# Synthesis and Biological Activity of $N^{\omega}$-Hemiphthaloyl-$\alpha,\omega$-diaminoalkanoic Acid Analogues of Aminopterin and 3′,5-Dichloroaminopterin[1]

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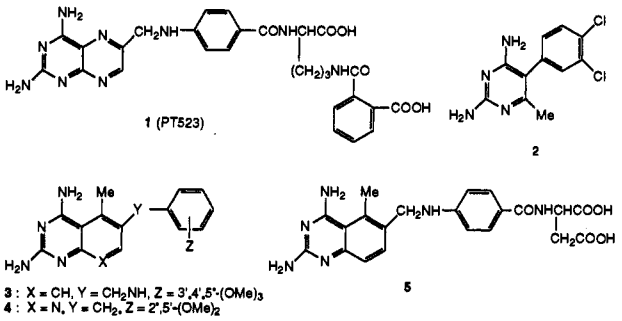
Analogues of $N^{\alpha}$-(4-amino-4-deoxypteroyl)-$N^{\delta}$-(hemiphthaloyl)-L-ornithine (PT523) with 3′,5′-dichloro substitution in the *p*-aminobenzoyl moiety or with one less or one more $CH_2$ group in the amino acid moiety were synthesized and tested as inhibitors of dihydrofolate reductase (DHFR) activity and cell growth. Replacement of L-ornithine in PT523 by L-2,4-diaminobutanoic acid or L-lysine did not decrease binding to human recombinant DHFR but resulted in some loss of activity against SCC25 human and SCC VII murine squamous cell carcinoma and against MCF-7 human breast carcinoma in culture. PT523 was several times more potent than methotrexate (MTX), aminopterin (AMT), or trimetrexate (TMQ). 3′,5′-Dichloro substitution did not decrease either DHFR binding or cytotoxicity. A new synthetic route to PT523 from 2,4-diamino-6-(hydroxymethyl)pteridine and methyl $N^{\alpha}$-(4-aminobenzoyl)-$N^{\delta}$-phthaloyl-L-ornithinate was investigated but was not found superior to previously described methods. In comparative experiments on the ability of PT523 and MTX to competitively inhibit the influx of (6*R*)-5,10-dideazatetrahydrofolate (DDATHF, lometrexol), used here as a surrogate for MTX and reduced folates, the $K_i$ of PT523 was lower than that of MTX in both wild-type CCRF-CEM human leukemic lymphoblasts and the transport- and polyglutamylation-defective subline CEM/MTX. The CCRF-CEM cells were 10-fold more sensitive to PT523 than to MTX, whereas the CEM/MTX cells were 240-fold more sensitive. However, in contrast to other MTX-resistant cells where collateral sensitivity to PT523 has been seen. CEM/MTX cells still showed substantial cross resistance to PT523 which may reflect an unusual heightened ability to utilize exogenous folic acid. The good correlation observed with both cell lines between the cytotoxicity of PT523 and MTX and the ability to inhibit DDATHF influx supported the view that PT523 and MTX share, at least in part, a common protein carrier for membrane transport.

$N^{\alpha}$-(4-Amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-L-ornithine (PT523, NSC633713, **1**) is a side chain-modified analogue of aminopterin (AMT) that can be viewed as a nonclassical antifolate because it cannot form polyglutamates.[2-6] In contrast to traditional nonclassical dihydrofolate reductase (DFHR) inhibitors like metoprine (**2**),[7] trimetrexate (**3**),[8] and piritrexim (**4**),[9] which lack COOH groups and are therefore very lipophilic and sparingly soluble in water, the two COOH groups in PT523 enable it to easily dissolve at pH 7. Thus PT523 may be described as a hydrophilic nonclassical antifolate to distinguish it from lipophilic nonclassical antifolates. Antitumor activity has been observed experimentally *in vitro* and *in vivo* with a number of other types of AMT and methotrexate (MTX) analogues containing two acid groups in the side chain which are not substrates for folylpolyglutamate synthetase.[10-13] However, apart from the quinazoline DHFR inhibitor methasquin (**5**),[14] the therapeutic potential of nonclassical pteridine and deazapteridine antifolates with a side chain other than glutamic acid remains clinically unexplored.

Despite its structural inability to form polyglutamates, PT523 has shown surprisingly high *in vitro* activity against a broad assortment of tumor cells, with $IC_{50}$ values in the low nanomolar and in some instances subnanomolar range.[2,5] More importantly, PT523 has been found to be




active against several cell lines selected for resistance to MTX by virtue of either a defect in MTX/reduced folate transport or an increase in DHFR activity.[2,5] *In vivo* activity has also been seen in several murine tumor models including L1210 leukemia,[2] M5076 ovarian reticulum cell sarcoma,[4,5] and SCC VII squamous cell carcinoma,[6] as well as against two human solid tumor xenografts in athymic nude mice.[15] Recent mechanistic studies offer strong evidence that the primary cellular target of PT523 is DHFR and, moreover, indicate that folypolyglutamate synthetase inhibition resulting from intracellular conversion of PT523 to $N^{\alpha}$-(4-amino-4-deoxypteroyl)-L-ornithine (APA-Orn)[16] does not contribute appreciably to cytotoxicity.[17,18] However, a curious feature of PT523 is that, even though its binding to dihydrofolate reductase (DHFR) is nearly stoichiometric, like that of MTX and AMT,[2] its potency as an inhibitor of cell growth and cellular DNA synthesis[17,18] is substantially greater. This suggests (a) more efficient influx via the carrier-mediated MTX/reduced

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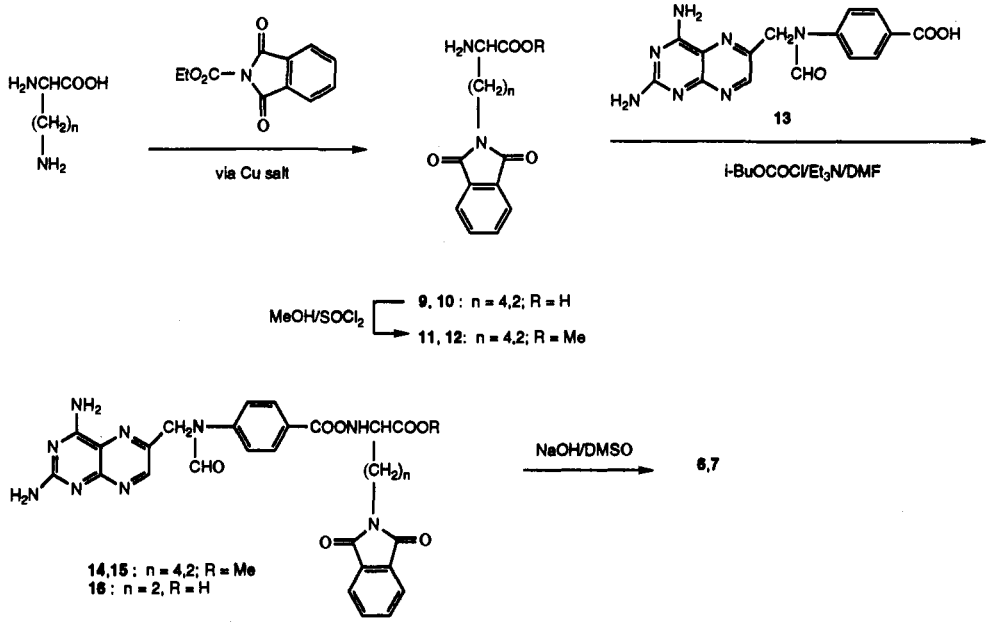
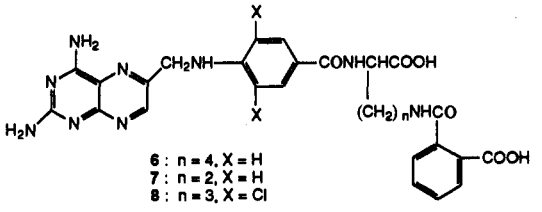
**Scheme 1**




folate active transport route and/or an alternative pathway, (b) less efficient efflux of nonbound drug, or (c) a combination of these effects.

Because of the very encouraging *in vitro* and *in vivo* antitumor activities shown thus far by PT523, we have embarked upon a program of analogue synthesis with a view to defining the optimal structural features for biological activity in this type of hydrophilic nonclassical antifolate. The present paper reports the synthesis and *in vitro* antitumor activity of the first PT523 analogues modified in the side-chain or phenyl moiety. An alternative route to PT523 is also described. The L-lysine (Lys) and L-2,4-diaminobutanoic acid (Daba) analogues **6** and **7** were made to evaluate the importance of the L-ornithine (Orn) side chain in PT523, whereas **8** was made to assess the effect of 3′,5′-dichloro substitution. Compound **8** was also of interest as a potential source of [$^3$H]PT523 via catalytic tritium exchange, an approach we had followed in the past to obtain $\gamma$-*tert*-butyl [$^3$H]MTX from $\gamma$-*tert*-butyl 3′,5′-dichloroMTX.[19]



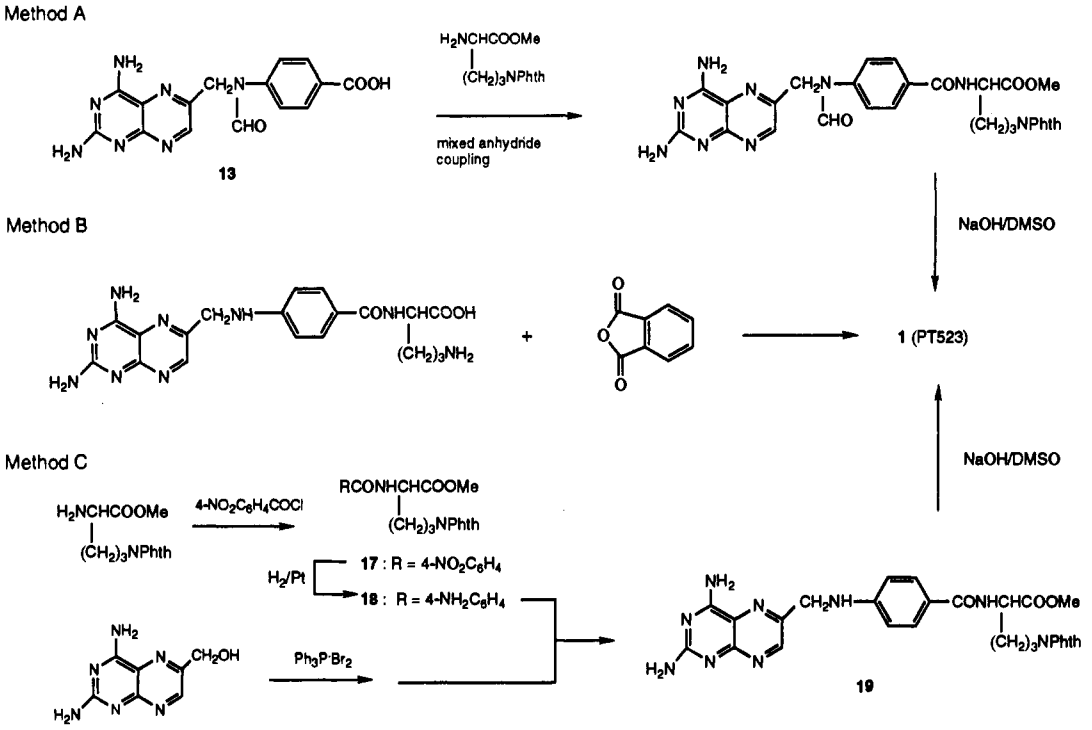

## Chemistry

The synthesis of the Lys and Dab derivatives **6** and **7** is summarized in Scheme 1. The previously undescribed starting materials $N^{\epsilon}$-phthaloyl-L-lysine (**9**) and $N^{\gamma}$-phthaloyl-L-2,4-diaminobutanoic acid (**10**), isolated as HCl salts, were obtained by reaction of *N*-carbethoxyphthalimide with the copper complexes of Lys and Daba, which we had made earlier in connection with other work.[16,20] Further reaction of **9** and **10** with MeOH/$SOCl_2$ afforded the esters **11** and **12**, respectively. Condensation of the esters with 4-amino-4-deoxy-$N^{10}$-formylpteroic acid (**13**) via an iterative mixed carboxylic–carbonic anhydride method we have utilized previously[12] then yielded **14** and **15**, the latter of which was accompanied by a small amount of the acid **16**. Brief treatment of **14** and **15** with NaOH in DMSO at room temperature[3] opened the phthaloyl ring with concomitant removal of the methyl ester and $N^{10}$-formyl groups to give the desired products **6** and **7** directly. Compound **6** was purified by gradient HPLC on a preparative-scale polystyrene–divinylbenzene gel column with 20–40% MeCN in 0.01 M $NH_4OAc$, pH 7.5, as the eluent. Compound **7** was isolated in sufficient purity to be used without further HPLC purification. Isolation of highly purified final product in the hydrolysis reaction required rigorous prior column chromatography of the precursors **14** and **15** on silica gel. Fortunately, this was facilitated by the favorable solubilizing properties of the $N^{10}$-formyl group, which had already been noted earlier in the synthesis of **1**.[2,3]

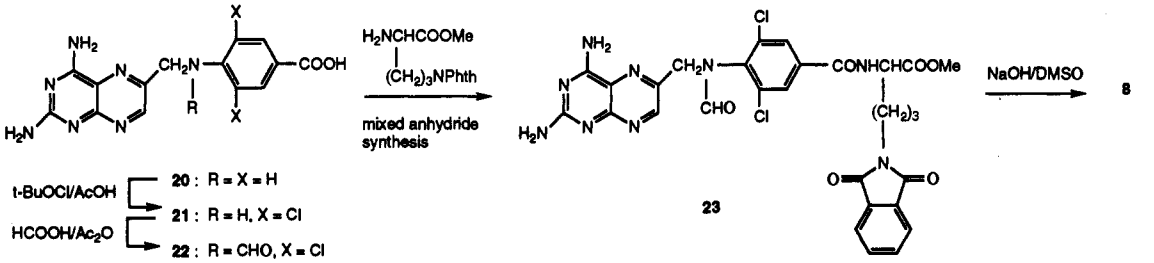
Previously reported routes to **1** have consisted of (a) coupling of **13** and methyl $N^{\delta}$-phthaloyl-L-ornithinate followed by removal of the $N^{10}$-formyl and methyl ester group and opening of the phthaloyl ring with NaOH in DMSO (Scheme 2, method A) or (b) acylation of $N^{\alpha}$-(4-amino-4-deoxypteroyl)-L-ornithine with phthalic anhydride (Scheme 2, method B).[2,3] An alternative route we investigated in the present work is depicted in Scheme 2, method C. Methyl $N^{\delta}$-phthaloyl-L-ornithinate was condensed with 4-nitrobenzoyl chloride and the resulting *N*-(4-nitrobenzoyl) derivative **17** was reduced catalytically to the amine **18**. Reaction of 2,4-diamino-6-(hydroxymethyl)-pteridine with $Ph_3P{\cdot}Br_2$ in DMAC,[21,22] followed by *in situ* addition of **18** and BaO without isolation of the brominated intermediate,[2,3] afforded the expected coupling product **19**. However, purification of **19** by silica gel column chromatography proved very difficult in comparison with that of the more soluble $N^{10}$-formyl derivative. Accordingly we elected to treat **19** with NaOH in DMSO directly, with purification on an anion-exchange column being left to the last step. Because of the purification problems caused by the poor solubility of **19** in all but the strongest solvents, we found method C to be less convenient for large-scale work than method B.

Two approaches were explored for the synthesis of the 3′,5′-dichloro derivative **8**. The first, based on a previously

**Scheme 2**




**Scheme 3**




described route to other 3',5'-dichloro derivatives of folates and antifolates,[19,23] involved treatment of preformed **1** with *t*-BuOCl in glacial AcOH. In the second approach (Scheme 3), 4-amino-4-deoxypteroic acid (**20**) was chlorinated with *t*-BuOCl and the resulting 3',5'-dichloro derivative **21** converted sequentially to the $N^{10}$-formyl derivative **22** and protected coupling product **23**. Further treatment with NaOH/DMSO in the usual manner[3] then gave **8**. Because of the small scale on which this sequence as carried out, it was expedient to use intermediates **21** and **22** without purification. The presence of 3',5'-dichloro substituents in **8** was evident from the $^1$H NMR spectrum (500 MHz, $D_2O$ solution), which showed the 2'- and 6'-protons as a sharp singlet at $\delta$ 7.77 and no upfield signals corresponding to 3'- and 5'-protons. In addition, there were present two doublets centered at $\delta$ 7.20 and 7.60 which we assigned to the protons ortho to the COOH and CONH groups of the phthaloyl moiety, respectively, and two triplets centered at $\delta$ 7.28 and 7.43 which were assigned to the other two ring protons. The UV spectrum at pH 7.4 showed maxima at 262 and 375 nm with only an inflection at 278 nm, whereas the spectrum of **1** showed three well-defined maxima at 260, 281, and 372 nm, a pattern likewise reported for the 3',5'-dichloro derivative of AMT.[24]

## Biological Activity

Compounds **6–8** were tested as inhibitors of human DHFR and as inhibitors of the growth of three tumor cell lines (SCC25 human and SCC-VII murine squamous cell carcinoma and MCF-7 human breast carcinoma) during 72-h drug exposure. As indicated in Table 1, the PT523 analogues **6** and **7**, with Lys and Daba replacing Orn in the side chain, were near-stoichiometric inhibitors of DHFR, as were PT523, the classical inhibitors MTX and AMT, and the lipophilic nonclassical inhibitor TMQ. Near-stoichiometric binding was indicated by the fact that all the titration curves were linear over a range of inhibitor concentrations averaging from 0 to >80% of equivalence and that the $IC_{50}$ value was almost exactly half the enzyme concentration. This stoichiometry was independent of whether the phthaloylated side chain contained two, three, or four $CH_2$ groups. This pattern was quite similar to what was observed previously in $\alpha$-aminoalkanedioic[10,12]

**Table 1.** Dihydrofolate Reductase and Cell Growth Inhibition Data for PT523 (**1**) and Compounds **6–8** in Comparison with MTX, AMT, and TMQ (**3**)

| | $IC_{50}$ (nM)[a] | | | |
|---|---|---|---|---|
| compound | DHFR | SCC25 cells | SCC VII cells | MCF-7 cells |
| PT523 (**1**) | 12.2 | 1.0 ± 0.4 | 0.89 ± 0.39 | 0.7 |
| **6** | 11.4 | 17.9 ± 2.6 | 4.0 ± 1.9 | 2.0 |
| **7** | 11.6 | 12.9 ± 1.4 | 19 ± 4.0 | 6.0 |
| $Cl_2$-PT523 (**8**) | 10.2 | 2.5 ± 0.2 | 1.6 ± 0.82 | |
| MTX | 11.2 | 7.5 ± 0.5 | 5.8 ± 2.0 | |
| AMT | 11.8 | 6.9 ± 0.8 | 4.0 ± 1.9 | |
| TMQ (**3**) | 12.2 | 8.0 ± 0.9 | 6.3 ± 1.5 | |

[a] See the Experimental Section for assay details.

Table 2. Growth Inhibition by PT523 and MTX and Relative Affinities for MTX/Reduced Folate Transport in Human Leukemic Lymphoblasts

| | cell growth inhibition ($IC_{50}$, nM)[a] | | [$^{14}$C]DDATHF influx inhibition ($K_i$, $\mu$M) | | | |
|---|---|---|---|---|---|---|
| cell line | PT523 | MTX[b] | PT523 | MTX[b] | leucovorin[b] | folic acid[b] |
| CCRF-CEM | 1.08 ± 0.18 | 10.5 ± 1.2 | 0.65 ± 0.05 | 4.48 ± 0.84 | 2.37 ± 0.78 | 167 ± 33 |
| CEM/MTX | 110 ± 10 | 2550 ± 140 | 0.44 ± 0.05 | 9.74 ± 1.52 | 0.74 ± 0.09 | 8.95 ± 2.30 |
| ratio[c] | 100 | 243 | 0.68 | 2.1 | 0.31 | 0.054 |

[a] Cells were incubated in a 5% $CO_2$ humidified atmosphere at 37 °C in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and counted after 72 h of continuous drug exposure. The CEM/MTX cells were maintained continuously in the presence of 1 $\mu$M MTX, but were kept out of MTX for 3–4 generations prior to carrying out growth inhibition and DDATHF influx inhibition assays.[36] Data are expressed as mean values ± standard deviation. [b] $K_i$ values are expressed as means ± SE for the inhibition of (6*R*)-[$^{14}$C]DDATHF (2 $\mu$M) influx into CCRF-CEM and CEM/MTX cells and were calculated from Dixon plots for two to seven separate experiments. The $K_i$ for inhibition of (6*R*)-[$^{14}$C]DDATHF influx by nonradioactive (6*R*)-DDATHF was 0.68 ± 0.11 $\mu$M for CCRF/CEM cells and 0.17 ± 0.07 $\mu$M for CEM/MTX cells.[36] The data for MTX, leucovorin [(6*R*,6*S*)-5-formyl-5,6,7,8-tetrahydrofolic acid], and folic acid are likewise taken from ref 36. [c] $IC_{50}$ or $K_i$ ratio for resistant CEM/MTX cells relative to wild-type CCRF-CEM cells.

and $\alpha,\omega$-diaminoalkanecarboxylic acid[16] acid analogues of MTX and AMT and provided additional evidence that the polarity of the charge on the terminal group and its distance from the $\alpha$-carbon are not critical for inhibition. It may be noted, on the other hand, that small, less than 2-fold, differences in DHFR inhibition have been noted among PT523 analogues in which the hemiphthaloyl moiety is replaced by a benzoyl, 4-chlorobenzoyl, or 3,4-dichlorobenzoyl group.[2] This suggests that some level of interaction probably does exist between the distal part of the side chain and hydrophobic residues in an outer domain of the active site.

Despite the fact that PT523 and its chain-modified analogues **6** and **7** were all essentially equipotent as DHFR inhibitors, and were as active as AMT, MTX, and TMQ, considerable differences were seen in their *in vitro* antitumor activity. As shown in Table 1, extending the side chain by one carbon (**6**) led to a 13-fold decrease in potency against SCC25 cells and somewhat smaller decreases against SCC VII and MCF-7 cells. Shortening the side chain by one carbon (**7**) led to a 9-fold decrease in potency against SCC25 and MCF-7 cells and a larger 21-fold decrease against SCC VII cells. Thus PT523 was more potent than AMT, MTX, and TMQ against all three cell lines, but **6** and **7** were not. These results revealed that three $CH_2$ groups were important for the potency of PT523 as a cell growth inhibitor. Compound **8** was likewise a near-stoichiometric inhibitor of purified human DHFR, with an $IC_{50}$ almost identical to that of the other antifolates tested (cf. Table 1), and was only slightly less active as an inhibitor of cell growth, suggesting that this change in the *p*-aminobenzoyl region is tolerated equally well in PT523 as it is in MTX.[25] Given that **6** and **7** bind essentially as tightly as PT523 to DHFR, it is reasonable to attribute their decreased potency against cultured cells to differences in cellular accumulation. The transport properties of these and other PT523 analogs being synthesized in our laboratory, including the critical issue of whether they are taken up by more than one mechanism, will be the subject of a future investigation.

PT523 itself has recently been shown to inhibit [$^3$H]-MTX and [$^3$H]folinic acid uptake into cultured rat hepatoma cells, suggesting that all three compounds share, at least in part, a common transport pathway; in contrast, [$^3$H]folic acid uptake is minimally affected.[18] To gain additional insight into the mode of uptake of PT523, an experiment was performed to determine its $K_i$ as a competitive inhibitor of [$^{14}$C]DDATHF influx into CCRF-CEM human leukemic lymphoblasts[26] and the MTX-resistant subline CEM/MTX,[27-29] which has normal DHFR activity but impaired MTX transport.[27,28]

Table 3. Relative *in Vitro* Potencies of PT523 (1) and Other Antifolates against CCRF-CEM Human Leukemic Lymphoblasts

| antifolate | primary target | $IC_{50}$ (nM)[a] | relative activity |
|---|---|---|---|
| PT523 (1) | DHFR | 1.1 | 100 |
| BW1843U89 | TS | 2.4 | 46 |
| edatrexate (10-EDAM) | DHFR | 2.6 | 42 |
| aminopterin (AMT) | DHFR | 3.0 | 37 |
| ICI D1694 | TS | 4.7 | 23 |
| lometrexol (DDATHF) | GAR FTase | 16.2 | 6.8 |

[a] Data for compounds other than PT523 are taken from ref 36.

DDATHF has been shown to be a substrate for the MTX/reduced folate carrier in CCRF-CEM cells.[35,36] The transport defect in CEM/MTX cells was recently shown to involve expression of an electrophoretically altered isoform of the glycoprotein membrane carrier for MTX and reduced folates.[28] The expression of a modified transporter in CEM/MTX cells was accompanied by significant changes in binding of assorted transport substrates. The $K_i$ values for PT523 as an inhibitor of [$^{14}$C]DDATHF influx into CCRF-CEM and CEM/MTX cells are given in Table 2, along with recently published values for MTX, leucovorin (5-formyl-(6*R*,6*S*)-5,6,7,8-tetrahydrofolate), and folic acid.[36] Also shown are $IC_{50}$ values for PT523 and MTX as inhibitors of the growth of CCRF-CEM and CEM/MTX cells. In agreement with our previous experience with a variety of other cell lines,[2,5,17] PT523 was 10-fold more potent than MTX against the parental CCRF-CEM cells ($IC_{50}$ = 1.08 vs 10.5 $\mu$M); moreover, its relative affinity for the MTX/reduced folate transport system, based on competitive inhibition of DDATHF influx, was 7-fold greater than that of MTX. With the CEM/MTX cells, the difference in the $IC_{50}$ for growth inhibition by PT523 and MTX was 23-fold (0.11 and 2.55 $\mu$M respectively), and the difference in the $K_i$ for DDATHF influx inhibition was 22-fold (0.44 and 9.74 $\mu$M, respectively). Thus, the increased potencies of PT523 over MTX for both wild-type CCRF-CEM and CEM/MTX lines are most likely due to greater uptake. It may be noted that PT523 shows excellent activity not only in comparison with MTX, but also several other polyglutamatable antifolates currently in clinical development. As shown in Table 3, the molar potency of PT523 against CCRF-CEM cells during 72 h of drug exposure was 3-fold greater than that of the DHER inhibitor edatrexate (10-EDAM), 2- and 5-fold greater than that of the TS inhibitors BW1843U89 and ICI D1694, respectively, and 16-fold greater that that of DDATHF.[36] Similarly low $IC_{50}$ values, in the low nanomolar or subnanomolar range, and consistently lower than those of MTX and several other standard antifolates, have also been obtained with PT523

against a variety of solid tumor cell lines in culture during 48 or 72 h of treatment.[5,15,18]

The finding that the CEM/MTX line was only partly cross-resistant to PT523 was qualitatively similar to our previous result using a wild-type human squamous cell carcinoma line (SCC15) and a subline (SCC15/$R_1$) resistant to MTX because of a transport defect.[37,38] SCC15/$R_1$ cells were 15-fold and 3.6-fold resistant to MTX and PT523, respectively. However, unlike CEM/MTX cells, the SCC15/$R_1$ subline was more sensitive to PT523 than the parental cells were to MTX. In our previous study, the MTX-resistant H35$R_{0.3}$ rat hepatoma subline was *less sensitive* to PT523 than to MTX.[18] These differences between cell lines probably reflect variations in affinity and/or capacity for antifolate transport. The striking difference between the reduced folate carriers of CEM/MTX and parental CCRF-CEM cells in their relative affinities for leucovorin and folic acid versus MTX is also observed with DDATHF and probably contributes, in part, to the disparate sensitivity of the CEM/MTX subline to this agent.[36] An analogous, albeit less pronounced, effect was observed with PT523 in the present study. Depending on its frequency in other MTX-resistant tumor cells, antifolates designed to exploit the transport phenotype expressed in CEM/MTX cells might offer a useful direction for future study.

## Experimental Section

IR spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer; peaks below 1600 $cm^{-1}$ are omitted. UV spectra were obtained on a Varian Model 210 spectrophotometer. $^1H$ NMR spectra were obtained at 60 MHz on a Varian EM360L spectrometer and at 500 MHz on a Varian Model XL500 instrument, using $Me_4Si$ as the reference. We thank Dr. Jonathan Lee, of the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, for his help in the use of the 500-MHz instrument. TLC analyses were done on fluorescent Baker Si250F silica gel plates, Eastman 13181 silica gel sheets, or Eastman 13254 cellulose sheets. Spots were visualized under 254-nm UV light, or with the aid of an iodine chamber or ninhydrin spray. Column chromatography was performed on Baker 60–200- or 70–230-mesh silica gel, Baker flash silica gel (40-$\mu$m particle size), or Whatman DE-52 preswollen DEAE-cellulose. Solvents for moisture-sensitive reactions were dried over Linde 4A molecular sieves (Fisher, Boston, MA). HPLC was on $C_{18}$ silica gel radial compression cartridges (Waters, Milford, MA; analytical: 5-$\mu$m particle size, 5 × 100 mm; preparative: 15-$\mu$m particle size, 25 × 100 mm) or a preparative polystyrene–divinylbenzene gel column (Phenomenex, Torrance, CA; Shodex RSpak DS-2013, 20 × 300 mm). Melting points (not corrected) were measured in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA) using Pyrex capillary tubes or in a Fisher-Johns apparatus (Fisher, Boston, MA). Microanalyses were done by Robertson Laboratory, Madison, NJ, and were within ±0.4% of theoretical values unless otherwise specified.

**$N^{\epsilon}$-Phthaloyl-L-lysine Hydrochloride (9·HCl).** L-Lysine dihydrochloride (3.65 g, 0.02 mol) was dissolved in $H_2O$ (35 mL), and solid NaOH (1.6 g, 0.04 mol) was added, followed by a solution of cupric nitrate trihydrate (2.41 g, 0.01 mol) in $H_2O$ (35 mL). The deep-blue solution was stirred, and to it were added $NaHCO_3$ (2.0 g, 0.023 mol) and then *N*-carbethoxyphthalimide (5.0 g, 0.023 mol). Stirring was continued for 1 h, and the precipitate was filtered and washed sequentially with $H_2O$, 95% EtOH, $CHCl_3$, and $Et_2O$. Drying *in vacuo* ($P_2O_5$, 50 °C) afforded the blue copper complex (5.48 g, 96%). To obtain **9**·HCl, the copper complex was pulverized in a mortar, stirred in 6 N HCl (40 mL) for 1 h, filtered, washed with 6 N HCl, and air-dried overnight. The HCl salt was redissolved in boiling MeOH (60 mL), and the solution was added quickly to EtOAc (200 mL). After 2 h at 0 °C, the solid was filtered, washed with EtOAc, and dried *in vacuo* ($P_2O_5$, 60 °C); first crop 4.45 g. Concentration of the mother liquor yielded a second crop weighing 0.16 g: total 4.61 g (74%); colorless plates; mp 221–223 °C; IR (KBr) $\nu$ 3420, 3140, 2950, 1770, 1710, 1705, 1610 $cm^{-1}$. Anal. ($C_{14}H_{17}ClN_2O_4$) C, H, Cl, N.

**$N^{\gamma}$-Phthaloyl-L-2,4-diaminobutanoic Acid Hydrochloride (10·HCl).** L-2,4-Diaminobutanoic acid dihydrochloride was converted to a pale-blue copper complex (49% yield) and the latter condensed with *N*-carbethoxyphthalimide as described above to obtain colorless plates (3.17 g, 48% yield): mp 194–195 °C; IR (KBr) $\nu$ 3160, 2960, 1770, 1740, 1705 $cm^{-1}$. Anal. ($C_{12}H_{13}ClN_2O_4$) C, H, Cl, N.

**Methyl $N^{\epsilon}$-Phthaloyl-L-lysinate Hydrochloride (11·HCl).** $SOCl_2$ (13.5 mL) was added in portions to a stirred solution of **9**·HCl (4.57 g, 14.6 mmol) in MeOH (100 mL) while maintaining the temperature between −20 and 0 °C. Stirring was continued for 21 h at 25 °C; a clear solution formed after 4 h. The solution was evaporated to dryness, and the solid residue was refluxed in a mixture of EtOAc (30 mL) and dry $Me_2CO$ (250 mL). The material which remained undissolved was dried *in vacuo* ($P_2O_5$, 80 °C); yield 3.33 g; mp 162–163 °C. Concentration of the mother liquor and cooling at −20 °C afforded a second crop weighing 1.20 g: total yield 4.53 g (95%); IR (KBr) $\nu$ 3460, 3050, 2960, 1770, 1750, 1710, 1590 $cm^{-1}$. Anal. ($C_{15}H_{19}ClN_2O_4$) C, H, Cl, N.

**Methyl $N^{\gamma}$-Phthaloyl-L-2,4-diaminobutanoate Hydrochloride (12·HCl).** Starting from $SOCl_2$ (4.9 mL) and **10**·HCl (1.49 g, 5.24 mmol) in MeOH (40 mL) at −20 °C, the same procedure as described above afforded colorless needles (1.48 g, 95%): mp 184–186 °C; IR (KBr) $\nu$ 2850, 2040, 1780, 1745, 1705 $cm^{-1}$. Anal. ($C_{13}H_{15}ClN_2O_4$) C, H, Cl, N.

**Methyl $N^{\alpha}$-(4-Amino-4-deoxy-$N^{10}$-formylpteroyl)-$N^{\epsilon}$-phthaloyl-L-lysinate (14).** i-BuOCOCl (185 mg, 1.35 mmol) was added to a stirred suspension of 4-amino-4-deoxy-$N^{10}$-formylpteroic acid (**13**) (496 mg, 1.35 mmol as the sesquihydrate)[2,3] in dry DMF (20 mL) containing $Et_3N$ (1.65 g, 11.5 mmol). After 15 min at room temperature, 11·HCl (441 mg, 1.35 mmol) was added, followed 10 min later by a second portion of i-BuOCOCl (93 mg, 0.68 mmol). After 20 min a second portion of 11·HCl (221 mg, 0.68 mmol) was added, followed by a third portion of i-BuOCOCl (46 mg, 0.34 mmol). After 15 min a third portion of 11·HCl (110 mg, 0.34 mmol) was added, and the final sequence of i-BuOCOCl and 11·HCl additions was repeated. After 1 h the reaction mixture was evaporated under reduced pressure, and the residue was dissolved in $CHCl_3$ (200 mL). The solution was washed with $H_2O$ (2 × 200 mL), dried ($MgSO_4$), and evaporated. The solid was redissolved in 2:5:5 MeOH–MeCN–$CHCl_3$ and the solution applied onto a silica gel column (40 g, 2 × 28 cm, 70–230 mesh), which was eluted with 1:5:5 MeOH–MeCN–$CHCl_3$. Fractions showing a blue-fluorescent TLC spot with $R_f$ 0.21 (silica gel, 2:5:5 MeOH–MeCN–$CHCl_3$) were pooled and evaporated, the residue was taken up in 2:1 $CHCl_3$–MeOH, and the solution was poured into stirred $Et_2O$ (100 mL). Filtration of the solid and drying *in vacuo* ($P_2O_5$, 80 °C) gave a pale-yellow powder (563 mg, 66% based on **13**): mp 132–133 °C; IR (KBr) $\nu$ 3340, 1770, 1740 infl, 1740, 1710, 1670, 1610 $cm^{-1}$; UV $\lambda_{max}$ (95% EtOH) 245, 265, 372 nm. Anal. ($C_{30}H_{29}N_9O_6 \cdot 0.6MeOH$) C, H, N.

**Methyl $N^{\alpha}$-(4-Amino-4-deoxy-$N^{10}$-formylpteroyl)-$N^{\gamma}$-phthaloyl-2,4-diaminobutanoate (15).** i-BuOCOCl (0.41 g, 3.0 mmol) was added to a stirred solution of **13**·1.5$H_2O$ (1.1 g, 3.0 mmol) in dry DMF (40 mL) containing $Et_3N$ (1.21 g, 12 mmol). After 15 min at room temperature, **12**·HCl (0.90 g, 3.0 mmol) was added, and 12 h later the volatile materials were evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ (200 mL) and the solution washed with $H_2O$ (200 mL). The dried ($MgSO_4$) organic phase was evaporated and the residue taken up in 2:5:5 MeOH–MeCN–$CHCl_3$, from which an orange-yellow solid crystallized out on standing. Filtration and drying *in vacuo* ($P_2O_5$, 60 °C) afforded 164 mg (9.5% yield) of $N^{\alpha}$-(4-amino-4-deoxy-$N^{10}$-formylpteroyl)-$N^{\gamma}$-phthaloyl-2,4-diaminobutanoic acid (**16**): mp 185–187 °C. Anal. ($C_{27}H_{23}N_9O_6 \cdot 0.75H_2O$) C, H, N. The filtrate from this solid was applied onto a silica gel column (80 g, 31 × 3 cm, 70–230 mesh), which was eluted with 2:5:5 MeOH–MeCN–$CHCl_3$. Fractions showing a blue-fluorescent TLC spot with $R_f$ 0.35 (silica gel, 2:5:5 MeOH–MeCN–$CHCl_3$) were pooled and further purified as in the preceding experiment to obtain ester **15** as a yellow powder (738 mg, 41%): mp 143–147

°C; IR (KBr) $\nu$ 3450, 3310, 3160, 2940, 1770, 1735 (infl), 1710, 1665, 1605 cm$^{-1}$. Anal. ($C_{28}H_{25}N_9O_6 \cdot 1.25H_2O$) C, H, N.

**$N^{\alpha}$-(4-Amino-4-deoxypteroyl)-$N^{\epsilon}$-hemiphthaloyl-L-lysine (6).** A stirred solution of 14 (204 mg, 0.33 mmol) in DMSO (2.0 mL) was treated dropwise with 2.5 N NaOH (0.8 mL) over a period of 30 s. After 5 min, the solution was diluted with $H_2O$ (10 mL), followed by dropwise addition of 1 N HCl (1.7 mL) until pH 4.3. The solid was filtered immediately, washed with $H_2O$ (50 mL), and freeze-dried to a yellow powder (179 mg), which was purified further by HPLC on a polystyrene–divinylbenzene gel column (0.01 M $NH_4OAc$, pH 7.5, 20–40% MeCN gradient over 45 min, 3 mL/min). The main peak was concentrated under reduced pressure and freeze-dried to a solid, which was redissolved in $H_2O$ (13 mL) by addition of a small amount of $NH_4OH$ and was reprecipated by adjusting to pH 4.5 with 10% AcOH. Filtration and drying *in vacuo* ($P_2O_5$, 90 °C) afforded a pale-yellow powder (129 mg, 64%): analytical HPLC 6.0 min (20% MeCN in 0.01 M $NH_4OAc$, pH 7.5, 1.5 mL/min); IR (KBr) $\nu$ 3400, 2930, 1770, 1705, 1640, 1610 cm$^{-1}$. Anal. ($C_{28}H_{28}N_9O_6 \cdot 1.4H_2O$) C, H, N.

**$N^{\alpha}$-(4-Amino-4-deoxypteroyl)-$N^{\gamma}$-hemiphthaloyl-L-2,4-diaminobutanoic Acid (7).** Saponification of 15 (204 mg, 0.337 mmol) as in the preceding experiment, followed by acid precipitation and freeze-drying yielded a yellow powder (196 mg, 86%): mp 167–170 °C dec; IR (KBr) $\nu$ 3340, 1645, 1635, 1605, 1520 cm$^{-1}$; analytical HPLC 6.0 min (20% MeCN in 0.01 $NH_4OAc$, pH 7.5, 1.5 mL/min). Anal. ($C_{26}H_{24}N_9O_6 \cdot AcOH \cdot 3H_2O$) C, H, N.

**Methyl $N^{\alpha}$-(4-Nitrobenzoyl)-$N^{\delta}$-phthaloyl-L-ornithinate (17).** $Et_3N$ (2.02 g, 0.02 mol) was added dropwise to a stirred suspension of methyl $N^{\delta}$-phthaloyl-L-ornithinate hydrochloride[2] (3.12 g, 0.01 mol) and 4-nitrobenzoyl chloride (1.86 g, 0.01 mol) in $CH_2Cl_2$ (70 mL) at 0 °C. After 1 h at 0 °C, stirring was continued at 20 °C for 15 min, at which point all the solid dissolved. After another 21 h at room temperature, the solution was washed with 50-mL portions of 0.1 N HCl, $H_2O$, and saturated $NaHCO_3$, respectively, dried ($MgSO_4$), and evaporated. The residue was redissolved in hot $CH_2Cl_2$ (70 mL), to which was added petroleum ether (bp 40–60 °C, 200 mL). Filtration gave colorless microcrystals, which were dried *in vacuo* ($P_2O_5$, 90 °C): yield 3.96 g (93%); mp 165–166 °C; $R_f$ 0.53 (silica gel, 95:5 $CHCl_3$–MeOH); IR (KBr) $\nu$ 3310, 2690, 1770, 1745, 1710, 1600 cm$^{-1}$. Anal. ($C_{21}H_{19}N_3O_7$) C, H, N.

**Methyl $N^{\alpha}$-(4-Aminobenzoyl)-$N^{\delta}$-phthaloyl-L-ornithinate (18).** A suspension of nitro ester 17 (3.95 g, 9.28 mmol) and $PtO_2$ monohydrate in a mixture of 95% EtOH (45 mL), glacial AcOH (15 mL), and $CH_2Cl_2$ (15 mL) was hydrogenated in a Parr apparatus (50 psi) for 5 h. The catalyst was filtered, the solvents were evaporated, and the residue was redissolved in $CHCl_3$. The solution was washed with $H_2O$ and saturated aqueous $NaHCO_3$, dried, and evaporated. The residue was taken up in 95:5 $CHCl_3$–MeOH (5 mL), the solution applied onto a silica gel column (70–230 mesh, 160 g, 56 × 3 cm), and the column eluted with the same solvent mixture. Fractions containing a TLC spot with $R_f$ 0.32 (silica gel, 95:5 $CHCl_3$–MeOH) were pooled and evaporated. The residue was dissolved in refluxing $CH_2Cl_2$ (125 mL), $C_6H_6$ (50 mL) was added, and the solution was concentrated to 50 mL and then 10 mL, yielding two crops of colorless rosettes of needles: total 1.42 g (39%); mp 187–188 °C; IR (KBr) $\nu$ 3480, 3390, 3340, 3220, 2950, 2370, 1770, 1735, 1715, 1605 cm$^{-1}$; NMR $\delta$ ($d_6$-DMSO, 60 MHz) 1.7–2.0 (m, 4H, $CH_2CH_2$), 3.57 (s, 5H, $OCH_3$, $NCH_2$), 4.33 (m, 1H, $\alpha$-CH), 5.56 (s, 2H, $NH_2$), 6.50 (d, 2H, 3′- and 5′-H), 7.53 (d, 2H, 2′- and 6′-H), 7.80 (s, 4H, phthaloyl protons), 8.16 (d, 1H, amide NH). Anal. ($C_{21}H_{21}N_3O_5$) C, H, N.

**$N^{\alpha}$-(4-Amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-L-ornithine (PT523, 1).** To a stirred solution of $Ph_3P$ (787 mg, 3 mmol) in dry DMAC (5 mL) at 0 °C were added successively $Br_2$ (480 mg, 3 mmol) and 2,4-diamino-6-hydroxymethylpteridine (193 mg, 1 mmol). Complete dissolution of the added pteridine occurred within 20 min. After 23 h at room temperature, BaO (153 mg, 1 mmol) and the amine 18 (395 mg, 1 mmol) were added, and the mixture was stirred under $N_2$ at 46 °C for 24 h. A small amount of MeOH was added, and the mixture was evaporated to dryness under reduced pressure (1.5 Torr). The residue was extracted with $CHCl_3$ (75 mL) and $H_2O$ (150 mL), the $CHCl_3$ layer was separated, and the aqueous suspension was washed again with $CHCl_3$ (75 mL). After removal of the organic phase, the solid was filtered, washed with $H_2O$, air-dried, and redissolved in MeOH. The volume of the solution was reduced to 6 mL by slow rotary evaporation at atmospheric pressure, the resulting slurry was diluted with i-ProH (6 mL), and the solid was filtered, washed successively with 1:3 and 1:1 MeOH–i-PrOH, and dried *in vacuo* ($P_2O_5$, 80 °C): crude yield 392 mg (69%); $R_f$ 0.23 (silica gel, 9:1 $CHCl_3$–MeOH). Silica gel chromatography of a small portion of the product, followed by recrystallization from MeOH–i-PrOH yielded 19 as a yellow powder: mp 197–198 °C; IR (KBr) $\nu$ 3350, 3150, 2940, 2570, 1780, 1740, 1710, 1640, 1610 cm$^{-1}$; UV $\lambda_{max}$ (95% EtOH) 242, 290, 336, 346 (infl), 380 (infl) nm.

A portion of nonpurified product 19 (103 mg, 0.18 mmol) was dissolved directly in DMSO (1 mL) and treated dropwise with 2.5 N NaOH (0.2 mL). The solution was kept at 20 °C for 4 min and diluted with $H_2O$ (5 mL). The precipitated solid was filtered and washed with $H_2O$ (2 mL), and the filtrate was acidified to pH 4.5 with 1 N HCl (ca. 0.4 mL). The resulting gelatinous solid was filtered, washed with $H_2O$, and freeze-dried to obtain 1 as a yellow powder whose IR spectrum was indistinguishable from that of the previously prepared reference specimen:[2,3] 51 mg (50% yield, 35% overall); UV $\lambda_{max}$ (pH 7.4) 260 nm ($\epsilon$ 30 514), 281 (20 121), 372 (10 410); analytical HPLC 7.0 min (10% MeCN in 0.1 M $NH_4OAc$, pH 6.0, 1.0 mL/min).

**$N^{\alpha}$-(4-Amino-4-deoxy-3′,5′-dichloropteroyl)-$N^{\delta}$-hemiphthaloyl-L-ornithine (8) (3′,5′-$Cl_2$-PT523). Method A.** *tert*-Butyl hypochlorite (3.53 $\mu$L, 3.38 mg, 0.0314 mmol) was added to a solution of 1 (10 mg, 0.0157 mmol) in glacial AcOH (3 mL), and the solution was kept at 25 °C for 4 h and freeze-dried. Analytical HPLC of the gummy product (15% MeCN in 0.01 M $NH_4OAc$, pH 7.0, 1 mL/min) showed one major peak with a retention time of 3.5 min. The product was purified by preparative HPLC (buffer A: 5% MeCN in 0.01 M $NH_4OAc$, pH 7.8; buffer B: 15% MeCN in 0.01 M $NH_4OAc$, pH 7.8; 0% to 66% buffer B over 30 min; 5 mL/min). The major peak (ca. 10 min) was collected and freeze-dried to obtain 8 as a yellow powder (4.1 mg). When the reaction was repeated on a 100-mg scale the yield was 12 mg: mp 200–205 °C; IR (KBr) $\nu$ 3470, 3330, 3400, 1635 cm$^{-1}$; UV $\lambda_{max}$ (pH 7.4 phosphate) 262 nm ($\epsilon$ 23 920), 278 (infl) (20 800), 375 (7800); $^1$H NMR ($D_2O$, 500 MHz) $\delta$ 1.69 (m, 2H, $CH_2$), 3.35 (t, 2H, $CH_2NHCO$), 4.33 (m, 1H, $\alpha$-CH), 4.84 (s, 2H, benzylic $CH_2NH$), 7.20 (d, 1H, phthaloyl proton ortho to COOH), 7.28 (t, 1H, phthaloyl proton para to COOH), 7.43 (t, 1H, phthaloyl proton para to CONH), 7.60 (d, 1H, phthaloyl proton ortho to CONH), 7.77 (s, 2H, 2′- and 6′-protons), 8.72 (s, 1H, $C_7$ proton).

**Method B.** Solid 4-amino-4-deoxypteroic acid (20) (1.64 g, 4.59 mmol as the dihydrate) was dissolved in TFA (15 mL), and *t*-BuOCl (1.85 mL, 1.14 g, 10.6 mmol) was added. The black solution was stirred at room temperature for 2 h and poured into 10% $NH_4OH$ (75 mL) to form a flocculent yellow solid. The pH was adjusted to 4.8 with $NH_4OH$, and after 1 h at 0 °C the solid was filtered and freeze-dried to obtain the 3′,5′-dichloro derivative 21 as a yellow powder (1.74 g). The crude product was added directly to a mixture of 95–97% $HCO_2H$ (90 mL) and $Ac_2O$ (45 mL) and the mixture refluxed for 2 h and evaporated to dryness. The residue was partly redissolved in $NH_4OH$, an insoluble gummy material was removed by filtration, and the filtrate was acidified to pH 5 with AcOH and left overnight at 0 °C. The solid was filtered and freeze-dried to obtain 22 as a yellow powder (1.48 g). The powder was suspended directly in dry DMF (55 mL), and the stirred mixture was treated with $Et_3N$ (2.08 $\mu$L, 1.52 g, 15 mmol) and i-BuOCOCl (0.47 $\mu$L, 0.49 g, 3.6 mmol). After 20 min at 25 °C, methyl $N^{\delta}$-phthaloyl-L-ornithinate hydrochloride (1.12 g, 0.36 mmol) was added, and stirring was continued for another 15 min. The solvent was removed under reduced pressure, the residue was dissolved in $CHCl_3$ (150 mL), and the solution was washed with $H_2O$ (2 × 50 mL). The organic phase, containing some suspended solid which formed during the extraction, was evaporated. The residue was taken up in 95:5 $CHCl_3$–MeOH (20 mL) and chromatographed on a silica gel column (60–200 mesh, 80 g, 37 × 3 cm) eluted with the same solvent. Fractions showing a TLC spot with $R_f$ 0.42 (silica gel, 2:5:5 MeOH–MeCN–$CHCl_3$) were combined and evaporated to a yellow solid (90 mg). Recrystallization from a concentrated $CHCl_3$–MeOH–$Me_2CO$ mixture afforded 23 as a yellow powder:

mp 175–178 °C; IR (KBr) $\nu$ 3460, 3320, 1770, 1740, 1710, 1620 cm$^{-1}$. Anal. ($C_{29}H_{25}Cl_2N_9O_6$) C, H, Cl, N.

Crude **23** (82 mg) was dissolved in DMSO (1 mL) and the solution treated dropwise with 2.5 N NaOH (0.3 mL). The mixture was placed in a sonication bath at room temperature for 5 min, diluted with $H_2O$ (2.5 mL), adjusted to pH 3.5 with 1 N HCl, and brought to a final volume of 20 mL with $H_2O$. The solid was filtered, washed with $H_2O$, and freeze-dried to a yellow powder (37 mg): $R_f$ 0.42 (silica gel, 5:4:1 $CHCl_3$–MeOH–$NH_4OH$); analytical HPLC 7.0 min (15% MeCN in 0.1 M $NH_4OAc$, pH 7.8, 1 mL/min). The product was then purified by preparative HPLC (11% MeCN in 0.01 M $NH_4OAc$, pH 7.8, 15 mL/min, 24–28 min). Lyophilization of pooled fractions afforded 8 as a yellow powder (21 mg). The IR spectrum of this material, as well the 500-MHz $^1$H NMR spectrum in NaOD–$D_2O$ solution, were virtually the same as those of the product from method A. Analysis by FAB–MS showed twin peaks at $m/z$ 641/642 (M + 1); calcd: 641/642. Anal. ($C_{27}H_{25}Cl_2N_9O_6$:$NH_4OAc$·2.5$H_2O$) C, Cl, N; H: calcd, 4.88; found, 4.37.

**DHFR Inhibition.** Human recombinant DHFR was expressed in *Escherichia coli* and purified by affinity chromatography on MTX-Sepharose and gel filtration as described.[39] Enzyme activity was measured at 22 °C by following the change in UV absorbance at 340 nm in an assay solution containing 60 $\mu$M NADPH, 50 $\mu$M dihydrofolate, and 3.6–5.6 munits of enzyme in 50 mM Tris-HCl, pH 7.0. The reaction was initiated with dihydrofolate after preincubating the other components for 2 min. The $IC_{50}$ values given in Table 1 are means of two to five replicate assays performed on different days.

**Cell Growth Inhibition.** Assays were performed with the following cell lines: SCC25, a human head and neck squamous cell carcinoma; SCC VII, a murine squamous cell carcinoma of abdominal wall origin; MCF-7, a human breast carcinoma; and CCRF-CEM and CEM/MTX, human leukemic lymphoblasts. The SCC25 and SCC VII cells were plated at 2 × 10$^3$/well into 96-well microtiter plates and incubated in Dulbecco's Modified Eagle's (DME) medium with 10% fetal bovine serum for 24 h at 37 °C in a 5% $CO_2$ humidified atmosphere. Drugs were added at 10 concentrations between 0.1 nM and 3 $\mu$M, and incubation was continued for an additional 72 h. Cell growth was determined by measurement of the 530-nm absorbance after protein staining with Sulforhodamine B according to a standard method.[40] Six or more replicate assays were performed with the SCC VII cells, and three to six assays with the SCC25 cells. The MCF-7 cells were grown in replicate 24-well plates in $\alpha$ Minimal Essential Medium containing 10% dialyzed fetal bovine serum supplemented with epithelial growth factor, hydrocortisone, and insulin.[41] After 72 h at 37 °C under 6.5% $CO_2$, the number of treated and control cells was determined electronically with a Coulter counter. The $IC_{50}$ values reported in Table 1 were estimated from semilog plots, and represent averages of three or more experiments on different days in the case of the SCC25 and SCC VII cells. The $IC_{50}$ values for MCF-7 cells are based on averaged data from a single experiment using replicate plates. The CCRF-CEM and CEM/MTX cells were incubated continuously with or without drugs for 72 h in RPMI 1640 medium containing 10% fetal bovine serum, and their numbers determined with a Coulter counter. The CEM/MTX cells were routinely maintained in culture in the presence of 1 $\mu$M MTX but were kept out of MTX for 3–4 generations prior to use in growth inhibition or transport experiments.[36]

**Transport Experiments.** Assays of the ability of PT523 to inhibit (6*R*)-[$^{14}$C]DDATHF influx into CCRF-CEM and CEM/MTX cells were carried out exactly as reported earlier;[36] cf. footnote *b*, Table 2. Nonlabeled and (6*R*)-[*benzoylcarboxyl*-$^{14}$C]DDATHF (13 $\mu$Ci/mg) were synthesized by Lilly Research Laboratories, Indianapolis, IN, and provided by Drs. Chuan Chih and Gerald Grindey.

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