

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

### 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).<sup>3,4</sup> Transformed cells were shaken at 240 rpm in LB media containing either 50 mg L<sup>-1</sup> kanamycin or 50 mg L<sup>-1</sup> streptomycin to OD<sub>600</sub> = 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  $\mu$ L) were performed in 400 mM potassium phosphate, 10 mM MgCl<sub>2</sub>, 10 mM ATP, 10 mM D-glucose, 0.5 mM NADP<sup>+</sup>, 0.2 mM CoA, 10 mM methylmalonate or <sup>13</sup>C methylmalonate, 5 mM TCEP, pH 7.5 with 10  $\mu$ M MatB, 10  $\mu$ M GDH, and 10  $\mu$ M 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  $\mu$ L). Precipitate was removed (15,000  $\times$  g for 5 min), and the supernatants were extracted with ethyl acetate (2  $\times$  200  $\mu$ L). The extract was dried *in vacuo*, dissolved in 100  $\mu$ L methanol, and analyzed by high-resolution mass spectrometry (HRMS) [6230 TOF LC/MS equipped with a Microsorb-MV 300-5 C<sub>18</sub> column (4.6  $\times$  250 mm) with a flow rate of 1 mL min<sup>-1</sup> (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<sup>-1</sup> kanamycin for 1-polypeptide synthases, 50 mg L<sup>-1</sup> kanamycin and 50 mg L<sup>-1</sup> 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<sup>-1</sup> yeast extract, 10 g L<sup>-1</sup> casein, 15 g L<sup>-1</sup> glycerol, 10 g L<sup>-1</sup> NaCl, and 100 mM potassium phosphate, pH 7.6 with 50 mg L<sup>-1</sup> kanamycin or 50 mg L<sup>-1</sup> kanamycin and 50 mg L<sup>-1</sup> streptomycin) in each 2.8 L non-baffled Fernbach flask. Cells were shaken at 240 rpm at 37 °C until OD<sub>600</sub> = 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  $\mu$ L concentrated HCl to 500  $\mu$ L culture broth, extracting twice with the same volume of ethyl acetate, and concentrating *in vacuo*. The extract was resuspended in 500  $\mu$ L of water and 10  $\mu$ L was

analyzed by HPLC [Waters 1525 HPLC system equipped with a Microsorb-MV 300-5 C<sub>18</sub> column (4.6 × 250 mm) with a flow rate of 1 mL min<sup>-1</sup> (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<sub>4</sub>, 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 isocratic 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* DH5 $\alpha$  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- $\beta$ -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<sub>3</sub> 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). <sup>1</sup>H and <sup>13</sup>C NMR spectra were collected on an Agilent 400-MR.

## **Bacterial strains**

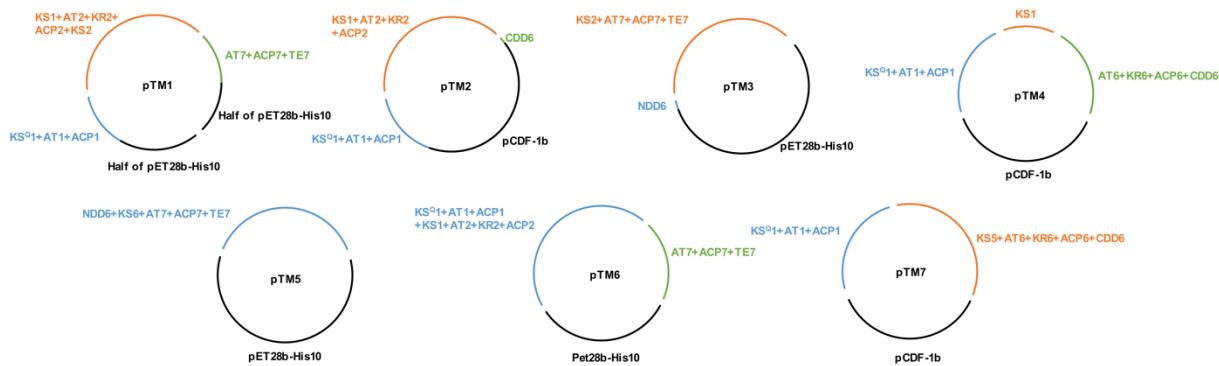
*E. coli* DH5 $\alpha$  and BL21 Star (DE3)pLysS were used for plasmid construction and protein expression (MatB and GDH), respectively. *E.coli* K207-3 was used for the expression of synthase polypeptides as well as the *in vivo* production of polyketides.<sup>5</sup>

**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<sub>10</sub> has been described<sup>2</sup>.

Plasmid	Method	Piece	Template	Primers
pTM1 (pET28b-His <sub>10</sub> vector) For updated 1-poly-peptide Pik127	3-piece Gibson	half of pET28b+ KS <sup>9</sup> 1+AT1+ACP1	pET28b-His <sub>10</sub> -PikAI/VemG (ref. 2)	TAAAGCTCATCAGCGTGGTCGTGAAGCGATTCA <u>CGGCCTCACACGCCCGAGCAAGCTCTCGATCTGTA</u>
		KS1+AT2+KR2+ ACP2+KS2	<i>S. venezuelae</i> ATCC 15439 genomic DNA	CCCGTACAGATCGAAGAGCTTGCTCGGGCGTGTG <u>GGCGGCTCGACGGCAGGGGAAGCAGCATCCGGCGCT</u> GCTCCA
		AT7+ACP7+TE7 + half of pET28b	pTM5	AGCAGGGGCCGGATGCTGCTTCCCCTGCCGTCGAGCC GCC CAGGCAGACATCTGTGAATCGCTTCACGACCACG
pTM2 (pCDF-1b vector) For 1 <sup>st</sup> poly-peptide of updated 2-poly-peptide Pik127 For 1 <sup>st</sup> poly-peptide of traditional 2-poly-peptide Pik127	2-piece Gibson	KS1+AT2+KR2+ ACP2	pTM1	CCCGTACAGATCGAAGAGCTTGCTCGGGCGTGTG <u>TCCCAGTCCGTGGGGCCGGCTCCTCGTCACCGAGGA</u> ACTCGCTGCGGA
		pCDF-1b+ KS <sup>9</sup> 1+AT1+ACP1 +CDD6	pTM4	AGTTCCCTCGGTGACGAGGGAGCCGGCCCCGACGGACTG GGAGGGGC <u>CGGCCTCACACGCCCGAGCAAGCTCTCGATCTGTA</u>
pTM3 (pET28b-His <sub>10</sub> vector) For 2 <sup>nd</sup> poly-peptide of updated 2-poly-peptide Pik127	2-piece Gibson	KS2+AT7+ACP7 +TE7	pTM1	GCCGGGCCGACCGTCGGCAGGATCCGATCGCGATCGT CGCGATGAGCT <u>TGGTGGTGGTGGTGCTCGAGCTTGCCCCCCCCCTCGA</u> TGCCCTCGAT
		pET28b-His <sub>10</sub> + NDD6	pTM5	GCATCGAGGGGGCGGGCAAGCTCGAGCACCACCA CCACCA <u>GCGACGATCGCGATCGGATCCTGCCGACGGTCGGCCC</u> GGCGA

<b>Plasmid</b>	<b>Method</b>	<b>Piece</b>	<b>Template</b>	<b>Primers</b>
pTM4 (pCDF-1b vector)  For 1 <sup>st</sup> poly-peptide of updated Pik167	4-piece Gibson	KS <sup>9</sup> 1+AT1+ACP1	<i>S. venezuelae</i> ATCC 15439 genomic DNA	<u>ACCACCATCACGTGGGTACCTCTTCAGCCGGAATTACC</u> <u>AGGACCGGT</u> <u>CGCCCGAGCAAGCTTTCGATCTGTA</u>
		KS1	<i>S. venezuelae</i> ATCC 15439 genomic DNA	<u>TCGAAGAGCTTGCTCGGGCGTGTG</u> <u>CGGGGGAGTCGACAACCACCGGGGCCTTCGA</u>
		AT6+KR6+ACP6 +CDD6	<i>S. venezuelae</i> ATCC 15439 genomic DNA	<u>GGTGGTTGTCGACTCCCCGGCCGTCGAGCCG</u> <u>GTTTCTTACAGACTCGAGTCAGGTGTTACGGGGGCC</u> <u>GAGAGCCAT</u>
		KpnI/XhoI-pCDF-1b	pCDF-1b	
pTM5 (pET28b-His <sub>10</sub> vector)  For 2 <sup>nd</sup> poly-peptide of updated Pik167  For 2 <sup>nd</sup> poly-peptide of traditional 2-poly-peptide Pik127  For 2 <sup>nd</sup> poly-peptide of traditional Pik167	2-piece SLiCE	NDD6+KS6+AT7 +ACP7+TE7	<i>S. venezuelae</i> ATCC 15439 genomic DNA	<u>CTTTAAGAAGGAGATATACCATGACGAGTTCCAACGAA</u> <u>CAGTTGGTGGAC</u> <u>TGGTGGTGGTGGTGCTCGAGCTTGCCCCCCCCCTCGAT</u> <u>GCCCTCGAT</u>
		NcoI/XhoI-digested pET28b-His <sub>10</sub>	pET28b-His <sub>10</sub>	

Plasmid	Method	Piece	Template	Primers
pTM6 (pET28b-His <sub>10</sub> vector)	3-piece Gibson	KS <sup>0</sup> 1+AT1+ACP1+KS1+AT2+KR2+ACP2	<i>S. venezuelae</i> ATCC 15439 genomic DNA	<u>TGGTGCCGCGCGCAGCCATATGTCTTCAGCCGAATT</u> ACCAGGACCGT <u>CCGACGATGCCATGGGCTCGTCGTCGGCATCGGTGCC</u> GGCGC
		KS6+AT7+ACP7+TE7	<i>S. venezuelae</i> ATCC 15439 genomic DNA	<u>CCGGCACCGATGCCGACGAGCCCATGGCGATCGTC</u> GGCATGAGCT <u>TGGTGGTGGTGGTGGCTCGAGCTTGCCCCGCCCCCTCGAT</u> GCCCT
		pET28b-His <sub>10</sub>	pET28b-His <sub>10</sub>	<u>GCATCGAGGGGGCGGGCAAGCTCGAGCACCAACCACAC</u> CACCACCATCAC <u>CTGGTAATCCGGCTGAAGACATATGGCTGCCGCGGG</u> CACCAGG
pTM7 (pCDF-1b vector)	3-piece Gibson	KS <sup>0</sup> 1+AT1+ACP1	<i>S. venezuelae</i> ATCC 15439 genomic DNA	<u>ACCACCATCACGTGGTACCTCTTCAGCCGAATT</u> TAC AGGACCGT <u>CCCACGATGCCACCGGCTCGACGGCACCGGCCGC</u> CGC
		KS5+AT6+KR6+ACP6+CDD6	<i>S. venezuelae</i> ATCC 15439 genomic DNA	<u>CGGCGGCCGGTGCCGTGACGAGCCGGTGGCGATCGTG</u> GGCATG <u>GTTTCTTACCACTCGAGTCAGGTGTTACGGGGGCC</u> GAGAGCCAT
		pCDF-1b	pCDF-1b	<u>TCGAGGGGGCGGGCAAGTGAACCTAGGCTGCTGCCACCG</u> CTGAG <u>CTGGTAATCCGGCTGAAGAGGTACCCACGTGATGGTG</u> GTGGTGA



**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
MGSSHHHHHHHHHSSGLVPRGSH <b>MSSAGITRTGARTPVTGRGAAAWDTGEVRVRGLPPAGPDHAHSFSRAPTG</b> <b>DVRAELIRGEMSTVSKSEEEFVSNSNDAGSAHGTAEPVAVVGISCRVPGARDPREFWELLAAGGQAVTDVPADRW</b> NAGDFYDPPRSAPGRSNSRWGGFIEDVDRDAAFFGISPREAAEMDPQQRLALELGWEALERAGIDPSSLTGTG <b>VFAGAIWDDYATLKHQRGGAAITPHTVTGLHRGIIANRLSYTLGLRGPSMVDSQSSSLVAVHLACESLRGESE</b> LALAGGVSLNLVPDSIIGASKFGGLSPDGRAYTFDARANGYVRGEFFFFVVLKRLSRAVADGDPVLA <b>IRGSANNGGAAQGMTPDAQAQEAVLREAHERAGTAPADVRYVELHGTGTPVGDPPIEAAALGAALGTGRPAGQPLLVGSVKTN</b> IGHLEGAAGIAGLIKA <b>VLA VRGRAL PASLN YETPNPAIPFEELNRVNTEYL PWEPEHDGQRMVVGSSFGMGGTN</b> AHVVLEEAPGVVEGAS <b>VVE</b> STVGGSAVGGGVWPVVSAKSAAALDAQIERLAA <b>FASR DRTDGV DAGA VDAG A VDAG A VARVLAGGRAQF EHRA VVVG SGP DDIAA ALA APE GLV RG VAS GV GR VAF V FP G Q GT Q WAG MG AE LL DSS AVFA AA</b> MAECEAALSPYDVWSLEAVVRQAPGAPTLERVDVVQPVTFAVMVSLARVWQHHGVTQAVVGHQSQEIAAAYVAGA LSLDDAARVTLRSKSIAAHLAGKGMLSALSEDAVLERLAGFDGLSVAAVNGPTATVSGDPVQIEELARACEA DGVRARVIPDYASHSRQVEIIIESELA <b>EVL AGL SPQ A PRV PFF STLEG AW I TE PV LD GG Y WYRN L R H RV G F A P A V E</b> T LAT DEG FTH F VEV SAH PV L T M A L P G T V T G L A T L R R D N G G Q D R L V A S L A E A W A N G L A V D W S P L L P S A T G H H S D L P T Y AF Q T E R H W L G E I E A L A P A G E P A V Q P A V L R T E A A E P A E L D R D E Q L R V I L D K V R A Q T A Q V L G Y A T G G Q I E V D R T F R E AGCTSLTVGDLRNRRINAAFGVRMAPSMIFDFPTPEALAEQLLLLHVGEAAANPAGAE <b>PAPVAAAGAVDEPVAIVGM</b> ACRLPGGVASPEDLWRLVAGGGDAISEFPQDRGWDVEGLYHPDPEHGTYSVRQGGFIENVAGFDAAFFGISPREA LAMDPQQRLLLETSWEAVEDAGIDPTSLRGRQGVFTGAMTHEYGPSLRDGGEGLDGYLLTGTASVMSGRVSYTL GLEGPALTVDTACSSLVALHLAQALRKGEVDMALAGGVAVMPTPGMFVEFSRQRLAGDGRSKAFAASADGTSW SEGVGVLVERLSDARRNGHQVLAVVRSASAVNQDGASNGLTAPNGPSQQRVIRRALADARLTTSDVDVVEAHGTGT RLGDPIEAQALIATYQGGRDDEQPLRLGSLKSIGHTQAAAGVSGVIKMVQAMRHGLPKTLHVDEPSDQIDWSAG <b>AVELLTEAVDWPEKQDGGLRRAAVSSFGISGTNAHVLEEAPVVVEGASVVEPSVGSAGVGGVTPWVSAKSAAA</b> LDAQIERLAA <b>FA S RD T DDADAGA DV A G A V D A G A V A H V L A D G R A Q F E H R A V A L G A G A D D L V Q A L A D P D G L I R G T A S G V G R V</b> AFVFPQGQTQWAGMGAE <b>LL DSS AV FA A A M A E C E A A L S P Y D V W S L E A V V R Q A P G A P T L E R V D V V Q P V T F A V M V S L A R</b> VWQHHGVTQAVVGHQSQEIAAAYVAGALPLDDAARVVTLRSKSIAAHLAGKGMLSALNEDAVLERLSDFDGLS VAAVNGPTATVSGDPVQIEELAQACKADGFRARIIPVDYASHSRQVEIIIESELAQVLAGLSPQAPRVPFFSTLEG TWITEPVLDGTYWYRNLRH RVGF A PAI ET LAVDEG FTH F VEV SAH PV L T M L PET V T G L G T L R RE QGG Q E R L V T S L AEAWVNGLPVAWTSILPATASRPGLPTYAFQAERYWLENTPAALATGDDWRYRIDWKRLPAAEGSERTGLSGRWLA VTPEDHSAQAAAVLTALVDAGAKVEVLTAGADDREALAARLTALT TDG DFTGVVSVSLLDGLVPQVAWVQALGDAGI KAPLWSVTQGAVSVGRDLTPADPDRAMLWGLRVVALEHPERWAGLVDPQAPDAAALAHLVTA LSGATGEDQIAI RTTGLHARRLARAPLHRRPTRDWQPHGTVLITGGT GALGSHAARWMAMHGAEHLLVSRSGEQAPGATQLTAE ASGARVTTAACDVADPHAMRTLDAI <b>PAETPLTAVVHTAGALDDGIVDTLAEQVRRRAHRAKAVGASVLDL</b> <b>TRDLDLDAFVLFSSVSSTLGI PGQGNYAPHNAYLDALAARRRATGRSAVSVAWGPWDGGGMAAGDGAERLRLNVPGMD</b> <b>PELALAALESALGRDETAITVADI DWDRFYLAYSSGRPQPLVEELPEVRRIIDARDSATSGQGGSAQGANPLAER</b> <b>LAAAAPGERTEIILLGLVRAQAAVLRMRSPEDVAADRAFKDIGFDLSAGVELRNRLTRATGLQLPATLVFDHPTPL</b> <b>ALVSSLRSEFLGDEETADARRSAALPATVGAGAGAGAGTDADDDPIAIVAMSCRYPGDIRSPEDLWRMLSEGGEGI</b> <b>TPFPPTDRGWLDLGLYDADPDALGRAYVREGGFLHDAEAEFDAEFFGVSPREALAMD</b> <b>PQQRMLLTT SWEA FERAGIEP ASLRGSSTGVFIGL SYQDYAARVNPAPRGVEGYLLTGSTPSVASGRIAYTFLEG PATTVDTACSSLTALH L A V R</b> <b>ALRS GECTM A LAGGVAMM ATPHMFVEFSRQRALAPDGRSKAFSADADGFAAEGVLLVERLSDARRNGHPVLA V</b> <b>VRGTAVNQDGASNGLTAPNGPSQQRVIRQALADARLAPGDIDAVETHGTGSLGDPIEAQGLQATYKG E R PA E R PL</b> <b>AIGSVKS NIGHTQAAAGAAGI IKMVLAMRHTLPKTLHADEPSPHVDWANSGLALVTEPIDWPAGTGPRRAVSSF</b> <b>GISGTNAHVVL E QAP DAA SPAVEPPAGGGVWPWPSAKTSAALDAQI QL A A Y A E D R T D V D P A V A A R A L V D S R T A M</b> <b>EHR A V A V G D S R E A L R D A L R M P E G L V R G T V T D P G R V A F V FP Q Q G T Q W A G M G A E L L D S S P E F A A M A E C E T A L S P Y V D</b> <b>W S L E A V V R Q A P S A P T L D R V D V V Q P V T F A V M V S L A K V W Q H G I T P E A V I G H S Q G E I A A A Y V A G A L T L D D A A R V V T L R</b> <b>SKSIAAHLAGKGGMISLALSEEATQRRIENLHGLSIAAVNGPTATVSGDPTQIQELAQACEADGIRARIIPVDYA</b> <b>SHSAH VETIENELADVLAGLSPQTQVPFSTLEGWITEPALDGGYWYRNLRH RVGF A P A V E T L A T D E G F T H F I E</b> <b>VSAHPVLTMTLPDKVTGLATLREDGGQHRLTTSLAEAWANGLADWASLPATGALSPAVPDPLPTYAFQHRSYWI</b> <b>SPAGPGEAPAHTASGREAVAETGLAWGPGAEDLDEEGRSAVLA M V M R Q A A S V L R C D S P E E V P V D R P L R E I G F D S L</b> <b>TAVDFRNVRNRLTGLQLPPTVVF</b> <b>E H P T P V A L A E R I S D E L A E R N W V A A E P S D H E Q A E E K A A P A G A R S G A D T G A G A</b> <b>GMFRALFRQAVEDDRGEFLDVLA  EASA  F RP Q F A S P E A C S E R L D P V L L A G G P T D R A E G R A V L V G C T G T A A N G G P H E</b> <b>FLRLSTS  F Q E E R D F L A V P L P G Y G T G T G T A L L P A D L D T A L D A Q A R A I L R A A G D A P V V L L G H S G G A L L A H E L A F R L</b> <b>ERA H G A P P A G I V L V D P Y P P G H Q E P I E V W S R Q L G E G L F A G E L E P M S D A R L L A M G R Y A R F L A G P R P G R S S A P V I L V R A</b> <b>S E P L G D W Q E E R G D W R A H W D L P H T V A D V P G D H F T M M R D H A P A V A E A V L S W L D A I E G I G A G K L E H H H H H H H H H H</b>

**1<sup>st</sup> polypeptide of updated 2-polypeptide Pik127 and 1<sup>st</sup> polypeptide of traditional 2-polypeptide  
Pik127, on pTM2**

MGSSHHHHHHHHHSSGLVPRGSHMSSAGITRTGARTPVTGRGAAAWDTGEVRVRRGLPPAGPDHAEHSFSRAPTG  
DVRAELIRGEMSTVKSEEEFVSVNSNDAGSAHGTAEPVAVVGISCRVPGARDPREFWELLAAGGQAVTDVPADRW  
NAGDFYDPDRSAPGRSNSRWCFFIEDVDRFDAAFFGISPREAAEMDPQQLALELGWEALERAGIDPSSLTGRTRG  
VFAGAIWDDYATLKHQRGGAAITPHTVTGLHRTIANRLSYTLGLRGPSMVDSGQSSLVAVHLACESLRGESE  
LALAGGVSLNLPDSIIGASKFGLSPDGRTFDARANGYVRGEFFFVVLKRLSRAVADGDPVLAVIRGSANNN  
GGAAQGMTPDAQAQEAVLREAHERAGTAPADVRYVELHGTGTPVGDPIEAAALGAALGTGRPAGQPLLVGSVKTN  
IGHLEGAAGIAGLIKAVLAVRGRALPASNYETPNPAIPFEELNRVNTEYLWPWEPEHDGQRMVVGVSFGMGGTN  
AHVVLEEAPGVVE GASVVESTVGGSAVGGVVPWVSAKSAAALDAQIERLAAFASRRTDGVDAGAVDAG  
AVARVLAGGRAQFEHRAVVVGSGPDLAALAALAAPEGLVRGVASGVGRVAFVFPQGQTQWAGMGAELLDS  
MAECEAALSPYVWDWSLEAVVRQAPGPTLERVDVVQPVTFAMVSLARVWQHHGVTQAVVGHQS  
GEIAAAYVAGA  
LSLDDAARVTLRSKSIAAHLAGKGGMMLSALSEDAVLERLAGFDGLSVAAVNGPTATVSGDPVQIEELARACEA  
DGVRARVIPDYASHSRQVEIIIESELAEVLAGLSPQAPRVPFFSTLEGAWITEPVLDGGYWYRNLHRVGFAPAVE  
TLATDEGFTHFVEVSAHPVLTMALPGTVTGLATLRRDNGGQDRLVASLAEAWANGLAVDWSPLLP  
SATGHHSIDLPT  
YAFQTERHWLGEIEALAPAGEPAVQPAVLTEAAEPAELDRDEQLRVIDKVRQTAQVLYATGGQIEVDRTFRE  
AGCTSLTVGDRLRNRNAFGVRMAPSMIFDFPTPEALAEQLLLHVGEAAANPAGAE  
PAPVAAAGAVDEPVAIVGM  
ACRLPGGVASPEDLWRLVAGGGDAISEFPQDRGWDVEGLYHPDPEHPGTSYVRQGGFIENVAGFDAAFFG  
ISPREA  
LAMDPQQLLLET SWEAVEDAGIDPTSLRGRQVGVFTGAMTHEYGPSLRDGGEGLDGYLLTGNTASVMSGRVSYTL  
GLEGPALTVDTACSSLVALHLAVQALRKGEVDMALAGGVAVMPTPGMFVEFSRQRLAGDGRSKAFAASADGT  
SW  
SEGVGULLVERLSDARRNGHQVLA VV RGS A V N Q D G A S N G L T A P N G P S Q Q R V I R R A L A D A R L T T S D V D V E A H G T  
T R L G D P I E A Q A L I A T Y Q Q G R D D E Q P L R L G S L K S N I G H T Q A A A G V S G V I K M V Q A M R H G L L P K T L H V D E P S D Q I D W S A G  
A V E L L T E A V D W P E K Q D G G L R R A A V S S F G I S G T N A H V V L E E A P V V V E G A S V V E P S V G G A V V G G V T P W V V S A K S A A A  
L D A Q I E R L A A F A S R D R T D D A D A G A V D A G A V A H V L A D G R A Q F E H R A V A L G A G A D D L V Q A L A D P D G L I R G T A S G V G R V  
A F V F P G Q G T Q W A G M G A E L L D S A V F A A A M A E C E A A L S P Y V D W S L E A V V R Q A P G A P T L E R V D V V Q P V T F A V M V S L A R  
V W Q H H G V T P Q A V V G H S Q G E I A A A Y V A G A L P L D D A A R V T L R S K S I A A H L A G K G G M L S L A L N E D A V L E R L S D F D G L S  
V A A V N G P T A T V V S G D P V Q I E E L A Q A C K A D G F R A R I I P V D Y A S H R Q V E I I E S E L A Q V L A G L S P Q A P R V P F F S T L E G  
T W I T E P V L D G T Y W Y R N L R H R V G F A P A I E T L A V D E G F T H F V E V S A H P V L T M T L P E T V T G L G T L R R E Q G G Q E R L V T S L  
A E A W V N G L P V A W T S L L P A T A S R P G L P T Y A F Q A E R Y W L E N T P A A L A T G D D W R Y R I D W K R L P A A E G S E R T G L S G R W L A  
V T P E D H S A Q A A V L T A L V D A G A K V E V L T A G A D D D R E A L A A R L T A L T G D G F T G V V S L L D G L V P Q V A W V Q A L G D A G I  
K A P L W S T Q G A V S V G R L D T P A P D D R A M I L W G L G R V V A L E H P E R W A G L V D L P A Q P D A A A L A H L V T A L S G A T G E D Q I A I  
R T T G L H A R R L A R A P L H G R R P T R D W Q P H G T V L I T G G T G A L G S H A A R W M A H H G A E H L L L V S R S G E Q A P G A T Q L T A E L T  
A S G A R V T I A A C D V A D P H A M R T L L D A I P A E T P L T A V V H T A G A D D D R E A L A A R L T A L T G D G F T G V V S L L D G L V P Q V A W V Q A L G D A G I  
D L D A F V L F S S V S T L G I P G Q G N Y A P H N A Y L D A L A A R R A T G R S A V S V A W G P W D G G G M A A G D G V A E R L R N H G V P G M D  
P E L A L A A L E S A L G R D E T A I T V A D I D W D R F Y L A Y S S G R P Q P L V E E L P E V R R I I D A R D S A T S G Q G G S S A Q G A N P L A E R  
L A A A P G E R T E I I L L G L V R A Q A A V L R M R S P E D V A A D R A F K D I G F D S L A G V E L R N R L T R A T G L Q L P A T L V F D H P T P L  
A L V S L L R S E F L G D E E P A P T D W E G R V R R A L A E L P L D R L R D A G V L D T V L R L T G I E P E P G S G G S D G G A A D P G A E P E A S I  
D D L D A E A L I R M A L G P R N T

**2<sup>nd</sup> polypeptide of updated 2-polypeptide Pik127, on pTM3**

MTSSNEQLVDALRASLKENEELRKESRRRADRRQDPPIAVAMSCRYPGDIRSPEDLWRMLSEGGEGITPFPTDRGW  
DLDGLYDADPDALGRAYVREGGLFLHDAAEFDAAEFFGVSPREALAMPQQRMLLTTSSWEAERAGIEPASLRGSSTG  
VFIGLSYQDYAARVNPAPRGVEGYLLTGSTPSVASGRIAYTFGLEGPATTVDTACSSLTALHLAVRALRSGETM  
ALAGGVAMMATPHMFVEFSRQRALAPDGRSKAFSADADFGAAEVGVLVERLSDARRNGHPVLA V R G T A V N Q D  
GASNGLTAPNGPSQQRVIRQALADARLAPGDIDAVEHTGTGTSLGDPIEAQGLQATYKERPAERPLAIGSVKSNI  
GHTQAAAGAAGTIKMLAMRHGTLPKTLADEPSPHDWANSGLALVTEPIDWPAGTGPRAA V S S F G I S G T N A H V  
V L E Q A P D A A S P A V E P P A G G G V V P W P V S A K T S A A L D A Q I G Q L A A Y A E D R T D V D P A V A A R A L V D S R T A M E H R A V A V G D  
S R E A L R D A L R M P E G L V R G T V T D P G R V A F V F P G Q G T Q W A G M G A E L L D S S P E F A A A M A E C E T A L S P Y V D W S L E A V V R Q  
A P S A P T L D R V D V V Q P V T F A V M V S L A K V W Q H H G I T P E A V I G H S Q G E I A A A Y V A G A L T L D D A A R V V T L R S K S I A A H L A  
G K G G M I S L A L S E E A T R Q R I E N L H G L S I A A V N G P T A T V V S G D P T Q I Q E L A Q A C E A D G I R A R I I P V D Y A S H S A H V E T I  
E N E L A D V L A G L S P Q T P Q V P F F S T L E G T W I T E P A L D G G Y W Y R N L R H R V G F A P A V E T L A T D E G F T H I E V S A H P V L T M  
T L P D K V T G L A T L R R E D G G Q H R L T T S L A E A W A N G L A L D W A S L L P A T G A L S P A V P D L P T Y A F Q H R S Y W I S P A G P G E A P  
A H T A S G R E A V A E T G L A W G P G A E D L D E E G R R S A V L A M V M R Q A A S V L R C D S P E E V P V D R P L R E I G F D S L T A V D F R N R V  
N R L T G L Q L P P T V V F E H P T P V A L A E R I S D E L A E R N W A V A E P S D H E Q A E E K A A A P A G A R S G A D T G A G A G M F R A L F R Q  
A V E D D R Y G E F L D V L A E A S A F R P Q F A S P E A C S E R L D P V L L A G G P T D R A E G R A V L V G C T G T A A N G G P H E F L R L S T S F Q  
E E R D F L A V P L P G Y G T G T G T A L L P A D L D T A L D A Q A R A I L R A A G D A P V V L L G H S G G A L L A H E L A F R L E R A H G A P P A

GIVLVDPYPPGHQEPIEVWSRQLGEGLFAGELEPMSDARLLAMGRYARFLAGPRPGRSSAPVLLVRASEPLGDWQE  
ERGDWRAHDLPTHADVPGDHTMMRDHAPEAVLSWLDIAEIGEAGKLEHHHHHHHH

### 1<sup>st</sup> polypeptide of updated 2-polypeptide Pik167, on pTM4

MAHHHHHVGTSSAGITRTGARTPVTGRGAAAWDTGEVRVRRGLPPAGPDHAEHSFSRAPTDVRAELIRGEMSTV  
SKSESEEFVSNSDAGSAHGTAEPVAVVGISCRVPGARDPREFWELLAAGGQAVTDVPADRNAGDFYDPDRSAPG  
RSNSRWGGFIEDVDRFDAAFFGISPREAAEMDPQQRLALELGWEALERAGIDPSSLTGTGTVFAGAIWDDYATLK  
HRQGGAAITPHTVTGLHRGITANRLSYTLGLRGPSMVDSGQSSLVAVHLACESLRRGESELALAGGVSLNLPD  
SIIGASKFGLSPDGGRAYTFDARANGYVRGEFFFVVLKRLSRAVADGDPVLAVIRGSANNGGAAQGMTPDAQA  
QEAVLREAHERAGTAPADVRYVELHGTGTPVGDPIEAAALGAALGTGRPAGQPLVGSVKTIGHLEGAAGIAGLI  
KAVLAVRGRALPASNYETPNPAIPFEELNRVNTEYLPWEPEHDGQRMVVGVSSFGMGGTNAHVVLEEAPGVVEG  
ASVVESTVGGSAVGGVVPWVSAKSAAALDAQIERLAAFASRRTDGVDAGAVDAGAVARVLAGGRAQFE  
HRAVVVGSGPDDLAALAALAAPEGLVRGVASGVGRVAFVFPGQGTQWAGMGAELLDSSAVFAAAMAECALSPYDVW  
SLEAVVRQAPGPTLERVDVQPVTFAMVSLARVWQHHGTPQAVVGHSGEIAAAYVAGALSDDAARVVTLRS  
KSIAAHLAGKGGMISLALSEDAVLERLAGFDGLSVAAVNGPTATVSGDPVQIEELARACEADGVRARVIPDYAS  
HSRQEVIIESELAEVLAGLSPQAPRVPFFSTLEGAWITEPVLDGGYWYRNLHRVGFAPAVETLATDEGFTHFVEV  
SAHPVLTMALPGTVTGLATLRRDNGGQDRLVASLAEAWANGLAVDWSPLPSATGHHSIDLPTYAFQTERHWLGEIE  
ALAPAGEPAVQPAVLRTEEAEPAELDRDEQLRVILDKVRAQTAQVLGYATGGQIEVDRTFREAGCTSLGVDLRN  
INAAGVVRMAPSMIFDFPTPEALAEQLLLHVGEAAANPAGAEAPVAAAGAVDEPVAIVGMACRLPGGVASPEDL  
WRLVAGGGDAISEFPQDRGWDVEGLYHPDPEHPGTSYVRQGGFIENVAGFDAAFFGISPREALAMDPPQRLLLETS  
WEAVEDAGIDPTSLRGRQVGFTGAMTHEYGPSLRDGEGLDGYLLTGNTASVMSGRVSYTLGLEGPALETVDTACS  
SSLVALHLAVQALRKGEVDMALAGGVAVMPTPGMFVEFSRQRLAGDGRSKAFAASADGTSWSEGVGVLLVERLSD  
ARRNNGHQVLAVVRGSAVNDGASNGLTAPNGPSQQRVIRRALADARLTTSDVDVVEAHGTGTRLGDPIEAQALIAT  
YGQGRDDEQPLRLGSLKSIGHTQAAAGVSGVIKMVQAMRHGLPKTLHVDEPSQIDWSAGAVELLTEAVDWPEK  
QDGGLRRAAVSSFGISGTNAHVLEEAPVVVDSPAVEPPAGGGVWPWPSAKTPAALDAQIQGQLAAYADGRTDVP  
AVAARALVDSRTAMEHRAAVAVGDSREALRDLRMPEGLVRGTSSDVGRAVFVFPQGTQWAGMGAELLDSSPEFAA  
SMAECETALSRYVWDLSLEAVVRQE PGAPTLDRDVVQPVTFAMVSLAKVWQHHGITPQAVVGHSGEIAAAYVAG  
ALTLDDAARVVTLRSKSIAAHLAGKGGMISLALDEAAVLKRLSDFDGLSVAAVNGPTATVSGDPTQIEELARTCE  
ADGVRARIIPVDYASHSRQVEIEKELAEVLAGLAPQAPHVPFSTLEGTWITEPVLDGTYWYRNLHRVGFAPAV  
ETLAVDGFTHFIEVSAHPVLTMTLPETVTGLGLRREQGGQERLVTSLAEAWANGLTIDWAPILTATGHHPELPT  
YAFQTERFWLQSSAPTSADDWRYRVEWKPLTASGQADLSGRWIVAVGSEPEAELLGALKAAGAEVDVLEAGADDD  
REALAARLTALTTGDGTGVVSSLDDILPVQAVWQALGDAGIKAPLWSVTQGAVSVGRLETPADPDRAMLWGLRV  
VALEHPERWAGLVDLPAQPDAALALAHLTALSGATGEDQIAIRRTGLHARRLARAPLHRRPTRDWQPHGTVLITG  
GTGALGSHAARWMAMHGAEHLLVRSRSGEQAPGATQLTAAELTASGARVTIAACDVADPHAMRTLDAIPAETPLTA  
VVHTAGAPGGDPLDVTGPEDIARIILGAKTSGAEVLDLLRGTPLDVFVLYSSNAGVGSGSQGVYAAANAHLDALA  
ARRRARGETATSAWGLWAGDGMGRGADDAYWQRRGIRPMSPDRALDELAKALSHDETFVAVADVDWERFAPAFTV  
SRPSLLLLDGVPEARQALAAPVGAPAPGDAAVAPTQGQSSALAAITALPEPERRPALLLVRTHAAVLGHSSPDRVA  
PGRAFTELGFDSLTAVQLRNQLSTVGNRLPATTVDHPTPAALAAHLHEAYLAPAEPAPTDWEGRVRRAELPL  
DRLRDAGVLDVLRLTGLEPEPGSGGSDGGAADPGAEPEASIDDDAEALIRMALGPRNT

### 2<sup>nd</sup> polypeptide of updated Pik167, 2<sup>nd</sup> polypeptide of updated 2-polypeptide Pik127, and 2<sup>nd</sup> polypeptide of traditional 2-polypeptide Pik127, on pTM5

MTSSNEQLVDALRASLKENEELKESRRRADRRQEPMIAVGMSCRFAGGIRSPELDIWDAVAAGKDLVSEVPEERGW  
DIDSPLYDPVPGRKGTIVRNAAFLDDAAGFDAAFFGISPREALAMDPPQQRQLLEASWEVFERAGIDPASVRGTDVG  
VYVGCGYQDYAPDIRVAPEGTGGYVVTGNSSAVASGRIAYSGLLEGPAVTVDTACSSLVALHLALKGLRNGDCST  
ALVGGVAVLATPGAFIEFSSQQAMAADGRTKGFASAADGLWGEVGAVLLERLSDARRKGHRLVAVVRGSAINQD  
GASNGLTAPHGPSQQLHIRQALADARLTSSDVEDVEGHGTGTRLGDPIEAQALLATYQGQGRAPGQPLRLGTLKSNI  
GHTQAAASGVAGVIKMVQALRHGVLPKTLHVDEPTDQDVDSAGSVELLTEAVDWPERPGRLRRAVGSAGVGCGTNAH  
VVLEEAPAVEESPANEPPAGGGVWPWPSAKTSAALDAQIQGQLAAYAEDRTDVPAVAARALVDSRTAMEHRAAV  
GDSREALRDLRMPEGLVRGTVTDPGRVAFVFPQGTQWAGMGAELLDSSPEFAAAMAECETALSPYVWDLSLEAVV  
RQAPSAPTLDRDVVQPVTFAMVSLAKVWQHHGITPEAVIGHSGEIAAAYVAGALTDDAARVVTLRSKSIAAH  
LAGKGGMISLALSEEATQRRIENLHGLSIAAVNGPTATVSGDPTQIQLAQACEADGIRARIIPVDYASHSAHVE  
TIENELADVLAGLSPQTQVPFSTLEGTWITEPALDGGYWYRNLHRVGFAPAVETLATDEGFTHFIEVSAHPV  
TMTPDKVTGLATLRRREDGGQHRLTTSLAEAWANGLALDWASLPPATGALSPAVPDLPTYAFQHRSYWI  
SPAFTGPEAPAHTASGREAVAETGLAWGPGAEDLDEEGRRAVLAMVMRQAAVLRCDSPEEVVDPLREIGFDSLTA  
VDFRN RVNRLTGLQLPPTVVFEHPTVALAERISDEAERNWA  
VAEP  
SDHEQAE  
EEEKA  
AAPAGA  
RSGAD  
TGAGAG  
MFRA  
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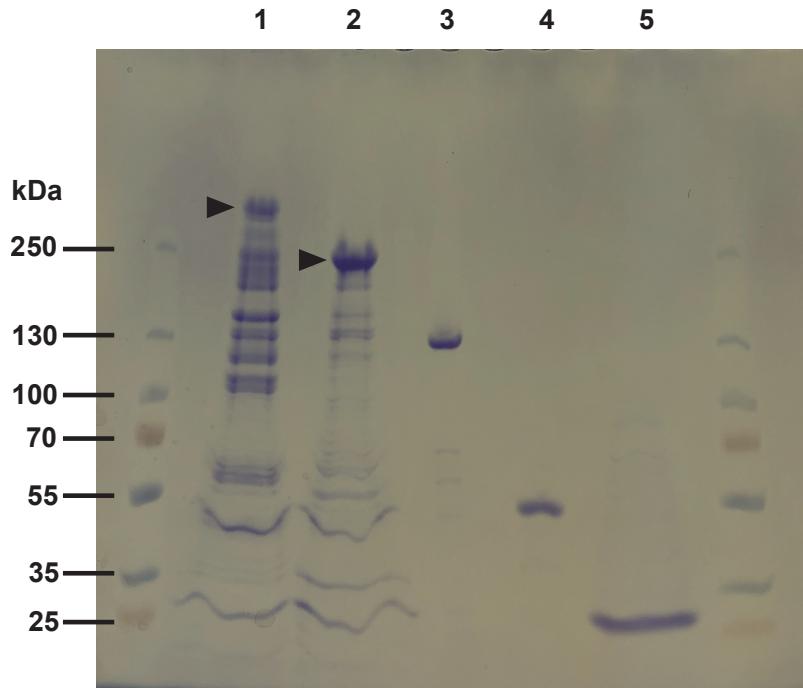
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## **Traditional 1-polypeptide Pik127, on pTM6**

MGSSHHHHHHHHSSGLVPRGSH**MSSAGITRTGARTPVTGRGAAAWDTGEVRVRRLGPLPPAGPDHAHSFSRAPTG**  
DVRAELIRGEMSTVKSEEEFVSVSNDAGSAHGTAEPVAVVGISCRVPGARDPREFWELLAAGGQAVTDVPADRW  
NAGDFYDPDRSAPGRSNRSWGGFIEDVDRFDAAFFGISPREAAEMDPQQLALELGWEALERAGIDPSSLTGTTRG  
VFAGAIWDDYATLKHQRGGAITPHTVTGLHRTIANRLSYTLGLRGPSMVVDSGQSSSLVAVHLACESLRRGESE  
LALAGGVSLNLVPDSIIGASKFGGLSPDGRAYTFDARANGYRGEFFFVVLKRLSRAVADGDPVLAVIRGSAVNN  
GGAAQGMTPDAQAQEAVLREAHERAGTAPADVRYVELHGTGTPVGDPIEAAALGAALGTGRPAGQPLLVGSVKTN  
IGHLEGAAGIAGLIKAFLAVRGRALPASLNYESPNPAIPFEELNLRVNTELYLPWEPEHDGQRMVGVSSFGMGGTN  
AHVVLEEAPGVVE**GASVVESTVGGSAVGGVWPWVSAKSAAALDAQIERLAAFASRDRTDGVDAGAVDAGAVDAG**  
AVARVLAGGRAQFEHRAVVVGSGPDDLAALAAPEGLVRGVASGVGRVAFFPGQGTQWAGMGAELLDSSAVFAAA  
MAECEAALSPYVWDWSLEAVVRQAPGAPTLERVDVVPQPTFAMVSLARVWQHHGVTQAVVGHSGQEIAAAYVAGA  
LSLDDAARVVTLSKSIAAHLAGKGGMISLALSEDADLERAFLAGFDGLSVAAVNGPTATVVSQDPVQIEELARACEA  
DGVRARVI PVDYASHSRQVEII ESELAEVLAGLSPQAPRVPFSTLEGAWITEPVLDGGYWYRNLRHVGFAPEAVE  
TLATDEGFTHFVEVSAHPVLTMALPGTVGLATLRRDNGGQDRLVASIAEAWANGLAVDWSPLLPSATGHHSDLPT  
YAFQTERHWLGEI EALAPAGEPAVQPAVLTEAAEPAELDRDEQLRVIDLKVRQAQVQLGYATGGQIEVDRTFRE  
AGCTSLTVVDLNRINAAFGVRMAPSMIFDFPTPEALAEQLLLHVGEAAAANPAGAEPAVAAAAGAVDEPVAIVGM  
ACRLPGGVASPEDILWRLVAGGGDAISEFPQDREGWDVEGLYHPDPEHPGTSYVRQGGFIENVAGFDAAFFGISPREA  
LAMDPQQLLLETSWEAVEDAGIDPTSLRGRQVGFTGAMTHEYGPSLRDGGEGLGYLLTGNTASVMSGRVSYTL  
GLEGPALTVDTACSSLVALHLAVQALRKGEVDMALAGGVAVMPTPGMFVEFSRQRLAGDGRSKAFAASADGTSW  
SEGVGVILVERLSDARRNGHQVLAVVRGSA**VNQDGASNGLTAPNGPSQQRVIRRALADARLTTSDVDVVEAHGTG**  
RLGDPPIEAQALIATYQGQRDDEQPLRLGSLKSIGHTQAAAGVSGVIKMVQAMRHGLPKTLHVDEPSDQIDWSAG  
AVELLTEAVDWPEKQDGGLRRAAVSSFGISGTNAHVLEEAPVVVEGASVVEPSVGGSAVGGVTPWVVAKSAAA  
LDAQIERLAAFASRDRDTDADAGAVDAGAVAHVLADGRAQFEHRAVALGAGADDLVQALADPDGLIRGTASGVGRV  
AFVFPQGTQWAGMGAELLDSSAVFAAAMAECCEAALSPYVWDLSLEAVVRQAPGAPTLERVDVVPQPTFAMVSLAR  
VWQHHGVTQAVVGHSGEIAAAYVAGALPLDDAARVVTLSKSIAAHLAGKGGMISLALNEDAVLERLSDFDGLS  
VAAVNGPTATVSGDPVQIEELAQACKADGFRARIIPVDYASHSRQVEII ESELAVQLAGLSPQAPRVPFSTLEG  
TWITEPVLDGTYWYRNLRHVGFAPAIETLAVDEGFTHFVEVSAHPVLTMLPETVTGLTLLREQGGQERLVTS  
AEAWVNGLPVAWTSLLPATASRPGLPTYAFQAEYWLENTPAALATGDDWRYIDWKRLPAAEGSERTGLSGRWLA  
VTPEDHSAQAAAALVLTALDAGAKVEVLTAGADDDREALAARLTALTTGDFGTGVVSLDGLVPQVAWVQALGDAGI  
KAPLWSVTQGAVSVGRDLTPADPDRAMIWGLRVVALEHPERWAGLVDPQDAALAHLVTLSGATGEDQIAI  
RTTGLHARRLARAPLHGRPTRDWQPHGTVLITGGT GALGSHAARWMAHHGAEHLLLVSRSGEQAPGATQLTAELT  
ASGARVTIAACDVADPHAMRTLLDAIPAETPLTAVVHTAGALDDGIVDILTAEQVRRAHRAKAVGASVLDELTRDL  
DLDASFVLFSSVSSTLGI PGQNYAPHNAYLDALAARRRATGRSAVSVAWGPWDGGMAAGDVAERLRNHGVPGMD  
PELALAALESALGDETAITVADIDWDRFYLAYSSGRPQPLVEELPEVRIIDARDSATSGQGGSSAQGANPLAER  
LAAAAPGERTEILLGLVRAQAAVLRMRSPEDVAADRAFKDIGFDLSAGVELRNRLTRATGLQLPATLVDHPTPL  
ALVSLLRSEFLGDEETADARRSAALPATVGAGAGAGT DADDEPMAIVGMSCRFAGGIRSPEDLWDAVAAGKDLV  
SEVPEERGWIDDSLYDPVPGRKGTTYVRNAAFLDDAAGFDAAFFGISPREALAMDQQRQLLEASWEVFERAGIDP  
ASVRGTDGVYVGCGYQDYAPDIRVAPEGTGGYVVTGNSSAVASGRIAYSLGLEPAVTVDTACSSLVALHLALK  
GLRNGDCSTALVGGAVLATPGAFIESSQQAMAADRTKGFASAADGLAWGEGVAVLLERLSDARRKGHRVLA  
VRGSAINQDGASNGLTAPHGPSQQLI RQALADARLTSSDVEDVVEGHGTGTRLGDPIEAQALLATYQGGRAPGQPL  
RLGTLKSNIGHTQAAASGVAGVIKMVQALRHGVLPKTLHVDEPTQVDWSAGSVELLTEAVDWPERPGRLLRAGVSA  
FGVGGTNAHVLEEAPAVEESPAVEPPAGGGVWPWVSAKTSALDAQIGQLAAYAEDRTDVPDAVAARALVDSRT  
AMEHRAVAVGDSREALRDALRMPGEGLVRGTVTDPGRAFVFPQGTQWAGMGAELLDSSPEFAAAMAECETALSPY  
VDWSLEAVVRQAPSAPTLDRVDVVPQPTFAMVSLAKVWQHHGITPEAVIGHSQGEIAAAYVAGALTDDAARVVT  
LSKSIAAHLAGKGGMISLALSEEATRQRIENLHGLSIAAVNGPTATVVSQDPTQIQELAQACEADGIRARIIPVD  
YASHSAHVENTIENELADVLAGLSPQTQVFFSTLEGWTIEPALDGGYWYRNLRHVGFAPEAVETLATDEGFTHF  
IEVSAHPVLTMTLPDKVTGLATLRRREDGGQHRLTTSLAEAWANGLDWASLLPATGALSPAVPDLPTYAFQHRSY  
WISPAGPGEAPAHTASGREAVAETGLAWGPGAEDLDEEGRRAVLAMVMRQAASVLCDSPEEVVPVDRPLREIGFD  
SLTAVDFRNVRNRLTGLQLPPTVVF**E**HPTPVALAERISDELAERNWAFAEPSDHEQAEEEKAAAPAGARSGADTG  
GAGMFRAFLRQAVEDDRYGEFLDVLAEEASAFRPQFASPEACSERLDPVLLAGGPTDRAEGRAVLVGCTGTAANGGP  
HEFLRLSTSFSQEEERDFLAVPLPGYGTGTGTALLPADLTDALDAQARAILRAAGDAPVVLGHSGGALLAHELAF  
RLERAHGAPPAGIVLVDPPYPPGHQEPIEVWSRQLGEGLFAGELEPMSDARLLAMGRYARFLAGPRPGRSSAPVLL  
RASEPLGDWQEERGDWRAHWDLPTHADVPGDHFTMMRDHAPAVAEAVLSWLDIAEGIEGAGKLEHHHHHHHHHH

**1<sup>st</sup> polypeptide of traditional 2-polypeptide Pik167, on pTM7**

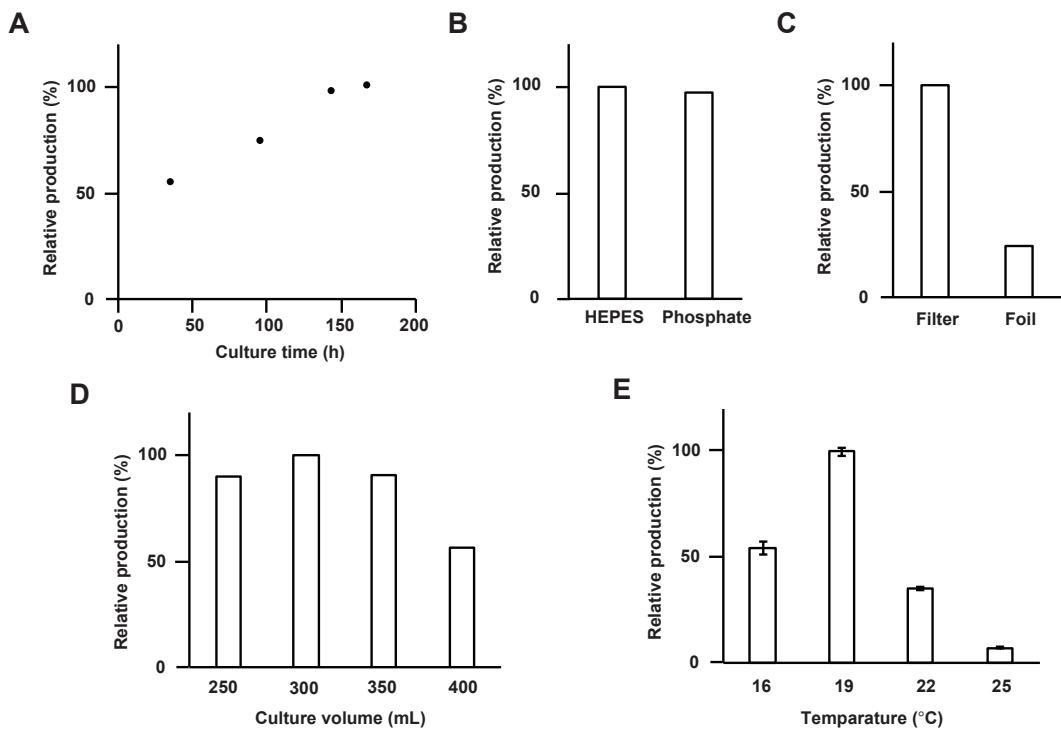
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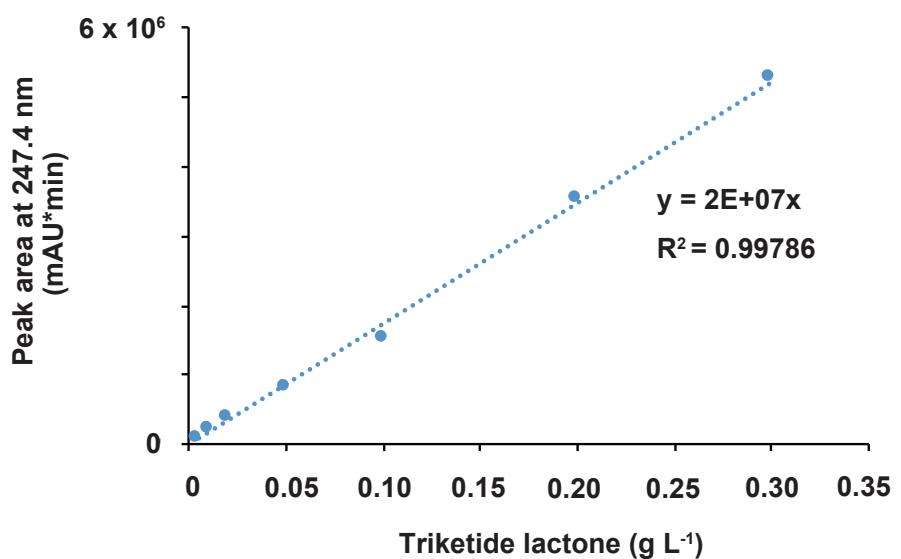
**Figure S1.** SDS-PAGE gel of nickel-NTA purified PKS polypeptides, MatB, and GDH.  
4-20% Tris-glycine gel (Thermo Fisher Scientific)

Lane 1: updated 1-polypeptide Pik127, 412 kDa  
Lane 2: 1<sup>st</sup> polypeptide of updated Pik167, 275 kDa  
Lane 3: 2<sup>nd</sup> polypeptide of updated Pik167, 143 kDa  
Lane 4 - MatB, 53 kDa  
Lane 5 - GDH, 30 kDa.

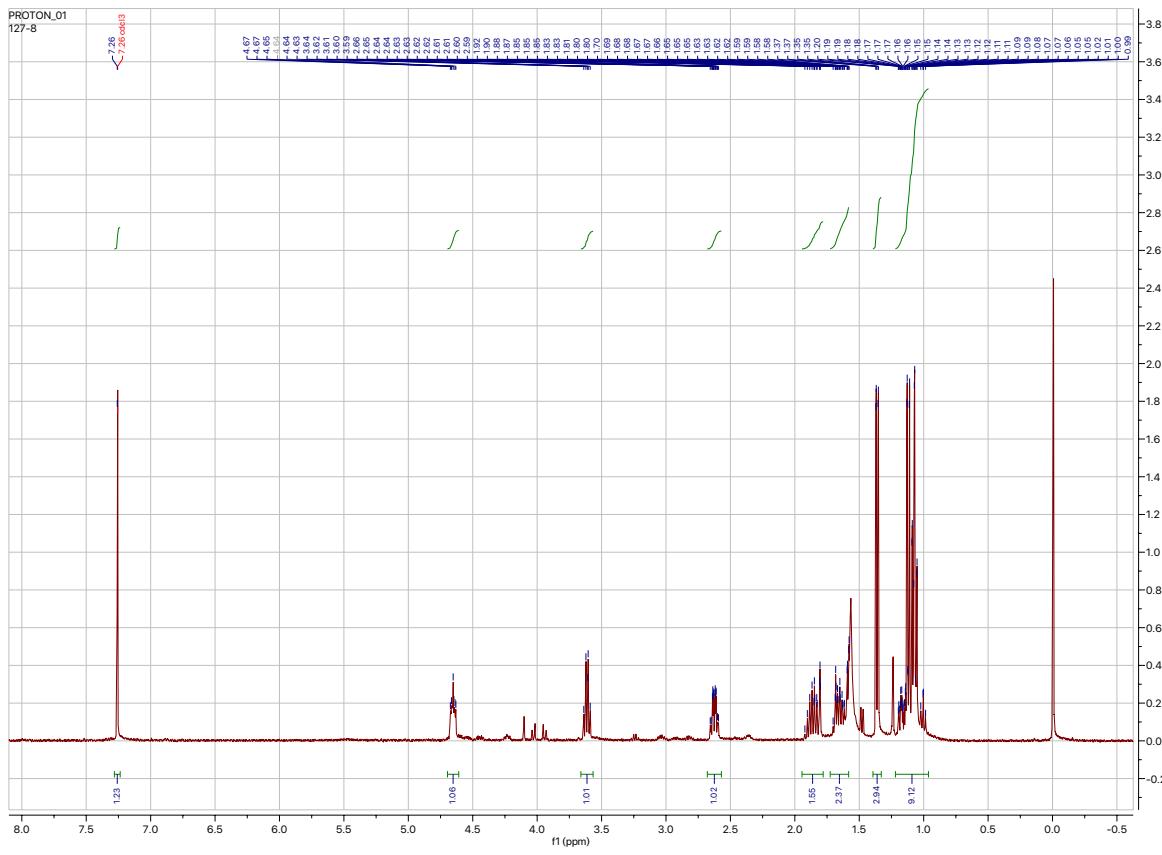
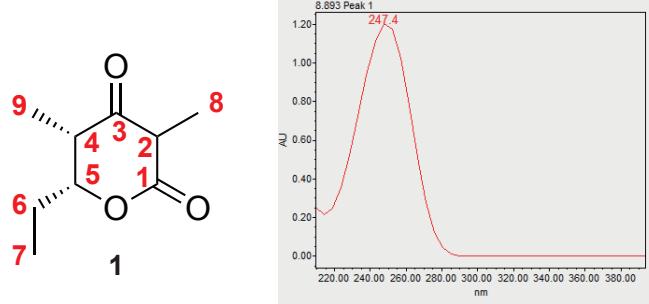
First and last lanes: PageRuler Plus Prestained Protein Ladder



**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 ( $\lambda=247$  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.

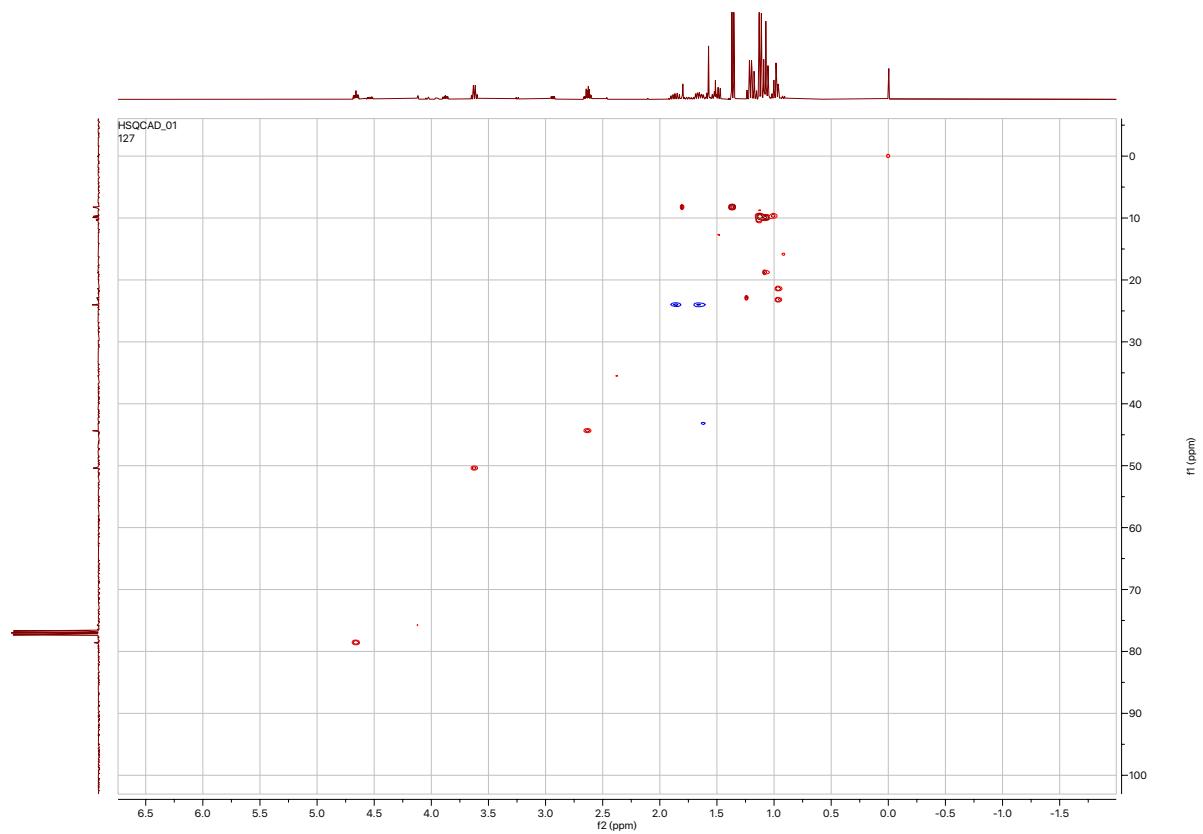


**Figure S3.** Triketide lactone calibration curve. Several concentrations of **1** dissolved in water (10  $\mu$ L) were analyzed by HPLC (conditions in Methods section).

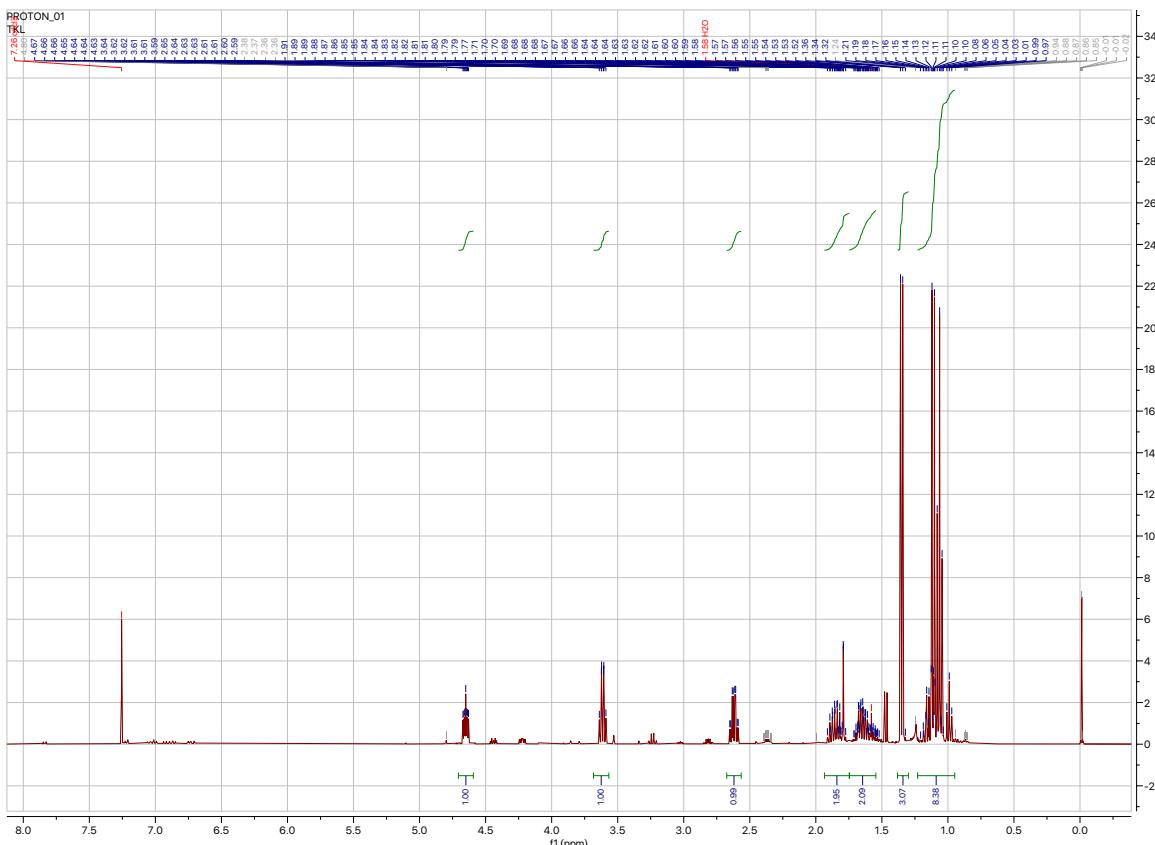
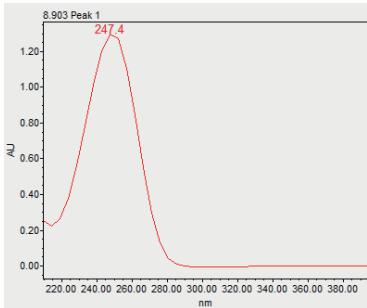
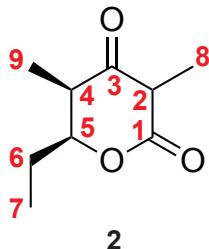


**Figure S4.**  $^1\text{H}$  NMR of **1** in  $\text{CDCl}_3$ .

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) 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<sub>9</sub>H<sub>14</sub>O<sub>3</sub> [M+Na]<sup>+</sup>, *m/z* 193.0835; found, *m/z* 193.0842. λ<sub>max</sub> = 247.4 nm. All characterization matched that from **1** in a previous study.<sup>6</sup>

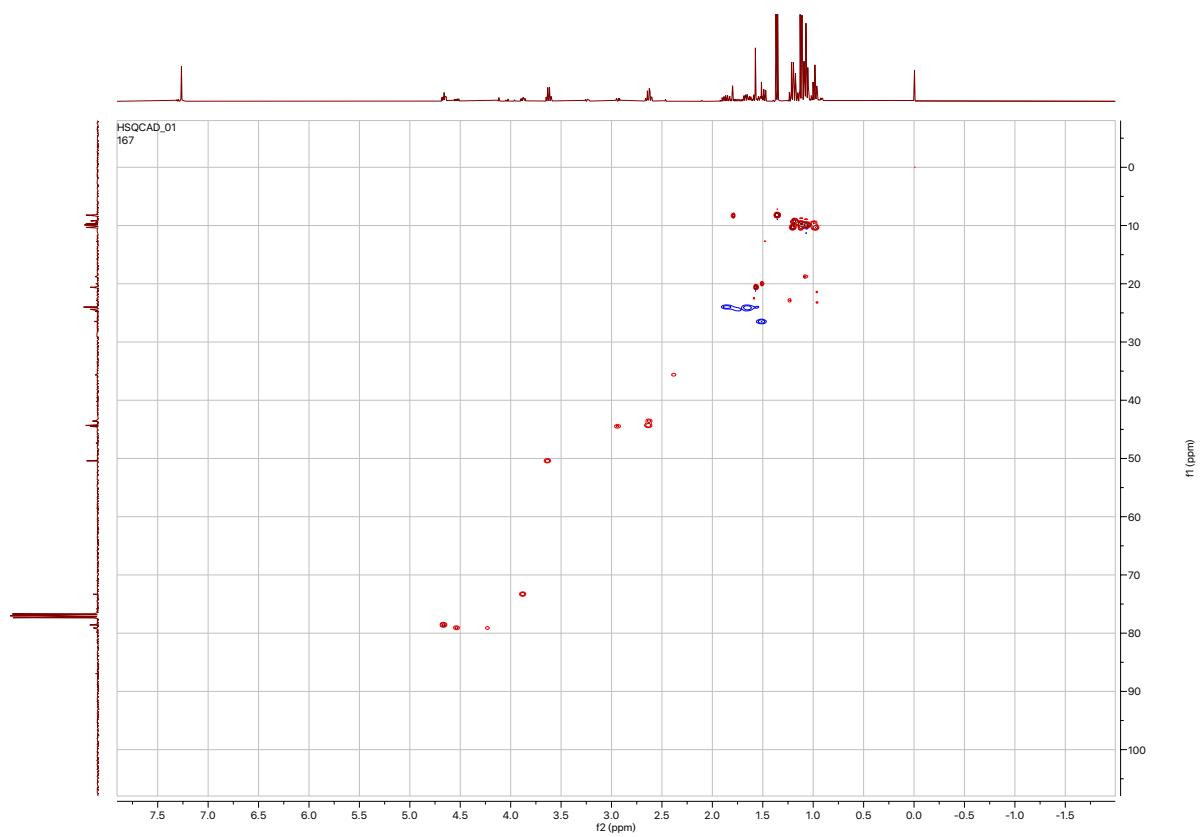


**Figure S5.**  $^1\text{H}$ - $^{13}\text{C}$ -HSQC of **1** in  $\text{CDCl}_3$ .

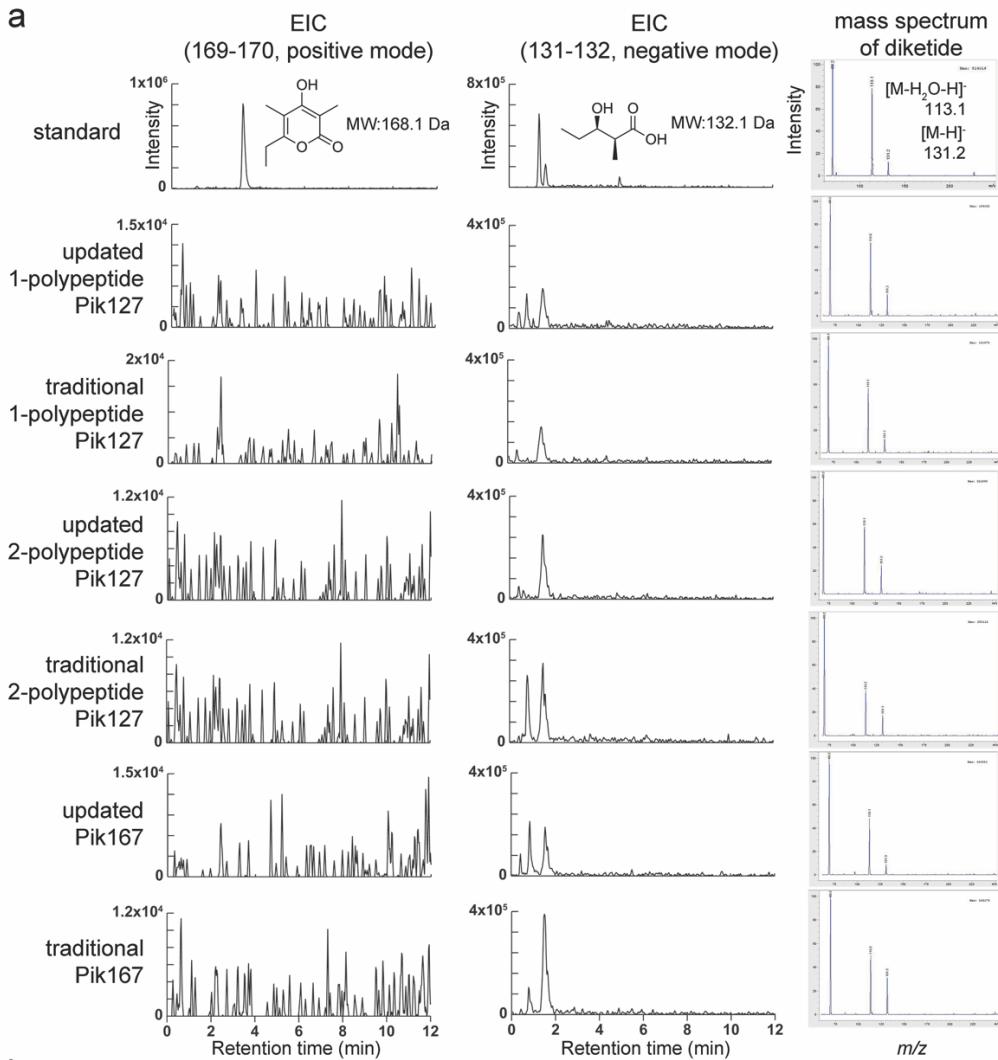


**Figure S6.**  $^1\text{H}$  NMR of **2** in  $\text{CDCl}_3$ .

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) 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<sub>9</sub>H<sub>14</sub>O<sub>3</sub> [M+Na]<sup>+</sup>, *m/z* 193.0835; found, *m/z* 193.0840. λ<sub>max</sub> = 247.4 nm. All characterization (except for chiral chromatography and crystallography) matched that of **1** from a previous study.<sup>6</sup>



**Figure S7.**  $^1\text{H}$ - $^{13}\text{C}$ -HSQC of **2** in  $\text{CDCl}_3$ .



**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<sub>18</sub> (2.1 × 50 mm) with a flow rate of 0.8 mL min<sup>-1</sup> (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<sup>4</sup>. 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 chromatography, a fraction containing **1** was crystallized in a glass vial.

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