# Synthesis and Antifolate Activity of New Pyrrolo[2,3-*d*]pyrimidine and Thieno[2,3-*d*]pyrimidine Inhibitors of Dihydrofolate Reductase



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Three previously undescribed dihydrofolate reductase (DHFR) inhibitors, $N^{\alpha}$-[4-[*N*-[(2,4-diaminopyrrolo[2,3-*d*]pyrimidin-5-yl)methyl]amino]benzoyl]-$N^{\delta}$-hemiphthaloyl-L-ornithine (**7**), $N^{\alpha}$-[4-[*N*-[(2,4-diaminothieno[2,3-*d*]pyrimidin-5-yl)methyl]amino]benzoyl]- $N^{\delta}$-hemiphthaloyl-L-ornithine (**8**), and *N*-[4-[*N*-[(2,4-diaminothieno[2,3-*d*]pyrimidin-5-yl)methyl]amino]benzoyl]-L-glutamic acid (**12**), were synthesized and their antifolate activity was assessed. The ability of **7** and **8** to bind to DHFR and inhibit the growth of CCRF-CEM human lymphoblastic leukemia cells in culture were dramatically reduced in comparison with the corresponding pteridine analogue, $N^{\alpha}$-(4-amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-L-ornithine (**1**, PT523). In a similar manner, the antifolate activity of **12** was markedly reduced in comparison with that of the corresponding glutamate analogue, aminopterin (**5**, AMT). In contrast, **7**, **8**, and **12** all displayed excellent affinity for the reduced folate carrier (RFC) of CCRF-CEM cells as measured by a standard competitive influx assay. Lack of a consistent correlation between the results of the growth inhibition assays and those of the DHFR and RFC binding assays results suggest that additional factors also play a role in the antifolate activity of these compounds.

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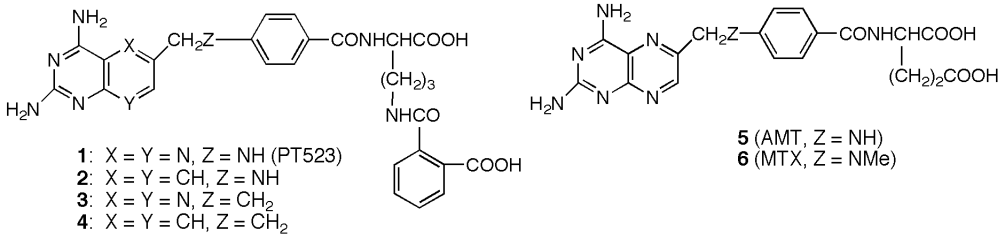
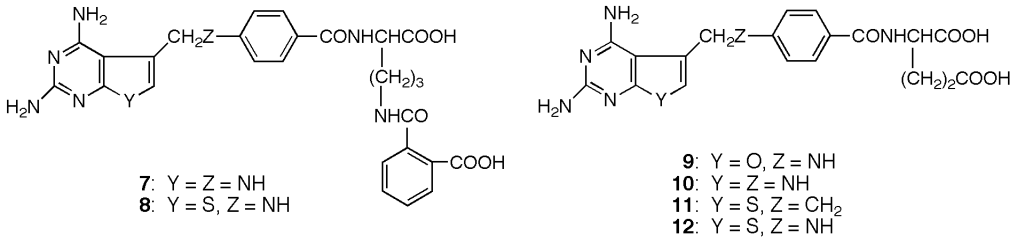
$N^{\alpha}$-(4-amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-L-ornithine (PT523, **1**) was synthesized in our laboratory a number of years ago [1a,b], and is currently in Phase 1 clinical trial. In contrast to classical antifolates with a glutamic acid side chain, the potent growth inhibitory activity of PT523 does not depend on the ability of a cell to form γ-polyglutamyl conjugates. It has been suggested that this property may confer a therapeutic advantage for PT523 in the treatment of tumors with low γ-folylpolyglutamate synthetase activity in comparison with dose-limiting tissues of the host [2].

As part of an ongoing effort to optimize the biological activity of PT523, we previously examined the effect of lengthening or shortening the amino acid side chain, moving the carboxyl group from the *ortho* to the *meta* or *para* position of the phthaloyl moiety, modifying the 9,10-bridge and 4-aminobenzoyl moiety comprising the central region of the molecule, and altering the electronic character of the B-ring by replacing either or both nitrogen atoms in the B-ring by carbon or by introducing Me or Cl substitution at the 5-position [3-7]. The various analogues were compared with respect to their ability to bind to dihydrofolate reductase (DHFR), serve as competitive inhibitors of the reduced folate carrier (RFC), and inhibit the growth of CCRF-CEM human leukemic lymphoblasts in culture [8,9]. Three of the more interesting members of the series thus far have been the 5,8-dideaza, 10-deaza-, and 5,8,10-trideaza derivatives **2**-**4**, all of which were superior to the classical DHFR inhibitors aminopterin (AMT, **5**) and methotrexate (MTX, **6**) in these assays.

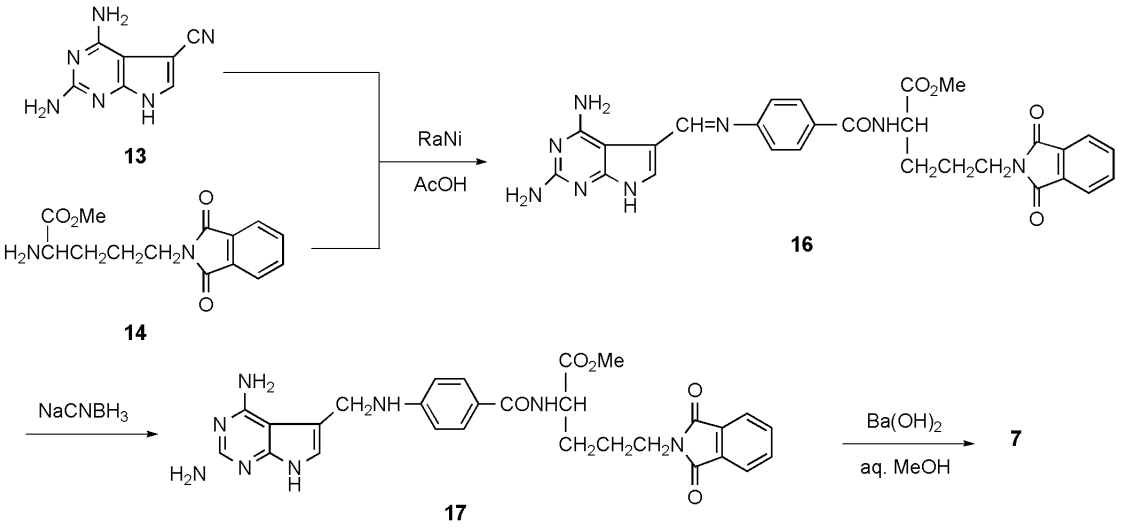
In the present paper we report the synthesis of the first two analogues of **1** in which *the B-ring is five-membered*, namely the pyrrolo[2,3-*d*]pyrimidine derivative **7** and the thieno[2,3-*d*]pyrimidine derivative **8**. Although the furo[2,3-*d*]pyrimidine and pyrrolo[2,3-*d*]pyrimidine derivatives **9** and **10** with a glutamate side chain and $CH_2NH$ bridge were known when this work began [10,11], and a synthesis of the thieno[2,3-*d*]pyrimidine derivative **11** with a glutamate side chain and $CH_2CH_2$ bridge had also been reported (albeit without biological data) [12], we were surprised to discover that the thieno[2,3-*d*]pyrimidine analogue **12** with a glutamate side chain and $CH_2NH$ bridge was unknown. Thus, we also synthesized **12** and compared its *in vitro* antifolate activity to that of **8**.

As shown in Scheme 1, the preparation of **7** began with the known compounds 2,4-diaminopyrrolo[2,3-*d*]pyrimidine-5-carbonitrile (**13**) [13] and methyl L-2-[4-aminobenzoyl)amino]-5-phthalimidopentanoate (**14**) [3], and was analogous to that of **10** from **13** and diethyl *N*-(4-(aminobenzoyl)-L-glutamate (**15**) described by Gangjee






Scheme 1



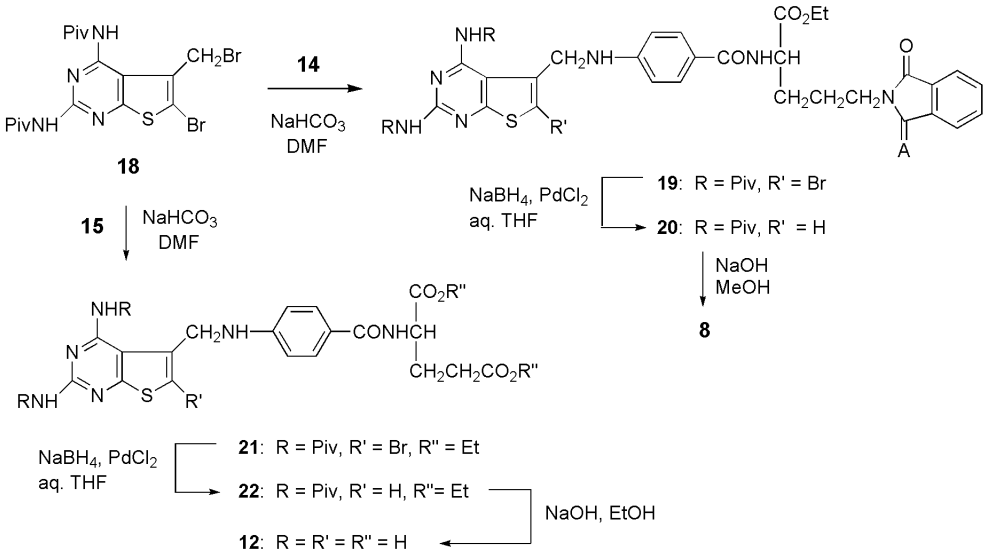

and coworkers [11]. The product was purified by flash chromatography on silica gel and characterized by its $^1$H NMR spectrum in DMSO-$d_6$ solution, which featured the expected singlets for the C6 and N7 protons of the pyrrole moiety and the CH=N proton of the bridge. The presence of a methyl ester group and closed phthalimide ring were also evident from the spectrum, and were supported by mass spectrometry. Treatment of **16** for 4 hours at room temperature with $NaCNBH_3$ in MeOH at pH 2 led to the desired reduction product **17**, whose $^1$H NMR spectrum showed the required loss of the bridge CH=N proton and confirmed that the methyl ester and closed phthalimide ring had remained intact. The identity of **17** was also supported by mass spectrometry and microchemical analysis. Deprotection of **17** was accomplished readily by treatment with barium hydroxide. The $^1$H NMR spectrum of the deprotected product in DMSO-$d_6$ solution again confirmed the presence of the C6 and N7 protons on the pyrrolo[2,3-*d*]pyrimidine moiety.

For the synthesis of the thieno[2,3-*d*]pyrimidine **8** (Scheme 2), we began with 2,4-dipivaloylamino-5-bromomethyl-6-bromothieno[2,3-*d*]pyrimidine (**18**) [14a,b]. Heating a mixture of **18** and **14** with dried sodium bicarbonate in dimethylformamide solution at 55 °C for 48 hours, followed by purification on a silica gel column afforded the bromo ester **19**, which was debrominated directly to **20** with sodium borohydride and palladium chloride in aqueous tetrahydrofuran at room temperature as described earlier. After purification to homogeneity, the product was treated directly with sodium hydroxide at room temperature for 5 days to cleave the methyl ester, remove the pivaloyl groups, and open the phthalimide ring. The same sequence starting from **15** instead of **14** afforded **12** *via* the bromo diester **21** and the debrominated diester **22**.

As in the synthesis of the pyrrolo analogue **7**, the identity of each compound in Scheme 2 was confirmed from $^1$H NMR spectra. Thus, reduction of **19** to **20** and of **21** to **22** was accompanied by the appearance of a distinctive vinyl singlet, and deprotection of **20** and **22** with sodium hydroxide was evident from the loss of the signals corresponding to the pivaloyl and methyl ester groups. Concomitant opening of the phthalimide ring was confirmed by the appearance of a new CONH peak along with the same change in appearance of the signal for the δ-$CH_2$ protons of the ornithine side chain as we had noted earlier in other examples of this cleavage reaction [6]. A small downfield shift for the C6 proton in **12** relative to **7** by ca. was consistent with the lower electronegative character of the sulfur atom.

The pyrrolo[2,3-*d*]pyrimidine **7** and thieno[2,3-*d*]pyrimidines **8** and **12** were tested for their ability to bind to recombinant human DHFR according to standard assays

Scheme 2




previously described [8,9]. As shown in Table 1, the potency of the pyrrolo[2,3-*d*]pyrimidine **7** was decreased 7-fold relative to PT523, whereas that of the thieno[2,3-*d*]pyrimidine **8** was decreased 60-fold, indicating that these structural modifications of the B-ring were both detrimental to binding. The weaker inhibition observed with **7** in comparison with PT523 was consistent with the results of Gangjee and coworkers [11], who found, using rat DHFR and $IC_{50}$ rather than $K_i$ values, that compound **10**, the analogue of **7** with a glutamate side chain, was 15-fold less potent than MTX (**6**). Although a three-dimensional structure for the ternary complex of **10** with NADPH and rat DHFR is not available, possible reasons for its relatively weak binding might be (i) that the change of the B-ring nitrogen atom from an H-bond acceptor to an H-bond donor is unfavorable and/or (ii) that the side chain is less optimally positioned for binding when the B-ring is five-membered than when it is six-membered. The weaker binding of **7** to human DHFR relative to PT523 presumably reflects steric and electronic differences analogous to those between **10** and MTX. In the case of the thienopyrimidine analogue **8**, we surmise that decreased affinity relative to **7** reflects (i) the inability of the sulfur atom to function as an H-bond acceptor and/or (ii) its larger size in comparison with nitrogen. As a consequence of the larger size of sulfur, this substitution in the five-membered ring might produce a less favorable orientation of the bridge, and hence the hemiphthaloylornithine side chain, than is the case in the pyrrole analogue **7** [16]. It was of interest to note, as well, that **8** was a 27-fold better inhibitor than **12**, in accord with our earlier findings with other types of B-ring analogues containing a hemiphthaloylornithine side chain as opposed to a glutamate side chain [8].

Table 1

Dihydrofolate Reductase (DHFR) Binding and CCRF-CEM Cell Growth Inhibition by Pyrrolo[2,3-*d*]pyrimidine and Thieno[2,3-*d*]pyrimidine Analogues of PT523 (**1**) and Aminopterin (**5**)

| Compound | DHFR Inhibition $K_i$ (pM) [a] | RFC binding $K_i$ (μM) [a] | Cell Growth $IC_{50}$ (_M, 72 h) [a] |
|---|---|---|---|
| **1** [b] | 0.30 ± 0.036 (n = 7) | 0.71 ± 0.12 (n = 3) | 1.5 ± 0.39 (n = 10) |
| **5** [b] | 3.7 ± 0.35 (n = 3) | 5.4 ± 0.09 (n = 3) | 4.4 ± 0.10 (n = 3) |
| **7** | 2.2 ± 0.51 (n = 4) | 0.14 ± 0.05 (n = 3) | 68 ± 0.11 (n = 6) |
| **8** | 18 ± 6.1 (n = 6) | 0.075 ± 0.01 (n = 3) | 120 ± 28 (n = 5) |
| **12** | 480 ± 110 (n = 3) [c] | 0.53 ± 0.12 (n = 3) | 43 ± 7.0 (n = 4) |

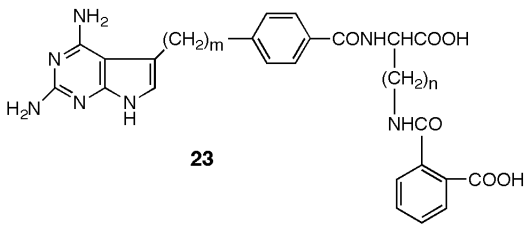
[a] Assays were carried out as described in reference 8 and 9. [b] Data for PT523 (**1**) and AMT (**5**) included for comparison are taken from reference 9. All numbers are rounded off to two significant figures and are followed by the standard deviation for n experiments performed on different days. [c] In contrast to **7** and **8**, the very weak inhibition of DHFR by **12** was typical of Zone B equilibrium binding [15].

In addition to their interaction with DHFR, we also examined the ability of **7**, **8**, and **12** to compete with [$^3$H]MTX for binding to the reduced folate carrier (RFC) of CCRF-CEM human lymphoblastic leukemia cells using the same standardized influx assay we had used earlier to compare the RFC binding of PT523 and its 5-deaza, 8-deaza, and 5,8-dideaza analogues [9]. As can be seen from Table 1, compounds **7** and **8** were both better inhibitors of RFC binding than either AMT or PT523, and in fact proved to have the lowest $K_i$s of any AMT analogues with a hemiphthaloylornithine side we have tested to date. Thus, in contrast to DHFR binding (see above), the five-membered B-ring appeared to be highly favorable for RFC binding [18]. Interestingly, while it was not quite as low as that of **8**, the $K_i$ of **12** was also lower than that of AMT,

providing further evidence of the advantage of a five-membered over a six-membered B-ring where binding to the RFC protein is concerned.

With regard to cell growth inhibition, **7** was 45-fold less potent than PT523, **8** was 80-fold less potent than PT523, and **12** was 10-fold less potent than AMT during 72 hours of continuous drug exposure, at which point the DHFR would presumably be fully saturated with inhibitor. Thus there was a greater decrease in growth inhibitory activity than could be accounted for by the $K_i$ differences in the DHFR and RFC binding assays alone. In the case of **7** and **8**, the fact that improved RFC binding was insufficient to offset decreased DHFR binding indicates that other factors are probably at work. For example, the steady-state level of *free* drug (*i.e.* not bound to DHFR) in the cell [19] might be lower with **7** and **8** than it is with PT523 because a difference in the ability of the RFC to act as a bidirectional pump mediating efflux as well as influx once the DHFR is saturated with drug [20]. In the case of **12**, a critical factor in addition to DHFR binding and cellular uptake of the monoglutamates form of the drug, is that polyglutamation (which we did not examine) might be less efficient than in the case of AMT.

It is of interest to note that, when our work on **7** began, we were unaware that a Japanese group was independently synthesizing DHFR inhibitors with a hemiphthaloylornithine or other non-glutamate side chain linked to a pyrrolo[2,3-*d*]pyrimidine scaffold [21]. Six compounds of general structure **23** were prepared in which the length of the $(CH_2)_m$ and $(CH_2)_n$ linkers was varied with a view to optimizing DHFR binding and cell growth inhibition. The analogue closest in structure to PT523, and the one that seemed to give the best combination of DHFR binding and cell growth inhibition, was **23** (m = 2, n = 3), with an $IC_{50}$ of 6.9 nM against bovine DHFR, an $IC_{50}$ of 1.5 nM against a mouse fibrosarcoma cell line (MethA), and an $IC_{50}$ of 7.5 nM against CCRF-CEM cells (72 h treatment) [21]. Thus, under incubation conditions essentially identical to ours, **23** was a more potent inhibitor of the growth of CCRF-CEM cells than **7** by almost one order of magnitude, showing that a $CH_2CH_2$ group is better than a $CH_2NH$ bridge when the heterocyclic scaffold is a pyrrolo[2,3-*d*]pyrimidine, just as we had observed, though to a lesser extent, when the scaffold was a pteridine (*i.e.*, in the case of the 10-deaza analogue of PT523) [6].




**23**

In summary, pyrrolo[2,3-*d*]pyrimidine and thieno[2,3-*d*]-pyrimidine analogues of the potent nonpolyglutamatable DHFR inhibitor PT523 were synthesized and tested *in vitro* as antifolates. Also synthesized was a previously unknown thieno[2,3-*d*]pyrimidine analogue of AMT. Replacement of the pteridine scaffold by these 6/5 ring systems with retention of the $CH_2NH$ bridge and hemiphthaloylornithine side chain was favorable for RFC binding but not for DHFR binding or cell growth inhibition. These results add to our understanding of the structure-activity correlations that have to be to be considered during the development of efficacious second-generation PT523 analogues.

## EXPERIMENTAL

2,4-Diaminopyrrolo[2,3-*d*]pyrimidine-5-carbonitrile (**13**) was synthesized from 2-amino-5-bromo-3,4-dicyanopyrrole [13] according to Gangjee and coworkers [11]. Methyl 2-L-[*N*-(4-aminobenzoyl)amino]-5-phthalimidopentanoate (**14**) was prepared as described earlier [3]. 2,4-Bis(pivaloylamino)-5-bromomethyl-6-bromothieno[2,3-*d*]pyrimidine (**18**) was the same minimally purified material we had used to prepare a series of lipophilic 2,4-diamino-5-thieno[2,3-*d*]pyrimidines [14a,b], and was obtained from 2,4-diamino-5-methylthieno[2,3-*d*]pyrimidine [22,23] by acylation with pivalic anhydride in pyridine followed by reaction with *N*-bromosuccinimide and dibenzoyl peroxide in chloroform. Diethyl *N*-(4-aminobenzoyl)-L-glutamate (**15**) and other chemicals were purchased from Aldrich, Milwaukee, WI. Other reagents were from Fisher, Boston, MA. Infrared spectra were obtained in potassium bromide disks with a Perkin-Elmer model 781 double-beam spectrophotometer. Only peaks with wave numbers greater than 1200 $cm^{-1}$ are reported. $^1$H-nmr spectra were recorded at 200 MHz on a Varian VX200 instrument. Fast-ion bombardment mass spectra (FABMS) were obtained by staff of the Dana-Farber Cancer Institute Molecular Biology Core Facility. High-resolution mass spectra (HRMS) were obtained by staff of the Department of Chemistry, Harvard University, Cambridge, MA. TLC analyses were performed on Whatman MK6F silica gel plates with UV illumination at 254 nm. Column chromatography was performed on Baker 7025 flash silica gel (40 μm particle size). HPLC separations were on $C_{18}$ silica gel radial compression cartridges (Millipore, Milford, MA; analytical, 5-μm particle size, 5 x 100 mm; preparative, 15-μm particle size, 25 x 100 mm). Melting points were measured in Pyrex capillary tubes in a Mel-Temp "Electrothermal" apparatus (Fisher), or on a hot-stage microscope apparatus (Fisher), and are not corrected. Elemental analyses (C, H, N) were performed by Robertson Laboratories, Madison, NJ, and were within ± 0.4% of theoretical values unless otherwise specified.

2,4-Diaminopyrrolo[2,3-*d*]pyrimidin-5-yl)methyl]amino]benzoyl]-amino]-N$^{\delta}$-hemiphthaloyl-L-ornithine (**7**).

Step 1. Raney nickel (3.3 g) was added to a solution of **13** (0.67 g, 3.88 mmole) and **14** (2.4 g, 5.82 mmole) in 80% acetic acid (65 mL) in a Parr apparatus and the mixture was shaken under hydrogen at 15 psi for 12 hours. The mixture was filtered through Celite, the filter pad was washed with 10% acetic acid, and the combined filtrate and wash solution were removed on the rotary evaporator. A cold solution of saturated aqueous sodium

bicarbonate was added to the residue, and the mixture was kept on ice for 1 hour. The yellowish-brown solid was collected by filtration, washed with water, dried in a vacuum oven, taken up in a minimal volume of 9:1 chloroform-methanol, and chromatographed on column of flash-grade silica gel packed with 95:5 chloroform-methanol and eluted sequentially with 95:5 and 93:7 chloroform-methanol. Fractions containing a major tlc spot at $R_f$ 0.31 (silica gel, 9:1 chloroform-methanol) along with a minor impurity were pooled and evaporated to dryness. The residue was dissolved in small volume of 9:1 chloroform-methanol, and the solution was poured into a large volume of ether. The precipitate was collected by filtration, washed well with ether, and dried in a vacuum oven to obtain Schiff's base **16** as yellow powder (0.475 g, 22%); ir: ν 3350, 3250, 2960, 1750, 1710, 1650, 1610, 1550, 1510, 1400, 1380, 1270, 1200 $cm^{-1}$; $^1$H-nmr: δ 1.70-1.78 (m, 4H, β- and γ-$CH_2$), 3.57-3.60 (m, 5H, $COOCH_3$ and δ-$CH_2$), 4.44 (m, H, α-CH), 5.67 (br s, $NH_2$), 6.70 (br s, $NH_2$), 7.22-7.26 (m, 2H, 3'- and 5'-H), 7.52 (s, H, 6-H), 7.80-7.89 (m, 6H, 2'-H, 6'-H, and phthalimide ring protons), 8.47 (s, H, C*H*=N), 8.63-8.67 (d, H, CONH), 11.51 (br s, H, 7-NH); FABMS: *m/e* 555 (M+H). This material was used directly in the next step.

Step 2. Solid sodium cyanoborohydride (70 mg, 1.12 mmole) was added to a stirred solution of **16** (450 mg, 0.81 mmole) in methanol (50 mL), and the pH of the solution was adjusted to 2 using 6 *N* hydrochloric acid. Stirring was continued for 4 hours at room temperature, and the precipitated solid was collected by filtration, washed with methanol, dried in a vacuum oven, and flash chromatographed on a silica gel column packed and eluted with 95:5 chloroform-methanol. Fractions containing a single tlc spot with $R_f$ 0.23 (silica gel, 9:1 chloroform-methanol) were pooled and evaporated to dryness. The residue was dissolved in a small volume of 9:1 chloroform-methanol and the solution was added to a large volume of ether. The precipitate was collected by filtration, washed with ether and dried in a vacuum oven to obtain **17** as pale-yellow powder (213 mg, 47%); mp 190 °C dec; ir: ν 3350, 3200, 1710, 1640, 1590, 1530, 1500, 1400, 1350, 1210 $cm^{-1}$; $^1$H-nmr ($d_6$-dimethylsulfoxide): δ 1.67-1.74 (m, 4H, β- and γ-$CH_2$), 3.57-3.61 (m, 5H, $COOCH_3$ and δ-$CH_2$), 4.31-4.38 (m, 3H, bridge C$H_2$NH and α-CH), 6.6 (m, H, bridge $CH_2$N*H*), 6.68-6.72 (m, 2H, 3'- and 5'-H), 6.95 (s, H, 6-H), 7.21 (br s, $NH_2$), 7.61-7.65 (m, 2H, 2'- and 5'-H), 7.80-7.85 (m, 4H, phthalimide ring protons), 7.9 (br s, $NH_2$), 8.23-8.27 (d, H, CONH), 11.62 (br s, H, 7-NH); ms: *m/e* 579 (M+Na).

Step 3. A solution of **17** (50 mg, 0.09 mmole) in methanol (25 mL) containing a few drops of water was diluted with additional water (25 mL), treated with solid barium hydroxide (80 mg, 0.25 mmole), and stirred at room temperature for 48 hours. Solid ammonium bicarbonate (100 mg) was then added, and stirring was continued for 30 minutes. The precipitated barium carbonate was filtered, the solvents were evaporated under reduced pressure, and the residue was dissolved in 10% ammonium hydroxide. The solution was cooled in ice and adjusted to pH 4.5 with 10% acetic acid. The precipitate was collected by filtration immediately, washed with water, and dried in a lyophilizer and then *in vacuo* at 50 °C over phosphorous pentoxide to obtain **7** as a white solid (30 mg, 70%); mp >200 °C, darkening above 190 °C; hplc: 21 min ($C_{18}$ silica gel, 1 to 25% acetonitrile gradient over 20 min in 0.1 *M* ammonium acetate, pH 7.5, 1 mL/min); ir: ν 3350. 2950, 1700, 1600, 1570, 1520, 1430, 1390 $cm^{-1}$; $^1$H-nmr ($d_6$-dimethylsulfoxide): δ 1.54-1.95 (m, 4H, β- and γ-$CH_2$), 3.16 (m, 2H, δ-$CH_2$), 4.24-4.26 (m, 2H, bridge C$H_2$NH), 4.35 (m, H, α-CH), 5.44 (br s, $NH_2$), 6.08 (br s, $NH_2$) 6.49 (t, H, bridge $CH_2$N*H*), 6.68 (s, H, 6-H), 6.71-6.75 (m, 2H, 3'- and 5'-H), 7.37-7.47 (m, 3H, phthaloyl ring protons), 7.63 (m, 1H, phthaloyl ring proton), 7.64-7.71 (m, 2H, 2'- and 6'-H), 8.03-8.06 (d, H, CONH), 8.36 (t, H, phthaloyl CONH), 10.53 (br s, H, 7-NH).

*Anal*. Calcd. for $C_{27}H_{28}N_8O_6 \cdot H_2O$; C, 56.05, H, 5.23, N, 19.37. Found; C, 55.72; H, 4.92; N, 19.49.

$N^{\alpha}$-[4-[*N*-[(2,4-Diaminothieno[2,3-*d*]pyrimidin-5-yl)methyl]-amino]benzoyl]-$N^{\delta}$-hemiphthaloyl-L-ornithine (**8**).

Step 1. Compound **14** (2.4 g, 6.1 mmole) and sodium bicarbonate (7.7 g, 92 mmole) were added sequentially to a solution of **18** (4.37 g, 9.2 mmole) in dry dimethylformamide (20 mL) and the reaction mixture was stirred at 55 °C for 48 hours. The solvent was removed by rotary evaporation, and the residue was taken up in ethyl acetate (200 mL). Some dark-brown insoluble material did not dissolve and was discarded. The solution was washed with water (100 mL), dried over anhydrous sodium sulfate, and evaporated. The product was chromatographed on a flash-grade silica gel column packed and eluted with 99:1 chloroform-methanol. Fractions whose tlc contained a spot with $R_f$ 0.50 (silica gel, 95:5 chloroform-methanol) were pooled and concentrated to dryness to obtain the bispivaloyated bromo ester **19** (690 mg, 10%); $^1$H-nmr (deuteriochloroform): δ 1.3 (m, 18H, 2 x t-Bu), 1.65-1.80 (m, 4H, β- and γ-$CH_2$), 3.65-3.72 (m, 5H, COOMe and δ-$CH_2$), 4.4-4.6 (m, 3H, α-CH and bridge C$H_2$NH), 6.3 (m, 1H, bridge $CH_2$N*H*), 6.65-6.9 (m, 2H, 3'- and 5'-H), 7.56-7.60 (m, 2H, 2'- and 6'-H), 7.68-7.82 (m, 4H, phthalimide ring protons), 8.0 (s, 1H, t-BuCON*H*), 8.2 (d, 1H, CONH), 8.65 (s, 1H, t-BuCON*H*); ms (FAB): *m/e* 844 (M+Na). This material was used directly in the next step.

Step 2. A stirred solution of **19** (650 mg, 0.79 mmole) in tetrahydrofuran (100 mL) was cooled to 0 °C, and treated sequentially with water (100 mL), palladium chloride (280 mg, 1.58 mmole), and sodium borohydride (299 mg, 7.9 mmole). After 20 minutes at 0 °C, the reaction mixture was left to stir at room temperature for 4 hours. Thin-layer chromatography showed two new spots with $R_f$ 0.40 and $R_f$ 0.23 (silica gel, 95:5 chloroform-methanol), and no starting material ($R_f$ 0.50). The reaction mixture was filtered through Celite, the filter pad was washed with 1:1 methanol-water, and the tetrahydrofuran and methanol were removed from the combined filtrate and wash by rotary evaporation. The remaining aqueous solution was diluted with water (100 mL) and extracted with chloroform (2 x 200 mL). The combined organic layers were washed with water (2 x 25 mL), dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was chromatographed on a column of flash-grade silica gel packed and eluted with 98:2 chloroform-methanol. Evaporation of appropriately pooled fractions containing a single tlc spot with $R_f$ 0.40 afforded the debrominated bispivaloyl ester **20** as a pale-yellow powder (98 mg, 17%); $^1$H-nmr (deuteriochloroform): δ 1.3 (s, 18H, 2 x t-Bu), 1.65-1.80 (m, 4H, β- and γ-$CH_2$), 3.65-3.70 (m, 5H, COOMe and δ-$CH_2$), 4.6-4.8 (m, 3H, α-CH and bridge C$H_2$NH), 5.8 (t, 1H, bridge $CH_2$N*H*), 6.56-6.62 (m, 2H, 3'- and 5'-H), 7.01 (s, 1H, 6-H), 7.59-7.63 (m, 2H, 2'- and 6'-H), 7.67-7.84 (m, 5H, t-BuCON*H* and phthalimide ring protons), 8.15 (d, 1H, CONH), 8.55 (s, 1H, t-BuCON*H*); FABMS: *m/e* 764 (M+Na). This material was used directly in the next step.

Step 3. A solution of **20** (98 mg, 0.13 mmole) in a mixture of methanol (12 mL) and 1 *N* sodium hydroxide (6 mL) was stirred at room temperature for 5 days, then concentrated to dryness on the

rotary evaporator. The residue was taken up in water (5 mL), and the solution was cooled on ice and adjusted to pH 4.5 with 1 *N* hydrochloric acid. The precipitate was filtered, washed with water, dried in a vacuum oven, and purified by preparative hplc ($C_{18}$ silica gel, 18% acetonitrile in 0.1 *M* ammonium acetate, pH 7.4, 10 mL/min). Appropriate fractions were pooled and lyophilized to obtain **8** as a pale-yellow solid (25 mg, 33%); mp >200 °C, darkening above 170 °C; analytical hplc: 8 min ($C_{18}$ silica gel, 18% acetonitrile in 0.1 *M* ammonium acetate, pH 7.4, 1 mL/min); ir: ν 3300, 2940, 1620, 1550, 1500, 1400, 1300, 1250 $cm^{-1}$; $^1$H-nmr ($d_6$-dimethylsulfoxide): δ 1.51-1.75 (m, 4H, β- and γ-$CH_2$), 3.13 (m, 2H, δ-$CH_2$), 4.31 (m, 3H, α-CH and bridge C*H*$_2$NH), 5.99 (br s, $NH_2$), 6.48 (br s, $NH_2$), 6.69-6.73 (m, 2H, 3'- and 5'-H), 6.77 (s, 1H, 6-H), 7.31-7.45 (m, 3H, phthaloyl ring protons), 7.65-7.69 (m, 3H, 2'-, 6'-, and phthaloyl ring proton), 8.15 (d, 1H, CONH), 8.22 (m, 1H, phthaloyl CONH); HRMS: *m/e* found 578.1823 (M+H), calcd 578.1822. Because it was purified to homogeneity by preparative hplc and its HRMS and $^1$H-nmr data were fully consistent with the assigned molecular structure, elemental microanalysis was omitted and the product was used for DHFR binding, transport, and cell growth assays directly.

*N*-[4-[[*N*-(2,4-Diaminothieno[2,3-*d*]pyrimidin-5-yl)methyl]-amino]benzoyl]-L-glutamic Acid (**12**).

Step 1. Diethyl *N*-(4-aminobenzoyl)-L-glutamate (**15**) (1.36 g, 4.22 mmole) and sodium bicarbonate (5.2 g, 63 mmole) were added sequentially to a solution of **18** (3 g, 6.3 mmole) in dry dimethylformamide (15 mL) and the reaction mixture was stirred at 55 °C for 24 hours. The solvent was removed by rotary evaporation, and the residue was taken up in ethyl acetate (200 mL). The solution was washed with water (100 mL), dried over anhydrous sodium sulfate, and evaporated. The product was taken up in a minimal volume of 99:1 chloroform-methanol and chromatographed on a flash-grade silica gel column packed and eluted with the same solvent mixture. Fractions containing a tlc spot with $R_f$ 0.48 (silica gel, 95:5 chloroform-methanol) and a faint slower-moving impurity were pooled and concentrated to dryness to obtain the bispivaloyl bromo diester **21** (327 mg, 10%); $^1$H-nmr (deuteriochloroform): δ 1.1 (m, 6H, 2 x C*H*$_3$CH$_2$), 1.3 (s, 18H, 2 x t-Bu), 1.8 (m, 2H, β-$CH_2$), 2.4 (t, 2H, γ-$CH_2$), 4.0-4.2 (m, 4H, 2 x $CH_3$C*H*$_2$), 4.4-4.6 (m, 3H, α-CH and bridge C*H*$_2$NH), 6.25 (m, 1H, bridge $CH_2$N*H*), 6.6 (m, 2H, 3'- and 5'-H), 7.4 (m, 2H, 2'- and 6'-H), 8.0 (s, 1H, t-BuCON*H*), 8.15 (d, 1H, CONH), 8.8 (s, 1H, t-BuCON*H*); ms (FAB); *m/e* 748 (M+H). This material was used directly in the next step.

Step 2. A stirred solution of **21** (433 mg, 0.58 mmole) in tetrahydrofuran (65 mL) was cooled to 0 °C, and treated sequentially with water (65 mL), palladium chloride (205 mg, 1.16 mmole), and sodium borohydride (219 mg, 5.8 mmole). After 20 minutes at 0 °C, the reaction mixture was left to stir at room temperature for 4 hours. Thin-layer chromatography showed a new spot with $R_f$ 0.40 (silica gel, 95:5 chloroform-methanol), along with disappearance of the starting material ($R_f$ 0.48). The reaction mixture was filtered through Celite, the filter pad was washed with 1:1 methanol-water, and the tetrahydrofuran and methanol were removed from the combined filtrate and wash by rotary evaporation. The remaining aqueous solution was diluted with water (50 mL) and extracted with chloroform (2 x 100 mL). The combined organic layers were washed with water (2 x 25 mL), dried over anhydrous sodium sulfate, and evaporated under reduced pressure to obtain the debrominated bispivaloyl ester **22** (301 mg, 77.5%); $^1$H-nmr (deuteriochloroform): δ 1.2 (m, 6H, 2 x C*H*$_3$CH$_2$), 1.3 (s, 18H, 2 x t-Bu), 1.8 (m, 2H, β-$CH_2$), 2.4 (t, 2H, γ-$CH_2$), 4.0-4.2 (m, 4H, 2 x $CH_3$C*H*$_2$), 4.6-4.8 (m, 3H, α-CH and bridge C*H*$_2$NH), 5.8 (t, 1H, bridge $CH_2$N*H*), 6.7 (m, 2H, 3'- and 5'-H), 6.95 (s, 1H, 6-H), 7.4 (m, 2H, 2'- and 6'-H), 8.0-8.2 (m, 1H, t-BuCON*H*); FABMS: *m/e* 669 (M+H). This material was used directly in the next step.

Step 3. A solution of **22** (300 mg, 0.4 mmole) in a mixture of 95% ethanol (40 mL) and 1 *N* sodium hydroxide (20 mL) was stirred at room temperature for 4 days, then concentrated to dryness on the rotary evaporator. The residue was taken up in water (10 mL), and the solution was cooled on ice and adjusted to pH 4.5 with 1 *N* hydrochloric acid. The precipitate was collected by filtration, washed with water, dried in a vacuum oven, and purified by preparative hplc ($C_{18}$ silica gel, 12% acetonitrile in 0.1 *M* ammonium acetate, pH 7.4, 10 mL/min). Appropriate fractions were pooled and lyophilized to obtain **12** as a pale-yellow solid (33 mg, 17%); mp >200 °C, darkening above 175 °C; ir: ν 3300, 1650, 1600, 1560, 1500, 1450, 1450, 1400, 1300, 1250 $cm^{-1}$; $^1$H-nmr ($d_6$-dimethylsulfoxide): δ 1.8-2.0 (m, 2H, β-$CH_2$), 2.3 (t, 2H, γ-$CH_2$), 4.0-4.2 (m, 3H, α-CH and bridge C*H*$_2$NH), 6.0 (br s, $NH_2$), 6.5 (br s, $NH_2$), 6.65 (t, 1H, bridge $CH_2$N*H*), 6.73-6.77 (m, 2H, 3'- and 5'-H), 6.82 (s, 1H, 6-H), 7.66-7.70 (m, 2H, 2'- and 6'-H); 8.1 (m, 1H CONH); FABMS: *m/e* 445 (M+H).

*Anal.* Calcd for $C_{19}H_{20}N_6O_5S\cdot 0.7H_2O$: C, 49.92; H, 4.71; N, 18.38. Found: C, 49.60; H, 4.53; N, 18.38.

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REFERENCES AND NOTES

[1a] A. Rosowsky, H. Bader, C. A. Cucchi, R. G. Moran, W. Kohler, and J. H. Freisheim, *J. Med. Chem.*, **31**, 1332 (1988); [b] A. Rosowsky, H. Bader, and R. A. Forsch, . *Pteridines*, **1**, 91 (1989).

[2] A. Rosowsky, *Current Med. Chem.*, **6**, 329 (1999).

[3] A. Rosowsky, H. Bader, J. E. Wright, K. Keyomarsi, and L. H. Matherly, *J. Med. Chem.*, **37**, 2167 (1994)

[4] A. Rosowsky, C. M. Vaidya, H. Bader, J. E. Wright, and B. A. Teicher, *J. Med. Chem.*, **40**, 286 (1997).

[5] A. Rosowsky, J. E. Wright, C. M. Vaidya, H. Bader, R. A. Forsch, C. Mota, J. Pardo, C. S. Chen, and Y. N. Chen, *J. Med. Chem.*, **41**, 5310 (1988)

[6] A. Rosowsky, J. E. Wright, C. M. Vaidya, R. A. Forsch, and H. Bader, *J. Med. Chem.*, **43**, 1620 (2000)

[7] C. Vaidya, J. E. Wright, and A. Rosowsky. *J. Med. Chem.*, **45**, 1690 (2002)

[8] J. E. Wright, C. M. Vaidya, Y. N. Chen, and A. Rosowsky, *Biochem. Pharmacol.,* **60**, 41 (2000).

[9] J. E. Wright, G. K. Yurasek, Y. N. Chen, and A. Rosowsky, *Biochem. Pharmacol.* **65**, 1427 (2003).

[10] A. Gangjee, R. Devraj, J. J. McGuire, R. L. Kisliuk, S. F. Queener, and L. R. Barrows, *J. Med. Chem.*, **37**, 1169 (1994).

[11] A. Gangjee, F. Mavandadi, S. F. Queener, and J. J McGuire, *J. Med. Chem.*, **38**, 2158 (1995).

[12] E. C. Taylor, H. Patel, G. Sabitha, and R. Chaudhari, R. *Heterocycles*, **4**, 349 (1996).

[13] E. E. Swayze, J. M. Hinkle, and L. B. Townsend, in *Nucleic Acid Chemistry, Part 4*; L. B. Townsend and R. S. Tipson, R., Eds.,

Wiley-Interscience, 1991, pp 16-17.

[14a] A. Rosowsky, A. T. Papoulis, and S. F. Queener, *J. Med. Chem.*, **40**, 3694 (1997); [b] A. Rosowsky, A. T. Papoulis, and S. F. Queener, *J. Med. Chem.*, **46**. 3424 (2003).

[15] O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).

[16] The spatial orientation of the side chain in the *furan* analogue **9** in its ternary complex with NADPH and human DHFR has been found by X-ray crystallographic analysis to be different from that of the side chain in MTX [17], and this may also be true in the case of the thiophene analogue **12**.

[17] V. Cody, N. Galitsky, J. R. Luft, W. Pangborn, A. Gangjee, R. Devraj, S. F. Queener, and R. L. Blakley, *Acta Crystal.*, **D53**, 638 (1997).

[18] Although this unanticipated result was gratifying, it does not necessarily signify that **7** and **8** accumulate to higher steady-state levels in the cell than PT523. High levels of cellular uptake of an antifolate *via* the RFC requires not only a high affinity (low $K_m$) for the carrier protein but also a high $V_{max}$, the latter of which is then used to calculate the more relevant first-order rate constant ($V_{max}/K_m$). Because $V_{max}$ is typically obtained using a radioactive substrate, and radiolabeled **7** and **8** were not available, measurement of the $V_{max}/K_m$ for these compounds was outside the scope of this work.

[19] Because the binding of diaminoheterocyclic antifolates to the active site of DHFR is thermodynamically reversible except in those instances where a covalent bond is formed, it is generally considered that an excess of free drug is important as a way to minimize dissociation of the antifolate from the enzyme, thereby maximally prolonging DNA synthesis inhibition.

[20] F. M. Sirotnak, *Pharm. Ther.*, **8**, 71 (1980).

[21] F. Itoh, Y. Yoshioka, K. Yukishige, S. Yoshida, K. Ootsu, and H. Akimoto, *Chem. Pharm. Bull.*, **49**, 1270 (2000).

[22] B. Roth, R. Laube, M. Y. Tidwell, and B. S. Rauckman, *J. Org. Chem.*, **45**, 3651 (1980).

[23] Although the preparation of 2,4-diamino-5-methylthieno[2,3-*d*]pyrimidine from 2,4-diamino-6-mercaptopyrimidine and chloroacetone described in ref. 14a was originally said to work with $KHCO_3$ as the base, this statement was an error [14b] and we subsequently returned to the original procedure using NaOMe [22].