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INFLUENCE OF A REMOTE OH GROUP ON THE STABILITY OF THE PHOSPHORAMIDE BOND IN AN ESTER OF URIDYLYL(5'→N)ALANINE

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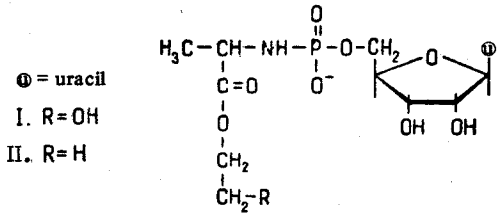
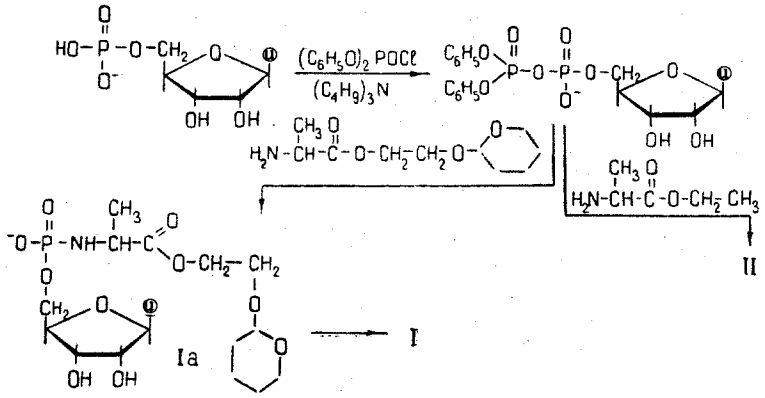
The influence of neighboring functional groups in molecules of the nucleotido(P→N) peptides on the properties of the nucleotidopeptide bond [1–3] has been studied by comparing the stability of the phosphoramide bond in the ethyl (II) and hydroxyethyl (I) esters of uridylyl(5'→N)alanine.



In previous papers [3, 4] it was shown that an OH group of ribose closely adjacent to the phosphoramide grouping [for example in uridylyl(3→N)-phenylalanine] considerably increases the lability of the phosphoramide bond in the region of neutral and slightly alkaline pH values where the isolated phosphoramide bond is very stable [1]. This activating influence of a neighboring cis hydroxyl is explained [1] by the intermolecular protonation of the amide nitrogen even at alkaline pH values.

In order to elucidate the analogous influence on the phosphoramide bond of a remote OH group which, however, may be spatially adjacent to the phosphoramide grouping because of free rotation about the ordinary bonds in the amino acid residue, we have studied the properties of compounds (I) and (II) differing by the presence or absence of a hydroxy group in the amino acid fragment.

Compounds (I) and (II) were obtained by the procedure developed previously [1] from the mixed anhydride of uridine-5' phosphate and diphenyl phosphate and the corresponding ester of alanine in the following way:



To obtain compound II, alanine ethyl ester was used as the amino component. The synthesis of substance I was effected from the tetrahydropyranyloxyethyl* ester of alanine, obtained by the action of dihydropyran on the hydroxyethyl ester of N-carbobenzoxyalanine with subsequent elimination of the cbz group by hydrogenation over palladium. The thp-oxyethyl ester of uridylyl(5'→N)alanine (Ia) was readily converted into substance I with the splitting out of the thp group in a weakly acid (pH 4.5) medium. Compounds I and II were isolated and purified by means of preparative chromatography and electrophoresis. The substances were characterized by their chromatographic and electrophoretic behavior, by the nucleotide: amino acid ratio, by reactions for functional groups, and also by the results of vigorous acid and alkaline hydrolysis (see table).

*The following abbreviations are used: thp—tetrahydropyranyl, cbz—carbobenzoxy [benzoxycarbonyl].

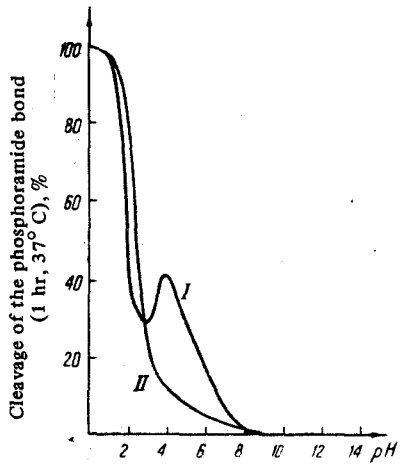
Some Characteristics of the Ethyl and Hydroxyethyl Esters of Uridylyl(5'→N)alanine

Compound	Yield, %	R_f in systems				Electrophoretic mobility relative to UMP-5' at pH 7.8	Ratio of base to amino acid	Reaction for a cis glycol grouping	Conditions for staining with ninhydrin	Hydrolysis products	
		1	2	3	4					0.1N HCl (1 hr, 37° C)	1N NaOH (1 hr, 37° C)
Hydroxyethyl ester of uridylyl(5'→N)alanine (I)	60	—	—	0.40	0.63	0.62	—	+		UMP-5', hydroxyethyl ester of alanine	—
Ethyl ester of uridylyl-(5'→N)alanine (II)	88	0.57	0.05	0.55	—	0.57	1 : 0.8	+	100° 30 min	UMP-5', ethyl ester of alanine	Uridylyl-(5'→N)-alanine
Tetrahydropyranyloxyethyl ester of uridylyl(5'→N)alanine (Ia)	77	—	0.08	0.70	0.89	0.57	1 : 0.8	+		UMP-5', hydroxyethyl ester of alanine	
Alanyloxyethyl ester of uridine-5' phosphate (III)	24	—	—	0.37	0.40	0.62	—	+	60°, 30 min		Alanine, UMP-5'

As in the case of other nucleotidyl(P→N)amino acids [1, 2, 4], the phosphoramide bond in the compounds obtained proved to be stable to alkali and unstable in an acid medium (see figure).

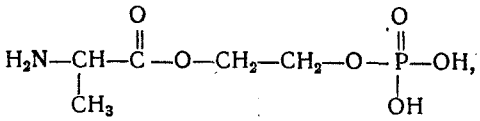
In a study of the comparative stabilities of compounds I* and II (pH 1–8) it was found that in the region of strongly acid pH values the hydrolysis of the phosphoramide bond in the ethyl ester of uridylyl(5'→N) alanine takes place considerably more readily than in compound (I).

Since the degree of hydrolysis of phosphoramides of this type is determined mainly by the ease of protonation of the nitrogen atom of the phosphoramide grouping, the stabilization of the phosphoramide linkage in compound I can be explained only by the presence in it of a fairly strong electron-accepting grouping drawing off electrons from the nitrogen atom and thereby opposing the protonation of this atom. Since the hydroxyethyl ester of uridylyl(5'→N)alanine (I) is one of the "active" esters the ester bond in which readily undergoes both alkaline and acid hydrolysis, the possibility cannot be excluded that in an acid medium hydrolysis of the ester linkage in compound I precedes hydrolysis of the phosphoramide linkage.

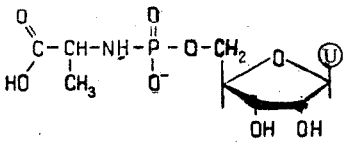


Degrees of hydrolysis of I and II as functions of the pH (37° C, 1 hr).

In order to test this supposition, we carried out the hydrolysis of the alanyloxyethyl ester of phosphoric acid [6]



which also contains a macroergic ester bond between the aniline and hydroxyethyl residue at pH 3.9 (1 hr; 37° C). It was found that under these conditions 20% hydrolysis of the ester linkage took place. This gives grounds for assuming that at pH 1–3 the results obtained apparently correspond to the hydrolysis not of the hydroxyethyl ester of uridyl(5'→N)alanine (I) but of uridylyl(5'→N)alanine containing a free carboxy group



which, because of the drawing off of electrons from the nitrogen atom, hinders the protonation of this atom and, consequently, the hydrolysis of the phosphoramide linkage. This type of stabilization of the phosphoramide linkage under the influence of a free carboxy group has been studied in detail by Halmann et al. [7].

However, when the pH is raised further, in spite of the decrease in the concentration of hydrogen ions (and, consequently, in the degree of protonation of the phosphoramide nitrogen), the degree of hydrolysis of the phosphoramide bond in compound I rises considerably, unlike the hydrolysis of this bond in compound II (see figure). This marked increase in the lability of the phosphoramide bonds is found in the pH range from 3 to 5. This effect is possibly connected with the fact that beginning at pH 3.0 the acid hydrolysis of the ester bond in compound I probably takes place considerably more slowly and does not outstrip the hydrolysis of the phosphoramide link. Consequently, it is possible to compare the hydrolytic stability of the phosphoramide links in I and II only in the region of weakly acidic and neutral pH values.

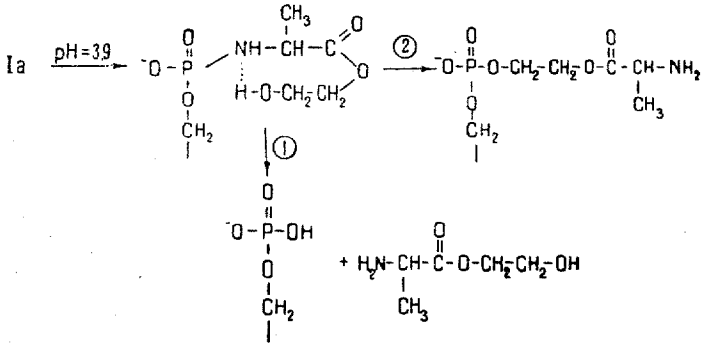
Like the 2'-hydroxy group of the ribose in uridylyl(3'→N)phenylalanine [2], the hydroxy group of the hydroxyethyl residue in compound I apparently favors the intramolecular protonation of the phosphoramide nitrogen atom. The weakening of the conjugation of the protons of the nitrogen and the phosphorus caused by this protonization makes the bond between these atoms unstable even in the region of pH values close to neutral. The considerable distance of the hydroxy

*In this case, the hydrolysis of the thp-oxyethyl ether of uridylyl(5'→N)-alanine (Ia) was studied, since it has been shown previously [5] that in the pH range from 1 to 7 the thp group is eliminated with considerably greater ease than the phosphoramide bond is cleaved.

group from the phosphoramide bond in compound I shows that such intramolecular protonation of the phosphoramide nitrogen takes place very readily even with the participation of a remote proton-donating group if for one reason or another it can approach the phosphoramide grouping.

The influence of the hydroxy group in the amino acid fragment on the phosphoramide bond is not exhausted by the protonation of the nitrogen atom. At pH 3.8 we isolated a product of the intramolecular rearrangement that may accompany this protonation—the alanyloxyethyl ester of uridine-5' phosphate (III). Substance III, isolated by preparative paper chromatography, was characterized from the results of chromatography, electrophoresis, reactions for functional groups, and the results of hydrolysis (see table). The alanyloxyethyl ester of uridine-5' phosphate gives a ninhydrin reaction under the conditions for revealing amino acids and is readily cleaved on mild alkaline hydrolysis (0.1 N NaOH, 37° C, 1 hr), forming the hydroxyethyl ester of uridine-5' phosphate and free alanine. Under more severe conditions (1 N NaOH, 37° C, 1 hr), compound (III) decomposes into alanine, uridine-5' phosphate, and ethylene glycol. Acid hydrolysis (0.1 N HCl, 37° C, 1 hr) leads to the cleavage of the two phosphate bonds and the formation of uridine-5' phosphate and alanine hydroxyethyl ester.

The formation of the alanyloxyethyl ester of uridine-5' phosphate (III), like the cleavage of the phosphoramide link by hydrolysis, is a secondary process accompanying the protonation of the phosphoramide nitrogen atom. Simultaneously with the loss of a proton from the OH group of the hydroxyethyl residue, there is an increase in the negative charge on the hydroxyl oxygen, and its spatial propinquity to the phosphorus atom leads to a nucleophilic attack on this by the hydroxyl oxygen. In this case, the protonization of the nitrogen facilitates (see Scheme) both the intermolecular attack on the phosphorus atom by a molecule



of water (route 1) and the intramolecular attack by the hydroxyl oxygen (route 2). The result of the intermolecular attack of such a phosphoramide by a water molecule is the splitting off of the amino acid component and the liberation of the nucleotide. In the case of the intramolecular reaction, a rearrangement takes place consisting in the cleavage of the phosphoramide bond and the formation of a phosphoric ester bond.

The results obtained show that a free hydroxy group in the amino acid part of the molecule of a nucleotidyl(P→N) amino acid has a considerable activating influence on the phosphoramide link, while the intramolecular protonation of the amide nitrogen may be accompanied by an intramolecular nucleophilic attack on the phosphorus atom, leading to the cleavage of the P→N bond and the formation of a new P→O bond. This influence of even a remote hydroxy group must be taken into account in studies of the properties of natural nucleotidopeptides containing a hydroxy amino acid.

Experimental

The following systems of solvents were used for chromatography: 1) isopropanol—concentrated ammonia—water (7 : 1 : 2); 2) water-saturated 1-butanol; 3) tertiary butyl alcohol—water (7: 3); and 4) ethanol—1 M ammonium acetate (5 : 2). Electrophoresis was carried out in a 0.05 M solution of triethylammonium bicarbonate (pH 7.8).

Hydroxyethyl ester of cbz-alanine [6]. 4.46 g (20 mmole) of cbz-alanine and 0.08 g (2 mmole) of caustic soda were added to a solution in 50 ml of water and 20 ml of ethylene oxide that had been redistilled over caustic potash. The reaction mixture was shaken for 8 hr and left at room temperature for 24 hr. Then another 10 ml of ethylene oxide was added to the solution and it was shaken again for 12 hr. The reaction mixture was evaporated until the ethylene oxide had been completely eliminated and was shaken with ether (4 × 25 ml), and the ethereal solution was rapidly washed with 1% sodium bicarbonate solution and with water. The solution was evaporated to the consistency of an oil, and traces of moisture were removed by azeotropic distillation with anhydrous benzene (3 × 5 ml). The white powder was dried in a vacuum desiccator over caustic potash. Yield 3.5 g (77%), mp 58° C, R_f 0.82 (system 2). Literature data—mp 57–58° C [6].

The thp-hydroxyethyl ester of alanine. Three capillary drops of an 8 N solution of HCl in anhydrous dioxane and 6 ml of dihydropyran that had been freshly distilled in caustic potash and cooled to 0° C were added to a cooled solution of 1 g of the hydroxyethyl ester of cbz-alanine (1.1 mole) in 8 ml of anhydrous dioxane. The reaction mixture was shaken and left for a day at room temperature, and then another 4 ml of dihydropyran was added and the mixture was again left for a day. The light yellow oil was dried by distillation with anhydrous dioxane, dissolved in 15 ml of anhydrous dioxane, and hydrogenated over Pd black for 20 hr at room temperature. The catalyst was filtered off and the solution was evaporated. The oil was dried by azeotropic distillation of the traces of moisture with anhydrous dioxane and benzene. Yield 85%; R_f 0.63, 0.5, 0.93 (systems 1–3).

Acid hydrolysis (0.1 N HCl, 37° C, 1 hr) formed the hydroxyethyl ester of alanine, a mixture of which with a reference sample [7] gave no depression of the melting point.

The R_f values obtained for the hydrolysis and the reference samples of the hydroxyethyl ester of alanine in various systems coincided.

Synthesis of uridylyl(5'→N)amino acid derivatives. The thp-hydroxyethyl ester of uridylyl(5'→N) alanine (I). By means of the resin "Dowex-50", 133 mg (0.2 mmole) of the barium salt of uridine-5' phosphate was converted into the H^+ form, and then the aqueous solution was evaporated to a volume of 5 ml and treated with 0.1 ml of tri-n-octylamine (0.2 mmole), the mixture was shaken and evaporated to dryness, and the residue was dissolved in anhydrous dioxane. The substance was carefully dried by repeated azeotropic distillation of traces of moisture with anhydrous dioxane and benzene, the residue was dissolved in 1 ml of anhydrous dioxane, and 0.08 ml (0.4 mmole) of diphenyl phosphorochloridate and 0.12 ml (0.45 mmole) of dry tri-n-butylamine were added. The solution was shaken, left for 3 hr, evaporated to small bulk, and shaken with 25 ml of cooled dry ether, and after 30 min the ether was decanted off. The oily residue was dissolved in 5 ml of anhydrous dioxane and the solution was evaporated to 2 ml, and then 0.13 g (0.6 mmole) of the thp-hydroxyethyl ether of alanine dissolved in 2 ml of anhydrous dioxane was added. After 12 hr, the reaction mixture was added in drops to absolute ether. The precipitate was eliminated by centrifuging and by paper electrophoresis in 0.05 M triethylammonium bicarbonate solution (pH 7.8). This gave the thp-hydroxyethyl ester of uridylyl(5'→N)alanine. Yield 77.5%. The ratio of base to amino acid was 1 : 0.8. UV spectrum: λ_{max} 261 mμ; K_1 the difference between the millimolar extinction coefficients at λ 261 and λ290, was 8.8. The other characteristics are given in the table.

Ethyl ester of uridylyl(5'→N) alanine (II). This was obtained similarly with a yield of 88% from 133 mg of the barium salt of uridine-5' phosphate and 117 mg (1 mmole) of alanine ethyl ester. The ratio of base to amino acid was 1 : 0.8. The other characteristics are given in the table.

Hydrolytic stability of the phosphoramide link in the ethyl and hydroxyethyl ester of uridylyl(5'→N)alanine. Hydrolysis was carried out in the following buffer solutions

pH	Composition of the mixture
0	1 N HCl
2.0	0.01 N HCl
2.9	0.001 N HCl
3.8	33.9 ml of 0.1 M citric acid + 16.1 ml of 0.2 M Na_2HPO_4 + H_2O to 100 ml
4.5	29.4 ml of 0.1 M citric acid −20.6 ml of 0.2 M Na_2HPO_4 + H_2O to 100 ml
5.3	24.3 ml of 0.1 M citric acid + 25.7 ml of 0.2 M Na_2HPO_4 +H_2O to 100 ml
5.85	17.9 ml of 0.1 M citric acid + 22.1 ml of 0.2 M Na_2HPO_4 + H_2O to 100 ml
6.4	9 ml of 0.1 M citric acid + 16 ml of 0.2 M Na_2HPO_4 + H_2O to 50 ml
7.42	3.3 ml of 0.1 M citric acid + 21.8 ml of 0.2 M Na_2HPO_4 + H_2O to 50 ml

A certain amount of the thp-hydroxyethyl ester of uridylyl(5'→N)alanine was dissolved in 0.5 ml of water, and 0.05-ml samples were added to 10 test-tubes. One tube was left as a control, and to each of the others was added 0.05 ml of a suitable buffer solution (pH 0.8). After incubation for 1 hr at 37° C, the samples were deposited completely on paper and were chromatographed in system 3 (descending chromatography). The hydrolysis products were eluted with 4 ml of 0.1 N hydrochloric acid and the percentages of the initial substance and the hydrolysis products were determined spectrophotometrically. The hydrolysis of the ether ester of uridylyl(5'→N)alanine was performed similarly.

Alanyloxyethyl ester of uridine-5' phosphate (III). A solution of 20 mg (42 mmole) of the thp-hydroxyethyl ester of uridylyl(5'→N)alanine in 0.5 ml of citrate buffer (pH 3.8) was kept at room temperature for 4 hr. Then it was deposited on a preparative chromatogram in system 3, and a narrow band with R_f 0.37 was cut out. The alanyloxyethyl ester of uridine-5' phosphate (III) was eluted with water into a tube, the solution was evaporated to dryness at a bath temperature of 25°C, and the residue was dissolved in the minimum amount of water and subjected to electrophoresis at pH of 7.8. Yield 24%. The characteristics of the alanyloxyethyl ester of uridine-5' phosphate are given in the table.

Summary

1. The ethyl and hydroxyethyl esters of uridylyl(5'→N) alanine have been synthesized and their comparative hydrolytic stabilities in the pH region from 0 to 8 have been studied.

2. It has been shown that the presence of a remote hydroxy group in the molecule of a uridylyl(5'→N)amino acid makes the phosphoramide link more labile in the region of weakly acid and neutral pH values.

3. At pH 3–5, the hydroxy ethyl ester of uridylyl(5'→N)alanine undergoes an intramolecular rearrangement with the cleavage of the phosphoramide linkage and the formation of a phosphoric diester linkage.

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