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The Synthesis of some B₆ Vitamin Halophosphates

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A series of new halo-derivates of pyridoxine-3-*O*- or 5'-*O*-phosphates and pyridoxal-3-*O*- or 5'-*O*-phosphates have been synthesized. In the reaction of partially protected pyridoxine and pyridoxal (3-5) with phosphorusoxychloride in the presence of triethylamine, the following dichlorophosphates were obtained: 3,4'-*O*-isopropylidene-pyridoxine-5'-*O*-dichlorophosphate (6), 4,5'-*O*-isobutylidene-pyridoxine-3-*O*-dichlorophosphate (7), and monoethylacetal-3-*O*-dichlorophosphate (8). Dichlorophosphates 6-8 reacted with NaF under catalytic action of 18-crown-6-ether, giving the corresponding difluorophosphates 14-16. The 3,4'-*O*-isopropylidene pyridoxine-5'-*O*-difluorophosphate (14), 4',5'-isobutylidene-3-*O*-difluoro-phosphate (15) and monoethylacetal-3-*O*-difluorophosphate (16), in the reaction with aniline, yielded the corresponding monofluorophosphates in the form of aniline salts (17-19). The direct fluorination of pyridoxal-5'-phosphate with dinitrofluorobenzene yielded pyridoxal-5'-*O*-monofluorophosphate (1) which was isolated as cyclohexylamine salt (2).

INTRODUCTION

Vitamin B₆ and its derivates are compounds of indisputable biological significance. Pyridoxal-5'-phosphate (PLP) is the prosthetic group of many enzymes important in amino acid metabolism, and its role is best characterized in aspartate aminotransaminase¹⁻⁵ and glycogen phosphorylase.^{6,7} Extensive research has been carried out in order to understand the role of PLP in the catalytic acts of these enzymes, as well as to define the structural determinants and the significance of individual functional groups within the PLP molecule. Minimum structural requirements for biologically ac-

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tive cofactor have been established. The 4-aldehyde, 3-phenol and the phosphate group are necessary in any PLP analogue if it is to function as an enzyme cofactor.⁸⁻¹²

Introducing new model modifications at various position of the molecule is a reasonable approach to learning more about their biological role. Among the many groups to be introduced into the native pyridoxal (PL) or PLP molecules, fluorine is the most interesting since, besides its effect in enzyme function, it can be easily identified by ¹⁹F NMR. Some fluoro derivatives of pyridoxine (PN) and PL have been already described and studied (fluorine in position 6).^{13, 14} However, the synthesis of 5'-fluorophosphate is potentially interesting as the phosphate group is necessary for binding in pyridoxal phosphate dependent enzymes.¹

In a previous paper,¹⁵ using Witman's¹⁶ procedure for the synthesis of alkyl or aryl fluorophosphates, we have reported the synthesis of small amounts of pyridoxal-5'-monofluorophosphate as cyclohexylamine salt. We now describe an improved synthesis of a variety of halophosphate derivatives based on fluorination of the PN and PLP molecule, and show that the monofluorophosphate derivatives are excellent cofactors for aspartate transaminase.

RESULTS AND DISCUSSION

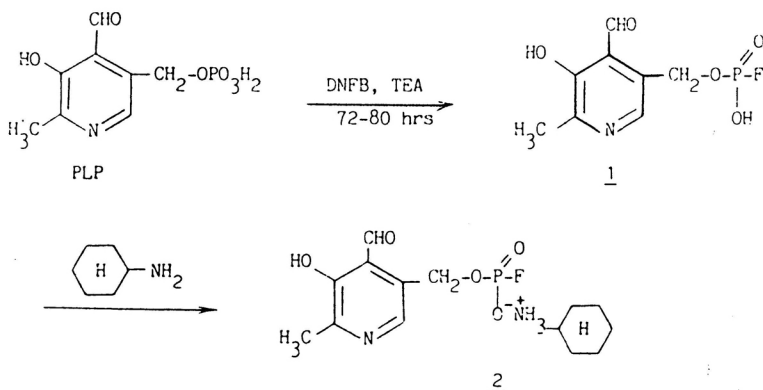
Fluorination of Free PLP

Pyridoxal-5'-fluorophosphate (5'-PLFP) (**1**) was prepared by fluorination of PLP with dinitrofluorobenzene (DNFB) in the presence of PLP triethylamine (TEA) in the molar ratio 1:4:8. 5'-PLFP was isolated as the stable cyclohexylamine salt **2** (Scheme 1).

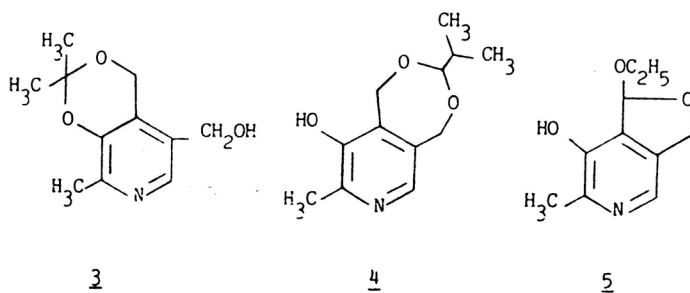
In addition, a new family of halogenated PLP derivatives as dichloro, difluoro, and monofluoro phosphates of pyridoxine and pyridoxal in 3-O- and 5'-O-position were prepared.

Difluoro and monofluoro phosphate were synthesized indirectly via dichlorophosphates.

A number of suitable partially protected derivatives of PN and PL were prepared providing either 3- or 5'-position ready to react, e.g. 3,4'-O-isopropylidene pyridoxin (**3**)¹⁷, 4',5'-O-isobutylidene pyridoxine (**4**)¹⁸ and monoethylacetate pyridoxal (**5**)¹⁹.



Scheme 1.

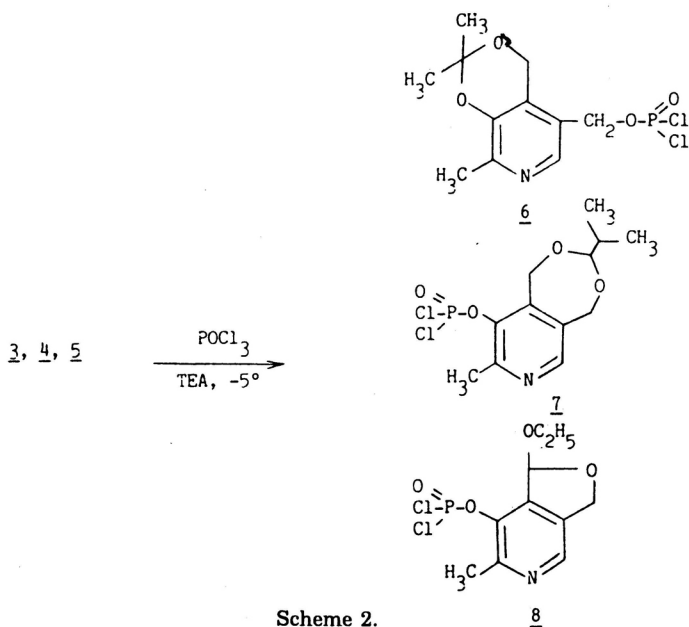


During the synthesis of compound 3,¹⁵ according to the method described by Korytnik²⁰, a mixture of 3 and of another isomer was obtained. When the reaction was carried out in $\approx 20\%$ HCl in dry acetone, almost pure 3 was isolated.

Dichlorophosphates of PL and PN Derivates

Synthesis of dichlorophosphates 6–8 was achieved by the following modified method by Heyl²¹ and Orloff²² for alkyl and aryl dichlorophosphates.

In our experiments, starting from compounds 3–5, the corresponding dichlorophosphates 6–8 were obtained when an excess of POCl_3 and TEA, were used (Scheme 2).



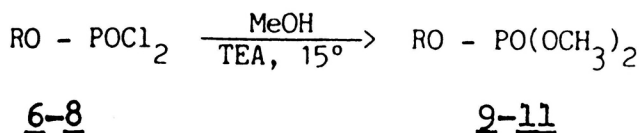
Scheme 2.

TABLE I
*P*dyridoxine and *P*yridoxal Dichlorophosphates 6–8

Start- ing Compound	Ob- tained	Ratio of Reagents in mmole to 1 mmole of Starting Compound		Yield (%)	Molecular Formula (Mw)	UV (CHCl ₃) λ/nm	IR (KBr) ν̄/cm ⁻¹
		POCl ₃	TEA				
3	6	2	4	80–85	C ₁₁ H ₁₄ Cl ₂ NO ₄ P (324.125)	220, 280	2980, 2840, 2700, 2000, 1640, 1550, 1480, 1400–1380, 1310–1280, 1070, 1030, 850, 805
4	7	3–3.5	5	75–80	C ₁₂ H ₁₆ Cl ₂ NO ₄ P (340.152)	228, 256	2950, 2940, 2790, 2680, 2500, 1680, 1470, 1400, 1390, 1290, 1260, 1080, 1040, 850, 802
5	8	31	5	80–82	C ₁₀ H ₁₂ Cl ₂ NO ₄ P (312.098)	222, 256	2960, 2620, 2500, 1570, 1470, 1400, 1270–1250, 1120, 1040, 850

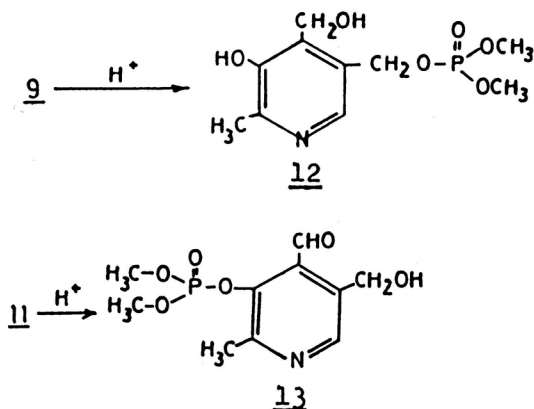
Compounds 6–8 were isolated as dark, oily and highly unstable substances that hydrolyze easily (Table I). The structures were proved by UV and IR spectra, as well as by conversion into the corresponding dimethylphosphates.

Different methods for the synthesis of dimethylphosphates have been reported,²²⁻²⁵ e. g. the described synthesis of aryldimethylphosphates by reaction of an excess of methanol in the presence of pyridine or TEA. In a similar way, using 7-fold excess TEA in absolute methanol, dichlorophosphates 6–8 were converted into the corresponding dimethylphosphates 9–11 (Scheme 3).



Scheme 3.

The addition of TEA was essential because the phosphate group was hydrolyzed in the absence of base. Hydrolysis of the protecting group of the isopropilidene derivative 9 with 1 M HCl at 40 °C,²⁶ produced pyridoxine-5'-dimethylphosphate (12). Similarly, monoethylacetal derivative 11 was converted to pyridoxal-3-O-dimethylphosphate (13)¹⁹ (Scheme 4).



Scheme 4.

Dimethylphosphates were isolated as oils and were fairly stable (Table II).

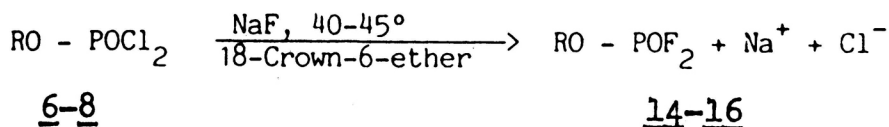
TABLE II
Pyridoxine and Pyridoxal Dimethyl Phosphates 9–13

Start- ing Compound	Ob- tained	Yield (%)	Molecular Formula (Mw)	Analysis Calc/Found:			UV (MeOH) λ /nm	IR (KBr) $\bar{\nu}$ /cm ⁻¹
				C	H	N (%)		
6	9	67	C ₁₃ H ₂₀ NO ₆ P (317.282)	49.21 49.05	6.35 6.68	4.41 4.17	197, 270	3000, 2980, 2860, 1600, 1450, 1420, 1380, 1300– 1200, 1100, 1070, 860, 790
7	10	65	C ₁₄ H ₂₂ NO ₆ P (331.309)	50.75 50.58	6.63 6.72	4.22 4.07	200, 263	3300, 3280, 2800, 1670, 1480, 1360, 1270–1250, 1220, 1030, 870, 800, 750
8	11	70	C ₁₂ H ₁₈ NO ₆ P (303.255)	47.52 47.38	5.98 6.20	4.62 4.51	198, 262	2960, 1650, 1450, 1400, 1270, 1210, 1030, 880, 860
9	12	54	C ₁₀ H ₁₆ NO ₆ P (277.222)	43.32 43.18	5.82 5.98	5.05 4.72	202, 368	3150–2540, 1620, 1500, 1450, 1350, 1230, 1050, 770
11	13	30	C ₁₀ H ₁₄ NO ₆ P (275.202)	43.64 43.51	5.12 5.33	5.09 4.81	200, 260	2980, 2870, 1670, 1450, 1420, 1500, 1200–1190, 1100, 1070–1000, 900, 850, 790

Diffluorophosphates of PN and PL Derivatives

Of the numerous methods²⁷⁻³⁰ available for the substitution of chlorine by fluorine, we followed the Effenberger method.³⁰ Alkyldichlorophosphates undergo a reaction with NaF (previously heated at 150 °C for 2–3 hours) in dry acetonitrile at 40–45 °C.

The reaction is catalyzed by addition of a small quantity of crown-ether. Following this procedure, dichlorophosphates 6-8 have been converted into difluoro derivatives 14-16 (Scheme 5).



Scheme 5.

The difluorophosphates 14-16 (Table III) are oily compounds, considerably more stable than their dichlorophosphate parent molecules, though not stable enough to be purified for elemental analysis. They were identified by UV and IR spectra as well as by ^{19}F NMR, and by conversion to the corresponding monofluorophosphates.

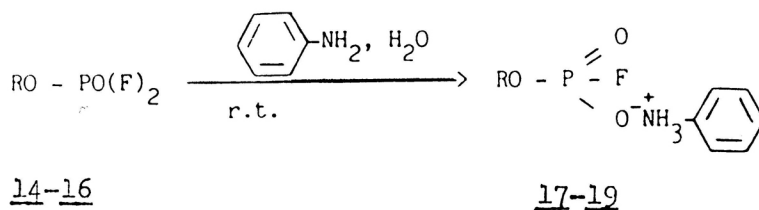
TABLE III
Pyridoxine and Pyridoxal Difluorophosphates 14-16

Start- ing Compound	Ob- tained	Yield (%)	Molecular Formula (<i>Mw</i>)	IR (KBr) $\bar{\nu}/\text{cm}^{-1}$	^{19}F NMR (DMSO-d ₆) δ/ppm	1J (P,F)/Hz
6	14	70	$\text{C}_{11}\text{H}_{14}\text{F}_2\text{NO}_4\text{P}$ (293.311)	2920, 2720-2680, 1550, 1470- 1450, 1400, 1360, 1320, 1280, 1060, 830	-78.8	952 (d)
7	15	65	$\text{C}_{12}\text{H}_{16}\text{F}_2\text{NO}_4\text{P}$ (307.238)	2920, 2860 (assoc.), 2720, 2680, 2600, 1650, 1470, 1450, 1400, 1350, 1330-1280, 1080, 830	-73.61	710.8 (d)
8	16	71	$\text{C}_{10}\text{H}_{12}\text{F}_2\text{NO}_4\text{P}$ (279.175)	2980, 2940, 2730, 2500, 1640, 1480, 1400, 1290-1170, 1040, 850, 805	-73.56	712 (d)

Difluorophosphates react readily with alcohols and primary amines³¹. Depending on the reaction conditions, it is possible to hydrolyze only one fluorine, thus yielding a corresponding amine salt of monofluorophosphate derivatives.

Monofluorophosphates of PN and PL Derivatives as Amine Salts

Ivanova and Kirsanov³¹ described the synthesis of aryl monofluorophosphates aniline salts by mixing aryldifluorophosphates with aniline in water. The same procedure, using difluoro compounds 14-16 (1 mmol) and aniline (2 mmol) in water at room temperature yielded the corresponding monofluorophosphates in the form of aniline salts 17-19 (Scheme 6). The salts were isolated as stable crystals which could be recrystallized from water and were identified by IR, ^{19}F NMR and elemental analysis. The data are summarized in Table IV.



Scheme 6.

TABLE IV

Pyridoxine and Pyridoxal Monofluorophosphates as aniline 17-19 and cyclohexylamine salts 2

Start- ing Compound	Ob- tained	Yield (%)	m. p. (°C)	Molecular Formula (Mw)	Analysis Calc/Found:			IR (KBr) ν/cm^{-1}	¹⁹ F NMR (DMSO-d ₆)	
					C	H	N %		δ/ppm	¹ J (P,F) /Hz
14	17	70	213- 214	C ₁₇ H ₂₂ FN ₂ O ₅ P (384.351)	53.12 53.00	5.72 6.03	7.28 7.20	3340, 3040, 2900- -2500 (assoc.), 1600, 1500, 1420, 1300, 1200, 1120, 1050, 840	-68.9	901.8 (d)
15	18	50	190 (decom p.)	C ₁₈ H ₂₄ FN ₂ O ₅ P (398.375)	54.27 54.23	6.07 5.88	7.03 7.19	3325, 3060, 2890, 2125, 1615, 1600, 1510, 1360, 1330, 1210, 1060, 940, 920, 820, 800, 740, 690	-69.72	711.5 (d)
16	19	50	217- -218	C ₁₆ H ₁₇ FN ₂ O ₅ P (370.323)	52.32 52.24	5.44 5.17	7.56 7.53	3340, 3040, 2940- -2500, 1600, 1420, 1300, 1260, 1210, 1040, 800, 740, 680	-68.54	712 (d)
PLP	2	63	178- -180 de- comp.	C ₁₄ H ₂₂ FN ₂ O ₅ P (348.317)	48.27 48.08	6.36 6.47	8.04 7.85	3450, 2940, 1590, 1410, 1390, 1280, 1210, 860, 830	-74.20	924.6 (d)

NMR Spectra

Fluorine-19 is one of the most used NMR probes, especially convenient for monitoring the process of fluorination. The existence of P-F bond in ¹⁹F NMR spectra is reflected as a doublet splitting of the signal due to $I = 1/2$ of ³¹P nucleus.

The ¹⁹F NMR data of compounds investigated here are presented in Tables III and IV. It can be recognized that one-bond P-F coupling constants are in a range of 900-950 Hz, for P-F bonded to oxygen at C-5' position. In difluoro compounds (PF₂ group), ¹J(P,F) has greater magnitude (20-50 Hz) than in the corresponding monofluoro ones as a consequence of the inductive effect of second fluorine atom. These values are in good agreement with the data published for some phospho-fluorine derivatives.³²

Difluoro molecules display only one doublet in ^{19}F NMR spectra, obviously due to the equivalency of fluorine nuclei. Chemical shifts, $\delta(\text{F})$, of C-5'-O-substituted compounds are in the range of (-)68 to (-)81 ppm. In difluoro molecules, the magnitudes of $\delta(\text{F})$ are greater (7–10 ppm) than in the corresponding monofluoro ones, which means that the second fluorine increases the shielding. The same effect has been found for some other tetracoordinated phospho-fluorine molecules although decreasing of shielding is frequently observed with increases of electronegativity of the substituent.³²

When P-F group is bonded to oxygen at C-3 position, $^1J(\text{P},\text{F})$ are ca 700 Hz. Interestingly, the second fluorine does not increase the magnitude of coupling here. However, $\delta(\text{F})$ in difluoro molecules are somewhat greater (4–7 ppm) than in monofluoro molecules, *i. e.* ^{19}F nuclei are more shielded than in the C-5'-fluorinated compounds. Generally, there is no significant difference of $\delta(\text{F})$ in C-5'-O- and C-3-O-PF derivatives, contrary to $^1J(\text{P},\text{F})$ where the differences are ca. 200 Hz.

Pyridoxal-5'-Fluorophosphate as Coenzyme

Incubation of stoichiometric amounts (1 μmole) of PLFP compound (2) and apoenzyme from cytosolic pig heart aspartate aminotransferase produces a complete enzyme (haloenzyme). At pH 8.0, the resulting haloenzyme has absorbance maxima at 360 nm due to the active-site bound compound (2). Binding to the enzyme is slow and in the presence of Tris-hydrochloride buffer, pH 8.0, takes about 2 hours to completion.

Binding of PLFP to the enzyme can be prevented by the native cofactor, PLP. Interestingly, upon addition of 50 mM glutamic acid, the enzyme with compound (2) at the active site converts to a form with absorbance maxima at 330 nm. The latter is characteristic of the conversion to a pyridoxamine 5' phosphate derivative. The enzyme with bound PLFP is also fully catalytically competent, indicating that substitution of the fluorine at the phosphate does not specifically impair the binding of the coenzyme derivative to the protein and that this binding is catalytically competent. Furthermore, the presence of fluorine as a P-F bond does not alter the coenzyme alignment in the protein cavity, nor does it have a deleterious effect on the rest of the coenzyme molecule's role in catalysis.

EXPERIMENTAL

Reagents: Anhydrous pyridoxal-5-phosphate was purchased from Sigma Chemical Company, St. Louis, Missouri, USA, and dinitrofluorobenzene from E. Merck, Darmstadt, Germany.

General Remarks: Melting points (*m. p.*) not corrected, were determined on Totolli apparatus. IR spectra were recorded as KBr pellets on a Perkin Elmer 457 grating spectrometer. The ^{19}F NMR spectra were recorded on a Varian 80 A FT NMR spectrometer operating at 74.84 MHz. The spectra widths 8000–2000 Hz and 16 K addresses were used, giving digital resolution of ± 0.02 ppm for chemical shifts and ± 1 Hz for coupling constants. The samples were dissolved in DMSO-d_6 using deuterium lock. All ^{19}F chemical shifts, expressed in ppm, are referenced to CFCl_3 as the internal standard, having a negative sign when upfield to CFCl_3 ; Machery-Nagel, SIL-NMR/UV₂₅₇ polygram plates (Germany) and Whatman, PE SIL G/UV₂₅₄ polyester plates were used for TLC. Elementary analyses were kindly performed by Dr. Herman, Institute Ruder Bošković, Zagreb.

Abbreviations: aq., aqueous; r. t. room temperature; sat., saturated; *i. v.*, *in vacuo*; TEA triethylamine; PL, pyridoxal; PN, pyridoxine; PLP, pyridoxal-5'-phosphate; DNFB, dinitrofluorobenzene; DNP, dinitrophenole; TLC, thin layer chromatography; Tris, tris(hydroxymethyl) amino methane.

Solvent System for TLC: isopropanol – ammoniumhydroxide – water, 7 : 1 : 2 (IAW), dichloromethane – methanol, 9 : 1 (DIM), benzene – dichloromethane, 1 : 1 (BED).

Pyridoxal-5'-monofluorophosphate Cyclohexylamine Salt (2): PLP (247 mg, 1 mmol) was suspended in acetonitrile (5 ml) together with TEA (1.2 ml, 8 mmol). Into the suspension, DNFB (744 mg, 4 mmol) was continuously added in small portions under stirring at r. t. Stirring was continued for an additional 24 hours at the same temperature, and the course of the reaction was monitored by TLC (DIM). The solvent was removed i. v., and 5 ml of water was added to the residue. The suspension was extracted with ether, which removed most unreacted DNFB and newly-formed DNP. A small amount (0.5 g) of ion exchange resin (Amberlite, IR-120, H⁺) was added to the water solution, shaken for a few minutes and filtered off. The aq.-solution was extracted with ether to remove traces of impurities. The water layer was carefully neutralized with cyclohexylamine and liophilized. Light orange colored crystals were thus obtained. TLC (IAW) showed a single component. In some experiments, a trace of unreacted PLP was detected. When necessary, the raw product was purified by recrystallization from acetonitrile. Yield: 218 mg (63%). IR, ¹⁹F NMR and elemental analyses value supported the proposed molecular structure of compound 2 (Table IV).

Dichlorophosphates 6–8 of Some PN and PL Analogues

General Procedure

The solution of protected PN 3,4 or PL 5 derivative (2 mmole) and TEA (7–10 mmole) in dry chloroform (2–3 ml) was gradually (20–25 minutes) added under stirring into the phosphorus oxychloride (0.63 ml, 7 mmole)-chloroform (3 ml) solution, keeping the temperature not higher than –5 °C. Stirring was continued for 30 minutes at r. t., and excess of reactants was evaporated in the presence of dry toluene i. v. The obtained material was suspended in dry acetonitrile (5 ml), and the precipitated TEA-hydrochloride was sucked off. The filtrate was concentrated, yielding a dark yellow product (Table I). Unstable dichlorophosphates were identified by UV, IR, and by conversion to the corresponding dimethylphosphate 9–11.

Methanolysis of Dichlorophosphates 6–8

General Procedure

Freshly prepared dichlorophosphate 6–8 (1 mmole) were quickly added into absolute methanol (5 ml) containing TEA (7–10 mmole). Reactants were stirred for 40–50 minutes below 15 °C. On evaporation of the solvent i. v. and addition of dry ether to the residue, precipitated TEA-hydrochloride was filtered off. The ether solution was thoroughly washed with water, dried over Na₂SO₄, and then flash-evaporated. Identification of the isolated oily dimethylphosphates 9–11 was carried out by TLC (DIM), elemental analysis, UV, IR (Table II).

Pyridoxine-5'-dimethylphosphate (12) and Pyridoxal-3-O-dimethylphosphate (13): they were isolated when protecting groups were hydrolysed by the following reaction procedure:

Pyridoxine-5'-dimethylphosphate (12): 3,4'-O-isopropylidene-pyridoxine-5'-dimethylphosphate (9) (317 mg, 1 mmole) was dissolved in 10 ml of 1 M hydrochloric acid and stirred for 2 hours at 40 °C. The reaction mixture was neutralized by solid K₂CO₃, and the solution was extracted with ether, dried over Na₂SO₄, and then concentrated to a small volume. A white fluffy crystalline product (152 mg, m. p. 140–142 °C) was obtained, which proved by TLC pure enough for all analytical purposes (Table II).

Pyridoxal-3-O-dimethylphosphate (13): The solution of monoethylacetalpyridoxal-3-O-dimethylphosphate (11) (303 mg, 1 mmole) in water (5 ml) was acidified with a few drops (2–3) of 6 M hydrochloric acid under stirring. The solution formed was kept for 5 minutes at 70–80 °C. Stirring was continued for an additional 30 minutes at r. t. The end point of hydrolysis was detected by TLC (DIM). The solution was then neutralised with solid K₂CO₃, extracted with ether, dried over anhydrous Na₂SO₄, and flash-evaporated. TLC pure, oily 13 (78 mg) was isolated (Table II).

Pyridoxine and Pyridoxal Difluorophosphates 14–16

General Procedure

Freshly prepared dichlorophosphates 6–8 (1 or 2 mmole) were dissolved in dry acetonitrile (5 ml) together with 18-crown-6-ether (0.15 or 0.3 mmole) under stirring at 40–45 °C. To the reaction mixture, sodium fluoride (2 to 4 mmole), previously dried at 150 °C for 2 hours, was added in small portions for the next 8 hours at 40–45 °C. The proceeding of the reaction was followed by TLC (solvent system IAW or DIM). Addition of ether to the reaction mixture precipitated the formed NaCl, which was filtered off, and the filtrate evaporated i. v. until dry. All the products were obtained as oily. Structures of difluorophosphates 14–16 were determined by IR and ¹⁹F NMR. The values of the characteristic absorbance are listed in Table III.

Monofluorophosphates 17–19 of Some Partially Protected PN and PL Derivatives as Aniline Salts

General Procedure

Difluorophosphates 14–16 obtained on the previous reaction step were converted to more stable monofluorophosphates as aniline salts by the following procedure: Into the suspension of aniline (0.2 ml, 2 mmole) and water (5 ml), a parent difluorophosphate 14–16 (1 mmole) was added dropwise under vigorous stirring for several minutes at r. t., pH of the clear solution was near 7.0. On cooling below 0 °C pale yellow crystals were formed. Separated products could be purified by careful recrystallisation from warm water, but further purification in most cases was not necessary (TLC, IAW). The raw compounds thus isolated proved pure enough by all analytical techniques, IR, ¹⁹F NMR, and elemental analysis. All physico-chemical data are listed in Table IV.

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SAŽETAK

Sinteza halofosfata iz reda vitamina B₆

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Sintetiziran je niz novih 3-O- i 5'-O- halofosfata piridoksina i piridoksala. Prikazani su reakcijski putovi za pripravu fluorofosfata, njihova izolacija i identifikacija. Sintetizirani fluorofosfati trebali bi poslužiti kao indikatori pri proučavanju vezanja fosfata na proteinske molekule.