

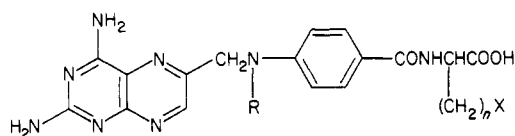
# Methotrexate Analogues. 26. Inhibition of Dihydrofolate Reductase and Folylpolyglutamate Synthetase Activity and in Vitro Tumor Cell Growth by Methotrexate and Aminopterin Analogues Containing a Basic Amino Acid Side Chain

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Analogues of the antitumor antifolate methotrexate (MTX) were synthesized in which the glutamate (Glu) moiety was replaced by ornithine (Orn), 2,4-diaminobutyric acid (Dab), or 2,3-diaminopropionic acid (Dap). An aminopterin (AMT) analogue with Orn in place of Glu was also synthesized. The MTX analogues were obtained (i) by reaction of 4-amino-4-deoxy-*N*<sup>10</sup>-methylpteroic acid (mAPA) and *N*<sup>ω</sup>-Boc- $\alpha,\omega$ -diaminoalkanoic acids in the presence of diethyl phosphorocyanidate, followed by deprotection with trifluoroacetic acid (TFA) or (ii) by reaction of *p*-nitrophenyl-mAPA and *N*<sup>ω</sup>-Boc- $\alpha,\omega$ -diaminoalkanoic acids and subsequent treatment with TFA. The AMT analogue (APA-Orn) was synthesized by reaction of *p*-nitrophenyl 4-amino-4-deoxy-*N*<sup>10</sup>-formylpteroate with silylated *N*<sup>6</sup>-Boc-L-ornithine in DMF at 55 °C for 3 days (45% yield), saponification (83%), and TFA cleavage (89%). APA-Orn was a potent inhibitor of both dihydrofolate reductase (DHFR) from L1210 mouse leukemia ( $IC_{50}$  = 0.072  $\mu$ M) and partly purified folylpolyglutamate synthetase (FPGS) from mouse liver ( $K_i$  = 0.15  $\pm$  0.06  $\mu$ M). The MTX analogue (mAPA-Orn) was likewise active against both enzymes, with an  $IC_{50}$  of 0.160  $\mu$ M for DHFR and a  $K_i$  of 20.4  $\pm$  7.7  $\mu$ M for FPGS inhibition. The other MTX analogues and the previously reported lysine derivative (mAPA-Lys) showed DHFR affinity similar to that of mAPA-Orn but lacked activity as FPGS inhibitors. The positively charged amino group appears to be detrimental to cellular uptake, as evidenced by the low cytotoxicity of these compounds ( $IC_{50}$  = 0.40–2.4  $\mu$ M) in comparison with MTX and AMT ( $IC_{50}$  = 0.002  $\mu$ M) against wild-type L1210 cells. On the other hand, mAPA-Orn and APA-Orn were both more potent than the corresponding Glu derivatives MTX and AMT against L1210/R81 cells, suggesting that in these MTX-resistant cells there may occur a "self-potential" process involving enhanced antifolate activity via interference with the polyglutamylation of reduced folates. APA-Orn is the most potent dual inhibitor of DHFR and FPGS discovered to date, but its effectiveness as a therapeutic agent may require some form of prodrug modification to neutralize the terminal amino group of the side chain.

As part of a larger research program on analogues of methotrexate (MTX, 1) as antifolates,<sup>1</sup> we synthesized *N*<sup>α</sup>-(4-amino-4-deoxy-*N*<sup>10</sup>-methylpteroyl)-L-lysine (2) and converted it to *N*<sup>ε</sup>-iodoacetyl<sup>2</sup> and *N*<sup>ε</sup>-(4'-fluoresceinyl)-thiocarbamoyl<sup>3</sup> derivatives. The iodoacetamide was found to be a potent active-site-directed irreversible inhibitor of dihydrofolate reductase (DHFR),<sup>2</sup> whereas the fluorescein derivative proved useful in detecting DHFR overproduction associated with gene amplification in MTX-resistant cells<sup>3,4</sup> and in identifying the presence of a defect in MTX transport.<sup>5</sup> Use of 2 and of the corresponding L-ornithine analogue 3 has been made to prepare fluorescent *N*<sup>6</sup>- and *N*<sup>ε</sup>-[[5-(*N,N*-dimethylamino)-1-naphthyl]sulfonyl] derivatives with strong DHFR affinity.<sup>6-8</sup>



- 1, R=Me; *n*=2; X=COOH (mAPA-L-Glu, MTX)
- 2, R=Me; *n*=4; X=NH<sub>2</sub> (mAPA-L-Lys)
- 3, R=Me; *n*=3; X=NH<sub>2</sub> (mAPA-L-Orn)
- 4, R=Me; *n*=2; X=NH<sub>2</sub> (mAPA-L-Dab)
- 5, R=Me; *n*=1; X=NH<sub>2</sub> (mAPA-L-Dap)
- 6, R=H; *n*=3; X=NH<sub>2</sub> (APA-L-Orn)

Analogues of 2 with fewer CH<sub>2</sub> groups in the side chain or with hydrogen in place of methyl at *N*<sup>10</sup> were required as synthetic intermediates in our program and were themselves of interest as potential inhibitors of DHFR and folylpolyglutamate synthetase (FPGS), another folate enzyme we have been studying as a potential target for chemotherapy.<sup>9-14</sup> Recently, Shane<sup>15</sup> reported that hog

liver FPGS is inhibited by pteroyl-L-ornithine and 5,6,7,8-tetrahydropteroyl-L-ornithine. The present paper describes the preparation of the L-ornithine, L-2,4-diaminobutyric acid, and L-2,3-diaminopropionic acid ana-

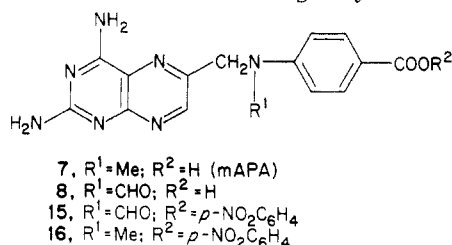
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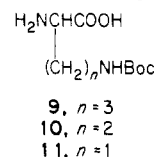
logues 3–5 or MTX, as well as the synthesis of the L-ornithine analogue 6 of aminopterin (AMT). Compounds 3–5 were prepared from 4-amino-4-deoxy-*N*<sup>10</sup>-methylpteroic acid (7)<sup>2,16</sup> while 6 was obtained from 4-amino-4-deoxy-*N*<sup>10</sup>-formylpteroic acid (8).<sup>11,16,17</sup> Compounds 3–6 were tested as inhibitors of DHFR from L1210 murine leukemia cells and of FPGS from normal mouse liver. In addition, the growth-inhibitory activity of 3–6 was evaluated in culture against L1210 and L1210/R81 cells, the latter of which are highly resistant to MTX. Although they show slightly lower activity than MTX or AMT as DHFR inhibitors, the L-ornithine derivatives 3 and 6 display striking activity against FPGS and may therefore be viewed as a lead for further analogue synthesis.



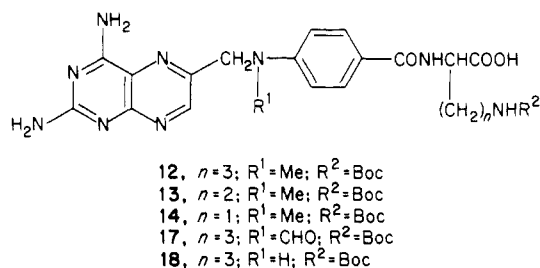
**Chemistry.** Because of our extensive prior experience with 7 as a precursor of MTX analogues with modified side chains,<sup>2,10,11,18–22</sup> we elected to prepare 3–5 from this compound and suitable *N*<sup>ω</sup>-protected α,ω-diaminoalkanoic acids. However, instead of using a benzyloxycarbonyl (Cbz) group to block the terminal NH<sub>2</sub> group of the amino acid, as we had done in the preparation of 2,<sup>2,3</sup> we used the *tert*-butoxycarbonyl (Boc) group, which Kumar and co-workers showed can be cleaved readily with trifluoroacetic acid.<sup>6</sup>

*N*<sup>δ</sup>-Boc-L-ornithine (9) was prepared from bis(L-ornithinato)copper(II)<sup>23</sup> in 60% overall yield by reaction with 2-[[*tert*-butoxycarbonyloxy]imino]-2-phenylacetonitrile in aqueous dioxane followed by copper sequestration with EDTA.<sup>24</sup> The same procedure was used to prepare *N*<sup>γ</sup>-Boc-L-2,4-diaminobutyric acid (10) but could not be applied to *N*<sup>δ</sup>-Boc-L-2,3-diaminopropionic acid (11) since the reaction of Cu(II) with L-2,3-diaminopropionic acid is known to produce a coordination complex involving the two amino groups, rather than the α-amino group and carboxy group, as ligands.<sup>25</sup> Compound 11 was therefore prepared from *N*<sup>α</sup>-tosyl-L-asparagine,<sup>26</sup> using the literature route<sup>27</sup> with the exception of the introduction of the *N*<sup>δ</sup>-Boc group, which was carried out with 2-[[*tert*-butoxy-

carbonyloxy]imino]-2-phenylacetonitrile<sup>24</sup> instead of *tert*-butoxycarbonyl azide. Compounds 9–11 gave a positive ninhydrin test and showed the expected NMR features including a δ 1.4 singlet which is characteristic of the Boc group. The *N*<sup>ω</sup>-Boc derivatives were of sufficient purity to be used directly in subsequent coupling reactions.



Condensation of the amino acid 9–11 with 7 was accomplished with the aid of the peptide-bond-forming reagent diethyl phosphorocyanidate (DEPC),<sup>28</sup> which we have used extensively in the past to prepare MTX analogues.<sup>2,3,10,11,18–22</sup> The reaction was conducted at room temperature in DMF, with 2 molar equiv each of DEPC and *N,N*-diisopropylethylamine. The carboxy group of the amino acid was left unprotected. Compounds 12–14 were obtained in yields that varied according to the number of methylene groups in the side chain (12, 87%; 13, 72%; 14, 34%), but there was a severe decrease in the yield for 14 in comparison with 12 and 13. This may reflect an inherent instability of the amino acid 11 under the conditions of the DEPC coupling reaction. One possibility is that 11 may suffer facile ring closure to an imidazolidin-2-one.



A modification of the usual workup procedure for DEPC reactions that is worth noting is that, in the isolation of 12–14, solid NaHCO<sub>3</sub> was added prior to the evaporation of the DMF and tertiary amine. A pH decrease is normally observed in this step, presumably because of the diethyl phosphate that is left behind. We reasoned that this could produce cleavage of the acid-labile Boc group and in fact observed such cleavage by TLC. When NaHCO<sub>3</sub> was included in the workup, loss of Boc was avoided and the product was isolated in pure state and higher overall yield.

Removal of the *N*<sup>ω</sup>-Boc group in 12–14 was accomplished easily with trifluoroacetic acid at room temperature, and the products (3–5) were purified by cation-exchange chromatography on sulfoxethylcellulose or anion-exchange chromatography on DEAE-cellulose, with 0.1 M NH<sub>4</sub>HCO<sub>3</sub> as the eluent in each system.<sup>29</sup>

An inherent drawback of DEPC coupling in the preparation of MTX analogues from 7 is that the amino acid component has to be used in excess. This is undesirable when the amino acid (e.g., 11) is in limited supply. In a recent paper<sup>22</sup> we reported that *p*-nitrophenyl 4-amino-4-deoxy-*N*<sup>10</sup>-formylpteroate (15) affords a 70% yield of

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**Table I.** Inhibition of Enzyme Activity and Cell Growth by Compounds 2-6

compd	enzymes		cells: <sup>a</sup> IC <sub>50</sub> , $\mu$ M	
	DHFR: <sup>b</sup> IC <sub>50</sub> , $\mu$ M	FPGS: <sup>c</sup> K <sub>i</sub> , $\mu$ M	L1210	L1210/R81
2	0.065	<i>d</i>	0.40	220
3	0.160	20.4 <sup>e</sup>	1.30	86
4	0.120	<i>d</i>	2.42	290
5	0.180	<i>d</i>	0.44	405
6	0.072	0.15 <sup>f</sup>	1.30	32
MTX	0.035	<i>g</i>	0.002	220
AMT	0.035	<i>g</i>	0.002	84

<sup>a</sup> Forty-eight hours of continuous drug exposure. <sup>b</sup> See ref 38 for assay method; IC<sub>50</sub> values are for DHFR concentrations of 0.07  $\mu$ M in the assay mixture. <sup>c</sup> See ref 10 for assay method. <sup>d</sup> <10% inhibition at 500  $\mu$ M inhibitor and 500  $\mu$ M folate. <sup>e</sup> Mean of three separate experiments; SD  $\pm$  7.7  $\mu$ M. <sup>f</sup> Mean of two separate experiments; SD  $\pm$  0.06  $\mu$ M. <sup>g</sup> The K<sub>m</sub> values of MTX and AMT as substrates for mouse liver FPGS were previously reported as 166  $\pm$  49 and 17.6  $\pm$  5.8  $\mu$ M, respectively; see ref 12.

coupling product on reaction with  $\gamma$ -*tert*-butyl L-glutamate (1 equiv) and Et<sub>3</sub>N (2 equiv) in DMF solution. In the present work, similar use of *p*-nitrophenyl 4-amino-4-deoxy-*N*<sup>10</sup>-methylpterolate (16) was made to prepare 3 and 5 in 55% and 50% yield, respectively. The coupling reaction was carried out at 55 °C for 3 days, and activated ester 16 was prepared in essentially quantitative yield from 7 and 2 molar equiv each of bis(*p*-nitrophenyl) carbonate and Et<sub>3</sub>N in DMF (55 °C, 5 h). Previous workers have described a synthesis of 16 from 7 and bis(*p*-nitrophenyl) sulfite and have reported subsequent conversion of 16 to MTX dimethyl ester, from which MTX could be obtained by saponification.<sup>30</sup> Protection of the carboxy group in the amino acid does not, however, seem to be required in this reaction.

The *p*-nitrophenyl ester method was likewise followed, with a slight but important procedural improvement, to obtain the AMT analogue 6 via the intermediates 17 and 18. Pterolate derivative 16<sup>22</sup> was treated briefly with 1 equiv each of trimethylsilyl chloride and Et<sub>3</sub>N in DMF at room temperature, and to this mixture was added directly a solution of the *O,N*-bis(trimethylsilyl) derivative of 9. Coupling was allowed to proceed at 55 °C for 3 days and the product (17, 45% yield) was purified by silica gel column chromatography with 15:6:1 CHCl<sub>3</sub>-MeOH-concentrated NH<sub>4</sub>OH as the eluent. When initial treatment of 16 with trimethylsilyl chloride was omitted, the yield of 17 was only 22%. This step presumably serves to remove water of hydration from 16, thereby minimizing its hydrolytic breakdown to 8 during the coupling step. With 17 in hand, the *N*<sup>10</sup>-formyl group was removed with dilute NaOH in aqueous MeOH at room temperature to obtain 18 (83%) and the *N*<sup>6</sup>-Boc group was removed with trifluoroacetic acid to obtain 6 (89%). Compound 6 could be obtained in analytically pure state by reprecipitation from AcOH with ammonia.

**Biological Activity.** The MTX analogues 2-5 and the AMT analogue 6 were assayed spectrophotometrically for their ability to inhibit dihydrofolate reduction by purified DHFR from L1210/R81 cells (Table I). The IC<sub>50</sub> for the *N*<sup>10</sup>-methyl compounds ranged from 0.065 (2) to 0.18  $\mu$ M (5). The IC<sub>50</sub> of MTX in the same experiment was 0.035  $\mu$ M. Thus, replacement of the  $\gamma$ -COOH by  $\gamma$ -NH<sub>2</sub> with no change in the number of CH<sub>2</sub> groups produces a 5-fold increase in IC<sub>50</sub>. However, an increase in distance between the terminal NH<sub>2</sub> and the  $\gamma$ -COOH seems to diminish this

effect. The AMT analogue 6 was twice as potent as the corresponding MTX analogue 3. A similar difference has been observed between the  $\gamma$ -*tert*-butyl esters of MTX and AMT<sup>22</sup> but not between the L-cysteic and L-homocysteic acid analogues.<sup>11</sup> It thus appears that AMT analogues may be more potent than MTX analogues in some instances but not in others, depending on the type of molecular modification.

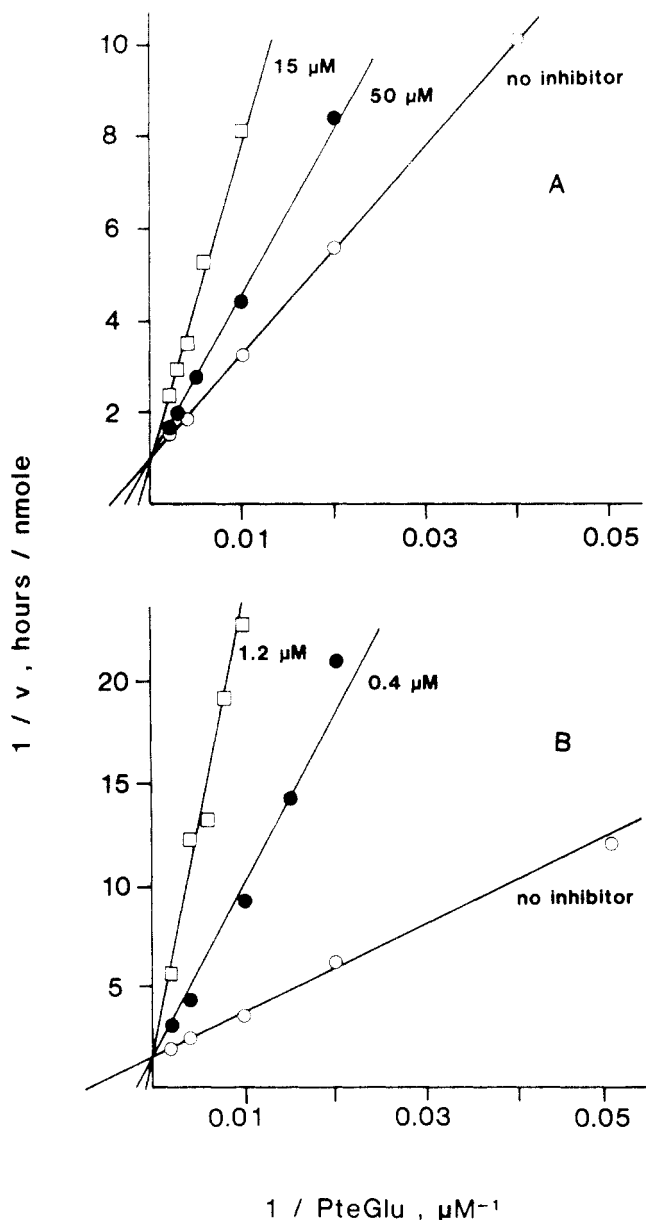
A correlation appears to exist between the IC<sub>50</sub>/[E] value and the degree of "curvilinearity" in the DHFR titration curve of antifolates.<sup>20</sup> In the absence of an actual K<sub>i</sub> determination, the IC<sub>50</sub>/[E] may be viewed as an indicator of the tightness of binding of the inhibitor to the enzyme. Tight-binding antifolates such as MTX and AMT have an IC<sub>50</sub>/[E] close to 0.5, whereas compounds for which there is greater departure from linearity in the enzyme titration curve have an IC<sub>50</sub>/[E] greater than 0.5. For such compounds there is a larger difference in the IC<sub>90</sub> than in the IC<sub>50</sub> in comparison with tight-binding analogues. The IC<sub>50</sub>/[E] for compounds 2-6 ranged from 0.93 to 2.6, suggesting that the presence of a basic NH<sub>2</sub> group in the side chain may result in a higher rate of dissociation from the enzyme, i.e., a higher "off-rate" or *k*<sub>off</sub>,<sup>31</sup> than is the case for MTX or AMT.

Compounds 2-6 were tested also as inhibitors of FPGS partially purified from mouse liver by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation.<sup>12,32</sup> Among the *N*<sup>10</sup>-methyl analogues, the L-ornithine derivative 3 showed surprisingly good activity. Lineweaver-Burk analysis of the kinetics of [<sup>3</sup>H]-L-glutamate incorporation into the charcoal-adsorbable fraction following incubation with 500  $\mu$ M folic acid<sup>12</sup> showed the K<sub>i</sub> of this compound to be 20.4  $\pm$  7.7  $\mu$ M (*n* = 3). As shown in Figure 1, inhibition was competitive, with double-reciprocal plots at different inhibitor concentrations intersecting the ordinate at a common point.<sup>33</sup> On the basis of K<sub>m</sub> values for folic acid as the substrate in the same experiments, a K<sub>i</sub>/K<sub>m</sub> of 0.091  $\pm$  0.034  $\mu$ M was calculated for 3. In an earlier paper,<sup>11</sup> we reported that the L-homocysteic acid analogue of MTX was a competitive inhibitor of FPGS with a K<sub>i</sub> of ca. 200  $\mu$ M. More recently we have found that the D,L-2-amino-4-phosphonobutyric acid analogue of MTX is likewise a competitive inhibitor and has approximately the same K<sub>i</sub> as the corresponding sulfonic acid.<sup>14</sup> It was therefore surprising to find that substitution of an aminomethylene group for the terminal carboxyl resulted in a 10-fold better inhibitor than did replacement of the carboxyl by a sulfonic or phosphonic acid group. Even higher potency was achieved with the L-ornithine analogue of AMT, compound 6, for which a K<sub>i</sub> of 0.15  $\pm$  0.06  $\mu$ M was observed. Since the corresponding L-homocysteic acid analogue had a K<sub>i</sub> of ca. 60  $\mu$ M,<sup>11</sup> the introduction of L-ornithine into AMT represented a 400-fold improvement. Compound 6 is the most potent FPGS inhibitor thus far encountered in the intensive search for such inhibitors that we<sup>10-14</sup> and others<sup>15,34-37</sup> have been conducting for several years.

An interesting aspect of the FPGS-inhibitory activity of 3 (and presumably of 6) is the exquisite specificity of its chain-length dependence. The shorter analogues 4 and

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**Figure 1.** Kinetics of inhibition of mouse liver FPGS by the L-ornithine analogues of MTX and AMT: panel A, 3; panel B, 6. Each point is the mean of two determinations from a representative experiment. The numbers next to each line are the concentrations of inhibitor used.

5 as well as the longer analogue 2 were essentially devoid of activity in this assay. This was in marked contrast to the behavior of the L-homocysteic and L-cysteic acid analogues of MTX and AMT, which show comparable activity even though they contain different numbers of  $\text{CH}_2$  groups in the side chain. We believe that compounds with an acidic group such as  $\text{SO}_3\text{H}$  in the side chain can bind to FPGS in such a way that this group forms an ionic bond to a basic residue in the enzyme active site and that there exists enough conformational flexibility in the enzyme-inhibitor complex to allow this interaction to occur regardless of whether the side chain contains two  $\text{CH}_2$  groups or only one. When a basic amino group is present in the side chain, on the other hand, it is projected in a different direction where it can interact with an acidic residue of the enzyme. It would appear that this acidic residue, which may be the one to which the amino group of the incoming glutamic acid residue must bind in the active site, is accessible to the inhibitor only when the side chain of the latter contains precisely three  $\text{CH}_2$  groups.

In order to determine whether compounds 2-6 are cytotoxic, we exposed cultured mouse L1210 cells to these agents for 48 h in culture (Table I). The  $\text{IC}_{50}$  values for the MTX analogues 2-5 ranged from 0.40 (2) to 2.42  $\mu\text{M}$  (4). There was a progressive decrease in potency as the side chain was shortened from four to two  $\text{CH}_2$  groups, but with a single  $\text{CH}_2$  group potency was restored to the level of the tetramethylene congener. The  $\text{IC}_{50}$  of the AMT analogue 6 was the same as that of the corresponding MTX analogue 3. Since the  $\text{IC}_{50}$  of MTX and AMT against these cells was 0.002  $\mu\text{M}$ , it would appear that the introduction of a terminal amino group produces a decrease in cytotoxicity of at least 200-fold. That this is not due merely to the inability of these compounds to form polyglutamates is indicated by the fact that the potency of the L-homocysteic acid analogue of MTX and the  $\gamma$ -tert-butyl ester of MTX, neither of which can be polyglutamylated, is decreased less than 30-fold in comparison with MTX.<sup>11,22</sup> It is likely that the positive charge on the terminal amino group of compounds 2-6 at physiologic pH interferes with cellular uptake. Amino-substituted prodrug derivatives of these compounds (especially the potent FPGS inhibitor 6) could be of interest as a means of overcoming this uptake problem.

In addition to the standard assays against MTX-sensitive L1210 cells, tests were carried out with cells (L1210/R81) that were previously shown to be 110 000-fold resistant to MTX<sup>22,38</sup> and 42 000-fold resistant to AMT<sup>22</sup> by virtue of a 35-fold increase in DHFR activity and a very severe defect in MTX active transport. The L1210/R81 cells have also been shown to be much less resistant to the  $\gamma$ -tert-butyl esters of MTX and AMT than they are to the parent acids,<sup>22</sup> and this incomplete cross-resistance has been posulated to be due to improved uptake of the esters. When MTX analogues 2-5 were assayed against the L1210/R81 cell line (Table I),  $\text{IC}_{50}$  values were found to vary from 86 (3) to 405  $\mu\text{M}$  (5). Thus, in contrast to L1210 cells, the L1210/R81 cells showed maximal sensitivity to the analogue with the L-ornithine side chain. The AMT analogue 6 was even more potent than 3 and was 7-fold more toxic than MTX. We believe that the decreased resistance of L1210/R81 cells to these L-ornithine derivatives may reflect the ability of the latter to not only inhibit DHFR but also block the polyglutamylated of exogenous folates. Since the affinity of 3 and 6 for FPGS is much lower than their affinity for DHFR, for which they probably have a  $K_i$  in the subnanomolar range, this "self-potential" effect would not be expected to play a role in MTX-sensitive cells. In DHFR-overproducing cells, however, where much higher concentrations of an antifolate have to be reached in order to saturate DHFR, enough drug may be present in the cytoplasm to interfere with polyglutamylated of the several tetrahydrofolate cofactors used for de novo synthesis of DNA precursors. The "self-potential" mechanism for dual inhibitors of DHFR and FPGS was first proposed in connection with MTX analogues in which the side chain contains a terminal sulfonate group.<sup>10,11</sup> The present findings support this concept and suggest further that "self-potentiating antifolates" need not be restricted to compounds with an acidic group at the end of the side chain.

### Experimental Section

Melting points were determined in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) and are not corrected. IR spectra were obtained on a Perkin-Elmer

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Model 781 double-beam spectrophotometer, and UV spectra were recorded on a Varian-Cary Model 210 instrument. NMR spectra were determined on a Varian T60A instrument with  $\text{Me}_4\text{Si}$  as the reference. TLC was performed on Whatman MK6F silica gel and Baker 250F silica gel plates containing a fluorescent indicator, and spots were visualized under 254-nm illumination. Column chromatography was carried out on Baker 3405 silica gel (60–200 mesh). Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, and Multi Chemical Laboratories, Lowell, MA, and were within  $\pm 0.4\%$  of the theoretical values unless otherwise noted. Reagent grade solvents were redistilled and stored over Davison 4A molecular sieves (Fisher Scientific, Boston, MA). *L*-Ornithine and *L*-2,4-diaminobutyric acid were purchased from Chemical Dynamics, South Plainfield, NJ. 2-[[*tert*-Butyloxycarbonyl]oxy]imino]-2-phenylacetonitrile, diethyl phosphorocyanidate, and bis(*p*-nitrophenyl) carbonate were from Aldrich, Milwaukee, WI. Bis(*L*-ornithinato)copper(II) dihydrochloride dihydrate and bis(*L*-2,4-diaminobutyrate)copper(II) dihydrochloride dihydrate were synthesized as described by Taurins.<sup>23</sup> *N* $\alpha$ -Tosyl-*L*-2,3-diaminopropionic acid was prepared from *N* $\alpha$ -tosyl-*L*-asparagine by the method of Zaoral and Rudinger.<sup>26</sup> 4-Amino-4-deoxy-*N*<sup>10</sup>-methylpteroic acid was obtained as previously reported,<sup>2</sup> using MTX kindly provided by Dr. Richard White, Lederle Laboratories, Pearl River, NY, and by Dr. V. L. Narayanan, National Cancer Institute, Bethesda, MD.

**Bis[*N* $\delta$ -(*tert*-butyloxycarbonyl)-*L*-ornithinato]copper(II).** To a solution of bis(*L*-ornithinato)copper(II) dihydrochloride dihydrate (3.27 g, 7.5 mmol) and 2-[[*tert*-butyloxycarbonyl]oxy]imino]-2-phenylacetonitrile (3.69 g, 15 mmol) in 1:1 dioxane- $\text{H}_2\text{O}$  (100 mL) was added (*i*-Pr)<sub>2</sub>NEt (3 mL), and the mixture was stirred at room temperature for 2 days. The precipitated product was collected, triturated with 95% EtOH (50 mL), filtered, washed with Et<sub>2</sub>O, and dried in vacuo to obtain a colorless powder (3.35 g, 80%); IR (KBr) 3320, 2980, 1680, 1620, 1530, 1450  $\text{cm}^{-1}$ . Anal. ( $\text{C}_{20}\text{H}_{38}\text{N}_4\text{CuO}_8$ ) C, H, N, Cu.

**Bis[*N* $\gamma$ -(*tert*-butyloxycarbonyl)-*L*-2,4-diaminobutyrate]copper(II).** This compound was prepared from bis(*L*-2,4-diaminobutyrate)copper(II) dihydrochloride dihydrate as described in the preceding experiment; yield 84%; IR (KBr) 3360, 2980, 1690, 1620, 1520, 1370  $\text{cm}^{-1}$ . Anal. ( $\text{C}_{18}\text{H}_{34}\text{N}_4\text{CuO}_8$ ) C, H, N.

***N* $\delta$ -(*tert*-Butyloxycarbonyl)-*L*-ornithine (9).** To a stirred suspension of bis[*N*-(*tert*-butyloxycarbonyl)-*L*-ornithinato]copper(II) (5 g, 9.5 mmol) in 150 mL of 0.1 M EDTA in 1 M  $\text{NH}_4\text{OH}$  was slowly added 60 mL of 95% EtOH. Stirring was continued at room temperature until all the solid dissolved, giving a blue solution. The volume was reduced to 25 mL by rotary evaporation, and the product was extracted into 2:1  $\text{CHCl}_3$ -EtOH (8  $\times$  100 mL). The combined extracts were evaporated, and the residue was dried in vacuo at room temperature to obtain a colorless solid (3.4 g, 75%); mp 220–222 °C dec; IR (KBr) 3360, 3300–2800, 1685, 1590, 1520, 1450; NMR ( $\text{D}_2\text{O}$ )  $\delta$  1.4 (s, 9 H, *t*-Bu), 2.0 (m, 4 H,  $\text{CH}_2\text{CH}_2$ ), 3.2 (t, 2 H,  $\text{CH}_2\text{N}$ ), 3.6 (t, 1 H,  $\alpha$ -CH). Anal. ( $\text{C}_{10}\text{H}_{20}\text{N}_2\text{O}_4 \cdot 0.25\text{H}_2\text{O}$ ) C, H, N.

***N* $\gamma$ -(*tert*-Butyloxycarbonyl)-*L*-2,4-diaminobutyric Acid (10).** This compound was prepared from bis[*N* $\gamma$ -(*tert*-butyloxycarbonyl)-*L*-2,4-diaminobutyrate]copper(II) as described in the preceding experiment; yield 54%; mp 205–208 °C; IR (KBr) 3380, 3300–2600, 1690, 1580, 1520, 1450; NMR ( $\text{D}_2\text{O}$ )  $\delta$  1.4 (s, 9 H, *t*-Bu), 2.0 (m, 2 H,  $\text{CH}_2$ ), 3.2 (t, 2 H,  $\text{CH}_2\text{N}$ ), 3.6 (s, 1 H,  $\alpha$ -CH). Anal. ( $\text{C}_9\text{H}_{18}\text{N}_2\text{O}_4 \cdot 0.2\text{H}_2\text{O}$ ) C, H, N.

***N* $\delta$ -(*tert*-Butyloxycarbonyl)-*L*-2,3-diaminopropionic Acid (11).** *N* $\alpha$ -Tosyl-*L*-2,3-diaminopropionic acid (2.58 g, 0.01 mol)<sup>26</sup> in 1 N NaOH (25 mL) was treated with 2-[[*tert*-butyloxycarbonyl]oxy]imino]-2-phenylacetonitrile (1.58 g, 0.015 mol) in dioxane (20 mL), and the mixture was stirred at 60 °C for 48 h. After removal of the dioxane by rotary evaporation, the solution was extracted with Et<sub>2</sub>O (2  $\times$  50 mL) and brought to pH 3–4 with concentrated citric acid while the temperature was maintained at 0–10 °C. The precipitated solid was extracted into EtOAc (100 mL), and the organic layer was washed with  $\text{H}_2\text{O}$  (2  $\times$  50 mL), dried over  $\text{Na}_2\text{SO}_4$ , and evaporated. The residue was applied onto a silica gel column (30  $\times$  5.5 cm) which was eluted with  $\text{CHCl}_3$  and then 4% MeOH in  $\text{CHCl}_3$ . Evaporation of pooled TLC-homogeneous fractions gave a white solid, which was recrystallized from 2:1 hexane-EtOAc; yield 2.37 g (66%); mp 127–128 °C (lit.<sup>39</sup>

mp 128–129 °C); TLC *R*<sub>f</sub> 0.52 (silica gel, 17:2:0.5  $\text{CHCl}_3$ -MeOH-AcOH). The product from several runs (7 g, 0.02 mol) was dissolved in liquid  $\text{NH}_3$  (300 mL) at –78 °C, and small pieces of metallic Na (3 g) were added with stirring until the blue color of the mixture persisted for 30 min. The reaction was quenched by addition of solid  $\text{NH}_4\text{OAc}$  (1 g), and the flask was fitted with a drying tube (NaOH pellets) and left overnight to allow the  $\text{NH}_3$  to evaporate. The solid was taken up in  $\text{H}_2\text{O}$  (250 mL), the pH was adjusted to neutrality with AcOH, and the cloudy solution was filtered through Celite before being concentrated to dryness on the rotary evaporator. Excess  $\text{NH}_4\text{OAc}$  was removed by repetitive rotary evaporation of an aqueous solution, and finally the product was extracted with several portions of 1-butanol. The aqueous layer was rinsed with Et<sub>2</sub>O between each extraction. The organic layers were pooled and evaporated, and the residue was taken up in  $\text{H}_2\text{O}$ . Freeze-drying left a colorless fluffy powder (4.4 g, 65% overall yield based on *N* $\alpha$ -tosyl-*L*-2,3-diaminopropionic acid); mp 168–170 °C dec; TLC *R*<sub>f</sub> 0.21 (silica gel, 15:5:1  $\text{CHCl}_3$ -MeOH-AcOH, positive ninhydrin test); NMR ( $\text{D}_2\text{O}$ )  $\delta$  1.43 (s, 9 H, *t*-Bu), 3.33 (s, 2 H,  $\text{CH}_2$ ), 5.5 (m, 1 H,  $\alpha$ -CH). This material was used with no further purification for coupling reactions (see below).

***N* $\alpha$ -(4-Amino-4-deoxy-*N*<sup>10</sup>-methylpteroyl)-*N* $\delta$ -(*tert*-butyloxycarbonyl)-*L*-ornithine (12). Method A.** To a suspension of 7·2 $\text{H}_2\text{O}$  (1 g, 2.77 mmol) in dry DMF (300 mL) were added (*i*-Pr)<sub>2</sub>NEt (0.96 mL, 5.5 mmol) and diethyl phosphorocyanidate (0.819 g, 5.54 mmol). Nearly complete dissolution occurred in ca. 15 min. After 4 h, the protected amino acid 9 (1.31 g, 5.53 mmol) was added as a solution in DMF (25 mL). A second portion of (*i*-Pr)<sub>2</sub>NEt (0.96 mL, 5.5 mmol) was added and stirring was continued at room temperature for 2 days. The reaction was terminated by adding solid  $\text{NaHCO}_3$  (0.45 g, 5.5 mmol) and the solvent was evaporated under reduced pressure (50–60 °C bath). The residue was applied onto a silica gel column (40  $\times$  2.5 cm) packed with  $\text{CHCl}_3$ , and the column was eluted with  $\text{CHCl}_3$  followed by 8:2:0.5  $\text{CHCl}_3$ -MeOH-concentrated  $\text{NH}_4\text{OH}$ . Pooled TLC-homogeneous fractions were evaporated to obtain 12 as a bright-yellow powder; yield 1.35 g (87%); TLC *R*<sub>f</sub> 0.35 (silica gel, 15:5:1  $\text{CHCl}_3$ -MeOH-AcOH); IR (KBr) 3390, 2990, 1640, 1550, 1510, 1450  $\text{cm}^{-1}$ . Anal. ( $\text{C}_{26}\text{H}_{33}\text{N}_9\text{O}_5 \cdot 1.25\text{H}_2\text{O}$ ) C, H, N.

**Method B.** Bis(*p*-nitrophenyl) carbonate (0.474 g, 1.56 mmol) and Et<sub>3</sub>N (0.12 g, 1.2 mmol) were added to a stirred suspension of 7·2 $\text{H}_2\text{O}$  (0.206 g, 0.6 mmol) in dry DMF (25 mL). The reaction mixture was heated to 55 °C for 5 h and monitored by TLC (silica gel, 15:5:1  $\text{CHCl}_3$ -MeOH-AcOH), which revealed nearly complete conversion of 7 to 15 (*R*<sub>f</sub> 0.75). After being cooled to room temperature, the mixture was added to *i*-PrOH (150 mL), and the precipitate was filtered, washed with *i*-PrOH, and dried; yield 0.24 g (86%). Without further purification, 15 was redissolved in dry DMF (25 mL), and to the solution were added 9 (0.122 g, 0.517 mmol) and Et<sub>3</sub>N (0.103 g, 1.03 mmol) with stirring. The reaction mixture was kept at 55 °C for 3 days, with TLC monitoring (silica gel, 15:5:1  $\text{CHCl}_3$ -MeOH-AcOH) of the disappearance of 15 (*R*<sub>f</sub> 0.75) and the formation of 12 (*R*<sub>f</sub> 0.38). After evaporation of the DMF under reduced pressure, the product was purified on a silica gel column (30  $\times$  3 cm), which was eluted first with  $\text{CHCl}_3$  to remove minor impurities and then with 8:2:0.5  $\text{CHCl}_3$ -MeOH-concentrated  $\text{NH}_4\text{OH}$  to elute the major yellow band. Appropriate fractions of the latter eluate were pooled and evaporated, and pure 12 was reprecipitated from MeOH-Et<sub>2</sub>O; yield 0.185 g (55% based on 7). The properties of this material and of the one obtained by method A were the same.

***N* $\alpha$ -(4-Amino-4-deoxy-*N*<sup>10</sup>-methylpteroyl)-*N* $\gamma$ -(*tert*-butyloxycarbonyl)-*L*-2,4-diaminobutyric Acid (13).** The procedure used to synthesize 12 (method A) was followed, except that the protected amino acid 10 was added as a solid. Mobile impurities were eluted from the silica gel column with 9:1  $\text{CHCl}_3$ -MeOH and 13 was eluted with 85:15  $\text{CHCl}_3$ -MeOH. Pooled TLC-homogeneous fractions were evaporated, and pure 13 was obtained as a bright-yellow powder by reprecipitation from MeOH-Et<sub>2</sub>O; yield 1.59 g (72%); TLC *R*<sub>f</sub> 0.3 (silica gel, 15:5:1  $\text{CHCl}_3$ -MeOH-AcOH); IR (KBr) 3410, 2990, 2940, 1600, 1430

cm<sup>-1</sup>. Anal. (C<sub>24</sub>H<sub>31</sub>N<sub>9</sub>O<sub>5</sub>·0.67CH<sub>3</sub>OH·H<sub>2</sub>O) C, H, N.

**N<sup>α</sup>-(4-Amino-4-deoxy-N<sup>10</sup>-methylpteroyl)-N<sup>β</sup>-(tert-butylloxycarbonyl)-L-2,3-diaminopropionic Acid (14).** Method A. To a stirred suspension of 7·2H<sub>2</sub>O (0.361 g, 1 mmol) in dry DMF (40 mL) was added (*i*-Pr)<sub>2</sub>NEt (0.53 mL, 3 mmol) followed by diethyl phosphorocyanidate (0.44 mL, 3 mmol). Stirring at room temperature was continued for 5 h, the protected amino acid 11 (0.24 g, 1 mmol) and a second portion of (*i*-Pr)<sub>2</sub>NEt (0.18 mL, 1 mmol) were added, and the reaction was allowed to proceed for 20 h. The product was isolated and purified as in the preceding experiment; yield 0.19 g (34%); TLC *R*<sub>f</sub> 0.3 (silica gel, 15:5:1 CHCl<sub>3</sub>-MeOH-AcOH); IR (KBr) 3400, 3200, 1640, 1610, 1510, 1450 cm<sup>-1</sup>; NMR (3:2 CD<sub>3</sub>OD-C<sub>6</sub>D<sub>6</sub>N) δ 1.4 (s, 9 H, *t*-Bu), 3.2 (s, 3 H, NMe), 3.8 (br, 2 H, CH<sub>2</sub>), 4.7 (br, 3 H, CH<sub>2</sub>N and α-CH), 6.7 (d, 2 H, 3'- and 5'-H), 8.0 (d, 2 H, 2'- and 6'-H), 8.6 (s, 1 H, 7-H). Anal. (C<sub>23</sub>H<sub>29</sub>N<sub>9</sub>O<sub>5</sub>·2H<sub>2</sub>O) C, H, N.

**Method B.** A mixture of the *p*-nitrophenyl ester 16 (90 mg, 0.2 mmol)<sup>22</sup> and 11 (44 mg, 0.2 mmol) in dry DMF (10 mL) containing Et<sub>3</sub>N (0.05 mL, 0.5 mmol) was stirred at 55 °C for 3 days and worked up as in the preparation of 14 from 7; yield 55 mg (50%). The properties of this material and the one obtained by method A were the same.

**N<sup>α</sup>-(4-Amino-4-deoxy-N<sup>10</sup>-methylpteroyl)-L-ornithine (3).** Compound 12 (1 g, 1.78 mmol) was dissolved in trifluoroacetic acid (8 mL) with occasional shaking, and after 3 h the solution was evaporated to dryness under a stream of nitrogen. The residue was taken up in MeOH, and Et<sub>2</sub>O was added. The precipitate was filtered, dried in vacuo at 40 °C, and redissolved in H<sub>2</sub>O (100 mL). The pH was adjusted to 10 with ammonia, the volume was reduced to 5–10 mL by rotary evaporation, EtOH (50 mL) and Et<sub>3</sub>N (5 mL) were added, and the mixture was evaporated to dryness. The crude product was applied onto a Whatman SE-53 sulfoethoxycellulose column (40 × 2.5 cm), which was packed in 0.1 NH<sub>4</sub>HCO<sub>3</sub> and eluted with the same buffer. Appropriate TLC homogeneous fractions (*R*<sub>f</sub> 0.15, silica gel, 3:1:1 BuOH-AcOH-H<sub>2</sub>O) were pooled and freeze-dried to obtain a bright-yellow powder; yield 0.64 g (80%); IR (KBr) 3340, 3200, 1600, 1450, 1380, 1200 cm<sup>-1</sup>. Anal. (C<sub>20</sub>H<sub>25</sub>N<sub>9</sub>O<sub>3</sub>·0.5H<sub>2</sub>O) C, H, N.

**N<sup>α</sup>-(4-Amino-4-deoxy-N<sup>10</sup>-methylpteroyl)-L-2,4-diaminobutyric Acid (4).** Compound 13 (1.13 g, 4 mmol) was treated with trifluoroacetic acid (10 mL) at 0 °C for 1.5 h, the solution was evaporated under a stream of nitrogen, and the gummy residue was dissolved in a mixture of EtOH (50 mL) and Et<sub>3</sub>N (5 mL). The solution was evaporated under reduced pressure, and the same process was repeated four times more. The crude product was redissolved in MeOH, a small amount of insoluble material was filtered off, and Et<sub>2</sub>O was added to the filtrate. The solid was collected and applied in 0.1 M NH<sub>4</sub>HCO<sub>3</sub> onto a Whatman DE52 DEAE-cellulose column packed in the same buffer. The column was eluted with 0.1 M NH<sub>4</sub>HCO<sub>3</sub>, and fractions were monitored by TLC (silica gel, 3:1:1 BuOH-AcOH-H<sub>2</sub>O). Several minor impurities preceded the product and were discarded. Fractions containing 4 were pooled and freeze-dried to obtain 0.63 g (65%) of bright-yellow powder; TLC *R*<sub>f</sub> 0.2 (silica gel, 3:1:1 BuOH-AcOH-H<sub>2</sub>O); IR (KBr) 3320, 3200, 1610, 1450, 1380, 1210 cm<sup>-1</sup>. Anal. (C<sub>19</sub>H<sub>23</sub>N<sub>9</sub>O<sub>3</sub>·3.25H<sub>2</sub>O) C, H, N.

**N<sup>α</sup>-(4-Amino-4-deoxy-N<sup>10</sup>-methylpteroyl)-L-2,3-diaminopropionic Acid (5).** Compound 14 (3.0 g, 5.48 mmol) was treated with trifluoroacetic acid (20 mL) as in the preparation of 4, and an identical workup was carried out to obtain 5 as a bright-yellow powder (1.85 g, 76%); TLC *R*<sub>f</sub> 0.18 (silica gel, 3:1:1 BuOH-AcOH-H<sub>2</sub>O); IR (KBr) 3340, 3200, 1610, 1450, 1370, 1200 cm<sup>-1</sup>. Anal. (C<sub>18</sub>H<sub>21</sub>N<sub>9</sub>O<sub>3</sub>·2H<sub>2</sub>O) C, H, N.

**N<sup>α</sup>-(4-Amino-4-deoxy-N<sup>10</sup>-formylpteroyl)-N<sup>β</sup>-(tert-butylloxycarbonyl)-L-ornithine (17).** To a stirred suspension of 9 (1.0 g, 0.004 mol assuming monohydrate formula) in dry benzene (20 mL) were added consecutively Et<sub>3</sub>N (1.78 g, 0.0176 mol) and trimethylsilyl chloride (1.91 g, 0.0176 mol). After being left in a closed flask at 25 °C for 20 h, the mixture was diluted with hexane (100 mL) and quickly filtered to remove Et<sub>3</sub>N·HCl. The solid was washed with hexane, and the combined filtrates were evaporated to an oil whose NMR spectrum was consistent with N<sup>α</sup>,O-bis(trimethylsilylation); yield 1.5 g (ca. 100%); NMR (CCl<sub>4</sub>) δ -0.03 (s, 9 H, Me<sub>3</sub>SiN), 0.2 (s, 9 H, Me<sub>3</sub>SiO), 1.33 (s, 9 H, *t*-Bu),

1.2–1.7 (m, 6 H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 3.0 (m, 1 H, α-CH).

To a suspension of 15 (1.84 g, 0.004 mol)<sup>22</sup> in dry DMF (100 mL) were added Et<sub>3</sub>N (0.4 g, 0.004 mol) and trimethylsilyl chloride (0.44 g, 0.004 mol). After 10 min of stirring at 25 °C, the silylated amino acid prepared above was added in a small volume of dry DMF, and the mixture was kept at 55 °C for 3 days. A homogeneous solution formed within 45 min. After 3 days, H<sub>2</sub>O (100 mL) was added, and solvents were removed on the rotary evaporator. The residue was rinsed twice with Et<sub>2</sub>O before being taken up in 15:6:1 CHCl<sub>3</sub>-MeOH-concentrated NH<sub>4</sub>OH and adsorbed onto a silica gel column (300 g), which was eluted with the same mixture. Fractions containing the product were pooled, evaporated, and redissolved in 10% NH<sub>4</sub>OH. Acidification to pH 4.5 with 10% AcOH gave a solid, which was filtered and dried, first in a lyophilizer and then at 100 °C over P<sub>2</sub>O<sub>5</sub>; yield 1.01 g (45%); TLC *R*<sub>f</sub> 0.24 (silica gel, 15:6:1 CHCl<sub>3</sub>-MeOH-concentrated NH<sub>4</sub>OH), *R*<sub>f</sub> 0.30 (silica gel, 15:5:1 CHCl<sub>3</sub>-MeOH-AcOH); IR (KBr) 1685, 1675, 1640, 1610 cm<sup>-1</sup>; NMR (Me<sub>2</sub>SO-*d*<sub>6</sub>) δ 1.35 (s, 9 H, *t*-Bu), 1.4–2.0 (m, 6 H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 5.2 (s, 2 H, 9-CH<sub>2</sub>), 7.51 (d, 2 H, 3'- and 5'-H), 7.79 (d, 2 H, 2'- and 6'-H), 8.55 (s, 1 H, CHO). Anal. (C<sub>25</sub>H<sub>31</sub>N<sub>9</sub>O<sub>6</sub>·0.5H<sub>2</sub>O) C, H, N.

**N<sup>α</sup>-(4-Amino-4-deoxypteroyl)-N<sup>β</sup>-(tert-butylloxycarbonyl)-L-ornithine (18).** A solution of 17 (0.93 g, 1.65 mmol) in a mixture of MeOH (50 mL) and 0.5 N NaOH (34 mL) was kept at 25 °C for 47 h. The progress of the reaction was followed by TLC (cellulose, pH 7.4 phosphate buffer), which showed disappearance of the fluorescent spot (*R*<sub>f</sub> 0.73) corresponding to 17 and the appearance of a UV-absorbing spot (*R*<sub>f</sub> 0.33) corresponding to product. The solution was acidified to pH 4.5 with 10% AcOH, and the precipitate was collected and dried, first in a lyophilizer and then at 100 °C over P<sub>2</sub>O<sub>5</sub>; yield 0.71 g. Concentration of the mother liquor and overnight storage at 0 °C produced an additional 0.025 g; total yield 0.73 g (83%); TLC *R*<sub>f</sub> 0.67 (silica gel, 5:4:1 CHCl<sub>3</sub>-MeOH-concentrated NH<sub>4</sub>OH); IR (KBr) 3325, 1685, 1640, 1610 cm<sup>-1</sup>. Anal. (C<sub>24</sub>H<sub>31</sub>N<sub>9</sub>O<sub>5</sub>·0.75H<sub>2</sub>O) C, H, N.

**N<sup>β</sup>-(4-Amino-4-deoxypteroyl)-L-ornithine (6).** Compound 18 (0.425 g, 0.788 mmol) was dissolved in trifluoroacetic acid (4.5 mL) at 0 °C and the solution was kept at 0 °C for 1.5 h before being added dropwise into stirred Et<sub>2</sub>O (100 mL). The precipitate was filtered, washed with Et<sub>2</sub>O, and dried in vacuo at 25 °C. For purification, the solid was redissolved in a mixture of H<sub>2</sub>O (8 mL) and AcOH (2 mL) at 80 °C, the solution was allowed to cool partly, and 30% NH<sub>4</sub>OH was added to bring the pH to 8.0. After 1 h at 0 °C, the precipitate was filtered, washed with H<sub>2</sub>O, and dried in a lyophilizer and then at 100 °C over P<sub>2</sub>O<sub>5</sub> to obtain a bright-yellow powder (0.322 g, 89%); TLC *R*<sub>f</sub> 0.49 (silica gel, 4:1 EtOH-concentrated NH<sub>4</sub>OH), *R*<sub>f</sub> 0.62 (silica gel, 5:4:1 CHCl<sub>3</sub>-MeOH-concentrated NH<sub>4</sub>OH); IR (KBr) 3400, 1630, 1610 cm<sup>-1</sup>. Anal. (C<sub>19</sub>H<sub>23</sub>N<sub>9</sub>O<sub>3</sub>·2H<sub>2</sub>O) C, H, N.

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**Registry No.** 3, 80407-73-4; 4, 100430-89-5; 5, 100484-53-5; 6, 100430-90-8; 7, 19741-14-1; 9, 13650-49-2; 9 N<sup>α</sup>,O-bis(trimethylsilyl) deriv., 100430-91-9; 10, 10270-94-7; 11, 74536-29-1; 12, 96846-16-1; 13, 100430-92-0; 14, 100484-54-6; 15, 95485-01-1; 16, 57245-77-9; 17, 100430-93-1; 18, 100430-94-2; bis(L-ornithinato)copper(II) dihydrochloride, 53109-82-3; 2-[[[tert-butylloxycarbonyl]oxy]imino]-2-phenylacetoneitrile, 58632-95-4; bis[N<sup>β</sup>-(tert-butylloxycarbonyl)-L-ornithinato]copper(II), 62125-49-9; bis[L-2,4-diaminobutyrate]copper(II) dihydrochloride, 15319-78-5; bis[N<sup>β</sup>-(tert-butylloxycarbonyl)-L-2,4-diaminobutyrate]copper(II), 100449-20-5; N<sup>α</sup>-tosyl-L-2,3-diaminopropionic acid, 21753-19-5; N<sup>β</sup>-(tert-butylloxycarbonyl)-N<sup>2</sup>-tosyl-L-2,3-L-2,3-diaminopropionic acid, 16947-86-7; bis(*p*-nitrophenyl) carbonate, 5070-13-3; dihydrofolate reductase, 9002-03-3; folypolyglutamate synthetase, 63363-84-8.