85375-85-5; Et **26**·HCl, 85375-14-0; Et **26**, 89203-62-3; Et (*R*)-**26**·HCl, 95274-16-1; Et (*S*)-**26**·HCl, 95274-17-2; **2c**, 95274-01-4; **2c**·HCl, 95274-00-3; Et **2c**·HCl, 95312-96-2; **2d**, 95274-03-6; **2d**·HCl, 95274-02-5; Et **2d**, 95274-04-7; **2e**, 95274-05-8; **2e**·HCl, 85375-40-2; Et **2e**, 85375-39-9; Me **3a**, 495-19-2; **3b**, 85375-88-8; **3b**·HCl, 85375-17-3; Me **3b**, 95274-06-9; Me 4a·HCl, 85375-51-5; **4b**, 85375-91-3; **4b**·HCl, 85375-53-7; Me **4b**, 85375-52-6; **5b**, 95274-08-1; **5b**·HCl, 95274-07-0; Et **5b**·HCl, 95274-09-2; **6b**, 95274-11-6; **6b**·HCl, 95274-10-5; GABA, 56-12-2; methyl 1-benzyl-3-pyrrolidineacetate cyclohexylsulfamate, 95274-13-8; 1-benzyl-3-pyrrolidineacetonitrile, 55278-09-6; methyl 3-pyrrolidineacetate cyclohexylsulfamate, 95274-15-0; 4,4-diphenyl-3-butenyl bromide, 6078-95-1; 3,3-diphenyl-2-propenyl bromide, 4801-15-4; 5,5-diphenyl-4-pentenyl bromide, 85375-38-8; 1-bromo-4-methoxybutane, 4457-67-4; benzophenone, 119-61-9; 1,1-diphenyl-5-methoxy-1-pentanol, 85375-37-7; methyl *cis*-1-benzyl-4-hydroxy-3-piperidinecarboxylate, 85375-46-8; methyl 1-benzyl-4-oxo-3-piperidinecarboxylate, 57611-47-9; methyl *trans*-1-benzyl-4-hydroxy-3-piperidinecarboxylate, 85375-47-9; ethyl *cis*-3-aminocyclohexanecarboxylate, 62456-14-8; 4,4-diphenyl-3-butenyl azide, 95274-18-3; 4,4-diphenyl-3-butenylamine hydrochloride, 93007-57-9; 4,4-diphenyl-3-butenylamine, 93007-58-0; *N*-benzyl-4,4-diphenyl-3-butenylamine hydrochloride, 95274-19-4; ethyl 4-bromobutyrate, 2969-81-5; ethyl *N*-benzyl-*N*-(4,4-diphenyl-3-butenyl]-γ-aminobutyrate, 95274-20-7; ethyl *N*-cyano-*N*-(4,4-diphenyl-3-butenyl)-γ-aminobutyrate, 95274-21-8.

# Methotrexate Analogues. 25. Chemical and Biological Studies on the γ-*tert*-Butyl Esters of Methotrexate and Aminopterin

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γ-*tert*-Butylaminopterin (γ-tBAMT), the first example of an aminopterin (AMT) γ-monoester, was synthesized, and new routes to the known $N^{10}$-methyl analogue γ-*tert*-butyl methotrexate (γ-tBMTX) were developed. The inhibitory effects of γ-tBAMT on the activity of purified dihydrofolate reductase (DHFR) from L1210 murine leukemia cells, the growth of L1210 cells and CEM human leukemic lymphoblasts in suspension culture, and the growth of several lines of human squamous cell carcinoma of the head and neck in monolayer culture were compared with the effects of γ-tBMTX and the parent acids AMT and methotrexate (MTX). Patterns of cross-resistance to γ-tBAMT, γ-tBMTX, and AMT among several MTX-resistant cell lines were examined. In vivo antitumor activities of γ-tBAMT and γ-tBMTX were compared in mice with L1210 leukemia. While the activity of γ-tBAMT was very close to that of γ-tBMTX in the DHFR inhibition assay, the AMT ester was more potent than the MTX ester against cells in culture and against L1210 leukemia in vivo. Only partial cross-resistance was shown against γ-tBMTX and γ-tBAMT in cultured cells that were resistant to MTX by virtue of a transport defect or a combination of defective transport and elevated DHFR activity.

γ-*tert*-Butyl *N*-(4-amino-4-deoxy-$N^{10}$-methylpteroyl)-L-glutamate (γ-tBMTX, 1), a sterically hindered lipophilic ester of the anticancer drug methotrexate (MTX), was synthesized in our laboratory[1] with the aim of evaluating its activity against MTX-resistant tumors with a defect in their transport mechanism for MTX. Decreased MTX uptake has been observed in a variety of mammalian tumor cell lines with induced MTX resistance[2-9] and is thought to also contribute to resistance in the clinic.[10] Since γ-esterification removes one of the negative charges from the glutamate side chain, we speculated that this would promote uptake into cells by passive transport. Moreover, we considered that intracellular drug retention, which in the case of MTX involves enzymatic conversion to nonefluxing γ-polyglutamates,[11-15] might be achieved in 1 via hydrophobic interaction of the ester alkyl group with lipid-rich sites within the cell. With regard to binding to dihydrofolate reductase (DHFR), the target enzyme for MTX and other antifolates,[10] it is known that this enzyme has considerable tolerance for structural changes in the γ-terminal region of the glutamate moiety,[16,17] and we therefore assumed that once the ester crossed the cell membrane it would probably bind nearly as well as MTX

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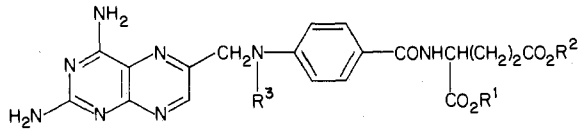
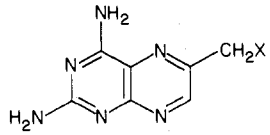
itself. These expectations were met when both the DHFR affinity and in vitro cytotoxicity of 1 proved to be comparable to those of MTX.[1] Recently we reported that MTX-resistant human squamous cell carcinoma (SCC) lines with a transport defect for MTX are only partly cross-resistant to 1 regardless of whether or not they also have elevated DHFR levels as a result of gene amplification.[9] These findings suggested that 1 or compounds of similar type could find therapeutic application in overcoming MTX resistance.

While the low cross-resistance of MTX and γ-tBMTX in vitro was encouraging, the potency of the ester against MTX-sensitive parental SCC cells was lower than desired. One possible way to overcome this problem might be to use an ester with a larger lipophilic group.[18] An alternative approach was to prepare the heretofore unknown analogue γ-*tert*-butyl *N*-(4-amino-4-deoxypteroyl)-L-glutamate (γ-tBAMT, **2**). The parent acid aminopterin (AMT) is known to be about 10 times more potent than MTX, and we had previously observed in at least one instance[19] that this difference persists in $N^{10}$-unsubstituted compounds even when polyglutamation is prevented by modification of the γ-position. In this paper we report the synthesis of **2** and also describe some improvements of our original method of preparation of 1. In addition, comparative data are presented for 1 and **2** with respect to DHFR binding, in vitro cytotoxicity, and in vivo antitumor activity.

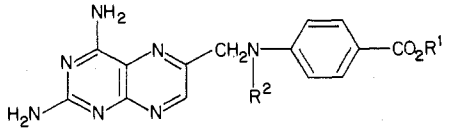


MTX, $R^1=R^2=H$; $R^3=Me$
AMT, $R^1=R^2=R^3=H$
1 (γ-tBMTX), $R^1=H$; $R^2=t$-Bu; $R^3=Me$
2 (γ-tBAMT), $R^1=R^3=H$; $R^2=t$-Bu

**Chemistry.** 2,4-Diamino-6-(hydroxymethyl)pteridine (**3**) was prepared in 75% yield from 2,4,5,6-tetraaminopyrimidine and 1,3-dihydroxyacetone in the presence of cysteine and $O_2$.[20] This process is superior to that of Baugh and Shaw[21] in that the product is uncontaminated by 2,4-diamino-6-methylpteridine or the 7-hydroxymethyl and 7-methyl compounds. Bromination of **3** with dibromotriphenylphosphorane in *N*,*N*-dimethylacetamide (DMA) at room temperature was performed essentially as described by Piper and Montgomery,[22] except that the isolation and purification of **4**·HBr was omitted. *p*-Aminobenzoic acid and BaO were added directly to the original reaction mixture, and after 24 h at 55 °C the crude product was isolated by pouring the mixture into $CH_2Cl_2$. Final traces of DMA and triphenylphosphine oxide were removed by washing with MeOH and the resulting 4-amino-4-deoxypteroic acid (**5**) was added directly to $HCO_2H$–AcOH. Dilute ammonia extraction removed the nonacidic byproducts, and pure **6** was recovered from the basic solution by adjusting the pH to 5.5 with 10% AcOH. Direct $N^{10}$-formylation of crude **5** as described here is very advantageous for large-scale work because it results in a product whose base solubility is greater than that of **5** and whose strong fluorescence makes it readily TLC identifiable.

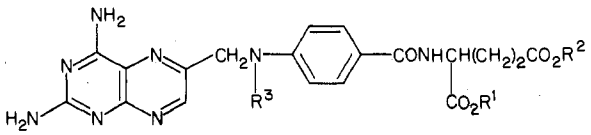


**3**, X = OH
**4**, X = Br



**5**, $R^1=R^2=H$
**6**, $R^1=H$; $R^2=CHO$
**9**, $R^1=C_6H_4NO_2$-*p*; $R^2=CHO$
**10**, $R^1=H$; $R^2=Me$
**18**, $R^1=Et$; $R^2=Me$

Carboxyl group activation in **6** was achieved with *i*-BuOCOCl and $Et_3N$ in DMF at room temperature, using the modified mixed anhydride technique we recently employed to prepare *N*-(4-amino-4-deoxypteroyl)-L-homocysteic acid.[19] After 15 min to allow anhydride formation, 1 equiv of α-methyl γ-*tert*-butyl L-glutamate hydrochloride[23] was added, and 1 min later another portion of *i*-BuOCOCl was added, followed after 15 min by an equivalent amount of amino diester. After two more cycles of sequential activation and amine addition, TLC showed that all of **6** had been consumed. An 82% yield of **7** was isolated after silica gel chromatography. A noteworthy difference between the process reported here and our earlier one[19] is that the rate of consumption of mixed anhydride to give **7** and **6** was so rapid that in situ reactivation could be performed in as little as 1 min. The entire four-cycle sequence required slightly over 1 h, with the rate-limiting step being the formation of the anhydride rather than its subsequent reaction with the amino ester.



**7**, $R^1=Me$; $R^2=t$-Bu; $R^3=CHO$
**8**, $R^1=H$; $R^2=t$-Bu; $R^3=CHO$
**14**, $R^1=R^3=Me$; $R^2=t$-Bu

Conversion of **7** to **2** required removal of the $N^{10}$-formyl and α-methyl groups. We originally had intended to accomplish this in one step with NaOH. However, trial experiments showed extensive AMT formation in the presence of excess NaOH, which was unexpected since this had not been observed with the $N^{10}$-methyl analogue 1.[1] In order to deprotect **7** under milder conditions, we elected to first saponify it with $Ba(OH)_2$. Treatment with 1.3 molar equiv of $Ba(OH)_2$ at room temperature for 18 h led to a 79% yield of **8**, with no cleavage of the $N^{10}$-formyl group. Conversion of **8** to **2** was then effected with 0.25 N NaOH at 25 °C. The reaction was terminated at 1.75 h, when all the **8** was consumed and only a small amount

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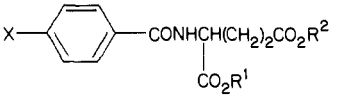
of AMT was detectable by TLC. Satisfactory purification of **2** was accomplished on a silica gel column with 5:4:1 $CHCl_3$–MeOH–concentrated $NH_4OH$ as the eluent. AMT remained firmly bound. Evaporation of pooled TLC-homogeneous eluates left a solid that gave a correct analysis for a partial ammonium salt of **2**. This material appeared to have a short shelf-life even at 4 °C and was therefore converted to a sodium salt that could be kept as a freeze-dried powder at −70 °C for at least 2 months without change. Unlike the $N^{10}$-unsubstituted monoester, the $N^{10}$-formyl derivative **8** was stable in the solid state even at room temperature. We are unable to explain the ease of ester cleavage in **2** in comparison with **8** and **1**. It is possible that $N^{10}$-substitution causes steric inhibition of resonance in the *p*-aminobenzoyl system, and that this diminishes the capacity for intramolecular attack of the γ-ester by the α-COOH group via a six-membered ring transition state. While we are unaware of any previous report of facile *γ-tert-butyl ester cleavage under nonacidic conditions* in N-substituted glutamic acids with a free α-COOH group, the fact that the α-amino group in this instance carries an *N*-aroyl as opposed to *N*-alkoxycarbonyl or *N*-(aryloxy)carbonyl substituent evidently makes **2** an atypical glutamic acid derivative in the context of peptide chemistry.

An alternative mode of activation of **6** was developed, which made it possible to use *γ-tert*-butyl L-glutamate without having to block the α-COOH group. Treatment of **6** with bis(*p*-nitrophenyl) carbonate in DMF at room temperature for 19 h gave a 94% yield of the *p*-nitrophenyl ester **9**. Reaction of **9** with *γ-tert*-butyl L-glutamate and 2 equiv of $Et_3N$ in DMA at 55 °C for 72 h afforded **8** in 74% yield. Similar use of a *p*-nitrophenyl ester has been made by others[24] to convert 4-amino-4-deoxy-$N^{10}$-methylpteroic acid (**10**) to MTX, with bis(*p*-nitrophenyl) sulfite being used as the activation reagent. Our combined two-step yield of **2** via this route was 70%.

In another route to **2** that did not require $N^{10}$-protection, the pteridine moiety was condensed with *γ-tert*-butyl *N*-(*p*-aminobenzoyl)-L-glutamate (**11**), which was prepared in four steps from α-methyl *γ-tert*-butyl L-glutamate hydrochloride.[1,23] Treatment of this diester with *p*-nitrobenzoyl chloride in $CH_2Cl_2$ containing 2 equiv of $Et_3N$ yielded the unknown *N*-(*p*-nitrobenzoyl) derivative **12** (100%) as an oil. Saponification followed by catalytic reduction afforded the crystalline nitro acid **13** (62%) and amino acid **11** (100%), respectively. Condensation of **11** with 2,4-diamino-6-(bromomethyl)pteridine hydrobromide (**4**·HBr)[22] in DMA containing 1 equiv of *i*-$Pr_2NEt$ (room temperature, 22 h) led to a mixture of products from which pure **2** could be isolated as the free acid. Purification was achieved by a modification of the method described above, which had given a partial ammonium salt. The crude coupling product was chromatographed initially on a conventional silica gel column with 5:4:1 $CHCl_3$–MeOH–concentrated $NH_4OH$ as the eluent. The monoester recovered from this column was taken up in aqueous ammonia, pH 10.9, and 10% AcOH was added to pH 5. The precipitated free acid was passed through a second silica gel column, this time in the flash chromatography mode with 14:6:1 $CHCl_3$–MeOH–concentrated $NH_4OH$ as the eluent. The final yield of very pure **2** via this method was 21%. Substantial cleavage of **2** to AMT occurred during chromatography, as evidenced by the fact that when **2**, which was AMT-free but still contained other minor impurities, was rechromatographed, the column became uniformly yellow (AMT formation) and retained its color even after all the ester had been eluted. Despite this low efficiency, silica gel chromatography was the best purification method we could find and was superior to ion-exchange chromatography (DEAE-cellulose, 3% $NH_4HCO_3$), which gave even more extensive degradation to AMT. Of the two routes to **2** described above, the mixed anhydride procedure appeared preferable, since it gave fewer byproducts and could be carried out without isolation of **4**·HBr.




**11**, X = $NH_2$; $R^1$ = H; $R^2$ = *t*-Bu
**12**, X = $NO_2$; $R^1$ = Me; $R^2$ = *t*-Bu
**13**, X = $NO_2$; $R^1$ = H; $R^2$ = *t*-Bu
**15**, X = NHMe; $R^1$ = H; $R^2$ = *t*-Bu
**16**, X = $N(COCF_3)Me$; $R^1$ = Me; $R^2$ = *t*-Bu
**17**, X = NHMe; $R^1$ = Me; $R^2$ = *t*-Bu

Improved routes to **1** were also sought in this work. A process for converting **3** to **10** without isolating **4**-HBr was developed. *p*-(Methylamino)benzoic acid and *i*-$Pr_2NEt$ were added directly to the flask in which the bromination of **3** had been carried out. After 25 h at 45 °C, the DMA solution was poured into a large volume of 0.33 N NaOH, and the insoluble products, including triphenylphosphine oxide and nonacidic pteridines, were removed by filtration. Acidification of the filtrate afforded analytically pure **10**·$2H_2O$ in 90% yield, based on **3**. Piper and Montgomery[22] reported a maximum combined yield of only 30% when **4**·HBr was isolated and purified prior to reaction with *p*-(methylamino)benzoic acid.

An improved process for coupling **10** and α-methyl *γ-tert*-butyl L-glutamate was also developed, which gave a higher yield of **14** than had been obtained earlier.[1] Both the molar proportions of **10** and diethyl phosphorocyanidate and the timing of addition of the amino ester were found to be important. The optimal amount of coupling reagent depends on the hydration state of the acid. Analyses showing 0.5–2.0 mol of $H_2O$ have been obtained for various batches of **10**, an it is therefore advisable to determine this value after every run. When diethyl phosphorocyanidate is in excess (i.e., if there is less $H_2O$ than expected) or the amine is added too early, a yellow byproduct is formed. We believe this product consists of the *N*-formamidino derivative **19** and/or **20** on the basis of microchemical and mass spectral analysis, a longer $\lambda_{max}$ in comparison with 2,4-diaminopteridines, and the presence in the NMR spectrum of a δ 6.7 singlet consistent with an *N*-formamidino proton (NHC*H*=NH). Further support for this structure was provided by the finding that **19**/**20**, upon saponification, yielded a monoester with one less nitrogen, presumably the *N*-formyl derivative **21** and/or **22**. The possibility of an isomer mixture was suggested by the presence, in the NMR spectrum of **19**/**20**, of two peaks at δ 8.23 and 8.36 consistent with two different $C_7$ protons. It is important to remove the *N*-formamidine(s) at the diester stage in order to have absolutely pure monoester after the next step. Optimal conditions for the coupling reaction were to use $x + 1$ equiv of diethyl phosphorocyanidate and $Et_3N$ per mole of **10**·$xH_2O$. After 4 h at room temperature to allow complete consumption of the diethyl phosphorocyanidate, 1 equiv of amino ester hydrochloride and 2 more equiv of $Et_3N$ were added, and the reaction was left at room temperature for 2 days. This afforded a 75% yield of extremely pure **14** after flash chromatography (silica gel, 5:5:1

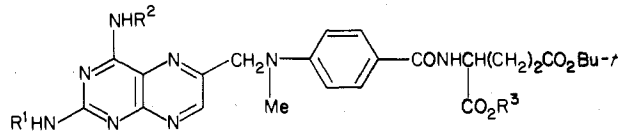
(24) Parry, D.; Moreau, J.; Bayle, M. *Eur. J. Med. Chem.-Chim. Ther.* **1975**, *10*, 147.

Table I. Activity of γ-*tert*-Butyl Methotrexate (1) and γ-*tert*-Butyl Aminopterin (2) against Methotrexate-Sensitive and Methotrexate-Resistant Human and Murine Cells in Culture

| | $IC_{50}$,[a] μM | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | human leukemic lymphoblasts | | human squamous cell carcinoma lines | | | | | | mouse leukemia cells | | |
| compd | CEM | CEM/MTX | SCC25 | SCC68 | SCC78 | SCC25/R1 | SCC68/R1 | SCC78/R1 | L1210 | L1210/R71 | L1210/R81 |
| 1 (γ-tBMTX) | 0.62 | 0.50 (0.81) | 0.40 | 0.37 | 0.48 | 0.78 (2.0) | 1.4 (3.8) | 1.4 (2.9) | 0.056 | 40 (710) | 25 (450) |
| 2 (γ-tBAMT) | 0.45 | 25 (56) | 0.066 | 0.19 | 0.080 | 1.8 (27) | 3.5 (18) | 0.43 (5.4) | 0.023 | 3.5 (150) | 6.5 (280) |
| MTX | 0.032 | 6.6 (210) | 0.014 | 0.032 | 0.013 | 0.15 (11) | 0.25 (7.8) | 0.071 (5.5) | 0.0020 | 19 (9500) | 220 (110000) |
| AMT | 0.0010 | 0.32 (320) | 0.0016 | 0.0037 | 0.0025 | 0.043 (27) | 0.29 (78) | 0.014 (5.6) | 0.0020 | 7.9 (4000) | 84 (42000) |

[a] Numbers in parentheses are normalized relative to the $IC_{50}$ for each compound against the appropriate parental cell.

$CHCl_3$–MeCN–MeOH). Upon treatment of **14** with $Ba(OH)_2$ in EtOH–$H_2O$, an 86% yield of **1** was obtained. The combined two-step yield of **1** starting from **10** was thus 65%, and the overall yield based on **3** was 54%.




**19**, $R^1$=CH=NH; $R^2$=H; $R^3$=Me
**20**, $R^1$=H; $R^2$=CH=NH; $R^3$=Me
**21**, $R^1$=CH=O; $R^2$=H; $R^3$=H
**22**, $R^1$=H; $R^2$=CH=O; $R^3$=H

As an alternative to the use of **10**, we also synthesized **1** from **3** and γ-*tert*-butyl *N*-[*p*-(methylamino)benzoyl]-L-glutamate (**15**), an unknown compound that was prepared in three steps from *p*-(methylamino)benzoic acid. *N*-Trifluoroacetylation followed by reaction with $SOCl_2$ and direct coupling to α-methyl γ-*tert*-butyl L-glutamate afforded the protected diester **16** in 84% overall yield. Simultaneous saponification and N-deprotection with NaOH gave **15** in quantitative yield. Addition of **15** and 2 equiv of *i*-$Pr_2NEt$ to a solution of **4** generated in situ gave **1** (54%). Similarly, coupling of ethyl *p*-aminobenzoate to the pteridine in the presence of BaO led to **18** (89%). We were therefore surprised to find, when the same procedure was tried with α-methyl γ-*tert*-butyl *N*-[*p*-(methylamino)benzoyl]-L-glutamate (**17**), that **14** was formed in only 10% yield. Compound **17** was prepared conveniently from **16** by treatment with $Na_2CO_3$, which leaves the α-methyl ester intact. While the reason for the low yield in the reaction of **4** and **17** is unclear, we suspect that there was significant loss of the amino diester due to self-condensation to form α-polyamides. Since **15** contains only an unreactive γ-*tert*-butyl ester group, this monoester is presumably not prone to undergo self-condensation and therefore gives a higher yield in the coupling reaction with **4**.

**Biological Activity.** A spectrophotometric enzyme inhibition assay was performed in order to compare the DHFR affinities of **1**, **2**, MTX, and AMT. The concentrations ($IC_{50}$) of **1** and **2** giving a 50% decrease in dihydrofolate reduction to tetrahydrofolate by purified DHFR from L1210 cells were 0.055 and 0.022 μM, while those of MTX and AMT were 0.067 and 0.025 μM. Thus, **2** binds to DHFR with virtually the same affinity as AMT, just as **1** binds with the same affinity as MTX. Interestingly, AMT appears to have somewhat lower affinity under our assay conditions than does MTX, and the same trend persists on esterification.

To compare the cytotoxicity of **2** with that of **1**, AMT, and MTX, we tested these compounds as inhibitors of the growth of human T-cell leukemic lymphoblasts (CEM cells) in suspension culture. As shown in Table I, the $IC_{50}$ of **2** against these cells was 0.45 μM, whereas that of **1** was 0.62 μM. In contrast to this small 1.4-fold difference between the esters, a 30-fold difference was observed between AMT and MTX. The latter was in agreement with the known higher toxicity of AMT in comparison with MTX,[25] which has been ascribed to a higher degree of intracellular polyglutamation.[26] Because of the larger difference in $IC_{50}$ between the acids, γ-esterification of AMT appears, paradoxically, to be more detrimental than that of MTX where the parental antifolate-sensitive line is concerned. When **1** and **2** were compared with MTX and AMT against CEM/MTX cells, which are MTX resistant by virtue of a profound transport defect,[5] unexpected patterns of cross-resistance were observed. While CEM/MTX cells were "collaterally sensitive" to **1** as previously reported,[5] they remained 56-fold resistant to **2**. Moreover, AMT resistance (320-fold) appeared to be greater than MTX resistance (210-fold). To our knowledge, this type of "collateral resistance" to AMT in MTX-resistant cells has not been recognized before.

To further compare the cytotoxic effects of **1** and **2**, we performed assays in monolayer culture against three lines of human squamous cell carcinoma (SCC25, SCC68, and SCC78) from head-and-neck cancer patients not previously exposed to MTX. Growth inhibition assays were also performed against three low-resistance sublines generated by stepwise selection in the presence of MTX.[9] The biochemical and biological changes associated with resistance in these sublines will be reported separately. As indicated in Table I, the $IC_{50}$ of MTX against the three parental lines was 0.01–0.04 μM, whereas the potency of AMT was 10-fold greater ($IC_{50}$ = 0.001–0.004 μM). On the average, the MTX ester **1** was about 25-fold less toxic ($IC_{50}$ = 0.3–0.5 μM) than MTX, whereas with **2** the potency decrease relative to MTX was closer to 10-fold ($IC_{50}$ = 0.06–0.2 μM). Thus, replacement of $N^{10}$-methyl by hydrogen appeared to increase potency but still did not give an ester that was more potent than MTX against MTX-sensitive cells.

Comparison of the effects of **1** and **2** against MTX-resistant SCC cells (Table I) again revealed unexpected cross-resistance patterns. In SCC78/R1 cells, which were only 5.5-fold MTX resistant, there was essentially the same degree of AMT resistance. However in SCC25/R1 and SCC68/R1 cells, where MTX resistance was 11-fold and 7.8-fold, AMT resistance was much greater. While this was unanticipated, it is understandable how the multiplicity of factors responsible for MTX resistance[27] could cause different MTX-resistant lines to respond differently to

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Table II. Antitumor Activity of Methotrexate and Aminopterin γ-*tert*-Butyl Esters against L1210 Leukemia in Mice

| compd | dose, mg/kg | 7-day wt change, % | survival, days | | % ILS |
|---|---|---|---|---|---|
| | | | mean | range | |
| 1 (γ-tBMTX) | 10, expt 1 | +10 | 10.2 | 9–12 | +13 |
| | 20 | +6 | 11.6 | 10–13 | +29 |
| | 40 | +2 | 13.8 | 13–17 | +53 |
| | 60, expt 2 | 0 | 16.8 | 15–19 | +87 |
| | 80 | +1 | 16.2 | 15–17 | +80 |
| 2 (γ-tBAMT) | 10, expt 3 | 0 | 15.2 | 12–20 | +69 |
| | 20 | −23 (toxic) | 10.6 | 7–14 | +17 |
| | 30 | −26 (toxic) | 8.6 | 7–10 | −4 |
| | 6, expt 4 | +3 | 12.6 | 12–14 | +40 |
| | 9 | +1 | 15.2 | 13–20 | +69 |
| | 12 | −8 | 15.6 | 13–19 | +73 |
| controls[a] | | +10 | 9.0 | 8–10 | |

[a] Survival value given is the mean of the controls in four experiments (SD ± 0.1 days).

AMT, whose cellular pharmacology is not identical with that of MTX. When the effect of 1 was compared with that of MTX, only 2- to 4-fold resistance was observed; i.e., there was only partial cross-resistance. We recently noted a similar relationship between 1 and MTX in another SCC line[9] and ascribed this to the fact that induced MTX resistance in these cells relates in part to a defect in MTX active transport that can be offset by the improved uptake of the more lipophilic ester analogue. When the $IC_{50}$'s of 1 and 2 were compared (Table I), the AMT ester 2 proved more potent only in those cells (SCC78/R1) against which AMT and MTX were equitoxic. In SCC25/R1 and SCC68/R1 cells, where there was "collateral resistance" to AMT, 2 was less potent than 1. It thus appears that, while 2 was consistently more toxic than 1 in MTX-sensitive parental cells, this was true in the MTX-resistant sublines only when collateral resistance to AMT was absent.

Cytotoxicity assays were also performed against L1210 murine leukemia cells and two highly MTX-resistant sublines, L1210/R71 and L1210/R81 (Table I). The L1210/R71 cells are a subline with 88-fold increased DHFR activity (J. H. Freisheim, unpublished results), while the L1210/R81 cells have a 35-fold increase in DHFR content but also a virtually total absence of MTX active transport.[28] As indicated in Table I, L1210/R71 cells were 9500-fold resistant to MTX but only 4000-fold resistant to AMT; similarly, L1210/R81 cells were 110 000-fold MTX-resistant but 42 000-fold AMT resistant. Thus, regardless of the basis of resistance in these cells, removal of the $N^{10}$-methyl group consistently leads to a 2.5-fold decrease in resistance. When the $IC_{50}$'s were compared for 1 and 2 relative to the parent acids, further decreases in resistance were noted, which now seemed to depend on the nature of the resistance. In the 9500-fold MTX-resistant L1210/R71 subline (DHFR overproduction only), there was 710-fold resistance to 1 (esterification) but only 150-fold resistance to 2 (esterification + $N^{10}$-demethylation). In the 110 000-fold MTX-resistant L1210/R81 subline (DHFR overproduction + defective transport), there was 450-fold resistance to 1 and 280-fold resistance to 2. Thus, while resistance to the esters was 0.01–0.1 of the MTX resistance level in L1210/R71 cells, it was <0.005 of this level in L1210/R81 cells. The toxicities of the esters were greater than those of the corresponding acids in L1210/R81 cells in both the MTX and AMT series, in qualitative agreement with our previous observations with other transport-defective SCC cells,[9] and in accord with the findings of others on the effects of *nonclassical* antifolates such as DDMP, trimetrexate, and BW301U against MTX-resistant cells.[6-8,29-31]

The in vivo antitumor activities of 1 and 2 were compared in L1210 leukemic mice according to the twice-daily schedule previously described for other nonpolyglutamated MTX and AMT analogues.[19] The results are presented in Table II. A therapeutic dose of 1 was found to be 60 mg/kg, which gave a +87% ILS. For the AMT ester 2, 12 mg/kg gave a comparable +73% ILS. Thus 2 is several times more potent than 1 in vivo. We recently reported that MTX on this schedule gave a +78% ILS at 0.5 mg/kg, while AMT gave a +75% ILS at 0.12 mg/kg.[19] Therefore, when the four compounds were compared at roughly equiactive doses, each ester had about 0.01 times the potency of the corresponding acid even under conditions of relatively frequent administration. In other experiments (data not shown), 80 mg/kg of 1 given once daily for 9 days afforded a +89% ILS. This represents a total administered dose of 720 mg/kg, as compared with 1200 mg/kg when 1 is given twice daily for 10 days. From this it appears that an increase in treatment frequency does not offer an advantage when nonpolyglutamation is the result of γ-esterification rather than replacement of γ-$CO_2H$ by γ-$SO_3H$[19] or insertion of extra $CH_2$ groups into the side chain.[16]

An important question relating to the biologically activity of 1 and 2 is whether it could reflect contamination by MTX or AMT, respectively. Consideration of the in vitro data (Table I) provides evidence that the activity of the esters is not an artifact. For example, the 5.6-fold difference in $IC_{50}$ between 1 and MTX in SCC78/R1 cells implies that if the activity of 1 were due only to an MTX impurity, the latter would have to comprise an improbable 18% of the bioassay sample. Similarly, the 12-fold difference in $IC_{50}$ between 2 and AMT in the same cells would require AMT to be present to the extent of 8.3%. HPLC analysis was carried out with both esters, and contamination by the parent acids was determined to be <0.5%. Thus the antitumor activity of 1 and 2 is unlikely to be due to MTX or AMT in the test sample. A possibility that cannot be ruled out, however, is that some ester hydrolysis occurs intracellularly and that the extent of cleavage varies among cells or that toxicity arises from some synergistic intracellular combination of ester and parent acid. While serum esterases do not cleave the sterically hindered *tert*-butyl group in these compounds (A. Rosowsky and D.

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Trites, unpublished results), it is conceivable that cleavage, especially of the relatively labile AMT ester **2**, could occur in the acidic milieu of lysosomes. Studies on the cellular pharmacology of **1** and **2** would be of interest in this regard.

## Experimental Section

IR spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer, and NMR spectra were measured on a Varian Model T60A instrument with chemical shifts ($\delta$) reported relative to $Me_4Si$. Mass spectra were obtained on a Finnigan MAT-312 instrument using reverse geometry with the magnetic sector preceding the electric sector. A Finnigan MAT-200 control system was used to process the data. TLC was performed on fluorescent Baker Si250F silica gel plates or Eastman 13254 cellulose sheets. Spots were visualized under 254-nm UV light or with the aid of $I_2$ or ninhydrin as appropriate. Column chromatography was carried out on Baker 3405 silica gel (60–200 mesh), Baker "Flash" silica gel (40 $\mu$m), or Whatman DE-52 cellulose. Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA) and are not corrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, and MCL Laboratories, Lowell, MA. Chemicals were purchased from Aldrich Chemical Co. Milwaukee, WI, Chemical Dynamics Corp., South Plainfield, NJ, and Vega Biochemicals, Tucson, AZ.

**2,4-Diamino-6-(hydroxymethyl)pteridine (3).** The following modification of the method of Boyle and Pfleiderer[20] was used. Solid 2,4,5,6-tetraaminopyrimidine sulfate monohydrate (70 g, 0.273 mol) was added to a stirred solution of $BaCl_2 \cdot 2H_2O$ (66.7 g, 0.273 mol) in $H_2O$ (1365 mL) at 85–90 °C. After 15 min at 90 °C, the mixture was cooled to 35 °C and filtered, and the filter cake was washed with warm (70 °C) $H_2O$. The filtrate was added to a solution of L-cysteine hydrochloride monohydrate (47.9 g, 0.273 mol), $NH_4Cl$ (700 g), and concentrated $NH_4OH$ (36.4 mL) in $H_2O$ (635 mL) in a three-necked flask equipped with a mechanical stirrer and sintered glass gas inlet tube. The solution was cooled to 5 °C and stirred while $O_2$ was introduced to saturate the system. Dihydroxyacetone dimer (73.8 g, 0.406 mol) was then added in one portion. After 20 min at 5 °C, the cooling bath was removed and stirring was continued at ambient temperature for 45 h. A slow $O_2$ stream was maintained, with occasional adjustment of the flow rate to minimize foaming. The reddish-yellow precipitate was collected, washed with $H_2O$ and 95% EtOH, and dried in vacuo at 100 °C to obtain **3**·HCl (46.8 g, 75%); NMR $\delta$ ($CF_3CO_2H$) 4.95 (s, 6-$CH_2OH$), 5.41 (s, 6-$CH_2OCOCF_3$, forming in the tube), 9.18 (s, $C_7$-H). A barely detectable singlet at $\delta$ 2.5 indicated virtual absence of 2,4-diamino-6-methylpteridine. A suspension of **3**·HCl (86.3 g) pooled from two runs was heated in a mixture of AcOH (150 mL) and $H_2O$ (1500 mL) at 78 °C until dissolution occurred. The solution was filtered through glass wool, and the filtrate was cooled to 45 °C and adjusted to pH 5.25 with concentrated $NH_4OH$. Filtration of the cooled mixture, washing of the solid with $H_2O$, and drying in vacuo at 100 °C over $P_2O_5$ yielded the free base **3** (63.2 g, 87%).

**4-Amino-4-deoxy-$N^{10}$-formylpteroic Acid (6).** $Br_2$ (3.08 mL, 0.06 mol) was added slowly at 0–5 °C to a stirred solution of triphenylphosphine (15.7 g, 0.06 mol) in dry DMA (48 mL). Solid **3** (3.86 g, 0.02 mol) was added and the mixture was stirred at 25 °C for 18 h. To the resulting solution were then added 4-aminobenzoic acid (4.11 g, 0.03 mol) and BaO (3.13 g, 0.02 mol), and the mixture was stirred at 55 °C for 24 h. After being cooled to room temperature, the mixture was poured into $CH_2Cl_2$ (500 mL) and MeOH (15 mL). The solid was collected, resuspended in $H_2O$, filtered again, washed with MeOH, and dried in vacuo at 100 °C to obtain a yellow solid (6.03 g). This solid was added directly to a solution obtained by adding $Ac_2O$ (115 mL) to 96% $HCO_2H$ (460 mL) and allowing the exothermic effect to subside. The formylation mixture containing the crude **5** was heated under reflux for 2.5 h and then evaporated to dryness under reduced pressure. The residue was taken up in a mixture of $H_2O$ (450 mL) and concentrated $NH_4OH$ (60 mL), a small amount of undissolved solid was removed, and the filtrate was adjusted to pH 5.5 with 10% AcOH. The precipitate was collected, washed with $H_2O$, and dried in vacuo at 100 °C over $P_2O_5$ to obtain **6** as a yellow powder (4.77 g, 65% based on **3**); UV $\lambda_{max}$ (pH 7.4) 259, 372 nm; IR $\nu$ (KBr) 1600–1680 $cm^{-1}$ (amide and acid C=O); $R_f$ 0.43, blue fluorescent spot (cellulose, pH 7.4 phosphate buffer). The chromatographic and spectral properties of this product were identical with those reported previously.[19] Anal. ($C_{15}H_{13}N_7O_3 \cdot 1.5H_2O$) C, H, N.

**$\alpha$-Methyl $\gamma$-*tert*-Butyl *N*-(4-Amino-4-deoxy-$N^{10}$-formylpteroyl)-L-glutamate (7).** *i*-BuOCOCl (0.52 mL, 0.004 mol) was added at room temperature to a stirred suspension of **6** (1.48 g, 0.004 mol) in dry DMF containing $Et_3N$ (1.62 g, 0.016 mol). After 15 min, $\alpha$-methyl $\gamma$-*tert*-butyl L-glutamate hydrochloride (1.01 g, 0.004 mol)[23] was added, followed after 1 min by another portion (0.26 mL, 0.002 mol) of *i*-BuOCOCl. After 15 min, a second portion of diester (0.51 g, 0.002 mol) was added, followed again after 1 min by *i*-BuOCOCl (0.13 mL, 0.001 mol). After 15 min, a third portion of diester (0.25 g, 0.001 mol) was added, followed 1 min later by *i*-BuOCOCl (0.13 mL, 0.001 mol). Finally, after another 15 min, a fourth portion of diester (0.25 g, 0.001 mol) was added. The reaction was monitored by TLC (silica gel, 9:1 $CHCl_3$–MeOH) to confirm the disappearance of **6** ($R_f$ 0.0) and the formation of **7** ($R_f$ 0.55, blue fluorescent spot). After removal of the DMF under reduced pressure, the residue was taken up in 9:1 $CHCl_3$–MeOH and applied onto a silica gel column (70 g) which was eluted with the same solvent mixture. Fractions containing the product were pooled and evaporated, and the residue was triturated with $Et_2O$ to obtain **7** as a yellow powder (1.83 g, 82%), mp 125–148 °C; IR $\nu$ (KBr) 1730 (ester C=O), 1660 (amide C=O) $cm^{-1}$. Anal. ($C_{25}H_{30}N_8O_6 \cdot H_2O$) C, H, N.

***p*-Nitrophenyl 4-Amino-4-deoxy-$N^{10}$-formylpteroate (9).** $Et_3N$ (60 mg, 0.6 mmol) and bis(*p*-nitrophenyl) carbonate (474 mg, 1.56 mmol) were added to a suspension of **6** (220 mg, 0.6 mmol based on sesquihydrate formula) in dry DMF (24 mL). Carbon dioxide evolution occurred immediately. After 18 h at 25 °C, the solvent was removed under reduced pressure and the residue was triturated with *i*-PrOH. Filtration gave **9** as a yellow solid (260 mg, 94%); $R_f$ 0.64, blue fluorescent spot (silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH); IR $\nu$ (KBr) 1737 (ester C=O), 1675 (amide C=O) $cm^{-1}$; NMR $\delta$ ($CF_3CO_2H$) 5.2 (s, 2 H, $CH_2$), 7.2 (m, 4 H, aromatic protons), 8.0 (m, 4 H, aromatic protons), 8.9 (s, 1 H, $C_7$-H), 9.3 (s, 1 H, NCHO). Anal. ($C_{21}H_{16}N_8O_5 \cdot 0.75H_2O$) C, H, N.

**$\gamma$-*tert*-Butyl *N*-(4-Amino-4-deoxy-$N^{10}$-formylpteroyl)-L-glutamate (8). Method A.** A solution of **7** (1.60 g, 1.46 mmol) in 50% EtOH (180 mL) was stirred at 25 °C with $Ba(OH)_2 \cdot 8H_2O$ (0.588 g, 0.947 mmol, 1.3 equiv) for 18 h. A solution of $NH_4HCO_3$ (0.162 g, 2.05 mmol) in $H_2O$ (3 mL) was added, and stirring was continued for another 0.5 h. After filtration, the $H_2O$ and EtOH were removed by rotary evaporation followed by freeze-drying to obtain **8** as a yellow powder (1.45 g, 79%); $R_f$ 0.7, blue fluorescent spot (silica gel, 5:4:1 $CHCl_3$–MeOH–concentrated $NH_4OH$); IR $\nu$ (KBr) 3400, 2980, 1720 (sh, ester C=O), 1655–1635 (amide C=O) $cm^{-1}$. Anal. ($C_{24}H_{28}N_8O_6 \cdot H_2O$) C, H, N.

**Method B.** A mixture of **9** (27.6 mg, 0.06 mmol) and $\gamma$-*tert*-butyl L-glutamate (12.2 mg, 0.06 mmol) in dry DMF (2.7 mL) containing $Et_3N$ (12 mg, 0.12 mmol) was stirred at 55 °C for 1 h, at which time a clear solution was formed. After another 72 h at 55 °C, the solvent was removed under reduced pressure, the residue was triturated with $Et_2O$, and the solid remaining after decantation of the $Et_2O$ was taken up in 6:1 $CH_2Cl_2$–MeOH (5 mL) and applied onto a silica gel column (2 g), which was eluted with the same solvent mixture. Fractions containing the product were pooled and evaporated, the residue was redissolved in dilute $NH_4OH$, and the pH was adjusted to 4.5 with dilute AcOH to obtain **8** as the free acid (25 mg, 74%); $R_f$ 0.19, blue fluorescent spot (silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH).

**$\alpha$-Methyl $\gamma$-*tert*-Butyl *N*-(*p*-Nitrobenzoyl)-L-glutamate (12).** $Et_3N$ (2.02 g, 0.02 mol) was added slowly at 5 °C to a stirred solution of *p*-nitrobenzoyl chloride (1.86 g, 0.01 mol) and $\alpha$-methyl $\gamma$-*tert*-butyl L-glutamate hydrochloride (2.54 g, 0.01 mol)[23] in $CH_2Cl_2$ (45 mL). Stirring was continued for 18 h, and the solution was washed successively with 0.1 N HCl (50 mL), $H_2O$ (50 mL), and saturated $NH_4HCO_3$ (50 mL). After drying ($MgSO_4$) and solvent evaporation, a yellow oil was obtained (3.66 g, 100%), which was dried in vacuo at 60 °C for 22 h before analysis; $R_f$ 0.56 (silica gel, $CHCl_3$); NMR $\delta$ ($CDCl_3$) 1.43 (s, 9 H, *t*-Bu), 2.1–2.6 (m, 4 H, $CH_2CH_2$), 3.8 (s, 3 H, OMe), 4.7 (t, 1 H, $\alpha$-CH), 8.0 (d, 2 H, aromatic protons), 8.3 (d, 2 H, aromatic protons). Anal. ($C_{17}H_{22}N_2O_5$) C, H, N.

**γ-*tert*-Butyl *N*-(*p*-Nitrobenzoyl)-L-glutamate (13).** A solution of **12** (3.6 g, 0.0098 mol) in a mixture of 1 N NaOH (12 mL), $H_2O$ (25 mL), and 95% EtOH (50 mL) was kept at 25 °C for 3.5 h and then acidified to pH 5.5 with 10% AcOH. After dilution with $H_2O$ to 200 mL, the solution was extracted three times with $CHCl_3$, and the combined extracts were dried ($MgSO_4$) and evaporated to an oil (3.16 g). Crystallization from benzene, followed by a second crop from benzene–ligroin (bp 65–90 °C), afforded 2.12 g (62%) of product, mp 145–146 °C dec; NMR δ ($CDCl_3$) 1.55 (s, 9 H, *t*-Bu), 2.1–2.7 (m, 4 H, $CH_2CH_2$), 4.8 (d, 1 H, α-CH), 8.0 (d, 2 H, aromatic protons), 8.3 (d, 2 H, aromatic protons); $R_f$ 0.32 (silica gel, 5:1 $CHCl_3$–MeOH). Anal. ($C_{16}H_{22}N_2O_5$) C, H, N.

**γ-*tert*-Butyl *N*-(*p*-Aminobenzoyl)-L-glutamate (11).** A solution of **13** (2.05 g, 0.005 82 mol) in 95% EtOH (15 mL) containing glacial AcOH (5 mL) was hydrogenated in the presence of $PtO_2$ (20 mg) in a Parr aparatus. Filtration of the catalyst and solvent evaporation under reduced pressure afforded a colorless solid (1.87 g, 100%), mp 172–173 °C. One recrystallization from *i*-PrOH–EtOAc gave mp 173.5–176 °C. Anal. ($C_{16}H_{22}N_2O_5$) C, H, N.

**γ-*tert*-Butyl *N*-(4-Amino-4-deoxypteroyl)-L-glutamate (γ-tBAMT, 2). Method A.** A solution of **8** (0.4 g, 0.68 mmol) in 0.25 N NaOH (40 mL) was stirred at 25 °C, with TLC monitoring of the disappearance of starting material ($R_f$ 0.62 for **8** and 0.16 for **2**; cellulose, pH 7.4 phosphate buffer) and the formation of **2** and AMT ($R_f$ 0.53 for **2** and 0.15 for AMT; silica gel, 5:4:1 $CHCl_3$–MeOH–concentrated $NH_4OH$). After 1.75 h, solid $NH_4HCO_3$ (0.95 g) was added, and the solution was freeze-dried. The residue was extracted with two portions (20 mL, 10 mL) of 5:4:1 $CHCl_3$–MeOH–concentrated $NH_4OH$, and a small amount of insoluble AMT was removed. The solution was applied onto a silica gel column (9 g) and the product was eluted with the same solvent mixture. Fractions containing pure **2** were pooled and evaporated to obtain a yellow powder (0.2 g, 52%); IR ν (KBr) 3380, 1710 (sh, ester C=O), 1640, 1610 $cm^{-1}$. Anal. ($C_{23}H_{28}N_8O_5 \cdot 0.25NH_3 \cdot 5H_2O$). Because this material was found to undergo partial cleavage to AMT upon storage at 4 °C for 1 month, it was converted to the more stable Na salt. The partial ammonium salt was suspended in $H_2O$ and an amount of NaOH just sufficient to give a clear solution was added. After filtration through Celite, the filter pad was washed with a small volume of MeOH, and the combined filtrate and wash solution were adjusted to pH 7.8–8.0 with Amberlite IR120($H^+$) C.P. resin. The resin was filtered off and the filtrate was concentrated to dryness by rotary evaporation followed by lyophilization. The product was dried over $P_2O_5$ in vacuo at 60 °C and stored immediately at −70 °C, where it remained stable for several months.

**Method B.** A mixture of the monoester **11** (161 mg, 0.5 mmol), *i*-$Pr_2NEt$ (65 mg, 0.5 mmol), and 2,4-diamino-6-(bromomethyl)-pteridine hydrobromide (191 mg, 0.5 mmol)[22] in dry DMA (1.5 mL) was stirred at 25 °C for 22 h. The resulting thick oil was diluted with $H_2O$ (12 mL) and the resultant precipitate was filtered, air-dried, and taken up in 5:4:1 $CHCl_3$–MeOH–concentrated $NH_4OH$ (10 mL) and applied onto a silica gel column (40 cm × 1 cm). The column was eluted with the same solvent mixture, and fractions were monitored by TLC. The fractions containing a major component (silica gel, 5:4:1 $CHCl_3$–MeOH–concentrated $NH_4OH$) were pooled and evaporated, and the residue was dissolved in aqueous $NH_4OH$, pH 10.9. Addition of 10% AcOH to pH 5 caused precipitation of a solid, which was isolated by centrifugation and then washed with a small amount of *i*-PrOH and $Et_2O$. Flash chromatography[32] on a silica gel column (17 cm × 1.1 cm) using 14:6:1 $CHCl_3$–MeOH–concentrated $NH_4OH$ gave TLC-homogeneous **2** (59 mg, 21%). The product was stored at once a −70 °C. Anal. ($C_{23}H_{28}N_8O_5 \cdot 3H_2O$) C, H, N.

***p*-[*N*-Methyl-*N*-(trifluoroacetyl)amino]benzoic Acid.** Trifluoroacetic anhydride (9.24 g, 0.044 mol) was added with stirring to an ice-cold suspension of *p*-(methylamino)benzoic acid (3.02 g, 0.02 mol) in $CHCl_3$ (40 mL). The solution was kept at 20 °C for 3 h and $H_2O$ (20 mL) was added. The organic layer was separated, washed with $H_2O$ (20 mL), dried, and evaporated. Recrystallization from benzene yielded colorless crystals (4.14 g, 84%), mp 174–176 °C; IR ν (KBr) 1705 (acid and amide C=O) $cm^{-1}$; NMR δ ($CDCl_3$ + $CF_3CO_2H$) 3.41 (s, 3 H, Me), 7.33 (d, $J$ = 8 Hz, aromatic protons), 8.15 (d, $J$ = 8 Hz, aromatic protons). Anal. ($C_{10}H_8F_3NO_3$) C, H, F, N.

Addition of a small amount of hexane to the mother liquor resulted in crystallization of *p*-[*N*-methyl-*N*-(trifluoroacetyl)-amino]benzoic anhydride (80 mg), mp 158–162 °C; IR ν (KBr) 1700 (amide C=O), 1800 (anhydride C=O) $cm^{-1}$. Anal. ($C_{20}H_{14}F_6N_2O_5$) C, H, N; F: calcd, 23.93; found, 23.28.

**α-Methyl γ-*tert*-Butyl *N*-[*p*-[*N*-Methyl-*N*-(trifluoroacetyl)amino]benzoyl]-L-glutamate (16).** A solution of *p*-[*N*-methyl-*N*-(trifluoroacetyl)amino]benzoic acid (4.1 g, 0.017 mol) in $SOCl_2$ (2.4 mL) was heated under reflux for 45 min. Excess $SOCl_2$ was evaporated under reduced pressure, and the residue was recrystallized from ligroin (bp 65–90 °C) to obtain the acid chloride in the form of colorless crystals (4.2 g, 94%), mp 68–70 °C; IR ν (KBr) 1770 (acid chloride C=O), 1700 (amide C=O) $cm^{-1}$. A solution of freshly prepared acid chloride (1 g, 0.004 mol) and α-methyl γ-*tert*-butyl L-glutamate hydrochloride (1 g, 0.004 mol)[23] in $CH_2Cl_2$ (18 mL) was cooled to 0 °C, and $Et_3N$ (0.81 g, 0.008 mol) was added over 5 min. After being stirred at room temperature for 17 h, the solution was extracted with 0.1 N HCl (20 mL), followed by saturated $NH_4HCO_3$ (20 mL). Drying ($MgSO_4$) and solvent evaporation gave the desired diester (1.85 g, 100%); IR ν (KBr) 1720–1740 (ester C=O), 1700 (amide C=O) $cm^{-1}$; NMR δ ($CDCl_3$) 1.43 (s, 9 H, *t*-Bu), 2.1–2.6 (m, 4 H, $CH_2CH_2$), 3.4 (s, 3 H, NMe), 3.8 (s, 3 H, OMe), 4.8 (t, 1 H, α-CH), 5.3 (s, 1 H, NH), 7.3 (d, 2 H, aromatic protons), 7.9 (d, 2 H, aromatic protons); $R_f$ 0.38 (silica gel, $CHCl_3$). Anal. ($C_{20}H_{25}F_3N_2O_6$) C, H, F, N.

**γ-*tert*-Butyl *N*-[*p*-(Methylamino)benzoyl]-L-glutamate (15).** A solution of **16** (1.55 g, 3.47 mmol) in a mixture of EtOH (50 mL), $H_2O$ (20 mL), and 1 N NaOH (10 mL) was kept at 20 °C for 2.5 h, at which time all the starting material was consumed (TLC). The solution was acidified to pH 5.5 with 10% AcOH (3.7 mL), diluted with $H_2O$ (200 mL), and extracted four times with $CHCl_3$. The combined $CHCl_3$ layers were dried ($MgSO_4$) and evaporated to an amorphous solid (1.17 g, 100%), mp 52–54 °C; NMR δ ($CDCl_3$) 1.4 (s, 9 H, *t*-Bu), 2.0–2.5 (m, 4 H, $CH_2CH_2$), 2.9 (s, 3 H, NMe), 4.6 (t, 1 H, α-CH), 6.5 (d, 2 H, aromatic protons), 7.6 (d, 2 H, aromatic protons), 7.6 (s, 1 H, NH, disappearing on addition of acetic acid-$d_4$). Anal. ($C_{17}H_{24}N_2O_5$) C, H, N.

The hydrochloride salt **15**·HCl, mp 91–95 °C, was formed in $Et_2O$; IR ν (KBr) 3400 (NH), 2650 ($NH^+$), 1725 (ester C=O), 1650 (amide C=O) $cm^{-1}$. Anal. ($C_{17}H_{25}ClN_2O_5 \cdot 0.75H_2O$) C, H, Cl, N.

**α-Methyl γ-*tert*-Butyl *N*-[*p*-(Methylamino)benzoyl]-L-glutamate (17).** To a stirred solution of **16** (1.8 g, 0.004 mol) in a mixture of EtOH (35 mL) and $H_2O$ (20 mL) was added slowly (1 h) a solution of 0.4 N $Na_2CO_3$ (11 mL) at a rate such that the pH was maintained at 9.5–10.5. After adjustment of the pH to 7.0 with 10% AcOH, the mixture was diluted with $H_2O$ (300 mL) and extracted 3 times with $CHCl_3$. The combined $CHCl_3$ layers were dried ($MgSO_4$) and evaporated to a clear oil (1.35 g, 96%); NMR δ ($CDCl_3$) 1.43 (s, 9 H, *t*-Bu), 2.1–2.5 (m, 4 H, $CH_2CH_2$), 2.9 (s, 1 H, NH), 3.4 (s, 3 H, NMe), 3.8 (s, 3 H, OMe), 4.8 (t, 1 H, α-CH), 7.3 (d, 2 H, aromatic protons), 7.9 (d, 2 H, aromatic protons). The hydrochloride salt **17**·HCl was prepared in $Et_2O$; mp 93 °C. Anal. ($C_{18}H_{27}ClN_2O_5$) C, H, Cl, N.

**α-Methyl γ-*tert*-Butyl *N*-(4-Amino-4-deoxy-$N^{10}$-methylpteroyl)-L-glutamate (14). Method A.** $Br_2$ (0.46 mL, 0.009 mol) was added slowly at 0 °C to a stirred solution of triphenylphosphine (2.36 g, 0.009 mol) in dry *N*,*N*-dimethylacetamide (10 mL). Solid **3** (0.58 g, 0.003 mol) was then added and the solution was stirred at 20 °C for 23 h. To this solution were added **17** (1.33 g, 0.0038 mol) and *i*-$Pr_2NEt$ (1.13 g, 0.0088 mol). The mixture was warmed to 45 °C and stirring was continued for 48 h. After being cooled to room temperature, the mixture was poured into a large volume of $H_2O$ and the solid was filtered, dried in vacuo, redissolved in $CHCl_3$, and chromatographed on silica gel with 95:5 $CHCl_3$–MeOH as the eluent to obtain **14** as a bright-yellow powder (0.16 g, 10%); $R_f$ 0.58 (silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH). This material and the product formed below (method B) were indistinguishable.

**Method B.** Acid **10** (1.3 g, 0.0037 mol based on sesquihydrate formula) was added in small portions to a stirred solution of diethyl phosphorocyanidate (1.8 g, 0.011 mol) and $Et_3N$ (1.12 g,

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0.011 mol) in dry DMF (250 mL). A clear solution was obtained within 20 min. After a total of 2.5 h at 25 °C, α-methyl γ-*tert*-butyl L-glutamate hydrochloride (0.94 g, 0.0037 mol)[23] and $Et_3N$ (0.75 g, 0.0074 mol) were added, and the solution was kept at 25 °C for 70 h. The solvent was evaporated in vacuo, the residue was taken up in $CHCl_3$ (200 mL), and the solution was extracted with 1% $NH_4OH$ (250 mL). The organic layer was evaporated to dryness and the product was subjected to flash chromatography[32] using 5:5:1 $CHCl_3$–MeCN–MeOH as the eluent. After removal of a forerun containing minor fast-moving impurities, two fractions were obtained. The first fraction ($R_f$ 0.5, silica gel, 5:5:1 $CHCl_3$–MeCN–MeOH) consisted of 75 mg (3.7%) of yellow crystals, mp 215 °C (MeOH); IR ν (KBr) 1745 (ester C=O), 1630 (amide C=O) $cm^{-1}$; UV $\lambda_{max}$ (95% EtOH) 281 nm (ε 35 700), 296 infl (33 500), 405 (9600); NMR δ ($Me_2SO$-$d_6$) 1.37 (s, 9 H, *t*-Bu), 3.18 (s, 3 H, NMe), 3.6 (s, 3 H, OMe), 4.9 (s, 3 H, α-CH and $NCH_2$), 6.7 (s, 1 H, NCH=N), 6.93 (d, 2 H, aromatic protons), 7.7 (d, 2 H, aromatic protons), 8.23 and 8.36 (s, s, 1 H total, $C_7$-H); MS, 552 (M + 1). This material was formulated tentatively as a mixture of the *N*-formamidino derivatives **19** and **20**. Anal. ($C_{26}H_{33}N_9O_5$) C, H, N.

The second fraction from the above flash chromatography ($R_f$ 0.24, silica gel, 5:5:1 $CHCl_3$–MeCN–MeOH; $R_f$ 0.64, silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH) consisted of 1.45 g (75%) of pure **14** indistinguishable from the material prepared by method A and from a reference sample.[1]

**Ethyl 4-Amino-4-deoxy-$N^{10}$-methylpteroate (18).** $Br_2$ (3.85 g, 0.075 mol), triphenylphosphine (19.7 g, 0.075 mol), and **3** (4.83 g, 0.025 mol) were added to dry DMA (70 mL) as in the preceding experiment, and to the resulting solution were added ethyl *p*-(methylamino)benzoate (4.48 g, 0.025 mol) and BaO (3.83 g, 0.025 mol). After being stirred at 45 °C for 21 h, the solution was poured into 0.005 N HCl (600 mL) and the precipitate was collected, dried in vacuo at 110 °C, and washed twice with hot benzene. The solid remaining after the benzene extractions (7.9 g, 89%) was indistinguishable from an authentic sample;[33] $R_f$ 0.64 (silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH).

**Alkaline Hydrolysis of Compounds 19/20. Formation of *N*-Formyl Esters 21/22.** A solution of the putative *N*-formamidines **19** and/or **20** (453 mg, 0.82 mmol) in a mixture of EtOH (30 mL) and $H_2O$ (15 mL) was stirred at 25 °C with $Ba(OH)_2 \cdot 8H_2O$ (364 mg, 1.15 mmol) for 22 h. A solution of $NH_4HCO_3$ (220 mg, 2.77 mmol) in $H_2O$ (2 mL) was then added, and after 30 min of stirring, the mixture was filtered. The filtrate was reduced in volume and acidified with 10% AcOH, and the precipitated solid was collected and dried in a lyophilization apparatus to obtain a bright-yellow powder (394 mg, 84%); $R_f$ 0.49 (silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH); IR ν (KBr) 3400, 1730 (ester C=O), 1620-1640 (amide C=O) $cm^{-1}$; UV $\lambda_{max}$ (0.1 N NaOH) 225 nm (ε 28 100), 254 (21 900), 306 (27 300), 348 (13 800); $\lambda_{max}$ (0.1 N HCl) 221 nm (ε 25 200), 248 (17 300), 310 (19 400), 340 infl (15 000). This product was formulated as the *N*-formyl derivatives **21** and/or **22**.

**γ-*tert*-Butyl *N*-(4-Amino-4-deoxy-$N^{10}$-methylpteroyl)-L-glutamate (1). Method A.** To a solution of **4** formed in situ as in the earlier preparation of the diester **14** were added with stirring **15** (1 g, 0.003 mol) and *i*-$Pr_2NEt$ (0.78 g, 0.006 mol). The reaction mixture was stirred at 45–50 °C for 25 h and then poured with stirring into a mixture of concentrated $NH_4OH$ (14 mL) and $H_2O$ (86 mL). The precipitate was filtered off, and the filtrate was acidified to pH 5.5 with 10% AcOH. Saturated $NH_4Cl$ was added, and after storage at 5 °C for 2 days the solid was collected; yield 0.83 g (54%); $R_f$ 0.34 (silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH).

**Method B.** A solution of **14** (3.09 g, 5.88 mmol) in $H_2O$ (220 mL) was stirred for 22 h with $Ba(OH)_2 \cdot 8H_2O$ (1.3 g, 4.11 mmol). To this was then added a solution of $NH_4HCO_3$ (0.4 g, 5.1 mmol) in $H_2O$ (3 mL), and after 30 min of stirring, the $BaCO_3$ was filtered off. Acidification of the filtrate to pH 4.5 with 10% AcOH, filtration of the solid, and drying in a lyophilization apparatus gave a bright-yellow solid (2.77 g, 86%); $R_f$ 0.29 (silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH). The properties of this material were identical with those of the product obtained above (method A) and with those of an authentic specimen.[1]

**Cell Growth Inhibition.** The cytotoxicity of **1** and **2** toward CEM and CEM/MTX cells in suspension culture was assayed as previously reported,[5] as was the cytotoxicity of these compounds toward L1210, L1210/R71, and L1210/R81 cells.[34,35]

In assays against human squamous cell carcinoma (SCC) lines in monolayer culture,[9] the cells were grown in Dulbecco's modified Eagle's medium containing 20% dialyzed fetal bovine serum and supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and hydrocortisone (0.4 μg/mL). Drugs were added 24 h after plating, and colonies were allowed to grow for about 2 weeks before being fixed, stained with methylene blue, and counted. The growth medium was replaced with fresh drug-containing medium every 3–4 days during the growth period.

**In Vivo Antitumor Assay.** The activity of **1** and **2** against L1210 leukemia in $B6D2F_1J$ mice was determined as previously described,[19] using a twice-daily schedule of ip injection for 10 days following ip tumor implantation ($10^5$ cells). Drugs were dissolved in sterile water with enough NaOH added to bring the pH to 7.5–8.0. Solutions for injection were freshly prepared prior to each dose.

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**Registry No.** **1**, 79640-76-9; **2**, 95484-98-3; **3**, 945-24-4; **3**·HCl, 73978-41-3; **5**, 36093-85-3; **6**, 89043-75-4; **7**, 95484-99-4; **8**, 95485-00-0; **9**, 95485-01-1; **10**, 54798-42-4; **11**, 95485-02-2; **12**, 95485-03-3; **13**, 95485-04-4; **14**, 79640-68-9; **15**, 95485-05-5; **16**, 95485-07-7; **17**, 95485-08-8; **17**·HCl, 95485-09-9; **18**, 43111-51-9; **19**, 95485-10-2; **20**, 95485-11-3; **21**, 95485-12-4; **22**, 95485-13-5; $HCO_2H$, 64-18-6; 1,3-dihydroxyacetone, 96-26-4; tetraaminopyrimidine, 1004-74-6; 4-aminobenzoic acid, 150-13-0; α-methyl γ-*tert*-butyl L-glutamate hydrochloride, 53838-27-0; bis(*p*-nitrophenyl) carbonate, 5070-13-3; γ-*tert*-butyl L-glutamate, 2419-56-9; *p*-nitrobenzoyl chloride, 122-04-3; 2,4-diamino-6-(bromomethyl)pteridine hydrobromide, 52853-40-4; trifluoroacetic anhydride, 407-25-0; *p*-(methylamino)benzoic acid, 10541-83-0; *p*-[(*N*-methyl-*N*-(trifluoroacetyl)amino]benzoic acid, 50734-07-1; *p*-[*N*-methyl-*N*-(trifluoroacetyl)amino]benzoyl chloride, 95063-86-8; **15**·HCl, 95485-06-6; ethyl *p*-(methylamino)benzoate, 10541-82-9; dihydrofolate reductase, 9002-03-3; *p*-[*N*-methyl-*N*-(triflouroacetyl)amino]benzoic anhydride, 95485-14-6.

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