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A Capture Strategy for the Identification of Thio-Templated Metabolites

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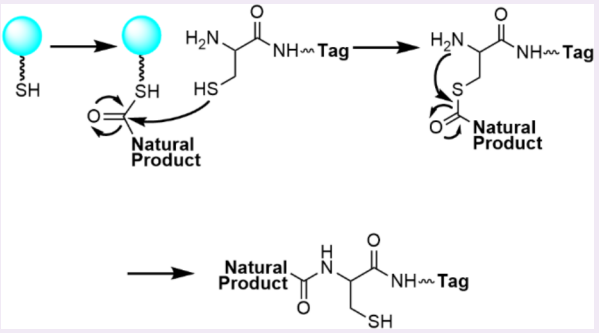
ABSTRACT: Nonribosomal peptide synthetase and polyketide synthase systems are home to complex enzymology and produce compounds of great therapeutic value. Despite this, they have continued to be difficult to characterize due to their substrates remaining enzyme-bound by a thioester bond. Here, we have developed a strategy to directly trap and characterize the thioester-bound enzyme intermediates and applied the strategy to the azinomycin biosynthetic pathway. The approach was initially applied *in vitro* to evaluate its efficacy and subsequently moved to an *in situ* system, where a protein of interest was isolated from the native organism to avoid needing to supply substrates. When the nonribosomal peptide synthetase AziA3 was isolated from *Streptomyces sahachiroi*, the capture strategy revealed AziA3 functions in the late stages of epoxide moiety formation of the azinomycins. The strategy was further validated *in vitro* with a nonribosomal peptide synthetase involved in colibactin biosynthesis. In the long term, this method will be utilized to characterize thioester-bound metabolites within not only the azinomycin biosynthetic pathway but also other cryptic metabolite pathways.



INTRODUCTION

Nonribosomal peptides (NRPs) and polyketides (PKs) represent two structurally diverse classes of natural products that include a large number of therapeutically relevant compounds such as antibiotics, antitumor agents, and immunosuppressants.[1−3] Nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) are multidomain proteins that employ carrier proteins with a phosphopantetheine arm to covalently bind substrates through a thioester bond.[3−5] PKSs utilize acyl-coenzyme A (CoA) substrates such as acetyl-, malonyl-, and methylmalonyl-CoA to assemble complex polyketides while NRPS substrates consist of amino acids and derivatives.[6,7] Once the catalytic cycle of the multidomain protein is complete, a thioesterase domain will hydrolyze the thioester bond between the phosphopantetheine arm and the substrate. This makes the metabolite available to the next NRPS/PKS in the biosynthetic pathway or releases it as the final product.[8−11]

Several methods are currently in use to characterize the enzymatic activity of these multidomain proteins and their thioester-bound enzyme intermediates. Thioesterase domains are used to catalyze product release *in vitro* but can be limited by identification of a thioesterase specific to the system.[12] Mass spectrometry based methods are used to produce a fragment containing the phosphopantetheine arm with the thioester-bound intermediate.[13−15] This method is limited by detection limits and the ability to produce this fragment, as many systems have been unable to do so. Acid/base hydrolysis can generate the free carboxylic acid of the intermediate. Not every system is susceptible to this hydrolysis or can withstand acid treatment without degradation. In turn, many of the free carboxylic acids can be difficult to detect and ionize by LC-MS.[16] Cleavage with a nucleophile has proven to be more effective than hydrolysis and allows for direct functionalization for LC-MS analysis. While these nucleophilic probes are more effective than hydrolysis, the sample will contain all compounds present in the reaction or cell environment, causing analysis to be difficult.[17−20] Here, we present a method in which a nucleophilic capture agent containing a purification tag is used to trap thioester-bound intermediates for characterization and apply this method to the azinomycin biosynthetic pathway.

The thioester intermediate capture strategy is based on native chemical ligation in which a cysteine thiol carries out a nucleophilic attack on the carboxy group of a thioester. A rearrangement then occurs to produce an amide bond.[21] The capture agent contains a cysteine attached to a tag, either an affinity tag or a fluorophore. The tag allows for purification or identification from a complex mixture of compounds that might be present in a protein reaction or cell lysate as well as

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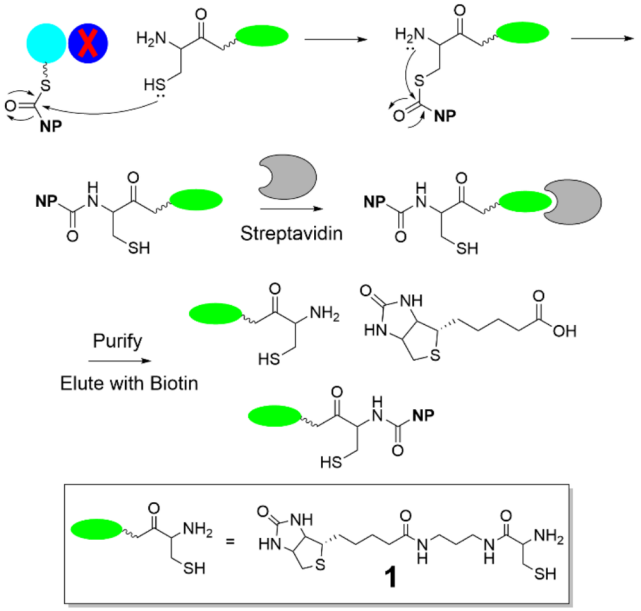
provides direct derivatization for LC-MS analysis. For this approach, the next protein in the biosynthetic pathway will be absent, causing a buildup of the intermediate on the protein of interest. This can be performed *in vitro* by leaving out the next protein in the pathway or by gene disruption in the native organism. Utilizing biotin as the tag allows for purification from the crude reaction using streptavidin (Scheme 1). Free biotin elutes the capture agents, producing a mixture of unreacted starting capture agent, biotin, and capture agent-trapped intermediate for analysis.

Scheme 1. Biotin-Cys Capture Strategy[a]



[a]NP = natural product.

The azinomycins (Figure 1) are NRP−PK and alkaloid hybrid natural products produced by the soil bacterium *Streptomyces sahachiroi* that exhibit potent antitumor activity.[22,23] Azinomycin A (**2**) exhibited an IC_{50} of 0.07 μg/mL against the leukemic cell line, L5178Y, while azinomycin B gave an IC_{50} of 0.11 μg/mL. Azinomycin B is more effective than azinomycin A *in vivo*, showing efficacy in 36 cases of malignant neoplasms as well as an increased life span of 193% at 32 μg/kg against P388 murine transplantable tumors.[24,25] Their activity comes from the ability to generate interstrand cross-links within the major groove of double-stranded DNA. The cross-links are formed between the electrophilic aziridine and epoxide rings of the azinomycins and the N7 position of the purine bases of DNA.[26−28] Time course analysis reveals the aziridine−DNA adduct forms first before the epoxide−DNA adduct.[29]

The naphthoate moiety of the azinomycins is produced by a Type I PKS, AziB, and a thioesterase, AziG. It is an unusual system as AziB alone produces a truncated, single ring product 2-methylbenzioic acid (**5**, Figure 1) and necessitates AziG to generate the full two-ring, naphthoate moiety.[30] On the basis of a feeding study with ^{13}C labeled substrates, biosynthesis of the epoxide moiety is thought to initiate from valine (Figure 1). Compounds **6**−**9** were each incorporated into the azinomycins, but the exact pathway from compound **9** to azinomycin is unknown.[31] A fermentation study using $^{18}O_2$

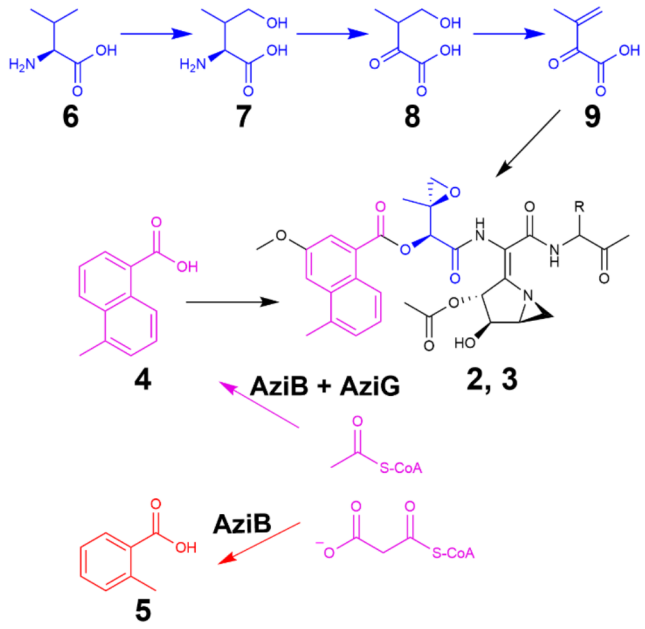


Figure 1. Azinomycins and biosynthetic origins of the naphthoate and epoxide moieties. Azinomycin A (**2**) R = H. Azinomycin B (**3**) R = CHOH.

suggests the epoxide is formed enzymatically, likely from a P450 or 2-oxoglutarate Fe-dependent oxygenase embedded within the azinomycin biosynthetic gene cluster.[32] Coupling of the epoxide moiety to that of the naphthoate necessitates that the ketone of **9** be reduced. The order of keto-reduction and epoxide formation is unknown.

RESULTS AND DISCUSSION

Development of the Capture Strategy. As an initial proof of principle experiment, we utilized the 2-methylbenzoic acid producer as an *in vitro* test system for the capture strategy. Four fluorophore capture agents were synthesized for evaluation (Figure 2). Two of the fluorophore capture agents (**10**, **11**) were shown to be prone to oxidation and unstable in the reaction buffer as observed by LC-MS (Figures S4 and S5). Pyrene-Cys (**12**) was the most stable, giving a reasonably clean

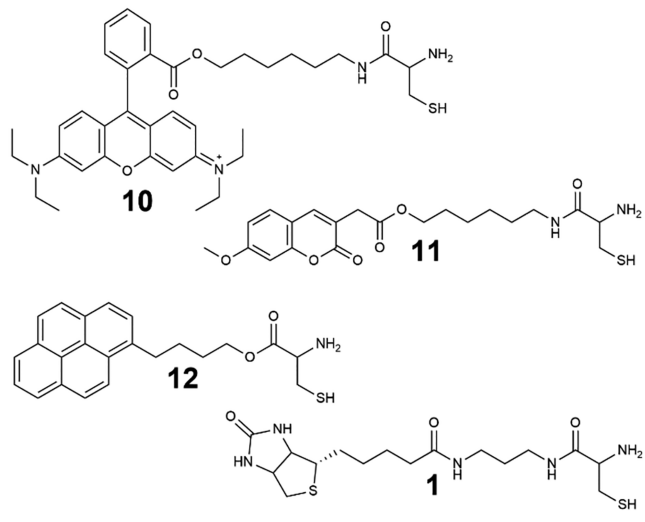


Figure 2. Capture agents.

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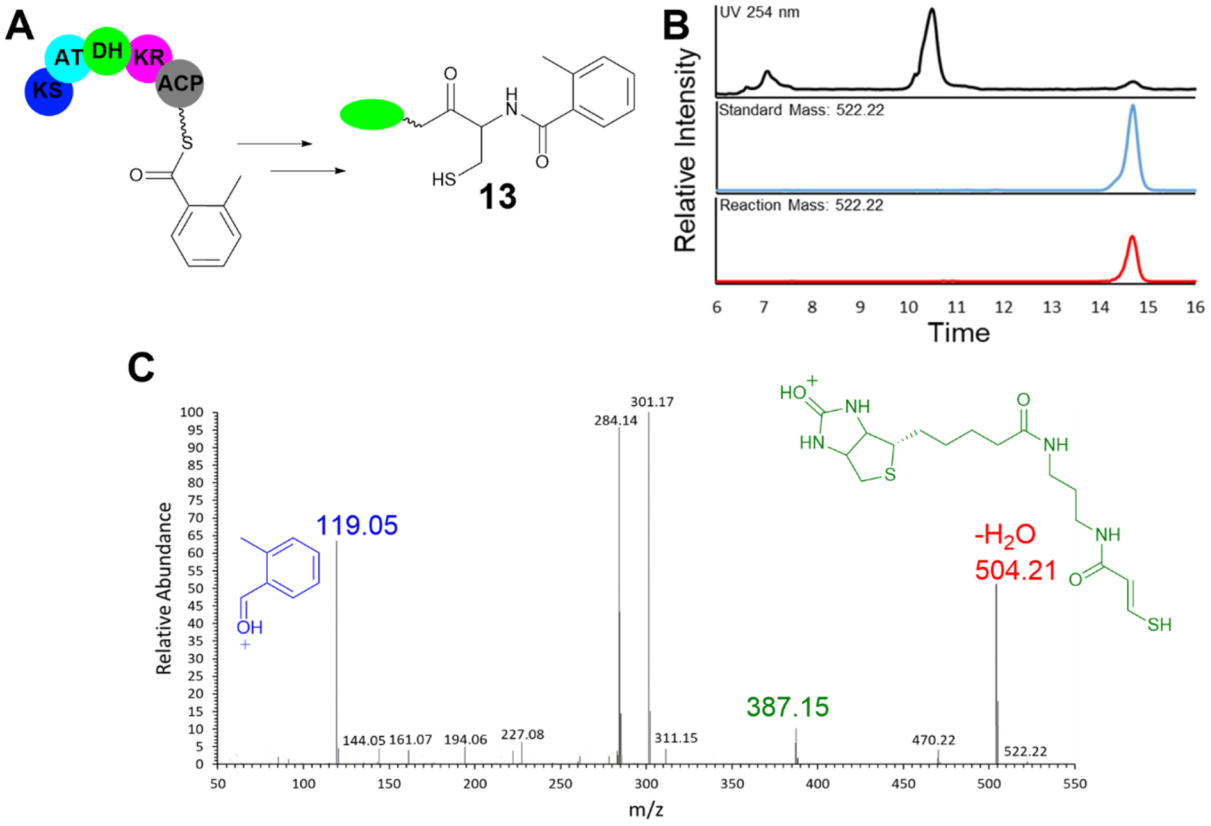

Figure 3. Biotin-Cys + AziB reaction and analysis. (A) Domain architecture of AziB with 2-methylbenzoic acid (**5**) bound to the acyl carrier protein domain. Domain abbreviations: ketosynthase (KS); acyltransferase (AT); dehydratase (DH); ketoreductase (KR); acyl carrier protein (ACP). (B) LC-MS traces of the biotin-Cys (**1**) reaction with AziB and synthetic standard of compound **13**. In the reaction trace, unreacted capture agent is seen at 7 min, and biotin from purification on the streptavidin column is seen at 10.5 min. (C) MS/MS fragmentation of 522.22 species at 14.7 min. Characteristic fragments are color coded.

profile. However, biotin-Cys (**1**) avoided these issues and offered an extra level of purification via streptavidin resin. It was, therefore, pursued as the primary capture agent.

Purified holo-AziB was incubated with its substrates to produce 2-methylbenzoic acid (**5**) bound to AziB as a thioester. Biotin-Cys (**1**) was then added to facilitate trapping of the intermediate, and the reaction passed through a streptavidin matrix for purification. The reaction was analyzed by LC-MS (Figure 3B). The formation of the product (**13**) was confirmed by LC-MS by mass ([M + H] 522.22) and by comparison to a synthetic standard of compound **13**. Additionally, MS/MS of the 522.22 species gives a characteristic fragmentation pattern for the expected compound (Figure 3C).

Evaluation of the Capture Strategy with AziA3. As the capture agent strategy was successful with AziB, we turned to evaluate an uncharacterized intermediate in the biosynthesis of the epoxide moiety. AziA3 is a NRPS containing a ketoreductase tailoring domain. On the basis of similarity to a NRPS in cereulide biosynthesis, it is believed AziA3 reduces the ketone of a small keto-acid to a hydroxy acid.[33] Due to low phosphopantetheinyl transferase post-translational modification efficiency with AziA3, *in vitro* reconstitution could not be pursued; additionally, the native substrate of AziA3 was unknown. To bypass identification of the AziA3 native substrate and *in vitro* post-translational modification, AziA3 was overexpressed and purified from *S. sahachiroi* with its intermediate of interest bound. To facilitate intermediate build up on AziA3, the gene for the thioesterase AziA6 was disrupted and AziA3 was overexpressed in the Δ*aziA6* strain (Figures S1−S3). Incubation of AziA3 with biotin-Cys (**1**) led to the trapping of compound **15** as characterized by compound **14** (Figure 4A). The presence of the cysteine thiol led to ring opening of the epoxide as confirmed by LC-MS ([M + H] 518.21) (Figure 4B), MS/MS (Figure 4C), and NMR (Figure S11); the presence of an electrophilic moiety lends itself to cyclization. The trapping of compound **15** demonstrates that AziA3 tethers the epoxide moiety and generates the alcohol, which would facilitate condensation with the naphthoate moiety.

Validation Outside the Azinomycin Pathway. To further evaluate the breadth of our approach, the biotin-Cys (**1**) capture agent was tested *in vitro* with the NRPS ClbN found in the colibactin biosynthetic pathway.[34] ClbN is responsible for production of the *N*-acyl-asparagine pro-drug scaffold, which is ultimately cleaved to give the final active molecule. This *N*-acyl asparagine pro-drug resistance mechanism is prevalent in NRPS containing gene clusters.[35,36] The NRPS ClbN accepts L-asparagine and performs a condensation reaction with an acyl-CoA substrate (Figure 5A). Purified ClbN was incubated with its substrates to produce octanoyl-asparagine as a thioester. The capture agent biotin-Cys (**1**) was subsequently added, and the resulting mixture was purified by streptavidin resin and analyzed by LC-MS (Figures S13−S16). Analysis confirmed the trapping of octanoyl-asparagine by the

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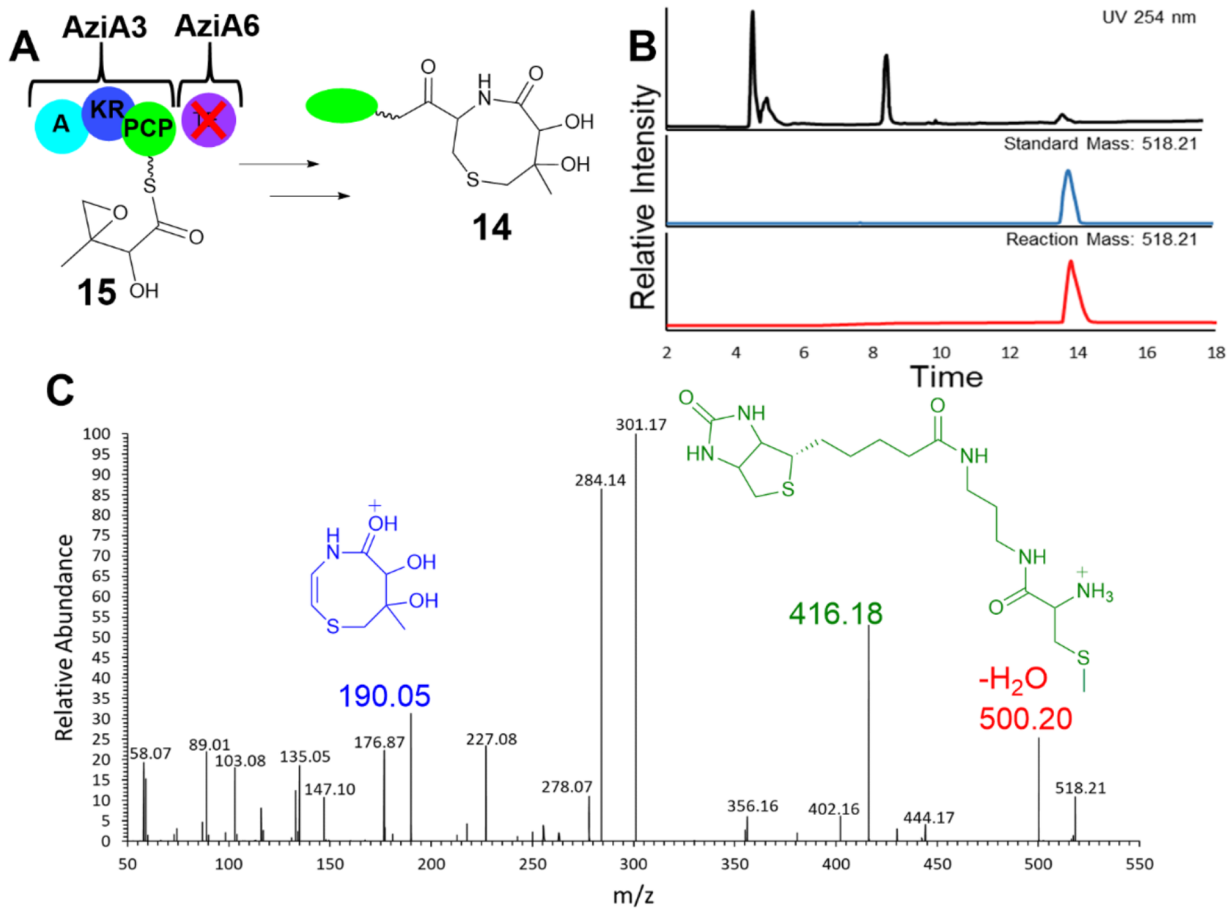

Figure 4. Biotin-Cys (**1**) + AziA3 reaction and analysis. (A) Domain architecture of AziA3 with compound **15** bound to the peptide carrier protein domain. Domain abbreviations: adenylation (A); ketoreductase (KR); peptide carrier protein (PCP). (B) LC-MS traces of the biotin-Cys (**1**) reaction with AziA3 and synthetic standard of compound **14**. In the reaction trace, the unreacted capture agent, disulfide dimer of the capture agent, and biotin from purification on the streptavidin column are seen at 4.2, 4.7, and 8.3 min, respectively. (C) MS/MS fragmentation of 518.21 species (compound **14**) at 13.8 min. Characteristic fragments are color coded.

capture agent in the form of compound **16** and the cyclized version compound **17** (Figure 5B).

CONCLUSION

We have developed an intermediate capture strategy that enables characterization of NRPS and PKS thioester-bound intermediates (*in vitro* or *in situ*) from their native organism. The presence of the biotin tag allows for the trapped intermediates to be purified from complex mixtures, greatly simplifying the analysis process. Application of this strategy to AziA3 and ClbN demonstrates the broad and general use of the approach. Using the intermediate capture strategy, AziA3 was determined to function in the final steps of epoxide moiety construction. Many factors hindered the *in vitro* identification of the AziA3 substrate and product, and the intermediate capture strategy provides an alternative method for characterization. This capture strategy will enable assignment of other enzymes within the azinomycin pathway that have eluded functional characterization as well as in the evaluation of metabolites from cryptic biosynthetic pathways. Ideally, this method will lend itself to the screening and identification of new bioactive natural products.

METHODS

General Procedures and Instrumentation. All chemicals were purchased from Sigma-Aldrich, and all bacterial media were purchased from Becton-Dickinson. Molecular biology reagents were obtained from New England Biolabs. RT-PCR reagents were purchased from Thermo-Fisher. ^{1}H and ^{13}C{^{1}H} NMR spectra were recorded on either a Bruker Avance 500 equipped with a cryoprobe or a Bruker Ascend 400. Deuterated solvents were purchased from Cambridge Isotope Laboratories. Yields are reported for chromatographically pure compounds unless otherwise stated. Mass spectra were obtained at the Laboratory for Biological Mass Spectrometry at the Department of Chemistry, Texas A&M University. A Phenomenex column (Prodigy 5 μm C18 150 Å, 150 × 4.6 mm) was used during HPLC and LC-MS purification and analysis. LC-MS was performed on a Thermo Scientific Ultimate 3000 UHPLC with a Thermo Fisher Scientific Q Exactive Focus mass spectrometer. Mass analysis of the synthesized compounds was performed on an ion trap mass spectrometer (LCQ-DECA, Thermo Fisher Scientific). HPLC purifications were performed on an Agilent 1260 HPLC with an automated fraction collector. The liquid chromatography solvent A consisted of water and 0.1% formic acid and solvent B consisted of 75% methanol, 24.9% isopropanol, and 0.1% formic acid. For method A, the column was pre-equilibrated with 80% A and 20% B. Solvent conditions were as follows: 0 min, 80% A, 20% B; 1 min, 80% A, 20% B; 23 min, 0% A, 100% B; 33 min, 0% A, 100% B; 35 min, 80% A, 20% B; 40 min, 80% A, 20% B. For the liquid chromatography method B, the column was pre-equilibrated with 90% A and 10% B.

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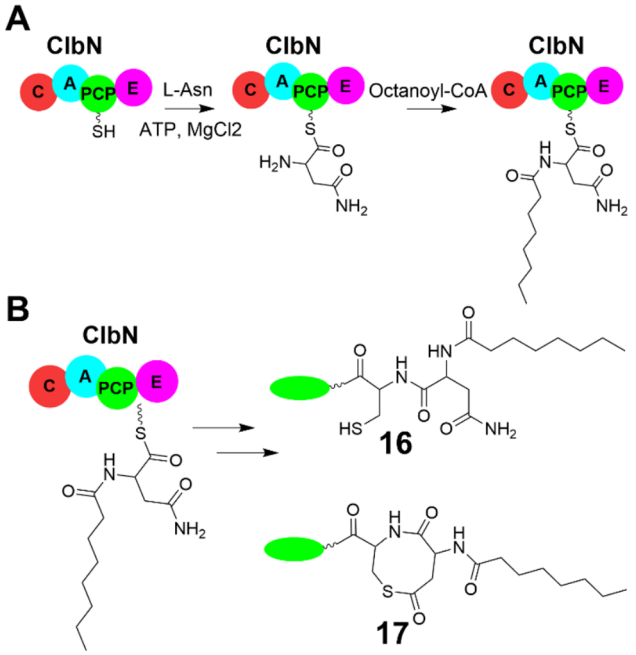

Figure 5. ClbN Reaction. (A) Condensation of L-Asn and octanoyl-CoA performed by ClbN. Domain abbreviations: condensation (C); adenylation (A); peptide carrier protein (PCP); epimerization (E). (B) ClbN reaction with biotin-Cys (**1**). Compound **16** ($[M + H]^+$ = 644.325) and compound **17** ($[M + H]^+$ = 627.299).

Solvent conditions were as follows: 0 min, 90% A, 10% B; 1 min, 90% A, 10% B; 5 min, 65% A, 35% B; 23 min, 5% A, 95% B; 28 min, 35% A, 65% B; 31 min, 90% A, 10% B; 40 min, 90% A, 10% B; flow rate, 0.75 μL min^{-1}.

Overexpression, Phosphopantetheinylation, and Purification of AziB. *E. coli* BL21 (DE3) containing pET-24-aziB was grown in LB medium with 50 μg mL^{-1} kanamycin overnight. The culture was used to inoculate 1 L of LB medium and cultured at 37 °C at 250 rpm until an OD_{600} of 0.6 was reached. Induction was performed by the addition of 1 mL of 1 M β-D-1-thiogalactopyranoside (IPTG). The cultures were incubated at 16 °C for 24 h at 250 rpm. The cells were harvested by centrifugation at 6500 rpm and resuspended in buffer containing 20 mM potassium phosphate, 500 mM NaCl, 1 mM dithiothreitol (DTT), 5 mM imidazole, and 20% glycerol, pH 7.4. Resuspended cells containing AziB were mixed with cells containing the phosphopantetheinyl transferase Svp. The cells were lysed by sonication and clarified using centrifugation. The supernatant containing AziB and Svp was used to post-translationally modify AziB to generate holo-AziB. Coenzyme A, 4 mg, was added to the supernatant, and the mixture was incubated at 28 °C for 1 h. After the reaction, the mixture was purified using a HisTrap FF 5 mL column. The purified protein was concentrated using a 50 kDa centrifugal ultrafiltration unit.

Capture Strategy with AziB and Rhodamine-Cys (6). Purified holo-AziB was buffer exchanged into reaction buffer containing 50 mM potassium phosphate, pH 7.5, and 20% glycerol. AziB (20 mg) was incubated with 50 μM acetyl coenzyme A, 250 μM malonyl coenzyme A, 10 μM dihydronicotinamide-adenine dinucleotide phosphate (NADPH), and 1 mM DTT at 28 °C for 12 h. Following incubation, guanidine hydrochloride (8 M) was used to denature the protein. Rhodamine-Cys (**10**) was added in a 10:1 molar excess to AziB alongside 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and the mixture was incubated in the dark for 12 h. The reaction was extracted with 10 mL of dichloromethane (DCM), and the solvent was removed *in vacuo*. As a control, rhodamine-Cys (**10**) was incubated in the dark for 12 h in reaction buffer with 1 mM TCEP and 8 M guanidine hydrochloride and extracted with DCM. The extracted compounds were analyzed by LC-MS using method A. The sample was monitored by UV at λ = 550 nm.

Evaluation of Fluorophore Capture Agents. To evaluate the stability of the fluorophore containing capture agents (**10**, **11**, **12**), each agent was incubated in reaction buffer with 1 mM TCEP for 12 h in the dark after which the reaction was lyophilized and analyzed by LC-MS following method A. The sample was monitored by UV (rhodamine-Cys (**10**) λ = 550 nm, coumarin-Cys (**11**) λ = 330 nm, pyrene-Cys (**12**) λ = 330 nm). The stability was determined by the presence of species other than the starting capture agent in the HPLC trace and by mass.

General Capture Strategy Protocol with Biotin-Cys (1). Purified holo-enzyme was buffer exchanged into reaction buffer containing 50 mM potassium phosphate, pH 7.5, and 20% glycerol. Following product formation, guanidine hydrochloride (8 M) was used to denature the protein. Biotin-Cys (**1**) was added in a 10:1 molar excess to the enzyme alongside 1 mM TCEP, and the mixture was incubated for 16 h. Biotinylated compounds were purified from the crude reaction mixture using Roche Streptavidin Mutein Matrix following the manufacturer's instructions. Eluted fractions were analyzed by LC-MS. The sample was monitored by UV at λ = 254 nm.

Capture Strategy with AziB and Biotin-Cys (1). AziB (20 mg) was incubated with 50 μM acetyl coenzyme A, 250 μM malonyl coenzyme A, 10 μM NADPH, and 1 mM DTT at 28 °C for 12 h for product formation. Eluted fractions were analyzed by LC-MS following method A. The reaction was compared to a control lacking AziB and a synthetic standard of compound **13**.

Construction of Disruption Plasmid pKCAziA6. An upstream fragment and downstream fragment to *aziA6* was amplified from the genomic DNA of *S. sahachiroi*. The upstream fragment was amplified using Taq 2× Master mix and primers AziA6UF/AziA6UR and AziA6DF/AziA6DR (Table S1). pGEM-Teasy cloning was utilized, and the upstream and downstream products were digested with XbaI/*Hin*dIII and XbaI/*Bam*HI, respectively, to clone into the corresponding site within the pKC1139 plasmid to generate pKCAziA6.

Generation of Δ*aziA6*. Intergenic conjugal transformation was used to introduce the pKCAziA6 plasmid to *S. sahachiroi* to facilitate gene deletion by homologous recombination. The pKCAziA6 plasmid was transformed into *E. coli* S-17 cells. An overnight culture of pKCAziA6/S17 cells was grown and washed twice with LB medium and resuspended in 600 μL of 2xYT medium. *S. sahachiroi* spores from 10 GYM agar plates were collected using 2xYT medium. The collected spores were subjected to heat shock at 65 °C for 10 min and incubated at 37 °C for 3 h. Following incubation, the spores were collected and washed with 20 mL of 2xYT two times and finally resuspended in 4 mL of 2xYT. The recipient cells were mixed with 600 μL of pKCAziA6/S17 donor cells, and 800 μL of the mixture was plated on ISP4 agar plates. The plates were incubated at 28 °C and overlaid with 1 mL of water containing nalidixic acid (500 μg) and apramycin (70 μg) to select for *S. sahachiroi* exconjugants after 24 h. Incubation continued for 10 days to allow for the appearance of apramycin resistant exconjugants. Exconjugants were screened for several generations until the double crossover allelic exchange was detected by the presence of apramycin sensitivity (Figure S2).

The Δ*aziA6* strain was confirmed by PCR, Southern Blot, and RT-PCR (Figure S3). PCR was used to screen for the loss of the apramycin resistance gene (AprF/AprR primers) as well as the *aziA6* gene (AziA6F/AziA6R primers). Southern Blot was performed using a Roche DIG-High Prime DNA Labeling and Detection Starter Kit I. Digestion of genomic DNA was performed with *Sal*I to generate characteristic DNA fragments. Taq 2× Master mix was used to generate the probe using primers AziA6-SBF and AziA6-SBR. RT-PCR was performed using Superscript IV First Strand Synthesis Kit. *S. sahachiroi* RNA was isolated following a previously published protocol.[37] Oligo$(dT)_{20}$ primer was used to generate cDNA. To evaluate the expression of *aziA6*, *aziA3* (upstream), *aziA4* (downstream), and *aziB* (control), cDNA was used as the template for PCR and primer pairs AziA6RTF, AziA6RTR AziA3F, AziA3R, AziA4F, AziA4R, AziBF, and AziBR were used (Table S1).

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Construction of Overexpression Plasmid pSETAziA3. Oligonucleotide primers pSETAziA3F and pSETAziA3R were used to amplify *aziA3* from *S. sahachiroi* genomic DNA. The primers incorporated restriction sites (XbaI and *Bgl*II) for cloning as well as a polyhistidine tag. PCR was performed with Phusion Master Mix polymerase. The PCR product and pSET152 plasmid were digested using XbaI and *Bgl*II and ligated together using T4 DNA ligase.

Generation of the pSETAziA3ΔaziA6 Strain. Intergenic conjugal transformation was used to introduce the pSETAziA3 plasmid into the Δ*aziA6* strain following the above protocol. The pSET152 backbone of the pSETAziA3 plasmid facilitated integration into the genome of *S. sahachiroi*, generating a stable apramycin resistant strain.

Overexpression and Purification of AziA3. A starter culture of the pSETAziA3ΔaziA6 strain in 2xYT medium was grown for 24 h at 28 °C. Protein expression was performed in YEME medium supplemented with 1% starter culture and 50 μg mL^{-1} apramycin. Cultures were grown at 28 °C for 120 h. The cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM β-mercaptoethanol (BME), 10% glycerol) with 5 mM imidazole and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted by sonication, and cell debris was removed by centrifugation. AziA3 was purified from the crude cell lysate on a HisTrap HP column. Pure protein was obtained in elution fractions containing 100 mM imidazole and 250 mM imidazole as observed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE).

Capture Strategy with AziA3 and Biotin-Cys (1). The general protocol was followed for AziA3. Eluted fractions were analyzed by LC-MS using method B. The reaction was compared to a control lacking AziA3 as well as a synthetic standard of compound **14**.

General Synthesis Procedures. Amide and ester coupling reactions were performed by dissolving 1 equiv of carboxylic acid in DMF at 4 °C. To it was added 2 equiv of 4-dimethylaminopyridine (DMAP), 1.3 equiv of *N*-(3-(dimethylamino)propyl)-*N*-ethylcarbodiimide (EDC), and 1.5 equiv of amine or ester. After 20 min, the reaction was warmed to RT and stirred for 12 h after which the solvent was removed. Boc deprotection was performed by dissolving the protected intermediate in 1:1 DCM/TFA for 12 h with stirring. The reaction was washed with 15 mL of saturated $NaHCO_3$ and extracted with ethyl acetate. The solvent was removed *in vacuo*. Boc and trityl deprotection was performed by dissolving the protected intermediate in water with 5 mL of trifluoroacetic acid (TFA) and 100 mg of triethylsilane for 24 h under nitrogen. The reaction mixture was evaporated *in vacuo* and dissolved in 15 mL of 1:1 water/dichloromethane. The mixture was extracted two times with dichloromethane, and the aqueous layer was lyophilized.

Synthesis of Biotin-Cys (1). *N*-(*tert*-Butoxycarbonyl)-*S*-trityl-L-cysteine was coupled to *N*-(3-aminopropyl)biotinamide trifluoroacetate following the above procedure. The crude reaction was dissolved in DCM and extracted with water to give the protected biotin-Cys(Boc)trityl after removal of the solvent. Boc and trityl deprotection was performed as above to give crude biotin-Cys (**1**).[38] Biotin-Cys (137 mg, 70.8%) was purified by HPLC using method A. ^{1}H NMR (400 MHz, D_2O) δ 4.61 (dd, 1H, J = 7.9, 4.8), 4.43 (dd, 1H, J = 7.8, 4.8), 4.14 (t, 1H, J = 6.0), 3.38−3.20 (m, 6H), 3.06 (dd, 1H, J = 9.4, 4.4), 3.00 (dd, 1H, J = 12.9, 4.8), 2.78 (d, 1H, J = 13.1), 2.27 (t, 2H, J = 7.4), 1.80−1.53 (m, 6H), 1.48−1.35 (m, 2H); ^{13}C{^{1}H} NMR δ 176.8, 167.8, 165.3, 62.0, 60.2, 55.3, 54.5, 39.6, 36.9, 36.4, 35.4, 27.9, 27.8, 27.6, 25.1, 24.8. HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{16}H_{30}N_5O_3S_2$, 404.1790; found, 404.1779.

Synthesis of Compound 13. Compound **13** was synthesized by coupling *o*-toluic acid with biotin-Cys (**1**). Purification was by HPLC method A to give compound **13** (134 mg, 85.9%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.26 (s, 0.5H), 7.72 (s, 0.5H), 7.49−7.18 (m, 4H), 6.34 (s, 1H), 6.29 (s, 1H), 4.34 (dd, 1H, J = 7.6, 4.7), 4.27 (dd, 1H, J = 7.6, 4.7), 4.09 (t, 1H, J = 6.0), 3.05−2.99 (m, 5H), 2.80−2.66 (m, 4H), 2.30 (s, 3H), 2.04−2.00 (m, 2H), 1.58−1.40 (m, 6H), 1.28−1.24 (m, 2H); ^{13}C{^{1}H} NMR δ 172.8, 169.6, 163.3, 161.7, 135.9, 132.1, 130.8, 129.9, 127.8, 125.9, 61.5, 59.7, 57.7, 55.8, 36.9, 36.6, 35.7, 29.5, 28.6, 28.5, 26.6, 25.7, 20.3. HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{24}H_{36}N_5O_4S_2$, 522.2209; found, 522.2206.

Synthesis of Compound 14. 2-Hydroxy-2-(2-methyloxiran-2-yl)acetic acid was synthesized following a previously established protocol.[31] HRMS (ESI) m/z [M − H]$^-$ calcd for $C_5H_7O_4$, 131.0344; found, 131.0336. ^{1}H NMR (400 MHz, D_2O) δ 1.24 (s, 3H), 2.72 (d, J = 4.12 Hz, 1H), 2.84 (d, J = 4.12, 1H), 4.23 (s, 1H); ^{13}C{^{1}H} NMR (400 MHz, D_2O) δ 17.9, 52.3, 57.9, 75.2, 170.4. 2-Hydroxy-2-(2-methyloxiran-2-yl)acetic acid was coupling to biotin-Cys (**1**). Compound **14** was purified by HPLC method B (29 mg, 39.2%). ^{1}H NMR (500 MHz, D_2O) δ 4.55 (dd, 1H, J = 7.7, 4.8), 4.36 (dd, 1H, J = 7.7, 4.8), 4.30 (s, 1H), 4.22 (t, 1H, J = 6.2), 3.31−3.15 (m, 5H), 3.08−3.05 (m, 2H), 2.99 (dd, 1H, J = 12.7, 4.9), 2.84 (d, 1H, J = 12.5), 2.73 (d, 1H, J = 12.5), 2.67 (d, 1H, J = 4.8), 2.20 (t, 2H, J = 7.5), 1.71−1.52 (m, 9H), 1.37−1.32 (m, 2H); ^{13}C{^{1}H} NMR δ 176.8, 174.5, 167.7, 164.9, 92.7, 76.9, 62.1, 60.3, 58.24, 55.4, 39.7, 39.2, 37.0, 36.6, 35.5, 27.9, 27.9, 27.7, 25.2, 22.3. HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{21}H_{36}N_5O_6S_2$, 518.2107; found, 518.2102

Synthesis of Rhodamine-Cys (10). Rhodamine B was coupled to 6-(Boc-amino)-1-hexanol. After 12 h, 5% HCl was added to the reaction, and DCM was used to extract the product. The rhodamine-linker-Boc intermediate was purified using silica column chromatography (ethyl acetate/ethanol 3:1). Boc deprotection then followed. The product was used directly in the coupling to *N*-(*tert*-butoxycarbonyl)-*S*-trityl-L-cysteine. The reaction was washed with 5% HCl and extracted with DCM. The extract was washed with saturated $NaHCO_3$ and NaCl. Protected rhodamine B-Cys intermediate was purified using silica column chromatography (ethyl acetate/ethanol 1:1), and Boc and trityl deprotection was performed, after which the solvent was removed and the product was purified with flash column chromatography (DCM/95% ethanol 9:2) to give a purple solid (48 mg, 49%).[39] ^{1}H NMR (400 MHz, D_2O) δ 8.04−8.03 (m, 1H), 7.65−7.59 (m, 2H), 7.24 (m, 1H), 6.63−6.36 (m, 6H), 4.16 (t, 2H, J = 6.1), 3.91 (m, 1H), 3.40−3.38 (m, 8H), 3.13−2.95 (m, 4H), 1.61−1.49 (m, 8H), 1.41−1.18 (m, 12H); ^{13}C{^{1}H} NMR δ 172.4, 169.7, 153.7, 149.9, 148.4, 134.2, 129.3, 129.2, 128.3, 125.2, 124.5, 108.4, 106.5, 97.5, 62.6, 55.8, 44.6, 40.2, 32.5, 30.3, 30.1, 26.4, 25.3, 12.6. HRMS (ESI) m/z [M]$^+$ calcd for $C_{37}H_{49}N_4O_4S^+$, 645.3469; found, 645.3416.

Synthesis of Coumarin-Cys (11). 7-Methoxycoumarin-4-acetic acid was coupled to 6-(Boc-amino)-1-hexanol in chloroform. The reaction was washed with 1 M HCl, saturated $NaHCO_3$, and saturated NaCl. The intermediate was purified using silica column chromatography (ethyl acetate/hexanes 3:1) to give a thick yellow gel. The Boc protecting group was removed, and the deprotected coumarin-linker was used directly. The deprotected intermediate was then coupled to *N*-(*tert*-butoxycarbonyl)-*S*-trityl-L-cysteine. The reaction was stirred at RT for 12 h and subsequently washed with 1 M HCl, saturated $NaHCO_3$, and saturated NaCl. The intermediate was purified using silica column chromatography (ethyl acetate/hexanes 3:1) to give a light green compound. Boc and trityl deprotection followed. The residue was rinsed with water and filtered to remove insoluble compounds, and the aqueous solution was lyophilized to give coumarin-Cys (**11**) (36 mg, 62%) as an off white solid. ^{1}H NMR (400 MHz, D_2O) δ 7.43 (s, 0.5H), 7.41 (s, 0.5H), 6.77 (m, 2H), 6.15 (s, 1H), 4.14−4.11 (m, 2H), 3.14−2.98 (m, 8H), 2.80 (s, 2H), 1.85−1.78 (m, 2H), 1.27−1.17 (m, 6H); ^{13}C{^{1}H} NMR δ 168.8, 168.8, 162.9, 160.8, 155.55, 148.3, 125.59, 113.8, 112.5, 112.5, 101.1, 65.7, 61.8, 55.8, 38.3, 34.9, 30.3, 29.9, 28.4, 26.3, 25.5. HRMS (ESI) m/z [M + H]$^+$ calcd for $C_{21}H_{29}N_2O_6S$, 437.1746; found, 437.1742.

Synthesis of Pyrene-Cys (12). *N*-(*tert*-Butoxycarbonyl)-*S*-trityl-L-cysteine was coupled to pyrene−butanol. The reaction was washed with 1 M HCl, followed by saturated $NaHCO_3$ and saturated NaCl. The solvent was removed *in vacuo*, and the protected intermediate was purified by silica column (ethyl acetate/hexanes 1:1) chromatography. Boc and trityl deprotection followed. The reaction was purified using silica column chromatography (ethyl acetate/hexanes/methanol 15:5:1) to give purified pyrene-Cys (**12**) (83 mg, 54.9%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.26−7.83 (m, 9H), 4.97 (s, 2H), 4.38−4.21 (m, 3H), 3.35 (d, 2H), 3.01 (t, 2H), 1.93−1.80(m,

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4H); $^{13}C\{^{1}H\}$ NMR δ 167.3, 136.1, 131.4, 130.9, 129.9, 128.4, 127.1, 127.1, 126.9, 126.3, 125.6, 124.8, 124.7, 124.5, 124.5, 124.4, 122.9, 66.3, 54.3, 32.32, 28.1, 27.7, 23.7. HRMS (ESI) m/z $[M + H]^+$ calcd for $C_{23}H_{24}NO_2S$, 378.1528; found, 378.1522.

Overexpression and Purification of ClbN. pET28a-ClbN-NHis was a gift from Emily Balskus (Addgene plasmid #51497; http://n2t.net/addgene:51497; RRID: Addgene_51497).[34] A starter culture of pET-28a-ClbN *E. coli* in LB medium with kanamycin was grown overnight at 37 °C. The starter culture was used to inoculate 1 L of LB medium with kanamycin. Cultures were incubated at 37 °C, induced with IPTG at OD_{600} = 0.6, and subsequently incubated at 16 °C for 16 h. The cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.8, 500 mM NaCl, 10 mM $MgCl_2$) with 5 mM imidazole and 1 mM PMSF. The cells were disrupted by sonication, and debris was removed by centrifugation. ClbN was purified on a HisTrap HP column. A 25 mM stepwise imidazole gradient ranging from 25 mM to 250 mM (two column volumes at each concentration) was used to purify and elute pure protein. Pure protein was obtained in elution fractions containing 100 to 250 mM imidazole as observed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE).

Capture Strategy with ClbN and Biotin-Cys (1). ClbN was desalted and buffer exchanged into ClbN reaction buffer (40 mM HEPES buffer, pH 7.5, 33 mM NaCl, 4 mM $MgCl_2$, 400 μM DTT). To post-translationally modify ClbN, 125 μM coenzyme A and 250 nM Sfp were added with ClbN, and the reaction mixture was incubated at RT for 1 h. To initiate the ClbN reaction, 4 mM L-Asn, 5 mM ATP, 900 μM octanoyl-CoA, and 6% DMSO were added and allowed to incubate for 3 h. The control reaction to verify product formation was quenched with methanol, and the products bound were hydrolyzed with KOH and HCl. Octanoyl-asparagine was detected by MS in the hydrolyzed positive control sample and not in the negative control sample lacking ClbN (Figure S12). Positive control octanoyl-asparagine HRMS (ESI) m/z $[M + H]^+$ calcd for $C_{12}H_{22}N_2O_4$, 259.1652; found, 259.1651.

The general protocol was followed for the capture strategy reaction. Eluted fractions were analyzed by the LC-MS method B. The reaction was compared to a negative control lacking ClbN and a synthetic standard reaction of biotin-Cys + octanoyl-asparagine containing compounds **16** and **17** (Figures S13–S16).

Synthesis of Compound 16 and Compound 17. Octanoyl-asparagine was synthesized following a previously established protocol.[34] HRMS (ESI) m/z $[M + H]^+$ calcd for $C_{12}H_{22}N_2O_4$, 259.1652; found, 259.1650. ^1H NMR (400 MHz, CD_3OD) δ 0.92 (t, J = 7.4 Hz, 3H), 1.34 (m, 8H), 1.64 (m, 2H), 2.26 (t, J = 7.1 Hz, 2H), 2.72 (m, 1H), 2.78 (dd, J = 5.3, 8.1 Hz, 1H), 4.75 (q, J = 7.4 Hz, 1H); $^{13}C\{^{1}H\}$ NMR (400 MHz, CD_3OD) δ 14.1, 22.4, 25.4, 28.6, 28.8, 31.4, 35.2, 36.6, 48.9, 171.2, 172.3, 173.2.

Octanoyl-asparagine was coupled to biotin-Cys (**1**), and the crude reaction was used directly. Compound **16**: HRMS (ESI) m/z $[M + H]^+$ calcd for $C_{28}H_{49}N_7O_6S_2$, 644.3259; found, 644.3246. Compound **17**: HRMS (ESI) m/z $[M + H]^+$ calcd for $C_{28}H_{46}N_6O_6S_2$, 627.2993; found, 627.2983.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00437.

LC-MS traces for fluorophore capture agents and ClbN products, NMR, mass spectra, and strain construction (PDF)

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Author Contributions

L.A.W. planned and performed all the experiments, except cloning of the pSETAziA3 construct, analyzed the data, and wrote the original draft of the manuscript. K.K.N. cloned the pSETAziA3 construct and assisted L.A.W. in the cloning of the pKCAziA6 construct. C.M.H.W. supervised the project and participated in planning the experiment and writing the manuscript.

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Notes

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■ ABBREVIATIONS

PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; PK, polyketide; NRP, nonribosomal peptide; CoA, coenzyme A; LC-MS, liquid chromatography−mass spectrometry; EIC, extracted ion chromatogram

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