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

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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 glycinamide 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-acyclotetrahydrofolate (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 Inglese 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]

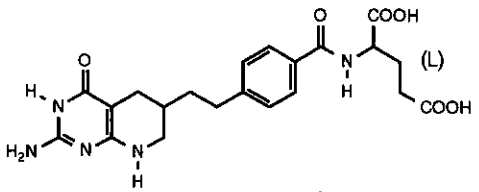



**Figure 1**

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-dideazafolate 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 multi-substrate analogue and its poor penetration into cells. Inhibition of carnitine acetyltransferase by this strategy had been previously reported by Chase and Tubs.[19]

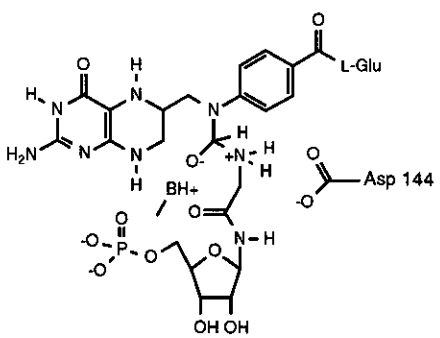



**Figure 2**

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-dideazafolate.[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.

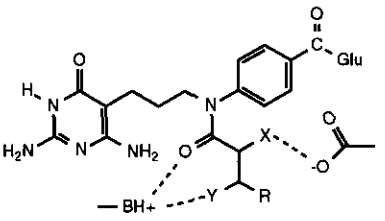
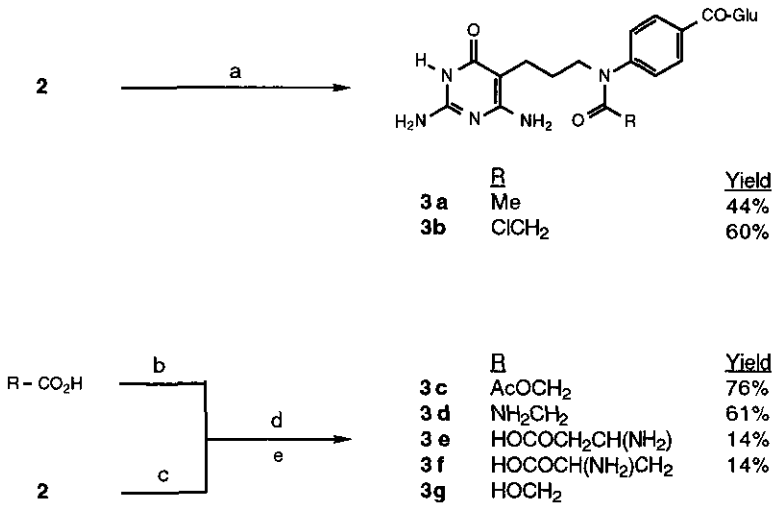


Figure 3

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

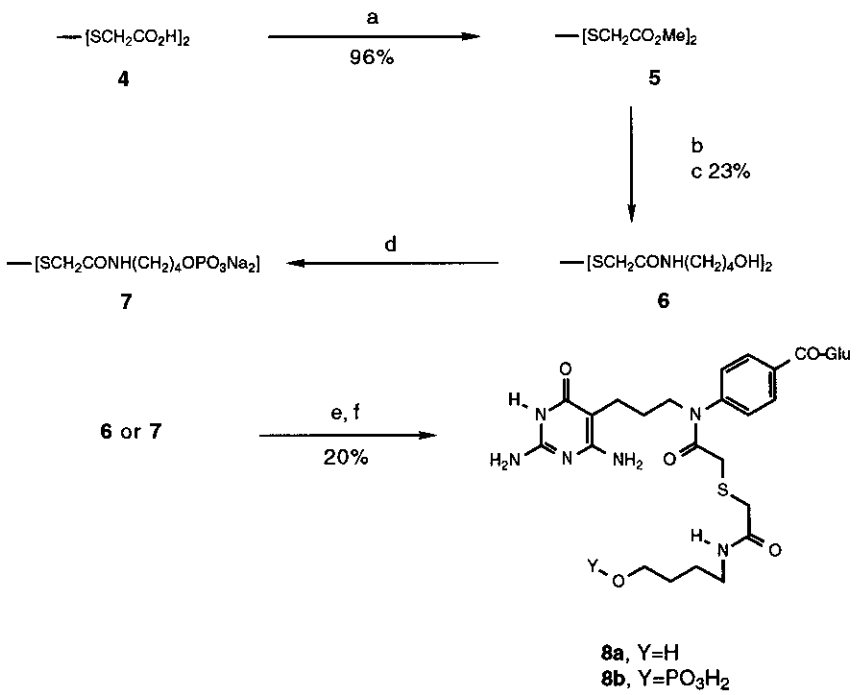



Reagents: (a) 4 RCOCl, DMAC, 6 h, room temperature (b) ClCOCOCl, DMF, -20°C (c) 3Å-Sieves, DMAC, 12 h, room temperature (d) -20°C. (e) deprotect

**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 Inglese 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 Inglese 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.

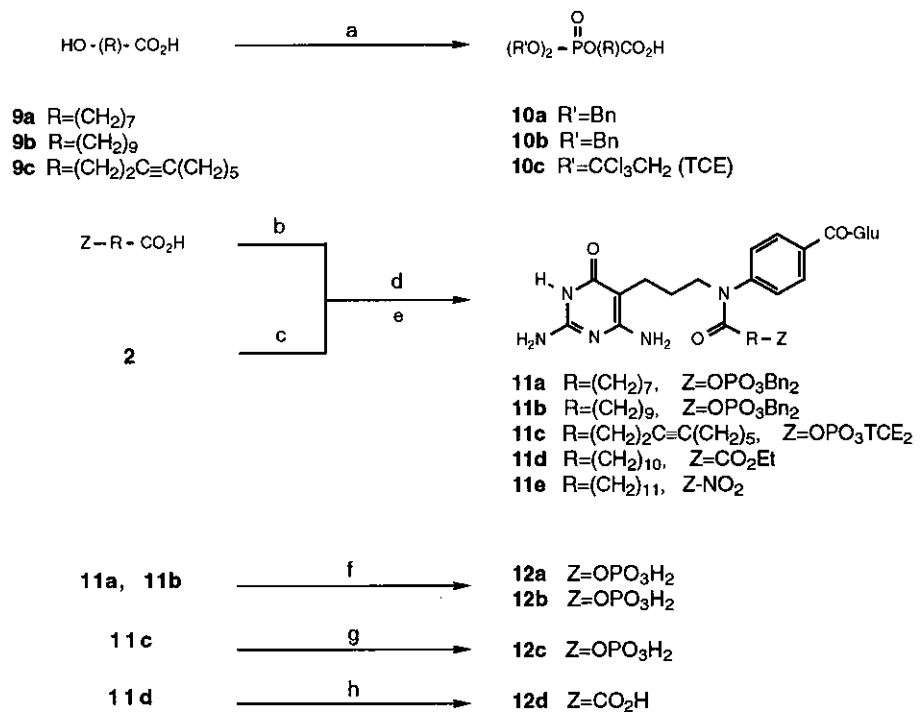



Reagents: (a) MeOH, HCl. (b) 2 $NH_2(CH_2)_4OH$, heat. (c) Kuglerohr distillation. (d) $POCl_3$, $(EtO)_3PO$, NaOH. (e) **3b**, $NaBH_4$. (f) Preparative C-18 chromatography.

**Scheme 2**

The structure was simplified further by replacing the thioether and amide linkers with methylene groups. Initially, ω-hydroxyalkanoic acids were phosphorylated ($POCl_3$, $(MeO)_3P{=}O$, 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 Vilsmeyer 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 Vilsmeyer coupling as shown in Scheme 3.

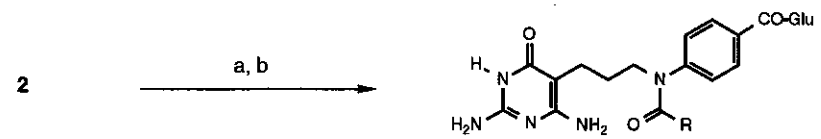



Reagents: (a) $(R'O)_2POCl$, pyridine, 20 h. (b)-(e) see Scheme 1. (f) 5% Pd/C, $H_2$, MeOH, $H_2O$, TFA. (g) Zn, 80% HOAc, 4 h. (h) 1N NaOH, EtOH.

**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. Inglese and Benkovic have demonstrated the MAMA approach in vitro,[15] but $N^{10}$-bromoacetyl dideazafolate 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 dimethylformimidate esters of monomethylfumaric 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.




| | | |
|---|---|---|
| **13a** | R=$MeCOCH_2$ | 66% |
| **13b** | R=MeOC(O) | 40% |
| **13c** | R=$MeO_2CCH$=CH | 51% |
| **13d** | R=MeC(O) | 42% |

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

**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 acetoxy (**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 $^3$H-**12b** was shown to bind to the cell membrane, only the dephosphorylated alcohol was found in cytosol (data not shown); and, in contrast to $^3$H-**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] Hog liver $IC_{50}$ μM | FPGS[b] Hog liver $V_{max}$%/Km | MTX Uptake[c] MOLT-4 $IC_{50}$ μM | Cell Growth[d] MCF7 $IC_{50}$ μM |
|---|---|---|---|---|
| **2** | 3.3 ± 0.1 | 14.5 | 1.20 | 0.037 |
| **3a** | 5.40 ± 0.05 | 7.3 | 3.30 | 3.10 |
| **3b** | 8.00 ± 0.75 | nd | 1.90 | na |
| **3c** | 12.4 ± 1.4 | 0.64 | 1.60 | 0.200 |
| **3d** | 1.99 ± 0.32 | 6.2 | 8.50 | 0.400 |
| **3e** | 31.9 ± 2.7 | 0.23 | 8.00 | 2.20 |
| **3f** | 70.7 ± 11.6 | 0.25 | 24.0 | 1.05 |
| **3g** | 2.28 ± 0.13 | 4.9 | 4.50 | 0.200 |
| **8a** | 10.6 ± 0.8 | 0.16 | 2.80 | 14%@0.1 |
| **8b** | 0.0037 ± 0.0003 | 1.0 | 2.50 | 1.00 |
| **12a** | 0.0217 ± 0.0027 | nd | 1.50 | nd |
| **12b** | 0.0046 ± 0.0004 | 1.5 | 2.20 | 2.500 |
| **12c** | 0.00569 ± 0.0004 | nd | 1.90 | 30.0 |
| **11d** | 1.50 ± 0.06 | 0.75 | 0.032 | na |
| **12d** | 0.648 ± 0.013 | 0.56 | 0.900 | 60.0 |
| **11e** | 0.506 ± 0.073 | 1.5 | 0.020 | na |
| **13a** | 16.5 ± 1.4 | nd | 2.20 | 0.060 |
| **13b** | 18.1 ± 1.4 | nd | 2.50 | 1.30 |
| **13c** | 71.0 ± 6.4 | nd | 0.450 | >50.0 |
| **13d** | 5.00 ± 0.12 | 1.4 | 2.40 | 0.140 |

[a]Hog liver GAR transformylase with (6R)-10-formyl-FH4 as cofactor.[7,9]

[b]Hog folylpolyglutamate synthase; $V_{max}$% is relative to aminopterin.[7,9]

[c]Inhibition of $^3$H-methotrexate transport into MOLT-4 T-cell leukemia cells.[25]

[d]Inhibition 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 | | | | | | |
|---|---|---|---|---|---|---|
| [13d] μM | 10-fTHFA | GAR | GAR-Tfase | Time (min) | Reaction Initiators | Percent Inhibition |
| 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 |

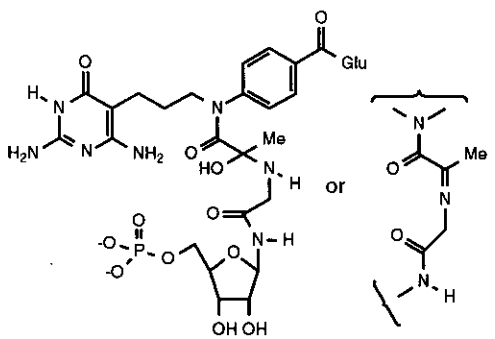



**Figure 4**

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 λmax are given in nm. $^1$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=carbobenzoxy, 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-DACTHF[7] (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%): $^1$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 $\lambda_{max}$ (ε): 274 (15600), 243 (14900). Anal. Calcd for $C_{21}H_{26}N_6O_7 \bullet 7/5\ H_2O$: 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%):* $^1$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 $\lambda_{max}$ (ε): 274 (16800), 240 (17900). Anal. Calcd for $C_{21}H_{25}N_6O_7Cl \bullet 1/2\ HCl \bullet 19/10\ H_2O$: 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 3Å 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→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. $^1$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, $NH_2$), 6.48 (br s, 2H, $NH_2$), 4.33 (m, 3H, NHCH and $CH_2OAc$), 3.63 (t, J=7 Hz, 2H, $CH_2NCO$), 2.35 (t, J=7.5 Hz, 2H, $CH_2$), 2.15 (t, J=7 Hz, 2H, $CH_2$), 2.1 (m, 2H, $CHCH_2$), 2.00 (s, 3H, $CH_3$), 1.42 (m, 2H, $CH_2CH_2CH_2$); uv $\lambda_{max}$ (ε): 241.6 (15100), 273.8 (14500); $\lambda_{min}$ (ε): 229.1 (13300), 259.2 (12700). Anal. Calcd for $C_{23}H_{28}N_6O_9 \cdot CF_3CO_2H \cdot 1.6\ H_2O$: 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 $H_2O$ 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 $NH_4HCO_3$ and chromatographed on DEAE Sephadex (400 ml column volume) with a gradient of 5 mM to 1.0 M $NH_4HCO_3$. Appropriate fractions (monitored by hplc, Supelco $C_{18}$, 20%MeCN / $H_2O$ / 0.1%TFA) were combined and lyophilized to give the product (**3d**) (0.36 g, 61%) as a white powder. $^1$H-Nmr (DMSO-$d_6$) 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, $NH_2$), 5.65 (br s, 2H, $NH_2$) 4.28 (M, 1H, CH), 3.65 (m, 2H, $CH_2N$), 3.20 (m, 2H, $COCH_2NH_2$), 2.25 (m, 2H, $CH_2CH_2CH_2N$) 2.15 (m, 2H, $CH_2CO_2H$), 1.90 (m, 2H, $CH_2CH_2CO_2H$), 1.40 (m, 2H, $CH_2CH_2CH_2$); 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 $C_{21}H_{27}N_7O_7 \cdot 7/2\ H_2O$: 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 $H_2O$ by addition of TFA (0.5 ml). Lyophilization gave the desired product **3e** as a white powder (0.26 g, 14%). $^1$H-Nmr ($D_2O$) 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-$NCH_2$), 2.90-2.50 (m, 4H, $CH_2$'s α to $CO_2H$'s on Asp and Glu), 2.50-2.10 (m, 4H, $CH_2$ α to pyrimidinone, $CH_2CH_2CO_2H$ to Glu $CO_2H$), 1.78 (m, 2H, $CH_2CH_2CH_2$); 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 $C_{23}H_{29}N_7O_9 \cdot 1.5H_2O \cdot 2.4$ 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 1.0 N NaOH and stored at 4°C overnight. The resulting suspension was filtered to give a white solid which was dissolved in 10 ml $H_2O$ by addition of TFA (0.5 ml). Lyophilization gave the desired product (**3f**) as a white powder (0.28 g, 14%). $^1$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, $CH_2CH_2CH_2N$), 2.80 (t, J=5 Hz, 2H, $NCOCH_2$) 2.57 (t, J=6 Hz, 2H, $CH_2COCH$), 2.50-2.10 (m, 4H, $CH_2CH_2CH_2H_2N$ and $CH_2CH_2CO_2H$), 1.75 (m, 2H, $CH_2CH_2CH)_2$, FABms m/z: 548 (M+H), 286 (M+H-Glu-Asp), 186 (M+H-Glu-Asp-Ph-CO); uv $\lambda_{max}$ (ε): 273 (18,000), 242 (16,600); $\lambda_{min}$ (ε): 257 (14,600), 230 (14,700). Anal. Calcd for $C_{25}H_{29}N_7O_9 \cdot 4.4\ H_2O$ 2.7 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 ~1/4 of its original volume, and the mixture was refrigerated overnight. The solid was filtered while still cold, washed with 3 x 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→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. $^1$H-Nmr (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, NHC**H**), 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, $CH_2CH_2CH_2$); uv $\lambda_{max}$ (ε): 243.8 (16800), 274.2 (17700); $\lambda_{min}$ (ε): 229.3 (14800), 258.4 (14900). Anal. Calcd for $C_{21}H_{26}N_6O_8 \cdot 1.35CF_3CO_2H \cdot 0.5\ H_2O$: 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. $^1$H-Nmr (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-$OCH_3$), 151 (M-$CO_2CH_3$). 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). $^1$H-Nmr (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 $C_{12}H_{14}N_2O_4S_2$: 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-((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 $NaBH_4$ (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 $C_{18}$ 20% MeCN/$H_2O$/0.1% TFA) followed by lyophilization to give the desired product (8a) (0.017 g, 6%) $^1$H-Nmr (DMSO-$d_6$/$D_2O$) 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, $CH_2$NPh), 3.45 (s, 2H, $CH_2$S), 3.20 (m, 2H, $CH_2$ OH), 3.10 (s, 2H, $CH_2$S), 2.98 (m, 2H, $CH_2$NH) 2.38 (t, J=7 Hz, 2H, $CH_2$ α to pyrimidinone), 2.20 (m, 2H, $CH_2CO_2H$), 2.15-2.00 (m, 2H, $CH_2CH_2CO_2H$), 1.45 (m, 2H, $CH_2CH_2CH_2$N-Ar), 1.4 (m, 4H, $CH_2CH_2CH_2OH$); FABms: calcd for $C_{27}H_{37}N_7O_9S$ M+H: 636.245. Found, 636.2449; uv $\lambda_{max}$,(ε): 272 (18,500), 243 (17,700); $\lambda_{min}$ (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 $POCl_3$ (1.03 ml, 11 mmol). After stirring for 4 1/2 h at 0 °C, the mixture was treated cautiously with 5 ml $H_2O$ 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 $H_2O$ (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 $NaBH_4$ (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 $C_{18}$ 10% →15% MeCN/$H_2O$/0.1% TFA) followed by lyophilization to give **8b** (0.27 g, 18%) as a white powder. $^1$H-Nmr ($D_2O$) 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, $CH_2$OP), 3.70 (t, J=7 Hz, 2H, $CH_2$NAr), 3.80, 3.70 (two s, 2H each, $CH_2SCH_2$), 3.09 (t, J=7 Hz, 2H, $NHCH_2$), 2.57 (t, J=7 Hz, 2H, $CH_2$ $CO_2H$), 2.40-2.10 (m, 2H, $CH_2CH_2CO_2H$), 2.23 (t, J=7.5 Hz, 2H, $CH_2$ α to pyrimidinone), 1.70 (t, J=6 Hz, 2H, $CH_2CH_2OP$), 1.50 (m, 4H, $CH_2CH_2CH_2$NPh and $CONHCH_2CH_2$;, FABms m/z: 716 (M+H); uv$\lambda_{max}$ (ε): 274 (16,400), 243 (15,900); $\lambda_{min}$ (ε): 259 (14,100), 230 (14,700). Anal. Calcd for $C_{27}H_{38}N_7O_{12}$ PS•2$H_2O$•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).** Dibenzyl phosphite (20.7 ml, 93.6 mmol) was stirred under $N_2$ in dry $CCl_4$ (300 ml) and treated dropwise with $SO_2Cl_2$ (9.8 ml, 122.0 mmol) over 10 min. Dry $N_2$ 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 $CCl_4$ (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 $CH_2Cl_2$ (500 ml) and washed with 1.0N HOAc (5x100 ml) and water (3x100 ml), dried ($CaSO_4$) and evaporated in vacuo. Chromatography ($SiO_2$ / $CH_2Cl_2$ / 5% MeOH) gave 10a as a colorless oil (7.17 g, 54%). $^1$H-Nmr (DMSO-$d_6$) δ: 12.00 (br s, 1H, $CO_2H$), 7.38 (s, 10H, Ph) 5.00 (d, J=8 Hz, 4H, Ph-$CH_2O$), 3.95 (dd, J=8 Hz, 2H, $POCH_2$

$CH_2$), 2.18 (t, J=7 Hz, 2H, $C\underline{H}_2CO_2H$), 1.50 (m, 4H, $POCH_2C\underline{H}_2$, $C\underline{H}_2CH_2CO_2H$), 1.22 (m, 6H, $(CH_2)_3$). 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%). $^1$H-Nmr (DMSO-$d_6$) δ: 7.35 (s, 10H, Ph), 5.00 (d, J=9 Hz, 4H, PO-$CH_2$-Ph), 3.92 (dd, J=8 Hz, 2H, PO-$C\underline{H}_2CH_2$), 2.17 (t, J=7.5 Hz, 2H, $CH_2CO_2H$), 1.50 (m, 4H, $POCH_2C\underline{H}_2$, $C\underline{H}_2CH_2CO_2H$), 1.20 (s, 1OH, $OCH_2CH_2$-$(C\underline{H}_2)_5$). 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 3Å 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 $C_{18}$ 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. $^1$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, $CH_2$-Ph), 7.00 (br, 2H, $NH_2$), 6.40 (br, 2H, NH2), 5.00 (d, J=7.1 Hz, 4H, $C\underline{H}_2$OP), 4.40 (m, 1H, methine), 3.90 (m, 2H, $POC\underline{H}_2CH_2$), 3.62 (dd, J=6.5 Hz, 2H, $CH_2N$), 2.37 (t, J=7.1 Hz, 2H, $C\underline{H}_2CH_2CH_2N$), 2.17 (t, J=6.5 Hz, 2H, $C\underline{H}_2CO_2H$), 2.30-1.90 (m, 4H, -$CHC\underline{H}_2$, $C\underline{H}_2CON$), 1.55-1.35 (m, 6H, $CH_2$ $C\underline{H}_2CH_2N$, $C\underline{H}_2CH_2CON$, $POCH_2C\underline{H}_2$-), 1.35-1.05 (m, 10H, -$(CH_2)_5$); 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 $C_{43}H_{55}N_6O_{11}P \cdot 1.9\ H_2O \cdot 0.7$ 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 3Å 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 dodecanedioate[26] (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. $^1$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, $CH_2CO_2Et$), 2.10 (m, 3H, $CH_2$), 2.00 (m, 3H, $CH_2$), 1.45 (m, 6H, $CH_2$), 1.15 (m, 15H, $CH_3$+ $CH_2$); uv $\lambda_{max}$ (ε): 242.9 (15200), 273.4 (16000); $\lambda_{min}$ (ε): 229.9 (13800), 257.7 (13800). Anal. Calcd for $C_{33}H_{48}N_6O_9 \cdot 0.6\ H_2O$: 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. $^1$H-Nmr (DMSO-$d_6$) δ: 12.4 (br s, 2H, $CO_2$H's), 9.80 (br s, 1H, pyrimidinone NH), 8.70 (d, J=8 $H_z$, 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, 2HNCOC$H_2$, $CH_2CH_2CO_2H$), 1.85 (m, 2H $CH_2CH_2CO_2H$); 1.5-1.35 (m, 4H, $NCOCH_2CH_2$, $O_2NCH_2$-$CH_2$), 1.30-1.10 (m, 16H, Ph-N-$CH_2$-$CH_2$ , $O_2NCH_2CH_2$-$CH_2)_7$); uv $\lambda_{max}$ (ε): 273 (17200), 241 (17300); $\lambda_{min}$ (ε): 259.1 (14700), 230 (16104). Anal. Calcd for $C_{31}H_{45}N_7O_9 \cdot H_2O \cdot 3/10\ C_3H_8O$: 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-(phosphonooxy)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% → 20% gradient of MeCN/$H_2O$ with 0.1% TFA) followed by lyophilization to give the desired product (**12a**) (0.116 g, 18%). $^1$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, $CH_2CH_2OP$), 3.62 (t, J=6.5 Hz, 2H, $CH_2N$), 2.37 (t, J=7.1 Hz, 2H, $CH_2CH_2CH_2N$), 2.18 (t, J=7.1 Hz, 2H, $CH_2CO_2H$), 2.15-2.05 (m, 2H, $NCOCH_2$), 2.05-1.9 (m, 2H, $CH_2CH_2CO_2H$), 1.55-1.35 (m, 6H,

$CH_2C\underline{H_2}CH_2N$, $NCOCH_2C\underline{H_2}$, $C\underline{H_2}CH_2OP$), 1.30-1.10 (m, 6H, $(CH_2)_3$) uv $\lambda_{max}$ (ε): 272.9 (18300, 242.5 (17100); $\lambda_{min}$ (ε): 257.0 (15300), 229.5 (15100). Anal. Calcd for $C_{27}H_{39}N_6O_{11}P \bullet 0.7$ TFA $\bullet 1.4$ $H_2O$: 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%). $^1$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, $C\underline{H_2}N$), 2.34 (t, J=7 Hz, 2H, $C\underline{H_2}CH_2CH_2N$), 2.15 (t, J=6 Hz, 2H, $C\underline{H_2}CO_2H$), 2.15-2.05 (t, J=6 Hz, 2H, $C\underline{H_2}CON$), 2.00-1.90 (m, 2H, $C\underline{H_2}CH_2CO_2H$), 1.50 (t, J=6.6 Hz, 2H, $C\underline{H_2}CH_2OP$), 1.50-1.32 (m, 4H, $C\underline{H_2}CH_2CON$ and $CH_2C\underline{H_2}$ $CH_2N$), 1.32-1.05 (m, 10H, $-(CH_2)_5$); uv $\lambda_{max}$ (ε): 274 (18050), 242 (17290); $\lambda_{min}$ (ε): 259 (15240), 230 (15345); FABms 683 (M+H) (100%). Anal. Calcd for $C_{29}H_{43}N_6O_{11}P \bullet H_2O \bullet$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 ($SiO_2$, ($CH_2Cl_2$ / 5% 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 3Å 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. $^1$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, $C\underline{H_2}CH_2OP$), 2.37 (t, J=6.7 Hz, $2H_2$, $C\underline{H_2}CH_2CH_2N$), 2.18 (m, 2H, $C\underline{H_2}$ $CO_2H$), 2.04 (m, 4H, $C\underline{H_2}CON$, $C\underline{H_2}C{\equiv}C$), 2.00 (m, 2H, $CHC\underline{H_2}$), 1.45 (m, 4H, $CH_2C\underline{H_2}CH_2N$, $NCOCH_2C\underline{H_2}$), 1.29 (m, 2H, $C{\equiv}CCH_2C\underline{H_2}$) 1.22 (m, 2H, $C{\equiv}CCH_2CH_2C\underline{H_2}$); FABms m/z 679 (M+H, 100%), 634 (M+H–$CO_2H$, 12%); uv $\lambda_{max}$ (ε): 274 (15400), 241 (15600); $\lambda_{min}$ (ε): 259 (13700), 229 (13900). Anal. Calcd for $C_{29}H_{39}N_6O_{11}P \bullet$TFA $\bullet H_2O$: 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. $^1$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, $NH_2$), 5.68 (br s, 2H, $NH_2$), 4.41 (m, 1H, NHC<u>H</u>), 3.63 (t, J=7 Hz, 2H, C<u>H</u>$_2$NCO), 2.37 (t, J=7.5 Hz, 2H, $CH_2$), 2.10 (m, 5H, $CH_2$), 1.95 (m, 3H, $CH_2$), 1.40 (m, 6H, $CH_2$), 1.15 (m, 12H, $CH_2$); uv $\lambda_{max}$ 242.4 (16500), 273.6 (17400); $\lambda_{min}$ (ε): 229.6 (14800), 257.9 (14800). Anal. Calcd for $C_{31}H_{44}N_6O_9 \cdot H_2O$: 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 $N_2$ for 23 h. *N*-Hydroxysuccinimidyl acetoacetate (2.13 g, 10.7 mmol) was added, and the mixture was stirred under $N_2$ 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/$H_2O$/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. $^1$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, $NH_2$), 7.42 (m, 2H, H-3′ and H-5′), 6.79 (br s, 2H, $NH_2$), 4.63 (s, <1H, vinyl of acetoacetyl enol tautomer), 4.41 (m, 1H, NHC<u>H</u>), 3.68 (m, 2H, C<u>H</u>$_2$NCO), 3.26 (s, <2H, acetoacetyl $CH_2$), 2.37 (t, J=7.4 Hz, 2H, $CH_2$), 2.20 (t, J=7 Hz, 2H, $CH_2$), 2.10 (m, 2H, CHC<u>H</u>$_2$), 2.00 (s, <3H, acetoacetyl $CH_3$), 1.77 (s, <1H, $CH_3$ of acetoacetyl enol tautomer), 1.44 (m, 2H, $CH_2$C<u>H</u>$_2$$CH_2$); uv $\lambda_{max}$ (ε): 244.3 (15600), 273.8 (17300); $\lambda_{min}$ (ε): 227.9 (13600), 257.1 (14200). Anal. Calcd for $C_{23}H_{28}N_6O_8 \cdot 1.5CF_3CO_2H$: 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 $N_2$ for 22 h. Methyl oxalyl chloride (393 µl, 4.27 mmol) was added, and the mixture was stirred under $N_2$ 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% $CH_3CN$/$H_2O$/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. $^1$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, $NH_2$), 7.44 (d, J=8.4 Hz, 2H, H-3′ and H-5′), 6.77 (br s, 2H, $NH_2$), 4.40 (m, 1H, NHC<u>H</u>), 3.80 (t, J=7.4 Hz, 2H, C<u>H</u>$_2$NCO), 3.51 (s, 3H, $CH_3$), 2.37 (t, J=7.5 Hz, 2H, $CH_2$), 2.22 (t, J=7.4 Hz, 2H, $CH_2$), 2.10 (m, 2H, CHC<u>H</u>$_2$), 1.48 (m, 2H, $CH_2$C<u>H</u>$_2$$CH_2$); uv$\lambda_{max}$ (ε): 244.3 (18100), 273.2 (19800); $\lambda_{min}$ (ε ): 229.8 (15500), 256.4 (17400). Anal. Calcd for $C_{22}H_{26}N_6O_9 \bullet 5/4\ CF_3CO_2H \bullet 4/3\ H_2O$: 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 $N_2$ 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 $N_2$ 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 $N_2$ 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% $CH_3CN/H_2O$/ 0.1% TFA). Appropriate fractions were combined and concentrated to a residue that was redissolved in 15% $MeCN/H_2O$/0.1% TFA and lyophilized to give **13c** (0.125 g, 51%) as a white solid. $^1$H-Nmr (DMSO-$d_6$) 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, $NH_2$), 6.70 (br s, 2H, $NH_2$), 6.65 (s, 2H, vinyl), 4.44 (m,1H, NHC<u>H</u>), 4.12 (q, J=7.1 Hz, C<u>H</u>$_2$CH$_3$), 3.81 (t, J=7 Hz, 2H, C<u>H</u>$_2$NCO), 2.39 (t, J=7.1 Hz, 2H, $CH_2$), 2.22 (t, J=7 Hz, 2H, $CH_2$), 2.10 (m, 2H, CHC<u>H</u>$_2$), 1.49 (m, 2H, $CH_2$C<u>H</u>$_2$$CH_2$), 1.20 (t J=7.1 Hz, 3H)); uv $\lambda_{max}$ (ε): 237.9 (sh) (18700), 274.8 (20600); $\lambda_{min}$ (ε): 255.0 (15400). Anal. Calcd for $C_{25}H_{30}N_6O_9 \bullet CF_3CO_2H \bullet 2H_2O$: 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)pyruvamido)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 $N_2$ 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 $N_2$ 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 $N_2$ 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% $MeCN/H_2O$/0.1% TFA, then 20% $MeCN/H_2O$/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. $^1$H-Nmr (DMSO-$d_6$) 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, $NH_2$), 6.70 (br s, 2H, $NH_2$), 4.38 (m, 1H, NHC<u>H</u>), 3.75 (t, J=7 Hz, 2H, C<u>H</u>$_2$NCO), 2.35 (t, J=7.6 Hz, 2H, $CH_2$), 2.24 (s, 3H, $CH_3$), 2.20 (t, J=7 Hz, 2H, $CH_2$), 2.10 (m, 2H, CHC<u>H</u>$_2$), 1.46 (m, 2H, $CH_2$C<u>H</u>$_2$$CH_2$); uv $\lambda_{max}$ (ε): 242.3 (17700), 273.2 (21100); $\lambda_{min}$ (ε): 230.3 (16100), 255.4 (17200). Anal. Calcd for $C_{22}H_{26}N_6O_8 \bullet 1.4CF_3CO_2H \bullet 0.25\ H_2O$: 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 = $IC_{30}$) were incubated at 30°C for 0-40 min. The reaction was started with $N^{10}$-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 $IC_{50}$ concentration was incubated at room temperature for 0-4 h and then diluted 17-fold into complete reaction mixtures containing $N^{10}$-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, $N^{10}$-pyruvoyl-5-DACTHF (**13d**) exhibited time-dependent inhibition (see Table 2).

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