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NUCLEOSIDE PHOSPHOROTHIOATES—I

THIOPHOSPHORYLATION OF NUCLEOSIDES WITH PHOSPHORUS THIOXY-ANIONS†

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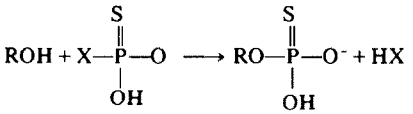
Abstract—The reactivity of the two phosphorus thioxy-anions, monothiophosphate and dithiophosphate towards nucleosides was investigated. Reaction of monothiophosphate, in its monoanionic form, with several nucleosides at 70° gave the corresponding nucleoside-O-phosphorothioates in yields ranging from 50 to 90%. The 5'-OH vs 3'-OH selectivity in thiophosphorylation was found to be 2:1, respectively. Evidence is presented to support the role of monothiopyrophosphate, derived from initial dimerization of monothiophosphate, as the active thiophosphorylating agent. Independently prepared monothiopyrophosphate was found to thiophosphorylate thymidine smoothly at room temperature in 63% yield. Reaction of dithiophosphate with several nucleosides at room temperature gave exclusively the corresponding nucleoside-O-phosphorothioates in yields ranging from 45 to 65%. The selectivity for 5'-OH vs 3'-OH thiophosphorylation using this reagent is 3:2, respectively.

Since their recent discovery,[1] nucleoside-O-phosphorothioates[1-7] as well as their cyclic[6,8] and oligomeric[9,10] derivatives have become an increasingly important class of nucleotide analogs, having found application[11] in studies related to enzyme mechanism and regulation in addition to possessing potentially useful biological activities. Previous methods developed for the preparation of nucleoside-O-phosphorothioates, from the corresponding nucleosides, have utilized triimidazolyl-1-phosphine sulfide,[6] thiophosphorylchloride[2-5] or DCC activated S-2-carbamoylethyl phosphorothioate[7] as the thiophosphorylating reagent. Although quite suitable for the preparation of simple nucleoside-O-phosphorothioates, the methods do not, however, provide the versatility of application to thiophosphorylations of nucleosides bearing labile substituents since each requires either acid or alkaline hydrolytic work-up of the crude reaction mixture to generate the desired nucleoside-O-phosphorothioate.

As a result, investigations directed at the development of methods for direct thiophosphorylation of both the primary and secondary nucleoside alcohol functions which employ exceptionally mild reaction conditions were initiated. Conceptually, such procedures could involve thiophosphoryl transfer using thiophosphate activated with a good leaving group. As illustrated below, reaction of a nucleoside with a reagent of this type would give directly the desired nucleoside-O-phosphorothioate, thus, eliminating the need for a hydrolytic work-up step.



X = a leaving group

Members of the phosphorus thioxy-anion series which includes tetrathiophosphate (PS_4^{3-}), trithiophosphate (POS_3^{3-}) dithiophosphate ($PO_2S_2^{3-}$), and monothiophosphate (PO_3S^{3-}) appear ideal for this purpose since they are easily prepared by known procedures,[12-14] show differing reactivity towards nucleophiles,[15] and possess the potential for generating nucleoside-O-phosphorothioates of varying sulfur content.

In this paper we report the results obtained from studies of the reactions of the phosphorus thioxy-anions, monothiophosphate and dithiophosphate, with several nucleosides.

RESULTS

Monothiophosphate. Reaction of six equivalents of monothiophosphate, in its monoanionic form, with thymidine in anhydrous dimethyl formamide at 70° for 12 hr gave thymidine 5'(3')-O-phosphorothioates (5'(3')-TMPS)[1,7] in 50% yield. The structural assignments of these products were based on both elemental analysis and comparison of physical and spectral properties with those obtained from authentic 5'(3')-TMPS, prepared from thymidine and thiophosphoryl chloride.[4] The isomeric ratio for the product mixture was determined by PMR spectroscopy to be 2:1 in favor of the 5'-isomer.§ Reaction of 2'-deoxycytidine and 2':3'-O-ethoxymethylene adenosine[16] separately with monothiophosphate under these same conditions gave 2'-deoxycytidine 5'(3')-O-phosphorothioates (5'(3')-dCMPS) (2:1 respective isomeric ratio determined by PMR spectral methods) and, after removal of the ethoxymethylene group from the second compound, adenosine 5'-O-phosphorothioate[2] (5'-AMPS) in respective 56% and 90% yields.

Strictly anhydrous conditions must be employed in these reactions in order to avoid formation of nucleoside monophosphates through hydrolysis of the nucleoside-O-phosphorothioates once formed in the reaction mixture. Although no side products were formed in the 2':3'-O-ethoxymethylene adenosine reaction, minor unidentified products totaling 7–10% of the crude reaction mixture were formed in the case of thymidine and 2'-deoxycytidine. The minor products as well as the unconsumed

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§The C_6-H resonance of the 5'-isomer appears downfield from that of the 3'-isomer.

starting material were, however, easily removed using DEAE-cellulose chromatography.

We next briefly explored the mechanism of this interesting and unusual thiophosphorylation process using thymidine as the model nucleoside. To determine if monothiophosphate was participating directly in the thiophosphoryl group transfer process or merely serving as a precursor for a more reactive thiophosphorylating agent, thymidine was heated (70°) with a mixture obtained from heating (70°) 6 equivalents of monothiophosphate alone in dimethyl formamide to complete conversion. Thymidine 5'(3')-O-phosphorothioates were formed in a 31% yield along with the previously observed minor products. The product composition of the mixture obtained by heating monothiophosphate alone, in dimethyl formamide, was then analyzed using anion exchange column chromatography. The elution profile obtained is shown in Fig. 1. By comparison with standard

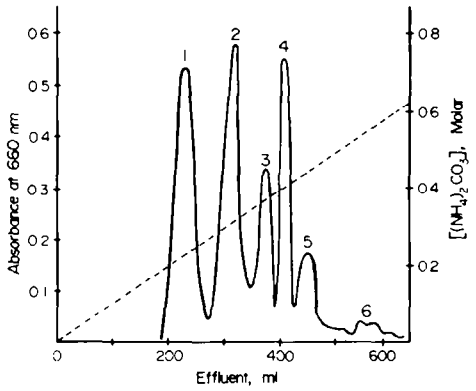


Fig. 1. Elution diagram from the anion exchange chromatography of the monothiophosphate thermolysis product mixture: (1) orthophosphate; (2) pyrophosphate; (3) monothiopyrophosphate; (4) triphosphate; (5) and (6) unidentified.

samples, peaks 1, 2 and 4 were identified as orthophosphate, pyrophosphate, and triphosphate, respectively. The sulfur-containing compound contained in peak 3 possessed an eluation volume consistent with that of monothiopyrophosphate,[17] and had the same electrophoretic mobility at three different pH's as independently prepared monothiopyrophosphate.[18]

It seemed likely that monothiopyrophosphate was the active thiophosphorylating agent in these reactions. As a result, the reactivity of independently prepared monothiopyrophosphate with thymidine was explored. Reaction of 3 equivalents of monothiopyrophosphate, in its dianionic form, under the same conditions employed in the monothiophosphate-thymidine reaction yielded 5'-(3')-TMPS in a respective 2:1 isomeric ratio. The rates of thymidine conversion with 3 equivts of monothiopyrophosphate and with 6 equivts of monothiophosphate, under similar conditions, were measured and are compared in Fig. 2. The very rapid conversion rate of thymidine in reaction with monothiopyrophosphate at 70° indicated that the utilization of this reagent for nucleoside thiophosphorylation at room temperature might be possible. Indeed, reaction of monothiopyrophosphate with thymidine at ambient temperature for 2 hr gave exclusively 5'(3')-TMPS (2:1 respective isomeric ratio) in a 63% yield.

Dithiophosphate. Independent reactions of 5 equivts of di-(triethylammonium) dithiophosphate with the

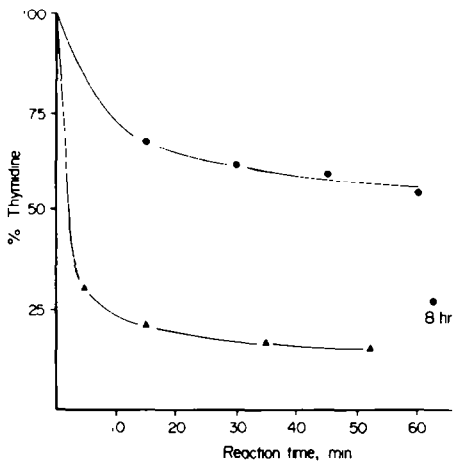
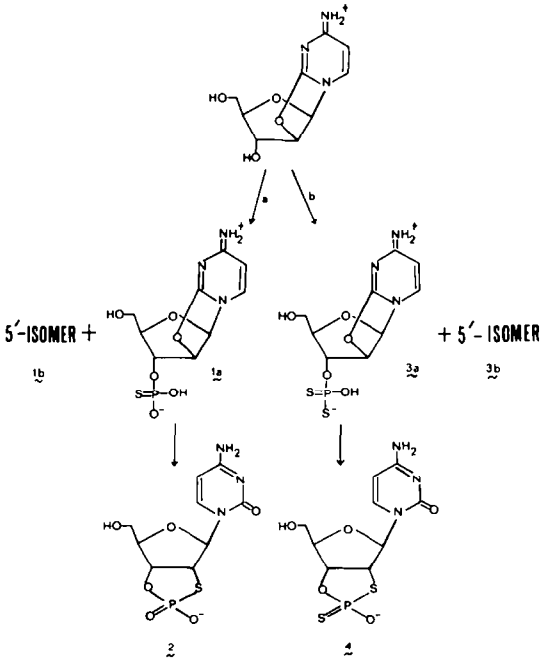


Fig. 2. Percent unreacted thymidine in the thymidine-monothiophosphate (6 equivalents) reaction mixture (●) and thymidine-monothiopyrophosphate (3 equivalents) reaction mixture (▲) vs reaction time.

nucleosides thymidine, 2'-deoxycytidine and 2':3'-O-ethoxymethylene adenosine in dimethyl formamide at room temperature for 12 hr gave the corresponding nucleoside-O-phosphorothioates, 5'(3')-TMPS, 5'(3')-dCMPS and, after removal of the ethoxymethylene group, 5'-AMPS in respective yields of 55%, 65% and 45%. The 5'-OH vs 3'-OH selectivity for thiophosphorylation of the two pyrimidine deoxynucleosides using this reagent is 3:2, respectively. No side products were detected in any of the three reactions.

Further investigations of the dithiophosphate reactions were conducted in order to determine if the nucleoside-O-phosphorothioates were primary products formed directly in the reaction mixture or if they were derived from initially formed nucleoside-O-phosphorodithioates during reaction work-up. The system chosen for this purpose was 2,2'-cyclocytidine. As shown in Scheme 1,



Scheme 1.

monothiophosphorylation (pathway a) of the 3'-OH of the cyclonucleoside would give **1a** which could undergo cyclization *in situ* to yield the monothiocyclic diester **2**, whereas, dithiophosphorylation (pathway b) of the 3'-OH would give **3a** which would lead ultimately to the dithiocyclic diester **4**. Thus, these two pathways might be distinguished on the basis of the sulfur content of the cyclic phosphorothioate produced since, in analogy to uridine 2':3'-O-phosphorothioate and -phosphorodithioate,[6] the exocyclic sulfur atom of **4** should be retained during the mild work-up conditions. The exclusive products formed from reaction of 2,2'-cyclocytidine with dithiophosphate are 2,2'-cyclocytidine 5'(3')-O-phosphorothioates (2:1 respective isomeric ratio) (**1**) and 2'-thio-2'-deoxycytidine 2':3'-phosphorothioate (**2**). The structure of **2** was ascertained by its independent synthesis from 2,2'-cyclocytidine and monothiophosphate.

DISCUSSION

Thiophosphorylation of several nucleosides using monothiophosphate in dimethyl formamide at 70° proceeds in yields ranging from 50 to 90%. The selectivity in thiophosphorylation of the 5'-OH vs 3'-OH functions in the two deoxynucleosides studied is 2:1, respectively.

The exclusive formation of nucleoside-O-phosphorothioates in the monothiophosphate reactions, appears to indicate that monothiophosphate serves as a precursor to the actual thiophosphorylating agent since its direct reaction with nucleosides might be expected to give the nucleoside monophosphates as products via phosphoryl transfer with loss of H_2S. Previous studies[18,19] have demonstrated that monothiophosphate, in its dianionic form, undergoes thermal dimerization to monothiopyrophosphate. Higher polymers are eventually formed when the monoanionic form is employed.[19] In light of these observations, it appeared likely that monothiophosphate, which is present in a 6-fold excess over the nucleoside, would undergo bimolecular reaction to produce the reactive thiophosphorylating agent, monothiopyrophosphate, under the conditions used for these reactions. Indeed, thymidine reacted smoothly with a thermolysis product mixture, prepared by heating monothiophosphate alone, and gave 5'(3')-TMPS in a 31% yield. The composition of the monothiophosphate thermolysis mixture which contains, therefore, the thiophosphorylating agent was determined using anion exchange column chromatography (Fig. 1). In addition to orthophosphate, pyrophosphate, and triphosphate, monothiopyrophosphate was isolated from this mixture.

The reactivity of independently prepared monothiopyrophosphate[18] (3 equivts) with thymidine was examined and compared with that observed for monothiophosphate (6 equivts) with thymidine. Interestingly, the minor products of both reactions were the same and the principle products of both reactions, 5'(3')-TMPS, were formed in the same respective ratio of 2:1. In addition, the rate of reaction of thymidine with monothiopyrophosphate is comparatively faster than with monothiophosphate (Fig. 2). These results seem to indicate that monothiopyrophosphate or possibly a monothiopyrophosphate-derived adduct, serves as the active thiophosphorylating agent in reactions of monothiophosphate with nucleosides. The mechanism by which monothiopyrophosphate directly or indirectly transfers exclusively a thiophosphoryl group is in itself quite interesting and is presently under study using simpler alcoholic substrates.

Thiophosphorylation of several nucleosides using dithiophosphate, in dimethyl formamide, proceeded smoothly at room temperature in yields ranging from 45 to 65%. The selectivity in thiophosphorylation of the 5'-OH vs 3'-OH in the two deoxynucleosides studied is 3:2, respectively, which is somewhat less than that observed using monothiopyrophosphate (or monothiophosphate).

We examined the dithiophosphate reaction further to determine if the nucleoside-O-phosphorothioate products are formed directly in the reaction via thiophosphoryl transfer or if they are derived from initially formed nucleoside-O-phosphorodithioates, resulting from dithiophosphoryl transfer, upon work-up of the reaction mixture. The method chosen to distinguish between these two possibilities was based upon the expectation that thiophosphorylation of the C_3-OH of 2,2'-cyclocytidine would ultimately lead to the 2',3'-cyclic adduct through internal displacement of the bridging oxygen at C_2· by the 3'-O-phosphorothioate sulfur, as illustrated in Scheme 1. Thus, reaction of dithiophosphate and 2,2'-cyclocytidine with monothiophosphoryl transfer would ultimately lead to the cyclic phosphorothioate **2**. On the other hand, the cyclized adduct derived from the phosphorodithioate monoester **3a** would contain two S atoms. Since, as previously mentioned, the exocyclic sulfur of the cyclic diester **4** should not be lost by hydrolysis during the purification of the reaction mixture, the nature of the cyclic product, whether it be **2** or **4**, isolated from reaction of dithiophosphate with 2,2'-cyclocytidine would indicate the nature of the thiophosphorylation process (i.e. monothiophosphoryl vs dithiophosphoryl transfer). Reaction of dithiophosphate with 2,2'-cyclocytidine gave exclusively the 2,2'-cyclocytidine 5'(3')-O-phosphorothioates (**1**) in an isomeric ratio of 2:1, respectively, and the cyclic adduct, 2'-thio-2'-deoxycytidine 2':3'-phosphorothioate (**2**). The greater ratio of the 5'-O-phosphorothioate monoester (**1b**) to the 3'-O-phosphorothioate monester (**1a**) relative to the corresponding 3:2 isomeric ratio obtained from thiophosphorylation of 2'-deoxynucleosides is consistent with the mechanism proposed in Scheme 1 for the formation of the cyclic products.† In addition, reaction of monothiophosphate with 2,2'-cyclocytidine also gave **2** as the exclusive cyclic product. These results indicate that reaction of dithiophosphate with nucleosides occurs via monothiophosphoryl transfer. Although the actual mechanism of the dithiophosphate-nucleoside reaction has not yet been subjected to detailed investigation, it is still interesting to note, at this time, that the active thiophosphorylating agent in this reaction transfers a thiophosphoryl group to the nucleoside with less selectivity (5'-OH vs 3'-OH) than that in the monothiopyrophosphate reaction.

In conclusion, both monothiopyrophosphate and dithiophosphate are ideal reagents for direct thiophosphorylation of nucleosides under exceptionally mild reaction conditions (dimethyl formamide, room temperature). Particularly attractive is the fact that nucleoside-O-phosphorothioates are formed in reasonably good yields and are uncontaminated with side products, which excludes the necessity for column purifications. Both procedures also seem applicable to

†The cyclic phosphorothioate **2** is stable under the conditions used to isolate the monoesters. It thus appears that **1a** does not undergo complete cyclization to **2** during the reaction.

the preparation of nucleoside 3'-O-phosphorothioates from appropriately protected nucleosides.

EXPERIMENTAL

General. High voltage electrophoresis was carried out on Whatman 3 MM paper with 0.05 M ammonium acetate buffer (unless stated otherwise) at the designated pH. TLC was carried out on Eastman silica gel chromagram sheets in solvent systems A (2-PrOH, H_2O, NH_4OH 7:2:1) and B [(1-butanol-H_2O 86:14), EtOH, 1 M ammonium acetate 6:3:1]. UV spectra were measured on a Beckman-Acta III UV spectrophotometer. ORD spectra were measured on a Cary 60 spectropolarimeter. PMR spectra of the nucleotides in D_2O were recorded at ambient temp. using both Varian HA-100 and T-60 spectrometers. ^{13}C-NMR spectra of the nucleotides in D_2O were recorded on a Jeol-PS100 NMR spectrometer at 25°. The multiplicity of the resonances were determined from the coupled spectra. IR spectra were recorded in KBr (pellets) on a DIGILAB FTS-20 IR spectrometer (*in vacuo*). Column chromatography was accomplished on DEAE-cellulose (Whatman, HCO_3^- form), Sephadex G-25 and G-10 (Pharmacia), and Amberlite CG-400 (Mallinckrodt) anion exchange resin (Cl^- form). Cation exchanges were made with Dowex 50W × 8 resin (Baker) and anion exchanges with Dowex1 × 8 anion exchange resin (Baker) in the forms designated. Quantitative phosphate determinations for column chromatography were performed by the general method of Bartlett.[20] Qualitative phosphate determinations of electrophoretic bands were made by spraying the dried paper with molybdate soln then developing under long wavelength UV light.[21] Qualitative sulfur determinations were made by digestion with 5 N HNO_3 then addition of 5% $AgNO_3$. All solvents used in the reactions were distilled and stored over Baker Type 4A molecular sieves. All nucleosides and nucleotides used were purchased from Sigma Chemical Co.

Mono-(tri-n-butylammonium) salt of monothiophosphate

Trisodium monothiophosphate, prepared from thiophosphoryl-chloride using the procedure described by Akerfeldt,[22] was passed through a cation exchange column (H^+ form) into an ice-cooled flask containing 1 equiv of tri-n-butylamine dissolved in pyridine. The resulting soln was concentrated *in vacuo* (bath temp. not exceeding 35°) then rendered anhyd by repeated evaporations with pyridine. The clear oil obtained was used in subsequent reactions.

Di-(tri-n-butylammonium) salt of monothiopyrophosphate

Tetrasodium monothiopyrophosphate was prepared in a manner similar to that summarized by Tridot and Tudo.[18] Trisodium monothiophosphate was converted to its free acid form by passage through an H^+ form cation exchange column with water. The monothiophosphoric acid was quickly converted to the disodium salt by titration with 0.2 N NaOH. The resulting solution was then lyophilized. The white powder obtained was dried under reduced pressure over P_2O_5 at room temp. for 6 hr, then heated to 153° for 1–2 hr. The monothiopyrophosphate was determined by electrophoretic analysis to be contaminated with approximately 10% pyrophosphate. The tetrasodium salt of monothiopyrophosphate was converted to the di-(tri-n-butylammonium) salt by passage through a pyridinium form ion exchange column into an ice-cooled flask containing 2 molar equivts of tri-n-butylamine dissolved in pyridine. The resulting soln was concentrated *in vacuo* (bath temp. not exceeding 35°) then rendered anhyd by repeated evaporations with pyridine. The clear oil obtained was used in subsequent reactions.

Di-(triethylammonium) salt of dithiophosphate

The triammonium salt of dithiophosphate was prepared from diphosphorus pentasulfide using the procedure described by Tridot and Tribodet.[14] To a freshly prepared sample dissolved in water, was added 1 part pyridine and 2 parts triethylamine. The solution was then concentrated *in vacuo* to the original volume. This sequence of addition and evaporation was repeated 5 times. To the resulting pyridine soln of di-(triethylammonium) dithiophosphate was added dimethyl formamide. The soln was then rendered anhyd by three evaporations with pyridine. This preparation was used in subsequent reactions.

Thiophosphorylation of thymidine, 2'-deoxycytidine and 2':3'-O-ethoxymethylene adenosine[16]

Method A. Monothiophosphate. The general procedure used in thiophosphorylation of these nucleosides with monothiophosphate is as follows. To the nucleoside (1.8 mmol) in 5–10 ml of dimethyl formamide was added 10.8 mmol of freshly prepared mono-(tri-n-butylammonium) monothiophosphate. The resulting soln was dried three times by evaporation of 2 ml portions of pyridine then concentrated *in vacuo* to a viscous oil which was heated to 70°, under argon, for 12 hr. The mixture was then dissolved in water (the 2':3'-O-ethoxymethylene adenosine mixture was acidified to pH 3 at this point for removal of the O-ethoxymethylene group), washed with carbon disulfide and chromatographed on a DEAE-cellulose column (97 × 3 cm) using a linear gradient of triethylammonium bicarbonate (1.5 liters 0.01 M → 1.5 liters 0.20 M) at pH 7.5. The pH of the combined fractions containing the nucleoside-O-phosphorothioate was adjusted to 6 with Dowex H^+ resin. The glass obtained by lyophilizing this soln was taken up in water and passed through a Dowex Na^+ column (16 × 1.7 cm). The product-containing eluate was lyophilized and rechromatographed for final purification on a Sephadex G-25 column (190 × 2.5 cm) in water. The reaction yield determined for 5'(3')-TMPS (2:1 respective isomeric ratio) is 50%, for 5'(3')-dCMPS (2:1 respective isomeric ratio) is 56% and for 5'-AMPS[2] is 90%.

Method B. Dithiophosphate. The general method employed in the thiophosphorylation of these nucleosides with dithiophosphate is as follows. To the nucleoside (1.76 mmol) in 5–10 ml of dimethyl formamide was added a dimethyl formamide solution of freshly prepared di-(triethylammonium) dithiophosphate (8.80 mmol). The resulting soln was dried by repeated evaporation with pyridine, concentrated to a viscous oil, then left at room temp. under agron for 12 hr. The thymidine and 2'-deoxycytidine mixtures were purified as follows. The crude mixture was dissolved in water, washed with carbon disulfide and chloroform, then concentrated to *ca* 5 ml. After adjusting the pH of the soln to 8 with ammonium hydroxide, 9 mmol of barium acetate dissolved in *ca* 5–10 ml water was added. After 1 hr at 4° the soln was filtered, then concentrated to 5 ml. The barium salt of the nucleoside-O-phosphorothioate was precipitated from this soln by addition of 2 parts EtOH. The filtered residue was dissolved in water and passed through a Na^+ form cation exchange column (16 × 1.7 cm). The eluate was lyophilized to a white powder which was crystallized to the pure nucleoside-O-phosphorothioate from an EtOH–H_2O mixture.

The 2':3'-O-ethoxymethylene adenosine reaction was purified as follows. The crude mixture was partially dissolved in 15–20 ml of water and filtered. The filtrate was then washed with CS_2 and $CHCl_3$ and concentrated to 5 ml. After adjusting the pH of the soln to 8 with ammonium hydroxide, 9.0 mmol of barium acetate, dissolved in *ca* 5–10 ml of water, was added. After standing 1 hr at 4° the soln was filtered. The filtrate was passed through a pyridinum form cation exchange column (16 × 1.7 cm) in water. The eluate was concentrated *in vacuo* to 15 ml and allowed to stand at room temp. for 2 hr to effect the hydrolysis of the O-ethoxymethylene protecting group. The soln was then co-evaporated 3 times with EtOH and passed through a Na^+ form cation exchange column (16 × 1.7 cm) in water. Lyophilization of the product-containing eluate gave a white powder which was crystallized from an EtOH–H_2O mixture to analytically pure sodium 5'-AMPS. The reaction yield determined for 5'(3')-TMPS (3:2 respective isomeric ratio) is 55%, for 5'(3')-dCMPS (3:2 respective isomeric ratio) is 65% and for 5'-AMPS is 45%.

The physical and spectral properties recorded for 5'(3')-TMPS are as follows: R_f (silica gel TLC, Sol A), 0.51; $\lambda_{max}^{H_2O}$ 265 nm; PMR (disodium salt form; TMS capillary), δ 8.15 (s, C_6-H of 5'-isomer), 8.02 (s, C_6-H of 3'-isomer), 6.67 (m, C_1'-H and C_5-H), 4.97 (m, C_3'-H), 4.40 (m, C_4'-H and C_5'-H), 2.73 (m, C_2'-H), 2.33 (s, CH_3). (Found: C, 29.25; H, 4.87; N, 6.69; P, 7.57; S, 7.84. Calc. for $C_{10}H_{14}N_2O_7PSNa\cdot(3)H_2O$: C, 28.99; H, 4.86; N, 6.76; P, 7.48; S, 7.74%).

The physical and spectral properties found for 5'(3')-dCMPS are as follows: R_f (silica gel TLC, Sol A), 0.38; $\lambda_{max}^{H_2O}$ 272 nm; PMR (disodium salt form; t-BuOH internal standard, δ 1.28), δ 8.07 (d, C_6-H of 5'-isomer), 7.80 (d, C_6-H of 3'-isomer), 6.16 (m, $C_{1'}$-H and $C_{3'}$-H), 4.80 (m, $C_{3'}$-H), 4.20 (m, $C_{5'}$-H and $C_{4'}$-H), 2.40 (m, $C_{2'}$-H). (Found: C, 27.50; H, 5.01; N, 10.65; P, 7.81; S, 7.93. Calc. for $C_9H_{13}N_3O_8PSNa\cdot(3)H_2O$: C, 27.35; H, 4.84; N, 10.63; P, 7.84; S, 8.11%).

The physical and spectral properties found for 5'-AMPS[2] are as follows: R_f (silica gel TLC, Sol A), 0.43; $\lambda_{max}^{H_2O}$ 259 nm; PMR (monosodium salt form; TMS capillary), δ 9.40 (s, C_8-H), 9.00 (s, C_2-H), 6.94 (d, $C_{1'}$-H), 5.40 (m, $C_{2'}$-H, $C_{3'}$-H, $C_{4'}$-H and $C_{5'}$-H). (Found: C, 26.66; H, 3.39; N, 15.44; P, 6.92; S, 6.91. Calc. for $C_{10}H_{12}N_5O_6PSNa_2\cdot(2)H_2O$: C, 27.09; H, 3.63; N, 15.79; P, 6.99; S, 7.23%).

Thiophosphorylation of 2,2'-cyclocytidine with monothiophosphate and dithiophosphate

Method A. Dithiophosphate. To a dimethyl formamide soln of freshly prepared di-(tri-ethylammonium) dithiophosphate (5.19 mmol) was added the proprionate salt of 2,2'-cyclocytidine[2,3] (1.73 mmol) in 50 ml of dimethyl formamide. The resulting soln was rendered anhyd. by repeated evaporations with pyridine and concentrated to 25 ml. The final soln was left at room temp., under argon, for 24 hr. Due to their different properties the cyclic **2** and **1** were isolated separately from two different mixtures. The cyclic **2** was isolated as follows. The crude mixture was dissolved in water, washed with CS_2, then chromatographed on a DEAE–cellulose column (115 × 3 cm) using a linear gradient (2 liter 0.01 M → 2 liter 0.10 M) of triethylammonium bicarbonate (pH 7.2) as eluant. The cyclic **2** was eluted at 0.07 M triethylammonium bicarbonate. The fractions containing **2** were combined, neutralized with Dowex H^+ resin, then lyophilized. The residue obtained was dissolved in water and passed through a Na^+ form cation exchange column. The product-containing eluate was lyophilized giving 86 mg (13% yield) of the salt **2**. An analytical sample was obtained by final purification using Sephadex G-25 column chromatography. The 2,2'-cyclocytidine 5'(3')-O-phosphorothioates were isolated from a mixture prepared in the same manner from 10.23 mmol of di-(triethylammonium) dithiophosphate and 2.05 mmol of the proprionate salt of 2,2'-cyclocytidine in 10 ml of dimethyl formamide. After standing 24 hr at room temp., the crude mixture was diluted with water and washed through an acetate form anion exchange column (16 × 2 cm) with 500 ml of 0.1% AcOH. The product-containing eluate was concentrated *in vacuo*, diluted with water, and then lyophilized. The resulting oil was dissolved in water and passed through a pyridinium form cation exchange column (11 × 2 cm). The product-containing eluate was lyophilized, giving a yellow oil which was dissolved in water and passed through a mixed-bed ion exchange column (Na^+ and Cl^- form). The column eluate was lyophilized to give **1** as a white powder weighing 174 mg (33% yield). An analytical sample of **1** was obtained by crystallization from a methanol–water–acetone mixture.

Method B. Monothiophosphate. To freshly prepared di-(tri-*n*-butylammonium) monothiophosphate (14.0 mmol) in pyridine was added the *p*-toluenesulphonate salt of 2,2'-cyclocytidine (3.25 mmol) in 40 ml of dimethyl formamide. The resulting mixture was rendered anhyd by repeated evaporations with pyridine then concentrated to 10 ml. The mixture, after 2 weeks at room temp., was purified in the same manner as described in Method A to give 78 mg (6.7% yield) of the sodium salt of **2**. No attempt was made to isolate the other reaction products, 2,2'-cyclocytidine 5'(3')-O-phosphorothioates (**1**).

The chromatographic properties of **2** are as follows: electrophoretic mobility, (pH 7.5) 0.95 times that of cytidine 2':3'-phosphate (2':3'-CMP) and (pH 4.5) 0.91 times that of 2':3'-CMP; silica gel TLC R_f (Sol B), 1.3 times that of 2':3'-CMP. The spectral data for the monosodium salt of **2** are as follows: UV, $\lambda_{max}^{H_2O}$ 272 nm (ϵ = 6224), $\lambda_{min}^{H_2O}$ 248 nm (ϵ 4394); ord, (H_2O) peak ($[M]^{290\,nm} = +2162$), trough ($[M]^{242.5\,nm} = -9921$), (0.10 M sodium phosphate buffer, pH 7.5) peak ($[M]^{290\,nm} = +3315$), trough ($[M]^{240\,nm} = -9944$); PMR (0.5% *t*-butyl alcohol as internal standard, δ 1.28), δ 7.95 (d, C_6-H), 6.21 (d, C_5-H and $C_{1'}$-H), 4.86 (m, $C_{2'}$-H and $C_{3'}$-H), 4.51 (q, $C_{4'}$-H), 3.93 (d, $C_{5'}$-H); ^{13}C-NMR (t-BuOH as internal standard, 31.6 and 68.7 ppm), ppm 56.61 (d, $C_{2'}$), 62.92 (t, $C_{5'}$), 80.30 (d, $C_{3'}$), 86.34 and 86.61 (d, $C_{1'}$ or $C_{4'}$),† 94.86 (d, $C_{1'}$ or $C_{4'}$), 98.05 (d, C_5), 145.31 (d, C_6), 153.38 (s, C_2), 164.19 (s, C_4); IR (KBr), 579 and 563 cm^{-1} (P–S–(C) stretch). (Found: C, 30.06; H, 3.71; N, 11.46; P, 8.47; S, 8.96. Calc. for $C_9H_{11}N_3O_6PSNa\cdot(1)H_2O$: C, 29.92; H, 3.63; N, 11.63; P, 8.57; S, 8.88%).

The spectral properties found for 2,2'-cyclocytidine 5'(3')-O-phosphorothioates (2:1 respective isomeric ratio) (**1**) are as follows: UV, $\lambda_{max}^{H_2O}$ 262 and 229 nm, $\lambda_{min}^{H_2O}$ 243 nm; PMR (TMS capillary), δ 8.66 (d, C_6-H of 3'-isomer), 8.62 (d, C_6-H of 5'-isomer), 7.18 (m, C_5-H and $C_{1'}$-H), 6.30 (d, $C_{2'}$-H of 3'-isomer), 6.14 (d, $C_{2'}$-H of 5'-isomer), 5.52 (d, $C_{3'}$-H of 3'-isomer), 5.28 (d, $C_{3'}$-H of 5'-isomer), 5.13 (m, $C_{4'}$-H), 4.44 (m, $C_{5'}$-H of 5'-isomer), 4.17 (m, $C_{5'}$-H of 3'-isomer); ^{13}C-NMR (t-BuOH as internal standard, 31.6 and 68.7 ppm), ppm 62.67 (t, $C_{5'}$ of 3'-isomer), 66.17 (t, $C_{5'}$-isomer), 77.05 (d, $C_{3'}$ of 5'-isomer), 80.36 (d, $C_{3'}$ of 3'-isomer), 89.80 and 90.18 (d, $C_{4'}$), 91.36 and 91.73 (d, $C_{1'}$), 92.30 (d, $C_{2'}$ of 5'-isomer), 93.30 (d, $C_{2'}$ of 3'-isomer), 104.68 and 105.12 (d, C_5), 141.94 (d, C_6), 161.63 and 162.32 (s, C_2), 169.44 (s, C_4). For aid in structure assignment the NMR spectral data for the chloride salt of 2,2'-cyclocytidine were measured under the same conditions and are as follows: PMR, δ 8.64 (d, C_6-H), 7.18 (d, C_5-H), 7.10 (d, $C_{1'}$-H), 6.10 (d, $C_{2'}$-H); 5.22 (d, $C_{3'}$-H), 4.96 (m, $C_{4'}$-H), 4.12 (d, $C_{5'}$-H); ^{13}C-NMR, ppm 62.27 (t, $C_{5'}$), 77.42 (d, $C_{3'}$), 92.24 (d, $C_{1'}$ and $C_{4'}$), 93.30 (s, $C_{2'}$), 104.62 (d, C_5), 142.06 (d, C_6), 162.32 (s, C_2), 169.26 (d, C_4). (Found: C, 31.62; H, 4.24; N, 12.19; P, 9.04; S, 9.29. Calc. for $C_9H_{12}N_3O_6PS\cdot(1)H_2O$: C, 31.86, H, 4.16; N, 12.39; P, 9.13; S, 9.45%).

Thiophosphorylation of thymidine with monothiopyrophosphate

To freshly prepared di-(tri-n-butylammonium) monothiopyrophosphate (3.0 mmol) in pyridine was added thymidine (1.0 mmol) in 5 ml of dimethyl formamide. The resulting soln, rendered anhyd by repeated evaporations with 1 ml portions of pyridine, was concentrated to a viscous oil and left at room temp under argon for 2 hr. Silica gel TLC (Sol A) of the crude mixture showed unreacted thymidine and TMPS. No side products were observed. The 5'(3')-TMPS, formed in 63% yield, was isolated in pure form using the barium acetate precipitation procedure described above. The isomeric ratio of the 5'(3')-TMPS product was determined from its PMR spectrum to be 2:1, respectively.

Rate investigation of the monothiophosphate-thymidine and monothiopyrophosphate-thymidine reactions. Two mixtures, one containing 6 mmol of mono-(tri-n-butylammonium) monothiophosphate and 1 mmol of thymidine and the other, containing 3 mmol of di-(tri-n-butylammonium) monothiopyrophosphate and 1 mmol of thymidine were prepared in the manner described above. The two mixtures were heated to 70° under argon. Samples were withdrawn at various time periods and analyzed using silica gel TLC (Sol A). The UV absorbing materials, corresponding to thymidine, 5'(3')-TMPS and the minor side products (which, as a mixture, gave a UV spectrum similar to thymidine), were isolated from the absorbant separately by elution with 10 ml of water. The absorbances of the resulting solns were measured at 260 nm. By assuming an equal ϵ^{260} for the compounds, the relative percentage of thymidine in the samples was calculated and plotted against the conversion time of the reaction. These results are shown in Fig. 2.

The selectivity in 5'-OH vs 3'-OH thiophosphorylation in the monothiopyrophosphate-thymidine reaction was determined from the PMR spectrum of the 5'(3')-TMPS to be 2:1, respectively. The 5'(3')-TMPS sample used for spectral analysis was obtained from the crude reaction mixture by preparative silica gel TLC in Sol A.

Monothiophosphate thermal decomposition products. Four mixtures, each containing 6 mmol of mono-(tri-n-butylammonium) monothiophosphate in dimethyl formamide, were pre-

†In the proton decoupled spectrum this resonance appears as a doublet.

pared in the manner previously described for the thiophosphorylation reactions with the exception that no nucleoside was added. A fifth mixture, containing 12 mmol of mono-(tri-n-butylammonium) monothiophosphate, was prepared in the same way. All 5 mixtures were heated at 70°. After a reaction period of 12, 24 and 38 hr, respectively, the first three reaction flasks were assayed, using electrophoretic analysis, for monothiophosphate content. After a 12 hr reaction period only a trace of monothiophosphate remained present and after 38 hr the monothiophosphate was completely consumed. After a 59 hr reaction period the fourth (6 mmol of monothiophosphate) and fifth (12 mmol of monothiophosphate) mixtures were allowed to cool to room temp. To the fourth reaction flask was added 1 mmol of thymidine. The mixture was then heated for an additional 60 hr. Analysis of this reaction mixture using silica gel TLC (Sol A) revealed unconsumed thymidine, 5'(3')-TMPS (31%) and the same minor products observed in the previous monothiophosphate-thymidine reaction mixture.

The remaining monothiophosphate (12 mmol) decomposition reaction mixture was analyzed in the following manner. The crude mixture was dissolved in water, washed with CS_2, then applied to an amberlite anion exchange column (60 × 2.5 cm) and eluted using a linear gradient of ammonium carbonate buffer (1 liter 0.001 M → 1 liter 1.0 M). The column fractions were analyzed by phosphate determination performed on 20 μl samples. The elution diagram obtained is shown in Fig. 1. The fractions comprising peak 1–4 were separately combined, neutralized, lyophilized and then desalted by passage through Sephadex G-10 columns (120 × 2.5 cm). Qualitative sulfur analysis was performed on all four compounds. The compound eluted as peak 3 contained sulfur whereas the other three compounds did not. The compounds contained in peaks 1, 2 and 4 were determined to be orthophosphate, pyrophosphate and triphosphate, respectively, by comparative electrophoresis at pH 5.0, 6.5 and 9.0 [0.05 M $(NH_4)_2CO_3$] with standard samples. The compound contained in peak 3 displayed the same electrophoretic mobilities at these three pH's as independently prepared[18] monothiopyrophosphate.

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