Supporting Information

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SI Materials and Methods

General. Oligonucleotide primers were synthesized by Sigma-Aldrich. Restriction enzymes were from New England Biolabs. Enzyme concentration was measured using the Coomassie (Bradford) protein assay kit (Thermo Scientific) using BSA as standard. DNA purification was performed using Qiagen kits. DNA sequencing to confirm accuracy of plasmid inserts was routinely performed by Beckman Coulter Genomics. Nickel–nitrilotriacetic acid–agarose (Ni-NTA) resin was purchased from Qiagen. SDS/PAGE and agarose gels were from Invitrogen. *Escherichia coli* TOP10 cells (Invitrogen) were used for routine cloning.

Construction of Gene Inactivation Plasmids. ***Dioxygenase gene fr9P.*** Two ∼700-bp-long DNA fragments upstream and downstream of the point of gene replacement were amplified by PCR (Pfu Ultra Polymerase; Promega) using FERM BP-3421 genomic DNA as template and primer pairs P1_diox (TGG CGA ACA GAT CGA GTT TG) and P2_diox (CTT GCG GAG AAC TGT GAA TGC GCA ATA GAA GCG CTG TCA TGG AAT G), and P3_diox (CCG AAA AGT GCC ACC TGA CGT CTA AGA TAA CTC GTG GAT ATT CGG CAA G) and P4_diox (AGA ATC CCG CGA TCC CAA C), respectively; the underlined bases represent homology regions to the tetracycline resistance (*tet*) marker. The *tet* marker was amplified by PCR using pEX18Tc (1) as template and primer pair Ptet_f (TTG CGC ATT CAC AGT TCT C) and Ptet_r (TCT TAG ACG TCA GGT GGC AC). The three fragments were assembled by SOE-PCR (using Pfu Ultra Polymerase; Promega) and ligated into the SmaI site of pEX100T (2) to generate inactivation plasmid pAE-PF12.

Cytochrome P450 fr9R. Two ∼700-bp-long DNA fragments upstream and downstream of the point of gene replacement were amplified by PCR (Pfu Ultra Polymerase; Promega) using FERM BP-3421 genomic DNA as template and primer pairs P1_P450 (GCA TCC AAT CAC TTG AAC AGG) and P2_P450 (CTT GCG GAG AAC TGT GAA TGC GCA AGC CAT CAT TCT CGA CAT TTC C), and P3_P450 (CCG AAA AGT GCC ACC TGA CGT CTA AGA AGA TTG TGA CGG TAC TGA AGC) and P4_P450 (AGA GAA CGA TCG CTC CAC AG), respectively; the underlined bases represent homology regions to the tetracycline resistance (*tet*) marker. The *tet* marker was amplified by PCR using pEX18Tc (1) as template and primer pair Ptet_f (TTG CGC ATT CAC AGT TCT C) and Ptet_r (TCT TAG ACG TCA GGT GGC AC). The three fragments were assembled by SOE-PCR (using Pfu Ultra Polymerase; Promega) and ligated into the SmaI site of pEX100T (2) to generate inactivation plasmid pAE-PF11.

FMO domain in fr9I. Two ∼700-bp-long DNA fragments upstream and downstream of the point of in-frame deletion were amplified by PCR (Pfu Ultra Polymerase; Promega) using FERM BP-3421 genomic DNA as template and primer pairs P1_OX (GCT CTA GAA CGG TCA ACG AGC ATA TCG AC; XbaI site underlined) and P2_OX (TGT CGC CTG CGA TTC TGA TG), and P3_OX (ATG CCC GAG TAT TTC CGA CG) and P4_OX (ATG GTG AAG CTT AGC AGA CGG TAG CGG ACC AC; HindIII site underlined), respectively. The product of the second reaction was used as template in a PCR (Phusion Hot start polymerase; Finnzymes) with primer pair P3H_OX (CGC ATC AGA ATC GCA GGC GAC AAT GCC CGA GTA TTT CCG ACG; the underlined bases represent homology region to the first fragment obtained with P1_OX and P2_OX). After gel purification, the two fragments were assembled by SOE-PCR (using Phusion Hot start polymerase; Finnzymes) and ligated into the XbaI and HindIII sites of pEX18Tc (1) to generate inactivation plasmid pAE-PF13.

PKS-NRPS fr9DEF module 2. Two ∼700-bp-long DNA fragments upstream and downstream of the point of gene replacement were amplified by PCR (Pfu Ultra Polymerase; Promega) using FERM BP-3421 genomic DNA as template and primer pairs P0175-NRPS-up_f (AAG CTT GCG TGA TCG ACA CGC TGT AC; HindIII site underlined) and P0175-NRPS-up_r (CCC GGG ACA CCA ACC CGC ACG CTG; SmaI site underlined), and P0175-NRPS-down_f (CCC GGG GGT CGA TCG AAC GCA CGA C; SmaI site underlined) and P0175-NRPS-down_r (TCT AGA CTT CCG AAC TTG TCC GCA TG; XbaI site underlined). Each fragment was separately ligated into in pCR2.1 vector (Invitrogen) to give pAE-PF1 and pAE-PF2, respectively. The 0.7-kb SmaI–XbaI fragment of pAE-PF2 was cloned into the same sites of pAE-PF1 to give pAE-PF5. A *tet* marker amplified from pEX18Tc using primers tet-pEX18Tc_f (CCC GGG TTG CGC ATT CAC AGT TCT C; SmaI site underlined) and tet-pEX18Tc_r (CCC GGG TCT TAG ACG TCA GGT GGC AC; SmaI site underlined) was ligated into the SmaI site of pAE-PF5 to give pAE-PF7. A 3.3-kb PvuII fragment of pAE-PF7 was ligated into the SmaI site of pEX100T to give inactivation plasmid pAE-PF9.

PKS fr9DEF module 4. Two ∼700-bp-long DNA fragments upstream and downstream of the point of gene replacement were amplified by PCR (Pfu Ultra Polymerase; Promega) using FERM BP-3421 genomic DNA as template and primer pairs C0646-5KS1-up_f (5′- AAG CTT GCC GCG CTC GAA CCG ATA CT-3′; HindIII site underlined) and C0646-5KS1-up_r (5′-CCC GGG GTT CGG CTC GGT TCG GAT TC-3′; SmaI site underlined), and C0646-5KS1-down_f (5′-CCC GGG CTG CCG TAT GGG ATC GGT AG-3′; SmaI site underlined) and C0646-5KS1-down_r (5′-TCT AGA CAC TTC CGG TTC ATC GGC TTC-3′; XbaI site underlined). Each fragment was separately ligated into in pCR2.1 vector (Invitrogen) to give pJEJ-PF1 and pJEJ-PF2, respectively. The 0.7-kb SmaI–XbaI fragment of pJEJ-PF2 was cloned into the same sites of pJEJ-PF1 to give pJEJ-PF5. A *tet* marker amplified from pEX18Tc using primers tet-pEX18Tc_f and r (see above) was ligated into the SmaI site of pJEJ-PF5 to give pJEJ-PF7. A 2.9-kb PvuII fragment of pJEJ-PF7 was ligated into the SmaI site of pEX100T to give inactivation plasmid pJEJ-PF9.

PKS fr9DEF module 5 through fr9GH module 6. Two ∼840-bp-long DNA fragments upstream and downstream of the point of gene replacement were amplified by PCR (Pfu Ultra Polymerase; Promega) using FERM BP-3421 genomic DNA as template and primer pairs C0397-12KS1-up_f (5′-AAG CTT ATC TCG ACA TCT TTG GGC AGG T-3′; HindIII site underlined) and C0397-12KS1-up_r (5′-CCC GGG CCG CAT CGG CCA GAT AGA AG-3′; SmaI site underlined), and C0397-12KS1-down_f (5′-CCC GGG TCT ACG AGC ACA CGA CAA TC-3′; SmaI site underlined) and C0397-12KS1-down_r (5′-TCT AGA AAC TGC GGC AAC GTC CTT C-3′; XbaI site underlined). Each fragment was separately ligated into in pCR2.1 vector (Invitrogen) to give pJEJ-PF3 and pJEJ-PF4, respectively. The 0.84-kb SmaI–XbaI fragment of pJEJ-PF4 was cloned into the same sites of pJEJ-PF3 to give pJEJ-PF6. A *tet* marker amplified from pEX18Tc using primers tet-pEX18Tc_f and r (see above) was ligated into the SmaI site of pJEJ-PF6 to give pJEJ-PF8. A 3.6-kb PvuII fragment of pJEJ-PF8 was ligated into the SmaI site of pEX100T to give inactivation plasmid pJEJ-PF10.

HMGS-like gene fr9K. Two ∼1-kb-long DNA fragments upstream and downstream of the point of gene replacement were amplified by PCR (Pfu Ultra Polymerase; Promega) using FERM BP-3421 genomic DNA as template and primer pairs P1181-HMGS-up_f (AAG CTT TGA GGC AGC ATC TCG AAG TG; HindIII site underlined) and P1181-HMGS-up_r (CCC GGG TCT CTG GAA ATT CAT ACG GAG; SmaI site underlined), and P1181-HMGS-down_f (CCC GGG TGA CGA TGG CTG AAT ACG ATG; SmaI site underlined) and P1181-HMGS-down_r (TCT AGA ATT GTG CGC CAC GTC ATG TC; XbaI site underlined). Each fragment was separately ligated into in pCR2.1 vector (Invitrogen) to give pAE-PF3 and pAE-PF4, respectively. The 1-kb HindIII–SmaI fragment of pAE-PF3 was cloned into the same sites of pAE-PF4 to give pAE-PF6. A *tet* marker amplified from pEX18Tc using primers tet-pEX18Tc_f and r (see above) was ligated into the SmaI site of pAE-PF6 to give pAE-PF8. A 4-kb PvuII fragment of pAE-PF8 was ligated into the SmaI site of pEX100T to give inactivation plasmid pAE-PF10.

Dioxygenase gene tstP. A 578-bp DNA fragment upstream of the point of in-frame deletion was amplified by PCR (Phusion Hot Start high fidelity polymerase; Finnzymes) using MSMB 43 genomic DNA as template and primer pair MSMB43-diox-del-up-F2 (5′-GCT CTA GAT CGC ATT TCA TAT CCC GCA AC-3′; XbaI site underlined) and MSMB43-diox-del-up-R2 (5′-ATT CTC GGC TCG TTC CGT ACT TCC-3′). A 644-bp fragment downstream of the point of in-frame deletion was amplified by PCR with primer pair MSMB43-diox-del-down-F (5′-AAT CAA CAC GAC GCC ATA GC-3′) and MSMB43-diox-del-down-R (5′-ACG GTA AGC TTA ACA CGC TTG ATA ACG CAG C-3′; HindIII site underlined). The product of the second reaction was used as template in PCR (Phusion Hot Start high-fidelity polymerase; Finnzymes) with primer pair MSMB43-diox-del-down-Fhom2 (CAT GGA AGT ACG GAA CGA GCC GAG AAT AAT CAA CAC GAC GCC ATA GC; the underlined bases represent homologous region to the first fragment obtained with MSMB43-diox-del-up-F2 and R2) and MSMB43-diox-del-down-R. After gel purification, the two fragments were assembled by SOE-PCR (Phusion Hot Start high-fidelity polymerase; Finnzymes) and ligated into the XbaI and HindIII sites of pEX18Tc (1) to generate inactivation plasmid pJEJ-PF11.

Isolation and Structure Elucidation of Spliceostatin Analog (7) from the cytochrome P450 *fr9R*$^{-}$ Mutant. A volume of 0.4 L of a 5-d-old production culture (2S4G medium) of the *fr9R*$^{-}$ mutant was centrifuged for 30 min at 5,000 × *g* to remove cells. Fifty grams of wet DIAION HP-20 resin was added to the supernatant, i.e., 12.5% (wt/vol), and the mixture was shaken at 200 rpm for 1 h. The compound-bound HP-20 was collected by centrifugation and then extracted twice with ethyl acetate (250 mL for each extraction). After drying the combined extracts with $MgSO_4$ (which was then removed by filtration with Whatman paper), the solvent was removed by evaporation under reduced pressure to afford a light yellow crude extract. One-half of the crude extract was purified by preparative, normal-phase HPLC [column: Princeton SFC 2-ethylpyridine, 250 × 21.2 mm, 5 μm; mobile phase A: heptane; mobile phase B: ethanol (denatured); gradient: 5% B for 1.5 min, to 100% B over 8.5 min, 100% B for 2 min, to 5% B over 0.5 min and 5% B for 2.5 min; flow rate: 27 mL/min]. The fractions with retention times of 6.58 and 8.18 min were collected and freeze-dried to afford **7** (163 mg, 89% pure as a very light, yellowish powder), and **2** (205 mg, 89% pure by UV), respectively. One-half of the 6.58-min fraction was purified by reverse-phase HPLC (column: Phenomenex Luna C18, 150 × 21.2 mm, 5 μm; mobile phase A: water; mobile phase B: acetonitrile; gradient: 20% B for 1.5 min, to 70% B over 8.5 min, to 100% B over 2 min, to 20% B over 0.5 min; flow rate: 27 mL/min). The fraction with retention time of 8.25 min was collected and freeze-dried to afford **7** (28 mg, 98.5% pure by UV) as a white powder. High-resolution electrospray ionization mass spectrometry (HRESIMS), *m/z* 492.296 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6, mult, *J* in Hz) δ 7.78 (d, *J* = 8.0 Hz, 1H), 6.35 (m, 1H), 6.21 (d, *J* = 15.8, 1H), 6.11 (dd, *J* = 0.9, 11.7, 1H), 5.85 (dd, *J* = 11.6, 7.5 Hz, 1H), 5.53 (m, 1H), 5.49 (m, 1H), 5.41 (d, *J* = 1.6 Hz, OH), 4.64 (m, 1H), 3.65 (m, 1H), 3.64 (m, 1H), 3.49 (m, 1H), 2.45 (m, 2H), 2.30 (m, 1H), 2.20 (m, 1H), 1.98 (s, 3H), 1.96 (m, 1H), 1.81 (m, 3H), 1.69 (s, 3H), 1.65 (m, 1H), 1.31 (s, 3H), 1.25 (m, 1H), 1.25 (d, *J* = 6.3 Hz, 3H), 1.14 (m, 1H), 1.07 (d, *J* = 6.5 Hz, 3H), 0.95 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 196.6, 146.6, 142.1, 134.1, 133.7, 128.2, 127.1, 122.6, 95.3, 79.5, 74.3, 67.6, 66.7, 54.5, 48.4, 46.1, 41.4, 37.5, 35.0, 31.1, 29.6, 28.5, 20.8, 19.5, 17.8, 13.9, 12.2.

Enzymatic Synthesis of 9. α-Ketoglutarate (0.8 mM final concentration, 2 eq), sodium ascorbate (0.08 mM, 0.2 eq), $NH_4Fe(II)SO_4$ (0.04 mM, 0.1 eq), and recombinant Fr9P (1.2 μM, 0.003 eq) were added to an aqueous solution of **2** (1 mg, 0.4 mM, 1 eq) in 50 mM 4-morpholinepropanesulfonic acid (MOPS) buffer, pH 7.5. After incubation at room temperature (RT) for 2 h, the reaction was acidified to pH ∼4–5 with acetic acid and extracted three times with equal volume of ethylacetate. The solvent was evaporated under reduced pressure, the residue resuspended in 0.25 mL of acetonitrile, filtered, and purified by reverse-phase HPLC [column: Cromolith RP-18e 100–10 mm; mobile phase A: 0.02% acetic acid in water; mobile phase B: 0.02% acetic acid in acetonitrile; gradient: 20–55% B over 30 min, 55–100% B over 4 min, 100–20% B over 2 min and 20% B for 2 min; flow rate: 2.5 mL/min; temperature: not controlled; detection: diode array detector (DAD) 230 nm; instrument: Agilent 1200 analytical HPLC]. The fraction with retention time of 18.5 min was collected and neutralized with ammonium hydroxide before it was concentrated under reduced pressure. The aqueous concentrate was acidified to pH ∼4 with acetic acid and extracted twice with equal volume of ethylacetate. The solvent was removed under reduced pressure to afford **9** as a white solid. Yield: 0.2 mg. HRESIMS, *m/z* 536.286 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6, mult, *J* in Hz) δ 12.24 (brs, OH), 8.00 (d, *J* = 8.0 Hz, 1H), 6.37 (m, 1H), 6.23 (d, *J* = 15.9, 1H), 6.12 (dd, *J* = 0.7, 11.5, 1H), 5.88 (dd, *J* = 11.6, 7.5 Hz, 1H), 5.54 (m, 1H), 5.50 (m, 1H), 4.67 (m, 1H), 3.66 (m, 2H), 3.51 (m, 1H), 2.60 (m, 1H), 2.53 (m, 1H), 2.33 (m, 1H), 2.31 (m, 1H), 2.20 (m, 1H), 2.00 (s, 3H), 1.84 (m, 1H), 1.81 (m, 2H), 1.70 (s, 3H), 1.67 (m, 1H), 1.39 (m, 1H), 1.26 (d, *J* = 6.6 Hz, 3H), 1.16 (m, 1H), 1.08 (d, *J* = 6.4 Hz, 3H), 0.96 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.7, 170.5, 165.0, 142.7, 134.4, 133.3, 128.5, 127.3, 122.6, 95.3, 79.9, 74.8, 67.9, 67.2, 46.7, 46.1, 38.9, 37.7, 35.0, 31.5, 28.7, 20.8, 19.7, 17.6, 14.2, 12.2.

Synthesis and Structure Elucidation of *n*-Propyl Amide Analog 12. Compound **8** (4.3 mg; 0.0080 mmol) was dissolved in dimethylformamide (DMF) (0.05 M), and cooled in an ice bath. 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) (1.4 eq) was added, and the resulting mixture was allowed to stir for 5 min. Then, *N*,*N*-diisopropylethylamine (DIPEA) (1 eq), followed by propylamine (1.5 eq) in DMF were added. The reaction was removed from the ice bath and allowed to stir at RT for 1 h, at which point partial conversion to the product was observed by liquid chromatography (LC)/MS. HATU (0.7 eq), DIPEA (1 eq), and propylamine (1 eq) were added at RT, and the mixture was stirred for an additional 30 min and analyzed by LC/MS, which showed complete conversion to the product. Total duration of the reaction was 1.5 h. The crude product was diluted with acetonitrile and filtered using a syringe filter before purification by HPLC (column: Cromolith RP-18e 100–10 mm; mobile phase A: water; mobile phase B: acetonitrile; gradient: 30–65% B over 20 min, 65–95% B over 1 min, 95–30% B over 2 min; flow rate: 2.5 mL/min; temperature: not controlled;

detection: DAD 230 nm; injection volume: variable, 0.025–0.1 mL; instrument: Agilent 1200 analytical HPLC; retention time: 8.60 min). The fraction containing the product was immediately frozen and lyophilized to afford **12** as a white solid (2.3 mg; yield, 49%). LC/MS, *m/z* 593 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6, mult, *J* in Hz) δ 8.01 (dd, *J* = 5.6, 5.6 Hz, 1H), 7.81 (d, *J* = 8.0 Hz, 1H), 6.36 (m, 1H), 6.24 (d, *J* = 16.1 Hz, 1H), 6.11 (dd, *J* = 12.1 Hz, 1H), 5.88 (dd, *J* = 11.7, 7.7 Hz, 1H), 5.67 (dd, 16.1, 4.4 Hz, 1H), 5.44 (m, 1H), 4.55 (d, 8.7 Hz, 1H), 4.33 (m, 1H), 3.65 (m, 1H), 3.64 (m, 1H), 3.48 (m, 1H), 3.33 (m, 1H), 3.07 (m, 1H), 3.00 (m, 1H), 2.73 (m, 1H), 2.39 (m, 2H), 2.31 (m, 1H), 2.29 (m, 1H), 2.27 (m, 1H), 2.19 (m, 1H), 1.98 (s, 3H), 1.80 (m, 2H), 1.69 (s, 3H), 1.64 (m, 1H), 1.49 (m, 1H), 1.41 (m, 2H), 1.25 (d, *J* = 6.3 Hz, 3H), 1.07 (d, *J* = 6.3 Hz, 3H), 0.95 (d, *J* = 7.2 Hz, 3H), 0.84 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.4, 169.6, 164.7, 143.4, 135.5, 134.0, 128.9, 126.3, 123.4, 95.7, 80.6, 75.4, 71.4, 68.4 (×2), 56.7, 46.7, 46.2, 45.9, 41.3, 40.6, 35.6, 32.1, 29.1, 22.8, 21.4, 20.2, 18.1, 14.6, 12.8, 11.8.

Synthesis and Structure Elucidation of *n*-Propyl Amide Analog 13. Compound **9** (4.0 mg; 0.0075 mmol) was dissolved in DMF (0.05 M), and cooled in an ice bath. HATU (1.4 eq) was added, and the mixture was allowed to stir for 5 min. DIPEA (1 eq), followed by propylamine (1.5 eq) in DMF were added. The reaction was removed the ice bath and allowed to stir at RT for 2 h. LC/MS analysis at 2-h time point indicated completion of the reaction. The crude product was diluted with acetonitrile and filtered using a syringe filter before purification by HPLC (column: Cromolith RP-18e 100–10 mm; mobile phase A: water; mobile phase B: acetonitrile; gradient: 30–65% B over 20 min, 65–95% B over 1 min, 95–30% B over 2 min; flow rate: 2.5 mL/min; temperature: not controlled; detection: DAD 230 nm; injection volume: variable, 0.025–0.1 mL; instrument: Agilent 1200 analytical HPLC; retention time: 10.8 min). The fraction containing the product was immediately frozen and lyophilized to afford **13** as a white solid (2.4 mg; yield, 60%). LC/MS, *m/z* 577 [M+H]+; ^{1}H NMR (400 MHz, DMSO-d_6, mult, *J* in Hz) δ 7.99 (dd, *J* = 5.5, 5.5 Hz, 1H), 7.80 (d, *J* = 8.0 Hz, 1H), 6.36 (m, 1H), 6.21 (d, *J* = 15.7 Hz, 1H), 6.11 (dd, *J* = 11.7, 0.9 Hz, 1H), 5.86 (dd, *J* = 11.6, 7.6 Hz, 1H), 5.53 (dd, 15.7, 6.0 Hz, 1H), 5.47 (m, 1H), 4.65 (m, 1H), 3.65 (m, 1H), 3.64 (m, 1H), 3.48 (m, 1H), 3.05 (m, 1H), 3.01 (m, 1H), 2.47 (m, 2H), 2.40 (m, 2H), 2.29 (m, 1H), 2.19 (m, 1H), 2.11 (m, 1H), 1.98 (s, 3H), 1.80 (m, 3H), 1.68 (s, 3H), 1.64 (m, 1H), 1.41 (m, 2H), 1.29 (m, 1H), 1.25 (d, *J* = 6.4 Hz, 3H), 1.17 (m, 1H), 1.07 (d, *J* = 6.7 Hz, 3H), 0.94 (d, *J* = 7.2 Hz, 3H), 0.84 (dd, *J* = 7.6, 7.6 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.0, 169.7, 164.4, 142.6, 133.9, 133.6, 128.4, 127.3, 122.6, 95.9, 79.7, 74.7, 66.8, 67.7, 54.2, 49.2, 46.0, 45.5, 39.8, 39.7, 37.8, 35.0, 31.4, 28.5, 22.0, 19.5, 17.4, 13.9, 12.6, 12.0, 10.9.

Half-Life Determination of 8 and Structure Elucidation of Degradation Products 10 and 11. **8** was obtained by incubating **3** (0.1 mM) with α-ketoglutarate (0.2 mM), $NH_4Fe(II)SO_4$ (0.01 mM), sodium ascorbate (0.02 mM), and recombinant Fr9P (250 nM) in 50 mM MOPS buffer, pH 7.5, at 30 °C for 1 h and 30 min. After the reaction had come to completion, it was divided into two aliquots, the pH of one of which was adjusted to 6.0; both were incubated at 25 °C and 200 rpm. Aliquots taken over time (2, 18, and 46 h) were quenched with 1 vol of acetonitrile, centrifuged, filtered, and analyzed by HPLC and LC/MS. See Fig. S6 for half-life calculation plots. For isolation of degradation products **10** and **11**, 4 mg of **8** was obtained as above (with 0.4 mM substrate, 20-mL reaction volume) and then incubated at 25 °C, 200 rpm for 3 d. The mixture was extracted three times with equal volume ethylacetate, and the extract dried under reduced pressure. The dried extract (5 mg) was resuspended in 0.25 mL of acetonitrile, filtered, and purified by reverse phase HPLC [C18 semiprep column YMC-Pack ODS-A 250 × 10 mm (S-5 μm, 12 nm); mobile phase: isocratic 45% aqueous acetonitrile (vol/vol) for 30 min; flow rate: 2.5 mL/min; temperature: not controlled; detection: DAD 230 nm; instrument: Agilent 1200 analytical HPLC]. The fraction with retention time of 20 min was collected and freeze-dried to afford **10** (0.7 mg, 99% pure by UV, UV_{max} = 230 nm) as a white powder. HRESIMS, *m/z* 490.280 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6, mult, *J* in Hz) δ 7.81 (d, *J* = 8.0, 1H), 7.27 (s, 1H), 6.36 (m, 1H), 6.17 (d, *J* = 15.6, 1H), 6.03 (s, 1H), 6.11 (dd, *J* = 1.3, 11.8, 1H), 5.86 (dd, *J* = 7.3, 11.8, 1H), 5.63 (dd, *J* = 6.1, 15.6, 1H), 5.44 (m 1H), 4.93 (d, *J* = 5.3, OH), 4.76 (d, *J* = 5.2, OH), 4.28 (m, 1H), 4.00 (m, 1H), 3.64 (m, 2H), 3.49 (m, 1H), 2.30 (m, 1H), 2.20 (s, 3H), 2.19 (m, 1H), 1.98 (s, 3H), 1.80 (m, 2H), 1.68 (m, 3H), 1.65 (m, 1H), 1.25 (d, *J* = 6.5, 3H), 1.07 (d, *J* = 6.3, 3H), 0.95 (d, *J* = 7.3, 3H). ^{13}C NMR (125 MHz, DMSO) δ 169.7, 164.6, 150.7, 142.7, 137.2, 134.7, 134.0, 128.0 (×2), 127.8, 122.7, 105.8, 79.9, 74.7, 74.6, 69.6, 67.7, 46.0, 35.0, 31.5, 28.6, 20.6, 19.6, 17.6, 14.1, 13.0, 12.3. The fraction with retention time of 24.9 min was collected and freeze-dried to afford **11** (1 mg, 98% pure by UV, UV_{max} = 280 nm) as a white powder. HRESIMS, *m/z* 378.229; error, 1.0 mDa $[M+H]^+$ (3).

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Fr9DEF_DH_4     (1) ----------PVWRFSRRLEGSEPFLRDHRVLGESWLPAVVYWEMTRWS
  Fr9I_DH_9     (1) HPLLHRLIPADRALRFLSTFSGDEPFFRDHRVRSAAMLPGAAYCEMFIAA
  PedF_DH_7     (1) ----QRNTSTLDRHCFESSFDGSEFFFRDHRVQGQPLLPAVAYLEWARAA
Fr9DEF_PS_4     (1) -HPLVHERVDGPDLRYRSEFIGDEFFLDGHRIAGVKILPAACYLEMAREV
  Fr9I_PS_9     (1) ----AVWRDEDGVRVYSCRFEPNDLYLRHHRIDGQALLPAVALLDLARVI
  PedF_PS_7     (1) ----SAASSTLVSTEGVSRFKGEEFFLRDHSG----MLPAAVYLEMVRAF
                                                 *
                      51                                                 100
Fr9DEF_DH_4    (40) ARQAAPEAP-ATDPVVLRDVIWQHPLS---LAAGPRVVEVRLERDAAVAL
  Fr9I_DH_9    (51) ATLAMPESERCSAGFRLEHLVWTRPLV---PSDGTADIAVTMEPAMTPAR
  PedF_DH_7    (47) AQIALGNAC-PDVALKLSNVVWIGPL----LAEQPIVGTITLQAREDRGI
Fr9DEF_PS_4    (50) SQRVGARGG------VLRDIFWLAPYQAPDLPGGARPAPITVQMEASGER
  Fr9I_PS_9    (47) ADTLPRDEAAAALR--ISRATWQKPVFVG---TPGRDAALRVAHRDGCWT
  PedF_PS_7    (43) AEGKHERKITG-----LSHVVWPKVLLVS---GEGREVRTCLTNVDRSAF
                      101                                                150
Fr9DEF_DH_4    (86) SPRFRFVIESRLAEPDDAA---ARLTHCSGSVEIMRTDDAGASCLLDLSA
  Fr9I_DH_9    (98) RAGIDGRDVFRLTVGDVRSNDAQPYAQAIVVPEQRDTMPVPPVVAVEQLA
  PedF_DH_7    (92) DYQISSVSAAGQQP----------VVHCQGIATTETEKEAAPVLDLDALR
Fr9DEF_PS_4    (94) HCRVVSTGADGRET-----------THFVGRWAQEAVPDAIGAVDVDAIL
  Fr9I_PS_9    (92) ATLESGNGMQGGVAP----------YAQCDLTWTADVPDADPLPDRARRA
  PedF_PS_7    (85) LISACEQSSEGPQE----------VTYCQGNLLLPEVMEEPGAALAIEAI
                      151                                                200
Fr9DEF_DH_4   (133) FRRQAEELDVAECYRAFAALGIEFGPSHRGLKKLYRYRDQLLAQVGR-SG
  Fr9I_DH_9   (148) VYSRGTRIDGARLYRRFAELGFEYGPSHRCIDALFYDGDEVLARMRP-YE
  PedF_DH_7   (132) SRLTQKEIGVERCYAALEAAGVNHGPAMRGLQAVSRNAEEVLATLRLPAE
Fr9DEF_PS_4   (133) RRTSTR--LARDAMNALVEATSSFGAPFQVMTWLQHGADEALAAYRL---
  Fr9I_PS_9   (132) GAPLSKADVAQRFAGSLVRESGNVLGLPGGIRIIGCESSADTTWLTLACE
  PedF_PS_7   (125) AYRCPSVLEAKQCDRLLQST---HGPALMSVQQLRYSDREALALLQLPDE
                      201                                                250
Fr9DEF_DH_4   (182) AAGIDDGYLLSPTITDAALQVHIGNVLGTGTDVLPLPYAMDSLTIVDAPS
  Fr9I_DH_9   (197) PDTHGTGYLLAPGLLDSALQASIG--------FDPAALDANHDGYLDGRL
  PedF_DH_7   (182) TVGEASAYVLHPAILDAALQASIA-------------------LTLRDDEV
Fr9DEF_PS_4   (178) PAGQSGPYHWHPGILSAALGAVEIWIAARG--ETGAHRLPYGIGSMIDHA
  Fr9I_PS_9   (182) DGNDDSAGGARSTAFGLLFAIVDLCGRDREAAGLAIPYGIDECLLYRAFP
  PedF_PS_7   (172) LQMGWDDYGWHPSLLNGAILASVVW-------------------------
                                  *
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Fig. S1. Protein sequence alignment of DH and PS domains. Nomenclature (sequence labels), protein name_domain type_PKS module number. PedF, PKS from the pederin biosynthetic gene cluster. Fr9, PKS from the spliceostatin biosynthetic gene cluster. Blue, PS domains (confirmed biochemically for pederin, and hypothetical for spliceostatin). Black, DH domains. Red, catalytic histidine that is conserved in both DH and PS domains, and catalytic aspartate that is conserved in DH domains only. Alignment carried out using vector NTI (Invitrogen). See also ref. 4.

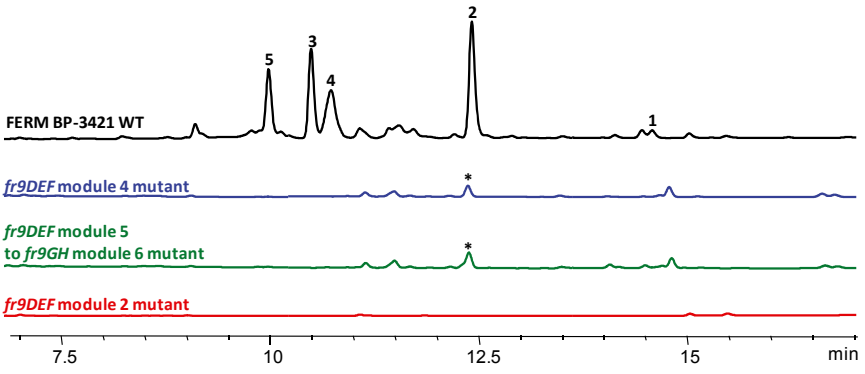


Fig. S2. Confirmation of spliceostatin biosynthetic gene cluster by gene deletion. Phenotypes of three different *fr9* PKS-NRPS deletion mutants compared with the wild-type (WT) strain. HPLC traces of culture supernatants with detection at 230 nm are shown. The peak marked with an asterisk was determined to not be related to spliceostatins by tandem-MS analysis.

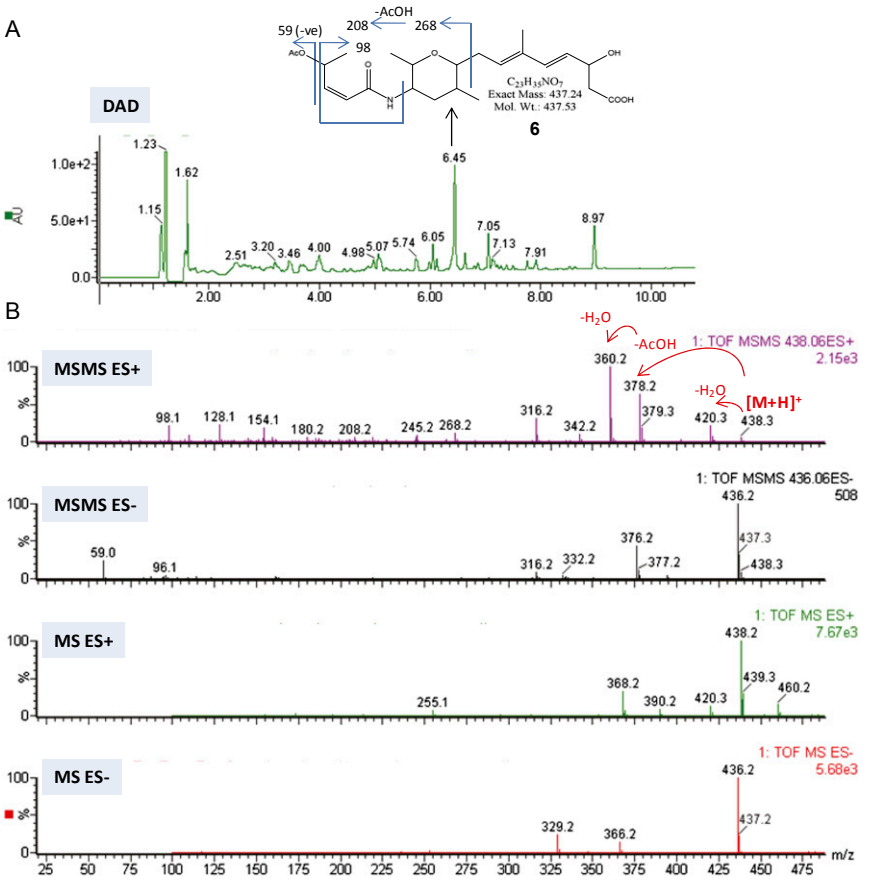


Fig. S3. Inactivation of HMGS-like gene *fr9K* leads to accumulation of a truncated spliceostatin analog. LC/MS and tandem-MS analyses of culture extracts, highlighting proposed structure of compound **6**. (*A*) UV trace; DAD detection at 210–400 nm. (*B*) Time of flight (TOF) mass spectra in positive and negative modes.

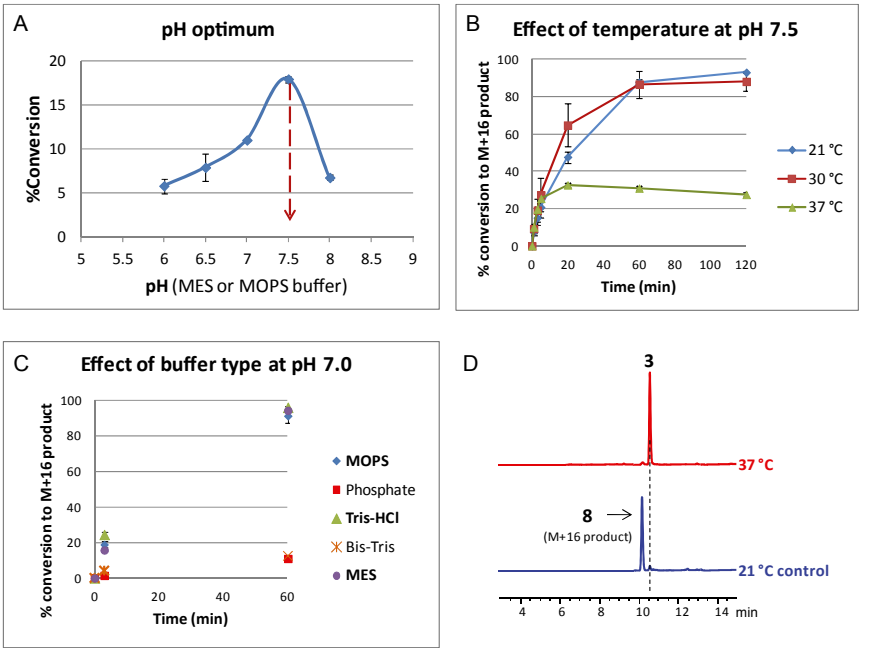


Fig. S4. Characteristics of recombinant Fe(II)/α-ketoglutarate–dependent dioxygenase Fr9P. (*A*) pH optimum. (*B*) Temperature. (*C*) Buffer type. (*D*) Fr9P is inactivated at 37 °C after 20 min. The enzyme was preincubated at 37 °C for 20 min and then used in a reaction at room temperature (red trace, 37 °C). The 21 °C control (blue trace) is the reaction without preincubation.

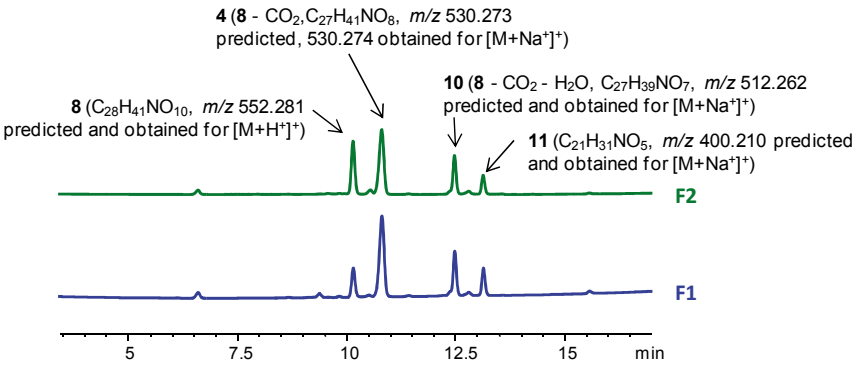


Fig. S5. Degradation of **8** upon freeze-drying. HPLC analysis of purified fractions after lyophilization overnight. F1, fraction from HPLC purification using acetonitrile/water as mobile phase without modifier. F2, fraction from HPLC purification using acetonitrile/water with 0.02% acetic acid as mobile phase, pH immediately adjusted to ~8 with NH_4OH after collection and before freeze-drying. High-resolution MS results for major peaks are indicated. Detection at 230 nm.

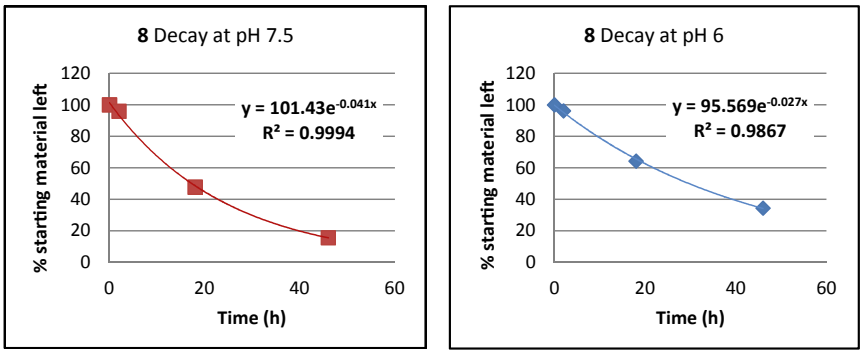


Fig. S6. Half-life of **8** at pH 7.5 and 6.0. The half-life of **8** was determined to be 17 h at pH 7.5 (*Left*) and 24 h at pH 6.0 (*Right*).

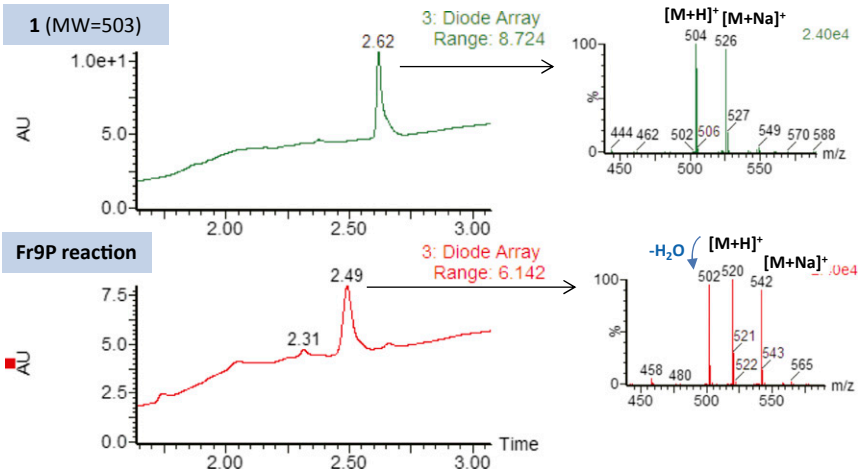


Fig. S7. Fr9P accepts spliceostatin congener **1** as substrate. LC/MS analysis of substrate **1** (*Upper*) and Fr9P-catalyzed reaction (*Lower*). Note the $-H_2O$ MS fragment of the product (*m/z* 502), which is characteristic of a hydroxylated compound.

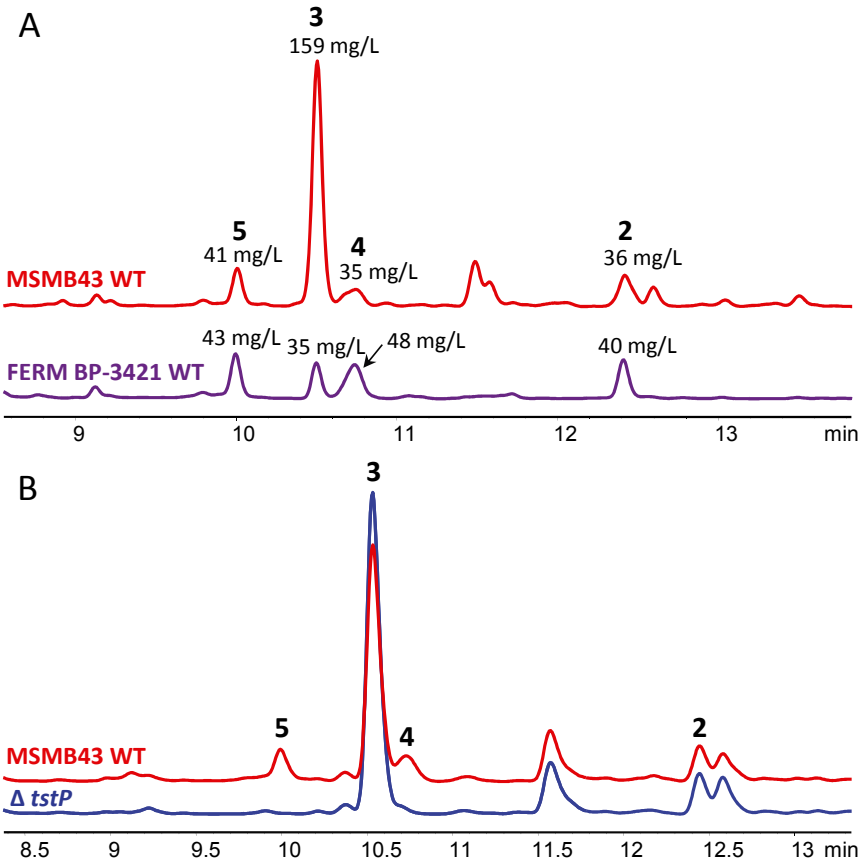
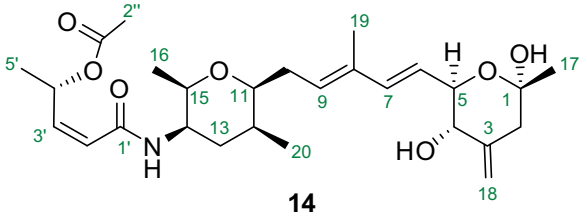


Fig. S8. Production of spliceostatins by *Burkholderia* sp. MSMB 43 and FERM BP-3421. (*A*) Comparative HPLC profiles of fermentation supernatants for FERM BP-3421 and MSMB43 cultured in parallel. Average titers of **2**, **3**, **4**, and **5** from duplicate fermentations are shown above their respective peaks. Detection at 230 nm. (*B*) Comparative HPLC profiles of fermentation supernatants for MSMB 43 wild type (WT) and the *tstP* in-frame deletion mutant (Δ*tstP*) cultured in parallel. Detection at 236 nm.

Table S1. One-dimensional and two-dimensional NMR assignments for 14 in DMSO-d_6



14

Position	δ_H, ppm; mult, (*J* in Hz)	δ_C, ppm	HSQC	COSY	HMBC
1	—	95.1	C (no signal)	—	
2	2.22 and 2.34 (overlapping with 10)	44.6	CH_2	2, 18	1, 3, 18, 4
3	—	145.3	C (no signal)	—	
4	3.55, br d (8.3)	71.8	CH	5, 18	None
5	4.01, br dd (9.1, 6.4)	74.5	CH	4, 6	7
6	5.66, dd (15.7, 6.2)	126.3	CH	7, 5	7
7	6.23, d (15.9)	135.1	CH	6, 5	8, 19, 5, 6
8	—	133.8	C (no signal)	—	
9	5.47, t (7.3)	128.1	CH	10, 19	19
10	2.21, m, and 2.29, m (overlapping with 2)	31.4	CH_2	10, 11, 9	11
11	3.51, m	79.8	CH	10, 12	20
12	1.65, m	28.5	CH	20	None
13	1.80, m	35.1	CH_2	13, 14	None
14	3.65, m	46.2	CH	NH, 13	16
15	3.64, m, overlapping with 14	74.7	CH	16	16
16	1.07, d (6.4)	17.6	CH_3	15, 14	15, 14
17	1.32, s	28.8	CH_3	None	1, 2, 3, 5
18	5.03, d (1.7), and 4.73, d (1.9)	106.9	CH_2	18, 4, 2	None
19	1.72, s	12.3	CH_3	10, 9	7, 9, 8, 6
20	0.95, d (7.3)	14.1	CH_3	12	11, 12, 13
1′	—	164.5	C (no signal)	—	
2′	6.11, dd (11.7, 1.2)	122.6	CH	3′, 4′	1′, 4′
3′	5.86, dd (11.7, 7.4)	142.5	CH	2′, 4′	1′, 5′, 4′
4′	6.36, m (6.4)	67.8	CH	5′, 3′, 2′	1″, 4′, 5′
5′	1.25, d (6.6)	19.8	CH_3	4′	4′, 3′
1″	—	169.5	C (no signal)	—	
2″	1.98, s	20.8	CH_3		1″, 4′
NH	7.81, d (7.8)	—	Exchangeable (no signal)	14	1′, 14, 15

2 OH were not clearly observable (could not be unambiguously assigned). Reference: DMSO solvent signal set at 2.50 and 39.5 ppm. COSY, correlation spectroscopy; HMBC, heteronuclear multiple-bond correlation spectroscopy; HSQC, heteronuclear single-quantum correlation spectroscopy.