Mechanistic Basis for ATP-Dependent Inhibition of Glutamine Synthetase by Tabtoxinine-β-Lactam

Supporting Information

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I. Supplementary Tables

Supplementary Table 1. Strains and plasmids used in this work.

Strain	Plasmid	Inducible Gene/Marker	Origin/Reference
Pseudomonas syringae pv. *tabaci* ATCC *11528*	None	Tabtoxin producer	ATCC
E. coli ATCC 29522	None	Antibiotic Susceptibility Testing	ATCC
E. coli TOP10	None	Cloning strain	Invitrogen
E. coli BL21 (DE3)	None	Protein expression strain	Agilent
E. coli BL21 (DE3)	pET28a	*GlnA* from *E. coli*	This Work
E. coli BL21 (DE3)	pET28a	*GlnA* from *S. aureus*	This work
E. coli BL21 (DE3)	pET28a	*GlnA* from *H. sapiens*	This work
E. coli TOP10	pET28a	*GlnA* from *E. coli*	This work
E. coli TOP10	pET28a	*GlnA* from *S. aureus*	This work
E. coli TOP10	pET28a	*GlnA* from *H. sapiens*	This work

Supplementary Table 2. Codon optimized glutamine synthetase genes used in this work.

Gene	Source	Codon Optimized Nucleotide Sequence
GlnA	*E. coli*	ATGAGCGCGGAGCACGTGCTGACCATGCTGAACGAGCACGAAGTGAAGTTCGTTGACCTGCGTTTTACCGATACCAAG GGTAAAGAGCAGCACGTGACCATCCCGGCGCACCAAGTTAACGCGGAATTCTTTGAGGAAGGTAAAATGTTTGACGGC AGCAGCATCGGTGGCTGGAAAGGCATTAACGAAAGCGACATGGTGCTGATGCCGGATGCGAGCACCGCGGTTATCGAC CCGTTCTTTGCGGATAGCACCCTGATCATTCGTTGCGATATTCTGGAGCCGGGTACCCTGCAGGGTTATGACCGTGAT CCGCGTAGCATCGCGAAACGTGCGGAAGACTATCTGCGTAGCACCGGTATTGCGGATACCGTGCTGTTTGGTCCGGAG CCGGAATTCTTTCTGTTCGACGATATCCGTTTTGGTAGCAGCATTAGCGGCAGCCACGTTGCGATCGACGATATTGAG GGTGCGTGGAACAGCAGCACCCAATACGAAGGTGGCAACAAGGGTCACCGTCCGGCGGTGAAAGGTGGCTATTTTCCG GTGCCGCCGGTTGACAGCGCGCAGGATATCCGTAGCGAGATGTGCCTGGTTATGGAACAAATGGGTCTGGTGGTTGAA GCGCACCACCATGAAGTTGCGACCGCGGGTCAGAACGAGGTTGCGACCCGTTTCAACACCATGACCAAGAAAGCGGAC GAAATCCAAATTTACAAGTATGTGGTTCACAACGTTGCGCACCGTTTCGGCAAGACCGCGACCTTTATGCCGAAACCG ATGTTCGGCGACAACGGTAGCGGCATGCACTGCCACATGAGCCTGAGCAAGAACGGTGTGAACCTGTTTGCGGGTGAT AAATACGCGGGCCTGAGCGAGCAGGCGCTGTACTATATCGGTGGCGTTATTAAGCACGCGAAAGCGATCAACGCGCTG GCGAACCCGACCACCAACAGCTACAAGCGTCTGGTGCCGGGTTATGAGGCGCCGGTTATGCTGGCGTATAGCGCGCGT AACCGTAGCGCGAGCATCCGTATTCCGGTGGTTAGCAGCCCGAAAGCGCGTCGTATTGAAGTTCGTTTTCCGGATCCG GCGGCGAACCCGTATCTGTGCTTTGCGGCGCTGCTGATGGCGGGTCTGGATGGCATCAAGAACAAAATTCACCCGGGC GAGGCGATGGACAAGAACCTGTATGATCTGCCGCCGGAGGAAGCGAAAGAAATTCCGCAAGTGGCGGGCAGCCTGGAG GAAGCGCTGAACGAGCTGGACCTGGATCGTGAATTTCTGAAAGCGGGTGGCGTTTTCACCGACGAAGCGATCGATGCG TACATTGCGCTGCGTCGTGAGGAAGACGATCGTGTGCGTATGACCCCGCACCCGGTTGAGTTCGAACTGTACTATAGC GTTTAA
GlnA	*S. aureus*	ATGCCGAAACGTACCTTCACCAAGGAAGACATTCGTAAATTTGCGGAGGAAGAGAACGTGCGTTACCTGCGTCTGCAG TTCACCGATATCCTGGGTACCATTAAGAACGTGGAAGTTCCGGTTAGCCAACTGGAAAAAGTTCTGGACAACGAGATG ATGTTCGATGGTAGCAGCATTGAGGGCTTTGTGCGTATCGAAGAGAGCGACATGTACCTGCACCCGGACCTGGATACC TGGGTGATTTTTCCGTGGACCGCGGGTCAGGGCAAGGTTGCGCGTCTGATCTGCGACGTGTATAAAACCGATGGTACC CCGTTTGAGGGCGATCCGCGTGCGAACCTGAAACGTGTTCTGAAGGAAATGGAGGACCTGGGTTTCACCGATTTTAAC CTGGGCCCGGAACCGGAGTTCTTTCTGTTCAAACTGGACGAAAAGGGCGAGCCGACCCTGGAACTGAACGACGATGGT GGCTACTTTGACCTGGCGCCGACCGATCTGGGTGAAAACTGCCGTCGTGATATTGTTCTGGAACTGGAGGACATGGGT TTTGATATCGAAGCGAGCCACCATGAGGTTGCGCCGGGTCAGCACGAAATCGACTTCAAATATGCGGATGCGGTGACC GCGTGCGACAACATTCAAACCTTTAAACTGGTGGTTAAGACCATCGCGCGTAAGCACAACCTGCACGCGACCTTCATG CCGAAACCGCTGTTTGGTGTTAACGGTAGCGGCATGCACTTCAACGTGAGCCTGTTTAAGGGTAAAGAGAACGCGTTC TTTGATCCGAACACCGAAATGGGCCTGACCGAGACCGCGTACCAATTCACCGCGGGTGTTCTGAAGAACGCGCGTGGC TTTACCGCGGTTTGCAACCCGCTGGTGAACAGCTATAAACGTCTGGTGCCGGGTTACGAAGCGCCGTGCTATATTGCG TGGAGCGGCAAGAACCGTAGCCCGCTGATCCGTGTTCCGAGCAGCCGTGGCCTGAGCACCCGTATTGAAGTTCGTAGC GTGGATCCGGCGGCGAACCCGTACATGGCGCTGGCGGCGATTCTGGAAGCGGGTCTGGATGGCATCAAGAACAAACTG AAGGTTCCGGAGCCGGTGAACCAGAACATTTACGAAATGAACCGTGAAGAGCGTGAGGCGGTTGGTATCCAAGACCTG CCGAGCACCCTGTATACCGCGCTGAAGGCGATGCGTGAAAACGAGGTGATCAAGAAAGCGCTGGGCAACCACATCTAT AACCAGTTCATTAACAGCAAAAGCATCGAATGGGACTACTATCGTACCCAAGTGAGCGAATGGGAGCGTGATCAGTAC ATGAAGCAATATTAA
GlnA	*H. sapiens*	ATGACCACCAGCGCGAGCAGCCACCTGAACAAGGGCATCAAACAGGTGTACATGAGCCTGCCGCAGGGTGAAAAGGTT CAAGCGATGTATATCTGGATTGACGGTACCGGCGAGGGTCTGCGTTGCAAGACCCGTACCCTGGATAGCGAACCGAAA TGCGTGGAGGAACTGCCGGAGTGGAACTTCGACGGCAGCAGCACCCTGCAAAGCGAAGGTAGCAACAGCGATATGTAC CTGGTTCCGGCGGCGATGTTCCGTGACCCGTTTCGTAAGGATCCGAACAAACTGGTGCTGTGCGAAGTTTTCAAGTAT AACCGTCGTCCGGCGGAGACCAACCTGCGTCACACCTGCAAACGTATCATGGACATGGTTAGCAACCAGCACCCGTGG TTTGGCATGGAGCAAGAATACACCCTGATGGGCACCGATGGTCACCCGTTCGGTTGGCCGAGCAACGGTTTTCCGGGT CCGCAGGGTCCGTACTATTGCGGTGTGGGTGCGGACCGTGCGTATGGCCGTGATATCGTTGAAGCGCACTACCGTGCG TGCCTGTATGCGGGCGTGAAAATTGCGGGTACCAACGCGGAAGTGATGCCGGCGCAGTGGGAATTCCAAATCGGTCCG TGCGAGGGCATTAGCATGGGTGACCACCTGTGGGTTGCGCGTTTTATTCTGCACCGTGTGTGCGAGGACTTCGGCGTT ATCGCGACCTTTGATCCGAAGCCGATTCCGGGTAACTGGAACGGCGCGGGTTGCCACACCAACTTCAGCACCAAGGCG ATGCGTGAGGAAAACGGTCTGAAATACATCGAGGAAGCGATTGAAAAGCTGAGCAAACGTCACCAATACCACATCCG TGCGTATGACCCGAAAGGTGGCCTGGATAACGCGCGTCGTCTGACCGGCTTCCACGAGACCAGCAACATTAACGACTT TAGCGCGGGTGTGGCGAACCGTAGCGCGAGCATCCGTATTCCGCGTACCGTTGGCCAGGAGAAGAAAGGTTACTTCGA AGACCGTCGTCCGAGCGCGAACTGCGATCCGTTTAGCGTGACCGAGGCGCTGATCCGTACCTGCCTGCTGAACGAGAC CGGTGATGAACCGTTTCAATATAAGAACTAA

Supplementary Table 3. Primary protein sequence of GS homologs used in this work.

Gene	Source	Primary Protein Sequences of *N*-His$_6$-GS Homologs[a]
GlnA	*E. coli*	MGSSHHHHHHSSGLVPRGSHMSAEHVLTMLNEHEVKFVDLRFTDTKGKEQHVTIPAHQVNAEFFEEGKMFDGSSI GGWKGINESDMVLMPDASTAVIDPFFADSTLIIRCDILEPGTLQGYDRDPRSIAKRAEDYLRSTGIADTVLFGPEPEFFLF DDIRFGSSISGSHVAIDDIEGAWNSSTQYEGGNKGHRPAVKGGYFPVPPVDSAQDIRSEMCLVMEQMGLVVEAHHHEV ATAGQNEVATRFNTMTKKADEIQIYKYVVHNVAHRFGKTATFMPKPMFGDNGSGMHCHMSLSKNGVNLFAGDKYAG LSEQALYYIGGVIKHAKAINALANPTTNSYKRLVPGYEAPVMLAYSARNRSASIRIPVVSSPKARRIEVRFPDPAANPYLC FAALLMAGLDGIKNKIHPGEAMDKNLYDLPPEEAKEIPQVAGSLEEALNELDLDREFLKAGGVFTDEAIDAYIALRREE DDRVRMTPHPVEFELYYSV
GlnA	*S. aureus*	MGSSHHHHHHSSGLVPRGSHMPKRTFTKEDIRKFAEEENVRYLRLQFTDILGTIKNVEVPVSQLEKVLDNEMMFDG SSIEGFVRIEESDMYLHPDLDTWVIFPWTAGQGKVARLICDVYKTDGTPFEGDPRANLKRVLKEMEDLGFTDFNLGPE PEFFLFKLDEKGEPTLELNDDGGYFDLAPTDLGENCRRDIVLELEDMGFDIEASHHEVAPGQHEIDFKYADAVTACDNI QTFKLVVKTIARKHNLHATFMPKPLFGVNGSGMHFNVSLFKGKENAFFDPNTEMGLTETAYQFTAGVLKNARGFTAV CNPLVNSYKRLVPGYEAPCYIAWSGKNRSPLIRVPSSRGLSTRIEVRSVDPAANPYMALAAILEAGLDGIKNKLKVPEPV NQNIYEMNREEREAVGIQDLPSTLYTALKAMRENEVIKKALGNHIYNQFINSKSIEWDYYRTQVSEWERDQYMKQY
GlnA	*H. sapiens*	MGSSHHHHHHSSGLVPRGSHMTTSASSHLNKGIKQVYMSLPQGEKVQAMYIWIDGTGEGLRCKTRTLDSEPKCVEE LPEWNFDGSSTLQSEGSNSDMYLVPAAMFRDPFRKDPNKLVLCEVFKYNRRPAETNLRHTCKRIMDMVSNQHPWFG MEQEYTLMGTDGHPFGWPSNGFPGPQGPYYCGVGADRAYGRDIVEAHYRACLYAGVKIAGTNAEVMPAQWEFQIGPC EGISMGDHLWVARFILHRVCEDFGVIATFDPKPIPGNWNGAGCHTNFSTKAMREENGLKYIEEAIEKLSKRHQYHIRAY DPKGGLDNARRLTGFHETSNINDFSAGVANRSASIRIPRTVGQEKKGYFEDRRPSANCDPFSVTEALIRTCLLNETGDEP FQYKN

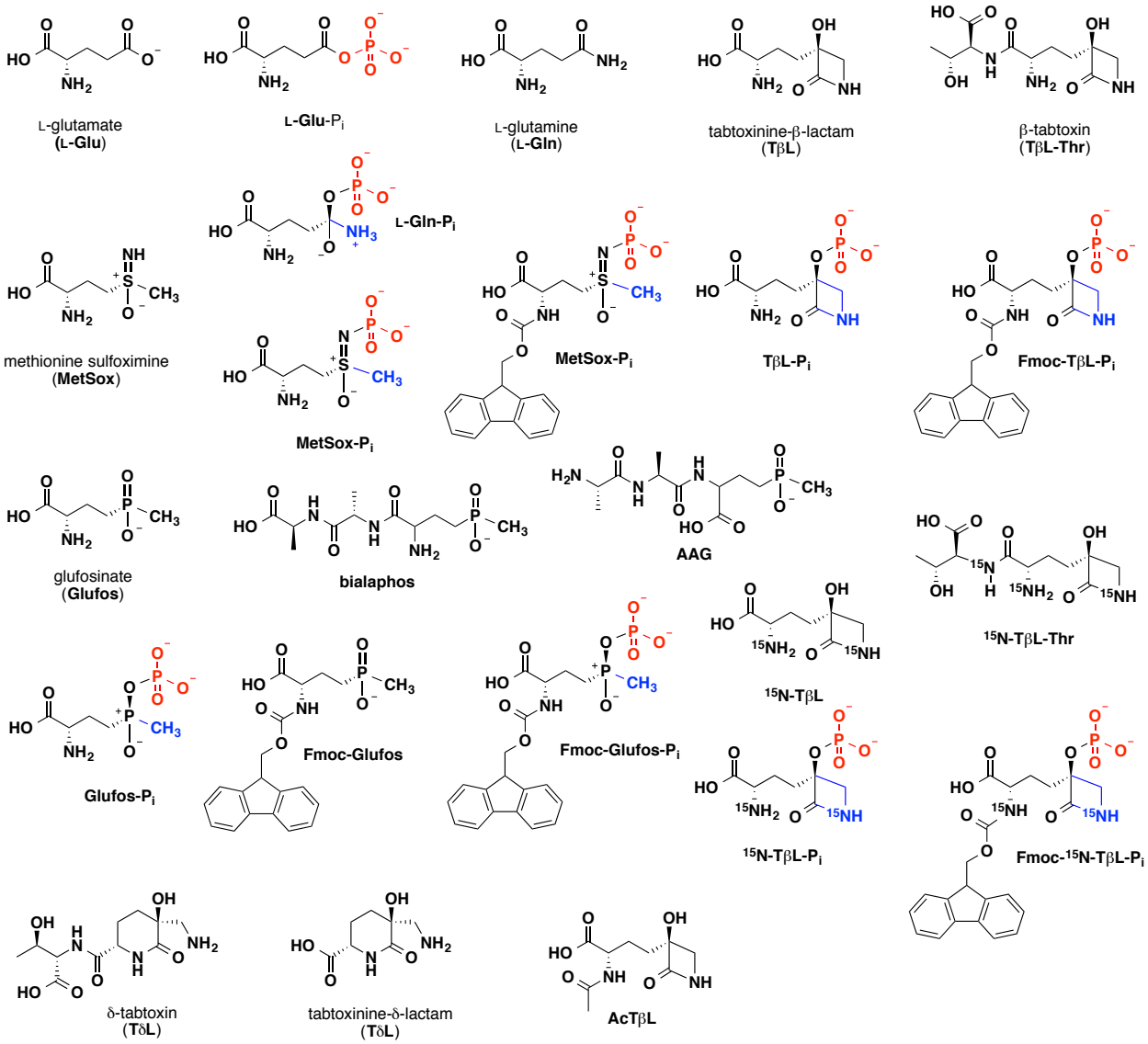
[a]Hexahistidine motif with thrombin cleavage site highlighted in yellow.

Supplementary Table 4. Percent sequence identity of GS homologs.

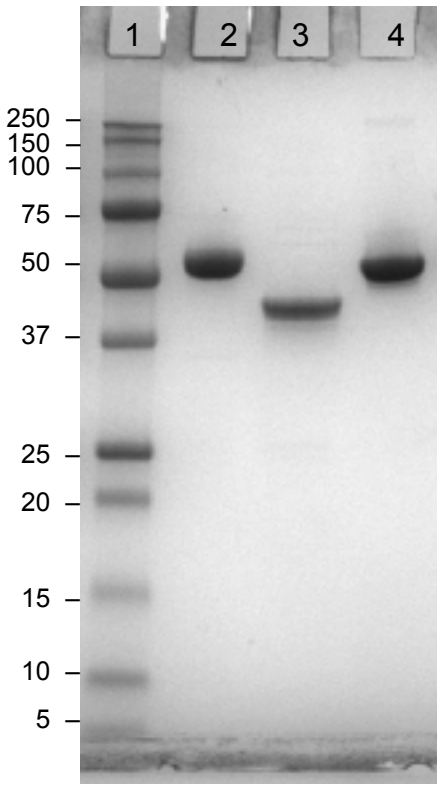
GS Homolog[a]	*E. coli*	*S. aureus*	Human
E. coli		40.5%	24.7%
S. aureus	40.5%		20.5%
Human	24.7%	20.5%	

[a]Percent sequence identities were determine using Clustal W.

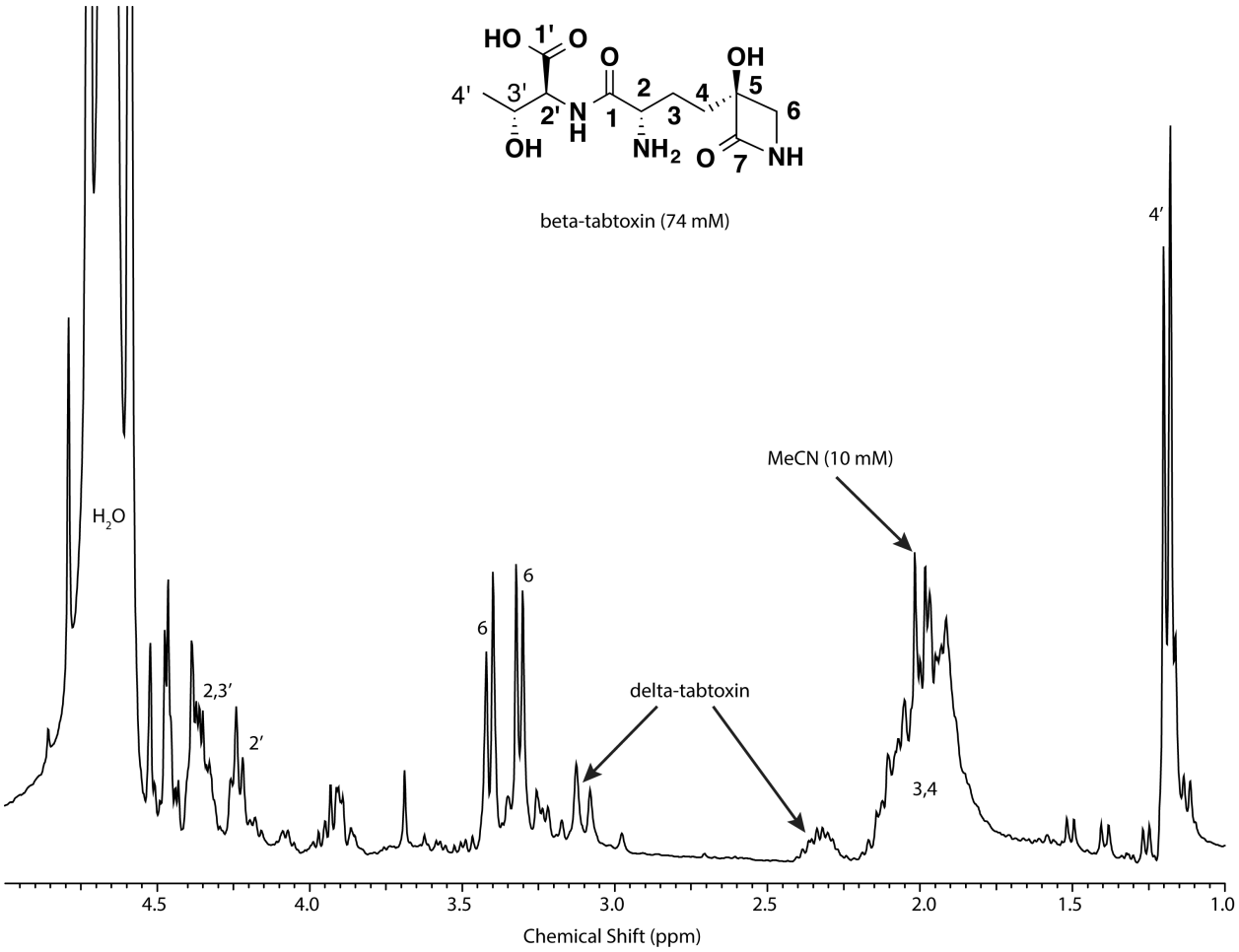
II. Supplementary Figures



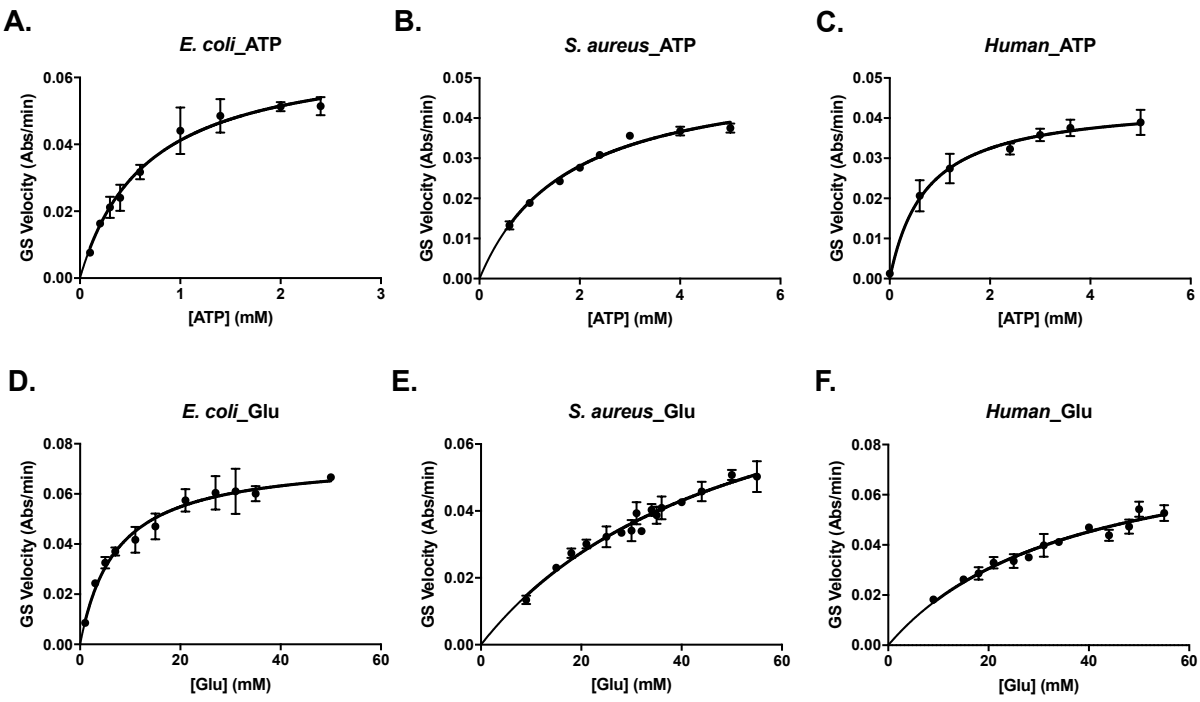
Supplementary Figure 1. Structures and abbreviations of compounds used in this work.



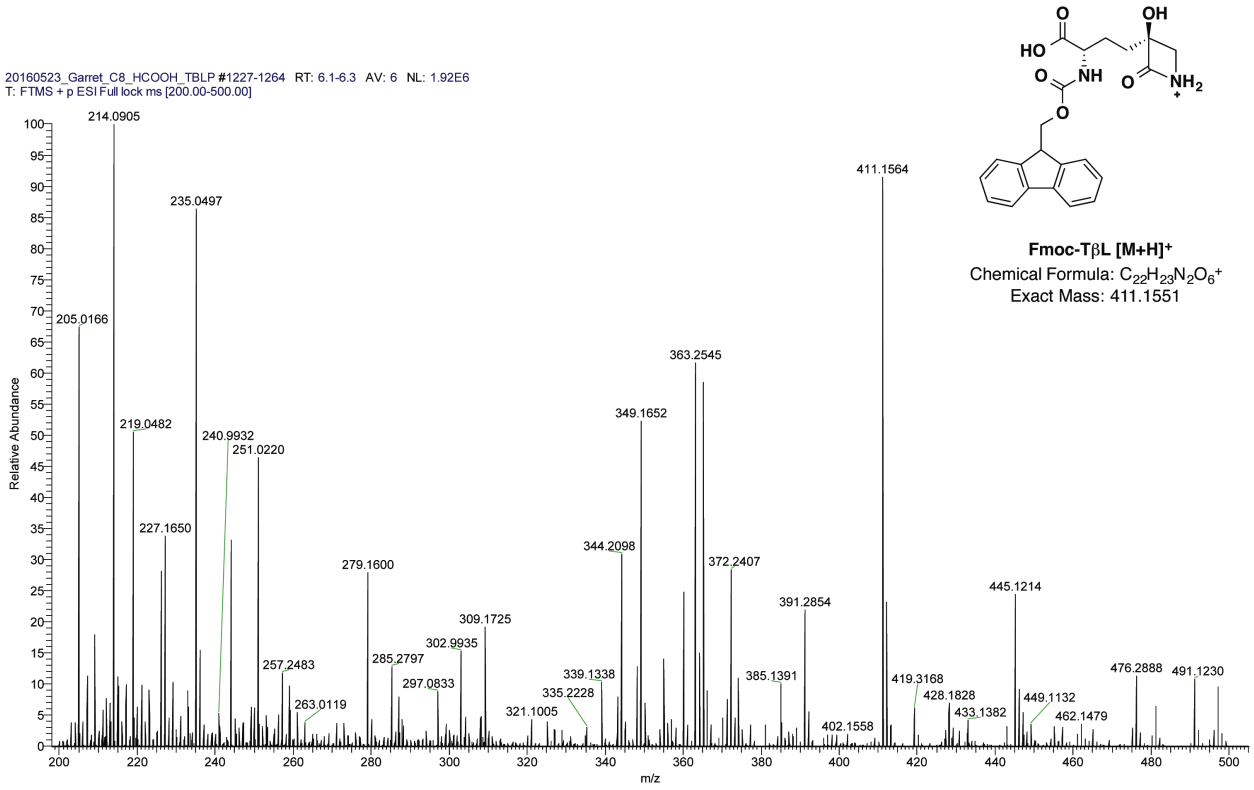
Supplementary Figure 2. SDS-PAGE analysis of purified *N*-His_6-GS homologs used in this work. SDS-PAGE gels (Any *k*D, Bio-Rad) were loaded with protein ladder (Precision Plus Protein Dual Xtra Prestained Protein Standards, Bio-Rad) in lane 1 and Ni-NTA elutions for recombinant *N*-His_6-GS from *E. coli* (54.1 kD), *H. sapiens* (44.2 kD), and *S. aureus* (53.0 kD) in lanes 2,3, and 4, respectively. Gel was stained with Coomassie blue.



Supplementary Figure 3. Representative ^{1}H-NMR (300 MHz) of quantified tabtoxin (74 mM) after HILIC chromatography taken in D_2O with an acetonitrile (10 mM) internal standard. For full purification and characterization of tabtoxin see our previous publication[1].



Supplementary Figure 4. Michaelis-Menten plots for GS homologs.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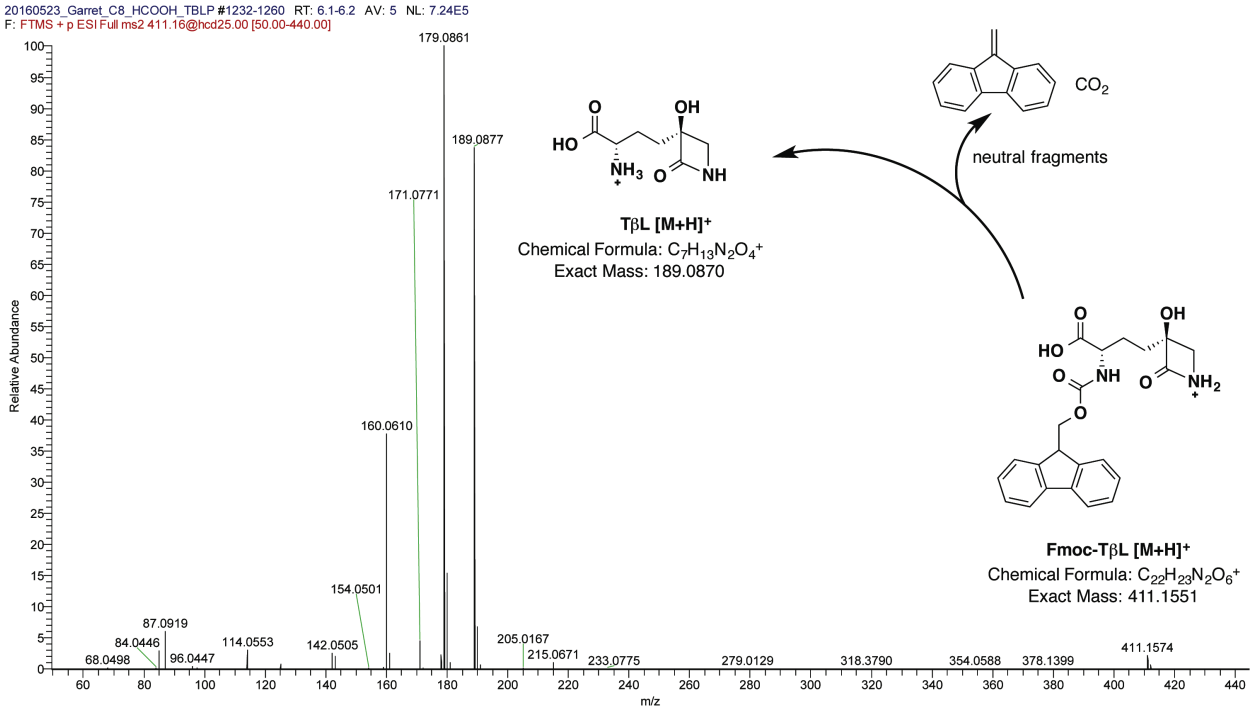
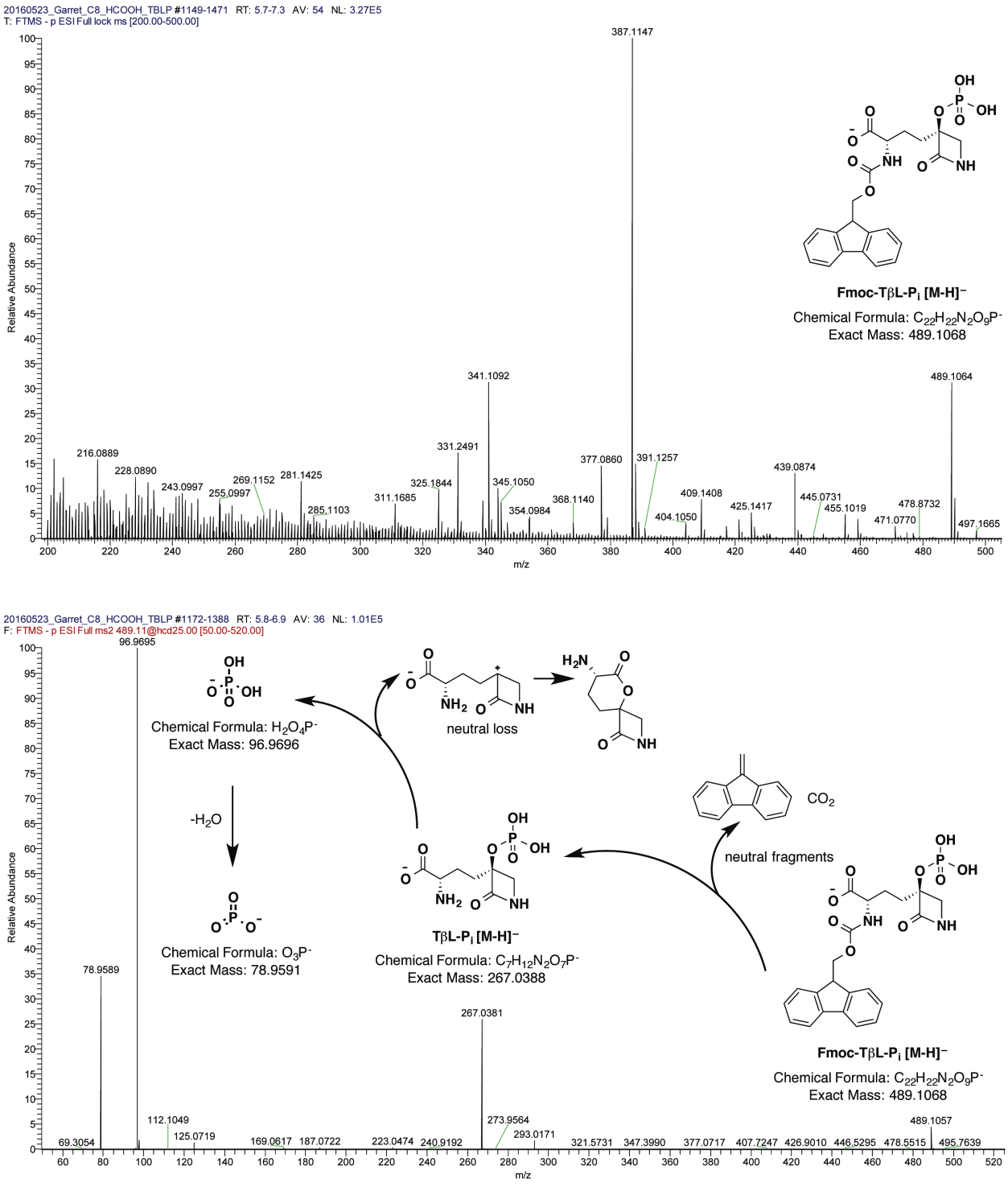
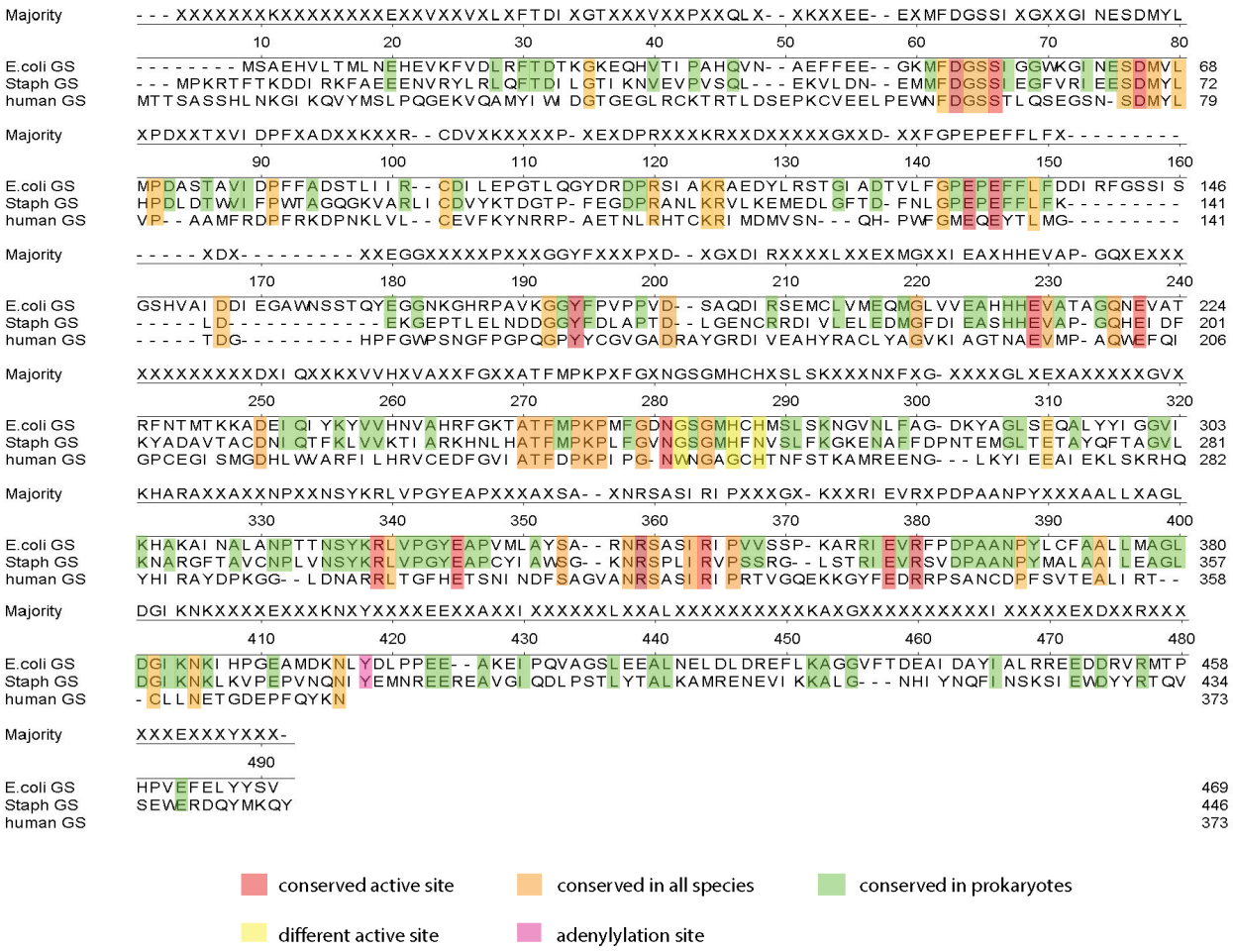
T: FTMS + p ESI Full lock ms [200.00-500.00]
Relative Abundance
m/z
214.0905
235.0497
411.1564
205.0166
363.2545
349.1652
219.0482
240.9932
251.0220
227.1650
344.2098
372.2407
279.1600
391.2854
445.1214
309.1725
302.9935
285.2797
257.2483
339.1338
385.1391
419.3168
476.2888
491.1230
297.0833
335.2228
428.1828
449.1132
263.0119
321.1005
433.1382
462.1479
402.1558
Fmoc-TβL [M+H]+
Chemical Formula: C22H23N2O6+
Exact Mass: 411.1551

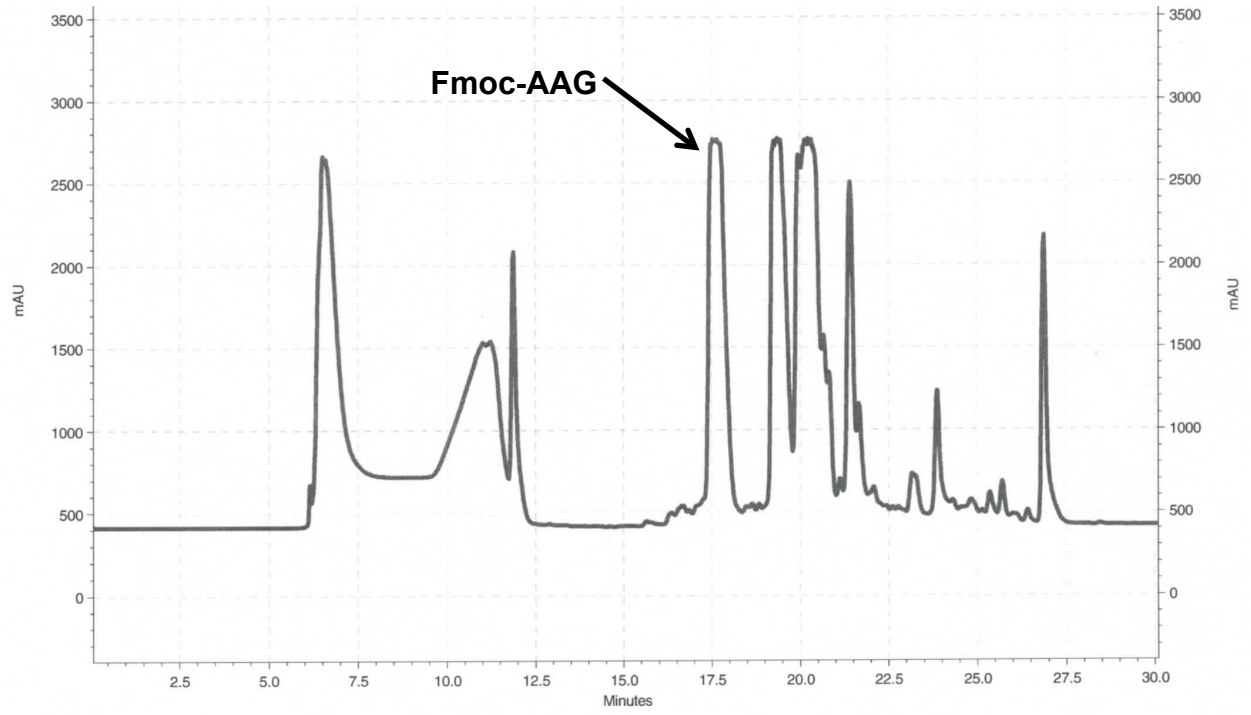

20160523_Garret_C8_HCOOH_TBLP #1232-1260 RT: 6.1-6.2 AV: 5 NL: 7.24E5
F: FTMS + p ESI Full ms2 411.16@hcd25.00 [50.00-440.00]
Relative Abundance
m/z
179.0861
189.0877
171.0771
160.0610
154.0501
87.0919
84.0446
68.0498
96.0447
114.0553
142.0505
205.0167
215.0671
233.0775
279.0129
318.3790
354.0588
378.1399
411.1574
TβL [M+H]+
Chemical Formula: C7H13N2O4+
Exact Mass: 189.0870
CO2
neutral fragments
Fmoc-TβL [M+H]+
Chemical Formula: C22H23N2O6+
Exact Mass: 411.1551



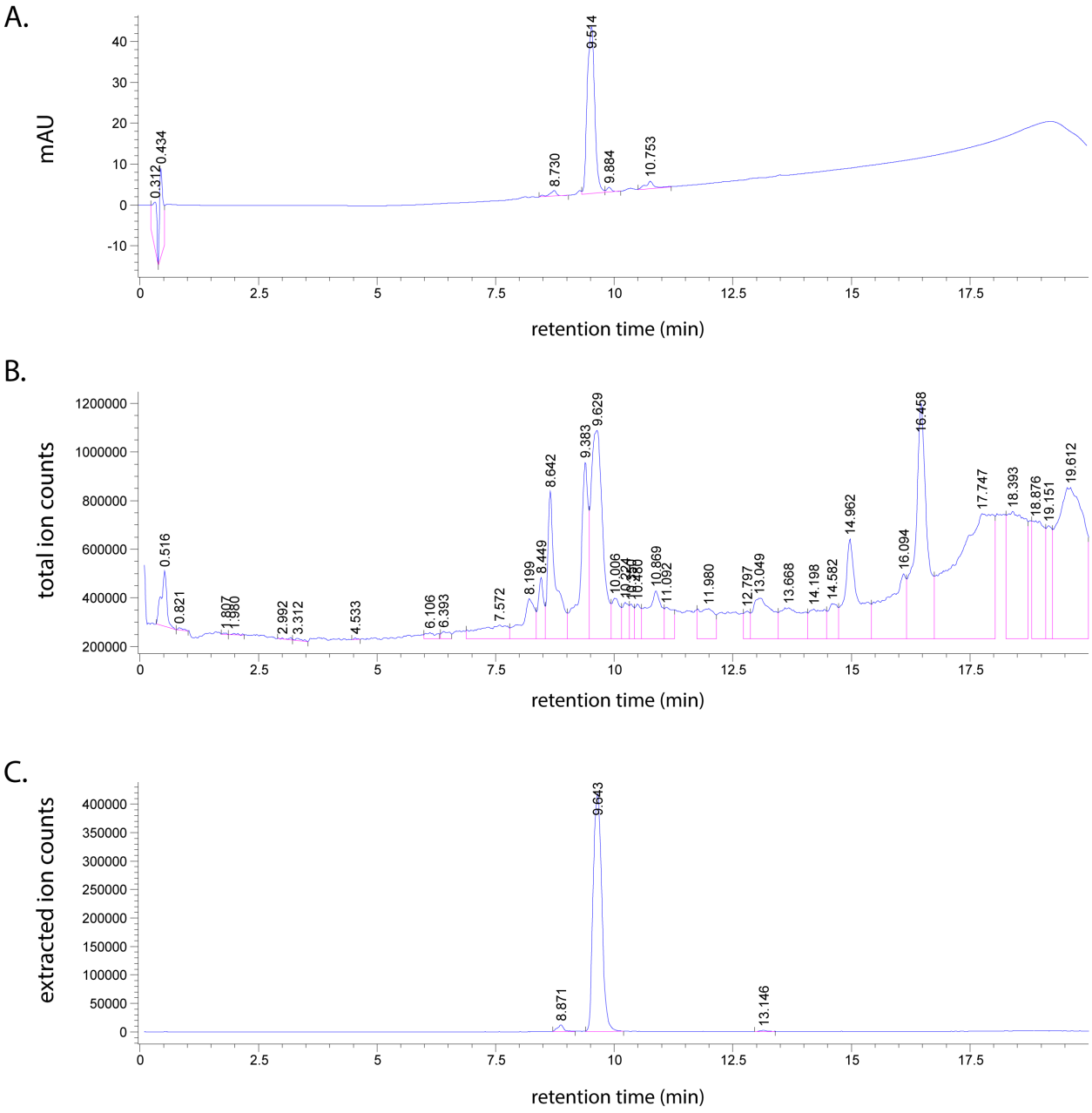
Supplementary Figure 5. High-resolution MS and MS/MS of **Fmoc-TβL** and **FmocTβL-P$_i$**.



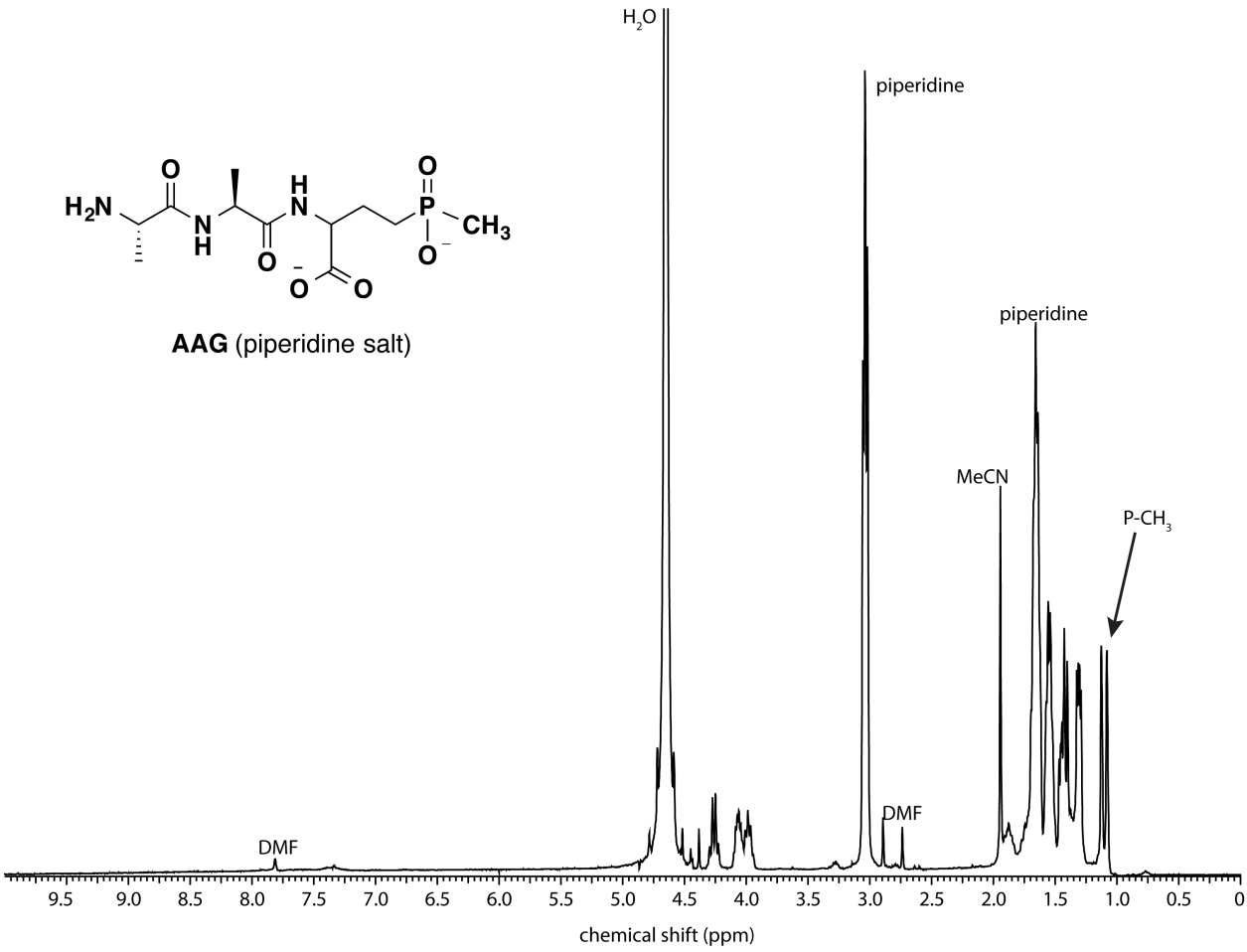
Supplementary Figure 6. Primary sequence alignments of *E. coli*, *S. aureus*, and Human GS performed using Clustal W.



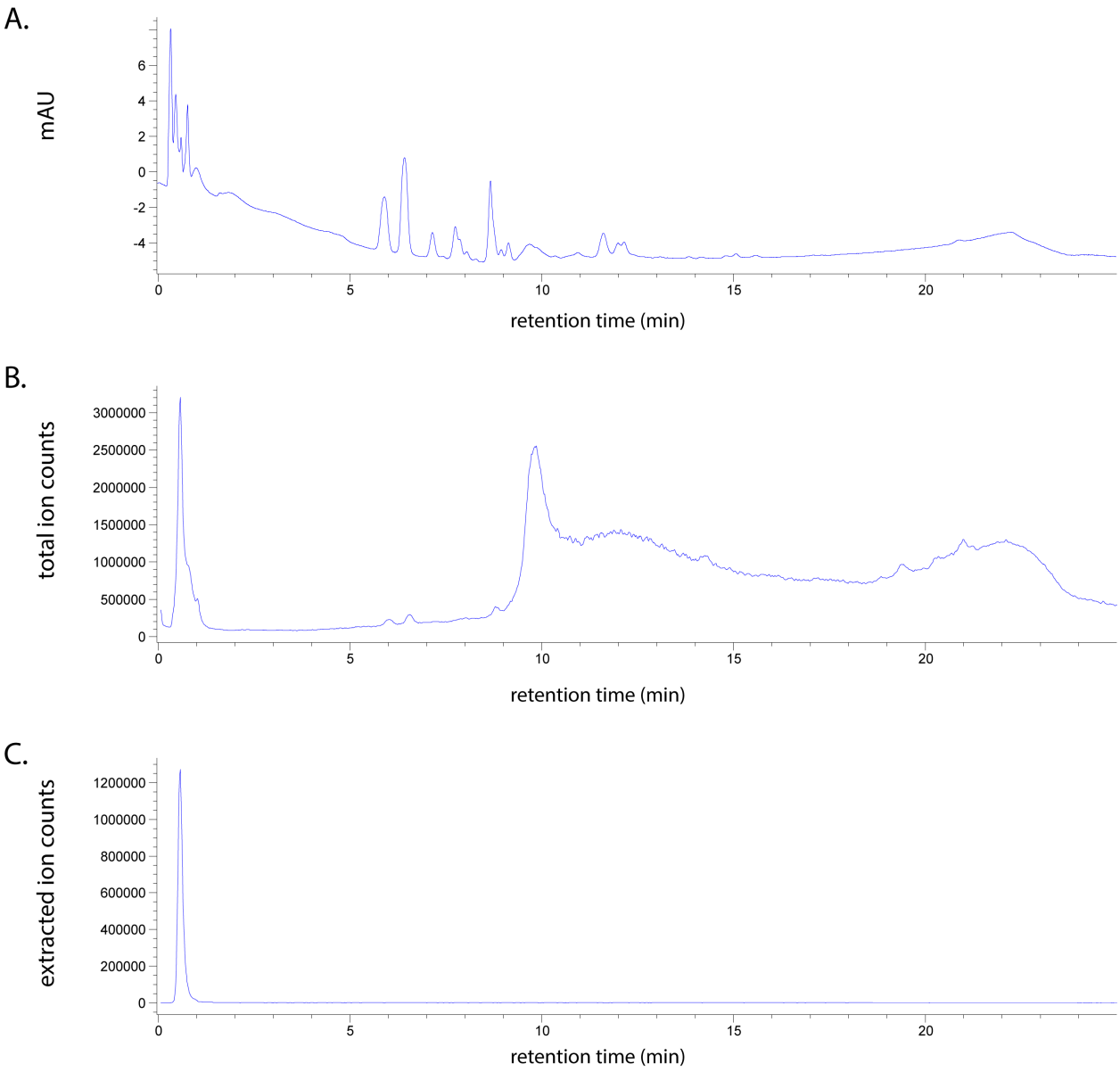
Supplementary Figure 7. Prep-HPLC chromatogram for purification of **Fmoc-AAG**. x-Axis represents retention time (min). y-Axis represents absorbance at 254 nm.



Supplementary Figure 8. LC-MS chromatograms of synthetic **Fmoc-AAG**. (**A**) Chromatogram of optical absorbance at 254 nm. (**B**) Total ion chromatogram (TIC). (**C**) Extracted ion chromatogram (EIC) for m/z = 546.2 corresponding to the $[M+H]^+$ ion of **Fmoc-AAG**.



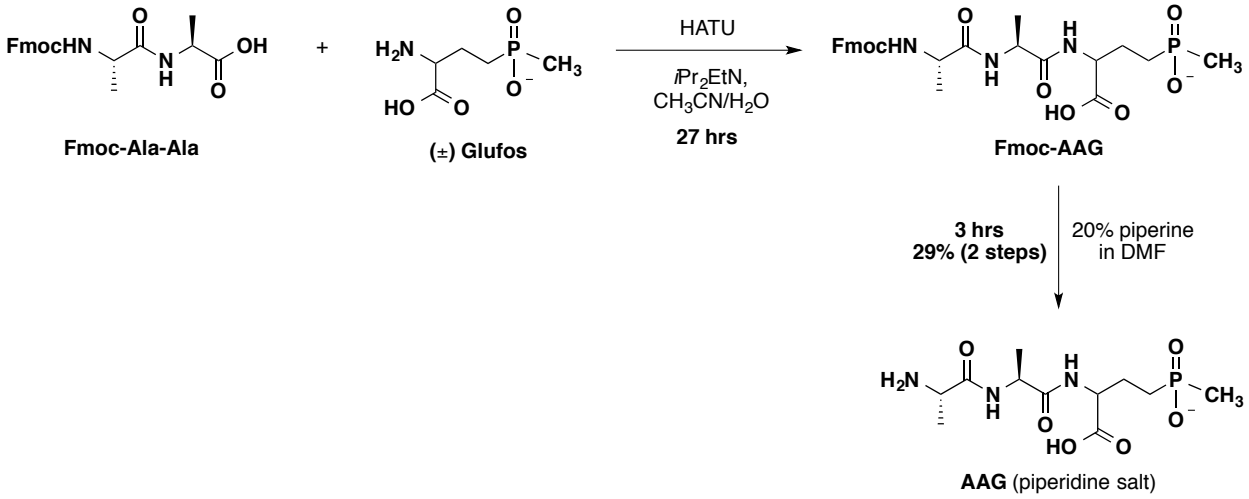
Supplementary Figure 9. ^{1}H-NMR (300 MHz) of **AAG** (piperidine salt) after Fmoc-deprotection in DMF. Spectrum was obtained in D_2O with an acetronitrile internal standard.



Supplementary Figure 10. LC-MS chromatograms of synthetic **AAG**. (**A**) Chromatogram of optical absorbance at 254 nm. (**B**) Total ion chromatogram (TIC). (**C**) Extracted ion chromatogram (EIC) for m/z = 324.1 corresponding to the $[M+H]^+$ ion of **AAG**.

III. Supplementary Schemes

AAG was synthesized in two steps from Fmoc-L-Ala-L-Ala and **(±) Glufos**. Fmoc-L-Ala-L-Ala (177 mg, 0.46 mmol) and *i*Pr_2EtN (0.45 mL) were dissolved in CH_3CN (20 mL) and H_2O (5 mL). The clear, yellow solution was stirred at rt for 20 min before addition of **(±) Glufos** (30 mg, 0.15 mmol). After 27 hrs, the reaction mixture was concentrated under reduced pressure. The crude material was dissolved in 40 mL of 50:50 CH_3CN:H_2O and purified by RP-C18 prep-HPLC using a linear gradient of 5% (B) to 95% (B) over 20 min. **Fmoc-AAG** eluted at ~17 min and the identity was confirmed by LC-MS (retention time = 9.7 min, MS (ESI) calculated for $C_{26}H_{33}N_3O_8P$: 546.2 $[M+H]^+$, found 546.2). Fractions containing pure **Fmoc-AAG** were combined and concentrated under reduced pressure before treatment with 20 mL of 20% piperidine in DMF (formation of white vapor was observed). After 3 hrs at rt, the DMF/piperidine were removed via rotary evaporation to give the **AAG** piperidine salt (14.3 mg, 0.37 mmol) in 29% overall yield. **AAG** purity was analyzed by LC-MS (retention time = 0.7 min, MS (ESI) calculated for $C_{11}H_{23}N_3O_6P$: 324.1 $[M+H]^+$, found 324.1) and ^{1}H-NMR (P-Me, 1.10 ppm, d, J = 13.4 Hz)[2,3].



Supplementary Scheme 1. Synthesis of L-Ala-L-Ala-Glufos (**AAG**).

IV. Acknowledgements

We thank A. D'Avignon (formerly WUSTL, Dept. of Chemistry; currently Sanford Burnham Medical Research Institute, Orlando, FL), J. Kao (WUSTL, Dept. of Chemistry), and B. Marsden (WUSTL, Dept. of Chemistry) for assistance in the acquisition of solution NMR spectra. We thank Dr. Brad Evans at the Proteomics & Mass Spectrometry Facility at the Donald Danforth Plant Science Center, St. Louis, MO for assistance with the acquisition of the QTRAP LC-MS/MS spectra (supported by the National Science Foundation under Grant No. DBI-0521250). We thank Margaret Reck (WUSTL, Dept. of Chemistry) for assistance purifying tabtoxin from *P. syringae* cultures. We thank Dr. Joe Jez and Cynthia Holland (WUSTL, Dept. of Biology) for diligent efforts crystallizing GS and analyzing diffraction patterns at the advanced photon source at Argonne National Laboratory.

V. References

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