SUPPLEMENTARY INFORMATION

Refactoring the pikromycin synthase for the modular biosynthesis of macrolide antibiotics in *E. coli*

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METHODS

Reagents and equipment

KOD One PCR master mix is from Toyobo. The KAPA HiFi DNA polymerase is from KAPA Biosystems. All restriction enzymes are from New England Biolabs. All primers are from Sigma-Aldrich. All gBlocks are from IDT. Luria-Bertani (LB) Miller Broth, sodium phosphate, potassium phosphate, sodium chloride, sodium propionate, and glycerol are from Fisher Scientific. Casein is from Spectrum. Yeast extract is from Gibco. Streptomycin sulfate, kanamycin sulfate, ampicillin sodium salt, chloramphenicol, and apramycin sulfate are from VWR. Isopropyl-β-D-thio-galactopyranoside (IPTG) is from Carbosynth. Ethyl acetate (EtOAc), acetonitrile, methanol, chloroform, and hexanes are from Fisher Scientific. $CDCl_3$ is from Cambridge Isotope Laboratories. SiliaFlash Silica Gels are from SiliCycle. Pikromycin is from AdipoGene. All strains, plasmids, and oligonucleotides are reported (Supplementary Tables 1-3).

High-resolution LC/MS data was obtained using an Agilent 6230 TOF LC/MS, containing a ZORBAX Eclipse Plus C18 column (50 x 2.1 mm, 1.8 μm). NMR data were obtained on Bruker AVANCE III 500 NMR or Bruker NEO 600 NMR spectrometers.

Construction of the 2-plasmid system

p**P1**-^{N}**P4** was constructed by Gibson assembly and SliCE (Figure S1a)[1]. First, amplicons of **P1** and ^{N}**P4** were generated by PCR using *Streptomyces venezuelae* ATCC 15439 genomic DNA and joined with KpnI/XhoI-digested pCDF-1b by Gibson assembly. Then, amplicons containing the BmtI site and **E3** ^{C}DD, generated using *Saccharopolyspora erythraea* NRRL 2338 genomic DNA as the template, were inserted into the XhoI site through SLiCE assembly.

p^{C}**P4**-**P7** was constructed by Gibson assembly (Figure S1b). An amplicon containing **E3** ^{N}DD was initially cloned into NcoI/XhoI-digested pET-28b. The resulting plasmid was used as a PCR template to generate an amplicon containing **E3** ^{N}DD. Amplicons containing ^{C}**P4** and **P7** were generated by PCR using *S. venezuelae* gDNA. These 3 amplicons were joined by Gibson assembly.

The sequences of p**P1**-^{N}**P4** and p^{C}**P4**-**P7** are reported (Figure S2).

Construction of the module-cloning plasmids

Most of the module-cloning plasmids were constructed in our previous report[2]. Briefly, the module-cloning plasmids contain DNA encoding the AT, processing enzymes, and ACP, and DNA encoding KS, separated by a "regulator" in a pUC19 vector. The regulator includes a T7 terminator, a T7 promoter, a lac operator, a ribosomal binding site, an ATG start codon, and cognate docking domains that enable the association of PKS polypeptides. Since the construction of heptaketide and octaketide synthases requires 5 and 6 sets of docking domain pairs, respectively, more regulators were created using gBlocks containing the **E3** and **A1** docking domains. Module-containing plasmids were constructed by swapping regulator sequences using the MfeI and BmtI sites. Construction of pUT21-P3, pUT21-P5, pUT21-P6, which respectively contain the native **P3**, **P5**, and **P6** docking domains, was performed by SLiCE so that the MfeI and BmtI sites are replaced by DNA encoding the native amino acids. pUT21-P6 (C to A) was constructed through site-directed mutagenesis. The gBlock sequences used to construct the regulators are provided (Figure S3).

Construction of hexaketide, heptaketide, and octaketide synthases

HindIII/XbaI-digested module-containing fragments were inserted between the HindIII and SpeI sites in both p**P1**-^{N}**P4** and p^{C}**P4**-**P7**. Since the insertions preserve the HindIII-N_{12}-SpeI sequence upstream of the inserted module and generate an inactive, SpeI/XbaI site downstream of the inserted module, subsequent insertions of module-containing fragments can be performed.

Construction of operons encoding desosamine biosynthesis/transfer and macrolide antibiotic resistance enzymes

The DNA encoding *desI*, *desII*, *desV*, *desVI* (D-desosamine biosynthesis); *desVII*, *desVIII* (D-desosamine transfer); and *pikC* (P450 monooxygenase that hydroxylates narbomycin and YC-17) was amplified by PCR using *S. venezuelae* gDNA as the template (Figure S32). The erythromycin resistance gene *ermE* was also amplified by PCR using *Saccharopolyspora erythraea* NRRL 2338 gDNA as the template. Each amplicon contains an SpeI site after the stop codon to enable XbaI/SpeI ligation as described below. The DNA encoding *desI* was cloned between the BamHI and EcoRI sites of pACYCDuet-1, while the DNA encoding each of *desII*, *desV*, *desVI*, *desVII*, *desVIII*, and *ermE* was cloned between the NdeI and EcoRI sites of pET-28b. To construct pDes, the plasmid encoding the D-desosamine biosynthesis/transfer pathway, DNA encoding *desI*, *desII*, *desV*, *desVI*, *desVII*, *desVIII*, and *ermE* was combined into single operon through the sequential ligation of XbaI/EcoRI inserts into the SpeI/EcoRI-digested pACYC-derived vector. To generate pDesPikC, an

equivalently-constructed *pikC* insert was further ligated into pDes. To construct pDesPikAV, the D-desosamine biosynthetic operon with *ermE* was amplified by PCR using pDes as a template and cloned between BamHI and EcoRI sites of pACYC-pikAV.

To express the D-desosamine biosynthetic genes from a BAC (copy number = 1 or 2), the operon was amplified by PCR and cloned between the BmtI and AvrII sites of pMKBAC02[3].

Construction of PikAV expression vectors

pikAV was amplified by PCR using *S. venezuelae* gDNA as the template and cloned between the EcoRI and HindIII sites of pACYCDuet-1 to yield pACYC-pikAV. One more copy of *pikAV* was cloned between the NdeI and XhoI sites to yield pACYC-pikAV-pikAV. To generate a set of pikAV expression plasmids with different expression levels, site-directed mutagenesis of the T7 promoters of pACYC-pikAV and pACYC-pikAV-pikAV was performed (Figure S13).

Integration of the D-desosamine biosynthesis/transfer pathway and *ermE* into the *E. coli* K207-3 genome

The genetic manipulation of *E. coli* K207-3 was performed with CRISPR/Cas9 genome editing tool pEcCas/pEcgRNA (Figure S53)[4,5]. To target the *cadA* locus for genome integration, CATCGCCGCAGCGGTTTCAGTGG, was selected as the target sequence. Oligonucleotides TAGTCATCGCCGCAGCGGTTTCAG and AAACCTGAAACCGCTGCGGCGATG were synthesized, annealed, and ligated into BsaI site of pEcgRNA to yield pEcgRNA-cadA.

Likely because each gene in the D-desosamine biosynthesis/transfer+*ermE* operon in pDes is preceded by an equivalent 104 bp sequence encoding a His-tag and ribosome binding site (from pET-28b), homologous recombination occurred when λ−red recombinase was expressed. To minimize the length of these equivalent sequences, each gene was amplified to include the minimal sequence (AAGGAGATATACCATG) and combined by overlap extension PCR to yield pEcgRNA-cadA.

1000 bp homology arms homologous to the upstream and downstream regions of the *cadA* locus were prepared by PCR using *E. coli* K207-3 gDNA as the template. The homology arms were combined by overlap extension PCR such that an XbaI site was created between them. The resulting amplicon was ligated between the EcoRI and HindIII sites of pEcgRNA-cadA to yield pEcgRNA-ΔcadA. The D-desosamine

synthesis operon was ligated into the XbaI site of the resulting plasmid to yield pEcgRNA-ΔcadA:desI,II,V,VI,VII,VIII,ermE. As linear DNA was found to be more effective guiding homologous recombination, it was generated by PCR using pEcgRNA-ΔcadA:desI,II,V,VI,VII,VIII,ermE as the template.

E. coli K207-3 harboring pEcCas was shaken at 240 rpm in 6 mL of LB media containing 50 mgL^{-1} kanamycin at 37 ºC. 1 mL of the culture was inoculated into 50 mL of LB media containing 50 mgL^{-1} kanamycin. Cells were shaken until OD_{600} = 0.2, and 10 mM arabinose was added to induce expression of the λ-red system. When OD_{600} = 0.6, cells were collected by centrifugation (4000 × g for 5 min, 4 ºC) and washed with 50 mL of ice-cold 10% (v/v) glycerol. After centrifugation (4000 × g for 5 min, 4 ºC), the cells were resuspended in 200 μL of ice-cold 10% (v/v) glycerol. 100 μL of the cells were mixed with 1 μg of the donor DNA and 100 ng of pEcgRNA-*cadA* and then electroporated in a 1 mene pulser cuvette (FisherBio) at 1.6 kV. 1 mL of LB medium was immediately added to the mixture after electroporation. The cells were allowed to recover in 1 mL of LB media at 37 ºC for 1 h before plating. The positive colonies were selected on an LB plate containing 50 mgL^{-1} kanamycin and streptomycin at 37 ºC. Genome integration was confirmed by colony PCR (Figure S53).

To cure pEcgRNA-*cadA* from the cells, single colonies of transformants were shaken at 240 rpm in 2 mL of LB media containing 50 mgL^{-1} kanamycin and 10 mM rhamnose at 37 ºC for 8 h. 200 μL of the cultures were inoculated into 2 mL of LB media and shaken at 240 rpm at 37 ºC for 3 h. To identify cells without pEcCas, they were spread on LB plates containing 5 gL^{-1} glucose and 5 gL^{-1} sucrose and incubated overnight at 37 ºC. Colonies sensitive to both kanamycin and streptomycin were selected – 3 out of 20 colonies grew in LB media without these antibiotics but did not in media with these antibiotics.

Inactivation of pathways competitive with D-desosamine biosynthesis

CRISPR/Cas9 genome-editing was performed as described above. To inactivate *vioAB*, *rmlC*, and *wecDE*, CATCGCCGCAGCGGTTTCAG, CTATGGAAAAAGGTATAAGA, and CGACTATATGCAGTCGGCAA were respectively selected as the target sites. Homology arms homologous to regions 500 bp upstream and downstream of the target sites were prepared by PCR using *E. coli* K207-3 gDNA as the template. These were combined by overlap extension PCR to yield the donor DNA that guides homologous recombination. 600 ng of the donor DNA and 100 ng of the targeting plasmid (pEcgRNA-rmlC, pEcgRNA-vioAB, pEcgRNA-

wecDE) were used for each electroporation. Plasmids were cured as above, and gene inactivations were confirmed by colony PCR (Figure S54).

Polyketide production and quantification

E. coli K207-3 (or TM1–7 derivative strains) transformed with PKS expression plasmids were shaken at 240 rpm in 6 mL LB media containing the appropriate antibiotics (50 mgL^{-1} kanamycin and 50 mgL^{-1} streptomycin, as well as 30 mgL^{-1} chloramphenicol when employing the desosamine biosynthesis/transfer plasmids) in a culture tube at 37 ºC. From these precultures, 0.3 mL was used to inoculate 30 mL of production media (5 gL^{-1} yeast extract, 10 gL^{-1} casein, 15 gL^{-1} glycerol, 10 gL^{-1} NaCl, and 100 mM potassium phosphate, pH 7.6 with appropriate antibiotics)[6] in 250 mL non-baffled Erlenmeyer flasks. Cells were cultured at 240 rpm at 37 ºC until OD_{600} = 0.6. They were then cooled to 16 ºC, supplied with 0.1 mM IPTG and 60 mM sodium propionate, and cultured for 10 d. 500 µL of the culture broth was extracted twice with the same volume of ethyl acetate and concentrated in *vacuo.* The extract was resuspended in 500 µL of methanol, and 1 µL samples were injected into a 6230 TOF LC/MS connected to a ZORBAX Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm) [Solvent A: water with 0.1% (v/v) formic acid; Solvent B: acetonitrile with 0.1% (v/v) formic acid. 5-100% B for 15 min, 100% B for 3 min, flow rate of 0.2 mL min^{-1}, positive mode, 180 V, 300 ºC].

To quantify polyketide production, EIC peak areas of the expected products were measured and compared with calibration curves generated by authentic standards (Figures S54-44, Table S4). A calibration curve generated using purified narbonolide was employed to quantify macrolactones and δ-lactones, while a calibration curve generated using commercial pikromycin was employed to quantify macrolides. When necessary, extracts were diluted with MeOH before quantification (10x dilution for narbomycin, 10-dml, and narbomycin/pikromycin/YC-17 derivatives, 100x dilution for narbomycin, pikromycin, and YC-17).

Isolation of narbonolide (1)

600 mL of culture broth from K207-3 cells expressing **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7** was extracted twice with the same volume of ethyl acetate, dried with $MgSO_4$, filtered, and concentrated *in vacuo.* The extract was subjected to a flash silica gel chromatography (hexanes:ethyl acetate = 30:70). Fractions containing narbonolide were further purified by semi-preparative HPLC [Waters 1525 HPLC system equipped with a Microsorb 300-5 C18 Dynamax column (250 × 10.0 mm) with a flow rate of 4 mL min^{-1} [solvent

A, water with 0.1% (v/v) formic acid; solvent B, acetonitrile with 0.1% (v/v) formic acid. 30–100% B for 15 min] to obtain 8.2 mg of narbonolide after lyophilization.

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.26 (s, 4H), 6.90 (dd, *J* = 16.3, 4.8 Hz, 1H), 6.10 (dd, *J* = 16.3, 1.9 Hz, 1H), 5.17 – 5.12 (m, 1H), 3.86 (q, *J* = 5.2 Hz, 1H), 3.71 (q, *J* = 7.0 Hz, 1H), 3.04 (q, *J* = 6.9 Hz, 1H), 2.73 – 2.64 (m, 1H), 2.11 (d, *J* = 5.1 Hz, 1H), 1.74 – 1.66 (m, 2H), 1.67 – 1.59 (m, 2H), 1.55 (s, 5H), 1.53 – 1.46 (m, 1H), 1.36 (d, *J* = 7.0 Hz, 3H), 1.14 (dd, *J* = 7.0, 4.0 Hz, 6H), 1.10 (d, *J* = 6.7 Hz, 4H), 0.96 – 0.89 (m, 7H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 207.61, 205.06, 171.06, 148.52, 129.00, 78.09, 77.21, 77.00, 76.78, 72.61, 50.28, 50.23, 39.63, 38.86, 36.45, 35.02, 24.22, 18.62, 18.31, 14.33, 10.87, 10.70, 10.41.

Isolation of 10-deoxymethynolide (4)

300 mL of culture broth from *E. coli* K207-3 cells expressing **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7** was extracted twice with the same volume of ethyl acetate, dried with $MgSO_4$, filtered, and concentrated *in vacuo.* The extract was subjected to flash silica gel chromatography (hexanes:ethyl acetate = 30:70). Fractions containing 10-dML were further purified by semi-preparative HPLC [Waters 1525 HPLC system equipped with a Microsorb 300-5 C18 Dynamax column (250 × 10.0 mm) with a flow rate of 4 mL min^{-1} [solvent A, water with 0.1% (v/v) formic acid; solvent B, acetonitrile with 0.1% (v/v) formic acid. 30–100% B for 15 min] to obtain 7.2 mg of 10-dml after lyophilization.

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.77 (dd, *J* = 15.7, 5.4 Hz, 1H), 6.44 (dd, *J* = 15.7, 1.3 Hz, 1H), 5.02 (ddd, *J* = 8.8, 5.5, 2.4 Hz, 1H), 3.58 (d, *J* = 10.4 Hz, 1H), 2.71 – 2.59 (m, 2H), 2.59 – 2.50 (m, 1H), 1.80 – 1.64 (m, 3H), 1.59 (s, 1H), 1.37 – 1.28 (m, 4H), 1.24 (d, *J* = 7.0 Hz, 3H), 1.14 (d, *J* = 6.8 Hz, 3H), 1.03 (d, *J* = 6.2 Hz, 3H), 0.94 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 204.98, 174.76, 147.14, 125.67, 78.25, 77.28, 77.02, 76.77, 73.73, 45.17, 43.37, 38.06, 33.24, 33.22, 25.17, 17.72, 17.47, 16.42, 10.34, 9.59.

Isolation of 10

900 mL of culture broth from *E. coli* K207-3 cells expressing **P1**-**P2**-**P5**-**P4**-**P5**-**P6**-**P7**+PikAV was extracted twice with the same volume of ethyl acetate, dried with $MgSO_4$, filtered, and concentrated *in vacuo.* The extract was subjected to flash silica gel chromatography (hexanes:ethyl acetate = 70:30 to 40:60). Fractions containing **10**

were further purified by HPLC [Waters 1525 HPLC system equipped with a Microsorb-MV 300-5 C_{18} column (250 × 4.6 mm) with a flow rate of 1 mL min^{-1} (solvent A, water with 0.1% formic acid; solvent B, acetonitrile with 0.1% formic acid. 10–90% B for 20 min)] to obtain 0.6 mg of **10** after lyophilization.

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.10 (dq, *J* = 7.8, 2.5 Hz, 1H), 3.97 (q, *J* = 3.6 Hz, 1H), 3.82 – 3.73 (m, 1H), 2.86 (d, *J* = 3.3 Hz, 1H), 2.80 (ddd, *J* = 16.1, 7.8, 4.6 Hz, 1H), 2.72 (dp, *J* = 7.0, 3.5 Hz, 1H), 2.69 – 2.56 (m, 1H), 1.97 – 1.85 (m, 1H), 1.82 (s, 2H), 1.81 – 1.61 (m, 3H), 1.55 (s, 2H), 1.44 – 1.27 (m, 6H), 1.17 (dd, *J* = 7.1, 0.7 Hz, 3H), 1.09 – 0.94 (m, 15H), 0.94 – 0.86 (m, 4H), 0.86 – 0.72 (m, 1H).

Isolation of 15

300 mL of culture broth from *E. coli* K207-3 cells expressing **P1**-**P2**-**P4**-**P5**-**P6**-**P7** was extracted twice with the same volume of ethyl acetate, dried with $MgSO_4$, filtered, and concentrated *in vacuo.* The extract was subjected to flash silica gel chromatography (hexanes:ethyl acetate = 60:40 to 50:50). Fractions containing **10** were further purified by HPLC [Waters 1525 HPLC system equipped with a Microsorb-MV 300-5 C_{18} column (250 × 4.6 mm) with a flow rate of 4 mL min^{-1} (solvent A, water with 0.1% formic acid; solvent B, acetonitrile with 0.1% formic acid. 30% B)] to obtain 9.3 mg of **15** after lyophilization.

^{1}H NMR (500 MHz, CDCl3) δ 4.35 (dd, *J* = 9.9, 2.6 Hz, 1H), 3.82 (ddtd, *J* = 10.1, 7.8, 5.1, 2.7 Hz, 1H), 3.63 (q, *J* = 6.7 Hz, 1H), 3.02 (ddt, *J* = 20.8, 14.0, 6.9 Hz, 1H), 2.85 (dqd, *J* = 14.3, 7.1, 2.9 Hz, 1H), 2.79 (s, 2H), 2.70 (qd, *J* = 7.5, 2.6 Hz, 1H), 2.32 – 2.20 (m, 1H), 1.90 (dtd, *J* = 9.9, 7.1, 4.7 Hz, 1H), 1.81 (s, 0H), 1.63 – 1.55 (m, 1H), 1.58 – 1.45 (m, 1H), 1.44 (td, *J* = 6.9, 5.1 Hz, 1H), 1.37 (d, *J* = 6.6 Hz, 3H), 1.26 – 1.16 (m, 5H), 1.15 (td, *J* = 7.6, 5.2 Hz, 8H), 1.00 (d, *J* = 7.4 Hz, 3H), 1.00 – 0.89 (m, 5H).

^{13}C NMR (126 MHz, CDCl3) δ 205.37, 169.55, 81.76, 81.01, 77.27, 77.01, 76.76, 72.79, 50.21, 47.90, 43.90, 43.18, 37.15, 31.65, 26.98, 17.51, 17.27, 15.97, 10.50, 9.86, 9.78, 8.08.

Isolation of narbomycin (2)

300 mL of culture broth from TM7 cells expressing **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7**+Des was adjusted to pH 9.5 with 1 M NaOH, extracted twice with the same volume of ethyl acetate, dried with $MgSO_4$, filtered, and concentrated *in vacuo.* The extract was

subjected to flash silica gel chromatography (chloroform:methanol = 95:5). Fractions containing narbomycin were further purified by semi-preparative HPLC [Waters 1525 HPLC system equipped with an Atlantis T3 OBST prep column (250 × 10.0 mm) with a flow rate of 4 mL min^{-1} (solvent A, water with 0.1% formic acid; solvent B, acetonitrile with 0.1% formic acid. 15–90% B for 15 min)] to obtain 3.1 mg of narbomycin after lyophilization.

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.70 (dd, *J* = 16.1, 6.1 Hz, 1H), 6.11 (dd, *J* = 16.2, 1.6 Hz, 1H), 4.96 (dt, *J* = 9.5, 3.9 Hz, 1H), 4.42 (d, *J* = 7.2 Hz, 1H), 4.21 (dd, *J* = 4.8, 2.4 Hz, 1H), 3.87 (q, *J* = 7.0 Hz, 1H), 3.66 (dqd, *J* = 11.4, 5.9, 2.7 Hz, 1H), 3.51 (dd, *J* = 10.1, 7.2 Hz, 1H), 3.22 (d, *J* = 12.1 Hz, 1H), 2.94 (qd, *J* = 7.3, 4.8 Hz, 1H), 2.86 – 2.69 (m, 3H), 2.74 (s, 5H), 1.98 (dt, *J* = 12.2, 2.6 Hz, 1H), 1.67 – 1.41 (m, 4H), 1.40 – 1.35 (m, 6H), 1.32 (d, *J* = 6.1 Hz, 3H), 1.27 – 1.14 (m, 1H), 1.13 (t, *J* = 6.5 Hz, 6H), 1.03 (d, *J* = 6.9 Hz, 3H), 0.92 (t, *J* = 7.3 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 208.86, 203.54, 169.47, 147.38, 128.07, 103.75, 78.94, 69.61, 68.27, 65.93, 50.46, 48.79, 41.77, 39.76, 38.35, 36.91, 36.31, 30.87, 22.91, 20.96, 17.05, 15.49, 14.21, 13.41, 12.36, 10.50.

Isolation of YC-17 (5)

600 mL of culture broth from TM7 cells expressing **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7**+Des was adjusted to pH 9.5 with 1 N NaOH, extracted twice with the same volume of ethyl acetate, dried with $MgSO_4$, filtered, and concentrated *in vacuo.* The extract was subjected to flash silica gel chromatography (chloroform:methanol = 95:5). Fractions containing YC-17 were further purified by semi-preparative HPLC [Waters 1525 HPLC system equipped with an Atlantis T3 OBST prep column (250 × 10.0 mm) with a flow rate of 4 mL min^{-1} (solvent A, water with 0.1% formic acid; solvent B, acetonitrile with 0.1% formic acid. 15–90% B for 15 min)] to obtain 3.8 mg of YC-17.

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.77 (dd, *J* = 15.7, 5.5 Hz, 1H), 6.45 (d, *J* = 15.7 Hz, 1H), 4.97 (ddd, *J* = 8.2, 5.6, 2.2 Hz, 1H), 4.33 (d, *J* = 7.2 Hz, 1H), 3.62 (d, *J* = 10.4 Hz, 1H), 3.56 (dt, *J* = 12.1, 6.0 Hz, 1H), 3.42 (dd, *J* = 10.2, 7.2 Hz, 1H), 3.02 (ddd, *J* = 14.1, 10.2, 4.2 Hz, 1H), 2.87 (dq, *J* = 10.5, 6.9 Hz, 1H), 2.65 (t, *J* = 6.8 Hz, 1H), 2.60 (s, 6H), 2.55 (ddd, *J* = 11.7, 6.8, 4.3 Hz, 1H), 1.90 – 1.82 (m, 1H), 1.71 (s, 1H), 1.58 (dq, *J* = 13.7, 7.0 Hz, 1H), 1.45 – 1.40 (m, 4H), 1.29 (d, *J* = 6.1 Hz, 3H), 1.22 (d, *J* = 6.9 Hz, 3H), 1.13 (d, *J* = 6.8 Hz, 3H), 1.02 (d, *J* = 6.7 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 205.28, 175.11, 146.97, 125.94, 104.39, 86.03, 73.71, 69.86, 68.38, 66.11, 45.11, 43.97, 39.72, 37.88, 34.08, 33.50, 29.96, 25.16, 20.98, 17.71, 17.40, 16.15, 10.29, 9.59.

Isolation of 17

2.4 L of culture broth from TM7 cells expressing **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7**+Des+PikAV was adjusted to pH 9.5 with1 N NaOH, extracted twice with the same volume of ethyl acetate, dried with $MgSO_4$, filtered, and concentrated *in vacuo.* The extract was subjected to flash silica gel chromatography (chloroform:methanol = 95:5). Fractions containing **17** were further purified by semi-preparative HPLC [Waters 1525 HPLC system equipped with an Atlantis T3 OBST prep column (250 × 10.0 mm) with a flow rate of 4 mL min^{-1} (solvent A, water with 0.1% formic acid; solvent B, acetonitrile with 0.1% formic acid. 15–70% B for 20 min)] to obtain 1.9 mg of **17** after lyophilization.

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.99 (ddd, *J* = 9.2, 4.6, 2.2 Hz, 1H), 4.39 (d, *J* = 7.3 Hz, 1H), 4.00 (dd, *J* = 4.8, 3.1 Hz, 1H), 3.83 (q, *J* = 7.1 Hz, 1H), 3.59 (dqd, *J* = 12.3, 6.0, 1.8 Hz, 1H), 3.41 (dd, *J* = 10.2, 7.3 Hz, 1H), 2.93 (qd, *J* = 7.1, 4.8 Hz, 1H), 2.77 (tp, *J* = 9.9, 3.1 Hz, 3H), 2.66 (ddd, *J* = 10.6, 6.8, 3.8 Hz, 2H), 2.48 (s, 6H), 2.04 (s, 1H), 1.83 (s, 1H), 1.73 – 1.61 (m, 2H), 1.58 – 1.49 (m, 2H), 1.41 (d, *J* = 7.1 Hz, 4H), 1.32 (d, *J* = 7.1 Hz, 4H), 1.26 (dd, *J* = 6.9, 3.2 Hz, 4H), 1.08 – 1.02 (m, 11H), 0.98 (d, *J* = 7.0 Hz, 4H), 0.90 (t, *J* = 7.4 Hz, 4H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 217.84, 209.09, 171.05, 105.05, 79.60, 77.87, 70.28, 69.35, 69.27, 65.35, 52.09, 46.85, 43.38, 42.34, 40.35, 36.11, 35.92, 35.82, 34.08, 30.16, 23.99, 21.17, 21.12, 17.50, 15.60, 15.46, 14.05, 13.89, 10.49.

Minimum inhibitory concentration (MIC) assay

The antibacterial activities of the purified compounds were evaluated against *Bacillus subtilis* strain 168 according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (Figure S57). *B. subtilis* was grown in Mueller-Hinton (M-H) medium at 37 °C overnight. The broth was diluted by the M-H medium (1:500) and used as an inoculum. 50 μL of the inoculum was mixed with 50 μL of M-H medium containing 2-fold serial dilutions of the compounds in 96-well plates. After incubating the cells for 24 h at 37 °C, each MIC was determined as the lowest concentration that prevented visible bacterial growth.

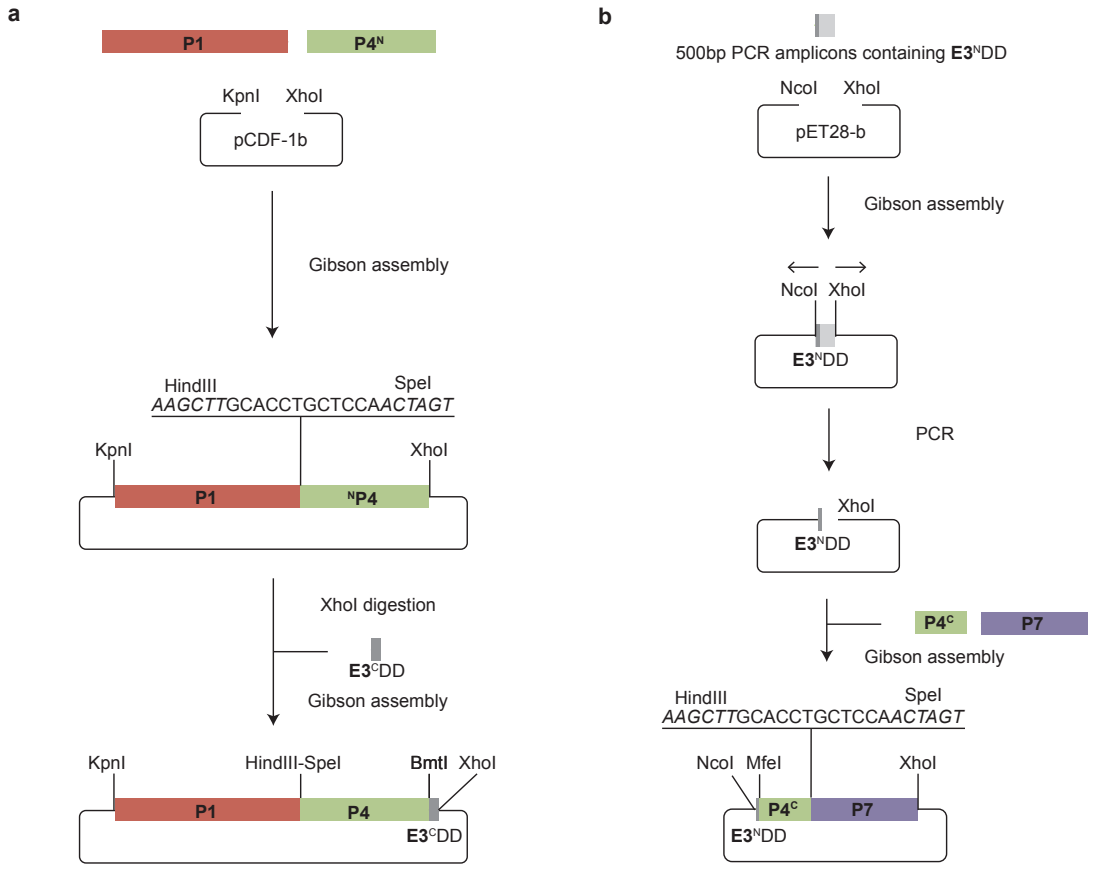

Figure S1. Construction of 2-plasmid system. a) Construction of p**P1**-^{N}**P4**. Amplicons of **P1** and ^{N}**P4** were joined with KpnI/XhoI-digested pCDF-1b by Gibson assembly. The HindIII-N_{12}-SpeI sequence between **P1** and ^{N}**P4** comes from the primers. An amplicon containing a BmtI site and **E3** ^{C}DD was inserted into the XhoI site through SLiCE assembly. b) Construction of p^{C}**P4**-**P7**. 500 bp PCR amplicons containing **E3** ^{N}DD was cloned into NcoI/XhoI-digested pET-28b. The resulting plasmid was used as a PCR template to generate an amplicon containing **E3** ^{N}DD. This amplicon and amplicons containing ^{C}**P4** and **P7** were joined by Gibson assembly. The HindIII-N_{12}-SpeI sequence between **P1** and ^{N}**P4** comes from the primers.

P1-^{N}P4-E3^{C}DD

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E3^{N}DD-^{C}P4-P7

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K

Figure S2. Sequences of **P1**-^{N}**P4**-**E3**^{C}DD and **E3**^{N}DD-^{C}**P4**-**P7**. Red, green, purple, and gray letters indicate **P1**, **P4**, **P7**, and **E3** DD, respectively (colored as in Fig. 1). Yellow letters indicate sequences encoded by HindIII-N_{12}-SpeI insertion sites.

S3 Regulator

ATAGACTACAAGCTTGCACCTGCTCCAACTAGTCATGTAGCGATTACACTTTGGCTAGCGCCGGGACATTC
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S5 Regulator

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S8 Regulator

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E5 Regulator

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P3 Regulator

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T

P5 Regulator

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P6 Regulator

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A1 Regulator

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TAGGCGAGCTGGAATCCCGCGACAACGAGCCAATTGCGTAGGTACCAGACTCTAGCTCTAGAGACATAAAT

AAGCTT- HindIII site

ACTAGT- SpeI site

GCTAGC- BmtI site

CTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTT- T7 terminator

TAATACGACTCACTATAGG- T7 promoter

GGAATTGTGAGCGGATAACAATTC- LacO

AAGGAG- RBS

CAATTG- MfeI site

TCTAGA- XbaI site

Figure S3. Regulator sequences. gBlocks were inserted between the HindIII and XbaI sites of pUC19 to construct cloning plasmids containing the **S3**, **S5**, **S8**, **E5**, **P3**, **P5**, **P6**, and **A1** docking domains.

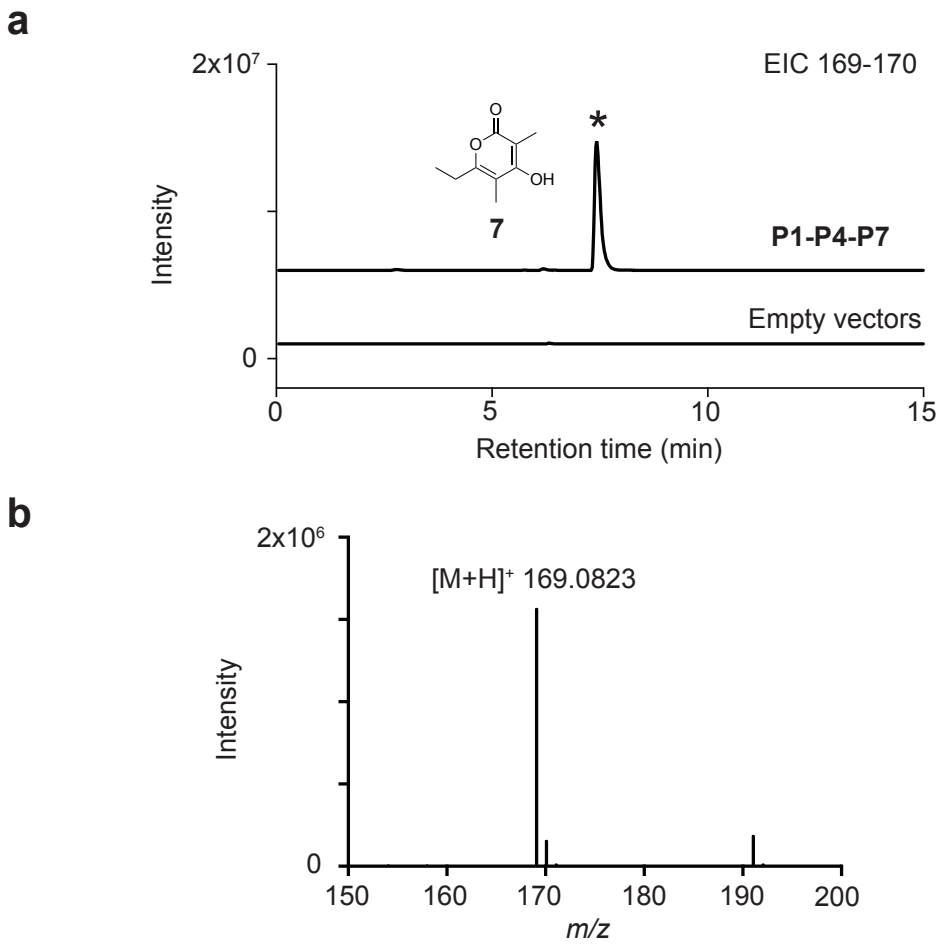


Figure S4. LC/MS analysis of the anticipated **P1**-**P4**-**P7** product, **7**. a) Extracted-ion chromatogram (EIC) for the $[M+H]^+$ of **7** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **7,** 169.0859; observed *m/z*, 169.0823 (2.3 ppm).

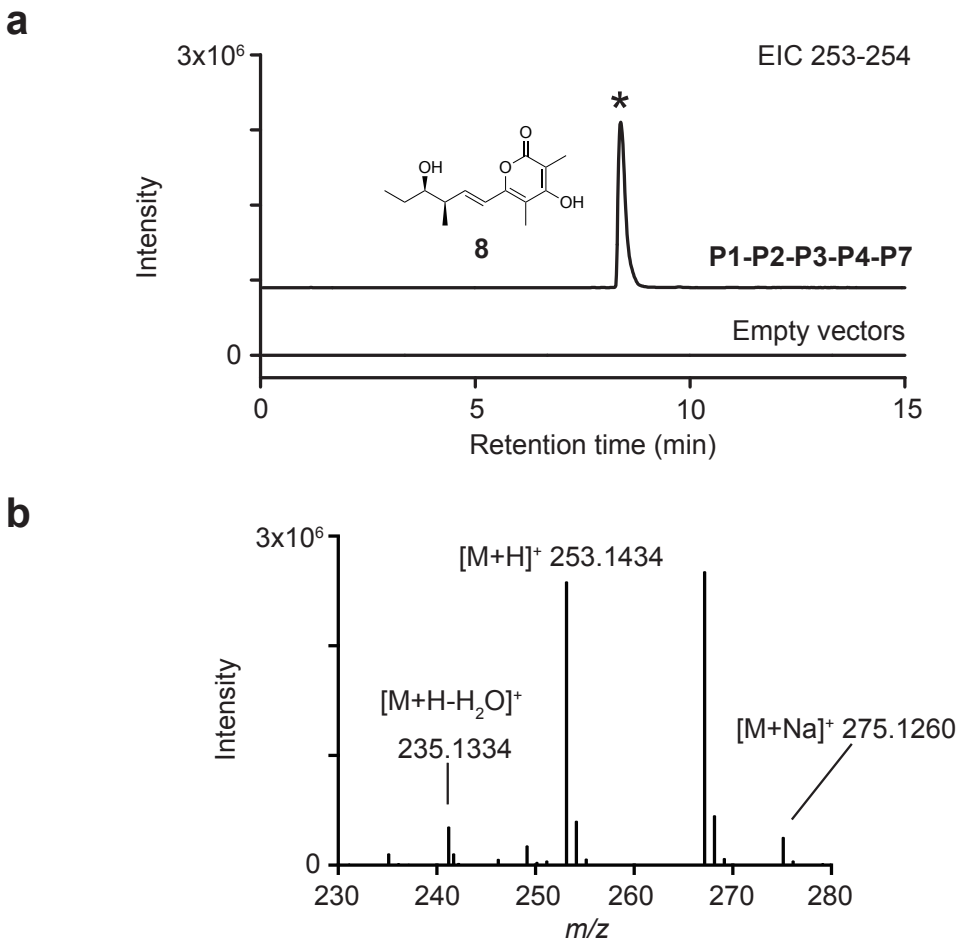


Figure S5. LC/MS analysis of the anticipated **P1**-**P2**-**P3**-**P4**-**P7** product, **8**. a) EIC for the $[M+H]^+$ of **8** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **8,** 253.1434; observed *m/z*, 253.1434 (0 ppm).

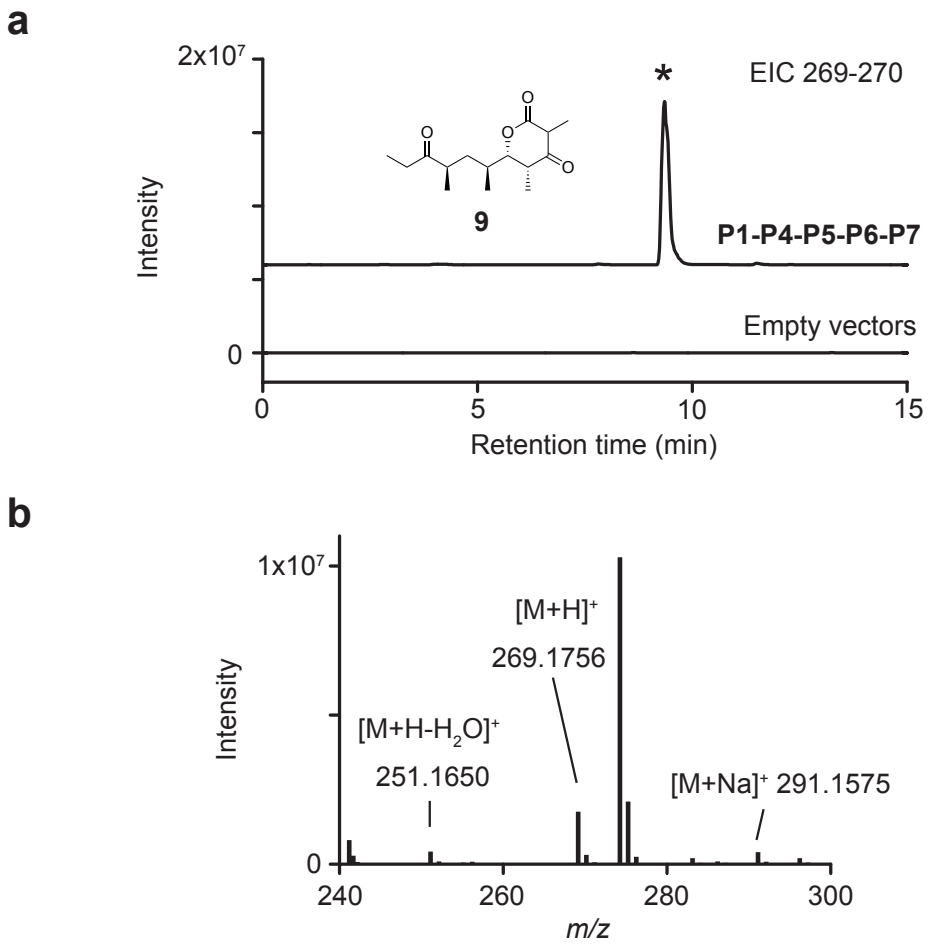


Figure S6. LC/MS analysis of the anticipated **P1**-**P4**-**P5**-**P6**-**P7** product, **9**. a) EIC for the $[M+H]^+$ of **9** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **9,** 269.1747; observed *m/z*, 269.1575 (3.3 ppm).

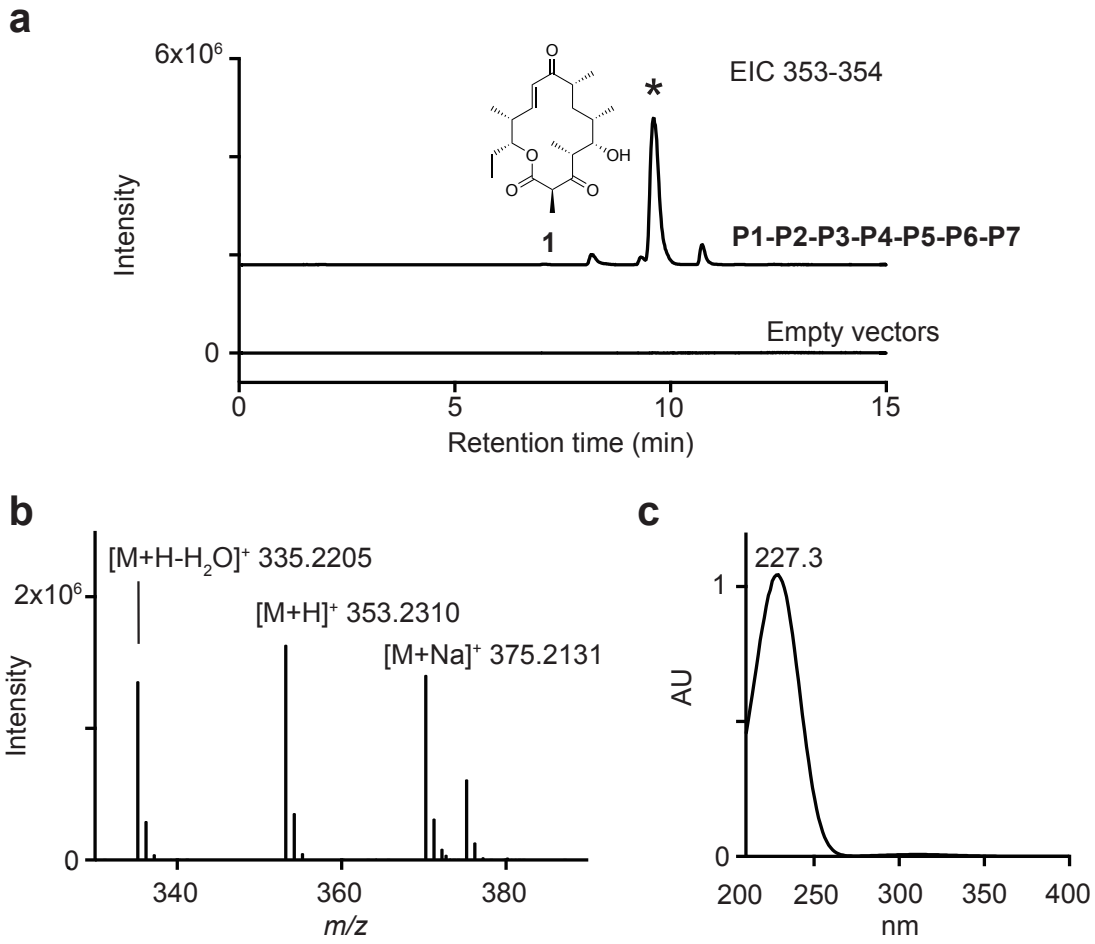


Figure S7. LC/MS analysis of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7** product, narbonolide (**1**). a) EIC for the $[M+H]^+$ of **1** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **1,** 353.2322; observed *m/z*, 353.2310 (-3.3 ppm). c) Absorbance spectrum of **1**.

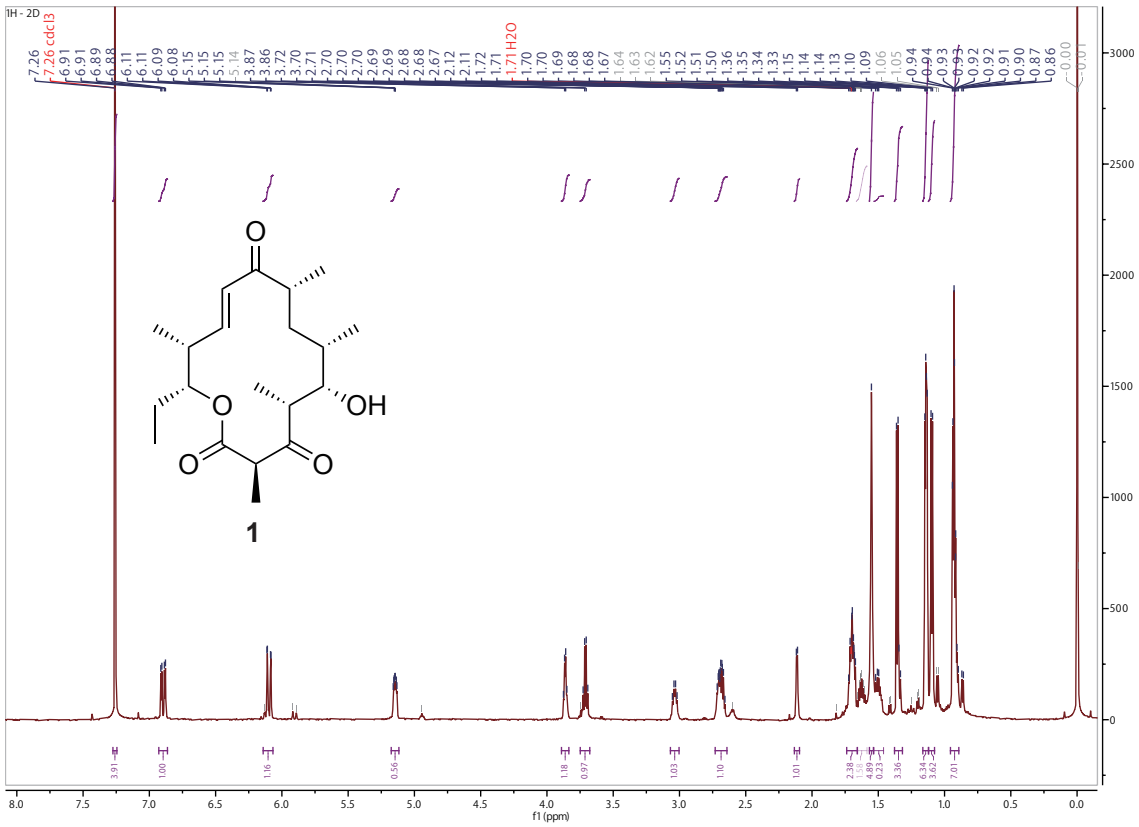


Figure S8. ^{1}H NMR spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7** product, narbonolide (**1**).

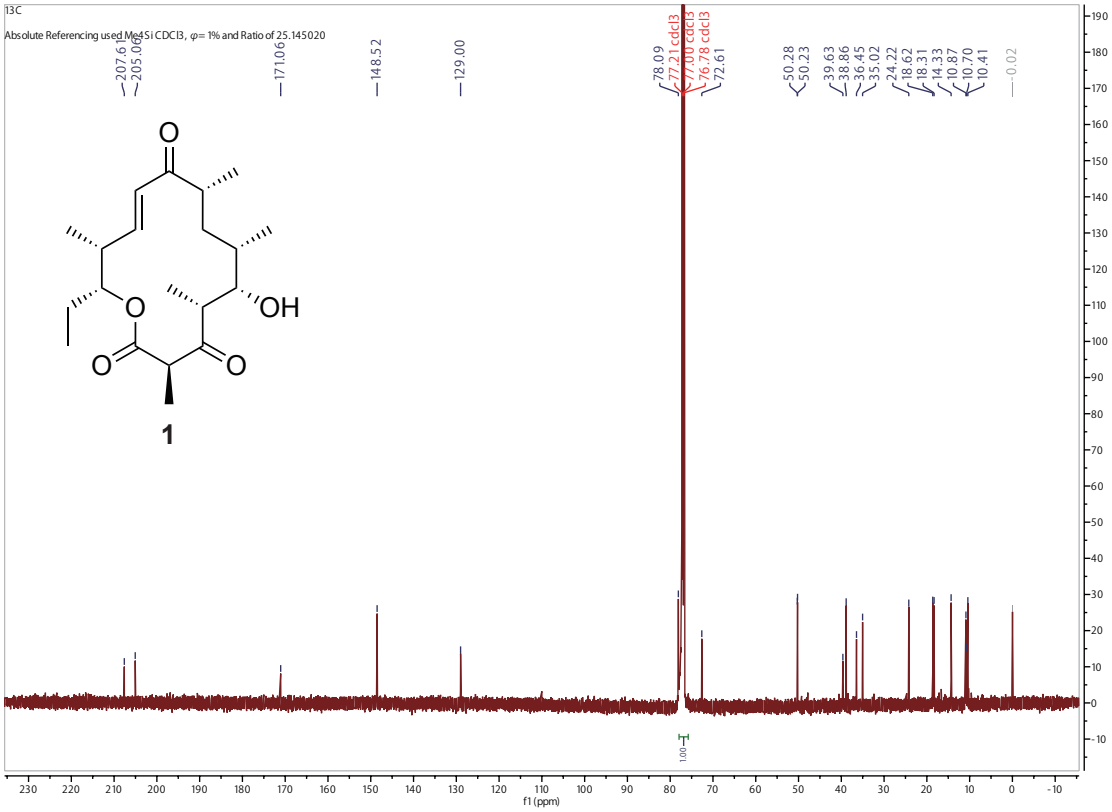


Figure S9. ^{13}C NMR spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7** product, narbonolide (**1**).

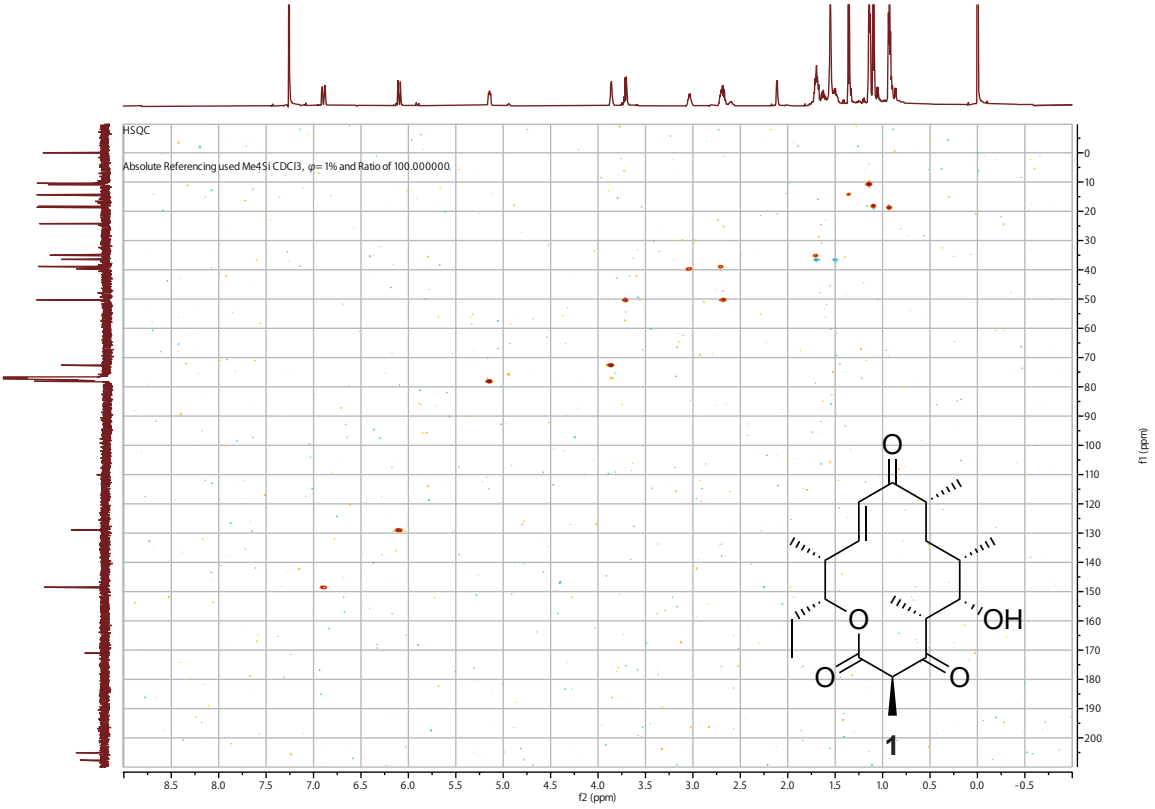


Figure S10. ^{1}H-^{13}C HSQC spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7** product, narbonolide (**1**).

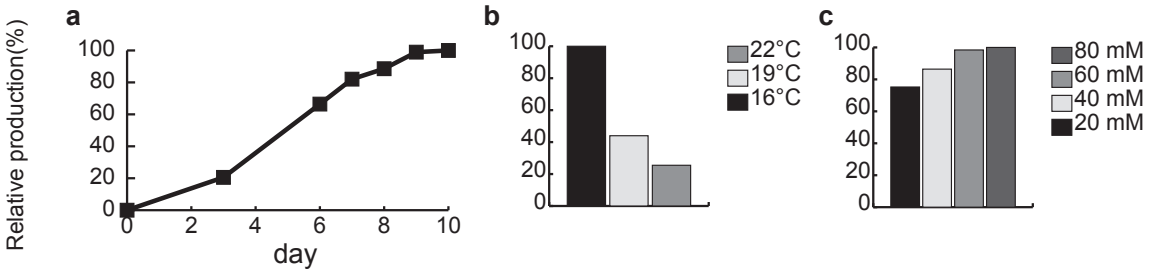


Figure S11. Optimization of narbonolide production. a) Time course of narbonolide production. b) Relative production of narbonolide at different temperatures. c) Relative production of narbonolide at different concentrations of sodium propionate. All data are collected from single experiment.

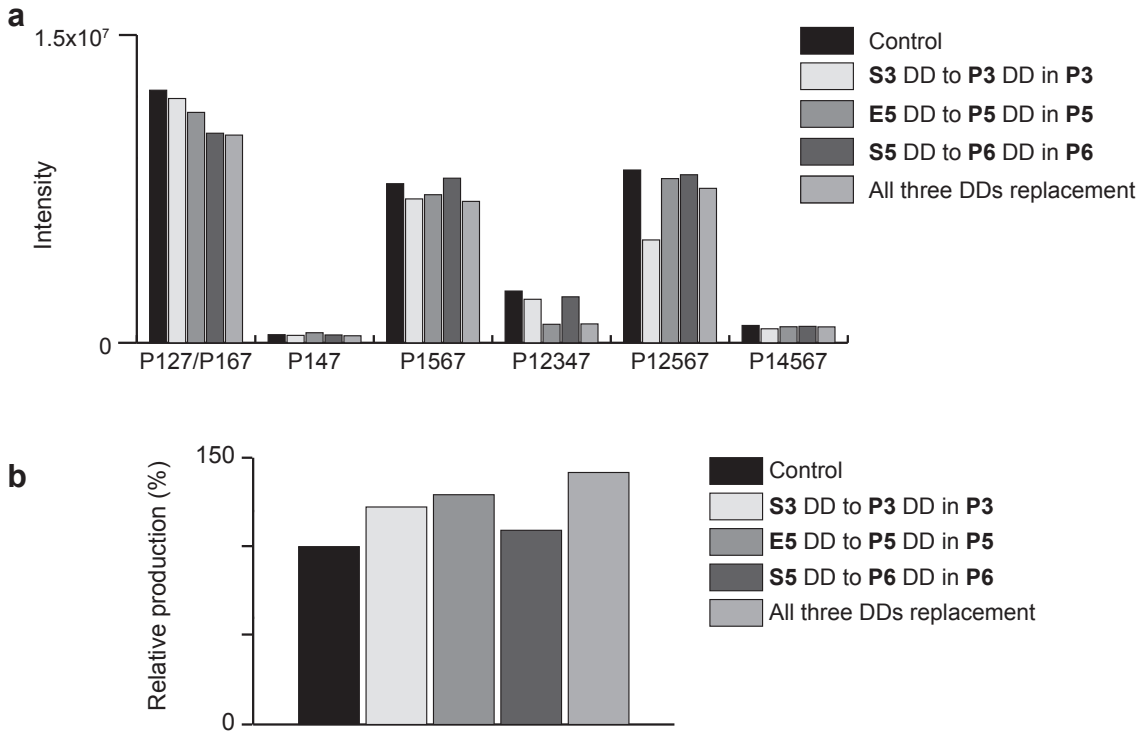


Figure S12. Replacement of the docking domain pair of the narbonolide synthases and analysis of shunt products. a) Shunt products analysis of the refactored narbonolide synthases and their docking domain(DD)-swapped variants. Shunt product accumulation were quantified based on the peak area of EIC of the shunt products. As an example of how shunt products are named, "P1567" is the **P1**-**P5**-**P6**-**P7** product[2]. b) Comparison of the narbonolide productivities between the DD-swapped variants. All data are collected from single experiment.

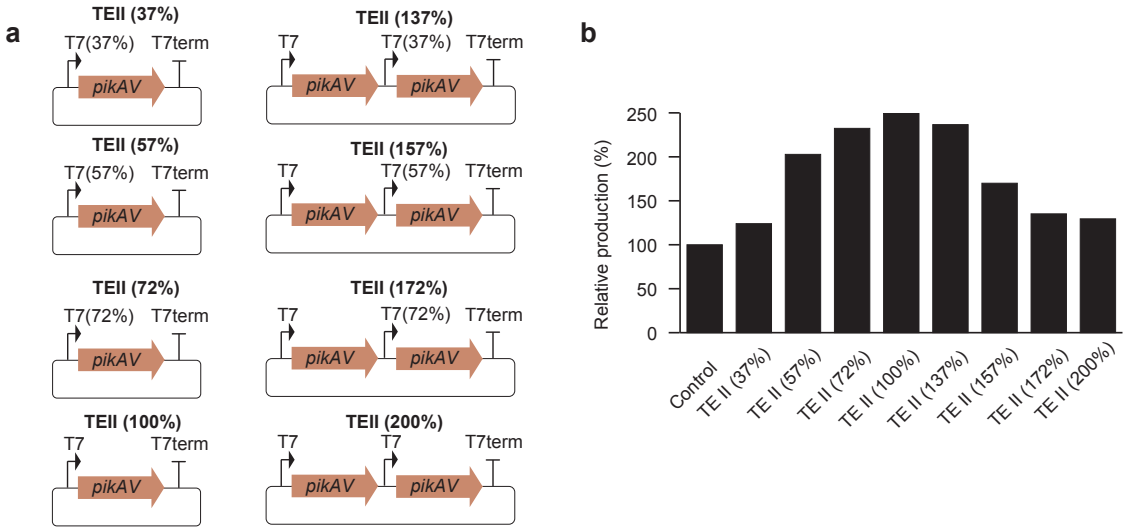


Figure S13. Analysis of the effect of TEII expression level on narbonolide production. a) A series of pACYC-derived *pikAV* expression vectors with different expression levels were co-transformed with the plasmids encoding the refactored pikromycin synthase. Expression of PikAV is controlled by the T7 promoter and attenuated T7 promotors[7]. b) Relative production of narbonolide was quantified by LC/MS.

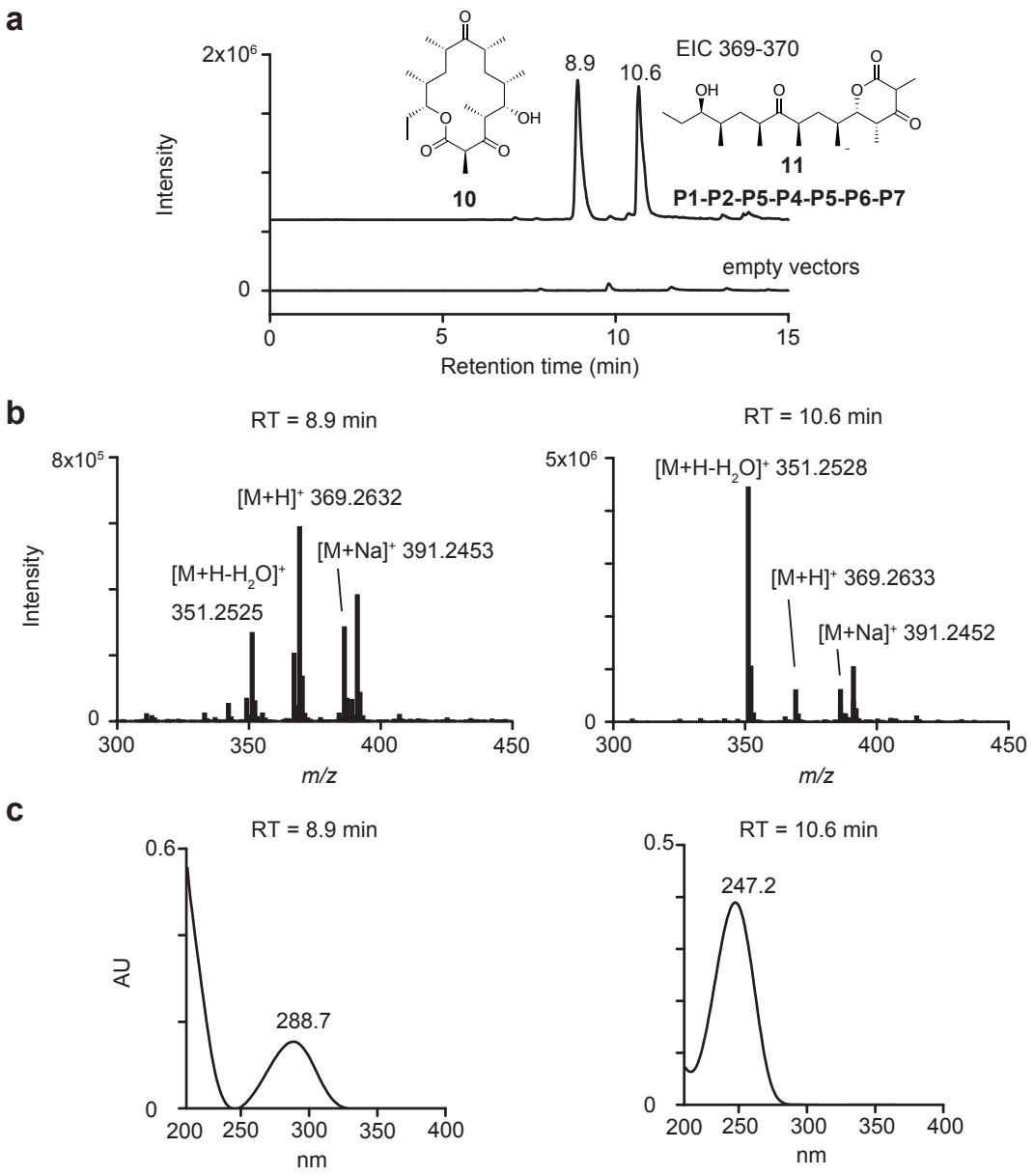


Figure S14. LC/MS analysis of the anticipated **P1**-**P2**-**P5**-**P4**-**P5**-**P6**-**P7** product, **10** and byproduct **11**. a) EIC for the $[M+H]^+$ of **10** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectra of the two main peaks (RT = 8.9 and 10.6 min). Theoretical $[M+H]^+$ *m/z* of **10**, 369.2635; observed *m/z*, 369.2632 (-0.8 ppm). Theoretical $[M+H]^+$ *m/z* of **11**, 369.2635; observed *m/z*, 369.2633 (-0.5 ppm). c) UV absorbance spectra correlated with the main peaks. The λ_{max} of **11** is characteristic of β-keto δ-lactones.

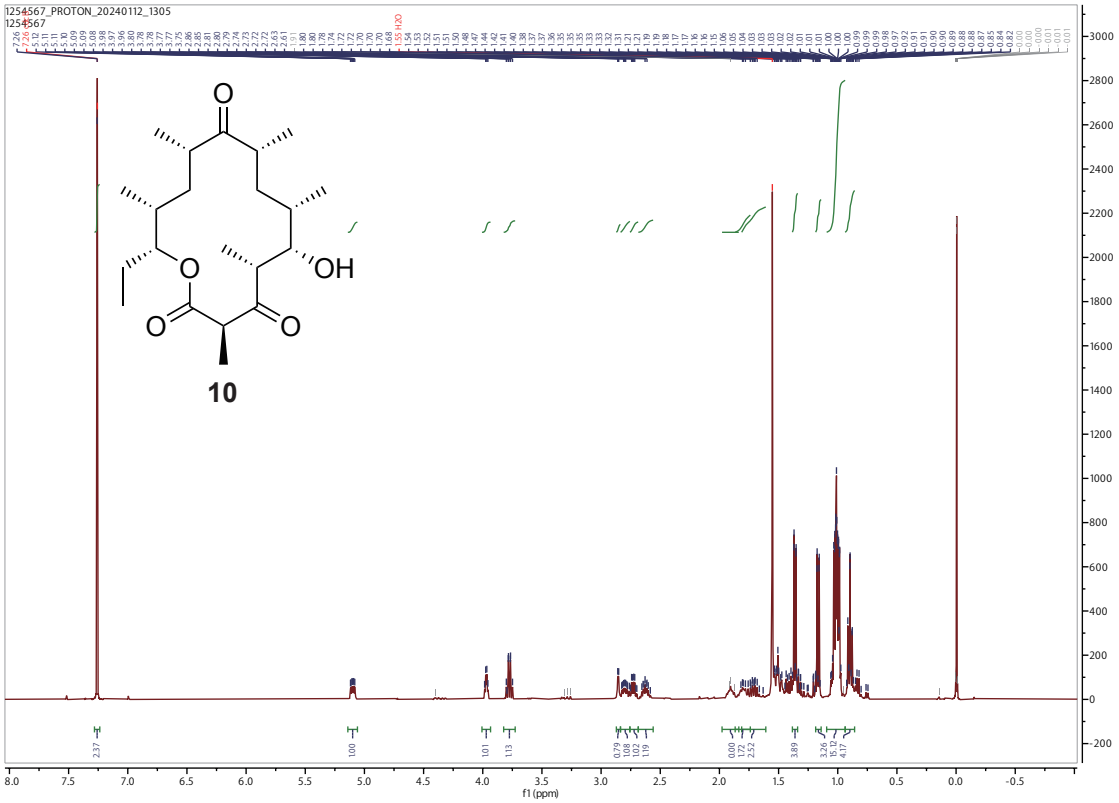


Figure S15. ^{1}H NMR spectrum of anticipated **P1**-**P2**-**P5**-**P4**-**P5**-**P6**-**P7** product, **10**.

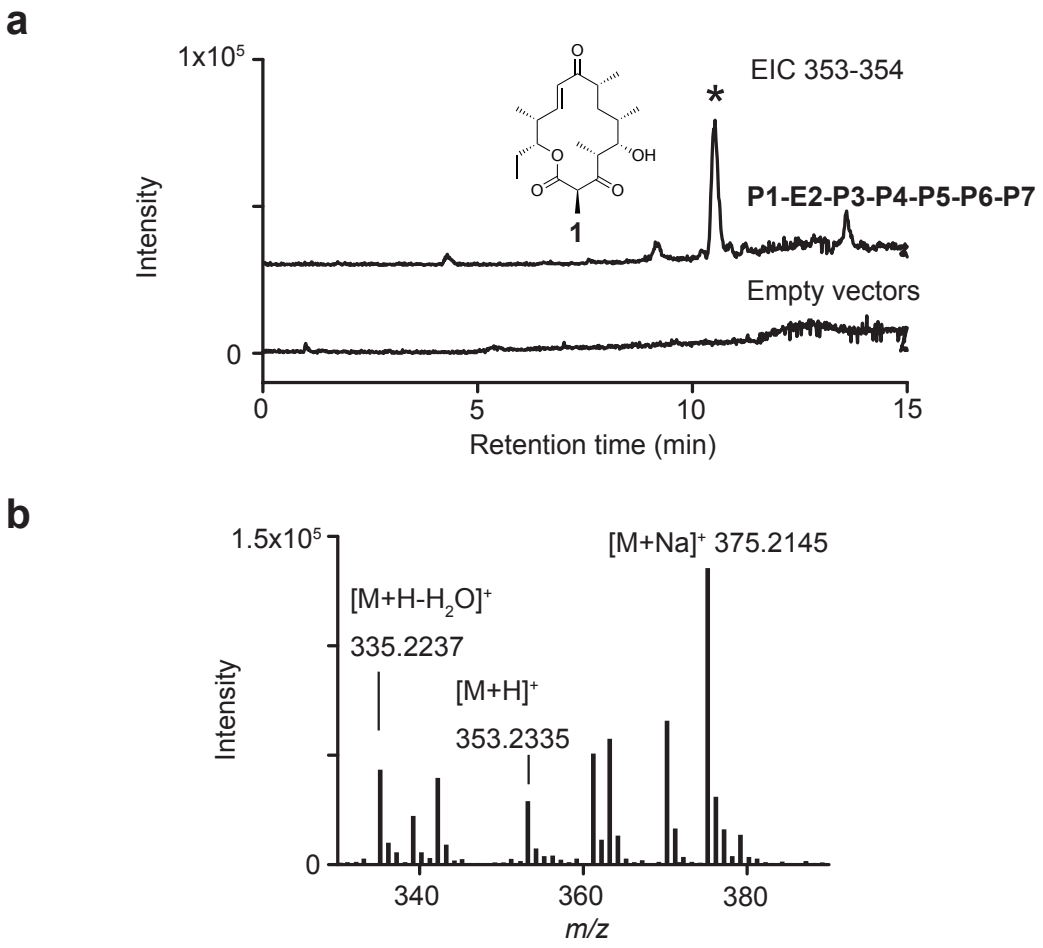


Figure S16. LC/MS analysis of the anticipated **P1**-**E2**-**P3**-**P4**-**P5**-**P6**-**P7** product, narbonolide (**1**). a) EIC for the $[M+H]^+$ of **1** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **1**, 353.2317; observed *m/z*, 353.2335 (3.6 ppm).

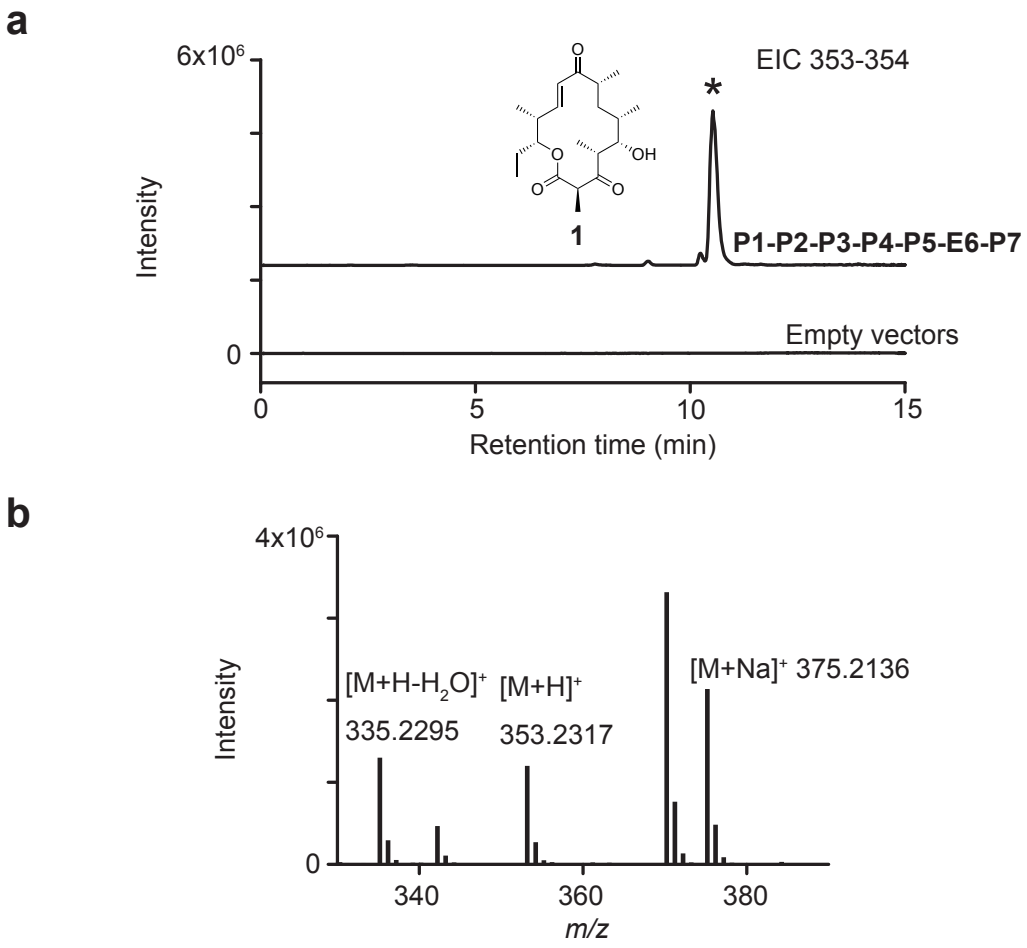


Figure S17. LC/MS analysis of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**E6**-**P7** product, narbonolide (**1**). a) EIC for the $[M+H]^+$ of **1** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **1**, 353.2317; observed *m/z*, 353.2317 (-1.4 ppm).

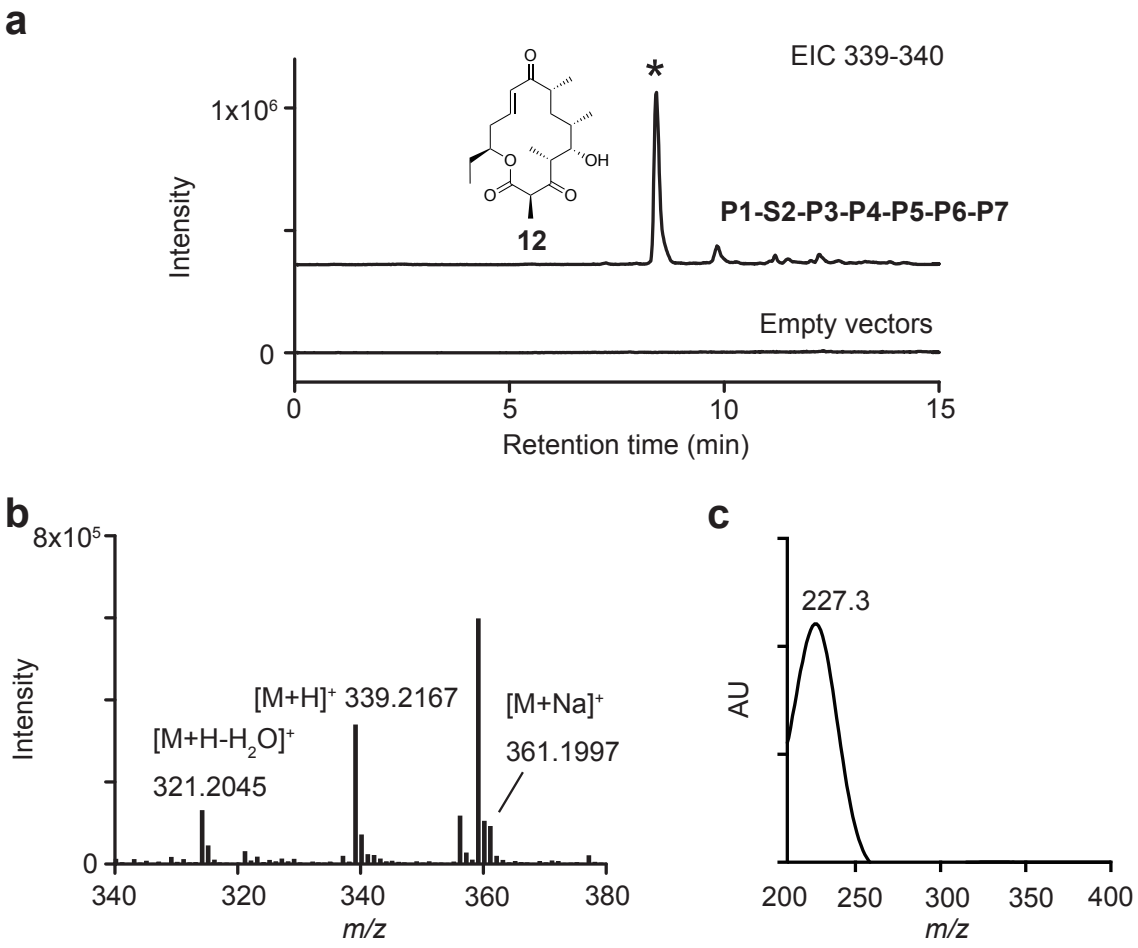


Figure S18. LC/MS analysis of the anticipated **P1**-**S2**-**P3**-**P4**-**P5**-**P6**-**P7** product, **12**. a) EIC for the $[M+H]^+$ of **12** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **12**, 339.2166; observed *m/z*, 339.2167 (0.29 ppm). c) UV absorbance spectrum for both of the main peaks. The λ_{max} of 227.3 nm is similar to that of narbonolide (Fig. S7c).

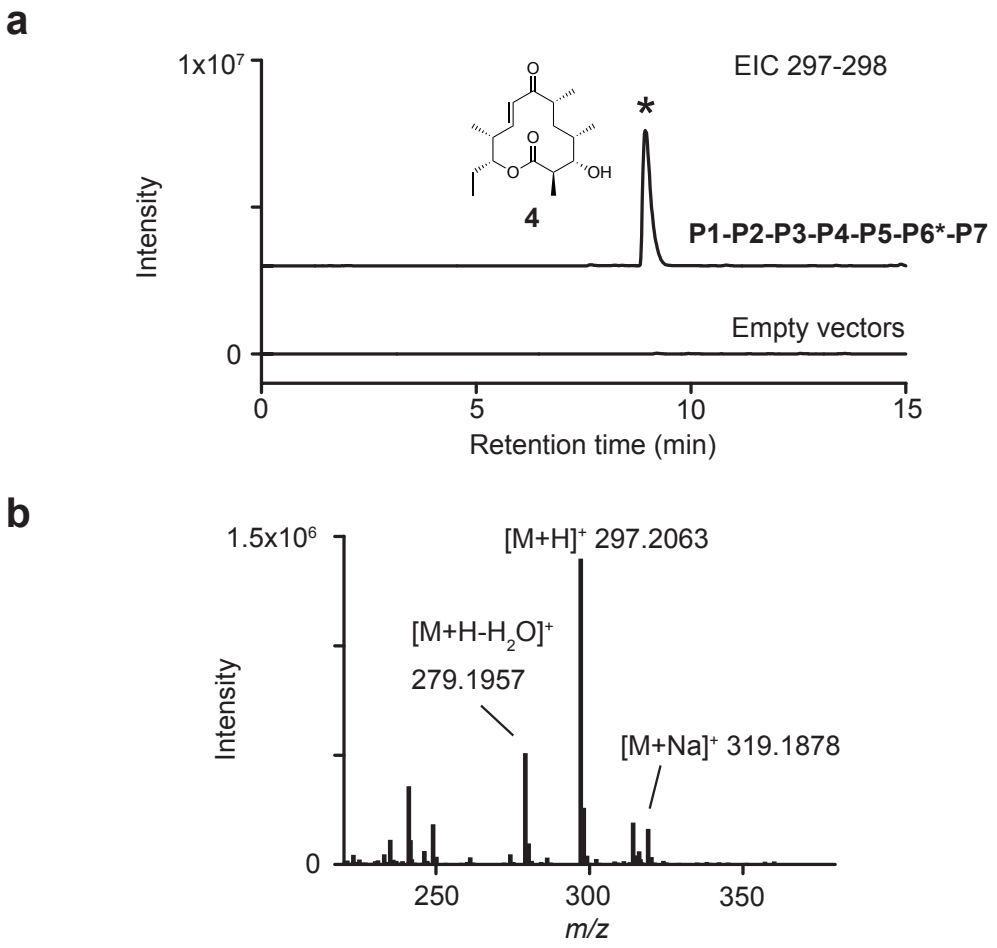


Figure S19. LC/MS analysis of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7** product, 10-dML (**4**). a) EIC for the $[M+H]^+$ of **4** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **4**, 297.2060; observed *m/z*, 297.2063 (1.0 ppm).

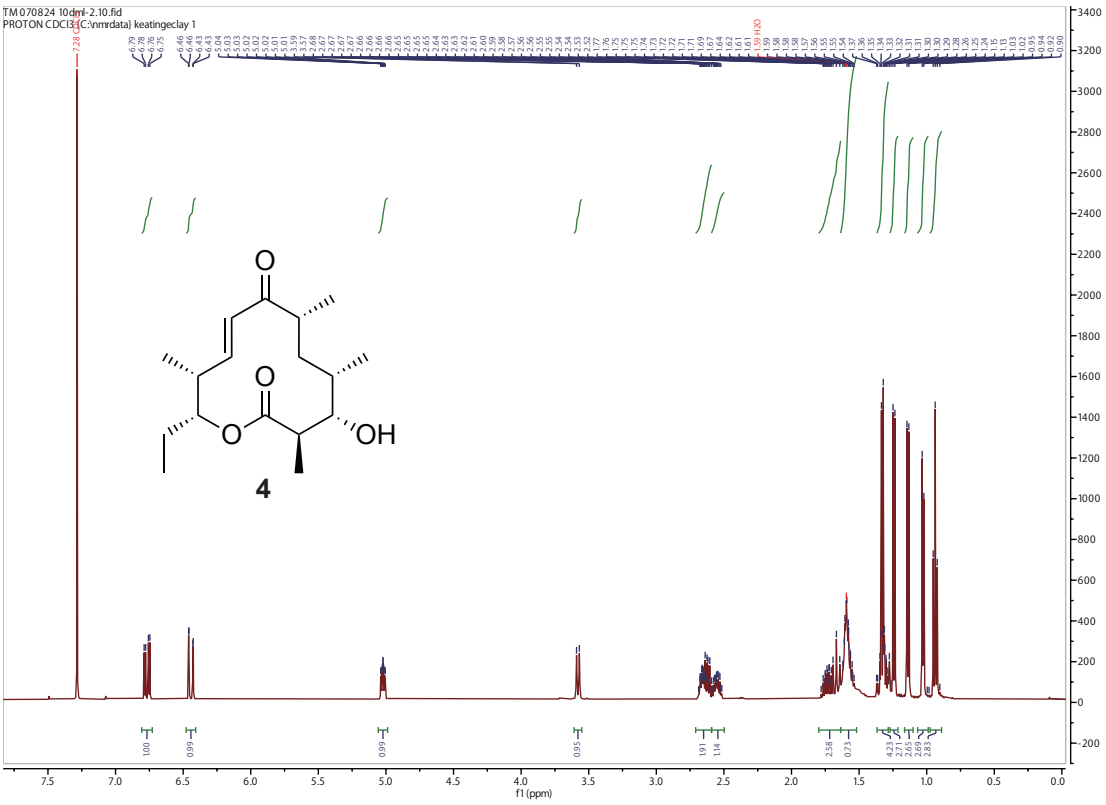


Figure S20. ^{1}H NMR spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7** product, **4** (10-dml).

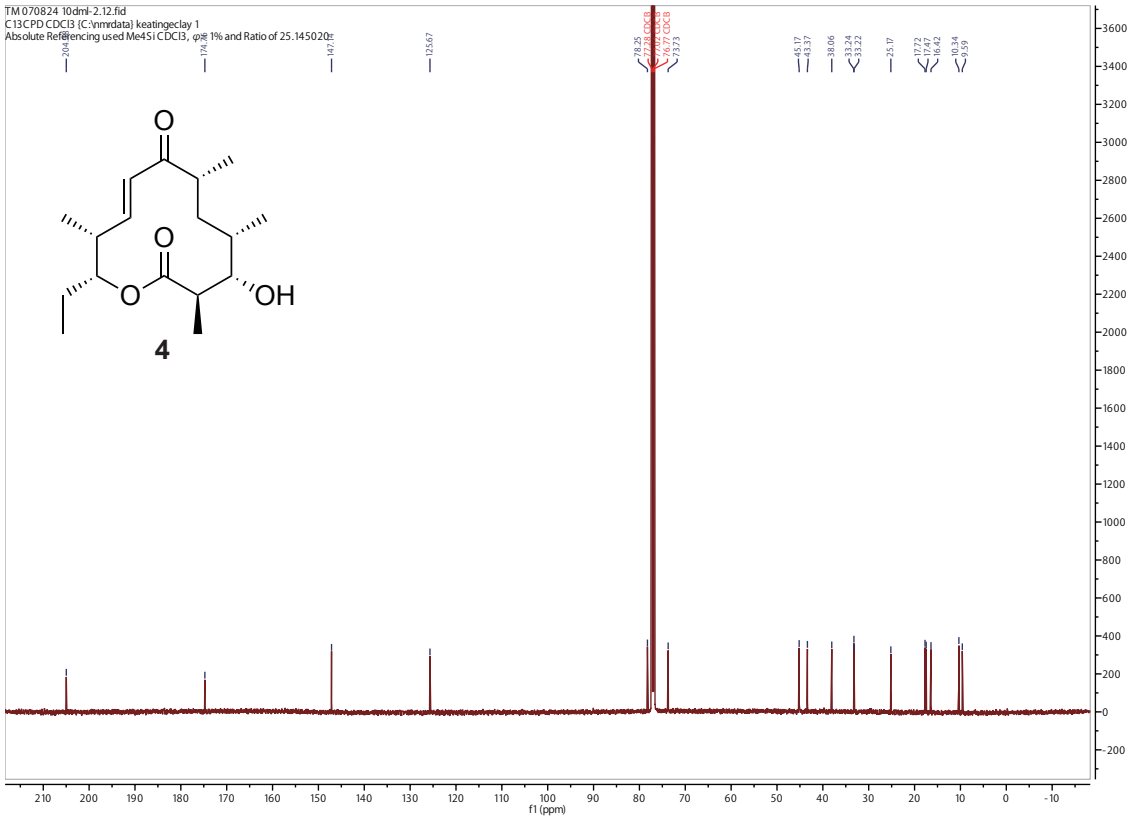


Figure S21. ^{13}C NMR spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7** product, **4** (10-dml).

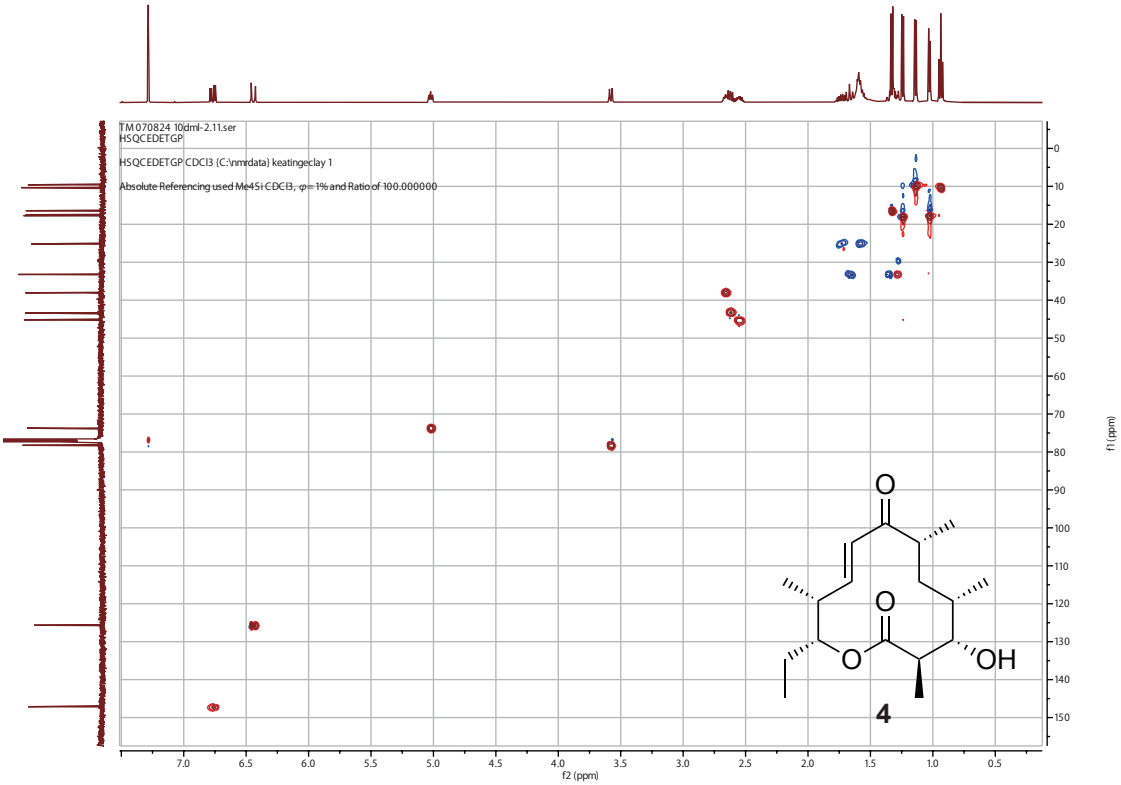


Figure S22. ^{1}H-^{13}C HSQC spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7** product, **4** (10-dml).

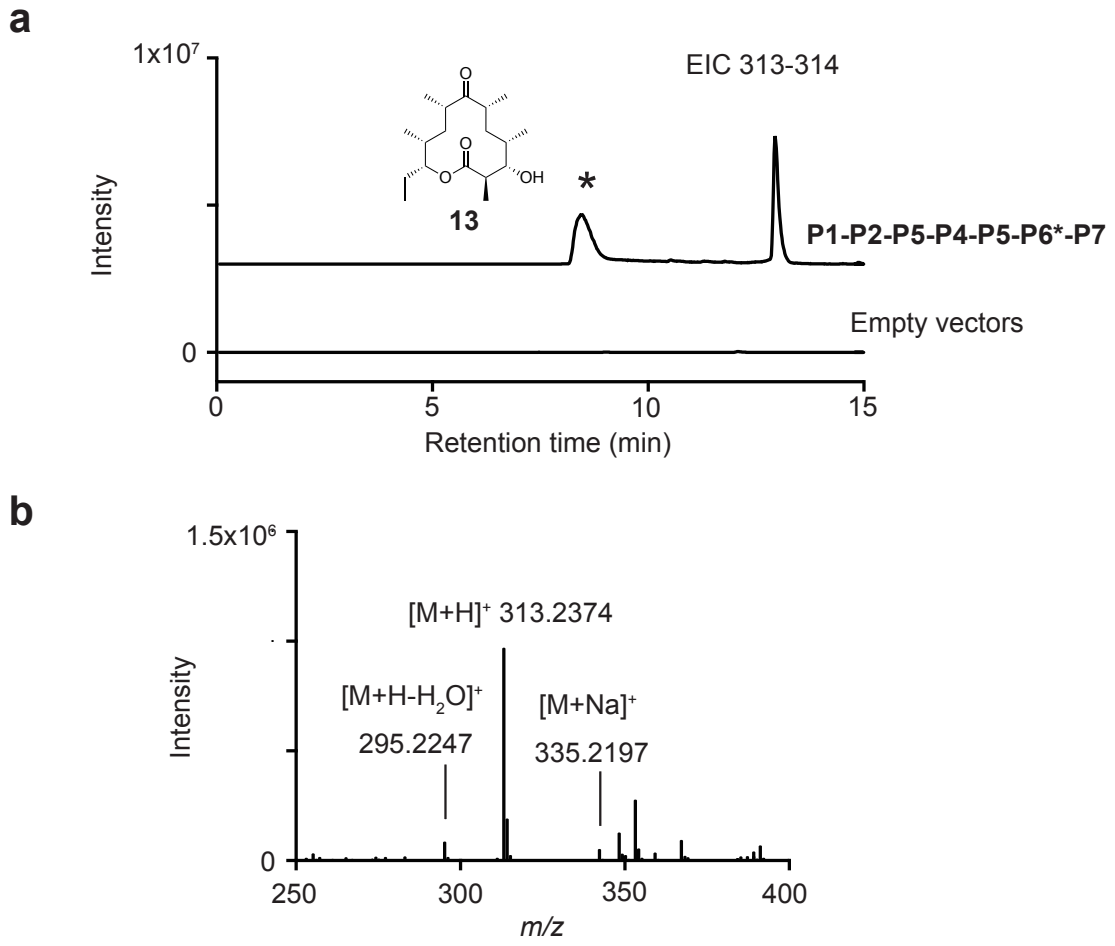


Figure S23. LC/MS analysis of the anticipated **P1**-**P2**-**P5**-**P4**-**P5**-**P6***-**P7** product, **13.** a) EIC for the $[M+H]^+$ of **13** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **13**, 313.2373; observed *m/z*, 313.2370 (0.31 ppm).

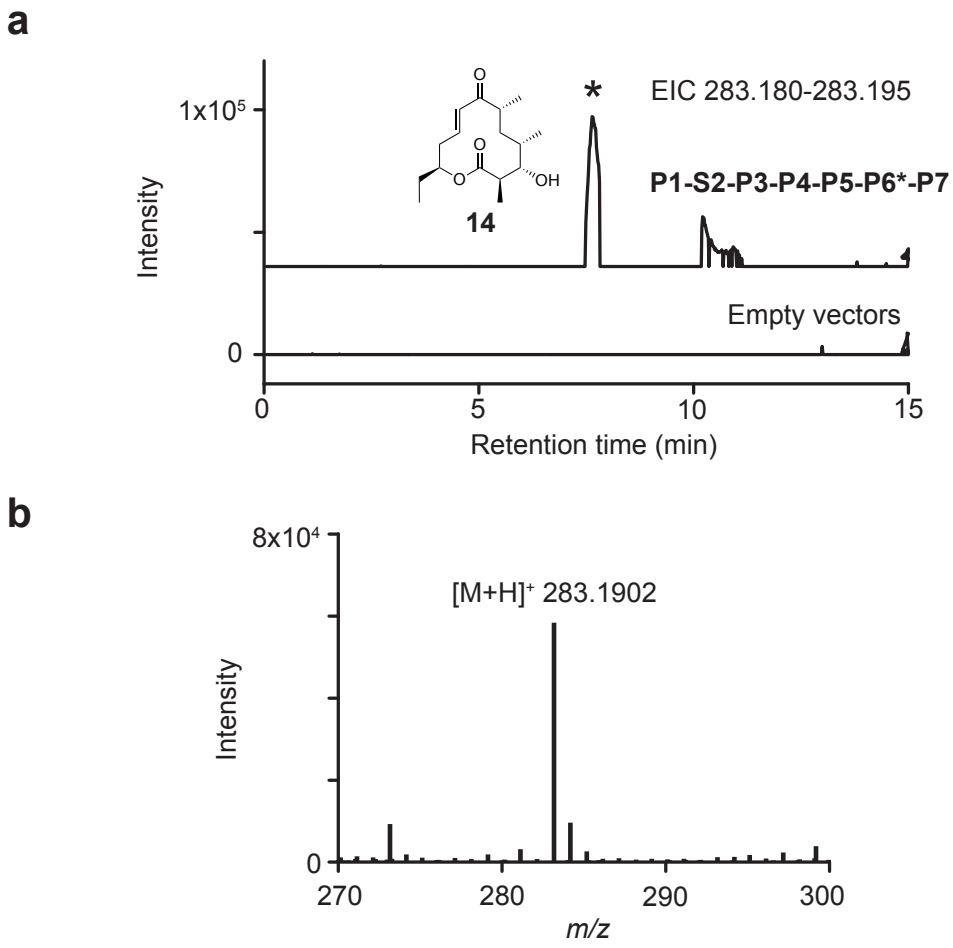


Figure S24. LC/MS analysis of the anticipated **P1**-**S2**-**P3**-**P4**-**P5**-**P6***-**P7** product, **14**. a) EIC for the $[M+H]^+$ of **14** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **4**, 283.1904; observed *m/z*, 283.1902 (-0.35 ppm).

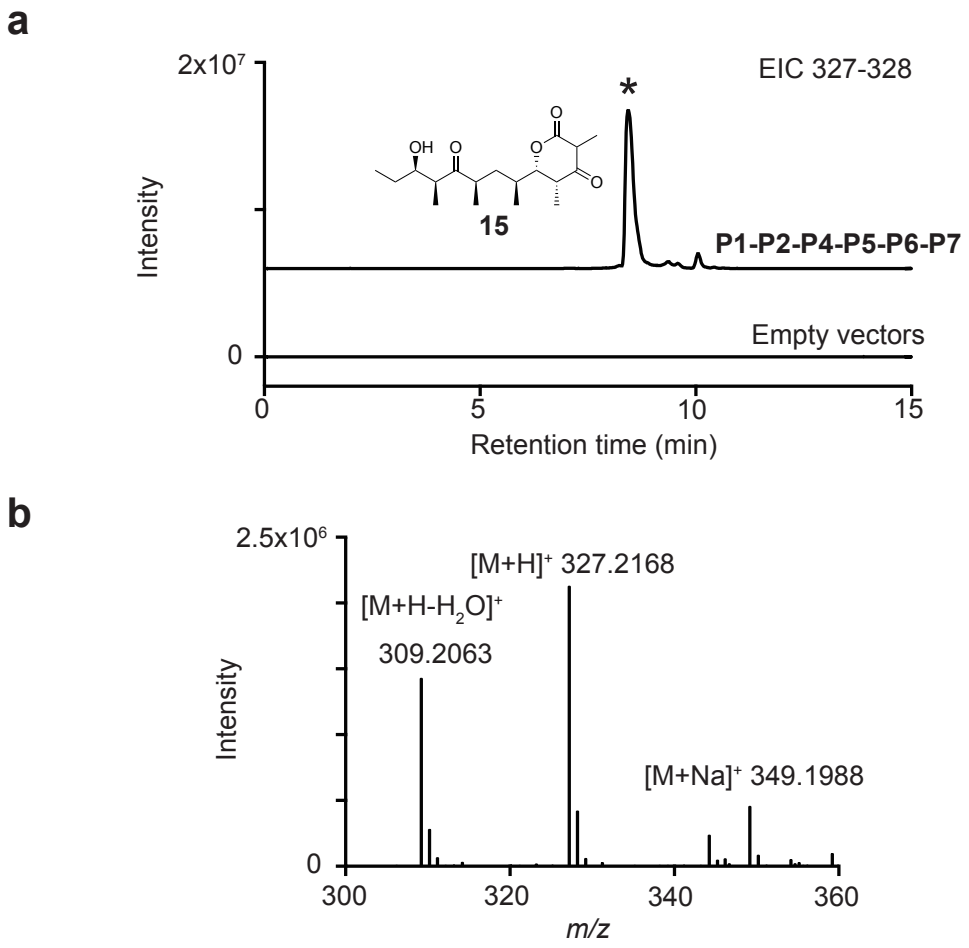


Figure S25. LC/MS analysis of the anticipated **P1**-**P2**-**P4**-**P5**-**P6**-**P7** product, **15**. a) EIC for the $[M+H]^+$ of **15** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **15**, 327.2166; observed *m/z*, 327.2168 (0.61 ppm).

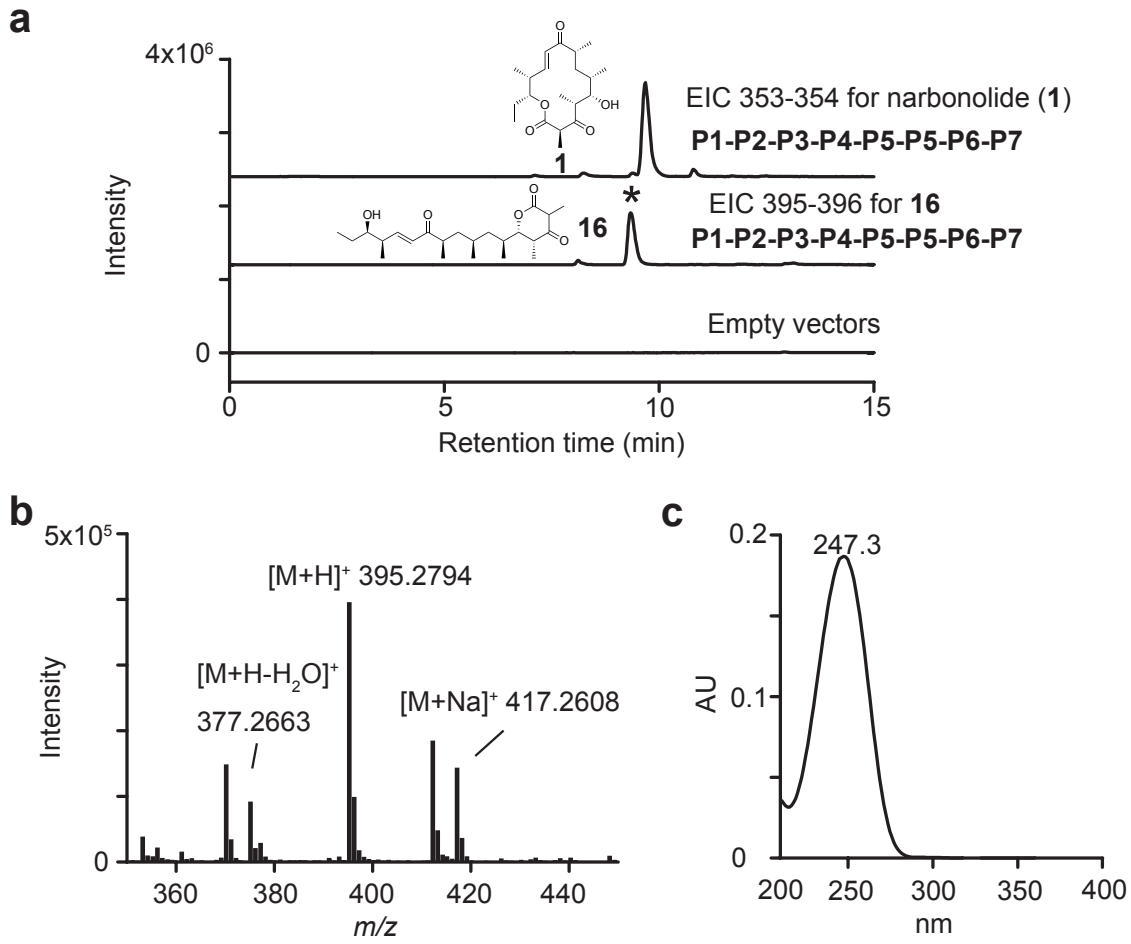


Figure S26. LC/MS analysis of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P5**-**P6**-**P7** product,**16**. a) EIC for the $[M+H]^+$ of **16** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the first peak (*). Theoretical $[M+H]^+$ *m/z* of **16**, 395.2792; observed *m/z*, 395.2794 (0.51 ppm). c) UV spectrum corresponding to the first peak (*) . Since its λ_{max} is equivalent to that of β-keto δ-lactones, the product of **P1**-**P2**-**P3**-**P4**-**P5**-**P5**-**P6**-**P7** product is **16**, rather than the anticipated octaketide macrolactone.

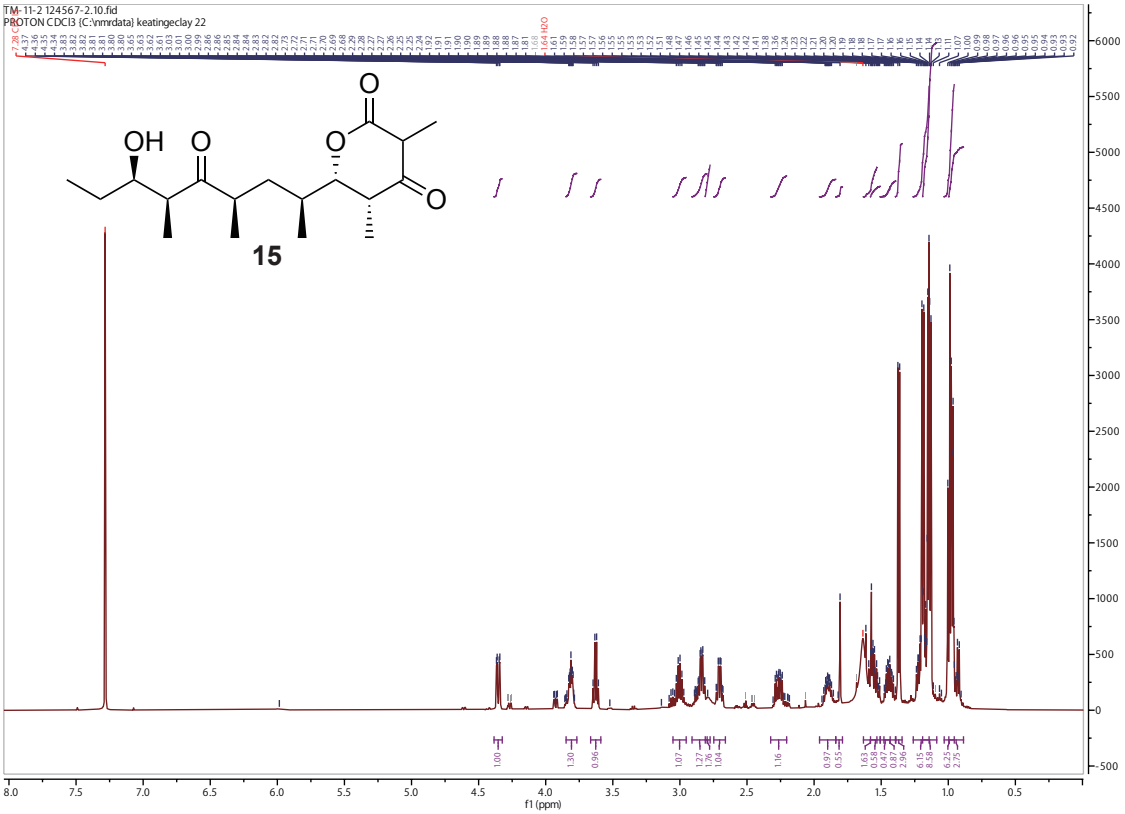


Figure S27. ^{1}H NMR spectrum of the anticipated **P1**-**P2**-**P4**-**P5**-**P6**-**P7** product, **15**.

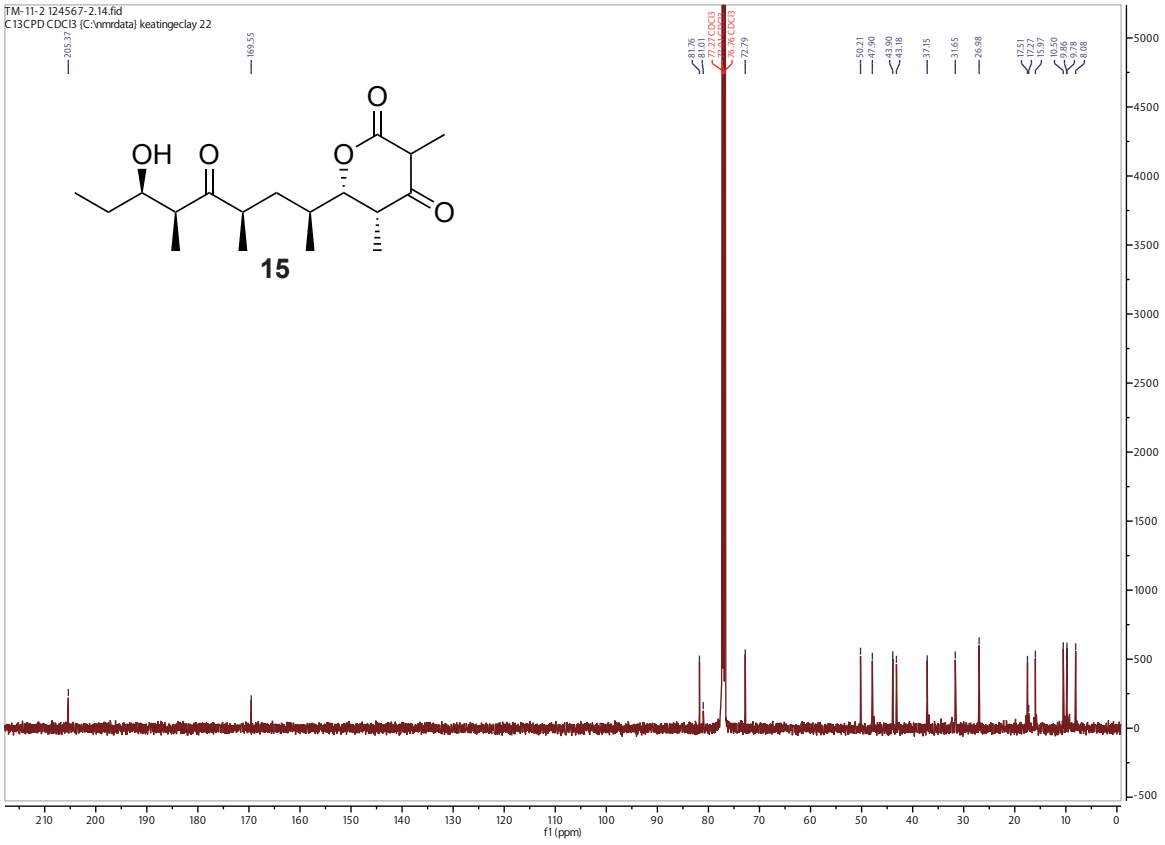


Figure S28. ^{13}C NMR spectrum of the anticipated **P1**-**P2**-**P4**-**P5**-**P6**-**P7** product **15**.

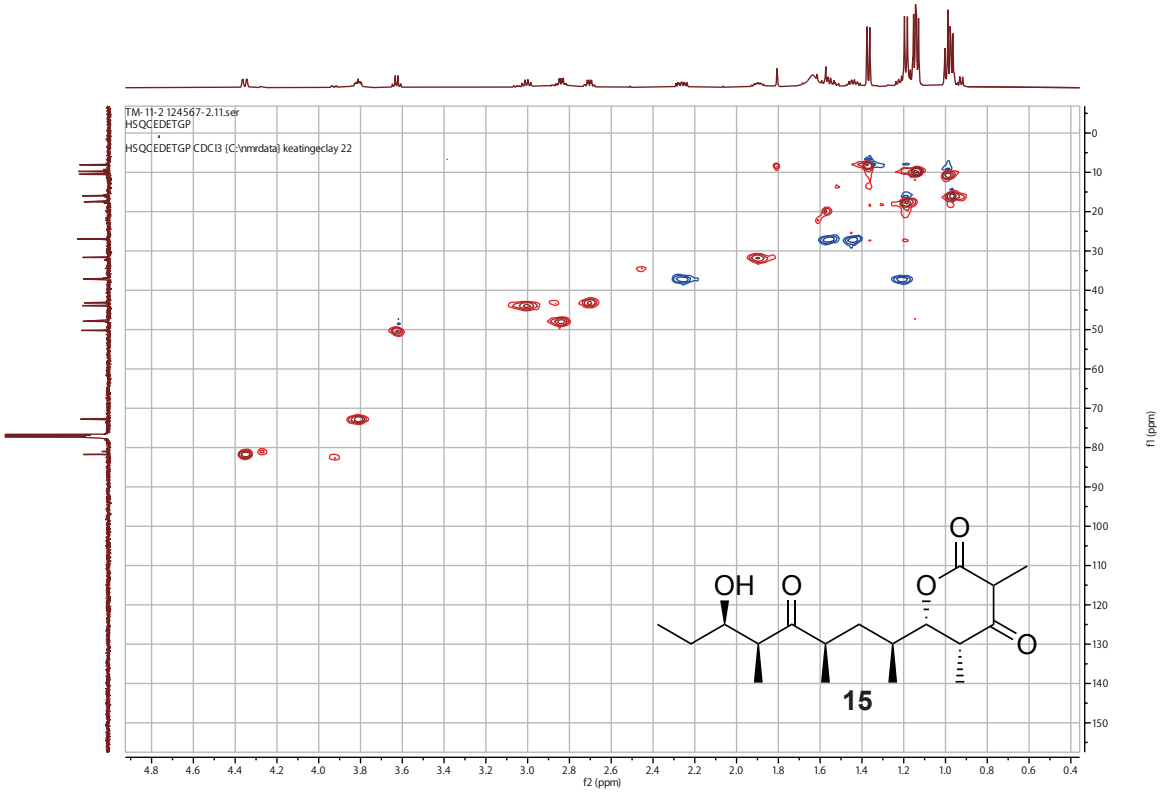


Figure S29. ^{1}H-^{13}C HSQC spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7** product, **15**.

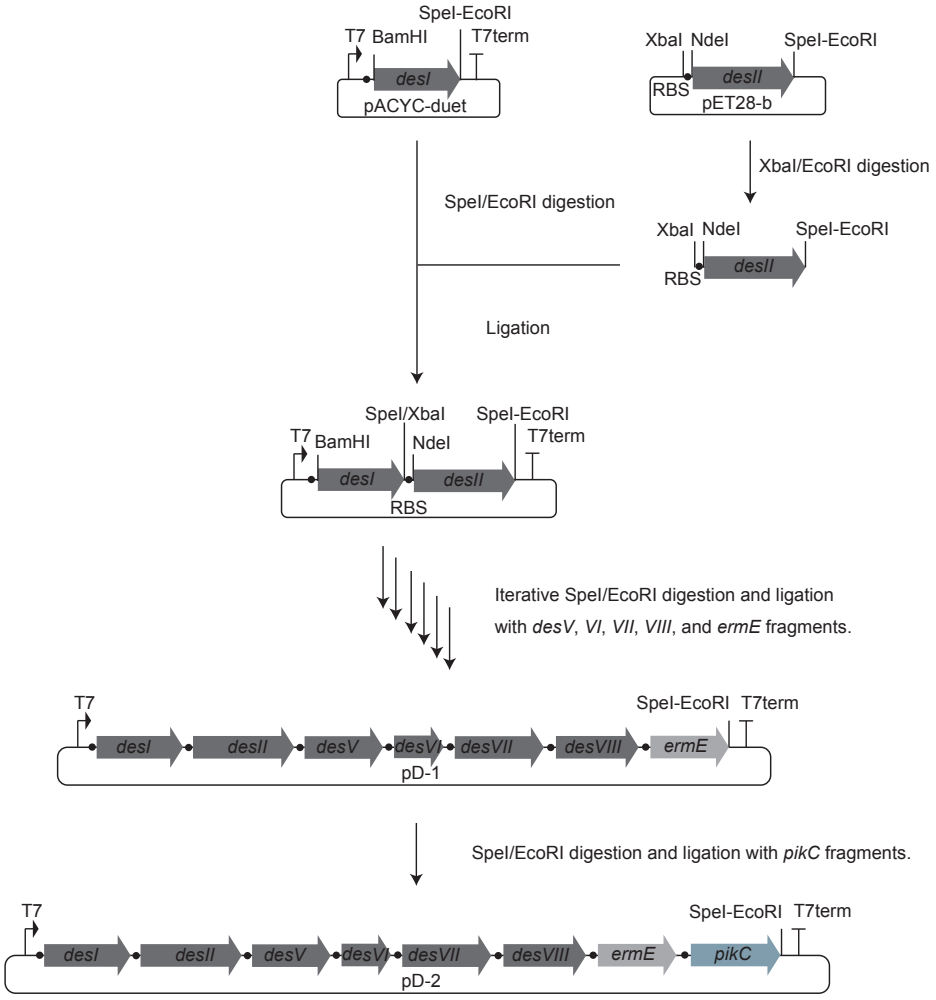


Figure S30. Construction of D-desosamine biosynthesis/transfer pathway. The *desI* gene with an SpeI site after its stop codon was cloned into the BamHI/EcoRI site of pACYC-duet vector, while *desII, V, VI, VII, VIII,* and *ermE* with SpeI sites after their stop codons were cloned into the NdeI/EcoRI site of a pET28b vector. The XbaI/EcoRI fragment containing *desII* was inserted into the SpeI/EcoRI site of the pACYC-*desI*. Since a nonfunctional SpeI/XbaI sites are formed through such ligations, XbaI/EcoRI-digested fragments can be sequentially inserted into the SpeI/EcoRI site of the pACYC plasmid. Iterative SpeI/EcoRI digestion and ligation of *desV*, *VI*, *VII*, *VIII*, and *ermE* fragments yielded pDes. The *pikC* gene was equivalently inserted into the Spe/EcoRI site of pDes to yield pDesPikC. Black dots indicate ribosome binding sites (RBSs).

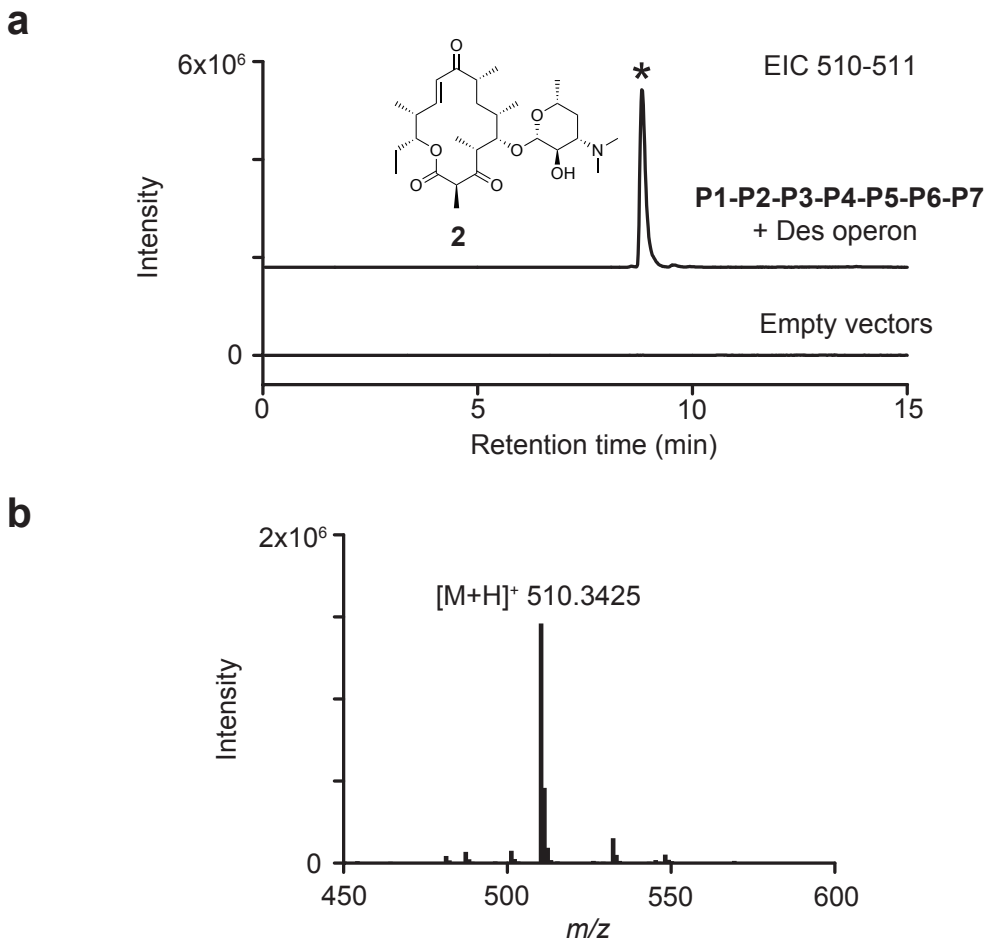


Figure S31. LC/MS analysis of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7**+Des product, narbomycin (**2**). a) EIC for the $[M+H]^+$ of **2** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **2**, 510.3425; observed *m/z*, 510.3425 (0 ppm).

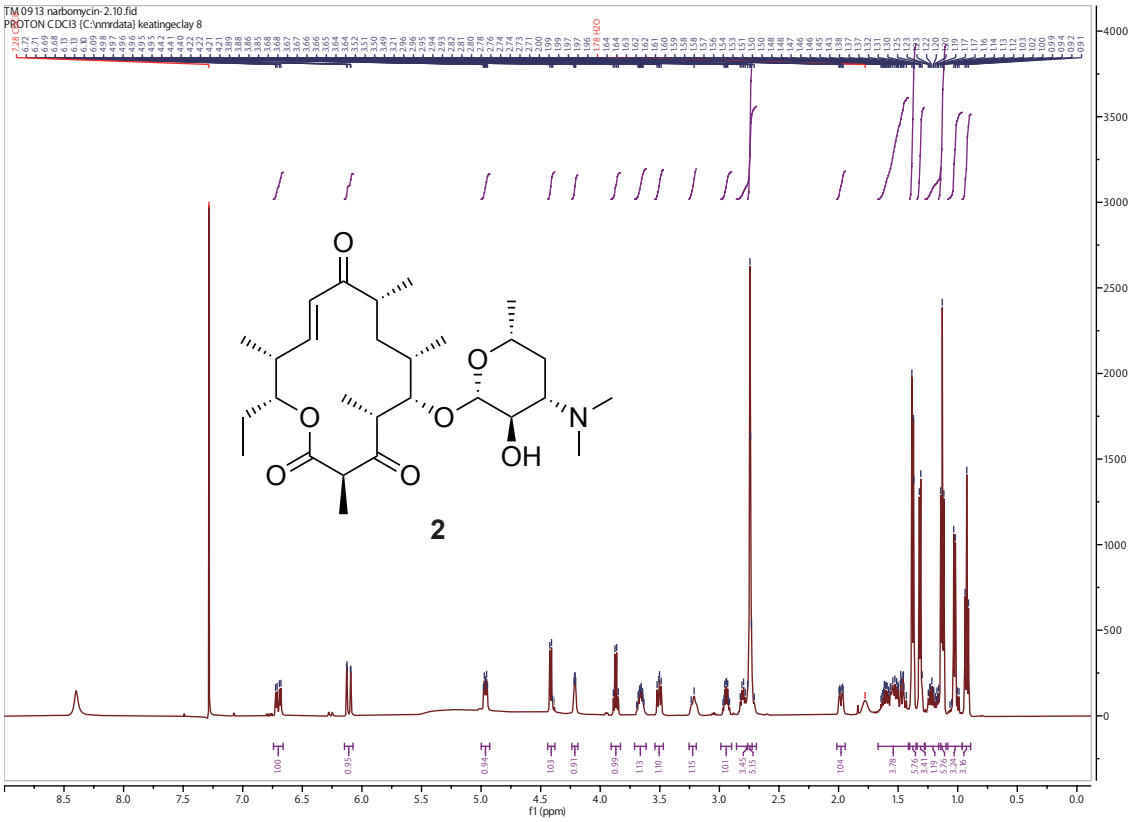


Figure S32. ^{1}H NMR spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7**+Des product, narbomycin (**2**).

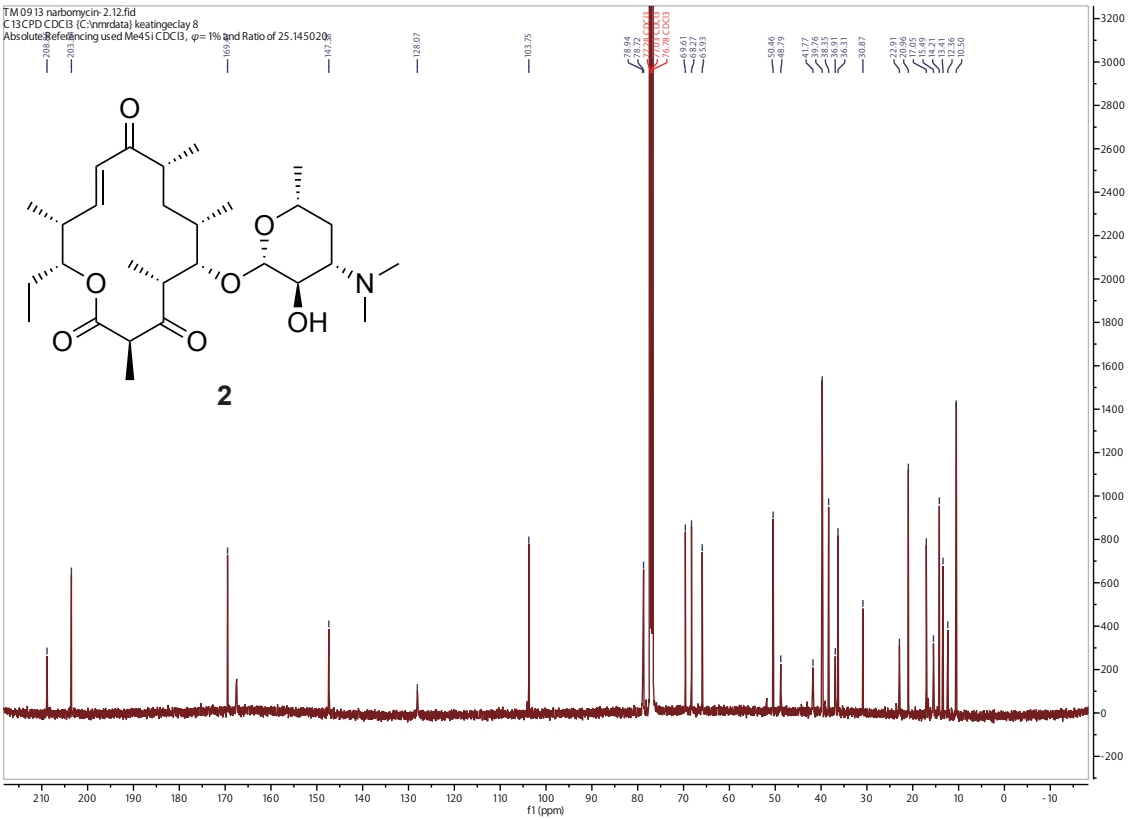


Figure S33. ^{13}C NMR spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7**+Des product, narbomycin (**2**).

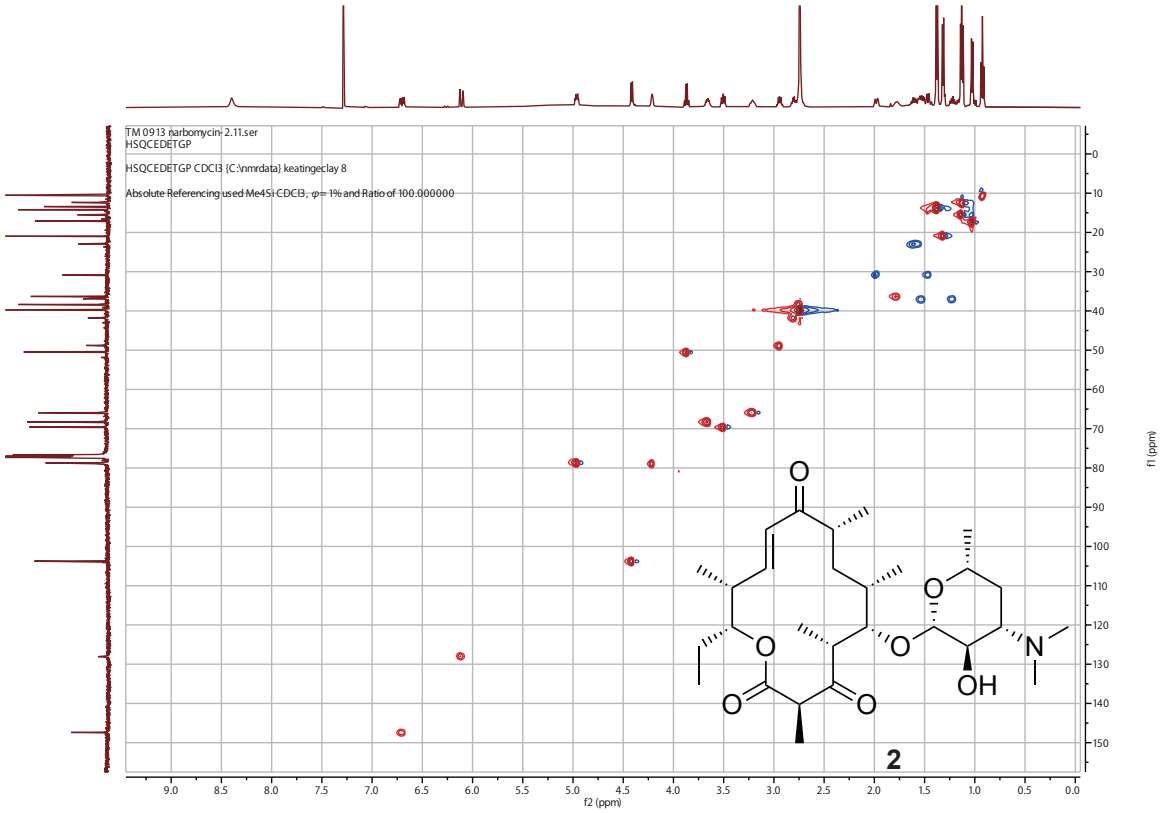


Figure S34. ^{1}H-^{13}C HSQC spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7**+Des, narbomycin (**2**).

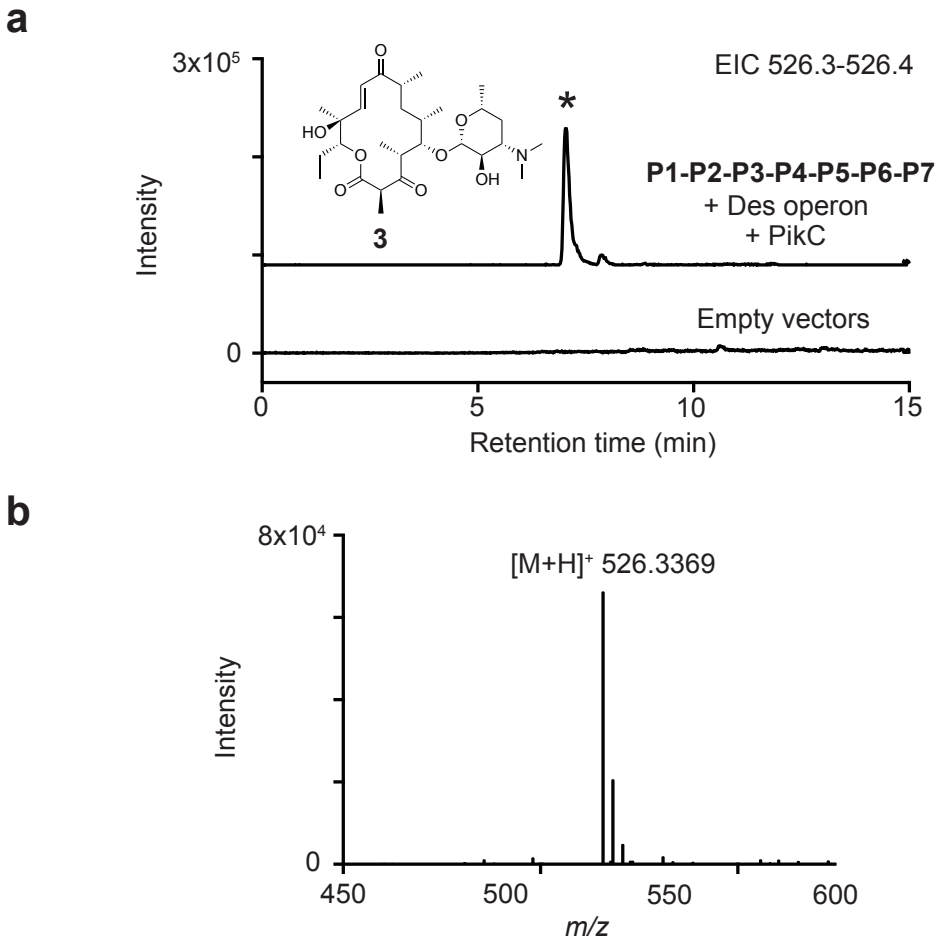
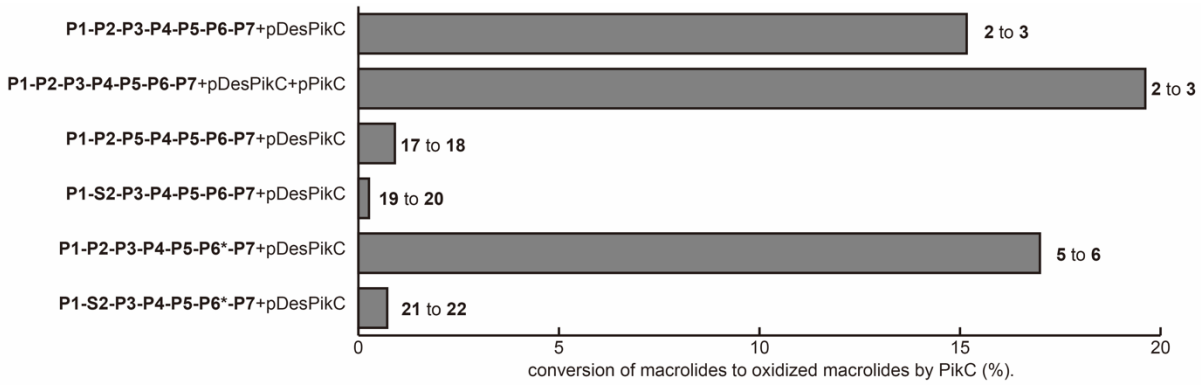


Figure S35. LC/MS analysis of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6**-**P7**+Des+PikC product, pikromycin (**3**). a) EIC for the $[M+H]^+$ of **3** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **3**, 526.3374; observed *m/z*, 526.3369 (-0.94 ppm).



	macrolide (mgL^{-1})	oxidized macrolide (mgL^{-1})	PikC conversion (%)
P1-P2-P3-P4-P5-P6-P7+pDesPikC	1.31	0.241	15.1
P1-P2-P3-P4-P5-P6-P7+pDesPikC+pPikC	1.21	0.305	19.6
P1-P2-P5-P4-P5-P6-P7+pDesPikC	0.772	0.00730	0.909
P1-S2-P3-P4-P5-P6-P7+pDesPikC	0.104	0.000284	0.263
P1-P2-P3-P4-P5-P6*-P7+pDesPikC	1.63	0.346	16.9
P1-S2-P3-P4-P5-P6*-P7+pDesPikC	0.0677	0.000508	0.718

Figure S36. PikC oxidation efficiency**.** PKS expression plasmids were co-transformed with pDesPikC or pDesPikC and pPikC into *E. coli* K207-3. Percent conversion was estimated using the EIC peak areas of the unoxidized and oxidized compounds. EIC ranges (*m*/*z*): 510-511 for narbomycin (**1**), 526-527 for pikromycin (**3**), 526-527 for **17**, 542-543 for **18**, 496-497 for **29**, 512-513 for **20**, 440-441 for **21**, 456-457 for **22**. All data are collected from single experiment.

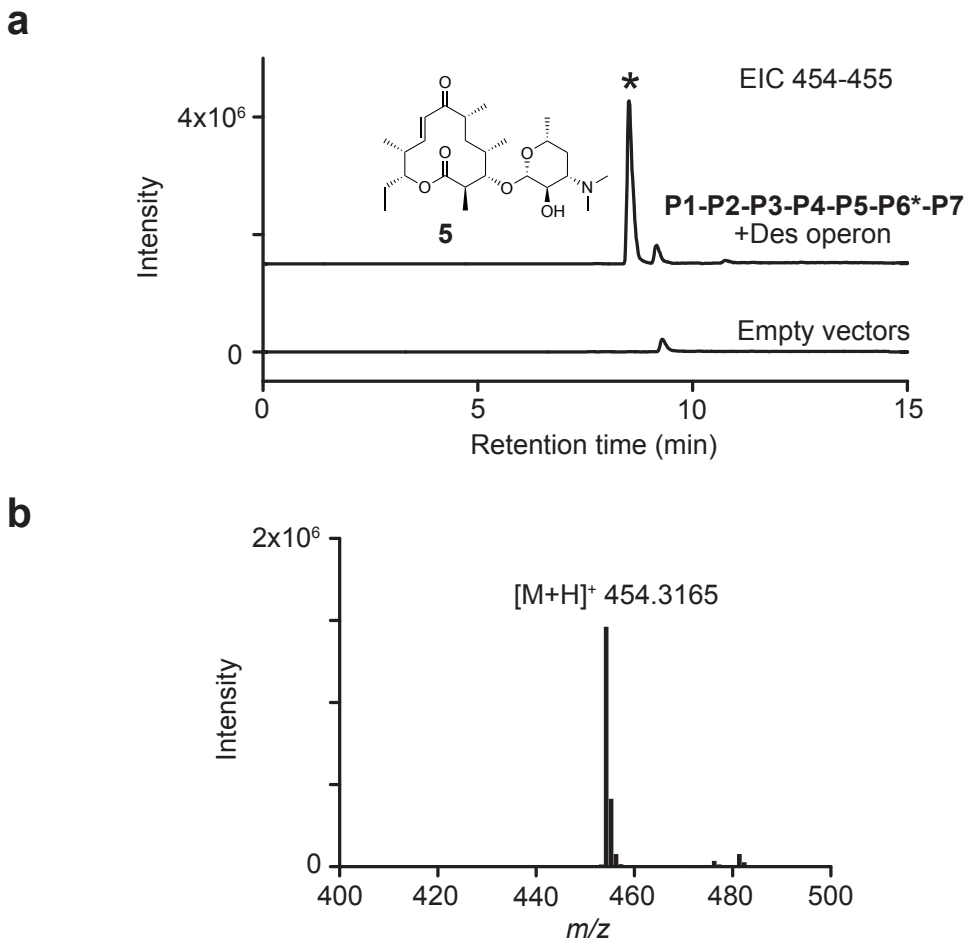


Figure S37. LC/MS analysis of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7**+Des product, YC-17 (**5**). a) EIC for the $[M+H]^+$ of **5** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **5**, 454.3163; observed *m/z*, 454.3165 (0.41 ppm).

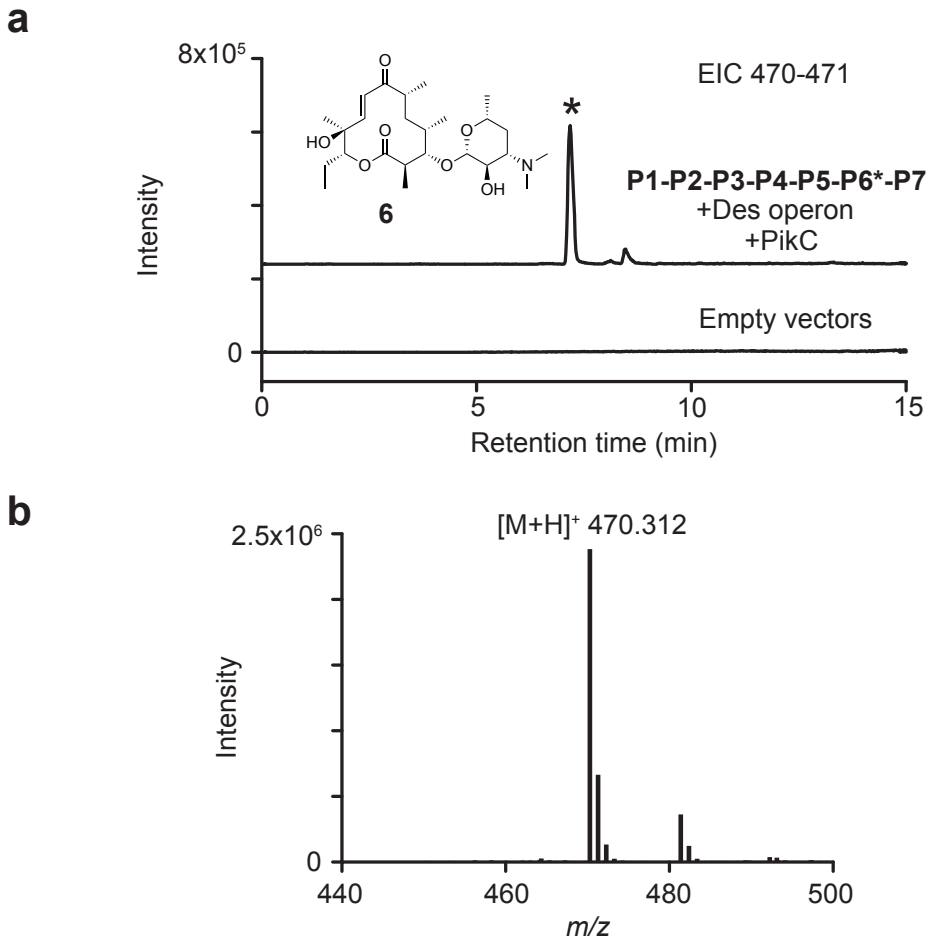


Figure S38. LC/MS analysis of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7**+Des+PikC product, methymycin (**6**). a) EIC for the $[M+H]^+$ of **6** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **6**, 470.3112; observed *m/z*, 470.3120 (1.6 ppm).

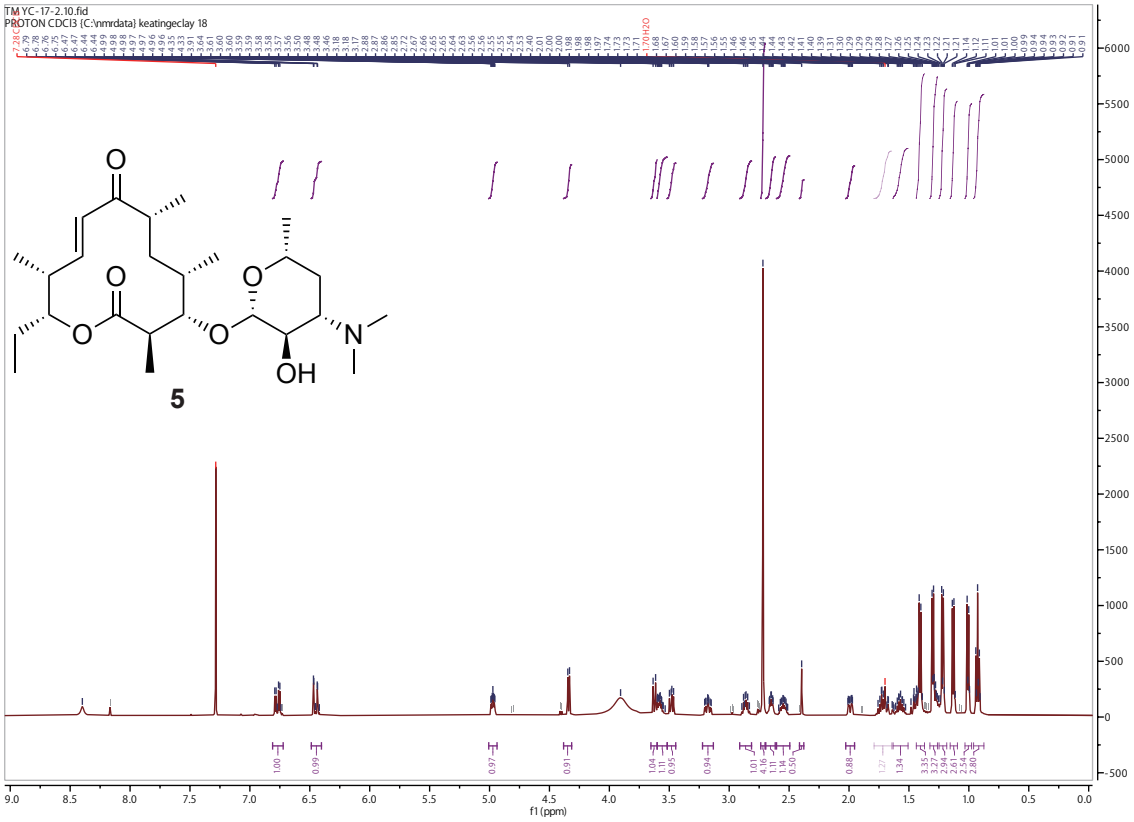


Figure S39. ^{1}H NMR spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7**+Des product, YC-17 (**5**).

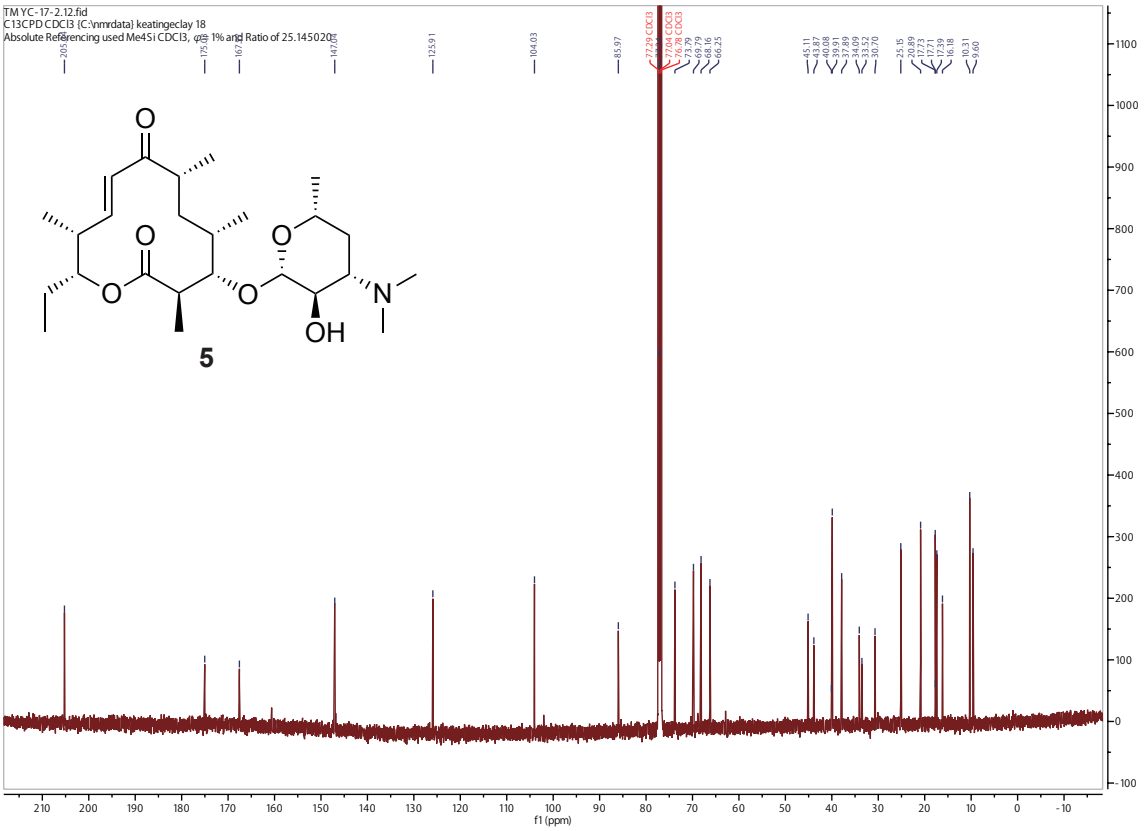


Figure S40. ^{13}C NMR spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7**+Des product, YC-17 (**5**).

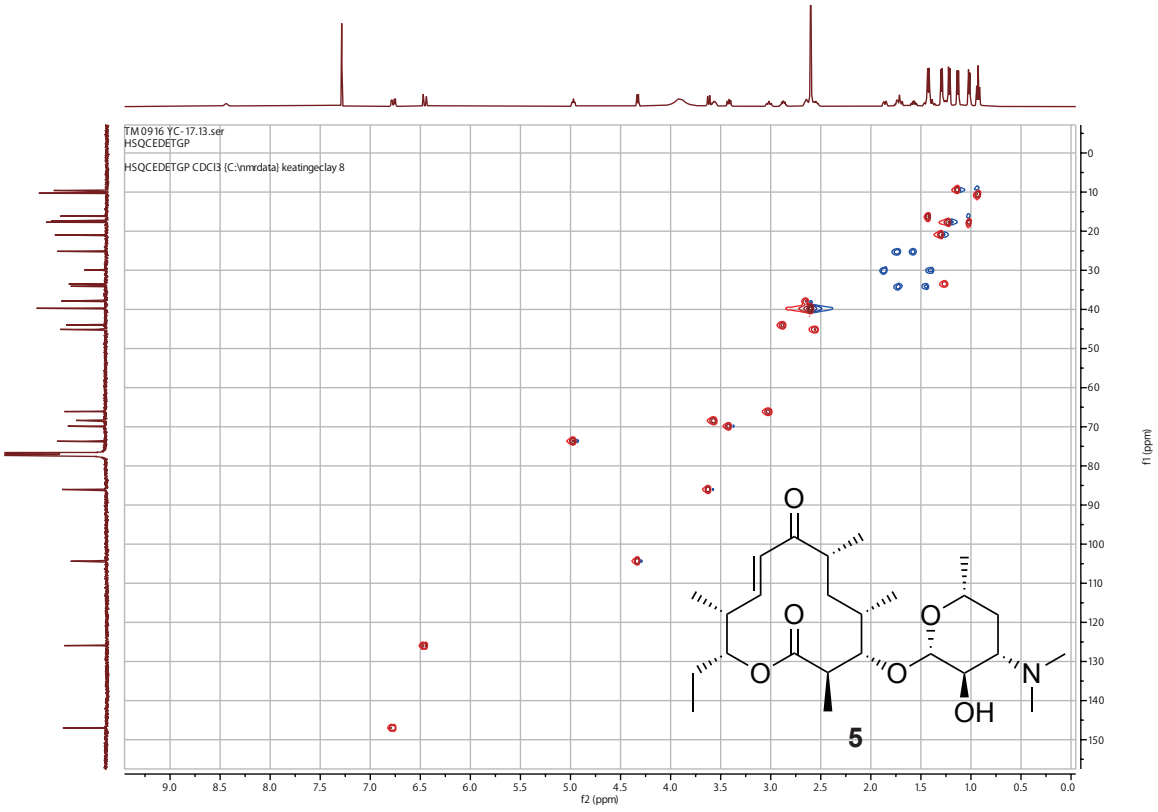


Figure S41. ^{1}H-^{13}C HSQC spectrum of the anticipated **P1**-**P2**-**P3**-**P4**-**P5**-**P6***-**P7**+Des product, YC-17 (**5**).

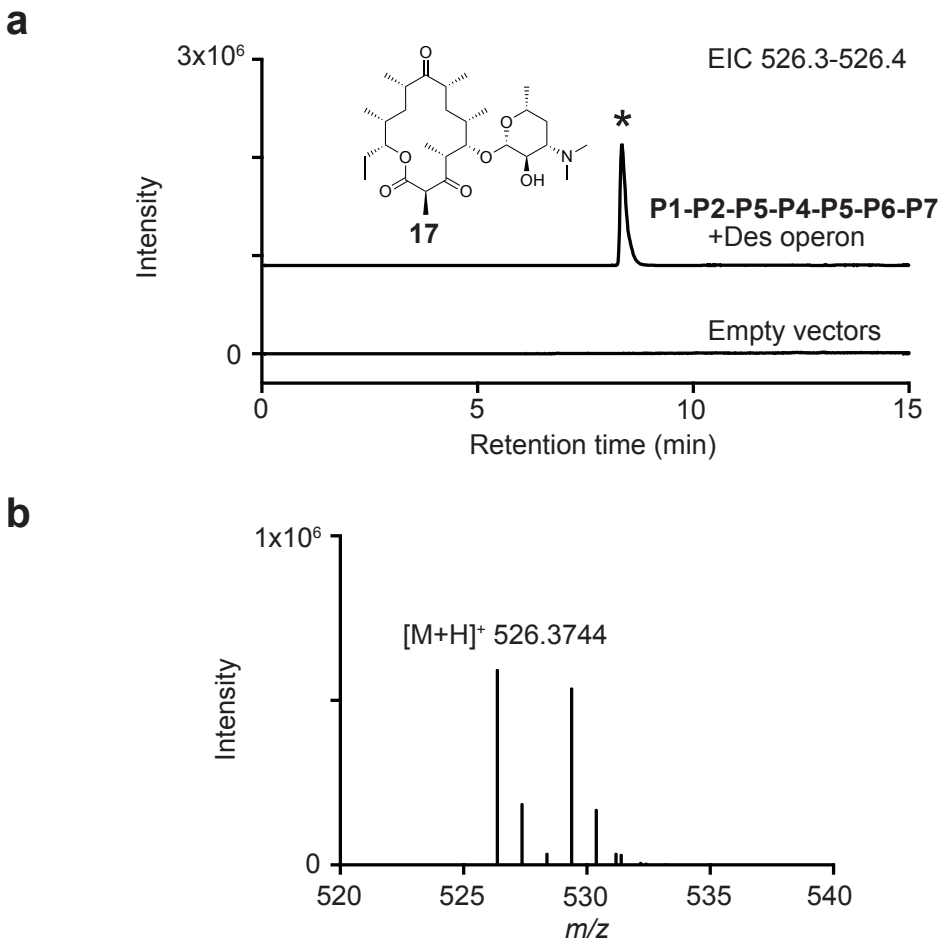


Figure S42. LC/MS analysis of the anticipated **P1**-**P2**-**P5**-**P4**-**P5**-**P6**-**P7**+Des product, **17**. a) EIC for the $[M+H]^+$ of **17** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **17**, 526.3738; observed *m/z*, 526.3744 (1.1 ppm).

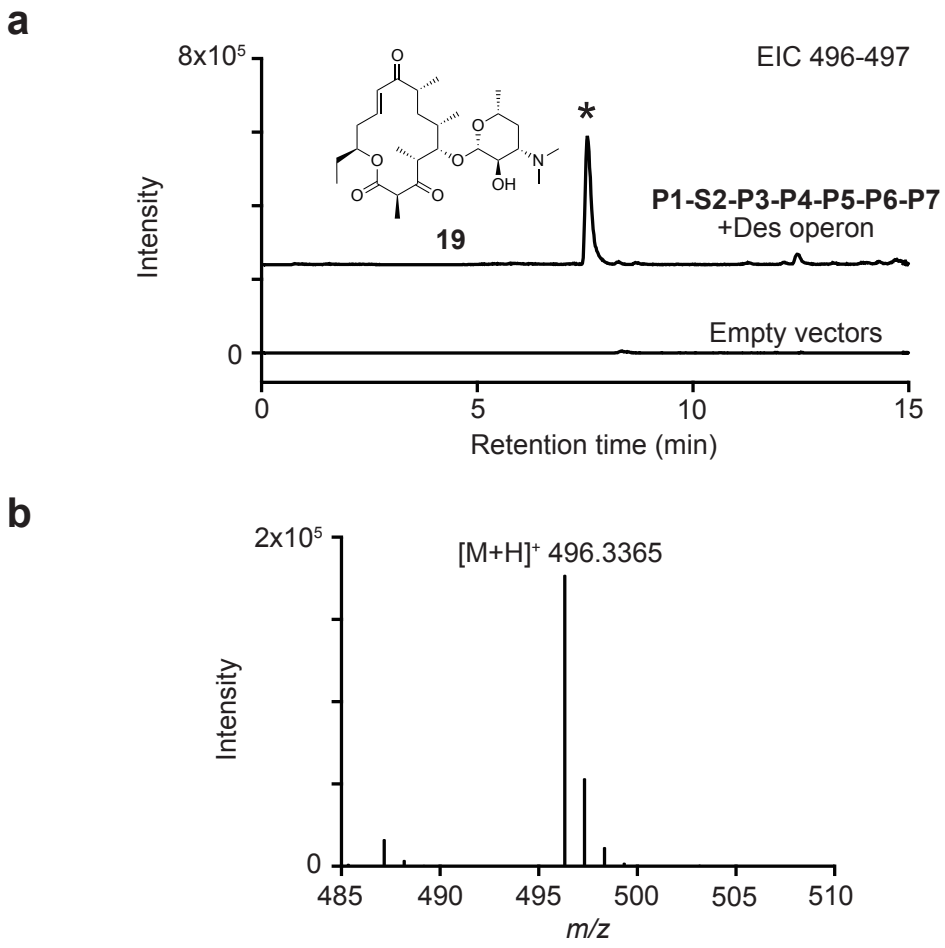


Figure S43. LC/MS analysis of the anticipated **P1**-**S2**-**P3**-**P4**-**P5**-**P6**-**P7**+Des product, **19**. a) EIC for the $[M+H]^+$ of **19** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **19**, 496.3268; observed *m/z*, 496.3271 (0.6 ppm).

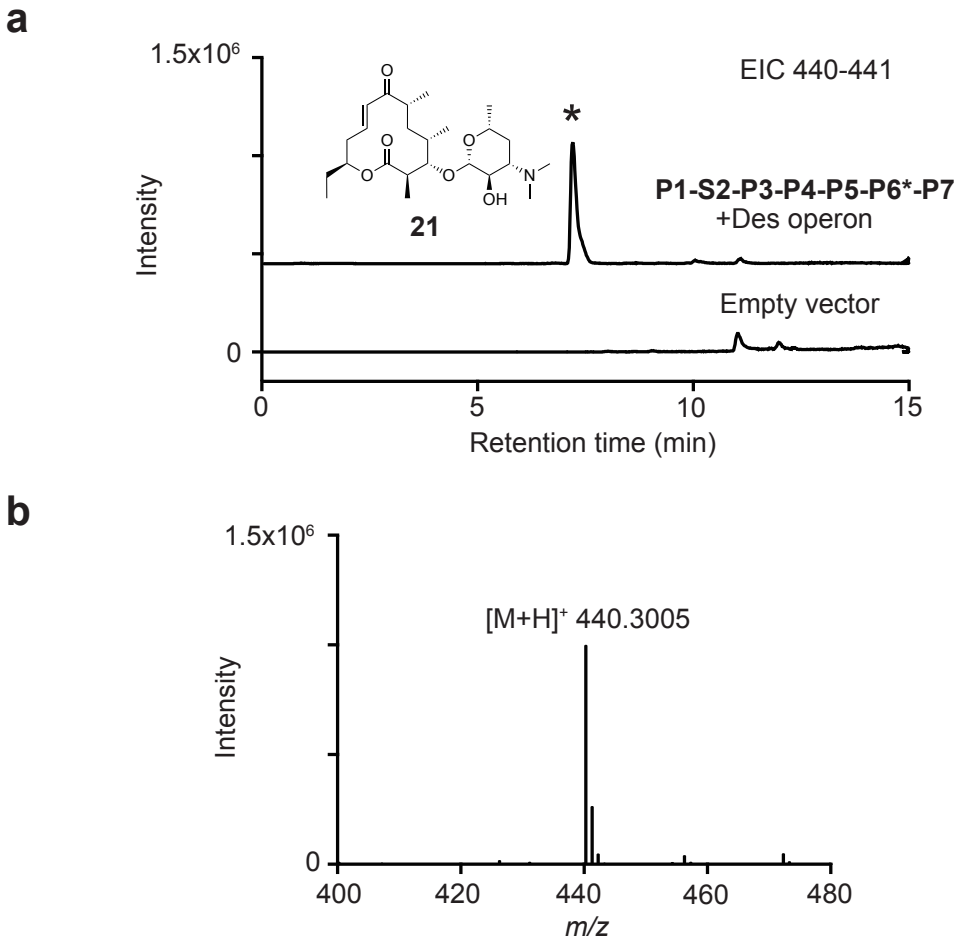


Figure S44. LC/MS analysis of the anticipated **P1**-**S2**-**P3**-**P4**-**P5**-**P6***-**P7**+Des product, **21**. a) EIC for the $[M+H]^+$ of **21** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **21**, 440.3006; observed *m/z*, 440.3005 (-0.22 ppm).

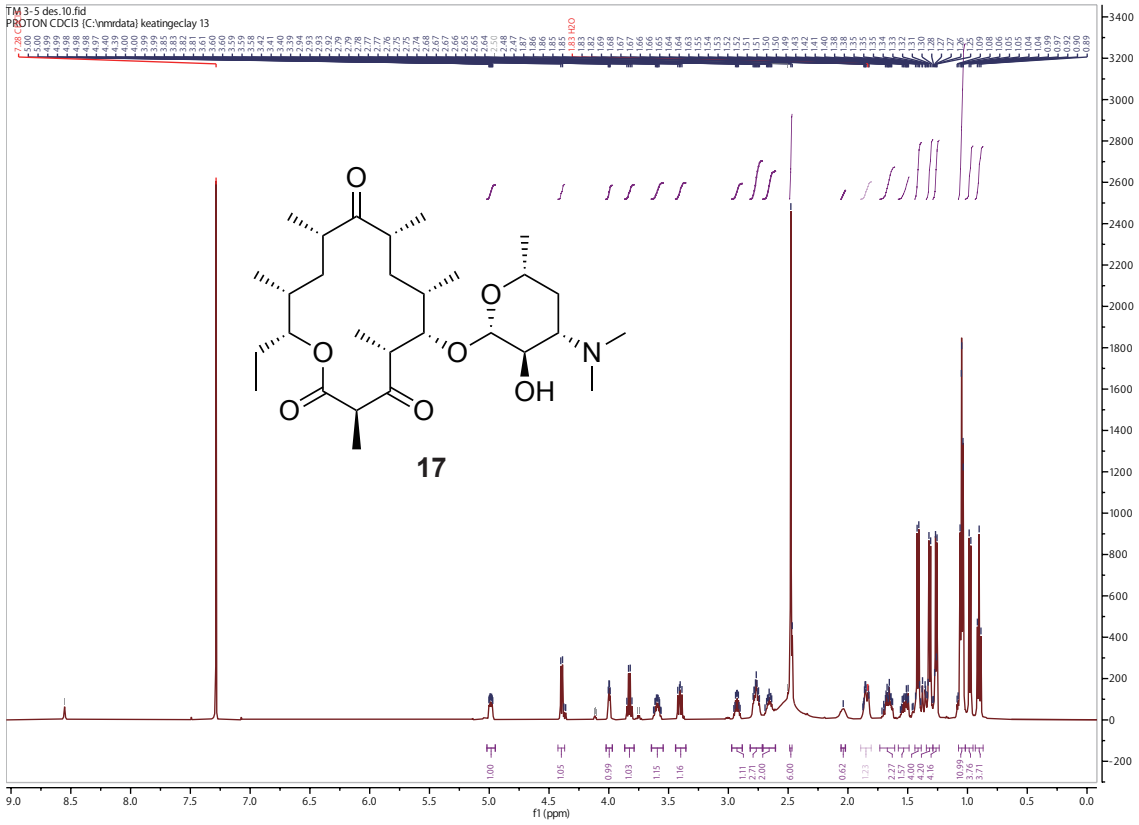


Figure S45. ^{1}H NMR spectrum of the anticipated **P1**-**P2**-**P5**-**P3**-**P4**-**P5**-**P6**-**P7**+Des product, **17**.

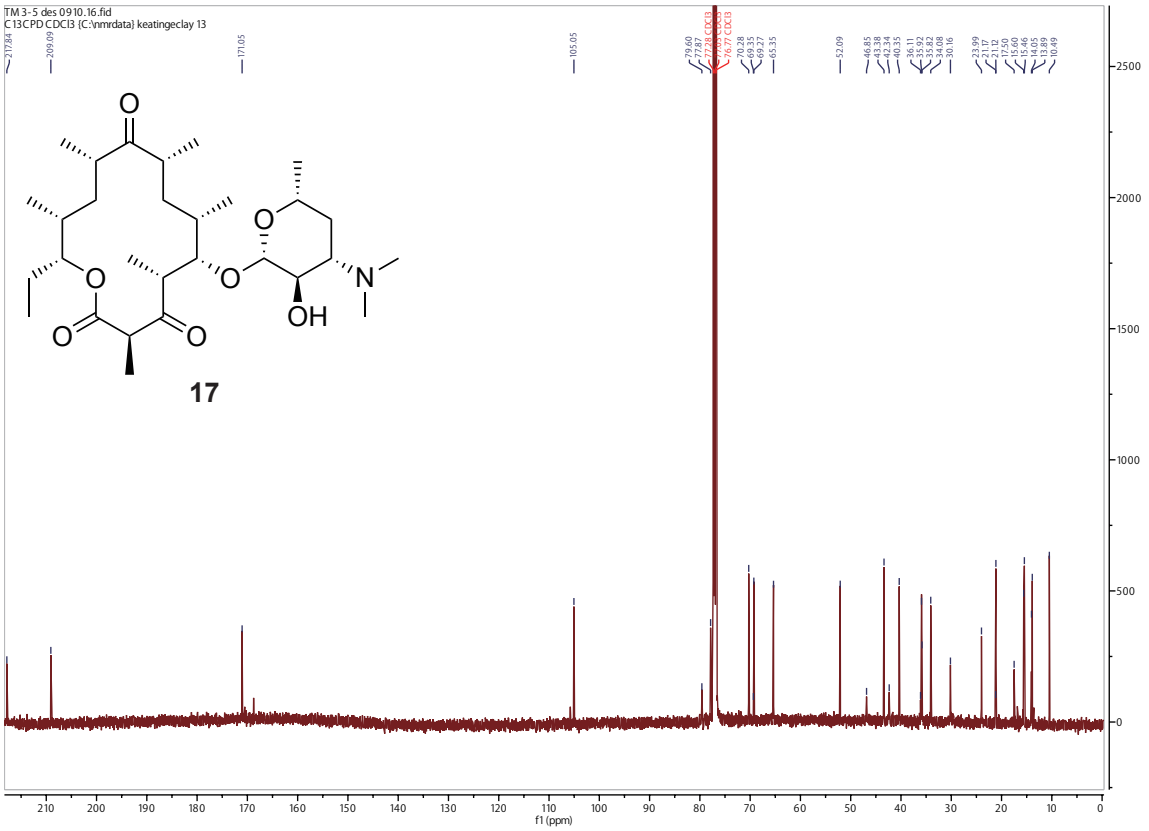


Figure S46. ^{13}C NMR spectrum of the anticipated **P1**-**P2**-**P5**-**P4**-**P5**-**P6**-**P7**+Des product, **17**.

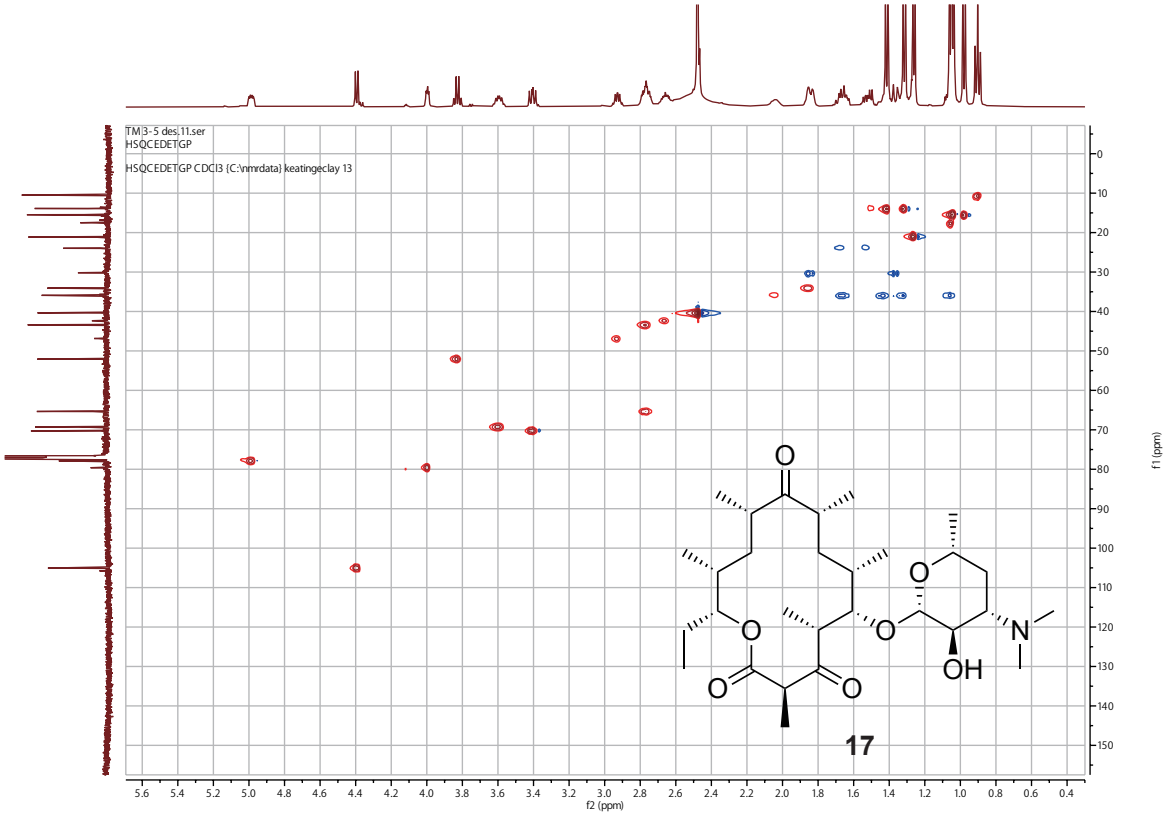


Figure S47. ^{1}H-^{13}C HSQC spectrum of the anticipated **P1**-**P2**-**P5**-**P4**-**P5**-**P6**-**P7**+Des product, **17**.

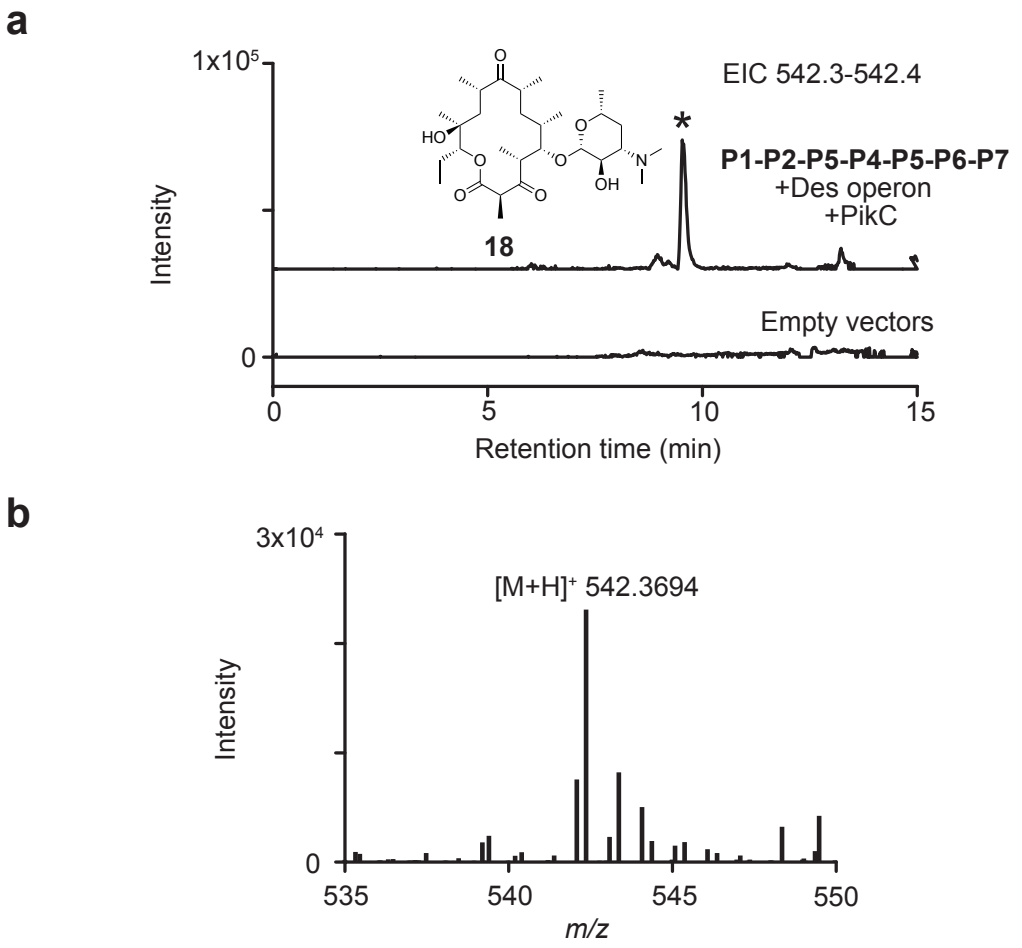


Figure S48. LC/MS analysis of the anticipated **P1**-**P2**-**P5**-**P4**-**P5**-**P6**-**P7**+Des+PikC, product,**18**. a) EIC for the $[M+H]^+$ of **18** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **18**, 542.3680; observed *m/z*, 542.3694 (1.2 ppm).

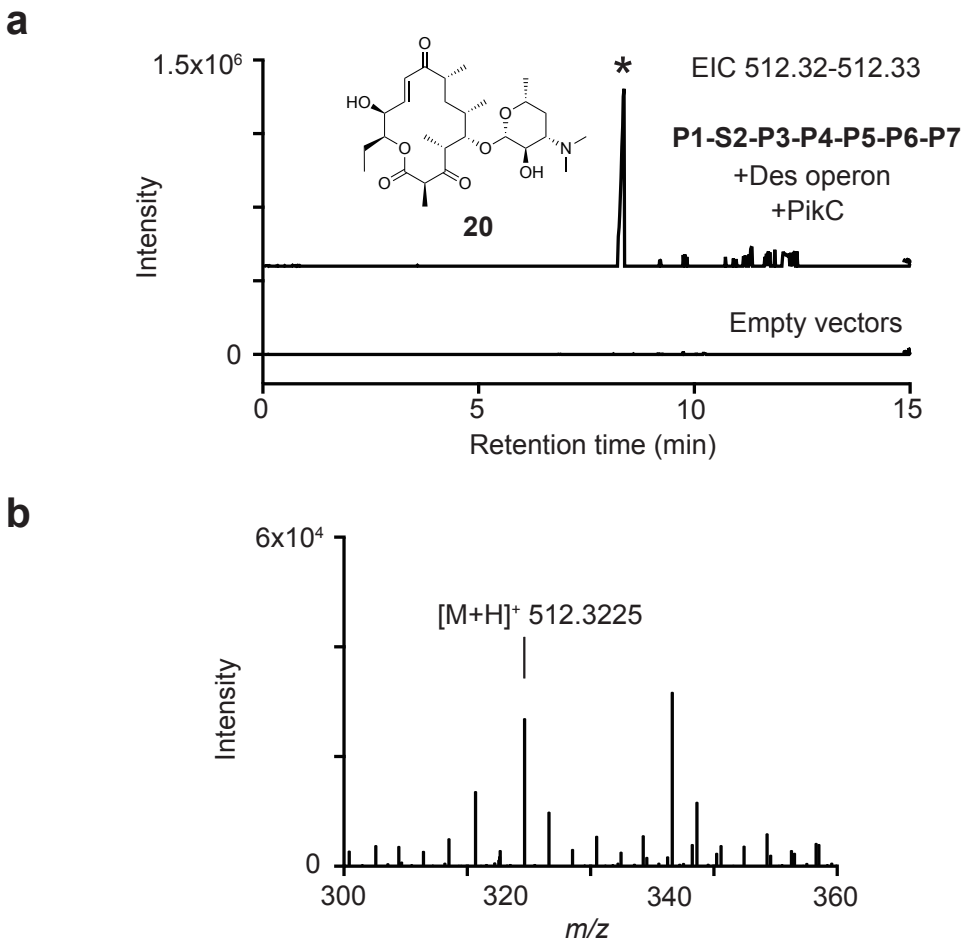


Figure S49. LC/MS analysis of the anticipated **P1**-**S2**-**P3**-**P4**-**P5**-**P6**-**P7**+Des+PikC product, **20.** a) EIC for the $[M+H]^+$ of **20** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **20**, 512.3217; observed *m/z*, 512.3225 (1.5 ppm).

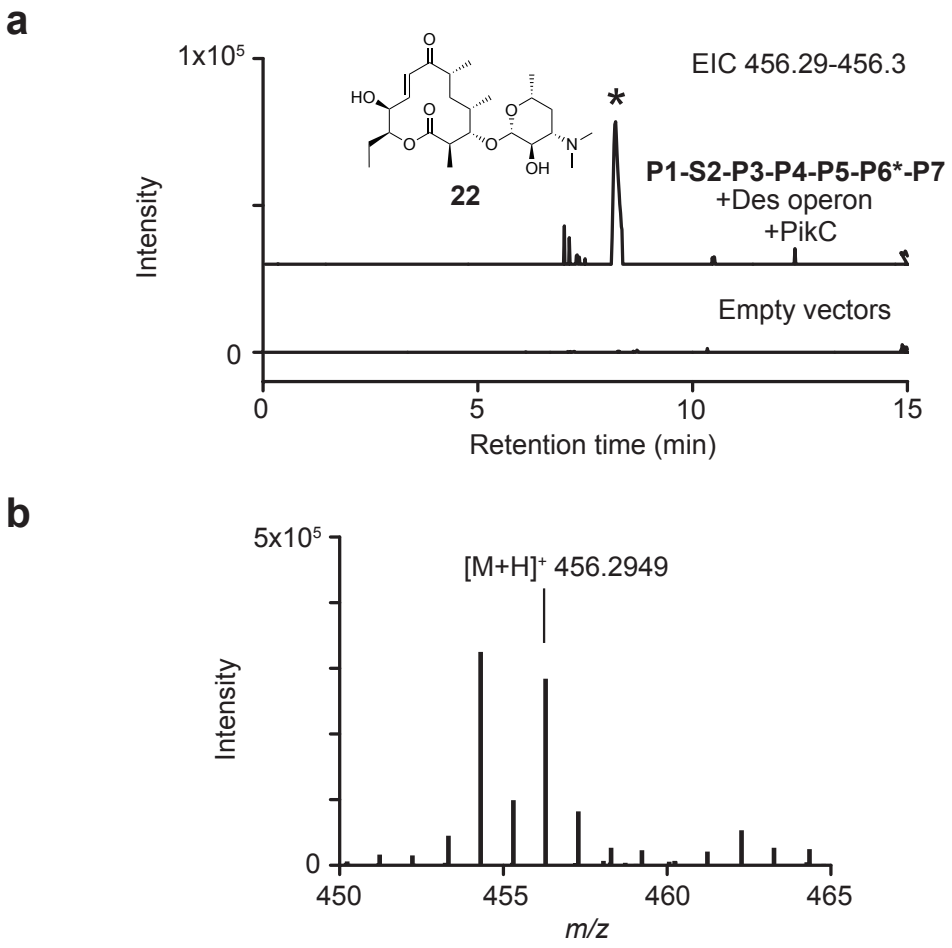


Figure S50. LC/MS analysis of the anticipated **P1**-**S2**-**P3**-**P4**-**P5**-**P6***-**P7**+Des+PikC product, **22**. a) EIC for the $[M+H]^+$ of **21** from the ethyl acetate extract of cells transformed with the PKS expression plasmids or corresponding empty vectors. b) MS spectrum of the main peak (*). Theoretical $[M+H]^+$ *m/z* of **21**, 456.2955; observed *m/z*, 456.2949 (-1.3 ppm).

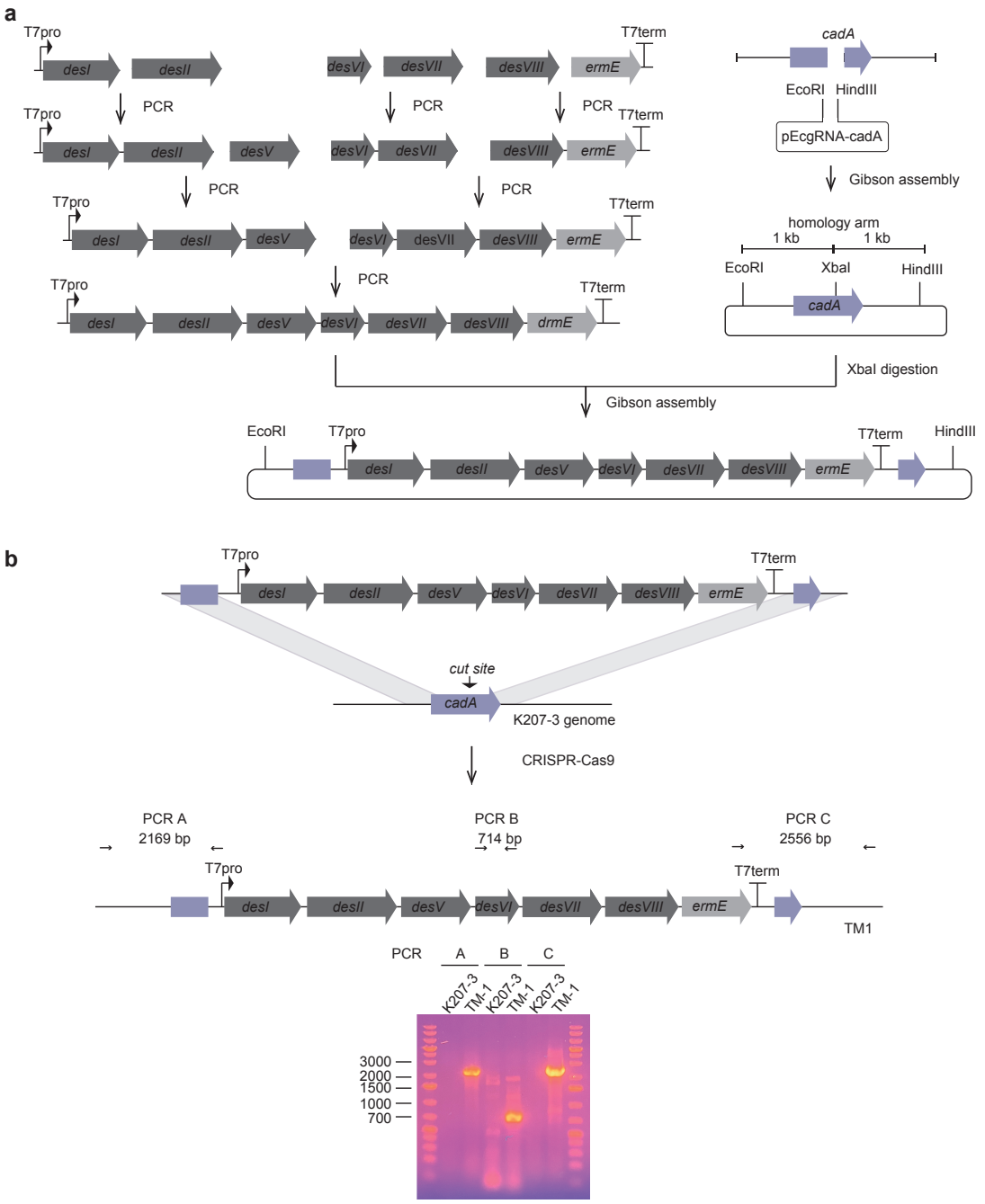


Figure S51. Integration of the desosamine biosynthesis/transfer (Des) operon into *cadA* locus of *E. coli* K207-3. a) Construction of pEcgRNA-ΔcadA:desI,II,V,VI,VII,VIII,ermE. To build the Des operon with short spacer sequences (aaggagatatacc, RBS underlined), each gene was amplified by PCR, and the amplicons were assembled through sequential overlap extension PCRs. The short spacer sequences are from the PCR primers. To construct plasmids that contain homology arms for *cadA* targeting, 1000 bp homology arms were amplified and assembled into the EcoRI/HindIII site of pEcgRNA-cadA through Gibson assembly. The Des operon was cloned into the XbaI site of the resulting plasmid by Gibson assembly to place the operon between the homology arms. The operon harboring the homology arms was cut from

the plasmid and used as the donor DNA for CRISPR/Cas9. b) Schematic representation of the genome integration by CRISPR/Cas9. PCRs A, B and C on genomic DNA confirmed the integration.

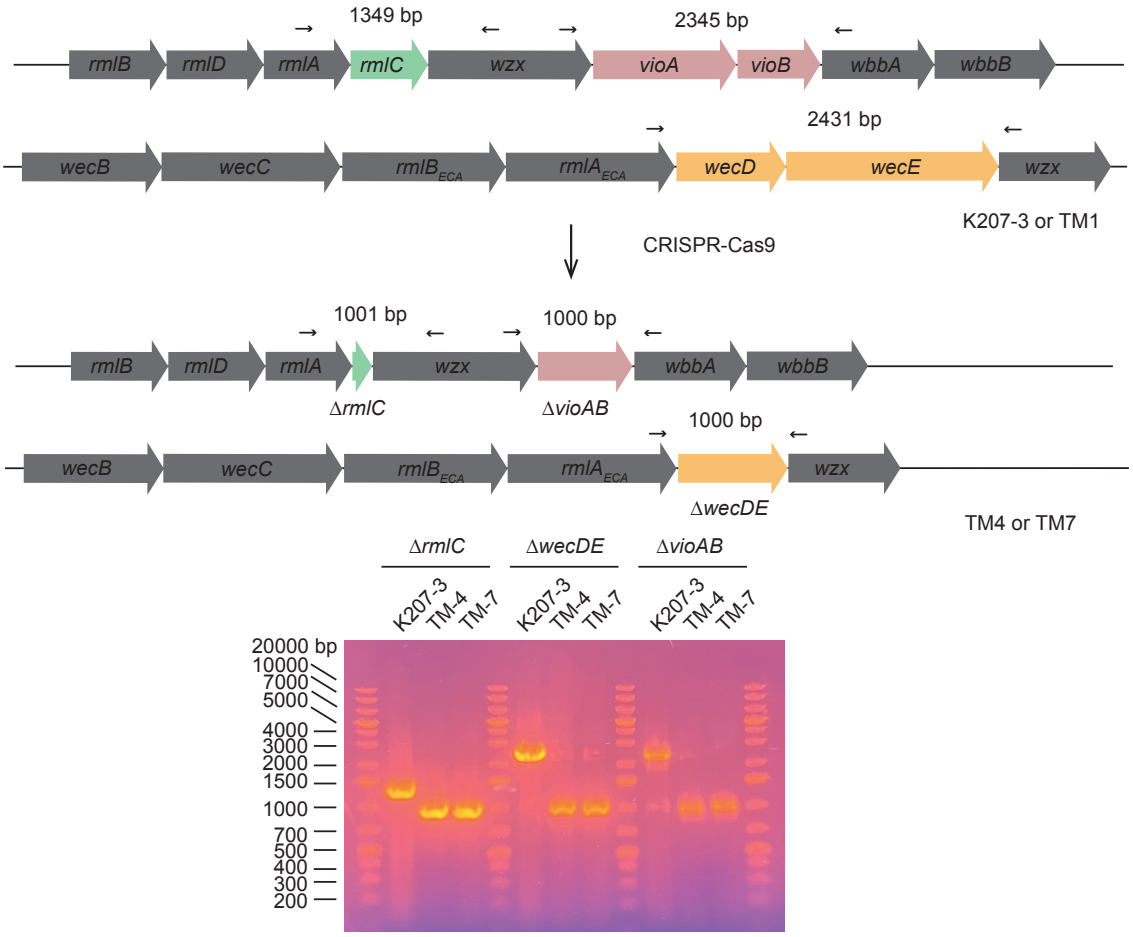
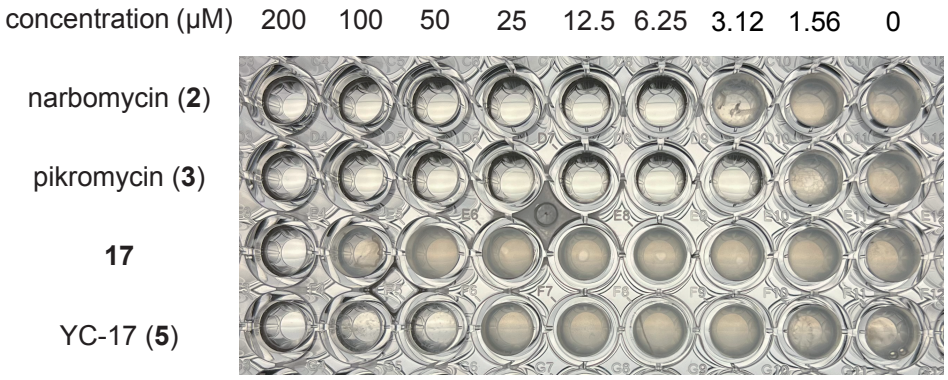


Figure S52. Inactivation of pathways competing with desosamine biosynthesis. Schematic for gene inactivation by CRISPR/Cas9. All gene inactivations are designed in frame to avoid the polar effect. Colony PCR confirmed gene inactivations.

a



b

	MIC values			
	narbomycin (**2**)	pikromycin (**3**)	**17**	YC-17 (**5**)
B. subtilis 168	6.25 μM	3.12 μM	200 μM	50 μM

Figure S53. MIC assay with *Bacillus subtilis* 168. Cells were grown in a 96-well plate in the presence of purified compounds (**2**, **5**, and **18** from *E. coli*, **3** from commercial source). Cells were grown in M-H medium containing 2-fold serial dilutions of the compounds. The lowest concentration that prevented visible bacterial growth in the wells is reported as the MIC. The MICs obtained for narbomycin, pikromycin, and YC-17 are similar to those previously reported for these compounds on bacteria from the Bacilli class[8,9].

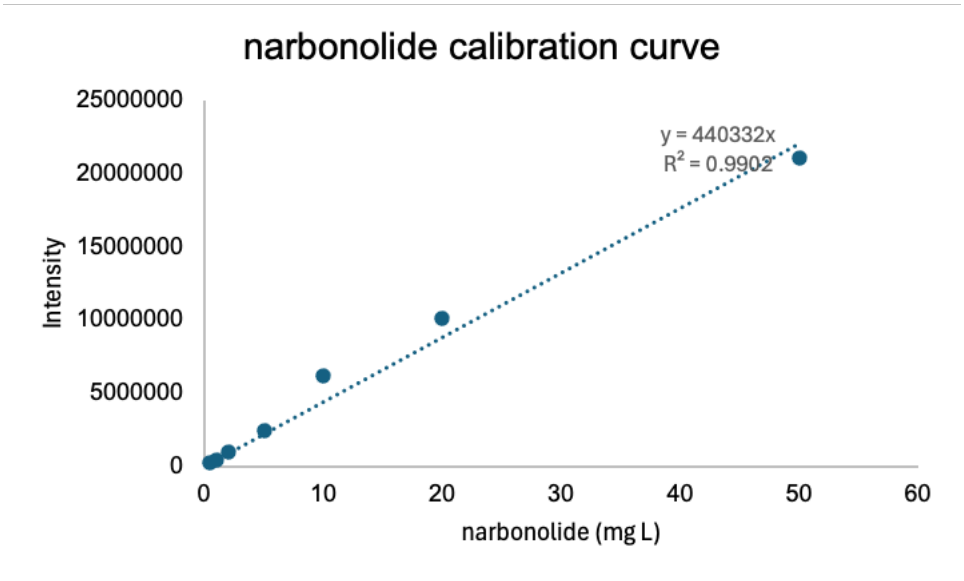


Figure S54. Calibration curve for quantification of narbonolide, 10-dml, and their derivatives. Narbonolide purified from K207-3 cells expressing **P1-P2-P3-P4-P5-P6-P7** was used to generate the calibration curve.

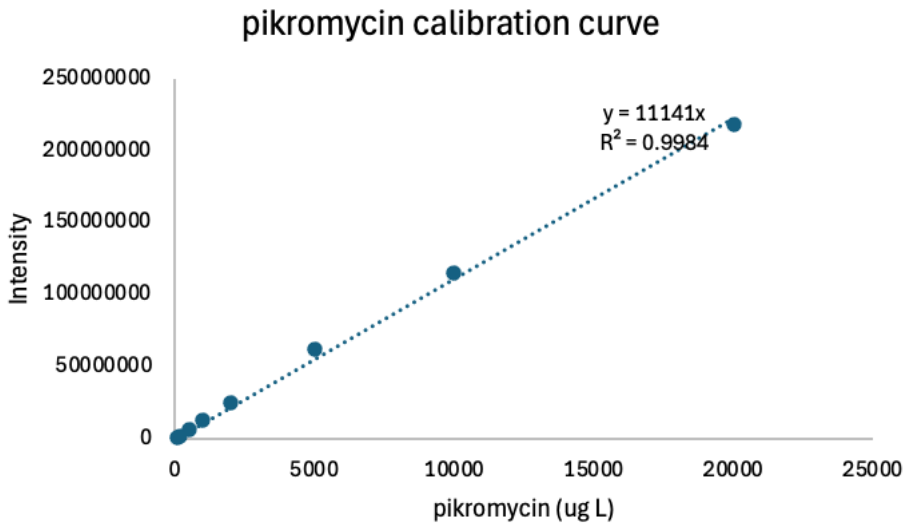


Figure S55. Calibration curve for quantification of narbomycin, pikromycin, YC-17, methymycin, and their derivatives. Commercial pikromycin was used to generate the calibration curve.

Table S1. Strains used in this study.

Strain	Genotype	Source
DH5α	*fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB
K207-3	F-*ompT hsdS*$_B$ *(r*$^-$*m*$^-$*), gal dcm* (DE3), *panD::panDS25A,* D*prpRBCD::*T7prom-*sfp,* T7prom-*prpE, ygfG::*T7prom-*accA1*-T7prom-*pccB*	Ref.[10]
TM1	K207-3 *cadA::*T7prom-*desI desII desV desVI desVII desVIII ermE*	This work
TM2	K207-3 Δ*rmlC*	This work
TM3	K207-3 Δ*rmlC wecDE*	This work
TM4	K207-3 Δ*rmlC wecDE vioAB*	This work
TM5	TM1 Δ*rmlC*	This work
TM6	TM1 Δ*rmlC wecDE*	This work
TM7	TM1 Δ*rmlC wecDE vioAB*	This work
Bacillus subtilis strain 168	*trpC2*	ATCC

Table S2. Plasmids used in this study. *str*, streptomycin resistance gene; *kan*, kanamycin resistance gene; *cat*, chloramphenicol acetyltransferase gene; *bla*, ampicillin resistance gene.

Plasmid	Description	Source
pUC19	*E. coli* cloing vector, ColE1 ori, *bla*	NEB
pCDF-1b	*E. coli* expression vector, CloDF13 ori, *str*	Novagene
pET28-b	*E. coli* expression vector, ColE1 ori, *kan*	Novagene
pACYCDuet-1	*E. coli* expression vector, p15A ori, *cat*	Novagene
pEcCas	*repA101*(Ts) *kan* P_{cas}-*cas9* P_{araB}-*Red lacI*^{q} P_{trc}-sgRNA-*pMB1*, sacB, P_{rhaB}-sgRNA-pMB1, pSC101	Addgene [5]
pEcgRNA	pMB1 *aadA* sgRNA, *ccdB, str*	Addgene [5]
pUT-21-S3DD	pUC19 containing S3 gBlocks between HindIII and XbaI	Ref.[2]
pUT-21-S5DD	pUC19 containing S5 gBlocks between HindIII and XbaI	Ref.[2]
pUT21-S8DD	pUC19 containing S8 gBlocks between HindIII and XbaI	Ref.[2]
pUT-21-E5DD	pUC19 containing E5 gBlocks between HindIII and XbaI	This work
pUT-21-P3DD	pUC19 containing P3 gBlocks between HindIII and XbaI	This work
pUT21-P5DD	pUC19 containing P5 gBlocks between HindIII and XbaI	This work
pUT21-P8DD	pUC19 containing P5 gBlocks between HindIII and XbaI	This work
pUT21-A1DD	pUC19 containing A1 gBlocks between HindIII and XbaI	This work
pUT21-S3DD	Cloning plasmid containing P2 (S3)	Ref.[2]
pUT21-P2 (S5)	Cloning plasmid containing P2 (S5)	Ref.[2]
pUT21-P2 (S8)	Cloning plasmid containing P2 (S8)	Ref.[2]
pUT21-P2 (E5)	Cloning plasmid containing P2 (E5)	This work
pUT21-P3 (S3)	Cloning plasmid containing P3 (S3)	Ref.[2]
pUT21-P3 (S5)	Cloning plasmid containing P3 (S5)	Ref.[2]
pUT21-P3 (S8)	Cloning plasmid containing P3 (S8)	Ref.[2]
pUT21-P3 (E5)	Cloning plasmid containing P3 (E5)	This work
pUT21-P3 (P3)	Cloning plasmid containing P3 (P3)	This work
pUT21-P4 (S3)	Cloning plasmid containing P4 (S3)	Ref.[2]
pUT21-P4 (S5)	Cloning plasmid containing P4 (S5)	Ref.[2]
pUT21-P4 (S8)	Cloning plasmid containing P4 (S8)	Ref.[2]
pUT21-P4 (E5)	Cloning plasmid containing P4 (E5)	This work
pUT21-P5 (S3)	Cloning plasmid containing P5 (S3)	Ref.[2]
pUT21-P5 (S5)	Cloning plasmid containing P5 (S5)	Ref.[2]
pUT21-P5 (S8)	Cloning plasmid containing P5 (S8)	Ref.[2]
pUT21-P5 (E5)	Cloning plasmid containing P5 (E5)	This work
pUT21-P5 (P3)	Cloning plasmid containing P5 (P3)	This work
pUT21-P5 (A1)	Cloning plasmid containing P5 (A1)	This work

pUT21-P6 (S3)	Cloning plasmid containing P6 (S3)	Ref.[2]
pUT21-P6 (S5)	Cloning plasmid containing P6 (S5)	Ref.[2]
pUT21-P6 (C to A, S5)	Cloning plasmid containing P6 (C to A, S5)	This work
pUT21-P6 (S8)	Cloning plasmid containing P6 (S8)	Ref.[2]
pUT21-P6 (E5)	Cloning plasmid containing P6 (E5)	This work
pUT21-P6 (P3)	Cloning plasmid containing P6 (P3)	This work
pCDF-P1-P4N	Expression plasmid containing P1-P4N	This work
pET28b-P4C-P7	Expression plasmid containing P4C-P7	This work
pCDF-P1-P2-P4N	Expression plasmid containing P1-P2-P4N	This work
pCDF-P1-P3-P4N	Expression plasmid containing P1-P3-P4N	This work
pCDF-P1-P2-P3-P4N	Expression plasmid containing P1-P2-P3-P4N	This work
pCDF-P1-P2-P3(P3)-P4N	Expression plasmid containing P1-P2-P3(P3)-P4N	This work
pCDF-P1-P3-P3-P4N	Expression plasmid containing P1-P3-P3-P4N	This work
pCDF-P1-P4-P3-P4N	Expression plasmid containing P1-P4-P3-P4N	This work
pCDF-P1-P5-P3-P4N	Expression plasmid containing P1-P5-P3-P4N	This work
pCDF-P1-P6-P3-P4N	Expression plasmid containing P1-P6-P3-P4N	This work
pCDF-P1-P2-P2-P4N	Expression plasmid containing P1-P2-P2-P4N	This work
pCDF-P1-P2-P4-P4N	Expression plasmid containing P1-P2-P4-P4N	This work
pCDF-P1-P2-P5-P4N	Expression plasmid containing P1-P2-P5-P4N	This work
pCDF-P1-P2-P5-P4N	Expression plasmid containing P1-P2-P5-P4N	This work
pCDF-P1-P2-P6-P4N	Expression plasmid containing 1-P2-P6-P4N	This work
pCDF-P1-E2-P3-P4N	Expression plasmid containing P1-E2-P3-P4N	This work
pCDF-P1-S2-P3-P4N	Expression plasmid containing P1-S2-P3-P4N	This work
pET28-P4C-P7	Expression plasmid containing P4C-P7	This work
pET28-P4C-P4-P7	Expression plasmid containing P4C-P4-P7	This work
pET28-P4C-P5-P7	Expression plasmid containing P4C-P5-P7	This work
pET28-P4C-P5(P3)-P7	Expression plasmid containing P4C-P5(P3)-P7	This work
pET28-P4C-P6-P7	Expression plasmid containing P4C-P6-P7	This work
pET28-P4C-P6(P6)-P7	Expression plasmid containing P4C-P6(P6)-P7	This work
pET28-P4C-P5-P6-P7	Expression plasmid containing P4C-P5-P6-P7	This work
pET28-P4C-P5(P3)-P6-P7	Expression plasmid containing P4C-P5(P3)-P6-P7	This work
pET28-P4C-P5-P6(P6)-P7	Expression plasmid containing P4C-P5-P6(P6)-P7	This work
pET28-P4C-P5(P5)-P6(P6)-P7	Expression plasmid containing P4C-P5(P5)-P6(P6)-P7	This work
pET28-P4C-P2-P6-P7	Expression plasmid containing P4C-P2-P6-P7	This work
pET28-P4C-P3-P6-P7	Expression plasmid containing P4C-P3-P6-P7	This work
pET28-P4C-P4-P6-P7	Expression plasmid containing P4C-P4-P6-P7	This work
pET28-P4C-P6-P6-P7	Expression plasmid containing P4C-P6-P6-P7	This work
pET28-P4C-P5-P2-P7	Expression plasmid containing P4C-P5-P2-P7	This work

pET28-P4C-P5-P3-P7	Expression plasmid containing P4C-P5-P3-P7	This work
pET28-P4C-P5-P4-P7	Expression plasmid containing P4C-P5-P4-P7	This work
pET28-P4C-P5-P5-P7	Expression plasmid containing P4C-P5-P5-P7	This work
pET28-P4C-P5-E6-P7	Expression plasmid containing P4C-P5-E6-P7	This work
pET28-P4C-P5(A1)-P5-P6-P7	Expression plasmid containing P4C-P5(A1)-P5-P6-P7	This work
pET28-P4C-P5-P6(C to A)-P7	Expression plasmid containing P4C-P5-P6(C to A)-P7	This work
pDes	pACYC-derived expression plasmid encoding desI ,II, V, VI, VII, VIII, ermE	
pDesPikC	pACYC-derived expression plasmid encoding desI ,II, V, VI, VII, VIII, ermE, and PikC	This work
pPikC	pET21 expression plasmid encoding pikC	This work
pDesPikAV	pACYC-derived expression plasmid encoding desI ,II, V, VI, VII, VIII, ermE, and pikAV	This work
pACYC-pikAV	Expression of plasmid encoding pikAV	This work
pACYC-T7prom(-5G)-pikAV	37% relative expression of pACYC-pikAV	This work
pACYC-T7prom(-13T)-pikAV	57% relative expression of pACYC-pikAV	This work
pACYC-T7prom(-16T)-pikAV	72% relative expression of pACYC-pikAV	This work
pACYC-pikAV,pikAV	200% relative expression of pACYC-pikAV	This work
pACYC-pikAV, T7prom(-5G)-pikAV	137% relative expression of pACYC-pikAV	This work
pACYC-pikAV, T7prom(-13T)-pikAV	157% relative expression of pACYC-pikAV	This work
pACYC-pikAV, T7prom(-16T)-pikAV	172% relative expression of pACYC-pikAV	This work
pEcgRNA-cadA	pEcgRNA sgRNA-*cadA*	This work
pEcgRNA-ΔcadA	pEcgRNA-*cadA* having 1000 bp homology arms upstream and downstream of an XbaI site	This work
pEcgRNA-ΔcadA: desI ,II, V, VI, VII, VIII, ermE	pEcgRNA-Δ*cadA:: desI ,II, V, VI, VII, VIII, ermE*	This work
pEcgRNA-rmlC	pEcgRNA sgRNA-*rmlC*	This work
pEcgRNA-vioAB	pEcgRNA sgRNA-*vioAB*	This work
pEcgRNA-wecDE	pEcgRNA sgRNA-*wecDE*	This work

Table S3. Oligonucleotides used in this study. Homology arms used for Gibson assembly, SLiCE, and ligation are in lowercase.

Target	Oligo name	Nucleotide sequence
P1	P1 F	tcaccaccaccatcacgtgggtaccTCTTCAGCCGGAATTACCAGGACCGG
	P1 R	gttggagcaggtgcaagcttGACAACCACCGGGGCCTCTTCGAGCAC
P4N	P4N F	ttgcacctgctccaactagtGAGACCCCTGCCTCCGAGGCGACCCC
	P4N R	gtttctttaccagactcgagACCGGCCTGCTCGCCCAGGATCT
Ery1CDD	Ery1CDD F	gcgagcaggccggtgctagcGGGGAGGCGCCGTCGGCCC
	Ery1CDD R	gtttctttaccagactcgagTCAATCGCCGTCGAGCTCCCGGCC
500 bp with Ery1CDD	Ery2NDD+500 F	taagaaggagatataccATGACTGACAGCGAGAAGGTGGCGGAGTACCT
	Ery2NDD+500 R	tggtggtggtggtgctcgagGCCACACCGGTGACCGAGTAGCCCT
pET-28b-Ery2NDD	pET-28-b F for	gcatcgaggggggcgggcaagCTCGAGCACCACCACCACCACCACCAT
	Ery2NDD R	catgccgacgatcgcaattgGGTCGGATTCCAGCTCGCGGATGCGC
Pik4C	Pik4C F	gctggaatccgacccaattgCGATCGTCGGCATGGCGTGCCGCCT
	Pik4C R	gttggagcaggtgcaagcttCTCGTCGACCGGGGCTTCTTCGAGCACGAT
Pik7	Pik7 F	ttgcacctgctccaactagtTCCCCTGCCGTCGAGCCGCCG
	Pik7 R	tggtggtggtggtgctcgagCTTGCCCGCCCCCTCGATGCCCTCGAT
P6C C to A half	P6 C to A F	ACACGGCG**GCC**TCCTCTTCGCTCGTC
	P6 C R	gtacccggggatccTCTAGACTCGACCGCCGGGGCCTCCTCC
P6C C to A half	P6 C F	ggagtcaggagccaattgccATCGTCGGCATGAGCTGCCGGTTCGCG
	P6 C to A R	CGAAGAGGA**GGC**CGCCGTGTCCACGG
Pik12DD	Pik12 F	agctgccgcgtggcgcatcgGACCAGGACGGAGCCGGGAACCGGAA
	Pik12 R	catgccgacgatcgccaccgGCTCGCCCGCCTTCGCCTCCA
Pik23DD	Pik23 F	aactcgccacggccgcgggcGGGTCCTGGGCGGAAGGCACCGG
	Pik23 R	gcaggccatgcccacgatcgCCACCGGCTCGTGCGTGCGTCC
Pik34DD	Pik34 F	gtacctcgcaccggccgagcCGGCCCCGACGGACTGGGAGG
	Pik34 R	cggcagctcatgccgacgatCGCCATGGGCTCCTGCCGACGGTC
Ery2 N	Ery2 N F	ttgcacctgctccaactagtGAAGGCGAGCGGGTCGAGGCCGGC
	Ery2 N R	gattcgatgccgttgctagcTCCGGTCGCACCGCCGAGTTCGGC
Ery2 C	Ery2 C F	ccaggagcacgacccaattgCGATCGTCGGCATGGCGTGCCG
	Ery2 C R	gtacccggggatcctctagaCTCCGGTTCCGGCGGCTCGGCGA
des1	Des1 F	atcatcaccacagccaggatAAAAGCGCCTTATCCGACCTCGCATTCTT
	DesI R	ggcgcgccgagctcgaattcACTAGTTCATCGGGAGCGTCCAATCGTGGG
desII	desII F	tgccgcgcggcagccatatgACCGCCCCCGCCCTTTCCGCCA
	desII R	tgtcgacggagctcgaattcACTAGTTCAGCGCAGGAAGCCGCGGGCCTC
desV	desV F	tgccgcgcggcagccatatgAGCAGCCGCGCCGAGACCCCCC

	desV R	tgtcgacggagctcgaattcACTAGTCTAGGCCTGGTCGACCCGCTCGG
desVI	desVI F	tgccgcgcggcagccatatgTACGAAGTCGACCACGCCGACGTCTACGA
	desVI R	tgtcgacggagctcgaattcACTAGTTCAGGCGGGGACGCCGACGAA
desVII	desVII F	tgccgcgcggcagccatatgCGCGTCCTGCTGACCTCGTTCGCA
	desVII R	tgtcgacggagctcgaattcACTAGTTCAGTGCCGGGCGTCGGCCG
desVIII	desVIII F	tgccgcgcggcagccatatgACCGACGACCTGACGGGGGCCCT
	desVIII R	ttgtcgacggagctcgaattcACTAGTTCAGGAGCTGCTGACCGGGACGCTCA
ermE	ermE F	tgccgcgcggcagccatatgAGCAGTTCGGACGAGCAGCCGCGCC
	ermE R	ttgtcgacggagctcgaattcACTAGTCTACCGCTGCCCGGGTCCGCCGC
desI with spacer	Des operon F	cggtatcgtggcgtccactgGGATCTCGACGCTCTCCCTTATGCGACT
	desI R spacer	cggtcatggtatatctccttTCATCGGGAGCGTCCAATCGTGGGC
desII with spacer	desII F spacer	ccgatgaaaggagatataccatgACCGCCCCCGCCCTTTCC
	desII R spacer	tgctcatggtatatctccttTCAGCGCAGGAAGCCGCGGG
desV with spacer	desV F spacer	gcgctgaaaggagatataccatgAGCAGCCGCGCCGAGACCCC
	desV R spacer	cgtacatggtatatctccttCTAGGCCTGGTCGACCCGCTCGG
desVI with spacer	desVI F spacer	ggcctagaaggagatataccatgACGAAGTCGACCACGCCGACGTC
	desVI R spacer	cgcgcatggtatatctccttTCAGGCGGGGACGCCGACGAA
desVII with spacer	desVII F spacer	cgcctgaaaggagatataccatgCGCGTCCTGCTGACCTCGTTC
	desVII R spacer	cggtcatggtatatctccttTCAGTGCCGGGCGTCGGCC
desVIII with spacer	desVIII F spacer	gcactgaaaggagatataccatgACCGACGACCTGACGGGGGC
	desVIII R spacer	tgctcatggtatatctccttTCAGGAGCTGCTGACCGGGACG
ermE with spacer	ermE F spacer	ctagaaggagatataccatgAGCAGTTCGGACGAGCAGCCGCG
	ermE R	ttgtcgacggagctcgaattcACTAGTCTACCGCTGCCCGGGTCCGCCGC
cadA CRISPR cut site	cadA target F	tagtCATCGCCGCAGCGGTTTCAG
	cadA target R	aaacCTGAAACCGCTGCGGCGATG
cadA up 1000 bp	cadA up F	tcggtgctttttttgaattcCTGCCGTTGTACGCGTTCGC
	cadA up R	cgccgcagcggttttctagaCAGTGGACGCCACGATACCG
cadA down 1000 bp	cadA down F	cgtggcgtccactgtctagaAAACCGCTGCGGCGATGA
	cadA down R	agggtaatagatctaagcttGCGGCTGTGAGGGTGTTTTC
2169bp fragments that include an integration site of cadA	cadA up check F	CGCAGCAGTAAGTACTGGTATGGTTAAA
	cadA up check R	CGAGGTCGGATAAGGCGCTTTT
2556 bp fragments that include an integration site of cadA	cadA down check F	CAATCGCGACGACGGACGAA
	cadA down check F	GCCAAAGGCAAACTTCAGCCAG
Des operon	Des operon F for BAC	tcgggccctggccagctagcGGATCTCGACGCTCTCCCTTATGCGACTCCTG
	Des operon R for BAC	agccctgtttaaaccctaggTACCGGAAGCAGTGTGACCGTGTGCTTCTC
vioAB CRISPR cut site	vioAB target F	tagtAAATAATTGATAGTCTTTACGTTT
	vioAB target R	aaacGTAAAGACTATCAATTATTTACTA

vioAB up 500 bp	VioAB up F	TTAGCCAGAAAGTATCAACGATCACAATAA
	VioAB up R	ctggtataccAAATGAATATGGTGTTGTTATTACCTCACC
vioAB down 500 bp	VIoAB down F	atattcatttGGTATACCAGCTAAACCAATATCTGA
	VioAB down R	TTTGATTTGACCAAGTTAAATATCTCATCA
rmlC CRISPR cut site	rmlC target F	tagtCTATGGAAAAAGGTATAAGA
	rmlC target R	aaacTCTTATACCTTTTTCCATAG
rmlC up 500 bp	rmlC up F	CTATGACAATGACGTTGTGGAAATGG
	rmlC up R	tcatgtctttATGGTTATCCTGAACAAATTCAACCTT
rmlC down 500 bp	rmlC down F	ggataaccatAAAGACATGAAACAACCATCATTCAGA
	rmlC down R	GCGATAAGTGAACTTTTAGCTTGGA
wecDE CRISPR cut site	wecDE target F	tagtAAATAATTGATAGTCTTTACGTTT
	wecDE target R	aaacGTAAAGACTATCAATTATTTACTA
wecDE up 500 bp	wecDE up F	TATCTCGCTGGAAGAAAAGCCAAAACA
	wecDE up R	cgttctgcacCGTCAGGAGCGGTGCTTCA
wecDE down 500 bo	wecDE down F	gctcctgacgGTGCAGAACGCGCATATGTTCTAC
	wecDE down R	GCTGCTGCGGATTATCATGGTAC
pikAV	pikAV EcoRI F	cacagccaggatccgaattcGACCGACAGACCTCTGAACGTGGAC
	pikAV HindIII R	cattatgcggccgcaagcttTCACCGTGGGTTCTGCCATCTCTTCG
pikAV	pikAV NdeI F	aagaaggagatatacatatgACCGACAGACCTCTGAACGTGGA
	pikAV XhoI R	gtttctttaccagactcgagTCACCGTGGGTTCTGCCATCTCTT
pikAV -5G (37%) -half of pACYC	pikAV-5G (37%) F	taggaaattaatacgactcaGTATAGGGGAATTGTGAGCG
	pACYC middle R	ccgggaagccctgggccaac
pikAV -5G (37%)-half of pACYC	pACYC middle F	acgtctcattttcgccaaaaGTTGGCC
	pikAV-5G (37%) R	cgctcacaattcccctatacTGAGTCGTATTAATTTCCTA
pikAV -13T(57%)- half of pACYC	pikAV-13T (57%) F	tcctgcattaggaaattaatTCGACTCACTATAGGGGAAT
	pACYC middle R	ccgggaagccctgggccaac
pikAV-13T (57%)- half of pACYC	pACYC middle F	acgtctcattttcgccaaaaGTTGGCC
	pikAV-13T (57%) R	attcccctatagtgagtcgaATTAATTTCCTAATGCAGGA
pikAV -16T(72%)- half of pACYC	pikAV-16T (72%) F	tcctgcattaggaaattaatTCGACTCACTATAGGGGAAT
	pACYC middle R	ccgggaagccctgggccaac
pikAV-16T (72%)- half of pACYC	pACYC middle F	acgtctcattttcgccaaaaGTTGGCC
	pikAV-16T (72%) R	attcccctatagtgagtcgaATTAATTTCCTAATGCAGGA

Table S4. Summary of polyketide production. A calibration curve generated using narbonolide (EIC peak areas) was employed for macrolactones and δ-lactones, while a calibration curve generated using pikromycin (EIC peak areas) was employed for desosaminylated macrolides (Figs. S54-55).

Strain	Product	Productivity (mgL^{-1})
K207-3 + **P1-P2-P3-P4-P5-P6-P7**	**1**	28.9
K207-3 + **P1-P2-P3(DD(P3))-P4-P5(DD(P5))-P6(DD(P6))-P7**	**1**	40.2
K207-3 + **P1-P2-P3-P4-P5-P6-P7**+pDesPikAV	**1**	71.2
K207-3 + **P1-P2-P3(DD(P3))-P4-P5(DD(P5))-P6(DD(P6))-P7**+pDesPikAV	**1**	84.6
K207-3 + **P1-P5-P3-P4-P5-P6-P7**	**10**	6.84
K207-3 + **P1-P5-P3-P4-P5-P6-P7**	**11**	6.91
K207-3 + **P1-S2-P3-P4-P5-P6-P7**	**12**	2.83
K207-3 + **P1-E2-P3-P4-P5-P6-P7**	**1**	0.989
K207-3 + **P1-P2-P3-P4-P5-E6-P7**	**1**	21.3
K207-3 + **P1-P2-P3-P4-P5-P6*-P7**	**4**	36.4
K207-3 + **P1-P5-P3-P4-P5-P6*-P7**	**13**	9.11
K207-3 + **P1-S2-P3-P4-P5-P6*-P7**	**14**	0.420
K207-3 + **P1-P2-P4-P5-P6-P7**	**15**	84.8
K207-3 + **P1-P2-P3-P4-P5-P5-P6-P7**	**1**	15.2
K207-3 + **P1-P2-P3-P4-P5-P5-P6-P7**	**16**	8.12
K207-3 + **P1-P2-P3-P4-P5-P6-P7** + pDes	**2**	1.30
K207-3 + **P1-P2-P3-P4-P5-P6-P7** + pDesPikC	**3**	0.241
K207-3 + **P1-P2-P3-P4-P5-P6-P7** + pDesPikC + pPikC	**3**	0.305
K207-3 + **P1-P2-P3-P4-P5-P6*-P7** + pDes	**5**	1.56
K207-3 + **P1-P2-P3-P4-P5-P6*-P7** + pDesPikC	**6**	0.347
K207-3 + **P1-P5-P3-P4-P5-P6-P7** + pDes	**17**	0.727
K207-3 + **P1-P5-P3-P4-P5-P6-P7** + pDesPikC	**18**	0.007
K207-3 + **P1-S2-P3-P4-P5-P6-P7** + pDes	**19**	0.848
K207-3 + **P1-S2-P3-P4-P5-P6-P7** + pDesPikC	**20**	0.0003
K207-3 + **P1-S2-P3-P4-P5-P6*-P7** + pDes	**21**	0.289
K207-3 + **P1-S2-P3-P4-P5-P6*-P7** + pDesPikC	**23**	0.0005
K207-3 + **P1-P2-P3-P4-P5-P6-P7** + pMKBAC02-Des operon	**2**	0.900
TM1 + **P1-P2-P3-P4-P5-P6-P7**	**2**	0.678
TM4 + **P1-P2-P3-P4-P5-P6-P7** + pDes	**2**	16.3
TM7 + **P1-P2-P3-P4-P5-P6-P7**	**2**	7.89
TM7 + **P1-P2-P3-P4-P5-P6-P7** + pDes	**2**	18.6
TM7 + **P1-P2-P3-P4-P5-P6-P7** + pDesPikAV	**2**	37.1

TM7 + **P1-P2-P3-P4-P5-P6*-P7** + pDesPikAV	**5**	17.2
TM7 + **P1-P2-P3-P4-P5-P6*-P7** + pDesPikC	**6**	2.13
TM7 + **P1-P2-P5-P4-P5-P6-P7** + pDesPikAV	**17**	13.8
TM7 + **P1-P2-P5-P4-P5-P6-P7** + pDesPikC	**18**	0.022
TM7 + **P1-S2-P3-P4-P5-P6-P7** + pDesPikAV	**19**	7.21
TM7 + **P1-S2-P3-P4-P5-P6-P7** + pDesPikC	**20**	0.012

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