Mechanism-Based Design of the First GlnA4-Specific Inhibitors

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γ-Glutamylamine synthetases are an important class of enzymes that play a key role in glutamate-based metabolism. Methionine sulfoximine (MSO) is a well-established inhibitor for the archetypal glutamine synthetase (GS) but inhibitors for most GS-like enzymes are unknown. Assuming a conserved catalytic mechanism for GS and GS-like enzymes, we explored if subtype-selective inhibitors can be obtained by merging MSO with the cognate substrates of the respective GS-like enzymes. Using GlnA4$_{Sc}$ from *Streptomyces coelicolor*, an enzyme recently shown to produce γ-glutamylethanolamine, we demonstrate that MSO can be reengineered in a straightforward fashion into potent and selective GlnA4$_{Sc}$ inhibitors. Linkage chemistry as well as linker length between the MSO moiety and the terminal hydroxyl group derived from ethanolamine were in agreement with the postulated phosphorylated catalytic intermediate. The best GlnA4 inhibitor **7b** potently blocked *S. coelicolor* growth in the presence of ethanolamine as the sole nitrogen source. Our results provide the first GlnA4$_{Sc}$-specific inhibitors and suggest a general strategy to develop mechanism-based inhibitors for GS-like enzymes.

Introduction

Glutamine synthetase (GS) is a highly conserved enzyme which is crucial for cellular nitrogen metabolism in all branches of life. GS catalyses the ATP-dependent conversion of glutamate to glutamine, thereby fixing free ammonia (Scheme 1a).

Many organisms, including major human pathogens, have evolved multiple additional GS-like enzymes to handle amine-containing substances. For example, *Mycobacterium tuberculosis* expresses three GS-like enzymes (GlnA2$_{Mt}$, GlnA3$_{Mt}$, GlnA4$_{Mt}$)[1] in addition to the archetypal GS (GlnA1$_{Mt}$ or MtGS) and *Pseudomonas aeruginosa* contains seven additional GS-like enzymes (PauA1-7).[2] Likewise, the actinobacterium *Streptomyces coelicolor* has three GS-like enzymes (GlnA2$_{Sc}$, GlnA3$_{Sc}$, GlnA4$_{Sc}$),[3] and the halophilic archaea *Haloferax mediterranei* harbours two GS-like enzymes (GlnA2$_{Hm}$, GlnA3$_{Hm}$).[4] GlnA3$_{Sc}$ and GlnA2$_{Hm}$ were recently shown to be putative glutamate polyamine ligases. We recently identified GlnA4 from *Streptomyces coelicolor* to be an ATP-dependent γ-glutamylethanolamine synthetase with a putative mechanism similar to GS (Scheme 1c).[5]

A universal inhibitor of GS is methionine sulfoximine (MSO **4**).[6] Inhibition of GS by MSO proceeds through a key intermediate, where MSO is N-phosphorylated by GS to produce P-MSO (**5**, Scheme 1b), which quasi-irreversibly traps GS in a non-processable transition state analogue-bound form.[7–10]

Intrigued by the putative mechanistic similarities between GS and GS-like enzymes we set out to explore if the peculiar inhibition mode of MSO could be extended to GS-like enzymes through tailored manipulation of the MSO scaffold (Scheme 1d).

Results and Discussion

Due to the known ATP-dependency of GlnA4$_{Sc}$ as well as high sequence homology in the ATP and glutamate binding sites of GlnA4$_{Sc}$ compared to GlnA1$_{Sc}$, GlnA4$_{Sc}$ was chosen as a model system for our studies. To achieve selectivity for GlnA4$_{Sc}$ we sought to address the postulated glutamate- and ethanolamine-recognizing subpockets of GlnA4$_{Sc}$ simultaneously. Towards this goal, two potential MSO modifications were considered: (i) Elongation of the methyl group of MSO by a 2-hydroxyethyl group (C-substituted MSO in Scheme 1d). This compound might be capable to be phosphorylated *in situ* by GlnA4$_{Sc}$ but it would lack the nitrogen corresponding to the amine of ethanolamine. (ii) Attachment of a 2-hydroxyethyl group to the sulfoximine nitrogen of MSO (N-substituted MSO in Scheme 1d), which would be a product analogue containing the joining nitrogen of γ-glutamylethanolamine. However, this compound would likely not be a substrate for *in situ* phosphorylation. *In silico* docking simulations with the target compound

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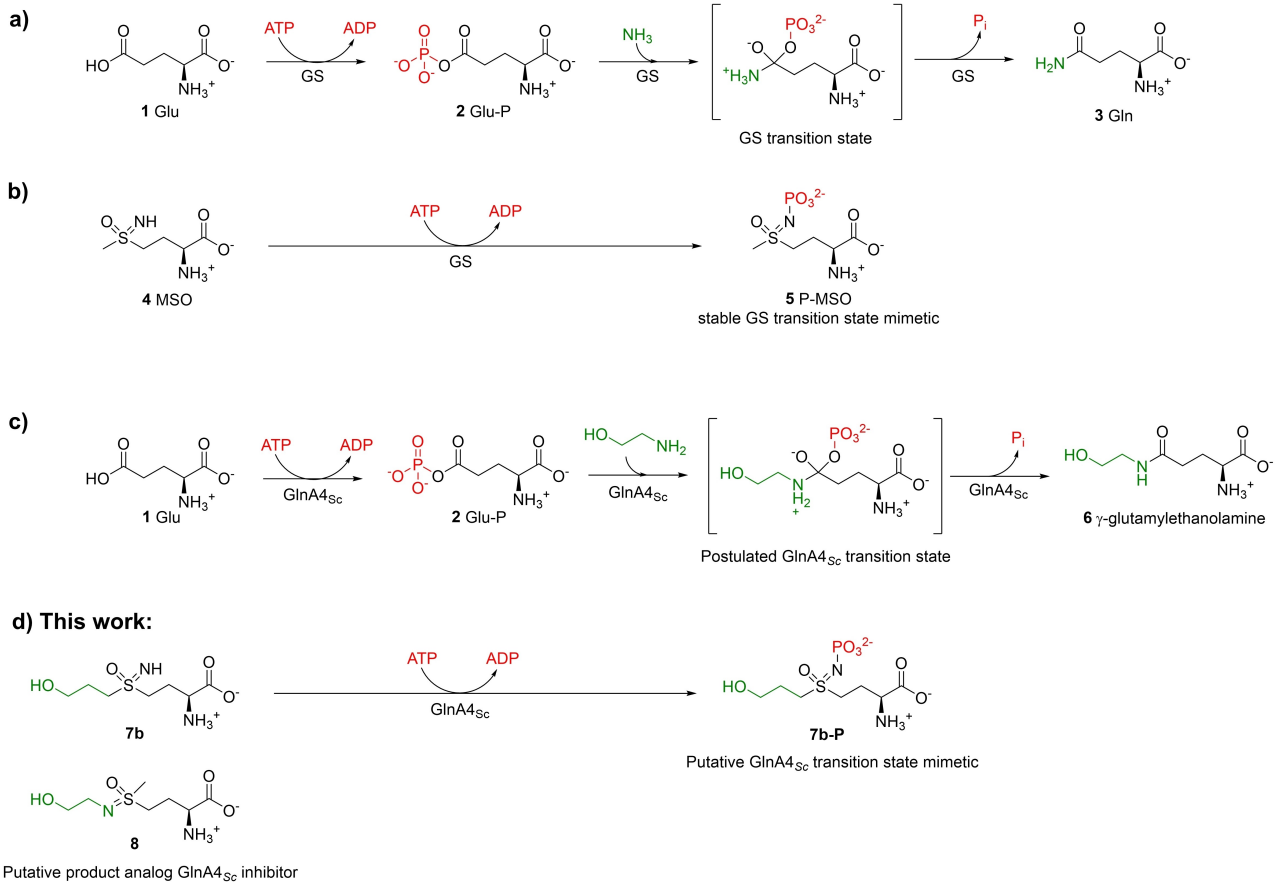
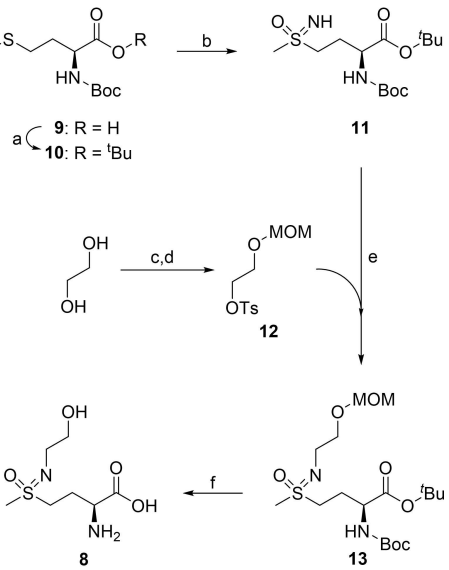


Scheme 1. a) Mechanism of GS-catalysed conversion of glutamate to glutamine. b) Mechanism of GS inhibition by MSO. c) Putative mechanism of GlnA4Sc-catalysed conversion of ethanolamine to γ-glutamylethanolamine. d) Proposed mechanism for putative GlnA4Sc inhibitors.

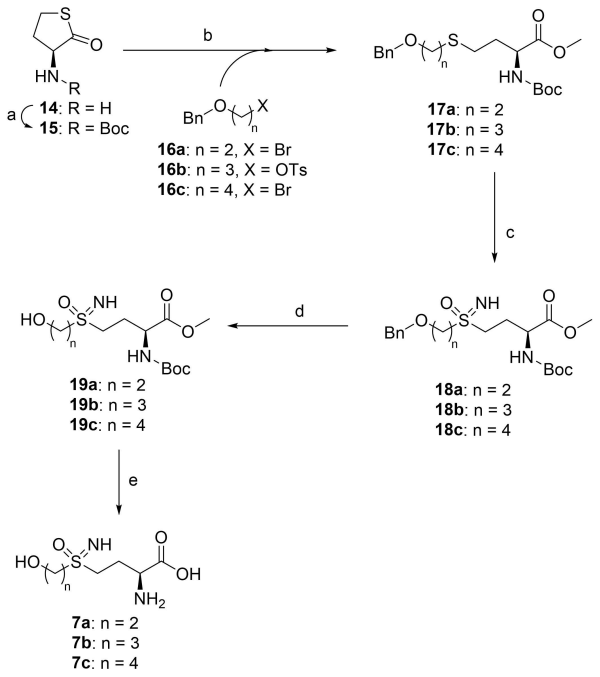
and a previously published $GlnA4_{Sc}$ model showed that our MSO analogues have the potential to bind the glutamate sub-pocket of $GlnA4_{Sc}$ in the same position as MSO with conserved putative key interactions. The designed inhibitor showed additional possible hydrogen bonds with its ethanolamine-mimicking moiety (Supporting Information Figure S1).

The N-substituted MSO-analogue **8** was synthesized from protected MSO, as shown in Scheme 2. Commercially available Boc-L-methionine **9** was protected as a tert-butyl ester, followed by oxidation and imination.[11] The diastereomers **11** (racemic at the sulfur) were alkylated with tosylate **12**. Acidic deprotection of 13 gave the N-substituted MSO-analogue **8** as a mixture of diastereomers (racemic at the sulfur atom). The diastereomeric ratio can be calculated from ^{13}C-NMR analysis (Supporting Information Figure S6), where most signals are shown as close duplicated peaks. Compound **8** showed a peak intensity ratio of 45:55.

The *C*-substituted MSO-analogues **7a–c** were synthesized from commercially available L-homocysteine thiolactone **14** (Scheme 3). After Boc-protection, thiolactone **15** was opened by sodium methoxide and directly alkylated with tosylate **16b** or bromides **16a** or **16c**. Transformation of the sulfides **17a–c** to the sulfoximines was performed according to Zenzola *et al.*,[11] again resulting in an inseparable mixture of diastereomers.



Scheme 2. Synthesis of *N*-substituted MSO **8**. Reagents and conditions: (a) ^{t}BuOH, DCC, DMAP, DCM, 0 °C–rt, 85%; (b) $MeCOONH_4$, PIDA, MeOH, rt, 87%; (c) TsCl, TEA, DCM, rt; (d) MOMCl, DIPEA, DCM, rt, 23% over two steps; (e) **12**, $NaHCO_3$, MeCN, reflux, 14%; (f) TFA, DCM, rt, 80%.



Scheme 3. Synthesis of C-substituted MSO variants **7a-c**. Reagents and conditions: (a) Boc_2O, $NaHCO_3$, THF/H_2O (1:1), 29%; (b) NaOMe, MeOH, rt, then $Br(CH_2)_2OBn$ (**16a**) or $TsO(CH_2)_3OBn$ (**16b**) or $Br(CH_2)_4OBn$ (**16c**), rt, 59–71%; (c) PIDA, $MeCOONH_4$, MeOH, rt; (d) Pd/C, H_2, EtOH, rt, 8–44% over 2 steps; (e) LiOH, THF/H_2O (1:1), rt, then HCl, rt, 40–85%.

18a–c were then benzyl deprotected using Pd/C and H_2. Finally, the methyl esters and Boc groups were cleaved under basic and acidic conditions, respectively, to yield the *C*-substituted MSO-analogues **7a–c** as mixtures of diastereomers (racemic at the sulfur atom). The diastereomeric ratio can again be calculated from ^{13}C-NMR (Supporting Information Figure S3-5), which is 40:60 for **7a**, 46:54 for **7b** and 43:57 for **7c**.

$GlnA4_{Sc}$ activity assay

The newly synthesized MSO-analogues **8** and **7a–c** were tested for their ability to inhibit purified $GlnA4_{Sc}$ in an enzymatic assay, in which the phosphate generation is colorimetrically measured to reflect enzyme activity.[3] Figure 1a shows the inability of MSO to inhibit $GlnA4_{Sc}$ activity, while $GlnA1_{Mt}$, an important glutamine synthetase representative, is inhibited with an IC_{50} of 201 μM (literature reference: 51 μM).[12] In contrast, compounds **7a–c** and **8** all inhibited $GlnA4_{Sc}$ (Figure 1b), whereas neither **7b** nor **8** showed activity against $GlnA1_{Mt}$ (Supporting Information Figure S2). This striking reversal of specificity suggests that the introduced 2-hydroxyethyl group is recognized by $GlnA4_{Sc}$ while it is sterically rejected by $GlnA1_{Mt}$.

The direct comparison of **7b** and **8** shows that a *C*-substituted attachment of the enanolamine mimetic is much better suited for $GlnA4_{Sc}$ inhibition than a *N*-substitution (IC_{50} of 12.3 μM (for **7b**) vs. 659 μM (for **8**), **7a–c** show IC_{50} values of 2166, 12.3 and 79.3 μM, respectively). This strongly suggests the generation of an intermediate with a phosphorylated sulfoximine nitrogen atom for **7b**, similar to the binding mode of MSO. This postulated phosphorylated sulfoximine species as a transition state mimetic seems to inhibit much stronger than the more product-like **8**, which contains a more authentic ethanolamine moiety. Among the three *C*-substituted MSO analogues, **7b** is the best inhibitor, where the three-carbon chain corresponds to the three-atom distance between the hydroxy group and Cγ in the product γ-glutamylethanolamine. A longer chain as in **7c** is also tolerated (IC_{50} = 79.3 μM) but a shortening to a two-carbon linker as in **7a** reduced inhibitory activity substantially (IC_{50} = 2166 μM). The structure-activity relationship is thus consistent with the recognition pattern of the authentic substrate γ-glutamylethanolamine.

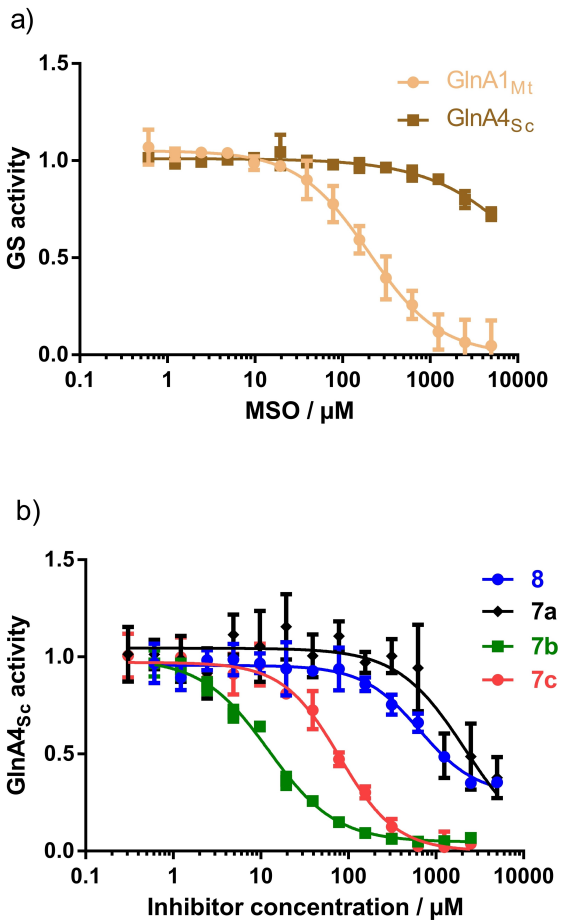


Figure 1. a) $GlnA1_{Mt}$ and $GlnA4_{Sc}$ activity at different MSO concentrations. b) $GlnA4_{Sc}$ activity at different inhibitor concentrations.

S. coelicolor growth assay

The antibacterial activity of the most potent $GlnA4_{Sc}$ inhibitor **7b** was analysed in a growth assay with *S. coelicolor*. Ethanolamine was provided as the sole nitrogen source to induce expression of GlnA4. Under these conditions growth of *S. coelicolor* depends on efficient ethanolamine utilization.[5] The

inhibitory effect of **7b** was visualised using phase-contrast microscopy (Figure 2).

Addition of **7b** reduced the size of the mycelial agglomerates already at the lowest tested concentration (0.78 μM, Figure 2B). The hyphae thickness can be used to quantify the growth of *S. coelicolor*. Without inhibitor (Figure 2A), the hyphae has an average thickness of 1.01 μm. At 0.78 μM **7b** (Figure 2B), it is only 0.77 μm thick. Higher concentrations dramatically blocked the growth of *S. coelicolor* and at 1.6 μM (Figure 2C) and above no mycelium can be observed, only spores or spore germination. The growth inhibition was clearly GlnA4-dependent, since additional glutamine as an alternative nitrogen source suppressed this effect, rescuing the growth of *S. coelicolor* at 100 μM **7b** (Figure 2J), with a hyphae thickness of 1.11 μm. This control experiment also excludes any non-specific toxic effects of compound **7b** even at 100 μM.

Conclusion

To summarize, we developed a generally applicable synthesis for MSO analogues bearing terminal substituents at the sulfoximine moiety. The attachment of a $GlnA4_{Sc}$-substrate mimicking moiety to MSO resulted in a novel, specific inhibitor of the γ-glutamylating enzyme $GlnA4_{Sc}$, homologs of which can be found in pathogenic actinobacteria like *M. tuberculosis*.

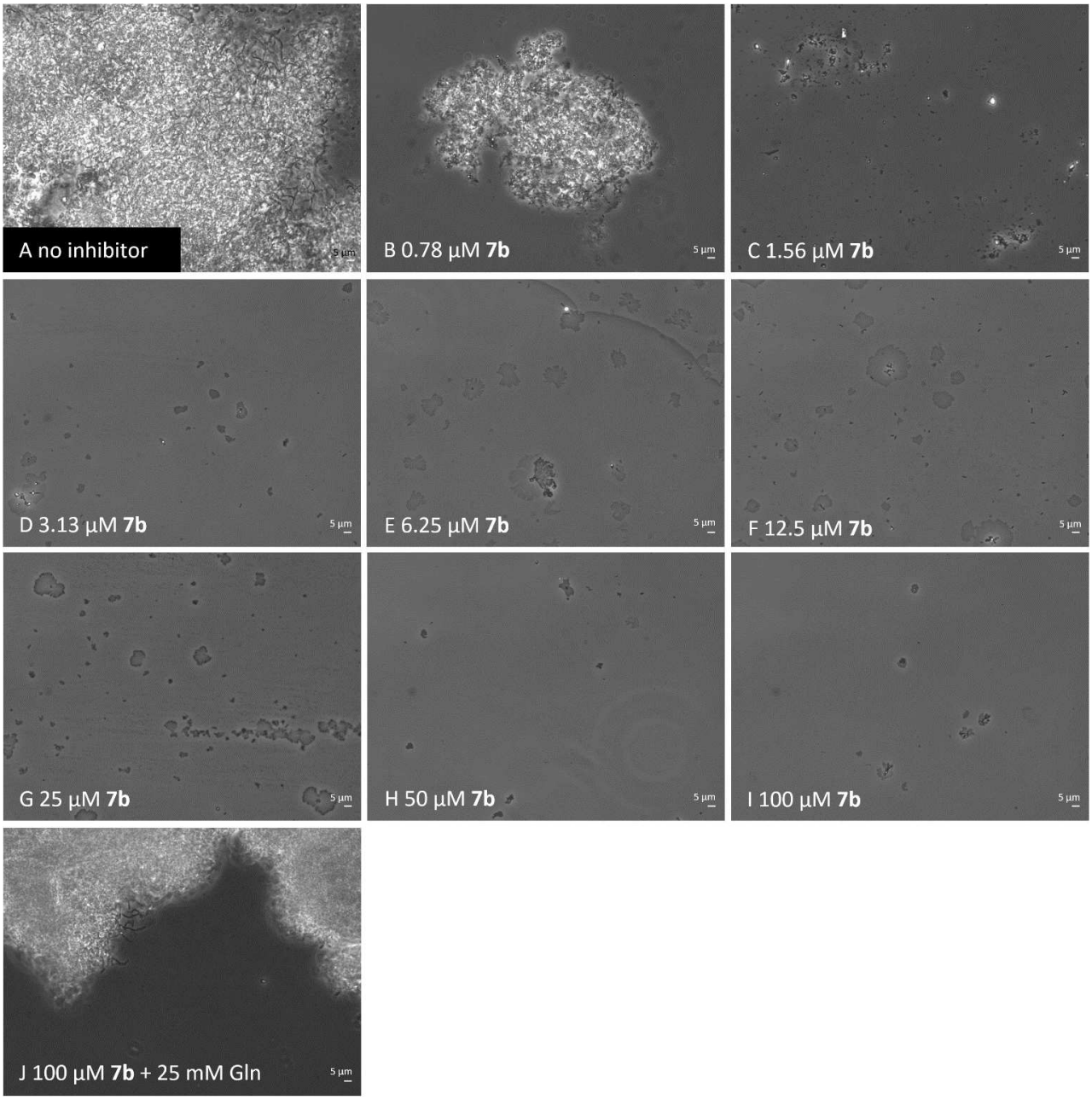


Figure 2. *S. coelicolor* growth with different concentrations of inhibitor **7b** using ethanolamine as a nitrogen source.

Sulfoximines have received substantial recent interest among medicinal chemists. Our results suggest that sulfoximines could have a broader and more active role as transition state mimetics for the class of ATP-dependent acid-amide synthases (EC 6.3). For GlnA4, the methyl group of the MSO sulfoximine emerged as a better attachment point for MSO analogues, underscoring the inhibitory power of the postulated phosphorylated intermediate. Our best $GlnA4_{Sc}$ ligand **7b** inhibits the enzyme with an IC_{50} value of 12.3 μM *in vitro* and stops *S. coelicolor* growth dose-dependently. Taken together, our compounds represent the first inhibitors for the GS-like enzyme GlnA4 and suggest a generally applicable strategy to design inhibitors for mechanistically similar enzymes.

Experimental Section

General information: Air- and water-sensitive reactions were performed under argon atmosphere with commercially available dry solvents. All commercially available chemicals and solvents were used as received. Silica gel column chromatography was performed on silica (SiO_2) from Macherey-Nagel (particle size 0.04–0.063 mm). Thin-layer chromatography (TLC) was performed on precoated aluminium plates with a fluorescence indicator from Merck (silica gel 60 F_{254}). ^{1}H-NMR and ^{13}C-NMR spectra were measured by the NMR Department of the Technical University Darmstadt, either on a 300 MHz Avance II spectrometer or a 500 MHz spectrometer DRX 500 from Bruker BioSpin GmbH. Chemical shifts are given in parts per million (ppm) and are referenced to residual solvents (^{1}H-NMR: $CDCl_3$, $\delta = 7.26$ ppm; D_2O, $\delta = 4.79$ ppm; ^{13}C-NMR: $CDCl_3$, $\delta = 77.16$ ppm.). Coupling constants (*J*) are given in hertz (Hz). All final compounds are >95% pure by ^{1}H-NMR. LC–MS (liquid chromatography - mass spectrometry) was performed on a Beckman Coulter System Gold 126 solvent module, Beckman Coulter System 508 autosampler and Beckman Coulter System Gold 166 detector over a YMC-Pack Pro C8 3 μm 120 A, 100x4.6 mm column connected to a Thermo Finnigan LCQ Deca XP Plus. Eluents were 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B), the used method was 5% B to 95% B in 19 min. High-resolution mass spectrometry (HRMS) was measured by the MS Department of the Technical University Darmstadt on a Bruker Daltonics Impact II mass spectrometer (quadrupole time-of-flight). Cation exchange solid phase extraction was performed on Dowex 50WX2 100–200 (H) resin. The resin was treated with 1 M NaOH, washed three times with water, then equilibrated with 1 M HCl and again washed three times with water. The sample was loaded in 0.1 M HCl, washed three times with water and eluted with 30% ammonia.

Enzyme activity assay: To determine the inhibitory effect of MSO and our GlnA4 inhibitors on the enzymatic reaction catalysed by $GlnA1_{Mt}$ or $GlnA4_{Sc}$, a modified GS activity assay previously described by Gawronski and Benson[13] was adopted. In brief, a serial dilution of the respective inhibitor in assay buffer (20 mM HEPES (pH 8.0), 50 mM $MgCl_2$) was placed in a 384-well microplate and incubated for 10 minutes with 40 nM purified His-$GlnA1_{Mt}$ or 2 μM purified His-$GlnA4_{Sc}$. To start the enzymatic reaction a substrate mixture containing 25 mM sodium glutamate monohydrate, 2.5 mM ATP and 50 mM ammonium chloride or 50 mM ethanolamine hydrochloride was added and the plate was incubated for 1 hour at 30 °C. The reaction was stopped by addition of an equal volume (30 μL) of a solution consisting of 2 parts 12% w/v L-ascorbic acid in 1 M HCl and 1 part 2% ammonium molybdate in H_2O. After 5 minutes colour development was stopped by addition of 30 μL of a solution containing 2% sodium citrate tribasic and 2% acetic acid in H_2O. Raw absorbance readings at 635 nm were normalized to enzymatic activity in the absence of inhibitors, and the 50% inhibitory concentration (IC_{50}) value was determined using 4PL-fit implemented in Prism 6.

Bacterial growth assay: The *S. coelicolor* M145 strain was grown in defined Evans medium (modified after Evans et al., 1970).[14] Evans medium was supplemented with 25 mM ammonium chloride, L-glutamine or ethanolamine hydrochloride as a sole nitrogen source (as described by Krysenko *et al.*[5]). Additionally, the media were supplemented with inhibitors in following final concentrations: 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.125 μM, 1.562 μM, 0.781 μM, 0 μM. Bacteria were incubated for 7 days at 30 °C on a rotary shaker (180 rpm). Phase-contrast microscopic pictures were taken under x400 magnification.

Molecular docking: Modelling of ligand-protein interactions was performed using the UCSF Chimera software[15] and AutoDockVina pipeline.[16] The $GlnA4_{Sc}$ model structure was based on the GSI_{St} template (PDB: 1FPY) and is identical with the model used in the study by Krysenko *et al.*[5] Structures were generated using MolView or PabChem Sketcher. The interaction network between residues at the active site of GlnA4 was analysed by searching for hydrophobic interactions inside respective structures as well as by computing possible contacts/clashes between molecules using UCSF Chimera software.

(*S*)-*tert*-Butyl 2-((*tert*-butoxycarbonyl)amino)-4-(methylthio)butanoate (10): Boc-L–Methionine (3.64 g, 14.60 mmol, 1.0 eq.) and DMAP (200 mg, 1.64 mmol, 0.1 eq.) were dissolved in DCM (150 mL) under argon atmosphere. *tert*-Butanol (2.64 mL, 28.19 mmol, 1.9 eq.) was added and the mixture was cooled to 0 °C. DCC (3.79 g, 18.37 mmol, 1.3 eq.) was added and the reaction mixture was allowed to warm to room temperature. After 18 h, the colourless precipitate was filtered off and the filtrate was washed with 1 M HCl, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (Cy/EA 9:1) to yield **10** (3.78 g, 85%) as a yellowish oil. $R_f = 0.44$ (Cy/EA 1:1); ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 1.14$ (s, 9H), 1.46 (s, 9H), 1.79–1.99 (m, 1H), 1.99–2.18 (m, 4H), 2.41–2.62 (m, 2H), 4.26 (d, 1H, $J = 4.6$ Hz), 5.10 ppm (d, 1H, $J = 5.2$ Hz); ^{13}C-NMR (75 MHz, $CDCl_3$): $\delta = 15.6$, 28.1, 28.5, 30.1, 32.8, 53.6, 79.9, 82.2, 155.4, 171.5 ppm.

(2*S*)-*tert*-Butyl 2-((*tert*-butoxycarbonyl)amino)-4-(methylsulfonimidoyl)butanoate (11): **10** (3.73 g, 12.21 mmol, 1.0 eq.), ammonium acetate (1.88 g, 24.42 mmol, 2.0 eq.) and PIDA (9.87 g, 30.64 mmol, 2.5 eq.) were dissolved in MeOH (150 mL) and stirred at room temperature. After 22 h the solvent was removed *in vacuo* and the residue was purified by silica gel column chromatography (EA) to afford **11** (3.58 g, 87%) as a yellowish solid. $R_f = 0.13$ (EA); ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 1.41$ (s, 9H), 1.44 (s, 9H), 2.04–2.18 (m, 1H), 2.26–2.44 (m, 1H), 2.97 (s, 3H), 3.02–3.27 (m, 2H), 4.14–4.36 (m, 1H), 5.21–5.44 ppm (m, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): $\delta = 26.6$, 28.0, 28.4, 43.0, 52.7, 53.5, 80.3, 83.0, 155.5, 170.5 ppm.

2-(Methoxymethoxy)ethyl 4-methylbenzenesulfonate (12): Ethylene glycol (4.40 mL, 78.68 mmol, 4.9 eq.) was dissolved in DCM (100 mL) under argon atmosphere. TEA (4.36 mL, 31.27 mmol, 1.9 eq.) and TsCl (3.07 g, 16.10 mmol, 1.0 eq.) were added and the reaction mixture was stirred at room temperature for 16 h. It was washed with water, the organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (Cy/EA 3:1–1:1) to afford 2-hydroxyethyl 4-methylbenzenesulfonate (1.44 g) as an intermediate. 2-hydroxyethyl 4-methylbenzenesulfonate (506 mg, 2.34 mmol, 1.0 eq.) was dissolved in DCM (25 mL) under argon atmosphere.

DIPEA (1.20 mL, 7.06 mmol, 3.0 eq.) and MOMCl (880 μL, 11.59 mmol, 5.0 eq.) were added and the reaction mixture was stirred at room temperature. After 26 h it was washed with water, the organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (Cy/EA 3:1) to afford **12** (319 mg, 23% over two steps). $R_f = 0.65$ (Cy/EA 1:1); ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 2.45$ (s, 3H), 3.31 (s, 3H), 3.71 (t, 2H, $J = 4.8$ Hz), 4.19 (t, 2H, $J = 4.8$ Hz), 4.56 (s, 2H), 7.34 (d, 2H, $J = 8.1$ Hz), 7.80 ppm (d, 2H, $J = 8.2$ Hz).

(2*S*)-*tert*-Butyl 2-((*tert*-butoxycarbonyl)amino)-4-(N-(2-(methoxymethoxy)ethyl)-methylsulfonimidoyl)butanoate (13): **11** (111 mg, 330 μmol, 1.0 eq.), **12** (118 mg, 453 μmol, 1.4 eq.) and K_2CO_3 (99 mg, 716 μmol, 2.2 eq.) were dissolved in MeCN (30 mL) and refluxed for 6 days. After cooling to room temperature, the precipitate was filtered off and the filtrate was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography three times (EA, DCM/MeOH 20:1, DCM/MeOH 30:1) to afford **13** (19 mg, 14%) as a colourless solid. $R_f = 0.47$ (DCM/MeOH 20:1); ^{1}H-NMR (500 MHz, $CDCl_3$): $\delta = 1.43$ (s, 9H), 1.46 (s, 9H), 2.04–2.18 (m, 2H), 2.26–2.43 (m, 2H), 2.93–2.98 (m, 3H), 3.26 (t, 2H, $J = 5.7$ Hz), 3.34–3.37 (m, 3H), 3.60-3.65 (m, 2H), 4.23 (s, 1H), 4.65 (d, 2H, $J = 3.9$ Hz), 5.36 ppm (dd, 1H, $J = 44.9/7.2$ Hz); ^{13}C-NMR (125 MHz, $CDCl_3$): $\delta = 26.9$, 28.1, 28.4, 40.1, 43.0, 51.0, 52.8, 55.4, 69.3, 80.3, 82.9, 96.7, 155.6, 170.6 ppm.

(2*S*)-2-Amino-4-(*N*-(2-hydroxyethyl)-methylsulfonimidoyl)butanoic acid (8): **13** (19 mg, 45 μmol, 1.0 eq.) was dissolved in DCM (5 mL) and TFA (2.0 mL, 26.0 mmol, 580 eq.) was added. The reaction mixture stirred at room temperature for 40 h, then the solvent was removed *in vacuo* and the residue was dissolved again in 15% aq. NH_4OH. After 15 h at room temperature, the solvent was removed *in vacuo* and the crude product was purified by cation exchange solid phase extraction to afford **8** (8 mg, 80%) as a colourless solid. ^{1}H-NMR (500 MHz, D_2O): $\delta = 2.16$–2.38 (m, 2H), 3.17 (s, 3H), 3.21 (td, 2H, $J = 5.8/2.2$ Hz), 3.39–3.58 (m, 2H), 3.68 (t, 2H, $J = 5.7$ Hz), 3.70–3.77 ppm (m, 1H); ^{13}C-NMR (125 MHz, D_2O): $\delta = 26.0$, 38.2, 44.6, 49.4, 53.6, 62.4 ppm; HRMS (ESI): m/z calculated for $C_7H_{17}N_2O_4S$ $[M+H]^+ = 225.09035$, found $[M+H]^+ = 225.09046$.

(*S*)-*tert*-Butyl (2-oxotetrahydrothiophen-3-yl)carbamate (15): L-Homocysteine thiolactone **14** (210 mg, 1.55 mmol, 1.0 eq.) was dissolved in THF/H_2O (1:1, 10 mL). $NaHCO_3$ (375 mg, 4.46 mmol, 2.9 eq.) and Boc_2O (350 μL, 1.64 mmol, 1.1 eq.) were added and the mixture was stirred at room temperature. After 18 h and then after another 7 h, additional Boc_2O (each 200 μL, 0.93 mmol, 0.6 eq.) was added. The reaction mixture was diluted with water and extracted with DCM. The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (Cy/EA 3:1) to afford **15** (107 mg, 29%) as a colourless solid. $R_f = 0.44$ (Cy/EA 2:1); ^{1}H-NMR (500 MHz, $CDCl_3$): $\delta = 1.42$ (s, 9H), 1.86–2.04 (m, 1H), 2.67–2.90 (m, 1H), 3.12–3.38 (m, 2H), 4.09–4.39 (m, 1H), 4.84–5.21 ppm (s, 1H) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): $\delta = 27.3$, 28.4, 32.1, 60.6, 80.4, 155.6, 205.3 ppm.

3-(Benzyloxy)propyl 4-methylbenzenesulfonate (16b): 3-(Benzyloxy)propan-1-ol (952 μL, 6.02 mmol, 1.0 eq.) was dissolved in DCM (40 mL) under argon atmosphere. Pyridine (1.20 mL, 14.87 mmol, 2.5 eq.) and TsCl (2.36 g, 12.38 mmol, 2.1 eq.) were added and the reaction mixture was stirred at room temperature. After 16 h, TsCl (1.03 g, 5.40 mmol, 0.9 eq.) and NaH (60% in mineral oil, 1.16 g, 29.30 mmol, 4.9 eq.) were added. The mixture was stirred for 3 days, then washed with water, the organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (Cy/EA 5:1–3:1) to afford **16b** (448 mg, 25%) as a red liquid. $R_f = 0.53$ (Cy/EA 3:1); ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 1.97$ (tt, 2H, $J = 6.1$ Hz), 2.45 (s, 3H), 3.53 (t, 2H, $J = 6.0$ Hz), 4.20 (t, 2H, $J = 6.2$ Hz), 4.44 (s, 2H), 7.24-7.40 (m, 7H), 7.82 ppm (d, 2H, $J = 8.4$ Hz).

(*S*)-Methyl 4-((2-(benzyloxy)ethyl)thio)-2-((*tert*-butoxycarbonyl)amino)butanoate (17a): **15** (255 mg, 1.17 mmol, 1.0 eq.) and NaOMe (382.1 mg, 7.07 mmol, 6 eq.) were dissolved in MeOH (20 ml) and stirred at room temperature. After 1 h, Benzyl-2-bromoethylether **16a** (273 mg, 1.27 mmol, 1.1 eq.) was added. After 16 h, the reaction mixture was extracted with ethyl acetate, the organic phase was dried over $MgSO_4$, the solvent evaporated *in vacuo* and the crude product was purified by silica gel column chromatography (Cy/EA 5:1) to afford **17a** (315 mg, 71%) as a colourless solid. $R_f = 0.20$ (Cy/EA 5:1); ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta =$ 1.37 (s, 9H), 1.73–1.95 (m, 1H), 1.96–2.11 (m, 1H), 2.53 (t, J = 7.6 Hz, 2H), 2.66 (t, 2H, $J = 6.6$ Hz), 3.56 (t, 2H, $J = 6.6$ Hz), 3.65 (s, 3H), 4.31 (s, 1H), 4.47 (s, 2H), 5.06 (s, 1H), 7.15-7.33 ppm (m, 5H); ^{13}C-NMR (75 MHz, $CDCl_3$): $\delta = 28.4$, 28.5, 31.8, 32.9, 52.4, 52.9, 70.0, 73.1, 80.1, 127.8, 128.5, 138.2, 155.4, 172.8 ppm.

(*S*)-Methyl 4-((3-(benzyloxy)propyl)thio)-2-((*tert*-butoxycarbonyl)amino)butanoate (17b): **15** (215 mg, 1.00 mmol, 1.0 eq.) and NaOMe (134 mg, 2.48 mmol, 2.5 eq.) were dissolved in dry MeOH (20 mL) under argon atmosphere. After 30 min at room temperature, **16b** (477 mg, 1.49 mmol, 1.5 eq.) was added. After 18 h, more NaOMe (145 mg, 2.68 mmol, 2.7 eq.) was added and the mixture was stirred for another 2 h. Water was added and it was extracted with EA. The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (Cy/EA 5:1) twice to afford **17b** (231 mg, 59%) as a yellowish oil. $R_f = 0.46$ (Cy/EA 3:1); ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 1.45$ (s, 9H), 1.82–2.00 (m, 3H), 2.03–2.20 (m, 1H), 2.55 (t, 2H, $J = 7.5$ Hz), 2.63 (t, 2H, $J = 7.3$ Hz), 3.56 (t, 2H, $J =$ 6.2 Hz), 3.74 (s, 3H), 4.41 (s, 1H), 4.51 (s, 2H), 5.14 (s, 1H), 7.21–7.42 ppm (m, 5H); ^{13}C-NMR (125 MHz, $CDCl_3$): $\delta = 28.0$, 28.4, 28.9, 29.8, 32.8, 52.5, 52.9, 68.8, 73.1, 80.1, 127.7, 127.7, 128.5, 138.5, 155.4, 172.9 ppm.

(*S*)-Methyl 4-((4-(benzyloxy)butyl)thio)-2-((*tert*-butoxycarbonyl)amino)butanoate (17c): **15** (250 mg, 1.15 mmol, 1.0 eq.) and NaOMe (193 mg, 3.57 mmol, 3.1 eq.) were dissolved in MeOH (20 ml) and stirred at room temperature. After 1 h, Benzyl-4-bromobutylether **16c** (330 μL, 1.73 mmol, 1.5 eq.) was added. After 19 h, NaOMe (113 mg, 2.09 mmol, 1.8 eq.) was added and it was stirred for another 5 h. The rection mixture was extracted with ethyl acetate, the organic phase was dried over $MgSO_4$, the solvent evaporated *in vacuo* and the crude product was purified by silica gel column chromatography (Cy/EA 9:1) to afford **17c** (281 mg, 59%) as a colourless oil. $R_f = 0.51$ (Cy/EA 9:1); ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 1.46$ (s, 9H), 1.60–1.79 (m, 4H), 1.83–1.99 (m, 1H), 2.03–2.18 (m 1H), 2.48–2.61 (m, 3H), 3.49 (t, 2H, J = 5.9 Hz), 3.74 (s, 3H), 4.32–4.47 (m, 1H), 4.51 (s, 2H), 5.09–5.21 (m, 1H), 7.25–7.38 ppm (m, 5H); ^{13}C-NMR (75 MHz, $CDCl_3$): $\delta = 26.3$, 27.8, 28.3, 28.9, 31.9, 32.7, 52.4, 52.8, 69.7, 72.9, 80.0, 127.5, 127.6, 128.4, 138.5, 155.3, 172.8 ppm.

(2*S*)-Methyl 4-(2-(benzyloxy)ethylsulfonimidoyl)-2-((*tert*-butoxycarbonyl)amino) butanoate (18a): **17a** (315 mg, 0.79 mmol, 1.0 eq.), $MeCOONH_4$ (126.24 mg, 1.64 mmol, 2.1 eq.) and PIDA (650 mg, 2.02 mmol, 2.6 eq.) were dissolved in methanol (25 ml) and stirred at room temperature for 18 h. The solvent was evaporated and the crude product was purified using silica gel column chromatography (Cy/EA 1:2-EA). Product **18a** (239 mg) remained impure and was used in the next reaction as it was.

(2S)-Methyl 4-(2-(benzyloxy)ethylsulfonimidoyl)-2-((*tert*-butoxycarbonyl)amino) butanoate (19a): Crude **18a** (239 mg, 0.58 mmol, 1.0 eq.) was dissolved in ethanol (20 ml), degassed with argon for 10 minutes and put under an argon atmosphere.

Palladium on carbon, 10 wt-% loading (613 mg, 0.58 mmol, 1.0 eq.) was added, hydrogen was introduced to the reaction mixture and it was stirred at room temperature. After 10 minutes, the mixture was put under a hydrogen atmosphere and continued to stir for additional 15 h. The palladium on carbon was then filtered over celite, the solvent evaporated and the crude product was purified using silica gel column chromatography (EA/MeOH 20:1) to afford **19a** (20 mg, 8% over two steps) as a colourless oil. $R_f = 0.15$ (EA/MeOH 20:1); ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 1.43$ (s, 9H), 2.10–2.26 (m, 1H), 2.25–2.54 (m, 1H), 3.11–3.47 (m, 6H), 3.76 (s, 3H), 3.96–4.24 (m, 2H), 4.40 (s, 1H), 5.45 ppm (s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): $\delta =$ 19.2, 28.4, 51.2, 52.3, 52.8, 54.5, 61.4, 80.4, 155.7, 172.1 ppm.

(2*S*)-Methyl 4-(3-(benzyloxy)propylsulfonimidoyl)-2-((*tert*-butoxycarbonyl)amino)butanoate (18b): **17b** (231 mg, 581 μmol, 1.0 eq.), $MeCOONH_4$ (99 mg, 1.28 mmol, 2.2 eq.) and PIDA (527 mg, 1.64 mmol, 2.8 eq.) were dissolved in MeOH (20 mL) and stirred at room temperature for 16 h. The solvent was removed *in vacuo* and the crude product was purified by silica gel column chromatography (Cy/EA 1:1 - EA). Product **18b** (302 mg) still showed some impurities and was used in the next step without further purification.

(2*S*)-Methyl 2-((*tert*-butoxycarbonyl)amino)-4-(3-hydroxypropylsulfonimidoyl)butanoate (19b): Crude **18b** (302 mg, 705 μmol, 1.0 eq.) was dissolved in EtOH (10 mL) under argon atmosphere and degassed by introducing argon in the solution for 10 min. Pd/C (10 wt-%, 618 mg, 581 μmol, 0.8 eq.) was added and H_2 was introduced in the solution for 10 min. The reaction mixture stirred for 1 h under H_2 atmosphere, then it was filtered over celite. The solvent was evaporated *in vacuo* and the crude product was purified by silica gel column chromatography (EA + 5% MeOH) to afford **19b** (86 mg, 44% over two steps) as a colourless oil. $R_f = 0.09$ (EA + 5% MeOH); ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 1.42$ (s, 9H), 2.03–2.46 (m, 4H), 3.16–3.26 (m, 3H), 3.26–3.40 (m, 2H), 3.75 (s, 3H), 4.38 (s, 1H), 5.54 ppm (s, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$): $\delta =$ 25.6, 28.4, 29.8, 51.7, 52.2, 52.3, 52.9, 60.5, 80.6, 155.7, 172.0 ppm.

(2*S*)-Methyl 4-(4-(benzyloxy)butylsulfonimidoyl)-2-((*tert*-butoxycarbonyl)amino) butanoate (18c): **17c** (281 mg, 0.70 mmol, 1.0 eq.), $MeCOONH_4$ (109 mg, 1.41 mmol, 2 eq.) and PIDA (576 mg, 1.79 mmol, 2.6 eq.) were dissolved in methanol (20 ml) and stirred at room temperature for 18 h. The solvent was evaporated *in vacuo* and the crude product was purified using silica gel column chromatography (Cy/EA 2:1-EA). Product **18c** (173 mg) remained impure and was used in the next reaction as it was.

(2*S*)-Methyl 2-((*tert*-butoxycarbonyl)amino)-4-(4-hydroxybutylsulfonimidoyl)butanoate (19c): Crude **18c** (173 mg, 0.39 mmol, 1.0 eq.) was dissolved in ethanol (20 ml), stirred at room temperature and argon was introduced for 10 min. The reaction mixture was held under an argon atmosphere and palladium on carbon, 10 wt-% loading (462 mg, 434 mmol, 1.1 eq.) was added. Hydrogen was introduced to the reaction mixture for 10 min and afterwards held under a hydrogen atmosphere and stirred at room temperature for 6 h. The solvent was evaporated *in vacuo* and the crude product was purified by silica gel column chromatography (EA/MeOH 20:1-EA/MeOH 10:1) to afford **19c** (53 mg, 22% over two steps) as a colourless oil. $R_f = 0.10$ (EA/MeOH 20:1); ^{1}H-NMR (300 MHz, $CDCl_3$): $\delta = 1.40$ (s, 9H), 1.57–1.74 (m, 2H), 1.83–1.98 (m, 2H), 2.06–2.21 (m, 1H), 2.25–2.44 (m, 1H), 2.95–3.22 (m, 5H), 3.56–5.69 (t, J = 6 Hz, 2H), 3.70–3.80 (s, 1H), 4.26 (s, 1H), 5.55–5.70 ppm (s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): $\delta =$ 19.03, 25.58, 28.20, 30.81, 51.07, 52.12, 52.60, 54.31, 61.19, 80.28, 155.49, 171.88 ppm.

(2*S*)-2-Amino-4-(2-hydroxyethylsulfonimidoyl)butanoic acid (7a): **19a** (20 mg, 0.06 mmol, 1.0 eq.) and LiOH (40 mg, 1.67 mmol, 28 eq.) were dissolved in a mixture of THF/H_2O (1:1, 5 ml) and stirred at room temperature for 18 h. The reaction mixture was acidified by addition of 1 M HCl and washed with EA. The aqueous phase was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (EA/MeOH 10:1 - MeOH/AcOH 50:1) and then using a cation exchange solid phase extraction to afford **7a** (11 mg, 85%) as a colourless solid. $R_f = 0.09$ (MeOH/AcOH 50:1); ^{1}H-NMR (500 MHz, D_2O): $\delta = 2.37–2.57$ (m, 2H), 3.42–3.67 (m, 4H), 3.90–4.00 (m, 1H), 4.07–4.17 ppm (m, 2H); ^{13}C-NMR (125 MHz, D_2O): $\delta =$ 23.8, 51.7, 53.3, 55.7, 56.1, 173.1 ppm. HRMS (ESI): m/z calculated for $C_6H_{15}N_2O_4S$ $[M+H]^+ = 211.07470$, found $[M+H]^+ = 211.07482$.

(2*S*)-2-Amino-4-(3-hydroxypropylsulfonimidoyl)butanoic acid (7b): **19b** (86 mg, 254 μmol, 1.0 eq.) was dissolved in THF/H_2O (1:1, 10 mL) and LiOH (16 mg, 668 μmol, 2.6 eq.) was added. After 14 h at room temperature, the solution was acidified by addition of 1 M HCl and washed with EA. The aqueous phase was concentrated *in vacuo*. The crude product was purified first by silica gel column chromatography (EA/MeOH/AcOH 95:5:1- MeOH/AcOH 50:1) and then by cation exchange solid phase extraction to afford **7b** (23 mg, 40%) as a colourless solid. $R_f = 0.14$ (MeOH/AcOH 50:1); ^{1}H-NMR (500 MHz, D_2O): $\boldsymbol{\delta} = 2.01–2.13$ (m, 2H), 2.29–2.46 (m, 2H), 3.31–3.54 (m, 4H), 3.76 (t, 2H, $J = 6.2$ Hz), 3.82–3.90 ppm (m, 1H); ^{13}C-NMR (125 MHz, D_2O): $\delta =$ 24.2, 24.7, 50.2, 50.8, 53.3, 59.7, 174.1 ppm. HRMS (ESI): m/z calculated for $C_7H_{17}N_2O_4S$ $[M+H]^+ = 225.0904$, found $[M+H]^+ = 225.0905$.

(2*S*)-2-Amino-4-(4-hydroxybutylsulfonimidoyl)butanoic acid (7c): **19c** (53 mg, 150 μmol, 1 eq.) was dissolved in THF/H_2O (1:1, 20 mL) and LiOH (10 mg, 417 μmol, 2.8 eq.) was added and stirred at room temperature for 3 h. The reaction mixture was acidified by addition of 1 M HCl and washed with EA. The aqueous phase was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (EA/MeOH 10:1 - MeOH/AcOH 50:1) and then using a cation exchange solid phase extraction to afford **7c** (22 mg, 61%) as a colourless solid. $R_f = 0.05$ (DCM/MeOH 10:1); ^{1}H-NMR (500 MHz, D2O): $\delta = 1.70$-1.81 (m, 2H), 1.84–1.97 (m, 2H), 2.30–2.46 (m, 2H), 3.26–3.44 (m, 3H) 3.44–3.55 (m, 1H), 3.68 (t, 2H, $J =$ 6.5 Hz), 3.88–3.97 ppm (m, 1H); ^{13}C-NMR (125 MHz, D2O): $\delta =$ 18.6, 23.7, 30.1, 50.0, 53.2, 53.4, 60.1, 173.1 ppm. HRMS (ESI): m/z calculated for $C_8H_{19}N_2O_4S$ $[M+H]^+ = 239.10600$, found $[M+H]^+ =$ 239.10606.

Abbreviations: MSO: L–Methionine sulfoximine; GS: Glutamine synthetase; *Sc*: *Streptomyces coelicolor*; ATP: Adenosine triphosphate; *Mt*: *Mycobacterium tuberculosis*; *Hm*: *Haloferax mediterranei*; *St*: *Salmonella typhimurium*.

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Conflict of Interest

The authors declare no conflict of interest.

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