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10-(2-Benzoxazolcarbonyl)-5,10-dideaza-acyclic-5,6,7,8-tetrahydrofolic Acid: A Potential Inhibitor of GAR Transformylase and AICAR Transformylase

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Abstract—The design and synthesis of 10-(2-benzoxazolcarbonyl)-DDACTHF (**1**) as an inhibitor of glycinamide ribonucleotide transformylase (GAR Tfase) and aminoimidazole carboxamide transformylase (AICAR Tfase) are reported. Ketone **1** and the corresponding alcohol **13** were evaluated for inhibition of GAR Tfase and AICAR Tfase and the former was found to be a potent inhibitor of recombinant human (rh) GAR Tfase ($K_i = 600$ nM).

Glycinamide ribonucleotide transformylase (GAR Tfase) and aminoimidazole carboxamide ribonucleotide transformylase (AICAR Tfase) are folate-dependent enzymes central to the de novo purine biosynthetic pathway. GAR Tfase utilizes the cofactor (6*R*)-N^{10}-formyltetrahydrofolate (Fig. 1) to transfer a formyl group to the primary amine of its substrate, glycinamide ribonucleotide (GAR, Fig. 1). This one carbon transfer incorporates the C-8 carbon of the purines and is the first of two formyl transfer reactions. The second formyl transfer reaction is catalyzed by the enzyme AICAR Tfase which also employs (6*R*)-N^{10}-formyltetrahydrofolate to transfer a formyl group to the C-5 amine of its substrate, aminoimidazole carboxamide ribonucleotide (AICAR, Fig. 1).[1] The discovery that (6*R*)-5,10-dideazatetrahydrofolate [Lometrexol, (6*R*)-DDATHF, Fig. 2] achieves its potent anticancer activity by selective GAR Tfase inhibition established GAR Tfase and the purine de novo biosynthetic pathway as viable targets for antineoplastic intervention.[2,3] Herein, we report the design, synthesis and evaluation of a novel folate analogue, 10-(2-benzoxazolcarbonyl)-DDACTHF (**1**, Fig. 2).

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Inhibitor design

The use of α-keto heterocycles as electrophilic, tight-binding reversible enzyme inhibitors was first disclosed by Edwards et al. in 1992.[4] Since then, a number of potent enzyme inhibitors have been disclosed based upon analogous design principles,[5] including our own work in the development of fatty acid amide hydrolase inhibitors.[6] In previous studies, we examined folate-based inhibitors which incorporated electrophilic functional groups that could potentially interact either with active site nucleophiles or the GAR/AICAR substrate amines.[7] It was envisioned that the properly positioned electrophilic carbonyl of an α-keto heterocycle could potentially form an imine or a tetrahedral adduct with these same potential nucleophiles or serve to stabilize gem diol formation of the electrophilic carbonyl and promote active site binding mimicking the tetrahedral intermediate of the formyl transfer reactions. This latter effect was observed with folate-based inhibitors bearing a nontransferable formyl group and has provided potent and efficacious GAR Tfase inhibitors.[7–9] In addition to the electrophilic carbonyl, an appropriately positioned nitrogen atom within the heterocyclic ring might form additional hydrogen bonds with an active site residue (e.g., protonated His-108), thereby further stabilizing the inhibitor complex.

Recently, we reported the synthesis and biological evaluation of 10-formyl-DDACTHF **2**.[8] This analogue was shown to be a potent GAR Tfase inhibitor ($K_i = 0.014$ μM against rhGAR Tfase) and an effective cytotoxic agent (CCRF–CEM $IC_{50} = 60$ nM).[8] Nonetheless, a facile oxidative decarbonylation of the formyl group conveyed a chemical instability to **2** precluding consideration for in vivo use. Consequently, we have examined a number of alternatives[5,7] to the incorporation of a formyl group. In the preceding article we detailed the preparation of an extensive series of simplified folate analogues bearing an electrophilic α-keto-heterocycle in place of the formyl group in structures which lack the benzoylglutamate subunit.[5] Herein, we report the synthesis and examination of **1**, 10-(2-benzoxazolcarbonyl)-DDACTHF which bears the additional benzoylglutamate subunit and may be representative of the entire series. In addition to active site interactions of the benzoxazole, the electrophilic carbonyl of the benzoxazolcarbonyl moiety in the DDACTHF scaffold could potentially interact with the active site of the enzyme either via formation of a covalent bond with the substrate GAR amine or by formation of hydrogen bonds (with its gem diol) with active site residues. Because of these possibilities, it was envisioned that **1** may exhibit interesting pharmacological properties in comparison to the formyl derivative **2**.[8,9]

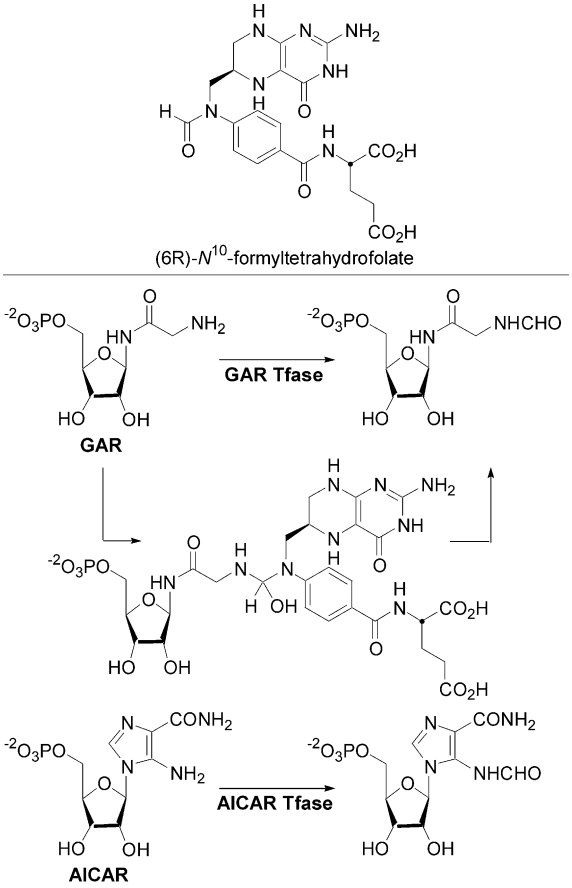


Figure 1.

Chemistry

The synthesis of 10-(2-benzoxazolcarbonyl)-DDACTHF (**1**) was accomplished in a convergent manner as shown in Schemes 1 and 2. Known aldehyde **3**[10] was converted to the corresponding cyanohydrin (KCN, THF/H_2O, 25 °C, 20 h). Following aqueous workup, the unstable crude cyanohydrin was converted into the corresponding ethyl imidate (HCl, anhydrous EtOH/$CHCl_3$, 25 °C, 12 h). Following concentration in vacuo, the unstable crude imidate was converted into benzoxazole **4** (2-aminophenol, anhydrous EtOH, 60 °C, 5 h).[11] Compound **4** was oxidized (Dess–Martin

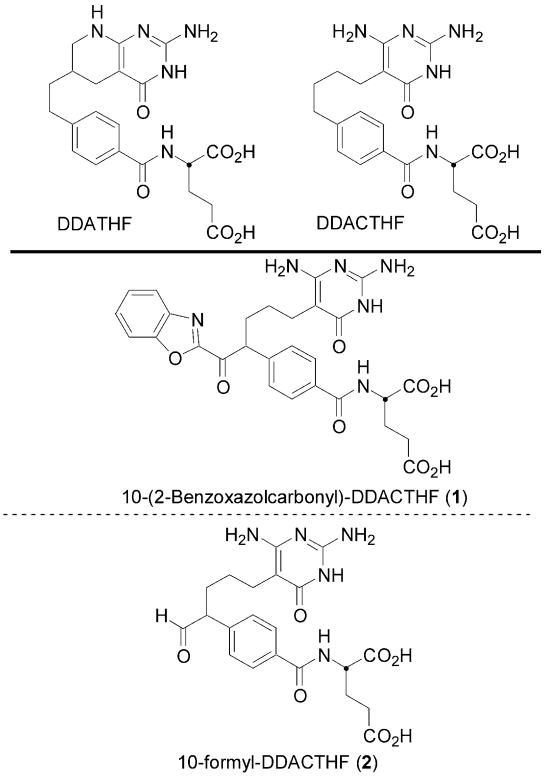
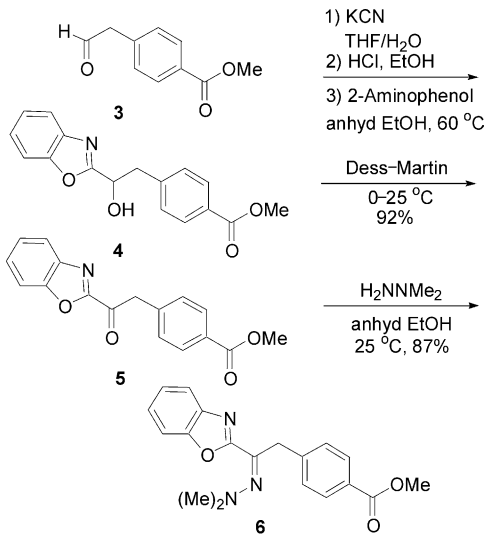


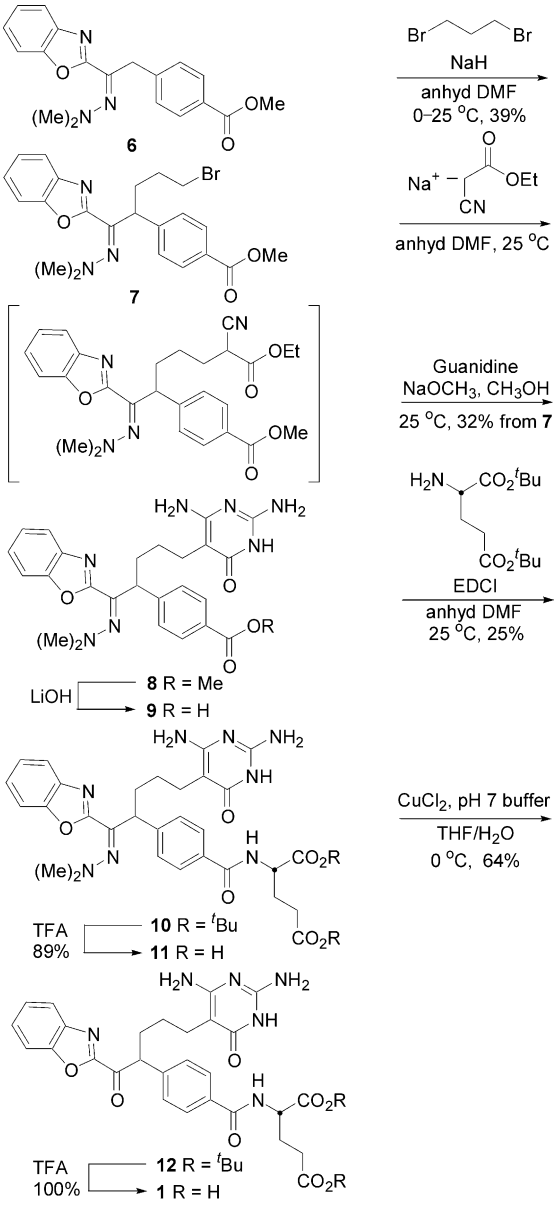
Figure 2.



Scheme 1.

periodinane, anhydrous CH_2Cl_2, 0–25 °C, 2 h, 92%) generating ketone **5**. The ^1H NMR ($CDCl_3$) of **5** clearly shows a peak corresponding to the benzylic methylene at δ 3.92, as well as the absence of a peak corresponding to an enol methine, indicating that this compound exists in the keto, rather than enol, form. Reaction of ketone **5** with *N*,*N*-dimethylhydrazine (glacial AcOH, anhydrous EtOH, 25 °C, 12 h, 87%) provided the key *N*,*N*-dimethylhydrazone **6** (Scheme 1). NaH deprotonation of **6** (DMF, 0 °C, 15 min) and subsequent treatment with excess 1,3-dibromopropane (10 equiv, DMF, 25 °C, 2 h, 39%) provided the mono-alkylation product **7**. The preformed sodium salt of ethyl cyanoacetate (NaH, DMF, 0 °C, 30 min) was alkylated with **7** (DMF, 25 °C, 2 h). Following aqueous workup, the crude alkylation intermediate was used without further purification. Cyclization with the free base of guanidine (1.2 equiv, CH_3OH, 25 °C, 1 h, 32% from **7**) under basic conditions gave the desired pyrimidinone **8**. Treatment of **8** with LiOH (2.1 equiv, 3:1 CH_3OH–H_2O, 25 °C, 24 h) cleanly provided the carboxylic acid **9** which was coupled with di-*tert*-butyl L-glutamate hydrochloride (EDCI, $NaHCO_3$, DMF, 25 °C, 24 h, 25%) to provide **10**. Acid-catalyzed deprotection of **10** (1:4 v/v TFA/$CHCl_3$, 24 h, 89%) provided **11**. Hydrolysis of the dimethylhydrazone by treatment with $CuCl_2$ (5.0 equiv, 0–25 °C, 7 h, 64%) in THF–H_2O buffered to pH 7,[12] provided ketone **12**. Deprotection of **12** was accomplished by treatment with trifluoroacetic acid (1:4 v/v TFA/$CHCl_3$, 25 °C, 12 h, 100%) to provide **1** (Scheme 2).

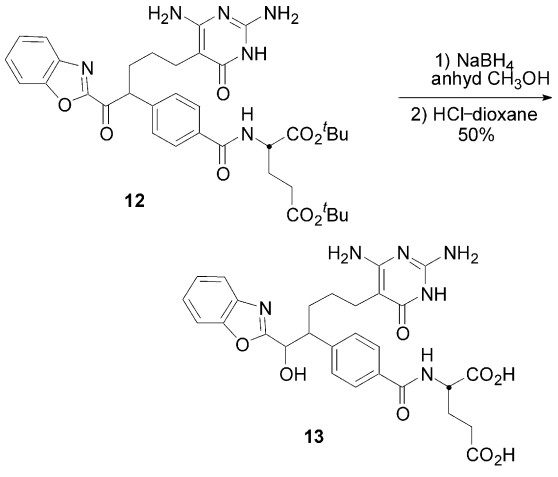
For comparative purposes, ketone **12** was reduced with $NaBH_4$ (2.0 equiv, CH_3OH, −20 °C, 30 min) followed by acid-catalyzed deprotection (4 N HCl–dioxane, 0–25 °C, 3 h, 50% from **12**) to provide alcohol **13** (Scheme 3).



Scheme 2.

GAR Tfase and AICAR Tfase Inhibition

Compounds **1**, **11** and **13** were tested for inhibition of GAR Tfase and AICAR Tfase and the results are presented in Table 1. The 10-(2-benzoxazolcarbonyl)-DDACTHF folate-based analogue (**1**) was the only derivative to show moderate activity against *Escherichia coli* GAR Tfase ($K_i = 11$ μM). The importance of the keto functionality in the binding affinity of **1** is apparent in the comparison of the analogous dimethylhydrazone (**11**) and hydroxy (**13**) derivatives which were 10-fold less potent ($K_i = 100$ μM) and inactive ($K_i > 100$ μM), respectively. Interestingly, of the three compounds examined for inhibition of rhAICAR Tfase, only the dimethylhydrazone **11** showed activity ($K_i = 30$ μM) whereas **1** and **13** were inactive ($K_i > 100$ μM). When tested against rhGAR Tfase, compound **1** showed an 18-fold increase in its potency (11 μM against *E. coli* vs 0.6 μM against rhuman GAR Tfase) now displaying a useful and potent level of activity. Although compound **11** was still inactive in this assay, the alcohol derivative **13** showed significant activity against rhGAR Tfase ($K_i = 1.8$ μM), while it was inactive against *E. coli* GAR Tfase. Consistent with the anticipated importance of the electrophilic carbonyl, this alcohol **13** was 3-fold less potent than the corresponding ketone **1** against rhGAR



Scheme 3.

Table 1. GAR and AICAR Tfase inhibition (K_i, μM)

Compd	*E. coli* GAR Tfase[a]	rhGAR Tfase[b]	rhAICAR Tfase[c]
1	11	0.6	>100
11	100	>100	30
13	>100	1.8	>100
Lometrexol	0.1	0.06[d]	nd[e]

[a]*E. coli* GAR Tfase.
[b]Recombinant human GAR Tfase.
[c]Recombinant human AICAR Tfase.
[d]Ref 13.
[e]nd, not determined.

Table 2. In vitro cytotoxic activity

Compd	CCRF–CEM (IC_{50}, μM)			
	(+) T, (+) H[a]	(−) T, (+) H	(−) T, (−) H	
1	>100	>100	>100	>100
11	>100	>100	>100	>100
13	>100	>100	>100	>100
Lometrexol	>100	>100	0.52	0.23

[a]T, thymidine; H, hypoxanthine.

Tfase. Although not easily anticipated, this preferential ca. 20-fold inhibition of rhGAR Tfase versus *E. coli* GAR Tfase and the relative potency of the series (**1** > **13** > **11**) is analogous to observations first made with **2** (10-formyl-DDACTHF).[8]

Cytotoxic Activity

Compounds **1**, **11** and **13** were examined for cytotoxic activity both in the presence (+) and absence (−) of added hypoxanthine against the CCRF–CEM cell line (Table 2). 10-(2-benzoxazolcarbonyl)-DDACTHF (**1**) and the related compounds (**11** and **13**) were inactive against the CCRF–CEM cell line in vitro. Thus, although compound **1** potently inhibits GAR Tfase in vitro (K_i = 600 nM), it does not display functional cytotoxic activity, perhaps due to ineffective cellular penetration or polyglutamation by FPGS.

Conclusions

10-(2-Benzoxazolcarbonyl)-DDACTHF (**1**), and the related dimethylhydrazone **11** and the hydroxy derivative **13** have been synthesized and evaluated as potential inhibitors of GAR Tfase and AICAR Tfase. Compound **1** was the most active derivative in the series exhibiting potent rhGAR Tfase inhibition (K_i = 600 nM) being 3-fold more potent than the corresponding alcohol (K_i = 1.8 μM), and >150-fold more potent than the corresponding *N*,*N*-dimethylhydrazone. Thus, the presence of a benzoxazolcarbonyl group on the DDACTHF framework produced folate analogues with potent enzyme inhibiting activity, but did not provide compounds that exhibited functional cytotoxic activity. The latter we suggest may be due to ineffective transport into the cell by the reduced folate carrier and/or lack of polyglutamation by FPGS.

Experimental

Methyl 4-(2-benzoxazol-2-yl-2-hydroxyethyl)benzoate (4). Known aldehyde **3**[10] (1.000 g, 5.612 mmol) was dissolved in a mixture of THF (25 mL) and H_2O (30 mL). Potassium cyanide (1.462 g, 22.45 mmol, 4.0 equiv) was added and the resulting solution was stirred at 25 °C for 20 h. The reaction mixture was partitioned between H_2O (100 mL) and EtOAc (400 mL). The organic layer was washed with H_2O (2×100 mL), saturated aqueous $NaHCO_3$ (1×100 mL), and saturated aqueous NaCl (1×100 mL) followed by concentration under reduced pressure. Due to instability, the crude cyanohydrin product was used without further purification. The crude cyanohydrin was dissolved in $CHCl_3$ (12 mL). Separately, anhydrous absolute EtOH (5.08 mL, 87.6 mmol, 25 equiv) was dissolved in $CHCl_3$ (9 mL) and cooled to 0 °C. Acetyl chloride (5.23 mL, 73.58 mmol, 21 equiv) was added dropwise. Following this addition, the cyanohydrin starting material in $CHCl_3$ solution was added dropwise and the resulting reaction mixture was allowed to warm to 25 °C. After stirring for 12 h, the reaction mixture was concentrated under reduced pressure. Due to instability, the crude imidate product was used without further purification. The crude imidate was dissolved in anhydrous absolute EtOH (15 mL). 2-Aminophenol (0.344 mg, 3.15 mmol, 0.90 equiv) was added and the resulting solution was warmed at 60 °C for 5 h. The reaction mixture was cooled to 25 °C and diluted with EtOAc (400 mL). The organic layer was washed with saturated aqueous $NaHCO_3$ (1×100 mL), 1 N aqueous HCl (3×100 mL), and saturated aqueous NaCl (1×100 mL) followed by concentration under reduced pressure. Chromatography (SiO_2, 1:1 EtOAc/hexanes) afforded **4** (0.250 g, 15% from **3**) as an orange solid: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.93 (d, J = 8.4 Hz, 2H), 7.69–7.61 (m, 1H), 7.58–7.50 (m, 1H), 7.41–7.28 (m, 4H), 5.21 (dd, J = 4.8, 7.7 Hz, 1H), 3.88 (s, 3H), 3.49–3.28 (m, 2H); MALDIFTMS (DHB) m/z 298.1065 ($M+H^+$, $C_{17}H_{15}NO_4$ requires 298.1074).

Methyl 4-(2-benzoxazol-2-yl-2-oxoethyl)benzoate (5). Dess–Martin periodane (1.028 g, 2.42 mmol, 3.0 equiv) was added to a stirred solution of **4** (0.240 g, 0.808 mmol) in anhydrous CH_2Cl_2 (13 mL) at 25 °C. The solution was stirred at 25 °C for 2 h. The reaction was diluted with Et_2O (50 mL) and quenched by the addition of 1:1 (v/v) aqueous $Na_2S_2O_3$/$NaHCO_3$ (50 mL) and stirred for 10 min. The resulting mixture was partitioned between Et_2O (400 mL) and H_2O (100 mL). The organic layer was washed with H_2O (2×100 mL) and saturated aqueous NaCl (1×100 mL) followed by concentration under reduced pressure affording **5** (0.219 g, 92%) as a yellow oil: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 8.10–7.39 (m, 8H), 3.92 (s, 2H), 3.89 (s, 3H); MALDIFTMS (DHB) m/z 296.0917 ($M+H^+$, $C_{17}H_{13}NO_4$ requires 296.0917).

Methyl 4-(2-benzoxazol-2-yl-2-dimethylhydrazonoethyl)-benzoate (6). Compound **5** (0.320 g, 1.08 mmol) was dissolved in anhydrous EtOH (20 mL). *N*,*N*-Dimethylhydrazine (0.42 mL, 5.42 mmol, 5.0 equiv) was added followed by glacial acetic acid (0.06 mL, 1.08 mmol, 1.0

equiv) and the resulting solution was stirred at 25 °C for 12 h. The reaction mixture was concentrated under reduced pressure. Chromatography (SiO_2, 1:2 EtOAc/hexanes) afforded **6** (0.318 g, 87%) as a red oil: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.92 (d, J = 8.4 Hz, 2H), 7.70–7.65 (m, 1H), 7.58–7.52 (m, 1H), 7.39–7.28 (m, 4H), 4.47 (s, 2H), 3.86 (s, 3H), 2.91 (s, 6H); MALDIFTMS (DHB) m/z 338.1486 ($M+H^+$, $C_{19}H_{19}N_3O_3$ requires 338.1499).

Methyl 4-[1-(benzoxazol-2-yl-2-dimethylhydrazonomethyl)-4-bromobutyl]benzoate (7). NaH (60% dispersion, 0.013 g, 0.313 mmol, 1.1 equiv) was added to a stirred solution of **6** (0.096 g, 0.285 mmol) in anhydrous DMF (1.5 mL) at 0 °C, and the solution was stirred at 0 °C for 15 min. 1,3-Dibromopropane (0.29 mL, 2.85 mmol, 10.0 equiv) was added to the reaction mixture and the cooling bath was removed. The solution was stirred at 25 °C for 2 h. The reaction was quenched by the addition of saturated aqueous NH_4Cl (1 mL). The resulting mixture was partitioned between EtOAc (100 mL) and H_2O (50 mL). The organic layer was washed with H_2O (2×50 mL) and saturated aqueous NaCl (1×50 mL) followed by concentration under reduced pressure. Chromatography (SiO_2, 1:2 EtOAc/hexanes) afforded **7** (0.051 g, 39%) as a yellow oil: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.98–7.90 (m, 2H), 7.75–7.70 (m, 1H), 7.60–7.52 (m, 1H), 7.48–7.29 (m, 4H), 4.22 (t, J = 7.2 Hz, 1H), 3.89 (s, 1H), 3.85 (s, 2H), 3.41 (t, J = 6.4 Hz, 2H), 3.03 (s, 1H), 2.90 (s, 3.5H), 2.70 (s, 1.5H), 2.38–2.23 (m, 1H), 2.09–1.79 (m, 3H); MALDIFTMS (DHB) m/z 458.1082 ($M+H^+$, $C_{22}H_{24}BrN_3O_3$ requires 458.1074).

Methyl 4-[1-(benzoxazol-2-yl-2-dimethylhydrazonomethyl)-4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)butyl]benzoate (8). A suspension of NaH (60% dispersion, 0.786 g, 19.7 mmol, 15 equiv) in anhydrous DMF (7.5 mL) at 0 °C was treated dropwise with ethyl cyanoacetate (2.09 mL, 19.7 mmol, 15 equiv). The solution was stirred at 0 °C for 30 min, forming the sodium salt as a clear solution. This was treated with a solution of **7** (0.600 g, 1.31 mmol) in anhydrous DMF (7.5 mL). The resulting reaction mixture was stirred at 25 °C for 2 h. The reaction was quenched by the addition of saturated aqueous NH_4Cl (5 mL). The reaction was diluted with EtOAc (200 mL) and washed successively with H_2O (3×50 mL) and saturated aqueous NaCl (50 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Sodium metal (1.108 g, 48.20 mmol, 2.3 equiv) was added to anhydrous CH_3OH (15 mL) and stirred at 25 °C for 10 min to generate $NaOCH_3$. Guanidine–HCl (2.40 g, 25.2 mmol, 1.2 equiv) was added to this solution and stirred at 25 °C for 30 min. Separately, the crude alkylation product was dissolved in anhydrous CH_3OH (20 mL) and the resulting solution quickly added to the stirring reaction mixture. The solution was stirred at 25 °C for 1 h. The reaction mixture was applied directly to a SiO_2 plug. After complete air evaporation of the reaction solvent, impurities were removed by washing with 5:1 hexanes/EtOAc. The product was subsequently eluted by washing the SiO_2 with 10:1 $CHCl_3/CH_3OH$ to afford **8** (0.210 g, 32% from **7**) as a yellow solid: ^{1}H NMR (CD_3OD, 250 MHz) δ 7.91–7.80 (m, 2H), 7.71–7.50 (m, 3H), 7.39–7.30 (m, 3H), 4.20 (t, J = 7.6 Hz, 1H), 3.85 (s, 1H), 3.83 (s, 2H), 2.79 (s, 4H), 2.70 (s, 2H), 2.42–2.22 (m, 1H), 2.35 (t, J = 7.4 Hz, 2H), 2.01–1.84 (m, 1H), 1.62–1.42 (m, 2H); MALDIFTMS (DHB) m/z 504.2349 ($M+H^+$, $C_{26}H_{29}N_7O_4$ requires 504.2354).

4-[1-(Benzoxazol-2-yl-dimethylhydrazonomethyl)-4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)butyl]benzoic acid (9). A solution of **8** (0.100 g, 0.199 mmol) in 3:1 $CH_3OH–H_2O$ (4.0 mL) was treated with $LiOH·H_2O$ (0.018 g, 0.417 mmol, 2.1 equiv) and the mixture was stirred at 25 °C for 24 h. The mixture was diluted with H_2O (15 mL) and the aqueous layer was washed with EtOAc (3×5 mL). The aqueous layer was acidified to pH = 4 by the addition of 1 N aqueous HCl. The solution was concentrated under reduced pressure and the residue was treated with toluene (3×10 mL) to remove traces of H_2O to provide crude **9** (0.090 g) which was used without further purification: ^{1}H NMR (CD_3OD, 250 MHz) δ 7.87–7.23 (m, 8H), 4.16 (t, J = 7.6 Hz, 1H), 2.76 (s, 4H), 2.69 (s, 2H), 2.45–2.22 (m, 2H), 2.34 (t, J = 7.4 Hz, 2H), 1.62–1.40 (m, 2H); MALDIFTMS (DHB) m/z 490.2205 ($M+H^+$, $C_{25}H_{27}N_7O_4$ requires 490.2197).

Di-*tert*-butyl *N*-{4-[1-(benzoxazol-2-yl-dimethylhydrazonomethyl)-4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)butyl]benzoyl}-L-glutamate (10). A solution of **9** (0.100 g, 0.204 mmol) and di-*tert*-butyl L-glutamate hydrochloride (0.091 g, 0.307 mmol, 1.5 equiv) in DMF (1.0 mL) was treated with $NaHCO_3$ (0.077 g, 0.920 mmol, 4.5 equiv) followed by EDCI (0.118 g, 0.613 mmol, 3.0 equiv). The reaction mixture was stirred at 25 °C for 24 h before the solvent was removed under reduced pressure. The resulting residue was suspended in $CHCl_3$ (50 mL) and washed with saturated aqueous $NaHCO_3$ (3×5 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Chromatography (SiO_2, 10:1 $CHCl_3/CH_3OH$) afforded **10** (0.038 g, 25%) as a yellow solid: ^{1}H NMR (CD_3OD, 250 MHz) δ 7.87–7.31 (m, 8H), 4.49–4.42 (m, 1H), 4.20 (t, J = 7.6 Hz, 1H), 2.79 (s, 4H), 2.71 (s, 2H), 2.50–2.12 (m, 4H), 2.35 (t, J = 7.4 Hz, 2H), 2.06–1.90 (m, 2H), 1.62–1.40 (m, 2H), 1.45 (s, 9H), 1.38 (s, 9H); MALDIFTMS (DHB) m/z 731.3901 ($M+H^+$, $C_{38}H_{50}N_8O_7$ requires 731.3875).

***N*-{4-[1-(Benzoxazol-2-yl-dimethylhydrazonomethyl)-4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)butyl]benzoyl}-L-glutamic acid (11)**. A solution of **10** (0.005 g, 0.007 mmol) in $CHCl_3$ (1.0 mL) cooled to 0 °C was treated with trifluoroacetic acid (0.25 mL). The solution was allowed to warm and stirred at 25 °C for 24 h. The reaction was concentrated under reduced pressure. Et_2O (1 mL) was added and a precipitate formed. The precipitate was triturated with Et_2O (3×1 mL) and dried in vacuo to give **11**–CF_3CO_2H (0.004 mg, 89%) as a gray solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.86 (d, J = 8.5 Hz, 1H), 7.70 (d, J = 7.9 Hz, 1H), 7.57 (d, J = 7.6 Hz, 1H), 7.42–7.35 (m, 4H), 4.62–4.51 (m, 1H), 4.31 (t, J = 7.5 Hz, 1H), 2.91 (s, 6H), 2.46–2.20 (m, 4H), 2.37 (t, J = 7.2 Hz, 2H), 2.09–1.90 (m, 2H), 1.58–1.40 (m, 2H);

MALDIFTMS (DHB) m/z 619.2596 ($M+H^+$, $C_{30}H_{34}N_8O_7$ requires 619.2623).

Di-*tert*-butyl *N*-{4-[1-(benzoxazol-2-carbonyl)-4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)butyl]benzoyl}-L-glutamate (12). A solution of **10** (0.030 g, 0.041 mmol) in THF (0.9 mL) and pH 7 aqueous phosphate buffer (0.2 mL) cooled to 0 °C was treated with a solution of $CuCl_2$ (0.028 g, 0.205 mmol, 5.0 equiv) in H_2O (0.3 mL). The solution was stirred at 0 °C for 1 h and then at 25 °C for 6 h before it was quenched by the dropwise addition of a pH 8 saturated aqueous NH_4Cl-NH_4OH solution (20 mL). The product was extracted with $CHCl_3$ (2×50 mL), purged with N_2, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Chromatography (SiO_2, 10:1 $CHCl_3/CH_3OH$) afforded **12** (0.018 g, 64%) as a white solid: ^{1}H NMR (CD_3OD, 250 MHz) δ 7.95–7.42 (m, 8H), 5.10 (d, $J=7.2$ Hz, 1H), 4.49–4.40 (m, 1H), 2.40–1.90 (m, 6H), 2.35 (t, $J=7.0$ Hz, 2H), 1.51–1.31 (m, 2H), 1.45 (s, 9H), 1.38 (s, 9H); MALDIFTMS (DHB) m/z 711.3109 ($M+Na^+$, $C_{36}H_{44}N_6O_8$ requires 711.3113).

***N*-{4-[1-(Benzoxazol-2-carbonyl)-4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)butyl]benzoyl}-L-glutamic acid (1).** A solution of **12** (0.005 g, 0.007 mmol) in $CHCl_3$ (1.0 mL) cooled to 0 °C was treated with trifluoroacetic acid (0.25 mL). The solution was allowed to warm and stirred at 25 °C for 12 h. The reaction was concentrated under reduced pressure. Et_2O (1 mL) was added and a precipitate formed. The precipitate was triturated with Et_2O (3×1 mL) and dried in vacuo to give **1**–CF_3CO_2H (0.005 mg, 100%) as a pale yellow solid: ^{1}H NMR (CD_3OD, 250 MHz) δ 7.95–7.42 (m, 8H), 5.10 (d, $J=7.4$ Hz, 1H), 4.62–4.52 (m, 1H), 2.44 (t, $J=7.2$ Hz, 2H), 2.37 (t, $J=7.4$ Hz, 2H), 2.32–2.20 (m, 2H), 2.15–1.90 (m, 2H), 1.55–1.35 (m, 2H); MALDIFTMS (DHB) m/z 577.2041 ($M+H^+$, $C_{28}H_{28}N_6O_8$ requires 577.2041).

***N*-{4-[1-(Benzoxazol-2-yl-2-hydroxymethyl)-4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)butyl]benzoyl}-L-glutamic acid (13).** A solution of **12** (0.010 g, 0.015 mmol) in anhydrous CH_3OH (0.5 mL) at −20 °C was treated with $NaBH_4$ (0.0011 g, 0.029 mmol, 2.0 equiv). The solution was stirred at −20 °C for 30 min before it was quenched by the addition of H_2O (0.5 mL). The reaction mixture was diluted with EtOAc (5 mL) and washed with H_2O (3×1 mL) and concentrated under reduced pressure. The resulting product (0.005 g, 0.007 mmol) was treated with 4 N HCl–dioxane (1.5 mL) at 0 °C. The solution was allowed to warm and stirred at 25 °C for 3 h. The reaction was purged with N_2 and then concentrated under reduced pressure. Et_2O (1 mL) was added and a precipitate formed. The precipitate was triturated with Et_2O (3×1 mL) and dried in vacuo to give **13**–HCl (0.004 mg, 50% from **12**) as a yellow solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.74 (d, $J=7.9$ Hz, 2H), 7.70–7.59 (m, 2H), 7.40–7.32 (m, 4H), 5.12 (d, $J=6.5$ Hz, 1H), 4.65–4.58 (m, 1H), 2.46 (t, $J=7.1$ Hz, 2H), 2.38–2.25 (m, 1H), 2.23 (t, $J=7.2$ Hz, 2H), 2.12–2.00 (m, 1H), 1.89–1.80 (m, 1H), 1.70–1.57 (m, 1H), 1.39–1.21 (m, 2H); MALDIFTMS (DHB) m/z 579.2211 ($M+H^+$, $C_{28}H_{30}N_6O_8$ requires 579.2198).

Cytotoxicity, GAR Tfase and AICAR Tfase inhibition. Cytotoxicity, rhGAR[14] and rhAICAR[15] Tfase inhibition studies were conducted as previously detailed[8] with the exception that the AICAR Tfase inhibition was conducted in the absence of 5 μM β-mercaptoethanol and screened with 10 nM enzyme, 25 μM inhibitor and 22.5 μM of cofactor.

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