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Monobactam formation in sulfazecin by a non-ribosomal peptide synthetase thioesterase

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Abstract

The *N*-sulfonated monocyclic β -lactam ring characteristic of the monobactams confers resistance to zinc metallo- β -lactamases and affords the most effective class to combat carbapenem-resistant enterobacteria (CRE). Here we report unprecedented non-ribosomal peptide synthetase activities where an assembled tripeptide is *N*-sulfonated *in trans* prior to direct synthesis of the β -lactam ring in a non-canonical, cysteine-containing thioesterase domain. This means of azetidinone synthesis is distinct from the three others known in nature.

Main text

The β -lactam antibiotics have been a frontline defense against infections by bacterial pathogens for more than 60 years. As a consequence of their long and widespread use, however, bacterial resistance has arisen, frequently conferred by β -lactamases that efficiently hydrolyze their azetidinone rings and render them completely ineffective¹. Four broad classes of these resistance proteins are known: Three classes consist of evolutionarily related serine hydrolases², for which inhibitors exist, and the fourth consists of Zn²⁺ metalloenzymes. Although long known, these zinc metallo- β -lactamases have recently emerged in the clinic to confer potent resistance to penicillins, cephalosporins and even carbapenems, the β -lactam antibiotics of last resort in human medicine³. The monobactams, however, having structurally distinct *N*-sulfonated β -lactam rings, are poor substrates for these metalloproteins, yet remain active against bacterial cell wall biosynthesis. We describe here the biosynthetic route to the monobactam sulfazecin, which is distinct from the three other known tactics in nature to generate β -lactam rings⁴.

Giant modular biosynthetic enzymes create the backbones of polyketide- and polypeptide-derived natural products, which have extensive structural diversity and important biological activities^{5–7}. The individual modules of non-ribosomal peptide synthetases (NRPSs) are

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Author Contributions

C.A.T. and R.A.O. designed and directed the study. R.A.O. carried out the syntheses and biochemical reactions. R.A.O. and R.F.L. cloned and expressed proteins. All authors analyzed the data and discussed the results. R.A.O., R.F.L., and C.A.T. prepared the manuscript.

Competing financial interests

The authors declare no competing financial interests.

composed of three core catalytic domains: an adenylation (A) domain that selects and binds a monomer building block, typically an α -amino acid, and carries out its adenylation in the presence of ATP. The activated substrate then reacts with an adjacent downstream pantetheinylated peptidyl-carrier protein (PCP) to present the substrate as its thioester for peptide bond formation in an intervening condensation (C) domain together with a similarly activated amino acid or peptide intermediate delivered from the upstream module. Modifying domains that perform reactions other than amino acid activation and condensation can also be present. For example, an epimerase (E) domain in module 2 (M2) accounts for the appearance of D-alanine in sulfazecin (Fig. 1). Recently, other previously unknown catalytic activities have been observed in the terminal NRPS module of nocardicin biosynthesis. The embedded β -lactam characteristic of this family of antibiotics is synthesized in the C domain from a tetrapeptide L-seryl residue delivered from the upstream module and an intramodule *p*-(hydroxyphenyl)glycine unit⁸. As for the seemingly related nocardicin G, the precursor polypeptide, all stereocenters and the β -lactam ring itself in sulfazecin are synthesized by NRPSs. However, in sulfazecin, the C-terminal *N*-sulfonated β -lactam ring emblematic of the monobactams is formed in a strikingly different manner.

The sulfazecin biosynthetic gene cluster (BGC) encodes two NRPS proteins, SulI and SulM (Fig. 1a). The former contains a single module (M1) that activates D-glutamate directly, which is readily available in bacteria as a fundamental component of the cell wall. The latter contains two complete modules (M2 and M3) terminating in a weakly predicted TE domain¹⁰. Further bioinformatics analysis¹¹ of the enigmatic TE suggested it was a member of the α/β -hydrolase superfamily where the highly conserved GxSxG active site signature is replaced by an AxCxG motif. M2 activates L-alanine, which is converted to the D-configuration by the E2 domain, and M3 activates L-2,3-diaminopropionate (L-Dap)¹². Upstream are two precursor biosynthetic genes *sulG* and *sulH*, whose protein products transform L-phosphoserine to L-Dap¹³. Immediately downstream of *sulM* lie *sulN*, *sulO* and *sulP*, which we demonstrate here encode the *N*-sulfotransferase, an α -ketoglutarate (α -KG)-dependent non-heme dioxygenase, and an *S*-adenosylmethione (SAM)-dependent methyltransferase, respectively^{12,14}. Our working mechanistic hypothesis was based on bioinformatics analysis of the proteins encoded by the BGC, experimental verification of the amino acids selectively activated by A₁, A₂ and A₃¹², and the chemical logic of antibiotic assembly. As illustrated in Figure 1b (path A), the biogenetic scheme posits assembly of the D-Glu–D-Ala–L-Dap tripeptide on PCP₃, which is transthioesterified to the unusual TE domain at the active site cysteine. This activated TE–acyl linkage would then be subject to intramolecular lactamization with the nucleophilic primary amine with concomitant release of the monocyclic β -lactam ring. Alternatively, if *N*-sulfonation occurs prior to transfer to the TE (Fig. 1b, path B), it may facilitate such a lactamization. An analogous acyl substitution reaction in nocardicin biosynthesis is carried out in a C domain to produce an integrated monocyclic β -lactam⁸.

To test the first hypothesis (path A), we purified recombinant sulfazecin TE as a stable, soluble monodomain. Synthetic D-Glu–D-Ala–L-Dap was used as its *N*-acetylcysteamine (SNAC) and pantetheine (SPANT) forms as well as converted to its coenzyme A (CoA) thioester and Sfp-loaded^{15,16} onto its native PCP₃, but all of these failed to give any detectable β -lactam product when exposed to the TE. To make peptidyl transfer

intramolecular, the *apo*-PCP₃-TE didomain was prepared and the tripeptide was similarly loaded onto the PCP₃ portion of the didomain with Sfp. Slow hydrolysis of the tripeptide-*holo*-PCP₃ thioester was observed relative to a C2818A TE mutant control. The TE C2818S mutant, on the other hand, efficiently hydrolyzed the bound tripeptide (Supplementary Fig. 1). Alignment of the sulfazecin TE with other NRPS TE domains (Supplementary Fig. 2) showed a conserved Asp among monobactam producers proximal to its expected location, suggesting at least partial restoration of a classical Ser-His-(Asp) catalytic triad in keeping with the observed tripeptide hydrolysis¹⁷. At first sight, hydrolytic release seems consistent with the native function of most TE domains—simple hydrolysis. But in this case it is also possible that if β -lactam formation indeed occurred initially, it could be followed by comparatively rapid hydrolysis and the azetidinone not detected.

To test path A (Fig. 1b) in an alternative manner, the proposed β -lactam product **1** was synthesized (Supplementary Note) and treated directly with the putative sulfotransferase, SulN, in the presence of adenosine 3'-phosphate 5'-phosphosulfate (PAPS), the universal sulfonyl donor and the cofactor supported by bioinformatics analysis. No *N*-sulfonation was detected by sensitive electrospray ionization mass spectrometric (ESI-MS) analysis, thus casting further doubt on path A. Therefore, sulfonation of the tripeptide L-Dap β -amine was considered (path B). The D-Glu-D-Ala-L-Dap tripeptide alone did not react with SulN and PAPS, but when first loaded on PCP₃, sulfonation *in trans* proceeded readily as monitored by ESI-MS (Fig. 2a,b and Supplementary Fig. 3). While *in cis* sulfonation has been studied in a model system representative of terminal alkene synthesis during curacin biosynthesis^{18,19}, *in trans* sulfamate formation on an NRPS has not been described, to our knowledge^{20,21}. Recently, native polyacrylamide gel electrophoresis (PAGE) mobility shift assays were used to demonstrate the association of cytochromes P450 with the “X domain” of NRPS enzymes responsible for glycopeptide antibiotic biosynthesis²². These protein-protein interactions allow critical oxidative crosslinking reactions to take place on NRPS-bound peptide intermediates. Titration, therefore, of SulN into a fixed concentration of *apo*-PCP₃-TE gave increasing amounts of a larger aggregate having an apparent mass consistent with a 1:1 stoichiometry (Fig. 2c). It is instructive that SulN binding to PCP₃-TE does not depend on the presence of the loaded tripeptide, an observation in parallel with the X domain above and recognition by its client P450s^{22,23}. Nonetheless, the presence of the native substrate would presumably amplify binding affinity.

Next, the entirety of M3, along with the E₂ and TE domains (E₂-C₃-A₃-PCP₃-TE) was expressed as a single His₆-fusion protein in *E. coli* and purified first by Ni-affinity chromatography and subsequently by fast protein liquid chromatography (FPLC). To insure full pantetheinylation of PCP₃, the pentadomain was treated with Sfp and CoA. Similarly, over-produced *apo*-PCP₂ was Sfp-loaded with D-Glu-D-Ala-CoA to reconstitute an *in vitro* biosynthetic system (Supplementary Fig. 4). The *holo*-E₂-M3-TE pentadomain was incubated with L-Dap and ATP with the presumption that A₃ would bind and activate its native amino acid substrate and present it on PCP₃ for peptide bond formation in the C₃ domain. To obtain a soluble M3-TE construct, it was necessary to include the preceding E₂ domain. To ensure stereochemically accurate synthesis, we loaded the pre-epimerized D-Glu-D-Ala diastereomer on *apo*-PCP₂. In keeping with the SulN experiments presented

above and in Figure 2a,b, no β -lactam product was detected, but the active site C2818S mutant did show hydrolysis of the expected tripeptide, demonstrating that L-Dap activation and condensation in M3 had taken place (Supplementary Fig. 4). Addition of sulfotransferase SulN and PAPS (Fig. 2d), however, dramatically changed the course of reaction, and at early time-points (10 min) β -lactam **2** was rapidly seen. With limiting dipeptide-*apo*-PCP₂, later time points (>30 min) gave increasing proportions of hydrolyzed β -lactam (Supplementary Figs. 5 and 6). Mutation of the TE active site in the pentadomain (C2818A or C2818S) led to slow or rapid hydrolytic release, respectively, of the sulfonated D,D,L -tripeptide **4** in analogous reactions (Supplementary Fig. 5). These findings underscore both the high substrate selectivity of the TE and its essential role in β -lactam synthesis.

That the unusual cysteine TE catalyzed both β -lactam formation and its hydrolysis posed the apparent contradiction of how the wild-type *Pseudomonas acidophila* manages to produce readily isolable amounts of sulfazecin. If the last step of antibiotic biosynthesis competes with TE-catalyzed hydrolysis, full reconstitution should produce the methoxylated β -lactam, presumably making it a poor substrate for the TE and, thus immune to hydrolysis. Indeed, full reconstitution with SulO and SulP, a dioxygenase and methyltransferase, respectively (Fig. 2e and Supplementary Figs. 7 and 8) produced sulfazecin but no detectable β -lactam hydrolysis product **4**, even after extended incubation. Meanwhile, the same reaction lacking the tailoring proteins SulO and SulP produced desmethoxylsulfazecin and approximately 12% hydrolyzed β -lactam **4** in 2 hours (Supplementary Figs. 6 and 9). A control reaction exposing sulfazecin to TE as the *apo*-PCP₃-TE exhibited no hydrolysis, whereas the same protein readily consumed β -lactam **2** (desmethoxylsulfazecin) under the same conditions (Supplementary Figs. 10 and 11).

In conclusion, we propose a new, fourth mechanism for β -lactam antibiotic biosynthesis that captures a previously unknown NRPS TE reaction type and *in trans* sulfamation to form a potentially activated β -lactam ring with deft efficiency. The pK_a of the sulfamate N-H is reported to be ca. 8 (ref. 24), a value readily accessible to physiological bases to mediate nucleophilic acyl substitution. Given the importance of monobactams for their low susceptibility to Zn²⁺-metallo- β -lactamases and their effectiveness against otherwise untreatable CRE infections, future work will examine how the natural biosynthetic machinery might be adapted to the production of semi-synthetic monobactams in a manner analogous to commercial penicillin production and new structures with improved antimicrobial properties.

Online Methods

UPLC-HRMS methods

Ultra-performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) experiments were carried out on a Waters Acquity H-class UPLC system in tandem with a Xevo-G2 high mass resolution Q-TOF MS/MS ESI system at the Johns Hopkins Mass Spectrometry Facility using one of the following UPLC-HRMS methods:

UPLC-HRMS Method A: ES- [binary gradient: water (solvent A), acetonitrile (solvent B), 0.3 mL/min]: 0–1 min isocratic 20% A; 1–7.5 min gradient 20% to 100% A; 7.5–8.4 min isocratic 100% A; 8.4–8.5 min gradient 100% to 20% A; 8.5–10 min isocratic 20% A. Waters ACQUITY UPLC BEH Amide Column, 130Å, 1.7 µm, 2.1 mm × 100 mm.

UPLC-HRMS Method B: ES+ [ternary gradient: water (solvent A), water (+1% formic acid (solvent B), acetonitrile (solvent C)], 0.3 mL/min]: 0–1 min isocratic 10% A, 10% B, 80% C; 1–7.5 min gradient 10% to 90% A, isocratic 10% B; 7.5–8.4 min isocratic 90% A, 10% B; 8.4–8.5 min gradient 90% to 10% A, isocratic 10% B; 8.5–10 min isocratic 10% A, 10% B, 80% C. Waters ACQUITY UPLC BEH Amide Column, 130Å, 1.7 µm, 2.1 mm × 100 mm.

UPLC-HRMS Method C: ES+ [ternary gradient: water (solvent A), water +1% formic acid (solvent B), acetonitrile (solvent C), 0.3 mL/min]: 0–1 min isocratic 90% A, 10% B; 1–7.5 min gradient 90% to 10% A, isocratic 10% B; 7.5–8.4 min isocratic 10% A, 10% B; 8.4–8.5 min gradient 10% to 90% A, isocratic 10% B; 8.5–10 min isocratic 90% A, 10% B. Waters ACQUITY UPLC Protein BEH C4 Column, 300Å, 1.7 µm, 2.1 mm × 50 mm. Protein mass analysis was carried out using either MassLynx^Ó or BiopharmaLynx^Ó software.

Cloning, expression, and purification of PCP₂, PCP₃, and PCP₃-TE

All three genes were PCR-amplified from pET28b/sulM¹² using the primers indicated in Supplementary Table 1. The PCR products were cloned into pGEM-5Z (Promega) and further digested with NdeI-HindIII and subcloned into pET28b (EMD Millipore) to give expression constructs pET28b/PCP₂, pET28b/PCP₃, and pET28b/PCP₃-TE, respectively. Overexpression was carried out in *E. coli* Rosetta2 (DE3) (EMD Millipore) cells. A 50 mL overnight culture was used to inoculate 5L of LB or TB medium in a bioreactor (Wheaton) supplemented with 50 µg/mL kanamycin and 25 µg/mL chloramphenicol. The secondary culture was quickly chilled in an ice-water bath for approximately 60 min at OD₆₀₀ = 0.6 and induced with 0.5 mM IPTG. The overexpression was continued for 15–20 h at 15 °C.

The *N*-terminal His₆ fusion proteins were purified using a modified standard Ni-NTA affinity chromatography procedure. 5–7 g of cell pellets were resuspended in 20–30 mL lysis buffer (50 mM NaH₂PO₄, 10 mM imidazole, 300 mM NaCl, 10% glycerol, 2 mg/mL lysozyme, pH = 8.0) and incubated on ice for 30 min before sonication (Cole-Parmer) (40% amplitude, 8 sec. on / 8 sec. off, 8 min). The cell-free-extract (CFE) was mixed with 3 mL Ni-NTA agarose nickel resin (MCLab) and rotated slowly at 4 °C for 1 hr. The protein-bound resin was homogenized and loaded onto a gravity column and washed with two volumes of wash buffer (50 mM NaH₂PO₄, 20 mM imidazole, 300 mM NaCl, 10% glycerol, pH = 8.0), and the *N*-His₆ fusion protein was eluted with 3 mL of elution buffer (50 mM NaH₂PO₄, 250 mM imidazole, 300 mM NaCl, 10% glycerol, pH = 8.0). The protein solution was desalted and buffer exchanged (Bio-Rad 10DG gravity desalting column) into 4 mL of assay buffer (50 mM KH₂PO₄ pH = 6.5) (Supplementary Figs. 12, 13, and 14).

Cloning, expression, and purification of SulN and SulP

Using the primers indicated (Supplementary Table 1), *sulN* and *sulP* were cloned from the sulfazecin gene cluster¹² by PCR amplification (Phusion high-fidelity DNA polymerase).

The PCR products were ligated into pET29b and co-transformed along with the chaperone-bearing plasmid pG-KJE8 (Takara) into *E. coli* Shuffle T7 (NEB). A 50 mL overnight seed culture was used to inoculate 4 L TB medium in a bioreactor containing 50 µg/mL kanamycin, 50 µg/mL chloramphenicol, 5 ng/mL tetracycline, and 0.5 mg/mL L-arabinose. Overexpression was induced with 0.5 mM IPTG at $OD_{600} = 0.6$ after the culture was chilled on ice for 40 min. Cells were harvested after overexpression was carried out for 24 h at 15 °C. SulN and SulP were purified in a fashion similar to that used for PCP₂, PCP₃, PCP₃-TE (Supplementary Figs. 12, 13, and 14).

Cloning, expression, and purification of SulO

SulO was cloned from the sulfazecin gene cluster by PCR amplification using the primers indicated in Supplementary Table 1. The PCR product was ligated into pBlueScript+ (Stratagene) and a positive clone was digested with NdeI and HindIII. The SulO gene was ligated into pET29b to create SulO/pET29b, which was transformed into *E. coli* Rosetta2(DE3). Overexpression was carried out in LB +1% glycerol for 20 h at 20°C. Purification was carried out in fashion similar to that reported above (Supplementary Figs. 12, 13, and 14).

Cloning, expression, and purification of module 3 and module 3 TE mutants

The gene-encoding module 3-TE (E₂C₃A₃PCP₃TE) was PCR amplified from the pET28b/sulM template using primers indicated (Supplementary Table 1). The PCR product was digested with NdeI-HindII and ligated into pET29b to generate expression vector pET29b/M3-TE. Alanine (C2818A) and serine (C2818S) TE active site mutants were generated from this expression vector using the primers indicated (Supplementary Table 1) with a Q5 site-directed mutagenesis kit (NEB). Overexpression and purification of wildtype M3-TE and M3-TE mutants were carried out similarly as described above. Further FPLC purification yielded protein with identical reactivity to that purified by conventional Ni-NTA agarose (Supplementary Figs. 12, 13, and 14).

Cloning, expression, and purification of PCP₃-TE* C2818A and C2818S mutants

Mutant PCP₃-TE constructs were generated by PCR amplification of PCP₃-TE/pET28b using the mutagenic primers listed in Supplementary Table 1 using a Q5 site-directed mutagenesis kit (NEB). Overexpression and purification of C2818A and C2818S PCP₃-TE constructs was carried out as described above (Supplementary Figs. 12, 13, and 14).

Module 3 *in vitro* β-lactam production

Dipeptide-*holo*-PCP₂ was generated by incubating *apo*-PCP₂ (70 µM), D-Glu-D-Ala-CoA (150 µM), and Sfp (2 µM) in assay buffer supplemented with 10 mM MgCl₂. The loading reaction was incubated at room temperature for 1 h, then buffer exchanged into assay buffer by three serial dilutions and concentration to 100 µL using an Amicon Ultra 3K (Millipore)⁸.

Both wild-type and mutant *holo*-M3 were generated by incubating the FPLC-purified M3 construct (7 – 46 µM) with coenzyme A (150 µM) and Sfp (2 µM) in assay buffer supplemented with 10 mM MgCl₂. The reaction was allowed to stand for 1 h at room

temperature then the excess CoASH and salts were removed through three serial dilutions and microfilter concentrations as before.

Dipeptide-*holo*-PCP₂ (200 μM) was added to a solution of *holo*-M3 (2 μM) in assay buffer supplemented with L-Dap (2 mM), ATP (2.5 mM), SulN (10 μM), and PAPS (2 mM) (all concentrations are final concentrations). The reactions were continued at room temperature for 2 h, then diluted with one volume of acetonitrile and filtered through an Amicon 3K (Millipore) filter. The filtrates were analyzed directly by UPLC-HRMS method A.

SulN sulfonation of tripeptide-*holo*-PCP₃

Apo-PCP₃ (100 μM) was incubated with D-Glu-D-Ala-L-Dap-CoA in assay buffer supplemented with MgCl₂ (1 mM). Conversion of PCP₃ to the tripeptide-*holo* form was initiated by addition of Sfp (5 μM). SulN (20 μM) and PAPS (750 μM) were subsequently added and incubated at room temperature for 4 h. A 10 μL aliquot of the reaction was diluted 10× with water and directly analyzed by UPLC-HRMS method C.

In vitro production of sulfazecin from D-Glu-D-Ala-PCP₂

Dipeptide-*holo*-PCP₂ and *holo*-M3 were generated as before. Dipeptide-*holo*-PCP₂ (200 μM) was added to a solution of *holo*-M3 (2 μM) in assay buffer supplemented with L-Dap (2 mM), ATP (2.5 mM), SulN (5 μM), PAPS (1 mM), ferrous ammonium sulfate (80 μM), α-ketoglutarate (8 mM), ascorbate (1 mM), SAM (2 mM), SulO (5 μM), and SulP (5 μM) (all concentrations are final concentrations). The reactions were continued at room temperature for 2 h, then diluted with one volume of acetonitrile and filtered through an Amicon 3K (Millipore) filter. The filtrates were analyzed directly by UPLC-HRMS method A.

Oxidation and methylation of desmethoxysulfazecin by SulO and SulP

A synthetic standard of desmethoxysulfazecin (500 μM) was incubated in assay buffer supplemented with ferrous ammonium sulfate (80 μM), α-ketoglutarate (6 mM), and sodium ascorbate (1 mM). SulO (20 μM) and *S*-adenosylmethionine (2 mM) were added and the reaction split into two tubes. To one tube was added SulP (10 μM) and the reactions were allowed to proceed at room temperature for 4 h. Acetonitrile was added to precipitate the proteins and the reaction mixtures were filtered through an Amicon Ultra 3K filter. The filtrate was analyzed directly by UPLC-HRMS method B.

Native PAGE SulN: PCP₃-TE interaction analysis

A 12% polyacrylamide native PAGE gel was prepared using Tris•HCl (pH = 8.8). Samples were prepared containing the indicated concentrations of *apo*-PCP₃-TE, SulN, and PAPS, and incubated 30 min. Following a standard protocol²⁵, loading buffer was then added and the samples loaded onto the gel, and run at 4 °C. The gel was subsequently visualized using Commassie Blue stain.

TE-catalyzed hydrolysis of desmethoxysulfazecin

Desmethoxysulfazecin **2** (1 mM) was incubated with *apo*-PCP₃-TE (10 μM) or catalytically inactive *apo*-PCP₃-TE C2818A (10 μM). 75 μL aliquots were taken at 1, 60, 120, and 240

min. Aliquots were diluted with 75 μ L acetonitrile and filtered through an Amicon Ultra 3k filter, then analyzed directly by UPLC-HRMS method A.

TE-catalyzed hydrolysis of sulfazecin

Sulfazecin purified from a fermentation of *P. acidophila*¹² (1 mM) was incubated with *apo*-PCP₃-TE (10 μ M) or catalytically inactive *apo*-PCP₃-TE C2818A (10 μ M). Aliquots (30 μ L) were taken at 1, 60, 120, and 240 min. The samples were diluted 5 \times with acetonitrile and filtered through an Amicon Ultra 3k filter for analysis directly by UPLC-HRMS method A.

SNAC and SPANT tripeptide assay with SulM TE

Either SNAC or SPANT tripeptide thioesters (Supplementary Note) (1 mM) were incubated with SulM TE (5 μ M) in assay buffer at room temperature. The reactions were left for 3 h, at which time they were diluted 2 \times with acetonitrile and microfiltered to remove protein. The crude filtrate was analyzed directly by UPLC-HRMS method B.

apo-PCP₃-TE loading and evaluation of products released from TE

Tripeptide CoA (Supplementary Note) (500 μ M) was incubated with either wildtype *apo*-PCP₃-TE, *apo*-PCP₃-TE* C2818A, or *apo*-PCP₃-TE* C2818S (100 μ M) in assay buffer supplemented with MgCl₂ (10 mM). Sfp was added (5 μ M) to initiate loading of the tripeptide onto the thiolation domain of the didomain constructs. The reactions were incubated at room temperature for 1 h then diluted 2 \times with acetonitrile, microfiltered to remove protein, and directly analyzed by UPLC-HRMS method B.

Sulfonation assay of β -lactam tripeptide 1 and D-Glu–D-Ala–L-Dap with SulN

Either tripeptide β -lactam or the unadorned D,D,L-tripeptide (Supplementary Note) (750 μ M) was incubated in assay buffer with SulN (20 μ M) in the presence or absence of PAPS (2 mM). The reactions were allowed to stand for 3 h before dilution with acetonitrile (2 \times). The reaction mixtures were then microfiltered to remove protein and directly analyzed by UPLC-HRMS method B.

Statistical Analysis

Data with error bars are expressed as mean \pm SD. In these cases the statistical analysis was calculated from 3 independent experiments.

Data availability

Sequences of constructs used in this study have been deposited in GenBank (accession numbers MF407278, MF359597, MF359598, MF359599), or may be found in the DNA sequence of the sulfazecin gene cluster (GenBank KX757706).

All data generated and analyzed during the course of this study and interpretation of results is available from the corresponding author at reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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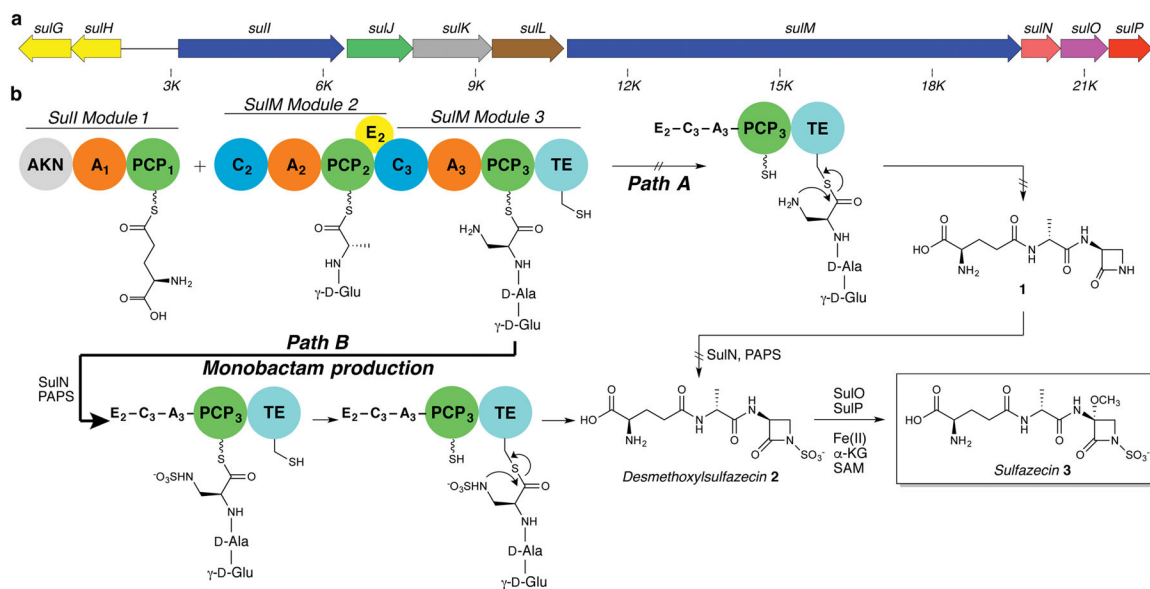


Figure 1. Alternative potential biosynthetic routes to monobactam synthesis in sulfazecin
(a) Biosynthetic gene cluster responsible for the production of sulfazecin in *P. acidophila*.
(b) Possible routes of β -lactam formation in sulfazecin. Path A involves β -lactam formation by the cysteine-containing thioesterase (TE) domain, whereas path B entails *N*-sulfonation of the NRPS-tethered tripeptide *in trans* before monobactam formation in the TE domain. AKN denotes the predicted adenylyl-sulfate kinase.

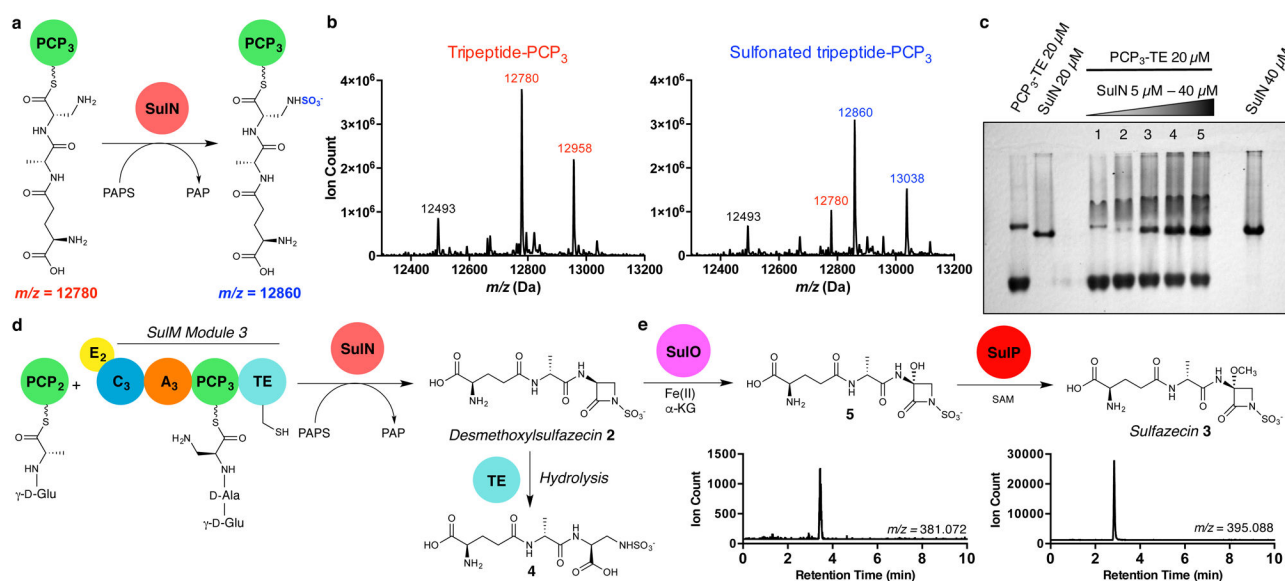


Figure 2. M3-catalyzed monobactam formation

(a) Sulfonation of tripeptide-*holo*-PCP₃ by SulN. (b) Mass spectra of tripeptide-*holo*-PCP₃ (MW = 12,780) and sulfonated tripeptide-*holo*-PCP₃ (MW = 12,860). The corresponding *N*-glucuronidated masses (12,958 and 13,038, respectively) are also present. (c) Native PAGE mobility shift assay showing the association between apo-PCP₃-TE and SulN. SulN concentrations of 5, 10, 20, 30, and 40 μM coincide with lanes 1, 2, 3, 4, and 5, respectively. (d) *In vitro* reconstitution of module 3 β-lactam formation activity. Upstream D-Glu-D-Ala-*holo*-PCP₂ was exposed to wildtype *holo*-module 3 in the presence of SulN, producing desmethoxysulfazecin followed by hydrolysis to **4**. (e) Addition of SulO and SulP to the *in vitro* reconstitution assay produced sulfazecin. Shown are the extracted ion chromatograms of both intermediate **5** (calc. 381.0722, found 381.0709) and sulfazecin (calc. 395.0878, found 395.0858).