Electronic Supplementary Material (ESI) for Chemical Communications.

SUPPLEMENTARY INFORMATION

Preparative production of an enantiomeric pair by engineered polyketide synthases

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METHODS

Construction of expression plasmids

Gibson assembly and SLiCE[1] were used to join fragments amplified from *Streptomyces venezuelae* ATCC 15439 genomic DNA with vectors (Table S1). Updated junctions were positioned between the 10th and 11th residues following the conserved GTNAH motif of KS; traditional junctions were positioned between the 1st and 2nd residues preceding the PIAIV motif of KS (Table S2).[2]

Protein expression and purification

All proteins were expressed in *E. coli* K207-3 except for *Streptomyces coelicolor* MatB and *Bacillus subtilis* glucose dehydrogenase (GDH), which were expressed in *E. coli* BL21(DE3).[3, 4] Transformed cells were shaken at 240 rpm in LB media containing either 50 mg L^{-1} kanamycin or 50 mg L^{-1} streptomycin to OD_{600} = 0.6 at 37 °C. Cultures were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 18 h at 15 °C. Cells were harvested (4000 x g for 20 min), resuspended in lysis buffer [50 mM potassium phosphate, 500 mM NaCl, 5 mM imidazole, 10% (v/v) glycerol, 1 mM TCEP, pH 7.5] and sonicated. After the cell debris was removed (30,000 x g for 30 min), the supernatant was applied to a Ni-NTA column (2 x 4 cm) and washed with 5 column volumes of lysis buffer containing 15 mM imidazole. Proteins were eluted with 2 column volumes of lysis buffer containing 250 mM imidazole. Each was concentrated (10-18 mg/mL) with an Amicon Ultra centrifugal filter (30 kD MWCO), and the buffer was exchanged for 400 mM potassium phosphate, 150 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP, pH 7.5 (synthase polypeptides) or 15 mM HEPES, 150 mM NaCl, 10% (v/v) glycerol, pH 7.5 (MatB and GDH). Each purification was assessed by SDS-PAGE (Figure S1).

***In vitro* assays**

Reactions (100 μL) were performed in 400 mM potassium phosphate, 10 mM $MgCl_2$, 10 mM ATP, 10 mM D-glucose, 0.5 mM $NADP^+$, 0.2 mM CoA, 10 mM methylmalonate or ^{13}C methylmalonate, 5 mM TCEP, pH 7.5 with 10 μM MatB, 10 μM GDH, and 10 μM of each synthase polypeptide. Synthase polypeptides were added after all other components had incubated for 5 min at 25 °C. After 1 h reactions were quenched through the addition of 70% (v/v) perchloric acid (5 μL). Precipitate was removed (15,000 × g for 5 min), and the supernatants were extracted with ethyl acetate (2 x 200 μL). The extract was dried *in vacuo*, dissolved in 100 μL methanol, and analyzed by high-resolution mass spectrometry (HRMS) [6230 TOF LC/MS equipped with a Microsorb-MV 300-5 C_{18} column (4.6 × 250 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. 5-100% B for 15 min, 100% B for 3 min), positive mode].

***In vivo* production in optimized conditions**

E. coli K207-3 cells transformed with PKS expression plasmids were shaken at 240 rpm in 50 mL LB media containing the appropriate antibiotics (50 mg L^{-1} kanamycin for 1-polypeptide synthases, 50 mg L^{-1} kanamycin and 50 mg L^{-1} streptomycin for 2-polypeptide synthases) in 250 mL flasks at 37 °C. From these precultures, 3 mL was used to inoculate 300 mL of production media (5 g L^{-1} yeast extract, 10 g L^{-1} casein, 15 g L^{-1} glycerol, 10 g L^{-1} NaCl, and 100 mM potassium phosphate, pH 7.6 with 50 mg L^{-1} kanamycin or 50 mg L^{-1} kanamycin and 50 mg L^{-1} streptomycin) in each 2.8 L non-baffled Fernbach flask. Cells were shaken at 240 rpm at 37 °C until OD_{600} = 0.6. They were then cooled to 19 °C, supplied with 0.1 mM IPTG and 20 mM sodium propionate, and cultured for 6 d. Time points were obtained by adding 5 μL concentrated HCl to 500 μL culture broth, extracting twice with the same volume of ethyl acetate, and concentrating *in vacuo*. The extract was resuspended in 500 μL of water and 10 μL was

analyzed by HPLC [Waters 1525 HPLC system equipped with a Microsorb-MV 300-5 C_{18} column (4.6 × 250 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. 5-100% B for 15 min, 100% B for 3 min)].

Purification of triketide lactones

Cultures broths were adjusted to pH 3 with HCl and extracted twice with the same volume of ethyl acetate, using centrifugation (4000 x g for 5 min in polypropylene bottles) to separate emulsions. The extract was dried with $MgSO_4$, filtered, and concentrated *in vacuo*. Propionic acid was removed by passing the extract through a silica gel plug (3 × 5 cm, EtOAc:hexanes = 30:70), and purification was performed using a silica gel column (1.5 × 15 cm, EtOAc:hexanes = 35:65).

Chiral chromatography

Purified triketide lactones were dissolved in 5% acetonitrile and injected onto an Agilent 6230 TOF LC/MS connected to a Chiralcel OD-RH column (2.1 x 150 mm) equilibrated with 5% acetonitrile and 0.5% formic acid. Elution was performed through isochratic flow with the same solvent system. Triketide lactones were observed at 247 nm and by ion count.

Crystallization

Purified triketide lactones were dissolved in EtOAc:hexanes = 5:95 at 40 °C and evaporated at room temperature over 3 h.

Reagents and equipment

KAPA HiFi DNA polymerase was from KAPA Biosystems. Restriction enzymes and the NEBuilder HiFi DNA Assembly Cloning Kit used for Gibson assembly reactions were from New England Biolabs. SLiCE extract was obtained from *E. coli* DH5α cells. Oligonucleotides were from Sigma-Aldrich. Luria-Bertani (LB) Miller Broth, potassium phosphate, sodium chloride, and HEPES were from Fisher Scientific. Kanamycin sulfate was from VWR. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Carbosynth. Ni-NTA affinity resin and tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) were from Thermo Fisher Scientific. Magnesium chloride was from MACRON. Magnesium sulfate, succinic acid, adenosine triphosphate (ATP), and glycine were from Sigma-Aldrich. Coenzyme A (CoA) was from Oriental Yeast Co., Ltd. Sodium malonate was from VWR. Amicon Ultra-4 centrifugal filters for protein concentration were from Millipore. Ethyl acetate (EtOAc), acetonitrile, methanol, chloroform, and hexane were from Fisher Scientific. $CDCl_3$ was from Cambridge Isotope Laboratories. SiliaFlash F60 was from SiliCycle. High-resolution mass spectral (HRMS) analyses were performed on a 6230 TOF LC /MS (Agilent Technologies). ^{1}H and ^{13}C NMR spectra were collected on an Agilent 400-MR.

Bacterial strains

E. coli DH5α and BL21 Star (DE3)pLysS were used for plasmid construction and protein expression (MatB and GDH), respectively. *E.coli* K207-3 was used for the expression of synthase polypeptides as well as the *in vivo* production of polyketides.[5]

Table S1. Expression plasmid construction. Primers, templates, and cloning methods used to obtain fragments and assemble expression plasmids. The underlined sequence indicates homology sequences. The construction of pET28-His_{10} has been described[2].

Plasmid	Method	Piece	Template	Primers
pTM1 (pET28b-His_{10} vector) For updated 1-poly-peptide Pik127	3-piece Gibson	half of pET28b+ KS^{Q}1+AT1+ACP1	pET28b-His_{10}-PikAI/VemG (ref. 2)	TAAAGCTCATCAGCGTGGTCGTGAAGCGATTCA CGGCCTCACACGCCCGAGCAAGCTCTTCGATCTGTA
		KS1+AT2+KR2+ ACP2+KS2	*S. venezuelae* ATCC 15439 genomic DNA	CCCGTACAGATCGAAGAGCTTGCTCGGGCGTGTG GGCGGCTCGACGGCAGGGGAAGCAGCATCCGGCGCCT GCTCCA
		AT7+ACP7+TE7 + half of pET28b	pTM5	AGCAGGCGCCGGATGCTGCTTCCCCTGCCGTCGAGCC GCC CAGGCAGACATCTGTGAATCGCTTCACGACCACG
pTM2 (pCDF-1b vector) For 1st poly-peptide of updated 2-poly-peptide Pik127 For 1st poly-peptide of traditional 2-poly-peptide Pik127	2-piece Gibson	KS1+AT2+KR2+ ACP2	pTM1	CCCGTACAGATCGAAGAGCTTGCTCGGGCGTGTG TCCCAGTCCGTCGGGGCCGGCTCCTCGTCACCGAGGA ACTCGCTGCGGA
		pCDF-1b+ KS^{Q}1+AT1+ACP1 +CDD6	pTM4	AGTTCCTCGGTGACGAGGAGCCGGCCCCGACGGACTG GGAGGGGC CGGCCTCACACGCCCGAGCAAGCTCTTCGATCTGTA
pTM3 (pET28b-His_{10} vector) For 2nd poly-peptide of updated 2-poly-peptide Pik127	2-piece Gibson	KS2+AT7+ACP7 +TE7	pTM1	GCCGGGCCGACCGTCGGCAGGATCCGATCGCGATCGT CGCGATGAGCT TGGTGGTGGTGGTGCTCGAGCTTGCCCGCCCCCTCGA TGCCCTCGAT
		pET28b-His_{10}+ NDD6	pTM5	GCATCGAGGGGGCGGGCAAGCTCGAGCACCACCACCA CCACCAC GCGACGATCGCGATCGGATCCTGCCGACGGTCGGCCC GGCGA

Plasmid	Method	Piece	Template	Primers
pTM4 (pCDF-1b vector) For 1st poly-peptide of updated Pik167	4-piece Gibson	KS^{Q}1+AT1+ACP1	*S. venezuelae* ATCC 15439 genomic DNA	ACCACCATCACGTGGGTACCTCTTCAGCCGGAATTACC AGGACCGGT CGCCCGAGCAAGCTCTTCGATCTGTA
		KS1	*S. venezuelae* ATCC 15439 genomic DNA	TCGAAGAGCTTGCTCGGGCGTGTG CCGGGGAGTCGACAACCACCGGGGCCTCTTCGA
		AT6+KR6+ACP6 +CDD6	*S. venezuelae* ATCC 15439 genomic DNA	GGTGGTTGTCGACTCCCCGGCCGTCGAGCCG GTTTCTTTACCAGACTCGAGTCAGGTGTTACGGGGGCC GAGAGCCAT
		KpnI/XhoI-pCDF-1b	pCDF-1b	
pTM5 (pET28b-His_{10} vector) For 2nd poly-peptide of updated Pik167 For 2nd poly-peptide of traditional 2-poly-peptide Pik127 For 2nd poly-peptide of traditional Pik167	2-piece SLiCE	NDD6+KS6+AT7 +ACP7+TE7	*S. venezuelae* ATCC 15439 genomic DNA	CTTTAAGAAGGAGATATACCATGACGAGTTCCAACGAA CAGTTGGTGGAC TGGTGGTGGTGGTGCTCGAGCTTGCCCGCCCCCTCGAT GCCCTCGAT
		NcoI/XhoI-digested pET28b-His_{10}	pET28b-His_{10}	

Plasmid	Method	Piece	Template	Primers
pTM6 (pET28b-His$_{10}$ vector) For traditional 1-poly-peptide Pik127	3-piece Gibson	KS^{Q}1+AT1+ACP1+KS1+AT2+KR2+ACP2	*S. venezuelae* ATCC 15439 genomic DNA	TGGTGCCGCGCGGCAGCCATATGTCTTCAGCCGGAATTACCAGGACCGGT CCGACGATCGCCATGGGCTCGTCGTCGGCATCGGTGCCGGCGC
		KS6+AT7+ACP7+TE7	*S. venezuelae* ATCC 15439 genomic DNA	CCGGCACCGATGCCGACGACGAGCCCATGGCGATCGTCGGCATGAGCT TGGTGGTGGTGGTGCTCGAGCTTGCCCGCCCCCTCGATGCCCT
		pET28b-His$_{10}$	pET28b-His$_{10}$	GCATCGAGGGGGCGGGCAAGCTCGAGCACCACCACCACCACCACCATCAC CTGGTAATTCCGGCTGAAGACATATGGCTGCCGCGCGGCACCAGG

pTM7 (pCDF-1b vector) For 1st poly-peptide of traditional Pik167	3-piece Gibson	KS^{Q}1+AT1+ACP1	*S. venezuelae* ATCC 15439 genomic DNA	ACCACCATCACGTGGGTACCTCTTCAGCCGGAATTACCAGGACCGGT CCCACGATCGCCACCGGCTCGTCGACGGCACCGGCCGCCGC
		KS5+AT6+KR6+ACP6+CDD6	*S. venezuelae* ATCC 15439 genomic DNA	CGGCGGCCGGTGCCGTCGACGAGCCGGTGGCGATCGTGGGCATG GTTTCTTTACCAGACTCGAGTCAGGTGTTACGGGGGCCGAGAGCCAT
		pCDF-1b	pCDF-1b	TCGAGGGGGCGGGCAAGTGACCTAGGCTGCTGCCACCGCTGAG CTGGTAATTCCGGCTGAAGAGGTACCCACGTGATGGTGGTGGTGA

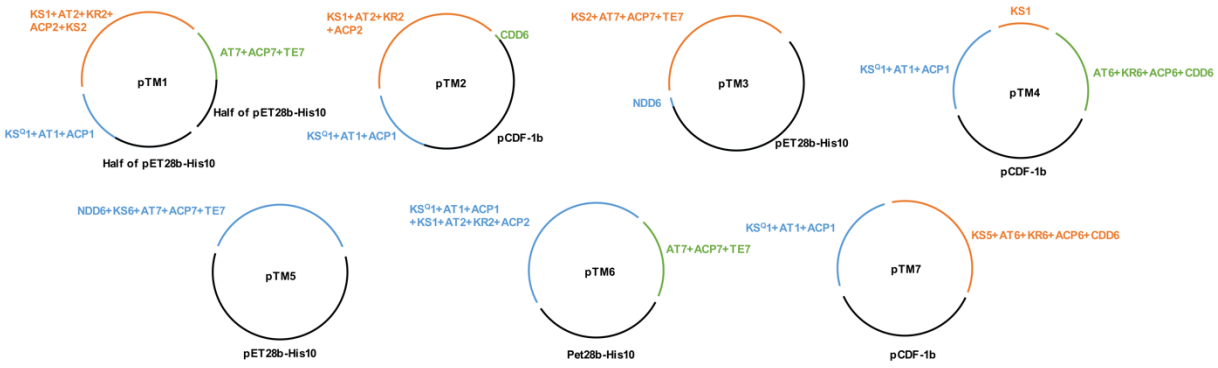

Table S2. Sequences of engineered PKSs. Red, orange, blue, purple, and gray letters indicate modules 1, 2, 5, 6, and 7 of the pikromycin PKS, respectively (colored as in Figure 1). Black letters indicate residues encoded by the vectors. Bold green indicates deviations from the published sequence.

Updated 1-polypeptide Pik127, on pTM1

MGSSHHHHHHHHHHSSGLVPRGSHMSSAGITRTGARTPVTGRGAAAWDTGEVRVRRGLPPAGPDHAEHSFSRAPTG
DVRAELIRGEMSTVSKSESEEFVSVSNDAGSAHGTAEPVAVVGISCRVPGARDPREFWELLAAGGQAVTDVPADRW
NAGDFYDPDRSAPGRSNSRWGGFIEDVDRFDAAFFGISPREAAEMDPQQRLALELGWEALERAGIDPSSLTGTRTG
VFAGAIWDDYATLKHRQGGAAITPHTVTGLHRGIIANRLSYTLGLRGPSMVVDSGQSSSLVAVHLACESLRRGESE
LALAGGVSLNLVPDSIIGASKFGGLSPDGRAYTFDARANGYVRGEGGGFVVLKRLSRAVADGDPVLAVIRGSAVNN
GGAAQGMTTPDAQAQEAVLREAHERAGTAPADVRYVELHGTGTPVGDPIEAAALGAALGTGRPAGQPLLVGSVKTN
IGHLEGAAGIAGLIKAVLAVRGRALPASLNYETPNPAIPFEELNLRVNTEYLPWEPEHDGQRMVVGVSSFGMGGTN
AHVVLEEAPGVVEGASVVESTVGGSAVGGGVVPWVVSAKSAAALDAQIERLAAFASRDRTDGVDAGAVDAGAVDAG
AVARVLAGGRAQFEHRAVVVGSGPDDLAAALAAPEGLVRGVASGVGRVAFVFPGQGTQWAGMGAELLDSSAVFAAA
MAECEAALSPYVDWSLEAVVRQAPGAPTLERVDVVQPVTFAVMVSLARVWQHHGVTPQAVVGHSQGEIAAAYVAGA
LSLDDAARVVTLRSKSIAAHLAGKGGMLSLALSEDAVLERLAGFDGLSVAAVNGPTATVVSGDPVQIEELARACEA
DGVRARVIPVDYASHSRQVEIIESELAEVLAGLSPQAPRVPFFSTLEGAWITEPVLDGGYWYRNLRHRVGFAPAVE
TLATDEGFTHFVEVSAHPVLTMALPGTVTGLATLRRDNGGQDRLVASLAEAWANGLAVDWSPLLPSATGHHSDLPT
YAFQTERHWLGEIEALAPAGEPAVQPAVLRTEAAEPAELDRDEQLRVILDKVRAQTAQVLGYATGGQIEVDRTFRE
AGCTSLTGVDLRNRINAAFGVRMAPSMIFDFPTPEALAEQLLLVVHGEAAANPAGAEPAPVAAAGAVDEPVAIVGM
ACRLPGGVASPEDLWRLVAGGGDAISEFPQDRGWDVEGLYHPDPEHPGTSYVRQGGFIENVAGFDAAFFGISPREA
LAMDPQQRLLLETSWEAVEDAGIDPTSLRGRQVGVFTGAMTHEYGPSLRDGGEGLDGYLLTGNTASVMSGRVSYTL
GLEGPALTVDTACSSSLVALHLAVQALRKGEVDMALAGGVAVMPTPGMFVEFSRQRGLAGDGRSKAFAASADGTSW
SEGVGVLLVERLSDARRNGHQVLAVVRGSAVNQDGASNGLTAPNGPSQQRVIRRALADARLTTSDVDVVEAHGTGT
RLGDPIEAQALIATYGQGRDDEQPLRLGSLKSNIGHTQAAAGVSGVIKMVQAMRHGLLPKTLHVDEPSDQIDWSAG
AVELLTEAVDWPEKQDGGLRRAAVSSFGISGTNAHVVLEEAPVVVEGASVVEPSVGGSAVGGGVTPWVVSAKSAAA
LDAQIERLAAFASRDRTDDADAGAVDAGAVAHVLADGRAQFEHRAVALGAGADDLVQALADPDGLIRGTASGVGRV
AFVFPGQGTQWAGMGAELLDSSAVFAAAMAECEAALSPYVDWSLEAVVRQAPGAPTLERVDVVQPVTFAVMVSLAR
VWQHHGVTPQAVVGHSQGEIAAAYVAGALPLDDAARVVTLRSKSIAAHLAGKGGMLSLALNEDAVLERLSDFDGLS
VAAVNGPTATVVSGDPVQIEELAQACKADGFRARIIPVDYASHSRQVEIIESELAQVLAGLSPQAPRVPFFSTLEG
TWITEPVLDGTYWYRNLRHRVGFAPAIETLAVDEGFTHFVEVSAHPVLTMTLPETVTGLGTLRREQGGQERLVTSL
AEAWVNGLPVAWTSLLPATASRPGLPTYAFQAERYWLENTPAALATGDDWRYRIDWKRLPAAEGSERTGLSGRWLA
VTPEDHSAQAAAVLTALVDAGAKVEVLTAGADDDREALAARLTALTTGDGFTGVVSLLDGLVPQVAWVQALGDAGI
KAPLWSVTQGAVSVGRLDTPADPDRAMLWGLGRVVALEHPERWAGLVDLPAQPDAAALAHLVTALSGATGEDQIAI
RTTGLHARRLARAPLHGRRPTRDWQPHGTVLITGGTGALGSHAARWMAHHGAEHLLLVSRSGEQAPGATQLTAELT
ASGARVTIAACDVADPHAMRTLLDAIPAETPLTAVVHTAGALDDGIVDTLTAEQVRRAHRAKAVGASVLDELTRDL
DLDAFVLFSSVSSTLGIPGQGNYAPHNAYLDALAARRRATGRSAVSVAWGPWDGGGMAAGDGVAERLRNHGVPGMD
PELALAALESALGRDETAITVADIDWDRFYLAYSSGRPQPLVEELPEVRRIIDARDSATSGQGGSSAQGANPLAER
LAAAAPGERTEILLGLVRAQAAAVLRMRSPEDVAADRAFKDIGFDSLAGVELRNRLTRATGLQLPATLVFDHPTPL
ALVSLLRSEFLGDEETADARRSAALPATVGAGAGAGAGTDADDDPIAIVAMSCRYPGDIRSPEDLWRMLSEGGEGI
TPFPTDRGWDLDGLYDADPDALGRAYVREGGFLHDAAEFDAEFFGVSPREALAMDPQQRMLLTTSWEAFERAGIEP
ASLRGSSTGVFIGLSYQDYAARVPNAPRGVEGYLLTGSTPSVASGRIAYTFGLEGPATTVDTACSSSLTALHLAVR
ALRSGECTMALAGGVAMMATPHMFVEFSRQRALAPDGRSKAFSADADGFGAAEGVGLLLVERLSDARRNGHPVLAV
VRGTAVNQDGASNGLTAPNGPSQQRVIRQALADARLAPGDIDAVETHGTGTSLGDPIEAQGLQATYGKERPAERPL
AIGSVKSNIGHTQAAAGAAGIIKMVLAMRHGTLPKTLHADEPSPHVDWANSGLALVTEPIDWPAGTGPRRAAVSSF
GISGTNAHVVLEQAPDAASPAVEPPAGGGVVPWPVSAKTSAALDAQIGQLAAYAEDRTDVDPAVAARALVDSRTAM
EHRAVAVGDSREALRDALRMPEGLVRGTVTDPGRVAFVFPGQGTQWAGMGAELLDSSPEFAAAMAECETALSPYVD
WSLEAVVRQAPSAPTLDRVDVVQPVTFAVMVSLAKVWQHHGITPEAVIGHSQGEIAAAYVAGALTLDDAARVVTLR
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SHSAHVETIENELADVLAGLSPQTPQVPFFSTLEGTWITEPALDGGYWYRNLRHRVGFAPAVETLATDEGFTHFIE
VSAHPVLTMTLPDKVTGLATLRREDGGQHRLTTSLAEAWANGLALDWASLLPATGALSPAVPDLPTYAFQHRSYWI
SPAGPGEAPAHTASGREAVAETGLAWGPGAEDLDEEGRRSAVLAMVMRQAASVLRCDSPEEVPVDRPLREIGFDSL
TAVDFRNRVNRLTGLQLPPTVVFEHPTPVALAERISDELAERNWAVAEPSDHEQAEEEKAAAPAGARSGADTGAGA
GMFRALFRQAVEDDRYGEFLDVLAEASAFRPQFASPEACSERLDPVLLAGGPTDRAEGRAVLVGCTGTAANGGPHE
FLRLSTSFQEERDFLAVPLPGYGTGTGTGTALLPADLDTALDAQARAILRAAGDAPVVLLGHSGGALLAHELAFRL
ERAHGAPPAGIVLVDPYPPGHQEPIEVWSRQLGEGLFAGELEPMSDARLLAMGRYARFLAGPRPGRSSAPVLLVRA
SEPLGDWQEERGDWRAHWDLPHTVADVPGDHFTMMRDHAPAVAEAVLSWLDAIEGIEGAGKLEHHHHHHHHHH

1st polypeptide of updated 2-polypeptide Pik127 and 1st polypeptide of traditional 2-polypeptide Pik127, on pTM2

MGSSHHHHHHHHHHSSGLVPRGSHMSSAGITRTGARTPVTGRGAAAWDTGEVRVRRGLPPAGPDHAEHSFSRAPTG
DVRAELIRGEMSTVSKSESEEFVSVSNDAGSAHGTAEPVAVVGISCRVPGARDPREFWELLAAGGQAVTDVPADRW
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VFAGAIWDDYATLKHRQGGAAITPHTVTGLHRGIIANRLSYTLGLRGPSMVVDSGQSSSLVAVHLACESLRRGESE
LALAGGVSLNLVPDSIIGASKFGGLSPDGRAYTFDARANGYVRGEGGGFVVLKRLSRAVADGDPVLAVIRGSAVNN
GGAAQGMTTPDAQAQEAVLREAHERAGTAPADVRYVELHGTGTPVGDPIEAAALGAALGTGRPAGQPLLVGSVKTN
IGHLEGAAGIAGLIKAVLAVRGRALPASLNYETPNPAIPFEELNLRVNTEYLPWEPEHDGQRMVVGVSSFGMGGTN
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MAECEAALSPYVDWSLEAVVRQAPGAPTLERVDVVQPVTFAVMVSLARVWQHHGVTPQAVVGHSQGEIAAAYVAGA
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DGVRARVIPVDYASHSRQVEIIESELAEVLAGLSPQAPRVPFFSTLEGAWITEPVLDGGYWYRNLRHRVGFAPAVE
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ACRLPGGVASPEDLWRLVAGGGDAISEFPQDRGWDVEGLYHPDPEHPGTSYVRQGGFIENVAGFDAAFFGISPREA
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SEGVGVLLVERLSDARRNGHQVLAVVRGSAVNQDGASNGLTAPNGPSQQRVIRRALADARLTTSDVDVVEAHGTGT
RLGDPIEAQALIATYGQGRDDEQPLRLGSLKSNIGHTQAAAGVSGVIKMVQAMRHGLLPKTLHVDEPSDQIDWSAG
AVELLTEAVDWPEKQDGGLRRAAVSSFGISGTNAHVVLEEAPVVVEGASVVEPSVGGSAVGGGVTPWVVSAKSAAA
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ASGARVTIAACDVADPHAMRTLLDAIPAETPLTAVVHTAGALDDGIVDTLTAEQVRRAHRAKAVGASVLDELTRDL
DLDAFVLFSSVSSTLGIPGQGNYAPHNAYLDALAARRRATGRSAVSVAWGPWDGGGMAAGDGVAERLRNHGVPGMD
PELALAALESALGRDETAITVADIDWDRFYLAYSSGRPQPLVEELPEVRRIIDARDSATSGQGGSSAQGANPLAER
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ALVSLLRSEFLGDEEPAPTDWEGRVRRALAELPLDRLRDAGVLDTVLRLTGIEPEPGSGGSDGGAADPGAEPEASI
DDLDAEALIRMALGPRNT

2nd polypeptide of updated 2-polypeptide Pik127, on pTM3

MTSSNEQLVDALRASLKENEELRKESRRRADRRQDPIAIVAMSCRYPGDIRSPEDLWRMLSEGGEGITPFPTDRGW
DLDGLYDADPDALGRAYVREGGFLHDAAEFDAEFFGVSPREALAMDPQQRMLLTTSWEAFERAGIEPASLRGSSTG
VFIGLSYQDYAARVPNAPRGVEGYLLTGSTPSVASGRIAYTFGLEGPATTVDTACSSSLTALHLAVRALRSGECTM
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GHTQAAAGAAGIIKMVLAMRHGTLPKTLHADEPSPHVDWANSGLALVTEPIDWPAGTGPRRAAVSSFGISGTNAHV
VLEQAPDAASPAVEPPAGGGVVPWPVSAKTSAALDAQIGQLAAYAEDRTDVDPAVAARALVDSRTAMEHRAVAVGD
SREALRDALRMPEGLVRGTVTDPGRVAFVFPGQGTQWAGMGAELLDSSPEFAAAMAECETALSPYVDWSLEAVVRQ
APSAPTLDRVDVVQPVTFAVMVSLAKVWQHHGITPEAVIGHSQGEIAAAYVAGALTLDDAARVVTLRSKSIAAHLA
GKGGMISLALSEEATRQRIENLHGLSIAAVNGPTATVVSGDPTQIQELAQACEADGIRARIIPVDYASHSAHVETI
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1st polypeptide of updated 2-polypeptide Pik167, on pTM4

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2nd polypeptide of updated Pik167, 2nd polypeptide of updated 2-polypeptide Pik127, and 2nd polypeptide of traditional 2-polypeptide Pik127, on pTM5

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PAGIVLVDPYPPGHQEPIEVWSRQLGEGLFAGELEPMSDARLLAMGRYARFLAGPRPGRSSAPVLLVRASEPLGDW
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Traditional 1-polypeptide Pik127, on pTM6

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1st polypeptide of traditional 2-polypeptide Pik167, on pTM7

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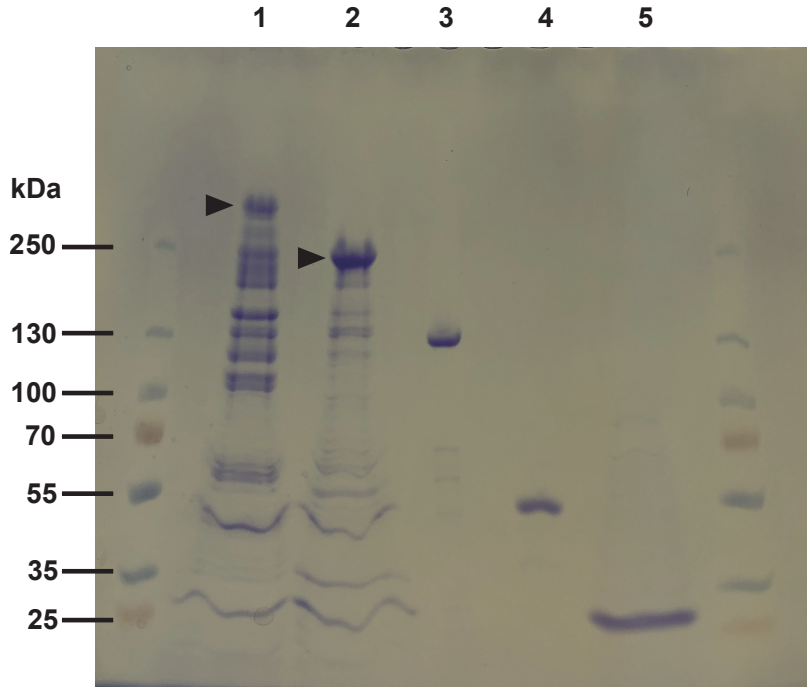


Figure S1. SDS-PAGE gel of nickel-NTA purified PKS polypeptides, MatB, and GDH. 4-20% Tris-glycine gel (Thermo Fisher Scientific)

Lane 1: updated 1-polypeptide Pik127, 412 kDa
Lane 2: 1st polypeptide of updated Pik167, 275 kDa
Lane 3: 2nd polypeptide of updated Pik167, 143 kDa
Lane 4 - MatB, 53 kDa
Lane 5 - GDH, 30 kDa.
First and last lanes: PageRuler Plus Prestained Protein Ladder

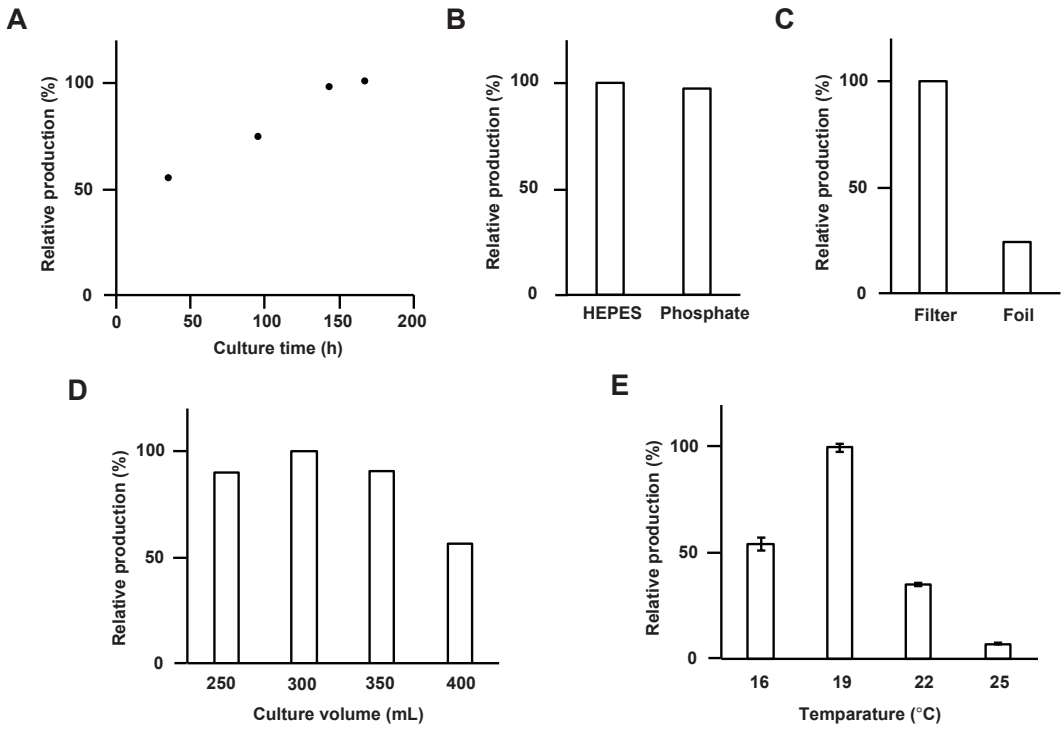


Figure S2. Optimization of *in vivo* triketide production. The optimization was performed by monitoring triketide production of **2** by Pik167 using peak areas from HPLC chromatograms (λ=247 nm). a) Time course of polyketide production, b) Comparison of HEPES and potassium phosphate buffers, c) Comparison of milk filter disk (Ken AG) and aluminum foil used to cover culture flasks, d) Comparison of culture volumes in a 2.8-L, non-baffled Fernbach flask, e) Comparison of growth temperatures after IPTG induction.

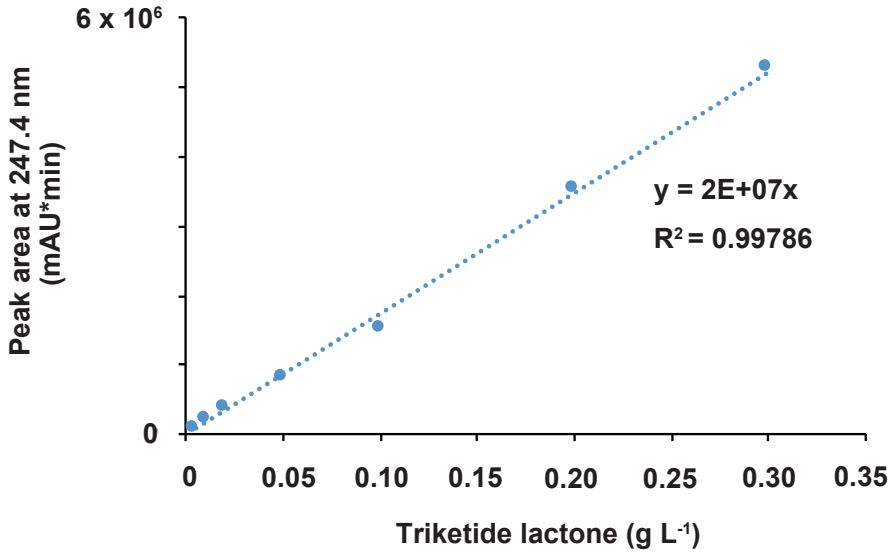
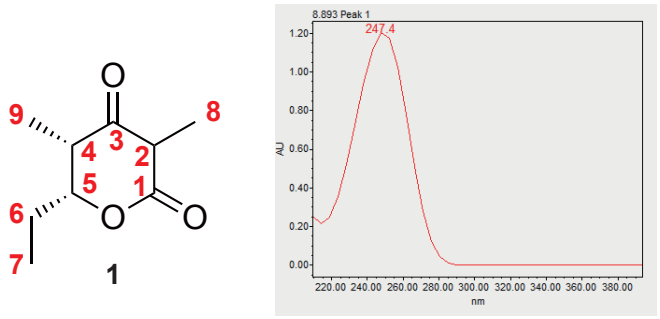


Figure S3. Triketide lactone calibration curve. Several concentrations of **1** dissolved in water (10 μL) were analyzed by HPLC (conditions in Methods section).



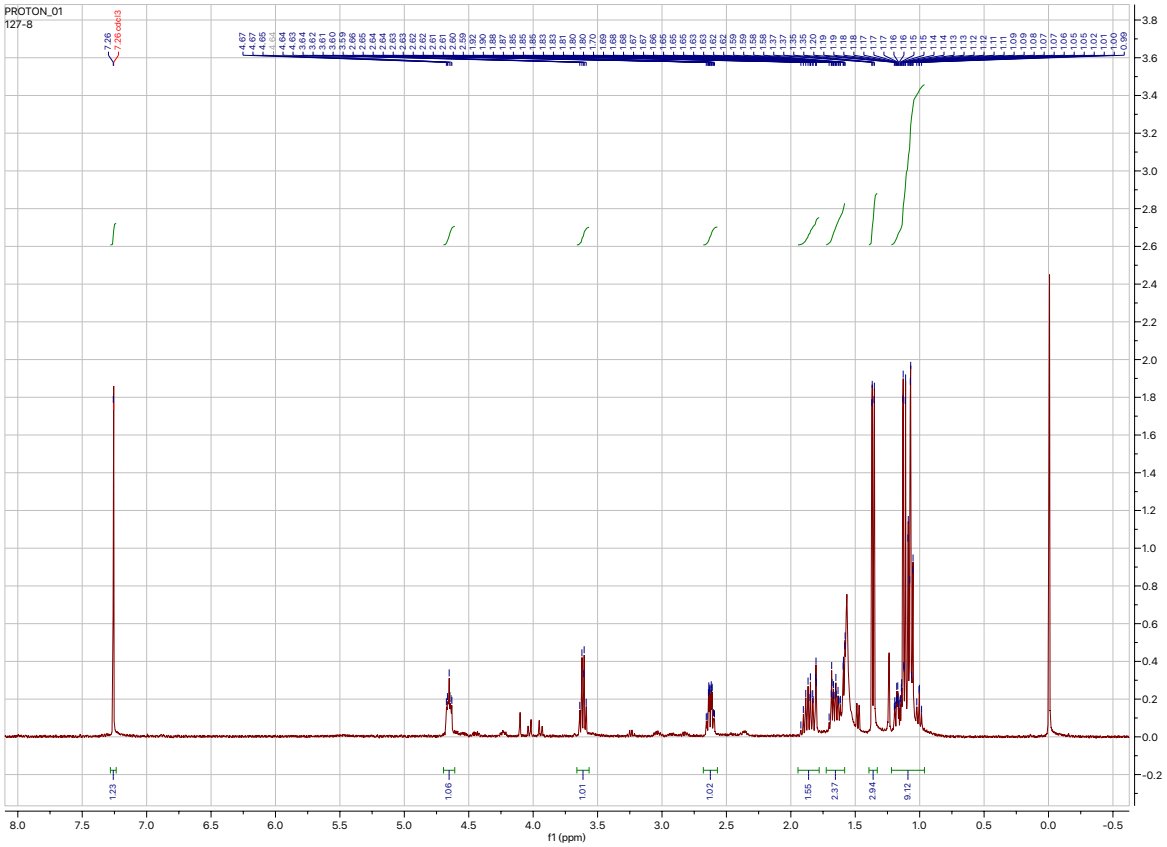


Figure S4. ^{1}H NMR of **1** in $CDCl_3$.

^{1}H NMR (400 MHz, $CDCl_3$) δ= 4.65 (m, 1H), 3.61 (q, J=7 Hz, 1H), 2.62 (dq, J=7 Hz, J=3.2 Hz, 1H), 1.85 (m, 1H), 1.65 (m, 1H), 1.36 (d, J=7 Hz, 3H), 1.12 (d, J=7 Hz, 3H), 1.07 (t, J=7 Hz, 3H) (only signals from keto form reported, although signals from enol form are present). HRMS: calcd. for $C_9H_{14}O_3$ $[M+Na]^+$, *m/z* 193.0835; found, *m/z* 193.0842. λ_{max} = 247.4 nm. All characterization matched that from **1** in a previous study.[6]

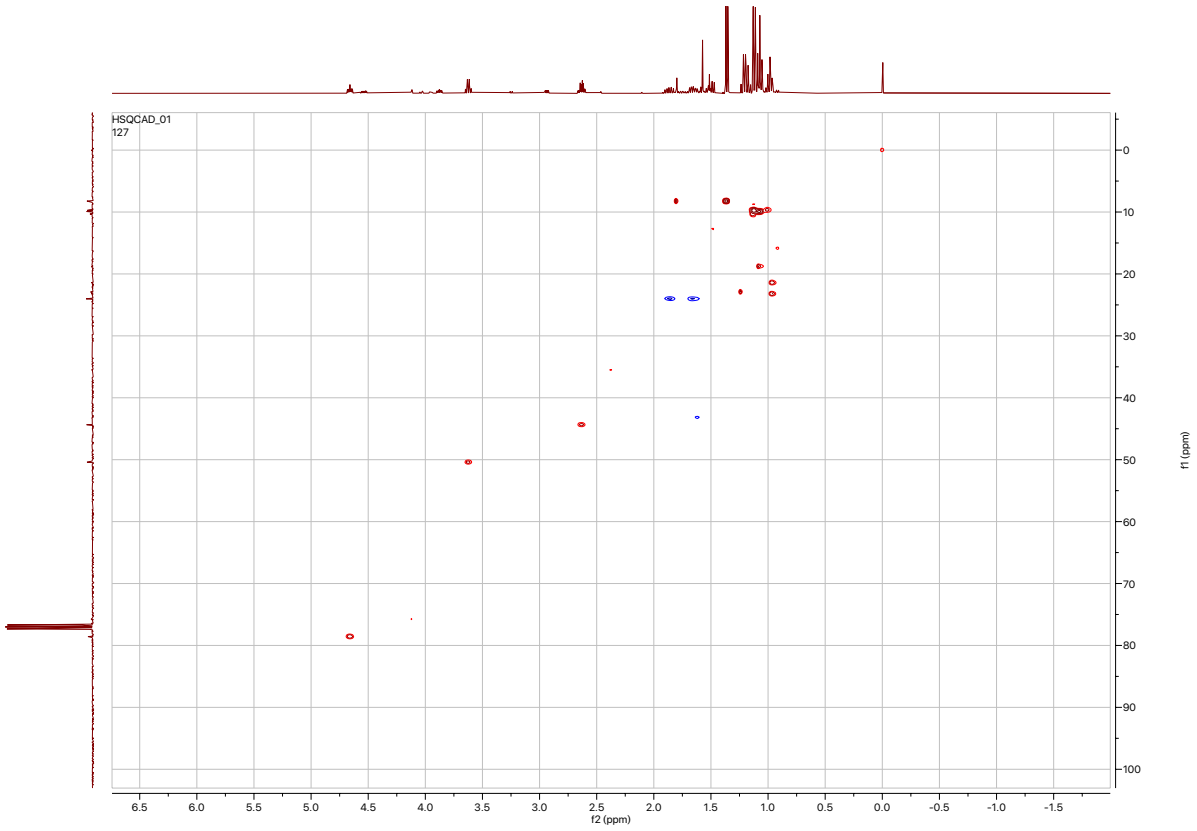
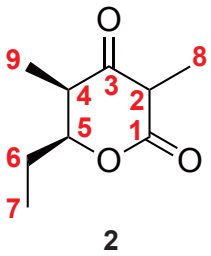
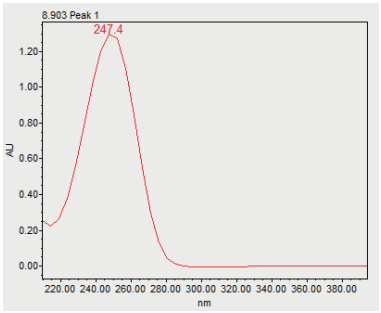


Figure S5. ^{1}H-^{13}C-HSQC of **1** in $CDCl_3$.



2



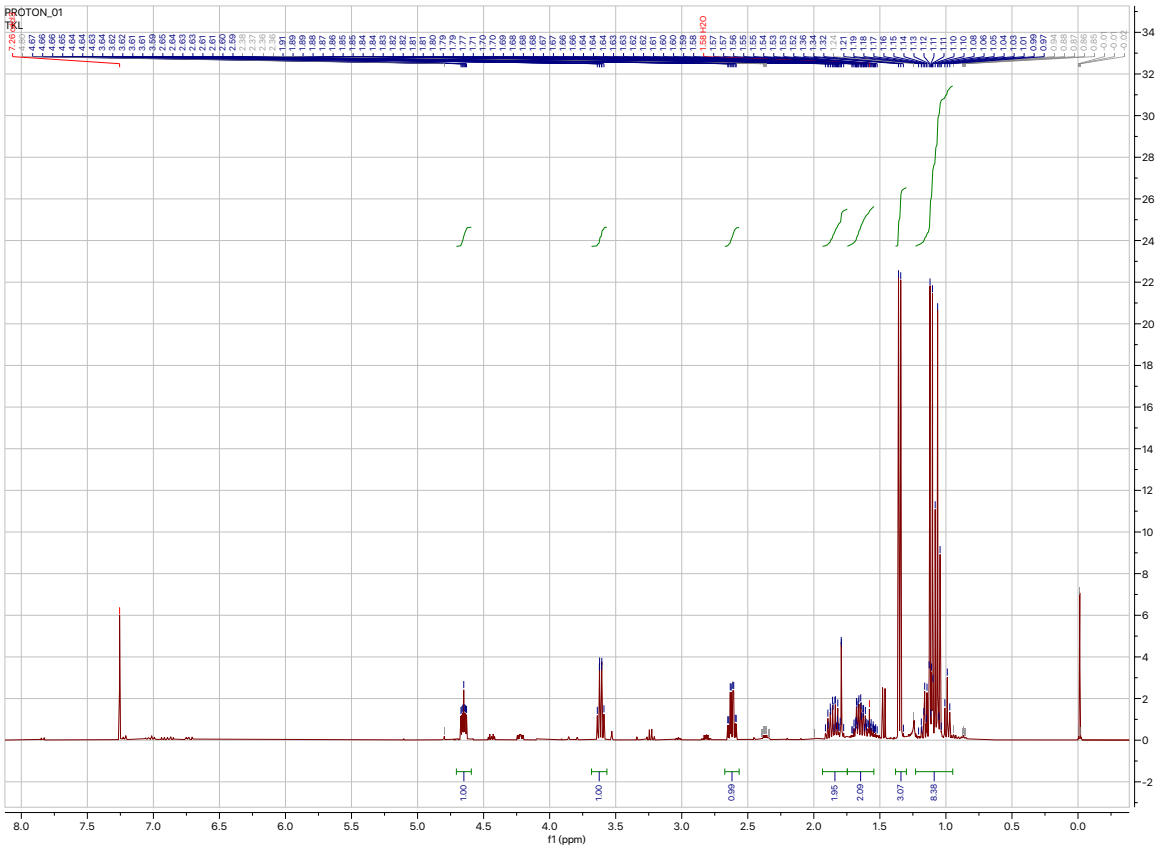


Figure S6. ^{1}H NMR of **2** in $CDCl_3$.

^{1}H NMR (400 MHz, $CDCl_3$) δ= 4.67 (m, 1H), 3.61 (q, J=7 Hz, 1H), 2.62 (dq, J=7 Hz, J=3.2 Hz, 1H), 1.85 (m, 1H), 1.65 (m, 1H), 1.36 (d, J=7 Hz, 3H), 1.12 (d, J=7 Hz, 3H), 1.07 (t, J=7 Hz, 3H) (only signals from keto form reported, although signals from enol form are present). HRMS: calcd. for $C_9H_{14}O_3$ $[M+Na]^+$, *m*/*z* 193.0835; found, *m*/*z* 193.0840. λ_{max} = 247.4 nm. All characterization (except for chiral chromatography and crystallography) matched that of **1** from a previous study.[6]

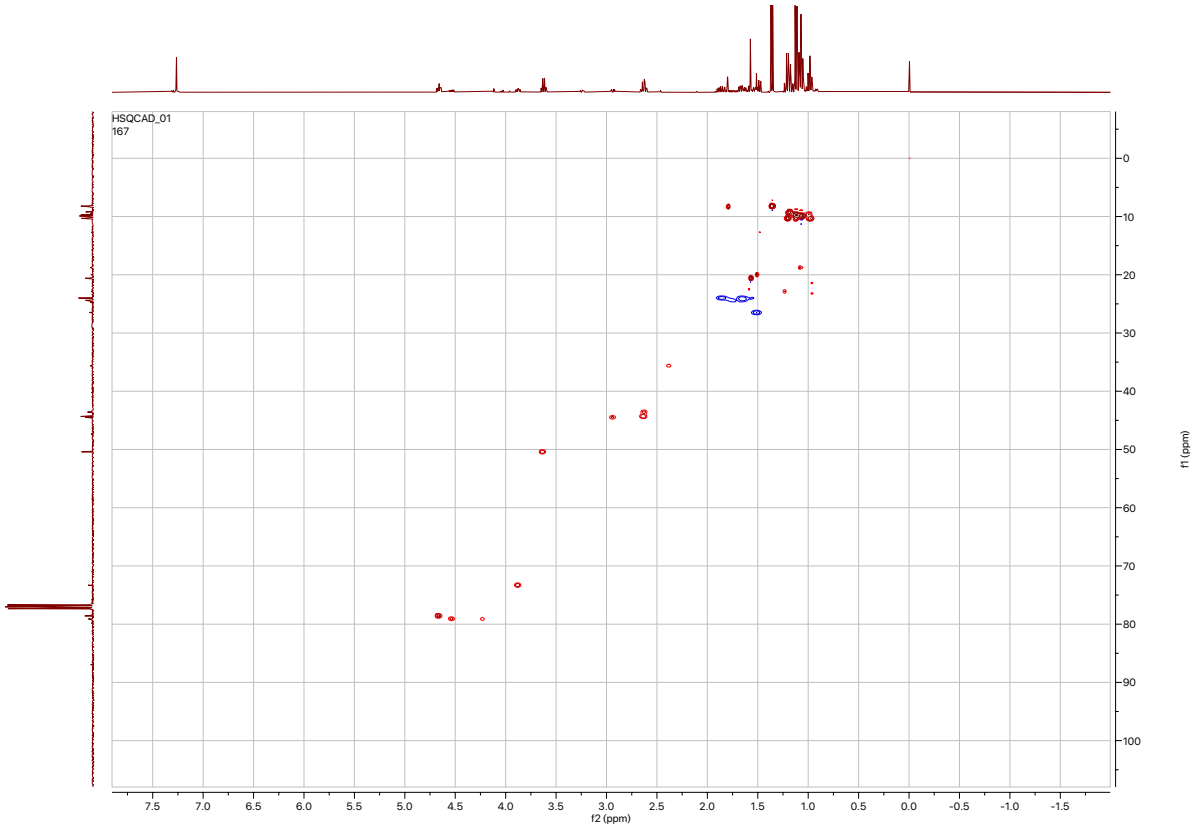


Figure S7. ^{1}H-^{13}C-HSQC of **2** in $CDCl_3$.

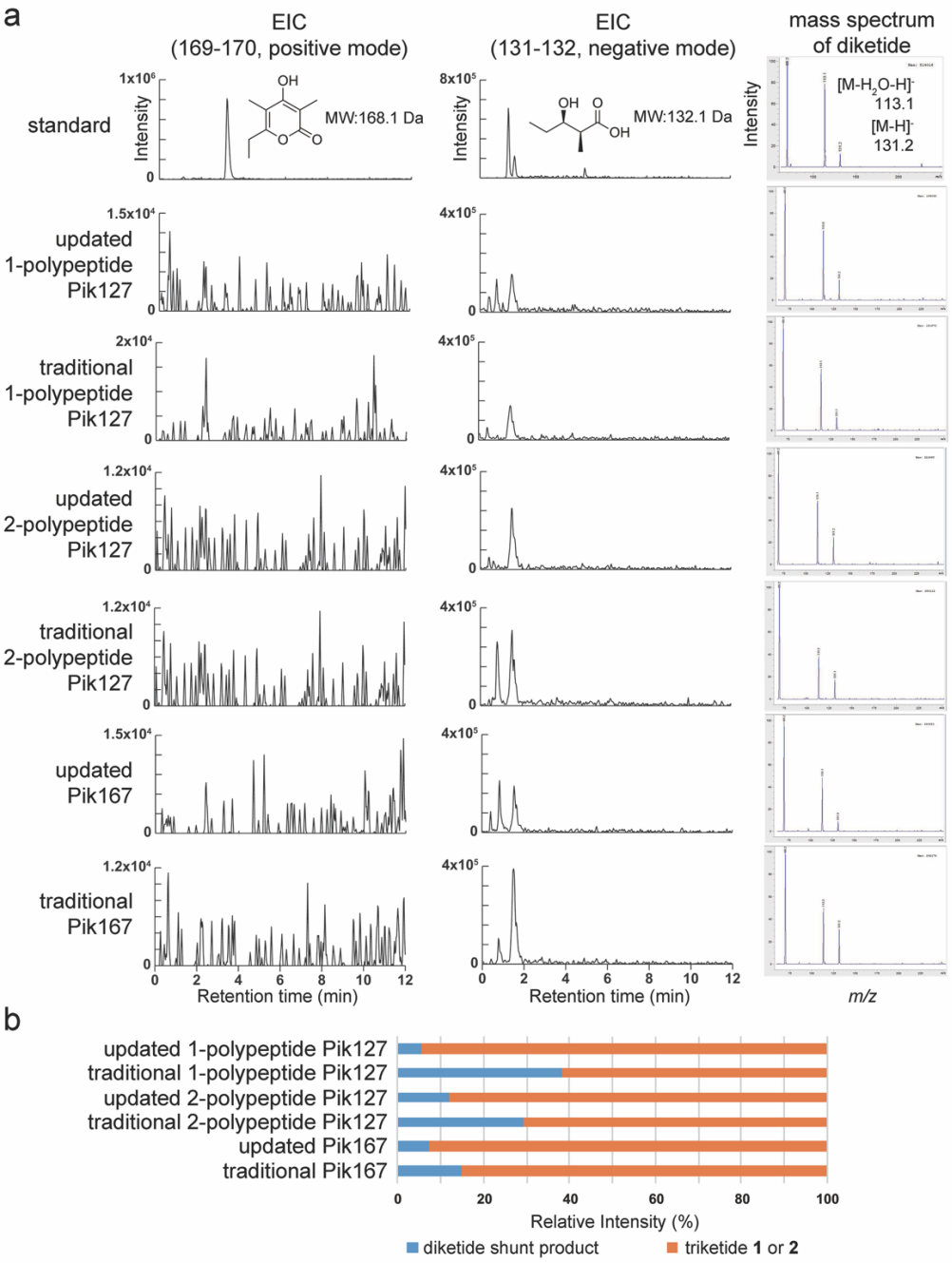


Figure S8. Analysis of shunt products. Ethyl acetate extracts from culture broths were analyzed by LC/MS. [Agilent 6120 system equipped with a ZORBAX Eclipse Plus C_{18} (2.1 × 50 mm) with a flow rate of 0.8 mL min^{-1} (solvent A, water with 0.1% formic acid; solvent B, acetonitrile with 0.1% formic acid. 5-100% B for 12 min)]. The pyrone standard is from an *in vitro* reaction of updated Pik167 without the NADPH regeneration system. The diketide standard, β-D-hydroxy-α-L-methylpentanoic acid, came from a previous study[4]. a) No pyrone was detected from any synthase (left). Diketide products [likely β-D-hydroxy-α-L-methylpentanoic acid for Pik127 synthases and β-L-hydroxy-α-D-methylpentanoic acid for Pik167 synthases] were observed from each synthase (mass spectra of the 1.3 min peak shown for each synthase). b) A comparison of the peak areas from the EIC of the diketide shunt product and the EIC of the triketide product shows that synthases designed with the updated module boundary form a smaller proportion of diketide shunt products.

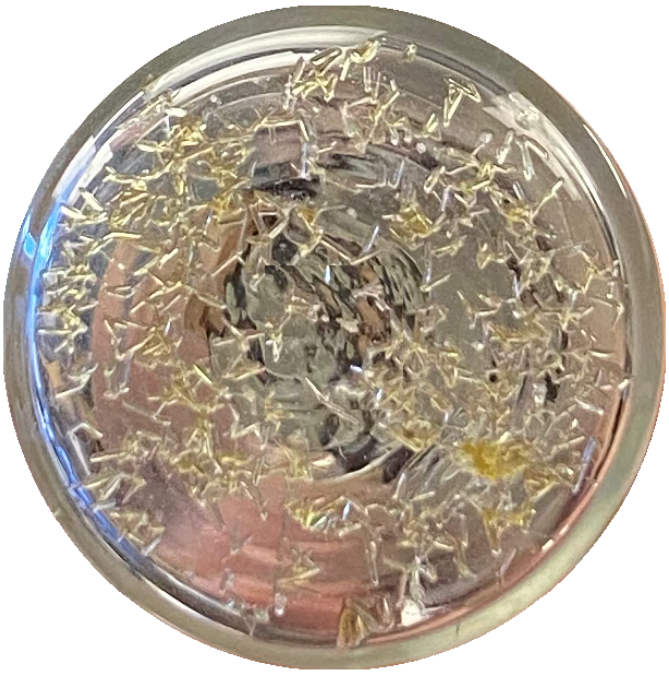


Figure S9. Triketide lactone crystals. After silica gel chromagraphy, a fraction containing **1** was crystallized in a glass vial.

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