Supporting Information

Antimicrobials inspired by nonribosomal peptide synthetase gene clusters

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Reagents, Glassware, and Instruments.

PEGA resins for peptide synthesis were purchased from EMD Millipore. Wang resins with preloaded amino acids were purchased from Matrix Innovation. Coupling reagents (PyBOP and Cl-HOBt) and *N*α-Fmoc/side-chain protected amino acids were purchased from P3 BioSystems, ChemPep Inc., and Chem-Impex International. Racemic mixture of 3-hydroxymyristic acid was purchased from TCI America and pure enantiomers were purchased from Santa Cruz Biotechnology. $DiBAC_4$, (Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol) was purchased from Thermo Fisher Scientific. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and all other chemical reagents were purchased from Sigma-Aldrich. Consumables were purchased from Fisher Scientific and VWR International. Reaction vessels (RV) for manual peptide synthesis were custom made by the Scientific Glassblowing Laboratory at the Department of Chemistry of Yale University. Peptides for primary screens were synthesized using a MultiPep RSi system (Intavis Bioanalytical Instruments AG). Peptides for secondary screens were synthesized either manually or using an Alstra Initiator+ (Biotage LLC). Fluorescence assays were performed on a SpectraMax M2e instrument (Molecular Devices, LLC).

Microbes, Cell Line, and Growth Media.

Microbes and human cell line were purchased from American Type Culture Collection (ATCC) and BEI Resources, and their corresponding growth media are listed in Table S4. Brain heart infusion (BHI), Luria-Bertani (LB), and yeast peptone dextrose (YPD) media were prepared directly from premixed powder purchased from Becton Dickinson and Co. (BD). RPMI 1640 medium was purchased from Thermo Fisher Scientific. Complete growth medium for HT29 cell cultures contains Modified Dulbecco Medium (ATCC) supplemented with 10% (v/v) of fetal bovine serum. YM+ medium (per liter) consists of yeast extract (3 g), malt extract (3 g), glucose (10 g), and peptone (5 g) supplemented with adenine, histidine, leucine, lysine, and uracil (225 mg of each supplement).

Bioinformatic Prediction of NRPs.

Bioinformatic predictions were performed as described previously.[1] Genome sequences were downloaded from the NCBI ftp website (ftp://ftp.ncbi.nih.gov/genomes/). The software package Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) v2.0 was used for the identification and prediction of NRP biosynthetic gene clusters (BGCs) encoded by these genomes. BGCs that contain either a starter condensation domain (C_s) or a thioesterase (TE) domain, indicating distinct start and termination sites respectively, were subjected to bioinformatic prediction. AntiSMASH consults three algorithms to predict the amino acid substrate of each adenylation domain (NRPSPredictor2, Stachelhaus code, and Minowa). Whenever possible we adhere to predictions made by NRPSPred2 as it is the latest and most intricate of the three. In cases where the NRPSPred2 algorithm predicts a cluster of amino acids – a small collection of amino acids with similar side-chain functionalities – we consult the Minowa and Stachelhaus algorithms to look for consensus predictions. An amino acid was chosen arbitrarily within the NRPSPred2 cluster if there was no consensus among the three algorithms. Among canonical aromatic amino acids Tyrosine (Tyr) and phenylalanine (Phe) predictions were chosen manually to override tryptophan (Trp) predictions as we noticed that Trp predictions are overrepresented. Non-proteinogenic amino acids with β-hydroxy and β-methyl modifications were replaced by the corresponding canonical amino acids that are commercially available as SPPS building blocks. The predicted syn-BNP outputs were name serially as *[X.Y]* (Table S1), where *X* indicates the plate number and *Y* indicates the position in a given plate (1 – 96).

Syn-BNPs for Primary Screen.

- **Linker synthesis.** The linker was synthesized based on a previously published procedure on polyethylene glycol amide resins (PEGA).[2] Monomethyl suberate (5 equiv.) was activated by PyBOP (4.95 equiv.), Cl-HOBt (5 equiv.), and DIEA (10 equiv.) in DMF and coupled to the PEGA resins (5 g, designated as 1 equiv.), followed by gentle agitation at room temperature for 2 h. Hydrolysis of the methyl ester was accomplished by immersing the resins in a 6:3:1 mixture of THF/MeOH/10M NaOH, followed by gentle agitation at 37°C for 30 min. The resins were neutralized by 1 M HCl, followed by washes with

water, methanol, dichloromethane, and DMF. The resin was coupled to β-alanine methyl ester (HCl salt), and then ethanolamine, by repeating the above procedure.

- **Amino acid loading.** *N*α-Fmoc/side-chain protected amino acids (8 eq.) were activated by DIC (4 eq.) in the presence of DMAP (0.1 eq.) in a 2:1 mixture of DCM/DMF for 15 min. The activated amino acids were added to the resins and shaken for 2 h. Loading yields were quantitated based on the amount of Fmoc adduct resulting from piperidine microcleavage. The extinction coefficient (ε) of the Fmoc-piperidine adduct at 300 nm is 6,234 $M^{-1}cm^{-1}$ in MeCN.[3] The loading step was repeated for batches with less than 75% yield.
- **Solid-phase peptide synthesis.** Syn-BNPs were synthesized in parallel at 6 to 8 μmol scale using in a 96-well plate format using standard Fmoc-^{t}Bu chemistry. C-terminal lysines were replaced with ornithines in the primary screen in order to streamline linker synthesis and resin loading. In each cycle Fmoc was removed by two rounds of treatment with 20% piperidine in DMF (10 min. each) followed by washing. Repetitive amino acid (300 μmol, designated as 1 equiv.) couplings were conducted using HATU (1 equiv.), Cl-HOBt (1 equiv.) and NMM (1.3 equiv.) in NMP for 90 min. Completed resin-bound peptides were washed and dried under vacuum prior to release (see Primary Screen). Syn-BNPs containing the 3-hydroxymyristic acid (HMA) modification were performed using a racemic mixture of HMA and the resulting diastereomeric mixture was used directly in the primary screens.

Primary Screen.

- **Microbial lawn on agar.** Bacteria / *C. albicans* cultures were grown at 37 / 30ºC to log-phase (OD_{600} 0.5 – 0.8) and added into molten LB / YPD agar at 55ºC to a final OD_{600} of 0.05. The molten agar/ microbial mixture was poured into 150 mm × 15 mm petri dishes (50 mL each) and left in a sterile laminar hood to dry for 1 h.
- **Syn-BNP release by hydrolysis.** The syn-BNPs were released from the PEGA resins by NaOH hydrolysis (100 mM, dissolved in 400 μL of 50% MeOH) with gentle agitation for 8 h. The resins were

removed using a vacuum manifold compatible with 96-well microtiter filter plates and washed with an additional 100 μL of 50% DMSO. The combined filtrate was neutralized by glacial acetic acid to pH 6 – 8. The resulting syn-BNP solutions were designated as "1×" and used directly for antibiosis screening. More than 90% (260/288) of SPPS including all primary and secondary hits, led to the desired syn-BNP detectable by LCMS or MALDI-MS.

- **Antibiosis screening.** Each syn-BNP was screened against seven microbes (the ESKAPE pathogens and *C. albicans*) in duplicate at three concentrations (1×, 1/3×, 1/9×). Aqueous DMSO (20% v/v) was used as the diluent to prepare the 1/3× and 1/9× solutions. Five microliter of each syn-BNP solution was spotted on solidified agar seeded with various microbes. After syn-BNP solutions were absorbed into the agar, all plates were incubated at 30°C and checked for growth inhibition zones the next day.

Syn-BNPs for Secondary Screen.

Syn-BNPs that inhibited microbial growth in the primary screen were subjected to resynthesis and validation (secondary screen). Resyntheses of syn-BNP **1** and its derivatives were outsourced from custom peptide synthesis companies (GenScript). Resynthesis of syn-BNP **2** and its derivatives were performed at 100 μmol scale (designated as 1 equiv.) on preloaded Wang resins using an automated microwave peptide synthesizer. In each cycle Fmoc was removed by two rounds of treatment with 20% piperidine in DMF (10 min. each) followed by washing. Repetitive amino acid (4 equiv.) couplings were conducted using PyBOP (3.95 equiv.), Cl-HOBt (8 equiv.) and DIEA (8 equiv.) in DMF at 70ºC for 15 min. Completed resin-bound peptides were washed and cleaved by TFA supplemented with water and TIPS (2.5% of each). The cleavage solution was then added into cold *tert*-butyl methyl ether and spun at 4,000 × g at 4°C for 15 min. The supernatant was discarded and the pellet was dissolved in 50% MeCN for HPLC purification. All peptides were verified by high-resolution mass spectrometry (HRMS, Table S2). Note that HMA-modified syn-BNPs tested in our primary screens are diastereomeric mixtures as a result of using a racemic mixture of HMA in the *N*-acylation reaction. For secondary screens, two different syn-BNP **2** were thus synthesized using the two enantiomers of HMA. These compounds were each purified to give separate diasteremoers of syn-BNP

2. The resulting compounds showed identical HPLC retention times and exhibited the same MICs against *C. albicans*. All additional analogs of syn-BNP **2** were thus synthesized using a racemic mixture of HMA and tested as diastereomeric mixtures.

Secondary Screen and Susceptibility Assays.

- **Standard assays.** Susceptibility assays were performed in in 96-well microtiter plates in accordance to protocols for the determination of MIC by broth microdilution method recommended by Clinical and Laboratory Standards Institute (CLSI).[4] DMSO stock solutions of syn-BNPs (12.8 mg/mL) were added into the first well of each row and serially diluted (1/2× per transfer) across the microtiter plate. The last 2 wells were reserved for positive (peptide-free) and negative (microbe-free) controls. Overnight bacteria / yeast cultures were diluted 5,000-fold and used as the inoculum (50 μL) to reach a final volume of 100 μL in each well. Minimum inhibitory concentrations (MIC) were determined by visual inspection after static incubation at 37°C (bacteria) / 30°C (yeast) for 18 h. All assays were done at least in duplicate. Microbial strains and media are listed in Table S4.
- **Assays with ergosterol and sorbitol supplements.** Susceptibility assays were setup as described above in the presence of ergosterol (400 μg/mL) or sorbitol (0.8 M) to investigate the mode of action of syn-BNP (**2**) on yeast. Both assays were done in duplicate.

Selection of *E. faecium* Mutants.

- **By direct plating.** A single *E. faecium* Com15 colony from a freshly struck plate was inoculated into LB medium and grown overnight at 37°C. The overnight culture was diluted 100-fold, supplemented with syn-BNP (**1N**) at 20 μg/mL (2.5× MIC), and distributed into 480 individual microtiter wells (100 μL per well). These microtiter plates were incubated at 37°C for 18 h and no resistant mutant could be raised.
- **By Serial Passage.** A single *E. faecium* Com15 colony (the mother) from a freshly struck plate was inoculated into LB medium and grown overnight at 37°C. The overnight culture was split into eight lineages. After 24 h of growth at 37°C, the bacterial culture from each lineage was diluted 200-fold into

LB (1 mL) containing the next higher concentration of syn-BNP **1N**. All lineages were passaged successively through 2, 4, 8, 12, 16, and 20 μg/mL of syn-BNP **1N**. Cultures at the end of the passage were plated onto antibiotic free LB agar, and the MIC of three colonies from each lineage were tested by broth microdilution. No colonies showed MIC values higher than that of the mother strain.

Cytotoxicity Assay.

Cytotoxicity of syn-BNPs were assessed using human HT29 cells. For each assay 5,000 HT29 cells per well were seeded in a microtiter plate and cultured for 24 h at 37°C in a 5% CO_2 atmosphere. Syn-BNPs were supplemented at concentrations ranging from 128 to 0.5 μg/mL and the cells were incubated for another 20 h. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).[5] For staining purposes growth media was removed and a solution of MTT (220 μL, 0.5 mg/mL in water) was added to each well. After a 4-hour incubation the MTT solution was removed and the wells were scored for cell presence by visual inspection. All assays were done in duplicate.

Membrane Depolarization Assay.[6]

Morphologically similar colonies of *A. baumannii* ATCC 17978 were scraped off LB agar plates and suspended in LB media to an OD_{600} of 0.5. The resulting suspension (1.8 mL) was mixed with $DiBAC_4$ (0.2 mL, 20 μg/mL solution in LB) and incubated at room temperature for 5 minute. The excitation, emission and filter wavelengths were set at 492, 518 and 515 nm, respectively. The mixtures of bacteria and fluorophore were first monitored for 1 min. Antibiotics (or DMSO) were then added to final concentrations that correspond to 3× their respective MICs and monitored for 5 min. Finally, polymyxin B was added as a positive control (2.54 μL, 12.8 mg/mL) to a final concentration of 125 μM and monitored for 3 min. The antibiotics were prepared as LB solutions (100 μL) with 8% DMSO (v/v) and fluorescence intensities were recorded at 2 second intervals. The same assay was performed on the *E. faecium* Com15 except that sodium deoxycholate was added as a positive control (50 μL, 1% (w/v)) to a final concentration of 0.05% (w/v).[7] Both assays were performed in duplicate.

Supporting References.

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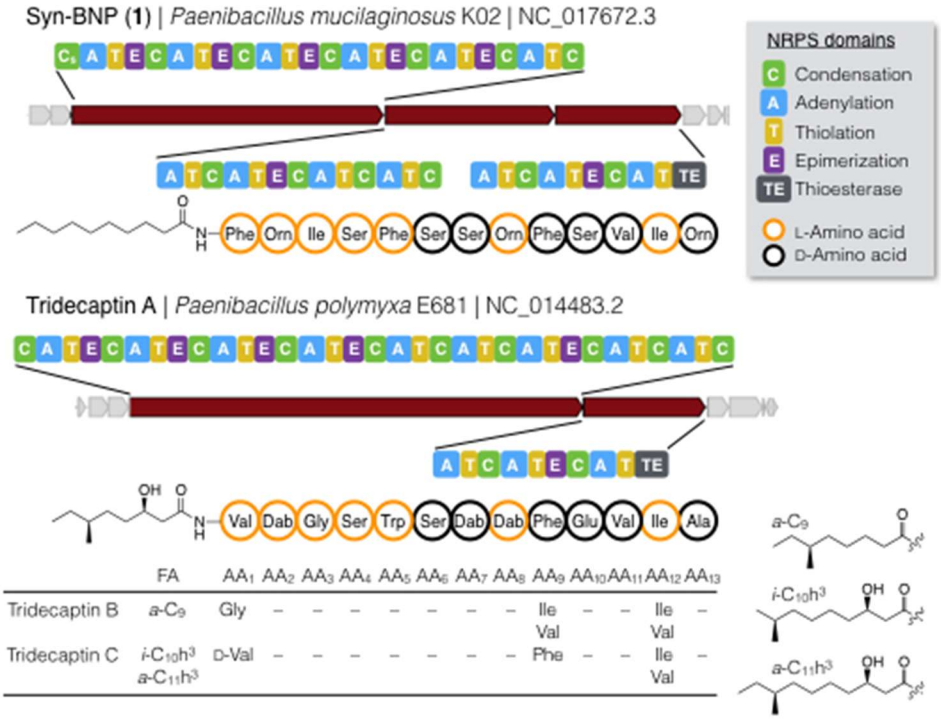
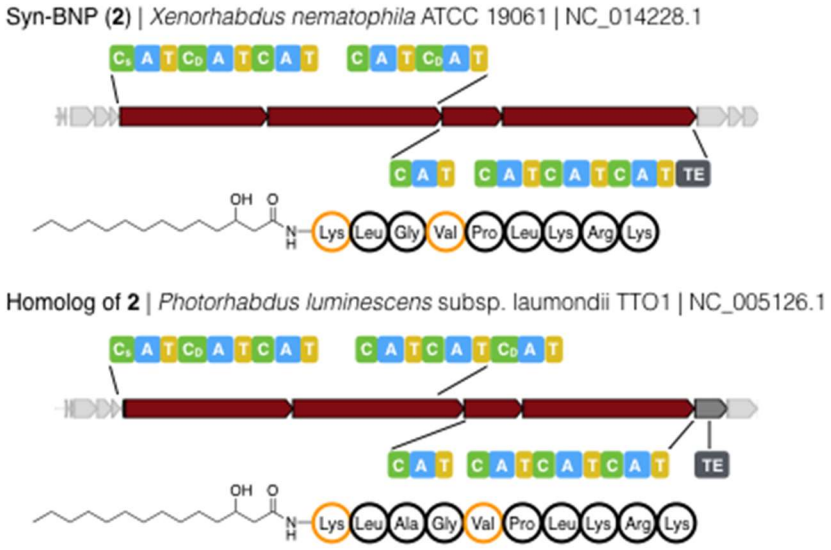
Figure S1. Comparison of NRPS BGCs encoding syn-BNP (**1**) and tridecaptin A-C.



	FA	AA1	AA2	AA3	AA4	AA5	AA6	AA7	AA8	AA9	AA10	AA11	AA12	AA13
Tridecaptin B	*a*-C9	Gly	–	–	–	–	–	–	–	Ile Val	–	–	Ile Val	–
Tridecaptin C	*i*-C10h3 *a*-C11h3	D-Val	–	–	–	–	–	–	–	Phe	–	–	Ile Val	–

Figure S2. Comparison of NRPS BGCs encoding syn-BNP (**2**) and its homolog.



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