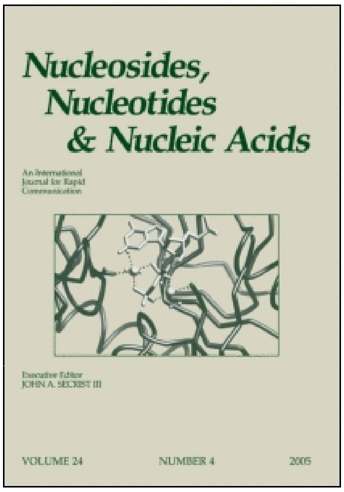

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P^1-(Thymidine 5'-)P^1-amino-triphosphate: Synthesis, Hydrolysis and Reaction with Cytidine in Alkali[1]

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P^1-(THYMIDINE 5'-)P^1-AMINO-TRIPHOSPHATE: SYNTHESIS, HYDROLYSIS AND REACTION WITH CYTIDINE IN ALKALI[1]

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Abstract. The title compound 1 is prepared from thymidine 5'-phosphorodiamidate (2) and inorganic pyrophosphate (3) in anhydrous DMF, at 30-32°C. The products of alkaline hydrolysis of 1, at room temperature, are: thymidine 5'-phosphoramidate (4), thymidine 3'-phosphoramidate (8) and thymidine (9) as well as 3 and inorganic trimetaphosphate (10). In 1 N NH_4OH, 1 reacts with cytidine (15) to form cytidylyl-/2'(3')-5'/-thymidine (16) and a mixture of cytidine 2',3'-cyclic phosphate (17) and 9.

INTRODUCTION

P^1-(thymidine 5'-)P^1-amino-triphosphate (1) and P^1-(adenosine 5'-) P^1-amino-triphosphate have been synthesized and described in a preliminary form recently, as the first representatives of a new type of nucleoside 5'-triphosphate analogue having P^1-atom in amide, anhydride and ester bonds[2]. Detailed hydrolysis studies on the adenosine derivative also were reported[3]. It was found by product analysis that in alkali, predominantly P^1-O-P^2 anhydride bond breaking occurs, and a very complex mixture of products is formed containing at least ten compounds, among others adenosine 2',3'-cyclic phosphate and dinucleotide derivatives. It has been assumed that cyclic phosphate formation is due to the intramolecular participation of 3'-OH group in anhydride bond breaking, while dinucleotide formation may be due to intermolecular nucleophilic attack of the ionized 2',3'-cis-diol group on the P^1-atom.

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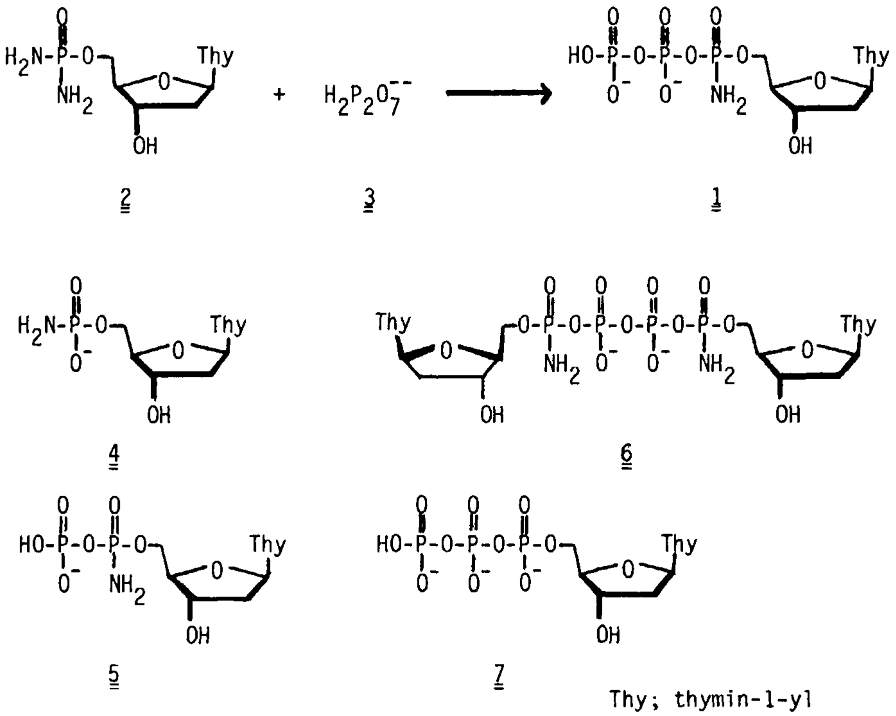
On this basis, in the alkaline hydrolysis of 1, participation of 3'-OH group is anticipated. At the same time, dinucleotide formation is not expected because of the lack of 2',3'-cis-diol group. Dinucleotides may be formed, however, in the presence of added ribonucleoside. Studies of the alkaline hydrolysis of 1 in the presence and absence of a ribonucleoside, thus seem to offer a possibility for checking the validity of the above assumption. The present paper reports the results of these studies in which cytidine was used as ribonucleoside.

RESULTS AND DISCUSSION

Synthesis and structure

Compound 1 was prepared from thymidine 5'-phosphorodiamidate (2) with a fivefold molar excess of bis-tri-n-butylammonium salt of inorganic pyrophosphate (3) in anhydrous N,N-dimethylformamide (DMF), at 30-32^{0}C, for 24 h (SCHEME 1). The reaction mixture always contained the following by-products: thymidine 5'-phosphoramidate (4), P^1-(thymidine 5'-)P^1-amino-diphosphate (5), P^1,P^4-di(thymidine 5'-)P^1,P^4-diamino-



SCHEME 1

tetraphosphate (6) and thymidine 5'-triphosphate (7).Product 1 was separated from unreacted starting materials and by-products by ion-exchange chromatography and was isolated as the solid sodium salt in 43% yield.

The structure of 1 was confirmed by phosphorus analysis, UV, CD and ^{31}P NMR spectroscopies as well as acid and enzymic hydrolyses. Product 1 contained three atoms of phosphorus per thymine residue. Its UV and CD spectra were identical with those of 7. The ^{31}P NMR spectrum was in agreement with the proposed structure and showed three characteristic differences in comparison with nucleoside 5'-triphosphates[4]: 1) The chiral P^{1} atom appeared as two doublets differing by 0.18 ppm. 2) These doublets were shifted by about 13 ppm downfield. A downfield shift of such magnitude is characteristic of a nitrogen-for-oxygen displacement on tetracoordinate phosphorus atom[5]. 3) $^{3}J_{P^{1}P^{2}}$ was about 2.5 Hz smaller than $^{3}J_{P^{2}P^{3}}$. This difference may be interpreted as a change in the conformation of triphosphate side chain[4]. Product 1 was quantitatively converted to 7 upon treatment with acid[6]. Escherichia coli alkaline phosphatase resulted in 4 formation with the simultaneous liberation of two thirds of total phosphorus as inorganic phosphate[7].

The structure of the by-products was verified by TLC comparison with authentic samples (4 and 7) or by chemical synthesis (5 and 6)[2].

Alkaline hydrolysis

The alkaline hydrolysis of 10^{-3}-10^{-1} M solutions of 1 was studied at pHs 10.0, 10.4, 11.7 and 14.0 and room temperature. As shown in TABLE 1 and FIG. 1, 4, thymidine 3'-phosphoramidate (8) and 3 were the

TABLE 1

UV absorbing products of the alkaline hydrolysis of P^{1}-(thymidine 5'-) P^{1}-amino-triphosphate (1), at room temperature[a].

No.	pH	~t½	Molar percentage of products			
			thymidine 5'-phosphoramidate(4)	thymidine 3'-phosphoramidate(8)	thymidine(9)	4/8
1.	10.0	30 h	74.7	25.3	<0.5[b]	2.95
2.	10.4	-	76.0	24.0	<0.5[b]	3.17
3.	11.7	2 h	72.9	23.5	3.6	3.10
4.	14.0	<1 min	72.7	24.2	3.1	3.00

[a] For details see Experimetal.
[b] Neglected for calculation.

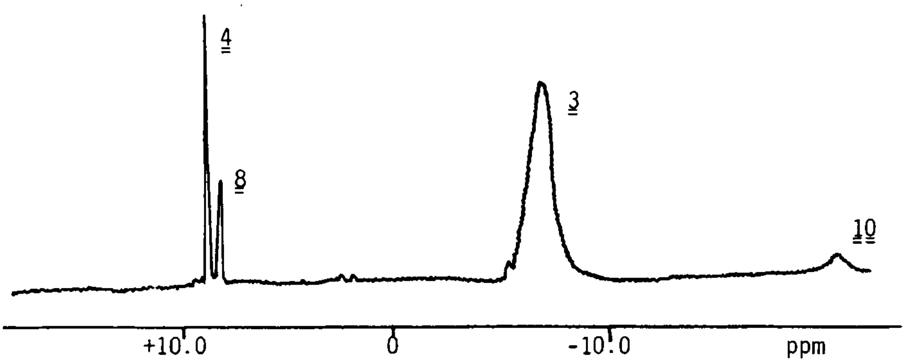


FIG. 1

^{31}P NMR spectrum of the hydrolysate at pH 11.7 of P^1-(thymidine 5'-)P^1-amino-triphosphate (1)[a].

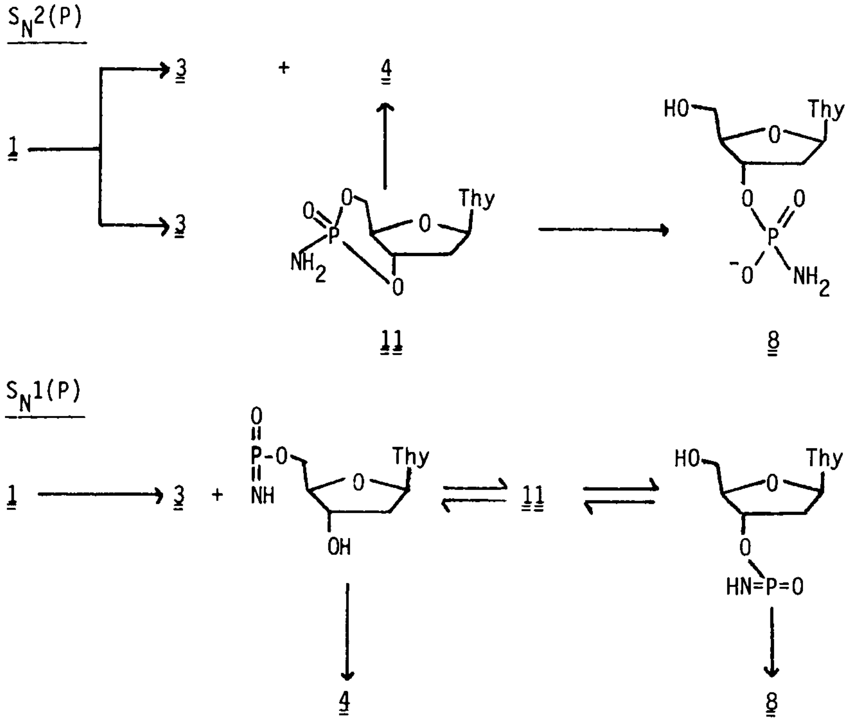
[a] Cf. expt.3. in TABLE 1. For details see Experimental.

main hydrolysis products. The 4/8 mole ratio was practically independent of pH and 1 concentration. At pH 11.7, small amounts of thymidine (9) and inorganic trimetaphosphate (10) were also formed. The quantity of 9 remained unaltered at pH 14.0.

The formation of 4, 8 and 3, i.e. the P^1-O-P^2 anhydride bond breaking may occur in principle according to either an $S_N2(P)$- or an $S_N1(P)$-mechanism[8] (SCHEME 2). The $S_N2(P)$-mechanism is likely to predominate at pH 10.0, while $S_N1(P)$-mechanism may operate particularly at higher alkalinity.This supposition is based on the comparison of ~t½ values in TABLE 1, by considering that in alkali, an $S_N1(P)$-mechanism may be responsible for the enhanced hydrolysis speed of those phosphoramidates having at least one ionizable hydrogen atom attached to the amide nitrogen atom[9].

Compound 8 is probably formed via thymidine 3',5'-cyclic phosphoramidate (11) by the intramolecular nucleophilic attack[10] /$S_N2(P)$-mechanism/ or trapping assistance[11] /$S_N1(P)$-mechanism/ by 3'-OH group.

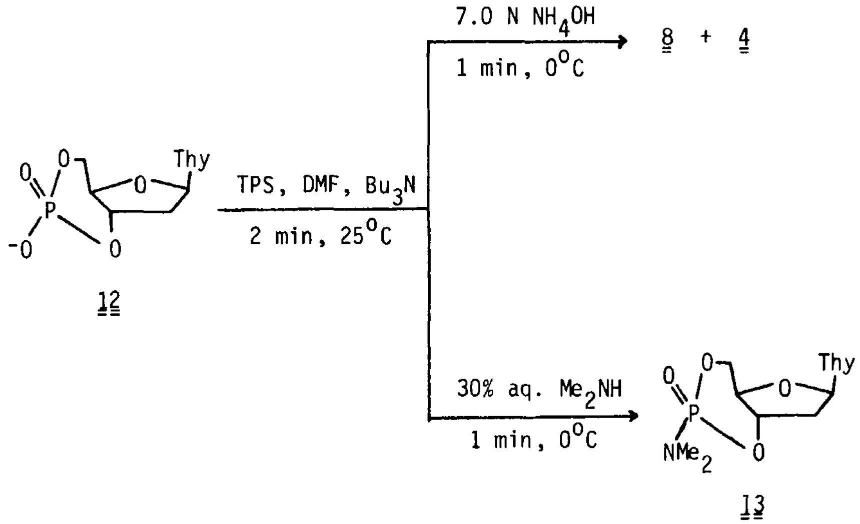
The presumed intermediate 11 is probably unstable under alkaline conditions and hydrolyzes to a mixture of 4 and 8[3]. As expected, attempts to prepare 11 from thymidine 3',5'-cyclic phosphate (12) with 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) in anhydrous DMF, followed by ammonolysis performed in situ with excess 7.0 N NH_4OH, met with failure. Instead, a 8/4 ~(3:1) mixture was formed in ~14% yield.



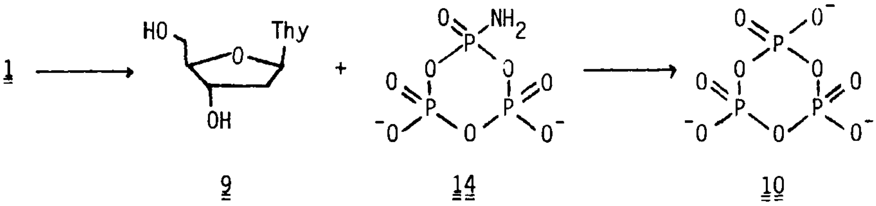
SCHEME 2

In a quite analogous reaction, thymidine 3',5'-cyclic N,N-dimethylphosphoramidate (13) was produced in ~18% yield (SCHEME 3). On this basis, it seems very likely that in the first case, 11 was indeed formed, but as a result of its high instability under alkaline conditions, it was instantaneously hydrolyzed - probably according to an $S_N1(P)$-mechanism - to a mixture of 8 and 4. The preferential formation of 8 over 4, i.e. the preferential P-O-$C^{5'}$ bond breaking, may be anticipated, on the basis of a similar preference observed in the alkaline hydrolysis of 12[10b].

The formation of 9 and 10, i.e. the P^1-O-$C^{5'}$ ester bond breaking, might be interpreted similarly to P^1-O-P^2 anhydride bond breaking. However, the absence of linear triphosphoric amide, $(O^-)_2(O)POP(O)(O^-)OP(O)(O^-)(NH_2)$, from the product mixture[12] and the approximate constancy of the quantity of 9 at pHs 11.7 and 14.0, may render probable that 9 and 10 are formed *via* inorganic trimetaphosphoramidate (14) by the intramolecular nucleophilic assistance of $P^3(O)(O^-)_2$ group[13] (SCHEME 4). The postulated decomposition 14⟶10 requires further studies.



SCHEME 3



SCHEME 4

Decomposition in 1 N NH_4OH in the presence of cytidine

Decomposition of 1 was studied in 1 N NH_4OH in the presence of 10^{-1}, 1.0 and 10.0 molar equivalents of cytidine (15), during 2 h, at room temperature. As shown in TABLE 2. 10^{-1} molar equivalent of 15 had practically no effect on the hydrolysis of 1 (expt. 6). The conversion did not alter even in the presence of 1.0 molar equivalent of 15, but two new UV absorbing products, cytidylyl-/2'(3')-5'/-thymidine (16) and cytidine 2',3'-cyclic phosphate (17) appeared, and the quantity of 9 significantly increased (expt. 7). The quantities of 16, 17 and 9 further increased in the presence of 10.0 molar equivalents of 15, and the conversion was also enhanced (expt. 8). Excess 9, i.e. the quantity of 9 formed in non-hydrolytic process(es), was equivalent with 17 in expt. 7, while a further 4-5% was produced in expt. 8. In expt. 7, 16 17 and excess 9 were formed at the expense of 4. In expt. 8, the formation of these compounds occurred mostly at the expense of 8.

TABLE 2

UV absorbing products formed in 10^{-2} M solutions of P^1-(thymidine 5'-)P^1-amino-triphosphate (1) in 1 N NH_4OH (pH 11.7) in the absence and presence of cytidine (15), during 2h, at room temperature[a].

No.	Conc. of 15 (M)	Molar percentage of products[b]					4/8	Conversion (%)
		thymidine phosphoramidates		thymidine(9)	cytidylyl-/2'(3')-5'/-thymidine(16)	cytidine 2',3'-cyclic phosphate (17)		
		5'(4)	3'(8)					
5.	0	69.2	27.2	3.6			2.54	47.0
6.	10^{-3}	67.7	28.7	3.6			2.36	47.0
7.	10^{-2}	58.7	29.5	9.5 (5.9)[c]	2.3	5.6	1.99	47.0
8.	10^{-1}	42.2	9.0	37.8 (34.2)[c]	11.0	29.3	4.69	62.5
9.[d]	10^{-1}	40.9	10.3	36.7 (33.1)[c]	12.7	29.3[e]	3.91	100.0

[a] For details see Experimental.

[b] The sum of molar quantities of thymidine containing products is taken as 100.0%. 17 is expressed in the percentage of thymidine containing products.

[c] Value in parentheses is excess 9: %9 - %9 of expt. 5.

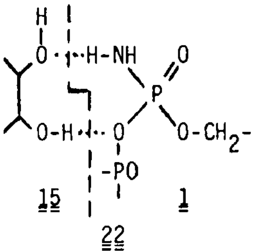
[d] The reaction time was 24 h.

[e] Mixture of 17 and cytidine 2'(3')-phosphate (18).

It should be noted that 16 was stable under the above conditions, i.e. 17 and excess 9 were not hydrolysis products of 16. Taking into consideration this fact,the results may best be interpreted as summarized in SCHEME 5.

The nucleophilic attack of partly ionized 2',3'-cis-diol group of 15 on the P^1-atom of 1 results in cytidylyl-/2'(3')-5'/-thymidine (P-N) amide (19) formation. 19 being a phosphoramidate with vicinal-cis-OH group, is unstable in alkaline solution and may be transformed either with the liberation of NH_3 via 20 cyclic triester into 16, or with the loss of 9 via cytidine 2',3'-cyclic phosphoramidate (21) into 17[14,15].

According to SCHEME 5, 15 should have no effect on the intramolecularly assisted hydrolysis of 1, since the cis-diol group of 15 competes with OH^- ion, but not with the 3'-OH group for the P^1-atom. This is the case in expt. 7, when 1 and 15 were present in equimolecular quantities. On the other hand, in expt. 8, the tenfold molar excess of 15 significantly repressed intramolecularly assisted hydrolysis. An essential prerequisite for the intramolecular reaction, is a change in the sugar puckering towards $^3T_4$, the sugar ring conformation of nucleoside 3',5'-cyclic phosphates[16]. When this conformational change is at least partly hindered, the intramolecularly assisted reaction will be repressed. Dreiding model suggests that such a hindrance for conformational change, might be, for example, a reversible complex formation between the cis-diol group of 15 and the phosphoramidate group of 1 to yield 22 and/or 23. At the same time, complexation may catalyze the formation of 16 and 17 as a result of the higher acidity of vicinal cis-diol group in comparison with that of $P\text{-}NH_2$ group (SCHEME 6). The production of 4-5% additional excess of 9 may similarly be interpreted, but via 23 (SCHEME 7). This explanation is purely speculative.



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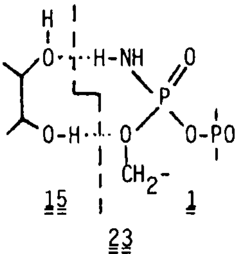

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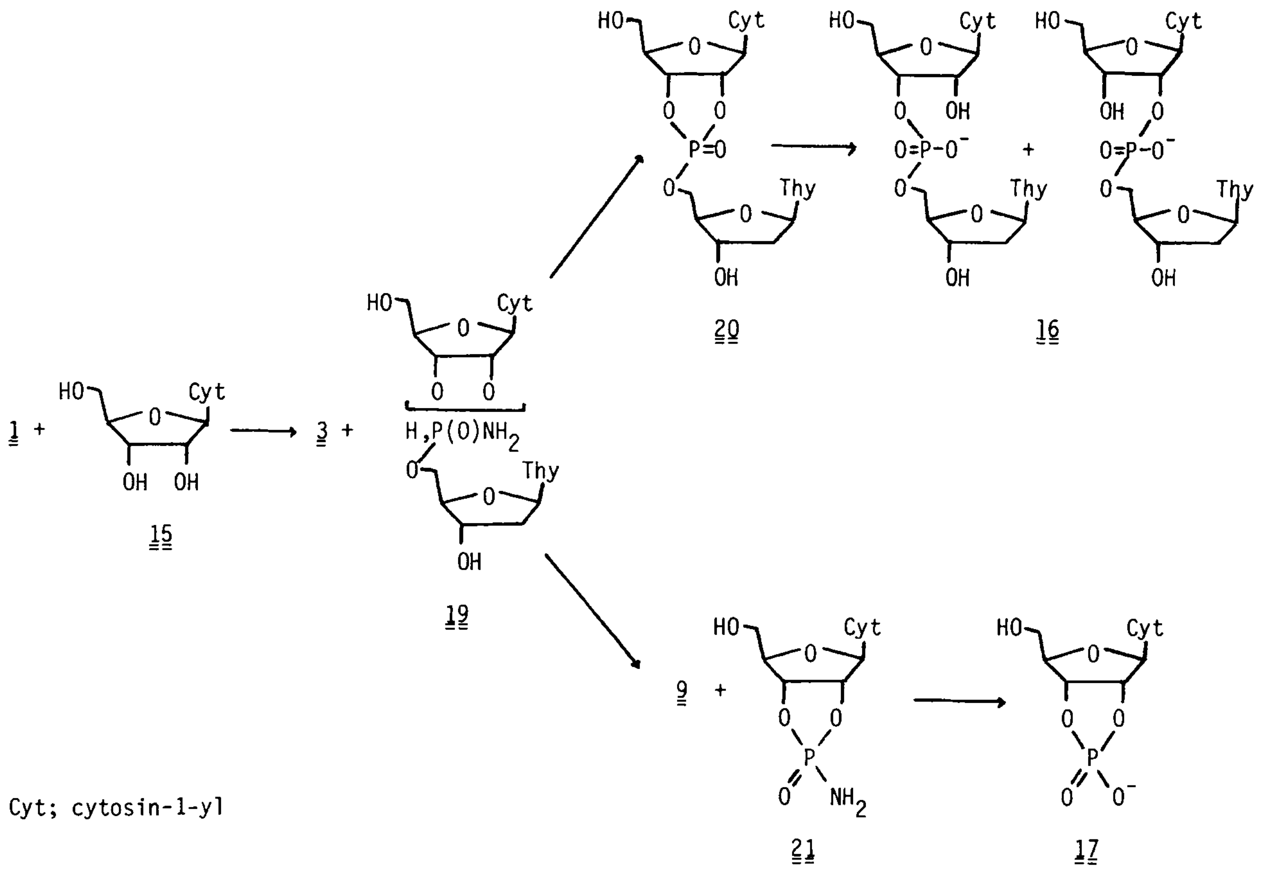
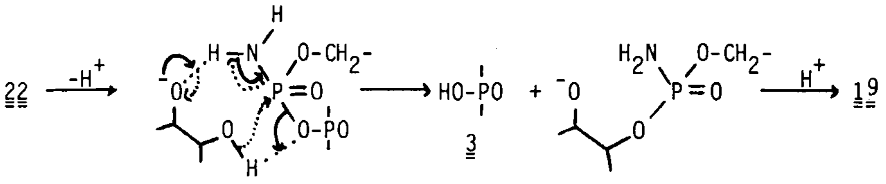
Experiments to determine whether this is a general reaction of vicinal cis-diols or restricted to ribonucleosides are in progress in our Laboratory.

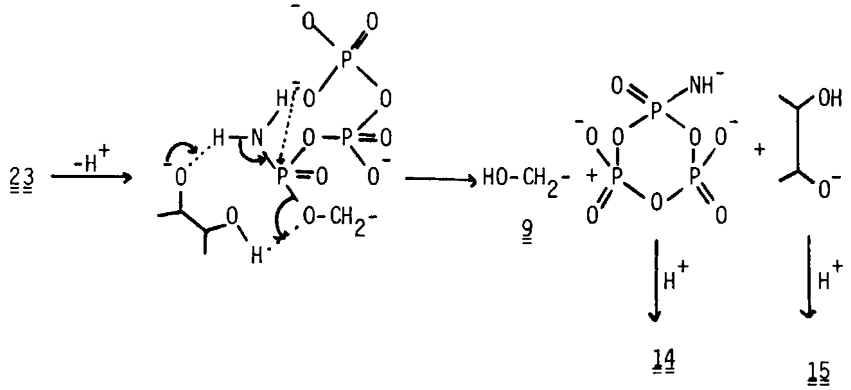


Cyt; cytosin-1-yl

SCHEME 5



SCHEME 6



SCHEME 7

EXPERIMENTAL

Materials. Compound 2 was prepared as described previously[17]. Compound 16 was synthesized according to Ref.18. Nucleosides, nucleotides and enzymes were obtained from Sigma. DMF was distilled from P_2O_5 and stored over 4Å molecular sieve. All other chemicals were of reagent grade, H_2O was deionized.

Methods. Evaporations were carried out using a rotary evaporator at about 13 Pa pressure with a bath temperature of 30°C. - UV spectra were recorded on a Cary 15 spectrophotometer at pHs 2.0 (10^{-2} N HCl), 7.0 (2×10^{-1} M sodium phosphate buffer) and 11.0 (3×10^{-3} N NaOH). For the determination of the molar quantities of UV absorbing products ε_{267}^{pH7}= 9650 (for 1, 4, 8, and 9)[19], ε_{270}^{pH7}=9000 (for 17 and 18)[20] and ε_{260}^{pH7}=15.200 (for 16)[21] were used. - CD spectra were obtained on a JASCO J-40C spectropolarimeter in 10^{-1} M Tris-HCl buffer, pH 8.0. - ^{31}P NMR spectra were obtained at 32.2 MHz on a Varian FT-80A NMR spectrometer in a mixture of 10^{-1} M Tris-HCl buffer, pH 8.0 - D_2O (7:3, v/v) unless otherwise stated. For 1, the buffer contained 10^{-3} M ethylenediaminetetraacetic acid. Positive chemical shift values are given for compounds that absorb at lower fields than the external standard 85% H_3PO_4 does. - TLC was performed on precoated cellulose chromatosheets (Cellulose F_{254}, Merck) in the solvent systems: S1, 1-propanol - cc. NH_4OH - H_2O (11:7:2, v/v), S2, sat. aqueous $(NH_4)_2SO_4$ - 10^{-1} M sodium acetate - 2-propanol (89:19:2), S3, 1-butanol - ethanol - 10^{-1} M TEAB (aqueous triethylammonium bicarbonate[22]) (16:2:5) and on precoated PEI-cellulose chromatoplates (Polygram Cel 300 PEI/UV_{254}, Macherey-Nagel & Co.) in the solutions: S4, 10^{-1} M NaCl and S5, 1.0 M NaCl. PEI-cellulose chromatosheets were pretreated[23]. Spots were visualized by UV absorption. R_f values are tabulated (TABLE 3). - Column chromatography was performed in the following systems: C1, column: DEAE-Sephadex A-25 (Sigma, HCO_3^- form[24], equilibrated with 10^{-1} M TEAB), eluent: linear gradient of 10^{-1}-2.5×10^{-1} M TEAB (1000 mL); C2, column: DEAE-cellulose (DE-32, Whatman, HCO_3^- form), eluent: linear gradient of 0.0-3×10^{-1} M TEAB (1000 mL); C3, column: cellulose (CC 31, Whatman), eluent: 1-butanol

TABLE 3

R_f values of compounds.

Compound	R_f values in systems				
	S1	S2	S3	S4	S5
1					0.33
4	0.57	0.44	0.30	0.62	0.93
7					0.19
8		0.40	0.35		
9	0.84	0.44		0.88	
12	0.69	0.33		0.53	
13	0.90				
15	0.73	0.63		0.82	
16	0.58	0.41 0.32^a	0.24 0.29^a	0.61	
17	0.67	0.49	0.28	0.41	
18	0.40	0.74 0.68^a		0.05	
24	0.38	0.51		0.14	
25	0.40	0.55		0.09	
thymine	0.81				

[a] The faster moving compound is the 2'-isomer.

- ethanol - 10^{-1} M TEAB (16:2:5), elution rate: 2.5 mL/20 min/fraction; C4, column: Dowex 50x8 (AG, 200-400 mesh, Serva, H^+ form), eluent: H_2O then 1.0 N HCl. Column size was 1.4x30.0 cm in each case. Elution rate was 12.0 mL/20 min/fraction for C1, C2 and C4. Temperature was $4^{o}C$ (for C1 and C2) or $25^{o}C$ (for C3 and C4). - Phosphorus was determined according to Eibl and Lands[25].

0.5 M stock solution of bis-tri-n-butylammonium pyrophosphate (3) in DMF

A solution of $Na_4P_2O_7.10H_2O$ (2.23 g, 5.0 mmol) in H_2O (50 mL) was passed through the column of system C4. The column was washed with H_2O until the eluate became neutral in such a way that the eluate was directly dropped into ethanolic tri-n-butylamine (2.37 mL, 10.0 mmol in 50-60 mL ethanol). The solution was evaporated. The residue was dissolved in DMF (10 mL), and the solution was again evaporated to remove last traces of H_2O. Finally, the residual oil was dissolved in and diluted to 10.0 mL with DMF. This solution was stored over 4 Å molecular sieve and used within one week.

P^1-(thymidine 5'-)P^1-amino-triphosphate (1)

Compound 2 (32 mg,10^{-1}mmol) was dissolved in H_2O (1 mL).The solution was freeze-dried. 0.5 M stock solution of bis-tri-n-butylammonium salt of 3 in DMF (1.0 mL) was added to the residue. The homogeneous solution obtained was set aside with the exclusion of atmospheric moisture at $30-32^{o}C$, for 24 h. During this time, the ammonium salt of 3 crystallized out of the solution and adhered firmly to the wall of the reaction flask. After cooling to $0^{o}C$, the solution was decanted from the crystal layer into ice-cold 10^{-1} M TEAB solution (10 mL). The crystal layer was washed with DMF (2x0.5 mL), and the washings were combined with the bulk. The resulting slightly turbid mixture was separated in system C1. Six UV absorbing compounds emerged from the column: 2 (in fractions 4-7, 13.8% of total A_{267}^{pH7} units eluted), 4 (10-14, 3.1%), 5 and 6 (double peak, 33-43. 10.6%), 1 (66-80, 70.9%) and 7 (91-94, 1.6%). 3 appeared after 1. Fractions of $A_{267}^{pH7} > 1.5$ of peak of 1 (68-78) were pooled. Dowex 50x8 (H^+) resin was added to the combined fractions in small portions under vigorous stirring and ice-cooling until the pH fell to 5.5. The resin was filtered with suction and the solution was evaporated to dryness. Traces of TEAB were removed by repeated evaporation with H_2O. The residue was dissolved in methanol (2.0 mL), and the solution was filtered through a G3 sintered glass filter into a centrifuge tube. A solution of NaI(60.0 mg) in acetone (6.0 mL) was added. The precipitated solid was collected by centrifugation and washed with a mixture of methanol - acetone (1:3, v/v, 8 mL) until the supernatant was free of iodide (3-4 washings). Finally, the white solid was washed with anhydrous ether and dried in a vacuum desiccator over P_2O_5, at room temperature, for 3-4 h. The whole isolation procedure should be performed as quickly as possible. Yield: 30 mg (∼43%), homogeneous by TLC. Analysis in system C2: 96.0% 1, 2.6% 4 and 1.4% 7. ^{31}P NMR, (ppm): 1.80 (d, $^{3}J_{P1P2}$=18.85 Hz), 1.62 (d, $^{3}J_{P1P2}$=18.15 Hz)/P^1/; -7.37 (d, $^{3}J_{P2P3}$=20.9 Hz)/P^3/; -22.90 (d of d)/P^2/. Thymine/P_{total}/$P_{enzyme\ labile}$= 1.00:2.91:1.99. For the determination of $P_{enzyme\ labile}$ 1 (1.0 μmol) was incubated with Escherichia coli alkaline phosphatase (1.0 unit) in $NaHCO_3$-Na_2CO_3, pH 10.4, buffer (200 μL), at 37°C, for 2 h. 10% aqueous trichloroacetic acid (200 μL) was then added. After centrifugation phosphorus was determined from the supernatant. The only UV absorbing product of enzymic hydrolysis was 4 as detected by TLC. In 5x10^{-1} N HCl 1 was quantitatively converted to 7, at room temperature, within 30 min as proved by TLC comparison with an authentic sample.

Alkaline treatment of P^1-(thymidine 5'-)P^1-amino-triphosphate (1)

The following solutions of 1 were prepared, at room temperature: 10^{-3} M in 10^{-1} M $NaHCO_3$-Na_2CO_3 buffers (expts. 1 and 2), 10^{-2} M in approx. 1 N NH_4OH (expts. 3 and 5-9) and 10^{-1} M in 1.0 N NaOH (expt. 4). In expts. 6-9, 1 N NH_4OH was 10^{-3} - 10^{-1} M in 15, as indicated in TABLE 2. 5 - 15 μmoles of 1 were used for each experiment. Reaction times were: 5 min (expt. 4), 2 h (expts. 5-8), 24 h (expts. 1, 3 and 9) or 116 h (expt. 2). The reaction mixtures were separated according to the flowsheets of FIG. 2. The UV absorbing products were identified (see later) and measured by spectrophotometry. Molar percentages of UV absorbing products are summarized in TABLES 1 and 2. Parallel with each experiment, identical quantity of unhydrolyzed 1 of the same batch was separated in system C2 and used as blank. Approx. t½ values were obtained from the pseudo-first-order rate constants calculated on the basis of the composition of solutions after 24 h (pH 10.0), 2 h (pH 11.7) and 1 min (pH 14.0).

For ^{31}P NMR studies expts. 3 and 9 were repeated. The solutions were evaporated to dryness. The residues were dissolved in the buffer (1.0 mL for each) and the ^{31}P NMR spectra were recorded. δ(ppm): 9.17 (4), 8.79 (8), -6.92 (3) and -21.42 (10) /for expt. 3/; 19.93 (17), 9.14 (4), 8.75 (8), 3.69, 3.50 (18), -0.77, -0.90 (16), -7.24 (3) and

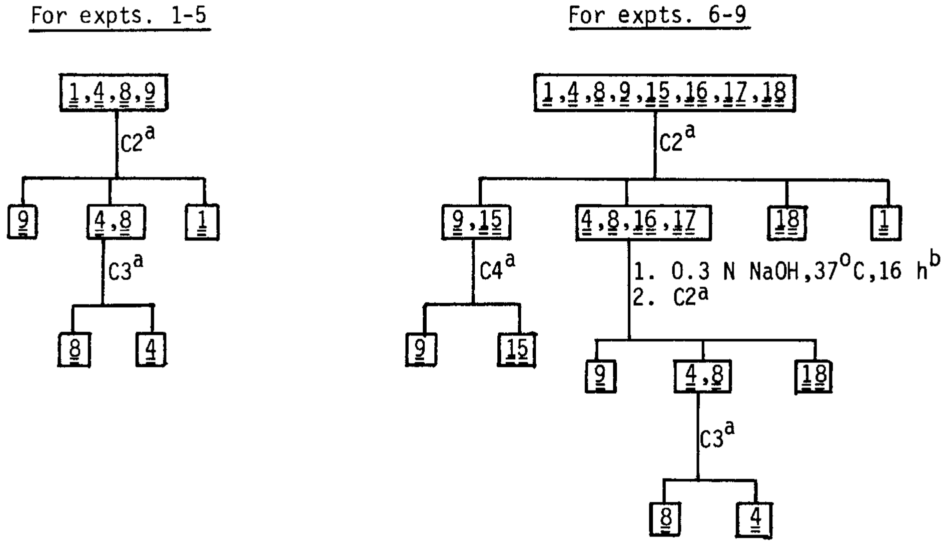


FIG. 2.

Flowsheets for the separation of UV absorbing products of the alkaline treatments of P^1-(thymidine 5'-)P^1-amino-triphosphate (1)

[a] For the column chromatographic systems C2, C3 and C4 see Methods. The setting of compounds from the left to the right corresponds to their elution order in the given system. Unresolved compounds are framed. Hydrolysis mixtures were subjected to separation by system C2 after tenfold dilution with H_2O (expts. 1, 2 and alkaline hydrolysates of products with -1 charge of expts. 6-9), or twentyfold dilution with H_2O and neutralization by acetic acid (expt. 4), or evaporation and dissolution in H_2O (1 mL)(expts. 3 and 5-9). The peaks of unresolved compounds in system C2 were pooled, evaporated, freed from TEAB by repeated evaporation with H_2O prior to the next separation or hydrolytic step.

[b] For 40 A_{267}^{pH7} units of the mixture of compounds, 500 µL NaOH was used. 4 and 8 remained unaltered under these conditions, while 16 and 17 were quantitatively hydrolyzed to a mixture of 9 + 18 and 18, respectively, as tested on authentic samples. The molar quantity of 9 formed in this process, was taken to be equal to that of 16.

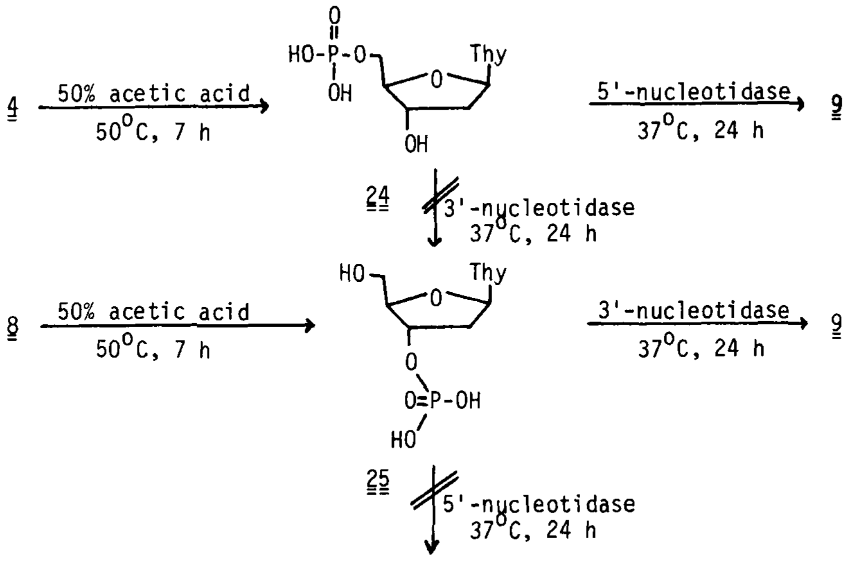
-21.56 (10) /for expt. 9/. Except of 4 and 8, compounds were identified by successively adding to the solutions authentic samples and repeating the spectrum. 4 and 8 were identified - among others - on the basis of proton coupled spectrum (see later).

Product identification

Products 9, 16, 17 and 18 were identified by UV photometry and TLC comparison with authentic samples. The structure of 4 and 8 was proved by ^{31}P NMR and hydrolytic degradations. ^{31}P NMR, δ(ppm): 4, 9.17 (t of ds, $^3J_{PH5'}$=5.02 Hz, $^3J_{PH5''}$=5.28 Hz, $^4J_{PH4'}$=1.27 Hz); **8**, 8.79 (d, $^3J_{PH3'}$= 8.41 Hz). Compounds (6.0 A_{267}^{pH7} units of each) were hydrolyzed first with acetic acid (100 μL), then with specific phosphatases as shown in SCHEME 8. Enzymic hydrolyses were performed with 2.0 A_{267}^{pH7} units of substrates and 0.5 units of enzymes in 40 μL volume. For incubation with 3'-nucleotidase from rye grass 10^{-1} M Tris-HCl, pH 7.5, for that with 5'-nucleotidase from snake venom 10^{-1} M glycine-NaOH, pH 9.0, were used. Hydrolyses were followed by TLC.

Thymidine 3',5'-cyclic N,N-dimethylphosphoramidate (**13**)

To a stirred solution of TPS (40.4 mg, 2×10^{-1} mmol) in DMF (1.0 mL), a solution of **12** triethylammonium salt (10^{-1} mmol) in DMF (1.0 mL) containing tri-n-butylamine (48.0 μL, 2×10^{-1} mmol) was dropped during 1 min, at room temperature. Stirring was continued with the exclusion of atmospheric moisture for an additional minute, then the solution was quickly poured into ice-cold 30% aqueous dimethylamine solution (10 mL) under vigorous stirring. The mixture was evaporated to dryness. The residue was dissolved in H_2O (50 mL) and the solution was extracted with ether (20 mL). After evaporation to a small volume (~1 mL), the aqueous phase was separated in system C2. Fractions containing neutral UV absorbing compounds (18.2% of the total A_{267}^{pH7} units applied, the other 81.8% was **12**), were pooled, evaporated and further purified in system C3 to yield 3.2 mg TLC pure, white solid. ^{31}P NMR (DMSO-d_6), δ(ppm):



SCHEME 8

7.47 and 6.25 (literature values[26]: 7.53 and 6.91). On standing in 5×10^{-1} N HCl, at room temperature, **13** was quantitatively converted to **12** and thymine[27] within 24 h, as detected by TLC.

Attempted synthesis of thymidine 3',5'-cyclic phosphoramidate (**11**)

The synthesis was performed on exactly the same manner as described for **13**, except that 7.0 N NH_4OH was used instead of 30% aqueous dimethylamine. The mixture of compounds with -1 charge obtained in system C2 was fractionated in system C3 to give 86.1% **12**, 10.4% **8** and 3.5% **4** in this elution order.

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