Supplementary Information

Epimerization and Substrate Gating by a TE-Domain in β-Lactam Antibiotic Biosynthesis

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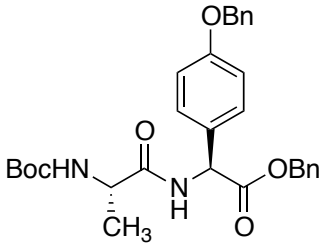
General Methods:

^{1}H-NMR spectra were recorded on a Bruker Avance (Billerica, MA) 400 or 300 MHz spectrometer or a Varian 500 MHz spectromenter. Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ 0.0 ppm) or with the solvent reference relative to TMS (H_2O, δ 4.79 ppm, $CHCl_3$, δ 7.26 ppm, DMSO-d_5, 2.50 ppm). Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration). ^{13}C-NMR spectra were recorded on a Bruker 400 (101 MHz) spectrometer with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the $(CD_3)_2SO$ (δ 39.52 ppm) or $CDCl_3$ (δ 77.16 ppm) as the internal standard. High-resolution mass spectrometry was performed by fast atom bombardment (FAB) or electrospray ionization (ESI) at the Mass Spectrometry Facility of the Johns Hopkins University. Column chromatography was carried out on Silica Gel 60 Merck (Whitehouse Station, NJ), 230-400 mesh ASTM. Reagents and chemicals were purchased from the Sigma-Aldrich Chemical Company (Milwaukee, WI) unless otherwise noted and used without further purification. Pearlman's catalyst (Pd-OH/C) was purchased and used without further purification from Strem Chemicals, Inc. (Newburyport, MA). All solvents used for reactions were distilled prior to use (THF over Na/benzophenone, CH_2Cl_2 and CH_3CN over CaH).

Preparative HPLC purifications were performed on an Agilent model 1100 (Santa Clara, CA) equipped with a multi-wavelength UV-Vis detector in conjunction with a reverse-phase Phenomenex Luna 10μ C18(2) 100 Å preparatory column (250 x 21.20 mm ID). Mobile phase conditions included one of the following: **Prep Method A** (water + acetonitrile (ACN) +0.1% TFA): 0–5 min isocratic 13% water 87% ACN + 0.1% TFA, 5-25 min gradient 13% to 50% ACN + 0.1% TFA, 25-30 min 50% ACN to 13% ACN, 30-35 min isocratic 13% water 87% ACN + 0.1% TFA. Flow rate = 6.5 mL/min. **Prep Method B** (water + ACN + 0.1% TFA): 0-25 min gradient 15-80% ACN + 0.1% TFA, 25-30 min 80% to 15% ACN + 0.1% TFA, 30-35 min 15% ACN + 85% water + 0.1% TFA. Flow rate = 6.5 mL/min.

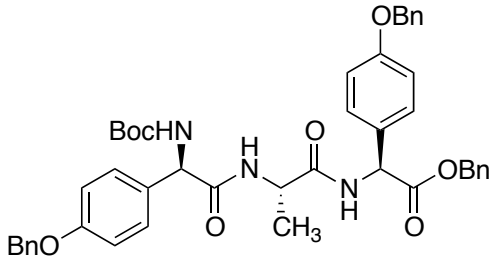
Synthesis of Compounds 4-13. Syntheses of compounds **4**-**9**, and **11** have been previously reported.[1] Compounds **10, 12,** and **13** are described below:



S1

***N-tert*-Butyloxycarbonyl-L-alanyl-L-[*p*-(benzyloxy)phenyl]glycine Benzyl Ester (S1).** To a 250 mL round-bottomed flask equipped with a magnetic stir bar, Boc-L-Ala (2.0 g, 10.57 mmol) and DIEA (1.37 mL, 10.57 mmol) were dissolved in 30 mL of freshly distilled DCM and 10 mL of reagent grade DMF and cooled to 0 °C in an ice-bath. In a separate flask benzyl-L-[*p*-(benzyloxy)phenyl]glycine toluenesulfonate[1] (5.49 g, 10.57 mmol) was dissolved in 30 mL of reagent grade DMF and to this suspension was added DIEA (2.78 mL, 21.14 mmol) which was cooled to 0 °C in an ice-bath. When both solutions had come to temperature, PyBOP (16.05 g, 11.63 mmol) was added to the flask containing Boc-L-Ala. After 1 min, the amine solution containing benzyl-L-[*p*-(benzyloxy)phenyl]glycine toluenesulfonate was added dropwise over 2 min to the activated carboxylic acid and the reaction mixture was allowed to stir at 0 °C to room temperature for 3 h. The solution was diluted with 200 mL of EtOAc and washed with sat. aq. NH_4Cl (2 x 75 mL), sat. aq. $NaHCO_3$ (2 x 75 mL) and brine (1 x 75 mL). The organic layer was concentrated *in vacuo* and the residue purified by silica gel chromatography with a gradient of 30:70 EtOAc:Hex to 50:50 EtOAc: Hex over 3 L to obtain the product as a white foam (4.44 g, 81%). **^{1}H-NMR** (400 MHz; DMSO-d_6): δ 8.54 (d, *J* = 6.8 Hz, 1H), 7.47-7.23 (m, 12H), 7.03 (d, *J* = 8.7 Hz, 2H), 6.96 (d, *J* = 7.8 Hz, 1H), 5.40 (d, *J* = 6.8 Hz, 1H), 5.13 (s, 1H), 5.12 (s, 1H), 4.12 (app. quintet, *J* = 7.2 Hz, 1H), 1.37 (s, 9H), 1.19 (d, *J* = 7.2 Hz, 3H). **^{13}C-NMR** (101 MHz, DMSO-d_6): δ 173.4, 171.0, 158.8, 155.5, 137.5, 136.2, 129.6, 128.9, 128.8, 128.52, 128.46, 128.3, 128.1,

128.0, 115.4, 100.0, 78.5, 69.7, 66.6, 56.2, 49.6, 28.7, 18.6. **HRMS** (FAB) calcd for $C_{30}H_{35}N_2O_6$ 519.24951; Found 519.24864 $[M+H]^+$.

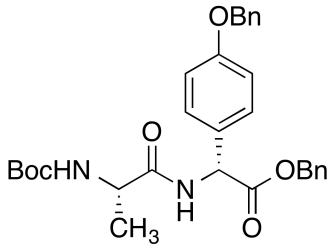


S2

***N-tert*-Butyloxycarbonyl-D-[*p*-(benzyloxy)phenyl]glycyl-L-alanyl-L-[*p*-(benzyloxy)-phenyl]glycine Benzyl Ester (S2).** To a 250 mL round-bottomed flask equipped with a magnetic stir bar, **S1** (2.00 g, 3.86 mmol) was dissolved in 100 mL of a 1:3 DCM:TFA solution and stirred at room temperature for 30 min. The solution was concentrated *in vacuo* and residual TFA was removed by azeotropic distillation with toluene (2 x 75 mL) and placed under high vacuum for 20 min.

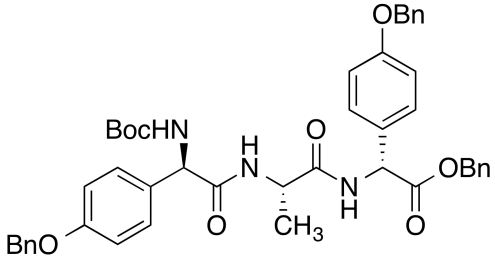
In a separate 250 mL round-bottomed flask equipped with a magnetic stir bar, *N-tert*-butyloxycarbonyl-D-*p*-(benzyloxy)phenyl]glycine[1] (1.52 g, 4.25 mmol) and DIEA (0.74 mL, 4.25 mmol) were dissolved in 30 mL of reagent grade DMF and cooled to 0 °C in an ice-bath. The freshly deprotected **S1** was separately dissolved in 30 mL of reagent grade DMF, DIEA (1.34 mL, 7.72 mmol) was added and the solution was cooled to 0 °C in an ice-bath. When both solutions were sufficiently cooled, PyBOP (2.21 g, 4.25 mmol) was added to the flask containing *N-tert*-butyloxycarbonyl-D-*p*-(benzyloxy)phenyl]glycine. After 1 min the solution containing **S1** was added dropwise over 2 min to the activated carboxylic acid and the solution was allowed to stir 0 °C to room temperature for 3 h. The reaction mixture was diluted with 200 mL of EtOAc and washed with sat. aq. NH_4Cl (2 x 75 mL), sat. aq. $NaHCO_3$ (2 x 75 mL) and brine (1 x 75 mL). The organic layer was concentrated *in vacuo* and the product was purified by silica gel chromatography using a gradient of 40:60 EtOAc:Hex to 70:30 EtOAc:Hex over 3 L to afford the product as a white foam (2.19 g, 75%). **^{1}H-NMR** (400 MHz; DMSO-d_6): δ 8.70 (d, *J* = 6.8 Hz, 1H), 8.32 (d, *J* = 7.3 Hz, 1H), 7.45-7.21 (m, 19H), 7.15 (d, *J* = 8.4 Hz, 1H), 7.01 (d, *J* = 8.8 Hz, 2H), 6.94 (d, *J* = 8.8 Hz, 2H), 5.38 (d, *J* = 6.8 Hz, 1H), 5.17

(d, *J* = 8.4 Hz, 1H), 5.11 (s, 4H), 5.07 (s, 2H), 4.36 (app. quintet, *J* = 7.1 Hz, 1H), 1.36 (s, 9H), 1.13 (d, *J* = 7.1 Hz, 3H). **^{13}C-NMR** (101 MHz, DMSO-d_6): δ 172.7, 170.9, 170.4, 158.8, 158.2, 155.2, 137.6, 137.4, 136.2, 131.8, 129.7, 128.9, 128.8, 128.5, 128.3, 128.1, 128.0, 115.4, 114.9, 78.9, 69.6, 66.6, 57.3, 56.4, 48.1, 28.6, 18.7. **HRMS** (FAB) calcd for $C_{47}H_{48}N_3O_8$ 758.34414; Found 758.34233 $[M+H]^+$.



S3

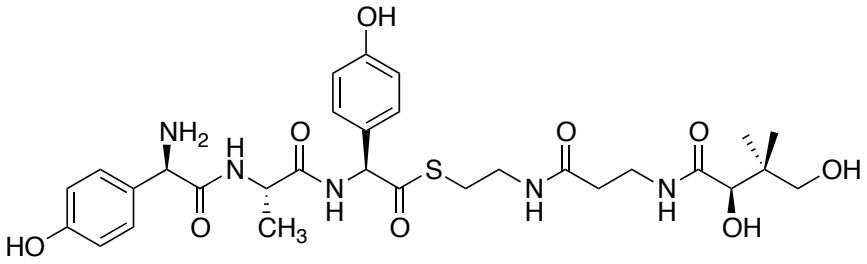
***N-tert*-Butyloxycarbonyl-L-alanyl-D-[*p*-(benzyloxy)phenyl]glycine Benzyl Ester (S3).** The title compound was prepare and purified analogously to compound **S1** by replacing benzyl-L-[*p*-(benzyloxy)phenyl]glycine toluenesulfonate with benzyl-D-[*p*-(benzyloxy)phenyl]glycine toluenesulfonate in the procedure. The product was obtained as an amorphous solid (4.69 g, 86%). **^{1}H-NMR** (400 MHz; DMSO-d_6): δ 8.52 (d, *J* = 6.9 Hz, 1H), 7.46-7.25 (m, 12H), 7.01 (d, *J* = 8.4 Hz, 2H), 6.93 (d, *J* = 7.6 Hz, 1H), 5.42 (d, *J* = 6.9 Hz, 1H), 5.17-5.08 (m, 4H), 4.13 (dt, *J* = 13.6, 6.5 Hz, 1H), 1.38 (s, 9H), 1.16 (d, *J* = 6.5 Hz, 3H). **^{13}CNMR** (101 MHz; DMSO-d_6): δ 173.2, 170.9, 158.7, 155.4, 137.4, 136.2, 129.3, 128.91, 128.81, 128.43, 128.31, 128.11, 128.01, 115.3, 100.0, 78.6, 69.7, 66.6, 56.1, 50.0, 28.7, 18.8. **HRMS** (FAB) calcd for $C_{30}H_{35}N_2O_6$ 519.24951; Found 519.2495 $[M+H]^+$.



S4

***N-tert*-Butyloxycarbonyl-D-[*p*-(benzyloxy)phenyl]glycyl-L-alanyl-D-[*p*-(benzyloxy)-phenyl]glycine Benzyl Ester (S4).** The title compound was prepared and purified analogously to compound **S2** by replacing **S1** with **S3** in the procedure. The product

was obtained as an amorphous solid (2.37 g, 81%). **^{1}H-NMR** (400 MHz; DMSO-d_6): δ 8.76 (d, *J* = 6.5 Hz, 1H), 8.39 (d, *J* = 6.9 Hz, 1H), 7.46-7.22 (m, 20H), 7.03 (d, *J* = 8.6 Hz, 2H), 6.96 (d, *J* = 8.6 Hz, 2H), 5.49 (s, 1H), 5.24 (d, *J* = 7.8 Hz, 1H), 5.22, 5.08 (ABq, J_{AB} = 12.4 Hz, 2H), 5.17 (s, 2H), 5.09 (s, 2H), 4.47 (dt, *J* = 13.4, 6.7 Hz, 1H), 1.37 (s, 9H), 1.15 (d, *J* = 6.7 Hz, 3H). **^{13}C-NMR** (101 MHz; DMSO-d_6): δ 172.6, 170.9, 170.5, 158.8, 158.3, 155.3, 137.55, 137.44, 136.2, 131.6, 129.4, 128.92, 128.90, 128.83, 128.70, 128.44, 128.32, 128.28, 128.11, 128.03, 115.4, 114.9, 78.9, 69.7, 66.7, 60.3, 57.6, 56.2, 48.5, 46.5, 28.6, 19.0. **HRMS** (ESI) calcd for $C_{47}H_{48}N_3O_8$ 758.34414; Found 758.3451 $[M+H]^+$.

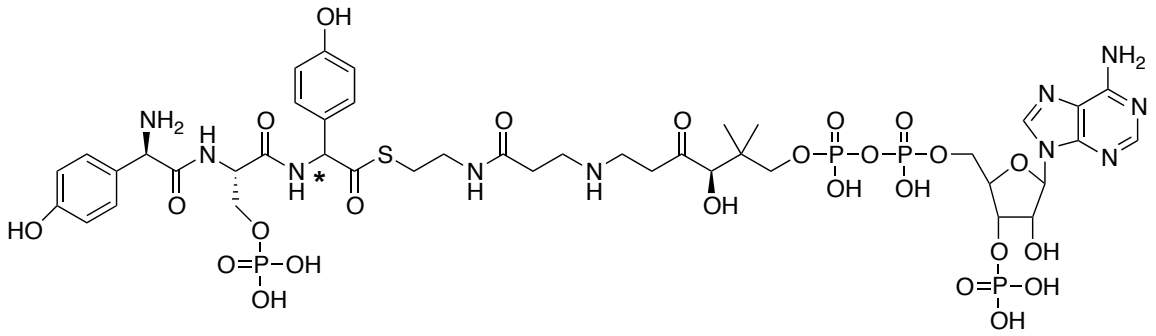


10

D-[*p*-(Hydroxy)phenyl]glycyl-L-alanyl-L-[*p*-(hydroxy)phenyl]glycyl-pantetheine (10). To a 250 mL pressure flask, protected tripeptide **S2** (568 mg, 0.75 mmol) was dissolved in 20 mL of reagent grade THF and to this solution was added a catalytic amount of Pd-OH/C. The mixture was vigorously shaken under 50 psi of H_2 overnight on a Parr hydrogenation apparatus. The mixture was filtered through Celite, which was washed with 100 mL of THF and concentrated *in vacuo* to a white foam and used in the next step without further purification.

To a 25 mL round-bottomed flask equipped with a magnetic stir bar, the freshly deprotected tripeptide was dissolved in 5 mL of reagent grade DMF. To this solution was added K_2CO_3 (291 mg, 2.27 mmol) and PyBOP (471 mg, 0.91 mmol) followed by pantetheine dimethyl ketal[1], and the reaction mixture was stirred at room temperature for 1 h. The solution was diluted with 35 mL of EtOAc and washed with sat. aq. NH_4Cl (2 x 15 mL), sat. aq. $NaHCO_3$ (1 x 15 mL) and concentrated *in vacuo*. The residue was redissolved in 2.0 mL of 1:1 ACN: H_2O and purified according to Prep Method B as a mixture of diastereomers. The product was collected, frozen on dry ice and lyophilized to dryness. The lyophilized powder was dissolved in TFA for 10 min, concentrated *in*

vacuo, re-dissolved in 2 mL of 80:20 H_2O:ACN with 0.1% TFA and purified by Prep Method B. The product was collected, frozen on dry ice and lyophilized to dryness to afford the product as the white TFA salt (199 mg, 41%). Isolation of 2 mg of diasteromerically pure material was achieved using Analytical Method A. **^{1}H-NMR** (400 MHz; D_2O): δ 7.25 (d, *J* = 8.6 Hz, 2H), 7.24 (d, *J* = 8.6 Hz, 2H), 6.85 (d, *J* = 8.6 Hz, 2H), 6.85 (d, *J* = 8.6 Hz, 2H), 5.43 (s, 1H), 4.98 (s, 1H), 4.29 (q, *J* = 7.2 Hz, 1H), 3.86 (s, 1H), 3.39 (d, *J* = 11.2 Hz, 1H), 3.31-3.21 (m, 4H) 3.27 (d, *J* = 11.2 Hz, 1H), 2.99 (t, *J* = 6.2 Hz, 2H), 2.18-2.15 (m, 2H), 1.27 (d, *J* = 7.2 Hz, 3H), 0.79 (s, 3H), 0.76 (s, 3H). **^{13}C-NMR** (101 MHz, D_2O): δ 201.6, 174.9, 174.6, 173.7, 168.3, 157.1, 156.5, 129.9, 129.8, 129.71, 129.69, 126.2, 123.6, 117.8, 116.3, 116.0, 114.9, 75.7, 68.4, 63.6, 56.0, 55.9, 49.8, 38.6, 38.4, 35.4, 35.1, 28.1, 20.5, 18.5, 16.4. **HRMS** (ESI) calcd for $C_{30}H_{42}N_5O_9S$ 648.2698; Found 648.2700 $[M+H]^+$.

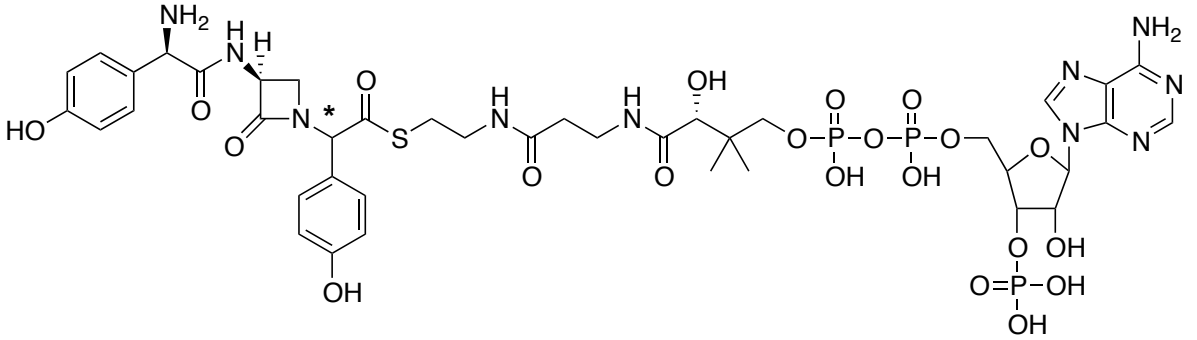


12
a = D, L, L*
b = D, L, D*

D-[*p*-(hydroxy)phenyl]glycine-L-*O*-phosphorylserine-L/D-[*p*-(hydroxy)phenyl]glycyl Coenzyme A (12a/b). To a 250 mL pressure flask, *N*-*tert*-butyloxycarbonyl-D-[*p*-(benzyloxy)phenyl]glycine-L-*tert*-butylphosphoseryl-L-benzyl-[*p*-(benzyloxy)phenyl]glycyl benzyl ester[1] (77 mg, 0.10 mmol) was dissolved in 10 mL of reagent grade THF and to it was added a catalytic amount of Pd-OH/C and the flask was vigorously shaken under 50 psi of H_2 for 12 h. The mixture was filtered through Celite, washed with 100 mL of THF, concentrated *in vacuo* to a white foam and used without further purification.

To a 10 mL round-bottomed flask equipped with a magnetic stir bar, the freshly hydrogenolyzed *O*-phosphoryl peptide (23 mg, 0.036 mmol) was added along with

K_2CO_3 (34 mg, 0.243 mmol) in 1 mL of freshly distilled THF. To this mixture was added PyBOP (38 mg, 0.072 mmol). In a separate 5 mL vial, coenzyme A sodium salt (55 mg, 0.072 mmol) was dissolved in 1 mL of HPLC grade H_2O. After 2 min, the coenzyme A solution was added to the flask containing pre-activated *N*-Boc-D-pHPG-L-tert-butylphosphoserine-L-pHPG and the reaction mixture was allowed to stir at room temperature for 30 min. The reaction was quenched by the addition of 2 µL of TFA and the product was purified by HPLC Prep Method A as the Boc-protected intermediate. Fractions containing the desired material were pooled, frozen on dry ice and lyophilized to dryness. The lyophilized powder was dissolved in 3 mL of anhydrous TFA and left to stand for 5 min. The TFA was removed *in vacuo* and the freshly deprotected product **12a/b** was purified as a mixture of diastereomers by HPLC Prep Method A (4.5 mg, 10%). **^{1}H-NMR** (400 MHz; D_2O): δ 8.58 (s, 1H), 8.34 (s, 1H), 7.29-7.18 (m, 4H), 6.88-6.78 (m, 4H), 6.14 (s, 1H), 5.44 (s, 1H), 5.10 (br. s, 1H), 4.23-4.20 (m, 1H), 4.09 (s, 1H), 3.96-3.92 (m, 2H), 3.83-3.78 (m, 2H), 3.77-3.56 (m, 1H), 3.58-3.55 (m, 2H), 3.26 (br. s, 4H), 3.01-2.97 (m, 2H), 2.21-2.17 (m, 2H), 0.85 (s, 3H), 0.73 (s, 3H). **HRMS** (ESI) calcd for $C_{40}H_{57}N_{10}O_{25}P_4S$ 1233.2162; Found 1233.2125 $[M+H]^+$.

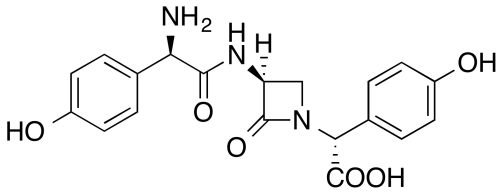


13
a = D, L, L*
b = D, L, D*

***epi*-Nocardicin G/Nocardicin G-coenzyme A (13a/b).** To a 10 mL round-bottomed flask equipped with a magnetic stir bar, *N-tert*-butyloxycarbonyl-nocardicin G[1] (40 mg, 0.081 mmol) and K_2CO_3 (34 mg, 0.243 mmol) was dissolved in 1 mL of freshly distilled THF and to this was added PyBOP (52 mg, 0.099). In a separate 5 mL vial, coenzyme

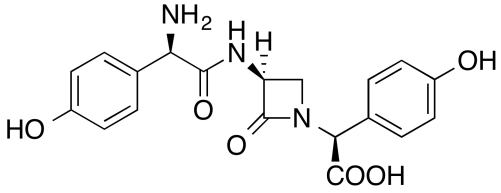
A sodium salt (76 mg, 0.099 mmol) was dissolved in 1 mL of HPLC grade H_2O. After 2 min, the coenzyme A solution was added to the flask containing pre-activated Boc-L-nocardicin G and the reaction mixture was allowed to stir at room temperature for 30 min. The reaction was quenched by the addition of 2 µL of TFA and the product was purified by HPLC Prep Method A as the Boc-protected intermediate. Fractions containing the desired material were pooled, frozen on dry ice and lyophilized to dryness. The lyophilized powder was dissolved in 3 mL of anhydrous TFA and left to stand for 5 min. The TFA was removed *in vacuo* and the freshly deprotected product **13a/b** was purified as a mixture of diastereomers by HPLC Prep Method A (12.0 mg, 13%). **^{1}H-NMR** (400 MHz; D_2O): δ 8.56 (s, 1H), 8.29 (s, 1H), 7.20 (d, *J* = 8.7 Hz, 2H), 7.14 (d, *J* = 8.7 Hz, 2H), 6.80 (d, *J* = 8.7 Hz, 2H), 6.79 (d, *J* = 8.7 Hz, 2H), 6.10 (d, *J* = 5.3 Hz, 1H), 5.55 (s, 1H), 4.96 (s, 1H), 4.81-4.78 (m, 1H), 4.51-4.50 (m, 1H), 4.18-4.17 (m, 2H), 3.92 (s, 1H), 3.76-3.72 (m, 2H), 3.50-3.44 (m, 1H), 3.30-3.28 (m, 4H), 3.19-3.14 (m, 1H), 3.03-2.99 (m, 2H), 2.27-2.26 (m, 2H), 0.82 (s, 3H), 0.66 (s, 3H). **HRMS** (ESI) calcd for $C_{40}H_{54}N_{10}O_{21}P_3S$ 1135.2393; Found 1135.2351 $[M+H]^+$.

Characterization of Standards. Analytical quantities of standards **1**, ***epi*-1**, **2a/b**, **3a/b**, **14, *epi*-14**, **15a** and **15b** (from **S2** and **S4**, respectively) were obtained by standard deprotection from the corresponding protected precursor.[1] ^{1}H-NMR and HRMS data are presented below:



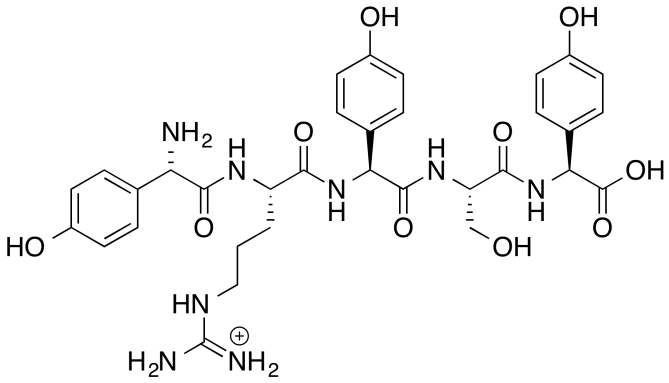
1

Nocardicin G (1). ^{1}H-NMR (400 MHz; D_2O): δ 7.21 (d, *J* = 8.7 Hz, 2H), 7.15 (d, *J* = 8.6 Hz, 2H), 6.85 (d, *J* = 8.7 Hz, 2H), 6.82 (d, *J* = 8.6 Hz, 2H), 5.25 (s, 1H), 4.96 (s, 1H), 4.79 (dd, *J* = 5.1, 2.5 Hz, 1H), 3.68 (t, *J* = 5.6 Hz, 1H), 2.98 (dd, *J* = 5.8, 2.4 Hz, 1H). **HRMS (ESI)** calcd for $C_{19}H_{20}N_3O_6$ 386.1347; Found 386.1346 [M+H].



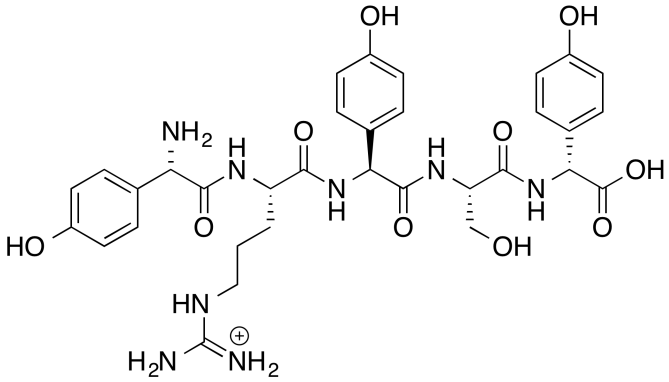
***epi*-1**

***Epi*-nocardicin G (*epi*-1). ^{1}H-NMR** (400 MHz; D_2O): δ 7.25 (d, *J* = 8.7 Hz, 2H), 7.14 (d, *J* = 8.7 Hz, 2H), 6.88 (d, *J* = 8.7 Hz, 2H), 6.83 (d, *J* = 8.7 Hz, 2H), 5.35 (s, 1H), 5.02 (s, 1H), 4.82 (dd, *J* = 4.8, 2.9 Hz, 1H), 3.37-3.33 (m, 2H). **HRMS (ESI)** calcd for $C_{19}H_{20}N_3O_6$ 386.1347 [M+H]+; Found 386.1347.



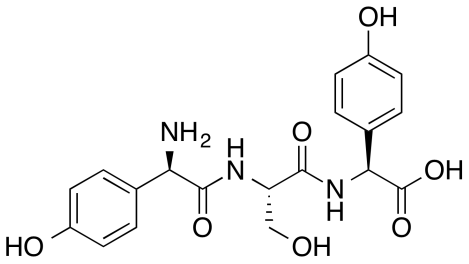
2a

L-pHPG-L-Arg-D-pHPG-L-Ser-L-pHPG pentapeptide (2a). **^{1}H-NMR** (400 MHz; D_2O): δ 7.16 (d, *J* = 8.5 Hz, 2H), 7.15 (d, *J* = 8.6 Hz, 2H). 7.04 (d, *J* = 8.5 Hz, 2H), 6.76 (d, *J* = 8.5 Hz, 4H), 6.70 (d, *J* = 8.7 Hz, 2H), 5.26 (s, 1H), 5.12 (s, 1H), 4.99 (s, 1H), 4.22 (t, *J* = 7.1 Hz, 1H), 3.72 (d, *J* = 5.6 Hz, 2H), 2.99 (app. t, *J* = 7.1 Hz, 2H), 1.64 (app. q, *J* = 7.6 Hz, 2H), 1.47-1.31 (m, 2H). **HRMS (ESI)** calcd for $C_{33}H_{41}N_8O_{10}$ 709.29402; Found 709.2933 [M+H]+.



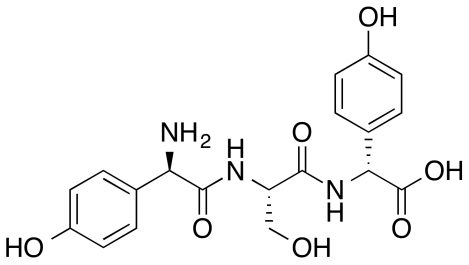
2b

L-pHPG-L-Arg-D-pHPG-L-Ser-D-pHPG pentapeptide (2b). **^{1}H-NMR** (400 MHz; D_2O): δ 7.14 (d, *J* = 8.6 Hz, 2H), 7.09 (d, *J* = 8.6 Hz, 4H), 6.79 (d, *J* = 8.9 Hz, 2H), 6.74 (d, *J* = 8.6 Hz, 2H), 6.68 (d, *J* = 8.6 Hz, 2H), 5.27 (s, 1H), 5.08 (s, 1H), 4.99 (s, 1H), 4.31 (t, *J* = 4.3 Hz, 1H), 3.72 (d, *J* = 4.7 Hz, 1H), 3.68 (d, *J* = 4.7 Hz, 1H), 2.98 (app. t, *J* = 7.6 Hz, 2H), 1.65-1.58 (m, 2H), 1.44-1.29 (m, 2H). **HRMS (ESI)** calcd for $C_{33}H_{41}N_8O_{10}$ 709.29402; Found 709.2935 [M+H]+.



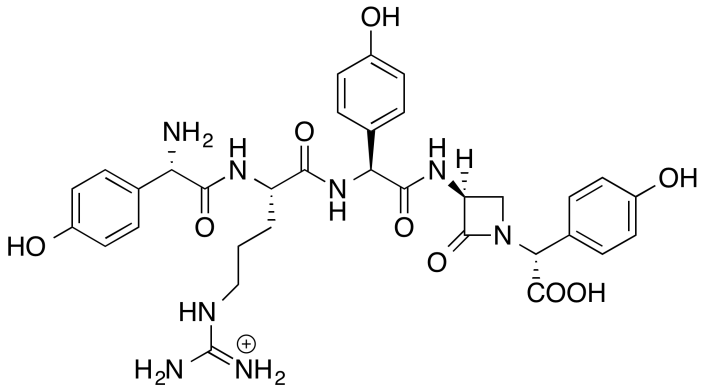
3a

D-pHPG-L-Ser-L-pHPG tripeptide (3a). ^{1}**H-NMR** (400 MHz; D_2O): δ 7.30 (d, *J* = 8.8 Hz, 2H), 7.27 (d, *J* = 8.8 Hz, 2H), 6.89 (d, *J* = 8.7 Hz, 2H), 6.88 (d, *J* = 8.7 Hz, 2H), 5.31 (s, 1H), 5.09 (s, 1H), 4.46 (t, *J* = 5.6 Hz, 1H), 3.74 (d, *J* = 5.6 Hz, 2H). **HRMS (ESI)** calcd for $C_{19}H_{22}N_3O_7$ 404.1452 [M+H]+; Found 404.1449.



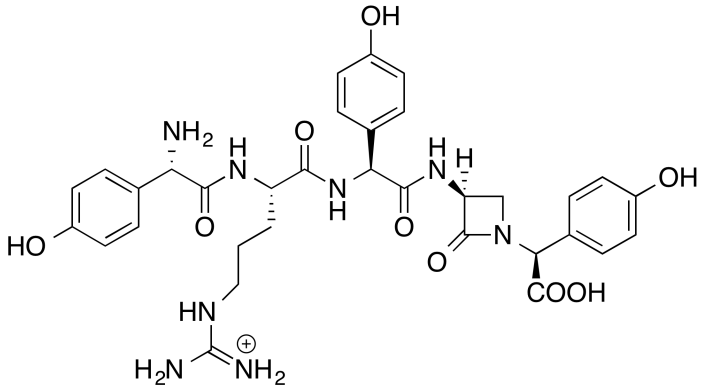
3b

D-pHPG-L-Ser-D-pHPG tripeptide (3a). ^{1}**H-NMR** (400 MHz; D_2O): δ 7.33 (d, *J* = 8.6 Hz, 2H), 7.26 (d, *J* = 8.6 Hz, 2H), 6.92 (d, *J* = 8.7 Hz, 2H), 6.87 (d, *J* = 8.7 Hz, 2H), 5.30 (s, 1H), 5.12 (s, 1H), 4.51 (t, *J* = 5.6 Hz, 1H), 3.66 (d, *J* = 5.6 Hz, 2H). **HRMS (ESI)** calcd for $C_{19}H_{22}N_3O_7$ 404.1452; Found 404.1448 [M+H]+.



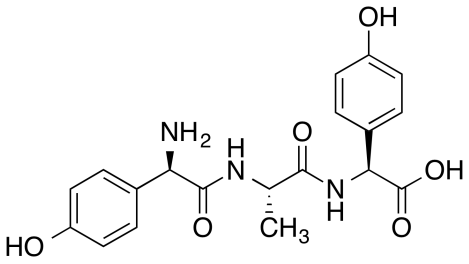
14

L-pHPG-L-Arg-nocardicin G (14). **^{1}H-NMR** (400 MHz; D_2O): δ 7.18 (d, *J* = 8.8 Hz, 2H), 7.15 (d, *J* = 8.7 Hz, 2H), 7.09 (d, *J* = 8.6 Hz, 2H), 6.84 (d, *J* = 8.6 Hz, 2H), 6.82 (d, *J* = 8.7 Hz, 2H), 6.75 (d, *J* = 8.7 Hz, 2H), 5.39 (s, 1H), 5.21 (s, 1H), 5.00 (s, 1H). 4.55 (dd, *J* = 5.3, 2.3 Hz, 1H), 4.27 (t, *J* = 7.3 Hz, 1H), 3.42 (dd, *J* = 5.5, 1.9 Hz, 1H), 3.29 (t, *J* = 5.8 Hz, 1H), 3.02 (t, *J* = 6.9 Hz, 2H), 1.65 (q, *J* = 8.6 Hz, 2H), 1.48-1.35 (m, 2H). **HRMS (ESI)** calcd for $C_{33}H_{39}N_8O_9$ 691.2835; Found 691.2838 [M+H]+.



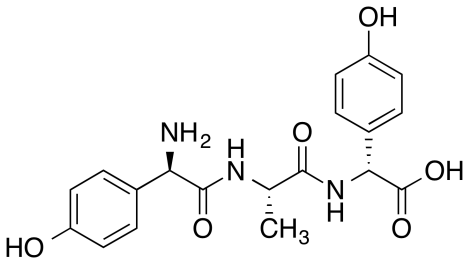
***epi*-14**

L-pHPG-L-Arg-*epi*-nocardicin G (*epi*-14). **^{1}H-NMR** (400 MHz; D_2O): δ 7.28 (d, *J* = 8.9 Hz, 2H), 7.21 (d, *J* = 8.9 Hz, 2H), 7.17 (d, *J* = 8.4 Hz, 2H), 6.91 (d, *J* = 8.4 Hz, 4H), 6.84 (d, *J* = 8.8 Hz, 2H), 5.28 (s, 1H), 5.10 (s, 1H), 4.97-4.95 (m, 1H), 4.36 (t, *J* = 7.5 Hz, 1H), 3.46-3.44 (m, 2H), 3.11 (t, *J* = 7.0 Hz, 2H), 1.78-1.72 (m, 2H), 1.57-1.46 (m, 2H). **HRMS (ESI)** calcd for $C_{33}H_{39}N_8O_9$ 691.2835; Found 691.2838 [M+H]+.



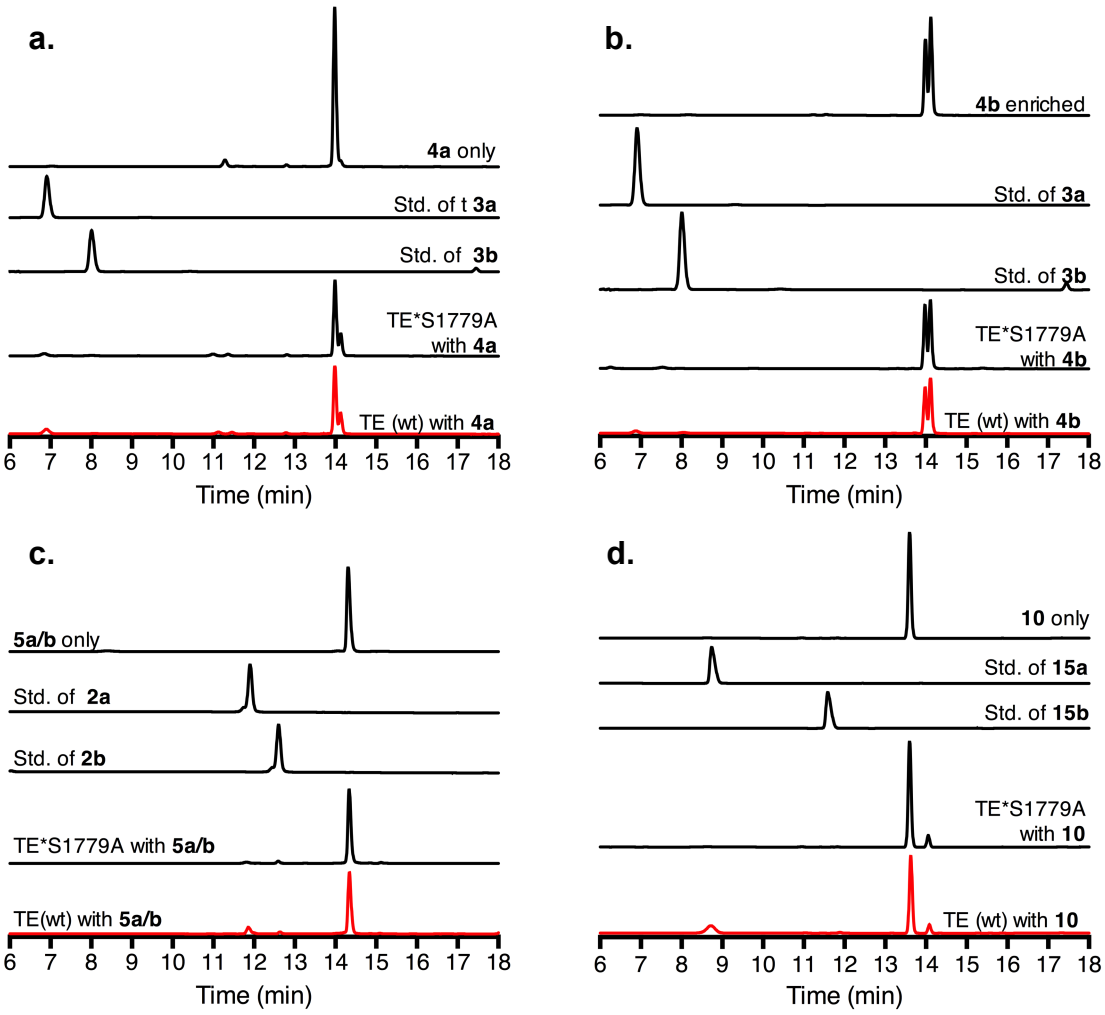
15a

D-pHPG-L-Ala-L-pHPG tripeptide (15a). ^{1}H-NMR (400 MHz; D_2O): δ 7.24 (d, *J* = 8.7 Hz, 2H), 7.22 (d, *J* = 8.7 Hz, 2H), 6.85 (d, *J* = 8.7 Hz, 2H), 6.83 (d, *J* = 8.7 Hz, 2H), 5.19 (s, 1H), 4.98 (s, 1H), 4.26 (q, *J* = 7.3 Hz, 1H), 1.21 (d, *J* = 7.3 Hz, 3H). **HRMS (ESI)** calcd for $C_{19}H_{22}N_3O_6$ 388.1503; Found 388.1503 [M+H]+.

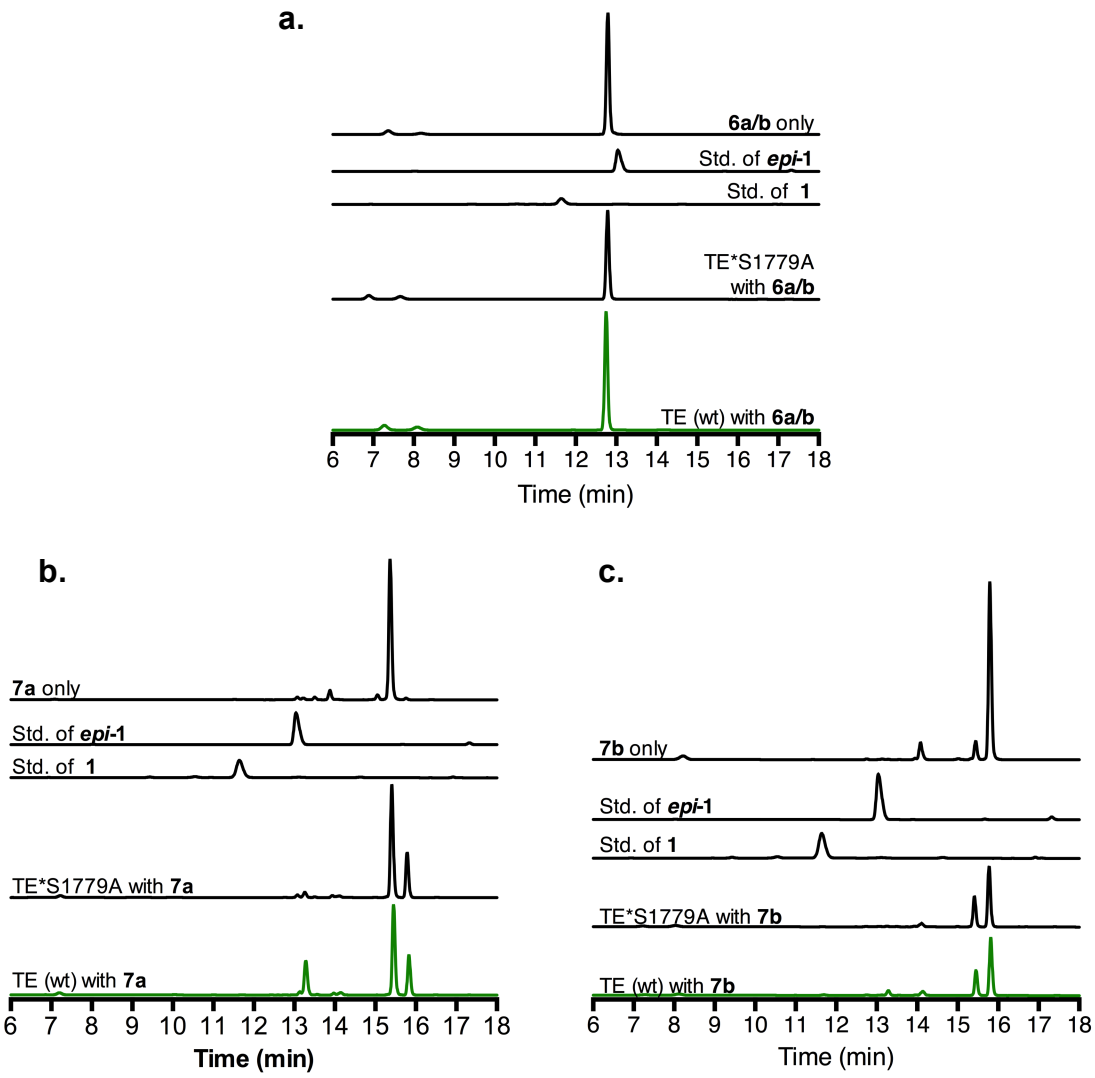


15b

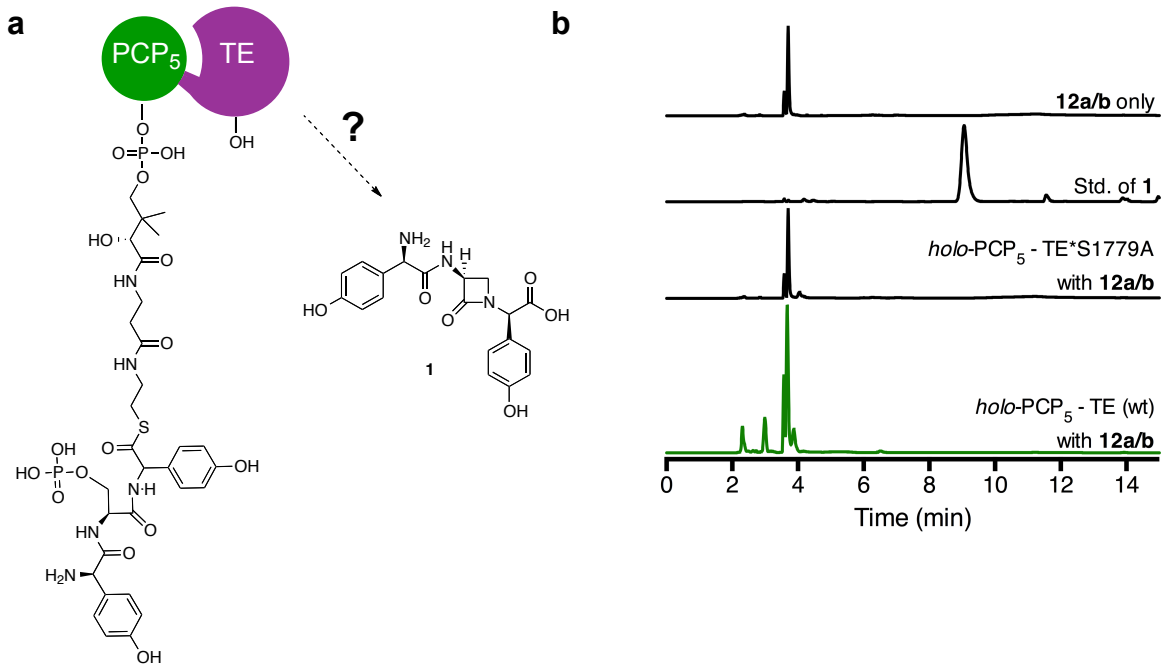
D-pHPG-L-Ala-D-pHPG tripeptide (15b). ^{1}H-NMR (400 MHz; D_2O): δ 7.26 (d, *J* = 8.7 Hz, 2H), 7.20 (d, *J* = 8.6 Hz, 2H), 6.86 (d, *J* = 8.7 Hz, 2H), 6.82 (d, *J* = 8.7 Hz, 2H), 5.25 (s, 1H), 4.99 (s, 1H), 4.32 (q, *J* = 7.2 Hz, 1H), 1.13 (d, *J* = 7.2 Hz, 3H). **HRMS (ESI)** calcd for $C_{19}H_{22}N_3O_6$ 388.1503; Found 388.1501 [M+H]+.



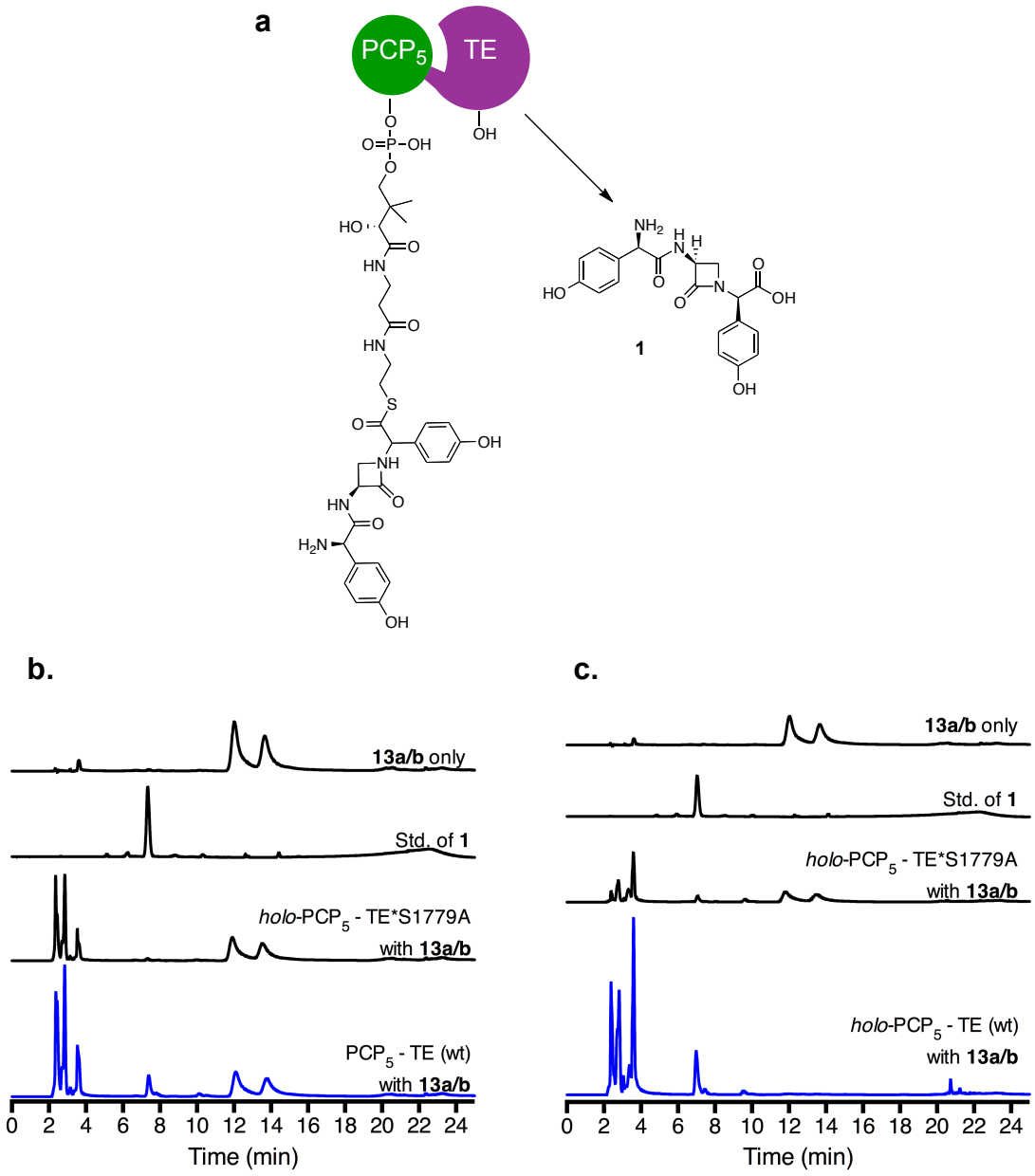
Supplementary Figure 1 | HPLC analysis of linear thioester substrates with NocTE. (a) HPLC analysis of NocTE incubated with tripeptide-pantetheine **4a** containing a *C*-terminal L-pHPG. Very little hydrolysis was observed after 3 h (< 5% turnover). NocTE preferentially hydrolyzed the tripeptide containing a *C*-terminal L-pHPG **3a**. **(b)** HPLC analysis of NocTE incubated with an enriched mixture of tripeptide-pantetheine **4b** containing a *C*-terminal D-pHPG. Very little hydrolysis was observed after 3 h (< 5% turnover). The tripeptide-thioester containing a *C*-terminal L-pHPG (**4a**) was preferentially hydrolyzed, forming **3a**. **(c)** HPLC analysis of NocTE incubated with pentapeptide-pantetheine **5a/b.** Very little hydrolysis was observed after 3 h (< 3% turnover). **(d)** HPLC analysis of NocTE incubated with alanine substituted tripeptide **10**. Very little hydrolysis was observed after 3 h (< 5% turnover) and only the product containing a *C*-terminal L-pHPG (**15a**) was detected.



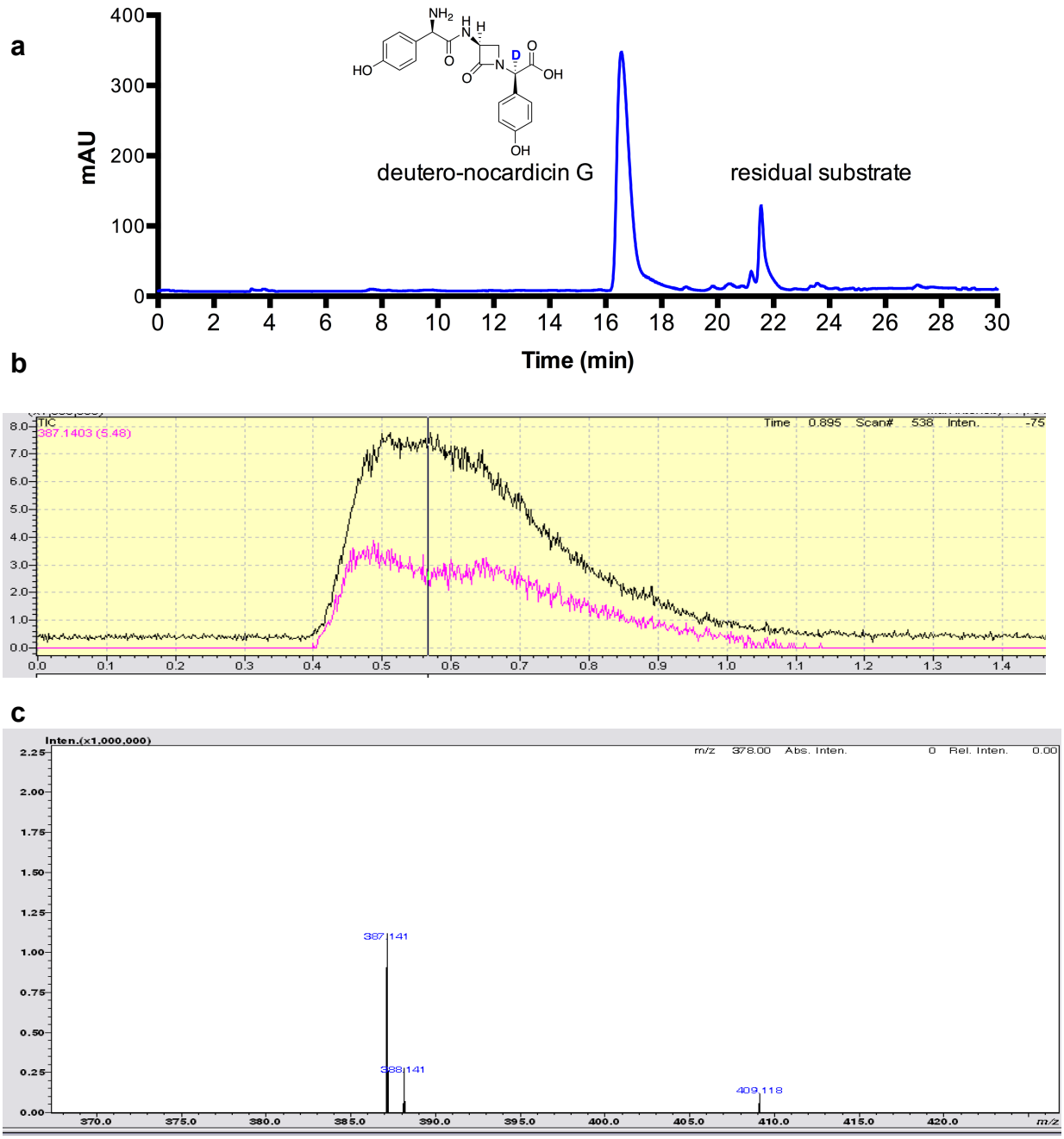
Supplementary Figure 2 | HPLC analysis of activated substrates with NocTE. (a) NocTE incubated with phosphoryl tripeptide-pantetheine **6a/b**. No observable hydrolysis above background was found and no β-lactam formation was detected. **(b)** HPLC analysis of NocTE incubated with *O*-acyl tripeptide-pantetheine **7a** substrate. Very little hydrolysis was observed after 3 h (< 5% turnover). NocTE hydrolyzed only the tripeptide thioester containing a *C*-terminal L-pHPG was observed by comparison with stereopure synthetic standard. **(c)** HPLC analysis of NocTE incubated with *O*-acetyl tripeptide-pantetheine **7b**. Very little hydrolysis was observed after 3 h (< 5% turnover).



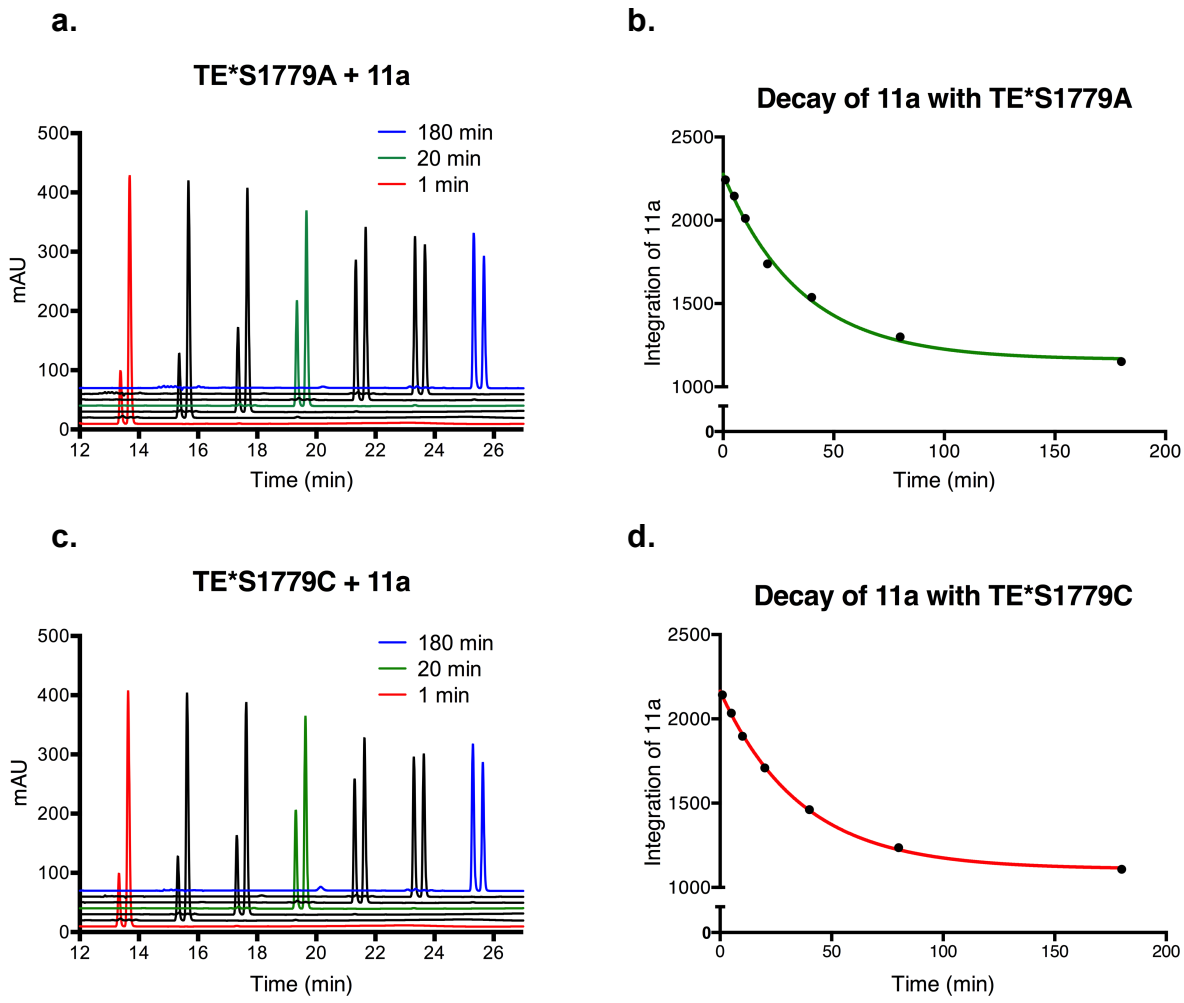
Supplementary Figure 3 | HPLC analysis of loaded *O*-phosphoryl tripeptide onto *apo*-PCP$_5$-TE to probe TE-catalyzed β-lactam ring closure. (a) Schematic of *holo*-PCP$_5$-TE construct covalently modified with an *O*-phosphoryl tripeptide on the active site serine of the PCP domain by an Sfp-mediated reaction with **12a/b**. **(b)** HPLC analysis of *holo*-PCP$_5$-TE modified construct. After 24 h incubation, nocardicin G (**1**) was not observed as determined by comparison with a synthetic standard of **1**. Simple hydrolysis of the *O*-phosphoryl tripeptide was observed only a small extent (peaks appear between 2 and 3.5 min on wild-type trace (in green).



Supplementary Figure 4 | HPLC analysis of covalently loaded *epi*-nocardicin G and nocardicin G onto *apo*-PCP$_5$-TE to observe turnover catalyzed by NocTE. (a) Schematic of *holo*-PCP$_5$-TE construct covalently modified with nocardicin G or *epi*-nocardicin G on the active site serine of the PCP domain by an Sfp-mediated reaction with **13a/b**. **(b)** HPLC analysis of the *holo*-PCP$_5$-TE construct modified with *epi*-nocardicin G or nocardicin G. After 3 h, the formation of **1** was observed. **(c)** HPLC analysis of 24 h incubation *holo*-PCP$_5$-TE covalently modified with *epi*-nocardicin G or nocardicin G. All bound β-lactam peptides and residual **13a/b** were converted to **1**.



Supplementary Figure 5 | HPLC and MS analysis of ^{1}H-NMR experiment with 12a/b in D_2O/KiPO4, pD 7.2. (a) Injection of 100 µL of 1 µM NocTE incubated with 10 mM **12a/b** after completion of an arrayed ^{1}H-NMR deuterium-exchange experiment. New product had formed at t_R = ~16 min. Product was isolated over multiple injections. HPLC analysis also revealed the presence of residual substrate **11a/b**. **(b)** Total ion chromatogram (TIC) of isolated product. **(c)** HRMS of isolated product, $[M]^+$, $[M + H]^+$ and $[M + Na]^+$ masses corresponding to monodeutero-nocardicin G were observed. $[M]^+$ calcd for $C_{19}H_{18}DN_3O_6$ 386.1337, [M + H]+ calcd for $C_{19}H_{19}DN_3O_6$ 387.1409, [M + Na]+ calcd for $C_{19}H_{18}DN_3NaO_6$ 409.1234.



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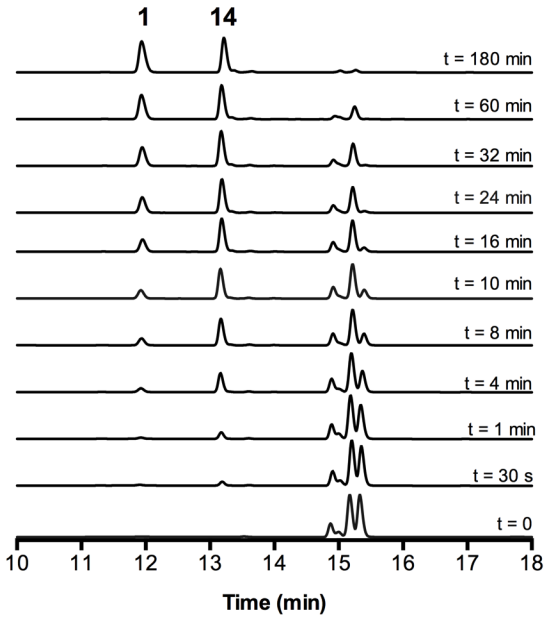
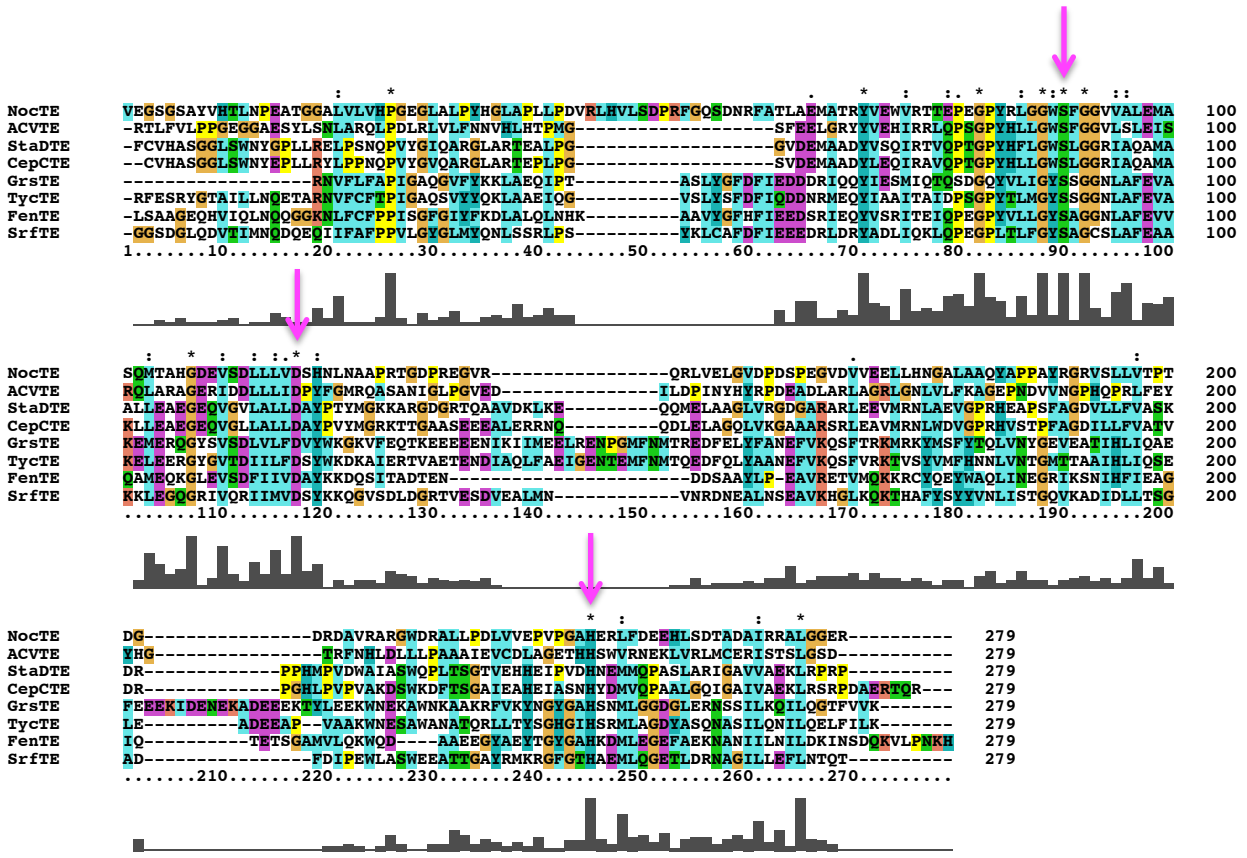
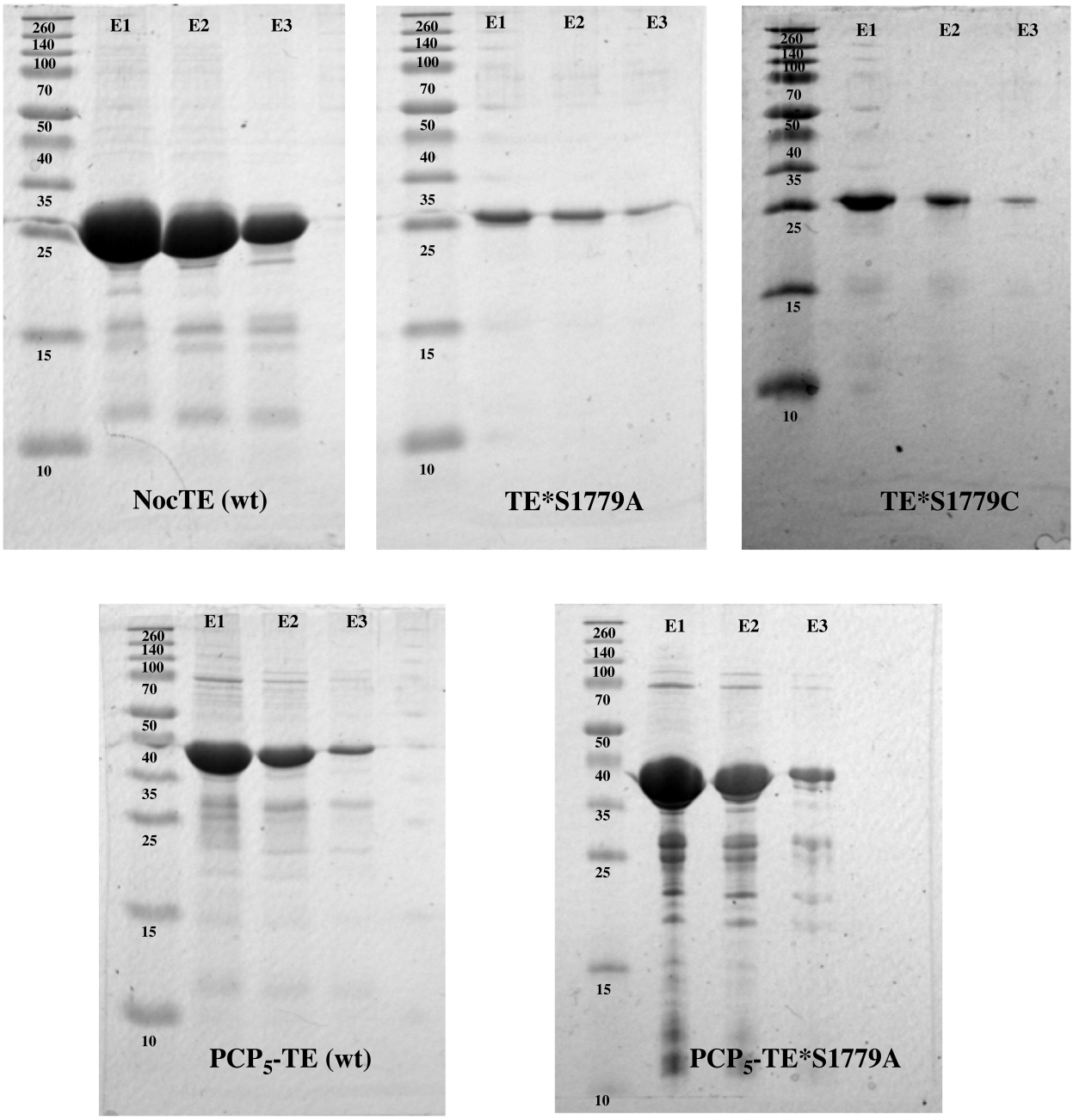
Supplementary Figure 6 | Time-course HPLC analysis of the conversion of 11a to 11b in the presence of TE*S1779A and TE*S1779C. (**a**) The rate of decay of **11a** in the presence of TE*S1779A was calculated to be ~3.0 x 10^{-2} min^{-1}, half life of **11a** was determined to be ~24 min. (b) The rate of decay of **11a** in the presence of TE*S1779C was calculated to be ~3.0 x 10^{-2} min^{-1}, half life of **11a** was determined to be ~24 min.



Supplementary Figure 7 | HPLC analysis of competition experiment between 11a and 9a. HPLC chromatograms represent quenched fixed-time points in the competition experiment between **11a** and **9a** in the presence of NocTE showing the appearance of **1** and **14**.



Supplementary Figure 8 | Primary sequence alignment of thioesterase domains. Multiple sequence alignment of NocTE with other TE hydrolase domains from the biosynthetic gene clusters of penicillin N (ACVTE),[2,3] A47934 (StaD),[4] chloroeremomycin (CepCTE)[5] and TE cyclase domains from gramicidin S (GrsTE),[6,7] tyrocidine (TycTE),[8] fengycin (FenTE)[9] and surfactin (SrfTE)[10]. The positions of the catalytic triad residues (Ser, His, Asp) are highlighted with arrows. Sequence alignment performed using CLUSTAL X.



Supplementary Figure 9 | SDS-PAGE gels of various protein constructs. Gels contain 15% acrylamide. E1, E2 and E3 correspond to elutions 1, 2 and 3 containing 100 mM, 150 mM and 200 mM imidazole respectively for the proteins indicated.

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