

**MULTISUBSTRATE ANALOGUE INHIBITORS OF GLYCINAMIDE
RIBONUCLEOTIDE TRANSFORMYLASE BASED ON 5-DEAZAACYCLO
TETRAHYDROFOLATE (5-DACTHF)**

Eric C. Bigham*, W. Revill Mallory, Steven J. Hodson, David S. Duch, Robert Ferone, and Gary K. Smith

Wellcome Research Laboratories, Burroughs Wellcome Co., 3030 Cornwallis Road,
Research Triangle Park, NC 27709, U.S.A.

Abstract- N^{10} -Substituted acetyl derivatives of 5-DACTHF are less active in general than the parent. However, multisubstrate analogue inhibitors that are 1000-fold more potent were synthesized, and N^{10} -pyruvoyl-5-DACTHF serves as a precursor for a metabolically assembled multisubstrate analogue.

Purine biosynthesis is currently under intensive investigation as a new target for the development of cancer chemotherapeutic agents. This interest stems from the role of de novo purine biosynthesis in providing purine nucleotides for DNA synthesis in rapidly growing cells.^{1,2} The most intensely studied target has been glycineamide ribonucleotide transformylase (GAR-Tfase, E.C.2.1.2.2), the first folate-requiring step in the biosynthetic pathway. The most promising compound in this area, 5,10-dideazatetrahydrofolate (DDATHF, 1), which was synthesized by Taylor et al., is now in clinical development at Eli Lilly and Co.³⁻⁵ Considerable effort has gone into SAR studies on this series, including acyclic 5,10-dideaza analogues.⁶

Recently, the synthesis of an acyclic analogue of 5-deazatetrahydrofolate, namely 5-deaza-acyclo-tetrahydrofolate (5-DACTHF, 2) and its activity as a GAR-Tfase inhibitor were reported by Kelley, Ferone, et al.^{7,8} Subsequently, the synthesis⁹ as well as in vitro, and in vivo antitumor activity¹⁰ of 5-DACTHF analogues was described. From this work we concluded that a more potent and less toxic GAR-Tfase inhibitor was needed.

The studies by Ingles and Benkovic on the mechanism of GAR-Tfase led to several new approaches for improving the potency and specificity of GAR-Tfase inhibitors.¹¹⁻¹⁵ Covalent labeling and site specific mutagenesis demonstrated the importance of Asp144 in stabilizing a tetrahedral intermediate (see Figure 2).¹¹⁻¹³ The formation of this intermediate in the active site prompted the synthesis of TGDDF ("ThiaGar-DiDeazaFolic acid") from N^{10} -bromoacetyl-5,8-dideazafolic acid and thioglycolamide ribonucleotide, the first successful multisubstrate analogue inhibitor (MAI) of GAR-Tfase.^{14,15} TGDDF is a slow, tight-binding inhibitor with $K_d = 0.25$ nM. The β anomer is much more active than the α , and the

phosphate is required for tight binding. The application of the MAI approach to folate-requiring enzymes has been reviewed by Broom.^{16, 17} The first attempted synthesis of an MAI for GAR-Tfase was not totally completed.¹⁸

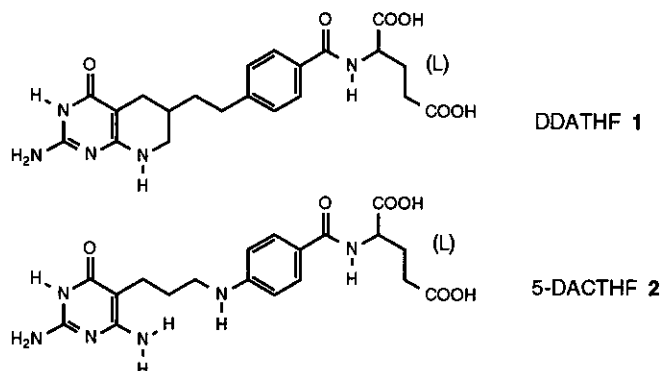


Figure 1

Inglese *et al.* also reported the metabolic assembly of a multisubstrate analogue (MAMA).¹⁵ Inglese showed that GAR-Tfase would catalyze the reaction of *N*¹⁰-bromoacetyl-5,8-dideazafofate with either GAR or "carba-GAR" to form a tight-binding MAI *in situ*. This approach avoids the problems associated with the complex synthesis of a complete multisubstrate analogue and its poor penetration into cells. Inhibition of carnitine acetyltransferase by this strategy had been previously reported by Chase and Tubs.¹⁹

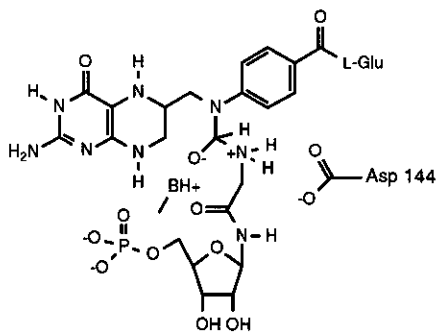


Figure 2

In this report, we describe the synthesis and biological activities of 5-DACTHF analogues utilizing the mechanism-based inhibitor, MAI, and MAMA approaches.

DESIGN AND SYNTHESIS

Mechanism Based Inhibitors. The possibility of additional polar binding sites in the vicinity of *N*-10 was suggested by the alkylation of Asp144 by *N*¹⁰-bromoacetyl-5,8-dideazafofate.¹¹⁻¹³ Furthermore, the work of Caperelli had shown that substitution on *N*-10 was not detrimental to binding even for relatively large groups.^{20, 21} Therefore, we prepared derivatives of 2 that could potentially interact with the active site as shown Figure 3.

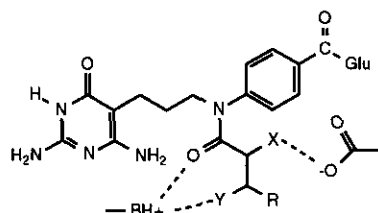
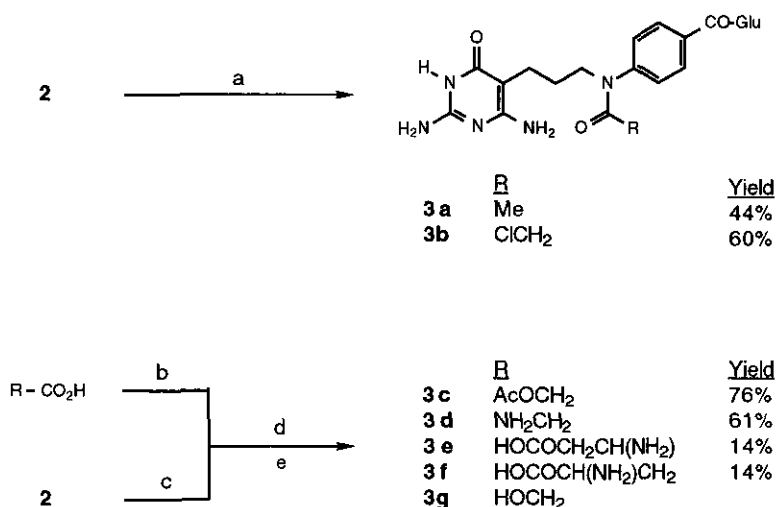


Figure 3

For this work, a method for the selective acylation of **2** at *N*-10 was required. Temple reported the selective acetylation on *N*-10 of folic acid with an excess of acetyl chloride.²² This method was satisfactory for the preparation of the acetyl (**3a**) and chloroacetyl derivatives (**3b**) of **2** (See Scheme 1). The use of four equivalents of acid chloride was undesirable in the case of complex or rare acyl groups. We discovered that the amount of acylating agent could be reduced to as little as 1.1 equivalents by drying a DMAC (dimethylacetamide) solution of **2** over activated 3Å molecular sieves for 8-12 h prior to addition of the acylating agent.

The acyl activation method was also important. Alternatives to the use of acid chlorides, which might be difficult to prepare in some cases, were investigated. Anhydrides were found to give mixtures of 2-amino and *N*-10 acylation. Dimethylformimidate esters, which can be formed under very mild conditions, gave good yields of *N*-10 acylated compounds in most cases (see Scheme 1).²³ In the acetoacetyl case, a pre-formed *N*-hydroxysuccinimide active ester gave good results (see below). The yields of the aspartyl derivatives (**3e**) and (**3f**) were low because of difficulties

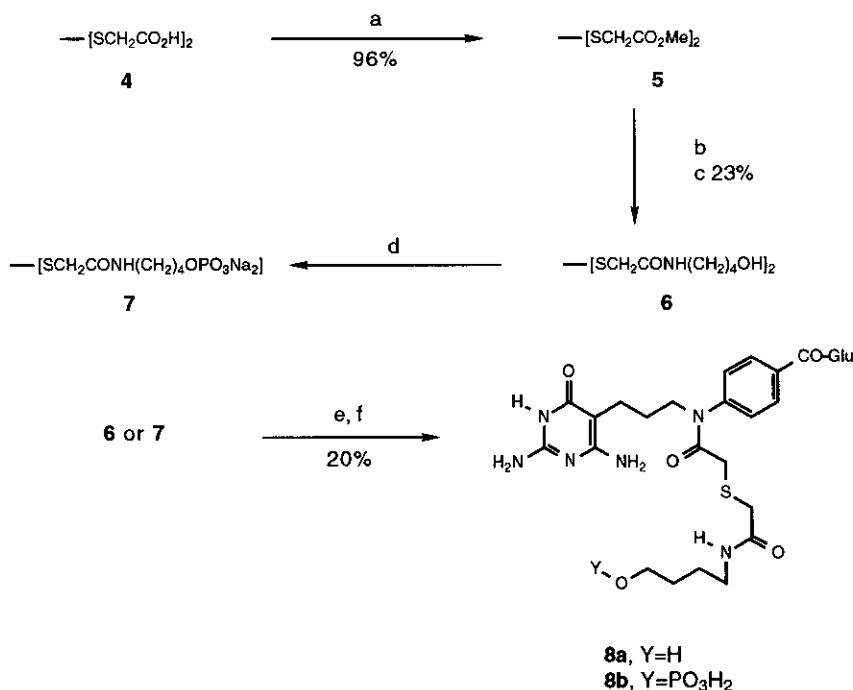


Reagents: (a) 4 RCOCl, DMAC, 6 h, room temperature (b) ClCOCOCl, DMF, -20°C (c) 3Å-Sieves, DMAC, 12 h, room temperature (d) -20°C. (e) deprotect

Scheme 1

encountered during the hydrogenation of the intermediate N-CBZ benzyl esters. The use of Dowex resins for neutralization of **3d** avoided having to separate the highly water soluble compound from inorganic salts.

Multisubstrate Analogue Inhibitors (MAI). Work by Ingles and Benkovic on TGDDF, an MAI of GAR-Tfase, showed that compounds with inhibitory potencies 1000-times greater than the best cofactor analogues are possible.¹⁴⁻¹⁵ Synthetic difficulties in the preparation of TGDDF directed us toward the synthesis of simpler compounds. As a starting point we chose 5-DACTHF (**2**) because it was a better substrate for the reduced folate transport system and a more potent inhibitor of tumor cell growth than was 5,8-dideazafolic acid.²⁴ The ribotide structure in TGDDF was replaced with a 4-carbon alkyl chain to give the stable "acyclo ribotide" (**8b**). The thioether and amide linkers were retained. In contrast to the Ingles synthesis,¹⁴ we protected the sulfur as a disulfide which was reduced to the sulfide in the presence of chloroacetyl-DACTHF (**3b**) (see Scheme 2). In this example the phosphate group needed no protection, and no sulfur protecting group fragments needed to be removed from the product.

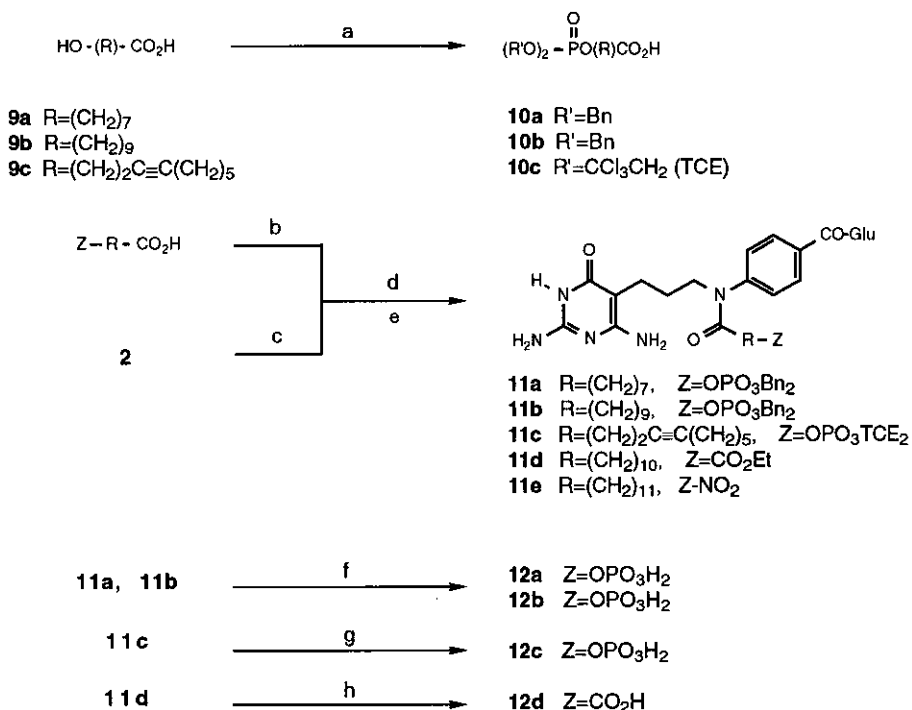


Reagents: (a) MeOH, HCl. (b) 2 NH₂(CH₂)₄OH, heat. (c) Kuglerrohr distillation. (d) POCl₃, (EtO)₃PO, NaOH. (e) **3b**, NaBH₄. (f) Preparative C-18 chromatography.

Scheme 2

The structure was simplified further by replacing the thioether and amide linkers with methylene groups. Initially, ω-hydroxyalkanoic acids were phosphorylated (POCl₃, (MeO)₃P=O, NaOH), but the resulting carboxyalkylphosphoric

acids could not be coupled to 5-DACTHF, **2**. Subsequently, the hydroxy acids were phosphorylated with dibenzyl or bis(2,2,2-trichloro)ethyl chlorophosphate, and the resulting intermediates (**10a-c**) were coupled to **2** by the Vilsmeier method (Scheme 3). The benzyl groups were hydrogenolyzed in the presence of trifluoroacetic acid to produce (**12a**) and (**12b**). The trichloroethyl groups of acetylenic analogue (**11c**) were removed with zinc in acetic acid to give (**12c**) (Scheme 3). Finally, compounds containing polar groups in place of phosphate (**11d**, **11e**, and **12d**) were also prepared via the Vilsmeier coupling as shown in Scheme 3.

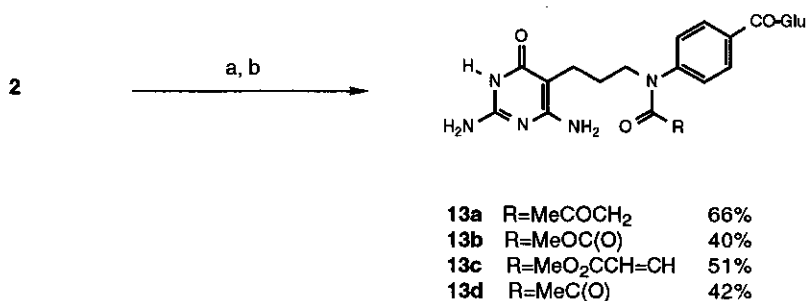


Reagents: (a) (R'O)₂POCl, pyridine, 20 h. (b)-(e) see Scheme 1. (f) 5% Pd/C, H₂, MeOH, H₂O, TFA. (g) Zn, 80% HOAc, 4 h. (h) 1N NaOH, EtOH.

Scheme 3

Metabolically Assembled Multisubstrate Analogues (MAMA). While extremely potent GAR-Tfase inhibitors can be designed with the MAI approach, these compounds have several drawbacks that limit their attractiveness as potential therapeutic agents: they are large, complex, difficult to synthesize, potentially unstable, and penetrate cells poorly. If one could discover a stable cofactor analogue that reacts covalently with GAR in the active site of GAR-Tfase to form a MAI in situ, then one could make use of the reduced folate transport system and folylpolyglutamate synthase (FPGS) to concentrate the folate cofactor analogue inside the cell. Ingles and Benkovic have demonstrated the MAMA approach in vitro,¹⁵ but *N*¹⁰-bromoacetyl dideazafofate is too reactive and too unstable to use as a drug. Therefore, we investigated the synthesis of carbonyl-containing derivatives of 5-DACTHF (2) that could react with the amino group of

GAR in the active site of GAR-Tfase to form an MAI. As shown in Scheme 4, the desired compounds (13a-d) were prepared by the methods described above. Thus acylation of a 3Å sieve-dried DMAC solution of **2** with the *N*-hydroxysuccinimide ester of acetoacetic acid, with methyl oxalyl chloride, and with the dimethylformimide esters of monomethylfumarcic acid and pyruvic acid gave the *N*-10 acyl derivatives (13a-d) in moderate yields. It should be noted that diketene failed to react with **2**, and a variety of attempts to make the glyoxamide (13, R = CH=O) failed.



Reagents: (a) 3Å molecular sieve, DMAC, 12 h. (b) RC(O)-Y, DMF or DMAC. Y=*N*-hydroxysuccinimide, Cl⁻, or dimethylformimide.

Scheme 4

BIOLOGICAL RESULTS AND DISCUSSION

The compounds described here were tested as inhibitors of hog liver GAR-Tfase and of growth of MCF-7 human breast adenocarcinoma in culture, as substrates for hog liver FPGS, and as inhibitors of methotrexate uptake into Molt-4 T-cell leukemia cells, which measured their affinity for the reduced folate transport system.^{7,9,25} These data are shown in Table 1.

Attempts to increase inhibitory potency through interaction with active site aspartate and histidine residues as pictured in Figure 2 were not successful. As data in Table 1 show, the simple *N*¹⁰-acetyl derivative (**3a**) was nearly 2-fold weaker vs. GAR-Tfase than **2**. Substitution on the acetyl group with chlorine (**3b**) or acetoxo (**3c**) further reduced the potency. The hydrogen-bond donor compounds (**3d**) and (**3g**) were slightly more potent than the parent compound (**2**). Aspartyl derivatives (**3e**) and (**3f**), were designed to improve potency by interactions with two active site groups as shown in Figure 3. These compounds were 10- to 20-fold weaker than 5-DACTHF (**2**). As can also be seen in Table 1, compounds (**3a**-**3g**) were generally poorer substrates for the reduced folate transport system and FPGS. Data in the last column show that the cumulative effect of these separate activities was a 5- to 80-fold reduction in tumor cell growth inhibition.

The MAIs (**8b**, **12b**, and **12c**) were exquisitely potent inhibitors of GAR-Tfase. The phosphate group was most important, since the corresponding alcohol (**8a**) was 2865-fold weaker. The substitution of other polar groups for the phosphate as in **11d**, **11e**, and **12d** resulted in better activity than the parent compound (**2**) but much weaker than the phosphate containing analogues. Replacement of the heteroatoms in the linking chain with methylene units, as in **12b**, only slightly affected its potency vs. GAR-Tfase. Shortening the chain length by two atoms reduced potency 5-fold (see **12a**).

The potent inhibition of GAR-Tfase *in vitro* by MAIs was not reflected in the tumor cell growth inhibition assay. Although the MAIs bind well to the reduced folate transport system (Table 1), they may not be concentrated in the cell. In fact, while ^3H -12b was shown to bind to the cell membrane, only the dephosphorylated alcohol was found in cytosol (data not shown); and, in contrast to ^3H -2, which accumulated 150-fold, the alcohol did not accumulate over extracellular levels.⁸ Furthermore, compounds (11d and e) bind so tightly (20-30 nM) that they could be inhibitors of the folate transport system. Finally, none of the MAIs were good substrates for FPGS.

Table 1. Biological Data for Purine Biosynthesis Inhibitors

Compound No.	GAR-Tfase ^a	FPGS ^b	MTX Uptake ^c	Cell Growth ^d
	Hog liver IC ₅₀ μM	Hog liver V _{max} %/Km	MOLT-4 IC ₅₀ μM	MCF7 IC ₅₀ μM
2	3.3 $\pm$ 0.1	14.5	1.20	0.037
3a	5.40 $\pm$ 0.05	7.3	3.30	3.10
3b	8.00 $\pm$ 0.75	nd	1.90	na
3c	12.4 $\pm$ 1.4	0.64	1.60	0.200
3d	1.99 $\pm$ 0.32	6.2	8.50	0.400
3e	31.9 $\pm$ 2.7	0.23	8.00	2.20
3f	70.7 $\pm$ 11.6	0.25	24.0	1.05
3g	2.28 $\pm$ 0.13	4.9	4.50	0.200
8a	10.6 $\pm$ 0.8	0.16	2.80	14% $\pm$ 0.1
8b	0.0037 $\pm$ 0.0003	1.0	2.50	1.00
12a	0.0217 $\pm$ 0.0027	nd	1.50	nd
12b	0.0046 $\pm$ 0.0004	1.5	2.20	2.500
12c	0.00569 $\pm$ 0.0004	nd	1.90	30.0
11d	1.50 $\pm$ 0.06	0.75	0.032	na
12d	0.648 $\pm$ 0.013	0.56	0.900	60.0
11e	0.506 $\pm$ 0.073	1.5	0.020	na
13a	16.5 $\pm$ 1.4	nd	2.20	0.060
13b	18.1 $\pm$ 1.4	nd	2.50	1.30
13c	71.0 $\pm$ 6.4	nd	0.450	>50.0
13d	5.00 $\pm$ 0.12	1.4	2.40	0.140

^aHog liver GAR transformylase with (6R)-10-formyl-FH4 as cofactor.^{7,9}

^bHog folylpolyglutamate synthase; V_{max}% is relative to aminopterin.^{7,9}

^cInhibition of ^3H -methotrexate transport into MOLT-4 T-cell leukemia cells.²⁵

^dInhibition of growth of MCF-7 human breast adenocarcinoma using 72 h of continuous exposure.

The compounds (13a-d), synthesized as potential MAMAs, were not only tested in the standard assays (see Table 1) but also for time dependent inhibition of GAR-Tfase. Inhibition by compounds (2, 3, and 13a-c) was not time dependent;

however, the N^{10} -pyruvoyl analogue (13d) was a time dependent inhibitor of GAR-Tfase (see Table 2). Furthermore, this time dependency required the simultaneous presence of inhibitor (13d) GAR-Tfase, and the substrate GAR during the incubations. Time dependent inhibition could have resulted from the formation of the potentially reversible MAMA (shown in Figure 4) in the active site. Although pyruvamide (13d) had reasonable affinity for the reduced folate transport system, it was only one-fourth as potent as 2 in the tumor cell growth assay.

Table 2. Time Dependent Inhibition of GAR-Tfase by Compound 13d.

Preincubation Components				Time (min)	Reaction Initiators	Percent Inhibition
[13d] μ M	10-fTHFA	GAR	GAR-Tfase			
2.8	+	+	-	5	GAR-Tfase	32
2.8	+	+	-	10	GAR-Tfase	27
2.8	-	-	+	10	GAR+10-fTHFA	27
2.8	-	+	+	5	10-fTHFA	64.4
2.8	-	+	+	10	10-fTHFA	68.3
2.8	-	+	+	20	10-fTHFA	69.2

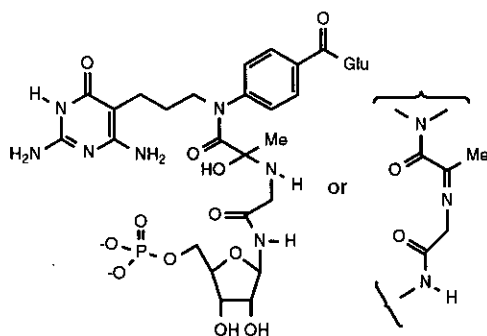


Figure 4

In conclusion, improved synthetic methods were developed for the selective acylation at N -10 of 5-DACTHF. Acyl groups containing polar, ionic, and hydrogen bonding groups failed to increase potency against GAR-Tfase. Multisubstrate analogue inhibitors, mimicking the bimolecular adduct formed during the transformylation, inhibit GAR-Tfase in the low nM ranges. The phosphate group is critical. A simpler compound, (13d), that could form an adduct with GAR in the active site of GAR-Tfase was synthesized and found to be a time dependent inhibitor as expected for a metabolically assembled multisubstrate analogue. However, the compounds were not found to have enhanced potency in cell culture; in at least one case this was due to poor cell penetration.

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 in pH 7 phosphate buffer on a Varian DMS 300

spectrometer, and $\lambda_{\max}$ are given in nm. $^1\text{H-Nmr}$ spectra were obtained on Varian XL200 or XL300 spectrometers. Chemical ionization mass spectra were obtained by Oneida Research Services, Whitesboro, NY, 13492. Mass spectral data are expressed as m/e (% Base, assignment). Elemental analyses (Atlantic Microlabs, Inc., Atlanta, GA) were within 0.4% of theoretical values. **Abbreviations:** TFA=trifluoroacetic acid, DMF=dimethylformamide, EtOAc=ethyl acetate, HOAc=acetic acid, THF=tetrahydrofuran, DMAC=dimethylacetamide, CIMS=chemical ionization mass spectrum, FABMS=fast atom bombardment mass spectrum, SiO_2 =silica gel, RT=room temperature, CBZ=carbobenzyloxy, DMSO=dimethyl sulfoxide.

***N*-[4-(Acetyl-(3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl]-L-glutamic Acid (3a).** A solution of acetyl chloride (0.135 ml, 1.9 mmol) in 1 ml of DMAC was added dropwise to solution of 5-DACTHF⁷ (2) (0.19 g, 0.41 mmol) in 3 ml of DMAC at room temperature. After 6 h, the solution was diluted with 20 ml of distilled H_2O , the pH was raised to 3.5 with 1N NaOH, this solution was evaporated to 1/4 volume and refrigerated overnight. A gum was isolated by decanting the water. The gum was twice reprecipitated by dissolution in 1-2 ml of hot water, cooling, and decanting the water phase. The tacky white solid was dried under high vacuum in a desiccator. Yield 0.091 g (44%): $^1\text{H-Nmr}$ (DMSO- d_6) δ : 1.40 (m, C- CH_2 -C), 1.75 (br s, MeCO), 2.00 (m, glu- β - CH_2), 2.105 (t, $J=7.6$ Hz, glu- γ - CH_2), 2.35 (t, $J=7.3$ Hz, pyrimidinyl- CH_2), 3.62 (t, $J=7.4$ Hz, N- CH_2), 4.38 (d of t, $J=7.7$ and $J=4.7$ Hz, glu- α -H), 5.63 (s, NH_2), 5.86 (s, NH_2), 7.40 (d, $J=8.4$ Hz, 3'-H and 5'-H), 7.89 (d, $J=8.4$ Hz, 2'-H and 6'-H), 8.64 (d, $J=7.73$ Hz, glu-NH), 9.65 (v br, NH), 12.40 (v br, OH); uv $\lambda_{\max}$ (e): 274 (15600), 243 (14900). Anal. Calcd for $\text{C}_{21}\text{H}_{26}\text{N}_6\text{O}_7 \cdot 7/5 \text{H}_2\text{O}$: C, 50.48; H, 5.81; N, 16.82. Found: C, 50.58; H, 5.84; N, 16.76.

***N*-(4-(2-Chloro-*N*-(3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)acetamido)benzoyl)-L-glutamic Acid (3b).** Compound (2) (1.0 g, 2.3 mmol) was acylated with chloroacetyl chloride (1.04 g, 9.2 mmol) by the same method used for 3a. An off-white solid, 3b, precipitated from water; yield 0.47 g (40%): $^1\text{H-Nmr}$ (DMSO- d_6) δ : 8.70 (d, $J=8$ Hz, 1H, NH), 7.90 (d, $J=8$ Hz, 2H, H-2' and H-6'), 7.50 (d, $J=8$ Hz, 2H, H-3' and H-5'), 5.90 (br s, 2H, NH_2), 5.70 (br s, 2H, NH_2), 4.20 (m, 1H, α -H), 4.00 (br, 2H, CH_2Cl), 3.80 (t, $J=7$ Hz, N- CH_2), 2.40 (t, $J=7$ Hz, 2H, pyrimidinyl- CH_2), 2.00-2.20 (m, 4H, CH_2 of Glu), 1.40 (m, C- CH_2 -C); uv $\lambda_{\max}$ (e): 274 (16800), 240 (17900). Anal. Calcd for $\text{C}_{21}\text{H}_{25}\text{N}_6\text{O}_7\text{Cl} \cdot 1/2 \text{HCl} \cdot 19/10 \text{H}_2\text{O}$: C, 44.93; H, 5.26; N, 14.97; Cl, 9.47. Found: C, 45.09; H, 4.95; N, 14.99; Cl, 9.36.

***N*-(4-(2-Acetoxy-*N*-(3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)acetamido)benzoyl)-L-glutamic Acid (3c).** A mixture of 2 (1.00 g, 2.13 mmol), activated 3 Å sieves (10 g), and dry DMAC (100 ml) was stirred while protected from light and under N_2 for 22 h and was then chilled to -20°C . Oxalyl chloride (268 μl , 3.07 mmol) was added dropwise to stirred dry DMF (28 ml) under N_2 at -20°C . Acetoxyacetic acid (0.324 g, 2.74 mmol) was added to the DMF mixture, and a solution formed during 28 min of stirring at -20°C . The DMF solution was added over 2 min to the DMAC mixture at -20°C . This mixture was allowed to warm to room temperature and stir under N_2 for 24.5 h. It was filtered through a bed of Celite, and the filtrate was concentrated under vacuum to a yellow oil that was solidified by the addition of acetonitrile (150 ml). The off-white solid was filtered, washed with acetonitrile (2 x 25 ml), and dried under vacuum; yield, 1.21 g of crude 3c. A 0.050-g sample was purified by reverse phase chromatography (Regis C18, 10 $\rightarrow$ 15% MeCN/ H_2O /0.1% TFA). Appropriate fractions were combined and concentrated to a residue that was dissolved in water and lyophilized to give 3c (0.045 g, 76%) as a white solid. $^1\text{H-Nmr}$ (DMSO- d_6) δ : 12.20 (br), 8.70 (d, $J=8.0$ Hz, 1H,

GluNH), 7.94 (d, $J=8.4$ Hz, 2H, H-2' and H-6'), 7.48 (d, $J=8.4$ Hz, 2H, H-3' and H-5'), 7.13 (br s, 2H, NH₂), 6.48 (br s, 2H, NH₂), 4.33 (m, 3H, NHCH₂ and CH₂OAc), 3.63 (t, $J=7$ Hz, 2H, CH₂NCO), 2.35 (t, $J=7.5$ Hz, 2H, CH₂), 2.15 (t, $J=7$ Hz, 2H, CH₂), 2.1 (m, 2H, CHCH₂), 2.00 (s, 3H, CH₃), 1.42 (m, 2H, CH₂CH₂CH₂); uv $\lambda_{\max}$ (ϵ): 241.6 (15100), 273.8 (14500); $\lambda_{\min}$ (ϵ): 229.1 (13300), 259.2 (12700). Anal. Calcd for C₂₃H₂₈N₆O₉·CF₃CO₂H·1.6 H₂O: C, 44.46; H, 4.81; N, 12.44. Found: C, 44.42; H, 4.77; N, 12.56.

N-(4-(2-Amino-N-(3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)acetamido)benzoyl)-L-glutamic Acid (3d). Pyrimidinone (2) (0.50 g, 1.07 mmol) in 50 ml of DMF was stirred for 18 h with 3Å sieves (5.0 g) then cooled to -20°C. Dry DMF (10 ml) was cooled to -20°C and treated with oxalyl chloride (0.14 ml, 1.60 mmol) followed by trifluoroacetyl glycine (0.275 g, 1.60 mmol). After 5 min the two solutions were mixed and allowed to warm to RT. After 1 h, the mixture was filtered and evaporated *in vacuo*. The residue was stirred in 10 ml of 0.5N NaOH for 1 h then diluted with 25 ml H₂O and the pH adjusted to 6.0 with DOWEX 50W X-8 (H⁺ form) (0.65 g). The resin was removed by filtration, the filtrate was evaporated to dryness, then the residue was dissolved in 100 ml of 5.0 mM NH₄HCO₃ and chromatographed on DEAE Sephadex (400 ml column volume) with a gradient of 5 mM to 1.0 M NH₄HCO₃. Appropriate fractions (monitored by hplc, Supelco C₁₈, 20%MeCN / H₂O / 0.1%TFA) were combined and lyophilized to give the product (3d) (0.36 g, 61%) as a white powder. ¹H-Nmr (DMSO-d₆) δ : 8.35 (d, $J=7$ Hz, 1H, GluNH), 7.90 (d, 2H, $J=8$ Hz, H-2' and H-6'), 7.45 (d, $J=8$ Hz, 2H, H-3' and H-5'), 6.0 (br s, 2H, NH₂), 5.65 (br s, 2H, NH₂), 4.28 (M, 1H, CH), 3.65 (m, 2H, CH₂N), 3.20 (m, 2H, COCH₂NH₂), 2.25 (m, 2H, CH₂CH₂CH₂N), 2.15 (m, 2H, CH₂CO₂H), 1.90 (m, 2H, CH₂CH₂CO₂H), 1.40 (m, 2H, CH₂CH₂CH₂); uv $\lambda_{\max}$ (ϵ): 273.0 (17,100), 241.5 (1670); $\lambda_{\min}$ (ϵ): 258.5 (14,700), 228.5 (14,500). Anal. Calcd for C₂₁H₂₇N₇O₇·7/2 H₂O: C, 45.65; H, 6.20; N, 17.74. Found: C, 45.56; H, 6.12; N, 17.95.

N-(4-(α -L-Aspartyl)-(3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutamic Acid (3e). Pyrimidinone (2) (1.0 g, 2.13 mmol) was dissolved in DMF (100 ml) treated with 3Å sieves (10.0 g) for 20 h, then cooled to -20°C. DMF (20 ml) was cooled to -20°C and treated with oxalyl chloride (0.241 ml, 2.76 mmol) followed by N-CBZ-L-aspartic acid β -benzyl ester (0.99 g, 0.276 mmol). The two solutions were mixed and allowed to warm to RT. After 1 h, the mixture was filtered, the filtrate evaporated *in vacuo*, and the resulting residue was dissolved in 200 ml of 1:1 EtOH/0.1N HCl. The mixture was treated with 5% Pd/C (0.5 g) and hydrogenated at 35 psi for 18 h. Catalyst was removed by filtration, and the filtrate was adjusted to pH 5.0 with 1.0 N NaOH and stored at 4 °C overnight. The resulting suspension was filtered to give a white solid which was dissolved in 10 ml H₂O by addition of TFA (0.5 ml). Lyophilization gave the desired product 3e as a white powder (0.26 g, 14%). ¹H-Nmr (D₂O) δ : 7.93 (d, $J=7$ Hz, 2H, H-2', H-6'), 7.54 (d, $J=7$ Hz, 2H, H-3' and H-5'), 7.87 and 7.40 (br doublets, 1/2 H each, NH of amide rotamers), 4.65 (m, 1H, Asp CH), 4.35 (m, 1H, Glu α -CH), 3.95 and 3.65 (m 1H each, rotamers of Asp-NCH₂), 2.90-2.50 (m, 4H, CH₂'s α to CO₂H's on Asp and Glu), 2.50-2.10 (m, 4H, CH₂ α to pyrimidinone, CH₂CH₂CO₂H to Glu CO₂H), 1.78 (m, 2H, CH₂CH₂CH₂); FABMs m/z: 548 (M+H), 432 (M+H-Aspartate), 401 (M+H-Glutamate), 286 (M+H-Glutamate-Aspartate); uv $\lambda_{\max}$ (ϵ): 274 (17200), 243 (17200); $\lambda_{\min}$ (ϵ): 258 (14700), 230 (15400). Anal. Calcd for C₂₃H₂₉N₇O₉·1.5H₂O·2.4 TFA: C, 39.37; H, 4.09; N, 11.56. Found C, 39.39; H, 4.16; N, 11.51.

N-(4-(β -L-Aspartyl)-(3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutamic Acid (3f). Pyrimidinone (2) (1.0 g, 2.13 mmol) was dissolved in DMF (100 ml) and treated with 3Å sieves (10.0 g) for 20 h then

cooled to -20°C . DMF (20 ml) was cooled to -20°C and treated with oxalyl chloride (0.24 ml, 2.76 mmol) followed by *N*-CBZ-aspartic acid α benzyl ester (0.99 g, 0.276 mmol). The two solutions were mixed and allowed to warm to RT. After 1 h, the mixture was filtered, the filtrate evaporated *in vacuo*, and the resulting residue was dissolved in 200 ml of 1:1 EtOH/0.1N HCl. The mixture was treated with 5% Pd/C (0.5 g) and hydrogenated at 35 psi for 18 h. Catalyst was removed by filtration, and the filtrate was adjusted to pH 5.0 with 1.0N NaOH and stored at 4°C overnight. The resulting suspension was filtered to give a white solid which was dissolved in 10 ml H_2O by addition of TFA (0.5 ml). Lyophilization gave the desired product (3f) as a white powder (0.28 g, 14%). $^1\text{H-Nmr}$ (D_2O) δ : 7.95 (d, $J=8$ Hz, 2H, H-2' and H-6'), 7.45 (d, $J=8$ Hz, 2H, H-3' and H-5'), 7.38 (d, $J=7$ Hz, 1H, GluNH), 4.68 (t, $J=5$ Hz, 1H, AspCH), 4.15 (t, $J=5$ Hz, 1H, GluCH), 3.73 (t, $J=7$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.80 (t, $J=5$ Hz, 2H, NCOCH_2), 2.57 (t, $J=6$ Hz, 2H, CH_2COCH), 2.50-2.10 (m, 4H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{H}_2\text{N}$ and $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 1.75 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$), FABms m/z : 548 (M+H), 286 (M+H-Glu-Asp), 186 (M+H-Glu-Asp-Ph-CO); $\text{uv } \lambda_{\text{max}}$ (ϵ): 273 (18,000), 242 (16,600); λ_{min} (ϵ): 257 (14,600), 230 (14,700). Anal. Calcd for $\text{C}_{25}\text{H}_{29}\text{N}_7\text{O}_9 \cdot 4.4 \text{ H}_2\text{O} \cdot 2.7 \text{ TFA}$: C, 36.28; H, 4.18; N, 10.71. Found: C, 36.50; H, 4.37; N, 10.49.

***N*-(4-((3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)(hydroxyacetyl)amino)benzoyl)-L-glutamic Acid (3g).**

A mixture of 0.847 g of crude 3c in 0.1N NaOH (81.4 ml) was stirred for 45 min. A small amount of undissolved solid was filtered, and the filtrate was brought to pH ~ 3 to 3.5 by the addition of HOAc and then concentrated HCl. The solution was concentrated under vacuum to $\sim 1/4$ of its original volume, and the mixture was refrigerated overnight. The solid was filtered while still cold, washed with 3×2 ml of cold H_2O , and dried under vacuum at room temperature; yield, 0.301 g of off-white solid. This solid was purified by reverse phase chromatography (Regis C18, 12% MeCN/ H_2O /0.1% TFA and then 8 $\rightarrow$ 15% MeCN/ H_2O /0.1% TFA). Appropriate fractions were combined and concentrated to a residue that was dissolved in water and lyophilized to give 3 g (0.223 g) as a white solid. $^1\text{H-Nmr}$ ($\text{DMSO}-d_6$) δ : 11.80 (br), 8.70 (d, $J=7.6$ Hz, 1H, Glu NH), 7.94 (d, $J=8.4$ Hz, 2H, H-2' and H-6'), 7.50 (br s, 2H, NH_2), 7.45 (d, $J=8.4$ Hz, 2H, H-3' and H-5'), 6.71 (br s, 2H, NH_2), 4.41 (m, 1H, NHCH), 3.67 (m, 4H, CH_2NCO and CH_2OH), 2.37 (t, $J=7.3$ Hz, 2H, CH_2), 2.20 (t, $J=7$ Hz, 2H, CH_2), 2.1 (m, 2H, CHCH_2), 1.45 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$); $\text{uv } \lambda_{\text{max}}$ (ϵ): 243.8 (16800), 274.2 (17700); λ_{min} (ϵ): 229.3 (14800), 258.4 (14900). Anal. Calcd for $\text{C}_{21}\text{H}_{26}\text{N}_6\text{O}_8 \cdot 1.35\text{CF}_3\text{CO}_2\text{H} \cdot 0.5 \text{ H}_2\text{O}$: C, 43.56; H, 4.37; N, 12.86. Found: C, 43.66; H, 4.66; N, 12.88.

Dimethyl Dithiodiglycolate (5). Dithiodiglycolic acid (4) (20.0 g, 0.11 mol) was stirred in dry MeOH (200 ml) and treated with acetyl chloride (5 ml, 0.07 mol). After 16 h at reflux under N_2 , the mixture was evaporated *in vacuo*, diluted with CH_2Cl_2 (150 ml), washed with saturated NaHCO_3 and dried (MgSO_4). Evaporation gave the desired diester (5) (22.1 g, 96%) as a colorless oil. $^1\text{H-Nmr}$ ($\text{DMSO}-d_6$) δ : 3.75 (s, 4H), 3.65 (s, 6H). CIMS (CH_4) m/z : 239 (M+29), 211 (M+H), 179 (M-OCH₃), 151 (M-CO₂CH₃). This product was used without further purification in the next reaction.

***N,N'*-Di(4-Hydroxy-*n*-butyl)dithiodiglycolamide (6).** Diester (5) (6.00 g, 28.5 mmol) was stirred in dry toluene (30 ml) and treated with 4-amino-1-butanol (6.0 ml, 65 mmol). After refluxing for 1.5 h under N_2 , the mixture was evaporated *in vacuo* and chromatographed (SiO_2 , EtOAc 80%/MeOH 20%) to give 6 (2.15 g, 23%). On a preparative scale, the diamide was purified by Kugelrohr distillation (120°C , 0.003 mm/Hg). $^1\text{H-Nmr}$ ($\text{DMSO}-d_6$) δ : 8.05 (t, $J=6$ Hz, 2H, NH), 4.48 (t, $J=5$ Hz, 2H, OH), 3.44 (s, 4H, CH_2S), 3.49 (m, 4H, CH_2N), 3.08 (m, 4H, CH_2O), 1.43 (m, 8H, CH_2CH_2). Anal. Calcd for $\text{C}_{12}\text{H}_{14}\text{N}_2\text{O}_4\text{S}_2$: C, 44.42; H, 7.46; N, 8.63; S, 19.77. Found: C, 44.42; H, 7.49; N, 8.62; S, 19.67.

N-(4-(N-(3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)-2-((4-hydroxybutyl)amino)-2-oxoethyl)thio)acetamido)benzoyl)-L-glutamic Acid (8a). Disulfide (6) (0.15 g, 0.46 mmol) was dissolved in EtOH (10 ml) and treated with NaBH₄ (0.023 g, 0.61 mmol). Compound (3b) (0.25 g, 0.46 mmol) was dissolved in DMF (10 ml) and treated with 1.0 N NaOH (1.0 ml) dropwise. After the two solutions were mixed and stirred for 1 h. The mixture was filtered, and the filtrate was evaporated *in vacuo*. The crude product was purified by reverse phase chromatography (Regis C₁₈ 20% MeCN/H₂O/0.1% TFA) followed by lyophilization to give the desired product (8a) (0.017 g, 6%) ¹H-Nmr (DMSO-d₆/D₂O) δ: 8.73 (d, *J*=7.5 Hz, 1/2H, GluNH), 7.96 (d, *J*=8.0 Hz, 2H, H-2' and H-6'), 7.46 (d, *J*=8.0 Hz, 2H, H-3' and H-5'), 4.40 (m, 1H, CH), 3.65, (t, *J*=7 Hz, 2H, CH₂NPh), 3.45 (s, 2H, CH₂S), 3.20 (m, 2H, CH₂OH), 3.10 (s, 2H, CH₂S), 2.98 (m, 2H, CH₂NH) 2.38 (t, *J*=7 Hz, 2H, CH₂ α to pyrimidinone), 2.20 (m, 2H, CH₂CO₂H), 2.15-2.00 (m, 2H, CH₂CH₂CO₂H), 1.45 (m, 2H, CH₂CH₂CH₂N-Ar), 1.4 (m, 4H, CH₂CH₂CH₂OH); FABms: calcd for C₂₇H₃₇N₇O₉S M+H: 636.245. Found, 636.2449; uv λ_{max}(ε): 272 (18,500), 243 (17,700); λ_{min} (ε): 257 (16,100), 230 (16,100).

N-(4-(3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl) 2-((2-oxo-2-((4-(phosphoryloxy)butyl)amino)-ethyl)thio)acetyl)amino)benzoyl)-L-glutamic Acid (8b). Disulfide 6 (1.49 g, 4.59 mmol) was dissolved in triethyl phosphate (30 ml) at 0 °C and treated with POCl₃ (1.03 ml, 11 mmol). After stirring for 4 1/2 h at 0 °C, the mixture was treated cautiously with 5 ml H₂O followed by 1.0N NaOH (~50 ml) to adjust the pH to 8.0. The mixture was evaporated *in vacuo* to 7. Compound (7) was diluted with water to 100 ml volume. Compound (3b) (2.46 g, 4.6 mmol) was stirred in H₂O (60 ml) and treated dropwise with 1.0 N NaOH (~4.6 ml) until the pH was 8.0. The diphosphate (7) solution and solution of 3b were mixed and treated with NaBH₄ (0.22 g, 5.95 mmol). The reaction mixture was stirred 1 h at RT, the pH was adjusted to 7.0 with 1.0 N HCl, and the solution was evaporated to dryness *in vacuo*. One third of the crude product was purified by reverse phase chromatography (Regis C₁₈ 10% →15% MeCN/H₂O/0.1% TFA) followed by lyophilization to give 8b (0.27 g, 18%) as a white powder. ¹H-Nmr (D₂O) δ: 7.90 (d, *J*=7.5 Hz, 2H, H-2' and H-6'), 7.42 (d, *J*=7.5 Hz, 2H, H-3' and H-5'), 4.60 (m, 1H, Glu α-CH), 3.80 (t, 2H, *J*=6 Hz, CH₂OP), 3.70 (t, *J*=7 Hz, 2H, CH₂NAr), 3.80, 3.70 (two s, 2H each, CH₂SCH₂), 3.09 (t, *J*=7 Hz, 2H, NHCH₂), 2.57 (t, *J*=7 Hz, 2H, CH₂CO₂H), 2.40-2.10 (m, 2H, CH₂CH₂CO₂H), 2.23 (t, *J*=7.5 Hz, 2H, CH₂ α to pyrimidinone), 1.70 (t, *J*=6 Hz, 2H, CH₂CH₂OP), 1.50 (m, 4H, CH₂CH₂CH₂NPh and CONHCH₂CH₂); FABms m/z: 716 (M+H); uv λ_{max} (ε): 274 (16,400), 243 (15,900); λ_{min} (ε): 259 (14,100), 230 (14,700). Anal. Calcd for C₂₇H₃₈N₇O₁₂ PS•2H₂O•TFA: C, 40.23; H, 5.01; N, 11.33; S, 3.70. Found: C, 40.09; H, 5.22; N, 11.32; S, 3.73.

8-Bisbenzyloxyphosphinoyloxyoctanoic Acid (10a). Dibenzyloxy phosphite (20.7 ml, 93.6 mmol) was stirred under N₂ in dry CCl₄ (300 ml) and treated dropwise with SO₂Cl₂ (9.8 ml, 122.0 mmol) over 10 min. Dry N₂ was bubbled through the mixture for 1.5 h, then the mixture was evaporated *in vacuo*. 8-Hydroxyoctanoic acid (5.00 g, 31.21 mmol) in pyridine (50 ml) and CCl₄ (450 ml) was cooled to 0°C and added to the chlorophosphoryl ester, and the mixture was stored at 4°C for 18 h. Water (50 ml) was added cautiously and the mixture evaporated *in vacuo* to about 50 ml. The mixture was dissolved in CH₂Cl₂ (500 ml) and washed with 1.0N HOAc (5x100 ml) and water (3x100 ml), dried (CaSO₄) and evaporated *in vacuo*. Chromatography (SiO₂ / CH₂Cl₂ / 5% MeOH) gave 10a as a colorless oil (7.17 g, 54%). ¹H-Nmr (DMSO-d₆) δ: 12.00 (br s, 1H, CO₂H), 7.38 (s, 10H, Ph) 5.00 (d, *J*=8 Hz, 4H, Ph-CH₂O), 3.95 (dd, *J*=8 Hz, 2H, POCH₂

CH_2), 2.18 (t, $J=7$ Hz, 2H, $\text{CH}_2\text{CO}_2\text{H}$), 1.50 (m, 4H, POCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 1.22 (m, 6H, $(\text{CH}_2)_3$). This intermediate was used without further purification.

10-Bisbenzyloxyphosphinoyloxydecanoic Acid (10b). Dibenzyl phosphite (17.5 ml, 79 mmol) was stirred under N_2 in dry CCl_4 (300 ml) and treated dropwise with SO_2Cl_2 (13.3 g, 9.9 mmol). After 15 min dry N_2 was bubbled through the solution for 1.5 h. The mixture was evaporated *in vacuo* and stored under high vacuum for 1 h then diluted with CCl_4 (100 ml) and cooled to 0°C . 10-Hydroxydecanoic acid (**9b**) (5.0 g, 26.5 mmol) was dissolved in pyridine (50 ml), diluted with 450 ml CCl_4 , and cooled to 0°C . The two solutions were mixed and stored at 4°C for 20 h. Water, 20 ml, was added cautiously, and the mixture evaporated *in vacuo*. The resulting residue was dissolved in CH_2Cl_2 (800 ml) and washed with 1.0 N HOAc (5 x 200 ml), water (5 x 200 ml), dried (CaSO_4) and evaporated *in vacuo*. Chromatography (SiO_2 , CH_2Cl_2) gave the desired product (**10b**). (8.85 g, 74%). $^1\text{H-Nmr}$ (DMSO-d_6) δ : 7.35 (s, 10H, Ph), 5.00 (d, $J=9$ Hz, 4H, $\text{PO-CH}_2\text{-Ph}$), 3.92 (dd, $J=8$ Hz, 2H, $\text{PO-CH}_2\text{CH}_2$), 2.17 (t, $J=7.5$ Hz, 2H, $\text{CH}_2\text{CO}_2\text{H}$), 1.50 (m, 4H, POCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 1.20 (s, 10H, $\text{OCH}_2\text{CH}_2\text{-(CH}_2)_5$). This product was used in the next reaction without further purification.

N-(4-(10-((Bis(benzyloxy)phosphinoyl)oxy)-N-(3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)-decanamido)benzoyl)-L-glutamic Acid (11b). Pyrimidinone (**2**) (0.96 g, 2.05 mmol) was dissolved in dry DMAC (100 ml) and treated with 3 Å sieves (10 g), while stirring under N_2 for 20 h, then the mixture was cooled to -20°C . Acid (**10b**) (1.38 g, 3.08 mmol) was dissolved in dry DMF (10 ml) and cooled to -20°C . Dry DMF (25 ml) was cooled to -20°C and treated dropwise with oxalyl chloride (0.27 ml, 3.08 mmol) followed by the solution of **10b**. After 5 min at -20°C , the entire mixture was added to the solution of **2** and allowed to warm to RT. After 1 h, the mixture was filtered, and the filtrate evaporated *in vacuo*. The residue was treated with H_2O (30 ml) and 1.0N NaOH (9.5 ml) to pH 10 and filtered. The filtrate was adjusted to pH 6.0 with dropwise addition of 1.0N HCl and eluted in several aliquots on a semi-preparative Supelco C₁₈ reverse phase column using a gradient of 40% MeCN/ H_2O to 75% MeCN/ H_2O . Evaporation of appropriate fractions gave **11b** (0.286 g, 0.33 mmol, 16%) as a white glass. $^1\text{H-Nmr}$ (DMSO-d_6) (D_2O exchanged) δ : 8.70 (d, $J=7$ Hz, 1H, GluNH), 7.95 (d, $J=7.8$ Hz, 2H, H-2' and H-6'), 7.40 (d, $J=7.8$ Hz, 2H, H-3' and H-5'), 7.35 (s, 10H, $\text{CH}_2\text{-Ph}$), 7.00 (br, 2H, NH_2), 6.40 (br, 2H, NH_2), 5.00 (d, $J=7.1$ Hz, 4H, CH_2OP), 4.40 (m, 1H, methine), 3.90 (m, 2H, POCH_2CH_2), 3.62 (dd, $J=6.5$ Hz, 2H, CH_2N), 2.37 (t, $J=7.1$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.17 (t, $J=6.5$ Hz, 2H, $\text{CH}_2\text{CO}_2\text{H}$), 2.30-1.90 (m, 4H, $-\text{CHCH}_2$, CH_2CON), 1.55-1.35 (m, 6H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$, $\text{CH}_2\text{CH}_2\text{CON}$, POCH_2CH_2), 1.35-1.05 (m, 10H, $-(\text{CH}_2)_5$); FABms m/z : 863 (M+H), 286 (M+H - Glutamic acid- N^{10} acyl-side chain), ms calcd: 863.3745. Found 863.3751. Anal. Calcd for $\text{C}_{43}\text{H}_{55}\text{N}_6\text{O}_{11}\text{P} \cdot 1.9 \text{H}_2\text{O} \cdot 0.7 \text{TFA}$: C, 54.59; H, 6.14; N, 8.60. Found: C, 54.31; H, 5.91; N, 8.88.

N-(4-(N-(3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)-11-(ethoxycarbonyl)undecanamido)benzoyl)-L-glutamic Acid (11d). A mixture of **2** (1.00 g, 2.13 mmol), activated 3 Å sieves (10 g), and dry DMAC (100 ml) was stirred while protected from light under N_2 for 22 h, then chilled to -20°C . Oxalyl chloride (268 μl , 3.07 mmol) was added dropwise to stirred dry DMF (28 ml) under N_2 at -20°C . Monoethyl dodecanedioate²⁶ (0.714 g, 2.74 mmol) was added to the DMF mixture, and a solution formed during 15 min of stirring at -20°C . The DMF solution was added over 2 min to the DMAC mixture at -20°C . This mixture was allowed to warm to room temperature and was stirred under N_2 for six

days. It was filtered through a bed of Celite, and the filtrate was concentrated under vacuum to a light yellow oil that was solidified by the addition of water (100 ml). The off-white solid was filtered, washed with water (2 x 10 ml), and dried under vacuum; yield, 1.43 g. This solid was recrystallized from EtOH/MeCN to give **11d** (0.962 g, 66%) as a white solid. $^1\text{H-Nmr}$ (DMSO- d_6) δ : 12.40 (br), 9.80 (br), 8.69 (d, $J=7.8$ Hz, 1H, GluNH), 7.92 (d, $J=8.4$ Hz, 2H, H-2' and H-6'), 7.41 (d, $J=8.4$ Hz, 2H, H-3' and H-5'), 6.02 (br s, 2H, NH_2), 5.74 (br s, 2H, NH_2), 4.41 (m, 1H, NHCH), 4.03 (q, $J=7.1$ Hz, 2H, CH_2CH_3), 3.63 (t, $J=7$ Hz, 2H, CH_2NCO), 2.37 (t, $J=7.4$ Hz, 2H, CH_2), 2.25 (t, $J=7.3$ Hz, 2H, $\text{CH}_2\text{CO}_2\text{Et}$), 2.10 (m, 3H, CH_2), 2.00 (m, 3H, CH_2), 1.45 (m, 6H, CH_2), 1.15 (m, 15H, $\text{CH}_3 + \text{CH}_2$); uv λ_{max} (ϵ): 242.9 (15200), 273.4 (16000); λ_{min} (ϵ): 229.9 (13800), 257.7 (13800). Anal. Calcd for $\text{C}_{33}\text{H}_{48}\text{N}_6\text{O}_9 \cdot 0.6 \text{H}_2\text{O}$: C, 57.98; H, 7.26; N, 12.30. Found: C, 57.93; H, 7.29; N, 12.34.

N-(4-(N-(3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)-12-nitrododecanamido)benzoyl)-L-glutamic Acid (11e). Pyrimidinone (**2**) (1.00 g, 2.13 mmol) was stirred for 18 h with activated 3Å sieves (10.0 g) in DMAC (100 ml), then the mixture was cooled to -20°C . Oxalyl chloride (0.24 ml, 2.77 mmol) was added dropwise to a stirred sample of DMF (25 ml) at -20°C . 12-Nitrododecanoic acid (0.68 g, 2.77 mmol) was added to the DMF suspension and the mixture stirred at -20°C for 5 min during which partial solution occurred. The DMF mixture was added to the DMAC solution and allowed to warm to RT. After 4 h, the mixture was filtered and the evaporated *in vacuo*. Addition of H_2O (50 ml) gave a solid which was collected by filtration, washed with water, and dried *in vacuo*. Crystallization from isopropanol gave pure product (**11e**) (0.57 g, 38%) as a first crop. $^1\text{H-Nmr}$ (DMSO- d_6) δ : 12.4 (br s, 2H, CO_2H 's), 9.80 (br s, 1H, pyrimidinone NH), 8.70 (d, $J=8$ Hz, 1H, GluNH), 7.93 (d, $J=8$ Hz, 2H, H-2' and H-6'), 7.42 (d, $J=8$ Hz, 2H, H-3' and H-5'), 6.00 (br s, 2H, NH_2), 5.50 (br s, 2H, NH_2), 4.52 (t, $J=7.5$ Hz, 2H, CH_2NO_2), 4.40 (m, 1H, CH), 3.63 (t, $J=7.5$ Hz, 2H, CH_2N), 2.38 (t, $J=7.5$ Hz, 2H, CH_2 α to pyrimidinone), 2.10 (m, 2H), 1.95 (m, 2HNCOCH_2 , $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 1.85 (m, 2H $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 1.5-1.35 (m, 4H, $\text{NCOCH}_2\text{CH}_2$, $\text{O}_2\text{NCH}_2\text{CH}_2$), 1.30-1.10 (m, 16H, Ph-N- CH_2CH_2 , $\text{O}_2\text{NCH}_2\text{CH}_2\text{CH}_2$); uv λ_{max} (ϵ): 273 (17200), 241 (17300); λ_{min} (ϵ): 259.1 (14700), 230 (16104). Anal. Calcd for $\text{C}_{31}\text{H}_{45}\text{N}_7\text{O}_9 \cdot \text{H}_2\text{O} \cdot 3/10 \text{C}_3\text{H}_8\text{O}$: C, 55.00; H, 7.16; N, 14.07. Found: C, 55.16; H, 6.96; N, 13.88.

N-(4-(N-(3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)-8-(phosphonoxy)octanamido)benzoyl)-L-glutamic Acid (12a). Pyrimidinone (**2**) (1.0 g, 2.1 mmol) was stirred in dry DMAC (100 ml) with 3Å sieves (10.0 g) under N_2 for 18 h then cooled to -20°C . Dry DMF (25 ml) was treated dropwise at -20°C with oxalyl chloride (1.16 g, 2.77 mmol) followed by **10a** (1.16 g, 2.77 mmol). After 5 min the two solutions were mixed and allowed to warm to RT. The mixture was filtered, and the filtrate was evaporated *in vacuo* to give a yellow glass (**11a**) that was treated with EtOH (60 ml), 1.0N HCl (30 ml) and 5% Pd/C (1.0 g) and hydrogenated at 40 psi for 18 h on a Parr apparatus. Catalyst was removed by filtration through Celite, and the filtrate was adjusted to pH 7.0 with 1.0N NaOH, and the solution was evaporated to dryness. The residue was dissolved in H_2O (100 ml) and treated dropwise with 1.0N HCl to pH 3.0, and the supernatant liquid decanted off leaving a tan resin (0.74 g after drying). A portion of this crude product (0.3 g) was purified by semi-preparative reverse phase chromatography (Regis C_{18} , 10% $\rightarrow$ 20% gradient of MeCN/ H_2O with 0.1% TFA) followed by lyophilization to give the desired product (**12a**) (0.116 g, 18%). $^1\text{H-Nmr}$ (DMSO- d_6) δ : 8.70 (d, $J=6.6$ Hz, 1H, GluNH), 7.93 (d, $J=7.8$ Hz, 2H, H-2' and H-6'), 7.40 (d, $J=7.8$ Hz, 2H, H-3' and H-5'), 7.00 (br s, 2H, NH_2), 6.45 (br s, 2H, NH_2), 4.40 (m, 1H, methine), 3.76 (dt, $J=6$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{OP}$), 3.62 (t, $J=6.5$ Hz, 2H, CH_2N), 2.37 (t, $J=7.1$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.18 (t, $J=7.1$ Hz, 2H, $\text{CH}_2\text{CO}_2\text{H}$), 2.15-2.05 (m, 2H, NCOCH_2), 2.05-1.9 (m, 2H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 1.55-1.35 (m, 6H,

$\text{CH}_2\text{CH}_2\text{CH}_2\text{N}, \text{NCOCH}_2\text{CH}_2, \text{CH}_2\text{CH}_2\text{OP}$, 1.30-1.10 (m, 6H, $(\text{CH}_2)_3$) $\text{uv } \lambda_{\text{max}}$ (e): 272.9 (18300), 242.5 (17100); λ_{min} (e): 257.0 (15300), 229.5 (15100). Anal. Calcd for $\text{C}_{27}\text{H}_{39}\text{N}_6\text{O}_{11} \text{P} \cdot 0.7 \text{ TFA} \cdot 1.4 \text{ H}_2\text{O}$: C, 44.90; H, 5.64; N, 11.06. Found: C, 44.87; H, 5.60; N, 11.17.

N-(4-(N-(3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)-10-(phosphooxy)decanamido)benzoyl)-L-glutamic Acid (12b). Phosphate triester (11b) (2.0 g, 2.32 mmol) was dissolved in MeOH (400 ml) and treated with H_2O (200 ml), TFA (0.8 ml), and 5% Pd/C (0.8 g) and the mixture was stirred under an H_2 atmosphere for 20 h. Additional catalyst (0.8 g) and TFA (0.4 ml) were added and the mixture was stirred under H_2 for another 20 h. The mixture was filtered and evaporated *in vacuo* to a viscous residue. Reverse phase semi-preparative chromatography (Regis C_{18} , 10% - 30% gradient MeCN / H_2O / 0.1% TFA), followed by lyophilization of appropriate fractions gave phosphate ester (12b) (0.38 g, 20%). $^1\text{H-Nmr}$ (DMSO-d_6) δ : 8.70 (d, $J=7.2$ Hz, 1H, GluNH), 7.93 (d, $J=7$ Hz, 2H, H-2' and H-6'), 7.41 (d, $J=7$ Hz, 2H, H-3' and H-5'), 7.20 (br, NH_2), 6.50 (br, 2H, NH_2), 4.40 (m, 1H, CH), 3.77 (dt, $J=7.5$ Hz, 2H, CH_2OP), 3.63 (t, $J=6$ Hz, 2H, CH_2N), 2.34 (t, $J=7$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.15 (t, $J=6$ Hz, 2H, $\text{CH}_2\text{CO}_2\text{H}$), 2.15-2.05 (t, $J=6$ Hz, 2H, CH_2CON), 2.00-1.90 (m, 2H, $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 1.50 (t, $J=6.6$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{OP}$), 1.50-1.32 (m, 4H, $\text{CH}_2\text{CH}_2\text{CON}$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 1.32-1.05 (m, 10H, $-(\text{CH}_2)_5$); $\text{uv } \lambda_{\text{max}}$ (e): 274 (18050), 242 (17290); λ_{min} (e): 259 (15240), 230 (15345); FABms 683 (M+H) (100%). Anal. Calcd for $\text{C}_{29}\text{H}_{43}\text{N}_6\text{O}_{11} \text{P} \cdot \text{H}_2\text{O} \cdot \text{TFA}$: C, 45.95; H, 5.77; N, 10.44. Found: C, 45.95; H, 5.88; N, 10.38.

N-(4-(N-(3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)-10-(phosphonooxy)-7-decynamido)benzoyl)-L-glutamic Acid (12c). Hydroxyacetylenic acid (9c)²⁷ (1.00 g, 5.43 mmol) was dissolved in dry pyridine (5 ml) and treated with bis(2,2,2-trichloro)ethyl chlorophosphate (2.67 g, 7.06 mmol). After 20 h, the mixture was evaporated *in vacuo* and the residue dissolved in CH_2Cl_2 (50 ml) and washed with 0.05 N HCl (3x5 ml) and H_2O (2x5 ml). The CH_2Cl_2 phase was dried (CaSO_4) and evaporated. The product was chromatographed (SiO_2 , $(\text{CH}_2\text{Cl}_2 / 5\% \text{ MeOH})$) to give 0.46 g (16%) of the intermediate phosphate triester (10c) which was used without further purification. Pyrimidinone (2) (0.40 g, 0.85 mmol) was dissolved in dry DMAC (40 ml) and treated with activated 3 Å sieves (4.0 g). The mixture was stirred for 20 h and then was cooled to -20°C . DMF (10 ml) was cooled to -20°C and treated with oxalyl chloride (80 ml, 0.92 mmol). After 5 min a solution of 10c (0.46 g, 0.87 mmol) in DMF (5 ml) was added. After 5 min this DMF solution was added to the pre-cooled DMAC solution, and the mixture was allowed to warm to RT. After 2 h, the mixture was filtered, and the filtrate evaporated *in vacuo* to dryness. The residue (11c) was dissolved in 80% HOAc (25 ml) and treated portion wise with activated Zn powder (0.80 g) over 4 h. The mixture was filtered, and the filtrate was evaporated *in vacuo*. The product was purified by reverse phase chromatography (Regis C_{18} 15→25% MeCN/ H_2O /0.1% TFA) followed by lyophilization of appropriate fractions to give 12c (0.11 g, 16%) as a white powder. $^1\text{H-Nmr}$ (DMSO-d_6) δ : 8.70 (d, $J=8$ Hz, 1H, GluNH), 7.95 (d, $J=7.8$ Hz, 2H, H-2' and H-6'), 7.38 (d, $J=7.8$ Hz, 2H, H-3' and H-5'), 7.15 (br, 2H, NH_2), 6.55 (br, 2H, NH_2), 4.40 (m, 1H, GluCH), 3.81 (dt, $J=7.1$ Hz, 2H, CH_2OP), 3.65 (t, $J=7.8$ Hz, 2H, CH_2N), 2.43 (t, $J=6.7$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{OP}$), 2.37 (t, $J=6.7$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.18 (m, 2H, $\text{CH}_2\text{CO}_2\text{H}$), 2.04 (m, 4H, CH_2CON , $\text{CH}_2\text{C}\equiv\text{C}$), 2.00 (m, 2H, CHCH_2), 1.45 (m, 4H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$, $\text{NCOCH}_2\text{CH}_2$), 1.29 (m, 2H, $\text{C}\equiv\text{CCH}_2\text{CH}_2$), 1.22 (m, 2H, $\text{C}\equiv\text{CCH}_2\text{CH}_2\text{CH}_2$); FABms m/z 679 (M+H, 100%), 634 (M+H- CO_2H , 12%); $\text{uv } \lambda_{\text{max}}$ (e): 274 (15400), 241 (15600); λ_{min} (e): 259 (13700), 229 (13900). Anal. Calcd for $\text{C}_{29}\text{H}_{39}\text{N}_6\text{O}_{11} \text{P} \cdot \text{TFA} \cdot \text{H}_2\text{O}$: C, 45.93; H, 5.22; N, 10.37. Found: C, 46.15; H, 5.29; N, 10.19.

***N*-(4-(11-Carboxy-*N*-(3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)-propyl)undecanamido)benzoyl)-L-glutamic Acid (12d).** A solution of 11d (0.500 g, 0.731 mmol) in 0.1N NaOH (43.9 ml, 4.39 mmol) was stirred while protected from light for 3 h before being brought to pH ~3 by the addition of 45 ml of 0.1N HCl. The resulting mixture was allowed to stand for 0.5 h, and precipitate was filtered, washed with water (3 x 5 ml), and dried under vacuum to give 12d (0.430 g, 89%) as a white solid. $^1\text{H-Nmr}$ (DMSO- d_6) δ : 12.20 (br), 9.70 (br), 8.69 (d, $J=7.5$ Hz, 1H, GluNH), 7.92 (d, $J=8.3$ Hz, 2H, H-2', H-6'), 7.41 (d, $J=8.3$ Hz, 2H, H-3', H-5'), 5.92 (br s, 2H, NH₂), 5.68 (br s, 2H, NH₂), 4.41 (m, 1H, NHCH), 3.63 (t, $J=7$ Hz, 2H, CH₂NCO), 2.37 (t, $J=7.5$ Hz, 2H, CH₂), 2.10 (m, 5H, CH₂), 1.95 (m, 3H, CH₂), 1.40 (m, 6H, CH₂), 1.15 (m, 12H, CH₂); uv λ_{max} 242.4 (16500), 273.6 (17400); λ_{min} (e): 229.6 (14800), 257.9 (14800). Anal. Calcd for C₃₁H₄₄N₆O₉·H₂O: C, 56.18; H, 7.00; N, 12.68. Found: C, 56.31; H, 7.03; N, 12.67.

***N*-(4-(Acetoacetyl 3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutamic Acid (13a).** A mixture of 2 (1.00 g, 2.13 mmol), activated 3Å sieves (10 g), and dry DMAC (120 ml) was stirred while protected from light and under N₂ for 23 h. *N*-Hydroxysuccinimide acetoacetate (2.13 g, 10.7 mmol) was added, and the mixture was stirred under N₂ for 25.5 h. The mixture was filtered through a bed of Celite, and the filtrate was concentrated under vacuum to a viscous residue that solidified upon the addition of acetonitrile (100 ml). The off-white solid was filtered, washed with acetonitrile (2 x 15 ml), and dried under vacuum at room temperature; yield, 1.09 g. A 0.200-g sample was purified by reverse phase chromatography (Regis C18, 12% MeCN/H₂O/0.1% TFA). Appropriate fractions were combined and concentrated to a residue that was dissolved in water and lyophilized to give 13a (0.179 g, 66%) as a white solid. $^1\text{H-Nmr}$ (DMSO- d_6) δ : 11.80 (br), 8.71 (d, $J=7.6$ Hz, 1H, GluNH), 7.96 (m, 2H, H-2' and H-6'), 7.63 (br s, 2H, NH₂), 7.42 (m, 2H, H-3' and H-5'), 6.79 (br s, 2H, NH₂), 4.63 (s, <1H, vinyl of acetoacetyl enol tautomer), 4.41 (m, 1H, NHCH), 3.68 (m, 2H, CH₂NCO), 3.26 (s, <2H, acetoacetyl CH₂), 2.37 (t, $J=7.4$ Hz, 2H, CH₂), 2.20 (t, $J=7$ Hz, 2H, CH₂), 2.10 (m, 2H, CHCH₂), 2.00 (s, <3H, acetoacetyl CH₃), 1.77 (s, <1H, CH₃ of acetoacetyl enol tautomer), 1.44 (m, 2H, CH₂CH₂CH₂); uv λ_{max} (e): 244.3 (15600), 273.8 (17300); λ_{min} (e): 227.9 (13600), 257.1 (14200). Anal. Calcd for C₂₃H₂₈N₆O₈·1.5CF₃CO₂H: C, 45.42; H, 4.32; N, 12.22. Found: C, 45.41; H, 4.34; N, 12.13.

***N*-(4-*N*-(3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)-*N*-methoxalyl)propylamino)benzoyl)-L-glutamic Acid (13b).** A mixture of 2 (1.00 g, 2.13 mmol), activated 3Å sieves (10 g), and dry DMAC (110 ml) was stirred while protected from light and under N₂ for 22 h. Methyl oxalyl chloride (393 μl , 4.27 mmol) was added, and the mixture was stirred under N₂ for 3.25 h. It was filtered through a bed of Celite, and the filtrate was concentrated under vacuum to an oil that was solidified by the addition of acetonitrile (100 ml). The light yellow solid was filtered, washed with acetonitrile (2x15 ml), and dried under vacuum; yield, 1.023 g. A 0.175-g sample was purified by reverse phase chromatography (Regis C18, 10% CH₃CN/H₂O/0.1% TFA). Appropriate fractions were combined and concentrated to a residue that was dissolved in water and lyophilized to give 13b (0.100 g, 40%) as a white solid. $^1\text{H-Nmr}$ (DMSO- d_6) δ : 11.80 (br), 8.74 (d, $J=7.4$ Hz, 1H, GluNH), 7.93 (d, $J=8.4$ Hz, 2H, H-2' and H-6'), 7.60 (br s, 2H, NH₂), 7.44 (d, $J=8.4$ Hz, 2H, H-3' and H-5'), 6.77 (br s, 2H, NH₂), 4.40 (m, 1H, NHCH), 3.80 (t, $J=7.4$ Hz, 2H, CH₂NCO), 3.51 (s, 3H, CH₃), 2.37 (t, $J=7.5$ Hz, 2H, CH₂), 2.22 (t, $J=7.4$ Hz, 2H, CH₂), 2.10 (m, 2H, CHCH₂), 1.48 (m, 2H, CH₂CH₂CH₂); uv λ_{max} (e): 244.3 (18100), 273.2 (19800); λ_{min} (e): 229.8 (15500), 256.4 (17400). Anal. Calcd for C₂₂H₂₆N₆O₉·5/4 CF₃CO₂H·4/3 H₂O: C, 42.95; H, 4.40; N, 12.27. Found: C, 42.91; H, 4.19; N, 12.27.

(E)-N-(4-((3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl) (4-ethoxy-4-oxo-2-butenoyl)amino)benzoyl)-L-glutamic Acid (13c). A mixture of **2** (2.00 g, 4.27 mmol), activated 3Å sieves (20 g), and dry DMAC (220 ml) was stirred while protected from light under N₂ for 26 h, then chilled to -20°C. Oxalyl chloride (0.54 ml, 6.14 mmol) was added dropwise to stirred dry DMF (56 ml) under N₂ at -20°C. Monoethyl fumarate (0.790 g, 5.48 mmol) was added to the DMF mixture, and a solution formed during 19 min of stirring at -20°C. The DMF solution was added over 2 min to the DMAC mixture at -20°C. This mixture was allowed to warm to room temperature and was stirred under N₂ for 24 h. It was filtered through a bed of Celite, and the filtrate was concentrated under vacuum to a residue that was solidified by the addition of acetonitrile (200 ml). The pale yellow solid was filtered, washed with acetonitrile (2 x 25 ml), and dried under vacuum; yield, 2.28 g. A 0.200-g sample was purified by reverse phase chromatography (Regis C18, 15% CH₃CN/H₂O/ 0.1% TFA). Appropriate fractions were combined and concentrated to a residue that was redissolved in 15% MeCN/H₂O/0.1% TFA and lyophilized to give **13c** (0.125 g, 51%) as a white solid. ¹H-Nmr (DMSO-d₆) δ: 12.00 (br), 8.77 (d, J=7.2 Hz, 1H, Glu NH), 7.98 (d, J=8.4 Hz, 2H, H-2', H-6'), 7.47 (d, J=8.4 Hz, 2H, H-3', H-5'), 7.40 (br s, 2H, NH₂), 6.70 (br s, 2H, NH₂), 6.65 (s, 2H, vinyl), 4.44 (m, 1H, NHCH), 4.12 (q, J=7.1 Hz, CH₂CH₃), 3.81 (t, J=7 Hz, 2H, CH₂NCO), 2.39 (t, J=7.1 Hz, 2H, CH₂), 2.22 (t, J=7 Hz, 2H, CH₂), 2.10 (m, 2H, CHCH₂), 1.49 (m, 2H, CH₂CH₂CH₂), 1.20 (t, J=7.1 Hz, 3H); uv λ_{max} (ε): 237.9 (sh) (18700), 274.8 (20600); λ_{min} (ε): 255.0 (15400). Anal. Calcd for C₂₅H₃₀N₆O₉ • CF₃CO₂H • 2H₂O: C, 45.76; H, 4.98; N, 11.86. Found: C, 45.70; H, 4.70; N, 11.85.

N-(4-(N-(3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)pyruvamide)benzoyl)-L-glutamic Acid (13d). A mixture of **2** (1.10 g, 2.35 mmol), activated 3Å sieves (11 g), and dry DMAC (110 ml) was stirred while protected from light under N₂ for 19.5 h and was then chilled to -20°C. Oxalyl chloride (295 μl, 3.38 mmol) was added dropwise to stirred dry DMF (31 ml) under N₂ at -20°C. Pyruvic acid (209 μl, 3.01 mmol) was added dropwise to the DMF mixture, and a solution formed during 15 min of stirring at -20°C. The DMF solution was added over 1 min to the DMAC mixture at -20°C. This mixture was allowed to warm to room temperature and was stirred under N₂ for 2 h 50 min. It was filtered through a bed of Celite, and the filtrate was concentrated under vacuum to an oil that was solidified by the addition of acetonitrile (100 ml). The pale yellow solid was filtered, washed with acetonitrile (2x10 ml), and dried under vacuum at 50°C; yield, 1.38 g. A 0.101-g sample was purified by reverse phase chromatography (Regis C18; 10→20% MeCN/H₂O/0.1% TFA, then 20% MeCN/H₂O/0.1% TFA). Appropriate fractions were combined and concentrated to a solid that was dissolved in water and lyophilized to give **13d** (0.074 g, 65%) as a white solid. ¹H-Nmr (DMSO-d₆) δ: 11.80 (br), 8.69 (d, J=7.9 Hz, 1H, GluNH), 7.87 (d, J=8.4 Hz, 2H, H-2' and H-6'), 7.43 (d, J=8.4 Hz, 2H, H-3' and H-5'), 7.40 (br s, 2H, NH₂), 6.70 (br s, 2H, NH₂), 4.38 (m, 1H, NHCH), 3.75 (t, J=7 Hz, 2H, CH₂NCO), 2.35 (t, J=7.6 Hz, 2H, CH₂), 2.24 (s, 3H, CH₃), 2.20 (t, J=7 Hz, 2H, CH₂), 2.10 (m, 2H, CHCH₂), 1.46 (m, 2H, CH₂CH₂CH₂); uv λ_{max} (ε): 242.3 (17700), 273.2 (21100); λ_{min} (ε): 230.3 (16100), 255.4 (17200). Anal. Calcd for C₂₂H₂₆N₆O₈ • 1.4CF₃CO₂H • 0.25 H₂O: C, 44.68; H, 4.22; N, 12.61. Found: C, 44.67; H, 4.35; N, 12.61.

Biological Tests. The details of the GAR-Tfase, AICAR-Tfase, and FPGS assays were reported previously.^{7,9} 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.²⁵

Metabolically assembled multisubstrate analogue formation was inferred by measuring a time-dependent increase of percent inhibition of enzyme activity upon co-incubation of inhibitor with GAR and enzyme.¹⁵ This procedure was based on the assumption that a MAI formed *in situ* would be a more potent inhibitor than the original folate analogue. The hog liver GAR-Tfase was partially purified by affinity chromatography. The enzyme concentration was estimated, based on specific activity measurements, to be approximately 1-3 nM in the assay.

Two procedures were used to test for time-dependent inhibition: (A) The buffer, 2-mercaptoethanol, bovine serum albumin, GAR, GAR-Tfase, and test compound (concentration = IC₃₀) were incubated at 30°C for 0-40 min. The reaction was started with N¹⁰-formyl tetrahydrofolic acid (10-fTHFA), and the absorbance at 295 nm was monitored. (B) A mixture as in A containing inhibitor at 17-times its IC₅₀ concentration was incubated at room temperature for 0-4 h and then diluted 17-fold into complete reaction mixtures containing N¹⁰-formyl tetrahydrofolic acid. No time dependency by method A was seen for compounds (2, 3c, 13b, and 13c). No time dependency by method B was seen for compounds 13a and 13c. However, N¹⁰-pyruvoyl-5-DACTHF (13d) exhibited time-dependent inhibition (see Table 2).

ACKNOWLEDGMENT

The authors wish to acknowledge Lisa Hefner, Gary Fay, Kristie Hardee and Patricia Parker for expert technical assistance; Dr. Gary Martin, Aris Ragouzeos, and Ron Crouch with ¹H-NMR studies; Dr. Lester Taylor and Robert Johnson with mass spectroscopic studies; and Randy Carmichael for preparing the manuscript. The authors also wish to thank Prof. Steve Benkovic and Dr. Jim Inglese for helpful discussions.

REFERENCES

1. G. Elion, *Science*, 1989, 244, 41.
2. B. Chabner, C. Allegra, and J. Baram, 'Antifolates: Expanding Horizons in 1986,' Chemistry and Biology of Pteridines, 1986, ed. by B. Cooper and V. Whitehead, de Gruyter, Berlin, 1986, pp. 945-951.
3. E. Taylor, P. Harrington, S. Fletcher, G. Beardsley, and R. Moran, *J. Med. Chem.*, 1985, 28, 914.
4. E. Taylor, J. Hamby, C. Shih, G. Grindey, S. Rinzel, G. Beardsley, and R. Moran, *J. Med. Chem.*, 1989, 32, 1517.
5. G. Beardsley, B. Moroson, E. Taylor, and R. Moran, *J. Biol. Chem.*, 1989, 264, 328.
6. E. Taylor, P. Harrington, and C. Shih, *Heterocycles*, 1989, 28, 1169. E. Taylor, T. Schrader, and L. Walensky, *Tetrahedron*, 1992, 48, 19. E. Taylor and P. Gillespie, *J. Org. Chem.*, 1992, 57, 5757.
7. J. Kelley, E. McLean, N. Cohn, M. Edelstein, D. Duch, G. Smith, M. Hanlon, and R. Ferone, *J. Med. Chem.*, 1990, 33, 561.
8. M. Hanlon, R. Ferone, R. Mullin, and B. Keith, *Cancer Res.*, 1990, 50, 3207.
9. E. Bigham, S. Hodson, W. Mallory, D. Wilson, D. Duch, G. Smith, and R. Ferone, *J. Med. Chem.*, 1992, 35, 1399.
10. R. Mullin, B. Keith, E. Bigham, D. Duch, R. Ferone, L. Heath, S. Singer, K. Waters, and H. Wilson, *Biochem. Pharmacol.*, 1992, 43, 1627.
11. S. Daubner, M. Young, R. Sammons, L. Courtney, and S. Benkovic, *Biochemistry*, 1986, 25, 2951.
12. J. Inglese, D. Johnson, A. Shiao, J. Smith, and S. Benkovic, *Biochemistry*, 1990, 29, 1436.

13. J. Inglese, J. Smith, and S. Benkovic, Biochemistry, 1990, 29, 6678.
14. J. Inglese, R. Blatchly, and S. Benkovic, J. Med. Chem., 1989, 32, 937.
15. J. Inglese and S. Benkovic, Tetrahedron, 1991, 47, 2351.
16. A. Broom, Fed. Proc., 1986, 45, 2779.
17. A. Broom, J. Med. Chem., 1989, 32, 2.
18. N. Licato, Diss. Abstracts Int. B., 1987, 47, 2918.
19. J. Chase and P. Tubbs, Biochem. J., 1969, 111, 225.
20. C. Caperelli, J. Med. Chem., 1987, 30, 1254.
21. C. Caperelli and J. Conigliaro, J. Med. Chem., 1986, 29, 2117.
22. C. Temple, L. Bennett, J. Rose, R. Elliott, J. Montgomery, and J. Mangum, J. Med. Chem., 1982, 25, 161.
23. P. Stadler, Helv. Chim. Acta, 1978, 61, 1675.
24. D. Duch unpublished results.
25. S. Patil, C. Jones, M. Nair, J. Galivan, F. Maley, R. Kisliuk, Y. Gaumont, D. Duch, and R. Ferone, J. Med. Chem., 1989, 32, 1284.
26. S. Swann, Jr., R. Oehler, and R. Buswell, Org. Synth. Coll. Vol. II, ed. by A. Blatt, John Wiley, New York, 1943, p. 276.
27. J. Cossy and J. Pete, Tetrahedron. Lett., 1986, 27, 573.

Received, 21st December, 1992