Investigation of the mechanism of the synthesis of oligonucleotides. IX*. \(^{31}\)P NMR spectra of the active dinucleotide derivatives and their analogs.

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ABSTRACT

The interaction of 3'-O-acetyldithymidilate (pdTp dt(Ac)), thymidine-3',5'-diphosphate (pdTp) and thymidine-3'-phenylphosphate-5'-phosphate (pdTpPh) with 2,4,6-triisopropylbenzene sulphonyl chloride (TPS) and H,N'-dicyclohexylcarbodiimide (DCC) in pyridine and dimethylformamide (DMF) was studied by pulsed NMR spectroscopy on phosphorus nuclei. Thymidine cyclic 3',5'-pyrophosphate and dimeric pyrophosphate derivatives were shown to be the main products of the reaction of pdTp with TPS and DCC. The former shows spin AB-system with the unusually large spin-spin coupling constant about 28 Hz upfield to the signals of the dimeric pyrophosphates in NMR spectrum. Analogous spin AB-systems with large spin-spin coupling constants (up to 32 Hz) were observed in the spectra of the reaction mixtures of pdTp dt(Ac) with TPS or DCC and of pdTpPh with TPS. These spin AB-systems were ascribed to 3',5'-cyclic pyrophosphate derivatives of pdTp dt(Ac) and pdTpPh.

INTRODUCTION

Recently it was found by pulsed \(^{31}\)P NMR spectroscopy that the interaction of mononucleotide with 2,4,6-triisopropylbenzenesulphonyl chloride (TPS) in pyridine results in the formation of highly reactive monomeric nucleotide derivative (compound B, nucleoside-5'-metaphosphate or its pyridinium derivative)\(^2\). This derivative reacts readily with phosphodiester groups forming trisubstituted pyrophosphates (compounds of type C). Thus the reaction of 5'-O-tritylthymidyl-(3'-5')-3'-O-acetyltymidine with the B type derivative of 3'-O-acytlythymidine-5'-phosphate results in the immediate formation of R-5'-O-tritylthymidine E, R-5'-bis-(3'-O-acetyltymidine)-pyrophosphate (C, R = 5'-O-tritylthymidine; R',R" = 3'-O-acetyltymidine)\(^2\).

*Part VIII: see Ref. 1
Compound C shows in $^{31}$P NMR spectrum multiplet which may be regarded as two partly overlapping spin AB-systems. Using a variety of model trisubstituted pyrophosphates it was demonstrated that in all cases when P₂ atom is asymmetric and one of the radicals is optically active the $^{31}$P NMR spectrum appears to be the sum of two overlapping spin AB-systems (e.g. $R$=5'-O-tritylthymidine, $R'$=3'-O-acetylthymidine, $R''$=phenyl), while in the absence of either asymmetric phosphorus (e.g. $R,R'=3'$-O-acetylthymidine, $R''$-phenyl) or asymmetric carbon atoms of definite configuration in the alkyl residues (e.g. $R$ = methyl, $R',R''$ = 2-cyanoethyl) the spectrum corresponds to one spin AB-system. These results suggest that the appearance of two spin AB-systems in $^{31}$P NMR spectra is due to the presence of two diastereoisomers.

In the synthesis of rather long oligonucleotides dinucleotides and even longer oligomers are usually used as the nucleotide components at the late steps of the synthesis. According to previous results a complex reaction system should be expected in the course of activation of these components due to simultaneous presence of phosphomonoester and phosphodiester groups in the same molecule. Our preliminary results demonstrated that $^{31}$P NMR spectrum of the reaction mixture of dinucleotide pdTpdT(Ac) with TPS is very complex.

The present paper deals with a detailed investigation of the structures of dinucleotide derivatives formed in the reaction mixtures under treatment of dinucleotides with moderate excesses of condensing reagents: TPS and N,N'-dicyclohexylcarbodiimide (DCC). In order to facilitate the interpretation of $^{31}$P NMR spectra we have used two kinds of model experiments. They were: (a) the use of the model compounds (dinucleotide analogs) and (b) the use in some experiments of dimethylformamide (DMF) as a solvent, for according to $^{3}$ $^{31}$P NMR signals of diastereoisomers in DMF in some cases...
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completely overlap and therefore the spectra are simpler than in pyridine solution.

METHODS AND MATERIALS

The $^{31}$P NMR spectra were taken with a Bruker HX-90 pulse spectrometer under conditions described earlier. The most spectra are recorded with heteronuclear spin-spin decoupling $^{31}$P-{$_H$}. The chemical shifts (see Table 1) are reported in ppm related to external 85% H$_3$PO$_4$ with the accuracy ± 0.05 ppm.

Table 1

$^{31}$P chemical shifts of the investigated compounds (relative to 85% H$_3$PO$_4$)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$\delta_{P_1}$ (ppm)</th>
<th>$\delta_{P_2}$ (ppm)</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p_4dTp_2dT(Ac)$</td>
<td>-0.7</td>
<td>1.2</td>
<td>pyridine</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>2.5</td>
<td>DMP</td>
</tr>
<tr>
<td>$p_4dTp_2$</td>
<td>0.1</td>
<td>0.9</td>
<td>DMP</td>
</tr>
<tr>
<td></td>
<td>-3.5</td>
<td>-3.0</td>
<td>water</td>
</tr>
<tr>
<td>$p_4dTp_2Ph$</td>
<td>-0.3</td>
<td>7.5</td>
<td>DMP</td>
</tr>
<tr>
<td></td>
<td>-3.0</td>
<td>5.3</td>
<td>water</td>
</tr>
<tr>
<td>$p_4dTp_2^*$</td>
<td>13.70</td>
<td>14.45</td>
<td>DMP</td>
</tr>
<tr>
<td></td>
<td>(calculated)</td>
<td>(calculated)</td>
<td></td>
</tr>
</tbody>
</table>

*Thymidine cyclic 3',5'-pyrophosphate $J_{POP} = 2.7.6 ± 0.2$ Hz

Electrophoretic mobilities ($E_r$) of the compounds were measured relative to pdT in 0.05M triethylammonium bicarbonate (Buffer $B_1$) at pH 7.5 and in 0.02M citrate Buffer ($B_2$) at pH 3.5 using apparatus for high voltage electrophoresis VEPA-5-0.35 (SKB BPA, USSR) (for $E_r$ values see Table 2).

Paper chromatography was performed by the descending technique on FN-1 paper in the solvent systems: $S_1$, ethanol - 1.0M ammonium acetate (7:3, v:v) pH 7.5 and $S_2$, isobutyric acid - 0.5M aqueous ammonia (10:6, v:v). Chromatographic mobilities ($R_f$, see Table 2) were measured relative to pdT.

2,4,6-triisopropylbenzenesulphonyl chloride $^6$ and $N,N'$-
Dicyclohexylcarbodiimide were used as a condensing reagent. Dimethylformamide and pyridine with water content less than 0.05% stored above molecular sieves 4Å were used as solvents.

3'-O-acetyldithymidilate (pyridinium and triethylammonium salts) was prepared according to 8.

Thymidine-3'-phenylphosphate-5'-phosphate (triethylammonium salt). A solution of 2.3 mmoles of thymidine-5'-(2-cyanoethyl)-phosphate and 3.6 mmoles of phenyl phosphoric acid in 30 ml of pyridine was concentrated in vacuo at 30°C to oil. The oil was then dissolved in 20 ml of anhydrous pyridine and the solution concentrated to dryness. The process was repeated twice. Solution of 7.4g of DCC in 30 ml of anhydrous pyridine was added to the dry residue, reaction flask tightly stoppered and was kept for 3 days at room temperature. To consume the excess of DCC 30ml of water was added and the mixture was left for 5 days. Pyridine was then removed by concentrating the solution to dryness in vacuo, the residue was taken up in 50 ml of water and filtered to remove dicyclohexylurea. Water was then removed in vacuo, 40 ml of concentrated aqueous ammonia was added and mixture was heated at 60°C for 5 hours. Ammonia was removed in vacuo, residue was diluted to 800 ml and applied to a column of Molselet-DEAE 25 (200 ml) ion exchange resin in HCO₃⁻-form. Column was eluted with the linear gradient of ammonium bicarbonate (0.01M to 0.5M; eluent volume was 3 liters). A peak corresponding
to compound with 3 charges was gathered, evaporated several times with water to remove the ammonium bicarbonate and diluted with water to 300 ml. Solution was then passed through a column of Dowex 50 x 8 (50 ml) ion exchange resin in \((\text{C}_2\text{H}_5)\text{NH}_4^+\) form and evaporated to dryness \textit{in vacuo} by adding absolute ethanol. Residue next was dissolved in the absolute ethanol (15 ml) and precipitated into absolute diethyl ether (400 ml). The precipitate was collected by centrifugation and dried \textit{in vacuo} above \(\text{P}_2\text{O}_5\). The yield was 1.0 g (65%).

The product was chromatographically and electrophoretically homogeneous (see Table 2) and had the following characteristics: (a) \(E_\varepsilon = 1.25\) (Buffer B,) and 1.8 (Buffer B,); (b) UV-spectrum corresponds to thymidine derivative; (c) \(^{31}\text{P}\) NMR spectrum shows signal of terminal phosphate group (Pm) and another resonance corresponding to the phosphophenyl ester group (Pg) \(^{10}\). \(^{31}\text{P}\) NMR spectrum recorded without heteronuclear spin-spin decoupling \(^{1}J_{^3^1\text{P}-^1\text{H}}\) shows for Pg a doublet \(J_{^3^1\text{P}(3')H} \approx 8\) Hz. According to these data the product obtained has been identified as thymidine-3'-phenylphosphate-5'-phosphate.

**Thymidine-3',5'-diphosphate (triethylammonium salt).** A solution of 4.1 mmole of thymidine and 16.4 mmole of 2-cyanoethylphosphate (pyridinium salt) (Reanal products) in 40 ml of pyridine was treated with DCC (23 g) by analogy with the preceding synthesis. The yield after chromatography was 1.2 g (50%). The \(E_\varepsilon\) and \(R_\varepsilon\) values for pdTp were identical with those given in the literature \(^{11}\).

**Interaction of pdTp with DCC in DMP.** A solution of 185 mg of pdTp (0.34 mmole) was treated with 694 mg of DCC (3.4 mmole) in 28 ml of DMP for 24 hours. Then 125 ml of water was added and unreacted DCC was extracted with diethyl ether. Dicyclohexylurea was filtered off and aliquot of the above mixture was analyzed by microcolumn version in Tomlinson-Te Ner system \(^{12}\). (Fig. 1). Next, the whole reaction mixture was applied to a column with Molselect-DEAE 25 ion exchange resin in \(\text{HCO}_3^-\) form (50 ml). Column was eluted with the linear gradient of ammonium bicarbonate (from 0.01M to 1.0M, eluent volume was 11). The elution profile was analogous to that in
Figure 1
Microcolumn chromatography of the reaction mixture (pdTp 0.01M with DCC 0.05M in DMP) after 24 hours at 30°C. DEAE-cellulose column 50 μl, h = 50mm. Linear gradient of NaCl 0 - 0.2M in 7M urea - 0.02M Tris·HCl pH 7.5, 300 μl/h.

the microcolumn version. Substance of peak 1 (I) was analyzed by paper chromatography and electrophoresis and was found to be homogeneous. UV-spectrum of I corresponds to thymidine derivative. Electrophoretic mobilities (see Table 2) and salt concentration of the column eluent (see Fig.1) for peak 1 demonstrate the presence of two ionized groups. $^{31}$P NMR spectrum of compound of peak 1 (Fig.2b) shows spin AB-system in the region of resonances for substituted pyrophosphates. According to these data substance of peak 1 was identified as thymidine cyclic $3',5'$-pyrophosphate.

RESULTS
As mentioned above, phosphorylating compounds of type B couldn't accumulate in the presence of phosphodiester groups ($P_d$), because of interaction of B and $P_d$ with the formation of trisubstituted pyrophosphates of type C. Since dinucleotides (e.g. pdTpT(Ac)) contain both phosphomono- ($P_m$) and phosphodiester groups the compounds of C type should be expected as the products of the reaction of dinucleotides with condensing reagents. These compounds were found to be unstable in the presence of nucleofiles such as amines, alcohols, water. Consequently, it seems to be difficult to analyze the reaction mixtures by the usual chemical means, e.g. electro-
phoresis and chromatography. Therefore, we undertook preliminary investigation of the interaction of DCC and TPS with the most simple model: thymidine-3',5'-diphosphate (pdTp). The molecule of pdTp doesn't contain Pd in contrast to pdTpdpT(Ac). Hence, all pyrophosphate fragments forming in the reaction mixtures should be disubstituted pyrophosphates. These pdTp derivatives must be stable in water solutions at neutral pH, therefore the reaction mixtures formed under activation of pdTp with either DCC or TPS, can be analyzed e.g. by the column chromatography.

After treatment of pdTp with DCC in DMF (or pyridine) (spectrum see Fig.2a) subsequent microcolumn chromatography in Tomlinson-Tener system showed a variety of peaks having 2, 4 and more charges (elution profiles were similar to Fig.1). Similar result was observed under treatment of pdTp with TPS in DMF. The first peak (see exp.) was separated and identified

![Figure 2](image)

**Figure 2**

$^{31}$P NMR spectra:

(a) of the reaction mixtures of pdTp (0.1M) with DCC (0.5M) in DMF after 24 hours;
(b) of the substance of the peak 1 (Fig.1) in H$_2$O;
(c) of the substances of the peak 2 (Fig.1) in H$_2$O;

(Note: scale relates to Fig.2a)

I, II - the components of spin AB-systems for compounds I ($J_{POP} = 25.0\pm0.2$Hz) and II ($J_{POP} = 19.8\pm0.2$Hz)

III - two singlets for compound III (Signal position of 5'-pyrophosphate bond for III overlaps with the position of one component of the spin AB-system for II).
according to its electrophoretic mobility (two charges) and UV-spectrum as thymidine cyclic 3',5'-pyrophosphate (I):

\[
\text{(I)}
\]

In accordance with that the \textsuperscript{31}P NMR spectrum of I shows spin AB-system in the region of resonances of substituted pyrophosphates (Fig.2b). The \( J_{\text{POP}} \) value amounts to 25.0 \( \pm \) 0.2 Hz in aqueous solution and 27.6 \( \pm \) 0.2 Hz in DMP solution that is considerably greater than those for linear asymmetric pyrophosphates of type C.

Two compounds with four charges could form from pdTdp. They are the compounds with asymmetric (II) and symmetric (III) pyrophosphate bonds.

\[
\text{(II)} \quad \text{(III)}
\]

Compound II must show in the \textsuperscript{31}P NMR spectrum spin AB-system corresponding to 3',5'-pyrophosphate groups, compound III - two singlets corresponding to symmetric 5',5'- and 3',3'-pyrophosphate groups. The experimental spectrum of the peak 2 substances (Fig.2c) is in a good agreement with the expected one and may be decomposed easily in two spectra (for II and III) assuming that signal position of 5',5'-pyrophosphate group of III overlaps with one component of spin AB-system for II. The coupling constant for II is 19.7 \( \pm \) 0.2 Hz and only slightly exceeds those for linear asymmetric pyrophosphates of type C.
It should be noted that the δ values for 3'-P and 5'-P of I (see Fig. 2a) is about 1.5 ppm upfield to those of II and III. The spectrum of the reaction mixture before chromatography (Fig. 2a) may be regarded as superposition of spectra for the separated individual compounds I, II and III (Fig. 2 b-c).

The polycharged compounds (6,8 charges) were discovered in the reaction mixtures too, but the total content of these substances never exceeded 20% (calculation based upon optical density). Therefore, they are neglected in the next considerations.

Assignments of signals for I-III were carried out using (a) the property of 3'-P to have a resonance at higher field than that of 5'-P (see Table 1) and (b) spectra recording without heteronuclear spin-spin decoupling $^3$P-{¹H}.

The activation of pdTp doesn't adequately reflect that of dinucleotides, because in contrast to two phosphomonoester groups in pdTp, there are two different types of phosphate groups in real dinucleotide (pdTpT(Ac)), $P_m$ and $P_d$. Unfortunately, the difference of the chemical shifts for pdTpT(Ac) $\Delta \delta = \delta_{P_d} - \delta_{P_m}$ is low and this circumstance embarrasses the interpretation of the $^3$P NMR spectra of the reaction mixtures forming under activation of pdTpT(Ac). Therefore, it is of interest to follow interaction of condensing reagents with dinucleotide analogs with larger $\Delta \delta$. Thymidine-3'-phenylphosphate-5'-phosphate (pdTpPh) was chosen for our experiments as a suitable model. In this compound the resonance of $P_d$ is shifted upfield (about 5ppm) as compared with the resonance of $P_d$ in pdTpT(Ac). When forming pyrophosphate bonds, resonance of phosphorus atom is known to be shifted by 10-13 ppm upfield $^{2,3}$. Hence, in the case of pdTpPh the resonances of the pyrophosphate $P_m$ and $P_d$ atoms must appear in the regions about 13 and 20 ppm respectively.

Figure 3 represents the $^3$P NMR spectrum of the reaction mixture forming under activation of pdTpPh with TPS in DMF. Signals 2 and 4 referred to (pdTpPh)$_2$ O-symmetric pyrophosphate. Two spin AB-systems (lines 9,10,18,21 and 11,12,22,23) are seen in the spectrum with anomalous coupling constants.
20.5 ± 0.2 Hz and 24.9 ± 0.2 Hz respectively. The signals of \( P_m \) and \( P_d \) of these AB-systems are shifted upfield to the rest signals of pyrophosphate \( P_m \) and \( P_d \) atoms of type C compounds (see Fig. 3). By analogy with the thymidine cyclic 3',5'-pyrophosphate these spin AB-systems may be related to two isomeric cyclic pyrophosphates IVa and IVb:

\[
\text{IVa, } R = \text{phenyl} \\
\text{Va, } R = 3'-O\text{-acetylthymidine} \\
\text{IVb, } R = \text{phenyl} \\
\text{Vb, } R = 3'-O\text{-acetylthymidine}
\]

Figure 3

\(^{31}P\) NMR spectrum of the reaction mixture of pdTpPh (0.1M) with TPS (0.4M) in DMP after 3.5 hours at 50°C.

According to the rest signals in the regions 12.5-14.5 ppm (\( P_m \)) and 18.5-20.5 ppm (\( P_d \)) were assigned to pyrophosphate fragments of linear or macrocyclic compounds of type C:

\[
\cdots \text{O}-P_d\text{-O-Ph} \\
\text{O} \\
\text{O} \\
\text{O} \\
\cdots \text{O}-P_m\text{-OT-}\cdots
\]

(Assignments of signals are based upon the chemical shifts of
P_m and P_d for pdTpPh and spectra without heteronuclear spin-spin decoupling $^{31}$P-(1H).

Using the above data we attempted to get some information from the spectra of the reaction mixtures forming under treatment of pdTpdt(Ac) with condensing reagents. Addition of 0.5 equiv TPS (or DCC) to pdTpdt(Ac) in pyridine solution results in the appearance in $^{31}$P NMR spectrum of the reaction mixture of a new singlet signal corresponding to symmetric 5',5'-pyrophosphate group ($\delta = 11.2$ ppm) and complex signals in the region of resonances of the type C compounds (about 11-13 ppm, Fig.4a). Therefore, the first stages of the activation of dinucleotide in pyridine lead to interaction of P_m as well as P_d groups. However in the presence of terminal phosphate groups compounds of type C are unstable and transform to 5',5'-pyrophosphate group, as may be seen from the growth of the signal with $\delta = 11.2$ ppm (see, Fig.4b).

Reaction proceeds more deeply, if greater starting excess of TPS (1.5-2 equiv.) (or DCC; 2-5 equiv.) is used. Figure 5 represents the spectra of the following reaction mixtures: pdTpdt(Ac) (pyridinium salt) with (a) TPS and (b) DCC in pyridine:
Figure 5  $^{31}P$ NMR spectra of the reaction mixtures:
(a) pdTpdT(Ac) (0.05M) with TPS (0.125M) after 70 min. at 30°C in pyridine;
(b) pdTpdT(Ac) (0.05M) with DCC (0.25M) after 16 hours at 30°C in pyridine;
(c) pdTpdT(Ac) (0.05M) with TPS (0.09M) after 2 hours at 30°C in DMF;
(d) pdTpdT(Ac) (0.07M) with DCC (0.35M) after 6 hours at 30°C in DMF.
(•) and (+) indicate the signals of the lowfield (L) and highfield (H) spin AB-systems respectively.

In pyridine solution these reactions are nearly complete in 3-5 hours. In both solvents two high field spin AB-systems with large coupling constants may be seen. (The $J_{\text{H}}$ and $\delta$ values are presented in Table 3). However, in pyridine solution the downfield parts of these spin AB-systems nearly overlap with the complex signals in the range of resonances of type C compounds. By analogy with the above data these AB-systems were assigned to isomeric cyclic 3',5'-pyrophosphate derivatives of pdTpdT(Ac) (Va and Vb).
Chemical shifts of P nuclei (δPm and δPd; calculated values) and coupling constants (Jpop) for two up field spin AB-systems (see Fig. 5 a-d) (cyclic intramolecular pyrophosphate derivatives of pdTpdT(Ac)).

<table>
<thead>
<tr>
<th>Spin AB system</th>
<th>Solvent and condensing reagent</th>
<th>Pyridine</th>
<th>Pyridine</th>
<th>DMF</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TPS</td>
<td>DCC</td>
<td>TPS</td>
<td>DCC</td>
</tr>
<tr>
<td>L</td>
<td>δPm (ppm)</td>
<td>13.00</td>
<td>13.30</td>
<td>14.95</td>
<td>13.65</td>
</tr>
<tr>
<td></td>
<td>δPd (ppm)</td>
<td>14.40</td>
<td>14.80</td>
<td>15.90</td>
<td>15.40</td>
</tr>
<tr>
<td></td>
<td>Jpop (Hz;±0.4)</td>
<td>23.4</td>
<td>24.9</td>
<td>22.7</td>
<td>29.3</td>
</tr>
<tr>
<td>H</td>
<td>δPm (ppm)</td>
<td>13.05</td>
<td>13.45</td>
<td>15.20</td>
<td>13.85</td>
</tr>
<tr>
<td></td>
<td>δPd (ppm)</td>
<td>16.55</td>
<td>16.85</td>
<td>18.25</td>
<td>17.30</td>
</tr>
<tr>
<td></td>
<td>Jpop (Hz;±0.4)</td>
<td>28.9</td>
<td>30.7</td>
<td>26.4</td>
<td>32.9</td>
</tr>
</tbody>
</table>

*L and H mean lowfield and highfield spin AB-systems marked (•) and (+) respectively at Figure 5 a-d.

The structure of the signals in the region of 11-13 ppm is rather complex. However at least two AB-systems may be seen in the spectrum (Fig. 6). These spin AB-systems may be related to isomeric dimers of the structure similar to II

Figure 6

$^3$P NMR spectrum of the reaction mixture of pdTpdT(Ac) (0.05M) with TPS (0.130M) after 90 min. at 28°C. (*) and (°) - indicate spin AB-systems refereed supposedly to compound VI.
with three different orientations of two 3'-O-acetylthymidine-5' residues towards the macrocycle (compound VII).

It should be noted that the signals of symmetric disubstituted pyrophosphate group (formed by P_m atoms) as well as signals of tetrasubstituted pyrophosphate group (formed by P_d atoms) are practically absent in the reaction mixtures under consideration (Fig. 6). Therefore macrocycle dimers with symmetric pyrophosphate bonds are not present in this case.

**DISCUSSION**

The data obtained demonstrate that thymidine cyclic 3',5'-pyrophosphate and similar derivative of pdTpdt(Ac) are among the main products of the interaction of the condensing reagents with pdTp and pdTpdt(Ac) respectively. The total contents of I as well as V(a,b) in reaction mixtures are of the same order about 20-25% (31P) in both cases at initial concentrations of pdTp or pdTpdt(Ac) 0.05M. These facts permit us to conclude that the mechanisms of the formation of the cyclic derivatives are nearly similar both for the activation of pdTp and pdTpdt(Ac). Therefore it is reasonable to suggest that the formation of cyclic dimers analogous to II and III takes place during the activation of pdTpdt(Ac). However, visible signals of tetrasubstituted pyrophosphate groups aren't observed in 31P NMR spectra of the reaction mixtures when moderate excesses of condensing reagents are used. Apparently, these derivatives being powerful phosphorylating species rearrange to compound with the type C fragments, e.g.:

![Diagram showing the rearrangement of cyclic dimers](attachment:image.png)
Next activation of this molecule may lead to the formation of cyclic dimer (VI) similar to II:

\[
\begin{align*}
\text{HO-P-OT0-P-OT-Ac} & \quad \rightarrow \quad \text{O-P-OT0-P-OT-Ac} \\
\text{(or DCG)} & \\
\text{TPS} & \quad \rightarrow \quad \text{AcT0-P-OT0-P-OT-Ac}
\end{align*}
\]

\[(\text{VI})\]

(T means thymidine residue with 3'-hydroxygroup to the right and 5'-hydroxygroup to the left).

It should be noted that the chemical shifts (\(\delta P_{d,a}\) and \(\delta P_{d,b}\)) of asymmetric phosphorus atoms (\(P_d\)) for isomers IVa and IVb as well as Va and Vb differ considerably (about 2-3 ppm), while those difference (\(\delta \delta P_{\alpha1,2} = \delta P_{\alpha1} - \delta P_{\alpha2}\)) for similar atoms of linear compounds of the type C (see page 2) never exceeded 0.2-0.7 ppm. Therefore, the appearance in the \(^{31}P\) NMR spectra of the upfield spin \(AB\)-systems with the large spin-spin coupling constants in the region of resonances of the substituted pyrophosphates among the products of activation of oligonucleotides indicates the presence of the cyclic 3',5'-pyrophosphate derivatives. Other signals in the pyrophosphate region may be related to the type C compounds, probably similar to VI.

The results of this paper are in a good agreement with the data published earlier. After treatment of the reaction mixture of pdTpT(Ac) with 2 eqv. of TPS with aniline the single compound of the nucleotide nature is formed, namely (PhNH)pdTpT(Ac) (VII). It is clear, that just formation of VII should be expected under treatment of Va, Vb and VI with amine.

As compared to the experiments with moderate excesses of TPS, signals at 5.5 ppm and 14.2 ppm (Fig.7) were observed in spectrum of reaction mixture with 5-fold excess of TPS. Therefore, the compounds with activated phosphomonoester groups (type B) and tetrasubstituted pyrophosphate fragments were present in the reaction mixture. Addition of amine to the above mixture (see, Fig.7) have led to the formation of VII and VIII.
Appearance of VIII indicates that active derivative containing both activated phosphomonoester and tetrasubstituted pyrophosphate fragments simultaneously were formed. The most simple structure of this derivative is as follow:

\[
\text{VII, } R = \text{phenyl or cyclohexyl}
\]

\[
\text{VIII, } R = \text{phenyl or cyclohexyl}
\]

**Figure 7**

$^31$P NMR spectrum of the reaction mixture of pdTpdpT (Ac) (0.15M) with TPS (0.75M) after 2 hours.

Compound of this type gave quantitative yield of (Tr)dTpdpT (Ac) after 42 hours reaction time (2 eqv. of (Tr)dT were used). However the use of high excess of TPS may lead to some side reactions.

The data obtained may be represented by following scheme (for moderate excesses of TPS):
pdTpR

ZpdTp

p means "activated" phosphoester groups.
The compounds taken in parenthesis are thought to be highly reactive intermediates present in the steady-state concentrations.

REFERENCES