OLIGONUCLEOTIDES AND NUCLEOTIDOPEPTIDES. XLII. EFFICACY OF THE INTRAMOLECULAR INFLUENCE OF THE CARBOXY GROUPS OF AMINO ACIDS IN NUCLEOTIDYL-(P → N)-AMINO ACIDS (PEPTIDES) AS A FUNCTION OF THEIR POSITIONS

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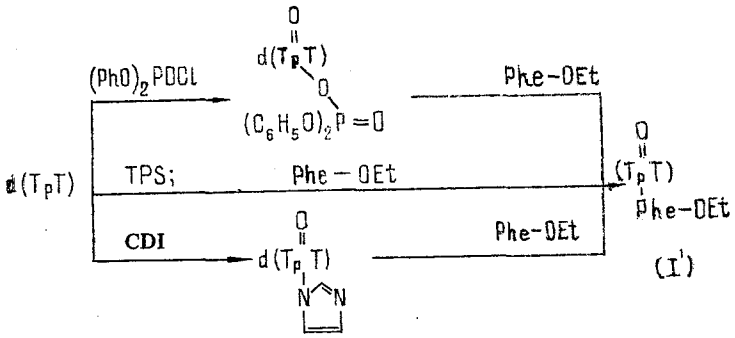
UDC 547.963.32.04

It has been established that remote carboxy groups in nucleotidyl-(5' → N)-β-alanine and -alanylalanine do not affect the mechanism of the cleavage of the phosphoramide center. The situation is different in the case of nucleotidyl-(5' → N^{ε})-lysine. It has been shown that in the 3'-phenylalanine derivative of dTMP, the influence of the α-carboxy group of the amino acid is only half as great as in the 5'-analog. The α-carboxy groups of the amino acids in oligonucleotidyl-(P^{in} → N)-amino acids also weaken the mechanism of the cleavage of the phosphoramide centers.

Recently, covalent nucleotide- and NA-protein complexes have been isolated from various sources (see the review [1]). In establishing the type of interbiopolymer bond, researchers have frequently made use of the treatment of the complexes with various chemical agents. It has now been shown [2] that such an approach is incorrect, since functional groups located adjacent to the interbiopolymer bond may have an intramolecular effect on the mechanism of its cleavage and complicate the interpretation of the results obtained. It is known [3] that the majority of α-carboxy groups of amino acids in nucleotidyl-(5' → N)-amino acids participate in intramolecular nucleophilic catalysis and are responsible for the appearance of nucleosides and phosphoric acid in their acid hydrolysates. In the present investigation it has been shown that the efficacy of intramolecular catalysis is affected by the distance of the carboxy groups of amino acids from the phosphoramide center and also by the position of the amino acid residue (a 5'-, a 3'-, or an internucleotide phosphate group).

The synthesis of esters of nucleotidyl-(P → N)-amino acids (dipeptides) was carried out by the dicyclohexylcarbodiimide method [4]. The ethyl ester of deoxythymidylyl-(3' → 5')-deoxythymidine-(P^{in} → N)-phenylalanine was obtained by the phosphate [5], triisopropylbenzenesulfonyl chloride, and carbonyldiimidazole methods.



Previously, for the synthesis of oligonucleotidyl-(P^{in} → N)-amino acid esters only the pyrophosphate method was used [5]. However, this method is laborious and the yields of reaction products are not constant, and therefore to activate the internucleotide phosphate

V. Kapsukas Vilnius State University. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 740-746, November-December, 1982. Original article submitted January 22, 1982.

0009-3130/82/1806-0703$07.50

TABLE 1. Some Characteristics of the Nucleotide Peptides of the Phosphoramide Type That Have Been Synthesized

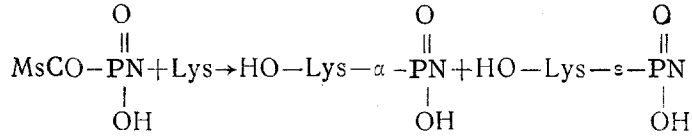
Compound	Yield, %	R_f in systems			U_{rel} pN,† pH 7.5	Base:phosphorus: amino acid ratio
		1	2	3		
HO-Phe-pdT (I)	100*	—	0,38	0,45	1,08	1:1,16:1,2
dTp-Phe-OH (II)	100*	—	0,4	0,48	1,06	1:1,03:0,96
HO-β-Ala-pdT (III)	96*		0,25	0,18	0,86	1:1,06:0,88
HO-Ala_2-pdT (IV)	100*		0,4	0,21	0,75	1:1,16:2,21
HO-Phe-(P^{in}→N)-d(TpT) (V)	90*	—	0,70	0,65	0,35	1,73:1:1
HO-Lys-α-pdT (VI)	27	0,35	—	0,09	0,2	0,9:1,1:1,2
HO-Lys-α-pA (VII)	28	0,24	—	0,08	0,25	1:1,2:1,1
HO-Lys-ε-pdT (VIII)	60	0,14	—	0,09	0,52	1:1,1:1,1
HO-Lys-ε-pA (IX)	65	0,16	—	0,08	0,45	1:1,23:1,08

*The yields of the saponification of the ester bonds in the corresponding esters were determined spectrophotometrically.
†Electrophoretic mobility relative to the nucleotide.

in d(TpT) we have also used triisopropylbenzenesulfonyl chloride (TPS), and carbonyldiimidazole (CDI).

The best method of synthesizing (I') proved to be the carbonyldiimidazole method, leading to an 80% yield of reaction product.

By saponifying with alkali the esters of nucleotidyl-(P → N)-amino acids (dipeptides) and (I'), we obtained the analogs with free carboxy groups. Deoxythymidylyl-(5' → N)-phenylalanine (I), deoxythymidylyl-(3' → N)-phenylalanine (II), deoxythymidylyl-(5' → N)-β-alanine (III), deoxythymidylyl-(5' → N)-alanylalanine (IV), and deoxythymidylyl-(5' → 3')-deoxythymidine-(P^{in} → N)-phenylalanine (V) were synthesized by this method. In the case of the synthesis of deoxythymidylyl-(5' → N)-lysine (VI), adenylyl-(5' → N^{α})-lysine (VII), deoxythymidylyl-(5' → N^{ε})-lysine (VIII), and adenylyl-(5' → N^{ε})-lysine (IX), the phosphoric acid residue of the nucleotide was activated with the aid of mesitylenecarbonyl chloride (MsCO—Cl) [6].



VI. N=dT; VII. N=A; VIII. N=dT; IX. N=A.

The reaction was performed at 37°C for two days. It was found that the composition of the reaction products depended greatly on the pH of the medium. We selected pH 10, at which considerable amounts not only of compounds (VI) and (VII) but also (VIII) and (IX) (30 and 65%, respectively) were obtained.

The yields and some characteristics of the compounds synthesized are given in Table 1.

We have shown previously [3] that α-carboxy groups of amino acids directly affect the mechanism of the cleavage of the phosphoramide centers in the nucleotidyl-(5' → N)-amino acids. To determine the influence of the distance of the carboxy group of an amino acid from the phosphoramide center we investigated the hydrolytic stabilities of compounds (III), (IV), and (VII) (Fig. 1).

It is not difficult to observe that the β-alanine and dipeptide derivatives of the nucleotide are cleaved in an acid medium only at the phosphoramide bond, i.e., in just the same way as analogs with a blocked carboxy group [2]. Thus, a carboxy group separated from the phosphoramide center of a nucleotidopeptide by two and four atoms does not affect the cleavage mechanism. Exceptions are formed by deoxythymidylyl- and adenylyl-(5' → N^{ε})-lysines (VIII and IX), in the acid hydrolysates of which, in addition to the nucleotide and the lysine, the nucleoside and inorganic phosphate were detected. It must be assumed that the remote carboxy group of lysine in the nucleotidylyl-(5' → N^{ε})-lysine (VIII and IX) participates in intramolecular catalysis. On the basis of the previously established fact [3] that

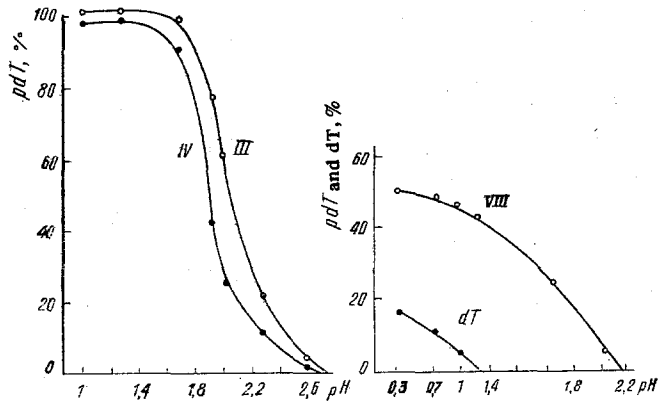


Fig. 1. Cleavage of the phosphoramide centers of (III), (IV), and (VIII) in an acid medium (1 h, 37°C).

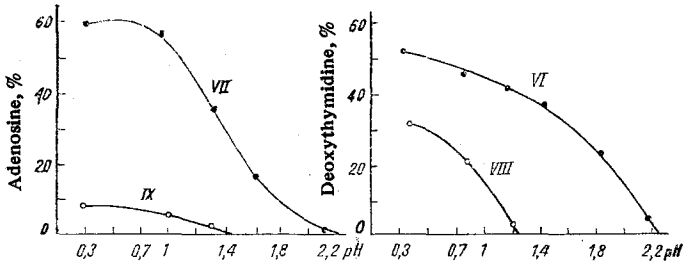
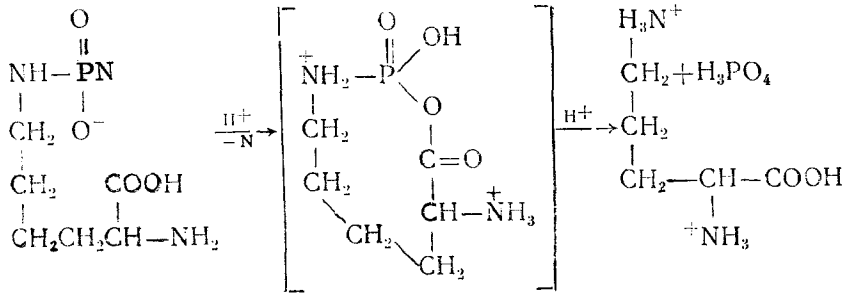


Fig. 2. Cleavage of (VII), (IX), (VI), and (VIII) to nucleotides as a function of the pH of the medium (1 h, 37°C).

the α-carboxy group of an amino acid participates in intramolecular nucleophilic catalysis, we assume a similar mechanism in the case of the nucleotidylyl-(5' → N^{ε})-lysines:



A comparison of the efficacies of the cleavage of the nucleotidylyl-(5' → N^{α})-lysines (VI and VII) and the N^{ε} analogs (VIII and IX) to the nucleosides (see Fig. 2) permits the statement that the distance of the carboxy groups in compounds (VIII) and (IX) of five atoms from the phosphoramide centers greatly lowers the efficacy of intramolecular catalysis. This is understandable, since the formation of a nine-membered intermediate ring in the case of the acid hydrolysis of the N^{ε} analogs (VIII) and IX) is far less favorable than that of a five-membered ring in the case of the N^{α}-lysine derivatives (V and VII).

A comparison of the hydrolytic stabilities of the phenylalanine phosphoramide derivatives of 5'-and 3'-deoxythymidylic acids (Fig. 3) has shown that compound (I) is cleaved to the nucleoside twice as easily as the 3'-analog (II). Thus, intramolecular nucleophilic catalysis with the participation of the α-carboxy group of phenylalanine is far more pronounced in the case of (I). An investigation of the conformation of compounds (I) and (II) with the aid of NMR spectroscopy [2] has shown that in the case of (I) there is an intramolecular interaction between the base of the nucleotide and the phenyl ring of phenylalanine, but this is practically absent in the case of (II). It is possible that this

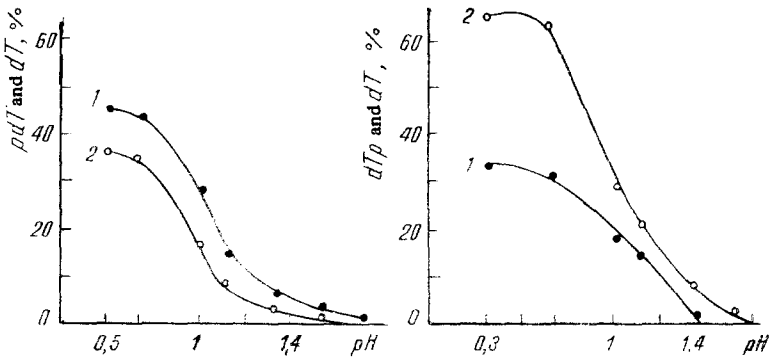


Fig. 3. Hydrolytic stabilities of (I) (a) and (II) (b) in an acid medium (1 h, 37°C): 1) dT; 2) pdT or dTp.

interaction is responsible for the appropriate position of the free carboxy group of the amino acid at the phosphoramide center in (I) and facilitates intramolecular catalysis with its participation.

In an investigation of the hydrolytic stability of [5] it was found that in an acid medium only the phosphoramide bond was cleaved. The first studies of oligonucleotidylyl-(p^{in} → N)-amino acids showed that the free carboxy group of an amino acid affects the mechanism of the cleavage of the phosphoramide center in diesters of phosphoramidic acid, as well [5]. A more detailed study of the hydrolytic stability of (V) showed that, in an acid medium, a number of cleavage products is formed. Figure 4 gives information on the accumulation of UV-absorbing cleavage products of (V) in an acid medium. The composition of the products of the acid hydrolysis of (V) shows that hydrolysis takes place in several directions:

$$\alpha(TpT)-(p^{in}\rightarrow N)\text{-Phe-OH} \longrightarrow \begin{cases} \xrightarrow{a} Phe + d(TpT) \\ \xrightarrow{b} dT + HO\text{-Phe-}pdT \rightarrow dT + pdT + Phe \\ \xrightarrow{c} dT + dTp\text{-Phe-OH} \rightarrow dT + dTp + Phe \end{cases}$$

Route "a" is the hydrolysis of the phosphoramide bond with the formation of d(TpT) and Phe, which also took place in the case of (I') [5]. Cleavage by this route takes place only to the extent of 5–8% over the whole pH range studied. With an increase in the concentration of acid, the cleavage of the phosphoramide bond decreases somewhat.

Route "b" is the cleavage of the phosphoric ester bond with the formation of dT + HO-Phe-pdT → dT + pdT + Phe.

Route "c" is the cleavage of the phosphoric ester bond with the formation of dT + dTp-Phe-OH → dT + dTp + Phe.

Route "b" predominates, since of the total amount of amides of mononucleotides 80% is made up of HO-Phe-pdT and only 20% of dTp-Phe-OH.

The phenylalanine amides of mononucleotides are cleaved further to dT and dpT (dTp). The mononucleotides are formed exclusively as the products of the hydrolysis of mononucleotide amides.

The cleavage of (V) by routes "b" and "c" is brought about by the free carboxy group of the amino acid residue. We assume that, as in the case of the nucleotidyl-(5' → N)-amino acids [3], intramolecular nucleophilic catalysis takes place.

The results obtained on the fundamental role of the free carboxy groups of the amino acid residues in the mechanism of the cleavage of the phosphoramide centers in synthetic nucleotidopeptides permits the assertion that a similar intramolecular influence may also be shown in the chemical treatment of natural nucleotide– and (nucleic acid)–protein complexes. Consequently, it is impossible to judge the type of interbiopolymer bond on the

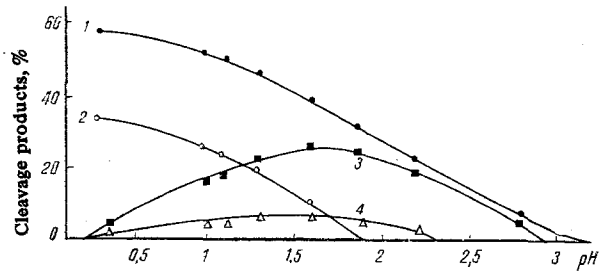


Fig. 4. Dependence of the rate of hydrolysis of (V) on the pH of the medium (37°C, 1 h): 1) dT; 2) pdT + dTp; 3) HO-Phe-pdT + dTp-Phe-OH; 4)d(TpT).

basis of the behavior of natural nucleotidopeptides with respect to chemical agents and the products of their hydrolysis.

EXPERIMENTAL

Deoxythymidine 5'-monophosphate of domestic production, carbonyldiimidazole from Fluka, N,N'-dicyclohexyldiimide from Ferak (Berlin), amino acids, DL-alanyl-DL-alanine, and Tris-HCl from Reanal, and triisopropylbenzenesulfonyl chloride from Merck were used.

Literature methods were used for the synthesis of the esters of the amino acids and the dipeptide [7], of dTp [8], of d(TpT) [9], and of (I') using the pyrophosphate method [5], and the nucleotidopeptide esters were saponified and the hydrolytic stabilities of the compounds were determined as we have described previously [3]. The compounds synthesized were isolated with the aid of preparative chromatography on FN-1 paper (rapid, GDR). For analytical purposes we used TLC on Silufol plates. The following solvent systems were employed: 1) ethanol—0.5 M ammonium acetate (5:2); 2) tert-butanol—water (7:3); and 3) isopropanol—concentrated ammonia—water (7:1:2). Paper electrophoresis was performed in 0.05 N triethylammonium bicarbonate buffer, pH 7.5 using a Labor instrument (Hungary). The structures of the nucleotidopeptides were shown by determining the base:phosphorus:amino acid ratio after complete acid hydrolysis (6 N HCl, 100°C, 24 h) [3].

Synthesis of the Nucleotidyl-(5' → N)-lysines. A solution of 0.1 mmole of the pyridinium salt of the appropriate nucleoside 5'-phosphate in 0.5 ml of anhydrous pyridine was cooled to 0°C, 0.07 ml of MsCOCl was added, and the mixture was kept at 0°C for 5 min, with careful stirring by rotation of the flask. Then 1 ml of water was added. A viscous protein residue deposited. The solution was extracted with ether (2 × 5 ml), and the aqueous layer was evaporated to small volume and chromatographed on paper in solvent system 3. This gave 70 μmole of MsCOpdT and 75 μmole of MsCOpA.

Then 50 μmole of the mixed anhydride of mesitylenecarboxylic acid and the appropriate nucleotide was dissolved in 1 ml of pyridine—water (1:5), the pH was brought to 10 with a few drops of 1 N NaOH, and 73 mg (0.5 mmole) of dry D,L-lysine hydrochloride was added. The reaction mixture was incubated at 37°C for 48 h. The reaction products were isolated with the aid of paper chromatography in solvent system 1. In this way, 13 mmole of deoxythymidylyl-(5' → N^{α})-lysine and 24 μmole of deoxythymidylyl-(5' → N^{ε})-lysine, and also 12 μmole of adenylyl-(5' → N^{α})-lysine and 24 μmole of adenylyl-(5' → N^{ε})-lysine, were obtained.

The yields, some characteristics, and proofs of the structures of the compounds obtained are given in Table 1.

Synthesis of d(TpT)-(P^{in} → N)-Phe-OEt (I'). Triisopropylbenzenesulfonyl Chloride Method. A solution of 20 μmole of the tri-n-octylammonium salt of d(TpT) in 2 ml of anhydrous pyridine was evaporated and the residue was dried by further distillation with anhydrous pyridine (4 × 2 ml). The oily residue was dissolved in 0.5 ml of anhydrous pyridine, and 0.02 ml of tri-n-octylamine and 40 μmole (13.2 mg) of freshly-recrystallized triisopropylbenzenesulfonyl chloride were added. The reaction mixture was kept at room temperature for 1 h, and then 0.1 mmole of phenylalanine ethyl ester in 0.3 ml of anhydrous pyridine was added and it was left at room temperature for 5 h. It was then deposited on a plate coated with silica gel (5 × 10 cm, layer thickness 3 mm) and chromatographed in the chloroform—methanol (9:1) system. The band with R_f 0.7 was eluted with 100 ml of chloroform—ethanol (1:1), and the eluate was evaporated, giving 6.6 μmole of (I').

Carbonyldiimidazole Method. A solution of 50 μmole of the tri-n-octylammonium salt of d(TpT) and 0.7 mmole of phenylalanine ethyl ester in 0.3 ml of anhydrous dimethylformamide was evaporated, and the residue was dried by the addition and distillation off of anhydrous dioxane (4 × 3 ml). Then 0.25 mmole (40 mg) of carbonyldiimidazole was added to the reaction mixture and it was left at room temperature for 12 h. The reaction product was isolated as described above, giving 44 mole of (I').

The yields and proofs of the structure of (I') have been given previously [2].

SUMMARY

1. A number of nucleotidopeptides of the phosphoramide type with free carboxy groups and with different distances of the carboxy groups of the amino acids from the phosphoramide center and different positions of attachment of the amino acid residue (at a 5'-, a 3'-, or an internucleotide phosphate group) have been synthesized.

2. The hydrolytic stability of the nucleotidopeptides has been investigated and it has been shown that:

The distance of the carboxy group of the amino acid residue from the phosphoramide centers affects the efficacy of the intramolecular catalysis in which it participates;

the free carboxy group of the phenylalanine residue in deoxythymidylyl-(5' → N)-phenylalanine participates far more effectively in intramolecular catalysis than in the case of the nucleotid-3'-yl analog; and

the free carboxyl groups of the amino acid residues in the oligonucleotidyl-(P^{in} → N)-amino acids are responsible for the cleavage of the phosphoric ester bonds in an acid medium.

3. The results obtained on the influence of a carboxy group of an amino acid residue in a nucleotidopeptide on the mechanism of the cleavage of the phosphoramide center must be taken into account in determining the type of interbiopolymer bond in covalent nucleotide—and nucleic acid—protein complexes.

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