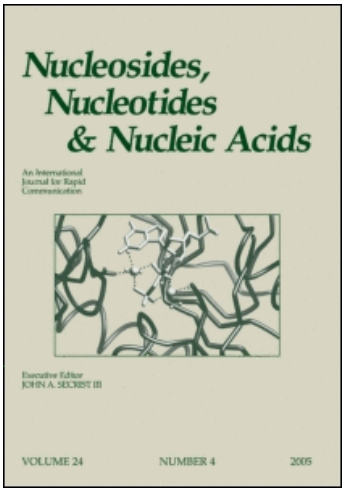

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Oligonucleotides and Nucleotide-Peptides. XXXIX. Synthesis and Properties of the Simplest Models of Covalent Nucleic Acid (Nucleotide)-protein Complexes With The Phosphodiester Bond Between The Components[I]

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OLIGONUCLEOTIDES AND NUCLEOTIDE-PEPTIDES. XXXIX. SYNTHESIS AND PROPERTIES OF THE SIMPLEST MODELS OF COVALENT NUCLEIC ACID (NUCLEOTIDE)-PROTEIN COMPLEXES WITH THE PHOSPHODIESTER BOND BETWEEN THE COMPONENTS[I]

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Abstract. A number of nucleotidyl-(5'→O)-amino acid (peptide) esters have been synthesized by the dicyclohexylcarbodiimide (DCC) and pyrophosphate methods. Their stability and mechanism of hydrolysis have been investigated.

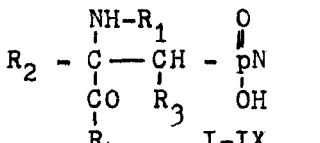
RNA- and DNA- protein complexes with a covalent bond between the components have recently been isolated from various sources[2]. To determine the biological functions of such mixed biopolymers, it is necessary to know their fine structure, including the nature of the bond between the individual fragments, which has been determined only in a few cases[2]. Investigation of the properties of synthetic model compounds, nucleotide-peptides, in which the nucleotide or the oligonucleotide is linked to the amino acid (peptide) by a covalent bond, is of great importance in solving these problems. A detailed study of nucleotide-peptides with the phosphoamide bond between the components has been made[3-6]. The hydrolytic stability of the phosphoamide bond was shown

to depend on the nature of the amino acid and the nucleotide[6], on the length of the peptide and oligonucleotide chains[4,5] and on the functional environment of the phosphorus atom[3,4]. A method for specific cleavage of the phosphoamide bond in nucleotide-peptides was worked out[4] and it has found application in determining the stucture of mixed biopolymers[7,8]. Some properties of nucleotide-peptides with the phosphodiester bond have been described earlier[4,9,10]. It is interesting to note that it was the phosphodiester bond that was found in some nucleic acid- and nucleotide-protein complexes[11-16].

The object of the present work was to synthesize nucleotidyl-(5'→0)-amino acids(peptides) and their esters and to determine the effect of the nature of amino acid containing the hydroxyl group and of the peptide chain length on the efficiency and mechanism of the hydrolysis of the phosphoester bond. A preliminary account of this work has been given earlier[17].

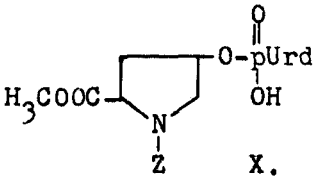
RESULTS AND DISCUSSIONS

The synthesis of nucleotidyl-(5'→0)-amino acid (dipeptide) esters was carried out using the pyrophosphate[9] and dicyclohexylcarbodiimide[10] methods, by direct addition of amino acid (dipeptide) esters to the nucleotide. As the dicyclohexylcarbodiimide method turned out to be the simpler one and to give higher yields, we applied it for preparative purposes. The nucleotide and the appropriately blocked amino acids (dipeptides) were dissolved in anhydrous pyridine, and the reaction solution was boiled in the presence of DCC. Esters of uridylyl-(5'→0)-N-Z-DL-serine (I), -DL-threonine (II), -2-methyl-DL-serine (III), -DL-serylglycine (IV), -DL-threonylglycine (V), -DL-alanyl-DL-serine (VI), deoxyadenylyl-(5'→0)-N-Z-serine (VII), deoxythymidylyl-(5'→0)-N-Z--serine (VIII), deoxycytidylyl-(5'→0)-N-Z-serine (IX) and uridylyl-(5'→0)-N-Z-L-hydroxyproline (X) were synthesized in this way.



I-IX.

I. R_1=Z; R_2=H; R_3=H; R_4=OCH_3; N=Urd; II. R_1=Z; R_2=H; R_3=CH_3; R_4=OCH_3; N=Urd; III. R_1=Z; R_2=CH_3; R_3=H; R_4=OCH_3; N=Urd; IV. R_1=Z; R_2=H; R_3=H; R_4=$NHCH_2COOC_2H_5$; N=Urd; V. R_1=Z; R_2=H; R_3=CH_3; R_4=$NHCH_2COOC_2H_5$; N=Urd; VI. R_1=$COCH(CH_3)NH$-Z; R_2=H; R_3=H; R_4=OCH_3; N=Urd; VII. R_1=Z; R_2=H; R_3=H; R_4=OCH_3; N=dAdo; VIII. R_1=Z; R_2=H; R_3=H; R_4=OCH_3; N=dThd; IX. R_1=Z; R_2=H; R_3=H; R_4=OCH_3; N=dCyd;



X.

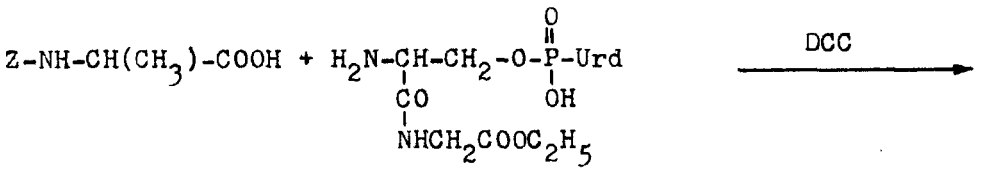
Z-benzyloxycarbonyl group.

A kinetic study has shown that the optimum reaction time was 30 min for all the compounds. The yields of the reaction products were 40-80%, depending on the nature of both the amino acid (peptide) and the nucleotide component.

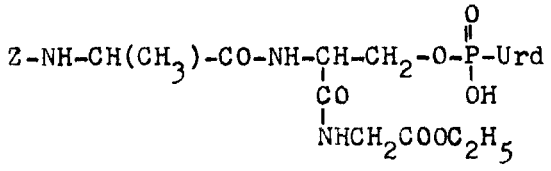
The benzyloxycarbonyl group in compound Z-Ser(OpU)-Gly--OEt (IV) was removed by HBr in anhydrous dioxane. H-Ser-(OpU)-Gly-OEt (XI) was obtained in this way.

The tripeptide derivative, ethyl ester of uridylyl-(5'→O)-(N-Z-DL-alanyl)-DL-serylglycine (XII), was synthesized by elongation of the peptide chain in an already prepared dipeptide derivative using DCC. The approach had been successful in our previous synthesis of nucleotidyl-(P→N)-peptides[5].

Uridylyl-(5'→O)-N-Z-DL-serine (XIII), -N-Z-DL-threonine (XIV), -(N-Z-DL-alanyl)-DL-serine (XV), -N-Z-DL-serylglycine (XVI) were obtained by alkaline hydrolysis of their esters. In view of the fact that saponification of the ester bond is accompanied by phosphodiester bond cleavage, the concentration of alkali was chosen in each particular case, depending on the behaviour of the ester analogues in an alkaline medium. For all the compounds investigated, the opti-



XI.



XII.

mum concentration of alkali did not exceed 0.04-0.1N, the reaction time being 0.5-1h, 37°C.

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

A study of the hydrolytic stability of the compounds synthesized (I-X and XII), which contain a blocked amino group, showed that the phosphodiester bond in nucleotidyl-(5'→O)-amino acid (peptide) esters is stable in acid (1 N HCl, 37°C, 1h) and it is hydrolyzed by alkali (see Fig.1). The stability toward acids is somewhat inconsistent with the findings published earlier[9], but it is in good agreement with the general characteristics of diesters of phosphoric acid[18,19]. The rate of alkaline hydrolysis depends considerably on the nature of aminoxydroxy acid involved in the formation of the phosphodiester bond: Z-Hyp(OpU)-OMe and Bz-Tyr(OpU)-OMe, which has been described previously[10], are hydrolyzed only under rigid conditions (30%; in 1 N NaOH, 100°C, 1h), whereas the amino-β-hydroxy acid analogues are much more labile (see Fig.1).

It has been found previously[4,5] that the elongation of the peptide chain in nucleotide-peptides of the phosphoamide type stabilizes the bond between the nucleotide and the peptide. The data on the hydrolysis of nucleotidyl-(5'→O)-peptide derivatives (see Fig.1 and 2) indicate that the ten-

TABLE 1

Some characteristics of synthesized compounds

Compound	Yield %[a]	R_f in systems A	B	C	UpN, pH 7.5	Ratio of base to phosphorus and to amino acid[9]
I	64	0.78	0.73	0.60	0.52	1:1.03:0.92
II	37	0.82	0.72	0.60	0.52	1:1.05:0.93
III	37	0.83	0.73	0.64	0.51	1:0.98[b]
IV	75	0.81	0.74	0.64	0.49	1:1.02:0.80:0.99
V	48	0.82	0.70	0.63	0.52	1:1.05:0.85:1.01
VI	76	0.83	0.74	0.62	0.50	1:1.03:0.88
VII	63	0.80	0.73	0.68	0.50	1:0.98:0.95
VIII	68	0.87	0.78	0.72	0.50	1:1.05:0.86
IX	36	0.78	0.75	0.60	0.49	1:1.03:0.78
X	60	0.82	0.59	0.51	0.52	1:1.03[b]
XI	90	0.57	0.35	0.35	0.33	1:0.97:0.85:0.95
XII	82	0.84	0.65	0.58	0.53	1:1.04:0.82:0.89:0.96
XIII	74[c]	0.57	0.27	0.52	0.92	1:0.92:0.89
XIV	74[c]	0.60	0.36	0.57	0.89	1:1.09:0.96
XV	77[c]	0.60	0.36	0.53	0.88	1:0.95:0.95:0.98
XVI	88[c]	0.60	0.30	0.51	0.88	1:1.07:0.80:0.94

a) DCC method.
b) The ratio of base to phosphorus was determined as in[9]. Amino acid was determined qualitatively.
c) Yields of saponification of ester bond under optimum conditions are shown.

dency is not observed in the case of nucleotide-peptides of the phosphodiester type.

The elongation of the peptide chain from the N-terminus of aminoxydroxy acid (compound VI) does not affect the stability of the phosphodiester bond, whereas the elongation of the peptide chain from the C-terminus of aminohydroxy acid (compounds IV, V and XII) even labilizes the

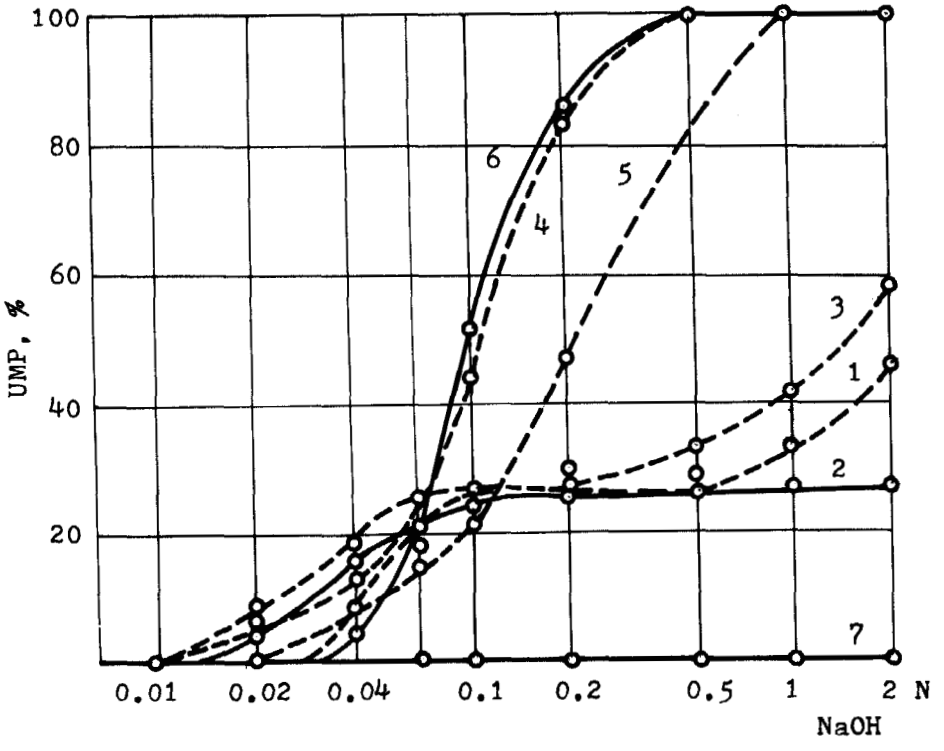


FIG.1. The stability of the phosphodiester bond in Z-Ser-(OpU)-OMe (1), Z-Thr(OpU)-OMe (2), Z-Ala-Ser(OpU)-OMe (3), Z-Ser(OpU)-Gly-OEt (4), Z-Thr(OpU)-Gly-OEt (5), Z-Ala-Ser-(OpU)-Gly-OEt (6) and Z-Hyp(OpU)-OMe (7) depending on NaOH concentration (37°C, 1h).

phosphodiester bond greatly. This suggests that the C-terminus of aminohydroxy acids is of particular importance in the mechanism of cleavage of nucleotidyl-(5'→O)-aminohydroxy acids (peptides). The dependence of the rate of phosphodiester bond cleavage on the state of the carboxy group (free or blocked) of amino-β-hydroxy acid becomes apparent on comparing alkaline hydrolysis of uridylyl-(5'→O)-amino acid (peptide) esters (see Fig.1) with that of their analogues with a free carboxy group (see Fig.2). Z-Ser(OpU)-OH (XIII), Z-Thr(OpU)-OH (XIV) and Z-Ala-Ser(OpU)-OH (XV) are much more stable than their ester analogues (I), (II) and (VI), whereas the rate of hydrolysis of Z-Ser(OpU)-Gly-OH (XVI) and Z-Ser(OpU)-Gly-OEt (IV) is the same. All this

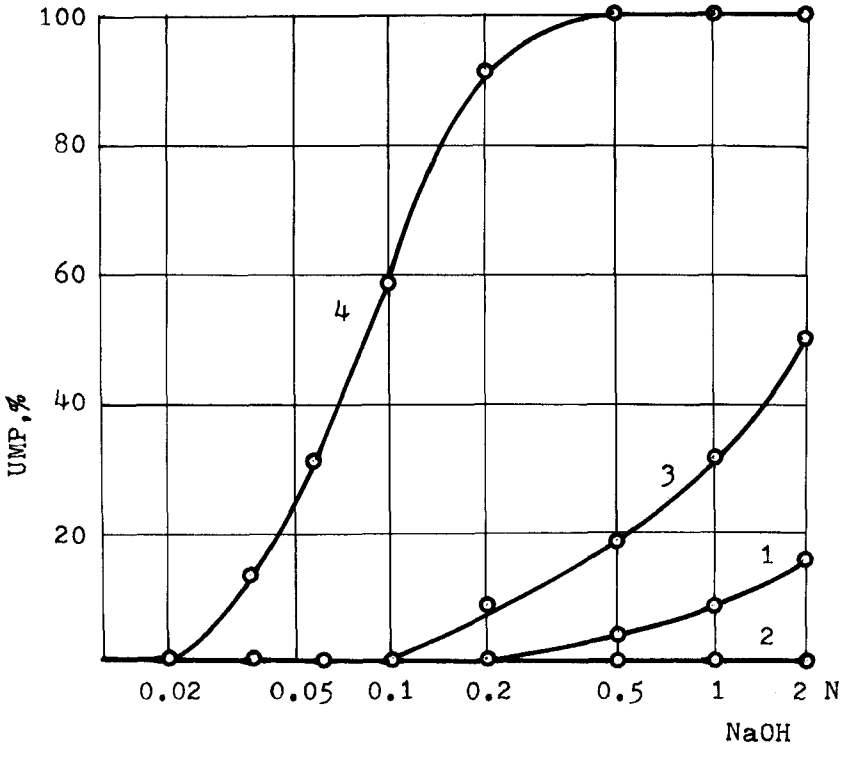


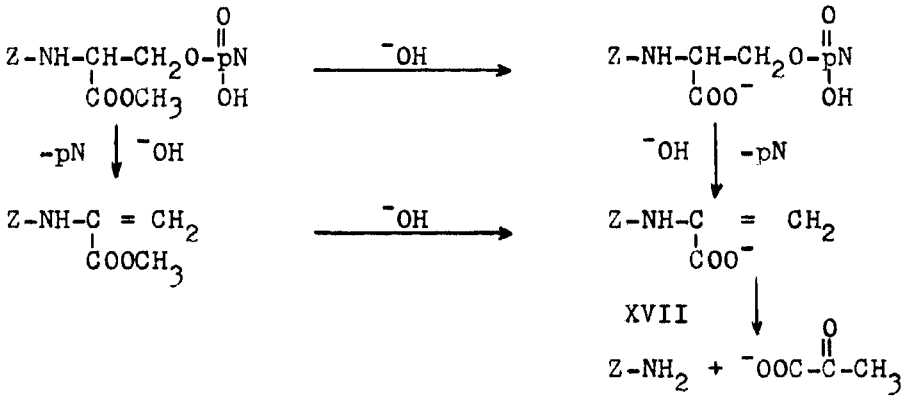
FIG.2. The stability of the phosphodiester bond in Z-Ser-(OpU)-OH (1), Z-Thr(OpU)-OH (2), Z-Ala-Ser(OpU)-OH (3), Z-Ser(OpU)-Gly-OH (4) depending on NaOH concentration (37°C, 1h).

indicates that the carboxy group of amino-β-hydroxy acids stabilizes the phosphodiester bond in uridylyl-(5→O)-aminohydroxy acids (peptides). The carboxy group, remote from the phosphodiester bond, does not affect its hydrolytic stability. This can account for the high lability of the phosphodiester bond in the peptide derivatives of UMP (IV, V, XII, XVI).

It may be inferred from the experimental data that the mechanism of phosphodiester bond cleavage in nucleotidyl-(5→O)-amino acids (peptides) depends on the structure of the amino acid (peptide) component. In the case of hydroxyproline (X) and the previously described[10] tyrosine derivatives of UMP, nucleophilic substitution at the phosphorus

atom involves participation of the hydroxide ion, and they behave in the way characteristic of diesters of phosphoric acid[18,19]. We have assumed that nucleotidyl-(5'→O)-N-Z-amino-β-hydroxy acid esters decompose in alkali by the β-elimination mechanism[20].

The maximum at 241 nm observed by us in the differential UV spectrum of the alkaline hydrolyzate of Z-Ser(OpU)--OMe serves as direct proof of this mechanism[21,22]. Another proof of β-elimination was the presence of pyruvic acid, the final product of hydrolysis of N-Z-aminoacrylic acid (XVII), in the alkaline hydrolyzate of Z-Ser(OpU)-OMe (I). Pyruvic acid was determined by lactate dehydrogenase. The total amount of pyruvic acid and N-Z-aminoacrylic acid turned out to be nearly equal to the amount of UMP formed. The final proof of the β-elimination mechanism was obtained during the study of the hydrolytic stability of methyl ester of uridylyl-(5'→O)-N-Z-2-methylserine (III). For β--elimination to proceed, the presence of a proton at the α-carbon atom of aminohydroxy acid is necessary, which is not the case with compound (III). The compound (III) is stable in the range from 1N HCl to 2N NaOH (37°C, 1h), i.e. it behaves in the same way as Z-Hyp(OpU)-OMe and Bz-Tyr-(OpU)-OMe which was described earlier[10]. The only explanation of the stability of the 2-methylserine derivative (III) and the difference between its properties and those



of the serine (I) and threonine (II) derivatives is the inability of compound (III) to undergo β-elimination.

A study of the hydrolysis of N-Z-serine derivatives of dTMP, dCMP and dAMP has shown that the nature of heterocyclic base does not affect the efficiency of phosphodiester bond cleavage.

Thus, the data obtained by us previously and in the course of the present work indicate that the stability and the mechanism of hydrolysis of the phosphodiester bond in nucleotidyl-(5$\rightarrow$O)-amino acids (peptides) depends on the nature of aminohydroxy acid. If hydroxyproline or tyrosine is involved in the formation of the phosphodiester bond, it is stable in moderately acidic and alkaline media, whereas under rigid alkaline conditions its degradation is analogous to that of simple diesters of phosphoric acid. When N-acyl-amino-β-hydroxy acids are involved in the formation of the phosphodiester bond, β-elimination takes place in an alkaline medium. The efficiency of β-elimination depends on whether the carboxy group of amino acids is free or blocked. In the case of nucleotidyl-(5$\rightarrow$O)-amino-β-hydroxy acids with a free amino group O$\rightarrow$N migration takes place[9].

The data on the hydrolytic stability of nucleotidyl--(5$\rightarrow$O)-amino acids (peptides) can be helpful in determining the nature of the bond between the nucleic acid (nucleotide) and the protein in covalent nucleic acid- and nucleotide - protein complexes. The stability of such complexes in an alkaline medium often leads to invoking the presence of the phosphoamide bond[23-26]. It should be kept in view that nucleotidyl-(P$\rightarrow$O)-amino acids, in which the hydroxyl group of tyrosine and hydroxyproline is involved in the formation of the phosphodiester bond, are also quite stable in an alkaline medium. To determine the type of a bond, it is particularly important that specific methods of degradation should be available. Phosphodiesterases are enzymes which specifically cleave the phosphodiester bond. All the above--mentioned synthesized compounds (I-XVI) were degraded by snake venom phosphodiesterase. However, the use of this en-

zyme does not make it possible to state what aminohydroxy acid is involved in the formation of the phosphodiester bond. The investigation of the hydrolytic stability of nucleotidyl-(P→O)-peptides of unknown structure in an alkaline medium makes it possible to distinguish the serine (threonine) derivatives of nucleotides from the tyrosine (hydroxyproline) analogues.

The presence of readily determinable pyruvic acid in the alkaline hydrolyzates suggests that serine is involved in the formation of the phosphodiester bond between the nucleotide (oligonucleotide) and the amino acid (peptide). Thus, the study of the properties of model nucleotidyl-(P→O)-amino acids (peptides) made it possible to find methods for identification of some aminohydroxy acids involved in the formation of the phosphodiester bond.

EXPERIMENTAL

The following reagents were used: uridine 5'-monophosphate disodium salt, NADH, DL-alanyl-DL-serine, N-benzyloxycarbonyl-DL-alanine, ethyl ester of N-benzyloxycarbonyl-DL-serylglycine, cyclohexylammonium salt of N-benzyloxycarbonyl-L-hydroxyproline (Reanal, Hungary), Dowex 50 (Serva, GFR), N, N'-dicyclohexylcarbodiimide (Ferak Berlin), snake venom phosphodiesterase (Koch-Light Laboratories Ltd.,UK), propargyl alcohol (Veb Laborchemie Apolda, GDR), amino acids and other reagents (USSR). The previously described methods were employed for the synthesis of compounds (I-X)[9,10], benzyloxycarbonyl chloride, N-benzyloxycarbonyl amino acids and N-benzyloxycarbonyl-DL-alanyl-DL-serine[27], diazomethane, methyl esters of N-benzyloxycarbonyl amino acids and of N-benzyloxycarbonyl-DL-alanyl-DL-serine[28], hydroxyacetone[29].

The hydrolysis of nucleotidyl-(P→O)-N-benzyloxycarbonyl amino acids (peptides) and their esters was carried out as described earlier[9]. The yields of the compounds synthesized (I-XVI) were determined spectrophotometrically. The stucture of nucleotidyl-(P→O)-amino acid (pepti-

de) derivatives was proved by means of degradation with snake venom phosphodiesterase and by determining the ratio of base to phosphorus and to amino acid after complete acid hydrolysis[9]. Nucleotidyl-(5→0)-amino acid derivatives were isolated by preparative chromatography on FN-1, FN-7 paper (rapid, Filtrak, GDR) and by thin-layer chromatography on Silpearl UV 254 silica gel (Sklarny Kavalier, Czechoslovakia). The following solvent systems were used; ethyl alcohol-1M ammonium acetate, 7:3 (A); tert.butyl alcohol-water, 7:3 (B); n-butyl alcohol-glacial acetic acid-water, 5:2:3 (C); Electrophoresis on FN-15 paper (slow, Filtrak, GDR) was performed in 0.05 M triethylammonium bicarbonate buffer, pH 7.5. A vertical high-voltage "Labor" device (Hungary) was employed.

2-methyl-DL-serine. 55 mL (0.78 mol) of hydroxyacetone, 45 mL (1.13 mol) of conc.ammonia, 5g (0.09 mol) of ammonium chloride and 6g (0.09 mol) of potassium cyanide was autoclaved at 50°C for 5h, evaporated to dryness in vacuo. 60 mL (1.9 mol) of conc. HCl was added, and the reaction mixture was refluxed for 2.5h. The brown residue obtained on evaporation was extracted with methyl alcohol (2x10mL). The cooled extract was filtered, tri-n-butylamine was added to the filtrate until the reaction was weakly alkaline and the filtrate was allowed to stand for 12 h at 8°C. The precipitate formed was twice recrystallized from 85% ethyl alcohol (yield 1.2g, 12%). M.p. 241-242°C (lit.[30] 242-243°C). Found, %: C 40.03; H 7.81; N 11.31; $C_4H_9N_1O_3$. Calculated,%: C 40.33; H 7.62; N 11.76.

Methyl ester of uridylyl-(5→0)-N-Z-DL-serine (I). Pyrophosphate method. 0.2 mmol of uridine 5'-monophosphate trioctylammonium salt was dissolved in 1 mL of anhydrous dioxane, freshly distilled diphenylchlorophosphate (0.4 mmol, 0.08 mL) was added first, and then a solution of dry tri-n-butylamine (0.45 mmol, 0.12 mL) in anhydrous dioxane (1 mL) was immediately added dropwise with vigorous stirring for some minutes. After 3h the reaction mixture was concentrated by evaporation to about 0.2 mL (at a temperature not

higher than 30°C), 25 mL of cold anhydrous ether was added, the mixture was shaken vigorously, allowed to stand at 8°C for 30 min, and the ether was decanted. The oily residue of p^1-uridine-5'-p^2-diphenylpyrophosphate was dissolved in 0.5 mL of anhydrous dioxane. o.5 mL of methyl ester of N--Z-DL-serine (1 mmol) in dioxane and 1 mmol of tri-n-butylamine or pyridine was added, and the reaction mixture was allowed to stand at room temperature for 12h. Methyl ester of uridylyl-(5'→0)-N-Z-DL-serine (I) was isolated by paper chromatography in solvent system B. The band with R_f 0.73 was eluted with water. 96 μmol of compound (I) was obtained in 48% yield.

Compounds (II-V) and (X) were synthesized analogously.

Some characteristics and proof of the structure of the synthesized compounds are given in Table 1.

DCC method. 0.2 mmol of uridine 5'-monophosphate trioctylammonium salt was dissolved in 2 mL of anhydrous pyridine, then 1 mmol of methyl ester of N-Z-DL-serine and 0.8 mmol of DCC was added. The reaction mixture was boiled for 2h, the precipitated dicyclohexyl urea was filtered off, and the reaction product was isolated by paper chromatography in solvent system A. The band with R_f 0.78 was eluted with water. 0.11 mmol of compound (I) was obtained in 55% yield.

Compounds (II-X) were prepared analogously.

Some characteristics and proof of the structure of the synthesized compounds are given in Table 1.

Ethyl ester of uridylyl-(5'→0)-DL-serylglycine (XI). To 0.015 mmol of ethyl ester of uridylyl-(5'→0)-N-benzyloxycarbonyl-DL-serylglycine (IV) 0.3 mL of 10% HBr in dioxane was added, and the mixture was incubated for 2h at room temperature. Then the reaction mixture was evaporated to dryness in vacuo. 0.2 mL of solvent system B was added, and the reaction mixture was chromatographed in the system on a thin-layer silica gel. The band with R_f 0.5, absorbing UV light and giving a positive ninhydrin reaction, was eluted with 50% isopropyl alcohol. The elu-

ate was concentrated by evaporation. 0.011 mmol ethyl ester of uridylyl-(5'→O)-DL-serylglycine was obtained in 74% yield.

Ethyl ester of uridylyl-(5'→O)-(N-Z-DL-alanyl)-DL-serylglycine (XII). 0.01 mmol of ethyl ester of uridylyl-(5'→O)-DL-serylglycine (XI) was dissolved in 1 mL of water, 0.04 mmol (0.02 mL) of tri-n-octylamine was added, the reaction solution was concentrated by evaporation and dried by azeotropic distillation with anhydrous benzene and anhydrous dioxane. The residue was dissolved in 0.3 mL of anhydrous dimethylformamide, 0.1 mmol (22 mg) of N-Z-alanine and 0.1 mmol (21 mg) of DCC was added, and the reaction mixture was allowed to stand for 3h at 37°C. The reaction product was isolated by paper chromatography in solvent system B. The band with R_f 0.65 was eluted with water. 6.5 μmol of compound (XII) was obtained in 65% yield.

Hydrolysis of uridylyl-(P→O)-amino acid derivatives by phosphodiesterase. To 0.05 mL of 6 mM solution of the compound under investigation 0.01 mL of snake venom phosphodiesterase solution was added (2 mg was dissolved in 1 mL of 0.05 M Tris-HCl buffer, pH 8.8, containing 0.03 M $MgCl_2$), and the reaction mixture was incubated for 2h at 37°C. Then it was subjected to paper chromatography in solvent system A. The UV-absorbing spots were identified by comparison with reference compounds.

Determination of pyruvic acid by lactate dehydrogenase. 0.05 mL of 0.1-2 N NaOH was added to 0.05 mL of 6mM solution of ethyl ester of uridylyl-(5'→O)-N-benzyloxycarbonyl-DL-serine, the reaction mixture was incubated for 1h at 37°C and neutralized by 0.05 mL of HCl of appropriate concentration. 2.75 mL of 0.05 M Tris-HCl buffer (pH 7.2), 0.05 mL of 6mM NADH and 0.04 mL of lactate dehydrogenase solution (25 mg/mL) was added. The reaction kinetics were observed by a change in optical density at 340nm. The amount of pyruvic acid formed corresponded to the decrease in NADH concentration.

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