Supporting Information for

Hybrid Biosynthesis of Roseobacticides from Algal and Bacterial Precursor Molecules

Mohammad R. Seyedsayamdost,[†,*] Rurun Wang,[†] Roberto Kolter,[‡] and Jon Clardy[§,*]

[†]Department of Chemistry, Princeton University, Princeton, NJ 08544
[‡]Department of Microbiology & Immunology and Department of Biological Chemistry & Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

*email: mrseyed@princeton.edu; jon_clardy@hms.harvard.edu

Reagents and general procedures. Yeast extract and tryptone were purchased from Becton Dickinson. Ring-$^{2}H_4$-L-tyrosine, ring-$^{2}H_5$-L-phenylalanine, ring-$^{2}H_5$-L-tryptophan, 2-^{13}C-malonic acid, and ring-$^{2}H_4$-4-hydroxybenzaldehyde were purchased from Cambridge Isotopes Laboratories. All other reagents, including sea salt, *p*-coumaric acid, sinapic acid, phenylacetic acid, 1,2-^{13}C-phenylacetic acid, ring-$^{2}H_5$-phenylacetic acid, 3-fluoro-DL-tyrosine, ring-$^{2}H_5$-phenylglycine, ring-$^{2}H_5$-indoleacetic acid, unlabeled L-phenylalanine, NAD, and phenylalanine dehydrogenase, the latter three required for the enzymatic synthesis of isotopically labeled phenylpyruvic acid, were obtained from Sigma-Aldrich. ^{34}S-L-Cysteine was a kind gift of Prof. Jeroen Dickschat.[1] UV-visible absorbance spectra were collected an Agilent Cary 60 Spectrophotometer. HPLC purifications were carried out on an Agilent 1200 Series analytical HPLC system equipped with a photo diode array detector and an automated fraction collector, or on an Agilent 1200 Series preparative HPLC system also equipped with the same modules. Low resolution HPLC-MS analysis was performed on an Agilent 1200 Series HPLC system equipped with a diode array detector and a 6130 Series ESI mass spectrometer using an analytical Phenomenex Luna C18 column (5 μm, 4.6 × 100 mm) operating at 0.7 mL/min with a gradient of 10 % MeCN in H_2O (+0.1% formic acid) to 100% MeCN (+0.1% formic acid) over 25 min. High resolution HPLC-ESI-MS (HR-MS) and tandem MS (MS/MS) were out on a 6540 UHD Accurate Mass Q-tof LC-MS system (Agilent), which consists of a 1260 Infinity Series HPLC system, an automated liquid sampler, a diode array detector, a JetStream ESI source, and the 6540 Series Q-tof. The HR-HPLC-MS was calibrated to within 1 ppm. Samples were resolved on a Poroshell 120 EC-C18 column (Agilent, 2.7 μm, 3 x 50 mm) operating at 0.4 mL/min with a gradient of 10% MeCN in H_2O to 95% MeCN in H_2O over 13 min. Both MeCN and H_2O contained 0.1% (v/v) formic acid. ^{1}H and 2D NMR spectra were recorded in the inverse-detection probe of a Varian Inova spectrometer (600 MHz for ^{1}H, 150 MHz for ^{13}C) at the Harvard Medical School NMR Facility. ^{13}C NMR spectra were recorded on the same instrument with a broad-band probe. NMR spectra of fluoro-roseobactcide A were recorded at the Princeton Chemistry NMR Facility on a Bruker Avance III 800 MHz spectrometer equipped with a cryoprobe for ^{1}H and ^{19}F measurements, and a Bruker Avance III 500 MHz instrument equipped with a ^{1}H-optimized cryoprobe for gCOSY experiments. Spectra were routinely obtained in MeOH-d_4, unless otherwise indicated, and the chemical shifts were referenced to the residual solvent peak(s).

Synthesis of isotopically-labeled precursors. Isotopically-labeled *p*-coumaric acid,[2] phenyl-pyruvic acid,[3] and phenylglyoxylic acid[4] were synthesized as previously described with minor modifications.

2-^{13}C-p-coumaric acid and ring-$^{2}H_4$-p-coumaric acid. To a 50 mL pear-shaped flask fitted with a stir bar were added 4.5 mL of acetic acid, 9 mmol of 4-hydroxybenzaldehye (1.1 g), and 9 mmol of morpholine (0.8 g). The mixture was stirred for 10 minutes before supplementing with 9 mmol of malonic acid (0.93 g). The reaction was refluxed for 1.5 h at 105°C, then cooled by stirring in ice-cold water for 15 min. To precipitate the product, the mixture was poured into 12 mL of ice-cold water, incubated on ice for 30 min, and then filtered. The residue was washed twice with a small amount of water and dried on the filter providing pure *p*-coumaric acid with a yield of 54%. 2-^{13}C-malonic acid or ring-$^{2}H_4$-hydroxybenzaldehyde was used to generate the desired isotopically-labeled *p*-coumaric acids, which were characterized by HPLC-MS using authentic standards, NMR, and HR-MS, as described above. Authentic pCA: HPLC-MS R_t = 8.0 min, λ_{max} = 310 nm, LR-ESI-MS $[M+H]^+_{expt}$ = 165.1, $[M+H]^+_{calc}$ = 165.1. 2-^{13}C-pCA: HPLC-MS R_t = 8.0 min, λ_{max} = 310 nm, LR-ESI-MS $[M+H]^+_{expt}$ = 166.1, $[M+H]^+_{calc}$ = 166.1; HR-MS $[M+H]^+_{expt}$ = 166.0577, $[M+H]^+_{calc}$ = 166.0585. Ring-$^{2}H_4$-pCA: HPLC-MS R_t = 7.9 min, λ_{max} = 310 nm, LR-ESI-MS $[M+H]^+_{expt}$ = 169.1; $[M+H]^+_{calc}$ = 169.1, HR-MS $[M+H]^+_{expt}$ = 169.0795, $[M+H]^+_{calc}$ = 169.0803; ^{1}H NMR (600 MHz, acetone-d_6) δ = 6.37 (d, 1H, C2-^{1}H, 15.9 Hz), δ = 7.65 (d, 1H, C3-^{1}H, 15.9 Hz).

Ring-$^{2}H_5$-phenylpyruvic acid. To a 50 mL polypropylene Falcon tube equipped with a stir bar were added 10 mL of glycine buffer (0.1 M glycine, pH 10.8), 165 μmol of ring-$^{2}H_5$-L-phenylalanine, 95 μmol of cofactor NAD and 12 U of phenylalanine dehydrogenase (~2 mg). The reaction was stirred overnight at room temperature and reaction progress monitored by TLC using a solvent system of MeCN/H_2O (4:1). After overnight incubation, the reaction was loaded onto a silica gel column (d=1.25 cm, l=18 cm), which had been equilibrated in EtOAc/MeOH/formic acid (98:1:1). The mixture was resolved isocratically and fractions containing ring-$^{2}H_5$-phenyl-pyruvic acid, as judged by HPLC-MS analysis, were pooled and concentrated in vacuo. The product was obtained in a 25% yield and was characterized by HPLC-MS using an authentic standard (phenylpyruvic acid) and by HR-MS. Authentic phenylpyruvic acid: HPLC-MS R_t = 11.9 min, λ_{max} = 289 nm, LR-ESI-MS $[M-H]^-_{expt}$ = 163.0, $[M-H]^-_{calc}$ = 163.0. Ring-$^{2}H_5$-phenylpyruvic acid: HPLC-MS R_t = 11.9 min, λ_{max} = 290 nm, LR-

ESI-MS $[M–H]^-_{expt}$ = 168.0, $[M–H]^-_{calc}$ = 168.0; HR-MS $[M–H]^-_{expt}$ = 168.0726, $[M–H]^-_{calc}$ = 168.0709.

1,2-$^{13}C_2$-phenylglycoxylic acid and ring-$^2H_5$-phenylglyoxylic acid. To a 50 mL pear-shaped flask fitted with a stir bar were added 8 mL of xylenes, 1.45 mmol of isotopically-labeled phenylacetic acid, and 1.6 mmol of SeO_2. The reaction was refluxed for 5 h at 155°C, cooled, and filtered to remove excess SeO_2. The solvent was azeotroped with water (2:3 water/xylenes by weight) and evaporated in vacuo. The resulting mixture was dissolved in a small volume of MeOH, supplemented with 1.5 g of silica gel and dry-loaded onto a silica gel column (20g, d=1.25 cm, l=30 cm) that had been equilibrated in CH_2Cl_2/MeOH/formic acid (95:5:0.5). The column was resolved isocratically in the same solvent, in which phenylacetic acid and phenylglyoxylic acid had R_f values of 0.4 and 0.12, respectively. This procedure afforded the corresponding product in a modest 10-20% yield. Despite the low yields, the procedure provided sufficient amounts of material to carry out the isotope feeding experiments. The isotopically-labeled precursors, 1,2-$^{13}C_2$-phneylacetic acid and ring-$^2H_5$-phenylacetic acid, were easily recovered during the work-up and purification in good yields. HPLC-MS analyses of the synthetic phenylglyoxylic acids and authentic, unlabeled phenylglyoxylic acid, as described above, confirmed the identity of the products. Authentic phenylglyoxylic acid: HPLC-MS R_t = 8.2 min, λ_{max} = 250 nm, 290 nm shoulder, LR-ESI-MS $[M–H]^-_{expt}$ = 149.0 $[M–H]^-_{calc}$ = 149.0. 1,2-$^{13}C_2$-phenylacetic acid: HPLC-MS R_t = 8.1 min, λ_{max} = 250 nm, 290 nm shoulder, LR-ESI-MS $[M–H]^-_{expt}$ = 151.0, $[M–H]^-_{calc}$ = 151.0. Ring-$^2H_5$-phenylglyoxylic acid: HPLC-MS R_t = 8.1 min, λ_{max} = 250 nm, 290 nm shoulder, LR-ESI-MS $[M–H]^-_{expt}$ = 154.0, $[M–H]^-_{calc}$ = 154.0; HR-MS $[M–H]^-_{expt}$ = 154.0559, $[M–H]^-_{calc}$ = 154.0553.

Growth of *P. inhibens* DSM 17395 and purification of roseobacticides. *P. inhibens* DSM 17395 was used throughout this study. This is the same strain used in our previous reports.[5,6] Formerly known as *Phaeobacter gallaeciensis* (BS107), it was recently renamed to *P. inhibens* DSM 17395.[7]

P. inhibens DSM 17395 was routinely cultured in half-strength YTSS media (referred to as YTSS throughout) consisting of the following (per L): 20 g Sigma sea salt, 2 g yeast extract, and 1.25 g tryptone. To initiate production cultures, wild-type *P. inhibens* DSM 17395 on a YTSS agar plate (YTSS containing 1.6% w/v agar) was used to inoculate a 5 mL YTSS overnight

culture in a 14 mL bacterial culture tube. After ~15 h at 30°C and 250 rpm, the culture was diluted 50-fold into a 500 mL Erlenmeyer flask containing 50 mL of YTSS media. Overnight growth of this culture at 30°C and 160 rpm provided the inoculum for large-scale cultures. The 50 mL overnight culture was diluted 50-fold into 4-6 x 4 L flasks containing 400 mL of YTSS medium, 1 mM sinapic acid or 1 mM pCA (in the case of L-Tyr isotopomers), and any of the following isotopically-labeled compounds: 0.4 mM ring-$^{2}H_5$-L-Phe, 0.5 mM ring-$^{2}H_5$-L-Trp, 0.4 mM ring-$^{2}H_4$-L-Tyr, 0.4 mM 2-^{13}C-L-Tyr, 0.4 mM 3-fluoro-DL-tyrosine, 0.4 mM 1,2-^{13}C-phenylacetic acid, or 0.4 mM ring-$^{2}H_5$-phenylacetic acid. When assessing incorporation of pCA into roseobacticides, 4-6 x 4 L Erlenmeyer flasks were used containing 400 mL YTSS and 1 mM ring-$^{2}H_4$-pCA or 1 mM 2-^{13}C-pCA. The large-scale cultures were grown at 160 rpm and 30°C for 3 days. Roseobacticides were isolated as previously described in detail.[5]

The fate of labeled phenylpyruvic acid, phenylglyoxylic acid, phenylglycine, and Cys were assessed by HPLC-MS from small production cultures. In this case, the 50 mL culture in a 500 mL Erlenmeyer flask contained 1 mM sinapic acid and 0.4 mM of either ring-$^{2}H_5$-phenylpyruvic acid, 1,2-^{13}C-phenyglyoxylic acid, ring-$^{2}H_5$-phenylglyoxylic acid, ring-$^{2}H_5$-phenylglycine, or ^{34}S-L-Cys. After 3 days, each culture was extracted once with an equal volume of EtOAc. The organic layer was separated, dried over Na_2SO_4, and evaporated to dryness in a speedvac. The dried residue was typically dissolved in 1 mL of MeOH, filtered, and 10-30 μL were subsequently analyzed on an Agilent low-resolution HPLC-ESI-MS and/or an Agilent Accurate Mass Q-tof HPLC-MS system, as described above.

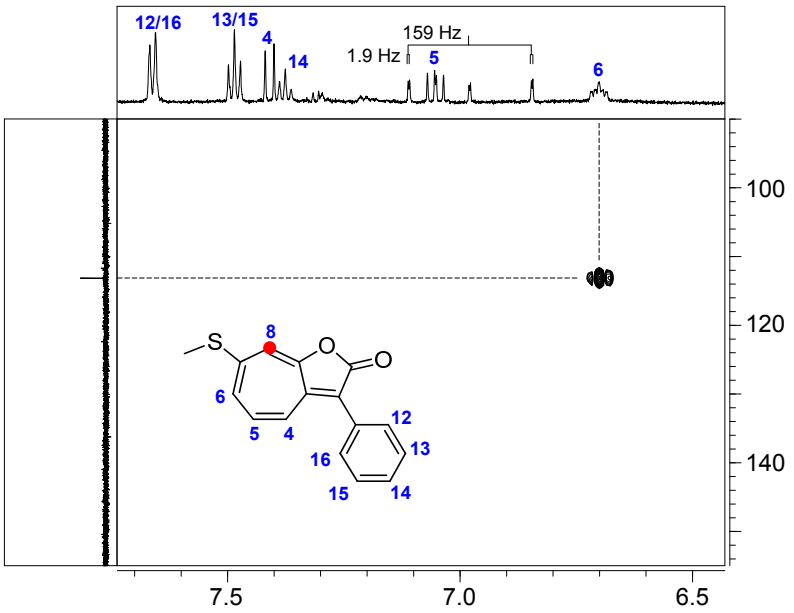


Figure S1. HMBC spectrum of roseobacticide B isolated from production cultures of *P. inhibens* DSM 17395 containing 0.4 mM 1,2-$^{13}C_2$-phenylacetic acid and 1 mM sinapic acid. The ^{1}H, ^{13}C, and HSQC spectra in Fig. 2 show that one ^{13}C is incorporated at position 8. The HMBC spectrum above further corroborates this conclusion by demonstrating a strong correlation between H6 and C8. The structure and numbering of roseobacticide B with a single ^{13}C at position 8 (red circle) is shown.

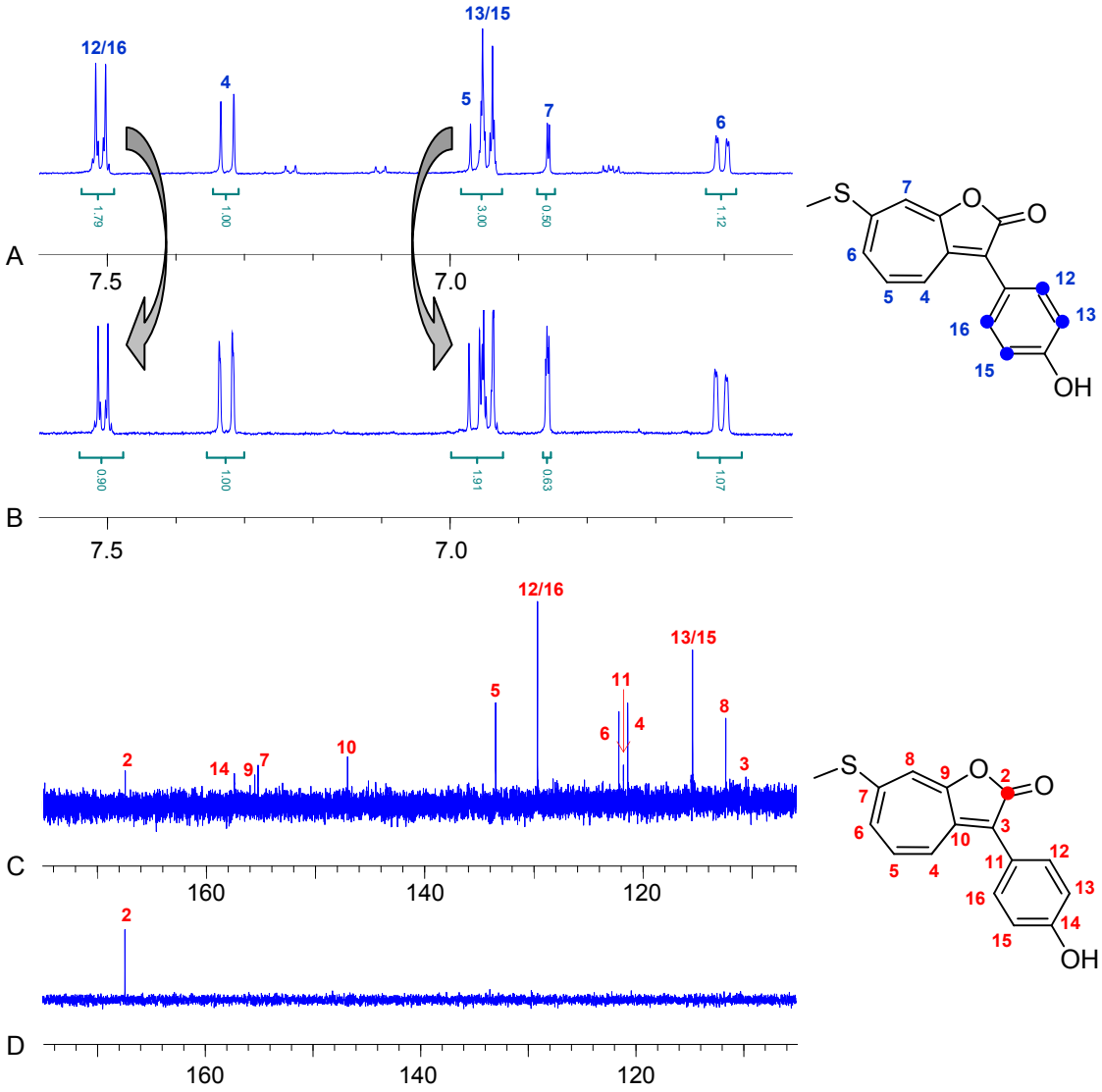


Figure S2. ^{1}H and ^{13}C spectra of roseobacticide A isolated from production cultures of *P. inhibens* DSM 17395 containing ring-$^{2}H_4$-L-tyrosine/pCA and 2-^{13}C-L-tyrosine/pCA, respectively. (A, B) ^{1}H NMR spectrum of authentic roseobacticide A (A) and of roseobacticide A isolated from cultures containing ring-$^{2}H_4$-L-tyrosine (B). The arrows indicate a reduction in the integral of the phenol aromatic peaks consistent with ^{2}H incorporation. See also Table S1 for HR-ESI-MS data, which further corroborate this conclusion. The structure and numbering of ring-$^{2}H_4$-roseobacticide A is shown. (C, D) ^{13}C NMR spectrum of authentic roseobacticide A (C) and of roseobacticide A isolated from cultures containing 2-^{13}C-L-tyrosine (D). Incorporation of ^{13}C into the carbonyl C of roseobacticide A is observed. The structure and numbering of 2-^{13}C-roseoabacticide A is shown. See Table S1 for HR-MS data.

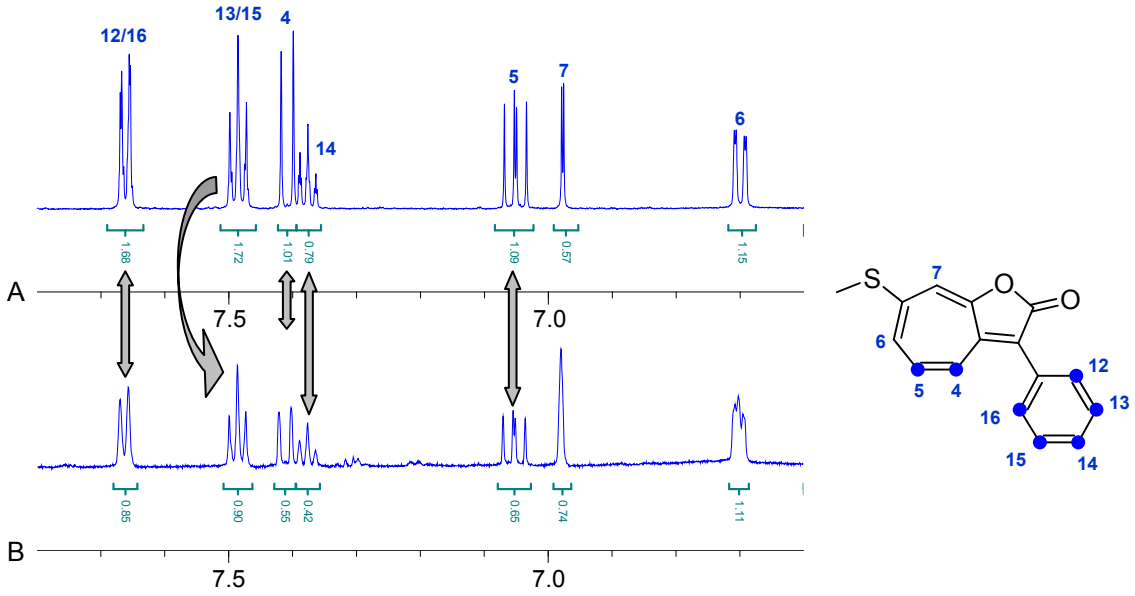


Figure S3. ^{1}H NMR spectrum of roseobacticide B isolated from production cultures of *P. inhibens* DSM 17395 containing ring-$^{2}H_5$-L-phenylalanine and sinapic acid. ^{1}H NMR spectrum of authentic roseobacticide B (A) and of roseobacticide B isolated from cultures containing ring-$^{2}H_5$-L-phenylalanine/sinapic acid (B). The arrows indicate a reduction in the integral of the phenyl aromatic peaks and the protons at positions 4 and 5 consistent with ^{2}H incorporation. The structure of the $^{2}H_7$-roseobacticide B isotopomer is shown. See also Table S1 for HR-ESI-MS data.

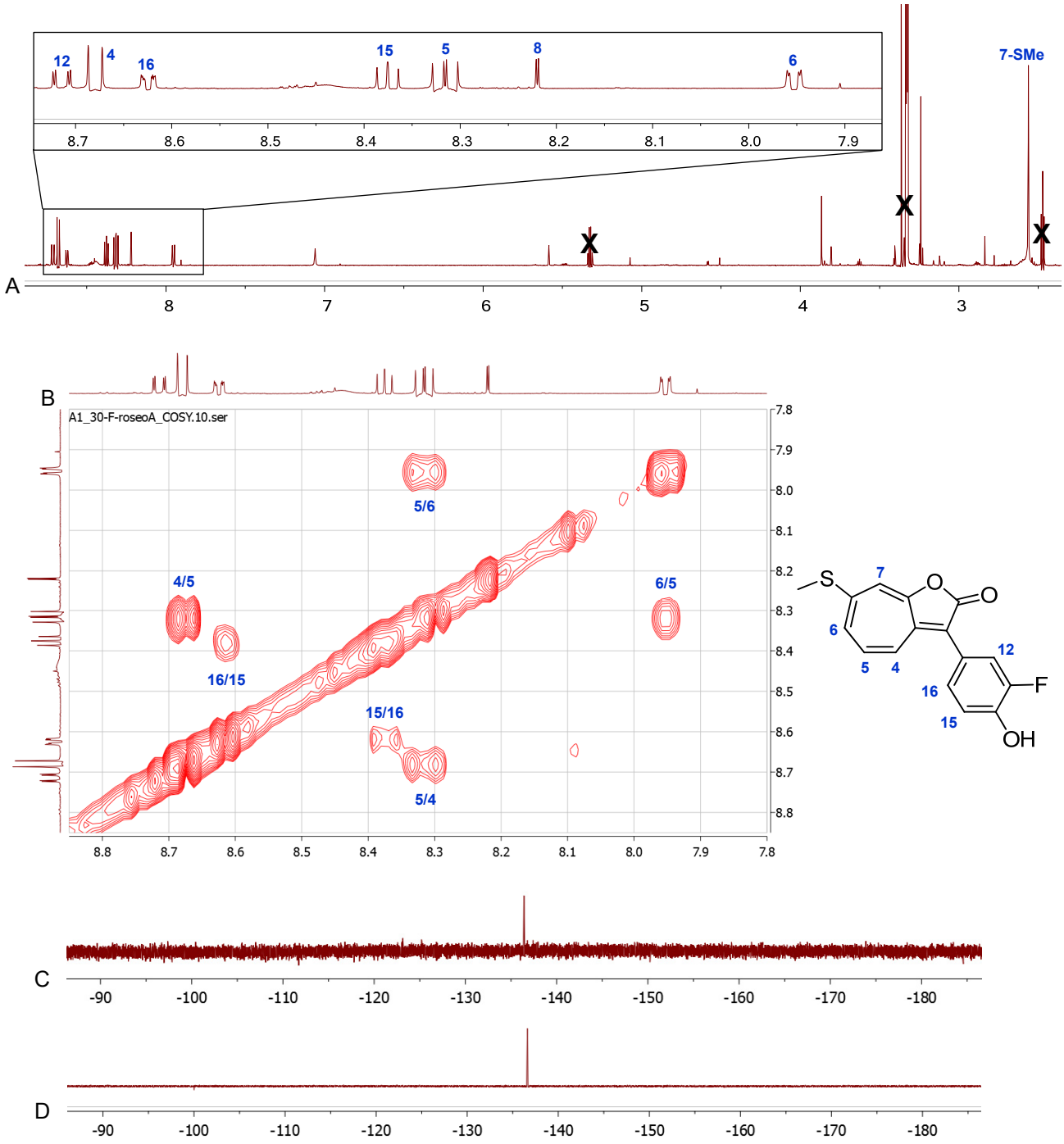


Figure S4. NMR characterization of 13-F-roseobacticide A. (A, B) ^{1}H (A) and gCOSY (B) NMR spectra of pure 13-F-roseobacticide A generated by culturing *P. inhibens* DSM 17395 in the presence of 3-fluoro-DL-tyrosine and pCA. The inset in panel (A) shows an enlarged view of the downfield region of the ^{1}H NMR spectrum. The observed peaks are assigned and the structure and numbering of 13-F-roseobacticide A is shown. (C, D) ^{19}F NMR spectra of pure 13-F-roseobacticide A (C) and 3-fluoro-DL-tyrosine (D). Both spectra contain a single peak at –136 ppm. See Table S1 for HR-MS data.

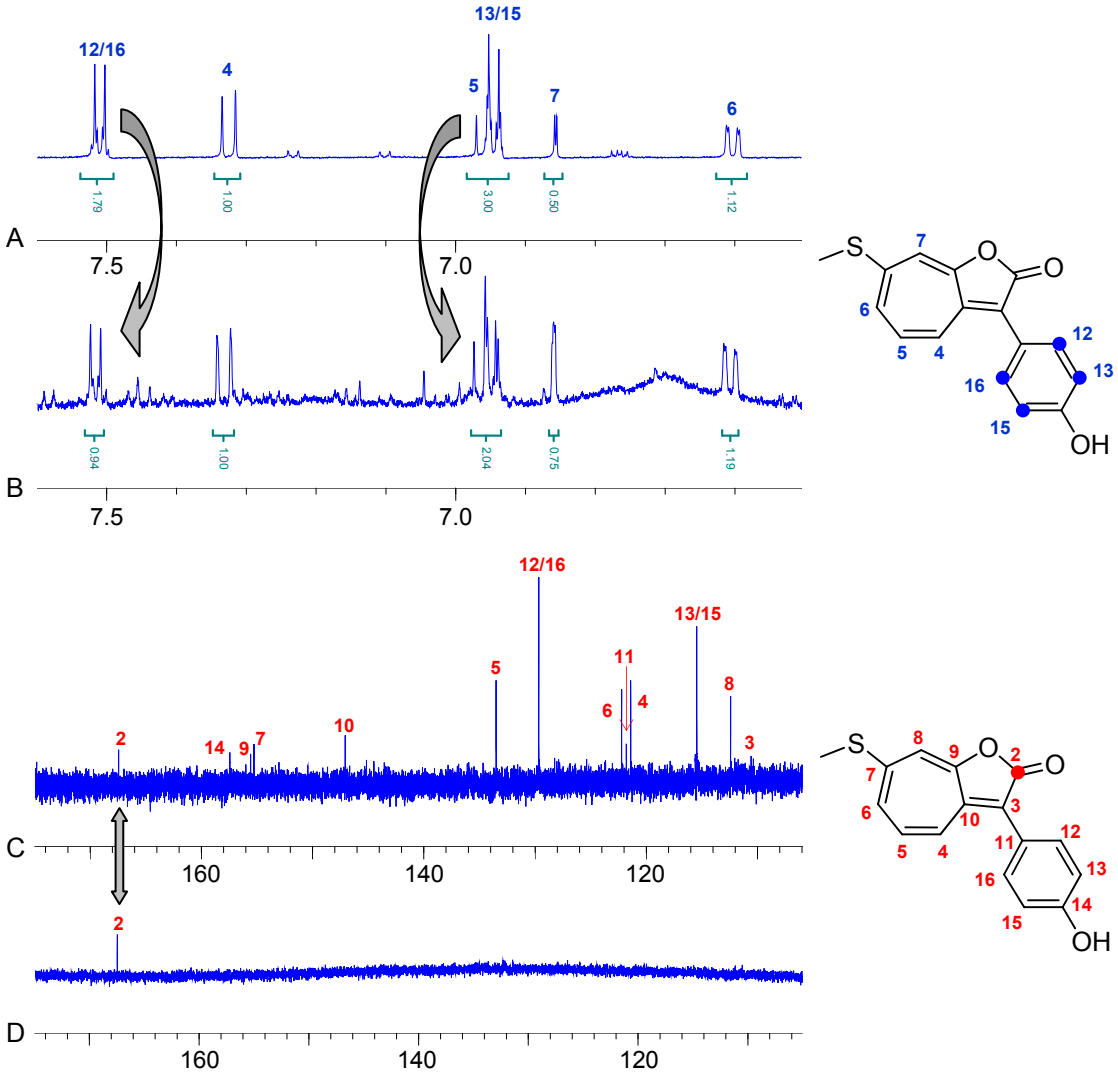


Figure S5. ^{1}H and ^{13}C spectra of roseobacticide A isolated from production cultures of *P. inhibens* DSM 17395 containing ring-$^{2}H_4$-pCA or 2-^{13}C-pCA, respectively. (A, B) ^{1}H NMR spectrum of authentic roseobacticide A (A) and of roseobacticide A isolated from cultures containing ring-$^{2}H_4$-pCA (B). The arrows indicate a reduction in the integral of the phenol aromatic peaks consistent with incorporation of ^{2}H at these positions. See also Table S1 for HR-ESI-MS data, which further corroborate this conclusion. The structure and numbering of $^{2}H_4$-roseobacticide A is shown. (C, D) ^{13}C NMR spectrum of authentic roseobacticide A (C) and of roseobacticide A isolated from cultures containing 2-^{13}C-pCA (D). Incorporation of ^{13}C into the carbonyl C of roseobacticide A is observed. The structure and numbering of 2-13C-roseobacticide A is shown. See Table S1 for HR-MS data.

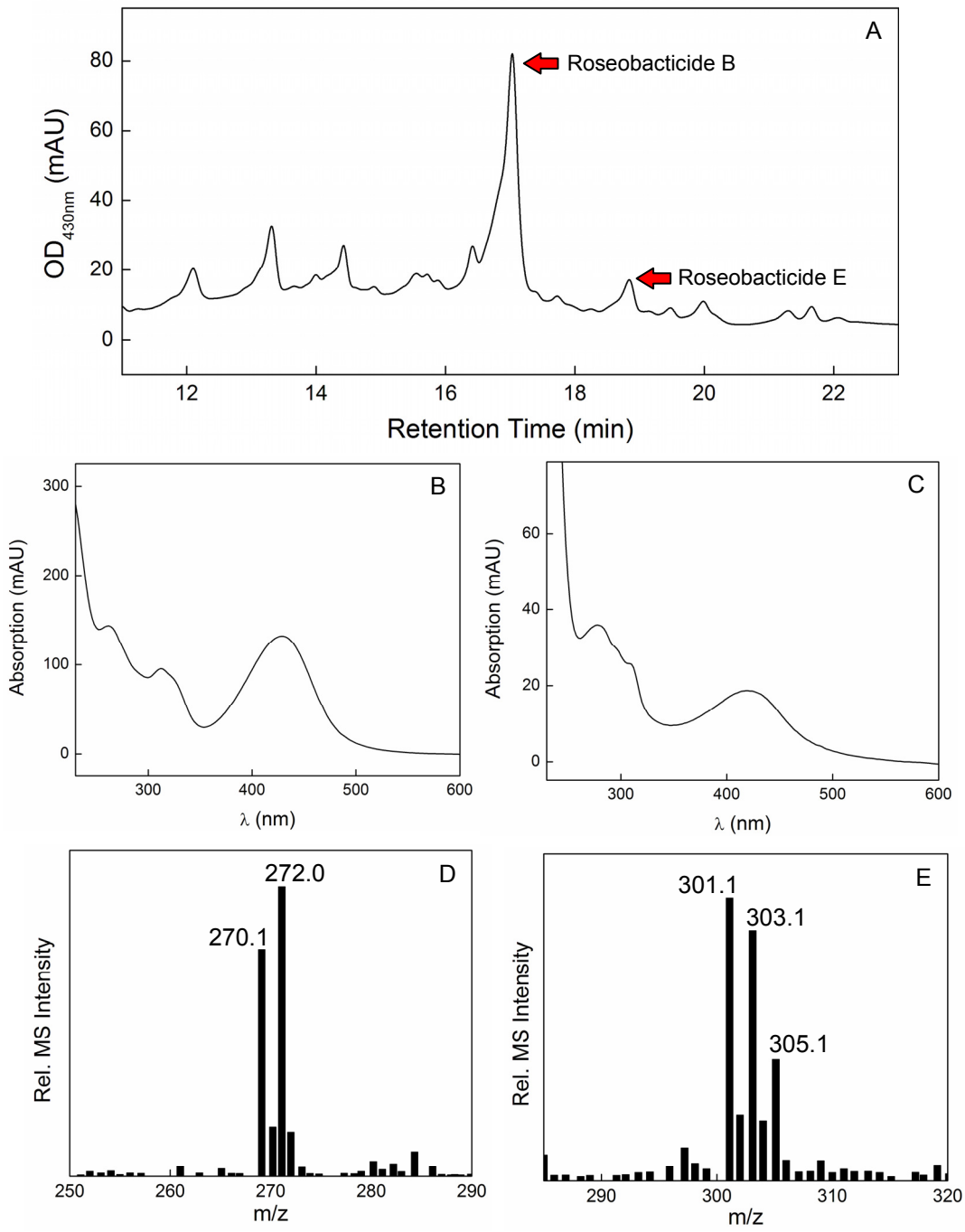
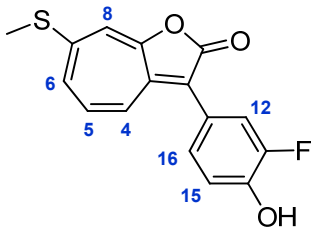


Figure S6. HPLC-ESI-MS analysis of ^{34}S-roseobacticide B and $^{34}S_2$-roseobacticide E. (A) Analysis of *P. inhibens* DSM 17395 cultures grown in the presence of ^{34}S-Cys and sinapic acid. The peaks corresponding to roseobacticides B and E, as compared with standards, are marked. The UV-vis and mass spectra of these two peaks are displayed (B-E). The UV-vis spectra of roseobacticide B (B) and roseobacticide E (C) show the signature features typical for these two analogs. The mass spectrum of roseobacticide B (D) shows both ^{32}S- and ^{34}S-bearing isotopomers. See Table S1 for HR-MS data. The mass spectrum of roseobacticide E shows a mixture of ^{32}S-, ^{34}S, and $^{34}S_2$-bearing roseobacticide E isotopomers.

Table S1. HR-ESI-MS data for roseobacticide isotopomers and analogs.

Experiment	Roseobacticide isotopomer/analog	$[M+H]^+_{calc}$	$[M+H]^+_{expt}$	Δppm	Molecular Formula
1,2-$^{13}C_2$-phenylacetic acid, sinapic acid	B	270.0670	270.0673	1.1	$C_{15}(^{13}C)H_{13}O_2S$
Ring-$^2H_5$-phenylacetic acid, sinapic acid	B	271.0762	271.0745	2.5	$C_{16}H_{11}(^2H)_2O_2S$
Ring-$^2H_5$-indoleacetic acid	C	308.0745	308.0747	0.7	$C_{18}H_{14}NO_2S$
Ring-$^2H_5$-L-tryptophan, sinapic acid	C	313.1059	313.1063	1.3	$C_{18}H_9(^2H)_5NO_2S$
Ring-$^2H_4$-L-tyrosine, pCA	A	289.0836	289.0838	0.7	$C_{16}H_9(^2H)_4O_3S$
2-^{13}C-L-tyrosine, pCA	A	286.0619	286.0620	0.3	$C_{15}(^{13}C)H_{13}O_3S$
Ring-$^2H_5$-L-phenylalanine, sinapic acid	B	271.0762	271.0764	0.7	$C_{16}H_{11}(^2H)_2O_2S$
		274.0950	274.0953	1.1	$C_{16}H_8(^2H)_5O_2S$
		276.1076	276.1078	0.7	$C_{16}H_6(^2H)_7O_2S$
Ring-$^2H_4$-pCA	A	289.0836	289.0841	1.7	$C_{16}H_9(^2H)_4O_3S$
Ring-$^2H_4$-pCA	D	321.0557	321.0557	0.0	$C_{16}H_9(^2H)_4O_3S_2$
2-^{13}C-pCA	A	286.0619	286.0623	1.4	$C_{15}(^{13}C)H_{13}O_3S$
3-F-DL-tyrosine, pCA	A	303.0491	303.0490	0.3	$C_{16}H_{12}FO_3S$
3-F-DL-tyrosine, pCA	D	335.0212	335.0207	1.5	$C_{16}H_{12}FO_3S_2$
Ring-$^2H_5$-phenylpyruvic acid, sinapic acid	B	274.0950	274.0954	1.5	$C_{16}H_8(^2H)_5O_2S$
Ring-$^2H_5$-phenylglyoxylic acid, sinapic acid	B	269.0636	269.0638	0.7	$C_{16}H_{13}O_2S$
Ring-$^2H_5$-phenylglycine, sinapic acid	B	269.0636	269.0639	1.1	$C_{16}H_{13}O_2S$
^{34}S-L-Cysteine	B	271.0594	271.0592	0.7	$C_{16}H_{13}O_2(^{34}S)$

Table S2. NMR data for 13-F-roseobacticide A.



C/H	δ_H	Integral, multiplicity (Hz)	δ_F	COSY
4	8.68	1H, d (11.7)		H5
5	8.31	1H, dd (9.5, 11.7)		H4, H6
6	7.95	1H, dd (1.9, 9.5)		H5, H8 (weak)
7-SMe	2.55	3H, s		
8	8.22	1H, d (1.9)		H6 (weak)
12	8.71	1H, dd (2.1, 12.4)		F13, H16 (weak)
13		1F, s*	-136.4	H12, H15
15	8.38	1H, dd (8.5, 9.3)		H16, F13
16	8.68	1H, ddd (1.0, 2.1, 8.5)		H12, H15

*^{19}F NMR spectrum collected in ^{1}H-decoupled mode

Supporting Information References

1- Brock, N. L.; Nikolay, A.; Dickschat, J. S. *Chem. Commun.* **2014**, *50*, 5487.

2- Robbins, R. J.; Schmidt, W. F. *J. Label. Compd. Radiopharm.* **2004**, *47*, 797.

3- Skowera, K.; Kanska, M. *J. Label. Compd. Radiopharm.* **2008**, *51*, 321.

4- Murata, S.; Sugiyama, K.; Tomioka, J. *J. Org. Chem.* **1993**, *58*, 1976.

5- Seyedsayamdost, M. R.; Carr, G.; Kolter, R.; Clardy, J. *J. Am. Chem. Soc.* **2011**, *133*, 18343.

6- Seyedsayamdost, M. R.; Case, R. J.; Kolter, R.; Clardy, J. *Nat. Chem.* **2011**, *3*, 331.

7- Buddruhs, N.; Pradella, S.; Göker, M.; Päuker, O.; Pukall, R.; Spröer, C.; Schumann, P.; Peterson, J.; Brinkhoff, T. *Int. J. Syst. Evol. Microbiol.* **2013**, *63*, 4340.

Complete citation for references (9) and (16) in the paper:

(9) Holligan, P. M.; Fernandez, E.; Aiken, J.; Balch, W. M.; Boyd, P; Burkill, P. H.; Finch, M.; Groom, S. B.; Malin, G.; Muller, K.; Purdie, D. A.; Robinson, C.; Trees, C. C.; Turner, S. M.; van der Wal, P. *Global Biogeochem. Cycles* **1993**, *7*, 879.

(16) Wagner-Döbler, I.; Ballhausen, B.; Berger, M.; Brinkhoff, T.; Buchholz, I.; Bunk, B.; Cypionka, H.; Daniel, R.; Drepper, T.; Gerdts, G.; Hahnke, S.; Han, C.; Jahn, D.; Kalhoefer, D.; Kiss, H.; Klenk, H. P.; Kyrpides, N.; Liebl, W.; Liesegang, H.; Meincke, L.; Pati, A.; Petersen, J.; Piekarski, T.; Pommerenke, C.; Pradella, S.; Pukall, R.; Rabus, R.; Stackebrandt, E.; Thole, S.; Thompson, L.; Tielen, P.; Tomasch, J.; von Jan, M.; Wanphrut, N.; Wichels, A.; Zech, H.; Simon, M. *ISME J.* **2010**, *4*, 61.