Synthesis and in Vitro Evaluation of Some Modified 4-Thiopyrimidine Nucleosides for Prevention or Reversal of AIDS-Associated Neurological Disorders

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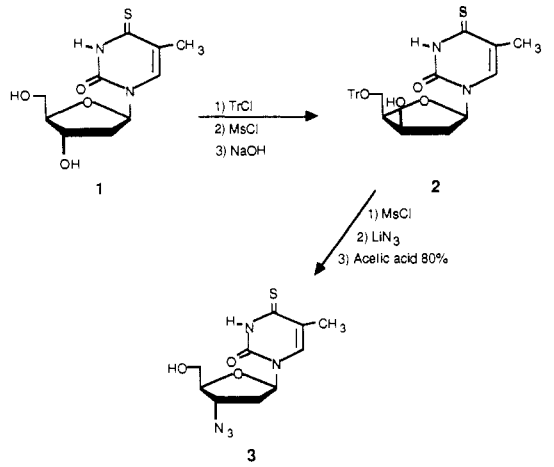
Oxygen–sulfur exchange at the C-4 carbonyl of several modified pyrimidine nucleosides, including 3′-azido-3′-deoxythymidine (AZT), is described in an effort to enhance the lipophilicity and, thereby, the delivery to the central nervous system of the sulfur analogues without compromising the anti-HIV activities of the parental structures. Preparation of 3′-azido-3′-deoxy-4-thiothymidine (**3**) proceeded from 4-thiothymidine (**1**) and utilized the same methodology developed for the initial synthesis of AZT. Thiation of 2′,3′-didehydro-3′-deoxythymidine (**4a**) and 2′,3′-didehydro-2′,3′-dideoxyuridine (**4c**) was carried out with Lawesson's reagent on the corresponding 5′-*O*-benzoate esters, **4b** and **4d**, to give **5a** and **5c**, respectively. The latter, on alkaline hydrolysis, gave 2′,3′-didehydro-3′-deoxy-4-thiothymidine (**5b**) and 2′,3′-didehydro-2′,3′-dideoxyuridine (**5d**), respectively. The same series of reactions were applied to the 5′-*O*-benzoate esters of 2′,3′-dideoxyuridine (**6a**) and 3′-deoxythymidine (**6b**) to give 2′,3′-dideoxy-4-thiouridine (**7d**) and 3′-deoxy-4-thiothymidine (**7b**), respectively. Characterization of the saturated and unsaturated thionucleosides included mass spectrometric studies. Under electron impact conditions, the thiated analogues gave more intense parent ions than the corresponding oxygen precursors. The lipophilicity of thymidine and the 3′-deoxythymidine derivatives are enhanced significantly, as indicated, by increases in corresponding *P* values (1-octanol–0.1 M sodium phosphate) upon replacement of the 4-carbonyl oxygens by sulfur. Compounds **5b**, **5d**, **7b**, and **7d** were evaluated for their effects on HIV-induced cytopathogenicity of MT-2 and CEM cells. Only **5b** and **7b** were moderately active in protecting both cell lines against the cytolytic effect of HIV. The inhibitory effects of analogues **5b**, **5d**, **7b**, and **7d** on thymidine phosphorylation by rabbit thymus thymidine kinase were evaluated. Only **3** showed moderate affinity (K_i = 54 μM) for the enzyme. The generally weak anti-HIV activities of the remaining thio analogues are consistent with correspondingly low susceptibilities to thymidine kinase phosphorylation as estimated from the respective K_i values of the synthetic nucleosides. However, the phosphorylation of the 5′-monophosphate derivatives to their respective 5′-triphosphates must also be considered in connection with the weak in vitro anti-HIV effects of these thiated compounds.

Infection with human immunodeficiency virus (HIV) is frequently complicated by a dementing syndrome, AIDS dementia complex, which is an important cause of morbidity in patients in advanced stages of infection.[1,2] It appears that the virus frequently invades the central nervous system (CNS) early in the course of systemic infection, even in the absence of symptoms.[1] Evidence is accumulating to support the concept that the predominant cell type in the brain that is infected with HIV is of monocyte/macrophage lineage.[3] These cells may serve as a reservoir for the virus because HIV replication in monocyte/macrophages is more prolonged and less cytolytic than in lymphocytes.[2] The longer survival of the virus in monocyte/macrophages, in turn, permits transport to other organs, e.g., lungs.

There is, then, a need for antiviral drugs that can penetrate the blood–brain and blood–cerebrospinal fluid (CSF) barriers. In this connection it may be noted that 3′-azido-3′-deoxythymidine, (AZT, N_3ddThd), the only antiretroviral agent approved to date by the Food and Drug Administration for treatment of AIDS patients and individuals with AIDS related complex (ARC), penetrates the CSF[4-6] and can, at least partly, reverse the neurological dysfunction due to HIV in some patients.[4,5]

It appears that N_3ddThd is highly unusual among nucleoside analogues in that it traverses the cell membrane chiefly by nonfacilitated diffusion and not via a nucleoside transport system.[7] The ability of N_3ddThd to permeate cells by passive diffusion is attributed to the considerable lipophilicity imparted to this molecule by replacement of the 3′-hydroxyl group of thymidine (dThd) by an azido substituent. Thus, the 1-octanol–buffer (pH7) partition coefficient, which essentially is a measure of lipophilicity, was determined to be 1.26 for N_3ddThd compared to a value of 0.064 for dThd.[7] The significantly greater lipophilicity of N_3ddThd correlates with a reversible permeability (freely enter and exit) to the CSF and its capacity to partially ameliorate AIDS-related neurological deficits.

Scheme I



In the present work we sought to enhance the lipophilicity[8] and, thereby, the delivery to the CNS of several

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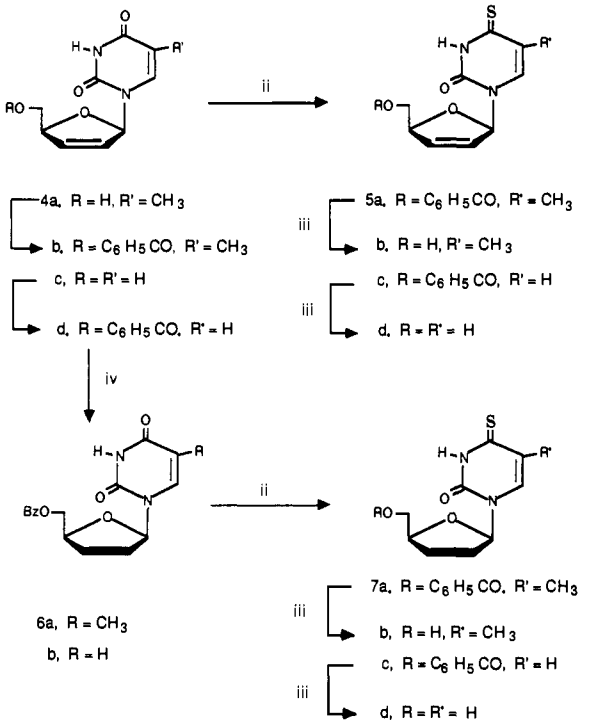
Scheme II[a]



[a] Reagents: i, C_6H_5 COCl/pyridine; ii, Lawesson's reagent; iii, NaOH; iv, H_2, 10% Pd/C.

modified pyrimidine nucleosides, including N_3ddThd, by effecting oxygen–sulfur exchange at the C-4 carbonyl moiety of the base without seriously compromising the anti-HIV activities of the parental structures.

Chemistry

The preparation of 3′-azido-3′-deoxy-4-thiothymidine (**3**, N_3ddThd^S) proceeded from 4-thiothymidine (dThd^S, 1)[9] and utilized essentially the same methodology[10] developed for the initial synthesis of N_3ddThd as shown in Scheme I. Thiation of 2′,3′-didehydro-3′-deoxythymidine (**4a**, ddeThd, Scheme II) and 2′,3′-didehydro-2′,3′-dideoxyuridine (**4c**, ddeUrd), which were both obtained by established methods,[11] was carried out with Lawesson's reagent [2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane 2,4-disulfide][12] on the corresponding 5′-*O*-benzoate esters, **4b** and **4d**, to give **5a** and **5c**, respectively. The latter, on hydrolysis with sodium hydroxide, gave the 4-thiopyrimidine 2′,3′-unsaturated nucleosides, ddeThd^S (**5b**) and ddeUrd^S (**5d**), respectively.

The 5′-*O*-benzoate esters of 3′-deoxythymidine (**6a**, ddThd) and 2′,3′-dideoxyuridine (**6b**, ddUrd), obtained on catalytic (10% Pd/C) hydrogenation of the corresponding unsaturated precursors (**4b** and **4d**, respectively), were subjected to the same oxygen–sulfur exchange and hydrolysis reactions to give 3′-deoxy-4-thiothymidine (**7b**, ddThd^S) and 2′,3′-dideoxy-4-thiouridine (**7d**, ddUrd^S).

The characterization of the thiated nucleosides included detailed mass spectrometric studies.[13] Compounds **6a** and **6b** and their thiated analogues, **7b** and **7d**, proved to be more stable than each of the corresponding 2′,3′-unsaturated analogues whether analyzed either by electron impact (EI) or positive chemical ionization (CI^+) techniques. The EI mass spectra of all the thiated derivatives prepared in this study showed the well-established fragmentation pattern characterized by cleavage of the glycosyl bond with formation of a protonated aglycon and a stabilized sugar moiety. The fragmentation of N_3ddThd and its sulfur analogue both showed the same pattern of glycosyl bond cleavage to form the protonated aglycon and a sugar residue that had lost a molecule of N_2. A relatively intense $M^{\bullet+}$ was observed with N_3ddThd, whereas the thiated analogue gave only a relatively weak $M^{\bullet+}$. Both compounds were examined with additional mass spectrometric techniques including CI^+, desorption chemical ionization, and thermospray (TSP) modes. Acceptable results were obtained with each but the TSP method, which gives rise to an intense molecular ion in both structures, would appear to be particularly useful.

Table I. Partition Coefficients (*P*) of Some Modified Pyrimidine Nucleosides[a]

nucleoside	*P*	*P* ratio[c]	nucleoside	*P*	*P* ratio[c]
dThd	0.055 (0.064)[b]		ddUrd	0.39	
dThd^S	0.855	15.5	ddUrd^S	0.61	1.56
ddThd	0.43		ddeUrd	0.43	
ddThd^S	2.76	6.41	ddeUrd^S	0.53	1.23
ddeThd	0.23		dCyd	0.024	
ddeThd^S	1.24	5.39	ddCyd	0.17	
N_3ddThd	1.68 (1.26)[b]		ddeCyd	0.20	
N_3ddThd^S	11.75	6.99			

[a] Measured in 1-octanol–0.1 M Na_3PO_4 (pH 7) according to the method described in ref 26. [b] See ref 7. [c] *P* ratio = $P_N{}^S/P_N$.

Partition Coefficients

The lipophilicities of the modified thymidine nucleosides are enhanced significantly, as indicated by the increases in corresponding *P* values (1-octanol–0.1 M sodium phosphate) upon replacement of the 4-carbonyl oxygen by sulfur (see Table I). Thus, oxygen–sulfur interchange leading to dThd^S is associated with a 16-fold increase in *P* value relative to that of dThd. The same conversion applied to ddThd, N_3ddThd, and ddeThd produced a 5–7-fold increase in the *P* value of the corresponding thiated analogues. In contrast, relatively modest (1.3–1.6-fold) increases in *P* values were noted in the sulfur exchange reaction leading to ddUrd^S and ddeUrd^S.

Table I includes, for purposes of comparison, the *P* values for dCyd, ddeCyd, and ddCyd. The latter, which mole for mole is about 10-fold more potent than N_3ddThd in arresting HIV replication,[14] manifests a comparatively limited capacity to penetrate the CNS.[15] The assumed absence, in the case of ddCyd, of a carrier-mediated mechanism of transport, of the types[7,16,17] utilized by physiological nucleosides, i.e., dCyd, would require that

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Table II. Comparative Potency and Selectivity of Some Modified Nucleosides as Inhibitors of HIV Replication in MT-2 and CEM Cells[a]

compound	cell culture	EC_{50}[b] μM	IC_{50},[c] μM
ddThd[d]	MT-2	62–250	>625
$ddThd^S$	MT-2	39	90
ddeThd[d]	MT-2	2.0	71–210
$ddeThd^S$	MT-2	27–157	>80
N_3ddThd[d]	MT-2	0.18	>970
ddThd[d]	CEM	42–333	>625
$ddThd^S$	CEM	31–35	93–175
ddeThd[d]	CEM	0.8–25	235–357
$ddeThd^S$	CEM	39	>430
N_3ddThd[d]	CEM	3.2×10^{-3}	>10

[a] Testing was performed in the National Cancer Institute's Developmental Therapeutics Program, AIDS antiviral screening program (see Experimental Section). All of the data listed above were compared with corresponding testing results for N_3ddThd which served as the treated control, performed at the same time. [b] Concentration of drug resulting in 50% reduction of the viral cytopathic effect. [c] Concentration of drug resulting in 50% growth inhibition of normal, uninfected cells. [d] The authors are indebted to Dr. Robert Schultz of the NCI's Developmental Therapeutics Program for this data.

the passage of this agent across cell membranes be effected by simple, nonfacilitated diffusion. The relatively small P value of ddCyd is consistent with the rather low CNS penetration of this antiretroviral agent.

The present findings indicate that the modified thiated pyrimidine nucleosides are all sufficiently lipophilic to permeate the brain. However, it is also recognized that, despite the improved efficiency with which these thiated analogues can be expected to enter the CNS, they may leave by the same mechanism with equal facility.

Anti-HIV Activities

The modified thiated nucleosides were evaluated for their effects on HIV-induced cytopathogenicity in two human T-lymphocyte cell lines, MT-2 and CEM, in the National Cancer Institute's Developmental Program AIDS antiviral screening program. The results are shown in Table II along with corresponding data on the parental structures.

$DdThd^S$ and $ddeThd^S$, like ddThd, were moderately active in protecting MT-2 and CEM cells against the cytolytic effect of the virus. By contrast, ddeThd showed an appreciably greater antiretroviral potency in both cell lines. The latter findings are in accord with other data which clearly indicate ddeThd to be a potent inhibitor of HIV in ATH8,[18] MT-4,[19] PBM,[20] and, as well, CEM cells.[21] The in vitro growth inhibition of MT-2 cells by $ddThd^S$, ddeThd, and $ddeThd^S$ were quite similar but significantly greater than ddThd. On the other hand, $ddeThd^S$ and ddThd both inhibited the growth of CEM at relatively high concentrations compared to both $ddThd^S$ and ddeThd.

Neither $ddUrd^S$ nor $ddeUrd^S$ showed any protective effect against HIV in either MT-2 or CEM cells (data not shown). The latter findings were not surprising in view of the fact that the corresponding oxygen analogues, ddUrd and ddeUrd, range from being minimally effective to essentially devoid of protecting activity of MT-4[22] and ATH8[18] cells against the cytopathic effect of the virus.

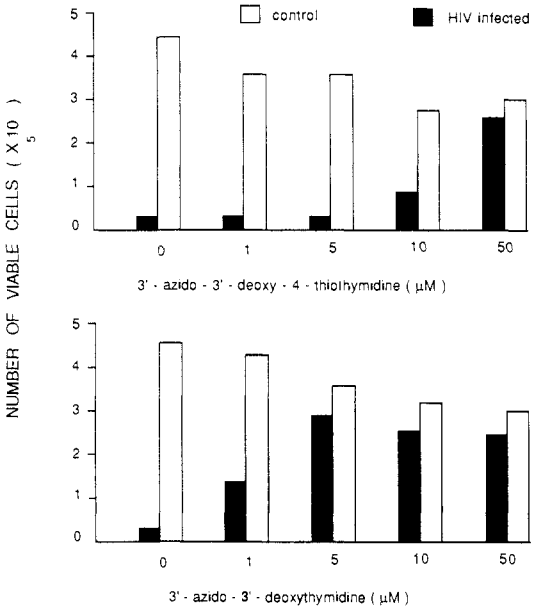

Figure 1. Inhibition of cytopathic effect of HIV against ATH8 cells by 3′-azido-3′-deoxy-4-thiothymidine and 3′-azido-3′-deoxythymidine (see ref 25).

Surprisingly, N_3ddThd^S failed to show any appreciable antiretroviral activity in either MT-2 or CEM cells (data not shown), but did manifest modest anti-HIV activity in ATH8 cells. However, when N_3ddThd^S and N_3ddThd were compared for their inhibitory effects on the cytopathogenicity of HIV in ATH8 cells, the sulfur analogue proved to be significantly less potent in protecting this culture against the virus (Figure 1). Thus, N_3ddThd at a concentration of 3–5 μM, affords virtually complete protection against the virus, whereas 50 μM N_3ddThd^S was required to achieve the same effect in these cells. Moreover, the sulfur analogue at this same concentration reduced ATH8 cell viability by approximately 30% in controls that were similarly treated but not exposed to the virus. It appears, then, that N_3ddThd^S, despite its enhanced lipophilicity, would offer no clinical advantage over N_3ddThd in the treatment of AIDS or associated neurological disorders.

Phosporylation Studies

It is now recognized that the initial phosporylation of deoxynucleosides analogues, although obligatory, may not be sufficient for potent anti-HIV activity.[22] Rather, the poor activity of certain pyrimidine deoxynucleosides may derive instead from their low affinity for pyrimidine nucleotide kinases, e.g., dThdMP/dUrdMP kinase, nucleoside diphosphate kinase, involved in the second or third phosporylation step or a poor affinity of the 5′-triphosphates for HIV-reverse transcriptase.[22] Nonetheless, it was of interest to determine whether the generally weak anti-HIV activities of the ddN^S and $ddeN^S$ (vide supra) reflect corresponding diminished affinities for thymidine kinase as the activating enzyme.

In this study we have estimated the affinity of each of the modified pyrimidine nucleosides and the corresponding thiated analogues for phosphorylation via thymidine kinase or deoxycytidine kinase by an indirect method, i.e., determination of their respective K_i values for the particular

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Table III. Inhibitory Effect of Some Modified Nucleosides on Phosphorylation by Rabbit Thymus Thymidine[a] and Deoxycytidine[b] Kinases

ddN	enzyme	K_i, μM	molt/4F kinase lit. K_i, μM
dThd^S	thymidine kinase	12	
ddThd		425	580,[c] 653[d]
ddThd^S		1600	
N_3ddThd	thymidine kinase	7.0	2.9[d]
N_3ddThd^S		54	
ddeThd		615	580[c]
ddeThd^S		1690	
ddUrd	thymidine kinase	975	>1000[d]
ddUrd^S		1275	
ddeUrd	thymidine kinase	445	
ddeUrd^S		1690	
ddCyd	deoxycytidine kinase	360	1000,[c] 1965[d]
ddeCyd		1100	4600[c]

[a] K_m value for [2-^{14}C]-dThd, 3.0 μM (Molt/4F; K_m = 4–5 μM).[18] [b] K_m value for [2-^{14}C]-dCyd 1.1 μM (Molt/4F; K_m = 2.8 μM).[18] [c] See ref 18. [d] See ref 22.

enzyme. The resulting data (Table III) provide an approximation of the expected rates of phosphorylation. The more direct and rigorous approach, involving a determination of K_m and V_{max} values for each of the compounds, which would have required the corresponding radioactive nucleosides, was not considered at this stage of the work.

There remained, then, the choice of an appropriate source of enzyme which is complicated by recent data indicating that the intracellular metabolism of modified nucleosides, such as N_3ddThd and ddCyd, to be highly dependent on cell species.[23] Moreover, the metabolism of, for example, ddCyd differs quantitatively among different human cell lines.[23]

In related studies we examined the utility of readily available rabbit thymus as a reproducible source of both dThd and dCyd kinases. Apparent K_m values for the corresponding physiological substrates were determined to be 3.0 and 1.1 μM, respectively (Table III). These values compare favorably with the same kinetic constants (K_m dThd = 4–5 μM; K_m dCyd = 2.8 μM) derived from a human T lymphoblast cell culture, Molt/4F.[18]

DdThd and ddeThd were then evaluated for their affinities for rabbit thymus dThd kinase. Both analogues were weakly inhibitory (K_i = 400–600 μM) against the enzyme with respect to dThd phosphorylation, but nonetheless comparative with their preferences for human T-lymphoblast (Molt/4F) dThd kinase (K_i = 580 μM).[18] By contrast, ddCyd and ddeCyd showed greater affinities for dCyd kinase of rabbit thymus origin than for enzyme of Molt/4F cells (Table II). This observation is in agreement with the comparison of K_m values for dCyd (noted above) with kinase from the two sources.

On the basis of these findings together with the observation that dThd^S is a reasonably good inhibitor of phosphorylation, as mediated by rabbit thymus dThd kinase and as estimated by its K_i value, it seemed reasonable to utilize this same source of enzyme to evaluate the inhibitory effects of the modified thiated pyrimidine nucleosides on dThd phosphorylation. Only N_3ddThd^S (K_i = 54 μM) showed moderate affinity for this dThd kinase, which is approximately 8-fold greater than the value (K_i = 7.0 μM) obtained for N_3ddThd with enzyme of the same source. The latter value, incidentally, is not markedly different from a K_i of 2 μM obtained with this analogue for the inhibition of dThd phosphorylation mediated by dThd kinase obtained from H9 cellular extracts.[24]

Both ddThd^S and ddeThd^S were only weakly inhibitory (K_i = 1600–1700 μM) to phosphorylation by rabbit thymus dThd kinase. Like ddUrd and ddeUrd, little inhibitory activity against dThd kinase could be detected for ddUrd^S and ddeUrd^S. The latter findings were not unexpected in view of the fact that ddUrd showed no inhibitory activity against Molt/4F dThd kinase.[18] This analogue and, as well, ddeUrd showed a similar lack of inhibitory activity against the rabbit thymus enzyme (Table II).

The generally weak anti-HIV activities observed with the modified thiated pyrimidine nucleosides are consistent with correspondingly low susceptibilities to dThd kinase phosphorylation as the activating enzyme. However, ddCyd, which shows comparably poor alternate substrate activity for phosphorylation by, for example, Molt/4F dCyd kinase,[18,22] is a potent inhibitor of the cytopathogenicity of HIV.[18] Clearly, the phosphorylation of 5′-monophosphate derivatives to their respective 5′-triphosphates must also be considered as responsible for differences in the in vitro anti-HIV effects of N_3ddThd and ddeThd vis a vis the corresponding sulfur analogues.[22]

Experimental Section

General Methods. Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. ^{1}H NMR spectra were obtained with a Nicolet QE-300 FT spectrometer. Mass spectra were obtained with a Kratos MS-80 RFA under the following conditions: source temperature, 300 °C; resolution, 1000; thermal vaporization, 0–400 °C; ionization potential under EI, 70 eV; under positive CI, 18 eV; reagent gas under positive CI, isobutane; thermal spray spectra were obtained with a Finnigan 4000/Veste thermospray instrument using 1:1 CH_3CN–NH_4OAc (0.1 M) as solvent; flow rate, 1.4 mL/min. Partition coefficients were determined in 1-octanol–0.1 M sodium phosphate (pH 7.0).[26]

4-Thiothymidine (1). The preparation of 1, described by Fox et al.,[9] provides the product in the form of a glass which is "at least 85% pure". An improved preparation is described which provides 1 in crystalline form and of correct elemental composition.

A mixture of 10 g of 3′,5′-dibenzoylthymidine[9] (22 mmol) and 14.5 g of P_4S_{10} (33 mmol) in 100 mL of pyridine, containing 0.1% of H_2O, was gently refluxed for 1.5 h. The reaction mixture was poured into 400 mL of ice–water and the product was collected by filtration. The solid was stirred at room temperature for 2 h with 200 mL of ethanol containing 2 g (45 mmol) of NaOH. The solution was neutralized with acetic acid and concentrated under vacuum. The dried solid was chromatographed on silica gel with ethyl acetate–hexanes (6:4) as eluent to give 3.1 g (30%) of crystalline material: mp 125–7 °C; ^{1}H NMR (DMSO) δ 1.949 (s, 3 H, CH_3), 2.12 (m, 1 H, $H_{2'}$), 3.57 (m, 1 H, $H_{4'}$), 3.60 (m, 1 H, $H_{5'}$), 3.78 (m, 1 H, $H_{5'}$), 4.23 (m, 1 H, $H_{3'}$), 5.50 (br s, 1 H, OH), 6.09 (t, 1 H, $H_{1'}$), 7.866 (s, 1 H, H_6), 12.60 (br s, 1 H, NH). Anal. ($C_{10}H_{14}N_2O_4S$) C, H, N, S.

3′-Azido-3′-deoxy-4-thiothymidine (3). A mixture of 6.86 g of 1 (26 mmol) and 9.0 g of trityl chloride (32 mmol) in 50 mL of anhydrous pyridine containing 100 mg of DMAP was heated at 80 °C for 30 min. To the reaction mixture cooled to 0 °C was added 2.43 mL (3.6 g) of mesyl chloride (31 mmol), and the solution was stored in a refrigerator overnight. The amber mixture was poured into 200 mL of ice–water and the solid was collected. This material, without further purification, was dissolved in 110

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mL of 90% ethanol containing 2.12 g of NaOH (53 mmol) and the mixture was refluxed for 2 h. The reaction mixture was cooled in an ice bath and acidified with 80% acetic acid. The precipitate was collected, washed thoroughly with water, and dried in vacuo: 3.02 g of crude 1-(5-*O*-trityl-2-deoxy-β-D-*threo*-pentofuranosyl)-4-thiothymine (**2**). An analytical sample of **2** was obtained by flash chromatography on silica gel (ethyl acetate–hexanes, 6:4, as the eluent): mp 137–9 °C; ^{1}H NMR ($CDCl_3$) δ 1.948 (s, 3 H, CH_3), 2.60 (m, 1 H, $H_{2'}$), 2.96 (m, 1 H, $H_{2'}$), 3.46 (m, 1 H, $H_{5'}$), 3.66 (m, 1 H, $H_{5'}$) 4.07 (m, 1 H, $H_{4'}$) 4.43 (m, 1 H, $H_{3'}$), 6.12 (q, 1 H, $H_{1'}$), 7.45 (m, 15 H), 7.663 (s, 1 H, H_6), 10.214 (br s, 1 H, NH). Anal. ($C_{29}H_{28}N_2O_4S$) C, H, N, S.

To a solution of 3 g of **2** (6 mmol) in 30 mL of anhydrous pyridine cooled to 0 °C was added 0.77 mL (1.14 g) of mesyl chloride (10 mmol) and the reaction mixture was kept at 0 °C overnight. The dark solution was poured into 200 mL of ice–water, and the dark brown solid was collected and dried in vacuo. The intermediate mesylate was dissolved in 50 mL of dry DMF and heated at 80 °C for 1 h with 0.7 g of LiN_3 (14 mmol). The solvent was removed in vacuo and the residual solid was heated with 80% acetic acid for 0.5 h. After evaporation of the solvent, the residue was chromatographed on silica gel (ethyl acetate–hexanes, 6:4) to give 0.81 g (11% overall yield from 1). An analytical sample of **3** was obtained on crystallization from EtOH: mp 82–5 °C; ^{1}H NMR ($CDCl_3$) δ 2.075 (s, 3 H, CH_3), 2.49 (m, 2 H, $H_{2'}$), 3.85 (m, 1 H, $H_{5'}$), 3.99 (m, 2 H, $H_{5'}$, $H_{4'}$), 4.40 (m, 1 H, $H_{3'}$), 6.064 (t, H, $H_{1'}$), 7.553 (s, 1 H, H_6), 10.20 (br s, 1 H, NH); UV (EtOH) λ max 340 nm (ε 18520), 242 (4000); IR (neat, cm^{-1}) 2100, 1685, and 1625; molecular ion expected 283.0739, found 283.0745. Anal. ($C_{10}H_{13}N_5O_3S$) C, H, N, S.

General Procedure for Benzoylation. The 2′,3′-unsaturated nucleosides **4a** and **4c** (2–3 mmol), dissolved in dry pyridine (10 mL), were treated with an equimolar amount of benzoyl chloride, and the mixture was kept at 0 °C for 24 h. The solution was poured into water (100 mL), and the mixture was extracted with CH_2Cl_2. The organic layer was washed with saturated aqueous $NaHCO_3$ (50 mL, 3×) and water (50 mL, 3×), and the solvent was removed in a rotary evaporator and dried in vacuo. Column chromatography on silica gel using ethyl acetate–hexanes (6:4) as the eluent provided an analytical sample.

5′-*O*-Benzoyl-2′,3′-didehydro-3′-deoxythymidine (4b): obtained in 85% yield; mp 151–3 °C; ^{1}H NMR (DMSO) δ 1.340 (s, 3 H, CH_3), 4.42 (q, 1 H, $H_{5'}$), 4.58 (q, 1 H, $H_{5'}$), 5.10 (t, 1 H, $H_{4'}$), 6.02 (m, 1 H, $H_{3'}$), 6.50 (m, 1 H, $H_{2'}$), 6.80 (m, 1 H, $H_{1'}$), 7.096 (s, 1 H, H_6), 7.51 (q, 2 H), 7.65 (m, 1 H), 7.91 (t, 2 H), 11.30 (s, 1 H, NH). Anal. ($C_{17}H_{16}N_2O_5$) C, H, N.

5′-*O*-Benzoyl-2′,3′-didehydro-2′,3′-dideoxyuridine (4d): obtained in 85% yield after further purification through preparative TLC using ethyl acetate–ethanol (8:2); mp 71–3 °C; ^{1}H NMR (DMSO) δ 4.46 (m, 2 H, $H_{5'}$), 5.11 (m, 1 H, $H_{4'}$), 5.16 (m, 1 H, $H_{3'}$), 6.03 (m, 1 H, $H_{2'}$), 6.51 (d, 1 H, H_6), 6.83 (d, 1 H, $H_{1'}$), 7.34 (d, 1 H, H_5), 7.52 (q, 2 H), 7.63 (m, 1 H), 7.91 (d, 2 H), 11.37 (s, 1 H, NH). Anal. ($C_{16}H_{14}N_2O_5$) C, H, N.

General Procedure for Reduction. To the 5′-O-benzoylated 2′,3′-unsaturated nucleosides **4a** and **4c** (3 mmol), dissolved in ethanol (150 mL), was added 10% Pd/C (150 mg), and the mixture was shaken with H_2 at 25 psi for 2 h. The suspension was filtered through a mixture of Celite/silica gel, the solvent removed under vacuum, and the solid chromatographed with ethyl acetate–hexanes (6:4) as eluent.

5′-*O*-Benzoyl-3′-deoxythymidine (6a): obtained in 85% yield; mp 57–9 °C; ^{1}H NMR (DMSO) δ 1.580 (s, 3 H, CH_3), 2.00 (m, 3 H, $H_{4'}$, $H_{2'}$), 4.40 (m, 4 H, $H_{3'}$, $H_{5'}$), 6.02 (t, 1 H, $H_{1'}$), 7.380 (s, 1 H, H_6), 7.49 (m, 2 H), 7.63 (m, 1 H), 7.94 (d, 2 H), 11.30 (s, 1 H, NH). Anal. ($C_{17}H_{18}N_2O_5$) C, H, N.

5′-*O*-Benzoyl-2′,3′-dideoxyuridine (6b): obtained in 75% yield; mp 81–3 °C (softens at 65 °C); ^{1}H NMR (DMSO) δ 2.50 (m, 3 H, $H_{4'}$, $H_{2'}$), 4.50 (m, 4 H, $H_{3'}$, $H_{5'}$), 5.50 (d, 1 H, H_6), 5.70 (d, 1 H, H_5), 6.05 (m, 1 H, $H_{1'}$), 7.43 (m, 2 H), 7.61 (m, 1 H), 8.06 (m, 2 H), 9.98 (s, 1 H, NH). Anal. ($C_{16}H_{16}N_2O_5$) C, H, N.

General Procedure for Thiation. The benzoylated nucleoside (15 mmol) was dissolved with heating and stirring in toluene (400 mL) and Lawesson's reagent (16 mmol) was added all at once. The solution was stirred at 80° C for 12 h under N_2, cooled, and filtered through a plug of silica gel, and the product then was eluted with ethyl acetate–hexanes (6:4).

5′-*O*-Benzoyl-2′,3′-didehydro-3′-deoxy-4-thiothymidine (5a): obtained in 94% yield; mp 173–4 °C; ^{1}H NMR ($CDCl_3$) δ 1.689 (s, 3 H, CH_3), 4.62 (m, 2 H, $H_{5'}$), 5.20 (m, 1 H, $H_{4'}$), 5.98 (m, 1 H, $H_{3'}$), 6.41 (m, 1 H, $H_{2'}$), 6.95 (m, 1 H, $H_{1'}$), 7.173 (s, 1 H, H_6), 7.45 (q, 2 H), 7.60 (m, 1 H), 7.99 (t, 2 H),11.076 (s, 1 H, NH). Anal. ($C_{17}H_{16}N_2O_4S$) C, H, N, S.

5′-*O*-Benzoyl-2′,3′-didehydro-2′,3′-dideoxy-4-thiouridine (5c): obtained in 90% yield; mp 75–7 °C; ^{1}H NMR ($CDCl_3$) δ 4.60 (m, 2 H, $H_{5'}$), 5.18 (m, 1 H, $H_{4'}$), 5.90 (m, 1 H, $H_{3'}$), 5.99 (m, 1 H, $H_{2'}$), 6.39 (d, 1 H, H_6), 6.95 (d, 1 H, $H_{1'}$), 7.18 (d, 1 H, H_5), 7.47 (q, 2 H), 7.58 (m, 1 H), 7.95 (d, 2 H), 11.097 (s, 1 H, NH). Anal. ($C_{16}H_{14}N_2O_4S$) C, H, N, S.

5′-*O*-Benzoyl-3′-deoxy-4-thiothymidine (7a): obtained in 85% yield; mp 125–7 °C; ^{1}H NMR (DMSO) δ 1.791 (s, 3 H, CH_3), 2.00 (m, 3 H, $H_{4'}$, $H_{2'}$), 4.45 (m, 4 H, $H_{3'}$, $H_{5'}$), 5.96 (t, 1 H, $H_{1'}$), 7.53 (m, 3 H, 2 arom + H_6), 7.66 (m, 1 H), 7.96 (m, 2 H). Anal. ($C_{17}H_{18}N_2O_4S$) C, H, N, S.

5′-*O*-Benzoyl-2′,3′-dideoxy-4-thiouridine (7c): obtained in 75% yield; mp 80–2 °C (softens at 65 °C); ^{1}H NMR (DMSO) δ 2.50 (m, 3 H, $H_{4'}$, $H_{2'}$), 4.50 (m, 4 H, $H_{3'}$, $H_{5'}$), 6.02 (m, 1 H, $H_{1'}$), 6.15 (d, 1 H, H_6), 6.28 (d, 1 H, H_5), 7.56 (m, 2 H), 7.67 (m, 1 H), 7.95 (m, 2 H). Anal. ($C_{16}H_{16}N_2O_4S$) C, H, N, S.

General Procedure for Hydrolysis of Benzoate Esters. The 5′-O-benzoylated nucleoside was stirred at room temperature with an excess of aqueous 1 N NaOH for 6 h. After acidification with acetic acid (80%), the solvent was removed in vacuo. The solid residue was dissolved in EtOH and filtered through a plug of silica gel. After removal of the solvent, the solid was purified by flash column chromatography using ethyl acetate–EtOH (8:2) as the eluent to provide the analytical sample.

2′,3′-Didehydro-3′-deoxy-4-thiothymidine (5b): obtained in 80% yield; mp 128–30 °C (lit.[11] mp 129–30 °C).

2′,3′-Didehydro-2′,3′-dideoxy-4-thiouridine (5d): obtained in 60% yield after preparative TLC (ethyl acetate–ethanol, 8:2); mp 85–7 °C; ^{1}H NMR (DMSO) δ 3.55 (m, 2 H, $H_{5'}$), 4.78 (m, 1 H, $H_{4'}$), 5.92 (m, 1 H, $H_{3'}$), 6.26 (d, 1 H, H_6), 6.39 (d, 1 H, $H_{2'}$), 6.753 (s, 1 H, $H_{1'}$), 7.62 (d, 1 H, H_5); UV (EtOH) λ max 334 nm (ε 20050), 240 (3850). Anal. ($C_9H_{10}N_2O_3S$) C, H, N, S.

3′-Deoxy-4-thiothymidine (7b): obtained in 86% yield; mp 103–5 °C; ^{1}H NMR (DMSO) δ 1.950 (s, 3 H, CH_3), 2.20 (m, 4 H, $H_{2'}$, $H_{3'}$), 3.60 (m, 2 H, $H_{5'}$), 4.04 (m, 2 H, $H_{4'}$), 5.11 (s, 1 H, OH), 5.88 (m, H, $H_{1'}$), 8.046 (s, 1 H, H_6); UV (EtOH) λ max 334 nm (ε 20520), 240 (3900). Anal. ($C_{10}H_{14}N_2O_3S$) C, H, N, S.

2′,3′-Dideoxy-4-thiouridine (7d): obtained in 70% yield; mp 66–8 °C; ^{1}H NMR (DMSO), 2.00 (m, 4 H, $H_{3'}$, $H_{2'}$), 3.38 (m, 2 H, $H_{5'}$), 4.39 (m, 1 H, $H_{4'}$), 4.794 (s, 1 H, OH), 5.91 (m, 1 H, $H_{1'}$), 6.26 (d, 1 H, H_6), 7.49 (d, 1 H, H_5); UV (EtOH) λ max 330 nm (ε 19900), 245 (4010). Anal. ($C_9H_{12}N_2O_3S$) C, H, N, S.

Partitioning. Nucleosides were partitioned between the phases of a mixture containing equal volumes of 1-octanol and 100 mM sodium phosphate, pH 7.0. The phases were separated by centrifugation, and drug concentration in each phase was determined by absorbance measurements with the wavelength determined from spectral studies.[26]

Enzyme Assays. Rabbit thymus acetone powder was homogenized in 100 volumes of 50 mM HEPES buffer, pH 7.2, containing 1 mM mercaptoethanol. Particulate matter was removed by centrifugation and the supernatant fluid mixed with 0.02 volume of 10% streptomycin to precipitate nucleic acids. These were removed by centrifugation, and the supernatant fluid was used as the source of both dThd and dCyd kinases.

K_i values for dThd, ddThd, ddeThd, and their 4-thio analogues were determined as described in ref 26. The substrate was [2-^{14}C]-dThd (50 mCi/mmol). Reactions were carried out in a 50-μL volume containing 50 mM HEPES buffer, pH 7.2, 3 mM $MgCl_2$, 0.1–20 labeled Thd, and 10–2000 μM levels of inhibitors. A similar reaction mixture was used to assess K_i values for dCyd, ddCyd, and ddeCyd, except that the labeled substrate was [2-^{14}C]-dCyd (25 mCi/mmol). After 30-min incubations, 25-μL portions of the reaction mixture were applied to 24-cm circles of DEAE-impregnated paper, and the unreacted labeled nucleosides were washed out with 10 mM ammonium formate. Radioactivity remaining on the disks corresponding to nucleosides was measured by liquid scintillation counting.

Anti-HIV Drug Testing System. The procedure used in the National Cancer Institute's assay basically involves the killing

of T4 lymphocytes by HIV. Small amounts of HIV are added to cells, and at least two complete cycles of virus reproduction are necessary to obtain the required cell killing. Agents which interact with virions, cells, or virus gene products to interfere with viral activities will protect cells from cytolysis. While every attempt is made to reduce variability, precise values for EC_{50} and IC_{50} are not possible. All tests are compared with a positive (AZT-treated) control done at the same time under identical conditions.

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Phosphoramidate Peptide Inhibitors of Human Skin Fibroblast Collagenase

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An extensive series of *N*-(monoethylphosphoryl)peptides was synthesized and their inhibition of purified human skin fibroblast collagenase examined. At the cleavage site S_1 all reported compounds have the (EtO)(OK)P(O) group and the peptide side chain extended toward the C-terminal end (up to P_5') of the substrate sequence. These phosphoramidates with a tetrahedrally hybridized phosphorus atom are thought to be transition state analogue inhibitors. They exhibited fair inhibitory potency against this vertebrate collagenase having K_i values in the micromolar range. The most potent of these, (EtO)(OK)P(O)-Ile-TrpNHCH$_3$ (**68**), inhibits with a K_i value of 1.5 μM and is nearly 100 times stronger than (EtO)(OK)P(O)-Ile-Ala-GlyOK (**51**) (K_i of 140 μM), which has the sequence matching that of the α_1(I) chain of collagen in P_1', P_2', P_3' after the cleavage site. Several compounds were prepared in an attempt to identify the nature of the S_2', S_3', and S_4' binding sites. Alanine at the P_2' position was replaced by leucine, phenylalanine, tryptophan, or tyrosine derivatives, resulting in K_i values in a significantly lower range, 1.0–40 μM, compared to **51**. No upper size limitation or specificity has been found at this position, yet similar replacements at the P_3' position, which is occupied naturally by a glycine residue, gave weaker inhibitors: (EtO)(OK)P(O)-Ile-Tyr(OBzl)-PheOK (**57**) had a K_i of 120 μM. Hexapeptide derivatives had weaker activities in the 270 μM–2 mM range. All inhibitors were evaluated by using the synthetic thio peptolide spectrophotometric assay.

Design of synthetic collagenase inhibitors is hindered not only because the three-dimensional structure of the enzyme is presently unknown but also because details of the interaction between collagen[1-6] and collagenase[7-13] have not been identified. Most synthetic substrates designed for collagenases have K_m values about 3 orders of magnitude greater than the K_m for collagen.[14-17] This suggests that simple peptides intended as substrates or inhibitors may not have a conformation that is complementary to the enzyme binding site.

A number of inhibitors of mammalian collagenases based on the natural substrate structure have been developed.[18] Peptides containing the thiol,[19] thiol with a methylene spacer,[20,21] *N*-carboxymethyl,[22,23] or hydroxamate[24] ligand group were introduced, giving compounds with activities generally below 10 μM. The best was a hydroxamate analogue of a tripeptide with a K_i of 5 nM[18] for synovial

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