Tabtoxinine-β-Lactam is a "Stealth" β-Lactam Antibiotic that Evades β-Lactamase-mediated Antibiotic Resistance

Supporting Information

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I. Bacterial Strains Used in this Work

Table S1. Bacterial strains and plasmids used in this work.

Strain	Plasmid	Inducible Gene/Marker	Origin/Reference
***E. coli* ATCC 25922**	none	control strain for antibiotic susceptibility testing	American Type Culture Collection
***E. coli* BL21(DE3)**	none	none	Agilent
+ TEM-1	pET24	TEM-1, Kan^R	This Work
+ TEM-1, no leader sequence	pET24	TEM-1 no leader, Kan^R	This Work
+ TEM-64	pET24	TEM-64, Kan^R	This Work
+ CTX-M-9	pET9	CTX-M-9, Kan^R	Yu Chen, University of South Florida[1]
+ CTX-M-9, no leader sequence	pET9	CTX-M-9 no leader, Kan^R	This Work
***E. coli* DH5α**	pUCP26	Tet^R	Pfizer[2]
+ TEM-1	pUCP26	TEM-1, Tet^R	Pfizer[2]
+ TEM-24	pUCP26	TEM-24, Tet^R	Pfizer[2]
+ CTX-M-15	pUCP26	CTX-M-15, Tet^R	Pfizer[2]
+ SHV-5	pUCP26	SHV-5, Tet^R	Pfizer[2]
+ SHV-12	pUCP26	SHV-12, Tet^R	Pfizer[2]
+ OXA-24/40	pUCP26	OXA-24/40, Tet^R	Pfizer[2]
+ KPC-3	pUCP26	KPC-3, Tet^R	Pfizer[2]
+ NDM-1	pUCP26	NDM-1, Tet^R	Pfizer[2]
+ VIM-2	pUCP26	VIM-2, Tet^R	Pfizer[2]

Kan^R: kanamycin resistance; Tet^R: tetracycline resistance

II. SDS-PAGE Analyses of β-Lactamases

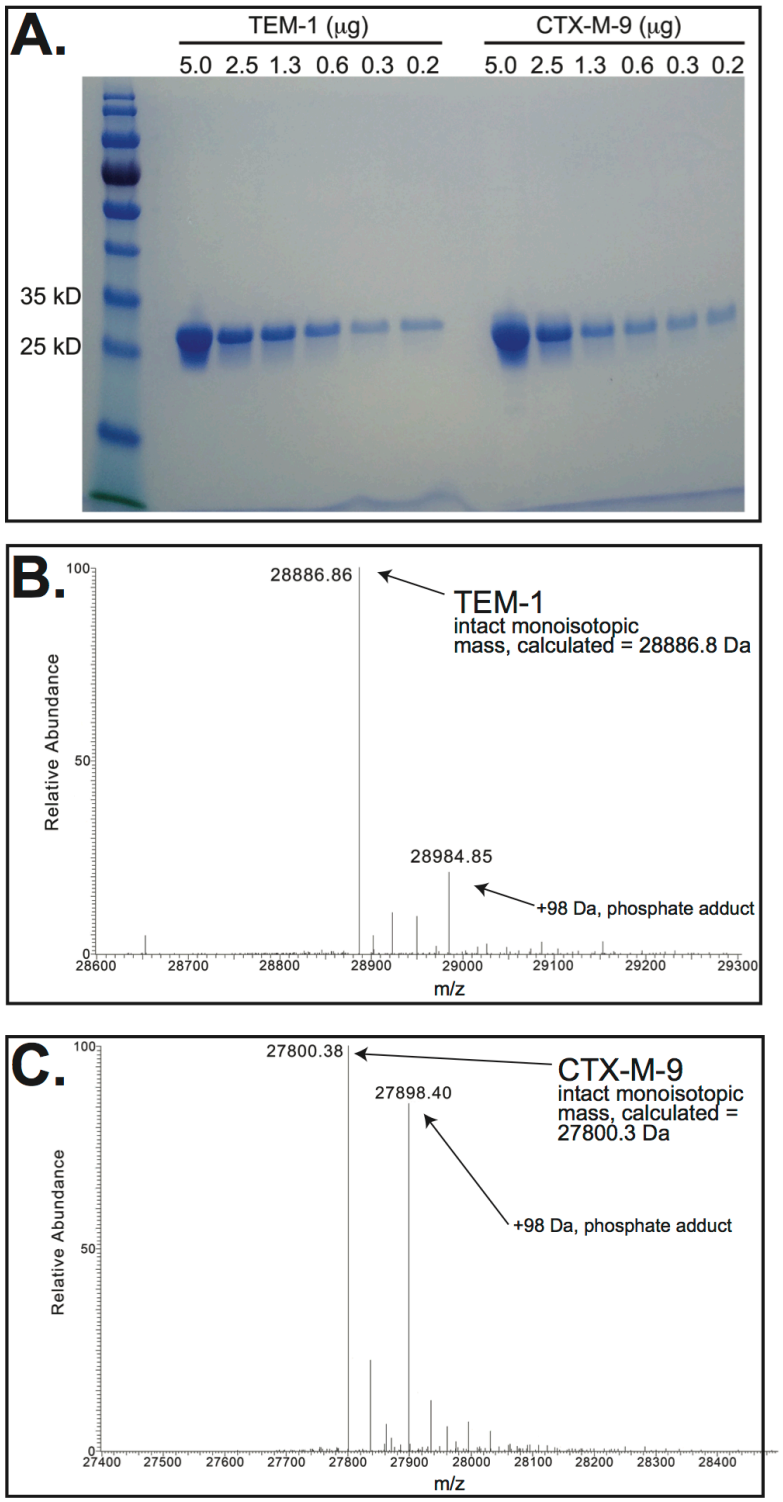
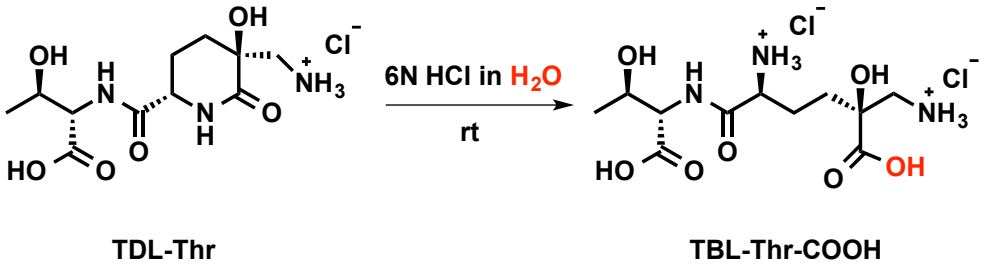


Figure S1. Confirming purity and identity of protein samples. (**A**) SDS-PAGE analysis of TEM-1 (28.9 kD) and CTX-M-9 (27.9 kD) after purification by ion exchange chromatography. (**B**) Electrospray mass spectrometry confirms masses of purified TEM-1 and (**C**) CTX-M-9.

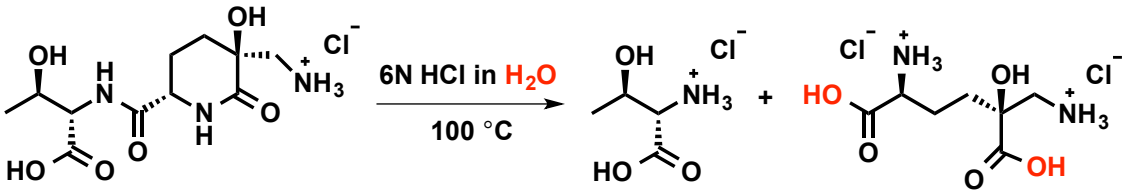
III. Synthesis of TβL-Thr-COOH and TβL-COOH Standards



TDL-Thr TBL-Thr-COOH

Scheme S1. Synthesis of TβL-Thr-COOH HCl salt from TδL-Thr.

Tabtoxinine-Threonine Dihydrochloride (TβL-Thr-COOH•2HCl). The dihydrochloride salt of tabtoxinine-threonine dipeptide (TβL-Thr-COOH•2HCl) was prepared via controlled hydrolysis of δ-tabtoxin (TδL-Thr) in aqueous 6N HCl at room temperature. Lyophilized δ-tabtoxin, from fermentation of *P. syringae* ATCC 11528, was redissolved at a concentration of 5 mg/ml in 6 N HCL and transferred to a 5 mm glass NMR tube. Hydrolysis was allowed to occur for 12 hours at room temperature. The sample was frozen, lyophilized, and dissolved in 600 µL 0.2 M phosphate buffer, pH 7.0, in deuterium oxide for analysis via NMR and use as a standard in analytical assays. See **Tables S2,S3** and **Figures S2-S8** for tabulated NMR, LCMS, and HPLC analytical characterization data and spectra.



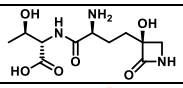
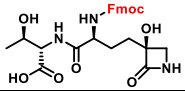
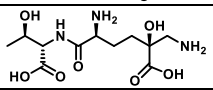
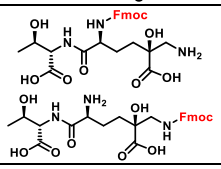
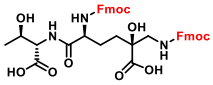
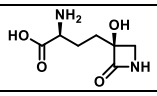
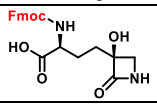
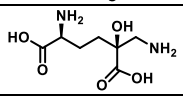
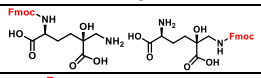
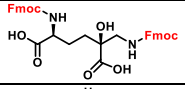
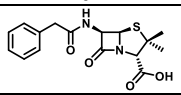
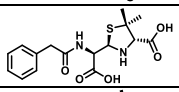
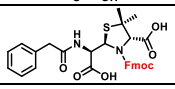
TDL-Thr Thr TBL-COOH

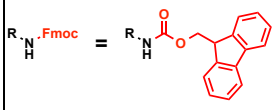
Scheme S2. Synthesis of TβL-COOH HCl salt from TδL-Thr.

Tabtoxinine Dihydrochloride (TβL-COOH•2HCl). The dihydrochloride salt of tabtoxinine (TβL-COOH•2HCl) was prepared using δ-tabtoxin (TδL-Thr) as starting material. Lyophilized δ-tabtoxin, from fermentations of *P. syringae* ATCC 11528, was dissolved at a concentration of 5 mg/ml in 6 N HCL and transferred to a 5 mm glass NMR tube. The tube was heated in a sand bath at 100 °C for 12 hours. The sample was frozen, lyophilized, and dissolved in 600 µL 0.2 M phosphate buffer, pH 7.0, in deuterium oxide for analysis via NMR and use as a standard in analytical assays. See **Tables S2,S3** and **Figures S2-S8** for tabulated NMR, LCMS, and HPLC analytical characterization data and spectra.

IV. HPLC and LCMS Traces for β-Lactamase Reactions

Table S2. Names, abbreviations, structures, masses, & retention times for compounds.

Compound Name	Compound ID	Structure	Chemical Formula	Molecular Weight	Calculated $[M+H]^+$	Observed $[M+H]^+$	LCMS[a] RT(min)	HPLC[b] RT(min)
β-tabtoxin	TβL-Thr		C11H19N3O6	289.29	290.13	NO[c]	NO	NO
Fmoc-β-tabtoxin	Fmoc-TβL-Thr		C26H29N3O8	511.53	512.20	511.0	5.9 & 6.5[d]	12.2
tabtoxinine-threonine dipeptide	TβL-Thr-COOH		C11H21N3O7	307.30	308.15	NO	NO	NO
monoFmoc-tabtoxinine-threonine dipeptide	Fmoc-TβL-Thr-COOH		C26H31N3O9	529.55	530.21	NO	NO	?
bisFmoc-tabtoxinine-threonine dipeptide	2Fmoc-TβL-Thr-COOH		C41H41N3O11	751.79	752.28	752.0	10.3	18.0
tabtoxinine-β-lactam	TβL		C7H12N2O4	188.18	189.09	NO	NO	NO
Fmoc-tabtoxinine-β-lactam	Fmoc-TβL		C22H22N2O6	410.43	411.43	411.0	6.1	12.8
tabtoxinine	TβL-COOH		C7H14N2O5	206.20	207.10	NO	NO	NO
monoFmoc-tabtoxinine	Fmoc-TβL-COOH		C22H24N2O7	428.44	429.17	429.0	3.0	?
bisFmoc-tabtoxinine	2Fmoc-TβL-COOH		C37H34N2O9	650.68	651.23	651.0	10.5	18.7
benzyl penicillin	Pen		C16H18N2O4S	334.39	335.11	334.8	3.7	12.1
benzyl penicilloic acid	Pen-COOH		C16H20N2O5S	352.41	353.12	352.8	0.85 & 1.6[e]	8.8 & 9.1
Fmoc-benzyl penicilloic acid	Fmoc-Pen-COOH		C31H30N2O7S	574.65	575.18	574.7	9.1 & 9.9[e]	16.7 & 17.1



[a]LCMS data were obtained on an Agilent 1200 series LCMS with 6130 quadrupole mass detector, G1322A degasser, G1311A quaternary pump, G1329A autosampler, G1316A column thermostat, and G1315A diode array detector. Compounds were separated using a Phenomenex Gemini C18 column (5μm, 110 Å, 2 x 50 mm, part #00B-4435-B0) with guard column assembly and mobile phases (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. A gradient was formed from 5% B to 95% B over 20 min at a flow rate of 0.5 mL/min. [b]HPLC data were obtained on a Beckman Coulter System Gold instrument with 126 solvent module, 168 diode array detector, and 508 autosampler. Compounds were separated using a Supelco Discovery C18 (2) column (5μm, 100 Å, 4.6 x 250 mm, part #504971) with guard column assembly and mobile phases (A) 0.1% trifluoroacetic acid in water and (B) 0.1% trifluoroacetic acid in acetonitrile. A gradient was formed from 20% B to 100% B over 25 min at a flow rate of 1 mL/min with detection at 263 nm. [c]NO: Not Observed. [d]Two peaks are observed because TβL-Thr isomerizes to stable TδL-Thr with a half-life of 37 hours at pH 7.2.[3] [e]Benzyl penicilloic acid is an equilibrating mixture of two diastereomers.[4]

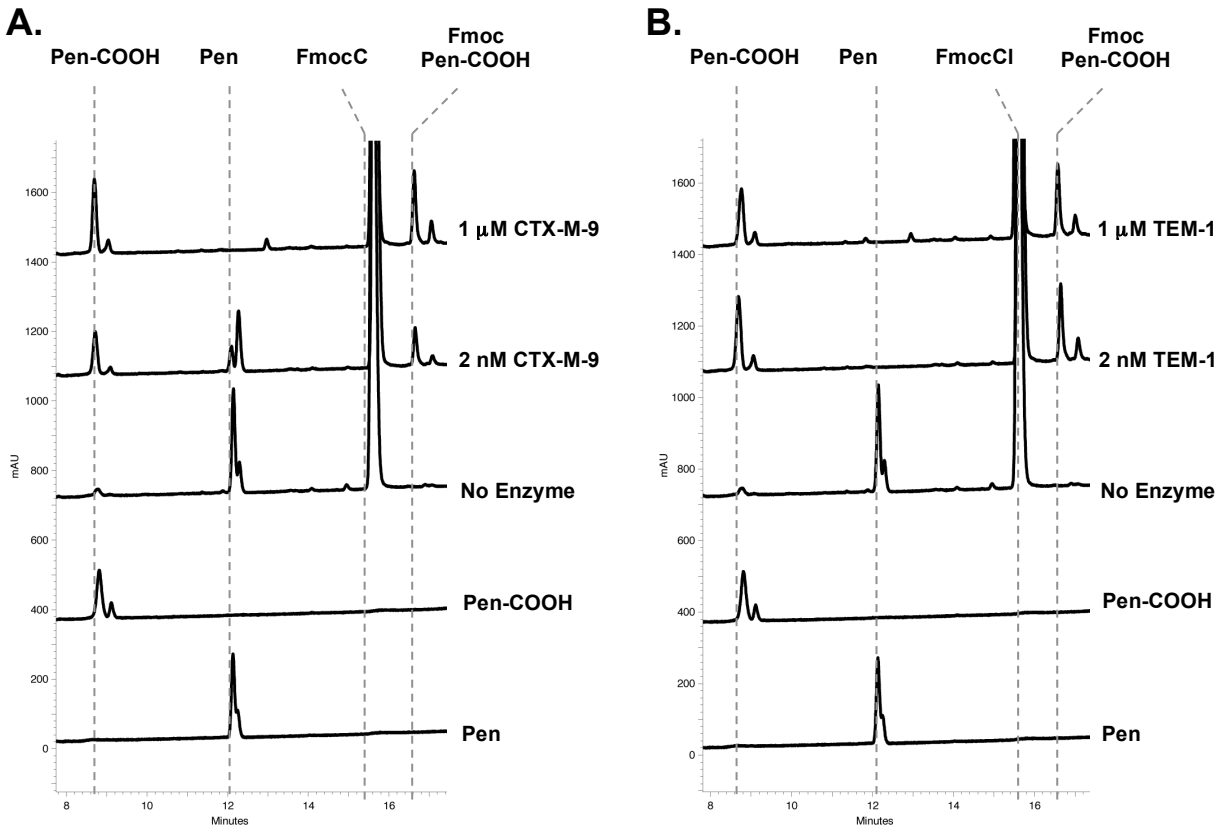


Figure S2. HPLC assay for β-lactamase activity. (**A**) Benzyl penicillin (Pen) + CTX-M-9; (**B**) Benzyl penicillin (Pen) + TEM-1. Reactions consisted of 1 mM substrate and 2 nM, 1 μM or no enzyme in 10% glycerol, 50 mM potassium phosphate buffer, pH 7. Reaction aliquots were taken after 30 minutes, treated with FmocCl, and analyzed by HPLC with detection at 263 nm according to the general protocol described in the main text.

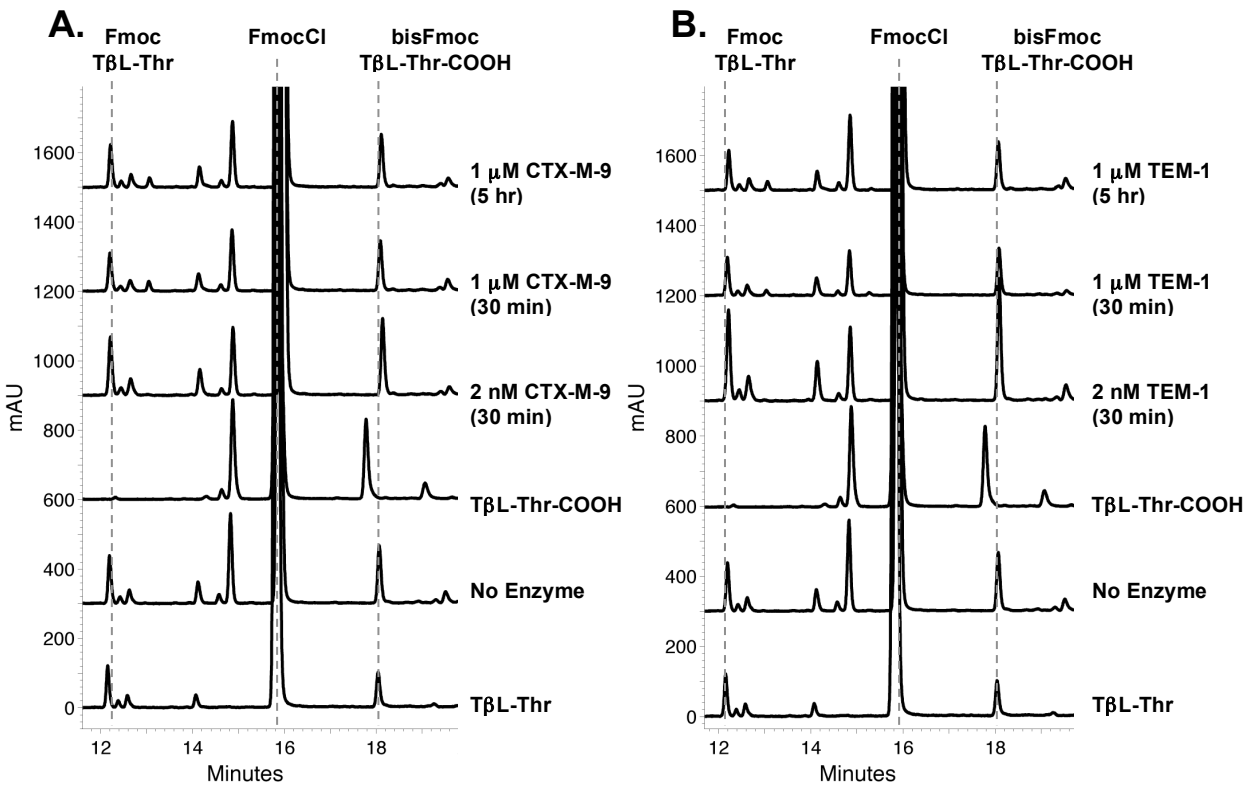


Figure S3. HPLC assay for β-lactamase activity. (**A**) Tabtoxin (TβL-Thr) + CTX-M-9; (**B**) Tabtoxin (TβL-Thr) + TEM-1. Reactions consisted of 1 mM substrate and 2 nM, 1 μM or no enzyme in 10% glycerol, 50 mM potassium phosphate buffer, pH 7. Reaction aliquots were taken after 30 min and 5 h, treated with FmocCl, and analyzed by HPLC with detection at 263 nm according to the general protocol described in the main text.

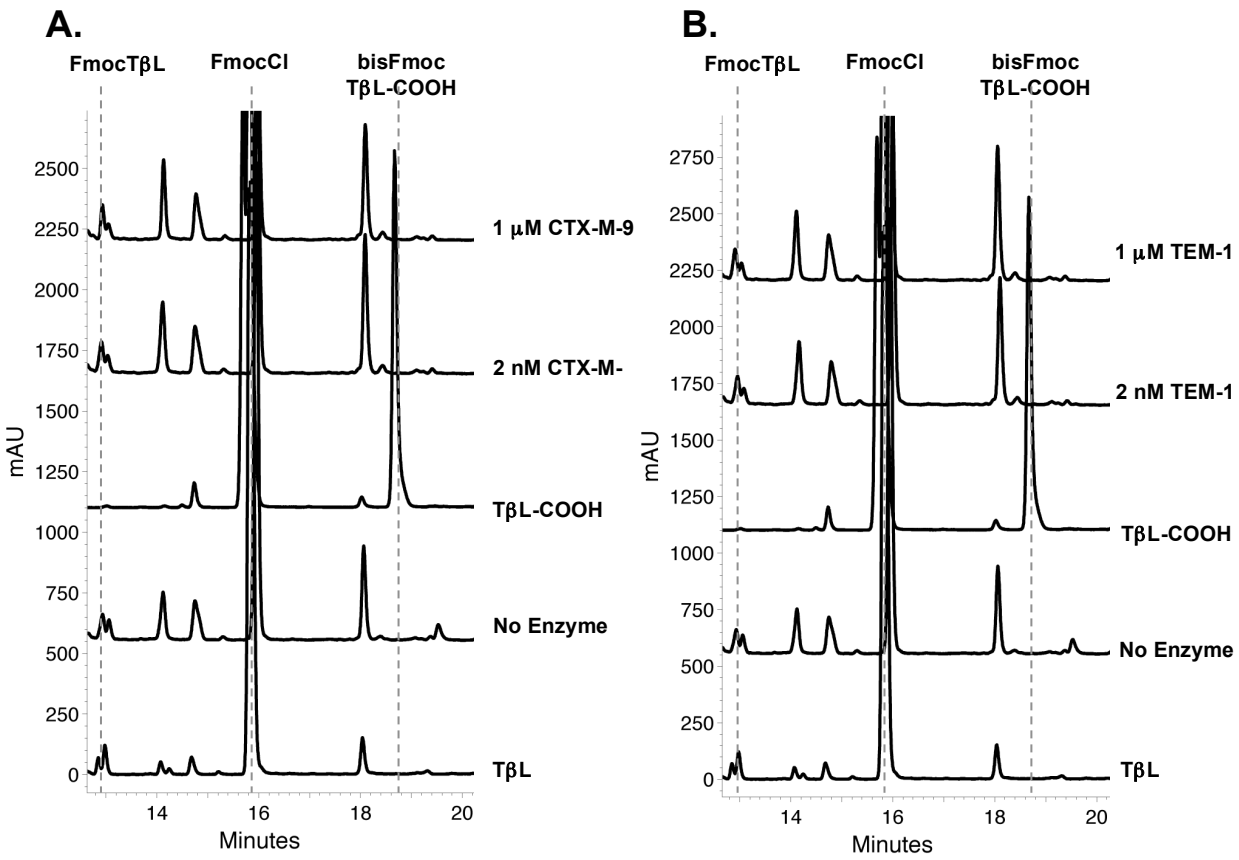


Figure S4. HPLC assay for β-lactamase activity. (**A**) Tabtoxinine-β-lactam + CTX-M-9; (**B**) Tabtoxinine-β-lactam + TEM-1. Reactions consisted of 1 mM substrate and 2 nM, 1 μM or no enzyme in 10% glycerol, 50 mM potassium phosphate buffer, pH 7. Reaction aliquots were taken after 30 min, treated with FmocCl, and analyzed by HPLC with detection at 263 nm according to the general protocol described in the main text. The peak at 18 min is FmocTβL-Thr-COOH. The sample of TβL used in this experiment was prepared from the sample of TβL-Thr that already contained some TβL-Thr-COOH impurity.

V. Additional Computational Docking Studies

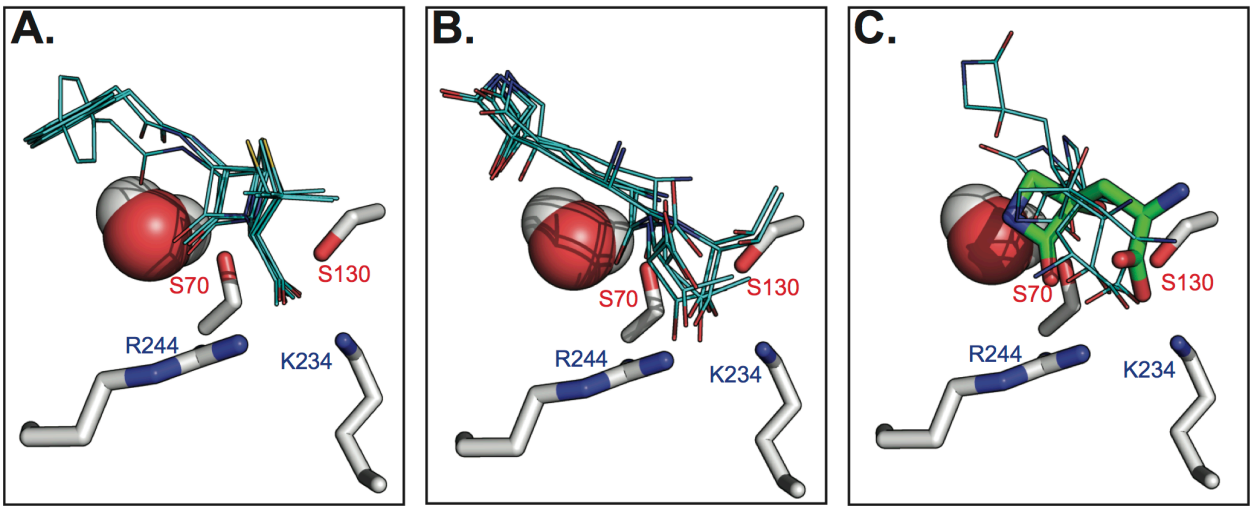


Figure S5. Lowest energy structures from molecular docking studies of (A) benzyl penicillin, (B) TβL-Thr and (C) TβL. Carbon atoms are shown in gray (protein) and cyan (antibiotic), and all other atoms are colored according to CPK conventions. The active site water is shown in space-filling mode. The second lowest energy pose, show in green, positions the β-lactam ring of TβL close to S70 but flipped 180° relative to the benzyl penicillin-bound structure, which prevents the necessary hydrogen bonding with S170.

VI. NMR Spectra and Tabulated Data for Purified Compounds

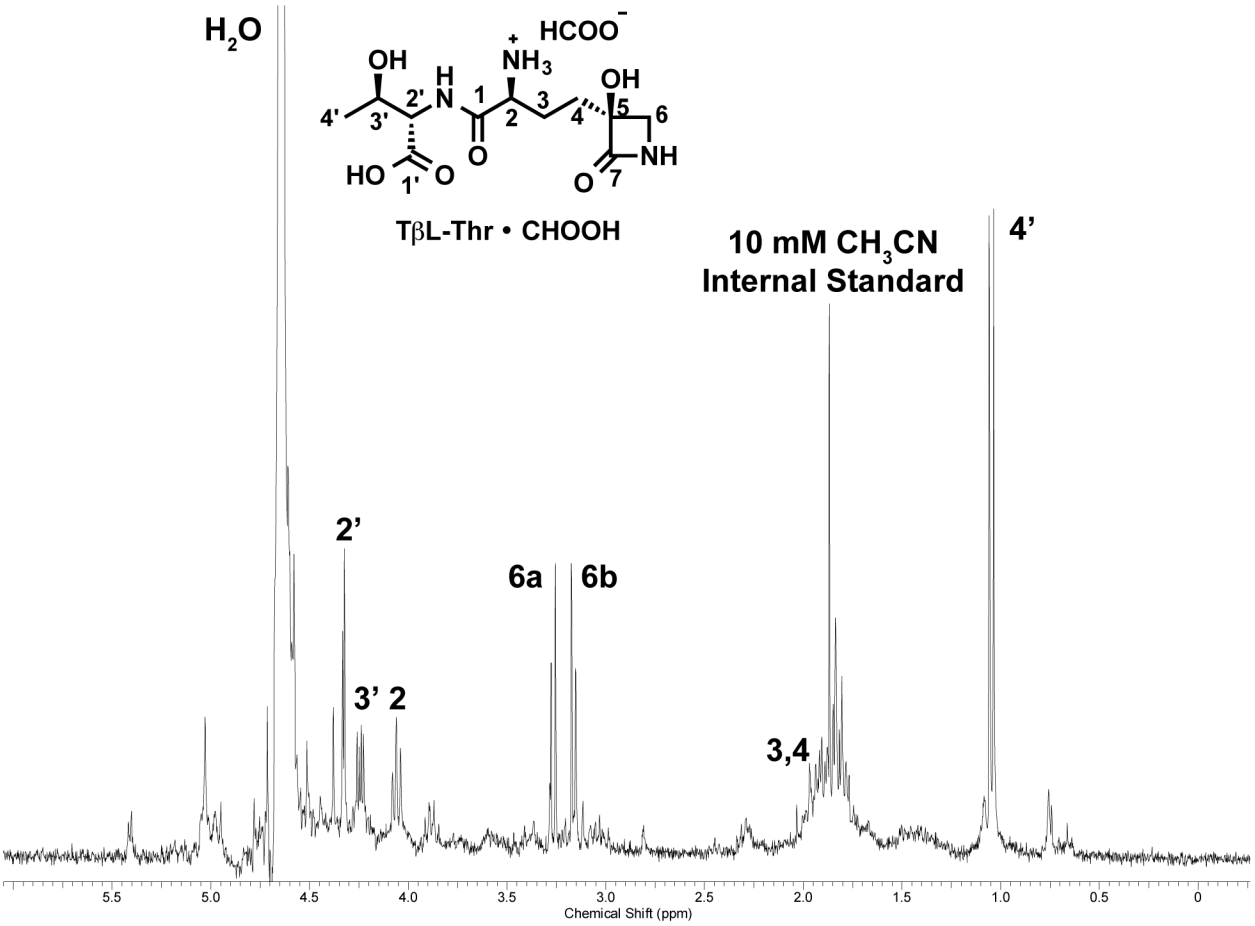
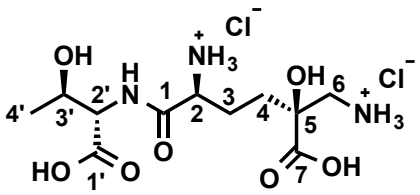


Figure S6. ^{1}H-NMR spectrum (300 MHz) of tabtoxin (TβL-Thr) formate salt in D_2O at pH 3.2 in ammonium formate buffer. The ammonium formate buffer is left over from HILIC Prep HPLC purification. This spectrum matched fully characterized samples previously reported by our laboratory.[3] An acetonitrile (CH_3CN) standard at 10 mM was included to quantify the sample. This sample was hydrolyzed with *P. syringae* cell lysate to give TβL which was titrated enzymatically as described previously[3] and used without NMR characterization.

Table S3. NMR spectral data (500 MHz) for tabtoxinine-threonine dipeptide (TβL-Thr-COOH) dihydrochloride salt in D_2O containing 0.2 M potassium phosphate at pH 7.0.



TβL-Thr-COOH • 2HCl

Atom	^{13}C (ppm)	^{1}H (ppm), multiplets in Hz	gHMBC ^{1}H-^{13}C couplings
1	169.5		
2	53.49	4.20-4.27 (m, 1 H)	1, 3, 4
3a	25.7	2.00-2.10 (m, 1 H)	1, 2, 4
3b		1.75-1.97 (m, 1 H)[a]	1, 2
4a	30.9	1.95-2.03 (m, 1 H)	3, 6
4b		1.75-1.97 (m, 1 H)[a]	7
5	75.3		
6a	51.6	3.28 (d, J = 12.9 Hz, 1 H)	4, 5, 7
6b		3.12 (d, J = 13.3 Hz, 1 H)	4, 5, 7
7	176.8		
1’	174.5		
2’	59.4	4.35-4.39 (m, 1 H)	1[b], 1’, 3’
3’	67.3	4.30-4.35 (m, 1 H)	1’, 4’
4’	19.0	1.21 (d, J = 6.1 Hz, 3 H)	2’, 3’

[a]Overlapping signals: 4b is primarily at the lower ppm range of this peak. [b]This is the key, strong correlation showing that the dipeptide bond is intact.

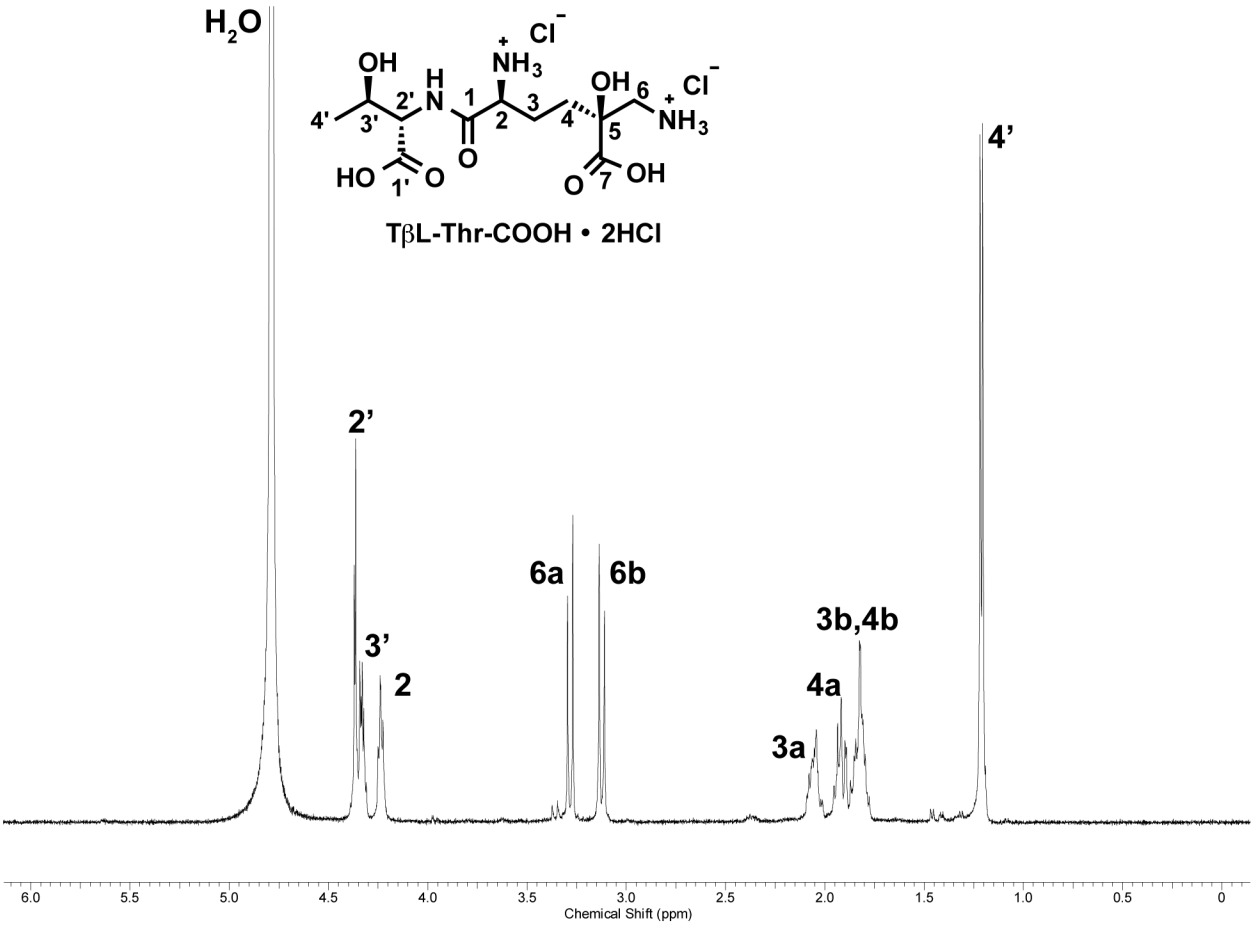
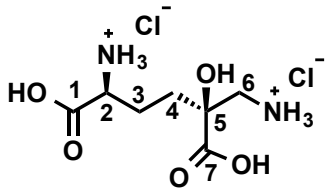


Figure S7. ^{1}H-NMR spectrum (500 MHz) of tabtoxinine-threonine dipeptide (TβL-Thr-COOH) dihydrochloride salt in D_2O containing 0.2 M potassium phosphate at pH 7.0.

Table S4. NMR spectral data (500 MHz) for tabtoxinine (TβL-COOH) dihydrochloride salt in D_2O containing 0.2 M potassium phosphate at pH 7.0.



TβL-COOH • 2HCl

Atom	^{13}C (ppm)	^{1}H (ppm), multiplets in Hz	gHMBC ^{1}H-^{13}C couplings
1	176.0		
2	56.3	3.92-4.00 (m, 1 H)	1, 3, 4
3a	27.2	2.00-2.12 (m, 1 H)	2
3b		1.78-1.90 (m, 1 H)	1, 2, 4
4a	34.6	1.95-2.05 (m, 1 H)	3
4b		1.68-1.77 (m, 1H)	3, 5, 6, 7
5	77.6		
6a	48.5	3.39 (d, J = 13.3 Hz, 1 H)	4, 7
6b		3.16 (d, J = 13.3 Hz, 1 H)	4, 5, 7
7	179.1		

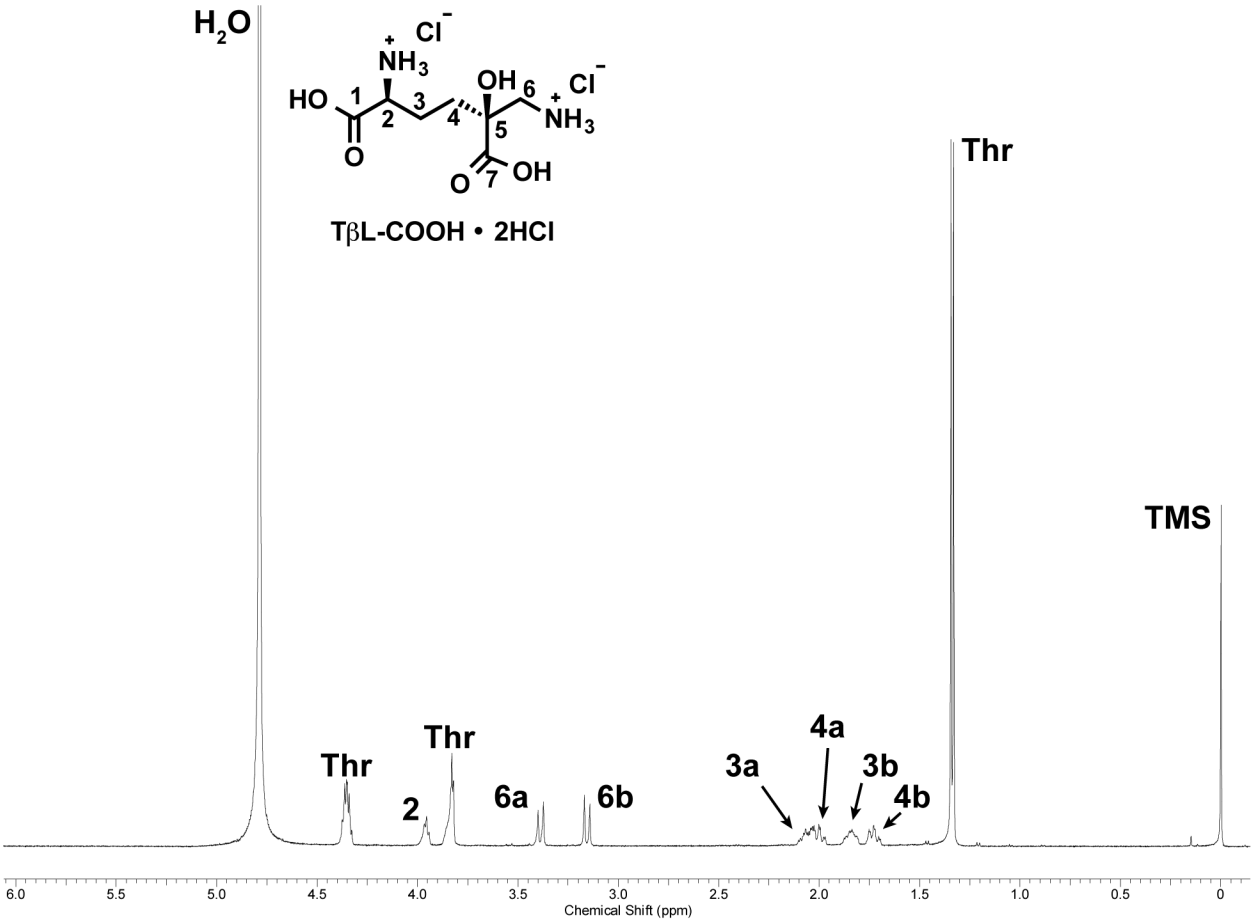


Figure S8. ^{1}H-NMR spectrum (500 MHz) of tabtoxinine (TβL-COOH) dihydrochloride in D_2O containing 0.2 M potassium phosphate at pH 7.0 an TMS internal standard. Signals at 4.35, 3.85, and 1.35 ppm are from L-Threonine as confirmed by spiking the sample with authentic L-Threonine.

VII. Acknowledgments

We thank Mr. Garrett Patrick (Wencewicz Lab) for providing authentic samples of TβL-COOH and TβL-Thr-COOH and assisting with preparation of TβL-Thr and TβL samples. We thank Dr. Andre D'Avignon (formerly WUSTL, Dept. of Chemistry; currently Sanford Burnham Medical Research Institute, Orlando, FL) and Dr. Jeff Kao (WUSTL, Dept. of Chemistry) for assistance provided in the acquisition and processing of 2D NMR spectra. We thank Dr. Yu Chen (University of South Florida) for the gift of the plasmid encoding CTX-M-9. We thank the Proteomics & Mass Spectrometry Facility at the Danforth Plant Science Center for analysis of purified proteins and whose work is supported by the National Science Foundation under Grant No.DBI-0922879 for acquisition of the LTQ-Velos Pro Orbitrap LC-MS/MS. We thank Pfizer for providing the library of *E. coli* DH5α β-lactamase expressing bacteria. GRB holds a Career Award at the Scientific Interface from the Burroughs Wellcome Fund.

VIII. References

[1] Chen, Y., Shoichet, B., and Bonnet, R. (2005) Structure, Function, and Inhibition along the Reaction Coordinate of CTX-M β-Lactamases, *Journal of the American Chemical Society 127*, 5423-5434.

[2] Starr, J., Brown, M. F., Aschenbrenner, L., Caspers, N., Che, Y., Gerstenberger, B. S., Huband, M., Knafels, J. D., Lemmon, M. M., Li, C., McCurdy, S. P., McElroy, E., Rauckhorst, M. R., Tomaras, A. P., Young, J. A., Zaniewski, R. P., Shanmugasundaram, V., and Han, S. (2014) Siderophore receptor-mediated uptake of lactivicin analogues in gram-negative bacteria, *Journal of medicinal chemistry 57*, 3845-3855.

[3] Wencewicz, T. A., and Walsh, C. T. (2012) *Pseudomonas syringae* self-protection from tabtoxinine-β-lactam by ligase TblF and acetylase Ttr, *Biochemistry 51*, 7712-7725.

[4] Levine, B. B. (1960) Degradation of benzylpenicillin at pH 7.5 to D-benzylpenicilloic acid, *Nature 187*, 939-940.