On the Inhibitory Activity of 4-Vinyl Analogues of Pyridoxal: Enzyme and Cell Culture Studies†

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ABSTRACT: Analogues of pyridoxal and of pyridoxal phosphate in which the 4-CHO group is replaced with $CH{=}CH_2$ were synthesized and were found to be potent inhibitors of pyridoxal kinase and pyridoxine phosphate oxidase of rat liver. They also inhibited the growth of mouse Sarcoma 180 and mammary adenocarcinoma TA3 in cell culture. Saturation of the vinyl double bond, replacement of the 5-CH_2OH with methyl, methylation of the phenolic hydroxyl, or conversion to the *N*-oxide resulted in diminution or loss of all these activities. Similarly, the introduction of a β-methyl group into the vinyl analogues of pyridoxal reduced all these inhibitory activities. The 4-vinyl analogue of pyridoxal was shown to be a substrate of pyridoxal kinase and the product a potent inhibitor of pyridoxine oxidase, competing with pyridoxal phosphate. The affinity of this phosphorylated pyridoxal analogue to some apoenzymes varied greatly, indicating striking differences among the cofactor binding sites of these enzymes. The growth inhibitory effects of these analogues on cells in culture correlated well with their effects on pyridoxal kinase and pyridoxine phosphate oxidase in cell-free systems.

Pyridoxal phosphate is the cofactor of numerous enzymes involved in interconversions of amino acids. The 4-formyl group of the cofactor is essential for these functions and for covalent attachment to the apoenzymes, being bound through aldimine linkage to the ϵ-amino group of a lysine residue at the cofactor binding site. During enzymatic reactions, this linkage is displaced by an aldimine linkage to the oncoming amino acid. The latter then undergoes a reaction such as transamination or decarboxylation (Snell and DiMari, 1970). One of the structures that may mimic some properties of the 4-formyl group and, at the same time, have a potential for irreversible reaction with the cofactor binding site of the enzyme is vinyl. It not only has the same size as the formyl group but it is also conjugated with the pyridine ring, and, hence, must be coplanar for maximum overlap of π electrons. Such a vinyl group in simple 4- and 2-vinylpyridines is known to be very reactive and is utilized, for example, in determining SH groups in proteins (Friedman and Krull, 1969; Friedman et al., 1970).

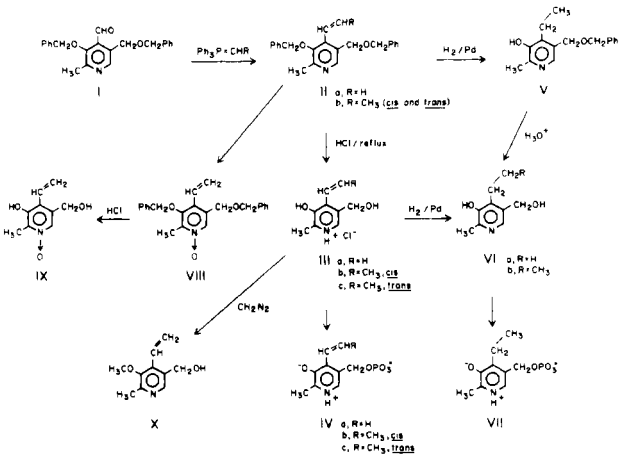
These considerations led to synthesis of a 4-vinyl analogue of pyridoxal (4-VPAL[1]; IIIa, Scheme I), which was found to be one of the most potent inhibitors of vitamin B_6 in vivo (Korytnyk et al., 1973). Binding of the 4-vinyl analogue of pyridoxal phosphate (IVa) and related compounds with apoasparate transaminase has been reported (Yang et al., 1975). The present paper describes our subsequent study of the mode of action of this antagonist and the synthesis of several related antagonists of vitamin B_6. Decreasing the content of the natural vitamin in the cell culture medium provided conditions for testing the growth-inhibitory effects of these compounds on cells in culture. These findings were correlated with studies of enzymes involved in the anabolism of pyridoxal phosphate, namely, pyridoxal phosphokinase and pyridoxine-phosphate oxidase. A brief report of some aspects of this study has appeared (Korytnyk et al., 1972).

Materials and Methods

Syntheses. Infrared spectra were determined with a Perkin-Elmer 457 spectrometer, and NMR spectra with a Varian A-60A instrument. Assignments of peaks were made on the basis of previous work (Korytnyk and Ahrens, 1970). Me_4Si or dioxane was used as an internal standard. Positions of peaks are given in ppm units (from Me_4Si). Thin-layer chromatography (TLC) was used routinely as described, and spots were detected by spraying with Gibb's reagent (Ahrens and Korytnyk, 1970). Measurement of radioactivity was carried out in a Packard Tri-Carb scintillation counter, using standard techniques. The structures of compounds are shown in Schemes I and II, and will be referred to by Roman numerals.

Triphenylmethyl[^{14}C]phosphonium Bromide. A sealed ampule of $^{14}CH_3Br$ (0.0174 mmol; total, 1 mCi; sp act., 57.5 mCi/mM; International Chemical and Nuclear Corporation, lot 7293-63) was cooled in a mixture of alcohol and dry ice and

Scheme I



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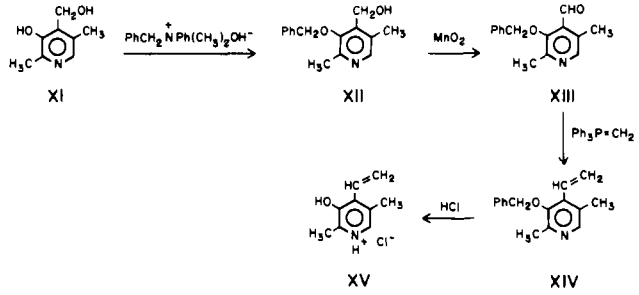
[1] Abbreviations used are: 4-VPAL, 4-vinyl-4-deformylpyridoxal (IIIa); 4-DOP, 4-deoxypyridoxine; ADP, ATP, adenosine di- and triphosphates; THF, tetrahydrofuran; NMR, nuclear magnetic resonance; IR, infrared; UV, ultraviolet; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

Scheme II



was then carefully opened, and a solution of triphenylphosphine (5.2 mg, 0.2 mmol) in benzene (0.5 ml, dry) was added. The ampule was resealed and was kept at room temperature for 1 week, while crystals of the product formed. Then, the reaction vessel was opened and its contents were centrifuged. The crystals were washed with benzene three times and the wash liquid was centrifuged. Radioactive triphenylmethylphosphonium bromide (4.3 mg, 0.012 mmol) thus obtained was diluted with the cold reagent (150 mg) and was crystallized from EtOH–Et_2O to constant specific activity (1.32 mCi/mmol). This material was further diluted with cold reagent; the final product (452 mg, ca. 0.4 mCi/mmol) was dried under high vacuum at 60 °C before the next step.

3,α^5-O-Dibenzyl-4-vinyl-4-deformyl[β^4-^{14}C]pyridoxal (IIa). The Wittig reaction was carried out under N_2, using 225 mg of labeled triphenylmethylphosphonium bromide prepared as just described. To a 1:1 mixture (200 mg) of potassium *tert*-butoxide and *tert*-butyl alcohol in anhydrous ether (5 ml), 1.6 M butyllithium in hexane was added dropwise for 10 min while the reaction vessel was cooled in an ice bath. Next, labeled triphenylmethylphosphonium bromide (0.63 mmol) was added and the reaction mixture was stirred at room temperature for 3 h. A solution of 3,α^5-*O*-dibenzylpyridoxal (150 mg, 0.43 mmol) in THF (1 ml, dry) was added to the ylide solution and was stirred at room temperature for 12 h; it was then diluted with ether and filtered. The filtrate was washed with saturated sodium bisulfite solution (to remove any unreacted aldehyde), 20% ammonium chloride solution, and water. After drying ($MgSO_4$) and evaporating, the oil was taken up in a small amount of EtOAc, and was chromatographed on a silica gel column (Bio-SIL "A", 100–200 mesh). Elution with EtOAc was followed by TLC; the combined fractions were evaporated, yielding 170 mg (56% based on the aldehyde used), with specific activity 0.43 mCi/mmol.

4-Vinyl-4-deformyl[β^4-^{14}C]pyridoxal (IIIa). Benzyl groups in IIa (169 mg, 0.49 mmol) were hydrolyzed by refluxing with 4 N HCl (15 ml) for 22 h. The solution was evaporated to dryness and was coevaporated with H_2O to remove the benzyl alcohol, and was crystallized from ethanol–acetone, yielding 78 mg (79%) of the labeled compound (sp act. 0.42 mCi/mM), identical with cold authentic material (Korytnyk et al., 1973) in TLC and IR spectra.

4-Vinyl-4-deformylpyridoxal 5′-Phosphate (IVa). Compound IIIa (150 mg, 0.75 mmol) was added to 1 ml of a phosphorylating mixture consisting of 1 part P_2O_5 and 1.3 parts 85% H_3PO_4, and the combined mixture was kept at 65–75 °C for 4–5 h, with occasional shaking, moisture being excluded (drying tube). Water (2 ml) was then added and the mixture was heated to 100 °C for 20 min. After the addition of 1 N HCl (8 ml), heating was continued for another 30 min.

After cooling, the mixture was diluted to 25 ml with H_2O and Darco G-60 charcoal (prewashed with 1 N HCl) was added. After being stirred for 10 min, the mixture was filtered and the charcoal was washed with H_2O (100 ml). The charcoal was now eluted with ammonia (2%) until no UV-absorbing material remained. The ammonia filtrate (ca. 100 ml) was concentrated to 3 ml and was chromatographed on an Amberlite CG-50 (H^+) column with H_2O as the eluent. Fractions with consistent UV absorptions were combined, filtered, and evaporated in vacuo. The oily residue crystallized on heating with alcohol. The yield was 120 mg (66%), mp (after crystallization from aqueous ethanol) 224 °C (dec). NMR (1 N NaOD) 2-CH_3 2.47, 5-CH_2 3.27 ($J_{P,H}$ = 4 Hz); 4-CH=CH_2 5.55–6.20 (m); 4-CH=CH_2 6.72–7.30 (m); 6-H 7.83.

Anal. Calcd for $C_9H_{12}NO_5P$: C, 44.09; H, 4.93; N, 5.71. Found: C, 44.06; H, 5.26; N, 5.86.

4-Vinyl-4-deformyl[β^4-^{14}C]pyridoxal 5′-phosphate (IVa) was obtained from 4-vinyl-4-deformyl[β^4-^{14}C]pyridoxal (25 mg, sp act. 0.42 mCi/mmol) by phosphorylation as described for the cold material and purification was followed by radioactivity measurements. The yield was 22 mg (66%), specific activity 0.42 mCi/mmol.

5-(Benzoxymethyl)-4-ethyl-3-hydroxy-2-methylpyridine (V). A solution of IIa (cold, 240 mg, 0.7 mmol) in methanol was hydrogenated at room temperature and atmospheric pressure in the presence of 40 mg of 5% Pd/C. After 1 h, H_2 ceased to be taken up. The reaction mixture was filtered and evaporated in vacuo. The oily residue crystallized, furnishing 130 mg (72%) of V, mp 140 °C (from ether–MeOH).

Anal. Calcd for $C_{16}H_{19}NO_2$: C, 74.68; H, 7.44; N, 5.44. Found: C, 73.63; H, 7.40; N, 5.85.

4-Ethyl-3-hydroxy-5-(hydroxymethyl)-2-methylpyridine Hydrochloride (VIa). The hydrochloride of IIa (200 mg, 0.5 mmol) was hydrogenated, as described for V, in the presence of 30 mg of Pd/C for 45 min. The reaction mixture was filtered and the filtrate was evaporated. The residue was taken up in 3 N HCl (5 ml) and MeOH (0.7 ml), and was kept under reflux for 22 h, evaporated to dryness, and crystallized from acetone. The overall yield was 90 mg (86%), mp 210 °C (dec, from methanol–acetone). NMR (D_2O) 4-CH_2CH_3 1.22 (t, J = 8); 2-CH_3 2.68; 4-CH_2-CH_3 2.92 (q, J = 8); 5-CH_2 4.88; 6-H 8.23.

Anal. Calcd for $C_9H_{14}NO_2Cl$: C, 53.08; H, 6.93; N, 6.88; Cl, 17.41. Found: C, 52.95; H, 6.95; N, 7.05; Cl, 17.40.

5′-Phosphate of VI (4-Ethyl-3-hydroxy-2-methyl-5-pyridylmethylphosphoric Acid, VII). Compound VIa was phosphorylated and the 5′-phosphate was purified as described for IVa. From 75 mg (0.4 mmol) of the hydrochloride of VI was obtained 77 mg (69%) of the 5′-phosphate, mp 240–242 °C (dec). NMR (D_2O) 2-CH_3 2.23; 4-CH_2 2.63 (q, J = 8); β^4-CH_3 1.02 (t, J = 8); 5-CH_2 4.03 ($J_{P,H}$ = 3); 6-H 7.58.

Anal. Calcd for $C_9H_{14}NO_5P$: C, 43.75; H, 5.70; N, 5.67. Found C, 43.95; H, 5.96; N, 5.43.

3,α^5-O-Dibenzyl-4-vinyl-4-deformylpyridoxal N-Oxide (VIII). To a solution of *m*-chloroperbenzoic acid (135 mg) in chloroform (5 ml) was added 5 ml of a chloroform solution of 3,α^5-*O*-dibenzyl-4-vinyl-4-deformylpyridoxal (IIa, 172 mg, 0.5 mmol) during 5 min. After the solution was stirred for 25 min, the reaction was complete (TLC). The solution was washed with a sodium sulfite solution and water, dried ($MgSO_4$), and evaporated to an oil (yield 174 mg). Adding ethereal HCl (anhydrous) to a solution of the *N*-oxide in ether converted the *N*-oxide to a hydrochloride and crystals formed. They were recrystallized from an ethanol–ether mixture; mp 127–128 °C. NMR (D_2O) 2-CH_3 2.83; 3 × CH_2 4.58, 4.70, 4.95; 2 × C_6H_5 7.50, 7.52; 4-CH=CH_2 5.67–7.00 (m); 4-CH=CH_2 6.63–7.33 (m); 6H 8.38, IR: ν_{max}^{KBr} 1475, (C=C),

1210 cm^{-1} (*N*-oxide).

Anal. Calcd for $C_{23}H_{24}ClNO_3$: C, 69.41; H, 6.08. Found: C, 69.13; H, 6.23.

4-Vinyl-4-deformylpyridoxal N-Oxide (IX) Hydrochloride. The benzyl groups in VIII were hydrolyzed on refluxing with 4 N HCl for 20 h. The debenzylated *N*-oxide IX was crystallized from ethanol–ether; mp 118–120 °C, yield 76%; NMR (D_2O) 2-CH_3 2.45; 5-CH_2 4.67; 4-CH=CH_2 5.67–6.00 (m); 4-C*H*=CH_2 5.33–6.47; 6-H 8.17. IR: ν_{max}^{KBr} 1460 (C=C), 1205 cm^{-1} (*N*-oxide).

Anal. Calcd for $C_9H_{12}ClNO_3$: C, 49.60; H, 5.55; N, 6.43. Found: C, 49.37; H, 5.79; N, 6.33.

3-O-Methyl-4-vinyl-4-deformylpyridoxal (X). The vinyl analogue IIIa (30 mg, 0.15 mmol) was suspended in a 1:1 mixture of *tert*-butyl alcohol and ether and cooled to −25 °C (ethanol and dry ice), and an ethereal solution of diazomethane was added dropwise during 1 h. The solution was allowed to warm up overnight and the solvent was evaporated. The oily residue was treated with 5% $NaHCO_3$ solution and then the product was extracted with ethyl acetate. Drying ($MgSO_4$) and evaporation yielded a semisolid, which was converted to the hydrochloride (ethereal HCl), and the hydrochloride was crystallized from ethanol–ether (yield 21 mg, 54%, mp 130–132 °C).

Anal. Calcd for $C_{10}H_{14}ClNO_2$: C, 55.70; H, 6.53. Found: C, 55.43; H, 6.39.

3,α^5-O-Dibenzyl-4-(2-methylvinyl)-4-deformylpyridoxal (IIb). 3,α^5-*O*-Dibenzylpyridoxal (I, 1.0 g, 2.8 mmol) was allowed to react with the ylide generated from ethyltriphenylphosphonium bromide (1.89 g, 4.9 mmol) as described earlier for the synthesis of IIa (Korytnyk et al., 1973). The yield was 780 mg (75%) of a mixture of cis and trans isomers (3:5 ratio), as determined by integration of the methyl peaks, NMR ($CDCl_3$) C*H*$_3$CH= 1.50, 1.61 (d, cis), 1.87, 1.90, 1.98 (t, trans); 2-CH_3 2.52; 3 × CH_2 2.93, 3.13; CH=CH 6.00–6.67; 2 × C_6H_5 7.43, C_6-H 8.45 and 8.48 (trans and cis, respectively); IR: ν_{max}^{neat} 1590 (C=C), 1200 cm^{-1} (C—O—C).

Anal. Calcd for $C_{24}H_{25}NO_2$: C, 80.24; H, 7.02; N, 3.90. Found: C, 79.63; H, 7.01; N, 3.26.

The hydrochloride of the mixture had a mp of 113–115 °C.

Anal. Calcd for $C_{24}H_{26}ClNO_2$: C, 72.77; H, 6.62; Cl, 8.96. Found: C, 72.53; H, 6.61; Cl, 9.12.

4-(2-Methylvinyl)-4-deformylpyridoxal Hydrochloride (IIIb,c). Compound IIb (500 mg, 1.39 mmol) was heated on a steam bath for 28 h. After the solvent was evaporated in vacuo, the product was crystallized from ethanol. The crystals obtained (160 mg, mp 208–210 °C, dec) were found to contain the trans isomer as the major component. The mother liquor was evaporated and the residue was crystallized from a mixture of ethanol and ethyl acetate. The resulting crystals (120 mg, mp 170–172 °C, dec) contained the cis isomer as the major product. Repeated crystallizations of the impure trans isomer (from ethanol) and the cis isomer (from a mixture of ethanol and ethyl acetate) yielded pure trans and cis forms, with the following characteristics: Trans isomer (IIIc), mp 219–221 °C dec; NMR (D_2O): C*H*$_3$-CH=CH 1.87, 1.95 (d); 2-CH_3 2.53; 5-CH_2OH 3.03; C*H*=C*H* 3.07–3.20; 6-H 8.15; IR ν_{max}^{KBr} 1612 (C=C), 966 cm^{-1} (very strong, HC=CH trans).

Anal. Calcd for $C_{10}H_{14}ClNO_2$: C, 55.72; H, 6.54; Cl, 16.45. Found: C, 55.44; H, 6.27; Cl, 16.30.

Cis isomer (IIIb), mp 184–185 °C; NMR (D_2O) C*H*$_3$CH=CH 1.40, 1.48; 2-CH_3 2.57; 5-CH_2 4.62; C*H*=C*H* 3.75–3.80; 6-H 8.20; IR ν_{max}^{KBr} 1620 cm^{-1}, no strong bond between 870–1000 cm^{-1}.

Anal. Calcd for $C_{10}H_{14}ClNO_2$: C, 55.72; H, 6.54; Cl, 16.45. Found: C, 55.44; H, 6.58; Cl, 16.72.

4-(2-trans-Methylvinyl)-4-deformylpyridoxal 5′-Phosphate (IVc). Compound IIIc (150 mg, 0.7 mmol) was heated with a phosphorylating mixture (1.5 ml) consisting of P_2O_5 (1 part) and 85% H_3PO_4 (1.3 parts) at 65–75 °C for 5 h. The reaction flask was protected with a drying tube and the mixture was shaken at intervals. It was diluted with water (2 ml) and kept on a steam bath for 30 min. Hydrochloric acid (1 N, 10 ml) was added and the heating was continued for another 30 min to effect the hydrolysis of polyphosphates. After cooling, the reaction mixture was diluted with water (20 ml), Darco G-60 charcoal (2 g, washed previously with 1 N HCl) was added, and the solution was stirred for 10 min. After filtration, the charcoal residue was washed with water (ca. 100 ml). Subsequent washing with 2% ammonia solution eluted the compound (absorption at 330 nm was monitored). The washings were evaporated in vacuo to about 3 ml and applied to an Amberlite CG-50 column (H^+ form), the phosphate was eluted with water, the solution was evaporated to dryness in vacuo, and the residue was crystallized from water–ethanol, yielding 130 mg (71%) of IVc, mp 248–250 °C (dec); NMR (1 N NaOD) C*H*$_3$CH=CH 1.78, 1.87; 2-CH_3 2.22; 5-CH_2 4.68, 4.77 (d, J_{HP} = 5 Hz), CH=CH 3.78–3.90 (m) 6-H 7.60; IR ν_{max}^{KBr} 1600 (C=C), 1100 (RO–C), 950 cm^{-1} (HC=CH trans).

Anal. Calcd for $C_{10}H_{14}NO_5P$: C, 46.36; H, 5.45; N, 5.41. Found: C, 46.12; H, 5.52; N, 5.20.

4-(2-cis-Methylvinyl)-4-deformylpyridoxal 5′-Phosphate (IVb). Compound IIIb (200 mg) was phosphorylated essentially the same as IIIc, yielding 153 mg (64%) of the phosphate IVb, mp 220–222 °C; NMR (0.1 N NaOD) CH_3CH=CH 1.37, 1.45; 2-CH_3 2.45; 5-CH_2 4.73–4.83 (d, J_{HP} = 6 Hz), CH=CH 6.10–6.20 (m) 6-H 7.67, IR ν_{max}^{KBr} 1640 (C=C), 1000 cm^{-1} (P–O–C).

Anal. Calcd for $C_{10}H_{14}NO_5P$: C, 46.36, H, 5.45; N, 5.41. Found: C, 46.09; H, 5.21; N, 5.15.

4-Propyl-4-deformylpyridoxal (VIb). Compound III (mixture of cis and trans isomers, 50 mg) was dissolved in ethanol (5 ml) and hydrogenated at atmospheric pressure over Pd/C (10%) as a catalyst for 20 h. TLC indicated that some of the *trans* isomer remained unhydrogenated. The mixture was filtered and VIb was crystallized from ethanol–ether; yield: 35 mg (71%), mp 169–171 °C (dec), NMR (D_2O) C*H*$_3$CH$_2$ 0.73, 0.85, 1.47; $CH_3CH_2CH_2$ 1.13–1.67 (m), $CH_3CH_2CH_2$ 2.60, 2.73, 2.87 (t); 5-CH_2OH 4.83; 6-H 7.97.

Anal. Calcd for $C_{10}H_{16}ClNO_2$: C, 55.20; H, 7.41. Found: C, 55.50; H, 7.10.

Hydrogenation for another 20 h resulted in hydrogenolysis of the 5′-hydroxyl, giving the 5′-deoxy analogue of VIb (mp 149–151 °C), the structure of which was shown by NMR and mass spectrometry.

3-O-Benzyl-5-deoxypyridoxol (XII). Benzyldimethylphenylammonium chloride (1.5 g) in methanol (3 ml) was added to a solution of 150 mg of sodium in methanol (3 ml). 5-Deoxypyridoxol (XI, 600 mg) in methanol (9 ml) was now added to the reaction mixture, was left standing for 20 min, and then was added to hot toluene (kept at ca. 100 °C) (75 ml), while the volatile material was being distilled off until 40 ml of residual toluene was left. After cooling, toluene was decanted, and the residue was extracted with toluene and filtered. The combined toluene solutions were evaporated in vacuo and the oil was crystallized from acetone, yielding 522 mg (55%), mp 115–116 °C; NMR ($CDCl_3$) 2 × CH_3 2.48, 2.52; 2 × CH_2 4.90, 4.63; C_6H_5 7.45; 6-H 490; mass spectrum: M^+ peak at

241 nm.

Anal. Calcd for $C_{15}H_{17}NO_2$: C, 74.11; H, 7.04; N, 5.76. Found: C, 74.18; H, 6.99; N, 5.54.

3-O-Benzyl-5-deoxypyridoxal (XIII). To a solution of XII (300 mg) in dry chloroform (15 ml) was added MnO_2 (1.8 g; prepared by heating $MnCO_3$ at 280–300 °C for 24–36 h), and the suspension was stirred for 20 h at room temperature until no starting material remained, as indicated by TLC (ethyl acetate used for development). After filtration (Celite filter aid), and washing with hot chloroform and methanol, the combined solutions were evaporated to an oil, which was crystallized by addition of ether, yielding 278 mg (92%) of crystalline material, mp 73–74 °C.

Anal. Calcd for $C_{15}H_{15}NO_2$: C, 74.72; H, 6.27; N, 5.81. Found: C, 74.62; H, 6.67; N, 5.24.

3-O-Benzyl-4-vinyl-4-deformyl-5′-deoxypyridoxal (XIV) Hydrochloride. A mixture of potassium *tert*-butoxide (220 mg) and *tert*-butyl alcohol (1:1 by weight) was suspended in ether (anhydrous, 4 ml) and added to a solution of 1.6 M *n*-butyllithium (1.0 ml) at 0 °C under N_2, while stirring was maintained. After the addition of triphenylmethylphosphonium bromide (500 mg), the reaction mixture was allowed to reach room temperature and was stirred for another 2 h. Now, a solution of 3-*O*-benzyl-5-deoxypyridoxal (170 mg) in tetrahydrofuran (1 ml) was added during 5 min and stirring was continued overnight. The reaction mixture was diluted with ether and filtered, and the filtrate was washed with water, saturated sodium bisulfite solution, 40% ammonium chloride solution, and, finally, water. After drying ($MgSO_4$) and evaporating, the oil was subjected to absorption chromatography (silica gel; ethyl acetate was the eluent). The yield of the oily product was 110 mg (65%). It was characterized as the hydrochloride (treatment of an ethereal solution of the oil with ethereal HCl), mp 171–172 °C (dec). NMR ($CDCl_3$) 2 × CH_3 2.37, 2.47; 3-OCH_2 4.95; 4-CH=CH_2 6.58–7.00 (m), C_6H_5 7.43; 6-H 8.23.

Anal. Calcd for $C_{16}H_{18}ClNO$: C, 69.68; H, 6.57; Cl, 12.86. Found: C, 69.83; H, 6.79; Cl, 13.03.

4-Vinyl-4-deformyl-5′-deoxypyridoxal (XV) Hydrochloride. Compound XIV (40 mg) was dissolved in 1 N HCl (3 ml) and was heated on a steam bath for 1.5 h. Evaporation and crystallization (from ethanol–ether) yielded 26 mg (84%), mp 210–212 °C (dec). NMR (D_2O) 2 × CH_3 2.30, 2.50; 4-CH=CH_2 5.53–6.05 (m); 4-CH=CH_2 6.50–7.00; 6-H 7.97. IR: ν_{max}^{KBr} 3100 cm^{-1} (OH), 1620 cm^{-1} (C=C).

Anal. Calcd for $C_9H_{12}ClNO$: C, 58.25; H, 6.48.

Cell Culture Studies. the growth inhibitory effects of 4-vinyl analogues of pyridoxal were tested using two mouse cell lines, Sarcoma 180 (S-180) and mammary adenocarcinoma (TA3). S-180 cells were grown in monolayer culture in Eagle's medium (Eagle, 1959) from which pyridoxal hydrochloride was omitted and which was supplemented with 5% of horse serum. TA3 cells were grown in suspension culture in Eagle's medium modified to contain 0.1 μM pyridoxal hydrochloride (in place of the usual 5 μM pyridoxal) and supplemented with 10% of horse serum. Details concerning other aspects of the testing procedure have been described for S-180 (Hakala, 1971) and for TA3 (Jonàk et al., 1971).

Enzyme Studies

Pyridoxal Phosphokinase. Preparation. Livers of five male Sprague-Dawley rats (ca. 50 g) were cut with scissors into small pieces and homogenized with 100–150 ml of 0.2 M potassium phosphate buffer, pH 6.8, in a cold Waring Blendor for 5 min in a cold room at 4 °C. The homogenate was chilled in an ice bath for 30–40 min and centrifuged at 70 000*g* for 4 h at 4 °C. The enzyme in the red supernatant, which precipitated between 35 and 65% saturation with ammonium sulfate, was separated by centrifugation and suspended in 24 ml of 0.2 M phosphate buffer, pH 6.8, containing 0.1 mM reduced glutathione (Sigma). The suspension was dialyzed overnight against 3 l. of the same buffer and filtered through one layer of lintless cloth (Miracloth). A Sephadex G-150 column (5 × 70 cm) using an upward-flow technique, with 0.2 M phosphate buffer, pH 6.8, as the eluent, was run at an operating pressure of approximately 15 cm (water-level difference) for 2 days, and 2.5-ml fractions were collected. Fractions having the highest enzyme were combined and used without further purification. The protein content averaged 5 mg/ml and the specific activity varied from 27 to 77 nmol of pyridoxal phosphate (mg of protein)$^{-1}$ h^{-1}, depending on the batch.

Enzyme Assay. When pyridoxal was used as the substrate, the activity of pyridoxal phosphokinase was determined by the method of Wada and Snell (1961), as modified by Tsubosaka and Makino (1969). The reaction mixture in a total volume of 2.5 ml contained 0.15 mM ATP, 0.01 mM $ZnSO_4$, 0.2 M potassium phosphate buffer, pH 5.52, kinase preparation, and 5–70 μM pyridoxal. It was shaken in a water bath at 37 °C for 1 h and the reaction was stopped by the addition of 0.3 ml of 100% (w/v) trichloroacetic acid. After centrifugation, 2.3 ml of cold water, 0.5 ml of 18 N H_2SO_4, and 0.2 ml of phenylhydrazine solution (1 g of recrystallized phenylhydrazine-HCl in 50 ml of 10 N H_2SO_4) were added to a 1-ml aliquot of the supernatant and the mixture was allowed to stand for 30 min in ice. Absorption at 410 nm was determined against a blank devoid of kinase.

Substrate Activity Determination. When 4-vinyl-4-deformyl[β^4-^{14}C]pyridoxal (0.42 mCi/mmol) was tested as a substrate of pyridoxal phosphokinase, the reaction mixture was identical to the one above, except that the reaction was stopped with 0.5 ml of 4.5 N $HClO_4$ and the resulting mixture was neutralized with 0.6 ml of 4.5 N KOH. The precipitated potassium perchlorate was removed by centrifugation and the supernatant was concentrated to a small volume (0.3–0.5 ml), so that most of the $KClO_4$ was still dissolved. The sample was applied to an Amberlite CG-50 (200–400 mesh) column in the H^+ form. The column, consisting of a Pasteur pipet (146 × 7 mm) with a glass-wool plug at the bottom, was eluted with distilled water until no more radioactive material was detected in the eluate and then with 0.1 N HCl until no more counts were detected. Aliquots of 0.1 ml were counted in 10 ml of Bray's solution (Bruno and Christian, 1961) and the counts were plotted against the eluted volume.

When nonradioactive analogues of pyridoxal were tested as substrates of pyridoxal phosphokinase, [γ-^{32}P]ATP (Amersham, 950 mCi/mmol) was used at the second substrate. The reaction mixture was concentrated as above and spotted on PEI-cellulose plates, developed with 1 M LiCl (Randerath and Randerath, 1964). The chromatograms, dried under cold air, were cut into 1-cm sections and counted in 10 ml of Bray's solution in a Packard Tri-Carb liquid scintillation spectrometer. The counts were plotted against the distance from the origin on the plates.

High-pressure liquid chromatography was employed for K_M determination. A Bondapak AX/Corasil anion-exchange column (2.3 mm i.d. × 610 mm length) was used in conjunction with a Waters ALC-202 high-pressure liquid chromatograph equipped with a UV detector operating at 254 nm. The eluent was 0.125 M $NH_4H_2PO_4$ buffer, pH 3, and the pressure was 580 psig, with a flow rate of 1 ml/min. A chromatogram

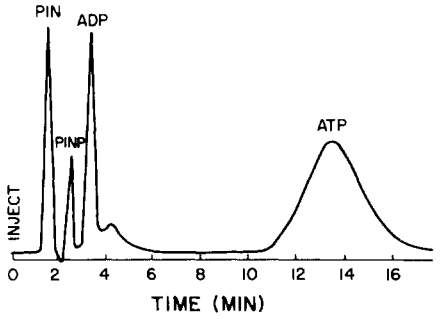


FIGURE 1: High-pressure liquid chromatogram of a mixture consisting of pyridoxine (PIN), pyridoxine 5′-phosphate (PINP), ADP, and ATP using a Bondapak AX/Corasil anion-exchange column.

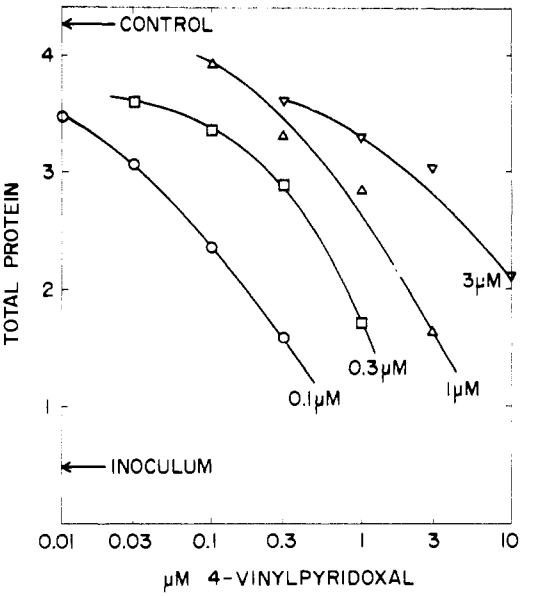


FIGURE 2: Competitive relationship between PAL and 4-VPAL with respect to the growth of TA3 cells in 4 days. The numbers next to the lines indicate the concentrations of PAL in the media. Control indicates the growth in 0.1 μM PAL in the absence of 4-VPAL and inoculum the amount of cells at the start. For details see Materials and Methods.

of an assay mixture is shown in Figure 1. The peaks were identified by cochromatography of known compounds.

The reaction, carried out as described, was stopped with $HClO_4$, followed by neutralization. After centrifugation, a 2.0-ml aliquot was evaporated to dryness at reduced pressure, a water pump being used at room temperature. The residue was dissolved in 0.5 ml of ammonium phosphate buffer, described above, and was injected into the column with a microsyringe. A standard curve for ADP, based on either the weight of the cut-out peaks, or their height, was prepared using known mixtures of ADP and ATP (without the enzyme) in identical conditions. The calibration curve for ADP, based on the height of the peaks, was chosen for convenience.

Pyridoxine-phosphate oxidase was prepared from rat liver as described earlier (Korytnyk et al., 1972). Pyridoxal phosphate formed has been estimated colorimetrically by the phenylhydrazine procedure of Wada and Snell (1961). The specific activity was 857 nmol of pyridoxal phosphate formed (mg of protein)$^{-1}$ h^{-1}.

Results

Synthesis. The syntheses described (Schemes I and II) utilized the general methods for modification of the 4 position of the pyridoxine molecule, made possible by the introduction of benzyl blocking groups in the 3 and 5 positions (Korytnyk and Ikawa, 1970).

^{14}C-labeled 4-VPAL was prepared by reacting radioactive Wittig reagent (obtained by reacting $C^{14}H_3Br$ with triphenylphosphine) with dibenzylpyridoxal (I), as shown in Scheme I. 4-VPAL (IIIa) was phosphorylated with polyphosphoric acid to the cofactor analogue IVa. The corresponding 4-ethyl analogue VIa was obtained by hydrogenation of the fully blocked intermediate IIa, yielding V, which was subsequently treated with acid to remove the 5-*O*-benzyl group. Alternatively, 4-VPAL (IIIa) was hydrogenated to give VIa, which was phosphorylated, giving VII. The *N*-oxide of IIIa, and its *O*-methyl derivative X, were obtained by modifications of standard methods. In order to explore the geometry of the binding sites of various enzymes, we have also used the Wittig reaction to prepare the *cis*- and *trans*-methylvinyl analogues (IIIb and IIIc) and their 5′-phosphates (IVb and IVc). Separation into pure cis and trans isomers (IIIb and IIIc) was achieved by fractional crystallization of the deblocked products. Assignment of structure was based primarily on the expected steric inhibition of resonance in the cis isomer, which is expressed in the shift of its UV-absorption bands towards shorter wavelengths (Yang et al., 1975) and the upfield shift of the β-methyl resonance in its NMR spectrum. A strong infrared absorption band at 966 cm^{-1} in the trans isomer and the absence of such a band in the cis isomer also lend support to the assignment (Rasmussen and Brattain, 1947). During hydrogenation of a mixture of cis and trans isomers (IIIb and IIIc) over 10% Pd/C as a catalyst, the cis form was completely converted to the saturated compound VIb, leaving behind the trans isomer partially unreacted. This result is due to the adsorption of the double bond on the catalyst being less hindered in the cis compound than in the trans isomer, and this finding again supports the assignment made.

Cell Culture Studies. Most of the pyridoxal analogues had no effect on cells grown in Eagle's medium, which normally contains 5 μM pyridoxal HCl. To observe any effect of these analogues on cellular multiplication, the medium had to be modified so as to contain limiting levels of the vitamin. It was found that S-180 cells grew normally, even in the total absence of pyridoxal, if the medium was supplemented with 5% of horse serum. It appears that the serum supplement provided an amount of vitamin B_6 adequate to support the growth of these cells. This was clearly indicated also by the observation that inhibition of the growth of S-180 cells by 4-deoxypyridoxine HCl in this medium was completely prevented either by raising the serum concentration or by adding pyridoxal into the medium (Hakala, unpublished). TA3 cells differed from S-180 by having a distinct requirement for 0.1 μM pyridoxal-HCl, in addition to 10% horse serum. Table I lists the concentrations of the compounds required to produce 50% inhibition of growth of the two cell lines and shows that S-180 cells were about ten times more sensitive than TA3 cells.

Results of a study of competition between 4-VPAL and PAL for the growth of TA3 cells are shown in Figure 2. This study demonstrated that, when the concentration of PAL in the culture medium was increased from 0.1 to 3 μM, the concentration of 4-VPAL had to be correspondingly increased to produce inhibition of growth. The average molar ratio of pyridoxal to 4-VPAL in these varied conditions was 0.7 when the growth was half of the control growth.

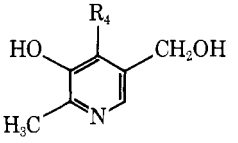
Pyridoxal Phosphokinase. [^{14}C]VPAL was found to be a substrate of pyridoxal phosphokinase, obtained from rat liver.

TABLE I: Growth Inhibition of Cultured Cells by 4-Vinyl Analogues and Related Compounds.[a]

Compound	R_4	R_5	Additional Groups	TA 3 Cells ID_{50} μM	S-180 Cells ID_{50} μM
4-Deoxypyridoxine	CH_3	CH_2OH		1.0	0.1
IIIa	$CH=CH_2$	CH_2OH		0.1	0.015
IVa	$CH=CH_2$	$CH_2OPO_3^{2-}$		Not tested	0.025
X	$CH=CH_2$	CH_2OH	3-O Me	100[b]	
XV	$CH=CH_2$	CH_3		100	
VIa	CH_2CH_3	CH_2OH		100	
VII	CH_2CH_3	$CH_2OPO_3^{2-}$		15	
IX	$CH=CH_2$	CH_2OH	N→O	56	
IIIc	*trans*-$CH=CHCH_3$	CH_2OH		0.72	
IVc	*trans*-$CH=CHCH_3$	CH_2OPO_3		3	
IIIb	*cis*-$CH=CHCH_3$	CH_2OH		32	
IVb	*cis*-$CH=CHCH_3$	$CH_2OPO_3^{2-}$		10	
VIb	$CH_2CH_2CH_3$	CH_2OH		70	

[a] ID_{50} indicates the concentration of the compound which caused 50% inhibition of growth in conditions described under Materials and Methods. [b] 25% inhibition.

TABLE II: Pyridoxal Phosphokinase: Substrate and Inhibition Data of Analogues.



Compound	R_4	Substrate Activity	K_i (μM)[a]	Type of Inhibition
4-Deoxypyridoxine	CH_3		360	Competitive[b]
IIIa	$CH=CH_2$	K_M 40 μM[c]	14	Competitive[b]
VIa	CH_2CH_3	K_M 69 μM[c]	>100	
XV	$CH=CH_2$ (deoxy)	Slight[d]	40	Competitive[b]
X	$CH=CH_2$ (3-O-Me)	Substrate[d]	240	Noncompetitive[b]
IX	$CH=CH_2$ (*N*-oxide)	Slight[d]	>100	
IVc	*trans*-$CH=CHCH_3$	Substrate[d]	18	Noncompetitive[b]
IVb	*cis*-$CH=CHCH_3$	Substrate[d]	35	Noncompetitive[b]

[a] Phenylhydrazine procedure was used (see Materials and Methods). [b] From Lineweaver–Burk plots. [c] From high-pressure liquid chromatography. The values are uncertain, due to substrate inhibition. [d] Qualitative studies on TLC (see Materials and Methods).

The identity of radioactive 5′-phosphate of 4-VPAL, isolated by chromatography on an Amberlite CG-50 column, was confirmed by comparing its R_f value on thin-layer chromatography (TLC) with that of chemically synthesized 5′-phosphate (Scheme I, IVa). The capacity of other analogues to act as substrates of this enzyme was examined by using [γ-^{32}P]ATP, as the record substrate followed by separation of the phosphorylated analogue by TLC, as described under Materials and Methods. In this way, it was shown that analogues IX, X, IVb, and IVc were also substrates. Surprisingly, the 5-deoxy analogue XV was also phosphorylated approximately 20% as compared with 4-VPAL, but the structure of the product was not established.

To follow the enzymatic phosphorylation quantitatively, a method using high-pressure liquid chromatography (HPLC) was developed. As seen in Figure 1, ADP was separated from ATP and the vitamin B_6 compounds, and could be quantitated by the height of the peak. Figure 3A shows how the reaction velocity measured using this method depended on the concentration of pyridoxal, and reveals a K_M of 180 μM, a value in good agreement with the 130 μM obtained colorimetrically using phenylhydrazine. Tsubosaka and Makino (1969) found K_M = 86 μM for this reaction. When HPLC was applied to 4-VPAL and to 4-ethyl-PAL for their K_M determination, the results show that, at 10 and 25 μM and above, respectively, these analogues inhibited their own phosphorylation. This phenomenon permitted only a rough estimation of the respective K_M values (Figure 3B).

Being a substrate of pyridoxal phosphokinase, 4-VPAL was expected also to compete with pyridoxal for phosphorylation. Indeed, 40 μM 4-VPAL, in the presence of varied concentrations of pyridoxal (42 to 330 μM), was a relatively potent competitive inhibitor, with a K_i of 14 μM, which has been determined from Lineweaver–Burk plots. The presence of pyridoxal analogues in the reaction mixture did not interfere with the colorimetric analysis. The discrepancy between the K_M (40 μM) and K_i (14 μM) may reflect the self-inhibitory property of the analogue. Table II summarizes both the substrate activity studies and the K_i values.

Pyridoxine-Phosphate Oxidase. The extent to which the phosphorylated pyridoxal analogues inhibit the oxidation of pyridoxine phosphate by pyridoxine-phosphate oxidase were determined using a partially purified preparation of the oxidase (Korytnyk et al., 1972). The Lineweaver–Burk plots show a competitive inhibition for 4-VPAL-5′-P (IVa, determined at 20 to −67 μM) and for *cis*- and *trans*-methylvinyl phosphates (IVb and IVc, determined at 100 μM each), VII (determined at 49 μM) was a poor inhibitor, and its inhibition was non-

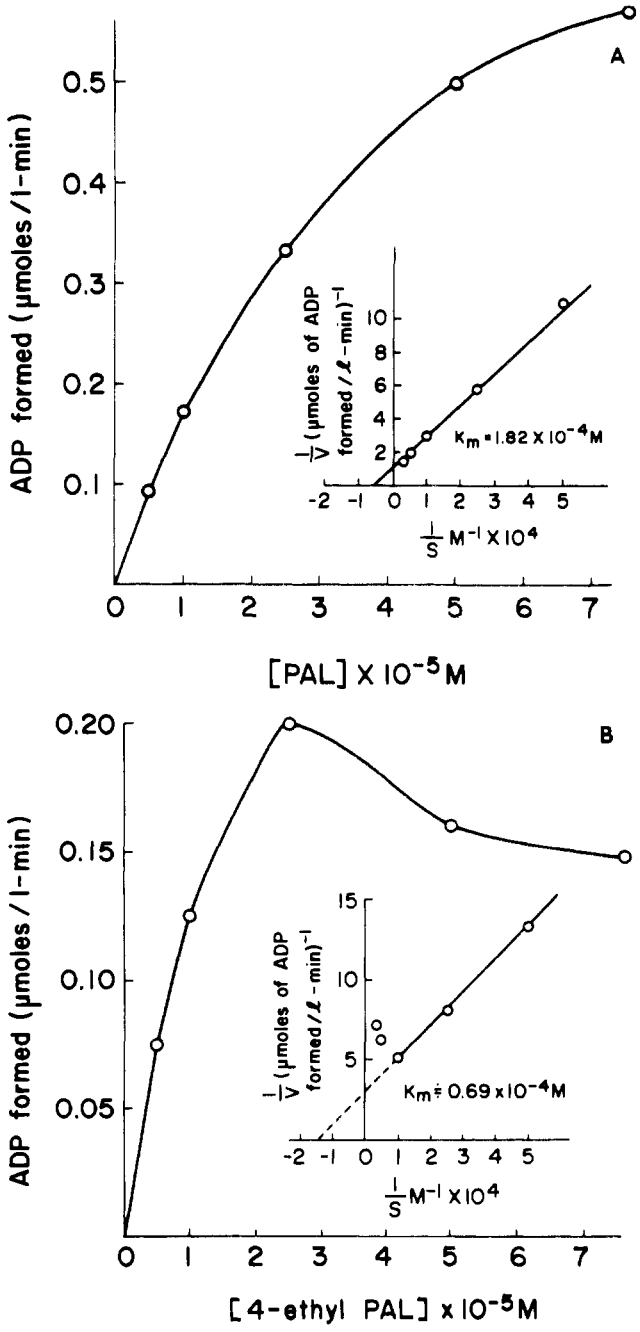


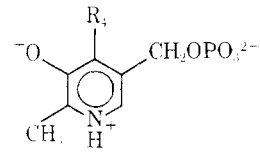
FIGURE 3: (A) The velocity of pyridoxal (PAL) phosphorylation catalyzed by rat liver pyridoxal phosphokinase (1.4 mg of protein/ml) as affected by substrate concentration. The determinations were carried out using HPLC as described under Materials and Methods. (B) Phosphorylation of 4-ethyl-PAL (VIa) as determined by HPLC method as in Figure 3A.

competitive. The results of these studies have been summarized and compared with the extent of inhibition by 4-DOP-5′-P in Table III.

Discussion

Adaptation of the cell-culture technique to the study of vitamin B_6 antagonists made possible the testing of hundreds of vitamin B_6 analogues that have been developed in the last 35 years. The most potent inhibitor found so far was 4-VPAL (IIIa). Its potency exceeds that of 4-DOP by one order of magnitude for the two cell lines investigated (Table I). An even greater potency of 4-VPAL, as compared with 4-DOP, is in

TABLE III: Pyridoxine Phosphate Oxidase: K_i Values of Analogues of Vitamin B_6 Modified at The 4-Position.



Compound	R_4	K_i (μM)	Type of Inhibition[a]
4-DOP-5′-P	CH_3	1.8	Competitive
IVa	$CH{=}CH_2$	0.53	Competitive
VII	CH_2CH_3	50	Noncompetitive
IVb	*cis*-$CH{=}CHCH_3$	53	Competitive
IVc	*trans*-$CH{=}CHCH_3$	25	Competitive

[a] Determined from Lineweaver–Burk plots.

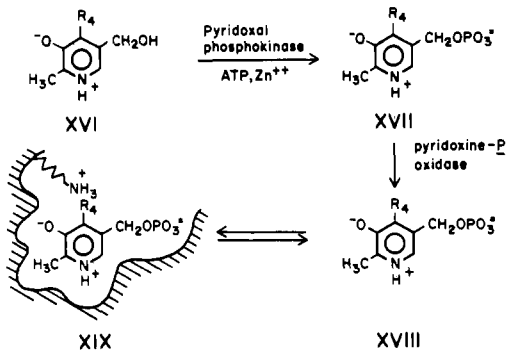
evidence from in vivo experiments (Korytnyk et al., 1973). Both analogues, however, can be readily reversed by vitamin B_6 both in vivo and in cell culture, and their activity in cell culture could be demonstrated only in media deficient in vitamin B_6. In a quantitative study of the reversal of growth inhibition by 4-VPAL (Figure 2), a family of dose–response curves for half-maximal growth was obtained which indicated a competitive relationship between pyridoxal and 4-VPAL. On the average, 0.7 mol of pyridoxal reversed the growth-inhibitory activity of 1 mol of 4-VPAL.

Since 4-VPAL was found to be a potent growth inhibitor of two mouse tumor cell lines, it was of interest to explore the basis and the structural specificity of the inhibition. As a growth inhibitor, the 5′-phosphate of 4-VPAL (IVa) was as active as the nonphosphorylated form. This may indicate dephosphorylation of the analogue at the cell surface by phosphatases, as has been shown for pyridoxal phosphate (Anderson et al., 1971). Methylation of the phenolic OH (X) almost abolished the growth-inhibitory activity, and N oxidation to IX reduced it considerably. Saturation of the double bond in 4-VPAL results in VIa, a homologue of 4-DOP. This change reduced the growth-inhibitory activity drastically. The phosphorylated 4-ethyl analogue VII was considerably more active as an inhibitor of growth than was the nonphosphorylated parent compound VIa. This increased activity suggests at least a partial penetration of the intact phosphorylated forms into the cells. Introduction of a methyl substituent on the β carbon of the vinyl group, as in IIIb and IIIc, decreased the growth-inhibitory activity of the parent compound. The extent of the decrease is dependent on the geometry of the substituent. The *trans*-methylvinyl compound IIIc, in which the coplanarity of the system is maintained, is still a potent growth inhibitor, whereas the *cis*-methylvinyl derivative (IIIb, out of plane) is considerably less so. Phosphorylation of the *trans* analogue decreased the growth-inhibitory activity of the analogue, whereas the opposite was true of the cis compound. Because the permeability characteristics and the metabolic fate of the phosphorylated analogues in cultured cells are not certain, they cannot be considered in regard to the structure–activity relationships in this system.

Replacement of the 5-OH in 4-VPAL with H, as in XV, also abolished the growth-inhibitory activity. This observation is consistent with the hypothesis that phosphorylation of the analogues is essential for inhibition of the growth of cells, and that the growth-inhibitory data could be rationalized in terms of the interactions of the analogues with enzymes involved in

the anabolism of pyridoxal phosphate. It has been established that the formation of pyridoxal phosphate in cells of higher organisms proceeds via the initial phosphorylation of the three forms of the vitamin (Scheme III, XIV, R_4 = CHO, CH_2NH_2, CH_2OH), which is followed by oxidation of the substituent in the 4 position to the formyl group (XVIII, R_4 = CHO). The pyridoxal phosphate thus formed binds to various apoenzymes, as in the case of XIX (Snell and Haskell, 1971). It has been shown that analogues of vitamin B_6, such as 4-DOP, are substrates of pyridoxal phosphokinase and, as such, they may effectively compete for the enzyme with the three forms of vitamin B_6 (McCormick et al., 1961). We have established that 4-VPAL (IIIa) and some of its analogues are substrates of pyridoxal phosphokinase (Table II). At higher substrate concentrations ($1-3 \times 10^{-5}$ M), both 4-VPAL and its saturated analogue (VIa) inhibited their own phosphorylation, as was shown in Figure 3B. A similar phenomenon was observed for 4-DOP by Tsubosaka and Makino (1969). When 4-VPAL was tested as an inhibitor of the phosphorylation of pyridoxal, the inhibition, as expected, was competitive (K_i = 14.3 μM). The ethyl analogue, on the other hand, did not inhibit the enzyme even at 100 μM. While 4-VPAL is the most potent inhibitor in this series, the *trans*-methylvinyl analogue (IIIc) was only slightly less active and the cis-isomer (IIIb) much less so.

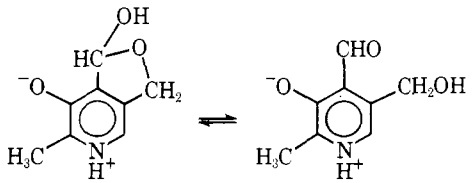
Scheme III



XVI XVII XIX XVIII

Structurally, 4-VPAL and its β-*trans*-methylvinyl derivative approach the open-chain form of pyridoxal (XXI). By both UV and NMR spectroscopy, however, it has been shown (Metzler and Snell, 1955; Korytnyk and Ahrens, 1970; Harris et al., 1976) that under physiological conditions pyridoxal exists predominantly in the cyclic hemiacetal form (XX). Although pyridoxal can be assumed to form the enzyme–substrate complex initially in the hemiacetal form (XX), during phosphorylation the transition state most likely resembles the open-chain form XXI. Thus, compounds resembling the π-electron system of XXI, such as 4-VPAL and oximes and hydrazides of pyridoxal (McCormick and Snell, 1961), could be regarded as the transition-state analogues of pyridoxal phosphokinase (Wolfenden, 1972; Lienhard et al., 1971). This may account for the superior inhibitory activities of these analogues and the loss of those activities on saturation of the double bond.

Scheme IV



XX XXI

The phosphorylation, even though slight, of the 5-deoxy analogue of 4-VPAL (XV) was surprising and may have been due either to a lack of specificity of the enzyme or to the presence of unrelated enzymes in the preparation. A similar observation regarding 5-deoxypyridoxine has been made by Tsubosaka and Makino (1969). Although the structures of these phosphorylated analogues have not been established, their dissimilarity from the natural cofactor precludes their interference with further metabolism of vitamin B_6.

Having established that 4-VPAL and its derivatives that have the 5′-OH group unsubstituted are substrates of pyridoxal phosphokinase, we proceeded to investigate the inhibition of pyridoxine-phosphate oxidase by the corresponding phosphorylated analogues. In these studies, a 184-fold purified preparation of the oxidase was used (Korytnyk et al., 1972); a higher purification of the enzyme has been achieved more recently (Kazarinoff and McCormick, 1973, 1975). 4-VPAL-5′-P (IVa) was found to be a competitive inhibitor of the enzyme, with a K_i value of 0.53 μM. Another potent inhibitor of the enzyme is the structurally related oxime of pyridoxal phosphate (Wada and Snell, 1961). Saturation of the double bond in VII decreased the binding to about 100th with respect to 4-VPAL-5′-P with a noncompetitive type of inhibition. Both *cis*- and *trans*-β-methylvinyl analogues were found to be competitive inhibitors, but much less potent. Since it has been established that the enzyme has a requirement for dianionic phosphate for both substrate and inhibitor activity (Wada and Snell, 1961; Korytnyk et al., 1972), in vivo phosphorylation of the analogue is the prerequisite for its becoming an active inhibitor against this enzyme in the cell. Pyridoxine-phosphate oxidase has been postulated to have an important regulatory function in vitamin B_6 metabolism (Snell and Haskell, 1971) and, hence, the effects of its inhibition in higher organisms could have profound effects.

Phosphorylated analogues can also effectively compete for the cofactor sites of many enzymes requiring pyridoxal phosphate (reviewed by Snell, 1970). 4-VPAL-5′-P (IVa) and its β-methyl derivatives (IVb and c) have been found to bind to apoaspartate aminotransferase (Yang et al., 1975). Under the usual conditions, the binding was found to be reversible. When the apoenzyme was pretreated with α-ketoglutarate, however, an irreversible inhibition with ^{14}C-labeled 4-VPAL-5′-P could be demonstrated by gel filtration, after which a considerable part of the analogue remained bound to the protein. Subsequent tryptic digestion under standard conditions did not lead to complete release of the ^{14}C label (Korytnyk et al., 1974). A tight, possibly irreversible binding of 4-VPAL-5′-P to glutamate decarboxylase has also been observed (Fonda, 1970). 4-VPAL-5′-P binds about as tightly to apo-L-arginine decarboxylase ($K_i = 2.5 \times 10^{-8}$ M), but no effect was seen with apo-D-serine dehydratase, even at a 500-molar excess of the analogue over pyridoxal phosphate (Dr. E. E. Snell, personal communication). Thus, the analogue shows considerable selectivity with respect to different apoenzymes.

In spite of a limited amount of data, we wish to propose two conditions that appear to dominate in determining which of the analogues in the present series has a substantial growth inhibitor activity: (1) substrate activity for pyridoxal phosphokinase with a relatively low K_M value, and (2) inhibition of pyridoxine-phosphate oxidase by the phosphorylated analogue with a low K_I value. These requirements ignore the role of cellular permeability and the possible interactions with various other enzymes requiring pyridoxal phosphate, factors

that are probably important but not dominating with respect to growth inhibition caused by this series of analogues. Thus, we have shown that 4-VPAL, as well as 4-DOP, satisfies these requirements and that 4-VPAL and its 5′-phosphate bind more tightly to pyridoxal phosphokinase and pyridoxine-phosphate oxidase, respectively, than do the corresponding 4-DOP analogues. Although the 4-ethyl analogue (VIa) is a substrate of pyridoxal phosphokinase, it binds poorly to the kinase and, likewise, its phosphate (VII) to the oxidase. The same thing is true of the 3-*O*-methyl derivative X and the *N*-oxide IX as far as the kinase is concerned and, hence, these analogues are poor growth inhibitors. The β-methylvinyl derivatives IIIb and IIIc satisfy the two requirements for inhibition, but show decreased binding to the two enzymes. This is particularly true for the cis isomer and this is reflected in relatively poorer growth inhibition of this isomer with respect to its trans form, and both isomers with respect to 4-VPAL.

While oximes and hydrazones of pyridoxal and isopyridoxal are known to be excellent inhibitors of pyridoxal kinase, they were shown to be generally poor growth inhibitors. This is probably due to their lack of substrate activity for this enzyme, again supporting the view that the inhibition of the oxidase is a prerequisite for the growth inhibitory activity.

Earlier microbial test systems, such as *S. carlsbergensis*, were used for testing of vitamin B_6 analogues. In this system, 4-DOP was found to be a potent antagonist. We have found that extension of the 5-hydroxymethyl group by one or two methylene groups resulted in antagonists which were more potent than 4-DOP (Korytnyk et al. 1967). However, these compounds were found to be ineffective as growth inhibitors in cell culture (M. T. Hakala, unpublished) and in vivo (E. Mihich, unpublished). This result is not surprising in view of the difference in metabolism of vitamin B_6 in microbial and mammalian systems (Snell and Haskell, 1971).

Although the 4-vinyl group in 4-VPAL and in its 5′-phosphate was found to be relatively inert with respect to the enzymes investigated, the enhanced potency of it, as compared with 4-DOP, is probably due to its close similarity to the 4-formyl group of pyridoxal and its 5′-phosphate in terms of both geometry and the π-electron system. The vinyl group in 4-VPAL is less reactive than in 2- and 4-vinylpyridines because of decrease of its nucleophilic character by the 3-phenolic group ortho to it. Subsequently, the acetylenic analogue of 4-VPAL-5′-P has been synthesized and found to bind irreversibly with apoaspartate transaminase (Yang et al., 1975).

Acknowledgments

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