Application of base-catalysed reaction to the synthesis of dinucleotides containing the four common deoxyribonucleosides and of oligodeoxythymidylates

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ABSTRACT

The phosphorylation of nucleosides by a nucleoside phosphorofluoridate in the presence of potassium tert-butoxide is a very effective reaction for internucleotide bond synthesis in the case of pyrimidine deoxynucleosides. However, for the purine deoxynucleosides, yields are reduced due to competing reactions. The method was applied to the stepwise synthesis of oligodeoxythymidylates. The yield of the trinucleotide was good whereas that of the tetranucleotide was reduced due to the insolubility of the intermediate trinucleotide in the presence of potassium tert-butoxide.

INTRODUCTION

A method for internucleotide bond synthesis by base catalysis has been developed recently. The model reaction involved suitably protected deoxythymidine and protected deoxythymidine phosphorofluoridate, the reaction being catalysed by potassium tert-butoxide in anhydrous dimethylformamide (1,2). The present investigation was undertaken to define the scope and limitations of this reaction for other deoxyribonucleosides and in stepwise oligodeoxyribonucleoside synthesis.

MATERIALS AND METHODS

General procedures were those described previously (2). The 5'-O-monomethoxytrityl derivatives of deoxyadenosine, deoxycytidine and deoxyguanosine where synthesized by the method of Schaller et al. (3). Preparations of 3'-O-monomethoxytritylthymidine-5' phosphorofluoridate, 5'-O-dimethoxytritylthymidine-3' phosphorofluoridate and 3'-O-monomethoxytritylthymidine have been described (2). Potassium tert-butoxide in hexamethylphosphoramide was used to catalyse the formation of the internucleotide bond from the phosphorofluoridate in dimethyl formamide.
by reaction for 15 minutes at 25° under the conditions prev-
iously described for the synthesis of thymidylyl-(5' → 3')-
thymidine (2), with the following modifications. Each reaction
contained 40 μmoles of 5'-O-methoxytrityldeoxynucleoside and
twice the amount of M potassium tert-butoxide in hexamethyl
phosphoramidite required to neutralize all ionizable groups was
used in the reaction.

5'-O-Dimethoxytrityl-3'-O-monomethoxytritylthymidylyl-(3' → 5')-
thymidylyl-(3' → 5')-thymidine (DMTrdT-dTMMTr). 3'-O-Monomethoxytritylthymidine (64 μmoles) and 5'-O-dimethoxytritylthymidine-3' phosphorfluoridate (118 μmoles) in dimethyl formamide (2 ml) were reacted with M potassium tert-butoxide in hexamethyl phosphoramidite (1.2 ml) for 15 minutes at 25°. After neutralization of the reaction with pyridinium AG50 x 2 resin (Bio-Rad), the products were separated on a column (25 x 200 mm) of DEAE-cellulose (carbonate form) using a linear elution gradient of 5 x 10^{-4} M NH₄HCO₃ in 50% ethanol (500 ml) to 0.1 M NH₄HCO₃ in 50% ethanol (500 ml). The desired product (48 μmoles), eluted by 0.05 M NH₄HCO₃ in 50% ethanol, was isolated after removal of solvent by rotary evaporation. It was characterized by removal of the protecting groups with 80% acetic acid and comparison with authentic dT-dT.

3'-O-Monomethoxytritylthymidylyl-(3' → 5')-thymidylyl-(3' → 5')-thymidine (dT-dTMMTr). DMTrdT-dTMMTr (30 μmoles) in H₂O (10 ml), ethanol (10 ml), glacial acetic acid (0.4 ml) was kept at 25° for 4 hrs. The solution was neutralized with aqueous NH₄OH and dimethoxytritylalcohol removed by extraction with diethyl ether. The solution was concentrated to dryness by rotary evaporation and taken up in 5 x 10^{-4} M NH₄HCO₃ (5 ml). This solution was passed through a column (25 x 950 mm) of Sephadex G-25 (fine grade) using 5 x 10^{-4} M NH₄HCO₃ as the eluent. Salt and traces of un-protected nucleotides were eluted at the usual position and the desired product emerged after elution with twice the volume of eluent required to elute salts. The nucleotide was isolated as its ammonium salt by freeze-drying. It was characterized as dT-dT after removal of the monomethoxytrityl group by treatment with 80% acetic acid.

5'-O-Dimethoxytrityl-3'-O-monomethoxytritylthymidylyl-(3' → 5')-
thymidylyl-(3' → 5')-thymidine (DMTrdT-dT-dTMMTr). dT-dTMMTr
(28 μmoles) and 5'-O-dimethoxytritylthymidine-3' phosphorofluoridate (55 μmoles) in dimethylformamide (2 ml) were reacted with M potassium tert-butoxide in hexamethyl phosphoramidate (1 ml) for 15 minutes at 25°. After neutralization of the reaction with pyridinium AG50 x 2 resin, the products were separated on a column (25 x 200 mm) of DEAE-cellulose in the carbonate form using a linear eluting gradient of 5 x 10^{-3} M NH₄HCO₃ in 50% ethanol (500 ml) and 0.12 M NH₄HCO₃, pH 9.75, in 50% ethanol (500 ml). The desired, major product was eluted last by 0.08 M NH₄HCO₃. It was isolated as the ammonium salt (17 μmoles) after removal of the solvent by rotary evaporation and was characterized after removal of protecting groups by 80% acetic acid by comparison with authentic dT-dT-dT.

3'-O-Monomethoxytritylthymidylyl-(3' → 5')-thymidylyl-(3' → 5')-thymidine (dT-dT-dTMMTr). DMTrdT-dT-dTMMTr (14 μmoles) was selectively hydrolyzed with dilute acetic acid and the product isolated on Sephadex-G-25 (fine grade) exactly as in the preparation of dT-dTMMTr. The dT-dT-dTMMTr eluted immediately after the salts and unprotected nucleotides. The protected oligonucleotide was isolated by freeze-drying (12 μmoles) and characterized by comparison with authentic dT-dT-dT.

5'-O-Dimethoxytrityl-3'-O-Monomethoxytritylthymidylyl-(3' → 5')-thymidylyl-(3' → 5')-thymidine (DMTrdT-dT-dTMMTr). DMTrdT-dT-dTMMTr (10 μmoles) and 5'-O-dimethoxytritylthymidine-3' phosphorofluoridate (30 μmoles) in dimethyl formamide (2 ml) were reacted with M potassium tert-butoxide in hexamethyl phosphoramidate (1 ml) for 15 minutes at 25° (a precipitate formed immediately on addition of the M potassium tert-butoxide). The product was worked up exactly as in the isolation of DMTrdT-dT-dTMMTr. The desired product which was eluted from DEAE-cellulose by 0.1 M NH₄HCO₃, was isolated (2 μmoles) after removal of the solvent by rotary evaporation. It was identified by removal of the protecting groups with 80% acetic acid and comparison with authentic dT-dT-dT-dT.

RESULTS AND DISCUSSION
Our initial studies with the base-catalysed phosphodiester synthesis of thymidine derivatives demonstrated that the 5'-hydroxyl function was phosphorylated most efficiently (2,4).
However, because of the ready availability of the 5'-O-monomethoxytrityl derivatives of deoxyadenosine, deoxycytidine and deoxyguanosine, their reaction with 3'-O-monomethoxytritylthymidine-5' phosphorofluoridate catalysed by potassium tert-butoxide was the route chosen to explore the potential of the new reaction. The results are summarized in Table I which also included comparable data on the preparation of dT-dT. It is clear that the reaction is also an excellent route to phosphodiesters involving deoxycytidine as exemplified by the yield of dC-dT. However, the yields of dA-dT and dG-dT were low, though significant. The possible reasons for this were further investigated in the case of dA-dT. Adenine was released from deoxyadenosine compounds at a significantly greater rate (approximately 3 fold) than was thymine from thymidine derivatives when treated with potassium tert-butoxide. In addition a major proportion of the phosphorofluoridate was converted to the phosphate. It is possible that this resulted from nucleophilic attack by the adenine.

### Table I

Reaction of 5'-O-monomethoxytrityldeoxynucleosides with 3'-O-monomethoxytritylthymidine-5' phosphorofluoridate catalysed by potassium tert-butoxide.

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Yield of dN-dT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-O-monomethoxytritylthymidine</td>
<td>45</td>
</tr>
<tr>
<td>5'-O-monomethoxytrityldeoxycytidine</td>
<td>96</td>
</tr>
<tr>
<td>5'-O-monomethoxytrityldeoxyadenosine</td>
<td>14</td>
</tr>
<tr>
<td>5'-O-monomethoxytrityldeoxyguanosine</td>
<td>22</td>
</tr>
</tbody>
</table>

The reaction conditions and removal of protecting groups are described under Materials and Methods. When the amount of M potassium tert-butoxide used in each reaction was doubled, the yields were increased; dT-dT (65%), dA-dT (28%).
because it was evident from spectral studies that the base is ionized in the presence of potassium tert-butoxide. These side reactions, together with others, not fully investigated, cumulatively resulted in the low yield of dA-dT and, by analogy, of dG-dT.

The potential of the base-catalysed reaction of nucleoside phosphorofluoridates for stepwise synthesis of oligodeoxynucleotides was investigated in the case of oligodeoxythymidylates. Essential features common to all stepwise chemical syntheses are protection of the deoxyribose hydroxyl of the incoming activated mononucleotide, protection of the deoxyribose hydroxyl at the non-growing end of the oligomer and the selective deprotection of the new growing point on the oligomer. Because of the strongly alkaline conditions for the present procedure, no base-labile protecting groups could be used. Hence, differential removal of base-stable, acid-labile protecting groups was investigated. The principal underlying this approach is illustrated in Table II.

**TABLE II**
Rates of hydrolysis of methoxytrityl derivatives by dilute acetic acid at 25°.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Half-life (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-O-dimethoxytrityl-thymidine-3' phosphorofluoridate</td>
<td>0.5</td>
</tr>
<tr>
<td>5'-O-monomethoxytrityl-thymidine-3' phosphorofluoridate</td>
<td>6.0</td>
</tr>
<tr>
<td>3'-O-monomethoxytrityl-thymidine-5' phosphorofluoridate</td>
<td>no hydrolysis</td>
</tr>
</tbody>
</table>
<pre><code>                                                                                 | (5 hrs)         |
</code></pre>

The nucleotides (10 umole) in H₂O (0.5 ml), ethanol (0.5 ml), glacial acetic acid (20 ml), pH 3.15 was kept at 25° and the hydrolysis of the methoxytrityl residues followed chromatographically in solvent 1. In 80% acetic acid at 25°, the methoxytrityl groups of all three compounds were completely hydrolyzed after 4 hours.
which presents data on the acid-lability of monomethoxytrityl and dimethoxytrityl groups attached to deoxyribonucleotide 3'-hydroxyl or 5'-hydroxyl groups. It is clear that a methoxytrityl group attached to a 5'-hydroxyl group is much more acid-labile than the same group attached to a 3'-hydroxyl group. The data in Table II also confirm the much greater lability of the dimethoxytrityl group relative to the monomethoxytrityl group (5). Thus, the most appropriate strategy for oligodeoxynucleotide synthesis involved protection of the non-growing 3'-hydroxyl end of the oligonucleotide using a monomethoxytrityl group and the use of a 5'-O-dimethoxytrityldeoxyribonucleoside-3' phosphoro-fluoridate to phosphorylate the unprotected 5'-hydroxyl of the oligonucleotide. The dimethoxytrityl could then be selectively removed to reveal the 5'-hydroxyl of the oligonucleotide. Application of this strategy to the stepwise synthesis of oligothymidylates resulted in a satisfactory yield on conversion of the dinucleotide to the trinucleotide. However, extension of the trinucleotide to the tetranucleotide was less efficient. The primary reason for this appeared to be the low solubility of the protected trinucleotide, dT-dT-dTMMTr under the conditions of the reaction. Presumably this problem, which was not investigated further, could be overcome by replacing potassium with a cation whose nucleotide salts are more soluble.

In summary, base-catalysed phosphorylation by a nucleoside phosphorofluoridate provides a very efficient route to the internucleotide bond in dinucleoside phosphates containing pyrimidine deoxynucleosides. The method also is effective in the stepwise synthesis of short pyrimidine oligodeoxynucleotides. Whilst the method can be applied to the synthesis of purine containing oligodeoxynucleotides, yields are reduced due to a variety of side reactions.

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1742
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