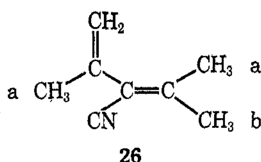


1,3-pentadiene (26): bp 71–74° (14.5 mm); ir (neat) 4.52 (C≡N), 6.08 and 6.14 μ (C═C); nmr (neat) δ 1.89 (split s, 6, a protons), 2.07 (s, 3, b protons), 4.87 (broad s, 1, vinyl proton), and 5.17 (m, 1, vinyl proton).

Anal. Calcd for $C_8H_{11}N$: C, 79.29; H, 9.15; N, 11.56. Found: C, 79.33; H, 9.61; N, 11.34.

Registry No.—**2,** 4786-24-7; **4,** 5631-82-3; **5,** 26157-47-1; **7,** 26157-48-2; **8,** 26154-39-2; **10,** 26157-49-3; **12,** 4786-26-9; **15,** 1885-38-7; **18,** 26157-51-7; **23,** 26157-52-8; **24,** 4786-37-2; **26,** 26154-42-7; 2,2,3-trimethylbutanenitrile, 26154-43-8.

Phosphoramidate Analogs of Oligonucleotides[1]

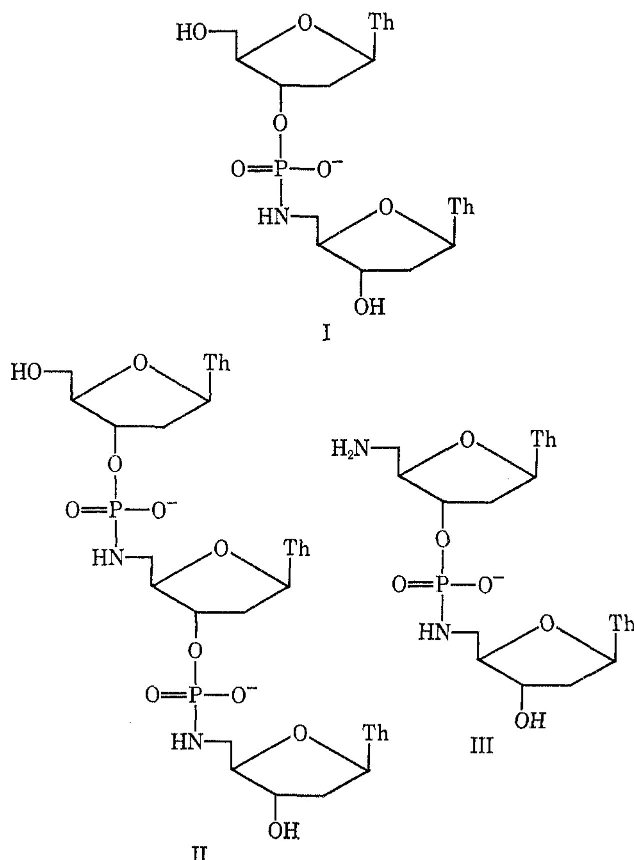
ROBERT L. LETSINGER AND WILLIAM S. MUNGALL[2]

Department of Chemistry, Northwestern University, Evanston, Illinois 60201

Received March 9, 1970

Some dinucleoside phosphate and trinucleoside diphosphate analogs that possess internucleotide phosphoramidate bonds [-OP(O)NH-] are described. These compounds are stable in neutral and alkaline solution, but they hydrolyze in acidic solutions and in solutions containing snake venom phosphodiesterase or spleen phosphodiesterase. A possible role for substances of this type in the synthesis of defined polynucleotides is suggested.

We describe in this paper the synthesis and some chemical properties of the oligonucleotide analogs thymidylyl-(3′–5′)-5′-amino-5′-deoxythymidine (Tp_NT, compound I), thymidylyl-(3′–5′)-5′-amino-5′-deoxythymidylyl-(3′–5′)-5′-amino-5′-deoxythymidine (Tp_NTp_NT, compound II), and 5′-amino-5′-deoxythymidylyl-(3′–5′)-5′-amino-5′-deoxythymidine ($_NTp_NT$, compound III). These compounds were prepared as



models to explore the accessibility and stability of polymers containing nucleoside units joined by O–P–N bonds. Our interest in this class of compounds was stimulated by the prospect that the stepwise chemical synthesis of such analogs might be more readily achieved than the synthesis of the natural polynucleotides and that the phosphoramidate analogs might serve as templates for enzymatic synthesis of defined polynucleotides from the nucleoside triphosphates and the polymerase enzymes.

The general synthetic approach was patterned after the phosphotriester method for oligonucleotides[3] as modified by Reese and Saffhill.[4] Thymidine was first protected by reaction with isobutyl chloroformate, a reagent that reacts selectively at the 5′ oxygen.[5] Treatment of the resulting ester, 5′-*O*-isobutyloxycarbonylthymidine, with phenyl phosphorodichloridate and pyridine in dioxane, followed by 5′-amino-5′-deoxythymidine[6] and triethylamine in dioxane, afforded the protected derivative, compound IV. This phosphoramidate was isolated in 83% yield by chromatography on silica gel. In agreement with expectations, the condensation of the phosphoryl monochloride with the amino group of 5′-amino-5′-deoxythymidine proceeded rapidly, being complete in less than 30 min. This feature is advantageous since it would facilitate the synthesis of a long chain poly(aminodeoxy nucleotide), both by reducing the time (relative to the time for synthesizing a natural polynucleotide *via* phosphoryl chlorides) and by eliminating the necessity for blocking the oxygen function at the 3′ position of the nucleosides.

One of the major questions concerning the utility of polynucleotide phosphoramidate analogs pertained to the stability of the internucleoside links. Simple phosphoramidates are known to be relatively labile; they hydrolyze readily in aqueous acid[7] and are suffi-

(1) Part XVI in series on Nucleotide Chemistry; for XV, see R. L. Letsinger, K. K. Ogilvie, and P. S. Miller, *J. Amer. Chem. Soc.*, **91**, 3360 (1969). This research was supported in part by a research grant (GM 10265) from the Division of General Medical Sciences of the National Institutes of Health.

(2) National Science Foundation Predoctoral Fellow, 1968–present.

(3) R. L. Letsinger and K. K. Ogilvie, *J. Amer. Chem. Soc.*, **91**, 3350 (1969); **89**, 4801 (1967).

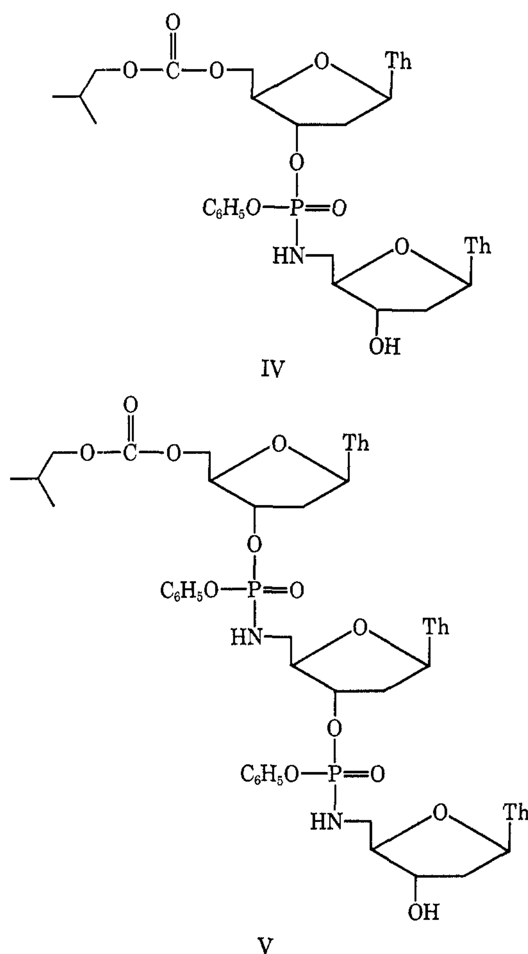
(4) C. B. Reese and R. Saffhill, *Chem. Commun.*, 767 (1968).

(5) K. K. Ogilvie and R. L. Letsinger, *J. Org. Chem.*, **32**, 2365 (1967).

(6) J. P. Horwitz, A. J. Tomson, J. A. Urbanski, and J. Chua, *ibid.*, **27**, 3045 (1962).

(7) (a) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. II, W. A. Benjamin, New York, N. Y., 1966, p 71; (b) A. W. Garrison and C. E. Boozer, *J. Amer. Chem. Soc.*, **90**, 3486 (1968).

ciently reactive toward nucleoside phosphates and pyrophosphate[8] to serve as reagents for preparation of dinucleoside pyrophosphates and nucleoside triphosphates. Jastorff and Hettler[9] recently synthesized an N-phosphorylated 5′-amino-5′-deoxy nucleoside and found that it hydrolyzed readily in neutral aqueous solution. Fortunately, the internucleoside phosphoramidate link in IV proved to be relatively stable. Thus we found that IV was unchanged for extended periods (48 hr) in anhydrous pyridine and in a mixture of 4 parts of pyridine and 1 part of acetic acid at room temperature. Furthermore, there was no evidence of decomposition when IV was chromatographed on silica gel with ethyl acetate.



On alkaline hydrolysis, the isobutyloxycarbonyl and phenoxy groups were cleanly removed from IV to give I. This substance did not degrade further when heated with 0.5 M aqueous sodium hydroxide at 98° for 1 hr. Compound I was also stable in 50% aqueous pyridine (64 hr) and in concentrated ammonium hydroxide (84 hr) at room temperature; however, it exhibited some degradation when heated with 50% aqueous pyridine at 100° for 25 min. That this latter reaction involves nucleophilic attack by pyridine rather than a base-catalyzed reaction is suggested by the observation that I is stable when heated with 50% aqueous 2,6-lutidine under the same conditions.[10] Especially important for the projected study of template activity of polynucleotide phosphoramidate analogs is the observation that I is stable in aqueous solutions containing phosphate (0.5 M at pH 8.6) or a nucleoside triphosphate (no reaction found over a period of 9 days for 10^{-2} M $\mathrm{Tp_NT}$ in the presence of 2×10^{-2} M adenosine triphosphate in 0.1 M Tris-HCl buffer at pH 8).

Successive treatment of compound IV with phenyl phosphorodichloridate and 5′-amino-5′-deoxythymidine afforded the protected trinucleotide analog, compound V. Like IV, this compound was relatively stable in neutral and in weakly alkaline solutions. On treatment with strong alkali (0.1 M sodium hydroxide in 50% aqueous dioxane), it was smoothly converted to II without any cleavage of P–N bonds.

Both I and II were completely hydrolyzed when heated at steam bath temperature in 80% aqueous acetic acid for 20 min. Compound I gave equimolar amounts of thymidine 3′-phosphate (Tp) and 5′-amino-5′-deoxythymidine ($\mathrm{_NT}$), as determined by the absorbance of material eluted from paper chromatograms. Compound II similarly afforded equimolar amounts of Tp, $\mathrm{_NT}$, and 5′-amino-5′-deoxythymidine 3′-phosphate ($\mathrm{_NTp}$).

Of especial interest are the hydrolyses effected by the exonucleases, snake venom phosphodiesterase and spleen phosphodiesterase. Whereas a P–O bond in the sequence O–P–O is broken in a reaction of a natural substrate, P–O or P–N in the sequence O–P–N must be broken if I or II is hydrolyzed. Both enzymes in fact attacked the phosphoramidates, though at a reduced rate relative to attack on TpT or TpTpT. Snake venom phosphodiesterase degraded both I and II completely, yielding T and $\mathrm{_NT}$ (1:1 ratio for I; 1:2 ratio for II). Formation of aminodeoxythymidine in these reactions may be rationalized on the basis that 5′-amino-5′-deoxythymidine 5′-phosphate, the expected product, hydrolyzes spontaneously in water to orthophosphate and aminodeoxythymidine.[9] Spleen phosphodiesterase, which attacks an oligonucleotide from the 5′ terminus, converted I to Tp and $\mathrm{_NT}$ (P–N cleavage). In the case of II, the first nucleotide unit was removed from the 5′ end; however, the reaction then slowed down markedly. At the end of 18 hr (compare with I, which was completely degraded within 12 hr) the products were Tp, $\mathrm{_NTp}$, $\mathrm{_NT}$, and a fourth substance which exhibited properties expected for $\mathrm{_NTp_NT}$. This result indicates that a terminal 5′-amino group inhibits the action of spleen phosphodiesterase.

To test the conclusion that a 5′-amino group in the nucleotide substrate inhibits the action of spleen phosphodiesterase, we prepared 5′-amino-5′-deoxythymidylyl-(3′–5′)-5′-amino-5′-deoxythymidine (III). This compound was made from 5′-*N*-isobutyloxycarbonyl-5′-amino-5′-deoxythymidine by the procedure used to prepare I from 5′-*O*-isobutyloxycarbonylthymidine. In agreement with expectations based on the behavior of II, compound III was completely hydrolyzed to 5′-amino-5′-deoxythymidine by an aqueous solution of snake venom phosphodiesterase, and the reaction with spleen phosphodiesterase was slow. With the spleen enzyme, 13% of the sample of III remained at the end of 18 hr; otherwise the hydrolysis proceeded normally, yielding equivalent amounts of 5′-amino-5′-deoxythymidine 3′-phosphate and 5′-amino-5′-deoxythymidine.

(8) J. G. Moffatt and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 649 (1961).
(9) B. Jastorff and H. Hettler, *Tetrahedron Lett.*, 2543 (1969).
(10) Compare ref 7a, p 78.

These synthetic and degradative experiments demonstrate that the phosphoramidate analogs of oligothymidylates can be made and that they are stable in aqueous neutral and alkaline solutions. Work is now in progress to synthesize long-chain oligonucleotide analogs to test the template activity of such compounds.

Experimental Section

Infrared spectra were recorded on a Beckman IR-5 spectrophotometer and ultraviolet spectra were recorded on a Cary 11 spectrophotometer. Melting points were determined with a Kofler hot-stage microscope apparatus and are uncorrected. Elemental analyses were made by Micro-Tech Laboratories, Skokie, Ill.

Reagent grade pyridine was distilled from *p*-toluenesulfonyl chloride, redistilled from calcium hydride, and stored over Linde 4-A molecular sieves. Reagent grade 1,4-dioxane was distilled from lithium aluminum hydride and stored over molecular sieves. Triethylamine was distilled from *p*-toluenesulfonyl chloride, redistilled from calcium hydride, and stored over barium oxide. DEAE-cellulose (0.69 equiv/g) was a product of Bio-Rad Laboratories. ChromAR 1000, a preparative thin layer chromatography (tlc) medium, was purchased from Mallinckrodt Chemical Co. For analytical tlc, Eastman 6060 sheets were used.

Paper electrophoresis was performed on a Savant flatbed apparatus at 2000 V using Whatman 3MM paper and a 0.05 *M* sodium phosphate buffer (pH 7.2). Nucleosides and their derivatives were located under uv light. In addition, 5′-amino-5′-deoxythymidine was detected by the 2,4-dinitrofluorobenzene color test.[11] Paper chromatography was carried out on Whatman 3MM paper by the descending technique. The solvent systems were: A, isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2 v/v/v); C, ethanol–1 *M* aqueous ammonium acetate (7:3, pH 7.5); F, *n*-propyl alcohol–concentrated ammonium hydroxide–water (55:10:35). For quantitative determinations the bands were cut out, eluted with water, and diluted to a known volume. Absorbances were determined with a Gilford spectrophotometer, and the values were corrected by subtraction of the absorbancy for a blank cut from the paper adjacent to the product spot. The extinction coefficients used in calculating yields are 9.7×10^3 at 267 nm for T, Tp, and pT[12] and 9.3×10^3 at 266 nm for $_N$T.[6]

Phenyl Ester of 5′-*O*-Isobutyloxycarbonylthymidylyl-(3′–5′)-5′-amino-5′-deoxythymidine (IV).—5′-*O*-Isobutyloxycarbonylthymidine[5] (68 mg, 0.20 mmol) was dried by evaporation of three 1-ml portions of pyridine under reduced pressure. The resulting gum was dissolved in 4 ml of dioxane and treated with pyridine (0.032 ml, 0.40 mmol) and phenyl phosphorodichloridate (0.031 ml, 0.20 mmol); then the solution was stirred 48 hr at room temperature. Triethylamine (0.056 ml, 0.40 mmol) and 5′-amino-5′-deoxythymidine[6] (72 mg, 0.30 mmol) in 18 ml of dioxane were added, the mixture was stirred for 30 min, aqueous sodium hydroxide (0.7 ml of 0.5 *M* solution) was added, and the solution was concentrated (<30°) at reduced pressure. Following addition of 2 ml of saturated aqueous sodium chloride, the mixture was extracted three times with 10-ml portions of ethyl acetate. On concentration of the ethyl acetate extracts and chromatography on two sheets of ChromAR 1000 (20 × 20 cm, developed twice with ethyl acetate), three bands were observed: a dark band at the origin consisting of excess $_N$T, a dark product band centered at R_f 0.3, and a faint band near the solvent front. Elution of the product band with tetrahydrofuran and precipitation by addition of hexane afforded 120 mg (83%) of compound IV, mp 108–111°, homogeneous on tlc in tetrahydrofuran (R_f 0.67), 1:1 tetrahydrofuran–ethyl acetate (R_f 0.44), acetone (R_f 0.36), and ethyl acetate (R_f 0.05). For analysis, a sample was reprecipitated from tetrahydrofuran with hexane, mp 108–111°, λ_{max} (CH_3OH) 265 nm (ϵ 1.9×10^4), and λ_{min} 234 nm.

Anal. Calcd for $C_{31}H_{40}N_5O_{13}P \cdot H_2O$: C, 50.33; H, 5.72; N, 9.47. Found: C, 50.29; H, 5.40; N, 9.49.

Thymidylyl-(3′–5′)-5′-amino-5′-deoxythymidine (I).—Compound IV (99 mg) was dissolved in 6 ml of 0.1 *M* sodium hydroxide in 50% aqueous dioxane and kept at room temperature for 6 hr. The solution was then neutralized with a minimum of pyridinium Dowex 50 resin, filtered to remove the resin (which was then washed with water), concentrated to 10 ml (<25°), diluted to 20 ml with water, concentrated again to remove dioxane, and applied to a DEAE-cellulose column (3 × 34 cm) in the bicarbonate form. The column was eluted with 2 l. of a linear gradient solution ranging from 10^{-3} *M* ammonium bicarbonate (in 10% aqueous ethanol adjusted to pH 8 with ammonium hydroxide) to 10^{-1} *M* ammonium bicarbonate (also in 10% aqueous ethanol, pH 8). Fractions of 13 ml were collected at the rate of 80 ml/hr. Two uv absorbing bands were eluted: the first (fractions 8–26) contained unhydrolyzed phenyl ester; the second (fractions 39–58) contained the desired Tp$_N$T (I). This material was obtained as the ammonium salt by concentrating the solution under reduced pressure, lyophilizing, redissolving the solid in water, and lyophilizing again to remove residual ammonium bicarbonate: weight 55 mg (70%); mp 205–208° dec; λ_{max} (H_2O) 267 nm (ϵ 1.9×10^4). It was homogeneous on chromatography in solvent A (R_f 0.28), C (R_f 0.55), F (R_f 0.67), and on electrophoresis (R_m 0.33 relative to pT).

Anal. Calcd for $C_{20}H_{31}N_6O_{11}P \cdot 3H_2O$: C, 38.96; H, 6.04; N, 13.63. Found: C, 39.34; H, 5.36; N, 13.38.

Diphenyl Ester of 5′-*O*-Isobutyloxycarbonylthymidylyl-(3′–5′)-5′-amino-5′-deoxythymidylyl-(3′–5′)-5′-amino-5′-deoxythymidine (V).—This compound was prepared from IV (361 mg, 0.50 mmol) by reaction with the same reagents in the same relative molar amounts as used for conversion of 5′-*O*-isobutyloxycarbonylthymidine to IV. On chromatography on a silica gel column (3 × 35 cm) with ethyl acetate and tetrahydrofuran, three substances were found: $_N$T, V, and IV. Compound V was eluted from the column with 50% ethyl acetate–tetrahydrofuran and precipitated with hexane, weight 320 mg (58%). It was homogeneous on tlc with acetone (R_f 0.19) and tetrahydrofuran–ethyl acetate (1:1, R_f 0.32).

Anal. Calcd for $C_{47}H_{58}N_8O_{19}P_2 \cdot H_2O$: C, 50.44; H, 5.40; N, 10.01. Found: C, 50.48; H, 5.35; N, 10.14.

Compound V (2 mg) was treated with 0.2 ml of 0.1 *M* sodium hydroxide in 50% aqueous dioxane for 6 hr at room temperature. The solution was carefully neutralized with 1 *M* hydrochloric acid and samples were withdrawn for testing. A single nucleotide spot was found on paper chromatography in solvents A (R_f 0.06) and C (R_f 0.32) and on paper electrophoresis (R_m 0.52 relative to pT). For enzyme assay, compound II was further purified by electrophoresis and by paper chromatography in solvent A and was collected as a dry powder by lyophilization.

Hydrolytic Degradation.—Acid-catalyzed hydrolyses were carried out by heating the nucleotidic material (1 mg) in a solution of acetic acid–water (80:20 v/v) at steam bath temperature for 20 min. The products were then separated by chromatography on paper with solvent A.

Enzymatic reactions were patterned after procedures described in the literature.[13] Standard solutions of the enzymes were prepared by dissolving commercial snake venom phosphodiesterase (200 units, Calbiochem) in 0.1 *M* Tris buffer (1 ml, adjusted to pH 9.2 with hydrochloric acid) and by adding spleen phosphodiesterase (10–15 units, Nutritional Biochemical Co.) to a 0.01 *M* sodium pyrophosphate buffer (1 ml, adjusted to pH 6.5 with phosphoric acid). For reaction with the snake venom enzyme, 0.1 ml of the standard enzyme solution was added to the solid nucleotidic sample (~10 OD units) and the solution was incubated at 37° for 12 hr. Additional enzyme solution (0.1 ml) was added and the reaction was continued for another 6 hr. The solution was then frozen, lyophilized, and subjected to chromatography with solvent A. The reactions with the spleen enzyme were similarly carried out by mixing 0.1 ml of the standard enzyme solution with a solution of the substrate (~10 OD units) in 0.2 ml of 0.5 *M* ammonium acetate buffer (pH 6.5). After 12 hr, additional enzyme solution (0.1 ml) was added and the reaction was continued another 6 hr before work-up. Data on the products are summarized in Tables I and II. When the reaction of I with the snake venom enzyme was terminated at the end of 12 hr, 13% of I remained. Also, the reaction of I with spleen phosphodiesterase was incomplete after 7 hr. In contrast, under the same conditions TpT and TpTpT were completely hy-

(11) N. D. Cheronis and J. B. Entrikin, "Identification of Organic Compounds," Interscience, New York, N. Y., 1963, p 131.

(12) H. G. Khorana and J. P. Vizsolyi, *J. Amer. Chem. Soc.*, **83**, 675 (1961).

(13) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961); M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *ibid.*, **84**, 430 (1962).

TABLE I
PROPERTIES OF HYDROLYTIC PRODUCTS OF I, II, III

Compd	R_f[a]	R_m[b]
T	0.60	−0.11
Tp	0.11	1.00
Tp_NT	0.28	0.33
Tp_NTp_NT	0.07	0.55
$_NT$	0.47	−0.69
$_NTp$	0.05	0.45
$_NTp_NT$	0.14	−0.10

[a] Paper chromatograph, solvent A, ~23°. [b] Electrophoretic mobility relative to pT.

dine (68 mg, 0.20 mmol) was used in place of 5′-*O*-isobutyloxycarbonylthymidine. The product was applied to two sheets of ChromAR 1000 (20 × 20 cm) and developed four successive times with ethyl acetate. At this point the desired material had moved about a quarter the length of the sheet. It was removed by elution with tetrahydrofuran and was precipitated by addition of hexane, weight 76 mg (53%), homogeneous on tlc with acetone (R_f 0.34), tetrahydrofuran-ethyl acetate (1:1, R_f 0.31), and tetrahydrofuran (R_f 0.65). The analytical sample was obtained by dissolving the sample in tetrahydrofuran, filtering, and precipitating with hexane, mp 128–130°.

Anal. Calcd for $C_{31}H_{41}N_6O_{12}P \cdot H_2O$: C, 50.40; H, 5.87; N, 11.38. Found: C, 50.55; H, 5.78; N, 11.15.

TABLE II
HYDROLYTIC PRODUCTS

Substrate	Catalyst	Relative molar amounts[a]				
		T	$_NT$	$_NTp_NT$	Tp	$_NTp$
Tp_NT	H+		1.0		0.91	
	Venom	0.92	1.0			
	Spleen		1.0		1.05	
Tp_NTp_NT	H+		1.0		1.07	0.99
	Venom	1.00	1.98			
	Spleen		0.42	0.38	1.00	0.49
$_NTp_NT$	H+		1.0			0.94
	Venom		1.0			
	Spleen		1.0	0.13		1.1

[a] Relative to one of the products, usually $_NT$.

drolyzed by both enzymes within 7 hr. Control experiments showed that no hydrolysis occurred when I or II was subjected to the action of the buffers in the absence of enzymes.

5′-*N*-Isobutyloxycarbonyl-5′-amino-5′-deoxythymidine.—To a solution of 5′-amino-5′-deoxythymidine (120 mg, 0.50 mmol) in pyridine (5 ml) was added isobutyl chloroformate (0.07 ml, 0.5 mmol). After standing 15 min at room temperature, the solution was mixed with water (0.5 ml), concentrated to a syrup, and mixed with ethyl acetate (60 ml) and water (5 ml). The organic layer was separated, washed with water, and evaporated. Recrystallization of the residue from acetonitrile afforded 147 mg (88%) of the title compound, mp 181-183°, homogeneous on tlc with ethyl acetate (R_f 0.16), acetone (R_f 0.61), and tetrahydrofuran–ethyl acetate (1:1, R_f 0.62). The analytical sample, mp 184–185°, was obtained by recrystallization from acetonitrile–ethyl ether (3:1).

Anal. Calcd for $C_{15}H_{23}N_3O_6$: C, 52.78; H, 6.79; N, 12.31. Found: C, 59.92; H, 6.72; N, 12.56.

5′-Amino-5′-deoxythymidylyl-(3′–5′)-5′-amino-5′-deoxythymidine (III).—The procedure used to prepare IV was followed except that 5′-*N*-isobutyloxycarbonyl-5′-amino-5′-deoxythymidine (68 mg, 0.20 mmol) was used in place of 5′-*O*-isobutyloxycarbonylthymidine.

For removal of the isobutyloxycarbonyl and phenyl groups, a sample (1.61 mg) was treated with 2 *M* aqueous sodium hydroxide (0.2 ml) for 2 hr on a steam bath. The solution was neutralized to a phenolphthalein end point with dilute hydrochloric acid and diluted with 2 vol of methanol to precipitate most of the sodium chloride. On application of the solution to paper (3MM) and development with solvent A, a single nucleotidic spot was observed (R_f 0.15–0.20). The band was cut out and compound III was eluted with water. It was homogeneous on paper chromatography in solvent A and C (R_f 0.23) and on electrophoresis (R_m −0.1 relative to pT). For hydrolytic studies, the sample was rechromatographed on paper with solvent F and recovered from the paper in the usual way. The reactions with acetic acid and the hydrolytic enzymes were carried out as in the case of compounds I and II (Table II).

Registry No.—I, 25383-42-0; II, 25383-43-1; phenyl ester of 5′-*N*-isobutyloxycarbonyl derivative of III, 25383-44-2; IV, 25442-41-5; V, 25383-45-3; 5′-*N*-isobutyloxycarbonyl-5′-amino-5′-deoxythymidine, 25383-46-4.