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Glutamyl-γ-boronate Inhibitors of Bacterial Glu-tRNA^{Gln} Amidotransferase

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Abstract—Analogues of glutamyl-γ-boronate (**1**) were synthesized as mechanism-based inhibitors of bacterial Glu-tRNA^{Gln} amidotransferase (Glu-AdT) and were designed to engage a putative catalytic serine nucleophile required for the glutaminase activity of the enzyme. Although **1** provides potent enzyme inhibition, structure–activity studies revealed a narrow range of tolerated chemical changes that maintained activity. Nonetheless, growth inhibition of organisms that require Glu-AdT by the most potent enzyme inhibitors appears to validate mechanism-based inhibitor design of Glu-AdT as an approach to antimicrobial development.

Many bacteria lack a synthetase to directly form glutamine (Gln)-loaded tRNA (Gln-tRNA^{Gln}). Instead, these organisms require a Glu-tRNA^{Gln} amidotransferase to transamidate mis-loaded Glu-tRNA^{Gln} and produce this critical precursor of protein synthesis.[1] Although a cognate to Glu-AdT is present in eukaryotic mitochondria,[2] its absence in mammalian cytoplasm and its essentiality for survival of many pathogenic bacteria implicate the enzyme as a target for antibiotic chemotherapy.

Glu-AdT catalyzes the amidolysis of L-Gln and transfer of NH_3 to transamidate Glu-tRNA^{Gln}. Amino acid sequence alignment with related amidases and site-directed mutagenesis studies[3] suggest that an active site serine is essential to the mechanism of Gln-dependent transamidation and implicate the hydroxyl group of this residue as the nucleophilic species in the amidolysis reaction step. This observation contrasts with the more typical transamidases which employ an active site cysteine (thiol nucleophile) as the displacing reactant.[1,4] In the absence of any structural information about the active site of Glu-AdT, we targeted substrate-based (Gln) derivatives that take advantage of the critical active site serine as inhibitors of the transferase activity.

A number of serine inactivators or 'serine traps' have been developed and successfully applied in the design of serine protease inhibitors. In general the serine trap contains an electrophilic center that is reactive toward a hydroxyl nucleophile. Ketones with alpha activating groups (electron withdrawing) such as esters, amides, halogen, phosphonates, β-lactams, and more sophisticated activated heterocycles have all been studied.[5] Specificity for a particular serine protease can usually be achieved by covalent attachment of a peptide sequence which is complementary to active site recognition elements in the protease. Boronic acids have been shown to be effective serine traps for a number of serine proteases. In general they display reversible inhibition kinetics and proceed through a serine-boronate acetal.[6] In light of our limited knowledge of the active site of Glu-AdT, we initially chose to study the boronic acid

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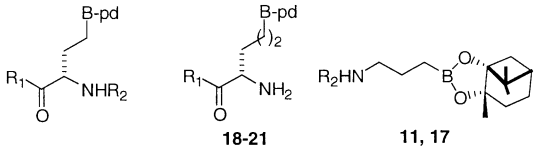
group because it is isosteric with the carboxamide present in the Gln substrate.

To test our design concept, Glu-γ-B(O_2R) was synthesized from (*S*)-*N*-Cbz-vinylglycine methyl ester as described previously.[7] This compound displayed IC_{50} values of 1.6 μM and 100 nM, respectively, against the glutaminase and transferase activities of Glu-AdT (Table 1). Despite its zwitterionic character, **1** displayed antibacterial activity against a host of relevant organisms (Table 2).

We next examined analogues of the parent in search of more active compounds. Scheme 1 describes the synthetic approach used to make analogues of the lead compound. Most compounds were synthesized and assayed directly as pinanediol boronate esters.[8] The synthesis of the protected vinyl glycine derivative **B** was accomplished in four steps from commercially available **A**.[9] Boron addition to the olefin of **B** was effected using BH_3–Me_2S followed by treatment with α-pinene/pinanediol to yield the key protected boronate **C**.[10] Selective manipulation of either the carboxy or amino protecting groups enabled the synthesis of a variety of analogues of interest (Table 1). Scheme 2 outlines the preparation of the one carbon homologues starting from the methyl or *tert*-butyl esters of L-allyl glycine (Cbz). The β-alanine derivative **6** was obtained from the Boc-β-lactam **E** as shown in Scheme 3.[11]

Table 1. In vitro glutaminase (GLA) and transferase (TRA) activity



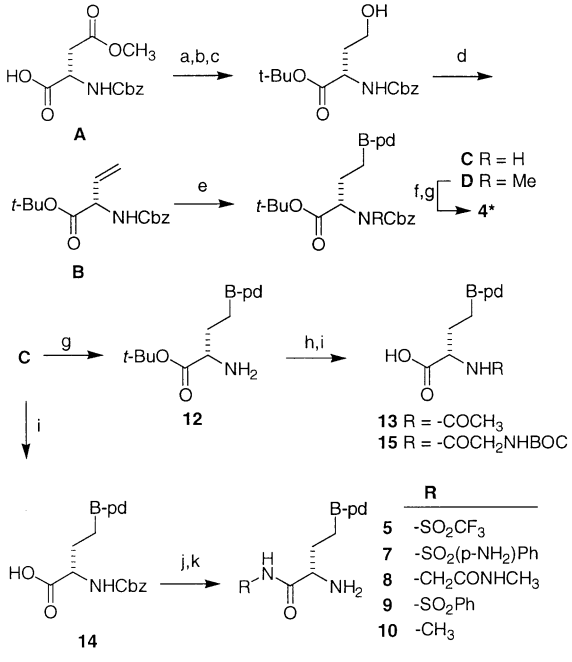
Compd	R_1	R_2	IC_{50} (μM)	
			GLA	TRA
1[ab]	–OH	–H	1.6	0.10
2[a]	–OCH_3	–H	1.3	0.05
3[b]	–OCH_3	–H	1.5	0.07
4	–OH	–CH_3	ND[c]	1.1
5	–$NHSO_2CF_3$	–H	55	4.7
6[d]	—	—	ND	6.5
7	–$NHSO_2BzNH_2$	–H	>80[e]	10
8	–$NHCH_2CONHCH_3$	–H	40	20
9	–$NHSO_2Bz$	–H	40	20
10	–$NHCH_3$	–H	>80	35
11	—	–H	>80	>20[f]
12	–OtBu	–H	>80	ND
13	–OH	–$COCH_3$	>80	>20
14	–OH	–Cbz	>80	ND
15	–OH	–$COCH_2NH_2$	>80	>20
16	–OCH_3	–Cbz	>80	>20
17	—	–CO_2tBu	>80	>20
18	–OH	Cbz	>80	>20
19	–OMe	-H	>80	>20
20	–OH	–H	>80	>20
21	–OMe	Cbz	>80	>20

[a]All compounds except **1** and **2** were prepared as pinanediol esters. For **1** and **2**, pd = $(OH)_2$.
[b]Based on multiple determinations for **1** and **2**, standard error of amidase-derived IC_{50} is ~25%; standard error in the transferase assay is ~20%.
[c]Not determined.
[d]See Scheme 2 for structure.
[e]Corresponds to <20% inhibition at single inhibitor concentrations of ≥20 μM for samples run in parallel with assays for **1** or **2**.
[f]Corresponds to <20% inhibition at a single inhibitor concentration of 5 μM for samples run in parallel with assays for **1**.

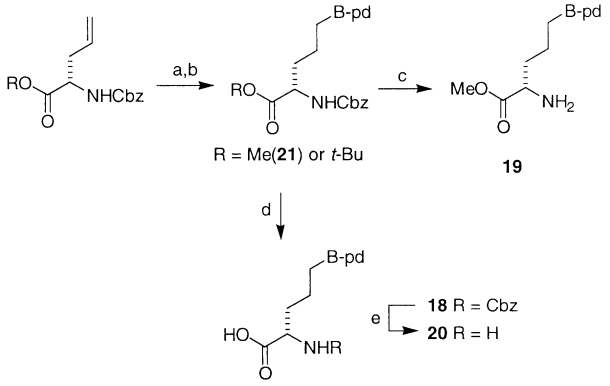
Table 2. Bacterial MIC determinations[a]

Compd	*S. pyo.*[b]	*S. pneu.*[b]	*S. aur.*[b]	*E. faec.*[b]	*H. pyl.*[b]	*E. coli*[c]
1[d]	8	3	64	8	32	>64
2[d]	6	2	64	3	32	>64
3[d]	8	2	>64	4	64	>64
8[d]	8	16	>64	16	16	8
10	32	32	>64	>64	>64	64
11	>64	ND[e]	>64	>64	>64	>64
12	>64	ND	>64	>64	>64	8
14	>64	ND	>64	>64	>64	3
15	>64	>64	>64	8	ND	>64
16	64	ND	>64	>64	32	>64
17	>64	ND	>64	>64	>64	8

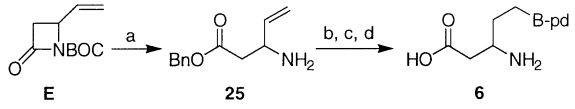
[a]Values are given in μg/mL. Highest concentration tested was 64 μg/mL; values >64 μg/mL indicate that no growth was observed at this level. Compounds **4**, **5**, **7**, **9**, **13**, and **18**–**20** exhibited MIC values >64 μg/mL for all test organisms. Determinations were not performed for **6**.
[b]Strains used: *Streptococcus pyogenes* ATCC 13709; *Streptococcus pneumoniae* ATCC 49619; *Staphylococcus aureus* ATCC 29213, *Enterobacter faecalis* ATCC 29212; *Helicobacter pylori* ATCC 43629.
[c]Identical values were obtained for *Escherichia coli* strains tolC and SM101. Both strains harbor functional Gln-$tRNA^{Gln}$ synthetase and do not express Glu-AdT.
[d]MIC >64 μg/mL for *S. aureus* ATTC 29213, 25923, and 43300, as well as *S. carnosus* ATCC 51365, and *S. epidermidis* ATCC strains 12228 and 51625.
[e]Not determined.



Scheme 1. (a) $CH_2C(CH_3)_2$, H_2SO_4, CH_2Cl_2, 78%; (b) LiOH, 1:1 THF/H_2O 95%; (c) NMM, CH_3CH_2OCOCl, $NaBH_4$ 45%; (d) 1-SeCN-2-$NO_2C_6H_4$, Bu_3P 71%; (e) BH_3·Me_2S, α-pinene, CH_3COH, (+)-pinanediol; (f) TFA 98%; (g) H_2, Pd-C, AcOH, MeOH 98%; (h) CH_3COCl, CH_2Cl_2/DMF 66% or HO_2CCH_2NHBOC, $NaHCO_3$, HOAt, EDC 64%; (i) TFA; (j) $NaHCO_3$, HN-R, HOAt, EDC, 30–83%; (k) H_2, Pd–C, AcOH/MeOH. pd = pinanediol. *See Table 1 for structure of **4**.



Scheme 2. (a) $CH_2C(CH_3)_2$, H_2SO_4, CH_2Cl_2, 98% or CH_3I, K_2CO_3, acetone, 83%; (b) $BH_3{\cdot}Me_2S$, α-pinene, CH_3COH; (+)-pinanediol, 65%; (c) (R = Me) H_2/Pd–C, HCl/MeOH, 74%; (d) (R = O*t*-Bu), TFA, 90%; (e) H_2, Pd–C, HCl/MeOH, 91%.



Scheme 3. (a) KCN, $C_6H_5CH_2OH$, DMF, 82%; (b) $BH_3{\cdot}Me_2S$, α-pinene, CH_3COH, (+)-pinanediol, 59%; (c) H_2, Pd–C; AcOH/MeOH; (d) TFA, 98%.

Holding the amine group constant, we first examined preliminary SAR around the carboxylate moiety of the lead. Methyl ester **2** proved to be equipotent with **1**, but we could not rule out ester hydrolysis under the assay conditions. *tert*-Butyl ester **12** was found to be more than 200-fold less potent than **1**. Interestingly, monomethylamine **4** was found to be an order of magnitude less potent than **1**. Removal of the carboxyl group entirely to give the proteo derivative **11**, and homologation of one methylene unit to give the β-amino acid **6** resulted in loss of activity. Similarly, carboxamide **10** was also inactive. We surmised that the placement of the negative charge must be important for activity and investigated an isoelectronic organic acid equivalent. We thus prepared acyl sulfonamides **5**, **7**, and **9**, and found that the most acidic derivative **5** did inhibit transferase activity with an IC_{50} of 4.7 μM; the other two in the series were active in the 10–20 μM range. The glycine derivative **8** was also moderately active with an IC_{50} of 20 μM.

All of the amine derivatives in which the carboxyl group was held constant resulted in inactive compounds (**13**–**15**). One-methylene homo-Gln derivatives of **1**, **3**, **14**, and **16** (**18**–**21**) were also made and found to be inactive against the enzyme.

Compounds were evaluated as in vitro inhibitors of both the glutaminase-only (absence of ATP and tRNA substrates) and Gln-dependent transferase activities of Glu-AdT (Table 1).[12] The rank order of inhibitor potencies are similar between glutaminase-only and transferase assays, as expected if the compounds inhibit transferase activity by blocking formation of NH_3 in the glutaminase reaction. The lower comparative IC_{50} values between glutaminase and transferase assays reflect both the use of a lower relative Gln concentration in the latter assay ($0.5\times K_m$ vs $2\times K_m$) and a mechanistic shift from time-dependent, tight-binding inhibition (glutaminase-only activity) to fast-binding, reversible inhibition (Gln-dependent transferase activity), as we have observed for **1**.[3]

As demonstrated in Table 1, Glu-AdT is generally intolerant of alterations to **1**. With the exception of the α-carboxymethyl derivatives (**2** and **3**), even modest changes to C_α substituents cause at least a 10-fold reduction in potency. Although a free α-carboxyl group may not be absolutely required for inhibition, proper orientation and electronic environment of the carbonyl group is essential: displacement by a methylene (**6**), alteration of pK_a (**5**, **7**, and **8**), alkylation with a larger group (**12**), or loss of the carboxyl (**11**) is not well tolerated. All but the sterically most simple modifications of the α-amino (–$NHCH_3$; **4**) were inactive as inhibitors. Thus, specific interactions between Glu-AdT and both C_α substituents of Gln-like analogues contribute to binding energy and specificity. This demonstrated binding specificity is consistent with other properties of Glu-AdT and related Gln-utilizing hydrolases. Although the three-dimensional structure of Glu-AdT has not been solved, the structures of several other Gln-utilizing hydrolases[13] reveal multiple active-site interactions with both α-amino and α-carboxyl groups of substrate. In addition, L-asparagine is utilized at 6-fold reduced k_{cat}/K_m by Glu-AdT (not shown). Extension of the backbone of **1** by a single carbon unit (**18**–**21**) may therefore disrupt interactions of the boronic acid with the catalytic serine, in addition to those of the C_α substituents.

The minimal growth-inhibition concentrations (MIC) for a representative panel of bacteria provides an index of whole cell antibacterial activity of these compounds (Table 2).[14] Included in this set is *S. pyogenes*, the natural source of the Glu-AdT utilized for in vitro enzyme assays. All of the test organisms except *E. coli* require Glu-AdT, as based on a functional or genomically-inferred lack of Glu-$tRNA^{Gln}$ synthetase. Hence, a specific in vivo inhibitor of Glu-AdT should impact all test organisms *except E. coli*.

The most potent Glu-AdT inhibitors (**1**–**3**) inhibited growth of most test bacteria that require Glu-AdT for growth (except *S. aureus*, vide infra). Notably, these compounds did not inhibit growth of *E. coli* tolC, a pump-defective strain,[15] or *E. coli* SM101 (lpxA2), an outer membrane permeability mutant,[16] consistent with the absence of Glu-AdT in this organism. Compounds with lesser in vitro efficacies showed little cellular activity except against *E. coli*. Likewise, **8**, which exhibited higher IC_{50} values than **1**–**3** and low MIC values for most organisms, also inhibited *E. coli*, indicating the activity may not be related to Glu-AdT inhibition. Thus, enzyme inhibition and bacterial growth inhibition correlate in this series, but only for inhibitors with $IC_{50} \leq 100$ nM.

A notable exception is the recalcitrance of multiple strains of *S. aureus* and other *Staphylococcus* species

towards even the best inhibitors. The basis of this effect is unclear. However, preliminary results show that **1** is a potent inhibitor of *S. aureus* Glu-AdT in vitro.[17] Therefore, the compounds must uniquely be exported, lack cell penetrance, and/or be metabolically processed by this genus. *H. pylori* is also less affected than anticipated, but qualitatively aligns with trends observed for *S. pyogenes*.

Compounds with (**1**–**3**) and without (**10**–**12**, **14**, and **16**) antibacterial properties were also surveyed for mammalian cellular toxicity.[18] IC_{50} values were >100 μM for all compounds in these assays.

In summary, replacement of the γ-carboxamide of Gln with boronic acid provides a route to potent inhibition of both glutaminase and (due to its reliance on Gln hydrolysis) net transferase activities of Glu-AdT. Indeed, **1** is the most potent inhibitor of Glu-AdT yet reported. Our results demonstrate a chemically narrow SAR with respect to the spacing of the boronic acid and C_α as well as simple modifications to the carboxyl and amino groups. In the absence of information on the three-dimensional active-site structure, further work is restricted on this class of compounds as antibacterials. However, we are encouraged by the apparent correlation between analyses for the best in vitro enzyme inhibitors, supportive of on-target in vivo effects, and the fact that mammalian cellular toxicity is not a generalized limitation of this compound class. Our results therefore validate Gln-AdT as a target and lend credence to a mechanism-based approach to the design of in vivo inhibitors of this enzyme.

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