EXPERIMENTAL

The elementary analyses of the compounds described corresponded to the calculated figures. N-Succinyl-L-methionine was obtained by a known method [4, 5].

Cobalt Salt of N-Succinyl-L-Methionine. A suspension of 5 g of N-succinyl-1-methionine, 2 g of cobalt hydroxide, and 40 ml of water was boiled for 2 h and was filtered hot from unchanged cobalt oxide. The latter was boiled with water three times, and the water was distilled off from the aqueous part in vacuum.

The lilac-colored residue was dissolved in 20 ml of methanol and the solution was filtered and evaporated to dryness.

In this way, 5.2 g (84.7% of theoretical) of the cobalt salt of N-succinyl-L-methionine was obtained. Lilac-colored crystals soluble in water and insoluble in organic solvents.

Copper Salt of N-Succinyl-L-methionine. A solution of 10.0 g of N-succinyl-L-methionine in 40 ml of water and 30 ml of ethanol was treated with a suspension of 10.0 g of copper hydroxide in 50 ml of water. The remainder of the synthesis was carried out in a similar manner to the preceding case. This gave 9.6 g (79.6% of theoretical) of the copper salt of N-succinyl-L-methionine, soluble in water and insoluble in organic solvents.

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OLIGONUCLEOTIDES AND NUCLEOTIDOPEPTIDES. LIII. SYNTHESIS AND PROPERTIES OF THE ETHYL ESTER OF THYMIDYL-(5′ → 3′)-THYMIDINE-(P_m → N)-SERINE

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The phosphoramide analog of the dinucleotide monophosphate EtO-Ser(P_m → N)-(TpT) has been synthesized. It has been shown that the serine amide of the dinucleoside monophosphate undergoes 20% N → O migration in an acid medium (0.1, 0.5, and 1 N HCl, 37°C, 1 h). In an alkaline medium, in contrast to other nucleopeptide phosphoramides, the phosphoramide bond is cleaved.

At the present time, natural covalent compounds of proteins and nucleic acids have been isolated from the most diverse prokaryotic and eukaryotic sources [1-5]. One of the possible types of covalent bond between proteins and nucleic acids is a bond between the functional group of an amino acid and an internucleotide phosphate group of an oligonucleotide. The possibility of the formation of such a bond has been reported previously [6] and has recently been discussed in several papers relating to eukaryotic cells [7]. In the elucidation of the physicochemical principles of the functioning of protein—NA structures a large role is played by synthetic model nucleotidopeptides.

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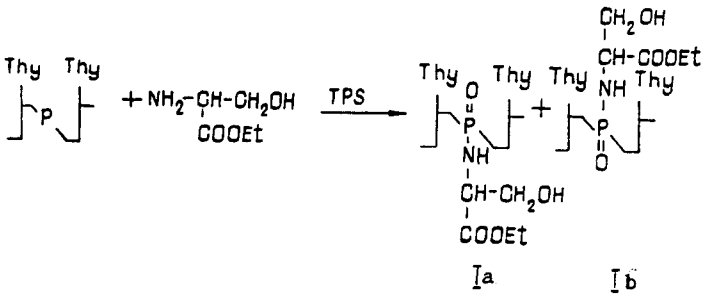
TABLE 1. Cleavage of EtO-Ser-(P_m → N)-(TpT) in an Alkaline Medium (37°C, 1 h)

Medium	Cleavage products, %					
	Ser-pT	(TpT)	T	Ser-(P_M → N)-(TpT)	EtO-Ser-(P_M → N)-(TpT)	$CH_3C(=O)COOH$
0,1 N NaOH	0	8,8	0	82	8	—
0,5 N NaOH	6,4	59	6	24	4	—
1 N NaOH	4,8	75	5	11	0	57

Model nucleotidopeptides in which the amino acid is attached to an internucleotide phosphate group have been studied in part previously [8]. However, in all cases the amino acids in the radicals contained no functional groups. It was found [3, 5, 9] that the functional groups of amino acids in mononucleotidyl-(P → N)-amino acids have a substantial effect on the mechanism of their hydrolysis. The aim of the present work was to synthesize and investigate the properties of serine amides of dinucleoside monophosphates.

Previously, the diphenyl phosphorochloridate [10] and the carbonyldiimidazole or triisopropylbenzenesulfonyl chloride [11, 12] methods have been used for the synthesis of amides of dinucleoside monophosphates. In the present work, to synthesize EtO-Ser-(P_m → N)-TpT* we have used a modified triisopropylbenzenesulfonyl chloride (TPS) method [13] with the addition to the reaction mixture as catalysts of 1-N-methylimidazole and triethylamine. The synthesis of EtO-Ser-(P_m → N)-(TpT) started from TpT, using a threefold excess of the serine ester:



The serine amide of the dinucleoside monophosphate was isolated by adsorption chromatography in a column of silica gel, in the form of an unresolved mixture of the diastereomers (Ia) and (Ib). Yield 38%. It was possible to separate the individual diastereomers only in an analytical variant using HPLC on an Ultrasphere ODS column.

We have investigated the nature of the transformation of the serine amide of TpT in the form of an unresolved mixture of diastereomers in both acid and alkaline media. It was difficult from the available information in the literature to predict what properties serine amides of nucleoside monophosphates would possess. It is known [12, 14] that phenylalanine amides of dinucleoside monophosphates are far more stable than a mononucleotide analog. These compounds are cleaved only in an acid medium at the phosphoramide bond, but when in addition to the phosphoramide center a free amino acid group is present, they are also cleaved at the phosphoric ester bonds.

Oligoribonucleotidyl-(P_m → N)-amino acids in which the free 2′-hydroxy group of ribose is present close to the phosphoramide center are cleaved mainly at the phosphoramide bond in acid and alkaline media [15]. It has been found that the serine amide of a dinucleoside monophosphate is fairly stable in an acid medium. On the action on the EtO-Ser-(P_m → N)-(TpT) of acid (0.1, 0.5, and 1 N HCl, 37°C, 1 h) a substance (18-20%) was formed which, from its chromatographic characteristics and behavior with chemical agents (it was not cleaved by hydroxylamine but was readily cleaved in neutral and alkaline media) was identified as EtO-Ser-(P_m → O)-(TpT). It may be assumed that in an acid medium the EtO-Ser-(P_m → N)-(TpT) compound undergoes N → O migration, as has been found in the case of N-diisopropylphospho derivatives of serine and threonine [16]. In the latter case, in addition to migration, cleavage of the P—O bond was also detected. This is apparently due to the severe conditions of hydrolysis (5-7% HCl, boiling).

*In the schemes and Table 1, the symbol "d" has been omitted.

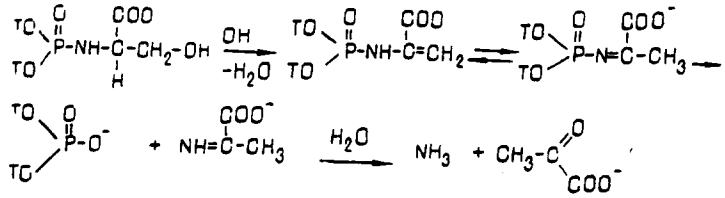
Under the same acid conditions, mononucleotidyl-(5′ → N)-serines are cleaved practically completely at the phosphoramide bond and partially at the phosphoric ester bonds [3, 5, 9]. Apparently, the difference in the properties in a strongly acidic medium (pH < 1) of the mononucleotidyl-(5′ → N)- and oligonucleotidyl-(P_m → N)-serines is due to different electrophilicities of the phosphorus atom in these compounds and to steric hindrance in the hydrolysis of the oligonucleotidyl-(P_m → N)-serines.

In the pH interval of 4-11 (37°C, 1 h), EtO-Ser-(P_m → N)-(TpT) did not undergo any chemical transformations whatever. Serine amides of mononucleotides are also stable under these conditions [15]. However, at higher concentrations of alkali (pH > 11) the serine amides of the dinucleoside monophosphates differed sharply in stability from their mononucleotide analogs. While in the case of nucleotidyl-(5′ → N)-serines in an alkaline medium (0.1, 0.5, and 1 N NaOH) only the phosphoric ester bond underwent cleavage (by 10-20%), the EtO-Ser-(P_m → N)-(TpT) compound was cleaved practically under the same conditions with the formation of the analog having a free carboxy group, together with TpT, thymidine, thymidylyl-(5 → N)-serine, ammonia, and pyruvic acid. The presence of pyruvic acid was established with the aid of lactate dehydrogenase [17], and ammonia was detected with the aid of the Nessler reagent [18].

It follows from the degradation products of EtO-Ser-(P_m → N)-(TpT) that in an alkaline medium both the phosphoramide and the phosphoric ester bonds are cleaved. Apparently, the unusual cleavage of EtO-Ser-(P_m → N)-(TpT) is due to the presence of the free hydroxy group of serine. As can be seen from Table 1, with an increase in the concentration of alkali the amount of TpT in a hydrolysate of EtO-Ser-(P_m → N)-(TpT) rises, which shows the participation of OH^- ions in the cleavage of the phosphoramide bond.

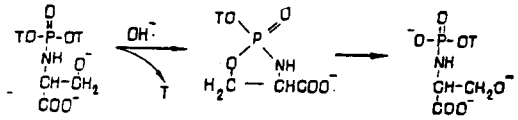
How does the cleavage of the phosphoramide take place in an alkaline medium? The presence of ammonia and pyruvic acid among the decomposition products shows that cleavage of the phosphoramide bond in EtO-Ser-(P_m → N)-(TpT) possibly takes place by a β-elimination mechanism. It is known that the cleavage of O-phosphoserine, O-phosphothreonine, and all the more, N-blocked derivatives of O-phosphoserine in an alkaline medium takes place by a β-elimination mechanism with the formation, in the final account, of ammonia and pyruvic acid [19, 20]. We have established that under the conditions of alkaline hydrolysis (1 N NaOH, 37°C, 1 h), serine and its ethyl ester, and also Ser-pT are not cleaved to form pyruvic acid.

We assume that the cleavage of the P—N bond in an alkaline medium takes place in the following way:



At alkaline pH values, the splitting out of mobile hydrogen from the α-carbon atom of serine followed by the β-elimination of the hydroxy group of the serine is observed. This forms an unsaturated compound which then breaks down to ammonia and pyruvic acid. As can be seen from Table 1, the cleavage of EtO-Ser-(P_m → N)-(TpT) by 1 N NaOH (37°C for 1 h) takes place with the formation of 75% of TpT and 57% of pyruvic acid. It is possible that under these conditions the unsaturated compound does not break down completely to ammonia and pyruvic acid and, therefore, the amount of pyruvic acid does not correspond to the amount of dinucleoside monophosphate.

EtO-Ser-(P_m → N)-(TpT) is also cleaved at the phosphoric ester bonds in an alkaline medium. This is shown by the appearance of Ser-pT and T (5-6%) in the hydrolysate. As in the case of the serine amides of mononucleotides, the cleavage of the phosphoric ester bonds apparently takes place as the consequence of an intramolecular attack by the hydroxy group of the serine on the phosphorus atom.



Thus, oligonucleotidyl-(P_m → N)-serines decompose differently from mononucleotidyl-(5′ → N)-serine. They are fairly stable in an acid medium and break down mainly at the phosphoramide bond in an alkaline medium, possibly by a β-elimination mechanism.

It is important to have information on such nontypical behavior of a phosphoramide in an alkaline medium in the investigation of the structures of natural covalent compounds of nucleic acids and proteins. The lability of such compounds

in an alkaline medium very frequently leads to the conclusion that the bond between the nucleic acid and the protein is a phosphoric ester bond. The results that we have obtained once more confirm the conclusion drawn previously [1], that it is impossible to speak of the type of covalent bond between a nucleic acid and a protein only from the results of the chemical hydrolysis of nucleic acid–protein structures.

EXPERIMENTAL

We used thymidine, TPS, and triethylamine from Merck (FRG), 1-methylimidazole from Fluka (Switzerland), silica gel from Pharmaceuticals [sic] (FRG), DEAE-cellulose from Reanal (Hungary), lactate dehydrogenase and thymidine 5′-phosphate from Reakhim (USSR), TpT obtained by the procedure of Moffatt and Khorana [21], and EtO-Ser-pT and Tp-Ser-OEt obtained as described in [22].

The mononucleotide derivatives were isolated by ion-exchange chromatography on a DEAE-cellulose column. Elution was performed with 0.02-0.2 M triethylammonium carbonate buffer.

Hydrolytic stability was studied as in [23]. The hydrolysis products were analyzed with the aid of paper chromatography on the papers FN-2 (fast), FN-4 (medium), and FN-16 (slow) (GDR). The chromatographic paper was previously washed with 2 N HCl and then with water to neutrality, and the chromatograms were run in the following systems: 1) isopropyl alcohol–NH_3–H_2O (7:1:2), 2) tert-butanol–H_2O (5:3), and 3) on Silufol, chloroform–methanol (9:1). Paper electrophoresis was carried out in 0.05 M triethylammonium bicarbonate buffer, pH 7.5. A Labor (Hungary) high-voltage instrument was used. UV spectra were recorded on a Specord UV-Vis spectrometer. Reversed-phase chromatography on a Beckman chromatograph (USA) was performed with a linear gradient of acetonitrile in water on a column of the resin Ultrasphere ODS (Beckman) with dimensions of 4.6 × 250 mm at a rate of chromatography of 1 ml/min. For analytical purposes, 0.01 OU of the substance under investigation was deposited on the column. The structures of the nucleotidopeptides were shown by determining the base–phosphorus–amino-acid ratio after complete hydrolysis (6 N HCl, 100°C, 24 h) and combustion (concentrated $HClO_4$, 180°C, 6 h) [23].

Determination of Pyruvic Acid in Hydrolysates of the Serine Derivatives. A mixture of 0.05 ml (0.25 μmole) of a solution of the substance under investigation and 0.05 ml of 1 N NaOH was incubated at 37°C for 1 h. Then it was neutralized with 0.05 ml of 1 N HCl, and was treated with 2.8 ml of an 0.05 M Tris-HCl buffer, pH 7.2, and 0.04 ml (0.25 μmole) of NADH. The optical density of the solution at λ 340 nm was measured, and then 0.02 ml (0.25 mg/ml) of a solution of lactate dehydrogenase was added. The change in the optical density was recorded over 3 min. The molar amount of NADH that had reacted was calculated from the results obtained:

$$M_{\text{NADH}} = \frac{\Delta A \cdot V}{\varepsilon},$$

where M is the number of micromoles of NADH; ΔA is the change in optical density in 3 min; ε is the molar extinction coefficient of NADH; and V is the volume of the solution.

The molar amount of NADH that had reacted is equal to the molar amount of pyruvic acid in the hydrolysate.

Synthesis of EtO-Ser-(P_m → N)-(TpT). A solution of 100 μmole (90 mg) of the tri-n-octylammonium salt of TpT and 300 μmole (40 mg) of EtO-Ser in 5 ml of absolute pyridine was evaporated in vacuum with absolute pyridine (10 ml × 4), the volume being brought to a minimum in the last evaporation, and after the addition of 0.5 ml of absolute acetonitrile, 0.4 mmole (130 mg) of TPS, 0.4 mole (32 μl) of 1-methyl-imidazole, and 0.4 mmole (28 μl) of triethylamine were added. The reaction was allowed to proceed at room temperature for 0.5 h, and then 2 ml of 50% pyridine in water was added, the mixture was kept at room temperature for 0.5 h and evaporated to dryness, and the residue was re-evaporated with chloroform (10 ml × 3) and was dissolved in 1 ml of chloroform. This solution was deposited on a column of silica gel (1 × 13 cm). Elution was performed with a chloroform–ethanol (20%) gradient (total volume 200 ml). The rate of elution was 1 ml/min. The EtO-Ser-(P_m → N)-(TpT) was eluted at a 9.5-12% concentration of ethanol in chloroform. These fractions were evaporated, and the residue was dissolved in 0.5 ml of chloroform and rechromatographed on a column of silica gel under similar conditions. This gave 38 μmole of a substance homogeneous chromatographically and electrophoretically.

Chromatographic characteristics of EtO-Ser-(P_m → N)-(TpT): R_f 0.91 in system 1 and 0.9 in system 2. Electrophoretic mobility relative to pT at pH 7.5: 0.16; ratio of thymine to phosphorus to serine: 2.2:1:0.9.

The individual diasterometers of EtO-Ser-(P_m → N)-(TpT) were seen on silica gel plates in the solvent system chloroform– methanol (9:1). R_f 0.45 and 0.49.

Analysis on an Ultrasphere ODS column (4.6 × 25 cm) was performed with the use of an acetonitrile–water (0-10%) gradient. Compounds (Ia) and (Ib) were eluted after 22.4 and 24.6 min, respectively.

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