Synthesis and Biological Activity of Open-Chain Analogues of 5,6,7,8-Tetrahydrofolic Acid—Potential Antitumor Agents

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This study describes the synthesis and in vitro antitumor activity of inhibitors of purine de novo biosynthesis that are analogues of *N*-[4-[[3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]benzoyl-L-glutamic acid (5-DACTHF). Benzene ring substituted analogues were synthesized from a protected pyrimidinyl propionaldehyde and a substituted benzoyl glutamate moiety by a key reductive amination step. Pyrimidine and linking chain substituted analogues were built up stepwise from *p*-aminobenzoic acid or analogues. The compounds were tested as inhibitors of methotrexate uptake as a measure of binding to the reduced folate transport system, as inhibitors of glycinamide ribonucleotide transformylase, as substrates for folylpolyglutamate synthetase, and as inhibitors of tumor cell growth in cell culture. With the exception of 2'-F substituent, the ring-substituted analogues are less active than the parent compound. Replacement of the 10-nitrogen by carbon, sulfur, or oxygen produced less than 2-fold changes to biological activity in vitro. A four-atom linking chain and an amino group at the 2-position on the pyrimidine ring are important for good activity.

During the past several decades, the role of folic acid analogues, especially methotrexate (MTX), in cancer chemotherapy has been intensively investigated.[1,2] The enzyme dihydrofolate reductase (DHFR) has been the primary target of this effort. The recent introduction of 10-ethyl-10-deazaaminopterin (10-EDAM), piritrexim (PTX), and trimetrexate (TMX) into clinical trials attests to the continued interest in this area.[3]

More recently, the search for new therapeutic targets has focused on the folate-utilizing enzymes in de novo purine and pyrimidine biosynthesis.[4-7] In the purine biosynthetic pathway, two enzymes utilize 10-formyl-5,6,7,8-tetrahydrofolate (10-CHO-THFA) as a cofactor: glycinamide ribonucleotide transformylase (GAR-Tfase, EC 2.1.2.2) and aminoimidazolecarboxamide ribonucleotide transformylase (AICAR-Tfase, EC 2.1.2.3).[8] It has been suggested that DHFR inhibitors such as MTX act in part through the inhibition of purine biosynthesis. This activity results from direct inhibition of AICAR-Tfase or indirectly through the buildup of inhibitory levels of dihydrofolate polyglutamates.[9,10]

The extent of selective toxicity is still unknown for specific purine biosynthesis inhibitors. Several groups have demonstrated higher levels of de novo purine biosynthesis in cancer cells than in normal cells.[5-7] Differences in polyglutamation between cancerous and normal cells also may form the basis for selectivity of folic acid analogues. The potential for serious side effects with these substances is still strong. Suppression of humoral immunity by MTX and TMX appears to be a result of inhibition of purine biosynthesis in B-cells.[11] Other inhibitors of purine base synthesis, such as the thiopurines, are immunosuppressive as a result of cytotoxic activities against lymphocytes and are antiinflammatory by virtue of inhibition of hematopoietic precursor replication.[12]

Recently, Taylor and co-workers reported the synthesis of 5,10-dideazatetrahydrofolic acid (DDATHF)[13] and 5-deaza-5,6,7,8-tetrahydrofolic acid (5-DATHF).[14] DDATHF inhibits de novo purine biosynthesis at the GAR-Tfase step and has potent in vitro and in vivo antitumor activity.[13,15] Subsequently, Taylor and collaborators described several new synthetic routes to DDATHF and analogues, including a stereospecific synthesis,[16] as well

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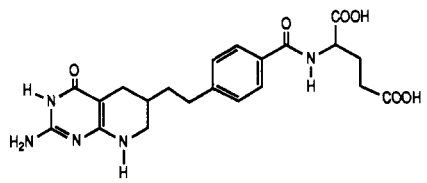

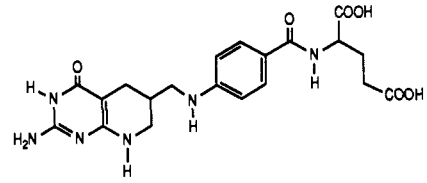

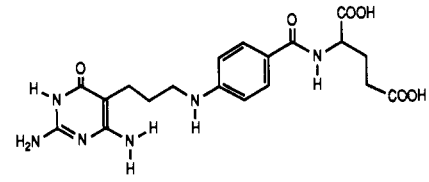

as the synthesis of a ring-opened analogue of DDATHF.[17] In addition, the synthesis of 10-methyl-DDATHF was reported by Taylor,[18] and the synthesis and biological activity of 10-alkyl-5,10-dideaza analogues of tetrahydrofolic acid (THFA) were described by DeGraw and coworkers.[19,20]

Reports on other inhibitors of purine biosynthesis have also appeared. MTX polyglutamates[21] and a metabolite of MTX, 7-hydroxymethotrexate pentaglutamate,[22] are inhibitors of AICAR-Tfase. Similarly, tetrahydrohomofolate polyglutamates are inhibitors of GAR-Tfase.[23] Various N-10-substituted derivatives of 5,8-dideazafolic acid (5,8-DDF) are potent inhibitors of GAR-Tfase,[24] but these compounds block other folate requiring enzymes as well. These studies highlighted the GAR-Tfase's tolerance to bulky substituents around N-10. Hynes et al. showed that 10-thia-5,8-DDF is a good inhibitor of GAR-Tfase.[25]

Two other papers describe compounds that have structures similar to those reported here. Kotva et al.[26] reported the synthesis of 4-[ω-(2-amino-6-hydroxy-4-oxo-3,4-dihydro-5-pyrimidinyl)alkyl]benzoic acids, which are open-chain analogues of pteroic acid that possess antineoplastic activity. A pyrimidine analogue of folic acid, namely *N*-[*p*-[[(2,4-diamino-6-hydroxy-5-pyrimidinyl)-methyl]amino]benzoyl]-L-glutamic acid, was found by Berezovskii and co-workers to inhibit DHFR.[27]

Kelley and co-workers recently described the synthesis and biological activity of a ring-opened analogue of 5-deaza-THFA, namely *N*-[4-[[3-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]benzoyl]-L-glutamic acid or 5-DACTHF (1).[28,29] This compound is a selective inhibitor of GAR-Tfase and, to a lesser extent, AICAR-Tfase, but is inactive against other folate-requiring enzymes. In cell culture, 1 inhibited tumor cell growth at concentrations below 100 nM. A chain-shortened analogue of 1 was also prepared and found to be less active in vitro.[31] Folylpolyglutamate synthetase (FPGS) converts the compound to longer polyglutamates[32] that are more potent GAR-Tfase and AICAR-Tfase inhibitors.[28] This result was confirmed by the chemical synthesis and biological evaluation of a series of polyglutamates of 1.[28,30] Tumor cells grown in vitro concentrated compound 1 300-fold, and the compound existed predominately as its polyglutamated

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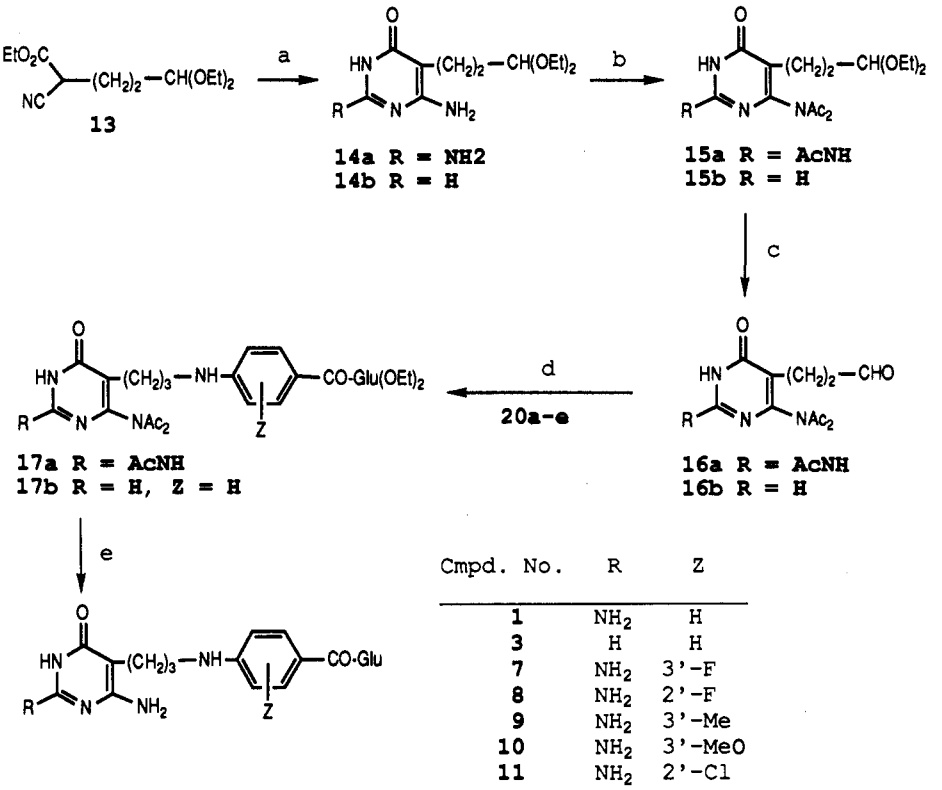
Scheme I[a]



Cmpd. No.	R	Z
1	NH_2	H
3	H	H
7	NH_2	3'-F
8	NH_2	2'-F
9	NH_2	3'-Me
10	NH_2	3'-MeO
11	NH_2	2'-Cl

[a] (a) Guanidine·HCl (series a) or formamidine·HOAc (series b), NaOEt, EtOH, heat. (b) 1:1 pyridine–Ac_2O, DMAP, N_2, 90 °C, 5 h. (c) H_2O, 52 °C, 3.5 h, N_2. (d) (1) NH_2-C_6H_3(Z)-CO-Glu$(OEt)_2$ (**20a–e**), EtOH, HOAc, 3-Å sieve, 25 °C, 3 h. (2) $NaBH_3CN$, 25 °C, 23 h. (e) 2:1 1 N NaOH–EtOH, 60 °C, 18 h, then HOAc. Glu$(OEt)_2$ = diethyl L-glutamate.

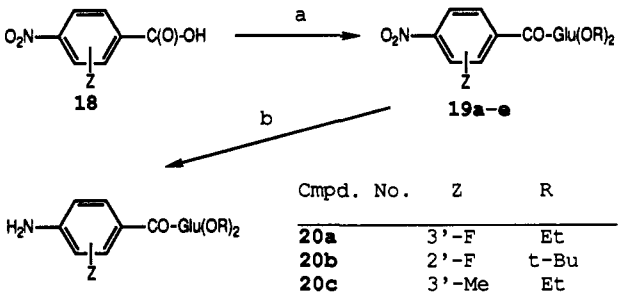
metabolites.[32] In mice, 5-DACTHF has a half-life of 2.15 h and is concentrated in kidney, pancreas, and liver to levels well above plasma concentrations.[32] From these data, it was concluded that cell-growth inhibition, for this class of compounds, depends not only on GAR-Tfase (or AICAR-Tfase) inhibition but also on transport and polyglutamation.

In this paper we describe the synthesis of 5-DACTHF analogues that are modified at the 2-position of the pyrimidine ring, at the 10-position, and on the benzoyl ring (see Table I) in an effort to define the requirements for inhibition of tumor cell growth.[33] The compounds were evaluated as substrates for the reduced folate transport system and FPGS, as inhibitors of GAR-Tfase and AICAR-Tfase, and as inhibitors of MCF-7 human breast carcinoma cell growth in culture so that the structure–activity relationship (SAR) could be studied at each of the important steps involved in antitumor activity.

Chemistry

The synthetic route used to prepare analogues of 5-DACTHF modified in the 2-position of the pyrimidine ring and substituted in the benzene ring was analogous to that reported by Kelley et al. (see Scheme I).[28,31] Thus, condensation of cyanoacetate ester **13** with formamidine acetate in refluxing NaOEt/EtOH gave the pyrimidine **14b** in 53% yield. Acetylation of the amino group with pyridine–acetic anhydride gave the acetal **15b** (67%).

Scheme II[a]



Cmpd. No.	Z	R
20a	3'-F	Et
20b	2'-F	t-Bu
20c	3'-Me	Et
20d	3'-MeO	Et
20e	2'-Cl	Et

[a] (a) L-Glu$(OEt)_2$·HCl, *N*-methylmorpholine, DCC, HOBT, DMF, CH_2Cl_2, N_2, RT. (b) 10% Pd/C, 40 psi H_2, 95% EtOH, 1.5 h, RT.

Very mild hydrolysis of the acetal in water at 50 °C gave the aldehyde **16b** in high yield. In the acetal hydrolysis step, heating an aqueous solution of **15a** on a steam bath also removed an *N*-acetyl moiety to produce a 2,4-bisacetamido intermediate, which exists as the cyclized hemiaminal, that could also be utilized in the reductive amination step. The facile hydrolysis of pyrimidylpropionaldehyde acetals has been described previously.[28,34] Partial hydrolysis of the imide group could provide HOAc as a catalyst, but this cannot be the only explanation since Baker's example had no imide. Perhaps the 6-oxo function assists with the hydrolysis by trapping the carbenium ion or, when in the lactim tautomer, by hydrogen bonding to the departing ethoxy group.

Ring-substituted (*p*-aminobenzoyl)glutamate esters **20a–e** were prepared as shown in Scheme II. The coupling

(33) A portion of this work was presented at the 9th International Symposium on Pteridines and Folic Acid Derivatives, Zurich, Switzerland, Sept. 3–8, 1989. See Bigham, E. C.; Duch, D.; Ferone, R.; Kelley, J.; Smith, G. K. Mono cyclic Tetrahydrofolate Analogues as Inhibitors of De Novo Purine Biosynthesis. *Chemistry and Biology of Pteridines, 1989*; Curtius, H.-Ch., Ghisla, S., Blau, N., Eds.; de Gruyter: Berlin, 1990; pp 961–4.

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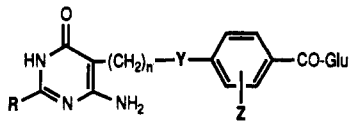
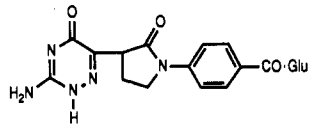
Table I. Structures and in Vitro Biological Testing Data



compd no.	structure R	n	Y	Z	IC_{50}, μM GAR-Tfase[a]	AICAR-Tfase[b] (or %I at [d])	MTX-uptake[c]	FPGS:[d] V_{max}/K_m, %/μM	cell growth:[e] IC_{50}, μM
1	NH_2	3	NH	H	3.0	94.0	1.2	106/7.3	0.037
2	NH_2	4	NH	H	18.2	ND	1.5	48/50	5.00
3	H	3	NH	H	52.0	26% at 123	2.0	131/13.4	>50
4	NH_2	3	S	H	2.6	25.8	0.8	125.5/26.7	0.023
5	NH_2	3	O	H	5.3	ND	1.1	124/47.6	0.085
6[f]	NH_2	3	CH_2	H	1.6	ND	0.9	132/46.9	0.061
7	NH_2	3	NH	3'-F	3.5	133.0	1.0	110/12.7	0.063
8	NH_2	3	NH	2'-F	3.1	79.3	0.7	107.3/4.3	0.033
9	NH_2	3	NH	3'-Me	6.1	ND	1.3	16.5/68	0.25
10	NH_2	3	NH	3'-MeO	18.7	ND	1.0	53.3/174	5.00
11	NH_2	3	NH	2'-Cl	6.6	182.0	0.7	99.6/7.4	0.115
12					>100	ND	>100	ND	>100
DDATHF[g]					0.22	130	1.5	97/17.5	0.018

[a] Hog liver GAR transformylase with (6R)-10-formyl-FH4 as cofactor.[28] [b] MOLT-4 T-cell leukemia cell AICAR transformylase.[28] [c] Inhibition of [^{3}H]methotrexate transport into MOLT-4 cells.[46] [d] Hog folylpolyglutamate synthase; V_{max}, %, is relative to aminopterin.[28] [e] Inhibition of growth of MCF-7 human breast adenocarcinoma using 72 h of continuous exposure. [f] Reference 17. [g] 5,10-Dideazatetrahydrofolic acid (ref 13).

of substituted 4-nitrobenzoic acids to diethyl glutamate by the DCC/HOBT method gave the nitro esters **19** in 50–60% yields. Subsequent catalytic hydrogenation in a Parr shaker gave amino esters **20** in very high yields.

The aldehyde **16a**[28] and anilines **20a–e** were subjected to reductive amination conditions. The 2-desamino aldehyde **16b** was reductively aminated with diethyl (*p*-aminobenzoyl)glutamate to produce **17b**. These reactions, despite considerable effort, gave products in yields ranging from 32% to 65%. Substitution ortho to the amino group was especially detrimental to the yield. We observed recently that heating the reductive amination mixture resulted in improved yield and purity of analogous products in similar reactions not reported here. Several alternative reductive amination procedures including (1) Me_2NH–BH_3 in HOAc, (2) imine formation in benzene or CH_2Cl_2 with *p*-toluenesulfonic acid followed by $NaBH_4$ reduction, and (3) the benzotriazole adduct method described by Katritzky et al.[35] were tried without success.

In the final step, the ester groups were hydrolyzed by warming the intermediates **17** in 1:1 EtOH–1 N NaOH for 2–18 h. We were concerned about the possibility of racemization of the glutamate moiety during the basic hydrolysis step. The parent compound **1** was shown to be a single peak by the chiral HPLC method of Cramer et al. which easily separated D- and L-methotrexate.[36] Because the peak for **1** was broad, the Cramer method was not suitable for the detection of small amounts of enantiomer. To confirm the enantiomeric purity, the glutamate groups from several examples were cleaved with 6 N HCl, and the liberated amino acid was derivatized with Marfey's reagent, then assayed by HPLC.[37] The samples contained about 5% D-glutamate, which is the same amount found in acid cleaved but non-base-hydrolyzed samples. We concluded that basic hydrolysis had not caused detectable racemization. The small amount of D-isomer that was found appears to have resulted from the acidic cleavage conditions.

Purification of the final products was often difficult. On a 100-mg scale, the compounds could be purified by preparative reversed-phase chromatography with 0.1% TFA (trifluoroacetic acid) in MeCN–H_2O as the eluting solvent. Compounds with low solubility in acid solutions benefited from reversed-phase chromatography using 0.1% triethylammonium trifluoroacetate in MeOH–H_2O as the mobile phase. For larger scale work, precipitation from weakly acidic solutions or recrystallization from methanol or H_2O was helpful in a few cases, but the purity of many compounds was not improved by this process. For these reasons, careful purification at the ester stage prior to hydrolysis and precipitation was important; but this step was often made difficult by the partial loss of the acetyl protecting groups during the reductive amination process and during chromatography on silica gel, resulting in complex mixtures of partially protected intermediates.

A slightly different scheme, based on the work of Baker et al.,[38] was developed for the preparation of the linking-chain-modified compounds **2**, **4**, and **5**. As shown in Scheme III, the tosylanilide **21a** was alkylated with 1,4-dibromobutane to produce the bromobutyl derivative **22a** (78% yield). Displacement by cyanoacetate anion in EtOH gave the protected cyano diester **23a** (50%). Guanidine

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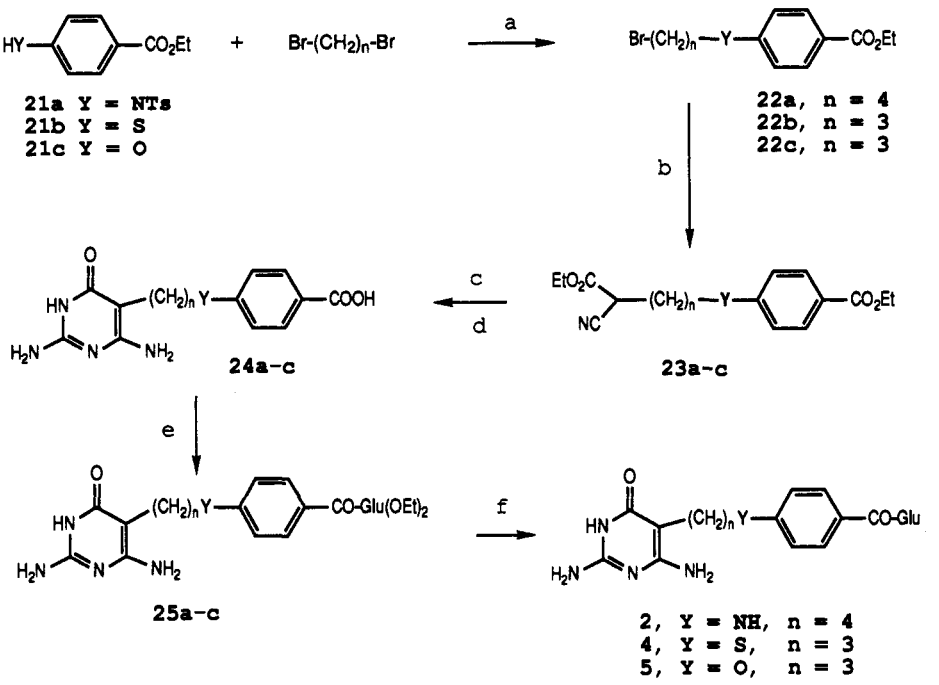
Scheme III[a]



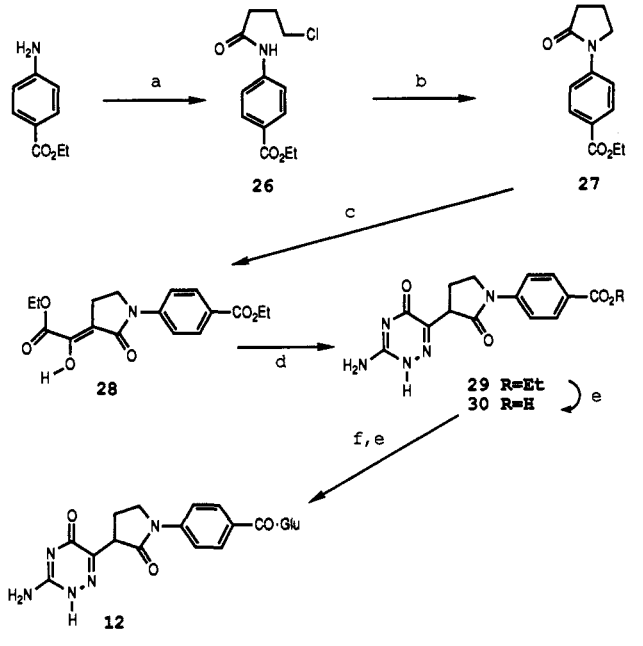
[a] (a) Base. (b) $NaCH(CO_2Et)CN$, EtOH, reflux, 6 h. (c) Guanidine·HCl, NaOEt, EtOH, reflux, (d) 1:1 1 N NaOH–EtOH, 60 °C, 18 h and then HCl. (e) L-Glu$(OEt)_2$·HCl, *N*-methylmorpholine, DCC, HOBT, DMF, N_2, RT, 12–59 h. (f) 0.1–1 N NaOH, EtOH, 40 °C, 2 h, N_2. (2) pH 3.5. Ts = tosyl.

cyclization and subsequent base hydrolysis proceeded in high yield to the benzoic acids **24**. Unfortunately, the benzoic acids were not good substrates for coupling with glutamate esters under normal peptide bond formation conditions. Dicyclohexylcarbodiimide (DCC) mediated coupling gave modest yields in this case. The tosyl group was cleaved with HBr–HOAc, and the esters were hydrolyzed with NaOH without purification.

Compounds with sulfur (**4**) and oxygen (**5**) in place of the anilino nitrogen were synthesized by a similar route (Scheme III). In these examples a thiophenol **21b** (from the disulfide by $NaBH_4$ reduction) or phenol **21c** was alkylated with dibromopropane to obtain the bromopropyl adducts **22b,c**. These intermediates were converted to the cyano esters **23b,c** and subsequently to the benzoic acids **24b,c** in good yields as described above. Much difficulty was experienced during the subsequent reactions, however. In the thioether example, glutamate coupling with DCC proceeded in 53% yield, which was typical for these compounds, but the ester hydrolysis gave an impure product that was difficult to purify. Ultimately, the desired compound was isolated in 41% yield after semipreparative reversed-phase chromatography. The 10-oxa compound proved to be even more difficult. Mixed anhydride coupling on **24c** gave only a 23% yield of ester **25c**. Furthermore, hydrolysis followed by reversed-phase chromatography gave the final product in only 24% yield.

The effects of changes in the pyrimidine portion of the molecule were also of interest. For the initial target, 3-amino-1,2,4-triazin-5-one **12**, an alkylated *p*-aminobenzoate with a terminal α-keto ester in the alkyl group was required. Toward this end, lactam **27**[45] was acylated with diethyl oxalate to obtain the lactam **28** in 59% yield as shown in Scheme IV. NMR revealed that this material existed predominately as the enol tautomer in DMSO. This substance was converted to the triazinone ester **29** by aminoguanidine cyclization (96%). Mild basic hydrolysis gave the benzoic acid **30** (56%), while prolonged heating in dilute base produced substantial amounts of

Scheme IV[a]



[a] (a) $Cl(CH_2)_3COCl$, Et_3N, CH_2Cl_2. (b) *t*-BuOK, THF. (c) NaH, THF, EtO_2CCO_2Et. (d) Aminoguanidine, EtOH, H_2O, heat. (e) (i) NaOH, (ii) HOAc. (f) L-Glu$(OEt)_2$·HCl, DCC, HOBT, *i*-Pr_2NEt.

lactam-opened diacid as a contaminant. Upon treatment with hot HOAc, the diacid relactamized to **30**. Attempted hydrolysis under more drastic conditions resulted in destruction of the triazinone ring. The diethyl glutamate coupling proceeded well under DCC or mixed anhydride conditions. Unfortunately, base hydrolysis of the ester groups and reversed-phase chromatography gave the desired compound **12** in low yield.

Attempts to prepare a 2-amino-4,6-dihydroxypyrimidinyl analogue by methods analogous to Schemes I or III were not successful.

Biological Results and Discussion

Smith et al. showed that GAR-Tfase has a low degree of control over the purine pathway in the uninhibited state.[39] Thus the enzyme must be inhibited substantially before any effect on purine biosynthesis would be seen. Hence, desirable traits for folate analogues that act as inhibitors of GAR-Tfase are that they be excellent substrates for the reduced folate transport system and for FPGS, which act in concert to accumulate and activate the inhibitors. The compounds described above were tested as inhibitors of hog liver GAR-Tfase, AICAR-Tfase from MOLT-4 leukemia T-cells, inhibition of MTX uptake into MOLT-4 cells, substrate activity on hog liver FPGS, and growth inhibition of MCF-7 human breast adenocarcinoma in culture.[28,46] The data from these studies are shown in Table I.

GAR-Tfase inhibition was moderately affected by substitution in the bridging group and benzene ring, but changes in the pyrimidine ring led to great reductions in activity. The best compounds in this series were 10- to 15-fold less potent than DDATHF against GAR-Tfase.

A bridging chain of four atoms seemed to be optimal for GAR-Tfase inhibition, but the type of atom in the 10-position was unimportant in this test. Lengthening the chain to five atoms, as in the acyclohomofolate analogue **2**, resulted in a 6-fold reduction in potency. Kelley et al. showed that a three-atom chain is also detrimental.[28,31] Replacement of the nitrogen at the 10-position of **1** by sulfur (**4**), oxygen (**5**), or carbon (**6**) had only a slight effect on the potency of the compounds. The result with the carbon isostere, **6**, was interesting because it should prefer an out-of-plane conformation around the C10-to-benzene bond, whereas the 10-N, 10-S, and 10-O compounds are probably coplanar. Thus GAR-Tfase did not appear to be very specific in its interactions around the 10-position of the inhibitors. This result is understandable because the 10-formyl group on the cofactor must be free to interact with the substrate during the enzymatic process.

Substitution on the benzene ring had interesting effects on GAR-Tfase inhibition. A small group, i.e. F, in the 2′- and 3′-positions (**8** and **7**), had no effect. Somewhat larger groups, i.e. 2′-Cl (**11**) and 3′-CH_3 (**9**), reduced potency 50%, and the addition of a 3′-OCH_3 resulted in a 6-fold loss of potency. Thus, in contrast to the freedom of modification at the 10-position, substituents on the benzene ring are limited to H or F. Alterations to the pyrimidine ring resulted in large losses of potency. Replacing the 2-NH_2 with H, as in **3**, raised the IC_{50} value 17-fold, and the triazinone analogue **12** was inactive.

These 5-deaza-THFA analogues are not particularly potent inhibitors of AICAR-Tfase. The 10-thia analogue, **4**, was the most potent AICAR-Tfase inhibitor, with an IC_{50} value = 25.8 μM. If one assumes that potency toward AICAR-Tfase increases 100-fold and toward GAR-Tfase 10-fold upon polyglutamation, as is the case with 5-DACTHF (**1**),[28] then both enzymes should be inhibited by 4 more or less equally within cells.

In contrast to inhibition of the transformylase enzymes, affinity for the reduced folate transport system was not significantly affected by the structural changes encompassed in close analogues of 5-DACTHF (**1**). IC_{50} values for inhibition of radiolabeled MTX uptake range from 0.7 to 2.0 μM. The more significant structural changes found in triazinone **12** resulted in loss of affinity for the transport protein.

On the other hand, FPGS activity was quite sensitive to structural modifications. Among the compounds tested in this study, the 2′-F derivative **8** appears to be the best substrate for FPGS. In comparison to **1**, compounds **3–6** showed only small changes in relative velocity but showed significant increases in K_m values, which resulted in lower pseudo rate constants V_{max}/K_m. Even the 3′-F compound **7** was 2-fold less active than 5-DACTHF, **1**. Compounds **2**, **9**, and **10** had reduced V_{max} values as well as increased K_m values. Because the FPGS assay conditions utilized in this study measured essentially the addition of one glutamate to the molecule, the results did not necessarily indicate the ability of the compounds to be converted to the important longer polyglutamates.

The inhibition of tumor cell growth in vitro is related to combined activity on GAR-Tfase, transport, and FPGS. The combination of reduced potency as GAR-Tfase inhibitors and greatly reduced activity as FPGS substrates resulted in much weaker tumor cell growth inhibition for **2**, **9**, and **10**. In spite of having 15-fold higher IC_{50} values against GAR-Tfase, the acyclo compounds **1**, **4**, and **8** are only 2-fold less potent than DDATHF as inhibitors of tumor cell growth in culture. This result might be explained by a combination of better transport and better polyglutamation, perhaps to higher polyglutamates, in the acyclo series. One might expect that small differences seen in cell culture experiments would be amplified in animal model experiments where exposure to the compounds is not constant.

In conclusion, potent inhibitors of tumor cell growth were prepared by the modification of 5-DACTHF. The substitution of carbon, oxygen, and sulfur for nitrogen at the 10-position or the addition of F at the 2′- or 3′-positions resulted in compounds that retained the potent activity of the parent. However, alterations in the bridging-chain length or in the pyrimidine ring resulted in loss of activity. None of these ring-opened analogues were as potent in vitro as DDATHF. The bicyclic ring system of DDATHF is more rigid than that of the open-chain compounds, suggesting that the design and synthesis of analogues of **1** that contain a more conformationally-restrained bridging chain is desirable.

Experimental Section

Melting points were obtained on a Thomas-Hoover capillary melting point apparatus. IR spectra (KBr) were recorded on a Perkin-Elmer 1470 spectrophotometer. UV spectra were obtained on a Varian DMS 300 spectrophotometer. NMR spectra were obtained on Varian XL200 or XL300 spectrometers. Chemical ionization mass spectra were obtained by Oneida Research Services, One Halsey Rd., Whitesboro, NY, 13492. Elemental analyses (Atlantic Microlabs, Inc., Atlanta, GA.) were within 0.4% of theoretical values unless noted otherwise. Several intermediates were used without complete purification as indicated by the absence of elemental analysis.

Abbreviations: PABG = *N*-(*p*-aminobenzoyl)-L-glutamic acid; $Glu(OEt)_2$ = diethyl L-glutamate; TFA = trifluoroacetic acid, DMF = dimethylacetamide; EtOAc = ethyl acetate; HOAc = acetic acid; THF = tetrahydrofuran; DCC = 1,3-dicyclohexylcarbodiimide; HOBT = *N*-hydroxybenzotriazole; CI-MS = chemical ionization mass spectrum; FAB-MS = fast-atom bombardment mass spectrum; SiO_2 = Silica Gel 60, RT = room temperature.

Ethyl 4-[(4-Bromobutyl)tosylamino]benzoate (22a). A solution of 10.00 g (0.0313 mol) of ethyl 4-(tosylamino)benzoate[38] (**21a**) in 55 mL of dry DMF was added dropwise over a 21-min period to 0.892 g (0.0372 mol) of NaH (from washing 1.11–1.12 g of an 80% oil dispersion with hexanes under N_2) in 26 mL of dry DMF. When the stirred mixture had cooled to RT, to it was

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added at one time 27.74 g (0.128 mol) of 1,4-dibromobutane. The stirred mixture was heated for 4.75 h at 80 °C, cooled to RT, and kept under N_2 overnight. The mixture was concentrated under vacuum at 40 °C to an oil–solid mixture, yield 22.37 g. EtOAc (100 mL) was added, and undissolved solid was filtered. The filtrate was purified by flash chromatography on 700 g of SiO_2 with 6:1 hexanes–EtOAc as the eluting solvent. The fractions containing pure product were combined and concentrated under vacuum to a colorless oil that solidified to a white solid: yield 10.91 g (77%); mp 52–54 °C; NMR (Me_2SO-d_6) δ 1.29 (t, J = 7.1 Hz, 3 H, Me), 1.3–1.5 (m, 2 H, NCH_2CH_2), 1.7–1.9 (m, 2 H, $BrCH_2CH_2$), 2.37 (s, 3 H, ArMe), 3.47 (t, J = 6.6 Hz, 2 H, NCH_2), 3.60 (t, J = 6.8 Hz, 2 H, $BrCH_2$), 4.29 (q, J = 7.1 Hz, 2 H, OCH_2), 7.23 (d, J = 8.5 Hz, 2 H, 3′- and 5′-H), 7.3–7.5 (AA′BB′, 4 H, tosyl), 7.91 (d, J = 8.5 Hz, 2 H, 2′- and 6′-H). Anal. ($C_{20}H_{24}BrNO_4S$) C, H, Br, N, S.

Ethyl 4-[(5-Cyano-6-ethoxy-6-oxohexyl)tosylamino]benzoate (23a). A mixture of 0.506 g (0.0220 mol) of Na in 10 mL of absolute EtOH under N_2 was refluxed briefly until a solution formed and then was cooled to RT. At that point, ethyl cyanoacetate (2.74 g, 0.0242 mol) was added dropwise over a 2-min period. The mixture was refluxed for 10 min and was allowed to cool to RT. Then to it was added over a 6-min period 5.00 g (0.0110 mol) of **22a** and 15 mL of rinse absolute EtOH. The mixture was refluxed under N_2 for 6 h, allowed to cool to RT, and allowed to stand overnight. Following neutralization with HOAc, the solution was concentrated under vacuum to a solvent damp solid. The solid was suspended in H_2O and extracted with Et_2O. The Et_2O washes were combined, dried ($MgSO_4$), filtered, and concentrated under high vacuum at 60 °C to a viscous, pink oil, yield 5.97 g. The oil was purified by flash chromatography on 400 g of SiO_2 with 3:1 hexanes–EtOAc as the eluting solvent. Fractions containing pure product were combined and concentrated under vacuum to a colorless, viscous oil: yield 2.76 g (50%); NMR (Me_2SO-d_6) δ 1.19 (t, J = 6.5 Hz, 3 H, OCH_2CH_3), 1.29 (t, J = 7.1 Hz, 3 H, $ArCOOCH_2CH_3$), 1.3–1.5 (m, 4 H, CCH_2CH_2C), 1.65–1.85 (m, 2 H, $CHCH_2$), 2.37 (s, 3 H, ArMe), 3.5–3.7 (m, 2 H, NCH_2), 4.14 (t, J = 6.6 Hz, 1 H, CH), 4.15 (q, J = 6.9 Hz, 2 H, OCH_2), 4.29 (q, J = 7.1 Hz, 2 H, $ArCOOCH_2$), 7.22 (d, J = 8.7 Hz, 2 H, 3′- and 5′-H), 7.3–7.5 (AA′BB′, 4 H, tosyl), 7.90 (d, J = 8.7 Hz, 2 H, 2′- and 6′-H).

4-[[4-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)butyl]tosylamino]benzoic Acid (24a). To a solution of NaOEt from 0.377 g (0.0164 mol) of Na in 8 mL of absolute EtOH under N_2 was added 1.04 g (0.0109 mol) of guanidine·HCl and 1 mL of rinse absolute EtOH. The mixture was stirred for 30 min, and to it was added a solution of 2.73 g (0.00546 mol) of **23a** in 6 mL of absolute EtOH and then 7 mL of rinse absolute EtOH. The mixture was refluxed for 4 h, cooled to RT, and kept under N_2 overnight. The reaction mixture was filtered by suction, and the filtrate was neutralized with 0.984 g (0.0164 mol) of glacial HOAc. The solution was concentrated under vacuum at 30 °C to a solid that was washed well with H_2O and dried under vacuum at 60 °C, yield 2.76 g. NMR (Me_2SO-d_6) and chemical ionization mass spectroscopy indicated a mixture of product and uncyclized intermediates. A 2.41-g sample of solid was added to a solution of 3.16 g (0.137 mol) of Na in 138 mL of absolute EtOH under N_2. The mixture was refluxed for 3 h 15 min. The slightly cloudy solution was allowed to cool to RT, and the resulting mixture was allowed to stand under N_2 overnight. The mixture was heated briefly back to reflux, and the resulting slightly cloudy, hot solution was neutralized with 8.3 g (0.138 mol) of glacial HOAc. The thick mixture was concentrated under vacuum at 35 °C to a white solid, 50 mL of H_2O was added, and the pH was adjusted to 5.5 with concentrated HCl. The mixture was filtered by suction, and collected solid was washed with H_2O and was dried under vacuum overnight at RT in a desiccator: yield 1.98 g (84%); mp 225–227 °C; NMR (Me_2SO-d_6) δ 1.2–1.4 (m, 4 H, CCH_2CH_2C), 2.06 (t, 2 H, $ArCH_2$), 2.37 (s, 3 H, $ArCH_3$), 3.54 (t, 2 H, NCH_2), 5.58 (br s, 2 H, NH_2), 5.89 (br s, 2 H, NH_2), 7.14 (d, J = 8.4 Hz, 2 H, 3′- and 5′-H), 7.3–7.5 (AA′BB′, 4 H, tosyl), 7.86 (d, J = 8.4 Hz, 2 H, 2′- and 6′-H), 9.75 (br). Anal. ($C_{22}H_{25}N_5O_5S·H_2O$) C, H, N, S.

Diethyl *N*-[4-[[4-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)butyl]tosylamino]benzoyl]-L-glutamate (25a). To a stirred mixture of 1.00 g (0.00209 mol) of benzoic acid derivative **24a** in 6 mL of dry CH_2Cl_2 and 6 mL of dry DMF under N_2 was added 0.433 g (0.00313 mol) of HOBT. After 17 min, 0.646 g (0.00313 mol) of DCC and then 6 mL of dry DMF and 6 mL of dry CH_2Cl_2 were added to the mixture. After 1 h, a cloudy solution of 0.750 g (0.00313 mol) of diethyl L-glutamate·HCl and 0.633 g (0.00626 mol) of 4-methylmorpholine in 6 mL of dry CH_2Cl_2 and 6 mL of dry DMF was added to the mixture. The mixture was stirred under N_2 for 59 h. To it was added an additional 0.431 g (0.00209 mol) of DCC, and the mixture was stirred for another 17 h. The mixture was concentrated under vacuum at 33 °C, and the residue was treated with 25 mL of MeOH. Undissolved white solid was collected by suction filtration, and the filtrate was separated by flash chromatography on 175 g of SiO_2 with 8:1 EtOAc–MeOH as the eluting solvent. Appropriate fractions were combined and concentrated under vacuum to an off-white solid: yield 0.74 g (50%); NMR (Me_2SO-d_6) δ 1.14 (t, J = 7 Hz, 3 H, OCH_2CH_3), 1.17 (t, J = 7 Hz, 3 H, OCH_2CH_3), 1.2–1.4 (m, 4 H, CCH_2CH_2C), 1.9–2.2 (m, 4 H, $ArCH_2$ and $CHCH_2$), 2.37 (s, 3 H, ArMe), 2.4 (t, J = 10 Hz, 2 H, $CH_2C{=}O$), 3.55 (t, 2 H, NCH_2), 4.03 (q, J = 7 Hz, 2 H, OCH_2), 4.10 (q, J = 7 Hz, 2 H, OCH_2), 4.41 (m, 1 H, CH), 5.58 (br s, 2 H, NH_2), 5.85 (br s, 2 H, NH_2), 7.15 (d, J = 8.5 Hz, 2 H, 3′- and 5′-H), 7.3–7.5 (AA′BB′, 4 H, tosyl), 7.81 (d, J = 8.5 Hz, 2 H, 2′- and 6′-H), 8.75 (d, 1 H, NHCH), 9.68 (br s, 1 H, N1-H); CI-MS m/e 657 (P + H, 1).

***N*-[4-[[4-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)butyl]amino]benzoyl]-L-glutamic Acid (2).** A solution of 0.313 g (0.00333 mol) of phenol in 4.7 mL of 31% HBr in HOAc was added to 0.7 g of **25a** under N_2. A complete solution formed within 20 min. After 1.5 h, 39 mL of Et_2O was added and a reddish orange oil formed. The oil was separated, washed five times with 10-mL portions of Et_2O, and dried under vacuum for 2 days. To the resulting oil–solid mixture was added 15 mL of 1 N NaOH, and the mixture was stirred under N_2 at 40 °C for 1 h 5 min. The mixture was filtered, and the filtrate was acidified to pH 3.5 with concentrated HCl. Precipitated solid was collected by suction filtration, washed five times with 5 mL of H_2O, and dried under vacuum overnight, yield 0.254 g. A 0.244-g sample was retreated with NaOH and was reprecipitated with concentrated HCl as above. Precipitated off-white solid was filtered, washed four times with 5 mL of H_2O, and dried overnight under vacuum, yield 0.214 g. A 40-mg sample was recrystallized from EtOH: yield 13 mg; NMR (Me_2SO-d_6) δ 1.2–1.6 (m, 4 H, CCH_2CH_2C), 1.8–2.1 (m, 2 H, $CH_2CH_2C{=}O$), 2.17 (t, J = 6 Hz, 2 H, $ArCH_2$), 2.31 (t, J = 7 Hz, 2 H, $CH_2C{=}O$), 2.9–3.1 (m, 2 H, NCH_2), 4.2–4.4 (m, 1 H, CH), 5.63 (br s, 2 H, NH_2), 5.87 (br s, 2 H, NH_2), 6.19 (m, 1 H, CH_2NH), 6.52 (d, J = 8.7 Hz, 2 H, 3′- and 5′-H), 7.62 (d, J = 8.7 Hz, 2 H, 2′- and 6′-H), 8.04 (d, J = 7.9 Hz, 1 H, CHNH), 9.75 (br s, 1 H, N1-H), 12.35 (br s, 2 H, COOH). The remaining sample was purified by reversed-phase chromatography with 0.1% TFA in 8% MeCN–H_2O as the mobile phase. The purified TFA salt was dried under high vacuum at 55 °C for 24 h; FAB-MS calcd M + H 447.1992, found 447.19780; UV (0.1 N NaOH) λ_{max} nm (ϵ) 276 (25 200), 293 sh (20 900). Anal. ($C_{20}H_{26}N_6O_6·2TFA·^1/_2H_2O$) C, H, N.

3-(4-Amino-1,6-dihydro-6-oxo-5-pyrimidinyl)propionaldehyde Diethyl Acetal (14b). To a solution of Na (2.73 g, 0.119 mol) in absolute EtOH (80 mL) under N_2 was added formamidine acetate (7.87 g, 0.0756 mol) and then cyanoacetate **13** (9.20 g, 0.0378 mol).[28] The stirred mixture was refluxed for 5.25 h and then was allowed to cool to RT. The mixture was filtered, and the dark filtrate was neutralized with HOAc. The resulting mixture was concentrated under vacuum to a dark brown solid, weight 14.43 g. This material was added to boiling MeCN (1 L), and the undissolved solid was filtered and rinsed twice with hot MeCN (100 mL). The combined filtrates were concentrated under vacuum to 9.48 g of a brown solid, which was recrystallized from CCl_4 after charcoal treatment and filtration. An off-white solid was collected, washed twice with CCl_4 (15 mL), and dried under vacuum at 45 °C: yield 4.79 g (53%); mp 108.5–111.5 °C; NMR (Me_2SO-d_6) δ 1.09 (t, J = 7 Hz, 6 H, Me), 1.55 (br q, 2 H, $ArCH_2CH_2$), 2.22 (br t, 2 H, $ArCH_2$), 3.48 (m, 4 H, OCH_2), 4.44 (t, J = 5.7 Hz, 1 H, CH), 6.05 (br s, 2 H, NH_2), 7.65 (s, 1 H, C2-H), 11.38 (br s, 1 H, N1-H); UV (0.1 N NaOH) λ_{max} nm (ϵ) 260 (7300). Anal. ($C_{11}H_{19}N_3O_3$) C, H, N.

3-[4-(Diacetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl]propionaldehyde Diethyl Acetal (15b). A stirred solution of pyrimidine **14b** (7.27 g, 0.0301 mol) in dry pyridine (32.3 mL) and

Ac_2O (32.3 mL) was heated under N_2 in a bath at 90 °C for 6 h. The mixture was cooled and stored at RT for 3 days. Then the mixture was concentrated to a viscous, dark orange oil, which was purified by flash chromatography on 350 g of SiO_2 with EtOAc as the eluent. The product-containing fractions were combined and concentrated under vacuum to a viscous, light brownish yellow oil: yield 6.88 g (67%); NMR (Me_2SO-d_6) δ 1.07 (t, J = 7 Hz, 6 H, Me), 1.62 (m, 2 H, $ArCH_2CH_2$), 2.18–2.24 (m, 8 H, Ac and $ArCH_2$), 3.45 (m, 4 H, OCH_2), 4.44 (t, J = 5 Hz, 1 H, CH), 8.15 (s, 1 H, C2-H), 12.84 (br s, 1 H, N1-H).

3-[4-(Diacetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl]-propionaldehyde (16b). A mixture of acetal **15b** (7.19 g, 0.0212 mol) in H_2O (162 mL) was filtered to remove undissolved, brown solid. The stirred filtrate was heated under N_2 for 3.5 h in a bath at 52 °C. The resulting cloudy solution was allowed to cool to RT and was filtered. The filtrate was concentrated under high vacuum to a light yellow solid, yield 5.34 g. A solution of this solid in CH_2Cl_2 (200 mL) was concentrated under vacuum to a light yellow solid: yield 5.58 g (95%); NMR (Me_2SO-d_6) δ 2.24 (m, 6 H, Ac), 2.4–2.65 (m, 4 H, CH_2CH_2), 8.18 (s, 1 H, C2-H), 9.63 (s, 1 H, CH=O), 12.9 (br s, 1 H, N1-H). This aldehyde was used quickly in the next step.

Diethyl *N*-[4-[[3-[4-(Diacetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl]propyl]amino]benzoyl]-L-glutamate (17b). A mixture of **16b** (1.08 g, 0.0039 mol) and diethyl *N*-(4-aminobenzoyl)-L-glutamate (1.54 g, 0.00478 mol) in dry EtOH (25 mL) was stirred under N_2 in a dry flask. Addition of glacial HOAc (1.1 mL) resulted in a complete solution within 30 s. After the addition of activated 3-Å sieves (5 g), the mixture was stirred for 2 h 40 min before $NaBH_3CN$ (0.286 g, 0.00455 mol) was added. After being stirred under N_2 at RT for 23 h the mixture was filtered to remove the sieves, and the filtrate was concentrated under vacuum to an off-white, hard foam (3.14 g). The foam was purified by flash chromatography on 170 g of SiO_2 with 10:1 EtOAc–MeOH as eluent. Appropriate fractions were combined and evaporated under vacuum to a hard, white foam: yield 0.55 g; NMR (Me_2SO-d_6) δ 1.15 (t, J = 7.1 Hz, 3 H, Me), 1.16 (t, J = 7.1 Hz, 3 H, Me), 1.65 (m, 2 H, $CH_2CH_2CH_2$), 2.0–2.1 (m, 2 H, $NCHCH_2CH_2$), 2.18 (s, 6 H, NAc), 2.2–2.5 (m, 4 H, $ArCH_2$ and CH_2C=O), 2.95–3.0 (m, 2 H, CH_2NH), 4.03 (q, J = 7.1 Hz, 2 H, OCH_2), 4.07 (q, J = 7.1 Hz, 2 H, OCH_2), 4.3–4.45 (m, 1 H, Cα-H), 6.25 (m, 1 H, CH_2NHAr), 6.51 (d, J = 8.7 Hz, 2 H, 3′- and 5′-H), 7.63 (d, J = 8.7 Hz, 2 H, 2′- and 6′-H), 8.15 (s, 1 H, C2-H), 8.21 (d, J = 7 Hz, 1 H, $CONHCH$), 12.6 (br, 1 H, N1-H).

***N*-[4-[[3-[4-Amino-1,6-dihydro-6-oxo-5-pyrimidinyl]-propyl]amino]benzoyl]-L-glutamic Acid (3).** A stirred solution of ester **17b** (0.37 g, 0.0006 mol) in absolute EtOH (14 mL) and 1 N NaOH (28.2 mL) was heated for 19 h under N_2 at 50 °C. The mixture was filtered, and the filtrate was concentrated to 7 mL under vacuum at 28 °C. The solution was adjusted to pH 3 with 1.24 mL of concentrated HCl. A cloudy supernatant was carefully pipetted from a gummy solid, which was washed with H_2O and dried under vacuum at RT, yield 0.13 g. The solid was dissolved in hot absolute EtOH (12.5 mL), treated with Darco G-60 activated carbon (0.02 g), and filtered through Celite. After addition of MeCN (65 mL), the solution was boiled down to 45 mL and allowed to cool to RT. An off-white solid was collected, washed five times with MeCN (2 mL), and dried under high vacuum at 60 °C: yield 0.072 g (25%); mp 128–175 °C dec; NMR (Me_2SO-d_6) δ 1.6 (m, 2 H, $CH_2CH_2CH_2$), 2.0 (m, 2 H, $NCHCH_2$), 2.31 (m, 4 H, $ArCH_2$ and CH_2C=O), 3.02 (br t, 2 H, CH_2NH), 4.33 (m, 1 H, Cα-H), 6.24 (m, 3 H, NH_2 and NHAr), 6.52 (d, J = 8.7 Hz, 2 H, 3′- and 5′-H), 7.64 (d, J = 8.7 Hz, 2 H, 2′- and 6′-H), 7.68 (s, 1 H, C2-H), 8.09 (d, J = 7.4 Hz, 1 H, CONH), 11.5 (br, 1 H, N1-H), 12.3 (br, OH); UV (0.1 N NaOH) λ_{max} nm (ε) 269.5 sh (17 300), 292 (22 000). Anal. ($C_{19}H_{23}N_5O_6 \cdot H_2O \cdot {}^3/_{10}MeCN \cdot {}^1/_{10}EtOH$) C, H, N.

Ethyl 4-[(3-Bromopropyl)thio]benzoate (22b). Diethyl 4,4-dithiobisbenzoate[40] (11.35 g, 31.3 mmol) was dissolved in EtOH (350 mL) and treated with $NaBH_4$ (2.4 g, 63.4 mmol) to generate **21b**. After 20 min, the solution was added to 1,3-dibromopropane (32 g, 158 mmol) in EtOH (200 mL), and the mixture was stirred for 18 h. The mixture was evaporated in vacuo to a viscous residue, which was purified by flash chromatography (700 g of SiO_2, 1:1 hexane–CH_2Cl_2) to give 6.01 g (32%) of compound **22b** as a colorless oil: NMR (Me_2SO-d_6) δ 1.3 (t, J = 7 Hz, 3 H, CH_3CH_2O), 2.1 (pent, J = 7 Hz, 2 H, $CH_2CH_2CH_2$), 3.15 (t, J = 7 Hz, 2 H, CH_2S), 3.6 (t, J = 7 Hz, 2 H, CH_2Br), 4.28 (q, J = 7 Hz, 2 H, CH_2O), 7.4 (d, J = 9 Hz, 2 H, 3′- and 5′-H's), 7.85 (d, J = 9 Hz, 2 H, 2′- and 6′-H).

Ethyl 4-[(4-Cyano-5-ethoxy-5-oxopentyl)thio]benzoate (23b). Ethyl cyanoacetate (6.3 g, 55.7 mmol) was alkylated as described for the synthesis of **23a**. The crude product was extracted from H_2O (50 mL) and Et_2O (50 mL), and the Et_2O phase was collected. The aqueous phase was washed four times with 50 mL of Et_2O, and the combined Et_2O fractions were dried ($CaSO_4$) and filtered. The filtrate was evaporated in vacuo, and the residue was purified by chromatography (400 g SiO_2, 3:1 hexane–EtOAc) to yield 6.06 g (70%) of **23b** as a pale yellow oil: NMR (Me_2SO-d_6) δ 1.17 (t, J = 7 Hz, 3 H, CH_3CH_2O), 1.28 (t, J = 7 Hz, 3 H, CH_3CH_2OCOAr), 1.7 (m, 2 H, $CH_2CH_2CH_2$), 1.95 (m, 2 H, $CH_2CH_2CH_2S$), 3.1 (t, J = 7 Hz, 2 H, CH_2S), 4.1 (m, 1 H, CH), 4.15 (q, J = 7 Hz, 2 H, CH_2O), 4.28 (q, J = 7 Hz, 2 H, CH_2OCOAr), 7.4 (d, J = 9 Hz, 2 H, 3′- and 5′-H), 7.85 (d, J = 9 Hz, 2 H, 2′- and 6′-H).

4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)-propyl]thio]benzoic Acid (24b). The cyanoacetate **23b** (6.00 g, 17.9 mmol) was allowed to react with dry guanidine·HCl (3.43 g, 35.9 mmol) under conditions analogous to compound **24a** above. The residue after workup was treated with H_2O (70 mL) and was filtered to give an off-white solid, which was washed three times with 30 mL of H_2O and three times with 30 mL of Et_2O. Then it was dried in vacuo to give crude intermediate ester (5.96 g, 95%) as a white solid: NMR (Me_2SO-d_6) δ 1.3 (t, J = 7 Hz, 3 H, Me), 1.65 (m, 2 H, $CH_2CH_2CH_2$), 2.3 (t, J = 7 Hz, 2 H, $CH_2CH_2CH_2S$), 2.95 (m, 2 H, CH_2S), 4.3 (q, J = 7 Hz, 2 H, OCH_2), 5.7 (br s, 2 H, 2-NH_2), 5.95 (br s, 2 H, 4-NH_2), 7.3 (d, J = 9 Hz, 2 H, 3′- and 5′-H), 7.95 (d, J = 9 Hz, 2 H, 2′- and 6′-H), 9.8 (br s, 1 H, N1-H). This ester (5.95 g, 17.1 mmol) was treated with 600 mL of 1:1 EtOH–1.0 N sodium hydroxide (v/v) and heated to 60 °C for 5 h. After cooling, the solution was treated with concentrated HCl to pH 3.5 and was filtered. The filtrate was reduced to 300 mL in volume and was refiltered to give an off-white solid, which was washed three times with 20 mL of H_2O. The solid was dissolved in 0.1 N sodium hydroxide (200 mL) and was reacidified to pH 3.5 with concentrated HCl. Filtration gave a white solid which was washed three times with 50 mL of H_2O and dried in vacuo at 50 °C to give 3.36 g (61%) of acid **24b** as a white solid: mp 245 °C dec; NMR (Me_2SO-d_6) δ 1.6 (m, 2 H, $CH_2CH_2CH_2$), 2.3 (t, J = 7 Hz, 2 H, $CH_2CH_2CH_2S$), 2.95 (t, J = 7 Hz, 2 H, CH_2S), 6.1 (br s, 2 H, NH_2), 6.5 (br s, 2 H, NH_2), 7.3 (d, J = 9 Hz, 2 H, 3′- and 5′-H), 7.8 (d, J = 9 Hz, 2 H, 2′- and 6′-H), 9.9 (br s, N1-H), OH not seen; UV (0.1 N NaOH) λ_{max} nm (ε) 272 (21 800), 230 sh (11 200); CI-MS m/e 349 (M + 29), 321 (M + 1), 167 (M–$SPhCO_2H$), 155 (H_2SPhCO_2H). Anal. ($C_{14}H_{16}N_4O_3S$) C, H, N, S.

Diethyl *N*-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]thio]benzoyl]-L-glutamate (25b). Compound **24b** (1.05 g, 3.27 mmol) was coupled to diethyl L-glutamate·HCl by the DCC/HOBT method in dry DMF (20 mL) in analogy to compound **25a**. The crude product was purified by chromatography on SiO_2 with 10:1 EtOAc–MeOH to yield **25b** (0.85 g, 53%) as a straw-colored glass: NMR (Me_2SO-d_6) δ 1.15 (2 t, 6 H, CH_3CH_2O), 1.65 (m, 2 H, $CH_2CH_2CH_2$), 2.1 (m, 2 H, $CHCH_2$), 2.3 (t, 2 H, $CH_2CH_2CH_2S$), 2.4 (t, 2 H, CH_2CO_2Et), 3.0 (m, 2 H, CH_2S), 4.05 (m, 4 H, CH_2O), 4.4 (m, 1 H, CH), 5.7 (s, 2 H, 2-NH_2), 5.9 (s, 2 H, 4-NH_2), 7.3 (d, 2 H, 3′- and 5′-H), 7.8 (d, 2 H, 2′- and 6′-H), 8.65 (d, 1 H, Glu NH), 9.75 (s, 1 H, N1-H).

***N*-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)-propyl]thio]benzoyl]-L-glutamic Acid (4).** Compound **25b** (0.50 g, 0.99 mmol) was stirred at 50 °C in 0.1 N NaOH (18 mL) for 2 h. The pH was adjusted to 8.0 with 1.0 N HCl, and the mixture was evaporated to dryness in vacuo. The resulting solid was dissolved in a minimum volume of 35% EtOH–H_2O and separated in five injections on a 2.1-cm × 50-cm, 10 μm, Regis C_{18} preparative reversed-phase chromatography column. The appropriate fractions were combined, reduced in vacuo to 5 mL

(40) Kim, Y. H.; Gaumont, Y.; Kisliuk, R. L.; Mautner, H. G. Synthesis and Biological Activity of 10-Thia-10-deaza Analogs of Folic Acid, Pteroic Acid, and Related Compounds. *J. Med. Chem.* **1975**, *18*, 776–80.

volume, and acidified with concentrated HCl to pH 3.0. Filtration gave a white solid, which was dried in vacuo at 50 °C to yield the title compound, 4, (0.185 g, 41%) as a white powder: mp 144–148 °C dec; NMR (Me_2SO-d_6) δ 1.6 (t, 2 H, $CH_2CH_2CH_2$), 2.0 (m, 2 H, $CH_2CH_2CO_2H$), 2.35 (m, 4 H, $CH_2CH_2CH_2S$ and CH_2CO_2H), 2.95 (t, 2 H, CH_2S), 4.35 (m, 1 H, methine), 5.8 (br, 2 H, NH_2), 6.1 (br, 2 H, NH_2), 7.3 (d, 2 H, 3′- and 5′-H), 7.75 (d, 2 H, 2′- and 6′-H), 8.55 (d, 1 H, Glu NH), 9.9 (br, 1 H, pyrimidine NH), 12.35 (br, 2 H, CO_2H's); UV (0.1 N NaOH) λ_{max} nm (ε) 216 (20 200), 273 (19 500), 295 sh (12 800). Anal. ($C_{19}H_{23}N_5O_6S \cdot {}^3/_2H_2O$) C, H, N, S.

Ethyl 4-[(4-Cyano-5-ethoxy-5-oxopentyl)oxy]benzoate (23c). Ethyl cyanoacetate (12.33 g, 109 mmol) was alkylated with ethyl 4-(3-bromopropoxy)benzoate[41] (14.06 g, 49 mmol) as described above for compound **23a**. The crude product was purified by chromatography (700 g SiO_2, 3:1 hexane–EtOAc) to obtain 7.00 g (45%) of **23c** as a colorless oil: NMR (Me_2SO-d_6) δ 1.20 (t, J = 6 Hz, 3 H, CH_3CH_2O), 1.29 (t, J = 6 Hz, 3 H, CH_3CH_2OCOAr), 1.88 (m, 2 H, $CH_2CH_2CH_2$), 2.0 (m, 2 H, $CHCH_2CH_2$), 4.35–5.05 (m, 7 H, CH_2OCO, CH, CH_2OAr), 7.0 (d, J = 9 Hz, 2 H, 3′- and 5′-H), 7.9 (d, J = 9 Hz, 2 H, 2′- and 6′-H).

4-[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propoxy]benzoic Acid (24c). Compound **23c** (7.0 g, 21.9 mmol) was cyclized with guanidine·HCl as described above for compound **24a**. The initial product was washed well with H_2O and dried at 50 °C in vacuo to give 6.76 g (93%) of ester as a white solid: NMR (Me_2SO-d_6) δ 1.28 (t, J = 6 Hz, 3 H, CH_3 of ester), 1.75 (m, 2 H, $CH_2CH_2CH_2$), 2.25 (t, J = 7 Hz, 2 H, $CH_2CH_2CH_2O$), 3.97 (t, J = 7 Hz, 2 H, CH_2O), 4.25 (q, J = 6 Hz, 2 H, CH_2O), 5.7 (s, 2 H, NH_2), 5.93 (s, 2 H, NH_2), 6.98 (d, J = 9 Hz, 2 H, 3′- and 5′-H), 7.85 (d, J = 9 Hz, 2 H, 2′- and 6′-H), 9.8 (br s, 1 H, N1-H). The ester (6.76 g, 20.3 mmol) was saponified 1:1 EtOH–1 N NaOH (v/v), precipitated with HCl, and washed with H_2O as described for compound **24b**. The solid was reprecipitated, washed with H_2O, and dried at 50 °C in vacuo to yield 3.57 g (58%) of **24c** as a white solid: mp 253 °C; NMR (Me_2SO-d_6) δ 1.8 (pent, J = 7 Hz, 2 H, $CH_2CH_2CH_2$), 2.3 (t, J = 7 Hz, 2 H, $CH_2CH_2CH_2O$), 4.0 (t, J = 7 Hz, 2 H, CH_2O), 5.7 (s, 2 H, 2-NH_2), 5.9 (s, 2 H, 4-NH_2), 7.0 (d, J = 9 Hz, 2 H, 3′- and 5′-H), 7.85 (d, J = 9 Hz, 2 H, 2′- and 6′-H), 9.8 (br s, N1-H), 12.6 (br, 1 H, CO_2H); FAB-MS 305 (M + 1), 167 (M–$OPhCO_2H$), 139 (H_2OPhCO_2H); UV (0.1 N NaOH) λ_{max} nm (ε) 248 (21 000), 265 sh (17 000). Anal. ($C_{14}H_{16}N_4O_4$) C, H, N.

Diethyl *N*-[4-[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propoxy]benzoyl]-L-glutamate (25c). Diethyl L-glutamate·HCl (0.45 g, 1.87 mmol) was dissolved in dry DMF (20 mL) containing triethylamine (0.25 mL, 1.86 mmol) and cooled to 0 °C. The mixture was treated with a slurry of DMF (35 mL) containing the mixed anhydride made from compound **24c** (0.50 g, 1.65 mmol), triethylamine (0.23 mL, 1.65 mmol), and isobutyl chloroformate (0.24 mL, 1.85 mmol) at 0 °C. After 30 min, the mixture was allowed to warm to RT and stirred for 2 h. The mixture was evaporated to dryness in vacuo, and the resulting residue was suspended in saturated $NaHCO_3$ solution (25 mL) and filtered. The resulting solid was treated twice more with saturated $NaHCO_3$ then washed with H_2O and dried to give 0.37 g (23%) of diethyl ester **25c**: NMR (Me_2SO-d_6) δ 1.16 (t, J = 6 Hz, 3 H, CH_3CH_2O), 1.19 (t, J = 6 Hz, 3 H, CH_3CH_2O), 1.8 (m, 2 H, $CH_2CH_2CH_2$), 2.0 (m, 2 H, $CHCH_2$), 2.3 (t, J = 7 Hz, 2 H, $CH_2CH_2CH_2O$), 2.45 (t, J = 7 Hz, 2 H, CH_2CO_2Et), 4.0 (m, 6 H, CH_2OAr and CH_2OCO), 4.4 (m, 1 H, CH), 5.7 (br s, 2 H, 2-NH_2), 5.95 (br s, 2 H, 4-NH_2), 6.97 (d, J = 10 Hz, 2 H, 3′- and 5′-H), 7.82 (d, J = 10 Hz, 2 H, 2′- and 6′-H), 8.55 (d, 1 H, NH of Glu), 9.8 (br, 1 H, N1-H).

***N*-[4-[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propoxy]benzoyl]-L-glutamic Acid (5).** Compound **25c** (0.366 g, 0.75 mmol) was hydrolyzed and purified as described for compound **25b**. The appropriate fractions were concentrated in vacuo to 3 mL and acidified with concentrated HCl to pH 3.0. Filtration gave a white solid which was dried in vacuo at 50 °C to yield **5** (80 mg, 24%) as a white powder: mp 194–200 °C dec; NMR (Me_2SO-d_6) δ 1.8 (pent, J = 6 Hz, 2 H, $CH_2CH_2CH_2$), 2.0 (m, 2 H, $CH_2CH_2CO_2H$), 2.3 (m, 4 H, CH_2CO_2H and $CH_2CH_2CH_2O$), 4.0 (t, J = 6 Hz, 2 H, CH_2O), 4.35 (m, 1 H, CH), 5.7 (s, 2 H, 2-NH_2), 5.98 (s, 2 H, 4-NH_2), 6.97 (d, J = 9 Hz, 2 H, 3′- and 5′-H), 7.82 (d, J = 9 Hz, 2 H, 2′- and 6′-H), 8.4 (d, J = 7 Hz, 1 H, NHCH), 9.8 (br s, 1 H, N1-H), 12.8 (br s, 2 H, CO_2H's); UV (0.1 N NaOH) λ_{max} nm (ε) 259 (30 300), 215 (30 600); CI-MS M + H calcd 434.1676, found 434.1675. Anal. ($C_{19}H_{23}N_5O_7 \cdot {}^{12}/_5H_2O$) C, H, N.

Diethyl *N*-(4-Nitro-3-fluorobenzoyl)-L-glutamate (19a). The DCC/HOBT method (see **25a**) was used to couple 3-fluoro-4-nitrobenzoic acid (6.00 g, 32.4 mmol) and diethyl L-glutamate·HCl (7.77 g, 32.4 mmol). The product was purified by extraction and flash chromatography to give a light yellow solid: yield 5.7 g (48%); mp 78–80 °C; NMR ($CDCl_3$) δ 1.25 (t, J = 7 Hz, 3 H, OCH_2CH_3), 1.31 (t, J = 7 Hz, 3 H, OCH_2CH_3), 2.2–2.3 (m, 2 H), 2.5–2.6 (m, 2 H), 4.14 (q, J = 7 Hz, 2 H, OCH_2), 4.25 (q, J = 7 Hz, 2 H, OCH_2), 4.71 (m, 1 H, CH), 7.58 (br d, J = 7 Hz, 1 H, CONH), 7.7 (m, 2 H, 2- and 6-H), 8.12 (t, J_{HH} and J_{HF} = 8 Hz, 1 H, 5-H); UV (MeOH) λ_{max} nm (ε) 253 (8000), 232 min (5890); CI-MS m/e 371 (M + 1, 100), 325 (33); IR (KBr) cm^{-1} 3314, 2985, 1735, 1645, 1601, 1542, 1347. Anal. ($C_{16}H_{19}FN_2O_7$) C, H, N.

Diethyl *N*-(4-Amino-3-fluorobenzoyl)-L-glutamate (20a). A solution of diethyl ester **19a** (5.3 g, 14 mmol) in 95% EtOH (150 mL) was shaken under a 40 psi H_2 atmosphere for 1.5 h with 10% Pd/C (0.4 g). The reaction mixture was filtered, spin evaporated, and dried to give a cream-colored solid: yield 4.7 g (98%); mp 92–97 °C; NMR ($CDCl_3$) δ 1.22 (t, J = 7 Hz, 3 H, OCH_2CH_3), 1.29 (t, J = 7 Hz, 3 H, OCH_2CH_3), 2.1–2.5 (m, 4 H, CCH_2CH_2CO), 3.9 (br s, 2 H, NH_2), 4.10 (q, J = 7 Hz, 2 H, OCH_2), 4.22 (q, J = 7 Hz, 2 H, OCH_2), 4.74 (m, 1 H, CH), 6.75 (t, J_{HH} and J_{HF} = 8 Hz, 1 H, 5′-H), 6.84 (d, J = 7 Hz, 1 H, CHN*H*), 7.4–7.5 (m, 2 H, 2′- and 6′-H); UV (MeOH) λ_{max} nm (ε) 281 (12 000), 210 sh (10 600); CI-MS m/e 341 (M + 1, 100), 138 (M–Glu, 64); IR (KBr) cm^{-1} 3456, 3361, 3308, 2977, 1734, 1636, 1625, 1507, 1201. Anal. ($C_{16}H_{21}FN_2O_5$) C, H, N.

Diethyl *N*-[4-[[3-[2-(Acetylamino)-4-(diacetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl]propyl]amino]-3-fluorobenzoyl]-L-glutamate (17a, Z = 3′-F). Reductive amination of ester **20a** (2.21 g, 6.49 mmol) and aldehyde **16a**[28] (2.00 g, 6.49 mmol) was carried out according to the procedure for compound **17b** above. The crude product was extracted from water with EtOAc and was purified by chromatography on SiO_2 (175 g) using an EtOAc wash (450 mL) followed by elution of the product with 5% MeOH in EtOAc. Appropriate fractions were combined, evaporated, and dried to give **17a** (Z = 3′-F) as a white solid: yield 2.66 g (65%); mp 76–84 °C dec; NMR (Me_2SO-d_6) δ 1.15 (t, J = 7 Hz, 3 H, OCH_2CH_3), 1.16 (t, J = 7 Hz, 3 H, OCH_2CH_3), 1.64 (m, 2 H), 2.00 (m, 2 H, $CHCH_2CH_2$), 2.11 (s, 3 H, N2-Ac), 2.20 (s, 6 H, N4-Ac), 2.27 (m, 2 H, $ArCH_2$), 2.40 (t, J = 7 Hz, 2 H, CH_2CO), 3.10 (m, 2 H, NCH_2), 3.9 (q, J = 7 Hz, 2 H, OCH_2), 4.1 (q, J = 7 Hz, 2 H, OCH_2), 4.35 (m, 1 H, CH), 6.10 (m, 1 H, NH), 6.66 (t, $J_0^{H,H}$ and $J_m^{F,H}$ = 9 Hz, 1 H, 5′-H), 7.5–7.6 (m, 2 H, 2′- and 6′-H), 8.35 (d, J = 7 Hz, 1 H, CON*H*CH), 11.8 (br s, 1 H, AcNH), 12.0 (br s, 1 H, N1-H); UV (MeOH) λ_{max} nm (ε) 299 (28 000), 257 min (7500); FAB-MS m/e 633 (M + 1, 23), 430 (M–Glu(OEt)$_2$, 100), 388 (87). Anal. ($C_{29}H_{37}FN_6O_9$) C, H, N.

***N*-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]-3-fluorobenzoyl]-L-glutamic Acid (7).** Ester **17a** (Z = 3′-F) (500 mg, 0.790 mmol) was hydrolyzed in 1.0 N sodium hydroxide (50 mL) at 55 °C under N_2 for 18 h. The crude product was precipitated as described for compound **2** to give a white solid (376 mg). A portion (200 mg) was recrystallized from DMF–H_2O to give a white solid: yield 119 mg (60%); mp 175 °C dec; NMR (Me_2SO-d_6) δ 1.56 (m, 2 H, $CH_2CH_2CH_2$), 1.8–2.1 (m, 2 H, $CHCH_2$), 2.1–2.4 (m, 4 H, $ArCH_2$ and CH_2CO), 3.07 (m, 2 H, NCH_2), 4.34 (m, 1 H, CH), 5.78 (s, 2 H, 2-NH_2), 5.93 (s, 2 H, 4-NH_2), 6.24 (br s, 1 H, ArNH), 6.70 (t, J_0^{HH} and J_m^{FH} = 9 Hz, 1 H, 5′-H), 7.55 and 7.56 (2d, J_0^{FH} = 12 Hz, J_0^{HH} = 9 Hz, 2 H, 2′- and 6′-H), 8.22 (d, J = 8 Hz, 1 H, CON*H*CH), 9.8 (v br s, 1 H, N1-H), 12.3 (v br s, 2 H, OH); UV (0.1 N NaOH) λ_{max} nm (ε) 295 (19 800), 273 (23 200), 220 max (18 800). Anal. ($C_{19}H_{23}FN_6O_6 \cdot 2H_2O$) C, H, N.

Di-*tert*-butyl *N*-[4-[[3-[2-(Acetylamino)-4-(diacetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl]propyl]amino]-2-

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fluorobenzoyl]-L-glutamate (17a, Z = 2'-F). Reductive amination of **16a**,[28] (1.00 g, 3.24 mmol) with di-*tert*-butyl *N*-(4-amino-2-fluorobenzoyl)-L-glutamate[42] (1.29 g, 3.24 mmol) was carried out according to the procedure for compound **17b** above. The product was purified by flash chromatography to give a white solid: yield 1.28 g (57%); mp 120 °C dec; NMR (Me_2SO-d_6) δ 1.36 (s, 9 H, *t*-Bu), 1.39 (s, 9 H, *t*-Bu), 1.62 (m, 2 H, $CH_2CH_2CH_2$), 1.8–2.4 (m, 6 H), 2.09 (s, 3 H, N2-Ac), 2.19 (s, 6 H, N4-Ac), 2.98 (m, 2 H, NCH_2), 4.31 (m, 1 H, $NCHCH_2$), 6.25 (d, $J_o^{F,H}$ = 15 Hz, 1 H, 3'-H), 6.38 (d, $J_o^{H,H}$ = 9 Hz, 1 H, 5'-H), 6.66 (br m, 1 H, ArNH), 7.44 (t, $J_o^{H,H}$ and $J_m^{F,H}$ = 9 Hz, 1 H, 6'-H), 7.68 (t, J_{HH} = 7 Hz, J_{HF} = 7 Hz, 1 H, CONHCH), 11–12 (v br s, 1 H); UV (MeOH) λ_{max} nm (ϵ) 299 (11900); FAB-MS *m/e* 689 (M + 1, 25), 430 (100), 388 (56), 277 (40). Anal. ($C_{33}H_{45}FN_6O_9 \cdot H_2O$) C, H, N.

***N*-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)-propyl]amino]-2-fluorobenzoyl]-L-glutamic Acid (8).** A solution of the di-*tert*-butyl ester (**17a**, Z = 2'-F) from above (0.70 g, 1.0 mmol) in freshly distilled TFA (10 mL) was stirred under N_2 for 1.5 **h**. The solution was spin evaporated and dried (high vacuum) to a light yellow residue that was mixed with 1.0 N NaOH (20 mL) and stirred under N_2 at 55 °C for 18 **h**. The reaction mixture was chilled (ice bath), neutralized by dropwise addition of glacial HOAc, and then adjusted to pH 2.5 by dropwise addition of 6 N HCl. After stirring cold for 2 h, the resulting suspension was filtered. The wet cake was recrystallized from DMF–H_2O to give a cream-colored solid: yield 0.26 g (57%); mp 195–200 °C; NMR (Me_2SO-d_6) δ 1.55 (m, 2 H, $CH_2CH_2CH_2$), 1.95 (m, 2 H, $CHCH_2CH_2$), 2.1–2.3 (2m, 4 H, pyrim-CH_2 and CH_2CO_2), 2.98 (m, 2 H, NCH_2), 4.36 (m, 1 H, $CHCH_2$), 5.75 (br s, 2 H, 2-NH_2), 5.92 (br s, 2 H, 4-NH_2), 6.27 (dd, J_o^{FH} = 15 Hz, J_m^{HH} = 2 Hz, 1 H, 3'-H), 6.39 (dd, J_o^{HH} = 9 Hz, J_m^{HH} = 2 Hz, 1 H, 5'-H), 6.56 (br m, 1 H, ArNH), 7.46 (t, J_o^{HH} and J_m^{FH} = 9 Hz, 1 H, 6'-H), 7.68 (t, J_{HH} = 7 Hz, J_{HF} = 7 Hz, 1 H, ArCONHCH), 9.8 (v br s, 1 H, N1-H), 12.4 (v br s, 2 H, CO_2H); UV (0.1 N NaOH) λ_{max} nm (ϵ) 295 (21400), 274 (24400), 220 (20400). Anal. ($C_{19}H_{23}FN_6O_6 \cdot {}^3/_2H_2O$) C, H, N.

Diethyl *N*-(4-Amino-3-methylbenzoyl)-L-glutamate (20c). Diethyl *N*-(3-methyl-4-nitrobenzoyl)-L-glutamate[43] (11.0 g, 30.0 mmol) was hydrogenated as for compound **20a** to obtain a beige solid: yield 10.1 g (100%); mp 79–82 °C; NMR (Me_2SO-d_6) δ 1.15 (t, *J* = 7 Hz, 3 H, Me), 1.16 (t, *J* = 7 Hz, 3 H, Me), 2.00 (m, 2 H, $CHCH_2CH_2$), 2.06 (s, 3 H, ArMe), 2.39 (t, *J* = 7 Hz, 2 H, CH_2CO_2), 4.0 (q, *J* = 7 Hz, 2 H, OCH_2), 4.06 (q, *J* = 7 Hz, 2 H, OCH_2), 4.33 (m, 1 H, $NHCHCH_2$), 5.40 (s, 2 H, NH_2), 6.56 (d, J_o = 8 Hz, 1 H, 5'-H), 7.47 (d, J_o = 8 Hz, 1 H, 6'-H), 7.50 (s, 1 H, 2'-H), 8.16 (d, *J* = 7 Hz, 1 H, CONHCH); UV (MeOH) λ_{max} nm (ϵ) 284 (17000); CI-MS *m/e* 337 (M + 1, 44), 134 (M–Glu(Et)$_2$, 100). Anal. ($C_{17}H_{24}N_2O_5$) C, H, N.

Diethyl *N*-[4-[[3-[2-(Acetylamino)-4-(diacetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl]propyl]amino]-3-methylbenzoyl]-L-glutamate (17a, Z = 3'-Me). Reductive amination of amino ester **20c** (2.18 g, 6.49 mmol) and aldehyde **16a**[28] (2.00 g, 6.49 mmol) was carried out as described for **17b**. The crude product was partitioned between EtOAc and H_2O. The extract was chromatographed on SiO_2 to give a colorless semisolid: yield 1.45 g (36%); NMR (Me_2SO-d_6) δ 1.15 (t, *J* = 7 Hz, 3 H, Me), 1.16 (t, *J* = 7 Hz, 3 H, Me), 1.66 (m, 2 H, $CH_2CH_2CH_2$), 2.00 (m, 2 H, $CHCH_2CH_2$), 2.08 (s, 3 H, ArMe), 2.11 (s, 3 H, N2-Ac), 2.19 (s, 6 H, N4-Ac), 2.29 (m, 2 H, pyrim-CH_2), 2.39 (t, *J* = 7 Hz, 2 H, CH_2CO_2), 3.11 (m, 2 H, NCH_2), 4.0 (q, *J* = 7 Hz, 2 H, OCH_2), 4.0 (q, *J* = 7 Hz, 2 H, OCH_2), 4.33 (m, 1 H, $NHCHCH_2$), 5.46 (m, 1 H, ArNH), 6.47 (d, J_o = 9 Hz, 1 H, 5'-H), 7.53 (s, 1 H, 2'-H), 7.57 (d, J_o = 9 Hz, 1 H, 6'-H), 8.20 (d, *J* = 7 Hz, 1 H, CONHCH), 11.8 (v br s, NH); UV (MeOH) λ_{max} nm (ϵ) 301 (31000); FAB-MS *m/e* 628 (M + 1, 8), 426 (M–Glu(OEt)$_2$, 100), 384 (95). Anal. ($C_{30}H_{40}N_6O_9 \cdot {}^3/_{10}H_2O \cdot {}^1/_2EtOAc$) C, H, N.

***N*-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)-propyl]amino]-3-methylbenzoyl]-L-glutamic Acid (9).** Ester **17a** (Z = 3'-Me) (0.70 g, 1.1 mmol) was saponified as before (see **3**). Recrystallization of the wet cake from DMF–H_2O failed, so the solution was spin evaporated to a clear oil. The oil was mixed with pH 2.1 aqueous HCl (20 mL) and sonicated 5 min to obtain a cream-colored solid, which was filtered and dried to give the product: yield 0.12 g (24%); mp 181 °C dec; NMR (Me_2SO-d_6) δ 1.58 (m, 2 H, $CH_2CH_2CH_2$), 1.95 (m, 2 H, $CHCH_2CH_2$), 2.12 (s, 3 H, ArMe), 2.2–2.4 (m, 4 H, pyrim-CH_2 and CH_2CO_2), 3.08 (m, 2 H, NCH_2), 4.34 (m, 1 H, NCH), 5.78 (br s, 2 H, 2-NH_2), 5.8 (v br s, ArNH), 5.94 (br s, 2 H, 4-NH_2), 6.52 (d, J_o = 9 Hz, 1 H, 5'-H), 7.53 (s, 1 H, 2'-H), 7.57 (d, J_o = 9 Hz, 1 H, 6'-H), 8.06 (d, *J* = 8 Hz, 1 H, CONH), 10.0 (v br s, 1 H, N1-H), 12.2 (v br s, 2 H, CO_2H); UV (0.1 N NaOH) λ_{max} nm (ϵ) 296 (18000), 274 (19900), 215 (21000); FAB-MS *m/e* 469.2 (M + Na, 6) 447.2 (M + H, 37), 300.1 (M–Glu, 53), 279.1 (3-MePABA-Glu, 20). Anal. ($C_{20}H_{26}N_6O_6 \cdot H_2O \cdot {}^2/_{10}DMF$) C, H, N.

Diethyl *N*-(3-Methoxy-4-nitrobenzoyl)-L-glutamate (19d). A solution of 3-methoxy-4-nitrobenzoic acid (10.0 g, 50.7 mmol) was coupled to diethyl L-glutamate·HCl by the DCC/HOBT method (see **25a**). The crude product was dissolved in CH_2Cl_2, washed, dried ($MgSO_4$), and filtered to give a brown-orange solution that showed a major spot on TLC (SiO_2, 2:1 hexane–EtOAc, R_f = 0.2). The solution was applied to a SiO_2 column (500 g, prewet with CH_2Cl_2) and eluted with CH_2Cl_2 (500 mL). The product was eluted by a stepwise gradient using 10% then 15% EtOAc–CH_2Cl_2 (eight 250-mL portions each). Appropriate fractions were combined, evaporated, and dried (high vacuum) to give a clear yellow oil: yield 11.5 g (59%); NMR (Me_2SO-d_6) δ 1.15 (t, *J* = 7 Hz, 3 H, Me), 1.18 (t, *J* = 7 Hz, 3 H, Me), 1.95 (m, 2 H, $CHCH_2CH_2$), 2.45 (t, *J* = 7 Hz, 2 H, CH_2CO_2), 3.98 (s, 3 H, OMe), 4.0 (2q, *J* = 7 Hz, 4 H, OCH_2), 4.46 (m, 1 H, NCH), 7.58 (dd, J_o = 8 Hz, J_m = 2 Hz, 1 H, 6'-H), 7.71 (d, J_m = 2 Hz, 1 H, 2'-H), 7.97 (d, J_o = 8 Hz, 1 H, 5'-H), 9.00 (d, *J* = 7 Hz, 1 H, NH); UV (MeOH) λ_{max} nm (ϵ) 327 (2940), 257 sh (6640), 235 sh (9140), 203 (29700); CI-MS *m/e* 383 (M + 1, 100). Anal. ($C_{17}H_{22}N_2O_8$) C, H, N.

Diethyl *N*-(4-Amino-3-methoxybenzoyl)-L-glutamate (20d). Diethyl ester **37** (11.0 g, 28.8 mmol) was hydrogenated (see **20a**) to obtain **20d** as a white wax: yield 9.95 g (99%); NMR (Me_2SO-d_6) δ 1.15 (t, *J* = 7 Hz, 3 H, Me), 1.17 (t, *J* = 7 Hz, 3 H, Me), 2.0 (m, 2 H, $NCHCH_2$), 2.40 (t, *J* = 7 Hz, 2 H, CH_2CO_2), 3.79 (s, 3 H, OMe), 4.1 (2q, *J* = 7 Hz, 4 H, OCH_2), 4.36 (m, 1 H, NCH), 5.29 (br s, 2 H, NH_2), 6.60 (d, J_o = 8 Hz, 1 H, 5'-H), 7.3–7.4 (m, 2 H, 2'- and 6'-H), 8.25 (d, *J* = 7 Hz, 1 H, CONH); UV (MeOH) λ_{max} nm (ϵ) 301 (17800), 280 sh (15700), 263 (13200), 256 sh (12500), 204 (40900); CI-MS *m/e* 353 (M + 1, 56), 150 (M–Glu(OEt)$_2$, 100). Anal. ($C_{17}H_{24}N_2O_6 \cdot {}^1/_{10}H_2O \cdot {}^3/_{10}EtOH$) C, H, N.

Diethyl *N*-[4-[[3-[2-(Acetylamino)-4-(diacetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl]propyl]amino]-3-methoxybenzoyl]-L-glutamate (17a, Z = 3'-MeO). Reductive amination of ester **20d** (3.52 g, 9.99 mmol) and aldehyde **16a**[28] (3.08 g, 9.99 mmol) was carried out as described for **17b**. The crude product was dissolved in EtOAc, washed, and purified by chromatography on SiO_2. The column was rinsed with EtOAc (400 mL) and then the product was eluted with 5% MeOH/EtOAc. The white solid was dried under high vacuum: yield 2.07 g (32%); mp 73–84 °C dec; NMR (Me_2SO-d_6) δ 1.15 (t, *J* = 7 Hz, 3 H, Me), 1.17 (t, *J* = 7 Hz, 3 H, Me), 1.65 (br m, 2 H, $CH_2CH_2CH_2$), 1.9–2.3 (br m, 4 H, pyrim-CH_2 and $CHCH_2$), 2.12 (s, 3 H, 2-NAc), 2.20 (s, 6 H, 4-NAc$_2$), 2.40 (t, *J* = 7 Hz, 2 H, CH_2CO_2), 3.10 (br m, 2 H, NCH_2), 3.80 (s, 3 H, OMe), 4.0–4.2 (2q, *J* = 7 Hz, 4 H, OCH_2), 4.39 (m, 1 H, NCH), 5.46 (br t, 1 H, ArNH), 6.47 (d, J_o = 8 Hz, 1 H, 5'-H), 7.29 (d, J_m = 2 Hz, 1 H, 2'-H), 7.42 (dd, J_o = 8 Hz, J_m = 2 Hz, 1 H, 6'-H), 8.28 (d, *J* = 7 Hz, 1 H, CONH), 11.9 (v br s, 2 H, NH); UV (MeOH) λ_{max} nm (ϵ) 306 (25800), 227 sh (17800), 205 (32400); FAB-MS *m/e* 645.6 (M + 1, 62), 442.5 (M–Glu(OEt)$_2$, 81), 400.1 (100). Anal. ($C_{30}H_{40}N_6O_{10}$) C, H, N.

***N*-[4-[[3-[2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl]-propyl]amino]-3-methoxybenzoyl]-L-glutamic Acid (10).** Diethyl ester **17a** (Z = 3'-MeO) (1.00 g, 1.55 mmol) was hydrolyzed and precipitated as in example **3**. The product was rinsed twice with 25 mL of pH 2.1 aqueous HCl and dried to give a white solid: yield 596 mg (83%); mp 165–184 °C dec; NMR (Me_2SO-d_6) δ 1.56 (m, 2 H, $CH_2CH_2CH_2$), 2.0 (br m, 2 H, $CHCH_2$), 2.2–2.4 (m, 4 H,

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pyrim-CH_2 and CH_2CO_2), 3.07 (m, 2 H, NCH_2), 3.81 (s, 3 H, OMe), 4.37 (m, 1 H, NCH), 5.6 (br s, 1 H, ArNH), 5.74 (s, 2 H, 2-NH_2), 5.93 (s, 2 H, 4-NH_2), 6.49 (d, J_o = 8 Hz, 1 H, 5'-H), 7.29 (d, J_m = 2 Hz, 1 H, 2'-H), 7.41 (dd, J_o = 8 Hz, J_m = 2 Hz, 1 H, 6'-H), 8.15 (d, J = 8 Hz, 1 H, CONH), 9.8 (v br s, 1 H, N1-H), 12.3 (v br s, 2 H, CO_2H); UV (0.1 N NaOH) λ_{max} nm (ϵ) 306 (15 100), 273 (15 200), 215 (25 800). Anal. ($C_{20}H_{28}N_6O_7 \cdot {}^7/_{10}H_2O$) C, H, N.

Diethyl *N*-[4-[[3-[2-(Acetylamino)-4-(diacetylamino)-1,6-dihydro-6-oxo-5-pyrimidinyl]propyl]amino]-2-chlorobenzoyl]-L-glutamate (17a, Z = 2'-Cl). Under reductive amination conditions (see **17b**), diethyl *N*-(4-amino-2-chlorobenzoyl)-L-glutamate[44] (2.84 g, 7.96 mmol) and aldehyde **16a**[28] (2.70 g, 8.76 mmol) gave a crude product that was dissolved in EtOAc, washed with 1:1 brine–H_2O, and chromatographed on SiO_2 with 5% MeOH in EtOAc to give a white solid: yield 2.28 g (44%); mp 63–72 °C dec; NMR (Me_2SO-d_6) δ 1.16 (t, J = 7 Hz, 3 H, Me), 1.18 (t, J = 7 Hz, 3 H, Me), 1.62 (m, 2 H, $CH_2CH_2CH_2$), 2.0 (br m, 2 H, $CHCH_2$), 2.12 (s, 3 H, 2-NAc), 2.23 (s, 6 H, 4-NAc_2), 2.26 (m, 2 H, pyrim-CH_2), 2.42 (t, J = 8 Hz, 2 H, CH_2CO_2), 2.99 (m, 2 H, NCH_2), 3.9–4.2 (2q, J = 7 Hz, 4 H, OCH_2), 4.34 (m, 1 H, NCH), 6.29 (br t, J = 5 Hz, 1 H, ArNH), 6.4–6.6 (m, 2 H, 3'- and 5'-H), 7.21 (d, J_o = 8 Hz, 1 H, 6'-H), 8.35 (d, J = 8 Hz, 1 H, CONH), 11.9 (br, 2 H, NH's); UV (MeOH) λ_{max} nm (ϵ) 289 (26 900), 207 (44 300); FAB-MS m/e 649 (M, 48), 615 (22), 446 (M–Glu(OEt)$_2$, 100), 412 (46). Anal. ($C_{29}H_{37}ClN_6O_9$) C, H, N, Cl.

***N*-[4-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]-2-chlorobenzoyl]-L-glutamic Acid (11).** Diethyl ester **17a**, (Z = 2'-Cl) (1.00 g, 1.54 mmol) was hydrolyzed (see **3**), precipitated, filtered, rinsed with cold pH 2.5 aqueous HCl, and dried to give a white solid: yield 437 mg (61%); mp 150–178 °C dec; NMR (Me_2SO-d_6) δ 1.55 (m, 2 H, $CH_2CH_2CH_2$), 1.95 (m, 2 H, $CHCH_2CH_2$), 2.1–2.4 (m, 4 H, pyrm-CH_2 and CH_2CO_2), 2.97 (m, 2 H, NCH_2), 4.32 (m, 1 H, NCH), 5.6–6.6 (v br s, 1 H, ArNH), 5.87 (br s, 2 H, NH_2), 6.12 (br s, 2 H, NH_2), 6.5 and 6.52 (d and s overlapped, 2 H, 3'- and 5'-H), 7.23 (d, J_o = 8 Hz, 1 H, 6'-H), 8.20 (d, 1 H, CONH), 10.0–12.6 (v br s, 3 H, NH and OH); UV (0.1 N NaOH) (ϵ) 271 (23 000), 217 (28 000). Anal. ($C_{19}H_{23}ClN_6O_6 \cdot {}^{13}/_{10}H_2O$) C, H, N, Cl.

Ethyl 4-(4-Chlorobutyramido)benzoate (26). To a solution of ethyl 4-aminobenzoate (50 g, 0.30 mol) in 400 mL of dry CH_2Cl_2 was added 0.3 mol of triethylamine. The solution was cooled to 3 °C, and a solution of 4-chlorobutyryl chloride (0.3 mol) in 100 mL of CH_2Cl_2 was added to it dropwise. The temperature was allowed to rise to RT, and the solution was stirred overnight. The solution was diluted to 900 mL with $CHCl_3$, and then it was washed with two 500-mL portions of 2 N HCl, two 500-mL portions of saturated $NaHCO_3$, and 500 mL of saturated NaCl. The washed organic layer was dried over $MgSO_4$, filtered, and evaporated. The product was obtained as an off-white solid: yield 77 g (94%); mp 91–93 °C; IR (KBr) cm^{-1} 3310, 1709, 1668, 1595, 1535, 1408, 1280, 1250, 1170, 1122, 1100, 770, 715, 670; NMR (Me_2SO-d_6) δ 1.29 (t, J = 7.1 Hz, Me), 2.02 (pent, J = 6.8 Hz, 2 H, $CH_2CH_2CH_2$), 2.51 (t, J = 7.2 Hz, CH_2CO), 3.69 (t, J = 6.40 Hz, $ClCH_2$), 4.26 (q, J = 7.03 Hz, OCH_2), 7.70 (d, J = 8.8 Hz, H-3',5'), 7.88 (d, J = 8.8 Hz, H-2',6'), 10.29 (br s, NH).

Ethyl [2-Oxo-1-pyrrolidinyl]benzoate (27).[45] Potassium *tert*-butoxide (*t*-BuOK) (2.2 g, 0.0196 mol, Aldrich) and 55 mL of dry THF were mixed in a dry apparatus. This hazy suspension was cooled with an ice bath and treated dropwise over 30 min with a solution of chloro amide **26** (4.94 g, 0.0183 mol) in 50 mL of dry THF. This mixture was stirred 2 h at 0 °C and 2 h at RT. The cyclization was not complete, so additional *t*-BuOK (1.1 g) was added, and the mixture was stirred overnight. The mixture was diluted to 400 mL with Et_2O, washed twice with 250 mL of 1 N HCl, twice with 250 mL of saturated $NaHCO_3$, and twice with 200 mL of saturated NaCl. An insoluble material was filtered. The Et_2O layer was dried ($MgSO_4$) and evaporated to a pale yellow solid: yield 3.0 g (70%); IR (KBr) cm^{-1} 3400 br, 2980, 1700 br, 1608, 1510, 1460, 1425, 1390, 1325, 1300, 1275, 1220, 1180, 1105, 1020, 850, 772, and 700 (showed loss of N–H stretch and C–Cl bands); NMR (Me_2SO-d_6) δ 1.30 (t, J = 7.05 Hz, Me), 2.06 (pent, J = 7.6 Hz, $CH_2CH_2CH_2$), 2.52 (t, J = 7.8 Hz, $CH_2CH_2C{=}O$), 3.86 (t, J = 7.2 Hz, NCH_2), 4.25 (q, J = 7.0 Hz, 2 H, OCH_2), 7.80 (d, J = 9.1 Hz, 3'- and 5'-H), 7.94 (d, J = 9 Hz, 2'- and 6'-H). A sample was recrystallized from EtOAc–hexanes to obtain colorless platelets: mp 93–94 °C. Anal. ($C_{13}H_{15}NO_3$) C, H, N.

Ethyl 4-[3-(2-Ethoxy-1-hydroxy-2-oxoethylidene)-2-oxo-1-pyrrolidinyl]benzoate (28). To a solution of distilled diethyl oxalate (1.2 mL, 8.9 mmol) and lactam **27** (1.0 g, 4.3 mmol) in 10 mL of dry THF was added a suspension of 80% NaH–mineral oil (0.25 g) in 5 mL of THF. The yellow mixture was heated briefly and then stirred at RT for 2 h. The mixture was treated with 0.5 mL of HOAc. The color faded. This mixture was diluted with 100 mL of $CHCl_3$ and was washed with H_2O, saturated $NaHCO_3$, and saturated NaCl. The yellow solution was dried ($MgSO_4$) and evaporated to a yellow powder. The above procedure was repeated, and the resulting products were combined and flash chromatographed on SiO_2 with 1% EtOH in $CHCl_3$ as eluent. The product-containing fractions were combined and evaporated to a pale yellow powder: yield 1.7 g (59%); mp 170–172 °C; NMR ($CDCl_3$) δ 1.39 (t, J = 7.13 Hz, 6 H, Me), 3.22 (t, J = 7.1, 2 H, CCH_2CH_2), 3.98 (t, J = 7.1 Hz, NCH_2), 4.37 (q, 4 H, OCH_2), 7.78 (d, J = 9.1, 2 H, 3'- and 5'-H), 8.075 (d, J = 9.1, 2 H, 2'- and 6'-H), 11.97 (s, 1 H, OH); CI-MS m/e 334 (M + 1, 100); UV (EtOH) λ_{max} nm (ϵ) 257 (6290), 322 (27 900). Anal. ($C_{17}H_{19}NO_6$) C, H, N.

Ethyl 4-[3-(3-Amino-2,5-dihydro-5-oxo-1,2,4-triazin-6-yl)-2-oxo-1-pyrrolidinyl]benzoate (29). The diester **28** (16.95 g, 51 mmol) and aminoguanidine carbonate (8.0 g, 59 mmol) were dissolved in 320 mL of 95% EtOH and 330 mL of H_2O. This solution was refluxed for 8 h and then stored at 5 °C overnight. The suspension was filtered to remove an insoluble yellow solid. This solid was washed twice with 95% EtOH and finally with Et_2O, and the resulting powder was dried at 50 °C under vacuum overnight, yield 11.74 g (67%). The reaction mother liquor was condensed to $^1/_3$ volume and refrigerated to obtain a second crop of solid that was washed and dried as before, yield 5.05 g (29%). The product was poorly soluble in DMSO, DMF, and EtOH; but it would dissolve in 0.1 N NaOH: mp >275 °C; NMR (Me_2SO-d_6) δ 1.3 (t, J = 7 Hz, Me), 2.3 (m, 2 H, $CHCH_2$), 3.63 (t, J = 9.11 Hz, 0.7 H, CH of lactam), 3.9 (m, 2 H, NCH_2), 4.28 (q, J = 7 Hz, OCH_2), 6.89 (br s, 0.7 H, NH), 7.6 (v br, 2 H, NH_2), 7.81 (d, J = 8.64 Hz, 2 H, 3'- and 5'-H), 7.95 (d, J = 9.1 Hz, 2 H, 2'- and 6'-H), 12.2 (br s, 0.3 H, NH or OH); CI-MS m/e 344 (M + 1, 100), 318 (M + 1-CN, 20), 259 (M + 1-HNCO–NH_2CN); UV (EtOH) λ_{max} nm (ϵ) 276; IR (KBr) cm^{-1} 3400s, 2960w, 1700s, 1625m, 1600s, 1575, 1510m, 1475w, 1425m, 1390s, 1275s, 1225w, 1180m, 1105m, 1015w, 850w, 770m. Anal. ($C_{16}H_{17}N_5O_4 \cdot {}^7/_{10}H_2O$) C, H, N.

4-[3-(3-Amino-2,5-dihydro-5-oxo-1,2,4-triazin-6-yl)-2-oxo-1-pyrrolidinyl]benzoic Acid (30). The triazinyl ester **29** (5.80 g, 16.9 mmol) was mixed with 177 mL of 1 N NaOH and stirred at RT for 17 h and then 75 °C for 2 h. The mixture was cooled, treated with Darco G-60 carbon, and filtered. The filtrate was adjusted to pH 3 with concentrated HCl. The mixture was refrigerated for 4 h and was then filtered. The finely divided solid was suspended in 95% EtOH and filtered. The cake was suspended in Et_2O and filtered to obtain **30**. The mother liquor and washings were combined and evaporated to 100 mL where a second crop of solid precipitated, but this material was found to be a 2:1 mixture of lactam-opened diacid and **30**. The lactam acid **30** was dried under vacuum overnight: yield 3.0 g (56%); mp >250 °C dec; NMR (Me_2SO-d_6) δ 2.33 (m, 2 H, $CHCH_2$), 3.88 (t, J = 9 Hz, CH), 3.93 (m, NCH_2), 6.92 (br s, NH_2), 7.815 (d, J = 8.92 Hz, 3'- and 5'-H), 7.965 (d, J = 8.9 Hz, 2'-H and 6'-H), 12.2 (br s, NH), 12.8 (br, COOH); UV (0.1 N NaOH) λ_{max} nm 273, 310 sh; CI-MS m/e 316 (5, M + H), 231 (10, M + H–[O=CNC(NH_2)-NH]), 206 (43, M + H-triazinone), 138 (89, PABA + H), 94 (100, aniline + H). Anal. ($C_{14}H_{13}N_5O_4 \cdot {}^3/_5H_2O$) C, H, N.

***N*-[4-[3-(3-Amino-2,5-dihydro-5-oxo-1,2,4-triazin-6-yl)-2-oxo-1-pyrrolidinyl]benzoyl]-L-glutamic Acid (12).** The benzoic acid **30** (1.26 g, 4 mmol) was coupled with diethyl L-glutamate·HCl by the DCC method (see **25a**). The crude product, a dark amber oil, was purified by flash chromatography on SiO_2

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with 5% then 10% MeOH in $CHCl_3$ as eluents. The intermediate diethyl ester was hydrolyzed (see 3). The crude product could not be precipitated or crystallized, but it was separated in 12 portions on a 21.1 × 250 mm^2 Regis C_{18} column. The column was eluted with a 5% to 15% MeCN gradient in 0.1% aqueous trifluoroacetic acid at 13.5 mL/min. The combined product was reeluted from the same column with 18% MeCN–0.1% TFA. The product-containing fractions were combined and lyophylized to a white powder: yield 0.53 g (12%); mp 150 °C dec; NMR (D_2O) δ 2.1 (m, 1 H, $CHCH_2CH_2$), 2.2 (m, β-H on Glu), 2.3 (m, 1 H, $CHCH_2CH_2$), 2.34 (t, J = 7.5 Hz, γ-CH_2 on Glu), 2.55 (m, β-H), 4.1 (m, CHC=O), 4.16 (t, J = 8.8 Hz, NCH_2), 4.35 (m, α-H), 7.71 (d, J = 8.6 Hz, 3′- and 5′-H), 7.91 (d, J = 8.6 Hz, 2′- and 6′-H); FAB-MS (thioglycerol) m/e 445 (M + 1), 429 (M + 1-NH_2); UV (0.1 N NaOH) λ_{max} (ε) 278 (16 500); IR (KBr) cm^{-1} 1689, 1655, 1638, 1608, 1570, 1502, 1426, 1400, 1294, 1201, 1140, 1109, 1025, 854, 805, 773, 725. Anal. ($C_{19}H_{20}N_6O_7 \cdot {}^3/_5TFA \cdot {}^{12}/_5H_2O$) C, H, N, F.

Biological Tests. The details of the GAR-Tfase, AICAR-Tfase, and FPGS assays were reported previously.[28]

MTX Uptake Assay. The ability of compounds to block the uptake of radiolabeled MTX into Molt-4 cells was used as a measure of the affinity of the test compounds for the reduced folate transport system as discussed in an earlier publication.[46]

Cell Culture Method for Evaluation of Compounds as Antitumor Agents. Cells and Medium. Molt-4 T-cell leukemia and MCF-7 human breast adenocarcinoma cells, obtained from the American Type Culture Collection (ATCC), were grown in RPMI 1640 medium supplemented with 10 nM calcium leucovorin as the folate source, 10% dialyzed fetal calf serum, penicillin, streptomycin, and, for MCF-7, sodium pyruvate (110 μg/mL).

Cytotoxicity Assay. Cells were seeded into 96-well plates using a Perkin-Elmer Pro/pette. MCF-7 cells were seeded at 15 000 cells per well in 150 μL of medium. Prior to the addition of drugs, cultures were incubated for 24 h at 37 °C. Compounds were added at 2× concentration in 150 μL of medium and each concentration was assayed in triplicate. Cultures were incubated for 72 h in a 37 °C humidified incubator at 5% CO_2. Inhibition of cell growth was measured using the MTT dye reduction assay.

MTT Dye Reduction Assay. Cell dilutions for a standard curve were prepared from a 72-h log-phase culture. Serial dilutions were seeded in triplicate in 96-well plates and incubated at 37 °C for 1 h. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was dissolved in phosphate buffered saline at 5 mg/mL and sonicated for 30 s. Using the Perkin-Elmer Pro/pette, 200 μL of medium was removed and 100 μL of MTT was added to the wells of the standard curve and test plates. Suspension cultures were spun for 5 min at 1000 rpm before removing medium from the wells. Plates were incubated for 1 h at 37 °C on a platform shaker. Following this incubation, 100 μL of medium was removed from the wells and 100 μL of dimethyl sulfoxide was added to each well. The plates were sonicated for approximately 10 s to solubilize the precipitated formazan dye. The absorbance of each well was measured using a Titertek Multiskan MC microtiter plate reader at 570 nm with a reference wavelength of 750 nm.

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Registry No. **2**, 126632-31-3; **2**·2TFA, 139348-96-2; **3**, 135439-31-5; **4**, 129166-73-0; **5**, 132497-50-8; **6**, 124656-55-9; **7**, 126632-27-7; **8**, 126632-26-6; **9**, 126632-28-8; **10**, 126632-29-9; **11**, 126632-30-2; **12**·${}^3/_5$TFA, 139348-92-8; **13**, 118252-48-5; **14b**, 139348-93-9; **15b**, 139348-94-0; **16a**, 118252-53-2; **16b**, 139348-95-1; **17a** (Z = 2′-F, di-*tert*-butyl ester), 126632-33-5; **17a** (Z = 3′-F), 126632-36-8; **17a** (Z = 3′-Me), 126632-38-0; **17a** (Z = 3′-MeO), 126632-41-5; **17a** (Z = 2′-Cl), 126632-42-6; **17b**, 139348-97-3; **18a**, 403-21-4; **18d**, 5081-36-7; **19a**, 126632-34-6; **19c**, 126632-47-1; **19d**, 126632-39-1; **20** (R = Et, Z = H), 13726-52-8; **20a**, 126632-35-7; **20b**, 85803-27-6; **20c**, 126632-37-9; **20d**, 126632-40-4; **20e**, 80014-92-2; **21a**, 739-33-3; **22a**, 126632-43-7; **22b**, 129166-68-3; **22c**, 74226-00-9; **23a**, 126632-44-8; **23b**, 129166-69-4; **23c**, 132497-46-2; **24a**, 126632-45-9; **24b**, 129166-71-8; **24b** (ethyl ester), 129166-70-7; **24c**, 132497-48-4; **24c** (ethyl ester), 132497-47-3; **25a**, 126632-46-0; **25b**, 129166-72-9; **25c**, 132497-49-5; **26**, 139348-98-4; **27**, 86364-65-0; **28**, 139348-99-5; **29**, 139349-00-1; **30**, 139349-01-2; MTX, 59-05-2; FPGS, 63363-84-8; GAR-Tfase, 9032-02-4; AICAR-Tfase, 9032-03-5; 4-$H_2NC_6H_4CO_2Et$, 94-09-7; (4-$EtO_2CC_6H_4S)_2$, 20057-83-4; $Br(CH_2)_4Br$, 110-52-1; $Br(CH_2)_3Br$, 109-64-8; $NCCH_2CO_2Et$, 105-56-6; $HN{=}C(NH_2)_2 \cdot HCl$, 50-01-1; $HN{=}CHNH_2 \cdot HOAc$, 40730-94-7; $HN{=}C(NH_2)NHNH_2 \cdot H_2CO_3$, 2582-30-1; H-Glu(OEt)-OEt·HCl, 1118-89-4; $Cl(CH_2)_3COCl$, 4635-59-0; $(CO_2Et)_2$, 95-92-1.

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Inhibition of Pig Kidney L-Aromatic Amino Acid Decarboxylase by 2,3-Methano-*m*-tyrosines

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Both racemic (*E*)- and (*Z*)-2,3-methano-*m*-tyrosines (**9***E* and **9***Z*) have been synthesized from a common intermediate, monoester (*Z*)-1-(ethoxycarbonyl)-2-[3-[(2-methoxyethoxy)methoxy]phenyl]cyclopropanecarboxylic acid (**5**). Quinine and ephedrine, respectively, were used to resolve their *N*-*tert*-butoxycarbonyl (Boc) derivatives. Among the compounds prepared, the (+)-(*E*)-diastereomer of **9** is the most potent inhibitor of L-aromatic amino acid decarboxylase (Dopa decarboxylase), having a K_i of 22 μM, with the (−)-*Z*-diastereomer (**9***Z*) second at K_i = 49 μM. (+)-**9***E* is a 45-fold more potent inhibitor of DDC than its acyclic analogue, D-*m*-tyrosine.

Introduction

L-Aromatic amino acid decarboxylase (Dopa decarboxylase, DDC) is a pyridoxal 5′-phosphate (PLP) dependent enzyme which catalyzes the decarboxylation of L-dopa and 5-hydroxy-L-tryptophan, thus playing a critical role in the biosynthesis of the important neurotransmitters, epinephrine, norepinephrine, and serotonin.[1] The possible involvement of catecholamines in a number of clinical

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