Synthesis of ppTppp via phosphotriester intermediates

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ABSTRACT

The bifunctional and crystalline phosphorylating agent morpholino-0,0-bis[1-benzotriazolyl]phosphate has been used for the preparation of a 3',5'-bis-phosphotriester intermediate of thymidine. The latter has been converted into ppTppp by the following consecutive steps; removal of the benzotriazolyl group followed by the addition of phosphoric acid and removal of the 2-(4-nitrophenyl)-ethyl group followed by the addition of pyrophosphoric acid.

INTRODUCTION

At the moment two types of monofunctional phosphorylating agents (i.e. 1 and 2a-c) are currently in use for the synthesis of mono-, di- and triphosphates of nucleic acids derivatives via phosphotriester intermediates. The introduction of the phosphate functions starting from the above mentioned phosphorylating agents is based on the following sequence of reactions. Phosphorylation of a hydroxyl group with either of the two types of agents affords stable triester intermediates carrying two different protective groups at phosphorus. Selective removal of one of these two protective groups, gives a phosphodies-
Apart from this, we demonstrated that the synthesis of the above-mentioned phosphate derivatives could be accomplished by applying, depending on the target molecule to be prepared, one of the three phosphorylating agents 2a-c. In this case, phosphorylation of a hydroxyl group with the agents 2a-c affords the corresponding stable triester intermediates. Selective removal of the alkyl (i.e. CH₂CBr₃) or aryl (i.e. 4-nitrophenyl or 2,4-dichlorophenyl) from the phosphotriesters gives in each case a morpholino phosphate derivative. The latter phosphoroamidate can be converted directly by hydrochloric acid, phosphoric or pyrophosphoric acid into mono-, di- or triphosphates of nucleic acids derivatives, respectively. We also realized the synthesis of an asymmetrically polyphosphorylated derivative of thymidine by a combined use of the two phosphorylating agents 2a and 2b.

We now wish to report that the bifunctional phosphorylating agent 3 is very suitable for the preparation of asymmetrically polyphosphorylated d-nucleosides. The latter will be demonstrated in the synthesis of ppTppp (i.e. compound 10 in Scheme II).

RESULTS AND DISCUSSION

Recently, we showed that phosphorylation of the 5'-OH of a suitably protected DNA fragment with morpholino-0,0-bis[1-benzotriazolyl]phosphate (3), which is easily accessible by the reaction of morpholinophosphorodichloridate with two equivalents of 1-hydroxybenzotriazole (HOBT) in the presence of two equivalents of pyridine, affords a relatively stable phosphotriester function which is protected with a morpholino and a benzotriazolyl group (e.g. compound 5 in Scheme I). An interesting property of the latter intermediate is that the benzotriazolyl group can be exchanged by another group (e.g. conversion of 5 into 6 in Scheme I). The above mentioned properties of phosphorylating agent 3, together with the finding that a benzotriazolyl group could be removed under very mild conditions (i.e. conversion of 7 in Scheme I into 8 in Scheme II), enabled us to synthesize an asymmetrically polyphosphorylated thymidine derivative (i.e. ppTppp compound 10 in Scheme II).

The synthesis of the thymidine 3',5'-bis-triester derivative 7, which is a key intermediate in the formation of 10 (Scheme II), is illustrated in Scheme I. In the first step, 5'-O-t-butyldimethylsilyl thymidine 4⁸ is converted by an excess of crystalline agent 3 into derivative 5. Addition of an excess of 2-(4-nitrophenyl)-ethanol⁹ to compound 5 in the presence of 1-methylimidazole gave, after work-up which included the removal of excess 2-(4-nitrophenyl)-ethanol with acetic anhydride in the presence of 4,4'-dimethylaminopyridine
Subsequent removal of the silyl protective group from 6 (R'=tBDMS) with p-toluenesulphonic acid, followed by phosphorylation of 6 (R'=H) with an excess of reagent 3, afforded 7 in an overall yield of 60% (based on 4). The homogeneity and identity of the bis-triester derivative 7 was unambiguously ascertained by $^1$H- and $^{31}$P-NMR spectroscopy. The $^{31}$P-NMR spectrum of compound 7 (see Fig. 1) shows two distinct sets of four resonances which are due to the presence of two diastereoisomeric phosphotriester functions. The particular choice of the 2-(4-nitrophenyl)-ethyl as a protective group for the 3'-phosphotriester function of 2 enabled us to deblock the benzotriazolyl group efficiently from the 5'-phosphotriester function of 2. The selective removal of the benzotriazolyl group from 2 was effected by treating 2 with triethylamine/water, to afford

Fig. 1 $^{31}$P($^1$H)-NMR spectrum of the 3',5'-bis-phosphotriester derivative 7.
the 5'-phosphodiester-3'-phosphotriester $\mathbf{8}$ in an excellent yield. Treatment of $\mathbf{8}$ thus obtained with the tri-n-butylammonium salt of phosphoric acid$^{10}$ in DMF for 16 h at 50°C, afforded, after purification on a column of DEAE-Sephadex A25, homogeneous $\mathbf{9}$ ($R^2=\text{CH}_2\text{CH}_2\text{C}_6\text{H}_4\text{NO}_2$) in 70% yield. The 2-(4-nitrophenyl)-ethyl group was now removed from $\mathbf{9}$ by treating it, under the conditions of Pfleiderer$^9$, with 1,5-diazabicyclo-[4,3,0]-non-5-ene (DBN) during 18 h at 20°C, to afford, after work-up, homogeneous $\mathbf{9}$ ($R^2=\text{H}$). A solution of the tri-n-butylammonium salt of pyrophosphoric acid$^{10}$ in DMF was now added to $\mathbf{9}$ ($R^2=\text{H}$) and left overnight at 20°C. Work-up and purification of crude $\mathbf{10}$ on a DEAE-Sephadex A25 column afforded, after passing purified $\mathbf{10}$ over a column of Dowex 50W (Na$^+$-form), the sodium salt of ppTppp in a yield of 52%. The structure of $\mathbf{10}$ was ascertained by $^1$H-, $^{13}$C- and $^{31}$P-NMR spectroscopy. For instance, the $^{31}$P-NMR spectrum of $\mathbf{10}$ (see Fig. 2) is in complete agreement with the presence

![Chemical Structure](image)
Table 1. $^{31}P$-NMR data [chemical shifts ($\delta$, ppm) and coupling constants (J, Hz)] of compounds \(\mathcal{Z}, \mathcal{B}, \mathcal{G}\) and \(\mathcal{Q}\).

<table>
<thead>
<tr>
<th>Compounds (conditions)</th>
<th>3'-Position</th>
<th>5'-Position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$-P</td>
<td>$\beta$-P</td>
</tr>
<tr>
<td>(\mathcal{Z}) ((CDCl_3))</td>
<td>7.65(^{b})</td>
<td>7.46(^{b})</td>
</tr>
<tr>
<td>(\mathcal{G}) ((D_2O+EDTA; pH 5.3))</td>
<td>7.84(^{b})</td>
<td>7.60(^{b})</td>
</tr>
<tr>
<td>(\mathcal{Z}) ((R_2=CH_2CH_2C_6H_4NO_2)) ((D_2O+EDTA; pH 5.2))</td>
<td>7.55</td>
<td>7.04</td>
</tr>
<tr>
<td>(\mathcal{G} (R_2=H)) ((D_2O+EDTA; pH 6))</td>
<td>6.77</td>
<td>-11.10(d)</td>
</tr>
<tr>
<td>(\mathcal{Q}) ((D_2O+EDTA; pH 5.2))</td>
<td>-12.24(d)</td>
<td>J=19.5 Hz</td>
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\(^{a}\) Mixture of diastereoisomers. \(^{b}\) Tentative assignment.

Of 5'-di- and 3'-triphosphate functions in \(\mathcal{Q}\). In this respect it is interesting to note that $^{31}P$-NMR spectroscopy proved to be a valuable tool for monitoring (see Table 1 for more detail) the different steps involved in the conversion of \(\mathcal{Z}\) into \(\mathcal{Q}\).

In conclusion, the synthesis of the asymmetrically phosphorylated thymidine derivative \(\mathcal{Q}\) clearly shows that the bifunctional phosphorylating agent \(\mathcal{Z}\) promises to be a powerful and versatile tool for the preparation of hitherto unaccessible polyphosphorylated nucleic acid derivatives.

EXPERIMENTAL PART

General methods and materials

High performance anion-exchange chromatography was performed with the strong anion-exchange resin Permaphase AAX (Dupont, USA) dry packed into a stainless-steel column (1 m x 2.1 mm).

Gradient elution was effected, starting with buffer A (0.005 M KH$_2$PO$_4$, pH 4.1) and applying 1% buffer B (0.1 M KH$_2$PO$_4$, 1.0 M KCl, pH 4.5) per min, or starting with buffer A and applying 3% buffer B (system II). A flow of 1 ml/min
at a pressure of 80 kp/cm² at 20°C was standard.

1H-NMR spectra were measured at 100 MHz with a Jeol JNMPS 100 spectrometer; shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard. 13C- and 31P-NMR spectra were measured at 25.15 MHz and 40.48 MHz, respectively, with a Jeol JNMPSFT 100 spectrometer equipped with an EC-100 computer, operating in the Fourier transform mode. Proton noise decoupling was used. 13C chemical shifts are given in ppm (δ) relative to tetrakis(dimethylamino)chloride (TDA) as internal standard and 31P chemical shifts in ppm (δ) relative to 85% H₃PO₄ as external standard. 31P-NMR spectroscopy of compounds containing phosphate functions (i.e. 8, 9 and 10) was performed in D₂O to which was added ethylenediamine tetra acetic acid (EDTA). The 31P-NMR spectroscopy data are recorded in Table 1. Short column chromatography was performed on Merck Kieselgel 60 (230-400 mesh ASTM). DEAE-Sephadex A25 and Sephadex G10 were purchased from Pharmacia (Upsala, Sweden). Schleicher & Schüll DC Fertigfolien F1500 LS254 were used for TLC in solvent systems A (chloroform/methanol, 88:12, v/v) and B (chloroform/methanol, 92:8, v/v).

Dioxane, acetonitrile, tetrahydrofuran, pyridine and triethylamine were dried by refluxing with CaH₂ for 16 h, and then distilled. Pyridine used in phosphorylation and condensation reactions was redistilled from p-toluenesulfonyl chloride (60 g per liter). Dimethylformamide was stirred with CaH₂ for 16 h and distilled under nitrogen and reduced pressure (15 mm Hg). All solvents were stored over molecular sieves. 1-Methylimidazole, 4,4'-dimethylaminopyridine and tri-n-butylamine were distilled under reduced pressure and stored over molecular sieves 4Å. 1-Hydroxybenzotriazole and 2-(4-nitrophenyl)-ethanol were purchased from Aldrich and dried in vacuo (P₂O₅) at 50°C. Morpholine was distilled from sodium. 1,5-Diazabicyclo[4,3,0]-non-ene (DBN) was purchased from Aldrich.

Morpholino-0,0-bis[1-benzotriazoly]phosphate (3)

A stock solution (0.2 M) of agent 3 in tetrahydrofuran, which was prepared as described in reference 7, was left at -20°C. Crude 3 was filtered off at 0°C and recrystallized from tetrahydrofuran/di-isopropylether (1:5, v/v) to give crystalline 3 in a yield of 75%. M.p. 128°C (dec.). 1H-NMR (CDCl₃): δ 8.2-7.3 (benzotriazoly, 8H, m); 3.9-3.7 and 3.6-3.4 (morpholino, 8H, m). 31P-NMR (CDCl₃): δ 9.52 ppm.

5'-O-t-Butyldimethylsilyl-thymidilyl-3'-morpholino-0-(1-benzotriazoly)-phosphate (5)

To a solution of 5'-O-t-butyldimethylsilyl-thymidine (4) (1.78 g, 5 mmole) in dry THF (5 ml) was added 50 ml of a 0.2 M solution of morpholino-0,0-bis-(1-
benzotriazolyl)-phosphate (3) (10 mmole) in THF and 1-methylimidazole (2.12 ml, 25 mmole). After standing for 1.5 h, TLC analysis (system A) showed the reaction to be complete. The reaction mixture was diluted with chloroform (250 ml) and extracted with cold 0.8 M triethylammonium bicarbonate solution (TEAB) pH 7.5 (3 x 50 ml) to remove excess 3 and successively with water (2 x 50 ml), aqueous KH$_2$PO$_4$ solution (0.5 M, pH 6.2; 2 x 50 ml) and water (2 x 50 ml). After drying (Na$_2$SO$_4$), the organic layer was concentrated to an oil, which was dissolved in chloroform (10 ml) and precipitated with petroleum-ether (40-60°, 3 times) to remove the last traces of pyridine and 1-methylimidazole. Yield 3.15 g of 5 (100%).

$^1$H-NMR (CDCl$_3$) of a mixture of diastereoisomers: δ 7.76 (H$_6$, s); 6.8-6.2 (4H, OBT, m); 5.28 (H$_1$, m); 4.45 (H$_3$, m); 3.25 (H$_5$, H$_5''$, m); 3.8-3.6 and 3.2-3.0 (8H, morpholine, m); 1.68 (CH$_3$, s) and 0.80 (t-butyl, s) ppm.

$^{31}$P-NMR (CDCl$_3$): δ 8.15 and 7.82 ppm.

5'-O-t-Butyldimethylsilyl-thymidilyl-3'-morpholino-2-(4-nitrophenyl)-ethylphosphate (6)

Compound 5 (3.15 g, 5 mmole) was coevaporated with toluene (3 x 50 ml) and dissolved in dry THF (12 ml). To the stirred solution was added 2-(4-nitrophenyl)-ethanol (2.5 g, 15 mmole) and 1-methylimidazole (1.7 ml, 20 mmole), and left for 24 h at 20°C. Chloroform (250 ml) was added to the reaction mixture and the organic layer was washed successively with aqueous M NaHCO$_3$ (3 x 50 ml), water (2 x 50 ml), KH$_2$PO$_4$ solution (pH 6.2; 2 x 50 ml), water (2 x 50 ml) and, finally dried with Na$_2$SO$_4$. Evaporation of the organic layer gave an oily residue, containing compound 6 and excess 2-(4-nitrophenyl)-ethanol. As the R$_f$-values of both products were nearly the same (system A), the 2-(4-nitrophenyl)-ethanol was acylated by treating the crude mixture in dioxane (50 ml) with Et$_3$N (5.6 ml, 40 mmole), DMAP (244 mg, 2 mmole) and acetic acid anhydride (2 ml, 20 mmole). The reaction was complete after standing for 1 h (TLC, system A). Water was then added, and most of the dioxane was evaporated off and the residue was dissolved in chloroform (250 ml). Further work-up was the same as described for the isolation of 5. After drying (Na$_2$SO$_4$) and evaporation of the organic layer the oily residue thus obtained was triturated three times with petroleum-ether (40-60°) and purified by column chromatography (Kieselgel column 6.2 cm x 25 cm$^2$, 50 g, suspended in chloroform). Elution of the column with chloroform/methanol (100:0 → 97.5: 2.5, v/v) and evaporation of the appropriate fractions, gave pure 6 as a light yellow powder. Yield 2 g (61%), R$_f$ 0.68 (system A).

$^1$H-NMR (CDCl$_3$) of a mixture of diastereoisomers: δ 9.58 (NH, s); 8.16 (2H,
To a solution of 6 (1.32 g, 2 mmole) in DMF (18 ml) was added water (3 ml), containing p-toluenesulfonic acid (1.9 g, 10 mmole). After standing for 30 min at 20°C, the acid was neutralized by adding imidazole (820 mg, 12 mmole) and the reaction mixture was concentrated under reduced pressure. The residue was dissolved in chloroform (150 ml), washed with aqueous NaHCO₃ (3 x 50 ml), water (2 x 50 ml) and dried (Na₂SO₄). After evaporation, the residue was dissolved in chloroform (10 ml) and added dropwise, with stirring to di-isopropylether (125 ml). The light yellow precipitate was collected by filtration. Yield 940 mg (86%) of 6 (R=H), Rf 0.48 (system A); m.p. 150-152°C (dec).

H-NMR (DMSO/d₆) of a mixture of diastereoisomers: δ 8.12 (2H, nitrophenyl, J = 8 Hz, d); 7.56 (H₄, s); 7.52 (2H, nitrophenyl, J = 8 Hz, d); 6.08 (H₁', m); 5.12 (H₃', m); 4.92 (H₅' and H₅", m); 4.72 (H₄', m); 4.18 (2H, α-CH₂, m); 3.40-3.20 and 2.92-2.72 (8H, morpholine, m); 3.04 (2H, α-CH₂, m); 2.10 (2H₂', m) and 1.76 (CH₃, s) ppm.


To a solution of compound 6 (R=H; 540 mg, 1.0 mmole) in pyridine (2 ml) a solution of 3 in THF (0.2 M, 10 ml) and 1-methylimidazole (0.26 ml, 3 mmole) were added. After standing for 2 h at 20°C, TLC analysis (system B) showed the reaction to be complete. Work-up of the reaction mixture was the same as described for the isolation of 5. Yield 800 mg (100%).

H-NMR (CDCl₃) of a mixture of 4 diastereoisomers: δ 9.00 (NH, s); 8.04 (2H, NO₂-phenyl, J = 8 Hz, d); 7.8-7.2 (4H, OBT, m); 7.36 and 7.28 (2H, NO₂-phenyl, d); 6.12 (H₁', m); 5.08 (H₃', m); 4.42 (H₅' and H₅", m); 4.28 (2H, α-CH₂, m); 3.64-3.44 and 3.10-2.90 (16H, morpholine, m); 3.28 (2H, α-CH₂, m); 2.18 (2H₂', m) and 1.93 (CH₃, s) ppm.

31P-NMR data are recorded in Table 1.
to the reaction mixture which was then carefully concentrated at reduced pressure (15 mm Hg) at 30°C, to a small volume (1 ml) which contained 8 as the TEA-salt. The solution thus obtained had a pH value of 6. $^{31}$P-NMR data are recorded in Table 1.

Thymidilyl-3'-morpholino-2-(4-nitrophenyl)-ethyl-phosphoryl-5'-diphosphate 9

(R$^2$=CH$_2$CH$_2$C$_6$H$_4$NO$_2$)

To a solution of 8 in DMF (8 ml) was added the tri-n-butyl-ammonium salt of phosphoric acid (0.5 M) in DMF (8 ml). The reaction mixture was dried by repeated coevaporation with toluene (3 x 25 ml). After 16 h at 50°C the pH of the solution (3.5) was brought to 8.0 by the addition of Et$_3$N, and evaporated under reduced pressure to afford an oil. Crude 9 thus obtained was applied to a column (25 cm x 6 cm$^2$) of DEAE-Sephadex A25 (HCO$_3^-$-form) suspended in 0.01 M TEAB. The column was eluted with a linear gradient of 0.01 - 0.8 M TEAB for 40 hrs with a flow rate of 36 ml per h. Fractions of 6 ml were collected. The appropriate fractions, as monitored by HPLC-analysis (system I) were pooled, concentrated to a small volume, coevaporated with water (4 x 100 ml) and finally lyophilized, to afford 540 mg (70%) of pure 9 (R$^2$=CH$_2$CH$_2$C$_6$H$_4$NO$_2$) as its TEA-salt. HPLC (system I) $R_t$ = 9.4 min. $^{31}$P-NMR data are recorded in Table 1.

Thymidilyl-3'-morpholino-phosphoryl-5'-0-diphosphate (9, R$^2$=H)

Compound 9 (R$^2$=CH$_2$CH$_2$C$_6$H$_4$NO$_2$) (250 mg, 0.25 mmole) was dissolved in acetonitrile (3.9 ml) to which was added DBN (3.25 mmole). After standing for 18 h at 20°C, the reaction mixture was diluted with acetonitrile (25 ml) and Dowex 50W, (HCO$_3^-$-form; 16 g) was added. The cation-exchange resin was filtered off after 10 min, washed with acetonitrile (2 x 20 ml); the major part of this solution, which was used for the synthesis of 10, was carefully coevaporated with toluene (25 ml) to a small volume (1 ml) at 15 mm Hg at 30°C. The remaining part of the solution containing 9 (R$^2$=H) was used for $^{31}$P-NMR spectroscopy. $^{31}$P-NMR data are recorded in Table 1.

Thymidilyl-3'-O-triphosphate-5'-O-diphosphate (10)

To the solution of 9 (R$^2$=H) obtained above (0.2 mmole) was added dry DMF (4 ml) and a solution of the tri-n-butylammonium pyrophosphoric acid in DMF (0.5 M, 40 ml). The reaction mixture was dried by repeated coevaporation with toluene (3 x 25 ml). After standing overnight at 20°C, further work-up and purification (DEAE-Sephadex A25; linear gradient 0.01 - 1.0 M TEAB) was the same as described for 9 (R$^2$=CH$_2$CH$_2$C$_6$H$_4$NO$_2$). The appropriate fractions were collected, concentrated and lyophilized. After G10 gel filtration of the triethylammonium salt of 10, it was brought into the sodium-salt by passing it
through a column (10 cm x 2 cm$^2$) of cation-exchange resin Dowex 50W (sodium-form). The eluate was lyophilized to give the sodium-salt of 10: 85 mg (52%).

HPLC-analysis (system II) $R_t$ = 9.6 min.

$^1$H-NMR (300 MHz) (D$_2$O; pH 7.5): $\delta$ 1.95 (s, 3H, CH$_3$ exocyclic base); 2.24 (m, 1H, H$_2$', J$_{1',2'}$ = 9 Hz, J$_{2',3'}$ = 6 Hz, J$_{2',2''}$ = 14 Hz); 2.57 (m, 1H, H$_2''$, J$_{1',2''}$ = 5.8 Hz, J$_{2''},3''$ = 1.8 Hz); 4.18 (s, broad, 2H, H$_5'$, H$_5''$); 4.43 (s, broad, 1H, H$_4'$); 5.01 (s, broad, 1H, H$_3'$); 6.42 (q, 1H, H$_1'$, J$_{1',2'}$ = 9 Hz, J$_{1',2''}$ = 5.8 Hz); 7.78 (s, 1H, H$_6$ exocyclic base) ppm.

$^{13}$C($^1$H)-NMR (D$_2$O): $\delta$ 12.6 (s, CH$_3$, exocyclic base); 38.6 (s, C$_2'$); 66.5 (s, C$_5'$, J$_{C_5'},{C_5'}_{-p}$ = 4.8 Hz); 77.5 (d, C$_3'$, J$_{C_3'C_3'}_{-p}$ = 5.3 Hz); 86.0, 85.6 (s, s, C$_1'$, C$_6'$); 112.7 (s, C$_6$); 138.4 (s, C$_5$); 152.7 (s, C$_4$); 167.5 (s, C$_2$) ppm.

$^{31}$P-NMR data are recorded in Table 1.

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REFERENCES