Supporting Information

Production of Stealthin C Involves an S–N-Type Smiles Rearrangement

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Supplementary Materials

1. General materials and methods

Oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Qiagen Miniprep kit and gel purification kit were used to carry out routine DNA manipulations. DNA sequencing were performed by Beckman Coulter Genomics (Danvers, MA). SDS-PAGE precast gels were purchased from Bio-Rad Laboratories. DU730 Life Sciences UV/Vis spectrophotometer (Beckman Coulter) was used to measure optical densities of *E. coli* cultures at 600 nm. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). HPLC analysis of *in vivo* and *in vitro* assays were performed on a Thermo Scientific Dionex Ultimate 3000 instrument and a Thermo Hypersil Golden aQ C18 reverse-phase column (3μ 3.0 x 150 mm). A linear gradient program 5 to 95% acetonitrile (v/v) in water (0.1% formic acid) was used to separate samples with flow rate of 0.4 mL/min for 30 minutes. MS analyses of *in vivo* experiments and *in vitro* assays were performed on an Advion high performance compact mass spectrometer with parameters: capillary voltage 180 V, capillary temperature 275 ºC, source voltage offset 20 V, source voltage span 30 V, source gas temperature 200 ºC, and electrospray ionization (ESI) voltage 3500 V. High-resolution MS analysis was performed on an Agilent 1290 Infinity UHPLC system (Agilent Technologies) coupled with a maXis impact UHR time-of-flight mass spectrometer system (Bruker Daltonics Inc), an Agilent 6220 TOF Mass Spectrometer equipped with an electrospray ionization (ESI), and a Thermo Scientific Dionex Ultimate 3000 uHPLC coupled to a Q Exactive Plus Orbitrap mass spectrometer system equipped with an electrospray ionization (ESI) source in the Small Molecule Mass Spectrometry Facility, FAS Division of Science. A Waters XTerra MS C18 analytical column (2.1 x 50 mm, 3.5 μm) was used for separation.

2. Bioinformatic analysis of the atypical angucycline biosynthetic gene clusters

The gene content of five atypical angucyclines biosynthetic gene clusters was analyzed: gilvocarcin (*gil*), jadomycin (*jad*), kinamycin from *Streptomyces ambofaciens* (*alp*), kinamycin from *Streptomyces murayamaensis* (*kin*), lomaiviticin (*lom*), and fluostatin (*AB649/1850*). The conserved enzymes hypothesized to be involved in the ring contraction reaction are labeled in red.

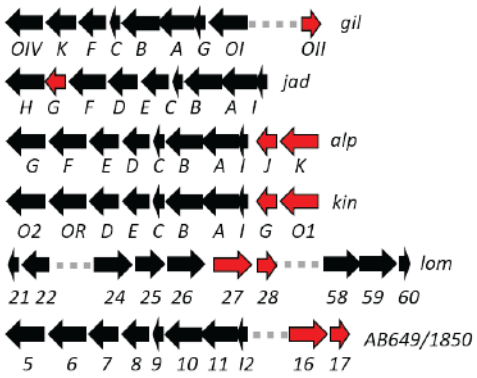


Figure S1. The organization of atypical angucycline biosynthetic gene clusters (BGC). The red-labeled conserved enzyme pairs AlpJ-K and their homologs are found in benzofluorene-type angucycline BGCs, whereas only AlpJ homologs are found in gilvocarcin and jadomycin BGCs.

3. Construction of the recombinant strain pAlpJK/TK64

Table S1. Primers used for cloning. Restriction sites are underlined

Primer Name	ORF Amplified	Primer Sequence (5' to 3')
AlpJ-NheI-NdeI-f	*alpJ*	GGAATT*GCTAGC*GGAGGAGCC*CATATG*CCCATCATCTCCGCCGAGG
AlpJ-AvrII-BamHI-r	*alpJ*	AA*GGATCC*GGAATT*CCTAGG*TCACTCGCCGGCCGAACGCGTGTGC.
AlpK-NheI-5	*alpK*	GGAATT*GCTAGC*GGAGGAGCCCATATGGAGTTCTACGATTCGGACG
AlpK-AvrII-EcoRI-f	*alpK*	AA*GAATTC*GGAATT*CCTAGG*TCAGGCGGTGGGGCCGAACCAGC

By using Q5® High-Fidelity 2x Master Mix (New England BioLabs) and C1000 Touch Gradient Cycler (Bio-Rad), AlpJ and AlpK encoding genes were amplified from genomic DNA of *Streptomyces ambofaciens* ATCC 23877 using the following parameters: denaturation for 5 min at 95 ºC, followed by 30 cycles of 30 sec at 95 ºC, 30 sec at gradient 55-65 ºC, 1 min (AlpJ) or 2 min (AlpK) at 72 ºC, and a final cycle 5 min at 72 ºC. Primers used in PCR reactions are shown in Table S1. All PCR reactions contained 50-100 ng DNA template, 2% (v/v) DMSO, 25 pmoles of each primer, and Q5® High-Fidelity 2X Master Mix in a total volume of 10 µL. The PCR products were analyzed by agarose gel electrophoresis and purified. The purified PCR product of *alpJ* was digested with *Nde*I and *BamH*I (New England BioLabs) for 2 hours at 37 ºC. The digestion reaction

contained 2 μL NEB buffer 3.1 (10x), 16 μL PCR product, 1 μL *Nde*I, and 1 μL *BamH*I. The digested *alpJ* product was purified by agarose gel electrophoresis. The purified *alpJ* was ligated into the same restriction sites of pUWL201PW to afford pAlpJ. The ligation reaction contained 1 μL T4 DNA ligase (New England BioLabs), 1 μL T4 DNA ligase buffer (10x), 2 μL linearized pUWL201PW, and 6 μL purified *alpJ* product in total volume of 10 μL. The ligation reaction mixture was incubated at room temperature overnight and 10 μL of the ligation was transformed into chemically competent *E. coli* TOP10 cells (Invitrogen). The PCR product of *alpK* was purified and ligated into pCR-Blunt vector (Invitrogen) to generate pCR-alpK. The ligation reaction contains 0.5 μL T4 DNA ligase (New England BioLabs), 0.5 μL T4 DNA ligase buffer (10x), 0.5 μL pCR-Blunt, and 3.5 μL purified *alpK* product in total volume of 5 μL. The ligation reaction mixture was incubated at room temperature overnight and 5 μL of the ligation was transformed into chemically competent *E. coli* TOP10 cells (Invitrogen). pCR-alpK was then digested by using *Nhe*I and *Avr*II for 2 hours at 37 ºC and cloned into *Avr*II site of pAlpJ to generate pAlpJK by using the same cloning protocol. The plasmid pAlpJK was transformed into *Streptomyces lividans* TK64 using the PEG-mediated protoplast transformation method[1] to generate the recombinant strain pAlpJK/TK64.

4. HPLC-HRMS analysis of *in vivo* feeding experiments

Recombinant strain cultures were prepared on a 20 mL scale in R5 media with 10 μg/mL thiostrepton (final concentration) at 28 ºC with shaking at 220 rpm. A total of 1 mg dehydrorabelomycin was added to each culture. L-cysteine and ^{15}N-L-cysteine were fed at a final concentration of ~11 mM to one culture. 500 μL of each culture was removed after a 24-hour incubation at 28 ºC with shaking at 220 rpm. Samples were quenched by adding a 1 mL mixture of 99% ethyl acetate and 1% acetic acid (v/v), followed by centrifugation at 13,000 rpm for 10 minutes. The supernatants were transferred to new 1.5 mL microcentrifuge tubes and dried *in vacuo* by using SpeedVac. The resulting dried extracts were dissolved in 50 μL methanol for further HPLC and LC-HRMS analysis. Compound **2** and **3** were separated and isolated on a Thermo Scientific Dionex Ultimate 3000 instrument and a Thermo Hypersil Golden aQ C18 reverse-phase column (3μ 3.0 x 150 mm). A linear gradient program 5 to 95% acetonitrile (v/v) in

water (0.1% formic acid) was used to separate samples with flow rate of 0.4 mL/min for 30 minutes. HRMS analyses of the isolated compounds were performed on an Agilent 6220 TOF Mass Spectrometer equipped with an electrospray ionization (ESI) by using flow injection method.

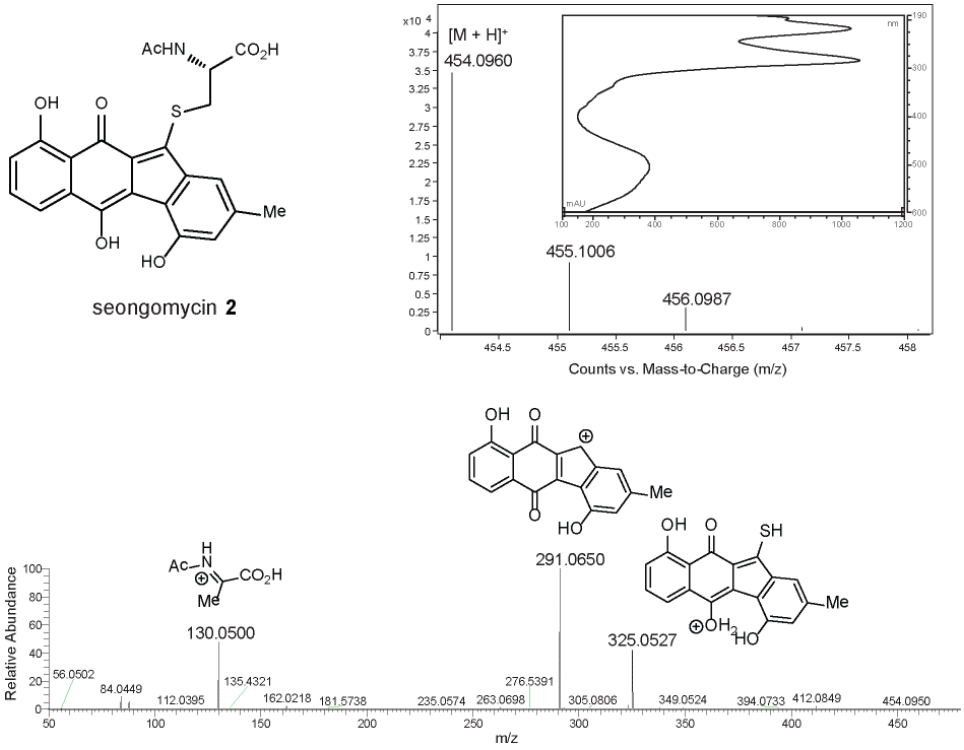


Figure S2. LC-HRMS data (positive mode), MS^2 fragmentation data (positive mode), and UV-vis spectrum for **2** from an *in vivo* experiment. $[M + H]^+$ Obs: 454.0960, Calc: 454.0955, Error (ppm): 1.10

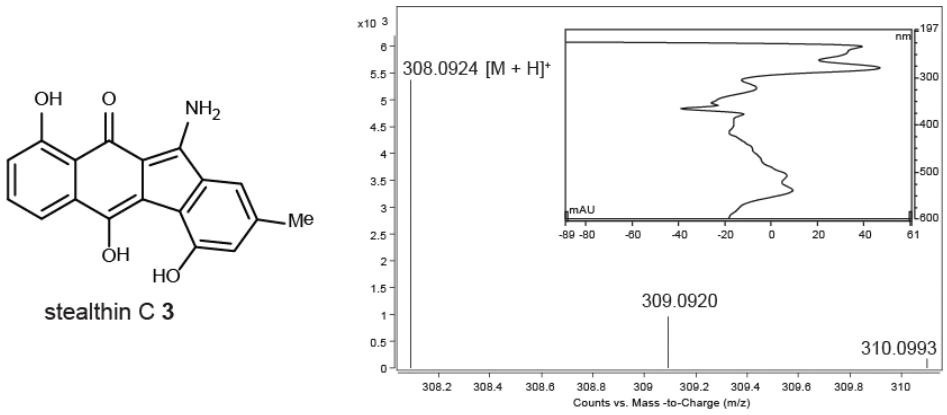


Figure S3. LC-HRMS data (positive mode) and UV-vis spectrum for **3** from an *in vivo* experiment. $[M + H]^+$ Obs: 308.0924, Calc: 308.0917, Error (ppm): 2.27.

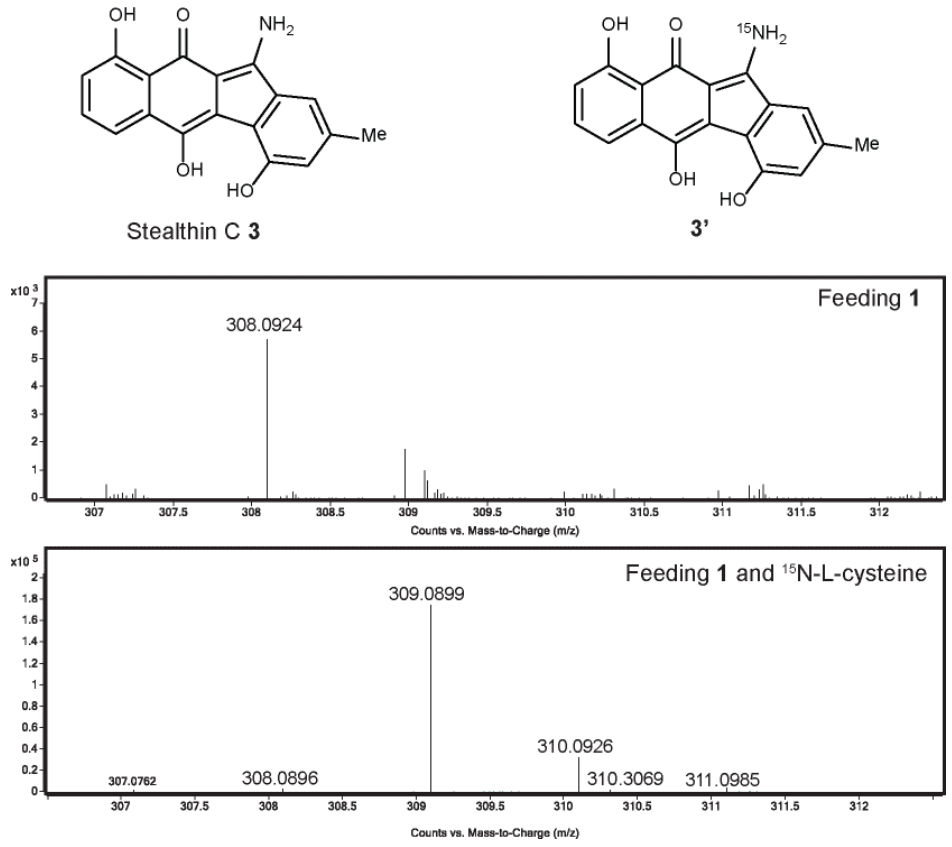


Figure S4. LC-HRMS data (positive mode) for the ^{15}N-L-cysteine feeding experiment.

5. Cloning, expression, and purification of AlpJ, KinO1, and Fre

Table S2. Primers used for cloning. Restriction sites are underlined.

Primer Name	ORF Amplified	Primer Sequence (5' to 3')
AlpJ-NdeI-f	*alpJ*	GGAATT*CATATG*CCCATCATCTCCGCCGAGG
AlpJN-XhoI-r	*alpJ*	GGAATT*CTCGAG*TCACTCGCCGGCCGAACGCGTGTGC.
KinO1-NdeI-f	*kinO1*	GGAATT*CATATG*GATAACTTTGACGCGGACG
KinO1-XhoI-r	*kinO1*	GGAATT*CTCGAG*TCAGTTGGGCCGGCCGAACCATCG
Fre-NdeI-f	*fre*	GGAATT*CATATG*ACAACCTTAAGCTGTAAAG
Fre-N-XhoI-r	*fre*	GGAATT*CTCGAG*TCAGATAAATGCAAACGCATCG.

The AlpJ encoding gene was amplified from *Streptomyces ambofaciens* ATCC 23877 genomic DNA, the KinO1 encoding gene was amplified from *Streptomyces murayamaensis* ATCC 21414 genomic DNA, and the Fre encoding gene was amplified from *Escherichia coli* BL21 genomic DNA using following parameters: denaturation for 1 min at 98 ºC, followed by 10 cycles of 15 sec at 98 ºC, 15 sec at 65 ºC to 55 ºC (–1 ºC each cycle) , 1 min at 72 ºC, additional 30 cycles of 15 sec at 98 ºC, 15 sec at 55 ºC, 1 min at 72 ºC and a final cycle 5 min at 72 ºC. Phusion® High-Fidelity DNA Polymerase (New England BioLabs) was used in all PCR reactions. The primers used for PCR are shown in Table S2. The PCR products of *alpJ*, *kinO1*, and *fre* were cloned into pET28a (Invitrogen) using *Nde*I and *Xho*I sites and the same cloning protocol described in Section 3. The resulting plasmids were then transformed into chemically competent *E. coli* BL21 for protein expression.

N-terminal hexahistidine tagged AlpJ, KinO1, and Fre were overexpressed in *E. coli* BL21. Overnight seed cultures were prepared in 5 mL LB broth with 35 µg/liter kanamycin. The overnight seed cultures were then inoculated into 500 mL LB broth in 2-liter baffled flasks and grown at 37 °C until a OD_{600} of 0.4 to 1.0. To induce protein expression, cultures were cooled to 15 °C, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 120 µM. The cultures were grown overnight at 15 °C. The cell pellets were harvested by centrifugation at 4 ºC at 4,000 rpm for 15 minutes and then re-suspended in 30 mL Buffer A (50 mM Tris-HCl, pH 7.9, 10 mM imidazole, and 50mM NaCl). The cells were disrupted by sonication and cell debris

removed by centrifugation at 12,000 rpm for 30 minutes at 4 ºC. 1 mL of nickel-nitrilotriacetic acid resin (GE Healthcare) was then added into the soluble supernatants and incubated at 4 ºC on a nutating mixer for at least 2 hours. The protein supernatants and resin mixture were then applied to a gravity flow column. The resin-bound protein was washed by using Buffer A with increasing concentrations of imidazole stepwise (10 mM and 20 mM). The target proteins were then eluted using 10 mL of Buffer A with 250 mM imidazole. 2 mM DTT was added in all the wash and elution buffers. Corning® Spin-X® UF concentrators were used to concentrate proteins and change protein buffer from Buffer A to Buffer B (50 mM Tris-HCl, pH 7.9, 2 mM EDTA, and 2 mM DTT). The concentrated proteins were stored at -80 ºC with 10% glycerol. Protein concentration was determined using a Nanodrop2000 instrument, with extinction coefficients calculated from http://web.expasy.org/protparam/.

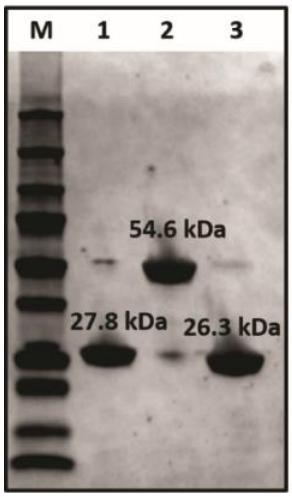


Figure S5. SDS-PAGE analysis of the recombinant proteins in this study. M. Precision Plus Protein™ all blue standards; 1. AlpJ; 2. KinO1; 3. Fre. All proteins are *N*-terminus polyhistidine tagged proteins.

6. Cofactor analysis of AlpJ and KinO1

50 uL of concentrated solutions of AlpJ and KinO1 were denatured using 100 uL methanol and centrifuged at 13,000 rpm for 10 minutes. The resulting supernatants were then submitted for LC-MS analysis. LC-MS analysis of the denatured KinO1 extract was performed on an Advion

high performance compact mass spectrometer with parameters: capillary voltage 180 V, capillary temperature 275 ºC, source voltage offset 20 V, source voltage span 30 V, source gas temperature 200 ºC, and electrospray ionization (ESI) voltage 3500 V. Thermo Hypersil Golden aQ C18 reverse-phase column (3µ 3.0 x 150 mm) was used. A linear gradient program 5 to 95% acetonitrile (v/v) in water (0.1% formic acid) was used to separate samples with flow rate of 0.4 mL/min for 30 minutes. LC-MS analysis of the denatured AlpJ extract was performed on an Agilent 1290 Infinity UHPLC system (Agilent Technologies, Palo Alto, CA) coupled to a 6460 Triple Quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto, CA) using an electrospray ionization (ESI) source with Agilent Jet Stream technology. The ion source parameters were as follows: drying nitrogen gas temperature 325 ºC, drying nitrogen gas flow 11 L/min, nebulizer pressure 30 psi, sheath nitrogen gas temperature 375 ºC, sheath nitrogen gas flow 11 L/min, capillary voltage 3000 V, and nozzle voltage 0 V. Waters XTerra MS C18 analytical column (2.1 x 100 mm, 3.5 µm) was used with the elution conditions: 2 min 100% solvent A, a gradient increasing solvent B from 0% to 52% over 3 min, a gradient increasing solvent B to 100% in 0.1 min, a gradient increasing solvent A to 100% in 0.1 min, and 100% solvent A for 1.8 min (solvent A: water with 0.2% v/v acetic acid, solvent B: acetonitrile with 0.2% v/v acetic acid) at a flow rate of 0.3 mL/min.

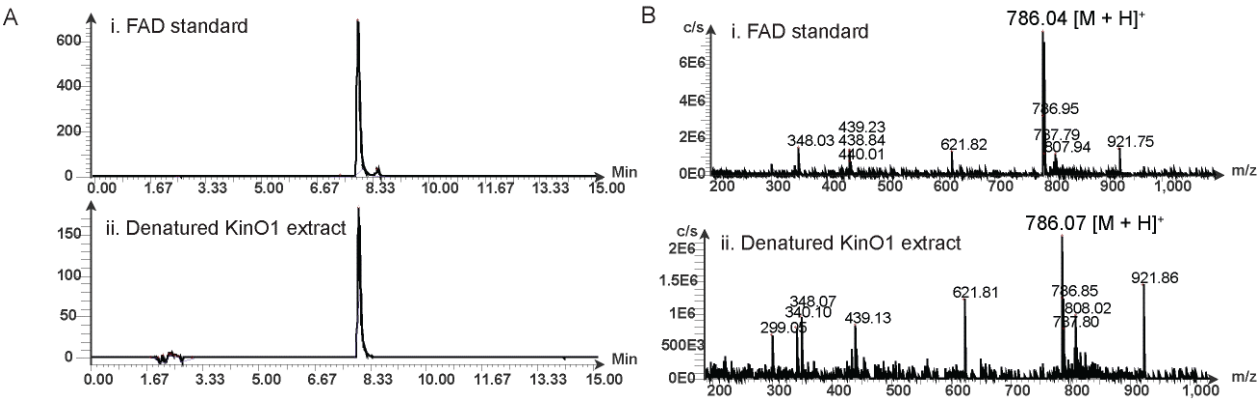


Figure S6. LC-MS analysis of the KinO1 flavin coenzyme. A) HPLC analysis (430 nm) of i, FAD standard; ii, supernatant extract from denatured KinO1; B) MS spectra of i, FAD standard; ii, supernatant extract from denatured KinO1.

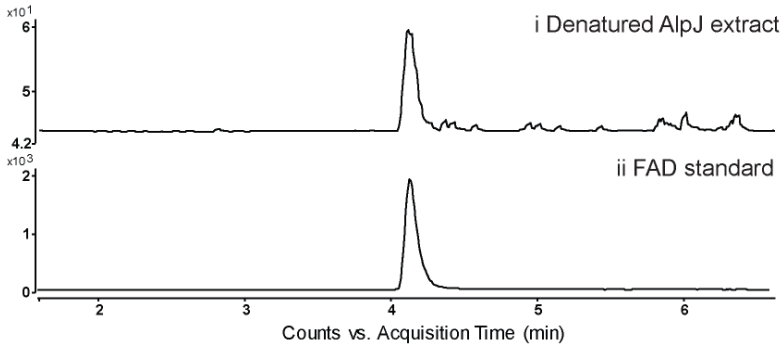


Figure S7. LC-MS analysis of the AlpJ flavin coenzyme. Extracted ion chromatogram *m/z* $[M - H]^-$ = 784.15 of i, supernatant extract from denatured AlpJ; ii, FAD standard.

7. HPLC-HRMS analysis of AlpJ *in vitro* assays

In vitro assays were carried out using the general conditions outlined here follow with specified modifications noted: A 100 μL *in vitro* reaction was set up by incubating 20 μM AlpJ, 40 μM Fre or KinO1, 2 mM NADPH, 20 μM FAD, 1 mM L-cysteine or other L-cysteine analogs, and 110 μM dehydrorabelomycin in 50 mM MOPS buffer pH 7.5 at room temperature for 2 hours. After 2 hours, the reactions were quenched by adding 400 μL of methanol followed by centrifugation at 13,000 rpm for 10 minutes. The supernatants were transferred to new 1.5 mL microcentrifuge tubes and dried *in vacuo* with a SpeedVac. Dried samples were dissolved in 50 μL methanol for further HPLC and LC-HRMS analysis. Waters XTerra MS C18 analytical column (2.1 x 50 mm, 3.5 μm) was used for LC-HRMS analyses with the different elution conditions.

Elution condition for compound **3'**and **3a'**: a gradient increasing solvent B from 20% to 60% over 2 min, a gradient increasing solvent B from 60% to 100% over 7.3 min, 100% solvent B for 3 min, 20% solvent B for 3 min (solvent A: water with 0.2% v/v acetic acid, solvent B: methanol) at a flow rate of 0.3 mL/min. Elution condition for compound **5**: a gradient increasing solvent B from 20% to 60% over 2 min, a gradient increasing solvent B from 60% to 71% over 7.2 min, a gradient increasing solvent B from 71% to 100% in 0.1 min, 100% solvent B for 3 min, a gradient increasing solvent A to 80% in 0.1 min and 80 % solvent A for 3 min (solvent A: water with 0.1% v/v formic acid, solvent B: acetonitrile with 0.1% v/v formic acid) at a flow rate of 0.3

mL/min. Elution condition for compound **10**, **11**, **13**, **14**, and **15**: a gradient increasing solvent B from 20% to 60% over 7.2 min, a gradient increasing solvent B from 60% to 71% over 2 min, a gradient increasing solvent B from 71% to 100% in 0.1 min, 100% solvent B for 3 min, a gradient increasing solvent A to 80% in 0.1 min and 80 % solvent A for 3 min (solvent A: water with 0.1% v/v formic acid, solvent B: acetonitrile with 0.1% v/v formic acid) at a flow rate of 0.3 mL/min. Elution condition for compound **12**: 2.4 min 100% solvent A, a gradient increasing solvent B from 0% to 100% over 6.3 min, 100% solvent B for 3.6 min, a gradient increasing solvent A to 100% in 0.1 min, 100% solvent A for 6.1 min. (solvent A: water with 0.1% v/v formic acid, solvent B: acetonitrile with 0.1% v/v formic acid) at a flow rate of 0.3 mL/min.

HRMS analysis of compound **3a** was performed on an Agilent 6220 TOF Mass Spectrometer equipped with an electrospray ionization (ESI). A Thermo Hypersil Golden aQ C18 reverse-phase column (3μ 3.0 x 150 mm) and a linear gradient program 5 to 95% acetonitrile (v/v) in water (0.1% formic acid) were used to separate sample with flow rate of 0.4 mL/min for 30 minutes.

Compound **2**, **3**, and **6** were separated and isolated on a Thermo Scientific Dionex Ultimate 3000 instrument and a Thermo Hypersil Golden aQ C18 reverse-phase column (3μ 3.0 x 150 mm). A linear gradient program 5 to 95% acetonitrile (v/v) in water (0.1% formic acid) was used to separate samples with flow rate of 0.4 mL/min for 30 minutes. HRMS analyses of the isolated compounds were performed on an Agilent 6220 TOF Mass Spectrometer equipped with an electrospray ionization (ESI) by using flow injection method.

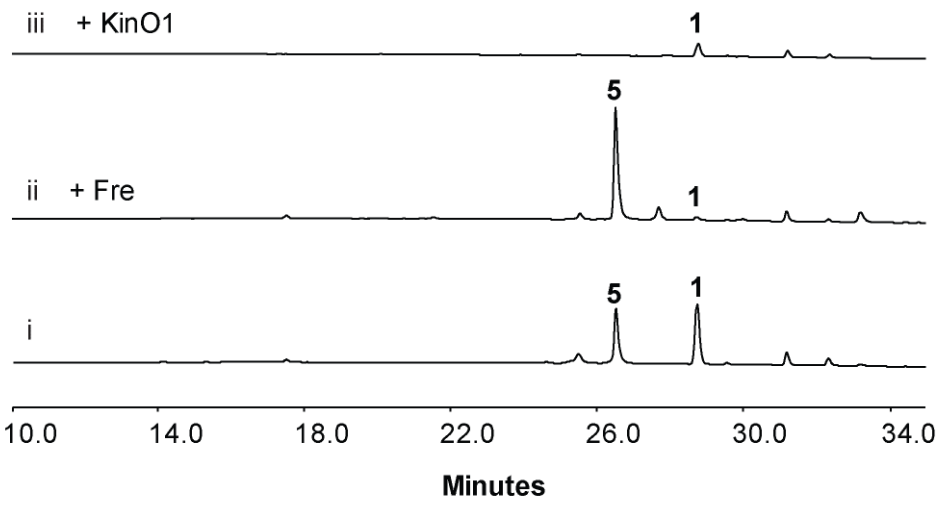


Figure S8. HPLC analysis (540 nm) of AlpJ *in vitro* assays without an amino acid co-substrate. i, AlpJ, **1**; ii AlpJ, Fre, **1**; iii, AlpJ, KinO1, **1**. All reactions contain 2 mM NADPH, 20 μM FAD in 50 mM MOPS buffer pH 7.5.

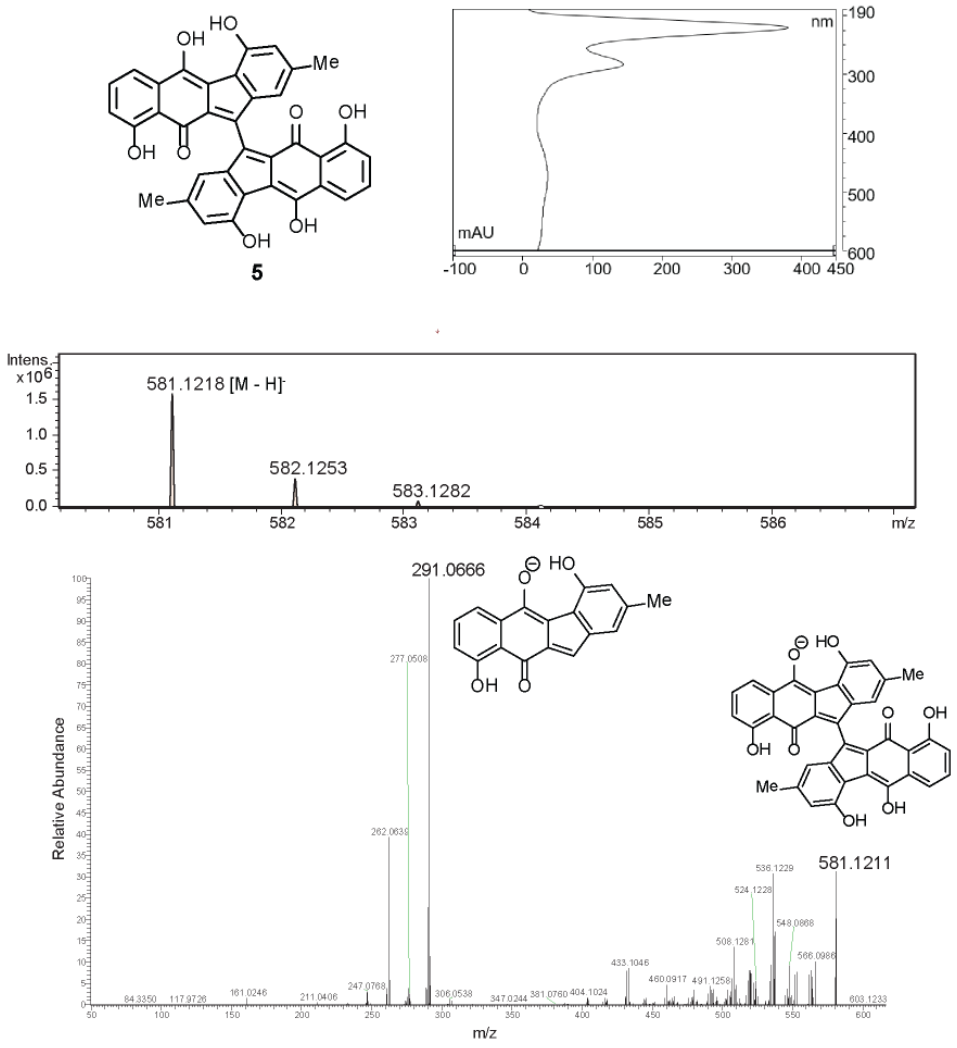


Figure S9. LC-HRMS data (negative mode), MS^2 fragmentation data (negative mode), and UV-vis spectrum for **5**. $[M - H]^-$ Obs: 581.1218, Calc: 581.1242, Error (ppm): -4.13.

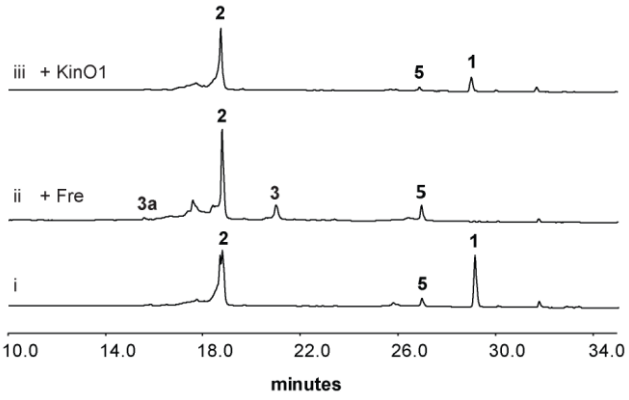


Figure S10. HPLC analysis (540 nm) of AlpJ *in vitro* assays containing *N*-acetyl-L-cysteine as a co-substrate. i, AlpJ, **1** and *N*-acetyl-L-cysteine; ii AlpJ, Fre, **1,** and *N*-acetyl-L-cysteine; iii, AlpJ, KinO1, **1**, and *N*-acetyl-L-cysteine. All reactions contain 2 mM NADPH, 20 μM FAD in 50 mM MOPS buffer pH 7.5. Note: The small amount of **3** and **3a** observed in trace ii is likely due to the trace amount of L-cysteine present in our stock of *N*-acetyl-L-cysteine.

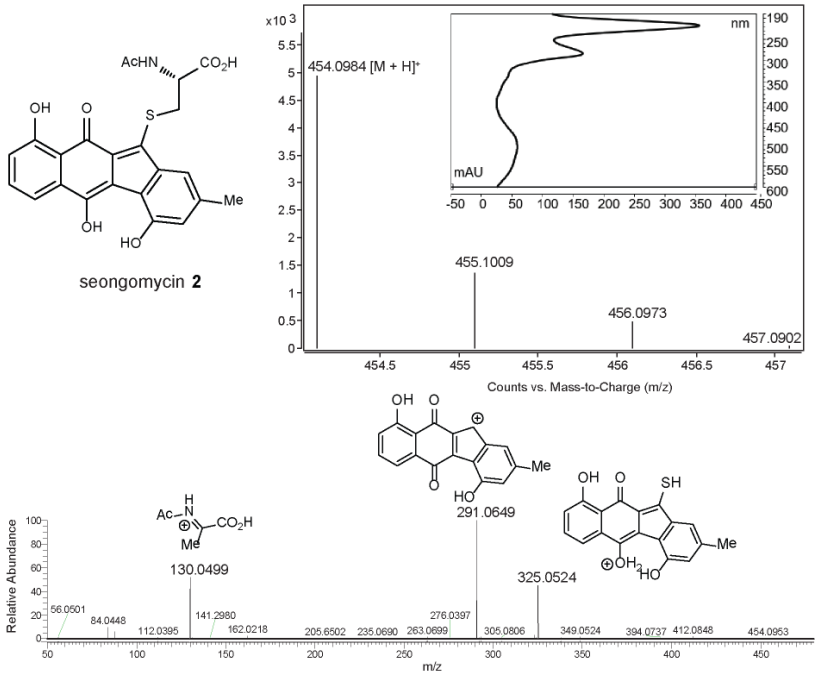
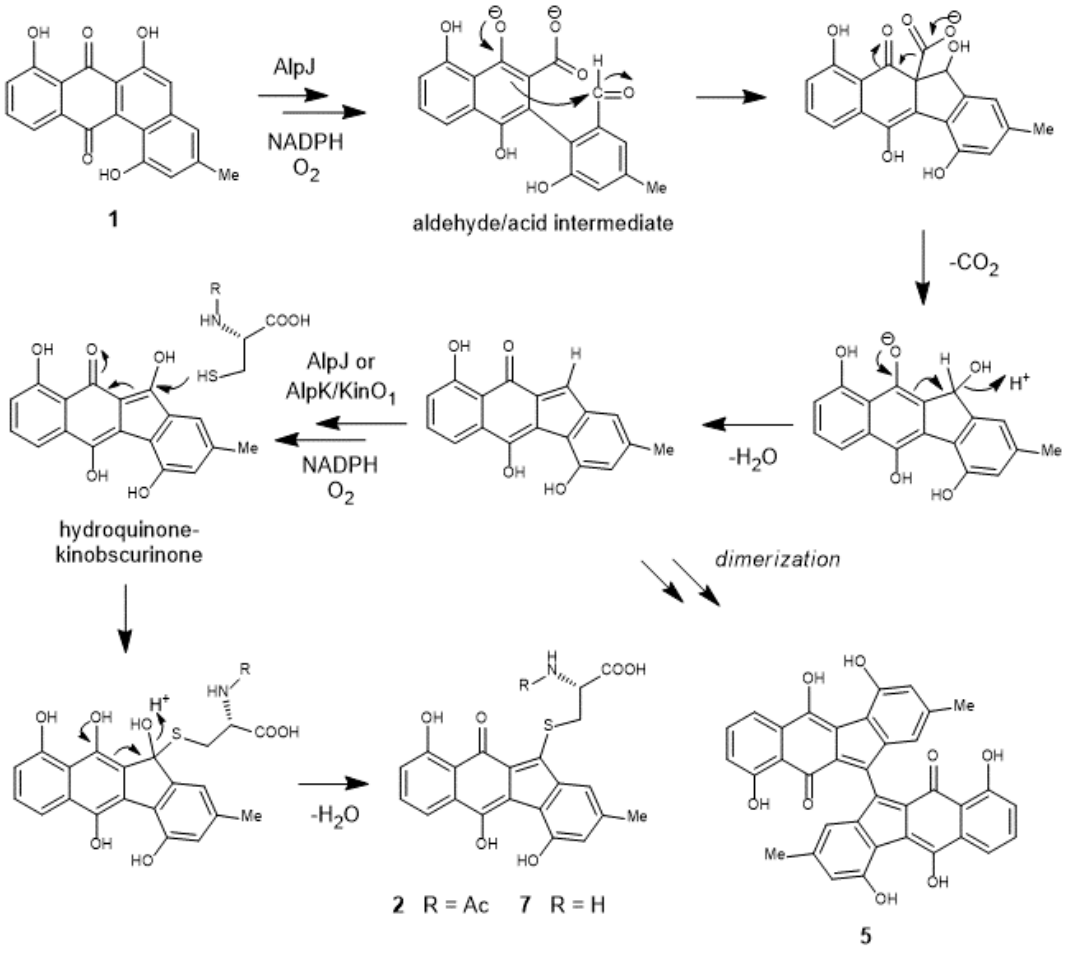


Figure S11. LC-HRMS data (positive mode), MS^2 fragmentation data (positive mode), and UV-vis spectrum for **2** from an *in vitro* assay. $[M + H]^+$ Obs: 454.0984, Calc: 454.0955, Error (ppm): 6.39.



Scheme S1. Proposed mechanism of AlpJ-derived formation of seongomycin **2**, dimeric benzofluorene **5**, and deacetylseongomycin **7**.

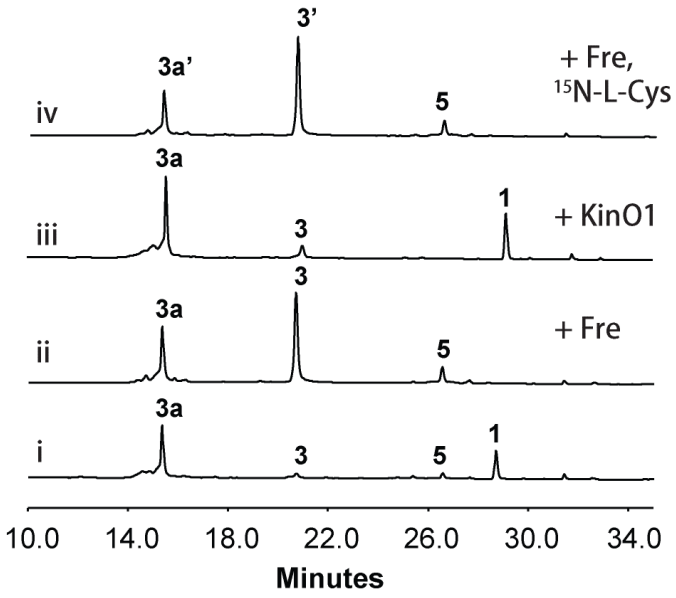


Figure S12. HPLC analysis (540 nm) of AlpJ *in vitro* assays containing L-cysteine as a co-substrate. i, AlpJ, **1**, and L-cysteine; ii AlpJ, Fre, **1**, and L-cysteine; iii, AlpJ, KinO1, **1**, and L-cysteine; iv, AlpJ, Fre, **1**, and ^{15}N-labeled L-cysteine. All reactions contain 2 mM NADPH, 20 μM FAD in 50 mM MOPS buffer pH 7.5.

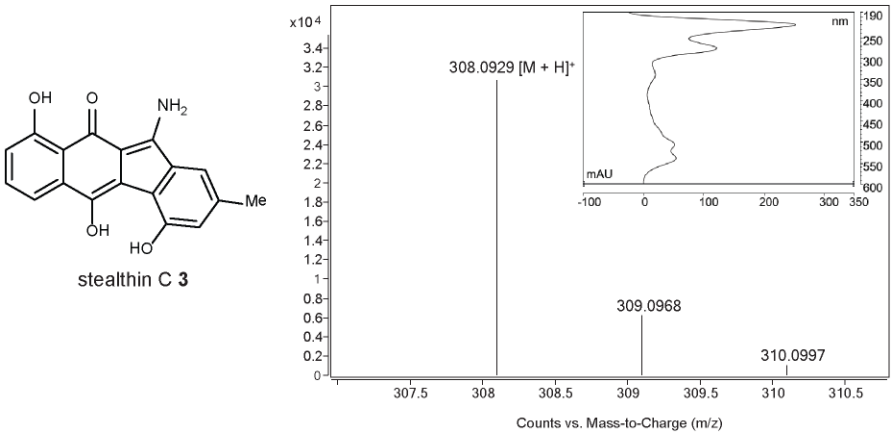


Figure S13. LC-HRMS data (positive mode) and UV-vis spectrum for **3** from an *in vitro* assay. $[M + H]^+$ Obs: 308.0929, Calc: 308.0917, Error (ppm): 3.89.

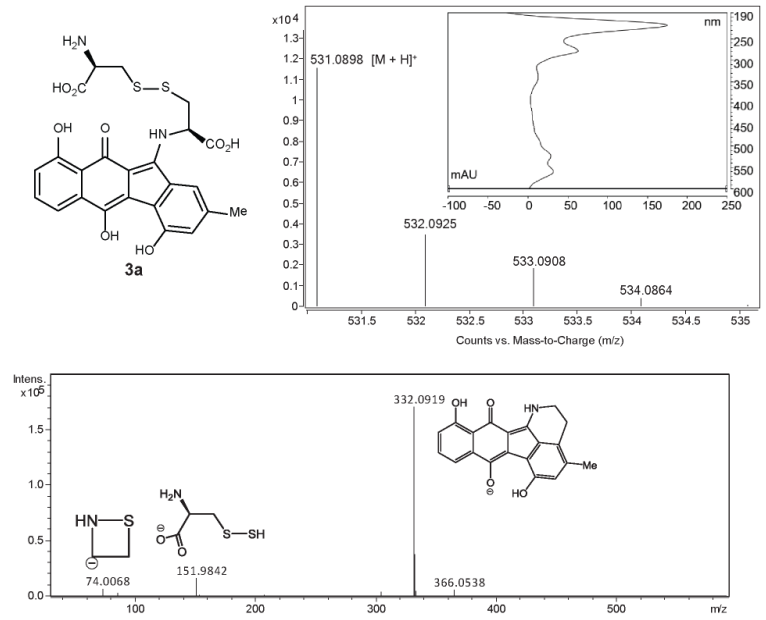


Figure S14. LC-HRMS data (positive mode), MS^2 fragmentation data (negative mode), and UV-vis spectrum for **3a**. $[M + H]^+$ Obs: 531.0898, Calc: 531.0890, Error (ppm): 1.51.

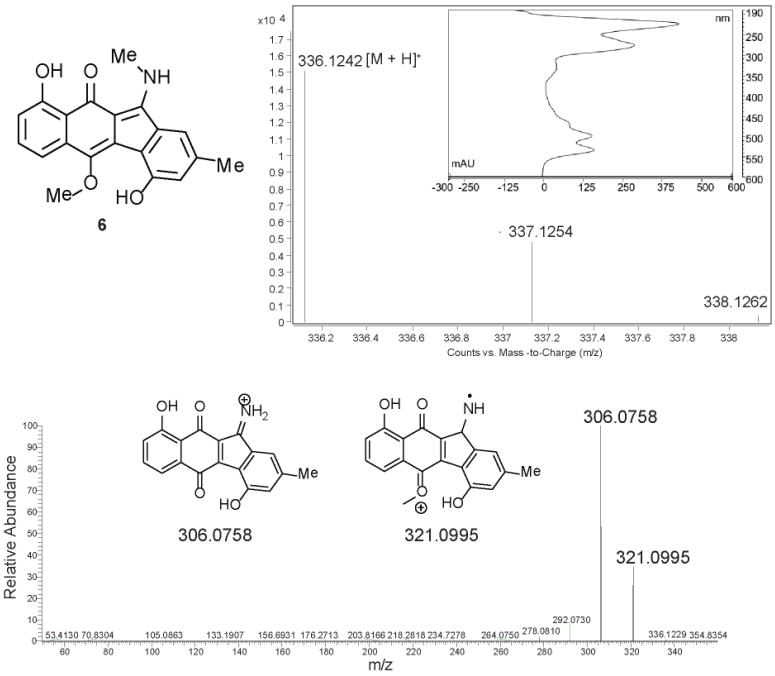


Figure S15. LC-HRMS data (positive mode), MS^2 fragmentation data (positive mode), and UV-vis spectrum of amine **6**. $[M + H]^+$ Obs: 336.1242, Calc: 336.1230, Error (ppm): 3.57.

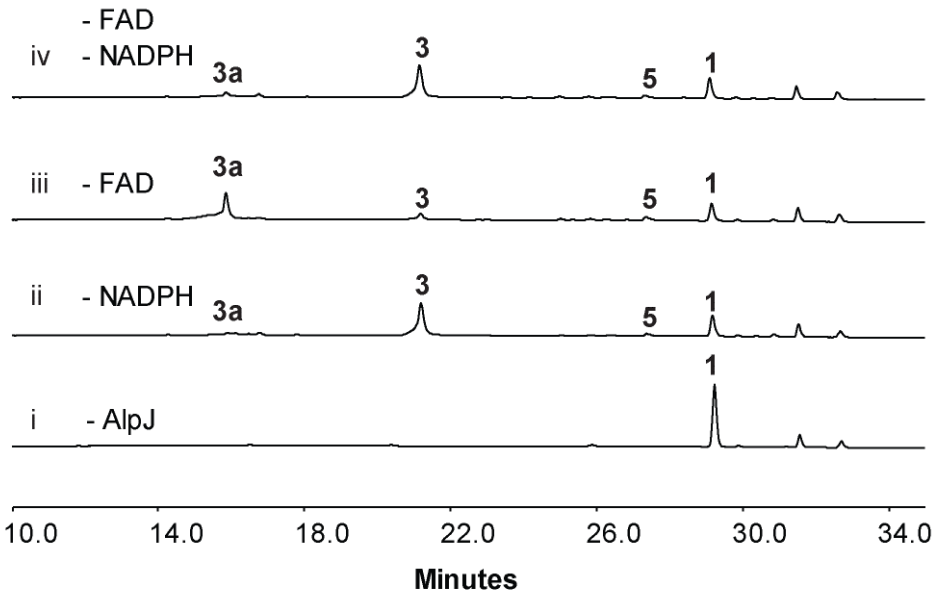


Figure S16. HPLC analysis (540 nm) of AlpJ *in vitro* assay controls. i, full reaction removing AlpJ; ii, full reaction removing NADPH; iii, full reaction removing FAD; iv, full reaction removing NADPH and FAD. Full reaction contains 20 μM AlpJ, 110 μM **1**, 1 mM L-cysteine, 2 mM NADPH, 20 μM FAD in 50 mM MOPS buffer pH 7.5.

We noticed that in the absence of NADPH, **3** and **3a** were still produced in AlpJ *in vitro* assays (Figure S16, trace ii). We hypothesized that the excess L-cysteine present in these assays could serve as an NADPH equivalent and enable the AlpJ reaction. This reactivity could resemble that of flavin-dependent dithiol oxidases that catalyze formation of disulfide bonds via thiol-mediated reduction of FAD to $FADH_2$.[2] Therefore, we performed ultraviolet-visible analysis of FAD reduction under the conditions of our *in vitro* assays (Figure S17). We observed a decreasing UV-vis absorbance at 450 nm, indicating the conversion from FAD to $FADH_2$ by L-cysteine with (red line, 12% decrease) and without AlpJ (green line, 13% decrease).

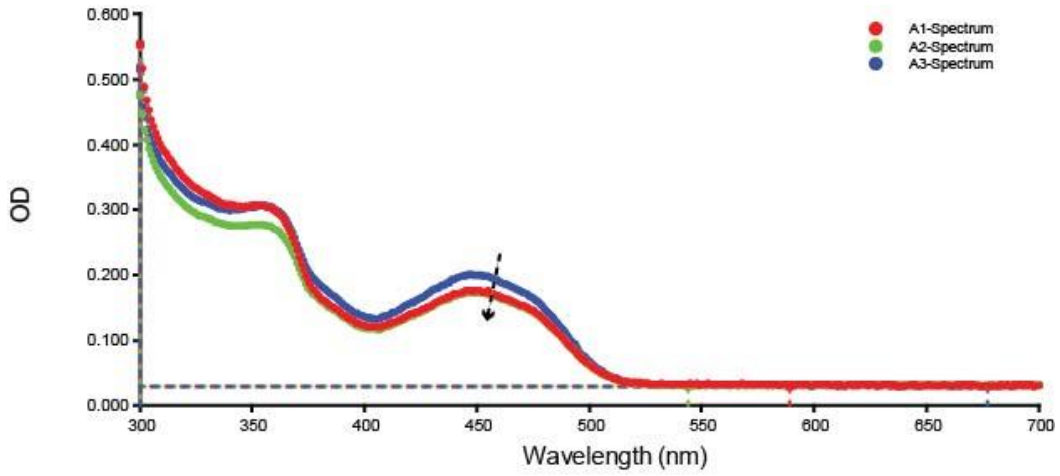


Figure S17. Ultraviolet-visible analysis of FAD reduction in *in vitro* assays. A1 (red line): AlpJ (20 μM), L-cysteine (2 mM), and FAD (100 μM). A2 (green line): L-cysteine (2 mM) and FAD (100 μM).; A3 (blue line): AlpJ (20 μM), and FAD (100 μM). All reactions were performed in MOPS buffer (50 mM, pH = 7.5). Reactions were set up at room temperature for 30 minutes. Ultraviolet-visible analysis was performed on BioTek Microplate Readers.

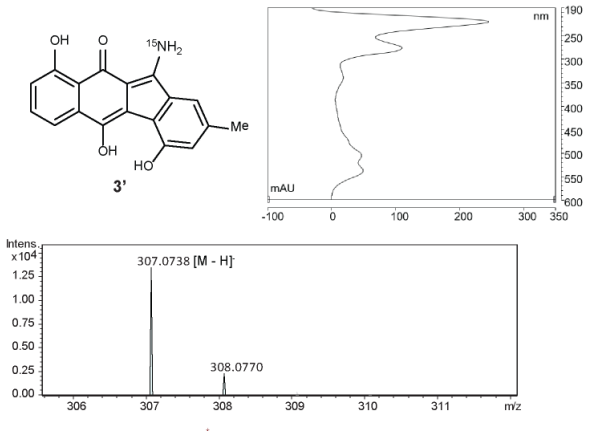


Figure S18. LC-HRMS data (negative mode) and UV-vis spectrum for **3'**. $[M - H]^-$ Obs: 307.0738, Calc: 307.0742, Error (ppm): -1.30.

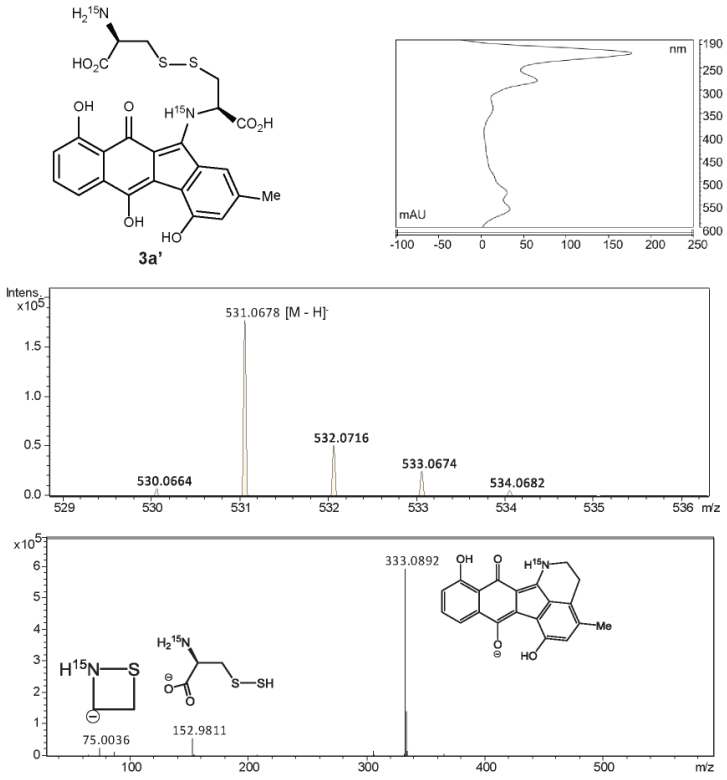


Figure S19. LC-HRMS data (negative mode), MS^2 fragmentation data (negative mode), and UV-vis spectrum for **3a'**. $[M - H]^-$ Obs: 531.0678, Calc: 531.0686, Error (ppm): -1.51.

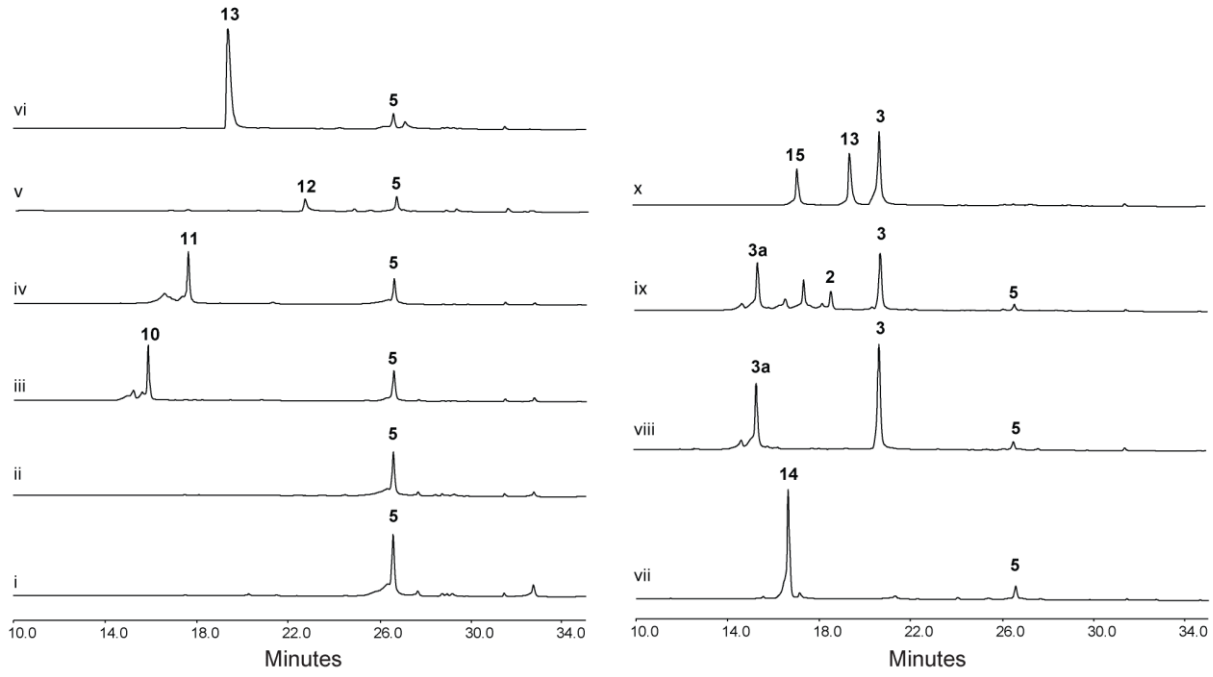


Figure S20. HPLC analysis (540 nm) of AlpJ *in vitro* assays with alternate substrates. i, L-serine; ii, *S*-methyl-L-cysteine; iii, γ-Glu-Cys; iv, *N*-carbamoyl-L-cysteine; v, *N*-methyl-L-cysteine; vi, cysteamine; vii, L-homocysteine; viii, D-cysteine; ix, L-cysteine and *N*-acetyl-L-cysteine; x, L-cysteine and cysteamine. All reactions contain 20 μM AlpJ, 110 μM **1**, 2 mM NADPH, 20 μM FAD in 50 mM MOPS buffer pH 7.5.

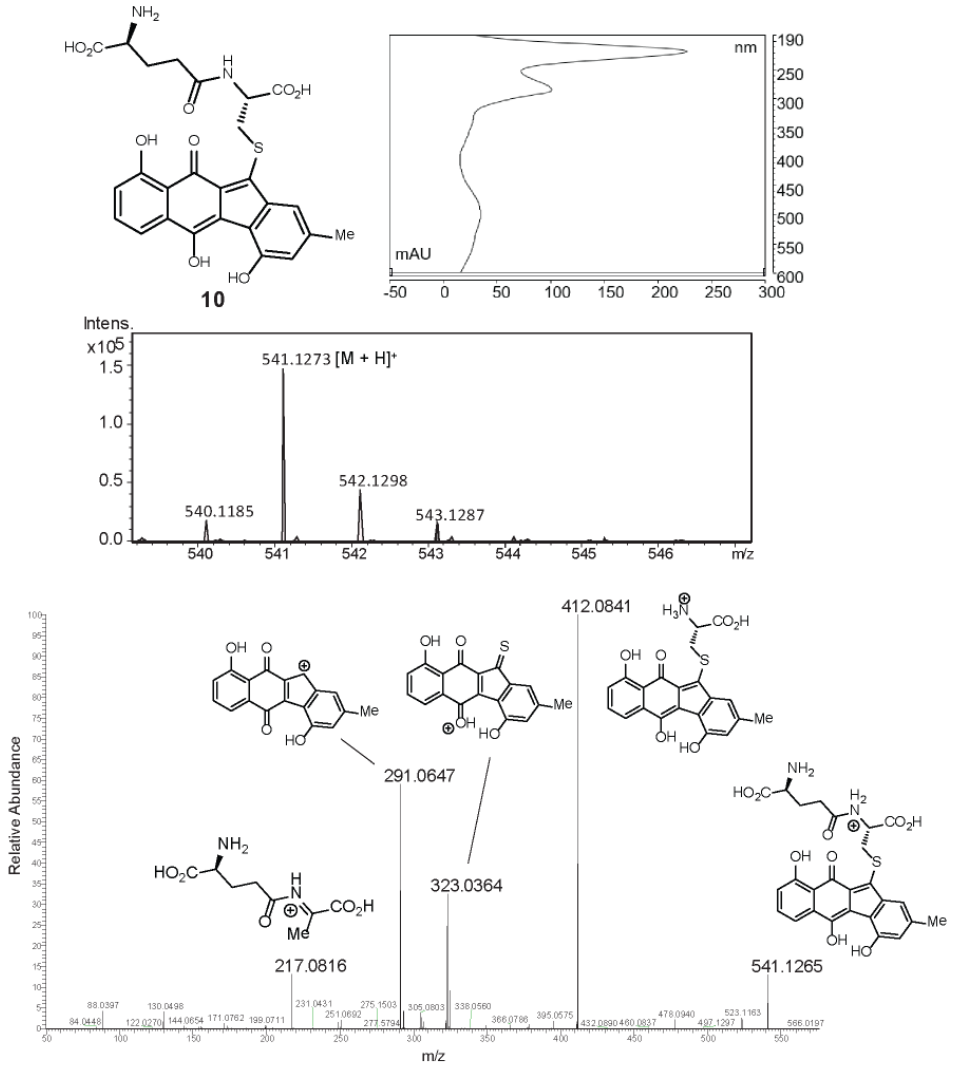


Figure S21. LC-HRMS data (positive mode), MS^2 fragmentation data (positive mode), and UV-vis spectrum for **10**. $[M + H]^+$ Obs: 541.1273, Calc: 541.1275, Error (ppm): -0.37.

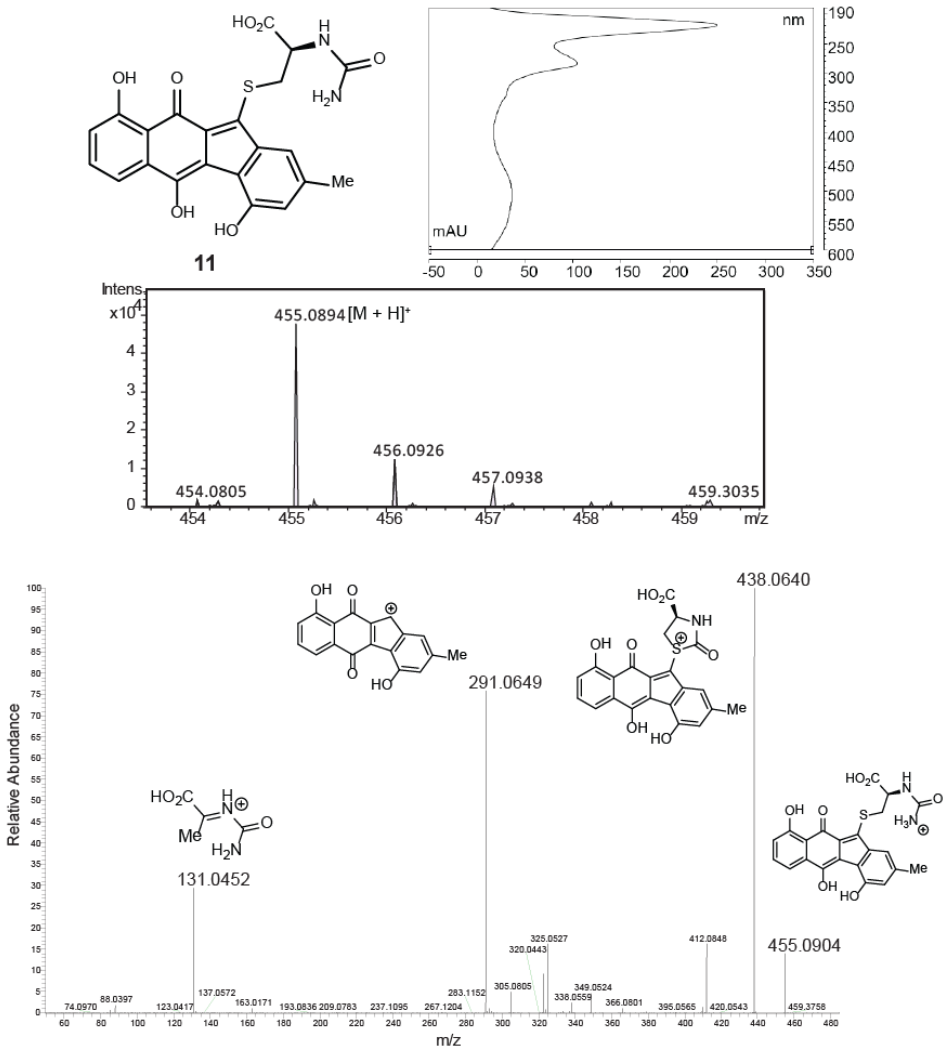


Figure S22. LC-HRMS data (positive mode), MS^2 fragmentation data (positive mode), and UV-vis spectrum for **11**. $[M + H]^+$ Obs: 455.0894, Calc: 455.0907, Error (ppm): -2.86.

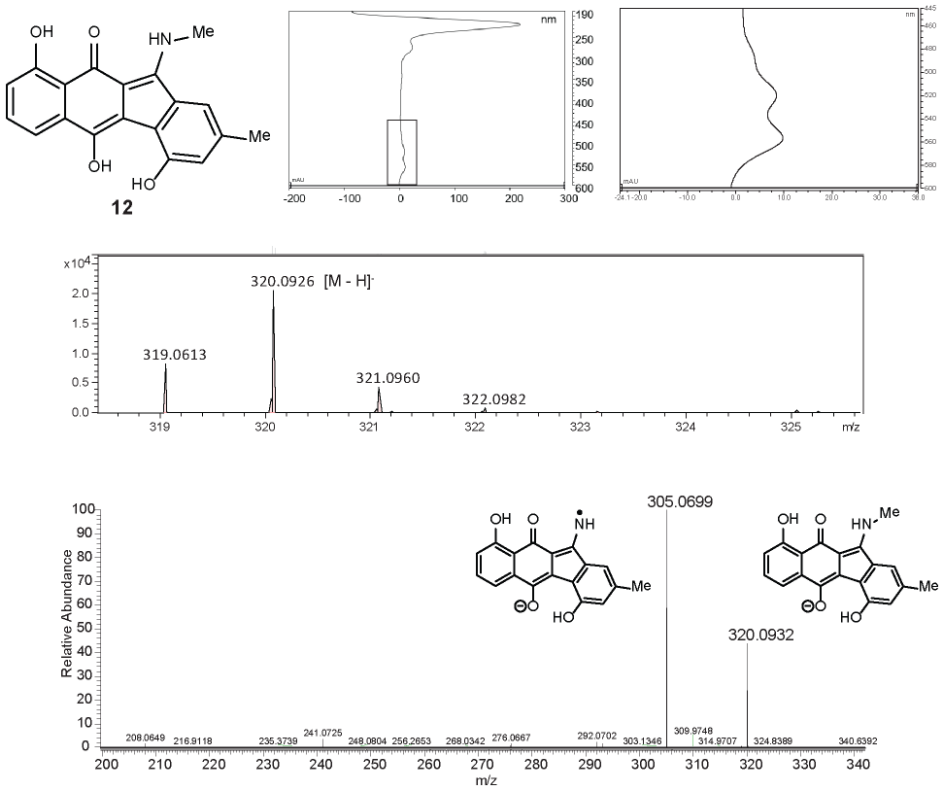


Figure S23. LC-HRMS data (negative mode), MS^2 fragmentation data (negative mode), and UV-vis spectrum for **12**. $[M - H]^-$ Obs: 320.0926, Calc: 320.0928, Error (ppm): -0.62.

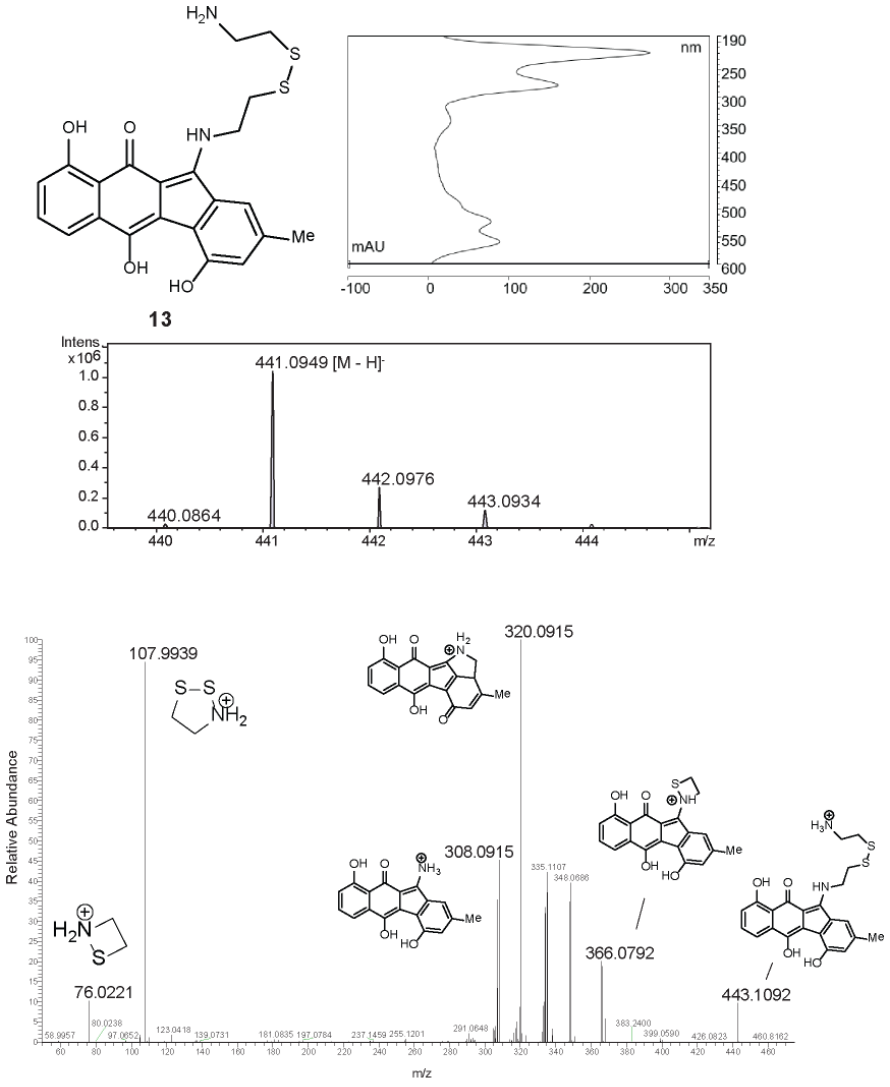


Figure S24. LC-HRMS data (negative mode), MS^2 fragmentation data (positive mode), and UV-vis spectrum for **13**. $[M - H]^-$ Obs: 441.0949, Calc: 441.0948, Error (ppm): 0.23.

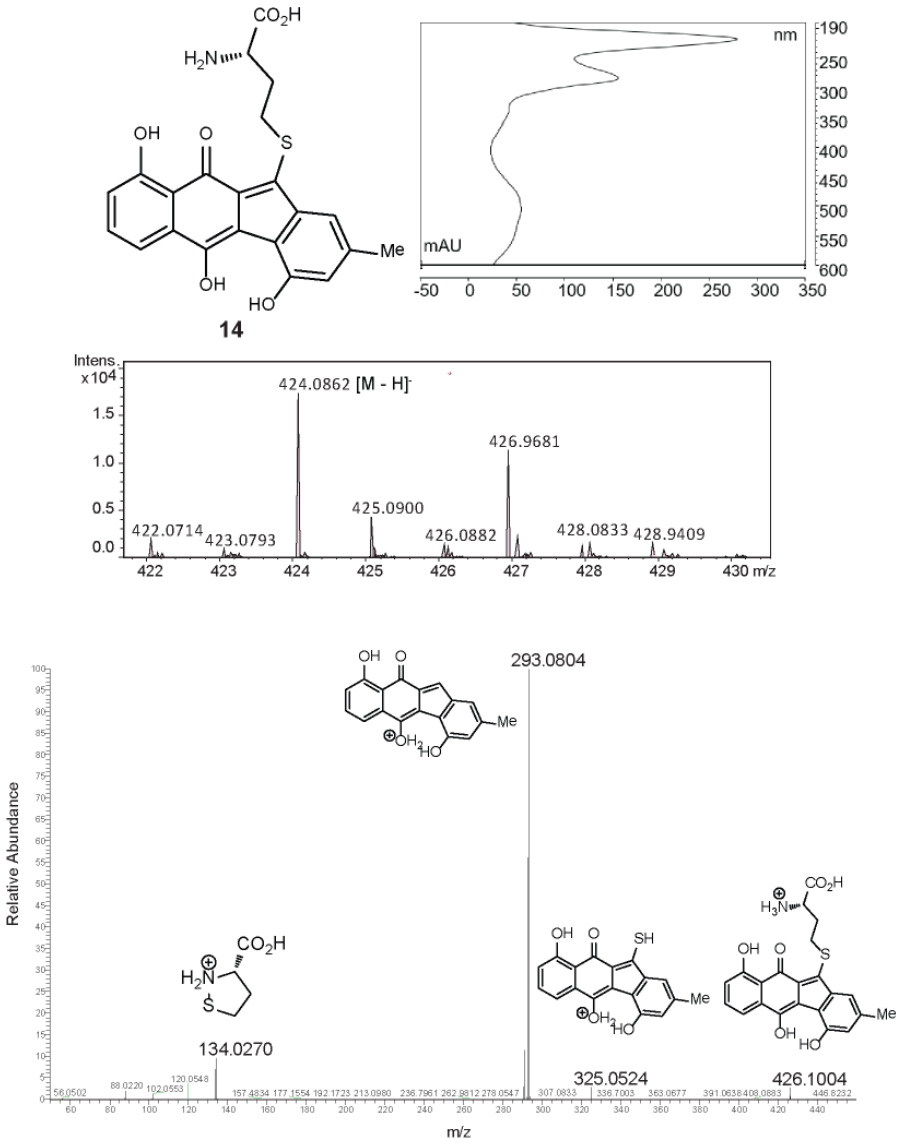


Figure S25. LC-HRMS data (negative mode), MS^2 fragmentation data (positive mode), and UV-vis spectrum for **14**. $[M - H]^-$ Obs: 424.0862, Calc: 424.0860, Error (ppm): 0.47.

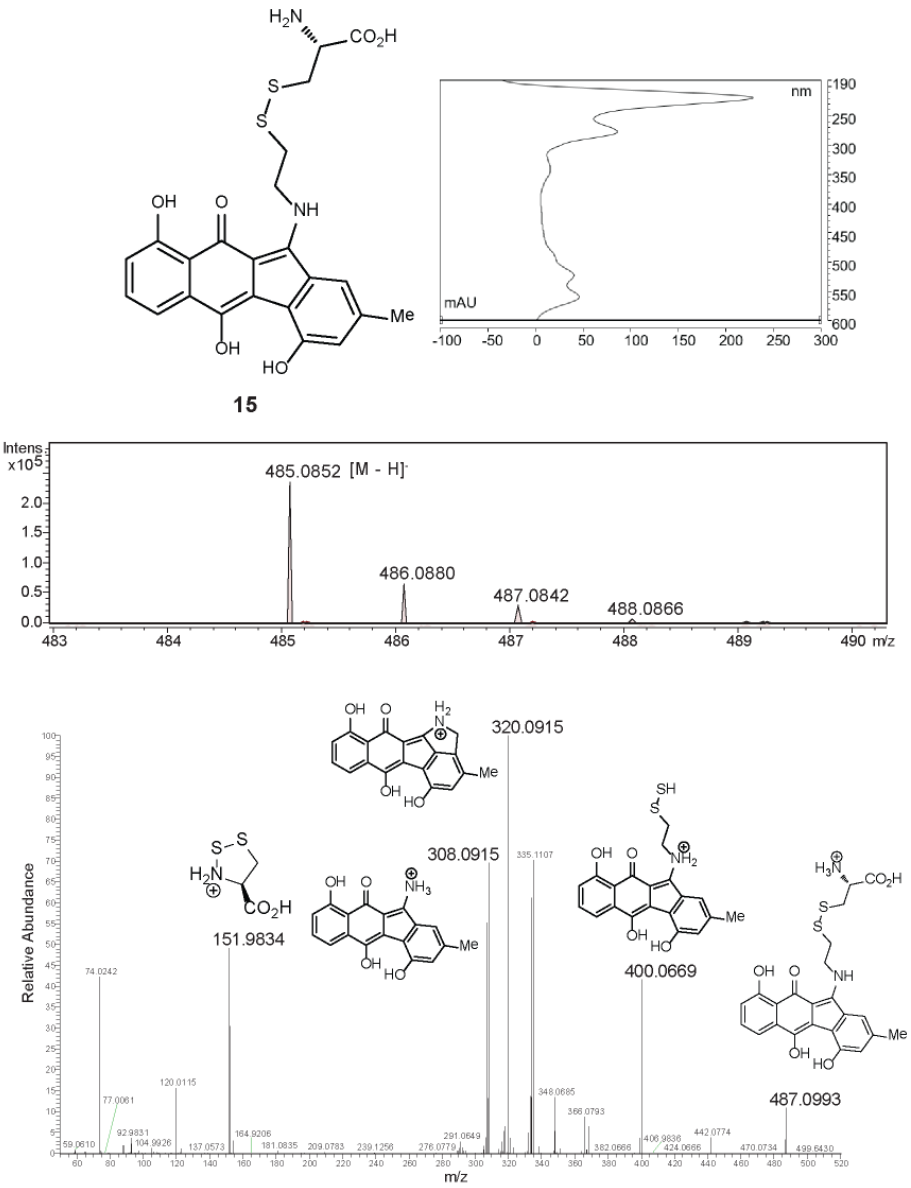


Figure S26. LC-HRMS data (negative mode), MS^2 fragmentation data (positive mode), and UV-vis spectrum for **15**. $[M - H]^-$ Obs: 485.0852, Calc: 485.0847, Error (ppm): 1.03.

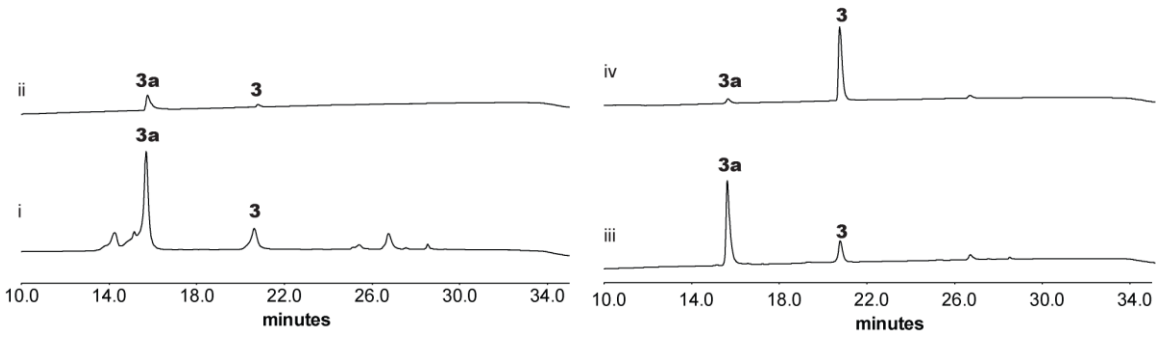


Figure S27. HPLC analysis (540 nm) of the non-enzymatic conversion of **3a** to **3**. i, AlpJ-Fre *in vitro* assays containing L-cysteine for 30 minutes; ii, **3a** was isolated and re-analyzed after 2 hours, iii, AlpJ-Fre *in vitro* assays containing L-cysteine for 30 minutes; iv, reaction was heated at 95 ºC for 10 minutes and re-analyzed after 2 hours.

8. Isolation of dehydrorabelomycin

The fluostatin biosynthetic machinery was used to produce the AlpJ substrate dehydrorabelomycin (**1**). A bacterial artificial chromosome (BAC) AB649/1850 containing a gene cluster from an uncultured bacterium was reported to generate fluostatins via the intermediacy of **1**.[3] The recombinant heterologous expression strain *Streptomyces lividans* K4-114 with the AB649/1850 BAC was cultured in modified SPYESS medium containing 5% HP-20 resin at 28 ºC and 220 rpm for two weeks. **1** was previously reported to be produced by *Streptomyces albus* harboring the AB649/1850 BAC.[3] The HP-20 resin was collected from a 12-day culture using centrifugation at 4,000 rpm for 15 minutes. The collected HP-20 resin was then washed with a 500 mL organic mixture consisting of 89% ethyl acetate, 10% methanol, and 1% acetic acid (v/v), followed by centrifugation at 4,000 rpm for 15 minutes. The resulting 500 mL of supernatant was dried *in vacuo* using rotary evaporation and then re-dissolved in 5 mL of chloroform. The chloroform extract was then loaded on a 40-gram silica gel P60 column (250 mL glass column) and eluted with a mixture of chloroform and methanol stepwise: 200-mL chloroform, 100-mL chloroform/methanol (90/10, v/v), and 100-mL chloroform/methanol (70/30, v/v). The fractions containing **1** were collected and dried *in vacuo* using rotary evaporation. The ^{1}H NMR spectrum of **1** matched that reported previously.[3]

9. Preparation of dimethylstealthin C

A large scale *in vitro* assay was prepared and incubated at room temperature overnight. This assay mixture contained 20 μM AlpJ, 20 μM Fre, 2 mM NADPH, 20 μM FAD, 200 μM **1**, and 1 mM L-cysteine in 50 mM MOPS buffer pH 7.5 in a total volume of 20 mL. The reaction was quenched by adding 80 mL methanol and aliquoted into 1.5 mL microcentrifuge tubes. The aliquoted samples were centrifuged at 13,000 rpm for 10 minutes. The supernatants were then transferred to new 1.5 mL microcentrifuge tubes and dried *in vacuo* using a SpeedVac. All of the sample pellets from AlpJ *in vitro* assays were combined and re-suspended in acetone (total volume 5 mL). K_2CO_3 powder (138 mg) was added, and N_2 was used to degas the mixture. Methyl iodide (0.600 mL) was added and the reaction mixture was stirred at room temperature for 6 days. The reaction mixture was neutralized with 1 M HCl and then 10 mL of ethyl acetate and 2 mL of methanol were added. The organic solvent then was removed *in vacuo* using rotary evaporation and the resulting crude material was extracted with ethyl acetate (~100 mL) until no pink color was left in the aqueous layer. The combined organic extracts were then dried *in vacuo* using rotary evaporation. A small amount of the extract (less than 1 mg) was purified on a Thermo Scientific Dionex Ultimate 3000 instrument and a Thermo Hypersil Golden aQ C18 reverse-phase column (3μ 3.0 x 150 mm). A linear gradient program 5 to 95% acetonitrile (v/v) in water (0.1% formic acid) was used to separate samples with a flow rate of 0.4 mL/min for 30 minutes. Using the HPLC method described above, we compared our reaction product with the authentic standard of dimethylstealthin C.[4] A purified sample was dried *in vacuo* using a SpeedVac and re-dissolved in 50 μL methanol. 10 μL of this purified sample was analyzed by HPLC. About 0.5 mg of the authentic standard of dimethylstealthin C was dissolved in 100 μL methanol and 20 μL of this solution was analyzed by HPLC. 20 μL of the purified sample and 40 μL of an authentic standard were mixed and 20 μL of the mixed sample was analyzed by HPLC. The co-elution of our sample and the authentic standard of dimethylstealthin C confirmed the chemical identity of **3**.

10. Preparation of *N*-methyl-L-cysteine

A flame-dried 25 mL round bottom flask containing a stir bar was cooled under vacuum and then flushed with nitrogen. NaH (60% in mineral oil, 0.206 g, 5.16 mmol, 2.40 equiv) was added to the flask followed by dry THF (2.15 mL, 1 M). The NaH suspension was cooled to 0 ºC for 10 minutes and then a solution of (*R*)-*N*-Boc-*S*-tritylcysteine (1.00 g, 2.15 mmol, 1.00 equiv) in THF (2.15 mL, 1 M) was added dropwise via syringe (**CAUTION**: H_2 gas evolution). After 5 min at 0 ºC, iodomethane (1.07 mL, 17.2 mmol, 8.00 equiv) was added and the reaction mixture was allowed to slowly warm to room temperature over approximately 12 hours. The progress of the reaction was then carefully monitored by LC-MS in 3-hour intervals. After a total reaction time of 19 hours, the reaction mixture was carefully quenched with methanol (5 mL) (**CAUTION**: H_2 gas evolution). The THF and methanol were removed *in vacuo* using a rotary evaporator and the resulting aqueous layer was extracted with Et_2O (2 x 5 mL). The aqueous layer was then acidified to pH = 4.0 with 1M HCl (aq.) and then extracted with EtOAc (3 x 10 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give known (*R*)-N-Boc-Me-Cys(Trt)-OH as a white solid (0.780 g, 76% yield). The crude product was taken to the next step without further purification.

To a solution of (*R*)-N-Boc-Me-Cys(Trt)-OH (0.390 g, 0.815 mmol, 1.00 equiv) in CH_2Cl_2 (1.63 mL, 0.5 M) was added triisopropylsilane (0.817 mL, 3.99 mmol, 4.90 equiv) and TFA (1.62 mL, 21.2 mmol, 26.0 equiv) under nitrogen. The resulting reaction mixture was stirred at room temperature and starting material consumption monitored by TLC (1:1: EtOAc/Hexanes w/ 1% acetic acid). After 2 hours, the reaction mixture was concentrated *in vacuo* and the resulting oily residue was washed with Et_2O (3 x 10 mL). The residue was then azeotroped with PhMe (3x) and placed under high vacuum for 1 hour to give free sulfide *N*-methyl-L-cysteine (0.079 g, 72% yield) as a fluffy yellow foam. The ^{1}H NMR spectrum of *N*-methyl-L-cysteine matched that reported previously.[5]

11. Feeding experiments in *Streptomyces murayamaensis*

Streptomyces murayamaensis (ATCC 21414) was obtained from the American Type Culture Collection. An authentic standard of kinamycin D was kindly provided by Prof. Philip J. Proteau (Oregon State University). A 20 mL *S. murayamaensis* seed culture was prepared in ISP medium 2 at 30 ºC with shaking at 220 rpm for 7 days. 50 µL of seed culture was inoculated into 5 mL of glycerol-ammonium sulfate medium (glycerol 3%, $(NH_4)_2SO_4$ 0.1%, K_2HPO_4 $3H_2O$ 0.1%, $CaCO_3$ 0.1%, $MgSO_4$ $7H_2O$ 0.01%, and $FeSO_4$ 7H2O 0.01%). Either ^{15}N-L-cysteine, $^{15}N_2$-L-asparagine, or $^{15}N_4$-L-arginine was fed at a final concentration of 0.5 mM. 500 µL of each culture was removed after incubating for 7 days at 30 ºC with shaking at 220 rpm. Samples were quenched by adding a 1 mL mixture of 99% ethyl acetate and 1% acetic acid (v/v), followed by centrifugation at 13,000 rpm for 10 minutes. The supernatants were transferred to new 1.5 mL microcentrifuge tubes and dried *in vacuo* using a SpeedVac. The resulting dried extracts were dissolved in 50 µL of methanol for further LC-HRMS-MS/MS analysis. LC-HRMS-MS/MS analyses of feeding experiments were performed on a Thermo Scientific Dionex Ultimate 3000 uHPLC coupled to a Q Exactive Plus Orbitrap mass spectrometer system equipped with an electrospray ionization (ESI) source by using a Waters XTerra MS C18 analytical column (2.1x50 mm, 3.5 µm). Elution condition: a gradient increasing solvent B from 20% to 60% over 7.2 min, a gradient increasing solvent B from 60% to 71% over 2 min, a gradient increasing solvent B from 71% to 100% in 0.1 min, 100% solvent B for 3 min, a gradient increasing solvent A to 80% in 0.1 min and 80 % solvent A for 3 min (solvent A: water with 0.2% v/v acetic acid, solvent B: methanol with 0.2% v/v acetic acid) at a flow rate of 0.3 mL/min. Enrichment was calculated by *m/z*.[6] Peak areas (arbitrary units) for unlabeled kinamycin D (from no feeding controls) were used to correct peak areas for kinamycin D produced in feeding experiments. The distribution of singly and doubly labeled kinamycin D species was computed using this formula: the corrected peak area of labeled species / the sum of all corrected peak areas.

Table S3. Incorporation percentages from feeding ^{15}N-labeled precursors.

Labeled precursors	Enrichment of Kinamycin D; singly/doubly labeled [%]
^{15}N-L-Cysteine	0.0/0.0
$^{15}N_2$-L-Asparagine•H_2O	0.0/0.2
$^{15}N_4$-L-Arginine•HCl	5.9/0.5

Average value from two independent experiments.

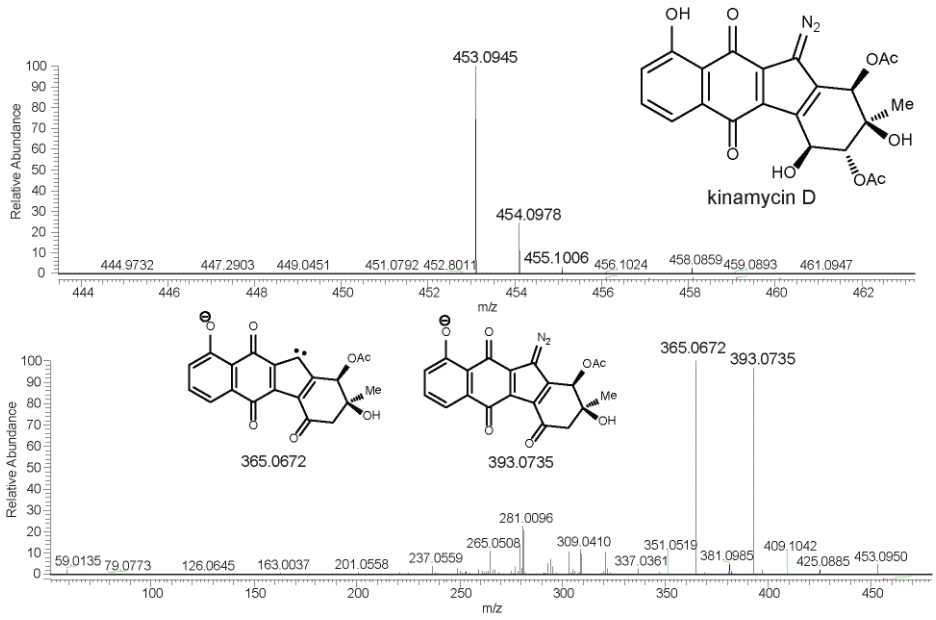


Figure S28. LC-HRMS data (negative mode) and MS^2 fragmentation data (negative mode) for kinamycin D standard. $[M - H]^-$ Obs: 453.0945, Calc: 453.0940, Error (ppm): 1.10.

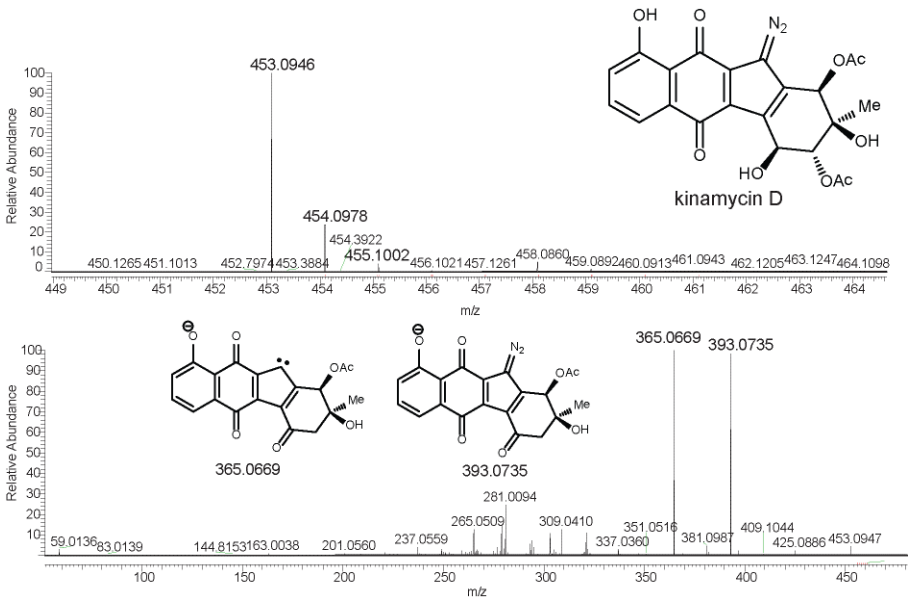


Figure S29. LC-HRMS data (negative mode) and MS^2 fragmentation data (negative mode) for kinamycin D from *S. murayamaensis* (no feeding). $[M - H]^-$ Obs: 453.0946, Calc: 453.0940, Error (ppm): 1.32.

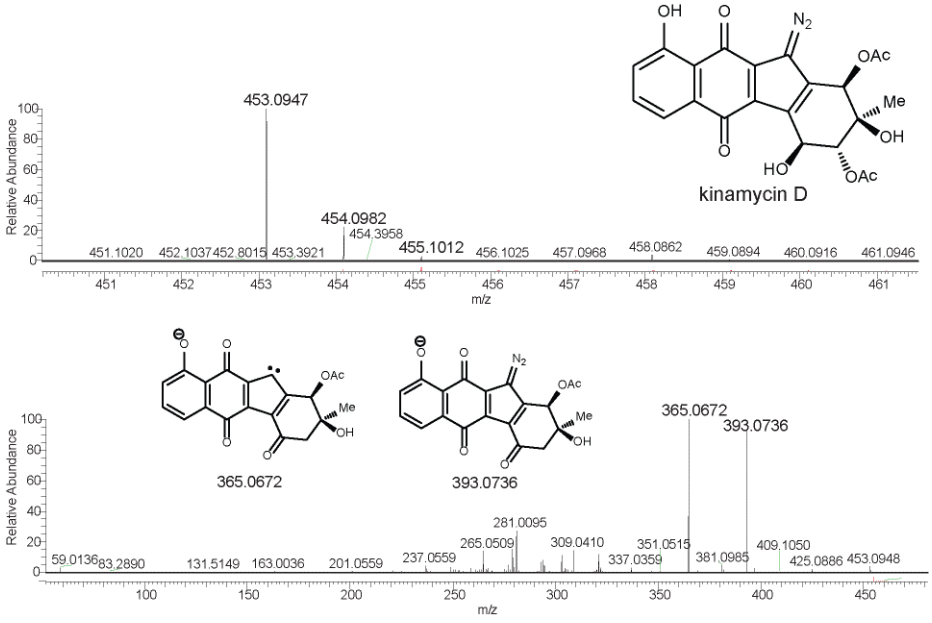


Figure S30. LC-HRMS data (negative mode) and MS^2 fragmentation data (negative mode) for kinamycin D from *S. murayamaensis* fed ^{15}N-L-cysteine. $[M - H]^-$ Obs: 453.0947, Calc: 453.0940, Error (ppm): 1.54.

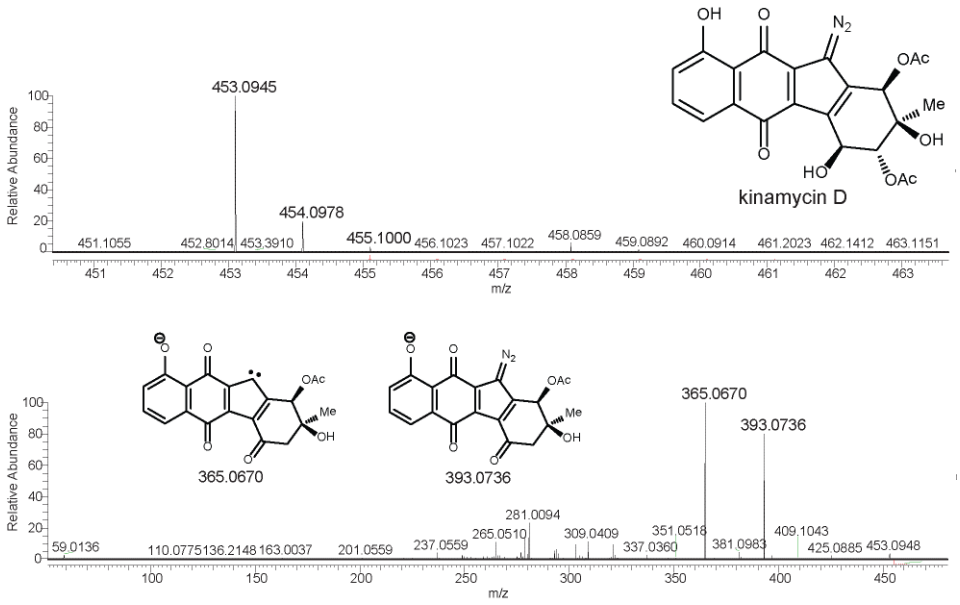


Figure S31. LC-HRMS data (negative mode) and MS^2 fragmentation data (negative mode) for kinamycin D from *S. murayamaensis* fed $^{15}N_2$-L-asparagine•H_2O. $[M - H]^-$ Obs: 453.0945, Calc: 453.0940, Error (ppm): 1.10.

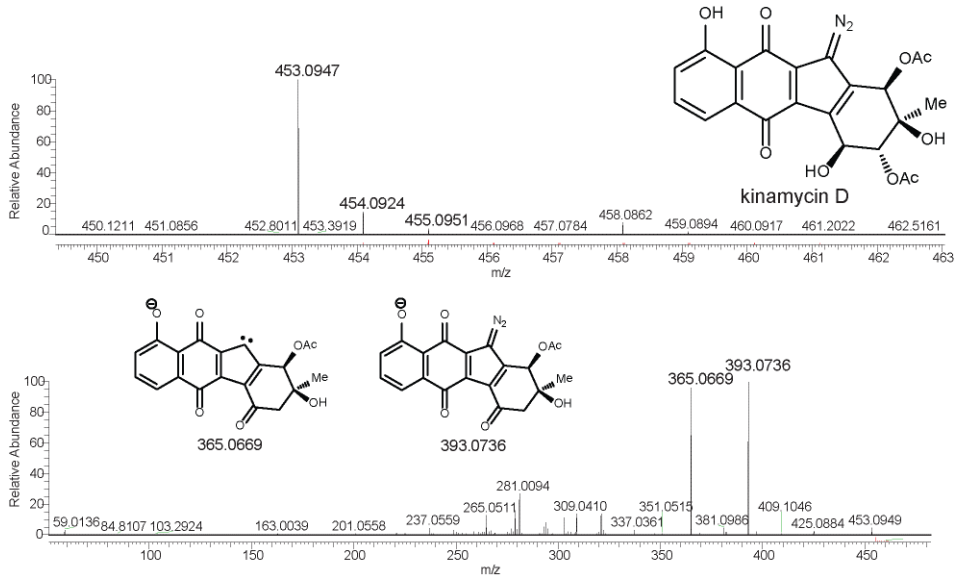


Figure S32. LC-HRMS data (negative mode) and MS^2 fragmentation data (negative mode) for kinamycin D from *S. murayamaensis* fed $^{15}N_4$-L-arginine•HCl. $[M - H]^-$ Obs: 453.0947, Calc: 453.0940, Error (ppm): 1.54.

12. Supplementary References

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