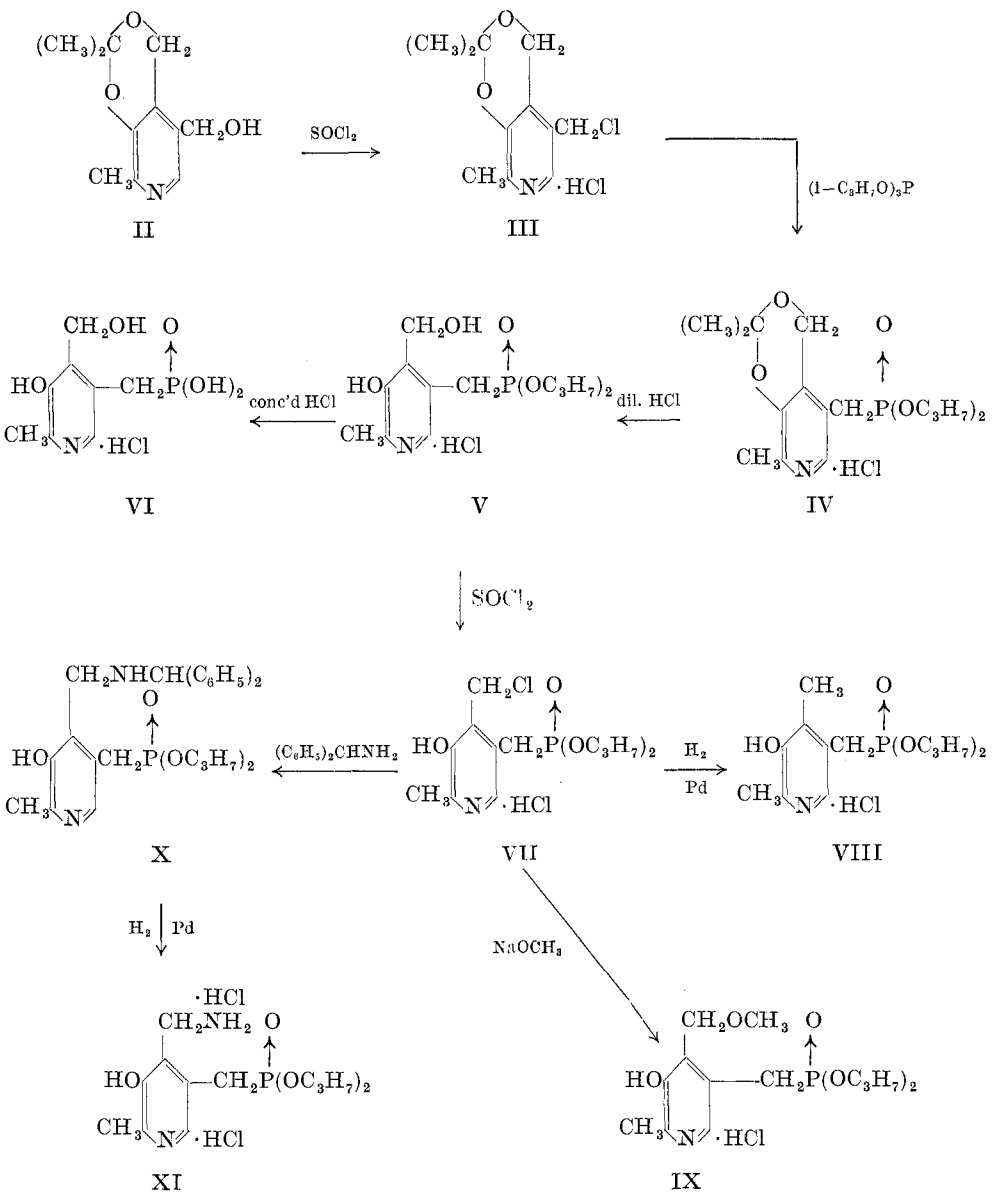
Chart I


SOCl2
(1-C3H7O)3P
conc'd HCl
dil. HCl
SOCl2
(C6H5)2CHNH2
H2
Pd
H2
Pd
NaOCH3
II
III
IV
V
VI
VII
VIII
IX
X
XI

The general method of synthesis involved first the replacement of the primary alcohol group in the 5-position of pyridoxine by a phosphonate ester via the chloride, followed by suitable alteration of the 4-hydroxymethyl group. The detailed scheme of synthesis is shown in Chart I.

To prepare the 5-chloromethyl compound necessary for introducing the phosphonate function it was essential that the 4-hydroxymethyl be blocked by a group which could be removed easily later; *iso*propylidenepyridoxine (II)[6] served these purposes. The reaction of II with thionyl chloride in benzene gave 69 per cent of 3,4-*iso*propylidene 5-chloromethyl-3-hydroxy-2-methyl-4-pyridinemethanol hydrochloride (III), which was subjected to a Michaelis–Arbuzov reaction with tri*iso*propyl phosphite. A yield of 61 per cent of di*iso*propyl *iso*propylidene 3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridinemethylphosphonate hydrochloride (IV) was obtained in this manner. Mild acid hydrolysis of IV removed the *iso*propylidene group and produced di*iso*propyl 3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridinemethylphosphonate hydrochloride (V) in 86 per cent yield. More drastic hydrolysis using concentrated hydrochloric acid converted V to 3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridinemethylphosphonic acid hydrochloride (VI).

Attempts to oxidize V to the pyridoxal analogue were without success; when V was treated with manganese dioxide in acid solution under conditions which converted pyridoxine phosphoric acid to pyridoxal phosphoric acid,[7,8] considerable decomposition occurred and no aldehyde could be isolated.

The remaining compounds were all made from di*iso*propyl 4-chloromethyl-3-hydroxy-2-methyl-5-pyridinemethylphosphonate hydrochloride (VII), which was obtained in 79 per cent yield by reacting V with thionyl chloride. Hydrogenation of VII gave 68 per cent of di*iso*propyl 2,4-dimethyl-3-hydroxy-5-pyridinemethylphosphonate hydrochloride (VIII). Di*iso*propyl 3-hydroxy-4-methoxymethyl-2-methyl-5-pyridinemethylphosphonate hydrochloride (IX) was prepared in 34 per cent yield by treatment of VII with sodium methoxide.

The synthesis of the pyridoxamine analogue XI from VII was achieved by the two-step method developed by Suter[9] for the preparation of primary amines from halides. Amination of

VII with benzhydrylamine yielded 62 per cent of di*iso*propyl 4-benzhydrylaminomethyl-3-hydroxy-2-methyl-5-pyridinemethylphosphonate (X), which upon catalytic hydrogenolysis gave di*iso*propyl 4-aminomethyl-3-hydroxy-2-methyl-5-pyridinemethylphosphonate dihydrochloride (XI) in 62 per cent yield.

The aqueous solutions of the hydrochlorides of five of the phosphonate analogues (V, VI, VIII, IX, XI) were tested for activity on a cell-free transaminase from beef heart. Even at concentrations as high as 4·5 g/l. V; 11 g/l. VI; 6·9 g/l. VIII; 2·9 g/l. IX; and 8·8 g/l. XI (at least 100 times those required for pyridoxal phosphoric acid) no activity was noted in the following tests.

(1) The complete unresolved transaminase system was not inhibited or stimulated by these compounds; i.e. they did not remove pyridoxal phosphate from the enzyme.

(2) The resolved system, i.e. the apoenzyme, was not stimulated by these materials when in contact with them for 5 min. This means they have no pyridoxal phosphate activity.

(3) When allowed contact with the apoenzyme for 10 min before adding pyridoxal phosphate, these substances did not inhibit the subsequent activity of pyridoxal phosphate; i.e. they did not compete with pyridoxal phosphate for a position on the enzyme.

Since all of these properties could also be attributed to pyridoxamine phosphoric acid which has some activity after a long contact with the apoenzyme,[10] the substances were incubated for 1 h with the apoenzyme before testing for activity. Here, a low order of activity was found, mostly for VIII. This observation will require further experimental clarification.

The inactivity of compounds V, VIII, IX, and XI could be due, in part, to the fact that these substances are phosphonate esters. This objection does not hold for 5-deoxypyridoxine-5-phosphonic acid (VI). By contrast, both pyridoxine phosphoric acid[10] and desoxypyridoxine phosphoric acid[11] inhibit transaminase when allowed contact with the apoenzyme prior to the addition of pyridoxal phosphate. This could indicate that the spatial arrangement of the pyridoxine and the phosphonate [$-P(O)(OH)_2$] moieties around the connecting oxygen atom in pyridoxine phosphoric acid is of crucial impact on activity. Since there is

no evidence for release of phosphate ions by vitamin B_6 5-phosphoric acids if activity is to be achieved, the concept of simple binding of the vitamins B_6 to an apoenzyme site by means of a 5-phosphoric acid group may have to be refined to include a description of steric conditions at the binding site.

Experimental*

Grateful acknowledgement is made to Dr. Karl Folkers of Merck Sharp and Dohme Company for the generous supply of pyridoxine used in this investigation.

Iso*Propylidene 5-chloromethyl-3-hydroxy-2-methyl-4-pyridinemethanol hydrochloride* (*III*). The procedure of Cohen and Hughes[6] in which *iso*propylidene pyridoxine was heated with thionyl chloride without a solvent yielded a product of unsatisfactory quality. Therefore, a solution of 9·5 ml (15·8 g, 145 mmoles) of thionyl chloride in 22 ml of dry benzene was added drop-wise to a hot solution of 14·7 g (70·3 mmoles) of *iso*propylidene pyridoxine[6] in 200 ml of benzene. The hydrochloride of the reaction product precipitated at once, and the mixture began to boil. After addition was completed the mixture was refluxed for another 15 min, cooled, the precipitate formed was collected and washed with benzene. It was then dissolved in warm ethanol, the solution was cleared with Darco, filtered, and the salt was re-precipitated with dry ether. The yield of colourless material (m.p. 187° dec.) was 12·8 g (69 per cent). The reported melting point[6] is 190° dec.

*Di*iso*propyl* iso*propylidene 3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridinemethylphosphonate hydrochloride* (*IV*). A suspension of 12·6 g of *iso*propylidene 5-chloromethyl-3-hydroxy-2-methyl-4-pyridinemethanol hydrochloride in 125 ml of tri*iso*propyl phosphite was heated to boiling. The solid material gradually went into solution, and the solution was refluxed for 10 h. After cooling the dark yellow solution was treated with Darco, filtered, and diluted with 800 ml of hexane. Dry hydrogen chloride was passed in, slowly at first, until a small amount of gummy yellow

* All melting points are corrected. Microanalysis by Mrs. M. Logan.

material precipitated, from which the solution was decanted. The hydrochloride was then completely precipitated by an excess of hydrogen chloride; the mixture was allowed to stand overnight to complete crystallization. The colourless crystals were filtered by suction, washed with petroleum ether, and dried in a vacuum desiccator to give 11·5 g (61 per cent) of colourless needles, m.p. 125–8°. Re-crystallization by dissolving in tetrahydrofuran, adding *cyclo*hexane short of turbidity, and standing at room temperature raised the melting point to 133–134°.

Anal. Calcd. for $C_{17}H_{29}ClNO_5P$: C, 51·85; H, 7·41. Found: C, 51·41; H, 7·66.

When triethyl phosphite was used in another run instead of tri*iso*propyl phosphite, only a low yield of crude diethyl *iso*propylidene 3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridinemethylphosphonate hydrochloride was realized, perhaps because of a side-reaction in which the phosphite was converted to diethyl ethylphosphonate.

*Di*iso*propyl 3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridinemethylphosphonate hydrochloride* (*V*). A solution of 9·2 g of di*iso*propyl *iso*propylidene 3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridinemethylphosphonate hydrochloride in 180 ml of 1 N hydrochloric acid was heated at 55° for 2 h. The cooled solution was neutralized with sodium bicarbonate and evaporated to dryness under reduced pressure. The solid residue was stirred into a paste with a little water and extracted with three 75 ml portions of hot benzene. The combined extracts were treated with Darco, filtered, and concentrated to 100 ml by distillation at atmospheric pressure. Dry hydrogen chloride was then passed into the filtrate and the crystals were filtered, washed with dry ether, and air-dried. The yield of colourless plates was 7·1 g (86 per cent). After two re-crystallizations from ethanol–ethyl acetate the melting point was 187·5–188° dec.

Anal. Calcd. for $C_{14}H_{25}ClNO_5P$: C, 47·51; H, 7·12. Found: C, 47·55; H, 7·34.

3-Hydroxy-4-hydroxymethyl-2-methyl-5-pyridinemethylphosphonic acid hydrochloride (*VI*). A solution of 2·0 g of di*iso*propyl 3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridinemethylphosphonate hydrochloride in 20 ml of concentrated hydrochloric acid was refluxed for 8 h, concentrated under reduced pressure until

crystallization began, and then cooled to 0°. The colourless crystals were filtered and re-crystallized twice from cold ethanol–ethyl acetate. The yield of pure material was 1·0 g (65 per cent), m.p. 194–195°.

Anal. Calcd. for $C_8H_{13}ClNO_5P$: C, 35·64; H, 4·85. Found: C, 35·48; H, 5·01.

*Di*iso*propyl 4-chloromethyl-3-hydroxy-2-methyl-5-pyridinemethylphosphonate hydrochloride* (*VII*). A hot solution of 3·0 g (8·5 mmoles) of di*iso*propyl 3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridinemethylphosphonate hydrochloride in 10 ml of water was treated with an excess of sodium bicarbonate; after shaking with four 25 ml portions of benzene, the combined extracts were concentrated to 30 ml by distillation at atmospheric pressure. To the solution was added drop-wise, over a period of 15 min, a solution of 3·0 ml (5·0 g, 42 mmoles) of thionyl chloride in 10 ml of dry benzene. After standing for 30 min. the mixture was diluted with 50 ml of dry ether and filtered. The crude yellow product was re-crystallized from ethanol–ethyl acetate–ether to give 2·5 g (79 per cent) of colourless needles, m.p. 170–172°. An analytical sample melted at 172–173° dec.

Anal. Calcd. for $C_{14}H_{24}Cl_2NO_4P$: C, 45·16; H, 6·51. Found: C, 45·08; H, 6·69.

*Di*iso*propyl 2,4-dimethyl-3-hydroxy-5-pyridinemethylphosphonate hydrochloride* (*VIII*). A solution of 1·20 g of di*iso*propyl 4-chloromethyl-3-hydroxy-2-methyl-5-pyridinemethylphosphonate hydrochloride in 30 ml of ethanol was shaken with 0·1 g of a 5 per cent palladium-on-carbon catalyst (from palladium chloride) under a hydrogen pressure of 3 atm. for 12 h. The catalyst was filtered off and the solvent removed under reduced pressure. The residue was re-crystallized from chloroform–ethyl acetate to give 0·75 g (68 per cent) of colourless prisms, m.p. 167–169°. Re-crystallization from ethanol–ethyl acetate raised the melting point to 170·5–171·5°.

Anal. Calcd. for $C_{14}H_{25}ClNO_4P$: C, 49·76; H, 7·46. Found: C, 50·03; H, 7·76.

*Di*iso*propyl 3-hydroxy-4-methoxymethyl-2-methyl-5-pyridinemethylphosphonate hydrochloride* (*IX*). To a solution of sodium methoxide, prepared from 0·21 g (9·1 mmoles) of sodium and 8·3 ml of methanol, was added 0·83 g (2·2 mmoles) of di*iso*propyl

4 - chloromethyl - 3 - hydroxy - 2 - methyl - 5 - pyridinemethylphosphonate hydrochloride and the mixture was refluxed for 6 h. After removal of the solvent, the residue was acidified with dilute hydrochloric acid and then neutralized with sodium bicarbonate. The solution was extracted exhaustively with benzene; the combined extracts were concentrated to 30 ml by distillation at atmospheric pressure and dry hydrogen chloride was passed in. After standing for several hours the benzene was decanted from a gummy yellow precipitate, which was dissolved in warm tetrahydrofuran. The product was then fractionally precipitated with dry ether, discarding the oily material which separated first, to give 0·28 g (34 per cent) of colourless crystals, m.p. 133–135°. Two more re-crystallizations from tetrahydrofuran–ether raised the melting point to 137–138°.

Anal. Calcd. for $C_{15}H_{27}ClNO_5P$: C, 48·99; H, 7·39. Found: C, 49·32; H, 7·69.

*Di*iso*propyl 4-benzhydrylaminomethyl-3-hydroxy-2-methyl-5-pyridinemethylphosphonate* (*X*). A solution of 2·4 g (13·1 mmoles) of benzhydrylamine, freshly prepared from the hydrochloride, in 8 ml of ethanol was added to a hot solution of 1·5 g (4·0 mmoles) of di*iso*propyl 4-chloromethyl-3-hydroxy-2-methyl-5-pyridinemethylphosphonate hydrochloride in 10 ml of ethanol. The mixture was heated on a steam bath for 15 h, and the solvent was evaporated under reduced pressure. The residue was extracted exhaustively with boiling *iso*propyl ether. The combined extracts were concentrated to 50 ml, cooled to 0°, and the crystalline precipitate filtered and re-crystallized from ethyl ether. The yield was 1·2 g (62 per cent), m.p. 171–174°. Two re-crystallizations from ether raised the melting point to 174–175·5°; the colourless crystals developed a pale yellow colour on standing.

Anal. Calcd. for $C_{27}H_{35}N_2O_4P$: C, 67·21; H, 7·31. Found: C, 67·30; H, 7·92.

*Di*iso*propyl 4-aminomethyl-3-hydroxy-2-methyl-5-pyridinemethylphosphonate dihydrochloride* (*XI*). A solution of 1·1 g of di*iso*propyl 4-benzhydrylaminomethyl-3-hydroxy-2-methyl-5-pyridinemethylphosphonate in 30 ml of ethanol was shaken with 0·2 g of a 5 per cent palladium-on-carbon catalyst (prepared *in situ* from palladium chloride on carbon) under a hydrogen pressure

of 4 atm at 65° for 12 h. The catalyst was filtered off and the filtrate was concentrated to 5 ml under reduced pressure. Enough alcoholic hydrogen chloride was added to make the solution strongly acidic and the product was precipitated by dry ether. One re-crystallization from ethanol–ethyl acetate yielded 0·55 g (62 per cent) of colourless powdery material, m.p. 186° dec.

Anal. Calcd. for $C_{14}H_{27}Cl_2N_2O_4P$: C, 43·18; H, 6·99. Found: C, 42·96; H, 7·30.

Summary. The syntheses of 5-deoxypyridoxine-5-phosphonic acid, and of the di*iso*propyl esters of 5-deoxypyridoxamine-5-phosphonic acid, 4,5-bis(deoxy)pyridoxine-5-phosphonic acid, and 5-deoxypyridoxine-5-phosphonic acid-4-methyl ether are described. These and some related compounds failed to exhibit either pyridoxal phosphate or competitive activity when tested with a cell-free transaminase.

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