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References

Supplementary Methods

Pacidamycin production and isolation. For PacB gene deletion experiment, two mutants were selected for pacidamycin production analysis after PCR verification (primers listed in table S1). Wild-type *S. coeruleorubidus* was grown side by side as controls with all of the mutants. Starter cultures in TSB medium were inoculated with spores and incubated at 30 °C, 200 rpm for 24 h. Production medium (20 mL; 20.99 g/L MOPS, 10 g/L lactose, 4.41 g/L K_2HPO_4, 2.14 g/L NH_4Cl, 0.6 g/L $MgSO_4$, 10 mg/L $FeSO_4{\cdot}2H_2O$, 10 mg/L $MnCl_2{\cdot}4H_2O$, 10 mg/L $ZnSO_4{\cdot}7H_2O$, 10 mg/L $CaCl_2$, pH 7.0) was inoculated with starter culture (0.5 mL) and incubated at 30 °C, 200 rpm for 5 d. Pacidamycins were extracted from the cell-free broth (10 mL) using XAD-16 resin (5% v/v). The resin was washed with water (20 mL) and the extract was eluted with methanol (5 mL). The solvent was removed by a rotary evaporator and the residue was redissolved in water/methanol 1:1 (500 µL) and subjected to LC-HRMS analysis (25 µL injection).

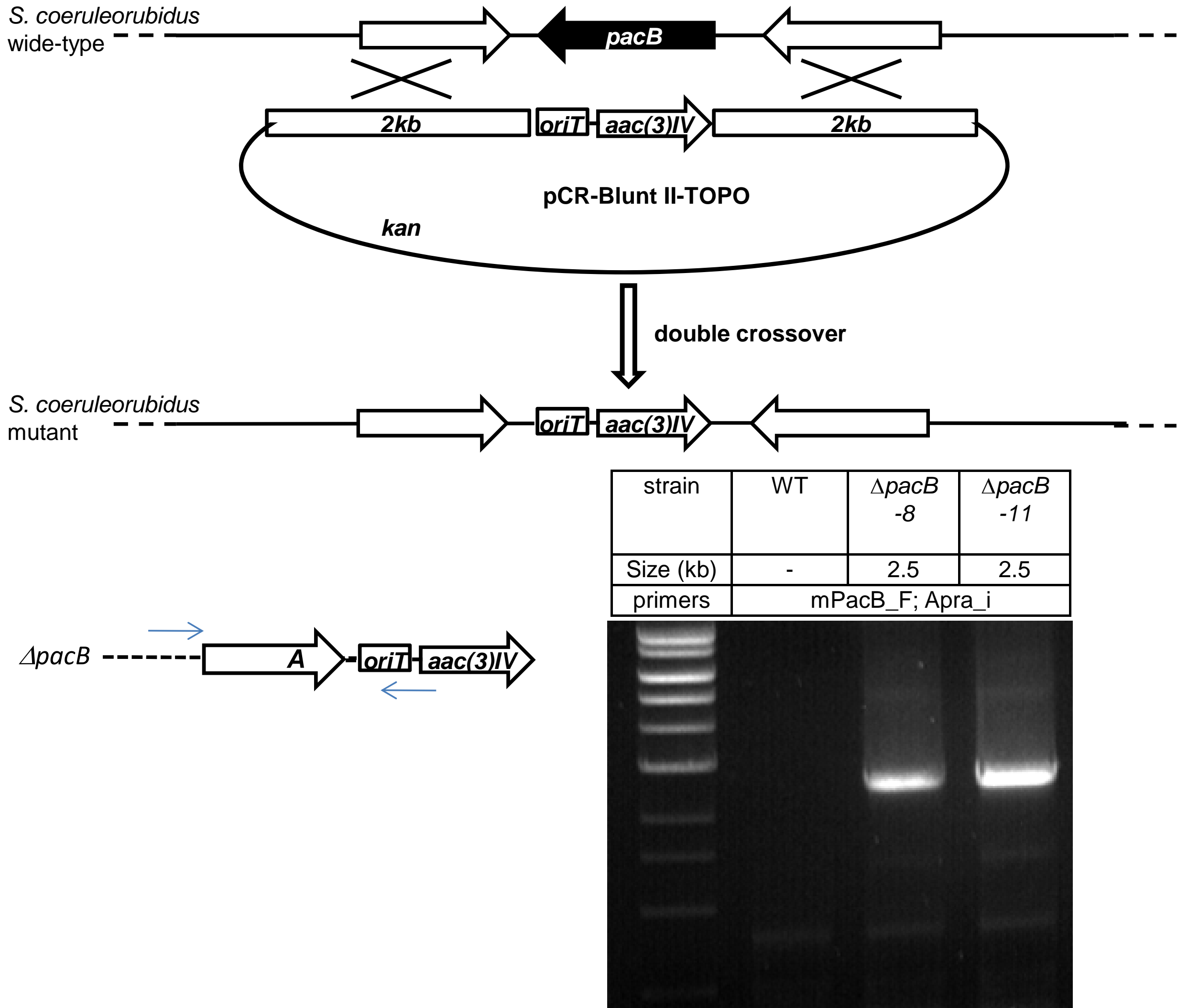
Cloning, Overexpression and Purification of Proteins. Cloning, expression, and purification of PacDHIJLNOPW have been reported previously (1). *pacB* was PCR amplified from genomic DNA and ligated to pET-30 Xa/LIC (Novagen) following the standard protocol and confirmed by DNA sequencing. The resulting expression construct was transformed into *E. coli* BL21 for protein expression. In 1 L of liquid culture, the cells were grown at 37 °C in LB medium with 50 µg/mL kanamycin to an OD600 of 0.4. The cells were cooled on ice for 10 min and then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 16 °C. The cells were harvested by centrifugation (6000 rpm, 6 min, 4 °C), resuspended in 30 mL lysis buffer (25 mM HEPES pH 8.0, 0.5 M NaCl, 5 mM imidazole) and lysed by sonication on ice. Cellular debris was removed by ultracentrifugation (35000 rpm, 35 min, 4 °C). Ni-NTA agarose resin was added to the supernatant (2 mL/L of culture) and the solution was nutated at 4 °C for 1 h. The protein resin mixture was loaded into a gravity flow column, and proteins were eluted with increasing concentrations of imidazole in Buffer A (50 mM HEPES, pH 8.0, 1 mM EDTA). Purified proteins were concentrated and buffer exchanged into Buffer A + 10% glycerol using Amicon Ultra filters. The final proteins were flash-frozen in liquid nitrogen and stored at -80 °C.

Supplementary Table

Table S1. Primers used in this study.

Primer	Sequence	Note
mPacB_F	5’- AAAACCGCCCTTGCCCGCGG-3’	Used for Δ*pacB* mutant construction; mPacB_F is also used for mutant verification
mPacB_R	5’- AACCGGATCCGGTAGGACAG-3’	
mPacBap_F	5’-CTCGCTGGTTGGACACTAGATCATGAGGGCTGATACatgATTCCGGGGATCCGTCGACC -3’	
mPacBap_R	5’-CCCGGTGGCTGCCGGCGGGTACCAGCCGCCTCCCTCttaTGTAGGCTGGAGCTGCTTC-3'	
Apra_i	5’-AGTTGTCTCTGACACATTCT-3’	Internal primer for mutant verification
PacB_PET30F	5’- GGTATTGAGGGTCGCATGGCTATTGGTTTTACCTC-3’	Used for PacB expression
PacB_PET30R	5’- AGAGGAGAGTTAGAGCCTTATGATTCGCTACAGGCAA-3’	

Supplementary Figures



strain	WT	Δ*pacB* -8	Δ*pacB* -11
Size (kb)	-	2.5	2.5
primers	mPacB_F; Apra_i		

Fig. S1. Inactivation of *pacB* in *S. coeruleorubidus*. *pacB* was deleted in-frame by homologous recombination as illustrated above. The genomic DNA of wide-type and two Δ*pacB* mutant strains was extracted and used as templates for subsequent PCR verification of mutants. PCR was carried out using one internal primer from *acc(3)IV-oriT* cassette and one external primer (Table S1). The size of PCR products was consistent with the expected size of Δ*pacB* mutant.

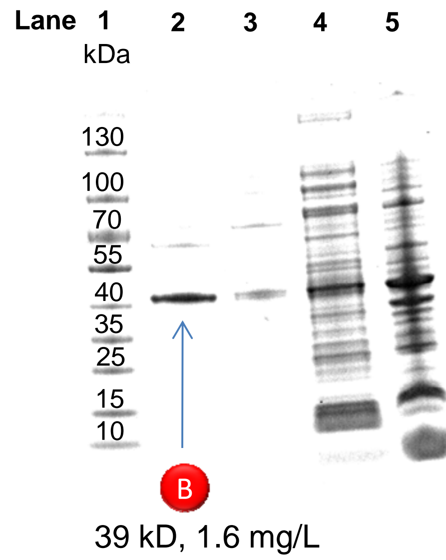


Fig. S2. SDS-PAGE analysis of the *E. coli* purified PacB. Lane 1: Ladder; Lane 2: Elution; Lane 3: Wash; Lane 4: Flowthrough; Lane 5: insoluble pellet.

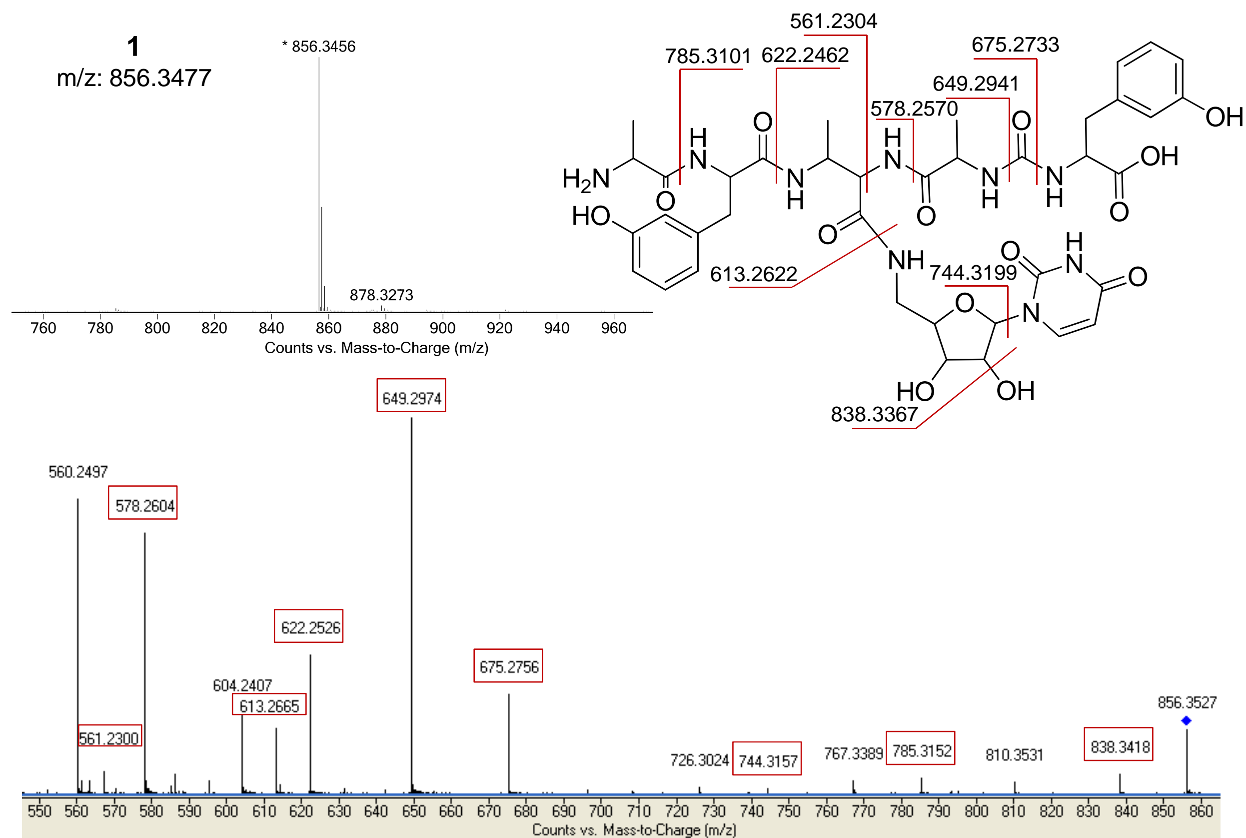


Fig. S3. HR-MS and HR-MS/MS of **1** measured during LC-MS.

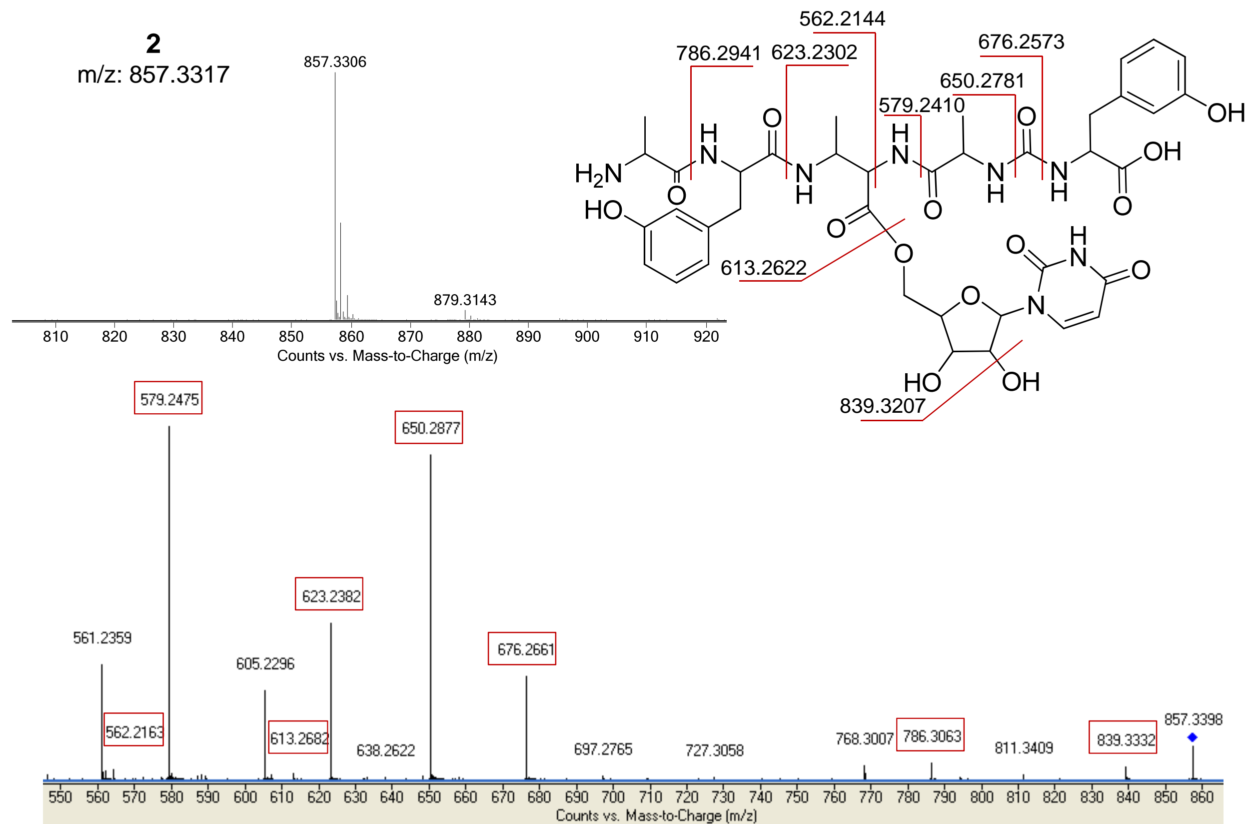


Fig. S4. HR-MS and HR-MS/MS of **2** measured during LC-MS.

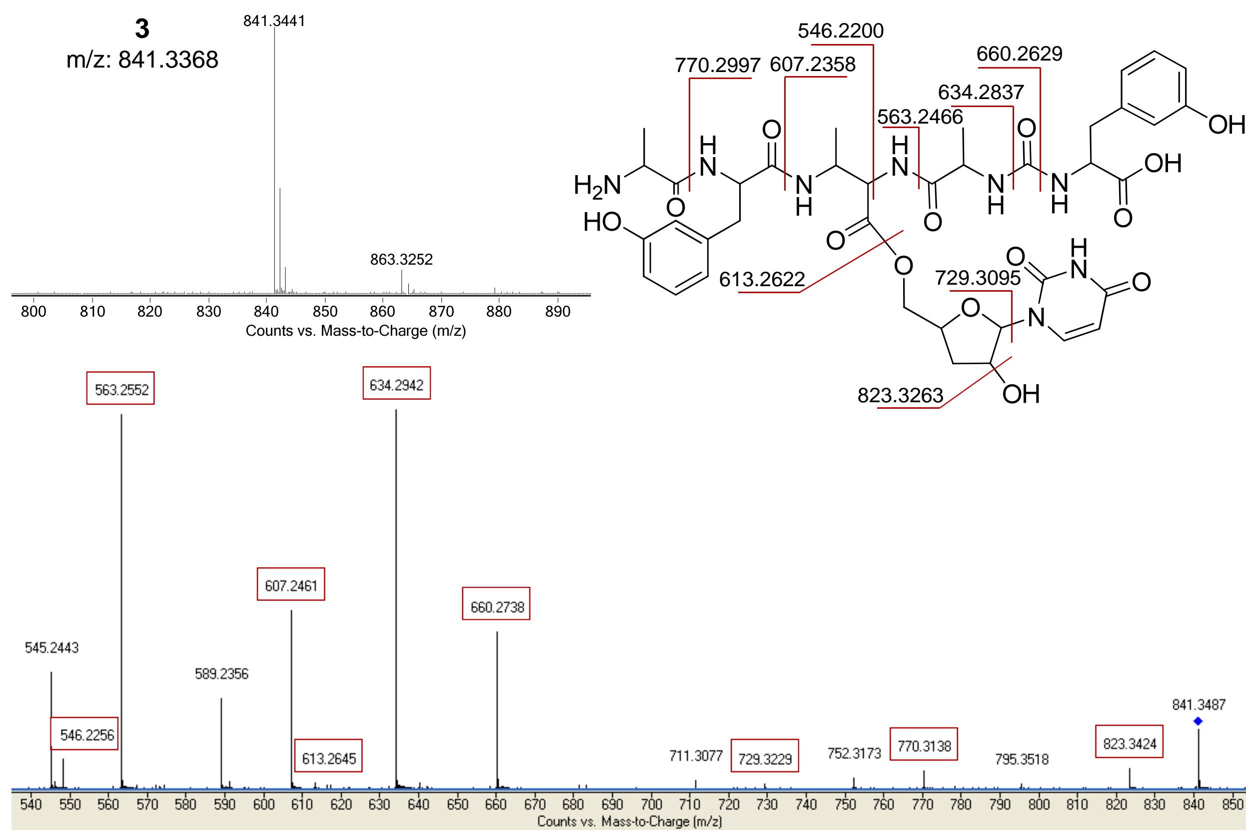


Fig. S5. HR-MS and HR-MS/MS of **3** measured during LC-MS.

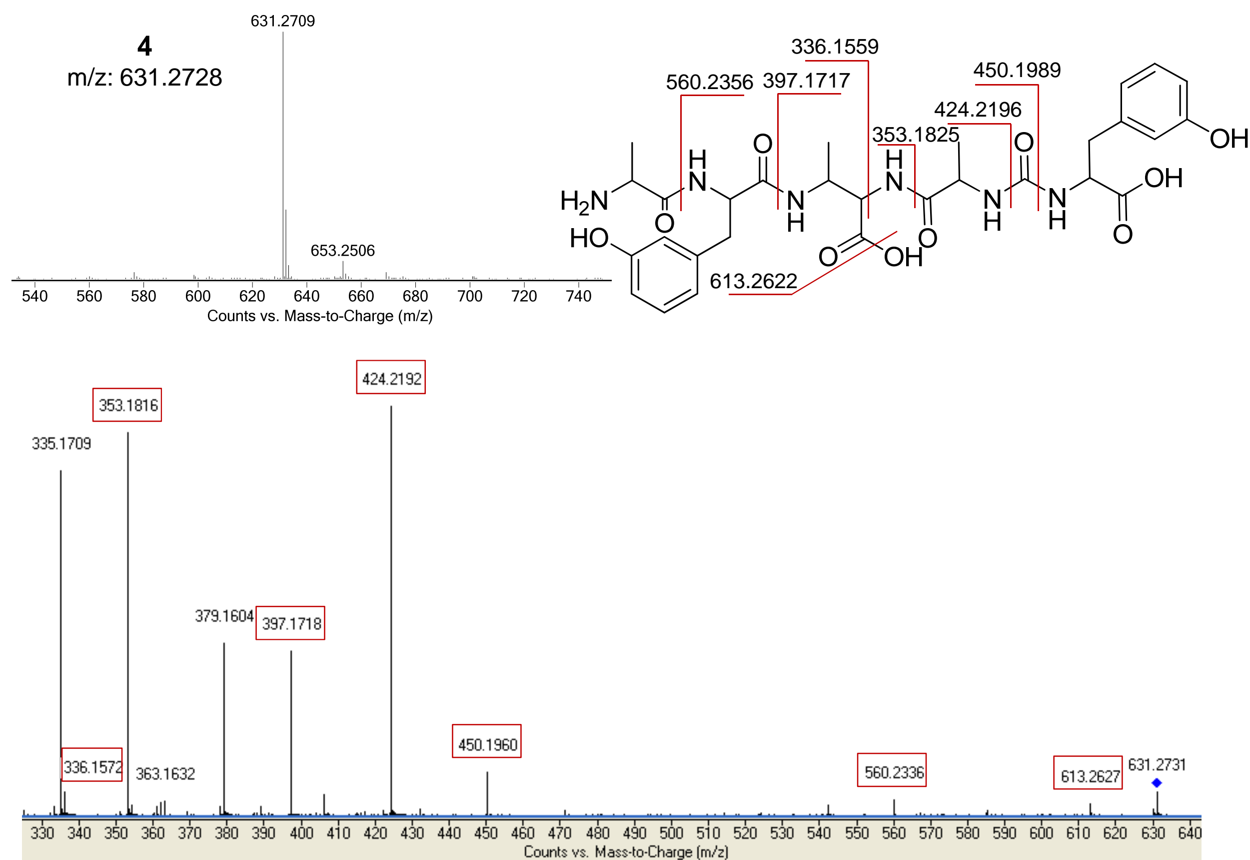


Fig. S6. HR-MS and HR-MS/MS of **4** measured during LC-MS.

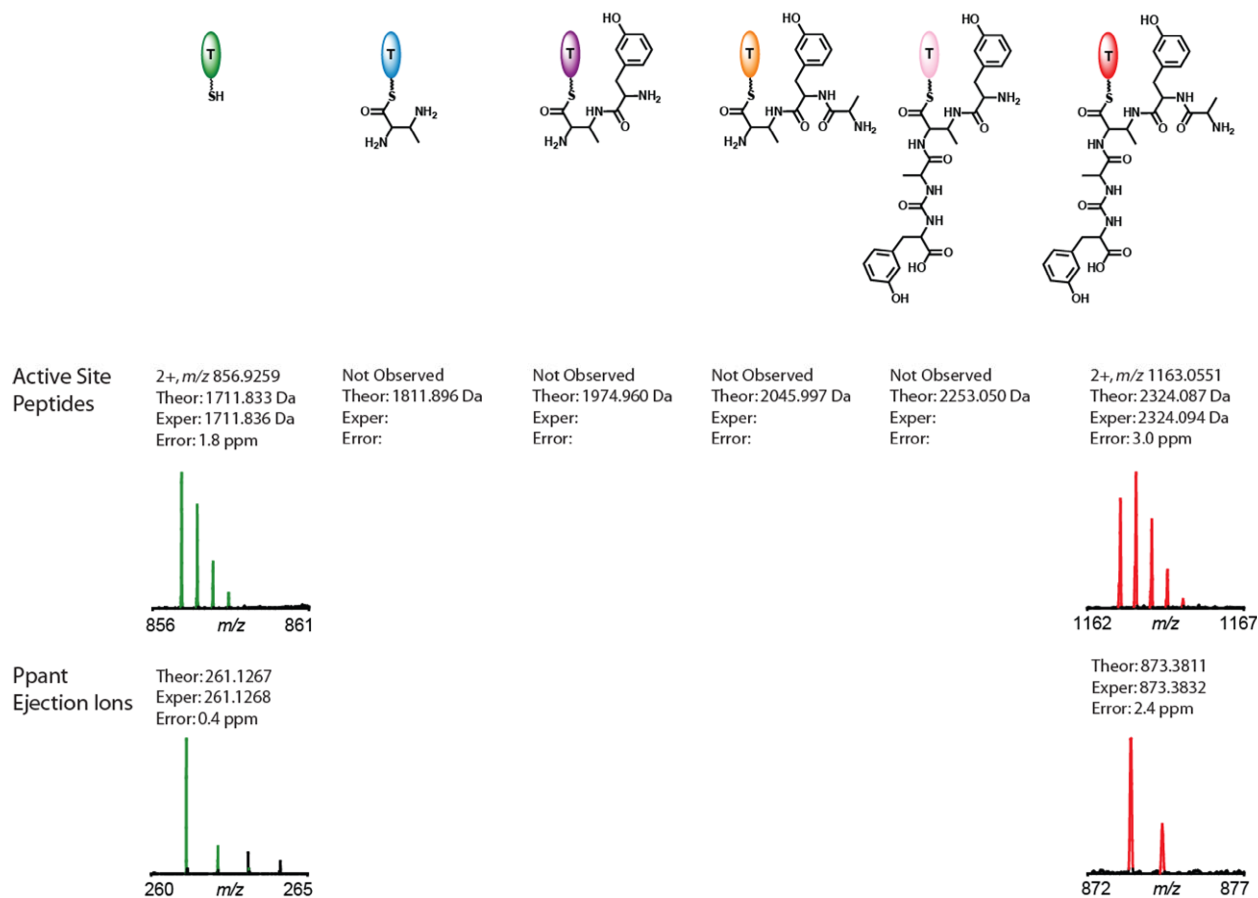


Fig. S7. FTMS and Ppant ejection analysis of the *in vitro* reaction generating PacH-bound Ala_1-$m Tyr_2$-$DABA_3$-Ala_4-CO-$m Tyr_5$. Enzymes and substrates: PacBDHJLNOPW, aatRS, tRNA, DABA, *m*-Tyr, Ala, ATP.

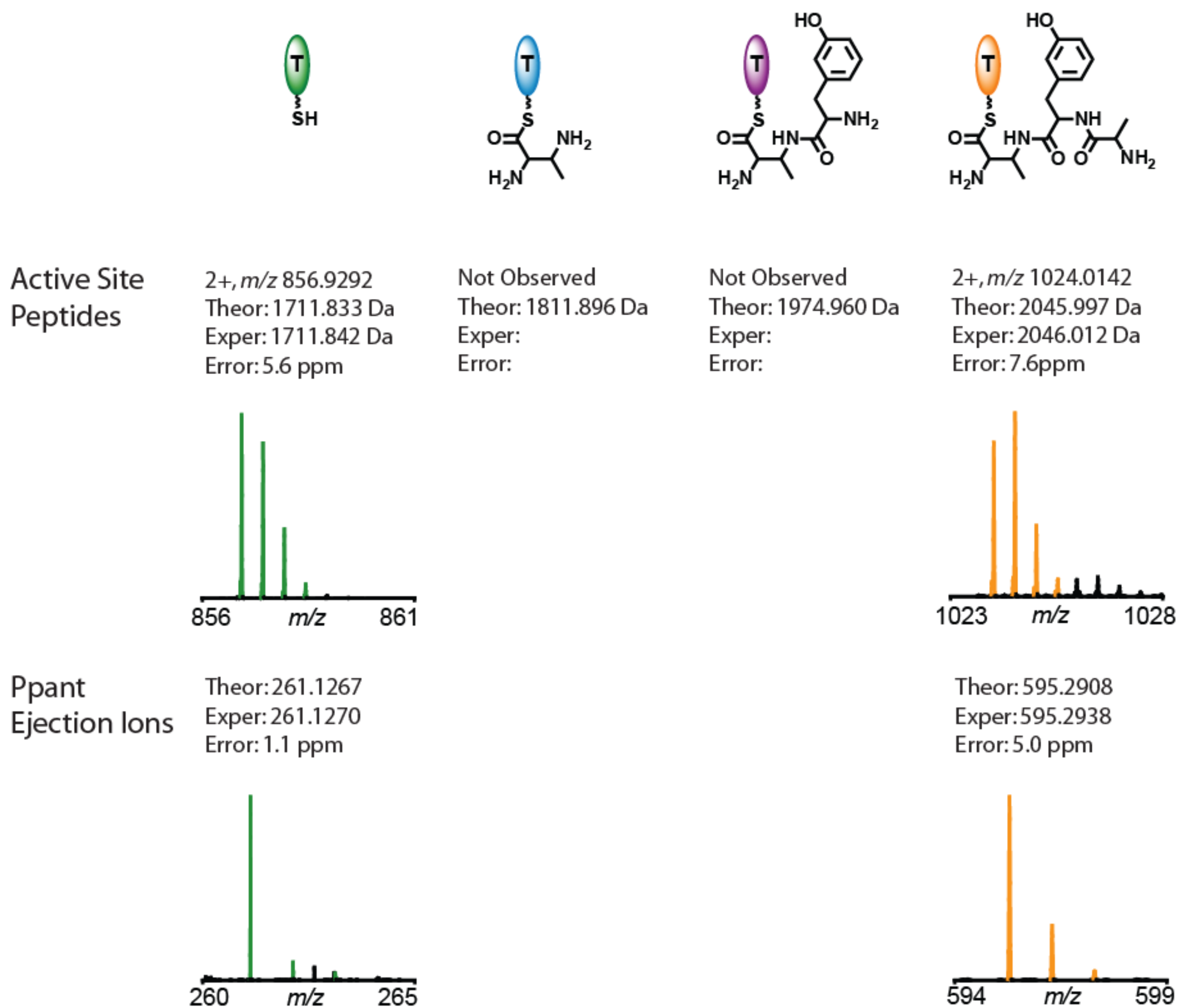


Fig. S8. FTMS and Ppant ejection analysis of the *in vitro* reaction generating Ala_1-*m*Tyr_2-$DABA_3$-*S*-PacH. Enzymes and substrates: PacBHPW, aatRS, tRNA, DABA, *m*-Tyr, Ala, ATP.

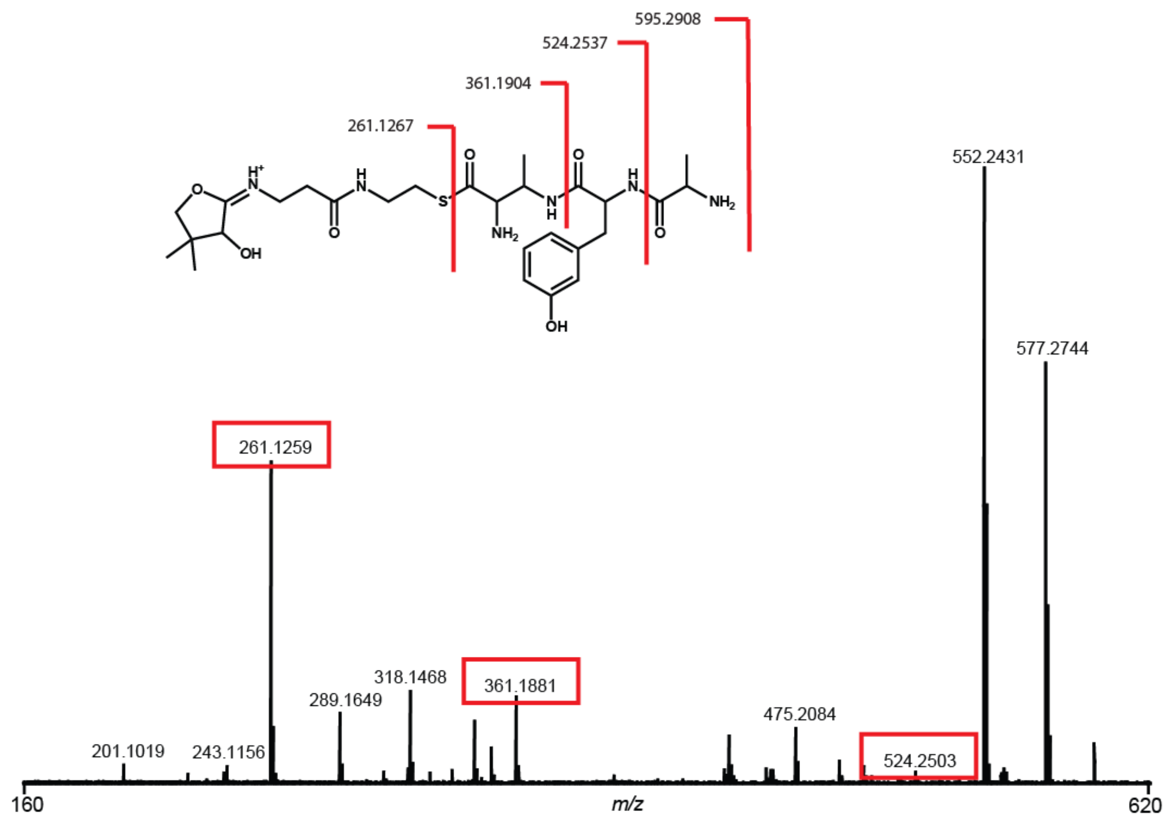


Fig. S9. MS/MS analysis of Ala_1-*m*Tyr_2-$DABA_3$-Pant ion in Ppant ejection assay.

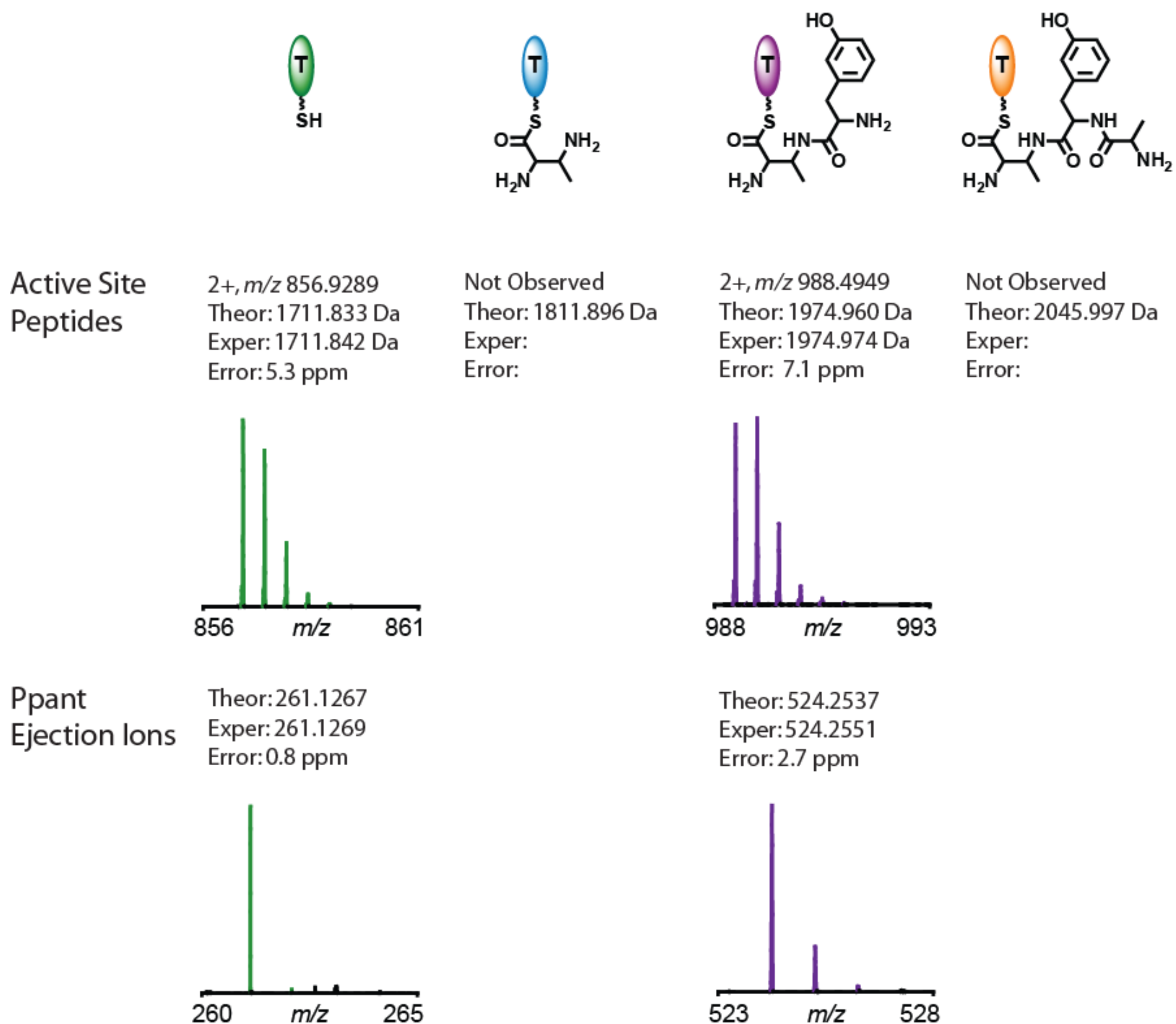


Fig. S10. FTMS and Ppant ejection analysis of the *in vitro* reaction generating *m*Tyr$_2$-DABA$_3$-*S*-PacH without tRNA. Enzymes and substrates: PacBHPW, aatRS, DABA, *m*-Tyr, Ala, ATP.

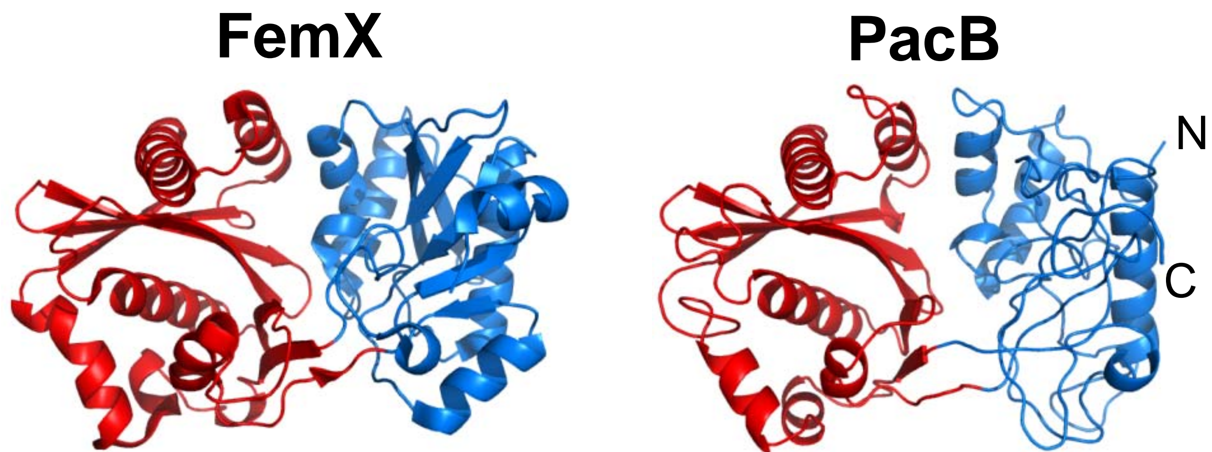


Fig. S11. Three-dimensional structural model of PacB in comparison to the reported structure of FemX (PDB ID 3GKR). Domain 1 is represented in blue and domain 2 in red. FemX domain 1: residues 1-145 and 317-335, FemX domain 2: residues 146-316. PacB domain 1: residues 1-164 and 332-350, PacB domain 2: residues 165-331.

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                1                          10             20             30             40             50
FemX  ......MPVLNLND.........PQAVERYEEFMRQSPYG.QVTQDLGWAKVKNNWEPVDVYLEDDQGAI
PacB  MAIGFTSAIADFDQKQFDALDTTAGAASAYSRLRQHEQDARWTSRYLGWFDGDEVRAAIPVYRYRMRSWP

             60             70             80             90            100            110            120
FemX  IAAMSMLLGDTPTDKKFAYASKGPVMDVTDVDLLDRLVDEAVKALDGRAYVLRFDPEVAYSDEFNTTLQD
PacB  DPSYDPRSWGLPDGIAEECSPRASLMVGGCIDRRTGFHVDAEARTPRELQRLLVEIAKHAADEDMCLTFP

            130            140            150            160            170            180            190
FemX  HGYVTRNRNVADAGMHATIQPRLNMVLDLTKFPDAKTTLDLYPSKTKSKIKRPFRDGVEVHSGNSATELD
PacB  YMYADAQSALAAATDDRIVWAELAREAHLFGLSDAQWESSLS.AKIRYRLRQDQRKIAAVPMTVGEVSWP

            200            210            220                       230            240            250
FemX  EFFKTYTTMAERHGITHRPIEYFQRMQAAFD.....ADTMRIFVAEREGKLLSTGIALKYGRKIWYMYAG
PacB  EVDTWASELISHHNASKGAHEHPEFVSFRYSGWQDNPDIDLMAFTARSAGLRGVETILLWENELEVYEVG

        260            270            280            290            300            310            320
FemX  SMDGNTYYAPYAVQSEMIQWALDTNTDLYDLGGIESESTDDSLYVFKHVFVKDAPREYIGEIDKVLDPEV
PacB  .MTGEESDERFALYLNLLFHLPIQYARARGIDHIRLGSKAETPKALRGAAFEN...LYGGVLSRAETKRL

        330
FemX  YAELVKD
PacB  ACSES..
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Fig. S12. Sequence alignment of PacB and FemX of *W. viridescens* by ClustalW analysis (2).

References

1. Zhang W, *et al.* (2011) Nine enzymes are required for assembly of the pacidamycin group of peptidyl nucleoside antibiotics. *J Am Chem Soc* 133:5240-5243.

2. Larkin MA, *et al.* (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947-2948.