Supplemental Material for "Marine Mammal Microbiota Yields Novel Antibiotic with Potent Activity Against *Clostridium difficile*"

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Materials and Methods

Phylogenetic Analysis

Evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model.[1] The bootstrap consensus tree inferred from 2000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrapped replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches (Fig. S1).[2,3] Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log-likelihood value. The analysis involved 44 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 1195 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.[4]

Extraction of Cultivated Isolates

Purified bacterial colonies were grown in 1 L of modified SYP broth (1 L Milli-Q water, 32.1 g Instant Ocean™, 10 g starch, 4 g peptone, 2 g yeast extract) with 20 g of Amberlite XAD-16 resin for 10 days at 27 °C. Culture broth and resin slurries were filtered through glass microfiber filters, washed with water (3×200 mL), and the cells, resin, and filter paper were extracted with 1:1 methanol/dichloromethane (250 mL). Organic fractions were dried *in vacuo* and subjected to solid phase extraction using Supelco-Discovery C_{18} cartridges (5 g), eluting with a step gradient of 40 mL MeOH/H_2O solvent mixtures (10%, 20%, 40%, 60%, 80%, 100% MeOH) and finally with EtOAc to afford seven fractions. The resulting fractions were dried *in vacuo*, resolubilized in 500 μL of dimethyl sulfoxide (DMSO), and transferred to deep-well 96-well plates for screening.

Fermentation and Isolation

The producing organism, MMA 6B HVS/10A, was isolated from the lower intestine of a harbor porpoise (specimen LMLPP2011SEP29) under permit number 151408SWR2011PR00001:SMW. The strain was originally isolated on HVS medium (18 g agar, 50 mg nalidixic acid, 50 mg cycloheximide, 10 g starch, 1.7 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g Na_2PO4, 3.0 g KNO_3, 0.5 mg thiamine (vitamin B1), 0.5 mg riboflavin (vitamin B2), 0.5 mg nicotonic acid (vitamin B3), 0.5 mg pyridoxine HCl (vitamin B6), 0.5 mg *p*-aminobenzoic acid, 0.5 mg myoinositol, 0.25 mg biotin, 20 mg $CaCO_3$, 750 mL 0.2-μm filtered seawater, 250 mL Milli-Q H_2O). All vitamins were dissolved in stock solutions, filtered, and added after autoclaving. Frozen stocks of environmental isolates were grown on fresh Marine Broth plates (37.4 g Difco Marine Broth, 18 g agar, 1 L Milli-Q water) and incubated at 25 °C until discrete colonies became visible. Selected colonies were inoculated into 10 mL modified saline SYP (mSYP) media (10 g starch, 4 g peptone, 2 g yeast extract, 31.2 g Instant Ocean in 1 L distilled H_2O). The

cultures were stepped up in stages at 7-day intervals by first inoculating 1.5 mL of the 10-mL cell cultures into 50 mL mSYP (medium scale), followed by inoculation of 40 mL of these medium-scale cell cultures into 1 L of the same broth also containing 20.0 g Amberlite XAD-16 adsorbent resin in 2.8 L Fernbach flasks for 7 days. All cultures were incubated at 25 °C containing glass beads for 10 mL cultures and stainless steel springs for 50 mL and 1 L cultures, and shaken at 200 rpm.

The cells and resin were collected from the bacterial extract by vacuum filtration using Whatman glass microfiber filters and washed with deionized water. This cell/resin slurry was extracted with 250 mL of 1:1 MeOH/CH_2Cl_2, and the organic extract was removed by vacuum filtration and concentrated to dryness *in vacuo*. The crude organic extract (code RLFI1036) was subjected to solid-phase extraction using a Supelco-Discovery C18 cartridge (10 g) and eluted using a step gradient of 80 mL MeOH/H_2O solvent mixtures (10% MeOH (A), 20% MeOH (B), 40% MeOH (C), 60% MeOH (D), 80% MeOH (E), 100% MeOH (F), and finally ethyl acetate (G)) to afford seven fractions designated as A−G. The seven fractions were dried *in vacuo*. The 100% MeOH prefraction, RLUS1036F, was subjected to reverse-phase HPLC (Phenomenex Synergi Fusion-RP 5 μm, 80 Å, 250×4.6 mm, 65:35 MeOH/H_2O isocratic run over 20 min, 1 mL/min flow rate) to produce phocoenamicin, which eluted at 15.7 min.

Flow Cytometry

Membrane potential was analyzed on a Becton Dickinson LSRII flow cytometer using the reagents and protocol from the BacLight™ Bacterial Membrane Potential Kit (B34950).

Bacterial Strains

Gram-positive: *Bacillus subtilis* 168*,* methicillin-susceptible *Staphylococcus aureus* (MSSA, ATCC 29213), *Staphylococcus epidermidis (*ATCC 14990), methicillin-resistant *Staphylococcus aureus* (MRSA, BAA-44), *Listeria ivanovii* (BAA-139), *Enterococcus faecium* (ATCC 6569), *Clostridium difficile* (ATCC 700057).

Gram-negative: *Escherichia coli* BW25113, *Providencia alcalifaciens* (ATCC 9886), *Ochrobactrum anthropi* (ATCC 49687), *Enterobacter aerogenes* (ATCC 35029), *Acinetobacter baumanii* (NCIMB 12457), *Vibrio cholerae* O1 biotype El Tor A1552, *Salmonella enterica* serovar Typhimurium LT2, *Pseudomonas aeruginosa* (ATCC 27853), *Yersinia pseudotuberculosis* (IP2666 pIBI).

High-Throughput Antibacterial Inhibition Assay

Bacterial test strains were grown on fresh agar plates and individual colonies were used to inoculate 3 mL sterile media. All *Staphylococcus* strains were grown in tryptic soy agar (TSA; 17 g tryptone, 3 g soytone, 2.5 g dextrose, 5 g NaCl, and 2.5 g dipotassium phosphate in 1 L distilled water; pH 7.5). *P. alcalifaciens*, *O. anthropi*, *E. aerogenes*, and *A. baumanii* were grown in nutrient broth agar (Difco, USA), and *B. subtilis*, *E. coli*, *V. cholerae*, *S.* Typhimurium, *P. aeruginosa*, and *Y. pseudotuberculosis* cultures were grown in Luria Broth (10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L distilled water; pH 7.5). *C.*

difficile was grown on TSA plates supplemented with 5% sheep blood (Hardy Diagnostics, VWR catalog number 89405-024). All *C. difficile* media were placed in anaerobic chambers 24 h prior to inoculation, and all culture conditions were made anaerobic using disposable chambers (BD Diagnostics, 90003-642). All in-house media were autoclaved at 121 °C for 30 min. Antimicrobial MICs were determined using Mueller Hinton Broth (VWR catalog number 90003-966). Inoculated cultures were grown overnight with shaking (200 rpm, 30 °C). Saturated overnight cultures were diluted 1:10,000, 1:1000, or 1:100 according to turbidity and dispensed into sterile clear polypropylene 384-well plates (30 μL screening volume). Optical density (OD_{600}) of cultures at a 1:100 dilution was recorded (Shimadzu UV-Visible Spectrophotometer), and cultures were further diluted on agar plates to calculate colony forming units (CFUs) per milliliter of culture. DMSO solutions of test compounds (200 nL) were pinned into each well prior to inoculation using a high-throughput pinning robot (Perkin Elmer Janus MDT). In the 384-well plates, lanes 1 and 2 were reserved for DMSO vehicle negative controls, while lanes 23 and 24 contained only culture medium and test organisms. After compound addition, screening plates were stacked in an automated plate reader/shaker (Perkin Elmer EnVision) and OD_{600} was measured every 1 h for ~18 h. The resulting growth curves for each dilution series were used to determine MIC values for all test compounds following standard procedures.

Structure Elucidation

Characterization of Phocoenamicin. White amorphous powder, $[\alpha]^{25}_D$ = -6.3, (c = 1.0, Acetone); UV(MeOH) λ_{max} (log ϵ) 232(3.34), 398(2.89), 314(2.82) nm; ^{1}H and ^{13}C see NMR table; HRMS [M-H]$^-$ 1069.4571 (calculated for $C_{56}H_{74}ClO_{18}$, 1069.4564).

Subunit A (Glycosylation). Initial evaluation of the ^{1}H and ^{13}C spectra, coupled with HRMS data, suggested a molecular formula of $C_{56}H_{75}ClO_{18}$. Examination of the HSQC spectrum suggested the presence of an aliphatic core, two sugar moieties, and a poly-substituted aromatic substituent (Fig. S3A). Evaluation of the COSY spectrum starting from the anomeric proton at 4.41 revealed a 6-deoxy-hexose sugar.

HMBC from δH 3.45 ppm on C3' to a new anomeric carbon C1" indicated a 1,3-linkage to a second sugar subunit. COSY correlations from this anomeric proton δH 4.61 on C1" revealed the presence of a second 6-deoxy-hexose sugar unit. HMBC from the proton at the 4 position (δH 4.93 on C4") to a new quaternary carbon C7" suggested the presence of a terminal substituent attached 1-4 to this sugar unit. HMBC correlations from δH 4.93 on C4" in the second sugar to the carbonyl C7" indicated an acetyl linkage to the aromatic core. HMBC correlations between aromatic proton δH 6.85 to C7" also confirmed the linkage. COSY correlations suggested adjacent aromatic protons. Comparison of ^{13}C chemical shifts to the known aromatic subunit of chlorothricin (Fig. S3C) as well as synthetic analogues of the aromatic core confirmed the regiochemistry of the ring. Together, these data identified subunit A.

Configurational Analysis

Based on the analyses described within the main text and the supplementary information the configuration of the stereogenic centers was found as follows: (4R, 5R, 6S, 8R, 9S, 10S, 13S, 14R, 18R, 21R, 23S*, 32S, 33R, 1'S, 2'R, 3'S, 4'R, 5'R, 1''S, 2''R, 3''S, 4''R, 5''R).

Sugars. Relative configuration was determined by ROE and $^3J_{HH}$ (approximate coupling constants were derived from the DQF-COSY). Key ROE correlations are represented in S3A. ROE correlations between H1' and H5' assigned them as axial protons on the top face of the sugar. Reciprocal ROE correlations between the methyl at C6' and both substituents on C4' (H4' and the δH 3.92 OH) suggested that the methyl was in the equatorial position, confirming the axial position of H5'. A large coupling constant of approximately 10 Hz between H5' and H4' set H4' as an axial proton on the bottom face of the sugar. A smaller coupling constant of approximately 4 Hz between H4' and H3' and a corresponding ROE placed H3' in the bottom face of the sugar. A coupling of approximately 5 Hz between H3' and H2' when considered with the ROE between H2' and H1' assigned H2' to the top face of the molecule in an equatorial position, completing the sugar composed of C1' to C6'. For the sugar containing C1'' to C6'', ROE correlations from H1'' to H2'' and H5'' defined the top face. ROE correlations from H4'' to H3'' and H6'' defined the bottom face.

Synthesis of D-Glucopyranoside, methyl 6-deoxy-, 2,3,4-triacetate. Phocoenamicin (10 mg, 0.009 mmol) was subjected to methanolysis by refluxing in 4 mL 1N HCl:MeOH for 2 h. Upon cooling, the reaction mixture was concentrated to dryness *in vacuo* and partitioned between H_2O (10 mL) and Et_2O (10 mL). The phases were separated and the aqueous phase extracted with Et_2O (2x 10 mL). The aqueous portion was lyophilized to dryness. To a mixture of the crude lyopholized product (1 mg) and $Y(CF_3SO_3)_3$ (0.1 mg) in dry DCM (5 mL) was added acetic anhydride (190 mL) at room temperature. After stirring for 2 h, the solution was quenched with saturated sodium bicarbonate solution, and the product was extracted with DCM three times. The combined organic extracts were washed with water and brine. The triacetate was purified using a Waters mass-directed preparative system equipped with an XBridge BEH130 5 µM 19 x 150 C18 column eluting with H_2O/acetonitrile. Triacetate 6-deoxy-D-glucopyranose was also synthesized from 6-deoxyglucopyranose using identical procedures. Optical rotation of the β anomer was consistent with synthetic compound β-D-Glucopyranoside, methyl 6-deoxy-, 2,3,4-triacetate (natural product): $[\alpha]^{25}_D$ = -4.2, (c =0.1, Chloroform), β-D-Glucopyranoside, methyl 6-deoxy-, 2,3,4-triacetate (synthetic standard): $[\alpha]^{25}_D$ = -4.7, (c =0.1, Chloroform).

Cytological Profile Screening and Image Analysis

Methods for cell culture and staining were as previously reported (36, 37). HeLa cells were plated into two clear-bottom 384-well plates at a target density of 2,500 cells per well. The plates were incubated for 24 h under 5% CO_2 at 37 °C, 150 nL of extract was pinned onto the culture plates, and the plates were incubated for 19 h under 5% CO_2 at 37 °C. The plates were then fixed and stained with sets of either cell-cycle or cytoskeletal stains, which report on the number of cells in S-phase or mitosis and the amount and distribution of tubulin and actin, respectively. Both stain sets also contained a nuclear stain (Hoechst), which was used to count the number of cells and segment the image. The plates were imaged with a 10X objective lens, acquiring four images per well. For each extract, 248 different parameters were measured from the images of each plate. Together, these values report on a diverse range of size and shape features, such as the total area and shape of the nuclei and the number of mitotic cells. Comparing extract-treated and DMSO-treated wells and reduction of these cellular metrics to population values for each well using a custom data management pipeline produced a 248-parameter fingerprint for each extract with attributes scaled from -1 to 1 (Fig. S4B).

**S1. Molecular phylogenetic analysis by maximum likelihood method.**
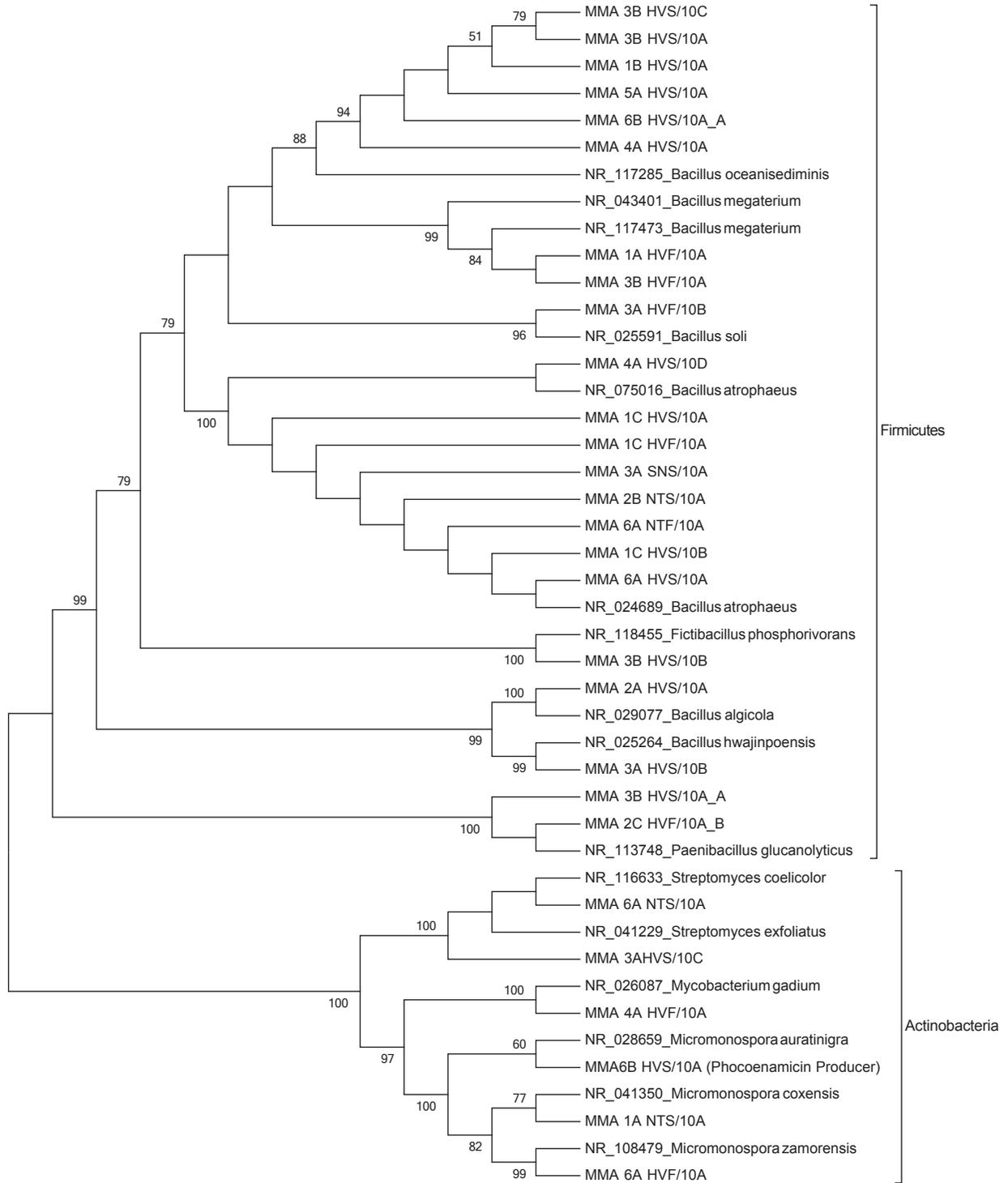
S2. Molecular networking of bacterial extracts. Colored nodes are metabolites produced only by one organism, nodes represented in grey are metabolites produced by many organisms.


Strain ID (# unique nodes)
MMA1029 (9)
MMA1020 (1)
MMA1028 (50)
MMA1031 (6)
MMA1030 (5)
MMA1027 (1)
MMA1025 (2)
MMA1022 (14)
MMA1021 (7)
MMA1010 (1)
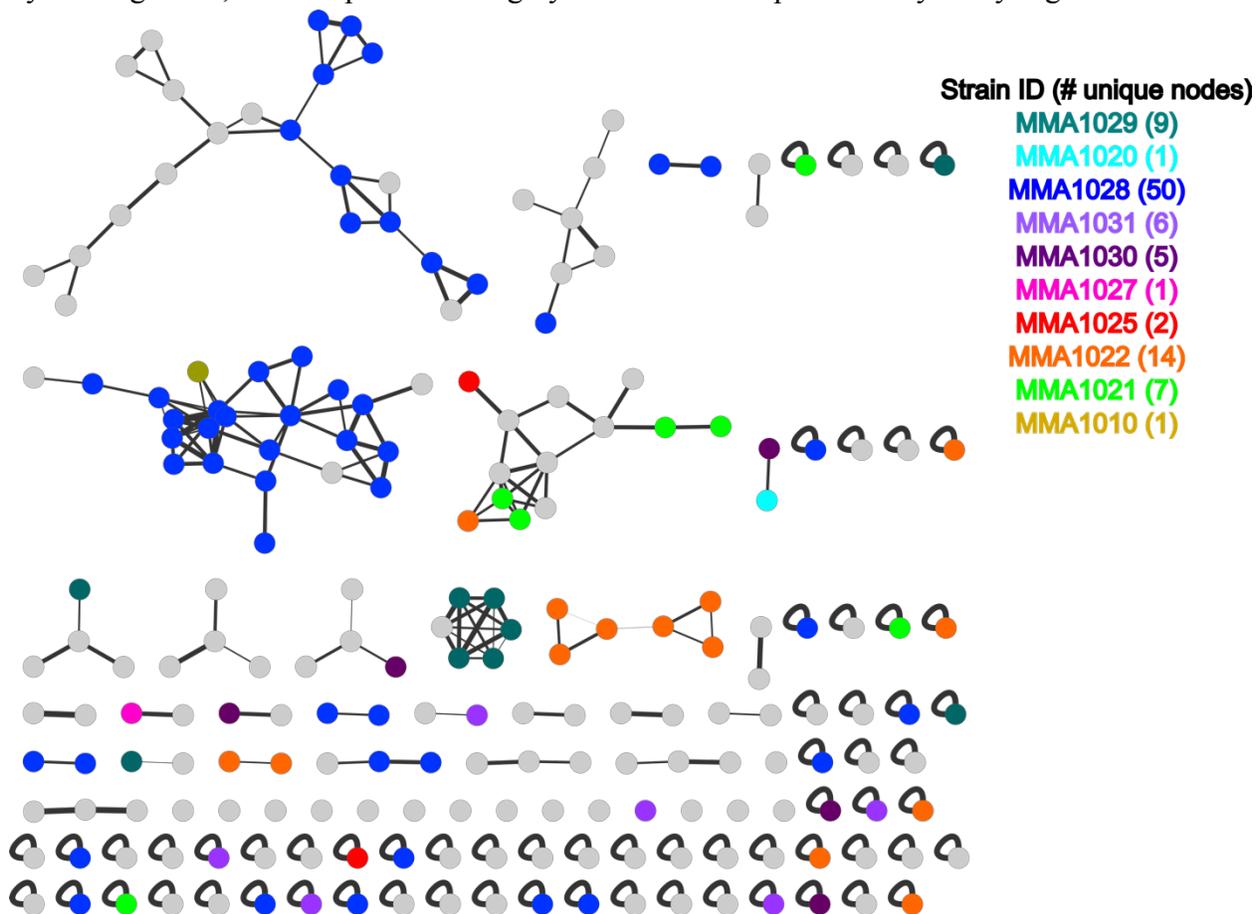
S3. Structure elucidation of phocoenamicin. (i) Key NMR correlations for subunits A (carbohydrates), B (aliphatic core), and C (cyclohexene unit). (ii) Key ROESY correlations for configurational analysis. (iii) HMBC correlations.

i. Subunits

Subunit A   Subunit B   Subunit C

B1 - Blue
B2 - Black
—— COSY
⌒ HMBC
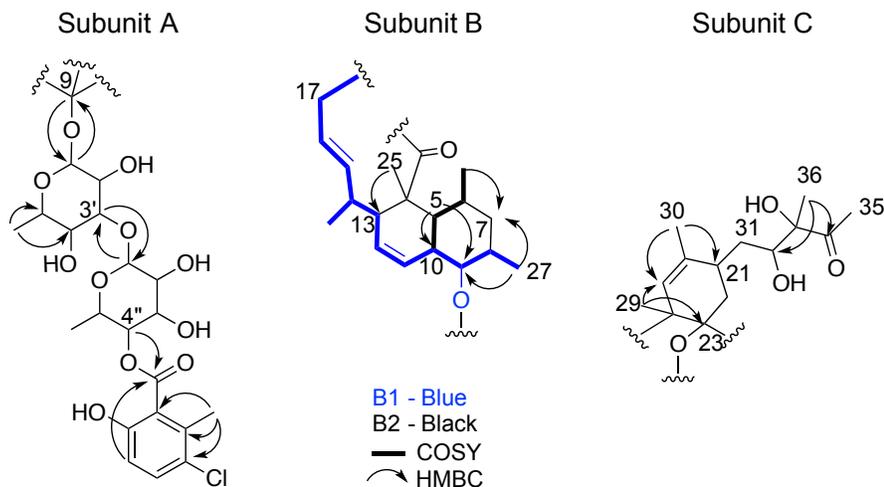
ii. ROESY Correlations

iii. HMBC Correlations

R = Subunit A

⌒ ROESY
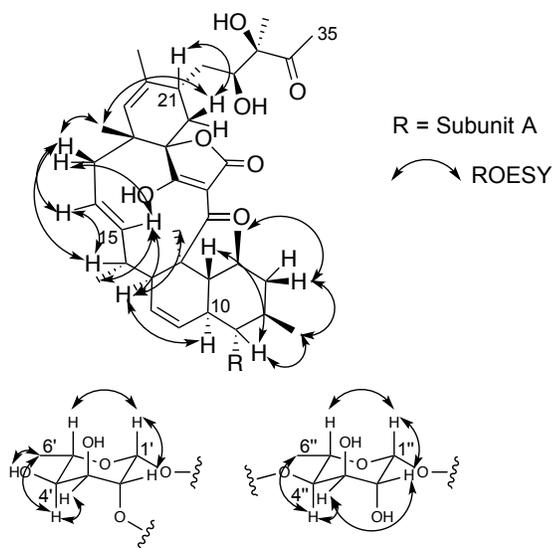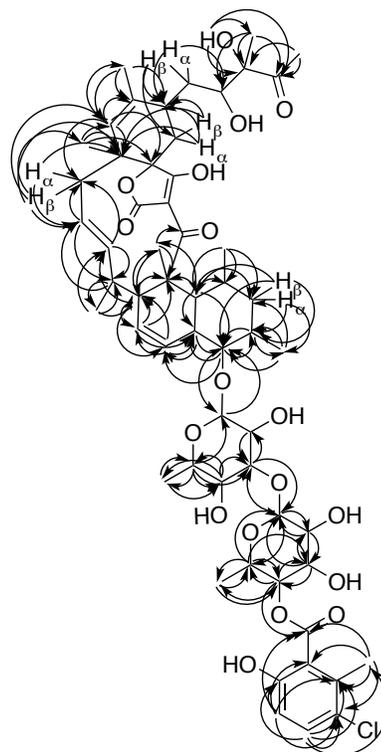
S4. ROE correlations of possible diastereomers. A combination of coupling constants and key ROE correlations indicated a configuration of 32S, 33R for the sidechain. To further justify this conclusion, all possible diastereomers of the side chain were modeled in Discovery Studio 4.0 (i, ii, iii). Except for the (32S, 33R) diastereomer shown in purple (iv), all other diastereomers were unable to reproduce all the observed ROE correlations. In any one confirmation, at least one observed ROE correlation was unobtainable in all the other models created. Examples of ROE correlations that were observed but not able to be replicated in the model without sacrificing other correlations are represented with yellow double-headed arrows. Correlations in the models consistent with the observed data are displayed with white double-headed arrows.

i) SS Stereoisomer: Observed ROE correlation not reproducible in model in yellow
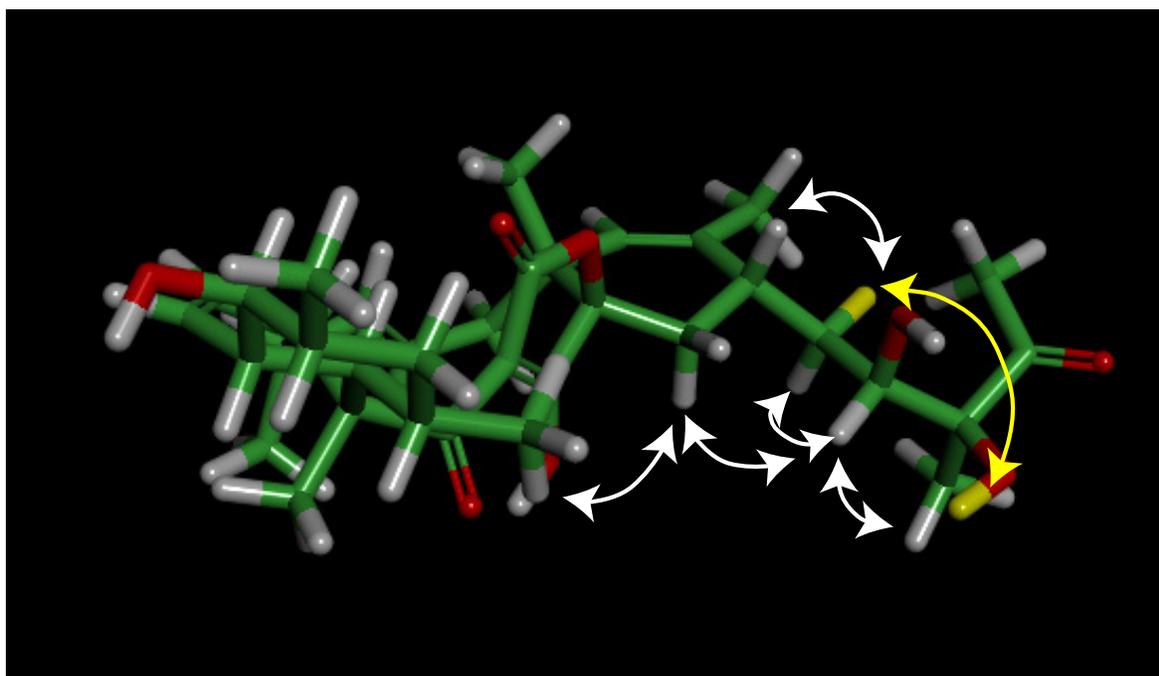
ii) SS Stereoisomer: Observed ROE correlation not reproducible in model in yellow
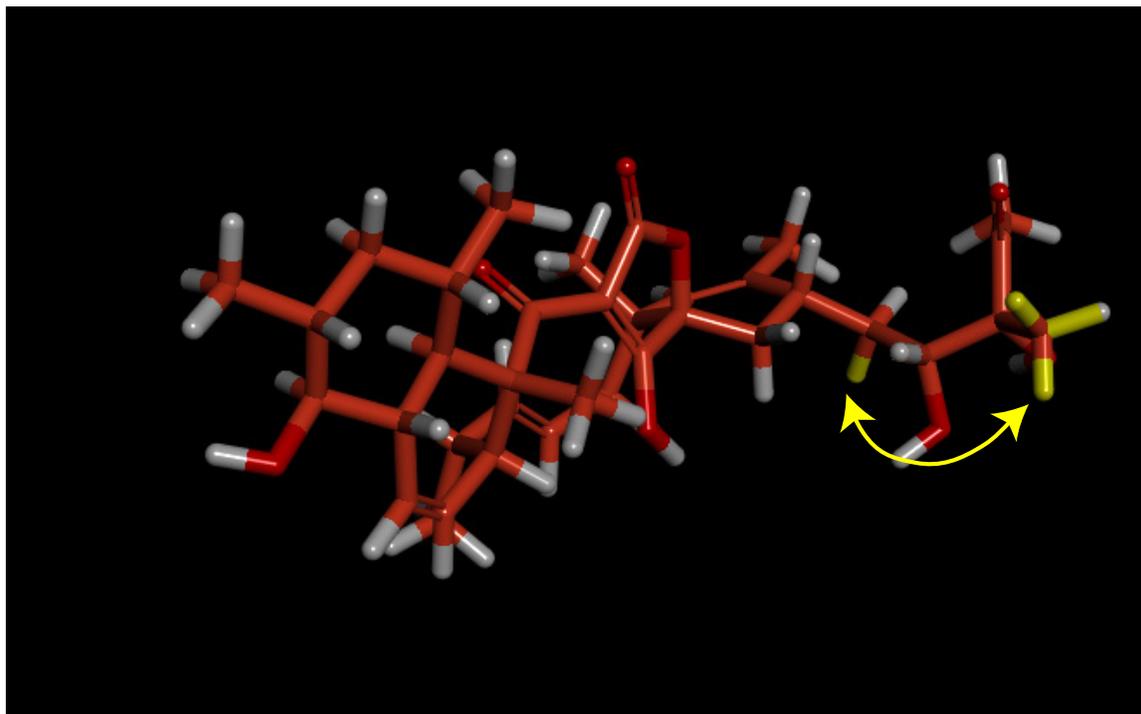

iii) RS Stereoisomer: Observed ROE correlation not reproducible in model in yellow
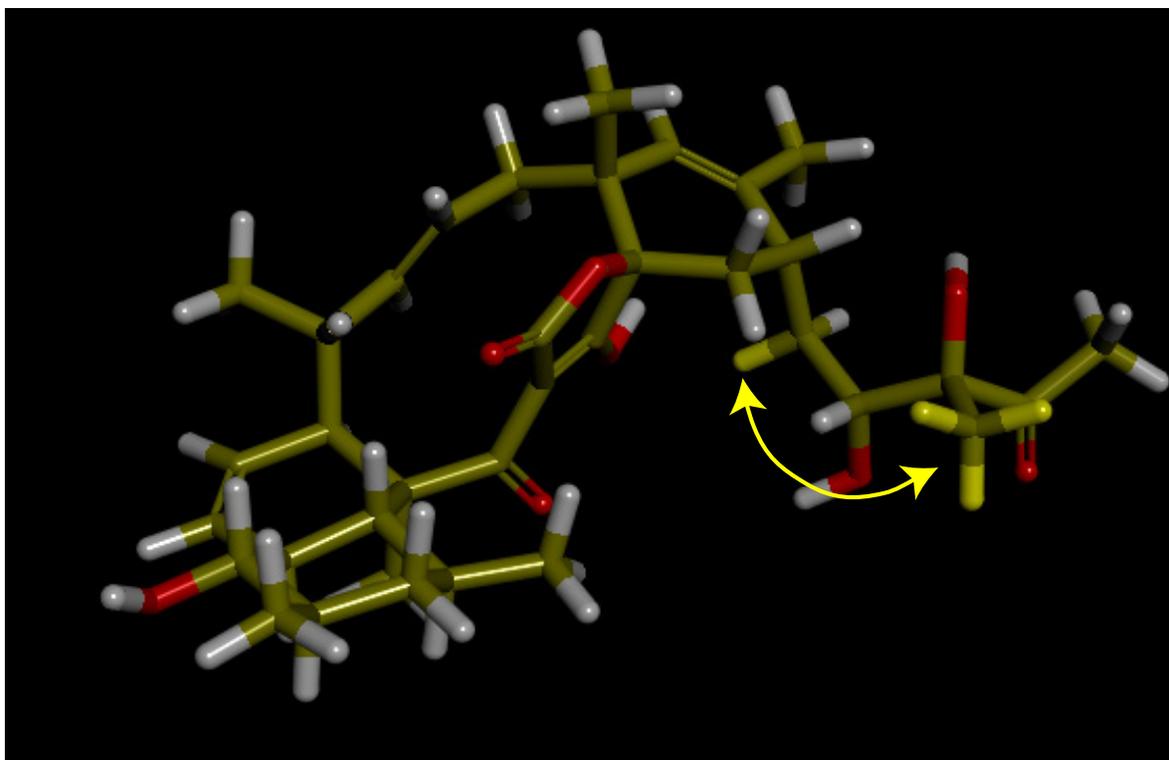
iv) SR: Matching NOE correlations to observed data in white
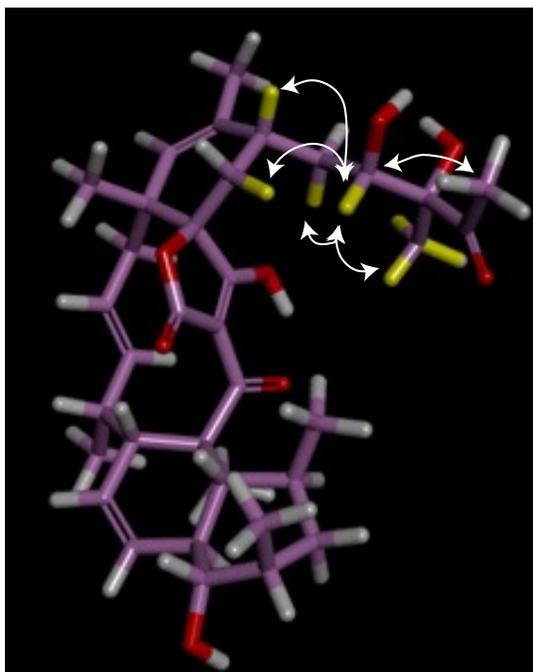

ROE correlations for proton on C32
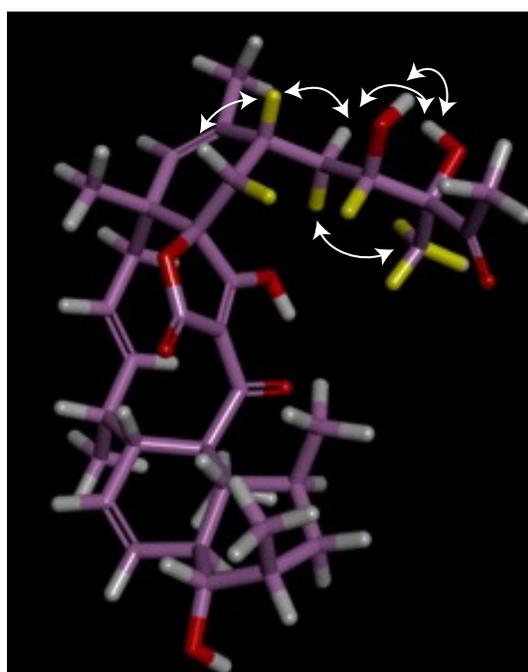

All other observed ROE correlations

S5. LCMS trace of crude extract, RLFI1036F. Spectra obtained using a gradient of MeCN:H2O + 0.02% formic acid (65% MeCN for 2 min, 65%-95% MeCN over 12 min) at a flow rate of 2 mL/min (Phenomenex Synergi Fusion-RP, 10 x 250 mm column).


Phocoenamicin, [M-H]⁻ 1069.4
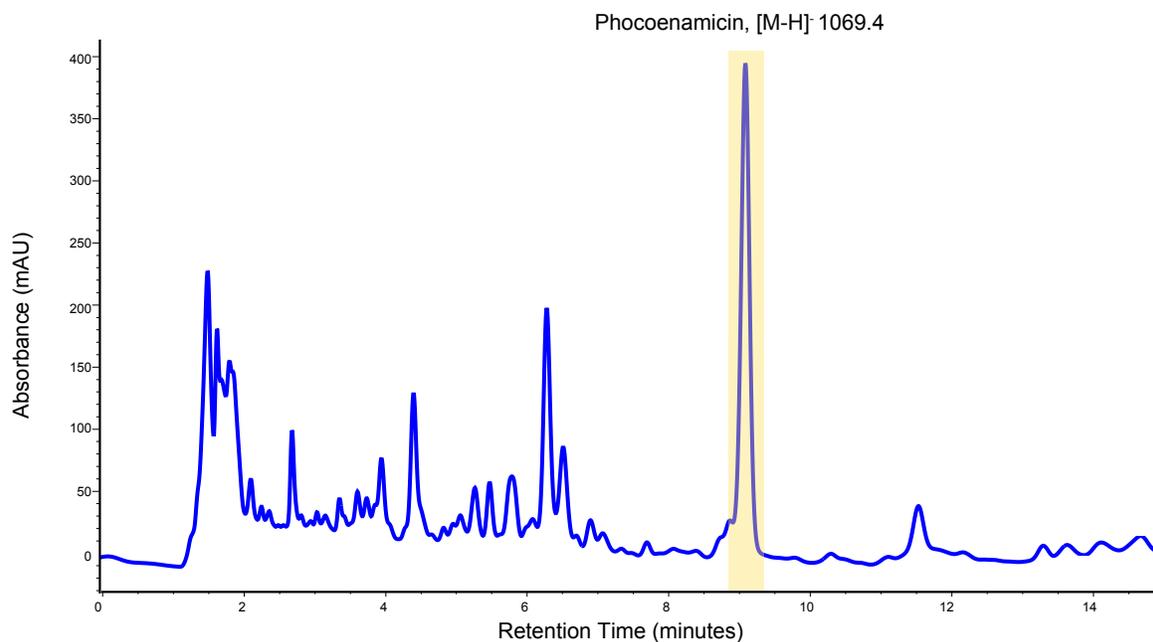
**S6A. ^{1}H of phocoenamicin at 600 MHz in acetone-d6.**
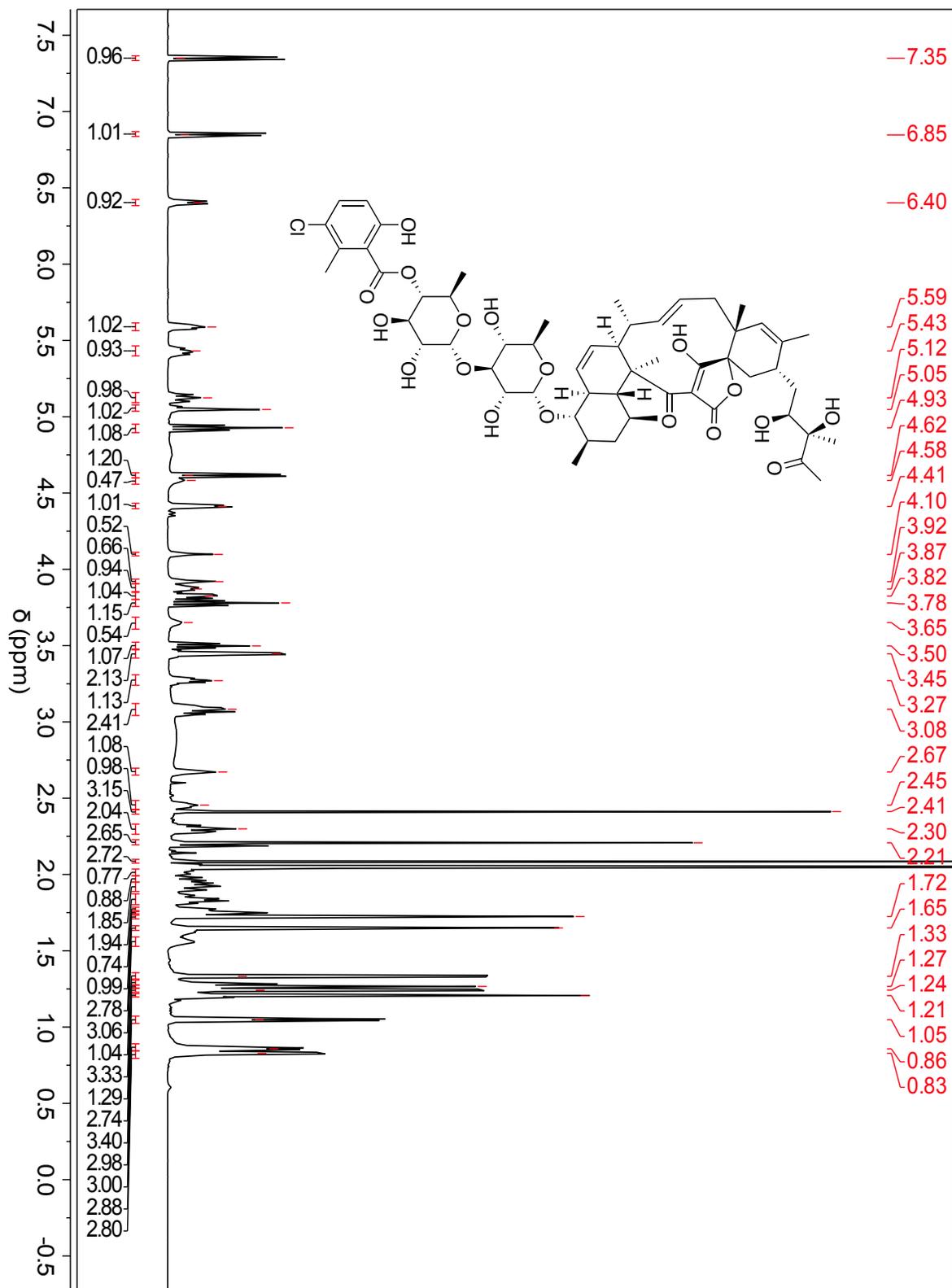
S6B. gCOSY of phocoenamicin at 600 MHz in acetone-d6.
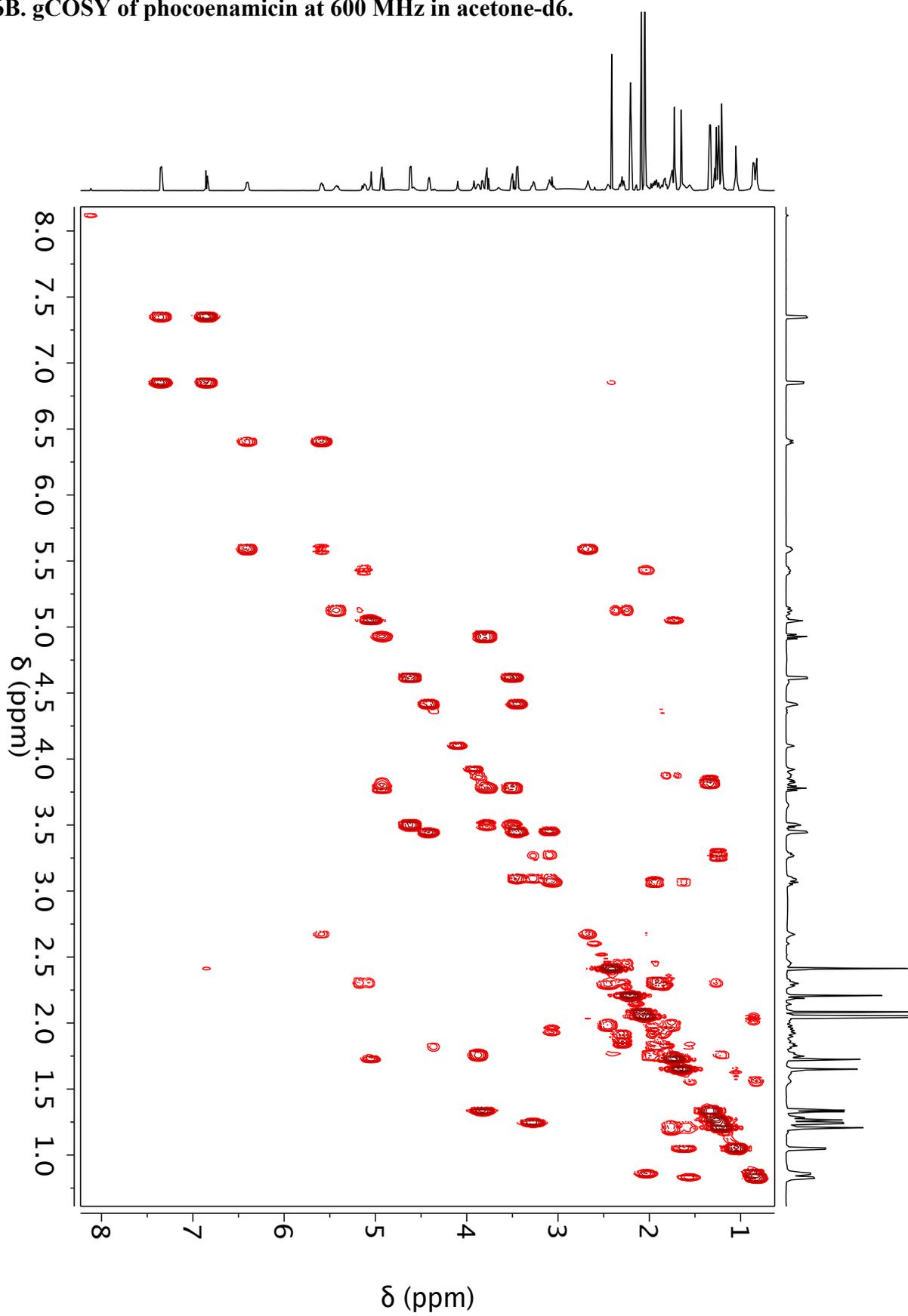
S6C. gTOCSY of phocoenamicin at 600 MHz in acetone-d6.
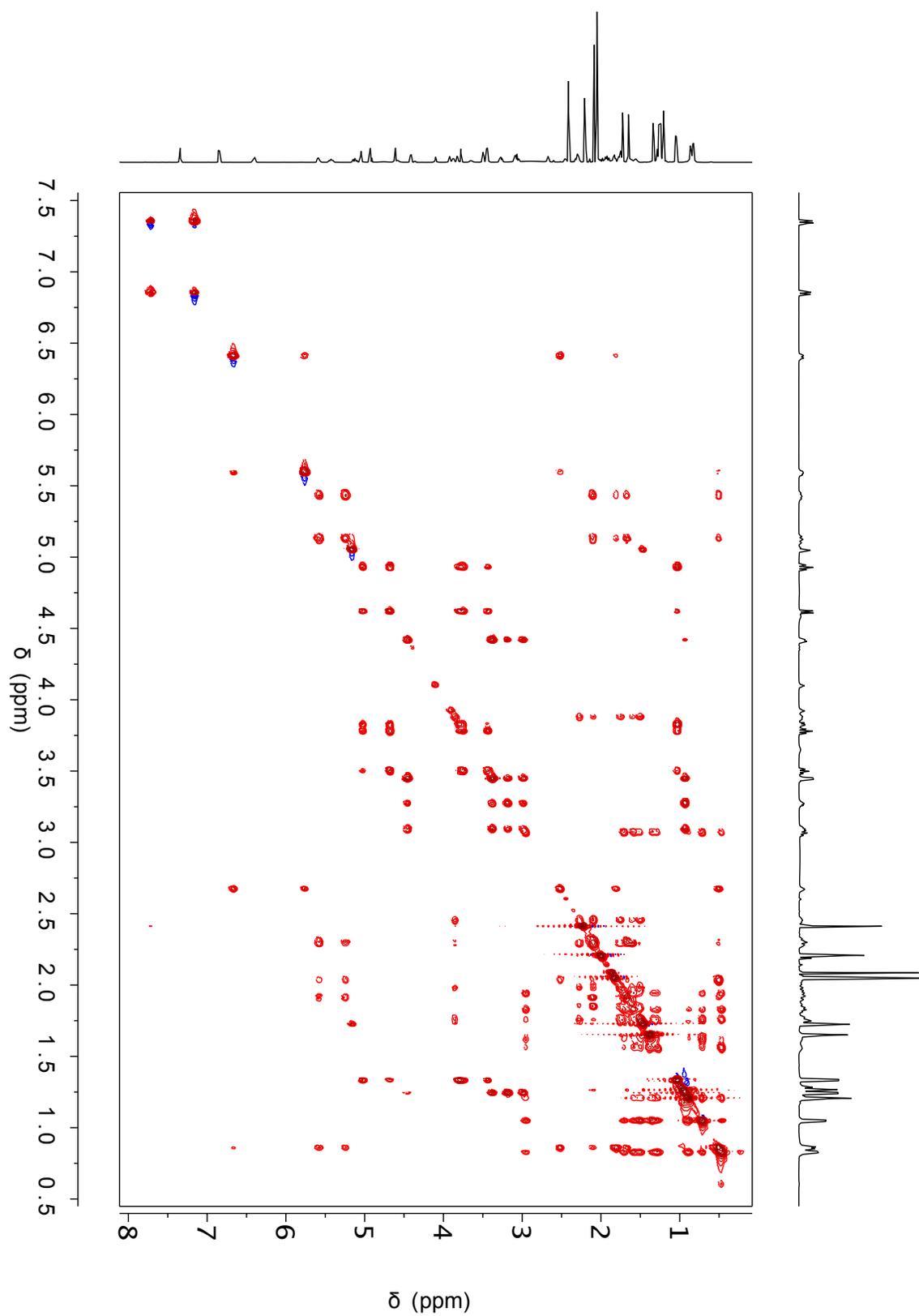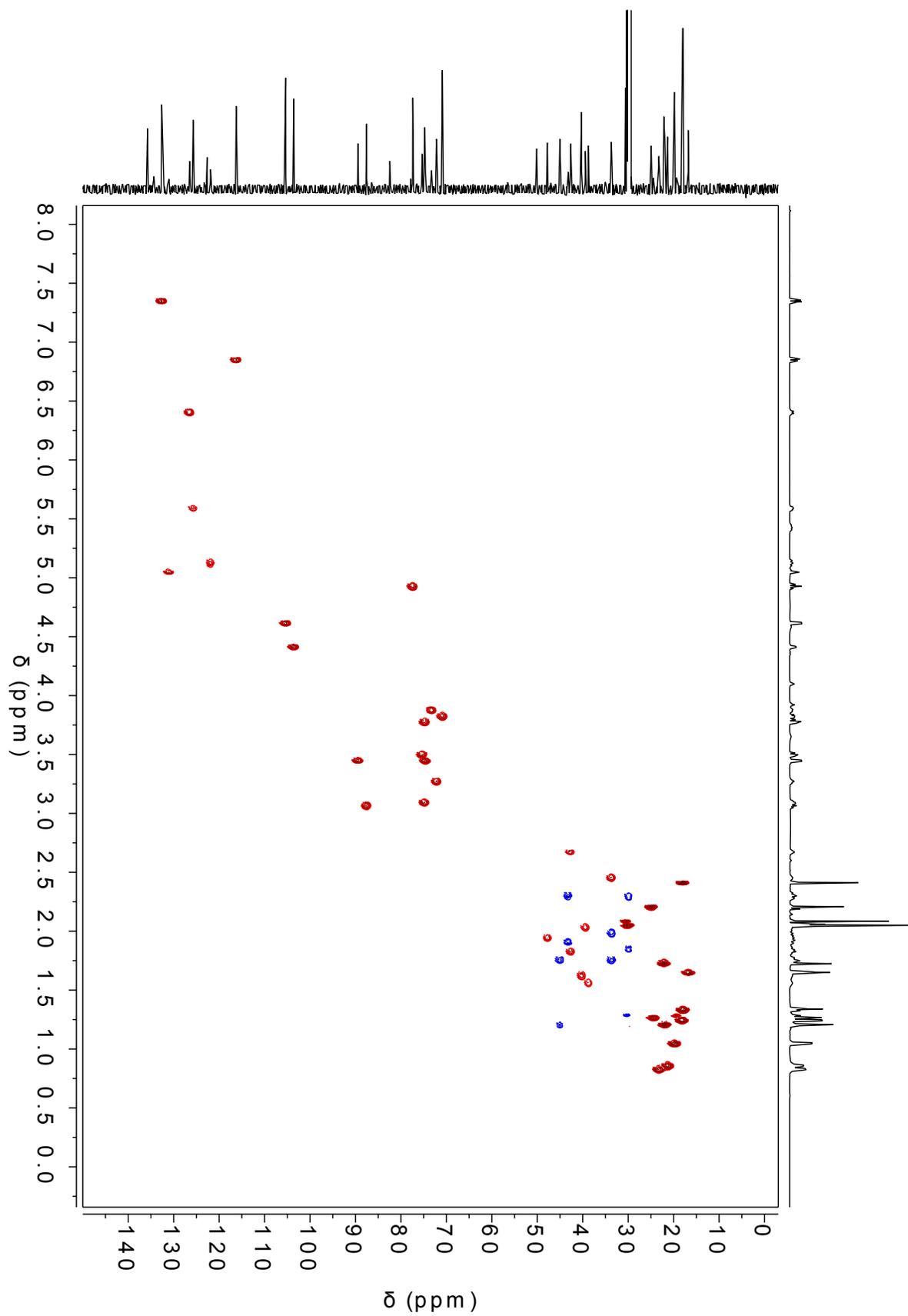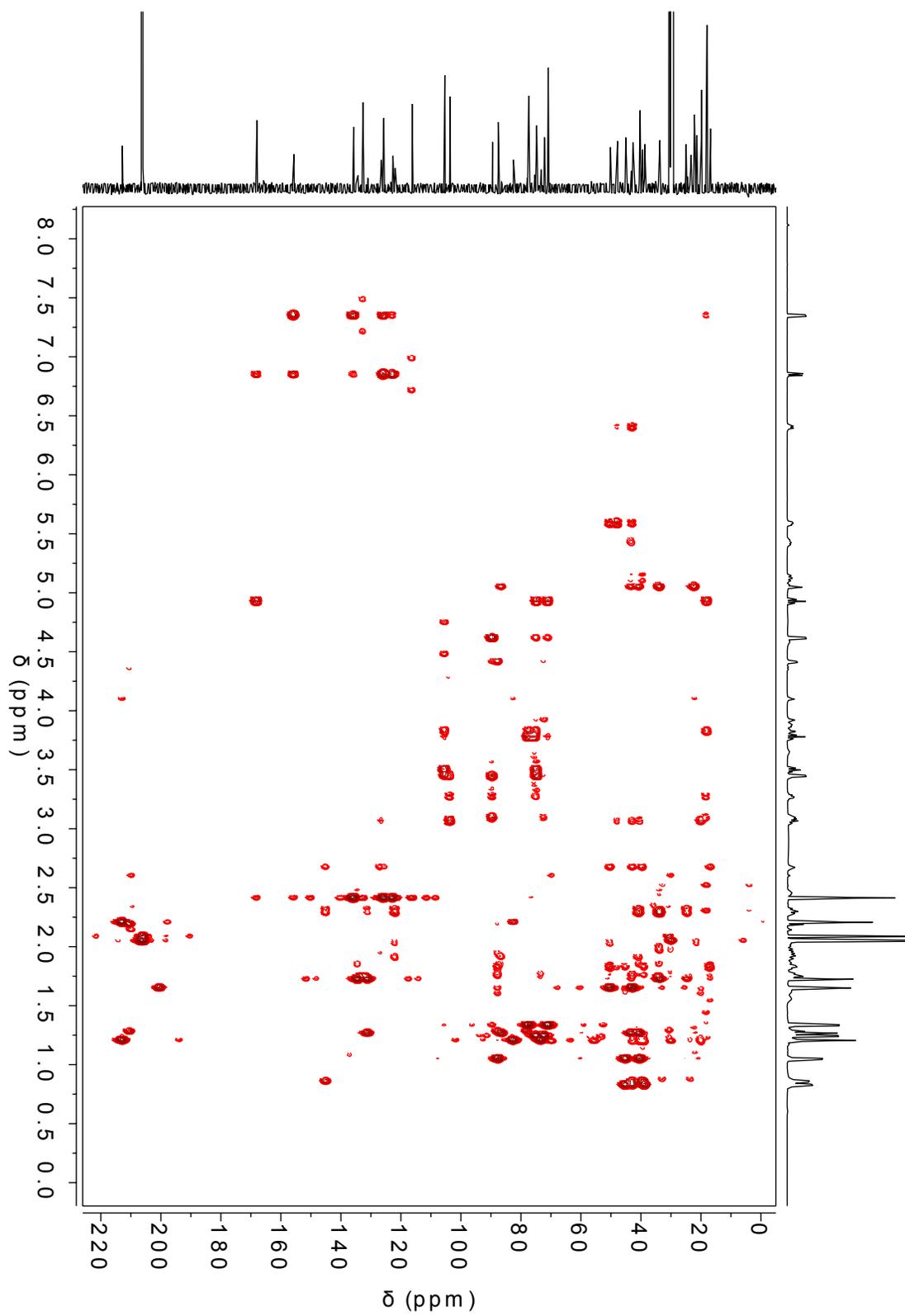
**S6F. ROESY of phocoenamicin at 600 MHz in acetone-d6.**
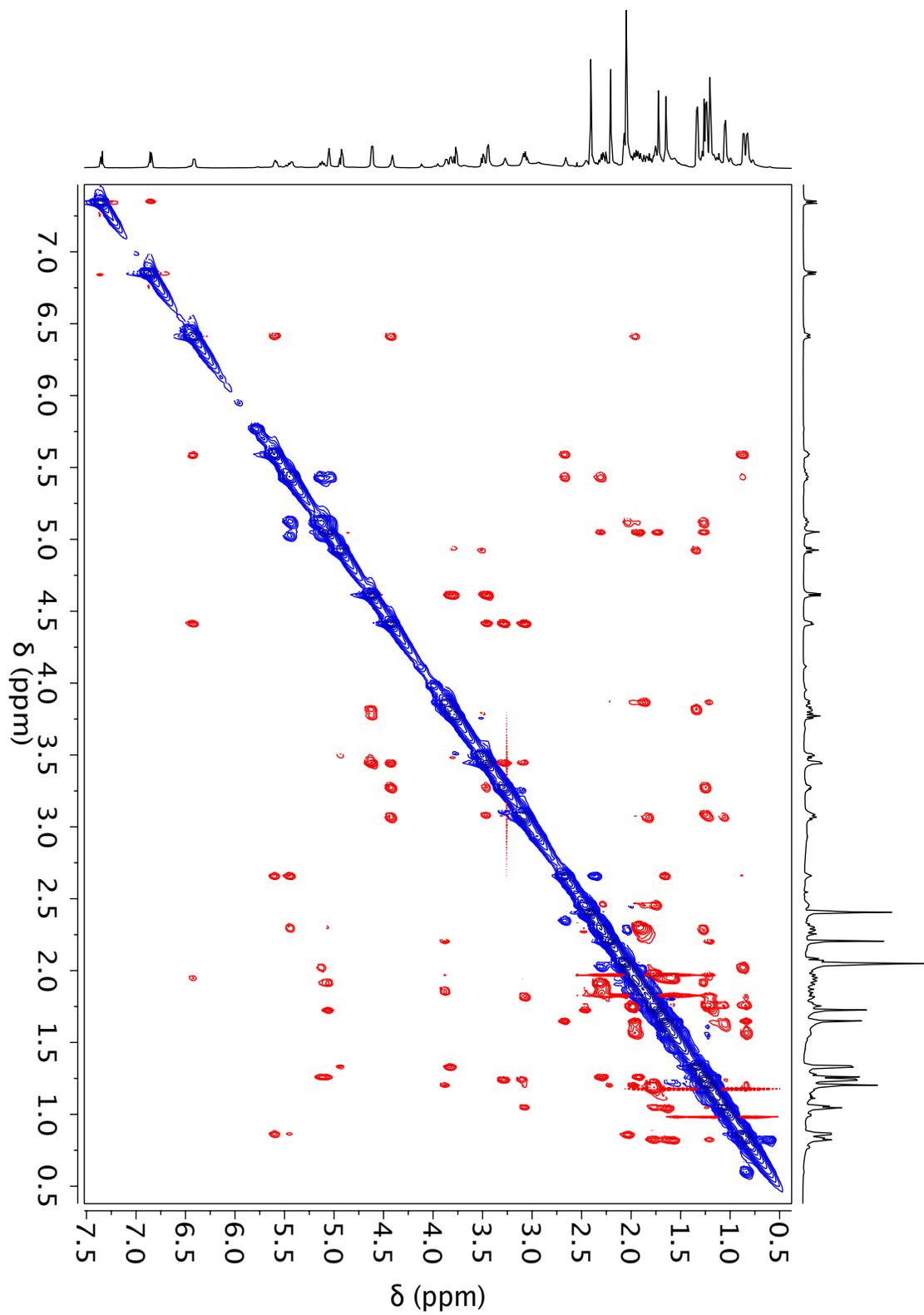
S6G. ^{13}C of phocoenamicin at 600 MHz in acetone-d6.
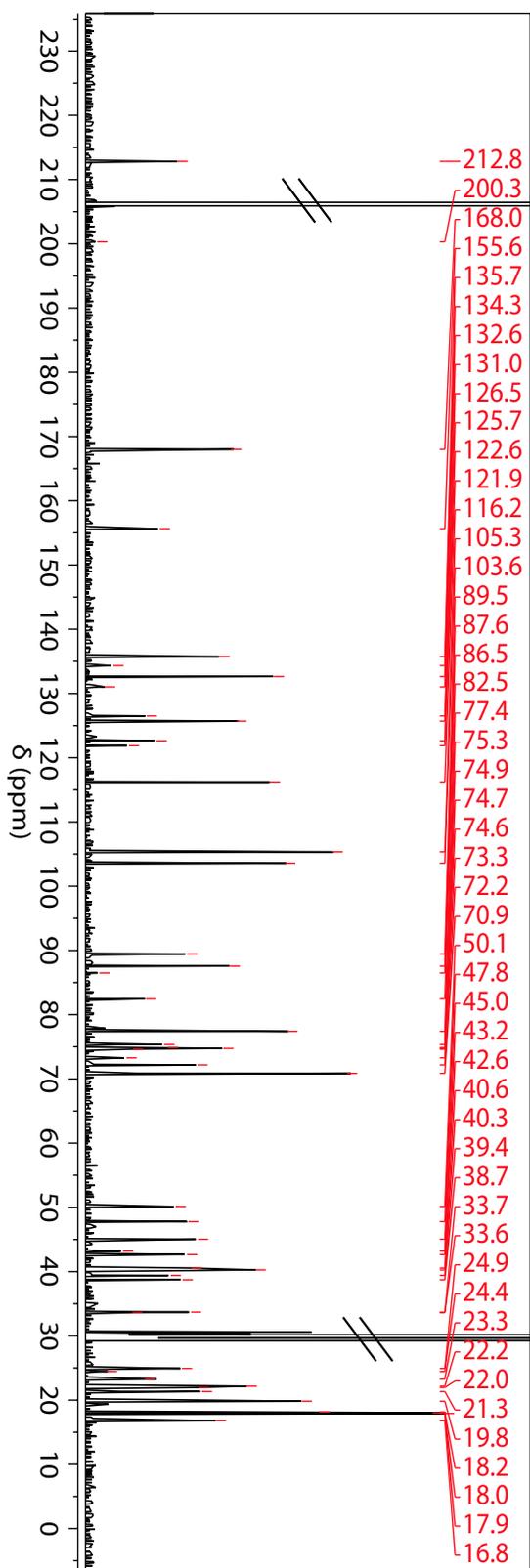
S6H. 1D-TOCSY of phocoenamicin at 600 MHz in acetone-d6, irradiated at 2.45 ppm.

—3.89

—3.65

—2.47

—2.30

—1.98
—1.88
—1.77

δ (ppm)
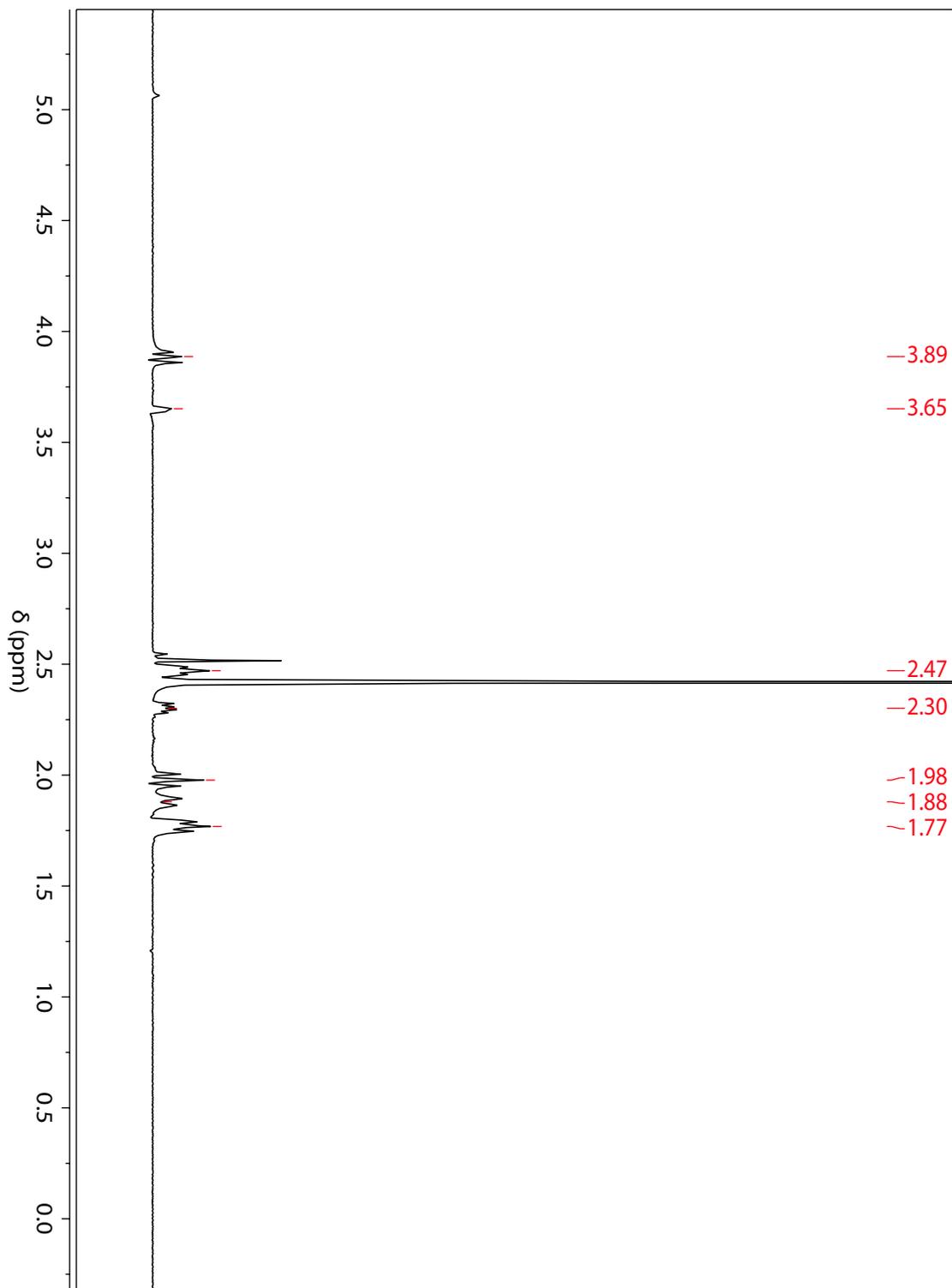
S6I. 1D-TOCSY of phocoenamicin at 600 MHz in acetone-d6 irradiated at 5.1 ppm.
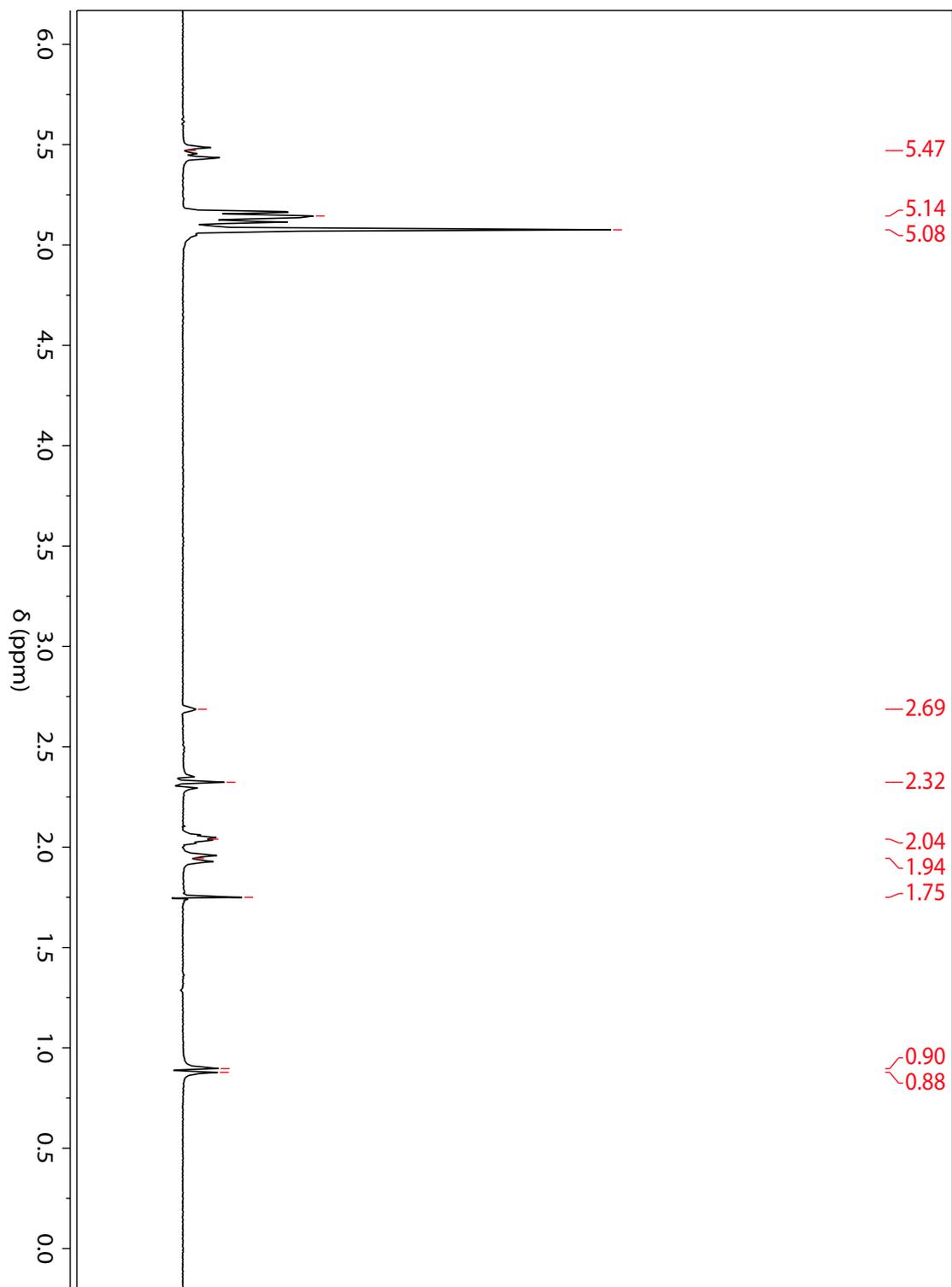
S7. ¹H of synthetic and isolated sugars at 600 MHz in chloroform-d1.

Isolated triacetate 6-deoxy- β-D-glucopyranose
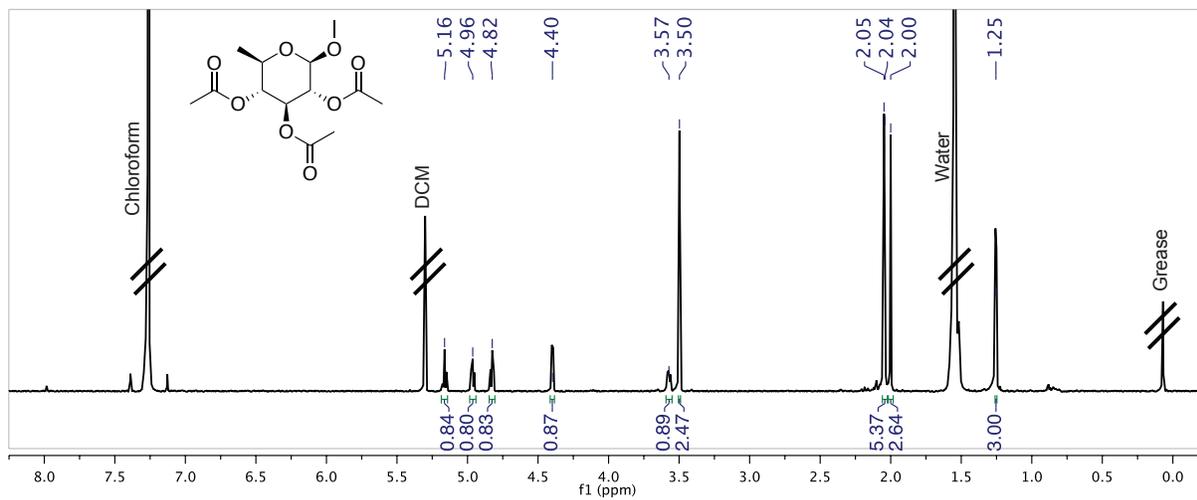
Synthetic triacetate 6-deoxy- β-D-glucopyranose
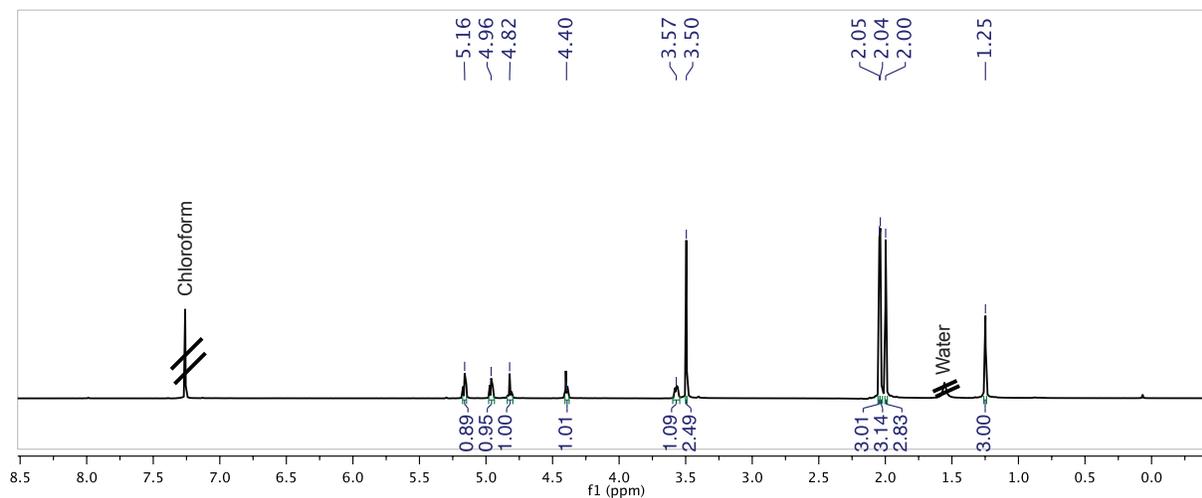
S8. Synthetic and isolated sugar co-injection data.

Isolated triacetate 6-deoxy- β-D-glucopyranose
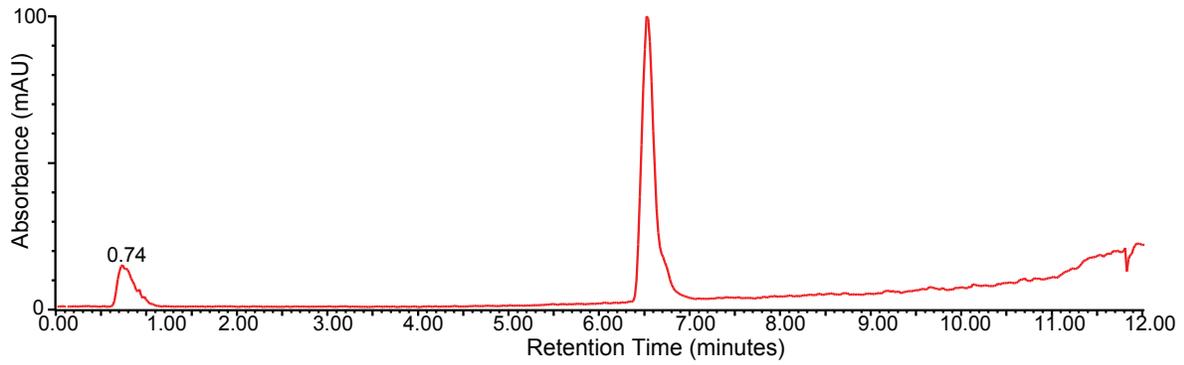
Synthetic triacetate 6-deoxy- β-D-glucopyranose
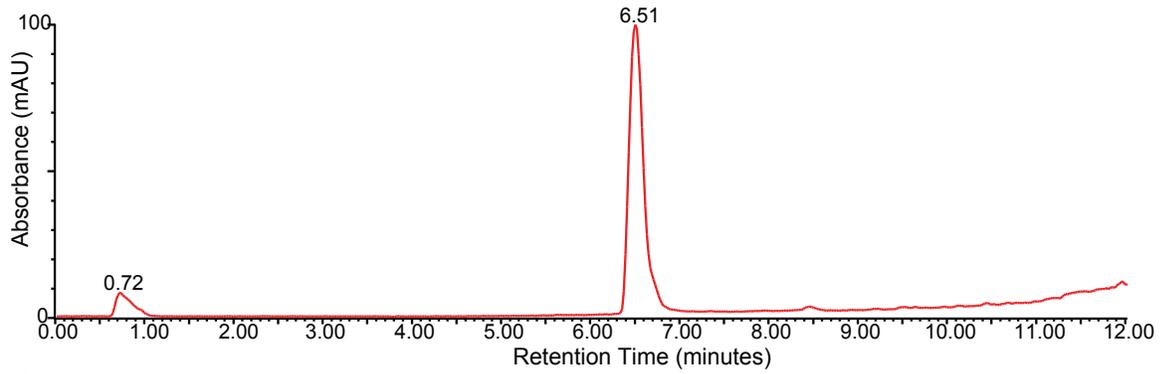
Co-injection
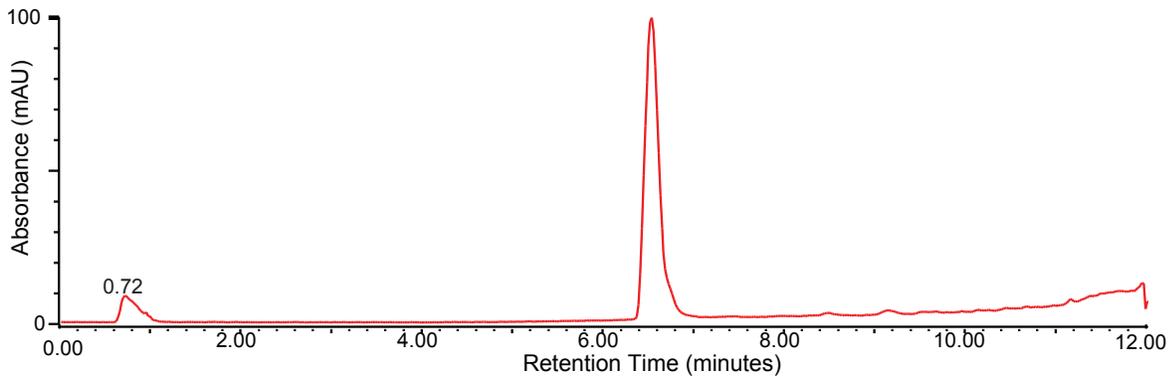
S9. Cytological profiling data. (A) Images of drug-treated HeLa cells stained with Hoechst dye (DNA), anti-phosphohistone H3 antibody (mitotic marker), and EDU (a clickable version of BrdU, a metabolically incorporated nucleoside analog used as an S-phase marker). Cells undergoing DNA synthesis are green, mitotic cells are pink, and cell nuclei are blue. (B) Color bar used to generate cytological profiling fingerprints in Fig. 5.

A   Monensin 9.6 µM   DMSO

Phocoenamicin 73 µM   DMSO

B

−1.00
−0.67
−0.33
0.00
0.33
0.67
1.00
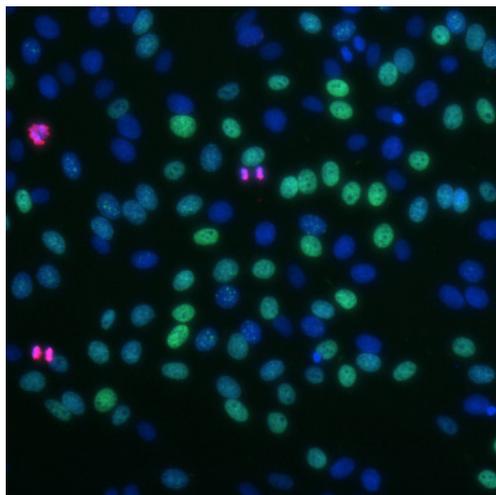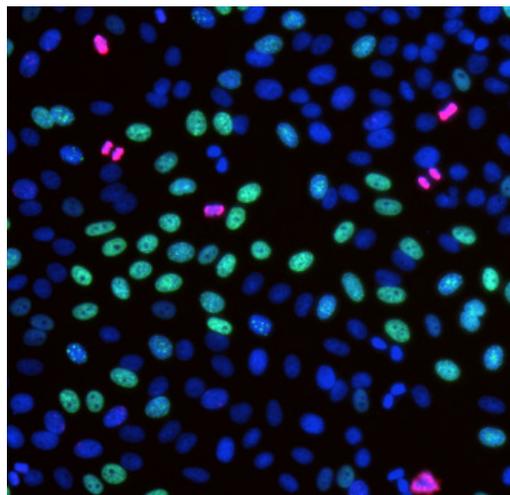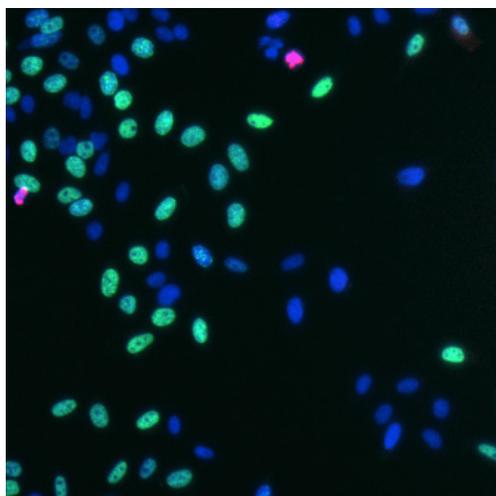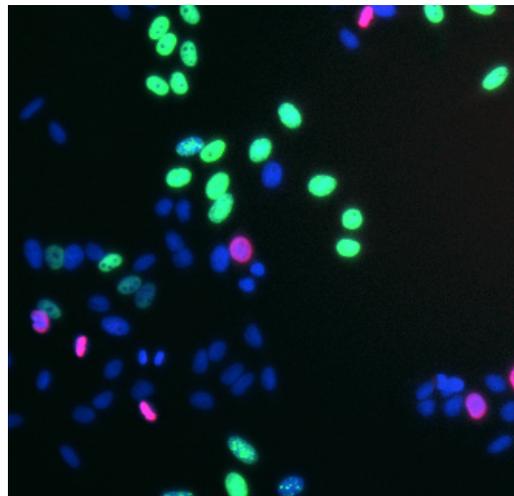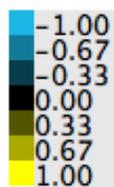
S10. Flow cytometry of *S. aureus* cells treated with phocoenamicin. Results were acquired in triplicate for cells incubated with the membrane potential dye (DiOC$_2$) and treated with CCCP or various concentrations of phocoenamicin around the MIC. Vertical axis represents cell count and is staggered by compound-treatment condition. Average red:green fluorescence ratio represented in bar graph, with error bars representing standard deviation across the triplicate runs.
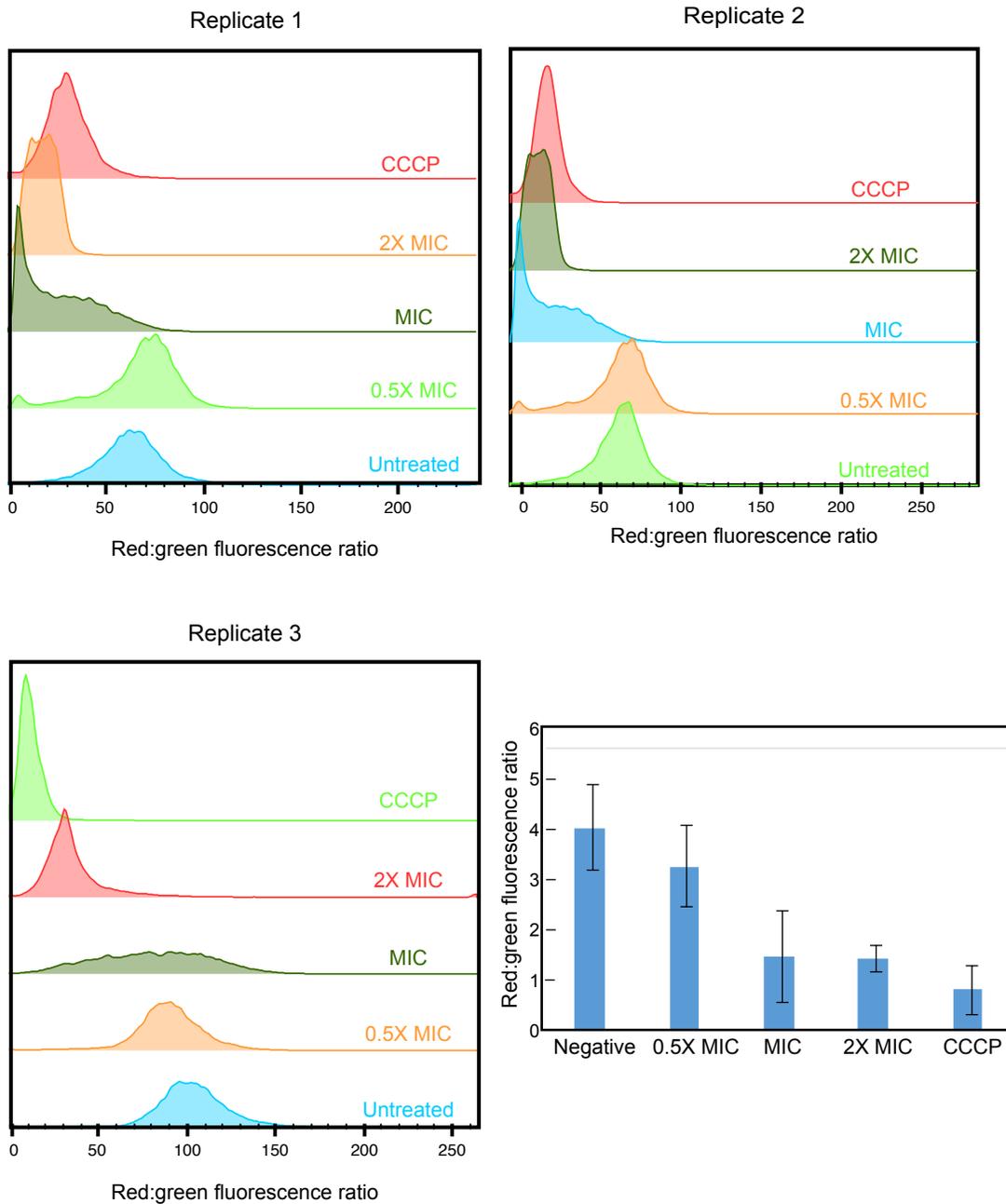
S11. Genetic screen attempting to identify the cellular target of phocoenamicin. (A) Screen of *Bacillus subtilis* essential gene knockdown library against phocoenamicin. Relative fitness distribution of CRISPRi knockdown strains grown on plates containing sub-inhibitory concentrations of trimethoprim (left) and phocoenamicin (right). Screens were performed and relative fitness determined as in previous protocols (38). Strains sensitized to the antibiotic by depletion of the essential gene target are expected to have a lower relative fitness. The target of trimethoprim was accurately identified as DfrA. By contrast, no apparent target was observed for phocoenamicin. (B) Identification of transposon-insertion sites of the strains showing resistance to phocoenamicin. *B. subtilis* transposon insertion library was plated on LB agar plates containing 4X MIC of phocoenamicin. After 16 h of incubation, 95 phocoenamicin-resistant colonies were isolated and the transposon-insertion site was mapped for 20 strains. All 20 transposon insertions were located upstream of the *mdtR-mdtP* operon, inducing overexpression of *mdtP*, which encodes a multidrug efflux pump. Inserted positions (red line) were labeled with coordinates and isolate number (in parentheses).

A

B

27
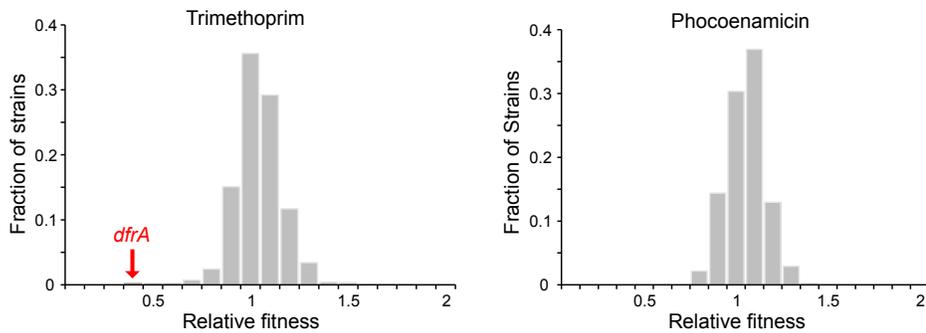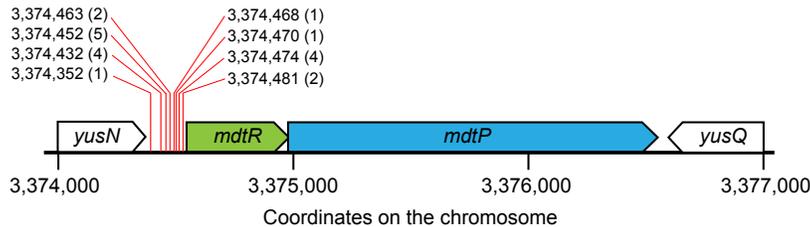
S12. Propidium iodide (PI) staining shows that phocoenamicin does not permeabilize the cell membrane of *S. aureus* cells. Cells were grown overnight in MB medium, back-diluted 1:100 in fresh MB, and then incubated in CellASIC microfluidic chips with the ONIX perfusion system at 37 °C while imaging. 75 µM PI was added to the perfusion medium and cells were treated with DMSO, 0.5X MIC phocoenamicin, or 75% ethanol. The amount of DMSO added was equivalent to the volume present in 0.5X MIC phocoenamicin. For ethanol, cells were treated in an Eppendorf tube, spun down, and then washed in medium before transfer to the CellASIC chip (to avoid ethanol disruption of the fluidics system). (A) Representative images of PI fluorescence in each treatment. Time is measured relative to the time treatment began. Only ethanol treatment cause PI staining. (B) Cell outlines were detected using *Morphometrics*.[5] Average PI fluorescence increased a few minutes after ethanol treatment, whereas fluorescence remained approximately constant after phocoenamicin and DMSO treatment. Error bars are standard deviation over *n* > 150 cells for each treatment.
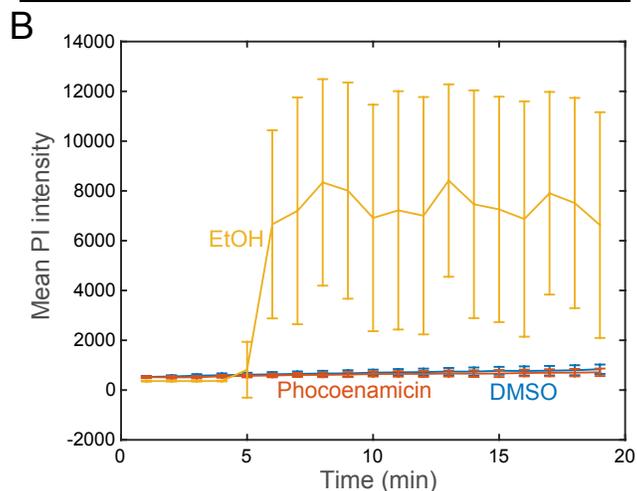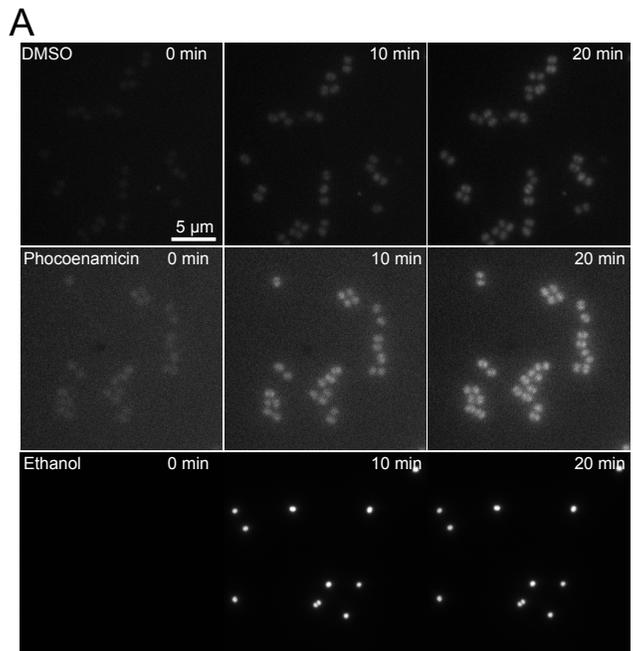
Table S1. Marine mammal necroscopy details. Whole dead intact mammals were recovered as part of the California Marine Mammal Stranding Network, and stored at -20 °C until dissection. All instruments were flame-sterilized prior to dissection and after each cut. The stomach and gastrointestinal tract were separated from the body cavity. 1 mL aliquots sampled along the digestive tract were transferred to sterile 10-mL Falcon tubes at discrete regions: the posterior portion of the intestine, the mid-intestine, the stomach, and the duodenum, as appropriate for each specimen.

Code	Specimen	Species	Common name	Details
MMA	LMLPP2011SEP29	*Phocoena phocoena*	Harbor porpoise	Adult female, killed by dolphins
MMB	LMLPV2013MAR03	*Phoca vitulina*	Harbor Seal	Adult female, possible infection
MMC	LMLZC2013APR10	*Zalophus californianus*	California Sea Lion	Adult female, emaciated
MMD	LMLPP2013JUL23	*Phocoena phocoena*	Harbor porpoise	Infant, killed by dolphins
MME	LMLDC2013JUL31	*Dolphinus capensis*	Common dolphin	Infant, death by separation

Table S2. 16S Sequencing data and organism nomenclature. % identity represents the sequence identity to the closest published strain in NCBI. All extracts are given MMAXX identification codes; only extracts prioritized for biological assays were given RLFI_XXXX codes.

Organism name	MMA10XX	RLFI_XXXX	Accession number	Closest NCBI strain accession number	Description	% identity
MMA 2A HVS/10A	MMA1001	RLFI_1034	KY580789	NR_029077.1	*Bacillus algicola*	98.6
MMA 1B HVS/10A	MMA1002	NA	KY580790	NR_025842.1	*Bacillus firmus*	99.9
MMA 3A HVF/10B	MMA1006	NA	KY580791	NR 025591.1	*Bacillus soli* strain R-16300	98.6
MMA 3A HVS/10C	MMA1009	NA	KY580792	NR_116633.1	*Streptomyces coelicolor* strain DSM	100.0
MMA 3A HVS/10B	MMA1010	RLFI_1038	KY580793	NR_025264.1	*Bacillus hwajinpoensis* strain SW-72	100.0
MMA 4A HVS/10A	MMA1011	NA	KY580794	NR_117285.1	*Bacillus oceanisediminis* strain H2	99.6
MMA 6B HVS/10A_A	MMA1012	NA	KY580795	NR_025842.1	*Bacillus firmus* strain IAM 12464	98.8
MMA 1C HVS/10A	MMA1013	NA	KY580796	NR_024689.1	*Bacillus atrophaeus*	99.8
MMA 1C HVF/10A	MMA1014	NA	KY580797	NR_024689.1	*Bacillus atrophaeus*	99.9
MMA 6A HVF/10A	MMA1017	RLFI_1043	KY580798	NR_108479.1	*Micromonospora zamorensis*	99.5
MMA 4A HVF/10A	MMA1019	RLFI_1044	KY580799	NR_026087.1	*Mycobacterium gadium* strain 1066	99.3
MMA 1A HVF/10A	MMA1020	NA	KY580800	NR_043401.1	*Bacillus megaterium* strain IAM 13418	99.6
MMA 3B HVF/10A	MMA1021	RLFI_1039	KY580801	NR_117473.1	*Bacillus megaterium* strain ATCC 14581	99.9
MMA 5A HVS/10A	MMA1023	NA	KY580802	NR_117285.1	*Bacillus oceanisediminis* strain H2	99.6
MMA 3B HVS/10C	MMA1024	NA	KY580803	NR_117285.1	*Bacillus oceanisediminis* strain H2	98.4
MMA 2B HVS/10A	MMA1025	NA	KY580804	NR_117285.1	*Bacillus oceanisediminis* strain H2	94.4
MMA 3B HVS/10B	MMA1027	RLFI_1040	KY580805	NR_118455.1	*Fictibacillus phosphorivorans*	99.5
MMA 6A NTS/10A	MMA1028	RLFI_1041	NA	NA	NA	NA
MMA 6A NTS/10A	MMA1029	RLFI_1042	KY580806	NR_041229.1	*Streptomyces exfoliatus* strain NBRC 13475	100.0
MMA 4A HVS/10D	MMA1033	NA	KY580807	NR_07016.1	*Bacillus atrophaeus* strain 1942	99.8
MMA 2C HVF/10A_B	MMA1035	NA	KY580808	NR_113748.1	*Paenibacillus glucanolyticus*	99.7
MMA 1A NTS/10A	MMA1036	RLFI_1035	KY580809	NR_041350.1	*Micromonospora coxensis*	98.8
MMA 6B HVS/10A	MMA1037	RLFI_1036	KY580810	NR_028659.1	*Micromonospora auratinigra* strain TT1-11	99.0
MMA 3B HVS/10A_A	MMA1039	RLFI_1037	KY580811	NR_113748.1	*Paenibacillus glucanolyticus* strain NBRC 15330	99.8
MMA 2A SNS/10A	MMA1041	NA	KY580812	NR_075016.1	*Bacillus atrophaeus* strain 1942	99.9
MMA 2B NTS/10A	MMA1042	NA	KY580813	NR_075016.1	*Bacillus atrophaeus* strain 1942	99.9
MMA 6A HTF/10A	MMA1043	NA	KY580814	NR_075016.1	*Bacillus atrophaeus* strain 1942	99.9
MMA 1C HVS/10B	MMA1044	NA	KY580815	NR_075016.1	*Bacillus atrophaeus* strain 1942	99.9
MMA 6A HVS/10A	MMA1045	NA	KY580816	NR_075016.1	*Bacillus atrophaeus* strain 1942	99.9

Table S3. NMR chemical shifts for phocoenamicin at 600 MHz in acetone-d6.

Position	δH (J in Hz)	δC
1	-	175.0, qC
2	-	106.9, qC
3	-	200.3, qC
4	-	50.1, qC
5	1.82, m	42.6, CH
6	1.56, m	38.7, CH
7α	1.75, m	45.0, CH₂
7β	1.20, m	-
8	1.62, m	40.3, CH
9	3.07, dd (10.2, 10.2)	87.6, CH
10	1.94, 1.94, m	47.8, CH
11	6.40, d (10.0)	126.5, CH
12	5.59, ddd (10.0, 6.0, 2.4)	125.5, CH
13	2.67, m	42.6, CH
14	2.03, m	39.4, CH
15	5.43, dd (15.0, 9.0)	144.7, CH
16	5.12, ddd (15.9, 10.8, 2.4)	121.9, CH
17α	2.30, dd (12.6, 10.8)	43.2, CH₂
17β	1.91, dd (12.6, 2.4)	-
18	-	40.6, qC
19	5.05, s	131.0, CH
20	-	134.3, qC
21	2.45, br ddd (9.6, 7.4, 3.1)	33.7, CH
22α	2.30, dd (13.9, 7.4)	29.9, CH₂
22β	1.85, m	-
23	-	86.5, qC
24	-	204.9, qC
25	1.65, s	16.8, CH₃
26	0.83, d (6.6)	23.3, CH₃
27	1.05, d (6.6)	19.8, CH₃
28	0.86, d (7.2)	21.3, CH₃
29	1.27, s	24.4, CH₃
30	1.72, s	22.2, CH₃
31α	1.98, ddd (13.2, 9.6, 4.8)	33.6, CH₂
31β	1.75, ddd (13.2, 10.5, 3.1)	-
32	3.87, d (10.2, 4.8)	73.3, CH
33	-	82.5, qC
34	-	212.8, qC
35	2.21, s	24.9, CH₃
36	1.21, s	22.0, CH₃
1'	4.41, d (6.0)	103.6, CH
2'	3.45, m	74.6, CH
3'	3.45, m	89.5, CH
4'	3.09, m	74.9, CH
5'	3.27, m	72.2, CH
6'	1.24, d (6.6)	18.2, CH₃
1"	4.62, d (7.9)	105.3, CH
2"	3.5, t (8.4)	75.3, CH
3"	3.78, t (9.6)	74.7, CH
4"	4.93, t (9.6)	77.4, CH
5"	3.82, dd (9.6, 6.0)	70.9, CH
6"	1.33, d (6.0)	17.9, CH₃
7"	-	168.0, qC
8"	-	122.6, qC
9"	-	155.6, qC
11"	7.35, d (8.4)	132.6, CH
10"	6.85, d (8.4)	116.2, CH
12"	-	125.7, qC
13"	-	135.7, qC
14"	2.41, s	18.0, CH₃
OH	4.10	-
OH	3.92	-
OH	3.65	-

Table S4. Cation and pH dependence of phocoenamicin MIC. No significant changes above or below a two-fold dilution were observed.

	Phocoenamicin MIC (μM)	Vancomycin MIC (μM)	Daptomycin Mic (μM)
+0 mM Ca	3.5	-	11
+0.5 mM Ca	4.2	-	0.5
+1.25 mM Ca	4.2	-	0.1
pH 7.0	1.3	1.3	-
pH 6.0	0.63	0.78	-
pH 5.0	0.53	1.1	-

Supplementary References

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(2) Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution 39*, 783–791.
(3) Tittensor, D. P., Mora, C., Jetz, W., Lotze, H. K., Ricard, D., Berghe, E. V., and Worm, B. (2016) Global patterns and predictors of marine biodiversity across taxa. *Nature 466*, 1098–1101.
(4) Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol. 28*, 2731–2739.
(5) Ursell, T., Lee, T. K., Shiomi, D., Shi, H., Tropini, C., Monds, R. D., Colavin, A., Billings, G., Bhaya-Grossman, I., Broxton, M., Huang, B. E., Niki, H., and Huang, K. C. (2017) Rapid, precise quantification of bacterial cellular dimensions across a genomic-scale knockout library. *BMC Biology 15*, 17.