NIH, Bethesda, MD). Animals that received no drug treatment died between 7 and 9 days after inoculation of L1210 cells. All animals were housed in central animal facilities having controlled temperature, relative humidity, and photoperiods.

Toxicological studies were done in male CD_1 albino mice (18–25 g). Compound 11 was administered intraperitoneally as a suspension in Klucel such that 0.1 mL of suspension/10 g of body weight delivered the desired dose. Two treatment schedules were evaluated, i.e., a single intraperitoneal injection or five daily intraperitoneal injections. Mice were observed daily for 14 days after the final injection. The LD_{10}, LD_{50}, and LD_{90} values were calculated for each treatment schedule using the probit analysis method of Finney.[13] Gross necropsy examination was performed on all mice that died during the observation period as well as those mice sacrificed at the completion of the study.

Acknowledgment. This work was supported by Grants CA 24543 and CA 22435 from the National Cancer Institute (Vermont) and grants from the American Cancer Society (27V) and the Hearst Foundation (SKI). We are grateful to Linda Mathews for exemplary experimental assistance.

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Affinity Therapeutics. 1. Selective Incorporation of 2-Thiouracil Derivatives in Murine Melanomas. Cytostatic Activity of 2-Thiouracil Arotinoids, 2-Thiouracil Retinoids, Arotinoids, and Retinoids

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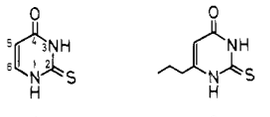
The incorporation of 2-[^{35}S]thiouracil and two of its derivatives into murine melanomas, in vivo, was studied. It was confirmed [J. R. Whittaker, *J. Biol. Chem.*, **246**, 6217–6226 (1971)] that 2-thiouracil has a marked affinity for melanin-producing tissue and that an affinity for such tissue could be sustained by 5-substituted 2-thiouracils. A series of derivatives of arotinoids and retinoids, with or without a 2-thiouracil group as a potential carrier to obtain affinity for melanomas, was examined for cytostatic activity, in vitro. None of these showed significant activity against murine melanomas.

The lack of tissue selectivity of the presently used cancer chemotherapeutic drugs constitutes a major problem. Consequently, the construction of chemotherapeutics that show specific affinity for, in casu, melanoma tissue would constitute an important improvement in such drugs.

It is known that 2-thiouracil (1) and 6-propyl-2-thiouracil



1 2

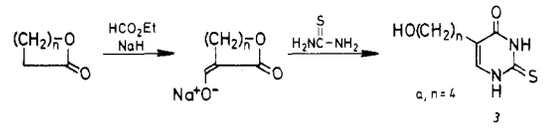
(2) exhibit marked affinities for melanin-producing tissue in vitro,[1] and 1 a similar affinity in vivo,[2] where they presumably act as false precursors for melanin.[1,2]

This affinity for melanin-producing tissue offers a possibility for preparing new potent drugs against malignant melanoma, where primary tumors and metastases often show a very high rate of melanin synthesis.

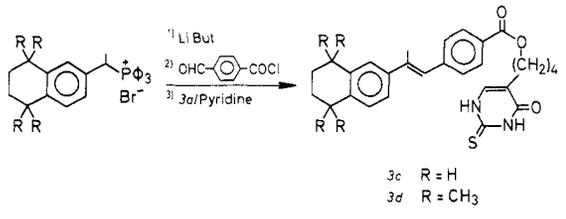
The necessary prerequisites for the development of an anticancer drug based on a carrier capacity of 2-thiouracil are (1) that 2-thiouracil can act as a carrier of substituents into the target tissue, i.e., that the affinity of the thiouracil moiety is of such a character that it is sustained in variously substituted derivatives, and (2) that substituents with, for example cytostatic properties or substituents capable of releasing, for example cytostatic drugs can be inserted into the thiouracil nucleus, etc.

We are attempting to develop such drugs, and our initial strategy was prompted by early reports that indicated that retinoids might cause regression of prenoplastic lesions and malignant skin lesions.[3–5] Furthermore, studies, in vitro, have shown that retinoids can inhibit cell proliferation and, idependently of this, stimulate melanogenesis.[6] This, combined with the fact that the incorporation of thiouracil was known to be related to the rate of melanin synthesis, constituted the basis for the present work, which indicates that it is possible to design derivatives of 2-thiouracil with affinity for murine melanomas.

Scheme I



Scheme II



Results

Chemistry. From a biochemical point of view, it appears most obvious to introduce substitutents in the 5- or

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(1) J. R. Whittaker, *J. Biol. Chem.*, **246**, 6217–6226 (1971).
(2) L. Dencker, B. Larsson, K. Olander, S. Ullberg, and M. Yokota, *Br. J. Cancer*, **39**, 449–452 (1979).
(3) W. Bollag, *Eur. J. Cancer*, **10**, 731–737 (1974).
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(5) W. Bollag and A. Matter, *Ann. N.Y. Acad. Sci.*, **359**, 9–23 (1981).
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Table I. Relative Tumor and Organ Incorporation of Radioactivity 48 h After a Single Intramuscular Injection of [^{35}S]Thiouracils[a]

tumor type	dose, μCi	drug	n	activity[a] liver	kidney	lung	spleen	eye	tumor[c]
A	5.1	1′	3	20.5	22.3	4.7	2.7	23.1	153.3
a				(19.5-23.5)	(17.8-25.3)	(3.9-5.2)	(2.1-3.0)	(20.0-27.5)	(104.8-253.2)
A	2.2	3a′	2	8.8	26.1	4.0	1.5	2.6	24.8
a				(8.0-9.6)	(23.8-28.5)	(3.6-4.3)	(1.4-1.6)	(2.4-2.8)	(16.2-49.2)
B	2.2	3a′	3	6.1	8.0	3.0	1.5	2.7	7.6
a				(4.2-8.5)	(5.7-10.6)	(2.2-3.7)	(1.1-2.0)	(2.2-3.4)	(3.2-16.1)
A	1.4	3c′	2	9.9	6.6	3.7	1.2	1.9	10.6
a				(7.8-12.0)	(6.5-6.7)	(3.3-4.0)	(1.2-1.3)	(1.7-2.0)	(9.8-12.0)
B	1.4	3c′	3	6.1	4.5	3.2	1.7	1.8	4.4
a				(5.8-6.4)	(4.1-5.1)	(3.1-3.3)	(1.5-1.9)	(1.5-2.1)	(3.4-5.9)

[a] Activity was measured as cpm/min/mg tissue (wet weight). Figures indicate the ratio and range, in parentheses, of uptake in the various organs vs muscle tissue of the same animal. A = Harding Passey melanoma (strongly melanized); B = Cloudman S 91 melanoma (moderately melanized). [b] Four tissue samples were taken from each tumor.

6-position of 2-thiouracil, since 6-propyl-2-thiouracil (**2**) was shown to be incorporated almost as effectively as the parent substance, in vitro.[1] Consequently, it was tempting to develop 6-substituted derivatives of **1**. However, 5-substituted 2-thiouracils appeared more advantageous from a synthetic point of view, since the synthesis of 5-(hydroxyalkyl)-2-thiouracils (**3**) is known[7] (Scheme I), and we decided to first test such compounds and their derivatives.

The next step, after synthesizing type **3** compounds, consists of introducing potential therapeutically active groups, and we initially chose a retinoid moiety. Furthermore, the so-called arotinoids, a group of vitamin A analogues with a very high biological activity,[8] appeared interesting as therapeutically active groups.

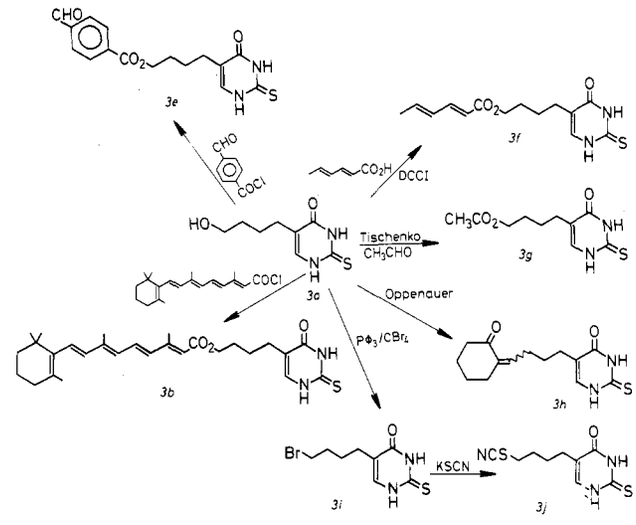
We succeeded in preparing a number of such compounds. Thus, the ester **3b** between 5-(4-hydroxybutyl)-2-thiouracil and *all-trans*-retinoic acid was prepared (Scheme III), but, unfortunately, we were not able to exchange ^{35}S for ^{32}S in this compound, which excluded an examination of its tissue distribution, in vivo. The next class of compounds prepared, i.e., esters **3c,d** between **3a** and the arotinoids **4** and **5** (Table II), turned out to be stable enough to allow for an exchange reaction with ^{35}S.[9] The preparation of these compounds is illustrated in Scheme II.[10,11]

Compounds **1**, **3a**, and **3c** were subjected to exchange with ^{35}S in order to determine their tissue distribution, in vivo. The exchange reaction, which was previously described as very selective,[9] in our hands led to a mixture of products. However, combined HPLC–TLC purification allowed the isolation of satisfactory exchanged samples, i.e., **1′**, **3a′** and **3c′**. Furthermore, a number of differently substituted 2-thiouracil derivatives (**3e–j**) was prepared (Scheme III) and examined for cytostaticity.

Discussion

The observed incorporation of 2-thiouracil in melanoma-bearing mice is in agreement with the results obtained by Dencker et al.[2,12] A direct comparison of these results with those presently reported is not possible, since the experimental details and procedures in this study differed from those applied previously. However, our observations

Scheme III



on [^{35}S]thiouracil incorporation in strongly melanized tissue, as well as the results with incorporation of the [^{35}S]thiouracil derivatives **3a′** and **3c′** into strongly and moderately melanized tumor tissue, are in agreement with the observations of Dencker et al.[12] on [^{35}S]thiouracil incorporation in melanotic and amelanotic tumors; they confirm that the degree of incorporation is dependent upon the rate of melanin synthesis. In the case of **3a′**, a considerable amount of label was found in the kidney of mice transplanted with the Harding Passey melanoma. A similar observation with mice carrying the same tumor was made by Dencker et al.,[12] who reported a higher relative uptake in the kidney for [^{35}S]thiouracil than for thio-[^{14}C]uracil. They interpreted this as a result of incorporation of [^{35}S]sulfate after loss of ^{35}S from the uracil moiety.

It is surprising that ^{35}S incorporation in the kidney was much lower in animals transplanted with Cloudman S 91 melanoma. However, in view of the limited number of observations, we refrain from any interpretation. It is noteworthy that the level of kidney and tumor labeling with **3a′** was similar for the two groups of animals.

In spite of the fact that the affinity for melanoma tissue of the 2-thiouracil derivatives, **3a** and **3c**, was reduced compared to that of the parent compound, **1**, we regard it as encouraging that considerable affinity was observed for **3a**. The tumor/eye ratio is 6.6 and 5.6 for **1** and **3c**, respectively, whereas it amounted to 9.5 for **3a**. Thus, a

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(8) P. Loeliger, W. Bollag, and H. Mayer, *Eur. J. Med. Chem.*, **15**, 9–15 (1980).
(9) J. Kolina, *Radioisotopy*, **12**, 989–996 (1971).
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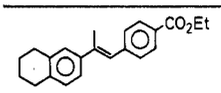
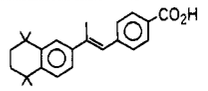
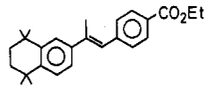
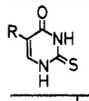
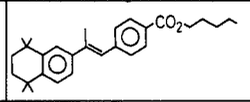
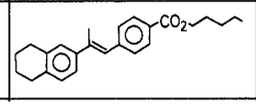
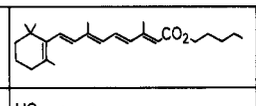
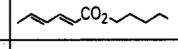
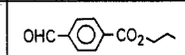
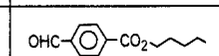
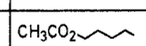
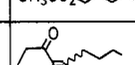
(11) F. Wätjen, O. Dahl, and O. Buchardt, unpublished results.
(12) L. Dencker, B. Larsson, K. Olander, and S. Ullberg, *Br. J. Cancer*, **45**, 95–104 (1982).

Table II. Growth Inhibition of Cloudman S91 Melanoma Cells[a]

Compound		Growth Conc: 1 μM, 10 μM	
All-trans-retinoic acid		11	A
		81	97
		122	98
		134	98
	R		
1	H	97	97
3d		146	84
3c		78	103
3b		73	A
3a		81	82
		134	106
3f		139	94
		82	83
3e		118	98
3g		90	94
3h		87	89
3j		93	100
3i		109	100

[a] The concentration of drug in the medium was 1 or 10 μM. Values are number of cells as percentage of control. A = not measured.

search for a compound with limited accumulation in the eyes and retained affinity for melanoma tissue would show that **3a** is better than the parent compound, **1**. In the case of **3c**, it appears that the introduction of the bulky group into the 5-position of 2-thiouracil strongly decreases the affinity for melanin-producing tissue. However, the degree of lipophilicity–hydrophilicity surely also plays a very important role in the biological behavior and utility of such compounds.

Conclusion

The presently reported results indicate that it may be possible to use 2-thiouracil as a carrier for a cytostatic agent. Although retinoids often are found to inhibit growth of melanoma cells, in vitro,[4] the retinoid derivatives tested in this work showed no such effect. Surprisingly, it was also observed that a series of arotinoids also did not show any appreciable effect on melanoma cells, even though in other cases such compounds are reported to be very active.[8]

Experimental Section

Biological Materials and Methods. Melanomas. An established line of melanoma S 91 (moderately melanized) was provided by the EG & G Mason Research Institute, DCT Tumor Bank, MA. The Harding Passey melanoma (strongly melanized) was kindly provided by Leo Pharmaceuticals, Hälsingborg, Sweden.

In Vivo. Inocula of 3×10^6 cultured S 91 cells were transplanted subcutaneously into the right flank of DBA/2 mice. Harding Passey melanoma tissue was minced with scissors, and tumor brei was transplanted subcutaneously into the right flank of DBA/2 mice. After 2 weeks, tumors measured approximately 6 × 7 mm.

The ^{35}S-enriched compounds **1'**, **3a'**, and **3c'**, corresponding to 11.2×10^6, 4.9×10^6, and 3×10^6 cpm, respectively, were injected intramuscularly into the right thigh. The animals were sacrificed 48 h after injection by cervical dislocation. The muscle tissue was obtained from the left thigh. Four tissue samples were taken from each tumor and one sample from each of a variety of tissues. All tissue samples were placed immediately in preweighed glass scintillation vials, and the amount of tissue was determined by reweighing. Tissues were solubilized in Lumasolve (Lumac) at 37 °C for 16 h. After bleaching with 2-propanol and hydrogen peroxide, Lipoluma (Lumac) was added as the scintillator. Radioactivity per milligram wet weight was calculated after counting in a Beckman LS 7000 scintillation counter.

In Vitro. Cloudman melanoma cells, 0.3×10^6, were seeded into T 25 culture flasks (Nunc), incubated for 48 h at 37 °C in Hams medium F 10, containing 15% horse serum and 2.5% fetal bovine serum, and supplemented with glutamine (8 mM), penicillin (25 IU/mL), and streptomycin (25 μg/mL). Control cultures were harvested by trypsinization (0.25% trypsin) for 1 min and counted visually. The medium was discarded and replaced with medium containing the compounds to be tested for cytostatic activity at final concentrations of 1 and 10 μM. Dimethyl sulfoxide at a final concentration of 0.5% was present in the media of the test, as well as of the control, culture. After a further 6 days of cultivation, cells were harvested by trypsinization and counted. The control culture, as well as the test culture, were in duplicate.

Chemistry. General. Melting points were determined on a Büchi melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 157 spectrometer. NMR spectra were recorded on a Varian T60A, JEOL FX 90Q, or Bruker HX 270. Chemical shifts are given in δ units (parts per million) with Me_4Si as internal standard; coupling constants are in hertz. Mass spectra were recorded on an AEI MS 902 instrument at 70 eV, using a direct-inlet system. The elemental analyses were within ±0.4% unless otherwise specified. CC purification refers to column chromatography using Merck silica 60 (70–230 mesh, ASTM). Evaporation of solvents was always performed under reduced pressure (ca. 10 mmHg).

5-(2-Hydroxypropyl)-2-thiouracil. A mixture of 4-methylbutyrolactone (40 g, 0.40 mol) and ethyl formate (48.4 mL, 0.60 mol) was added dropwise during 16 h at room temperature to a well-stirred suspension of sodium hydride (14.0 g, 0.58 mol; the mineral oil was removed by treatment with ether) in deoxygenated ether (600 mL) under nitrogen. This was followed by reflux for 8 h and evaporation of the solvent. The remaining residue was dissolved in ethanol (600 mL) at 0 °C, thiourea (26 g, 0.34 mol) was added, and the reaction mixture was refluxed for 16 h. The ethanol was evaporated, and the residue was dissolved in water (600 mL). This solution was neutralized with concentrated hydrochloric acid, which resulted in precipitation of crude product. Recrystallization from water yielded 14.8 g (23%) of the title compound: mp 189–191 °C; ^1H NMR (60 MHz, CF_3CO_2H) δ 1.50 (d, J = 6 Hz, 3 H), 2.95 (m, 2 H), 5.50 (m, 1 H), 7.45 (s, 1 H). Anal. ($C_7H_{10}N_2O_2S$) C, H, N; S: calcd, 17.18; found, 18.29.

5-[4-(2,4-Hexadienoyloxy)butyl]-2-thiouracil (3f). A solution of sorbic (2,4-hexadienoic) acid (1.12 g, 10 mmol), 5-(4-hydroxybutyl)-2-thiouracil[7] (**3a**; 2 g, 10 mmol), and dicyclohexylcarbodiimide (2.1 g, 11 mmol) in pyridine (50 mL) was

refluxed for 2 h, followed by cooling in ice–water and removal of the precipitated dicyclohexylurea by filtration. The solvent was evaporated from the filtrate, and the remaining oil was purified by CC with acetone/methylene chloride/triethylamine (50:50:1) as the eluent to give 1.0 g (34%) of crude **3f**, which was recrystallized from water: mp 136–139 °C; ^{1}H NMR (90 MHz, Me_2SO-d_6) δ 1.55 (m, 4 H), 1.82 (d, J = 4 Hz, 3 H), 2.23 (t, J = 6 Hz, 2 H), 3.27 (br, 2 NH), 4.08 (t, J = 4 Hz, 2 H), 5.76–6.31 (m, 3 H, ethylenic), 7.27 (s, 1 H), 6.98–7.62 (m, 1 H, ethylenic). Anal. ($C_{14}H_{18}N_2O_3S$) C, H, N, S.

5-(4-Acetoxybutyl)-2-thiouracil (3g). A mixture of **3a**[7] (1.0 g, 5 mmol) and aluminum isopropoxide (1.0 g, 5 mmol) in acetaldehyde (10 mL) was stirred for 1 h, during which time an exothermic reaction took place. The solvent was removed by evaporation to give an oil, which was purified by CC with acetone/methylene chloride (1:1) as the eluent. Evaporation of the solvents again gave an oil, which, however, solidified after crystallization from water: yield 0.6 g (50%); mp 145–147 °C; ^{1}H NMR (60 MHz, $CDCl_3$–Me_2SO-d_6) δ 1.55 (m, 4 H), 2.05 (s, 3 H), 2.30 (5, $J \approx 6$ Hz, 2 H), 4.08 (t, $J \approx 6$ Hz, 2 H), 7.21 (s, 1 H), 12.25 (br, 2 NH). Anal. ($C_{10}H_{14}N_2O_3S$) C, H, N, S.

5-[4-[(4-Formylbenzoyl)oxy]butyl]-2-thiouracil (3e). A solution of 4-formylbenzoyl chloride (3.6 g, 20 mmol) in toluene (20 mL) was added dropwise to a 90 °C stirred solution of **3a**[7] (4.0 g, 20 mmol) in pyridine (50 mL). After addition, the reaction mixture was stirred for an additional period of 10 min at 90 °C and cooled to ca. 0 °C, the precipitate was removed, and the filtrate was concentrated to 10 mL by evaporation, followed by addition of ether (100 mL). The semicrystalline precipitate was isolated and triturated with ethyl acetate to give crude **3e** (5.0 g, 75%), which was purified by recrystallization from acetone/pyridine (50:1) and water: mp 204–207 °C ^{1}H NMR (60 MHz, $CDCl_3$–Me_2SO-d_6) δ 1.80 (m, 4 H), 2.45 (t, $J \approx 6$ Hz, 2 H), 4.45 (t, $J \approx 6$ Hz, 2 H), 7.22 (s, 1 H), 7.90–8.60 (m, 4 H, aromatic), 10.16 (s, 1 H), 12.40 (br, 2 NH). Anal. ($C_{16}H_{16}N_2O_4S$) N, H, S; C: calcd, 57.83; found, 56.59.

5-[2-[(4-Formylbenzoyl)oxy]ethyl]-2-thiouracil. This compound was prepared analogously to **3e** by replacing **3a** with 5-(2-hydroxyethyl)-2-thiouracil.[7] Purification was by CC in the solvent mixture acetone/methylene chloride/triethylamine (50:50:1): yield 1 g (33%); mp 217–220 °C; ^{1}H NMR (60 MHz, Me_2SO-d_6) δ 2.70 (t, $J \approx 6$ Hz, 2 H), 3.35 (br, HOD), 4.45 (t, $J \approx 6$ Hz, 2 H), 7.45 (s, 1 H), 8.0–8.4 (m, 4 H, aromatic), 10.10 (s, 1 H), 12.43 (br, 1 NH). Anal. ($C_{14}H_{12}N_2O_4S$) C, H, N, S.

5-(4-Bromobutyl)-2-thiouracil (3i). A mixture of **3a**[7] (5.5 g, 27.5 mmol), tetrabromomethane (13 g, 40 mmol), and triphenylphosphine (10.5 g, 40 mmol) was dissolved in dimethylformamide (DMF, 25 mL) with stirring. The reaction took place immediately upon dissolution, and the temperature rose to 50–60 °C. The solution was subsequently stirred for 1 h and cooled in ice. Addition of methanol (10 mL), followed by ether (50 mL), allowed the isolation of a crystalline precipitate (12.0 g) consisting of **3i** (yield 84%) and triphenylphosphine oxide. The ratio varied between 0.84:1 and 1:1 in different experiments. In order to isolate **3i**, the mixture was chromatographed (CC) in the solvent mixture acetone/methylene chloride (1:1): mp 130–133 °C; ^{1}H NMR (60 MHz, $CDCl_3$–Me_2SO-d_6) δ 1.80 (m, 4 H), 2.35 (t, J = 6 Hz, 2 H), 3.50 (t, J = 6 Hz, 2 H), 7.2 (d, J = 6 Hz, 1 H), 12.20 (br, 2 NH). Anal. ($C_8H_{11}N_2OSBr$) C, H, N, S, Br.

5-(4-Thiocyanatobutyl)-2-thiouracil (3j). A solution of potassium thiocyanate (110 mg, 1.2 mmol) and **3i** (200 mg, 0.76 mmol) in acetone (10 mL) was refluxed for 3 h, followed by cooling in ice. The precipitated potassium bromide was filtered off, the solvent was removed by evaporation, and the residue was recrystallized from water to yield 120 mg (66%) of **3j**: mp 128–129 °C; IR (KBr) 3400 (NH), 3050 (CH), 2900 (CH), 2150 (sharp, SCN), 1650 (C=O), 1570 (C=S) cm^{-1}; MS, m/z 241 (M^+, 11.7), 208 (6.7), 183 (5.0), 156 (5.0), 154 (5.0), 141 (15), 82 (20.0), 76 (38.3), 59 (100). Anal. ($C_9H_{11}N_3OS_2$) C, H, N, S.

5-[4-(2-Oxocyclohexylidenyl)butyl]-2-thiouracil (3h). A solution of **3a** (2.0 g, 10 mmol) in cyclohexanone (25 mL), pyridine (100 mL), and toluene (100 mL) was concentrated to 160 mL by distillation, whereby all water present was removed. To this solution was added aluminum isopropoxide (2.5 g, 12 mmol), and it was refluxed for 3 h, followed by evaporation of the excess volatile material. The remaining oil was purified by CC in the solvent mixture acetone/chloroform (1:1) to give 156 mg (5%) of **3h**: mp 169–171 °C; ^{1}H NMR (270 MHz, Me_2SO-d_6) δ 1.44–1.82 (m, 6 H), 2.00–2.44 (m, 8 H), 6.30 (t, J = 6 Hz, 1 H, ethylenic), 7.20 (s, 1 H), 12.13 (br, 1 NH), 12.67 (br, 1 NH); MS, m/z 278 (M^+, 20), 216 (13), 155 (22), 142 (53), 137 (53), 79 (100). Anal. ($C_{14}H_{18}N_2O_2S$) C, H, N.

5-[4-(Retinoyloxy)butyl]-2-thiouracil (3b). To a stirred suspension of *all-trans*-retinoic acid (1.0 g, 3.3 mmol) in toluene (10 mL) was added phosphorous trichloride (0.2 mL), which after 1.5 h resulted in a homogeneous solution. This solution was added dropwise to a solution of **3a**[7] (0.70 g, 3.3 mmol) dissolved in *N,N*-dimethyl-4-toluidine at 60 °C, and stirring was continued for 30 min. The solvents were removed by evaporation, and the residue was purified by CC in the solvent mixture acetone/methylene chloride/triethylamine (50:50:1) to give 0.5 g (31%) of **3b** in the form of a yellow oil: The ^{1}H NMR (90 MHz, $CDCl_3$) spectrum was in excellent agreement with the assigned structure; MS, m/z 482 (M^+, 81.5), 282 (31.6), 267 (23.6), 201 (23.5), 183 (55.4), 159 (79), 141 (63.4), 133 (47.5), 82 (100). Anal. ($C_{28}H_{38}N_2O_3S$) H, N, S; C: calcd, 69.68; found, 67.70.

(*E*)-5-[4-[[4-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-2-methylethenyl]benzoyl]oxy]butyl]-2-thiouracil (3d). A solution of (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-2-methylethenyl]benzoyl chloride[11] (360 mg, 1 mmol) in toluene (5 mL) was added dropwise to a solution of **3a**[7] in pyridine (5 mL) heated to 100 °C; the mixture was stirred for an additional 20 min at this temperature, cooled in ice, and filtered, and the solvents were evaporated. The residue was crystallized from methanol to give 110 mg (19%) of **3d**; mp 140–142 °C; ^{1}H NMR (90 MHz, $CDCl_3$) δ 1.29 (s, 6 H), 1.32 (s, 6 H), 1.70 (s, 4 H), 1.76 (m, 1 H), 2.27 (d, J = 1.5 Hz, 3 H), 2.44 (t, J = 6 Hz, 2 H), 4.35 (t, J = 6 Hz, 2 H), 6.80 (br, 1 H, ethylenic), 6.95 (br, 2 NH), 7.36 (s, 1 H), 7.25–8.06 (m, 7 H, aromatic). Anal. ($C_{32}H_{28}N_2O_3S \cdot H_2O$) C, H, N, S.

(*E*)-5-[4-[[4-[2-(5,6,7,8-Tetrahydro-2-naphthyl)-2-methylethenyl]benzoyl]oxy]butyl]-2-thiouracil (3c). Compound **3c** was prepared analogously to **3d** from (*E*)-4-[2-(5,6,7,8-tetrahydro-2-naphthyl)-2-methylethenyl]benzoyl chloride[11] and **3a**:[7] yield 42%; mp 168–171 °C (MeOH); ^{1}H NMR (90 MHz, $CDCl_3$) δ 1.80 (m, 8 H), 2.25 (d, $J \approx 1$ Hz, 3 H), 2.34 (t, J = 6 Hz, 2 H), 2.78 (m, 4 H), 4.35 (t, J = 6 Hz, 2 H), 6.79 (br, 1 H), 7.10–8.06 (m, 8 H aromatic and H-6 from the thiouracil moiety). Anal. ($C_{28}H_{30}N_2O_3S$) C, H, N, S.

Ethyl (*E*)-4-[2-(5,6,7,8-Tetrahydro-2-naphthyl)-2-methylethenyl]benzoate. [1-(5,6,7,8-Tetrahydro-2-naphthyl)-ethyl]triphenylphosphonium bromide[8] (4.8 g, 10 mmol) was suspended in dry ether (30 mL) and treated, dropwise, under nitrogen, with a 2 M solution of butyllithium in hexane (5 mL, 10 mmol) at room temperature, and the dark red solution was stirred for 1 h. To this solution was added dropwise, with stirring, a solution of ethyl 4-formylbenzoate (1.8 g, 10 mmol) in deoxygenated dry ether (20 mL). Cooling with water was necessary to maintain a temperature of ca. 20 °C. A heavy white precipitate was formed during the reaction. The stirring was continued for 1 h, the precipitate was removed by filtration, and the filtrate was concentrated to dryness to give the title compound, which was recrystallized from methanol: mp 81–83 °C; ^{1}H NMR (90 MHz, $CDCl_3$); 1.47 (t, J = 8 Hz, 3 H), 1.80 (m, 4 H), 2.26 (*d*, $J \approx 1$ Hz, 3 H), 2.78 (m, 4 H), 4.37 (q, J = 8 Hz, 2 H), 6.80 (br, 1 H), 7.09–8.07 (m, 7 H, aromatic). Anal. ($C_{22}H_{24}O_2$) C, H.

^{35}S Enrichment of 1. ^{35}S-enriched elemental sulfur in toluene (Amersham, specific activity 103 Ci/g of S) was used; 20 mCi of the sulfur dissolved in toluene (0.5 mL) was placed in a glass ampule (1 mL), and the solvent was blown off with a gentle stream of nitrogen. After this, a solution of 2-thiouracil (0.65 mg, 5 μmol) in pyridine (0.3 mL) was added, and the ampule was cooled to ca. 70 K, evacuated (0.5 mmHg), sealed, and heated at 100 °C for 24 h. The ampule was cooled and opened, and the reaction mixture was injected directly into an HPLC column [RP-18 Lichrosorp, mobile phase methanol/acetonitrile/H_2O (10:7:3)] and thereby partially separated into its components. The fraction containing the enriched thiouracil (1′) was further purified by TLC [HP-TLC-Alufoil, silica gel 60 F 254, mobile phase acetone/methylene chloride (1:1)]. The recovery of 1 in the form of 1′ was 80%, calculated on the basis of its UV extinction. The specific activity was 43 mCi/mmol.

^{35}S Enrichment of 3a. This was done exactly as described above for 1. The recovery of **3a** in the form of **3a'** was 80%, and the specific activity was 41 mCi/mmol.

^{35}S Enrichment of 3c. This was done analogously to the method described for 1, with the exception that a different TLC solvent mixture was used, i.e., methylene chloride/ether (1:1). The recovery in the form of **3c'** was 80% with a specific activity of 34 mCi/mmol.

The initial attempts to find a method for ^{35}S enrichment of **3b**, which were analogous to those described above, but under milder conditions, all resulted in the destruction of **3b**.

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Methotrexate Analogues. 15. A Methotrexate Analogue Designed for Active-Site-Directed Irreversible Inactivation of Dihydrofolate Reductase†

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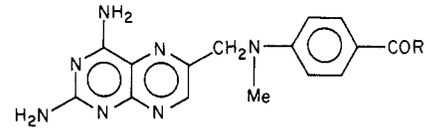
N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-(iodoacetyl)-L-lysine (1) was synthesized as a potential active-site-directed irreversible inhibitor of dihydrofolate reductase (DHFR). In an ultraviolet spectrophotometric assay of dihydrofolate reduction by *Lactobacillus casei* DHFR, 1 and methotrexate (MTX, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid) had ID_{50} values of 4.5 and 6.2 nM. The corresponding ID_{50} values in a competitive radioligand binding assay against [^{3}H]MTX were 31 and 16 nM. Thus, as reversible inhibitors of this enzyme over a short exposure time, 1 and MTX had comparable activity. On the other hand, when *L. casei* DHFR was incubated for up to 6 h with 0.1 or 1.0 μM 1, a progressive decrease in the ability of [^{3}H]MTX to subsequently displace the drug was observed. When MTX itself was used at the same concentrations, the extent of displacement of [^{3}H]MTX did not decrease with time. These results were consistent with rapid reversible binding of 1 to the enzyme, followed more slowly by covalent bond formation near the active site. The pH profile for this effect followed a curve with a sigmoidal shape. The apparent inflection point near pH 7.2 was consistent with alkylation of a histidine residue.

The development of a comprehensive model for the interaction of the antitumor agent methotrexate (MTX, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid) with its target enzyme dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:NADPH oxidoreductase EC 1.5.1.3) in intact mammalian cells has been a longstanding goal in a number of laboratories. An important contribution to the understanding of the mechanism of this interaction came from the demonstration that "free" exchangeable MTX in excess of the amount needed to saturate the enzyme and intercept any newly synthesized enzyme is an essential cytotoxic determinant.[1-7] Since even stoichiometrically bound MTX undergoes slow dissociation from the enzyme over time and since even a small fraction (<5%) of the total intracellular DHFR can generate enough tetrahydrofolate to support thymidylate synthesis adquate for cell growth,[6] one reason for seeking to maintain a critical level of free MTX in cells is to prevent this dissociation to the maximum possible extent. It has also been postulated that excess MTX may be necessary to saturate a low-affinity form of the enzyme, possibly associated with inadequate levels of NADPH, that can continue to convert dihydrofolate to tetrahydrofolate even when the high-affinity form is saturated.[3] The existence of low-affinity variants of DHFR in MTX-resistant mammalian cells has in fact been observed in several studies[8-12] and lends support to this concept.

The development of agents that bind more tightly than MTX to both low-affinity and high-affinity forms of DHFR would be a desirable goal in the search for improved folate antagonists.[3] Thus, we became interested in preparing MTX analogues with side-chain functional groups capable of reacting *covalently* with DHFR once the initial reversible enzyme–inhibitor complex has formed. In this paper we report the synthesis of N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-(iodoacetyl)-L-lysine (1), an example



1, R = NHCHCO$_2$H
(CH$_2$)$_4$NHCOCH$_2$I

2, R = OH

3, R = NHCHCO$_2$Me
(CH$_2$)$_4$NHCbz

4, R = NHCHCO$_2$H
(CH$_2$)$_4$NHCbz

5, R = NHCHCO$_2$H
(CH$_2$)$_4$NH$_2$

of a "classical antifol" structurally modified with a view

†This paper has been presented in part. See "Abstracts of Papers", 182nd National Meeting of the American Chemical Society, New York, NY, Aug 23–28, 1981, American Chemical Society, Washington, DC, 1981, Abstr MEDI 87.

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