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A trans-acting cyclase off-loading strategy for non-ribosomal peptide synthetases

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Abstract

The terminal step in the biosynthesis of non-ribosomal peptides is the hydrolytic release, and frequently, macrocyclization of an aminoacyl-S-thioester by an embedded thioesterase. The surugamide biosynthetic pathway is composed of two NRPS assembly lines where one produces surugamide A, which is a cyclic octapeptide, and the other produces surugamide F, a linear decapeptide. The terminal module of each system lacks an embedded thioesterase, which led us to question how the peptides are released from the assembly line (and cyclized in the case of surugamide A). We characterized a cyclase belonging to the β-lactamase superfamily (SurE) in vivo and established that it is a trans-acting release factor for both compounds and verified this functionality in vitro with a thioester mimic of linear surugamide A. Using bioinformatics, we estimate that ~11% of filamentous Actinobacteria harbor an NRPS system lacking an embedded thioesterase and instead employ a trans-acting cyclase. This study expands the paradigmatic understanding of how non-ribosomal peptides are released from the terminal PCP and adds a new dimension to the synthetic biology toolkit.

Non-ribosomal peptides (NRPs) are a large family of structurally complex and diverse natural products, often with biologically and therapeutically relevant activities. They are synthesized by large multifunctional enzymes called non-ribosomal peptide synthetases (NRPSs), which are organised into relatively independently functioning modules that work in an assembly line-like manner until the final polypeptide structure is generated.[1] During biosynthesis, the growing peptide chain remains covalently linked to the 4´-phosphopantetheinyl cofactor of the peptidyl carrier protein (PCP) domains. The terminal module usually possesses a C-terminal thioesterase (TE) domain, which off-loads the polypeptide intermediate from the PCP on to a conserved serine residue whereby either a hydrolytic or macrocylization reaction occurs to produce the mature peptide.[2]

Surugamide A (**1**) and associated minor products B-E (**2-5**) are cyclic octapeptides initially identified from Streptomyces sp. JAMM992 as inhibitors of cathepsin B, which also possess antibacterial activity.[3,4] Identification of the surugamide (sur) biosynthetic gene cluster (BGC) from this organism revealed the presence of four NRPS genes (*surABCD*), which encoded 18 biosynthetic modules (**Figure 1, Scheme 1**). The eight modules encoded by SurAD were consistent with the biosynthesis of **1-5**, whereas the remaining 10 modules encoded by SurBC were shown to direct the biosynthesis of an unrelated linear decapeptide named, surugamide F (**7**), which is structurally similar to gramicidin A.[5] S. albus J1074 was recently shown to produce surugamides, including an unusual derivative named acyl-surugamide A (**6**), which has antifungal bioactivity.[6,7] We identified the same BGC in a related strain, S. albus S4[8] and verified that it produced the major products **1** and **7** using LC-HRMS (**Figures S1-S5**). An intriguing feature of the sur BGC harbored by these organisms is that the terminal biosynthetic modules of SurC and SurD lack an integrated thioesterase domain and alternative off-loading mechanisms such as hydrolytic condensation or reductase domains.

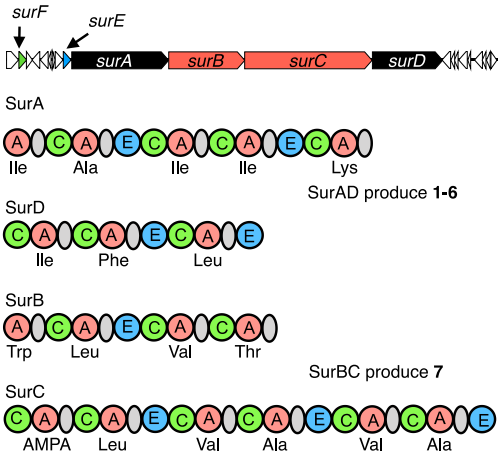
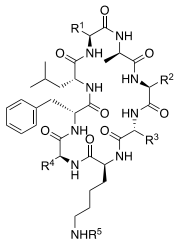
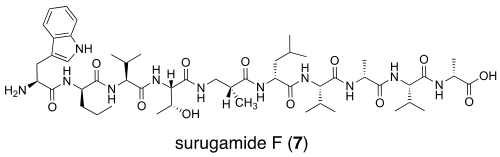


Figure 1. The surugamide (sur) biosynthetic gene cluster (BGC). Schematic representation of the sur BGC and domain organization of SurAD, which make cyclic surugamides (**1-6**) and SurBC, which makes surugamide F (**7**). A, adenylation domain; C, condensation domain; E, epimerase domain; AMPA, 3-amino-2-methylpropionic acid. Grey ovals indicate peptidylcarrier proteins.



	R^1	R^2	R^3	R^4	R^5
surugamide A (**1**)	L-Ile	D-Ile	L-Ile	L-Ile	H
surugamide B (**2**)	L-Ile	D-Val	L-Ile	L-Ile	H
surugamide C (**3**)	L-Val	D-Ile	L-Ile	L-Ile	H
surugamide D (**4**)	L-Ile	D-Ile	L-Val	L-Ile	H
surugamide E (**5**)	L-Ile	D-Ile	L-Ile	L-Val	H
acyl-surugamide A (**6**)	L-Ile	D-Ile	L-Ile	L-Ile	butryl



surugamide F (**7**)

Scheme 1. Structures of surugamides.

The absence of an obvious cis-acting mechanism for off-loading the final peptide from the terminal PCP domains of SurC and SurD led us to hypothesize that one or more trans-acting release factors may be encoded by genes within the sur BGC. Inspection of other genes within the BGC revealed two candidates, which we named surF and surE, that may perform this role. The surF gene product belongs to InterPro Family IPR029058, which includes enzymes with the characteristic alpha/beta hydrolase fold harbored by thioesterases.[9] Standalone proofreading thioesterases (so-

called type II thioesterases) are relatively common within NRPS biosynthetic systems, where they remove aberrant thioester intermediates from the assembly line.[10] However, SurF is bioinformatically inconsistent with this functionality. For instance, SurF belongs to IPR029058, while type II thioesterases belong to IPR1223; it also possesses a hydrolase signature motif (GxSxG) of GTSLG, which differs to that of GHSMG for established type II thioesterases.[11] Therefore, we investigated the possibility that surF encoded a trans-acting release factor. If this were the case, then its deletion would preclude detection of mature surugamides. To test this hypothesis, we deleted surF in S. albus S4 and assessed the resulting mutant strain (ΔsurF) for its ability to produce **1** and **7.** LC-HRMS analysis of chemical extracts prepared from the ΔsurF strain revealed that surF is not essential for the production of **1** or **7** (**Figure S6**), indicating that SurF is unlikely to be a factor that off-loads the terminal amino-acyl-S-thioester intermediates from the assembly line, but instead may be a type II thioesterase with an unusual hydrolase signature motif.

The surE gene encodes a protein belonging to InterPro Family IPR012338, indicating it is a member of the β-lactamase superfamily, which includes transpeptidases and esterases. Analysis of SurE indicates that it possesses the Ser-Lys-Tyr-His tetrad active site motif the characteristic of Class C β-lactamases (**Figure S7**) and that it is phylogenetically distinct from type I and type II thioesterases (**Figure S8**). A recent genetic study of mannopeptimycin biosynthesis suggested that a member of this protein family, MppK (39% shared amino acid identity with SurE) functioned as a termination/cyclization protein.[12] Therefore, in order to test whether surE encodes a trans-acting off-loading factor, we deleted surE in S. albus S4 and tested the resulting mutant strain for its ability to produce **1** and **7** by LC-HRMS. This revealed that only trace amounts of **1** and **7** were detectable in the extract prepared from the ΔsurE strain (**Figure 2**). Complementation of the ΔsurE mutant with pDT2, which contained the surE gene under the control of the constitutive ermE* promoter, restored production of both **1** and **7** to near wild-type levels and verified that loss of their production was not due to other mutational events (**Figure 2**). Taking these findings together, we conclude that SurE is required for the production of both **1** and **7**, presumably because it off-loads the linear aminoacyl-S-

thioester intermediate of surugamide A and performs a head-to-tail cyclisation reaction to result in **1** and simply hydrolytically releases the linear aminoacyl-S-thioester intermediate of surugamide F to result in **7**.

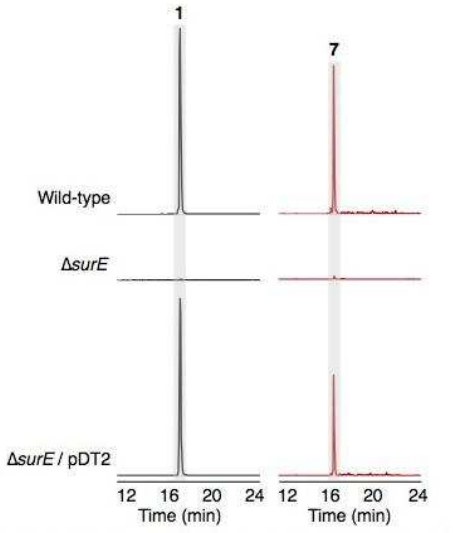


Figure 2. surE is essential for the production of surugamide A and surugamide F. LC-HRMS analysis of chemical extracts prepared from the indicated strains. The m/z corresponding to the $[M+H]^+$ ions derived from **1** (black, $C_{48}H_{81}N_9O_8$) and **7** (red, $C_{52}H_{85}N_{11}O_{12}$) are shown. The intensity scale for EICs is $1x10^6$ for **1** and is $1x10^5$ for **7.**

We sought to reconstitute these reactions in vitro to corroborate our in vivo data and to unambiguously determine the function of SurE. SurE was overproduced and purified from E. coli as an N-terminal hexahistidine tagged variant ($(His)_6$SurE, **Figure S9**). The cost of the AMPA residue within **7** led us to first verify the functionality of SurE using a thioester mimic of linear **1** before embarking on synthesis of the equivalent mimic for **7.** Thus, we synthesized an N-acetylcysteamine (SNAC) thioester mimic of **1** (**Figures S10-S13**) and assessed the ability of SurE to utilise this substrate. When SurE is incubated with SNAC-surugamide A (**8**) it catalyzes the formation of **1** as judged by LC-HRMSMS (**Figure 3, Figure S14-S16**). These data were recently corroborated by a report in which an ortholog of SurE from S. albidoflavus NBRC12854 catalyzed the same reaction in vitro with the SNAC thioester mimic of **2,** a shunt product of the pathway.[13] While our chemical synthesis of a thioester mimic of **7** was in progress, its synthesis and utilization by SurE was reported elsewhere.[14] Surprisingly, surugamide F was not the major product in vitro and instead SurE produced

a head-to-tail cyclized variant named cyclosurugamide F, which was also retrospectively observed in low abundance within a crude chemical extract generated from the producing organism. [14] The authors proposed an intriguing possibility that a cytosolic peptidase may hydrolyze the bond generated by SurE to result in **7.** [14] Taken together, our data and that of others unambiguously demonstrate that SurE is a trans-acting cyclase that off-loads the terminal linear aminoacyl-S-thioester intermediates of surugamides A and F and performs head-to-tail cyclisation reactions to result in **1** and **7.**

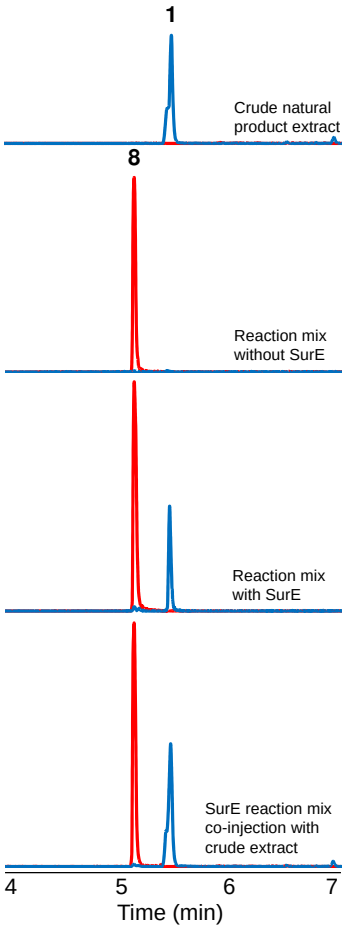


Figure 3. LC-HRMS analysis of in vitro SurE reaction with SNAC-surugamide A. (The m/z corresponding to the $[M+H]^{2+}$ ions derived from **1** (blue, $C_{48}H_{81}N_9O_8$) and **8** (red, $C_{52}H_{90}N_{10}O_9S$) are shown. The intensity scale for all EICs is $1x10^6$.

Although the hypothesis was not rigorously evaluated, MppK was suggested to perform a function analogous to SurE during the biosynthesis of mannopeptimycin[12] and putative orthologs have been observed within the desotamide and ulleungmycin BGCs.[15,16] This led us to evaluate how widespread the SurE off-loading mechanism may be within NRPS biosynthetic systems. To do this,

we identified organisms harboring sententious orthologs of SurABCD and SurE using a multigeneblast database that we previously built with 1,421 filamentous Actinobacterial genomes.[17,18] Next, the candidate genomes that arose from this analysis were subjected to analysis by antiSMASH 4.1.0[19] and the corresponding output was used to manually curate a list of NRPS biosynthetic systems lacking an embedded TE domain, but harboring an adjacent SurE ortholog. These criteria were satisfied by 174 NRPS systems harbored by 166 organisms in our dataset, suggesting that overall, ~11% of filamentous Actinobacteria harbor an NRPS biosynthetic system that utilizes a release strategy involving a trans-acting cyclase belonging to the β-lactamase superfamily.

Anecdotal observations during the manual curation of antiSMASH analyses suggested that several of the identified biosynthetic systems were related to one another. In order to explore this possibility, we used BiG-SCAPE[20] to generate a BGC similarity network of the NRPS systems identified above (**Figure 4, Table S1**). The final network was grouped into 15 subnetworks and 12 singletons. In addition to 174 nodes for the BGCs identified here, three additional nodes were generated from entries within the MIBiG database of experimentally characterized BGCs[21]; these three nodes correspond to BGCs that direct the biosynthesis of surugamides[5], ulleungmycin[16] and dechlorocuracomycin[22]. Surprisingly, the BGCs for desotamide[15] and mannopeptimycin[12], which are also present within the MIBiG database, were not resolved within the network, implying that they are distantly related systems. The number of modules encoded within a biosynthetic system, which ranged from 4-10 modules and is color-coded within Figure 4, was a major factor that influenced subnetwork formation (**Figure 4, Table S1**). SurE itself is capable of accepting amino-S-acyl thioester substrates 8 and 10 amino acid residues in length, however our bioinformatics analyses suggests that SurE and/or its orthologs may be able to utilise substrates composed of just four amino acid residues. Future studies will interrogate the molecular rules underpinning substrate recognition by SurE.

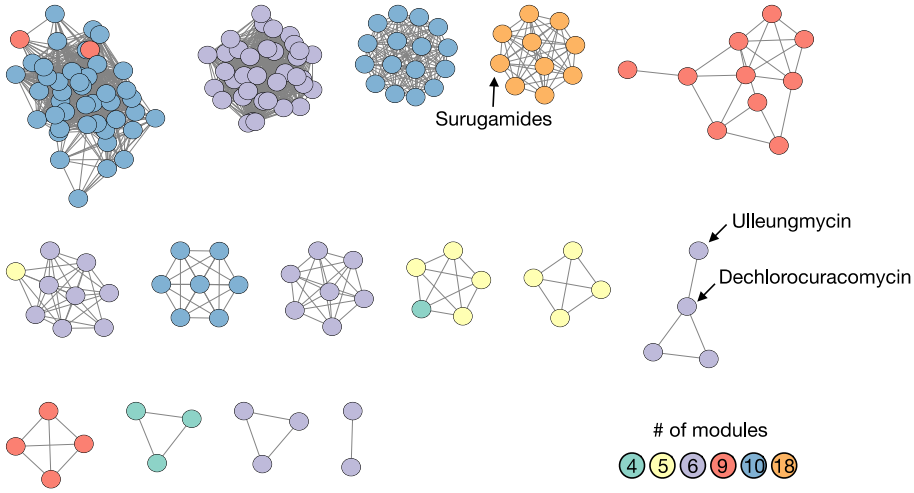


Figure 4. Gene cluster similarity network for NRPS BGCs without an embedded thioesterase but encoding a SurE ortholog. Each node represents a BGC and is color-coded based on the number of its putative biosynthetic modules. The node table is shown in **Table S1.**

In summary, we have identified and characterized in vivo and in vitro a trans-acting release factor, SurE, that off-loads surugamides via macrolactamization from their respective assembly lines. Based on our bioinformatic analyses, we propose that this release mechanism is relatively widespread among Actinobacteria. This work expands the paradigmatic understanding of how non-ribosomal peptides are off-loaded from terminal PCP domains in NRPS assembly lines and in the process has identified a standalone (thereby more amenable to augmentation) release factor that could aid reengineering of non-ribosomal peptide biosynthesis. As access to genomic data increases and bioinformatics approaches become more robust and sophisticated, we should expect and indeed look forward to discovering the myriad of ways nature has evolved biosynthetic processes.

Associated content

Supporting information

The supporting information is available free of charge via the internet at http://pubs.ac.org.

Experimental details, LC-MS and NMR spectra and supporting figures and tables (PDF)

Acknowledgements

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Supporting Information

A trans-acting cyclase off-loading strategy for non-ribosomal peptide synthetase

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Growth media, strains, cosmids, plasmids and other reagents. Escherichia coli strains were cultivated using Lennox agar (LA) or broth (LB) and Streptomyces strains were propagated on mannitol-soya flour agar or ISP2 broth.[1] Culture media was supplemented with antibiotics as required at the following concentrations: apramycin (50 μg/ml), carbenicillin (100 μg/ml), hygromycin (75 μg/ml), kanamycin (50 μg/ml), nalidixic acid (25 μg/ml). Enzymes were purchased from New England Biolabs unless otherwise stated. Oligonucleotides were purchased from Integrated DNA Technologies and are described in **Table S2.** The DNA constructs and bacterial strains used in this study are listed in **Table S3.**

Construction and complementation of mutant strains. The surE and surF genes were deleted using the ReDirect PCR targeting system[2] with Cos994. Cos994 was identified by screening a previously constructed S. albus S4 cosmid library[3] by PCR using primers DT159 and DT160, which targeted an internal fragment of surF. Cos994 was insert-end sequenced using primers RFS184 and RFS185 and the resulting reads were mapped to the S. albus S4 genome[4,5] in order to establish the sequence of the cloned insert. Gene disruption cassettes consisting of an oriT and the apramycin resistance gene, aac(3)IV from pIJ773[2] were generated by PCR using primers DT161 and DT162 (surE), and DT167 and DT168 (surF) containing 40 nt of homology that included the start and stop codons of each gene and 37 nt upstream or downstream. The resulting gel purified PCR products were used to delete surE or surF from Cos994 by recombineering with E. coli GB05-red[6]. Mutagenised cosmids were electroporated into E. coli ET12567/pUZ8002 and subsequently mobilized to S. albus S4 by conjugation as described.[1] Transconjugants were selected for apramycin resistance and kanamycin sensitivity. The integrity of mutant strains was verified by PCR. The complemented ΔsurE strain was generated as follows. The surE gene PCR amplified from S. albus S4 genomic and cloned into pIJ10257[7] at the NdeI and HindIII sites. The resulting plasmid (pDT2) was sequenced using RFS582 and DT186 to verify the integrity of cloned insert and mobilised by conjugal transfer into the S. albus S4 ΔsurE. The resulting strain was verified by PCR.

Generation of chemical extracts and LC-HRMS analysis. S. albus strains were first cultured in 10 ml of LB while shaking (220 rpm) at 30 °C for 3 days, at which point the entire culture was added to ISP2 broth (50 ml in a 250 ml flask) and incubated at 30 °C with shaking (180 rpm) for 4 days. Bacterial cells were removed by centrifugation, and metabolites were extracted from the supernatant twice with two volumes of ethyl acetate. Extracts were pooled and concentrated in vacuo. The remaining residue was resuspended in 0.7 ml of 100% MeOH. Two microliters of methanolic extract were injected into a Bruker MaXis Impact II mass spectrometer coupled with a Dionex 3000 RS UHPLC equipped with an Agilent C18 column (2.1 × 100 mm). The solvent system was as follows: solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile. The following gradient was used: 0-5 min, 5% B; 5-20 min, 5-100% B; 20-25 min, 100% B; 25-27 min, 100-5% B. The flow rate was 0.2 ml/min. Calibration was achieved with 1 mM sodium formate at the beginning of each run.

Overproduction and Purification of Recombinant Protein. The surE PCR amplified from extracted S. albus S4 genomic DNA using primers DT206 and DT213 (**Table S2**). The resulting PCR product was gel purified and digested with NdeI and XhoI endonucleases, and ligated with T4 DNA ligase to pET28a cut with the same enzymes to result in pET28-surE. The integrity of the cloned insert was verified by DNA sequencing and subsequently used to transform E. coli BL21-Gold(DE3) (Agilent Technologies). For overproduction of the recombinant protein, a starter culture E. coli cells harboring pET28-surE were cultured overnight in 10 ml LB supplemented with kanamycin while shaking (180 rpm) at 37 °C. Five milliliters of the starter culture were used to inoculate 500 ml of Super Broth auto-induction media (Formedium) and cultured for 48 hours while shaking (180 rpm)

at 16°C. Bacterial cells were collected by centrifugation, resuspended in lysis buffer (50 mM HEPES, 500 mM NaCl, 10 mM imidazole, 1 mM EDTA, protease inhibitor, DNAse I, pH 7.5) and lysed using an ultrasonic probe (40% power, 2 x× 90 seconds, 1 pulse s^{-1}). Lysates were clarified by centrifugation and passed through a 20 ml BioRad Econo Pac column loaded with 3 ml Ni-NTA resin (Generon) by gravity flow. The resin was washed with two column volumes of wash buffer (50 mM HEPES, 200 mM NaCl, 50 mM imidazole, 1 mM EDTA, pH 7.5). Protein was eluted from the resin with elution buffer (50 mM HEPES, 200 mM NaCl, 300 mM imidazole, 1 mM EDTA, pH 7.5) and analysed by SDS-PAGE. Elution fractions containing the protein of interest were pooled and concentrated using an Amicon spin filter (Merck) and subjected to buffer exchange by dialysis into storage buffer (50 mM HEPES, 200 mM NaCl, pH 7.5).

Chemical Synthesis of SNAC-surugamide A. Linear surugamide A was synthesised as a protected peptide as follows. 2-Chlorotrityl resin (100 mg, 1.00-1.80 mmol g^{-1}) was swollen in DMF (10 ml) for 30 minutes. The resin was transferred to the peptide synthesiser and coupled twice with Fmoc D- Leu (1 mmol) using DIC (1 mmol) and Oxyma (1 mmol) for 30 minutes with microwave irradiation. The resin was washed with DMF (2 × 10ml) after the coupling step. Fmoc deprotection was conducted using 20% pipiridine in DMF (2 × 10ml) and washed with DMF (2 × 10ml). The coupling and deprotection cycle was repeated using Fmoc D-Phe, Fmoc L-Ile, Fmoc L-Lys(Boc), Fmoc D-Ile, Fmoc L-Ile, Fmoc D-Ala and Boc L-Ile. The resin was filtered and washed successively with DMF (2 × 10ml) and DCM (2 × 10ml). The 2-chlorotrityl resin was deprotected using hexafluoroisopropanol in DCM (1:4, 3ml) for 2 hours after which time the resin was filtered and washed with DCM (3 × 10ml). The solvents were removed in vacuo resulting in an off-white solid which was used without further purification.

In vitro SNAC assay. The ability of $(His)_6$SurE to utilise SNAC-surugamide A was assessed in vitro using the following reaction conditions in a final volume of 100 µl: 50 mM HEPES (pH 7.5), 0.1 mM SNAC-surugamide A, 20 µg of $(His)_6$SurE. The enzymatic reaction or SNAC-surugamide A alone were incubated at 30 °C. After 5 hrs of incubation, samples were treated with 100 µL of 0.05% TFA and extracted with Phenomenex Strata-XL C18 (100 µm, 30 mg, 1 ml) solid-phase extraction column and a vacuum manifold. Metabolites were eluted from the column with 120 µl of 100 % methanol. The methanolic extract was either diluted 1:1 with 100% methanol or mixed 1:1 with crude natural product prior to centrifugation for 10 mins at 16,000g to remove any insoluble material before analysis by LC-HR-ESI MSMS on a Bruker MaXis Impact II TOF mass spectrometer equipped with a Dionex UltiMate 3000 high-performance liquid chromatography apparatus with conditions described previously[8].

The crude protected peptide was dissolved in DCM (2 ml) to which was added DIC (63 mg, 0.5 mmol), HOBT (67 mg, 0.5 mmol) and N-acetylcysteamine (60 mg, 0.5 mmol). The solution was stirred at room temperature overnight followed by removal of solvent in vacuo. The crude thioester was globally deprotected by dissolving in 9:1 TFA:DCM and stirred at 0 ºC to room temperature for 3 hours. The volatiles were removed in vacuo and the crude sample dissolved in cold water (10 ml). ice cold diethyl ether was used to precipitate the peptide which was collected by centrifugation (4 ºC). The crude SNAC peptide was purified by preparative HPLC using Agilent 1260 Mass Directed Preparative HPLC instrument equipped with a Phenomenex Kinetex EVO C18 column (5 µm, 100 × 21.2 mm). The solvent system was as follows: solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile. The following gradient was used: 0-2 min, 5% B; 2-12 min, 5-95% B; 12-14 min, 95% B; 14-15 min, 100-5% B. The flow rate was 20 ml/min.) and solvent removed in vacuo resulting in the pure peptide as a white solid (8 mg). The final peptide was subjected to NMR spectroscopy on a JEOL ECA600 series II 600MHz NMR spectrometer ^{1}H NMR (600 MHz, D_2O)

7.29 – 7.26 (m, 2H), 7.23 – 7.19 (m, 3H), 4.68 (m, 1H), 4.41 (dd, J = 10.1, 3.9 Hz, 1H), 4.35 (q, J = 7.2 Hz, 1H), 4.25 (dd, J = 9.4, 5.3 Hz, 1H), 4.21 (d, J = 7.1 Hz, 1H), 4.02 (d, J = 8.0 Hz, 1H), 3.93 (d, J = 7.9 Hz, 1H), 3.70 (s, 1H), 3.24 (t, J = 6.3 Hz, 2H), 3.20 (dd, J = 14.1, 5.5 Hz, 1H), 2.96 – 2.82 (m, 6H), 1.86 (s, 3H), 1.80 (ddd, J = 9.9, 7.4, 4.5 Hz, 2H), 1.71 (tt, J = 11.4, 5.6 Hz, 1H), 1.67 – 1.52 (m, 8H), 1.45 – 1.32 (m, 4H), 1.31 (d, J = 7.2 Hz, 3H), 1.29 – 1.24 (m, 1H, 1.20 (ddd, J = 13.6, 7.5, 3.5 Hz, 1H), 1.17 – 1.02 (m, 4H), 0.94 (dt, J = 13.7, 7.8 Hz, 1H), 0.89 (d, J = 6 9 Hz, 3H), 0.85 – 0.80 (m, 14H), 0.77 (ddd, J = 7.8, 6.5, 1.3 Hz, 10H), 0.66 (t, J = 7.4 Hz, 3H), 0.49 (d, J = 6.9 Hz, 3H).0.5 (d, J = 6.9 Hz, 3H); HMRS $C_{52}H_{91}N_{10}O_9S^+$ $[M+H]^+$ requires 1031.6686; found 1031.6712 (Δ 2.5ppm).

Bioinformatics analysis. A bespoke multigeneblast[9] database of 1,421 Prokka-annotated[10] Actinobacterial genomes (Genera: Actinobactera, Actinomadura, Actinospica, Amycolatopsis, Kitasatospora, Micromonospora, Nocardia, Saccharopolyspora, Planomonospora, Pseudonocardia, Salinispora, Streptacidiphilus and Streptomyces) was previously constructed[11]. SurABCDE were used as a multigeneblast query with the default settings. The resulting output was inspected manually to identify genomes harboring putative NRPS systems with an adjacent SurE ortholog. The resulting complete genomes and draft genome contigs harboring such systems were subjected to antiSMASH analysis (webserver version 4.1.0).[12] The resulting output was manually inspected to determine if the putative terminal biosynthetic module harbored an embedded TE domain. Systems harbored near the end of a contig were discarded in order to remove truncated genes from future analyses. In total, 174 NRPS BGCs were identified that appeared to lack an embedded thioesterase domain and harbor an adjacent SurE ortholog. The corresponding antiSMASH-generated GenBank files for these systems were redacted to remove all genes except those encoding the putative chemical scaffold and off-loading factor (i.e. putative NRPS genes and the SurE ortholog) in order to make generation the BGC similarity network more coherent. BiG-SCAPE[13] was used with the redacted GenBank files to generate a BGC similarity network. The graphic displayed in Figure 4 was generated using Cytoscape 3.7.1[14] and the raw BiG-SCAPE is available from via Figshare (https://doi.org/10.6084/m9.figshare.7665494.v1). The multi-sequence alignment depicted in Figure S7 was generated using the Geneious R8.1.7 implementation of MUSCLE. [15] The phylogeny of SurE, β-lactamses and thioesterases was constructed from a MUSCLE multi-sequence alignment using the Geneious R8.1.7 implementation of PhyML 3.2.2.[16] The thioesterase sequences used to construct the phylogeny were obtained from accession provided by.[17]

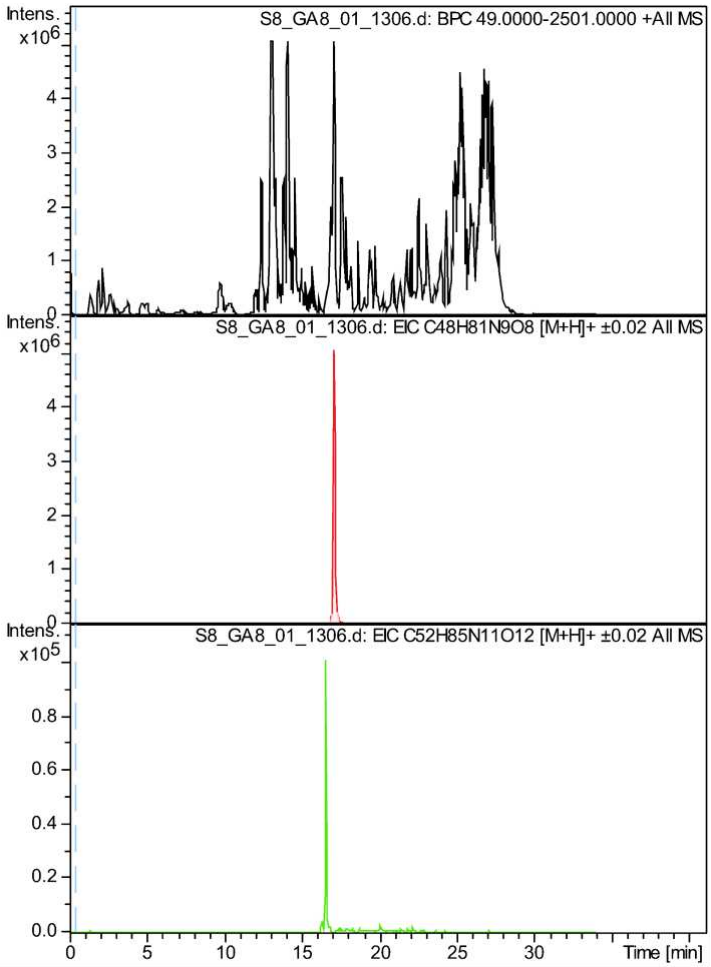

Figure S1. Streptomyces albus S4 produces surugamide A (**1**) and surugamide F (**7**). **(A)** LC-HRMS analysis of chemical extracts prepared from S. albus S4. The base peak chromatogram (BPC) and the m/z corresponding to the $[M+H]^+$ ions derived from surugamide A (red, $C_{48}H_{81}N_9O_8$) and surugamide F (green, $C_{52}H_{85}N_{11}O_{12}$) are shown.

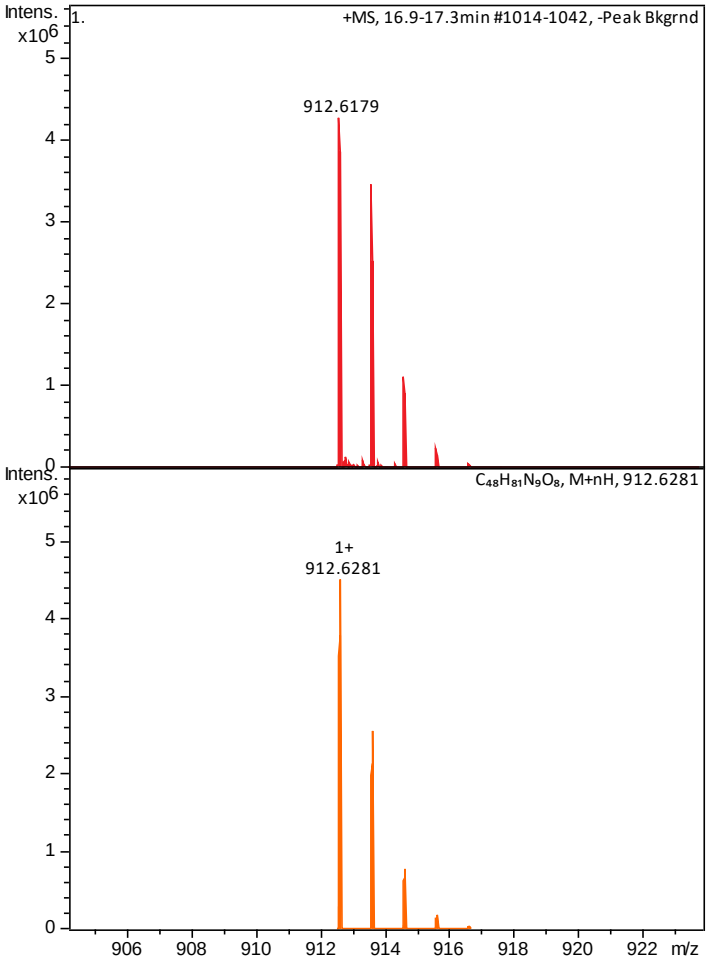

Figure S2. The measured (red) and simulated (orange) $[M+H]^+$ mass spectra for surugamide A.

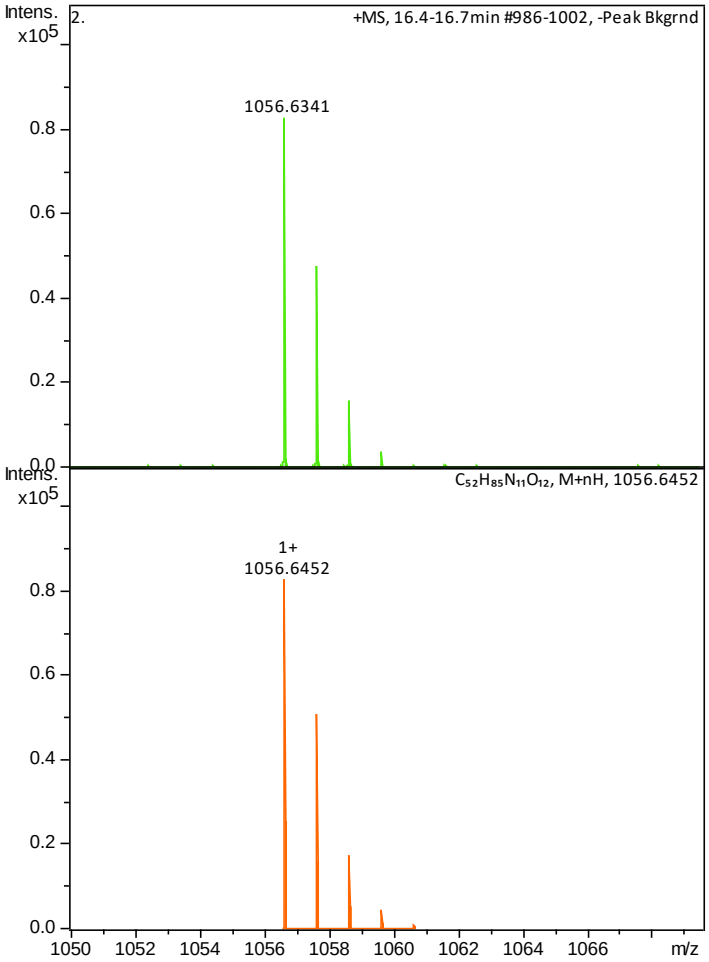

Figure S3. The measured (green) and simulated (orange) $[M+H]^+$ mass spectra for surugamide F.

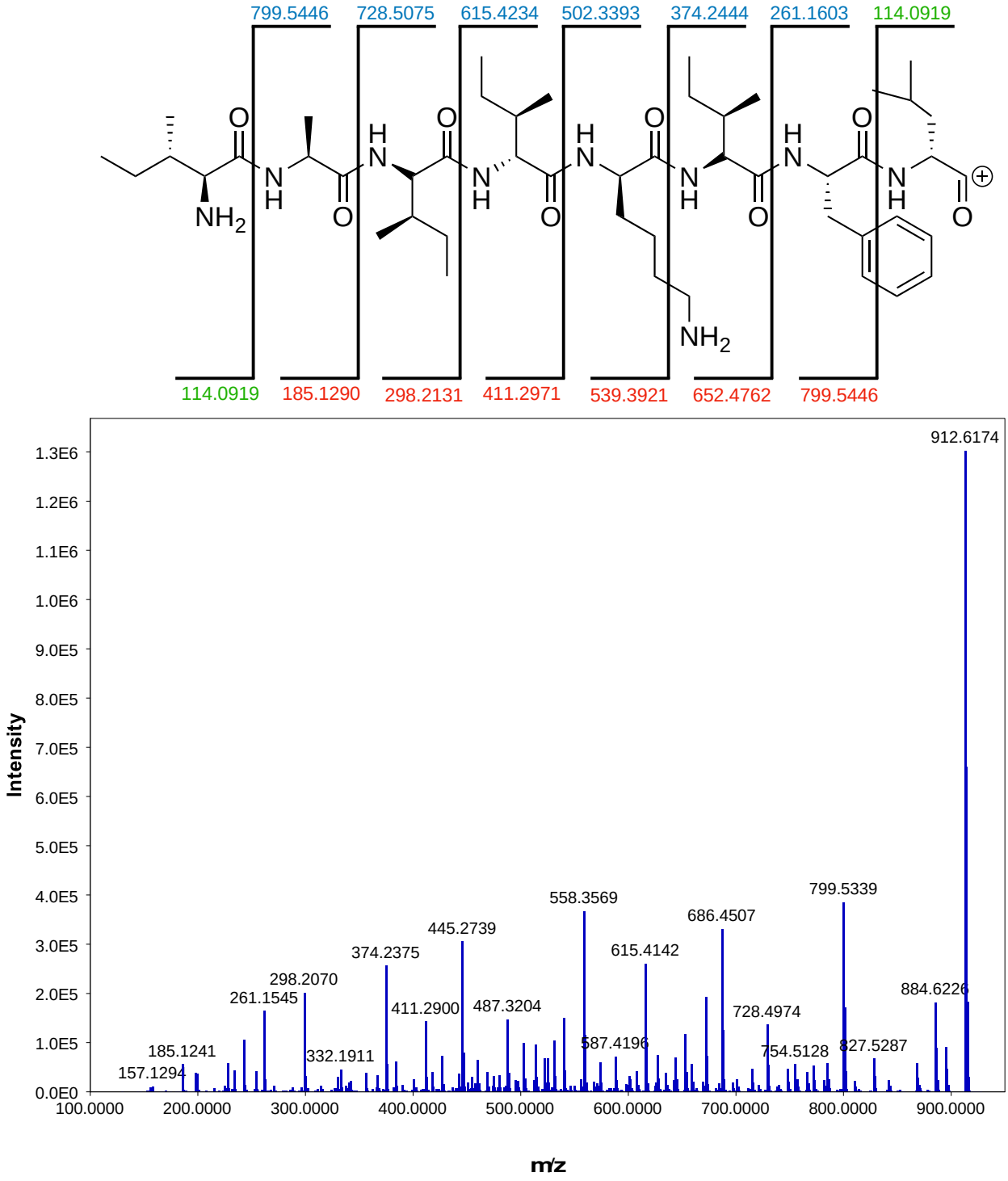

Figure S4. MS2 fragmentation of surugamide A. Mapped fragments are color-coded. Green text indicates an unobserved ion.

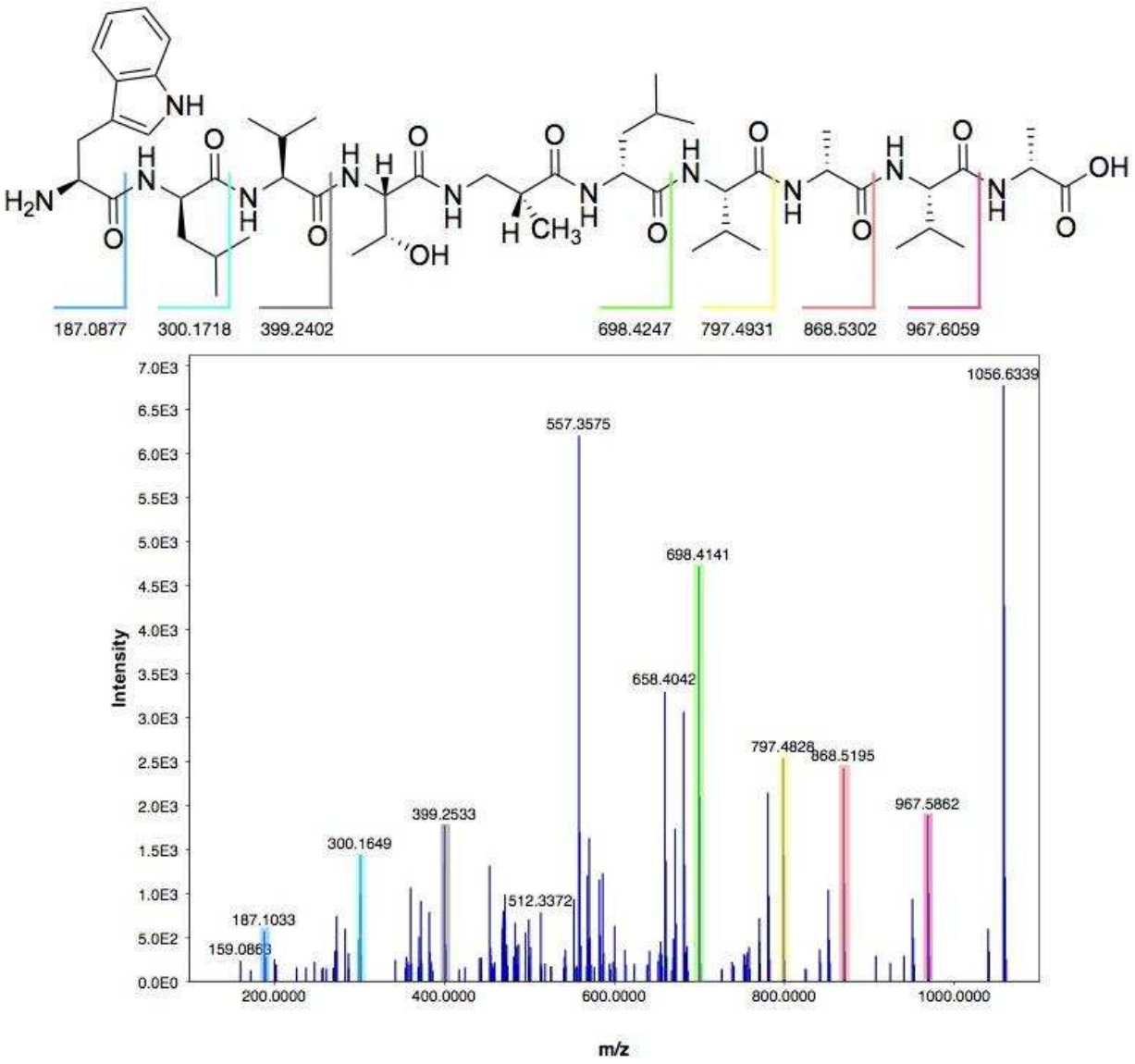

Figure S5. MS2 fragmentation of surugamide F. Mapped fragments are color-coded.

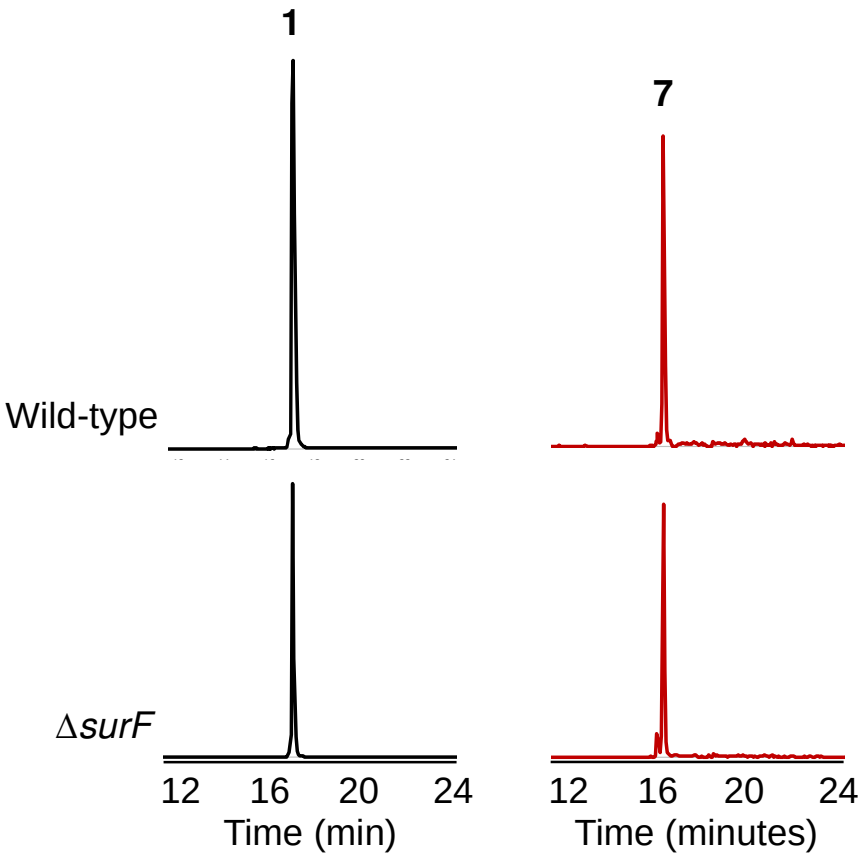

Figure S6. SurF is not essential for the production of **1** or **7.** LC-HRMS analysis of chemical extracts prepared from the indicated strains. The m/z corresponding to the $[M+H]^+$ ion derived from **1** ($C_{48}H_{81}N_9O_8$) and **7** ($C_{52}H_{85}N_{11}O_{12}$) are shown. The intensity scale is $1x10^6$ for EICs of **1** and $1x10^5$ for EICs of **7.**

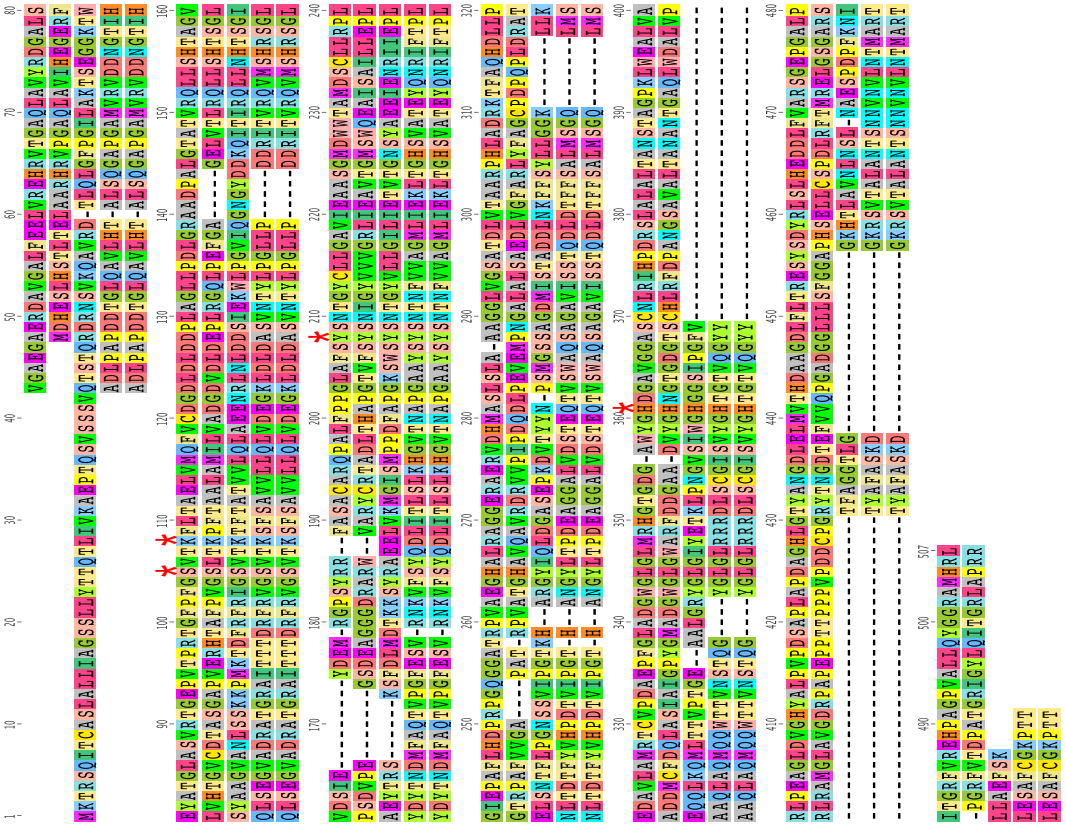

Figure S7. MUSCLE alignment of SurE with closely related proteins. MppK (discussed in the text) is a proposed cyclase involved of mannopeptimycin. 4Y7P, 1IKI, 1CEF are PDB accessions for structurally characterised D-peptidases. The active site tetrad Ser-Lys-Tyr-His is indicated by red asterisks.

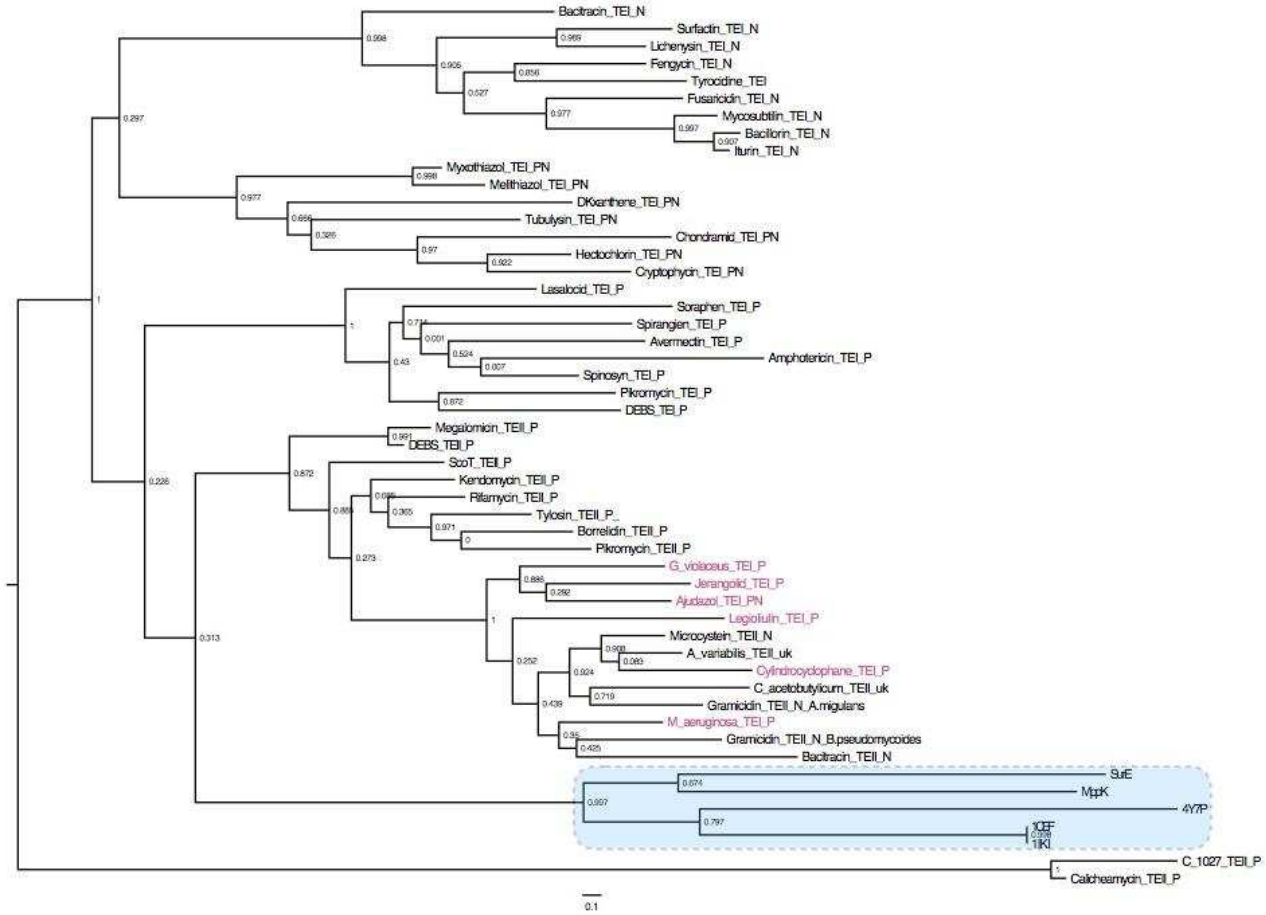

Figure S8. Maximum likelihood phylogeny of SurE and related proteins. The mid-root phylogeny is based on SurE, type I and Type II thioesterases and β-lactamases. SH-like support values are indicated at nodes as decimal values. The amino acid sequences used here, with the exception of SurE, MppK and the β-lactamase PDB entries (shaded blue), were obtained from. [17]. Pink text indicates cis-encoded type II thioesterases. TE_I, type I thioesterase; TE_II, type II thioesterase; P = from PKS system; N = from NRPS system; PN = from PKS-NRPS system; uk = unknown. The scale bar represents 10% sequence divergence.

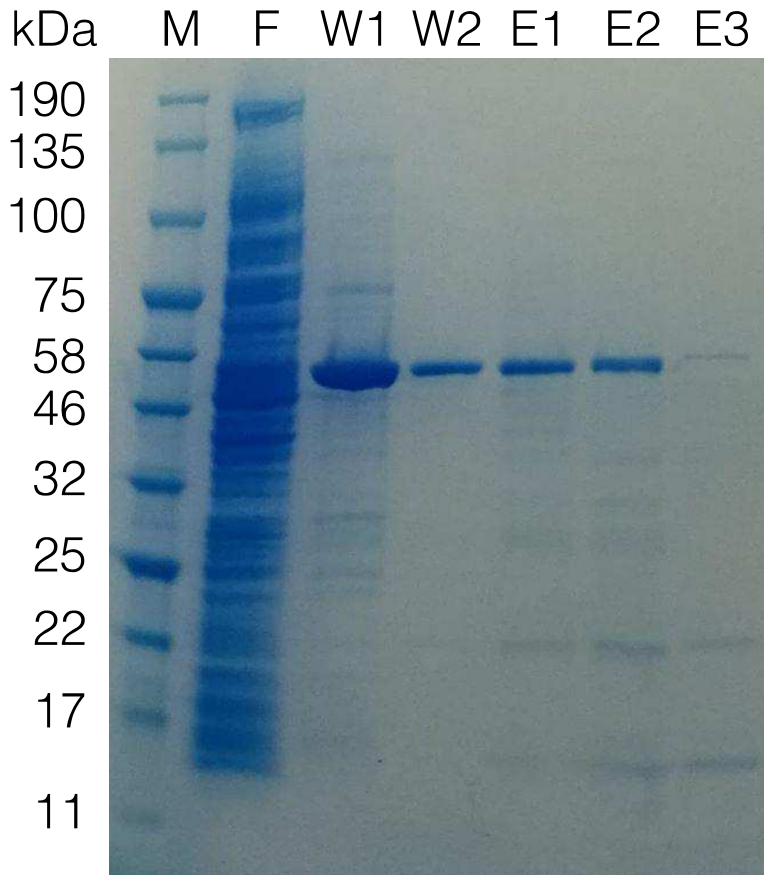

Figure S9. SDS-PAGE analysis of purified $(His)_6$SurE from E. coli BL21-Gold(DE3). M, marker; F, Ni-NTA column flow through; W1, first wash; W2, second wash; E1-E3, elutions 1-3. Buffers for washing and elution indicated in the methods section.

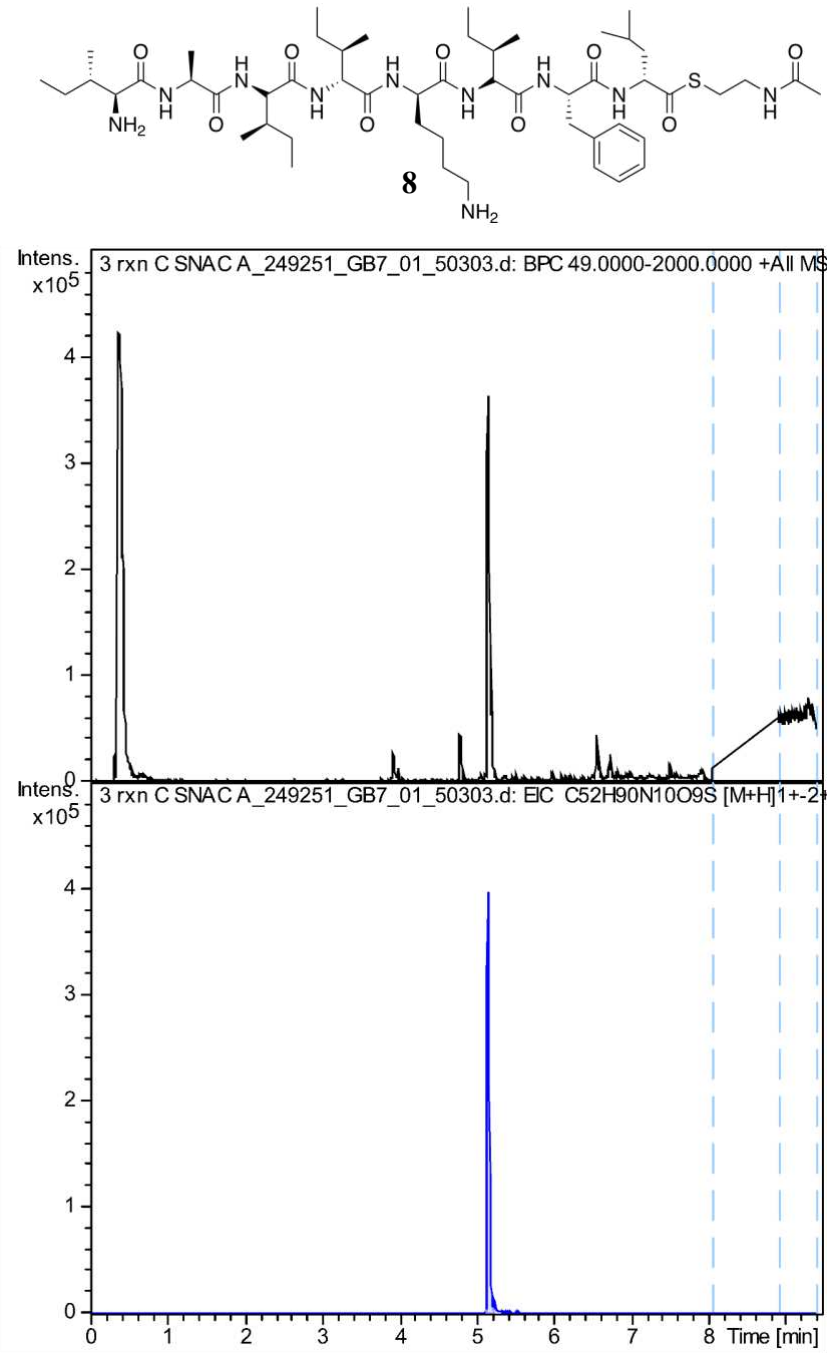

Figure S10. LC-HRMS analysis of SNAC-surugamide A (**8**). The base peak chromatogram (BPC) and the m/z corresponding to the $[M+H]^+$ ion derived from **8** ($C_{52}H_{90}N_{10}O_9S$) is shown in blue.

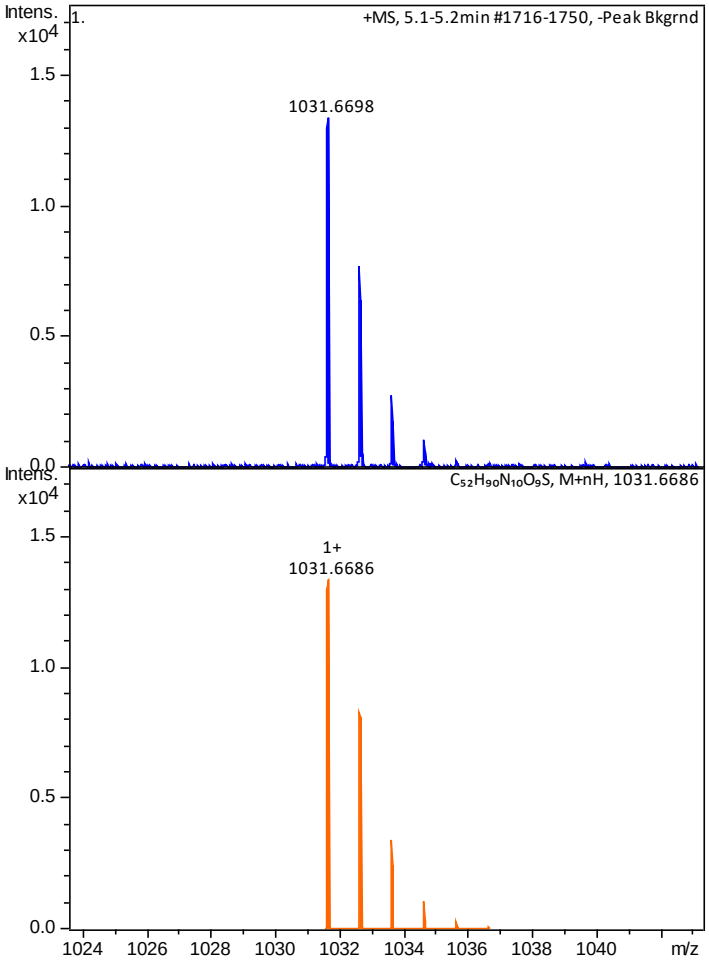

Figure S11. The measured (blue) and simulated (orange) $[M+H]^+$ mass spectra for SNAC-surugamide A.

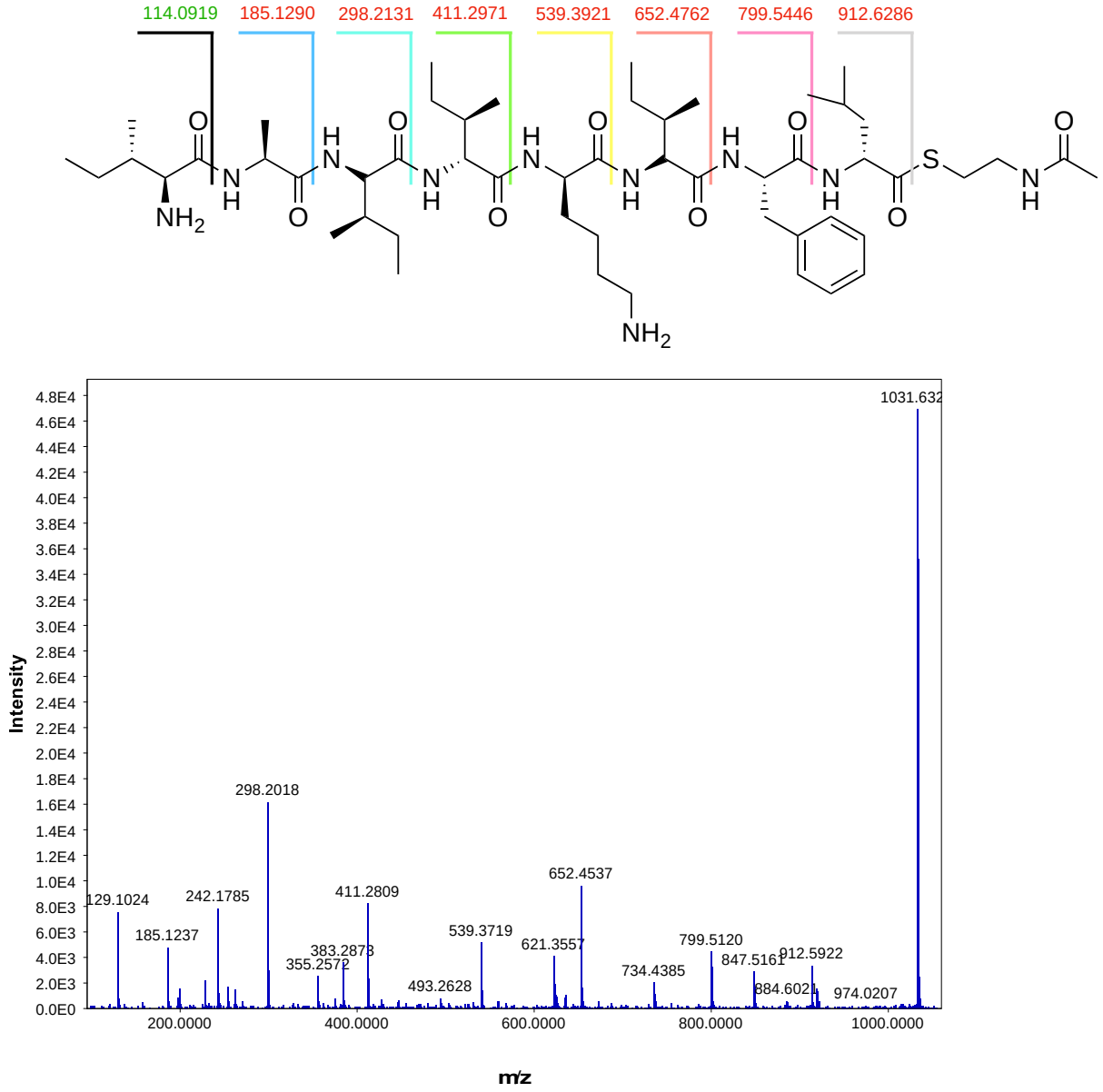

Figure S12. MS2 fragmentation of SNAC-surugamide A (**8**). Mapped fragments are color-coded. Green text indicates an unobserved ion.

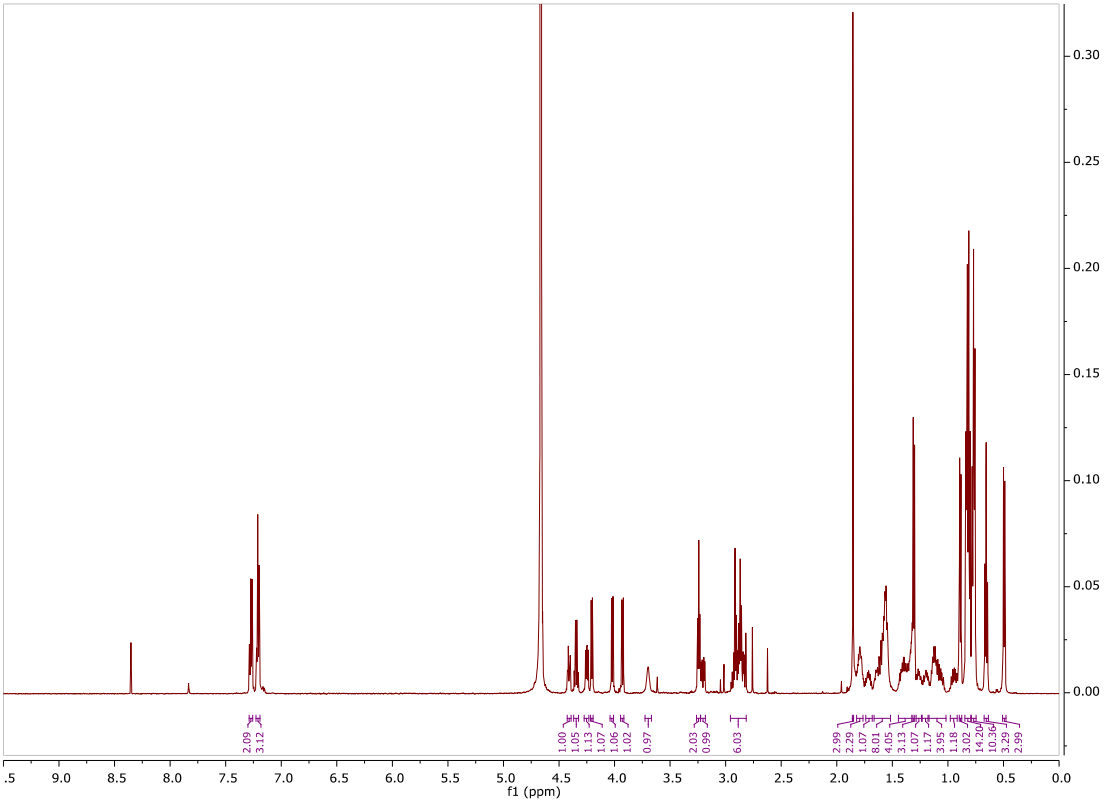

Figure S13. ^{1}H NMR of SNAC-surugamide A.

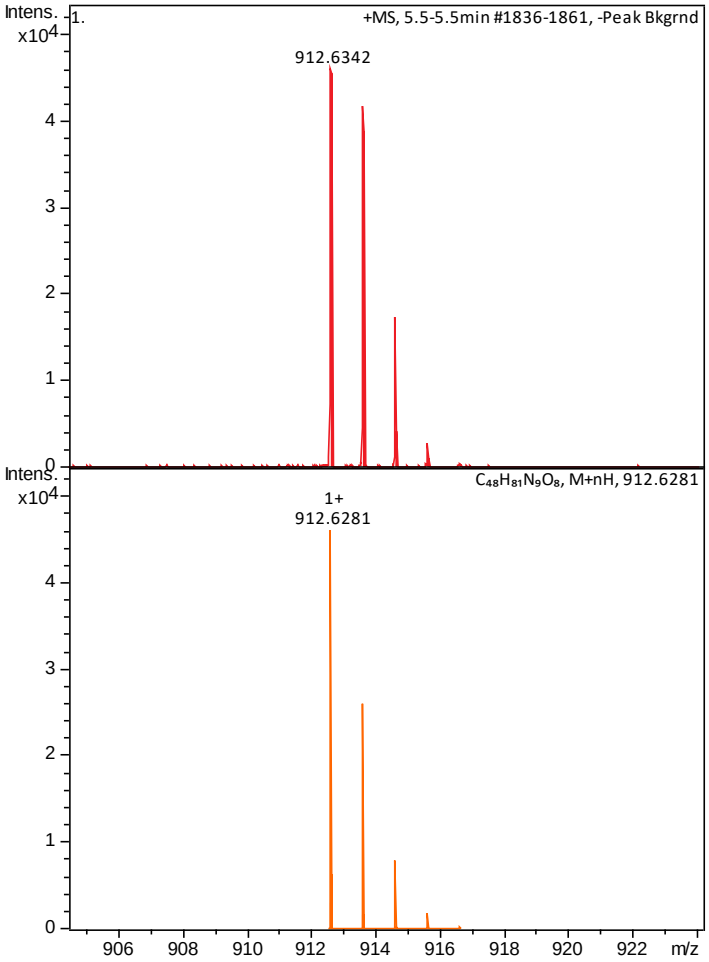

Figure S14. The measured (red) and simulated (orange) $[M+H]^+$ mass spectra for surugamide A produced by SurE in vitro.

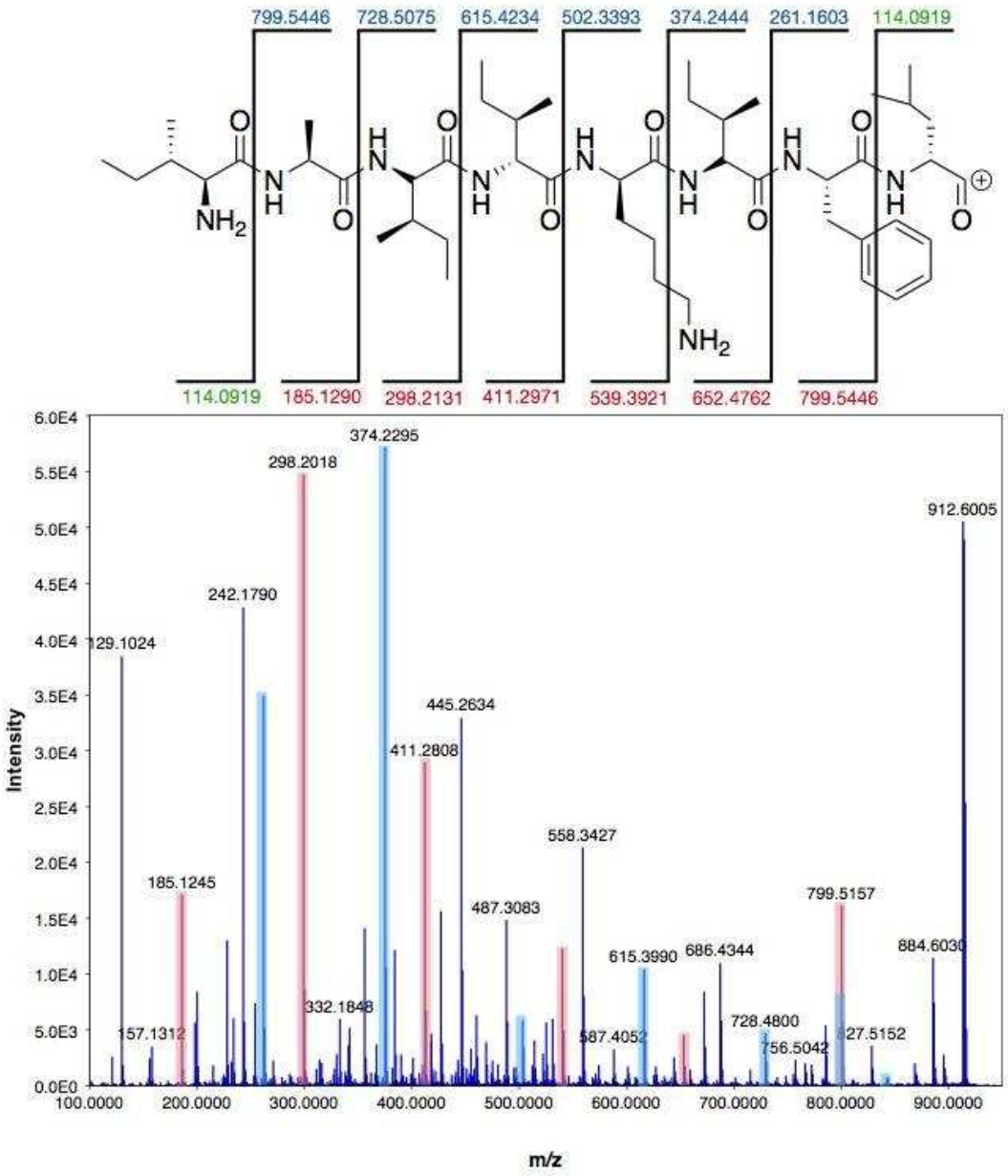

Figure S15. MS2 fragmentation of surugamide A produced by SurE in vitro. Green text indicates an unobserved ion.

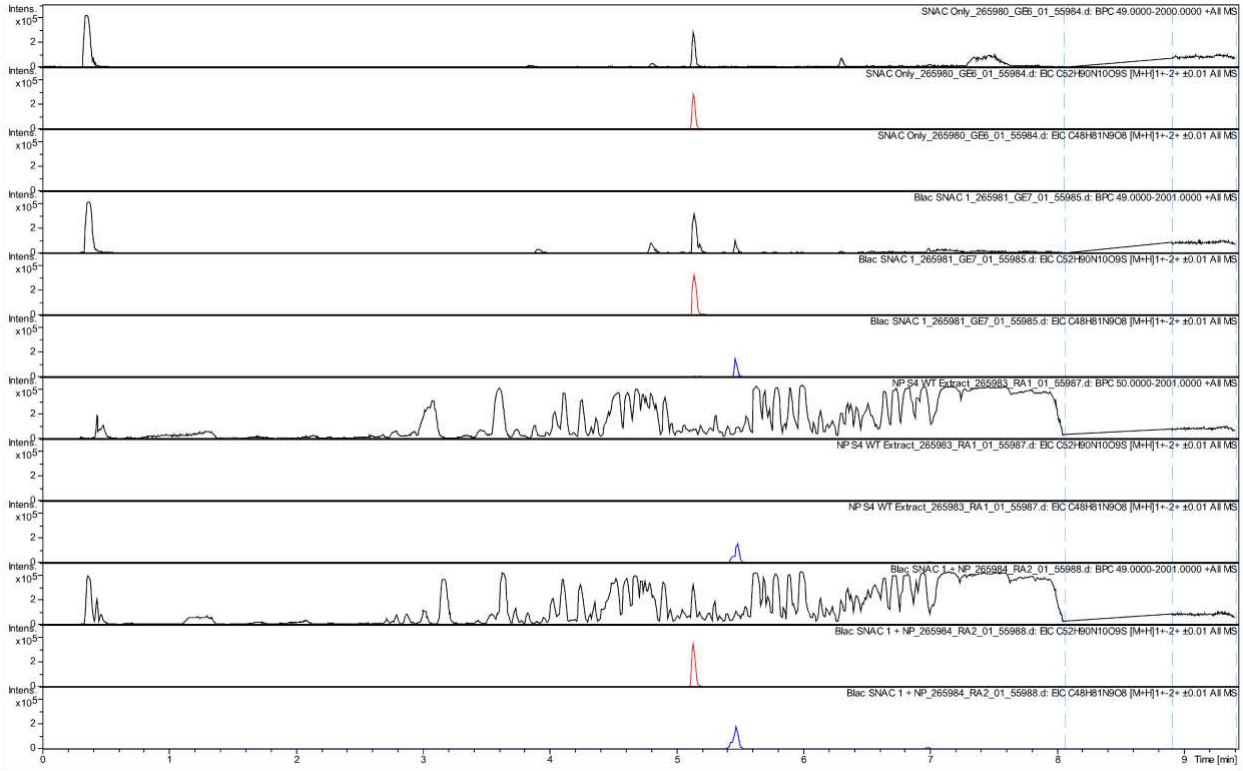

Figure S16. LC-HRMS analysis of in vitro reactions with **8.** The BPC and EICs for **8** (red, $C_{52}H_{90}N_{10}O_9S$, $[M+H]^{2+}$) and **1** (blue, $C_{48}H_{81}N_9O_8$, $[M+H]^{2+}$) for each sample. Samples include (from top down): **8** alone, **8** + SurE, crude extract from S. albus WT, co-injection of **8** + SurE with crude extract from S. albus WT.

Table S1. NRPS biosynthetic systems lacking an embedded thioesterase domain and with an adjacent SurE ortholog

Accession	Taxonomy	Number of Modules	BiG-SCAPE gene cluster family
BGCs in the network			
JOCL01000027.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3877	6	1899
LGCU01000069.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3898	6	1899
JNWV01000002.1	Streptomyces flaveolus strain NRRL B-1589	10	1915
KQ948566.1	Streptomyces longwoodensis strain DSM 41677	10	1838
FKJH01000063.1	Streptomyces sp. F-7	10	1838
LN831790.1	Streptomyces leeuwenhoekii	10	1838
JNWX01000027.1	Streptomyces rimosus subsp. rimosus strain NRRL B-16073	6	1899
JOBS01000025.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3558	6	1899
LMWF01000128.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3897	6	1899
JMGX01000001.1	Streptomyces rimosus strain R6-500MV9	6	1899
JOCN01000027.1	Streptomyces lavendulae subsp. lavendulae strain NRRL WC-3532	6	1899
JOJH01000022.1	Streptomyces sp. NRRL WC-3725	10	1915
LWAB01000002.1	Streptomyces sp. H-KF8	10	1838
JODT01000010.1	Streptomyces achromogenes subsp. achromogenes strain NRRL B-2120	10	1915
JOJG01000030.1	Streptomyces sp. NRRL WC-3683	10	1915
JOEX01000030.1	Streptomyces lavendulae subsp. lavendulae strain NRRL B-2775	6	1899
LGCR01000071.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3875	6	1899
CM002273.1.1	Streptomyces sp. PVA 94-07	6	1904
JOGU01000009.1	Streptomyces griseus subsp. griseus strain NRRL F-5621	6	1904
BBNN01000019.1	Streptomyces sp. NBRC 110035	10	1838
CP013219.1	Streptomyces hygroscopicus subsp. limoneus	10	1915
CP016174.1	Amycolatopsis orientalis B-37	9	1851
FOPQ01000016.1	Amycolatopsis regifaucium strain DSM 45072	9	1851
LJJF01000001.1.2*	Streptomyces sp. MJM8645	10	1915
CP006259.1	Streptomyces collinus Tu 365	10	1838
JOIC01000025.1	Streptomyces sp. NRRL F-3307	10	1915
KQ948863.1	Streptomyces bungoensis strain DSM 41781	10	1838
LGUX01000036.1	Streptomyces antibioticus strain NRRL B-2032	10	1915
LMTQ02000010.1	Streptomyces sp. AVP053U2	10	1838
BBOU01000038.16	Streptomyces hygroscopicus subsp. hygroscopicus	10	5
BCAN01000035.1	Streptomyces hygroscopicus subsp. hygroscopicus	10	5
JOBN01000024.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3925	6	1899
LBDA02000012.1	Streptomyces malaysiense strain MUSC 136	10	1915
LGDZ01000181.1	Streptomyces sp. NRRL B-3648	10	1915
FNTM01000001.1	Streptomyces sp. 2133.1	10	1849
BBUZ01000026.1	Streptomyces sp. NBRC 110028	10	5
LAKD02000032.1	Streptomyces antioxidans strain MUSC 164	10	5
JOCM01000023.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3876	6	1899
JOET01000024.1	Streptomyces rimosus subsp. rimosus strain NRRL B-2661	6	1899

JNYK01000022.1	Streptomyces rimosus subsp. rimosus strain NRRL B-8076	6	1899
JOCK01000027.1	Streptomyces peucetius strain NRRL WC-3868	6	1899
KQ948353.1	Streptomyces corchorusii strain DSM 40340	10	1915
LIPD01000005.1	Streptomyces sp. CB02056	9	1915
JOCJ01000019.1	Streptomyces sp. NRRL WC-3744	10	1915
JOCB01000020.1	Streptomyces sp. NRRL S-31	10	1915
JOJJ01000019.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3929	6	1916
CM002271.1.2*	Streptomyces sp. GBA 94-10	18	1821
CP004370.1.2*	Streptomyces albus J1074	18	1821
LGCV01000363.1	Streptomyces rimosus subsp. pseudoverticillatus strain NRRL WC-3896	6	1899
JOBO01000008.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3927	6	1916
CM002271.1.1	Streptomyces sp. GBA 94-10	6	1904
LGCP01000253.1	Streptomyces sp. NRRL WC-3701	6	1899
JOIV01000032.1	Streptomyces sp. NRRL S-1896	10	1915
JOCP01000022.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3880	6	1899
LGUY01000146.1	Kitasatospora aureofaciens strain NRRL B-2658	6	1899
CP004370.1.1	Streptomyces albus J1074	6	1820
CP009802.1.1	Streptomyces sp. FR-008	6	1820
FMZK01000015.1	Streptomyces emeiensis strain CGMCC 4.3504	10	1838
LFVR01000013.1	Streptomyces regensis strain NRRL B-11479	10	1915
JOCO01000023.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3882	6	1899
JOCR01000021.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3900	6	1899
JOIU01000014.1	Streptomyces sp. NRRL S-1022	10	1915
JNWP01000024.1	Streptomyces capuensis strain NRRL B-12337	6	1899
JOBW01000024.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3924	6	1916
CP015588.1	Streptomyces alfalfae ACCC40021	10	1915
JOCH01000022.1	Streptomyces sp. NRRL WC-3703	6	1899
KV757140.1	Streptomyces sporocinereus strain OsiSh-2	10	5
LXIC01000010.1	Streptomyces sp. RTd22	10	5
JOCU01000001.1	Streptomyces flaveus strain NRRL WC-3505	10	1915
LN997842.1	Streptomyces reticuli	10	1915
JNYR01000027.1	Streptomyces rimosus subsp. rimosus strain NRRL ISP-5260	6	1899
CP003720.1	Streptomyces hygroscopicus subsp. jinggangensis TL01	10	1915
JOHT01000002.1	Streptomyces sp. NRRL F-5053	6	1906
JODF01000025.1	Streptomyces sp. NRRL S-920	10	1915
FOWC01000006.1	Amycolatopsis rubida strain DSM 44637	9	1918
KB913032.1	Amycolatopsis alba DSM 44262	9	1918
JOBP01000016.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3930	6	1916
JOFU01000020.1	Streptomyces griseofuscus strain NRRL B-5429	10	1915
LGUU01000283.1	Streptomyces decoyicus strain NRRL 2666	10	1849
FNSG01000001.1	Streptomyces sp. 2112.2	10	1849
JOIX01000003.1	Streptomyces sp. NRRL S-337	10	1915
LGDX01000188.1	Streptomyces sp. NRRL WC-3723	10	1915
CP019457.1	Streptomyces lydicus GS93	5	1830

JNZA01000037.1	Streptomyces lydicus strain NRRL ISP-5461	5	1830
JOCC01000027.1	Streptomyces sp. NRRL WC-3702	6	1899
JNWN01000025.1	Streptomyces sp. NRRL B-11253	6	1899
LGCO01000080.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3909	6	1899
FNSO01000004.1	Amycolatopsis tolypomycina strain DSM 44544	10	1919
JPRA01000062.1	Amycolatopsis mediterranei strain HP-130	10	1919
CP009802.1.2*	Streptomyces sp. FR-008	18	1821
KB898235.1	Streptomyces sp. CNY228	18	1821
FNKS01000001.1	Streptomyces sp. KS_16	10	1849
LLZJ01000403.1	Streptomyces violaceusniger strain NRRL F-8817	10	5
FNST01000002.1	Streptomyces melanosporofaciens strain DSM 40318	10	5
BDFA01000034.1	Streptomyces sp. SPMA113	10	5
CP015726.1	Streptomyces sp. RTd22	10	5
CP009438.1	Streptomyces glaucescens	10	1838
JOJM01000018.1	Streptomyces sp. NRRL B-1347	9	1917
FMBX01000012.1	Streptomyces sp. ScaeMP-6W	18	1821
MNPQ01000023.1	Streptomyces albidoflavus strain OsiLf-2	18	1821
JOEO01000025.1	Streptomyces rimosus subsp. rimosus strain NRRL B-2626	6	1899
FNTP01000001.1	Streptomyces sp. 2112.3	10	1849
MIBiG_BGC0001792.1	Surugamide A/D (from Streptomyces albus)	18	1821
CP016824.1.1	Streptomyces sampsonii KJ40	18	1821
LGCT01000071.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3899	6	1899
JOFM01000024.1	Streptomyces capuensis strain NRRL B-3501	6	1899
JOCQ01000017.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3904	6	1916
JNWZ01000005.1	Kitasatospora phosalacinea strain NRRL B-16230	4	1893
JODW01000005.1	Streptomyces globisporus subsp. globisporus strain NRRL B-2293	4	1893
FNUJ01000015.1	Amycolatopsis pretoriensis strain DSM 44654	9	1918
MQUQ01000027.1	Amycolatopsis coloradensis strain DSM 44225	9	1918
CP019458.1	Streptomyces autolyticus CGMCC0516	10	5
JJNO01000003.1	Streptomyces rimosus R6-500	6	1899
JNYY01000016.1	Amycolatopsis vancoresmycina strain NRRL B-24208	10	1919
KB913037.1	Amycolatopsis balhimycina FH 1894 strain DSM 44591	10	1919
JOBD01000008.1	Streptomyces lavenduligriseus strain NRRL ISP-5487	10	1915
JOIK01000003.1	Streptomyces hygroscopicus subsp. hygroscopicus strain NRRL B-1477	10	5
LGCS01000311.1	Streptomyces rimosus subsp. rimosus strain NRRL WC-3873	6	1899
CP018627.1	Streptomyces hygroscopicus XM201	10	5
LLZK01000014.1	Streptomyces sp. NRRL S-1521	10	1915
JOGD01000011.1	Streptomyces sp. NRRL S-1868	6	1906
KB891896.1	Streptomyces sp. MspMP-M5	5	1830
BBUZ01000046.1	Streptomyces sp. NBRC 110028	6	1936
CP020567.1	Kitasatospora aureofaciens strain DM-1	6	1936
CP006567.1	Streptomyces rapamycinicus NRRL 5491	10	5
LOBU02000031.1	Amycolatopsis regifaucium strain GY080	9	1851
JTCK01000091.1	Streptomyces sp. CNQ431	6	1904

CM002273.1.2*	Streptomyces sp. PVA 94-07	18	1821
JOER01000020.1	Kitasatospora aureofaciens strain NRRL B-2657	6	1936
JOFK01000006.1	Streptomyces avellaneus strain NRRL B-3447	6	1936
LAYX01000005.1	Streptomyces sp. KE1	5	1904
CP003777.1	Amycolatopsis mediterranei RB	10	1919
CP016824.1.2*	Streptomyces sampsonii KJ40	5	1855
JOII01000014.1	Streptomyces albidoflavus strain NRRL B-1271	5	1855
JOGG01000034.1	Streptomyces sp. NRRL B-5680	6	1936
FNSJ01000001.1	Streptomyces sp. 2314.4	10	1849
FNSB01000002.1	Streptomyces sp. 2224.1	10	1849
KN549147.1	Streptomyces sp. M10	6	1904
JABQ01000025.1	Streptomyces sp. PRh5	10	5
JPLW01000002.1	Amycolatopsis sp. MJM2582	9	1918
CP003410.1	Amycolatopsis orientalis HCCB10007	9	1918
CP019779.1	Streptomyces sp. MOE7	4	1830
JOGN01000041.1	Streptomyces griseus subsp. griseus strain NRRL F-5618	5	1855
MIBiG_BGC0001569.1	Dechlorocuracomycin (from Streptomyces noursei)	6	1810
CP011533.1	Streptomyces noursei ATCC 11455	6	1810
FNTA01000002.1	Amycolatopsis lurida strain DSM 43134	9	1918
JPRF03000087.1	Kitasatospora aureofaciens strain ATCC 10762	6	1936
LBHA01000110.1	Kitasatospora aureofaciens strain NRRL 2209	6	1936
CP017157.1	Streptomyces lydicus 103	5	1830
MVFC01000034.1	Streptomyces tsukubensis strain F601	6	1904
JMQJ01000071.1	Amycolatopsis mediterranei strain DSM 40773	10	1919
JOIQ01000012.1	Streptomyces sp. NRRL F-3213	9	1918
LK022848.1	Streptomyces iranensis	10	5
JODE01000022.1	Streptomyces sp. NRRL S-515	6	1889
JODJ01000024.1	Streptomyces sp. NRRL S-575	6	1889
CP014485.1.2*	Streptomyces albus SM254	18	1821
JMQG01000012.1	Amycolatopsis mediterranei strain DSM 46096	10	1919
LQCI01000007.1	Amycolatopsis regifaucium strain GY080	9	1851
LWSF01000010.1	Amycolatopsis sp. M39	9	1918
JHDU01000021.1	Streptomyces wadayamensis strain A23	5	1855
CP003987.1	Streptomyces sp. 769	6	1810
CP014485.1.1	Streptomyces albus SM254	6	1820
FNSM01000001.1	Streptomyces sp. TLI_105	4	1893
MIBiG_BGC0001814.1	Ulleungmycin (from Streptomyces sp. KCB13F003)	6	1810
FPJG01000006.1	Amycolatopsis australiensis strain DSM 44671	9	1918
Singletons			
ASHX02000001.1	Streptomyces thermolilacinus SPC6	4	0
CP017316.1	Streptomyces rubrolavendulae MJM4426	6	1828
CP019798.1	Streptomyces sp. fd1-xmd	5	1833
CP020563.1	Kitasatospora albolonga YIM 101047	6	1834
FNON01000001.1	Amycolatopsis xylanica strain CPCC 202699	6	1840

JNXP01000027.1	Streptomyces seoulensis strain NRRL B-24310	4	1865
JOHO01000003.1	Streptomyces sp. NRRL S-350	6	1905
KB890716.1	Streptomyces sp. LaPpAH-202	6	1922
KB897508.1	Salinispora pacifica CNY331	5	1924
LGDY01000106.1	Nocardia sp. NRRL S-836	7	1946
LJJF01000001.1	Streptomyces sp. MJM8645	6	1952
NBCN01000013.1	Streptomyces sp. CB03238	5	1969

[a]Accession numbers provided are for whole genomes or draft genome contigs harboring the BGC

*Denotes accession numbers which have been appended with ".2" in cases where a whole genome harbors more than one SurE-containing BGC.

Table S2. Oligonucleotide primers used in this study

Primer name	Sequence (5'- 3')	Description
DT045	tgaccgggaacaccgtgctcac	Hygromycin resistance cassette
DT046	cggaaggcgttgagatgcagtt	Hygromycin resistance cassette
DT159	aacgtgtttcacctgggctc	Identification of Cos994
DT160	acggccgaaaagaagggcaa	Identification of Cos994
DT161	**agggtccggtgggttgcagtgggcgcaggggacgcagtg**attccggggatccgtcgacc	ReDirect primers for deleting surE
DT162	**gcgtcgcggggcgcccggccggccgagtggggcgggtca**tgtaggctggagctgcttc	ReDirect primers for deleting surE
DT163a	agctggtcatgcagttcgtct	Verification of Δ*surE*
DT164a	atcgtgggtcaccatcagctc	Verification of Δ*surE*
DT167	**cggatcacgcgggacggccgaaaagaagggcaagttgtg**attccggggatccgtcgacc	ReDirect primer for deleting surF
DT168	**acgccgcgcgaggcgtctgccggccccccctgccggttcat**gtaggctggagctgcttc	ReDirect primer for deleting surF
DT171	acttcctcaaccacaccacg	Verification of ΔsurF
DT172	cggttcaacgtgtttcacct	Verification of ΔsurF
DT186	tggacagttgcctgctgcgt	Sequencing surE in pDT2
DT206	**atct**catatggtgggtgccgaggggggcgga	Construction of pET28-surE
DT210	**atca**catatggtgggtgccgaggggggcgga	Construction of pDT2
DT211	**atct**aagctttcagagccggtgcatggccc	Construction of pDT2
DT213	**atca**ctcgagtcagagccggtgcatggccc	Construction of pET28-surE
RFS115	cattcttcgcatcccgcct	Apramycin resistance cassette
RFS116	ctccttccgtagcgtcc	Apramycin resistance cassette
RFS184	ccattattatcatgacattaa	Cos994 insert-end sequencing
RFS185	gtccgtggaatgaacaatgg	Cos994 insert-end sequencing
RFS582	agcccgacccgagcacg	Sequencing surE in pDT2

*Non-homologous sequences are bolded and restriction endonuclease sites are underlined

Table S3. Bacterial strains, cosmids and plasmids used in this study

Strain/cosmid/plasmid	Description	Reference
Streptomyces		
S. albus S4	Streptomyces albus S4 wildtype	[18]
ΔsurE	S4 surE mutant, Apr^R	This study
ΔsurE attB ΦBT1::pDT2	S4 surE mutant complemented with surE expressed from the ermE* promoter; Apr^R, Hyg^R	This study
ΔsurF	S4 surF mutant, Apr^R	This study
Escherichia coli		
BL21-Gold(DE3)	Protein production host	Agilent Technologies
GB0R-red	RecET recombineering host	[6]
ET12567	Non-methylating host to for conjugal transfer of DNA into Streptomyces (dam, dcm, hsdM); Cam^R	[19]
XL10-Gold	General cloning host	Agilent Technologies
Cosmids		
Supercos1	Cosmid backbone for S. albus S4 Cos994; $Carb^R$, Kan^R	Stratagene
Cos994	Supercos1 derivative spanning the surugamide gene cluster from surF into surB; $Carb^R$, Kan^R	This study
Cos994-ΔsurE	Cos994 derivative with surE deletion; $Carb^R$, Kan^R, Apr^R	This study
Cos994-ΔsurF	Cos994 derivative with surF deletion; $Carb^R$, Kan^R, Apr^R	This study
Plasmids		
pDT2	pIJ10257 derivative containing the surE coding sequence cloned into the NdeI-HindIII sites; Hyg^R	This study
pET28a	Commercial protein production vector, Kan^R	Agilent Technologies
pET28-surE	pET28a derivative containing the surE gene cloned into the NdeI-XhoI restriction sites; Kan^R	
pIJ773	ReDirect PCR template for the aac(3)IV + oriT cassette	[2]
pIJ10257	pMS81 derivative containing ermE*p, integrates into the ΦBT1 attB site in Streptomyces; Hyg^R	[7]
pUZ8002	Encodes the conjugation machinery for mobilisation of plasmids from E. coli to Streptomyces; Kan^R	[19]

Apr, apramycin; Hyg, Hygromycin; Cam, chloramphenicol; Kan, kanamycin; Carb, carbenicillin; oriT, origin of conjugal transfer

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