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Methods.
No unexpected safety hazards were encountered during the experiments conducted for this study. Reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was performed using a Bruker UltrafleXtreme MALDI-TOF/TOF mass spectrometer in reflector positive mode at the University of Illinois School of Chemical Sciences Mass Spectrometry Laboratory. Calibration was performed using a peptide calibration kit (AnaSpec Peptide Mass Standard Kit). Electrospray ionization (ESI)-MS/MS analyses were performed using a ThermoFisher Scientific Orbitrap Fusion ESI-MS with an Advion TriVersa Nanomate 100.

Bioinformatic analysis of glycosylated polyene macrolide biosynthetic gene clusters.
Position-iterative BLAST (PSI-BLAST) was performed on the NCBI non-redundant database using known glycosylated polyene macrolide (GPM) protein sequences from the amphotericin, natamycin, and candicidin biosynthetic gene clusters (BGCs). Sequences retrieved in October 2019 using three or less iterations with an expectation values lower than 0.05 were retained. A non-redundant list of P450, AmphI, glycosyltransferase, and aminotransferase (mycosamine synthase) orthologs were compiled (Supplementary File 1) and used as input for the bioinformatics program RODEO (www.ripprodeo.org). Custom profile Hidden Markov Models (pHMMs) were generated for each class of post-PKS tailoring enzyme that has been characterized by compiling a list of all verified GPM clusters as well as closely-related gene clusters, aligning sequences through MAFFT and converting into pHMMs using the HMMER3 software package. The resulting pHMMs are available as a part of Supplementary File 2. These pHMMs were used in conjunction with RODEO to analyze open reading frames (ORFs) near each input protein. BGCs with co-occurrence of multiple GPM modifying enzymes as well as polyketide synthase (PKS) enzymes were included for further analysis. Putative complete GPM BGC (i.e. all genes expected to encode the PKS, modifying enzymes, and transporters) were downloaded in fasta format from NCBI and submitted for analysis by antiSMASH and PRISM to evaluate PKS module identity. Predicted macrolide scaffolds were constructed via module by module analysis of PKS ORFs obtained from antiSMASH/PRISM. All maximum-likelihood trees were created by aligning sequences using MAFFT 7.394 with the G-INS-I option and transformed into maximum-likelihood trees using FastTree 2.1 with default Jones-Taylor-Thornton (JTT) model. Trees were visualized on the Interactive Tree of Life (iTOL) website (http://itol.embl.de).7

Whole-genome sequencing.
Streptomyces eurocidicus ISP-5604 and Streptomyces “eurocidicus” B-1677 are known eurocidin producers. These strains were grown for 5 d in ATCC 172 liquid medium and 10 mL portion of the cultures were harvested by centrifugation (4,000 × g, 15 min at 4 °C) with the supernatant discarded. The genetic DNA was extracted using a Qiagen DNeasy UltraClean Microbial Kit. Genomic libraries were prepared with an Illumina-Compatible KAPA DNA Library Preparation Kit (Kapa Biosystems). Paired-end sequencing was performed on an Illumina MiSeq with 250 nt reads by the Roy J. Carver Biotechnology Center (University of Illinois, IL, USA). Genomic assembly was performed with CLC Genomic Workbench (CLC Bio) and genomes were uploaded to the NCBI database under the following identifying codes:

| Bacterial Strain          | BioProject | Accession      |
|---------------------------|------------|----------------|
| *Streptomyces eurocidicus* ISP-5604 | PRJNA593613 | WPBY00000000 |
This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accessions WPBY00000000 and WPBZ00000000. *Streptomyces* “eurocidicus” NRRL B-1677 was determined to be misidentified and thus has been renamed as *Streptomyces* sp. NRRL B-1677. The versions described in this paper are versions WPBY01000000 and WPBZ01000000, respectively.

Entries for eurocidin D/E, rimocidin, and lucensomycin have been deposited into the Minimum Information about a Biosynthetic Gene cluster (MIBiG) repository under the accession numbers BGC0002104, BGC0002105, and BGC0002106, respectively.

**General preparation of cell extracts for screening.**

Actinomycetes were optimized for secondary metabolite production on one of the following five media:

- **ATCC #172 medium** (1 L contains: 10 g glucose, 20 g soluble starch, 5 g yeast extract, 5 g N-Z Amine, 1 g CaCO$_3$, 15 g agar)
- **ISP4 medium** (1 L contains: 10 g soluble starch, 1 g K$_2$HPO$_4$, 1 g MgSO$_4$, 1 g NaCl, 2 g (NH$_4$)$_2$SO$_4$, 2 g CaCO$_3$, 1 mg FeSO$_4$$\cdot$7H$_2$O, 1 mg MnCl$_2$$\cdot$7H$_2$O, 1 mg ZnSO$_4$$\cdot$7H$_2$O, 15 g agar)
- **altMS medium** (1 L contains: 10 g mannitol, 10 g soya flour (Wel-Pac brand), 10 g malt extract, 15 g agar)
- **ISP2 medium** (1 L contains: 4 g yeast extract, 10 g malt extract, 4 g dextrose, 15 g agar)
- **SGG medium** (1 L contains: 10 g starch, 10 g glucose, 10 g glycerol, 2.5 g corn steep powder, 5 g peptone, 2 g yeast extract, 1 g NaCl, 3 g CaCO$_3$, 15 g agar).

Actinomycetes seed cultures were grown in 5 mL liquid ATCC 172 medium for 3 d at 30 °C then transferred onto ATCC 172 solid agar medium and grown for 7 d at 30 °C. Afterwards, the cells were scraped from the surface of the agar and extracted at room temperature with methanol and agitation for 2 h. The extract was separated from the cell mass by centrifugation (4000 × g, 15 min at 4 °C). The extracts were then analyzed by MALDI-TOF-MS.

**Isolation of GPMs.**

**Tetrazine labeling.**

Extract labeling reactions were performed in methanol unless otherwise stated. Labeled compounds exhibit a mass shift of either + 206 Da (re-aromatized adduct) or +208 Da (tautomericized adduct, Figure 3). In labeling reactions performed in methanol, 5 µL extract was mixed with 20 µL of 10 mM 3,6-di-2-pyridyl-1,2,4,5-tetrazine 1 in a 0.65 mL microfuge tube at a final concentration of 8 mM tetrazine in 25 µL volume. The extracts were then reacted at room temperature for 12 h followed by analysis by MALDI-TOF-MS. Commercially available compounds were labeled in a similar manner. For chloroform-soluble compounds, a 40 µL solution was prepared in a 0.4 mL reactivial containing a final concentration of 1 mM compound and 8 mM 3,6-di-2-pyridyl-1,2,4,5-tetrazine. Compounds used in scope of reactivity testing were obtained from the open compound repository of the National Cancer Institute through the developmental therapeutics program.

**Kineosporicin:** *Actinokineospora spheciospongiae* DSM 45935 was grown on altMS solid medium for 7 d at 30 °C (15 cm sterilized petri dishes, n = 450). Cells were harvested by scraping cell mass from plates with a sterile razor blade, followed by 3 extractions with 1 L MeOH and shaking at room temperature for 2 h per extraction. The methanolic extracts were vacuum-filtered and dried under reduced pressure onto 20 g of Celite 545 adsorbent. Purification employed a Teledyne Isco CombiFlash EZprep equipped with a RediSep Rf C18 cartridge (130 g, 60 Å pore size, 40-63 µm particle size, 230-400 mesh) with a mobile phase of 10 mM aq. NH$_4$HCO$_3$/MeOH at 75 mL min$^{-1}$ over a gradient from 10-90% MeOH (10 column
volumes), then a 90% MeOH hold (5 column volumes). Fractions were analyzed by MALDI-TOF-MS and kineosporicin-containing fractions dried under reduced pressure.

Semi-pure kineosporicin was then dissolved in 10 mL of 80/20 10 mM aq. NH₄HCO₃/MeCN and 0.22 µm syringe filtered (BD technologies) prior to purification using a Perkin Elmer Flexar HPLC equipped with a Betasil C18 (ThermoFisher Scientific) reverse phase column (250 × 10 mm, 5 µm particle size). 2 mL dissolved kineosporicin was injected with a mobile phase of 10 mM aq. NH₄HCO₃/MeCN with a gradient from 20-60% MeCN over 20 min followed by 60-95% MeCN over 5 min. Sample absorbance was monitored at 220, 280, and 320 nm and kineosporicin-containing fractions were dried under reduced pressure, dissolved in water (3 mL), flash frozen, and lyophilized overnight to dryness. Kineosporicin was collected as a slightly yellow tinted powder. Post-purification yield for kineosporicin was ~16 µg/15 cm petri dish.

**Lucensomycin:** *Streptomyces viridosporus* NRRL ISP-5243 was grown on ISP2 liquid medium for 7 d at 30 °C (6 L). Cells were harvested by centrifugation (11,000 × g, 25 minutes at 4 °C). Aqueous supernatant was separated from cell pellet and frozen prior to lyophilization. The cell mass was extracted twice (1 L MeOH with shaking at room temperature) and the MeOH dried under reduced pressure adsorbed onto 20 g Celite 545. The lucensomycin on adsorbed Celite was then purified using conditions similar to those for kineosporicin. Lucensomycin was collected as a slight yellow-tinted powder. Post-purification yield for lucensomycin was ~1.5 mg/L culture.

**High-resolution MS analysis.**
Kineosporicin and lucensomycin were desalted using a ZipTip and eluted into 75% aq MeCN. Samples were directly infused onto a ThermoFisher Scientific Orbitrap Fusion ESI-MS using an Advion TriVersa Nanomate 100. MS calibration was performed with Pierce LTQ Velos ESI Positive Ion Calibration Solution (ThermoFisher). The MS was operated using the following parameters: 100,000 resolution, 2 m/z isolation width (MS/MS), 35 normalized collision energy (MS/MS), 0.4 activation q value (MS/MS), and 30 ms activation time (MS/MS). Fragmentation was performed using collision-induced dissociation (CID) at 70%. Data analysis was conducted using the Qualbrowser application of Xcalibur software (ThermoFisher Scientific).

**NMR spectroscopic characterization.**
HPLC-purified GPMs were dissolved in 600 µL of 1:1 v/v CD₃OD 0.05% TMS:C₅D₅N (CD₃OD: Cambridge Isotope Labs, >99.8% atom %D, C₅D₅N: Cambridge Isotope Labs, >99.5% atom %D). NMR spectra were recorded on an Agilent VNMRS 750 MHz narrow bore magnet spectrometer equipped with a 5 mm triple resonance (1H-13C-15N) triaxial gradient probe and pulse-shaping capabilities. The samples were kept at 25 °C during acquisition. Standard Varian pulse sequences were used for each of the following experiments: 1H, 1H-1H TOCSY (80 ms mixing time), 1H-1H NOESY (400 ms mixing time). Solvent suppression was not used for 1D or 2D experiments. Spectra were recorded in VNMRJ 3.2A and data processing was done using MestReNova version 8.11.

**Quantitative NMR for extinction coefficient determination.**
The concentration of HPLC purified kineosporicin was determined via quantitative NMR on a Bruker Carver B500 NMR spectrometer equipped with a 5 mm broadband Cryoprobe. An unknown amount of kineosporicin was dissolved in 300 µL CD₃OD (Cambridge Isotope Labs, >99.8% atom %D) and UV-vis absorbance spectra were obtained on a Cary 4000 UV–vis spectrophotometer (Varian). This solution of kineosporicin was then diluted to 600 µL 1:1 v/v CD₃OD:C₅D₅N (CD₃OD: Cambridge Isotope Labs, >99.8% atom %D, C₅D₅N: Cambridge Isotope Labs, >99.5% atom %D) with a final concentration of 10 mM dimethyl terephthalate as calibrant. Samples were held at 25 °C during acquisition and standard Bruker pulse sequences were used with 30 s T₁ relaxation times to acquire 1H-NMR spectra over 128 scans.
Kineosporicin concentrations were determined by comparing the ratios of the dimethyl terephthalate aromatic proton integrals with those of the mycosamine methyl proton integrals. This was performed in triplicate and the averaged values were used to determine the extinction coefficients at each absorbance maximum.

**Antibacterial assay.**
Microbroth dilution assays were used to determine the minimum inhibitory concentration (MIC) of kineosporicin against the following strains: *Staphylococcus aureus* USA300, *Pseudomonas aeruginosa* PA01, *Bacillus anthracis* strain Sterne, *Escherichia coli* MC4100, and *Enterococcus faecium* U503. These bacteria were grown in 5 mL brain heart infusion (BHI) broth (BD Sciences) overnight at 37 °C to stationary phase. Cultures were then diluted 50-fold in 5 mL of fresh BHI and grown back to mid-exponential phase (OD$_{600}$ = 0.4) and diluted to OD$_{600}$ = 0.015. Kineosporicin was serially diluted (2-fold) with BHI in a 96-well microtiter plate and an equal volume of bacterial culture in BHI was added to each well. Cultures were grown with shaking at 37 °C and the MIC reported is that which resulted in no visible growth after 18 h.

**Antifungal assays.**
The protocol for MIC determination on *Candida* sp. was adapted from CLSI publication M27, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts 4th Ed. *Candida* sp. were subcultured on Difco Sabouraud dextrose agar (SDA; Becton, Dickinson, and Co.) plates at 35 °C for 24 h. Single colonies were selected and suspended in 1 mL sterile saline (85% w/v) and diluted 1:10 in HyClone RPMI-1640 medium (GE Healthcare Life Sciences) with 165 mM 3-(N-morpholino)propanesulfonic acid (MOPS, Fisher Scientific), pH 7.0. Cell density was determined using a hemocytometer with the final inoculum diluted using RPMI medium to $1 \times 10^3$ colony forming units (CFU)/mL. Compounds tested were prepared in a dilution series using a solution of dimethyl sulfoxide (DMSO, D6-99.9%; Cambridge Isotope Laboratories). Each dilution series was made at 100x final concentration and diluted again in 1:2.5 RPMI medium. DMSO without GPM was used as negative control for culture growth. Each GPM concentration was then diluted 1:40 in the final cell suspension inoculum to a volume of 200 µL in a round-bottom, 96-well plate (Corning) in duplicate. Similarly, DMSO alone was diluted in RPMI medium for the negative control. 96-well plates were then incubated statically at 35 °C for 24 h. Immediately after incubation, MICs were determined by visual inspection at the concentration where no difference was noticed compared to the negative control when looking from the bottom of the plate, averaging the values of both replicates.

The protocol for MIC determination on *Aspergillus* sp. contained changes based on CLSI publication M38, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi 3rd Ed. *Aspergillus* sp. were also subcultured on Difco Sabouraud dextrose agar (SDA; Becton, Dickinson, and Co.) plates at 35 °C for 5 d. Following growth, 1 mL sterile saline (85% w/v) with 0.1% Tween 20 was pipetted onto the plate and fungal spores were resuspended with mild agitation and transferred to a separate tube. This spore suspension was then diluted 1:1000 in RPMI medium and density determined using a hemocytometer with a final concentration of $1 \times 10^4$ spores/mL in RPMI medium. Following dilution of GPM compounds in the final inoculum, the resulting 96-well plates were incubated at 35 °C for 2 d.

**Isothermal Titration Calorimetry.**

**General Information.**
Experiments were performed using a NanoITC isothermal titration calorimeter (TA Instruments, Wilmington, DE). Solutions of the GPMs to be tested were prepared by diluting a 15.0 mM stock solution of the compound in DMSO to 150 µM with K buffer (5.0 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES)/ potassium 4-(2-hydroxyethyl)-1-piperazinethanesulfonate (KHEPES), pH = 7.4) and a final concentration of DMSO of 1% v/v 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) large unilamellar vesicles (LUVs) were prepared and phosphorus and ergosterol content quantified as described below. LUV solutions were diluted with K buffer and DMSO to give a final phospholipid concentration of 8.0 mM in 1% DMSO/K buffer solution. Immediately prior to use, all
solutions were degassed under vacuum at 25 °C for 10 min and the reference cell of the instrument (0.191 mL) was filled with a solution of 1% v/v DMSO/K buffer.

**LUV Preparation.**

POPC was obtained as a 20 mg/mL solution in CHCl₃ (Avanti Polar Lipids) and was stored at -20 °C under an atmosphere of dry argon and used within 1 month. A 4 mg/mL solution of cholesterol in CHCl₃ was also prepared monthly and stored at 4 °C under an atmosphere of dry argon. Prior to preparing a lipid film, the solutions were warmed to ambient temperature to prevent condensation contamination. A 13 × 100 mm test tube was charged with 800 µL POPC and 230 µL ergosterol solution. For ergosterol-free liposomes, a 13 × 100 mm test tube was charged with 800 µL POPC. The solvent was removed with a gentle stream of nitrogen and the resulting lipid film was stored under high vacuum for a minimum of 8 h prior to use. The film was then hydrated with 1 mL K buffer and vortexed vigorously for approximately 3 min to form a suspension of multilamellar vesicles (MLVs). The resulting lipid suspension was pulled into a Hamilton 1 mL gastight syringe and the syringe was placed in an Avanti Polar Lipids Mini-Extruder. The lipid solution was then passed through a 0.20 µm Millipore polycarbonate filter 21 times, with the newly formed LUV suspension being collected in the syringe that did not contain the original suspension of MLVs to prevent carryover of MLVs into the LUV solution.

**Determination of Phosphorus Content.**

Determination of total phosphorus was adapted from a previous report.⁹ The LUV solution was diluted tenfold with K buffer and three 10 µL samples of diluted LUV suspension were added to three separate 7 mL vials. Subsequently, solvent was removed with a stream of N₂. To each dried LUV film, and a fourth vial containing no lipids that was used as a blank, 450 µL of 8.9 M H₂SO₄ was added. The four samples were incubated open to ambient atmosphere in a 225 °C aluminum heating block for 25 min and then moved to 23 °C and cooled for 5 min. After cooling, 150 µL of 30% w/v aq. hydrogen peroxide was added to each sample, and the vials were returned to the 225 °C heating block for 30 min. The samples were then moved to 23 °C and cooled for 5 min before the addition of 3.9 mL water. Then 500 µL of 2.5% w/v ammonium molybdate was added to each vial and the resulting mixtures were then vortexed briefly and vigorously five times. Subsequently, 500 µL of 10% w/v ascorbic acid was added to each vial and the resulting mixtures were then vortexed briefly and vigorously five times. The vials were enclosed with a Polytetrafluoroethylene (PTFE) lined cap and then placed in a 100 °C aluminum heating block for 7 minutes. The samples were then removed to 23 °C and cooled for approximately 15 min prior to analysis by UV/Vis spectroscopy. Total phosphorus was determined by observing absorbance at 820 nm and comparing to a standard curve obtained through this method and a standard phosphorus solution of known concentration.

**Determination of Ergosterol Content.**

Ergosterol content was determined spectrophotometrically. A 50 µL portion of the LUV suspension was added to 450 µL 2:18:9 hexane:isopropanol:water (v/v/v). Three independent samples were prepared and then vortexed vigorously for approximately 1 min. The solutions were then analyzed by UV/Vis spectroscopy and the concentration of ergosterol in solution was determined by the extinction coefficient of 10,400 L mol⁻¹ cm⁻¹ at the UV absorbance max of 282 nm and was compared to the concentration of phosphorus to determine the percent sterol content. The extinction coefficient was determined independently in the above ternary solvent system. LUVs prepared by this method contained between 7 and 14% ergosterol.

**Titration Experiments.**

Titrations were performed by injecting the LUV suspension at ambient temperature into the sample cell (volume = 0.191 mL) which contained the 150 µM solution of the GPM in question at 25 °C. The volume of the first injection was 0.23 µL. Consistent with standard procedure, due to the large error commonly associated with the first injection of ITC experiments, the heat of this injection was not included in the analysis of the data.¹⁰ Next, nineteen 2.49 µL injections of the LUV suspension were performed. The time
interval between each injection was 300 s to ensure a stable baseline prior to the subsequence injection. The rate of stirring for each experiment was 350 rpm.

**Data Analysis.**
NanoAnalyze software (TA Instruments) was used for baseline determination and integration of the injection heats, and Microsoft Excel was used for subtraction of dilution heats and the calculation of overall heat evolved. To correct for dilution and mixing heats, the heat of the final injection from each run was subtracted from all the injection heats for that particular experiment. By this method, the overall heat evolved during the experiment was calculated using the following formula:

$$\text{ucal}_{\text{overall}} = \sum_{i=1}^{n} (\Delta h_{\text{injection}}^{i} - \Delta h_{\text{injection}}^{n})$$

where $i =$ injection number, $n =$ total number of injections, $\Delta h_{\text{injection}}^{i} =$ heat of the $i$th injection $\Delta h_{\text{injection}}^{n} =$ the heat of the final injection of the experiment.

**Potassium Efflux Assay.**
An overnight culture of *C. albicans* SN250 in RPMI was centrifuged at 300 × g for 5 minutes at 23 °C. The supernatant was decanted, and the cells were washed twice with sterile water. After the second wash step, the cells were suspended in 150 mM NaCl, 5 mM HEPES pH 7.4 (Na buffer) to an OD$_{600}$ of 1.5 (∼1 × 10$^9$ CFU/mL) as measured by a Thermo Scientific NanoDrop spectrophotometer. A 3 mL sample of the cell suspension was taken to a glass vial held in an aluminum block with stirring for approximately 5 minutes before data collection. The probe was then inserted, and data collected for 5 minutes before adding 30 µL of the GPM compound in question as a 0.3 mM solution in DMSO, resulting in a final concentration of 3 µM GPM in cell suspension. The cell suspension was stirred, and data collected for 10 minutes and then 30 µL of a 1% aqueous solution of digitonin was added to complete potassium release. The endpoint readout was recorded after another 15 minutes. The difference between the initial and endpoint readout was used as 100% potassium release.
Supplemental Figure S1: Structural diversity in reported glycosylated polyene macrolide (GPMs). a, Canonical GPMs, featuring a mycosamine sugar and combinations of backbone hydroxylation, epoxidation, and hemiketal exocyclic methyl group carboxylation. b, Structurally divergent GPMs, featuring carboxamide installation (rimocidin B), diglycosylation (NPP A1 and selvamicin), para-aminobenzoic acid N-methylation, perosamine sugar (perimycin), and divergent hydroxylation (selvamicin). A highly conserved carbon framework encompassing the last alkene in the polyene region through the hemiketal is highlighted in blue. Prior to this study, perimycin and selvamicin were the only non-mycosamine containing GPMs.
Supplemental Figure S2: Phylogenetic trees of GPM tailoring enzymes. Trees were constructed from homologs of AmphL (polyol backbone hydroxylation or epoxidation), AmphDII (aminotransferase for mycosamine or perosamine formation), and AmphN (hemiketal exocyclic methyl group oxidation). Trees are annotated with the predicted GPM type. Cases classified as “indeterminate” were from incomplete BGCs where insufficient information could be gleaned from neighboring genes. AHBA: 3-amino-5-hydroxybenzoic acid.
Supplemental Figure S3: Maximum likelihood phylogenetic tree of GPM thioesterases. The tree is rooted with the thioesterase of linear polyene polyol natural product, mediomycin A (NCBI accession ID: MF139773.1). The thioesterase of linear glycosylated polyene meiijiemycin is also featured in grey as the closest relative to the mediomycin A thioesterase. GPM-forming thioesterases from reported BGCs are included with thioesterases from newly predicted GPM BGCs.
Supplemental Figure S4: Bioinformatic analysis of predicted methyl-substituted tetraene GPMs. a, Representative BGC of predicted methyl-substituted tetraene GPM from *Pseudonocardia*. b, Functional prediction of PKS modules predictions from the *Actinokineospora mzaensis* BGC. Red, segment of polyketide backbone derived from methylmalonyl-CoA. Ketoreductase (KR) domain with dashed outline is predicted to be catalytically inactive akin to KR5 of Amph1 (see Figure 1). c, Predicted structures with potential modifications predicted by pHMM analysis of tailoring enzymes shown in grey (i.e., oxidation and glycosylation). The appended sugar is predicted by profile hidden Markov model to be perosamine (4,6-dideoxy-4-aminomannose, see Supplemental File 2).
Supplemental Figure S5: Bioinformatic analysis of predicted AHBA-heptaene GPMs. a, Representative BGCs of predicted AHBA-loading heptaene macrolides from *Pseudonocardia*. b, Functional prediction of PKS modules predictions from the *Lentzea waywayandensis* BGC. All other non-truncated AHBA-containing heptaene BGCs harbor an identical 26-module architecture. Ketoreductase (KR) domains with dashed outlines are predicted to be catalytically inactive akin KR5 of AmphI (see Figure 1). c, Linear ACP-bound structure as predicted by PKS modules and post-TE macrolide product, with potential modifications predicted by pHMM analysis of tailoring enzymes shown in grey (i.e. oxidation and glycosylation).
**Supplemental Figure S6: Bioinformatic analysis of predicted hexaene GPMs.**

*a*, Representative BGCs. *b*, Bioinformatic predictions of PKS modules from the *Amycolatopsis jejuniensis* BGC; modules are identical to *Amycolatopsis saalfeldensis* BGC. Colorless, dotted modules are inactive. *c*, Linear ACP-bound structure as predicted by PKS modules and post-TE macrolide product, with potential modifications predicted by HMM analysis of tailoring enzymes shown in grey.
Supplemental Figure S7: Probe labeling of terminal olefin- and polyene-containing compounds.
Labeling reactions of pure amphotericin B (amB), erlotinib, FK506, quinine (previous page), NSC 761910, and everolimus (current page) with 1. In all cases, 8 mM 1 was reacted with 1 mM compound in MeOH for 10 h. Ions are [M+H]^+ unless otherwise noted. Shown in red are the predicted tetrazine-labeled structural moieties and the tetrazine-labeled mass peak. All sterically unhindered alkenes demonstrated labeling with probe 1.
Supplemental Figure S8: Lack of labeling for electron-deficient and sterically occluded compounds.
In all cases, 8 mM 1 was reacted with 1 mM himbacine, colubrine, bryostatin I, and streptovaricin C in MeOH for 10 h. No labeling was observed for any of these compounds. Expected mass of labeled species is marked by a red asterisk and dashed lines. No labeling was observed for sterically hindered alkenes.
Supplemental Figure S9: UV-Vis absorbance and HRMS/MS data for kineosporicin. 

a, UV spectrum acquired in MeOH with absorbance maxima and extinction coefficients of 295 nm (57,100 L mol\(^{-1}\) cm\(^{-1}\)), 307 nm (85,400 L mol\(^{-1}\) cm\(^{-1}\)), 321 nm (72,600 L mol\(^{-1}\) cm\(^{-1}\)). 
b, Kineosporicin parent ion observed by high-resolution mass spectrometry. 
c, Tandem MS spectrum of kineosporicin (collision-induced dissociation). Assigned daughter ions are provided in tabular form.
Table S1. NMR assignments for lucensomycin from *S. viridosporus*.

Abbreviations: s, singlet; d, doublet; m, multiplet

| Carbon Number | 1H, δ, ppm   | 13C, δ, ppm | HMBC |
|---------------|--------------|-------------|------|
| 1             | -            | 166.3       |      |
| 2             | 6.24 d (15.9)| 124.7       | 1, 4 |
| 3             | 6.65 dd (15.9, 6.4) | 145.3 | 1, 2, 4 |
| 4             | 3.27 dd (6.4, 2.2) | 55     | 2, 3 |
| 5             | 3 ddd (2.2, 8.5, 1.7) | 59.6   | 6   |
| 6             | 1.37 ddd (8.5, 11.2, 15.1) | 42     | 5, 7 |
| 6_a          | 2.03 m       | 42          |      |
| 6_b          | 2.03 m       | 42          |      |
| 7             | 4.56 m       | 67.9        |      |
| 8_a          | 1.7 m        | 48.1        | 9    |
| 8_b          | 1.84 m       | 48.1        | 7, 9 |
| 9             | -            | 98.4        |      |
| 10_a         | 2.31 dd (4.7, 12.1) | 45.4 | 9, 11, 12 |
| 10_b         | 1.58 m       | 45.4        | 9, 12 |
| 11            | 4.78 m       | 67.3        |      |
| 12            | 2.44 dd (10.5, 10.5) | 61.4   | 13, 30 |
| 13            | 4.84 (10.5, 8.5) | 67.2   | 15   |
| 14_a         | 1.91 m       | 39.1        | 13   |
| 14_b         | 2.79 m       | 39.1        | 12   |
| 15            | 4.71 m       | 78.3        |      |
| 16            | 6.31 m       | 137         | 19   |
| 17            | ~6.26-6.3    | 129.9       |      |
| 18            | ~6.26-6.3    | 129.9       |      |
| 19            | 6.57 dd (9.9, 14.7) | 134.4 | 22   |
| 20            | 6.22 m       | 132.7       |      |
| 21            | 6.17 m       | 132.7       | 20, 22 |
| 22            | 6.07 dd (9.2, 15.1) | 136.7   | 21   |
| 23            | 5.62 ddd (15.1, 9.8, 5.1) | 129.4 |      |
| 24_a         | 2.39 m       | 38.6        | 22, 23 |
| 24_b         | 2.18 m       | 38.6        | 22   |
| 25            | 4.88 m       | 74          |      |
| 26_a         | 1.69 m       | 35          | 25, 27, 28 |
| 26_b         | 1.57 m       | 35          | 27, 28 |
| 27_a         | ~1.3 m       | 28.2        | 28   |
| 27_b         | ~1.3 m       | 28.2        | 28   |
| 28            | ~1.3 m       | 23.2        | 27   |
| 29            | ~1.27 m      | 23.2        | 27   |
| 30            | -            | 179.5       |      |
| Mycosamine   |              |             |      |
| 1'           | 4.98 s       | 98.7        | 15, 2' |
| 2'           | 4.53 d (3.2) | 69.4        | 3'   |
| 3'           | 3.58 dd (3.2, 9.7) | 57.2 | 3', 5', 6' |
| 4'           | 3.79 dd      | 71.1        | 3', 5', 6' |
| 5'           | 3.64 m       | 74.4        | 4'   |
| 6'           | 1.45 d (6.0) | 18.2        | 4', 5' |
Figure S10: NMR Spectra for lucensomycin from *S. viridosporus*. All spectra were acquired in 1:1 CD$_3$OD and C$_5$D$_5$N. a, 1H NMR b, 1H-1H COSY c, 1H-13C HSQC d, 1H-13C HMBC
b

1H-1H COSY NMR, 750 MHz

Figure S10 cont.
1H-13C HSQC NMR, 750 MHz

Figure S10 cont.
Figure S10 cont.
Supplemental Figure S11: Bioinformatic analysis of the kineosporicin BGC. 

**a.** The GPM BGCs from *Actinokineospora mzabensis* and *Actinokineospora spheciospongiae* are virtually identical, though the BGC from *A. spheciospongiae* is incomplete owing to the draft status of the genome. Both BGCs likely encode kineosporicin. The gene-naming convention was adapted from the pimaricin BGC. 

**b.** Biosynthetic analysis and structure of kineosporicin, as determined by NMR and HRMS/MS with tailoring modifications shown in cyan.
### Table S2. NMR assignments for kineosporicin from *A. spheciospongiae*.

Abbreviations: s, singlet; d, doublet; m, multiplet

| Carbon Number | 1H, δ, ppm | 13C, δ, ppm | HMBC | NOE |
|---------------|------------|-------------|------|-----|
| 1             | -          | 167.8       | -    | -   |
| 2             | 3.76 d (1.9) | 51.7       | 1, 3, 4 | 4 |
| 3             | 2.85 dd (8.1, 1.9) | 58         | 1, 2, 4 | - |
| 4             | 2.46 dd (8.1, 2.1) | 55.9     | 3, 5 | 2, 6a |
| 5             | 2.87 m     | 54.4       | 3, 6, 7 | - |
| 6a            | 1.19 ddd (15.1, 9.9, 9.9) | 41.2      | 5, 7, 8 | 6b |
| 6b            | 1.98 m     | 41.2       | - | 7, 5, 8b, 6a |
| 7             | 4.46 m     | 67.9       | - | 5, 6b |
| 8a            | 2.38 dd (14.3, 11.1) | 44        | 6, 7, 9 | 8b, 6a |
| 8b            | 1.72 dd (14.3, 1.9) | 44        | 9 | 8a, 6b, 10, 7 |
| 9             | -          | 100        | - | - |
| 10            | 3.45 d (9.4) | 77.7       | 8, 9, 11 | - |
| 11            | 4.64 m     | 71.9       | 10, 29 | 13 |
| 12            | 2.78 dd (10.6, 10.6) | 59.2      | 11, 13 | - |
| 13            | 4.96 m     | 66.3       | 15 | 11, 17 |
| 14a           | 1.99 m     | 38.5       | 13 | 14a, 15 |
| 14b           | 2.65 m     | 38.5       | 15 | 14a |
| 15            | 4.82 m     | 77         | - | 18, 17, 14b |
| 16            | 6.25 dd (15.4, 8.4) | 136.5     | 18 | 18 |
| 17            | ~6.32 m    | 130.3      | 15 | 15 |
| 18            | 6.57 dd (15.4, 10.2) | 129.9     | 16, 20 | 16, 30 |
| 19            | ~6.32 m    | 137.3      | 17, 21, 30 | 21 |
| 20            | -          | 135.5      | - | - |
| 21            | 6.08 d (11.4) | 131.3     | 19, 30 | 19, 23, 22 |
| 22            | 6.44 dd (14.7, 11.4) | 129.7    | 20, 21, 24 | 24, 30 |
| 23            | 5.44 dd (14.7, obs) | 137.5    | 21, 24, 31 | 21 |
| 24            | 2.63 m     | 41.1       | 22, 23, 31, 25 | 22 |
| 25            | 5.09 d (10.6) | 84.1      | 23, 24, 31, 29, 27 | 27, 24 |
| 26            | -          | 132.5      | - | - |
| 27            | 5.68 q (6.8) | 127.4     | 25, 29, 28 | 25, 28 |
| 28            | 1.6 d (6.8) | 13.2       | 31, 27 | 27 |
| 29            | -          | 176.3      | - | - |
| 30            | 1.92 s     | 13.1       | 19, 20, 21 | 18, 22, 3 |
| 31            | 0.88 d (6.9) | 16.7      | 23, 24, 27 | 23, 25, 24 |
| 32            | 1.64 s     | 10.8       | 24, 27, 28 | 24 |

**Perosamine**

|      |      |    |      |      |
|------|------|----|------|------|
| 1'   | 4.91 s | 98.2 | 2', 16 | 3', 5', 14b |
| 2'   | 4.38 d (3.1) | 71.2 | 3', 4' | 3' |
| 3'   | 3.96 dd (10.3, 3.1) | 72.3 | 4' | 1', 2' |
| 4'   | 3.36 t (10.3) | 56.1 | 3', 5', 6' | - |
| 5'   | 3.81 dq (6.1, 10.3) | 70.8 | - | 1' |
| 6'   | 1.51 d (6.1) | 18.4 | 4', 5' | - |
Supplemental Figure S12: NMR Spectra for kineosporicin. All spectra were acquired in 1:1 CD$_3$OD and C$_5$D$_5$N. a, 1H NMR b, 1H-1H COSY c, 1H-1H TOCSY d, 1H-13C HSQC e, 1H-13C HMBC f, 1H-1H NOESY.
Figure S12 cont.
1H-1H TOCSY NMR, 750 MHz

Figure S12 cont.
d

1H-13C HSQC NMR, 750 MHz

Figure S12 cont.
1H-13C HMBC NMR, 750 MHz

Figure S12 cont.
1H-1H NOESY NMR, 750 MHz

Figure S12 cont.
Supplemental Figure S13: Kineosporicin NMR correlations. 

a, Key COSY, TOCSY, and HMBC correlations.
b, Key NOESY correlations supporting the relative stereochemistry of kineosporicin C2-13.
c, NMR correlations and coupling constants supporting the assigned stereochemistry.
Supplemental Figure S14: Chemical shift data of consecutive diepoxides alpha to carbonyl moieties. The 1H and 13C chemical shift values for kineosporicin and previously reported synthetic diepoxidated carbonyl-containing compounds.\textsuperscript{12}
Supplemental Figure S15: 1H-1H COSY correlation of kineosporicin perosamine. COSY correlations show C3’ H shift of 3.96 ppm and C4’ H shift of 3.36 ppm.
Supplemental Figure S16: Assignment of eurocidin, rimocidin and lucensomycin BGCs. a, Confirmation of rimocidin and eurocidin production by *S. eurocidicus* ISP-5604 and *S. rimosus* subsp. *rimosus* WC-3558. b, Assigned eurocidin, rimocidin and lucensomycin BGCs.
Table S3. NMR assignments for Eurocidin D from *S. eurocidicus*.

Abbreviations: s, singlet; d, doublet; m, multiplet

| Carbon Number | 1H, δ, ppm | 13C, δ, ppm | HMBC | NOE |
|---------------|-------------|-------------|------|-----|
| 1             | -           | 171.4       | -    | -   |
| 2a            | 2.57 m      | 44.3        | 1, 3, 4 | -   |
| 2b            | 2.75 m      | 44.3        | 1, 3, 4 | 4b  |
| 3             | 4.62 dddd (3.7, 5.7, 7.3, 9.4) | 65.4 | - | 4b |
| 4a            | 2.59 m      | 50.1        | 3, 5  | 2b  |
| 4b            | 2.76 m      | 50.1        | 3, 5  | 4a  |
| 5             | -           | 210.2       | -    | -   |
| 6a            | 2.6 m       | 42.5        | 3, 5, 7 | 6b  |
| 6b            | 2.95 ddd (4.1, 10.4, 18.9) | 42.5 | 5, 7, 8 | 6a  |
| 7a            | 1.69 m      | 27.8        | -    | 7b, 9|
| 7b            | 2.22 m      | 27.8        | -    | 7a  |
| 8             | 3.33 d (2, 2, 11.4) | 74.2 | - | 9, 10a |
| 9             | 4.31 d (2, 2, 11.4) | 72.2 | - | 7a, 8, 18 |
| 10a           | 1.74 dd (1.9, 14.2) | 44.2 | 11 | 8, 10b, 12a |
| 10b           | 2.31 m      | 44.2        | 9, 11 | 10a |
| 11            | -           | 98.6        | -    | -   |
| 12a           | 1.62 dd (11.6, 11.9) | 45.6 | 10, 11, 13, 14 | 10b, 12b |
| 12b           | 2.34 dd (4.7, 11.9) | 45.6 | 11, 13, 14 | 12a, 13 |
| 13            | 4.78 ddd (4.7, 10.1, 11.6) | 67.5 | 34 | 12b |
| 14            | 2.48 dd (10.5, 10.1) | 61.3 | 12, 13, 16, 34 | 12a |
| 15            | 4.84 dd (8.1, 10.5) | 67.4 | 17 | 18 |
| 16a           | 1.96 m      | 39.1        | 13   | 16a, 17 |
| 16b           | 2.74 m      | 39.1        | 14, 17, 18 | 16b, 17 |
| 17            | 4.73 m      | 78.1        | 15   | 16a, 16b, 18 |
| 18            | 6.25 m      | 137.3       | -    | -   |
| 19-24         | 6.17-6.47   | 130-134.8   | -    | -   |
| 25            | 6.38 m      | 134         | -    | -   |
| 26            | 6.17 m      | 134.8       | -    | -   |
| 27            | 5.69 ddd    | 130.7       | -    | 25  |
| 28a           | 2.26 m      | 36.6        | 26, 27, 29, | 26, 33 |
| 28b           | 2.32 m      | 36.6        | 26, 27 | 27, 29, 30, 33 |
| 29            | 5.04 ddd (2, 4.4, 10.9) | 76.4 | 1, 27, 31, 33 | 27, 30 |
| 30            | 1.53 m      | 39.9        | -    | 29, 30 |
| 31a           | 1.13 qdd    | 26.5        | 29, 30, 32, 33 | 31a, 32 |
| 31b           | 1.43 m      | 26.5        | 29, 30, 32, 33 | 31a, 32 |
| 32            | 0.89 t (7.4) | 11.9 | 29, 30, 31 | 31a, 31b |
| 33            | 0.92 d (6.8) | 14.5 | 30, 31 | 28a, 30 |
| 34            | -           | 179.6       | -    | -   |

**Mycosamine**

| Carbon Number | 1H, δ, ppm | 13C, δ, ppm | HMBC | NOE |
|---------------|-------------|-------------|------|-----|
| 1'            | 5.01 s      | 98.7        | 2', 5', 17 | 2', 3', 5', 16b, 17 |
| 2'            | 4.53 d (3.1) | 69.4 | 3', 4' | 1', 3' |
| 3'            | 3.61 dd (3.1, 10.3) | 57.2 | 4' | 1', 2', 4' |
| 4'            | 3.8 dd (9.1, 10.3) | 71 | 3', 5', 6' | 5', 6' |
| 5'            | 3.67 dq (6.1, 9.1) | 74.4 | 4' | 1', 4', 6' |
| 6'            | 1.45 (6.1) | 18.1 | 4', 5' | 4', 5' |
Supplemental Figure S17: NMR Spectra for Eurocidin D. All spectra were acquired in 1:1 CD$_3$OD and C$_5$D$_5$N. a, $^1$H NMR b, $^1$H-$^1$H COSY c, $^1$H-$^1$H TOCSY d, $^1$H-$^{13}$C HSQC e, $^1$H-$^{13}$C HMBC f, $^1$H-$^1$H NOESY
b

1H-1H COSY NMR, 750 MHz

Figure S17 cont.
1H-1H TOCSY NMR, 750 MHz

Figure S17 cont.
1H-13C HSQC NMR, 750 MHz

Figure S17 cont.
Figure S17 cont.
$f$

**1H-1H NOESY NMR, 750 MHz**

Figure S17 cont.
| Strain                                | Kineosporicin MIC |
|---------------------------------------|-------------------|
| *Staphylococcus aureus* NRS384/USA300 | >32               |
| *Enterococcus faecium* U503           | >32               |
| *Bacillus anthracis* strain Sterne    | >32               |
| *Pseudomonas aeruginosa* PAO1         | >32               |
| *Escherichia coli* MC4100             | >32               |

**Supplemental Figure S18: Kineosporicin antibacterial assay.** Minimum inhibitory concentrations (MIC, in µM, *n* = 3) were determined for kineosporicin by the microbroth dilution method.
Supplemental Figure S19: Potassium efflux upon addition of various GPMs. Potassium ion release was monitored as a function of time with addition of GPM or DMSO control after 5 minutes of equilibration. All GPMs were tested at 3 µM.
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