PRACTICAL SYNTHESIS OF 2'(3')-O-AMINOACYL DERIVATIVES OF *Cp*A*

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N-Tert-butyloxycarbonyl-L-phenylalanine reacts with 1 equivalent of 1,1'-carbonyldiimidazole and 5'-O-dimethoxytrityl-2'-tetrahydropyranyl-N^4-dimethylaminomethylenecytidylyl-(3'→5')-N^6--dimethylaminomethyleneadenosine (*I*) and affords 2'(3')-O-(N-tert-butyloxycarbonyl)-L-phenylalanyl derivative *II*. The compound *II* gives by the action of formic acid in aqueous 1-butanol the 2'-O-tetrahydropyranylcytidylyl-(3'→5')-2'(3)-O-(tert-butyloxycarbonyl)-L-phenylalanyladenosine (*III*), which affords cytidyl-(3'→5')-2'(3')-O-L-phenylalanyladenosine (*IV*) by treatment with 95% aqueous trifluoroacetic acid.

Interest in the particular processes of protein biosynthesis has stimulated the synthesis of various aminoacyl derivatives of nucleosides and nucleotides[1-16]. Smaller number of papers has been devoted to the more difficult synthesis of aminoacyl derivatives of dinucleoside phosphates[17-21] and trinucleoside diphosphate[22]. Two general procedures served for the preparation of the N-protected esters of amino acids. The first method used *in situ* generated symmetrical N-protected anhydrides with pyridine[1] or 4-N,N-dimethylaminopyridine[12] as catalysts, the second one used imidazolidates[4]. The orthoester synthesis[3] did not find general application.

Nucleoside esters of amino acids containing free *cis*-hydroxyl function (riboside derivatives) are known to be quite unstable in aqueous solution at near neutrality[1,2] and relatively stable in acid solution. Therefore the last deblocking step was carried out by hydrogenolysis of N-benzyloxycarbonyl derivatives in aqueous acetic acid. The products were isolated as "stock solutions" in aqueous acetic acid or freeze--dried powders. A considerable improvement was achieved by utilisation of relative stability of O-aminoacyl derivatives in strong acid solutions. Mercer and Symons[22] described the deblocking of O-(N-tert-butyloxycarbonyl)aminoacyl derivatives of ribooligonucleotides by trifluoroacetic acid. In the same time Pozdnyakov and coworkers[15] reported the same procedure for the preparation of aminoacyl nucleoside with blocked *cis*-hydroxyl function. Trifluoroacetic acid was used recently

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for the preparation of aminoacyl derivatives of ribonucleoside 5'-phosphates[16]. In their synthesis of O-aminoacyl-O-methyladenosines, Robins and coworkers[14] used formic acid for the deblocking of N-tert-butyloxycarbonyl derivatives. They abandoned the use of trifluoroacetic acid since the trifluoracetate anions present in the products might have interfered in biochemical evaluation.

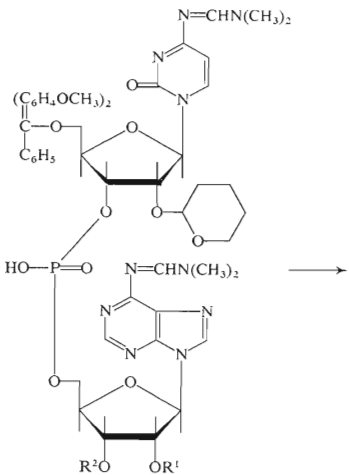
For studies on functions of the elongation factor T_u aminoacyl derivatives of the t-RNA terminal sequence were required. It was desirable to obtain these substances in stable solid form. We supposed that the salt of O-aminoacyl-dinucleoside phosphate with trifluoroacetic acid might fullfill these requirements. We found recently that the trifluoroacetate anion to the concentration $2{\cdot}5 \cdot 10^{-3}$M does not influence T_u factor activity[23].

On the ground of our previous work on the synthesis of 2'-O- (and 3'-O-)-N-formylmethionyl derivatives of cytidylyl-(3'→5')-3'-O-(and 2'-O)methyladenosines[24] and on the ground of relative stability of ribo-internucleotidic bond in trifluoroacetic acid[25] we designed the synthetic approach outlined in the Scheme 1. Dinucleoside phosphate fully protected by acidolabile groups except for adenosine *cis*-diol is esterified by N-tert-butyloxycarbonylphenylalanine imidazolidate in anhydrous medium. The deblocking proceeds in two steps. In the first step, formic acid in aqueous 1-butanol (according[24]) splits off dimethoxytrityl and dimethylaminomethylene groups, in the second step, 95% aqueous trifluoroacetic acid splits off the tetrahydropyranyl and tert-butyloxycarbonyl groups.

Two preliminary tests were carried out. The first one concerned the stability of optical configuration in N-tert-butyloxycarbonylamino acids during imidazolidate formation. Although N-tert-butyloxycarbonylamino acids have been considered to be resistant to racemisation we found that L-phenylalanyl derivative lost entirely the optical activity by the action of 2 equivalents of 1,1'-carbonyldiimidazole after 20 h. In the presence of 1 equivalent of the reagent, the optical activity was preserved. The result showed that the excess of 1,1'-carbonyldiimidazole should be avoided. The second test should verify the stability of ribo-internucleotidic bond in cytidylyl--adenosine in trifluoroacetic acid. It was known[25], that uridylyl-(3'→5')-uridine after 10 min treatment with 95% aqueous trifluoroacetic acid gave 10% products of splitting along with 90% of starting substance containing 0·1% of 2',5'-isomer. Mercer and Symons[22] found no more than 3% of 2',5'-isomers in their aminoacyl derivatives of CpA. Our findings confirmed their results. It was found that CpA structure is more stable in strong acid medium than UpU. 2'-O-Tetrahydropyranyl-cytidylyl-(3'→5')-adenosine gave, after 6 min treatment with 95% aqueous trifluoroacetic acid and precipitation with ether, cytidylyl-(3'→5')-adenosine as sole product containing no more than 1% of ribonuclease resistant material.

The key intermediate for the synthesis of acidolabile protected diribonucleoside phosphate derivative *I* was 5'-O-acetyl-2'-O-tetrahydropyranyl-N^4-acetyl-cytidine 3'-phosphate[24,26]. Pyridinium salt of this nucleotide was condensed with N,N,$O^{2'}$,$O^{3'}$-

-tetrabenzoyladenosine by means of N,N′-dicyclohexylcarbodiimide[27]. The reaction product was treated with ammonia to split off the acyl groups and 2′-O-tetrahydropyranylcytidylyl-(3′→5′)-adenosine was isolated in the form of triethylammonium salt. This salt was reacted with dimethylformamide dimethylacetale[28] to protect the heterocyclic amino groups. Treatment of the product with dimethoxytrityl chloride afforded the substance *I*, which may be used generally for the preparation of 2′(3′)-O-acyl derivatives on adenosine moiety.



I, $R^1 = R^2 = H$

II. R^1 or R^2 = —COCHCH$_2$C$_6$H$_5$ (NHCOOC(CH$_3$)$_3$); R^2 or R^1 = H

III, R^1 = —O— (tetrahydropyranyl); R^2 or R^3 = —COCHCH$_2$C$_6$H$_5$ (NHCOOC(CH$_3$)$_3$); R^3 or R^2 = H

IV, $R^1 = H$; R^2 or R^3 = —COCHCH$_2$C$_6$H$_5$ (NH$_2$); R^3 or R^2 = H

Compound *I* was treated with 1,1 equivalent of N-tert-butyloxycarbonyl-L-phenylalanyl-imidazol in dimethylformamide solution. TLC showed after 20 h almost quantitative transformation of dimethoxytrityl containing material from R_F 0·2

to 0·8 in chloroform–methanol (7 : 3). The reaction mixture was diluted with 50% aqueous pyridine and the dimethoxytrityl containing material extracted to chloroform. The crude product was treated with formic acid in aqueous 1-butanol and then with 95% aqueous trifluoroacetic acid. The amino acid determination showed, however, that the product contained only 9% of phenylalanine. The result indicated that the primary fast moving reaction product of substance *I* and imidazolidate was not O-aminoacyl derivative but P—O-acyl derivative. An excess of aminoacylation reagent (5 equivalents) and longer reaction time (1 week) was therefore used. The product of aminoacylation (*II*), isolated by solvent extraction, was codistilled with toluene to remove last traces of pyridine. The first step of deblocking was carried out by formic acid in aqueous 1-butanol, according to *loc. cit.*[19]. The resulting 2'-O-tetrahydropyranylcytidylyl-(3'→5')-2'(3')-O-(N-tert-butyloxycarbonyl)-phenylalanyl-adenosine (*III*) was purified by solvent extraction. The final deblocking by 95% aqueous trifluoroacetic acid and precipitation with ether gave the product *IV* as solid trifluoroacetate containing 20% of CpA. The stable salt is readily soluble in water to a solution of pH 3—4. The method was used for the synthesis of different aminoacyl derivatives of CpA for elongation factor T_u testing[28].

EXPERIMENTAL

Thin-layer chromatography was performed on ready-for-use Silufol UV_{254} silica gel foil (Kavalier Glassworks, Votice, Czechoslovakia) in the solvent systems S1, 2-propanol–conc. ammonia–water (7 : 1 : 2), S2, 1-butanol–acetic acid–water (4 : 3 : 3). Column chromatography was performed on macroporous silica gel (produced by Service Laboratory, Institute of Organic Chemistry and Biochemistry, Prague, Suchdol). Unless stated otherwise, solutions were taken down on a rotatory evaporator equipped with dry-ice condenser at 1 Torr.

2'-O-Tetrahydropyranylcytidylyl-(3'→5')-adenosine

A solution of the triethylammonium salt of 5'-O-acetyl-2'-O-tetrahydropyranyl-N^4-acetylcytidine 3'-phosphate[24] (11 mmol) in 50% aqueous pyridine (20 ml) is passed through a column (60 ml) of pyridinium Dowex 50 ion exchange resin and the column is eluted with precooled (0°C) 50% aqueous pyridine (200 ml). The eluates are combined and concentrated to one fifth of the original volume. The concentrate is coevaporated with six 60 ml portions of pyridine to sirupy consistence and dissolved in pyridine (60 ml). To the solution, N,N,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (10 mmol) and dry pyridinium Dowex 50 ion exchange resin (8 g) are added, and the solution evaporated to a sirup. Pyridine (60 ml) and N,N'-dicyclohexylcarbodiimide (11 g) are added and the mixture is kept at 37°C for 2 days. Water (10 ml) is added followed (after 10 min) by methanol (80 ml), conc. aqueous ammonia (80 ml), and cyclohexane (80 ml). The whole is briefly shaken, left overnight, and filtered. The lower layer of the filtrate is concentrated (40°C, 15 Torr) to the volume of about 50 ml, the concentrate decanted from the resinous material, and applied to a column (2 l) of DEAE-cellulose (HCO_3^-). The column is washed with water (1 l) and then eluted with the linear gradient of triethylammonium hydrogen carbonate (4 l of water ← 4 l 0·15M of the buffer). The peak eluate (at about 0·07M buffer concentration) is evaporated, the residue coevaporated with three portions of ethanol and dried under diminished pressure. Yield 5·6 g (60%) of triethylammonium salt. R_F 0·55 (S1).

Cytidylyl-(3′→5′)-adenosine

Triethylammonium salt of 2′-O-tetrahydropyranylcytidylyl-(3′→5′)-adenosine (0·3 g) is shaken with 95% trifluoroacetic acid (3 ml). After 6 min, ether (30 ml) is added and the whole is briefly shaken. The precipitate is collected with suction, washed with ether and dried under diminished pressure. Yield 0·19 g (95%). R_F 0·44 (S1), 0·25 (S2). Molecular weight as determined spectrophotometrically, 575. Pancreatic ribonuclease degradation proceeded to 99%.

5′-O-Dimethoxytrityl-2′-O-tetrahydropyranyl-N^4-dimethylaminomethylene-cytidylyl-(3′→5′)--N^6-dimethylaminomethylene-adenosine (*I*)

Triethylammonium salt of 2′-O-tetrahydropyranylcytidylyl-(3′→5′)-adenosine (7 mmol) is dissolved in dimethylformamide (30 ml) and the solution evaporated. The residue is dissolved in dimethylformamide (20 ml), dimethylformamide dimethylacetate (7 ml) is added and the mixture, after 20 h at 20°C, evaporated. The residue is dissolved in pyridine (30 ml), dimethoxytrityl chloride (2·45 g) is added, the mixture shaken for several minutes and then allowed to stand overnight. Water (10 ml) is added and, after 10 min, the mixture diluted with water (100 ml) and extracted with 1-butanol (150 ml). The butanol layer is clarified by anhydrous magnesium sulfate (3 g), filtered and evaporated to a volume of 50 ml. The concentrate is diluted with pyridine (10 ml) and the solution added dropwise with stirring into ether (800 ml). The precipitate is collected with suction, washed with ether and dried under diminished pressure. Yield 4·31 g (52%). R_F 0·62 (S1). UV-spectrum (methanol): λ_{max} 315 nm. For $C_{55}H_{61}N_{10}O_{14}P$ (1069) calculated: 13·10% N, 2·80% P; found: 12·64% N, 2·98% P.

2′-O-Tetrahydropyranylcytidylyl-(3′→5′)-2′(3′)-O-(N-tert-butyloxycarbonyl)--L-phenylalanyl-adenosine (*III*)

A suspension of dicyclohexylammonium salt of N-tert-butyloxycarbonyl-L-phenylalanine (2·5 mmol) and Dowex 50 (H^+) ion exchange resin (4 ml) in 50% aqueous methanol (20 ml) is stirred for 1 h, filtered, the resin washed with 50% aqueous methanol (20 ml), and the filtrate evaporated (40°C, 15 Torr). The residue is evaporated with two 20 ml portions of toluene–ethanol (1 : 1). The residue is evaporated with dimethylformamide (10 ml), dissolved in dimethylformamide (2·5 ml), and 1,1′-carbonyldiimidazole (2·4 mmol) is added. After 5 min, the substance *I* (0·5 mmol) is added and the mixture shaken 2 h and allowed to stand for 6 days. 50% Aqueous pyridine (10 ml) in added, and, after 20 h, the mixture is extracted with chloroform (30 ml). The chloroform extract is washed twice with water (20 ml) and evaporated (40°C, 15 Torr). The residue is evaporated with three 10 ml portions of toluene and the crude *II* dissolved in a mixture of 1-butanol (16 ml), water (3 ml) and formic acid (1 ml). After 20 h, the solution is evaporated (1 Torr) and then triturated with ether (30 ml). The precipitate is collected by suction, washed with ether and dried under diminished pressure. Yield 130 mg. R_F 0·64 (S1). The product is not degraded by pancreatic ribonuclease and does not give ninhydrin reaction.

Cytidylyl-(3′→5′)-2′(3′)-O-L-phenylalanyl-adenosine (*IV*)

The substance *III* (130 mg) is shaken with 95% aqueous trifluoroacetic acid (2 ml) for 5 min. Ether (30 ml) is added, the whole briefly shaken, and the precipitate collected by suction, washed with ether and dried under diminished pressure. Yield 130 mg. R_F 0·30 (S2), ninhydrin positive. The product contains 20% of CpC R_F 0·25 (S2). UV spectrum (pH 1): λ_{max} 263 nm, λ_{min} 235 nm. Molecular weight as determined spectroscopically 840. Pancreatic ribonuclease degradation

affords cytidine 3′-phosphate, adenosine and phenylalanine. Triethylamine in water splits the product to CpA and phenylalanine (ratio 1 : 0·8). No decomposition was observed after several months storage at 0°C.

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