solution was evaporated to dryness. The residue was dissolved in ethanol (15 mL) and adsorbed on a portion of silica gel (5 g, 50–100 mesh) by evaporation. The dried gel was applied onto a silica gel short column (3.5 × 8 cm). Elution was performed with chloroform–methanol (6:1); 12-mL fractions were collected. Fractions 13–29 contained chromatographically pure product **6** as a white powder (212 mg, 77%) after evaporation and drying. An analytical sample was crystallized from ethanol, which gave fine crystals of **6**, mp 256 °C dec. Anal. ($C_{12}H_{15}N_5O_3$) C, H, N.

***N*-2-Methyl-9-[(2-hydroxyethoxy)methyl]guanine (4).** To a solution of **6** (138.6 mg, 0.50 mmol) in water (10 mL) was added *N*-bromosuccinimide (98 mg, 0.55 mmol) with stirring. After 25 min of being stirred, the reaction mixture was alkalized with concentrated aqueous ammonia (15 mL) and left at room temperature for 30 min. The solution was concentrated to a volume of 15 mL, diluted with ethanol (20 mL), and evaporated with a portion of silica gel (3 g, 50–100 mesh). The dried gel was applied onto a silica gel short column (2.5 × 5 cm). Elution was performed with solvent C (see above), and 10-mL fractions were obtained. Fractions 12–28 containing the main product were concentrated to a volume of ca. 100 mL and left at 5 °C for 2 days. The resulting crystalline material was collected by filtration, washed with ether, and dried in vacuo to give 82.1 mg of **7** (68%), mp 232–237 °C (dec without melting point). Anal. ($C_9H_{13}N_5O_3 \cdot 1.5H_2O$) C, H; N: calcd, 26.30; found, 25.69.

7-Methyl-9-[(2-hydroxyethoxy)methyl]-1,*N*-2-isopropenoguanine (7). A solution of **5** (105.3 mg, 0.40 mmol) in dimethylformamide (2 mL) was treated with dimethyl sulfate (50 mg, 0.48 mmol). After it had been left for 30 h at room temperature, the reaction mixture was diluted with acetone (5 mL) and ethyl ether (2 mL), cooled to −5 °C, and adjusted to pH 9.5 with concentrated aqueous ammonia. The resulting white precipitate was immediately collected by filtration on a suction funnel, washed with acetone and ethyl ether, and dried under diminished pressure to give **7** as a white powder (102.3 mg, 92%) homogeneous by TLC, mp 139 °C. Anal. ($C_{12}H_{15}N_5O_3$) H, N; C: calcd, 51.98; found, 52.46.

Biological Activity Evaluation. The assays used for measuring antiviral activity (based on inhibition of either virus-induced cytopathogenicity or plaque formation), antimetabolic activity (based on inhibition of DNA, RNA, and protein synthesis), and antitumor cell activity (based on inhibition of tumor cell proliferation) have been described previously.[32,33]

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cis-4-Carboxy-6-(mercaptomethyl)-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one, a Potent Inhibitor of Mammalian Dihydroorotase

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A series of *cis*- and *trans*-4-carboxy-3,4,5,6-tetrahydropyrimidin-2(1*H*)-ones possessing either a carboxy, hydroxymethyl, or mercaptomethyl substituent at C-6 were prepared and tested for their ability to inhibit mammalian dihydroorotase. Of these compounds, only the *cis*-6-mercaptomethyl compound, *cis*-**1**, was found to be a potent competitive inhibitor of the enzyme (K_i = 140 nM at pH 7.4 and 8.5) when assayed in the direction of dihydro-L-orotate hydrolysis. These results suggest that the inhibition arises from the ligation of the thiolate to the zinc atom which is thought to be located in the enzyme's active site.[2,3] Although analysis of *cis*-**1** with 2,2′-dithiobis(5-nitrobenzoic acid) revealed significant loss of the free thiol group under enzymatic assay conditions, the addition of the reducing agent, dithiothreitol, to the enzymatic reaction mixtures afforded *cis*-**1** complete protection against this chemical decomposition, as evidenced by lowering of the inhibition constant in the presence of dithiothreitol. Compound *cis*-**1** had no significant antiproliferative activity against B16 melanoma cells in tissue culture, possibly due to the rapid decomposition of the compound or poor permeability into cells.

The mammalian CAD protein is a trifunctional protein containing the first three enzymes of the de novo pyrimidine nucleotide biosynthetic pathway: carbamoylphosphate synthetase II, aspartate transcarbamoylase (ATCase), and dihydroorotase. The elevation in neoplastic tissues of the enzymatic activities of this complex as well as other key enzymes involved in the biosynthesis of DNA has been noted as an important consequence of the biochemical commitment of cancer cells to replicate.[4] This observation suggests that inhibition of these key enzymes may be a valid approach for the design of new antineoplastic agents.

N-(Phosphonoacetyl)-L-aspartate (PALA) is an extremely potent inhibitor of ATCase and is highly effective in a number of experimental tumor models.[5,6] However,

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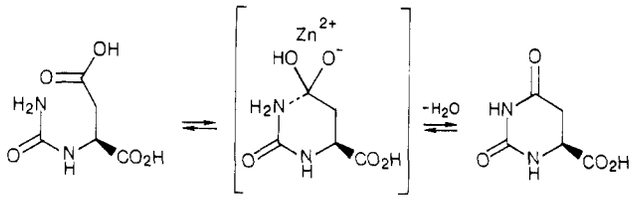
Scheme I

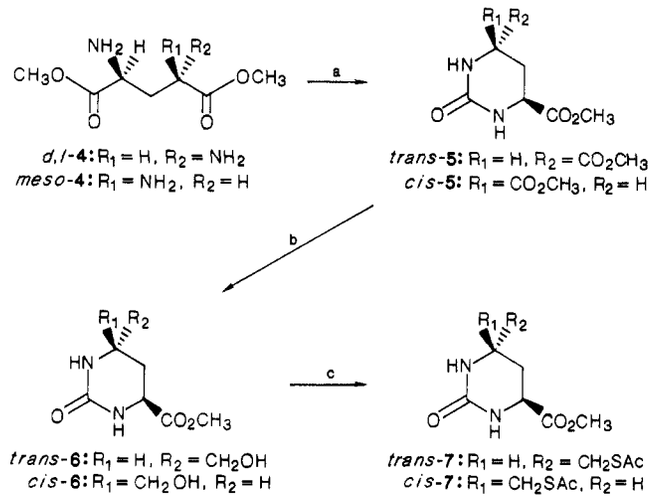


PALA has proven to be of limited clinical utility, possibly due to one or more of the following: (a) ATCase activities in normal and neoplastic tissues greatly exceed those of the other five enzymes in the de novo pyrimidine biosynthetic pathway;[7] (b) synthesis of additional CAD protein in response to the PALA-induced depletion of the pyrimidine pool, thereby producing an increase in ATCase and more carbamoyl phosphate to effectively compete with PALA at the active site of ATCase;[8-10] or (c) provision via the salvage pathways to maintain a sufficient supply of pyrimidine nucleosides to maintain nucleotide pools at an adequate level for survival.[11] Therapeutic approaches to overcome these latter two mechanisms of resistance are being explored in clinical trials.[12] For example, PALA is being administered in combination with acivicin, an irreversible inhibitor of carbamoyl-phosphate synthetase II (the first and rate-limiting enzyme of the metabolic pathway) and certain other glutamine-dependent synthetases.[13] Acivicin is currently under investigation as a single therapeutic agent in cancer patients as it has also demonstrated significant activity in animal tumor models.[14]

Other enzymes in the de novo pyrimidine nucleotide biosynthetic pathway might also serve as targets for antitumor agents. As the activity of dihydroorotase is quite low relative to the other enzymes in this pathway,[7] this enzyme might prove to be a valid target for chemotherapeutic intervention. To that end, we sought to design and synthesize analogues of dihydro-L-orotate that would effect potent inhibition of this enzyme.

Dihydroorotase catalyzes the cyclization of *N*-carbamoyl-L-aspartate to dihydro-L-orotate, a reaction that is mechanistically similar to that of a protease operating in reverse (Scheme I). Early mechanistic studies of the enzyme strongly suggested that zinc was an essential active-site cofactor,[2] and more recent work has conclusively demonstrated the presence of zinc in the enzymes of both mammalian and prokaryotic origin.[3] Several strategies for the inhibition of zinc proteases have been elucidated and the application of this knowledge was a logical starting point for inhibitor design. The thiols *cis*-**1** and *trans*-**1** were chosen on the basis of captopril, a potent inhibitor of the zinc protease angiotensin converting enzyme (ACE),[15] and the known sensitivity of dihydroorotase to thiols such as cysteine.[2] The diacids *cis*-**3** and *trans*-**3** were based on another ACE inhibitor, enalaprilat, which contains a carboxylic acid moiety to complex zinc and a basic amine to engage the acidic residue involved in protonation of the departing amine.[16] Finally the hydroxy compounds *cis*-**2** and *trans*-**2** were included in order to evaluate the role of the sulfur and carboxylate in **1** and **3**. Because all the proposed inhibitors possess a sp^3 carbon at C-4, they can be viewed as analogues of the presumed transition state for the reaction. This mimicry of the transition state should further enhance binding.[17]

Scheme II[a]



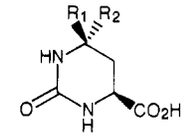
d,l-**4**: $R_1 = H$, $R_2 = NH_2$
meso-**4**: $R_1 = NH_2$, $R_2 = H$

trans-**5**: $R_1 = H$, $R_2 = CO_2CH_3$
cis-**5**: $R_1 = CO_2CH_3$, $R_2 = H$

trans-**6**: $R_1 = H$, $R_2 = CH_2OH$
cis-**6**: $R_1 = CH_2OH$, $R_2 = H$

trans-**7**: $R_1 = H$, $R_2 = CH_2SAc$
cis-**7**: $R_1 = CH_2SAc$, $R_2 = H$

[a] (a) CDI, Et_3N/DMF; (b) $BH_3 \cdot SMe_2$/DMF; (c) diisopropyl azodicarboxylate, triphenylphosphine, CH_3COSH/THF.



trans-**1**: $R_1 = H$, $R_2 = CH_2SH$
cis-**1**: $R_1 = CH_2SH$, $R_2 = H$
trans-**2**: $R_1 = H$, $R_2 = CH_2OH$
cis-**2**: $R_1 = CH_2OH$, $R_2 = H$
trans-**3**: $R_1 = H$, $R_2 = CO_2H$
cis-**3**: $R_1 = CO_2H$, $R_2 = H$

Results and Discussion

Chemistry. The method of Elberson was used to prepare a nearly equal diastereomeric mixture of *d,l*- and *meso*-α,α'-diaminoglutaric acid,[18] which following esterification afforded the diester dihydrochloride **4**. The cyclization of **4** to **5** (Scheme II) was best accomplished by the slow addition of a solution of carbonyldiimidazole (CDI) to a mixture of **4** and Et_3N in DMF, thereby avoiding the activation of both amines by CDI. In this manner, good yields of the isomeric mixture were obtained. These isomers were exceedingly difficult to separate by flash chromatography. Fortunately this separation could be avoided by selective recrystallization of **4**. Cyclization of these enriched diastereomeric mixtures of **4** followed by a simple recrystallization then gave both *trans*-**5** and *cis*-**5** in >98% isomeric purity. Saponification of the diesters

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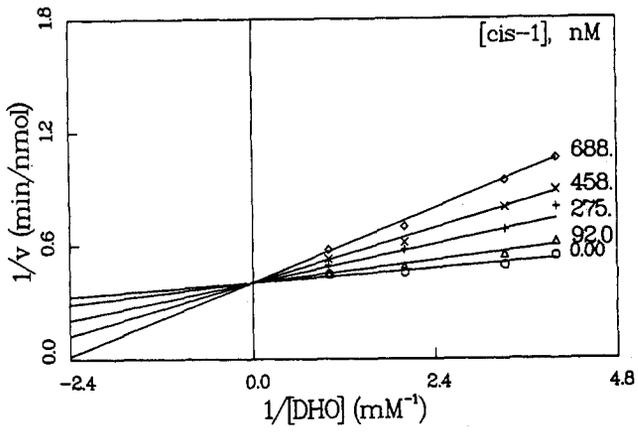

Figure 1. Double-reciprocal plot of dihydroorotase activity at variable concentrations of dihydro-L-orotate (0.25, 0.3, 0.5, 1.0 mM) and at changing fixed levels of (±)-*cis*-1 (0, 92.0, 275.0, 458.0, and 688.0 nM) in 0.167 M Tris·HCl (pH 8.5)–1 mM dithiothreitol. The initial velocities are in units of nanomoles of *N*-carbamoylaspartate/minute. The lines drawn through the experimental points (the average of triplicate determinations) were obtained from fitting of the data to eq 1. Other experimental conditions are described in the Experimental Section.

gave the corresponding diacids, *trans*-**3** and *cis*-**3**.

The direct reduction of the diesters **5** to the hydroxy esters **6** with borane–dimethyl sulfide proceeded with reasonable selectivity for the trans compound, but was far less satisfactory for the cis, yielding as the major product the diol expected from overreduction. The introduction of sulfur was facilely accomplished by using a modification of the Mitsunobu reaction employing thioacetic acid[19] and gave the thioacetate methyl esters *trans*-**7** and *cis*-**7**. Basic hydrolysis of the isomerically pure thioacetates **7** and hydroxy esters **6** provided respectively *trans*- and *cis*-**1** and *trans*- and *cis*-**2**.

Enzymology. At a concentration of dihydro-L-orotate of 0.1 mM (K_m = 69 ± 8 μM, pH 8.5), the cis and trans forms of compounds **2** and **3** did not effect any inhibition of the dihydroorotase activity at concentrations of 0.1–1000 μM. While the trans form of compound **1** did not inhibit the enzyme at an identical range of concentrations, *cis*-**1** proved to be a very potent inhibitor of dihydroorotase activity. A double-reciprocal plot of *cis*-**1** vs dihydro-L-orotate (data not shown) demonstrated that the compound was a competitive inhibitor (K_i = 200 nM) of the dihydroorotase reaction.

However, following prolonged storage of *cis*-**1** at 4 °C, no inhibition of dihydroorotase was observed. Quantitation with 2,2′-dithiobis(5-nitrobenzoic acid) (DTNB) indicated that the loss of inhibition was directly related to the disappearance of the free thiol group. During the course of the enzymatic reaction at pH 8.5, 37 °C, approximately 33% of the thiol group of *cis*-**1** was lost. However, as demonstrated by titration with DTNB and spectrophotometric data, storage of 1–6 mM solutions of *cis*-**1** in 0.1 mM dithiothreitol (DTT) at −20 °C afforded complete protection against thiol loss, and the inhibitor was stable under enzymatic reaction conditions in the presence of 1 mM DTT.

A double-reciprocal plot of *cis*-**1** vs dihydro-L-orotate (Figure 1) in the presence of 1 mM dithiothreitol also conformed to competitive inhibition, but a lower dissociation constant for the inhibitor was obtained (K_i = 143 ± 18 nM), suggesting that the added thiol prevented the facile decomposition of the inhibitor. At pH 7.4, the dissociation constant for *cis*-**1** was unchanged (K_i = 140 ± 11 nM). Inasmuch as the *cis*-**1** compound actually consists of a pair of enantiomers, should one of the pair not inhibit the enzyme, then the true dissociation constant of the inhibitor is 3 orders of magnitude lower than the K_m of the substrate (K_m(DHO) = 0.07 mM, pH 8.5).

Table I. Evaluation of Antiproliferative Activities of *cis*-**1** and *cis*-**7** against Murine B16 Melanoma Cells in Vitro[a]

compd	concn, mM	growth inhibition, %	
		no uridine	+1 mM uridine
cis-1	2	22	18
	1	17	12
	0.5	14	4
	0.25	9	3
	0.12	4	0
cis-7	2	96	98
	1	94	83
	0.5	28	9
	0.25	2	2
	0.12	0	0
PALA	10	84	10
	1	86	9
	0.1	79	6
	0.01	16	6

[a] Values shown for *cis*-**1** and PALA are averages from three and four experiments, respectively, each of which included triplicate determinations for each data point. Values for *cis*-**7** are from a single experiment with triplicate determinations.

Biological Evaluation. The biological activity of *cis*-**1** was assessed by determining whether it inhibited the proliferation of murine B16 melanoma cells in tissue culture (Table I). PALA was utilized as a positive control, and compounds were evaluated in the absence or presence of 1 mM uridine to assure that the observed antiproliferative effects were due to blockade of the de novo pyrimidine nucleotide biosynthetic pathway. At concentrations of ≥0.1 mM, PALA inhibited cell proliferation by about 80%; uridine effectively reversed the inhibitory effects of PALA even at an antimetabolite concentration of 10 mM. Uridine has been shown to be effective at circumventing the effects in cultured cells of other inhibitors of the de novo pathway. At a concentration of up to 2 mM *cis*-**1** had very little effect on proliferation of B16 melanoma cells. The slight but concentration-dependent inhibition that was observed was unaffected by exogenous uridine, suggesting that this effect was not due to inhibition of de novo pyrimidine biosynthesis.

The failure of *cis*-**1** to inhibit cell proliferation despite its potent inhibition of dihydroorotase may have resulted from either the compound's rapid decomposition as discussed above or poor permeability into cells as a carboxylate. In an attempt to circumvent these proposed limitations, the *S*-acetyl methyl ester (*cis*-**7**), was evaluated in the cellular system. This compound produced essentially complete inhibition of proliferation at ≥1 mM (Table I). However, the antiproliferative effects of *cis*-**7** were not substantially modified by uridine supplementation, indicating that the observed in vitro activity was not due to inhibition of dihydroorotase.

Conclusion. The thiol *cis*-**1** is the most potent inhibitor of mammalian dihydroorotase yet reported. The K_i of racemic *cis*-**1**, 143 nM, is approximately 2.5 orders of magnitude lower than the K_m of dihydro-L-orotate. Since the inhibition is competitive in nature, the compound can reasonably be assumed to be binding at the active site, the thiolate acting as a ligand for zinc. Furthermore, since the NMR coupling constant data (see the Experimental Section) demonstrate the equatorial disposition of the mercaptomethyl substituent in both *cis*- and *trans*-**1**, the

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preference for an equatorial carboxylate in the transition state leading to cyclization is clearly suggested. In light of this result, the total lack of activity of the diacid *cis*-**3** is surprising. Extrapolating from the results seen with ACE in going from captopril to a carboxylate analogue (substitution of CO_2H for SH raises the K_i of captopril from 1.7 nM to 2.5 μM[16]), *cis*-**3** would be expected to have an inhibition constant in the low millimolar range, and if binding interactions were analogous to that of enalaprilat, a result similar to that of *cis*-**1** would be expected.

The lack of antiproliferative activity of *cis*-**1** may be due to the inability of the compound to reach the target enzyme and/or be maintained at an inhibitory concentration for a sufficient period to produce a growth-suppressive depletion of pyrimidine nucleotide pools.[20] Enzymatic or nonenzymatic instability, e.g. sulfhydryl oxidation, could result in the rapid inactivation of the compound. The appropriateness of dihydroorotase as a target for antineoplastic chemotherapy remains open. However, this study indicates that it is feasible to design potent inhibitors of dihydroorotase.

Experimental Section

Synthesis. Melting points were determined on samples dried at 70 °C and 1 mmHg with a Thomas-Hoover melting point apparatus and are uncorrected. Infrared spectra were recorded in KBr on a Perkin-Elmer Model 783 spectrophotometer. Proton NMR were recorded on either a JEOL-GX-270 MHz or a Bruker WM-360 MHz instrument. Chemical shifts are reported in parts per million on the δ scale relative to tetramethylsilane as internal standard or in the case of D_2O as solvent, as an external standard.

THF was distilled from $LiAlH_4$. Anhydrous DMF and CH_3CN were Aldrich Gold Label grade. All other solvents were reagent grade and used without further purification. Chromatography refers to purification by flash chromatography[20] on E. Merck silica gel 60 (230–400 mesh). The eluants are listed in order of the sequence used to elute the product. Unless otherwise noted, all reactions were run under argon at 20 °C.

***d,l*- and *meso*-α,α'-Diaminoglutaric Acid Dimethyl Ester Dihydrochloride (*trans*- and *cis*-4).** To a stirring, ice-cooled solution of 36.43 g (225 mmol) of α,α'-diaminoglutaric acid[18] in 650 mL of dry methanol was added dropwise 100 mL of thionyl chloride. Upon completion of the addition, the cooling bath was removed and the reaction was continued for 24 h at 20 °C. The solvent was removed at reduced pressure, and the resulting foam was redissolved in 70 mL of methanol. The addition of 120 mL of methylene chloride induced crystallization. After being allowed to stand overnight at −5 °C the solution was filtered to yield, after washing with methanol, 28.7 g of a beige solid. After the addition of the MeOH washings to the filtrate, ether was added to yield a second crop of 18.9 g. Analysis of these materials by high-field proton NMR showed the first crop to be a 87:13 mixture of *d,l*:meso isomers and the second crop to be a 90:10 mixture of meso:*d,l*.

Recrystallization of a portion of each of these solids (CH_2Cl_2/MeOH) gave analytical samples of the isomeric α,α'-diaminoglutaric acid dimethyl ester dihydrochloride.

d,l-**4**: mp 191 °C dec; IR 3400 (N—H), 1740 (C=O) cm^{-1}; NMR (360 MHz, D_2O) δ 2.56 (t, 2 H, H-3, J = 6.9 Hz), 3.90 (s, 6 H, OCH_3), 4.53 (t, 2 H, H-2,4). Anal. ($C_7H_{16}N_2O_4Cl$) C, H, N, Cl.

meso-**4**: mp 220 °C dec; IR 3250 (N—H), 1740 and 1760 (C=O); NMR (360 MHz, D_2O) δ 2.41 (dt, 1 H, H-3, J = 15.1 and 6.2 Hz), 2.79 (dt, 1 H, H-3′, J = 15.1 and 7.8 Hz), 3.89 (s, 6 H, OCH_3), 4.48 (dd, 2 H, H-2,4). Anal. ($C_7H_{16}N_2O_4Cl_2$) C, H, N, Cl.

***trans*-4,6-Dicarbomethoxy-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*trans*-5).** To a stirring suspension containing 2.0 g (8.47 mmol) of *d,l*-**4** (92:8 *d,l*:meso) and 2.6 mL of Et_3N (18.7 mmol) in 25 mL of DMF was added dropwise over a 30-min period 1.85 g (11.4 mmol) of carbonyldiimidazole in 20 mL of DMF. After the mixture was stirred overnight, the reaction was quenched with 1 mL of water, and the bulk of the DMF was removed at reduced pressure. The resulting residue was dissolved in 50 mL of water and Dowex-50X8-100 (H^+) was added until the pH of the solution was 2–3. Upon filtration, concentration (60 °C and 20 mmHg) to a volume of 10 mL, and cooling, the product crystallized to yield 880 mg (54%) of pure **5** as a white crystalline solid. Column chromatography (5–7%, MeOH/$CHCl_3$) of the filtrate gave an additional 478 mg of product (total yield 83%). Analysis of these two materials by HPLC [4.6mm × 50 cm Zorbax CN; eluting with 8:2 (MeOH–H_2O) at 0.7 mL/min; monitoring absorbance at 210 nm] showed the initial solid to be a 98:2 (trans:cis) isomeric mixture and the chromatographed product a 87:13 (trans:cis) mixture. Subsequently, only the isomerically purer material obtained in the initial recrystallization was characterized and used: mp 208–209 °C; IR 3350 (N—H), 1745 and 1685 (C=O) cm^{-1}; NMR (270 MHz, CD_3OD) δ 2.28 (t, 2 H, H-5, J = 6.0 Hz), 3.77 (s, 6 H, OCH_3), 4.13 (t, 2 H, H-4,6). Anal. ($C_8H_{12}N_2O_5$) C, H, N.

***cis*-4,6-Dicarbomethoxy-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*cis*-5).** By use of the above procedure 5.21 g of **5** (35% yield as a 98:2 cis:trans mixture) was obtained from 17.5 g (66.6 mmol) of *meso*-**4** (90:10 meso:*d,l*) as a white solid: mp 182–186 °C; IR 3350 (N—H), 1750 and 1690 (C=O) cm^{-1}; NMR (270 MHz, $CDCl_3$) δ 2.02 (dt, H-5_{ax}, J = 10.2 and 13.2 Hz), 2.68 (dt, H-5_{eq}, J = 4.3 and 13.2 Hz), 3.80 (s, 6 H, OCH_3), 4.19 (dd, 2 H, H-4,6). Anal. ($C_8H_{12}N_2O_5$) C, H, N.

***trans*-4,6-Dicarboxy-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*trans*-3).** A mixture of 1.03 g (4.76 mmol) of *trans*-**5** and 11 mL of 1 N LiOH was stirred for 1 h and upon acidification with 6 N HCl the diacid spontaneously crystallized. Following filtration and drying, 652 mg (73%) of *trans*-**3** was obtained as a white solid: mp 217 °C dec; IR 3360 (N—H), 1710 and 1620 (C=O); NMR (270 MHz, D_2O) δ 2.38 (t, H-5, J = 6.1 Hz), 4.19 (t, H-4 and 6). Anal. ($C_6H_8N_2O_5$) C, H, N.

***cis*-4,6-Dicarboxy-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*cis*-3).** Similarly, 595 mg of *cis*-**5** produced 390 mg (75%) of *cis*-**3**: mp 229 °C dec; IR 1730 and 1620 (C=O) cm^{-1}; NMR (270 MHz, D_2O–NaOD) δ 1.95 (dt, H-5_{ax}, J = 9.4 and 13.2 Hz), 2.52 (dt, H-5_{eq}, J = 4.7 Hz), 4.09 (dd, H-4,6). Anal. ($C_6H_8N_2O_5$) C, H, N.

***trans*-4-Carbomethoxy-6-(hydroxymethyl)-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*trans*-6).** A mixture of 4.98 g (23.0 mmol) of *trans*-**5** and 5.4 mL of 10 M BH_3·SMe_2 in 300 mL of CH_3CN was heated and stirred at 60 °C for 1.5 h, at which point the reaction was quenched by the addition of MeOH. The bulk of the solvent was removed at reduced pressure, the resulting residue was redissolved in MeOH and again concentrated, and the product was then chromatographed, eluted sequentually with 90:10:1, 80:20:2, and 70:30:3 ($CHCl_3$–MeOH–H_2O). The desired alcohol **6**, 2.30 g (53%), eluted shortly after unreacted **5** and was followed by 530 mg (14%) of diol derived from overreduction. Analytically pure *cis*-**6**, 1.70 g, was obtained following recrystallization from MeOH–2-propanol: mp 166–168 °C; IR 3390 (N—H), 1750 and 1690 (C=O) cm^{-1}; NMR (360 MHz, CD_3OD) δ 1.91 (ddd, H-5_{ax}, $J_{5,5}$ = 13.6 Hz, $J_{4,5}$ = 5.5 Hz, $J_{5,6}$ = 9.5 Hz), 2.12 (dt, H-5_{eq}, $J_{5,6}$ = $J_{4,5}$ = 4.2 Hz), 3.37 (m, H-6), 3.50 (m, CH_2OH), 3.75 (s, CO_2CH_3), 4.17 (dd, H-4). Anal. ($C_7H_{12}N_2O_4$) C, H, N.

***cis*-4-Carbomethoxy-6-(hydroxymethyl)-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*cis*-6).** In a similar manner 1.93 g (8.93 mmol) of *cis*-**5** was converted to 362 mg (22%) of *cis*-**6** and 590 mg (41%) of the overreduced diol. Analytically pure product, 250 mg, was obtained as a white solid following recrystallization: mp 162–163 °C dec; IR 3360 and 3240 (N—H), 1740 and 1650 (C=O) cm^{-1}; NMR [270 MHz, $(CD_3)_2SO$] δ 1.51 (m, H-5_{ax}), 2.13 (dt, H-5_{eq}, $J_{5,5}$ = 13.2 Hz, $J_{4,5}$ = $J_{5,6}$ = 4.4 Hz), 3.1–3.4 (m, 3 H + solvent), 3.66 (s, CO_2CH_3), 4.06 (dd, H-4, $J_{4,5ax}$ = 10.3 Hz), 6.07 (s, NH), 6.18 (s, NH). Anal. ($C_7H_{12}N_2O_4$) C, H, N.

***trans*-4-Carboxy-6-(hydroxymethyl)-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*trans*-2).** A solution of 118 mg (0.63 mmol) of *trans*-**6** in 1 mL of 1 N LiOH was allowed to stand for 1 h and then desalted by passage through a column of Dowex-50X8 (H^+), eluting with water. The concentrated eluate was recrystallized from 2-propranol to yield 48 mg (44%) of *trans*-**2** as a

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white solid: mp 178 °C dec; IR 3340 (N—H), 1680 (C=O) cm^{-1}; NMR (360 MHz, D_2O) δ 2.03 (m, H-5_{ax}), 2.20 (dt, H-5_{eq}, $J_{5,5}$ = 13.7 Hz, $J_{4,5} = J_{5,6}$ = 4.4 Hz), 3.48 (m, H-6), 3.62 (m, CH_2OH), 4.27 (t, H-4). Anal. ($C_6H_{10}N_2O_4$) C, H, N.

***cis*-4-Carboxy-6-(hydroxymethyl)-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*cis*-2).** In a similar manner 66 mg (0.35 mmol) of the *cis*-6 was converted to 41 mg (67%) of a white solid: mp 250–300 °C (slow dec); IR 3280 (N—H), 1710 and 1610 (C=O) cm^{-1}; NMR (360 MHz, D_2O) δ 1.80 (m, H-5_{ax}), 2.32 (dt, H-5_{eq}, $J_{5,5}$ = 13.2 Hz and $J_{4,5} = J_{5,6}$ = 4.5 Hz), 3.55 (m, H-6), 3.62 (m, CH_2OH), 4.24 (dd, H-4, $J_{4,5ax}$ = 10.1 Hz), 5.99 (s, NH), 6.07 (s, NH). Anal. Calcd for $C_6H_{10}N_2O_4$: C, 41.38; H, 5.79; N, 16.08. Found: C, 41.91; H, 5.71; N, 16.69 ($C_6H_{10}N_2O_4 \cdot 2H_2O$).

***trans*-4-Carbomethoxy-6-[(thioacetoxy)methyl]-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*trans*-7).** To a stirred solution of 2.1 g (8.0 mmol) of triphenylphosphine in 20 mL of THF at 0 °C was slowly added 1.6 mL (8.0 mmol) of diisopropyl azodicarboxylate producing a copious precipitate. Thirty minutes later a solution containing 0.72 g (0.38 mmol) of *trans*-6, 0.57 mL (8.0 mmol) of thioacetic acid in 10 mL of DMF, and 20 mL of THF was added dropwise to the stirring suspension. The cooling bath was removed, and after 4 h at 20 °C, the solvent was removed at reduced pressure. The resulting solid was triturated ($CHCl_3$–MeOH, 20:1) and filtered to give 540 mg of product. Chromatography (20:1, 10:1 $CHCl_3$–MeOH) resulted in the isolation of additional 7 (237 mg, total yield 83%). Recrystallization (MeOH) gave 600 mg of pure *trans*-7 as a white solid: mp 201–202 °C; IR 3280 (N—H), 1760, 1745, and 1695 (C=O) cm^{-1}; NMR (270 MHz, CD_3OD) δ 1.91 (ddd, H-5_{ax}, $J_{5,5}$ = 13.2 Hz, $J_{4,5}$ = 9.6 Hz, $J_{5,6}$ = 5.1 Hz), 2.11 (dt, H-5_{eq}, $J_{4,5} = J_{5,6}$ = 4.2 Hz), 2.36 (s, $SCOCH_3$), 3.07 (m, CH_2S), 3.49 (m, H-6), 3.75 (s, CO_2CH_3), 4.16 (t, H-4). Anal. ($C_9H_{14}N_2O_4S$) C, H, N, S.

***cis*-4-Carbomethoxy-6-[(thioacetoxy)methyl]-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*cis*-7).** In a similar procedure 148 mg of *cis*-6 afforded 143 mg (74%) of pure *cis*-7: mp 161 °C; IR 3400 (N—H), 1755 and 1680 (C=O) cm^{-1}; NMR (270 MHz, CD_3OD) δ 1.68 (dt, H-5_{ax}, $J_{5,5}$ = 13.1 Hz, $J_{4,5} = J_{5,6}$ = 10.2 Hz), 2.29 (dt, H-5_{eq}, $J_{4,5} = J_{5,6}$ = 4.5 Hz), 2.36 (s, $SCOCH_3$), 3.06 (m, CH_2S), 3.64 (m, H-6), 3.77 (s, CO_2CH_3), 4.17 (dd, H-4). Anal. ($C_9H_{14}N_2O_4S$) C, H, N, S.

***trans*-4-Carboxy-6-(mercaptomethyl)-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*trans*-1).** Compound *trans*-7 (322 mg, 1.31 mmol) was dissolved in 4 mL each of MeOH and 1 N LiOH and then acidified 1 h later with 6 N HCl. The solvent was removed at reduced pressure and the resulting residue recrystallized from H_2O–MeOH to yield 217 mg (87%) of *trans*-1 as a white solid: mp 194 °C dec; IR 1710 and 1650 (C=O) cm^{-1}; NMR (360 MHz, D_2O) δ 2.06 (ddd, H-5_{ax}, $J_{5,5}$ = 13.7 Hz, $J_{4,5}$ = 5.3 Hz, $J_{5,6}$ = 9.5 Hz), 2.23 (dt, H-5_{eq}, $J_{4,5} = J_{5,6}$ = 5.3 Hz), 2.68 (m, CH_2SH), 3.47 (m, H-6), 4.22 (t, H-4). Anal. ($C_6H_{10}N_2O_3S$) C, H, N, S.

***cis*-4-Carboxy-6-(mercaptomethyl)-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one (*cis*-1).** In a similar manner 133 mg of *cis*-7 gave 61 mg (60%) of *cis*-1 as a white solid: mp 201–202 °C dec; IR 3300 (N—H), 1715 and 1610 (C=O) cm^{-1}; NMR (360 MHz, D_2O) δ 1.89 (dt, H-5_{ax}, $J_{5,5}$ = 13.3 Hz and $J_{4,5} = J_{5,6}$ = 9.6 Hz), 2.39 (dt, H-5_{eq}, $J_{4,5} = J_{5,6}$ = 4.4 Hz), 2.69 (m, CH_2SH), 3.64 (m, H-6), 4.22 (dd, H-4). Anal. ($C_6H_{10}N_2O_3S$) C, H, N, S.

Enzymology. Source and Assay of Dihydroorotase. The dihydroorotase used in these studies was isolated from 165-23 cells, a line of SV40-transformed Syrian hamster kidney cells that are highly resistant to PALA.[22] The enzyme was purified as the trifunctional protein CAD by the method of Coleman[23] with several minor modifications as described.[24] The purified enzyme could be stored indefinitely in 20 mM Hepes (pH 7.4), 4 mM aspartate, 0.1 mM EDTA, 1 mM DTT, 30% (v/v) dimethyl sulfoxide, and 5% (w/v) glycerol at −80 °C. Initial velocities for dihydroorotase were determined in the more easily measurable reverse direction, that is, the enzyme-catalyzed hydrolysis of dihydro-L-orotate to *N*-carbamoyl-L-aspartate. Inhibition constants obtained for competitive inhibitors at a given pH should be invariant for either reaction direction for the unireactant enzyme. Initial velocities were determined in 0.167 M Tris·HCl (pH 8.5 and 7.4) at 37 °C.[25] Solutions (0.6 mL) containing buffer, various concentrations of dihydro-L-orotate, and inhibitors were preincubated at 37 °C for 5 min, followed by the initiation of reaction by the addition of dihydroorotase. Compound *cis*-1 was added to reaction mixtures just prior to the addition of enzyme. Levels of the product *N*-carbamoyl aspartate were determined after reaction for 30 min by the colorimetric method of Prescott and Jones.[26] Blank samples contained no enzyme. Duplicate or triplicate determinations were made for each data point.

The inhibitory properties of the synthetic compounds **1–3** were initially assessed at micromolar concentrations in the presence of dihydro-L-orotate (0.1 mM) and dihydroorotase. Initial rate data (in which less than 10% of dihydro-L-orotate had been hydrolyzed), apparently conforming to competitive inhibition, were fitted to eq 1 using the Fortran program of Cleland.[27] In eq 1, v is the measured initial rate, V is the maximal rate, V_{max}, K is the Michaelis constant for dihydro-L-orotate, I is the inhibitor concentration, A is dihydro-L-orotate concentration, and K_{is} is the slope inhibition constant or K_i. The concentration of free

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (1)$$

thiol in solutions of *cis*-1 was determined with 2,2′-dithiobis(5-nitrobenzoic acid) (DTNB) according to the procedure of Habeeb.[28] The apparent rate of disappearance of thiol in a 1 mM solution of *cis*-1 (k = 0.0033 min^{-1} in 0.2 M Tris, pH 8.5, 37 °C) was reflected in the rate of change of the compound's absorbance at 269 nm (k = 0.0042 min^{-1}). No change in absorbance at 269 nm could be detected in a solution containing 1 mM *cis*-1 upon the addition of 1 mM DTT, indicating protection by DTT of *cis*-1 against its decomposition.

Biological Evaluation. Antiproliferative activity of test compounds was assessed against B16-F10 murine melanoma cells growing in continuous monolayer culture as described previously.[29] Cells were in logarithmic growth phase at the time of drug addition and were exposed continuously for 72 h, at which time cell number was determined by a colorimetric method.[30] For assessment of the reversibility of drug effects by uridine, cells were exposed to various drug concentrations in the presence of medium containing 1 mM uridine (added at the time of drug addition). The appropriateness of the uridine concentration was determined from preliminary experiments in which it was found that the antiproliferative effects of PALA, even at high concentrations (i.e. 10 mM), were completely blocked by uridine at 0.1, 1, and 10 mM.

Registry No. (±)-*trans*-1, 114132-83-1; (±)-*cis*-1, 114132-84-2; (±)-*trans*-2, 114132-79-5; (±)-*cis*-2, 114132-80-8; (*d,l*)-*trans*-3, 114132-75-1; (*meso*)-*cis*-3, 114132-76-2; (*d,l*)-4, 114132-72-8; (*meso*)-4, 114182-30-8; (*d,l*)-*trans*-5, 114132-73-9; (*meso*)-*cis*-5, 114132-74-0; (±)-*trans*-6, 114132-77-3; (*d,l*)-*trans*-6 (diol), 114155-58-7; (±)-*cis*-6, 114132-78-4; (*meso*)-*cis*-6 (diol), 114155-59-8; (±)-*trans*-7, 114132-81-9; (±)-*cis*-7, 114132-82-0; dihydroorotase, 9024-93-5.

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