OLIGONUCLEOTIDES AND NUCLEOTIDOPEPTIDES

XV. SYNTHESIS AND SOME PROPERTIES OF THE METHYL ESTER OF URIDYLYL-(5' → O)-N-BENZOYL-L-TYROSINE

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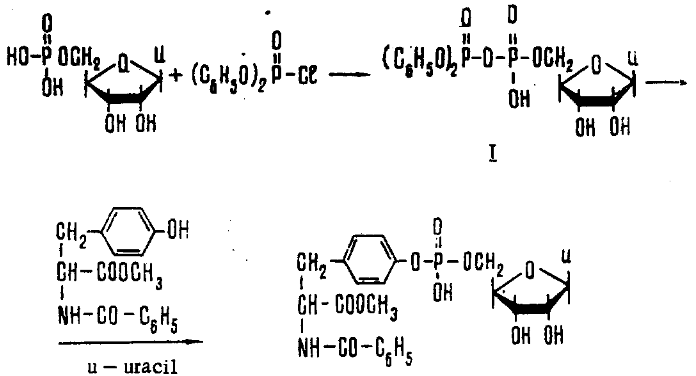
In a study of the mechanism of the action and regulation of some enzymes, it was found that in a number of cases nucleotides are covalently attached to the enzymes and either activate [1] or inactivate [2] them. Interesting in this respect is the enzyme glutamine synthetase from E. coli. It catalyzes a very important reaction of the metabolism of amino acids and nitrogen – the biosynthesis of glutamine from glutamic acid and ammonia. It has been shown [2] that the regulation of this enzyme involves a complex protein system inactivating glutamine synthetase by the covalent additon to it of adenosine 5'-phosphate (AMP). It has been established [3] that AMP adds to the enzyme through the hydroxy group of the tyrosine by means of a phosphoric ester bond. It is assumed [2] that the covalent addition to the protein of uridine 5'-phosphate (UMP) also participates in the complex system of regulation. It may be that an analogous phenomenon is observed in the case of RNA polymerase [4].

The present paper describes the chemical synthesis and some properties of the methyl ester of uridylyl-(5' →O)-N-benzoyl-L-tyrosine, the simplest model of this protein nucleotide interaction.

The synthesis was performed by the mixed-anhydride and dicyclohexylcarbodiimide (DCC) methods. The mixed-anhydride method was based on the use of the high reactivity of an unsymmetrical ester of pyrophosphoric acid – P^1-diphenyl P^2-uridin-5'-yl pyrophosphate (I).



I

u – uracil

II

The reaction was performed in absolute dioxane [5]. We have previously synthesized by this method nucleotide phosphoric esters of hydroxy amino acids and peptides [6]. However, in all cases, the yield was low. Consequently, to synthesize compound (II) we used the DCC method. The trioctylammonium or pyridinium salt of uridine 5'-phosphate, a fivefold excess of the methyl ester of N-benzoyl-L-tyrosine, and a fourfold excess of DCC were boiled in absolute pyridine for 3 h. The yield of methyl ester of uridylyl-(5' →O)-N-benzoyl-L-tyrosine was 75%. The reaction mixture was found to contain, in addition to the main product, about 20% of uridine and 5% of an unidentified compound with R_f 0.35 and 0.14 in solvent systems 1 and 2, respectively.

Control experiments performed with the trioctylammonium salt of uridine 5'-phosphate and compound (II) under the reaction conditions (without DCC) showed that the uridine found in the reaction mixture is the product of the partial cleavage of the nucleotide.

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TABLE 1. Some Characteristics of the Reaction Products and the Initial Compounds

Compound	Yield by method*		R_f in systems				U rel. UMP†		Ratio‡ of base to phosphorus to amino acid
	mixed anhydride	DCC	1	2	3	4	pH 3,5	pH 7,6	
Uridine 5'-phosphate	—	—	0,29	0,08	0,09	0,04	1,0	1,0	—
Uridine	—	—	0,71	0,48	0,74	0,42	0,17	0,15	—
Methyl ester of uridylyl-(5'→O)-N-benzoyl-L-tyrosine	25	75	0,54	0,21	0,19	0,08	1,0	0,83	1:0,95:0,94
Methyl ester of N-benzoyl-L-tyrosine	—	—	0,95	0,91	0,98	0,94	0	0	—
Ethyl ester of L-tyrosine	—	—	0,90	0,79	0,83	0,56	0,18	0,45	—
L-Tyrosine	—	—	0,66	0,33	0,40	0,32	0,16	0,19	—

*Yield determined spectrophotometrically.

†Electrophoretic mobility relative to UMP.

‡ Determined after hydrolysis with 6 N HCl at 105°C for 24 h.

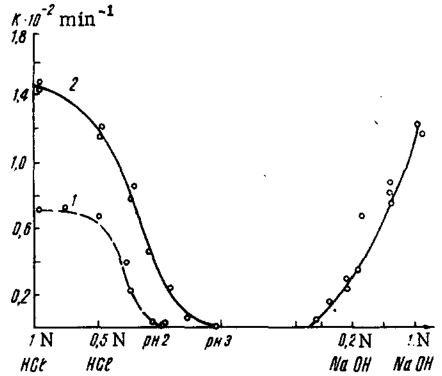

Fig. 1. Hydrolytic stability of compound (II) as a function of the pH (100°C, 60 min): 1) uridine; 2) uridine 5'-phosphate.

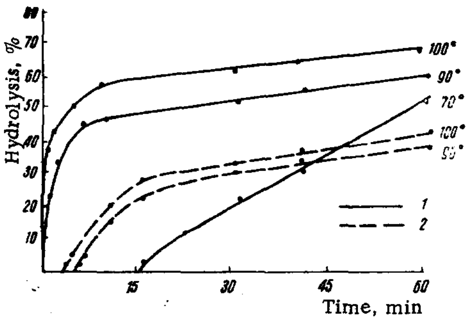

Fig. 2. Kinetics of the hydrolysis of compound (II) at 70, 90, and 100°C (2 N HCl). 1) Uridine 5'-phosphate; 2) uridine.

To avoid the decomposition of the UMP and the formation of byproducts, the reaction was performed in absolute dioxane, in a mixture of tert-butanol and water (1 : 1), and in absolute dimethylformamide. However, in the first case the yield of compound (II) was only 20%, and the reaction did not take place in the mixture of tert-butanol and water. In absolute dimethylformamide, polymerization of the UMP took place, which gave many different reaction products. In this reaction, apparently, the pyridine did not only play the role of a passive solvent. Some characteristics of the reaction products and other substances used for the investigation are given in Table 1.

The results of a study of the hydrolysis of compound (II) as a function of the pH (Fig. 1) shows that it is fairly stable in both acid and alkaline media. Hydrolysis takes place only under severe conditions (100°C, 60 min). Such stability is characteristic for the majority of diesters of phosphoric acid [7]. It is possible that $p_\pi - d_\pi$ conjugation between the oxygen atoms of the substituents and the phosphorus atom opposes nucleophilic attack on the tetrahedral phosphorus atom. However, information on the hydrolytic stability of esters of uridylyl-(5'→ O)-oxy amino acids (peptides) that we have studied previously permit the conclusion that the stability and nature of the cleavage of the phosphoric ester center in nucleotidyl-(P→O)-oxy amino acids depends strongly on the nature of the amino acid fragment. A particular influence is shown by the amino groups of hydroxy amino acids located adjacent to the phosphoric ester bond. As has been shown [6], in this case O → N migration takes place in an alkaline medium. As can be seen from Fig. 1, compound (II) is cleaved into uridine 5'-phosphate and uridine in an acid medium, i.e., both phosphoric ester bonds hydrolyze, and in an alkaline medium it forms the nucleotide. A similar picture is observed in a study of the kinetics of the hydrolysis of compound (II) in acid (Fig. 2) and alkaline (Fig. 3) media. The curves of the decomposition of the methyl ester of uridylyl-(5' →O)-N-benzoyl-L-tyrosine were plotted as a function of the accumulation of uridine and uridine 5'-phosphate in the hydrolyzates in an acid medium and of uridine 5'-phosphate in an alkaline

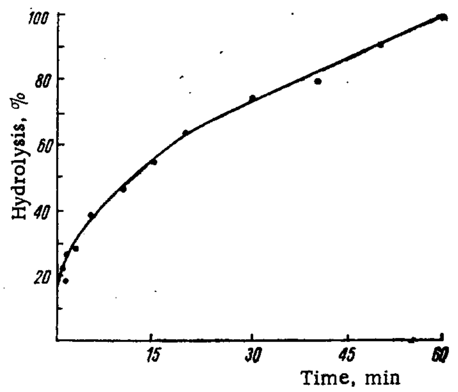

Fig. 3. Kinetics of the hydrolysis of compound (II) in an alkaline medium (2N NaOH, 100°C).

medium and, consequently, characterize only the cleavage of the phosphoric diester center. The activation energies were found from the kinetic results obtained in the hydrolysis of compound (II) in an acid medium at three different temperatures (see Fig. 2): 10 kcal/mole in hydrolysis to the nucleotide and 56 kcal/mole in hydrolysis to the nucleoside.

The hydrolytic results show that if uridine 5'-phosphate adds to a protein by means of a phosphoric ester bond formed between the hydroxy group of the tyrosine and the phosphoric acid residue of the nucleotide, this bond should be fairly stable both in acid and alkaline media. This statement is the more valid [6, 8] in that a lengthening of the peptide chain in nucleotidopeptides stabilizes both the phosphoramide and the phosphoric ester bonds.

EXPERIMENTAL

Chromatography was performed on German chromatographic papers FN7, FN12, and FN16 in the following solvent systems: 1) ethanol – 1 M ammonium acetate (7 : 3); 2) isopropanol – concentrated ammonia–water (7 : 1 : 2); 3) tert-butanol–water (7 : 3); and 4) n-butanol–glacial acetic acid–water (4 : 1 : 5). Paper electrophoresis was performed with the aid of a high-voltage vertical instrument on the firm of "Labor" (Hungary). Acetate (pH 3.5) and phosphate (pH 7.6) buffers were used. The amount of tyrosine was determined from a calibration curve and the amount of phosphorus by the method of Lowry and Lewis in Skulachev's modification [9]. In the spectrophotometric determination of the amount of uridylic acid we used Cowgill's calculating factors [10].

The experiments were performed with the disodium salt of uridine 5'-phosphate ("Reanal") and the methyl ester of N-benzoyl-L-tyrosine ("Reanal"). The diphenylphosphorochloridate was obtained by the method of Brigl and Muller [11].

The methyl ester of uridylyl-(5' → O)-N-benzoyl-L-tyrosine was synthesized by the mixed-anhydride method and was hydrolyzed by a method described previously [6].

Synthesis of Compound (II) by the Dicyclohexylcarbodiimide Method. Uridine 5'-phosphate (0.2 mmole of the disodium salt) was dissolved in 0.5 ml of distilled water and passed through a column of Dowex-50 (H^+ form). The aqueous solution was evaporated (all evaporation operations were performed in a rotary evaporator under reduced pressure) to small volume, tri-n-octylamine (0.1 ml; 0.22 mmole) was added, the mixture was shaken and evaporated to dryness, and the residue was dissolved in absolute dioxane. The clear solution was again evaporated to dryness, and the residue was dried by repeated azeotropic distillation with dioxane and benzene at 30-35°C. The dry vitreous mass was dissolved in 2 ml of absolute pyridine, and 0.299 g (1 mmole) of the methyl ester of N-benzoyl-L-tryosine and 0.164 g (0.8 mmole) of N,N'-dicyclohexylcarbodiimide were added. The reaction mixture was boiled and after 2 h the precipitated dicyclohexylurea was filtered off and the clear reaction mixture was chromatographed on paper in system 1. The zone with R_f 0.54, absorbing in UV light, was cut out and eluted with distilled water, and the solution was evaporated to the required concentration. The yield of the methyl ester of uridylyl-(5' → O)-N-benzoyl-L-tyrosine was 75%.

The reaction took place similarly in absolute dioxane, absolute dimethylformamide, and a mixture of tert-butanol and water (1 : 1).

CONCLUSIONS

1. The methyl ester of uridylyl-(5' → O)-N-benzoyl-L-tyrosine has been obtained by the mixed-anhydride method and by the dicyclohexylcarbodiimide method.

2 The model synthesized is fairly stable both in acid and in alkaline media. Its hydrolysis takes place only under severe conditions (1 h, 100°C). The products of hydrolysis in an acid medium are uridine and uridine 5'-phosphate, and in an alkaline medium only uridine 5'-phosphate.

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