acid which has a pK_a of 4.2 has its K_a value lowered by a factor of 3.13 in heavy water.[30]

Conclusions

The unimolecular decarboxylation of the zwitterionic carbamic acid species is the main mode of decomposition of carbamates. Direct transfer of CO_2 from arylamine to an acceptor molecule such as hydroxide ion, amine, or sulfhydryl does not appear to be important. The mode of decomposition is similar to the mode of decomposition of phosphates,[31] phosphoramidates,[32] and sulfates,[33] all of which feature leaving group protonation and loose transition states which can only weakly incorporate a molecule of nucleophile. The positive values of $\Delta S^{\pm}$ for carbamate and dithiocarbamate decomposition are consistent with a transition state with unimolecular character.[34] Enzymes containing biotin are able to transfer CO_2 from *N*-carboxybiotin to appropriate acceptors. The enzyme must therefore increase the specificity of the CO_2 transfer reaction by correct positioning of acceptor and biotin-bound CO_2, or by increasing the susceptibility of *N*-carboxybiotin to nucleophilic attack by the acceptor. It is quite likely that metal ions play an important role in complexing with *N*-carboxybiotin in such a way to fulfill this role.

Acknowledgments. We wish to thank Mrs. Carol Guilbert and Mrs. Viera Knoppe for carrying out some of the kinetic determinations. This investigation was supported by the National Science Foundation.

(30) C. K. Rule and V. K. LaMer, *J. Amer. Chem. Soc.*, 60, 1974 (1938).
(31) A. J. Kirby and W. P. Jencks, *ibid.*, 87, 3209 (1965); A. J. Kirby and A. G. Vargolis, *ibid.*, 89, 415 (1967).
(32) W. P. Jencks and M. Gilchrist, *ibid.*, 87, 3199 (1965).
(33) S. J. Benkovic and P. A. Benkovic, *ibid.*, 88, 5504 (1966); S. J. Benkovic, *ibid.*, 88, 5510 (1966).
(34) The $\Delta S^{\pm}$ values for the k_H terms of diethyldithiocarbamate[35] and morpholine carbamate[4] decarboxylation are 23.9 and 2.4 eu, respectively, and the water term for *N*-carboxyimidazolidone decarboxylation[4] has a $\Delta S^{\pm}$ value of 13.9 eu.
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Nucleoside *S*-Alkyl Phosphorothioates. V.[1] Synthesis of a Tridecadeoxyribonucleotide

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Abstract: The tridecadeoxyribonucleotide d-pTpTpApApTpCpCpApTpApTpGpC has been chemically synthesized using the *S*-ethyl group for 5′-phosphate protection. The synthetic approach involved sequential addition of oligonucleotides of size 2–4 monomer units to the growing nucleotide chain containing the *S*-ethyl group. The condensations employed mesitylenesulfonyl chloride as the condensing agent, and the yields were in the range of 17–48%. The *S*-ethyl group was stable under the conditions employed in the synthetic scheme, and was retained until the oligonucleotide chain was completed. It was finally removed from the completed tridecamer by treatment with aqueous iodine. The trinucleotide d-pC^{An}pA^{Bz}pT-OAc and the tetranucleotide d-pA^{Bz}pTpG$^{i\text{-}Bu}$pC^{An}-OAc, both of which were required in the synthesis of the tridecamer, were also prepared using the *S*-ethyl method for 5′-phosphate protection. For comparison, the trimer was also prepared using the cyanoethyl group as the phosphate-protecting group, and the overall yields were closely similar.

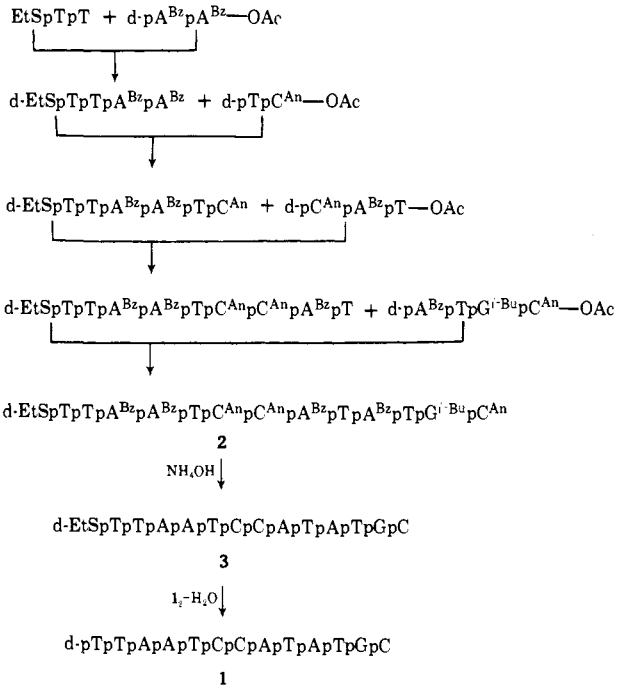
In previous papers from this laboratory,[1-3] the activation and protection of the phosphate groups of nucleotides *via* their alkythio derivates have been described. The activating feature of the *S*-ethyl group lies in its susceptibility to cleavage by a wide variety of nucleophiles in the presence of mild oxidizing agents; in this way a number of nucleotide derivatives have been prepared by variation of the nucleophilic agent.[2] The protecting feature of this group lies in the relative stability of these compounds under the conditions encountered during oligonucleotide synthesis. Thus, an *S*-ethyl group on the 5′-terminus of a growing nucleotide chain protects this chain against self-condensation until completion of the reaction sequence. It is also stable to the alkaline conditions required for removal of 3′-*O*-acyl groups prior to chain extension, and thus obviates the necessity for reprotection of the 5′-phosphate after each elongation, as is required for the cyanoethyl group. Applications to di- and trinucleotide synthesis have been described.[3] A similar approach to oligonucleotide synthesis involving protection of the 5′-phosphate by phosphoramidate formation has recently been reported.[4] The purpose of this paper is to extend the range of the *S*-ethyl phosphorothioate technique to the synthesis of the tridecanucleotide d-pTpTpApApTpCpCpApTpApTpGpC (**1**), an oligonucleotide of sufficient size to permit its efficient coupling with other oligonucleotides to form larger sequences *via* template-guided joining by the enzyme polynucleotide ligase.[5] The particular sequence chosen for synthesis

(1) Paper IV in this series: A. F. Cook, *J. Amer. Chem. Soc.*, 92, 190 (1970).
(2) A. F. Cook, M. J. Holman, and A. L. Nussbaum, *ibid.*, 91, 1522 (1969).
(3) A. F. Cook, M. J. Holman, and A. L. Nussbaum, *ibid.*, 91, 6479 (1969).
(4) E. Ohtsuka, M. Ubasawa, and M. Ikehara, *ibid.*, 92, 5507 (1970).

is one of several being undertaken in this laboratory, the ultimate aim being to prepare, by a combination of chemical and enzymatic methods, a double-stranded deoxynucleotide sequence with coding properties for a derivative of the S peptide of ribonuclease A. Further reports on the overall strategy will be provided at a later date.

The tridecanucleotide in question was synthesized by addition of oligonucleotides of size 2–4 monomer units to the growing chain (Chart I), rather than by stepwise

Chart I. Synthesis of the Tridecanucleotide



addition. This approach is more practical in terms of conservation of the growing nucleotide chain, since the number of condensations is reduced, and the incoming oligonucleotides can readily be prepared in sufficiently large amounts to provide an excess at each step. Larger oligomers (up to 20 monomer units) have been synthesized chemically,[5] but the existing techniques for isolation and identification of these materials become less efficient with increasing size. The protected mononucleotide required for chain initiation of the oligomer in question was thymidine 5′-*S*-ethyl phosphorothioate (EtSpT).[6] The original synthesis of this compound[2] has been improved so that 30- to 40-mmol quantities can be routinely prepared in yields of around 65%. One notable improvement lies in the use of a Dowex 1 (bicarbonate) column for the chromatography of the reaction mixture. This column allows for much greater loading capacity than was previously possible using a DEAE-cellulose column. The dinucleotide EtSpTpT was prepared by two methods. The first involved the condensation of EtSpT with pT-OAc, using mesitylenesulfonyl chloride (MS) as the condensing agent. Although a paper chromatographic examination of the reaction mixture indicated that the reaction was successful, problems were encountered during the column purification procedure. Previous work[3] had indicated that pure dimer could be obtained as its 3′-*O*-acetyl derivative by chromatography on DEAE-cellulose in the acetate form. A scale-up of this procedure did indeed give the dimer, but its separation from pT-OAc was incomplete. Similar results were obtained with the deacetylated derivatives using the bicarbonate form of DEAE-cellulose; a mixture of EtSpTpT and pT was obtained. This mixture was successfully resolved by further chromatography on a silica gel column. Upon elution of the column with methanol–ethyl acetate (3:2), the dimer was almost completely freed from pT, the latter being retained longer on the column. Due to these separation problems, an alternate synthesis of EtSpTpT was studied. This involved initial formation of the dinucleoside phosphate derivative TpT-OAc[7] by condensation of 5′-*O*-tritylthymidine with pT-OAc followed by detritylation of the product. This procedure could be performed on a very large scale since no column purification was necessary, the product being isolated by extraction procedures. A condensation of TpT-OAc with *S*-ethyl phosphorothioate using dicyclohexylcarbodiimide (DCC) as the condensing agent gave, after ammonia treatment, EtSpTpT and, in this case, the column purification was relatively simple, and pure material was obtained in 43% yield. This method would be the method of choice for the preparation of EtSpTpT. The tetramer d-EtSpTpTpA^{Bz}pA^{Bz} was synthesized in 21% yield by reaction of EtSpTpT (5.9 mmol) with the dinucleotide d-pA^{Bz}pA^{Bz}OAc[8] (6.4 mmol) using mesitylenesulfonyl chloride as the condensing agent. For synthesis of the hexamer, d-EtSpTpTpA^{Bz}pA^{Bz}pTpC^{An}, the tetramer was condensed with a threefold excess of the dinucleotide d-pTpC^{An}-OAc.[9] After column purification and precipitation from pyridine–ether, the hexamer was isolated in 48% yield.

For elongation of the chain to the nonamer level, the trimer d-pC^{An}pA^{Bz}pT-OAc was required. For comparison, this material was synthesized by two methods: (a) using the cyanoethyl group for 5′-phosphate protection, and (b) using the *S*-ethyl group. In the former method, the dinucleotide d-CEpC^{An}pA^{Bz} was prepared as described in the literature[9] and coupled with pT-OAc using mesitylenesulfonyl chloride as the condensing agent.[10] After partial hydrolysis and O-acetylation the overall yield of d-pC^{An}pA^{Bz}pT-OAc from protected mononucleotide was 16%. The second method involved d-EtSpC^{An} as the starting material—this was obtained from 3′-*O*-acetyl-*N*-anisoyldeoxycytidine by the conventional method.[3] Condensation of d-EtSpC^{An} with d-pA^{Bz}-OAc in the usual way gave, after partial hydrolysis, the protected dinucleotide d-EtSpC^{An}pA^{Bz}. A further condensation with pT-OAc gave the *S*-ethyl trinucleotide, from which the *S*-ethyl group was removed by treatment with iodine in water–acetone–pyridine. After acetylation the overall yield of trimer from monomer was 14%, in comparison to the yield of

(5) K. L. Agarwal, *et al.*, *Nature (London)*, **227**, 27 (1970).

(6) The abbreviated nomenclature follows that described in *J. Biol. Chem.*, **241**, 527 (1966); H. Schaller and H. G. Khorana, *J. Amer. Chem. Soc.*, **85**, 3841 (1963). In addition, EtSp- refers to 5′-*S*-ethyl phosphorothioate.

(7) T. M. Jacob and H. G. Khorana, *ibid.*, **87**, 368 (1965). The preparation for TrTpT was employed, except that a reaction with 80% acetic acid at 100° was substituted for the final alkaline treatment.

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

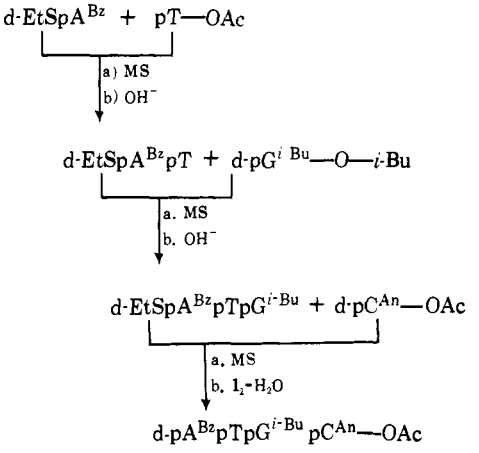
(9) A. Kumar and H. G. Khorana, *ibid.*, **91**, 2743 (1969).

(10) We are indebted to Mr. G. Mack and Mrs. E. McElligott Clericuzio for assistance in the preparation of this material, as well as some of the protected mononucleotides.

16% obtained by the cyanoethyl method. In this case the *S*-ethyl group must be removed before coupling to the growing chain, and since the cyanoethyl derivative of d-pC^{An} is more accessible than the *S*-ethyl derivative, the *S*-ethyl method offers no particular advantage for the synthesis of short fragments of this kind.

The *S*-ethyl nonanucleotide d-EtSpTpTpA^{Bz}pA^{Bz}pTpC^{An}pC^{An}pA^{Bz}pT was prepared by condensation of the hexamer (0.2 mmol) with a fivefold excess of the previously prepared d-pC^{An}pA^{Bz}pT-OAc. After a brief treatment of the reaction mixture with alkali to remove the 3′-O-acetyl groups, the nonamer was isolated by DEAE-cellulose ion-exchange chromatography in 17% yield. The corresponding completely unprotected nonamer had also been prepared in this laboratory by another method, and used for gel permeation studies.[11] For extension of the chain to the tridecamer level, the blocked tetranucleotide d-pA^{Bz}pTpG$^{i\text{-}Bu}$pC^{An}-OAc was required. This material was also prepared using the *S*-ethyl as the protecting group for 5′-phosphate (Chart II), the required starting material being

Chart II. Synthesis of d-pA^{Bz}pTpG$^{i\text{-}Bu}$pC^{An}-OAc



d-EtSpA^{Bz}. Our previous work[2] had reported on the small-scale synthesis of this material by condensation of 3′-*O*-acetyl-*N*-benzoyldeoxyadenosine with *S*-ethyl phosphorothioate. The reported synthesis of the acetylated nucleoside from *N*-benzoyldeoxyadenosine[12] involved successive di-*O*-para-methoxytritylation, acetylation, and removal of the trityl group. In our hands the last step in this sequence was difficult to control, since the acid conditions necessary for removal of the trityl group also caused extensive depurination. We have, therefore, found it more convenient to prepare d-EtSpA^{Bz} from *N*-benzoyldeoxyadenosine[13] rather than from its 3′-*O*-acetyl derivative. As expected, the yields of d-EtSpA^{Bz} from this reaction were generally lower, the main contaminant being the 3′,5′-di-*S*-ethyl phosphorothioate, but the lower yields were offset by the greater accessibility of starting material. The product was shown to be free of the 3′-*S*-ethyl isomer since a snake venom diesterase digest of an ammonia hydrolysate completely degraded the material to d-pA. After isolation of d-EtSpA^{Bz} as its triethylammonium salt, it was coupled with pT-OAc using mesitylenesulfonyl chloride, and after the usual brief alkaline treatment the dinucleotide d-EtSpA^{Bz}pT was isolated by DEAE-cellulose column chromatography. Condensation with d-pG$^{i\text{-}Bu}$-O-*i*-Bu produced the trinucleotide, but the isolation of this material presented problems. Paper chromatographic examination of the eluates from a DEAE-cellulose column indicated that the trimer d-EtSpA^{Bz}pTpG$^{i\text{-}Bu}$ was impure and a second column was therefore required for isolation of pure material. Preparation of the tetramer d-pA^{Bz}pTpG$^{i\text{-}Bu}$pC^{An}-OAc was achieved in 30% yield by condensation of the trimer (1 mmol) with a fivefold excess of d-pC^{An}-OAc. After an aliquot of the reaction mixture had been hydrolyzed and examined by paper chromatography to confirm the presence of tetramer, the bulk was treated with aqueous iodine to remove *S*-ethyl groups, and purified by column chromatography. Although the iodine treatment has been shown not to affect acyl-protecting groups, it was thought necessary to include an acetylation step after column chromatography due to the mildly alkaline conditions encountered using the bicarbonate buffer. The final chain-elongation step, *i.e.*, synthesis of the tridecamer, was achieved by condensation of the nonamer (23 μmol) with a sevenfold excess of d-pA^{Bz}pTpG$^{i\text{-}Bu}$pC^{An}-OAc, and the mixture was applied to a DEAE-cellulose column in the usual way. The protected tridecamer (**2**) was isolated from the column in 23% yield. A sample was treated with ammonium hydroxide to remove all protecting groups except *S*-ethyl, and this material (**3**) was purified using a DEAE-cellulose (chloride) column in 7 *M* urea.[14] The isolation of a single peak from this column confirmed the homogeneity of the original material. Another portion of **2** was treated with ammonium hydroxide, followed by aqueous iodine and purified in the same way to give the completely unprotected tridecamer **1**. Since it was not possible to determine the extent of removal of the *S*-ethyl group from **3** by paper chromatographic methods, an alternate method involving enzymatic techniques was employed. The 5′-terminal phosphomonoester was removed from the product using bacterial alkaline phosphatase,[15] and the liberated 5′-hydroxyl group was phosphorylated using γ-^{32}P-ATP and the enzyme polynucleotide kinase.[16] The extent of incorporation of ^{32}P into the oligonucleotide indicated that greater than 90% of the original material was present as the 5′-monoester **1** rather than its *S*-ethyl derivative **3**. In addition the labeled tridecamer obtained by this process was degraded to its component mononucleotides using snake venom diesterase, and the monomers were resolved on an ion-exchange column.[17] The only mononucleotide containing ^{32}P was found to be pT, thus confirming the homogeneity of the 5′ terminus of the chain. Further confirmation of the structure of tridecamer came from molecular weight determinations:[18] both the unprotected oligonucleotide and its *S*-ethyl derivative gave the expected values within acceptable error.

(11) A. Ramel, E. Heimer, S. Roy, and A. L. Nussbaum, *Anal. Biochem.*, in press.
(12) M. W. Moon, S. Nishimura, and H. G. Khorana, *Biochemistry*, **5**, 937 (1966).
(13) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *J. Amer. Chem. Soc.*, **85**, 3821 (1963).
(14) G. M. Tener, *Methods Enzymol.*, 12, 398 (1967).
(15) *S*-Ethyl derivatives are not degraded by this enzyme.
(16) B. Weiss, T. R. Live, and C. C. Richardson, *J. Biol. Chem.*, **243**, 4530 (1968).
(17) Performed by Mr. T. Gabriel. Details will be provided at a later date.
(18) By a sedimentation equilibrium method in the Spinco Model E ultracentrifuge equipped with ultraviolet scanning optics; details are to be described by P. Bartl and D. Luk of these laboratories.

Table I. Spectral Characteristics of Protected Oligonucleotides

Compound	λ_{max}, nm	λ_{min}, nm	260/280	280/300
d-pC^{An}pA^{Bz}pT-OAc	281	240	0.77	1.33
d-EtSpA^{Bz}pTpG$^{i\text{-}Bu}$	262, 276	233	1.01	2.73
d-pA^{Bz}pTpG$^{i\text{-}Bu}$pC^{An}-OAc	262, 279	235	0.92	1.47
d-EtSpTpTpA^{Bz}pA^{Bz}	275	233	0.86	3.11
d-EtSpTpTpA^{Bz}pA^{Bz}pTpC^{An}	276	236	0.85	1.80
d-EtSpTpTpA^{Bz}pA^{Bz}pTpC^{An}pC^{An}pA^{Bz}pT	278	242	0.79	1.59
d-EtSpTpTpA^{Bz}pA^{Bz}pTpC^{An}pC^{An}pA^{Bz}pTpA^{Bz}-pTpG$^{i\text{-}Bu}$pC^{An}	277	239	0.91	1.47

Table II. Paper Chromatography of Oligonucleotides, R_f Values

	Solvent system		
	A	B	C
EtSpT	0.75	0.88	0.74
d-EtSpC	0.71	0.82	0.75
d-EtSpA	0.68	0.77	0.78
EtSpTpT	0.59	0.78	0.64
d-EtSpCpA	0.37	0.65	0.62
d-EtSpApT	0.47	0.70	0.65
d-EtSpCpApT	0.21	0.59	0.50
d-EtSpApTpG	0.15	0.51	0.37
d-EtSpTpTpApA	0.40[a]	0.45	0.41
d-pApTpGpC	0.03[a]	0.21	0.42[a]
d-EtSpTpTpApApTpC	0.05[a]	0.28	0.67[a]
d-EtSpTpTpApApTpCpCpApT		0.07	0.29[a]
d-EtSpTpTpApApTpCpCpApTpApTpGpC			0.07[a]

[a] Calculated with respect to pT.

I lists uv characteristics of protected oligomers. Paper chromatography was carried out by the descending technique, using Whatman No. 1 paper. The following solvent systems were routinely employed: solvent A, ethanol–1 *M* ammonium acetate, pH 7 (7:3, v/v); solvent B, ethanol–1 *M* ammonium acetate, pH 7 (1:1, v/v); solvent C, 1-propanol–concentrated ammonium hydroxide–water (55:10:35, v/v/v). Table II lists pertinent R_f values. Detection of sulfur-containing spots on paper chromatograms was carried out as described by Wieland and Lambert.[20] This technique was not applicable to the larger oligonucleotides due to their smaller sulfur content. The base compositions of the oligonucleotides were determined by ammonia hydrolysis followed by enzymatic digestion with snake venom diesterase. The mononucleotides produced were assayed either by paper chromatography followed by extraction and uv measurement of the appropriate spots, or by separation on an analytical ion-exchange column.[17] In addition, the presence of an *S*-ethyl group could be detected by the smell of ethanethiol liberated during the digestion. See analyses listed in Table III.

Table III. Venom Diesterase Digestion of Oligonucleotides

Compound	Ratio of mononucleotides (theory)			
	pT	d-pC	d-pA	d-pG
d-EtSpCpA		1 (1)	1.1 (1)	
d-EtSpApT	1.1 (1)		1 (1)	
d-EtSpCpApT	1 (1)	1.0 (1)	0.95 (1)	
d-EtSpApTpG	1.1 (1)		0.9 (1)	1 (1)
d-EtSpTpTpApA	1 (1)		1.0 (1)	
d-pApTpGpC	1.2 (1)	1 (1)	1.0 (1)	1.0 (1)
d-EtSpTpTpApApTpC	2.8 (3)	1.0 (1)	2 (2)	
d-EtSpTpTpApApTpCpCpApT	4.0 (4)	2.3 (2)	3 (3)	
d-EtSpTpTpApApTpCpCpApTpApTpGpC	4.6 (5)	2.8 (3)	4 (4)	1.0 (1)

Experimental Section

General Methods. Pyridine was purified by treatment with chlorosulfonic acid (15 ml/l.) followed by distillation, redistillation over potassium hydroxide pellets, and storage over Linde Molecular Sieve Type 4A. Mesitylenesulfonyl chloride was freshly crystallized from dry pentane for each condensation. Materials were made anhydrous before each condensation reaction by repeated evaporation of dry pyridine over the mixture (at least three times), and additions of reagents were made in a drybox. DEAE-cellulose columns employed DE 23 cellulose (Whatman) and were packed and preequilibrated in the starting buffer to be used in the gradient. Unless otherwise stated, the bicarbonate form of DEAE-cellulose was used with a linear gradient of aqueous triethylammonium bicarbonate, pH 7.5, and columns were run at 5°. Convex gradients employed a constant volume mixing chamber. Fractions of 20 ml were collected. Evaporation of column fractions was carried out at a temperature not exceeding 25°, with occasional addition of pyridine. Precipitations were carried out by dropwise addition of solution of the material in anhydrous pyridine (or methanol) to a large volume of vigorously stirred anhydrous ether. The precipitate was collected by centrifugation and washed three times by centrifugation with ether. The yields of condensation reactions were calculated without regard to the hypochromicity of the products. The extinction values employed at 260, 280, and 300 mμ, respectively, in neutral solution were as follows: T (8800, 6400, 200); C^{An} (12,800, 17,700, 22,300); A^{Bz} (10,800, 18,300, 5900); G$^{i\text{-}Bu}$ (16,700, 11,500, 4000).[19] Ultraviolet measurements were determined using a Cary Model 15 spectrophotometer. Table

EtSpT. An anhydrous mixture of 3′-*O*-acetylthymidine (17 g, 60 mmol) in pyridine (250 ml) was treated with DCC (100 g) in anhydrous pyridine (250 ml) with shaking for 24 hr at room temperature. The reaction mixture was treated with concentrated ammonium hydroxide (500 ml) overnight and filtered, and pyridine and ammonia were removed from the filtrate by evaporation. The aqueous solution was diluted to 7 l. and filtered, and the filtrate was applied to a Dowex 1-X8 column (55 × 3.5 cm, bicarbonate form). The column was eluted at room temperature with a convex gradient of bicarbonate buffer (9 l. of 0.05 *M* in the mixing chamber, and 0.3 *M* buffer in the reservoir). Fractions of 300 ml were collected, and fractions 40–60 were evaporated to dryness, dissolved in water, and converted into the sodium salt by passage over a column of Dowex 50 resin (sodium form). The eluate was evaporated to dryness, dried by evaporation of ethanol over the residue, and dissolved in dry methanol (150 ml). Precipitation occurred when this solution was added dropwise with stirring to dry ether (6 l.). The precipitate was collected, washed with ether, and dried *in vacuo* to give 16.3 g (38.6 mmol, 64%) of EtSpT as the sodium salt.

EtSpTpT. (a) From EtSpT. An anhydrous solution of EtSpT (15 mmol, pyridinium salt) and pT-OAc (30 mmol) in pyridine (50 ml) was treated with mesitylenesulfonyl chloride (11.5 g) for 2 hr at room temperature. Water (50 ml) was added with cooling to 0°, and the mixture was stored overnight, diluted to 1 l., and applied to

(19) M. Poonian, personal communication.
(20) T. Wieland and R. Lambert, *Chem. Ber.*, 89, 3476 (1956).

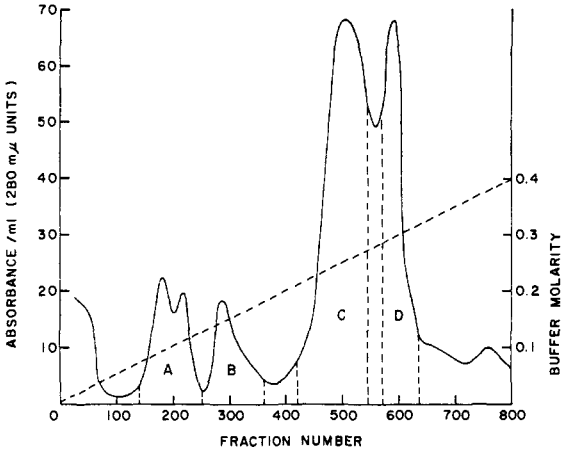


Figure 1. Condensation of EtSpTpT with d-pA^{Bz}pA^{Bz}-OAc. Separation of the products on a DEAE-cellulose column. For identification of the peaks, see text.

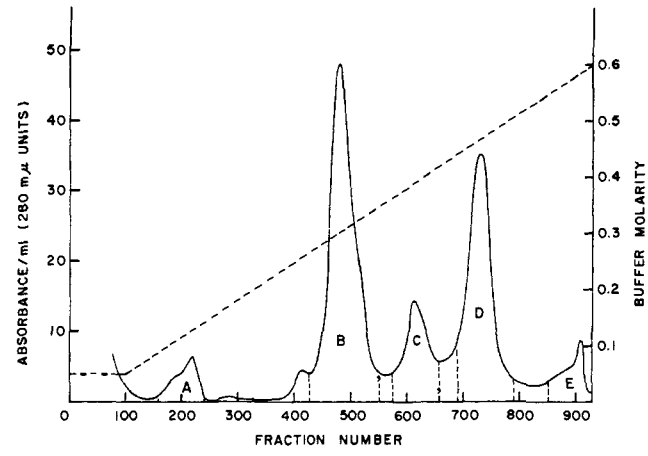


Figure 2. Condensation of d-EtSpTpTpA^{Bz}pA^{Bz} with d-pTpC^{An}-OAc. Chromatographic separation of the products on a DEAE-cellulose column. For explanation, see text.

a DEAE-cellulose column (85 × 6.5 cm). The column was eluted with a convex buffer gradient (6 l. of 0.05 *M* in the mixing vessel and 0.2 *M* in the reservoir). The required product was eluted in fractions 301–490, but paper chromatography of aliquots indicated that it was contaminated with mononucleotide. The mixture was therefore treated with concentrated ammonium hydroxide (200 ml) overnight, and evaporated to dryness. The residue was dried by evaporation of ethanol over the residue, and dissolved in methanol–ethyl acetate (3:2, v/v, 400 ml). The solution was applied to a silica gel column (70 × 4.5 cm) which was eluted with the same solvent. Fractions 96–132, which contained pure EtSpTpT, were evaporated to dryness and precipitated from pyridine (90 ml) with ether (3.5 l.) in the normal way. The precipitate was collected, washed, and dried to give 5.7 mmol (38%) of EtSpTpT.

(b) From TpT-OAc. TrTpT-OAc was prepared as described in the literature[7] and detritylation of this compound was carried out with 80% acetic acid at 100° for 30 min. The solution was evaporated to dryness and made anhydrous using pyridine, and TpT-OAc was obtained in solid form by precipitation from pyridine–ether in the usual way.

An anhydrous solution of *S*-ethyl phosphorothioate (33.5 mmol, pyridinium salt) and TpT-OAc (13.4 mmol) in pyridine (100 ml) was treated with a solution of DCC (13.4 g) in anhydrous pyridine (60 ml). The mixture was shaken at room temperature for 60 hr, and water (40 ml) was added. After 8 hr, concentrated ammonium hydroxide (200 ml) was added, and the mixture was stored overnight at room temperature. The solids were filtered off and washed with 50% aqueous pyridine, and the filtrate was evaporated to remove ammonia and pyridine. Filtration through Celite was necessary at this stage to remove fine particles. The filtrate was diluted to 1 l. and applied to a DEAE-cellulose column (90 × 4.5 cm), which was eluted at 25° with a gradient of 8 l. of 0.005 *M* buffer in the mixing vessel and 8 l. of 0.25 *M* buffer in the reservoir. Fractions 240–430 were evaporated to dryness, dissolved in anhydrous methanol (25 ml), and precipitated by addition to ether (1500 ml) in the usual way. The solid was washed and dried *in vacuo* to give 5.8 mmol (43%) of EtSpTpT.

d-EtSpTpTpA^{Bz}pA^{Bz}. A mixture of EtSpTpT (5.9 mmol) and d-pA^{Bz}pA^{Bz}-OAc[8] (6.4 mmol) in pyridine (40 ml) was treated with mesitylenesulfonyl chloride (6.6 g) for 2.5 hr. Aqueous pyridine (50%, 40 ml) was added to the cooled reaction mixture, and after storage overnight, the solution was treated with aqueous sodium hydroxide (2 *N*, 80 ml) at 0° for 10 min. The solution was neutralized by addition of excess Dowex 50 resin (pyridinium form), and the resin was filtered off and washed with 50% aqueous pyridine. The filtrate was diluted to 1 l. and applied to a DEAE-cellulose column (95 × 6 cm). The conditions and chromatographic pattern are shown in Figure 1. The contents of the fractions were identified by hydrolysis of aliquots with ammonia, followed by paper chromatographic examination using solvent systems A and C. Peak A contained mesitylenesulfonic acid; peak B contained EtSpTpT; peak C consisted of d-pA^{Bz}pA^{Bz}; peak D contained the required tetranucleotide d-EtSpTpTpA^{Bz}pA^{Bz} together with traces of d-pA^{Bz}pA^{Bz}. After evaporation of peak D and precipitation from pyridine (20 ml) with ether (1 l.) in the usual way, 1.24 mmol (21%) of tetramer was isolated.

Preparation of d-EtSpTpTpA^{Bz}pA^{Bz}pTpC^{An}. An anhydrous solution of d-EtSpTpTpA^{Bz}pA^{Bz} (1 mmol) and d-pTpC^{An} (3 mmol)[9] in pyridine (10 ml) was treated with mesitylenesulfonyl chloride (2.3 g) for 2.5 hr at room temperature. The product was cooled to 0°, and diisopropylethylamine in pyridine (1 *M*, 20 ml) and water (20 ml) were added. After storage overnight at 5°, the solution was diluted with water (25 ml) and treated at 0° with an ice-cold solution of sodium hydroxide (2 *N*, 80 ml) for 20 min. Excess Dowex 50 resin (pyridinium form) was added with stirring, and the neutral solution was filtered and diluted to 500 ml with water. Ethanol was added to give a 20% (v/v) ethanolic solution, and the product was applied to a DEAE-cellulose column (77 × 4.5 cm). The chromatographic elution pattern is shown in Figure 2. Peak A contained mesitylenesulfonic acid, and peak B contained d-pTpC^{An}. Peak C consisted of starting tetranucleotide, d-EtSpTpA^{Bz}pA^{Bz}-pTpC^{An} was located in peak D, and peak E was unidentified. The yield of hexanucleotide, after precipitation in the usual way, was 48%.

d-pC^{An}pA^{Bz}pT-OAc, Using *S*-Ethyl Method. (a) d-EtSpC^{An}pA^{Bz}. A mixture of d-EtSpC^{An} (2.1 mmol)[3] and d-pA^{Bz}-OAc (3.5 mmol) in pyridine (10 ml) was treated with mesitylenesulfonyl chloride (1.35 g) for 3 hr at room temperature. Water (10 ml) was added with cooling, and after 18 hr the solution was treated with aqueous sodium hydroxide (2 *N*, 50 ml) for 25 min at room temperature. After neutralization with Dowex 50 resin (pyridinium form) the solution was filtered, diluted to 150 ml with water, and applied to a DEAE-cellulose column (50 × 4.5 cm). A gradient of 4 l. of 0.05 *M* buffer in the mixing vessel and 4 l. of 0.3 *M* buffer in the reservoir was applied. Fractions 230–304 contained d-EtSpC^{An}pA^{Bz} (0.59 mmol, 28%).

(b) d-pC^{An}pA^{Bz}pT-OAc. An anhydrous solution of d-EtSpC^{An}-pA^{Bz} (0.5 mmol) and pT-OAc (1.5 mmol) in pyridine (4 ml) was treated with mesitylenesulfonyl chloride (440 mg) for 2.5 hr, cooled to 0°, and treated with water (4 ml). After 18 hr at room temperature, the solution was diluted to 100 ml with water and applied to a DEAE-cellulose column (50 × 3.5 cm, acetate form). The column was eluted with a gradient of 4 l. of 0.05 *M* triethylammonium acetate, pH 6, in the mixing chamber and 4 l. of 0.4 *M* acetate buffer, pH 6, in the reservoir. d-EtSpC^{An}pA^{Bz}pT-OAc (0.29 mmol, 58%) was eluted in fractions 263–340. These fractions were evaporated to dryness, dissolved in water (20 ml) and pyridine (10 ml), and treated with a solution of iodine (600 mg) in acetone (20 ml). After storage overnight at room temperature, the solution was briefly evaporated to remove acetone, and diluted to 100 ml with water. This solution was applied to a DEAE column (50 × 2.3 cm) which was eluted with a gradient of 2 l. of 0.005 *M* buffer in the mixing vessel and 2 l. of 0.5 *M* buffer in the reservoir. d-pC^{An}-pA^{Bz}pT (0.20 mmol, 88%) was eluted in fractions 100–125. After evaporation to dryness, the product was acetylated using acetic anhydride (2 ml) in pyridine (10 ml) for 18 hr at 5°, and precipitated in the usual way.

d-pC^{An}pA^{Bz}pT-OAc *via* Cyanoethyl Method.[10] A mixture of d-CEpC^{An}pA^{Bz} (3 mmol)[9] and pT-OAc (15 mmol) in pyridine (30 ml) was treated with mesitylenesulfonyl chloride (10 g) for 2 hr at room temperature. The solution was cooled to 0° and treated with diisopropylethylamine in pyridine (1 *M*, 90 ml) and water (20 ml).

After storage overnight at 5°, an equal volume of aqueous sodium hydroxide (2 *N*) was added, followed by ethanol until a clear solution was obtained. The solution was allowed to stand at 0° for 20 min and then neutralized by addition of Dowex 50 (pyridinium form). The neutral solution was filtered and the resin was washed with 10% aqueous pyridine. The filtrate and washings (volume 1200 ml) were applied to a DEAE-cellulose column (100 × 5 cm) and a convex buffer gradient was started (4 l. of water in the mixing vessel and 0.25 *M* bicarbonate buffer in the reservoir). Fractions 550–740 contained pure trinucleotide as shown by paper chromatography of hydrolyzed aliquots in solvent system A. After evaporation of the pooled fractions to dryness, the residue was treated with acetic anhydride (10 ml) in dry pyridine (30 ml) at room temperature for 4 hr. The product was cooled to 0°, treated with 50% aqueous pyridine (20 ml), and stored at 5° overnight. After evaporation of the solvents, the residue was made anhydrous, dissolved in pyridine (20 ml), and precipitated by addition with stirring to ether (800 ml). The precipitate was collected, washed with ether, and dried *in vacuo* to give 1.9 mmol (63%) of d-pC^{An}-pA^{Bz}pT-OAc.

d-EtSpTpTpA^{Bz}pA^{Bz}pTpC^{An}pC^{An}pA^{Bz}pT. An anhydrous solution of d-EtSpTpTpA^{Bz}pA^{Bz}pTpC^{An} (0.2 mmol) and d-pC^{An}pA^{Bz}-pT-OAc (1 mmol) in pyridine (10 ml) was treated with mesitylenesulfonyl chloride (0.95 g) for 2 hr at room temperature, and then cooled to 0°. A solution of diisopropylethylamine in pyridine (1 *M*, 8.6 ml) was added, followed by water (10 ml), and the mixture was stored overnight at 5°. The solution was treated at 0° for 20 min with an equal volume of ice-cold aqueous sodium hydroxide (2 *N*) and neutralized with Dowex 50 resin in the usual way. After filtration, the filtrate was diluted to 400 ml with water, and applied to a DEAE-cellulose column (88 × 4.5 cm). A convex buffer gradient was employed, consisting of 6 l. of 0.005 *M* buffer in 20% ethanol in the mixing vessel and 0.45 *M* buffer containing 20% ethanol in the reservoir. The chromatographic elution pattern is shown in Figure 3. Peak A contained mesitylenesulfonic acid, and peak B consisted of recovered trinucleotide d-pC^{An}pA^{Bz}pT. Peak C contained starting hexanucleotide, and peak D the symmetrical pyrophosphate of d-pC^{An}pA^{Bz}pT. Peak E was not identified and peak F contained the required nonanucleotide. The yield was 34 μmol (17%).

d-EtSpA^{Bz}. A mixture of *N*-benzoyldeoxyadenosine[12] (7.1 g, 20 mmol) and pyridinium *S*-ethyl phosphorothioate (30 mmol) in pyridine (60 ml) was treated with a solution of DCC (20.6 g) in pyridine (80 ml) and shaken vigorously for 18 hr at room temperature. The reaction mixture was cooled and allowed to stand at 5° after addition of water (150 ml). The solids were filtered through Celite and washed with water, and the filtrate and washings were combined and diluted to 2 l. This solution was adjusted to pH 7 with triethylamine and applied to a DEAE-cellulose column (89 × 5 cm). The column was eluted with a gradient of 12 l. of 0.005 *M* bicarbonate buffer in the mixing vessel and 12 l. of 0.1 *M* buffer in the reservoir. Fractions 341–520 contained pure d-EtSpA^{Bz} as determined by paper chromatography. These fractions were evaporated to dryness, made anhydrous, and precipitated from pyridine (150 ml) with ether (4 l.). d-EtSpA^{Bz} (6.6 mmol, 33%) was obtained as a white solid.

d-EtSpA^{Bz}pT. A mixture of d-EtSpA^{Bz} (6.6 mmol) and pT-OAc (11.1 mmol) in pyridine (20 ml) was treated with mesitylenesulfonyl chloride (7.4 g) for 2.5 hr at room temperature. The solution was cooled in an ice bath and diisopropylethylamine in pyridine (*M*, 20 ml) and water (25 ml) were added. After storage overnight, the solution was treated at 0° with an equal volume of ice-cold aqueous sodium hydroxide (2 *N*) for 20 min. Excess Dowex 50 resin (pyridinium form) was added with stirring, and the neutral solution was filtered and the resin washed with water. The filtrate and washings were combined, diluted to 1 l. with water, and applied to a DEAE-cellulose column (94 × 6.5 cm). The column was eluted with a gradient of 9 l. of 0.005 *M* buffer in the mixing chamber and 9 l. of 0.3 *M* buffer in the reservoir. Fractions 640–730 contained pure d-EtSpA^{Bz}pT as shown by paper chromatography of aliquots. These fractions were combined, evaporated to dryness, made anhydrous with pyridine, and precipitated by addition of a pyridine solution (25 ml) to ether (1800 ml). The precipitate was washed and dried to give 2.74 mmol (42%) of d-EtSpA^{Bz}pT.

d-EtSpA^{Bz}pTpG$^{i\text{-}Bu}$. A mixture of d-EtSpA^{Bz}pT (4.2 mmol) and d-pG$^{i\text{-}Bu}$-O-*i*-Bu (12.6 mmol) in pyridine (15 ml) was treated with mesitylenesulfonyl chloride (5.5 g) for 2.5 hr at room temperature. The solution was cooled to 0° and treated with diisopropylethylamine in pyridine (1 *M*, 50 ml) followed by water (35 ml). After storage overnight at 5° the solution was diluted to 150 ml with 50% aqueous pyridine and treated at 0° for 5 min with an equal volume of precooled sodium hydroxide solution (2 *N*). The product was neutralized with Dowex 50 resin (pyridinium form) and the resin was filtered off and washed with water. The filtrate and washings were diluted to 800 ml, and ethanol (200 ml) was added. The mixture was applied to a DEAE-cellulose column (93 × 6.5 cm) and the column was eluted with a gradient of 9 l. of 0.05 *M* bicarbonate buffer containing 20% ethanol in the mixing vessel and 9 l. of 0.4 *M* buffer in 20% ethanol in the reservoir. Fractions 700–920 contained the trinucleotide, but paper chromatography of hydrolyzed aliquots in system A demonstrated that substantial amounts of an unidentified impurity were present. These fractions, therefore, were evaporated and reapplied to a DEAE-cellulose column (88 × 4.5 cm, acetate form). This column was eluted with a gradient of 8 l. of 0.1 *M* triethylammonium acetate, pH 6, in the mixing chamber, and 8 l. of 0.3 *M* buffer in the reservoir. Pure d-EtSpA^{Bz}pTpG$^{i\text{-}Bu}$ was obtained from fractions 570–670, and, after removal of buffer, the residue was precipitated from pyridine (15 ml)–ether (900 ml) in the usual way, to give 1.02 mmol (24%) of d-EtSpA^{Bz}pTpG$^{i\text{-}Bu}$.

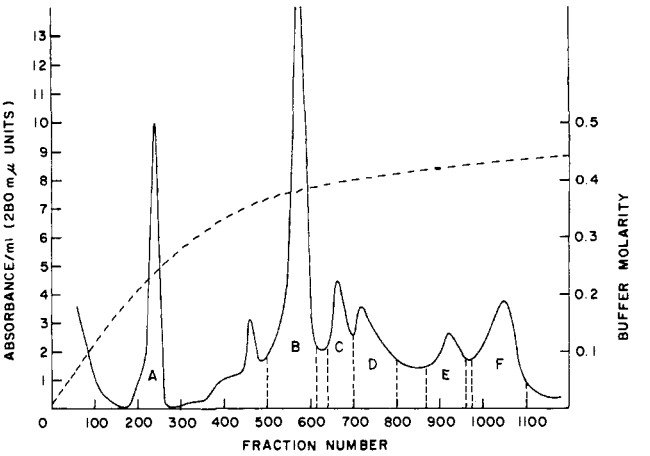


Figure 3. Condensation of the hexanucleotide d-EtSpTpTpA^{Bz}-pA^{Bz}pTpC^{An} with d-pC^{An}pA^{Bz}pT-OAc. Chromatographic elution pattern from a DEAE-cellulose column. For identification of peaks, see text.

d-pA^{Bz}pTpG$^{i\text{-}Bu}$pC^{An}-OAc. An anhydrous solution of d-EtSpA^{Bz}-pTpG$^{i\text{-}Bu}$ (1 mmol) and d-pC^{An}-OAc (5 mmol) in pyridine (100 ml) was treated with mesitylenesulfonyl chloride (2 g) for 2 hr. The product was cooled in an ice bath and treated with diisopropylethylamine in pyridine (1 *M*, 18 ml) and water (80 ml). After storage overnight, the solution was treated with iodine (5 g) in acetone (200 ml) for 18 hr, diluted with water (400 ml), and extracted with ether (700 ml). The aqueous layer was adjusted to pH 7.5 with triethylamine and applied to a DEAE-cellulose column (86 × 6.5 cm). The column was eluted with a convex gradient of 6 l. of 0.1 *M* triethylammonium bicarbonate buffer in the mixing vessel and 0.35 *M* buffer in the reservoir. Three nucleotidic peaks were eluted from the column; fractions 391–470 contained d-pC^{An}, fractions 521–610 contained d-pA^{Bz}pTpG$^{i\text{-}Bu}$, and 691–760 contained the tetranucleotide d-pA^{Bz}pTpG$^{i\text{-}Bu}$pC^{An}. Fractions 691–760 were evaporated, made anhydrous with pyridine, and acetylated using acetic anhydride (2 ml) in pyridine (10 ml) overnight at room temperature. The solution was cooled to 0° and treated with methanol (4 ml) and after 2 hr, water (4 ml) was added. After storage overnight, the solution was evaporated to dryness and precipitated from pyridine (4 ml)–ether (200 ml). The precipitate was washed and dried to give 301 μmol (30%) of d-pA^{Bz}pTpG$^{i\text{-}Bu}$-pC^{An}-OAc.

d-EtSpTpTpA^{Bz}pA^{Bz}pTpC^{An}pC^{An}pA^{Bz}pTpA^{Bz}pTpG$^{i\text{-}Bu}$pC^{An}. A mixture of the *S*-ethyl nonamer (23 μmol) and d-pA^{Bz}pTpG$^{i\text{-}Bu}$pC^{An}-OAc (170 μmol) in pyridine (3 ml) was treated with mesitylenesulfonyl chloride (170 mg) for 2 hr. The product was cooled to 0° and treated with diisopropylethylamine in pyridine (1 *M*, 1.6 ml) and water (5 ml). After storage overnight at 5° the reaction mixture was diluted to 20 ml with 50% aqueous pyridine, cooled to 0°, and treated with an equal volume of ice-cold aqueous sodium hydroxide (2 *N*) for 20 min. Excess Dowex 50 resin (pyridinium) was added with stirring, and the resin was filtered off and washed with 10% aqueous pyridine. The filtrate and washings were ap-

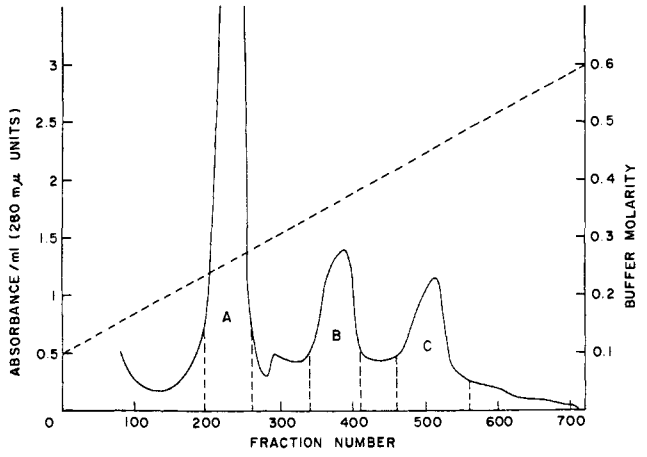


Figure 4. Chromatography of the reaction mixture in the preparation of the tridecanucleotide d-EtSpTpTpA^{Bz}pA^{Bz}pTpC^{An}pC^{An}-pA^{Bz}pTpA^{Bz}pTpG^{i-Bu}pC^{An}. For explanation of the peaks, see text.

plied to a DEAE-cellulose column (65 × 2 cm) which was eluted with a gradient of 6 l. of 0.1 *M* bicarbonate buffer in the mixing vessel and 6 l. of 0.6 *M* buffer in the reservoir. The chromatographic pattern is shown in Figure 4. Paper chromatographic examination of hydrolyzed aliquots in system C for 3 days showed that peak A contained d-pA^{Bz}-pTpG^{i-Bu}pC^{As}, peak B contained the starting nonamer, and C the required tridecamer. Peak C was evaporated to dryness to give 5.3 μmol (23%) of **2**.

Preparation of *S*-Ethyl Tridecamer 3. A sample of **2** (100 OD_{280} units) was hydrolyzed using concentrated ammonium hydroxide (3 ml) for 18 hr at room temperature, and then evaporated to dryness. The residue was dissolved in 75 ml of a solution containing 0.05 *M* sodium chloride, 7 *M* urea, and 0.2 *M* Tris, pH 7.4, and applied to a DEAE column (52 × 2 cm, chloride form) which had been preequilibrated with the same buffer. A linear gradient was immediately applied, consisting of 1 l. of the original buffer in the mixing vessel and 1 l. of a solution containing 0.3 *M* sodium chloride, 7 *M* urea, and 0.2 *M* Tris, pH 7.4 in the reservoir. The column was run at room temperature and 6-ml fractions were collected. Tubes 301–329 contained the only nucleotidic peak to be eluted from the column (57 OD_{260} units). Aliquots (60 ml) of this solution were freed from salts and urea by passage through a column of Bio-Gel P-2 (130 × 2 cm, 100–200 mesh, Bio-Rad Labs) which was eluted with water. In this way, 40 OD_{260} units of *S*-ethyl tridecamer **3** were obtained. Molecular weight[18] was 4200 (calculated 4036).

Unprotected Tridecamer 1. A sample (100 OD_{280} units) of **2** was treated with concentrated ammonium hydroxide (3 ml) overnight at room temperature, and evaporated to dryness. Aqueous pyridine (50%, 1 ml) was added, followed by a solution of iodine (10 mg) in acetone (1 ml). After storage overnight, the solution was diluted to 25 ml with a buffer solution containing 0.05 *M* sodium chloride, 7 *M* urea, and 0.2 *M* Tris, pH 7.4, and applied to a DEAE-cellulose column (52 × 2 cm, chloride form). A gradient was applied, consisting of 1.5 l. of the original buffer in the mixing vessel, and 1.5 l. of a solution containing 0.5 *M* sodium chloride, 7 *M* urea, and 0.2 *M* Tris, pH 7.4, in the reservoir. The nucleotidic material was obtained in fractions 250–270. Salts and urea were removed using a Bio-Gel P-2 column as described in the preparation of **3**. Fourteen OD_{260} units of the unprotected tridecamer **1** were obtained. Molecular weight[18] was 4180 (calculated 3992).

Acknowledgments. We are indebted to Mr. T. F. Gabriel for analytical data, to Dr. C. L. Harvey for enzymological experiments, and to Dr. P. Bartl and Mr. D. Luk for molecular weight determinations.

Crystal and Molecular Structure of an Antiinflammatory Agent, Indomethacin, 1-(*p*-Chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic Acid[1]

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Received June 28, 1971

Abstract: The crystal and molecular structure of the antiinflammatory agent indomethacin has been determined by single-crystal X-ray diffraction methods. Crystals of indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid, were grown from anhydrous acetonitrile. The crystals are triclinic, space group $P\bar{1}$, with cell constants: $a = 9.295$ (2) Å, $b = 10.969$ (1) Å, $c = 9.742$ (1), Å, $\alpha = 69.38$ (1)°, $\beta = 110.79$ (1)°, $\gamma = 92.78$ (1)°, and $Z = 2$. The calculated density is 1.37 g/cm^3; the observed value is 1.38 (1) g/cm^3. The structural solution was obtained by a routine application of the symbolic-addition method of direct sign determination. Full-matrix least-squares refinement based on 3678 counter-collected X-ray intensities gave a final *R* index of 0.059. The indole, *p*-chlorophenyl, and carboxylic acid groups are each nearly planar. The relative orientation of the *p*-chlorophenyl and indole groups is unusual and may play an important role in the chemistry of indomethacin. The crystal structure exhibits the expected hydrogen bonding of the carboxylic acid groups about centers of inversion to form molecular dimers.

The role of nonsteroidal aryl acids in antiinflammatory chemotherapy is well known. Whitehouse[3] has given a general review of the biochemical and pharmacological properties of antiinflammatory drugs. In particular, Shen[4] has recently summarized these properties for the antiinflammatory agent in-

(1) Contribution No. 4262 from the Arthur Amos Noyes Laboratory. This investigation was supported in part by Public Health Service Research Grant No. GM 16966, from the National Institute of General Medical Sciences, National Institutes of Health, and in part by a National Science Foundation Postdoctoral Fellowship (T. J. K.)

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