PHOSPHODIANILIDATES IN THE SYNTHESIS OF THE DEOXYRIBOOLIGONUCLEOTIDIC CHAIN*

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Reaction of 5'-O-dimethoxytritylthymidine with the dianilidophosphochloridate *I* affords the 5'-O-dimethoxytritylthymidine 3'-phosphodianilidate (*II*) which is converted to 5'-O-dimethoxytritylthymidine 3'-phosphate (*III*) on treatment with 3-methylbutyl nitrite and to thymidine 3'-phosphodianilidate (*IV*) by the action of 90% aqueous acetic acid. Condensation of compounds *III* and *IV* by the action of 2,3,5-triisopropylbenzenesulfonyl chloride followed by treatment with 2-cyanoethanol gives 5'-O-dimethoxytritylthymidylyl-(3' → 5')-thymidine 3'-phosphodianilidate [P-(2-cyanoethyl) ester] (*V*). Compound *V* is converted into thymidylyl-(3' → 5')-thymidine-3'-phosphodianilidate [P^1-(cyanoethyl) ester] (*VI*) and into 5'-O-dimethoxytritylthymidylyl-(3' → 5')-thymidine 3'-phosphate [P^1-(2-cyanoethyl) ester] (*VII*). Condensation of compounds *VI* and *VII* and treatment with 2-cyanoethanol as above affords 5'-O-dimethoxytritylthymidylyl-(3' → 5')-thymidylyl-(3' → 5')-thymidylyl-(3' → 5')-thymidine 3'-phosphodianilidate [P^1,P^2,P^3-tris(2-cyanoethyl) ester] (*VIII*) and 5'-O-dimethoxytritylthymidylyl-(3' → 5')--thymidylyl-(3' → 5')-thymidylyl-(3' → 5')-thymidine 3'-phosphate (*IX*).

The determining step in the synthesis of polynucleotidic chains consists in an effective synthesis of shorter segments of a known sequence. Condensation of these shorter segments may afford products, the properties of which are sufficiently different from those of the starting components and which are easy to separate. The starting substance in the synthesis of such segments must contain a suitably protected phosphoryl group which would not interfere in the formation of the particular internucleotidic bonds of the segment and which could be deblocked without affecting the remaining protecting groups. In the diester synthesis, the phosphoryl group was protected in the form of 2-cyanoethyl esters[1,2], 2,2,2-trichloroethyl esters[3], S-alkyl phosphorothioates[4], aromatic phosphoroamidates[5,6], N-phenyl-3-hydroxypropionamide and benzaldoxime derivatives, and esters with 2-(phenylthio)ethanol[7,8]. All these derivatives as well as the diesters or esteramidates of phosphoric acid contain an unprotected ionisable function which might interfere in the triester or combined synthesis. In the case of the triester synthesis, the phosphoryl group was

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therefore simultaneously protected with 2-cyanoethanol and 2,2,2-trichloroethanol in the form of a triester[9].

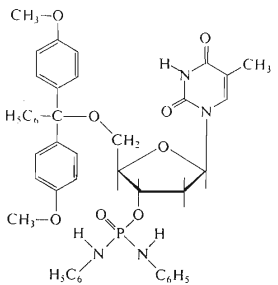
In our approach (combined synthesis: the internucleotidic bond is synthesized in the form of a diester which is *in situ* converted by the action of 2-cyanoethanol to the triester), the intermediates contain both the acidolabile groups such as the dimethoxytrityl and tetrahydropyranyl groups, and the alkalilabile 2-cyanoethyl group in the phosphotriester. For the sake of the connection of segments, the phosphoryl group must be consequently removed under practically neutral conditions. This requirement is fulfilled *e.g.* by bis(2,2,2-trichloroethyl) esters which have been recently used in this Laboratory in the case of the *ribo* series[10]. Another alternative in blocking of the phosphoryl group removable under neutral conditions consists in phosphoroamidates which are split by the action of 3-methylbutyl nitrite in an 1 : 1 pyridine–acetic acid mixture[5]. The requirement of neutral intermediates in the combined synthesis makes necessary the use of neutral ester diamidates. Substances of this type are accessible by reaction of nucleoside derivatives bearing a single free hydroxylic function, with dianilidophosphochloridate (*I*); the cleavage with 3-methylbutyl nitrite is equally easy as in the case of the monoester anilidates[11].

$$\mathrm{ROH} + \underset{I}{\mathrm{Cl{-}P(O)(NHC_6H_5)_2}} \rightarrow \mathrm{R{-}O{-}P(O)(NHC_6H_5)_2} \rightarrow \mathrm{R{-}O{-}P(O)(OH)_2}$$

In order to verify the applicability of phosphodianilidates in the synthesis of oligonucleotides, we have now effected the synthesis of a thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine 3′-phosphate (TpTpTpTp) derivative (see Scheme 1) starting from 5′-O-dimethoxytritylthymidine 3′-phosphodianilidate (*II*). The starting compound *II* was prepared (as reported in a preliminary communication[11]) by reaction of 5′-O-dimethoxytritylthymidine with 1,1 equivalent of dianilidophosphochloridate (*I*) in pyridine. After two days at 20°C, the reaction is practically quantitative. The excess reagent *I* is hydrolysed with aqueous potassium acetate and the product is isolated by extraction with chloroform. The aromatic phosphomonoester amidates are cleaved with 3-methylbutyl nitrite for the period of twenty hours[5,6]. In the 1 : 1 mixture of pyridine and acetic acid, the cleavage of compound *II* is quantitatively accomplished in the course of 2–3 hours, as shown by thin-layer chromatography. The preparative yields of the pyridinium salt of 5′-O-dimethoxytritylthymidine 3′-phosphate (*III*) are about 80%.

Compound *II* was then used in the preparation of the hydroxylic component for the synthesis of the internucleotidic bond. The dimethoxytrityl group was removed by the action of 90% aqueous acetic acid and the resulting thymidine 3′-phosphodianilidate (*IV*) was isolated in 82% yield. The phosphate *IV* (1·5 equivalent) was condensed with the hydroxylic derivative *III* by the action of 2,3,5-triisopropylbenzenesulfonyl chloride with the formation of the intermediary phosphodiester *Va* which was *in situ*

converted by treatment with 2-cyanoethanol into 5'-O-dimethoxytritylthymidylyl-(-3' → 5')-thymidine 3'-phosphodianilidate [P¹-(1-cyanoethyl) ester] (*Vb*) in 54% yield. On successive treatment with 3-methylbutyl nitrite (cleavage of anilidates), ammonia (removal of the 2-cyanoethyl group), and 90% aqueous acetic acid (removal of the dimethoxytrityl group), compound *Vb* was converted into thymidylyl-(3' → 5')-thymidine 3'-phosphate of R_{Up} 0·6 in 7 : 1 : 2 2-propanol–aqueous ammonia–water (paper Whatman No 1).



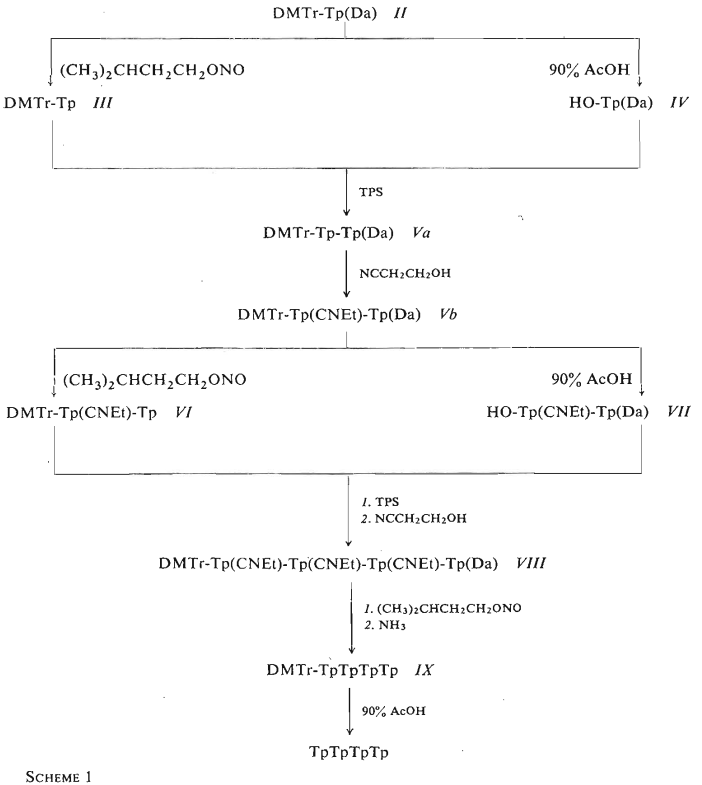
= DMTr—Tp(Da)

II

The dinucleotidic derivative *Vb* can be again subjected to two types of the selective deblocking. Thus, the treatment with 3-methylbutyl nitrite affords 5'-O-dimethoxytritylthymidylyl-(3' → 5')-thymidine 3'-phosphate [P¹-(2-cyanoethyl) ester] (*VI*) which was isolated in the form of the pyridinium salt in 59% yield. On the other hand, the treatment with 90% aqueous acid removes the dimethoxytrityl group with the formation of thymidylyl-(3' → 5')-thymidine 3'-phosphodianilidate [P¹-(2-cyanoethyl) ester] (*VII*) in 91% yield.

The two thus-prepared dinucleotidic segments with a triester-protected internucleotidic bond carry either a phosphoryl group or a free hydroxylic function. Condensation of these two segments was performed with the use of 1·5 equivalent of the phosphate component and 3·3 equivalent of 2,3,5-triisopropylbenzenesulfonyl chloride for 3 days. The reaction mixture was then treated with additional 3·3 equivalent of 2,3,5-triisopropylbenzenesulfonyl chloride and with 2-cyanoethanol to form the triester on the diester bond obtained by condensation. To exclude any removal of the dimethoxytrityl group on the silica gel layer, the sulfonicacid-containing mixture was diluted prior to chromatography with 1M tributylammonium acetate in pyridine in such an amount which was equivalent to the amount of the sulfonyl chloride reagent. The further procedure was analogous to that in the case of dinucleotide *Vb*. The tetranucleotide derivative *VIII* was obtained in 49% yield. Com-

pound *VIII* was then treated with 3-methylbutyl nitrite and the deamidation product subjected without isolation to the action of ethanolic ammonia. The resulting DMTr-TpTpTpTp (*IX*) was isolated by preparative paper chromatography as the ammonium salt. The structure of the final product was confirmed by a further degrada-



SCHEME 1

tion. The dimethoxytrityl group of compound *IX* was removed, the resulting TpTpTpTp isolated by preparative paper chromatography and subjected to the bacterial alkaline phosphatase degradation. The degradation product was again

isolated by preparative paper chromatography and finally subjected to the snake venom diesterase degradation to afford thymidine 5'-phosphate and thymidine in the ratio 2·75 : 1. As indicated by this ratio and by chromatographic properties of the degradation products, the product *VIII* possesses the expected structure.

The above result may be compared with the analogous synthesis[12] of pTpTpTpT where the starting components contained phosphodiester bonds. With the use of 3 equivalents of the phosphate component, the yield of the tetranucleotide was 39·4%. The yield obtained by the present procedure was 10% higher and the ratio of reactants was more economic (1·5 equivalent of the phosphate component). Moreover, the preparation of the starting dinucleotidic components is neither laborious nor time consuming.

EXPERIMENTAL

Thin-layer chromatography was performed on ready-for-use Silufol UV_{254} silica gel foils (Kavalier Glassworks, Votice, Czechoslovakia) in the solvent systems S_1, chloroform–methanol (9 : 1), and S_2, 2-propanol–aqueous ammonia–water (7 : 1 : 2). Thick-layer preparative chromatography was performed on fluorescent-indicator-containing loose silica gel according to Pitra (produced by Service Laboratories of this Institute, Prague-Suchdol) in the solvent systems S_3, chloroform–pyridine–methanol (8 : 1 : 1), and S_4, chloroform–pyridine–methanol (6 : 2 : 2). The elution of compounds from preparative chromatograms was performed with the solvent system S_6, chloroform–methanol (1 : 1). Descending paper chromatography was carried out on Whatman papers in the solvent systems S_2 and S_5, 1-propanol–aqueous ammonia–water (55 : 10 : 35). The dimethoxytrityl-positive bands were identified by pressing strips of paper to the moist preparative layers and spraying the strips with 10% perchloric acid in 30% aqueous acetic acid. Solutions were taken down on a rotatory evaporator equipped with dry ice condenser; the atmospheric pressure was restored through a column packed with blue silica gel. Unless stated otherwise, the R_F values refer to Silufol UV_{254}.

Enzymes. The alkaline phosphatase (suspension in ammonium sulfate) was purchased from Sigma, St. Louis, Missouri, USA. The phosphodiesterase (solution in glycerol) was purchased from C. F. Boehringer and Sons, Mannheim. Federal Republic Germany.

5'-O-Dimethoxytritylthymidine 3'-Phosphodianilidate (*II*)

A mixture of 5'-O-dimethoxytritylthymidine (10·35 g; 19 mmol), pyridine (120 ml), and dianilidophosphochloridate (5·55 g; 20·8 mmol) is briefly shaken and the resulting homogeneous solution kept at room temperature for 2 days. Aqueous potassium acetate (100 ml of a 5% solution) is then added with stirring, the mixture is stirred for additional 20 min, and extracted with two 150 ml portions of chloroform. The extract is dried over anhydrous magnesium sulfate and evaporated under diminished pressure. The residue is coevaporated three times with toluene and finally kept at 40°C/1 Torr to remove solvents. Yield, 13·7 g (95%) of compound *II* in the form of a solid foam; R_F 0·73 (in S_1). For $C_{43}H_{43}N_4O_8P$ (774·8) calculated: 7·24% N, 4·00% P; found: 7·44% N, 4·16% P.

Thymidine 3′-Phosphodianilidate (*III*)

A solution of compound *II* (5·5 g; 7 mmol) in 90% aqueous acetic acid (100 ml) is kept at room temperature for 90 min and evaporated at 20°C/1 Torr. The residue is coevaporated with three portions of 1-butanol and finally dissolved in chloroform (20 ml). The chloroform solution is added dropwise into cyclohexane (300 ml). After several hours at 0°C, the precipitate is collected with suction, washed with a cyclohexane–benzene mixture, and dried under diminished pressure. The crude product is dissolved in a mixture of chloroform (10 ml) and pyridine (0·5 ml) and the solution is added dropwise with stirring into a mixture (500 ml) of cyclohexane–ether (1 : 4). The precipitate is collected with suction, washed with cyclohexane–ether, and dried under diminished pressure. Yield, 2·78 g (82%) of compound *III*, m.p. 116—119°C; R_F 0·30 (in S_1). Mol. weight from A_{260} (methanol): 472. For $C_{22}H_{25}N_4O_6P$ (472·4) calculated: 11·82% N, 6·56% P; found: 11·85% N, 6·61% P.

5′-O-Dimethoxytritylthymidine 3′-Phosphate (*IV*) Pyridinium Salt

To a solution of compound *II* (6·2 g; 8 mmol) in an 1 : 1 pyridine–acetic acid mixture (160 ml) there is added 3-methylbutyl nitrite (21·4 ml). The mixture is stirred at room temperature for 3 h and evaporated first at 20°C/15 Torr and then at 18°C/1 Torr under the addition of four 50 ml portions of pyridine. The residue is dissolved at 0°C in 50% aqueous pyridine (50 ml) and the solution is extracted with chloroform (100 ml and 50 ml). The extract is washed with ice-cold water (50 ml), dried over anhydrous magnesium sulfate, and evaporated first at 20°C/15 Torr and then at 20°C/1 Torr. The residue is coevaporated with pyridine and dissolved in a mixture (50 ml) of pyridine and chloroform (4 : 1). The solution is added dropwise with stirring into ether (1 200 ml), the precipitate collected with suction, washed with ether, and dried under diminished pressure. Yield, 4·51 g (80%) of the pyridinium salt of compound *IV*, R_F 0·35 (in S_2). For $C_{31}H_{33}N_2O_{10}P.C_5H_5N$ (703·7) calculated: 5·98% N, 4·41% P; found: 6·21% N, 4·44% P.

5′-O-Dimethoxytritylthymidylyl-(3′→5′)-thymidine 3′-Phosphodianilidate [P^1-(2-Cyanoethyl) Ester] (*V*)

A mixture of the pyridinium salt of compound *IV* (4·21 g; 6 mmol) and compound *III* (2·1 g; 4·4 mmol) is coevaporated with three 20 ml portions of pyridine and the residue is treated with 2,3,5-triisopropylbenzenesulfonyl chloride (2·42 g; 8 mmol) and pyridine (20 ml). The mixture is then shaken for several minutes and evaporated to the incipient crystallisation. After 3 days, the mixture is shaken with additional 2,3,5-triisopropylbenzenesulfonyl chloride (2·42 g) and pyridine (20 ml) for several minutes, evaporated to the incipient crystallisation, the concentrate kept with 2-cyanoethanol (2 ml; 32 mmol) for 20 h at room temperature, diluted with dichloromethane (5 ml), and chromatographed on five 20 × 20 × 0·6 cm layers of loose silica gel in the solvent system S_3. The dimethoxytrityl-positive bands (distance, 9—16 cm) are eluted with S_e, the eluate evaporated (first at 20°C/15 Torr, finally at 20°C/1 Torr,) the residue dissolved in chloroform (5 ml), and the solution diluted with ether (300 ml). The precipitate is collected with suction, washed with a chloroform–ether (1 : 9) mixture, and air-dried. Yield, 2·68 g (54%) of compound *V*, R_F 0·38 (in S_1). For $C_{56}H_{59}N_7O_{15}P$ (1132) calculated: 8·64% N, 5·48% P; found: 8·56% N, 5·67% P.

5′-O-Dimethoxytritylthymidylyl-(3′ → 5′)-thymidine 3′-Phosphate [P^1-(2-Cyanoethyl) Ester] (*VI*)

To a solution of compound *V* (3·4 g; 3 mmol) in a pyridine-acetic acid (1 : 1) mixture (60 ml) there is added 3-methylbutyl nitrite (8·1 ml). The reaction mixture is stirred at room temperature for 2·5 h and then evaporated under occasional additions of pyridine (first at 20°C/15 Torr and finally at 20°C/1 Torr). The residue is dissolved at 0°C in 50% aqueous pyridine (100 ml) and the solution is extracted with chloroform (100 ml and 50 ml). The extract is dried over anhydrous magnesium sulfate and evaporated, first at 20°C/15 Torr and finally at 18°C/1 Torr. The residue is dissolved in a pyridine–chloroform (1 : 4) mixture (25 ml) and the solution is added dropwise with stirring into ether (880 ml). The precipitate is cooled down to 0°C, collected with suction, washed with ether, and dried under diminished pressure. The crude product is dissolved in an equal amount of the pyridine–chloroform mixture and the precipitation with ether is repeated once more. The precipitate is collected by centrifugation and dried. Yield, 1·87 g (59%) of the pyridinium salt of compound *VI*. For $C_{44}H_{49}N_5O_{17}P_2.C_5H_5N$ (1061) calculated: 7·91% N, 5·84% P; found: 8·24% N, 5·61% P.

Thymidylyl-(3′ → 5′)-thymidine 3′-Phosphodianilidate [P^1-(2-Cyanoethyl) Ester] (*VII*)

A solution of compound *V* (1·73 g; 1·53 mmol) in 90% aqueous acetic acid is kept at room temperature for 1 h and evaporated at 20°C/1 Torr. The residue is coevaporated with 1-butanol, dissolved in chloroform (3 ml), and the solution precipitated with ether (200 ml). The precipitate is collected with suction, washed with ether, and dried under diminished pressure. Yield, 1·16 g (91%) of compound *VII*, R_F 0·24 (in S_1). For $C_{35}H_{41}N_7O_{13}P_2$ (829·6) calculated: 11·81% N, 7·48% P; found: 11·52% N, 7·25% P.

5′-O-Dimethoxytritylthymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine 3′-Phosphodianilidate [P^1,P^2,P^3-tris(2-Cyanoethyl) Ester] (*VIII*)

A mixture of the pyridinium salt of compound *VI* (320 mg; 0·3 mmol) and compound *VII* (167 mg; 0·2 mmol) is coevaporated three times with pyridine, the residue is shaken with 2,3,5-triisopropylbenzenesulfonyl chloride (300 mg) and pyridine (10 ml) for several minutes, and the whole is evaporated to the incipient crystallisation. After three days at room temperature, the concentrate is shaken with additional 2,3,5-triisopropylbenzenesulfonyl chloride (300 mg) and pyridine (10 ml) for several minutes, evaporated to the incipient crystallisation, and treated finally with 2-cyanoethanol (0·4 ml; 6 mmol). The whole mixture is kept at room temperature for 20 h, diluted with 1M tributylammonium acetate in pyridine (2 ml), and chromatographed on one layer (20 × 20 × 0·6 cm) of loose silica gel in the solvent system S_4. The dimethoxytrityl-positive band (distance, 11 – 14 cm) is eluted with S_e and the eluate evaporated, first at 20°C/15 Torr and finally at 20°C/1 Torr. The residue is coevaporated with one portion of toluene, dissolved in chloroform (2 ml), and the solution precipitated with ether (100 ml). The precipitate is kept at 0°C for 2 h, collected with suction, and washed with ether. Yield, 180 mg (49%) of compound *VIII*, R_F 0·16 (in S_1).

5′-O-Dimethoxytritylthymidylyl-(3′ → 5′)thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)--thymidine 3′-Phosphate (*IX*) Ammonium Salt

To a solution of compound *VIII* (59 mg) in a pyridine–acetic acid (1 : 1) mixture (0·7 ml) there is added 3-methylbutyl nitrite (0·09 ml), the mixture kept at room temperature for 2 h, diluted with pyridine (2 ml), and evaporated at 20°C/1 Torr. The residue is dissolved in a conc. aqueous

ammonia–ethanol (1 : 1) mixture (2 ml), the solution kept at room temperature for 1 h and chromatographed on a half-sheet of paper Whatman 3 MM in the solvent system S_2. The dimethoxytrityl-positive band (R_F 0·8) is eluted with a conc. aqueous ammonia–ethanol (1 : 2) mixture and the eluate is evaporated under diminished pressure. Yield, 28 mg (54%) of the ammonium salt of compound *IX*, R_F 0·30 (in S_2).

Enzyme degradation. Compound *IX* (10 mg) was dissolved in 90% aqueous acetic acid (1 ml), the solution kept at room temperature for 1 h, and chromatographed on a 10 cm wide strip of paper Whatman 3 MM in the solvent system S_2 for 80 h. The UV-absorbing band (R_{Up} 0·15) was eluted with water (0·1 ml), the eluate diluted with 0·05M-Tris-HCl buffer solution pH 9·5 (0·05 ml), and treated with the bacterial alkaline phosphatase solution (0·01 ml). After the incubation (2 h at 37°C), the mixture was chromatographed on a 10 cm wide strip of paper Whatman 3 MM in the solvent system S_5. The UV-absorbing band (R_{Up} 1·16) was eluted with water (0·3 ml), the eluate diluted with 0·05M-Tris-HCl buffer solution pH 9 (0·025 ml) and treated with the snake venom diesterase solution (0·025 ml). After the incubation (4 h at 37°C), the mixture was chromatographed on a 10 cm wide strip of paper Whatman No 1 in the solvent system S_2. The UV-absorbing bands (corresponding to thymidine 5′-phosphate and thymidine) were eluted with equal volumes of 0·01M-HCl. As shown by extinction at 260 nm, thymidine 5′-phosphate and thymidine were formed in the ratio 2·75 : 1·00.

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