Chemical Synthesis of 80-mer Thymidylic Acid

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Chemical synthesis of long-chain oligodeoxyribonucleotides was studied by the method of block condensation in a liquid phase. A method for elongating the chain and the synthesis of oligothymidylic acids up to 80-mer are described.

Chemical synthesis of oligonucleotides has attracted much attention from the standpoint of its application to gene engineering. The phosphotriester approach introduced by Letsinger and Ogilvie\(^1\) has the advantages of an unambiguous formation of the internucleotide phosphate linkage and of easier isolation of the product by chromatography on silica gel. Based on this methodology, a variety of modified procedures involving improved phosphorylating agents, condensing agents or protecting groups have been established.\(^2\)

Some of them using solid support allow rapid preparation of reasonable quantities of oligonucleotide fragments which can be used in studies of molecular biology. On the other hand, liquid-phase condensations of oligonucleotide blocks are still useful when larger amounts of material are needed.

In the chemical synthesis of higher oligomers, more difficulties might be anticipated, especially in effecting the stoichiometric reactions and isolating the desired oligomers from the reaction mixture. Sood and Narang have reported 38-mer thymidylic acid by coupling a 5'-hydroxyl component of 14-mer to a 3'-phosphodiester of 24-mer in solution.\(^3\)

Recently, Chakhmakhcheva et al. obtained 62-mer oligodeoxyribonucleotide by sequentially adding oligomer blocks of not longer than 10-mer length to the 5'-terminus.\(^4\)

Solid-phase approaches have also succeeded in the synthesis of higher oligomers including 31-mer,\(^5\) 42-mer\(^4\) and 51-mer.\(^6\)

However, few papers have discussed the maximum molecular size of an oligonucleotide block that can be used in the condensation reactions. We now describe our findings in the block condensation of protected oligothymidylic acid up to 80-mer by employing the strategy of convergent synthesis.

RESULTS AND DISCUSSION

In our synthetic studies, we adopted the most commonly used protecting groups: dimethoxytrityl (DMTr), for 5'-hydroxyl protection, o-chlorophenyl for internucleotide phosphate, and β-cyanoethyl for temporary protection of the phosphate at the 3'-terminus.

The monomer and dimer of thymidylic acid protected in these ways were prepared according to the literature.\(^7,8\)

After removing a dimethoxytrityl or a β-cyanoethyl group from the fully protected nucleotides under the established conditions,\(^9,3,10\) both components (1 and 2) were condensed together in pyridine with the help of mesitylenesulfonyl 3-nitrotriazolide (MSNT), a readily available and widely used condensing agent.\(^11\)

In the cases of oligomers higher than trimer, the conventional work-up procedures for reactions (condensation and de-dimethoxytritylation), which involve a washing step
with aqueous NaHCO₃, often cause low yields due to their low solubility in chloroform. Therefore, we omitted such a troublesome step when removing the dimethoxytrityl group. Instead, after completing the reaction, benzenesulfonic acid was removed from the mixture by treating with an anion exchange resin, Amberlite IR 45, and the 5'-hydroxyl free oligomers were precipitated with an ether-hexane mixture. The products were isolated quantitatively by this procedure and proved to be pure from an examination of the thin layer chromatogram.

To remove mesitylenesulfonic acid from the condensation reaction mixture, the ion-exchange procedure was also proved to be more efficient than by washing with aqueous bicarbonate, and the isolated yields of condensation products were much improved.

A summary of the condensation is given in Table I, in which the entries with the asterisk (*) employed this modified work-up procedure, the others resulting from conventional extraction.

The chromatographic behaviors of the product and the unreacted 5'-hydroxyl component were very similar on silica gel in a preparation of 40-mer, so further purification was performed by using gel permeation under medium pressure in chloroform–methanol. The synthetic oligomers obtained were analyzed by HPLC, using a gel permeation column.

They were deprotected according to the known procedures and the validity of their postulated structures was fully supported by an electrophoretic analysis.

An approach to 80-mer by coupling two 40-
mer components was unsuccessful, despite the prolonged exposure to a large excess of MSNT (20 equivalent to 1, 26 hr). In the reaction, the isolated product bearing the DMTr group moved faster than the 5'-hydroxyl component of 40-mer on TLC. The electrophoretic analysis of its deprotected derivative, however, suggested it to be a 40-mer. It is conceivable that the isolated material was an azolide derivative of 40-mer 1, which was not hydrolyzed during the work-up procedure.

The phosphodiester group at the 3'-terminus is always subject to two chemical processes during a condensation reaction. One involves activation of the phosphate by the condensing agent and the other is nucleophilic attack by the hydroxyl group of the 5'-hydroxyl component. It is probable that the bulk of the 3'-phosphodiester component of 40-mer prevented the unimpeded process of at least one of these reactions.

In order to overcome this difficulty, we planned an alternative approach using repeated runs with 20-mer of the phosphodiester component instead of 40-mer. In addition, we introduced an alternative protecting group, p-tert-butylanilino group, for phosphate at the 3'-terminus, the introduction of which facilitated confirmation of the structure of the product in each condensing step by non-destructive analysis. The intensive signal of the tert-butyl group was easily distinguishable in the C-13 NMR spectrum as well as some signals arising from the dimethoxytrityl group. Therefore, the coexistence of these signals in the carbon-13 spectrum offered corroborating evidence to conclusively prove condensation when the product was chromatographically homogeneous. The preparation of 2-chlorophenyl \(N-(p\text{-}\text{tert-butylphenyl})\)phosphoramidochloridate and its phosphorylation of 5'-dimethoxytritylthymidine are depicted in Fig. 2. Analogously to the other anilino group, the protecting group was removed by treating with isoamyl nitrite.

The carbon-13 spectrum (in pyridine-\(d_5\)) of the protected thymidylic acid 8 shows the characteristic signals at 55.3 and 113.8 ppm for DMTr, and that at 31.5 ppm for tert-Bu. Starting from 8, elongation of the oligomer chain was carried out in a similar manner to that already described, the results being summarized in Table II.

All the condensation products exhibited
both signals of DMT\textsubscript{r} and \textit{tert}-Bu in their carbon-13 spectra, although their relative intensity decreased with the increasing number of thymidylic acid units.

The purification of higher oligomers than 40-mer was carried out again by a combination of column chromatography on silica gel and gel permeation on Toyopearl HW50F. As shown in Table II, the synthesis of 80-mer material was achieved in a moderate yield by an coupling 20-mer with 60-mer. Figure 4 shows the C-13 NMR spectrum of the synthetic 80-mer. Heptactamer was also obtained by coupling 30-mer with 40-mer. However, the isolated yield was lower than those from reactions using 20-mer as the 3'-phosphodiester component I. An attempt to condense the 40-mer component 1 with 9 of 40-mer under similar conditions (1:9 = 2.0:1, MSNT:1 = 10:1, 6 hr) was again unsuccessful.

Only a trace amount of compound corresponding to 80-mer was detected by an HPLC analysis of the crude reaction products.

The synthetic oligomers were deprotected by successive treatment with isamyl nitrite, 2-pyridinedaldoximate and 80% acetic acid, and characterized by their electrophoretic behaviors.

In conclusion, we were able to show that the reactivity of the 3'-phosphodiester component I markedly decreased by changing its molec-

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** Table II.**

<table>
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<tr>
<th>Entry</th>
<th>1 (mm)**</th>
<th>9 (mm)**</th>
<th>1/9</th>
<th>MSNT/1</th>
<th>Reaction time (hr)</th>
<th>10</th>
<th>Yield (%)</th>
</tr>
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<tr>
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<td>T1 (66)</td>
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<td>1.5</td>
<td>3</td>
<td>T5</td>
<td>87</td>
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<tr>
<td>2</td>
<td>T5 (104)</td>
<td>T5 (75)</td>
<td>1.4</td>
<td>1.5</td>
<td>4</td>
<td>T10</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>T10 (74)</td>
<td>T10 (61)</td>
<td>1.2</td>
<td>2.0</td>
<td>5</td>
<td>T20</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>T20 (37)</td>
<td>T20 (31)</td>
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<td>3.0</td>
<td>6</td>
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<td>72</td>
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<tr>
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<td>T20 (15)</td>
<td>T40 (12)</td>
<td>1.3</td>
<td>7.3</td>
<td>8</td>
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<td>58</td>
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<tr>
<td>6</td>
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<td>T40 (6)</td>
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<td>9.9</td>
<td>5</td>
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<td>41</td>
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<tr>
<td>7</td>
<td>T20 (23)</td>
<td>T60 (11)</td>
<td>2.1</td>
<td>8.0</td>
<td>5</td>
<td>T80</td>
<td>51</td>
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** Milli-molar concentration in the reaction mixture (pyridine).
ular size from 30-mer to 40-mer, and that by using 20-mer instead, led to oligomers up to 80-mer. Further elongation will be possible using 20-mer, although the reactivity of the 5'-hydroxyl component might decrease by increasing its molecular size.

EXPERIMENTAL

$^{13}$C-NMR spectra were measured with a JEOL JNM FX90Q spectrometer. HPLC analyses were carried out on a Hitachi 655 instrument with columns of GL-A130 and GL-A140 (Hitachi) in a solvent system of CHCl₃-MeOH (95:5). Column chromatography and thin layer chromatography were performed on Kieselgel 60, 60 ~230 mesh, respectively, in chloroform containing from 3 to 10% methanol with or without 0.5% pyridine. Purification by gel permeation was carried out under medium pressure with a glass column (φ22 × 500 mm) packed with Toyopearl HW50F. Purification of the deprotected oligomer was performed by column chromatography on silanized silica gel in acetonitrile-0.1 m triethylammonium acetate (4:1).

Removal of dimethoxytrityl groups. According to the literature, the oligomer (3 or 10) was stirred with 1% benzenesulfonic acid in chloroform-methanol (7:3), (BSA soln: 3 or 10 = 4~30: 1 ml/g) on an ice-cooled water bath for 1~2 hr. When TLC showed the completion of the reaction, the mixture was diluted with an appropriate amount of chloroform-methanol (7:3) and stirred with Amberlite IR 45 (approx. 1.5 g for 1 ml of 1% BSA soln.) for 15 min. During this period, the color of the DMTr cation disappeared. The mixture was then filtered to remove the resin and the filtrate was concentrated in vacuo.

Removal of the β-cyanoethyl group. According to the literature, the oligomer was treated with triethylamine in dry pyridine. After completing the reaction (3~6 hr), the mixture was concentrated in vacuo. To remove the contaminating triethylamine, the residue was co-evaporated twice with an appropriate quantity of dry pyridine. The resulting material was used for the next condensation.

5'-O-(Di-p-methoxytrityl)thymidine-3'-(2-chlorophenyl)phosphoro-p-tert-butylanilidate 8. A mixture of 5.44 g of 5'-O-dimethoxytritylthymidine 7, 10.4 g of 6, and 0.25 g of 4-N,N-dimethylaminophosphorylpyridine in 150 ml of dry pyridine was stirred overnight at room temperature. The mixture was then stirred with 20 ml of water for 30 min and concentrated in vacuo. The residue was dissolved in chloroform, washed with 0.1 m triethylammonium bicarbonate solution, and dried over anhydrous Na₂SO₄. After removing the solvent, the crude product was chromatographed on 750 g of silica gel. Elution with a mixed solvent of chloroform-methanol-pyridine (100:3:0.5) afforded a syrupy product, which was then dissolved in a minimum quantity of chloroform and precipitated with hexane. Filtration followed by drying in vacuo gave 6.1 g of 8 as a powder (70.5%).

Removal of the 3'-phosphodiester component (1) and a 5'-hydroxyl component (2 or 9) was dried three times by evaporation of pyridine and dissolved in dry pyridine. Reaction was carried out at room temperature under the conditions shown in Tables I and II, and then stopped by adding water. After stirring for 15 min, the mixture was diluted with chloroform-methanol (7:3) and stirred with Amberlite IR 45 (25~30 g for 1 mmol of MSNT) for 10~20 min, and then filtered. The filtrate was concentrated in vacuo and the residue was chromatographed on silica gel to isolate the condensed product. In the case of the high oligomer (3, n = 40; 10, n = 60, 70, 80), further purification was performed by using a gel permeation column under medium pressure in chloroform-methanol (85:15).

Deprotection of oligothymidylic acid. 10 mg of the oligomer was stirred with 0.1 ml of isoamyl nitrite in 0.2 ml of a mixture of pyridine and acetic acid (1:1) at room temperature for 24 hr. The mixture was diluted...
with 1 ml of chloroform and added dropwise to a vigorously stirred mixture of ether–hexane (1:1, 10 ml). The precipitate was collected by filtration, and washed successively with ether, 80% aqueous acetone and acetone. The powdered material on the filter was then dissolved in chloroform–methanol, and the solution was concentrated in vacuo. The residue was treated with 1 ml of a 0.5 M solution of tetramethylguanidinium 2-pyridinealdoximate at room temperature for 7 days. After removing the volatile materials in vacuo, the residue was chromatographed on silanized silica gel. The fraction containing the material with dimethoxytrityl was concentrated and the residue was treated with 1.5 ml of 80% aqueous acetic acid at room temperature for 1.5 hr. The mixture was concentrated and purified on a silanized silica gel column. The samples obtained in this way were characterized by an analysis based on electrophoresis.

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REFERENCES AND NOTES

12) In our previous report, the synthesis of 80-mer was incorrectly stated. Y. Nakahara and T. Ogawa, Nucleic Acids Res., Symp. Ser., 12, 59 (1983).