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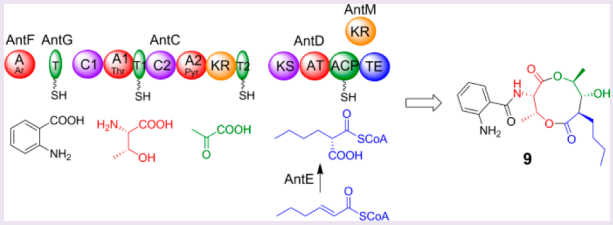
Enzymatic Synthesis of Dilactone Scaffold of Antimycins

Moriah Sandy, Zhe Rui, Joe Gallagher, and Wenjun Zhang*

Department of Chemical and Biomolecular Engineering and Energy Biosciences Institute, University of California, Berkeley, California 94720, United States

Supporting Information

ABSTRACT: Antimycins are a family of natural products possessing outstanding biological activities and unique structures, which have intrigued chemists for over a half century. The antimycin structural skeleton is built on a nine-membered dilactone ring containing one alkyl, one acyloxy, two methyl moieties, and an amide linkage connecting to a 3-formamidosalicylic acid. Although a biosynthetic gene cluster for antimycins was recently identified, the enzymatic logic that governs the synthesis of antimycins has not yet been revealed. In this work, the biosynthetic pathway for antimycins was dissected by both genetic and enzymatic studies for the first time. A minimum set of enzymes needed for generation of the antimycin dilactone scaffold were identified, featuring a hybrid nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) assembly line containing both *cis*- and *trans*-acting components. Several antimycin analogues were further produced using *in vitro* enzymatic total synthesis based on the substrate promiscuity of this NRPS-PKS machinery.



Antimycins are a family of natural products produced by various *Streptomyces* species and share a common structural skeleton: a nine-membered dilactone ring substituted with one alkyl (C-7), one acyloxy (C-8), two methyl moieties (C-4 and C-9), and an amide linkage (C-3) connecting to a 3′-formamidosalicylic acid (Figure 1). Since the first isolation in 1949, more than 40 antimycin-type compounds varying in the alkyl and acyl chains have been reported.[1−6] The unique structures of antimycins suggest novel chemical logic and enzymatic machinery for the scaffold assembly.

Antimycins have exhibited significant bioactivities, including antifungal, insecticidal, and nematocidal properties.[1−3] They are widely known as inhibitors of mitochondrial electron transport chains by binding to cytochrome c oxidoreductase and are active ingredients in Fintrol, a chemical piscicide.[7,8] Recently, potent and selective inhibition of Bcl2/BclX_L related antiapoptotic proteins by antimycins was reported, rendering antimycins as promising new anticancer agents.[9,10] Elucidation of the enzymatic mechanism for the antimycin scaffold assembly will therefore facilitate the generation of antimycin analogues exhibiting improved pharmaceutical properties through combinatorial biosynthesis.

The recently identified antimycin biosynthetic gene cluster from *Streptomyces* S4,[11] together with feeding studies with labeled precursors,[12−14] have allowed us and other researchers to propose a putative enzymatic pathway for antimycin biosynthesis (Figure 1).[15] The dilactone scaffold of antimycins is presumably generated through a hybrid NRPS-PKS assembly line-based mechanism. The assembly line starts with the activation of anthranilic acid, a tryptophan degradation product, by an acyl-CoA ligase homologue AntF through adenylation, and the adenylated acid is loaded onto a carrier protein AntG for further processing. AntHIJKL shows high sequence similarity to PaaABCDE, a multicomponent oxygenase catalyzing the epoxidation of the aromatic ring of phenylacetyl-CoA.[16−18] AntHIJKL presumably catalyzes the formation of the 3-aminosalicyloyl-*S*-AntG from anthraniloyl-*S*-AntG by epoxidation to 6-amino-1,2-epoxycyclohexa-3,5-dienecarboxylic acid moiety followed with a 1,2-shift of the thioester group, which was suggested by recent feeding experiments of isotope- and fluorine-labeled precursors.[13] AntC is a dimodule NRPS with domains organized into C1-A1-T1-C2-A2-KR-T2 (C, condensation; A, adenylation; T, thiolation; KR, ketoreduction). The A1 domain is predicted to activate L-Thr, which is then condensed with 3-aminosalicylate via an amide bond catalyzed by C1; A2-KR-T2 is a typical α-hydroxy acid specifying module homologous to the first module of CesA and CesB in cereulide synthesis.[19] The A2 domain is then proposed to activate α-keto acid pyruvate, which is stereoselectively reduced by the KR domain and condensed to threonine via an ester bond promoted by C2. The PKS AntD has domains organized into KS-AT-ACP-TE (KS, ketosynthase; AT, acyltransferase; ACP,acyl carrier protein; TE, thioesterase). The AT domain transfers a 2-carboxylated acyl moiety from CoA to ACP, and it lacks a sequence motif that correlates with specificity for methylmalonate (signature motif: YASH) or malonate (signature motif: HAFH), the most typical extender units for PKS. The AT domain of AntD is proposed to select atypical extender units such as butylmalonyl-CoA, which is generated from reductive carboxylation of 2*E*-hexenoyl-CoA

Received: August 7, 2012
Accepted: September 12, 2012
Published: September 12, 2012

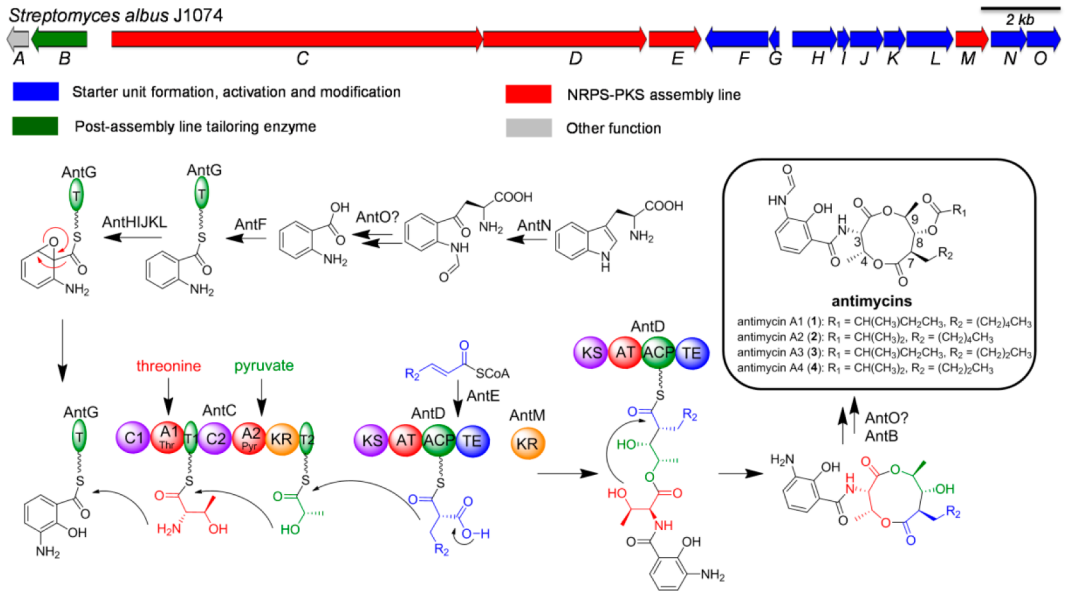


Figure 1. Map of antimycin gene cluster and proposed biosynthetic pathway. The proposed functions of proteins encoded by the antimycin biosynthetic gene cluster are summarized in Table S2, Supporting Information.

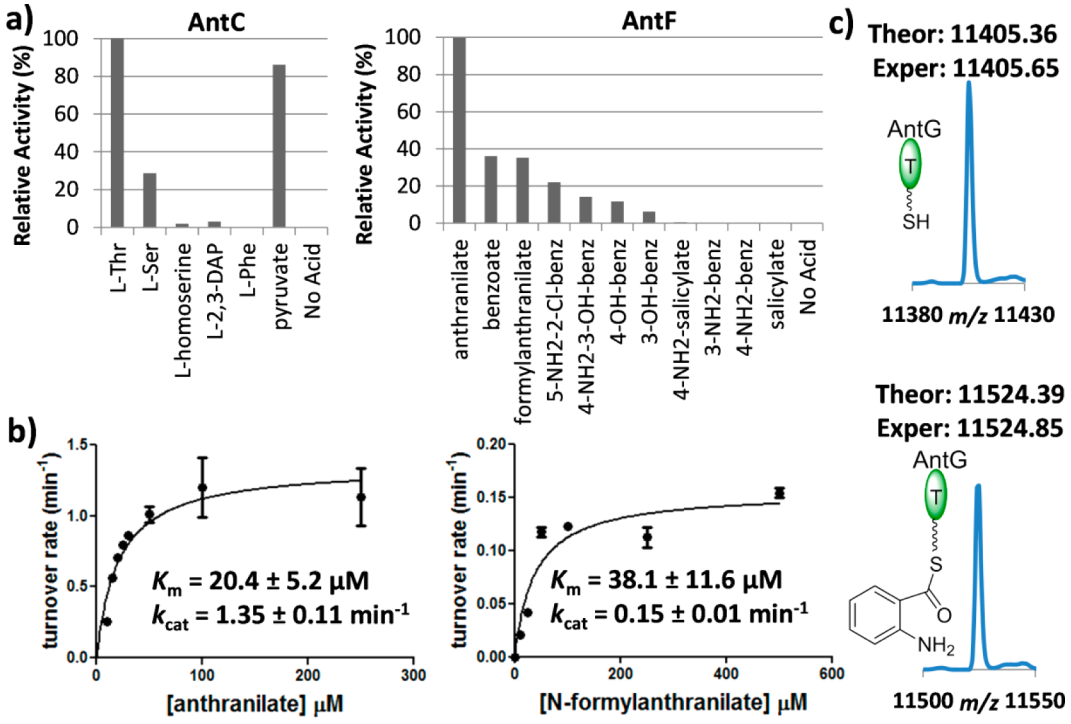


Figure 2. Characterization of AntC, F, and G. (a) A-domain activities of AntC and AntF. A relative activity of 100% for L-Thr- and anthranilate-dependent exchange corresponds to 270k and 195k cpm, respectively. DAP: diaminopropionate. (b) Determination of AntF kinetic parameters by ATP-[^{32}P]PP$_i$ exchange assays. Error bars represent standard deviations from at least three independently performed experiments. (c) Detection of holo-AntG without AntF and anthraniloyl-*S*-AntG in the AntF reaction by HRMS with deconvolution.

by AntE, an enzyme of the crotonyl-CoA reductase (CCR) family.[20−22] The KS domain catalyzes the decarboxylative condensation between the aminoacyl-*S*-T2 domain of AntC and the 2-carboxy-acyl-*S*-ACP domain of AntD, followed by stereoselective reduction of the *β*-keto functionality by a 3-oxoacyl-ACP reductase homologue AntM, and regiospecific macrolactone cyclization and release of the nine-membered dilactone product promoted by the TE domain of AntD. A lipase homologue AntO and an acyltransferase homologue AntB presumably catalyze the installation of an *N*-formyl group and the transesterification on the C-8 hydroxyl group during or after the assembly line, respectively.

On the basis of the proposed biosynthetic pathway for antimycins, 11 proteins (AntCDEFGHIJKLM) are needed for generating the antimycin dilactone scaffold from building monomers anthranilate, L-Thr, pyruvate, and 2,3-unsaturated acyl-CoA. Their functions have been dissected in this work mainly by *in vitro* characterization using purified proteins from

dx.doi.org/10.1021/cb300416w | *ACS Chem. Biol.* 2012, 7, 1956−1961

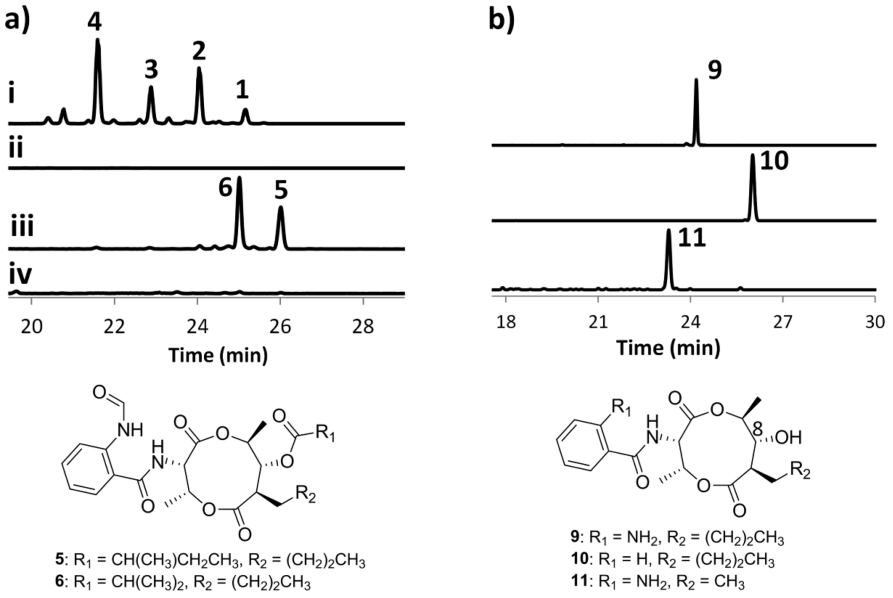


Figure 3. Extracted ion chromatograms showing production of antimycin analogues. (a) *In vivo* production of antimycins from wild-type (trace i) but not from Δ*antH* of *S. albus* (trace ii), and production of deoxy-isoantimycins from Δ*antH* (trace iii) but not from wild-type of *S. albus* (trace iv). (b) *In vitro* production of antimycin analogues containing the dilactone scaffold. The calculated mass with 10 ppm mass error tolerance was used. The tentative structures are shown in the bottom.

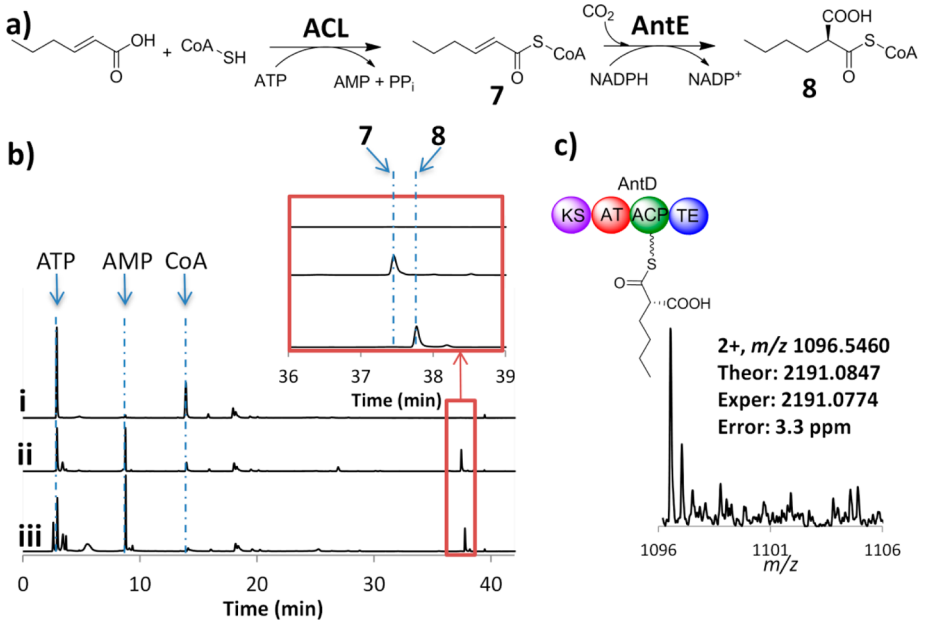


Figure 4. Characterization of AntE and D. (a) Schematic of 2*E*-hexenoyl-CoA (**7**) preparation and conversion to butylmalonyl-CoA (**8**). (b) HPLC traces (260 nm) of no enzyme control (trace i), ACL reaction to generate **7** (trace ii), and ACL/AntE reaction to generate **8** (trace iii). (c) Detection of butylmalonyl-*S*-AntD by HRMS after trypsin digestion.

Escherichia coli (Figure S1, Supporting Information). *E. coli* BAP1 strain that contains a chromosomal copy of the phosphopantetheinyl transferase Sfp was used for AntC, D, and G expression to ensure their posttranslational modification to the pantetheinylated forms.[23]

The ability of AntC and AntF to reversibly adenylate various acids was tested using the classical ATP-[^{32}P]PP$_i$ exchange assay.[24] As expected, AntC demonstrated a strong preference for the activation of L-Thr and pyruvate, with modest activation of L-Ser. AntF activated various aryl acids, with anthranilate being the most preferred one (Figure 2a). Since contradictory results were reported regarding the incorporation of C-2 of the tryptophan indole ring into the 3′-formamido carbonyl of antimycins by labeling experiments,[12,13] we compared the kinetic parameters of AntF toward anthranilate (k_{cat}/K_m = 0.066 min^{-1} μM^{-1}) and *N*-formylanthranilate (k_{cat}/K_m = 0.004 min^{-1} μM^{-1}) (Figure 2b). The preferential activation of anthranilate by AntF, with a specificity constant (relative k_{cat}/K_m) of 16-fold, is in agreement with the conclusions reported by Spiteller group that a formyl group is reintroduced later in the biosynthesis of antimycins.[13] Aryl-CoA products were not detected upon addition of CoA to the enzymatic reactions of AntF, suggesting that AntF could function similarly to an A domain without forming a CoA thioester intermediate. AntG

dx.doi.org/10.1021/cb300416w | *ACS Chem. Biol.* 2012, 7, 1956−1961

was then tested as a putative cognate loading partner of AntF. Indeed, anthraniloyl-*S*-AntG was formed and detected by high-resolution mass spectrometry (HRMS) (Figures 2c and S2, Supporting Information).

Anthraniloyl-*S*-AntG is presumably oxidized by AntHIJKL and rearranges to yield 3-aminosalicyloyl-*S*-AntG (Figure 1). Since reconstituting the activities of multicomponent oxygenase AntHIJKL *in vitro* has not yet been successful, a gene disruption experiment in the antimycin-producing organism *S. albus* J1074 was carried out to probe the function of oxygenase in the biosynthesis of antimycins. AntH was deleted in-frame through double crossover, which completely abolished the production of antimycins that could be identified from the culture of wild-type strain (Figures 3a and S3, Supporting Information). Careful analysis of the mutant culture by liquid chromatography (LC)-HRMS revealed the production of two new compounds in trace amounts, and their identities were indicated to be deoxy-isoantimycins by comparing HRMS and HRMS/MS data with those of antimycin standards (Figures 3a and S6−11, Supporting Information). Compounds **5** and **6** were quickly deformylated during purification, and the presence of an anthraniloyl moiety was confirmed by HRMS/MS and NMR spectroscopic analysis (Figures S16−17 and S19, Supporting Information). The lower yields of new compounds (>1000-fold decrease compared to wild-type) suggest that the oxygenation of the anthraniloyl moiety happens in the early stage on the assembly line as shown in Figure 1. Furthermore, the production of deoxy-isoantimycins demonstrated the tolerance of the NRPS-PKS assembly line toward the aryl starter unit,[13,15] suggesting that the activity of AntHIJKL could be circumvented for enzymatic synthesis of antimycin dilactone scaffold.

The formation of atypical PKS extender units by AntE and the subsequent loading onto AntD were also biochemically characterized. AntE belongs to a growing family of CCR enzymes that furnish unusual carboxylated extender units to PKS systems from unsaturated precursors.[20−22] The putative substrate 2*E*-hexenoyl-CoA was enzymatically prepared using 2*E*-hexenoic acid, CoA, ATP, and a promiscuous acyl-CoA ligase (ACL) characterized previously.[25] *In vitro* analysis of AntE confirmed that it is able to reductively carboxylate 2*E*-hexenoyl-CoA in an NADPH-dependent manner, yielding the product butylmalonyl-CoA (Figure 4a,b). AntE demonstrated relaxed substrate specificity toward the side chain of the respective unsaturated fatty acid precursor and was able to take crotonoyl- and 2*E*-octenoyl-CoA and form ethylmalonyl- and hexylmalonyl-CoA, respectively (Figure S4, Supporting Information), consistent with the natural variations on C-7 alkyl group of antimycins isolated from a single producer. When AntD was included in the reaction, the acylmalonyl moiety could be transferred from CoA to holo-ACP of AntD as confirmed by HRMS after trypsin digestion (Figures 4c and S5, Supporting Information).

We then performed enzymatic total synthesis of the antimycin dilactone scaffold by mixing purified enzymes (AntC, D, E, F, G, and M) and building monomers (anthranilate, L-Thr, pyruvate, and 2*E*-hexenoyl-CoA). The expected product **9** was successfully formed and confirmed using ^{13}C-labeled substrate in UV, HRMS, and HRMS/MS analyses (Figures 3b, S12−13, and S18, Supporting Information). Control experiments without ATP or NADPH did not yield the product, consistent with the cofactor requirement of AntC and F for ATP, and AntC, E, and M for NADPH. The formation of **9** required all six proteins and four substrates, and no 8-carbonyl version of **9** could be detected in the reaction omitting AntM. AntM was thus strongly indicated to be a *trans*-acting KR domain, which reduces the β-keto group of the AntD-tethered intermediate before release through AntD-TE catalyzed macrolactone cyclization (Figures 1 and S20, Supporting Information). The assembly line exhibited relaxed substrate specificity since alternative substrates, such as benzoate and crotonoyl-CoA, could also be utilized to generate dilactone scaffolds (Figures 3b and S14−15, Supporting Information).

In summary, we have dissected the hybrid NRPS-PKS assembly line that builds the dilactone scaffold of antimycins. The unique dilactone core is constructed upon four distinct monomers: an aminobenzoate, a natural amino acid, an α-keto acid, and an acylmalonyl moiety. Modular NRPS and PKS with linearly organized catalytic domains are employed in the biosynthetic system, along with a highly dissociated loading module and a KR domain acting in trans. The dilactone connectivity is formed by two different enzymatic activities: a C domain of NRPS and a TE domain of PKS. The NRPS-PKS assembly line has high tolerance toward various building blocks, as demonstrated by this work and previous feeding studies.[13,15] Our work provides the basis for rational reprogramming of the antimycin assembly line toward the biosynthesis of antimycin analogues.

METHODS

ATP-PP$_i$ Exchange Assays. The assays were performed in 100 μL of reaction buffer (50 mM Tris-HCl/2 mM $MgCl_2$, pH 8) containing 5 mM ATP, 1 mM $Na_4[^{32}P]$-pyrophosphate (PP_i) (~4 × 10^6 cpm mL^{-1}), 1 mM TCEP, 5 mM substrate, and 5 μM enzyme. Reactions were incubated at 25 °C for 40 min then quenched by the addition of charcoal suspension (1.6% w/v activated charcoal, 0.1 M Na_4PP_i, 3.5% $HClO_4$). Free $[^{32}P]PP_i$ was removed by centrifugation of the sample followed by washing twice with wash solution (0.1 M Na_4PP_i and 3.5% $HClO_4$). Charcoal-bound radioactivity was measured on a Beckman LS 6500 scintillation counter.

LC-HRMS Analysis of AntG-Bound Biosynthetic Intermediate. Assays were performed in 100 μL of 50 mM Tris pH 8, 2 mM $MgCl_2$, 1 mM TCEP, 2 mM ATP, 5 mM acid substrate, and 4 μM AntF, G. Reactions were incubated for 0.5−2 h and immediately analyzed by nanocapillary LC-HRMS using a chip column (Agilent Zorbax 300SB-C18 5 μm; separation, 43 mm × 75 μm; enrichment, 4 mm 40 nL) in-line with a QTOF (Agilent 6510 Q-TOF LC/MS). A linear gradient of 3−95% CH_3CN over 9 min in H_2O with 0.1% formic acid at a flow rate of 0.6 μL min^{-1} was used for analysis. All data were analyzed using MassHunter Qualitative Analysis, part of the Agilent software packaged with the Accurate-Mass Q-TOF LC/MS. Theoretical mass calculations are shown in Figure S2, Supporting Information.

Gene Disruption in *S. albus* and Mutant Analysis. *In vivo* generation of targeted mutations in *S. albus* was achieved by conjugative transfer of disruption plasmids from *E. coli* WM6026 to *S. albus* according to general protocols.[26] The knockout cassettes were constructed using the ReDirect technology.[27] In summary, a 5.8 kb fragment containing *antH* flanked by 2 kb arms was PCR amplified from genomic DNA and inserted into a PCRBlunt vector. This plasmid was introduced into *E. coli* BW25113/pIJ790 by electroporation. The *acc*(3)*IV-oriT* cassette amplified by PCR from pIJ773 was then introduced to replace the entire *antH* using PCR targeting and λ-red-mediated recombination. The resulting knockout cassette was transformed into *E. coli* WM6026 (a diamino-pimelic acid auxotroph) for conjugation with *S. albus*. The double-crossover strain was obtained from antibiotic selection ($Apra^RKan^S$) and confirmed by PCR (Figure S3, Supporting Information). See the Supporting Information for details of mutant analysis. LC-HRMS analysis was

performed on an Agilent Technologies 6520 Accurate-Mass Q-TOF LC-MS instrument and an Agilent Eclipse Plus C18 column (4.6 × 100 mm). A linear gradient of 25 to 95% CH_3CN (v/v) over 20 min in H_2O supplemented with 0.1% (v/v) formic acid at a flow rate of 0.5 mL min^{-1} was used.

Enzymatic Synthesis of Acylmalonyl-CoAs by ACL and AntE. Reactions were performed at room temperature (RT) in 100 μL of 50 mM HEPES (pH 8.0) containing 4 mM ATP, 4 mM $MgCl_2$, 5 mM acid substrate (2*E*-hexenoic acid or 2*E*-octenoic acid), 2 mM CoA, 33 mM $NaHCO_3$, 2 mM NADPH, and 50 μM enzymes. Crotonoyl-CoA (Sigma) was directly used for the AntE reaction without ACL. Reactions were incubated for 1 h, and quenched by adding equal volume of 10% trichloroacetic acid (TCA). Precipitated proteins were removed by centrifugation, and the supernatant was analyzed by HPLC and LC-HRMS. HPLC analysis was normally performed using an Intertsil ODS-4 column (4.6 mm i.d., 250 mm L, GL Sciences Inc.) with a linear gradient of 2 to 12% CH_3CN (v/v) over 30 min, 12 to 95% CH_3CN (v/v) over 5 min, and 95% CH_3CN (v/v) for a further 10 min in H_2O with 0.1% (v/v) TFA at a flow rate of 1 mL min^{-1}. LC-HRMS analysis was normally performed using an Agilent Eclipse Plus C18 column (4.6 × 100 mm) with a linear gradient of 2 to 98% CH_3CN (v/v) over 30 min in H_2O with 0.1% (v/v) formic acid, at a flow rate of 0.5 mL min^{-1}. HRMS spectra for carboxylated products are shown in Figure S4, Supporting Information.

LC-HRMS Analysis of AntD-Bound Biosynthetic Intermediate. Assays were performed in 100 μL of 50 mM HEPES (pH 8.0) containing 4 mM ATP, 4 mM $MgCl_2$, 1 mM TCEP, 4 mM NADPH, 33 mM $NaHCO_3$, 1 mM CoA, 5 mM 2*E*-hexenoic acid, 0−5 μM AntDE, and 50 μM ACL. After 1−4 h incubation at 25 °C, 0.1 M NH_4HCO_3, 100 μM DTT, and trypsin (1:5 w/w trypsin/total protein) were added and further incubated at 30 °C for 0.25−12 h. The reactions were quenched with equal volume of 25% formic acid and analyzed by nanocapillary LC-ESIMS using a chip column (Agilent Zorbax 300SB-C18 5 μm; separation, 43 mm × 75 μm; enrichment, 4 mm 40 nL) in-line with a QTOF (Agilent 6510 Q-TOF LC/MS). A linear gradient of 10−55% CH_3CN over 30 min in H_2O with 0.1% formic acid at a flow rate of 0.6 μL min^{-1} was used for analysis. All data were analyzed using MassHunter Qualitative Analysis, part of the Agilent software packaged with the Accurate-Mass Q-TOF LC/MS (Figure S5, Supporting Information).

LC-HRMS Product Assays from Enzymatic Total Synthesis. Assays were performed in 100 μL of 50 mM HEPES (pH 8.0) containing 4 mM ATP, 4 mM $MgCl_2$, 1 mM TCEP, 4 mM NADPH, 33 mM $NaHCO_3$, 1 mM CoA, 5 mM acid substrates, and 0−10 μM enzymes. After 4 h incubation at 25 °C, the reactions were extracted with 2 volumes of ethyl acetate. The organic layer was dried under vacuum; the residue was then redissolved in 50 μL MeOH and was subjected to LC-HRMS and HRMS/MS analysis using an Agilent Technologies 6520 Accurate-Mass Q-TOF LC-MS instrument and an Agilent Eclipse Plus C18 column (4.6 × 100 mm). A linear gradient of 2 to 98% CH_3CN (v/v) over 30 min in H_2O with 0.1% (v/v) formic acid at a flow rate of 0.5 mL min^{-1} was used for analysis. A collision energy of 22 V was used for all HRMS/MS experiments. The assays were scaled up to 500 μL for HPLC analysis using an Agilent Eclipse Plus C18 column (4.6 × 100 mm). A linear gradient of 25 to 95% CH_3CN (v/v) over 20 min, and 95% CH_3CN (v/v) for a further 10 min in H_2O with 0.1% (v/v) TFA at a flow rate of 1 mL min^{-1} was used.

■ ASSOCIATED CONTENT

Supporting Information

SDS-PAGE analysis of purified proteins, MS calculation of AntG-bound and AntD-bound biosynthetic intermediates, *antH* inactivation, compound characterizations, and biosynthetic pathway schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: wjzhang@berkeley.edu.

Notes

The authors declare no competing financial interests.

■ ACKNOWLEDGMENTS

We thank B. Shen (The Scripps Research Institute) for providing *S. albus* J1074, R. Sarpong (UC Berkeley) for providing various benzoic acids, and J. Pelton (UC Berkeley) for helping with NMR spectroscopic analysis. This work was supported by Berkeley and EBI startup funds, and the Pew Scholars Program.

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