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Abstract

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SYNTHESIS OF NUCLEOTIDYL-(P–N)-PHENYLALANINE BY THE PYROPHOSPHATE METHOD

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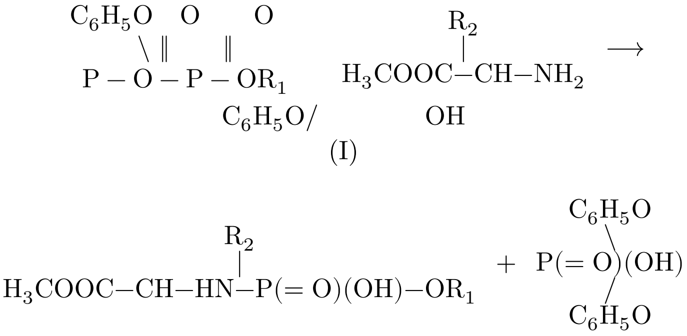
Continuing our studies of nucleotidyl-amino acids containing a phosphamide bond (1–3), we have proposed a convenient method for the synthesis of nucleotidyl-(P–N)-amino acids (“the pyrophosphate method”), based on the use of the high reactivity of triesters of pyrophosphoric acid:



$$\begin{array}{c} C_6H_5O \quad O \qquad O \\ \backslash \ \| \qquad \| \\ P - O - P - OR_1 \\ C_6H_5O/ \end{array} \quad \begin{array}{c} R_2 \\ | \\ H_3COOC-CH-NH_2 \\ OH \end{array} \longrightarrow$$

(I)

$$\begin{array}{c} R_2 \\ | \\ H_3COOC-CH-HN-P(=O)(OH)-OR_1 \end{array} + \begin{array}{c} C_6H_5O \\ \backslash \\ P(=O)(OH) \\ / \\ C_6H_5O \end{array}$$

where R_1 is a nucleoside.

The application of the pyrophosphate method made it possible to obtain, in high yields, methyl esters of uridylyl-(5′–N)-phenylalanine (II), adenylyl-(5′–N)-phenylalanine (III), guanylyl-(5′–N)-phenylalanine (IV), and 2′,5′-di-O-acetyl-uridylyl-(3′–N)-phenylalanine (V):

II R_3 = uracil,
III R_3 = adenine, (V)
IV R_3 = guanine.

For the synthesis of nucleotidyl-(P–N)-amino acids we used nucleoside-diphenyl pyrophosphates (I), which, owing to the stability of the anion of diphenylphosphoric acid, are very reactive phosphorylating agents (4–8). P^1-nucleoside-5′(3′)-P^2-diphenyl pyrophosphate (I, R_1–uridine, adenosine, guanosine, or 2′,5′-di-O-acetyluridine) is obtained by condensation of diphenyl chlorophosphate with a nucleotide in the presence of tri-*n*-butylamine in an inert anhydrous solvent. Dioxane is the best solvent to use. The nucleotide is introduced into the condensation in the form of the mono-(tri-*n*-octylammonium) salt. Tri-*n*-octylammonium salts

uridine 5′-phosphate and 2′,5′-di-*O*-acetyluridine 3′-phosphate are readily soluble in anhydrous dioxane. The corresponding salts of adenosine 5′- and guanosine 5′-phosphates are much less soluble in dioxane, and therefore dimethylformamide, or a mixture of it with dioxane, is preferable as the solvent. 5′-Nucleotides may be introduced into the reaction without protective groups, since diphenyl chlorophosphate reacts with the phosphate group much more rapidly than with the OH groups of ribose. 3′-Nucleotides must have the ribose OH group adjacent to the phosphorus residue protected, in order to avoid cyclophosphate formation and the consequent cleavage of the amino acid. The amino group of cytidylic acid must be protected beforehand, for example by acetylation.

P^1-Nucleoside-P^2-diphenyl pyrophosphate (I) is washed with ether to remove excess diphenyl chlorophosphate (a 1-2-fold excess of the latter is necessary to prevent symmetrization of the resulting pyrophosphoric triester into the inert symmetrical diester) and, without purification, is used directly in the reaction with the amino acid ester. In dioxane or dimethylformamide medium the reaction proceeds in 12 h at room temperature. Isolation and purification of nucleotidyl-(P—N)-amino acids are carried out by preparative paper chromatography (after washing the reaction mixture with ether to remove the excess amino acid ester), followed by elution of the corresponding zone of the paper with 98% methanol.

All the compounds obtained (II—V) do not react with ninhydrin. Derivatives of nucleotidyl-(5′—N)-phenylalanine give a positive reaction for the bis-glycol grouping.

The phosphoamide bond in the synthesized nucleotidyl-(5′—N)-phenylalanines shows the hydrolytic properties usual for amides of phosphoric acid: stability at alkaline pH values and easy hydrolyzability in acidic medium. For complete cleavage of the P—N bond in the methyl ester of nucleotidyl-(5′—N)-phenylalanine, heating at pH 1 is sufficient (15 min at 70° or 60 min at 37°); however, within the range pH 5.4-14 (other conditions being equal) the P—N bond is practically resistant to hydrolysis (only 3-5% is hydrolyzed).

The stability of the P—N bond in uridylyl-(3′—N)-phenylalanine depends on the presence of a protective group on the adjacent (2′) hydroxyl of ribose: removal of the acetyl protection causes rapid hydrolysis of the P—N bond even in alkaline medium, which is detected by the appearance, immediately after hydrolysis, of an intense positive ninhydrin reaction.

Experimental Part

Chromatography and electrophoresis were carried out on Leningrad (fast) chromatographic paper or Whatman 3MM (preparatively). Phenylalanine was determined quantitatively by the Giri–Bode method as modified by Zaitseva (9); phosphorus was determined by the method of Lowry and Lopez as modified by Skulachev (10). In the spectrophotometric determination of uridylic acid, the conversion coefficients according to Cohn (11) were used; for guanylic acid, those according to Spirin (12). Solutions were evaporated under reduced pressure in a rotary evaporator. The analytical data and the chromatographic and electrophoretic characteristics of the compounds obtained are given in Table 1.

Methyl ester of uridyl-(5′–N)-phenylalanine (II). Uridine 5′-phosphate (0.2 mmole of the barium salt) is desalted by passage through a small column of Dowex-50 (H^+ form), the aqueous solution is evaporated to a small volume, tri-*n*-octylamine (0.1 ml, 0.22 mmole) is added, the mixture is shaken, evaporated to dryness, and dissolved in anhydrous dioxane. The clear solution is again evaporated to dryness, and the residue is dried by repeated azeotropic distillation with anhydrous dioxane, benzene, and toluene at 30–35°. The resulting glassy dry mass is dissolved in 1 ml of anhydrous dioxane, and to the clear solution is added first

Table 1

Substance	Yield*, %	Ratio** compound : phosphorus : phenylalanine	R_f in systems*** tert.-C_4H_9OH – NH_4OH (1 N) – H_2O (7 : 0.1 : 3)	R_f in systems*** iso-C_3H_7OH – NH_4OH (conc.) – H_2O (7 : 1 : 2)	R_f in systems*** n-C_4H_9OH – CH_3COOH – H_2O (4 : 1 : 5)	R_f in systems*** C_2H_5OH – $^{1M}CH_3COONH_4$ (pH 7.5) (5 : 2)	R_f in systems*** iso-C_3H_7OH – 1% $(NH_4)_2SO_4$ (2 : 1)	R_f in systems*** n-C_4H_9OH – acetic acid – H_2O	Relative mobility in electrophoresis pH 3.5 (acetate)	Relative mobility in electrophoresis pH 8.9 (phosphate)	Relative mobility in electrophoresis pH 9.0 (borate)
Uridine			0.60	0.47	0.40	0.64	0.62	0.21	0.11	0.35	0.76
UMP-5′			0.12	0.08	0.07	0.20	0.38	0.00	1	1	1
II	95	1 : 0.96 : 0.95	0.56	0.54	0.34	0.73	0.70	0.11	0.81	0.63	0.87

Substance	Yield*, %	Ratio** compound : phosphorus : phenylalanine	R_f in systems*** tert.-C_4H_9OH — NH_4OH (1 N)— H_2O (7 : 0.1 : 3)	R_f in systems*** iso-C_3H_7OH — NH_4OH (conc.) — H_2O (7 : 1 : 2)	R_f in systems*** n-C_4H_9OH — CH_3COOH — H_2O (4 : 1 : 5)	R_f in systems*** C_2H_5OH — $^{1M}CH_3COONH_4$ (pH 7.5) (5 : 2)	R_f in systems*** iso-C_3H_7OH — 1% $(NH_4)_2SO_4$ (2 : 1)	R_f in systems*** n-C_4H_9OH — acetic acid — H_2O	Relative mobility in electrophoresis pH 3.5 (acetate)	Relative mobility in electrophoresis pH 8.9 (phosphate)	Relative mobility in electrophoresis pH 9.0 (borate)
2′,5′-di-O-acetyl-UMP-3′			0.22		0.25	0.53			1	1	1
V	85	1 : 0.98 : 1.10	0.75		0.61	0.95		0.25	0.84	0.62	0.84
Guanine				0.34	0.27	0.52	0.46	0.00	0	0.14	0.23
Guanosine				0.36	0.32	0.62	0.50	0.02	0.13	0.29	0.77
GMP-5′				0.04	0.04	0.19	0.32	0.00	1	1	1
IV	75	1 : 0.97 : 0.94		0.47	0.41	0.68	0.60	0.00	0.98	0.68	0.88
Phenylalanine			0.60	0.64							
Methyl ester of phenylalanine			0.95	0.86							
Diphenyl phosphate			0.80								

* Determined spectrophotometrically.

** Determined after hydrolysis with 1 N HCl, 100°, 60 min (in the case of IV—0.1 N HCl, 50°, 30 min).

*** Detection under UV light; phosphorus with ninhydrin; ascending chromatography.

diphenyl chlorophosphate (0.08 ml, 0.4 mmole), and then immediately a solution of dry tri-*n*-butylamine (0.12 ml, 0.45 mmole) in anhydrous dioxane (1 ml) is added dropwise with thorough stirring over 3 min. After 3 h the reaction mixture is evaporated to a small volume (at a temperature not above 30°), 25 ml of dry cooled ether is added, the mixture is shaken vigorously, and after standing for 30 min in a refrigerator the ether is decanted. The oil is dissolved in anhydrous dioxane, the clear solution is evaporated to a small volume (about 1 ml), and a solution of dry methyl ester of phenylalanine (1 mmole) in anhydrous dioxane (1 ml) is added. After 12 h the clear reaction mixture is chromatographed on paper in the system isopropanol—NH_4OH (conc.)—H_2O (7 : 1 : 2). The zone with R_f 0.50, absorbing in UV light, is cut out and eluted with 98% methanol. The eluate is evaporated to a small volume and poured dropwise into dry ether. The precipitated white solid is separated by centrifugation and dried over P_2O_5 and paraffin. One obtains 100 mg of the methyl ester of uridylyl-(5′—N)-phenylalanine as a white powder, readily soluble in water, homogeneous electrophoretically and chromatographically in various systems (see Table 1). At pH 7.0 $\lambda_{\max}$ 261.5 mμ, ε 9000; $\lambda_{\min}$ 232 mμ, ε 2200. At pH 12.5 $\lambda_{\max}$ 261.5 mμ, ε 7200; $\lambda_{\min}$ 242 mμ, ε 5000.

Methyl ester of 2′,5′-di-O-acetyl-uridylyl-(3′—N)-phenylalanine (V). Obtained from 2′,5′-di-O-acetyluridine-3′-phosphate* (13) (free acid) by the method described above. The substance is isolated by preparative descending chromatography on paper in the system tert.-butanol—NH_4OH (1 N)—H_2O (7 : 0.1 : 3). Eluted with 98% methanol. Methyl ester of 2′,5′-di-O-acetyl-uridylyl-(3′—N)-phenylalanine repre—

* The starting preparation of uridine-3′-phosphate contained a small admixture of uridine-2′-phosphate.

is a white, nonhygroscopic, readily water-soluble amorphous powder. At pH 7.0, $\lambda_{\max}$ 262 mμ, ε 11,000; $\lambda_{\min}$ 232 mμ, ε 3400. At pH 12.5, $\lambda_{\max}$ 262 mμ, ε 9000; $\lambda_{\min}$ 242 mμ, ε 7900.

Methyl ester of guanylyl-(5′—N)-phenylalanine (IV). The mono-(tri-*n*-octylammonium) salt of guanosine 5′-phosphate (obtained analogously to the corresponding salt of UMP-5′; see above) is dried by repeated azeotropic distillation with anhydrous dimethylformamide and dissolved in dimethylformamide. Further treatment is carried out in the same way as in the preparation of uridylyl-(5′—N)-phenylalanine, replacing dioxane everywhere by dimethylformamide. The methyl ester of guanylyl-(5′—N)-phenylalanine is a water-soluble grayish powder, homogeneous chromatographically and electrophoretically (see Table 1). At pH 7.0, $\lambda_{\max}$ 252 mμ, ε 14,200; $\lambda_{\min}$ 226 mμ, ε 6500. At pH

1.0, $\lambda_{\max}$ 256 mμ, ε 13,800; $\lambda_{\min}$ 228 mμ, ε 5800. At pH 12.0, $\lambda_{\max}$ 265 mμ, ε 13,400; $\lambda_{\min}$ 232 mμ, ε 6500.

Methyl ester of adenylyl-(5′—N)-phenylalanine (III). Obtained in the same way as the methyl ester of guanylyl-(5′—N)-phenylalanine and identified by comparison with an authentic sample ([2]) by electrophoresis, chromatography in various systems, and the kinetics of hydrolysis of the P—N bond. Yield 80%.

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named after M. V. Lomonosov

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CITED LITERATURE

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