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## Asymmetric Synthesis of Inhibitors of Glycinamide Ribonucleotide Transformylase

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### Abstract

Glycinamide ribonucleotide transformylase (GAR Tfase) catalyzes the first of two formyl transfer steps in the de novo purine biosynthetic pathway that require folate cofactors and has emerged as a productive target for antineoplastic therapeutic intervention. The asymmetric synthesis and evaluation of the two diastereomers of 10-methylthio-DDACTHF (10*R*-**3** and 10*S*-**3**) and related analogues as potential inhibitors of GAR Tfase are reported. This work, which defines the importance of the C10 stereochemistry for this class of inhibitors of GAR Tfase, revealed that both diastereomers are potent inhibitors of rhGAR Tfase (10*R*-**3**  $K_i = 210$  nM, 10*S*-**3**  $K_i = 180$  nM) that exhibit effective cell growth inhibition (CCRF-CEM  $IC_{50} = 80$  and 50 nM, respectively) which is dependent on intracellular polyglutamation by folylpolyglutamate synthetase (FPGS), but not intracellular transport by the reduced folate carrier.

### Introduction

Glycinamide ribonucleotide transformylase (GAR Tfase) is a folate-dependent enzyme central to the de novo purine biosynthetic pathway.<sup>1,2</sup> GAR Tfase utilizes the cofactor (6*R*)-N<sup>10</sup>-formyltetrahydrofolic acid (10-formyl-THF) to transfer a formyl group to the primary amine of its substrate,  $\beta$ -glycinamide ribonucleotide ( $\beta$ -GAR) (Figure 1). This one carbon transfer provides the C-8 carbon of the purines and is the first of two formyl transfer reactions enlisted in the biosynthesis of purines. Inhibitors of folate dependent enzymes including GAR Tfase have provided important compounds for cancer chemotherapy as a result of their inhibition of the biosynthesis of nucleic acid precursors.<sup>3,4</sup> Validation of GAR Tfase as a useful anticancer target emerged with the discovery of the first potent and selective inhibitor, 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF).<sup>5</sup> Its selective toxicity has been attributed in part to the reliance of sensitive tumor cells on de novo purine synthesis, whereas the salvage pathway is an available and primary source of purines in normal untransformed cells.<sup>6,7</sup>

In previous studies, we reported the synthesis and biological evaluation of two unique folate-based inhibitors of GAR Tfase. 10-Formyl-DDACTHF<sup>8</sup> (**1**) and 10-CF<sub>3</sub>CO-DDACTHF<sup>9</sup> (**2**), bearing a nontransferable formyl or trifluoroacetyl group, proved to be potent inhibitors of GAR Tfase (**1**,  $K_i = 0.014$   $\mu$ M against rhGAR Tfase; **2**,  $K_i = 0.015$   $\mu$ M against rhGAR Tfase). Both inhibitors have been shown through X-ray and NMR studies to bind GAR Tfase as the *gem*-diols.<sup>9,10</sup> The formation of the *gem*-diol mimics the formyl transfer reaction tetrahedral intermediate and provides strong stabilizing interactions between the inhibitor and the active site catalytic residues of the protein. In both instances, the inhibitors C10 diastereomers rapidly interconvert preventing independent assessment of the individual diastereomers.

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In a more recent study designed to explore the ability of 10-methanesulfonyl-DDACTHF to also mimic such active site binding interactions, we reported the synthesis and biological activity of its precursor **3**, 10-methylthio-DDACTHF, which proved to be a surprisingly potent inhibitor of GAR Tfase ( $IC_{50} = 100$  nM, CCRF-CEM;  $K_i = 250$  nM, rhGAR Tfase).<sup>11</sup> Although the activity of **3** was unexpected since it does not contain a C10 tetrahedral intermediate mimic, the thiomethyl moiety does incorporate a potential hydrogen bond acceptor and presents a soft hydrophobic substituent potentially stabilizing active site binding. The introduction of sulfur within inhibitors of GAR Tfase is well established. The known inhibitors LY309887 (**4**), AG2034 (**5**), and AG2037 (**6**) all incorporate sulfur into their structures although none do so in a manner analogous to **3** (Figure 2),<sup>12-14</sup> and it has been reported that analysis of the GAR Tfase active site using the program GRID suggested that sulfur atoms should have particular affinity for two regions of the folate cofactor binding site.<sup>13</sup>

The original synthesis of **3** was racemic and the derivative was evaluated as an equi-mixture of C10-diastereomers. However, and unlike **1** and **2**, the individual C10-diastereomers of **3** would not be expected to easily interconvert and could be anticipated to be individually evaluated revealing the importance of the C10 stereochemistry to the properties of the candidate inhibitors. Herein, we report an asymmetric synthesis of 10-methylthio-DDACTHF (**3**) permitting this investigation into the importance of the C10 stereochemistry and report the biological properties of each C10-diastereomer of 10-thiomethyl-DDACTHF (**3**) along with that of a series of key analogues that further define the importance of the C10 substituent itself: 10-hydroxy-DDACTHF (10*R*-**7** and 10*S*-**7**) and 10-methoxy-DDACTHF (10*R*-**8** and 10*S*-**8**) and C10 substituted dithiane **9**.

## Inhibitor Synthesis

Starting from methyl 4-formylbenzoate, the aldehyde was converted to dithiane **11** upon reaction with 1,3-propanedithiol (Scheme 1). The resulting dithiane was alkylated with 1-chloro-3-iodopropane to give **12**, and subsequent removal of the dithiane using bis(trifluoro)acetoxyiodobenzene (PIFA) provided **13**. Ketone **13** was reduced using the *R*-Me-CBS<sup>15</sup> catalyst in high yield (97%) and high enantiomeric excess (99% ee) to yield alcohol *S*-**14**. The absolute stereochemistry of *S*-**14** and its subsequent derivatives were assigned based on the well-established stereochemical preferences of the reagent,<sup>16</sup> and confirmed in X-ray structures of both 10*R*-**3** and 10*S*-**3** bound to recombinant human GAR Tfase. The details of these latter studies will be disclosed elsewhere. Similarly, the *S*-Me-CBS catalyst was utilized to provide the enantiomer of **14** (not shown) and characterization data for this enantiomeric series is provided in the Supporting Information.

The preformed sodium salt of ethyl cyanoacetate was alkylated with *S*-**14** to give the common intermediate *S*-**15** for all three optically active inhibitors. Treatment of *S*-**15** with thioacetic acid under modified Mitsunobu conditions provided *R*-**16** with inversion of C10 stereochemistry (Scheme 2). Compound *R*-**16** was subsequently deprotected and the free thiol was methylated to yield *R*-**18**. Intermediate *R*-**18** was treated with the free base of guanidine under basic conditions to form the pyrimidine *R*-**19** and this intermediate was subsequently saponified to give *R*-**20**. Compound *R*-**20** was coupled with di-*tert*-butyl L-glutamate and subsequently deprotected using trifluoroacetic acid to provide final inhibitor 10*R*-**3**. The C10 diastereomer 10*S*-**3** was prepared using an analogous route employing the enantiomeric *R*-Me-CBS catalyst to provide *R*-**14** (See Supporting Information).

The inhibitor 10*S*-**7** bearing a C10-hydroxyl substituent, was prepared from the common intermediate *S*-**15** (Scheme 3). Compound *S*-**15** was treated with guanidine hydrochloride to give the corresponding pyrimidone *S*-**22** which subsequently was saponified using lithium

hydroxide to afford **S-23**. Intermediate **S-23** was then coupled with dimethyl *L*-glutamate and deprotected under basic conditions to provide the final inhibitor **10S-7**.

A key compound for comparison is the C10-methoxy derivative **8** where an oxygen replaces the sulfur of **3** as a probe for its unique importance. The synthesis of inhibitor **10S-8**, bearing the C10-methoxy substituent, was accomplished by methylation of the common intermediate **S-15** using TMSCHN<sub>2</sub> under acidic conditions<sup>17</sup> to give **S-25** (Scheme 4). Intermediate **S-25** was carried forward employing an analogous route, starting with formation of pyrimidone **S-26** and saponification to give **S-27**. Compound **S-27** was coupled with di-*tert*-butyl *L*-glutamate hydrochloride and deprotected using trifluoroacetic acid to provide **10S-8**.

Finally, **9**, which incorporates two C10 sulfur atoms and, in a sense, simultaneously incorporates both C10-diastereomers of **3**, was judged as an attractive additional derivative to examine that avoids the presence of two C10-diastereomers. Synthesis of inhibitor **9** started with alkylation of **12** using ethyl cyanoacetate to give **29** (Scheme 5). Intermediate **29** was converted to **9** using the same route detailed for the previous inhibitors, starting with formation of pyrimidine **30** and saponification to give **31**. Compound **31** was coupled with di-*tert*-butyl *L*-glutamate and deprotected using trifluoroacetic acid to provide **9**.

## Results and Discussion

Each diastereomer of **3**, **7**, **8**, and **9** was examined for inhibition of rhGAR Tfase and aminoimidazole carboxamide ribonucleotide transformylase (rhAICAR Tfase), and the results are summarized in Table 1. Remarkably, both diastereomers of the thiomethyl derivative **3** exhibited potent activity against rhGAR Tfase with **10S-3** being slightly more potent than **10R-3** ( $K_i = 180$  nM vs  $K_i = 210$  nM, respectively). This nearly indistinguishable activity of the two C10-diastereomers of **3** is only consistent with both possessing near equivalent capabilities for binding at the GAR Tfase active site and suggests that the flexible linker region joining the pyrimidine and phenyl ring conformationally adjusts to allow a comparable positioning of the thiomethyl substituent in the formyl transfer pocket of the GAR Tfase active site. Inhibitors **10R-7** and **10S-7** displayed very modest activity ( $K_i = 1060$  nM and  $1210$  nM, respectively) against rhGAR Tfase. This represents a 6-fold reduction in activity relative to **3** and again both diastereomers proved active, although it was the C10-*R* diastereomer that was slightly more potent than the corresponding C10-*S* diastereomer. Even more interestingly, methoxy derivatives **10R-8** and **10S-8** were found to inhibit rhGAR Tfase with potencies nearly equivalent to the corresponding alcohols ( $K_i = 850$  nM and  $1240$  nM, respectively). Again, while both diastereomers exhibited near equivalent potencies, the C10-*R* diastereomer was slightly more effective, and the 4-6 fold distinction relative to **3** highlights the special nature of the thiomethyl versus methoxy interaction at the enzyme active site. Inhibitor **9** also exhibited a reduced binding to rhGAR Tfase ( $K_i = 1060$  nM), illustrating that optimally one, but not two, thioalkyl substituents may enhance binding at the enzyme active site. These results define a clear role and remarkable stereochemical accommodation of the C10-substituents on DDACTHF with a unique stabilization attributable to the C10-thiomethyl group.

All inhibitors were also examined for activity against rhAICAR Tfase. The only compound in the series to show any activity against the enzyme was dithiane **9** ( $K_i = 20$   $\mu$ M). Each diastereomer of the thiomethyl, hydroxyl, and methoxy inhibitors was inactive against rhAICAR Tfase ( $K_i > 100$   $\mu$ M) illustrating that they disrupt de novo purine biosynthesis by selectively targeting GAR Tfase.

The compounds were also examined for cytotoxic activity (growth inhibition) both in the presence (+) and absence (-) of added hypoxanthine (purine) or thymidine (pyrimidine) against the CCRF-CEM cell line (Table 2). All the inhibitors exhibited activity that paralleled their

potency against rhGAR Tfase. Of all the inhibitors, only 10R-3 and 10S-3 exhibited potent activity in this cell-based assay ( $IC_{50} = 80$  and  $50$  nM, respectively), being only 3-5 times less potent than **2**, the most potent inhibitor of GAR Tfase disclosed to date. While both C10-diastereomers displayed this potent cytotoxic activity, the 10S-diastereomer proved to be slightly more active than the 10R-diastereomer, and both display a potency that slightly exceeds that observed on the isolated enzyme. Compounds 10R-7, 10S-7, 10R-8, and 10S-8 were all approximately ten times less potent than the thiomethyl inhibitors. All still showed modest activity and there was little distinction between the individual C10 diastereomers. Inhibitor **9**, on the other hand, showed no activity in this cell-based assay. An especially interesting set of comparisons are the activities of the two diastereomers of **3** and the related inhibitors with DDACTHF lacking a C10 substituent. These comparisons indicate that each of the substituents actually enhance, or at least maintain, the GAR Tfase inhibition of DDACTHF and enhance and maintain its corresponding cell growth inhibitory activity. Thus, the differences observed among one another and relative to DDACTHF may be attributed to relative binding enhancements provided by the key C10 substituents and not to destabilizing interactions they introduce at the enzyme active site. Finally, in the presence of thymidine, a pyrimidine, all inhibitors retained their activity, whereas the inhibitors were inactive in the presence of added hypoxanthine. This indicates that these compounds, like **2**, inhibit cell growth by selectively inhibiting an enzyme within the purine biosynthetic (not pyrimidine) pathway consistent with their potent inhibition of GAR Tfase.

All seven inhibitors were subsequently examined in mutant CCRF-CEM cell lines that lack folypolyglutamate synthetase (CCRF-CEM/FPGS<sup>-</sup>) or the reduced folate carrier (CCRF-CEM/MTX) (Table 3). Like the past inhibitors of GAR Tfase that we have examined, all inhibitors including **3** lost activity against the cell line that lacks FPGS (ca. 100-fold for **3**), indicating that they benefit from intracellular polyglutamation. This observation suggests that the potent activity of both diastereomers of **3** in the cell growth assays, which exceed their inhibition of GAR Tfase roughly 3-fold, may be attributed to their FPGS polyglutamation and the resulting intracellular accumulation or enhanced target affinity. In contrast, none of the inhibitors disclosed herein exhibited altered activity against the cell line lacking the reduced folate carrier indicating its transport of the inhibitors is not essential to their activity. Thus, cell lines and tumors that derive their resistance to antifolates through down regulation of the reduced folate carrier would be expected to remain sensitive to the two diastereomers of **3**.

## Conclusions

Examination of the importance of the C10 stereochemistry of DDACTHF-based inhibitors was addressed with the asymmetric synthesis of the two diastereomers of 10-methylthio-DDACTHF (**3**) and a series of key analogues incapable of in situ racemization of the C10 center. Remarkably, the activities of the C10-substituted DDACTHF derivatives proved to be essentially independent of the C10 stereochemistry and those bearing a C10-thiomethyl substituent were surprisingly potent and efficacious. Presumably this reflects the conformational flexibility of the linking region between the pyrimidine and phenyl group which contains the C10-substituent that allows a comparable positioning of the thiomethyl group in the formyl transfer pocket of the GAR Tfase active site. In addition to being substantially more potent than the corresponding C10-methoxy derivatives highlighting the unique properties embodied in **3**, the two diastereomers of **3** proved especially efficacious in inhibiting cell growth (CCRF-CEM  $IC_{50} = 80$  and  $50$  nM for 10R-3 and 10S-3, respectively) that could be rescued by added purine (but not pyrimidine) and that relies on intracellular FPGS polyglutamation, but not intracellular transport by the reduced folate carrier. Similarly, both diastereomers of **3** were found to be potent and essentially indistinguishable inhibitors of rhGAR Tfase ( $K_i = 210$  and  $180$  nM for 10R-3 and 10S-3, respectively), albeit with potencies that are slightly lower than their actual cell growth inhibitory activity. This latter observation

suggests that the required intracellular polyglutamation observed with **3** functionally enhances its potency in the cell-based assays.

## Experimental Section

Characterization of the compounds with the alternative C10-stereochemistry is provided in the Supporting Information. Our use of “concentrated” in the following experimental refers to evaporation to dryness on a rotary evaporator.

### Methyl 4-(1,3-Dithian-2-yl)benzoate (**11**)

A solution of methyl 4-formylbenzoate (14.10 g, 85.9 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (282 mL) was treated with 1,3-propanedithiol (9.48 mL, 94.5 mmol, 1.1 equiv) and the mixture was stirred for 1 h at room temperature. The solution was cooled to 0 °C and boron trifluoride diethyl etherate was added (11.9 mL, 94.5 mmol, 1.1 equiv). The reaction mixture was stirred and allowed to warm to room temperature overnight. The mixture was diluted with  $\text{CH}_2\text{Cl}_2$  and quenched with the addition of saturated aqueous  $\text{NaHCO}_3$ . The organic layer was washed with  $\text{H}_2\text{O}$  and saturated aqueous  $\text{NaCl}$ , dried over  $\text{Na}_2\text{SO}_4$ , and concentrated (evaporated to dryness on a rotary evaporator). Column chromatography ( $\text{SiO}_2$ , 25-50% EtOAc/hexanes gradient) yielded **11** (21.0 g, 96%) as a white solid: mp 132-134 °C;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  8.02 (d, 2H,  $J = 8.3$  Hz), 7.55 (d, 2H,  $J = 8.3$  Hz), 5.22 (s, 1H), 3.92 (s, 3H), 3.10-3.04 (m, 2H), 2.96-2.92 (m, 2H), 2.22-2.19 (m, 1H), 1.99-1.95 (m, 1H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  166.8, 144.2, 130.3 (2C), 130.2, 128.1 (2C), 52.4, 51.3, 32.1 (2C), 25.2; IR (film)  $\nu_{\text{max}}$  2903, 1707, 1272, 1108  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  255.0507 ( $\text{M} + \text{H}^+$ ,  $\text{C}_{12}\text{H}_{14}\text{O}_2\text{S}_2$  requires 255.0508).

### Methyl 4-(2-(3-Chloropropyl)-1,3-dithian-2-yl)benzoate (**12**)

A solution of **11** (20.96 g, 82.4 mmol) in THF (629 mL) cooled to -78 °C was treated with  $\text{NaHMDS}$  (2 M in THF, 51.5 mL, 103 mmol, 1.25 equiv) dropwise. The solution was stirred for 30 min followed by the addition of 1-chloro-3-iodopropane (44.3 mL, 412 mmol, 5.0 equiv). The reaction mixture was allowed to warm to room temperature overnight and was quenched with the addition of saturated aqueous  $\text{NH}_4\text{Cl}$ . The mixture was diluted with EtOAc and subsequently washed with saturated aqueous  $\text{NaCl}$ , dried over  $\text{Na}_2\text{SO}_4$ , and concentrated. Column chromatography ( $\text{SiO}_2$ , 0-10% EtOAc/hexanes gradient) yielded **12** (21.73 g, 80%) as a white solid: mp 75-77 °C;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  8.05 (d, 2H,  $J = 8.7$  Hz), 8.00 (d, 2H,  $J = 8.7$  Hz), 3.92 (s, 3H), 3.40 (t, 2H,  $J = 6.4$  Hz), 2.75-2.60 (m, 4H), 2.17-2.14 (m, 2H), 1.96-1.93 (m, 2H), 1.75-1.70 (m, 2H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  166.8, 147.2, 130.0 (2C), 129.2, 129.0 (2C), 58.1, 52.3, 44.7, 42.3, 27.8 (2C), 27.3, 25.0; IR (film)  $\nu_{\text{max}}$  2944, 1723, 1277  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  331.0587 ( $\text{M} + \text{H}^+$ ,  $\text{C}_{15}\text{H}_{19}\text{ClO}_2\text{S}_2$  requires 331.0588).

### Methyl 4-(4-Chlorobutanoyl)benzoate (**13**)

A solution of **12** (10.79 g, 32.6 mmol) in 9:1 MeCN/ $\text{H}_2\text{O}$  (163 mL) was treated with bis(trifluoro)acetoxyiodobenzene (21.0 g, 48.9 mmol, 1.5 equiv) and allowed to stir for 30 min. The reaction mixture was quenched with the addition of saturated aqueous  $\text{NaHCO}_3$  and diluted with EtOAc. The mixture was extracted with  $\text{H}_2\text{O}$  (2 $\times$ ) and saturated aqueous  $\text{NaCl}$ , dried over  $\text{Na}_2\text{SO}_4$ , and concentrated. Column chromatography ( $\text{SiO}_2$ , 20% EtOAc/hexanes) provided **13** (6.37 g, 81%) as a white solid: mp 45-46 °C;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  8.15 (d, 2H,  $J = 8.3$  Hz), 8.04 (d, 2H,  $J = 8.2$  Hz), 3.96 (s, 3H), 3.70 (t, 2H,  $J = 6.3$  Hz), 3.22 (t, 2H,  $J = 6.9$  Hz), 2.28-2.22 (m, 2H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  198.6, 166.3, 140.0, 134.1, 130.0 (2C), 128.0 (2C), 52.6, 44.6, 35.8, 26.7; IR (film)  $\nu_{\text{max}}$  2944, 1723, 1682, 1272, 1108  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  241.0624 ( $\text{M} + \text{H}^+$ ,  $\text{C}_{12}\text{H}_{13}\text{ClO}_3$  requires 241.0626).



### Methyl (S)-4-(4-Chloro-1-hydroxybutyl)benzoate (S-14)

A solution of *R*-Me-CBS (1 M in toluene, 1.09 mL, 1.09 mmol, 0.1 equiv) in anhydrous THF (10.1 mL) cooled to 0 °C was treated with borane dimethylsulfide complex (2 M in THF, 6.80 mL, 13.6 mmol, 1.25 equiv) and stirred for 15 min at 0 °C. A solution of **13** (2.62 g, 10.9 mmol) in THF (54.4 mL) was added dropwise to the reaction mixture and stirred for 1 h, allowing the reaction mixture to warm to room temperature. The reaction was quenched with the addition of MeOH and allowed to stir for 30 min at room temperature. The mixture was diluted with EtOAc and washed with 1 N HCl (3×), H<sub>2</sub>O (2×), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. Column chromatography (SiO<sub>2</sub>, 40% EtOAc/hexanes) afforded *S*-**14** (2.55 g, 97%) as a clear oil: <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.04 (d, 2H, *J* = 8.2 Hz), 7.44 (d, 2H, *J* = 8.4 Hz), 4.81 (t, 1H, *J* = 5.9 Hz), 3.92 (s, 3H), 3.60-3.53 (m, 2H), 1.97-1.82 (m, 4H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 167.0, 149.6, 130.0 (2C), 129.5, 125.8 (2C), 73.4, 52.2, 45.0, 36.3, 28.8; IR (film)  $\nu_{\max}$  2923, 1748, 1277 cm<sup>-1</sup>; ESI-TOF *m/z* 243.0781 (M + H<sup>+</sup>, C<sub>12</sub>H<sub>15</sub>ClO<sub>3</sub> requires 243.0782); [α]<sub>D</sub> -30 (c 1.0, CHCl<sub>3</sub>). Enantiomeric excess was determined (99% ee) using a Chiralcel AD-H analytical column (250 mm × 4.6 mm, 3% *i*-PrOH/hexane, 1 mL/min, α = 1.07).

### Methyl 4-((1S)-5-Cyano-6-ethoxy-1-hydroxy-6-oxohexyl)benzoate (S-15)

A suspension of NaH (60% dispersion, 1.11 g, 27.7 mmol, 3.0 equiv) in anhydrous DMF (27.7 mL) was treated with ethyl cyanoacetate (2.96 mL, 27.7 mmol, 3.0 equiv) at 0 °C. The reaction mixture was stirred for 30 min while allowing the mixture to warm to room temperature, forming the sodium salt as a clear solution. A solution of *S*-**14** (2.24 g, 9.24 mmol) in DMF (18.5 mL) was added at room temperature and the reaction mixture was stirred at 60 °C for 12 h. The reaction mixture was cooled to room temperature and quenched with the addition of saturated aqueous NH<sub>4</sub>Cl. The mixture was concentrated and then diluted with EtOAc. The organic layer was washed with H<sub>2</sub>O and saturated aqueous NaCl, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. Column chromatography (SiO<sub>2</sub>, 50% EtOAc/hexanes) provided *S*-**15** (1.76 g, 60%) as a yellow oil: <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.04 (d, 2H, *J* = 8.2 Hz), 7.43 (d, 2H, *J* = 8.2 Hz), 4.78 (t, 1H, *J* = 6.0 Hz), 4.26/4.25 (two q, 2H, *J* = 7.1 Hz), 3.92 (s, 3H), 3.49/3.48 (two t, 1H, *J* = 6.9 Hz), 2.00-1.97 (m, 2H), 1.85-1.65 (m, 4H), 1.31/1.30 (two t, 3H, *J* = 7.1 Hz); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 167.0, 166.1, 149.6, 130.0 (2C), 129.6, 125.8 (2C), 116.5, 73.6, 63.0, 52.2, 38.1, 37.6, 29.8, 23.2, 14.1; IR (film)  $\nu_{\max}$  3467, 2923, 1744, 1713, 1282, 1195, 1113 cm<sup>-1</sup>; ESI-TOF *m/z* 342.1309 (M + Na<sup>+</sup>, C<sub>17</sub>H<sub>21</sub>NO<sub>5</sub> requires 342.1312).

### Methyl 4-((1R)-1-(Acetylthio)-5-cyano-6-ethoxy-6-oxohexyl)benzoate (R-16)

A cooled (0 °C) solution of triphenylphosphine (358 mg, 1.36 mmol, 2.0 equiv) in THF (3.4 mL) was treated with DIAD (270 μL, 1.36 mmol, 2.0 equiv). The reaction mixture was stirred at 0 °C for 30 min before a solution of *S*-**15** (218 mg, 0.68 mmol) and thiolacetic acid (121 μL, 1.36 mmol, 2.0 equiv) in THF (1.7 mL) was added dropwise. The mixture was stirred for 1 h, while allowing it to warm to room temperature. The reaction mixture was diluted with EtOAc, washed with saturated aqueous NaHCO<sub>3</sub> (3×), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. Column chromatography (SiO<sub>2</sub>, 30% EtOAc/hexanes) yielded *R*-**16** (246 mg, 95%) as a yellow oil: <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.00 (d, 2H, *J* = 8.3 Hz), 7.36 (d, 2H, *J* = 8.2 Hz), 4.60 (t, 1H, *J* = 7.7 Hz), 4.25/4.24 (two q, 2H, *J* = 7.1 Hz), 3.91 (s, 3H), 3.47-3.43 (m, 1H), 2.31 (s, 3H), 2.02-1.94 (m, 4H), 1.54-1.43 (m, 2H), 1.29/1.28 (two t, 3H, *J* = 7.0 Hz); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 194.4, 166.8, 165.9, 146.6, 130.2 (2C), 129.5, 127.7 (2C), 116.4, 63.0, 52.3, 47.2, 37.4, 35.1, 30.6, 29.4, 24.7, 14.1; IR (film)  $\nu_{\max}$  3733, 2974, 1733, 1641, 1256, 1108 cm<sup>-1</sup>; ESI-TOF *m/z* 400.1191 (M + Na<sup>+</sup>, C<sub>19</sub>H<sub>23</sub>NO<sub>5</sub>S requires 400.1189).

**Methyl 4-((1*R*)-5-Cyano-6-ethoxy-1-mercapto-6-oxohexyl)benzoate (*R*-17)**

A solution of *R*-16 (2.01 g, 5.3 mmol) in EtOH (26.6 mL) was treated with K<sub>2</sub>CO<sub>3</sub> (3.68 g, 26.6 mmol, 5.0 equiv) and stirred for 12 h. The reaction mixture was diluted with EtOAc and washed with H<sub>2</sub>O and saturated aqueous NaCl, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. Column chromatography (SiO<sub>2</sub>, 30% EtOAc/hexanes) yielded the unstable free thiol *R*-17 (291 mg, 16%) as a colorless oil: <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 8.00 (d, 2H, *J* = 7.8 Hz), 7.38 (d, 2H, *J* = 8.2 Hz), 4.26-4.21 (m, 2H), 4.01-3.98 (m, 1H), 3.91 (s, 3H), 3.47-3.43 (m, 1H), 1.99-1.92 (m, 4H), 1.96 (d, 1H, *J* = 5.6 Hz), 1.66-1.58 (m, 1H), 1.49-1.40 (m, 1H), 1.30-1.26 (m, 3H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 166.7, 165.9, 149.4, 130.3 (2C), 129.4, 126.9 (2C), 116.4, 63.0, 52.2, 43.4, 38.6, 37.4, 29.4, 25.2, 14.1; IR (film) ν<sub>max</sub> 2933, 1718, 1436, 1271, 1184, 1102 cm<sup>-1</sup>; ESI-TOF *m/z* 336.1267 (M + H<sup>+</sup>, C<sub>17</sub>H<sub>21</sub>NO<sub>4</sub>S requires 336.1264).

**Methyl 4-((1*R*)-5-cyano-6-ethoxy-1-(methylthio)-6-oxohexyl)benzoate (*R*-18)**

Free thiol *R*-17 (291 mg, 0.87 mmol) dissolved in MeOH (8.68 mL) was treated with MeI (542 μL, 8.68 mmol, 10 equiv) and NaHCO<sub>3</sub> (80 mg, 0.97 mmol, 1.1 equiv) and the mixture was stirred for 3 h at room temperature. The reaction mixture was diluted with EtOAc and washed with H<sub>2</sub>O and saturated aqueous NaCl, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. Column chromatography (SiO<sub>2</sub>, 30% EtOAc/hexanes) provided *R*-18 (238 mg, 79%) as a colorless oil: <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 8.01 (d, 2H, *J* = 7.3 Hz), 7.37 (d, 2H, *J* = 7.8 Hz), 4.26-4.20 (m, 2H), 3.91 (s, 3H), 3.69 (t, 1H, *J* = 7.4 Hz), 3.46-3.41 (m, 1H), 1.99-1.87 (m, 3H), 1.85 (s, 3H), 1.64-1.56 (m, 1H), 1.50-1.45 (m, 1H), 1.31-1.26 (m, 3H); <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>) δ 166.9, 166.0, 147.4, 130.1 (2C), 129.3, 127.9 (2C), 116.4, 63.0, 52.2, 50.9, 37.5, 35.1, 29.6, 25.1, 14.4, 14.1; IR (film) ν<sub>max</sub> 3358, 2923, 1718, 1431, 1267, 1097, 1010 cm<sup>-1</sup>; ESI-TOF *m/z* 372.1252 (M + Na<sup>+</sup>, C<sub>18</sub>H<sub>23</sub>NO<sub>4</sub>S requires 372.1240).

**Methyl (*R*)-4-(4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(methylthio)butyl)benzoate (*R*-19)**

A solution of NaOMe (60 mg, 1.12 mmol, 3.0 equiv) in anhydrous MeOH (3 mL) was treated with guanidine hydrochloride (107 mg, 1.12 mmol, 3.0 equiv) at room temperature. After the reaction mixture was stirred for 30 min, a solution of *R*-18 (130 mg, 0.372 mmol) in MeOH (2.10 mL) was added. The reaction mixture was warmed at reflux for 16 h before being quenched with acetic acid after cooling to room temperature. After concentration, column chromatography (SiO<sub>2</sub>, 10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) gave *R*-19 (39 mg, 29%) as a white solid: mp 180-185 °C; <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 7.96 (d, 2H, *J* = 8.4 Hz), 7.43 (d, 2H, *J* = 8.4 Hz), 3.89 (s, 3H), 3.81 (t, 1H, *J* = 6.7 Hz), 2.29 (t, 2H, *J* = 7.2 Hz), 1.97-1.86 (m, 2H), 1.84 (s, 3H), 1.53-1.47 (m, 1H), 1.42-1.34 (m, 1H); <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD) δ 168.4, 165.2, 164.1, 154.8, 150.2, 130.6 (2C), 129.8, 129.2 (2C), 89.8, 52.6, 52.2, 36.3, 27.2, 23.1, 14.2; IR (film) ν<sub>max</sub> 3353, 2933, 1713, 1610, 1431, 1282, 1108 cm<sup>-1</sup>; ESI-TOF *m/z* 363.1489 (M + H<sup>+</sup>, C<sub>17</sub>H<sub>22</sub>N<sub>4</sub>O<sub>3</sub>S requires 363.1485); [α]<sub>D</sub><sup>20</sup> +70 (c 0.1, MeOH).

**(*R*)-4-(4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(methylthio)butyl) benzoic Acid (*R*-20)**

A solution of *R*-19 (39 mg, 0.11 mmol) in MeOH (1.5 mL) was treated with LiOH monohydrate (23 mg, 0.54 mmol, 5 equiv) in water (840 μL), and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with H<sub>2</sub>O, washed with EtOAc, acidified to pH 4 by the addition of aqueous 1 N HCl, and concentrated. The resulting white solid, *R*-20 (38 mg, 100%), was used directly in the next step: mp 210 °C (dec); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 7.97 (d, 2H, *J* = 8.3 Hz), 7.43 (d, 2H, *J* = 8.4 Hz), 3.81 (t, 1H, *J* = 6.8 Hz), 2.35-2.31 (m, 2H), 1.97-1.87 (m, 2H), 1.85 (s, 3H), 1.55-1.48 (m, 1H), 1.43-1.36 (m, 1H); <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD) δ 169.6, 164.3, 153.4, 152.6, 149.8, 130.8 (2C), 130.5, 129.1 (2C), 89.6,

52.0, 36.2, 26.9, 22.5, 14.3; IR (film)  $\nu_{\max}$  3356, 1569, 1405, 1241  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  349.1336 ( $M + H^+$ ,  $C_{16}H_{20}N_4O_3S$  requires 349.1329);  $[\alpha]_D +30$  ( $c$  0.1, MeOH).

**Di-*tert*-butyl (S)-2-(4-((R)-4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(methylthio)butyl)benzamido)pentanedioate (10R-21)**

A solution of *R*-**20** (38 mg, 0.11 mmol), di-*tert*-butyl L-glutamate hydrochloride (48 mg, 0.16 mmol, 1.5 equiv) and  $\text{NaHCO}_3$  (27 mg, 0.32 mmol, 3.0 equiv) in DMF (1.08 mL) was treated with EDCI (62 mg, 0.32 mmol, 3.0 equiv). The reaction mixture was stirred for 24 h at room temperature before the addition of  $\text{CHCl}_3$ . The resulting solution was washed with saturated aqueous  $\text{NaHCO}_3$  (2 $\times$ ), dried over  $\text{Na}_2\text{SO}_4$ , and concentrated. Column chromatography ( $\text{SiO}_2$ , 10% MeOH/ $\text{CH}_2\text{Cl}_2$ ) provided the protected glutamate intermediate *10R*-**21** (27 mg, 43%) as a white solid: mp 108-112  $^\circ\text{C}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  7.80 (d, 2H,  $J = 8.3$  Hz), 7.43 (d, 2H,  $J = 8.3$  Hz), 4.52-4.49 (m, 1H), 3.81 (t, 1H,  $J = 6.8$  Hz), 2.40 (t, 2H,  $J = 7.4$  Hz), 2.30 (t, 2H,  $J = 7.6$  Hz), 2.24-2.17 (m, 1H), 2.06-1.99 (m, 1H), 1.97-1.86 (m, 2H), 1.84 (s, 3H), 1.53-1.50 (m, 1H), 1.49 (s, 9H), 1.44 (s, 9H), 1.41-1.36 (m, 1H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  173.9, 172.7, 170.2, 165.1, 164.2, 154.8, 148.6, 133.7, 129.2 (2C), 128.6 (2C), 89.8, 83.0, 81.9, 54.5, 52.1, 36.4, 32.9, 28.3 (6C), 27.6, 27.3, 23.1, 14.2; IR (film)  $\nu_{\max}$  2913, 1733, 1631, 1149  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  590.2999 ( $M + H^+$ ,  $C_{29}H_{43}N_5O_6S$  requires 590.3007);  $[\alpha]_D +36$  ( $c$  0.1, MeOH).

**(S)-2-(4-((R)-4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(methylthio)butyl)benzamido)pentanedioic Acid (10R-3)**

A solution of *10R*-**21** (21 mg, 0.035 mmol) in  $\text{CHCl}_3$  (1.26 mL) was treated with trifluoroacetic acid (1 mL) at 0  $^\circ\text{C}$ . The reaction mixture was allowed to warm to room temperature and stirred overnight. The solution was concentrated and triturated with  $\text{Et}_2\text{O}$  to give *10R*-**3** (20 mg, 100%) as a white solid: mp 190  $^\circ\text{C}$  (dec);  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  7.82 (d, 2H,  $J = 8.4$  Hz), 7.42 (d, 2H,  $J = 8.3$  Hz), 4.66-4.63 (m, 1H), 3.81 (t, 1H,  $J = 6.6$  Hz), 2.49 (t, 2H,  $J = 7.5$  Hz), 2.35-2.28 (m, 3H), 2.15-2.07 (m, 1H), 1.98-1.91 (m, 1H), 1.88-1.82 (m, 1H), 1.84 (s, 3H), 1.54-1.47 (m, 1H), 1.42-1.33 (m, 1H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  176.6, 175.1, 170.2, 164.6, 154.8, 153.0, 148.4, 133.8, 129.2, 128.6, 89.6, 53.8, 52.0, 36.3, 31.5, 27.6, 26.9, 22.6, 14.3; IR (film)  $\nu_{\max}$  2964, 1653, 1544, 1210  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  478.1752 ( $M + H^+$ ,  $C_{21}H_{27}N_5O_6S$  requires 478.1755);  $[\alpha]_D +32$  ( $c$  0.1, MeOH). Agilent 1100 LC/MS: reverse phase (2-40% acetonitrile/water/0.1% formic acid, flow rate 0.75 mL/min);  $t_R$ , 12.0 min; purity, 99.2%.

**Methyl (S)-4-(4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-hydroxybutyl)benzoate (S-22)**

A solution of NaOMe (270 mg, 5.01 mmol, 3.0 equiv) in anhydrous MeOH (13.8 mL) was treated with guanidine hydrochloride (478 mg, 5.01 mmol, 3.0 equiv) at room temperature. After the reaction mixture was stirred for 30 min, a solution of *S*-**15** (533 mg, 1.67 mmol) in MeOH (9.4 mL) was added. The reaction mixture was warmed at reflux for 16 h and quenched with acetic acid after cooling to room temperature. After concentration, column chromatography ( $\text{SiO}_2$ , 10% MeOH/ $\text{CH}_2\text{Cl}_2$ ) gave *S*-**22** (336 mg, 61%) as a white solid: mp 120  $^\circ\text{C}$  (dec);  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  7.97 (d, 2H,  $J = 8.4$  Hz), 7.46 (d, 2H,  $J = 8.2$  Hz), 4.73 (t, 1H,  $J = 7.4$  Hz), 3.89 (s, 3H), 2.35-2.32 (m, 2H), 1.77-1.74 (m, 2H), 1.61-1.52 (m, 1H), 1.50-1.42 (m, 1H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  168.0, 164.7, 163.7, 152.1, 152.0, 130.0 (2C), 129.9, 126.6 (2C), 89.5, 74.1, 52.0, 39.1, 24.9, 22.7; IR (film)  $\nu_{\max}$  3333, 2912, 1718, 1615, 1436, 1287, 1102  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  333.1562 ( $M + H^+$ ,  $C_{16}H_{20}N_4O_4$  requires 333.1557);  $[\alpha]_D -8$  ( $c$  0.1, MeOH).



**(S)-4-(4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-hydroxybutyl)benzoic Acid (S-23)**

A solution of *S*-22 (36 mg, 0.11 mmol) in MeOH (1.5 mL) was treated with LiOH monohydrate (23 mg, 0.54 mmol, 5 equiv) in water (840  $\mu$ L), and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with H<sub>2</sub>O, washed with EtOAc, acidified to pH 4 by the addition of aqueous 1 N HCl, and concentrated. The resulting white solid, *S*-23 (35 mg, 100%), was used directly in the next step: mp 220 °C (dec); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD)  $\delta$  7.98 (d, 2H, *J* = 8.3 Hz), 7.45 (d, 2H, *J* = 8.4 Hz), 4.73 (t, 1H, *J* = 7.3 Hz), 2.36 (t, 2H, *J* = 7.4 Hz), 1.78-1.73 (m, 2H), 1.61-1.55 (m, 1H), 1.50-1.44 (m, 1H); <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD)  $\delta$  169.8, 165.2, 163.5, 158.4, 152.2, 130.8 (2C), 130.5, 127.0 (2C), 89.9, 74.6, 39.4, 25.2, 22.8; IR (film)  $\nu_{\max}$  3374, 2923, 1636, 1390 cm<sup>-1</sup>; ESI-TOF *m/z* 319.1405 (M + H<sup>+</sup>, C<sub>15</sub>H<sub>18</sub>N<sub>4</sub>O<sub>4</sub> requires 319.1401); [ $\alpha$ ]<sub>D</sub> -12 (c 0.1, MeOH).

**Dimethyl (S)-2-(4-((S)-4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-hydroxybutyl)benzamido)pentanedioate (10S-24)**

A solution of *S*-23 (29 mg, 0.090 mmol), dimethyl L-glutamate hydrochloride (29 mg, 0.14 mmol, 1.5 equiv) and NaHCO<sub>3</sub> (23 mg, 0.27 mmol, 3.0 equiv) in DMF (0.90 mL) was treated with EDCI (52 mg, 0.27 mmol, 3.0 equiv). The reaction mixture was stirred for 24 h at room temperature before being concentrated. Column chromatography (SiO<sub>2</sub>, 20% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) provided 10S-24 (31 mg, 72%) as a white solid: mp 105-110 °C; <sup>1</sup>H NMR (600 MHz, CD<sub>3</sub>OD)  $\delta$  7.82 (d, 2H, *J* = 8.4 Hz), 7.46 (d, 2H, *J* = 7.8 Hz), 4.73 (t, 1H, *J* = 6.0 Hz), 4.65-4.63 (m, 1H), 3.75 (s, 3H), 3.65 (s, 3H), 2.50 (t, 2H, *J* = 7.2 Hz), 2.35-2.27 (m, 3H), 2.14-2.09 (m, 1H), 1.79-1.74 (m, 2H), 1.57-1.54 (m, 1H), 1.46-1.43 (m, 1H); <sup>13</sup>C NMR (150 MHz, CD<sub>3</sub>OD)  $\delta$  174.9, 173.7, 170.3, 165.2, 164.3, 154.8, 151.2, 133.5, 128.5 (2C), 127.1 (2C), 90.0, 74.7, 60.4, 53.7, 52.8, 52.2, 39.7, 31.3, 27.4, 25.5, 23.3; IR (film)  $\nu_{\max}$  3364, 1723, 1631, 1441 cm<sup>-1</sup>; ESI-TOF *m/z* 476.2142 (M + H<sup>+</sup>, C<sub>22</sub>H<sub>29</sub>N<sub>5</sub>O<sub>7</sub> requires 476.2140); [ $\alpha$ ]<sub>D</sub> -18 (c 0.1, MeOH).

**(S)-2-(4-((S)-4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-hydroxybutyl)benzamido)pentanedioic Acid (10S-7)**

A solution of 10S-24 (26 mg, 0.055 mmol) in MeOH (0.55 mL) was treated with aqueous 1 N NaOH (0.22 mL, 4 equiv). The reaction mixture was stirred for 18 h and then acidified with the addition of Dowex 50X8-200. The solution was filtered and concentrated to give 10S-7 (21 mg, 87%) as a white solid: mp 200 °C (dec); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD)  $\delta$  7.82 (d, 2H, *J* = 8.3 Hz), 7.45 (d, 2H, *J* = 8.3 Hz), 4.72 (t, 1H, *J* = 6.1 Hz), 4.64-4.61 (m, 1H), 2.40-2.24 (m, 5H), 2.17-2.08 (m, 1H), 1.79-1.74 (m, 2H), 1.59-1.54 (m, 1H), 1.49-1.42 (m, 1H); <sup>13</sup>C NMR (150 MHz, CD<sub>3</sub>OD)  $\delta$  176.7, 175.1, 174.3, 170.3, 163.7, 163.6, 151.0, 133.8, 128.5 (2C), 127.1 (2C), 89.9, 74.6, 53.8, 39.4, 35.4, 31.0, 25.2, 23.0; IR (film)  $\nu_{\max}$  3333, 1600, 1451, 1405, 1318, 1108 cm<sup>-1</sup>; ESI-TOF *m/z* 448.1836 (M + H<sup>+</sup>, C<sub>20</sub>H<sub>25</sub>N<sub>5</sub>O<sub>7</sub> requires 448.1827); [ $\alpha$ ]<sub>D</sub> +4 (c 0.1, MeOH). Agilent 1100 LC/MS: reverse phase (2-40% acetonitrile/water/0.1% formic acid, flow rate 0.75 mL/min); *t*<sub>R</sub>, 9.9 min; purity, 99.5%.

**Methyl 4-((1S)-5-Cyano-6-ethoxy-1-methoxy-6-oxohexyl)benzoate (S-25)**

A solution of *S*-15 (246 mg, 0.77 mmol), 49% aqueous fluoroboric acid (97  $\mu$ L, 1.54 mmol, 2 equiv) in CH<sub>2</sub>Cl<sub>2</sub> (3.08 mL) was cooled to 0 °C. TMSCHN<sub>2</sub> (2 M in hexanes, 0.385 mL, 0.77 mmol, 1 equiv) was added to the mixture and stirring was continued at 0 °C. Three further portions of TMSCHN<sub>2</sub> (0.193 mL (0.5 equiv), 0.097 mL (0.25 equiv), and 0.097 mL (0.25 equiv)) were added dropwise at intervals of 20 min. The reaction mixture was stirred a further 30 min, poured into H<sub>2</sub>O and extracted with EtOAc. The organic layer was washed with H<sub>2</sub>O, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. Column chromatography (SiO<sub>2</sub>, 30% EtOAc/hexanes) gave *S*-25 (110 mg, 43%) as a colorless oil: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.04 (d, 2H, *J* = 8.2 Hz), 7.36 (d, 2H, *J* = 8.1 Hz), 4.28-4.22 (m, 2H), 4.18-4.15 (m, 1H), 3.92 (s, 3H),

3.49-3.45 (m, 1H), 3.22 (s, 1.5H), 3.21 (s, 1.5H), 1.98-1.93 (m, 2H), 1.82-1.78 (m, 1H), 1.71-1.63 (m, 2H), 1.54-1.48 (m, 1H), 1.32-1.28 (m, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  166.9, 166.0, 147.3, 129.9 (2C), 129.7, 126.5 (2C), 116.5, 83.1, 62.8, 57.0, 52.1, 37.6, 37.2, 29.7, 23.2, 14.0; IR (film)  $\nu_{\text{max}}$  3405, 1708, 1272, 1103  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  356.1465 ( $\text{M} + \text{Na}^+$ ,  $\text{C}_{18}\text{H}_{23}\text{NO}_5$  requires 356.1468).

**Methyl (S)-4-(4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-methoxybutyl)benzoate (S-26)**

A solution of NaOMe (44 mg, 0.81 mmol, 3.0 equiv) in anhydrous MeOH (2.2 mL) was treated with guanidine hydrochloride (77 mg, 0.81 mmol, 3.0 equiv) at room temperature. After the reaction mixture was stirred for 30 min, a solution of *S*-25 (90 mg, 0.27 mmol) in MeOH (1.53 mL) was added. The reaction mixture was warmed at reflux for 16 h and quenched with acetic acid after cooling to room temperature. After concentration, column chromatography ( $\text{SiO}_2$ , 10% MeOH/ $\text{CH}_2\text{Cl}_2$ ) gave *S*-26 (27 mg, 29%) as a white solid: mp 160-164 °C;  $^1\text{H}$  NMR (600 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  8.00 (d, 2H,  $J = 8.4$  Hz), 7.41 (d, 2H,  $J = 7.8$  Hz), 4.28-4.25 (m, 1H), 3.90 (s, 3H), 3.20 (s, 3H), 2.30 (t, 2H,  $J = 7.5$  Hz), 1.84-1.78 (m, 1H), 1.68-1.62 (m, 1H), 1.56-1.52 (m, 1H), 1.43-1.36 (m, 1H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  168.4, 165.1, 164.0, 154.8, 149.3, 130.7 (2C), 130.4, 127.9 (2C), 89.8, 84.4, 57.1, 52.6, 38.5, 25.4, 23.2; IR (film)  $\nu_{\text{max}}$  3354, 2933, 1610, 1431, 1282  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  347.1729 ( $\text{M} + \text{H}^+$ ,  $\text{C}_{17}\text{H}_{22}\text{N}_4\text{O}_4$  requires 347.1714);  $[\alpha]_{\text{D}} -30$  ( $c$  0.1, MeOH).

**(S)-4-(4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-methoxybutyl)benzoic Acid (S-27)**

The intermediate pyrimidine *S*-26 (30 mg, 0.086 mmol) in MeOH (1.2 mL) was treated with LiOH monohydrate (18 mg, 0.43 mmol, 5 equiv) in water (675  $\mu\text{L}$ ), and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with  $\text{H}_2\text{O}$ , washed with EtOAc, acidified to pH 4 by the addition of 1 N aqueous HCl, and concentrated. The resulting white solid, *S*-27 (29 mg, 100%), was used directly in the next step: mp 175 °C (dec);  $^1\text{H}$  NMR (600 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  7.95 (d, 2H,  $J = 8.2$  Hz), 7.32 (d, 2H,  $J = 8.2$  Hz), 4.24 (t, 1H,  $J = 5.8$  Hz), 3.19 (s, 3H), 2.30 (t, 2H,  $J = 7.5$  Hz), 1.84-1.78 (m, 1H), 1.70-1.63 (m, 1H), 1.56-1.52 (m, 1H), 1.42-1.36 (m, 1H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  173.2, 165.1, 164.0, 154.9, 147.3, 135.4, 130.6 (2C), 127.4 (2C), 90.0, 85.2, 57.0, 38.5, 25.5, 23.3; IR (film)  $\nu_{\text{max}}$  3385, 2923, 1626, 1395, 1246, 1087  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  333.1563 ( $\text{M} + \text{H}^+$ ,  $\text{C}_{16}\text{H}_{20}\text{N}_4\text{O}_4$  requires 333.1557);  $[\alpha]_{\text{D}} -20$  ( $c$  0.1, MeOH).

**Di-tert-butyl (S)-2-(4-((S)-4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-methoxybutyl)benzamido)pentanedioate (10S-28)**

A solution of *S*-27 (29 mg, 0.086 mmol), di-*tert*-butyl *L*-glutamate hydrochloride (38 mg, 0.13 mmol, 1.5 equiv) and  $\text{NaHCO}_3$  (22 mg, 0.26 mmol, 3.0 equiv) in DMF (0.86 mL) was treated with EDCI (50 mg, 0.26 mmol, 3.0 equiv). The reaction mixture was stirred for 24 h at room temperature before the addition of  $\text{CHCl}_3$ . The resulting solution was washed with saturated aqueous  $\text{NaHCO}_3$  (2 $\times$ ), dried over  $\text{Na}_2\text{SO}_4$ , and concentrated. Column chromatography ( $\text{SiO}_2$ , 10% MeOH/ $\text{CH}_2\text{Cl}_2$ ) provided 10*S*-28 (14 mg, 28%) as a white solid: mp 120-125 °C;  $^1\text{H}$  NMR (600 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  7.83 (d, 2H,  $J = 7.8$  Hz), 7.40 (d, 2H,  $J = 7.8$  Hz), 4.51-4.49 (m, 1H), 4.27 (t, 1H,  $J = 7.4$  Hz), 3.21 (s, 3H), 2.40 (t, 2H,  $J = 7.2$  Hz), 2.30 (t, 2H,  $J = 7.2$  Hz), 2.24-2.18 (m, 1H), 2.05-1.99 (m, 1H), 1.85-1.79 (m, 1H), 1.70-1.64 (m, 1H), 1.56-1.52 (m, 1H), 1.49 (s, 9H), 1.44 (s, 9H), 1.41-1.37 (m, 1H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  173.9, 172.6, 170.3, 165.2, 164.3, 154.8, 148.1, 134.4, 128.6 (2C), 127.9 (2C), 90.0, 85.0, 83.0, 81.9, 57.0, 54.5, 38.5, 32.9, 28.3 (6C), 27.5, 25.4, 23.3; IR (film)  $\nu_{\text{max}}$  3344, 2923, 1723, 1626, 1446, 1369, 1149  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  574.3234 ( $\text{M} + \text{H}^+$ ,  $\text{C}_{29}\text{H}_{43}\text{N}_5\text{O}_7$  requires 574.3235);  $[\alpha]_{\text{D}} -48$  ( $c$  0.1, MeOH).

**(S)-2-(4-((S)-4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-methoxybutyl)benzamido)pentanedioic Acid (10S-8)**

The protected glutamate 10S-28 (8 mg, 0.015 mmol) in  $\text{CHCl}_3$  (0.52 mL) was treated with trifluoroacetic acid (0.5 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solution was concentrated and triturated with  $\text{Et}_2\text{O}$  to give 10S-8 (6 mg, 91%) as a white solid: mp 130 °C (dec);  $^1\text{H}$  NMR (600 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  7.84 (d, 2H,  $J = 8.4$  Hz), 7.40 (d, 2H,  $J = 8.4$  Hz), 4.65-4.63 (m, 1H), 4.26 (t, 1H,  $J = 7.2$  Hz), 3.20 (s, 3H), 2.50-2.47 (m, 2H), 2.33-2.30 (m, 3H), 2.14-2.08 (m, 1H), 1.85-1.79 (m, 1H), 1.70-1.64 (m, 1H), 1.58-1.51 (m, 1H), 1.44-1.37 (m, 1H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  176.7, 175.1, 170.2, 165.0, 159.3, 154.0, 147.9, 134.4, 128.6 (2C), 127.9 (2C), 90.0, 85.0, 57.0, 53.8, 38.3, 31.6, 27.6, 25.1, 23.0; IR (film)  $\nu_{\text{max}}$  3354, 2933, 1636, 1426, 1205  $\text{cm}^{-1}$ ; ESI-TOF  $m/z$  462.1997 ( $\text{M} + \text{H}^+$ ,  $\text{C}_{21}\text{H}_{27}\text{N}_5\text{O}_7$  requires 462.1983);  $[\alpha]_{\text{D}} -22$  ( $c$  0.1, MeOH). Agilent 1100 LC/MS: reverse phase (2-40% acetonitrile/water/0.1% formic acid, flow rate 0.75 mL/min);  $t_{\text{R}}$ , 10.9 min; purity, 97.1%.

**Methyl 4-(2-(4-Cyano-5-ethoxy-5-oxopentyl)-1,3-dithian-2-yl)benzoate (29)**

A suspension of NaH (60% dispersion, 9.19 g, 230 mmol, 3.5 equiv) in anhydrous DMF (202 mL) was treated with ethyl cyanoacetate (24.5 mL, 230 mmol, 3.5 equiv) at 0 °C. The reaction mixture was stirred for 30 min while allowing the mixture to warm to room temperature, forming the sodium salt as a clear solution. A solution of **12** (21.7 g, 65.7 mmol) in DMF (122 mL) was added at room temperature and the reaction mixture was stirred at 60 °C for 12 h. The reaction mixture was cooled to room temperature and quenched with the addition of saturated aqueous  $\text{NH}_4\text{Cl}$ . The mixture was concentrated and then diluted with EtOAc. The organic layer was washed with  $\text{H}_2\text{O}$  and saturated aqueous NaCl, dried over  $\text{Na}_2\text{SO}_4$ , and concentrated. Column chromatography ( $\text{SiO}_2$ , 15-50% EtOAc/hexanes gradient) followed by vacuum distillation provided **29** (14.06 g, 53%) as a white solid: mp 54-56 °C;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  8.06 (d, 2H,  $J = 8.6$  Hz), 8.00 (d, 2H,  $J = 8.6$  Hz), 4.21 (q, 2H,  $J = 7.1$  Hz), 3.93 (s, 3H), 3.37 (t, 1H,  $J = 7.6$  Hz), 2.70-2.63 (m, 4H), 2.03 (t, 2H,  $J = 8.3$  Hz), 1.97-1.93 (m, 2H), 1.84-1.81 (m, 2H), 1.57-1.42 (m, 2H), 1.26 (t, 3H,  $J = 7.2$  Hz);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  166.8, 165.9, 130.1 (2C), 129.2, 129.0 (2C), 116.3, 62.9, 58.4, 52.3, 44.2, 37.4, 29.8, 27.8 (2C), 25.0, 21.6, 14.1; IR (film) 2924, 2360, 1719, 1276, 1105, 1017  $\text{cm}^{-1}$ ; ESI-FTMS  $m/z$  408.1292 ( $\text{M} + \text{H}^+$ ,  $\text{C}_{20}\text{H}_{25}\text{NO}_4\text{S}_2$  requires 408.1298).

**Methyl 4-(2-(3-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)propyl)-1,3-dithian-2-yl)benzoate (30)**

A solution of NaOMe (3.26 g, 60.4 mmol, 3.5 equiv) in anhydrous MeOH (166 mL) was treated with guanidine hydrochloride (5.77 g, 60.4 mmol, 3.5 equiv) at room temperature. After the reaction mixture was stirred for 30 min, a solution of **29** (7.03 g, 17.25 mmol) in MeOH (97.5 mL) was added. The reaction mixture was warmed at reflux for 16 h and quenched with acetic acid after cooling to room temperature. After concentration, column chromatography ( $\text{SiO}_2$ , 10% MeOH/ $\text{CH}_2\text{Cl}_2$ ) gave **30** (6.04 g, 83%) as a white solid: mp dec 150 °C;  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  8.00 (s, 4H), 3.91 (s, 3H), 2.74-2.59 (m, 4H), 2.19 (t, 2H,  $J = 7.2$  Hz), 2.10-2.05 (m, 2H), 1.94-1.88 (m, 2H), 1.42-1.36 (m, 2H);  $^{13}\text{C}$  NMR (125 MHz, DMSO)  $\delta$  165.9, 162.3, 161.4, 152.9, 147.7, 129.3 (2C), 128.7 (2C), 128.1, 86.8, 58.1, 52.1, 43.2, 27.0 (2C), 24.6, 22.9, 21.9; IR (film)  $\nu_{\text{max}}$  3361, 1711, 1646, 1435, 1282, 1111  $\text{cm}^{-1}$ ; ESI-FTMS  $m/z$  421.1364 ( $\text{M} + \text{H}^+$ ,  $\text{C}_{19}\text{H}_{24}\text{N}_4\text{O}_3\text{S}_2$  requires 421.1363).

**4-(2-(3-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)propyl)-1,3-dithian-2-yl)benzoic Acid (31)**

A solution of **30** (2.01 g, 4.78 mmol) in MeOH (36 mL) was treated with LiOH monohydrate (1.00 g, 23.9 mmol, 5 equiv) in water (12 mL), and the reaction was stirred at room temperature

for 24 h. The reaction mixture was diluted with H<sub>2</sub>O, washed with Et<sub>2</sub>O, acidified to pH 4 by the addition of aqueous 1 N HCl, and concentrated. The resulting white solid, **31** (1.48 g, 76%), was used directly in the next step: <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 8.04 (d, 4H, *J* = 16.6 Hz), 2.72-2.62 (m, 4H), 2.22 (t, 2H, *J* = 7.3 Hz), 2.07-2.04 (m, 2H), 1.95-1.89 (m, 2H), 1.41-1.38 (m, 2H); <sup>13</sup>C NMR (125 MHz, DMSO) δ 167.8, 167.1, 151.0, 151.4, 147.2, 129.7 (2C), 129.5, 128.6 (2C), 87.2, 58.0, 42.9, 27.2 (2C), 24.7, 22.6, 21.3.

**Di-*tert*-butyl (S)-2-(4-(2-(3-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)propyl)-1,3-dithian-2-yl)benzamido)pentanedioate (32)**

Compound **31** (1.48 g, 3.65 mmol), di-*tert*-butyl L-glutamate hydrochloride (1.62 g, 5.48 mmol, 1.5 equiv) and NaHCO<sub>3</sub> (675 mg, 8.03 mmol, 2.2 equiv) in DMF (32 mL) were treated with EDCI (1.40 g, 7.30 mmol, 2.0 equiv). The reaction mixture was stirred for 24 h at room temperature before the addition of CHCl<sub>3</sub>. The resulting solution was washed with saturated aqueous NaHCO<sub>3</sub> (2×), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. Column chromatography (SiO<sub>2</sub>, 10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) provided **32** (1.76 g, 75%) as a white solid: mp 120-124 °C; <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 8.00 (d, 2H, *J* = 8.5 Hz), 7.84 (d, 2H, *J* = 8.5 Hz), 4.53-4.50 (m, 1H), 2.72-2.62 (m, 4H), 2.41 (t, 2H, *J* = 6.7 Hz), 2.37-2.30 (m, 2H), 2.19 (t, 2H, *J* = 7.5 Hz), 2.10-2.06 (m, 2H), 1.97-1.91 (m, 2H), 1.49 (s, 9H), 1.45 (s, 9H), 1.42-1.36 (m, 2H); <sup>13</sup>C NMR (125 MHz, DMSO) δ 171.5, 171.0, 166.4, 161.4, 157.1, 153.0, 145.5, 132.5, 128.2 (2C), 127.6 (2C), 86.8, 80.6, 79.7, 58.2, 52.5, 43.3, 31.4, 30.9, 27.7 (3C), 27.6 (3C), 27.0, 25.9, 23.0, 22.0; IR (film) ν<sub>max</sub> 3349, 2961, 1728, 1617, 1440, 1364, 1183 cm<sup>-1</sup>; ESI-FTMS *m/z* 648.2890 (M + H<sup>+</sup>, C<sub>31</sub>H<sub>45</sub>N<sub>5</sub>O<sub>6</sub>S<sub>2</sub> requires 648.2884).

**(S)-2-(4-(2-(3-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)propyl)-1,3-dithian-2-yl)benzamido)pentanedioic Acid (9)**

A solution of **32** (54 mg, 0.083 mmol) in CHCl<sub>3</sub> (833 μL) was treated with trifluoroacetic acid (1 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solution was concentrated and triturated with Et<sub>2</sub>O to give **9** (41 mg, 91%) as a white solid: mp dec 125-130 °C; <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 7.98 (d, 2H, *J* = 8.6 Hz), 7.84 (d, 2H, *J* = 8.5 Hz), 4.68-4.65 (m, 1H), 2.72-2.62 (m, 4H), 2.51-2.48 (m, 2H), 2.43-2.39 (m, 2H), 2.19 (t, 2H, *J* = 7.1 Hz), 2.15-2.11 (m, 2H), 1.94-1.98 (m, 2H), 1.40-1.37 (m, 2H); <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD) δ 176.9, 175.8, 170.0, 164.9, 154.0 (2C), 147.7, 133.7, 130.2 (2C), 128.5 (2C), 87.9, 59.5, 53.9, 44.8, 31.1, 28.6 (2C), 27.6, 26.9, 23.2, 22.7; IR (film) ν<sub>max</sub> 3380, 2961, 1718, 1634 cm<sup>-1</sup>; ESI-FTMS *m/z* 536.1625 (M + H<sup>+</sup>, requires C<sub>23</sub>H<sub>29</sub>N<sub>5</sub>O<sub>6</sub>S<sub>2</sub> 536.1632). Agilent 1100 LC/MS: reverse phase (2-40% acetonitrile/water/0.1% formic acid, flow rate 0.75 mL/min); *t<sub>R</sub>*, 13.7 min; purity, 99.5%.

**GAR and AICAR Tfase Assay**

Enzyme assays for rhGAR and rhAICAR were performed as described previously.<sup>8,9</sup> Kinetics of the enzyme reactions were monitored for 2 min after initiation of the reaction. *K<sub>i</sub>*'s of the inhibitors were calculated using Dixon plots.

**Cytotoxic Activity**

The cytotoxic activity of the compounds was measured using the CCRF-CEM human leukemia cell lines as described previously.<sup>8,9,19</sup>

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgement

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## Abbreviations

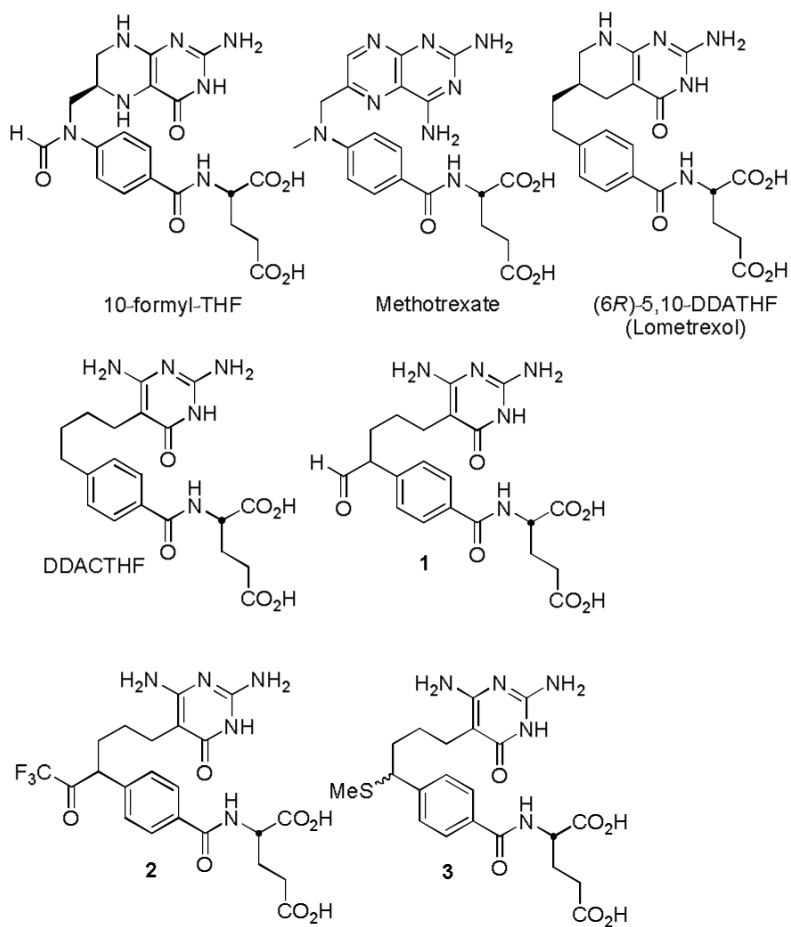
GAR Tfase, glycinamide ribonucleotide transformylase; rhGAR Tfase, recombinant human GAR Tfase; FPGS, folypolyglutamate synthetase; 10-formyl-THF, 10-formyltetrahydrofolic acid; MTX, methotrexate; AICAR, aminoimidazole carboxamide ribonucleotide transformylase; rhAICAR Tfase, recombinant human AICAR Tfase; DDACTHF, dideaza-acyltetrahydrofolic acid.

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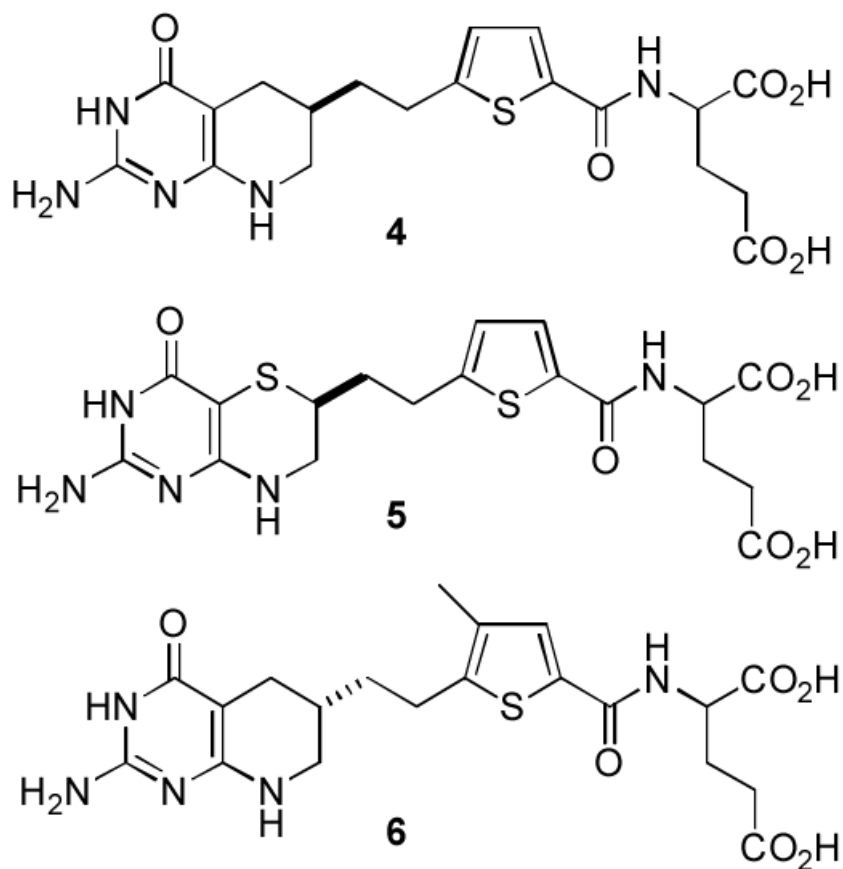
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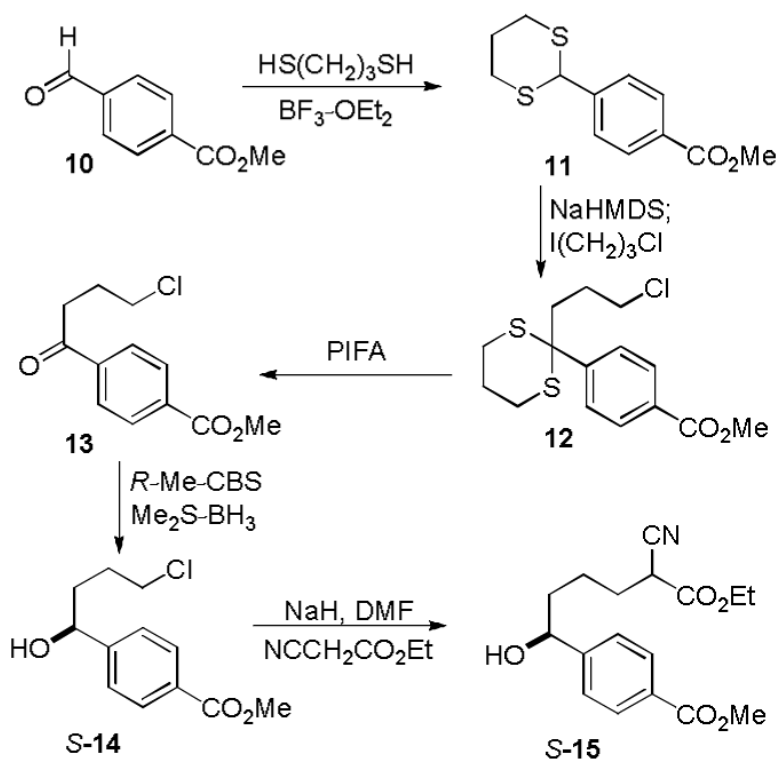
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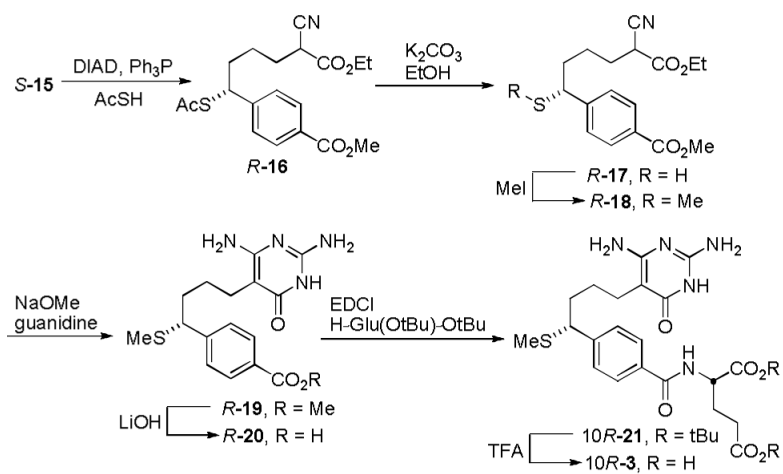
**Figure 1.** Cofactor and representative inhibitors of folate-dependent enzymes.



**Figure 2.**  
Inhibitors of GAR Tfase that contain sulfur atoms.

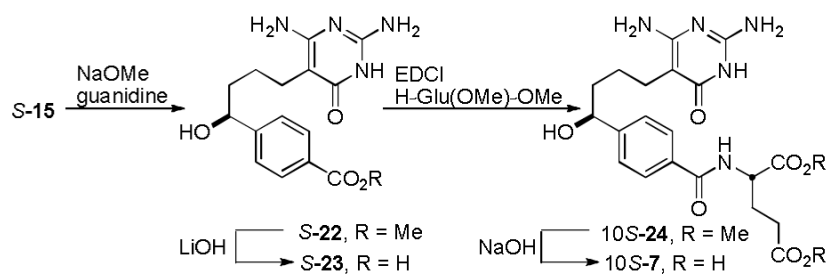


Scheme 1.

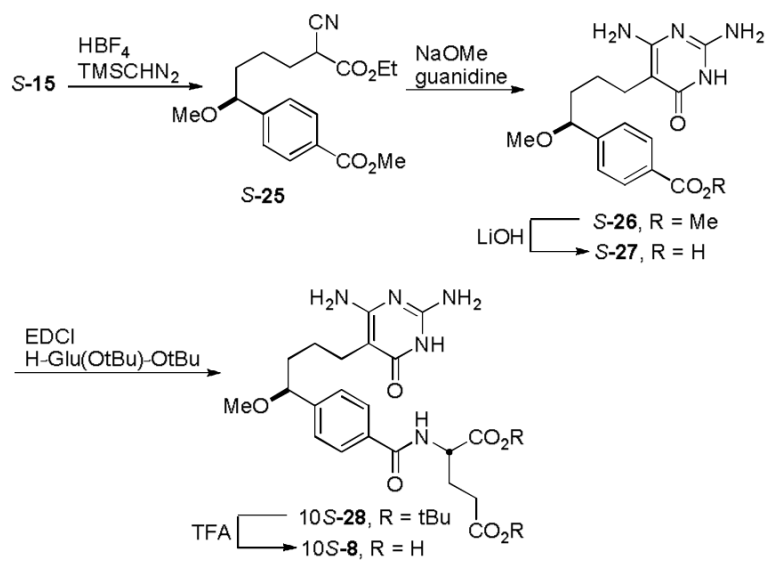


Scheme 2.

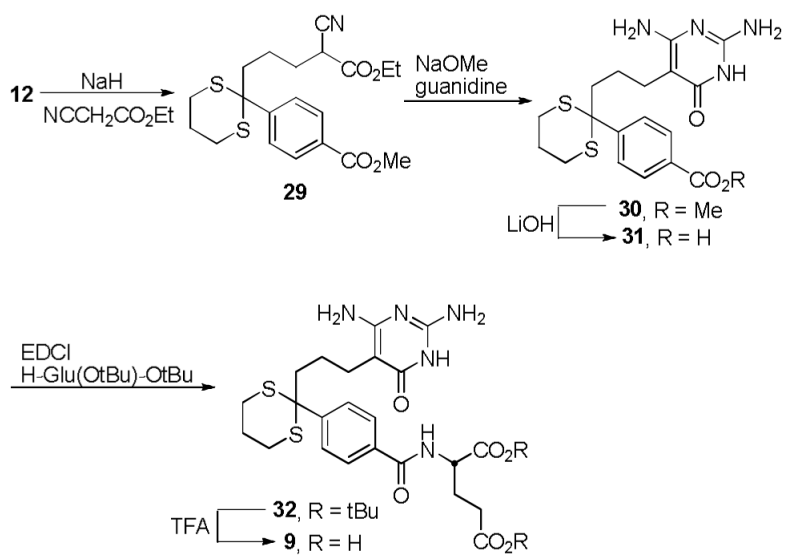




Scheme 3.



Scheme 4.



Scheme 5.

**Table 1**GAR and AICAR Tfase Inhibition ( $K_i$ ,  $\mu\text{M}$ )

Compound	rhGAR Tfase	rhAICAR Tfase
10R-3	0.21	> 100
10S-3	0.18	> 100
10R-7	1.1	> 100
10S-7	1.4	> 100
10R-8	0.85	> 100
10S-8	1.2	> 100
<b>9</b>	1.1	20
DDACTHF	1.7	20
<b>2</b>	0.03	> 100
Lometrexol	0.06 <sup>a</sup>	> 100

<sup>a</sup>Ref 18

**Table 2**

## In vitro Cytotoxic Activity

Compound	CCRF-CEM (IC <sub>50</sub> μM)		
	(-) T, (-) H	(+) T, (-) H	(-) T, (+) H
10R-3	0.08	0.08	>10
10S-3	0.05	0.07	>10
10R-7	2.4	5.7	>10
10S-7	0.5	0.6	>10
10R-8	0.5	0.7	>10
10S-8	0.7	0.9	>10
<b>9</b>	>100	>100	>10
DDACTHF	2.7	3.6	>10
<b>2</b>	0.016	0.017	>10
Lometrexol	0.2	0.2	>10



**Table 3**

## In vitro Cytotoxic Activity in Mutant Cell Lines

Compound	IC <sub>50</sub> , $\mu$ M [(-)T, (-)H]		
	CCRF-CEM	CCRF-CEM/FPGS <sup>b</sup>	CCRF-CEM/MTX
10R-3	0.09	5.5	0.07
10S-3	0.06	5.0	0.06
10R-7	5.50	>10	4.4
10S-7	0.60	>10	0.6
10R-8	0.60	6.0	0.6
10S-8	0.80	>10	0.9
<b>9</b>	>100	nd	nd
DDACTHF	2.7	>10	>100
<b>2</b>	0.06	>10	nd
Lometrexol	0.20	>10	nd (>100) <sup>a</sup>

<sup>a</sup>Ref 18