Methotrexate Analogues. 29. Effect of γ-Aminobutyric Acid Spacers between the Pteroyl and Glutamate Moieties on Enzyme Binding and Cell Growth Inhibition

Andre Rosowsky,*† Ronald A. Forsch,† James H. Freisheim,‡ Peter V. Danenberg,§ Richard G. Moran,⊥ and Michael M. Wick†

Dana-Farber Cancer Institute and the Departments of Pharmacology and Dermatology, Harvard Medical School, Boston, Massachusetts 02115, Department of Biochemistry, Medical College of Ohio, Toledo, Ohio 43699, Department of Biochemistry and the Comprehensive Cancer Center, University of Southern California School of Medicine, Los Angeles, California 90033, and Division of Hematology/Oncology, Children's Hospital of Los Angeles, Los Angeles, California 90027.
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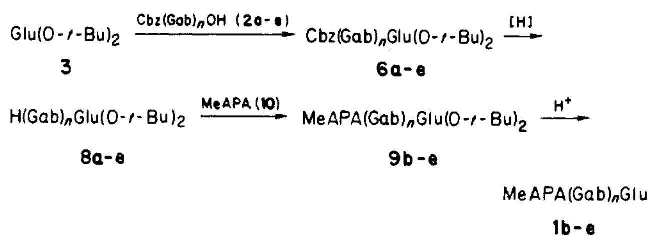
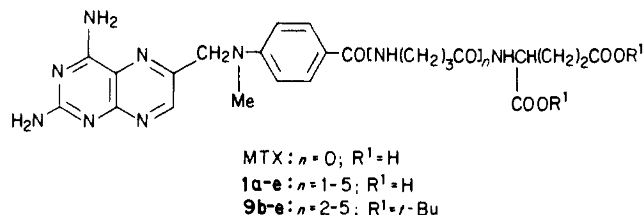
A series of "stretched" methotrexate (MTX) analogues containing up to five 4-aminobutyryl (Gab) spacers between the 4-amino-4-deoxy-N^{10}-methylpteroyl (MeAPA) moiety and the glutamate (Glu) side chain was prepared. Interest in these compounds stemmed from their relationship to MTX γ-polyglutamates, from which they differ only in lacking "internal" α-carboxyl groups. The ability of the MeAPA-Gab_n-Glu derivatives to inhibit dihydrofolate reductase (DHFR) and thymidylate synthase (TS) in vitro and to inhibit the growth of tumor cells in culture was evaluated. The IC_{50} for DHFR inhibition increased progressively from 0.082 to 0.84 μM as the number of Gab spacers was varied from one to five. At the same time the introduction of Gab spacers was found to produce substantial TS inhibition (K_i 0.1–0.4 μM) similar to that reported for MTX polyglutamates. Despite the activity of the MeAPA-Gab_n-Glu derivatives as combined inhibitors of TS and DHFR, there was a steep loss of cell growth inhibitory potency as the number of Gab spacers was increased. This most likely reflects low cell uptake and the fact that when $n > 1$ there is almost total abolition of substrate activity for folylpolyglutamate synthetase, which had previously been observed with $n = 1$.

As part of a larger program of study of substrates and inhibitors of the enzyme folylpolyglutamate synthetase (FPGS),[1-6] we recently synthesized *N*-[4-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)amino]butyryl]-L-glutamic acid (MeAPA-Gab-Glu, **1a**) and showed that this "stretched" analogue of methotrexate (MTX) is a substrate for mouse liver FPGS in vitro.[7] In contrast, substrate activity was not observed with an isomeric analogue (MeAPA-Glu-Gab) that contained 4-aminobutyric acid at the terminal position and L-glutamic acid as the "internal" residue. A model was proposed, wherein binding of the α-carboxyl in the terminal residue of the growing oligoglutamate was a requisite for further catalytic activity. A larger series of stretched MTX analogues with spacers between the pteroyl and glutamate moieties was sought as a means of testing this hypothesis. 4-Aminobutyryl (Gab) spacers were attractive because the resultant compounds, $MeAPA(Gab)_nGlu$, would represent MTX oligoglutamate analogues with all but one α-carboxyl group deleted. Further interest in these compounds stemmed from the possibility that (i) they might cross the cell membrane more effectively than the corresponding oligoglutamates, which carry multiple negative charges, and (ii) they might interact with enzymes other than FPGS, such as dihydrofolate reductase (DHFR), thymidylate synthase (TS), or AICAR transformylase. While the binding affinity of the lower oligoglutamates of MTX to DHFR is not greatly different from that of MTX itself,[8] a 40-fold increase in affinity has been reported for the pentaglutamate MTX-Glu_5 in comparison with MTX.[9] This explains the fact that MTX-Glu_5 can gradually replace MTX as the DHFR-bound species in cells treated with MTX. Recent work on TS and AICAR transformylase indicates that, especially under conditions of limited availability of reduced folate oligoglutamates as competitive substrates, the inhibition of these enzymes by MTX oligoglutamates increases by as much as three orders of magnitude upon addition of four glutamyl residues to the side chain.[10,11] The present paper describes

Scheme I



the synthesis and in vitro biological evaluation of the MeAPA-Gab_n-Glu derivatives **1b–e** in comparison with **1a**.



† Dana-Farber Cancer Institute.
‡ Medical College of Ohio.
§ University of Southern California.
⊥ Children's Hospital of Los Angeles.

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Table I. Protected 4-Aminobutyric Acid Oligomers

compd	starting material(s)	method[a]	yield, %	mp, °C	empirical formula	anal.
7b	**2a**, **4**[b]	A	85	86–87	$C_{17}H_{24}N_2O_5$	C, H, N
7c	**2a**, **5**[c]	A	33	137–138	$C_{21}H_{31}N_3O_6$	C, H, N
7d	**2b**, **5**	A	78	170–171	$C_{25}H_{38}N_4O_7$	C, H, N
7e	**2d**, **4**	A	73	193–195	$C_{29}H_{45}N_5O_8$	C, H, N
2b	**7b**	B	97	114.5–115	$C_{16}H_{22}N_2O_5$	C, H, N
2c	**7c**	B	89	149.5–150.5	$C_{20}H_{29}N_3O_6$	C, H, N
2d	**7d**	B	97	168–170[d]	$C_{24}H_{36}N_4O_7$	C, H, N
2e	**7e**	B	90	187–189[d]	$C_{28}H_{43}N_5O_8$	C, H, N

[a] Key: A, mixed carboxylic carbonic anhydride synthesis (i-BuOCOCl/Et_3N); B, saponification with $Ba(OH)_2$/aqueous EtOH. [b] Compound **4** was prepared from 4-aminobutyric acid by reaction with MeOH and $SOCl_2$ as previously described[7] and was used as the HCl salt (**4**·HCl). [c] Compound **5** was prepared from **7b** by catalytic hydrogenolysis (H_2/Pd–C) in the presence of HCl (Table II, method E), and was used directly as the HCl salt (**5**·HCl). The product obtained after filtration of the catalyst and evaporation of the MeOH/HCl was crystalline but very hygroscopic. For best results in subsequent mixed anhydride coupling reactions (compare **7d** and **7e** with **7c**); the crystalline form of **5**·HCl was heated strongly in vacuo to constant weight, whereupon it became a glassy solid or gum. The dried compound was used as soon as possible to avoid reabsorption of water and possible hydrolysis. [d] These melting point values were found to depend on the rate of heating and could vary by several degrees.

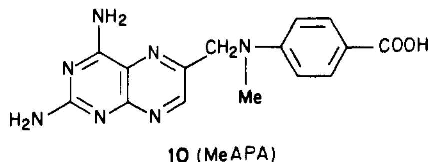
Table II. Methotrexate Analogues with Oligomeric 4-Aminobutyric Acid Spacers

compd	method(s)[a,b]	mp, °C	empirical formula	anal.
9b	**2b**, **3** (C, 88); **6b** (D, 96); **8b**, **10** (F, 45)	122–132	$C_{36}H_{52}N_{10}O_7·CHCl_3·2CH_3OH$	C, H, N
9c	**2c**, **3** (C, 99); **6c** (D, 56); **8c**, **10** (F, 28)	119–128	$C_{40}H_{59}N_{11}O_8·0.7CHCl_3·CH_3OH$	C, H, N
9d	**2d**, **3** (C, 100); **6d** (E, 98); **8d**, **10** (F, 53)	192–194[c]	$C_{44}H_{66}N_{12}O_9·CHCl_3·CH_3OH$	C, H, N
9e	**2a**, **3** (A, 100); **6a** (E, 100); **8a**, **2d** (C, 47); **6e** (E, 100); **8e**, **10** (F, 21)	192–194[c]	$C_{48}H_{73}N_{13}O_{10}·0.75CHCl_3·0.25CH_3OH$	C, H, N
1b	**9b** (G, 81)	*d*	$C_{28}H_{36}N_{10}O_7·1.75H_2O$	C, H, N
1c	**9c** (G, 66)	*d*	$C_{32}H_{43}N_{11}O_8·3H_2O$	C, H, N
1d	**9d** (G, 90)	*d*	$C_{36}H_{50}N_{12}O_9·1.5NH_3·3.5H_2O$	C, H, N
1e	**9e** (G, 71)	*d*	$C_{40}H_{57}N_{13}O_{10}·5H_2O$	C, H, N

[a] Individual compounds in the synthetic sequence from **2a–e** to **9b–e** are given, with yields after each method in parentheses. [b] Key: A, mixed carboxylic carbonic anhydride synthesis (i-BuOCOCl/Et_3N); C, diphenylphosphoryl azide coupling (DPPA/Et_3N); D, catalytic hydrogenolysis (H_2/Pd–C/AcOH); E, catalytic hydrogenolysis (H_2/Pd–C/HCl); F, diethyl phosphorocyanidate coupling (DEPC/Et_3N); G, trifluoroacetic acid (TFA) cleavage. [c] Decomposition (gas evolution). [d] These compounds decomposed over a broad temperature range starting at ca. 200 °C and therefore could not be assigned melting points.

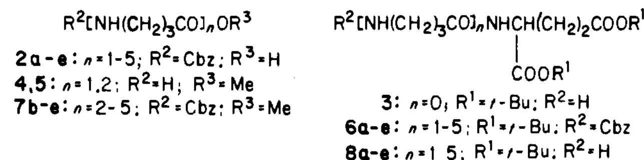
Chemisty

An overall outline of the synthetic route to compounds **1b–e** is given in Scheme I. A series of C-protected $(Gab)_nGlu$ fragments was prepared, and each compound was condensed with 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA) by the diethyl phosphorocyanidate method,[12] which we have used extensively in the past to obtain MTX analogues.[13-17] The C-terminal glutamic acid residue was protected as a di-*tert*-butyl ester, and the (benzyloxy)carbonyl (Cbz) group was used for N-protection of the aminobutyric acid residue. Detailed aspects of the syntheses are discussed below and in Tables I and II.



10 (MeAPA)

$Cbz(Gab)_nOH$ derivatives **2b–e** were synthesized by $Ba(OH)_2$ hydrolysis of the corresponding methyl esters **7b–e**, which were obtained via mixed carboxylic–carbonic anhydride synthesis (i-BuOCOCl/Et_3N/DMF) from appropriate Cbz-blocked precursors and either methyl 4-aminobutyrate (**4**) [**2a** → **7b** (85%) → **2b** (89%); **2d** → **7e** (73%) → **2e** (90%)] or methyl 4-[*N*-(4-aminobutyryl)-amino]butyrate (**5**) [**2a** → **7c** (33%) → **2c** (89%); **2b** → **7d** (78%) → **2d** (97%)]. Dipeptide **5** was prepared by similar



mixed anhydride coupling of *N*-Cbz-4-aminobutyric acid (**2a**) with **4**, followed by catalytic hydrogenolysis. The latter reaction was performed in the presence of HCl, which led to isolation of **5** as a crystalline but highly solvated salt. When solvated **5**·HCl was used in a mixed carboxylic–carbonic anhydride reaction, a poor yield was obtained (see **2a** → **7c**). However, when **5**·HCl was heated strongly under vacuum in order to desolvate it, a glass was obtained which afforded an improved yield in the coupling step (see **2b** → **7d**). Esters **7b–e** were purified by crystallization from MeOH or mixtures of MeOH and a hydrocarbon. Acids **2b–e** were precipitated from alkaline solution with HOAc and, except for freeze-drying, required no additional handling. The shorter-chain diepeptide **2b** and tripeptide **2c** were very DMF soluble; the tetrapeptide **2d** was soluble at room temperature but not at 0 °C, but the pentapeptide **2e** was insoluble even at room temperature and was therefore not used in further coupling reactions.

Coupling of the deprotected oligomers $H(Gab)_nGlu(O\text{-}t\text{-}Bu)_2$ to MeAPA was performed with the aid of diethyl phosphorocyanidate in DMF solution containing excess

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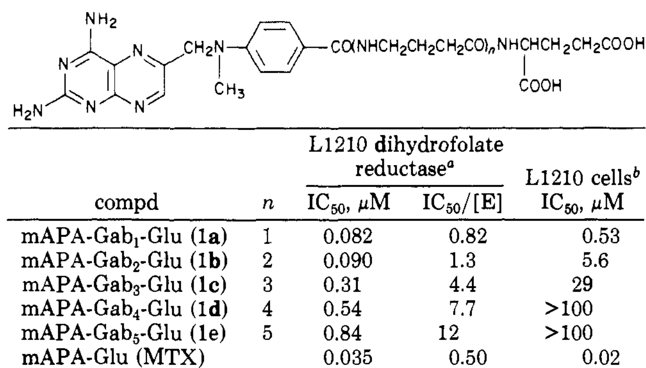
Table III. Dihydrofolate Binding and Cell Growth Inhibition by Stretched Analogues of Methotrexate



compd	n	L1210 dihydrofolate reductase[a] IC$_{50}$, μM	IC$_{50}$/[E]	L1210 cells[b] IC$_{50}$, μM
mAPA-Gab$_1$-Glu (**1a**)	1	0.082	0.82	0.53
mAPA-Gab$_2$-Glu (**1b**)	2	0.090	1.3	5.6
mAPA-Gab$_3$-Glu (**1c**)	3	0.31	4.4	29
mAPA-Gab$_4$-Glu (**1d**)	4	0.54	7.7	>100
mAPA-Gab$_5$-Glu (**1e**)	5	0.84	12	>100
mAPA-Glu (MTX)		0.035	0.50	0.02

[a] The enzyme concentration was 0.1 μM in the assay of **1a** and 0.07 μM in the assays of the other analogues; see ref 19 for assay conditions. [b] Cells were exposed to drug for 48 h; see ref 2 for assay conditions.

Et_3N. Three molar equivalents of activation reagent were used, and the H(Gab)$_n$Glu(O-*t*-Bu)$_2$ component was added only when disappearance of all the MeAPA had been confirmed by TLC. Coupling was allowed to proceed at room temperature for 3 days, and products were purified by silica gel column chromatography. Nonoptimized yields were 45% (**9b**), 28% (**9c**), 53% (**9d**), and 47% (**9e**). These yields, based on MeAPA, were somewhat lower than we have observed in other diethyl phosphorocyanidate reactions and are probably a result of the fact that intermediates **6a–e** and **8a–e** were carried through to the coupling step without purification. It is likely that accumulated impurities present during the coupling reaction contributed to our low yields. A characteristic of the diesters **9b–e**, attesting to their high degree of lipophilicity, was their apparent tendency to form solvates with $CHCl_3$ and MeOH. Although rigorous proof of the formation of such solvates was not sought for these diesters because our intent was to immediately convert them to diacids, we have previously observed tenacious $CHCl_3$ retention, as evidenced by Cl analytical data, with other lipophilic MTX ester derivatives.[17a]

Removal of the *tert*-butyl ester groups in **9b–e** was achieved readily by treatment with trifluoroacetic acid at room temperature for 10 min. The products (**1b–e**) were purified as ammonium salts on a DEAE-cellulose column, with 3% NH_4HCO_3 as the eluent after an initial wash with distilled water to remove salts. Upon freeze-drying, eluates of **1b**, **1c**, and **1e** yielded hydrated free acids (cf. Table II); compound **1d**, on the other hand, retained ammonia in addition to water.

Biological Activity

The ability of the stretched MTX analogues **1a–e** to inhibit purified DHFR from murine L1210 cells was evaluated spectrophotometrically.[18] The enzyme was obtained from an overproducing cell line (L1210/R81) and was purified by affinity chromatography.[19] The kinetic properties of the enzyme from L1210/R81 cells are the same as those of the enzyme from the wild-type line. Enzyme activity was plotted as a function of inhibitor concentration at a given DHFR concentration [E], and the IC$_{50}$ (50% inhibitory concentration) was determined. The IC$_{50}$/[E] ratio was also calculated, since this provides a convenient qualitative measure of the extent of deviation of the inhibition curve from linearity at high inhibitor concentrations.[6,16] As indicated in Table III, there was only a 2- to 3-fold difference in IC$_{50}$ between MTX and the analogues **1a** and **1b**, which contain one and two Gab spacers, respectively. With the introduction of a third Gab spacer (**1c**), the IC$_{50}$ increased 10-fold in comparison with that of MTX, and by the time five Gab spacers were added (**1e**) the IC$_{50}$ increase was 24-fold. Although the stepwise increases with each added Gab spacer did not follow an absolutely uniform pattern, the average increase per spacer was 2-fold. The IC$_{50}$/[E] ratio also increased progressively as spacers were added. These results clearly demonstrate that the spatial location of the glutamate side chain in relation to the pteridine moiety plays a major role in DHFR binding. It is known that the γ-carboxyl is much less important than the α-carboxyl in the binding of MTX to DHFR.[20,21] From this it seems reasonable to conclude that the main reason for the progressive loss of DHFR affinity in the stretched analogues **1a–e** is that it becomes increasingly difficult for the α-carboxyl in the Glu residue of these compounds to reach, and interact with, the invariant arginine residue to which the α-carboxyl of MTX normally binds.[22]

Table IV. Inhibition of *L. casei* Thymidylate Synthase by Stretched Methotrexate Analogues

compd	K_i,[a] μM
1a	0.336
1b	0.205
1c	0.130
1d	0.189
1e	0.172

[a] 5,10-Methylenetetrahydrofolate (K_m = 10 μM) was used as the variable substrate; inhibition was competitive with each compound. Assay conditions were as described in ref 26; see text (Biological Activity) for published data on MTX as an inhibitor of bacterial[23] and mammalian[10] thymidylate synthase.

In light of the important finding that MTX polyglutamates become better inhibitors of TS as the number of glutamates is increased[10,23,24] we were interested in determining whether a similar trend would be followed by analogues lacking "internal" γ-carboxyls. Compounds **1a–e** were tested as inhibitors of TS isolated from MTX-resistant *Lactobacillus casei* as previously described.[25] The rate of conversion of deoxyuridylate to thymidylate in the presence of 5,10-methylenetetrahydrofolate as the variable substrate was measured at 340 nm, and K_i's were obtained from slopes of double-reciprocal plots. As shown in Table IV, the K_i's of the stretched MTX analogues were in the 0.1–0.4 μM range. Binding appeared to increase somewhat as the number of Gab spacers went from one to three but did not increase thereafter. Inhibition was competitive with respect to 5,10-methylenetetrahydrofolate, as judged

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from the fact that double-reciprocal plots intersected the ordinate at a common point (data not shown). Our results compared well with K_i's reported recently for the binding of MTX polyglutamates to TS from a human cell (MCF-7 carcinoma).[10] With 5,10-methylenetetrahydrofolate as the variable substrate, the K_i's for MTX mono-, di-, tri-, and tetra-γ-glutamates (MTX-Glu_{2-5}) were in the 0.05–0.2 μM range, while the K_i for MTX was 13 μM.[10] Although comparable K_i's are not available for the inhibition of *L. casei* enzyme, IC_{50}'s of 0.2 μM are reported[23] for MTX-Glu_2 and MTX-Glu_4 as compared with 44 μM for MTX. The TS binding of **1a–e** therefore seems to approximate that of MTX polyglutamates of comparable chain length and to be substantially greater than that of MTX. Our results indicate that, while insertion of one or more Gab spacers results in some loss of DHFR inhibition as discussed above, this structure modification has a favorable effect in terms of TS inhibition and produces compounds with the potential of kill cells via combination of DHFR and TS inhibition.

In light of our finding that **1a–e** could compete with 5,10-methylenetetrahydrofolate for binding to TS, it was of interest to examine whether these compounds could form a ternary complex with TS and 5-fluoro-2′-deoxyuridylate (FdUMP) in the absence of reduced folate. In a nitrocellulose filter assay designed to measure tight binding of radioactive FdUMP to TS,[26] no counts above background were detected, indicating that the stretched MTX analogues cannot replace the natural folate cofactor in forming a ternary complex with TS and FdUMP.

In addition to the enzyme assays described above, growth inhibition assays against murine L1210 cells in culture were carried out. Cells were treated continuously with drugs for 48 h, and IC_{50}'s were obtained from the growth curves. As shown in Table III, a single Gab spacer (**1a**) produced a 25-fold decrease in potency relative to MTX. Addition of two spacers (**1b**) caused activity to decrease another 10-fold, and a third spacer (**1c**) led to a further 5-fold decrease. With four and five spacers (**1d**, **1e**) the IC_{50} surpassed 100 μM, and higher concentrations were not tested.

Comparison of the data for DHFR inhibition and cell growth inhibition showed that the latter dropped off more steeply than could be explained on the basis of DHFR binding alone. In the extreme case of **1e** vs. **1a** there was only a 10-fold difference in IC_{50} for DHFR inhibition but a >200-fold difference in IC_{50} for cell growth. One possible explanation for this discrepancy was that an increase in the Gab spacers in MeAPA-Gab_n-Glu to $n > 1$ results in abolition of substrate activity for folylpolyglutamate synthetase; another was that this chain lengthening has a retardant effect on cell uptake. In order to evaluate the first possibility, we compared the substrate activities of **1b–e** toward mouse liver FPGS with those of MTX and **1a**. While we had observed earlier that 1 mM **1a** reacts at a rate of 0.4 nmol/h in the presence of FPGS and 1 mM [^{3}H]glutamate as compared with 1.7 nmol/h for MTX under the same conditions,[7] **1b–e** were neither substrates nor inhibitors of FPGS in our assay. Thus, while FPGS-catalyzed addition of glutamate to **1a** may contribute to its activity, this is unlikely to be a factor with analogues containing more than one Gab spacer. Since differences in polyglutamylation clearly cannot account for the differences in IC_{50} for growth inhibition among compounds **1b–e**, we believe that their low potency is most likely due to decreased affinity for the carrier protein that mediates MTX uptake through the cell membrane. Poor uptake of these compounds would be consistent with a model of the carrier protein requiring binding sites for both the pteroyl moiety and the α-carboxyl.

It was proposed by us previously[7] that in the formation of MTX di-γ-glutamate from MTX mono-γ-glutamate the "inner" glutamate residue may form a loop to allow the α-carboxyl of the "outer" glutamate to bind to a putative basic amino acid residue in the FPGS active site. The present results demonstrating lack of FPGS substrate activity polyglutamate analogues lacking "inner" α-carboxyls is relevant to this model in that it suggests that when the loop forms it may project into a region of the enzyme that contains additional basic amino acids. The latter are perhaps regularly spaced so as to allow the repeating α-carboxyls in the loop to contribute binding energy even though they are no longer in the active site at the time. Further studies in this area would be of interest.

Experimental Section

4-[*N*-(Benzyloxycarbonyl)amino]butyric acid (**2a**) was from Sigma (St. Louis, MO), di-*tert*-butyl L-glutamate·HCl (**3**·HCl) was from Bachem (Torrance, CA), and 4-aminobutyric acid, diphenylphosphoryl azide, and isobutyl chloroformate were from Aldrich (Milwaukee, WI). MeAPA (**10**) and diethyl phosphorocyanidate were prepared as previously described.[13] *N*,*N*-Dimethylformamide (DMF) used in coupling reactions was dried over Linde molecular sieves. Ion-exchange chromatography was carried out on Whatman DE-52 (diethylamino)ethyl cellulose (preswollen), silica gel chromatography was carried out on Baker 3405 silica gel (60–200 mesh), and TLC was carried out on Eastman 13181 silica gel or Eastman 13254 cellulose sheets containing a fluorescent indicator. Infrared spectra (KBr or thin-film as appropriate) were obtained for all the protected amino acid and peptide derivatives as well as for the pteridines on a Perkin-Elmer Model 781 double-beam recording spectrophotometer and were consistent with assigned structures. NMR spectra, obtained for $CDCl_3$-soluble amino acid and peptide derivatives with a Varian T60A instrument, were similarly in agreement with structure assignments. Melting points were determined in a Mel-Temp apparatus (Laboratory Devices, Cambridge, MA) in open Pyrex capillary tubes and are not corrected. Microanalyses were performed by Galbraith Laboratories, Knoxville TN, and by Multichem Laboratories, Lowell, MA, and were within 0.4% of the theoretical values for C, H, and N.

Mixed Carboxylic Carbonic Anhydride Coupling (Method A). Preparation of 7b–e and 6a. A solution of **2a** or **2b** (0.2 M) in dry DMF at room temperature was treated successively with Et_3N (1 equiv) and *i*-BuOCOCl (1 equiv). After 20 min of stirring, **3**·HCl, **4**·HCl, or **5**·HCl (1 equiv) was added, followed by Et_3N (1 equiv). Stirring was continued for another 30 min, and the DMF was evaporated under reduced pressure (35–40 °C). Products **7b** and **7c** were partitioned between EtOAc or $CHCl_3$ and H_2O, and the organic layer was washed with H_2O, 5% $NaHCO_3$, and saturated NaCl and evaporated. Compounds **7d** and **7e** were triturated with Et_2O to remove the final traces of DMF and were then recrystallized from MeOH. Compound **7b** was recrystallized from a mixture of benzene and *n*-hexane, and compound **7c** was recrystallized from benzene and MeOH. Compound **6a** was an oil and was used without further purification.

Saponification of Esters with Barium Hydroxide (Method B). Preparation of 2b–e. A solution of **7b–e** (0.01–0.1 M) in 50% EtOH was treated with $Ba(OH)_2 \cdot 8H_2O$ (0.66 equiv), and the mixture was stirred overnight at room temperature. A slight excess of NH_4HCO_3 in a small volume of H_2O was added, and after 5 min of vigorous stirring, the $BaCO_3$ precipitate was filtered off. The filtrate was concentrated under reduced pressure to remove the alcohol, and the solution was acidified to pH 5–6 with 10% AcOH and chilled. The precipitate was collected and dried with the aid of a lyophilizer.

Diphenylphosphoryl Azide Coupling (Method C). Preparation of 6a–e. A solution of **2b–e** (0.06 M) and **3**·HCl (0.06 M) in dry DMF was cooled to 0 °C and treated with DPPA (1

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equiv) and Et_3N (3 equiv). The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure (35–40 °C), the residue was redissolved in EtOAc, and the solution was washed sequentially with H_2O, 0.1 N HCl, and saturated NaCl before being evaporated. The products were all glassy solids or gums and were used without additional purification.

Catalytic Hydrogenolysis in the Presence of AcOH (Method D). Preparation of 8b and 8c. A solution of **6b** or **6c** (0.03 M) and AcOH (equal weight) in MeOH was shaken with 5% Pd–C (20 wt %) under H_2 (3–4 atm) in a Parr apparatus overnight. The catalyst was removed, and the filtrate was concentrated to a small volume. After partitioning between CH_2Cl_2 and excess aqueous K_2CO_3, the organic layer was evaporated to a foamy gum which was used directly in the next reaction.

Catalytic Hydrogenolysis in the Presence of HCl (Method E). Preparation of 8a, 8d, and 8e. A solution a **6a**, **6d**, or **6e** (0.01 M) in MeOH containing 1 equiv of HCl gas was hydrogenated for 1 h in the presence of 5% Pd–C (20–30 wt %). The catalyst was filtered off, the solvent was evaporated, and the residual HCl salts (foamy gums) were used without further treatment.

Diethyl Phosphorocyanidate Coupling (Method F). Preparation of 9b–e. To a solution of DEPC (0.09 M) and Et_3N (0.09 M) in DMF were added small portions of compound **10** (0.33 equiv). The solution was left overnight and checked by TLC (silica gel, 4:1 $CHCl_3$–MeOH) to confirm that activation was complete. If unreacted **10** was detected, additional equivalent amounts of DEPC and Et_3N were added, and the mixture was left to stand for several more hours. To the solution was then added the amine **8b** or **8c** (or the salt **8a**, **8d**, or **8e** with an extra equiv of Et_3N), and the mixture was left for 3 days. After evaporation of the DMF under reduced pressure (35–40 °C), the residue was partitioned between $CHCl_3$ and dilute NH_4OH (with some MeOH included in the workup of the product from **8e**). The organic layer was evaporated, and the residue was chromatographed on silica gel. For compounds **9b** and **9c**, the column was eluted with 19:1 followed by 9:1 $CHCl_3$–MeOH. For **9d**, 9:1 and 6:1 $CHCl_3$–MeOH were used, and for **9e**, 6:1 $CHCl_3$–MeOH was used.

Ester Cleavage with Trifluoroacetic Acid (Method G). Preparation of 1b–e. The diester (**9b–e**) was dissolved in TFA (5–10 v/w), and the solution was kept at room temperature for 10 min before being evaporated under reduced pressure. The residue was triturated with Et_2O to remove final traces of TFA, and the Et_2O was decanted off. The remaining powder was taken up in a large volume of NH_4OH adjusted to pH 8, and the solution was desalted on a DEAE-cellulose column (75–100 w/w, HCO_3^- form) by elution with H_2O. When desalting was complete, the product was eluted with 3% NH_4HCO_3. TLC homogeneous fractions were pooled and freeze-dried. Compounds **1b–d** had R_f 0.7–0.8 (cellulose, pH 7.4 phosphate buffer) and compound **1e** had R_f 0.6 (cellulose, 3% NH_4Cl).

Enzyme Inhibition. DHFR inhibition was measured spectrophotometrically at 340 nm by using purified enzyme from L1210/R81 cells as previously described.[19] The cuvette contained 0.07 μM DHFR, 75 μM NADPH, 50 μM dihydrofolate reductase, and 50 mM Tris-Cl, pH 7.5, in a volume of 1 mL. The reaction was initiated by adding the dihydrofolate to the other components after a 2-min preincubation at 22 °C.

FPGS inhibition assays were carried out as previously reported,[7] by using a partially purified enzyme preparation from mouse liver. The compounds (0–1.0 mM) were incubated with enzyme for 1 h at 37 °C in a mixture consisting of 1.0 mM L-[^{3}H]glutamic acid (4 mCi/mmol), 5 mM ATP, 10 mM $MgCl_2$, 30 mM KCl, 20 mM α-thioglycerol, and 200 mM Tris, pH 8.6, in a volume of 0.25 mL.

The TS assays were performed spectrophotometrically at 340 nm according to the method of Wahba and Friedkin.[26] The enzyme was obtained from MTX-resistant *L. casei* as previously described,[25] by using 5,10-methylenetetrahydrofolate prepared by DHFR reduction of dihydrofolate followed by reaction with formaldehyde.[27] The initial reaction mixture consisted of 0.008 unit of enzyme, 5,10-methylenetetrahydrofolate (2–20 μM), and various concentrations of inhibitor in 100 mM Tris, pH 7.5, containing 20 mM 2-mercaptoethanol in a total volume of 0.95 mL. The solution was prewarmed to 37 °C for 5 min, and then 0.05 mL of a 2 mM solution of dUMP was added to initiate the reaction.

In the formation of ternary complexes of TS, FdUMP, and compounds **1a–e**, a mixture of TS (0.1 μM), [6-^{3}H]FdUMP (1.0 μM), and the analogue (10 μM) was incubated at 32 °C for 30 min in 0.2 mL of Tris, pH 7.5, containing 20 mM 2-mercaptoethanol. Binding of ligands to TS was then determined by nitrocellulose filtration assay as previously described.[26]

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Registry No. **1a**, 91425-22-8; **1b**, 103321-29-5; **1c**, 103321-30-8; **1d**, 103321-31-9; **1d**·1.5NH_3, 103321-48-8; **1e**, 103321-32-0; **2a**, 5105-78-2; **2b**, 1489-76-5; **2c**, 90139-74-5; **2d**, 103321-33-1; **2e**, 103321-34-2; **3**·HCl, 32677-01-3; **4**·HCl, 13031-60-2; **5**·HCl, 103321-35-3; **6a**, 103321-36-4; **6b**, 103321-37-5; **6c**, 103346-42-5; **6d**, 103346-43-6; **6e**, 103346-44-7; **7b**, 90139-76-7; **7c**, 90139-67-6; **7d**, 90139-68-7; **7e**, 103321-38-6; **8a**·HCl, 103321-39-7; **8b**, 103321-40-0; **8c**, 103321-41-1; **8d**·HCl, 103321-42-2; **8e**·HCl, 103321-43-3; **9b**, 103321-44-4; **9c**, 103321-45-5; **9d**, 103321-46-6; **9e**, 103321-47-7; **10**, 19741-14-1; dihydrofolate reductase, 9002-03-3; thymidylate synthase, 9031-61-2.

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