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Functionalized Analogues of 5,8,10-Trideazafolate as Potential Inhibitors of GAR Tfase or AICAR Tfase

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Abstract—A series of TDAF-based analogues of 10-formyl-tetrahydrofolic acid are examined in efforts to explore the formyl transfer region of GAR Tfase and AICAR Tfase. © 1997 Elsevier Science Ltd.

Introduction

In the preceding article,[1] we detailed the preparation and evaluation of 10-formyl-5,8,10-trideazafolate (**2**, 10-formyl-TDAF)[2] as a potent inhibitor of glycinamide ribonucleotide transformylase (GAR Tfase, K_i = 0.26 μM) 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 (Figure 1).[3–18] Herein we report the preparation and evaluation of the additional series of potential GAR and AICAR Tfase inhibitors **3–11** derived from further functionalization of this core inhibitor. The inhibitors **3–11** constitute the introduction of the first of two[19] fundamentally different types of electrophilic centers with the intention of establishing whether they might serve to capture the substrate primary amine at the enzyme active site and thus serve as enzyme-assembled tight binding inhibitors of either GAR Tfase or AICAR Tfase.[20] In this first set of inhibitors, the primary alcohol **3** derived from the reduction of **2** was further functionalized with acyl groups bearing electrophilic carbonyls incapable of transfer to the substrates or which would do so less effectively than the natural cofactor. The potential inhibitor **11** could serve as a chemically stable precursor to the relatively unstable aldehyde **2** with in vitro/in vivo release, as an effective competitive inhibitor in its own right by virtue of mimicking the formyl transfer tetrahedral intermediate, or as a suicide cofactor analogue by virtue of active site oxonium ion generation and subsequent trap by the enzyme-bound substrate or active site nucleophiles (eq 1). Inhibitor **9** constitutes a cofactor analogue incorporating a reactive D-bromoacetate capable of electrophilic trap of the substrate amines and inhibitor **10** incorporates the glycinamide side-chain of the substrate GAR.

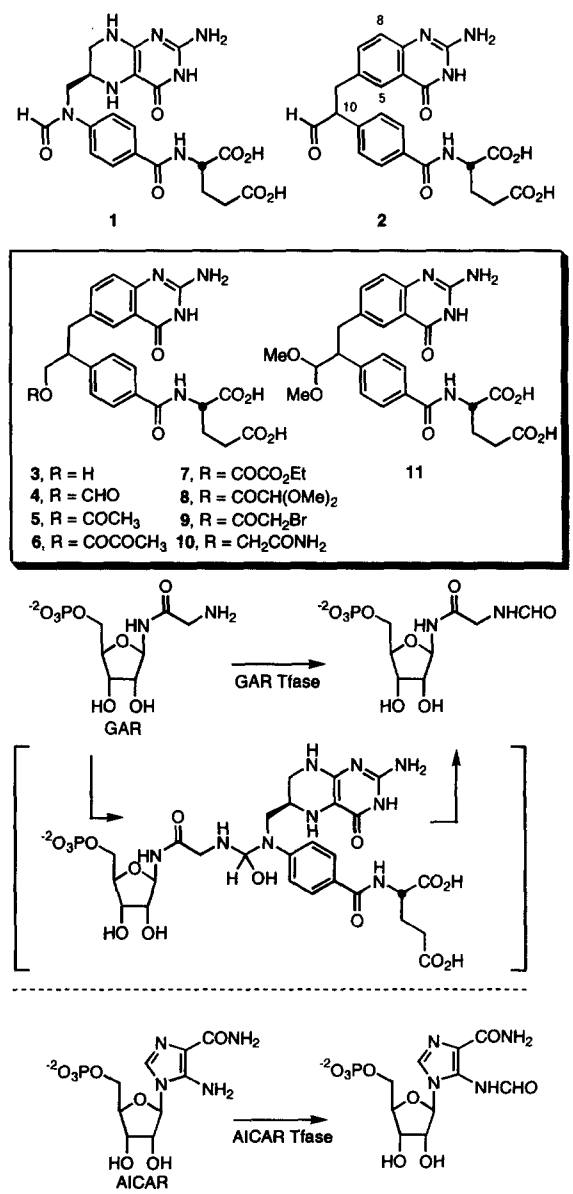
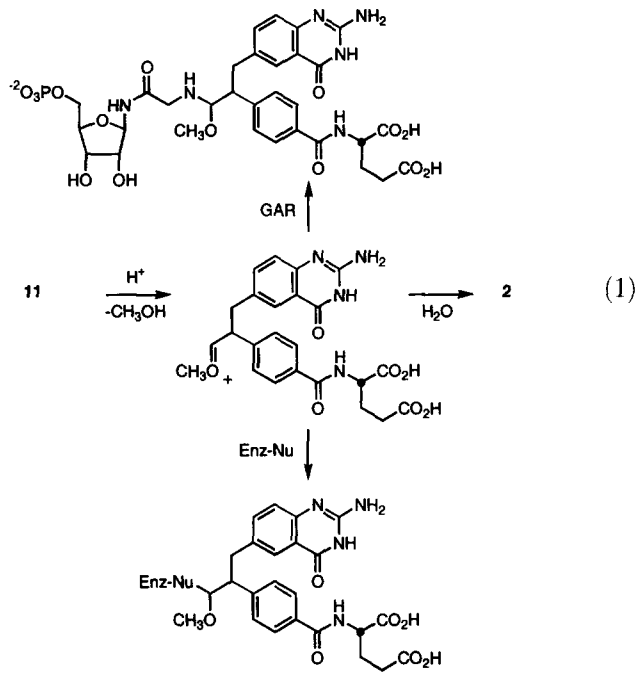
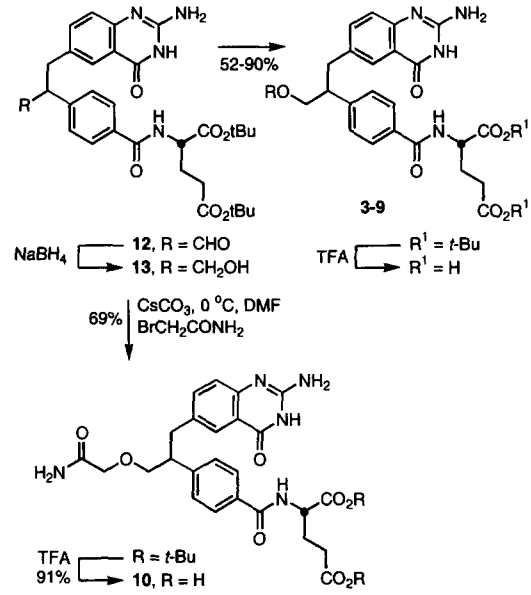
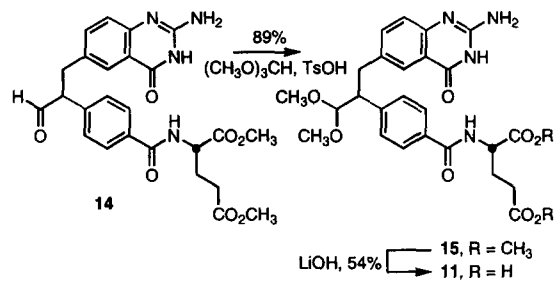


Figure 1.





Scheme 1.



Scheme 2.

Chemistry

The potential inhibitors **3–10** were prepared as a mixture of diastereomers by reduction (0.4 equiv $NaBH_4$, EtOH, 0 °C, 10 min) of **12**,[1] the immediate precursor to **2**, followed by acylation (**4–9**, 52–90%) or alkylation (**10**; 1 equiv $CsCO_3$, DMF, 0 °C, 18 h, 69%) of the resulting alcohol **13** and subsequent acid-catalyzed deprotection of the *tert*-butyl esters (TFA, $CHCl_3$, 0–25 °C, 18 h, 53–91%; Scheme 1).

That acylation occurred on the primary alcohol and not the quinazolinone C2-amine was established by the ^1H NMR spectroscopic properties of **4–9** in which the chemical shift of the alcohol α-CH_2 underwent a diagnostic 0.55–0.82 ppm downfield shift on acylation of **13**. Similarly, alkylation of **13** with α-bromoacetamide did not alter the C2 amine (2H, 6.77 ppm) establishing alkylation of the primary alcohol. Although this was not pursued exhaustively, efforts to cleave the dimethyl acetal **8** to provide the corresponding aldehyde were not successful. Treatment with TFA simply removed the *tert*-butyl esters but did not cleave the dimethyl acetal. In addition, the α-bromoacetate **9** proved sensitive to nucleophilic displacement by other halides[21] establishing its electrophilic character.

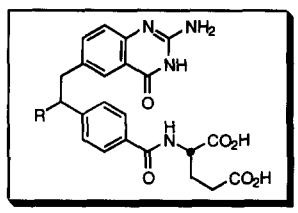
The acetal **11** was prepared as a mixture of diastereomers from **14**[1] by acid-catalyzed dimethyl acetal formation (TsOH, $(CH_3O)_3CH$, 25 °C, 20 min, 89%) followed by base-catalyzed removal of the methyl esters (4 equiv LiOH, THF–H_2O–CH_3OH, 25 °C, 48 h, 54%; Scheme 2).

Enzyme inhibition studies

Compounds **2–11** were tested for inhibition of GAR Tfase and AICAR Tfase and the results are presented in Table 1. As described in the preceding paper,[1] compound **2** exhibits potent inhibition of GAR Tfase, with K_i = 0.26 ± 0.05 μM. Substitution of the aldehyde group in **2** by alcohol **3** results in the loss of approximately one order of magnitude of binding affinity, with K_i = 3.1 ± 0.8 μM (Table 1). These compounds serve as a basis for extending this substituent group, resulting in compounds **4–11**. Agents **4–10** are all carbonyl-based electrophilic compounds designed to react with the substrate GAR and AICAR at the enzyme active sites with the resulting adducts potentially serving as tight binding inhibitors.

Agents **4**, **5**, **6**, **8**, **9**, and **11** all demonstrate approximately the same binding to GAR Tfase, with a K_i range of 17–35 μM. This is comparable to the binding of the cofactor used in the kinetic studies of GAR Tfase, 10-formyl-5,8-dideazafolate (K_m = 17 μM) and is one to two orders of magnitude lower than agents **2** and **3**. This would appear to indicate that the side-chains of these agents have no positive effect on binding and that they may have lost key contacts used by **2** and **3** for tight binding. It is interesting that **10**, which incorporates the acetamide side-chain of GAR into the inhibitor structure, shows essentially no inhibition. Similarly, **11**

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



Agent	GAR Tfase, K_i	AICAR Tfase, K_i	R
2	0.26 ± 0.05 μM	7.6 ± 1.5 μM	CHO
3	3.10 ± 0.81 μM	30 ± 7 μM	CH_2OH
4	19 ± 2 μM	70 ± 20 μM	CH_2OCHO
5	31 ± 4 μM	50 ± 10 μM	CH_2OCOCH_3
6	17 ± 4 μM	6.5 ± 1.7 μM	$CH_2OCOCOCH_3$
7	5.81 ± 1.5 μM	26 ± 6 μM	CH_2OCOCO_2Et
8	21 ± 3 μM	60 ± 20 μM	$CH_2OCOCH(OMe)_2$
9	35 ± 15 μM	22 ± 5 μM	CH_2OCOCH_2Br
10	>100 μM	23 ± 7 μM	$CH_2OCH_2CONH_2$
11	34 ± 6 μM	35 ± 10 μM	$CH(OMe)_2$

[a]*pur*N GAR Tfase, avian AICAR Tfase.

proved to be approximately 100 times less effective than **2**, and 10 times less potent than **3** indicating that even small perturbations in the inhibitor structure at this position can have a profound effect on potency. The only compound of this series to show comparable binding to **3** is **7**, with K_i = 5.8 ± 1.5 μM, and this agent was still 20 times less potent than **2**. Thus, it would appear that the disruptions to the formyl oxygen in **2** described here results in significantly weaker binding to GAR Tfase. Substitution of an alcohol (**3**) in place of the aldehyde (**2**) results in the loss of one order of magnitude in binding affinity, while conversion of this aldehyde to an ether, ester, or acetal causes the loss of two orders of magnitude or more in binding affinity.

For AICAR Tfase, agent **6** shows the tightest binding, with K_i = 6.5 ± 1.7 μM. This value compares quite favorably with the K_i = 7.6 ± 1.5 determined for agent **2**. All of the other compounds **3–5**, **7–11** show four- to 10-fold weaker binding, with a range in K_i from 22–70 μM (Table 1).

Time-dependent inhibition (adduct formation)

Compounds **2–11** were tested for the possibility of enzyme-assembled stable adduct formation with the substrates of GAR Tfase and AICAR Tfase. Data for the time dependence of inhibition are presented in Table 2 for GAR Tfase and Table 3 for AICAR Tfase. None of these compounds showed any changes in inhibition levels over time for AICAR Tfase that would indicate the possibility of the formation of an adduct. In the case of GAR Tfase, only **9** showed any significant changes over time. The decrease in activity seen with **9** could indicate either the formation of a multisubstrate adduct with substrate GAR, or alkylation of an active site residue by **9**. Either of these possibilities would result in the time-dependent decrease in measured activity. If a multisubstrate adduct were forming, the resulting adduct should bind much more tightly than **9**, leading to increased levels of inhibition over time as the concentration of the adduct increases. On the other hand, if **9** acts by alkylating the enzyme and either preventing substrate binding or catalysis, the result also would be decreased levels of enzyme activity over time. The corresponding bromoacetyl derivative of 5,8-dideazafolate acts both as an active site directed suicide inhibitor, alkylating Asp-144 in the absence of substrate GAR, and as a component of a multisubstrate adduct that forms in the presence of GAR whose K_i is in the picomolar range.[23] The time-dependent inhibition demonstrated by **9** indicates that at least one of these possibilities is also occurring. It is quite possible that both adduct formation and active site alkylation are occurring and contributing to the decrease in activity observed over time.

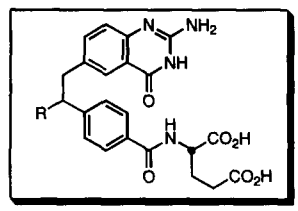
Cytotoxic activity

The results of the testing for cytotoxic activity of **3–11** are summarized in Table 4. From these comparisons, several important conclusions may be drawn about the behavior of **2–11**. With the exception of **3**, which exhibited a modest sensitivity to the presence of medium purines in the CCRF-CEM cell line, the cytotoxic activity of each of the agents was comparable whether the test was conducted in the presence or absence of medium purine (hypoxanthine). Thus, although the cytotoxic potencies of the agents **4–11** are roughly of the same order of magnitude as the GAR or AICAR K_i values, the observation that the cytotoxic activity is relatively insensitive to the presence or absence of purine in the medium indicates that the activity of the agents is not derived selectively from inhibition of purine synthesis and, thus, not derived uniquely from inhibition of GAR or AICAR Tfase. This is especially interesting in light of the comparatively successful and analogous studies with 5-DACTHF.[20]

Experimental

(2*RS*)-*N*-[4-{3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(hydroxy)prop-2-yl}benzoyl]-L-glutamic acid (3). A solution of crude **12** (54.4 mg, 0.09 mmol) in EtOH (0.94 mL) at 0 °C was treated with $NaBH_4$ (2.0 mg, 0.05 mmol) and the mixture was stirred at 0 °C for 10 min before being quenched by the dropwise addition of H_2O. The mixture was poured onto H_2O (30 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 **13** (22.6 mg, 41% overall) as a white solid: mp 119–121 °C; ^1H NMR (CD_3OD, 400 MHz) δ 7.73 (1H, d, *J* = 1.6 Hz, H-5), 7.70 (2H, d, *J* = 8.3 Hz), 7.35 (1H, dd, *J* = 1.9, 8.4 Hz, H-7), 7.27 (2H, d, *J* = 8.3 Hz), 7.09 (1H, d, *J* = 8.4 Hz, H-8), 4.44 (1H, m, $NHCHCO_2C(CH_3)_3$), 3.78 (1H, d, *J* = 6.5 Hz, CH_2), 3.16 (1H, m, CH), 3.25 (1H, dd, *J* = 8.9, 13.4 Hz,

Table 2. Time-dependent GAR Tfase inhibition[a]



Agent	Enzyme activity (%)				R
	t = 3 min	30 min	3 h	6 h	
None	100	100	96	92	—
2	3	3	7	9	CHO
3	51	61	63	65	CH_2OH
4	71	71	67	66	CH_2OCHO
5	89	89	86	83	CH_2OCOCH_3
6	77	75	73	71	$CH_2OCOCOCH_3$
7	46	43	41	40	CH_2OCOCO_2Et
8	90	90	83	78	$CH_2OCOCH(OMe)_2$
9	80	72	63	51	CH_2OCOCH_2Br
10	108	108	100	103	$CH_2OCH_2CONH_2$
11	105	107	106	—	$CH(OMe)_2$

[a]*pur*N GAR Tfase (2 nM), 10 μM inhibitor, 26 °C.

C*H*H), 2.90 (1H, dd, J = 9.7, 13.6 Hz, CH*H*), 2.32 (2H, t, J = 7.7 Hz, CH_2), 2.16 (1H, m, C*H*H), 1.98 (1H, m, CH*H*), 1.42 (9H, s, $COC(CH_3)_3$), 1.41 (9H, s, $COC(CH_3)_3$); ^{13}C NMR (CD_3OD, 100 MHz) δ 172.5, 172.0, 167.4, 162.8, 152.4, 147.7, 135.8, 132.8, 128.9, 127.89, 127.84, 126.5, 126.3, 124.8, 117.9, 81.2, 80.4, 74.6, 66.2, 53.5, 51.2, 45.9, 38.0, 32.2, 28.0, 27.0; IR (neat) ν_{max} 3325, 3170, 2931, 1728, 1660 cm^{-1}; FABHRMS (NBA–NaI) *m/z* (M^+ + H, $C_{31}H_{40}N_4O_7$ requires 581.2975).

A solution of **13** (7.8 mg, 0.01 mmol) in $CHCl_3$ (76 mL) cooled to 0 °C was treated with 25 mL trifluoroacetic acid 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 (6.3 mg, 80%) as a white solid: mp >300 °C; ^1H NMR (DMF-d_7, 400 MHz) δ 9.24 (2H, br s, NH_2), 7.94 (2H, d, J = 8.2 Hz), 7.90 (1H, s, H-5), 7.69 (1H, d, J = 8.6 Hz, H-7), 7.50 (2H, d, J = 8.3 Hz), 7.38 (1H, d, J = 8.4 Hz, H-8), 4.72 (1H, t, J = 5.4 Hz, CH_2), 3.29 (1H, m, NHC*H*CO_2H), 3.71 (1H, m, CH), 3.34 (1H, dd, J = 6.6, 14.4 Hz, C*H*H), 3.23 (1H, dd, J = 8.9, 13.9 Hz, CH*H*), 2.50 (2H, t, J = 7.1 Hz, CH_2), 2.24 (1H, m, C*H*H) 2.08 (1H, m, CH*H*); ^{13}C NMR (DMF-d_7, 100 MHz) δ 174.6, 174.1, 167.1, 160.6, 152.9, 144.5, 138.3, 137.3, 136.7, 133.7, 128.9, 128.3, 127.9, 126.5, 117.9, 71.6, 66.3, 53.0, 46.2, 37.6, 27.1; IR (neat) ν_{max} 3351, 2917, 1668, 1529 cm^{-1}; FABHRMS (NBA) *m/z* 469.1720 (M^+ + H, $C_{23}H_{24}N_4O_7$ requires 469.1723).

(2*RS*)-*N*-[4-{3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(formyloxy)prop-2-yl}benzoyl]-L-glutamic acid (4). Acetic anhydride (0.7 μL, 0.007 mmol) and formic acid (0.3 μL, 0.007 mmol) were added to a solution of **13** (3.6 mg, 0.007 mmol) in CH_3CN (35 μL) at 25 °C. The mixture was stirred at 25 °C for 5 h

Table 3. Time-dependent AICAR Tfase Inhibition[a]

Agent	Enzyme activity (%)				R
	t = 3 min	30 min	3 h	6 h	
None	100	100	100	100	—
2	58	—	—	46	CHO
3	78	—	—	83	CH_2OH
4	94	—	—	100	CH_2OCHO
5	88	—	—	95	CH_2OCOCH_3
6	55	—	—	57	$CH_2OCOCOCH_3$
7	70	—	—	83	CH_2OCOCO_2Et
8	87	—	86	99	$CH_2OCOCH(OMe)_2$
9	81	—	79	77	CH_2OCOCH_2Br
10	80	—	—	74	$CH_2OCH_2CONH_2$
11	81	—	—	86	$CH(OMe)_2$

[a]Avian AICAR Tfase (100 nM), 10 μM inhibitor, 26 °C.

Table 4. Cytotoxic activity (μM)[a]

Agent	L-1210	CCRF-CEM
(6*R*)-DDATHF	>225, 0.07	>225, 0.05
Methotrexate	0.05, 0.05	0.06, 0.07
2	60, 50	860, 430
3	17, 10	80, 20
4	9, —	—, 13
5	11, —	—, 17
6	10, —	—,13
7	23,15	5, 3
8	4, —	—,12
9	9, 5	10, 6
10	>10, —	—, 66
11	>100, >100	>100, >100

[a]Dialyzed FBS, RPMI-1640 medium, with (+), and without (−) hypoxanthine.

at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded di-*tert*-butyl *N*-[4-{3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(formyloxy)prop-2-yl}benzoyl]-L-glutamate (2.4 mg, 52%) as a white solid; ^{1}H NMR (DMF-d_7, 400 MHz) δ 8.20 (1H, s, CHO), 7.89 (2H, d, J = 8.1 Hz), 7.75 (1H, d, J = 1.9 Hz, H-5), 7.44 (2H, d, J = 8.1 Hz), 7.38 (1H, dd, J = 1.5, 8.5 Hz, H-7), 7.08 (1H, d, J = 8.2 Hz, H-8), 6.50 (2H, br s, NH_2), 4.49 (1H, m, NHC*H*$CO_2C(CH_3)_3$), 4.40 (2H, d, J = 5.7 Hz, CH_2), 3.9 (1H, m, CH), 3.18 (1H, dd, J = 6.5, 13.7 Hz, C*H*H), 3.04 (1H, dd, J = 8.9, 13.7 Hz, CH*H*), 2.42 (2H, t, J = 7.3 Hz, CH_2), 2.14 (1H, m, C*H*H), 2.03 (1H, m, CH*H*), 1.42 (9H, s, $CO_2C(CH_3)_3$), 1.39 (9H, s, $CO_2C(CH_3)_3$); IR (neat) ν_{max} 3356, 2927, 1723, 1678, 1658 cm^{-1}; FABHRMS (NBA–NaI) *m/z* 609.2920 (M^+ + H, $C_{32}H_{40}N_4O_8$ requires 609.2924).

A solution of the di-*tert*-butyl ester (3.6 mg, 0.005 mmol) in $CHCl_3$ (474 μL) cooled to 0 °C was treated with 118 μL trifluoroacetic acid 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 (3.2 mg, 88%) as a white solid: mp >300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 9.11 (1H, br s, NH), 8.20 (1H, s, CHO), 7.92 (2H, d, J = 8.2 Hz), 7.85 (1H, s, H-5), 7.60 (1H, d, J = 8.2 Hz, H-7), 7.45 (2H, d, J = 8.2 Hz), 7.23 (1H, d, J = 8.3 Hz, H-8), 4.60 (1H, m, NHC*H*CO_2H), 4.41 (2H, m, CH_2), 3.71 (1H, m, CH), 3.26 (1H, dd, J =6.4, 13.8 Hz, C*H*H), 3.11 (1H, dd, J = 9.5, 13.5 Hz, CH*H*), 2.50 (2H, t, J = 6.9 Hz, CH_2), 2.23 (1H, m, C*H*H), 2.09 (1H, m, CH*H*); IR (KBr) ν_{max} 2945, 1715, 1678, 1553 cm^{-1}; FABHRMS (NBA) *m/z* 497.1650 (M^+ + H, $C_{24}H_{24}N_4O_8$ requires 497.1672).

(2*RS*)-*N*-[4-{1-(Acetoxy)-3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)prop-2-yl}benzoyl]-L-glutamic acid (5). Pyridine (0.2 μL, 0.002 mmol) and acetic anhydride (0.2 μL, 0.002 mmol) were added to a solution of **13** (1.5 mg, 0.002 mmol) in CH_3CN at 25 °C and the solution was stirred at 25 °C for 1 h at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded di-*tert*-butyl *N*-[4-{1-(acetoxy)-3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)prop-2-yl}-benzoyl]-L-glutamate (1.0 mg, 62%) as a white solid: mp >300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 11.04 (1H, br s, NH), 7.89 (2H, d, J = 8.3 Hz), 7.75 (1H, d, J = 2.1 Hz, H-5), 7.43 (2H, d, J = 8.3 Hz), 7.37 (1H, dd, J = 2.1, 8.4 Hz, H-7), 7.07 (1H, d, J = 8.3 Hz, H-8), 6.47 (2H, br s, NH_2), 4.49 (1H, m, NHC*H*$CO_2C(CH_3)_3$), 4.27 (2H, d, J = 6.7 Hz, CH_2O), 3.42 (1H, m, CH), 3.17 (1H, dd, J = 6.6, 13.7 Hz, C*H*H), 3.03 (1H, dd, J = 8.7, 14.0 Hz, CH*H*), 2.43 (2H, t, J = 6.6 Hz, CH_2), 2.14 (1H, m, C*H*H), 2.02 (1H, m, CH*H*), 1.95 (3H, s, CH_3CO), 1.42 (9H, s, $CO_2C(CH_3)_3$), 1.39 (9H, s, $CO_2C(CH_3)_3$); IR (neat) ν_{max} 3156, 2970, 2914, 1726, 1612 cm^{-1}; FABHRMS (NBA–NaI) *m/z* 623.3080 (M^+ + H, $C_{33}H_{42}N_4O_8$ requires 623.3081).

A solution of the di-*tert*-butyl ester (1.0 mg, 0.001 mmol) in $CHCl_3$ (9 μL) cooled to 0 °C was treated with 3 μL trifluoroacetic acid and the solution 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 **5**·CF_3CO_2H (0.8 mg, 79%) as a white solid: mp >300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 7.91 (2H, d, J = 8.4 Hz), 7.80 (1H, d, J = 1.9 Hz, H-5), 7.53 (1H, d, J = 6.8 Hz, H-7), 7.44 (2H, d, J = 8.3 Hz), 7.17 (1H, d, J = 8.2 Hz, H-8), 4.62 (1H, m, NHC*H*CO_2H), 4.28 (2H, d, J = 6.5 Hz, CH_2), 3.90 (1H, m, CH), 3.21 (1H, dd, J = 6.7, 13.9 Hz, C*H*H), 3.08 (1H, dd, J = 9.0, 14.2 Hz, CH*H*), 2.50 (2H, t, J = 7.5 Hz, CH_2), 2.25 (1H, m, C*H*H), 2.08 (1H, m, CH*H*), 1.96 (3H, s, CH_2); IR (KBr) ν_{max} 3156, 2970, 2914, 1726, 1612 cm^{-1}; FABHRMS (NBA) *m/z* 511.1850 (M^+ + H, $C_{25}H_{26}N_4O_8$ requires 511.1829).

(2*RS*)-*N*-[4-{3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(pyruvoyloxy)prop-2-yl}benzoyl]-L-glutamic acid (6). DCC (0.9 mg, 0.004 mmol) and HOBt (0.5 mg, 0.003 mmol) followed by **13** (2 mg, 0.003 mmol) in CH_3CN (50 μL) was added to pyruvic acid (0.4 mg, 0.004 mmol) at 25 °C. This slurry was stirred at 25 °C for 5 h at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded di-*tert*-butyl *N*-[4-{3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(pyruvoyloxy)prop-2-yl}benzoyl]-L-glutamate (1.5 mg, 67%) as a yellow oil; ^{1}H NMR (DMF-d_7, 400 MHz) δ 7.90 (2H, d, J = 8.3 Hz), 7.77 (1H, s, H-5), 7.58 (2H, d, J = 8.4 Hz), 7.40 (1H, dd, J = 2.3, 5.3 Hz, H-7), 7.08 (1H, d, J = 8.4 Hz, H-8), 6.41 (2H, br s, NH_2), 4.48 (2H, m, CH_2), 4.48 (1H, m, NHC*H*$CO_2C(CH_3)_3$), 3.60 (1H, m, CH), 3.23 (1H, m, C*H*H), 3.08 (1H, m, CH*H*), 2.42 (2H, t, J = 7.7 Hz, CH_2), 2.36 (3H, s, CH_3), 2.17 (1H, m, C*H*H), 2.05 (1H, m, CH*H*), 1.37 (9H, s, $CO_2C(CH_3)_3$), 1.36 (9H, s, $CO_2C(CH_3)_3$); IR (neat) ν_{max} 2921, 2856, 1722, 1662, 1605 cm^{-1}; FABHRMS (NBA) *m/z* 651.3045 (M^+ + H, $C_{34}H_{42}N_4O_9$ requires 651.3030).

A solution of the di-*tert*-butyl ester (2.8 mg, 0.004 mmol) in $CHCl_3$ (345 μL) cooled to 0 °C was treated with 86 μL trifluoroacetic acid 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 **6**·CF_3CO_2H (1.5 mg, 53%) as a white solid: mp >300 °C; ^1H NMR (DMF-d_7, 400 MHz) δ 7.94 (2H, d, J = 5.8 Hz), 7.86 (1H, d, J = 1.8 Hz, H-5), 7.61 (1H, d, J = 8.0 Hz, H-7), 7.49 (2H, d, J = 8.5 Hz), 7.23 (1H, d, J = 8.2 Hz, H-8), 4.62 (1H, m, NHC*H*CO_2H), 4.46 (2H, m, CH_2O), 4.33 (1H, m, CH), 3.31 (1H, dd, J = 6.5, 14.2 Hz, C*H*H), 3.18 (1H, dd, J = 8.9, 14.1 Hz, CH*H*), 2.50 (2H, t, J = 7.9 Hz, CH_2), 2.38 (3H, s, CH_3), 2.25 (1H, m, C*H*H), 2.08 (1H, m, CH*H*); IR (KBr) ν_{max} 2921, 2856, 1722, 1662, 1605 cm^{-1}; Ionspray MS (NBA) *m/z* 539 (M^+ + H, $C_{26}H_{26}N_4O_9$ requires 539).

(2*RS*)-*N*-[4-{3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(ethoxalyoxy)prop-2-yl}benzoyl]-L-glutamic acid (7). Pyridine (0.4 μL, 0.003 mmol) and ethyl oxalyl chloride (0.3 μL, 0.003 mmol) were added to a solution of **13** (2.3 mg, 0.003 mmol) in CH_3CN at 25 °C. The solution was stirred at 25 °C for 15 min at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded di-*tert*-butyl *N*-[4-{3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(ethoxalyoxy)prop-2-yl}benzoyl]-L-glutamate (1.6 mg, 59%) as a yellow oil; ^1H NMR (DMF-d_7, 400 MHz) δ 11.08 (1H, br s, NH), 7.90 (2H, d, J = 8.4 Hz), 7.76 (1H, d, J = 2.2 Hz, H-5), 7.47 (2H, d, J = 8.3 Hz), 7.38 (1H, dd, J = 2.2, 8.5 Hz, H-7), 7.06 (1H, d, J = 8.4 Hz, H-8), 6.50 (2H, br s, NH_2), 4.54 (2H, t, J = 7.4 Hz, CH_2), 4.53 (1H, m, NHC*H*$CO_2(CH_3)_3$), 4.26 (2H, q, J = 7.1 Hz, CH_2), 3.55 (1H, m, CH), 3.24 (1H, m, C*H*H), 3.08 (1H, m, CH*H*), 2.42 (2H, t, J = 7.8 Hz, CH_2), 2.13 (1H, m, C*H*H), 2.02 (1H, m, CH*H*), 1.42 (9H, s, $CO_2C(CH_3)_3$), 1.39 (9H, s, $CO_2C(CH_3)_3$), 1.27 (3H, t, J = 7.1 Hz, CH_2CH_3); IR (neat) ν_{max} 3385, 2957, 2923, 1722, 1688, 1658 cm^{-1}; FABHRMS (NBA–NaI) *m/z* 681.3140 (M^+ + H, $C_{35}H_{44}N_4O_{10}$ requires 681.3136).

A solution of the di-*tert*-butyl ester (3.0 mg, 0.004 mmol) in $CHCl_3$ (353 μL) cooled to 0 °C was treated with 88 μL trifluoroacetic acid 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 **7**·CF_3CO_2H (2.1 mg, 70%) as a white solid: mp >300 °C; ^1H NMR (DMF-d_7, 400 MHz) δ 7.92 (2H, d, J = 8.3 Hz), 7.85 (1H, d, J = 1.5 Hz, H-5), 7.59 (2H, d, J = 6.8 Hz, H-7), 7.48 (2H, d, J = 8.4 Hz), 7.22 (1H, d, J = 8.2 Hz, H-8), 4.62 (1H, m, NHC*H*CO_2H), 4.53 (2H, t, J = 12.9 Hz, OCH_2), 4.27 (2H, q, J = 7.1 Hz, CH_2), 3.50 (1H, m, CH), 3.30 (1H, dd, J = 6.4 , 14.4 Hz, C*H*H), 3.17 (1H, dd, J = 9.4, 13.9 Hz, CH*H*), 2.50 (2H, t, J = 7.3 Hz, CH_2), 2.25 (1H, m, C*H*H), 2.08 (1H, m, CH*H*CH_2CO_2H), 1.27 (3H, t , J = 7.1 Hz, CH_3); IR (KBr) ν_{max} 3447, 3260, 2914, 1605 cm^{-1}; Ionspray MS (NBA) *m/z* 567 (M^+ + H, $C_{27}H_{28}N_4O_8$ requires 567).

(2*RS*)-*N*-[4-{3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(1,1-dimethoxyacetoxy)prop-2-yl}benzoyl]-L-glutamic acid (8). A solution of methyl dimethoxyacetate (200 μL, 1.63 mmol) in THF:H_2O:CH_3OH (3:1:1, 4 mL) was treated with aqueous 1 N LiOH (1.96 mL, 1.96 mmol). The solution was stirred at 25 °C for 24 h. Additional aqueous 1 N LiOH (1.96 mL, 1.96 mmol) was added and the solution 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 aqueous 10% HCl (1 mL, pH 1). The precipitated product was collected by filtration to give dimethoxyacetic acid (350 mg, 71%) as a white solid. A solution of dimethoxyacetic acid (0.8 mg, 0.006 mmol) in CH_2Cl_2 (30 μL) at 0 °C was treated with iPr_2NEt (3 μL, 0.017 mmol), **13** (3.1 mg, 0.006 mmol) in CH_2Cl_2 (30 μL), BOPCl (2.2 mg, 0.008 mmol) and the mixture was stirred at 0 °C for 18 h at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded di-*tert*-butyl *N*-[4-{3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(1,1-dimethoxyacetoxy)-prop-2-yl}benzoyl]-L-glutamate (2.1 mg, 57%) as a white solid: mp >300 °C; ^1H NMR (DMF-d_7, 400 MHz) δ 10.90 (1H, br s, NH), 7.89 (2H, d, J = 8.3 Hz), 7.75 (1H, d, J = 1.9 Hz, H-5), 7.44 (2H, d, J = 8.3 Hz), 7.37 (1H, dd, J = 2.1, 8.5 Hz, H-7), 7.07 (1H, d, J = 8.4 Hz, H-8), 6.43 (2H, br s, NH_2), 4.80 (1H, s, CH), 4.47 (1H, m, NHC*H*$CO_2C(CH_3)_3$), 4.45 (2H, m, CH_2), 4.01 (1H, m, CH), 3.29 (3H, s, CH_3), 3.27 (3H, s, CH_3), 3.39 (1H, m, C*H*H), 3.17 (1H, m, CH*H*), 2.42 (2H, t, J = 7.7 Hz, CH_2), 2.14 (1H, m, C*H*H), 2.02 (1H, m, CH*H*), 1.42 (9H, s, $CO_2C(CH_3)_3$), 1.39 (9H, s, $CO_2C(CH_3)_3$); IR (neat) ν_{max} 2966, 2925, 2853, 1724, 1659, 1639, 1611 cm^{-1}; FABHRMS (NBA) *m/z* 683.3268 (M^+ + H, $C_{35}H_{46}N_4O_{10}$ requires 683.3292).

A solution of the di-*tert*-butyl ester (1.9 mg, 0.002 mmol) in $CHCl_3$ (223 μL) cooled to 0 °C was treated with trifluoroacetic acid (56 μL) and the solution was stirred at 0 °C for 1 h and at 25 °C for 12 h. Et_2O (1 mL) was added and a white precipitate formed. The precipitate was triturated with Et_2O (3 × 1 mL) and dried in vacuo to give **8**·CF_3CO_2H (1.4 mg, 73%) as a white solid: mp >300 °C; ^1H NMR (DMF-d_7, 400 MHz) δ 7.92 (2H, d, J = 5.8 Hz), 7.86 (1H, d, J = 1.7 Hz, H-5), 7.60 (1H, d, J = 8.5 Hz, H-7), 7.46 (2H, d, J = 8.3 Hz), 7.26 (1H, d, J = 8.4 Hz, H-8), 4.82 (1H, s, CH), 4.60 (1H, m, NHC*H*CO_2H), 4.46 (2H, m, CH_2), 3.54 (1H, m, CH), 3.30 (3H, s, CH_3), 3.28 (3H, s, CH_3), 3.28 (1H, m, C*H*H), 3.15 (1H, dd, J = 13.9, 8.6 Hz, CH*H*), 2.50 (2H, t, J = 4.5 Hz, CH_2), 2.25 (1H, m, C*H*H), 2.08 (1H, m, CH*H*); IR (KBr) ν_{max} 2966, 2925, 2853, 1724, 1659, 1639, 1611 cm^{-1}; Ionspray MS (NBA) *m/z* 571 (M^+ + H, $C_{27}H_{30}N_4O_{10}$ requires 571).

(2*RS*)-*N*-[4-{3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(bromoacetoxy)prop-2-yl}benzoyl]-L-glutamic acid (9). A solution of **13** (1.2 mg, 0.002 mmol) in CH_2Cl_2 (10.3 μL) was cooled to 0 °C, was treated with bromoacetyl bromide (ca. 0.2 μL, 0.002 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 di-*tert*-butyl *N*-[4-{3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1-(bromoacetoxy)-prop-2-yl}benzoyl]-L-glutamate (1.3 mg, 89%) as a white solid: mp >300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 7.89 (2H, d, J = 8.2 Hz), 7.76 (1H, d, J = 4.6 Hz, H-5), 7.45 (2H, d, J = 8.2 Hz), 7.38 (1H, dd, J = 1.9, 8.5 Hz, H-7), 7.08 (1H, d, J = 8.1 Hz, H-8), 6.41 (2H, br s, NH_2), 4.48 (1H, m, NHC*H*$CO_2C(CH_3)_3$, 4.38 (2H, d, J = 6.6 Hz, CH_2), 4.15 (2H, s, CH_2), 3.47 (1H, m, CH), 3.21 (1H, m, C*H*H), 3.07 (1H, m, CH*H*), 2.42 (2H, t, J = 6.7 Hz, CH_2), 2.15 (1H, m, CH), 2.02 (1H, m, CH), 1.42 (9H, s, $CO_2C(CH_3)_3$), 1.39 (9H, s, $CO_2C(CH_3)_3$); IR (neat) ν_{max} 2928, 2852, 2134, 1729 cm^{-1}; FABHRMS (NBA–CsI) *m/z* 833.1150 (M^+ + H, $C_{33}H_{41}N_4O_8Br$ requires 833.1162).

A solution of the di-*tert*-butyl ester (2.9 mg, 0.004 mmol) in $CHCl_3$ (331 μL) cooled to 0 °C was treated with 83 μL trifluoroacetic acid 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 **9**·CF_3CO_2H (2.0 mg, 69%) as a white solid: mp >300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 12.83 (2H, br s, NH_2), 7.92 (2H, d, J = 8.2 Hz), 7.83 (1H, d, J = 1.6 Hz, H-5), 7.55 (1H, d, J = 8.4 Hz, H-7), 7.47 (2H, d, J = 8.4 Hz), 7.20 (1H, d, J = 8.3 Hz, H-8), 4.60 (1H, m, NHC*H*CO_2H), 4.39 (2H, d, J = 6.3 Hz, CH_2), 4.16 (2H, s, CH_2), 3.50 (1H, m, CH), 3.27 (1H, dd, J = 6.4, 13.7 Hz, C*H*H), 3.13 (1H, dd, J = 8.9, 13.6 Hz, CH*H*), 2.50 (2H, t, J = 7.4 Hz, CH_2), 2.26 (1H, m, CH), 2.08 (1H, m, CH); IR (KBr) ν_{max} 3027, 2249, 1718, 1502 cm^{-1}; FABHRMS (NBA) *m/z* 589.0906 (M^+ + H, $C_{25}H_{25}N_4O_8Br$ requires 589.0934).

(2*RS*)-*N*-[4-{1-(Acetamideoxy)-3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)prop-2-yl}benzoyl]-L-glutamic acid (10). Bromoacetamide (0.8 mg, 0.005 mmol) and $CsCO_3$ (1.85 mg, 0.005 mmol) were added to **13** (3.3 mg, 0.005 mmol) in DMF (25 μL) at 0 °C and the mixture was stirred at 0 °C for 18 h at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded di-*tert*-butyl *N*-[4-{1-(acetamideoxy)-3-(2-amino-3,4-dihydro-4-oxo-quinazolin-6-yl)prop-2-yl}benzoyl]-L-glutamate (2.5 mg, 69%) as a white solid: mp >300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 7.85 (2H, d, J = 8.2 Hz), 7.73 (1H, br s, NH), 7.76 (1H, d, J = 2.0 Hz, H-5), 7.36 (2H, d, J = 8.1 Hz), 7.35 (1H, dd, J = 2.1, 6.5 Hz, H-7), 7.18 (1H, br s, NH), 7.04 (1H, d, J = 8.4 Hz, H-8), 6.75 (2H, br s, NH_2), 4.80 (2H, s, CH_2), 4.48 (1H, m, NHC*H*$CO_2C(CH_3)_3$), 3.71 (2H, t, J = 5.9 Hz, CH_2), 3.23 (1H, dd, J = 6.0, 13.0 Hz, C*H*H), 3.17 (1H, m, CH), 2.94 (1H, dd, J = 8.4, 13.2 Hz, CH*H*), 2.42 (2H, t, J = 7.7 Hz, CH_2), 2.13 (1H, m, CH*H*), 2.02 (1H, m, CH*H*), 1.42 (9H, s, $CO_2C(CH_3)_3$), 1.39 (9H, s, $CO_2C(CH_3)_3$); IR (neat) ν_{max} 3325, 2928, 1677 cm^{-1}; FABHRMS (NBA–NaI) *m/z* 660.2985 (M^+ + Na, $C_{33}H_{43}N_5O_8$ requires 660.2986).

A solution of the di-*tert*-butyl ester (2.5 mg, 0.003 mmol) in $CHCl_3$ (314 μL) cooled to 0 °C was treated with 88 μL trifluoroacetic acid and the solution was stirred at 0 °C for 2 h and at 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 **10**·CF_3CO_2H (2.3 mg, 91%) as a white solid: mp >300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 12.70 (1H, br s, NH), 7.94 (2H, d, J = 8.1 Hz), 7.93 (1H, s, H-5), 7.77 (1H, d, J = 8.6 Hz, H-7), 7.51 (2H, d, J = 8.4 Hz), 7.25 (d, 1H, J = 8.2 Hz, H-8), 4.86 (2H, s, CH_2), 4.72 (2H, t, J = 7.3 Hz, CH_2), 4.60 (1H, m, NHC*H*CO_2H), 3.71 (1H, m, CH), 3.33 (1H, dd, J = 6.6, 14.3 Hz, C*H*H), 3.12 (1H, dd, J = 4.9, 14.2 Hz, CH*H*), 2.50 (2H, t, J = 7.5 Hz, CH_2), 2.24 (1H, m, C*H*H), 2.09 (1H, m, CH*H*); IR (KBr) ν_{max} 3004, 1783, 1683, 1537 cm^{-1}; Ionspray MS (NBA) *m/z* 526 (M^+ + H, $C_{25}H_{27}N_5O_8$ requires 526).

(2*RS*)-*N*-[4-{3-(2-Amino-3,4-dihydro-4-oxo-quinazolin-6-yl)-1,1-(dimethoxy)prop-2-yl}benzoyl]-L-glutamic acid (11). A solution of **14**[1] (2.3 mg, 0.004 mmol) and *p*-TsOH (cat) in trimethylorthoformate (10 μL, 0.04 mmol) was stirred at 25 °C for 20 min at which time the solvent was removed under reduced pressure. PCTLC (SiO_2, 1 mm plate, 10% CH_3OH–$CHCl_3$) afforded **15** (2.3 mg, 89%) as a yellow solid: mp >300 °C; ^{1}H NMR (DMF-d_7, 400 MHz) δ 11.00 (1H, br s, NH), 7.82 (2H, d, J = 8.3 Hz), 7.64 (1H, d, J = 1.7 Hz, H-5), 7.33 (2H, d, J = 8.3 Hz). 7.25 (1H, dd, J = 2.0, 8.3 Hz, H-7), 7.01 (1H, d, J = 8.3 Hz, H-8), 6.42 (1H, br s, NH_2), 4.60 (1H, d, J = 6.5 Hz, CH), 4.57 (1H, m, NHC*H*CO_2CH_3), 3.66 (3H, s, CO_2CH_3), 3.59 (3H, s, CO_2CH_3), 3.40 (3H, s, OCH_3), 3.36 (1H, m, C*H*H), 3.24 (3H, s, OCH_3), 3.22 (1H, m, CH*H*), 2.99 (1H, m, CH), 2.51 (2H, t, J = 7.6 Hz, CH_2), 2.19 (1H, m, C*H*H), 2.10 (1H, m, CH*H*); IR (neat) ν_{max} 2914, 2850, 1731, 1712, 1693, 1650, 1633 cm^{-1}; FABHRMS (NBA) *m/z* 541.2310 (M^+ + H, $C_{27}H_{32}N_4O_8$ requires 541.2298).

A solution of **15** (3.9 mg, 0.007 mmol) in THF:H_2O:CH_3OH (3:1:1, 18 μL) was treated with aqueous 1 N LiOH (14 μL, 0.01 mmol). The solution was stirred at 25 °C for 24 h. Additional aqueous 1 N LiOH (14 μL, 0.01 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 aqueous 10% HCl (1 mL, pH 1). The precipitated product was collected by filtration to give **12**·HCl (2 mg, 54%) as a white solid: mp > 250 °C; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.72 (2H, dd, J = 1.8, 8.4 Hz), 7.66 (1H, d, J = 1.2 Hz, H-5), 7.21 (2H, d, J = 8.3 Hz), 7.16 (1H, dd, J = 1.9, 8.4 Hz, H-7), 6.99 (1H, d, J = 8.4 Hz, H-8), 4.59

(1H, d, J = 6.3 Hz, CH), 4.38 (1H, m, NHC*H*CO_2H), 3.43 (3H, s, OCH_3), 3.34 (1H, m, CH_2), 3.25 (3H, s, OCH_3), 2.94 (1H, t, J = 12.4 Hz, CH), 2.20 (m, 4H, $(CH_2)_2$); IR (KBr) ν_{max} 3380, 3035, 1649, 1558 cm^{-1}; Ionspray MS (NBA) m/z 513 (M^+ + H, $C_{25}H_{28}N_4O_8$ requires 513).

GAR and AICAR Tfase inhibition

The evaluations of **3–11** were conducted as detailed in the preceding article.[1]

Cytotoxic activity

The cytotoxic testing was conducted following protocols we have described in detail.[1]

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