A Dideazatetrahydrofolate Analogue Lacking a Chiral Center at C-6, *N*-[4-[2-(2-Amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic Acid, Is an Inhibitor of Thymidylate Synthase

Edward C. Taylor* and Dietmar Kuhnt

Department of Chemistry, Princeton University, Princeton, New Jersey 08544

Chuan Shih, Sharon M. Rinzel, and Gerald B. Grindey

Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, Indiana 46285

Julio Barredo, Mehrdad Jannatipour, and Richard G. Moran

Department of Biochemistry, University of Southern California, Los Angeles, California 90033

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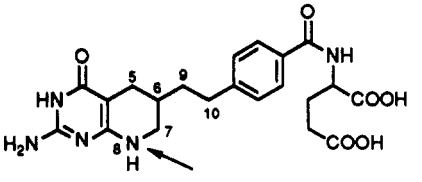
N-[4-[2-(2-Amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid (**15**), prepared in five steps from 2-pivaloyl-7-deazaguanine, has been found to be an antitumor agent with its primary site of action at thymidylate synthase rather than purine synthesis. This compound appears to be a promising candidate for clinical evaluation.

Introduction

5,10-Dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF, Lometrexol, 1) was synthesized in 1985[1] and immediately recognized as the first representative of a new class of potent folate antimetabolites that were active antitumor agents due to their effects on de novo purine synthesis.[2] Its target has been shown to be glycinamide ribonucleotide formyltransferase (GARFT, EC 2.1.2.1), the first folate cofactor-dependent formyl transfer step in the de novo purine biosynthetic pathway.[3] Because DDATHF is not



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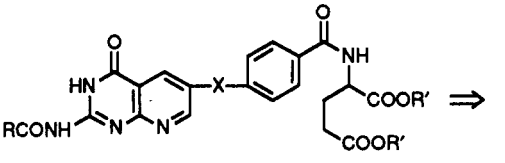
a dihydrofolate reductase (DHFR) inhibitor, it is fully active against tumors resistant to methotrexate due to amplification of the DHFR gene.[2b,4] DDATHF was advanced to clinical trial on the basis of its profile of therapeutic activity against several murine solid tumors and human tumor xenografts. It has demonstrated activity against human disease in the course of four Phase 1 clinical trials.[5]

The penultimate step in the majority of the DDATHF syntheses described thus far is reduction of an aromatic pyridine intermediate such as **2** (X = $-CH_2CH_2-$), resulting in a mixture of two DDATHF diastereomers differing in chirality at C-6.[1] Subsequent resolution of these mixtures



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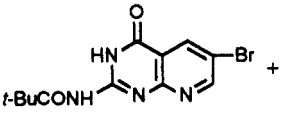

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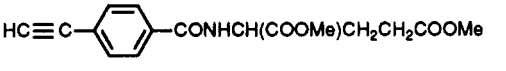

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leads to the 6*S* and 6*R* diastereomers,[3c] the latter of which

(1) Taylor, E. C.; Harrington, P. J.; Fletcher, S. R.; Beardsley, G. P.; Moran, R. G. Synthesis of the Antileukemic Agents 5,10-Dideazaaminopterin and 5,10-Dideaza-5,6,7,8-tetrahydroaminopterin. *J. Med. Chem.* **1985**, *28*, 914–921. (b) For a discussion of this development in antifolate chemistry, see: Taylor, E. C. New Pathways from Pteridines. Design and Synthesis of A New Class of Potent and Selective Antitumor Agents. *J. Heterocycl. Chem.* **1989**, *27*, 1–12.

(2) (a) Moran, R. G.; Taylor, E. C.; Beardsley, G. P. 5,10-Dideaza-5,6,7,8-tetrahydrofolic Acid (DATHF), A Potent Antifolate Inhibitory To de novo Purine Synthesis. *Proc. Am. Assoc. Cancer Res.* **1985**, *26*, 231. (b) Beardsley, G. P.; Taylor, E. C.; Shih, C.; Poore, G. A.; Grindey, G. B.; Moran, R. G. A New Class of Antifolates. 5,10-Dideazatetrahydrofolic Acid (DDATHF), An Inhibitor Of GAR Transformylase With Broad in vivo Activity. *Proc. Am. Assoc. Cancer Res.* **1986**, *27*, 259.

(3) (a) Beardsley, G. P.; Moroson, B. A.; Taylor, E. C.; Moran, R. G. A New Folate Antimetabolite, 5,10-Dideaza-5,6,7,8-tetrahydrofolate, Is A Potent Inhibitor Of de novo Purine Synthesis. *J. Biol. Chem.* **1989**, *264*, 328–333. (b) Moran, R. G.; Baldwin, S. W.; Taylor, E. C.; Shih, C. The 6S- and 6R-Diastereomers of 5,10-Dideaza-5,6,7,8-tetrahydrofolate Are Equiactive Inhibitors of de Novo Purine Synthesis. *J. Biol. Chem.* **1989**, *264*, 21047–21051. (c) Baldwin, S. W.; Tse, A.; Gossett, L. S.; Taylor, E. C.; Rosowsky, A.; Shih, C.; Moran, R. G. Structural Features of 5,10-Dideaza-5,6,7,8-tetrahydrofolate That Determine Inhibition of Mammalian Glycinamide Ribonucleotide Formyltransferase. *Biochemistry* **1991**, *30*, 1997–2006.

(4) Pizzorno, G.; Moroson, B. A.; Cashmore, A. R.; Taylor, E. C.; Beardsley, G. P. Biochemical Determinants Of Sensitivity And Resistance To 5,10-Dideaza-5,6,7,8-tetrahydrofolic Acid, DDATHF, In CCRF-CEM Cells. *Proc. Am. Assoc. Cancer Res.* **1988**, *29*, 281.

(5) (a) Ray, M.; Muggia, F. M.; Leichman, G. C.; Grunberg, S. G.; Dyke, R.; Moran, R. G. Phase I Study Of (6R) 5,10-Dideaza-5,6,7,8-tetrahydrofolate (Lometrexol): A Folate Antimetabolite Inhibitory To de novo Purine Synthesis. Submitted. (b) Young, C.; Currie, V.; Baltler, L.; Trochanowski, B.; Eton, O.; Dyke, D.; Bowsher, R. Phase 1 And Clinical Pharmacologic Study Of LY264618, 5,10-Dideazatetrahydrofolate. *Proc. Am. Assoc. Cancer Res.* **1990**, *31*, 177. (c) Nelson, R.; Butler, F.; Dugan, W., Jr.; Davis-lung, C.; Stone, M.; Dyke, R. Phase 1 Trial of LY264618 (Dideazatetrahydrofolate, DDATHF). *Proc. Am. Soc. Clin. Oncol.* **1990**, *9*, 293. (d) Pagani, O.; Sessa, C.; deJong, J.; Kem, H.; Hatty, S.; Schmitt, H.; Cavalli, F., Phase 1 Studies of Lometrexol (DDATHF) Given In Combination With Leucovorin. *Proc. Am. Assoc. Clin. Oncol.* **1992**, *11*, 109.

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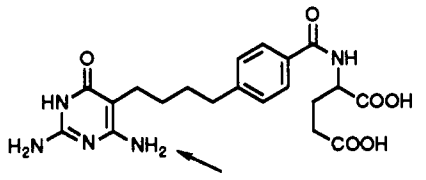
was selected for clincal trial. Cell culture and enzyme kinetic studies[3b,c] have shown that both DDATHF diastereomers are potent and near-equiactive inhibitors of de novo purine biosynthesis; however, they possess slightly different activities against different neoplasms in vivo. Thus, the 6*S* diastereomer shows little activity against B-16 melanoma and C3H mammary adenocarcinoma but is highly active against 6C3HED lymphosarcoma, while the 6*R* diastereomer shows excellent activity against all three tumors.[6] It is therefore of considerable interest that the enantiomerically homogeneous "open chain" DDATHF isomer **5**, in which the C-6 chiral center has been removed



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by excision of the C-7 methylene group, was inactive in vivo, although it was almost as active in vitro as the mixture of diastereomers of DDATHF.[7] Since studies on model compounds have indicated that an NH grouping attached to the pyrimidine C-6 position (arrows, formulas **1** and **5**) is mandatory for activity as an inhibitor of GARFT,[3c] we were particularly interested in examining an analogue possessing each of the following characteristics: (1) a left-hand heterocyclic moiety which would simulate the rigidity of the bicyclic 6-6 ring system of DDATHF, (2) the hydrogen-bonding NH grouping (arrows above) but (3) no chiral center at the position joining rings A/B to the ethano bridge. The present paper describes the synthesis of the pyrrolopyrimidine DDATHF analogue **15** which fulfills all of the above structural requirements. To our surprise, although **15** is quite active as an inhibitor of tumor growth in vitro and in vivo, it is almost inactive against GARFT and its activity is primarily the result of inhibition of thymidylate synthase.

Chemistry

We have recently described a convenient and convergent synthesis of DDATHF which gave the key intermediate **2** (R = *t*-Bu, X = –C≡C–) by a palladium-assisted carbon–carbon coupling of 2-pivaloyl-6-bromo-5-deazapterin (**3**) with dimethyl (4-ethynylbenzoyl)glutamate (**4**).[8] It appeared that our target analogue **15** might be available by a similar strategy, which would thus require a 7-halo-7-deazaguanine as the coupling partner. However, attempts to brominate 7-deazaguanine (**6**)[9a,b] proved fruitless; the reaction mixture turned deep blue and no brominated product(s) could be recovered from the reaction mixture. This blue product presumably arises as a consequence of oxidation; attempted nitrosation of 7-deazaguanine likewise produced a blue solution. In an attempt to circumvent these problems, 7-deazaguanine (**6**) was pivaloylated in order to improve its solubility,[8,10] and the resulting 2-pivaloyl derivative **7** was treated with bromine in acetic acid to give a mixture of unreacted starting material, and (in low yield) the 7- or 8-monobromo derivative (**8**) together with the 7,8-dibromo derivative **9**. However, both products were unstable and decomposed even at room temperature.

Attention was then turned to iodination. Treatment of **7** with 1 equiv of *N*-iodosuccinimide in DMF gave a mixture of the diiodo compound **10** and the 8- and 7-monoiodo derivatives (**11** and **12**, respectively), in addition to considerable starting material. However, increasing the amount of *N*-iodosuccinimide to 2.2 equiv led directly and in 89% yield to the 7,8-diiodo derivative **10**. This compound could be smoothly and regioselectively monodeiodinated with zinc and acetic acid to give the desired 2-pivaloyl-7-iodo-7-deazaguanine **12** (see Scheme I).[11]

The 7-iodo intermediate **12** was then successfully subjected to a palladium-catalyzed carbon–carbon coupling reaction with dimethyl (4-ethynylbenzoyl)glutamate (**4**)[8] utilizing tetrakis(triphenylphosphine)palladium, copper(I) iodide, and triethylamine in DMF as solvent. Selective hydrogenation of the triple bond in the resulting coupling product **13**, without reduction of the pyrrole ring, was then smoothly accomplished with hydrogen and 3% palladium-on-charcoal catalyst in a mixture of methylene chloride and methanol. Treatment of the resulting ethano-bridged intermediate **14** with 1 N sodium hydroxide at room temperature for 3 days resulted both in saponification of the ester functionalities and removal of the pivaloyl protecting/solubilizing grouping at N-2 to give the target DDATHF analogue **15**.

Biological Evaluation

Compound **15** inhibited the growth of both mouse L1210 and human CCRF-CEM lymphoblastic leukemic cells with half-maximum inhibition observed at 2.2 and 1.6×10^{-8} M, respectively (Figure 1). Growth inhibition by **15** was prevented either by the reduced folate compound folinic acid (leucovorin, (6-*R*,*S*)-5-formyltetrahydrofolate) (Figure 1B) or by the combination of thymidine and hypoxanthine as a source of thymidylate and purine nucleotides (Figure 1A,C). This constitutes strong evidence that the activity of **15** against tumor cells was the result of inhibition of folate metabolism. The concentration of **15** required for growth inhibition increased with increasing concentration of folinic acid in the medium, a clear indication of competition between **15** and reduced folates. Hypoxan-

(6) Shih, C.; Grindey, G. B.; Houghton, P. J.; Houghton, J. A. In Vivo Antitumor Activity of 5,10-Dideazatetrahydrofolic Acid (DDATHF) and Its Diastereomeric Isomers. *Proc. Am. Assoc. Cancer Res.* **1988**, *29*, 283.

(7) Shih, C.; Gossett, L. S.; Worzalla, J. F.; Rinzel, S. M.; Grindey, G. B.; Harrington, P. M.; Taylor, E. C. Synthesis and Biological Activity of Acyclic Analogues of 5,10-Dideaza-5,6,7,8-tetrahydrofolic Acid. *J. Med. Chem.* **1992**, *35*, 1109–1116.

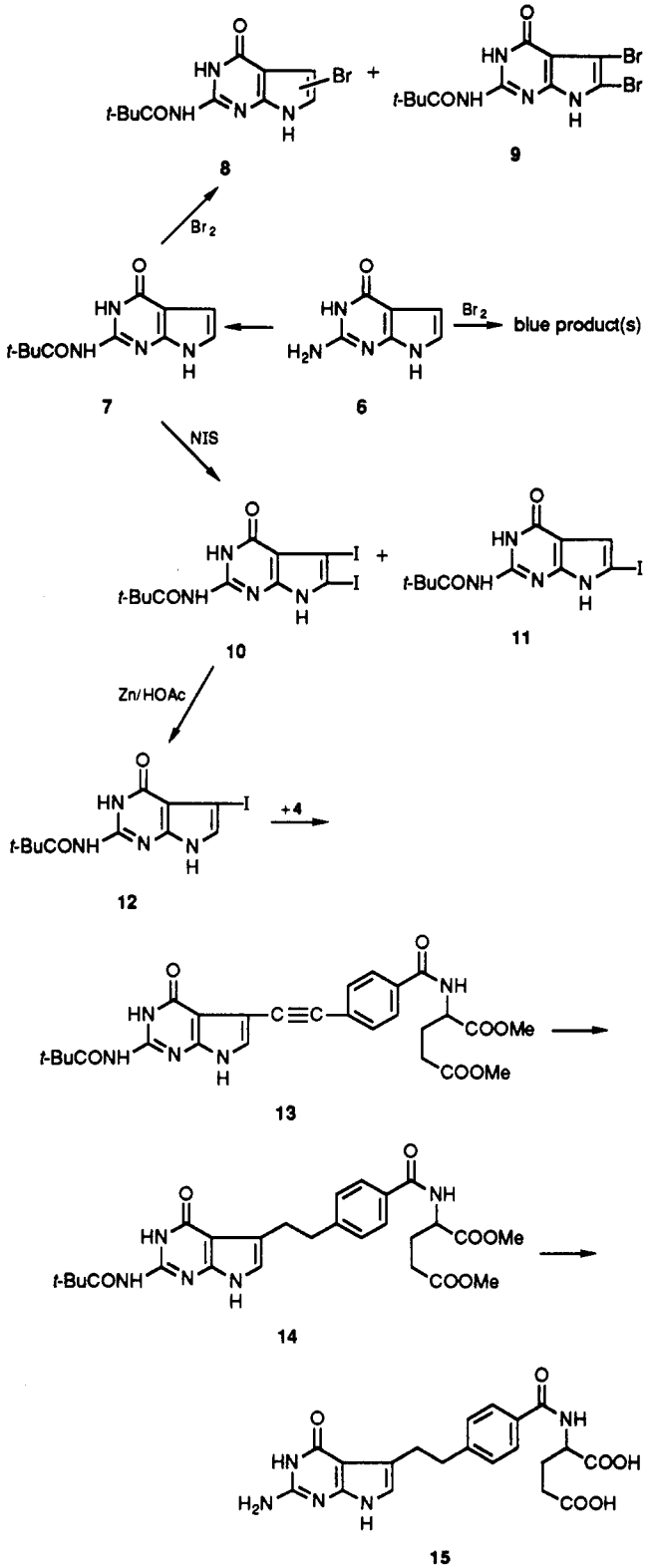
(8) Taylor, E. C.; Wong, G. S. W. Convergent and Efficient Palladium-Effected Synthesis of 5,10-Dideaza-5,6,7,8-tetrahydrofolic Acid (DDATHF). *J. Org. Chem.* **1989**, *54*, 3618–3629.

(9) (a) Davoll, J. Pyrrolo[2,3-*d*]pyrimidines. *J. Chem. Soc.* **1960**, 131–138. (b) Lüpke, U.; Seela, F. Mannich-Reaction of 2-Amino-3,7-dihydropyrrolo[2,3-*d*]pyrimidin-4-one, the Chromophore of Ribonucleoside "Q". *Chem. Ber.* **1977**, *110*, 1462–1469.

(10) The formation of soluble pivaloyl derivatives from insoluble amino-substituted heterocycles has been described earlier by us; see, for example: (a) Taylor, E. C.; Yoon, C.-M. A Convenient Synthesis of 6-Formyl-5-deazapterin. *Synth. Commun.* **1988**, *18*, 1187–1191. (b) Taylor, E. C.; Ray, P. S. Pteridines. 51. A New And Unequivocal Route to C-6 Carbon-Substituted Pterins and Pteridines. *J. Org. Chem.* **1987**, *52*, 3997–4000. (c) Taylor, E. C.; Hamby, J. M.; Shih, C.; Grindey, G. B.; Rinzel, S. M.; Beardsley, G. P.; Moran, R. G. Synthesis And Antitumor Activity Of 5-Deaza-5,6,7,8-tetrahydrofolic Acid (5-DATHF) and N^{10}-Substituted Analogues. *J. Med. Chem.* **1989**, *32*, 1517–1522.

(11) 2-Pivaloyl-7-deazaguanine (**7**) is thus considerably more reactive to electrophilic substitution than is 2-methylthio-6-methoxy-9-(deoxyribosyl)-7-deazapurine, which undergoes only mono-iodination with an excess of N-iodosuccinimide; Cocuzza, A. J. Total Synthesis Of 7-Iodo-2',3'-dideoxy-7-deazapurine Nucleosides, Key Intermediates In The Preparation Of Reagents For The Automated Sequencing Of DNA. *Tetrahedron Lett.* **1988**, *29*, 4061–4064.

Scheme I



thine or aminoimidazolecarboxamide alone could not protect leukemic cells from the growth-inhibitory effect of this agent (Figure 1A,C). This was unexpected, because such reversal experiments had indicated that most of the 5-deaza- and 5,10-dideazatetrahydrofolic acid analogues evaluated to date have been primarily inhibitors of purine synthesis. However, thymidine was able to substantially decrease the cytotoxicity of **15** at concentrations up to 2×10^{-4} M in CCRF-CEM cells (Figure 1A). Likewise, the growth inhibition of L1210 cells was reversed at low

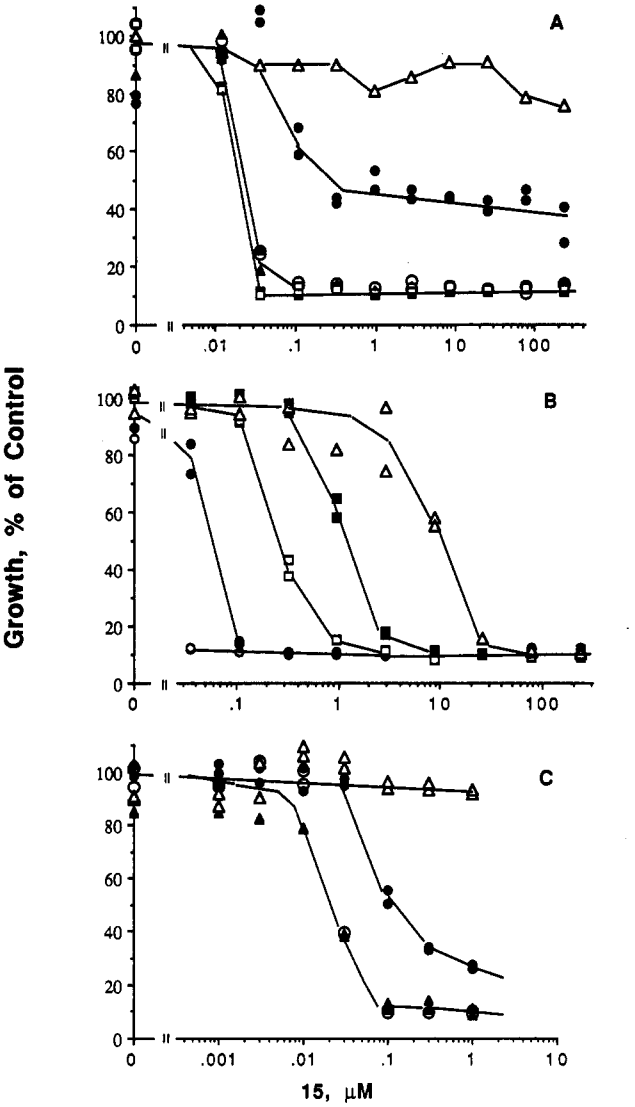


Figure 1. Inhibition of the growth of leukemic cells by compound **15**. The indicated concentrations of **15** were incubated with CCRF-CEM human (A, B) or L1210 mouse (C) leukemic cells for 72 h in the presence of the reduced folate, folinic acid (B) or of a source of purines and/or thymidylate (A, C). The compounds used were as follows: (A, C), no additions (○), 5 μM thymidine (●), 100 μM hypoxanthine (▲), 320 μM aminoimidazole carboxamide (□), or 100 μM hypoxanthine plus 5 μM thymidine (△); (B), folinic acid ((6-*R*,*S*)-5-formyltetrahydrofolate) at 0 (○), 0.05 (●), 0.5 (□), 1.6 (■), or 16 μM (△). In A and C, the data for **15** with either hypothanthine or aminoimidazole carboxamide are superimposed on that for **15**.

concentrations by thymidine alone, but could be prevented at higher concentrations only by a combination of thymidine and hypoxanthine (Figure 1C). These results suggested that the prime therapeutic target of this pyrrolopyrimidine antifolate is thymidylate synthase but that **15** (or its metabolites) has (have) inhibitory activity against other folate-requiring enzymes.

Compound **15** was found to be an inhibitor of recombinant mouse thymidylate synthase purified from a bacterial expression system.[12] Inhibition was competitive with variable 5,10-methylenetetrahydrofolate (Figure 2) with a $K_{i,slope}$ of 0.34 ± 0.15 μM (n = 3). In contrast, **15** had little inhibitory activity against GARFT purified from

(12) Zhang, H.; Cisneros, R. J.; Dunlap, R. B.; Johnson, L. F. Efficient Synthesis Of Mouse Thymidylate Synthase In Escherichia coli. *Gene* **1989**, *84*, 487–491.

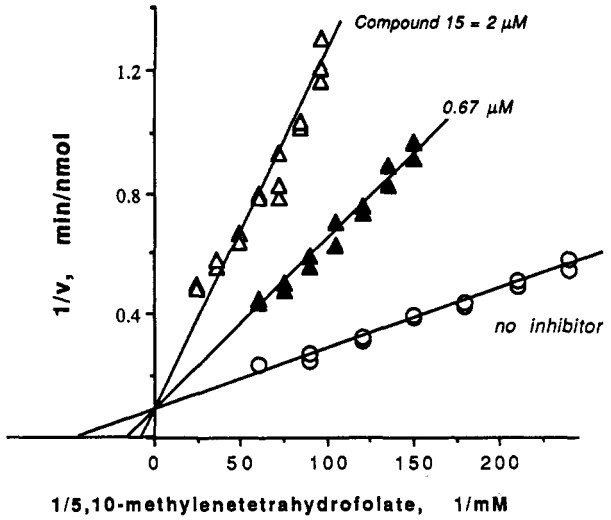


Figure 2. Inhibition of recombinant mouse thymidylate synthase by compound **15**. The inital rate of the thymidylate synthase reaction was measured spectrophotometrically as a function of the concentration of the substrate (6*R*)-5,10-methylenetetrahydrofolate in the presence or absence of **15**. This plot of the reciprocal of reaction rates vs the reciprocal of substrate concentration demonstrates that **15** competes with the folate substrate for binding to enzyme. Each point represents a single determination from a representative experiment.

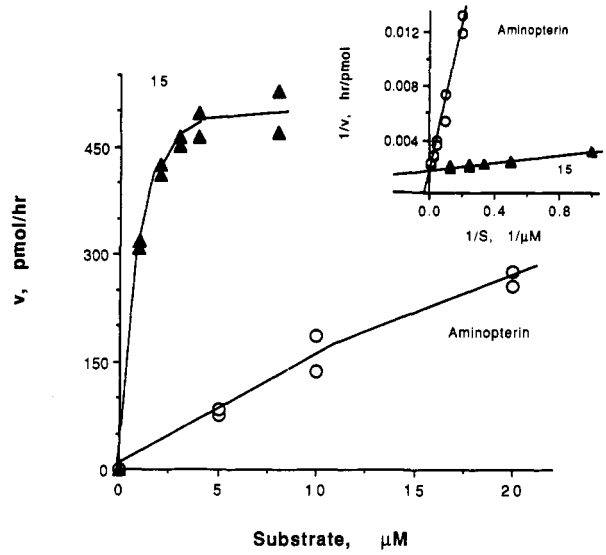


Figure 3. Substrate activity of compound **15** for mouse liver folylpolyglutamate synthetase. The rate of formation of diglutamate product was followed in vitro as previously described with the modifications listed in the text. The inset demonstrates that **15** and aminopterin have equivalent V_{max} values for this enzyme.

mouse L1210 cells (<30% inhibition at 30 μM; extrapolated K_i >8 μM). On the other hand, (6-*R*)DDATHF, which we have previously shown to be inhibitory to GARFT (K_i = 0.03 μM),[3b,c] did not inhibit mouse thymidylate synthase except at very high concentrations (<20% inhibition at 30 μM; extrapolated K_i >10 μM). Hence, these structural changes about the 5, 6, and 7 positions of DDATHF could specifically direct inhibition either to thymidylate synthase and away from GARFT or vice versa.

The potency of **15** against intact tumor cells growing in culture was much higher than would be expected from the K_i found for isolated mouse thymidylate synthase. This comparison suggested that metabolism of **15** to polyglutamate derivatives was involved in its antitumor activity. In support of this concept, it was found that compound **15** was an exceptionally efficient substrate for mouse liver folylpolyglutamate synthetase (Figure 3). Comparing the conversion of **15** to its diglutamate in an in vitro reaction with an internal standard of aminopterin, the maximum velocities of this reaction utilizing these two substrates were equivalent, but the K_m of **15** (0.8 ± 0.2 μM; n = 3) was much lower than that of aminopterin (21 μM)[13a] (Figure 3). The first-order rate constant (which we commonly express relative to that of folic acid)[13a] of **15** (400 ± 86) was substantially higher than that of methotrexate (0.9), aminopterin (10.1), CB3717 (2.7), or DDATHF (32.) and was in the range of those of the most efficient substrates we have studied to date, 5-deazatetrahydrofolate (170) and ICI-D1694.[3a,b,10c,13] In view of this substrate activity for FPGS, the pentaglutamate of **15** was synthesized and studied as an inhibitor of thymidylate synthase. The strength of binding of this compound to TS was too tight to allow measurement of a K_i using Michealis–Menten kinetics. However, an estimate of the tightness of this interaction was made by fitting inhibition data to the equation of Morrison for tight-binding inhibitors,[14] which takes into account depletion of inhibitor by binding to enzyme. Assuming competitive inhibition, we estimated the K_i as 3.4 ± 1.7 nM (n = 3), with the data fitting this model very well for 2 mol of binding sites per mole of enzyme, but poorly for a binding stoichiometry of 1:1.

Compound **15** was active against a thymidine kinase and hypoxanthine-guanine phosphoribosyltransferase-deficient murine lymphoma (LY5178Y/TK$^-$/HX$^-$) when administered ip daily for 8 consecutive days. Complete inhibition of the growth of the tumor was achieved at doses from 12.5 mg/kg to 200 mg/kg and an excellent therapeutic index is seen in this model. By comparison, the potent TS inhibitor, 10-propargyl-5,8-dideazafolic acid (PDDF, CB3717), was almost inactive at the highest tolerated dose (100 mg/kg) under the same protocol (Figure 4). Compound **15** is also very effective against the VRC5 and HXGC3 human colon xenografts in nude mice; good growth inhibition (>80%) was observed when mice were treated at 25 mg/kg and 50 mg/kg (ip, daily ×10 beginning 7 days after implantation of tumor).

In summary, substitution of a 5-membered pyrrole ring for the fused tetrahydropyridine ring of 5,10-dideazatetrahydrofolic acid (DDATHF) has led to the discovery of a new agent, **15** with a locus of action distinctly different from that of DDATHF. The primary enzymatic target of this compound is thymidylate synthase, with secondary effects elsewhere in the folate pathways observed at high drug concentrations. Excellent in vivo antitumor activities were observed against LY5178Y/TK$^-$/HX$^-$ tumors as well as two human colon xenografts. This novel 2-amino-4-oxopyrrolopyrimidine thus represents a new class of folate antimetabolite and is an interesting candidate for clinical evaluation.

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(14) Morrison, J. F. The Kinetics Of The Reversible Inhibition Of Enzyme Catalyzed Reactions By Tight-binding Inhibitors. *Biochem. Biophys. Acta* **1969**, *185*, 269–286.

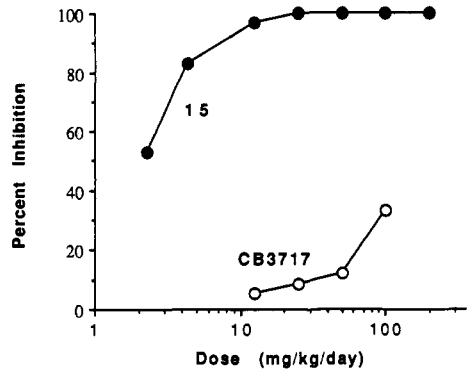

Figure 4. The comparative antitumor activity of compound 15 and CB 3717 against the L5178Y/TK⁻/HX⁻ tumor. The indicated doses of 15 and CB3717 were administered to groups of mice bearing a subline of the L5178Y lymphoma lacking expression of thymidine and hypoxanthine-guanine phosphoribosyltransferase.

Experimental Section

2-Pivaloyl-7-deazaguanine (7). A mixture of 3.0 g (0.02 mol) of 7-deazaguanine (6) and 8.4 g (0.07 mol) of pivaloyl chloride in 40 mL of pyridine was stirred for 30 min at 80–90 °C. The solution was evaporated to dryness and the residue was dissolved in 30 mL of methanol. Precipitation with 10% ammonia in water gave 4.2 g (89%) of 7, which was purified by filtration through silica gel with 8% methanol in CH_2Cl_2: mp 295 °C; ^1H NMR (d_6-DMSO) δ 1.20 (s, 9 H), 6.37 (d, J = 3.4 Hz, 1 H), 6.92 (d, J = 3.4 Hz, 1 H), 10.78 (s, 1 H), 11.56 (s, 1 H), 11.82 (s, 1 H). Anal. Calcd for $C_{11}H_{14}N_4O_2$: C, 56.40; H, 6.02; N, 23.92. Found: C, H, N.

7,8-Diiodo-2-pivaloyl-7-deazaguanine (10). To a solution of 4.7 g (20 mmol) of 7 in 200 mL of DMF was added 9.9 g (44 mmol) of *N*-iodosuccinimide, and the reaction mixture was stirred at room temperature for 18 h in the dark. Most of the DMF was then removed by evaporation under reduced pressure, and the remaining slurry (ca. 30 mL) was poured into 300 mL of water. The resulting precipitate was filtered off and dried in vacuo over P_2O_5; yield 8.7 g (89%). Chromatography of a sample of the crude product through a silica column with 2.5% methanol in CH_2Cl_2 gave the analytical sample: mp >250 °C dec (loses I_2); ^1H NMR (d_6-DMSO) δ 1.18 (s, 9 H), 10.85 (s, 1 H), 11.85 (s, 1 H), 12.42 (s, 1 H). Anal. Calcd for $C_{11}H_{12}N_4O_2I_2$: C, 27.18; H, 2.49; N, 11.53; I, 52.22. Found: C, H, N, I.

7-Iodo-2-pivaloyl-7-deazaguanine (12). To a mixture of 4.86 g (10 mmol) of crude 10 dissolved in 100 mL of glacial acetic acid and 25 mL of water was added 1.3 g (20 mmol) of zinc powder, and the mixture was stirred at room temperature for 18 h. The solution was then diluted with 500 mL of water and cooled and the resulting precipitate collected by suction filtration. The crude product was dried in vacuo over P_2O_5 to give 3.18 g (88%) of 12, a sample of which was purified by chromatography through a silica gel column using 2.5% methanol in CH_2Cl_2: mp >240 °C dec; ^1H NMR (d_6-DMSO) δ 1.20 (s, 9 H), 7.12 (d, J = 1.8 Hz, 1 H) 10.82 (s, 1 H), 11.79 (s, 1 H), 11.89 (s, 1 H). Anal. Calcd for $C_{11}H_{13}N_4O_2I$: C, 36.69; H, 3.64; N, 15.56; I, 35.24. Found: C, H, N, I.

***N*-[4-[2-[2-(Pivaloylamino)-3,4-dihydro-4-oxo-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl]ethynyl]benzoyl]-L-glutamic Acid Dimethyl Ester (13).** To a solution of 3.6 g (10 mmol) of crude, well-dried 7 in 40 mL of DMF were added 4.0 g (13.19 mmol) of dimethyl (4-ethynylbenzoyl)glutamate (4), 0.38 g of copper(I) iodide, 3 mL of triethylamine, and 1.0 g of tetrakis(triphenylphosphine)palladium. The mixture was stirred at room temperature for 2 h and poured into 500 mL of water. The precipitate was collected by filtration and air-dried. The crude product was refluxed in 200 mL of methanol, cooled, and filtered, and the collected solid was dissolved in 2 L of a mixture of 10% methanol in CH_2Cl_2 (2 L). Filtration through silica gave several very dark fractions and several colorless fractions which contained the desired product. The black fractions were again chromatographed, and the combined colorless eluates were evaporated under reduced pressure to give a crude solid which was recrystallized from 1:1 CH_3OH/CH_2Cl_2 to give 3.5 g (65%) of 13: mp 280–285 °C dec; ^1H NMR (d_6-DMSO) δ 1.21 (s, 9 H), 1.96–2.15 (m, 2 H), 2.44 (t, J = 7.5 Hz, 2 H), 3.56 (s, 3 H), 3.62 (s, 3 H), 4.40–4.45 (m, 1 H), 7.43 (s, 1 H), 7.53 (d, J = 8.4 Hz, 2 H), 7.87 (d, J = 8.4 Hz, 2 H), 8.82 (d, J = 7.4 Hz, 1 H), 10.95 (s, 1 H), 11.95 (s, 1 H). Anal. Calcd for $C_{27}H_{29}N_5O_7$: C, 60.56; H, 5.46; N, 13.08. Found: C, H, N.

***N*-[4-[2-[2-(Pivaloylamino)-3,4-dihydro-4-oxo-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl]ethyl]benzoyl]-L-glutamic Acid Dimethyl Ester (14).** To a solution of 1.0 g of 13 in 250 mL of 1:1 CH_2Cl_2/CH_3OH was added 0.8 g of palladium-on-carbon (3%), the mixture was hydrogenated at 50 psi for 3 h and filtered through Celite, and the filtrate was concentrated under reduced pressure. The solid which separated was collected by filtration and dried: yield 0.72 g (72%); mp 247 °C; ^1H NMR (d_6-DMSO) δ 1.21 (s, 9 H), 1.90–2.12 (m, 2 H), 2.42 (t, J = 7.4 Hz, 2 H), 2.92 (t, J = 4 Hz, 2 H), 2.97 (t, J = 4 Hz, 2 H), 3.55 (s, 3 H), 3.61 (s, 3 H), 4.38–4.45 (m, 1 H), 6.61 (s, 1 H), 7.27 (d, J = 8.2 Hz, 2 H), 7.75 (d, J = 8.2 Hz, 2 H), 8.64 (d, J = 7.4 Hz, 1 H), 10.75 (s, 1 H), 11.22 (s, 1 H), 11.75 (s, 1 H). Anal. Calcd for $C_{27}H_{33}N_5O_7$: C, 60.10; H, 6.17; N, 12.98. Found: C, H, N.

***N*-[4-[2-(2-Amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic Acid (15).** A solution of 1.5 g of 14 in 10 mL of 1 N NaOH was stirred at room temperature for 3 days and neutralized with glacial acetic acid, and the precipitate which separated was collected by filtration and recrystallized from methanol/acetone: yield 0.8 g (67%); ^1H NMR (d_6-DMSO) δ 1.80–2.00 (m, 2 H), 2.10–2.30 (m, 2 H), 2.77–2.82 (m, 2 H), 2.89–2.93 (m, 2 H), 4.13–4.19 (m, 1 H), 6.25 (d, J = 1.3 Hz, 1 H), 6.36 (s, 2 H), 7.23 (d, J = 8.1 Hz, 2 H), 7.69 (d, J = 8.1 Hz, 2 H), 8.13 (d, J = 6.7 Hz, 1 H), 10.55 (s, 1 H), 11.02 (s, 1 H). Anal. Calcd for $C_{20}H_{21}N_5O_6$: C, 56.20; H, 4.95; N, 16.38. Found: C, H, N.

Cell Culture and Enzymatic Studies. The conditions used for culture of human CCRF-CEM and mouse L1210 cells have previously been described. The IC_{50} determinations were performed by addition of increasing concentrations of drug to RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum at time zero and then assessment of the growth following 72 h of continuous exposure to drug, leucovorin, and thymidine and/or hypoxanthine.

Recombinant mouse thymidylate synthase was purified from sonicates of *E. coli* BL21(DE3)[pLYsS] transfected with plasmid pETSM following induction of plasmid growth and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 6 h. Enzyme was purified on a column of phosphocellulose eluted with a step gradient of phosphate buffer and was found to be >95% pure by SDS polyacrylamide gel electrophoresis. The purification procedures for mouse L1210 leukemia cell GARFT and for mouse liver FPGS and the design of kinetic experiments were as previously described.[3c,13a] The activity of mouse thymidylate synthase was followed spectrophotometrically during kinetic experiments, with a total enzyme concentration of between 50 and 80 nM. For the determination of K_i values for the pentaglutamate form of 15, 78 nM enzyme was used, and data sets of initial enzyme velocity as a function of inhibitor concentration were fit to the Morrison equation as previously described[3c,14] with proportional weighting. The techniques previously used to measure kinetic constants for substrates for the FPGS reaction were modified to allow determination of the low K_m for 15. The reaction volume of these reactions was increased to 1 mL and total enzyme activity was decreased to prevent excessive consumption of substrate during estimation of initial velocities. Even with these modifications, consumption of 15 at the lowest concentrations used was approximately 35%. Standard incubations for thymidylate synthase and GARFT utilized 10 μM (6*R*)-5,10-methylenetetrahydrofolate and 11 μM 10-formyl-5,8-dideazafolate, respectively.

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