Nucleoside S-Alkyl Phosphorothioates. VI.[1] Synthesis of Deoxyribonucleotide Oligomers

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Abstract: The chemical synthesis of several deoxyribooligonucleotides *via* the phosphorothioate method is described. The dodecanucleotide d-pTpGpCpTpApApApTpTpTpGpA constitutes a fragment of a projected larger, double-stranded, array of DNA coding for a derivative of S-peptide of ribonuclease A.

The normal course of events in natural products chemistry has been the isolation of a new substance, its purification to homogeneity as adjudged by current standards, the determination of its structure by analytical techniques, and eventually its chemical synthesis—the successful outcome of which may be final proof of those features of the structure left equivocal by the preceding analysis. This tight correlation between analytical and synthetic aspects begins to relax in the case of nucleic acid chemistry. For instance, a synthetic[2] polydeoxyribonucleotide duplex corresponding (in the base-pairing sense of Watson and Crick) to the known sequence of alanine transfer RNA[3] does not in fact directly specify the actual polyribonucleotide sequence of the latter. Such tRNA molecules contain a substantial number of nucleotides in which the four major bases are altered by substitutions, changes in oxidation state, or other structural variation. The synthetic DNA correlate (the "gene") does not reflect these alterations which are considered to be modifications suffered subsequent to the biosynthesis of the polyribonucleotide.[4]

This alienation of the synthetic objective from the available analytical data becomes more stringent, in a different sense, when it is desired to synthesize a molecule comprising the "genetic instruction" for a messenger in turn coding for a given amino acid sequence. Here, each amino acid is specified by a ribonucleotide triplet by means of the genetic code.[5] However, because of the degeneracy of the code, a unique deduction of ribonucleotide (and, hence, DNA) sequence from peptide structure is precluded; it will presumably have to come from analysis of isolated messenger, a formidable task. Nevertheless, possible solutions can be constructed[6] which are in agreement with code specifications and facilitate the synthetic effort.

The present paper describes a synthesis of the dodecadeoxyribonucleotide d-pTpGpCpTpApApApTpTpTpGpA, which is to form part of the nonsense strand

of a DNA duplex coding for a simplified S-peptide[7] derived from ribonuclease A.[8] It will be seen (Table I) that the completed codons (the nonsense strand being

Table I

Nonsense strand	5'-T G C T A A A T T T G A
Sense strand	3'-A C G A T T T A A A C T
Messenger RNA	5'--U/G C U/A A A/U U U/G A--
Peptide	(ala) ala lys phe (glu)

identical with the corresponding messenger, *mutatis mutandis*, i.e., T = U) are GCU for alanine, AAA for lysine, and UUU for phenylalanine. The first two assignments have been confirmed *in vivo*,[9] whereas the last is, of course, the classical phe codon. These three amino acids occupy sites 6, 7, and 8 of S-peptide.[10]

The strategy for the synthesis of the various oligonucleotides here described is outlined in Charts I and II. The anchoring d-EtSpA^{Bz} (1), described in the preceding paper,[1,11] was condensed (Chart I) with d-pA^{Bz}-OAc[12] to give protected dimer 2, which was in turn similarly elongated to trimer 3. Each of these condensations occurred in yields of ca. 30%. Further condensation with thymidylate trimer 4[13] gave the pivotal hexamer 5 in similar yield.

A tetramer 8 was constructed along conventional lines. 5' Terminally protected dimer 6 with incoming dimer 7[14] gave this tetramer, although in lower yield (17.2%) than preceding condensations. Decamer 9 was obtained in 9.2% yield by condensing the hexamer 5 with this tetramer.

Characterization of fragments of this size consisted of several steps; thus, the fully protected decamer 9 was deacylated, and the resulting 10 was shorn of its 5'-terminal blocking group to give the deoxyribodecanucleotide 11 proper. Both 10 and 11 gave acceptable monomer compositions after complete enzymatic hydrolysis with snake venom diesterase. The decamer

(1) Paper V in this series: A. F. Cook, D. T. Maichuk, M. J. Holman, E. P. Heimer, and A. L. Nussbaum, *J. Amer. Chem. Soc.*, **94**, 1334 (1972).

(2) K. L. Agarwal, H. Buchi, M. H. Caruthers, N. Gupta, H. G. Khorana, K. Kleppe, A. Kumar, E. Ohtsuka, U. L. Rajbhandary, J. H. van de Sande, V. Sgaramella, H. Weber, and T. Yamada, *Nature (London)*, **227**, 27 (1970).

(3) R. W. Holley, J. Apgar, G. A. Everett, J. T. Madison, M. Marquisee, S. H. Merrill, J. R. Penswick, and A. Zamir, *Science*, **147**, 1462 (1965).

(4) H. Zachau, *Angew. Chem., Int. Ed. Engl.*, **8**, 711 (1969).

(5) "The Genetic Code," *Cold Spring Harbor Symp. Quant. Biol.*, **21** (1966).

(6) The problem has been considered by S. A. Narang and S. K. Dheer, *Biochemistry*, **8**, 3443 (1969); see also H. Kössel, *Umschau*, 525 (1970).

(7) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Amer. Chem. Soc.*, **88**, 3633 (1966).

(8) F. M. Richards, *Proc. Nat. Acad. Sci. U.S.*, **44**, 162 (1958).

(9) Y. Ocada, S. Amagase, and A. Tsugita, *J. Mol. Biol.*, **54**, 219 (1970).

(10) M. S. Doscher and C. H. W. Hirs, *Biochemistry*, **6**, 304 (1967).

(11) See also that paper for nomenclature and abbreviations.

(12) R. K. Ralph and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 2926 (1961).

(13) S. A. Narang, J. J. Michniewicz, and S. K. Dheer, *ibid.*, **91**, 936 (1969).

(14) S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, **89**, 2158 (1967).

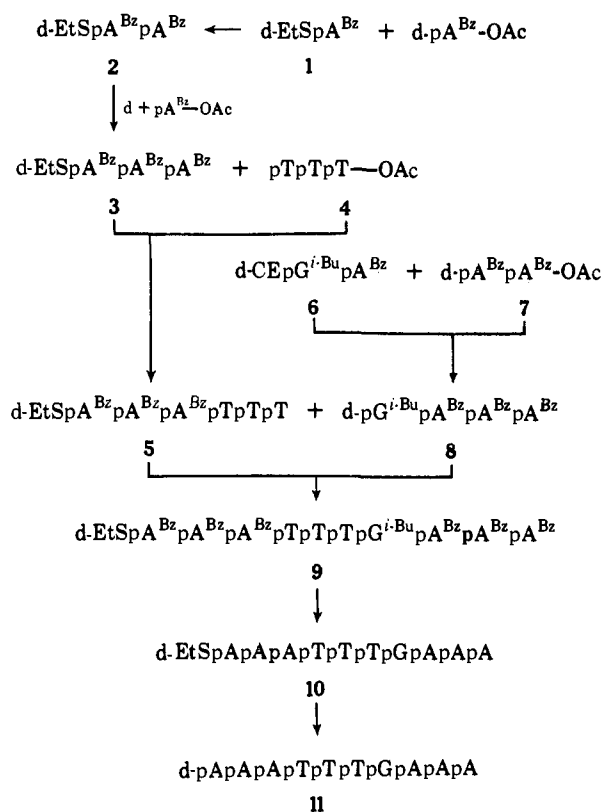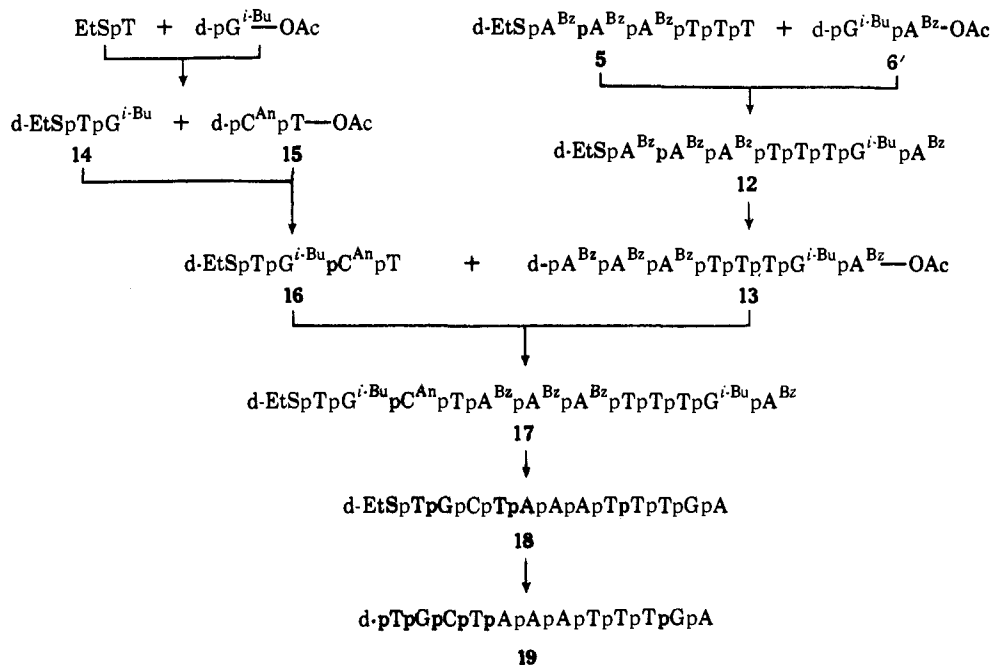Chart I. Synthesis of the Decanucleotide

$$d\text{-EtSpA}^{Bz}pA^{Bz} \longleftarrow d\text{-EtSpA}^{Bz} + d\text{-pA}^{Bz}\text{-OAc}$$
$$\mathbf{2} \qquad\qquad\qquad \mathbf{1}$$

$$\downarrow d + pA^{Bz}\text{-OAc}$$

$$d\text{-EtSpA}^{Bz}pA^{Bz}pA^{Bz} + pTpTpT\text{—OAc}$$
$$\mathbf{3} \qquad\qquad\qquad\qquad \mathbf{4}$$

$$d\text{-CEpG}^{i\text{-Bu}}pA^{Bz} + d\text{-pA}^{Bz}pA^{Bz}\text{-OAc}$$
$$\mathbf{6} \qquad\qquad\qquad \mathbf{7}$$

$$d\text{-EtSpA}^{Bz}pA^{Bz}pA^{Bz}pTpTpT + d\text{-pG}^{i\text{-Bu}}pA^{Bz}pA^{Bz}pA^{Bz}$$
$$\mathbf{5} \qquad\qquad\qquad\qquad\qquad \mathbf{8}$$

$$d\text{-EtSpA}^{Bz}pA^{Bz}pA^{Bz}pTpTpTpG^{i\text{-Bu}}pA^{Bz}pA^{Bz}pA^{Bz}$$
$$\mathbf{9}$$
$$\downarrow$$

$$d\text{-EtSpApApApTpTpTpGpApApA}$$
$$\mathbf{10}$$
$$\downarrow$$

$$d\text{-pApApApTpTpTpGpApApA}$$
$$\mathbf{11}$$

Chart II. Synthesis of a Dodecanucleotide

$$\text{EtSpT} + d\text{-pG}^{i\text{-Bu}}\text{-OAc}$$

$$d\text{-EtSpTpG}^{i\text{-Bu}} + d\text{-pC}^{An}pT\text{—OAc}$$
$$\mathbf{14} \qquad\qquad\qquad \mathbf{15}$$

$$d\text{-EtSpTpG}^{i\text{-Bu}}pC^{An}pT$$
$$\mathbf{16}$$

$$d\text{-EtSpA}^{Bz}pA^{Bz}pA^{Bz}pTpTpT + d\text{-pG}^{i\text{-Bu}}pA^{Bz}\text{-OAc}$$
$$\mathbf{5} \qquad\qquad\qquad\qquad\qquad \mathbf{6'}$$

$$d\text{-EtSpA}^{Bz}pA^{Bz}pA^{Bz}pTpTpTpG^{i\text{-Bu}}pA^{Bz}$$
$$\mathbf{12}$$
$$\downarrow$$

$$d\text{-pA}^{Bz}pA^{Bz}pA^{Bz}pTpTpTpG^{i\text{-Bu}}pA^{Bz}\text{—OAc}$$
$$\mathbf{13}$$

$$d\text{-EtSpTpG}^{i\text{-Bu}}pC^{An}pTpA^{Bz}pA^{Bz}pA^{Bz}pTpTpTpG^{i\text{-Bu}}pA^{Bz}$$
$$\mathbf{17}$$
$$\downarrow$$

$$d\text{-EtSpTpGpCpTpApApApTpTpTpGpA}$$
$$\mathbf{18}$$
$$\downarrow$$

$$d\text{-pTpGpCpTpApApApTpTpTpGpA}$$
$$\mathbf{19}$$

cal ultracentrifuge by a sedimentation equilibrium method (standard error $\pm 5\%$; details to be published), and found to give a satisfactory value. Application of these criteria—enzymatic susceptibility, composition, end group determination, and size—are taken as proof of structure. Homogeneity and migration rate by column and paper chromatography are additional, though inconclusive, evidence.

It had originally been planned to construct a fragment of which decamer 11 was the 5'-terminal moiety. Subsequent reexamination of the fitness of this fragment to serve as an appropriate substrate for joining with another fragment by means of the enzyme polynucleotide ligase, especially in view of the findings of Sgaramella, et al.,[16] forced a revision of this approach as will be discussed in a future publication.

Synthesis of dodecamer 17 is shown in Chart II; octamer 12, which has been obtained from an alternative approach toward the now obsolete decamer 9 in 28% yield, was selectively deprotected at its 5'-terminus and blocked at the opposite end, 13, and used as the *incoming* fragment in an atypical condensation with tetramer 16. The latter was prepared from EtSpT[17] and d-pG$^{i\text{-Bu}}$-OAc, the resulting dimer 14 serving as the receiving portion of a second condensation with protected dimer[18] 15. These two condensations proceeded in 47 and 30% yields, respectively.

The final condensation, octamer 13 into tetramer 16, was carried out, in atypical fashion, with the

was then dephosphorylated with bacterial alkaline phosphatase, and this phosphomonoester at the 5'-terminus reestablished by means of polynucleotide kinase[15] and ATP-γ^{32}P. The resulting labeled oligomer was again subjected to diesterase breakdown, and the location of the radioactivity was determined. Essentially all of the latter was found in the deoxyadenylate fraction; that is, where expected. Finally, the molecular weight of 11 was determined in the analyti-

tetramer present in excess. Two condensations gave the desired product 17 with yields of 5 and 12%. The reason for the discrepancy in these figures is unknown. Conversion of the blocked dodecamer into deacylated 18 and totally unblocked species 19 proceeded normally; characterization of this and other oligomers is described in the Experimental Section.

(15) C. C. Richardson, *Proc. Nat. Acad. Sci. U. S.*, **54**, 158 (1965).

(16) V. Sgaramella, J. H. van de Sande, and H. G. Khorana, *ibid.*, **67**, 1468 (1970).

(17) A. F. Cook, M. J. Holman, and A. L. Nussbaum, *J. Amer. Chem. Soc.*, **91**, 1522 (1969).

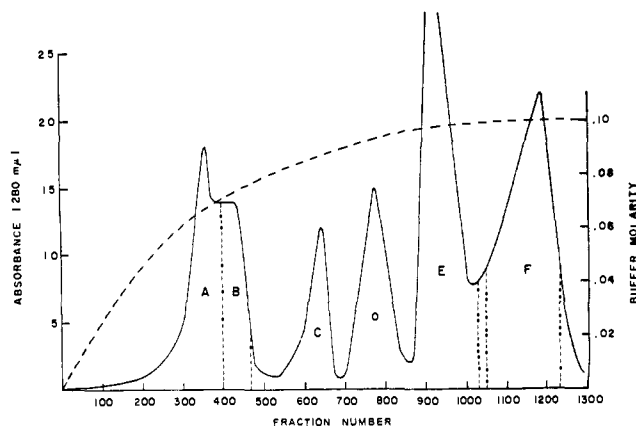(18) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3828 (1963).

Figure 1. DEAE-cellulose chromatography of d-EtSpA^{Bz}pA^{Bz} (2); column size 7 × 92 cm; convex gradient; reservoir, 6 l. of 0.1 M triethylammonium bicarbonate; mixing chamber, 6 l. of distilled water.

Figure 2. DEAE-cellulose chromatography of d-EtSpA^{Bz}pA^{Bz}-pA^{Bz} (3); column size 5 × 100 cm (Pharmacia K 50/100); convex gradient; reservoir, 4 l. of 0.15 M triethylammonium bicarbonate; mixing chamber, 4 l. of distilled water.

The phosphorothioate method in oligonucleotide synthesis has proved to be useful and convenient. Its major drawback at the present time lies in the need to synthesize S-alkyl phosphorothioates by condensation with free nucleosidic 5′-hydroxyl, either in the nucleoside itself, or in oligonucleotides not phosphorylated at the 5′ terminus,[1] since we have been unsuccessful in preparing phosphorothioates from the nucleotides themselves. In this respect it is at a disadvantage as compared to the recently published phosphoramidate[19] procedure. Unlike with the latter, however, deprotection can be carried out with the deacylated oligomer, and again unlike with cyanoethylated intermediates,[13] the protecting group, once affixed, can be carried throughout the entire synthesis.

Experimental Section[20]

Condensation. General Method. The procedures of Khorana were followed closely. The amounts of reagents, condensing agent, and solvent (dry pyridine) are indicated in stylized format under the specific descriptions of the individual substances. The nucleotidic components were rendered anhydrous by several concentrations to dryness in vacuo, the final concentration being carried out in a drybox; the appropriate quantity of condensing agent (mesitylenesulfonyl chloride) was added and the mixture was allowed to stand at room temperature with occasional agitation. After 2 hr the solvent was largely removed in vacuo and the residual viscous syrup was allowed to react another hour. At the end of that period the reaction vessel was removed from the drybox, and an indicated quantity of a cooled solution of 1 M diisopropylethylamine (DIEA) in pyridine was added; this was followed by cold 50% aqueous pyridine. The resulting suspension was allowed to stay at 4° overnight. Selective removal of the 3′-acetate was brought about by adding sufficient 2 N sodium hydroxide to make the mixture 1 N with respect to that base; sometimes a small amount of ethanol had to be added to achieve a clear solution. The solution was left at 0° for 10 min and then rapidly neutralized by the addition of Dowex-50 (pyridinium cycle) until the pH dropped to 6.5. The resin was removed by filtration and washed well with 10% aqueous pyridine.[21] Combined filtrate and wash were adjusted

with water to a dilution with a conductivity equivalent to 0.025 M triethylammonium bicarbonate[20] and charged onto an appropriately sized DEAE-cellulose column in the bicarbonate cycle. The column was washed with water until the eluate was free of pyridine. An appropriate constant (mixing chamber) volume gradient of triethylammonium bicarbonate, pH 7.5, was then applied, and 20-ml fractions were collected at flow rates of 2-3 ml/min.[22] Column chromatography was carried out at 4°, unless otherwise indicated. Eluates were monitored by uv spectrophotometry, paper chromatography, and sulfur tests.[23] Appropriate peaks were combined and concentrated in vacuo to remove water and volatile buffer. The residual material was dissolved in a small volume of dry pyridine and precipitated from a large excess of dry ether. The fine solid was recovered by centrifugation, washed with another portion of ether, and again subjected to centrifugation. The material was dried in a vacuum desiccator over P_2O_5 and stored at −70°. Yields were calculated on the basis of the limiting component and determined by uv spectroscopy of weighed aliquots. Hyperchromicity was disregarded. Details are given below. Analyses and physical characterization are summarized in the appropriate tables.

d-EtSpA^{Bz}pA^{Bz} (2). Condensation: d-EtSpA^{Bz} (1),[1] 9 mmol; d-pA^{Bz}-OAc, 15 mmol; mesitylenesulfonyl chloride (MSC), 45 mmol; pyridine, 50 ml. Work-up: diisopropylethylamine (DIEA), 90 ml; 50% aqueous pyridine, 250 ml; 2 N sodium hydroxide, 400 ml; ethanol, 8 ml. Chromatography: see Figure 1 and Table II.

Table II. Composition of Peaks from the Chromatography in the Synthesis of d-EtSpA^{Bz}pA^{Bz} (2)^a

Peak	Composition
A	Sulfonic acid
B	Largely d-EtSpA^{Bz}
C	dpA
D	Partially unblocked dimer
E	d-pA^{Bz}
F	d-EtSpA^{Bz}pA^{Bz}

^{a} Peak F (fractions 1050-1230) gave 1.33 mmol (29.8%) of desired product.

d-EtSpA^{Bz}pA^{Bz}pA^{Bz} (3). Condensation: d-EtSpA^{Bz}pA^{Bz} (2), 5.5 mmol; d-pA^{Bz}-OAc, 16.5 mmol; MSC, 33 mmol; pyridine, 50 ml. Work-up: 1 M DIEA in pyridine, 70 ml; water, 80 ml; 2 N sodium hydroxide, 200 ml. Chromatography: see Figure 2 and Table III.

(19) E. Ohtsuka, M. Ubasawa, and M. Ikehara, J. Amer. Chem. Soc., 92, 5507 (1970); K. L. Agarwal, A. Yamazaki, and H. G. Khorana, ibid., 93, 2754 (1971).

(20) Purification of solvents and reagents, DEAE-cellulose and paper chromatography, work-up of products, ultraviolet spectroscopy, and enzymology followed the procedures described in ref 1, with the following additional measures: mesitylenesulfonyl chloride (MSC) was decolorized with charcoal during recrystallization. Buffer concentration was measured via conductivity with a CDM2 instrument, Radiometer, Copenhagen, as calibrated with standards of triethylammonium bicarbonate, pH 7.

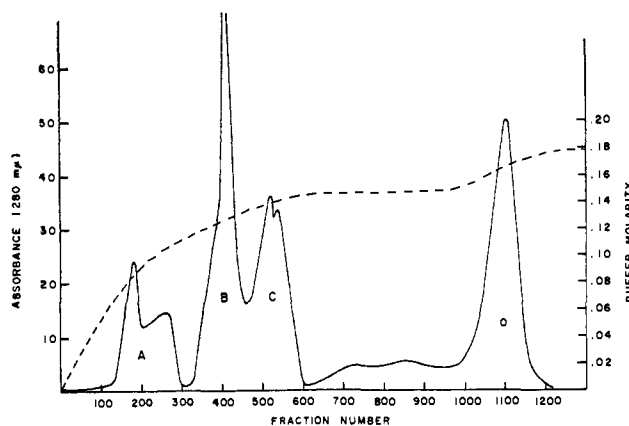(21) At this stage, small aliquots of the total were completely hy-

drolyzed in concentrated ammonia at 60° for 2 hr and monitored by rapid paper chromatography; see T. F. Gabriel, J. Chromatogr., 36, 518 (1968). In the case of larger oligomers, system A was used.

(22) R. M. Bock and N. W. Ling, Anal. Chem., 26, 1541 (1954).
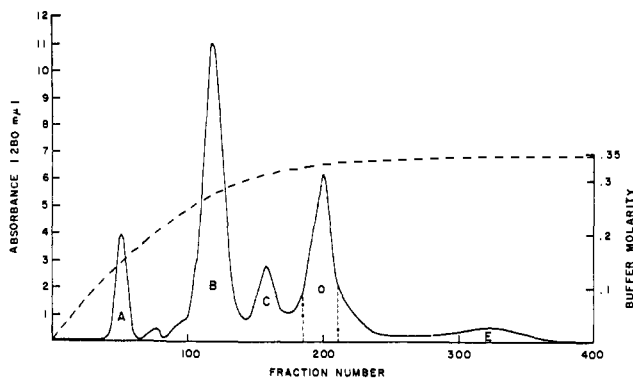(23) T. Wieland and R. Lambert, Chem. Ber., 89, 2476 (1956).

Figure 3. DEAE–cellulose chromatography of d-EtSpA^{Bz}pA^{Bz}-pA^{Bz}pTpTpT (5); column size 2.5 × 100 cm (K 25/100, Pharmacia); convex gradient; reservoir 1.5 l. of 0.35 M triethylammonium bicarbonate; mixing chamber, 1.5 l. of distilled water; fraction size, 18.5 ml.
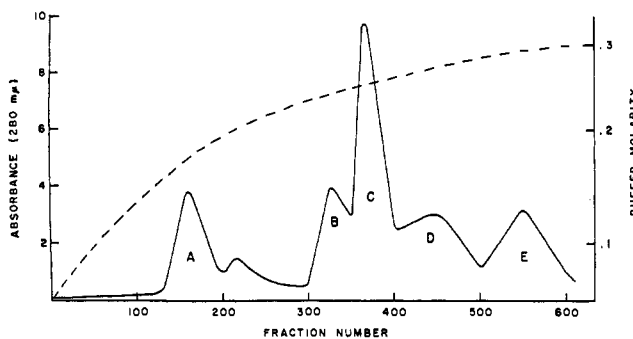

Figure 4. DEAE–cellulose chromatography of d-pG^{i-Bu}pA^{Bz}-pA^{Bz}pA^{Bz} (8); column size 5 × 100 cm (Pharmacia K 50/100); convex gradient; reservoir, 4 l. of 0.3 M triethylammonium bicarbonate; mixing chamber, a like volume of water.

d-EtSpA^{Bz}pA^{Bz}pA^{Bz}pTpTpT (5). Condensation: d-EtSpA^{Bz}-pA^{Bz}pA^{Bz} (3), 0.12 mmol; pTpTpT-OAc, 0.2 mmol; MSC, 0.6 mmol; pyridine, 1 ml. Work-up as usual. Chromatography: see Figure 3 and Table IV.

Table III. Composition of Peaks from the Chromatography in the Synthesis of d-EtSpA^{Bz}pA^{Bz}pA^{Bz} (3)[a]

Peak	Composition
A	Sulfonic acid
B	d-pa^{Bz}
C	Mixture of d-pA^{Bz}, its pyrophosphate, and d-EtSpA^{Bz}pA^{Bz}
D	d-EtSpA^{Bz}pA^{Bz}pA^{Bz}

[a] Peak D (fractions 1035–1140) gave 1.47 mmol of product (27%).

Table IV. Composition of Peaks from the Chromatography in the Synthesis of d-EtSpA^{Bz}pA^{Bz}pA^{Bz}pTpTpT (5)[a]

Peak	Composition
A	Sulfonic acid
B	Both starting trimers
C	Unidentified
D	Hexamer

[a] Peak D (fractions 185–210) gave the hexamer in 32% yield.

d-CEpG^{i-Bu}pA^{Bz} (6). Condensation: d-CEpG^{i-Bu}, 10 mmol; d-pA^{Bz}OAc, 11.3 mmol; MSC, 30 mmol; pyridine, 40 ml. Work-up: 60 ml of 1 M DIEA in pyridine, 20 ml of water. After the usual overnight storage at 4°, the volume was adjusted to 200 ml with cold water, and partial hydrolysis was carried out by the
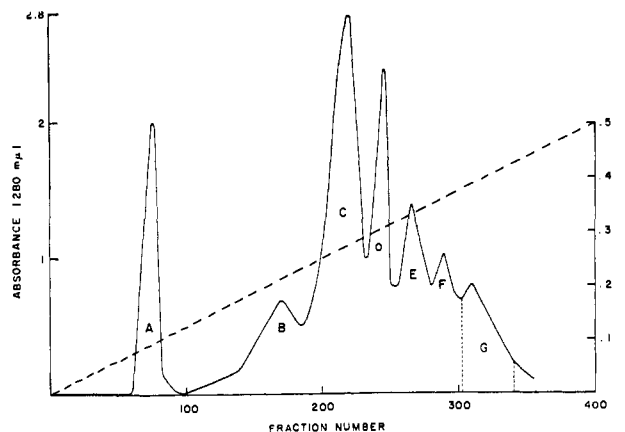
Figure 5. DEAE-cellulose chromatography of d-EtSpA^{Bz}pA^{Bz}-pA^{Bz}pTpTpTpG^{i-Bu}pA^{Bz}pA^{Bz}pA^{Bz} (9); column size 2.5 × 100 cm; linear gradient; reservoir, 4 l. of 0.5 M triethylammonium bicarbonate; mixing chamber, 4 l. of water.

addition of 200 ml of 2 N sodium hydroxide. The solution was allowed to react for 10 min at 0° and worked up as usual. Chromatography on DEAE-cellulose, bicarbonate form (Pharmacia, K50/100), by means of a convex gradient consisting of 4 l. of 0.25 M triethylammonium bicarbonate in the reservoir and 4 l. of water in the mixing chamber gave the desired d-pG^{i-Bu}pA^{Bz} in fractions 350–420. The material was isolated as usual; a yield of 2.9 mmol (29%) was obtained.

Conversion to the cyanoethyl derivative was as follows: a solution of 2.8 mmol of the foregoing product in the pyridinium form (passage over Dowex-50, pyridinium, followed by concentration and drying), 8 ml of hydracylonitrile, 7 g of dicyclohexylcarbodiimide, and 4 g of Dowex-50 (pyridinium) in 30 ml of anhydrous pyridine was allowed to shake in the dark at room temperature. At the end of 24 hr, the reaction mixture was cooled in ice, 30 ml of water was added with agitation, and the resulting suspension was stored at room temperature overnight. Assay by rapid paper chromatography showed the reaction to be complete (RpG^{i-Bu}pA^{Bz} = 1.7 in the 70–30 system).[21] The precipitated dicyclohexylurea was filtered off, the cake was washed with 10% aqueous pyridine, and the combined filtrate and washings were extracted with cyclohexane. The aqueous portion was concentrated to dryness *in vacuo* with the help of several added portions of pyridine, dissolved in a small amount of the latter solvent, and precipitated from anhydrous ether. Precipitation was repeated to give 2.6 g (2 mmol) of a tan powder; the overall yield was 22%.

d-pG^{i-Bu}pA^{Bz}pA^{Bz}pA^{Bz} (8). Condensation: d-CEpG^{i-Bu}pA^{Bz}, 0.35 mmol; d-pA^{Bz}pA^{Bz}-OAc, 0.5 mmol; MSC, 1.5 mmol; pyridine, 5 ml. Work-up: 1 M DIEA in pyridine, 3 ml; water, 1 ml; water overnight storage, total volume is adjusted to 20 ml with water; sodium hydroxide (2 M), 20 ml. Chromatography: see Figure 4 and Table V.

Table V. Composition of Peaks from the Chromatography of d-pG^{i-Bu}pA^{Bz}pA^{Bz}pA^{Bz} [a]

Peak	Composition
A	Sulfonic acid
B + C	Starting dimers
D	d-pA^{Bz}pA^{Bz} and its pyrophosphate
E	Desired tetramer

[a] Peak E (fractions 500–600) gave the tetramer in 17.2% yield (0.06 mmol).

Acetylation. The protected tetramer was rendered anhydrous by several evaporations with pyridine *in vacuo*, dissolved in 10 ml of anhydrous pyridine, and treated with 3 ml of acetic anhydride. After 5 hr at room temperature, the solution was cooled in ice and methanol (3 ml) was added. After standing at room temperature for 15 min, it was cooled again and 3 ml of water was added. After overnight storage, the solution was concentrated, made anhydrous with pyridine, dissolved in 5 ml of dry pyridine, and pre-
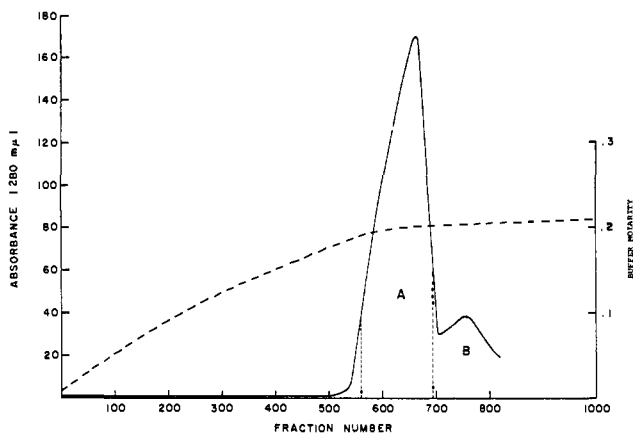
Figure 6. DEAE-cellulose chromatography of d-EtSpTpG^{i-Bu} (14). Details are given in the text.
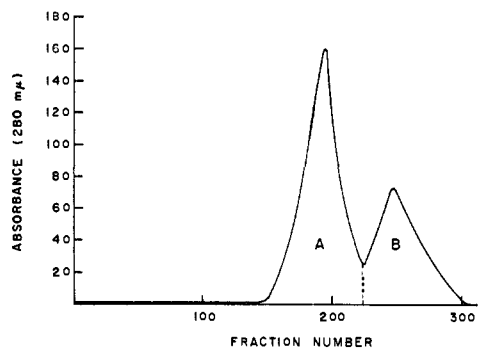

Figure 7. G-25 Sephadex chromatography of d-EtSpTpG^{i-Bu}-pC^{An}pT (16). Details in text.

cipitated in 500 ml of anhydrous ether. After decantation of the ether, the gummy residue was redissolved in 10 ml of dry pyridine and again precipitated in 1 l. of ether. The material was collected as usual; yield was quantitative.

d-EtSpA^{Bz}pA^{Bz}pA^{Bz}pTpTpTpG^{i-Bu}pA^{Bz}pA^{Bz}pA^{Bz} (9). Condensation: d-EtSpA^{Bz}pA^{Bz}pA^{Bz}pTpTpT, 24 μmol; d-pG^{i-Bu}pA^{Bz}-pA^{Bz}pA^{Bz}-OAc, 54 μmol; MSC, 0.5 mmol; pyridine, 0.4 ml. Work-up: 1 ml of 1 M DIEA in pyridine, 0.4 ml of water, then made up to 2 ml with water; 2 ml of 2 N sodium hydroxide. Chromatography: see Figure 5 and Table VI.

Table VI. Composition of Peaks from the Chromatography of d-EtSpA^{Bz}pA^{Bz}pA^{Bz}pTpTpTpG^{i-Bu}pA^{Bz}pA^{Bz}pA^{Bz} ^{a}

Peak	Composition
A	Sulfonic acid
B	Unidentified
C	Tetramer
D	Hexamer
E	Mixture of protected and partially unprotected (overhydrolysis)
F	Unidentified, probably pyrophosphate of tetramer
G	Decamer

^{a} Peak G (fractions 310–340) gave the decamer, 2.2 μmol (9.2%).

Small Scale Preparation of d-pApApApTpTpTpGpApApA (11). An aliquot (10 A_{280}) of 9 was hydrolyzed with 1 ml of concentrated ammonia at 60° for 2 hr. After concentration, the residue was dissolved in 1 ml of 10% aqueous pyridine, and 1 ml of acetone containing 10 mg of iodine was added. After 14 hr at room temperature, the mixture was concentrated, 1 ml of water was added, and the mixture was extracted with three 1-ml portions of ether. The aqueous portion was concentrated and streaked on paper (Whatman no. 1, previously washed with methanol). After de-
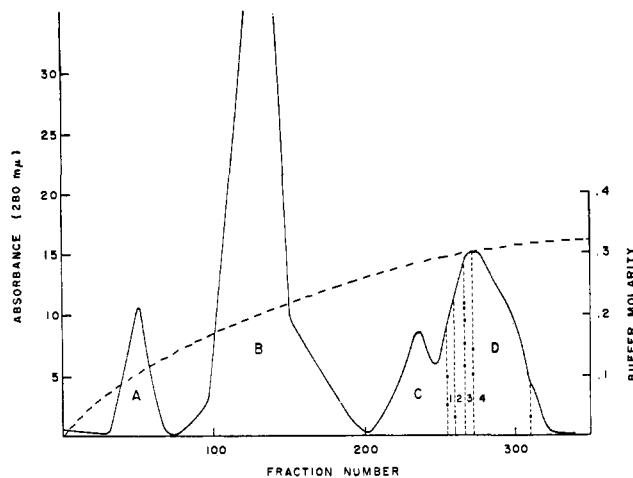

Figure 8. DEAE–cellulose chromatography of d-EtSpA^{Bz}pA^{Bz}-pA^{Bz} pTpTpTpG^{i-Bu}pA^{Bz} (12); column size, 2.5 × 100 cm; convex gradient; triethylammonium bicarbonate (0.38 M, 4 l.) in the reservoir; 4 l. of water in the mixing chamber; fraction size 23 ml.
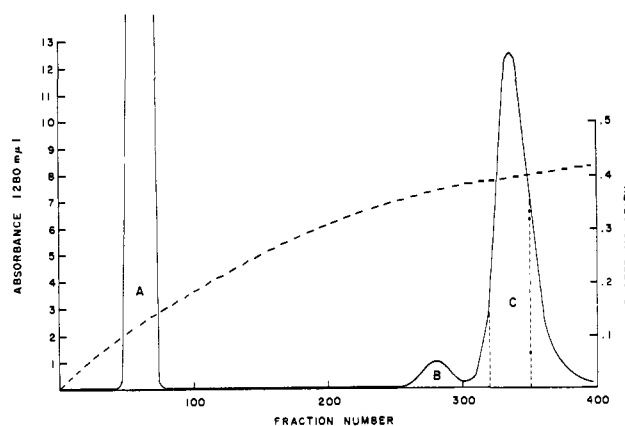

Figure 9. DEAE–cellulose chromatography of octamer pA^{Bz}-pA^{Bz}pA^{Bz}pTpTpTpG^{i-Bu}pA^{Bz} (13). Details in text.

veloping for 3 days in system C, 4 A_{260} were extracted and characterized as to composition after venom diesterase digestion (see Table VIII). Removal of 5'-phosphate and labeling with ATP-γ-^{32}P resulted in label of d-pA to a degree of above 90%. Molecular weight (ultracentifuge): 3120 daltons (calcd 3137).

d-EtSpTpG^{i-Bu} (14). Condensation: EtSpT, 28.5 mmol; d-pG^{i-Bu}-O-i-Bu, 25 mmol; MSC, 85 mmol; pyridine, 85 ml. Work-up: triethylamine, 5 ml; 50% aqueous pyridine, 200 ml; total volume adjusted to 450 ml with 50% aqueous pyridine after the usual overnight period at 4°; 2 N sodium hydroxide, 450 ml.

A first chromatography (5 × 100 cm of DEAE-cellulose connected to a like column in series; reservoir, 9 l. of 0.25 M triethylammonium bicarbonate; mixing chamber, 9 l. of distilled water) failed to resolve the product from the incoming monomer; it was necessary to combine appropriate fractions (410–560), carefully concentrate them in vacuo (frequent addition of pyridine), and subject them a second time to chromatography on a column of DEAE-cellulose, 9 × 110 cm, by a convex gradient consisting of 12 l. of 0.05 M triethylammonium bicarbonate in 20% ethanol in the mixing chamber, and 12 l. of the same buffer (0.3 M) in the reservoir (see Figure 6). Peak A contained the desired dimer (11.8 mM, 47.2%), peak B was monomer d-pG^{i-Bu}.

d-EtSpTpG^{i-Bu}pC^{An}pT (16). Condensation: EtSpTpG^{i-Bu}, 11.8 mmol; d-pC^{An}pT-OAc, 14.1 mmol; MSC, 42 mmol; pyridine, 50 ml. Work-up: 1 M DIEA in pyridine, 85 ml; 50% aqueous pyridine, 85 ml. After overnight standing at 4°, the volume was adjusted to 355 ml with 50% aqueous pyridine; 355 ml of 2 N sodium hydroxide; hydrolysis, 7 min. A preliminary chromatography on DEAE-cellulose (5.5 × 121 cm; convex gradient; reservoir, 8 l. of 0.25 M triethylammonium bicarbonate; mixing chamber, 8 l. of water) failed to separate the desired tetramer from d-pC^{An}pT.
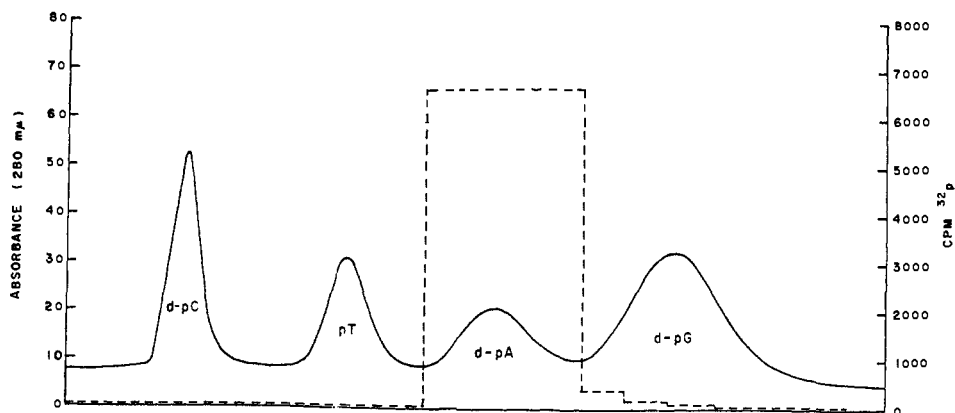
Figure 10. High-pressure liquid chromatography of venom diesterase digest of **13** labeled with ^{32}P at its 5' terminus. The digest was supplemented with the mononucleotides as markers. Solid line, absorbance; dashed line, radioactivity.
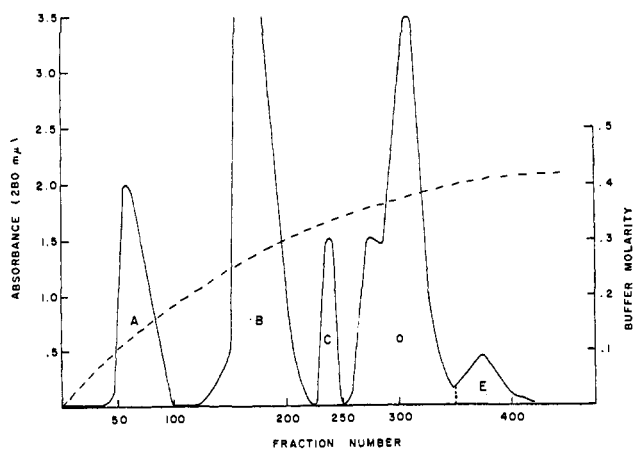

Figure 11. DEAE–cellulose chromatography of d-EtSpTpG^{i-Bu}-pC^{A}pTpA^{Bz}pA^{Bz}pA^{Bz}pTpTpTpG^{i-Bu}pA^{Bz} (**17**). Column size, 2.5 × 100 cm; convex gradient; reservoir 4 l. of 0.5 M triethylammonium bicarbonate; mixing chamber, 4 l. of water.

The peak containing the mixture (fraction 650–910) was further purified in two portions on a G-25 Sephadex (superfine)[14,24] column (see Figure 7), 10 × 100 cm (bed volume, 7 l.), elution with 0.2 M triethylammonium bicarbonate, pH 7.4. Peak A was pure tetramer, peak B the dimer. The yield was 3.5 mmol (30%). A small sample was deacylated and converted to the free 5'-phosphate for characterization. Ultracentrifugation gave a molecular weight of 1280 daltons (theory 1244).

Table VII. Composition of Peaks from the Chromatography of the Octamer **12**

Peak	Composition
A	Sulfonic acid
B	Dimer
C	Hexamer and octamer
D-1	Mixture
-2	80% octamer
-3	90% octamer
-4	Pure octamer

d-EtSpa^{Bz}pA^{Bz}pA^{Bz}pTpTpTG^{i-Bu}pA^{Bz} (**12**). Condensation: d-EtSpA^{Bz}pA^{Bz}pA^{Bz}pTpTpT (**5**), 0.3 mmol; d-pG^{i-Bu}pA^{Bz}-OAc, 2.0 mmol; MSC, 6.0 mmol; pyridine, 5 ml. Work-up: 1 M DIEA in pyridine, 15 ml of 50% aqueous pyridine; made up to 50 ml; 50 ml of 2 N sodium hydroxide. Chromatography: see Figure 8

(24) See also A. Ramel, E. Heimer, S. Roy, and A. L. Nussbaum, *Anal. Biochem.*, **41**, 323 (1971).

and Table VII; obtained 0.09 mmol (28%) of pure octamer from fractions 275–310.

Removal of Alkylthio Substituent. 8000 A_{280} of **12** was dissolved in 50 ml of 10% aqueous pyridine, and 260 mg of iodine in 50 ml of acetone was added. The solution was left at room temperature overnight, and then diluted to 800 ml with water and charged onto a column of DEAE–cellulose in the bicarbonate cycle (dimensions 2.5 × 100 cm). The column was washed with 400 ml of water, followed by a convex gradient consisting of 4 l. of water in the mixing chamber and 0.5 M triethylammonium bicarbonate, pH 7.4, in the reservoir. Flow rate and fraction size were as usual. See Figure 9 for elution profile. Peak A was iodide ion, peak B was not identified (probably the fully protected octamer), and peak C (fractions 320–350) was the desired N-protected octamer with a free 5'-terminal phosphate. The yield was 4800 A_{280} (60%).

Acetylation (13). The foregoing material was dissolved in dry pyridine (5 ml) and cooled in an ice bath. Acetic anhydride (2 ml) was added and the solution was allowed to remain at room temperature for 2 hr and at 4° overnight. It was then concentrated *in vacuo* with frequent additions of pyridine and finally made anhydrous by several coevaporations with pyridine. The residue (no smell of acetic acid) was dissolved in 2 ml of dry pyridine and added dropwise to 300 ml of anhydrous ether. The fine precipitate was collected by centrifugation and dried *in vacuo* over P_2O_5.

An aliquot of **13** was hydrolyzed with concentrated ammonia, dephosphorylated with bacterial alkaline phosphatase, and re-phosphorylated at the 5' terminus with ATP-γ-^{32}P by means of polynucleotide kinase. The labeled oligonucleotide was hydrolyzed to monomers with venom diesterase, and the monomer fractions separated on a high-pressure liquid chromatography apparatus. Assay of radioactivity showed essentially all the radioactivity to be associated with d-pA. Figure 10 is included as typical for analyses of this kind.

d-EtSpTpG^{i-Bu}pC^{A}pTpA^{Bz}pA^{Bz}pA^{Bz}pTpTpTpG^{i-Bu}pA^{Bz} (**17**). Condensation: d-EtSpTpG^{i-Bu}pC^{A}pT (**16**), 290 μmol; octamer acetate **13**, 38.2 μmol; MSC, 90 mg; pyridine, 1 ml. Work-up: 1 M DIEA in pyridine, 1 ml; 50% aqueous pyridine, 1 ml. After the usual overnight standing, the volume was brought up to 8 ml with 50% aqueous pyridine, 2 N sodium hydroxide, 8 ml; ethanol, 2 ml. Chromatography: see Table VIII and Figure 11. The

Table VIII. Composition of Peaks from the Chromatography of Dodecamer **17**

Peak	Composition
A	Sulfonic acid
B	Tetramer
C	Unidentified
D	Octamer
E	Dodecamer

dodecamer was obtained in a yield of 15.4%. A sample of **17** was deacylated and converted into the free 5'-phosphate as above. Radioactive labeling (as described for Figure 10) gave mostly ^{32}pT (>90%). A molecular weight determination gave 3890 daltons (theory 3737) (Tables IX–XI).

Table IX. Nucleotide Compositions in Mole %[a] (Theory)

	pT	d-pA	d-pC	d-pG
d-EtSpTpG (**14**)[b]	51 (50)			49 (50)
d-EtSpTpGpCpT (**16**)	48.6 (50)		27.1 (25)	24.3 (25)
d-EtS(pA)₃(pT)₃ (**5**)	50.8 (50)	49.1 (50)		
d-(pA)₃(pT)₃pGpA (**13**)	37.6 (37.5)	49.3 (50)		13.2 (12.5)
d-EtSpTpGpCpT(pA)₃(pT)₃pGpA (**18**)	41.7 (41.6)	32.3 (33.3)	8.9 (8.4)	16.3 (16.6)
d-EtS(pA)₃(pT)₃pGpA (**12**)	38.6 (37.5)	51.3 (50)		10.1 (12.5)
d-pTpGpCpT(pA)₃(pT)₃pGpA (**19**)	43.8 (41.7)	29.6 (33.3)	10.9 (8.3)	15.8 (16.7)
d-EtS(pA)₃(pT)₃pG(pA)₃ (**11**)	32.8 (30)	58.9 (60)		8.4 (10)
d-pG(pA)₃ (**8**)		75.5 (75)		24.5 (25)

[a] Determined by high-pressure liquid chromatography after deacylation and snake venom diesterase digestion. [b] Numbers refer to the corresponding (generally acylated) derivatives in Charts I and II.

Table X. Paper Chromatography[a]

	A	B	C	D
EtSpApA	1.25	0.96	1.25	
EtSpTpG	1.4	1.2	1.5	
pGpA	0.25	0.54	0.76	0.91
EtSpApApA	0.54	0.69	0.94	
pGpApApA	0.04	0.22	0.30	
EtSpTpGpCpT	0.41	0.76	1.1	
EtS(pA)₃(pT)₃	0.09	0.42	0.78	0.79
EtS(pA)₃(pT)₃pGpA	0.03	0.14	0.37	0.63
(pA)₃(pT)₃pGpA		0.07	0.15	
EtS(pA)₃(pT)₃pG(pA)₃		0.07	0.16	0.39
(pA)₃(pT)₃pG(pA)₃			0.07	
EtSpTpGpCpT(pA)₃(pT)₃pGpA		0.04	0.14	
pTpGpCpT(pA)₃pGpA			0.05	

[a] Systems A, B, and C as in ref 1. System D is isobutyric acid–0.5 M ammonia, 10:6 v/v. Values are relative to pT.

Table XI. Uv Characteristics of Blocked Oligomers[a]

Compound	λ_{max}[b]	λ_{min}[b]	260/280	280/300
EtSpTpG[i-Bu]	260, 280 (shoulder)	231	1.48	3.25
EtSpTpG[i-Bu]pC[An]pT	262, (305 shoulder)	234	1.2	1.36
EtSpA[Bz]pA[Bz]	280	245	0.55	2.4
EtSpA[Bz]pA[Bz]pA[Bz]	280	243–245	0.62	2.34
EtS(pA[Bz])₃(pT)₃	276, 260 (shoulder)	236	0.88	2.76
pGpA[Bz]pA[Bz]pA[Bz]	280, 260	235	0.75	2.22
EtS(pA[Bz])₃(pT)₃pG[i-Bu]pA[Bz]	277	233	0.83	2.73
EtS(pA)₃(pT)₃pG[i-Bu](pA[Bz])₃	286	241	0.91	2.7
EtSpTpG[i-Bu]pC[An]pT(pA[Bz])₃(pT)₃pG[i-Bu]pA[Bz]	275, 262	240	1	2.0

[a] At pH 7. [b] nm.