The Post-translational Modification Cascade to the Thiopeptide Antibiotic Berninamycin Generates Linear Forms and Altered Macrocyclic Scaffolds

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SUPPORTING INFORMATION APPDENDIX

Contents:

1. Figures and tables mentioned in the main text:

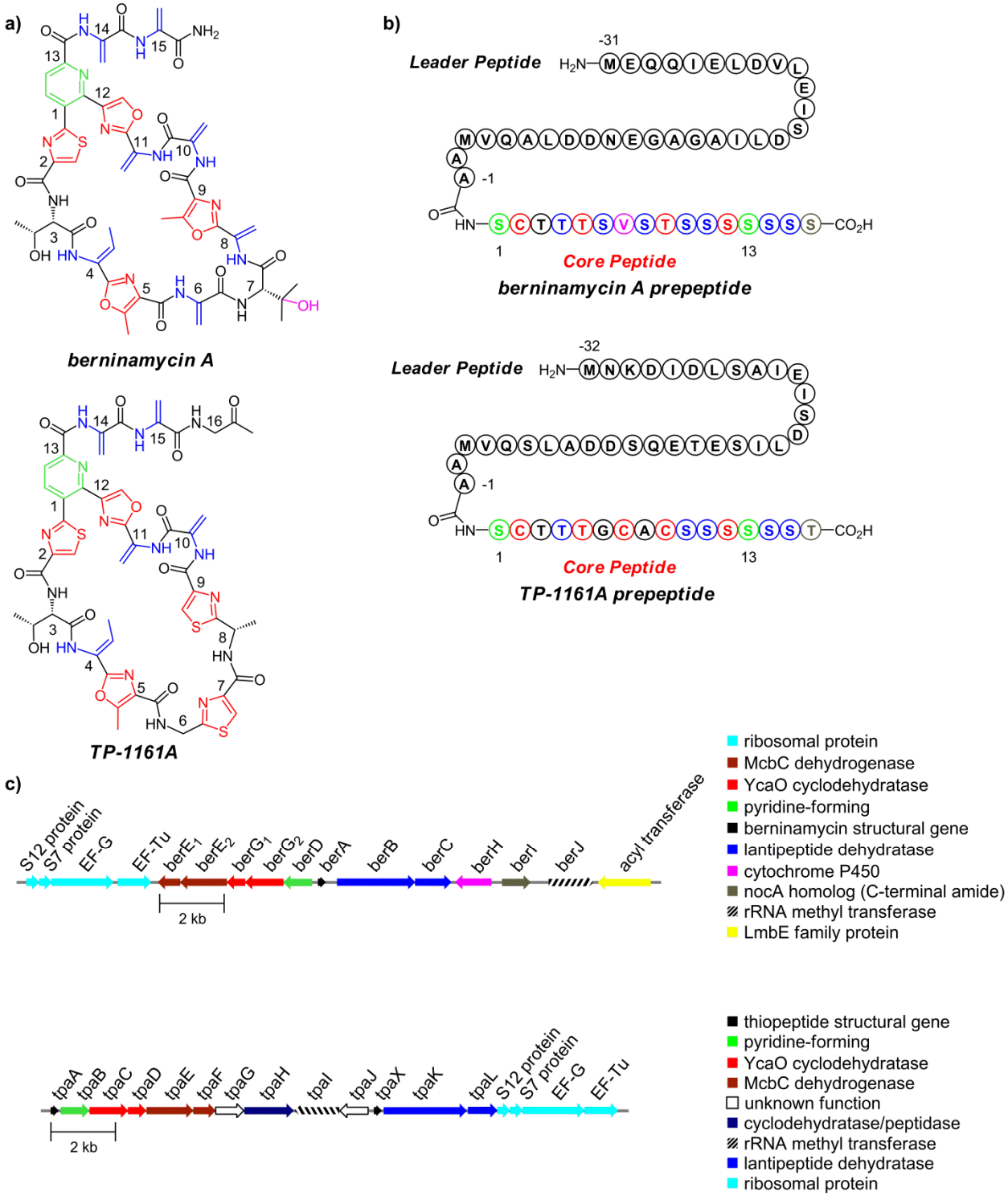

Fig. S1. Comparison of 35-membered ring thiopeptides berninamycin A and TP-1161A. a) Structures of thiopeptides berninamycin A and TP-1161A. b) Berninamycin A and TP-1161A prepeptides, each comprised of a leader peptide and core peptide region. c) Comparison of the berninamycin A and TP-1161A gene clusters.

Table S1. Bioinformatic analysis of the berninamycin A gene cluster.

Gene	Size of protein[a]	Protein homolog[b,c]	Source of protein homolog[b]	% identity/ % similarity[b]	Proposed function[b]	Homolog(s) in another cluster(s)[b,c,d]
berA	47				berninamycin prepeptide	
berB	885	Lantibiotic dehydratase domain-contaning protein [EJJ05682]	*Streptomcyes auratus* AGR0001	50/65	lantipeptide dehydratase	TpaK [ADO67781], NocE [ADR01081], NosE [ACR48334], TclK [NP_834752]
berC	348	Hypothetical protein [YP_006266718]	*Actinoplanes* sp. SE50/110	46/61	lantipeptide dehydratase	TpaL [ADO67782], NocD [ADR01080], NosD [ACR48333], TclL [NP_834751]
berD	384[e]	Hypothetical protein [EJJ05679]	*Streptomcyes auratus* AGR0001	41/57	pyridine/macrocycle formation	TpaB [ADO67771], NocO [ADR01090], NosO [ACR48344], TclM [NP_834750]
berE$_1$	233	Hypothetical protein [EJJ05685]	*Streptomcyes auratus* AGR0001	54/69	McbC dehydrogenase	TpaF [ADO67775]
berE$_2$	542	TpaE [ADO67774]	*Nocardiopsis* sp. TFS65-07	53/66	McbC dehydrogenase	TsrF [ACN52296], SioM [ACN80650], NosF [ACR48335]
berG$_1$	203	TpaD [ADO67773]	*Nocardiopsis* sp. TFS65-07	47/67	YcaO cyclodehydratase	
berG$_2$	448	TpaC [ADO67772]	*Nocardiopsis* sp. TFS65-07	62/73	YcaO cyclodehydratase	TclJ [NP_834753], NocG [ADR01083], NosG [ACR48336], TpaH [ADO67777]
berH	400	epothilone B hydroxylase [ABG22116]	*Amycolaptosis orientalis*	58/73	Val$_7$ hydroxylase	
berI	153	Hypothetical protein [EJJ05676]	*Streptomcyes auratus* AGR0001	65/75	C-terminal amide formation	NocA [ADR01069], NosA [ACR48330], TpdK [ACS83773]
berJ	267	Thiostrepton resistance rRNA methyl transferase [P52393]	*Streptomcyes laurentii*	79/89	23S rRNA methyl transferase	TpaI [ADO67778]

[a] Number of amino acids. [b] Determined by NCBI BLAST search. [c] Accesion number in brackets. [d] From known thiopeptide-producing strains. [e] We are unsure of the exact start codon for this protein as there are several candidate leucines or valines with similar probability of being correct; there is no appropriate methionine in frame in this region.

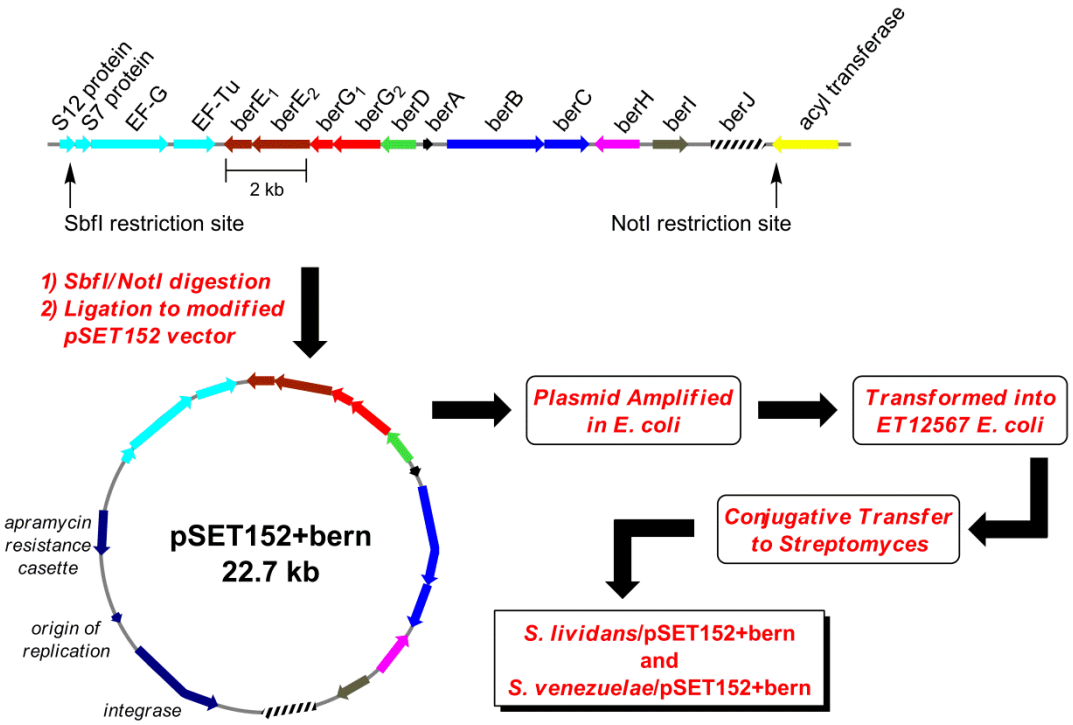

Fig. S2. Cloning strategy for synthesis of the pSET152+bern plasmid and conjugative transfer to *Streptomyces* species.

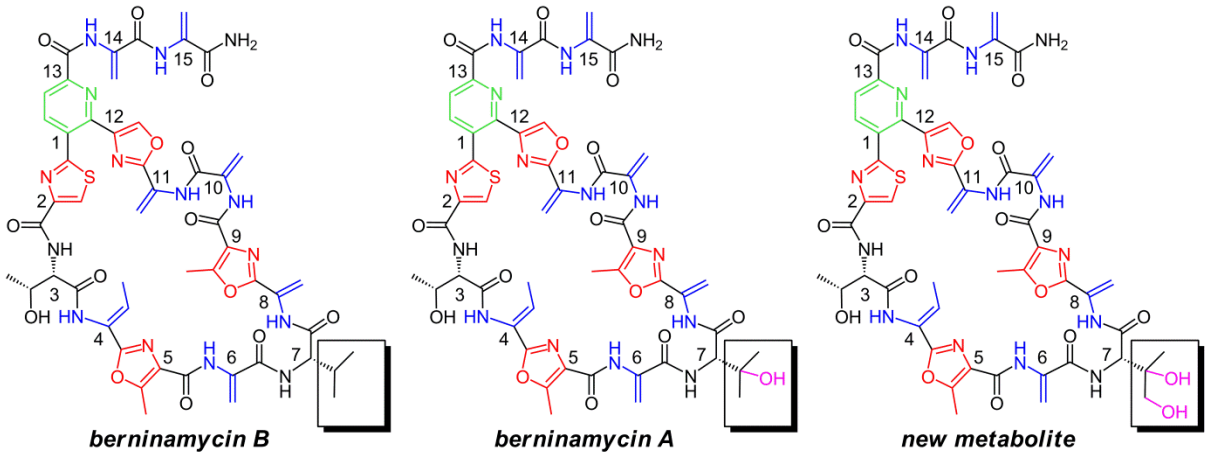

Fig. S3. Three metabolites from *S. lividans*/pSET152+bern expression, varying only in the oxidation state of the Val_7 side chain. The doubly-hydroxylated species is a new metabolite; the stereochemistry at C_β of Val_7 in this compound has not been determined.

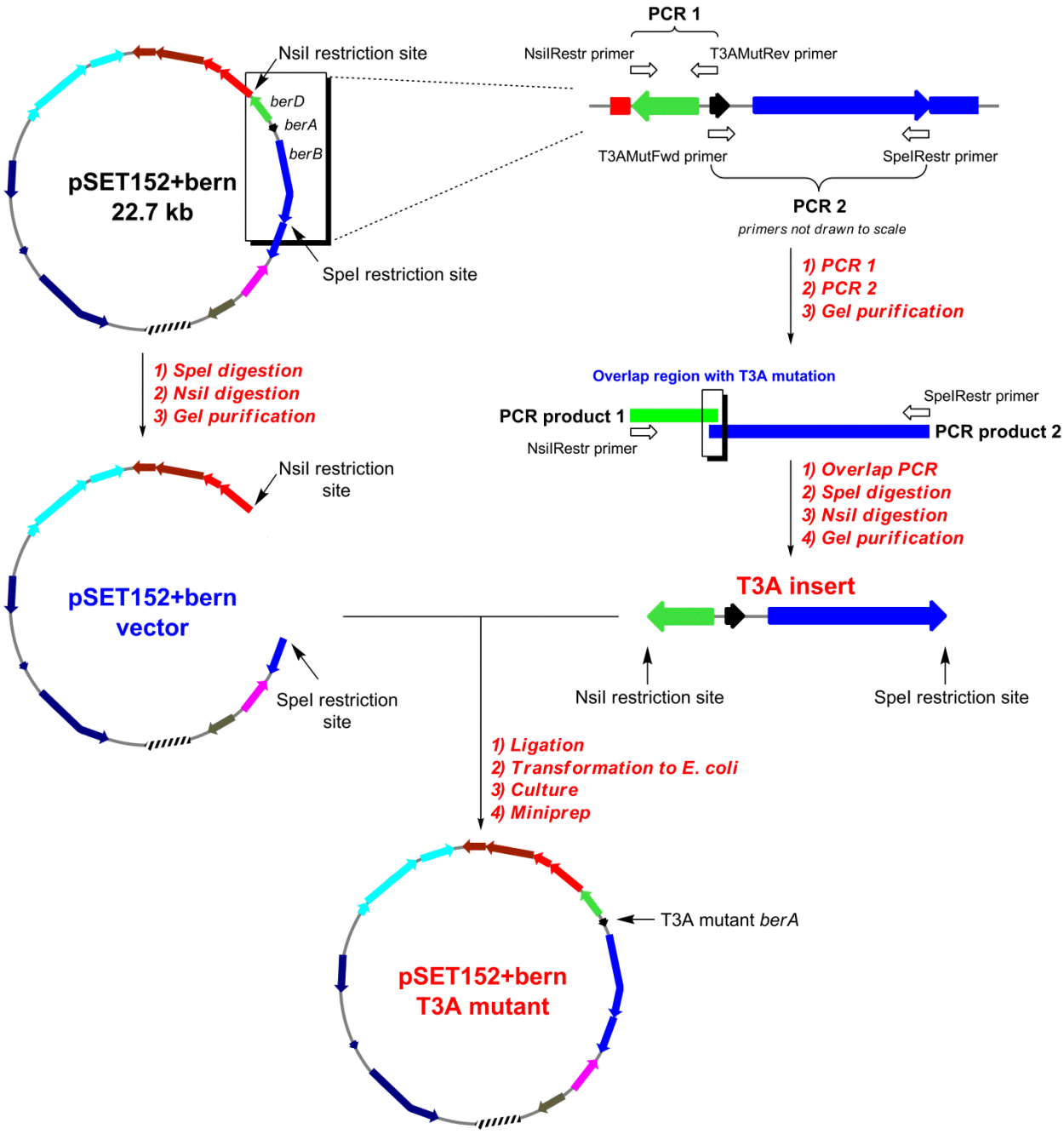

Fig. S4. PCR-directed mutagenesis to introduce mutations to the *berA* gene for cloning, here shown for the T3A mutation.

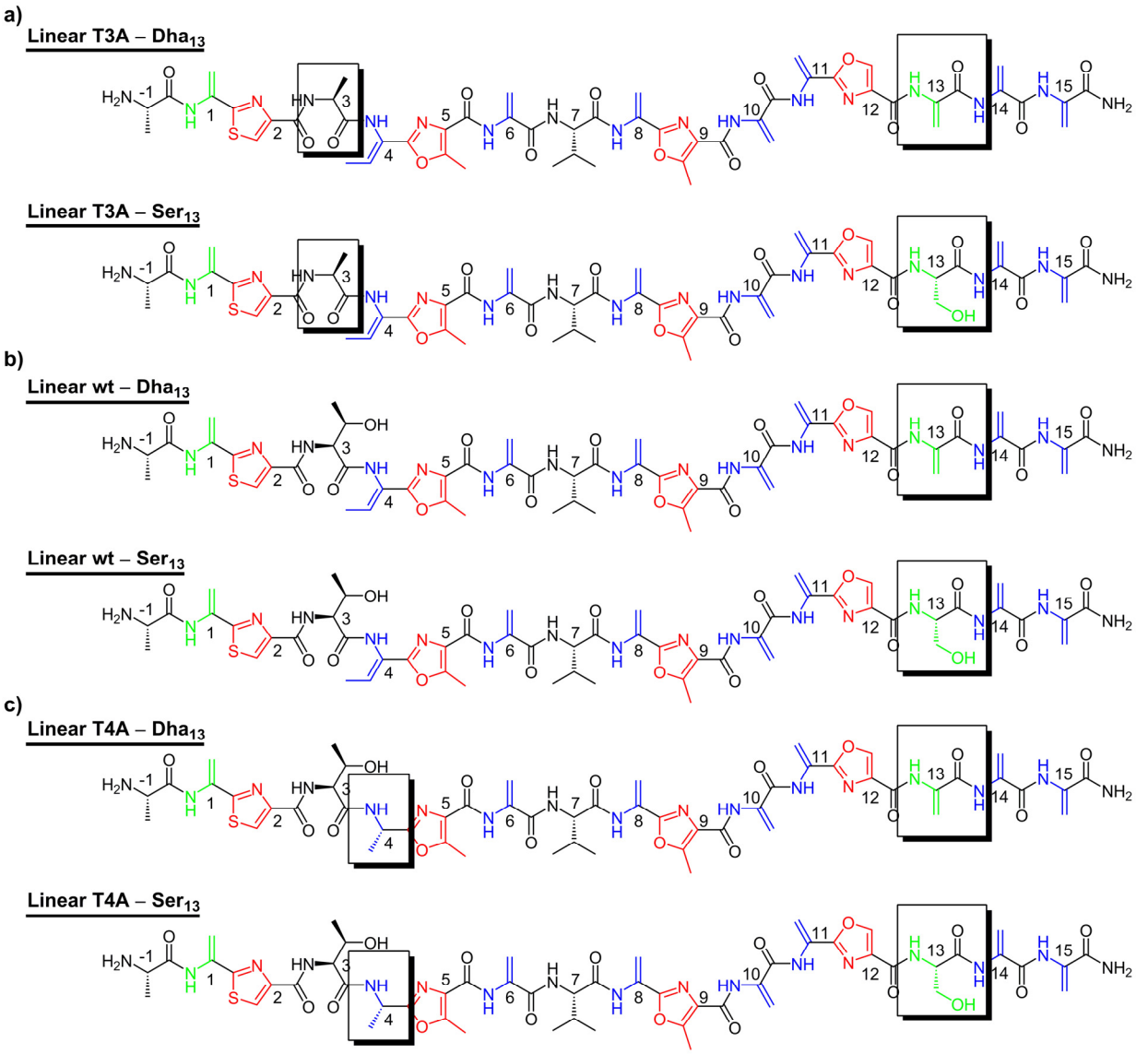

Fig. S5. Linear compounds obtained from heterologous expressions of *S. lividans*/pSET152+bern variants. a) Linear precursors to the T3A mutant macrocycle – Dha_{13} and Ser_{13}. b) Linear precursors to the wild type macrocycle – Dha_{13} and Ser_{13}. c) Linear precursors to the T4A mutant macrocycle – Dha_{13} and Ser_{13}. The stereochemistry of these linear precursors is assumed based on the structure of berninamycin and its prepeptide and has not been proven spectroscopically.

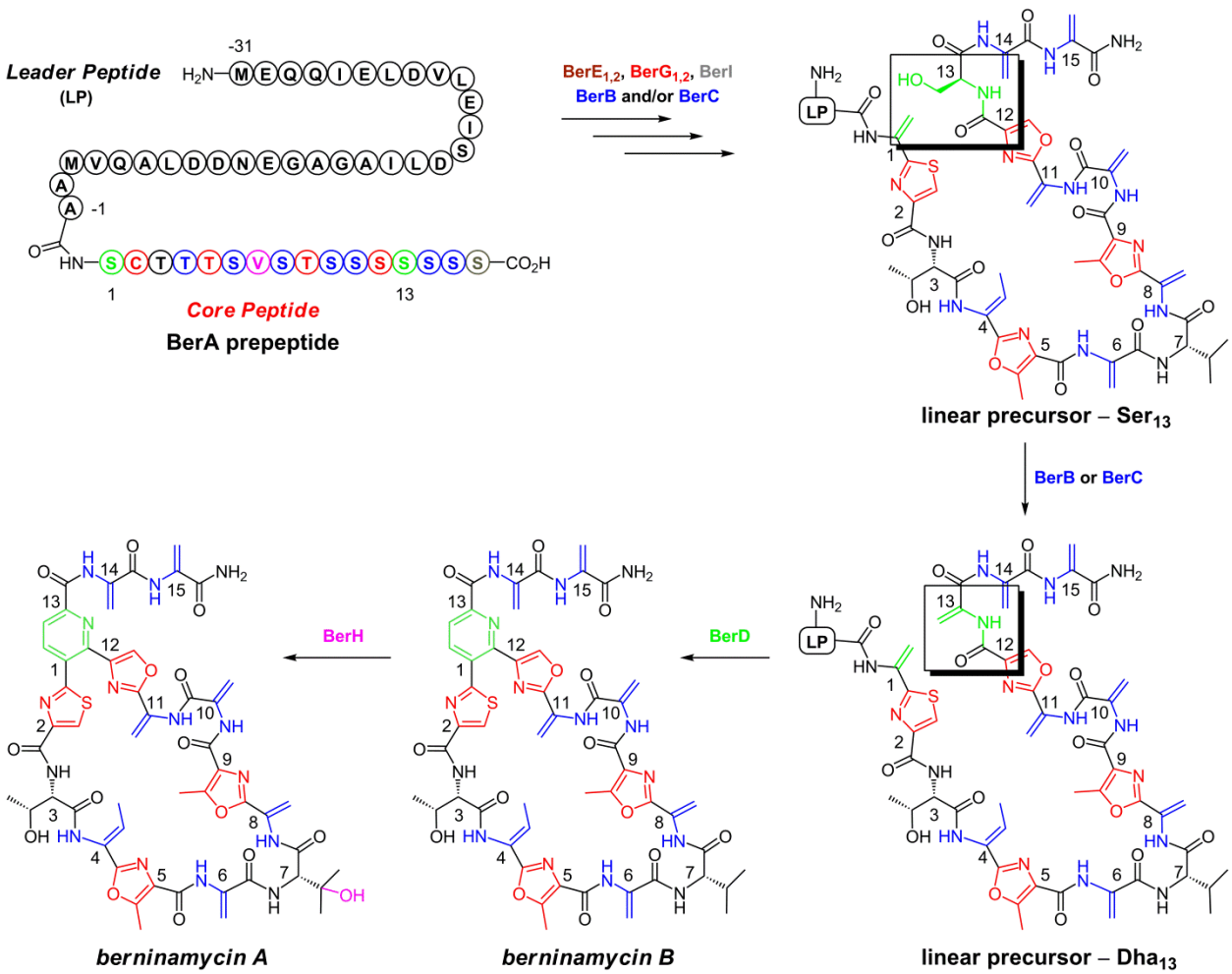

Fig. S6. Proposed pathway for berninamycin A biosynthesis from the BerA prepeptide, featuring a late-stage Dha_{13} formation, pyridine cyclization, and Val_7 hydroxylation.

2. Abbreviations:

LC-MS = liquid chromatography – mass spectrometry

MS = mass spectrometry

Q-TOF = quadrupole time-of-flight

HPLC = high-performance liquid chromatography

NMR = nuclear magnetic resonance

DMSO = dimethylsulfoxide

MeCN = acetonitrile

MeOH = methanol

EtOAc = ethyl acetate

3. General information: High resolution LC-MS and MS/MS fragmentation were performed on an Agilent 6520 Accurate-Mass Q-TOF mass spectrometer using an electrospray ionization

(ESI) source in positive ion mode. MS conditions were identical to those used previously (1). A 5 μm C18 column (2.0 mm x 50 mm, Phenomonex Gemini-NX) was used with the elution monitored at 220, 254, 290, and 350 nm UV detection wavelengths. Preparative HPLC purification was carried out on a Waters preparative HPLC with a 5 μm C18 column (19 mm x 250 mm, Waters SunFire Prep^{TM}) with the elution monitored at 254 and 350 nm UV detection wavelengths. NMR spectra were recorded on a Varian 600 MHz spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance resulting from incomplete deuteration as the internal reference (d_6-DMSO: δ 2.50). For ^{1}H NMR, multiplicity is designated as follows: s = singlet, d = doublet, q = quartet, and m = multiplet). PCR was performed on a Bio-Rad MyCycler thermal cycler. DNA sequencing was carried out at the Molecular Biology Core Facilities at the Dana Farber Cancer Institute (Boston, MA) or at Genewiz, Inc. (Cambridge, MA). Streptomyces fermentations were carried out at the Harvard Medical School NERCE/BEID Biomolecule Production Laboratory.

4. Materials: The CopyControl™ Fosmid Library Production Kit was purchased from Epicentre Biotechnologies (Madison, WI). DNA clean and concentration kits (5, 25, and 100) were purchased from Zymo Research. Miniprep, Maxiprep, and agarose Gel Purification kits were purchased from Qiagen.

PCR primers were purchased from Integrated DNA Technologies (IDT); see *SI Appendix*, Table S2 below. Platinum PCR Supermix was purchased from Invitrogen, Phusion High-Fidelity PCR Mastermix with HF buffer from New England Biolabs, and PfuTurbo DNA polymerase (and buffer) from Stratagene. dNTP mix (10 mM) was purchased from Invitrogen.

1. Acker MG, Bowers AA, Walsh CT (2009) Generation of thiocillin variants by prepeptide gene replacement and in vivo processing by *Bacillus cereus*. *J Am Chem Soc* 131:17563–17565.

Table S2. PCR and sequencing primers.

Primer Name	Primer Sequence (5' to 3')	SI Section
BernContigAFor	CCTTCGGACGGTTCACCGAGATCG	8
BernContigARev	TGAGTTCGGAGAAGTGTCCGCGTAGC	8
5'Walk	ATTCCCTGCTGGCCACCACATACGCCCGGC	8
EFTuFwd1	CTTTAGGCTTGTCACCGGAGC	8
EFTuRev1	TGACCTTCAGGACACCACGCTC	8
EFTuFwd2	AGATCCTGGAGCTCGTCGAGC	8
EFTuRev2	CTGCTTGGTGCTGTGCGCTC	8
P450Fwd2	TCGTGACCGCCGACCAGGAGG	8
P450Rev2	TGGTGATCCCGGAGTTCACCGTCAAG	8
S12Fwd	GTGCAGTATTCGACACAC	8
S7Rev	TGTTGATCAGCGAGGTGAC	8 and 11
3EndFwd	AGGCCAGAGTGACCTTGGTGATC	8
pSETQkchngFwd1	AATCCCGCATTTGGACGGC	9
pSETQkchngRev1	TCGCCCTGCTGGCGAAGATC	9
pSETQkchngFwd2	TGACGCCGTTGGATACACCAAG	9
pSETQkchngRev2	TGACCTGGGAAAACGTGAAG	9
pSETbernSbfI2	GAGTGCTCCTGCAGGAATGGCGCCTGATGCGGTATTTTCTCC	9
pSETbernNotI2	CGCTACCGCGGCCGCGCGCGATATCGAATTCGTAATCATGTC	9
CloneChk1	GAAGTTTCTGATCGAAAAGTTCG	11
CloneChk2	TTAGGCACCCCAGGCTTTAC	11
tsrFwd	CGAGTCCCTCAACGTATC	11
NsiIRestr	TGTTCAGATGCATGTGGTTCTTC	13
SpeIRestr	TCTGCCCACTAGTTGTCGGTACAC	13
T3AMutFwd	CCTCGTGCGCGACCACCAGTGTTTCGACGAGTTCTTCGTCGTCCTCGTCC	13
T3AMutRev	ACACTGGTGGTCGCGCACGAGGCGGCCATCACCTGCGCCAGGTCATCGTTC	13
T4AMutFwd	CGTGCACGGCCACCAGTGTTTCGACGAGTTCTTCGTCGTCCTCGTCCTG	13
T4AMutRev	AACACTGGTGGCCGTGCACGAGGCGGCCATCACCTGCGCCAGGTCATCGTTC	13
T5AMutFwd	GCACGACCGCCAGTGTTTCGACGAGTTCTTCGTCGTCCTCGTCCTGAATC	13
T5AMutRev	GAAACACTGGCGGTCGTGCACGAGGCGGCCATCACCTGCGCCAGGTCATC	13
StrucFwd1	ACCGAGATAGGCCAGCTGGAC	13
StrucRev1	TCGGACCGGAGCAGCGGCTCGACC	13
BernContig2F	AGCTCGCCCAGTGGGAGTCTGC	13
tclMFwd	GAGCCGCTTGGAGGAAGC	13

Lysozyme was purchased from VWR. Proteinase K and T4 DNA ligase (and buffer) were purchased from New England Biolabs. The following restriction enzymes and their recommended buffers were purchased from New England Biolabs: BamHI-HF, EcoRI-HF, NotI-HF, SbfI-HF, SpeI, NsiI, and DpnI. BSA and CIP were purchased from New England Biolabs.

The following antibiotics were used (all purchased from Aldrich): apramycin (50 μg/mL), chloramphenicol [25 μg/mL (in ethanol)], kanamycin (50 μg/mL), nalidixic acid (25 μg/mL (prepared in 0.3 M aqueous NaOH)). N.B. In some instances, the antibiotics were used at half the listed concentrations as noted in specific procedures (*vide infra*).

All chemicals were purchased from Aldrich or Fisher with the following exceptions: agarose was purchased from VWR, low-melt agarose was purchased from Bio-Rad, SDS was purchased from VWR, phenol/chloroform mixture was purchased from VWR, and d_6-DMSO was purchased from Cambridge Isotope Labs. ColiRollers glass plating beads were purchased from Novagen.

One Shot Top10 Chemically Competent *E. coli* cells were purchased from Invitrogen. ET12567/pUZ8002 *E. coli* were obtained from the *E. coli* Genetic Stock Center (CGSC) at Yale University. *S. bernensis* UC 5144 was obtained from the NRRL culture collection (NRRL 3575). *S. lividans* TK24 and the pSET152 plasmid were generously donated by Suzanne Walker (Harvard Medical School). *S. venezuelae* ATCC 10712 was generously donated by Mervyn Bibb and the John Innes Centre (Norwich, UK). *B. subtilis* PY79 and MRSA MW2 (number BAA-1707) were obtained from ATCC.

5. Buffers and other solutions: Buffers and solutions were prepared as follows and were sterile filtered (except for trace elements solution):

SET buffer: 75 mM NaCl, 25 mM EDTA (pH = 8.0), 20 mM Tris-HCl (pH = 7.5)

TE buffer: 1 mM EDTA (pH = 8.0), 10 mM Tris-HCl (pH = 7.5)

Hutner's trace elements solution: to deionized water (100 mL), add $ZnSO_4 \cdot 7H_2O$ (2.2 g), H_3BO_3 (1.1 g), $MnCl_2 \cdot 4H_2O$ (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.5 g), $CoCl_2 \cdot 6H_2O$ (0.16 g), $CuSO_4 \cdot 5H_2O$ (0.16 g), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.11 g), EDTA (disodium salt, 5.0 g, Fluka); heat to boiling and then allow to cool to 60 °C before adjusting the pH to 6.5–6.8 (discard if pH exceeds 7.0); solution turns purple on standing for several days and a white precipitate forms.

6. Media: LB, 2xYT, and LB agar were purchased from VWR. The remaining media were prepared as follows (1000 mL final volume) before autoclaving:

Bennett's broth: maltose (10.0 g, Aldrich); yeast extract (1.0 g, VWR); beef extract (1.0 g, Aldrich); N-Z amine, type AS (2.0 g, Aldrich); for agar, add Bacto agar (15.0 g, VWR); deionized water.

Seed: glucose (20.0 g, VWR); yeast extract (5.0 g, VWR); meat extract (5.0 g, VWR); peptone (5.0 g, VWR); casein, acid hydrolysate (3.0 g, Aldrich); NaCl (1.5 g, Aldrich); tap water.

AF/MS: glucose (20.0 g, VWR); yeast extract (2.0 g, VWR); organic soy flour (8.0 g, Arrowhead Mills); $CaCO_3$ (4.0 g, Aldrich); NaCl (1.0 g, Aldrich); tap water.

GYM: glucose (4.0 g, VWR); yeast extract (4.0 g, VWR); malt extract (10.0 g, Fluka); N-Z amine, type AS (1.0 g, Aldrich); NaCl (2.0 g, Aldrich); deionized water; pH adjusted to 7.2 with 2 M aqueous NaOH prior to autoclaving.

SFM: organic soy flour (20.0 g, Arrowhead Mills); mannitol (20.0 g, Aldrich); Bacto Agar (20.0 g, VWR); Hutner's trace elements solution (1.0 mL); deionized water.

7. Isolation of *S. bernensis* genomic DNA and preparation of an *S. bernensis* spore stock (2): A stab of a -80 °C stock of *S. bernensis* UC 5144 (NRRL 3575) was used to inoculate 5 mL LB medium in a 14-mL plastic culture tube, which was shaken (200 rpm, 28 °C) for six days. An inoculation loop was dipped in the culture and used to streak out the *S. bernensis* cells on an LB

2. The procedure was adapted from Kieser T, Bibb MJ, Chater KF, Hopwood DA (2000) *Practical Streptomyces Genetics* (John Innes Foundation: Norwich, UK).

agar plate; the plate was then incubated at 30 °C for two days. A single colony was then picked from the LB plate and used to inoculate deionized water (200 μL), which was divided in half and evenly spread out on two Bennett's agar plates. The plates were incubated at 30 °C for four days at which point both plates had a uniformly gray lawn of *S. bernensis* spores.

A small portion of the spores from one plate were picked with an inoculation loop and used to inoculate 30 mL of LB medium in a 250-mL baffled Erlenmeyer flask with ca. 30 glass plating beads. The culture was placed in a 30 °C shaker (220 rpm) for three days. The cell culture was then transferred to a 50-mL plastic Corning tube and centrifuged (3750 rpm for 20 min); the liquid was decanted. Lysozyme (5 mg), dissolved in deionized water (100 μL), was added to SET buffer (5 mL) and the solution transferred to the cell pellet, which was broken up by repeated pipetting until a homogenous suspension was obtained. The mixture was transferred to a 15-mL plastic Corning tube and placed in a 37 °C water bath for 1 h.

Proteinase K (140 μL, 20 mg/mL) and 10% aqueous SDS (600 μL) were added and the mixture inverted several times until the solution became homogenous and clear. The solution was placed in a 55 °C water bath for 2 h. Aqueous NaCl (2 mL, 5.0 M) was added to the mixture and a precipitate formed; placed in a 37 °C water bath for 5 min. Chloroform (5 mL) was added and the layers mixed by inverting the tube periodically over 50 min. The mixture was then centrifuged (3750 rpm for 30 min); solids remained in the top water layer, which was aliquoted (1 mL portions) into eight Eppendorf tubes and centrifuged (13K rpm, 5 min). The water layer was transferred to a 50-mL plastic Corning tube and isopropanol (3.9 mL) was added; the DNA precipitated from solution. The tip of a glass Pasteur pipet was sealed in a flame, and once cool the DNA was spooled on it. The DNA was washed by immersing it in 70% ethanol (5 mL, then 2 x 1 mL) for 2 min each and then was allowed to air dry for 15 min. The genomic DNA was then re-dissolved in TE buffer (1 mL) in an Eppendorf tube, which was subsequently heated to 55 °C for 1 h before being stored at 4 °C.

To the other Bennett's agar plate of *S. bernensis* was added deionized water (5 mL) and the spores were harvested from the plate's surface by scraping with an L-shaped spreader. The cell suspension was filtered through glass wool into a 15-mL plastic Corning tube. Additional water (3 mL) was added to the plate's surface and the remaining cells were harvested and similarly filtered. The cell suspension was centrifuged (3750 rpm, 10 min) and the liquid decanted (ca. 60 μL of liquid remained). Deionized water (400 μL) was added to the spores, which were evenly suspended by pipetting; the resulting cell suspension was added to 50% glycerol (460 μL), mixed by pipet, and stored at -80 °C.

8. Illumina DNA sequencing and initial assembly of the berninamycin gene cluster: A library was constructed from fragmented *S. bernensis* genomic DNA, obtained above and randomly mechanically sheared into 200 ± 50 bp fragments, and sequenced using the Illumina Genome Analyzer IIx. Paired-end reads were obtained for 16,571,771 amplicons (33,143,542

65nt reads). Translated-query BLAST (BLASTx) (3) was used to align reads to a custom database of proteins that we hypothesized to have homologs in the putative berninamycin synthesis pathway: TsrC and TsrF from *Streptomyces laurentii* (GenBank accessions ACN52293 and ACN52296, respectively) (4), a lanthionine biosynthesis protein and NADH oxidase from *Bacillus cereus* (AAP11953 and NP_834749, respectively) (5), dehydratase from a *Nonomuraea* sp. (ACS83782) (6), and nitroreductase from a *Micromonospora* sp. (ZP_04607686); as well as the following proteins from *Propionibacterium acnes*: lanthionine biosynthesis and hypothetical proteins PPA0859 (AAT82614), PPA0860 (AAT82615), PPA0861 (AAT82616), PPA0862 (AAT82617), PPA0863 (AAT82618), PPA0864 (AAT82619), and PPA0865 (AAT82620) (7). This protein set includes putative orthologs, so reads were sought for BLAST-generated alignments of at least 15 aa (expect values were ≤0.1) to at least two proteins from this database. The 1437 reads meeting those criteria were used as seeds for targeted, localized genome assembly using an alpha version of the Paired-Read Iterative Contig Extension (PRICE) software (open-source and available for free download at http://derisilab.ucsf.edu/software/price/index.html) (8). Eight cycles of contig extension generated 1,217 contigs, eight of which generated alignments with expect values $\leq 10^{-3}$ with at least one of the custom database proteins from above. Further assembly cycles seeded with those eight contigs generated three contigs of 9926 nt, 1808 nt, and 97 nt; the translated sequences of the two longer contigs contained significant similarity to proteins from our custom database. Those contigs were connected through PCR amplification and extended by primer walking as described below.

9. Genomic DNA library construction/screening and primer walking: Genomic DNA was mechanically sheared by pipetting into fragments that were ca. 40 kb in length. These fragments were purified by low-melt agarose gel electrophoresis and isolated from the gel according to instructions from the Epicentre Biotechnologies fosmid library preparation manual. A genomic DNA fosmid library of approximately 10,000 clones was prepared from the pCC2FOS vector.

A portion of the library was screened by colony PCR (Platinum PCR Supermix) by a strategy that has been previously reported (9), checking for the presence of a portion of the *berB* gene

3. Altschul SF *et al.* (1997) Gapped BLAST and PSI–BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.
4. Engelhardt K, Degnes KF, Zotchev SB (2010) Isolation and characterization of the gene cluster for biosynthesis of the thiopeptide antibiotic TP-1161. *Appl Environ Microbiol* 76:7093–7101.
5. Ivanova N *et al.* (2003) Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. *Nature* 423:87–91.
6. Morris RP *et al.* (2009) Ribosomally synthesized thiopeptide antibiotics targeting elongation factor Tu. *J Am Chem Soc* 131:5946–5955.
7. Brüggemann H *et al.* (2004) The complete genome sequence of *Propionibacterium acnes*, a commensal of human skin. *Science* 305:671–673.
8. Ruby JG, Bellare P, DeRisi JL (2013) PRICE: Software for the Targeted Assembly of Components of (Meta)Genomic Sequence Data. *G3* doi: 10.1534/g3.113.005967.
9. Young TS, Walsh CT (2011) Identification of the thiazolyl peptide GE37468 gene cluster from *Streptomyces* ATCC 55365 and heterologous expression in *Streptomyces lividans*. *Proc Natl Acad Sci USA* 108:13053–13058.

(see *SI Appendix*, Fig. S1) to identify positive clones (BernContigAFor + BernContigARev primers). BamHI-HF and EcoRI-HF digestion revealed six of the clones to be unique.

Primer walking (5'Walk primer, Genewiz) off the *berE1* gene, outward from the cluster, revealed the presence of a gene encoding EF-Tu, which allowed the cluster to be connected with another contig from the Illumina sequencing. [The Illumina sequencing was corrected at two nucleotides by PCR amplification (primer combinations were EFTuFwd1 + EFTuRev1, EFTuFwd2 + EFTuRev2) of the EF-Tu gene in the fosmid DNA and traditional sequencing (Genewiz).] This contig contained genes encoding for EF-G and the ribosomal S7 and S12 proteins. Five out of the six clones, however, did not contain the full cytochrome P450 gene, *berH* (*SI Appendix*, Fig. S1). Fortunately, one clone contained both the P450 gene (confirmed by PCR, P450Fwd2 + P450Rev2 primers), the EF-Tu gene (EFTuFwd1 + EFTuRev1 primers) and the S12 gene (S12Fwd + S7Rev primers).

This one clone was cultured overnight in 1000 mL LB media [2.8-L baffled flask, 2 mL induction solution (Epicentre), 12.5 μg/mL chloramphenicol, 37 °C, 250 rpm]. After 18 h, the culture was centrifuged (6000 rpm for 15 min) and the liquid decanted. The fosmid was isolated from the cell pellet by Qiagen Maxiprep Kit.

Primer walking off the fosmid obtained above (3EndFwd primer, Genewiz) from *berH* away from the cluster showed that it contained the *berI* and *berJ* genes as well as an LmbE family acyl transferase, which is presumably not related to berninamycin production. This one clone thus appeared to contain all the genes necessary for berninamycin synthesis in *S. bernensis* and greatly resembled the TP-1161A (35-membered ring thiopeptide) gene cluster reported previously (*SI Appendix*, Fig. S1 and Table S1) (4,10).

10. Synthesis of the pSET152+bern plasmid: The sequencing effort also showed an SbfI restriction site in the S12 protein and a NotI site at the C-terminus of the acyl transferase, neither of which was present anywhere within the berninamycin gene cluster. We took advantage of these natural restriction sites surrounding the gene cluster to clone it into the pSET152 shuttle vector (*SI Appendix*, Fig. S2).

A portion of the ca. 48 kb fosmid containing the entire berninamycin cluster, which was obtained through the Maxiprep discussed above, was subjected to restriction digest with SbfI-HF and NotI-HF restriction endonucleases: DNA template (27 μL, 750 ng/μL, ca. 20.25 μg), deionized water (309 μL), NEBuffer 4 (40 μL, 10X), BSA (4 μL, 100X), SbfI-HF (10 μL, 20U/μL), NotI-HF (10 μL, 20U/μL) were mixed in an Eppendorf tube and incubated for 1 h at 37 °C. The enzymes were then heat inactivated for 10 min at 65 °C and the mixture purified by agarose gel electrophoresis. The portion of the gel that contained DNA that was ca. 17.3 kb in length was cut and the DNA isolated by Qiagen Gel Extraction Kit. The sample was then purified and concentrated by Zymo Clean and Concentrator-5 Kit.

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10. Engelhardt K *et al.* (2010) Production of a new thiopeptide antibiotic, TP-1161, by a marine *Nocardiopsis* species. *Appl Environ Microb* 76:4969–4976.

To introduce the cluster into the pSET152 vector, two SbfI restriction sites first had to be removed from the pSET152 plasmid and the SbfI and NotI sites introduced into the *lacZ* gene. All four mutations were accomplished by Quikchange [pfuTurbo polymerase; transformation into chemically competent One Shot Top10 *E. coli*; plating (LB agar containing apramycin) and culturing of the *E. coli* found to have apramycin resistance; and isolation of the plasmid by Qiagen Miniprep Kit]. The following primers were used: pSETQkchngFwd1 + pSETQkchngRev1 to remove the first SbfI site, pSETQkchngFwd2 + pSETQkchngRev2 to remove the second SbfI site, and pSETbernSbfI2 + pSETbernNotI2 to introduce the restriction sites into *lacZ*. Successful introduction of the mutations was confirmed at each stage by DNA sequencing (Genewiz).

Restriction digest was then carried out on the modified pSET152 plasmid with the SbfI-HF and NotI-HF restriction endonucleases analogously to the fosmid digestion outlined above except that DpnI (2 μL) and CIP (2 μL) were also added to the reaction. The mixture was then purified by agarose gel electrophoresis, the portion of the gel that contained the desired pSET152 vector was excised, and the DNA isolated by Qiagen Gel Extraction Kit. The sample was then purified and concentrated by Zymo Clean and Concentrator-25 Kit.

Ligation of the berninamycin gene cluster (929 ng "insert", ca. 17.3 kb) to the pSET152 vector (77 ng, ca. 5.4 kb) was accomplished with T4 DNA ligase (0.3 μL) on a 10 μL scale (1 μL T4 ligase buffer, 10X); this corresponds to roughly a 4:1 molar ratio of insert:vector. The reaction was set up at 0 °C and run for 12 h at 16 °C before heat inactivation of the enzyme at 70 °C for 10 min. The ligation reaction was then stored at 4 °C until transformation by electroporation (Section 12).

11. Preparation of electrocompetent One Shot Top10 *E. coli* and ET12567/pUZ8002 *E. coli*: Four starter cultures of One Shot Top10 *E. coli* (no antibiotic used) or ET12567/pUZ8002 *E. coli* (chloramphenicol and kanamycin at half concentrations) were begun by inoculating LB media (5 mL) in 14-mL plastic Corning tubes; the cultures were placed in a roller drum in a 37 °C incubator overnight. The starter cultures were then used to inoculate LB media (500 mL) in two 2.8-L baffled flasks such that the starting concentration had an OD_{600} of ca. 0.07. The flasks were placed in a 37 °C shaker (250 rpm) until the OD_{600} was ca. 0.50 (N.B. do not exceed an OD_{600} of 0.60).

The cultures were then transferred into two sterile 500-mL centrifuge bottles and the cells pelleted by centrifugation (SLA-3000 rotor, 6000 rpm, 7 min, 4 °C). The supernatant was decanted and cold, sterile 10% glycerol (500 mL) added to each pellet; the suspension was shaken vigorously to break up the pellet. The cells were again pelleted by centrifugation as above and the process repeated once more. After the second 10% glycerol wash, the two cell pellets were combined in one centrifuge bottle, washed with 10% glycerol, and the cell pellet broken up as above. The cells were pelleted by centrifugation once again and the supernatant decanted such that a small amount of liquid remained to resuspend the cells.

The cells were stored on ice and were gently resuspend by pipet. The 10% glycerol suspension of electrocompetent cells was then aliquoted into 50-μL portions in sterile Eppendorf tubes, which were then frozen on dry ice. The frozen cell suspensions were stored at –80 °C until needed (stored for up to six months).

12. Isolation of pSET152+bern from *E. coli* and transformation to ET12567/pUZ8002 cells: A portion of the ligation reaction (2 μL) to furnish the pSET152+bern plasmid (Section 10) was added to electrocompetent One Shot Top10 *E. coli* (25 μL of cells) and gently mixed on ice. The entire suspension was transferred to a cold 1 mm gap electroporation cuvette (Fisher Scientific) and the DNA transformed by electroporation (1.8 V, 200 Ω resistance, 25 C capacitance). The cells were then recovered in LB or 2xYT media (500 μL) in a 14-mL Falcon tube for 1 h at 37 °C in a roller drum. A portion of the recovery solution (100 μL) was plated (LB agar with apramycin) and the plate incubated at 37 °C overnight. Resistant colonies were picked and cultured overnight (5 mL LB medium with apramycin, 37 °C) and the pSET152+bern plasmid isolated from each by Qiagen Miniprep Kit. The synthesis and isolation of the pSET152+bern plasmid was confirmed by PCR amplification of the two ligation sites (CloneChk1 + S7Rev and tsrFwd + CloneChk2 primers) and DNA sequencing (Genewiz).

Successfully prepared pSET152+bern plasmid (1 μL) was then added to electrocompetent ET12567/pUZ8002 *E. coli* (25 μL of cells) and gently mixed on ice. Transformation by electroporation was carried out as above and the cells were recovered as above for 1 h. At that time, 20 μL of the recovery solution, as well as 20 μL of 10X and 100X dilutions of the recovery solution, were streaked out onto three separate and roughly equal portions of an LB agar plate (contains half concentrations of apramycin, kanamycin, and chloramphenicol); the plate was incubated at 37 °C.

After 24 h, a single colony was picked and used to inoculate LB or 2xYT media (1 mL, with half concentrations of apramycin, kanamycin, and chloramphenicol) in a 14-mL Falcon tube, which was placed in a roller drum at 37 °C for 9 h. At that time, the entire cell suspension was plated (LB agar, half concentrations of apramycin, kanamycin, and chloramphenicol) and the plate placed in a 37 °C incubator overnight. Alternatively, a single colony could be picked and used to inoculate LB or 2xYT media (100 μL), which could be immediately plated (LB agar, with half concentrations of apramycin, kanamycin, and chloramphenicol) and the plate placed in a 37 °C incubator overnight. Either procedure produced a lawn of ET12567/pUZ8002, which contained the pSET152+bern plasmid.

13. Conjugative transfer of pSET152+bern to *Streptomyces lividans* TK24 and *Streptomyces venezuelae* ATCC 10712 (2): A lawn of *Streptomyces lividans* TK24 (*S. lividans*) or *Streptomyces venezuelae* ATCC 10712 (*S. venezuelae*) spores was prepared on SFM agar plates (incubation at 30 °C for 3–4 days). A suspension of these spores was prepared by adding LB or 2xYT media (2 mL) to each plate and scraping the spores with an L-shaped spreader; the cell suspension was transferred to a sterile Eppendorf tube by pipet. The suspension was then centrifuged (8000 rpm for 1 min) and the supernatant discarded. The cells were resuspended in

LB or 2xYT (1 mL) and again centrifuged, subsequently discarding the supernatant. The procedure was repeated once more before resuspending the spores in LB or 2xYT (500 μL) and placing the Eppendorf and its contents at 50 °C for 10 min. The cell suspension was then allowed to cool to 22 °C.

During the heat shock of the *Streptomyces*, a suspension of ET12567/pUZ8002/pSET152+bern *E. coli* was prepared by adding LB or 2xYT media (2 mL) to each plate and scraping the spores with an L-shaped spreader; the cell suspension was transferred to a sterile Eppendorf tube by pipet. The suspension was then centrifuged (8000 rpm for 1 min) and the supernatant discarded. The cells were resuspended in LB or 2xYT (1 mL) and again centrifuged, subsequently discarding the supernatant. The procedure was repeated once more before resuspending the *E. coli* in LB or 2xYT (250 μL).

A portion of the *Streptomyces* spore suspension (250 μL) was added to the suspension of ET12567/pUZ8002/pSET152+bern and gently mixed by pipet. The suspension was centrifuged (8000 rpm for 1 min) and the supernatant discarded. The cells were resuspended in LB or 2xYT (100 μL), serial dilutions prepared of each mixture (10X, 100X, and 1000X), and a portion of each concentration of cells (10 μL) streaked on its own quadrant of an SFM agar plate (supplemented with 10 mM $MgCl_2$). The plates were incubated at 30 °C for 16–20 h at which time a 1 mL solution of antibiotics was overlaid on the plate (0.5 mg/mL nalidixic acid and 1.25 mg/mL apramycin). The plates were then incubated at 30 °C for 4 days. Two colonies were picked from each plate and restreaked on SFM plates (containing apramycin and nalidixic acid). The plates were incubated at 30 °C for 4 days.

14. PCR-directed mutagenesis to introduce the T3A, T4A, and T5A mutations into the *berA* gene on the pSET152+bern plasmid: To obtain the mutant plasmids, an insert (contains *berD*, the mutant *berA*, and *berB*) was ligated to the pSET152+bern vector (consists of the pSET152+bern plasmid minus *berD*, *berA*, and *berB*), both prepared by the procedure outlined below and illustrated for the T3A mutation in *SI Appendix*, Fig. S4.

For the T3A mutant insert, two PCRs were run where one primer in each reaction contained the T3A mutation and the other primer overlapped with one of two restriction sites: NsiIRestr + T3AMutRev and SpeIRestr + T3AMutFwd (200 μL reactions for each with HF-Phusion Mastermix and 3% DMSO added; pSET152+bern plasmid as the template in each case). The PCR products were purified by agarose gel electrophoresis, the portion of the gel that contained the desired product was excised, and the DNA isolated by Qiagen Gel Extraction Kit. The sample was then purified and concentrated by Zymo Clean and Concentrator-5 Kit.

A PCR was then run to link the two PCR products obtained above: the two PCR products were mixed in approximately equimolar amounts along with the NsiIRestr and SpeIRestr primers (400 μL reaction with HF-Phusion Mastermix and 3% DMSO added). The product of this overlap PCR was then subjected to SpeI restriction digest (10-fold over-digestion with SpeI restriction endonuclease in NEBuffer 4 with BSA) at 37 °C for 2 h; the solution was then purified by Zymo

Clean and Concentrator-5 Kit. The obtained solution was then subjected to NsiI restriction digest (10-fold over-digestion with NsiI restriction endonuclease in NEBuffer 3) at 37 °C for 2 h. Subsequently, the DNA was purified by agarose gel electrophoresis, the portion of the gel that contained the desired product was excised, and the DNA isolated by Qiagen Gel Extraction Kit. The sample was then purified and concentrated by Zymo Clean and Concentrator-5 Kit.

To obtain the pSET152+bern vector, the pSET152+bern plasmid was first subjected to SpeI restriction digest (10-fold over-digestion with SpeI restriction endonuclease in NEBuffer 4 with BSA) at 37 °C for 2 h; the solution was then purified by Zymo Clean and Concentrator-5 Kit. The obtained solution was then subjected to NsiI restriction digest (10-fold over-digestion with NsiI restriction endonuclease in NEBuffer 3; 3 μL of CIP was also added to the mixture) at 37 °C for 2 h. Subsequently, the DNA was purified by agarose gel electrophoresis, the portion of the gel that contained the desired product was excised, and the DNA isolated by Qiagen Gel Extraction Kit. The sample was then purified and concentrated by Zymo Clean and Concentrator-5 Kit.

The T3A mutant insert (ca. 54.0 ng) was ligated to the pSET152+bern vector (ca. 54.0 ng) with T4 DNA ligase (0.3 μL) on a 10 μL scale (1 μL T4 ligase buffer); this corresponds to roughly a 4:1 molar ratio of insert:vector. The reaction was set up at 0 °C and run for 12 h at 16 °C before heat inactivation of the enzyme at 70 °C for 10 min. The ligation reaction was then stored at 4 °C until transformation to electrocompetent One Shot Top10 *E. coli* by electroporation as in section 11. The integrity of the ligation sites was checked by DNA sequencing (Genewiz) the plasmid obtained from the One Shot Top10 *E. coli* (StrucFwd1 and StrucRev1 primers); the successful introduction of the mutation was also checked by DNA sequencing (Genewiz) with the BernContig2F and tclMFwd primers.

The pSET152+bern T3A mutant plasmid was isolated from One Shot Top10 *E. coli* and transformed to electrocompetent ET12567/pUZ8002 *E. coli* as described in section 12. Conjugative transfer of the plasmid to *S. lividans* TK24 was accomplished as described in section 13. An analogous procedure to prepare the T4A and T5A mutants was also carried out with the following primers used to introduce the mutations to the *berA* gene: NsiIRestr + T4AMutRev and SpeIRestr + T4AMutFwd for the T4A mutation and NsiIRestr + T5AMutRev and SpeIRestr + T5AMutFwd for the T5A mutation.

15. Small scale heterologous expressions of *S. bernensis*, *S. venezuelae*/pSET152+bern (wt), *S. venezuelae*/pSET152, *S. lividans*/pSET152+bern (wt, T3A, T4A, and T5A), and *S. lividans*/pSET152: 25 mL Seed media (no antibiotic) in a 250-mL baffled Erlenmeyer flask, containing approximately 20 glass beads, was inoculated with spores from a plate of *S. bernensis* grown on Bennett's agar. Similar inoculations of Seed media (with apramycin) were carried out for the *S. venezuelae*/pSET152+bern (wt), *S. venezuelae*/pSET152, *S. lividans*/pSET152+bern (wt, T3A, T4A, and T5A), and *S. lividans*/pSET152 strains with spores taken directly from an agar plate. All cultures were incubated at 30 °C for three days with 250 rpm shaking.

At that point, 500 μL of the *S. bernensis* culture was used to inoculate 50 mL AF/MS media (no antibiotic) in a 250-mL baffled Erlenmeyer flask, containing approximately 20 glass beads. Similarly, AF/MS media (with apramycin) was inoculated with *S. venezuelae*/pSET152+bern (wt), *S. venezuelae*/pSET152, *S. lividans*/pSET152+bern (T4A or T5A), or *S. lividans*/pSET152 from the starter cultures. Additionally, 50 mL GYM media (with apramycin) was similarly inoculated with *S. lividans*/pSET152+bern (wt or T3A) and *S. lividans*/pSET152 from the starter cultures (11). All cultures were incubated at 30 °C for four days with 250 rpm shaking.

At that time, 10 mL of each culture was transferred to a 50-mL plastic vial, the vial and contents centrifuged (3750 rpm for 20 min), and the supernatant decanted. Approximately 10 glass beads, a copious amount of anhydrous Na_2SO_4, and 10 mL acetone were added to each vial, and the mixture was shaken and vortexed vigorously for 30 min. Then, the mixture was filtered through a cotton plug and the volatiles removed by rotary evaporation. The remaining residue was dissolved in 500 μL of 50:50 H_2O:MeCN and filtered through a 0.45 μM filter for High Resolution LC-MS analysis (*SI Appendix*, Table S3).

It should be noted that the quantity of macrocyclic thiopeptides produced by the *S. lividans*/pSET152+bern (wt and T3A) strains decreases over time as the strain is stored, and eventually the strains stop producing thiopeptides altogether. Conversely, the quantity of linear precursors produced by the strain remains relatively constant. Thus, in order to obtain the largest quantity of macrocycles from an expression, it is important to have freshly prepared the strain through conjugative transfer. This behavior was not exhibited by the *S. venezuelae* heterologous host.

LC-MS Elution Method A:

For the *S. bernensis*, *S. venezuelae*/pSET152+bern (wt), *S. venezuelae*/pSET152, *S. lividans*/pSET152+bern (wt and T3A), and *S. lividans*/pSET152 samples, 10 μL of each sample was injected onto the C18 column and eluted according to the following program: 0.4 mL/min elution; 98:2 H_2O:MeCN isocratic for 2 min; then increase to 50:50 H_2O:MeCN over 10 min, followed by isocratic elution for 2 min; then increase to 100% MeCN over 5 min, followed by isocratic elution for 2 min. Each solvent contained 0.1% formic acid.

LC-MS Elution Method B:

For the *S. lividans*/pSET152+bern (T4A and T5A) samples, 10 μL of each sample was injected onto the C18 column and eluted according to the following program: 0.4 mL/min elution; 98:2 H_2O:MeCN isocratic for 2 min; then increase to 100% MeCN over 10 min, followed by isocratic elution for 2 min. Each solvent contained 0.1% formic acid.

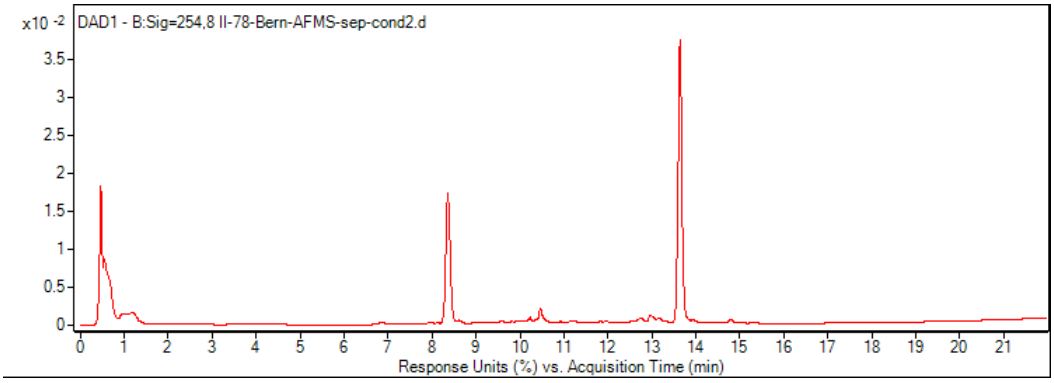
11. GYM was eventually found to be the optimal media for expression from *S. lividans* strains (roughly ten times the amount produced in AF/MS) whereas *S. bernensis* and *S. venezuelae* gave optimal production in AF/MS. The same metabolites are produced by the *S. lividans* strains, regardless of the media employed, including the control strain *S. lividans*/pSET152.

Table S3. LC-MS analysis of metabolites produced by expression of the berninamycin gene cluster.[a]

Strain	Compound Identification	RetentionTime (min)	Calc. Mass (m/z)[b]	Found Mass (m/z)[b]	Error (ppm)
S. bernensis[c]	linear–Dha_{13}	10.43	1236.4276	1236.4351	6.1
	dbl-hydroxylated berninamycin	12.94	1162.3432	1162.3485	4.6
	berninamycin D	13.01	1008.3053	1008.3066	1.3
	berninamycin C	13.56	1077.3268	1077.3264	0.4
	berninamycin A	13.61	1146.3483	1146.3524	3.6
	berninamycin B	14.78	1130.3533	1130.3532	0.1
S. venezuelae/pSET152+bern (wt)[c]	dbl-hydroxylated M+2 berninamycin	13.21	1164.3588	1164.3589	0.1
	M+2 berninamycin D	13.42	1010.3210	1010.3207	0.3
	M+2 berninamycin A	13.84	1148.3639	1148.3617	1.9
	M+2 berninamycin C	13.85	1079.3424	1079.3401	2.1
S. lividans/pSET152+bern (wt)[c]	linear–Ser_{13}	10.01	1254.4381	1254.4409	2.2
	linear–Dha_{13}	10.50	1236.4276	1236.4275	0.1
	dbl-hydroxylated berninamycin	12.91	1162.3432	1162.3430	0.2
	berninamycin D	12.98	1008.3053	1008.3068	1.5
	berninamycin A	13.60	1146.3483	1146.3506	2.0
	berninamycin B	14.76	1130.3533	1130.3522	1.0
S. lividans/pSET152+bern (T3A)[c]	T3A linear–Ser_{13}	10.07	1224.4276	1224.4322	3.8
	T3A linear–Dha_{13}	10.55	1206.4170	1206.4229	4.9
	T3A dbl-hydroxylated berninamycin	13.05	1132.3326	1132.3381	4.9
	T3A berninamycin D	13.08	978.2948	978.2966	1.8
	T3A berninamycin A	13.70	1116.3377	1116.3404	2.4
	T3A berninamycin B	14.81	1100.3428	1100.3438	0.9
S. lividans/pSET152+bern (T4A)[d]	T4A linear–Ser_{13}	7.78[e]	1242.4381	1242.4361	1.6
	T4A linear–Dha_{13}	8.16	1224.4276	1224.4278	0.2

[a] Analyzed by detection at λ = 254 nm and by the total ion chromatogram and specific mass ion extracted chromatograms. Metabolites related to berninamycin production have not been detected from fermentations of *S. lividans*/pSET152+bern (T5A) nor the control strains *S. lividans*/pSET152 and *S. venezuelae*/pSET152. [b] Mass of the protonated compound. [c] Elution method A. [d] Elution method B. [e] This metabolite co-elutes with a much more abundant and unrelated compound and is thus obscured at 254 nm.

a)



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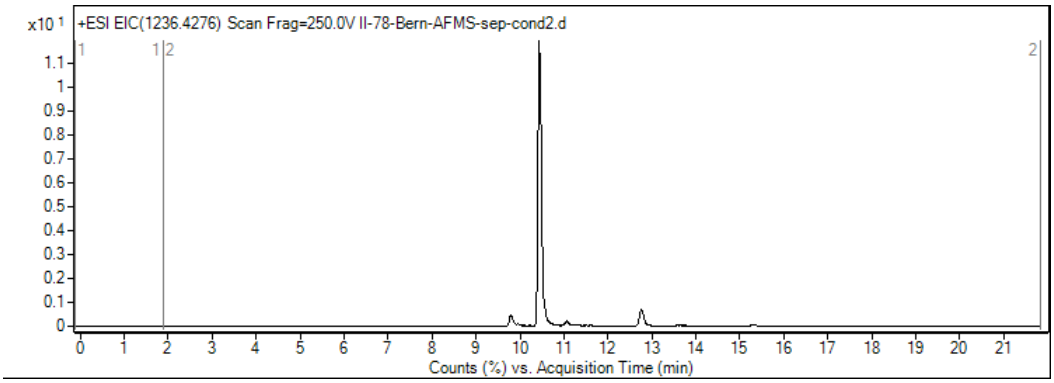
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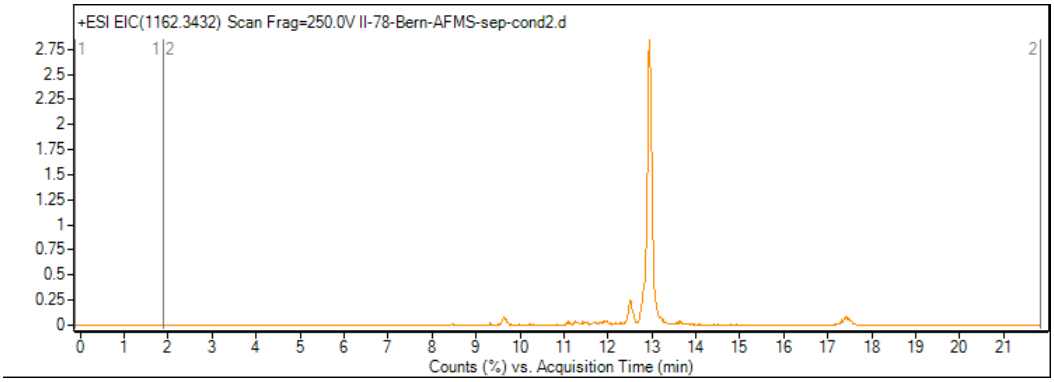
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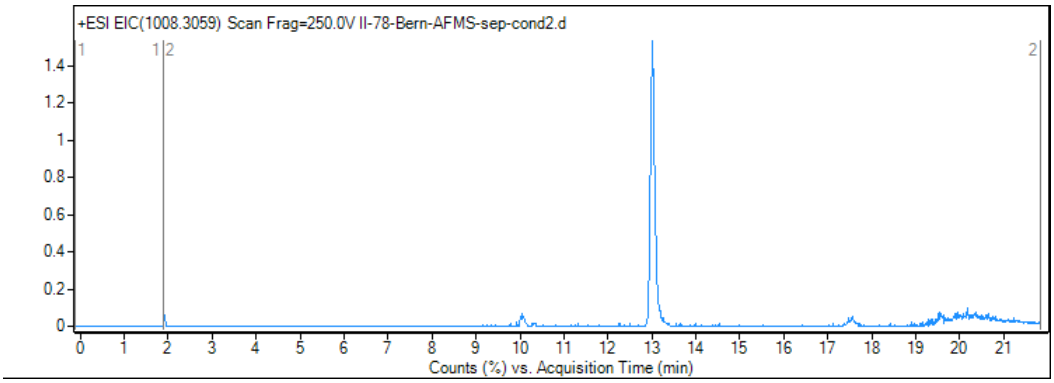
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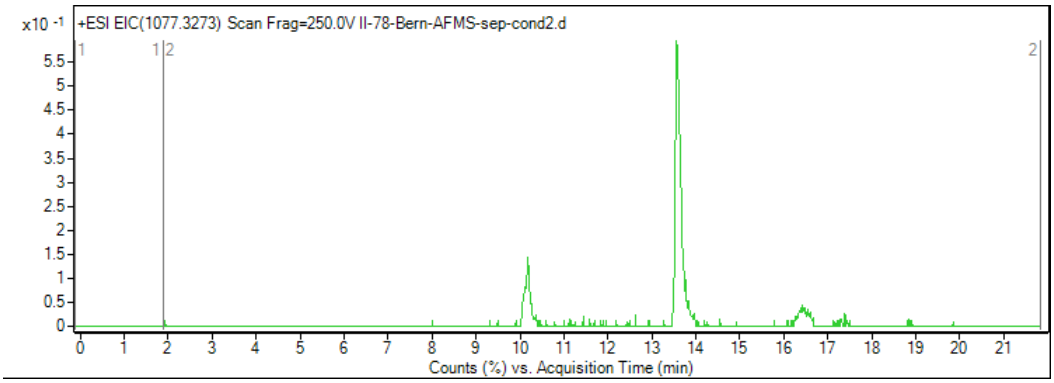
e)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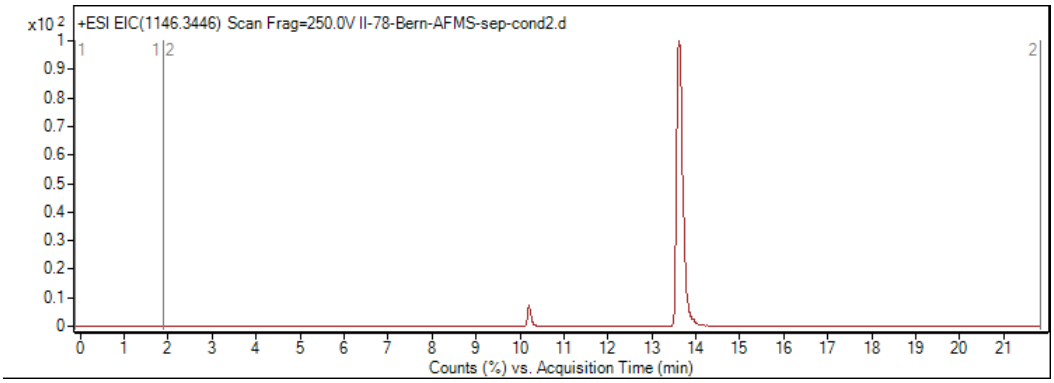

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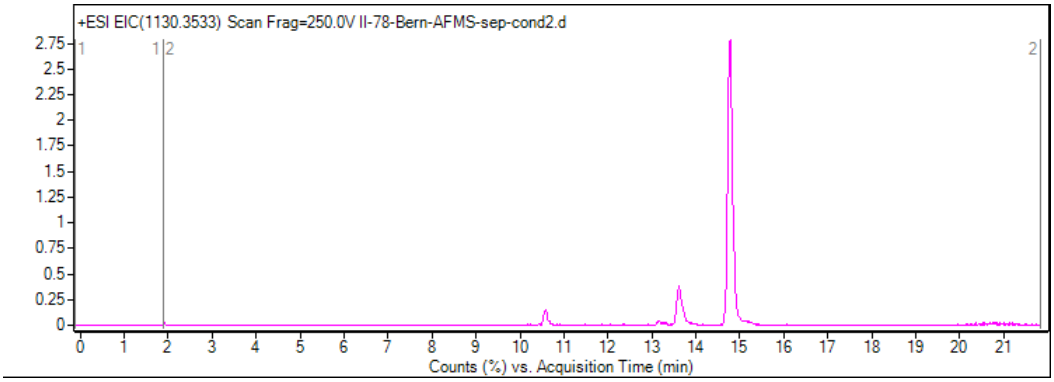
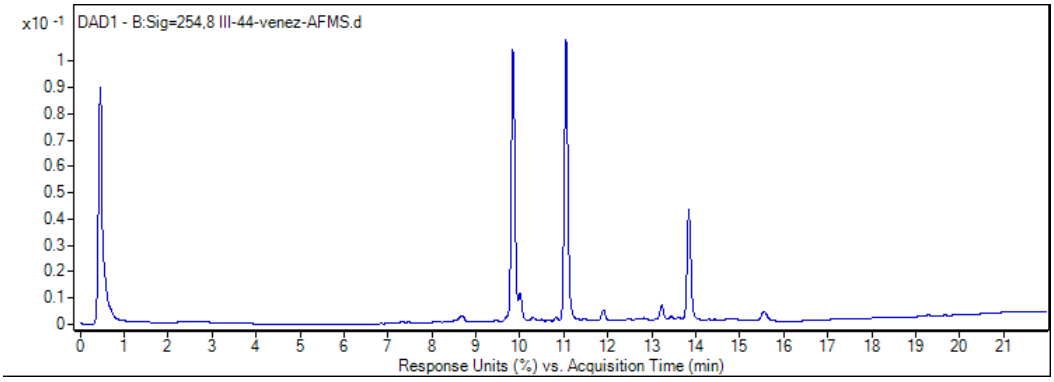


Fig. S7. LC-MS traces for *S. bernensis* expression in AF/MS media. a) UV trace (λ = 254 nm). b) Extracted ion chromatogram for linear–Dha_{13}. c) Extracted ion chromatogram for doubly-hydroxylated berninamycin. d) Extracted ion chromatogram for berninamycin D. e) Extracted ion chromatogram for berninamycin C. f) Extracted ion chromatogram for berninamycin A. g) Extracted ion chromatogram for berninamycin B.

a)



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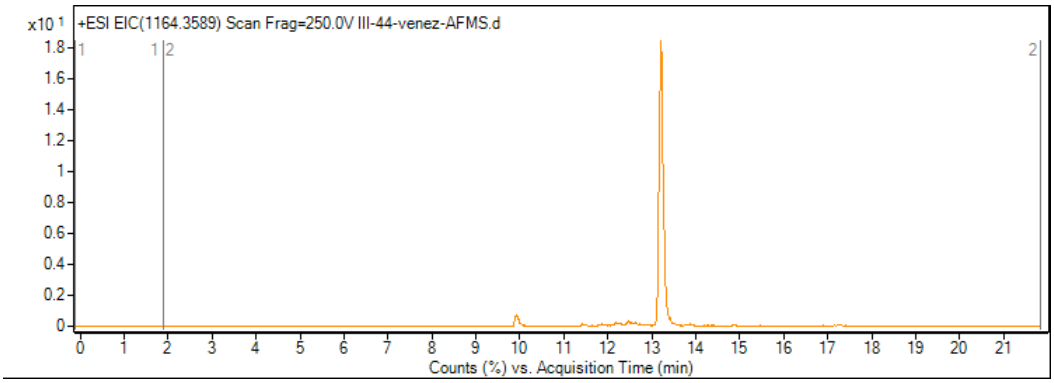
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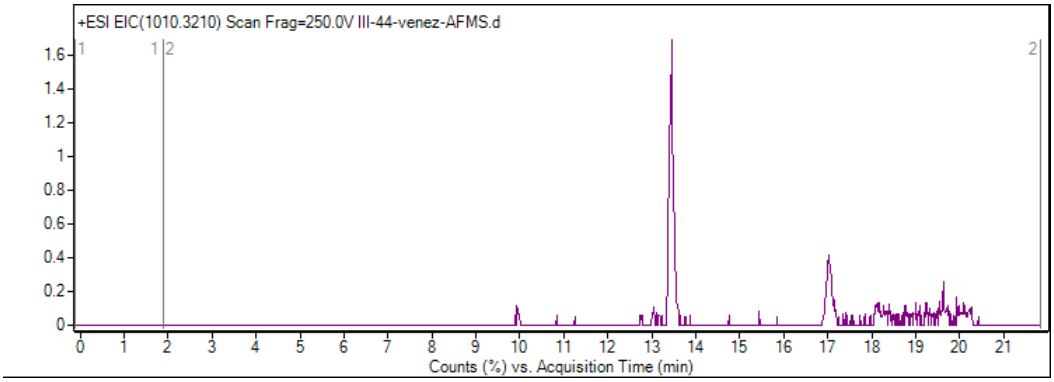
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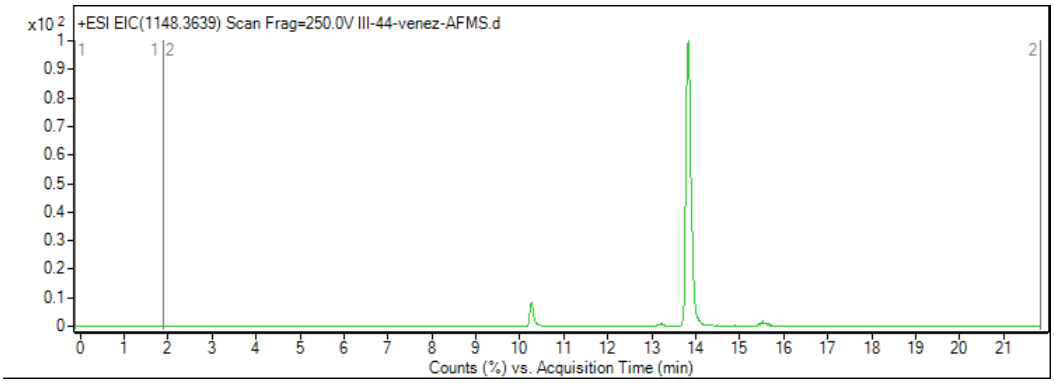
d)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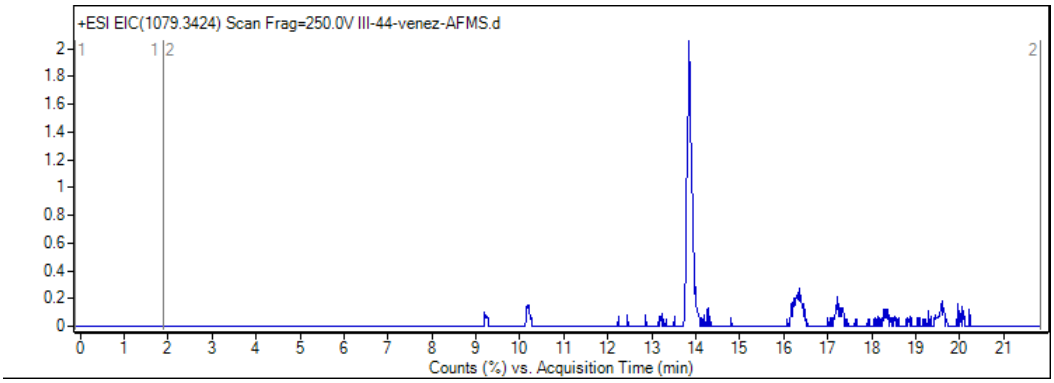
e)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Counts (%) vs. Acquisition Time (min)

Fig. S8. LC-MS traces for *S. venezuelae*/pSET152+bern expression in AF/MS media. a) UV trace (λ = 254 nm). b) Extracted ion chromatogram for doubly-hydroxylated M+2 berninamycin. c) Extracted ion chromatogram for M+2 berninamycin D. d) Extracted ion chromatogram for M+2 berninamycin A. e) Extracted ion chromatogram for M+2 berninamycin C.

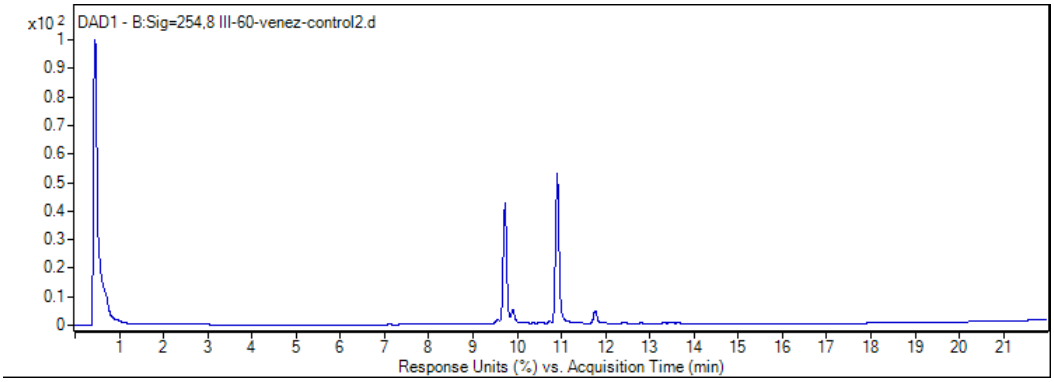
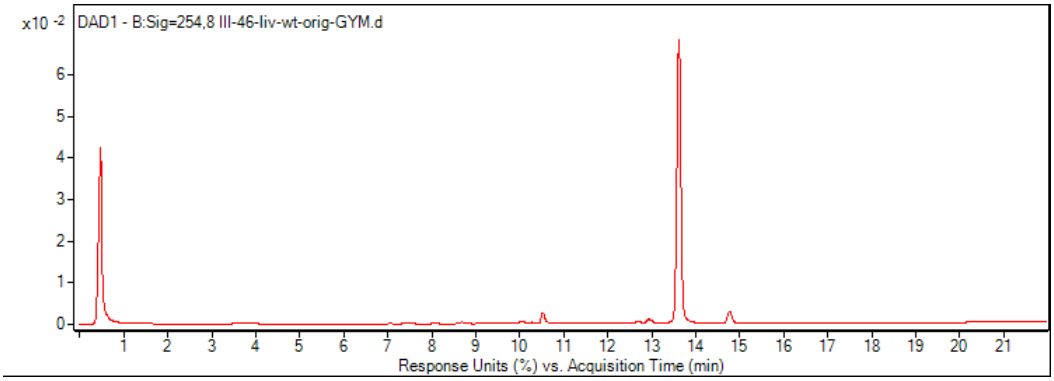
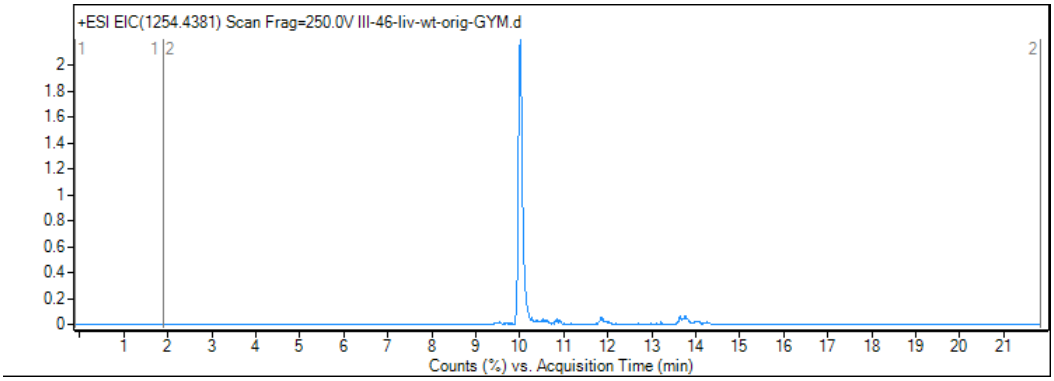


Fig. S9. UV trace (λ = 254 nm) from the *S. venezuelae*/pSET152 control expression in AF/MS media, showing a lack of any berninamycin-related metabolites.

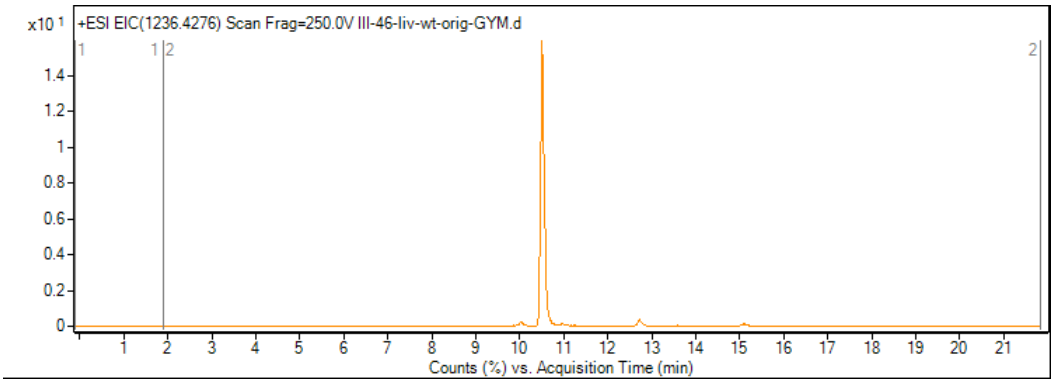
a)



b)



c)


x10 1
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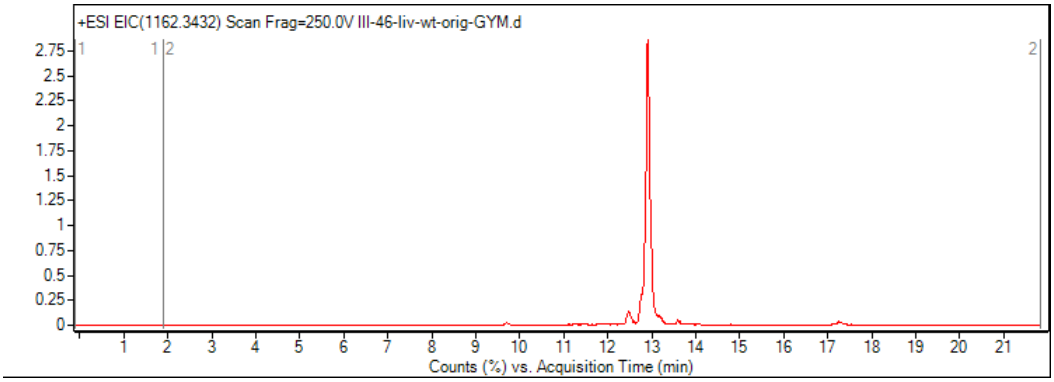
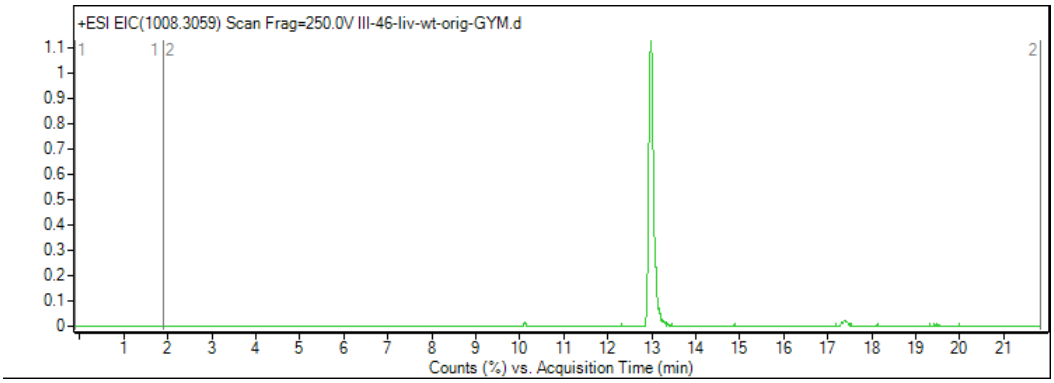
Counts (%) vs. Acquisition Time (min)

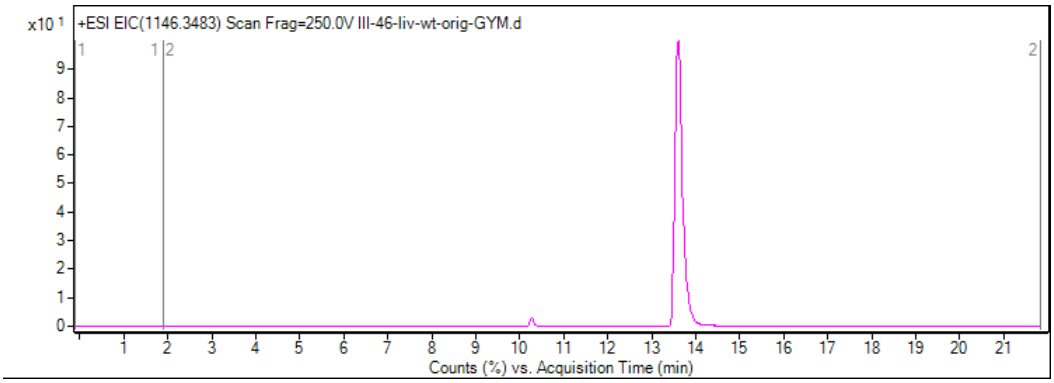
d)


+ESI EIC(1162.3432) Scan Frag=250.0V III-46-liv-wt-orig-GYM.d
Counts (%) vs. Acquisition Time (min)

e)


+ESI EIC(1008.3059) Scan Frag=250.0V III-46-liv-wt-orig-GYM.d
Counts (%) vs. Acquisition Time (min)

f)


x10 1
+ESI EIC(1146.3483) Scan Frag=250.0V III-46-liv-wt-orig-GYM.d
Counts (%) vs. Acquisition Time (min)

g)

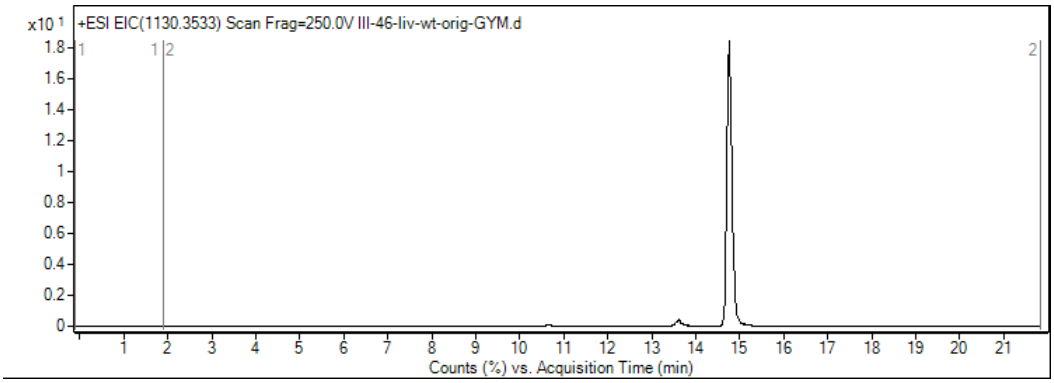
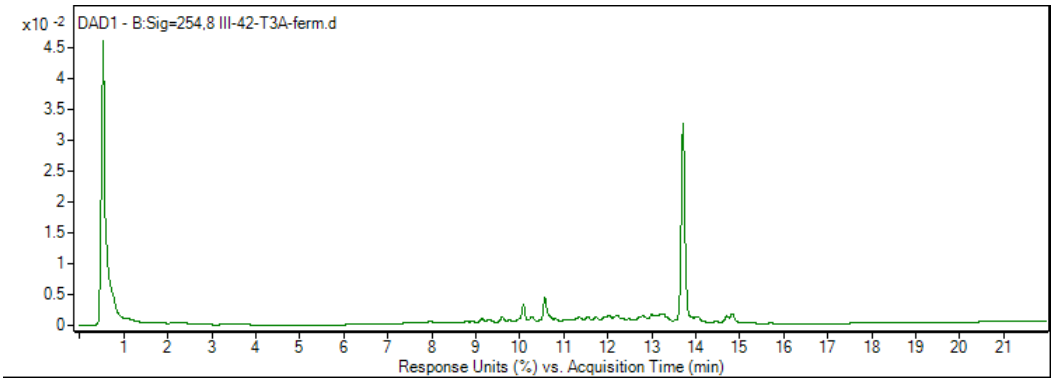
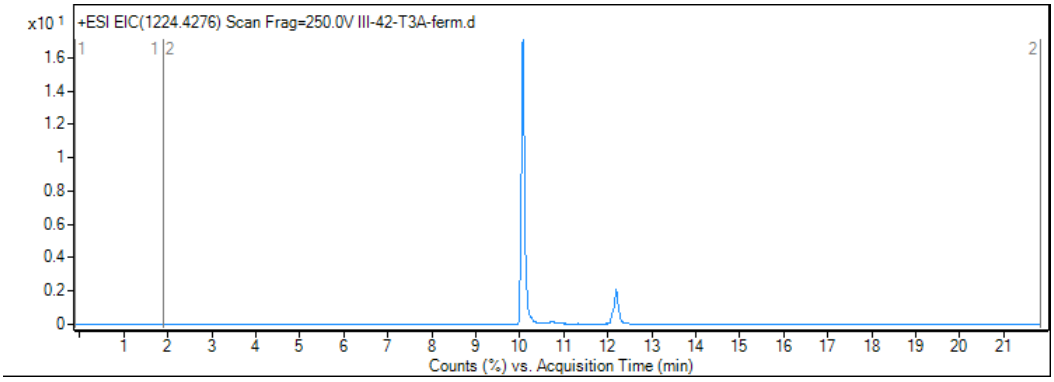


Fig. S10. LC-MS traces for *S. lividans*/pSET152+bern (wt) expression in GYM media. a) UV trace (λ = 254 nm). b) Extracted ion chromatogram for linear–Ser_{13}. c) Extracted ion chromatogram for linear–Dha_{13}. d) Extracted ion chromatogram for doubly-hydroxylated berninamycin. e) Extracted ion chromatogram for berninamycin D. f) Extracted ion chromatogram for berninamycin A. g) Extracted ion chromatogram for berninamycin B.

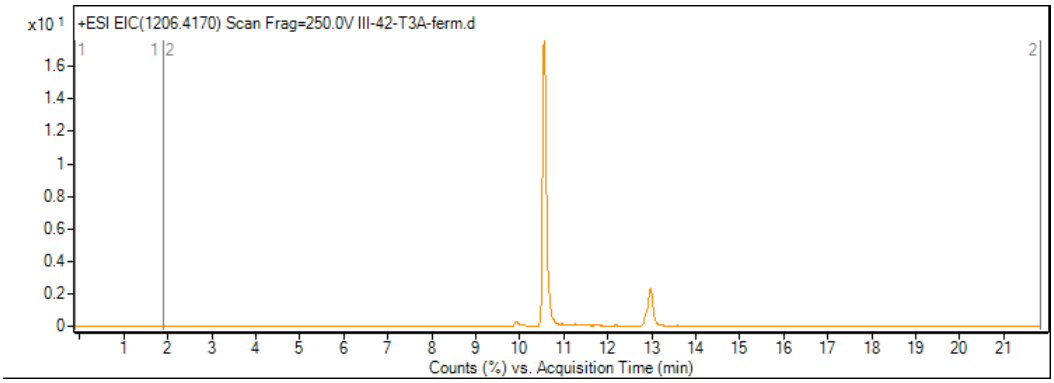
a)



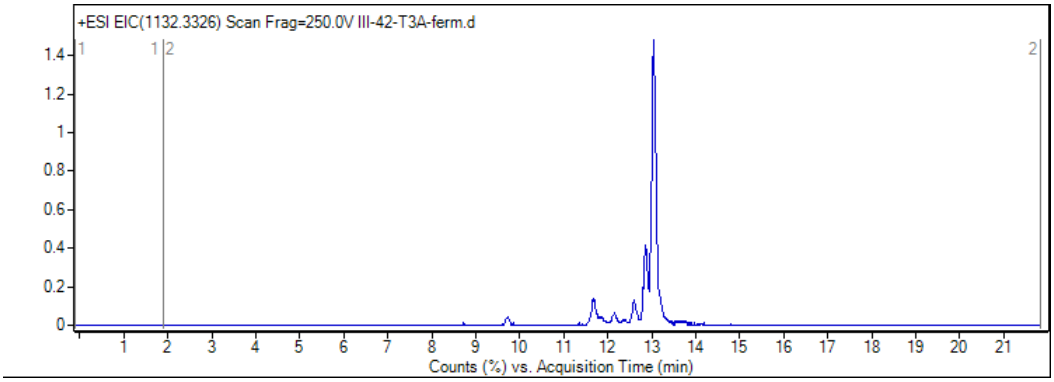
b)



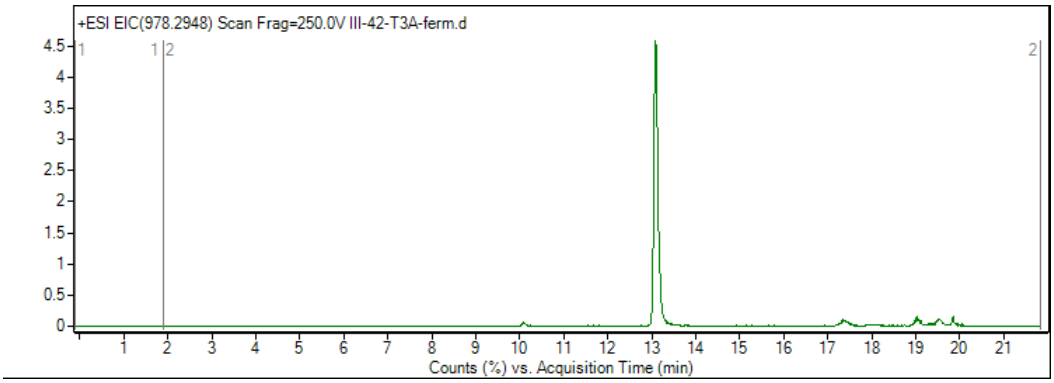
c)


x10 1
+ESI EIC(1206.4170) Scan Frag=250.0V III-42-T3A-ferm.d
Counts (%) vs. Acquisition Time (min)

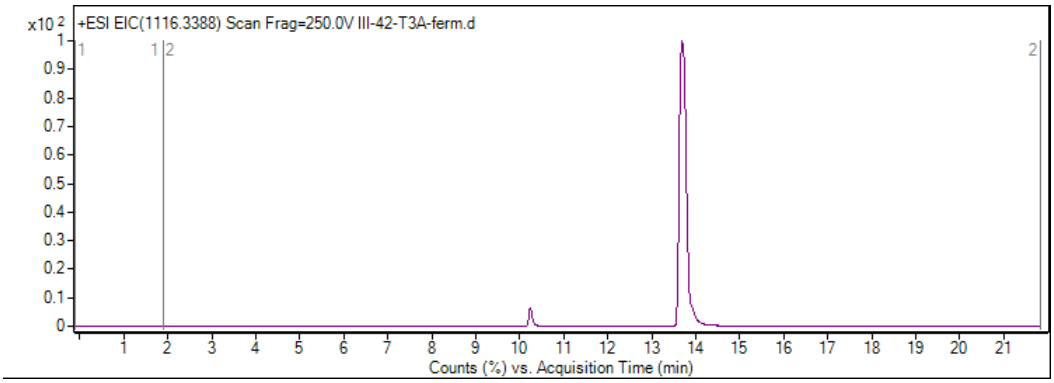
d)


+ESI EIC(1132.3326) Scan Frag=250.0V III-42-T3A-ferm.d
Counts (%) vs. Acquisition Time (min)

e)


+ESI EIC(978.2948) Scan Frag=250.0V III-42-T3A-ferm.d
Counts (%) vs. Acquisition Time (min)

f)


x10 2
+ESI EIC(1116.3388) Scan Frag=250.0V III-42-T3A-ferm.d
Counts (%) vs. Acquisition Time (min)

g)

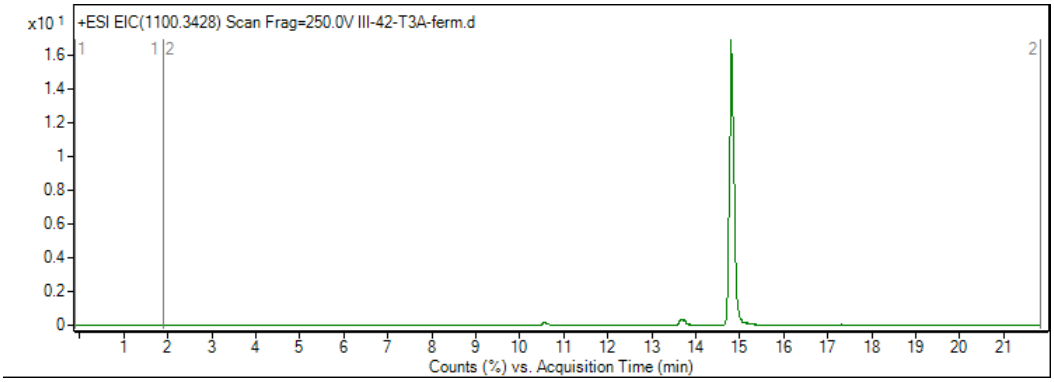
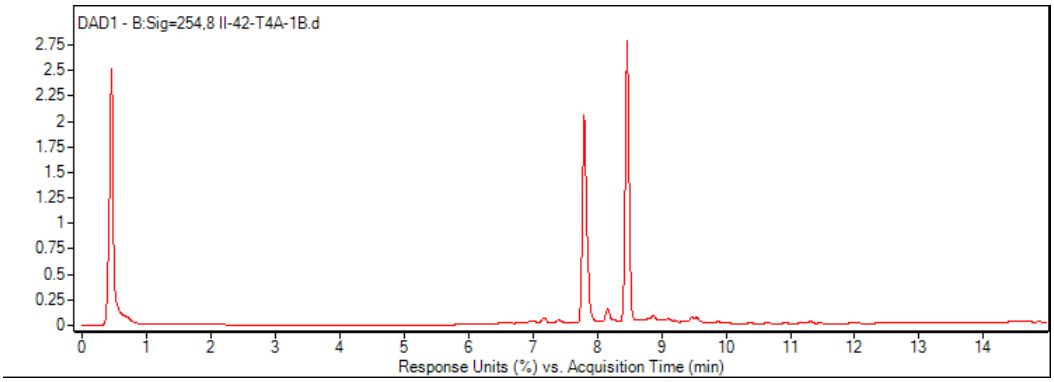
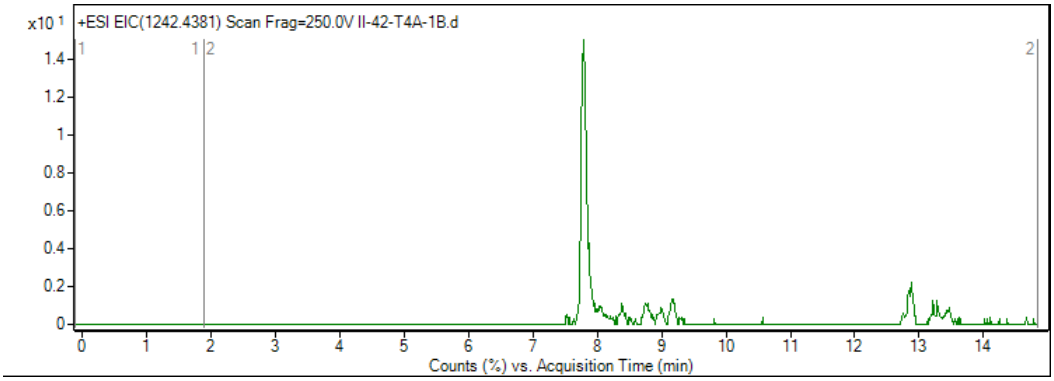


Fig. S11. LC-MS traces for *S. lividans*/pSET152+bern (T3A) expression in GYM media. a) UV trace (λ = 254 nm). b) Extracted ion chromatogram for T3A linear–Ser_{13}. c) Extracted ion chromatogram for T3A linear–Dha_{13}. d) Extracted ion chromatogram for T3A doubly-hydroxylated berninamycin. e) Extracted ion chromatogram for T3A berninamycin D. f) Extracted ion chromatogram for T3A berninamycin A. g) Extracted ion chromatogram for T3A berninamycin B.

a)



b)



c)

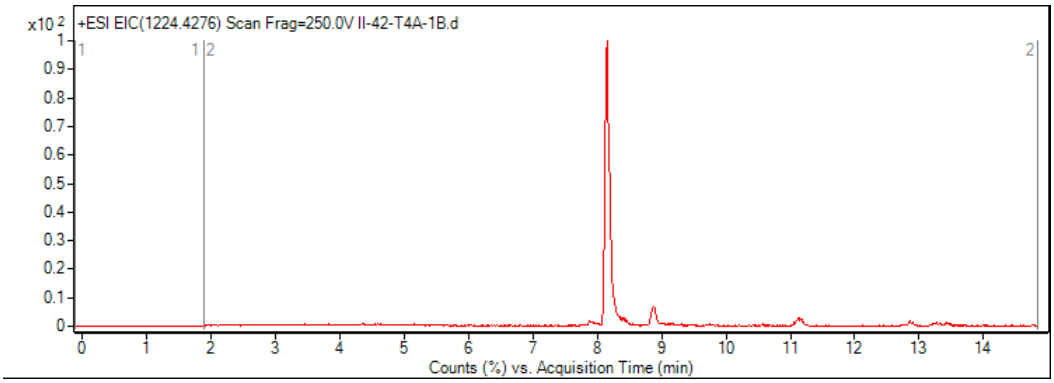


Fig. S12. LC-MS traces for *S. lividans*/pSET152+bern (T4A) expression in AF/MS media. a) UV trace (λ = 254 nm). b) Extracted ion chromatogram for T4A linear–Ser_{13}. c) Extracted ion chromatogram for T4A linear–Dha_{13}.

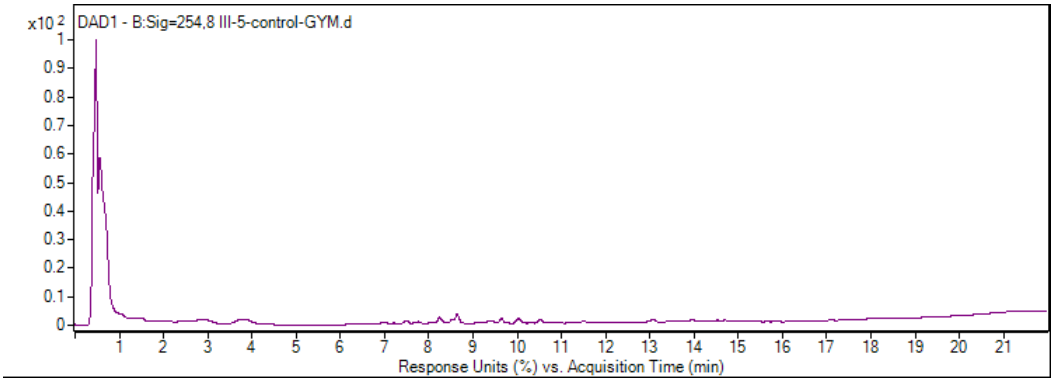


Fig. S13. UV trace (λ = 254 nm) from the *S. lividans*/pSET152 control expression in GYM media, showing a lack of any berninamycin-related metabolites.

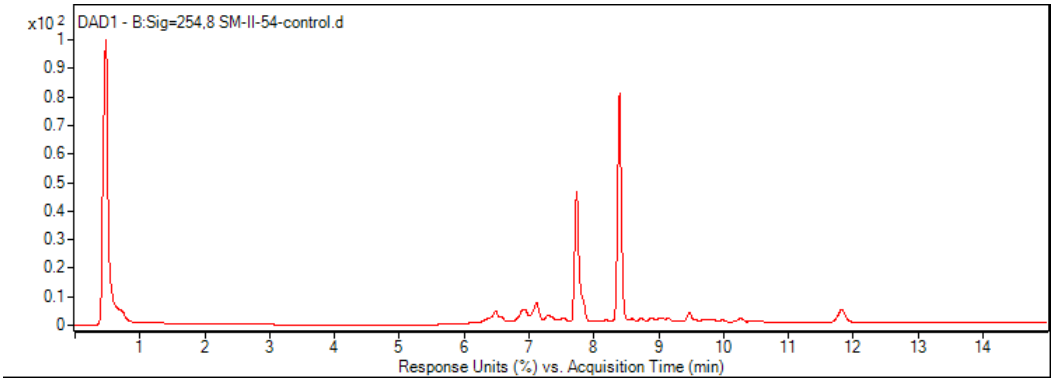
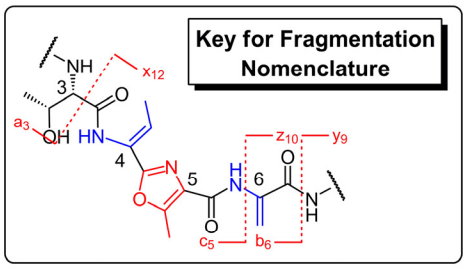


Fig. S14. UV trace (λ = 254 nm) from the *S. lividans*/pSET152 control expression in AF/MS media, showing a lack of any berninamycin-related metabolites.

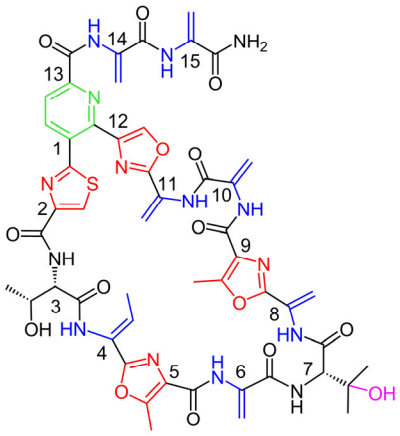
MS/MS fragmentation data were consistent with the structures shown in the main text and in the the *SI Appendix*. A number of the fragments observed for berninamycin A (*SI Appendix*, Table

S4) had been observed previously by Rinehart and coworkers (12). The data are tabulated in *SI Appendix*, Tables S4, S5, S6, S7, S8, S9, S10, S11, S12, and S13 below. The stereochemistry of all compounds (except berninamycins A and B) is assumed based on the parent compound and its prepeptide and has not been proven spectroscopically.

Table S4. MS/MS fragmentation analysis for berninamycin A.[a]

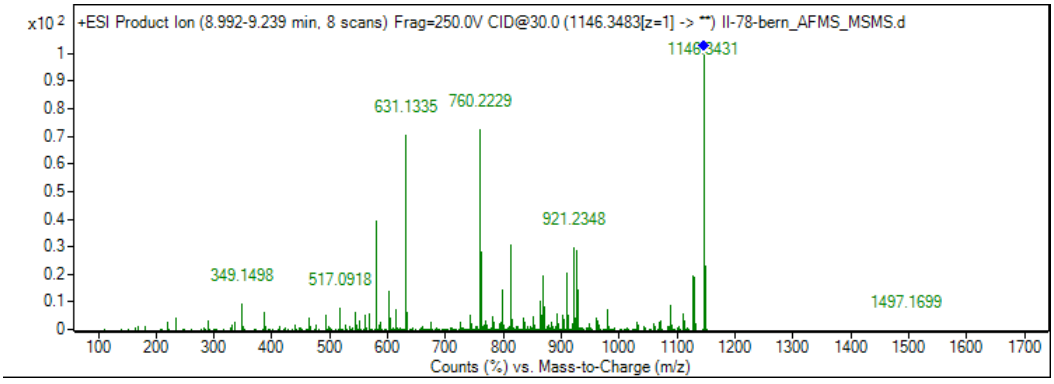


Fragmentation Description[b]	Calc. Mass (m/z)	Found Mass (m/z)	Error (ppm)
$(b_7\text{-}y_8\text{-}b_{10}\text{-}y_5)^+$	927.2839[c]	927.2805[d]	3.67
$(b_5\text{-}y_{10}\text{-}a_8\text{-}x_7 + H)^+$	921.2369	921.2348[d]	2.28
$(b_6\text{-}y_9\text{-}b_{10}\text{-}y_5)^+$	812.2205[c]	812.2202[e]	0.369
$(b_3\text{-}y_{12}\text{-}b_7\text{-}y_8)^+$	798.2049	798.2026[d]	2.88
$(c_5\text{-}z_{10}\text{-}b_{10}\text{-}y_5)^+$	760.2256	760.2229[d]	3.55
$(b_5\text{-}y_{10}\text{-}b_{10}\text{-}y_5)^+$	743.1991[c]	743.1991[d]	0.00
$(b_3\text{-}y_{12}\text{-}c_9\text{-}z_6 - H)^+$	631.1354	631.1335[e]	3.01
$(a_3\text{-}x_{12}\text{-}c_9\text{-}z_6 - H)^+$	603.1405	603.1389[e]	2.65
$(b_3\text{-}y_{12}\text{-}b_{10}\text{-}y_5)^+$	579.1405[c]	579.1388[d]	2.94
$(a_3\text{-}x_{12}\text{-}b_{10}\text{-}y_5)^+$	551.1456	551.1459[e]	0.544
$(b_3\text{-}y_{12}\text{-}b_7\text{-}y_8)^+$	349.1506	349.1498[d]	2.29
$(b_3\text{-}y_{12}\text{-}b_6\text{-}y_9)^+$	234.0873	234.0864[d]	3.84



[a] 1146.3483 m/z targeted and fragmented with a CID energy of either 30 or 45 eV in positive ion mode. [b] Numbering of amino acid residues follows standard thiopeptide convention; all other descriptors follow standard peptide fragmentation nomenclature; see the key for details. [c] Previously observed fragment. [d] CID = 30 eV. [e] CID = 45 eV.

a)



12. Lau RCM, Rinehart KL (1995) Biosynthesis of berninamycin: incorporation of ^{13}C-labeled amino acids. *J Am Chem Soc* 117:7606–7610.

b)

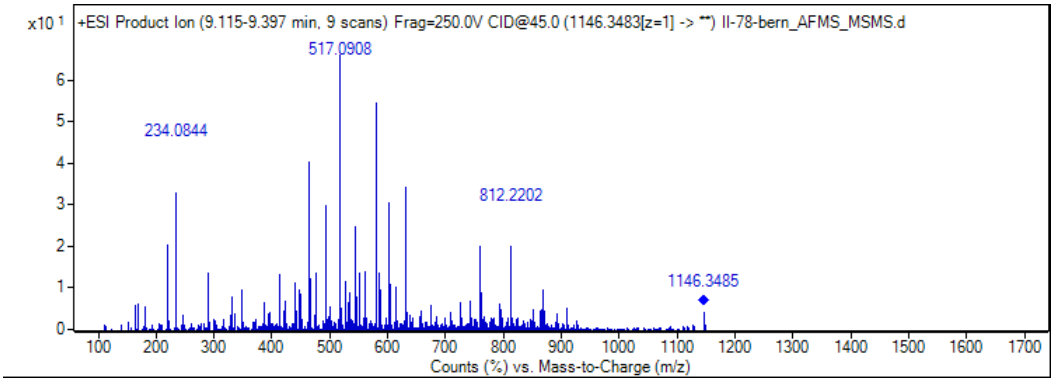
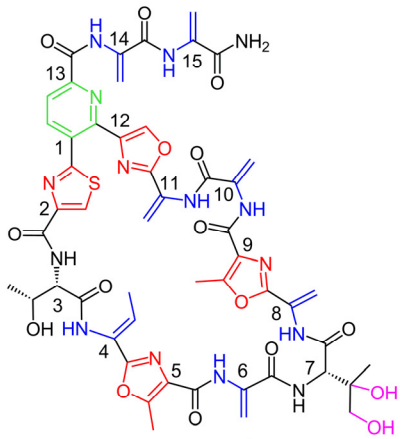


Fig. S15. MS/MS fragmentation spectra of berninamycin A. a) CID = 30 eV. b) CID = 45 eV.

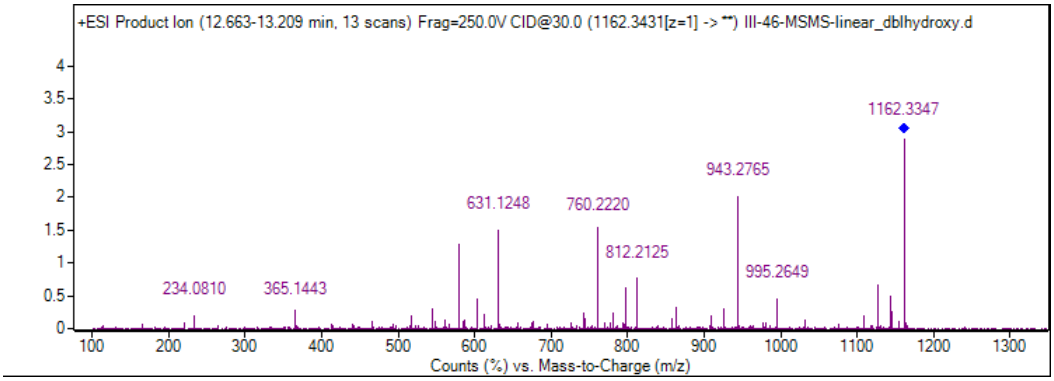
Table S5. MS/MS fragmentation analysis for doubly-hydroxylated berninamycin A.[a]

Fragmentation Description[b]	Calc. Mass (m/z)	Found Mass (m/z)	Error (ppm)
$(b_7\text{-}y_8\text{-}b_{10}\text{-}y_5)^+$	943.2788	943.2765[c]	2.44
$(b_6\text{-}y_9\text{-}b_{10}\text{-}y_5)^+$	812.2205	812.2125[c]	9.85
$(b_3\text{-}y_{12}\text{-}b_7\text{-}y_8)^+$	798.2049	798.1981[c]	8.52
$(c_5\text{-}z_{10}\text{-}b_{10}\text{-}y_5)^+$	760.2256	760.2220[c]	4.74
$(b_5\text{-}y_{10}\text{-}b_{10}\text{-}y_5)^+$	743.1991	743.1936[c]	7.40
$(b_3\text{-}y_{12}\text{-}c_9\text{-}z_6\text{-}H)^+$	631.1354	631.1248[c]	16.8
$(a_3\text{-}x_{12}\text{-}c_9\text{-}z_6\text{-}H)^+$	603.1405	603.1326[d]	13.1
$(b_3\text{-}y_{12}\text{-}b_{10}\text{-}y_5)^+$	579.1405	579.1363[c]	7.25
$(a_3\text{-}x_{12}\text{-}b_{10}\text{-}y_5)^+$	551.1456	551.1376[d]	14.56
$(b_3\text{-}y_{12}\text{-}b_7\text{-}y_8)^+$	365.1456	365.1443[c]	3.56
$(b_3\text{-}y_{12}\text{-}b_6\text{-}y_9)^+$	234.0873	234.0814[d]	25.2



[a] 1162.3432 m/z targeted and fragmented with a CID energy of either 30 or 55 eV in positive ion mode. [b] Numbering of amino acid residues follows standard thiopeptide convention; all other descriptors follow standard peptide fragmentation nomenclature. [c] CID = 30 eV. [d] CID = 55 eV.

a)



b)

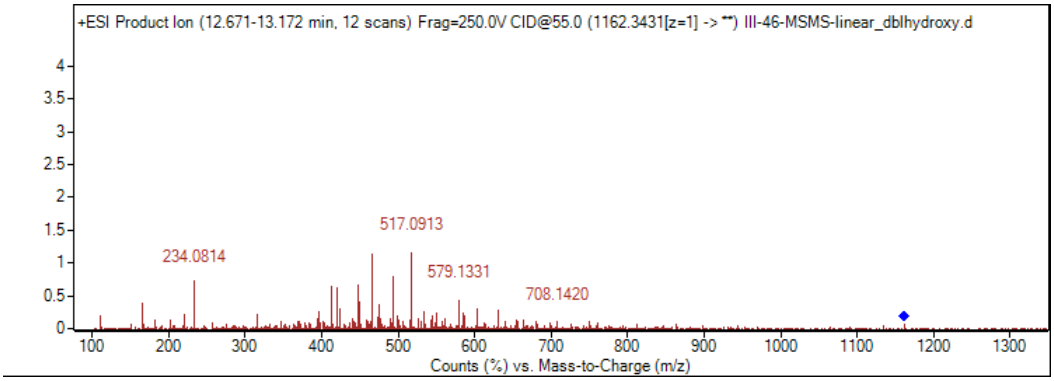
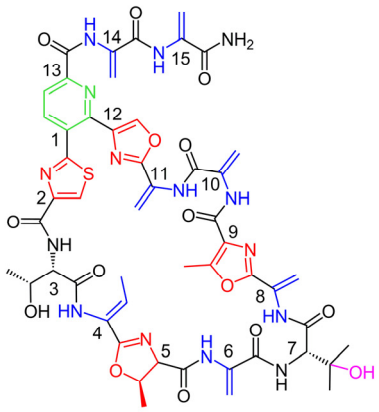


Fig. S16. MS/MS fragmentation spectra of doubly-hydroxylated berninamycin A. a) CID = 30 eV. b) CID = 55 eV.

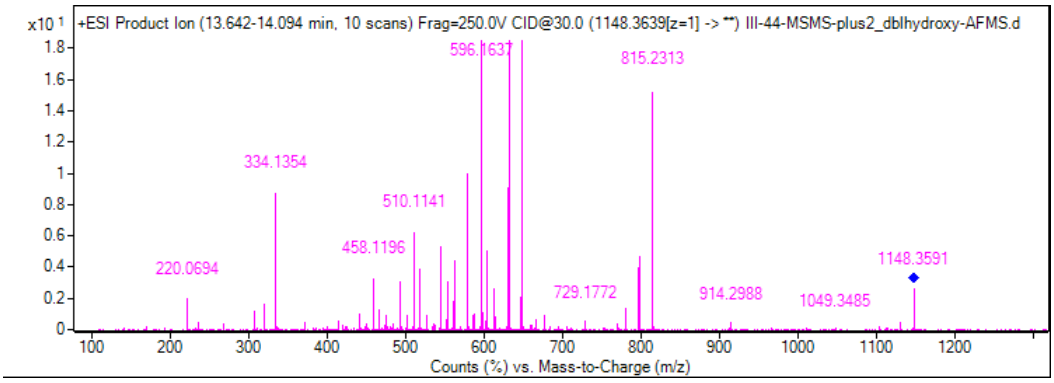
Table S6. MS/MS fragmentation analysis for M+2 berninamycin A.[a]

Fragmentation Description[b]	Calc. Mass (m/z)	Found Mass (m/z)	Error (ppm)
$(c_3\text{-}z_{12}\text{-}b_7\text{-}y_8\text{ -H})^+$	815.2314	815.2313[c]	0.123
$(b_3\text{-}y_{12}\text{-}b_7\text{-}y_8)^+$	798.2049	798.1952[c]	12.2
$(b_3\text{-}y_{12}\text{-}b_9\text{-}y_3)^+$	648.1620	648.1596[c]	3.70
$(b_3\text{-}y_{12}\text{-}c_9\text{-}z_6\text{ -H})^+$	631.1354	631.1322[c]	5.07
$(a_3\text{-}x_{12}\text{-}c_9\text{-}z_6\text{ -H})^+$	603.1405	603.1392[d]	2.16
$(b_3\text{-}y_{12}\text{-}c_9\text{-}z_6\text{ -H})^+$	596.1670	596.1637[c]	5.54
$(c_3\text{-}z_{12}\text{-}b_{10}\text{-}y_5)^+$	551.1456	551.1410[d]	8.35
$(c_3\text{-}z_{12}\text{-}b_7\text{-}y_8\text{ -H})^+$	334.1397	334.1354[c]	12.9

[a] 1148.3639 m/z targeted and fragmented with a CID energy of either 30 or 55 eV in positive ion mode. [b] Numbering of amino acid residues follows standard thiopeptide convention; all other descriptors follow standard peptide fragmentation nomenclature. [c] CID = 30 eV. [d] CID = 55 eV.



a)



b)

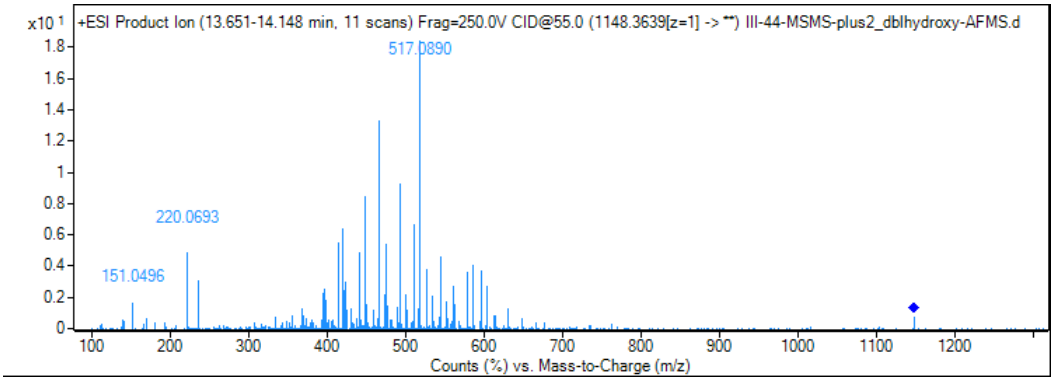
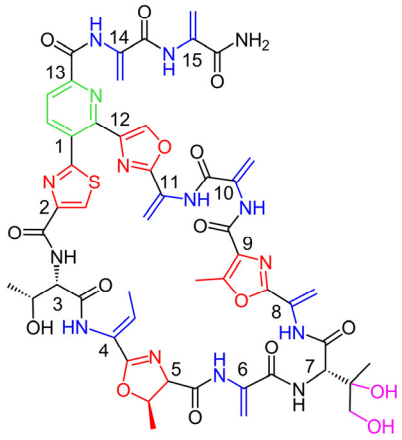


Fig. S17. MS/MS fragmentation spectra of M+2 berninamycin A. a) CID = 30 eV. b) CID = 55 eV.

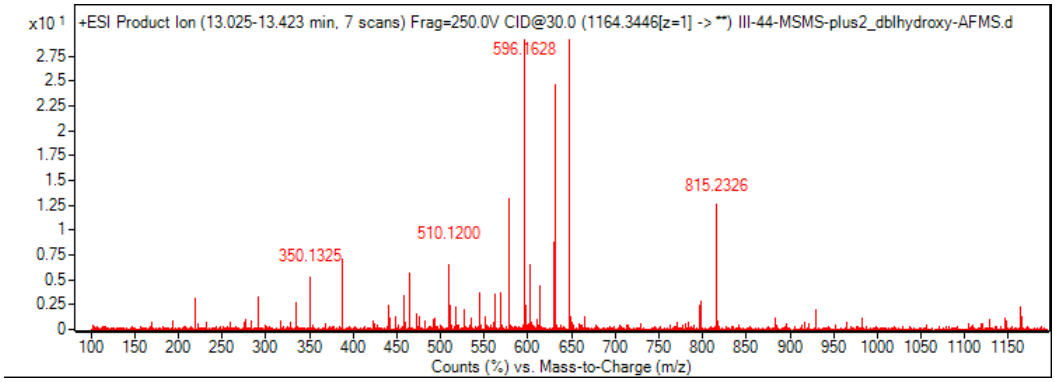
Table S7. MS/MS fragmentation analysis for M+2 doubly-hydroxylated berninamycin A.[a]

Fragmentation Description[b]	Calc. Mass (m/z)	Found Mass (m/z)	Error (ppm)
$(c_3\text{-}z_{12}\text{-}b_7\text{-}y_8\text{ -H})^+$	815.2314	815.2326[c]	1.47
$(b_3\text{-}y_{12}\text{-}b_7\text{-}y_8)^+$	798.2049	798.1959[c]	11.3
$(b_3\text{-}y_{12}\text{-}b_9\text{-}y_3)^+$	648.1620	648.1653[c]	5.09
$(b_3\text{-}y_{12}\text{-}c_9\text{-}z_6\text{ -H})^+$	631.1354	631.1305[c]	7.76
$(a_3\text{-}x_{12}\text{-}c_9\text{-}z_6\text{ -H})^+$	603.1405	603.1273[d]	21.9
$(b_3\text{-}y_{12}\text{-}c_9\text{-}z_6\text{ -H})^+$	596.1670	596.1628[c]	7.05
$(c_3\text{-}z_{12}\text{-}b_{10}\text{-}y_5)^+$	551.1456	551.1383[c]	13.2
$(c_3\text{-}z_{12}\text{-}b_7\text{-}y_8\text{ -H})^+$	350.1347	350.1325[c]	6.28

[a] 1164.3588 m/z targeted and fragmented with a CID energy of either 30 or 55 eV in positive ion mode. [b] Numbering of amino acid residues follows standard thiopeptide convention; all other descriptors follow standard peptide fragmentation nomenclature. [c] CID = 30 eV. [d] CID = 55 eV.



a)



b)

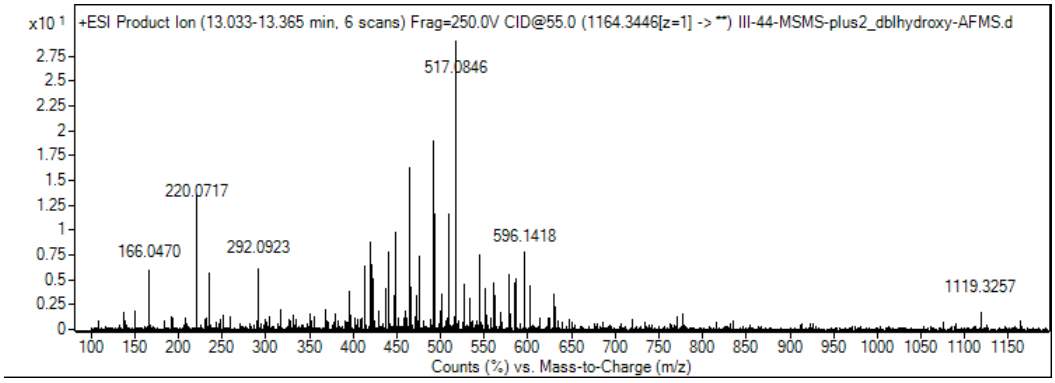
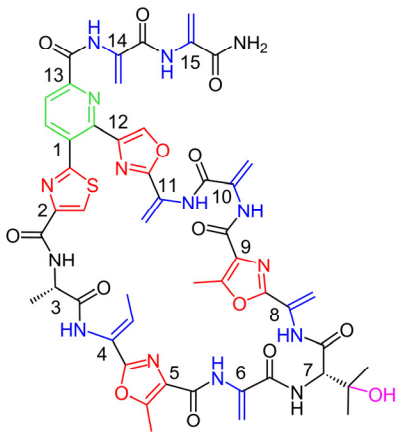


Fig. S18. MS/MS fragmentation spectra of M+2 doubly-hydroxylated berninamycin A. a) CID = 30 eV. b) CID = 55 eV.

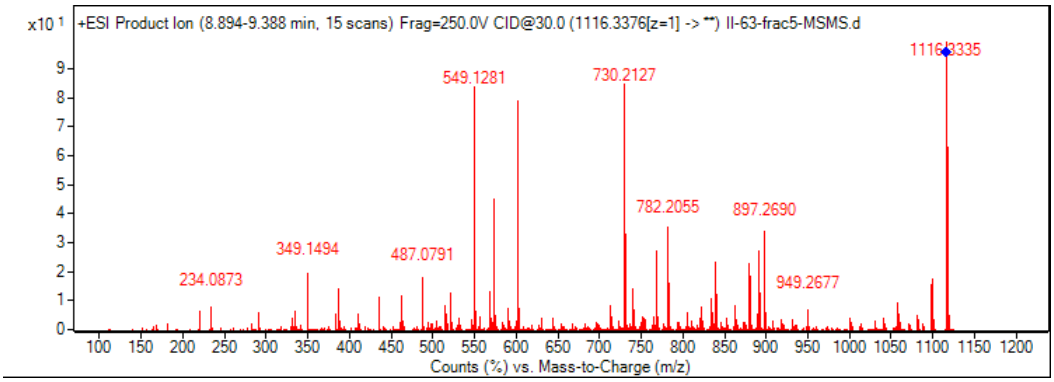
Table S8. MS/MS fragmentation analysis for T3A berninamycin A.[a]

Fragmentation Description[b]	Calc. Mass (m/z)	Found Mass (m/z)	Error (ppm)
$(b_7\text{-}y_8\text{-}b_{10}\text{-}y_5)^+$	897.2733	897.2690[c]	4.79
$(b_5\text{-}y_{10}\text{-}a_8\text{-}x_7 + H)^+$	891.2263	891.2245[c]	2.02
$(b_6\text{-}y_9\text{-}b_{10}\text{-}y_5)^+$	782.2100	782.2055[c]	5.75
$(b_3\text{-}y_{12}\text{-}b_7\text{-}y_8)^+$	768.1943	768.1916[c]	3.51
$(c_5\text{-}z_{10}\text{-}b_{10}\text{-}y_5)^+$	730.2151	730.2127[c]	3.29
$(b_5\text{-}y_{10}\text{-}b_{10}\text{-}y_5)^+$	713.1885	713.1862[c]	3.22
$(b_3\text{-}y_{12}\text{-}c_9\text{-}z_6 - H)^+$	601.1248	601.1236[c]	2.00
$(a_3\text{-}x_{12}\text{-}c_9\text{-}z_6 - H)^+$	573.1299	573.1285[c]	2.44
$(b_3\text{-}y_{12}\text{-}b_{10}\text{-}y_5)^+$	549.1299	549.1292[d]	1.27
$(a_3\text{-}x_{12}\text{-}b_{10}\text{-}y_5)^+$	521.1350	521.1330[d]	3.84
$(b_3\text{-}y_{12}\text{-}b_7\text{-}y_8)^+$	349.1506	349.1494[c]	3.44
$(b_3\text{-}y_{12}\text{-}b_6\text{-}y_9)^+$	234.0873	234.0873[c]	0.00



[a] 1116.3377 m/z targeted and fragmented with a CID energy of either 30 or 45 eV in positive ion mode. [b] Numbering of amino acid residues follows standard thiopeptide convention; all other descriptors follow standard peptide fragmentation nomenclature. [c] CID = 30 eV. [d] CID = 45 eV.

a)



b)

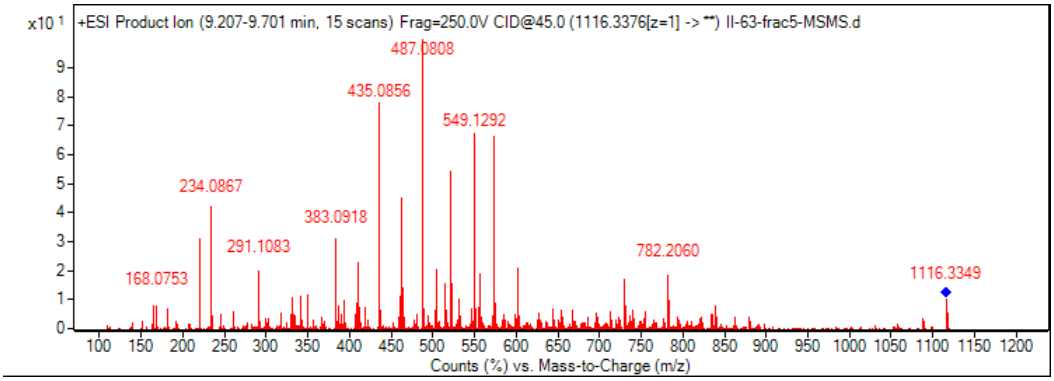
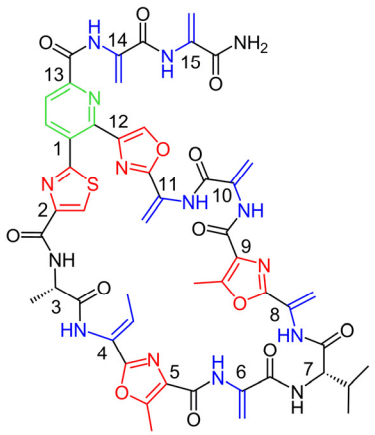


Fig. S19. MS/MS fragmentation spectra of T3A berninamycin A. a) CID = 30 eV. b) CID = 45 eV.

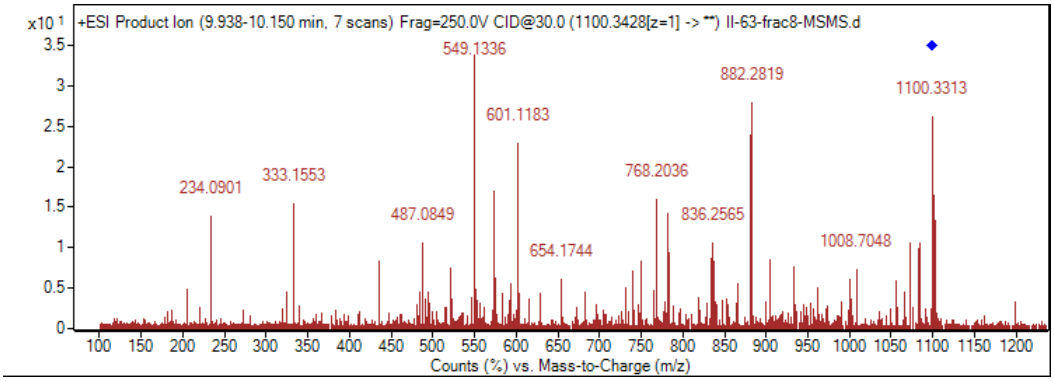
Table S9. MS/MS fragmentation analysis for T3A berninamycin B.[a]

Fragmentation Description[b]	Calc. Mass (m/z)	Found Mass (m/z)	Error (ppm)
$(b_7\text{-}y_8\text{-}b_{10}\text{-}y_5)^+$	881.2784	881.2689[c]	10.8
$(b_6\text{-}y_9\text{-}b_{10}\text{-}y_5)^+$	782.2100	782.2141[c]	5.24
$(b_3\text{-}y_{12}\text{-}b_7\text{-}y_8)^+$	768.1943	768.2036[c]	12.1
$(b_3\text{-}y_{12}\text{-}c_9\text{-}z_6\text{ -H})^+$	601.1248	601.1183[c]	10.8
$(a_3\text{-}x_{12}\text{-}c_9\text{-}z_6\text{ -H})^+$	573.1299	573.1357[c]	10.1
$(b_3\text{-}y_{12}\text{-}b_{10}\text{-}y_5)^+$	549.1299	549.1336[c]	6.74
$(a_3\text{-}x_{12}\text{-}b_{10}\text{-}y_5)^+$	521.1350	521.1382[d]	6.14
$(b_3\text{-}y_{12}\text{-}b_7\text{-}y_8)^+$	333.1557	333.1553[c]	1.20
$(b_3\text{-}y_{12}\text{-}b_6\text{-}y_9)^+$	234.0873	234.0871[d]	0.854

[a] 1100.3428 m/z targeted and fragmented with a CID energy of either 30 or 45 eV in positive ion mode. [b] Numbering of amino acid residues follows standard thiopeptide convention; all other descriptors follow standard peptide fragmentation nomenclature. [c] CID = 30 eV. [d] CID = 45 eV.



a)



b)

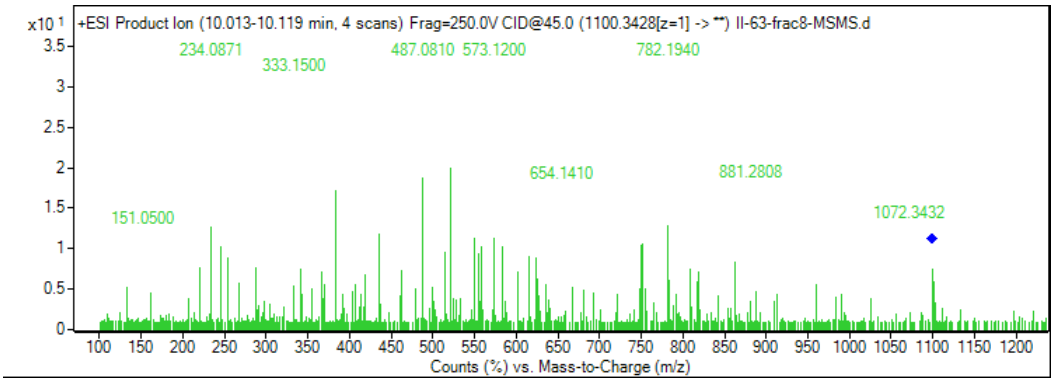
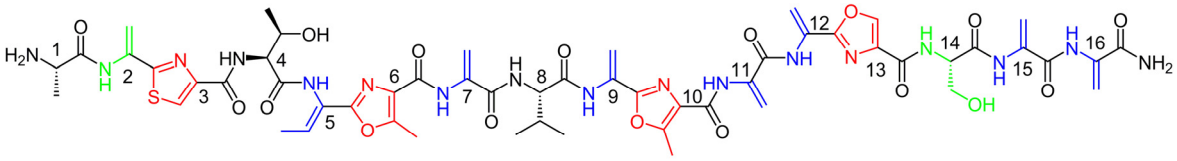


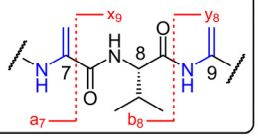
Fig. S20. MS/MS fragmentation spectra of T3A berninamycin B. a) CID = 30 eV. b) CID = 45 eV.

Table S10. MS/MS fragmentation analysis for the linear wt–Ser_{13} metabolite.[a]



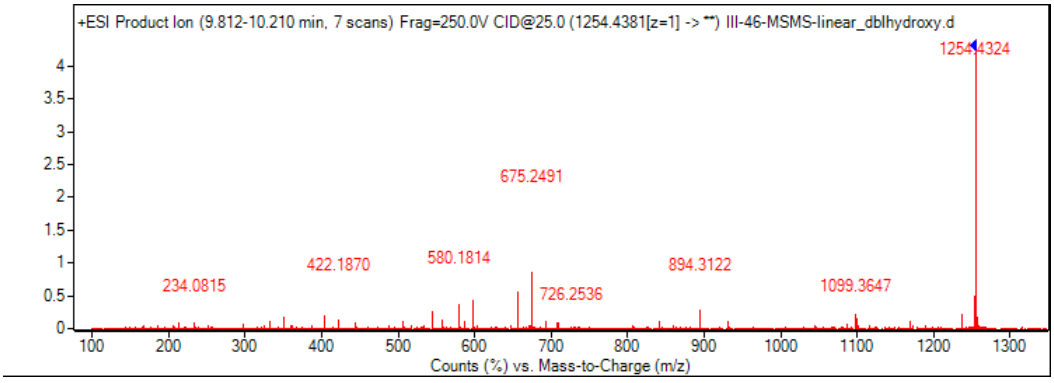
Fragmentation Description[b]	Calc. Mass (m/z)	Found Mass (m/z)	Error (ppm)
$(b_{15}\text{-}y_1)^+$	1168.3901	1168.3604[c]	25.4
$(b_{14}\text{-}y_2)^+$	1099.3687	1099.3647[c]	3.64
$(b_4\text{-}y_{12})^+$	930.3489	930.3391[c]	10.5
$(b_{11}\text{-}y_5)^+$	876.3093	876.3457[d]	41.5
$(b_8\text{-}y_8)^+$	657.2450	657.2423[c]	4.11
$(a_8\text{-}x_8)^+$	629.2500	629.2445[c]	8.74
$(b_8\text{-}y_8)^+$	598.2004	598.1898[c]	17.7
$(b_7\text{-}y_9)^+$	558.1765	558.1727[c]	6.81
$(b_4\text{-}y_{12}\text{-}b_{11}\text{-}y_5)^+$	552.2201	552.2147[c]	9.78
$(a_2\text{-}x_{14}\text{-}b_8\text{-}y_8 + H)^+$	545.1813	545.1783[d]	5.50
$(a_7\text{-}x_9)^+$	530.1816	530.1782[d]	6.41
$(b_1\text{-}y_{15}\text{-}b_7\text{-}y_9)^+$	487.1394	487.1407[c]	2.67
$(a_2\text{-}x_{14}\text{-}b_7\text{-}y_9 + H)^+$	446.1129	446.1031[d]	22.0
$(b_4\text{-}y_{12}\text{-}b_8\text{-}y_8)^+$	333.1557	333.1509[d]	14.4
$(b_4\text{-}y_{12})^+$	325.0965	325.0894[d]	21.8
$(b_{10}\text{-}y_6\text{-}b_{13}\text{-}y_3)^+$	206.0560	206.0827[d]	130

Key for Fragmentation Nomenclature



[a] 1254.4381 m/z targeted and fragmented with a CID energy of either 25 or 45 eV in positive ion mode. [b] For the purposes of this analysis, the 16 aa peptide was labeled consecutively from N-terminus to C-terminus with Ala_{-1} labeled as residue 1 and Dha_{15} labeled as residue 16. All other descriptors follow standard peptide fragmentation nomenclature; see the key for details. [c] CID = 25 eV. [d] CID = 45 eV.

a)



b)

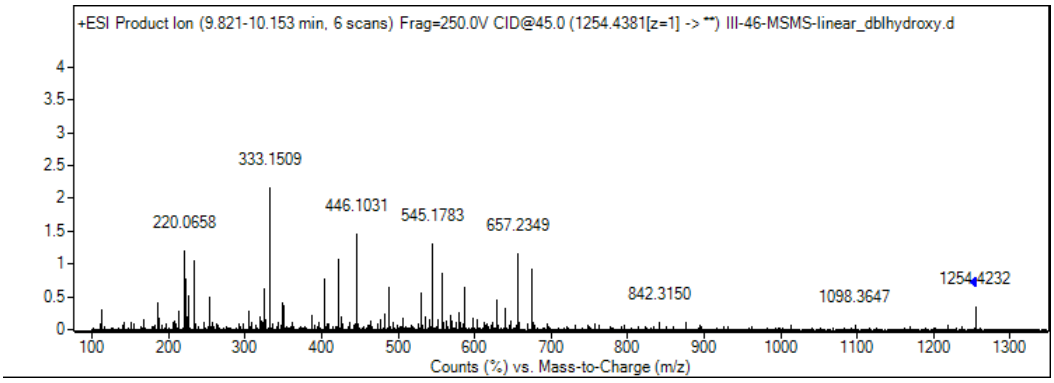
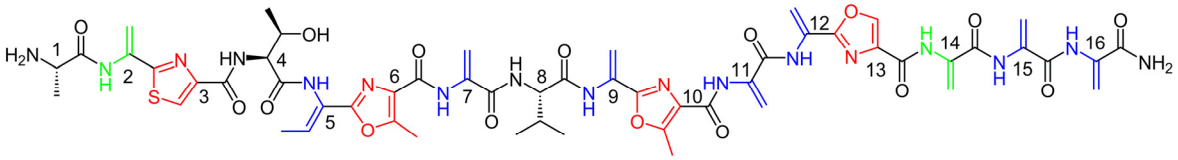


Fig. S21. MS/MS fragmentation spectra of linear wt-Ser_{13}. a) CID = 25 eV. b) CID = 45 eV.

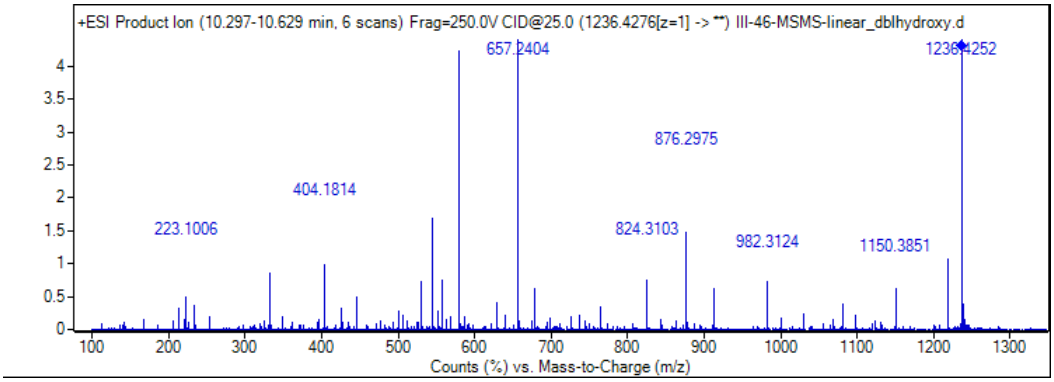
Table S11. MS/MS fragmentation analysis for the linear wt–Dha_{13} metabolite.[a]



Fragmentation Description[b]	Calc. Mass (m/z)	Found Mass (m/z)	Error (ppm)
$(b_{15}\text{-}y_1)^+$	1150.3769	1150.3851[c]	7.13
$(b_{14}\text{-}y_2)^+$	1081.3581	1081.3592[c]	1.02
$(b_4\text{-}y_{12})^+$	912.3383	912.3265[c]	12.9
$(b_{11}\text{-}y_5)^+$	876.3093	876.3102[d]	1.03
$(b_8\text{-}y_8)^+$	657.2450	657.2404[c]	7.00
$(a_8\text{-}x_8)^+$	629.2500	629.2471[d]	4.61
$(b_8\text{-}y_8)^+$	580.1899	580.1813[c]	14.8
$(b_7\text{-}y_9)^+$	558.1765	558.1695[d]	12.5
$(b_4\text{-}y_{12}\text{-}b_{11}\text{-}y_5)^+$	552.2201	552.2147[c]	9.78
$(a_2\text{-}x_{14}\text{-}b_8\text{-}y_8 + H)^+$	545.1813	545.1722[d]	16.7
$(a_7\text{-}x_9)^+$	530.1816	530.1759[c]	10.8
$(b_1\text{-}y_{15}\text{-}b_7y_9)^+$	487.1394	487.1328[d]	13.5
$(a_2\text{-}x_{14}\text{-}b_7\text{-}y_9 + H)^+$	446.1129	446.1029[c]	22.4
$(b_4\text{-}y_{12}\text{-}b_8\text{-}y_8)^+$	333.1557	333.1516[c]	12.3
$(b_4\text{-}y_{12})^+$	325.0965	325.0887[d]	24.0
$(b_{10}\text{-}y_6\text{-}b_{13}\text{-}y_3)^+$	206.0560	206.0511[d]	23.8

[a] 1236.4276 m/z targeted and fragmented with a CID energy of either 25 or 45 eV in positive ion mode. [b] For the purposes of this analysis, the 16 aa peptide was labeled consecutively from N-terminus to C-terminus with Ala_1 labeled as residue 1 and Dha_{15} labeled as residue 16. All other descriptors follow standard peptide fragmentation nomenclature. [c] CID = 25 eV. [d] CID = 45 eV.

a)



b)

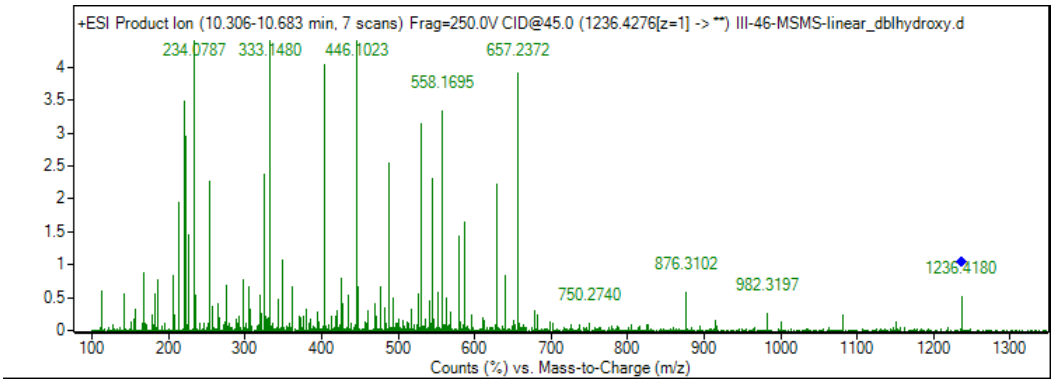
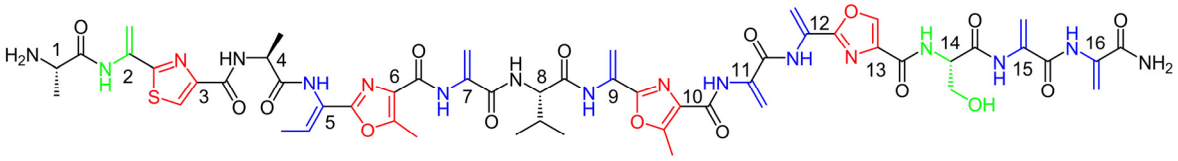


Fig. S22. MS/MS fragmentation spectra of linear wt-Dha$_{13}$. a) CID = 25 eV. b) CID = 45 eV.

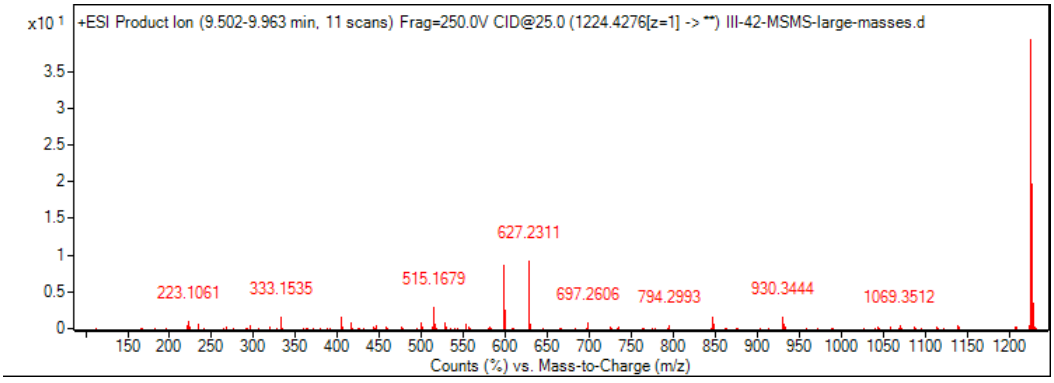
Table S12. MS/MS fragmentation analysis for the linear T3A–Ser_{13} metabolite.[a]



Fragmentation Description[b]	Calc. Mass (m/z)	Found Mass (m/z)	Error (ppm)
$(b_{15}\text{-}y_1)^+$	1138.3796	1138.3707[c]	7.81
$(b_{14}\text{-}y_2)^+$	1069.3581	1069.3512[c]	6.45
$(b_4\text{-}y_{12})^+$	930.3489	930.3444[c]	4.84
$(b_{11}\text{-}y_5)^+$	846.2988	846.2918[c]	8.27
$(b_8\text{-}y_8)^+$	627.2344	627.2311[c]	5.26
$(a_8\text{-}x_8)^+$	599.2395	599.2316[d]	13.2
$(b_8\text{-}y_8)^+$	598.2004	598.1984[c]	3.34
$(b_7\text{-}y_9)^+$	552.2201	552.2145[c]	10.1
$(b_4\text{-}y_{12}\text{-}b_{11}\text{-}y_5)^+$	528.1660	528.1656[d]	0.757
$(a_2\text{-}x_{14}\text{-}b_8\text{-}y_8 + H)^+$	515.1707	515.1679[c]	5.44
$(a_7\text{-}x_9)^+$	500.1711	500.1712[d]	0.200
$(b_1\text{-}y_{15}\text{-}b_7y_9)^+$	457.1289	457.1281[d]	1.75
$(a_2\text{-}x_{14}\text{-}b_7\text{-}y_9 + H)^+$	416.1023	416.1008[d]	3.60
$(b_4\text{-}y_{12}\text{-}b_8\text{-}y_8)^+$	333.1557	333.1539[d]	5.40
$(b_4\text{-}y_{12})^+$	295.0859	295.0848[d]	3.73
$(b_{10}\text{-}y_6\text{-}b_{13}\text{-}y_3)^+$	206.0560	206.0549[d]	5.34

[a] 1224.4276 m/z targeted and fragmented with a CID energy of either 25 or 50 eV in positive ion mode. [b] For the purposes of this analysis, the 16 aa peptide was labeled consecutively from N-terminus to C-terminus with Ala_{-1} labeled as residue 1 and Dha_{15} labeled as residue 16. All other descriptors follow standard peptide fragmentation nomenclature. [c] CID = 25 eV. [d] CID = 50 eV.

a)



b)

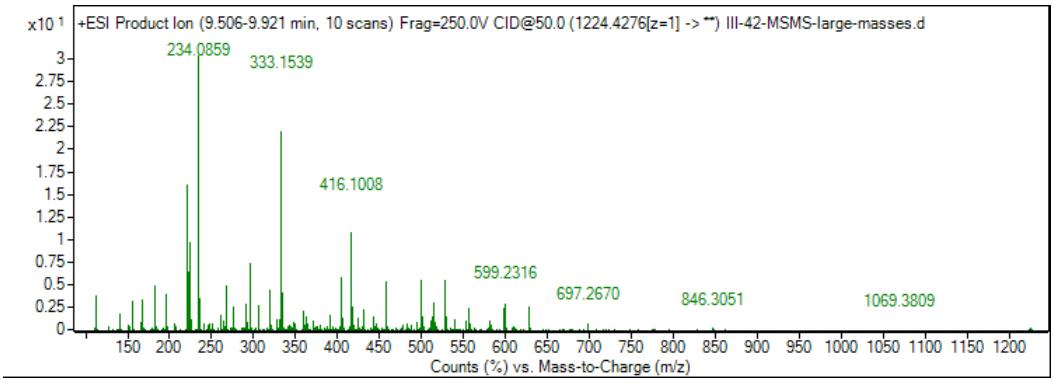
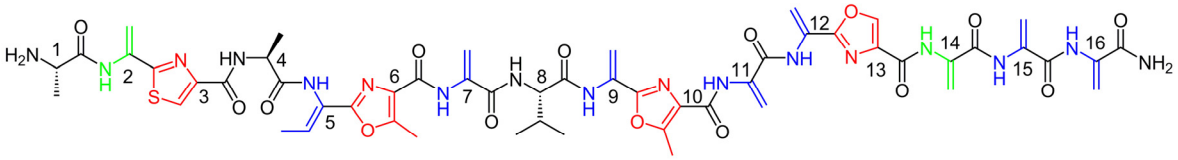


Fig. S23. MS/MS fragmentation spectra of linear T3A-Ser$_{13}$. a) CID = 25 eV. b) CID = 50 eV.

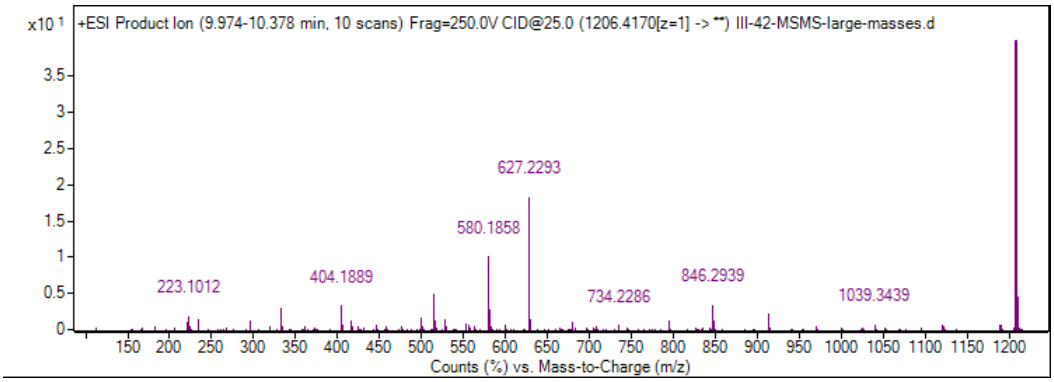
Table S13. MS/MS fragmentation analysis for the linear T3A–Dha$_{13}$ metabolite.[a]



Fragmentation Description[b]	Calc. Mass (m/z)	Found Mass (m/z)	Error (ppm)
$(b_{15}\text{-}y_1)^+$	1120.3690	1120.3832[c]	12.7
$(b_{14}\text{-}y_2)^+$	1051.3475	1051.3396[c]	7.51
$(b_4\text{-}y_{12})^+$	912.3383	912.3333[c]	5.48
$(b_{11}\text{-}y_5)^+$	846.2988	846.2939[c]	5.79
$(b_8\text{-}y_8)^+$	627.2344	627.2293[c]	8.13
$(a_8\text{-}x_8)^+$	599.2316	599.2313[c]	0.501
$(b_8\text{-}y_8)^+$	580.1899	580.1858[c]	7.07
$(b_7\text{-}y_9)^+$	552.2201	552.2124[c]	13.9
$(b_4\text{-}y_{12}\text{-}b_{11}\text{-}y_5)^+$	528.1660	528.1634[d]	4.929
$(a_2\text{-}x_{14}\text{-}b_8\text{-}y_8 + H)^+$	515.1707	515.1657[c]	9.71
$(a_7\text{-}x_9)^+$	500.1711	500.1665[d]	9.20
$(b_1\text{-}y_{15}\text{-}b_7y_9)^+$	457.1289	457.1255[d]	7.44
$(a_2\text{-}x_{14}\text{-}b_7\text{-}y_9 + H)^+$	416.1023	416.0976[d]	11.3
$(b_4\text{-}y_{12}\text{-}b_8\text{-}y_8)^+$	333.1557	333.1510[d]	14.1
$(b_4\text{-}y_{12})^+$	295.0859	295.0804[d]	18.6
$(b_{10}\text{-}y_6\text{-}b_{13}\text{-}y_3)^+$	206.0560	206.0544[c]	7.76

[a] 1206.4170 m/z targeted and fragmented with a CID energy of either 25 or 50 eV in positive ion mode. [b] For the purposes of this analysis, the 16 aa peptide was labeled consecutively from N-terminus to C-terminus with Ala$_1$ labeled as residue 1 and Dha$_{15}$ labeled as residue 16. All other descriptors follow standard peptide fragmentation nomenclature. [c] CID = 25 eV. [d] CID = 50 eV.

a)



b)

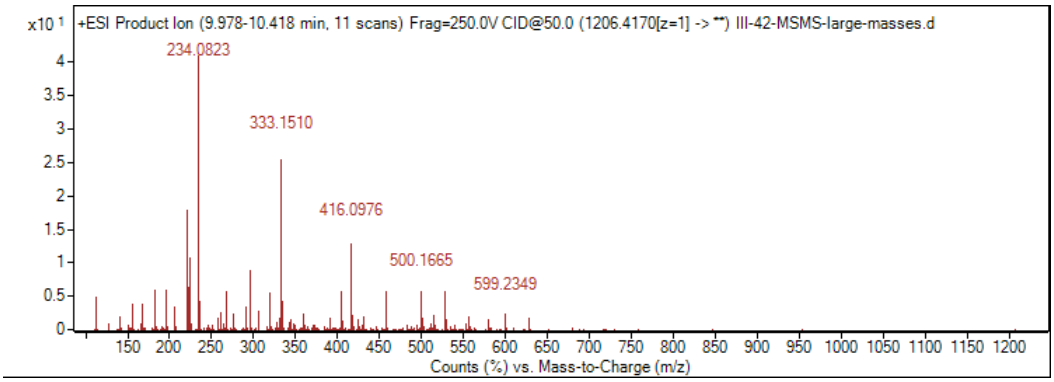


Fig. S24. MS/MS fragmentation spectra of linear T3A-Dha$_{13}$. a) CID = 25 eV. b) CID = 50 eV.

For the M+2 berninamycin A analog, MS/MS fragmentation data restricted the site that differed from berninamycin A to the fifth amino acid residue; the 2 Da increase in mass indicated that the residue had been dehydrated but not oxidized. This change compared to the wild type compound could either arise from conversion of Thr$_5$ to a methyloxazoline, but incomplete processing to the methyloxazole, or from the transformation of Thr$_5$ to a Dhb moiety.

To distinguish between these two possibilities, berninamycin A and its M+2 analog were separately subjected to nickel boride reduction by adapting a known procedure (13): in a 4-mL glass vial, a solution of the thiopeptide in DMSO (20 µL, ca. 231 µg) was added to a suspension of nickel (II) chloride hexahydrate (3.0 mg) in 50:50 MeOH:H_2O (250 µL). An excess amount of sodium borohydride was added (ca. 15.0 mg) and the vial was loosely capped; the mixture became black immediately and H_2 gas rapidly evolved. After the reaction stood for 1 h, the contents were transferred to an Eppendorf tube and centrifuged to pellet the insoluble material.

13. Martin NI *et al.* (2004) Structural characterization of lacticin 3147, a two-peptide lantibiotic with synergistic activity. *Biochemistry* 43:3049–3056.

A portion of the supernatant (100 μL) was added to 50:50 MeCN:H_2O (200 μL) and filtered through a 0.45 μM filter for High Resolution LC-MS analysis (10 μL of this solution injected).

For berninamycin A, all seven alkenes within the natural product were hydrogenated; the thiazole was also completely reduced and desulfurized (see *SI Appendix*, Fig. S24), delivering a macrocycle with an 1134.5327 Da mass (M+H, calculated). For the M+2 analog, the same sequence of events occurred to furnish an 1136.5483 Da compound (M+H, calculated); see *SI Appendix*, Table S14 for analysis. This confirms that M+2 berninamycin A contains a methyloxazoline at residue 5. If there had been a Dhb at position 5, an 1138.5640 Da compound would have been obtained from these reductive conditions.

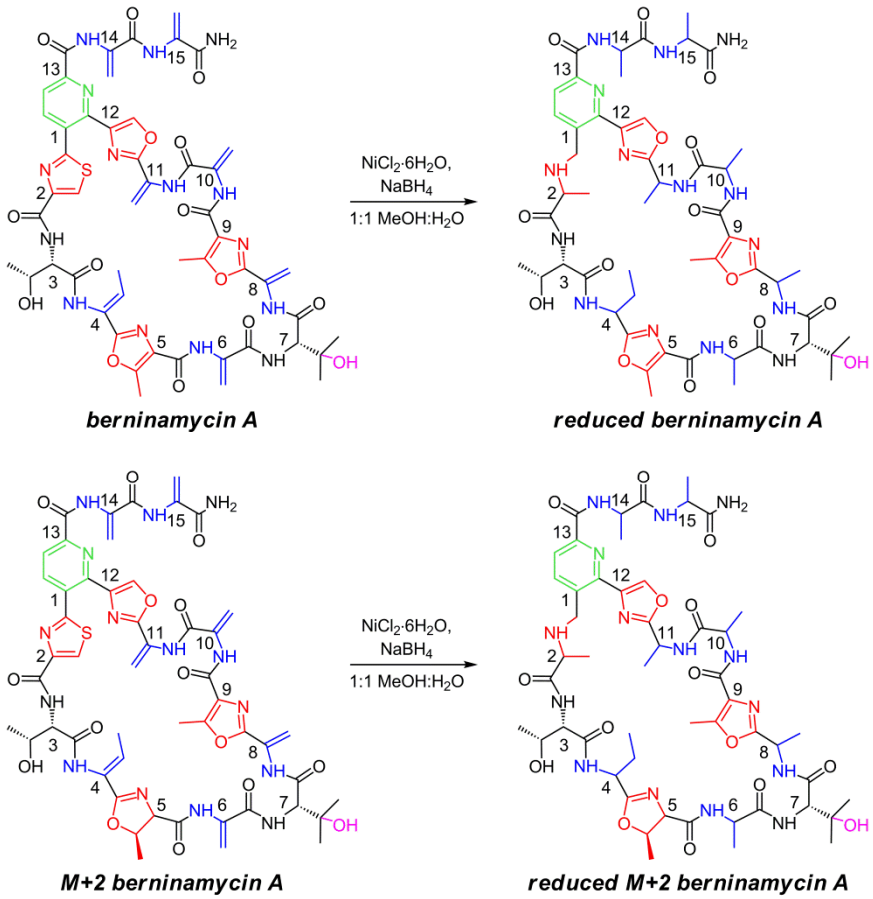
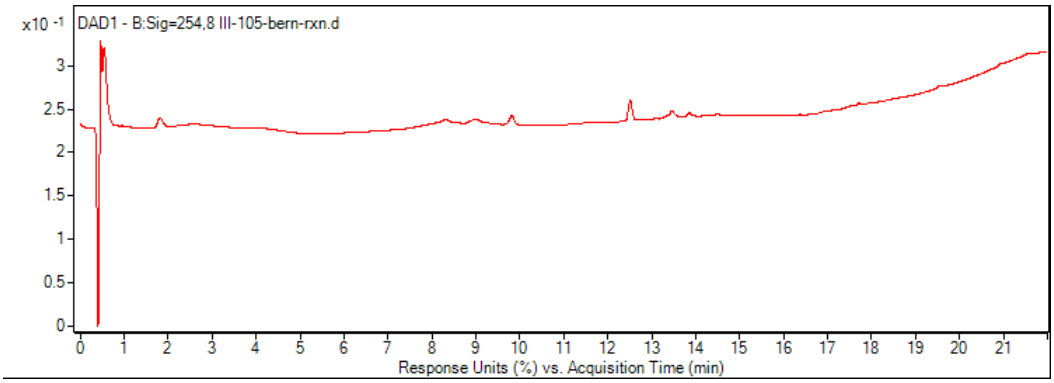
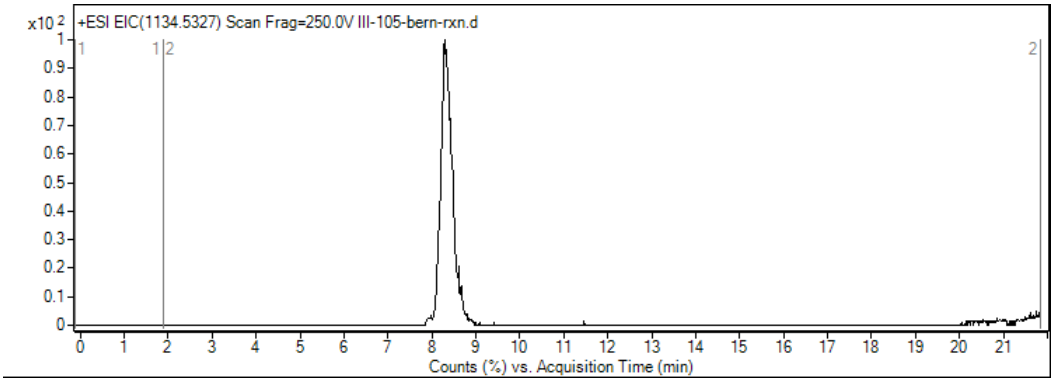


Fig. S25. Ni-catalyzed reduction of berninamycin A and M+2 berninamycin A to determine the identity of the functional group at position 5 in the M+2 compound.

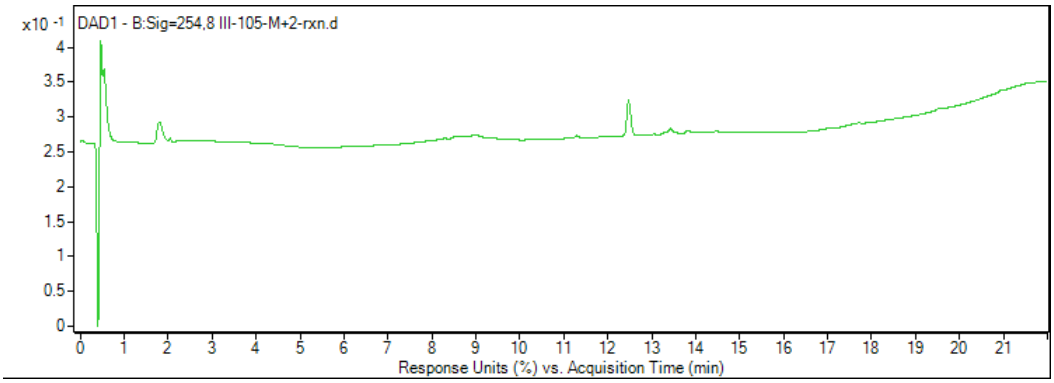
a)


x10 -1
DAD1 - B:Sig=254,8 III-105-bern-rxn.d
Response Units (%) vs. Acquisition Time (min)

b)


x10 2
+ESI EIC(1134.5327) Scan Frag=250.0V III-105-bern-rxn.d
Counts (%) vs. Acquisition Time (min)

c)


x10 -1
DAD1 - B:Sig=254,8 III-105-M+2-rxn.d
Response Units (%) vs. Acquisition Time (min)

d)

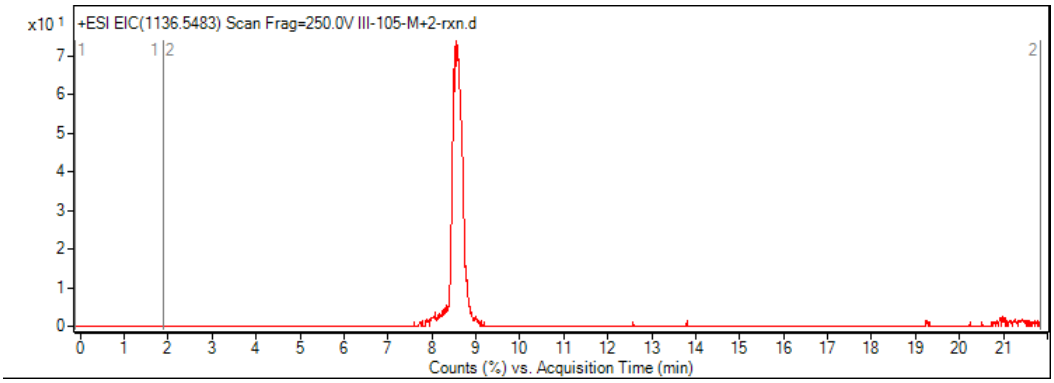

x10 1
+ESI EIC(1136.5483) Scan Frag=250.0V III-105-M+2-rxn.d
Counts (%) vs. Acquisition Time (min)

Fig. S26. LC-MS traces for the Ni-catalyzed reductions of berninamycin A and M+2 berninamycin A. a) UV trace for reduced berninamycin A (λ = 254 nm). b) Extracted ion chromatogram for reduced berninamycin A. c) UV trace for reduced M+2 berninamycin A (λ = 254 nm). d) Extracted ion chromatogram for reduced M+2 berninamycin A.

Table S14. LC-MS analysis of the products of Ni-catalyzed reductions of berninamycin A and M+2 berninamycin A.[a]

Compound Identification	RetentionTime (min)	Calc. Mass (m/z)[b]	Found Mass (m/z)[b]	Error (ppm)
reduced berninamycin A	8.22	1134.5327	1134.5426	8.7
reduced M+2 berninamycin A	8.51	1136.5483	1136.5501	1.6

[a] Analyzed by detection at λ = 254 nm and by the total ion chromatogram and specific mass ion extracted chromatograms. Elution method A used. [b] Mass of the protonated compound.

16. Large scale fermentations of *S. bernensis*, *S. venezuelae*/pSET152+bern (wt), and *S. lividans*/pSET152+bern (T3A) and isolation of major metabolites: For *S. bernensis* expression, 50 mL Seed media (no antibiotic) in a 250-mL baffled Erlenmeyer flask, containing approximately 20 glass beads, was inoculated with spores of *S. bernensis* (20 μL of the -80 °C 25% glycerol stock prepared in section 7). Seven more identical inoculations were carried out. All cultures were incubated at 30 °C for four days with 250 rpm shaking.

Four fermenters, each containing 5-L of AF/MS, were each inoculated with 500 mL of starter culture. The cultures were maintained at pH = 7 with mixing at 500 rpm for the first 68 h and then 950 rpm for the last 96 h. Oxygen was kept in excess by infusion of air (or O_2 when necessary). Cells were harvested by centrifugation at 6600g and the supernatant decanted; 338 g of cell mass and insoluble portions of the media was obtained as 24 separate pellets, which were frozen at –80 °C.

The 24 approximately equal fractions (ca. 14.1 g each) were placed in 500-mL plastic centrifuge bottles and broken up with a spatula. Approximately 50 glass beads, a copious amount of anhydrous Na_2SO_4, and 200 mL of acetone were added to each bottle, and the mixture was shaken and vortexed vigorously for 3.5 h. At that time, the mixture was vacuum-filtered through Whatman-1 paper and the volatiles removed by rotary evaporation. To what remained was added 125 mL saturated aqueous NaCl and 150 mL EtOAc; the mixture was shaken and the layers separated. The water layer was washed with additional EtOAc (2 X 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4, vacuum-filtered through Whatman-1 paper, and the volatiles removed by rotary evaporation to deliver a viscous brown oil.

The oil was dissolved in 20 mL 50:50 H_2O:MeCN (many solids did not dissolve) and filtered by syringe through a 0.45 μM filter, which was subsequently rinsed with 6 mL 50:50 H_2O:MeCN. 2 mL of the sample was then purified by preparative HPLC: 15 mL/min elution; 75:25 H_2O:MeCN isocratic for 2 min; then increase to 40:60 H_2O:MeCN over 3 min, followed by isocratic elution for 2 min; then increase to 15:85 H_2O:MeCN over 15 min, followed by isocratic elution for 2 min. Each solvent contained 0.1% formic acid. The peak corresponding to berninamycin A was collected by hand. The remainder of the sample was purified in six equal portions of 4 mL each. The combined fractions of berninamycin A were concentrated *in vacuo*

to remove the volatiles and the water was lyophilized. The white powder was then redissolved in 3 mL 50:50 H_2O:MeCN and again purified by preparative HPLC (1-mL batch and then a 2-mL batch): 15 mL/min elution; 75:25 H_2O:MeCN isocratic for 2 min; then increase to 55:45 H_2O:MeCN over 8 min, followed by isocratic elution for 3 min; then increase to 15:85 H_2O:MeCN over 7 min, followed by isocratic elution for 3 min. Each solvent contained 0.1% formic acid. The peak corresponding to berninamycin A was once again collected by hand. The combined fractions of berninamycin A were concentrated *in vacuo* to remove the volatiles and the water was lyophilized. Berninamycin A (130 mg) was collected as a white powder.

For *S. venezuelae*/pSET152+bern (wt) expression, 50 mL Seed media (with apramycin) in a 250-mL baffled Erlenmeyer flask, containing approximately 20 glass beads, was inoculated with spores of *S. venezuelae*/pSET152+bern (wt) taken directly from an agar plate. Seven more identical inoculations were carried out. All cultures were incubated at 30 °C for three days with 250 rpm shaking.

Four fermenters, each containing 5-L of GYM (with apramycin), were each inoculated with 500 mL of starter culture. The cultures were maintained at pH = 7 with mixing at 500 rpm for the first 68 h and then 950 rpm for the last 96 h. Oxygen was kept in excess by infusion of air (or O_2 when necessary). Cells were harvested by centrifugation at 6600g and the supernatant decanted; 136 g of cell mass was obtained as 24 separate pellets, which were frozen at –80 °C.

The 24 approximately equal fractions (ca. 5.7 g each) were placed in 500-mL plastic centrifuge bottles and broken up with a spatula. Extractions of the cell pellets were performed as was done for the *S. bernensis* fermentation to deliver an orange-brown semi-solid.

The oil was dissolved in 5 mL 50:50 H_2O:MeCN (many solids did not dissolve) and filtered by syringe through a 0.45 μM filter, which was subsequently rinsed with 5 mL 50:50 H_2O:MeCN. 2 mL of the sample was then purified by preparative HPLC: 15 mL/min elution; 75:25 H_2O:MeCN isocratic for 2 min; then increase to 40:60 H_2O:MeCN over 3 min, followed by isocratic elution for 2 min; then increase to 15:85 H_2O:MeCN over 15 min, followed by isocratic elution for 2 min. Each solvent contained 0.1% formic acid. The peak corresponding to M+2 berninamycin A was collected by hand as was the doubly-hydroxylated version of M+2 berninamycin A. The remainder of the sample was purified in four equal portions of 2 mL each. The combined fractions of M+2 berninamycin A were concentrated *in vacuo* to remove the volatiles and the water was lyophilized; the same was done for the doubly-hydroxylated version of M+2 berninamycin A. The brown powder for M+2 berninamycin A was then redissolved in 3 mL 50:50 H_2O:MeCN and again purified by preparative HPLC (1-mL batch and then a 2-mL batch): 15 mL/min elution; 75:25 H_2O:MeCN isocratic for 2 min; then increase to 55:45 H_2O:MeCN over 8 min, followed by isocratic elution for 3 min; then increase to 15:85 H_2O:MeCN over 7 min, followed by isocratic elution for 3 min. Each solvent contained 0.1% formic acid. The same was done for the doubly-hydroxylated version of M+2 berninamycin A. The peak corresponding to M+2 berninamycin A was once again collected by hand as was the peak for the doubly-hydroxylated version of M+2 berninamycin A. The combined fractions of

M+2 berninamycin A were concentrated *in vacuo* to remove the volatiles and the water was lyophilized. M+2 berninamycin A was collected as a light brown powder. Similarly, doubly-hydroxylated M+2 berninamycin A was collected as a light brown powder.

For *S. lividans*/pSET152+bern (T3A) expression, 50 mL Seed media (with apramycin) in a 250-mL baffled Erlenmeyer flask, containing approximately 20 glass beads, was inoculated with spores of *S. lividans*/pSET152+bern (T3A) taken directly from an agar plate. One additional identical inoculation was carried out. The cultures were incubated at 30 °C for three days with 250 rpm shaking.

A fermenter, containing 5-L of GYM (with apramycin), was inoculated with 50 mL of starter culture. The cultures were maintained at pH = 7 with mixing at 500 rpm for the first 68 h and then 950 rpm for the last 96 h. Oxygen was kept in excess by infusion of air (or O_2 when necessary). Cells were harvested by centrifugation at 6600g and the supernatant decanted; the six approximately equally-sized pellets were not massed and were frozen at –80 °C.

The six pellets were transferred to 500-mL plastic centrifuge bottles and broken up with a spatula. Extractions of the cell pellets were performed as was done for the *S. bernensis* fermentation to deliver a viscous brown oil.

The oil was dissolved in 5 mL 50:50 H_2O:MeCN (many solids did not dissolve) and filtered by syringe through a 0.45 μM filter, which was subsequently rinsed with 5 mL 50:50 H_2O:MeCN. 2 mL of the sample was then purified by preparative HPLC: 15 mL/min elution; 75:25 H_2O:MeCN isocratic for 2 min; then increase to 40:60 H_2O:MeCN over 3 min, followed by isocratic elution for 2 min; then increase to 15:85 H_2O:MeCN over 15 min, followed by isocratic elution for 2 min. Each solvent contained 0.1% formic acid. The peak corresponding to T3A berninamycin A was collected by hand. The remainder of the sample was purified in four equal portions of 2 mL each. The combined fractions of T3A berninamycin A were concentrated *in vacuo* to remove the volatiles and the water was lyophilized. The white powder was then redissolved in 3 mL 50:50 H_2O:MeCN and again purified by preparative HPLC (1-mL batch and then a 2-mL batch): 15 mL/min elution; 75:25 H_2O:MeCN isocratic for 2 min; then increase to 55:45 H_2O:MeCN over 8 min, followed by isocratic elution for 3 min; then increase to 15:85 H_2O:MeCN over 7 min, followed by isocratic elution for 3 min. Each solvent contained 0.1% formic acid. The peak corresponding to T3A berninamycin A was once again collected by hand. The combined fractions of T3A berninamycin A were concentrated *in vacuo* to remove the volatiles and the water was lyophilized. T3A berninamycin A was collected as a white powder.

17. Minimum inhibitory concentration assays: Minimum inhibitory concentration (MIC) assays were performed as described previously (1). Berninamycin A, its T3A analog, the M+2 analog of berninamycin A, and the double hydroxylated variant of the M+2 compound were screened against the strains *B. subtilis* PY79 and MRSA MW2 (see *SI Appendix*, Table S15). The concentrations of all four compounds as solutions in DMSO were determined by integration of each compound's peak from an LC-MS elution (measured at λ = 254 nm) in comparison with

the standard curve established for berninamycin A (*vide infra*); for the purposes of this assay, the extinction coefficients of the four compounds were presumed to be approximately equal.

Table S15. MIC of berninamycin and related analogues against susceptible Gram-positive organisms.

Compound	*B.subtilis* PY79	MRSA MW2
berninamycin A	6.3 μM	10.9 μM
T3A berninamycin A	>400 μM	ND
M+2 berninamycin A	>200 μM	ND
dbl-hydroxylated M+2 berninamycin A	>200 μM	ND

ND = not determined.

The concentration of berninamycin A in solution was determined by standard curve according to the following protocol: 1.9 mg of berninamycin A was dissolved in MeCN (950 μL) and DMSO (500 μL); H_2O (450 μL) was added to give a final concentration of 1.0 mg/mL. Similarly, 4.0 mg of berninamycin A was dissolved in MeCN (1000 μL) and DMSO (500 μL); H2O (500 μL) was added to give a final concentration of 2.0 mg/mL. Each sample was diluted 10-fold with 50:50 MeCN:H_2O. A portion of each sample (10 μL, corresponding to 1.0 μg and 2.0 μg of berninamycin from each sample) was separately injected onto the C18 column and eluted on the High Resolution LC-MS. The peak corresponding to berninamycin A was integrated at 254 nm for each sample: from the 1.0 mg/mL sample, 1.0 μg of berninamycin A integrated for 10,752.49 units, and from the 2.0 mg/mL sample, 2.0 μg of berninamycin A integrated for 16,533.43 units. Thus, the average integration for 1.0 μg of berninamycin A at 254 nm = 9,419.6 units.

18. ^{1}H and ^{13}C NMR data for berninamycin A: The NMR data obtained closely matched that obtained by Rinehart and coworkers (12); see *SI Appendix*, Tables S16 and S17 below.

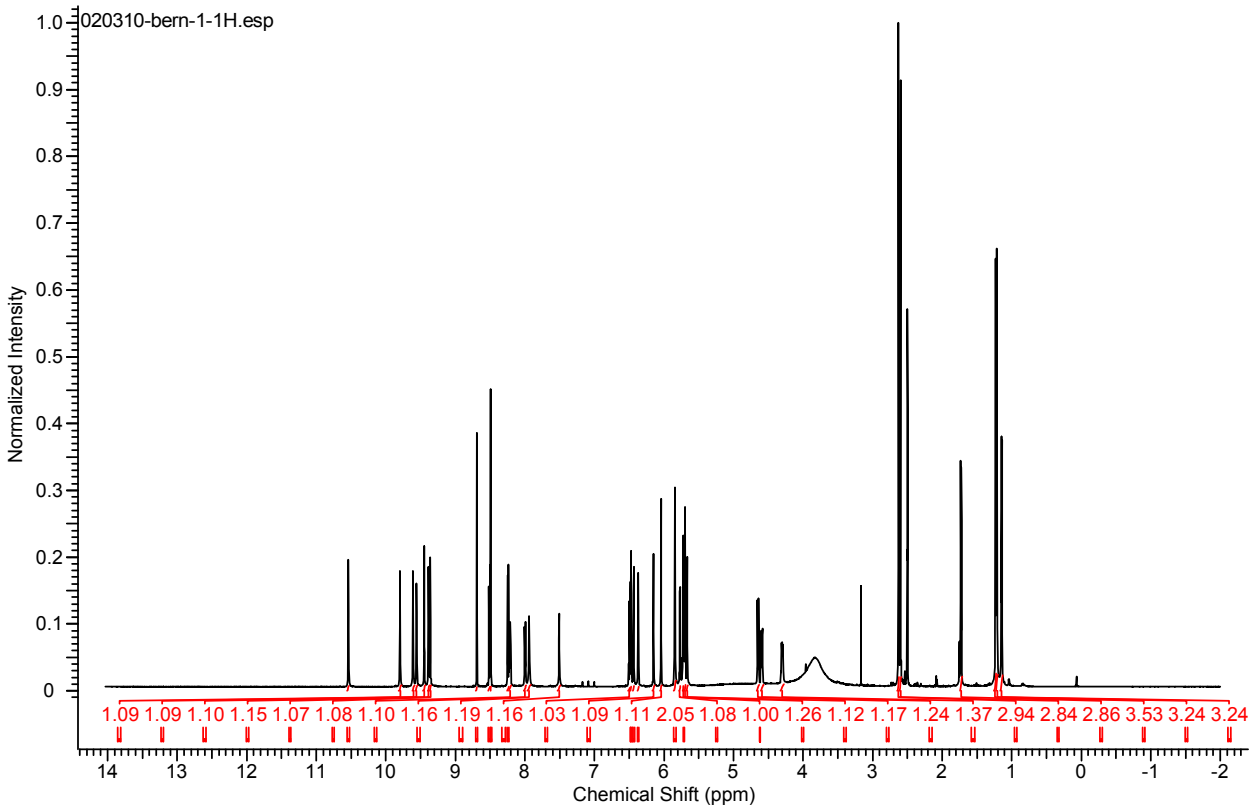


Fig. S27. ^{1}H NMR spectrum of berninamycin A (d_6-DMSO, 600 MHz).

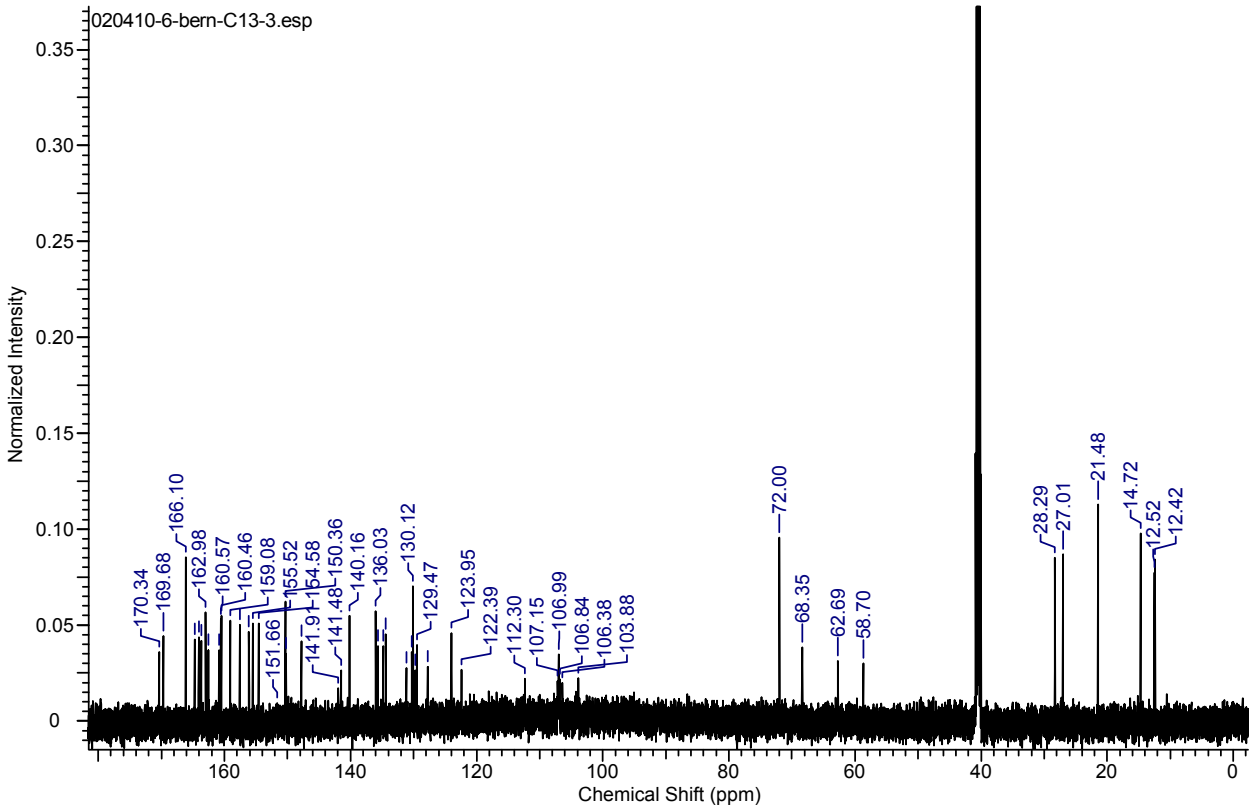
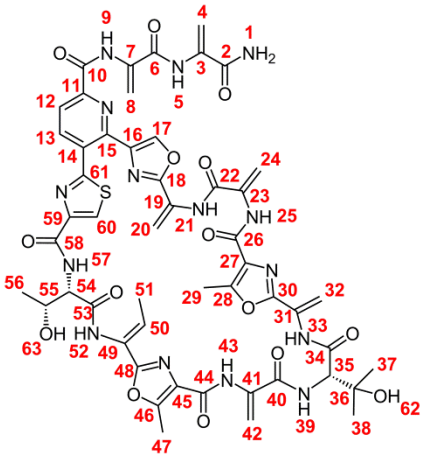


Fig. S28. ^{13}C NMR spectrum of berninamycin A (d_6-DMSO, 150 MHz).



berninamycin A

Table S16. Berninamycin A ^{13}C resonances, which lack attached ^{1}H, and NMR structural assignment.[a]

^{13}C NMR (δ)[b]	Atom #	^{13}C NMR (δ)[b]	Atom #
170.34	34	150.36	11, 15, 16, or 61
169.68	53	150.20	11, 15, 16, or 61
166.10	2	147.80	11, 15, 16, or 61
164.73	40	140.16	11, 15, 16, or 61
164.09	58	136.03	3
163.67	22	135.64	7
162.98	6	134.81	23
162.52	10	134.42	41
160.87	59	131.16	14
160.57	44	130.29	19
160.46	26	130.12	28 or 46
159.08	18	130.10	28 or 46
157.60	48	129.47	31
156.10	30	123.95	49
155.52	27 or 45	72.00	36
154.58	27 or 45		

[a] Spectrum was recorded from a sample prepared in d_6-DMSO. [b] Chemical shift in ppm from tetramethylsilane.

Table S17. Berninamycin A NMR structural assignment for ^{1}H and their correlated ^{13}C resonances.[a]

^{1}H NMR (δ, #, mult, *J*-value)[b]	COSY	HSQC	HMBC	ROESY	Atom #
10.54, 1, s			162.98, 107.15		9
9.79, 1, s			163.67, 159.08, 112.30	5.77	21
9.61, 1, s			170.34, 156.10, 106.38	4.65	33
9.56, 1, s			169.68, 129.73	4.59, 4.33	52
9.45, 1, s			166.10, 162.98, 106.99	6.04, 5.84	5
9.39, 1, s			164.73, 160.57, 103.88		43
9.36, 1, s			163.67, 160.46, 106.84		25
8.69, 1, s		141.48	159.08		17
8.51, 1, d, 8.4 Hz	8.24	141.91	150.20, 147.80, 141.48		13
8.49, 1, s		127.78	164.09		60
8.24, 1, d, 7.8 Hz	8.51	122.39	131.16		12
8.22, 1, d, 8.4 Hz	4.65		164.73	5.84	39
8.00, 1, d, 8.4 Hz	4.59		160.87		57
7.94, 1, s			166.10	7.51, 5.70	1
7.51, 1, s			166.10, 136.03	7.94, 5.70	1
6.49, 1, q, 7.2 Hz	1.73	129.73	157.60, 123.95	1.73	50
6.48, 1, s		103.88	164.73, 134.42	5.84	42
6.44, 1, s		107.15	162.98, 135.64	5.84	8
6.37, 1, s		106.84	163.67, 134.81	5.77	24
6.15, 1, s		106.38	156.10, 129.47	5.67	32
6.04, 1, s		106.99	166.10, 136.03	9.45, 5.70	2
5.84, 2, s		103.88, 107.15	164.73, 162.98	9.45, 6.48, 6.44	8, 42
5.77, 1, s		106.84	163.67	9.79, 6.37	24
5.72, 1, s		112.30	159.08, 130.29		20
5.71, 1, s		112.30	159.08, 130.29		20
5.70, 1, s		106.99	166.10, 136.03	6.04	2
5.67, 1, s		106.38	156.10	6.15	32
4.65, 1, d, 8.4 Hz	8.22	62.69	170.34, 164.73, 72.00, 28.29, 27.01	9.61	35
4.59, 1, dd, 9.0, 3.6 Hz	8.00, 4.33–4.27	58.70		9.56	54
4.33–4.27, 1, m	4.59, 1.14	68.35		9.56	55
2.63, 3, s		12.42	155.52, 130.10 or 130.12		29 or 47
2.60, 3, s		12.52	154.58, 130.10 or 130.12		29 or 47
1.73, 3, d, 8.4 Hz	6.49	14.72	157.60, 129.73, 123.95		51
1.23, 3, s		28.29	72.00, 62.69, 27.21		37 or 38
1.21, 3, s		27.01	72.00, 62.69, 28.29		37 or 38
1.14, 3, d, 6.6 Hz	4.33–4.27	21.48	68.35, 58.70		56

[a] All spectra were recorded from a sample prepared in d_6-DMSO. [b] δ = chemical shift in ppm from tetramethylsilane; # = number of protons; mult = multiplicity; *J*-value = coupling constant.