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Functionalized Analogues of 5,8,10-Trideazafolate: Development of an Enzyme-Assembled Tight Binding Inhibitor of GAR Tfase and a Potential Irreversible Inhibitor of AICAR Tfase

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Abstract—A set of inhibitors **3** and **4** of GAR and AICAR Tfase based on the TDAF core which contain an sp^2 C-10 carbon atom replacing N-10 of the natural cofactor are detailed. Both possess electrophilic olefins and the potential of trapping the reacting amine of the substrates GAR and AICAR by a Michael addition at the enzyme active site to provide an enzyme-assembled tight binding inhibitor. While these agents did not display such characteristics and served as simple competitive inhibitors of GAR Tfase and AICAR Tfase, inhibitor **15** prepared in the conversion of **3** to **4** may provide an enzyme-assembled tight binding inhibitor of GAR Tfase upon reaction with the substrate GAR and may inactivate AICAR Tfase by virtue of alkylation of an active site residue.

Introduction

In the two preceding articles[1] we detailed the preparation and evaluation of 10-formyl-5,8,10-trideazafolate (**2**)[2] and a series of functionalized analogues as potential inhibitors of glycinamide ribonucleotide transformylase (GAR Tfase) and aminoimidazole carboxamide ribonucleotide transformylase (AICAR Tfase), folate-dependent enzymes utilizing **1** as the cofactor which are responsible for the transfer of a formyl group to GAR and AICAR in the de novo synthesis of purines (Fig. 1).[3–18] Here we report the preparation and evaluation of the additional potential inhibitors **3** and **4** derived from further functionalization of this core with removal of the chiral center and the introduction of electrophilic centers potentially capable of trapping the substrate reactive amine by a Michael addition at the enzyme active site providing enzyme-assembled tight binding inhibitors. In this class, the sp^2 nature of C-10 may mimic N-10 of the natural cofactor and, thus, potentially provide insights into the conformational characteristics within the formyl transfer region of the enzymes.

Chemistry

The first of the two inhibitors was prepared by two complementary approaches. The first approach, which anticipated that the quinazolinone C-2 amine might compete with alcohol mesylation and suffer glutamate racemization upon exposure to the strongly basic conditions required of elimination, is summarized in

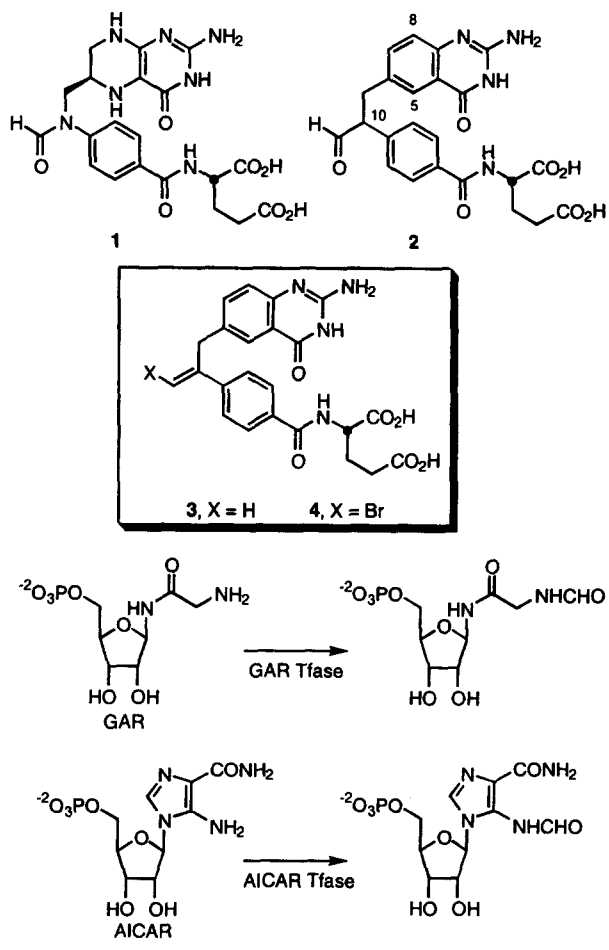


Figure 1.

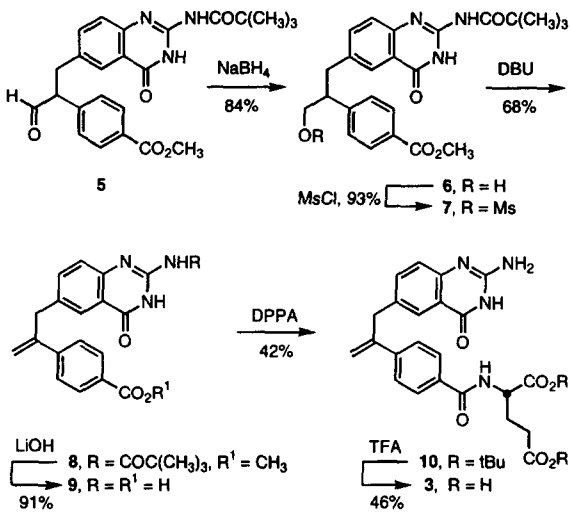
Scheme 1. Reduction of the sensitive aldehyde **5**[1] (0.4 equiv $NaBH_4$, EtOH, 0 °C, 84%) followed by formation of the mesylate **7** (1.8 equiv MsCl, 3.6 equiv Et_3N, CH_2Cl_2, 0 °C, 10–15 min, 94%) and base-catalyzed elimination (5 equiv DBU, DMF, 55 °C, 8 h, 68%) cleanly provided the olefin **8**. Hydrolysis of the methyl ester and concurrent removal of the pivaloylamide (4 equiv LiOH, THF:-CH_3OH:H_2O 3:1:1, 25 °C, 48 h, 91%) followed by coupling with di-*tert*-butyl L-glutamate hydrochloride[19] (1.2 equiv DPPA, 3.1 equiv Et_3N, 10% DMF–THF, 0 °C, 24 h, 42%) and acid-catalyzed deprotection (20% TFA–$CHCl_3$, 0–25 °C, 12 h) of the *tert*-butyl esters of **10** provided **3** (46%).

The inhibitor **3** was also prepared from **11**[1] itself by clean formation of the mesylate **12** (1 equiv MsCl, 1 equiv Et_3N, CH_2Cl_2, 0 °C, 5 min, 72%) and base-catalyzed elimination (5 equiv DBU, DMF, 85 °C, 18 h, 84%) to afford the same key olefin **10** (Scheme 2). Not only did the quinazoline C-2 amine not interfere with the mesylate formation despite the reactivity of the sulfene intermediate, but **10** resulting from base-catalyzed elimination suffered little or no racemization as judged from the optical rotations measured for olefin **10** obtained from the two routes.

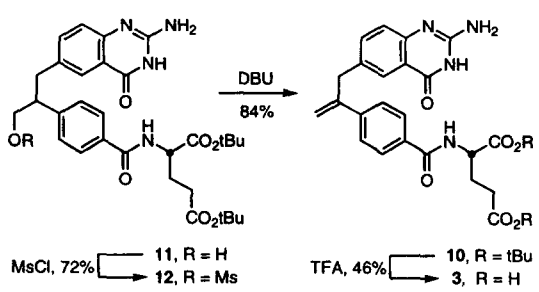
After a number of unsuccessful efforts to directly convert **2** to a range of substituted olefinic analogues of **3**, the vinyl bromide **4** was successfully prepared from **10** by sequential bromination–dehydrobromination (Scheme 3). Although the origin of the unusual and perhaps unexpected regioselectivity of the base-catalyzed elimination of HBr is not clear, the structure of vinyl bromide **14** as well as the olefin **10** were unambiguously established by the observation of several significant NOEs in the ^1H–^1H ROESY NMR. The terminal olefin of **10** was confirmed by the presence of two individual proton signals, H_a and H_b, and two C9 methylene proton signals. Assignment of H_a was based on a strong NOE to olefinic proton H_b as well as to H-7 and to the C9 methylene protons. The assignment of H_b was based on strong NOEs to H_a and to two aromatic protons on the phenyl ring system. A summary of the diagnostic NOEs of **10** may be found in Table 1.

In the DBU promoted elimination of HBr from **13**, two different olefins can be formed. The formation of the terminal olefin was confirmed by the presence of the two methylene protons at C9 and the absence of an NOE from the olefinic proton to the heterocycle. The position of the bromide was determined by the strong NOEs of the olefin proton to an aromatic proton on the phenyl ring. The diagnostic NOEs in of the ^1H–^1H ROESY NMR of **14** are summarized in Table 2.

For a comparison examination that proved more interesting than anticipated, acid-catalyzed deprotection of **13** provided **15** (Scheme 3) for analysis alongside **3** and **4**.



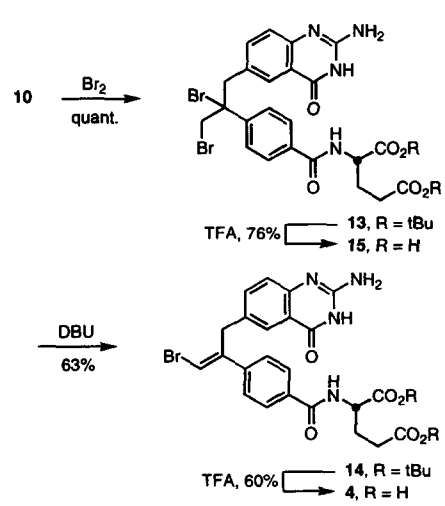
Scheme 1.



Scheme 2.

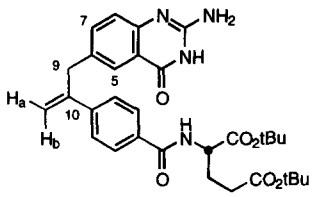
Inhibitor studies

Compounds **3**, **4**, and **15** were tested for the inhibition of GAR Tfase and AICAR Tfase and the results are summarized in Table 3. All three compounds demonstrated binding approximately two orders of magnitude worse than **2** toward GAR Tfase, with a range in K_i of 18–21 μM. Binding of these compounds appears to be quite similar to the binding of the cofactor used in the kinetic studies of GAR Tfase, 10-formyl-5,8-dideazafo-



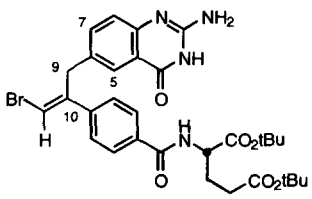
Scheme 3.

Table 1. ^{1}H–^{1}H NOEs observed with **10** (DMF-d_7)



Proton	δ	NOE with	δ
H_a	5.25	H_b	5.64
	5.25	9-CH_2	3.97
	5.25	H-7	7.47
H_b	5.64	H_a	5.25
	5.64	Ar-H	7.64
	5.64	Ar-H	7.90
9-CH_2	3.97	H_a	5.25
	3.97	H-5	7.83
	3.97	H-7	7.47

Table 2. ^{1}H–^{1}H NOEs observed with **14** (DMF-d_7)



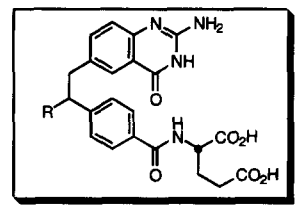
Proton	δ	NOE with	δ
H	7.15	Ar-H	7.60
9-CH_2	4.21	Ar-H	7.60
	4.21	H-5	7.81
	4.21	H-7	7.43

late (K_m = 17 μM) and analogous to the C10 sp^3 inhibitors detailed in the preceding study. Similar results were seen for AICAR Tfase, with a K_i range of 16–35 μM. Thus, the introduction of an sp^2 C10 into the TDAF core did not improve or alter the binding affinity of the inhibitors, although **3** and **4** may mimic the conformational characteristics of the cofactor N-10 more closely than the preceding inhibitors.[1]

One compound, **15** demonstrated time-dependent inhibitory properties. Data from a preliminary study of the time dependence of inhibition toward GAR Tfase are presented in Table 4, and that toward AICAR Tfase are presented in Table 5.

The decrease in activity seen with agent **15** towards GAR Tfase and AICAR Tfase could indicate either the formation of a multisubstrate adduct with the substrates GAR and AICAR, or alternatively, alkylation of an active site residue by **15**. Either of these possibilities would result in the time-dependent decrease in measured activity shown in Tables 4 and 5. If multisubstrate adducts were forming, the resulting adducts should bind much more tightly than **15** itself, leading to

Table 3. GAR and AICAR Tfase inhibition, K_i (μM)



Agent	R	GAR Tfase[a]	AICAR Tfase[b]
2	–CHO	0.26 ± 0.05	7.6 ± 1.5
3	$=CH_2$	21 ± 3	35 ± 10
4	=CHBr	18 ± 3	20 ± 5
15	Br, CH_2Br	20 ± 4	16 ± 4

[a]*pur*N GAR Tfase.
[b]Avian AICAR Tfase.
Preincubation time was 2 min at 26 °C.

Table 4. Time-dependent GAR Tfase inhibition[a]

	Enzyme activity (%)				
Agent	***t* = 3 min**	**15 min**	**90 min**	**3 h**	**6 h**
None	100	100	—	96	92
2	3	3	5	7	9
3	82	88	91	87	76
4	80	83	86	75	68
15	81	80	63	43	21

[a]*pur*N GAR Tfase (2 nM), 10 μM inhibitor.

Table 5. Time-dependent AICAR Tfase inhibition[a]

	Enzyme activity (%)				
Agent	***t* = 3 min**	**15 min**	**90 min**	**3 h**	**6 h**
None	100	—	—	100	100
2	58	—	—	—	46
3	95	—	—	86	99
4	80	—	—	82	82
15	72	80	—	62	62

[a]Avian AICAR Tfase (100 nM), 10 μM inhibitor.

increased levels of inhibition over time as the concentration of the adducts increase. On the other hand, if **15** acts by alkylating the enzymes and either preventing substrate binding or catalysis, the result also would be decreased levels of enzyme activity over time. To distinguish between these possibilities, further studies were undertaken in which the enzymes and **15** were incubated in the presence and absence of substrates GAR and AICAR. These data are presented in Tables 6 and 7 and illustrated in Figure 2.

The data in Table 6 strongly suggest the formation of a multisubstrate adduct between GAR and agent **15**. When **15** and GAR are incubated with GAR Tfase, one observes a time-dependent decrease in activity. After 6 h of incubation with **15** and GAR, only about one fourth of the enzyme activity remains. In contrast, only negligible decreases in enzyme activity are observed

Table 6. Time-dependent GAR Tfase inhibition[a]

Incubation conditions	*t* (min) = 2	30	60	140	220	280	360	460	520	1120	1800	3200	5600
E + GAR (control)	99	105	103	106	99	101	99	100	98	94	85	78	67
E + GAR + **15**	89	81	75	63	49	37	27	18	14	1.7	1.4	0.7	0.18
E + **15**	88	90	94	93	94	94	91	92	90	79	81	62	60
GAR + **15**	90	87	86	82	80	79	78	77	77	64	—	—	38
E + fDDF + **15**	99	96	101	105	109	104	104	106	107	104	108	100	98

[a]2 nM *pur*N GAR Tfase (E), 10 μM inhibitor (**15**), 20 μM fDDF, 50 μM GAR, percent enzyme activity vs time, 2 min preincubation.

Table 7. Time-dependent AICAR Tfase inhibition[a]

Incubation conditions	*t* (min) = 3	180	345	1410	2800	6900
E + AICAR (control)	104	98	102	86	91	91
E + AICAR + **15**	81	50	51	36	26	23
E + **15**	77	78	76	57	4	0

[a]100 nM avian AICAR Tfase (E), 10 μM inhibitor (**15**), 50 μM AICAR, 50 μM fTHF, percent enzyme activity vs time, 2 min preincubation.

when the enzyme and **15** were incubated in the absence of GAR, indicating that the decrease in activity is dependent upon the presence of GAR. A mixture of **15** and GAR that is allowed to incubate for 6 h prior to the addition of enzyme shows only a slight decrease in enzyme activity levels, indicating that while **15** and GAR may be forming an adduct even in the absence of enzyme, this is substantially slower than the enzyme catalyzed rate of adduct formation. When these incubations are allowed to continue for up to 4 days, the data still strongly support adduct formation. After a 4-day incubation of GAR Tfase, GAR, and **15**, only 0.18% of the enzyme activity remains. In contrast, 38% of the enzyme activity remains when enzyme is added to a solution in which **15** and GAR have been allowed to react nonenzymatically for 4 days. Without the presence of GAR in the incubation, a mixture of enzyme and **15** alone still demonstrates 60% activity after a 4-day incubation. This would strongly suggest that the decrease in activity is not due to alkylation of the enzyme by **15** alone. Nearly the same decrease is observed when the enzyme is incubated with GAR over 4 days without the presence of inhibitor. The presence of the 10-formyl-5,8-dideazafolate cofactor prevents this loss of activity. After 4 days of incubation, no significant alterations in activity levels were seen with a mixture of enzyme, inhibitor, and cofactor.

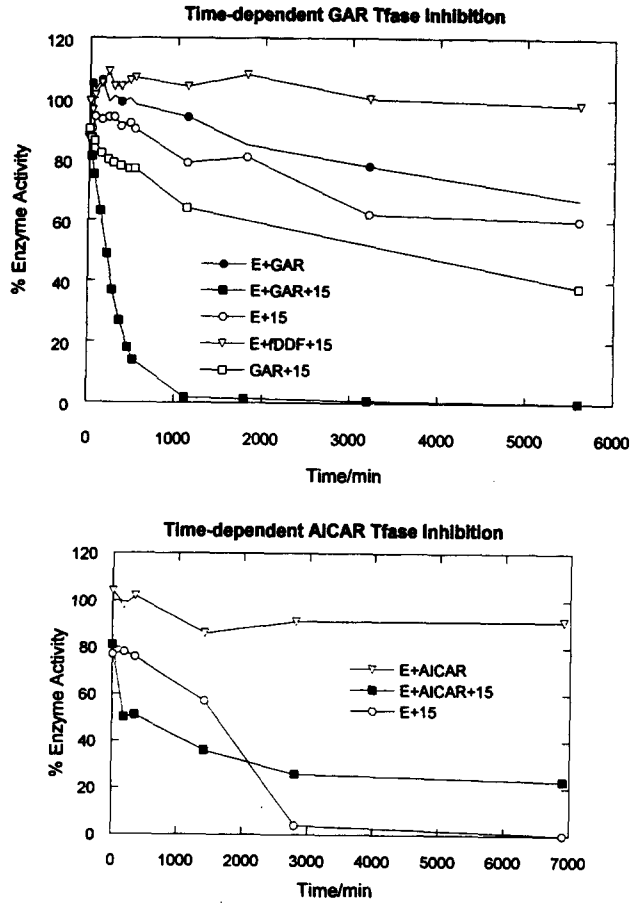
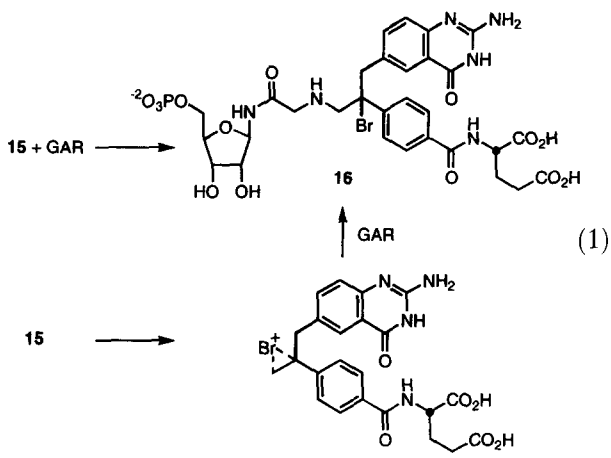


Figure 2.

The time dependence in inhibition observed requires GAR, **15**, and enzyme. Only an insignificant decrease in activity levels is seen if GAR is not present. These results are consistent with, and strongly suggest the formation of a multisubstrate adduct between **15** and GAR when bound by the enzyme.

Although we do not yet have chemical evidence for the nature of the reaction leading to such a multisubstrate adduct, and the direct alkylation leading to **16** could be envisioned, it is also possible that generation of an intermediate bromonium ion via loss of the tertiary bromide may be responsible for this unique behavior of **15** (eq 1).



The data for AICAR Tfase and agent **15** present a very different scenario (Table 7 and Fig. 2). With the AICAR Tfase, if enzyme and **15** are incubated in the absence of substrate AICAR, the measurable activity falls to only 4% within 2 days and is essentially nonexistent after 5 days. If AICAR is present during this incubation, the measurable activity is 26% after 2 days and still 23% even after 5 days. The most likely explanation for these results is that agent **15** is alkylating an active site residue of AICAR Tfase. The presence of substrate AICAR has the effect of protecting the enzyme from attack by **15**, supporting the hypothesis that the enzyme group affected is an active site residue. This makes **15** quite valuable in mapping active site residues of AICAR Tfase in the absence of a crystal structure. After attack by **15**, the enzyme group(s) alkylated could be identified, assisting in the identification of active site residues. Confirmation of the importance of such residues could be accomplished by site-directed mutagenesis, as was previously done for the GAR Tfase prior to the solving of a crystal structure.[5,6]

The reasons are not yet clear why **15** can form a multisubstrate adduct with GAR in GAR Tfase but does not do so with AICAR in AICAR Tfase, and why **15** can serve to alkylate an active site residue of AICAR Tfase but does not do so with GAR Tfase. In addition to the structural differences between the active sites of these two enzymes, the reacting amine of AICAR is much less nucleophilic than that of GAR and would be expected to be less prone to form a multisubstrate adduct.

Cytotoxic activity

The inhibitors were examined for cytotoxic activity both in the presence (+) and absence (−) of added hypoxanthine against both L-1210 and CCRF-CEM cell lines cultured in medium in which purines were removed from the FBS supplement by dialysis (Table 8). All three inhibitors, **3**, **4**, and **15**, exhibited comparable and modest cytotoxic activity with **3** being the most potent in the series. All three exhibited cytotoxic activity at concentrations comparable to that required to inhibit either GAR Tfase or AICAR Tfase. However, none of the agents exhibited a sensitivity to the presence of medium purines suggesting that they are not deriving their biological activity solely from the selective inhibition GAR Tfase or AICAR Tfase and purine synthesis.

Table 8. Cytotoxic activity (IC_{50}, μM)[a]

Agent	L1210[b]	CCRF-CEM[b]
(6*R*)-DDATHF	>225, 0.07	>225, 0.05
Methotrexate	0.05, 0.05	0.06, 0.07
3	16, 12	5, 4
4	30, 30	75, 50
15	50, 40	60, 50

[a]Dialyzed FBS, RPMI-1640 medium.
[b]With + hypoxanthine, − hypoxanthine.

Experimental

Methyl (2*R)-4-[1-Hydroxy-3-(2-trimethylacetimido-3,4-dihydro-4-oxo-quinazolin-6-yl)prop-2-yl]benzoate (6).** A solution of **5**[1] (12.1 mg, 0.03 mmol) in EtOH (278 μL) at 0 °C was treated with $NaBH_4$ (0.4 mg, 0.01 mmol) and the solution was stirred at 0 °C for 5 min before being quenched by the dropwise addition of H_2O. This mixture was poured into H_2O (10 mL). The product was extracted into $CHCl_3$ (3 × 40 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded **6** (10.2 mg, 84%) as a white solid: mp 159–160 °C; ^1H NMR (DMF-d_7, 400 MHz) δ 7.85 (2H, d, J = 8.2 Hz), 7.82 (1H, d, J = 1.9 Hz, H-5), 7.53 (1H, dd, J = 2.1, 8.4 Hz, H-7), 7.42 (2H, d, J = 8.2 Hz), 7.33 (1H, d, J = 8.4 Hz, H-8), 4.88 (1H, t, J = 5.4 Hz, $HOCH_2$), 3.84 (3H, s, OCH_3), 3.75 (2H, t, J = 5.7 Hz, $HOCH_2$), 3.33 (1H, dd, J = 5.6, 13.4 Hz, C*H*H), 3.24 (1H, m, CH), 3.03 (1H, dd, J = 9.0, 13.4 Hz, CH*H*), 1.33 (9H, s, $COC(CH_3)_3$); ^{13}C NMR (DMF-d_7, 100 MHz) δ 167.1, 162.9, 149.5, 138.1, 136.5, 129.7, 129.3, 128.6, 127.8, 126.9, 126.5, 126.1, 126.0, 66.1, 52.2, 51.2, 40.9, 38.0, 26.7, 20.7; IR (neat) ν_{max} 3435, 3216, 1716, 1661, 1626 cm^{-1}; FABHRMS (NBA–NaI) *m/z* 438.2045 (M^+ + Cs, $C_{24}H_{27}N_3O_5$ requires 438.2045).

Methyl (2*R)-4-[1-(Methanesulfonyl)oxy-3-(2-trimethylacetimido-3,4-dihydro-4-oxo-quinazolin-6-yl)-prop-2-yl]benzoate (7).** A solution of **6** (19 mg, 0.04 mmol) in CH_2Cl_2 (400 μL) was treated with Et_3N (22 μL, 0.15 mmol) and methanesulfonyl chloride (6.0 μL, 0.07 mmol). The solution was stirred at 0 °C for 10 min at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 20% hexane–EtOAc) afforded **7** (20.1 mg, 94%) as a yellow solid: mp 173–175 °C, ^1H NMR (DMF-d_7, 400 MHz) δ 7.89 (2H, d, J = 8.3 Hz), 7.85 (1H, d, J = 1.9 Hz, H-5), 7.57 (1H, dd, J = 2.1, 8.4 Hz, H-7), 7.52 (2H, d, J = 8.3 Hz), 7.35 (1H, d, J = 8.4 Hz, H-8), 4.56 (2H, d, J = 7.0 Hz, $MsOCH_2$), 3.85 (3H, s, OCH_3),

3.66 (1H, m, CH), 3.33 (1H, dd, J = 6.2, 13.8 Hz, C*H*H), 3.16 (1H, dd, J = 8.0, 15.2 Hz, CH*H*), 3.16 (3H, s, CH_3SO_2), 1.33 (9H, s, $COC(CH_3)_3$); ^{13}C NMR (DMF-d_7, 100 MHz) δ 182.8, 166.9, 161.0, 148.3, 146.7, 144.8, 137.2, 136.5, 129.9, 129.4, 127.8, 126.8, 126.3, 120.3, 83.9, 73.4, 52.2, 47.3, 42.8, 40.9, 37.7, 36.7, 32.3, 26.7, 20.6; IR (neat) ν_{max} 2960, 2364, 1721, 1668, 1638, 1616 cm^{-1}; FABHRMS (NBA–CsI) *m/z* 648.0793 (M^+ + Cs, $C_{25}H_{29}N_3O_7S$ requires 648.0781).

Methyl 4-[3-(2-Trimethylacetimido-3,4-dihydro-4-oxo-quinazolin-6-yl)prop-1-ene-2-yl]benzoate (8). A solution of **7** (17.6 mg, 0.03 mmol) in DMF (342 μL) was treated with DBU (17 μL, 0.11 mmol) and warmed at 55 °C for 8 h at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 20% hexane–EtOAc) afforded **8** (9.8 mg, 68%) as a white solid: mp 227–229 °C; ^1H NMR (DMF-d_7, 400 MHz) δ 12.20 (1H, br s, NH), 10.98 (1H, br s, NH), 7.96 (1H, d, J = 2.1 Hz, H-5), 7.90 (2H, d, J = 8.6 Hz), 7.71 (2H, d, J = 8.6 Hz). 7.66 (1H, dd, J = 2.3, 8.4 Hz, H-7), 7.40 (1H, d, J = 8.2 Hz, H-8), 5.72 (1H, d, J = 1.1 Hz, HC=C), 5.34 (1H, d, J = 1.2 Hz, HC=C), 4.09 (2H, s, CH_2), 3.85 (3H, s, OCH_3), 1.32 (9H, s, $COC(CH_3)_3$); ^{13}C NMR (DMF-d_7, 100 MHz) δ 166.7, 162.7, 148.3, 146.9, 145.3, 136.9, 136.2, 130.0, 129.6, 129.4, 127.3, 126.8, 126.4, 117.2, 73.4, 52.3, 47.3, 40.7, 37.3, 26.7, 20.7; IR (neat) ν_{max} 3239, 2957, 1654 cm^{-1}; FABHRMS (NBA) *m/z* 420.1930 (M^+ + H, $C_{24}H_{25}N_3O_4$ requires 420.1923).

4-[3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-prop-1-ene-2-yl]benzoic acid (9). A solution of **8** (4.6 mg, 0.01 mmol) in THF:H_2O:CH_3OH (3:1:1, 0.4 M) was treated with aqueous 1 N LiOH (22 μL, 0.02 mmol). The solution was stirred at 25 °C for 24 h. Additional aqueous 1 N LiOH (22 μL, 0.02 mmol) was added and the solution was stirred at 25 °C for an additional 24 h. The mixture was diluted with H_2O (20 mL) and the aqueous layer was washed with EtOAc (3 × 20 mL) and acidified with the addition of 10% aqueous HCl (1 mL, pH 1). The precipitated product was collected by filtration to give **9** (3.2 mg, 91%) as an off-white solid: mp > 250 °C; ^1H NMR (DMF-d_7, 400 MHz) δ 7.92 (2H, d, J = 7.9 Hz), 7.79 (1H, d, J = 1.8 Hz, H-5), 7.45 (2H, d, J = 8.0 Hz), 7.41 (1H, dd, J = 1.7, 8.4 Hz, H-7), 7.24 (2H, br s, NH_2), 7.09 (1H, d, J = 8.4 Hz, H-8), 5.53 (1H, s, HC=C), 5.15 (1H, s, HC=C), 3.91 (2H, s, CH_2); ^{13}C NMR (DMF-d_7, 100 MHz) δ 178.0, 172.0, 162.9, 148.0, 145.8, 138.7, 135.0, 133.0, 131.1, 127.8, 126.3, 125.8, 118.0, 114.7, 49.3, 41.1, 24.6, IR (neat) ν_{max} 3376, 1644, 1573, 1556, 1414 cm^{-1}; Ionspray MS (NBA) *m/z* 322 ($M–H^+$, $C_{18}H_{15}N_3O_3$ requires 322).

Di-*tert*-butyl *N*-[4-{3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-propene-2-yl}benzoyl]-L-glutamate (10). Method A: a slurry of **9** (6.7 mg, 0.02 mmol) and di-*tert*-butyl L-glutamate hydrochloride[19] (7.5 mg, 0.02 mmol) in 10% DMF–THF (2.1 mL) cooled to 0 °C was treated with Et_3N (9 μL, 0.02 mmol) followed by diphenylphosphoryl azide (DPPA, 5.4 μL, 0.02 mmol) and the reaction mixture was stirred at 0 °C for 24 h. The solvent was removed under reduced pressure. PCTLC (1 mm plate, 10% CH_3OH–$CHCl_3$) afforded **10** (5.0 mg, 42%) as a white solid: mp > 300 °C; $[\alpha]^{23}_D$ +2.5 (*c* 0.04, CH_3OH).

Method B: a solution of **12** (7.6 mg, 0.03 mmol) in DMF (115 μL) was treated with DBU (8.6 μL, 0.03 mmol) and warmed at 55 °C for 8 h at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded **10** (5.5 mg, 84%) as a white solid: mp > 300 °C; $[\alpha]^{23}_D$ +2.8 (*c* 0.10, CH_3OH); ^1H NMR (DMF-d_7, 400 MHz) δ 11.00 (1H, br s NH), 7.90 (2H, d, J = 8.4 Hz), 7.83 (1H, s, H-5), 7.64 (2H, d, J = 8.4 Hz), 7.47 (1H, dd, J = 2.0, 8.4 Hz, H-7), 7.13 (1H, d, J = 8.4 Hz, H-8), 6.45 (2H, br s, NH_2), 5.64 (1H, d, J = 1.0 Hz, HC=C), 5.25 (1H, d, J = 1.1 Hz, HC=C), 4.48 (1H, m, $NHCHCO_2C(CH_3)_3$), 3.97 (2H, s, CH_2), 2.41 (2H, t, J = 7.2 Hz, $CH_2CH_2CO_2C(CH_3)_3$), 2.16 (1H, m, $CHHCH_2CO_2C(CH_3)_3$), 2.05 (1H, m, $CHHCH_2CO_2C(CH_3)_3$), 1.41 (9H, s, $CO_2C(CH_3)_3$), 1.39 (9H, s, $CO_2C(CH_3)_3$); ^{13}C NMR (DMF-d_7, 100 MHz) δ 173.9, 172.6, 170.1, 160.6, 147.9, 145.1, 136.6, 135.6, 134.1, 129.4, 128.7, 128.5, 127.5, 127.2, 117.7, 116.6, 82.9, 81.9, 54.5, 41.8, 32.8, 28.2, 27.4; IR (neat) ν_{max} 3355, 2928, 1731, 1646, 1633 cm^{-1}; FABHRMS (NBA) *m/z* 585.2699 (M^+ + Na, $C_{32}H_{38}N_4O_6$ requires 585.2689).

***N*-[4-{3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-prop-1-ene-2-yl}benzoyl]-L-glutamic acid (3)**. A solution of **10** (2.4 mg, 0.004 mmol) in $CHCl_3$ (342 μL) cooled to 0 °C was treated with trifluoroacetic acid (85 μL) and the solution was stirred at 0 °C for 2 h and 25 °C for 12 h. Et_2O (1 mL) was added to the reaction mixture and a white precipitate formed. The precipitate was triturated with Et_2O (3 × 1 mL) and dried in vacuo to give **3**·$CF_3CO_2H_3$ (1.1 mg, 46%) as a white solid: mp > 300 °C ; ^1H NMR (DMF-d_7, 400 MHz) δ 9.13 (1H, br s, NH), 7.92 (2H, d, J = 7.8 Hz), 7.92 (1H, s, H-5), 7.75 (1H, dd, J = 1.7, 8.4 Hz, H-7), 7.66 (2H, d, J = 8.3 Hz), 7.35 (1H, d, J = 8.4 Hz, H-8), 5.68 (1H, s, HC=C), 5.30 (1H, s, HC=C), 4.60 (1H, m, $NHCHCO_2H$), 2.49 (2H, t, J = 7.4 Hz, $CH_2CH_2CO_2H$), 2.23 (1H, m, $CHHCH_2CO_2H$), 2.08 (1H, m, $CHHCH_2CO_2H$); IR (neat) ν_{max} 3431, 2958, 1702, 1684 cm^{-1}; FABHRMS (NBA) *m/z* 451.1625 (M^+ + Cs, $C_{23}H_{22}N_4O_6$ requires 451.1618).

Di-*tert*-butyl *N*-[4-{3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(methanesulfonyl)oxy-prop-2-yl}benzoyl]-L-glutamate (12). A solution of **11**[1] (7 mg, 0.012 mmol) in CH_2Cl_2 (60 μL) at 0 °C was treated with Et_3N (1.7 μL, 0.012 mmol) and methanesulfonyl chloride (1 μL, 0.012 mmol) and the solution was stirred at 0 °C for 10 min at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded **12** (5.7 mg, 72%) as a yellow solid: mp > 300 °C; ^1H NMR (CD_3OD, 400 MHz) δ 7.74 (2H, d, J = 8.3 Hz), 7.74 (1H, d, J = 2.3 Hz, H-5), 7.36 (1H, dd, J = 2.0, 8.4 Hz, H-7), 7.34 (2H, d, J = 8.3 Hz), 7.10 (1H, d, J = 8.4 Hz,

H-8), 4.45 (2H, d, J = 10.0 Hz, MsOCH_2), 4.45 (1H, m, NHCHCO$_2$C(CH$_3$)$_3$), 3.47 (1H, m, CH), 3.24 (1H, dd, J = 5.9, 13.9 Hz, CHH), 3.03 (1H, dd, J = 9.1, 13.9 Hz, CHH), 2.94 (3H, s, CH_3SO_2), 2.36 (2H, t, J = 7.2 Hz, $CH_2CH_2CO_2C(CH_3)_3$), 2.16 (1H, m, CHHCH$_2$CO$_2$C(CH$_3$)$_3$), 1.99 (1H, m, CHHCH$_2$CO$_2$C(CH$_3$)$_3$), 1.41 (9H, s, $CO_2C(CH_3)_3$), 1.42 (9H, s, $CO_2C(CH_3)_3$); ^{13}C NMR (CD_3OD, 100 MHz) δ 172.5, 172.0, 167.2, 162.9, 152.5, 151.2, 151.1, 145.0, 135.8, 133.6, 132.9, 128.9, 128.2, 126.5, 125.1, 118.0, 81.3, 80.4, 73.6, 53.6, 47.3, 37.8, 36.7, 32.2, 28.0, 27.0; IR (neat) ν_{max} 2979, 2928, 1731, 1651, 1567 cm^{-1}; FABHRMS (NBA) m/z 791.1738 (M^+ + Cs, $C_{32}H_{42}N_4O_9S$ requires 791.1727).

Di-*tert*-butyl (2*RS*)-*N*-[4-{3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1,2-dibromo-prop-2-yl}benzoyl]-L-glutamate (13). A solution of **10** (8.9 mg, 0.02 mmol) in CCl_4 (300 μL) at 0 °C was treated with bromine (3.3 μL, 0.063 mmol) in CCl_4 (126 μL) and the solution was stirred at 0 °C for 30 min before being quenched by the dropwise addition of aqueous sodium bisulfite (10 mL). The solution was extracted with EtOAc (3 × 10 mL), dried (Na_2SO_4), filtered, and the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded **13** (12 mg, quant.) as a yellow solid: mp > 300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 8.07 (1H, d, J = 2.0 Hz, H-5), 8.05 (2H, d, J = 8.5 Hz), 7.91 (2H, d, J = 8.5 Hz), 7.50 (1H, dd, J = 1.8, 8.4 Hz, H-7), 7.16 (1H, d, J = 4.8 Hz, H-8), 6.70 (2H, br s, NH_2), 4.62 (1H, d, J = 10.8 Hz, CHHBr), 4.52 (1H, m, NHCHCO$_2$C(CH$_3$)$_3$), 4.16 (1H, d, J = 10.8 Hz, CHHBr), 3.92 (1H, d, J = 14.5 Hz, CHHCBr), 3.81 (1H, d, J = 14.5 Hz, CHHCBr), 2.40 (2H, t, J = 7.4 Hz, $CH_2CH_2CO_2C(CH_3)_3$), 2.17 (1H, m, CHHCH$_2$CO$_2$C(CH$_3$)$_3$), 2.05 (1H, m, CHHCH$_2$CO$_2$C(CH$_3$)$_3$), 1.44 (9H, s, $CO_2C(CH_3)_3$), 1.41 (9H, s, $CO_2C(CH_3)_3$); IR (neat) ν_{max} 3338, 3151, 2858, 1728, 1607 cm^{-1}; FABHRMS (NBA) m/z 853.0235 (M^+ + Cs, $C_{31}H_{38}N_4O_6Br_2$ requires 853.0212).

Di-*tert*-butyl *N*-[4-{3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-bromo-1-propene-2-yl}benzoyl]-L-glutamate (14). A solution of **13** (6.6 mg, 0.01 mmol) in DMF (114 μL) was treated with DBU (5.6 μL, 0.04 mmol) and warmed at 40 °C for 8 h at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 20% hexane–EtOAc) afforded **14** (3.6 mg, 63%) as a yellow oil: ^{1}H NMR (DMF-d_7, 400 MHz) δ 7.89 (2H, d, J = 8.5 Hz), 7.81 (1H, d, J = 2.0 Hz, H-5), 7.60 (2H, d, J = 8.5 Hz), 7.43 (1H, dd, J = 2.2, 8.4 Hz, H-7), 7.35 (1H, d, J = 8.4 Hz, H-8), 7.15 (1H, s, HC=C), 6.43 (2H, br s, NH_2), 4.48 (1H, m, NHCHCO$_2$C(CH$_3$)$_3$), 4.21 (2H, s, CH_2), 2.41 (2H, t, J = 7.6 Hz, $CH_2CH_2CO_2C(CH_3)_3$), 2.13 (1H, m, CHHCH$_2$CO$_2$C(CH$_3$)$_3$), 2.01 (1H, m, CHHCH$_2$CO$_2$C(CH$_3$)$_3$), 1.41 (9H, s, $CO_2C(CH_3)_3$), 1.38 (9H, s, $CO_2C(CH_3)_3$); IR (neat) ν_{max} 3320, 2971, 2972, 1721, 1480 cm^{-1}; FABHRMS (NBA) m/z 663.1780/665 (M^+ + Na, $C_{31}H_{37}N_4O_6Br$ requires 663.1794/665).

***N*-[4-{3-(2-2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(bromo)prop-1-ene-2-yl}benzoyl]-L-glutamic acid (4).** A solution of **14** (1.5 mg, 0.002 mmol) in $CHCl_3$ (187 μL) cooled to 0 °C was treated with trifluoroacetic acid (47 μL) and the solution was stirred at 0 °C for 2 h and 25 °C for 12 h. Et_2O (1 mL) was added to the reaction mixture and a white precipitate formed. The precipitate was triturated with Et_2O (3 × 1 mL) and dried in vacuo to give **4**·CF_3CO_2H (0.9 mg, 60%) as a white solid: mp > 300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 7.93 (2H, d, J = 8.5 Hz), 7.87 (1H, s, H-5), 7.62 (2H, d, J = 8.5 Hz), 7.38 (1H, d, J = 8.5 Hz, H-7), 7.25 (1H, d, J = 8.6 Hz, H-8), 7.19 (1H, s, HC=C), 4.60 (1H, m, NHCHCO$_2$H), 4.27 (2H, s, CH_2), 2.49 (2H, t, J = 7.4 Hz, $CH_2CH_2CO_2H$), 2.24 (1H, m, CHHCH$_2$CO$_2$H), 2.09 (1H, m, CHHCH$_2$CO$_2$H); IR (neat) ν_{max} 3417, 2945, 1695, 1651 cm^{-1}; Ionspray MS (NBA) m/z 527/529 ($M - H^+$, $C_{23}H_{21}N_4O_6Br$ requires 527/529).

(2*RS*)-*N*-[4-{3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1,2-dibromo-prop-2-yl}benzoyl]-L-glutamic acid (15). A solution of **13** (3.0 mg, 0.004 mmol) in $CHCl_3$ (332 μL) cooled to 0 °C was treated with trifluoroacetic acid (83 μL) and the solution was stirred at 0 °C for 2 h and 25 °C for 12 h. Et_2O (1 mL) was added to the reaction mixture and a white precipitate formed. The precipitate was triturated with Et_2O (3 × 1 mL) and dried in vacuo to give **15**·CF_3CO_2H (2.3 mg, 76%) as a white solid: mp > 300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 9.04 (1H, br s, NH), 8.14 (1H, d, J = 1.9 Hz, H-5), 8.05 (2H, d, J = 8.6 Hz), 7.92 (2H, d, J = 8.6 Hz), 7.73 (1H, d, J = 9.0 Hz, H-7), 7.35 (1H, d, J = 8.4 Hz, H-8), 4.66 (1H, m, NHCHCO$_2$H), 4.64 (1H, d, J = 11.5 Hz, CHHBr), 4.15 (1H, d, J = 11.0 Hz, CHHBr), 4.01 (1H, d, J = 14.5 Hz, CHHCBr), 3.89 (1H, d, J = 14.5 Hz, CHHCBr), 2.54 (2H, t, J = 7.5 Hz, $CH_2CH_2CO_2H$), 2.27 (1H, m, CHHCH$_2$CO$_2$H), 2.21 (1H, m, CHHCH$_2$CO$_2$H); IR (neat) ν_{max} 3415, 2925, 2846 cm^{-1}; Ionspray MS (NBA) m/z 607/609/611 ($M–H^+$, $C_{23}H_{22}N_4O_6Br_2$ requires 607/609/611).

GAR and AICAR Tfase inhibition

The evaluation of **3**, **4**, and **15** was conducted as detailed in the accompanying article.[1]

Cytotoxicity testing

The cytotoxic activity of **3**, **4**, and **15** was determined following protocols described in detail.[1]

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