Total Synthesis of the Structural Gene for the Precursor of a Tyrosine Suppressor Transfer RNA from Escherichia coli

5. SYNTHESIS OF THE DEOXYRIBOPOLYNUCLEOTIDE SEGMENTS REPRESENTING THE NUCLEOTIDE SEQUENCE 71–103*

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Chemical syntheses of the pentadecanucleotide, d(G-G-T-G-G-G-T-T-C-C-C-G-A-G), the undecanucleotides, d(G-G-T-G-G-G-G-T-T-C-C) and d(C-C-C-A-C-C-A-C-G-G), the deca nucleotide, d(G-T-A-T-G-C-T-T-T), and the nonanucleotides, d(A-T-T-A-C-C-C-G-T) and d(A-G-T-A-A-A-A-G-C) are described. The deoxyribopolynucleotides together represent the DNA duplex corresponding to the nucleotide sequence 71–103 (from the 3'-end) of the gene for the tyrosine suppressor tRNA. Synthesis of the guanine-rich undecanucleotide d(G-G-T-G-G-G-G-T-T-C-C) was performed by the use of a new protecting group for the guanine ring, the methylbutyryl group. The heptanucleotide d[(MeOTr)mbG-mbG-mbG-mbG-mbG], prepared by the new method, was condensed with the tetranucleotide d[pacC-anC-T-A(Nc)]. All of the condensations described followed previously developed chemical principles and started with the N- and 5'-protected deoxyribonucleosides. Successive condensations at the 3'-end with protected mononucleotides, preformed di-, tri-, or tetranucleotides gave products which were separated by anion exchange chromatography and characterized by chemical and enzymatic methods.

The plan adopted for the total synthesis of the DNA corresponding to the precursor for the Escherichia coli tyrosine tRNA required the chemical synthesis of a total of 26 short deoxyribopolynucleotide segments (2). In the preceding three papers (1, 3, 4), chemical syntheses of the segments 1 to 15 have been described. The present paper documents the synthesis of segments 16 to 21 shown in Fig. 1. The following paper describes the synthesis work dealing with segments 22 to 25 (5) and, therefore, completes the task of the chemical synthesis of all the segments.

In the present work, segment 16 consisted of only the tetranucleotide d(C-G-A-G) (Fig. 1). While it was hoped that this short oligonucleotide would undergo joining reactions when used together with the adjacent segments, it was considered worthwhile, as a safe alternative, to chemically join segment 16 to segment 17 and to use the resulting pentadecanucleotide in the joining reactions. As described later, segment 16 could indeed be used reasonably well when segments 14 and 17 as well as the segments in the complementary strand were present in the ligase-catalyzed joining reactions. On the other hand, difficulty was experienced in the chemical synthesis of the pentadecanucleotide containing both segments 16 and 17 (Fig. 1). Therefore, in the enzymatic work, both of these segments were necessary.

Chemical synthesis of the very guanine-rich segment 17 presented considerable difficulties. During this work, a new protecting group (2-methylbutyryl) was used for the guanine ring. Using this group, synthesis of the heptanucleotide d(G-G-T-
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G-G-G-G) and then of the complete segment 17 was accomplished. Synthesis of segment 18 necessitated only the addition of the dinucleotide, d(G-G), to the fully protected nonanucleotide, d(C-C-C-A-C-C-A-C). This had already been synthesized.

Discussion of Experimental Methods

Synthesis of Undecanucleotide, d(G-G-T-G-G-G-G-T-C-C) (Segment 17)—The undecanucleotide contains an unusually high content of the guanine nucleotide and because of the problem of cumulative partial deisobutyrylation during synthetic work, an alternative protecting group, the 2-methylbutyryl group, was investigated and used for the guanine amino function. This group was found to be more stable than iso-butyl group during the standard synthetic operations and, therefore, offered a distinct advantage. However, subsequent experience in the use of this group in syntheses of different oligonucleotides has also shown the necessity for a prolonged and more forcible ammoniacal treatment for its subsequent removal.

The plan adopted for the synthesis of the undecanucleotide is shown in Diagram 1. Since deoxyguanosine constitutes the 5' terminus of the undecanucleotide, a method for the preparation of N-methylbutyryl-5'-O-monomethoxyltrityl deoxyguanosine was worked out. Preparation of N-methylbutyryl deoxyguanosine 5'-phosphate is also described under "Experimental Section." The dinucleotide, d(MeOtTrmbG-mbG), was prepared by condensing d(MeOtTrmbG-mbG) in the presence of TPS. The dinucleotide was isolated by organic solvent extraction and was further condensed with the mononucleotides d[pT(Ac)] and d[pmbG(Ac)], respectively. The products at each step were isolated by organic solvent extraction procedure. The tetrancanucleotide, d(MeOtTrmbG mbG T mbG), which was contaminated with a trace of trinucleotide, was purified by silica gel column chromatography in aqueous acetonitrile. The tetranucleotide was further condensed with d[pmbG(Ac)] and the reaction mixture was first purified by trityl-cellulose (6) followed by silica gel chromatography to give homogeneous pentanucleotide in 56% yield. Up to this step of the synthesis, no loss of methylbutyryl was observed. The above pentanucleotide was again condensed with d[pmbG(Ac)] and the reaction products were purified by anion exchange chromatography. However, two modifications were introduced in the standard procedure which uses DEAE-cellulose as the exchanger and TEAB as the eluting agent.

1See Footnote 1 of Ref. 3.

In the synthesis of these and other oligonucleotides, thin layer chromatography has been extensively used for monitoring the condensation reactions, following organic solvent extraction step, and for judging the purity of the components. The solvent used for developing thin layer chromatographies was acetonitrile with varying amounts of water. In this solvent system, oligonucleotides of chain lengths up to five could be easily resolved by adjusting the amount of water in acetonitrile. On this concept, the silica gel column chromatographic procedure was developed and used successfully for purification up to the pentanucleotide.

ECTEOLA (epichlorohydrintriethanolamine)-cellulose was used in place of DEAE-cellulose and the eluting buffer used was diisopropylethylammonium bicarbonate in place of TEAB. These modifications minimized the loss of the protecting group on the guanine ring. The hexanucleotide was obtained (Fig. 3) in 67% yield. Further condensation of the hexanucleotide with d[pmbG(Ac)] and separation of the products by ECTEOLA-cellulose column (see Fig. 3) gave the heptanucleotide in 71% yield. The protected heptanucleotide was condensed with the protected tetranucleotide, d[pT-T-anC-anC(Ac)], and the products were separated first at the fully protected stage by anion exchange chromatography (ethanol and salt gradients) (Fig. 4). The undecanucleotide was obtained in 49% yield. Further purification was achieved by two successive chromatographic steps after removal of the protecting groups (Figs. 5 and 6). It is worthy to note that in order to obtain sharp elution of the guanine-rich product, elutions were performed at an elevated temperature using the denaturing agent, guanidine hydrochloride (7).

Synthesis of Tetranucleotide, d[pC-G-A-G] (Segment 16)—The dinucleotide, d[panC-mbG], was prepared by condensation of d[(TPM)panC] with d[pmbG(Ac)] followed by the removal of the protecting group. The yield was 62%. The dinucleotide, d[panC-mbG], was prepared similarly in 56% yield. The dinucleotide d[panC-mbG] was converted to the cyanoethyl derivative and the latter was condensed with d[pza-A-mbG(Ac)] using TPS as the condensing agent. After alkaline hydrolysis (0°, 10 min), the protected tetranucleotide, d[panC-mbG-bza-mbG], was isolated by anion exchange chromatography (Fig. 7) in 33% yield. The deprotected product obtained after the standard procedure was further characterized by chromatography on a DEAE-cellulose column in the presence of 7 M urea (Fig. 8) and by enzymatic degradation.

Pentadecanucleotide, d[G-G-T-G-G-G-G-T-C-C-C-C-C] (Segments 16 + 17)—Condensation of the protected undecanucleotide described above with d[panC-mbG-bza-mbG(Ac)] was performed using a 15-fold excess of the latter. A preliminary separation of the product and the undecanucleotide from the smaller compounds was effected on a short DEAE-cellulose column. The separation of the undecanucleotide and the pentadecanucleotide at the unprotected stage was achieved only after two successive chromatographic steps (Figs. 9 and 10A). The purity of the final product was checked by phosphorylation of the 5'-OH group with polynucleotide kinase and [γ-32P]ATP followed by electrophoresis on polyacrylamide gel.

As mentioned above, chemical synthesis of the pentadecanucleotide was carried out as a safeguard against the uncertainty in the enzymatic joining of the tetranucleotide d(C-G-A-G) to the undecanucleotide (segment 17) on one side and the segment 14 on the other. As it turned out, the latter joinings proceeded reasonably well and the use of the pentadecanucleotide proved unnecessary. In fact, due to a reason as yet not understood, the pentadecanucleotide failed to give any significant joining.
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Diagram 1. Synthesis of the tetranucleotide, d(C-G-A-G) (Segment 16), the undecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C) (Segment 17), and the pentadecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C-C-G-A-G) (segments 16 + 17).

Synthesis of Undecanucleotide, d(C-C-C-A-C-C-A-C-G-G) (Segment 18)—The synthesis of the protected nonanucleotide d[(MeOTr)anC-anC-anC-anC-bzA-anC-anC-bzA-anC-bzA] has been described in connection with the synthesis of segment 2 (3). For the present purpose, the single step involving the condensation of d[pibG-ibG(Ac)] with the above protected nonanucleotide was necessary. This was performed as described under “Experimental Section” and the product was purified by two-step chromatography at the fully protected and unprotected stage (Fig. 11). The final yield of the pure product was 17%.

Synthesis of Decanucleotide, d(G-T-A-A-T-G-C-T-T-T) (Segment 20)—The condensation steps used in the synthesis of this segment are shown in Diagram 3. Thus, the sequence involved addition of a mononucleotide, a dinucleotide, another dinucleotide, and, finally, a tetranucleotide. The primary reasons for this sequence were (a) relative ease in obtaining large amounts of the respective oligonucleotide blocks containing 5'-phosphate groups and (b) ease in separation of the oligonucleotide intermediates. Thus, solvent extraction was used successively up to the tetranucleotide, d[panC-T-T-T(Ac)]. At the hexanucleotide stage, a trityl column was used for separation of the trityl-containing compounds from the non-trityl components. In the final condensation step, the pyrimidine tetranucleotide, d[panC-T-T-T(Ac)] (Fig. 16) was used as the incoming block to facilitate the separation of the

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d(MeOTr)bzA

\[ \xrightarrow{+ d[pT(Ac)] + TPS} \]

\( \Rightarrow \)

\( \xrightarrow{d([MeOTr]bzA\cdot T)} \)

\( \xrightarrow{d([MeOTr]bzA\cdot T\cdot T\cdot T\cdot T}) \)

\( \xrightarrow{1. \text{NH}_2\text{OH}} \)

\( \xrightarrow{2. \text{H}^+} \)

\( \xrightarrow{3. \text{Column chromatography}} \)

d(A-T-T-A-C-C-C-G-T)

(\text{Segment 19})

\text{Diagram 2. Steps in the synthesis of the nonanucleotide d(A-T-T-A-C-C-C-G-T) (Segment 19).}

decanucleotide from the unreacted hexanucleotide. It is noteworthy that only the modest 8-fold excess of the tetranucleotide block was used and the yield of the decanucleotide (Fig. 17) was 42%. The fully deprotected product was purified by DEAE-cellulose chromatography in the presence of 7 M urea (Fig. 18).

\text{Synthesis of Nonanucleotide, d(A-G-T-A-A-A-A-G-C) (Segment 21)—As seen in Diagram 4, the steps in the synthesis of the nonanucleotide involved four condensations with the protected mononucleotides and two condensations with the protected dinucleotides. Early condensations with the mononucleotides proceeded in high yields and the products were isolated by solvent extraction methods. Thus, d([MeOTr]bzA-mbG]) was obtained in 91% yield and the trimucleotide, d([MeOTr]bzA-mbG-T), was isolated in 69% yield although d[pT(Ac)] was used in only 50% excess. In the next step, d([MeOTr]bzA-mbG-T) was condensed with d[pbzA(Ac)] (4-fold excess only) and the required tetranucleotide was isolated by extraction in 76% yield. The yield at the next stage of pentanucleotide synthesis was lower, anion exchange chromatography being necessary for purification of the products (Fig. 19). The next steps involved successive condensations with the protected dinucleotides, d[pbzA-bzA(Ac)] and d[pmbG-anC(Ac)], and the yields after the chromatographic steps were 62% (Fig. 20) and 48% (Fig. 22), respectively. Both the hepta- and the nonanucleotides were further subjected to chromatography in the presence of 7 M urea after removal of the protecting groups (Figs. 21 and Fig. 23).}

\text{Comment—It had originally been hoped that segments 16 and 17 could be combined and the pentadecanucleotide would be chemically synthesized. Unfortunately, difficulties were experienced in the chemical synthesis of this guanine-rich polynucleotide. There, as described later, the plan for the joining of this part of the gene dealt mainly with the question of joining segments 14, 16, and 17 to one another. Although yield was not very high, the objective nonetheless was realized. The problem of the protection of the guanine ring is still not solved completely satisfactorily. Methylbutyryl group appears to be much too stable although no side reactions were detected during a careful investigation of the conditions necessary for its removal. Isobutyryl group, which has been used previously, continues to be used currently in synthetic work. Since detectable loss of this group may occur during prolonged exposure to triethylammonium bicarbonate, an alternative approach has been developed. Anion exchange}
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TABLE I
Chromatographic properties of methoxytrityl oligonucleotides

All of the nucleotides are deoxynucleotides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent A</th>
<th>Solvent B</th>
<th>Solvent C</th>
<th>Solvent D</th>
<th>Other Solvents*</th>
</tr>
</thead>
<tbody>
<tr>
<td>d(MeOTr)mbG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.88(J)</td>
<td></td>
</tr>
<tr>
<td>d(MeOTr)mbG-mbG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.66(J), 0.85(K)</td>
<td></td>
</tr>
<tr>
<td>d(MeOTr)mbG-mbG-T</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.12(J), 0.65(K)</td>
<td></td>
</tr>
<tr>
<td>d(MeOTr)mbG-mbG-T-bzA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.32(K)</td>
<td></td>
</tr>
<tr>
<td>d(MeOTr)mbG-mbG-T-bzA-bzA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.10(K), 0.14(L)</td>
<td></td>
</tr>
<tr>
<td>d(MeOTr)mbG-mbG-T-bzA-bzA-bzA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.13(L), 0.28(M)</td>
<td></td>
</tr>
<tr>
<td>d(MeOTr)mbG-mbG-T-bzA-bzA-bzA-bzA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.24(M)</td>
<td></td>
</tr>
<tr>
<td>d(MeOTr)mbG-mbG-T-bzA-bzA-bzA-bzA-bzA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.90(M)</td>
<td></td>
</tr>
</tbody>
</table>

Rf values relative to pdT.

* Other solvent systems used are shown in brackets.

** Absolute Rf values on silica gel thin layer chromatography plates.

* Chromatographed on DE81 paper.

Characterization of Synthetic Deoxyribopolynucleotides

The characterization of the synthetic intermediates and final products was carried out by extensive monitoring of ultraviolet spectrophotometric changes, anion exchange chromatography both at protected and unprotected stages, and by paper chromatography and thin layer chromatography as far as possible. The Rf values in different solvents are given in Tables I and II. Further characterization was obtained by degradation of the unprotected purified oligonucleotides by venom phosphodiesterase. The results are in Table III. Before use in the ligase reactions, it was necessary to phosphorylate the 5'-ends of the synthetic segments using γ-32P-labeled ATP and the polynucleotide kinase. This step afforded a high sensitive method to check their purity.

TABLE II
Chromatographic properties of unprotected oligonucleotides

All of the nucleotides are deoxynucleotides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent A</th>
<th>Solvent B</th>
<th>Solvent C</th>
<th>Solvent D</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpc-G-A-G</td>
<td>-</td>
<td>0.35</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>dG-T</td>
<td>-</td>
<td>-</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>dG-T-A-A</td>
<td>-</td>
<td>-</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>dG-T-A-A-G</td>
<td>-</td>
<td>-</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>dpc-T-T-T</td>
<td>-</td>
<td>0.67</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>dA-G</td>
<td>-</td>
<td>1.15</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>dA-G-T</td>
<td>-</td>
<td>0.79</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>dA-G-T-A</td>
<td>-</td>
<td>0.60</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>dA-G-T-A-A-A</td>
<td>-</td>
<td>0.67</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>dA-G-T-A-A-A-G-C</td>
<td>-</td>
<td>-</td>
<td>0.41</td>
<td></td>
</tr>
</tbody>
</table>

Rf values relative to pdT.

* Methods for the preparation of protected deoxyribonucleosides, di- and trinucleotides, for condensation reactions, chromatog-
A synthesis of undecanucleotide, d(G-G-T-G-G-G-T-T-C-C-C) (Segment 17)

N-2-Methylbutyryl Deoxyguanosine 5'-phosphate, d(pmbG)

An anhydrous pyridine solution (200 ml) of deoxyguanosine 5'-phosphate (10 mmol, pyridinium or ammonium salt) and DIEA (100 mmol) was treated with 2-methylbutyryl chloride (10 ml, 80 mmol) at room temperature. After 2 hours the reaction mixture was concentrated under vacuum and evaporated to a gum. This was then dissolved in CHCl₃ (300 ml) and washed with ice water (300 ml). The organic layer was dried over anhydrous sodium sulfate and evaporated to a syrup under reduced pressure. The syrup was dissolved in pyridine/ethanol (1/1, 100 ml) and cooled in a dry ice bath (-50°C), a solution of sodium hydroxide (2N, 100 ml) was added dropwise keeping the temperature of the reaction mixture below -30°C. After the addition was complete, the reaction mixture was neutralized by the addition of pyridinium Dowex 50 resin (200 ml); the resin was removed by filtration and washed with aqueous pyridine (20%, 600 ml). The combined filtrate and washings were concentrated under vacuum and the residue was dissolved in water (200 ml) and kept at 0°C for 20 min. The solution was concentrated and the residue was dissolved in pyridine (5 ml) and kept at 0°C for 20 min. The solution was then concentrated by evaporation and dissolved in pyridine/ethanol (1/1, 50 ml). Sodium hydroxide solution (2N, 50 ml) was added dropwise to the above solution (-50°C) maintaining the solution at -30°C. After the addition was complete, the solution was brought to 0°C and kept at that temperature for 40 min. The solution was then neutralized by the addition of pyridinium Dowex 50 resin (100 ml); the resin was removed by filtration and washed with 20% aqueous pyridine. The combined filtrate and washings were concentrated under vacuum and the residue was taken up in dry pyridine (50 ml) and precipitated into petroleum ether (1.5 liters). The product, d(pmbG) (yield 93%) was homogeneous on thin layer chromatography in solvent spJ and K (Rₑ, 0.69).

5'-O-Monomethoxytrityl-N-2-methylbutyryl Deoxyguanosine, d(MeOTr)pmbG—An anhydrous pyridine solution (30 ml) of d(pmbG) (4 mmol) was reacted with d(MeOTr)Cl (4.8 mmol) for 24 hours at room temperature. The reaction mixture was treated with ethanol (2 ml) in an ice bath and kept at room temperature for 1 hour. The solution was then concentrated and dissolved in CH₄Cl₃ (50 ml) and washed with 0.2 M TEAB (3 x 50 ml). The reaction mixture was chromatographed on silica gel column (150 g, 3.5 x 32 cm) packed in CHCl₃. The product (yield 76%) was eluted with chloroform/methanol (98/2, v/v) and was homogeneous by thin layer chromatography; Rₑ 0.3 (F), Rₑ 0.4 (L).

Dinucleotide, d([MeOTr]pmbG-mbG)—An anhydrous pyridine solution (18 ml) of d(MeOTr)pmbG (1.86 mmol) and pyridinium d[pmbG(Ac)] (0.78 mmol) was reacted with TPS (7.44 mmol) for 5 hours at room temperature. After usual work up (termination and sodium hydroxide treatment of the reaction mixture by standard procedure), the reaction mixture was taken up in TEAB (150 ml, 0.2 M) and extracted first with ether (3 x 100 ml) and then with CHCl₃ (3 x 100 ml). Ether extract contained d[MeOTr]pmbG and chloroform extract contained d([MeOTr]pmbG-mbG) as judged by thin layer chromatography. Chloroform solution was concentrated in presence of pyridine and precipitated into anhydrous ether (1.5 liters). The product (yield 99%) was homogeneous on thin layer chromatography, Rₑ 0.6 (J) and had τ₂₀₀,₄₁₂ = 0.60.

Trinucleotide, d([MeOTr]pmbG-mbG-T)—An anhydrous pyridine solution (20 ml) of d([MeOTr]pmbG-mbG) (1.5 mmol) and pyridinium d[pT(Ac)] (3 mmol) was reacted with TPS (6.0 mmol) for 5 hours at room temperature. After termination and usual workup of the reaction mixture, the solution was concentrated and the residue was dissolved in TEAB (0.2 M, 100 ml). The dinucleotide, d([MeOTr]pmbG-mbG), was extracted with ethyl acetate/butanol-1 (3 x 100 ml, 9/1, v/v) from aqueous TEAB solution. The trinucleotide, d([MeOTr]mbG-mbG-T), was extracted from aqueous TEAB solution by dichloromethane/butanol-1 (4 x 50 ml, 8/2, v/v). The product was isolated (73% yield) after the usual drying and precipitation of its pyridine solution into ether (1 liter). The trinucleotide was homogeneous by thin layer chromatography, Rₑ 0.65 (H) and had τ₂₀₀,₄₁₂ = 0.72.

Tetranucleotide, d([MeOTr]mbG-mbG-T-mbG)—An anhydrous pyridine solution (3 ml) of d([MeOTr]mbG-mbG-T) (0.2 mmol) was isolated on silica gel column. The product was purified on silica gel column (150 g, 3.5 x 32 cm) packed in CHCl₃. The product (yield 90%) was homogeneous by thin layer chromatography, Rₑ 0.6 (J) and had τ₂₀₀,₄₁₂ = 0.60.
and concentration, the residue was dissolved in TEAB (0.2 M, 50 ml) and extracted with ethyl acetate (3 × 100 ml). The aqueous solution was further extracted with dichloromethane/butanol-1 (7/3, 3 × 50 ml, v/v). After removal of the solvent, the product was chromatographed on silica gel column (50 g). Elution of column with 7% water in acetonitrile to yield 8% of trinucleotide, d[(MeOTr)mbG-mbG-T]. The tetranucleotide (74% yield) was eluted with 12% water-acetonitrile and was judged homogeneous by thin layer chromatography.

**Pentanucleotide, d[(MeOTr)mbG-mbG-T-mgG-mbG]—The above tetranucleotide (0.14 mmol) and pyridinium d[pmbG-(Ac)] (0.7 mmol) was treated with TPS (1.4 mmol) in anhydrous pyridine (2 ml) for 5 hours at room temperature. After termination and usual work up, the residue was dissolved in TEAB (0.2 m, 50 ml) and extracted with ethyl acetate/hutanol-1 (8/2, v/v). The aqueous 0.2 M TEAB solution was diluted to 0.05 M TEAB and applied to a trityl-cellulose column (2 × 20 cm). The column was washed with 0.05 m TEAB containing 20% ethanol. After all of the nonmethoxytrityl-containing components were eluted off, the column was washed with 90% aqueous ethanol containing 0.05 m TEAB (500 ml). The eluates containing the methoxytrityl components were concentrated in the presence of pyridine followed by precipitation into ether. This mixture was further purified by silica gel (50 g) column chromatography as described above. The tetranucleotide (8%) was eluted with 8% aqueous acetonitrile and the pure pentanucleotide (56%) was eluted with 12% aqueous acetonitrile.

**Hexanucleotide, d[(MeOTr)mbG-mbG-T-mbG-mbG-mbG]—The above pentanucleotide (0.06 mmol) and pyridinium d[pmbG-(Ac)] (0.6 mmol) was reacted with TPS (0.60 mmol) in anhydrous pyridine (2 ml) for 5 hours at room temperature. After the usual workup, the solution (5% aqueous pyridine, 10% ethanol, and 0.05 M TEAB (200 ml)) was applied to an ECTEOLA-cellulose column (11 × 60 cm) pre-equilibrated with 0.05 M TEAB containing 5% ethanol. After washing with 0.05 M diethylammonium bicarbonate containing 10% ethanol (1 liter), a linear gradient was applied as shown in Fig. 2. Peak I contained protected hexanucleotide and Peak II contained the required hexanucleotide (56% yield).

**Heptanucleotide, d[(MeOTr)mbG-mbG-T-mbG-mbG-mbG-mbG]-An anhydrous pyridine solution (1.5 ml) of the heptanucleotide, d[(MeOTr)mbG-mbG-T-mbG-mbG-mbG-mbG] (24 µmol) and pyridinium d[pT-T-anC-anC(Ac)] (0.13 mmol) was allowed to react with TPS (0.65 m) for 5 hours at room temperature. After the usual workup and alkaline hydrolysis, one third of the solution was applied to an ECTEOLA-column (1.5 × 80 cm) in the presence of 2% pyridine, 10% ethanol, and 0.05 m TEAB (300 ml). After washing the column with 10% ethanol/0.05 m TEAB (1 liter), the column was eluted with the gradients shown in Fig. 4. Fractions of 8 ml was collected. Peak III contained a mixture of the unreacted heptanucleotide and the heptanucleotide, and Peak IV contained the desired heptanucleotide (yield 71%).

**Undecanucleotide, d[(MeOTr)mbG-mbG-T-mbG-mbG-mbG-mbG-mbG-mbG-T-T-anC-anC]-An anhydrous pyridine solution (1.5 ml) of the heptanucleotide, d[(MeOTr)mbG-mbG-T-mbG-mbG-mbG-mbG-mbG] (24 µmol) and pyridinium d[pT-T-anC-anC(Ac)] (0.60 mmol) was allowed to react with TPS (0.65 m) for 5 hours at room temperature. After the usual workup and alkaline hydrolysis, one third of the solution was applied to an ECTEOLA-column (1.5 × 80 cm) in the presence of 2% pyridine, 10% ethanol, and 0.05 m TEAB (300 ml). After washing the column with 10% ethanol/0.05 m TEAB (1 liter), the column was eluted with the gradients shown in Fig. 4. Fractions of 8 ml was collected. Peak III contained a mixture of the unreacted undecanucleotide and the heptanucleotide, and Peak IV contained the desired undecanucleotide, d[(MeOTr)-

![Fig. 2. Separation of the products formed in the condensation of the pentanucleotide, d[(MeOTr)mbG-mbG-T-mbG-mbG], with d[pmbG(Ac)] on ECTEOLA-cellulose column (1.5 × 80 cm). Peak I contained unreacted pentanucleotide and Peak II contained the required hexanucleotide.](http://www.jbc.org/)

![Fig. 3. Chromatography of the reaction product obtained in the condensation of the hexanucleotide, d[(MeOTr)mbG-mbG-T-mbG-mbG-mbG-mbG], with d(pmbG) on ECTEOLA-cellulose column (1.4 × 90 cm). Peak I contained the unreacted hexanucleotide and Peak II was the required heptanucleotide.](http://www.jbc.org/)

![Fig. 4. Separation of the products in the condensation of the heptanucleotide, d[(MeOTr)mbG-mbG-T-mbG-mbG-mbG-mbG-mbG], with the tetra block, d[pT-T-anC-anC(Ac)] on ECTEOLA column (1.5 × 80 cm). Peak I was the unreacted heptanucleotide, d[(MeOTr)mbG-mbG-T-T-anC-anC]; Peak II was the unreacted heptanucleotide, d[(MeOTr)mbG-mbG-T-mbG-mbG-mbG-mbG]; Peak III contained a mixture of the heptanucleotide and the undecanucleotide, while Peak IV contained the undecanucleotide, d[(MeOTr)mbG-mbG-T-mbG-mbG-mbG-mbG-mbG-mbG-T-T-anC-anC).](http://www.jbc.org/)
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mbG-mbG-T-mbG-mbG-mbG-mbG-T-T-C-C]. The undecanucleotide had the ratio \( t_{260}/t_{440} \) of 2.8 and \( t_{260}/t_{200} \) of 1.79. The overall yield of the undecanucleotide after precipitation from an excess of ether was 49%.

**Undecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C)**—The fully protected undecanucleotide (15 \( A_{260} \)) was first treated with concentrated ammonium hydroxide (1 ml) for 18 hours at 50° and then with the pyridine/acetic acid/water buffer (1/14/3), v/v at room temperature for 48 hours. The fully deprotected undecanucleotide was subjected to DE23 column chromatography in the presence of 7 M urea at 47°. The undecanucleotide in 7 M urea, 0.02 M Tris buffer, pH 7.8, and 0.002 M EDTA was heated at 90° for 2 min prior to applying onto the column. The elution pattern is shown in Fig. 5. Fractions of 2 ml were collected. The major peak (7.0 \( A_{260} \)) as indicated by the dotted lines was pooled and desalted on a Bio-Gel P-2 column.

**Rechromatography of Undecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C)**—The undecanucleotide from the previous DE23-urea column (Fig. 2) was subjected to rechromatography under the identical conditions in DE23 cellulose in the presence of 7 M urea described above. A symmetrical peak was not obtained (Fig. 6). After desalting on Bio-Gel P-2 column, 6.8 \( A_{260} \) units of the undecanucleotide was recovered. The ratio of the constituent nucleotides after snake venom degradation was as expected for the undecanucleotide (Table III).

**Tetranucleotide, d[panC-mbG-bza-mbG(Ac)]**—An anhydrous pyridine solution (20 ml) of the dinucleotide d[(CNEt)anC-mbG] (0.44 mmol) and d[bpzA-mbG(Ac)] (0.45 mmol) was treated with TPS (2.3 mmol) for 5 hours at room temperature. After the usual workup, the reaction mixture was chromatographed on a DEAE-cellulose column (bicarbonate form, 2.4 x 100 cm), pre-equilibrated with 0.05 M TEAB containing 10% ethanol. The elution pattern obtained is shown in Fig. 7. The Peak IV (Fractions 270 to 300) contained the desired tetranucleotide and the compound after deprotection with ammonia, was homogeneous in solvents C and D. The \( R_f \) values of the protected and unprotected compounds are given in Tables I and II. The yield of the protected tetranucleotide was 33%.

**Unprotected Tetranucleotide, d(pC-G-A-G)**—After treatment with ammonia, the tetranucleotide was purified by chromatography as described in Fig. 8.

**Unprotected Pentadecanucleotide, d(G-G-T-G-G-G-T-T-C-C-G-A-G)**—An anhydrous pyridine solution of the undecanucleotide d[(MeOTr)mbG-T-mbG-mbG-anC-anC] (2 \( \mu \)mol) and d[panC-mbG-bza-mbG(Ac)] (30 \( \mu \)mol) was treated with TPS (170 \( \mu \)mol) for 6 hours at room temperature. After the usual workup, the reaction mixture was chromatographed on a DEAE-cellulose (bicarbonate form, 1.7 x 100 cm), pre-equilibrated with 0.05 M TEAB containing 10% ethanol. After pyridine had been washed off with the same solvent, a linear salt gradient was started with 600 ml of 0.05 M TEAB containing 10% ethanol.
 containing 10% ethanol in the mixing vessel and 600 ml of 0.15 M TEAB containing 10% ethanol in the reservoir. After washing the column with 600 ml of 0.2 M TEAB containing 10% ethanol, the reaction mixture containing the unreacted undecanucleotide and the product, pentadecamer, was eluted with 0.3 M TEAB containing 50% ethanol and 1 M TEAB containing 40% ethanol, respectively. A total of 280 A260 units was obtained. After complete deprotection as described under “General Methods,” a portion of this reaction mixture (1.2 ml) was applied to a column of DEAE (chloride form, 0.6 x 90 cm) in the presence of 7 M urea.

Rechromatography of Pentadecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C-C-G-A-G).—The isolated pentadecamer from the previous DE23 urea column was subjected to rechromatography twice in DE23 column (chloride form, 0.6 x 90 cm) in the presence of 7 M urea. The conditions used and the elution pattern obtained are shown in Fig. 9. Peak IV (Fractions 125 to 180) as indicated by the dotted lines was pooled and desalted using a Bio-Gel P-2 column.

Rechromatography of Pentadecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C-C-G-A-G).—The isolated pentadecamer from the previous DE23 urea column was subjected to rechromatography twice in DE23 column (chloride form, 0.6 x 90 cm) in the presence of 7 M urea. The conditions used and the elution pattern obtained are shown in Fig. 9. Peak IV (Fractions 125 to 180) as indicated by the dotted lines was pooled and desalted using a Bio-Gel P-2 column.

Rechromatography of Pentadecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C-C-G-A-G).—The isolated pentadecamer from the previous DE23 urea column was subjected to rechromatography twice in DE23 column (chloride form, 0.6 x 90 cm) in the presence of 7 M urea. The conditions used and the elution pattern obtained are shown in Fig. 9. Peak IV (Fractions 125 to 180) as indicated by the dotted lines was pooled and desalted using a Bio-Gel P-2 column.

Rechromatography of Pentadecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C-C-G-A-G).—The isolated pentadecamer from the previous DE23 urea column was subjected to rechromatography twice in DE23 column (chloride form, 0.6 x 90 cm) in the presence of 7 M urea. The conditions used and the elution pattern obtained are shown in Fig. 9. Peak IV (Fractions 125 to 180) as indicated by the dotted lines was pooled and desalted using a Bio-Gel P-2 column.

Rechromatography of Pentadecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C-C-G-A-G).—The isolated pentadecamer from the previous DE23 urea column was subjected to rechromatography twice in DE23 column (chloride form, 0.6 x 90 cm) in the presence of 7 M urea. The conditions used and the elution pattern obtained are shown in Fig. 9. Peak IV (Fractions 125 to 180) as indicated by the dotted lines was pooled and desalted using a Bio-Gel P-2 column.

Rechromatography of Pentadecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C-C-G-A-G).—The isolated pentadecamer from the previous DE23 urea column was subjected to rechromatography twice in DE23 column (chloride form, 0.6 x 90 cm) in the presence of 7 M urea. The conditions used and the elution pattern obtained are shown in Fig. 9. Peak IV (Fractions 125 to 180) as indicated by the dotted lines was pooled and desalted using a Bio-Gel P-2 column.

Rechromatography of Pentadecanucleotide, d(G-G-T-G-G-G-G-T-T-C-C-C-G-A-G).—The isolated pentadecamer from the previous DE23 urea column was subjected to rechromatography twice in DE23 column (chloride form, 0.6 x 90 cm) in the presence of 7 M urea. The conditions used and the elution pattern obtained are shown in Fig. 9. Peak IV (Fractions 125 to 180) as indicated by the dotted lines was pooled and desalted using a Bio-Gel P-2 column.
peak I. Peak VI was the undecanucleotide.

was at 65° using a linear gradient of sodium chloride in 7 M urea and 0.02 M Tris hydrochloride (pH 8.0). Elution was at 65° using a linear gradient of sodium chloride in 7 M urea and 0.02 M Tris hydrochloride (pH 8.0). Nonnucleotidic material was in Peak I. Peak VI was the undecanucleotide.

Precipitation from dry ether) was determined spectrophotometrically and was found to be 84%.

Trinucleotide, d([MeOTr]bzA-T-T)—An anhydrous pyridine solution (15 ml) of d([MeOTr]bzA-T) (1.65 mmol) and d[pT(Ac)] (3.3 mmol) was allowed to react with TPS (8.25 mmol) for 4.5 hours at room temperature. After the usual workup, the reaction mixture was concentrated under vacuum, the residue was taken into TEAB (0.2 M) and unreacted dinucleotide was extracted with ethyl acetate. The product, d([MeOTr]bzA-T-T), was then extracted from the aqueous phase by dichloromethane/butanol-1 (7/3, v/v). The yield was 94%. The Rf values of the product are given in Table I.

Pentanucleotide, d([MeOTr]bzA-T-T-bzA-anC)—An anhydrous pyridine solution (17 ml) of d([MeOTr]bzA-T) (1.65 mmol) and d[panC-anC(Ac)] (3.4 mmol) was allowed to react with MS (9.25 mmol) for 2.5 hours at room temperature. After the usual workup, the reaction mixture was chromatographed on a DEAE-cellulose column (3.2 x 82 cm) pre-equilibrated with 0.05 M TEAB containing 10% ethanol. The chromatographic pattern is shown in Fig. 12. The purity of the compound in various peaks was checked by paper chromatography after concentrated ammonia treatment. Peak IV contained the desired pure pentanucleotide, d([MeOTr]bzA-T-T-bzA-anC). Peaks were pooled as shown by vertical broken lines.

Nonanucleotide, d([MeOTr]bzA-T-T-bzA-anC-anC-anC-anC-anC-anC-anC-anC-anC-anC)*—d[panC-anC(Ac)] was prepared using the TPSM method (10).

mbG-T)—An anhydrous pyridine solution (10 ml) of d([MeOTr]bzA-T-T-bzA-anC-anC-anC-anC-anC-anC-anC-anC-anC-anC-anC-anC-anC-anC-anC-anC-anC)* (0.067 mmol) and d[pmbG-T(Ac)] (1.22 mmol) was treated with TPS (4 mmol) for 5 hours at room temperature. The chromatographic pattern obtained is shown in Fig. 14.

Representative fractions from Peaks I to V were checked on paper chromatography, after treatment with concentrated aqueous ammonia solution along with the marker of the methoxytritylheptanucleotide, Peak IV contained the pure nonamer, Peak III contained mostly nonamer and some of the heptamer, whereas Peak II is mostly the heptanucleotide. The yield of the nonanucleotide was 40%.

Nonanucleotide, d(A-T-T-A-C-C-C-G-T)—A sample (160 A260) of the nonanucleotide (Peak IV, Fig. 14) was treated with concentrated ammonia (15 hours 50°) to remove N-protecting groups, followed by treatment with pyridine/acetic acid/water...
(1/14/3, v/v) at room temperature (88 hours) to effect detritylation. The fully unprotected nonanucleotide was subjected to DE-23 column chromatography in the presence of 7 M urea. The elution pattern is shown in Fig. 15.


Dinucleotide, d[(MeOTr)mbG-T-bzA]—d(MeOTr)mbG was synthesized as described above. An anhydrous pyridine solution (10 ml) of d[(MeOTr)mbG] (1.87 mmol) and d[pT(Ac)] (2.25 mmol) was allowed to react with TPS (4.5 mmol) for 5½ hours at room temperature. The reaction was stopped by cooling in a dry ice-ethanol bath with the addition of a pyridine solution of DIEA (8 ml of 1M) followed by water (8 ml). After overnight, the solution was neutralized by the addition of excess pyridine and treated with alkali for 6 min at 4°. The solution was neutralized by the addition of an excess pyridinium Dowex 50 resin (100 ml). The resulting total aqueous pyridine solution was made to 70% in pyridine and concentrated to 20 ml with the addition of excess pyridine, treated with alkali by the standard method, and the alkaline solution neutralized with pyridinium Dowex 50 ion exchange resin. The clear supernatant was made up to 70% in pyridine and concentrated to a gum. The latter was dissolved in 50 ml of 0.2 M TEAB and the unreacted d(MeOTr)mbG-T was extracted with ethyl acetate/butanol-1 (9/1, v/v; 4 × 300 ml). The tetranucleotide, d[(MeOTr)mbG-T-bzA-bzA], was extracted in turn with CH₂Cl₂/butanol-1 (4/1, v/v; 3 × 200 ml). The latter solutions were pooled, concentrated with excess pyridine to 50 ml and added dropwise to 1.2 ml of ethyl ether. The tetranucleotide (0.57 mmol) d[(MeOTr)mbG-T-bzA-bzA] was obtained in 44% yield. Its spectrum at neutral pH showed λmax at 262 nm with a shoulder at 275 nm; the ratio at 260/280 nm was 0.95.

Hexanucleotide, d[(MeOTr)mbG-T-bzA-bzA-T-G(T)]—An anhydrous pyridine solution (10 ml) of d[(MeOTr)mbG-T-bzA-bzA] (0.57 mmol) and d[pT-ibG(Ac)] (3 mmol) was allowed to react with TPS (10.28 mmol) for 31 hours at room temperature. The reaction was stopped by cooling in a dry ice-ethanol bath and by the addition of 1 mL pyridine solution (22 mL) of DIEA followed by water (22 ml). After 18 hours, the solution was concentrated to 20 ml with the addition of excess pyridine, treated with alkali by the standard method, and the alkaline solution neutralized with pyridinium Dowex 50 ion exchange resin. The clear supernatant was made up to 70% in pyridine and concentrated to a gum. The latter was dissolved in 50 ml of 0.2 M TEAB and the unreacted d(MeOTr)mbG-T was extracted with ethyl acetate/butanol-1 (9/1, v/v; 4 × 300 ml). The hexanucleotide, d[(MeOTr)mbG-T-bzA-bzA-T-G(T)], was separated by preparative thin-layer chromatography. Fractions 181 to 300 eluted (97% yield). The spectrum at neutral pH gave a λmax at 262 nm with a shoulder at 277 nm; the ratio at 260/280 nm was 0.95.
was 1.14 and the ratio at 260/472 nm was 1.31.

**Tetranucleotide, d[panC-T-T-T-T]**—An anhydrous pyridine solution (10 ml) of d[(CNEt)-mC-T] (1.15 mmol) and d[pT-T](Ac) (1.40 mmol) was treated with TPS (5.61 mmol) for 2.5 hours. After the usual workup, including aqueous pyridine, saponification with 1 N NaOH at room temperature for 25 min and neutralization with pyridinium Dowex 50, the solution was concentrated with excess pyridine to a gum. This was taken up in 1 liter of 0.05 M TEAB in 10% ethanol and applied to a DEAE-cellulose column (DE23, 3.2 x 82 cm, equilibrated with 0.05 M TEAB in 10% ethanol). The gradient was from 0.05 to 0.25 M TEAB in a total volume of 4 liters of 75% ethanol. Fractions of 10 ml were collected. The trityl-positive fractions were pooled, concentrated with excess pyridine to 15 ml, and precipitated into 400 ml of ethyl ether. Approximately 220 A\textsubscript{260} units of decanucleotide (18 \textmu mol) was obtained corresponding to a yield of 42%. The spectrum at neutral pH showed \(\lambda_{\text{max}}\) at 260/280 nm ratio of 1.32 and a 260/472 nm ratio of 2.10.

**Unprotected Decanucleotide, d(G-T-A-A-A-T-G-C-T-T-T)**—Three hundred A\textsubscript{260} units of the protected decanucleotide were deprotected in the usual way. Prior to applying the deprotected product to DEAE-cellulose, the mixture was passed through a Bio-Gel P-2 column (2 x 100 cm, 100 to 200 mesh, equilibrated with weak aqueous ammonia) to remove pyridine and other low molecular weight compounds. The Bio-Gel effluent containing the decanucleotide was concentrated to dryness (with 0.1 ml of 1 M Tris, pH 7.5, added to maintain the pH), taken up in 5 ml of 7 M urea + 0.2 M Tris, and applied to a DEAE-cellulose column (DE23, 0.7 x 84 cm equilibrated with 7 M urea, 0.05 M NaCl, and 0.02 M Tris, pH 7.5). A salt gradient from 0.05 to 0.50 M NaCl to 0.02 M Tris, pH 7.5 in 7 M urea (total volume 500 ml) was applied as shown in Fig. 18. Fractions 130 to 150 were pooled and applied to a Bio-Gel P-2 column (2 x 100 cm); 130 A\textsubscript{260} units of the decanucleotide were obtained. The spectrum at neutral pH showed a \(\lambda_{\text{max}}\) at 259 nm with a 260/280 nm ratio of 1.71.


**Dinucleotide, d[(MeOTr)mbG-bzA-mbG]**—The protected deoxyribonucleoside, 5'-O-methoxytrityl-N-benzoyldeoxyadenosine, d(MeOT)bzA, was prepared as previously described (11).

An anhydrous pyridine solution (15 ml) of d[(MeOT)bzA] (1.2 mmol) and pyridinium d(pmbG(Ac)) (1.44 mmol) was allowed to react with TPS (2.28 mmol) for 5 hours in the dark at room temperature. The reaction was terminated by cooling in an ethanol-dry ice bath followed by the addition of a pyridine solution (6 ml of 1 M of DIEA and then water (6 ml). After 18 hours, the mixture was concentrated to 10 ml, ethanol (10 ml) was added, and the solution was treated at 0°C for 5 min with 2 N NaOH (20 ml). After the standard workup, the combined filtrate and washings were concentrated (10 ml) under vacuum after dilution with pyridine to give a 70%
aqueous pyridine solution. The residue was taken up in 0.2 M TEAB (100 ml) and was extracted with ether (3 x 100 ml) to remove the trace amount of unreacted d(MeOTr)bzA (silica gel thin layer chromatography in 8% water/acetonitrile mixture, Rf 0.8). The desired dinucleotide, d[(MeOTr)bzA-mbG] (silica gel thin layer chromatography in 8% water/acetonitrile, Rf 0.2; 15% water/acetonitrile, Rf 0.5), was isolated by extraction with a mixture of butanol-1 ethyl acetate (2/8, v/v; 3 x 50 ml). Pyridine (150 ml) was added to the combined organic extracts, the solution evaporated under vacuum and dried. The residue was taken up in anhydrous pyridine (50 ml) and precipitated from an excess of anhydrous ether (2000 ml). The Rf values of the protected and unprotected compounds are given in Tables I and II. The yield as determined spectrophotometrically was 91%.

**Trimucleotide, d[(MeOTr)bzA-mbG-T]**—An anhydrous pyridine solution (10 ml) of d[(MeOTr)bzA-mbG] (0.8 mmol) and d[pT(Ac)] (1.2 mmol) was allowed to react with TPS (2.4 mmol) for 5 hours at room temperature. After the usual workup and alkaline hydrolysis, the residue after evaporation was taken up in 0.2 M TEAB (100 ml). The unreacted dinucleotide, d[(MeOTr)bzA-mbG] was removed by extraction with a mixture of butanol-1/ethyl acetate (1/9, v/v, 4 x 100 ml). The trimucleotide d[(MeOTr)bzA-mbG-T] (silica gel thin layer chromatography 15% water/acetonitrile, Rf 0.25) was isolated by extraction with a mixture of butanol-1/dichloromethane (4/6, v/v, 4 x 50 ml). After evaporation of the solvents and drying, the residue was taken up in anhydrous pyridine (30 ml) and precipitated from ether (1500 ml). The Rf values of the protected and unprotected compound are given in Tables I and II. The yield as determined spectrophotometrically was 69%.

**Tetranucleotide, d[(MeOTr)bzA-mbG-T-bzA]**—An anhydrous pyridine solution (6 ml) of d[(MeOTr)bzA-mbG-T] (0.5 mmol) and d[pbzA(Ac)] (1.5 mmol) was allowed to react with TPS (5 mmol) for 5 hours at room temperature. After the usual workup and alkaline hydrolysis, the residue after evaporation was taken up into 0.2 M TEAB (100 ml). The trimucleotide d[(MeOTr)bzA-mbG-T-bzA] was removed by extraction with a mixture of butanol-1 ethyl acetate (3/7, v/v, 2 x 50 ml). After evaporation of the solvents and drying, the compound was precipitated from a mixture of 20 ml of pyridine and 1000 ml of anhydrous ether. The Rf values of the protected and unprotected compounds are given in Tables I and II. The yield as determined spectrophotometrically was 76%.

**Pentanucleotide, d[(MeOTr)bzA-mbG-T-bzA-bzA]**—An anhydrous pyridine solution (6 ml) of d[(MeOTr)bzA-mbG-T-bzA] (0.3 mmol) and d[pbzA(Ac)] (1.5 mmol) was allowed to react with TPS (3.0 mmol) for 5 hours at room temperature. After the usual workup, the mixture was evaporated to a gum and was taken up in 0.2 M TEAB (50 ml). Both the unreacted d[(MeOTr)bzA-mbG-T-bzA] and the product, d[(MeOTr)bzA-mbG-T-bzA-bzA(Ac)], were extracted into a mixture of butanol-1/dichloromethane (4/6, v/v, 3 x 100 ml). After backwashing of the combined organic phase with 0.2 M TEAB (2 x 50 ml), pyridine was added, evaporated, and hydrolyzed with NaOH by the usual method. The compounds in a 2% pyridine, 0.05 M TEAB, and 10% ethanol mixture (500 ml) were applied to an ECTEOLA column (2.5 x 80 ml) pre-equilibrated with 0.05 M TEAB and 10% ethanol. After washing with more of the 0.05 M TEAB and 10% ethanol mixture (2000 ml), which removed the pyridine and all the nonnucleotide material, an ethanol gradient followed by a salt gradient was applied as shown in Fig. 19. Fractions of 10 ml were collected. Peak I contained mainly the tetranucleotide d[(MeOTr)bzA-mbG-T-bzA], Peak II contained the pentanucleotide d[(MeOTr)bzA-mbG-T-bzA-bzA(Ac)], which was homogeneous on silica gel thin layer chromatography (20% water/acetonitrile, Rf 0.6). The mobilities on paper chromatography are shown in Tables I and II. The yield of the pentanucleotide after precipitation was 41%, somewhat lower than expected because of low recovery from a newly packed column.

**Heptanucleotide, d[(MeOTr)bzA-mbG-T-bzA-bzA-bzA-bzA]**—An anhydrous pyridine solution (4 ml) of d[(MeOTr)bzA-mbG-T-bzA-bzA-bzA-bzA] (100 pmol) and pyridinium d[pbzA-bzA(Ac)] (806 pmol) was allowed to react with TPS
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(2.4 mmol) for 5 hours at room temperature. After the usual workup and alkaline hydrolysis, the reaction mixture, in 2% pyridine, 0.05 M TEAB, and 10% ethanol, was applied to a DEAE-column (2.5 x 80 cm) pre-equilibrated with 0.05 M TEAB and 10% ethanol. After washing with more of 0.05 M TEAB and 10% ethanol (2000 ml), the column was eluted with the gradients shown in Fig. 20. Fractions of 8 ml were collected. Peak II contained the pentanucleotide d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA]. Peaks III and IV contained pure heptanucleotide, d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA-bZA-bZA], as shown by paper chromatography after N-deprotection, and by chromatography of the fully deprotected sample from Peak III and Peak IV on DE23 column using 7 M urea as eluant. The gradient used is shown in Fig. 21. The main peak was desalted (Bio-Gel P-2). The product was analyzed for constituent nucleotides by venom phosphodiesterase degradation, the result is shown in Table III. The yield of the heptanucleotide was 62% as determined spectroscopically after precipitation.

Nonanucleotide, d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA-mbG-anC]—An anhydrous pyridine solution (1.5 ml) of the heptanucleotide d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA-bZA-bZA] (24 pmol, 1600 A260) and pyridinium d[pmbG-bZA(Ac)] (0.3 mmol) was allowed to react with TPS (0.75 mmol) for 5 hours at room temperature. After the usual workup and alkaline hydrolysis, the reaction mixture was applied to a DEAE-cellulose column (25 x 80 cm) pre-equilibrated with 0.05 M TEAB and 10% ethanol. The column was first washed with 0.05 M TEAB and 10% ethanol (2000 ml) followed by the gradients shown in Fig. 22. Fractions of 8 ml were collected. Peak III was shown to contain pure nonanucleotide d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA-bZA-bZA-bZA-mbG-anC]. The Rf values of the protected and unprotected compounds are shown in Tables I and II. The yield of the

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**Fig. 20.** Separation of the products in the condensation of the pentanucleotide, d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA], with the dinucleotide, d[pbZA-bZA(Ac)], on a DEAE-cellulose column (2.5 x 80 cm). Peak I, dinucleotide d[pbZA-bZA]; Peak II, pentanucleotide d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA]; Peak III and Peak IV, the heptanucleotide d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA-bZA-bZA-bZA].

**Fig. 21.** Urea column chromatography of the heptanucleotide, dA-G-T-A-A-A-A, on a DE23 cellulose column (0.5 x 100 cm) in 7 M urea. The gradient of sodium chloride was as shown in the figure. The main peak as shown by the dotted lines contained the pure heptanucleotide.

**Fig. 22.** Separation of the products in the condensation of the heptanucleotide, d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA-bZA-bZA], with the dinucleotide, d[pmbG-anC(Ac)], on a DEAE-cellulose column (2.5 x 80 cm). Peak I, dinucleotide d[pmbG-anC]; Peak II, heptanucleotide d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA-bZA-bZA-bZA]; Peak III, nonanucleotide d[(MeOTr)bZA-mbG-T-bZA-bZA-bZA-bZA-bZA-mbG-anC].

**Fig. 23.** Urea column chromatography of the nonanucleotide, dA-G-T-A-A-A-A-G-C, on a DE23 cellulose column (0.5 x 100 cm) in 7 M urea.
nonanucleotide was 48% after precipitation as determined spectroscopically.

Nonanucleotide, \(d(A-G-T-A-A-A-A-G-C)\)—The protected nonanucleotide from Peak III of Fig. 4 (80 \(A_{260}\)) was successively treated with concentrated ammonium hydroxide and then pyridine/acetic acid/water (1/14/3, v/v) at room temperature for 48 hours. The fully unprotected nonanucleotide was subjected to DE23 column chromatography in the presence of 7 M urea. The elution pattern is shown in Fig. 23. The major peak contained 32 \(A_{260}\). Salt and urea were removed by Bio-Gel P-2 as described under “General Methods.” The ratio of the constituent nucleotides after snake venom degradation was as expected for the nonanucleotide (Table III).

REFERENCES
Total synthesis of the structural gene for the precursor of a tyrosine suppressor transfer RNA from Escherichia coli. 5. Synthesis of the deoxyribopolynucleotide segments representing the nucleotide sequence 71-103.

E Jay, P J Cashion, M Fridkin, B Ramamoorthy, K L Agarwal, M H Caruthers and H G Khorana