(6R,6S)-5,8,10-Trideaza-5,6,7,8-tetrahydrofolate and (6R,6S)-5,8,10-Trideaza-5,6,7,8-tetrahydropteroyl-L-ornithine as Potential Antifolates and Antitumor Agents[1]

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(6R,6S)-5,8,10-Trideaza-5,6,7,8-tetrahydropteroic acid was synthesized in several steps from 4,4-(ethylenedioxy)-cyclohexanone and [4-(*tert*-butyloxycarbonyl)benzyl]triphenylphosphonium bromide and was elaborated to (6R,6S)-5,8,10-trideaza-5,6,7,8-tetrahydropteroyl-L-glutamic acid (1) and (6R,6S)-5,8,10-trideaza-5,6,7,8-tetrahydropteroyl-L-ornithine (2). Compound 1 was found to be a good substrate for partially purified mouse liver folylpolyglutamate synthetase (FPGS), with a Michaelis constant (K_m = 15 μM) comparable to that reported for the reduced folate substrate (6S)-5,6,7,8-tetrahydropteroyl-L-glutamic acid and for (6R,6S)-5,10-dideaza-5,6,7,8-tetrahydropteroyl-L-glutamic acid (DDATHF). However, in striking contrast to DDATHF, which is potently cytotoxic, 1 failed to inhibit tumor cell growth in culture at concentrations of up to 100 μM. These results suggested that the NH at position 8 of DDATHF is important for cytotoxic activity but not for polyglutamylation. Just as 1 was a good substrate for FPGS, the ornithine analogue 2 proved to be among the more potent competitive inhibitors of this enzyme discovered to date, with a $K_{i,s}$ of 10 μM. While the binding affinity of 2 was lower than that reported for 5,6,7,8-tetrahydropteroyl-L-ornithine (H_4PteOrn), very substantial FPGS inhibition was observed even though N^5, N^8, and N^{10} in H_4PteOrn were replaced by carbon. Binding to FPGS thus appears to be tolerant of bioisosteric replacements made simultaneously in ring B and the bridge region. Neither 1 nor 2 was active in preventing cell growth in culture at concentrations of up 100 μM. The N^δ-hemiphthaloyl derivative of 2, synthesized as a potential prodrug, was also inactive.

Structural analogues of folic acid modified in the pyrazine ring and/or C^9–N^{10} bridge region by carbon–nitrogen substitution have been of interest as potential inhibitors of folate metabolism for almost 40 years.[2] Among the analogues of this type studied to date have been 5-deaza-,[3,4] 8-deaza-,[5-7] 10-deaza-,[8] 5,8-dideaza-,[9,10] 5,10-dideaza-,[11] 8,10-dideaza-,[12] and 5,8,10-trideazafolic acid.[13] While none of these parent deaza analogues showed significant antitumor activity, one compound in the 5,8-dideaza series, N^{10}-propargyl-5,8-dideazafolic acid (CB3717),[14] proved to be an exceptionally potent inhibitor of thymidylate synthase (TS).[14,15] This led to intensive preclinical studies,[16-18] and CB3717 eventually became the first antifolate acting at a site other than dihydrofolate reductase (DHFR) to reach clinical trial.[19]

Because the active folate cofactors in the biosynthesis of the purine and pyrimidine nucleotide precursors of DNA are in the 5,6,7,8-tetrahydro form, analogous reduced derivatives of the deaza- and dideazafolates have also attracted interest. As early as 1958, Baker and co-workers,[20] recognizing that 5,6,7,8-tetrahydro-5,8-dideazafolic acid had the potential to serve as an antagonist of tetrahydrofolate, synthesized the 6R,6S form of this molecule. More recently described have been the (6R,6S)-5,6,7,8-tetrahydro derivatives of 8-deazafolic acid[5,7] and 5,10-dideazafolic acid.[11] The latter compound is especially interesting because of its potent antipurine activity at the level of glycinamide ribotide transformylase (GAR transformylase) and because of its therapeutic efficacy against a number of experimental tumors with a poor response to methotrexate (MTX).[21]

This paper reports the first synthesis of (6R,6S)-5,8,10-trideaza-5,6,7,8-tetrahydrofolic acid (1), which was prepared with a view to assessing whether N^8 in 5,10-dideaza-5,6,7,8-tetrahydrofolic acid can be replaced by carbon without loss of biological activity. Also described are the side chain analogue (6R,6S)-5,8,10-trideaza-5,6,7,8-tetrahydropteroyl-L-ornithine (2), which was designed as a potential inhibitor of folylpolyglutamate synthetase (FPGS), and the N^δ-hemiphthaloyl derivative 3, whose

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(1) Paper 35 in this series, for previous paper, see: Rosowsky, A.; Bader, H.; Kohler, W.; Freisheim, J. H.; Moran, R. G. *J. Med. Chem.* **1988**, *31*, 1338.
(2) For a useful overview of this field, see: Montgomery, J. A.; Piper, J. R. In *Folate Antagonists as Therapeutic Agents*; Sirotnak, F. M., Burchall, J. J., Ensminger, W. D., Montgomery, J. A., Eds.; Academic Press, Orlando, FL, 1984; pp 219–260.
(3) Temple, C., Jr.; Elliott, R. D.; Montgomery, J. A. *J. Org. Chem.* **1982**, *47*, 761.
(4) Taylor, E. C.; Palmer, D. C.; George, T. J.; Fletcher, S. R.; Tseng, C. P.; Harrington, P. J.; Beardsley, G. P. *J. Org. Chem.* **1983**, *48*, 4852.
(5) De Graw, J. I.; Kisliuk, R. L.; Gaumont, Y.; Baugh, C. M. *J. Med. Chem.* **1974**, *17*, 470.
(6) Srinivasan, A.; Broom, A. D. *J. Org. Chem.* **1981**, *46*, 1777.
(7) Temple, C., Jr.; Kussner, C. L.; Rose, J. D.; Smithers, D. L.; Bennett, L. L., Jr.; Montgomery, J. A. *J. Med. Chem.* **1981**, *24*, 1254.
(8) Struck, R. F.; Shealy, Y. F.; Montgomery, J. A. *J. Med. Chem.* **1971**, *14*, 693.
(9) (a) Davoll, J.; Johnson, A. M. *J. Chem. Soc.* **1970**, 997. (b) Bird, A. D.; Vaitkus, J. W.; Clarke, J. *Mol. Pharmacol.* **1970**, *6*, 573.
(10) Acharya, S. P.; Hynes, J. B. *J. Heterocycl. Chem.* **1975**, *12*, 1283.
(11) (a) Taylor, E. C.; Harrington, P. J.; Fletcher, S. R.; Beardsley, G. P.; Moran, R. G. *J. Med. Chem.* **1985**, *28*, 914. (b) Beardsley, G. P.; Moroson, B. A.; Taylor, E. C.; Moran, R. G. *J. Biol. Chem.*, in press.
(12) De Graw, J. I.; Brown, V. H.; Kisliuk, R. L.; Gaumont, Y.; Sirotnak, F. M. In *Chemistry and Biology of Pteridines*; Kisliuk, R. L., Brown, G. M., Eds; Elsevier/North Holland: New York, 1979; pp 229–234.
(13) Oatis, J. E., Jr.; Hynes, J. B. *J. Med. Chem.* **1977**, *20*, 1393.
(14) Jones, T. R.; Calvert, A. H.; Jackman, A. L.; Brown, S. J.; Jones, M.; Harrap, K. R. *Eur. J. Cancer* **1981**, *17*, 11.
(15) Pogolotti, A. L., Jr.; Danenberg, P. V.; Santi, D. V. *J. Med. Chem.* **1986**, *29*, 478.
(16) (a) Jackson, R. C.; Jackman, A. L.; Calvert, A. H. *Biochem. Pharmacol.* **1983**, *32*, 3783. (b) Jackman, A. L.; Taylor, G. A.; Calvert, A. H.; Harrap, K. R. *Biochem. Pharmacol.* **1984**, *33*, 3269.

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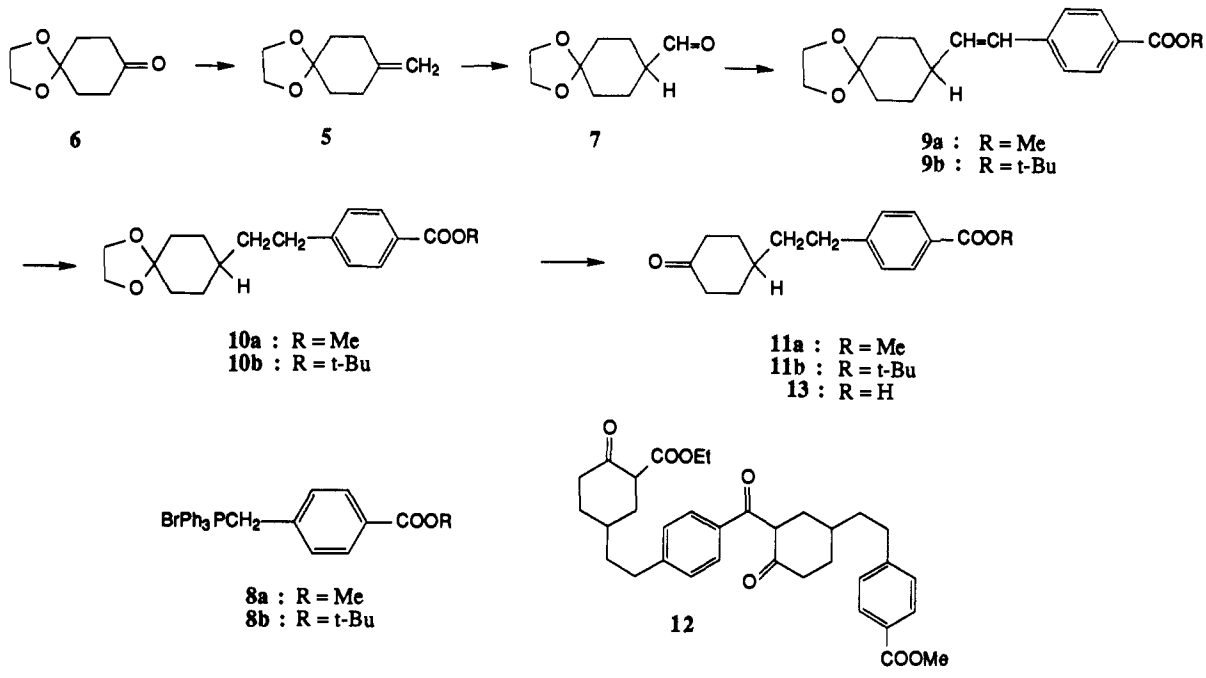
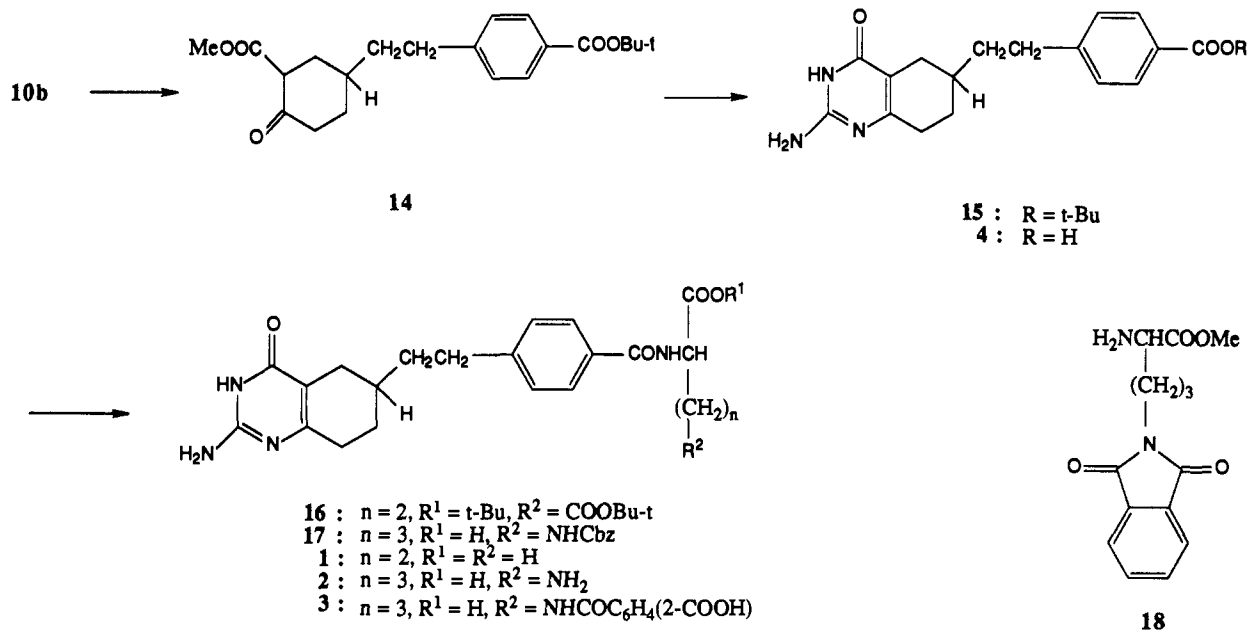
Scheme I



Scheme II

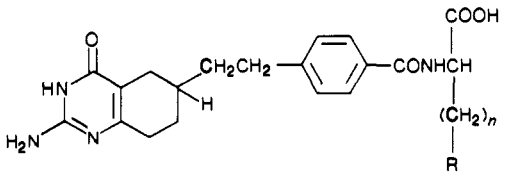


synthesis was prompted by the possibility that it might serve as a prodrug.[1] Our choice of ornithine to replace glutamic acid was based on previous work showing that potent inhibition of this enzyme is achieved when tetrahydrofolate,[22] aminopterin (AMT),[23] and to a lesser degree MTX[23,24] are modified in this fashion.

Chemistry

The synthetic plan we chose to follow involved condensation reactions of appropriately blocked L-glutamic acid or L-ornithine derivatives with the key intermediate (6*R*,6*S*)-5,8,10-trideaza-5,6,7,8-tetrahydropteroic acid (**4**) (Scheme II). An attractive starting point for the prepa-

(17) Cheng, Y. C.; Dutschmann, G. E.; Stearns, M. C.; Fisher, M. H.; Nanavathi, N. T.; Nair, M. G. *Cancer Res.* **1985**, *45*, 598.
(18) (a) Alison, D. L.; Newell, D. R.; Sessa, C.; Harland, S. J.; Hart, L. I.; Harrap, K. R.; Calvert, A. H. *Cancer Chemother. Pharmacol.* **1985**, *14*, 265. (b) Newell, D. R.; Alison, D. L.; Calvert, A. H.; Harrap, K. R.; Jarman, M.; Jones, T. R.; Manteuffel-Cymborowska, M.; O'Connor, P. *Cancer Treatment Rep.* **1986**, *70*, 971.
(19) (a) Calvert, A. H.; Alison, D. L.; Harland, S. J.; Robinson, B. A.; Jackman, A. L.; Jones, T. R.; Newell, D. R.; Siddik, Z. H.; Wiltshaw, E.; McElwain, T. J.; Smith, I. E.; Harrap, K. R. *J. Clin. Oncol.* **1986**, *4*, 1245. (b) Cantwell, B. M. J.; Earnshaw, M.; Harris, A. L. *Cancer Treatment Rep.* **1986**, *70*, 1335.
(20) Koehler, R.; Goodman, L; De Graw, J.; Baker, B. R. *J. Am. Chem. Soc.* **1958**, *80*, 5779.
(21) Beardsley, G. P.; Taylor, E. C.; Shih, C.; Poore, G. A.; Grindey, G. B.; Moran, R. G. *AACR Proc.* **1986**, *27*, 259.
(22) George, S.; Cichowicz, D. J.; Shane, B. *Biochemistry* **1987**, *26*, 522.
(23) Rosowsky, A.; Freisheim, J. H.; Moran, R. G.; Solan, V. C.; Bader, H.; Wright, J. E.; Radlike-Smith, M. *J. Med. Chem.* **1986**, *29*, 655.
(24) McGuire, J. J.; Hsieh, P.; Franco, C. T.; Piper, J. R. *Biochem. Pharmacol.* **1986**, *35*, 2607.



1: n = 2, R = COOH
2: n = 3, R = NH_2
3: n = 3, R = $NHCOC_6H_4(2\text{-}COOH)$

ration of **4** was the olefin **5** (Scheme I), which is easily accessible from the commercially available starting material 4,4-(ethylenedioxy)cyclohexanone (**6**) via a Wittig reaction.[25] The olefin was subjected to hydroboration and direct oxidation with pyridinium chlorochromate[26] to obtain the aldehyde **7** (66%). Condensation of **7** with the ylide of the phosphonium salt **8a**[27] afforded the olefin **9a**, whose complex pattern of vinyl proton signals in the NMR at δ 5.6–6.4 indicated a probable mixture of cis and trans isomers. Catalytic reduction of **9a** afforded the ketal ester **10a** (68%), and selective hydrolysis of the ketal with retention of the aromatic ester group was performed with dilute trifluoroacetic acid in aqueous THF, giving the keto ester **11a** (84%). We next intended to form an α-carbethoxy derivative of **11a** with diethyl carbonate and sodium hydride in benzene according to a standard procedure in alicyclic chemistry,[28] but could not prevent the reaction from giving a crystalline product which we believe to be the Claisen-type dimer **12**. Support for this putative structure was provided by the NMR spectrum, which revealed the presence of one methyl ester, one ethyl ester, and two para-substituted phenyl rings, rather than one. Acylation was attempted also with diethyl pyrocarbonate and potassium hydride in benzene,[29] but C-acylation was found to be accompanied by substantial O-acylation. This was surprising in view of the report[29] that diethyl pyrocarbonate is selective for C-acylation. Faced with the apparent unsuitability of keto ester **11a** as a C-acylation substrate, we hydrolyzed it to keto acid **13** with the aim of acylating the latter in the presence of an extra equivalent of base. This alternative method failed when the dipotassium salt of **13** proved to be insoluble in the low-temperature reaction mixture.

Success was achieved finally when the *tert*-butyl ester **11b** was used in place of **11a** with the aim of preventing the Claisen-type dimerization reaction. Aldehyde **7** was condensed with the ylide of [4-(*tert*-butyloxycarbonyl)-benzyl]triphenylphosphonium bromide (**8b**), and the resulting cis/trans olefin mixture **9b** was reduced directly to give the ketal ester **10b** in 45–50% overall yield. The phosphonium salt **8b**, a previously undescribed compound, was prepared in 72% yield from *tert*-butyl α-bromo-*p*-toluate and triphenylphosphine. We had some initial concerns about whether it would be possible to selectively cleave the ketal group while retaining the *tert*-butyl ester function. Fortunately this was accomplished in reasonable yield by treatment with ca. 0.2 N HCl in dioxane–H_2O at room temperature, which afforded the crystalline keto ester **11b** in 92% total yield. Acylation of **11b** was found to proceed satisfactorily with methyl cyanoformate.[30] The

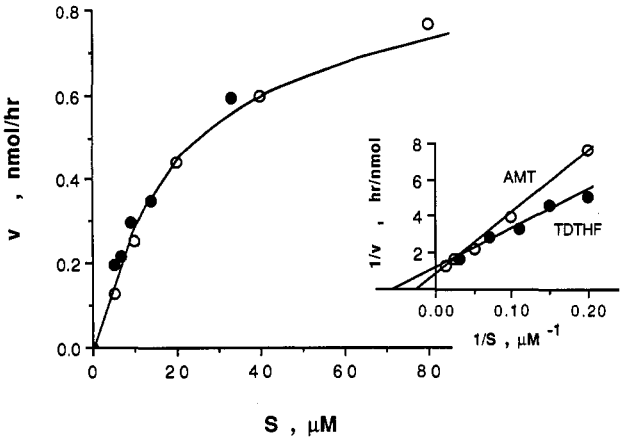


Figure 1. Substrate activity of (6*R*,6*S*)-5,8,10-trideazatetrahydrofolate (1) for mouse liver FPGS. The amount of product formed during a 1-h incubation of AMT (○) or 1 (●) with partially purified enzyme is indicated as a function of concentration. Each symbol represents the mean of two determinations from a representative experiment. Inset: double-reciprocal transformation of the data in the main figure.

literature method[30] was followed, except that the temperature was kept at −78 °C not only during enolate formation but throughout the reaction. If the temperature was allowed to rise from −78 to 0 °C during addition of the methyl cyanoformate, a dark red color developed and a second product formed, whose structure was not determined. However; by rigorously controlling the temperature it was possible to limit the red color to a faint trace and obtain the keto ester **14** in 49% yield. Condensation of **14** with guanidine carbonate in refluxing MeOH afforded a 76% yield of a high-melting white solid whose analysis and UV spectrum were consistent with the 2-amino-4-(3*H*)-oxopyrimidine structure **15**. Acidolysis of the *tert*-butyl ester was accomplished in 83% yield by brief treatment with trifluoroacetic acid at room temperature, giving the acid **4**. Coupling of di-*tert*-butyl L-glutamate to **4** was performed by the mixed carboxylic–carbonic anhydride method and the resulting diester (**16**, 51% yield) was hydrolyzed with trifluoroacetic acid (room temperature, 5 min) to obtain **1** in 45% yield after purification on a DEAE-cellulose column.

For the preparation of the ornithine analogue **2**, acid **4** was activated for mixed-anhydride coupling in the usual manner (i-BuOCOCl/Et_3N) and treated with trimethylsilylated N^δ-Cbz-L-ornithine. The crude coupled product was hydrolyzed directly with 30% HBr in acetic acid, and the deprotected final product **2** precipitated out of dilute HCl as a sodium salt on neutralization to pH 7. For the preparation of the N^δ-hemiphthaloyl derivative **3**, acid **4** was coupled with N^δ-phthaloyl-L-ornithine methyl ester (**18**), and the phthaloyl ring was opened by treatment with NaOH in DMSO at room temperature for 5 min.

Biological Evaluation

Compound **1** was tested as a substrate for partially purified mouse liver FPGS as described earlier[31] and was found to be an excellent substrate (Figure 1), with an apparent K_m of 15.4 ± 6.9 μM (n = 3). The apparent K_m values of MTX, AMT, and (6*S*)-tetrahydrofolate as substrates for this enzyme have been reported as approximately 165, 20, and 8 μM respectively.[31] Thus, the substrate activity of **1** is intermediate between that of a natural

(25) Nicolaou, K.; Magolda, R.; Claremon, D. *J. Am. Chem. Soc.* **1980**, *102*, 1404.
(26) Brown, H. C.; Kulkarni, S.; Rao, C. *Synthesis* **1980**, 151.
(27) Struck, R.; Shealy, Y.; Montgomery, J. A. *J. Med. Chem.* **1971**, *14*, 693.
(28) Krapcho, A. P.; Diamanti, J.; Cayen, C.; Bingham, R. *Organic Syntheses*; Wiley: New York, 1973; Collect. Vol. V, p 198.
(29) Hellou, J.; Kingston, J.; Fallis, A. *Synthesis* **1984**, 1014.
(30) Mander, L.; Sethi, S. *Tetrahedron Lett.* **1983**, *24*, 5425.
(31) (a) Moran, R. G.; Colman, P. D.; Rosowsky, A.; Forsch, R. A.; Chan, K. K. *Mol. Pharmacol.* **1985**, *27*, 156. (b) Moran, R. G.; Colman, P. D. *Biochemistry* **1984**, *23*, 4580.

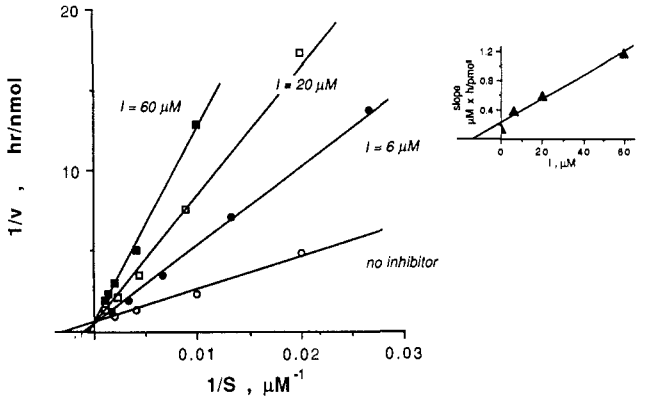


Figure 2. Inhibition of the FPGS reaction by (6*R*,6*S*)-5,8,10-trideazatetrahydropteroyl-L-ornithine (**2**). Mouse liver enzyme was incubated with varying concentrations of folic acid and **2** for 1 h at 37 °C and the amount of folyldiglutamate product formed was determined. The concentration of **2** used in each set of assays is indicated. Each point represents the mean of two determinations from a representative experiment. Inset: secondary replot of the slope of each line in the main figure as a function of inhibitor concentration.

reduced folate and that of AMT, which is one of the best substrates among the antifolates for which data have been reported to date. On this basis, it was expected that, upon being taken up into cells, **1** would be converted to non-effluxing polyglutamates. The potently cytotoxic 5,10-dideaza analogue, DDATHF,[11,32] to which **1** is structurally related, is an equivalent substrate for FPGS to compound **1**. The effect of **1** on the growth of CEM human lymphoblasts and L1210 mouse leukemia cells in culture was tested over a range of concentrations from 1 nM to 100 μM, but to our surprise no activity was observed. This was striking in view of the fact that DDATHF has been found to have an IC_{50} of 20–60 nM against murine and human leukemic cells.[11] Our results would appear to indicate that N^8 in DDATHF plays an essential and heretofore unrecognized role either on drug uptake or on subsequent inhibition of cellular GAR transformylase activity and purine synthesis by the parent drug and its polyglutamates.[21,32] On the other hand, two other bioisosteres, (6*R*,6*S*)-8-deaza-5,6,7,8-tetrahydrofolate[33] and (6*R*,6*S*)-5,8-dideaza-5,6,7,8-tetrahydrofolate,[20] have been reported to possess only minimal biological activity. It thus appears that N^8 deletion, $N^5 + N^8$ deletion, or $N^5 + N^8 + N^{10}$ deletion from tetrahydrofolate produces analogues without significant cytotoxic activity, while $N^5 + N^{10}$ deletion results in excellent activity.

As we had hoped on the basis of the reported anti-FPGS activity of (6*R*,6*S*)-5,6,7,8-tetrahydropteroyl-L-ornithine (H_4PteOrn),[22] compound **2** proved to be an excellent inhibitor of this enzyme (Figure 2), with an apparent $K_{i,s}$ of 10.1 ± 3.3 μM (n = 3). Compound **2** behaved as a competitive inhibitor of the FPGS reaction (relative to the folyl substrate) at most concentrations, but measurable effects on V_{max} were observed when the concentration **2** exceeded the $K_{i,s}$ by a factor of 5 or more (Figure 2). While a number of side chain altered compounds have now been demonstrated to possess significant anti-FPGS activity,[1,23,24,34-36] these have been mainly 2,4-diaminopteridine derivatives, and have therefore been inhibitors of both FPGS and DHFR. Although **2** is considerably less potent than either H_4PteOrn,[2] the latter is not stable in oxygenated solution. Hence, **2** is notable as being, thus far, the most potent known stable inhibitor of FPGS that does not have other sites of action. The basic inactivity of this ring system as an inhibitor of folate-dependent enzymes other than FPGS was clear from the lack of cytotoxicity of compound **1**. Hence, **2** was judged to be a promising compound as a pure inhibitor of FPGS, provided that the problem of membrane penetration stemming from replacement of the glutamic acid moiety by ornithine could be overcome.

We have recently shown[1] that the N^δ-hemiphthaloyl derivative of N^α-(4-amino-4-deoxypteroyl)-L-ornithine is much more cytotoxic than the parent amine and have attributed this to improved uptake as a result of the elimination of the positive charge on the terminal nitrogen and the concomitant introduction of a second carboxyl group in the side chain. The N^δ-hemiphthaloyl derivative **3** was synthesized in the hope of accomplishing a similar increase in uptake for **2**. However, in assays against CEM human leukemic lymphoblasts (suspension culture, 48-h treatment) and SCC15 human squamous carcinoma cells (monolayer culture, 14-day treatment), neither **2** nor **3** inhibited growth at concentrations of up to 100 μM. These results suggested either that N^δ-hemiphthaloylation does not increase the uptake of **2** or, as seems more likely, that **3** enters the cells but does not yield enough **2** by deacylation to significantly influence cellular FPGS activity. It appears that other more labile blocking groups for the terminal amino group of **2** will need to be developed before the therapeutic potential of this compound can be properly assessed.

Experimental Section

Infrared spectra were obtained on a Perkin-Elmer Model 781 double-beam spectrophotometer (only peaks in the 3500–1400-cm^{-1} region are reported). Ultraviolet spectra were obtained on a Varian Model 215 UV/vis spectrophotometer. NMR spectra were recorded on a Varian T60 instrument using Me_4Si as the internal reference standard. Melting points were determined on a Fisher-Johns apparatus (corrected) or in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) (not corrected). TLC was performed on plastic sheets (Eastman silica gel 13181 or cellulose 13254) containing a fluorescent indicator. Spots were visualized under 254-nm illumination. Column chromatography was on Baker 3405 silica gel (60–200 mesh) or Whatman DE-52 preswollen DEAE-cellulose. A Waters C_{18} reversed-phase radial compression cartridge (5-μm particle size, 0.5 × 10 cm) was used for HPLC. 8-Methylene-1,4-dioxaspiro[4.5]decane (**5**) was synthesized from 4,4-(ethylenedioxy)-cyclohexanone (**6**) (Aldrich) according to Nicolaou and co-workers,[25] methyl α-bromotoluate according to Julia and Chastrette,[38] [4-(methoxycarbonyl)benzyl]triphenylphosphonium bromide (**8a**) according to Struck and co-workers,[27] methyl cyanoformate according to Childs and Weber,[39] and N^δ-phthaloyl-L-ornithine according to Bodanszky and co-workers.[40] Other chemicals were

(32) Moran, R. G.; Taylor, E. C.; Shih, C.; Beardsley, G. P. *AACR Proc.* **1987**, *28*, 274.
(33) Temple, C., Jr.; Kussner, C. L.; Rose, J. D.; Smithers, D. L.; Bennett, L. L., Jr.; Montgomery, J. A. *J. Med. Chem.* **1981**, *24*, 1254.
(34) Rosowsky, A.; Forsch, R. A.; Freisheim, J. H.; Moran, R. G.; Wick, M. *J. Med. Chem.* **1984**, *27*, 600.
(35) Rosowsky, A.; Moran, R. G.; Forsch, R. A.; Radlike-Smith, M.; Colman, P. D.; Wick, M. M.; Freisheim, J. H. *Biochem. Pharmacol.* **1986**, *35*, 3327.
(36) (a) Rosowsky, A.; Bader, H.; Forsch, R. A.; Moran, R. G.; Freisheim, J. H. *AACR Proc.* **1988**, *29*, 288. (b) Rosowsky, A.; Forsch, R. A.; Moran, R. G.; Kohler, W.; Freisheim, J. H. *J. Med. Chem.* **1988**, *31*, 1326.
(37) McGuire, J. J.; Bolanowska, W.; Piper, J. R. *AACR Proc.* **1988**, *29*, 285.
(38) Julia, M.; Chastrette, F. *Bull. Soc. Chim. Fr.* **1962**, 2247.
(39) Childs, M.; Weber, W. *J. Org. Chem.* **1976**, *41*, 3486.
(40) Bodanszky, M.; Ondetti, M. A.; Birkhimer, C. A.; Thomas, P. L. *J. Am. Chem. Soc.* **1964**, *86*, 4452.

from Aldrich, Milwaukee, WI, Chemical Dynamics, South Plainfield, NJ, Sigma, St. Louis, MO, and Fisher, Boston, MA. Et_3N and solvents used for moisture-sensitive reactions were dried over Davison 4A molecular sieves unless otherwise stated. Microchemical analyses were by Robertson Laboratory, Madison, NJ, and were within ±0.4% of theory unless otherwise specified.

1,4-Dioxaspiro[4.5]decane-8-carboxaldehyde (7). A solution of 8-methylene-1,4-dioxaspiro[4.5]decane (**5**)[25] (12.6 g, 0.082 mol) in dry THF (80 mL, distilled from Na) under N_2 at 0 °C was treated with BH_3 in THF (36 mL of 0.098 M solution, 0.035 mol), and after 30 min the solvent was evaporated under reduced pressure and replaced with CH_2Cl_2 (50 mL). This solution was then added dropwise to a vigorously stirred mixture of pyridinium chlorochromate (45.3 g, 0.21 mol) and CH_2Cl_2 in a second flask equipped with a reflux condenser. After the initial exothermic reaction subsided, the mixture was refluxed for 2 h, cooled to room temperature, concentrated to 100 mL, and rediluted with Et_2O (100 mL). The resulting cloudy solution was filtered through a bed (0.5 cm) of silica gel, the filtrate evaporated, a large volume of petroleum ether added to the residue, and the mixture left to stand for 1 h until an oil settled out. The supernatant was decanted and evaporated to a colorless liquid (9.2 g, 66%): IR (NaCl) 1750 cm^{-1} (aldehyde C=O); NMR ($CDCl_3$) δ 1.4–2.4 (m, 8 H, cyclohexyl), 3.90 (s, 4 H, ketal), 9.57 (s, 1 H, C*H*=O). This material was used without further purification.

[4-(*tert*-Butyloxycarbonyl)benzyl]triphenylphosphonium Bromide (8b). *p*-Toluoyl chloride (200 g, 1.3 mol) was added in one portion to a mixture of *t*-BuOH (125 mL) and pyridine (125 mL). After 3 days at room temperature, enough H_2O and EtOAc were added to dissolve all the solids. The organic layer was washed with 0.5 N HCl until the aqueous phase became acidic and was then rinsed with H_2O, 5% $NaHCO_3$, and H_2O before being evaporated to dryness under reduced pressure. On standing overnight, some *p*-toluic anhydride was deposited. Decantation of the liquid and vacuum distillation gave *tert*-butyl *p*-toluate (151 g, 61%), bp 70–72 °C (0.75 Torr) (lit.[41] bp 114 °C, (11 Torr)); IR (NaCl) 1715 cm^{-1} (ester C=O); NMR ($CDCl_3$) δ 1.63, (s, 9 H, *t*-Bu), 2.41 (s, 3 H, Me), 7.27 (d, J = 8 Hz, 2 H, aromatic), 7.97 (d, J = 8 Hz, 2 H, aromatic). A mixture of the ester (19.2 g, 0.1 mol), *N*-bromosuccinimide (17.8 g, 0.1 mol), and dibenzoyl peroxide (0.2 g) in CCl_4 (100 mL) was refluxed until all the *N*-bromosuccinimide was replaced by succinimide, which floated to the surface as it formed. After heating was discontinued, the solid was filtered and washed with CCl_4, the combined filtrates were evaporated, and the residue was stirred in vacuo at room temperature until crystallization occurred. The solid was triturated with boiling petroleum ether (bp 30–60 °C) until all but 0.9 g of it dissolved, and the decantate was evaporated to obtain a white solid (25 g, 92%); mp 46–49 °C. Recrystallization from MeOH gave a purer sample: mp 50–52 °C; IR (KBr) 1700 cm^{-1} (ester C=O); NMR ($CDCl_3$) δ 1.60 (s, 9 H, *t*-Bu), 4.48 (s, 2 H, benzylic CH_2), 7.40 (d, J = 8 Hz, 2 H, aromatic), 7.95 (d, J = 8 Hz, 2 H, aromatic). A solution of the crude bromide (22.5 g, 0.083 mol) (*caution*: lachrymator) and Ph_3P (21.0 g, 0.08 mol) in PhMe (100 mL) was heated in an oil bath at 110 °C for 18 h. After cooling, the precipitated solid was broken up, washed with PhMe, and dried in vacuo at 60 °C to obtain a white solid (30.6 g, 72%): mp >280 °C (bumping at 160–180 °C due to pyrolysis of the *tert*-butyl ester); IR (KBr) 1710 cm^{-1} (ester C=O). Anal. ($C_{30}H_{30}O_2PBr$) C, H.

Methyl 4-[2-(1,4-Dioxaspiro[4.5]dec-8-yl)ethyl]benzoate (10a). A solution of the phosphonium salt **8a** (34.4 g, 0.07 mol) in dry DMSO (150 mL) under N_2 was treated with NaOMe (3.78 g, 0.07 mol) in a single portion, and the mixture was stirred at room temperature for 15 min before being chilled and treated with the aldehyde **7** (9.2 g, 0.054 mol), likewise in a single portion. The clear solution was allowed to stand at room temperature for 1 day and then partitioned between Et_2O and 10% AcOH. The aqueous layer was extracted thoroughly with Et_2O and discarded. The combined Et_2O extracts were washed consecutively with H_2O, 5% $NaHCO_3$, and saturated NaCl and finally dried over $MgSO_4$ and concentrated to a small volume. Addition of a generous amount of petroleum ether, filtration of the precipitated triphenylphosphine oxide, and evaporation of the filtrate gave a gum, which was purified by chromatography on silica gel (120 g, 4 × 28 cm). The material eluting with 4:1 hexane–Et_2O was still impure and was therefore passed through a second column (60 g, 3 × 24 cm) with the same eluent to obtain **9a** as a soft white solid (11.1 g, 68%): IR (NaCl melt) 1715 cm^{-1} (ester C=O); NMR ($CDCl_3$) δ 1.7 (m, 9 H, cyclohexyl), 3.88 and 3.93 (overlapping singlets, 7 H, MeO and ketal), 5.6–6.4 (complex m, 2 H, vinyl), 7.3 (m, 2 H, C_3 and C_5 aromatic), 7.9 (d, J = 8 Hz, 2 H, C_2 and C_6 aromatic). A solution of this compound (974 mg, 3.23 mmol) in MeOH (50 mL) was shaken with 5% Pd–C (200 mg) under H_2 (3 atm) overnight in a Parr apparatus, the catalyst was removed, the filtrate was evaporated, and the residue was crystallized from MeOH to obtain **10a** as glistening white flakes (667 mg, 68% yield): mp 72–73 °C; R_f 0.30 (silica gel, 2:1 hexane–Et_2O); IR (KBr) 1720 cm^{-1} (ester C=O); NMR ($CDCl_3$) δ 1.6 (m, 11 H, cyclohexane and nonbenzylic CH_2), 2.65 (m, 2 H, benzylic CH_2), 3.85 and 3.93 (overlapping singlets, 7 H, OMe and ketal), 7.18 (d, J = 8 Hz, 2 H, C_3 and C_5 aromatic), 7.90 (d, J = 8 Hz, 2 H, C_2 and C_6). Anal. ($C_{18}H_{24}O_4$) C, H.

Methyl 4-[2-(4-Oxocyclohexyl)ethyl]benzoate (11a). A solution of ketal **10a** (7.89 g, 0.026 mol) in a mixture of THF (100 mL), H_2O (25 mL), and trifluoroacetic acid (10 mL) was heated in an oil bath at 60–65 °C for 2 h, then cooled, and partitioned between Et_2O and H_2O. The Et_2O layer was washed with 5% $NaHCO_3$ until the aqueous layer was alkaline, then rinsed with saturated NaCl, dried over $MgSO_4$, and evaporated. Recrystallization of the product from Et_2O gave white flakes (5.68 g, 84%): mp 56–57 °C; R_f 0.15 (silica gel, 2:1 hexane–Et_2O); IR (KBr) 1720 cm^{-1} (ester C=O); NMR ($CDCl_3$) δ 1.1–2.9 (complex m, 13 H, cyclohexyl, nonbenzylic CH_2, benzylic CH_2), 3.87 (s, 3 H, MeO), 7.20 (d, J = 8 Hz, C_3 and C_5 aromatic), 7.92 (d, J = 8 Hz, C_2 and C_6 aromatic). Anal. ($C_{16}H_{20}O_3$) C, H.

***tert*-Butyl 4-[2-(4-Oxocyclohexyl)ethyl]benzoate (11b).** A solution of **8b** (30.6 g, 0.057 mol) in dry DMSO (120 mL) was obtained by gentle warming under N_2 and was then cooled to room temperature before adding NaOMe (3.08 g, 0.057 mol) in a single portion. After 15 min of stirring at room temperature, the mixture was cooled in an ice bath and aldehyde **7** (8.84 g, 0.052 mol) was added, likewise in a single portion. The mixture was left at room temperature for 18 h, quenched by pouring into ice, and partitioned between Et_2O and H_2O. The aqueous phase was extracted with Et_2O, and the combined Et_2O layers were washed with H_2O and saturated NaCl, dried over $MgSO_4$, concentrated to near dryness, and treated with light petroleum ether (bp 30–60 °C). The precipitated triphenylphosphine oxide was removed, the filtrate evaporated, and the residue passed through a silica gel column (120 g, 4 × 29 cm) with 4:1 hexane–Et_2O as the eluent to obtain olefin **9b** as an oil (8.29 g, 46%): IR (NaCl) 1715 cm^{-1} (ester C=O); NMR ($CDCl_3$) δ 1.4–2.1 (m, 18 H, cyclohexyl and *t*-Bu), 3.98 (s, 4 H, ketal), 6.3–6.6 (m, 2 H, vinyl), 7.40 (m, 2 H, C_3 and C_5 aromatic), 7.97 (m, 2 H, C_2 and C_6 aromatic). The entire sample of **9b** in EtOAc (100 mL) was stirred with 5–10 g of Davison sponge Ni and, after filtration, was reduced in the presence of 5%, Pd–C (300 mg) in a Parr bottle at 50 psi H_2 for 16 h. Filtration of the catalyst and solvent evaporation afforded ketal **10b** as an oil (8.23 g, 99%): IR (NaCl) 1715 cm^{-1} (ester C=O); NMR ($CDCl_3$) δ 1.1–2.0 (20 H, cyclohexyl, nonbenzylic CH_2, and *t*-Bu), 2.70 (m, 2 H, benzylic CH_2), 3.97 (s, 4 H, ketal), 7.25 (d, J = 8 Hz, 2 H, C_3 and C_5 aromatic), 7.97 (d, J = 8 Hz, 2 H, C_2 and C_6 aromatic). The entire sample of **10b** in dioxane (125 mL) was treated with 0.75 N HCl (50 mL) and the mixture was stirred at room temperature for 7 h. The solution was neutralized with NaOH, the solvent evaporated under reduced pressure, and the residue partitioned between CH_2Cl_2 and H_2O. The residue after evaporation of the organic phase was recrystallized from H_2O–MeOH to obtain a first crop of white flakes (2.85 g, 40%): mp 71–73 °C. Two additional crops of slightly

(41) Vowinkel, E. *Ber.* **1967**, *100*, 16.

(42) After completion of this work, we learned that **1** has been synthesized independently by Dr. M. G. Nair, Department of Biochemistry, University of South Alabama, Mobile, AL, and that the compound was tested by Dr. R. Ferone, Burroughs-Wellcome Co., Research Triangle Park, NC, and found to be inactive as an inhibitor of GAR transformylase in vitro. We are grateful to Drs. Nair and Ferone for personally communicating their findings, which confirm the critical role of N^8 for binding to GAR transformylase.

less pure material were obtained to bring the total to 6.6 g (92%): IR (KBr) 1715 (ketone C=O), 1700 (ester C=O) cm^{-1}; NMR ($CDCl_3$) δ 1.2–2.0 (m, 16 H, cyclohexyl, CH_2, *t*-Bu), 2.1–2.9 (m, 6 H, benzylic CH_2 and CH_2CO), 7.28 (d, J = 8 Hz, 2 H, C_3 and C_5 aromatic), 8.00 (d, J = 8 Hz, 2 H, $C_2$and C_6 aromatic). Anal. ($C_{19}H_{26}O_3$), C, H.

4-[2-(4-Oxocyclohexyl)ethyl]benzoic Acid (13). A solution of the ester **11a** (300 mg, 1.15 mmol) in a mixture of MeOH (15 mL) and H_2O (2 mL) was treated with KOH (ca. 200 mg) and heated in an oil bath at 60 °C for 40 min. The MeOH was evaporated and replaced with H_2O, the solution acidified with HCl, and the precipitate collected and recrystallized from H_2O–MeOH to obtain colorless crystals (157 mg, 55%): mp 177–179 °C; IR (KBr) 3440 (br), 1715 cm^{-1} (carboxyl C=O); NMR ($CDCl_3$) δ 1.2–2.9 (complex m, 13 H, cyclohexyl, nonbenzylic CH_2, benzylic CH_2), 7.22 (d, J = 8 Hz, 2 H, C_3 and C_5 aromatic), 7.97 (d, J = 8 Hz, 2 H, C_2 and C_6 aromatic). Anal. ($C_{15}H_{18}O_3$) C, H.

***tert*-Butyl (6*R*,6*S*)-5,8,10-Trideaza-5,6,7,8-tetrahydropteroate (15).** A solution of *i*-Pr_2NH (840 mL, 606 mg, 6.0 mmol) in dry THF (15 mL) under N_2 at −78 °C was treated with 2.5 M *n*-BuLi in hexane (2.4 mL, containing 6.0 mmol), and after 40 min of stirring, ketone **10b** (1.57 g, 5.2 mmol) was added. The solution was kept at −78 °C for 20 min and then treated consecutively with hexamethylphosphorotriamidate (1.04 mL, 1.07 g, 6.0 mmol) and methyl cyanoformate (510 mg, 6.0 mmol). After another 10 min of stirring, the mixture was poured into 5% $NaHCO_3$ and the product extracted into Et_2O. The Et_2O layer was evaporated and the residue purified on a silica gel column (60 g, 3 × 30 cm) with 9:1 hexane–EtOAc as the eluent to obtain an oil (910 mg, 49%): IR (NaCl) 1750 sh (ketone C=O), 1715 (ester C=O) cm^{-1}; NMR ($CDCl_3$) δ 1.2–2.9 (m, 20 H, cyclohexyl, benzylic and nonbenzylic CH_2, *t*-Bu), 3.78 (s, 3 H, OMe), 7.25 (d, J = 8 Hz, 2 H, C_3 and C_5 aromatic), 7.95 (d, J = 8 Hz, 2 H, C_2 and C_6 aromatic), 12.18 (s, 0.75 H, enol). A mixture of the keto ester **14** (910 mg, 2.53 mmol) and guanidine carbonate (540 mg, 3 mmol) in dry MeOH (20 mL) was refluxed for 18 h, cooled, and filtered. The solid was washed with cold MeOH and H_2O, dried in vacuo at 60 °C over P_2O_5; yield 710 mg (76%): mp >300 °C; IR (KBr) 3440, 3120, 2990, 2950, 1705 (ester C=O), 1670, 1655, 1615, 1505, 1440, 1420, 1400 cm^{-1}. Anal. ($C_{21}H_{27}N_3O_3 \cdot 0.25H_2O$) C, H, N.

(6*R*,6*S*)-5,8,10-Trideaza-5,6,7,8-tetrahydropteroic Acid (4). A solution of ester **15** (725 mg, 1.96 mmol) in trifluoroacetic acid (5 mL) was allowed to stand at room temperature for 10 min and evaporated to dryness under reduced pressure. The residue was dissolved in a minimal volume of 1 N NaOH and the solution acidified with AcOH to obtain a solid, which was collected and dried in vacuo at 100 °C over P_2O_5: yield 586 mg (96%); mp >300 °C; IR (KBr) 3380, 3180, 3100, 2950, 1675, 1615, 1580, 1535 sh, 1515, 1455, 1420 cm^{-1}; HPLC 26.1 min (0.1 M NH_4OAc, pH 7.0, with 1–15% MeCN gradient over 30 min, 1.0 mL/min flow rate). Anal. ($C_{17}H_{19}N_3O_3 \cdot 0.25H_2O$) C, H, N.

(6*R*,6*S*)-5,8,10-Trideaza-5,6,7,8-tetrahydrofolic Acid (1). A suspension of acid **4** (94 mg, 0.3 mmol) in dry DMF (5 mL) containing Et_3N (140 mL, 101 mg, 1.0 mmol) was treated with *i*-BuOCOCl (44 mL, 46 mg, 0.34 mmol) and placed in an sonication bath to improve solubility. After 15 min, when only a fine precipitate of $Et_3N \cdot HCl$ remained suspended in the reaction mixture, di-*tert*-butyl L-glutamate hydrochloride (117 mg, 0.4 mmol) was added. After a few minutes, when complete dissolution had occurred, the solvent was evaporated under reduced pressure. The residue was partitioned between $CHCl_3$ and H_2O, and the cloudy $CHCl_3$ layer was dried over $MgSO_4$ and evaporated. The product was purified by chromatography on silica gel (7 g, 1.5 × 15 cm) with 19:1 $CHCl_3$–MeOH as the eluent. A fast-moving impurity with R_f 0.8 (silica gel, 19:1 $CHCl_3$–MeOH) was eluted first, followed by the main product, R_f 0.5. Fractions containing only the product were pooled and evaporated, and the residue was dried in vacuo at 60 °C over P_2O_5 to obtain the diester **16** as a white solid (85 mg, 51%): mp 198–201 °C (dec with gas evolution); IR (KBr) 3430, 3130, 2990, 2940, 1735 (ester C=O), 1670, 1655, 1575, 1500, 1455, 1425 cm^{-1}. A solution of **16** (64 mg, 0.12 mmol) in trifluoroacetic acid (1 mL) was allowed to stand at room temperature for 5 min. After evaporation to dryness, the residue was purified by ion-exchange chromatography on DEAE-cellulose (1.5 × 27 cm, HCO_3^- form). The column was eluted first with 0.2 M NH_4HCO_3 (200 mL) and then with 0.3 M NH_4HCO_3. Fractions were monitored by HPLC (see below), and those containing the product were pooled and freeze-dried. The residue was added to a small volume of H_2O, a few drops of concentrated NH_4OH were added, a small amount of material remaining undissolved was removed, the filtrate was acidified with 10% AcOH, and the precipitate was collected and dried in vacuo at 60 °C over P_2O_5 to obtain a white powder (23 mg, 45%): mp >200 °C dec; IR (KBr) 3430, 2940, 1695, 1645, 1555, 1505, 1450sh cm^{-1}; UV λ_{max} (EtOH) 234 nm (ϵ 23300), 286 (8300), λ_{max} (0.1 N NaOH) 237 nm (ϵ 21700), 269 infl (8300), λ_{max} (0.1 N HCl) 238 nm (ϵ 22600); HPLC 18.1 min (0.1 M NH_4OAc, pH 7.0, with 1–15% MeCN gradient over 20 min, 1.0 mL/min flow rate). Anal. ($C_{22}H_{26}N_4O_6 \cdot 1.25H_2O$) C, H, N.

(6*R*,6*S*)-*N*$^{\alpha}$-(5,8,10-Trideaza-5,6,7,8-tetrahydropteroyl)-L-ornithine (2). A suspension of *N*$^{\delta}$-Cbz-L-ornithine (67 mg, 0.25 mmol) in CH_2Cl_2 (2 mL) was treated with Et_3N (60 mL, 50 mg, 0.5 mmol) and Me_3SiCl (64 mL, 55 mg, 0.5 mmol) and stirred at room temperature in a stoppered flask for 30 min, with occasional sonication to improve solubility. In a separate flask, a suspension of acid **4** (62 mg, 0.2 mmol) in dry DMF (5 mL) was treated with Et_3N (35 mL, 25 mg, 0.25 mmol) and *i*-BuOCOCl (32 mL, 34 mg, 0.25 mmol). Solubilization required gentle warming (40 °C) and sonication. When a clear solution was obtained, the contents of the first flask were added, and the mixture was concentrated on the rotary evaporator to remove the CH_2Cl_2. The remaining DMF solution was then stirred at room temperature overnight and finally at 70 °C for 6 h. After cooling, 2 drops of H_2O were added, and the solution was evaporated to dryness. The residue, consisting of the Cbz derivative **17** was treated with AcOH (1 mL) and 30% HBr in AcOH (1 mL), and the course of hydrolysis of the Cbz group was monitored by TLC on cellulose with pH 7.4 phosphate buffer as the developing solvent. The Cbz derivative **17** gave an immobile spot, whereas the deblocked product **2** had R_f 0.3. At the end of the reaction, the pH was adjusted to 8 with HCl and the precipitate was collected. The solid was added to 0.01 N HCl, a small amount of undissolved material removed, the filtrate adjusted to pH 7, and the precipitated product collected and dried in vacuo at 60 °C over P_2O_5 to obtain the Na salt of **2** as a white powder (15 mg, 16% overall yield from **4**): IR (KBr) 3440, 2940, 1650, 1620, 1575, 1545, 1460 cm^{-1}; HPLC 8.9 min (0.1 M NH_4OAc, pH 7.0, with 12% MeCN, 1.0 mL/min flow rate). Anal. ($C_{22}H_{28}N_5O_4Na \cdot 1.4H_2O$) C, H, N.

Methyl *N*$^{\delta}$-Phthaloyl-L-ornithinate Hydrochloride (18). A well-stirred suspension of *N*$^{\delta}$-phthaloyl-L-ornithine[40] (1.3 g, 5 mmol) in MeOH (50 mL) in an ice bath was treated dropwise with thionyl chloride (5 mL) at a rate such that the internal temperature remained below 12 °C. After addition was complete, the bath was removed and the solution left at room temperature for 18 h. The solvent was evaporated under reduced pressure, the residue was dissolved in a minimum amount of MeOH, and a large volume of EtOAc was added to produce crystallization. The product was collected and dried in vacuo at 60 °C over P_2O_5: yield 1.53 g (98%); mp 192–193 °C (dec, gas evolution); α^{20}_D = +15.7° (c = 2, H_2O); NMR (D_2O) δ 1.93 (m, 4 H, CH_2CH_2), 3.43 (t, J = 7 Hz, 2 H, CH_2N), 3.85 (s, 3 H, CH_3O), 4.18 (m, 1 H, α-CH), 7.60 (m, 5 H, aryl). Anal. ($C_{14}H_{17}ClN_2O_4$) C, H, Cl, N.

(6*R*,6*S*)-*N*$^{\delta}$-Hemiphthaloyl-*N*$^{\alpha}$-(5,8,10-trideaza-5,6,7,8-tetrahydropteroyl)-L-ornithine (3). A suspension of **4** (63 mg, 0.2 mmol) in dry DMF (5 mL) was treated with Et_3N (20 mg, 28 mL, 0.2 mmol) and *i*-BuOCOCl (27 mg, 26 mL, 0.2 mmol), and the mixture was sonicated until all the solid dissolved. To the solution were then added 18·HCl (63 mg, 0.2 mmol) followed by another portion of Et_3N (20 mg, 28 mL, 0.2 mmol). The solvent was evaporated under reduced pressure and the residue passed through a column of silica gel (6 g, 1 × 10 cm), which was eluted first with 19:1 $CHCl_3$–MeOH and then with up to 4:1 $CHCl_3$–MeOH until no more UV-absorbing material eluted. The crude product (170 mg) was dissolved in DMSO (1 mL) and treated dropwise with stirring with 1 N NaOH (1 mL). After 5 min the pH was adjusted to 9 with 1 N HCl and the solution was applied onto a DEAE-cellulose column (1.5 × 17 cm, HCO_3^- form). The column was eluted first with a large volume of H_2O to remove salts and DMSO and then with 0.4 M NH_4HCO_3 to elute two impurities followed by a major band with R_f 0.7 (cellulose, pH 7.4 phosphate buffer). Freeze-drying of this material afforded

a colorless solid (50 mg) whose HPLC (15% MeCN in 0.1 M NH_4OAc, pH 7, flow rate 1.0 mL/min) revealed two major peaks (4:1 ratio) with retention times of 15.3 and 20 min, respectively. HPLC-homogeneous product was obtained by repeated passage of small samples (1–2 mg) through the analytical column and collecting the faster moving peak. Freeze-drying of pooled eluates afforded **3** as a water-soluble white solid (10 mg): IR (KBr) 3440, 2930, 2860, 1635, 1505, 1445, 1405, 1385, 1310, 1265, 1100, 800, 760 cm^{-1}; UV λ_{max} (95% EtOH) 230 nm (ϵ 30900), 279 inf (9160), λ_{max} (0.1 N HCl) 233 nm (ϵ 29000); high-resolution MS, M + 1 = 576.2479 (calcd 576.2458). The elemental analysis of the product was consistent with a hydrated ammonium salt, but suggested probable contamination with a small amount (5%) of silica from the HPLC column. Since the mass spectrum firmly supported the structure and the C/N ratio was correct, this material was judged to be suitable for bioassays. Sufficient material was not available for repurification. Anal. ($C_{30}H_{33}N_5O_7 \cdot NH_3 \cdot 3H_2O \cdot 0.05SiO_2$) C, H, N. C/N: calcd, 5.00; found, 5.05.

Bioassays. The ability of **1** to serve as a substrate and of **2** to serve as an inhibitor of mouse liver FPGS was evaluated as previously described.[31] Growth inhibition assays against CEM human leukemic lymphoblasts, L1210 murine leukemia cells, and SCC15 human squamous carcinoma cells were also carried out as previously reported.[23]

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Registry No. (6*R*)-L-**1**, 118537-33-0; (6*S*)-L-**1**, 118537-50-1; (6*R*)-L-**2**, 118537-34-1; (6*R*)-L-**2**·Na, 118537-57-8; (6*S*)-L-**2**, 118537-51-2; (6*S*)-L-**2**·Na, 118537-58-9; (6*R*)-L-**3**, 118537-35-2; (6*R*)-L-**3**·NH_3, 118537-59-0; (6*S*)-L-**3**, 118537-52-3; (6*S*)-L-**3**·NH_3, 118537-60-3; (6*RS*)-**4**, 118537-36-3; **5**, 51656-90-7; **6**, 4746-97-8; **7**, 93245-98-8; **8a**, 1253-46-9; **8b**, 118537-46-5; *cis*-**9a**, 118537-37-4; *trans*-**9a**, 118537-53-4; *cis*-**9b**, 118537-47-6; *trans*-**9b**, 118537-54-5; **10a**, 118537-38-5; **10b**, 118537-48-7; **11a**, 118537-39-6; **11b**, 118537-49-8; **13**, 118537-40-9; **14**, 118537-41-0; (6*RS*)-**15**, 118537-42-1; (6*R*)-L-**16**, 118537-43-2; (6*S*)-L-**16**, 118537-55-6; (6*R*)-L-**17**, 118537-44-3; (6*S*)-L-**17**, 118537-56-7; **18**, 118537-45-4; FPGS, 63363-84-8; 4-MeC_6H_4COCl, 874-60-2; 4-MeC_6H_4COOBu-*t*, 13756-42-8; 4-$BrCH_2C_6H_4CH_2COOMe$, 7398-42-7; Ph_3P, 603-35-0; H-Glu(OEt)-OEt·HCl, 1118-89-4; H-Orn(Cbz)-OH, 3304-51-6; N^{δ}-phthaloyl-L-ornithine, 7780-77-0; folic acid, 59-30-3.

Modification of the Hydroxy Lactone Ring of Camptothecin: Inhibition of Mammalian Topoisomerase I and Biological Activity

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Several camptothecin derivatives containing a modified hydroxy lactone ring have been synthesized and evaluated for inhibition of topoisomerase I and cytotoxicity to mammalian cells. Each of the groups of the hydroxy lactone moiety, the carbonyl oxygen, the ring lactone oxygen, and the 20-hydroxy group, were shown to be critical for enyzme inhibition. For example the lactol, lactam, thiolactone, and 20-deoxy derivatives did not stabilize the covalent DNA–topoisomerase I complex. With a few exceptions, those compounds that did not inhibit topoisomerase I were not cytotoxic to mammalian cells. Two cytotoxic derivatives that did not inhibit topoisomerase I were shown to produce non-protein-associated DNA single-strand breaks and are likely to have a different mechanism of action. One of these compounds was tested for antitumor activity and was found to be inactive. The present findings, as well as other reports that the hydroxy lactone ring of camptothecin is critical for antitumor activity in vivo, correlate with the structure–activity relationships at the level of topoisomerase I and support the hypothesis that antitumor activity is related to inhibition of this target enzyme.

Camptothecin (**1**) is a naturally occurring compound isolated from *Camptotheca acuminata* that exhibits antitumor activity in several experimental tumors including human colon, lung, and mammary tumor lines.[1,2] Several lines of evidence suggest that DNA topoisomerase I is the cellular locus at which camptothecin exerts its antineoplastic effects. Camptothecin and structurally related compounds stabilize a DNA–topoisomerase I covalent complex in which one strand of the DNA helix is broken.[3-6] Studies of camptothecin analogues have suggested a correlation between the ability to induce DNA breakage and antitumor activity.[7] The recent finding that camptothecin-resistant cells contain an altered topoisomerase I that is not inhibited by the drug strongly supports the hypothesis that camptothecin kills cancer cells by inhibiting this enzyme.[8,9]

(1) Wall, M. E.; Wani, M. C.; Cook, C. E.; Palmer, K. H.; McPhail, A. T.; Sim, G. A. *J. Am. Chem. Soc.* **1966**, *88*, 3888.
(2) Suffness, M.; Cordell, G. A. In *The Alkaloids: Chemistry and Pharmacology*; Brossi, A. Ed.; Academic Press: Orlando, FL, 1985; Vol. 25, p 73.
(3) Hsiang, Y.-H.; Hertzberg, R.; Hecht, S.; Liu, L. F. *J. Biol. Chem.* **1985**, *260*, 14873.
(4) Thomsen, B.; Mollerup, S.; Bonven, B. J.; Frank, R.; Blocker, H.; Nielsen, O. F.; Westergaard, O. *EMBO J.* **1987**, *6*, 1817.
(5) Hertzberg, R. P.; Holden, K. G.; Hecht, S. M.; Mattern, M. R.; Faucette, L. F.; Johnson, R. K. *Proc. Am. Assoc. Cancer Res.* **1987**, *28*, 27.
(6) Mattern, M. R.; Mong, S.-M.; Bartus, H. F.; Mirabelli, C. K.; Crooke, S. T.; Johnson, R. K. *Cancer Res.* **1987**, *47*, 1793.
(7) Horwitz, S. B. In *Antibiotics III. Mechanism of Action of Antimicrobial and Antitumor Agents*; Corcoran, J. W., Hahn, F. E., Eds.; Springer: New York, 1975; p 48.

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