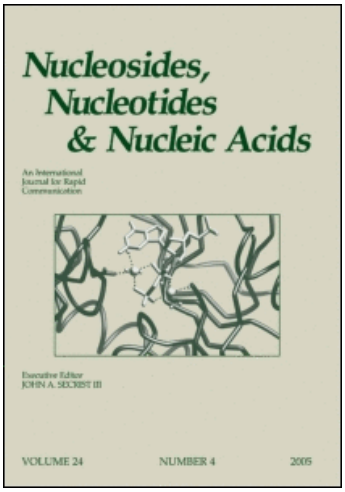

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SYNTHESIS OF SOME NUCLEOSIDE CYCLIC ETHYLENE PHOSPHOTHIONATES

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Abstract. This communication describes the synthesis of 5'-deoxy-5'-chloro-3'-(2-thio-1,3,2-dioxaphosphorinanyl)thymidine, N^4,2',3'-triacetyl-5'-(2-thio-1,3,2-dioxaphosphorinanyl)-1-β-D-arabinosyl-cytosine and N^4-acetyl-5'-(2-thio-1,3,2-dioxaphosphorinanyl)-1-β-D-arabinosylcytosine.

Recent efforts to obtain suitable transport or depot forms of biologically active nucleotides have pointed to the usefulness of the cyclic phosphotriester group. A number of phosphotriesters of cyclic AMP, i.e. a 6-member cyclic phosphate, were synthesized[1,2] and found to be rather stable. The phosphodiester, cyclic AMP, could be released by nucleophilic attacks on the exocyclic carbon which may cause undesirable alkylation reactions. We have now continued this approach with the synthesis of other nucleoside phosphotriesters which are less stable and are hydrolysed via P-O cleavage. The great lability of cyclic ethylene phosphotriesters and the mechanism of their hydrolysis is well known[3]. No 5-member ring phosphate ester of nucleosides has ever been described although the 2':3'-5' phosphotriesters of RNA[4] were believed to exist in anhydrous solvents. We sought to stabilize the cyclic ethylene phosphate moiety against hydrolysis in the form of a phosphothionate. This communication describes the synthesis of 5'-deoxy-5'-chloro-3'-(2-thio-1,3,2-dioxaphosphorinanyl) thymidine (3), N^4,2',3'-triacetyl-5'(2-thio-1,3,2-dioxaphosphorinanyl)-1-

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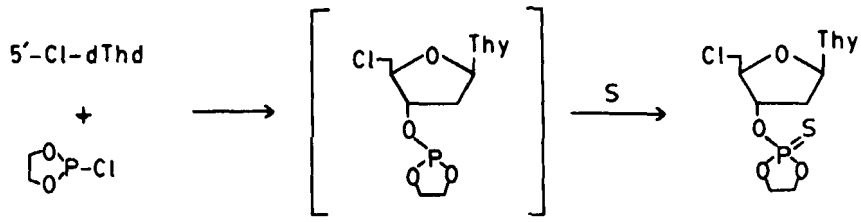
β-D-arabinosylcytosine (5) and N^4-acetyl-5'-(2-thio-1,3,2-dioxaphosphorinanyl)-1-β-D-arabinosylcytosine (7). These triesters were stable enough to be observed in aqueous solvents.

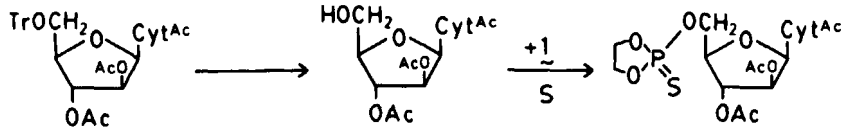
The reaction of 5'-deoxy-5'-chlorothymidine with 2-chloro-1,3,2-dioxaphospholane (1, scheme 1) in dioxane at 0°C afforded a neutral compound which could be observed on TLC in aprotic solvents. Attempts to isolate this intermediate (2) in pure form have failed. Intermediate 2 reacted with sulfur to yield the desired product 3 in 30% yield. Compound 3 appeared neutral on electrophoresis, gave excellent PMR and MS data and elemental analysis.

Once the synthetic utility of the proposed route was demonstrated satisfactorily, it was applied to 1-β-D-arabinosylcytosine (araCyt). Reaction of unprotected araCyt with 1 under similar conditions gave several products, presumably due to the nonselective reaction of 1 with not only the desired 5'hydroxy group but also with the 4-amino and 2' and 3' hydroxy groups. In order to remove this problem, the 4-amino and 2' and 3' hydroxy groups were blocked by acetyl groups in several steps according to the method of Wechter[5]. The reaction of N^4,2',3'-triacetyl-araCyt (4, scheme 2) with 1 in a fashion similar to the one described above once again gave a phosphite intermediate which was



1 2 3

Scheme 1



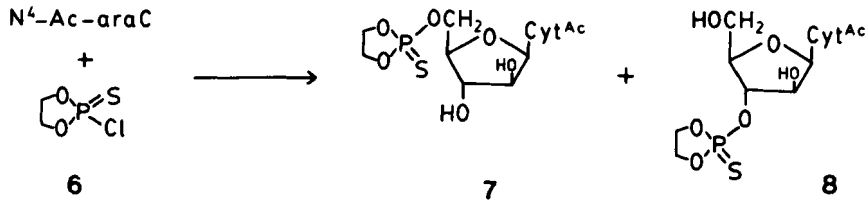
4 5

Scheme 2

reacted in situ with sulfur and yielded the desired compound 5 in 30% yield. Compound 5 was neutral on electrophoresis and gave the expected PMR and MS data as well as elemental analysis. As anticipated, a selective removal of the acetyl groups was not possible.

Because of the greater interest in the unprotected nucleoside phosphotriester, attempts were undertaken to achieve specific 5' phosphorylation of unprotected araCyt using the less reactive and sterically somewhat hindered reagent, 2-thio-2-chloro-1,3,2-dioxaphospholane (6). Unexpectedly, the major product of this reaction contained an altered chromophore (UV max 262 nm) indicative of anhydro-araCyt formation. To our knowledge, this reactivity of araCyt has not been observed before. Apparently, the most nucleophilic center of araCyt is the C-2 carbonyl group, and the phosphorylation of C=O may provide the driving force to cyclisation. Ring closure was even more rapid when the C^4-NH_2 group was protected by the electron-donating N,N-dimethylamino methylene group. This finding led to the conclusion that the amino group must be protected by an electron attracting group.

Reaction of N^4-acetyl-araCyt and 6 (scheme 3) in dry pyridine and triethylamine at 4° followed by a rapid silica gel chromatography with 20% MeOH in $CHCl_3$ gave the desired cyclic triester 7 in about 30% yield and 90% purity. Further purification was achieved by preparative TLC at 4°C using 20% MeOH in $CHCl_3$. A minor product (3-5%), possibly the 3'-isomer 8, had close values in several solvent systems and could not be separated completely. Compound 7 was neutral on electrophoresis. Upon standing in 0.05 M bicarbonate buffer (pH 7.5), it hydrolyzed mainly to a phosphodiester but significant amount of a new compound, which was also a phosphotriester, was also observed. This compound could have been a 2':5' or 3':5'-cyclic phosphotriester formed by transesterification.



Scheme 3

Triester 7 was characterized by its mass spectrum and NMR spectroscopy. The PMR data were in excellent agreement with the proposed product structure. ^{31}P NMR showed the expected splitting pattern for 7: a septet due to the six equivalent protons linked by three CH_2-O-P bridges centered near -53.5 ppm with coupling constant 11.0 Hz. It also indicated the presence of two other triesters in less than 5% of the total sample.

The rates of hydrolysis of 3, 5, and 7 were determined in 0.1 N KOH, 0.05 M $NaHCO_3$, pH 7.2 and in 0.05 M Tris-HCl, pH 7.05. While 3 and 5 had a half-life of about 5 min in 0.1 N KOH, 7 was almost completely hydrolyzed in less than 5 min. In bicarbonate and Tris-HCl buffers, 3 and 5 had a half-life of about 1 hr. The half-life of 7 could not be determined accurately because of the contaminating transesterification product; it is between 1 and 2 hr. The new triester is more stable ($t_{1/2}$ ca 20 hr). The relatively small amount of nucleoside observed after several days points to a predominant ring opening in the hydrolysis of 3, 5, and 8.

These results show that cyclic triesters of phosphothionate may possess sufficient stability to be suitable transport forms of some nucleotide analogs. However, their usefulness may be limited by undesirable transesterification reactions in the case of araCyt.

EXPERIMENTAL

Materials and Methods. 1-β-D-arabinosylcytosine was a gift from Dr. M.V.Nadkarni, The National Cancer Institute. 5'-Deoxy-5'-chlorothymidine was from a stock left over from an earlier work[6]. Analytical grade solvents were dried over molecular sieve type 4A. Eastman Chromagram sheets were used for thin-layer chromatography on silica gel in the solvent mixtures of $CHCl_3$-methanol (3:1 and 4:1) and 1-butanol-H_2O (8.5:1.5). Paper electrophoresis was performed in a Savant flat-plate apparatus in 0.05 M triethylammonium bicarbonate, pH 7.5. NMR spectra were recorded on a Varian FT-80 spectrometer in acetone-d_6 and methanol-d_4 with TMS as external standard. Mass spectra were obtained on a ^{252}Cf plasma desorption mass spectrometer[7] or on a CEC 20-110 instrument at 70 eV. Elemental analyses were carried out by Galbraith Laboratories, Inc.

5'-Deoxy-5'-chloro-3'-(2-thio-1,3,2-dioxaphosphorinanyl) thymidine (3):

To a solution of 5'-deoxy-5'-chlorothymidine[6] (300 mg, 1.15 mmol) in 100 mL of dioxane and 1.6 mL of pyridine was added 2-chloro-1,3,2-dioxaphospholane[8] (1, 0.3 mL, 2.4 mmol) at 0°. After 15 min, 0.05 M triethylammonium bicarbonate, pH 7.2 (200 mL) was added and the phosphite ester 2 was extracted with toluene (3 x 25 mL). The organic phase was dried over $MgSO_4$ and treated with sulfur (100 mg) overnight at room temperature. After filtration and concentration, the solution was applied to a silica gel column (35 x 2 cm) and the product was eluted with a mixture of ethylacetate-diethylether, 1:1. The main fraction (from 60 to 160 mL) was concentrated to 10 mL and crystalised by the addition of ether. Yield 158 mg (0.41 mmol, 35%), mp 144-145° (decomp). ^{1}H-NMR in acetone-d_6: 7.53 (d, 1, C_6H), 6.31 (t, 1, $C_1'H$), 4.62 (d, 2, $C_3'H$, $C_4'H$), 4.50 (m, 2-CH_2), 3.92 (d, 2, $C_5'H$), 2.57 (m, 2, $C_2'H$) 1.82 (d, 3, CH_3).Anal calcd $C_{12}H_{16}ClN_2O_6PS$: C 37.65, H 4.21, N 7.32, P 8.90; found C 37.68, H 4.29, N 7.28, P 8.12. ^{252}Cf plasma desorption mass spectroscopy of underivatised compound: 383.2 $(MCl^{37}-H)^-$, 381.2 $(MCl^{35}-H)^-$, 357.1 $(HO_3PSRCl^{37})^-$, 355.2 $(HO_3PSRCl^{35})^-$, 139.0 $(C_2H_4O_3PS)^-$.

N^4, 2', 3'-Triacetyl-5'-(2-thio-1,3,2-dioxaphosphorinanyl) araCyt (5):

This compound was prepared in a fashion similar to that described for the synthesis of 3 using 359 mg of 4 (0.97 mmol), 1.8 mL dry pyridine and 0.95 mL of 1 in 100 mL dry dioxane, followed by sulfur treatment of the intermediate and chromatography. Yield 150 mg, (0.30 mmol, 30%). Calculated for $C_{17}H_{22}N_3O_{10}PS$: C 41.51, H 4.47, N 8.55, P 6.30. Found: C 41.52, H 4.56, N 8.40, P 6.37. ^{1}H NMR in acetone-d_6: 8.15 (d, 1, C_6H, J_{5-6} 6Hz), 7.35 (d, 1, C_5H), 6.30 (d, 1, $C_1'H$, $J_{1'-2'}$4Hz), 5.45 (t, 1, $C_3'H$),5.18 (t, 1, $C_2'H$), 4.50 (m, 7, $C_4'H$, $C_5'H_2$, 2-CH_2). ^{252}Cf plasma desorption mass spectroscopy of underivatised compound: 490.4 $(M-H)^-$, 464.4 $(M-C_2H_4+H)^-$, 448.3 $(M-CH_3CO)^-$.

N^4-Acetyl-5'-(2-thio-1,3,2-dioxaphosphorinanyl) araCyt (7):

A flask containing 0.4 mL of 2-thio-2-chloro-1,3,2-dioxaphosphorinane[9],2.50 mL of dry pyridine, and 0.8 mL of dry triethylamine was cooled to 0°C. N^4-Acetyl araCyt[10], 285 mg (14,800 OD_{248} units, 1 mmol)

was added in one portion and the solution was stirred at 0°C for 15 min. The excess phosphorochloridate was destroyed with methanol (1.0 mL) and the mixture was immediately placed on a silica gel column (1 x 15 cm) prepared at 0°C with $CHCl_3$. First it was washed with 100 ml $CHCl_3$ at the rate of 3-4 mL/min to remove pyridine and Et_3N. The triester 7 was eluted by 100 mL of 25% methanolic $CHCl_3$. It was slightly contaminated by pyridinium salts and degradation products. The solvent was removed under reduced pressure at 10°C. The residue was taken up in 4 mL of dry methanol and purified on 2 preparative silica gel plates (20 x 20 cm, 2000 μ thickness) using 25% methanolic $CHCl_3$ at 0°C. The product had an R_f of 0.45 and was eluted from the silica using methanol. Yield 4,350 OD_{248} units, 0.29 mmol, 134 mg, 29%, fluffy white crystals. Efforts to remove the nucleotidic contamination (5%) led to further degradation. We could not obtain satisfactory elemental analyses because of contaminating silica. Two nonionic impurities, presumably isomers, had close R_f values of 0.43 and 0.38 resp. ^{1}H-NMR (methanol-d_4, TMS): 8.22 (d, 1, C_6-H), 7.40 (d, 1, J 7.1 Hz, C_5-H), 6.30 (d, 1, J 4.5 Hz, C_1'-H), 5.05 (m, 1, C_3'-H), 4.90 (t, 1, C_2'-H), 4.45 (m, 7, C_4'-H, $C_5'H_2$, 2-CH_2).

The ^{31}P-NMR was carried out in acetone-d_6 with inorganic phosphate as reference, and the spectrum revealed the expected septet at -53.5 ppm ($J_{P,C}$ 11.0 Hz) with minor triester phosphorus also present at -63 ppm. For the mass spectrum, 7 was dissolved in pyridine and reacted with an excess of bis-trimethylsilyl acetamide. The main positive ions found on electron impact mass spectroscopy: 581 (P, parent ion $C_{11}H_{13}N_3O_7PS(Me_3Si)_3$), 566 (P-Me), 508 (P-$Me_3Si$), 399 (P-base), 139 (ethylene phosphate).

The stability of triesters 3, 5, and 7 was explored via TLC analysis following incubation at 20° in 0.05 M $NaHCO_3$, pH 7.2, and 0.05 M Tris-HCl, pH 7.05. Both the diester spots and the neutral material were eluted, quantitated by UV and spotted again in TLC. All three compounds had a half-life of approximately 1 hr, the hydrolysis product being a corresponding nucleoside phosphodiester. In the case of 7, however, the main product was a new phosphotriester (R_f 0.38) which comprised about 45% of the total material after 24 hr. For the alkaline hydrolysis of triesters 3, 5, and 7, samples (2 mg) of triesters were dissolved in 1 mL of 0.1 N KOH and aliquots were

analyzed by electrophoresis in 0.05 M Tris-HCl pH 7.0. Triester 7 was almost completely hydrolyzed in 5 min to the diester, while prolonged hydrolysis produced increasing amounts of araC. The triesters 3, 5, and the putative transesterification product of 7 were only slightly more stable and all were completely hydrolyzed in 30 min.

Attempted Phosphorylations of araCyt and N^4-dimethylaminomethylene-araCyt:

An attempt to introduce the phosphothionate triester via phosphorylation with 6 was made under the conditions as described for the preparation of 7. In the case of araCyt, only minor amounts of phosphorylated products were observed, and these were in the diester form. The main product was O^2:2'-anhydro-araCyt which was identified by its characteristic UV spectrum (λ max 262) and cationic behavior on electrophoresis. N^4-Dimethylaminomethylene-araCyt prepared by the general method of Zemlicka[11] gave similar results. A characteristic UV shift to λ 325 nm took place indicating anhydronucleoside formation. Both products were identified by comparison with specimens obtained by our previously published procedure[12].

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