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Discovery of a diazo-forming enzyme in cremeomycin biosynthesis

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Abstract

The molecular architectures and potent bioactivities of diazo-containing natural products have attracted the interest of synthetic and biological chemists. Despite this attention, the biosynthetic enzymes involved in diazo group construction have not been identified. Here, we show the ATP-dependent enzyme CreM installs the diazo group in cremeomycin via late-stage N–N bond formation using nitrite. This finding should inspire efforts to use diazo-forming enzymes in biocatalysis and synthetic biology and enable genome-based discovery of new diazo-containing metabolites.

TOC Graphic:

Diazo-containing compounds are exceptionally useful reagents and intermediates in synthetic organic chemistry. The diazo group enables a wide scope of unique transformations including 1,3-dipolar cycloadditions, alkylations, and carbene insertions (Figure 1A).^{1–3} Recently, diazo compounds have been used extensively as carbene precursors in biocatalysis applications employing engineered heme-Fe and porphyrin-Ir based metalloenzymes for a variety of X–H (X = C, N, B, Si and S) insertions and

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications Website at DOI:

Additional information and data related to *S. lividans* heterologous system, cremeomycin degradation, non-enzymatic diazotization, quantification of cremeomycin and 2,4-HMBA production levels in *E. coli*, 2,4-HMBA production in *E. coli* lysate and *in vitro* experiments, multiple sequence alignment of CreM, structural homology model of CreM, and partial purification of CreM. ¹H and ¹³C NMR spectra for cremeomycin. Primers, plasmids and strain descriptions.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

cyclopropanations (Figure 1A).^{4–10} Diazo-containing natural products are made by soil- and marine-derived bacteria (Figure 1B),¹¹ were first isolated in the 1950s, and have been evaluated as chemotherapeutics in clinical trials.^{12–16} More recently, the exceptional potency of the polyketide lomaiviticin A has attracted considerable attention from biological and synthetic chemists.^{17–23} Despite significant interest among the chemical community in diazo compounds, no diazo-forming enzyme(s) has been identified or proposed. Discovering these enzymes could advance biocatalysis and synthetic biology by providing the tools for accessing both reagents and potential therapeutic candidates. Furthermore, elucidating the genes required for diazo biosynthesis would enable genome mining efforts to uncover novel and potentially bioactive diazo-containing small molecules.

Synthetic chemists have developed numerous methods for diazo group installation, including approaches involving C–N and N–N disconnection strategies.^{2, 24–25} Biosynthetic studies suggest diazo biosynthesis may exhibit some parallels. Early feeding studies and genetic disruption experiments with the producers of the diazo-containing kinamycin D and pyridazine-containing azamerone have implicated a late-stage N–N bond forming, diazotization reaction with an aryl amine intermediate.^{26–29} However, recent *in vitro* characterization of enzymes from the hydrazide-containing fosfazinomycin pathway and identification of homologous enzymes in diazofluorene gene clusters imply an alternative diazo-forming strategy involving formation of a discrete, hydrazide intermediate and late-stage C–N bond formation.^{30–32}

Additional insight into diazo biosynthesis was obtained from the discovery and characterization of the cremeomycin (*cre*) gene cluster (Figure 2A). Cremeomycin is a light-sensitive, antibacterial *o*-diazquinone natural product isolated in 1967.^{33–34} *In vitro* enzyme characterization, feeding studies with ¹⁵N-labeled intermediates, and genetics experiments have demonstrated the final transformation in the pathway is the diazotization of 3-amino-2-hydroxy-4-methoxybenzoic acid (3,2,4-AHMBA) (Figure 2B).^{35–36} However, many questions remain regarding the details of this late-stage N–N bond forming reaction. Nitrite is the proposed source of the distal diazo group nitrogen atom, as two enzymes from the *cre* cluster, the flavin-dependent *N*-monooxygenase CreE and the aspartase homolog CreD, produce nitrite from L-aspartate (Figure 2B).³⁶ Feeding studies have also implicated nitrite in the biosynthesis of the diazo-containing azamerone intermediate A80915D.³⁷ This biosynthetic logic is analogous to well-established synthetic methods for diazotization of aryl amines with nitrite under acidic conditions.^{29,38} However, neither CreD nor CreE catalyzed diazotization of 3,2,4-AHBMA *in vitro*, and genetic disruption experiments by the Ohnishi group did not reveal any candidate diazotizing enzyme(s).³⁶ Given that nitrite-based diazotization of aryl amines requires acidic conditions and the culture medium remains at pH ~7 throughout cremeomycin production, it appeared an enzyme would be required to catalyze diazotization of 3,2,4-AHMBA.

Here, we report the discovery and preliminary characterization of a diazo-forming enzyme from cremeomycin biosynthesis. Using an *Escherichia coli*-based heterologous expression system, lysate experiments, site-directed mutagenesis, and biochemical reconstitution with partially-purified enzyme, we demonstrate the fatty acid-coenzyme A (CoA) ligase homolog CreM catalyzes diazotization of 3,2,4-AHMBA with nitrite to afford cremeomycin *in vivo*

and *in vitro*. *In vitro* biochemical reconstitution and *in vivo* site-directed mutagenesis experiments suggest CreM uses ATP to promote diazotization. Additionally, we provide evidence potentially explaining why previous efforts failed to identify this enzyme.³⁶

Our search for a diazo-forming enzyme(s) was informed by the need to activate nitrite to afford the electrophilic nitrosonium ion required for diazotization. After eliminating genes within the *cre* cluster that encoded enzymes with previously characterized roles in cremeomycin biosynthesis, we identified three candidate diazo-forming enzymes, including CreM, a predicted fatty acid-CoA ligase from the ANL superfamily (Figure S1). These ligases use ATP to activate the fatty acid carboxylate group and catalyze thioesterification with CoA. We hypothesized the general catalytic capabilities of this enzyme, the activation of a negatively charged, heteroatom-containing functional group with ATP, could be used to promote diazotization.³⁹ Specifically, we predicted ATP could activate nitrite to afford an electrophilic intermediate that could be attacked directly by 3,2,4-AHMBA or generate a nitrosonium ion. Activation with ATP could also facilitate the dehydration of subsequent N–N linked intermediates, which would parallel the role of acid catalysis in synthetic methods. The nitrite and N–N linked intermediate(s) could be activated as phosphate derivatives (using the γ -phosphate of ATP) or as adenosine monophosphate (AMP) mixed anhydrides, both well-established mechanisms for ATP activation (Figure 2C).⁴⁰ However, previous studies demonstrated that the genetic disruption of *creM* did not abolish cremeomycin production in a heterologous expression system, suggesting CreM is not responsible for diazo formation.³⁶ Therefore, the role of CreM in diazo biosynthesis remained unclear.

We began by reconstituting the final diazotization step *in vivo* using a *Streptomyces lividans* TK64 heterologous host expressing the nitrite-generating enzymes CreD and CreE as well as the putative diazo-forming enzyme CreM. Feeding 3,2,4-AHMBA to this strain followed by culture supernatants analysis by liquid chromatography-mass spectrometry (LC-MS) demonstrated the production of cremeomycin and its known, two-electron reduced degradation product 2-hydroxy-4-methoxybenzoic acid (2,4-HMBA) in several growth media (Figure S2).³⁵ However, the production of these metabolites also occurred in an *S. lividans* strain that expressed only CreD and CreE. Importantly, the pH of the buffered media remained near neutral during the fermentation. We reasoned components in the complex growth media could catalyze non-enzymatic diazotization of 3,2,4-AHMBA with the nitrite generated by CreD and CreE. Indeed, incubating 500 μ M 3,2,4-AHMBA and 500 μ M nitrite for 24 h at 30 °C in sterile, pH ~7 buffered R5, Q and FM media resulted in varying levels of cremeomycin production in all media (Figure S3). No cremeomycin was detected when nitrite or 3,2,4-AHMBA were removed. The ability of Q media to promote the non-enzymatic diazotization of 3,2,4-AHMBA with nitrite may explain why cremeomycin was previously detected in a *creM* mutant of a heterologous host.³⁶

Since identifying a medium for *S. lividans* that did not promote diazotization would likely be difficult, we moved our *in vivo* system into *E. coli*. Importantly, standard *E. coli* LB growth medium did not catalyze the non-enzymatic diazotization of 3,2,4-AHMBA with nitrite (Figure S3). Culturing different *E. coli* strains (Strains 1–8, Table S3) expressing various combinations of CreD, CreE, and CreM in LB media supplemented with 3,2,4-AHMBA revealed CreM-dependent production of 2,4-HMBA (Figure 3A, 3B). The pH of

the culture remained at pH 7 over the course of the experiment. While cremeomycin was not detected, we are unaware of any biological route to 2,4-HMBA in *E. coli*, suggesting cremeomycin is being produced but subsequently degraded. When 500 μ M of cremeomycin standard was incubated with *E. coli* in LB under the expression conditions, 2,4-HMBA was detected (Figure S4). Together, these results implicate CreM in diazo formation, but suggest low production levels and chemical instability prevent cremeomycin detection.

We next sought to increase cremeomycin titers and more directly determine the role of CreM in diazotization. When an *E. coli* strain expressing a maltose binding fusion construct of CreM (MBP-CreM) was cultured in LB supplemented with 100 μ M 3,2,4-AHMBA and 200 μ M nitrite, we observed CreM-dependent production of cremeomycin and 2,4-HMBA (Figure 3C, Figure S5).

Removal of 3,2,4-AHMBA or nitrite abolished production of both products. *E. coli* strains expressing an empty pET28a vector or the soluble protein CreB did not generate either metabolite, demonstrating that MBP-CreM expression was required for diazotization. Quantification of cremeomycin and 2,4-HMBA showed a conversion to both products of approximately 7% from the starting material 3,2,4-AHMBA (Figure S6).

Additionally, we sought to confirm that the catalytic activity of CreM was responsible for diazo formation by mutating a key active site residue in CreM. Members of the fatty acid-CoA ligase family contain a highly conserved glutamate required to coordinate an essential Mg^{2+} ion necessary for ATP binding.^{39,41–42} Using multiple sequence alignments and structural homology modeling, we identified E352 as the conserved glutamate in CreM (Figure S7 and S8). Upon mutating E352 to alanine (MBP-CreM_E352A) neither cremeomycin nor 2,4-HMBA could be detected. Taken together, these results demonstrate CreM catalyzes diazotization of 3,2,4-AHMBA with nitrite to generate cremeomycin and suggest ATP is required for this transformation.

To assess the stability of MBP-CreM to purification, we incubated cell lysates of *E. coli* expressing MBP-CreM with 3,2,4-AHMBA and nitrite in Tris-HCl pH 7.0 aqueous buffer. These reactions produced both cremeomycin and 2,4-HMBA, demonstrating MBP-CreM remains active in *E. coli* lysates (Figure 4B, Figure S9). In agreement with our previous experiments, lysates from strains expressing CreB or MBP-CreM_E352A did not catalyze the reaction. The substrates nitrite and 3,2,4-AHMBA were also required. Using clarified lysate abolished production of both metabolites suggesting MBP-CreM may be improperly folded, aggregated, and/or membrane-bound, potentially explaining the relatively low conversion rate seen *in vivo*.

We next sought to biochemically reconstitute CreM's activity *in vitro*, but despite numerous attempts, CreM always expressed as insoluble inclusion bodies in *E. coli*. Expression of N-terminal His₆-tagged CreM in *S. lividans* TK64 yielded protein that could be partially purified using metal ion affinity chromatography (Figure S10). Incubation of partially-purified CreM with 100 μ M 3,2,4-AHMBA, 200 μ M nitrite, and 200 μ M ATP in Tris-HCl pH 7.0 aqueous buffer yielded both cremeomycin and 2,4-HMBA (Figure 4C, Figure S11). This activity required CreM, the substrates 3,2,4-AHMBA and nitrite, ATP, and was

abolished when CreM was heated prior to use in the assay. Furthermore, assays run with the more hydrolytically stable ATP analogue γ -S-ATP did not yield cremeomycin and produced ~40-fold less 2,4-HMBA. These results provide additional evidence that CreM is a diazo-forming enzyme and suggest ATP is involved in the transformation. Unfortunately, the low *in vitro* activity of the CreM preparation prevented labeling studies (e.g. with ^{18}O -labeled nitrite) to gain deeper insights into the diazotization mechanism. Future efforts are aimed at improving the purification of CreM to characterize the mechanism and structure of this enzyme.

In summary, our identification of the diazo-forming enzyme CreM has revealed a strategy for diazo group construction in natural product biosynthesis, addressing a significant challenge in the field. This work represents a critical step for future biochemical and structural investigations aimed at understanding this chemistry in greater mechanistic detail. Additionally, the work described here now informs investigations of other diazo-containing and related N–N bond-containing natural products. This discovery will also facilitate efforts to use CreM and homologs in synthetic biology and biocatalysis applications to produce diazo-containing compounds. Finally, the *creDEM* genes can now be used to mine sequenced microbial genomes for gene clusters encoding putative diazo-containing natural products. Initial mining of sequencing data from the National Center for Biotechnology Information revealed greater than 90 gene clusters encoding predicted diazo natural products. Characterizing the products of these gene clusters will not only provide access to new, potentially bioactive compounds but may also afford additional CreM homologs that could provide starting points for enzyme engineering.

EXPERIMENTAL SECTION

General Materials and Methods.

DNA polymerases, restriction enzymes, and T4 ligase used for cloning were purchased from New England BioLabs. Recombinant plasmid DNA was purified with a QIAprep Kit from Qiagen or with a E.Z.N.A. Plasmid DNA Mini Kit I from Omega Bio-Tek. Gel extraction of DNA fragments and restriction endonuclease clean up were performed using an Illustra GFX PCR DNA and Gel Band Purification kit from GE Healthcare. DNA sequencing was performed by Beckman Coulter Genomics (Danvers, MA) and Eton Bioscience (Charlestown, MA). Nickel-nitrilotriacetic acid-agarose (Ni-NTA) resin was purchased from Qiagen and Thermo Scientific. SDS–PAGE gels (4 – 15% Mini-Protean TGX) were purchased from Bio-Rad. *Streptomyces lividans* TK64 was obtained from Prof. Wenjun Zhang at the University of California, Berkeley. The vector pET28-MBP was obtained from Prof. Doug Mitchell at the University of Illinois Urbana-Champaign.

Optical densities of *Escherichia coli* cultures were determined with a DU 730 Life Sciences (Beckman Coulter) or a Genesys 20 UV/Vis spectrophotometer (Thermo Scientific) by measuring absorbance at 600 nm. Thermocycling was carried out in a C1000 Gradient Cycler (Bio-Rad). Cell lysis by disruption was performed using an Avestin EmulsiFlex-C3. Cell lysis by sonication was performed using a Branson Digital Sonifier Model 450. High-resolution mass spectral data were obtained at the Small Molecule Mass Spectrometry Facility, FAS Division of Science, Harvard University. Detection of cremeomycin and 2-

hydroxy-4-methoxybenzoic acid (2,4-HMBA) by liquid chromatography-mass spectrometry (LC-MS) were carried out on a Thermo Scientific Dionex UltiMate 3000 UHPLC coupled to a Thermo Q Exactive Plus mass spectrometer system (Thermo Fisher Scientific Inc, Waltham, MA) equipped with an HESI-II electrospray ionization (ESI) source. LC-MS data were analyzed with Chromeleon Xpress software for UHPLC and Thermo Xcalibur software version 3.0.63 for mass spectrometry and processed with Thermo Xcalibur Qual Browser software version 4.0.27.19. 8 μ L of each sample was injected onto the UHPLC including a HPG-3400RS binary pump with a built-in vacuum degasser and a thermostatted WPS-3000TRS high performance autosampler. An XTerra MS C18 analytical column (2.1 $\times$ 100 mm, 3.5 μ m) from Waters Corporation (Milford, MA) was used at a flow rate of 0.3 mL/min with 0.2% acetic acid in water as mobile phase A and 0.2% acetic acid in acetonitrile as mobile phase B. The column temperature was maintained at room temperature. The following gradient was applied: 0–3 min: 5% B isocratic, 3–12 min: 5–100% B, 12–14 min: 100% B isocratic, 14–14.5 min: 100–5% B, 14.5–20.5 min: 5% B isocratic. The MS conditions were as follows: positive ionization mode for cremeomycin and negative ionization mode for 2,4-HMBA; full scan mass range, m/z 50 to 750; resolution, 70.00; AGC target, 1e6; maximum IT, 220 ms; spray voltage, 3500 V; capillary temperature, 280 $^{\circ}$ C; sheath gas, 47.5; Aux gas, 11.25; probe heater temperature, 412.5 $^{\circ}$ C; S-Lens RF level, 50.00. A mass window of $\pm$ 5 ppm was used to extract the ion of $[M+H]^+$ for cremeomycin and $[M-H]^-$ for 2,4-HMBA. Targets were considered detected when the mass accuracy was less than 5 ppm, there was a match of isotopic pattern between the observed and theoretical ions, and there was a match of retention time with the standards.

Proton nuclear magnetic resonance (1H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on Varian Inova-500 (500 MHz, 125 MHz) NMR spectrometers. Chemical shifts are reported in parts per million downfield from tetramethylsilane using the solvent resonance as internal standard for 1H ($CDCl_3$ = 7.26 ppm) and ^{13}C ($CDCl_3$ = 77.25 ppm). Data are reported as follows: chemical shift, integration multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, q=quartet, qt=quintet), coupling constant, integration, and assignment. NMR spectra were processed using iNMR Reader, version 5.3.4.

Synthetically prepared cremeomycin³⁸ and commercially available 2,4-HMBA from Sigma-Aldrich were used as standards in all LC-MS analyses. The 3,2,4-AHMBA used in all *in vivo* heterologous expression experiments and *in vitro* assays was synthetically prepared following the previous report by Varley et al.³⁸ Our synthesis and characterization of 3,2,4-AHMBA following Varley's protocol was reported previously by our lab.³⁵

As cremeomycin is light sensitive, all procedures undertaken in which cremeomycin was potentially present (i.e. *in vivo* heterologous expression experiments in *S. lividans* and *E. coli*, *in vitro* experiments, non-enzymatic diazotization experiments, and cremeomycin degradation experiments) were carried out with exclusion of as much light as possible. Lights were turned off while working, culture flasks and tubes were wrapped in tinfoil during incubations, incubator windows were covered in tinfoil, Eppendorf tubes were wrapped in tinfoil, centrifuges were covered in tinfoil, and amber, light-excluding LC-MS sample vials were used for analysis.

Creomeomycin

^1H NMR (500 MHz, CDCl_3) δ 8.36 (d, J = 8.55 Hz, 1H, aromatic CH), δ 5.97 (d, J = 8.57 Hz, 1H, aromatic CH), δ 4.05 (s, 3H, OCH_3); ^{13}C NMR (125 MHz, CDCl_3) δ 177.16 (C2), 165.79 (COOH), 161.87 (C6), 146.47 (C4), 114.67 (C1), 94.87 (C5), 57.55 (CH3). HRMS (ESI): calc'd m/z for $\text{C}_8\text{H}_7\text{N}_2\text{O}_4^+$ $[\text{M}+\text{H}]^+ = 195.0400$; found, 195.0401. ^1H and ^{13}C NMR data matched those previously reported by Moody.³⁸ No paramagnetic reagent was added; therefore the diazo carbon was silent in the ^{13}C NMR.³⁸

Generation of *S. lividans* TK64 strains expressing CreD, CreE, and CreM.

The genes *creD*, *creE*, and *creM* were each cloned using the 'compatible cohesive ends' method (using *AvrII* and *NheI* restriction sites) to insert each gene in succession into the pUWL201PW *Streptomyces-E. coli* shuttle vector⁴³ to generate final vectors containing either *creD* and *creE* (pUWL201PW-*creDE*) or *creD*, *creE*, and *creM* (pUWL201PW-*creDEM*) using the primers shown in Table S1. All cloning was performed in *E. coli* TOP10 cells. Plasmids were sequenced for verification. The pUWL201PW vector confers ampicillin resistance (100 $\mu\text{g}/\text{mL}$) in *E. coli* and thiostrepton resistance (50 $\mu\text{g}/\text{mL}$) in *Streptomyces* species. Briefly, *creD* was first cloned into pUWL201PW using restriction sites *NdeI* (5') and *BamHI* (3'). After purification and sequence verification of pUWL201PW-*creD*, the plasmid was digested with *AvrII* and *BamHI* while *creE* was cloned into the plasmid using *NheI* (5') and *BamHI* (3') sites (*AvrII* and *NheI* have compatible ends) to generate pUWL201PW-*creDE*. After purification and sequence verification of pUWL201PW-*creDE*, the plasmid was digested using *AvrII* and *BamHI* while *creM* was cloned into the plasmid using *NheI* (5') and *BamHI* (3') sites to generate pUWL201PW-*creDEM*. The vector backbone contains a ribosome binding site (RBS) for the first gene *creD* and a constitutive promoter, *ermE*, to drive transcription of the entire *creDEM* operon. A RBS with the sequence GGAGGAGCC was inserted three nucleotides upstream of both *creE* and *creM* for translation of these genes. *Streptomyces lividans* TK64 protoplasts were generated and transformed with plasmid DNA according previous reports in Practical Streptomyces Genetics.⁴⁴ After protoplasts were transformed, the viable ex-transformants were streaked on R5 agar plates with 50 $\mu\text{g}/\text{mL}$ thiostrepton to confirm resistance through the plasmid. Confirmed ex-transformants were stored as spores or mycelial stocks in 25% glycerol at -80°C .

In vivo production of creomeomycin and 2,4-HMBA from *S. lividans* TK64 strains expressing CreD, CreE, and CreM.

Stocks of *S. lividans* TK64[pUWL201PW-*creDE*] and *S. lividans* TK64[pUWL201PW-*creDEM*] stored at -80°C were streaked on R5 agar plates⁴⁴ containing 50 $\mu\text{g}/\text{mL}$ thiostrepton and incubated at 30°C for approximately 4–6 days until robust mycelial growth and sporulation occurred. *S. lividans* TK64 wild-type stored at -80°C was streaked on R5 agar plates containing no antibiotics. The mycelia/spores from approximately $\frac{1}{4}$ of an agar plate were removed with a cotton swab tip and used to inoculate 50 mL of seed culture of R5, Q or FM liquid media containing 50 $\mu\text{g}/\text{mL}$ thiostrepton (for strains expressing CreDE or CreDEM) or no antibiotics (wild-type strain). Cultures were incubated at 30°C with shaking at 220 rpm for 3 days. These seed cultures were used to inoculate 50 mL of fresh

R5, Q or FM liquid media supplemented with 1 mM 3,2,4-AHMBA and 50 µg/mL thiostrepton for CreDE- and CreDEM-expressing strains at an inoculum of 1:100 and incubated at 30 °C with shaking at 220 rpm for 36 h. The pH of the buffered fermentation culture remained at pH 7 over the course of the fermentation. After 36 h, 1 mL of culture was removed and centrifuged at 4 °C at 16,000 x *g* for 5 min. A 200 µL aliquot of clarified supernatant was removed and quenched with 400 µL of acetone. This mixture was centrifuged at 4 °C at 16,000 x *g* for 15 min. 200 µL of clarified supernatant was removed and analyzed by LC-MS as described in General Methods.

Non-enzymatic diazotization of 3,2,4-AHMBA in various bacterial growth media.

Lysogeny broth (LB), R5⁴⁴, Q³⁶ and fermentation media³³ (FM) media were prepared, adjusted to pH ~7 and sterilized by autoclaving. To 500 µL of each medium ampicillin was added at 100 µg/mL to prevent bacterial growth during subsequent incubation plus 500 µM of 3,2,4-AHMBA and 500 µM sodium nitrite. Additional controls were prepared in which 3,2,4-AHMBA or sodium nitrite was not added. Samples were wrapped in tinfoil and incubated at 30 °C for 24 h. After 24 h, samples were centrifuged at 4 °C at 16,000 x *g* for 15 min. A 200 µL aliquot of clarified supernatant was removed and quenched with 400 µL of acetone. This mixture was centrifuged at 4 °C at 16,000 x *g* for 15 min. 200 µL of clarified supernatant was removed and analyzed by LC-MS as described in General Methods.

Generation of *E. coli* BL21 strains expressing CreD, CreE, and CreM.

The genes *creD*, *creE*, and *creM* were cloned into various vectors (DUET vectors: pCOLA (kanamycin resistance), pETDUET (ampicillin resistance), pACYC (chloramphenicol resistance) and maltose binding protein fusion vector: pMAL-c2x (ampicillin resistance)) via either PCR and ligation or subcloning. Plasmids were subsequently transformed into *E. coli* BL21 and Tuner in a variety of combinations to generate Strains 1–8 as described in Table S2. Primers used for this cloning are given in Table S3. All cloning was performed in *E. coli* TOP10 cells. After cloning and sequence verification of each plasmid, the plasmid combinations were transformed into *E. coli* BL21 or Tuner via heat shock or electroporation. The *E. coli* Strains 1–8 were stored in 25% glycerol stocks at –80 °C.

In vivo production of 2,4-HMBA from *E. coli* strains expressing of CreD, CreE, and CreM.

Glycerol cell stocks of *E. coli* Strain 1–8, as well as wild-type *E. coli* BL21 and Tuner strains, were used to inoculate 5 mL of LB media containing the appropriate antibiotics (refer to Table S2 for resistance profile of each strain) and incubated at 37 °C overnight on a rotating drum. These seed cultures were used to inoculate 5 mL of fresh LB media containing the appropriate antibiotics at an inoculum of 1:100, which were then incubated at 37 °C on a rotating drum. After approximately 2 h, the strains were transferred to an incubator at 15 °C and incubated for approximately one additional hour with shaking. When the OD₆₀₀ of the cultures reached 0.5–0.6, 100 µM of 3,2,4-AHMBA and 500 µM of IPTG was added and the strains were incubated overnight at 15 °C with shaking. The pH of the *E. coli* culture remained at pH 7 over the course of the fermentation. After approximately 15 h, the cultures were centrifuged at 4 °C at 3,200 x *g* for 5 min. A 400 µL aliquot of supernatant was removed and quenched with 800 µL of acetone. This mixture was centrifuged at 4 °C at

16,000 x *g* for 15 min. 200 μ L of clarified supernatant was removed and analyzed by LC-MS as described in General Methods.

Creomeomycin degradation experiments in presence of *E. coli* in LB media.

A glycerol stock of *E. coli* BL21 was used to inoculate 5 mL of LB media and incubated at 37 °C overnight on a rotating drum. This seed culture was used to inoculate 5 mL of fresh LB media at an inoculum of 1:100 and incubated at 37 °C on a rotating drum. After approximately 2 h, the strain was transferred to an incubator at 15 °C and incubated for approximately one additional hour with shaking. When the OD₆₀₀ of the culture reached 0.5–0.6, 500 μ L of culture was transferred to an Eppendorf tube (with holes punctured in the top to allow for aeration) and 500 μ M of synthetically prepared creomeomycin was added to the culture. The tube was covered in tinfoil and placed horizontally on the platform in the incubator and allowed to shake overnight for 15 h. Additional controls were set up in which creomeomycin was added to LB in the absence of *E. coli* cells or where creomeomycin was added to water alone. After 15 h, the culture was centrifuged at 4 °C at 16,000 x *g* for 5 min. A 200 μ L aliquot of supernatant was removed and quenched with 400 μ L of acetone. This mixture was centrifuged at 4 °C at 16,000 x *g* for 15 min. 200 μ L of clarified supernatant was removed and analyzed by LC-MS as described in General Methods.

Generation of *E. coli* BL21 strains for MBP-CreM expression experiments.

The *creM* gene was cloned into pET28-MBP vector (constructed and received from Prof. Douglas Mitchell, University of Illinois, Urbana-Champaign) using the BamHI (5') and XhoI (3') restriction sites to create an in-frame N-terminal MBP fusion construct with *creM*. The gene *creM* was amplified from a bacterial artificial chromosome (BAC) clone from an *S. cremeus* genomic library constructed previously using the primers shown in Table S4.³⁵ The gene *creB* was cloned into pET28a using the NdeI (5') and XhoI (3') restriction sites to give an N-terminal His₆-tagged protein. The gene *creB* was amplified from *S. cremeus* genomic DNA using the primers shown in Table S4. The pET28-MBP-*creM*_E352A plasmid was constructed by site-directed mutagenesis through whole plasmid amplification with the primers shown in Table S4. All cloning was performed in *E. coli* TOP10 cells. After cloning and sequence verification of each plasmid, the plasmids were transformed into chemically-competent *E. coli* BL21 and stored in 25% glycerol stocks at –80 °C. For the 'empty vector' control, pET28a was transformed into *E. coli* BL21 and for the 'no vector' control wild-type *E. coli* BL21 was used.

In vivo production of creomeomycin and 2,4-HMBA from *E. coli* strains expressing MBP-CreM.

Glycerol cell stocks of the *E. coli* BL21 strains were used to inoculate 5 mL of LB media containing kanamycin (50 μ g/mL final concentration) where appropriate and incubated at 37 °C overnight on a rotating drum. These seed cultures were used to inoculate 5 mL of fresh LB media containing kanamycin (50 μ g/mL final concentration) where appropriate and incubated at 37 °C on a rotating drum. After approximately 2 h, the strains were transferred to an incubator at 15 °C and incubated for approximately one additional hour with shaking. When the OD₆₀₀ of the cultures reached 0.5–0.6, 100 μ M of 3,2,4-AHMB, 200 μ M sodium nitrite, and 250 μ M of IPTG were added, and the strains were incubated overnight at 15 °C

with shaking. The pH of the *E. coli* culture remained at pH 7 over the course of the fermentation. After approximately 15 h, the cultures were centrifuged at 4 °C at 3,200 x *g* for 5 min. A 400 µL aliquot of supernatant was removed and quenched with 800 µL of acetone. This mixture was centrifuged at 4 °C at 16,000 x *g* for 15 min. 200 µL of clarified supernatant was removed and analyzed by LC-MS as described in General Methods.

Quantification of cremeomycin and 2,4-HBMA production levels from *E. coli* expressing MBP-CreM.

Dilution curves for cremeomycin and 2,4-HMBA were prepared using authentic standards in the concentration ranges of 0.1 µM – 25 µM and 0.01 µM – 25 µM, respectively. Each concentration was prepared in triplicate and analyzed by LC-MS to generate a standard curve for each compound as shown in Figure S5. This standard curve was used to calculate the concentrations of cremeomycin and 2,4-HMBA in triplicate samples in a typical *in vivo* experiment of *E. coli* BL21 expressing MBP-CreM.

Lysate diazotization experiments with *E. coli* BL21 expressing MBP-CreM.

Glycerol cell stocks of the *E. coli* BL21 strains were used to inoculate 5 mL of LB media containing kanamycin (50 µg/mL final concentration) where appropriate and incubated at 37 °C overnight on a rotating drum. These seed cultures were used to inoculate 50 mL of fresh LB media containing kanamycin (50 µg/mL final concentration) where appropriate and incubated at 37 °C on a rotating drum. After approximately 2 h, the strains were transferred to an incubator at 15 °C and incubated for approximately one additional hour with shaking. When the OD₆₀₀ of the cultures reached 0.5–0.6, 250 µM of IPTG was added, and the strains were incubated overnight at 15 °C with shaking. After approximately 15 h, the cultures were centrifuged at 4 °C at 3,200 x *g* for 5 min to collect the cells. The pellets were each resuspended in 1 mL of ice-cold lysis buffer (25 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM MgCl₂, Thermo Scientific Pierce Protease inhibitor cocktail Prod #A32963) and kept on ice. The *E. coli* cell suspensions were lysed by sonication (Branson Digital Sonifier Model 450; Method: 10 s on, 30 s off, 40 s total, 25% duty cycle) on ice. Lysate assays were setup in 100 µL reactions (final volume) in buffer (50 mM Tris-HCl pH 7.0, 0.1 mM MgCl₂, 10 mM NaCl) containing 100 µM 3,2,4-AHMB, 200 µM sodium nitrite, and 200 µM ATP. Reactions were initiated with the addition of 20 µL non-clarified *E. coli* cell lysate, mixed, and then incubated at room temperature overnight. After approximately 12 h, the reactions were quenched with 200 µL of acetone and vortexed to mix vigorously. This mixture was centrifuged at 4 °C at 16,000 x *g* for 15 min. 150 µL of clarified supernatant was removed and analyzed by LC-MS as described in General Methods.

Cloning of N-terminal His₆-CreM from *S. lividans* TK64.

The *creM* gene was cloned into the *Streptomyces-E. coli* shuttle vector pUWL201PW using the restriction sites NdeI (5') and EcoRI (3'). As the pUWL201PW encodes no purification tags, an N-terminal His₆ was engineered into the primers as shown in Table S5. All cloning was performed in *E. coli* TOP10 cells. Plasmids were sequenced for verification. The pUWL201PW vector confers ampicillin resistance (100 µg/mL) in *E. coli* and thiostrepton resistance (50 µg/mL) in *Streptomyces* species. The vector backbone contains a ribosome binding site (RBS) for *creM* and a constitutive promoter, *ermE*, to drive transcription.

Streptomyces lividans TK64 protoplasts were generated and transformed with pUWL201PW-N-His₆-creM according to previous reports in Practical Streptomyces Genetics.⁴⁴ After protoplasts were transformed, the viable ex-transformants were streaked on R5 agar plates with 50 µg/mL thiostrepton to confirm resistance through the plasmid. Confirmed ex-transformants were stored as spore or mycelial stocks in 25% glycerol at –80 °C.

Expression and partial purification of N-terminal His₆-CreM from *S. lividans* TK64.

Stocks of *S. lividans* TK64[pUWL201PW-N-His₆-creM] stored at –80 °C or from fresh transformations were streaked on to R5 agar plates containing 50 µg/mL thiostrepton and incubated at 30 °C for approximately 4–6 days until robust mycelial growth and sporulation occurred. The mycelia/spores from approximately ¼ of an agar plate were removed with a cotton swab tip and used to inoculate 4 × 50 mL seed cultures in R5 liquid media containing 50 µg/mL thiostrepton. Cultures were incubated at 30 °C with shaking at 220 rpm for 3 days. These seed cultures were then used to inoculate 4 × 500 mL of fresh R5 liquid media at an inoculum of 1:50 and incubated at 30 °C with shaking at 220 rpm for 3 days. After 3 days, cells from each culture were centrifuged separately at 4 °C at 6,500 × *g* for 30 min and taken through the following purification separately. The cell pellets were resuspended in 100 mL of ice-cold lysis buffer (25 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM MgCl₂, 1 mg/mL lysozyme, 1 µg/mL DNase, Thermo Scientific Pierce Protease inhibitor cocktail Prod #A32963) and kept on ice. The cells were lysed by passage through a cell disruptor (Avestin EmulsiFlex-C3) twice at 20,000–25,000 psi and the lysate was clarified by centrifugation at 20,000 × *g* for 45 min. Imidazole (10 mM final concentration) was added to the supernatant and flowed over a glass column containing 5 mL of Ni-NTA resin and allowed to exit the column by gravity flow. The column was washed with 20 mL each of wash buffers (25 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM MgCl₂) containing 25 mM imidazole and 50 mM imidazole. Protein was eluted with elution buffer (25 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM MgCl₂) containing 200 mM imidazole. Collected fractions were analyzed by SDS-PAGE to ascertain the presence and purity of protein in each fraction. Fractions containing N-His₆-CreM were pooled and the buffer was exchanged using an Illustra NAP-10 desalting column (GE Healthcare Prod #17-0854-02) into 25 mM Tris-HCl pH 7.5, 50 mM NaCl, 10% glycerol. This provided a partially-purified protein extract (Figure S9) for use in *in vitro* biochemical assays. Solutions containing protein were frozen in liquid nitrogen and stored at –80 °C.

In vitro biochemical reconstitution of diazotization of 3,2,4-AHMBAs using partially-purified CreM.

In vitro biochemical assays were setup in 100 µL reactions (final volume) in buffer (50 mM Tris-HCl pH 7.0, 0.1 mM MgCl₂, 10 mM NaCl) containing 100 µM 3,2,4-AHMBAs, 200 µM sodium nitrite, and 200 µM ATP. Reactions were initiated with the addition of 10 µL of partially purified CreM, mixed, and incubated at room temperature overnight. For the heated control, ~100 µL of CreM was heated at 95 °C for 5 min and then centrifuged at 16,000 × *g* for 15 min. The resulting clarified supernatant was used in the assay after cooling to room temperature. After approximately 12 h, the reactions were quenched with 200 µL of acetone and vortexed to mix vigorously. This mixture was centrifuged at 4 °C at 16,000 × *g* for 15

min. 150 μ L of clarified supernatant was removed and analyzed by LC-MS as described in General Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Transformations of diazo compounds and natural products.

A) Synthetic and biocatalytic transformations involving diazo compounds. Proposed metallocarbenoid intermediate involved in heme-based biocatalysts. B) Examples of natural products containing a diazo group and the pyridazine natural product azamerone with its diazo-containing precursor A80915D.

Figure 2. The predicted ATP-dependent enzyme CreM is a candidate diazo-forming enzyme. A) The *cre* biosynthetic gene cluster. Blue = characterized genes; Green = predicted regulatory/resistance genes; Grey = genes with unknown functions. B) Cremeomycin biosynthesis involves late-stage diazotization of 3,2,4-AHMB with nitrite. C) Proposed mechanisms by which CreM may use ATP to activate nitrite or an *N*-hydroxydiazonyl intermediate.

Figure 3. CreM expression reconstitutes diazo formation *in vivo* in *E. coli*

A) *In vivo E. coli* diazo formation heterologous expression system. B) LC-MS analysis showing CreM-dependent production of 2,4-HMBA in *E. coli* expressing either CreDE or CreDEM in 3,2,4-AHMBA-supplemented LB. C) LC-MS analysis demonstrating CreM-dependent production of cremeomycin in *E. coli* expressing MBP-CreM in nitrite- and 3,2,4-AHMBA-supplemented LB medium. EIC = extracted ion chromatogram.

Figure 4. Reconstitution of diazo formation with lysate and partially-purified CreM. A) Reconstitution conditions for diazo formation using lysate or partially-purified CreM. B) LC-MS analysis demonstrating diazotization activity in MBP-CreM *E. coli* lysates. C) LC-MS analysis showing *in vitro* biochemical reconstitution of diazo formation with partially-purified N-His₆-CreM. heated = CreM protein heat denatured before use in assay.