

# Studies on Polynucleotides. LXV.<sup>1</sup> The Synthesis of Deoxyribopolynucleotides Containing Repeating Tetranucleotide Sequences (2)<sup>2</sup>

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**Abstract:** Syntheses of the following three deoxyribopolynucleotides containing repeating tetranucleotide sequences have been accomplished: (1) a dodecanucleotide containing the repeating sequence thymidyldeoxyadenylthymidyldeoxycytidyl, (2) an octanucleotide containing the repeating sequence thymidyldeoxyadenyldeoxyguanylyldeoxyadenyl, and (3) another octanucleotide containing the repeating sequence thymidyldeoxyadenyldeoxyadenyldeoxyguanylyl. The general procedure used involved the stepwise condensation of preformed protected dinucleotides with the 3'-hydroxyl end of growing deoxyoligonucleotide chains. The starting blocks containing 3'-hydroxyl end groups were 5'-O-monomethoxytrityl- or 5'-dimethoxytritylthymidyl-(3'→5')-N-benzoyldeoxyadenosine (d-MMTr-TpA<sup>Bz</sup> or d-DMTr-TpA<sup>Bz</sup>). The protected dinucleotide blocks containing 5'-phosphomonoester groups were: 5'-O-phosphorylthymidyl-(3'→5')-N-anisoyl-3'-O-acetyldeoxycytidine (d-pTpC<sup>An</sup>-OAc), 5'-O-phosphorylthymidyl-(3'→5')-N-benzoyl-3'-O-acetyldeoxyadenosine (d-pTpA<sup>Bz</sup>-OAc), 5'-O-phosphoryl-N-acetyldeoxyguanylyl-(3'→5')-N-benzoyl-3'-O-acetyldeoxyadenosine (d-pG<sup>Ac</sup>pA<sup>Bz</sup>-OAc), and 5'-O-phosphoryl-N-benzoyldeoxyadenyl-(3'→5')-N-benzoyl-3'-O-acetyldeoxyguanosine (d-pA<sup>Bz</sup>pG<sup>Bz</sup>-OAc). An increasing excess of the protected dinucleotide with an increase in the chain length of the deoxyribopolynucleotides was used. 2,4,6-Triisopropylbenzenesulfonyl chloride was used as the condensing agent. After each condensation step, the terminal 3'-O-acetyl group was selectively removed from the protected oligo- or polynucleotides by a mild alkaline treatment, and the latter products were purified by chromatography on DEAE-cellulose, anion-exchange columns.

As discussed in an accompanying paper,<sup>4</sup> ribopolynucleotides containing repeating tetranucleotide sequences are of potential interest for further studies of several aspects of the genetic code and of protein biosynthesis. The general method developed for the preparation of ribopolynucleotides with repeating nucleotide sequences requires the chemical synthesis of complementary sets of short chain deoxyribopolynucleotides.<sup>1,4</sup> The present paper records chemical syntheses of a dodecanucleotide containing the repeating sequence thymidyldeoxyadenylthymidyldeoxycytidyl, and of two octanucleotides containing the sequences thymidyldeoxyadenyldeoxyguanylyldeoxyadenyl and thymidyldeoxyadenyldeoxyadenyldeoxyguanylyl. The present work and the synthesis of the hexadecanucleotide described in the succeeding paper<sup>5</sup> complete the preparation of two sets of complementary deoxyribopolynucleotides. Both sets have been shown to serve as templates for the DNA polymerase of *Escherichia coli*, and the products have been characterized as DNA-like polymers containing the repeating tetranucleotide sequences originally present in the short-chain templates.<sup>6</sup> Brief reports of the present work have already appeared.<sup>7,8</sup>

The general approach used in the present work has been that of stepwise synthesis involving condensation of suitably protected dinucleotides with the 3'-hydroxyl ends of growing oligonucleotide chains. This approach was recently studied for the synthesis of thymidine oligonucleotides and gave encouraging results.<sup>9</sup> It was considered that the use of this approach in the synthesis of deoxyribopolynucleotides containing different nucleotides would provide further information of fundamental interest in synthetic work, in addition to making available deoxyribopolynucleotides of interest in enzymatic work.

Deoxyribopolynucleotides, whose synthesis formed the subject of the present work, all contained a free 5'-hydroxyl group at one end and a free 3'-hydroxyl group at the opposite end. Synthesis of such a polynucleotide containing a repeating tetranucleotide sequence required, as starting materials, a protected dinucleoside phosphate containing a free 3'-hydroxyl group and two protected dinucleotides containing 5'-phosphomonoester groups. The protected dinucleoside phosphates used were 5'-O-monomethoxytritylthymidyl-(3'→5')-N-benzoyldeoxyadenosine (d-MMTr-TpA<sup>Bz</sup>)<sup>10</sup> and the corresponding 5'-O-dimethoxytrityl derivative (d-DMTr-TpA<sup>Bz</sup>).<sup>10</sup> As described previously,<sup>11</sup> these compounds were prepared by the condensation of 5'-O-methoxytritylthymidine with 3'-O-

(1) Preceding paper in this series: T. M. Jacob, S. A. Narang, and H. G. Khorana, *J. Am. Chem. Soc.*, **89**, 2177 (1967).

(2) This work has been supported by grants from the National Science Foundation (Grant No. GB-3342), the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service (Grant No. CA-05178), and the Life Insurance Medical Research Fund (Grant No. G-62-54).

(3) H. K. wishes to acknowledge the receipt of a postdoctoral fellowship (1964-1966) from the Deutscher Akademischer Austauschdienst, Bad Godesberg, Germany.

(4) H. G. Khorana, H. Büchi, T. M. Jacob, H. Kössel, S. A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, **89**, 2154 (1967).

(5) E. Ohtsuka and H. G. Khorana, *ibid.*, **89**, 2195 (1967).

(6) R. D. Wells, H. Büchi, H. Kössel, and H. G. Khorana, *J. Mol. Biol.*, in press.

(7) R. D. Wells, T. M. Jacob, H. R. Kössel, A. R. Morgan, S. A.

Narang, E. Ohtsuka, and H. G. Khorana, *Federation Proc.*, **25**, 404 (1966).

(8) H. G. Khorana, H. Büchi, H. Ghosh, N. Gupta, T. M. Jacob, H. Kössel, R. Morgan, S. A. Narang, E. Ohtsuka, and R. D. Wells, *Cold Spring Harbor Symp. Quant. Biol.* (June 1966) in press.

(9) H. Kössel, M. W. Moon, and H. G. Khorana, *J. Am. Chem. Soc.*, **89**, 2148 (1967).

(10) The system of abbreviations is as has been described and used in the preceding papers.

(11) See, e.g., G. Weimann, H. Schaller, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3835 (1963).

Table I. Components, Products, and Yields of Condensations by Addition of Dinucleotide Blocks

3'-Hydroxyl-containing oligonucleotide component	Amount, $\mu$ moles	Dinucleotide component	Amount, $\mu$ moles	TPS, $\mu$ moles	Products	Purified product yield, %
Synthesis of the Protected Dodecanucleotide						
d-MMTr-TpA <sup>Bz</sup>	158	d-pTpC <sup>An</sup> -OAc	314	1250	d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup>	45
d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup>	71.6	d-pTpA <sup>Bz</sup> -OAc	250	1000	d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup>	47
d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup>	20	d-pTpC <sup>An</sup> -OAc	120	720	d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup>	45
d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup>	13	d-pTpC <sup>An</sup> -OAc	78	400	d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup>	42
d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup>	7.1	d-pTpA <sup>Bz</sup> -OAc	60	240	d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup>	47
d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup>	3.0	d-pTpC <sup>An</sup> -OAc	30	120	d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup>	33
Synthesis of the Protected Octanucleotide						
d-MMTr-TpA <sup>Bz</sup>	650	d-pG <sup>Ac</sup> pA <sup>Bz</sup> -OAc	660	2650	d-MMTr-TpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup> pTpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup>	25
d-MMTr-TpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup>	62	d-pTpA <sup>Bz</sup> -OAc	250	1000	d-MMTr-TpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup> pTpA <sup>Bz</sup>	25
d-MMTr-TpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup> pTpA <sup>Bz</sup>	1.9	d-pG <sup>Ac</sup> pA <sup>Bz</sup> -OAc	17	70	d-MMTr-TpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup> pTpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup>	35
Synthesis of the Protected Octanucleotide						
d-DMTr-TpA <sup>Bz</sup>	48	d-pA <sup>Bz</sup> pG <sup>Bz</sup> -OAc	97	194	d-DMTr-TpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Bz</sup> pTpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Bz</sup>	22
d-DMTr-TpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Bz</sup>	25	d-pTpA <sup>Bz</sup> -OAc	102	400	d-DMTr-TpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Bz</sup> pTpA <sup>Bz</sup>	54
d-DMTr-TpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Bz</sup> pTpA <sup>Bz</sup>	11	d-pA <sup>Bz</sup> pG <sup>Bz</sup> -OAc	205	410	d-DMTr-TpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Bz</sup> pTpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Bz</sup>	40

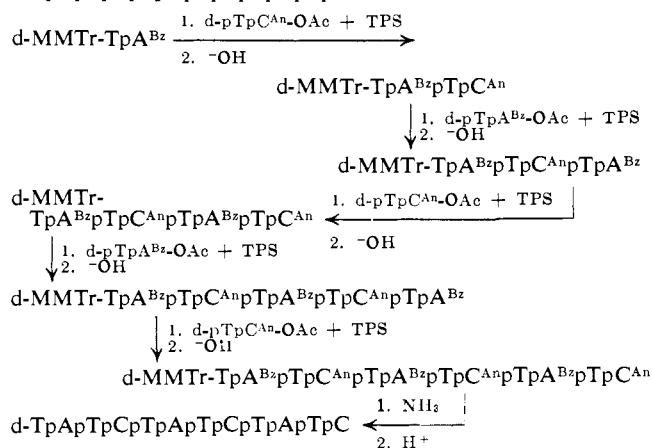
acetyl-N-benzoyldeoxyadenosine 5'-phosphate in the presence of dicyclohexylcarbodiimide followed by the removal of the 3'-O-acetyl group. The protected dinucleotide blocks used were 5'-O-phosphorylthymidyl-(3'→5')-N-anisoyl-3'-O-acetyldeoxycytidine (d-pTpC<sup>An</sup>-OAc),<sup>10</sup> 5'-O-phosphorylthymidyl-(3'→5')-N-benzoyl-3'-O-acetyldeoxyadenosine (d-pTpA<sup>Bz</sup>-OAc), 5'-O-phosphoryl-N-acetyldeoxyguanylyl-(3'→5')-N-benzoyl-3'-O-acetyldeoxyadenosine (d-pG<sup>Ac</sup>pA<sup>Bz</sup>-OAc), and 5'-O-phosphoryl-N-benzoyldeoxyadenylyl-(3'→5')-N-benzoyl-3'-O-acetyldeoxyguanosine (d-pA<sup>Bz</sup>pG<sup>Bz</sup>-OAc). The general method used in the synthesis of all of the dinucleotides was as has been developed and used extensively in previous work.<sup>12</sup> Thus, for example, the synthesis of d-pTpC<sup>An</sup>-OAc involved (1) the condensation of the cyanoethyl ester of d-pT with the protected mononucleotide d-pC<sup>An</sup>-OAc, (2) an alkaline treatment which removed the cyanoethyl and 3'-O-acetyl groups, and (3) reacetylation of the terminal 3'-OH group.

The condensing agent used in all of the present work was triisopropylbenzenesulfonyl chloride (TPS).<sup>13</sup> An increasing excess of the incoming dinucleotide component was used with an increase in the chain length of the oligonucleotide component bearing the 3'-hydroxyl end group. In this way, reasonable yields were obtained with respect to the latter component at successive condensation steps. A summary of the pertinent reaction conditions used in different syntheses is shown in Table I.

**Synthesis of the Dodecanucleotide d-TpApTpCpTpApTpCpTpApTpC.** The steps used in the synthesis of the dodecanucleotide are shown in Chart I. After each step, the products were separated by anion-exchange chromatography on DEAE-cellulose columns. The elution patterns obtained are shown in Figures 1-5. While the tetranucleotide, d-MMTr-TpA<sup>Bz</sup>pTpC<sup>An</sup>, emerged as a broad peak (Figure 1) from the column (evidently due to lower alcohol concentration in the

eluting buffer), higher protected oligonucleotides (Figures 2-5) appeared as rather sharp peaks. The desired products as obtained directly from the columns were pure except for the dodecanucleotide which necessitated

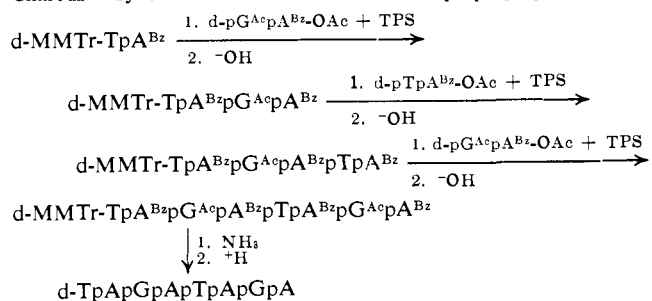
Chart I. Stepwise Synthesis of the Dodecanucleotide d-TpApTpCpTpApTpCpTpApTpC



further purification by paper chromatography after removal of the protecting groups (see Experimental Section). The yields of the pure products at various synthetic steps were in the range 33-54% (Table I).

**Synthesis of the Octanucleotide d-TpApGpApTpApGpA.** The steps used for the synthesis of this octanucleotide are shown in Chart II. While, as seen in

Chart II. Synthesis of the Octanucleotide d-TpApGpApTpApGpA



(12) See, for example, E. Ohtsuka, M. W. Moon, and H. G. Khorana, *J. Am. Chem. Soc.*, **87**, 2956 (1965); S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, **89**, 2158 (1967).

(13) R. Lohrmann and H. G. Khorana, *ibid.*, **88**, 829 (1966).

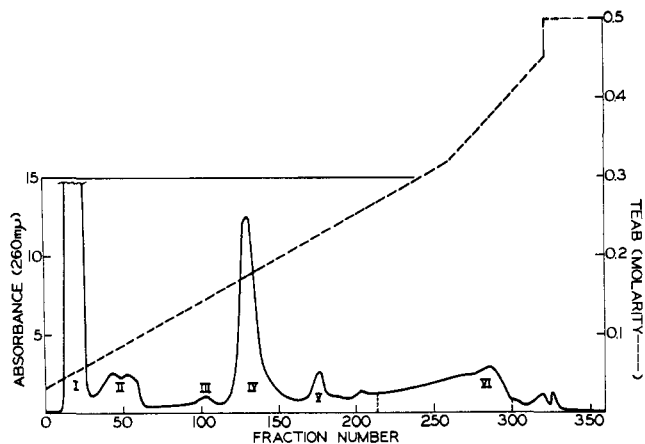


Figure 1. Chromatography of the reaction products in the preparation of the tetranucleotide  $d\text{-MMTr-TpA}^{Bz}_p\text{TpC}^{An}$  on a DEAE-cellulose (carbonate form) column ( $2.4 \times 55$  cm) by using a linear gradient of triethylammonium bicarbonate with 3 l. of 0.03  $M$  TEAB in the mixing vessel and 3 l. of 0.40  $M$  TEAB in the reservoir. All solutions contained 20% ethyl alcohol. Fractions of  $\sim 18$  ml were collected every 15 min. At fraction 260, the gradient was steepened by using 0.5 l. of 0.32  $M$  TEAB-20% ethyl alcohol in the mixing vessel and 0.5 l. of 0.5  $M$  TEAB-50% ethyl alcohol in the reservoir. At fraction 322 elution was continued without a gradient using 0.5  $N$  TEAB-50% ethyl alcohol. Peak VI contained the desired tetranucleotide.

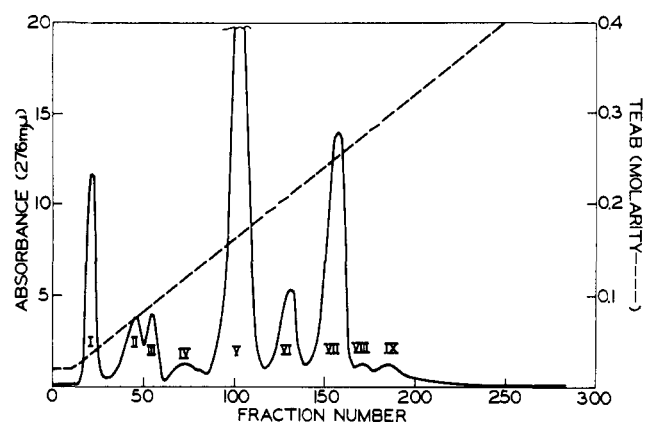


Figure 2. Chromatography of the reaction products in the preparation of the hexanucleotide  $d\text{-MMTr-TpA}^{Bz}_p\text{TpC}^{Anp}\text{TpA}^{Bz}$  on a DEAE-cellulose (carbonate form) column ( $2.4 \times 60$  cm) by using a linear gradient of triethylammonium bicarbonate with 2.5 l. of 0.01  $M$  TEAB-40% ethyl alcohol in the mixing vessel and 2.5 l. of 0.50  $M$  TEAB-50% ethyl alcohol in the reservoir. Fractions of  $\sim 18$  ml were collected every 15 min. Peak VII contained the desired hexanucleotide.

Table I, the general principles used in the condensation reactions were similar to those used above, the yields of the pure products were much lower (20–30%) than those obtained in the series described above. Further, as seen from the elution patterns obtained on column chromatography (Figures 6–8), there was increased formation of side products in this series. It seems possible that the side products were partly due to some loss of *N*-benzoyladenine (depurination) during aqueous pyridine treatment after the condensation reactions.<sup>14</sup> Another probable factor appears to be the partial loss of the *N*-acetyl group on the guanine ring during work-up and/or column chromatography.<sup>15</sup> Thus, for

(14) Cf. S. A. Narang, T. M. Jacob, and H. G. Khorana, *J. Am. Chem. Soc.*, **89**, 2167 (1967).

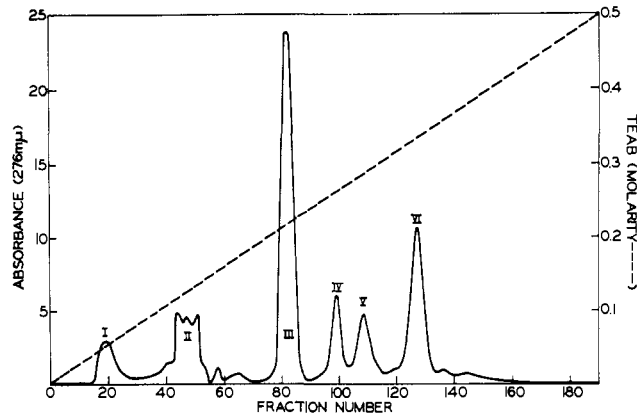


Figure 3. Chromatography of the reaction products in the preparation of the octanucleotide  $d\text{-MMTr-TpA}^{Bz}_p\text{TpC}^{Anp}\text{TpA}^{Bz}_p\text{TpC}^{An}$  on a DEAE-cellulose (carbonate form) column ( $2.3 \times 44$  cm) by a linear gradient of triethylammonium bicarbonate using 1.5 l. of 0.01  $M$  TEAB-40% ethyl alcohol in the mixing vessel and 1.5 l. of 0.50  $M$  TEAB-50% ethyl alcohol in the reservoir. Fractions of 15 ml were collected every 10 min. Peak VI contained the desired octanucleotide.

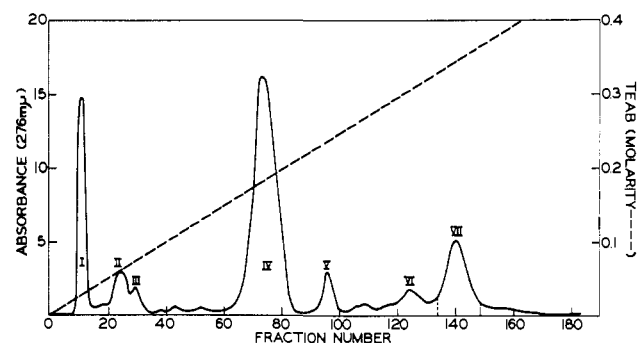


Figure 4. Chromatography of the reaction products in the preparation of the decanucleotide  $d\text{-MMTr-TpA}^{Bz}_p\text{TpC}^{Anp}\text{TpA}^{Bz}_p\text{TpC}^{Anp}\text{TpA}^{Bz}$  on a DEAE-cellulose (carbonate form) column ( $1 \times 50$  cm) by a linear gradient of triethylammonium bicarbonate using 1 l. of 0.01  $M$  TEAB-40% ethyl alcohol in the mixing vessel and 1 l. of 0.5  $M$  TEAB-50% ethyl alcohol in the reservoir. Fractions of  $\sim 10$  ml were collected every 15 min. Peak VII contained the desired decanucleotide.

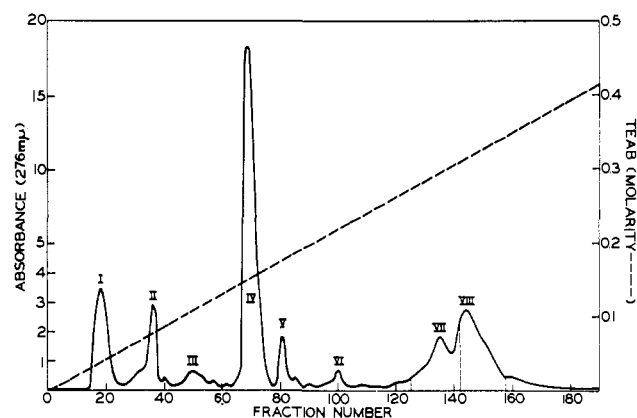


Figure 5. Chromatography of the reaction products in the preparation of the dodecanucleotide  $d\text{-MMTr-TpA}^{Bz}_p\text{TpC}^{Anp}\text{TpA}^{Bz}_p\text{TpC}^{Anp}\text{TpA}^{Bz}_p\text{TpC}^{Anp}\text{TpA}^{Bz}_p\text{TpC}^{An}$  on a DEAE-cellulose (carbonate form) column ( $0.7 \times 40$  cm) by a linear gradient of triethylammonium bicarbonate using 800 ml of 40% ethyl alcohol in the mixing vessel and 800 ml of 0.5  $M$  TEAB-50% ethyl alcohol in the reservoir. Fractions of  $\sim 8$  ml were collected every 10 min. Peak VIII contained the desired dodecanucleotide.

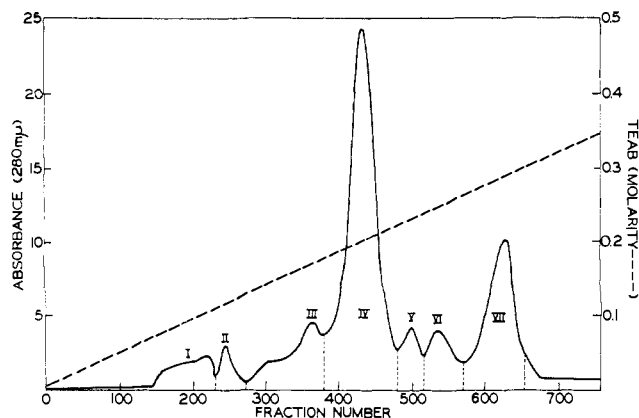


Figure 6. Chromatography of the reaction products in the preparation of the tetranucleotide  $d\text{-MMTr-TpA}^{Bz}pG^{Ac}pA^{Bz}$  on a DEAE-cellulose (carbonate form) column (1600 ml). After washing the column with 2.5 l. of 20% aqueous ethyl alcohol to remove pyridine a linear gradient of triethylammonium bicarbonate was started with 4 l. of 20% aqueous ethyl alcohol in the mixing vessel and 4 l. of 0.2 *M* TEAB-30% ethyl alcohol in the reservoir. Then the gradient was continued using 4 l. of 0.2 *M* TEAB-30% ethyl alcohol in the mixing vessel and 4 l. of 0.4 *M* TEAB-40% ethyl alcohol in the reservoir. Fractions of  $\sim 20$  ml were collected every 15 min. Peak VII contained the desired tetranucleotide.

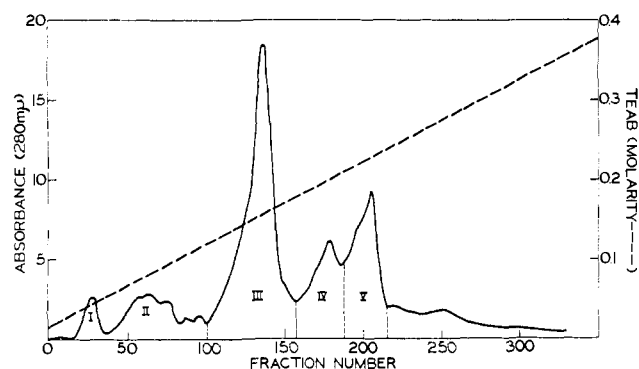


Figure 7. Chromatography of the reaction products in the preparation of the hexanucleotide  $d\text{-MMTr-TpA}^{Bz}pG^{Ac}pA^{Bz}pTpA^{Bz}$  on a DEAE-cellulose (carbonate form) column ( $2.4 \times 60$  cm) by a linear gradient of triethylammonium bicarbonate using 2.5 l. of 0.01 *M* TEAB-40% ethyl alcohol in the mixing vessel and 2.5 l. of 0.50 *M* TEAB-50% ethyl alcohol in the reservoir. Fractions of  $\sim 15$  ml were collected every 15 min. Peak V contained the desired hexanucleotide.

example, peak VI of Figure 6 contained as a major (60%) product a trityl-containing compound which was characterized as  $d\text{-MMTr-TpA}^{Bz}pGpA^{Bz}$ . Thus, its mobility (0.62) on chromatography in solvent B was slightly less than that of  $d\text{-MMTr-TpA}^{Bz}pG^{Ac}pA^{Bz}$  (0.63). Both products gave, after removal of the *N*-protecting groups, the same product,  $d\text{-MMTr-TpApGpA}$  (identical mobility of 0.15 in solvent A). Again, acidic removal of the methoxytrityl group gave, in both cases, the tetranucleotide,  $d\text{-TpApGpA}$ , which was characterized by chromatography and by analysis of products obtained on degradation by spleen and venom phosphodiesterase.

While the protected tetranucleotide,  $d\text{-MMTr-TpA}^{Bz}pG^{Ac}pA^{Bz}$ , obtained by column chromatography (Figure

(15) Partial loss of the *N*-acetyl group was also observed in the preparation of  $d\text{-pG}^{Ac}pA^{Bz}$  and during the recovery of the unreacted  $d\text{-pA}^{Bz}pG^{Ac}$  in the condensation of the latter with the hexanucleotide  $d\text{-DMTr-(pA}^{Bz})_2pG^{Bz}pTpA^{Bz}$  (see Experimental Section).

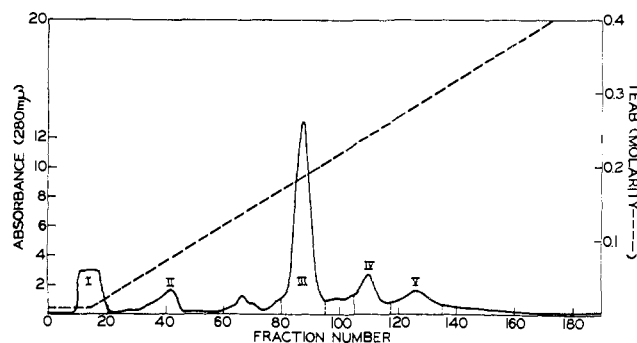


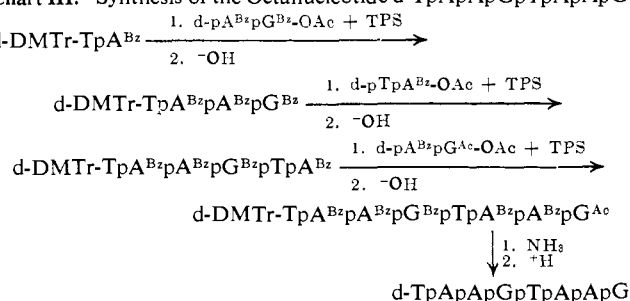
Figure 8. Chromatography of the reaction products in the preparation of the octanucleotide  $d\text{-MMTr-TpA}^{Bz}pG^{Ac}pA^{Bz}pTpA^{Bz}pG^{Ac}pA^{Bz}$  on a DEAE-cellulose (carbonate form) column (40 ml) by a linear gradient of triethylammonium bicarbonate using 500 ml of 0.01 *M* TEAB-40% ethyl alcohol in the mixing vessel and 500 ml of 0.50 *M* TEAB-50% ethyl alcohol in the reservoir. Fractions of 5 ml were collected every 12 min. Peak V contained the desired octanucleotide.

6) was pure, the protected hexanucleotide obtained after similar chromatography (Figure 7) contained considerable amount of impurities and for characterization it was purified by paper chromatography as described in the Experimental Section. However, the protected hexanucleotide as obtained after column chromatography was used directly in the next condensation step. The protected octanucleotide obtained after chromatography (peak V, Figure 8) was purified by paper chromatography after successive removal of the protecting groups (see Experimental Section).

Synthesis in this series was not taken beyond the octanucleotide stage because the octanucleotide in combination with the complementary dodecanucleotide above described served very well as template for the DNA polymerase catalyzed reaction.

**Synthesis of the Octanucleotide  $d\text{-TpApApGpTpApApG}$ .** The reaction sequence used is shown in Chart III.  $d\text{-DMTr-TpA}^{Bz}$  was used in place of  $d\text{-$

Chart III. Synthesis of the Octanucleotide  $d\text{-TpApApGpTpApApG}$



$\text{MMTr-TpA}^{Bz}$  as one of the starting components in order to eliminate any depurination during the acidic removal of the methoxytrityl group. Furthermore, in view of the above-described experience the more stable benzoyl group was used in place of the acetyl group for the protection of the guanine ring. Thus, the protected dinucleotide  $d\text{-pA}^{Bz}pG^{Bz}\text{-OAc}$  was used for synthesis of the protected tetranucleotide from  $d\text{-DMTr-TpA}^{Bz}$ . However, in the last step (hexanucleotide to octanucleotide) the protected dinucleotide ( $d\text{-pA}^{Bz}pG^{Ac}\text{-OAc}$ ) with the *N*-acetylguanine moiety was used without disadvantage. Another precaution taken to prevent any depurination during the work-up after condensation steps was to add routinely

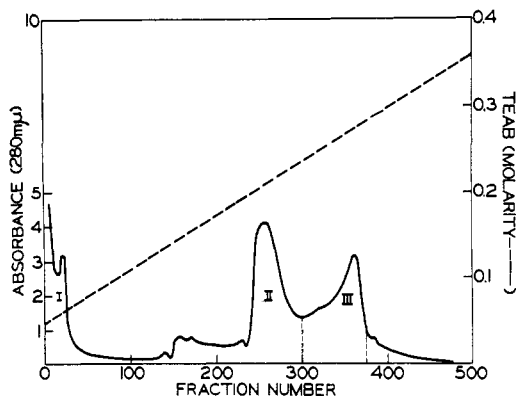


Figure 9. Chromatography of the reaction products in the preparation of the tetranucleotide  $d\text{-DMTr-TpA}^{Bz}pA^{Bz}pG^{Bz}$  on a DEAE-cellulose (carbonate form) column ( $2.2 \times 70$  cm). After the material was applied to the column it was washed with 0.5 l. of 0.05 *M* TEAB-40% alcohol. Then a linear gradient of triethylammonium bicarbonate was started with 3 l. of 0.05 *M* TEAB-20% alcohol in the mixing vessel and 3 l. of 0.42 *M* TEAB-45% alcohol in the reservoir. Fractions of 10 ml were collected at 10-15-min intervals. Peak III contained the desired tetranucleotide.

the hindered trialkylamine, *N,N*-diisopropyl-*N*-ethylamine, during the aqueous pyridine treatment. With these precautions and using the molar proportions and reaction conditions shown in Table I, higher yields (40-54%) of the pure desired products were again obtained in this series. An exception (Table I) was at the step of the protected tetranucleotide synthesis where the low yield was probably due to the lack of separation (Figure 9) of the protected tetranucleotide from the unreacted protected dinucleotide and its pyrophosphate. As a result, the main peak corresponding to the product from the first chromatography (Figure 9) was rechromatographed, thus lowering the yield of the purified tetranucleotide. Elution patterns obtained on chromatography of the hexa- and the octanucleotides are shown in Figures 10 and 11. Separation of the desired products in these cases was satisfactory; the oligonucleotides thus obtained were homogeneous.

**General Comments.** All of the deoxyribopolynucleotides obtained at successive synthetic steps were characterized by their ultraviolet absorption spectra and by their mobilities on paper chromatograms (1) when they were in their fully protected form (Table II), (2) after an ammoniacal treatment to remove the *N*-protecting groups (Table III), and (3) after an acidic treatment to remove the methoxytrityl groups (Table IV). Wherever necessary, further purification was carried out by paper chromatography. Finally, samples of all of the deoxyribopolynucleotides were characterized by their susceptibility toward spleen phosphodiesterase and by analysis of the resulting nucleosides and mononucleotides (Table V).

The total experience in the present work has further shown the practical usefulness of the approach using preformed dinucleotide blocks in the stepwise synthesis of deoxyribopolynucleotides. In the following paper this study of the use of preformed blocks was extended to an investigation of preformed tri- and tetranucleotide blocks.

## Experimental Section

**General Methods and Materials.** Paper chromatography was carried out by the descending technique using Whatman No. 40 or

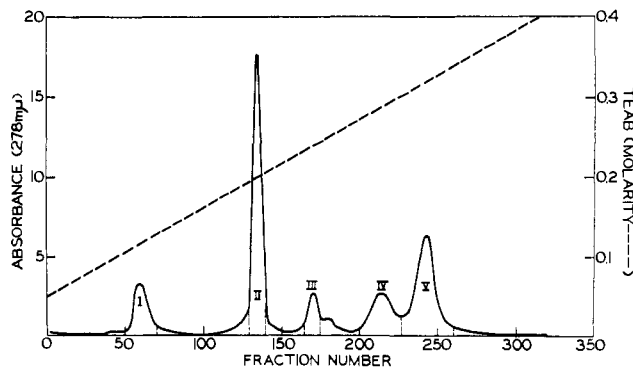


Figure 10. Chromatography of the reaction products in the preparation of the hexanucleotide  $d\text{-DMTr-TpA}^{Bz}pA^{Bz}pG^{Bz}pTpA^{Bz}$  on a DEAE-cellulose (carbonate form) column ( $2.2 \times 70$  cm). After pyridine was washed off with 0.05 *M* TEAB-40% alcohol a linear gradient of triethylammonium bicarbonate was started with 2 l. of 0.05 *M* TEAB-40% alcohol in the mixing vessel and 2 l. of 0.50 *M* TEAB-40% alcohol in the reservoir. Fractions of 10 ml were collected every 18 min. Peak V contained the desired hexanucleotide.

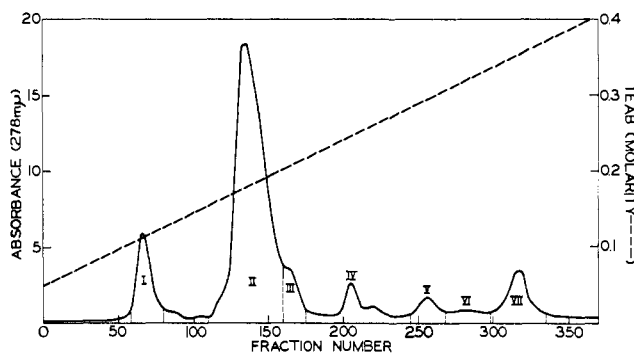


Figure 11. Chromatography of the reaction products in the preparation of the octanucleotide  $d\text{-DMTr-TpA}^{Bz}pA^{Bz}pG^{Bz}pTpA^{Bz}pA^{Bz}pG^{Ac}$  on a DEAE-cellulose (carbonate form) column ( $2.2 \times 70$  cm). After washing off pyridine with 0.05 *M* TEAB-40% alcohol a linear gradient of triethylammonium bicarbonate was started with 2.5 l. of 0.05 *M* TEAB-40% alcohol in the mixing vessel and 2.5 l. of 0.53 *M* TEAB-40% alcohol in the reservoir. Fractions of 10 ml were collected every 18 min. Peak VII contained the desired octanucleotide.

Whatman No. 1 paper. The solvent systems used were: solvent A, isopropyl alcohol-concentrated ammonia-water (7:1:2, v/v); solvent B, ethyl alcohol-ammonium acetate 1 *M* (pH 7.5) (7:3, v/v); solvent C, *n*-propyl alcohol-concentrated ammonia-water (55:10:35, v/v); solvent D, isobutyric acid-concentrated ammonia-water (66:1:33, v/v). The paper chromatographic mobilities of different compounds are listed in Tables II, III, and IV.

The presence of the trityl group in different compounds was detected by spraying the chromatograms with 10% aqueous perchloric acid and drying in warm air. The trityl-containing compounds appeared yellow (monomethoxytrityl) or orange (dimethoxytrityl).

The  $\epsilon$  values used for the protected nucleotides at neutral pH are as follows:  $d\text{-pT}$ , 8700 (276  $m\mu$ ) and 6400 (280  $m\mu$ );  $d\text{-pC}^{An}$ , 16,000 (276  $m\mu$ ) and 22,400 (302  $m\mu$ );  $d\text{-pA}^{Bz}$ , 17,000 (276  $m\mu$ ), 18,300 (280  $m\mu$ ), and 5500 (302  $m\mu$ );  $d\text{-pG}^{Ac}$ , 11,800 (280  $m\mu$ );  $d\text{-pG}^{Bz}$ , 13,000 (276  $m\mu$ ) and 14,000 (280  $m\mu$ ). The abbreviations  $OD_{276}$ ,  $OD_{280}$ , etc., refer to the extinction of the nucleotide solution using a 1-cm light-path quartz cell, the number in subscript being the wavelength used. For yield calculations hypochromicity was neglected.

Enzymic degradations of the synthetic products using spleen phosphodiesterase and purified venom phosphodiesterase were performed as described previously.<sup>16</sup>

(16) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 686 (1961).

Table II. Spectral and Chromatographic Properties of Fully Protected Polynucleotides

Compound	$\lambda_{\max},^a$	$\lambda_{\min},^a$	$\epsilon_{280}^a/$	$\epsilon_{302}^a/$	$R_f,$ solvent B
	m $\mu$	m $\mu$	$\epsilon_{260}$	$\epsilon_{216}$	
d-MMTr-TpA <sup>Bz</sup>	276	250	1.30	0.28	0.85
d-DMTr-TpA <sup>Bz</sup>	276	256	1.28	...	0.78
d-pTpA <sup>Bz</sup>	276	235	1.30	0.28	0.34
d-pTpA <sup>Bz</sup> -OAc	276	235	1.30	0.28	0.45
d-pA <sup>Bz</sup> pG <sup>Bz</sup>	281,237	255,229	1.46	...	0.30
d-pA <sup>Bz</sup> pG <sup>Bz</sup> -OAc	281,237	255,229	1.46	...	0.36
d-pA <sup>Bz</sup> pG <sup>Ac</sup>	278,261	268,231	1.10	...	0.32
d-pA <sup>Bz</sup> pG <sup>Ac</sup> -OAc	278,261	268,231	1.10	...	0.42
d-pG <sup>Ac</sup> pA <sup>Bz</sup>	280,257	268,231	1.07	...	0.32
d-pG <sup>Ac</sup> pA <sup>Bz</sup> -OAc	280,257	268,231	1.07	...	0.44
d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup>	276	245	1.26	0.58	0.62
d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup>	276	243	1.21	0.48	0.46
d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup>	276	243	1.22	0.57	0.27
d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup>	...	...	...	0.53	0.15
d-MMTr-TpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup> pTpA <sup>Bz</sup> pTpC <sup>An</sup>	...	...	...	0.58	0.11
d-MMTr-TpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup>	278	245	1.21	...	0.70
d-MMTr-TpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup> pTpA <sup>Bz</sup>	276	248	1.15	...	0.48
d-MMTr-TpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup> pTpA <sup>Bz</sup> pG <sup>Ac</sup> pA <sup>Bz</sup>	...	...	1.21	...	...
d-DMTr-TpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Bz</sup>	278	256	1.33	...	0.53
d-DMTr-TpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Bz</sup> pTpA <sup>Bz</sup>	278	248	1.25	...	0.5
d-DMTr-TpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Bz</sup> pTpA <sup>Bz</sup> pA <sup>Bz</sup> pG <sup>Ac</sup>	278	247	1.17	...	0.1

<sup>a</sup> At neutral pH.

Table III. Spectral and Chromatographic Properties of Trityl Polynucleotides

Compound	$\lambda_{\max},^a$	$\lambda_{\min},^a$	$\epsilon_{280}^a/$	$\epsilon_{250}^a/$	$R_f, \text{solvent}$		
	m $\mu$	m $\mu$	$\epsilon_{260}$	$\epsilon_{260}$	A	B	C
d-MMTr-TpA	...	...	...	...	...	...	0.81
d-MMTr-TpApTpCpTpA	264	235	0.55	0.81	0.02	0.15	...
d-MMTr-TpApTpCpTpApTpC	...	...	...	...	...	...	0.34
d-MMTr-TpApTpCpTpApTpCpTpA	...	...	...	...	...	...	0.28
d-MMTr-TpApTpCpTpApTpCpTpApTpC	263	234	0.59	0.80	...	...	0.19
d-MMTr-TpApGpA	258	229	0.48	0.91	0.15	...	0.68
d-MMTr-TpApGpApTpA	...	...	...	...	...	...	0.56
d-MMTr-TpApGpApTpApGpA	...	...	...	...	...	...	0.35
d-DMTr-TpApApG	254	227	0.50	1.03	...	...	0.72
d-DMTr-TpApApGpTpA	...	...	...	...	...	...	0.57
d-DMTr-TpApApGpTpApApG	...	...	...	...	...	...	0.28

<sup>a</sup> At neutral pH.

Table IV. Spectral and Chromatographic Properties of Unprotected Polynucleotides

Compound	$\lambda_{\max},^a$	$\lambda_{\min},^a$	$\epsilon_{280}^a/$	$\epsilon_{250}^a/$	$R_f, \text{solvent}$	
	m $\mu$	m $\mu$	$\epsilon_{260}$	$\epsilon_{260}$	C	D
d-TpA	261	231	0.37	0.75	0.56	0.68
d-pTpA	261	231	0.37	0.75	0.39	...
d-pApG	254	226	0.41	1.07	0.28	...
d-pGpA	256	226	0.42	0.98	0.28	0.36
d-TpApTpCpTpA	263	233	0.53	0.76	0.23	...
d-TpApTpCpTpApTpC	264	233	0.60	0.79	0.14	0.26
d-TpApTpCpTpApTpCpTpA	263	233	0.57	0.88	0.07	0.19
d-TpApTpCpTpApTpCpTpApTpC	...	...	...	...	0.04	...
d-TpApGpA	257	226	0.41	0.89	0.32	0.45
d-TpApGpApTpA	...	...	0.40	0.85	0.21	0.37
d-TpApGpApTpApGpA	259	234	0.44	0.90	0.08	0.25
d-TpApApG	256	228	0.45	0.99	0.28	...
d-TpApApGpTpA	257	230	0.40	0.92	0.17	...
d-TpApApGpTpApApG	257	230	0.44	0.95	0.1	0.16

<sup>a</sup> At neutral pH.

Reagent grade pyridine was dried, distilled over chlorosulfonic acid (15 ml/l. of pyridine) and redistilled over potassium hydroxide. It was stored in the dark over Molecular Sieve beads (4A) from the Linde Co. for several weeks. 2,4,6-Triisopropylbenzenesulfonyl chloride was prepared as described earlier.<sup>13</sup>

The pyridinium salts of d-pA<sup>Bz</sup>-OAc, d-CE-pT, d-pA<sup>Bz</sup>pG<sup>Ac</sup>-d-pTpA<sup>Bz</sup>, and d-pTpC<sup>An</sup>-OAc were prepared as described previously.<sup>17</sup> Pyridinium d-pG<sup>Ac</sup> was prepared by the method de-

scribed previously<sup>18</sup> except for ether-precipitation steps which were carried out directly after acetylation and after removal of the O-acetyl group. 5'-O-Mono-p-methoxytrityldeoxythymidine was prepared as described earlier<sup>19</sup> except for the aluminum oxide column step which was found to be unnecessary and for the pre-

(18) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 1983 (1963).(19) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *ibid.*, **85**, 3821 (1963).

Table V. Spleen Phosphodiesterase Degradation of Synthetic Polynucleotides

Degraded Compd	Products, OD <sub>260</sub> units <sup>d</sup>							Ratios <sup>e</sup>		
	d-Tp	d-Cp	d-Ap	d-Gp	d-C	d-A	d-G	Theor	Found	
d-TpApTpCpTpApTpC <sup>a</sup>	2.31	0.54	1.88	...	0.42	...	...	d-Tp:d-Cp:d-Ap:d-C	4:1:2:1	4.0:1.0:1.9:0.9
d-TpApTpCpTpApTpCpTpApTpC <sup>a</sup>	1.35	0.41	1.11	...	0.18	...	...	d-Tp:d-Cp:d-Ap:d-C	6:2:3:1	6.0:2.1:2.8:0.9
d-TpApGpA <sup>b</sup>	0.63	...	1.10	0.96	...	0.93	...	d-Tp:d-Ap:d-Gp:d-A	1:1:1:1	1.0:1.1:1.1:0.9
d-TpApGpApTpA <sup>b</sup>	1.42	...	2.58	0.96	...	1.05	...	d-Tp:d-Ap:d-Gp:d-A	2:2:1:1	2.0:2.1:1.0:0.8
d-TpApGpApTpApGpA <sup>b</sup>	0.96	...	2.36	1.18	...	0.62	...	d-Tp:d-Ap:d-Gp:d-A	2:3:2:1	2.1:3.0:2.0:0.8
d-TpApApG <sup>a</sup>	0.47	...	1.84	...	...	...	0.63	d-Tp:d-Ap:d-G	1:2:1	1.0:2.3:1.0
d-TpApApGpTpA <sup>c</sup>	3.77	...	6.36	2.47	...	2.84	...	d-Tp:d-Ap:d-Gp:A	2:2:1:1	2.0:2.0:1.0:0.9
d-TpApApGpTpApApG <sup>a</sup>	1.56	...	5.08	0.88	...	...	1.10	d-Tp:d-Ap:d-Gp:G	2:4:1:1	2.1:4.0:1.1:0.9

<sup>a</sup> The digestion products were separated by chromatography in solvent B; fairly good separation of d-Cp and d-Ap was obtained in this system only if a maximum time was used. A mixture of d-Ap and d-Gp had to be separated further by chromatography in solvent A.

<sup>b</sup> The digestion products were separated by chromatography in solvent C; fairly good separation of d-Ap and d-Tp was achieved running the chromatography for a maximum time. <sup>c</sup> The digestion products were separated by chromatography in solvent A. After having separated d-A, the remaining nucleotides were separated from each other by a prolonged run (2 days) in the same solvent. <sup>d</sup> The OD<sub>260</sub> units were determined by elution with water and spectrophotometric analysis at neutral pH using appropriate blanks. The products were identified by their  $\epsilon_{280}/\epsilon_{260}$  and  $\epsilon_{230}/\epsilon_{260}$  ratios or ultraviolet spectra. <sup>e</sup> For calculation of the molar ratios of the products  $\epsilon_{260}$  values of  $9.6 \times 10^3$ ,  $7.4 \times 10^3$ ,  $15.4 \times 10^3$ , and  $11.7 \times 10^3$  were used for the chromophores of T, C, A, and, G, respectively.

cipitation step which was carried out using petroleum ether instead of ether.

Pyridinium d-MMTr-TpA<sup>Bz</sup> was prepared according to the method described for the preparation of d-DMTr-TpA<sup>Bz11</sup> except for a modification of the column procedure. After having washed the DEAE column with 1.5 l. of 20% aqueous ethyl alcohol elution was continued using a linear gradient with 2 l. of 20% aqueous ethyl alcohol in the mixing vessel and an equal volume of 0.20 M ammonium bicarbonate containing also 20% ethyl alcohol in the reservoir. The desired product appeared as a single broad peak.

**5'-O-Dimethoxytritylthymidyl-(3'→5')-N-benzoyldeoxyadenosine, d-DMTr-TpA<sup>Bz</sup>.** A solution of 5'-O-dimethoxytritylthymidine (2.6 mmoles) and N-benzoyl-3'-O-acetyldeoxyadenosine 5'-phosphate (1.3 mmoles) in pyridine was rendered anhydrous by repeated addition and evaporation of dry pyridine and concentrated to a volume of 10 ml. Dry Dowex W50 X2 ion-exchange resin (620 mg) and DCC (2.8 g, 14 mmoles) were added, and the reaction mixture was shaken for 5 days at room temperature in the dark under exclusion of moisture. Water (10 ml) was added in the cold and the mixture kept at room temperature overnight and extracted with pentane (three 20-ml portions). Resin and dicyclohexylurea were filtered off and washed with some 20% aqueous pyridine, and the filtrate was concentrated in the presence of an excess of pyridine to a volume of 20 ml at low temperature. A sodium hydroxide solution (20 ml, 2 N) was added in the cold and the mixture kept for 10 min at 0°. An excess of pyridinium Dowex 50 ion-exchange resin was added quickly with shaking. After 10 min the resin was filtered off and rinsed with 50% aqueous pyridine (150 ml) and the total filtrate (pH 7) concentrated at low temperature to a volume of 40 ml. Alcohol (360 ml) was added and the solution applied to the top of a DEAE-cellulose, anion-exchange column in the carbonate form (3.6 × 87 cm) which had been preequilibrated with 0.05 M TEAB (triethylammonium bicarbonate) in 40% ethanol. Chromatography was carried out at 4°. After pyridine and the nucleoside were washed off with 90% aqueous alcohol (2.8 l.), a linear salt gradient of 4 l. of 90% alcohol in the mixing vessel and 4 l. of 0.05 M TEAB in 90% alcohol in the reservoir was started. Fractions of about 20 ml were collected every 10 min. The product appeared as a sharp peak in the fractions 140–240 which were pooled and evaporated in the presence of an excess of pyridine. The residue was dissolved in anhydrous pyridine (30 ml) and precipitated twice by dropwise addition to anhydrous ether (600 ml). The precipitate was collected by centrifugation, washed with ether three times, and dried overnight *in vacuo* at room temperature. Triethylammonium d-DMTr-TpA<sup>Bz</sup> (73 mg) (54% based on d-pA<sup>Bz</sup>) was obtained. The product gave a single spot on paper chromatography in solvent B.

**5'-O-Phosphoryl-N-benzoyldeoxyadenyl-(3'→5')-N-benzoyl-3'-O-acetyldeoxyguanosine, d-pA<sup>Bz</sup>pG<sup>Bz</sup>OAc.** Benzoylation of d-pApG. d-pA<sup>Bz</sup>pG<sup>Ac</sup>OAc<sup>12</sup> (6200 OD<sub>278</sub> units) and d-pA<sup>Bz</sup>pG<sup>Ac</sup> (3900 OD<sub>278</sub> units) recovered from a preparation of the octanucleotide DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup>pT(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Ac</sup> (a total of 0.34 mmole) were treated with a 1:1 mixture (v/v) of *n*-butylamine and methanol for 4 days at room temperature. The solvent was evaporated at room temperature and the residue taken up in dry pyridine (20 ml). Benzoyl chloride (1.0 ml, 8.6 mmoles) was added and

the mixture shaken in the dark for 2.75 hr under exclusion of moisture. Water (20 ml) and 2 N sodium hydroxide solution (40 ml) were added at 0°, and the mixture was kept at this temperature for 20 min with occasional shaking. Pyridinium Dowex 50 ion-exchange resin (200 ml; 0.14 mmole) was added quickly with vigorous shaking. The neutral slurry (pH 6.8) was poured onto an additional 100 ml of the same resin in a column (i.d. 4 cm). The resin was filtered off and washed slowly with 20% aqueous pyridine (1 l.) in the cold. Chromatography of the filtrate was carried out on a DEAE-cellulose, anion-exchange column in the carbonate form (89 × 2.4 cm) preequilibrated with 0.01 M TEAB in 20% aqueous alcohol at 4°. A linear salt gradient of 3 l. of 0.01 M TEAB in the mixing vessel and 3 l. of 0.30 M TEAB in the reservoir was used, both buffers containing 20% aqueous alcohol. Fractions of about 10 ml were collected. The product was obtained between a salt concentration of 0.22 and 0.27 M. The yield of the pooled fractions was 7500 OD<sub>260</sub> units, corresponding to 0.23 mmole (68%). Paper chromatography in solvent B showed essentially one spot with a bluish fluorescence. The product fractions were evaporated in the presence of an excess of pyridine at low temperature and finally rendered anhydrous by continuous evaporation of dry pyridine from the residue.

The product obtained was treated with acetic anhydride (2.5 ml) in anhydrous pyridine (10 ml) for 4.5 hr at room temperature in the dark. Methanol (5 ml) was added with cooling and the mixture kept at room temperature for 3.5 hr. Water (10 ml) was added and the mixture kept overnight at room temperature. Evaporation of the solvent with continuous addition of dry pyridine afforded an anhydrous pyridine solution (8 ml), which was precipitated by dropwise addition to anhydrous ether (500 ml). The precipitate was collected by centrifugation, washed three times with dry ether, and dried *in vacuo*. d-pA<sup>Bz</sup>pG<sup>Bz</sup>OAc (0.22 g) was obtained, and proved to be homogeneous on paper chromatography in solvent B.

**β-Cyanoethyl-N-acetyldeoxyguanosine 5'-Phosphate, d-CE-pG<sup>Ac</sup>.** A mixture of pyridinium N-acetyldeoxyguanosine 5'-phosphate (5 mmoles), hydracrylonitrile (13 ml, 180 mmoles), pyridine (20 ml), dimethylformamide (5 ml), and DCC (13 g, 62 mmoles) was shaken at room temperature for 3 days. Then 40 ml of water was added with cooling in an ice bath. After 1 hr at 0° dicyclohexylurea was removed by filtration. The urea was washed three times with 20 ml of 50% pyridine. The total filtrate was evaporated at low temperature (<10°) to a final volume of about 20 ml. After addition of water (100 ml) the solution (pH 6) was allowed to stand at room temperature for 5 days. After repeated evaporation at low temperature with dry pyridine, an anhydrous pyridine solution (60 ml) was obtained, which was added dropwise to 600 ml of dry ether. The ether was decanted after centrifugation and the precipitate dissolved in 50 ml of pyridine and precipitated a second time by addition to 500 ml of ether. The precipitate was washed with dry ether and centrifuged three times to yield 2.75 g of fine dry powder. Paper chromatography in solvent B at this stage showed complete disappearance of N-acetyldeoxyguanylic acid, the products being β-cyanoethyldeoxyguanosine 5'-phosphate and a trace of the neutral, faster moving di-β-cyanoethyl-N-acetyldeoxyguanosine 5'-phosphate. The product also contained unidentified fluorescent material.



**5'-O-Phosphoryl-N-acetyldeoxyguanylyl-(3'→5')-N-benzoyl-3'-O-acetyldeoxyadenosine, d-pG<sup>A</sup>pA<sup>Bz</sup>-OAc.** A mixture of 2.7 g of pyridinium CE-pG<sup>A</sup> (5.2 mmoles) and 2.9 g pyridinium pA<sup>Bz</sup>-OAc (5.2 mmoles) in 20 ml of dry pyridine was treated with 7.9 g of TPS (25 moles) for 9 hr at room temperature in the dark. After addition of 20 ml of water in the cold, the reaction mixture was allowed to stand at room temperature overnight. Then 80 ml of cold 40% aqueous alcohol was added followed by addition of 125 ml of cold 2 N sodium hydroxide. After 10 min at 0°, an excess of pyridinium Dowex 50 X8 ion-exchange resin was added to neutralize the reaction mixture. The resin was removed by filtration. An extensive wash of the resin with 20% aqueous alcohol yielded a final volume of the combined solutions of 2500 ml. The pH was adjusted to 7.8 by careful addition of a few drops of triethylamine. The total solution was applied to a DEAE-cellulose, anion-exchange column (1500 ml of carbonate form, 20% alcohol) in the cold (4°). A linear salt gradient was used with 4 l. of 0.01 M triethylammonium bicarbonate in the mixing vessel and 4 l. of 0.15 M triethylammonium bicarbonate in the reservoir. The gradient was continued with 4 l. of 0.15 M salt in the mixing vessel and 4 l. of 0.30 M salt in the reservoir. All buffer solutions contained 20% alcohol. Fractions of about 18 ml were collected every 10 min. The absorbance at 280 m $\mu$  was measured.

The desired product (fractions 260–520) appeared as a broad peak. Fractions 300–500 were combined to yield 66,000 OD<sub>280</sub> units (40%). Paper chromatography in solvent B showed complete absence of d-pA<sup>Bz</sup>, d-pG<sup>A</sup>pA<sup>Bz</sup> being the major product. A side product amounting to about 20% in terms of OD<sub>280</sub> units moved slightly slower with R<sub>f</sub> 0.23. After ammonia treatment as well as after acetylation a homogeneous product was obtained (solvent B); evidently the side product was d-pGpA<sup>Bz</sup>, owing to partial loss of the N-acetyl group on the guanine moiety. Ammonia treatment and chromatography in solvent B and solvent D showed also complete absence of d-pA and d-pG.

The pooled fractions were evaporated and several times reevaporated with additional pyridine to remove triethylammonium bicarbonate. The remaining gum was finally dissolved in 300 ml of 5% aqueous pyridine and passed through a column of pyridinium Dowex 50 X8 ion-exchange resin (100 ml) in the cold. The total eluate (320 ml) was lyophilized to give a slightly brownish powder, which was dissolved in about 50 ml of 70% aqueous pyridine followed by evaporation to about 10 ml. After addition of 50 ml of dry pyridine and reevaporation to about 10 ml, 30 ml of dry pyridine was added and the solution added dropwise to 1 l. of dry ether. The precipitate was collected by centrifugation and washed three times with dry ether. A fine white powder was obtained (2.1 g, 40% yield).

All of the material was treated with a mixture of 50 ml of pyridine and 15 ml of acetic anhydride for 30 hr at room temperature. Then 50 ml of methanol was added in the cold. After 1.5 hr the mixture was evaporated to about 40 ml and added dropwise to 1 l. of dry ether to yield a white precipitate which was centrifuged and resuspended in dry ether three times. Paper chromatography in solvent B at this stage showed a single peak (R<sub>f</sub> 0.44). The final yield was 2.05 g (43,500 OD<sub>280</sub> units), corresponding to 1.44 mmoles (28%).

**5'-O-Phosphorylthymidylyl-(3'→5')-N-benzoyl-O-acetyldeoxyadenosine, d-pTpA<sup>Bz</sup>-OAc.** The preparation of d-pTpA<sup>Bz</sup> is described in an accompanying paper.<sup>14</sup> Acetylation of this compound (0.49 mmole) was achieved by treatment with a mixture of 10 ml of dry pyridine and 3 ml of acetic anhydride at room temperature overnight. Then 10 ml of water was added in the cold, and the reaction mixture was allowed to stand at room temperature for 2 hr. After repeated evaporation with dry pyridine the gum obtained was dissolved in 10 ml of dry pyridine and the solution added dropwise to 150 ml of dry ether. The precipitate was centrifuged and washed three times with dry ether to yield finally 420 mg of a dry, white powder (10,400 OD<sub>276</sub> units) corresponding to 0.45 mmole (92%).

**General Method of Condensation.** The condensation of the protected oligonucleotides bearing the free 3'-hydroxyl group with the protected dinucleotide blocks was carried out by the following general method. Both the reaction components were dissolved in pyridine, and the mixture was rendered anhydrous by repeated evaporation of added dry pyridine *in vacuo*, each time the flask being opened to dry air of a drybox containing phosphorus pentoxide. After the last evaporation the final minimal amount of dry pyridine necessary for complete solubilization of the reaction components was added followed by addition of triisopropylbenzenesulfonyl chloride (TPS). To obtain a clear solution, the sealed reaction mixture had to be shaken thoroughly by means of a vibrator in some cases. The reaction was stopped by addition of an

equal amount of water under cooling. The resulting mixture was allowed to stand at room temperature overnight and was then treated with an amount of 2 N sodium hydroxide sufficient to give over-all molarity of 1 M. The alkaline mixture was kept for 10 min at 0° and the sodium ions were then removed by the rapid addition of an excess of pyridinium Dowex 50 ion-exchange resin. The resin was removed and washed thoroughly with aqueous alcohol containing 5% pyridine. The alcohol concentrations were the same as used during the DEAE-cellulose column chromatography. The total final volume, which was large enough to render an over-all anion molarity lower than 0.03, then was applied directly to a DEAE-cellulose column preequilibrated with triethylammonium bicarbonate buffer (0.01–0.03 M) in 20–40% alcohol. Appropriate gradients of triethylammonium bicarbonate and of alcohol were started immediately. All the column procedures were carried out at 0–4°.

**Isolation of the Protected Oligonucleotides.** Appropriate fractions containing the desired polynucleotide were pooled and, after addition of equal amounts of pyridine, the solvent was removed by evaporation at 10° or below under reduced pressure. The syrupy residue was rendered anhydrous by repeated evaporation of added dry pyridine and was then taken up in 10–50 ml of 30% aqueous pyridine and passed through a column of pyridinium Dowex 50 X8 ion-exchange resin (at least 20 times the theoretical amount necessary to remove triethylammonium ions) in the cold. After the column was washed with 2–3 bed volumes of 30% aqueous pyridine, the combined eluates were adjusted to 50% pyridine by addition of more pyridine and evaporated to a dry gum. Repeated evaporation of added dry pyridine and final solution in a known small amount (5–20 ml) of this solvent rendered stock solutions of the desired oligonucleotides which were used directly for further condensation.

**Removal of Protecting Groups.** The removal of the ring protecting groups from protected oligonucleotides was effected by treating a pyridine solution of the compound with a large excess (five- to tenfold) of saturated methanolic ammonia<sup>20</sup> for 2 days or with a 1:1 mixture (v/v) of *n*-butylamine and methanol for 4 days at room temperature. The compounds then were chromatographed in solvent C. The trityl groups were removed (only after the removal of the ring protecting groups and after chromatography) by treating the products with an excess of 80% acetic acid for 45 min (monomethoxytrityl) or 20 min (dimethoxytrityl) or with a mixture of acetic acid–pyridine–water, 14:1:3 (v/v/v), for 24 hr at room temperature (monomethoxytrityl).<sup>6</sup> After evaporation of the solvent under reduced pressure at 20° or below, final purification of the polynucleotide was accomplished by paper chromatography in solvent C or D. Except for the fully protected compounds, elution of the products from paper was carried out using water containing 1% concentrated ammonia. In order to obtain good recovery the paper areas had to be eluted twice. After evaporation of the aqueous ammonia the compounds were dissolved in distilled water and kept frozen.

**The Tetranucleotide d-MMTr-TpA<sup>Bz</sup>-pTpC<sup>An</sup>.** An anhydrous pyridine solution (1.5 ml) of pyridinium d-MMTr-TpA<sup>Bz</sup> (4000 OD<sub>276</sub> units, 158  $\mu$ moles), pyridinium d-pTpC<sup>An</sup>-OAc (7000 OD<sub>302</sub> units, 314  $\mu$ moles), and TPS (370 mg, 1.25 mmoles) was kept at room temperature for 8 hr under exclusion of moisture. The subsequent work-up was as described above under General Methods. Column chromatography of the reaction mixture was carried out as shown in Figure 1. Peaks I and II contained mainly pyridine and triisopropylbenzenesulfonic acid. Peak IV (3600 OD<sub>302</sub> units) contained the excess of the dinucleotide d-pTpC<sup>An</sup> and peak V (380 OD<sub>302</sub> units) mainly the symmetrical pyrophosphate of d-pTpC<sup>An</sup>. Peak VI had a ratio of  $\epsilon_{302}/\epsilon_{280}$  of 0.7–0.8 and contained the desired product in virtually pure form. Paper chromatography in solvent B showed a broad band of d-MMTr-TpA<sup>Bz</sup>pTpC<sup>An</sup> and a nonnucleotidic fluorescent side product (R<sub>f</sub> 0.62,  $\lambda_{max}$  242) which amounted to 3% in terms of OD<sub>max</sub> units. The yield of the pooled fractions was 3120 OD<sub>276</sub> units, corresponding to 72  $\mu$ moles (45%).

**The Hexanucleotide d-MMTr-TpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup>.** An anhydrous pyridine solution (1.2 ml) of pyridinium d-MMTr-TpA<sup>Bz</sup>-pTpC<sup>An</sup> (3100 OD<sub>276</sub> units, 71.6  $\mu$ moles), pyridinium d-pTpA<sup>Bz</sup>-OAc (6450 OD<sub>276</sub> units, 250  $\mu$ moles), and TPS (302 mg, 1.0 mmole) was kept at room temperature for 8 hr under exclusion of moisture. The subsequent work-up was as described above under General Methods. Column chromatography of the reaction mixture was

(20) If this solvent caused precipitation of the compound, an equal amount of aqueous concentrated ammonia was added to give complete solubilization.



carried out as shown in Figure 2. Peaks I, II, and III contained mainly pyridine and triisopropylbenzenesulfonic acid. Peak V (4540 OD<sub>276</sub> units) contained the excess of d-pTpA<sup>Bz</sup> and peak VI (930 OD<sub>276</sub> units) mainly the symmetrical pyrophosphate of d-pTpA<sup>Bz</sup>. Peak VII had a constant ratio of  $\epsilon_{302}/\epsilon_{276}$  (0.48) throughout its fractions and contained pure d-MMTr-TpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup>. The product was homogeneous in solvent B and after ammonia treatment also in solvents A and B. The yield of the pooled fractions was 2570 OD<sub>276</sub> units, corresponding to 34  $\mu$ moles (47%).

**The Octanucleotide d-MMTr-TpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup>pTpC<sup>An</sup>.** An anhydrous pyridine solution (1.0 ml) of pyridinium d-MMTr-TpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup> (1520 OD<sub>276</sub> units, 20  $\mu$ moles), pyridinium d-pTpC<sup>An</sup>-OAc (2900 OD<sub>276</sub> units, 120  $\mu$ moles), and TPS (218 mg, 720  $\mu$ moles) was kept at room temperature for 6.25 hr under exclusion of moisture. The subsequent work-up was as described above under General Methods. Column chromatography of the reaction mixture was carried out as shown in Figure 3. Peaks I and II contained mainly pyridine and triisopropylbenzenesulfonic acid. Peak III (2160 OD<sub>276</sub> units) contained the excess of d-pTpC<sup>An</sup> and peak IV (380 OD<sub>276</sub> units) the symmetrical pyrophosphate of d-pTpC<sup>An</sup>. Peak V (412 OD<sub>276</sub> units) contained partly the unreacted hexanucleotide. Peak VI had a constant ratio of  $\epsilon_{302}/\epsilon_{276}$  (0.59) throughout its fractions and contained the octanucleotide, homogeneous in solvent B and after ammonia treatment in solvent C. The yield of the pooled fractions was 890 OD<sub>276</sub> units, corresponding to 8.9  $\mu$ moles (45%).

**The Decanucleotide d-MMTr-TpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup>.** An anhydrous pyridine solution (0.5 ml) of pyridinium d-MMTr-TpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup>pTpC<sup>An</sup> (720 OD<sub>276</sub> units, 7.1  $\mu$ moles), pyridinium d-pTpA<sup>Bz</sup>-OAc (1490 OD<sub>276</sub> units, 60  $\mu$ moles), and TPS (73 mg, 240  $\mu$ moles) was kept at room temperature for 6.25 hr under exclusion of moisture. The subsequent work-up was as described above under General Methods. Column chromatography of the reaction mixture was carried out as shown in Figure 4. Peaks I and II contained mainly pyridine and triisopropylbenzenesulfonic acid. Peak IV (1140 OD<sub>276</sub> units) contained the excess of d-pTpA<sup>Bz</sup> and peak V mainly the symmetrical pyrophosphate of d-pTpA<sup>Bz</sup>. The unreacted octanucleotide was in peak VI. Peak VII had a constant ratio of  $\epsilon_{302}/\epsilon_{276}$  (0.54) throughout its fractions and contained the desired decanucleotide which was shown to be homogeneous in solvents B and C. After ammonia treatment, the product was virtually pure in solvent C showing only a very faint side band ( $R_f$  0.35) possibly due to some unreacted octanucleotide. Subsequent acid treatment also gave a homogeneous product in solvent C. The yield of the pooled fractions was 420 OD<sub>276</sub> units, corresponding to 3.3  $\mu$ moles (47%).

**The Dodecanucleotide d-MMTr-TpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup>pTpC<sup>An</sup>.** An anhydrous pyridine solution (0.3 ml) of pyridinium d-MMTr-TpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup>pTpC<sup>An</sup>pTpA<sup>Bz</sup> (380 OD<sub>276</sub> units, 3.0  $\mu$ moles), pyridinium d-pTpC<sup>An</sup>-OAc (740 OD<sub>276</sub> units, 30  $\mu$ moles), and TPS (36 mg, 120  $\mu$ moles) was kept at room temperature for 6 hr under exclusion of moisture. Then 50% aqueous pyridine (2 ml) was added, and the resulting mixture was kept at room temperature overnight. After dilution with 40% aqueous alcohol (20 ml) the total solution was applied directly to the column and eluted as described in Figure 5. Peaks I and II contained mainly pyridine and triisopropylbenzenesulfonic acid. Peak IV (650 OD<sub>276</sub> units) contained the excess of pTpC<sup>An</sup>. Peak V (37 OD<sub>276</sub> units) contained mainly the symmetrical pyrophosphate of d-pTpC<sup>An</sup>. Peaks VII (138 OD<sub>276</sub> units) and VIII (181 OD<sub>276</sub> units) had a ratio of  $\epsilon_{302}/\epsilon_{276}$  which varied between 0.54 and 0.62. The total material of both the peaks was treated separately with ammonia and chromatographed in solvent C. In terms of OD<sub>260</sub> units 60% of peak VII was unreacted decanucleotide and 6% was the desired dodecanucleotide, the rest being an unidentified trityl-containing side product ( $R_f$  0.39). Peak VIII contained the desired dodecanucleotide to an amount of 75% in terms of OD<sub>260</sub> units, the remaining 25% accounting for an unidentified trityl-containing side product ( $R_f$  0.28). The total yield of the fully protected dodecanucleotide was calculated from this analysis to be 144 OD<sub>276</sub> units (8 units in peak VII, 136 units in peak VIII), which corresponds to 0.95  $\mu$ moles (33%). The free dodecanucleotide finally was obtained by acid treatment and rechromatography in solvent C.

**The Tetranucleotide d-MMTr-TpA<sup>Bz</sup>pG<sup>Ac</sup>pA<sup>Bz</sup>.** An anhydrous pyridine solution (4.0 ml) of pyridinium d-MMTr-TpA<sup>Bz</sup> (16,000 OD<sub>280</sub> units, 650  $\mu$ moles), pyridinium d-pG<sup>Ac</sup>pA<sup>Bz</sup>-OAc (20,000 OD<sub>280</sub> units, 660  $\mu$ moles), and TPS (800 mg, 2.65 mmoles) was kept at room temperature for 11.75 hr under exclusion of moisture. The subsequent work-up was as described above under General Meth-

ods. Column chromatography of the reaction mixture was carried out as shown in Figure 6. Peaks I and II contained mainly pyridine and triisopropylbenzenesulfonic acid. Peak IV (18,800 OD<sub>280</sub> units) contained mainly unreacted starting materials and peak V (1760 OD<sub>280</sub> units) mainly the symmetrical pyrophosphate of d-pG<sup>Ac</sup>pA<sup>Bz</sup>.

In terms of OD<sub>280</sub> units 60% of peak VI (2280 OD<sub>280</sub> units) consisted of a trityl-positive compound moving with  $R_f$  0.62 in solvent B. After treatment with ammonia the product had the same mobility ( $R_f$  0.15) in solvent A as d-MMTr-TpApGpA (material of peak VII, ammonia treated). Subsequent acid treatment yielded a product with the same mobility ( $R_f$  0.32) as d-TpApGpA in solvent C. Degradation of this material by spleen phosphodiesterase followed by chromatography in solvent B showed complete digestion to d-Tp, d-Ap, d-Gp, and d-A with the molar ratio of 1.0:1.2:1.2:0.8; degradation by purified venom phosphodiesterase followed by chromatography in solvent B showed complete digestion to d-pA, d-pG, and d-T with the molar ratio of 2.0:1.2:1.0. Evidently the main product of peak VI was d-MMTr-TpA<sup>Bz</sup>pGpA<sup>Bz</sup> because of the loss of the acetyl group at the guanine moiety of the desired tetranucleotide. Peak VII contained the fully protected tetranucleotide homogeneous in solvent B and after ammonia treatment also homogeneous in solvent A. The yield of the pooled fractions excluding peak VI was 7400 OD<sub>280</sub> units, corresponding to 165  $\mu$ moles (25%).

**The Hexanucleotide d-MMTr-TpA<sup>Bz</sup>pG<sup>Ac</sup>pA<sup>Bz</sup>pTpA<sup>Bz</sup>.** An anhydrous pyridine solution (1.2 ml) of pyridinium d-MMTr-TpA<sup>Bz</sup>-pG<sup>Ac</sup>pA<sup>Bz</sup> (3400 OD<sub>280</sub> units, 62  $\mu$ moles), pyridinium d-pTpA<sup>Bz</sup>-OAc (6200 OD<sub>280</sub> units, 250  $\mu$ moles), and TPS (302 mg, 1.0 mmole) was kept at room temperature for 8.25 hr under exclusion of moisture. Then pyridine (1.2 ml) and water (2.4 ml) were added in the cold, and the mixture was allowed to stand at room temperature overnight. Subsequent work-up was as described above under General Methods. Column chromatography of the reaction mixture was carried out as shown in Figure 7. Peaks I and II contained mainly pyridine and triisopropylbenzenesulfonic acid. Peak III (4200 OD<sub>280</sub> units) contained mainly unreacted d-pG<sup>Ac</sup>pA<sup>Bz</sup>. Peak V (1880 OD<sub>280</sub> units) contained the desired hexanucleotide. Treatment of an aliquot of the combined fractions of peak V with ammonia followed by chromatography in solvent C gave only one nucleotidic band ( $R_f$  0.56, trityl positive). This material was treated with a mixture of acetic acid-pyridine-water as described above under General Methods. Subsequent chromatography in solvent C yielded a single nucleotidic band ( $R_f$  0.20) whereas by chromatography in solvent D two bands ( $R_f$  0.53 and 0.37) were obtained. The faster moving band amounted to 35% of the material in terms of OD<sub>260</sub> units and presumably is due to partial depurination of the desired hexanucleotide during work-up ( $\epsilon_{250}/\epsilon_{260} = 0.51$ ,  $\epsilon_{230}/\epsilon_{260} = 0.80$ ). The slower moving band amounting to 65% of the material in terms of OD<sub>260</sub> units was the unreacted desired hexanucleotide ( $\epsilon_{280}/\epsilon_{260} = 0.40$ ,  $\epsilon_{230}/\epsilon_{260} = 0.85$ ) as shown by degradation with spleen phosphodiesterase. Thus the yield of the protected hexanucleotide was 1220 OD<sub>280</sub> units, which corresponds to 15.5  $\mu$ moles (25%).

**The Octanucleotide d-MMTr-TpA<sup>Bz</sup>pG<sup>Ac</sup>pA<sup>Bz</sup>pTpA<sup>Bz</sup>pG<sup>Ac</sup>pA<sup>Bz</sup>.** An anhydrous pyridine solution (0.1 ml) of pyridinium d-MMTr-TpA<sup>Bz</sup>pG<sup>Ac</sup>pA<sup>Bz</sup>pTpA<sup>Bz</sup> [230 OD<sub>280</sub> units of the preparation obtained in the preceding experiment which contained 150 OD<sub>280</sub> units (1.9  $\mu$ moles) of pure hexanucleotide], pyridinium d-pG<sup>Ac</sup>pA<sup>Bz</sup>-OAc (525 OD<sub>280</sub> units, 17  $\mu$ moles), and TPS (21 mg, 70  $\mu$ moles) was kept at room temperature for 7.5 hr under exclusion of moisture. Then pyridine (1 ml) and water (1 ml) were added in the cold and the resulting mixture was worked up further as described above under General Methods. Column chromatography of the reaction mixture was carried out as shown in Figure 8. Peaks I and II contained mainly pyridine and triisopropylbenzenesulfonic acid. Peak III (450 OD<sub>280</sub> units) contained mainly the excess of d-pG<sup>Ac</sup>pA<sup>Bz</sup>. Peak IV (141 OD<sub>280</sub> units) contained unreacted hexanucleotide together with the above-mentioned side product. Peak V (102 OD<sub>280</sub> units) contained the desired octanucleotide. The pooled fractions of peak V were treated with ammonia and chromatographed in solvent C to give a single nucleotidic band ( $R_f$  0.35, trityl positive). Subsequent treatment of the product with a mixture of acetic acid-pyridine-water and chromatography in solvent C gave a single band ( $R_f$  0.08), whereas chromatography in solvent D showed a nucleotidic side product ( $R_f$  0.37,  $\epsilon_{250}/\epsilon_{260} = 0.47$ ) which amounted to 31% in terms of OD<sub>260</sub> units. The yield of the desired protected octanucleotide was calculated to be 71 OD<sub>280</sub> units, which corresponds to 0.67  $\mu$ mole (35%).

**The Tetranucleotide d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup>.** An anhydrous pyridine solution (2.5 ml) of triethylammonium d-DMTr-TpA<sup>Bz</sup>

(1580 OD<sub>271</sub> units, 48.5  $\mu$ moles), triethylammonium d-pA<sup>Bz</sup>pG<sup>Bz</sup>-OAc (3300 OD<sub>281</sub> units, 97  $\mu$ moles), and TPS (59 mg, 194  $\mu$ moles) was kept at room temperature for 5 hr under exclusion of moisture. N,N,N-Diisopropylethylamine (0.39 mmole) in pyridine (0.39 ml) and water (2 ml) were then added in the cold, and the reaction mixture was kept at room temperature for 11 hr, then diluted to 5 ml with pyridine, then cooled to 0°. Water (6 ml) and ice-cold 2 N sodium hydroxide solution (10 ml) were added. The clear solution obtained upon shaking was kept at 0° for 20 min. Pyridinium Dowex 50 ion-exchange resin (20 ml) was added. The mixture (pH 6.9) was poured onto an additional 20 ml of the same resin in a column (i.d. 2 cm), and the resin was filtered off and washed with 20% pyridine (150 ml). Chromatography of the reaction mixture was carried out on a DEAE-cellulose column in the carbonate form, preequilibrated to 0.05 M TEAB containing 40% ethanol as shown in Figure 9. Before the gradient was started the column was washed with the same buffer (400 ml). Peak I contained some trityl-positive material besides pyridine, and peak II mainly the excess of d-pA<sup>Bz</sup>pG<sup>Bz</sup>. Peak III contained essentially all the tetranucleotide d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup> as shown by paper chromatography in solvent B but also some dinucleotide d-pA<sup>Bz</sup>pG<sup>Bz</sup>. The product peak fractions were pooled, evaporated in the presence of pyridine, and rechromatographed on the same column with a linear salt gradient of 2 l. of 0.05 M TEAB containing 20% ethanol in the mixing vessel and 2 l. of 0.40 M TEAB containing 40% ethanol in the reservoir. Again a splitting into two peaks was observed: dinucleotide d-pA<sup>Bz</sup>pG<sup>Bz</sup> (between salt concentration of 0.26 and 0.29) and the desired tetranucleotide (between salt concentration of 0.29 and 0.35). The fractions of the second peak were pooled in a way to avoid contamination of the desired product as much as possible. Paper chromatography in solvent B showed two spots:  $R_f$  0.62, d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup> (95% in terms of OD<sub>278</sub> units). After the standard amine treatment paper chromatography in solvent C showed again two spots:  $R_f$  0.72, d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG, traveling at the same position as a marker of the same tetranucleotide obtained through another pathway, and a spot with  $R_f$  0.28 (d-pApG). The faster traveling spot was acid treated as usual and gave one spot on chromatography in system C at the same position as d-T(pA)<sub>2</sub>pG obtained as above. The yield of the pooled fractions of d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup> was 680 OD<sub>278</sub> units, corresponding to 11  $\mu$ moles (22%).

**The Hexanucleotide d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup>pTpA<sup>Bz</sup>.** An anhydrous pyridine solution (2 ml) of triethylammonium d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup> (1600 OD<sub>278</sub> units, 25  $\mu$ moles), triethylammonium d-pTpA<sup>Bz</sup>-OAc (2600 OD<sub>278</sub> units, 102  $\mu$ moles), and TPS (122 mg, 400  $\mu$ moles) was kept at room temperature for 3.75 hr under exclusion of moisture. N,N,N-Diisopropylethylamine (0.80 mmole) in pyridine (0.80 ml) and water (2 ml) were added, and the reaction mixture was kept at room temperature overnight. The mixture was diluted to 23 ml with pyridine and treated with 2 N sodium hydroxide solution (25 ml) for 10 min at 0°. In order to obtain a homogeneous solution some more water was added during the reaction. Pyridinium Dowex 50 ion-exchange resin (30 ml, 50 mmoles) in 10% pyridine was quickly added. The slurry (pH 6.8–7.0) was passed onto an additional 30 ml of the same resin in a column (i.d. 2 cm), and the resin was filtered off and washed with 20% pyridine (250 ml). Chromatography of the filtrate was carried out as shown in Figure 10 on a DEAE-cellulose column in the carbonate form, preequilibrated with 0.05 M TEAB containing 40%

alcohol. Before the salt gradient was started, the column was washed with the same buffer until no further pyridine appeared in the effluent. Peak I (114 OD<sub>278</sub> units) contained probably cyclo-d-pTpA<sup>Bz</sup>, peak II (1230 OD<sub>278</sub> units) 47% of the original d-pTpA<sup>Bz</sup>, and peak III mainly the symmetrical pyrophosphate of d-pTpA<sup>Bz</sup>. Peak IV (540 OD<sub>278</sub>) corresponded to 34% of the original d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup>. Peak V contained the desired hexanucleotide d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup>pTpA<sup>Bz</sup>. The yield of the pooled fractions was 1080 OD<sub>278</sub> units, corresponding to 14  $\mu$ moles (54%). Paper chromatography in solvent B gave one streaking spot which was eluted from the paper and treated with amine as usual. A sample thus obtained was tested on paper chromatography and showed essentially one spot with  $R_f$  0.57 and two faint traces with  $R_f$  0.20 and 0.12 in solvent C. Another aliquot was treated with acid and gave one spot on paper chromatography in solvent C.

**The Octanucleotide d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup>pTpA<sup>Bz</sup>.** An anhydrous pyridine solution (2 ml) of triethylammonium d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup>pTpA<sup>Bz</sup> (1000 OD<sub>278</sub> units, 11  $\mu$ moles), triethylammonium d-pA<sup>Bz</sup>pG<sup>Ac</sup>-OAc (6000 OD<sub>278</sub> units, 205  $\mu$ moles), and TPS (124 mg, 410  $\mu$ moles) was kept at room temperature for 7 hr under exclusion of moisture. N,N,N-Diisopropylethylamine (0.82 mmole) in pyridine (0.82 ml) and water (2 ml) were added at 0°. The mixture was kept at room temperature overnight and then diluted to 10.5 ml with more pyridine. Sodium hydroxide solution (12 ml, 2 N) and water (8 ml) were added at 0° and the homogeneous mixture was kept at this temperature for 10 min. Pyridinium Dowex 50 resin (30 ml, 54 mmoles) in 10% pyridine was quickly added. The slurry (pH 6.8–7.0) was poured onto an additional 30 ml of the same resin in a column (i.d. 2 cm); the resin was filtered off and washed with 20% pyridine (200 ml). The total filtrate was applied to the top of a DEAE-cellulose column in the carbonate form, preequilibrated with 0.05 M TEAB containing 40% ethanol. Before the gradient was started, the column was washed with the same buffer until no further pyridine was eluted. The elution pattern is shown in Figure 8. Peak I (660 OD<sub>278</sub> units) contained probably cyclo-d-pA<sup>Bz</sup>pG<sup>Bz</sup> and peak II (3900 OD<sub>278</sub> units) 65% of the original d-pA<sup>Bz</sup>pG<sup>Ac</sup>. The shoulder III (280 OD<sub>278</sub> units) contained additional 5% of the starting dinucleotide in the form of pA<sup>Bz</sup>pG ( $\lambda_{max}$  278, 256 m $\mu$ ,  $\lambda_{min}$  265, 234 m $\mu$ ). A superposition of pA<sup>Bz</sup> and pG shows the following ultraviolet data:  $\lambda_{max}$  278, 257 m $\mu$ ,  $\lambda_{min}$  265, 230 m $\mu$ . Peak IV contained mainly the symmetrical pyrophosphate of d-pA<sup>Bz</sup>pG<sup>Bz</sup>. Peaks V–VII were trityl positive. Peak V (235 OD<sub>278</sub> units) contained 23% of the original hexanucleotide, d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup>pTpA<sup>Bz</sup>, and peak VII the desired octanucleotide, d-DMTr-T(pA<sup>Bz</sup>)<sub>2</sub>pG<sup>Bz</sup>pTpA<sup>Bz</sup>. The yield of the pooled fractions was 520 OD<sub>278</sub> units, corresponding to 4.4  $\mu$ moles (40%). The product showed on paper chromatography in solvent B a broad streak up to  $R_f$  0.25. Part of the product was treated with *n*-butylamine-methanol as described above and stored as such for several days at –20°. Paper chromatography in solvent C gave the main trityl-positive spot ( $R_f$  0.28) and a faint additional spot ( $R_f$  0.05) which proved not to be identical with fully unprotected octanucleotide upon further chromatography. The main spot was eluted and treated with acid as usual. Paper chromatography in solvent D for prolonged times showed one spot plus some trailing material, which was cut off before the material was eluted with 1 N ammonia. On rechromatography in solvent C the product was homogeneous in a prolonged run.