2-Thio Derivatives of dUrd and 5-Fluoro-dUrd and Their 5'-Monophosphates: Synthesis, Interaction with Tumor Thymidylate Synthase, and *in Vitro* Antitumor Activity

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A convenient synthesis of 5-fluoro-2-thiouracil (11) is based on hydrolytic deamination of 5-fluoro-2-thiocytosine (9). Lewis acid-catalyzed condensation of di-TMS-5-fluoro-2-thiouracil (13) or di-TMS-2-thiouracil (14) with 2-deoxy-3,5-di-*O*-*p*-toluyl-D-ribofuranosyl chloride (15) led to mixtures of the β- and α-anomers of 3',5'-toluylated 2'-deoxy-5-fluoro-2-thiouridine (16 and 18) or 2'-deoxy-2-thiouridine (17 and 19), each of which was deblocked with MeOH–NH_3 to give the desired free anomeric nucleoside pairs 1, 5 and 3, 7, respectively. These were selectively converted to the corresponding 5'-monophosphates 2, 6 and 4, 8, with the aid of the wheat shoot phosphotransferase system. Conformations of the nucleosides 1, 3, 5, 7 are deduced from ^{1}H NMR spectra, and circular dichroism spectra for nucleotide anomeric pairs 2, 6 and 4, 8 are reported. Whereas β-2-thio-dUMP (4) was a good substrate ($K_m \approx 10^{-5}$ M), β-5-fluoro-2-thio-dUMP (2) proved to be a potent competitive, slow-binding inhibitor ($K_i \approx 10^{-8}$ M) of the purified enzymes from Ehrlich ascites carcinoma and L1210 cells. The α-anomer 6 was a weak inhibitor, with K_i in the mM range, and its congener 8 hardly interacted with the enzyme. The β-anomer 1 exhibited antitumor activity in a mouse leukemic cell line L5178Y ($IC_{50} \approx 10^{-6}$ M), hence 40–100-fold weaker than 5-fluoro-dUrd. Its α-anomer 5 was 10-fold less active, but exhibited at least 10-fold higher selectivity with respect to the tumor cells than the β-anomer 1.

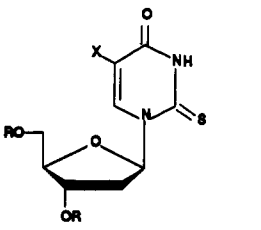
Thymidylate synthase (EC 2.1.1.45) catalyzes methylation of dUMP with concomitant conversion of N^5,N^{10}-methylenetetrahydrofolate to dihydrofolate.[1] The sole reported modification of the pyrimidine base of dUMP, with retention of substrate activity, is replacement of the C(4)-oxygen by sulfur to give 4-thio-dUMP.[2] Similar modification of 5-fluoro-dUMP (FdUMP), the substrate analogue which is a potent inhibitor of the enzyme and an active antitumor and antifungal chemotherapeutic agent,[3,4] led to 5-fluoro-4-thio-dUMP (S^4FdUMP), a strong slow-binding inhibitor of the enzyme from mammalian sources;[5] while 5-fluoro-4-thio-dUrd (S^4FdUrd) proved to be an effective inhibitor of proliferation of several mammalian tumor cell lines.[6]

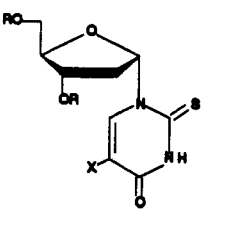
The foregoing pointed to the utility of examining the substrate and inhibitory properties of the hitherto unknown 2-thio derivatives of dUMP and FdUMP and their α-anomers, as well as the influence of the corresponding parent nucleosides on the growth of normal and tumor cell lines.

Chemistry

Although a large number of analogues of FdUMP have been synthesized, in efforts to obtain more effective antitumor agents as well as to facilitate studies on the mechanism of inhibition of thymidylate synthase, no attention appears to have been devoted to 2-thio derivatives of 5-fluoro-dUrd (1) and its 5'-phosphate (2). It is, in fact, surprising that the synthesis of 2-thio-dUMP (4) has not been reported. The parent nucleoside 2'-deoxy-2-thiouridine (3) was obtained by a biochemical procedure,

Chart I





via glycosylation of 2-thiouracil (12) with the aid of thymidine phosphorylase,[7,8] but with only superficial characterization of the product. The α-anomers 5–8 are consequently also unknown.

The foregoing is due to several factors which limit a direct approach to the synthesis of 2'-deoxy-5-fluoro-2-thiouridine (1). Thiation of uracil nucleosides at C(2) or C(2), C(4) requires elevated temperatures not applicable to deoxynucleosides because of the lability of the glycosidic bond. Attempted thiation of thymidine with P_2S_5 in refluxing tetralin led to thymine as the sole product.[9] Thiation based on the action of H_2S on 5',O_2-anhydronucleosides[10,11] led, in our hands, to a mixture of compounds including defluorinated product, as previously noted in attempts to obtain 5-fluoro-2-thio-ara-C by treatment of 2,2'-anhydro-5-fluoro-ara-C with H_2S.[12] These negative results pointed to the need for some condensation procedure. The starting aglycon for such condensation, 5-fluoro-2-thiouracil (11), hitherto prepared by ring closure with the use of the highly toxic ethyl fluoroacetate,[13,14] was now obtained by acid-catalyzed deamination of 5-fluoro-2-thiocytosine[15] (9) (Scheme I) in

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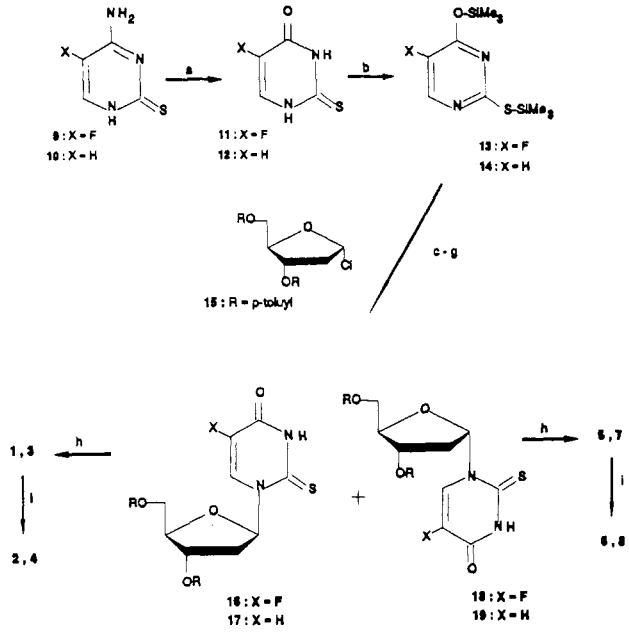
Scheme I[a]



[a] Reagents: (a) 1 N HCl, 100 °C, 24 h; (b) HMDS/TCS, 1,4-dioxane, reflux; (c) **13**, TMS triflate/CH_2Cl–CH_2Cl, 10% yield, **16**:**18** = 1:3; (d) **13**, $SnCl_4$/CH_2Cl–CH_2Cl, 30% yield, **16**:**18** = 1:2; (e) **13**, $TiCl_4$/CH_2Cl–CH_2Cl, 70% yield, **16**:**18** = 3:1; (f) **13**, $TiCl_4$/CH_3CN, 40% yield, **16**:**18** = 1:4; (g) **14**, $TiCl_4$/CH_2Cl–CH_2Cl, 60% yield, **17**:**19** = 4:1; (h) NH_3/MeOH, 20 °C; (i) *p*-nitrophenyl phosphate/wheat shoot phosphotransferase, 37 °C.

a single step in 64% yield. The same procedure converted 2-thiocytosine (**10**) to 2-thiouracil (**12**) in 50% yield. Initial trials at condensation of the di-TMS derivative of 5-fluoro-2-thiouracil (**13**) with 2-deoxy-3,5-di-*O*-*p*-toluyl-α-D-ribofuranosyl chloride (**15**) in 1,2-dichloroethane in the presence of TMS triflate, a weak Lewis acid, resulted in only a low yield (≈10%) of a mixture of the blocked nucleoside anomers **16** and **18**. Replacement of the triflate with stronger Lewis acids gave, with $SnCl_4$, about 30% of desired products, and with $TiCl_4$ about 70% yield. In the latter case, the use of a more polar solvent (acetonitrile) decreased the yield to 45%. The ratio of the β- to the α-anomer in the moderately polar dichloroethane solvent increased with the strength of the Lewis acid catalyst, being 1/3 with TMS-triflate, 1/2 with $SnCl_4$, and 3/1 with $TiCl_4$. Replacement of dichloroethane by acetonitrile decreased not only the overall yield, but also the ratio β/α. It was long ago reported that condensation of the di-TMS congener of 2-thiothymine with the chloride **15** in benzene, and $AgCl_4$ as catalyst, led to formation predominantly of the α-anomer (β/α ≈ 1/2).[16] It is clear that, in our case, changing both the solvent and catalyst may significantly increase the yield of the β-anomer. Similar results were obtained in the condensation reaction with the di-TMS derivative of 2-thiouracil (**14**), the overall yield for which was 60%, with β/α (**17**/**19**) = 4/1. The foregoing blocked mixtures of anomers, following separation by crystallization and TLC, were deblocked with methanolic ammonia to the corresponding β- and α-2′-deoxy-5-fluoro-2-thiouridines (**1** and **5**) and β- and α-2′-deoxy-2-thiouridines (**3** and **7**). These nucleosides were isolated in the form of hygroscopic glasses, which could not be crystallized. However, they were homogeneous in several solvent systems, with the expected precise masses on high-resolution LSIMS mass spectrometry (see the Experimental Section). Their NMR and pH-dependent UV spectra, as well as the CD spectra of their phosphorylated derivatives, were also fully consistent with their structures.

Table I. Solution Conformations of 2′-Deoxy-2-thiouridines Calculated from 500-MHz ^1H NMR Spectra[a]

	conformations								
compd	S	g+	t	g−	Ng+	Nt	Sg+	St	Sg−
1	0.59	0.56	0.32	0.12	0.28	0.13	0.29	0.19	0.12
5	0.87	0.40	0.41	0.19	0.08	0.05	0.32	0.36	0.19
3	0.60	0.52	0.37	0.11	0.25	0.15	0.27	0.22	0.11
7	0.90	0.42	0.40	0.19	0.06	0.04	0.35	0.36	0.19
FdUrd	0.63	0.52	0.36	0.12	0.24	0.13	0.28	0.23	0.12
22	0.84	0.43	0.40	0.17	0.09	0.06	0.34	0.34	0.17

[a] Spectra obtained in D_2O.

Our previous observation that S^4FdUrd is a good substrate for wheat shoot nucleoside phosphotransferase[5] prompted us to apply the same procedure to the 2-thio analogues. Such enzymatic phosphorylation of the β-nucleosides **1** and **3** led to the 5′-phosphates **2** and **4** in 60% yield, and was fully stereoselective (no 3′-phosphates or 3′,5′-diphosphates were formed), the products being quantitatively converted to the parent nucleosides by 5′-nucleotidase. However, the corresponding α-anomers **5** and **7** were poorer substrates, and were converted to the 5′-phosphates in only 15% yield; these were also dephosphorylated quantitatively, but at a much slower rate, by 5′-nucleotidase. Phosphorylation of the α-5-fluoro-dUrd (**22**) to α-5-fluoro-dUMP (**23**) by this procedure was negligible, and this compound was phosphorylated chemically with $POCl_3$ in triethyl phosphate. Since this procedure occasionally gives small admixtures of the 3′-phosphate and 3′,5′-diphosphates,[17] the product was isolated by TLC and shown to be hydrolyzed, albeit very slowly, by 5′-nucleotidase.

^1H NMR and CD Spectra

The conformations and configurations of the nucleosides **1**, **3**, **5**, **7**, and **22** were deduced from the ^1H NMR (500 MHz) spectra, the data for which (see the Experimental Section) are consistent with the proposed structures. In the ^1H NMR spectra, the change in location of the anomeric proton on going from the β- to the α-anomer, resulting from the change in orientation of the pentose ring with respect to the aglycon ring currents, is the source of the shifts of the H(2′) and H(4′) signals to lower field (0.4 ppm), and H(2″) to higher field (0.3 ppm). The lower vicinal coupling constants $J_{1',2''}$ for the α-anomers are due to the virtual cisoidal conformation of the H(1′)–C(1′)–C(2′)–H(2″) fragment and permit unequivocal assignments of the anomeric H(1′). Pseudorotational analysis (Table I), based on measurements of $J_{3',4'}$, pointed to enhanced stabilization of the S form of the pentose ring of the α-anomers, with no significant change in conformation of the exocyclic CH_2OH. In the case of the β-anomers the form S (2′-*endo*) was only slightly favored over the form N (3′-*endo*).

The CD spectra of the nucleotide anomeric pairs **2**, **6** and **4**, **8** are, as might be anticipated, virtually mirror images of each other (Figure 1). The β-anomers **2** and **4** exhibit a strong positive Cotton effect for the principal B_{2u} band (270–272 nm), while their α-anomers **6** and **8** display a comparable, negative effect at these wavelengths. A characteristic feature of these nucleotides is, apparently, the additional long-wavelength (320–322 nm) weaker n-π* band with a sign opposite to that of the principal B_{2u} band.

It should be noted that **3** (pK_a = 8.0 for dissociation of

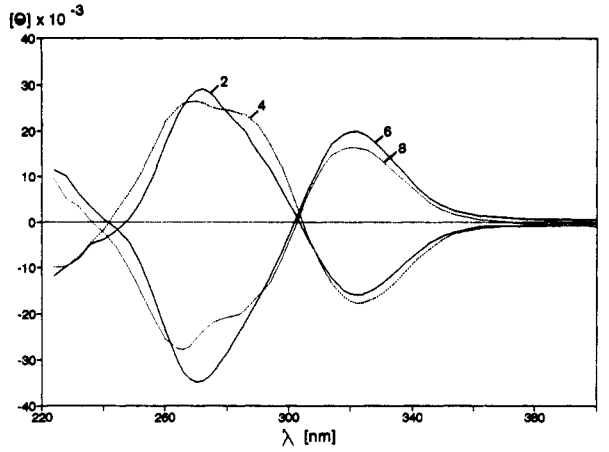


Figure 1. CD spectra of β- and α-anomers of 2-thio-FdUMP (2 and 6) and 2-thio-dUMP (4 and 8) in 0.05 M acetate buffer, at pH 4 (neutral form).

the ring N-3 proton) and 1 (pK_a = 6.5), and hence also their nucleotides, contain aglycons with markedly enhanced acidities relative to the parent compounds dUrd (pK_a = 9.3) and FdUrd (pK_a = 7.8) and their nucleotides. This is due to the weaker distribution of charge in the thiouracil residues, related to their lower dipole moments and resultant poorer ability to pair with a complementary base such as adenine.[18] A similar situation prevails for the corresponding 4-thio congeners, i.e. 2'-deoxy-4-thiouridine (pK_a = 8.3) and S⁴FdUrd (pK_a = 6.8).

Biological Results

Interaction with Thymidylate Synthase. Spectrophotometric monitoring at 340 nm of the reaction mixture with β-2-thio-dUMP (4) revealed it to be a good substrate for the enzyme from both Ehrlich carcinoma and L1210 cell lines. The dependence of reaction velocity, determined spectrophotometrically by monitoring A_{340} in the presence of 0.5 mM (6*RS*,α*S*)-N^5,N^{10}-methylenetetrahydrofolate (CH_2FH_4), on substrate concentration (varied within the ranges 4–83 μM for β-2-thio-dUMP and 4–33 μM for dUMP) was analyzed by means of Lineweaver–Burk plots. With the enzyme from both sources, apparent K_m values for β-2-thio-dUMP and dUMP were 0.02 and 0.01 mM, respectively, while V_{max} was the same with both substrates (not shown).

With the Ehrlich carcinoma enzyme, no time-dependent increase of A_{340} was observed with 0.15 mM α-2-thio-dUMP (8), pointing to the need of the β-anomeric structure for substrate activity, previously demonstrated for dUMP.[19] Nonetheless, when the enzyme-catalyzed release of tritium from [5-³H]dUMP (20 μM) was followed, 0.3 mM α-2-thio-dUMP caused 60% inhibition, indicating feeble interaction of the analogue with the enzyme.

Inhibition of both enzymes by β-5-fluoro-2-thio-dUMP (2) was examined by varying the dUMP concentration with different concentrations of inhibitor, added simultaneously to the reaction mixture. Competitive inhibition, reflected by intersection at the ordinates of Lineweaver–Burk plots (not shown), led to apparent K_i values 3-fold and 14-fold higher than those found for FdUMP with the enzymes from L1210 and Ehrlich carcinoma cells, respectively (Table II). Similarly, α-5-fluoro-2-thio-dUMP (6) behaved as a very weak competitive inhibitor *vs* dUMP, with an apparent K_i of 1.6 mM.

β-5-Fluoro-2-thio-dUMP (2), when preincubated with the enzyme from both cell lines, in the presence of CH_2FH_4, led to time-dependent inactivation (Figure 2), consistent with its being a slow-binding inhibitor.[20] The inactivation rate plots were biphasic (Figure 2) and similar to these obtained with some other dUMP analogues,[21] suggesting differing interactions of the inhibitor with the two known binding sites on the enzyme molecule. Consequently, inhibition constants and inactivation rate constants, calculated for initial and later phases separately, are K_i' and k_2', and K_i'' and k_2'', respectively (see Table III).

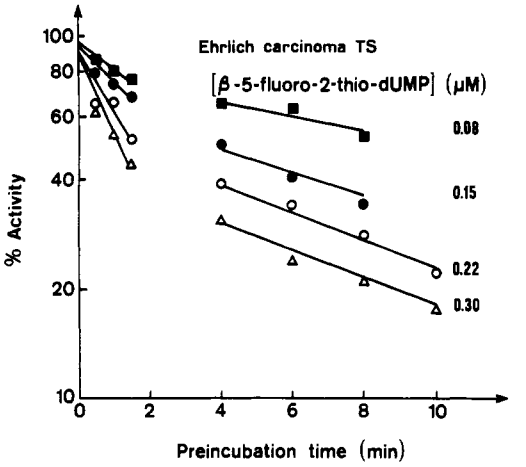


Figure 2. Time-dependent inactivation of Ehrlich carcinoma thymidylate synthase by β-2-thio-FdUMP.

Table II. Apparent K_i Values for Competitive Inhibition of Thymidylate Synthases from Ehrlich Carcinoma and L1210 Cells by FdUMP and 2-Thio-FdUMP (2)

	K_i (μM)	
analogue	Ehrlich carcinoma	L1210
FdUMP	0.01[a]	0.02[b]
2	0.14	0.07

[a] Jastreboff *et al.*[26] [b] Rode *et al.*[21]

Table III. Parameters for Inactivation of Ehrlich Carcinoma and L1210 Thymidylate Synthases by β-Anomers of FdUMP and 2-Thio-FdUMP (2)

analogue	K_i' (nM)	K_i'' (nM)	k_2' (min⁻¹)	k_2'' (min⁻¹)
		Ehrlich Carcinoma Enzyme		
FdUMP[a]	5.5	71	0.18	0.17
2	79	49	0.36	0.06
		L1210 Enzyme		
FdUMP[b]	1.8 ● 0.4(6)[c]	20 ● 5(4)	0.17 ± 0.02(6)	0.12 ± 0.04(5)
2	41 ± 9(3)	46 ± 25(3)	0.12 ± 0.02(3)	0.02 ± 0.01(3)

[a] Rode *et al.*[24] [b] Rode *et al.*[21] [c] Results are presented as means ± SEM, followed by the number of separate experiments in parentheses.

Inhibition constants of 2 were in the range 10^{-8} M with both enzymes (Table III), hence an order of magnitude higher than for FdUMP[5,21] and slightly lower than for S⁴FdUMP.[5]

The α-anomers 6 and 23, when preincubated with the Ehrlich carcinoma enzyme and CH_2FH_4, also led to time-dependent inactivation, but, in contrast to the β-anomers, the plots of log (remaining activity) *vs* time were linear (Figure 3). This provides additional assurance that the α-anomers were not contaminated with traces of the corresponding β-anomers which might account for the observed inhibitory effect (considering the 10^4–10^6-fold higher K_i values describing inhibition by the α-anomers relative to the β-anomers).

2-Thio-dUMP (4) appears to be a somewhat better substrate ($K_m \approx 20$ μM) than its 4-thio congener with the

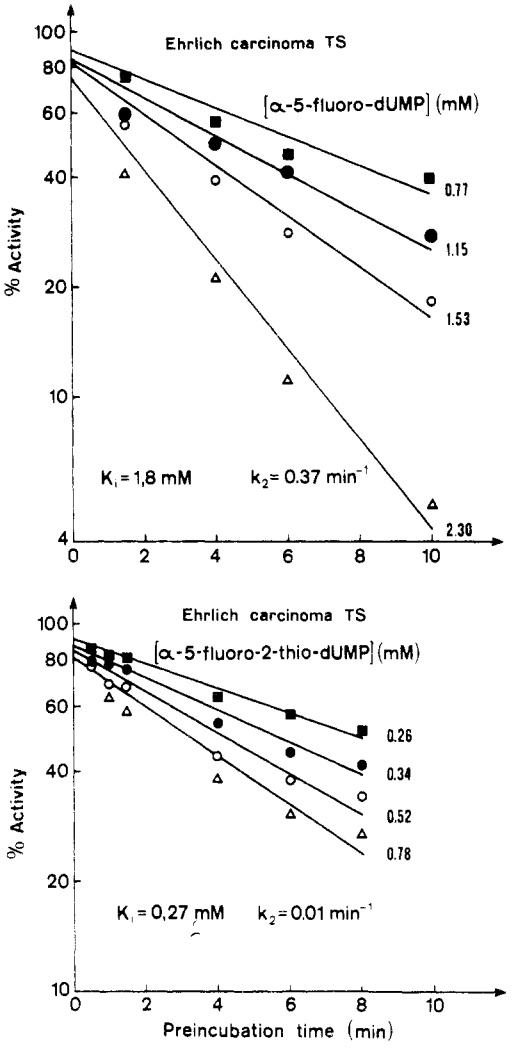


Figure 3. Time-dependent inactivation of Ehrlich carcinoma thymidylate synthase by α-anomers of FdUMP and 2-thio-FdUMP.

bacterial enzyme ($K_m \approx 70\,\mu M$).[2] A nondissociated N(3)-H was postulated to strengthen binding to the enzyme,[5] subsequently ascribed to the specificity of binding of the pyrimidine moiety to an asparagine residue.[22] The higher acidity of this proton in 2-thio-dUMP, relative to 4-thio-dUMP, should then result in weaker substrate properties, if binding of the pyrimidine were due solely to this effect. Hence the 2-thio analogue appears to possess some other advantage over the 4-thio analogue in interaction with the enzyme.

***In Vitro* Antitumor Activity.** β-5-Fluoro-2-thio-dUrd (**1**) was a good inhibitor of L5178Y and 3T3 cell growth, with IC_{50} values in the μM range, while β-FdUrd was a 10^2-fold stronger inhibitor of L5178Y, but not of 3T3, cell growth (Table IV).

The α-anomers **22** and **5** feebly inhibited growth of both cell lines, the effect being stronger with the tumor line and the difference between IC_{50} values, describing cell growth inhibition by α- and β-anomers, distinctly larger for the 5-fluoro- than for the 5-fluoro-2-thio- pairs of congeners (Table IV).

Conclusions

The presently described 2-thio, together with the previously described 4-thio, congeners of dUMP and FdUMP constitute an interesting class of substrates and inhibitors, respectively, of thymidylate synthase. 2-Thio-dUMP is a good substrate, its affinity for the enzyme being only 2-fold lower than that of dUMP, and 2-thio-FdUMP is a potent, competitive (*vs* dUMP) slow-binding inhibitor, only 1 order of magnitude weaker than FdUMP, and slightly more effective than S^4FdUMP.[5] The β-anomer of 2-thio-FdUrd exhibited marked antitumor activity, albeit 40–100-fold less effectively than FdUrd. Although, as expected, α-2-thio-FdUrd is a much weaker inhibitor of tumor cell growth, it is of interest that it is 10-fold more selective *vs* mouse tumor (L5178Y) than normal (3T3) cells.

Experimental Section

General Methods. Melting points (uncorrected) were measured on a Boetius microscopic hot stage; UV spectra were recorded on Cary 3 instrument using 10-mm-pathlength cuvettes, acetate buffers in the pH range 3–4, and phosphate buffers in the range 6–8.4. Extremes of pH made use of standard solutions of HCl and NaOH. A Cole-Parmer instrument with combination electrode was employed for pH measurements. CD spectra were obtained with an AVIV 62DS instrument, using 10-mm-pathlength cuvettes. High-resolution EI mass spectra for pyrimidines were carried out on a Finigan MAT spectrometer and liquid matrix secondary ion mass spectra (LSIMS) for nucleosides with an AMD-604 spectrometer. High-resolution ^{1}H NMR spectra were recorded on a Bruker 500-MHz instrument in D_2O with DSS as internal standard. All evaporations were under vacuum at 35 °C. TLC on Merck silica gel F_{254} glass plates (DC, 20 × 20 cm, 0.25 mm, No. 5715) made use of the following solvents (v/v): (A) $CHCl_3$–MeOH, 9:1; (B) C_6H_6–EtOAc, 8:2; (C) $CHCl_3$–Me_2CO, 96:4; (D) $C_6H_6CH_3$–$CHCl_3$–EtOAc, 8:1:1; (E) EtOAc–i-PrOH–H_2O, 75:16:9. TLC on Merck cellulose F glass plates (DC, 20 × 20 cm, 0.1 mm, No. 5718) made use of solvents: (F) i-PrOH–concentrated NH_4OH–H_2O, 7:1:2; (G) *n*-BuOH–Me_2CO–80% CH_3COOH–5% NH_4OH–H_2O, 35:25:15:15:10.

Biology. (a) Reagents. (6*RS*,α*S*)-Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid (Fluka, Buchs, Switzerland) as previously described[23] except that 2-mercaptoethanol (Serva, Heidelberg, GFR) was used in place of 2,3-dimercaptopropanol. [5-^{3}H]dUMP (≈15 Ci/mmol) from Amersham (Amersham, UK) was purified as previously described.[24] L-[^{14}C]leucine and [*methyl*-^{3}H]thymidine were from UVVR (Prague, Czechoslovakia). Fischer's and Dulbecco's media, fetal and newborn calf sera, sodium bicarbonate, glutamine, HEPES, trypsin, and agar were from GIBCO (Paisley, Scotland).

(b) Cell Lines. Mouse Ehrlich ascites carcinoma and leukemia L1210 cells were maintained, harvested, and stored as previously described.[25] L5178Y cells (mycoplasma-free with the GIBCO MycoTect test) were grown as a suspension in Fischer's medium, supplemented with 8% newborn calf serum and 0.01 M HEPES, in 5% CO_2 atmosphere at 37 °C (doubling time: 8 h). 3T3 mouse fibroblasts (mycoplasma-free) were grown as a monolayer in Dulbecco's medium, supplemented with 10% fetal calf serum and 2% glutamine, in a 5% CO_2 atmosphere at 37 °C (doubling time: 14 h).

(c) *In Vitro* Cell Growth Inhibition. The influence of each drug on cell viability, colony formation, and [^{14}C]leucine and [^{3}H]thymidine incorporation was followed, and IC_{50} values were determined. In the cell viability test, L5178Y and 3T3 cells were plated at densities of 5×10^5 and 10^5 cells/mL, respectively, grown for 4 h and exposed to drug (in five replicates for each drug concentration) for 48 h. Cells, stained with 0.02% trypan blue, were counted directly under an inverted light microscope. Each experiment was repeated three times. In the colony formation test, L5178Y and 3T3 cells were plated at densities of 5×10^6 and 10^6 cells/mL, respectively, grown for 4 h, exposed to drug (in triplicate for each drug concentration) for 48 h, harvested, and seeded in drug-free medium at a density of 800 cells/mL. The L5178Y cell cultures included 0.2% agar. After 5 days at 37 °C in a 5% CO_2 atmosphere, all cultures were stained with 2% crystal violet and colonies (≈0.25-mm diameter) counted. Each experiment was repeated three times. For L-[^{14}C]leucine incorporation,

Table IV. Inhibition of Cell Growth by 5-Fluoropyrimidine Deoxynucleosides

drug	IC_{50}[a] (μM)			
	growth assay	clonal assay	[^{14}C]Leu incorporation	[^{3}H]Thd incorporation
		L5178Y Cells		
FdUrd	0.025 ± 0.001	0.02 ± 0.002	0.009 ± 0.0	0.015 ± 0.001
1	2.00 ± 0.20	4.50 ± 0.40	1.0 ± 0.1	1.4 ± 0.1
22	5.0 ± 0.40	5.0 ± 0.50	2.0 ± 0.4	4.0 ± 0.4
5	25 ± 4	20.0 ± 2.5	10.0 ± 1.5	10.0 ± 4
		3T3 Cells		
FdUrd	1.0 ± 0.1	0.60 ± 0.02	3.2 ± 0.14	0.50 ± 0.03
1	4.0 ± 0.1	7.00 ± 0.5	5.0 ± 0.25	4.0 ± 0.1
22	150 ± 1.0	60.0 ± 4.8	250 ± 11	60.0 ± 0.4
5	400 ± 3.0	500 ± 21	2000 ± 100	100 ± 3

[a] IC_{50} is drug concentration required for 50% reduction in cell number, colonies formed, [^{14}C]Leu or [^{3}H]Thd incorporation.

L5178Y and 3T3 cells were plated and, after 4 h, exposed to drug as in the cell viability test; but, 24 h after addition of drug, 1 μM L-[^{14}C]leucine (2 Ci/mmol) was added, and after an additional 24 h, cells were harvested, washed twice with ice-cold 0.85% saline, and precipitated with ice-cold 10% TCA. The pellet was transferred to a glass microfiber filter (Whatman GF/C) and washed twice with 3-mL portions of 5% TCA and then three times with 2-mL portions of 1% TCA, and radioactivity was determined. Each experiment was repeated three times in duplicate. Thymidine incorporation was followed using 2 μM [^{3}H]thymidine (5 mCi/mmol) in place of L-[^{14}C]leucine.

(d) Thymidylate Synthase. Electrophoretically homogeneous preparations of the enzyme from Ehrlich carcinoma and L1210 cells were obtained as previously reported.[25,26]

(e) Enzyme Assays. [5-^{3}H]dUMP tritium release was determined in triplicate as previously described.[25] For substrate activity of 4, the analogue was substituted for dUMP in the enzyme reaction and absorbance at 338 nm monitored.[27] 4 and 2, or their α-anomers (8 and 6), were added to the reaction mixture as neutral aqueous solutions.

(f) Kinetic Studies. To identify the type of inhibition involved, the effect of 2 on the dependence of reaction rate on dUMP concentration, was analyzed by means of Lineweaver–Burk plots, as previously reported.[25] Thymidylate synthase inhibition by 2, leading to time-dependent inactivation of the enzyme, was analyzed as elsewhere described.[21,24]

Chemistry. 5-Fluoro-2-thiouracil (11). A sealed ampoule containing 725 mg (5 mmol) of 5-fluoro-2-thiocytosine (**9**)[15] in 25 mL 1 N HCl was heated for 20 h at 100 °C. The reaction mixture was brought to dryness several times from toluene, and the residue crystallized from methanol to obtain 467 mg (64%) of 11: mp 220–223 °C (lit.[14] mp 225 °C). R_f (A): 0.53. UV: λ_{max} (pH 2) 214.5 nm (ε 12 200), 273.5 nm (ε 13 400); λ_{max} (pH 7) 212 nm (ε 9500), 266.5 nm (ε 10 300); λ_{max} (pH 12) 217 nm (ε 13 700), 256.5 nm (ε 9600). MS: *m/z* (M^+ − H) calcd 144.98716, found 144.98533.

2-Thiouracil (12). This was obtained from 2-thiocytosine (**10**) by the procedure described for 11, above, followed by TLC isolation in solvent A, in 50% yield: mp 315 °C dec (lit.[29] mp 315 °C dec). UV: λ_{max} (pH 2) 274 nm (ε 13 800), λ_{max} (pH 7.4) 271 nm (ε 12 200). Lit.[30] λ_{max} (pH 2) 273.5 nm (ε 14 000), λ_{max} (pH 7.4) 270 nm (ε 12 400).

1-(2-Deoxy-3,5-di-*O*-*p*-toluyl-β-D-ribofuranosyl)-5-fluoro-2-thiouracil (16) and Its α-Anomer (18). To a suspension of 365 mg (2.5 mmol) of 5-fluoro-2-thiouracil (11) in 25 mL of 1,4-dioxane was added 10 mL of HMDS and 1 mL of TCS, and the mixture was heated under reflux for 20 h. The mixture was brought to dryness to give an oily residue, to which was added a solution of 970 mg (2.5 mmol) of 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluyl-α-D-ribofuranose (**15**) in 30 mL of dichloroethane. To the cooled solution was added 2.5 mL (2.5 mmol) of a 1 M solution of $TiCl_4$ in 1,2-dichloroethane. The course of the reaction was followed by TLC (silica gel) with solvents B and C. After 1 h the reaction mixture was diluted by addition of 30 mL of $CHCl_3$ and extracted with a saturated solution of $NaHCO_3$. The resulting suspension was filtered through Hyflo-Supercel, and the organic phase was washed with water, dried over anhydrous Na_2SO_4, and then brought to dryness. The residue was crystallized from ethanol to give 940 mg (75%) of a mixture of the anomers 16 and 18. Recrystallization yielded 375 mg (30%) of pure 16 as checked by TLC. The resulting filtrate, containing a mixture of 16 and 18, was subjected to preparative chromatography on silica gel with solvent D to give 188 mg each of 16 and 18. Total yield of 16, 563 mg (45%): mp 200–202 °C. R_f (B): 0.62, (C): 0.42, (D) 0.22. Anal. ($C_{25}H_{23}FN_2O_6S·H_2O$) C, H, N. 18: Yield of 188 mg (15%), mp 190–192°C. R_f (B): 0.70, (C): 0.51, (D): 0.30. Anal. ($C_{25}H_{23}FN_2O_6S·H_2O$) C, H, N.

1-(2-Deoxy-3,5-di-*O*-*p*-toluyl-β-D-ribofuranosyl)-2-thiouracil (17) and Its α-Anomer (19). 17 and 19 were obtained from 12 and 15 as described in the previous paragraph for 16 and 18. Yield of 17 41%, mp 175–177°C. R_f (D): 0.12. Anal. ($C_{25}H_{24}N_2O_6S·H_2O$) C, H, N. Yield of 19 10%, mp 97–99 °C. R_f (D): 0.17. Anal. ($C_{25}H_{24}N_2O_6S·H_2O$) C, H, N.

2'-Deoxy-5-fluoro-2-thiouridine (1). To 150 mg (0.3 mmol) of 16 was added 25 mL of methanol saturated with NH_3 at 0 °C, and the mixture was stirred overnight at room temperature. The mixture was brought to dryness, and the residue was dissolved in water and extracted with ether. The aqueous phase was concentrated under vacuum and subjected to preparative chromatography on silica gel with solvent A to yield 52 mg (66%) of 1. R_f (A): 0.33; (F): 0.72; (G): 0.85. UV: λ_{max} (pH 2) 219 nm (ε 16 300); 272.5 nm (ε 14 800); λ_{max} (pH 12) 239 nm (ε 20 800). ^{1}H NMR (D_2O): δ 2.30 (1 H, m, $J_{1'2''}$ = 6.39 Hz, 2'-H), 2.64 (1 H, m, $J_{1',2''}$ = 6.37 Hz, 2''-H), 3.83 (1 H, dd, $J_{4',5''}$ = 4.53 Hz, 5''-H), 3.91 (1 H, dd, $J_{4',5'}$ = 3.43 Hz, 5'-H), 4.11 (1 H, m, $J_{4',3'}$ = 4.07 Hz, 4'-H), 4.45 (1 H, m, $J_{2',3'}$ = 6.46 Hz, $J_{2'',3'}$ = 4.54 Hz, 3'-H), 6.89 (1 H, br t, 1'-H), 8.28 (1 H, d, 6-H). MS (LSIMS): m/z 263 ($M+H^+$).

α-2'-Deoxy-5-fluoro-2-thiouridine (5). This was obtained from 18 as described for 1 in the previous section to give 14 mg (66%) of 5. R_f (A): 0.33; (F): 0.74; (G): 0.85. UV: λ_{max} (pH 2) 219 nm (ε 16 300); 272.5 nm (ε 14 800); λ_{max} (pH 12) 239 nm (ε 20 800). ^{1}H NMR (D_2O): δ 2.28 (1 H, br d, $J_{1',2''}$ = 2.0 Hz, 2''-H), 2.85 (1 H, m, $J_{1',2'}$ = 6.83 Hz, 2'-H), 3.64 (1 H, a dd, $J_{4',5''}$ = 5.56 Hz, 5''-H), 3.72 (1 H, a dd, $J_{4',5'}$ = 4.03 Hz, 5'-H), 4.43 (1 H, m, $J_{2'',3'}$ = 2.0 Hz, $J_{2',3'}$ = 5.8 Hz, 3'-H), 4.56 (1 H, m, $J_{3',4'}$ = 2.0 Hz, 4'-H), 6.75 (1 H, br d, 1'-H), 8.11 (1 H, d, 6-H). MS (LSIMS): *m/z* 263 ($M+H^+$).

2'-Deoxy-2-thiouridine (3). This was prepared from 17 as described for 1, in the form of a hygroscopic glass, which could not be crystallized (yield 70%). R_f (A): 0.30; (E): 0.83; (G): 0.76. UV λ_{max} (pH 2) 216 nm (ε 18 100); 275 nm (ε 14 800); λ_{max} (pH 7) 218 nm (ε 15 100); 274 nm (ε 13 400); λ_{max} (pH 12) 239 nm (ε 20 700); 270 nm (ε 13 700). ^{1}H NMR (D_2O): δ 2.38 (1 H, m, $J_{1'2'}$ = 6.59 Hz, 2'-H), 2.70 (1 H, m, $J_{1',2''}$ = 6.28 Hz, 2''-H), 3.88 (1 H, a dd, $J_{4',5''}$ = 5.00 Hz, 5''-H), 3.97 (1 H, a dd, $J_{4',5'}$ = 3.37 Hz, 5'-H), 4.19 (1 H, m, $J_{4',3'}$ = 4.05 Hz, 4'-H), 4.52 (1 H, m, $J_{2',3'}$ = 6.62 Hz, $J_{2'',3'}$ = 4.93 Hz, 3'-H), 7.03 (1 H, t, 1'-H), 8.12 (1 H, d, 6-H). MS (LSIMS): *m/z* ($M+H^+$) for $C_9H_{13}N_2O_4S$ calcd 245.05960, found 245.05973.

α-2'-Deoxy-2-thiouridine (7). This was prepared from 19 as described for preparation of 1, to give a hygroscopic glass in 68% yield. R_f (A): 0.30; (E): 0.81; (G): 0.76. UV λ_{max} (pH 2) 215 nm (ε 18 100); 273 nm (ε 14 800); λ_{max} (pH 7) 217 nm (ε 15 300); 273 nm (ε 13 400); λ_{max} (pH 12) 238 nm (ε 20 700); 270 nm (ε 13 700). ^{1}H NMR (D_2O) δ 2.41 (1 H, br d, $J_{1',2''}$ = 1.80 Hz, 2''-H), 2.90 (1 H, m, $J_{1',2'}$ = 6.90 Hz, 2'-H), 3.72 (1 H, a dd, $J_{4',5''}$ = 5.42 Hz, 5''-H), 3.80 (1 H, a dd, $J_{4',5'}$ = 4.02 Hz, 5'-H), 4.51 (1 H, m, $J_{2'',3'}$ = 1.80 Hz, $J_{2',3'}$ = 5.93 Hz, 3'-H), 4.64 (1 H, br t, $J_{3',4'}$ = 1.80 Hz, 4'-H),

6.78 (1 H, br d, 1'-H), 8.09 (1 H, d, 6-H). MS (LSIMS): m/z ($M+H^+$) for $C_9H_{13}N_2O_4S$ calcd 245.05960, found 245.05968.

2'-Deoxy-5-fluoro-2-thiouridine 5'-Phosphate (2). To a solution of 5.2 mg (0.02 mmol) of 1 in 0.6 mL of 0.1 M acetate buffer pH 4 was added 112 mg (0.3 mmol) of *p*-nitrophenyl phosphate, and the pH was brought to 4 by addition of concentrated acetic acid. To this was added 0.6 mL of a crude extract of wheat shoot nucleoside phosphotransferase.[31] The mixture was incubated at 37 °C for 40 h, concentrated to half-volume, and extracted three times with ether. The aqueous layer was subjected to chromatography on Whatman paper 3MM with solvent F. The band with R_f 0.10 was eluted with water, and the eluate was brought to dryness to yield 3.3 mg (48%) of **2**. R_f (F): 0.10, (G): 0.56; CD (pH 4) [θ]: +29 200 (272 nm), −17 000 (320 nm). UV λ_{max} (pH 4) 220 nm, 275 nm (ϵ 14 800); λ_{max} (pH 8) 240 nm (ϵ 17 800).

Enzymatic Hydrolysis. To a solution of 40 μL of 0.1 M Tris/HCl buffer pH 8.8 + 20 μL of 0.1 M $MgCl_2$ was added 0.05 μmol of **2**, followed by 5 μL of a 10 mg/mL stock solution of *Crotalus adamantaeous* (EC 3.1.3.5) snake venom. Following a 1-h incubation at 37°C, **2** underwent 60% hydrolysis to the nucleoside 1, while 2'-deoxy-5-fluorouridine-5'-phosphate (5-FdUMP) was quantitavely converted to the nucleoside. An additional control, 2'(3')-GMP, was anaffected, pointing to the absence of nonspecific phosphatases.

α-2'-Deoxy-5-fluoro-2-thiouridine 5'-Phosphate (6). This was prepared from **5** in the same way as the β-anomer **2**, above, to give 1.7 mg (24%) of **6**. R_f (F): 0.08 and (G): 0.58; CD (pH 4) [θ]: −34 000 (270 nm), +19 000 (322 nm). UV: λ_{max} (pH 4) 220 nm (ϵ 14 800), 273 nm (ϵ 14 800); λ_{max} (pH 8) 238 nm (ϵ 17 800). The nucleotide **6** was converted to the parent nucleoside **5** by 5'-nucleotidase much more slowly than **2**, as expected. During a1-h incubation, **2** was hydrolyzed to the extent of 60%, but **6** only 5%. Following overnight incubation both **2** and **6** were quantitatively converted to the nucleosides.

2'-Deoxy-2-thiouridine 5'-Phosphate (4). This was prepared by enzymatic phosphorylation of **3**, as described for **2**, in 55% yield. R_f (G): 0.53; CD (pH 4) [θ]: +27 000 (270 nm), −17 500 (322 nm). UV: λ_{max} (pH 4) 220 nm (ϵ 18 100), 273 nm (ϵ 14 800). Compound **4** was quantitatively converted by 5'-nucleotidase to the parent nucleoside **3**, as described for **2**.

α-2'-Deoxy-2-thiouridine 5'-Phosphate (8). **8** was prepared from **7** as described for **2**, in 15% yield. R_f (G): 0.48; CD (pH 4) [θ]: −27 000 (268 nm), +16 500 (321 nm). UV: λ_{max} (pH 4) 220 nm (ϵ 18 100), 273 nm (ϵ 14 800). The compound **8** was hydrolyzed by 5'-nucleotidase to the parent nucleoside **7** quantitatively, but much more slowly than the β-anomer **4**.

1-(2-Deoxy-3,5-di-*O*-*p*-toluyl-β-D-ribofuranosyl)-5-fluorouracil (20) and Its α-Anomer (21). Both of these were obtained from 2,4-di-*O*-TMS-5-fluoropyrimidine and **15** by the procedure of Freskos.[32] Yield of **20**, 80%, mp 225–227°C (lit.[33] mp 229 °C). R_f (D): 0.05. Yield of **21**, 9%, mp 210–212°C (lit.[33] mp 214–215°C). R_f (D): 0.06.

α-2'-Deoxy-5-fluorouridine (22). This was obtained by treatment of **21** as in the procedure for **1** to yield 92 mg (69%), mp 150°C (lit.[33] mp 150–151 °C). R_f (A): 0.21. ^{1}H NMR (D_2O): δ 2.20 (1 H, m, $J_{1',2''}$ = 2.44 Hz, 2''-H), 2.73 (1 H, m, $J_{1',2'}$ = 7.12 Hz, 2'-H), 3.62 (1 H, a dd, $J_{4',5''}$ = 5.42 Hz, 5''-H), 3.71 (1 H, a dd, $J_{4',5'}$ = 3.85 Hz, 5'-H), 4.44 (1 H, m, $J_{2'',3'}$ = 2.33 Hz, $J_{2',3'}$ = 5.86 Hz, 3'-H), 4.45 (1 H, m, $J_{3',4'}$ = 2.20 Hz, 4'-H), 6.18 (1 H, dq, 1'-H), 8.11 (1 H, d, 6-H).

α-2'-Deoxy-5-fluorouridine 5'-Phosphate (23). This was prepared by chemical phosphorylation as described by Scheit and Faerber[34] and isolated on Whatman 3MM paper with solvent F to yield 41.7 mg (54%). R_f (F): 0.11; (G): 0.43. The corresponding R_f's for the β-anomer were 0.10 and 0.37, respectively. UV: λ_{max} (pH 7) 271 nm (ϵ 8200); λ_{max} (pH 12) 270 nm (ϵ 7300). CD (pH 7): [θ] −6800 (270 nm); for the β-anomer [θ] +6000 (271 nm). Compound **23** was converted to the nucleoside **22** by 5'-nucleotidase, but at a rate much slower than for the β-anomer.

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