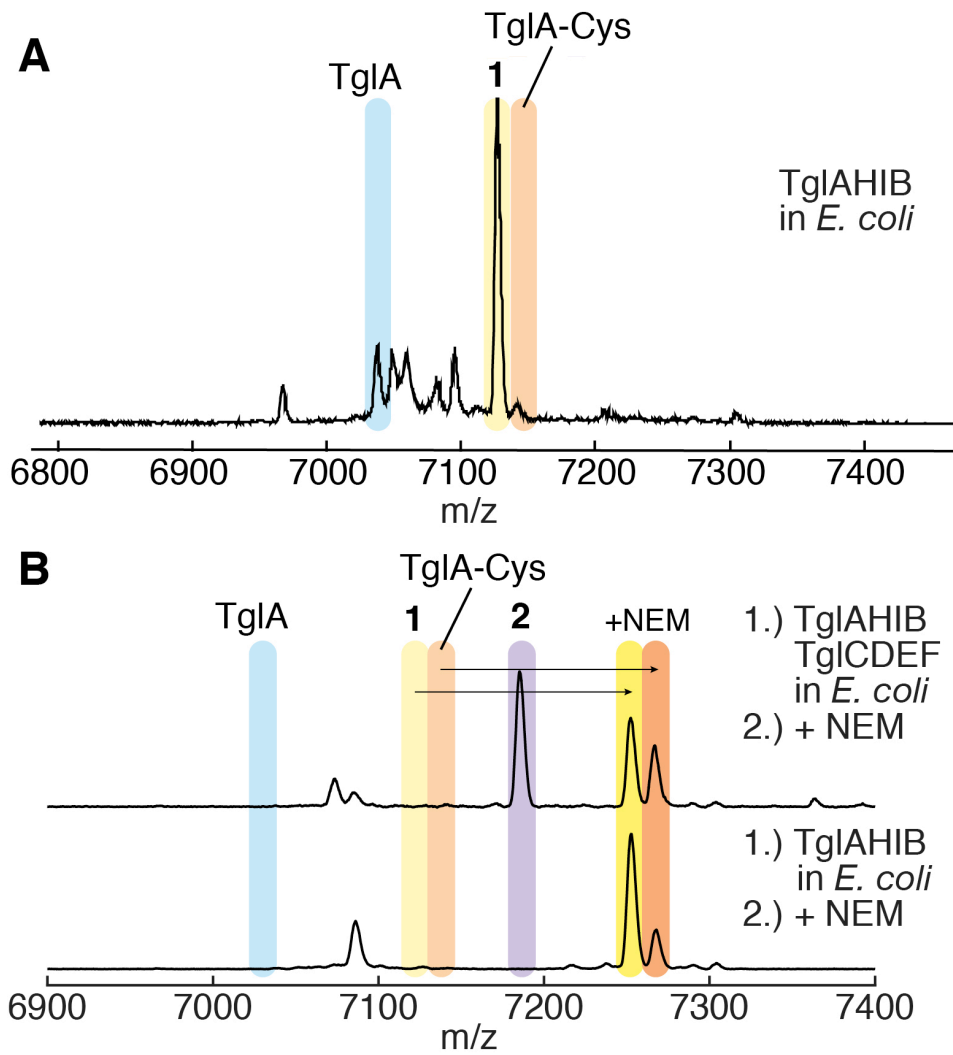
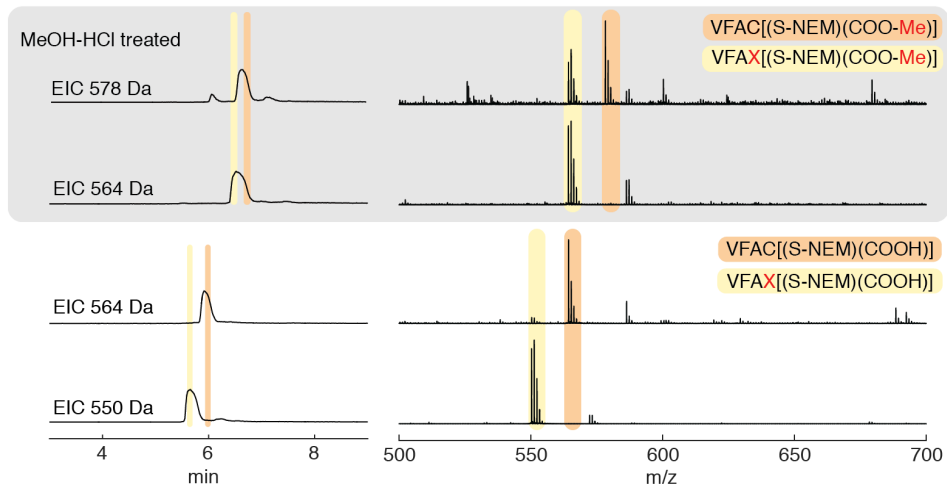


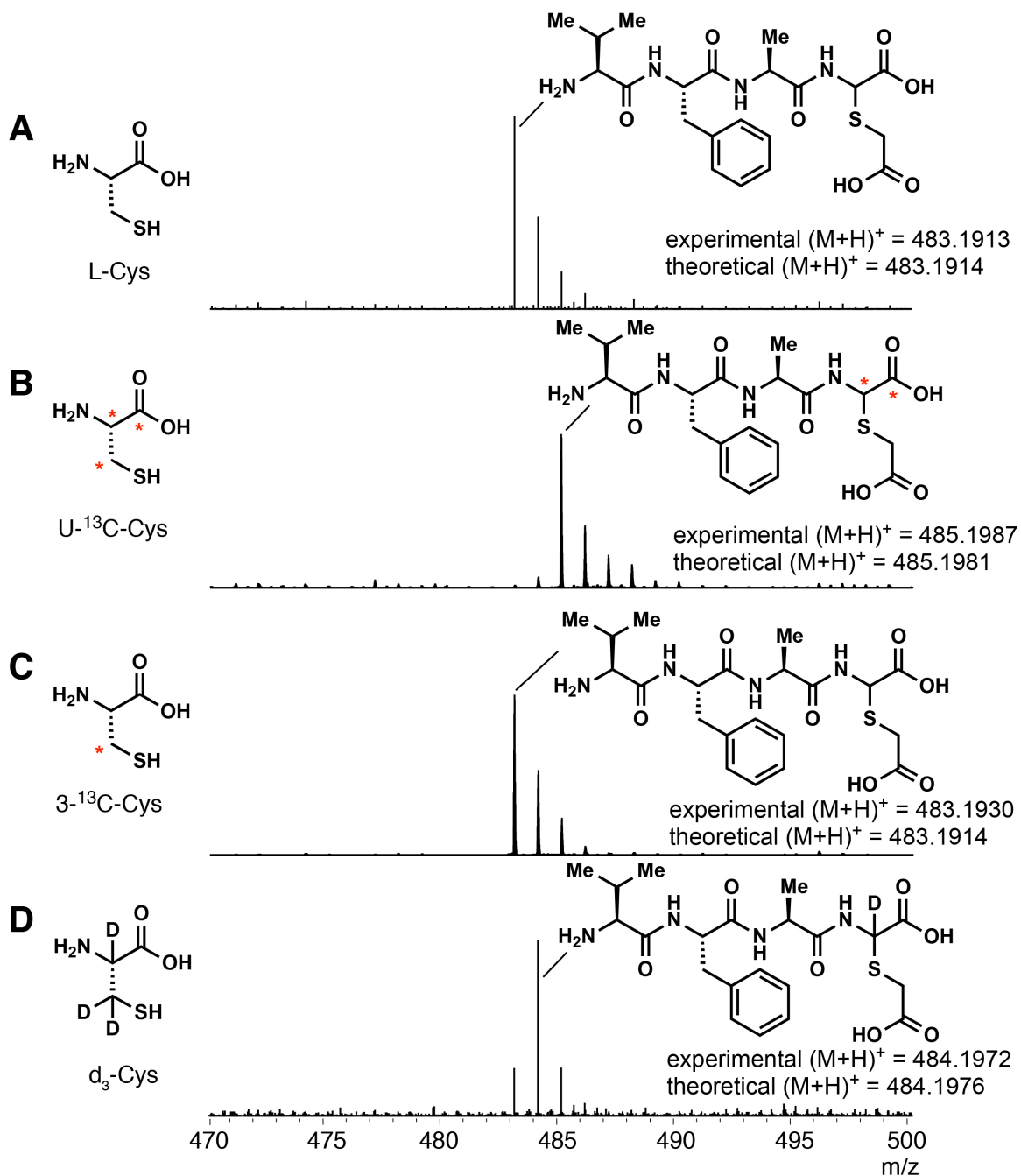
**Figure S1. *In vitro* cysteine addition by TglB to the precursor peptide TglA.** (A) MALDI-TOF mass spectra of TglB *in vitro* reaction with TglA confirm the reaction is tRNA and ATP-dependent. For the complete reaction conditions, see Methods. Various components were omitted from the assays as indicated or RNase was added prior to initiating the reaction (top mass spectrum). (B)  $^{31}\text{P}$  NMR analysis shows ADP and phosphate as products of the TglB reaction. (C) MALDI-TOF MS of TglA-Cys prepared by *in vitro* TglB reaction in 85%  $\text{H}_2^{18}\text{O}$  results in  $^{18}\text{O}$  enrichment in product. (D). MALDI-TOF mass spectra of the  $\text{NH}_2\text{OH}$  quenching assay and ESI-MS/MS analysis of the endoproteinase GluC digested hydroxamate product. (E) Top, mechanism 1: The reaction begins by phosphorylation of the C-terminal carboxylate of TglA followed by amide bond formation to the amino group of Cys-tRNA<sup>Cys</sup>. Finally, tRNA is hydrolyzed by water to produce TglA-Cys. This mechanism is consistent with the data presented. Bottom, mechanism involves the intermediacy of an anhydride which then subsequently rearranges (either by direct O-to-N migration or indirectly through a thioester) to the product. Mechanism 2 does not require ATP and does not result in incorporation of oxygen from solvent in the product. Hence, this mechanism is not operational. (F) MALDI-TOF mass spectra of TglB *in vitro* reaction with TglA 12-mer. (G) Kinetic experiments showing that TglB has a turnover number of  $28.2 \text{ min}^{-1}$ .



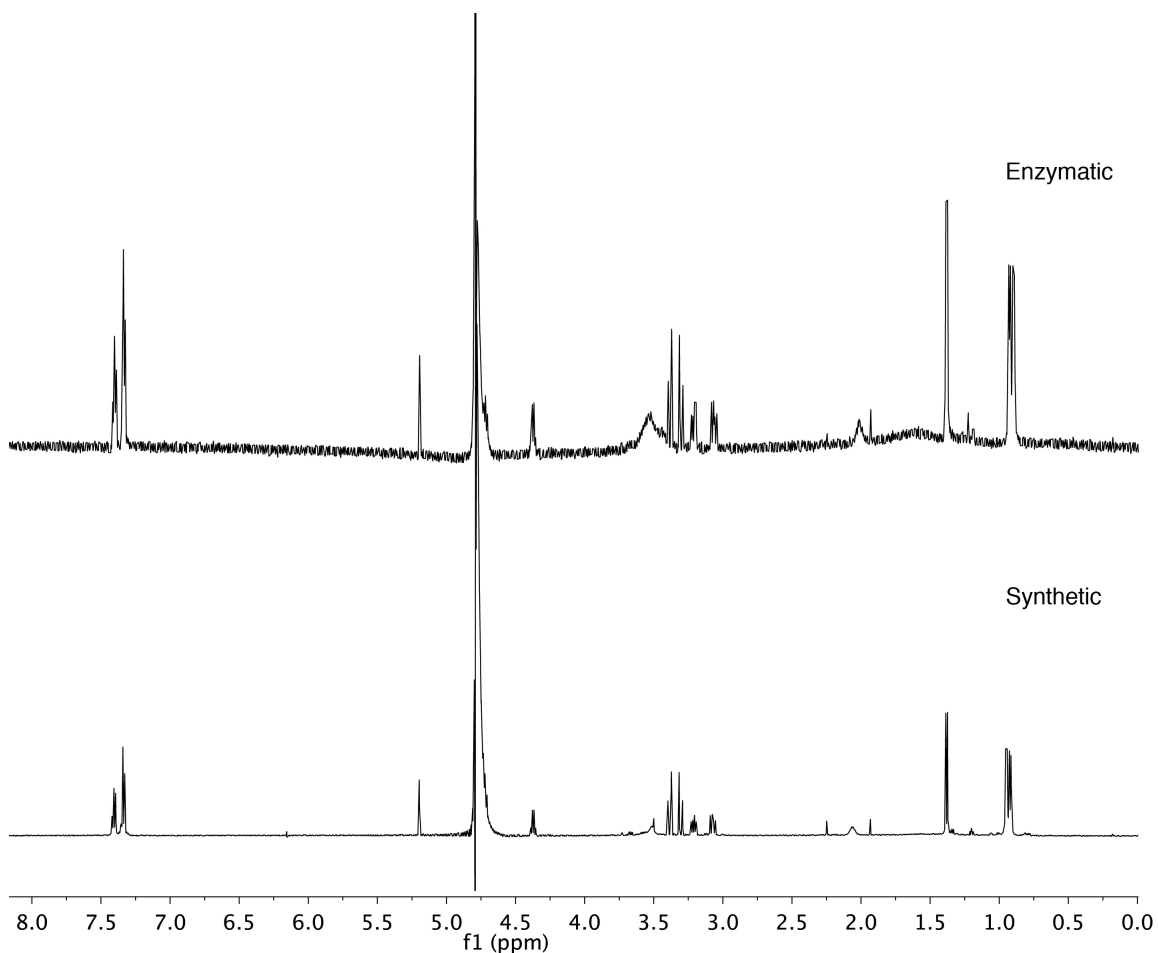
**Figure S2. MALDI-TOF mass spectra of TglA co-expressed with other proteins from the *tgl* cluster in *E. coli*.** (A) TglA co-expressed with TglBHI results in peptide 1 as major product. (B) TglA co-expressed with TglBCDEFHI results in TglA-thiaGlu (2) as major product. Peptides were modified by N-ethylmaleimide as indicated.



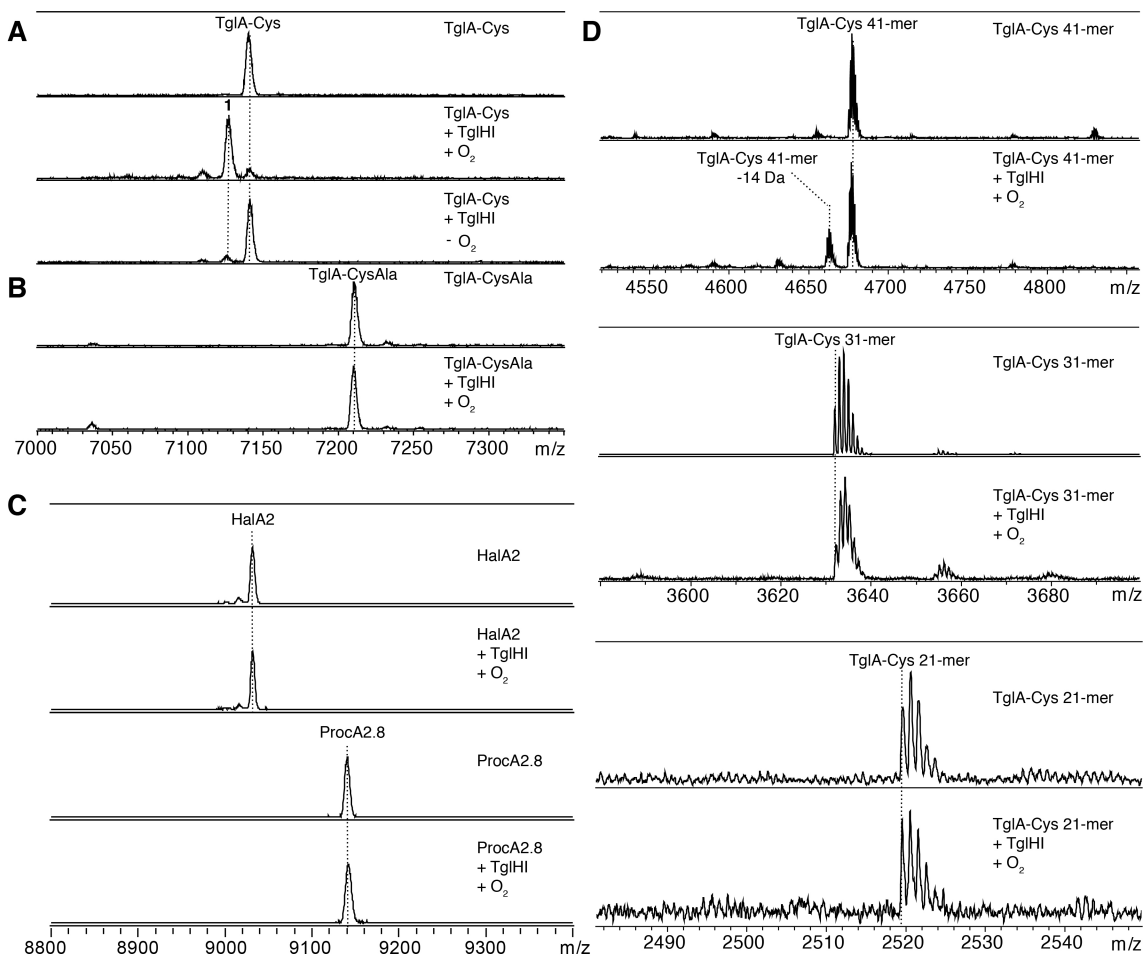
**Figure S3. Esterification of TgIA-Cys and the TgIHI-modified peptide following consecutive NEM and trypsin treatment demonstrate preservation of the carboxylate group in the tetrapeptide VFAX (3).** Shaded panel at the top represent experiments that were MeOH/HCl treated, bottom panel are controls that were not MeOH/HCl treated. Extracted ion chromatograms are shown (left) with the corresponding mass spectra of the peaks as marked (right). The observed methylation and NEM-alkylation illustrate that VFAX still contains thiol and carboxylate groups.



**Figure S4. LC-MS spectra (normalized intensity) of trypsin-digested TglA-Cys modified by TglHI.** <sup>13</sup>C-Enriched peptides were prepared by co-expression of His<sub>6</sub>-TglA-Cys and TglHI in a cysteine auxotrophic strain of *E. coli* grown with (A) unlabeled L-cysteine, (B) U-<sup>13</sup>C-L-cysteine, and (C) 3-<sup>13</sup>C-L-cysteine. (D) Deuterium labeled **1** was prepared by reaction of d<sub>3</sub>-TglA-Cys and TglHI *in vitro*. Peptides were derivatized with iodoacetic acid prior to analysis.

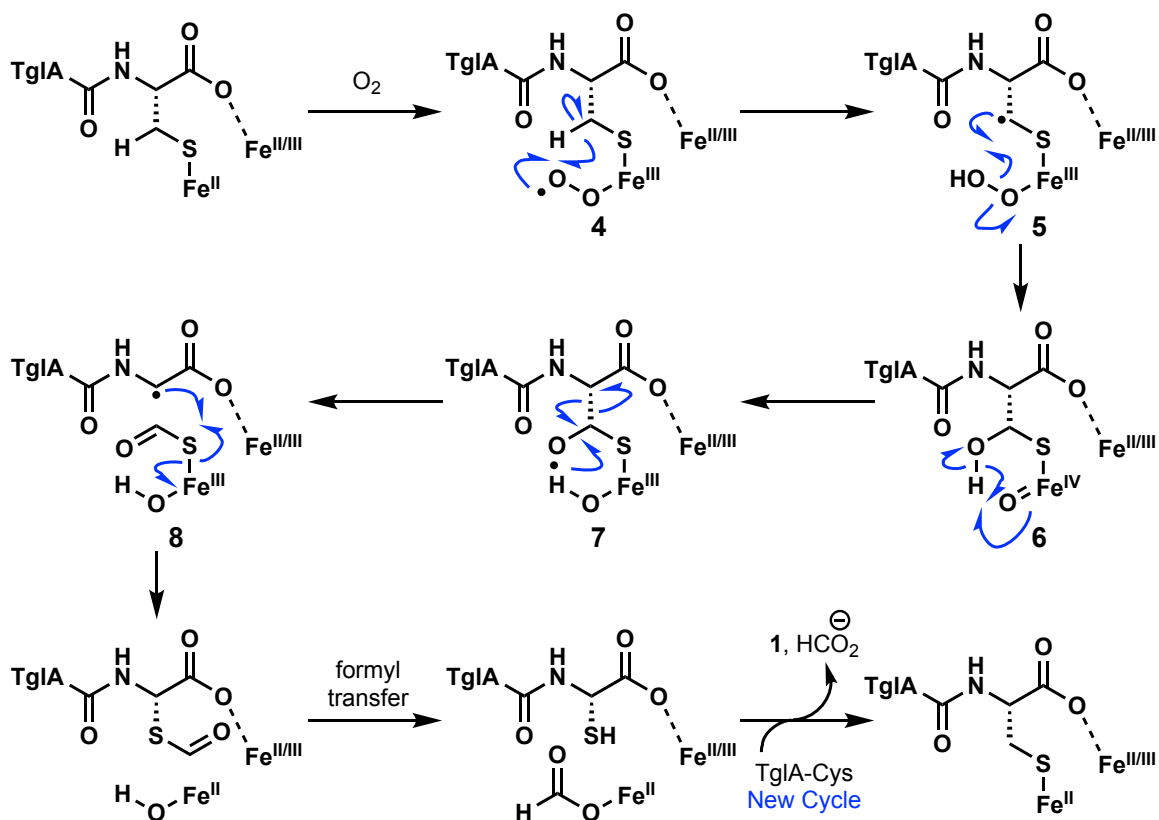


**Figure S5.  $^1\text{H}$  NMR spectra of VFA-thiaGlu tetrapeptide.** Top:  $^1\text{H}$  NMR spectrum of VFA-thiaGlu prepared by coexpression of TglAHIB, subsequent purification and modification by iodoacetic acid, and trypsin digestion. Bottom:  $^1\text{H}$  NMR spectrum of VFA-L-thiaGlu (L-3) prepared by chemical synthesis (See supplementary information). The signal at 5.2 ppm corresponding to the thioaminal proton is diagnostic.

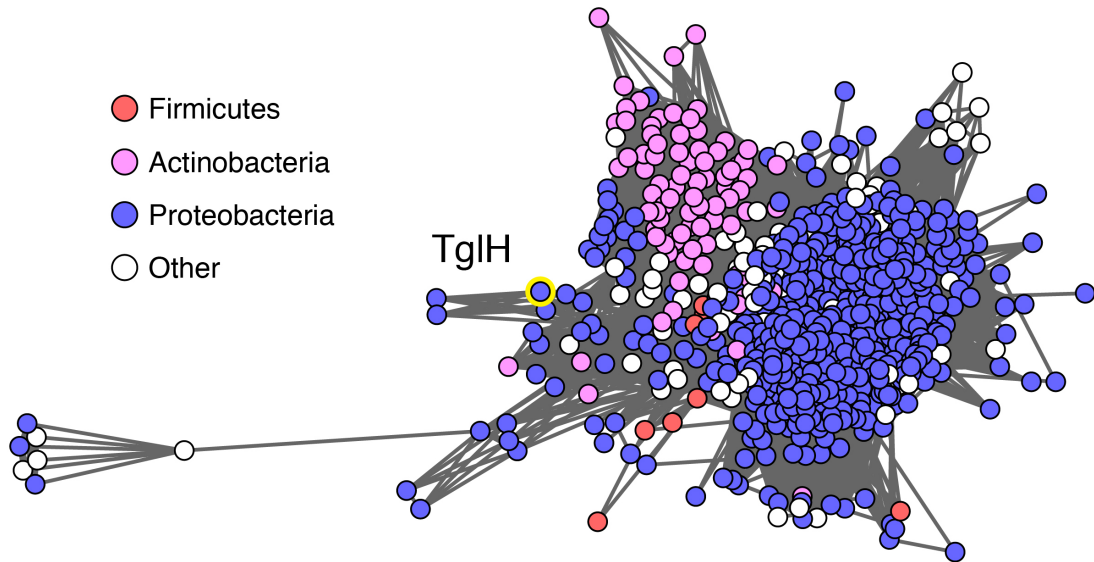
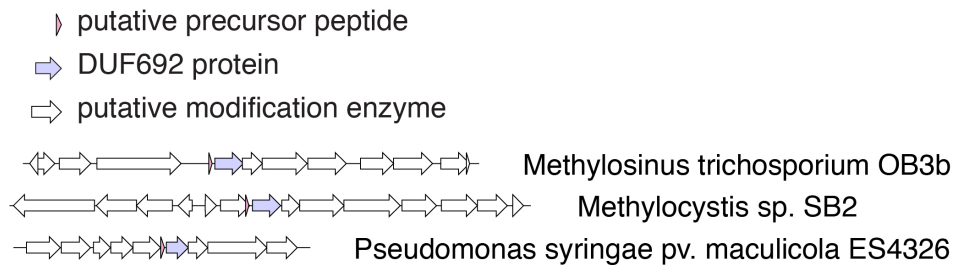


HalA2 GSSHHHHHHSSGLVPRGSHMVNSKDLRNPEFRKAQGLQFVDEVNEKELSSLAGSGDIE  
 GRTTWPcATVGVSVAlcPTTKcTSQC  
ProcA2.8 GSHMSEELKAFLLTKVQADTSLQEQLKIEGADVVAIAKAAGFSITTEDLNshRQNLSD  
 DELEGVAGGAACHNHAPSMPPSYWEGEC  
TglA-Cys 41mer SNQQASGDVkdLENTpQATEEALFEFDLDDIEVIESKVFAC  
TglA-Cys 31mer SLENTpQATEEALFEFDLDDIEVIESKVFAC  
TglA-Cys 21mer SALFEFDLDDIEVIESKVFAC

**Figure S6. Oxygen-dependency and substrate scope of TglHI.** (A) MALDI-TOF MS spectra of TglA-Cys before and after reaction with TglHI in phosphate buffer. Compound **1** (-14 Da) was observed as the major product along with complete consumption of TglA-Cys. Low conversion of TglA-Cys to **1** was observed at low oxygen concentrations (see Methods). (B) MALDI-TOF MS spectra of TglA-CysAla before and after reaction with TglHI in phosphate buffer. (C) MALDI-TOF mass spectra of HalA2 and ProcA2.8, two lanthipeptide precursors ending in Cys, before and after reaction with TglHI in phosphate buffer. (D) MALDI-TOF mass spectra of N-terminal truncants of TglA-Cys before and after reaction with TglHI in phosphate buffer. Diminished TglHI activity was observed for the TglA-Cys 41-mer and no reaction was observed for the other truncants.

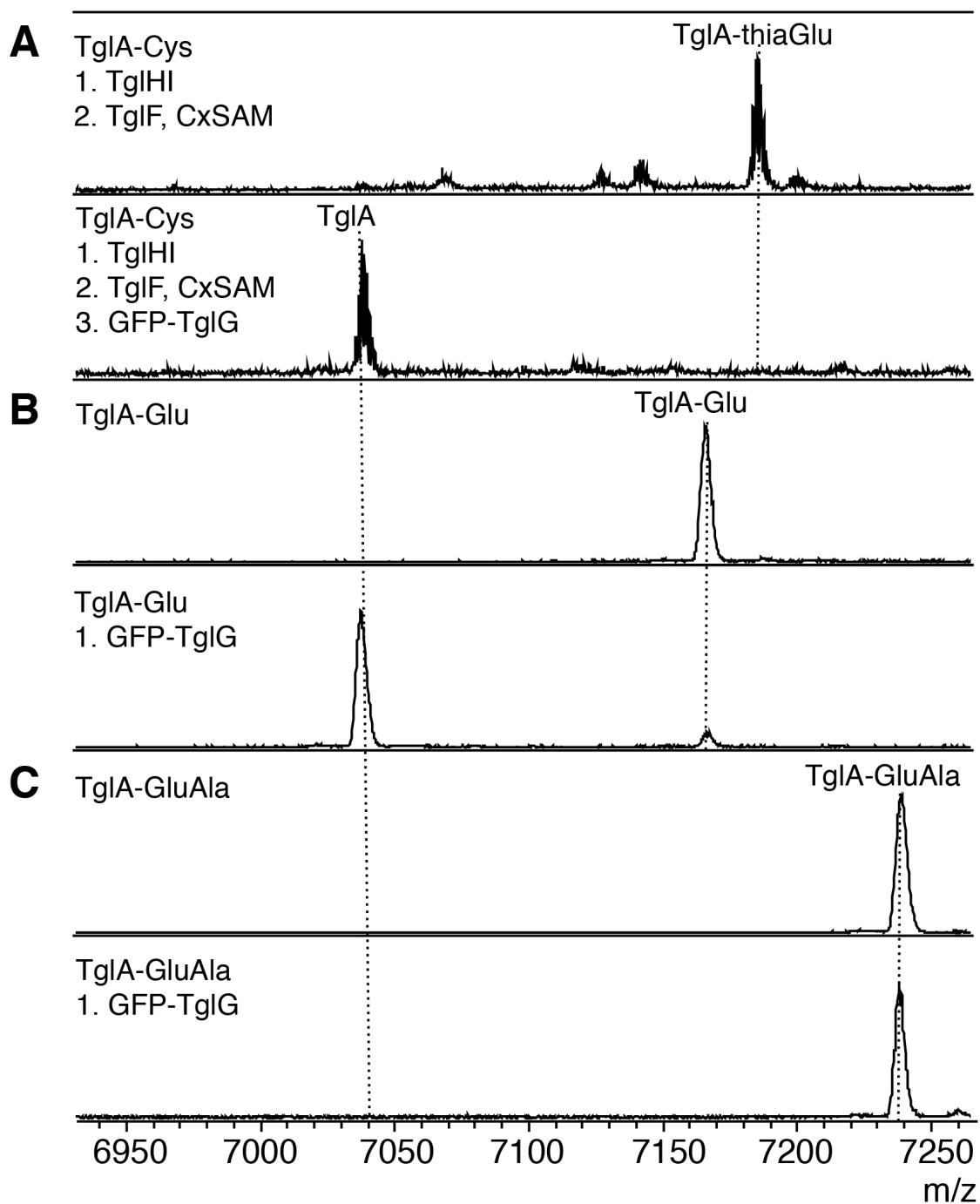


**Figure S7. Proposed mechanism for the carbon excision reaction catalyzed by TglHI.** The two iron atoms might be connected by bridging ligands and their oxidation states are currently not defined. Similar to the non-heme diiron enzyme *myo*-inositol oxygenase (MIOX) (44, 45), the reaction of TglHI is likely initiated via hydrogen atom abstraction from substrate by a Fe<sup>III</sup>-superoxo species (**4** → **5**). The retention of deuterium at the  $\alpha$ -carbon suggests abstraction from the  $\beta$ -carbon. Conversion of **4** to **5** could also involve electron transfer from the radical to the Fe(III), thus generating a thioaldehyde and a Fe(II)-hydroperoxo species. The latter could expel hydroxide and form a ferryl, and the hydroxide could hydrate the thioaldehyde, arriving again at intermediate **5**. A related transformation is invoked in HEPD (17). Hydroxylation by the Fe<sup>III</sup>-hydroperoxo intermediate would produce a thioacetal bound to a Fe<sup>IV</sup>=O complex (**5** → **6**). Carbon-carbon bond fragmentation would be triggered by proton-coupled electron transfer from the hydroxyl group by the ferryl intermediate to generate an oxygen radical, which undergoes  $\beta$ -scission (**6** → **7** → **8**). This process resembles a step in the reaction catalyzed by 2-hydroxyethylphosphonate dioxygenase (HEPD) (46). Radical recombination with the thioformate ligand forges the carbon-sulfur bond in **1** with retention of configuration and regenerates the Fe<sup>II</sup> complex, a step with some similarity to thioether formation by isopenicillin N synthase (47). Finally, hydrolysis of the thioformyl group by the hydroxide ligand on iron produces both formate and **1** after ligand exchange, closing the catalytic cycle.

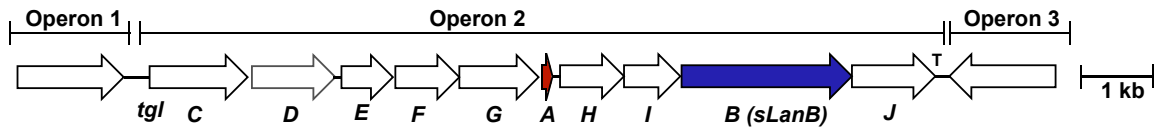
**A****B**

**Figure S8. TglH is a divergent member of a large group of putative enzymes within the DUF692 family.** (A) Network of DUF692 (TglH-related) enzymes, annotated by phylogeny and clustered at 40% sequence identity(48). A cluster of nodes (N=667) was identified at an E value cutoff of -30. Edges (N=100,893) densely connect nodes at this cutoff. TglH is, however, distinct from the associated group, with only 8 connecting nodes at this threshold. (B) Two methanobactin biosynthetic gene clusters are shown for comparison to the *tgl* cluster. For sequence similarity networks of the entire DUF692 family, see reference by Kenney and co-workers (19).

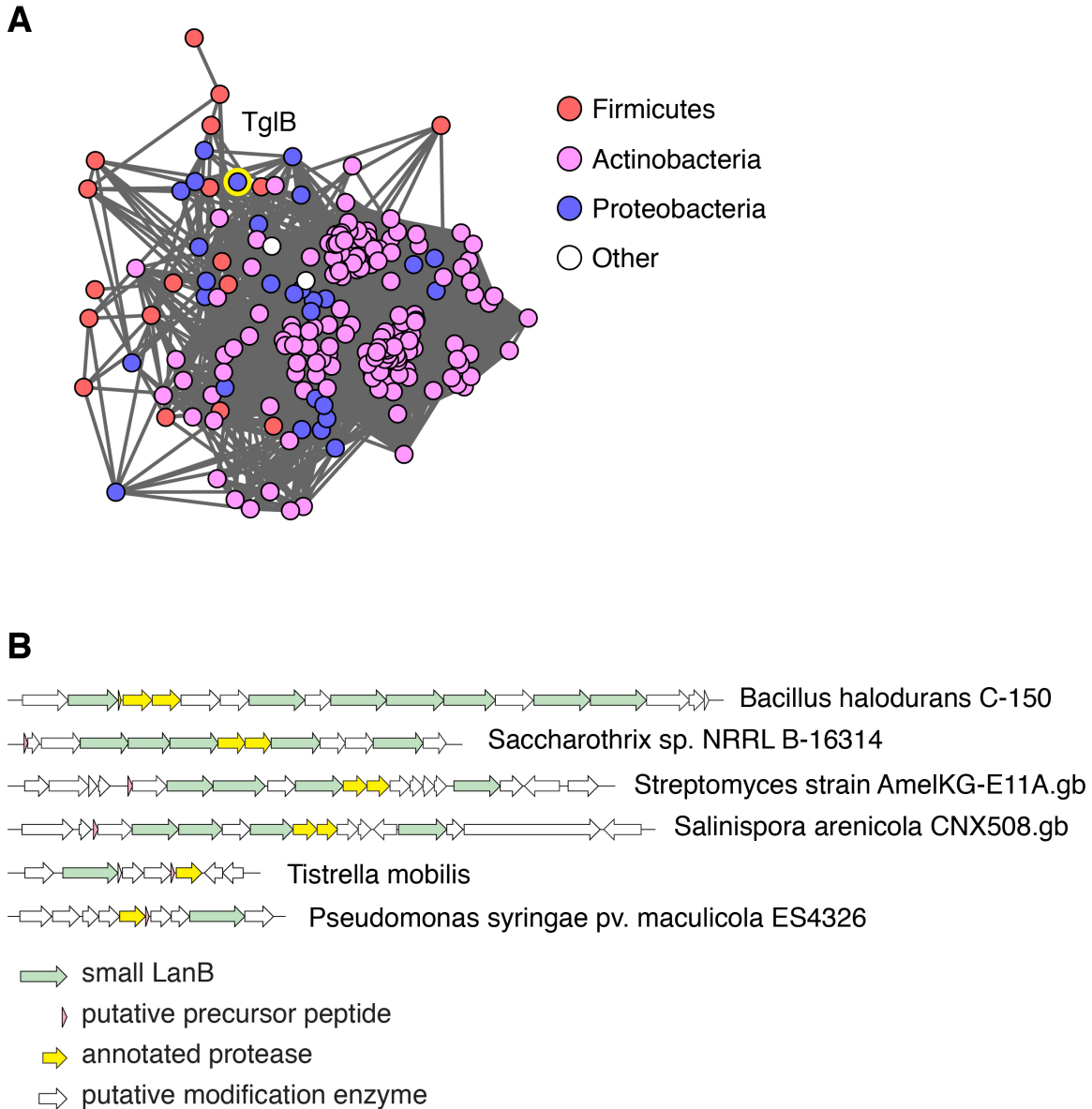




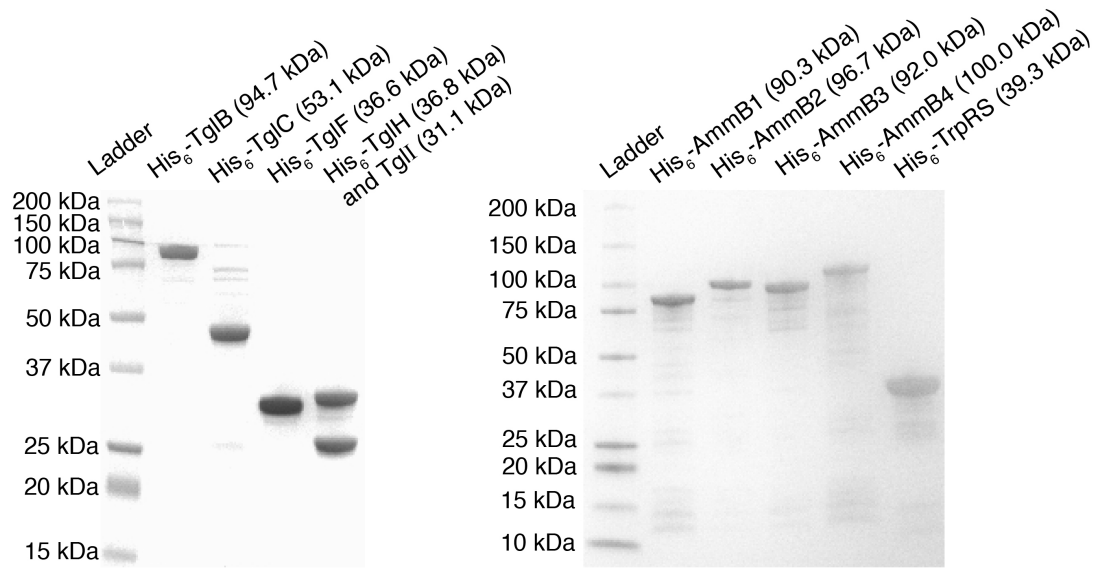
**Figure S9. TglG cleaves off a single amino acid from the C-terminus of the TglA-3-thiaGlu.** (A) MALDI-TOF MS spectra of TglA-thiaGlu (2) before and after reaction with *E. coli* cell lysate expressing GFP-TglG. (B) MALDI-TOF MS spectra of TglA-Glu before and after exposure to GFP-TglG cell lysate. (C) MALDI-TOF MS spectra of TglA-GluAla before and after exposure to GFP-TglG cell lysate.



**Figure S10. Operon predictions for *tgl*.** Operon predictions for the cluster in *P. syringae* pv. *maculicola* ES4326 based on FGENESB (36, 49).



**Figure S11. Small LanB enzymes are diverse and are found in many gene contexts.** (A) Sequence similarity network (48) of small LanB enzymes, annotated by phylogeny. Nodes (circles, N=214) represent sequences clustered at 40% sequence identity. Lines (N=12279) represent connections drawn at an alignment score E value of -20. Network was generated from sequences in PFAM group PF04738 at an E value of -5 and trimmed to remove clusters containing full length LanB enzymes at an E value of -20. Colors are assigned based on phylum level annotation. The node containing *TglB* is highlighted in yellow and has 10 neighbors at the cut-off. (B) Example biosynthetic gene clusters containing *tglB*-related genes. Five examples were chosen for each analysis based on diversity of the cluster architecture; the *tgl* cluster is shown at the bottom for comparison.



**SDS-PAGE analysis of all proteins used in this study.**

**Table S1. Primers used in this study.**

Primer name	Sequence
pET28b_plasmid(tglF)_f	AGTCCACGGTCTCGCATAGCTCGAGCACCACCAC
pET28b_plasmid(tglF)_r	AGTCCACGGTCTCGTTCATATGGCTGCCGCGCGG
pET28b_tglF_f	AGTCCACGGTCTCGTGAACGATTGGGCTATCAGC
pET28b_tglF_r	AGTCCACGGTCTCGTATGATGGCAACACCAGCGC
pET28b_plasmid(cysRS)_f	TCACAGCGGTCTCCGACTCGAGCACCACCACCAC
pET28b_plasmid(cysRS)_r	TCACAGCGGTCTCCTATGGCTGCCGCGCGGCAC
pET28b_cysRS_f	TCACAGCGGTCTCCCATATGCTTTCTATCTACAA CACGCTCACCA
pET28b_cysRS_r	TCACAGCGGTCTCCAGTCAGTCCGCCAGACGCCA
pACYC_Duet_plasmid(tglB)_f	GTGGACAGGTCTCCAAGCTTGCGGCCGCATAATG
pACYC_Duet_plasmid(tglB)_r	GTGGACAGGTCTCCATCGGATCCTGGCTGTGGTG
pACYC_Duet_tglB_f	GTGGACAGGTCTCGCGATGAAAGCTCACACTATTTTC
pACYC_Duet_tglB_r	GTGGACAGGTCTCGGCTTATTCATCACGCTTACTC
pRSF_Duet_tglA(frameshift)_f	CATCACACAGCCAGGATCCATGGGACAACCCAACGTG
pRSF_Duet_tglA(frameshift)_r	CATTATGCGGCCGCAAGCTTCAGGCAAAGACCTTGCTC
pRSF_Duet_tglA(corrected)_f	CCAGGATCCGATGGGACAACC
pRSF_Duet_tglA(corrected)_r	GGTTGTCCCATCGGATCCTGG
pRSF_Duet_plasmid(tglAHIB)_f	CGCTTTGGGTCTCCTCGGATCCTGGCTGTGGTG
pRSF_Duet_plasmid(tglAHIB)_r	CGCTTTGGGTCTCCTAAGCGGCCGCATAATGCTTA
pRSF_Duet_tglAHIB_f	CGCTTTGGGTCTCCCCGATGGGACAACCCAACGTG
pRSF_Duet_tglAHIB_r	CGCTTTGGGTCTCCCTTATTCATCACGCTTACTCC
pET28b_GFPplasmid(tglG)_f	GAGGACTGGTCTCCGACTCGAGCACCACCACCAC
pET28b_GFPplasmid_r	GAGAGTGGGTCTCCATCTTGTACAGCTCGTCCATGCC
pET28b_GFPtglG_f	GAGAGTGGGTCTCCAGATGACACACGCTTCCGAT
pET28b_tglG_r	GAGGACTGGTCTCCAGTCACTGGTTATGAAATACACG
pACYC_tglA_C51_f	CGCTTTGGGTCTCCTAAGCGGCCGCATAATGCTTA
pACYC_tglA_C51_r	CGCTTTGGGTCTCCTCGGATCCTGGCTGTGGTG
pACYC_Duet_tglA_C51_f	CGCTTTGGGTCTCCCCGATGGGACAACCCAACGTG
pACYC_Duet_tglA_C51_r	CGCTTTGGGTCTCCCTTATCAGCAGGCAAAGACCTTG
pACYC-Duet_(tglAC51HI)_r-2	CGCTTTGGGTCTCCCTTACTACAAAGGCTTCCTTATCGCG
pET15b-TglHI_f	AGCGGCCTGGTGCCGCGCGGCAGCCATATG ATGCCTGATTCGTCAAGC
pET15b-TglHI_r	TCGGGCTTTGTTAGCAGCCGGATCCTCGAG CTACAAAGGCTTCCTTATCGC
pACYC-TglA(CA)_f	AGGTCTTTGCCTGCGCCTGATAAGCGGCCGC
pACYC-TglA(CA)_r	GCGGCCGCTTATCAGGCGCAGGCAAAGACCT
pACYC-TglA(E51)_f	CATTATGCGGCCGCTTATCATT CGGCAAAGACCTTGCTCTCGA
pACYC-TglA(E51)_r	TCGAGAGCAAGGTCTTTGCCGA ATGATAAGCGGCCGCATAATG
pACYC-TglA(EA)_f	AGAGCAAAGGTCTTTGCCGAAGCCTGATAAGCGGCCG
pACYC-TglA(EA)_r	CGGCCGCTTATCAGGCTTCGGCAAAGACCTTGCTCT

41mer_F	GAGAACCTGTACTTCCAATCCAACCAGCAAGCGTCC
TEV_R	GGATTGGAAGTACAGGTTCTCcggatcctggctgtg
31mer_F	GAGAACCTGTACTTCCAATCCCTCGAAAACACTCCGCAG
21mer_F	GAGAACCTGTACTTCCAATCCGCGTTGTTTGAAGAGTTTGAC C
pACYC-ammA_f	CTTGCCCTGAAAGAGTCCCGGTCCCTGGTGAtaagcggccgca taatgc
pACYC-ammA_r	GTCTGTCTCTGTAACCTGTGTTTCGGACATcggatcctggct gtgg
AmmA-stop_f	ccctggcacttgcctgactgaaagagtcccg
AmmA-stop_r	cgggactctttcagtcaggcaagtgccaggg
tRNA_Trp_F	AATTCCTGCAGTAATACGACTCACTATAAGGGTCGTAGCTCA ATTGGTAGAGCACTGG
tRNA_Trp_R	mUmAGCAGGGCCGGAGGGACTTGAACCCCCAACCCTGGTTT TGGAGACCAGTGCTCT

**Table S2. Sequence of synthetic genes optimized for *E. coli* expression.**

*ammA* (codon optimized sequence)

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*ammB1* (codon optimized sequence)

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*ammB2* (codon optimized sequence)

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ammB3 (codon optimized sequence)

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ammB4 (codon optimized sequence)

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*trpRS* from *Streptomyces* sp. CNR698 (codon optimized sequence)

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**Table S3. MicroED Crystallographic Table**

Stoichiometric formula	C <sub>21</sub> N <sub>4</sub> O <sub>7</sub> S <sub>1</sub>
Space group	P 2 <sub>1</sub>
Unit cell lengths a, b, c (Å)	9.660(2), 9.580(2), 10.790(2)
angles $\alpha$ , $\beta$ , $\gamma$ (°)	90.00(3), 94.00(3), 90.00(3)
Reflections (#)	5689 (777)
Unique reflections (#)	2262 (308)
R <sub>obs</sub>	21.6 (40.5)
R <sub>meas</sub>	23.6 (57.5)
CC <sub>1/2</sub>	92.7 (61.4)
Resolution (Å)	1.0
Completeness (%)	95.8 (96.6)
Total exposure (e <sup>-</sup> Å <sup>-2</sup> )	<1
R	0.23
wR2	0.4503
GooF	1.756