Synthesis and Biological Evaluation of *N*-[4-(2-*trans*-[([2,6-Diamino-4(3*H*)-oxopyrimidin-5-yl]methyl)thio]cyclobutyl)benzoyl] -L-glutamic acid a Novel 5-Thiapyrimidinone Inhibitor of Dihydrofolate Reductase

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The synthesis and biological evaluation of *N*-[4-(2-*trans*-[([2,6-diamino-4(3*H*)-oxopyrimidin-5-yl]methyl)thio]cyclobutyl)benzoyl]-L-glutamic acid (**1**) is reported. Compound **1** is a potent dihydrofolate reductase (DHFR) inhibitor (K_i = 12 n*M*) with excellent *in vitro* cell culture growth inhibition (L1210, IC_{50} = 29 n*M*). Protection experiments showed that the cell growth inhibitory activity was due to DHFR inhibition. The key step in the synthesis was the coupling of a cyclobutylmethylthiol with the 5-bromo-2,6-diamino-4-oxopyrimidine **8**.

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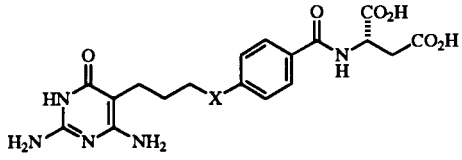
Introduction.

The essential vitamin folic acid in its various forms is utilized as a cofactor by a large number of metabolic enzymes in the transfer of one-carbon units [1]. Antagonists of folic acid have long been seen as clinically useful antitumor agents. The classical, glutamate containing, antifolates have seen a resurgence in the recent past due in part to the interesting clinical activity of a number of second and third generation inhibitors of thymidylate synthase (TS), glycinamide ribonucleotide formyl transferase (GARFT), and dihydrofolate reductase (DHFR) [2,3].

We have had an ongoing interest in the design and synthesis of novel inhibitors of GARFT and as part of this program have synthesized the glutamate containing 5-thiapyrimidinone **1**. This compound is related in part to the previously reported acyclic folate analogues **2a** and **2b** shown in Figure 1 [4,5]. Compound **1** has as its linker between the benzoylglutamate and the pyrimidinone a conformationally constrained *trans* substituted cyclobutyl group. Biological testing revealed that compound **1** was indeed a modest inhibitor of GARFT, but more importantly was a potent and selective inhibitor of DHFR. The synthesis and biological activity of compound **1** are herein reported.



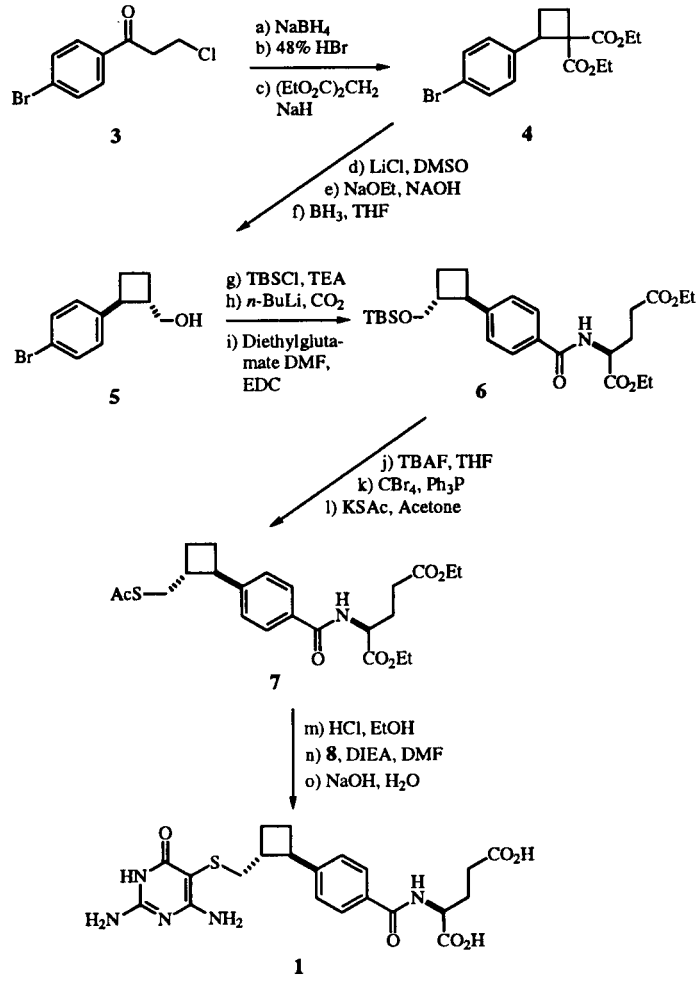
2a X = NH
2b X = CH_2

Figure 1

Chemistry.

The synthesis of compound **1** is shown in Scheme 1.

Scheme 1



The synthesis begins with the commercially available chloropropanone **3** [6], and the cyclobutanedicarboxylate **4** was prepared by double alkylation of diethyl malonate

Table I

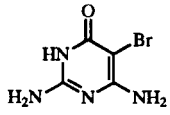
Biochemical and Biological Evaluation of Compound **1** [a]

Human TS K_i, μM	Human GARFT K_i, μM	Human DHFR K_i, μM	L1210 IC_{50}, μM	CCRF-CEM IC_{50}, μM	Thymidine Shift [b]	Hypoxanthine Shift [c]	Thymidine/ Hypoxanthine Shift [d]
40	0.93	0.012	0.029	0.077	4.1	13.1	>5334

[a] See Experimental for a detailed description. [b] Expressed as the ratio of the L1210 IC_{50} in the presence of 20 μM thymidine divided by the IC_{50} with no thymidine added. [c] Expressed as the ratio of the L1210 IC_{50} in the presence of 100 μM of hypoxanthine divided by the IC_{50} with no hypoxanthine added. [d] Expressed as the ratio of the L1210 IC_{50} in the presence of both 20 μM thymidine and 100 μM of hypoxanthine divided by the IC_{50} with neither added.

using the bromochloropropane intermediate in a 44% yield from **3**. Hydrolysis and decarboxylation provided the *trans* substituted cyclobutane acid which was reduced with borane to give the pure *trans* hydroxymethylcyclobutane **5** in a 54% yield from **4**. This sequence is similar to the previously described methods used to prepare 2-phenylcyclobutylmethanol [7,8]. The *para* carboxy functionality was introduced by first protecting the primary hydroxy group as the *t*-butyldimethylsilyl ether followed by lithiation and trapping with gaseous carbon dioxide. The crude carboxylic acid was peptide coupled to diethyl-L-glutamate to give diester **6** in 35% yield from alcohol **5**. The poor yield through these three steps was due to a 37% conversion in the peptide coupling step.

Introduction of the sulfur atom was accomplished by deprotection of the silyl group with tetrabutylammonium fluoride and conversion of the resulting alcohol to the bromide using carbon tetrabromide and triphenylphosphine followed by displacement with potassium thioacetate to give compound **7** in a 53% overall yield from ether **6**. The thiol used in the key coupling step was prepared by acid hydrolysis of the acetyl group. Base catalyzed coupling of the thiol to 5-bromo-2,6-diamino-4(3*H*)-pyrimidinone (**8**) [9] using diisopropylethylamine in dimethylformamide proceeded in 27% yield [10]. Base hydrolysis of the ethyl esters completed the synthesis of diacid **1**.



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Biology and Biochemistry.

Compound **1** was tested for inhibition of the three human folate binding enzymes TS, GARFT, and DHFR. Shown in Table I are the results indicating that compound **1** had weak TS inhibition (K_i = 40 μM), moderate GARFT inhibition (K_i = 0.93 μM), and potent DHFR inhibition (K_i = 12 nM).

Compound **1** was also tested against L1210 and CCRF-CEM cells and displayed potent cell growth inhibition against both cell lines with IC_{50} values of 29 nM and 77 nM respectively. Cell growth inhibition protection experiments were initiated to elucidate the intracellular site of action using thymidine, hypoxanthine, and a combination of the two. As shown in Table I, neither thymidine nor hypoxanthine alone protected against cell growth inhibition activity, thus eliminating TS and GARFT as the site of action, but the combination of both thymidine and hypoxanthine gave complete protection. These results are consistent with that seen with DHFR inhibitors [11] and coincides well with the potent *in vitro* enzyme DHFR inhibition shown by compound **1**.

In conclusion, we have described the synthesis and biological evaluation of *N*-[4-(2-*trans*-[([2,6-diamino-4(3*H*)-oxopyrimidin-5-yl]methyl)thio]cyclobutyl)benzoyl]-L-glutamic acid (**1**) a novel and potent inhibitor of human DHFR. Cell culture protection experiments have established that the potent cell growth inhibition activity is consistent with DHFR inhibition.

EXPERIMENTAL

Proton magnetic resonance spectra were determined using a General Electric QE-300 spectrometer operating at a field strength of 300 MHz. Chemical shifts are reported in parts per million (δ) and setting the references such that in deuteriochloroform the chloroform is at 7.26 ppm and in DMSO-d_6 the DMSO is at 2.49 ppm. Infrared absorption spectra were taken on either a Perkin-Elmer 457 spectrometer or a MIDAC Corporation FTIR. Elemental microanalysis were performed by Atlantic Microlab Inc., Norcross, Georgia. *N,N*-Dimethylformamide (DMF) was dried over activated (250°) 4-Å molecular sieves; *N,N*-dimethylacetamide (DMA) (Aldrich Gold Label grade) was similarly dried. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl under nitrogen. Ether refers to diethyl ether and DIEA refers to diisopropylethylamine. Flash chromatography was performed using silica gel 60 (Merck Art 9385). Thin layer chromatographs (tlc) were performed on precoated sheets of silica 60 F_{254} (Merck Art 5719). Melting points were determined on a Mel-Temp apparatus and are uncorrected.

1-(4-Bromophenyl)-3-chloro-1-propanol.

A solution of 44.56 g (0.18 mole) of 1-(4-bromophenyl)-3-chloro-1-propanone (**3**) [6] dissolved in 150 ml of THF was diluted with 150 ml of ethanol. To this cooled (-10°) solution was added 7.19 g (0.19 mole) of sodium borohydride, in portions, as the solid over a 10 minute interval. The reaction mixture was stirred for an additional 10 minutes at -5°, then cautiously poured into a mixture of 500 ml of saturated, aqueous ammonium chloride and 250 g of ice. The layers were separated and the aqueous phase was extracted with ether (2 x 250 ml). The combined organic extracts were dried (sodium sulfate) and evaporated to give 44.5 g of a brown gum which was purified on a silica gel column (7.5 cm x 22 cm). Elution of the column with hexane:ethyl acetate (85:15, v:v) and evaporation of the appropriate fractions gave 42.26 g (94%) of a yellow oil; ^{1}H nmr (deuteriochloroform): δ 7.49 (d, 2H, J = 8.4 Hz), 7.25 (d 2H, J = 8.4 Hz), 4.93 (dd, 1H, J = 4.6, 8.5 Hz), 3.74 (ddd, 1H, J = 5.4, 8.3, 11.0 Hz), 3.58-3.51 (m, 1H), 2.25-2.14 (m, 1H), 2.10-1.99 (m, 1H).

Anal. Calcd. for $C_9H_{10}OBrCl$: C, 43.32; H, 4.04. Found: C, 43.21; H, 4.08.

1-Bromo-1-(4-bromophenyl)-3-chloropropane.

A mixture of 42.26 g (0.17 mole) of 1-(4-bromophenyl)-3-chloro-1-propanol and 550 ml of 48% aqueous hydrobromic acid was stirred at ambient temperature for 3 hours, then cautiously poured into a mixture of 150 g of potassium carbonate and 1 kg of ice. Additional solid potassium carbonate (~150 g) was cautiously added, portionwise, to this aqueous mixture to attain a neutral *p*H. The resultant mixture was extracted with ether (2 x 350 ml). The combined organic extracts were dried (magnesium sulfate) and evaporated to give 52 g of a yellow oil which was purified on a silica gel column (7.5 cm x 21 cm). Elution of the column with hexane:ethyl acetate (95:5, v:v) and evaporation of the appropriate fractions gave 49.59 g (94%) of a yellow oil; ^{1}H nmr (deuteriochloroform): δ 7.49 (d, 2H, J = 8.5 Hz), 7.29 (d, 2H, J = 8.5 Hz), 5.17 (dd, 1H, J = 5.7, 9.0 Hz), 3.72 (ddd, 1H, J = 5.0, 7.9, 11.2 Hz), 3.60-3.52 (m, 1H), 2.73-2.61 (m, 1H), 2.49-2.37 (m, 1H).

Anal. Calcd. for $C_9H_9Br_2Cl$: C, 34.60; H, 2.90. Found: C, 34.69; H, 2.88.

Diethyl 2-(4-Bromophenyl)cyclobutane-1,1-dicarboxylate (**4**).

A solution of 49.59 g (0.16 mole) of 1-bromo-1-(4-bromophenyl)-3-chloropropane and 27.22 g (0.17 mole) of diethyl malonate in 550 ml of anhydrous 1,4-dioxane was heated to reflux, under an argon atmosphere, prior to the cautious addition of 6.6 g (0.165 mole) of sodium hydride (60% by weight mineral oil dispersion), in portions, over a 10 minute interval. After one hour at reflux, an additional 6.6 g (0.165 mole) of sodium hydride was added to the reaction mixture which was heated at reflux for a further 5 hours. Upon cooling to room temperature, the crude reaction mixture was filtered and the insoluble material was washed with ether (3 x 250 ml). Evaporation of the combined filtrates gave 60 g of a yellow oil which was purified on a silica gel column (7.5 cm x 28 cm). Elution of the column with hexane:ethyl acetate (97:3, v:v) and evaporation of the appropriate fractions gave 28.35 g (50%) of a colorless oil; ^{1}H nmr (deuteriochloroform): δ 7.40 (d, 2H, J = 8.5 Hz), 7.18 (d, 2H, J = 8.5 Hz), 4.33-4.17 (m, 3H), 3.85-3.66 (m, 2H), 2.71-2.49 (m, 2H), 2.30-2.10 (m, 2H), 1.27 (t, 3H, J = 7.1 Hz), 0.82 (t, 3H, J = 7.1 Hz).

Anal. Calcd. for $C_{16}H_{19}O_4Br$: C, 54.10; H, 5.39; Br, 22.49. Found: C, 54.19; H, 5.34; Br, 22.63.

trans-1-(4-Bromophenyl)-2-(hydroxymethyl)cyclobutane (**5**).

A mixture of 14.21 g (40 mmoles) of diethyl 2-(4-bromophenyl)cyclobutane-1,1-dicarboxylate (**4**), 3.60 g (85 mmoles) of lithium chloride, 0.8 ml (44 mmoles) of water and 70 ml of DMSO was heated at reflux for 4 hours. After cooling to room temperature, this mixture was diluted with 350 ml of ether and 150 ml of hexane, then washed sequentially with 100 ml of brine, 5 x 100 ml of water and 100 ml of brine. The organic phase was dried (magnesium sulfate) and evaporated to give 8.17 g (72%) of an amber oil. This crude ethyl 2-(4-bromophenyl)cyclobutane-1-carboxylate was employed directly in the subsequent reaction without further purification.

A solution of 8.16 g (29 mmoles) of crude ethyl 2-(4-bromophenyl)cyclobutane-1-carboxylate in 80 ml of 2 *M* sodium ethoxide was stirred under argon at ambient temperature for 24 hours. This solution was then diluted with 50 ml of water and left stirring for an additional 48 hours. The ethanol was removed by concentration, *in vacuo*, and the aqueous residue was diluted with 100 ml of 1*N* sodium hydroxide. After stirring for a further 5 hours, the aqueous mixture was washed with 100 ml of hexane, then acidified to *p*H 1 by addition of 6*N* hydrochloric acid and extracted with 3 x 100 ml of ether. The combined ether extracts were dried (sodium sulfate) and evaporated to give 5.85 g (80%) of an orange oil which solidified on standing. This crude *trans*-2-(4-bromophenyl)cyclobutane carboxylic acid was employed directly in the subsequent reaction without any further purification.

To a cooled (-5°) solution of 5.61 g (22 mmoles) of crude *trans*-2-(4-bromophenyl)cyclobutane carboxylic acid in 90 ml of THF was added, dropwise, 25 ml of a 1.0 *M* solution of borane in THF. The reaction mixture was stirred for 1 hour at 0° and 15 hours at ambient temperature, under an argon atmosphere, then diluted with 150 ml of saturated, aqueous ammonium chloride. The layers were separated and the aqueous phase was extracted with 150 ml of ether. The combined organic extracts were dried (sodium sulfate) and evaporated to give 5.6 g of a yellow oil which was purified on a silica gel column (4 cm x 24 cm). Elution of the column with hexane:ethyl acetate (85:15, v:v) and evaporation of the appropriate fractions, gave 4.92 g (93%) of a pale yellow oil; ^{1}H nmr (deuteriochloroform): δ 7.41 (d, 2H, J = 8.4 Hz), 7.13 (d, 2H, J = 8.4 Hz), 3.69 (dd, 2H, J = 1.3, 5.9 Hz), 3.28-3.19 (m, 1H), 2.63-2.50 (m, 1H), 2.31-2.20 (m, 1H), 2.08-1.95 (m, 2H), 1.86-1.72 (m, 1H).

Anal. Calcd. for $C_{11}H_{13}OBr$: C, 54.79; H, 5.43; Br, 33.14. Found: C, 54.87; H, 5.46; Br, 33.04.

trans-1-(4-Bromophenyl)-2-[(*t*-butyldimethylsilyloxy)methyl]-cyclobutane.

A solution of 4.75 g (19.7 mmoles) of *trans*-1-(4-bromophenyl)-2-(hydroxymethyl)cyclobutane (**5**), 3 ml (21.5 mmoles) of triethylamine, 3.24 g (21.5 mmoles) of *t*-butyldimethylchlorosilane and 75 mg of 4-(dimethylamino)pyridine in 70 ml of methylene chloride was stirred at ambient temperature for 16 hours, then poured into 100 ml of water. The layers were separated and the organic phase was washed sequentially with 100 ml of 0.5 *N* hydrochloric acid, 100 ml of brine and 75 ml of saturated, aqueous sodium bicarbonate, then dried (magnesium sulfate) and evaporated to give 6.8 g of a yellow oil which

was purified on a silica gel column (4 cm x 25 cm). Elution with hexane:ethyl acetate (95:5, v:v) and evaporation of the appropriate fractions gave 6.64 g (95%) of a colorless oil; ^{1}H nmr (deuteriochloroform): δ 7.39 (d, 2H, J = 8.3 Hz), 7.13 (d, 2H, J = 8.3 Hz), 3.63 (dd, 2H, J = 1.7, 5.6 Hz), 3.30-3.21 (m, 1H), 2.56-2.47 (m, 1H), 2.28-2.17 (m, 1H), 2.03-1.92 (m, 2H), 1.89-1.71 (m, 1H), 0.88 (s, 9H), 0.03 (s, 6H).

Anal. Calcd. for $C_{17}H_{27}OBrSi$: C, 57.45; H, 7.66; Br, 22.48. Found: C, 57.60; H, 7.71; Br, 22.31.

Diethyl *N*-[4-(*trans*-2-[(*t*-Butyldimethylsilyloxy)methyl]cyclobutyl)benzoyl]-L-glutamate (**6**).

A solution of 1.42 g (4.0 mmoles) of *trans*-1-(4-bromophenyl)-2-[(*t*-butyldimethylsilyloxy)methyl]cyclobutane in 25 ml of THF was added, dropwise, to 3.4 ml of a precooled (-78°) solution of 2.5 *M* *n*-butyllithium in hexane. The reaction was warmed to -10° and dry carbon dioxide was bubbled through this mixture for 45 minutes. The crude reaction mixture was then poured into 50 ml of saturated, aqueous ammonium chloride and extracted with 2 x 50 ml of ether. The combined organic extracts were dried (sodium sulfate) and evaporated to give 1.35 g of a yellow gum. This crude 4-(*trans*-2[(*t*-butyldimethylsilyloxy)methyl]cyclobutyl)benzoic acid was employed directly in the subsequent reaction without further purification.

A solution of 1.35 g (4 mmoles) of the crude 4-(*trans*-2[(*t*-butyldimethylsilyloxy)methyl]cyclobutyl)benzoic acid, 595 mg (4.4 mmoles) of 1-hydroxybenzotriazole, 1.05 g (4.4 mmoles) of L-glutamic acid diethyl ester hydrochloride, 0.8 ml (4.6 mmoles) of diisopropylethylamine and 844 mg (4.4 mmoles) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in 15 ml of DMF was stirred at ambient temperature for 18 hours, then poured into 50 ml of brine, diluted with 20 ml of water and extracted with 3 x 30 ml of ether. The combined organic extracts were washed with water (40 ml), dried (magnesium sulfate) and evaporated to give 1.5 g of a yellow oil which was purified on a silica gel column (3 cm x 25 cm). Elution of the column with hexane:ethyl acetate (80:20, v:v) and evaporation of the appropriate fractions gave 742 mg (37%) of a colorless oil; ^{1}H nmr (deuteriochloroform): δ 7.74 (d, 2H, J = 8.2 Hz), 7.33 (d, 2H, J = 8.2 Hz), 6.95 (d, 1H, J = 7.6 Hz), 4.79 (ddd, 1H, J = 4.8, 7.6, 12.5 Hz), 4.24 (q, 2H, J = 7.1 Hz), 4.11 (q, 2H, J = 7.1 Hz), 3.65 (dd, 2H, J = 1.6, 5.6 Hz), 3.40-3.31 (m, 1H), 2.60-1.78 (m, 9H), 1.30 (t, 3H, J = 7.1 Hz), 1.22 (t, 3H, J = 7.1 Hz), 0.89 (s, 9H), 0.04 (s, 6H).

Anal. Calcd. for $C_{27}H_{43}NO_6Si$: C, 64.13; H, 8.57; N, 2.77. Found: C, 64.18; H, 8.73; N, 2.65.

Diethyl *N*-[4-(*trans*-2-[Hydroxymethyl]cyclobutyl)benzoyl]-L-glutamate.

To a solution of 1.73 g (3.4 mmoles) of diethyl *N*-[4-(*trans*-2-[(*t*-butyldimethylsilyloxy)methyl]cyclobutyl)benzoyl]-L-glutamate (**6**) in 20 ml of THF was added 9 ml of a 1.0 *M* solution of tetrabutylammonium fluoride in THF. The reaction was stirred for one hour at ambient temperature, then diluted with 40 ml of brine. The layers were separated and the aqueous phase was extracted with 3 x 25 ml of methylene chloride. The organic extracts were combined, washed with 2 x 40 ml of water, dried (sodium sulfate) and evaporated to give 2.3 g of a yellow gum which was purified on a silica gel column (4 cm x 24 cm). Elution of the column with hexane:ethyl acetate (40:60, v:v) and evaporation of the appropriate fractions gave 8.74 mg (65%) of a colorless gum, ^{1}H nmr (deuteriochloroform): δ 7.76 (d, 2H, J = 8.2 Hz), 7.33 (d, 2H, J = 8.2 Hz), 6.98 (d, 1H, J = 7.5 Hz),4.79 (ddd, 1H, J = 4.8, 7.5, 12.5 Hz), 4.24 (q, 2H, J = 7.1 Hz), 4.11 (q, 2H, J = 7.1 Hz), 3.72 (dd, 2H, J = 1.6, 5.9 Hz), 3.39-3.30 (m, 1H), 2.69-2.56 (m, 1H), 2.54-2.41 (m, 2H), 2.39-2.23 (m, 2H), 2.19-1.97 (m, 3H), 1.89-1.75 (m, 1H), 1.30 (t, 3H, J = 7.1 Hz), 1.22 (t, 3H, J = 7.1 Hz).

Anal. Calcd. for $C_{21}H_{29}NO_6$: C, 64.43; H, 7.47; N, 3 58. Found: C, 64.30; H, 7.50; N, 3.51.

Diethyl *N*-(4-[*trans*-2-(Bromomethyl)cyclobutyl]benzoyl)-L-glutamate.

To a cooled (-5°) solution of 906 mg (2.3 mmoles) of diethyl *N*-(4-[*trans*-2-(hydroxymethyl)cyclobutyl]benzoyl)-L-glutamate and 929 mg (2.8 mmoles) of carbon tetrabromide in 15 ml of methylene chloride was added a solution of 734 mg (2.8 mmoles) of triphenylphosphine in 10 ml of methylene chloride. The reaction mixture was stirred for 1 hour at 0°, then 15 hours at ambient temperature. The solvent was removed by concentration, *in vacuo,* to give 2.2 of a yellow gum which was purified on a silica gel column (4 cm x 24 cm). Elution of the column with hexane:ethyl acetate (70:30, v:v) and evaporation of the appropriate fractions gave 938 mg (89%) of a waxy, white, solid, mp 63-66°; ^{1}H nmr (deuteriochloroform): δ 7.76 (d, 2H, J = 8.2 Hz), 7.30 (d, 2H, J = 8.2 Hz), 7.01 (d, 1H, J = 7.5 Hz),4.78 (ddd, 1H, J = 4.8, 7.5, 12.5 Hz), 4.23 (q, 2H, J = 7.1 Hz), 4.11 (q, 2H, J = 7.1 Hz), 3.55 (dd, 1H, J = 5.9, 10.0 Hz), 3.46 (dd, 1H, J = 7.6, 10.0 Hz), 3.33-3.24 (m, 1H), 2.83-2.70 (m, 1H), 2.56-2.41 (m, 2H), 2.39-2.22 (m, 2H), 2.19-1.97 (m, 3H), 1.86-1.74 (m, 1H), 1.30 (t, 3H, J = 7.1 Hz), 1.22 (t, 3H, J = 7.1 Hz).

Anal. Calcd. for $C_{21}H_{28}NO_5Br$: C, 55.51; H, 6.21; N, 3.08; Br, 17.59. Found: C, 55.62; H, 6.19; N, 3.05; Br, 17.65.

Diethyl *N*-[4-(*trans*-2-[(Acetylthio)methyl]cyclobutyl)benzoyl]-L-glutamate (**7**).

A mixture of 1.73 g (3.8 mmoles) of diethyl *N*-(4-[*trans*-2-(bromomethyl)cyclobutyl]benzoyl)-L-glutamate and 868 mg (7.6 mmoles) of potassium thioacetate in 25 ml of acetone was heated at reflux for 25 minutes. After cooling to room temperature, the insoluble solid was removed by filtration. The filtrate was concentrated, *in vacuo,* and the residue obtained was partitioned between ether and water (30 ml each). The layers were separtated and the aqueous phase was extracted with 30 ml of ethyl acetate. The combined organic extracts were dried (magnesium sulfate) and evaporated to give 1.8 g of an orange gum which was purified on a silica gel column (4 cm x 25 cm). Elution of the column with hexane:ethyl acetate (70:30, v:v) and evaporation of the appropriate fractions gave 1.58 g (92%) of a yellow oil; ^{1}H nmr (deuteriochloroform): δ 7.76 (d, 2H, J = 8.3 Hz), 7.30 (d, 2H, J = 8.3 Hz), 6.97 (d, 1H, J = 7.5 Hz), 4.79 (ddd, 1H, J = 4.7, 7.5, 12.4 Hz), 4.24 (q, 2H, J = 7.1 Hz), 4.11 (q, 2H, J = 7.1 Hz), 3.25-3.15 (m, 1H), 3.12 (dd, 1H, J = 7.7, 13.5 Hz), 3.01 (dd, 1H, J = 7.4, 13.5 Hz), 2.66-1.90 (m, 11H), 1.77-1.68 (m, 1H), 1.30 (t, 3H, J = 7.1 Hz), 1.22 (t, 3H, J = 7.1 Hz).

Anal. Calcd. for $C_{23}H_{31}NO_6S$: C, 61.45; H, 6.95; N, 3.12; S, 7.13. Found: C, 61.25; H, 7.01; N, 3.10; S, 7.08.

Diethyl *N*-[4-(*trans*-2-(mercaptomethyl)cyclobutyl)]benzoyl)-L-glutamate.

A solution of 1.49 g (3.3 mmoles) of diethyl *N*-[4-*trans*-2-

[(acetylthio)methyl]cyclobutyl)benzoyl]-L-glutamate in 30 ml of 0.5 *N* ethanolic hydrogen chloride was heated at reflux for 2 hours. After cooling to room temperature, the reaction was diluted with 20 ml of water. The ethanol was removed by concentration, *in vacuo*, and the aqueous residue was extracted with 2 x 40 ml of ether. The combined organic extracts were dried (sodium sulfate) and evaporated to give 1.31 g (97%) of a pale orange oil, ^{1}H nmr (deuteriochloroform): δ 7.76 (d, 2H, J = 8.2 Hz), 7.31 (d, 2H, J = 8.2 Hz), 6.99 (d, 1H, J = 7.5 Hz), 4.79 (ddd, 1H, J = 4.7, 7.5, 12.4 Hz), 4.24 (q, 2H, J = 7.1 Hz), 4.11 (q, 2H, J = 7.1 Hz), 3.25-3.16 (m, 1H), 2.80-1.93 (m, 10H), 1.77-1.63 (m, 2H), 1.30 (t, 3H, J = 7.1 Hz), 1.22 (t, 3H, J = 7.1 Hz).

Anal. Calcd. for $C_{21}H_{29}NO_5S$: C, 61.89; H, 7.17; N, 3.44; S, 7.87. Found: C, 61.85; H, 7.20; N, 3.45; S, 7.94.

Diethyl *N*-[4-(*trans*-2-[([2,6-Diamino-4(3*H*)-oxopyrimidin-5-yl]thio)methyl]cyclobutyl)benzoyl]-L-glutamate.

Argon was bubbled through a suspension of 574 mg (2.8 mmoles) of 5-bromo-2,6-diamino-4(3*H*)-pyrimidinone (**8**) [9] and 1.23 g (3.0 mmoles) of diethyl *N*-(4-[*trans*-2-(mercaptomethyl)cyclobutyl]benzoyl)-L-glutamate in 25 ml of DMF for 45 minutes. To this mixture was added 1.1 ml of diispropylethylamine and the reaction was heated at 85° for 50 minutes. After cooling to room temperature, the reaction was diluted with 150 ml of brine and extracted with 120 ml of ethyl acetate. The organic extract was diluted with 40 ml of ether, washed with 6 x 100 ml of water, dried (sodium sulfate) and evaporated to give 1.2 g of a yellow gum which was purified on a silica gel column (4 cm x 22 cm). Elution of the column with chloroform:methanol (94:6, v:v) and evaporation of the appropriate fractions gave 404 mg (27%) of a white solid, mp 94-98°; ^{1}H nmr (deuteriochloroform): δ 7.73 (d, 2H, J = 8 .0 Hz), 7.25 (d, 2H, J = 8.0 Hz), 7.21 (d, 1H, J = 7.6 Hz), 6.18 (broad, 2H), 5.35 (broad s, 2H), 4.78 (ddd, 1H, J = 4.8, 7.6, 12.5 Hz), 4.22 (q, 2H, J = 7.1 Hz), 4.10 (q, 2H, J = 7.1 Hz), 3.18-3.09 (m, 1H), 2.80 (dd, 1H, J = 6.0, 12.6 Hz), 2.68 (dd, 1H, J = 7.5, 12.6 Hz), 2.56-2.41 (m, 2H), 2.37-1.93 (m, 6H), 1.73-1.63 (m, 1H), 1.29 (t, 3H, J = 7.1 Hz), 1.21 (t, 3H, J = 7.1 Hz).

Anal. Calcd. for $C_{25}H_{33}N_5O_6S \cdot 0.3H_2O$: C, 55.91; H, 6.31; N, 13.04; S, 5.97. Found: C, 55.96; H, 6.35; N, 12.83; S, 5.86.

N-[4-(*trans*-2-[([2,6-Diamino-4(3*H*)-oxopyrimidin-5-yl]thio)-methyl]cyclobutyl)benzoyl]-L-glutamic Acid (**1**).

A suspension of 336 mg (0.63 mmole) of diethyl *N*-[4-(*trans*-2-[([2,6-diamino-4(3*H*)-oxopyrimidin-5-yl]thio)methyl]-cyclobutyl)benzoyl]-L-glutamate in 25 ml of 1 *N* aqueous sodium hydroxide was stirred at ambient temperature for 18 hours, then filtered. The filtrate was acidified to *p*H 5 by addition of 6 *N* aqueous hydrochloric acid. The precipitate that formed was collected by filtration and washed with 2 x 10 ml of water to give 223 mg (74%) of a white solid, mp 159-163°; ^{1}H nmr (DMSO-d_6): δ 12.39 (broad, 2H), 10.31 (broad, 1H), 8.52 (d, 1H, J = 7.7 Hz), 7.78 (d, 2H, J = 8.2 Hz), 7.29 (d, 2H, J = 8.2 Hz), 6.66 (broad, 2H), 6.37 (broad, 2H), 4.37 (ddd, 1H, J = 4.9, 7.7, 9.6 Hz), 3.24-3.15 (m, 1H), 2.73 (dd, 1H, J = 4.8, 12.5 Hz), 2.57 (dd, 1H, J = 9.0, 12.5 Hz), 2.34 (t, 2H, J = 7.3 Hz), 2.19-1.86 (m, 6H), 1.73-1.63 (m, 1H).

Anal. Calcd. for $C_{21}H_{25}N_5O_6S \cdot 0.75H_2O$: C, 51.57; H, 5.46; N, 14.32; S, 6.56. Found: C, 51.54; H, 5.33; N, 14.28; S, 6.61.

Biochemical Assays.

Human thymidylate synthase (TS) activity was measured spectrophotometrically essentially by the method of Daron and Aull [12]. The racemic cofactor (6*R*, 6*S*)-5,10-methylenetetrahydrofolic acid was generated *in situ* by the reaction of tetrahydrofolate with formaldehyde [13]. The reaction mixture contained TS, 0-1.2 *mM* compound **1**, 50 μ*M* dUMP, 20 μ*M* racemic tetrahydrofolic acid, 15 *mM* formaldehyde, 25 *mM* magnesium chloride, 1 *mM* EDTA, and 20 *mM* 2-mercaptoethanol. Reactions were initiated by the addition of TS and were conducted at 30° for 5 minutes. The TS catalyzed production of dihydrofolate was monitored by the appearance of absorbance at 340 nm using a millimolar change in the extinction coefficient for the reaction of 6.4 cm^{-1}.

The inhibition constant (K_i) was determined by computer assisted non-linear least squares fitting of the initial rates to the michaelis equation describing competitive inhibition. The michaelis constant (K_m) for racemic methylene-tetrahydrofolate was determined independently by saturation in the presence of saturating dUMP to be 10 μM (data not shown). Compound **1** was assumed to be a competitive inhibitor although some human thymidylate synthase inhibitors have been demonstrated to display competitive, non-competitive, and mixed non-competitive inhibitory behavior [14-17]. This behavior is a result of the two non-equivalent binding sites on the dimeric TS protein and not due to non-specific sites of binding.

The glycinamide ribonucleotide formytransferase (GARFT) assay method of Young [18] was modified and used as described below. Reactions mixtures contained the catalytic domain of the human GARFT [19], 0-150 μ*M* compound **1**, 20 μ*M* α,β-GAR, 10 or 20 μ*M* N^{10}-formyl-5,8-dideazafolate (FDDF), 50 *mM* HEPES-KOH, *p*H 7.5, and 50 *mM* KCl. The reaction was initiated with the addition of enzyme and followed by monitoring the increase in absorbance at 294 nm at 20° using a change in the millimolar extinction coefficient for the reaction of 18.9 cm^{-1}.

The GARFT K_i for compound **1** was determined from the dependence of the steady-state catalytic rate upon inhibitor and substrate concentration. The type of inhibition observed was determined to be competitive with respect to FDDF by the dependence of the apparent K_i ($K_{i,app}$) on the concentration of FDDF and was shown to be described by $K_{i,app} = K_i + (K_i/K_m)[FDDF]$. The michaelis constant for FDDF, K_m, was determined independently by the dependence of the catalytic rate upon FDDF concentration and shown to be 0.6 μ*M* (data not shown). Data for both the K_m and K_i determinations were fitted by non-linear methods to the Michaelis equation or the Michaelis equation for competitive inhibition as appropriate.

Human dihydrofolate reductase (DHFR) activity was determined as previously described [20,21]. The loss of NADPH was monitored at 30° by the decrease in absorbance at 340 nm using a millimolar extinction coefficient for the reaction of 12.8 mM^{-1}. Reaction mixtures contained 50 *mM* MES, 25 *mM* Tris, 25 *mM* ethanolamine, *p*H 7.5, 100 *mM* NaCl, 0.02% sodium azide, 50 μ*M* NADPH and 20 μ*M* dihydrofolate. The DHFR K_i for compound **1** was determined from the dependence of the steady-state catalytic rate upon inhibitor and substrate concentration. The michaelis constant for DHF, K_m, was 0.1 μ*M*, as previously reported [22]. Initial rate data were fit by non-linear methods to the Michaelis equation for competitive inhibition.

Biological Assays.

L1210 cell growth inhibition was measured by a modification

of the method of Mosmann [23]. Mid-log phase cells were diluted to 18,500 cells/ml in fresh RPMI growth medium (Mediatech, Washington, DC) supplemented with dialyzed fetal calf serum (Hyclone Laboratories Inc., Logan, UT) and then aliquoted into columns 2 through 12 of 96 well microtiter plates. Column 1 was filled with the same volume, 135 µl, of fresh medium, without cells, for use as a blank. The plates were then placed in a 37°C, 5% air-carbon dioxide incubator. After 1 to 4 hours, plates were removed from the incubator followed by addition of drug, 15 µl/well in binary dilutions, to columns 12 to 4. Wells containing compound **1** were prepared in duplicate or quadruplicate on duplicate plates. 15 µl of media, without compound **1**, was added to the wells in column 1 of the plates. The cells were then returned to the incubator. On day 3, 50 µl of 0.8 mg/ml MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma catalog No. M2128) dissolved in tissue culture medium was added to each well of all plates after which cells were returned to the incubator. After 4 hours all plates were removed from the incubator and centrifuged at 1200 rpm for 7 minutes. Media was siphoned off and 150 µl of DMSO was added to each well of all plates. Plates were then mixed at slow speed on a vortex mixer for 1 hour in the dark at room temperature. The extent of metabolized MTT was measured spectrophotometrically at 540 nm on a Molecular Devices Vmax™ kinetic microplate reader. The concentration of drug required to reduce cell growth by 50% as measured by MTT metabolism was determined by interpolation between the O.D. (minus blank) immediately above and below 50% of control O.D. (minus blank). CEM cell growth inhibition was measured as for L1210 cells except that the exposure time was for 120 hours.

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