stirred for 18 h with a mixture of 80 mL of 0.1 N NaOH and 25 mL of acetonitrile. The pH of the hydrolysate was adjusted to 7.5 with 1 N HCl, concentrated by rotary evaporation to about 25 mL, cooled to ~10 °C, and acidified with glacial HOAc to pH 3.5 and the resultant yellow precipitate was filtered, washed, and dried. HPLC analysis revealed that the precipitate was a mixture of unreacted pteroic acid (7) and the desired product 4. The crude product was dissolved in 15 mL of 5% NH_4OH and evaporated under reduced pressure to a residue which was dissolved in 10 mL of distilled water and applied on a column made of 15 g of C_{18} silica gel that was equilibrated with 10% acetonitrile in water. The product was eluted with 10% acetonitrile in water, the fractions containing the fast moving band on the column were pooled, and the pH was adjusted to 3.5 to precipitate product 4. The light yellow precipitate was separated by filtration, washed with distilled water, and dried: yield 180 mg (40%); mp 291-295 °C dec; UV (0.1 N NaOH) λ_{max} 254 nm (ϵ = 30 704), 369 (ϵ = 6859); NMR (TFA) δ 2.95 (c, 6 H, CH_2), 4.82 (c, 1 H, CH), 5.74, 6.3 (d, 2 H, olefinic), 7.02, 7.39 (q, 4 H, aromatic), 8.37 (s, 1 H, C^7H); MS m/z 452 (MH^+) (calcd for $C_{21}H_{21}N_7O_5$ 451). Anal. C, H, N.

4-Amino-4-deoxy-10-ethyl-10-deazapteroyl-γ-methyleneglutamic Acid (5). The pteroic acid analogue 8 was prepared according to the procedure of Nair.[19] The mixed anhydride 11 was prepared exactly as described for 10 with 338 mg (1 mmol) of 8. After coupling of 11 with a neutralized solution of 19, the reaction product 5 was isolated and purified as described above for 4: yield 215 mg (45%); mp 253-257 °C dec; UV (0.1 N NaOH) λ_{max} 253 nm (ϵ = 31 806), 370 (ϵ = 7491); MS (FAB) m/z 480 (MH^+) (calcd for $C_{23}H_{25}N_7O_5$ 479). Anal. C, H, N.

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Synthesis and Biological Activity of the 2-Desamino and 2-Desamino-2-methyl Analogues of Aminopterin and Methotrexate[1]

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The previously undescribed 2-desamino and 2-desamino-2-methyl analogues of aminopterin (AMT) and methotrexate (MTX) were synthesized from 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile. The AMT analogues were obtained via a three-step sequence consisting of condensation with di-*tert*-butyl *N*-(4-aminobenzoyl)-L-glutamate, heating with formamidine or acetamidine acetate, and mild acidolysis with trifluoroacetic acid. The MTX analogues were prepared similarly, except that 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile was condensed with 4-(*N*-methylamino)benzoic acid and the resulting product was annulated with formamidine or acetamidine acetate to obtain the 2-desamino and 2-desamino-2-methyl analogues, respectively, of 4-amino-4-deoxy-N^{10}-methylpteroic acid. Condenstion with di-*tert*-butyl L-glutamate in the presence of diethyl phosphorocyanidate followed by ester cleavage with trifluoroacetic acid was then carried out. Retention of the L configuration in the glutamate moiety during this synthesis was demonstrated by rapid and essentially complete hydrolysis with carboxypeptidase G_1 under conditions that likewise cleaved the L enantiomer of MTX but left the D enantiomer unaffected. The 2-desamino and 2-desamino-2-methyl analogues of AMT and MTX inhibited the growth of tumor cells, but were very poor inhibitors of dihydrofolate reductase (DHFR). These unexpected results suggested that activity in intact cells was due to metabolism of the 2-desamino compounds to polyglutamates.

The 2-amino group in the classical folic acid analogues methotrexate (MTX, 1) and aminopterin (AMT, 2) is generally assumed to be essential for biological activity; indeed, among the hundreds of AMT and MTX analogues synthesized and tested over the past 40 years, virtually all have retained the fused 2,4-diaminopyrimidine moiety, which is considered responsible for the tight binding of these molecules to the target enzyme dihydrofolate reductase (DHFR).[2] Analogues with the 2-amino group replaced by a 2-(dimethylamino)[3] or 2-methylthio[4] group were shown to have very little antifolate activity, supporting the concept that 2,4-diamino substitution is necessary for tight binding to DHFR. More direct evidence has been provided by X-ray crystallographic studies,[5-7] which reveal that the 2-amino group is hydrogen bonded to a highly conserved aspartic or glutamic acid residue in the active site of DHFR, as well as to a water molecule hydrogen bonded to a threonine residue. Replacement of the aspartic acid residue with serine by site-directed mutagenesis has been shown to produce a 3000-fold increase in the K_D for MTX binding as measured by equilibrium dialysis.[8,9] This substantial change corresponds to a decrease of 4.4 kcal mol^{-1} in the binding energy. In another study,[10] replacement of the threonine by valine, whose side

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(1) Paper 42 in this series; for previous paper, see: Rosowsky, A.; Forsch, R. A.; Moran, R. G. *Pteridines*, in press.
(2) For general reviews of strategies used in the design of classical antifolates, see: (a) Piper, J. R.; Montgomery, J. A. In *Folate Antagonists as Therapeutic Agents*; Sirotnak, F. M., Burchall, J. J., Ensminger, W. D., Montgomery, J. A. Eds.; Academic Press: Orlando, 1984; pp 219-260. (b) Rosowsky, A. *Progr. Med. Chem.* **1989**, *26*, 1.
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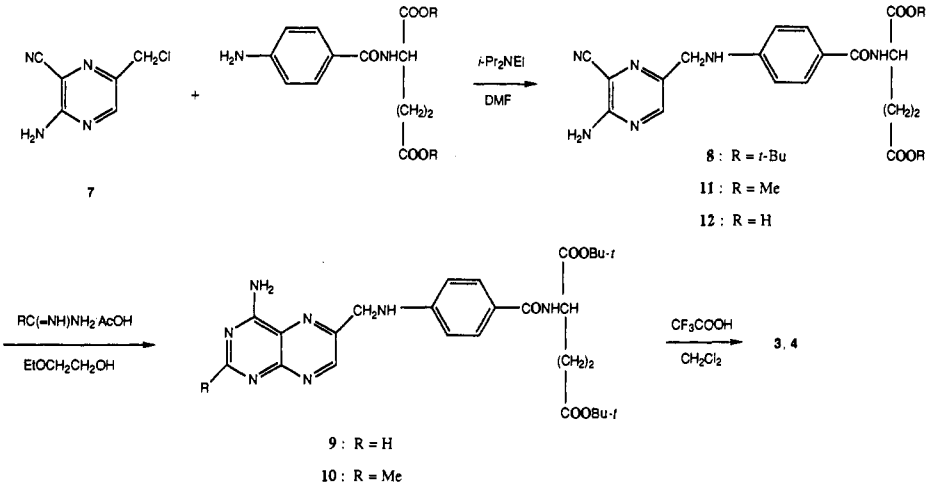
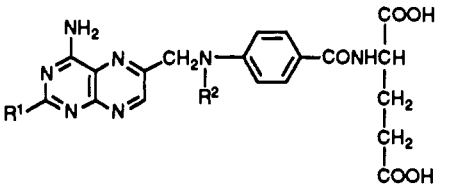
Scheme I



chain cannot participate in hydrogen bonding, was found to produce a 25-fold decrease in the K_D for MTX, corresponding to a free energy difference of almost 2 kcal mol^{-1}. A further important role for the 2-amino group is to provide resonance stabilization of the positive charge when N^1 becomes protonated during binding.[11] Thus, the 2-amino group participates in ionic as well as hydrogen bonding to the active site of DHFR, and its deletion would ordinarily be regarded as an unlikely avenue to new antifolates targeted against this enzyme.

Our interest in the effect of 2-amino group deletion on DHFR binding evolved from our discovery,[12] subsequently confirmed by others,[13] that N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine, a structural analogue of AMT with the side-chain CH_2CH_2COOH group replaced by $CH_2CH_2CH_2NH_2$, inhibits not only DHFR but also folylpolyglutamate synthetase (FPGS), an enzyme thought to be essential for the growth and survival of cells[14] and a potential target for chemotherapy.[15] As part of a broader program of design and synthesis of folate analogues, we were intrigued by the possibility that removal of the 2-amino group from AMT and MTX might result in loss of DHFR inhibition activity without loss of FPGS substrate activity. Accordingly, we undertook to synthesize 2-desaminoaminopterin (**3**), 2-desamino-2-methylaminopterin (**4**), 2-desaminomethotrexate (**5**), and 2-desamino-2-methylmethotrexate (**6**). The synthesis and biological activity of **3** and **4** have been already reported in an abstract[16a] and brief communication.[16b] The present paper gives a detailed account of the synthesis of these AMT analogues, as well as of the MTX analogues **5** and **6**. All four compounds were poor DHFR inhibitors in comparison with **1** and **2**. However, even though the MTX analogues (**5**, **6**) were less potent than the AMT analogues (**3**, **4**) as inhibitors of cell growth, inhibition of cell growth was greater in every instance than could be explained on the basis of DHFR inhibition data alone. While the exact biochemical target of these compounds remains to be determined, growth inhibition by these desamino compounds is likely to involve intracellular metabolism by FPGS.[16b]



1: $R^1 = NH_2$, $R^2 = Me$
2: $R^1 = NH_2$, $R^2 = H$
3: $R^1 = R^2 = H$
4: $R^1 = Me$, $R^2 = H$
5: $R^1 = H$, $R^2 = Me$
6: $R^1 = R^2 = Me$

Chemistry

Our synthesis of the N^{10}-unsubstituted analogues **3** and **4**, outlined in Scheme I, is an adaptation of the Taylor route to 6-substituted 2,4-diaminopteridines.[17] 2-Amino-5-(chloromethyl)-3-cyanopyrazine (**7**)[18,19] was condensed with 1 molar equiv of di-*tert*-butyl *N*-(4-aminobenzoyl)-L-glutamate[20] in DMF solution in the presence of *i*-Pr_2NEt to obtain a mixture of the *N*-alkyl and *N*,*N*-dialkyl derivatives. After separation of this mixture by column chromatography on neutral alumina, pure **8** was isolated in 59% yield. Ring closure of the amino nitrile **8** was then achieved in 59% yield by treatment with a 4-fold molar excess of formamidine acetate[21] in refluxing 2-ethoxyethanol. Acidolysis of the resulting diester **9** proceeded smoothly at room temperature in 2.5 h in a 2:1 mixture of CH_2Cl_2 and CF_3COOH, giving **3** ("2-desamino-AMT") as a monohydrate. The UV absorption spectrum of **3** [λ_{max} (pH 7.4) 246, 285, 333 nm; λ_{max} (0.1

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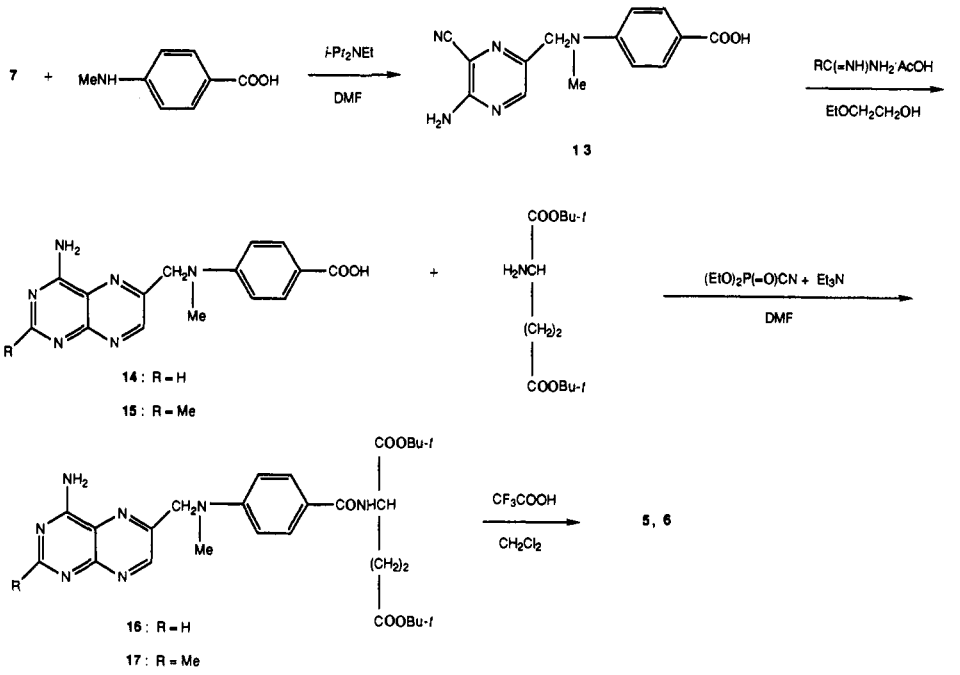
Scheme II



N HCl) 293 nm] exhibited the expected hypsochromic shift for a 4-aminopteridine in comparison with a 2,4-diaminopteridine.[22] The NMR spectrum, in D_2O containing enough K_2CO_3 to dissolve the sample, contained two downfield singlets at δ 8.85 and 8.33 corresponding to hydrogens at position 7 and 2, respectively. Replacement of formamidine acetate with acetamidine acetate[21] in the foregoing sequence afforded the diester **10** (54% yield), which on treatment with 2:1 CH_2Cl_2–CF_3COOH was converted to **4** (88% yield). The UV spectrum of **4** [λ_{max} (pH 7.4) 247, 285, 337 nm; λ_{max} (0.1 N HCl) 219, 293 nm] was consistent with its assigned structure as a 4-amino-2-methylpteridine. The NMR spectrum of the diester **10**, in $CDCl_3$ solution, showed the 2-methyl group as a singlet at δ 2.68.

Two alternative routes to **3** were initially explored and abandoned because they were less satisfactory than the one described above. In one approach, **7** was condensed with dimethyl *N*-(4-aminobenzoyl)-L-glutamate[23] instead of with the di-*tert*-butyl ester. The expected dimethyl ester **11** was obtained in 52% yield, but unfortunately when **11** was allowed to react with formamidine acetate in 2-ethoxyethanol a mixture of transesterified products was formed which made purification difficult. Furthermore, when the crude product mixture was treated with 0.1 N NaOH to hydrolyze the ester groups, there was a change in UV spectral absorption indicating probable cleavage of the pyrimidine ring. While ester cleavage could be effected more satisfactorily with $Ba(OH)_2$, the yield of **3** was still only 33%. An attempt was also made to carry out the formamidine acetate reaction with diacid **12**, which could be obtained either from **7** and *N*-(4-aminobenzoyl)-L-glutamic acid (31% yield) or from **8** by acidolysis with 2:1 CH_2Cl_2–CF_3COOH. While it appeared that **3** was formed from **12** in small amount, there was again a large number of byproducts which made purification difficult. It was clear from these experiments that annulation would best carried out with the carboxyl groups blocked, and that the best blocking group would be a *tert*-butyl ester, which would be more stable than a methyl ester to transesterification and would be removable at the end with acid as opposed to alkali.

We had anticipated that removal of the electron-donating group from the 2-position would decrease the stability of the pteridine ring not only to alkaline but also to acid conditions. This was borne out by the finding that, in contrast to our previous experience with di-*tert*-butyl esters of analogues containing an intact 2,4-diaminopteridine ring,[24] treatment of **8** with neat CF_3COOH resulted in extensive decomposition. Fortunately, when the reaction was conducted in a 2:1 mixture of CH_2Cl_2 and CF_3COOH, the 4-aminopyrimidine moiety remained intact and the *tert*-butyl esters were cleaved in high yield.

The synthesis of the N^{10}-methyl analogues **5** and **6** is depicted in Scheme II, and differs from the synthesis of **3** and **4** in that the pyrazine **7** was used to alkylate 4-(*N*-methylamino)benzoic acid instead of di-*tert*-butyl *N*-(4-aminobenzoyl)-L-glutamate. Two useful features of this approach are that (a) the amino acid side chain could be introduced without protection of N^{10} with a formyl or other base-labile group, and (b) the alkylation of 4-(*N*-methylamino)benzoic acid by **7**, unlike that of di-*tert*-butyl *N*-(4-aminobenzoyl)-L-glutamate, was not accompanied by dialkylation. The amino nitrile **13**, obtained in 66% yield, was found to react efficiently with formamidine and acetamidine acetate without protection of the COOH group; in fact, these reactions were much more efficient than those of the N^{10}-unsubstituted analogue **8**, giving the 2-desamino and 2-desamino-2-methyl analogues **14** and **15** in yields of 85–90%. Condensation of **14** and **15** with di-*tert*-butyl L-glutamate in the presence of diethyl phosphorocyanidate afforded the diesters **16** and **17** (65–70% yield), and removal of the ester groups with 2:1 CH_2Cl_2–CF_3COOH, as in the synthesis of **3** and **4** discussed above, gave the diacids **5** and **6** (60–65% yield). The diacids were purified by two-stage ion-exchange chromatography, first on Dowex 50W-X2(H^+), which allowed removal of CF_3COOH by washing with water and 3% NH_4OH, and then on [(diethylamino)ethyl]cellulose with 3% NH_4HCO_3 as the

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eluent. The UV absorption spectra of **5** [λ_{max} (pH 7.4) 219, 246, 303, 345 (inflection) nm; λ_{max} (0.1 N HCl) 223, 305, 343 (inflection) nm] and **6** [λ_{max} (pH 7.4) 218, 247, 303, 345 (inflection) nm; λ_{max} (0.1 N HCl) 222, 305 (inflection), 345 (inflection) nm] were similar, and also showed a consistent trend relative to the spectra of **3** and **4**, in that a new short-wavelength band was present and that the long-wavelength bands underwent substantial bathchromic displacement.

Critically important to the successful purification of **5** and **6** on [(diethylamino)ethyl]cellulose was that the fractions were freeze-dried as soon as possible after collection. When the 3% NH_4HCO_3 eluates were left to stand overnight in the refrigerator prior to freeze-drying, extensive decomposition occurred. Similar base-labile properties were observed for the AMT analogues **3** and **4**; when solutions of these compounds in 0.1 N NaOH were examined by HPLC, rapid appearance of new peaks indicating a chemical reaction was observed. Solutions in pH 7.4 phosphate buffer, on the other hand, were stable at room temperature for at least 24 h, and only after some time ($t_{1/2} \approx 3$ days) began to show UV changes suggestive of pyrimidine ring cleavage. This slow ring opening at physiologic pH could prove to be a useful property, since it might minimize long-term accumulation of these compounds (or their presumably more toxic polyglutamates) in liver and kidney.

A potential problem that was considered in connection with Scheme I was whether the use of formamidine or acetamidine at the relatively high temperature of refluxing 2-ethoxyethanol might cause racemization of the esterified amino acid side chain, resulting in a DL mixture whose biological activity would be compromised. This problem has been recognized previously when annulation is effected with guanidine in refluxing ethanol,[25] and the enantiomeric purity of MTX is known to contribute to antitumor activity.[26,27] Evidence that compounds **3** and **4** were, in fact, enantiomerically pure was obtained by treatment with the enzyme carboxypeptidase G_1,[28] which cleaves L-MTX to 4-amino-4-deoxy-N^{10}-methylpteroic acid under conditions that leave D-MTX unaffected.[27] Brief incubation of the 2-desamino compounds with carboxypeptidase G_1 as previously described[29] and analysis of the course of reaction by HPLC showed rapid disappearance of all but a few percent of the starting material, as did incubation of commercial MTX. By contrast, D-MTX generated previously by treatment of commercial MTX with the enzyme remained unchanged. We conclude from these results that our desamino analogues are essentially pure L enantiomers. Use of carboxypeptidase G_1 appears to be a convenient method for the determination of enantiomeric purity in synthetic antifolates containing a glutamate side chain. The fact that annulation with formamidine and acetamidine acetate in refluxing 2-ethoxyethanol did not cause significant racemization validated our choice of the synthetic plan in Scheme I.

Biological Activity

Compounds **2–5** were tested as inhibitors of purified DFHR from human leukemic lymphoblasts (WI-L2/M4 cells).[30] As shown in Table I, replacement of the 2-amino group in AMT led to a >1000-fold increase in IC_{50} from 0.025 to 19 μM, whereas the same structure modification in MTX led to a smaller, but still considerable, 250-fold increase from 0.024 to 6 μM. Replacement of hydrogen by methyl resulted in a still greater loss of inhibitory activity. These results suggested that there is little bulk tolerance at position 2 and provided direct experimental validation of the prediction based on X-ray crystallographic[5-7] and mutagenesis studies[8,9] that the 2-amino group in classical 2,4-diamino antifolates is a major contributor to the binding of these compounds to the enzyme active site.

The fact that removal of the 2-amino group had a smaller effect on the interaction of MTX than AMT with DHFR was interesting in view of our previous findings that replacement of the L-glutamate moiety by L-homocysteic acid[31] or long-chain amino dicarboxylic acids containing six to ten CH_2 groups[32] likewise resulted in a smaller change in the binding of MTX than AMT. It appears that very subtle distinctions exist between the binding of AMT and MTX analogues to DHFR. Although these distinctions can probably be traced ultimately to the effect of N^{10}-methyl substitution on the orientation of the phenyl and pteridine rings relative to the C^9–N^{10} bond, the nature of this effect is not entirely clear. Moreover, differences between pairs of AMT and MTX analogues are not necessarily observed in every instance; for example, a greater change in DHFR binding occurs in MTX than in AMT when glutamic acid is replaced with 2-amino-4-phosphonobutyric acid.[33] It is also important to note that changes of substituents at the 2-position of the pteridine ring in antifolates do not necessarily affect enzyme interaction to the same degree. Thus, a <10-fold decrease in binding to TS has been observed on replacement of the 2-amino group in N^{10}-propargyl-5,8-dideazafolate (PDDF) by hydrogen or a methyl group,[34,35] and relatively small changes have been noted in the interaction of the 2-desamino analogues of other 2-aminoquinazolin-4(3*H*)-one antifolates with both thymidylate synthase (TS)[36] and DHFR.[37] In contrast, our findings are that replacement of the 2-amino group by hydrogen or methyl in AMT and MTX elicit a very substantial decrease in DHFR interaction. A marked effect on ligand interaction with another pivotal enzyme of the folate pathway, glycinamide ribotide formyltransferase (GAR TFase), has likewise been noted on replacement of the 2-amino group by methyl in 5,8-dideaza-5,6,7,8-tetrahydrofolate (DDATHF) (R. L. Kisliuk, personal communication). It appears from these findings that a 2-amino group contributes more to the energy of binding of 4-oxo compounds to GAR TFase, and of 4-amino compounds to DHFR, than it does to the energy

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Table I. Dihydrofolate Reductase Inhibition and Cell Growth Inhibition by 2-Desamino Analogues of Aminopterin and Methotrexate

compd	DHFR inhibn:[a,c] IC_{50}, μM	growth inhibition (IC_{50}, μM)[b,c] L1210 cells	WI-L2 cells
MTX (1)	0.024	0.009	0.013
AMT (2)	0.025	0.002	0.007
3	19	0.082	0.081
4	>50	0.042	0.028
5	6	0.36	0.23
6	>20	0.22	0.33

[a] Activity was measured spectrophotometrically at 340 nm as previously described,[33] using purified enzyme from human lymphoblasts (WI-L2/M4). Data are reported for a DHFR concentration of 0.05 μM as measured by MTX titration, and are the means of triplicate experiments. [b] Assays were performed as previously described, using the MTT method:[47] cells were counted after 48 h of drug treatment. [c] IC_{50} values for MTX, 3, and 4 were reported previously,[16b] and are included for comparison.

of binding of 4-oxo compounds to TS or DHFR. Crystallographic and other biophysical studies using 2-desamino analogues as ligands to various enzymes of the folate pathway would be of interest.

While the low potency of compounds 3–6 as DHFR inhibitors was expected to be accompanied by correspondingly low activity against cells in culture, it was surprising to find that, in fact, 3 and 4 inhibited the growth of murine (L1210) and human (WI-L2) leukemic cells with IC_{50} values in the 0.01–0.1 μM range (Table I). Although compounds 5 and 6 were somewhat less potent, with IC_{50} values only in the 0.2–0.4 μM range, they too were much more active than would be anticipated from their weak interaction with purified DHFR. The greatest disparity between cell growth inhibition and enzyme inhibition was observed with 4, for which the ratio IC_{50}(cell growth inhibition)/IC_{50}(enzyme inhibition) was >1000:1. In the least favorable case (6), this ratio was still 26:1. It was evident that the 2-desamino analogues were all more potent than would be anticipated from the DHFR data alone, and that additional work was needed to establish the basis of this unusual biological activity.

Since it was possible that cell growth inhibition was unrelated to antifolate activity, protection experiments were performed with 3 and 4. As reported earlier,[16b] when L1210 or WI-L2 cells were incubated for 48 h with a range of concentrations of these,compounds in the prsence of a combination of 10 μM thymidine (dThd) and 100 μM hypoxanthine (Hx) they were fully protected (IC_{50} > 100 μM). Similar protection was afforded to cells treated with MTX, and only partial protection was afforded by 10 μM dThd alone or 100 μM Hx alone. Since abrogation of the toxicity of the desamino compounds required that the cells be provided with both dThd and Hx in the medium, their mode of action appears to resemble that of MTX in that it involves interference with the biosynthesis of both pyrimidine and purine precursors of DNA, rather than interference with some biochemical pathway not related to folate metabolism.

The question of how the 2-desamino analogues of AMT and MTX inhibit cell growth is still undetermined. From what is known about the action of MTX,[38] whose effect on L1210 and WI-L2 cell growth was completely abrogated only by a combination of dThd and Hx,[16b] inhibition of purine nucleotide synthesis by the 2-desamino compounds could occur at the level of GAR TFase, whereas inhibition of thymidylate (dTMP) synthesis could occur either indirectly as a result of DHFR inhibition (tetrahydrofolate depletion) or directly as a result of TS inhibition. The importance of TS and GAR TFase as biochemical targets in antifolate chemotherapy has come to be widely appreciated as a result of extensive work on the TS inhibitor PDDF[39] and the GAR TFase inhibitors DDATHF[40] and 5-deaza-5,6,7,8-tetrahydrofolate.[41] Compounds 3 and 4 were tested as inhibitors of purified TS as well as purified GAR TFase at concentrations as high as 100 μM, but were inactive (results not shown); inhibition of these enzymes by MTX is likewise weak in comparison with PDDF and DDATHF.[39,40] However, it is known that polyglutamates of MTX are much more active than the monoglutamate as inhibitors of both TS[42] and GAR TFase.[43] Moreover, replacement of the 2-amino group by hydrogen or methyl in PDDF is well tolerated in terms of FPGS substrate activity.[44] Since 3 and 4 are likewise good FPGS substrates, with kinetic constants similar to those of AMT,[16b] it is very possible that TS, GAR TFase, and perhaps other folate-requiring enzymes become inhibited when polyglutamate metabolites of these compounds are formed inside the cell. Thus, since non-polyglutamylated MTX is already a potent drug by itself, the desamino compounds may more truly represent FPGS-activated "prodrugs" than does MTX.[45]

In summary, we have synthesized analogues of the well-known anticancer drugs aminopterin and methotrexate in which the 2-amino group is replaced by nonpolar hydrogen and methyl substituents. These analogues represent a novel structural modification designed to abrogate DHFR binding while retaining good FPGS substrate activity. We are currently synthesizing the di- through pentaglutamates of compound 4 with a view to determining how these putative active metabolites interact with key enzymes of the folate pathway, including DHFR, TS, and GAR TFase. The synthesis of radiolabeled 4 is also planned, so as to allow transport and polyglutamylation studies to be performed in intact cells. It is hoped that these studies will lead to a clearer understanding of how this compound, and its congeners, act at the cellular level.

Experimental Section

IR spectra were recorded on a Perkin-Elmer Model 781 double-beam spectrophotometer (only peaks with wave numbers greater than 1250 cm^{-1} are listed). UV spectra were obtained on a Cary Model 210 instrument and ^1H NMR spectra on Varian Model T60 and EM360 instruments with Me_4Si or MeSi-$(CH_2)_3SO_3Na$ as the reference. TLC was performed on 250-μm silica gel plates (Analabs, North Haven, CT) or silica gel sheets (Eastman 13181) containing a fluorescent indicator. Spots were visualized under ordinary laboratory light or with 254-nm ul-

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traviolet illumination in a viewing box. Unless otherwise specified, column chromatography was carried out on Baker 3405 silica gel (60–200 mesh) or Whatman DE-52 preswollen [(diethylamino)-ethyl]cellulose (DEAE-cellulose). HPLC was performed on a Waters radial compression cartridge (C_{18} silica gel, 5-μm particle size, 0.5 × 10 cm) connected to a Waters Model 400 instrument equipped with a Model 490 programmable multiwavelength detector and Model 660 programmable solvent gradient system. Retention times are given in all instances for an eluent flow rate of 1.0 mL/min. Melting points were taken in Pyrex capillary tubes in a Mel-Tamp apparatus (Cambridge Laboratory Devices, Inc.) and are not corrected. Starting materials were purchased from Aldrich, Milwaukee, WI, or synthesized according to the literature as indicated. Carboxypeptidase G_1[28] and clinical grade MTX were generously provided by the National Cancer Institute, Bethesda, MD. DMF was dried over Davison 4A molecular sieves (Fisher, Boston, MA). Elemental analyses were performed by MultiChem Laboratories, Lowell, MA, or Robertson Laboratory, Inc., Madison, NJ, and the values reported are within ±0.4% of theoretical value unless otherwise specified.

Di-*tert*-butyl *N*-[4-[[(2-Amino-3-cyanopyrazin-5-yl)-methyl]amino]benzoyl]-L-glutamate (8). 2-Amino-5-(chloromethyl)pyrazine-3-carbonitrile (7) (1.68 g, 0.01 mol)[18] was added in small portions over 10 min to a stirred solution of di-*tert*-butyl *N*-(4-aminobenzoyl)-L-glutamate (3.78 g, 0.01 mol)[20] and *i*-Pr_2NEt (1.74 mL, 1.29 g, 0.01 mol) in dry DMF (25 mL). After 20 h at room temperature, the solvent was evaporated under reduced pressure and the residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was evaporated and the product purified by column chromatography on a column of neutral Al_2O_3 (120 g, 3 × 24 cm) with 50:1 $CHCl_3$–MeOH as the eluent. Fractions containing 8 (R_f 0.4, Al_2O_3, 50:1 $CHCl_3$–MeOH) were allowed to evaporate passively in the hood until yellow crystals were formed: yield 3.60 g (59%); mp 71–78 °C; IR (KBr) ν 3400, 3210, 2990, 2940, 2230 (C≡N), 1730 (ester C=O), 1635, 1615, 1575, 1515, 1495 sh, 1460, 1420, 1395, 1375, 1335, 1315, 1285, 1260 cm^{-1}; NMR ($CDCl_3$) δ 1.41 (s, γ-*t*-OBu), 1.48 (s, α-*t*-OBu), 2.0–2.4 (m, CH_2CH_2), 4.37 (m, 2 H, CH_2N), 5.35 (m, α-CH and NH), 6.61 (d, *J* = 9 Hz, 3′- and 5′-H), 7.67 (d, 9 Hz, 2′- and 6′-H), 8.22 (s, 6-H). Anal. ($C_{26}H_{34}N_6O_5 \cdot 0.8CHCl_3$) C, H, N.

Di-*tert*-butyl *N*-[4-[[(4-Aminopteridin-6-yl)methyl]-amino]benzoyl]-L-glutamate (9). A mixture of 8 (2.55 g, 0.005 mol), formamidine acetate (Aldrich, 2.08 g, 0.02 mol), and 2-ethoxyethanol (30 mL) was refluxed for 45 min, the solvent was evaporated under reduced pressure, and the dark residue was partitioned between $CHCl_3$ and H_2O. The emulsion was allowed to settle, and the residue after evaporation of the $CHCl_3$ layer was passed through a silica gel column (70 g, 3 × 35 cm), which was eluted first with 19:1 $CHCl_3$–MeOH to sequentially remove a brown impurity and yellow impurity, and then with 15:1 $CHCl_3$–MeOH to obtain the product. TLC-homogeneous fractions (R_f 0.5, Analabs silica gel plates, 9:1 $CHCl_3$–MeOH) were combined, care being taken to exclude a colored impurity immediately following the product, and the solvent was evaporated to obtain 9 as a tan powder (1.33 g, 58% yield); mp 103–110 °C, with sintering at lower temperature; IR (KBr) ν 3370, 2980, 2940, 1730 (ester C=O), 1635, 1610, 1565, 1555, 1515, 1455, 1420, 1395, 1370, 1350, 1310, 1265 cm^{-1}; UV λ_{max} (95% EtOH) 248 nm (ε 18 200), 289 (23 000), 338 (7390); NMR ($CDCl_3$) δ 1.40 (s, γ-*t*-OBu), 1.47 (s, α-*t*-OBu), 2.32 (m, CH_2CH_2), 4.70 (m, CH_2N), 5.58 (m, α-CH), 6.60 (d, *J* = 8 Hz, 3′- and 5′-H), 6.8–7.3 (m, NH_2 and CONH), 7.67 (d, *J* = 8 Hz, 2′- and 6′-H), 8.73 (s, 2-H), 9.05 (s, 7-H). Anal. ($C_{27}H_{35}N_7O_5 \cdot 0.25H_2O$) C, H, N.

Di-*tert*-butyl *N*-[4-[[(4-Amino-2-methylpteridin-6-yl)-methyl]amino]benzoyl]-L-glutamate (10). A mixture of 8 (2.55 g, 0.005 mol), acetamidine acetate (Aldrich, 2.95 g, 0.025 mol), and 2-ethoxyethanol (25 mL) was refluxed for 40 min, the solvent was evaporated, and the residue was partitioned between $CHCl_3$ and H_2O. The residue after evaporation of the organic layer was purified on a silica gel column (75 g, 3 × 35 cm), which was eluted with 19:1 $CHCl_3$–MeOH to obtain crude 10 as a glass (1.8 g). Recrystallization from MeCN afforded pure 10 as a yellow powder (two crops totaling 1.44 g, 60% yield); mp 105–110 °C; IR (KBr) 3380, 2980, 2940, 1730 (ester C=O), 1630 sh, 1610, 1570, 1550, 1515, 1455, 1420, 1395, 1370, 1355, 1335, 1305, 1280, 1260 cm^{-1}; UV λ_{max} (95% EtOH) 249 nm (ε 20 200), 290 (23 800), 341 (7370); NMR ($CDCl_3$) δ 1.42 (s, γ-*t*-OBu), 1.48 (s, α-*t*-OBu), 1.9–2.4 (m, CH_2CH_2), 2.68 (s, 2-Me), 4.72 (m, CH_2N and α-CH), 5.28 (m, NH), 6.65 (d, *J* = 9 Hz, 3′- and 5′-H, and overlapping s, NH_2), 7.72 (d, *J* = 9 Hz, 2′- and 6′- H), 9.23 (s, 7-H). Anal. ($C_{28}H_{37}N_7O_5 \cdot H_2O$) C, H, N.

Dimethyl *N*-[4-[[(2-Amino-3-cyanopyrazin-5-yl)methyl]-amino]benzoyl]-L-glutamate (11). A stirred solution of dimethyl *N*-(4-aminobenzoyl)-L-glutamate (3.23 g, 0.011 mol)[46] and *i*-Pr_2NEt (2.84 g, 3.84 mL, 0.022 mol) in dry DMF (50 mL) was treated in small portions over 15 min with amino nitrile 7 (1.69 g, 0.01 mol). After 18 h, the solvent was evaporated under reduced pressure and the residue partitioned between CH_2Cl_2 and H_2O. Evaporation of the organic layer followed by chromatography on a neutral Al_2O_3 column (120 g, 3 × 21 cm) with 50:1 $CHCl_3$–MeOH as the eluent afforded a yellow foam: mp 55–62 °C, R_f 0.3 (Al_2O_3, 50:1 $CHCl_3$–MeOH); IR (KBr) 3410, 3220, 3010, 2970, 2860, 2240 (C≡N), 1740 ester (C=O), 1635, 1615, 1580, 1520, 1490 sh, 1445, 1420, 1395, 1340, 1315, 1285, 1270 cm^{-1}; NMR ($CDCl_3$) δ 2.0–2.4 (m, CH_2CH_2), 3.67 (s, γ-OMe), 3.78 (s, α-OMe), 4.42 (m, CH_2NH, collapsing to CH_2N singlet on exchange with D_2O), 4.6–5.2 (m, α-CH and CONH), 5.43 (m, NH_2, exchangeable with D_2O), 6.65 (d, *J* = 9 Hz, 3′- and 5′-H), 7.73 (d, *J* = 9 Hz, 2′- and 6′-H), 8.32 (s, 6-H). Anal. ($C_{20}H_{22}N_6O_5 \cdot 0.2CHCl_3$) C, H, N.

***N*-[4-[[(2-Amino-3-cyanopyrazin-5-yl)methyl]amino]-benzoyl]-L-glutamic Acid (12). A.** Amino nitrile 7 (0.084 g, 0.5 mmol) was added in small portions over 5 min to a stirred solution of *N*-(4-aminobenzoyl)-L-glutamic acid (0.16 g, 0.6 mmol)[47] and *i*-Pr_2NEt (0.232 g, 0.314 mL, 1.8 mmol) in dry DMF (5 mL). After 20 min, the solvent was evaporated under reduced pressure and the residue applied onto a DEAE-cellulose column (1.5 × 25 cm, HCO_3^- form) which was eluted first with a large volume of H_2O to remove salts and then with 0.2 M NH_4HCO_3. Fractions containing the product along with some remaining impurities were rechromatographed. Lyophilization of appropriately pooled eluates afforded 12 as a yellow solid (0.062 g, 31% yield) identical with the material described in procedure B.

B. Diester 8 (2.35 g, 4.61 mmol) was dissolved in cold CF_3COOH (10 mL) and after 5 min was evaporated under reduced pressure. The residue was washed several times with Et_2O and then dissolved in H_2O. The solution was adjusted to pH 6–7 with NH_4OH and applied onto a column of Dowex 50W-X2 (2 × 16 cm, H^+ form) which was washed first with a large volume of water until all the CF_3COOH was removed and then with 3% NH_4OH. Freeze-drying of pooled eluates afforded a yellow solid (1.75 g, 96% yield). The analytical sample was obtained by rechromatographing a portion (120 mg) of this material on a DEAE-cellulose column (1.5 × 23 cm, HCO_3^- form) with 0.2 M NH_4HCO_3 as the eluent. Several colorless impurities eluted ahead of the product, and the latter was obtained, as a partial ammonium salt, by freeze-drying of TLC-homogeneous yellow fractions: yield 69 mg (55% yield); mp 130–140 °C (shrinking above 110 °C); IR (KBr) ν 3410, 3220, 2970, 2600 sh, 2330 (C≡N), 1720, 1635, 1610, 1575, 1545, 1520, 1455, 1400, 1340, 1265 cm^{-1}; NMR (D_2O + K_2CO_3) δ 2.30 (m, CH_2CH_2), 4.28 (m, CH_2NH), 6.67 (d, *J* = 9 Hz, 3′- and 5′-H), 7.68 (d, *J* = 9 Hz, 2′- and 6′-H), 8.22 (s, 6-H). Anal. ($C_{18}H_{18}N_6O_5 \cdot 0.5NH_3 \cdot 1.25H_2O$) C, H, N.

4-[[(2-Amino-3-cyanopyrazin-5-yl)methyl]methyl-amino]benzoic Acid (13). Compound 7 (3.38 g, 0.02 mol) was added in small portions over 5 min to a stirred solution of 4-(*N*-methylamino)benzoic acid (3.02 g, 0.02 mol) and *i*-Pr_2NEt (6.96 mL, 5.16 g, 0.04 mol) in dry DMF (40 mL). After 1.5 h at room temperature, the solution was concentration to ca. 15 mL under reduced pressure and was added dropwise with stirring to H_2O (200 mL). After addition of 1 M HCl (2 mL, 0.02 mol), the mixture was stirred for 5 min, and the solid was collected and added to H_2O (100 mL) along with enough concentrated NH_4OH to make the solution strongly alkaline. Undissolved material was filtered off, the filtrate was acidified with 10% AcOH, and the precipitate was collected and dried in vacuo at 90 °C over P_2O_5 to obtain 13 as a light-yellow powder (3.72 g, 66%). The product was of sufficient purity to be used directly in the next step, but an

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analytical sample was obtained by applying portion of this material (0.2 g) on a DEAE-cellulose column (1.5 × 25 cm, HCO_3^- form), washing with a large volume of H_2O to remove salts, and eluting the product with 0.2 M NH_4HCO_3. Fractions homogeneous by HPLC (10% MeCN in 0.1 M NH_4OAc, pH 7.5, retention time 14.1 min) were pooled and freeze-dried, and the residue was taken up in dilute NH_4OH, reprecipitated with 10% AcOH, and dried as above; dec >250 °C; IR (KBr) ν 3400, 3200, 2930, 2840, 2670, 2570, 2245 (C≡N), 1680, 1615, 1570, 1535, 1495, 1435, 1390, 1335, 1305, 1280 cm^{-1}; NMR (d_6-DMSO) δ 3.03 (s, Me), 4.51 (s, CH_2), 6.67 (d, J = 9 Hz, 3′- and 5′-H), 7.15 (s, NH_2), 7.67 (d, J = 9 Hz, 2′- and 6′-H), 8.10 (s, 6-H). Anal. ($C_{14}H_{13}N_5O_2 \cdot 0.15\ H_2O$) C, H, N.

4-[[(4-Aminopteridin-6-yl)methyl]methylamino]benzoic Acid (14). A mixture of **13** (1.73 g, 6.05 mmol) and formamidine acetate (3.18 g, 30.6 mmol) in 2-ethoxyethanol (30 mL) was placed in an oil bath preheated to 135 °C. The mixture became homogeneous after a few minutes, but gradually a precipitate appeared. After being heated for 1 h, the mixture was concentrated to ca. 10 mL under reduced pressure and diluted with H_2O (50 mL). The solid was collected, taken up in dilute NH_4OH, reprecipitated with 10% AcOH, filtered, and dried on a lyophilizer to obtain **14** as a light-yellow powder (1.71 g, 89%): dec >250 °C; HPLC 10% MeCN in 0.1 M NH_4OAc, pH 7.5, retention time 10.2 min; IR (KBr) ν 3460, 2940 sh, 2670 sh, 1675 sh, 1640, 1615, 1595 sh, 1570, 1535, 1495, 1430, 1405 sh, 1395, 1370, 1335, 1305, 1270, 1250 sh cm^{-1}; NMR (d_6-DMSO) δ 3.27 (s, Me), 4.95 (s, CH_2), 6.83 (d, J = 9 Hz, 3′- and 5′-H), 7.75 (s, J = 9 Hz, 2′- and 6′-H), 7.8–8.2 (broad m, NH_2), 8.52 (s, 2-H), 8.93 (s, 7-H). Anal. ($C_{15}H_{14}N_6O_2 \cdot 0.5H_2O$) C, H, N.

4-[[(4-Amino-2-methylpteridin-6-yl)methyl]methylamino]benzoic Acid (15). Reaction of **13** (1.42 g, 5 mmol) with acetamidine acetate (2.95 g, 25 mmol) in 2-ethoxyethanol (25 mL) exactly as in the synthesis of **14** gave **15** as a light-yellow powder (1.46 g, 86%): dec >250 °C; IR (KBr) ν 3450, 3220 sh, 2950 sh, 2650, 2520 sh, 1920–1850 broad, 1670 sh, 1635, 1610, 1585, 1570, 1530, 1485, 1450 sh, 1420, 1390, 1360, 1340 sh, 1325, 1300, 1285, 1255 cm^{-1}; NMR (d_6-DMSO) δ 2.45 (s, 2-Me), 3.23 (s, N^{10}-Me, overlapping another s, H_2O), 4.88 (s, CH_2), 6.78 (d, J = 9 Hz, 3′- and 5′-H), 7.68 (d, J = 9 Hz, 2′- and 6′-H, overlapping a broad m, NH_2), 8.83 (s, 7-H). Anal. ($C_{16}H_{16}N_6O_2 \cdot H_2O$) C, H, N.

Di-*tert*-butyl *N*-[4-[[(4-Aminopteridin-6-yl)methyl]methylamino]benzoyl]-L-glutamate (16). Compound **14** (160 mg, 0.5 mmol) was added in portions over 3 min to a solution of diethyl phosphorocyanidate (98 mg, 0.6 mmol) and Et_3N (278 μL, 202 mg, 2 mmol) in dry DMF (10 mL). After 20 min at room temperature, di-*tert*-butyl L-glutamate hydrochloride (177 mg, 0.6 mmol) was added in a single portion and the reaction was monitored by TLC (silica gel, 19:1 $CHCl_3$–MeOH) to follow the disappearance of activated intermediate (R_f 0.4) and formation of product (R_f 0.5). When reaction was complete, the solution was evaporated under reduced pressure and the residue partitioned between $CHCl_3$ and dilute NH_4OH. The $CHCl_3$ layer was evaporated and the residue purified by chromatography on a silica gel column (15 g, 1.5 × 23 cm) with 19:1 $CHCl_3$–MeOH as the eluent. Pooled pure fractions were concentrated and transferred to a vial with the aid of $CHCl_3$. The solvent was evaporated under a stream of air and the residue dried in vacuo at 60 °C to obtain **16** as a hardened orange foam (181 mg, 66%): mp 88–93 °C; IR (KBr) ν 3440, 3230 sh, 2990, 2940, 1735, 1635, 1615, 1565 sh, 1560, 1555 sh, 1515, 1455, 1425, 1390, 1375, 1355 sh, 1310, 1260 cm^{-1}; NMR ($CDCl_3$) δ 1.42 (s, γ-*t*-OBu), 1.48 (s, α-*t*-OBu), 1.8–2.4 (m, CH_2CH_2), 3.23 (s, N^{10}-Me), 4.60 (broad m, 1 H, α-CH), 4.87 (s, CH_2N), 6.73 (d, J = 9 Hz, 3′- and 5′-H, overlapping a broad m, NH_2), 7.73 (d, J = 9 Hz, 2′- and 6′-H), 8.75 (s, 2-H), 8.93 (s, 7-H). Anal. ($C_{28}H_{37}N_7O_5 \cdot 0.75H_2O$) C, H, N.

Di-*tert*-butyl *N*-[4-[[(4-Amino-2-methylpteridin-6-yl)methyl]methylamino]benzoyl]-L-glutamate (17). Compound **15** (171 mg, 0.5 mmol) was treated with diethyl phosphorocyanidate (163 mg, 1 mmol) and Et_3N (278 μL, 202 mg, 2 mmol) as described in the preceding experiment, and di-*tert*-butyl L-glutamate (296 mg, 0.5 mmol) was added after 1 h. TLC (silica gel (19:1 $CHCl_3$–MeOH) was used to monitor for the formation of product (R_f 0.6) and consumption of activated intermediate (R_f 0.5). An extra 100-μL portion of Et_3N had to be added to drive the reaction to completion. After a workup identical with the one for **16**, diester **17** was obtained as a hardened orange foam (200 mg, 69%): mp 91–97 °C; IR (KBr) ν 3440, 3240 sh, 2990, 2940, 1735 (ester C=O), 1635, 1615, 1570, 1515, 1455, 1420, 1395, 1375, 1340, 1300, 1260 cm^{-1}; NMR ($CDCl_3$) δ 1.40 (s, γ-*t*-OBu), 1.47 (s, α-*t*-OBu), 2.1–2.4 (m, CH_2CH_2), 2.67 (s, 2-Me), 3.22 (s, N^{10}-Me), 4.60 (broad m, α-CH), 4.85 (s, CH_2N), 6.73 (d, J = 9 Hz, 3′- and 5′-H, overlapping a broad m, NH_2), 7.72 (d, J = 9 Hz, 2′- and 6′-H), 8.88 (s, 7-H). Anal. ($C_{29}H_{39}N_7O_5 \cdot 0.75H_2O$) C, H, N.

***N*-[4-[[(4-Aminopteridin-6-yl)methyl]amino]benzoyl]-L-glutamic Acid ("2-DesaminoAMT", 3).** **A.** A solution of **9** (1.23 g, 2.27 mmol) in 2:1 CH_2Cl_2–CF_3COOH (15 mL) was left to stand at room temperature for 2.5 h, and the progress of acidolysis was followed by TLC and HPLC. When no more diester (TLC: R_f 0.5, silica gel, 9:1 $CHCl_3$–MeOH) or intermediate monoester (HPLC: 20% MeCN in 0.1 M NH_4OAc, pH 7.5, retention time 11.0 min) could be detected, the reaction mixture was poured into a separatory funnel containing 5% NH_4OH (100 mL) and $CHCl_3$ (50 mL). After some time to allow partitioning, the aqueous layer was reduced in volume under reduced pressure and the pH adjusted to 5 with 10% AcOH. A very fine precipitate formed and was collected by centrifugation, washed with a small volume of cold H_2O, and redissolved in 5% NH_4OH. Reacidification to pH 5, followed again by centrifugation and finally drying of the solid on a lyophilizer afforded **3** as a dark yellow powder (0.4 g, 40% yield) [Note: part of product appeared to be lost in the supernatants and wash solution, which all had a some yellow color]: mp >300 °C; HPLC 5% MeCN in 0.1 M NH_4OAc, pH 7.0, retention time 12.2 min; IR (KBr) ν 3450, 2930, 1715, 1635, 1610, 1565, 1520, 1455, 1390, 1355, 1285, 1265 cm^{-1}; UV: λ_{max} (pH 7.4) 246 nm (ϵ 19 200), 285 (21 200), 333 (7240), λ_{max} (0.1 N NaOH) 246 nm (ϵ 19 400), 285 (21 200), 333 (7270), λ_{max} (0.1 N HCl) 293 (ϵ 16 700); NMR (D_2O + K_2CO_3) δ 2.28 (m, CH_2CH_2), 4.47 (m, CH_2N and α-CH), 6.55 (d, J = 8 Hz, 3′- and 5′-H), 7.53 (d, J = 8 Hz, 2′- and 6′-H), 8.33 (s, 2-H), 8.85 (s, 7-H). Anal. ($C_{19}H_{19}N_7O_5 \cdot H_2O$) C, H, N.

B. A mixture of **11** (213 mg, 0.5 mmol), formamidine acetate (260 mg, 2.5 mmol), and 2-ethoxyethanol (5 mL) was refluxed for 30 min, after which the solvent was evaporated and the residue partitioned between $CHCl_3$ and H_2O. The organic layer was concentrated to dryness and the product chromatographed on a column of silica gel (13 g, 1.5 × 20 cm) with 19:1 $CHCl_3$–MeOH as the eluent. A relatively broad band was obtained, which on evaporation afforded 94 mg of a yellow solid; R_f 0.5 (silica gel, 9:1 $CHCl_3$–MeOH). In addition to signals at δ 3.65 (s, γ-OMe) and 3.75 (s, α-OMe), the NMR spectrum of this material contained a strong signal at δ 1.15 (t, J = 7 Hz) indicative of partial transesterification to α- and/or β-(2-ethoxyethyl) esters. In a large run using 3.84 g (1.63 mmol) of **7**, completely transesterification was observed. A mixture of the transesterified annulation product (543 mg, 0.95 mmol) and $Ba(OH)_2 \cdot 8H_2O$ (315 mg, 1 mmol) in 50% EtOH (20 mL) was stirred at room temperature overnight and then treated with Na_2SO_4 (142 mg, 1 mmol) in a small volume of H_2O. The $BaSO_4$ was filtered off and filtrate concentrated to a small volume and applied onto a DEAE-cellulose column (1.5 × 25 cm, HCO_3^- form) which was eluted first with H_2O to remove salts and then with 0.2 M NH_4HCO_3. Pooled pure fractions were freeze-dried, the residue was taken up in a small volume of dilute NH_4OH, and AcOH was added until precipitation occurred. The filtered yellow solid was dried with the aid of a lyophilizer; yield 134 mg (33%). The product was identical with that obtained by procedure A.

***N*-[4-[[(4-Amino-2-methylpteridin-6-yl)methyl]amino]benzoyl]-L-glutamic Acid ("2-Desamino-2-methylAMT", 4).** Acidolysis of diester **10** (1.27 g, 2.22 mol) was performed as described for **9** except that the product after acidification to pH 5 was much easier to collect due to its lower solubility: yield 0.9 g (88%); mp >300 °C; HPLC 10% EtOH in 0.1 M NH_4OAc, pH 7.5, retention time 10.2 min; IR (KBr)ν 3420, 3110, 2940, 2860, 1640, 1610, 1570, 1520, 1450, 1390, 1355, 1335, 1305, 1285, 1260 cm^{-1}; UV λ_{max} (pH 7.4) 247 (ϵ 22 100), 285 (22 700), 337 (7670), λ_{max} (0.1 N HCl) 219 nm (ϵ 20 900), 293 (19 700). Anal. ($C_{20}H_{21}N_7O_5 \cdot 1.5H_2O$) C, H, N.

***N*-[4-[[(4-Aminopteridin-6-yl)methyl]methylamino]benzoyl]-L-glutamic Acid ("2-DesaminoMTX", 5).** A solution of diester **16** (180 mg, 0.319 mmol) in 2:1 CH_2Cl_2–CF_3COOH (3 mL) was allowed to stand at room temperature for 3 h. The

dark-purple solution was then poured into a separatory funnel containing $CHCl_3$ (20 mL) and 5% NH_4OH (20 mL). After partitioning, the aqueous layer was concentrated to ca. 10 mL, and the solution was applied onto a Dowex 50W-X2 column (2 × 25 cm, H^+ form) which was eluted first with a large volume of H_2O and then with 3% NH_4OH. Collected fractions were freeze-dried to a solid which was purified further on a DEAE-cellulose column (1.5 × 25 cm, HCO_3^- form) with 0.2 M NH_4HCO_3 as the eluent. HPLC-pure fractions were pooled and *promptly* freeze-dried to obtain **5** as a light-yellow solid (92 mg, 61%); dec >300 °C; HPLC 10% MeCN in 0.1 M NH_4OAc, pH 7.0, retention time 5.7 min; IR (KBr) ν 3450, 2990 sh, 1710 sh, 1645, 1620, 1570, 1525, 1465, 1395, 1370 sh, 1315, 1270 cm^{-1}; NMR (d_6-DMSO) δ 1.8–2.4 (m, CH_2CH_2), 3.20 (s, N^{10}-Me), 4.90 (s, CH_2N, overlapping another s, H_2O), 6.78 (d, J = 9 Hz, 3′- and 5′-H), 7.65 (d, J = 9 Hz, 2′- and 6′-H), 7.8–8.2 (broad m, NH_2), 8.47 (s, 2-H), 8.88 (s, 7-H); UV λ_{max} (pH 7.4) 219 nm (ϵ 19700), 246 (19400), 303 (24000), 345 infl (6900), λ_{max} (0.1 N HCl) 223 nm (ϵ 21200), 305 (23600), 343 infl (9000). Anal. ($C_{20}H_{21}N_7O_5 \cdot 0.5NH_3 \cdot 1.5H_2O$) C, H, N.

***N*-[4-[[(4-Amino-2-methylpteridin-6-yl)methyl]methylamino]benzoyl]-L-glutamic Acid (6) ("2-Desamino-2-methylMTX").** Diester **17** (195 mg, 0.345 mmol) was hydrolyzed exactly as in the preceding experiment to obtain **6** as a light-yellow solid (109 mg, 65%); dec >300 °C; HPLC 10% MeCN in 0.1 M NH_4OAc, pH 7.0, retention time, 8.6 min; IR (KBr) ν 3420, 3220 sh, 2950 sh, 2600 br, 1910 br, 1700 br, 1635, 1610, 1570, 1560, 1520, 1455 sh, 1420 sh, 1395, 1345, 1305, 1255 cm^{-1}; NMR (d_6-DMSO) δ 1.8–2.4 (m, CH_2CH_2), 2.45 (s, 2-Me), 3.22 (s, N^{10}-Me), 4.90 (s, CH_2N, overlapping another s, H_2O), 6.80 (d, J = 8 Hz, 3′- and 5′-H), 7.68 (d, J = 8 Hz, 2′- and 6′-H), 7.7–8.1 (broad m, NH_2), 8.87 (s, 7-H); UV λ_{max} (pH 7.4) 218 nm (ϵ 20300), 247 (21300), 303 (24200), 345 infl (7100) λ_{max} (0.1 N HCl) 222 nm (ϵ 21300), 305 (23400), 345 infl (8900). Anal. ($C_{21}H_{23}N_7O_5 \cdot 0.5NH_3 \cdot 2H_2O$) C, H, N.

Hydrolysis with Carboxypeptidase G_1. Small samples (1 mg) of compounds **3** and **4** were treated at room temperature in 1 M NaOAc (10 mL) containing $ZnCl_2$ (10 mg) with freshly thawed enzyme solution (0.3 μL, 4500 units/mL). In less than 15 min, glutamate hydrolysis was nearly complete according to HPLC analysis (20% MeCN in 0.1 M NH_4OAc, pH 7.5), which showed the disappearance of >95% of the starting material (**3**, 2.5 min; **4**, 2.89 min) and the appearance of new peaks (3.0 and 3.3 min) assumed to be the 2-desamino and 2-desamino-2-methyl derivatives of 4-amino-4-deoxypteroic acid, respectively. Under identical conditions, clinical grade MTX (mainly the L form, 3.0 min) was cleaved to 4-amino-4-deoxy-N^{10}-methylpteroic acid (4.0 min), whereas D-MTX (3.0 min, preformed from clinical grade MTX by carboxypeptidase G_1 treatment)[27] was resistant to further treatment with the enzyme.

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Registry No. **3**, 118869-51-5; **4**, 118869-52-6; **5**, 129780-79-6; **6**, 129780-80-9; **7**, 40127-91-1; **8**, 118869-53-7; **9**, 118869-54-8; **10**, 118869-55-9; **11**, 118869-56-0; **12**, 118869-57-1; **13**, 43111-44-0; **14**, 129780-81-0; **15**, 129780-83-2; **16**, 129780-82-1; **17**, 129780-84-3; DHFR, 9002-03-3; 4-H_2NC_6HyCO-Glu(ONu-*t*)-OBu-*t*, 76282-66-1; 4-H_2NC_6HyCO-Hlu(OMe)-OMe, 52407-60-0; 4-H_2NC_6HyCO-Glu-OH, 4271-30-1; HN=$CHNH_2$·AcOH, 3473-63-0; HN=$CMeNH_2$·AcOH, 36896-17-0; 4-(MeNH)$C_6HyCOOH$, 10541-83-0; H-Glu(OBu-*t*)-OBu-*t*·HCl, 32677-01-3.

Nucleosides and Nucleotides. 94. Radical Deoxygenation of *tert*-Alcohols in 1-(2-*C*-Alkylpentofuranosyl)pyrimidines: Synthesis of (2′*S*)-2′-Deoxy-2′-*C*-methylcytidine, an Antileukemic Nucleoside[1]

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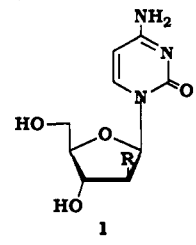
(2′*S* and 2′*R*)-2′-Deoxy-2′-*C*-methylcytidine (**1i** and **15**) and (2′*S*)-2′-deoxy-2′-*C*-ethylcytidine (**1j**) have been synthesized from the corresponding 2′-*C*-alkylarabinofuranosyl- or -ribofuranosylpyrimidine derivatives **3** and **4** by radical deoxygenation of the methyl oxalyl esters of the 2′-*tert*-alcohol, followed by sequential deblocking and amination at the 4-position. (2′*S*)-2′-Deoxy-2′-*C*-methyl-5-methyluridine (**8**) has also been synthesized in a similar manner. Among them, compound **1i** exhibits the most potent cytotoxicity to L1210 cells with potency comparable to that of 1-β-D-arabinofuranosylcytosine (**1a**). The size of the 2′-substituents and the configuration at the 2′-position are the most important for the cytotoxicity. Cytotoxicity in vitro of **1i** against various human cancer cell lines was also examined and compared with that of **1a** and 5-fluorouracil.

1-β-D-Arabinofuranosylcytosine (*ara*-C, **1a**, Chart I) is one of the most effective drugs for the treatment of human acute myeloblastic leukemia.[2-4] Its usefulness is, however, limited by several drawbacks: a short half-life time in plasma due in part to the deamination to inactive 1-β-D-arabinofuranosyluracil by the action of cytidine deaminase, development of drug resistance, and ineffectiveness to solid tumors.[5-7] Consequently, with the objective of overcoming these problems, efforts have been made to develop a large number of prodrugs[8] of *ara*-C or introduce certain other substituents into the 2′-*arabino* position in place of the hydrogen atom of 2′-deoxycytidine. As a latter approach, 2′-azido- and 2′-amino-2′-deoxy-β-D-arabinofuranosylcytosines (**1b,c**) were found to be resistant to cytidine deaminase and also potent inhibitors of mouse leukemic cells L1210 in vitro as well as in vivo.[9,10] Among a series of 2′-deoxy-2′-halo-β-D-arabinofuranosylcytosines (**1d**–

Chart I



1

R	
a	OH
b	N_3
c	NH_2
d	F
e	Cl
f	Br
g	I
h	SCH_3
i	CH_3
j	CH_2CH_3

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