petroleum ether. The resulting product was removed by filtration, washed twice with 30-ml portions of petroleum ether, and dried *in vacuo* at room temperature to afford 9.2 g (65% yield) of product, $[\alpha]^{25}_{589}$ −89.3° (*c* 1.00, CH_2Cl_2). The rotation remained essentially unchanged (−89.7°) on recrystallization from the same solvent system. *Anal.* Calcd for $C_{15}H_{17}NO_5$: C, 61.85; H, 5.88; N, 4.81; CO_2, 15.1. Found: C, 61.62; H, 5.89; N, 4.56; CO_2, 15.0.

Treatment of the above mother liquor with an additional 200 ml of petroleum ether gave 3.1 g (22% yield) of the diastereoisomer as a crystalline product, $[\alpha]^{25}_{589}$ −177.0° (*c* 1.00, CH_2Cl_2); one further recrystallization from methylene chloride–ether brought the rotation to −178.2° *Anal.* Calcd for $C_{15}H_{17}NO_5$: C, 61.85; H, 5.88; N, 4.81; CO_2, 15.1. Found: C, 61.92; H, 5.71; N, 5.27; CO_2, 15.00.

O-Acetyl-L-tyrosine NCA (XVb). To a suspension of 2.072 g of L-tyrosine NCA, prepared as described above, in 35 ml of freshly distilled THF which had been cooled in an ice bath was added from a syringe, with exclusion of moisture and vigorous stirring, 0.711 ml of acetyl chloride and then 0.8 ml of anhydrous pyridine in 15 ml of THF. The reaction mixture was stirred at 0–5° for 30 min. During this time a yellow gum which had separated was transformed into a white solid. The mixture was allowed to come to room temperature and was filtered under nitrogen pressure and exclusion of moisture to remove insolubles. The filtrate was concentrated to dryness *in vacuo* and the residue was treated with 20 ml of ethyl acetate followed by 10 ml of hexane. The supernatant was decanted from the solid which had separated. The supernatant was then treated with 20 ml of hexane to give a solid which crystallized on standing in a dry atmosphere. After crystallization had occurred, an additional 130 ml of hexane was added slowly with continued stirring. The crystalline solid was removed by filtration and was washed with a mixture of hexane–ethyl acetate (8:1). The resulting product (0.78 g, 31% yield) gave a negative Beilstein test and showed only weak absorption at 2.7 μ (OH) in the infrared in CH_3CN solution. The product was dissolved in 10 ml of ethyl acetate and treated with 10 ml of hexane. The supernatant was decanted from a small amount of gummy solid which adhered to the flask. The solution was then treated with an additional 50 ml of hexane and the resulting crystalline product was removed by filtration and washed with hexane–ethyl acetate (6:1) to afford 0.7 g; dec pt 122° (lit. 99–100°,[34] dec pt 122–123° [35]); $[\alpha]^{25}_{589}$ −37.2° (*c* 1.06, CH_3CN); ir (CH_3CN) 2.95 (NH), 5.37, 5.59, 5.62, 8.38 μ. *Anal.* Calcd for $C_{12}H_{11}NO_5$: C, 57.82; H, 4.45; N, 5.62. Found: C, 57.77; H, 4.40; N, 5.61.

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Studies on Polynucleotides. XCVIII.[1] A Convenient and General Method for the Preparation of Protected Dideoxyribonucleotides Containing 5′-Phosphate End Groups[2]

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Contribution from The Institute for Enzyme Research of the University of Wisconsin, Madison, Wisconsin 53706. *Received August 24, 1970*

Abstract: Protected dinucleotides carrying 5′-phosphomonoester end groups (*e.g.*, $pC^{An}pA^{Bz}$) are required as intermediates in the blockwise synthesis of deoxyribopolynucleotides of defined sequence. A rapid and general method for their preparation is now described, the primary feature of which is that it obviates the time-consuming separation by anion exchange or gel filtration columns and, instead, uses convenient solvent extraction procedures. The synthetic steps used were as follows. (1) The N-protected 5′-deoxyribomononucleotides were converted to the corresponding 5′-phosphoramidates by reaction with dicyclohexylcarbodiimide and the highly lipophilic aromatic amine, *p*-aminophenyltriphenylmethane. The phosphoramidates were obtained in 60–65% yield. (2) The phosphoramidates were condensed with suitably protected mononucleotides (*e.g.*, pA^{Bz}-OAc) using triisopropylbenzenesulfonyl chloride as the condensing agent. The resulting dinucleotide phosphoramidates were isolated by solvent extraction in yields of 50–65%. The phosphoramidate protecting group was selectively removed by treatment with isoamyl nitrite in a pyridine–acetic acid mixture. All of the required 16 dinucleotides were thus prepared in reasonably satisfactory yields (50–65%).

Synthesis of high molecular weight double-stranded DNA's with defined nucleotide sequences has been accomplished by a combination of chemical and enzymatic methods.[3–7] Thus, when chemically synthesized short-chain deoxyribopolynucleotides with repeating nucleotide sequences are used as templates for the DNA polymerase of *E. coli*, macromolecular DNA-like polymers containing the repeating sequences present in the short templates are obtained. The availability

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(1) Paper XCVII is by R. C. Miller, P. Besmer, H. G. Khorana, M. Fiandt and W. Szybalski, *J. Mol. Biol.*, **56**, 363 (1971).

(2) This work has been supported by grants from the National Cancer Institute (Grant No. CA-05178) of the National Institutes of Health, U. S. Public Health Service, The National Science Foundation (Grant No. GB-7484X), Washington, and the Life Insturance Medical Research Fund (Grant No. 65-44).

(3) H. G. Khorana, *Pure Appl. Chem.*, **17**, 349 (1968).

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(6) H. G. Khorana, H. Buchi, T. M. Jacob, H. Kössel, S. A. Narang, and E. Ohtsuka, *ibid.*, **89**, 2154 (1967).

(7) 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).

of these polymers has permitted extensive studies of the cell-free protein synthesis and of the genetic code.[3-6] In further work aimed at the total synthesis of a bihelical DNA containing specific nonrepeating nucleotide sequences, the synthesis of the gene corresponding to the principal yeast alanine tRNA has recently been accomplished.[7] The general strategy developed during this work for the construction of bihelical DNA's comprises the following three steps: (1) chemical synthesis of deoxyribopolynucleotide segments of chain length in the range of 8–12 units only with free 3′- and 5′-hydroxyl groups; the segments should represent the entire two strands of the intended DNA and those belonging to the complementary strands should have an overlap of four to five nucleotide units; (2) the phosphorylation of the 5′-hydroxyl group with ATP using the T4 phage polynucleotide kinase; and (3) the head-to-tail joining of the appropriate segments when they are aligned to form bihelical complexes using the T4 polynucleotide ligase. These general principles of synthesis seem to offer a variety of opportunities for further studies of the biological functions of DNA, *e.g.*, (1) systematic studies of the structure–function relationship in the tRNA's by introducing changes at the gene level and by preparation of "mixed" tRNA genes and (2) studies of the start and stop signals for the expression of genetic information by "welding" of suitable deoxypolynucleotide sequences to either end of the tRNA structural genes.[3,7]

In view of the above development in the methodology for DNA synthesis and of the essentially unlimited scope of the synthetic approach in the study of gene function, rapid and efficient chemical synthesis of deoxyribopolynucleotide segments assumes an ever-increasing importance. Indeed, the availability of the short segments with defined nucleotide sequences is at present the progress-determining factor in the synthesis of high molecular weight DNA's of defined nucleotide sequences possessing specific biological functions. (It should be particularly noted that, fortunately, the problem of the purely chemical synthesis of deoxypolynucleotides is now limited to chain lengths in the range of 8–12 units only.) Therefore, we are attempting to introduce newer innovations in the synthetic methodology presently available with the hope that time and effort may be reduced and that the efficiency in the synthetic steps may be increased.

Easily the greater part of the effort, which is currently required for the synthesis of a deoxyribopolynucleotide, is spent on the purification (a) of the di- and trinucleotide blocks which serve as intermediates and (b) of the growing oligonucleotide chain carrying the 3′-hydroxyl end group. Methods of purification which are commonly used involve prolonged chromatography on anion exchanger columns[8] and/or gel filtration on Sephadex columns.[9,10] Clearly, a marked advantage would accrue if the protected dinucleotide blocks, which are the most frequently used synthetic intermediates and of which there are only a maximum number of sixteen, could be prepared in bulk by rapid and convenient methods. In the present paper we report results of an investigation carried out with this aim in view. A very lipophilic protecting group is introduced at the 5′-phosphate group of the four suitably protected deoxyribonucleotides. This enables the rapid isolation by solvent extraction of the resulting protected mononucleotides and, more importantly, of the protected dinucleotides after condensation of the former with the 3′-*O*-acetyl protected mononucleotides. All of the 16 possible dinucleotides have been prepared in the suitably protected form without resort to column chromatography. It is also clear that this type of method lends itself readily to a substantial increase in the scale whereas there is a serious practical limit on the amounts of the dinucleotide blocks which can be prepared in any one run by the anion exchange chromatographic methods.

Preparation of the Nucleotide Phosphoramidates. While the β-cyanoethyl group[11] has been used most commonly so far for protection of the 5′-phosphomonoester group in mononucleotides,[12] a number of other groups have been proposed more recently for the purpose.[13] The groups investigated in the present work have been the phosphoramides.[13d] The two principal reasons for their choice were as follows. Firstly, methods for their one-step preparation from the 5′-nucleotides were available. Indeed a number of these were prepared in connection with synthesis of nucleotide coenzymes and a great deal was then learned about their properties.[14] Thus, they were expected to be stable during the usual synthetic operations in the deoxyoligonucleotide field. Secondly, it has been recently demonstrated by Ohtsuka and coworkers[13c] that the amidates derived from the aromatic amines, *e.g.*, aniline, can be readily cleaved to give the parent 5′-phosphate group by treatment with isoamyl nitrite under mild conditions (pyridine–acetic acid mixture at room temperature). No loss of the N-protection groups or any other side reaction was observed. (The complete specificity of the isoamyl nitrite reagent under the conditions used for the removal of the phosphoramidate has been confirmed in the present work.)

For developing isolation procedures using the principle of partition, it was desirable to use highly lipophilic substituents[15] on the amine used. In initial experiments, thymidine 5′-(α-naphthyl)phosphoramidate was prepared but solubility tests soon showed that the gain in its preference for organic solvents was not adequate. Attempts toward the synthesis of thymidine 5′-triphenylmethylphosphoramidate from thymidine 5′-

(8) See, *e.g.*, T. M. Jacob and H. G. Khorana, *J. Amer. Chem. Soc.*, 87, 2971 (1965).

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(12) S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, 89, 2158 (1967); unpublished work of H. Buchi, H. Weber, A. Kumar, and others as referred to in ref 10.

(13) (a) A. Franke, F. Eckstein, K. Scheit, and F. Cramer, *Chem. Ber.*, 101, 944 (1968); (b) A. F. Cook, M. J. Holman, and A. L. Nussbaum, *J. Amer. Chem. Soc.*, 91, 6479 (1969); (c) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *ibid.*, 92, 3441 (1970); (d) S. A. Narang, O. S. Bhanot, J. Goodchild, and J. Michniewic, *Chem. Commun.*, 516 (1970).

(14) J. G. Moffatt and H. G. Khorana, *J. Amer. Chem. Soc.*, 83, 649 (1961).

(15) For example, protected di- and oligonucleotides containing the very lipophilic 5′-monomethoxytrityl group have often been purified from the condensation reaction mixtures simply by extraction procedures: T. M. Jacob and H. G. Khorana, *ibid.*, 87, 368 (1965), and unpublished work of Dr. H. Weber, A. Kumar, M. H. Caruthers, and J. H. Van de Sande.

Table I. Experiments on the Synthesis of Thymidine 5′-Phosphoramidate (II)

Amount of d-pT, mmol	Amount of the amine I, mmol	Solvent	Volume, ml	Condensing agent (mmol)	Recovered pT, %	Yield, %
0.1	0.4	Pyridine	4.0	DCC (1) + pyridinium Dowex-50 (300 mg)	26	62
0.1	0.4	Pyridine	4.0	DCC (1) + pyridinium hydrochloride (0.4)	30	64
0.1	0.4	HMPA[a]	4.0	DCC (1) + pyridinium Dowex-50 (300 mg)	35	56
0.1	0.2	Pyridine	3.0	IV (1)	40	48 + some impurity
0.1	0.4	DMF[b]	1.0	VII (1)	78	20
0.1	0.4	Pyridine	1.0	VII (1) + catalytic pyridinium hydrochloride	70	22
0.1	0.4	Pyridine	1.0	CCl_3CN (0.5)	85	10
0.5	1.0	Pyridine	5.0	DCC followed by TPS (0.25)	25	63
0.1	0.2	Pyridine	3.0	V (1.0)	44	42

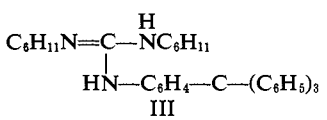
[a] Abbreviation for hexamethylphosphoric triamide. [b] Abbreviation for dimethylformamide.

phosphate and tritylamine were unsuccessful, probably due to the steric hindrance in the latter compound. Therefore, the amine next investigated and successfully used in the present work was *p*-aminophenyltriphenylmethane (I). This amine was readily synthesized by

I

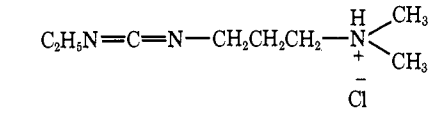
II, R = thymine, *N*-anisoylcytosine, *N*-benzoyladenine, or *N*-isobutyrylguanine

the published procedure.[16] The preparation of the 5′-phosphoramidate II by reaction of d-pT[17] with the amine I proceeded in low yield when dicyclohexylcarbodiimide (DCC) was added to a pyridine solution of the nucleotide and the amine. The yield was improved to 65% when an anhydrous solution of the nucleotide alone was first treated with DCC for some 10 min and this preactivation was followed by the addition of the amine. The best yields in all the subsequent experiments in the present work have been between 60 and 65% and all attempts (Table I) at further improvement have been unsuccessful. It is known from the earlier work[11] that a side reaction occurs during the above synthesis which leads to the formation of the adduct between DCC and the amine. In the present case, the guanidine to be expected would be III and its accumulation would be expected to inhibit the activation of the 5′-phosphate group. Attempts to neutralize the strongly basic guanidine formed

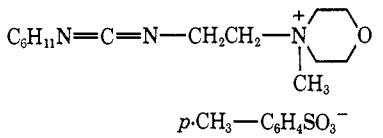


III

during the condensation reaction by the addition of pyridinium Dowex-50 or pyridine hydrochloride and thus to drive the condensation reaction further were unsuccessful (Table I). Similarly, the use of alternative activating agents, such as the water-soluble 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (IV) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide *p*-toluenesulfonate (V), the isoxazolium salt VI,[18] trichloroacetonitrile,[19] and carbonyldiimidazole[20] (VII), consistently gave yields lower than those obtained by using DCC (Table I). Replacing pyridine by hexamethylphosphoric triamide or dimethylformamide as the solvent failed to improve the yields (Table I). Prior activation of the phosphate group in d-pT with mesitylenesulfonyl chloride (MS) and then the amine I gave the required phosphoramidate again in



IV



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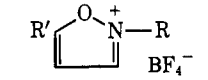
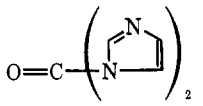
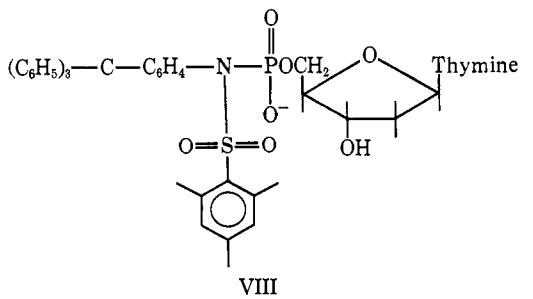


VI, R = ethyl; R′ = phenyl



VII



VIII

(16) B. Witten and E. Reid, "Organic Syntheses," Collect. Vol. IV, Wiley, New York, N. Y., 1962, p 47.

(17) For the system of abbreviations used see H. Schaller and H. G. Khorana, *J. Amer. Chem. Soc.*, **85**, 3841 (1963). The additional abbreviations used in this paper and not defined in the text are: iB, the isobutyryl group on the guanine ring; TPM-*p*-dT, thymidine 5′-phosphoro-(*p*-triphenylmethyl)anilidate (general structure II as given in text). The corresponding phosphoramidate derivatives of other protected mononucleotides are abbreviated similarly.

(18) (a) R. B. Woodward and R. A. Olofson, *ibid.*, **83**, 1007 (1961); (b) R. B. Woodward, R. A. Olofson, and H. Mayer, *ibid.*, **83**, 1010 (1961). We thank Professor R. B. Woodward for the gift of the reagent used in the above studies.

(19) F. Cramer and G. Weimann, *Chem. Ber.*, **94**, 996 (1961).

(20) H. Schaller, H. A. Staab, and F. Cramer, *ibid.*, **94**, 1621 (1961).

Table II. Spectral and Chromatographic Properties of Protected and Unprotected Compounds

Compd	λ_{max}, nm	R_f,[a] solvent A	R_f,[a] solvent B	R_f,[a] solvent C	R_f,[a] solvent D	R_f,[a] paper electrophoresis (pH 7.1)
TPM-pC^{An}	211, 270	3.54	1.53	1.97		
TPM-pT	210, 252	6.45	1.30	2.19		
TPM-pA^{Bz}	210, 256 275	6.02	1.76	2.08		
TPM-pG^{iB}	211, 254	5.9	1.42	2.42		
TPM-pC^{An}pC^{An}-OAc		6.95	1.84	2.28		
pC^{An}pC^{An}-OAc	302	2.28	1.24	1.79		
pCpC		0.22	0.41	0.69	0.97	0.94
TPM-pC^{An}pT-OAc		6.73	1.69	1.62		
pC^{An}pT-OAc	275, 301	1.39	1.24	1.42	0.63	
pCpT		0.31	0.66	0.81	0.91	0.86
TPM-pC^{An}pG^{iB}-OiB		6.90	1.70	1.61		
pC^{An}pG^{iB}-OiB	257, 285	2.49	1.66	1.91	0.69	0.69
pCpG		0.18	0.35	0.56	0.81	0.88
TPM-pC^{An}pA^{Bz}-OAc		6.82	1.69	1.60		
pC^{An}pA^{Bz}-OAc	285	2.13	1.33	1.90	1.43	
pCpA		0.25	0.56	0.73	1.37	0.90
TPM-pT		6.45	1.30	2.19		
TPM-pTpT-OAc		4.38	1.55	2.04		
pTpT-OAc		0.72	1.07	1.06	1.21	0.96
pTpT	267	0.46	0.88	0.87	0.78	0.94
TPM-pTpC^{An}-OAc			1.32	1.97		0.78
pTpC^{An}-OAc	272, 303	1.45	1.16	1.39	1.92	0.61
pTpC		0.26	0.71	0.81	0.91	0.96
TPM-pTpA^{Bz}-OAc			1.38	2.16		
pTpA^{Bz}-OAc	276	1.60	1.23	1.35	1.93	0.73
pTpA			0.70	0.88	1.16	1.16
TPM-pTpG^{iB}-OiB		6.00	1.32	2.19		
pTpG^{iB}-OiB	261	1.42	1.44	1.57		0.79
pTpG		0.22	0.62	0.75	0.90	0.89
TPM-pA^{Bz}pA^{Bz}-OAc		5.92	1.41	2.23		
pA^{Bz}pA^{Bz}-OAc	283	2.06	1.34	1.83		
pApA		0.21	0.53	0.79	1.36	0.92
TPM-pA^{Bz}pC^{An}-OAc		6.46	1.68	1.49		
pA^{Bz}pC^{An}-OAc	286	2.09	1.3	1.8		
pApC		0.25	0.52	0.68	1.33	0.86
TPM-pA^{Bz}pT-OAc		6.32	1.32	2.06		
pA^{Bz}pT-OAc	279	1.50	1.36	1.42		0.80
pApT			0.75	0.82	1.26	1.02
TPM-pA^{Bz}pG^{iB}-OiB		5.90	1.60	1.72		
pA^{Bz}pG^{iB}-OiB	282	1.68	1.46	1.59		
pApG			0.60	1.32	1.20	0.96
TPM-pG^{iB}pA^{Bz}-OAc		5.80	1.74	1.62		
pG^{iB}pA^{Bz}-OAc	282	1.60	1.55	1.70		
pGpA			0.68	1.43	1.30	1.0
TPM-pG^{iB}pC^{An}-OAc		6.8	1.72	1.52		
TPM-pG^{iB}pT-OAc		6.2	1.43	2.4		
TPM-pG^{iB}pG^{iB}-OAc		5.9	1.56	2.36		
pG^{iB}pC^{An}-OAc	257, 285	2.6	1.56	1.83		
pGpC		0.23	0.46	0.62	0.87	0.93
pG^{iB}pT-OAc	263	1.36	1.58	1.60		0.83
pGpT		0.28	0.64	0.78	0.95	0.9
pG^{iB}pC^{An}-OAc	257, 285	2.36	1.22	1.82		0.63
pGpC		0.20	0.46	0.72	0.85	0.95

[a] R_f values were recorded relative to pT.

35% yield but, in addition, a side product was formed. The latter is tentatively concluded to be VIII on the basis of (1) its spectrophotometric properties, (2) its chromatographic behavior, (3) its resistance to the isoamyl nitrite reagent, and (4) a positive test for the presence of sulfur.

Following the method discussed above for the preparation of the phosphoramidate of d-pT, the corresponding derivatives of the other three protected deoxyribonucleotides,[17] d-pC^{An}, d-pA^{Bz}, and d-pG^{iB}, were prepared in similar yields. All of the phosphoramidates II were isolated by the solvent extraction procedure described in the Experimental Section. As in earlier work[11] the phosphoramidates were obtained evidently as salts of the guanidine III. In support of this conclusion, on paper or thin-layer chromatography in ammonical solvents, invariably two ultraviolet absorbing spots were obtained, one corresponding to the ammonium salt of the phosphoramidate and a second faster traveling spot. That the latter material was not the amine I was shown by their different mobilities on paper chromatograms in solvent J.

Analysis showed that preparations of the phosphoramidates were contaminated by some dicyclohexylurea. The latter did not interfere with the quantitative determination of the phosphoramidates by uv absorption

Table III. The Synthesis of Protected Dinucleotides Containing 5′-Phosphate Groups. Summary of Conditions and Yields of Condensation Reactions

Dinucleotide	3′-OH containing component	Amount, mmol	5′-Phosphate containing component	Amount, mmol	TPS amount, mmol	Pyridine, ml	Time, hr	Yield,[a] %
d-pTpT-OAc	TPM-*p*-dT[b]	0.25	d-pT-OAc	0.50	1.0	3.0	2.5	68
d-pTpA^{Bz}-OAc	TPM-*p*-dT	0.22	d-pA^{Bz}-OAc	0.75	1.5	3.5	2.5	56
d-PTpC^{An}-OAc	TPM-*p*-dT	0.25	d-pC^{An}-OAc	0.50	1.0	3.5	2.5	54
d-pTpG^{iB}-OiB	TPM-*p*-dT	0.25	d-pG^{iB}-OiB	0.75	1.5	3.0	3.0	66
d-pA^{Bz}pA^{Bz}-OAc	TPM-*p*-dA^{Bz}[b]	0.25	d-pA^{Bz}-OAc	0.50	1.0	3.5	3.0	58
d-pA^{Bz}pC^{An}-OAc	TPM-*p*-dA^{Bz}	0.25	p-pC^{An}-OAc	0.50	1.0	3.5	3.0	60
d-pA^{Bz}pT-OAc	TPM-*p*-dA^{Bz}	0.25	d-pT-OAc	0.50	1.25	3.5	3.0	71
d-pA^{Bz}pG^{iB}-OiB	TPM-*p*-dA^{Bz}	1.00	d-pG^{iB}-OiB	2.00	5.0	10.0	3.0	58
d-pC^{An}pC^{An}-OAc	TPM-*p*-dC^{An}	0.20	d-pC^{An}-OAc	0.40	0.90	3.0	3.0	56
d-pC^{An}pA^{Bz}-OAc	TPM-*p*-dC^{An}	0.20	d-pA^{Bz}-OAc	0.40	0.90	3.0	3.0	46
d-pC^{An}pT-OAc	TPM-*p*-dC^{An}	0.20	d-pT-OAc	0.40	0.90	3.0	3.0	51
d-pC^{An}pG^{iB}-OiB	TPM-*p*-dC^{An}	0.20	d-pG^{iB}OiB	0.40	0.90	3.0	3.0	52
d-pG^{iB}pA^{Bz}-OAc	TPM-*p*-dG^{iB}	0.25	d-pA^{Bz}-OAc	0.50	1.0	4.0	3.0	62
d-pG^{iB}pC^{An}-OAc	TPM-*p*-dG^{iB}	0.25	d-pC^{An}-OAc	0.50	1.0	4.0	3.0	56
d-pG^{iB}pT-OAc	TPM-*p*-dG^{iB}	0.25	d-pT-OAc	0.5	1.0	4.0	3.0	60
d-pG^{iB}pG^{iB}-OiB	TPM-*p*-dG^{iB}	0.25	d-pG^{iB}-OiB	0.6	1.3	4.5	3.0	58

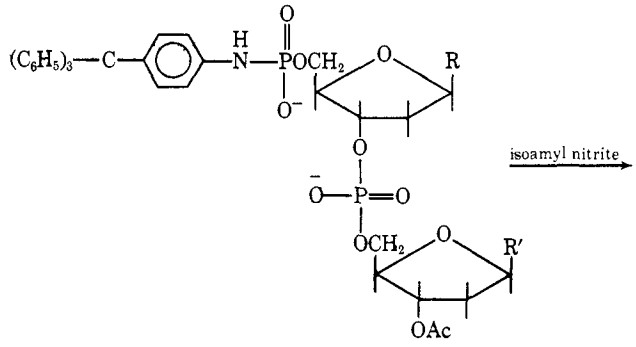
[a] Yields were determined spectrophotometrically in water. [b] See ref 17.

nor did it interfere in the subsequent condensation reactions to form the dinucleotides. Treatment of the protected nucleotide phosphoramidates with isoamyl nitrite in pyridine–acetic acid regenerated the parent protected nucleotide quantitatively. The N-protected deoxynucleoside 5′-phosphates thus obtained were checked for purity by paper chromatography using a variety of solvent systems (R_f's in Table II). The protected nucleotides on further treatment with concentrated ammonia under the standard conditions gave the fully unprotected mononucleotides which were again checked for homogeneity in two solvent systems. The total analysis thus gave assurance that the treatment with isoamyl nitrite brought about specific and quantitative regeneration of the 5′-phosphomonoester group without any accompanying side reaction.

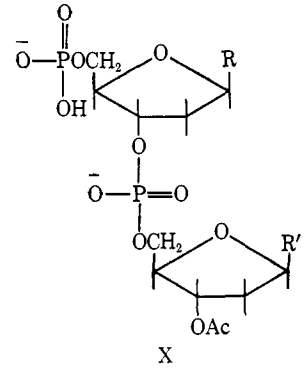
Synthesis of the Protected Dinucleotides Carrying 5′-Phosphate End Groups X. Condensation of the phosphoramidates with the protected mononucleotides (*e.g.*, d-pA^{Bz}-OAc) proceeded smoothly to give the fully protected dinucleotides **IX** in yields of 55–70% as based on the phosphoramidate. A comprehensive summary of the conditions used in the condensation reactions and the yields obtained is given in Table III. TPS is so far the reagent of choice for these condensations. When DCC or mesitylenesulfonyl chloride (MS) was used, the yields were lower. The reason for the lower yields with DCC is not quite clear except for the possibility that the presence of the strongly basic guanidine III in the phosphoramidates II inhibited the reaction.[21] The use of MS led to a considerable amount of N sulfonylation of the phosphoramidate group in the mononucleotides as well as in the dinucleotides. The extent of the latter reaction was very much reduced in the condensations when TPS was used as the condensing agent.

The dinucleotide phosphoramidates **IX** were isolated by extraction of the reaction mixture (in aqueous pyridine) with methylene dichloride–*n*-butyl alcohol (7:3, v/v) mixture or chloroform. The organic solvent also extracted the unreacted phosphoramidate and the

(21) One experiment was carried out with DCC in the presence of added pyridinium hydrochloride, but the yields remained unchanged.



IX



X

R or R′ = thymine, *N*-benzoyladenine, *N*-anisoylcytosine, or *N*-isobutyrylguanine

N-sulfonylated derivatives of both the mono- and dinucleotide. The next step was selective precipitation of the dinucleotide phosphoramidate from methylene dichloride–*n*-butyl alcohol or chloroform extract. This was accomplished by addition of an ethyl acetate–ether (1:1) mixture or ethyl acetate alone. The composition of the solvent used for the precipitation step was influenced by the nature of the nucleotide units. Thus, the phosphoramidates of the mono- and dinucleotides derived from d-pA^{Bz}, d-pC^{An}, and d-pG^{iB} were more soluble in the organic solvents than those derived from the thymidine nucleotide alone. The isolated yields of the dinucleotide phosphoramidates were in the range of 50–65%.

The fully protected dinucleotide phosphoramidates **IX** often obtained as off-white, nonhygroscopic powders

were homogeneous on paper chromatography (see Experimental Section).

Sometimes a small contamination by the N-sulfonylated derivatives of IX was detected. Thus, when the phosphoramidates were treated with the isoamyl nitrite reagent and the products were examined by paper chromatography, the *N*-sulfonyl derivatives showed up as the unchanged starting material. This contamination did not cause any serious problem since it could be readily removed after the isoamyl nitrite treatment. Thus, following the latter treatment, the N-sulfonylated product as well as the diazo derivative arising from the amine I could be selectively extracted from the aqueous phase with a methylene chloride–*n*-butyl alcohol mixture while the desired protected dinucleotide blocks stayed in the aqueous phase. The products (general structure X) were homogeneous by paper chromatography (Table II) and had the expected uv absorption characteristics. Sometimes traces (easily less than 5%) of impurities were detected when the protected dinucleotides were further characterized by chromatography after complete deprotection. The trace contaminations, which were detected when paper chromatography was performed using 10–20 absorbance units (260 mμ) per spot, are unlikely to cause any practical difficulty in the use of the dinucleotide blocks.

Concluding Remarks. The main accomplishment of the present work has been that time- and effort-saving procedures for all the 16 protected deoxyribodinucleotide blocks have been described. The fact that, in the preparation of the phosphoramidates from the N-protected mononucleotides, the yields (about 65%) were less than ideal is of secondary importance because the protected mononucleotides can be prepared routinely in large quantities (preparations on 10–20-mmol scale are standard). It should be noted that the yields at the dinucleotide stage, although again leaving room for improvement, were, in general, higher than those that have been usually obtained in the past using the β-cyanoethyl protecting group. The overriding question in the chemical synthesis of short deoxyribopolynucleotides now is that of speed and convenience, and we believe that the present work has made a significant contribution toward this goal.

Experimental Section

Materials and General Methods. Pyridinium salts of the protected nucleotides, d-pT-OAc, d-pC^{An} and d-pC^{An}-OAc, d-pA^{Bz} and d-pA^{Bz}-OAc, and d-pG^{iB} and d-pG^{iB}-OiB, were prepared as described previously. Large scale preparations of d-pC^{An}, d-pA^{Bz}, and d-pG^{iB} were also carried out for us by Collaborative Research, Waltham, Mass. The following molar extinction values were used for the protected mononucleotides in aqueous solution: d-pC^{An}, 22,500 (302 mμ); d-pA^{Bz}, 18,300 (280 mμ); d-pG^{iB}, 16,700 (259 mμ). Calculation of the yields after condensation reactions are based on uv absorption spectra theoretically derived by summation of the spectra of all the protected dinucleotides.

TPS, MS, and DCC were commercial products (Aldrich Chemicals). TPS was freshly crystallized from dry *n*-pentane before use. *p*-Aminophenyltriphenylmethane was prepared by the method of Witten and Reid[16] and was crystallized from toluene before use. Glacial acetic acid and isoamyl nitrite were purified by fractional distillation. Pyridine was treated with a small amount of chlorosulfonic acid before distillation, redistilled from potassium hydroxide, and stored over molecular sieves. All the condensation reactions were carried out by repeated evaporation *in vacuo* of a solution of the components with added dry pyridine (at least four times) and the reaction flask was opened into a drybox which had a positive pressure of dry nitrogen. Paper chromatography was performed by the descending technique using Whatman No. 1 or No. 40 paper. Solvent systems used were: solvent A, 2-propanol–concentrated ammonia–water (7:1:2, v/v); solvent B, ethanol–ammonium acetate (1 *M*, pH 7.5, 7:3, v/v); solvent C, 1-propanol–concentrated ammonia–water (55:10:35, v/v); solvent D, isobutyric acid–concentrated ammonia–water (66:1:33, v/v); solvent J, ethanol–water–4 *N* hydrochloric acid (6:3:1, v/v).

The R_f values of different compounds are listed in Table II. Thin-layer chromatography (tlc) was carried out on precoated silica gel plates containing fluorescence indicator (Brinkmann F_{254}). The solvent systems used for tlc were: solvent E, chloroform–methanol (50:50, v/v); solvent F, chloroform–methanol (70:30, v/v); solvent G, acetonitrile–water (80:20, v/v); solvent H, acetonitrile–water (90:10, v/v); solvent I, acetonitrile–concentrated ammonia (9:1, v/v).

General Procedure for Removal of Phosphoramidate Protecting Group from Mono- and Dinucleotides. Phosphoramidate derivatives of mono- and dinucleotides (1 mmol) were dissolved in a mixture of pyridine–acetic acid (50:50, 20 ml) and isoamyl nitrite (10 mmol) was added. The homogeneous reaction mixture was allowed to stand at room temperature overnight before being evaporated repeatedly to a syrup by frequent addition of pyridine. The residual gum was dissolved in chloroform, in the case of the mononucleotide, or in chloroform–*n*-butyl alcohol (70:30), in the case of dinucleotides, and extracted with 0.2 *M* triethylammonium bicarbonate (TEAB) (two 3-ml portions) and the combined aqueous extracts were concentrated and examined by tlc. Determination of the OD_{260} units present in the aqueous solution gave the yield of the mono- and dinucleotides as based on the parent nucleotides.

Thymidine 5′-Phosphoro-(*p*-triphenylmethyl)anilidate (TPM-p-dT). To a dry solution of pyridinium thymidine 5′-phosphate (0.5 mmol) in pyridine (5.0 ml) was added a solution of DCC (5 mmol) in pyridine (3.0 ml) and the reaction mixture was concentrated *in vacuo* to about 5 ml. After 5 min at room temperature, a solution of triphenyl-*p*-aminophenylmethane (335 mg, 1 mmol) in pyridine (4.0 ml) was added and the total reaction mixture concentrated to 6 ml before being allowed to stand at room temperature overnight. Water (2 ml) was added and the reaction mixture kept overnight at room temperature. The resulting dicyclohexylurea was removed by filtration and washed with aqueous pyridine (50%; two 10-ml portions). The combined filtrate and washings were concentrated, the residual syrup was dissolved in chloroform (25 ml), and the solution washed with 0.2 *M* triethylammonium bicarbonate (TEAB) (two 10-ml portions). The chloroform solution was concentrated and on trituration with a mixture of toluene and petroleum ether (1:1, 5 ml) gave a white powder which was further washed with the same solvent (three 4-ml portions) and finally with ether. The yield of the thymidine phosphoramidate was 340 mg. This material represented a total of 3000 OD_{267} units of d-pT as determined after conversion of a portion to d-pT by treatment with isoamyl nitrite as described below. The yield of the phosphoramidate thus determined was 63%.

Conversion of TPM-p-dT to p-dT. The above phosphoramidate II (11.3 mg) was dissolved in pyridine (0.5 ml) and acetic acid (0.5 ml) and isoamyl nitrite (0.02 ml) was added. After keeping the reaction mixture overnight, the solvent was evaporated repeatedly to a syrup by addition of pyridine. Chloroform (1 ml) solution was extracted with 0.1 *M* TEAB (three 0.5-ml portions) and the combined aqueous extracts were once again washed with chloroform (0.5 ml). The aqueous extract was concentrated to a known volume. Spectrophotometric determination showed that the solution contained a total of 100.1 OD_{267} units. The d-pT thus obtained was found to be chromatographically homogeneous. Estimation of the OD units (100.1 OD_{267}) gave the yield of chromatographically homogeneous d-pT.

***N*^{6}-Benzoyldeoxyadenosine 5′-Phosphoro-(*p*-triphenylmethyl)-anilidate (TPM-p-dA^{Bz}).** An anhydrous solution of pyridinium d-pA^{Bz} (1.0 mmol) in dry pyridine (6.0 ml) was treated with DCC (5.0 mmol) at room temperature for 10 min. This was then treated with a solution of triphenyl-*p*-aminophenylmethane (2.0 mmol) in dry pyridine (10 ml). The pink-colored solution was concentrated to 6 ml and then shaken overnight at room temperature. Water (3.0 ml) was then added and the reaction mixture kept for a further period of 12 hr before being concentrated to a thick syrup. This was taken up in 75 ml of ethyl acetate and the precipitated dicyclohexylurea was filtered off and washed with the same solvent. The combined filtrate and washings were washed with 0.2 *M* TEAB (three 30-ml portions). Tlc examination in solvent G showed that the organic phase contained the required phosphoramidate along

with some of the unreacted amine I whereas the aqueous phase contained the unreacted nucleotide and the corresponding symmetrical pyrophosphate. The organic phase was concentrated to about 4 ml and the solution was added dropwise to petroleum ether (200 ml, bp 60–110°). The precipitated phosphoramidate was collected by centrifugation (800 mg). The yield (64%, 11,700 OD_{280}) was determined after isoamyl nitrite treatment as described in the general procedure. Unreacted d-pA^{Bz} (24%) was recovered from the aqueous solution.

Conversion of TPM-*p*-dA^{Bz} to *p*-dA^{Bz}. To a solution of the phosphoramidate (20 mg, 280 OD_{280}) in pyridine (0.3 ml) and glacial acetic acid (0.3 ml) was added freshly distilled isoamyl nitrite (0.06 ml) and the homogeneous reaction mixture was allowed to stand at room temperature for 12 hr. After the usual work-up as described in the general procedure, the aqueous solution was examined by paper chromatography in the solvent B. The only nucleotidic product obtained was d-pA^{Bz} which was identical in R_f with the authentic d-pA^{Bz}. Similarly, both the authentic sample of d-pA^{Bz} and the sample obtained above gave identical paper chromatographic pattern in solvents A, C, and D and after treatment with concentrated ammonia to remove the *N*-benzoyl group.

***N*-Isobutyryldeoxyguanosine 5′-Phosphoro-(*p*-triphenylmethyl)-anilidate (TPM-*p*-dG^{iB}).** An anhydrous pyridine solution (8.0 ml) of pyridinium d-pG^{iB} (1.0 mmol) was treated with DCC (1.03 g, 5.0 mmol) for 10 min at room temperature under exclusion of moisture. A dry solution of the amine I (670 mg, 2.0 mmol) in pyridine (10.0 ml) was added to the above solution and the reaction mixture concentrated to 6.0 ml before being shaken at room temperature overnight. Water (6.0 ml) was then added and after being kept for 12 hr at room temperature, the reaction mixture was concentrated to a thick syrup. The syrup was taken up in ethyl acetate (50 ml) and the precipitated dicyclohexylurea was filtered off and washed with the same solvent. The combined filtrate and washings were washed with 0.2 M TEAB (three 25-ml portions). The ethyl acetate solution was concentrated (6.0 ml) and the product was precipitated by addition of the solution to petroleum ether (300 ml, bp 60–110°). The phosphoramidate (900 mg) was collected by centrifugation. The yield (60%, 10,000 OD_{260}) was determined after conversion to the parent nucleotide by isoamyl nitrite treatment. Unreacted d-pG^{iB} (30%) was recovered from the aqueous solution.

Conversion of TPM-*p*-dG^{iB} to *p*-$dG.^{iB}$. The phosphoramidate (20.0 mg, 140 OD_{260}) was dissolved in pyridine (0.3 ml) and acetic acid (0.3 ml) and the solution treated with freshly distilled isoamyl nitrite (0.06 ml). After 12 hr at room temperature the reaction was complete as judged by tlc. The reaction mixture was concentrated, 0.2 *M* TEAB (1.0 ml) was added, and the solution was extracted with chloroform (1.0 ml). The chloroform extract was washed back with aqueous TEAB. The combined aqueous solution was concentrated and checked by paper chromatography in solvent B. The mobility of the nucleotidic product was identical with that of d-pG^{iB}. The above aqueous solution was concentrated and treated with concentrated ammonia at 50° for 3 hr. Paper chromatography in solvents A, C, and D showed the nucleotide to be identical with the d-pG obtained by treating another sample of d-pG^{iB} with concentrated ammonia.

***N*-Anisoyldeoxycitidine 5′-Phosphoro-(*p*-triphenylmethyl)anilidate (TPM-*p*-dC^{An}).** An anhydrous pyridine solution (10.0 ml) of pyridinium d-pC^{An} (1.5 mmol) was treated with DCC (1.54 g, 7.5 mmol) for 10 min at room temperature under exclusion of moisture. A dry solution of the amine I (1.0 g, 3.0 mmol) in pyridine (10.0 ml) was added to the above solution and the reaction mixture concentrated to 8.0 ml and shaken at room temperature for 12 hr. Water (8.0 ml) was then added and after a further 12 hr at room temperature the reaction mixture was concentrated and treated with ethyl acetate (50 ml). The precipitated dicyclohexylurea was filtered off and washed with ethyl acetate. The combined filtrate and washings were washed with 0.2 *M* TEAB (three 30-ml portions). The ethyl acetate solution was concentrated (4.0 ml) and the product precipitated by dropwise addition of the solution to toluene–petroleum ether (50:50, 200 ml). The precipitate was collected by centrifugation and washed with petroleum ether (three 30-ml portions). The phosphoramidate (1.6 g) was obtained in the yield of 60% (20,007 OD_{302}). (The yield was determined after conversion to the starting nucleotide by treatment with isoamyl nitrite.)

Conversion of TPM-*p*-dC^{An} to *p*-dC^{An}. The phosphoramidate (20.0 mg) was treated with isoamyl nitrite (0.06 ml) in pyridine (0.3 ml) and glacial acetic acid (0.3 ml) for 12 hr at room temperature. After the usual work-up, the d-pC^{An} obtained was found to give identical chromatographic behavior with an authentic sample of d-pC^{An}. Both authentic d-pC^{An} and d-pC^{An} obtained from the phosphoramidate on ammonia treatment gave d-pC identical in paper chromatographic behavior in solvents A, C, and D (Table II).

The Dinucleotide, d-pTpT-OAc. An anhydrous pyridine solution (2.5 ml) of pyridinium d-pT-OAc (0.5 mmol, 4800 OD_{267}) was treated with TPS (303 mg, 1 mmol) for 5 min at room temperature. A dry solution of TPM-*p*-dT (0.25 mmol, 2400 OD_{267} based on pT) in pyridine (3.0 ml) was then added and the reaction mixture was concentrated to 3.0 ml and kept at room temperature for 2.5 hr. A 1 *N* solution of diisopropylethylamine in pyridine (3.25 ml) and water (3.25 ml) was then added under cooling and the solution kept at room temperature overnight.[22] After concentration and addition of water (10 ml) the solution was extracted with chloroform to remove unreacted TPM-*p*-dT and an additional product which remains unidentified. The protected dinucleotide IX (R,R′ = thymine) was present in the aqueous phase and was extracted into CH_2Cl_2–*n*-butyl alcohol (70:30, three 10-ml portions). The combined organic phase was washed with 0.2 *M* TEAB (two 7-ml portions) and concentrated with frequent addition of pyridine to 2.0 ml. The product was precipitated by dropwise addition of the concentrated pyridine solution to an excess of dry ether and was collected and washed by centrifugation (293 mg).

The above product IX (R,R′ = thymine) was dissolved in pyridine (5.0 ml) and glacial acetic acid (5.0 ml) and the solution treated with isoamyl nitrite (234 mg, 2 mmol) for 12 hr at room temperature. The solvent was evaporated with frequent addition of pyridine to remove acetic acid. Water (10 ml) was added and the aqueous solution was extracted with chloroform (three 10-ml portions). The aqueous solution was concentrated by frequent addition of pyridine and the product was precipitated by the dropwise addition of the pyridine solution to an excess of dry ether. The pyridinium salt of the dinucleotide was collected by centrifugation; the yield (3020 OD_{267}) corresponded to 68% as based on the amount of the phosphoramidate. The dinucleotide so obtained was homogeneous on paper chromatography in solvents A and B (R_f's are in Table II).

The Dinucleotide, d-pTpC^{An}-OAc. Activation of the pyridinium d-pC^{An}-OAc (0.5 mmol, 11,150 OD_{302}) was carried out with TPS (303 mg, 1 mmol) in dry pyridine (3.0 ml) for 5 min. This was then treated with a solution of TPM-*p*-dT (0.25 mmol, 2400 OD_{267} based on pT) in pyridine (4.0 ml) and the total reaction mixture was concentrated to 3.5 ml. After being kept at room temperature for 2.5 hr, the reaction mixture was worked up by addition of a 1 *M* solution of diisopropylethylamine in pyridine (3.3 ml) followed by water. The solution was concentrated to a thick syrup and after the addition of water (10 ml) the solution was extracted with chloroform (two 30-ml portions) followed by chloroform–*n*-butyl alcohol (9:1, two 30-ml portions). The combined organic extract was washed with 0.2 *M* TEAB (three 10-ml portions) and then concentrated in the presence of pyridine. The protected dinucleotide was precipitated by the addition of ethyl acetate (40 ml), collected by centrifugation, and washed with ether. The protected dinucleotide (301 mg) was treated with isoamyl nitrite as described for the dinucleotide pTpT-OAc. The yield of the dinucleotide (as pyridinium salt), d-pTpC^{An}-OAc, was 3220 OD_{260} (54%).

The Dinucleotide, d-pTpA^{Bz}-OAc. An anhydrous solution of d-pA^{Bz}-OAc (443 mg, 0.75 mmol) in pyridine (25 ml) was first treated with TPS (453 mg, 1.5 mmol) for 5 min at room temperature and then with a solution of TPM-*p*-dT (0.221 mmol; 2120 OD_{267} based on pT) in dry pyridine (5.0 ml). The total reaction mixture was concentrated to 3.5 ml and kept at room temperature for 2.5 hr. After the work-up as described for d-pTpT-OAc (see ref 22), the aqueous solution (20 ml) was extracted with chloroform (two 30-ml portions) and washed with 0.1 *M* TEAB (two 10-ml portions). The combined chloroform extracts were concentrated (5.0 ml) in the presence of pyridine and the product was precipitated by addition to an excess of ether. The precipitate contained some TPM-*p*-dT which was removed by reprecipitation (addition of a chloroform solution to an excess of ethyl acetate–ether (50:50)). The pro-

(22) The standard work-up procedure described in the dinucleotide synthesis was as follows. After the condensation reaction (2.5–3 hr at room temperature), the mixture was cooled in Dry Ice–ethanol mixture (−20°). Diisopropylethylamine (2 mmol/mmol of TPS) was added as a 1 *M* solution in pyridine, followed by the same amount of water. The solution was then left at room temperature overnight.

tected dinucleotide TPM-*p*-dTpA^{Bz}-OAc (350 mg) on treatment with isoamyl nitrite gave the dinucleotide (as triethylammonium salt), d-pTpA^{Bz}-OAc (3060 OD_{280}, 56%). It was homogeneous as judged by paper chromatography in solvents A and B.

The Dinucleotide, d-pTpG^{iB}-OiB. TPS (453 mg, 1.5 mmol) was added to an anhydrous pyridine solution (2.0 ml) of d-pG^{iB}-OiB (389 mg, 0.75 mmol) and the reaction mixture kept for 5 min with exclusion of moisture. To this reaction mixture was then added a solution of TPM-*p*-dT (0.25 mmol, 2400 OD_{267} based on pT) in pyridine (5.0 ml). The total reaction mixture was concentrated (3 ml) before being kept at room temperature for 3 hr. After following the standard work-up procedure[22] (see above for d-pTpT-OAc) the aqueous solution (20 ml) was extracted with chloroform (three 20-ml portions). The combined chloroform extracts were washed with 0.2 *M* TEAB (2 × 10 ml) followed by concentration (4 ml) and precipitation of the product from an excess of ether. The precipitated material was once again dissolved in chloroform (2 ml) and precipitated from ethyl acetate–ether (50:50, 40 ml). On isoamyl nitrite treatment, the fully protected dinucleotide (345 mg) gave d-pTpG^{iB}-OiB (2930 OD_{280}, 66%) which was isolated as triethylammonium salt.

The Dinucleotide, d-pA^{Bz}pA^{Bz}-OAc. To an anhydrous mixture of TPM-p-dA^{Bz} (0.25 mmol, 4575 OD_{280} based on pA^{Bz}) and d-pA^{Bz}-OAc (260 mg, 0.5 mmol) in dry pyridine (5.0 ml) was added TPS (303 mg, 1.0 mmol) and the total reaction mixture concentrated (3.5 ml) and kept at room temperature for 3.0 hr. After standard work-up[22] the aqueous solution (10 ml) was extracted with methylene chloride–*n*-butyl alcohol (7:3 v/v, three 20-ml portions). The organic phase was then washed with 0.2 *M* TEAB (two 30-ml portions) and concentrated (6.0 ml). The product was precipitated into an excess of ethyl acetate–ether (50:50) and was then washed with ether and collected by centrifugation. The protected dinucleotide TPM-*p*-dA^{Bz}pA^{Bz}-OAc (405 mg) on treatment with isoamyl nitrite gave the dinucleotide (isolated as triethylammonium salt), pA^{Bz}pA^{Bz}-OAc (58%, 5309 OD_{280}), which was homogeneous by paper chromatography (see Table II).

The Dinucleotide, d-pA^{Bz}pT-OAc. An anhydrous pyridine solution (3.5 ml) of TPM-*p*-dA^{Bz} (0.25 mmol, 4575 OD_{302} based on pA^{Bz}) and d-pT-OAc (0.5 mmol) was treated with TPS (377 mg, 1.25 mmol) at room temperature for 3.0 hr with exclusion of moisture. After following the work-up procedure described for pTpT-OAc (see ref 22), the aqueous solution (20 ml) was extracted with methylene chloride–*n*-butyl alcohol (7:3, three 25-ml portions). The organic phase was washed with 0.2 *M* TEAB (two 40-ml portions) and then concentrated (4.0 ml). The product was precipitated into ethyl acetate–ether (50:50), washed with ether, and collected by centrifugation. The protected dinucleotide (300 mg) on treatment with isoamyl nitrite gave the dinucleotide d-pA^{Bz}-pT-OAc (71%, 4369 OD_{260}) which was homogeneous by paper chromatography (for R_f's see Table I).

The Dinucleotide, d-pA^{Bz}pC^{An}-OAc. An anhydrous solution of d-pC^{An}-OAc (0.5 mmol, 11,500 OD_{302}) and TPM-p-dA^{Bz} (0.25 mmol, 4575 OD_{280} based on pA^{Bz}) in pyridine (3.5 ml) was treated with TPS (303 mg, 1.0 mmol) at room temperature for 3 hr with exclusion of moisture. After following the work-up procedure described, the aqueous solution (15 ml) was extracted with methylene chloride–*n*-butyl alcohol (7:3, three 20-ml portions). The organic phase was washed with 0.2 *M* TEAB (two 15-ml portions) and then concentrated (6.0 ml). The product was precipitated into excess of ethyl acetate–ether (50:50), washed with ether, and collected by centrifugation. The protected dinucleotide (250 mg) on treatment with isoamyl nitrite gave the dinucleotide (as isolated as triethylammonium salt), pA^{Bz}pC^{An}-OAc (60%, 5400 OD_{280}), which was homogeneous by paper chromatography in solvents A and B.

The Dinucleotide, d-pA^{Bz}pG^{iB}-OiB. TPS (1.5 g, 5 mmol) was added to an anhydrous pyridine solution (8 ml) of d-pG^{iB}-OiB (1.04 g, 2.0 mmol) and the reaction mixture was allowed to stand at room temperature for 5 min with exclusion of moisture. The reaction mixture was further treated with TPM-*p*-dA^{Bz} (1 mmol, 18,300 OD_{280} based on pA^{Bz}) in pyridine (5.0 ml) and the total reaction mixture was concentrated (10 ml) and kept at room temperature for 2.5 hr. After following the work-up procedure described,[22] the aqueous solution (30 ml) was extracted with methylene chloride–*n*-butyl alcohol (7:3, three 30-ml portions) and the organic phase was washed with 0.2 *M* TEAB (three 20-ml portions) before being concentrated (10 ml). The product was precipitated by addition to an excess of ethyl acetate–ether (50:50), washed with ether, and collected by centrifugation. The protected dinucleotide (1.3 g) on treatment with isoamyl nitrite gave the dinucleotide (isolated, as triethylammonium salt), d-pA^{Bz}pG^{iB}-OiB (58%, 16,000 OD_{260}). The dinucleotide was homogeneous by paper chromatography (see Table II).

The Dinucleotide, d-pC^{An}pT-OAc. An anhydrous solution of d-pT-OAc (0.4 mmol, 3840 OD_{267}) and TPM-p-dC^{An}-OH (0.2 mmol, 4500 OD_{302} based on pC^{An}) in pyridine (3.0 ml) was treated with TPS (273 mg, 0.9 mmol) at room temperature for 3 hr. After following the work-up procedure described,[22] the reaction mixture was concentrated to a thick syrup. This was dissolved in chloroform (20 ml) and washed with 0.2 *M* TEAB (four 15-ml portions). The aqueous phase containing the dinucleotide was further extracted with methylene chloride–*n*-butyl alcohol (7:3, v/v, four 20-ml portions) and the organic phase was backwashed with 0.2 *M* TEAB (two 20-ml portions). Chloroform and *n*-butyl alcohol–methylene chloride extracts were combined and concentrated (3.0 ml) in the presence of pyridine. The pyridine solution was added dropwise to an excess of ethyl acetate–ether (50:50) and the precipitated dinucleotide was washed with the same solvent mixture and then with ether. The precipitated dinucleotide phosphoramidate (162 mg) was treated with isoamyl nitrite as described in the general procedure. After this treatment the aqueous solution (10 ml) was extracted with *n*-butyl alcohol–methylene chloride (3:7); if the amount of organic phase exceeded 2.0 ml then some product was lost in the organic layer as shown by tlc. The dinucleotide (triethylammonium salt), after usual work-up, was precipitated into ether and dried (2470 OD_{280}, 51%). The dinucleotide was homogeneous by paper chromatography in solvents described in Table II.

The Dinucleotide, pC^{An}pC^{An}-OAc. An anhydrous pyridine solution (2.0 ml) of pyridinium d-pC^{An}-OAc (0.4 mmol, 9600 OD_{302}) was treated with TPS (273 mg, 0.9 mmol) for 5 min at room temperature. To this reaction mixture was then added a dry solution of TPM-pC^{An} (0.2 mmol, 4500 OD_{302} as based on pC^{An}) in pyridine (4.0 ml). The total reaction mixture was concentrated (3 ml) and kept for 3 hr at room temperature with exclusion of moisture. After the usual aqueous pyridine treatment,[22] the reaction mixture was concentrated and the oily residue dissolved in chloroform (30 ml). Chloroform extract was then washed with 0.2 *M* TEAB (four 15-ml portions) and the aqueous phase was further extracted with *n*-butyl alcohol–methylene chloride (3:7, four 20-ml portions). On examination of chloroform and *n*-butyl alcohol–methylene chloride extracts it was found that the product was present in the chloroform phase whereas the sample from *n*-butyl alcohol–methylene chloride phase did not give any dinucleotide on treatment with isoamyl nitrite but an unidentified side product was obtained. Chloroform solution of the required product was concentrated and dried by repeated evaporation with pyridine to 3.0 ml. The solution was precipitated into ethyl acetate–ether (70:30). The precipitate was collected and washed with the above mixture and finally with ether. The dinucleotide phosphoramidate (135 mg) was obtained as a light brown powder which on treatment with isoamyl nitrite gave the dinucleotide (triethylammonium salt), d-pC^{An}pC^{An}-OAc (56%, 4950 OD_{302}).

The Dinucleotide, pC^{An}pA^{Bz}-OAc. An anhydrous pyridine solution (3.0 ml) of d-pA^{Bz}-OAc (0.4 mmol, 7320 OD_{280}) and TPM-pC^{An} (0.2 mmol, 4500 OD_{302} based on pC^{An}) was treated with TPS (273 mg, 0.9 mmol) at room temperature for 3 hr. After the usual work-up[22] and aqueous pyridine treatment, the solution was concentrated to an oil and then dissolved in chloroform (30 ml). The chloroform solution was extracted with 0.2 *M* TEAB (four 15-ml portions). The organic phase contained the required product and was concentrated after addition of pyridine. The residue was dissolved in chloroform (3 ml) and precipitated into ethyl acetate–ether (50:50). The precipitate was collected by centrifugation and washed with ethyl acetate–ether (50:50) and then with ether. The dinucleotide phosphoramidate (177 mg) was treated with isoamyl nitrite by standard procedure to yield the dinucleotide (as triethylammonium salt), pC^{An}pA^{Bz}-OAc (3280 OD_{280}, 46%).

The Dinucleotide, pC^{An}pG^{iB}-OiB. Activation of d-pG^{iB}-OiB (0.4 mmol, 4600 OD_{280}) was carried out with TPS (273 mg, 0.9 mmol) in dry pyridine (3 ml) for 5 min at room temperature. This was further treated with a solution of TPM-p-C^{An} (0.2 mmol, 4500 OD_{302} based on pC^{An}) in dry pyridine (4 ml) and the total reaction mixture (3 ml) was allowed to stand at room temperature for 3 hr. After the usual work-up,[22] the reaction mixture was concentrated and dissolved in chloroform (25 ml). Chloroform solution was then washed with 0.2 *M* TEAB (four 15-ml portions), concentrated, and rendered anhydrous by repeated evaporation of pyridine. The pyridine solution (3 ml) was precipitated into ethyl acetate–ether (50:50) and the precipitate was washed with this

solvent three times before being washed with ether. The dinucleotide phosphoramidate (slightly brown-colored powder, 164 mg) gave dinucleotide (as triethylammonium salt) $pC^{An}pG^{iB}$-OiB (3040 OD_{280}, 52%) on treatment with isoamyl nitrite.

The Dinucleotide, d-pG^{iB}pT-OAc. An anhydrous pyridine solution (4.0 ml) of TPM-p-G^{iB} (0.25 mmol, 4175 (OD_{260} based on pG^{iB}) and d-pT-OAc (0.5 mmol, 4800 OD_{267}) was treated with TPS (303 mg, 1.0 mmol) at room temperature for 3 hr with exclusion of moisture. After usual work-up the aqueous solution (15 ml) was extracted with *n*-butyl alcohol–methylene chloride (three 20-ml portions, 3:7). The organic phase was washed with 0.2 *M* TEAB (two 40-ml portions) and then concentrated (4.0 ml) in the presence of pyridine. The product was precipitated into ethyl acetate–ether (50:50) and washed with the same solvent once more and then with ether. The protected dinucleotide phosphoramidate (300 mg) on treatment with isoamyl nitrite gave the dinucleotide (as triethylammonium salt), d-pG^{iB}pT-OAc (60%, 2750 OD_{280}).

The Dinucleotide, d-$pG^{iB}pA^{Bz}$-OAc. An anhydrous solution of of d-pA^{Bz}-OAc (9150 OD_{280}, 0.5 mmol) and TPM-p-G^{iB} (0.25 mmol, 4175 OD_{260} based on pG^{iB}) in pyridine (4.0 ml) was treated with TPS (303 mg, 1.0 mmol) at room temperature for 3 hr. After following the standard work-up procedure,[22] the reaction mixture was concentrated to a thick oil which was treated with *n*-butyl alcohol–methylene chloride (15 ml, 3:7) and 0.2 *M* TEAB (10 ml). The aqueous phase was further extracted with the same organic solvent as described above (two 15-ml portions) and the combined extracts were washed with 0.2 *M* TEAB (three 10-ml portions). Concentration (4 ml) of combined organic extracts was carried out in the presence of pyridine. The concentrated pyridine solution was then precipitated into ethyl acetate–ether (50:50) to give the dinucleotide phosphoramidate (270 mg). This on treatment with isoamyl nitrite yielded the dinucleotide (as triethylammonium salt), d-$pG^{iB}pA^{Bz}$-OAc (4600 OD_{280}, 62%).

The Dinucleotide, d-$pG^{iB}pG^{iB}$-OiB. TPS (400 mg, 1.3 mmol) was added to an anhydrous pyridine solution (4.5 ml) of TPM-p-G^{iB} (0.25 mmol, 4175 OD_{260} based on pG^{iB}) and d-pG^{iB}-OiB (6850 OD_{280}, 0.6 mmol). The total reaction mixture was allowed to stand at room temperature for 3 hr with exclusion of moisture. After the usual work-up,[22] the aqueous solution (15 ml) was extracted with *n*-butyl alcohol–methylene chloride (3:7, three 20-ml portions). The organic phase was further extracted with 0.2 *M* TEAB (three 20-ml portions) and then concentrated in the presence of pyridine to a solution of 3 ml. This was then precipitated into ethyl acetate–ether (50:50) and the precipitate collected by centrifugation. The dinucleotide phosphoramidate (290 mg) was treated with isoamyl nitrite as described in the general method to give the dinucleotide (as triethylammonium salt), d-$pG^{iB}pG^{iB}$-OAc (58%, 3306 OD_{280}).

The Crystal Structure of a Thymine Trimer, $C_{15}H_{20}N_6O_7 \cdot H_2O$, a Photoproduct of Thymine

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Abstract: A hydrated trimer has been isolated by Varghese and Wang from the uv irradiation of a frozen aqueous solution of thymine. The most common products of the irradiation are the cyclobutyl-type dimers. The X-ray crystal-structure analysis has established the stereoconfiguration of the trimer and has shown that it is hydrated (see VII). Similarities to both the *cis-syn*-cyclobutyl dimers and to the thymine–thymine adduct are apparent. The trimer crystallizes in the triclinic space group $P\bar{1}$ with one molecule of H_2O of crystallization per molecule of trimer. Cell parameters are $a = 9.373$, $b = 14.387$, and $c = 7.201$ Å, $\alpha = 103.2$, $\beta = 100.0$, and $\gamma = 91.8°$. The molecules are held together in the crystal by an extensive system of intra- and intermolecular hydrogen bonds. The structure was solved directly by means of the symbolic addition procedure.

Irradiation of frozen aqueous solutions of thymine, uracil, and some of their derivatives has yielded several different kinds of photoproducts such as the cyclobutyl dimers[1,2] and, more recently, the thymine–thymine adduct.[3] For products I,[4–7] II,[8,9] IV,[10,11] and V,[12,13] or simple derivatives thereof, the molecular formulas have been confirmed and the stereoconfigurations have been established by crystal-structure analyses using X-ray diffraction.

Ultraviolet irradiation of [*2-*^{14}*C*]- or [*5-methyl-*^{3}*H*]-thymine in frozen aqueous solution produces photoproducts designated in the literature[14] as PT_1 and PT_2. The product PT_2 has been shown to be a mixture of the *cis-syn*-cyclobutyl dimer and thymine–thymine adduct[3] while PT_1 appeared to be a trimer of thymine.[14b] The trimer has the characteristics of the adduct, the photoreversibility of the cyclobutyl dimers, and the instability of the hydration products. In the present study, an X-ray analysis of a single crystal of the trimer has established its molecular formula and stereoconfiguration. A preliminary note on the structure has been reported.[15]

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