SYNTHESIS OF DIRIBONUCLEOSIDE PHOSPHO-(P → N)-AMINO ACID DERIVATIVES*

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Starting from completely protected uridylyl-(3′→ 5′)-uridine and adenylyl-(3′→ 5′)-uridine derivatives and using mixed anhydrides with bis(p-nitrophenyl) phosphoric acid as intermediates, the derivatives of uridylyl-uridine (P→ N)-cyclohexylamide (*II*), uridylyl-uridine (P→ N)-glycine ethyl ester (*III*), and adenylyl-uridine (P→ N)-D,L-alanine tert-butyl ester (*V*) have been prepared. Compounds *II* and *III* have been also obtained with the use of 2,3,5-triisopropylbenzenesulfonyl chloride or dimethylchloromethyleneammonium chloride. The stability of the phosphodiester amidate system in the above compounds has been discussed.

In the living matter proceeds a continuous noncovalent interaction between proteins and nucleic acids. The covalently bound amino acid material has been found in some nucleic acids[1-3] and the nucleotides covalently bound by a phosphoamidic bond are intermediates of some enzymatical processes[4,5]. These findings have aroused interest in the chemical synthesis of substances containing amino acids and peptides bound by a P—N bond to mono or oligonucleotides[6-8]. Our efforts have been directed to the synthesis of unprotected compounds bearing amino acids bound through nitrogen to the ribointernucleotidic bond. The phosphodiester amidate system of substances of this type may be connected with new problems concerning the choice of protecting groups.

The phosphodiester amidate grouping is known to decompose in acid media under the formation of a phosphodiester while a phosphomonoester amidate is formed in alkaline media[9]. Despite the known instability of phosphodiester amidates[9], a preparation of N-(dialkoxyphosphoryl)amino acids has been recently reported, consisting in alkaline saponification of the corresponding esters[10,11]. The 2′,5′-di-O-acetyluridylyl-(3′ → 5′)-2′,3′-O-isopropylideneuridine-(P→ N)-phenylalanine methyl ester has been recently shown[12] to undergo a fission mainly at the P—N bond even at pH 10·5. The ready fission is explained by a cyclic mechanism under the participation of the $C_{(2')}$-hydroxylic function, set free during the reaction. The influence of the amino acid carboxyl has not been considered.

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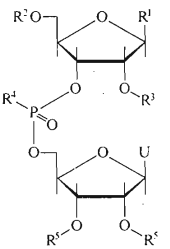
To estimate the influence of the carboxylic function of the amino acid residue (the participation of the *cis*-hydroxylic function being excluded) on the stability of the phosphodiester amidate grouping, two model derivatives of uridylyl-(3′ → 5′)-uridine have been prepared. The $C_{(2')}$-hydroxylic function of these derivatives is protected by an alkalistable group (tetrahydropyranyl) and the phosphorus atom carries an amidically bound cyclohexylamine (in compound *II*) or glycine ethyl ester (in compound *III*). The effect of the $C_{(2')}$-hydroxylic function (the participation of the carboxyl being excluded) on the stability of the phosphodiester amidate grouping has been examined on a adenylyl-(3′ → 5′)-uridine and D,L-alanine derivative; the $C_{(2')}$-hydroxyl of this derivative was protected by an alkalilabile group (acetyl) and the amino acid carboxyl was esterified by the alkalistable tert-butyl group (compound *V*). In the preparation of compounds *II* and *III*, 5′-O-dimethoxytrityl-2′-O--tetrahydropyranyluridylyl-(3′ → 5′)-2′,3′-O-ethoxymethyleneuridine (*I*) has been used as the starting material. Compound *I* was obtained from 5′-O-dimethoxytrityl-



I; R^1 = uracilyl, R^2 = dimethoxytrityl, R^3 = tetrahydropyranyl, R^4 = OH, $2\,R^5 = >C<^{H}_{OC_2H_5}$

II; R^1 = uracilyl, R^2 = dimethoxytrityl, R^3 = tetrahydropyranyl, $R^4 = -NHC_6H_{11}$, $2\,R^5 = >C<^{H}_{OC_2H_5}$

III; R^1 = uracilyl, R^2 = dimethoxytrityl, R^3 = tetrahydropyranyl, $R^4 = -NHCH_2COOC_2H_5$ $2\,R^5 = >C<^{H}_{OC_2H_5}$

IV; R^1 = N^6-acetyladenosyl, R^2 = dimethoxytrityl, R^3 = acetyl, R^4 = OH, R^5 = benzoyl

V; R^1 = N^6-acetyladenosyl, R^2 = dimethoxytrityl, R^3 = acetyl, $R^4 = -NHCH(CH_3)-COOC(CH_3)_3$, R^5 = benzoyl

-2′-O-tetrahydropyranyluridine 3′-phosphate[13] and 2′,3′-O-ethoxymethyleneuridine by the action of 2,3,5-triisopropylbenzenesulfonyl chloride[14]. 5′-O-Dimethoxytrityl--N^6-acetyl-2′-O-acetyladenylyl-(3′ → 5′)-2′,3′-di-O-benzoyluridine (*IV*), the starting material in the synthesis of the diester amidate *V*, has been prepared analogously.

The dinucleoside phosphates are converted to the corresponding phosphodiester amidates by the method of mixed anhydrides[14,15]. This conversion may be also effected by the action of 2,3,5-triisopropylbenzenesulfonyl chloride or dimethylchloromethyleneammonium chloride (a compound of dimethylformamide and phosgene[16]) as exemplified in the present paper on the preparation of compounds *II* and *III*; the yields vary between 10 and 20%. N,N′-Carbonyldiimidazole proved ineffective in this respect. From the preparative point of view, best yields (30 – 60%) of the phosphodiester amidates *II*, *III*, and *V* have been obtained by reaction of the corresponding amino compounds with mixed anhydrides of bis(*p*-nitrophenyl) phosphoric acid and dinucleoside phosphates and the subsequent chromatography on a loose layer of silica gel. The mobility of compounds *II*, *III*, and *V* on thin layers is almost the same as that of the analogous dinucleoside alkyl phosphates[13].

As shown by preliminary investigations on the behaviour of compounds *II* and *III* in acidic media (80% aqueous acetic acid), a rapid fission of the P—N bond takes place in both cases. A qualitatively different between the cyclohexylamide *II* and the glycine methyl ester derivative III has been observed in alkaline media (0·5M-KOH in 80% aqueous ethanol at 50°C). Under these conditions, compound *II* is stable while compound *III* undergoes a rapid fission under the predominant formation of glycine and the dinucleoside phosphate *I*. Consequently, the phosphoamidate bond is labilised by the amino acid carboxylic group. The effect of the free $C_{(2')}$-hydroxylic function on the stability of the phosphodiester amidate system (the influence of the carboxyl being excluded) has been examined with the use of compound *V* in alkaline medium (15% methanolic ammonia; 50°C; 30 min); under these conditions, uridine was formed as the predominant product under the fission of the P—O_C5′ bond.

As shown by the above preliminary results, the phosphodiester amidates derived from diribonucleoside phosphates and amino acids are split in alkaline media under the participation of both the $C_{(2')}$-hydroxylic function and the carboxylic group at the amino acid residue. In view of this instability both in acidic and alkaline media, the synthesis of diribonucleoside phosphodiester amidates will require the choice of such protecting groups which may be removed under neutral conditions.

EXPERIMENTAL

Preparative and analytical thin-layer chromatography was performed similarly to an earlier paper[13] in the solvent systems T_1, chloroform–methanol–pyridine (90 : 5 : 5); T_2, chloroform–methanol (95 : 5); T_3, chloroform–methanol–pyridine (95 : 5 : 1); T_4, chloroform–methanol (9 : 1); T_5, chloroform–methanol (8 : 2); and T_6, 2-propanol–conc. NH_4OH–water (7 : 1 : 2). T_e, chloroform–methanol (1 : 1), was used as eluant.

5′-O-Dimethoxytrityl-2′-O-tetrahydropyranyluridylyl-(3′→ 5′)-2′,3′-O-ethoxymethylene-uridine-(P→ N)-cyclohexylamide (*II*)

A. To a solution of 5′-O-dimethoxytrityl-2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-2′,3′-O--ethoxymethyleneuridine (*I*) triethylammonium salt (62 mg; 0·05 mmol) in dioxane (3 ml), there is added tri-n-butylamine (0·02 ml) and chloroform (3 ml), the mixture shaken for several minutes, and the resulting solution taken down to dryness under diminished pressure. The residue is dried by repeated coevaporations with dioxane. The final residue is dissolved in dioxane (0·5 ml) and the solution is treated with tri-*n*-butylamine (0·04 ml) and bis(*p*-nitrophenyl) phosphochloridate (36 mg; 0·1 mmol). After 3 h at 20°C, cyclohexane (25 ml) is added and the whole mixture kept at 0°C for 30 min. The solution is decanted and the residue is dried under diminished pressure. The dry residue is dissolved in dioxane (0·5 ml), the solution treated with cyclohexylamine (0·02 ml; 0·25 mmol), the whole kept at room temperature for 15 h, diluted with chloroform, and chromatographed on a 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_1. The dimethoxytrityl-positive band (R_F 0·47) is eluted with T_e and the eluate taken down to afford 19 mg (30%) of compound *II*, R_F 0·50 (in T_2). The chromatographic mobility of *II* on thin layers is similar to that of phosphotriesters[13]. By the action of a mixture (1 : 1) of 0·3M-HCl and methanol, compound *II* is quantitatively converted (10 min at 50°C) to UpU.

B. To a solution of the dinucleoside phosphate *I* (the same amount as in paragraph *A*) in pyridine (5 ml) there is added 2,3,5-triisopropylbenzenesulfonyl chloride (30 mg), the mixture concentrated to the volume of about 0·5 ml, the concentrate kept at room temperature for 3 h, treated with cyclohexylamine (0·02 ml), kept for additional 15 h, and processed similarly to paragraph *A*. Yield, 16 mg (26%) of compound *II*.

C. A solution of the dinucleoside phosphate *I* (the same amount as in paragraph *A*) in pyridine (5 ml) is evaporated to dryness, the residue dissolved in pyridine (0·2 ml), and the solution treated at −30°C with 2M dimethylchloromethyleneammonium chloride in dimethylformamide (0·075 ml). After 1 h at room temperature, cyclohexylamine (0·02 ml) is added, the mixture kept for additional 15 h, and processed by thin-layer chromatography to afford 15 mg (20%) of compound *II*.

5′-O-Dimethoxytrityl-2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-2′,3′-O-ethoxymethylene-uridine-(P → N)-glycine Ethyl Ester (*III*)

A solution of the triethylammonium salt of compound *I* (189 mg; 0·17 mmol) in a mixture of dioxane (5 ml), chloroform (5 ml) and tri-n-butylamine (0·06 ml) is taken down to dryness, the residue coevaporated repeatedly with dioxane, and finally dissolved in dioxane (1 ml). The solution is kept with bis(*p*-nitrophenyl) phosphochloridate (108 mg; 0·3 mmol) at room temperature for 3 h, evaporated, the residue shaken at 0°C with cyclohexane (30 ml), and the mixture kept at 0°C for 30 min. The solution is then decanted, the residual product dried under diminished pressure, dissolved in dioxane (2 ml), and treated with a solution of glycine ethyl ester (prepared from 210 mg of the hydrochloride in chloroform presaturated with ammonia). The reaction mixture is filtered, the filtrate evaporated, the residue dissolved in dioxane (0·5 ml), the solution kept at 20°C for 15 h, diluted with chloroform (3 ml), and chromatographed on a 20 × 20 × 0·6 cm layer of loose silica gel in T_1. The dimethoxytrityl-positive band (R_F 0·59) affords the crude substance *III*, contaminated by glycine ethyl ester. Pure *III* is obtained by rechromatography on a 30 × 15 × 0·6 cm layer of loose silica gel in T_3 (R_F 0·39); yield, 132 mg (63%); R_F 0·54 (in T_2). When the thin layer is treated with a spray of 0·3M-HCl, dried, and eluted with T_2 to remove dimethoxytriphenylmethanol, the original spot of compound *III* gives a positive ninhydrin test. UpU and glycine ethyl ester are obtained on keeping compound *III* in a mixture (1 : 1) of 0·3M-

HCl and methanol (30 min at 20°C). Glycine and the dinucleoside phosphate *I* are obtained on treatment of compound *III* with 0·5M-KOH in 80% aqueous ethanol (1 h at 50°C). Compound *III* was also prepared with the use of 2,3,5-triisopropylbenzenesulfonyl chloride or dimethylchloromethyleneammonium chloride in yields of about 20%.

5′-O-Dimethoxytrityl-2′-O-acetyl-N^6-acetyladenylyl-(3′→ 5′)-2′,3′-di-O-benzoyluridine-(P→ N)-D,L-alanine tert-Butyl Ester (*V*)

A mixture of 5′-O-dimethoxytrityl-2′-O-acetyl-N^6-acetyladenosine 3′-phosphate pyridinium salt[14] (252 mg; 0·25 mmol) and 2′,3′-di-O-benzoyluridine (226 mg; 0·5 mmol) is coevaporated three times with pyridine. The final residue is dissolved in pyridine (5 ml), the solution shaken for several minutes with 2,3,5-triisopropylbenzenesulfonyl chloride (227 mg), and evaporated to the consistence of a sirup which is kept at 0°C for 20 h. The sirup is diluted with 50% aqueous pyridine (20 ml) and extracted with chloroform in the presence of sodium chloride (50 mg). The extracts are dried over anhydrous magnesium sulfate and evaporated first at 20°C/15 Torr and finally at 20°C/1 Torr. The residue is coevaporated with two portions of pyridine and the final residue is dissolved in 5 ml of pyridine. The solution is added dropwise with stirring into ether (300 ml), the precipitate collected by centrifugation, and washed with ether. The ether-containing precipitate is dissolved in a mixture of pyridine (5 ml) and tri-n-butylamine (2·5 ml), and the solution is taken down to dryness at 20°C/1 Torr. The residue is coevaporated three times with pyridine and treated with a solution of thoroughly dried (*in vacuo*) bis(*p*-nitrophenyl) phosphochloridate (180 mg; 0·5 mmol) in dioxane (2 ml). The mixture is shaken for 3 h, evaporated, and the residue treated at 0°C with cyclohexane (50 ml). After 30 min at 0°C, the solution is removed by decantation, the residual matter dried under diminished pressure, and treated with a solution of D,L-alanine tert-butyl ester ditosylimide salt (370 mg; kindly provided by Dr K.Poduška, Department of Peptides) in dioxane (2 ml) and tri-n-butylamine (0·3 ml). The whole mixture is shaken for several minutes, kept at 20°C for 15 h, diluted with chloroform (5 ml). and chromatographed on a 15 × 30 × 0·6 cm layer of loose silica gel in T_1. The dimethoxytrityl-positive band (R_F 0·40) is eluted with the solvent mixture T_e to afford 100 mg (30%) of compound *V*, R_F 0·55 (in T_2). By the action (30 min at 50°C) of a mixture (1 : 1) of conc. NH_4OH and methanol, compound *V* affords as the main products uridine and a substance (R_F 0·87 in T_6; 0·1 in T_5) which is dimethoxytrityl- and phosphorus-positive and which is ascribed the structure of 5′-O-dimethoxytrityladenylyl-3′-(P→ N)-alanine tert-butyl ester on the basis of the electrophoretical mobility (E_{Up} 0·50) after removal of the dimethoxytrityl group by the action of 80% aqueous acetic acid at 0°C. As by-products, 5′-O-dimethoxytrityladenylyl-(3′→ 5′)-uridine and alanine tert-butyl ester are formed in small amounts.

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