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Polyketide Ring Expansion Mediated by a Thioesterase, CEC Domain, in Azinomycin Biosynthesis: Characterization of AziB and AziG

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Abstract

The azinomycins are a family of potent anti-tumor agents with the ability to form interstrand crosslinks with DNA. This study reports on the unusual biosynthetic formation of the 5-methyl naphthoate moiety, which is essential for effective DNA association. While sequence analysis predicts that the polyketide synthase (AziB) catalyzes the formation of this naphthoate, 2-methylbenzoic acid, a truncated single-ring product, is formed instead. We demonstrate that the thioesterase (AziG) acts as a chain elongation and cyclization (CEC) domain and is required for the additional two rounds of chain extension to form the expected product.

Polyketide synthases (PKSs) are megaenzymes, involving multiple catalytic domains, whose complex chemistry involves such processes as decarboxylative condensation, reduction, and dehydration and parallel that observed in fatty acid biosynthesis.^{1,2} Starter units (*e.g.* acetyl-CoA) and extender units (*e.g.* malonyl-CoA) are homologated to form ketide chains through Claisen-like condensations. In both fatty acid and polyketide biosynthesis, an acyl transferase (AT) domain transfers the starter and extender units onto an acyl carrier protein (ACP), which contains a phosphopantetheine arm enabling this domain to shuttle intermediates through the catalytic cycle. The ketosynthase (KS) domain accepts the starter unit from the ACP and subsequently catalyzes the condensation reaction with malonyl-CoA.

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Primer sequences, strains and plasmids, SDS-PAGE of AziB, phosphopantetheinylation of AziB, reconstitution of PKS activity with *S. sahachiroi* cell free extract, confirmation of PKS by MALDI-MS, NMR of 2-methylbenzoic acid as formed by AziB, SDS-PAGE of AziG, demonstration of 2-methylbenzoic acid and 5-methylnaphthoic acid as formed by AziB by LC/MS, LC/MS traces of AziB + AziA6, AziA7, AziA8 product mixtures, gel filtration studies of AziB and AziB/AziG, Kinetic studies of AziG and its mutants, AziG data collection and refinement statistics. The atomic coordinates for AziG structures have been deposited in the Protein Data Bank as entries 5HMB (apo) and 5HMC (5-methyl naphthoic acid complex).

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information. Additional supplemental material. The Supporting Information is available free of charge on the ACS Publications website at: <http://pubs.acs.org>.

In fatty acid biosynthesis, the process is completed by full reduction of the β -ketone in stepwise reactions involving ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains resulting in an inert hydrocarbon chain. The thioesterase (TE) domain promotes hydrolysis, releasing the fatty acid from the protein. In contrast, PKSs are selective and may or may not utilize reductive and/or dehydrative processing steps giving four possible functional states at the β -carbon during polyketide assembly. This enables the formation of different scaffolds and cyclization patterns.

It has long been held that aromatic polyketides are generated by Type I iterative PKSs in higher organisms where catalytic activities are housed in a single, multidomainal polypeptide chain. In contrast, in bacterial systems, aromatic polyketides have been thought to be biosynthesized by Type II PKSs, where each catalytic unit is discretely expressed and represents free-standing proteins that physically associate to perform the chemistry. In this report, we describe unforeseen chemistry exhibited by the azinomycin PKS.

The azinomycins (A and B, Figure 1) are anti-tumor agents produced by the terrestrial microorganism *Streptomyces sahachiroi*.³ In an early phase clinical investigation, azinomycin B has shown favorable results against 36 cases of malignant neoplasms, with the most promising effects observed against squamous cell carcinoma.⁴ *In vitro* studies suggest their biological activity resides in their ability to cross-link double stranded DNA through the actions of the electrophilic aziridine and epoxide moieties of the molecule.^{3,5,6} Non-covalent interactions made by the 5-methylnaphthoate and DNA are also essential for effective association and subsequent crosslinking of DNA.^{7,8} The formation of the 5-methylnaphthoate moiety (Figure 1), would be expected to form by homologation of 1 acetyl CoA unit with 5 malonyl CoA units, as mediated by a polyketide synthase. The 3'-methoxy group of the naphthoate is generated by oxidation and methylation as mediated by tailoring enzymes.³

MATERIALS AND METHODS

Materials

Streptomyces sahachiroi (NRRL 2485) was obtained from the American Type Culture Collection (ATCC). Cloning steps were performed in *E. coli* DH5 α or *E. coli* TOP10. *E. coli* Rosetta (DE3) was used for protein expression. PCR was performed using Phusion High-Fidelity DNA Polymerase® (New England BioLabs). TOPO® XL PCR Cloning Kit (Invitrogen) was used for the cloning of genes. Plasmids used for gene expression were pET-24a(+) and pET-21a(+) purchased from Novagen. Plasmid preparation and DNA purification were carried out using commercial kits (Qiagen). Restriction enzymes and other molecular biology reagents were from commercial sources (New England BioLabs). Radiolabeled coenzyme A (CoA), acetyl coenzyme A and malonyl coenzyme A substrates were purchased from PerkinElmer. Unlabeled coenzyme A (CoA), acetyl coenzyme A, malonyl coenzyme A substrates, β -nicotinamide adenine dinucleotide phosphate reduced form (NADPH) and S-(5'-adenosyl)-L-methionine chloride (SAM) were from Sigma Aldrich. 5-Bromo-1-naphthoic acid was purchased from Ark Pharm, Inc. The deuterated solvents for NMR experiments were purchased from Cambridge Isotope Laboratories and all other solvents were purchased from Fisher Scientific at the highest available grade. The

liquid scintillation cocktails, Opti-Fluor and Opti-Fluor O, were purchased from PerkinElmer.

Instrumentation

Autoradiographic analyses were obtained on a BASReader, BAS-5000 phosphorimager (Fujifilm, Tokyo, Japan). Fermentations were carried out with a Fermentation Design Inc. Model # MS21 (Allentown, PA, USA), 15 L total capacity. Sample collection by normal phase HPLC was carried out with a Varian Prostar HPLC System with 230 Pump and 330 UV/Vis Detector (Palo Alto, CA, USA) equipped with a Luna 5 μm Silica 100 $\text{\AA}$, LC Column 250 $\times$ 10 mm (Phenomenex, Torrance, CA, USA). Reversed Phase HPLC experiments were performed with a Waters 600 Controller (Milford, MA, USA) equipped with a Prodigy 5 μm ODS-2 150 $\text{\AA}$, LC Column 250 $\times$ 4.6 mm (Phenomenex) and a Waters 2487 dual λ absorbance detector. LC-MS was carried out on a Bruker MicroTOF-QII mass spectrometer coupled to an Agilent 1200 Infinity series liquid chromatography system (Agilent Technologies) with a Prodigy 5 μm ODS-2 150 $\text{\AA}$, LC Column 250 $\times$ 4.6 mm (Phenomenex). NMR spectra were acquired in CDCl_3 with a Varian Mercury 300 spectrometer or Bruker Advance III 500 MHz spectrometer equipped with a 5 mm H-C-N cryoprobe.

Cloning of *aziRB* into Expression Vector pET-24a

The PKS gene (*aziB*) was amplified by PCR using *Streptomyces sahachiroi* genomic DNA as a template. The PCR reaction was performed in a 50 μL reaction mixture consisting of 2.5 U Phusion DNA Polymerase, 10 μL 5X Phusion GC Reaction Buffer, 0.25 mM each of the four dNTPs, 100 pmol of each primer (PKS-FP and PKS-RP, Table S1), and ~50–100 ng of *Streptomyces* genomic DNA. The PCR program consisted of denaturation (95 $^{\circ}\text{C}$ for 5 min), followed by 30 cycles of denaturation (95 $^{\circ}\text{C}$ for 0.5 min), annealing (55 $^{\circ}\text{C}$ for 0.5 min), and extension (72 $^{\circ}\text{C}$ for 5 min), and amplification was ended after a final extension. The resulting PCR product (5.5kb) was cloned into the PCR $\text{\textcircled{R}}$ XL-TOPO Vector using the TOPO $\text{\textcircled{R}}$ XL PCR Cloning Kit (Invitrogen). Taking advantage of the naturally occurring *NotI* site at the end of the PKS open reading frame (ORF), the complete PKS ORF was isolated by *EcoRI/NotI* digestion, and inserted between the *EcoRI/NotI* sites of the *E. coli* expression vector pET-24a (EMD Millipore) to obtain pET-24a-*aziB*. Cloning of the gene was confirmed by DNA sequencing using BigDye $\text{\textcircled{R}}$ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The cloned plasmid was transformed into *E. coli* expression strain BL21(DE3) for overexpression. The resulting plasmid pET24a-*aziB* carries the kanamycin resistant gene, T7 promoter, *lac* operator, RBS site for the expression of the PKS and a 6xHis-tag at the 3'-end.

Cloning of *aziA6* into Expression Vector pET-24b

Thioesterase gene, *aziA6*, was amplified from *S. sahachiroi* genomic DNA by PCR with primers AziA6-F and AziA6-R (Table S1). The PCR reaction was performed in a 25 μL reaction mixture consisting of: 0.5 μL Phusion High-Fidelity DNA Polymerase, 4 μL Phusion GC Buffer, 15 ng of *S. sahachiroi* genomic DNA, 0.5 μL of each 20 mM forward and reverse primer, 0.5 μL of 20 mM dNTP, 1.25 μL of DMSO, and water to a total volume

of 25 μ L. The PCR program utilized an initial denaturation at 98 °C (30 s), 25 cycles of 98 °C (10 s), 75 °C for 20 s, and 1 min at 72 °C, and a final extension for 10 min at 72 °C. The DNA fragment of *aziA6* was purified by agarose gel extraction using the QIAquick Gel Extraction Kit (Qiagen, Venlo, Netherlands), and subsequently digested with restriction enzymes *Hind*III and *Xho*I. Following purification by agarose gel extraction, the DNA fragment was ligated into the *Hind*III/*Xho*I multiple cloning site of expression plasmid vector pET-24b, which contains a 6xHis-tag at the 3'-end. Cloning of the gene was confirmed by DNA sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit and the cloned plasmid transformed into *E. coli* expression strain BL21(DE3) for overexpression.

Overexpression of Apo-AziB

The *E. coli* strain harboring expression vector pET-24a-aziB was cultivated in 5 mL LB medium with 50 μ g/mL kanamycin overnight. The bacterial cultures were inoculated into 1 L LB medium and cultured at 37 °C with shaking at 250 rpm until OD₆₀₀ 0.6–0.8. After induction with the addition of 1 mM β -D-1-thiogalactopyranoside (IPTG), the cultures were incubated at 16 °C at 250 rpm for 24 h. The resulting cells were harvested by centrifugation at 7000 rpm for 15 min and washed with deionized distilled water and buffer. AziB buffer included: 20 mM potassium phosphate, 500 mM NaCl, 1 mM dithiothreitol (DTT), 5 mM imidazole, 20 % glycerol, pH 7.4. The cell pellet was resuspended in 30 mL of buffer. After the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF), the cells were disrupted by sonication utilizing the following program: Branson Sonifier 450 (Branson Ultrasonics) fitted with a 5 mm micro-tip, output setting 6, duty cycle 60%, and 5 cycles of 30 s each. The cell debris was removed by centrifugation at 9,500 rpm for 30 min and filtered with a 0.4 μ m syringe filter. The AziB cell extract was applied to pre-equilibrated HisTrap FF 5 mL columns (GE Healthcare Life Sciences) at 0.25 mL/min. The loaded column was washed with 150 mL of the column buffer with 40 mM imidazole. The protein was eluted by the column buffer with 250 mM imidazole. The buffer was exchanged into PPT buffer and concentrated to 10 mg/mL using a 10 kDa centrifugal ultrafiltration unit (Amicon Ultra - 15 Centrifugal Filter Unit, Millipore).

Streptomyces sahachiroi Cell-Free Extract Preparation

Streptomyces sahachiroi (inoculated from spore stock derived from dehydrated plates) was grown on GYM plates for 5–7 days at 28 °C until sporulation. A 1 cm² piece of the GYM plate was used to inoculate 100 mL of PS5 media in a 250 mL Erlenmeyer flask. The culture was incubated at 28 °C for 24 h at 250 rpm (first stage culture). The first stage culture (50 mL) was utilized to inoculate 600 mL of PS5 in 2 L baffled Erlenmeyer flasks. The cultures were grown for about 24 h at 28 °C, 250 rpm (second stage culture). Two 600 mL second stage cultures were used to inoculate the 15 L fermenter containing 10 L of the reduced PS5 medium. Following inoculation, the fermenter was agitated at 300 rpm and aerated with sterile filtered air (8 L/min) for 48 h. The mycelium was harvested by centrifugation and frozen in liquid nitrogen. The cell-free extract was prepared by combining frozen mycelium (20 g), glass beads (28 g, 0.1 mm), and cell-free extract buffer (100 mM potassium phosphate pH 7.5, 50% glycerol, 2 mM dDTT, and 1 mM EDTA, 80 mL, 4 °C) in a bead beater equipped with an ice water jacket. The cells were pulverized employing ten 1 min

cycles separated by 1 min intervals to prevent warming of the protein extracts and subsequently centrifuged (7,660 g, 15 min) to give the crude cell-free extract. GYM plates contained per liter: 4 g glucose monohydrate, 4 g yeast extract (Difco), 10 g malt extract (Difco), 2 g CaCO₃, 15 g Bacto-agar (Difco), and tap water; adjusted to pH 6.8 prior to sterilization. PS5 medium contained per liter 5 g Pharmamedia (yellow cotton seed flour), 5 g soluble starch, pH 7.0 prior to sterilization. Reduced PS5 medium contained per liter 1.25 g Pharmamedia, 1.25 g soluble starch, pH 7.0 before sterilization.

Reconstitution of the PKS, AziG with *S. sahachiroi* Cell Free Extract

The conversion of the heterologously expressed apo-PKS to the holo-PKS was initially carried out with *S. sahachiroi* cell free extract (CFE), which contained all enzymes involved in azinomycin biosynthesis, including the PKS specific phosphopantetheinyl transferase(s). The expressed and purified apo-PKS (25 mg) and 0.5 mg coenzyme A were added to 15 mL of cell free extract and incubated at 28 °C for 60 min. To detect the activity of the expressed “activated” PKS within the CFE, four reaction systems were set up as follows and each of them carried out in triplicate:

1. Boiled CFE (5.0 mL) + Co-factor solution 80 µL, acetyl-CoA (1 mg/mL) 5 µL, ¹⁴C-malonyl CoA 0.25 µCi, CFE Buffer 1.0 mL; negative control
2. Boiled CFE (5.0 mL) + Co-factor solution 80 µL, acetyl-CoA (1 mg/mL) 5 µL, ¹⁴C-malonyl CoA 0.25 µCi + Purified PKS 0.2 mg (in 1 mL CFE buffer)
3. CFE (5.0 mL) + Co-factor solution 80 µL, acetyl-CoA (1 mg/mL) 5 µL, ¹⁴C-malonyl CoA 0.25 µCi
4. CFE (5.0 mL) + Co-factor solution 80 µL, acetyl-CoA (1 mg/mL) 5 µL, ¹⁴C-malonyl CoA 0.25 µCi + Purified PKS 0.2 mg (in 1 mL CFE buffer)

Following incubation at 28 °C for 24 h, the samples were vortexed with dichloromethane (DCM; 5 mL) and microcentrifuged for 5 min at 14,000 rpm. Organics were transferred to fresh tubes and evaporated to dryness. The organic residues were resolubilized in a minimal volume of dichloromethane (60 µL) and applied to TLC (dichloromethane : methanol = 95 : 5). The naphthoate spots ($R_f = 0.3$) were scraped from the TLC plates and transferred to vials containing *OPTI-Fluor O* for scintillation counting analysis. To confirm the results the organic residues from the reaction systems were also analyzed by normal phase HPLC (Phenomenex silica column, 250 × 10 mm) and reverse phase HPLC (Phenomenex C8 column, 250 × 4.6 mm). The normal phase HPLC analysis conditions were 0–6.0% MeOH/DCM in 60 min, 3 mL/min. The reverse phase HPLC conditions were 20–100% acetonitrile in 15 min, followed by 100% acetonitrile for 5 min, and 100–20% acetonitrile in 15 min; 1.0 mL/min. The corresponding peaks were respectively collected, evaporated, and transferred to vials containing *OPTI-Fluor O* for scintillation counting analysis.

Cloning of *svp* into Expression Vector pET-21a

The PPTase gene *svp* was amplified by PCR from pBS18 (provided by Prof. Ben Shen, Scripps Florida). The PCR reaction mixture contained 2.5 U Phusion DNA polymerase, 10 µL 5X Phusion GC Reaction Buffer, 0.25 mM each of the four dNTPs, 100 pmol of each

primer (SVP-F and SVP-R, Table S1), and ~50–100 ng of pBS18 DNA in a final volume of 50 μ L. The PCR program consisted of denaturation at 95 °C for 5 min, 29 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C, and 10 min at 72 °C. The resultant PCR product was digested and cloned into the *EcoRI/XhoI* sites of the expression vector pET-21a to yield pET-21a-SVP in which Svp will be produced with a 6xHis tag at the C-terminus. Cloning of the gene was confirmed by DNA sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit.

Overexpression of Svp

The constructed vector pET-21a-SVP was introduced into the host strain *E. coli* Rosetta DE3 giving a lawn of cells. LB broth (5 mL) containing 100 μ g/mL ampicillin was inoculated with a single colony and incubated overnight at 37 °C, 250 rpm. The 5 mL overnight culture was used to inoculate 1 L of LB medium and the culture was grown utilizing the same conditions until OD₆₀₀ = 0.8. The culture was induced with 1 mM IPTG and incubated at 16 °C, 250 rpm for 24 h. The cells were harvested by centrifugation at 7000 rpm for 15 min and washed with deionized distilled water and phosphopantetheinylation (PPT) buffer (5 mM DTT, 10 mM MgCl₂, 75 mM Tris-HCl, pH 8.0, and 20% glycerol). After the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF), the cells were disrupted by sonication utilizing the following program: Branson Sonifier 450 (Branson Ultrasonics, Danbury, CT) fitted with a 5 mm micro-tip, output setting 6, duty cycle 60%, and 5 cycles of 30 s each. The cell debris was removed by centrifugation at 9,500 rpm for 30 min and filtered with a 0.4 μ m syringe filter.

Phosphopantetheinylation of AziB with ³H-pantetheine-CoA

The 400 μ L crude solution of Svp was mixed with 100 μ L (1 mg) of purified apo-AziB solution. Phosphopantetheinylation was initiated by adding 0.8 μ Ci [³H-pantetheine]-CoA and carried out at 28 °C for 2 h. The reaction mixture was concentrated to 100 μ L with a centrifugal ultrafiltration unit (Amicon Ultra 15; 0.5 mL), and 12 μ L of each sample evaluated on a 12 % SDS-PAGE gel. The resulting gel was washed with deionized distilled water, dried between two sheets of cellulose membrane, exposed to X-ray film for a week (Imaging Plate BAS-MS 2025, Fujifilm), and visualized with a phosphorimager.

Reconstitution of holo-PKS AziB Activity and Analysis by Autoradiography

Cells from 5 L PKS expression cultures and 3 L Svp expression cultures were collected by centrifugation. The cultures were mixed and resuspended in 100 mL of PPT buffer and subsequently lysed by sonication. The cellular debris was removed by centrifugation at 9,500 rpm for 30 min. The supernatant, which contained both the apo-PKS and Svp, was filtered with a 0.2 μ m syringe filter to give ~90 mL of filtered protein extract. To posttranslationally modify the PKS and generate the holo-form, 4 mg pantetheine-CoA was added to the supernatant and the mixture was incubated at 28 °C for 1 h. After the reaction, the mixture was applied to a pre-equilibrated His-Trap FF 5 mL column (GE Healthcare Life Science), and using the same purification procedures for the apo-PKS, about 100 mg of purified holo-PKS was obtained. The purified holo-PKS was buffer exchanged into PKS reaction buffer (75 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 5 mM DTT, and 20% glycerol) by

centrifugal ultrafiltration (Amicon Ultra-15 Centrifugal Filter Unit) to a final concentration of 1 mg/mL.

To investigate the activity of the purified holo-PKS, 10 mg of the purified holo-PKS (in 10 mL PKS reaction buffer) was incubated with 150 μ L of co-factor solution (FAD, 1 mg; SAM, 1 mg; NADPH, 1 mg in 1 mL of deionized distilled water) and radiolabeled substrates (14 C-acetyl-CoA 0.1 μ Ci, 14 C-malonyl CoA 0.5 μ Ci) at 28 °C for 2 h. The reaction system was adjusted to pH 12 by treating with 0.5 M NaOH and incubated at room temperature for about 10 min to hydrolyze the thioester. The pH of the mixture was adjusted to pH 5 with 1 M HCl and extracted with 5 mL of dichloromethane. Organics were transferred to fresh tubes and evaporated to dryness. The organic residues were resolubilized in a minimal volume of dichloromethane (60 μ L) and applied to TLC (DCM : MeOH = 95 : 5). The PKS reaction products were visualized by autoradiography on X-ray film (Fuji Medical system).

Reconstitution of 2-Methylbenzoic Acid Formation by Phosphopantetheinylated AziB

To identify the major product produced by the holo-PKS, 80 mg of the purified holo-PKS (in 80 mL PKS reaction buffer) was incubated with 3 mg of unlabeled acetyl-CoA, 15 mg of malonyl-CoA and 1.0 mL of co-factor solution. The resulting reaction mixture was incubated at 28 °C for 12 h. The reaction system was adjusted to pH 12 by treating with 0.5 M NaOH and incubated at room temperature for about 10 min to hydrolyze the thioester. The pH of the mixture was adjusted to pH 5 with 1 M HCl and extracted with dichloromethane (100 mL). Organics were transferred to fresh tubes and evaporated to dryness for NMR analysis. The product was visualized by TLC by resolubilizing the compound in a minimal volume of dichloromethane and applying it to TLC (dichloromethane : methanol = 95 : 5).

Confirmation of the PKS, AziB by MALDI-MS

To confirm the identity of the PKS by MS, approximately 2 mm protein bands were excised from a polyacrylamide gel stained with Coomassie Blue. The excised gel bands were digested following standard protocols. Briefly, the method involved a series of washing and dehydration steps with 25 mM ABC and ACN, prior to digestion with trypsin at 37 °C for 4–12 h. The resulting tryptic peptides were combined with matrix (α -cyano-4-hydroxycinnamic acid), mixed thoroughly and spotted onto a MALDI plate for analysis. MS data were acquired using the reflection detector in positive mode using 25 laser shots (25 shots per sub-spectrum). Tandem mass spectra were acquired in positive ion mode using a Model 4800 Proteomics Analyzer (Applied Biosystems, Framingham, Massachusetts, USA). CID acceleration of 2kV was used and the collision gas was atmospheric. The mass spectra obtained in these experiments were analyzed using Data ExplorerTM. Fragment ions are labeled using the nomenclature suggested by Roepstorff and Fohlman as modified by Biemann. All MS and MS/MS data were searched against the Swiss-Prot protein sequence database using the GPS Explorer (Applied Biosystems) software

Cloning of *aziA7*

Two oligonucleotides, AziA7-F and AziA7-R (Table S1), containing *Eco*RI and *Hind*III sites, respectively, were used to amplify the *aziA7* gene from the genomic DNA of *S.*

sahachiroi. The PCR conditions were as follows: 4 µL of 5X Phusion buffer, 0.4 µL of 10 mM of dNTPs, 1 µL of each primer (10 µM), 0.2 µL Phusion High Fidelity DNA Polymerase, 1 µL of *S. sahachiroi* genomic DNA (15 ng) and nuclease free water to 20 µL. The PCR thermocycler conditions were as follows: initial denaturation at 98°C for 30 sec, 30 cycles of denaturation at 98°C for 10 sec, annealing at 62°C for 1 min, and polymerization at 72°C for 1 min. The DNA fragment of *aziA7* was purified by agarose gel extraction using the QIAquick Gel Extraction Kit (Qiagen), and subsequently digested with restriction enzymes *EcoRI* and *HindIII*. Following purification by agarose gel extraction, the DNA fragment was ligated into the *EcoRI/HindIII* multiple cloning site of expression plasmid vector pET-24a, which contains a 6xHis-tag at the 3'-end. Cloning of the gene was confirmed by DNA sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit and the cloned plasmid transformed into *E. coli* expression strain BL21(DE3) for overexpression.

Cloning of *aziA8*

Oligonucleotide primers, AziA8F and AziA8R (Table S1), containing *EcoRI* and *HindIII* sites, respectively, were used to amplify the *aziA8* gene from the genomic DNA of *S. sahachiroi*. The PCR conditions were as follows: 4 µL of 5X Phusion buffer, 0.4 µL of 10 mM of dNTPs, 1 µL of each primer (10 µM), 0.2 µL Phusion High Fidelity DNA Polymerase, 1 µL of *S. sahachiroi* genomic DNA (15 ng) and nuclease free water to 20 µL. The PCR thermocycler conditions were as follows: initial denaturation at 98 °C for 30 sec, 30 cycles of denaturation at 98 °C for 10 sec, annealing at 62 °C for 1 min, and polymerization at 72 °C for 1 min. The DNA fragment of *aziA8* was purified by agarose gel extraction using the QIAquick Gel Extraction Kit (Qiagen), and subsequently digested with restriction enzymes *EcoRI* and *HindIII*. Following purification by agarose gel extraction, the DNA fragment was ligated into the *EcoRI/HindIII* multiple cloning site of expression plasmid vector pET-24a, which contains a 6xHis-tag at the 3'-end. Cloning of the gene was confirmed by DNA sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit and the cloned plasmid transformed into *E. coli* expression strain BL21(DE3) for overexpression.

Cloning of *aziG*

Two oligonucleotides, AziG-F and AziG-R (Table S1), containing *EcoRI* and *HindIII* sites, respectively, were used to amplify the *aziG* gene from the genomic DNA of *S. sahachiroi*. The PCR conditions were as follows: 4 µL of 5X Phusion buffer, 0.4 µL of 10 mM of dNTPs, 1 µL of each primer (10 µM), 0.2 µL Phusion High Fidelity DNA Polymerase, 1 µL of *S. sahachiroi* genomic DNA (15 ng) and nuclease free water to 20 µL. The PCR thermocycler conditions were as follows: initial denaturation at 98 °C for 30 sec, 30 cycles of denaturation at 98 °C for 10 sec, annealing at 62 °C for 1 min, and polymerization at 72 °C for 1 min. The DNA fragment of *aziG* was purified by agarose gel extraction using the QIAquick Gel Extraction Kit (Qiagen), and subsequently digested with restriction enzymes *EcoRI* and *HindIII*. Following purification by agarose gel extraction, the DNA fragment was ligated into the *EcoRI/HindIII* multiple cloning site of expression plasmid vector pET-24a, which contains a 6xHis-tag at the 3'-end. Cloning of the gene was confirmed by DNA

sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit and the cloned plasmid transformed into *E. coli* expression strain BL21(DE3) for overexpression.

X-ray Crystallographic Analysis of AziG

A. AziG Overexpression, and Purification—The pET-24a-aziG vector was transformed into BL21(DE3) and plated on selective kanamycin (30 µg/mL) containing LB agar and incubated at 37 °C overnight. For each 1.5 L volume to be grown, a single colony was selected and transferred to 10 mL sterile LB medium supplemented with 30 µg/mL of kanamycin and shaken overnight at 37 °C. After approximately 18 h, 10 mL of overnight starter culture was added directly to 1.5 L volumes of sterile LB. The flasks were shaken at 200 rpm at 37 °C until the media reached an OD₆₀₀ of ~0.6, at which point the incubator temperature was turned down to 15 °C. After the incubator reached the induction temperature of 15 °C and the media OD₆₀₀ was ~0.8, solid IPTG was added to give a final concentration of 0.5 mM. After induction of protein expression by addition of IPTG, the cells were incubated with shaking for approximately 18 h prior to harvesting. The cells were harvested by centrifugation at 2000 g for 15 min and subsequently stored in 3 L aliquots, frozen at –20 °C until purification.

To purify the AziG protein, the 3 L cell pellet was thawed and resuspended in 45 mL of lysis buffer (50 mM Tris-HCl, 300 mM NaCl, and 20 mM imidazole at pH 7.5). The resuspended cells were lysed on ice with three rounds of sonication for 3 min, with 10 min off after each round. The lysed cell suspension was centrifuged at 40,000g for 30 min at 4 °C. The cleared lysate was decanted and loaded onto a 2 mL Ni-NTA column (Qiagen) pre-equilibrated with lysis buffer. Following loading of the cleared lysate, the column was washed with 50 mL of lysis buffer at a flow rate of 1 mL/min to remove non-specifically bound contaminants.

To elute AziG, 20 mL of elution buffer (50 mM Tris-HCl, 300 mM NaCl, and 250 mM imidazole at a final pH of 7.5) was loaded onto the column and the volume containing AziG was collected. AziG purity was assessed by SDS-PAGE gel electrophoresis. The sample was buffer exchanged into 10 mM Tris-HCl, 50 mM NaCl at a pH of 7.5 by overnight dialysis and concentrated to 10 mg/ml using a centrifugal filter device with a molecular weight cut off of 3,000 Da. The purified protein was aliquoted and flash frozen into liquid nitrogen for storage at –80 °C. This purification process yielded approximately 25 mg/L of purified cell culture.

B. Crystallization of AziG—The hanging drop vapor diffusion method was used for crystallization. In this method, the frozen protein aliquots were thawed on ice and equal volumes of protein and reservoir solution were mixed and equilibrated at 22 °C against a total volume of 500 µL well solution. Using the commercially available sparse matrix Wizard screens (Emerald Biosystems), three crystallization conditions were identified. Crystals were observed in Wizard I condition 13 (1.32 M ammonium sulfate and 0.1 M cacodylate pH 6.5), Wizard II condition 39 (0.1 M CAPS pH 10.5, 0.2 M NaCl, and 20% PEG 8,000), and Wizard III condition 33 (1.6 M magnesium sulfate and 0.1 M MES pH 6.5). Optimized conditions are: (1) 1.25 M ammonium sulfate and 0.1 M cacodylate pH 6.0; (2) 1.6 M magnesium sulfate and 0.1 M MES pH 5.5; and (2) 0.1 M CAPS pH 10.8, 0.2 M

NaCl, and 14% PEG 8000. In all three conditions, crystals with similar morphologies appeared overnight. The crystals were square bipyramidal and grew to maximum dimensions of $60\ \mu\text{m} \times 40\ \mu\text{m} \times 40\ \mu\text{m}$ in two weeks. The crystals were cryoprotected for data collection in a solution composed of their mother liquor supplemented with 10% (v/v) glycerol and 10% (v/v) ethylene glycol before flash freezing in liquid nitrogen. For the ligand complex, the crystals were soaked with 10 mM 5-methylnaphthoic acid for 30 min before cryoprotection and flash freezing.

C. X-ray Data Collection and Processing—X-ray diffraction experiments for the unliganded structure were conducted at CHESS (Cornell High Energy Synchrotron Source) beamline F1 using a Q270 detector (Area Detector Systems Corporation) at a distance of 305 mm and wavelength of $0.91830\ \text{\AA}$. The oscillation method was used with cryocooling with a 1° rotation and 10 s exposures per frame for a total of 90° . Crystals grown in all three conditions were tested for diffraction quality. The data sets reported are for crystals grown in the ammonium sulfate containing condition. The diffraction data for the 5-methyl naphthoic acid complex were collected at APS beamline 24-ID-C with cryocooling using a PILATUS 6MF pixel array detector (Dectris) at a distance of 400 mm and wavelength of 0.97919 , with 0.2° oscillation and 0.2 s exposure time per frame for a total of 90° . HKL2000⁹ and NE-CAT-developed automatic data processing pipeline RAPD were used for indexing, integration, and data scaling. Complete data collection and refinement statistics are listed in Table S3.

D. Structure Determination, Model Building, and Refinement—The SsAziG crystal belongs to space group $I4_122$ and contains one promoter per asymmetric unit and a Matthews's coefficient¹⁰ of $2.73\ \text{\AA}^3/\text{Da}$, corresponding to a solvent content of 54.9%. The structures of SsAziG were determined using the molecular replacement method, with *Mycobacterium tuberculosis* thioesterase (PDB ID: 3S4K) as the initial model. The program Chainsaw¹¹ was used to create a search model from 3S4K. Molecular replacement using this search model was conducted with MolRep.¹² Iterative rounds of model building were conducted using COOT,¹³ with refinement using RefMac5¹⁴ in initial rounds, and PHENIX.refine¹⁵ in final rounds. The quality of the final structure was assessed using MolProbity.¹⁶ Structure refinement for AziG and AziG/5-methyl naphthoic acid complex converged with $R_{\text{work}}/R_{\text{free}}$ of 22.2%/26.2% and 18.5%/21.8%, respectively. Complete refinement statistics for both structures are listed in Table S3.

Gel Filtration Analysis of AziG: Evaluation of its Native State

The native form of AziG in solution was assessed by gel filtration (size-exclusion) chromatography. Protein samples (standards and AziG, 1 mg of each) were injected into a pre-equilibrated (10 mM phosphate and 150 mM NaCl at pH 7.2) gel filtration column (Superdex 75 PC HiLoad 16/60, GE Healthcare) at room temperature. The elution volume was measured by detecting the sample elution at a UV absorbance of $\lambda\ 280\ \text{nm}$. The calibration curve was generated with protein standards: bovine serum albumin (molecular mass, 67 kDa; elution volume, 55.6 mL), ovalbumin (45 kDa; 61.0 mL), carbonic anhydrase (29 kDa; 69.2 mL), and cytochrome C (13 kDa; 81.6 mL) and plotting elution volume (mL) vs. molecular mass of standard (kDa).

The elution volume of native AziG was 55.6 mL, thus the molecular mass was calculated as 62 kDa. Since the theoretical mass of expressed AziG (containing plasmid restriction site and 6 × Histidine) is 15.8 kDa, it is predicted that the native state of AziG in solution is that of a tetramer (± 2 % error).

Gel Filtration Analysis of AziB-AziG Mixture: Evaluation of Stable Complex Formation

It was assessed by gel filtration whether AziB and AziG stably interact with each other in solution. Protein samples (AziB and AziB+AziG, 0.5 mg of each) were injected into a pre-equilibrated (10 mM phosphate, 150 mM NaCl, and 20% glycerol at pH 7.4) gel filtration column (Superdex 200 PG HiLoad 16/60, GE Healthcare) at room temperature. The elution volume was measured by detecting the sample elution at a UV absorbance of λ 280 nm.

Site-directed Mutagenesis of *aziG*

H44, H48, E57, S58 and S61 of AziG were mutated to alanine using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) on plasmid pET-24a-AziG. Primers containing point mutations were used to amplify mutated plasmids for each (Table S1). Primers for H44A, E57A, S58A, and S61A mutations were AziG-H44A-F and AziG-H44A-R, AziG-H48A-F and AziG-H48A-R, AziG-E57A-F and AziG-E57A-R, AziG-S58A-F and AziG-S58A-R, and AziG-S61A-F and AziG-S61A-R, respectively. The PCR reaction was performed in a 50 μ L reaction mixture consisting of: 1 μ L of *PfuUltra* HF DNA polymerase (Agilent Technologies, Santa Clara, CA, USA), 5 μ L of 10X reaction buffer, 1 μ L of dNTP mix, 10 ng of pET-AziG, 125 ng of each forward and reverse primer, and water to 50 μ L. PCR reactions utilized the following program: initial denaturation for 30 s at 95 °C and 12 cycles of 30 s at 95 °C, 1 min at 55 °C, and 6 min at 68 °C. The amplified plasmids were treated with *DpnI* restriction enzyme for 3 h to degrade the parent, wild-type methylated DNA. The mutated plasmids were transformed into *E. coli* DH10b by electroporation, and the mutations were confirmed by DNA sequencing. The plasmids were subsequently transformed into BL21(DE3) for overexpression.

Sample Preparation for Analysis by LC/MS and Evaluation of Kinetics

A. Overexpression of AziB, Svp, AziA6, AziA7, AziA8, AziG, and AziG Mutants
—AziB, Svp, AziA6, AziA7, AziA8, AziG, and AziG mutants were each overexpressed in the same manner. *E. coli* strains that contained their respective expression plasmids were cultured in 5×5 mL LB media for AziB, 3×5 mL for Svp, 4×5 mL for AziA6, and 5 mL for AziA7, AziA8, and AziGs overnight supplemented with the appropriate antibiotics (50 μ g/mL kanamycin for AziB, AziA6, AziA7, AziA8, and AziGs and 100 μ g/mL ampicillin for Svp). ~Note: the differing amounts of each culture reflect optimization to account for differences in expression levels and loss of protein during purification. Each of the 5 mL overnight cultures was inoculated into 1 L LB medium and grown until OD₆₀₀ 0.6–0.8. Production of proteins was induced with 1 mM IPTG and incubated at 16 °C, 250 rpm for 22 h. The resulting cells were harvested by centrifugation at 7000 rpm for 15 min. The cells were washed with deionized distilled water and the appropriate buffer: PPT buffer for AziB and Svp and column buffer (1 mM DTT, 500 mM NaCl, 5 mM imidazole, 50 mM potassium phosphate, pH 7.5, and 20% glycerol) for AziA6, AziA7, AziA8, and AziGs. The remaining

steps were performed on ice. The cells were resuspended in their respective buffers: AziA6 (30 mL), AziA7 (30 mL), AziA8 (30 mL), each AziG (30 mL) and AziB + Svp (a total of 100 mL), which was subsequently mixed together. PMSF was added to give a final concentration of 1 mM shortly before the cells were lysed by sonication. Sonication was carried out with a Branson Sonifier 450 (Branson Ultrasonics) fitted with a 5 mm micro-tip, output setting 6, duty cycle 60%, and 5 cycles for 30 s each. The cell debris was removed by centrifugation at 9,900 rpm for 45 min, twice and subsequently passed through a 0.4 μ m syringe filter. The protein solution was stored at 4 °C.

B. Posttranslational Modification of AziB with Svp and Coenzyme A—

Approximately 90 mL of apo-AziB and Svp crude protein extract was obtained following cell lysis and centrifugation. Apo-AziB was converted to the holo-form by adding 5 μ mol of coenzyme A, giving a final concentration of ~5.5 μ M. The sample was incubated at 28 °C, 250 rpm for 1 h. Any resulting precipitation formed during incubation was removed by centrifugation, 9,900 rpm for 45 min. Holo-AziB/Svp were purified by nickel affinity chromatography. A HisTrap FF 5 mL column was equilibrated with PPT buffer and subsequently loaded with the protein mixture at a rate of 0.25 mL/min. The column was washed with 200 mL of PPT column buffer (1 mM DTT, 500 mM NaCl, 40 mM imidazole, 75 mM Tris-HCl, pH 8.0, and 20% glycerol) and eluted with 30 mL of PPT column buffer supplemented with 250 mM imidazole. The buffer was exchanged to the AziB-AziG reaction buffer (50 mM potassium phosphate, pH 7.5, and 20 % glycerol). The purity and quantity of holo-AziB and Svp were assessed by 8 % (v/v) SDS-PAGE and Bradford assay utilizing the Bio-Rad Protein Assay kit (Bio-Rad Laboratories); bovine serum albumin (BSA) was used as a standard.

C. Purification of AziA6, AziA7, AziA8, AziG, and AziG mutants—The crude solutions, which contained AziA6, AziA7, AziA8 or AziGs were applied to pre-equilibrated HisTrap FF 5 mL columns at 0.25 mL/min. The loaded columns were treated as follows:

For AziA6, the column was washed with 200 mL of column buffer supplemented with 40 mM imidazole, and eluted with 30 mL of column buffer supplemented with 250 mM imidazole

For AziA7 and AziA8 the columns were washed with 240 mL of column buffer supplemented with 10 mM and 20 mM imidazole each and eluted with 50 mL of column buffer supplemented with 100 mM imidazole

For AziG and its mutants the column was washed with 200 mL of column buffer supplemented with 60 mM imidazole and eluted with 30 mL of column buffer supplemented with 250 mM imidazole

The buffer was exchanged to AziB-AziG reaction buffer (potassium phosphate, 50 mM; glycerol, 20%; pH 7.5) by centrifugal ultrafiltration units for 10 kDa (Amicon Ultra - 15) except for AziA6. An additional purification step was required for AziA6. The buffer was exchanged to sephadex column buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.5). A Sephadex G-50 fine size exclusion column, 1 m height, with ~200 mL bed volume was equilibrated with Sephadex column buffer. The AziA6 solution was concentrated to 1.5 mL

and loaded onto the column. Fractions were collected every 3 mL and were later analyzed for quantity and purity by Bradford assay and 12% (v/v) SDS-PAGE. The purified AziA6 fractions were combined together and the buffer was exchanged to AziB-AziG reaction buffer.

Reconstitution of 5-Methylnaphthoic Acid Formation

Acetyl CoA (50 μ M), malonyl CoA (250 μ M), NADPH (10 μ M), AziB-Svp (10 mg, section B), and ~10 mg of AziA6 or AziG were combined in 25 mL of AziB-AziG reaction buffer. The reaction was incubated at 30 °C overnight at 250 rpm. The reaction solution was filtered with centrifugal ultrafiltration units (3 kDa MW cutoff) and the filtrate was extracted with an equal volume of ethyl acetate three times. After drying the organics over anhydrous sodium sulfate, the sample was evaporated to dryness with a gentle flow of dry nitrogen gas.

LC/MS Analysis of *In vitro* Reaction Products

Product formation was analyzed by LC/MS. Samples were dissolved in 100 μ L of methanol, and 10–30 μ L of the sample was injected onto the reversed-phase LC/MC equipped with a Phenomenex column (Columbus 5 μ C8 100A, 250 $\times$ 3.20 mm 5 μ), which was pre-equilibrated with 80% A (water) and 20% B (75% methanol and 25% isopropanol). LC/MS conditions were as follows: Time 0 min A-80% B-20%; 1 min A-80% B-20%; 15 min A-0% B-100%; 30 min A-0% B-100%; 32 min A-80% B-20% till 40 min, flow rate 0.75 μ L/min. Sample elution was monitored by UV at λ 254 nm and by mass, 136 m/z for 2-methylbenzoic acid and 187 m/z for 5-methyl-1-naphthoic acid.

The synthetic compound standard, 5-methylnaphthoic acid, was either treated directly with AziB-AziG reaction buffer or treated first with acid or base prior to the addition of the buffer. These three samples and untreated compound were analyzed by LC/MS as detailed above. The base treated sample gave a single peak, which was also shown in the AziB-AziG reaction sample; co-injection confirmed the identity of the peak as 5-methylnaphthoic acid.

Synthesis of 5-Methyl-1-Naphthoic Acid

tert-Butyl 5-bromo-1-naphthoate¹⁷—Oxalyl chloride (0.4 mL) was added to a suspension of 5-bromo-1-naphthoic acid (1.08 g) in dichloromethane (9.3 mL) containing 3 drops of dimethylformamide and the reaction mixture was stirred at ambient temperature for 6 h. The solution was concentrated to afford a white solid, which was re-dissolved in tetrahydrofuran (THF). The solution was cooled to 2 °C and a solution of potassium tert-butoxide (0.72 g) in THF (14.8 mL) was added dropwise and stirred at ambient temperature for 3 h. The solution was poured onto ice (20 g) and extracted with ethyl acetate (2 $\times$ 10 mL). The combined organic extracts were washed with water (10 mL), dried over sodium sulfate, and concentrated in vacuo. The residue was purified by flash column chromatography (cyclohexane:ethyl acetate = 1:1) to give 5-bromo-1-naphthoic acid tert-butyl ester in 91 % yield (12 g). ¹H NMR (300 MHz, CDCl₃): δ _H 8.86 (d, J = 9.0 Hz, 1H), 8.48 (d, J = 8.7 Hz, 1H), 8.13 (dd, J = 7.3, J = 0.9 Hz, 1H), 7.86 (dd, J = 7.5, J = 0.9 Hz, 1H), 7.62 (dd, J = 8.6, J = 7.5 Hz, 1H), 7.45 (dd, J = 8.7, J = 7.5 Hz, 1H), 1.71 (s, 9H).

tert-Butyl 5-methyl-1-naphthoate¹⁸—To a solution of 5-bromo-1-naphthoic acid tert-butyl ester (1.00 g, 3.26 mmol) in THF (10 mL), -78°C , was added n-butyllithium (2.5 M in hexanes, 1.56 mL) over a period of 2 min. After stirring for 40 min, methyl iodide (0.55 g) was added. This reaction mixture was allowed to warm to room temperature over 1.5 h. The reaction was quenched with water (10 mL) and partitioned between water and diethyl ether (3×50 mL). The combined organic extracts were dried over MgSO_4 and concentrated *in vacuo*. Purification of the residue by flash-chromatography (hexanes:EtOAc = 98:2) gave compound (3) in 84% yield (660 mg). $^1\text{H-NMR}$ (300 MHz, CDCl_3): δ_{H} 8.70 (d, $J = 8.7$ Hz, 1H), 8.20 (d, $J = 8.7$ Hz, 1H), 8.07 (dd, $J = 7.3$, $J = 1.2$ Hz, 1H), 7.56 (d, $J = 7.2$ Hz, 1H), 7.54–7.48 (m, 1H), 7.42–7.37 (m, 1H), 2.75 (s, 3H), 1.72 (s, 9H).

5-Methyl-1-naphthoic acid¹⁸—5-Methynaphthalene-1-carboxylic acid tert-butyl ester (1.37 g, 4.45 mmol) was dissolved in dichloromethane (10 mL), and trifluoroacetic acid (3 mL) was added. After stirring for 16 h, the reaction was concentrated *in vacuo* to give compound (4) in quantitative yield. $^1\text{H-NMR}$ (300 MHz, CD_3OD): δ_{H} 8.73–8.74 (d, $J = 8.7$ Hz, 1H), 8.28 (d, $J = 8.4$ Hz, 1H), 8.18 (dd, $J = 7.2$, $J = 1.2$ Hz, 1H), 7.58 (dd, $J = 8.5$, $J = 7.2$ Hz, 1H), 7.52–7.44 (m, 1H), 7.44–7.36 (m, 1H), 2.73 (s, 3H). $^{13}\text{C NMR}$ (125 MHz, CD_3OD): δ_{C} 20.0, 125.0, 125.5, 125.8, 128.0, 128.1, 130.0, 130.4, 132.6, 134.3, 135.9, 171.5. MS (ESI): m/z calculated for $[\text{M-H}^+]$ $\text{C}_{12}\text{H}_9\text{O}_2$ 185.0603, found 185.0611.

Kinetics of Hydrolysis Reaction Catalyzed by AziG—The kinetic parameters of AziG and its mutants were measured as the formation of 2-nitro-5-thiobenzoic acid (TNB) at 412 nm ($\epsilon = 8,857.1 \text{ M}^{-1}\text{cm}^{-1}$) as a function of time. The reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent), reacts with free thiol produced by hydrolysis of 5-methyl-1-naphthoate-SNAC by AziGs, resulting in the TNB product. Absorbance readings were measured on a GENESYSTM 2 UV-Vis Spectrometer (Thermo Fisher Scientific). The reaction was carried out in AziB-AziG reaction buffer without glycerol, which contains 0.4 mM of DTNB, 20 μM of AziGs, 0.1–0.5 mM of the substrate, and 5% DMSO.

Three types of buffered solutions consisting of DTNB alone, AziG + DTNB, or substrate + DTNB were used to establish a control because DTNB and the substrate are chemically degraded by water molecules and AziG contains cysteine residues, which can react with DTNB. The effect of chemical degradation of DTNB, which was counted twice on the absorbance of two solutions ($A_{\text{AziG-DTNB}}$ and $A_{\text{substrate-DTNB}}$) can be eliminated by utilizing the following equation:

$$A_{\text{control}} = A_{\text{AziG-DTNB}} + A_{\text{substrate-DTNB}} - A_{\text{DTNB}}$$

The control was used to correct these undesired effects with the following equation:

$$A_{\text{corrected}} = A_{\text{AziG+substrate+DTNB}} - A_{\text{control}}$$

The absorbance was observed continuously every 30 s, and the rates of hydrolysis were determined from the initial linear component of the curves. The assays were performed at

least 4 times for each data point. DMSO was used to solubilize the substrate. Since the solubility was highly limited even in 5% DMSO, the velocity could be calculated only at low substrate concentrations. Therefore, the limited case of Michaelis–Menten kinetics where $[S] \ll [K_m]$ was applied to obtain k_{cat}/K_m values as the slope of the plotted linear graph of $V/[E]$ vs. $[S]$ by the following equation:

$$\frac{V}{[E]} = \frac{k_{cat}}{K_m} [S]$$

where V is velocity, $[E]$ is concentration of AziGs, and $[S]$ is concentration of the substrate.

Molar absorptivity of DTNB:

The molar absorptivity of TNB was calculated with the Beer-Lambert law:

$$A = \epsilon cl$$

where A is the absorbance, ϵ is the molar absorptivity ($M^{-1} \text{ cm}^{-1}$), c is the concentration (M), and l is the path length (cm). Since the path length is 1 cm, the molar absorptivity was calculated as the slope of a linear graph of A vs. c .

The absorbance of TNB (412 nm) was measured, varying the substrate (5-methyl-naphthoate-SNAC) concentration. The reaction mixture contained 50 mM potassium phosphate at pH 7.5, 0.4 mM DTNB, 5% DMSO, and varying concentrations of 5-methyl-naphthoate-SNAC (0.1, 0.25, 0.4, and 0.5 mM).

Assaying for Background Hydrolytic Activity—As a negative control, to assay for contaminating hydrolytic activity, the AziG purification procedure was carried out with *E. coli* BL21(DE3) harboring pET-24a (no insert). The eluted column buffer solution was subjected to kinetic analysis as detailed above.

Synthesis of 5-Methyl-1-Naphthoate-SNAC Ester—5-methyl-1-naphthoic acid (55 mg, 0.3 mmol), (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (114 mg, 0.6 mmol), DMAP (37 mg, 0.3 mmol), N-acetylcysteamine (SNAC) (35 mg, 0.3 mmol) and triethylamine (60 mg, 0.6 mmol) were stirred in dichloromethane (10 mL) under argon at room temperature overnight. The organic layer was washed with saturated NaHCO_3 solution, 0.1 N HCl solution and brine (10 mL of each). The organics were dried over anhydrous sodium sulfate, concentrated *in vacuo* and purified by flash column chromatography (dichloromethane) to provide pure white solid product in 81% yield (70 mg). ^1H NMR (300 MHz, CDCl_3): δ 1.99 (s, 3H), 2.71 (s, 3H), 3.27 (t, $J = 6.3$ Hz, 2H), 3.59 (q, $J = 6.3$ Hz, 2H), 6.11 (s, 1H), 7.38 (d, $J = 7.2$ Hz, 1H), 7.44–7.55 (m, 2H), 8.01 (d, $J = 7.2$ Hz, 1H), 8.19 (d, $J = 8.4$ Hz, 1H), 8.28 (d, $J = 8.7$ Hz, 1H). ^{13}C NMR (75 MHz, CDCl_3): 195.05, 170.4, 135.7, 134.7, 132.9, 129.2, 129.0, 127.7, 127.5, 127.3, 124.3, 123.2, 39.7, 29.5, 23.3, 19.8. MS (ESI): m/z calculated for $[\text{C}_{16}\text{H}_{17}\text{NO}_2\text{S} + \text{Li}^+]$ 294.1140, found 294.113

RESULTS AND DISCUSSION

Evaluation of the azinomycin biosynthetic gene cluster by BLAST analysis reveals the presence of a Type I iterative PKS, denoted *aziB* containing KS-AT-DH-KR-ACP catalytic domains.^{3,19} *In vivo* experiments on this PKS have suggested that AziB alone is responsible and sufficient for the biosynthesis of 5-methylnaphthoate.²⁰ The AziB protein was successfully cloned and overexpressed in *E. coli* giving a 187 kDa His-tagged apo-enzyme form of the PKS that was successfully purified by Ni-NTA chromatography (Fig. S1). Reconstitution of PKS activity was initially demonstrated by incubation of AziB with *S. sahachiroi* cell-free extracts (CFE). Conversion to the azinomycin naphthoate was detected by the incorporation of ¹⁴C-malonyl coenzyme A (MAL CoA) as shown by TLC/autoradiographic analysis and HPLC/scintillation counting (Figure 2 and Fig. S3–S4).

An enhancement in naphthoate production was observed when apo-AziB was added to CFE. As PKS activity was successfully reconstituted by incubation with *S. sahachiroi* CFE, we sought to activate the PKS directly with a phosphopantetheinyl transferase (PPTase). Activation was successfully achieved by incubation of AziB with the PPTase from *Streptomyces verticillus*²¹ and coenzyme A as shown by use of ³H-pantetheine CoA and autoradiographic analysis (Fig. S2). The AziB holo-PKS was evaluated for the production of 5-methylnaphthoic acid by incubation with radiolabeled precursors and NADPH (Figure 3A). Following treatment of the reaction mixture with base and subsequent neutralization, the resulting metabolites were extracted and the products visualized by TLC/autoradiography. In comparison to the control lanes, lanes 5 and 6 (duplicate samples) showed the appearance of a prominent spot (highlighted in red); the R_f value was significantly lower than that shown for naphthoic acid shown in lane 7. The *in vitro* reaction was thus performed in large scale with unlabeled substrates, which led to the production of one primary metabolite (Figure 3B). Structural characterization of the compound by NMR (Fig. S6–S7) and MS revealed the compound as 2-methylbenzoic acid, a truncated PKS product. Evaluation of AziB by MALDI-MS (Fig. S5) revealed that the protein was expressed intact and was not frame shifted or mutated. Hence, we hypothesized that a domain was likely missing; in the absence of the domain, we obtain 2-methylbenzoic acid as a result of condensation between an acetate unit and 3 malonate units. In contrast, in the presence of the domain, 5-methylnaphthoic acid is generated, formed through condensation of an acetate unit with 5 malonate units. As thioesterase (TE) domains typically catalyze the release of a nascent fatty acid or polyketide chain from that of an FAS or PKS by direct hydrolysis or cyclization, we envisioned that a TE domain might catalyze this process.²² Evaluation of the azinomycin biosynthetic gene cluster reveals the presence of four putative TE domains: AziA6, AziA7, AziA8 and AziG, none of which are physically associated with the PKS. AziA6 (29.6 kDa) and AziA7 (27.4 kDa) are adjacent or associated with NRPS genes AziA3 and AziA4, respectively. AziA6, AziA7 and AziA8 (27.8 kDa) are each putative Type II thioesterases based upon protein sequence homology to known thioesterases. They each exhibit at least 32% identity and 47% similarity to a Type II thioesterase from *Amycolatopsis mediterranei*.²³ The TE domains are discrete units with proposed editing functions, *i.e.* possess the ability to release any mis-bound substrates or intermediates.²⁴ AziG is a comparatively smaller protein (14.4 kDa) of the thioesterase

superfamily. All four TE domains were individually cloned with His-tags, expressed in *E. coli* and subsequently purified by Ni-NTA chromatography. LC/MS analysis was performed on the holo-PKS reaction with and without each of the TE domains (Figure 4). Analysis of AziB alone gives an m/z of 137.07 [M + H], corresponding to 2-methylbenzoic acid and was confirmed by co-injection (Fig. S9). Incubation of AziB with AziG showed a decrease in the formation of the 2-methylbenzoic acid peak with a new peak forming with an m/z of 187.14 [M + H]. LC/MS analysis of a synthetic standard utilizing the same buffer conditions, confirmed the production of 5-methylnaphthoic acid. This result was further corroborated by co-injection (Fig. S10). None of the other TE domains (AziA6, AziA7, AziA8) were capable of catalyzing the reaction (Fig. S11). Gel filtration analysis of AziB with AziG does not reveal a stable complex, which suggests that the complex is formed transiently or is below our level of detection (Fig. S14). The few available crystal structures of TE domains reveal an α/β hydrolase fold with the presence of a GX SXG motif and three canonical TE catalytic triad residues within the active site consisting of amino acids Ser-His-Asp.²⁵ Serine serves as the nucleophile to which the polyketide would be covalently bound, histidine serves as a general base deprotonating the serine and aspartate in its deprotonated state neutralizes the developing charge on the histidine.

By primary sequence comparisons, we could not identify a GX SXG motif or the Ser-His-Asp catalytic triad in AziG. The X-ray crystal structure of AziG was determined at 2.15 Å. As our biochemical assays suggested that AziG might be involved in generating the azinomycin naphthoate, AziG crystals were soaked in a solution containing 5-methylnaphthoic acid. Data were collected to 2.2 Å for the AziG/5-methylnaphthoic acid complex. AziG forms a tetramer with four 5-methylnaphthoic acid binding sites at the interface of two AziG chains (Figure 5A), as supported by gel-filtration analysis (Fig. S12–S13). The 5-methylnaphthoate bound structure reveals the active site locations at the AB and CD interfaces, with two binding sites in each interface. Each cavity is approximately 7 Å in diameter and 20 Å deep. The AziG protomer displays the characteristic hotdog fold in which the antiparallel β -sheet surrounds a long α -helix (Figure 5B). Three additional α -helices 1, 2 and 4 are short secondary structural elements of only three to five amino acids found within the long loop regions of AziG.

Within the active site, the 5-methylnaphthoic acid carboxy group interacts with Ser61, Glu57, Ser58, His48', and His44' (Figure 6, the prime designates amino acid residues within the opposing subunit). The AziG/5-methylnaphthoic acid bound structure enables us to identify possible catalytic residues. We have considered three possible mechanisms. We evaluated a serine hydrolase mechanism as TE domains from bacterial modular PKSs, fungal PKSs, NRPSs and human FAS have each been reported to follow a serine hydrolase mechanism involving Ser-His-Asp.^{25–28} BLAST analysis and a DALI structural search alignment against the PDB, however, do not reveal PKS, NRPS, or FAS TE domains as close relatives. Based upon these analysis tools AziG is most highly related to PaaI, a phenylacetic acid degradation protein from *Thermus thermophilus* (PDB ID: 1WLV),²⁹ and 4-hydroxybenzoyl-CoA thioesterase, from *Anthrobacter* sp. SU (PDB ID: 1Q4S), resulting in RMSD values of 1.1 and 1.3 Å, respectively.³⁰ These thioesterases both share 31%

sequence identity with AziG. As such we considered both a base catalyzed hydrolytic mechanism and a hydrolytic cleavage of an enzyme-anhydride intermediate mechanism.

All of the polar residues in the *aziG* active site shell were individually mutated. Hydrolytic activity of wild-type AziG and its respective mutants was assayed utilizing a DTNB (5,5'-dithio-2-nitrobenzoic acid) assay and the *N*-acetylcysteamine (NAC) analog of the azinomycin naphthoate. NAC thioesters have been shown to be effective surrogates for the natural ACP-bound substrates, where the NAC serves as a mimic of the phosphopantetheine (PPT) arm of the ACP. DTNB allows for the detection of released free thiol by monitoring the progress of the reaction spectrophotometrically at 412 nm. The reaction was carried out in the absence of any thiol reducing agents to decrease background absorbance. An assay of this type was utilized to evaluate the TE domain of the erythromycin polyketide synthase.³¹ However, the catalytic activity of wild-type AziG and its corresponding mutants was low (Table 1, Fig. S15–S16)). To further substantiate these results, we assayed for contaminating hydrolytic activity. No contaminating background activity was observed. Mutants resulted in a 2–3 fold reduction in rate and were not distinguishable. The low catalytic competency for the AziG catalyzed reaction ($k_{cat}/K_m = 1.1 \text{ M}^{-1}\text{s}^{-1}$), suggests that the active hydrolase requires association between AziB and AziG to form the fully active enzyme. Additional evidence for a complex, albeit a low affinity one, comes from the observation that the product of AziB is different in the presence and absence of AziG. We have tested 5-methylnaphthoyl-NAC as a substrate for AziB and see no clear enhancement in the hydrolysis rate suggesting that AziB requires covalently attached product in order for AziG activation to occur. However, we are currently unable to test this due to the low levels of phosphopantetheinylation of AziB. Experiments designed to evaluate these hypotheses are currently in progress.

CONCLUSION

The azinomycin PKS system is unique. While DNA annotation would lead one to expect that the polyketide synthase AziB would catalyze the formation of the naphthoate ring system (a two-membered aromatic ring system), a truncated single-ring product, 2-methylbenzoic acid, is formed instead. The thioesterase AziG behaves as a chain elongating and cyclization (CEC) domain and works in concert with AziB to enable the additional two rounds of chain extension needed to form the correct product, 5-methylnaphthoic acid. From an evolutionary perspective, the azinomycin PKS system appears to be a hybrid involving a Type I PKS and a Type II thioesterase domain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CEC domain	chain elongating and cyclization
PKS	polyketide synthase
FAS	fatty acid synthase
AT	acyl transferase
ACP	acyl carrier protein
KS	ketosynthase
KR	ketoreductase
DH	dehydratase
ER	enoyl reductase
TE	thioesterase
NRPS	non-ribosomal peptide synthetase
CFE	cell free extract
PPTase	phosphopantetheinyltransferase
SVP	<i>Streptomyces verticillus</i>
CoA	coenzyme A
NADPH	nicotinamide adenine dinucleotide phosphate
NAC	N-acetylcysteamine
DMAP	4-dimethylaminopyridine
DTNB	5,5'-dithio-2-nitrobenzoic acid
TNB	2-nitro-5-thiobenzoic acid
PDB	protein data bank
BLAST	basic local alignment search tool
TLC	thin layer chromatography
HPLC	high performance liquid chromatography
LC/MS	liquid chromatography/mass spectrometry

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Figure 1.
Chemical structures of the azinomycins with the naphthoate portion highlighted in red. The naphthoate is assembled by homologation of an acetyl CoA starter unit with 5 malonyl CoA units.

Figure 2.

Evaluation of PKS Activity with *S. sahachiroi* Cell-Free Extracts: [1] Boiled CFE + ^{14}C MAL CoA, [2] Boiled CFE + PKS + ^{14}C MAL CoA, [3] CFE + ^{14}C MAL CoA, [4] CFE + PKS + ^{14}C MAL CoA; assays run in triplicate (S1–S3)

Figure 3.

Reconstitution of AziB Activity *in vitro*. Panel A: [1] ^{14}C Ac CoA, [2] ^{14}C MAL CoA, [3] Boiled AziB + ^{14}C Ac CoA + ^{14}C MAL CoA, [4] Boiled AziB + ^{14}C Ac CoA + ^{14}C MAL CoA, [5] AziB + ^{14}C Ac CoA + ^{14}C MAL CoA, [6] AziB + ^{14}C Ac CoA + ^{14}C MAL CoA, [7] naphthoic acid control; Panel B: Iodine stained TLC plate showing a single primary product formed upon scale up and incubation with unlabeled substrates.

Figure 4. Ion selected chromatograms of PKS reaction mixtures: [A] 2-methylbenzoic acid peak, 137.07 [M + H] [B] 5-methylnaphthoic acid peak, 187.14 [M + H]. The orange trace in both panels represent the PKS, AziB alone and the green trace represents the PKS, AziB + TE domain, AziG.

Figure 5.
X-ray crystal structure of AziG. [A] The biological tetramer formed by AziG. [B] The monomer adopts the characteristic hotdog fold with the β -sheet of each monomer facing the core and the main α -helices facing outward.

Figure 6.
Depictions of AziG active site. 5-methylnaphthoic acid within the AziG active site

Table 1

Kinetic Analysis of wild-type AziG and its Mutants

AziG Thioesterase	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1}\text{s}^{-1}$)
WT	1.1 ± 0.04
H44A	0.56 ± 0.02
H48A	0.36 ± 0.03
E57A	0.40 ± 0.03
S58A	0.37 ± 0.03
S61A	0.46 ± 0.04