SYNTHESIS AND PROPERTIES OF THYMIDYLYL-(P → N) PHENYLALANINES

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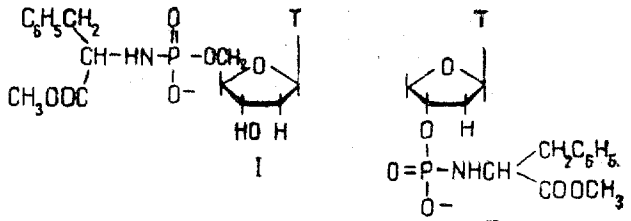
Continuing a study of the influence of the amino acid and the nucleotide moieties in the molecules of nucleotido-(P→N)-peptides on the properties of the nucleotidopeptide link [1-3], we have synthesized the methyl esters of thymidylyl-(5' → N)-phenylalanine (I) and thymidylyl-(3' → N)-phenylalanine (II), and have investigated their properties.



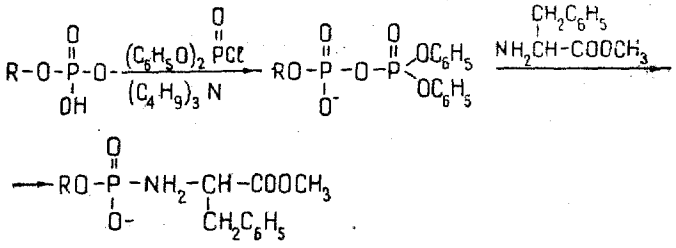
I

II

T-thymine

In contrast to the phenylalanine derivatives of ribonucleotides studied previously [1, 2] (for example, uridylic acid), compounds (I) and (II) lack the 2'-hydroxyl group which, as we showed previously, has a fundamental influence on the stability of the phosphoramide bond [2].

The synthesis of the thymidylphenylalanines (I) and (II) was carried out by the method that we have developed [1, 4] from a mixed anhydride for the corresponding nucleotide and the methyl ester of phenylalanine as follows:



I—R = thymidine-5', II—R = thymidine-3'

Compounds (I) and (II) were isolated and purified by preparative chromatography in the isopropanol-NH_4OH-H_2O (7:1:2) system and by electrophoresis (pH 8.9) (table).

The phenylalanine derivatives (I) and (II) are not shown up by ninhydrin; they have UV absorption spectra typical for thymidylic acid. On electrophoresis in an acid medium, the thimidylylphenylalanines (I) and (II) migrate more slowly than TMP (see Table). This is apparently due to the fact that at pH 3.5 the amide nitrogen atoms of compounds (I) and (II) are partially protonated and the molecule of the nucleotidyl amino acid bears a smaller negative charge than thymidylic acid, in which one hydroxyl group of the phosphate residue is in the dissociated state at this pH. In a study of the hydrolytic stability of the phosphoramide bond in the thymidylylphenylalanines (I) and (II) in relation to pH (37°C, 60 min), the nucleotidopeptide bond in both compounds was found to hydrolyze rapidly in an acid medium (Fig. 1).

The values of $\tau/2$ for the thymidylylphenylalanines (I) and (II) on hydrolysis with 0.05 N HCl at 37°C (Fig. 2) were, respectively, 3.5 and 3.0 min, i.e., the rate of hydrolysis in an acid medium is practically unaffected by the position of the phosphoramidic acid residue in the thymidine molecule.

The phosphoramide bond is stable in the pH range from 4 to 12 regardless of the position of the phosphoramidic acid residue in the thymidine (positions 5' and 3') (Fig. 1). It is an interesting fact that the stability of the phosphoramide bond of the corresponding derivatives of uridylic acid depends markedly on the position of the phosphoramido acid residue. We have shown previously [2] that only uridylyl-('5' → N)-phenylalanine (methyl ester) is stable at pH → 4, while uridylyl-(3'→N)-phenylalanine, in which there is a hydroxyl group adjacent to the phosphoramide grouping, is very unstable not only in an acid medium but also at alkaline pHs. This instability of uridylyl-(3'→N)-phenylalanine

is due to electrophilic-nucleophilic catalysis by the cis-hydroxyl group, which leads to nucleophilic substitution on the phosphorus atom with the formation of uridine-2', 3' cyclic phosphate with the simultaneous cleavage of the nucleotidopeptide bond.

Some Chacteristics of the Methyl Esters of Thymidylyl-(5'→N)- and Thimidylyl-(3'→N)-phenylalanine

Substance	R_f in systems				Rel. mobility on electrophoresis		Ratio of base: phosphorus: phenylalanine
	isopropanol – NH_4OH-H_2O (7:1:2)	butan-1-ol – CH_3COOH-H_2O (4:1:5)	tert-butanol – NH_4OH (1N) – H_2O (7:0.1:3)	butanol saturated with water	pH 3.5, acetate	pH 8.9, phosphate	
Thymidine	0.68	0.50	0.68	0.41	0	0	—
Thymidine-5' phosphate	0.19	0.10	0.12	0.00	1,0	1,0	—
Methyl ester of thymidylyl-(5'→N)-phenylalanine	0.69	0.42	0.43	0.15	0,84	0,50	1:0.95:0.94
Thymidine-3' phosphate	0,19	0.10	0.12	0.00	1,00	1,00	—
Methyl ester of thymidylyl-(3'→N)-phenylalanine	0.67	0.42	0.62	0.16	0,84	0,50	1:0.96:0.93
Thymidine-3', 5' cyclic phosphate	0.40	—	—	—	1,00	0,70	—
Phenyalanine	0.70	—	—	—	—	—	—
Methyl ester of phenylalanine	0.89	—	—	—	—	—	—

The stability of the thymidylyl-(3'→N)-phenylalanine ester, which lacks a hydroxyl group in position 2', at pH 4 confirms once again the existence of nucleophilic-electrophilic catalysis by the cis-hydroxyl group in ribonucleotidyl-(3'→N)-peptides.

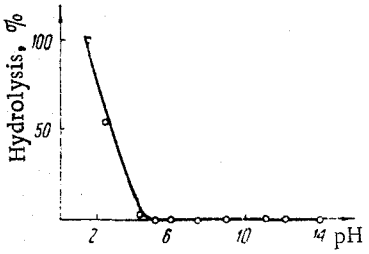


Fig. 1. Rate of hydrolysis of the phosphoramide bond in the methyl esters of thymidylyl-(3'→N)-and thymidylyl-(5'→N)-phenylalanines as a function of the pH (37°C, 60 min).

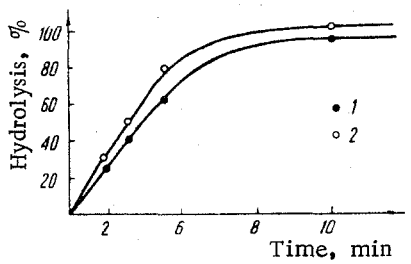
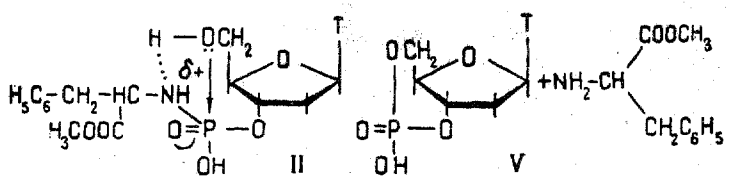


Fig. 2. Hydrolysis of the phosphoramide bond in the methyl esters of thymidylyl-(P→N)-phenylalanines (0.05 N HCl, 37°C): 1) thymidylyl-(5'→N)-phenylalanine; 2) thymidylyl-(3'→N)-phenylalanine.

We have observed a similar intramolecular catalysis by a hydroxyl group in the case of thymidylyl-(3'→N)-phenylalanine ester (II). On standing (20°C, 5 days) or on heating (70°C, 5 hr) in absolute dimethylformamide, compound (II) was converted into thymidine-3', 5' cyclic phosphate, and phenylalanine ester accumulated in the reaction mixture. The thymidine-3', 5' cyclic phosphate was isolated and purified by preparative paper chromatography and was shown to be identical (see table) with the compound obtained synthetically from thymidine-5' phosphate by the carbodiimide method [5]. This reaction, as the scheme given below shows is due to the intramolecular protonation of the amide nitrogen with the participation of the 5'-hydroxyl group, followed by attack on the electrophilic phosphorus atom by the oxygen of this group. The attack is facilitated by the fact that the conjugation between the nitrogen and the phosphorus is decreased through the protonation of the nitrogen and also by the increase in the nucleophilic properties of the 5'-hydroxyl group because of the propinquity of the proton-accepting amide group. A fact confirming this mechanism of the nucleophilic attack on the phosphorus atom is a comparison of the molar amounts of cyclic phosphate (V) and phenylalanine ester in

the reaction medium.



The results obtained indicate the considerable activating influence of an amino acid residue on the nucleotidic phosphorus under conditions when there is a proton-donating grouping in the neighborhood. This grouping may be not only an adjacent cis-hydroxyl group but also a hydroxyl in the trans position, which shows the ease with which not only the protonation of the amide nitrogen in nucleotido-(P→N)-peptides but also the nucleophilic substitution of the nucleotidic phosphorus atom connected with the amino acid take place.

Experimental

The quantitative determination of phenylalanine was carried out by the Giri-Bode method as modified by G. N. Zaitseva [6]. The phosphorus was determined by Berenblum and Chain's method as modified by Kulaev [7]. In the spectrophotometric determination of thymidylic acid, Cowgill and Pardee's calculation factors [8] were used. The solutions were evaporated in a rotary apparatus under reduced pressure.

The thymidine-5' and -3' phosphates were obtained by the phosphorylation of 3'-acetylthymidine and 5'-trityl-thymidine, respectively, using β-cyanoethyl phosphate [9], with subsequent elimination of the protecting groups. The following systems were used for chromatography: 1) isopropanol-ammonia-water (7:1:2), 2) butan-1-ol-acetic acid-water (4:1:5), 3) tert-butanol-NH_4OH (1N)-H_2O (7:0.1:3), 4) butan-1-olsaturated with water.

Methyl ester of thymidylyl-(5'→N)-phenylalanine (I). Thymidine-5' phosphate (0.1 mmole of the barium salt) was desalted by passage through a column of "Dowex-50" (H^+ form). The aqueous solution was concentrated to small bulk, tri-n-octylamine (0.1 ml, 0.22 mmole) was added, the mixture was evaporated to dryness, and the residue was dissolved in anhydrous dioxane. The clear solution was evaporated and dried by repeated distillation with absolute benzene and toluene at 30-35°C. The residue was dissolved in 1 ml of anhydrous dioxane and diphenyl phosphorochloridate (0.04 ml, 0.2 mmole) was added, followed in drops with careful stirring over 3 min, by a solution of dry tri-n-butylamine (0.06 ml, 0.45 mmole) in absolute dioxane (0.5 ml). After 3 hr, the reaction mixture was concentrated to small bulk (< 30°C), 25 ml of dry cooled ether was added, the mixture was shaken and left for 30 min (0°C), and the ether was decanted off. The oil was dissolved in absolute dioxane, the solution was reduced to small bulk by distillation, and a solution of the dry methyl ester of phenylalanine (0.5 mmole) in absolute dioxane (0.5 ml) was added. After 12 hr, the reaction mixture was chromatographed on Leningrad type "B" or "Whatman-3 mm" paper in system 1. The zone with R_f 0.69, which absorbed in UV light, was cut out and eluted with 98% methanol. The eluate was concentrated to small bulk and was added in drops to dry ether. The precipitate was separated by centrifuging and was dried over P_2O_5. The dry powder was dissolved in methanol and the solution was deposited on an electrophoregram (paper of the same grade). The substance was purified from phenylalanine and its ester by electrophoresis in a phosphate buffer at pH 7-8. The zone corresponding to substance (I) was cut out and eluted. The eluate was evaporated, and the residue was dissolved in absolute methanol and precipitated with absolute ether. This gave the methyl ester of thymidylyl-(5'→N)-phenylalanine in the form of a white powder readily soluble in water and alcohol. Yield 50-60%.

Methyl ester of thymidylyl-(3'→N)-phenylalanine (II). The compound was obtained from thymidine-3' phosphate by the method described above with a yield of 50%. The substance was isolated preparatively by means of ascending paper chromatography in system 1 and was purified by electrophoresis on paper in a phosphate buffer at pH 7-8. Compound (II) was eluted with 98% methanol, the eluate was evaporated, and the residue was dissolved in absolute methanol and precipitated with absolute ether. This gave substance (II) as a white powder readily soluble in water and alcohol. Yield 50-60%.

Thymidine-3', 5' cyclic phosphate. On standing in absolute dimethylformamide (5 days, 20°C), the methyl ester of thymidylyl-(3'→N)-phenylalanine formed thymidylyl-3', 5' cyclic phosphate, shown to be identical with the synthetic cyclic phosphate [5]. Thymidylyl-3', 5' cyclic phosphate was also formed in a yield of 30% on heating (70°C) for 5 hr in absolute dimethylformamide.

Hydrolysis of compounds (I) and (II) at various pHs. 0.1 ml of an aqueous solution of compound (I) or (II) (the concentration was determined spectrophotometrically) was mixed with 0.1 ml of acid or alkali or a buffer solution, and the mixture was placed in a thermostat (37°C). After the end of the incubation, the mixture was cooled and was deposited on a chromatogram (ascending chromatography in system 1). As a control, one sample was deposited on the chroma-

togram without the addition of a buffer. The degree of hydrolysis was determined spectrophotometrically from the amount of thymidylic acid formed.

Hydrolysis of compounds (I) and (II) with 0.05 N HCl. Several 0.1-ml samples of an aqueous solution of compound (I) or (II) were each treated with 0.1 ml of 0.1 N HCl (the concentration of nucleotide was determined spectrophotometrically). Hydrolysis was carried out in the thermostat at 37°C and the samples were deposited on a chromatogram (system 1). The degree of hydrolysis was determined spectrophotometrically from the amount of thymidylic acid formed. The following results were obtained:

Substance, mmole	Times, min			
	2	3	5	10
(I) 0.334	0.116	0.165	0.278	0.330
(II) 0.219	0.066	0.092	0.131	0.197

Summary

1. The methyl esters of thymidylyl-(5'→N)-and thymidylyl-(3'→N)-phenylalanines have been obtained from the mixed anhydrides of the corresponding nucleotides with diphenyl phosphate with yields of 50-60%.

2. The esters of the thymidylyl-(P→N)- phenylalanines are readily hydrolyzed in an acid medium. The phosphoramide bond in the thymidylylphenylalanines is stable in the pH range from 4 to 12.

3. The conversion of the ester of thymidylyl-(3'→N)-phenylalanine into thymidine-3', 5' cyclic phosphate shows the ease of protonation of the amide nitrogen with the participation of the 5'-hydroxyl group and subsequent nucleophilic substitution on the nucleotidic phosphorus atom.

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