Methotrexate Analogues. 33. N^{δ}-Acyl-N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine Derivatives: Synthesis and in Vitro Antitumor Activity[1]

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N^{δ}-Acyl derivatives of the potent folylpolyglutamate synthetase (FPGS) inhibitor N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine (APA-L-Orn) were synthesized from N^{α}-(4-amino-4-deoxy-N^{10}-formylpteroyl)-L-ornithine by reaction with an *N*-(acyloxy)succinimide or acyl anhydride, followed by deformylation with base. The N^{δ}-hemiphthaloyl derivative was also prepared from 4-amino-4-deoxy-N^{10}-formylpteroic acid by reaction with persilylated N^{δ}-phthaloyl-L-ornithine, followed by simultaneous deformylation and ring opening of the N^{δ}-phthaloyl moiety with base. The products were potent inhibitors of purified dihydrofolate reductase (DHFR) from L1210 murine leukemia cells, with IC_{50}'s ranging from 0.027 and 0.052 μM as compared with 0.072 μM for APA-L-Orn. Several of the N^{δ}-acyl-N^{10}-formyl intermediates also proved to be good DHFR inhibitors. One of them, N^{α}-(4-amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-(4-chlorobenzoyl)-L-ornithine, had a 2-fold lower IC_{50} than its deformylated product, confirming that the N^{10}-formyl group is well tolerated for DHFR binding. While N^{δ}-acylation of APA-L-Orn did not significantly alter anti-DHFR activity, inhibition of FPGS was dramatically diminished, supporting the view that the basic NH_2 on the end of the APA-L-Orn side chain is essential for the activity of this compound against FPGS. N^{δ}-Acylation of APA-L-Orn markedly enhanced toxicity to cultured tumor cells. However, N^{δ}-acyl derivatives also containing an N^{10}-formyl substituent were less cytotoxic than the corresponding N^{10}-unsubstituted analogues even though their anti-DHFR activity was the same, suggesting that N^{10}-formylation may be unfavorable for transport. Two compounds, the N^{δ}-benzoyl and N^{δ}-hemiphthaloyl derivatives of APA-L-Orn, with IC_{50}'s against L1210 cells of 0.89 and 0.75 nM, respectively, were more potent than either methotrexate (MTX) or aminopterin (AMT) in this system. These compounds were also more potent than MTX against CEM human lymphoblasts and two human head and neck squamous cell carcinoma cell lines (SCC15, SCC25) in culture. Moreover, in assays against SCC15/R1 and SCC25/R1 sublines with 10–20-fold MTX resistance, the N^{δ}-hemiphthaloyl derivative of APA-L-Orn showed potency exceeding that of MTX itself against the parental cells. Although other mechanisms cannot be ruled out, a possible explanation for the fact that these N^{δ}-acyl derivatives of APA-L-Orn are much more potent in the cell growth assay than in the DHFR assay is that they are efficiently taken up by the cell and are then cleaved to APA-L-Orn, which can simultaneously inhibit DHFR and FPGS, thereby acting as a "self-potentiating antifolate". According to this view, blockade of cellular FPGS activity should complement DHFR inhibition by diminishing the cell's ability to convert tetrahydrofolate monoglutamate cofactors to polyglutamates, which are the most efficiently used species for DNA precursor synthesis.

N^{α}-(4-Amino-4-deoxypteroyl)-L-ornithine (APA-L-Orn, 1) has been reported to be a potent inhibitor of dihydrofolate reductase (DHFR) and folylpolyglutamate synthetase (FPGS) but is relatively inactive as an inhibitor of cell growth in culture,[2-4] presumably because the primary amino group on the end of the side chain, which is positively charged at physiologic pH, prevents efficient transport of the drug into the cell. Interest in APA-L-Orn stems from the fact that this compound has the potential to act as a "self-potentiating antifolate" by not only binding tightly to DHFR but also interfering with the cell's ability to convert reduced folates to polyglutamates.[5,6] Polyglutamate derivatives of reduced folates are utilized more efficiently as cofactors than are the corresponding monoglutamates, which tend to have higher K_m and/or lower V_{max} values as substrates for their respective enzymes and are less well retained in the cell.[7] By inducing a folate-deficient state, FPGS inhibition may make the cell more antifolate-sensitive. These considerations prompted us to investigate the possibility that APA-L-Orn derivatives with potentially cleavable N^{δ}-acyl groups on the terminal amino group might have greater cell growth inhibitory activity than APA-L-Orn itself. This paper reports the synthesis and biological activity of several such derivatives (**2–7**), the most active of which proved to be N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (**7**). This compound was a 300-fold poorer inhibitor of FPGS than APA-L-Orn but was 1700-fold more potent as an inhibitor of L1210 cell growth in culture. Moreover, **7** was 6-fold more potent than methotrexate (MTX) and 3-fold more potent than aminopterin (AMT) in cell culture despite the fact that the IC_{50}'s of all three compounds against purified DHFR from L1210 cells were practically the same. Biological activity data are also presented for several N^{δ}-acyl-N^{10}-formyl intermediates, some which were as potent as their N^{10}-unsubstituted counterparts.

Chemistry. Our approach to preparing N^{δ}-acyl-N^{α}-(4-amino-4-deoxypteroyl)-L-ornithines **2-7** was to condense N^{α}-(4-amino-4-deoxy-N^{10}-formylpteroyl)-L-ornithine (**14**)[2] with anhydrides, or in the case of **2** with *N*-acetoxysuccinimide, to form N^{δ}-acyl-N^{α}-(4-amino-4-deoxy-N^{10}-formylpteroyl)-L-ornithines **8–13**, and to treat the latter under mild alkaline conditions that we had previously found would cleave the N^{10}-formyl group selectively in the

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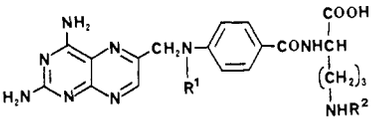

	R^1	R^2
1	H	H
2	H	$COCH_3$
3	H	$COCH_2CH_2COOH$
4	H	COC_6H_5
5	H	COC_6H_4Cl-4
6	H	$COC_6H_3Cl_2$-3,4
7	H	COC_6H_4COOH-2
8	CHO	$COCH_3$
9	CHO	$COCH_2CH_2COOH$
10	CHO	COC_6H_5
11	CHO	COC_6H_4Cl-4
12	CHO	$COC_6H_3Cl_2$-3,4
13	CHO	COC_6H_4COOH-2
14	CHO	H
15	CHO	COOBu-t

presence of other side-chain amide groups.[8] Compound 14 was used as its trifluoroacetate salt, **14**·TFA, prepared in quantitative yield by treatment of N^{α}-(4-amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-(*tert*-butyloxycarbonyl)-L-ornithine (**15**) with cold trifluoroacetic acid. Except for the reaction of **2** with *N*-acetoxysuccinimide, which was carried out in water, acylations of **14** with anhydrides were carried out in *N*-methylpyrrolidinone, which proved to be a better solvent than DMF. The previously undescribed compound 3,4-dichlorobenzoic anhydride (**16**), which was used to form **6**, was synthesized in 76% yield from 3,4-dichlorobenzoyl chloride and 3,4-dichlorobenzoic acid.

N^{δ}-Acyl-N^{10}-formyl derivatives were purifed by column chromatography on silica gel with $CHCl_3$–MeOH–NH_4OH mixtures as eluents or, in some cases, after the initial passage through silica gel, by chromatography on a DEAE-cellulose column eluted with 3% NH_4HCO_3. Tenacious solvation of the N^{δ}-acyl-N^{10}-formyl compounds was consistently observed even after rigorous drying, and one compound (**9**) was isolated as a hydrated partial ammonium salt.

With the exception of **13**, whose deformylation was accompanied by cleavage to **1** in dilute base, removal of the N^{10}-formyl group was complete after 5–7 h in 0.25 N NaOH at room temperature, affording yields of 85–90% in most cases. Compound **7**, after separation from **1** by chromatography, was isolated in only 25–30% yield. Acidification of the hydrolysis mixture to ca. pH 4 with AcOH caused the deformylated product to precipitate, generally in the form of a gel, which was most easily harvested after being disaggregated with water and stored at 0 °C to increase its granularity. Rigorous purification of the N^{10}-formyl intermediates made it possible to obtain all the final products except **7** in analytically pure state by simple washing and drying. In addition to elemental microanalysis, HPLC on a C18-bonded silica gel column with NH_4OAc buffer containing MeCN as the eluent was used to confirm that all compounds submitted for biological evaluation were >95% pure. In one case (**12**) the final sample was also preparatively purified by HPLC to remove a trace of 3,4-dichlorobenzoic acid that had coprecipitated on acidification of the deformylation reaction.

In an alternative route to **7** (Scheme I), condensation of N^{10}-formyl-4-amino-4-deoxypteroic acid (**17**)[9] with the N^{α},*O*-bis(trimethylsilyl) derivative **18** of N^{δ}-phthaloyl-L-ornithine[10] was carried out by our previously described modification[4,5,7] of the mixed anhydride coupling procedure to obtain the N^{δ}-phthaloyl derivative **19** in 40% yield, with 21% recovery of unchanged **17**. Interestingly, the phthalimide ring in **19** opened during N-deformylation to give **13** (26% yield) in one step. Thus, N^{δ}-acyl derivatives of **1** are accessible either via preformed **14** or more directly, and in higher overall yield, via **17**.

Scheme I

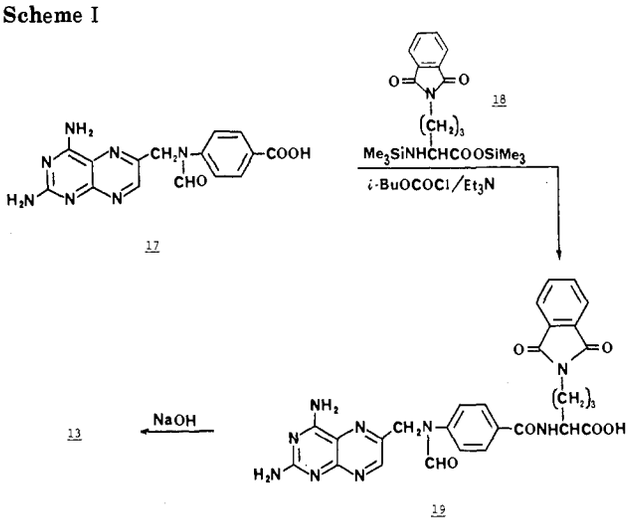

Table I. Biological Activity of N^{δ}-Acyl Derivatives of N^{α}-(4-Amino-4-deoxypteroyl)-L-ornithine (APA-L-Orn, **1**)

	enzyme inhibition		cell-growth inhibition[c,d]	
compd	DHFR[a]	FPGS[b]	L1210	L1210/R81
MTX	0.025	*e*	0.0046 (1)	200 (1)
AMT	0.035	*e*	0.0020 (2.3)	84 (2.4)
1	0.072	0.15	1.3 (0.0035)	32 (6.3)
2	0.028	68	0.017 (0.27)	81 (2.5)
3	0.032	44	0.037 (0.12)	54 (3.7)
4	0.027	>100	0.00089 (5.2)	17 (12)
5	0.028	*f*	0.0032 (1.4)	27 (7.4)
6	0.045	*f*	0.032 (0.14)	127 (1.6)
7	0.052	*f*	0.00075 (6.1)	52 (3.8)
8	0.053	*f*	0.41 (0.011)	230 (0.87)
9	*f*	39	*f*	*f*
10	0.034	>1000	0.020 (0.23)	156 (1.3)
11	0.015	>100	0.20 (0.023)	>200 (<1.0)
12	0.056	*f*	3.2 (0.0014)	>200 (<1.0)
14	*f*	0.65	*f*	*f*

[a] IC_{50} concentrations (μM) were determined spectrophotometrically at 340 nm as described in ref 6. [b] K_i values (μM) were determined as described in ref 13, with partially purified enzyme from mouse liver in the presence of folic acid or AMT as the variable substrate. The K_i listed for **1** is taken from ref 2. [c] IC_{50} concentrations (μM). Numbers in parentheses are normalized for each cell line with respect to MTX. Numbers greater than one indicate potency greater than that of MTX. Data for **4** and **7** are the means of six separate experiments on different days. [d] Three compounds were also tested against human CEM and CEM/MTX lymphoblasts. The IC_{50}'s (μM) against CEM cells, with normalized values relative to MTX given in parentheses, were as follows: **4**, 0.0066 (4.8); **5**, 0.27 (0.12); **6**, 7.4 (0.0043); **7**, 0.0042 (7.6). The IC_{50}'s (μM) and MTX-normalized values against CEM/MTX cells were as follows: **4**, 1.1 (6.0); **5**, 1.0 (6.6); **6**, 10 (0.66); **7**, 0.42 (16). [e] MTX and AMT are substrates for mouse liver FPGS; see ref 13. [f] Not tested.

Biological Activity. As shown in Table I, the N^{δ}-acyl derivatives **2**–**7** of **1** were all potent inhibitors of DHFR from leukemic murine cells, with IC_{50}'s ranging from 0.028 μM for **2** to 0.052 μM for **7**. The molar potency of the N^{δ}-acyl derivatives was comparable to, or slightly lower

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than, that of MTX and AMT and slightly higher than that of 1. The diminished potency of 1 in comparison with AMT was ascribed earlier[2,4] to the basicity of the terminal NH_2 group, which would result in a positive charge at physiologic pH. This is supported by the present finding that acylation of the nitrogen increases binding affinity 2–3-fold. The N^{δ}-hemisuccinoyl derivative **3** was a good DHFR inhibitor even though the end of the side chain now carried a potential negative charge. Since the two COOH groups in **3** are separated by a total of eight atoms, this compound may be viewed as a chain-extended analogue of AMT.

The N^{10}-formyl derivatives **8** and **10–12** were also good DHFR inhibitors. The 4-chlorobenzoyl congener **11**, which was the best inhibitor, had an IC_{50} lower than those of the nonformylated compounds and lower even than those of MTX and AMT. The fact that the IC_{50}'s of pairs of compounds with the same N^{δ}-substituent (e.g., **2** and **8**) differed by no more than 2-fold ruled out the possibility that the activity of the formyl intermediates was due to contamination by the deformylated products or vice versa. In assays against DHFR in crude extracts from mouse liver, Soucek and co-workers[11] found N^{10}-formylaminopterin to be less active than aminopterin, though the difference in IC_{50} was only 2-fold. It thus appears that N^{10}-formylation of a 4-amino-4-deoxypteroyl derivative is tolerated by the enzyme irrespective of whether or not the side chain is also modified. Interestingly, N^{10}-formylfolic acid has likewise been reported to be a tight-binding inhibitor of DHFR.[12]

Compounds **2** and **3** showed some in vitro activity against murine FPGS in the presence of folate or aminopterin as competitive substrates. Although activity was low in comparison with 1, it was higher than that of a number of other side chain modified analogues.[13] Because 1 is so potent in the FPGS assay, however, we could not rule out that the apparent activity of **2** and **3** was due to contamination of the test samples by traces of 1 that had escaped HPLC detection. From the K_i values for 1 and **2**, for example, a 0.22% contamination by 1 could lead to the observed FPGS inhibition. The N^{δ}-benzoyl and N^{10}-formyl-N^{δ}-hemisuccinoyl derivatives **4** and **9** were inactive below 100 μM, and the N^{δ}-benzoyl-N^{10}-formyl derivative **10** was inactive below 1000 μM. We conclude from these results that acylation of the terminal NH_2 group in 1 leads to essentially complete abrogation of FPGS binding. This is consistent with the role we postulated earlier for the side chain NH_2 group in the highly specific interaction of 1 with FPGS.[2-4] Interestingly, however, the N^{10}-formyl derivative **14** had a K_i of 0.65 μM and was therefore among the most potent FPGS inhibitors we have tested to date.

In contrast to the low activity (IC_{50} = 1.3 μM) of 1 as an inhibitor of L1210 cell growth in culture, all the N^{δ}-acyl derivatives had IC_{50}'s of <0.05 μM, and two of them, the N^{δ}-benzoyl and N^{δ}-hemiphthaloyl derivatives **4** (IC_{50} = 0.89 nM) and **7** (IC_{50} = 0.75 nM), were more potent than either MTX (IC_{50} = 4.6 nM) or AMT (IC_{50} = 2.0 nM). The N^{δ}-(4-chlorophenyl) derivative **5** (IC_{50} = 3.2 nM) and N^{δ}-(3,4-dichlorophenyl) derivative **6** (IC_{50} = 32 nM) were also good inhibitors but less potent than the nonhalogenated congener **4**. The high activity of compounds **4** and **7** was remarkable in view of the fact that other members of the series were 10–100-fold less active even though they had virtually the same affinity for purified murine DHFR. In the case of **7**, which showed a <2-fold increase in DHFR affinity relative to 1, there was a $>10^3$-fold increase in growth-inhibitory potency. These results are consistent with a prodrug mechanism wherein the N-acyl group promotes uptake of the drug into the cell and is subsequently cleaved to give free 1. The latter would then function as a "self-potentiating antifolate" by concomitantly binding to DHFR and interfering with the cell's ability to polyglutamylate reduced folate cofactors used for de novo DNA precursor synthesis. If this mechanism were operative, it might be expected to enhance cytotoxicity relative to "classical" antifolates such as MTX or AMT. Two possibilities that concerned us were that these compounds might be killing cells (a) by inhibiting thymidylate synthase (TS) or (b) by a mechanism unrelated to folate metabolism. The first possibility was ruled out on the basis of a direct assay of the ability of **4** and **7** to inhibit purified TS from L1210 cells. The IC_{50}'s of both compounds in this assay were >100 μM, a concentration 5 orders of magnitude higher than the IC_{50} for cell-growth inhibition. The second possibility was equally excluded by demonstrating that 10 nM **4** and **7** were completely nontoxic to L1210 cells in the presence of 10 μM thymidine plus 100 μM hypoxanthine. The toxicity of antifolates to cultured mammalian cells is known to be fully prevented by supplying thymidine and hypoxanthine at these concentrations in the growth medium.[14] A third alternative not requiring intracellular conversion to 1 is that the N^{δ}-acyl derivatives are simply able to accumulate to very high levels in the cell, resulting in less dissociation of bound drug from DHFR. Studies to distinguish between this mechanism and FPGS inhibition following intracellular formation of 1 are in progress and will be reported elsewhere.

While N^{10}-formylation had a negligible effect on binding to DHFR (see above), this modification was detrimental to activity against intact cells. For example, **8** was 24-fold less toxic than **2** and **10** was 33-fold less toxic than **4**. It may be concluded from these results than N^{10}-formylation is probably unfavorable for transport into the cell.

As part of a continuing search for compounds with the potential to overcome MTX resistance based on transport, the N^{δ}-acyl derivatives of 1 were tested as inhibitors of the growth of L1210/R81 cells, which are >40 000-fold resistant to MTX and AMT. These cells have been shown to owe their resistance to a severe transport defect combined with 35-fold overproduction of kinetically normal DHFR.[15] Compounds **2–7**, with IC_{50}'s in the 10–100 μM range, were somewhat more active than MTX (IC_{50} = 200 μM) as inhibitors of the growth of L1210/R81 cells but remained much less active against these cells than against the parent line. Once again, each N^{10}-formyl derivative tested was less active than its nonformylated counterpart.

Four compounds were also tested in culture against CEM lymphoblasts.[16] While the IC_{50} of MTX against these cells was 0.032 μM, that of the N^{δ}-benzoyl derivative **4** was 0.0066 μM, corresponding to a ca. 5-fold enhancement in potency. Thus, this compound was more active than MTX against the human as well murine leukemic cells. The N^{δ}-(4-chlorobenzoyl) and N^{δ}-(3,4-dichlorobenzoyl) derivatives **5** (IC_{50} = 0.27 μM) and **6** (IC_{50} = 7.4

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Table II. Growth-Inhibitory Activity of N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (**7**) against MTX-Sensitive and MTX-Resistant Human Squamous Cell Carcinoma Lines in Culture

	cells and growth inhibition: IC_{50},[a] μM			
compd	SCC15	SCC15/R1	SCC25	SCC25/R1
MTX	0.038 (1)	0.58 (0.07)	0.0075 (1)	0.15 (0.05)
tBMTX	0.60 (0.06)	1.3 (0.03)	0.40 (0.02)	0.78 (0.01)
7	0.0011 (35)	0.0040 (9.5)	0.00096 (7.8)	0.0013 (5.8)

[a] Cell monolayers were exposed continuously to drug for ca 2 weeks.[21] Numbers in parentheses are normalized relative to the IC_{50} of MTX against the *parental* SCC15 cells (columns 1 and 2) or SCC25 cells (columns 3 and 4). Data for MTX and tBMTX were taken from ref 21 and 9, respectively. IC_{50} = concentration needed to inhibit cell growth by 50% relative to controls. Numbers greater than one indicate potency greater than that of MTX.

μM) were considerably less active than **4** even though their DHFR affinities, at least for the murine enzyme, were similar. Piper and co-workers[17] reported that the N^{10}-methyl analogue of **5** inhibits L1210 cells with an IC_{50} of 0.0017 μM and human epithelial carcinoma H.ep.-2 cells with an IC_{50} of 0.004 μM. Compounds **5** and **6** were difficulty soluble and might have formed microprecipitates upon addition to growth medium. Compounds **4–6** were also assayed against CEM/MTX cells.[18] These cells are ca. 200-fold MTX resistant by virtue of a severe transport defect,[18] as well as an inability to polyglutamylate MTX even at extracellular drug concentrations high enough to overcome the transport defect.[19] Compounds **4** and **5** had IC_{50}'s of ca. 1 μM against these cells and were thus several times more potent than MTX, while **6** had an IC_{50} of 10 μM. Compound **7**, with an IC_{50} of 0.42 μM, was 16-fold more potent than MTX against CEM/MTX cells and its potency against these resistant cells was only 13-fold lower than that of MTX against the parental MTX-sensitive line itself.

Finally, the N^{δ}-hemiphthaloyl derivative **7** was tested against two human cell lines with 10- to 20-fold resistance to MTX. While this level of resistance is lower than that of L1210/R81 or CEM/MTX cells, it is felt to be clinically relevant because the steep nature of the MTX dose–response in humans means that when a tumor reaches this level of resistance in a patient, further escalation of the MTX dose is not possible. Data documenting the growth inhibitory activity of **7** against cultured SCC15/R1 and SCC25/R1 cells and two head and neck squamous cell carcinoma (SCC) sublines with "low-level" resistance[20,21] are presented in Table II, along with data obtained with the MTX-sensitive parent lines SCC15 and SCC25. Also included for comparison are MTX and the lipophilic γ-*tert*-butyl ester of MTX (tBMTX).[9] The SCC15/R1 and SCC25/R1 cells have previously been shown to be only partly cross-resistant to tBMTX,[9] and we have proposed that this relates to the efficiency with which this active DHFR inhibitor penetrates the cell even when MTX uptake is poor.[22] Compound **7** was 10–30-fold more potent than MTX against the parental SCC15 and SCC25 cells, while tBMTX was less than one-tenth as potent as MTX. Moreover, while tBMTX was at least 30-fold less potent than MTX against the MTX-resistant SCC15/R1 and SCC25/R1 sublines, the potency of **7** against both resistant cells *exceeded that of MTX against the parental cells.* This was an unexpected and gratifying observation for which there is no precedent among the many side chain altered analogues of MTX we have studied up to now.

In summary, it is possible to substantially increase the cytotoxicity of the DHFR and FPGS inhibitor APA-L-Orn (**1**) by acylation of the terminal NH_2 group. While the number of derivatives we have studied thus far is limited, it appears that aromatic N^{δ}-acyl groups are the most promising type for further exploration. Folate analogue inhibitors of FPGS are of current interest not only as probes of the enzyme active site but also as potential therapeutic agents in their own right.[23-27] We have previously reported that N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-ornithine (mAPA-L-Orn), which is an analogue of MTX rather than AMT, is a competitive inhibitor of mouse liver FPGS with a K_i of 20.4 μM.[2] McGuire and co-workers[26] recently reported that mAPA-L-Orn has an apparent K_i in the 3-4 μM range against FPGS from human myeloblasts (K562) and lymphoblasts (CEM) and from rat liver. APA-L-Orn, with a K_i in the submicromolar range,[2] is clearly more potent than mAPA-L-Orn. In fact, apart from APA-L-Orn, the only other folate analogues reported to date to have a K_i below 1 μM against FPGS are 7,8-dihydropteroyl-L-ornithine and 5,6,7,8-tetrahydropteroyl-L-ornithine.[26] These compounds both have a K_i of 0.2 μM against hog liver FPGS and are therefore of similar potency as APA-L-Orn. In view of the results reported here, cytotoxicity studies with aromatic N^{δ}-acyl derivatives of the reduced pteroyl-L-ornithines would be of interest. Studies on the in vivo metabolism and antitumor activity of these compounds are planned.

Experimental Section

Melting points were determined in a Fischer-Johns hot stage apparatus and are corrected. IR spectra were obtained on a Perkin-Elmer Model 781 double-beam spectrophotometer. Analytical TLC was done on Whatman MK6F silica gel, Baker 250F silica gel, or Eastman 13254 cellulose (with fluorescent indicator). Preparative TLC was done on Analtech 1000 μm GF silica gel plates. Spots were visualized under 254-nm illumination. Plates were developed with the following solvent systems: 4:1 MeOH–NH_4OH (system A), 4:1 EtOH–NH_4OH (system B), 15:5:1 $CHCl_3$–MeOH–AcOH (system C), 10:6:1 $CHCl_3$–MeOH–NH_4OH (system D), 5:4:1 $CHCl_3$–MeOH–NH_4OH (system E), 14:6:1 $CHCl_3$–MeOH–NH_4OH (system F), pH 7.4 phosphate buffer (system G), 28:12:1 $CHCl_3$–MeOH–NH_4OH (system H). Column chromatography was on Baker 3405 silica gel (60–200 mesh) or Whatman DE52 [(diethylamino)ethyl]cellulose (DEAE-cellulose). Several of the TLC solvent systems were also used for column chromatography. HPLC purifications were performed on a Waters Model 400 instrument equipped with a Model 660 solvent programmer, a Model 440 UV absorbance detector set at 280 nm,

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and an RCM100 radial compression module with a 0.5 × 10 cm C18 column of 5-μm particle size. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, and MultiChem Laboratories, Lowell, MA, and were within ±0.4% of theory unless otherwise indicated. Solvents were stored over Davison 4A molecular sieves (Fisher, Boston, MA). 4-Amino-4-deoxy-N^{10}-formylpteroic acid sesquihydrate (**17**) and N^{α}-(4-amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-(*tert*-butyloxycarbonyl)-L-ornithine (**15**) were synthesized as previously described.[2,9] N^{δ}-Phthaloyl-L-ornithine was prepared according to Bodanszky and co-workers.[10] Other chemicals were purchased from Chemical Dynamics, South Plainfield, NJ, and Aldrich, Milwaukee, WI.

N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-L-ornithine Trifluoroacetate (14·TFA). Compound **15** (2.03 g, 3.61 mmol) was added to stirred TFA (10 mL) at −20 °C, and after 1.5 h at 0–5 °C the solution was poured into stirred Et_2O (200 mL). Stirring was continued for 15 min, and the mixture was cooled to 0 °C and filtered. The solid was dried in vacuo at 50 °C over P_2O_5 overnight to obtain an off-white powder (2.43 g, 100% yield): mp 135–140 °C dec; R_f 0.81 (silica gel, system A), 0.02 (silica gel, system C); IR (KBr) 3400, 3250, 1680, 1640, 1610 cm^{-1}. Anal. ($C_{20}H_{23}N_9O_4 \cdot 1.8CF_3COOH \cdot 0.8H_2O$) C, H, F, N.

N^{δ}-Acetyl-N^{α}-(4-amino-4-deoxy-N^{10}-formylpteroyl)-L-ornithine (8). A solution of **14**·TFA (300 mg, 0.445 mmol) in warm H_2O (50 mL) was adjusted to pH 7.5 with aqueous $NaHCO_3$, and to it were added *N*-acetoxysuccinimide (87 mg, 0.556 mmol) followed by solid $NaHCO_3$ (93 mg, 1.11 mmol). A clear solution formed after 10 min. The reaction mixture was kept at room temperature for 1 h, and a second portion of *N*-acetoxysuccinimide (175 mg, 1.11 mmol) was added, followed by $NaHCO_3$ (116 mg, 1.38 mmol). The progress of the reaction was monitored by TLC on silica gel (system A), which showed **8** and **14** as blue fluorescent spots with R_f 0.68 and 0.36, respectively. After 18 h, the solution was acidified to pH 3.5 with AcOH and evaporated to dryness under reduced pressure. The residue was chromatographed on a silica gel column (27 × 2.0 cm) packed and eluted with solvent system D. Fractions giving a single TLC spot (R_f 0.35, silica gel, system D) were pooled, evaporated by rotary evaporation and freeze-drying, and rechromatographed on a DEAE-cellulose column (27 × 2.0 cm) with 3% NH_4HCO_3 as the eluent. TLC-homogeneous fractions were pooled and lyophilized, and the product was dried in vacuo at 60 °C over P_2O_5 to obtain a yellow powder (195 mg, 84% yield). The analytical sample was recrystallized from EtOH: mp 168 °C; IR (KBr) 3400, 1635, 1610 cm^{-1}. Anal. ($C_{22}H_{25}N_9O_5 \cdot 0.25C_2H_5OH \cdot 0.8H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-hemisuccinyl-L-ornithine (9). Et_3N (182 mg, 1.8 mmol) was added to a solution of **14**·TFA (303 mg, 0.45 mmol) and succinic anhydride (54 mg, 0.45 mmol) in *N*-methylpyrrolidinone (25 mL), and after 18 h at 25 °C the solvent was distilled off under reduced pressure. The residue was triturated with Et_2O and applied onto a silica gel column (25 × 2.0 cm) packed and eluted with solvent system E. Appropriately pooled fractions were evaporated and dried with the aid of a lyophilizer to obtain a dark yellow powder (186 mg, 65% yield): mp 164–166 °C; R_f 0.29 (silica gel, system E), 0.61 (silica gel, system A); IR (KBr) 3400, 1650, 1610 cm^{-1}. Anal. ($C_{24}H_{27}N_9O_7 \cdot 0.6NH_3 \cdot H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-benzoyl-L-ornithine (10). A solution of **14**·TFA (303 mg, 0.45 mmol), benzoic anhydride (153 mg, 0.675 mmol), and Et_3N (136 mg, 1.35 mmol) in *N*-methylpyrrolidinone (25 mL) was kept at 25 °C for 90 min and worked up as in the preparation of **9** except that solvent system F was used to elute the silica gel column: yield 143 mg (55%); mp 176–177 °C; R_f 0.20 (silica gel, system F), 0.44 (silica gel, system D); IR (KBr) 3350, 1640, 1610 cm^{-1}. Anal. ($C_{27}H_{27}N_9O_5 \cdot H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-(4-chlorobenzoyl)-L-ornithine (11). Under the conditions of the preceding experiment, acylation of **14**·TFA with 4-chlorobenzoic anhydride gave a 37% yield of **11** as a yellow powder, sintering from 170 °C: R_f 0.23 (silica gel, system F); IR (KBr) 3350, 1640, 1610 cm^{-1}. Anal. ($C_{27}H_{26}ClN_9O_5 \cdot 0.2CHCl_3 \cdot 1.33H_2O$) C, H, Cl, N.

3,4-Dichlorobenzoic Anhydride (16). A solution of 3,4-dichlorobenzoyl chloride (2.09 g, 0.01 mol) in Et_2O (15 mL) was added dropwise with stirring to a solution of 3,4-dichlorobenzoic acid (1.91 g, 0.01 mol) and pyridine (1 mL) in Et_2O (50 mL) at 5 °C. After being stirred at 25 °C for 2 h, the reaction mixture was filtered. The filtrate was evaporated to dryness, and the residual solid was triturated with hot benzene (30 mL). The benzene was decanted from a heavy insoluble oily residue and was left to stand. The product crystallized in the form of needles, which were collected and dried over P_2O_5 at 50 °C: yield 2.76 g (76%); mp 147.5–149.5 °C; IR (KBr) 1790, 1725 cm^{-1} (anhydride C=O). Anal. ($C_{14}H_6Cl_4O_3$) C, H, Cl.

N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-(3,4-dichlorobenzoyl)-L-ornithine (12). A solution of **14**·TFA (303 mg, 0.45 mmol) in dry DMF (10 mL) was treated with Et_3N (136 mg, 1.36 mmol) followed consecutively by *N*-methylpyrrolidinone (30 mL) and 3,4-dichlorobenzoic anhydride (246 mg, 0.675 mmol). The resulting solution was kept at 25 °C for 4 h, and the reaction mixture was worked up as in the synthesis of **10** except that the product was chromatographed on a silica gel column (27 × 2.0 cm), which was packed and eluted with solvent system H. Fractions containing a single TLC spot (R_f 0.27, silica gel, system H) were pooled and evaporated to obtain the analytical sample as a yellow powder (57 mg), mp 171 °C. Impure column fractions were purified further by preparative TLC (system H) to obtain another 21 mg of product: total 78 mg (25% yield); IR (KBr) 3400, 1645, 1610 cm^{-1}. Anal. ($C_{27}H_{25}Cl_2N_9O_5 \cdot 2H_2O \cdot 0.8CH_3OH$) C, H, N.

N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (13). Under the conditions of the synthesis of **10**, acylation of **14**·TFA with phthalic anhydride and chromatography on a silica gel column (26 × 2.0 cm) with system E as the eluent gave a product that was still not TLC-homogeneous and had to be further purified on a DEAE-cellulose column (28 × 2.0 cm) with 3% NH_4CO_3 as the eluent. The pure product weighed 116 mg (40% yield): mp 182 °C; R_f 0.58 (silica gel, system B); IR (KBr) 3400, 1645, 1610 cm^{-1}. Anal. ($C_{28}H_{27}N_9O_7 \cdot 2.5H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-phthaloyl-L-ornithine (19). To a suspension of **18** (1.19 g, 4 mmol) in CH_2Cl_2 (25 mL) were consecutively added Et_3N (0.89 g, 8.8 mmol) and Me_3SiCl (0.96 g, 8.8 mmol). The mixture was stirred at 25 °C for 18 h and evaporated to dryness under reduced pressure, and the residue was redissolved in DMF (30 mL) at the reflux temperature. The DMF solution (solution A) was kept warm throughout the operations described below.

i-BuOCOCl (273 mg, 2 mmol) was added to a suspension of **17**·1.5H_2O (0.732 g, 2 mmol) in dry *N*-methylpyrrolidinone (35 mL) containing Et_3N (0.809 g, 8 mmol), and after 15 min of stirring at ambient temperature, half of solution A was added. After 10 min, a second portion of *i*-BuOCOCl (137 mg, 1 mmol) was added, followed 20 min later by one-fourth of solution A. After another 10 min, a third portion of *i*-BuOCOCl (68 mg, 0.5 mmol) was added, followed 15 min later by one-eighth of solution A. The last sequence was repeated with *i*-BuOCOCl (68 mg, 0.5 mmol) and the final portion of solution A. After 1 h, MeOH was added, and all volatile materials were removed under reduced pressure. The residue was triturated with Et_2O (150 mL), and the insoluble material was taken up in solvent system E and applied onto a silica gel column (29 × 3.0 cm), which was packed and eluted with system F. Fractions containing a major spot at R_f 0.41 (silica gel, system D), along with other lesser spots, were pooled and rechromatographed on a second column (36 × 3.0 cm), which was eluted with solvent system H. Evaporation of pooled fractions containing a single spot at R_f 0.32 (silica gel, system D) yielded starting material (155 mg, 21% recovery). Pooled fractions containing a single spot at R_f 0.41 were evaporated and redissolved in a small volume of MeOH, from which a portion of the product crystallized on standing. The remainder of the product was reprecipitated by adding the mother liquor to Et_2O: total yield 494 mg (40%); mp 235 °C dec; IR (KBr) 3430, 1710 (imide C=O), 1670, 1645 cm^{-1}. Anal. ($C_{28}H_{25}N_9O_6 \cdot 2H_2O$) C, H, N.

N^{α}-Acetyl-N^{δ}-(4-amino-4-deoxypteroyl)-L-ornithine (2). A solution of **8** (167 mg, 0.32 mmol) in 0.25 N NaOH (10 mL) was kept at 25 °C for 5.5 h, acidified to pH 4.5 with 10% AcOH, cooled to 0 °C, and filtered. The solid was washed with H_2O and dried, first with the aid of a lyophilizer and then in vacuo overnight at 100 °C over P_2O_5, to obtain a yellow powder (134 mg, 85% yield): mp 197–200 °C; R_f 0.21 (silica gel, system D). Anal. ($C_{21}H_{25}N_9O_4 \cdot 0.5H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemisuccinoyl-L-ornithine (3). A solution of **9** (186 mg, 0.292 mmol) in 0.25 N NaOH (9.3 mL) was kept at 25 °C for 7 h and acidified with 10% AcOH to form a gel. Ammonia was added to redissolve the gel, and the solution was applied onto a DEAE-cellulose column (23 × 1.5 cm), which was eluted first with H_2O to remove salts and then with 3% NH_4HCO_3. Fractions giving a single TLC spot (R_f 0.28, silica gel, system E) were pooled, evaporated, and dried in a lypohilizer and in vacuo over P_2O_5 at 100 °C overnight to obtain a yellow powder (113 mg, 68% yield): mp 181 °C; IR (KBr) 3350, 1650, 1610 cm^{-1}. Anal. ($C_{23}H_{27}N_9O_6 \cdot 2.4H_2O$) C, N; H: calcd, 5.63; found 5.16.

N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-benzoyl-L-ornithine (4). A solution of **10** (120 mg, 0.208 mmol) in 0.25 N NaOH was kept at 25 °C for 7 h and acidified to pH 4 with 10% AcOH. Water (40 mL) was added to the resultant gel, and the mixture was stirred for 50 h, cooled to 0 °C, and filtered. The dried solid weighed 103 mg (88% yield): mp 175–180 °C; R_f 0.75 (cellulose, system G); IR (KBr) 3400, 1645, 1635, 1610 cm^{-1}. Anal. ($C_{26}H_{27}N_9O_4 \cdot 1.75H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-(4-chlorobenzoyl)-L-ornithine (5). A solution of **11** (80 mg, 0.12 mmol) in 0.25 N NaOH (3.4 mL) was kept at 25 °C for 2 h, when another 2 mL of 0.25 N NaOH was added to dilute the gel that initially formed. After a total of 5 h, water (10 mL) was added, followed by acidification to pH 4 with 10% AcOH and the usual workup: yield 62 mg (85%); mp 180 °C dec; R_f 0.51 (silica gel, system E). Anal. ($C_{26}H_{26}ClN_9O_4 \cdot 2H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-(3,4-dichlorobenzoyl)-L-ornithine (6). A solution of **12** (62 mg, 0.1 mmol) in 0.25 N NaOH (5 mL) was kept at 25 °C for 5 h, another 1 mL of 0.25 N NaOH was added to redissolve the initially formed gel, and after a total of 7 h, water (4 mL) was added, followed by acidification to pH 4 with 10% AcOH. The mixture was stirred overnight, filtered, and worked up as usual to obtain a yellow powder (41 mg, 61% crude yield). This material was subjected to further purification by preparative HPLC with 0.1 M NH_4OAc, pH 6, containing 19% MeCN, as the eluent. Fractions corresponding to the main peak were pooled, evaporated under reduced pressure, redissolved in a small volume of H_2O, and reevaporated to constant weight: yield 17 mg (23%); mp 255 °C dec; R_f 0.67 (silica gel, system E); IR (KBr) 3400, 1645, 1630, 1610 cm^{-1}. Anal. ($C_{26}H_{25}Cl_2N_9O_4 \cdot 0.8CH_3COOH \cdot 3.5H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (7). Method A. A solution of **13** (152 mg, 0.235 mmol) in 0.25 N NaOH (7.5 mL) was kept at 25 °C for 9 days with TLC monitoring (cellulose, system G). When the spot corresponding to starting material (R_f 0.68, blue fluorescent) disappeared, the solution was acidified to pH 4 with 10% AcOH, evaporated to dryness, redissolved in 3% NH_4HCO_3, and applied onto a DEAE-cellulose column (23 × 1.5 cm), which was eluted first with distilled H_2O to remove salts and then with 3% NH_4HCO_3. TLC-homogeneous fractions were combined, evaporated by lyophilization, and dried in vacuo over P_2O_5 at 100 °C to obtain a yellow powder (44 mg, 29% yield): mp 208 °C dec; R_f 0.30 (silica gel, system E); IR (KBr) 3400, 1650, 1610 cm^{-1}.

Method B. A solution of **19** (469 mg, 0.757 mmol) in 0.25 N NaOH was kept at 25 °C for 6.5 h, and the pH was adjusted to 4.3 with 10% AcOH. The gel was stirred overnight, separated by centrifugation, redissolved in system E, and applied onto a silica gel column (26 × 2.0 cm), which was then eluted with system D. Pooled TLC-homogeneous fractions were evaporated to dryness, and the residue was crystallized from EtOH, 122 mg (26% yield). Anal. ($C_{27}H_{27}N_9O_6 \cdot H_2O$) C, H, N.

The bioassay sample was obtained from a 40-mg portion of the product purified further by preparative HPLC with 0.1 M NHOAc, pH 7.8, containing 8% MeCN as the eluent.

Dihydrofolate Reductase Inhibition. The spectrophotometric assay procedure described earlier[28] was used to determine the IC_{50} of the *N*-acyl derivatives as inhibitors of DHFR purified by MTX affinity chromatography from L1210/R81 cells. The results are given in Table I.

Folylpolyglutamate Synthetase Inhibition. Compounds **2–4** and **10** were tested as inhibitors of partially purified FPGS from mouse liver as described earlier.[13]

Thymidylate Synthase Inhibition. Compounds **4** and **7** were tested as inhibitors of purified enzyme from TS-overproducing L1210 cells by the method of Roberts.[29] The cells were generously provided by Drs. Ann Jackman and Hilary Calvert, Institute of Cancer Research Sutton, Surrey, U.K. Purification of the enzyme was accomplished by affinity chromatography as described by Rode and co-workers.[30]

Cell-Growth Inhibition. A cytotoxicity assay based on a published method[31] that uses a color reaction to measure the number of viable cells was used to obtain IC_{50} values for the compounds listed in Table I. The culture conditions for the L1210 and L1210/R81 cells used in the assay were those described earlier.[9] Serial dilutions of each compound in RPMI 1640 medium containing 10% fetal bovine serum were added in 50-μL aliquots to individual wells of a 96-well tissue culture plate (Costar 3596), leaving one row for controls (no drug) and one row for blanks (no cells). The cells, in log phase growth, were diluted to 1.2×10^5/mL, and 50 μL, or 6×10^3 cells, was added to each well except in the blank row. After 48 h of incubation in an 8% CO_2 humidified atmosphere, 10 μL of a 5 mg/mL solution of the tetrazolium salt MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide], dissolved in 10 mM sodium phosphate buffer/150 mM NaCl, pH 7.4 and sterile-filtered, was added to each well. During the ensuing 3-h incubation, the mitochondrial dehydrogenases in the viable cells cleaved the tetrazolium ring, resulting in formation of dark blue formazan crystals. The crystals were dissolved by adding 100 μL of 0.04 N HCl in *i*-PrOH to each well, with thorough mixing as needed. The optical density of each well was then measured at 570 nm on an ELISA plate reader. The plates were read within 1 h following addition of the HCl and *i*-PrOH.

Assays of growth inhibition against cultured CEM and CEM/MTX cells were carried as described earlier.[9] Drug exposure was for 48 h. It should be noted that we have consistently found the activity of antifolates to be lower against CEM cells than against L1210 cells. This is presumably due to the fact that different culture conditions are used in the two assays, and the possibility that the cell lines have different folate requirements for growth. In the assays reported here, the L1210 and L1210/R81 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), while the CEM and CEM/MTX cells were cultured in Dulbecco's modified Eagle's medium (DME) containing 10% FBS.

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