# SYNTHESIS OF ANALOGS OF PYRIDOXAL 5'-PHOSPHATE AND PYRIDOXAMINE 5'-PHOSPHATE

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The following analogs of pyridoxamine 5'-phosphate (PAMP) have been synthesized by the direct phosphorylation of the corresponding amines: 2-nor-PAMP, 6-methyl-2-nor-PAMP, and 6-methyl-PAMP. A method for the synthesis of analogs of pyridoxal 5'-phosphate (PLP) by the phosphorylation of the Schiff's bases of the corresponding aldehydes with p-phenetidine and subsequent hydrolysis on a sulfonated resin has been worked out. 2-Nor-PLP, 6-methyl-2-nor-PLP, and 6-methyl-PLP have been obtained with yields of 58–73%. The spectral properties of the compounds obtained have been investigated.

Pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PAMP) form the coenzymes of a large number of enzymes catalyzing various transformations of amino acids [1, 2]. In order to study the nature of the bond of PLP with the apoenzyme, we undertook the synthesis of analogs of PLP and PAMP modified at positions 2 and 6 of the pyridine ring.

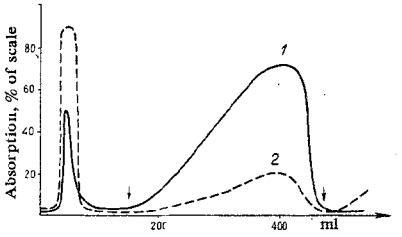
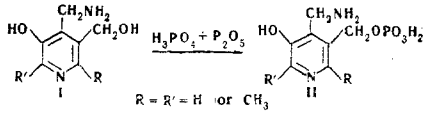



Fig. 1. Isolation of 2-nor-PLP on Dowex 50W × 4 in the acid form: 1) absorption at 295 nm; 2) electrical conductivity.

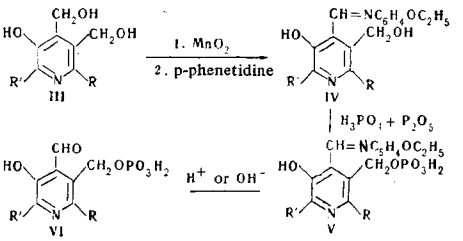
The preparation of PAMP, which, together with PLP, is a coenzyme of the transaminases, by the direct phosphorylation of pyridoxamine with a yield of about 70% has been described in the literature [3, 4]. So far as concerns PLP itself, none of the methods described can give it with a yield of more than 40%, and in a number of cases they start from the more accessible PAMP [3]. The synthesis of C-substituted analogs of PLP and PAMP has not been effected*.

It appeared to us to be desirable to start from the preparation of the analogs of PAMP, which were synthesized by the phosphorylation of the previously-obtained amines [6] in the following way:




The presence of two ortho substituents (positions 2 and 6) creates definite difficulties in phosphorylation. For the synthesis of the PLP analogs we used a method proposed previously [7–9] for obtaining PLP itself—the phosphorylation of Schiff's bases (SB) of pyridoxal. As the initial materials we used pyridoxine analogs [6].

The diol III (R = R' = H or $CH_3$) was oxidized with manganese dioxide in dilute sulfuric acid, and by adding a mixture of p-phenetidine and sodium acetate to the resulting mixture the SB was isolated with a yield of 38–79%, calculated on the diol.




The phosphorylation of the SB was effected with a mixture of 85% phosphoric acid and phosphorus pentoxide (1 : 3 : 1.0). The conditions for performing the reaction depend on the structure of the SB. The presence of a methyl group in position 6 of the pyridine ring creates steric hindrance in esterification. 6-Methylpyridoxylidene-p-phenetidine (IV, R = R' = $CH_3$) was phosphorylated by heating at 60° C for 6 hr, while for

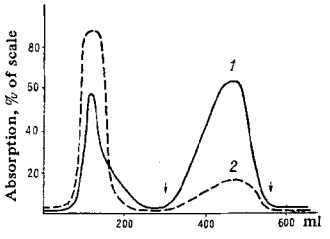



Fig. 2. Isolation of 6-methyl-PLP on Dowex 50W × 4 in the acid form. Symbols as for Fig. 1.

the phosphorylation of 2-norpyridoxylidene-p-phenetidine (IV, R = R' = H) the optimum conditions were heating at 40° C for 4 hr. The pyrophosphates formed were hydrolyzed by heating with 0.1 N hydrochloric acid at 60° C. It was possible to precipitate the SB phosphate (V, R = R' = H) in the solid form by bringing the pH of the reaction mixture to 3.

In 2 N NaOH solution compound V decomposes and by extraction with ether it is possible to remove the

*When the present work was completed, we received a letter from Prof. E. E. Snell (USA) [5] on the synthesis of 2-nor-PLP and 2-ethyl-2-nor-PLP.

Table 1
UV Spectra of Analogs of PLP and PAMP

| Compound | $\lambda_{max}$, nm ($\varepsilon \cdot 10^{-3}$) | | |
|---|---|---|---|
| | 0.1 *N* HCl | pH 6.9** | 0.1 *N* KOH |
| Pyridoxal 5′-phosphate* monohydrate | 295 (6.7)<br>338 (1.4) | 330 (2.500)<br>388 (4.900) | 305 (1.1)<br>388 (6.6) |
| 2-Norpyridoxal 5′-phosphate monohydrate | 292 (6.0)<br>332 (1.1) | 330 (2.7)<br>383 (3.2)*** | 305 (1.0)<br>386 (5.6) |
| 6-Methyl-2-norpyridoxal 5′-phosphate monohydrate | 298 (6.2) | 330 (4.4)<br>390 (1.1)*** | 312 (3.9)<br>394 (2.8) |
| 6-Methylpyridoxal 5′-phosphate monohydrate | 302 (7.1)<br>350 (1.3) | 332 (4.6)<br>390 (2.5) | 313 (2.7)<br>398 (4.3) |
| Pyridoxamine 5′-phosphate dihydrate* | 293 (9.0) | 253 (4.7)<br>325 (8.3) | 245 (6.7)<br>308 (8.0) |
| 2-Norpyridoxamine 5′-phosphate dihydrate | 292 (7.1) | 248 (3.7)<br>288 (3.4)<br>324 (3.6) | 243 (7.3)<br>307 (5.2) |
| 6-Methyl-2-norpyridoxamine 5′-phosphate dihydrate | 299 (6.3) | 252 (3.7)<br>297 (1.9)<br>333 (3.7) | 246 (7.0)<br>313 (5.0) |
| 6-Methylpyridoxamine 5′-phosphate dihydrate | 302 (9.7) | 252 (6.3)<br>336 (8.9) | 248 (7.7)<br>314 (8.0) |

*Spectra taken from the literature [12].

**0.1 N phosphate buffer.

***Inflection on the curve.

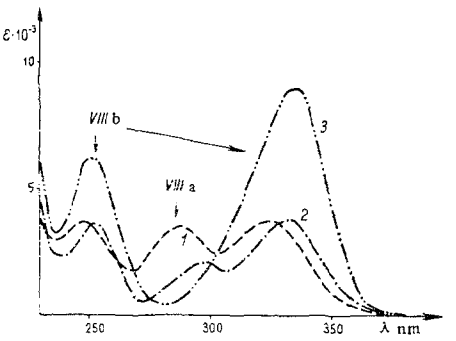



Fig. 3. UV spectra of analogs of pyridoxamine 5-phosphate at pH 7: 1) 2-nor-PAMP; 2) 6-methyl-2-nor-PAMP; 3) 6-methyl-PAMP.

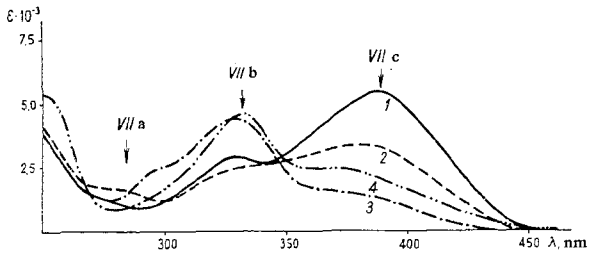



Fig. 4. UV spectra of pyridoxal 5-phosphate and its analogs at pH 7; 1) PLP (commercial sample); 2) 2-nor-PLP; 3) 6-methyl-2-nor-PLP; 4) 6-methyl-PLP.

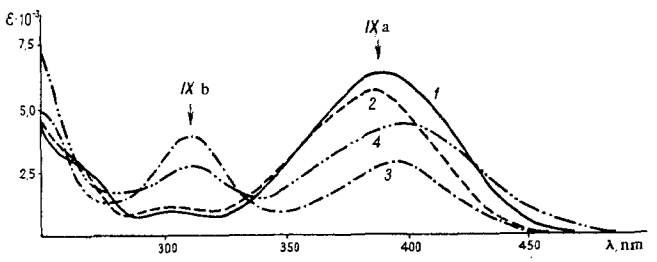



Fig. 5. UV spectra of PLP and its analogs in 0.1 N KOH. Symbols the same as in Fig. 4.

Table 2
Absorption of the PLP Analogs in Acid Phenylhydrazine at 410 nm

| Compound | ε |
|---|---|
| Pyridoxal 5′-phosphate | 22000* |
| 2-Norpyridoxal 5′-phosphate | 18900 |
| 6-Methyl-2-norpyridoxal 5′-phosphate | 20300 |
| 6-Methylpyridoxal 5′-phosphate | 20000 |

*Commercial sample.

Table 3
Analogs of Pyridoxamine 5′-Phosphate

| Compound | Empirical formula | Found, % | | | Calculated, % | | | Yield, % |
|---|---|---|---|---|---|---|---|---|
| | | C | H | N | C | H | N | |
| 2-Norpyridoxamine 5′-phosphate | $C_7H_{11}N_2O_5P \cdot 2H_2O$ | 30.88 | 5.69 | 10.11 | 31.12 | 5.60 | 10.36 | 65 |
| 6-Methyl-2-norpyridoxamine 5′-phosphate dihydrate | $C_8H_{13}N_2O_5P \cdot 2H_2O$ | 33.39 | 5.84 | 9.51 | 33.82 | 6.03 | 9.85 | 51 |
| 6-Methylpyridoxamine 5′-phosphate dihydrate | $C_9H_{15}N_2O_5P \cdot 2H_2O$ | 35.95 | 6.12 | 9.20 | 36.24 | 6.42 | 9.39 | 53 |

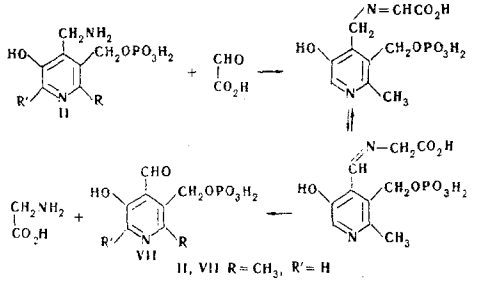
Table 4
Analogs of Pyridoxal 5′-phosphate

| Compound | Method of preparation | Temperature, °C (time of phosphorylation, hr | Empirical formula | Found, % | | | Calculated, % | | | Yield, % |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | C | H | N | C | H | N | |
| 2-Norpyridoxal 5′-phosphate monohydrate | a | 45 (6) | $C_7H_8NO_6P \cdot H_2O$ | 33.22 | 4.26 | 5.35 | 33.48 | 4.02 | 5.58 | 70 |
| | b | 40 (4) | | | | | | | | 73 |
| 6-Methyl-2-norpyridoxal 5′-phosphate monohydrate | c | — | $C_8H_{10}NO_6P \cdot H_2O$ | 35.87 | 4.28 | 5.03 | 36.24 | 4.56 | 5.28 | 67 |
| 6-Methylpyridoxal 5′-phosphate monohydrate | b | 60 (6) | $C_9H_{12}NO_6P \cdot H_2O$ | 38.44 | 4.92 | 4.76 | 38.72 | 5.05 | 5.02 | 53 |

p-phenetidine from the 2-nor-PLP (VI, R = R' = H). To remove salts, after neutralization the aqueous layer was chromatographed on a sulfonated resin in the acid form, and 2-nor-PLP was obtained with a yield of 70%. The separation curve is given in Fig. 1.

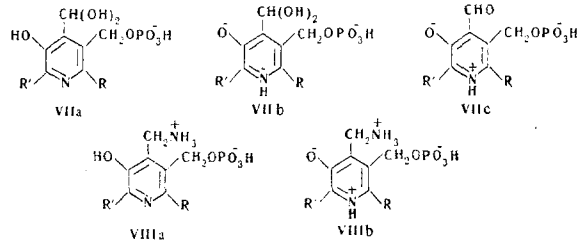
It is more convenient to hydrolyze the SB phosphates directly on a sulfonated resin in the acid form. Under these conditions, the yields of 2-nor-PLP and of 6-methyl-PLP were 73 and 53%, respectively (Fig. 2). However, in those cases where the SB is obtained in low yield and inadequate purity, this method does not give satisfactory results. Consequently, to obtain 6-methyl-2-nor-PLP (VII) we used the transamination reaction of the corresponding readily-accessible amine with glyoxylic acid, which was used successfully for the synthesis of the coenzyme itself [4, 10].

As the UV spectra show (Table 1), the properties of the compounds obtained depend to a considerable extent on the position of the methyl group.



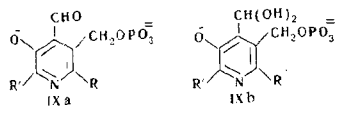
II, VII R = $CH_3$, R' = H

Like the nonphosphorylated derivatives [6], at pH 6.9 the spectra of the PLP and PAMP analogs having no substituent in position 2 show an absorption maximum corresponding to the forms VIIa, VIIb, and VIIc and VIIIa and VIIIb (Figs. 3 and 4).

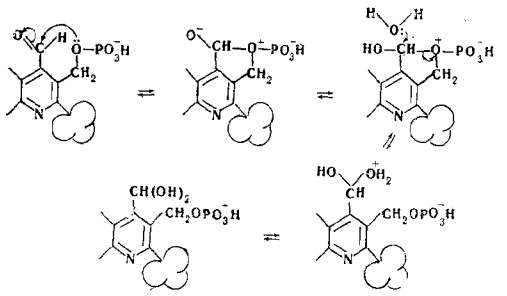


The possible causes of this phenomena have been discussed previously [6].

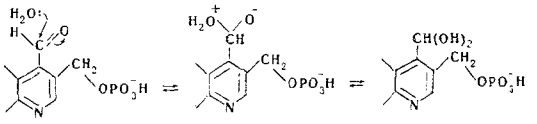
The increased degree of hydration of the aldehyde group of the 6-methyl analogs of PLP as compared with PLP itself was somewhat unexpected. The spectra given in Figs. 4 and 5 show increased absorption at 390 nm corresponding to the free aldehyde form IXa and, at the same time, increased absorption of the hydrate IXb at 330 nm.



This fact can be explained if we assume the presence of anchimeric cooperation of the phosphate group:



The presence of a methyl group in position 6 substantially hinders rotation round the $C_{(5)}$—$C_{(5')}$ bond, and the carbon of the formyl group and the 5'-oxygen atom are close in space, which favors cooperation. Conversely, for the 6-unsubstituted aldehydes the conformation in which the phosphate group is remote from the aldehyde group is the predominating one. In this case, the usual nucleophilic addition of water to the carbonyl group takes place:



The fact that the 5'-oxygen atom has a greater nucleophilic activity than water and the advantageousness of the five-membered rigid cyclic system formed as an intermediate leads to an increased degree of hydration for the 6-methyl analogs of PLP. It is natural that on passing to an alkaline medium, i.e., on replacing a weak nucleophile (water) by a stronger one (hydroxyl) the difference in the degree of hydration of the PLP analogs must be levelled down. And, in actual fact, the UV spectra in 0.1 N KOH differ slightly from one another. Measurements were carried out on the absorption of PLP analogs in acid phenylhydrazine at 410 nm by the method of Wada and Snell [11]. The results of the determination are given in Table 2.

At the present time the compounds obtained are being studied as cofactors of L-aspartate:2-oxoglutarate aminotransferase.

## EXPERIMENTAL

The Schiff's bases with p-phenetidine were obtained as described in the preceding paper [6].

**Analogs of pyridoxamine 5'-phosphate.** To a mixture of 0.52 g of 85% phosphoric acid and 0.4 g of $P_2O_5$ was added 0.46 mM of the dihydrochloride of an analog of pyridoxamine, and the mixture was kept at room temperature until the evolution of HCl had eased. Then it was heated at 60° C for 2 hr, and cooled, and 3 ml of ethanol followed by 8 ml of ether were added. The liquid was decanted off, the residue was dissolved in 5 ml of 1 N HCl, and the solution was heated in the boiling water bath for 20 min and evaporated in vacuum to 1 ml. The pH of the solution was brought to 5 with concentrated ammonia and it was deposited on a 1.6 × 54 cm column of Amberlite IRC-50 in the acid form. Elution was carried out with water at the rate of 20 ml/hr. The fractions containing the PAMP analog were evaporated in vacuum to small volume and cooled. The crystals that deposited were washed with a small amount of cold ethanol and with ether. The compounds obtained are given in Table 3.

**Analogs of pyridoxal 5'-phosphate.** A) To a mixture of 4.25 g of 85% phosphoric acid and 3.48 g of $P_2O_5$ was added 1.35 mole of a

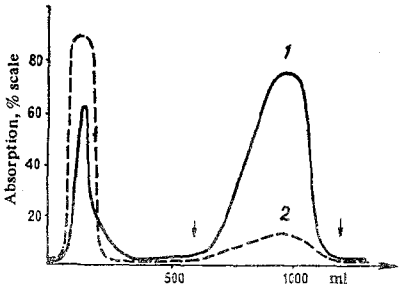



Fig. 6. Isolation of 6-methyl-2-nor-PLP on Dowex 50W × 4 in the acid form. Symbols as in Fig. 1.

Schiff's base. The dark red mixture obtained was heated at 45° C for 6 hr and was then cooled, and 1.4 ml of 0.1 N HCl was added and the mixture was again heated at 60° C for 15 min. The mixture was cooled and brought to pH 3 with 30% NaOH. The precipitate of the 5'-phosphate of the Schiff's base was centrifuged, washed with water, and dried.

Then it was dissolved in 2.5 ml of 2 N NaOH and the p-phenetidine was extracted with ether. The aqueous layer was treated with 2.5 ml of 2 N HCl and the solution was deposited on a 1.37 × 40 cm column of Dowex 50W × 4 in the acid form. Elution was carried out with water at the rate of 20 ml/hr. The elution curve is shown in Fig. 1. The fractions containing the PLP analog were concentrated in vacuum at 40° C to 30 ml and were then freeze-dried.

B) The Schiff's base (2 mM) was dissolved in a mixture of 3.72 g of 85% phosphoric acid and 2.86 g of $P_2O_5$. The mixture was heated (see Table 4) and then, with cooling, 6.0 ml of 0.1 N HCl was added and it was heated again at 60° C for 15 min. After cooling, the mixture was chromatographed as in (A). The elution curve is given in Fig. 2.

C) One mole of an analog of pyridoxamine 5'-phosphate was dissolved in 7 ml of water and 1 ml of 2 N NaOH. The resulting solution was treated with 240 mg (2.5 mM) of sodium glyoxylate and the mixture (pH 9) was stirred for 10 min. Then its pH was brought to 5 with glacial acetic acid and it was stirred for another 10 min and 3 ml of a 0.25 M solution of copper acetate was slowly added (pH of the reaction mixture 6) and stirring was continued at room temperature for 30 min. Then the mixture was deposited on a 1.4 × 40 cm column of Dowex 50W × 4 in the acid form. The subsequent isolation procedure was the same as in (A). The elution curve is given in Fig. 6.

The PLP analogs obtained are given in Table 4. All the compounds obtained were homogeneous on chromatography in a superfine layer in the ethanol–butanol–5% ammonia–glacial acetic acid (10 : 10 : 10 : 1) system [13] and on electrophoresis.

The UV spectra were taken on an SF-4A instrument at a layer thickness of 1 cm using concentrations of $10^{-4}$ M.

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