Supporting Appendix for

Elucidating the mechanism of fluorinated extender unit loading for improved production of fluorine-containing polyketides

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Materials and Methods

Supplementary Results

Materials and Methods

Commercial materials. Luria-Bertani (LB) Broth Miller, LB Agar Miller, Terrific Broth (TB), and glycerol were purchased from EMD Biosciences (Darmstadt, Germany). Carbenicillin (Cb), isopropyl-β-D-thiogalactopyranoside (IPTG), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), sodium chloride, dithiothreitol (DTT), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), magnesium chloride hexahydrate, kanamycin (Km), acetonitrile, dichloromethane, ethyl acetate, ethylene diamine tetraacetic acid disodium dihydrate (EDTA), and restriction enzymes were purchased from Fisher Scientific (Pittsburgh, PA). Coenzyme A sodium salt (CoA), malonyl-CoA, methylmalonyl-CoA, diethylfluoromalonate, malonic acid, diethylmethylmalonate, tris(2-carboxyethyl)phosphine (TCEP) hydrochloride, phosphoenolpyruvate (PEP), adenosine triphosphate sodium salt (ATP), nicotinamide adenine dinucleotide reduced form dipotassium salt (NADH), nicotinamide adenine dinucleotide sodium salt hydrate (NAD^+), nicotinamide adenine dinucleotide phosphate sodium salt hydrate ($NADP^+$), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), myokinase, pyruvate kinase, lactate dehydrogenase, α-Ketoglutarate Dehydrogenase, poly(ethyleneimine) solution (PEI), β-mercaptoethanol, thiamine pyrophosphate, alpha ketoglutaric acid, sodium phosphate dibasic hepthydrate, ^{13}C-iodomethane, cysteamine, acetic anhydride, (1-^{13}C)propionic acid (3-^{13}C)sodium propionate, (2-^{13}C)sodium acetate, 1-ethyl-3-(-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 4-dimethylaminopyridine (DMAP), *o*-benzyl hydroxylamine, N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED), acetonitrile, dimethyl sulfoxide (DMSO), ammonium acetate, and ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid was purchased from Acros Organics (Morris Plains, NJ). Acrylamide/Bis-acrylamide (30%, 37.5:1), electrophoresis grade sodium dodecyl sulfate (SDS), Bio-Rad protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, CA). Restriction enzymes, T4 DNA ligase, Phusion DNA polymerase, amylose resin, and Taq DNA ligase were purchased from New England Biolabs (Ipswich, MA). Deoxynucleotides (dNTPs) were purchased from Invitrogen (Carlsbad, CA). PageRuler™ Plus prestained protein ladder was purchased from Fermentas (Glen Burnie, Maryland). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA), resuspended at a stock concentration of 100 μM in 10 mM Tris-HCl, pH 8.5, and stored at either 4°C for immediate use or -20°C for longer term use. DNA purification kits and Ni-NTA agarose were purchased from Qiagen (Valencia, CA). Complete EDTA-free protease inhibitor was purchased from Roche Applied Science (Penzberg, Germany). Amicon Ultra 3,000 MWCO, 10,000 MWCO centrifugal concentrators, and 30,000 MWCO centrifugal concentrators were purchased from EMD Millipore (Billerica, MA). Deuterium oxide, DMSO-*d*$_6$, and chloroform-*d* were purchased from Cambridge Isotope Laboratories (Andover, MA). ^{19}F, ^{13}C, and ^{1}H NMR

spectra were collected at 25ºC on Bruker AV-600 spectrometers at the College of Chemistry NMR Facility at the University of California, Berkeley or on a Bruker Biospin 900 MHz spectrometer at the QB3 Central California 900 MHz NMR Facility NMR. Assignments were made based on literature president and reference spectra from authentic standards, where appropriate. High-resolution mass spectral analyses were carried out on a 6530 QTOF Accurate Mass spectrometer purchased from Agilent Technologies.

Bacterial strains. *E. coli* DH10B-T1^R and BL21(de3)T1^R were used for plasmid construction and heterologous protein expression, respectively. *E. coli* BAP1 (1) cells were used for heterologous expression of DEBS modules.

Gene and plasmid construction. Standard molecular biology techniques were used for plasmid construction. All PCR amplifications were done using Phusion High Fidelity DNA polymerase. For amplification of GC-rich sequences from *S. erythraea*, PCR reactions were supplemented with DMSO (10% *v/v*) using the standard buffer rather than GC buffer with primer annealing temperatures 6-8°C below the T_m with a maximum temperature of 72ºC. All constructs were verified by sequencing (Quintara Biosciences; Berkeley, CA).

pET21c-His$_6$-Mod3$_{TE}$ACP^0-His$_6$ and pET21c-His$_6$-Mod3$_{TE}$AT^{0}ACP^0-His$_6$ were constructed by amplification from pRGS34 (2) and pAYC136 (3), respectively. For pET21c-His$_6$-Mod3$_{TE}$ACP^0-His$_6$, the S1430A mutation (based on EryAII numbering) was introduced by amplifying pRGS34 with Mod3$_{TE}$ACP^0 F1/R1 and pAYC136 with Mod3$_{TE}$ACP^0 F2/R2 (*Table S1*), which contained a 60 bp overlap. The two PCR fragments where inserted into the BsiWI-EcoRI sites of pRGS34 using the Gibson Protocol. For pET21c-His$_6$-Mod3$_{TE}$AT^{0}ACP^0-His$_6$, the S1430A (based on EryAII numbering) mutation was introduced by amplifying pAYC136 with Mod3$_{TE}$ACP^0 F1/R1 and Mod3$_{TE}$ACP^0 B F2/R2 (*Table S1*), which contained a 60 bp overlap. The two PCR fragments where inserted into the BsiWI-EcoRI sites of pAYC136 using the Gibson protocol (4).

pET21c-KSAT^0-His$_6$ and pET21c-KS^{0}AT^0-His$_6$ and pET21c-KS^{0}AT-His$_6$ were constructed by amplification from pAYC02 (5). For pET21c-KSAT^0-His$_6$, the S651A (based on EryAII numbering) mutation was introduced by amplifying pAYC02 with KS^{0}AT^0 F1 and KS^{0}AT^0 R2 (*Table S1*). The PCR fragment was inserted into the NdeI-BsiWI sites of pAYC02 using the Gibson Protocol. For pET21c-KS^{0}AT-His$_6$, the C202A (based on EryAII numbering) mutation was introduced by amplifying pAYC02 with KS^{0}AT^0 F1/R1 and KS^{0}AT^0 F2/R2 (*Table S1*), which contained a 60 bp overlap. The two PCR fragments where inserted into the NdeI-BsiWI sites of pAYC2 using the Gibson protocol. For pET21c-KS^{0}AT^0-His$_6$, the S651A and C202A mutation

were introduced by amplifying pET21c-KS^{0}AT-His$_6$ with KS^{0}AT^0 F1 and KS^{0}AT^0 R2 (*Table S1*). The PCR fragment was inserted into the NdeI-BsiWI sites of pAYC02 using the Gibson Protocol (4).

pET28a-His$_6$-KSAT2 was constructed by amplification from pSV272-His$_6$-MBP-DEBS$_{Mod2}$ using the primers pET28a KSAT2 F/R (*Table S1*). The fragment was insterted into the BsaI-BsaI site of pET28agg-RFP-NHis using the Golden Gate method (6).

pET28a-His$_6$-PIKSKR1 was constructed by ligating PIKS-KR1-block1 and PIKS-KR1-block3 into the BsaI-BsaI site of pET28agg-NHis using Golden Gate method (6). Amplification of PIKS-KR1-block2 with PIKS _KR1 F/R (in order to correct for an error in the initial design of the gBlock) enabled the ligation of the linear product of the Golden Gate reaction with the amplified DNA using the Gibson Protocol (4).

Expression of His-tagged and MBP-tagged proteins. TB (1 L) in a 2.8 L Fernbach baffled shake flask was inoculated to OD_{600} = 0.05 with an overnight TB culture of freshly transformed *E. coli* containing the appropriate overexpression plasmid. The cultures were grown at 37°C at 200 rpm to OD_{600} = 0.6 to 0.8 at which point cultures were cooled on ice for 20 min, followed by induction of protein expression with 0.25 mM IPTG and overnight growth at 16°C. Cell pellets were harvested by centrifugation at 9,800 × *g* for 7 min at 4ºC and stored at -80ºC.

Purification of His$_6$-rpMatB, His$_6$-Epi,DszAT-His$_6$, and His$_6$-PiksKR1. Frozen cell pellets were thawed and resuspended at 5 mL/g cell paste with Buffer A (50 mM sodium phosphate, 300 mM sodium chloride, 20% (*v/v*) glycerol, 20 mM BME, pH 7.5) containing imidazole (20 mM). Complete EDTA-free protease inhibitor cocktail (Roche) was added to the lysis buffer before resuspension. The cell paste was homogenized before lysis by passage through a French Pressure cell (Thermo Scientific; Waltham, MA) at 14,000 psi. The lysate was centrifuged at 15,300 × *g* for 20 min at 4°C to separate the soluble and insoluble fractions. DNA was precipitated in the soluble fraction by addition of 0.015% (*w/v*) poly(ethyleneimine). The precipitated DNA was removed by centrifugation at 15,300 × *g* for 20 min at 4°C. The remaining soluble lysate was diluted three-fold with Buffer A containing imidazole (20 mM) and loaded onto a Ni-NTA agarose column (Qiagen, 1 mL resin/g cell paste) by gravity flow or on an ÄKTA purifier FPLC (2 mL/min; GE Healthcare; Piscataway, NJ). The column was washed with Buffer A until the eluate reached an $A_{280\ nm} < 0.05$ or was negative for protein content by Bradford assay (Bio-Rad).

His_6-Epi and His_6-MatB, and DszAT-His_6. His_6-Epi, His_6-MatB, and DszAT-His_6 were eluted using a linear gradient from 0 to 300 mM imidazole in Buffer A over 30 column volumes.

His_6-PIKS KR1. The column was washed with 5 to 10 column volumes of Buffer A supplemented with 20 mM imidazole. The protein was then eluted with 250 mM imidazole in Buffer A.

Fractions containing the target protein were pooled by $A_{280\ nm}$ and concentrated using either an Amicon Ultra spin concentrator (3 kDa MWCO, Millipore) or an Amicon ultrafiltration cell under nitrogen flow (65 psi) using a membrane with an appropriate nominal molecular weight cutoff (Ultracel-5 or YM10, Millipore). Protein was then exchanged into Buffer C (50 mM HEPES, 100 mM sodium chloride, 2.5 mM EDTA, 2.5 mM DTT, 20% (*v/v*) glycerol, pH 7.5) using a Sephadex G-25 column (Sigma-Aldrich, bead size 50-150 μm, 10 mL resin/mL protein solution), then concentrated again before storage.

Final protein concentrations before storage were estimated using the $\varepsilon_{280\ nm}$ calculated by ExPASY ProtParam as follows: His_6-MatB: 34.2 mg/mL ($\varepsilon_{280\ nm}$ = 40,340 $M^{-1}\ cm^{-1}$), His_6-Epi: 10.5 mg/mL ($\varepsilon_{280\ nm}$ = 11,460 $M^{-1}\ cm^{-1}$), DszAT-His_6: 13.3 mg/mL ($\varepsilon_{280\ nm}$ = 17,420 $M^{-1}\ cm^{-1}$), His_6-$PIKS_{Mod1}$ KR: 15.4 mg/mL ($\varepsilon_{280\ nm}$ = 73,450 $M^{-1}\ cm^{-1}$) All proteins were aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C.

Purification of His_6-KSAT2, KSAT3-His_6, $KSAT3^0$-His_6, KS^0AT3-His_6, KS^0AT3^0-His_6 and KSAT6-His_6. The KSAT didomains were heterologously expressed in *E. coli* BL21(de3) $T1^R$ as described above. Frozen cell pellets were thawed and resuspended at 5 mL/g cell paste with Buffer A containing imidazole (10 mM). Complete EDTA-free protease inhibitor cocktail (Roche) was added to the lysis buffer before resuspension. The cell paste was homogenized before lysis by passage through a French Pressure cell (Thermo Scientific; Waltham, MA) at 14,000 psi. The lysate was centrifuged at 15,300 × *g* for 20 min at 4°C to separate the soluble and insoluble fractions. DNA was precipitated in the soluble fraction by addition of 0.015% (w/v) poly(ethyleneimine). The precipitated DNA was removed by centrifugation at 15,300 × *g* for 20 min at 4°C. The remaining soluble lysate was diluted three-fold with Buffer B containing 10 mM imidazole and loaded onto a Ni-NTA agarose column (Qiagen, 1 mL resin/g cell paste) by gravity flow. The column was washed with Buffer A until the eluate reached an $A_{280\ nm} < 0.05$ or was negative for protein content by Bradford assay (Bio-Rad). The column was washed with 20 column volumes with Buffer A supplemented with 25 mM imidazole. The protein was then eluted with Buffer D (50 mM sodium phosphate, 50 mM sodium chloride, 20% (*v/v*) glycerol, 20 mM BME, pH 7.5) containing 250 mM Imidazole. Fractions containing the target protein were pooled by A_{280}

$_{nm}$ and concentrated using an Amicon Ultra spin concentrator (30 kDa MWCO, Millipore). Protein was then exchanged into Buffer C (50 mM HEPES, 100 mM sodium chloride, 2.5 mM EDTA, 2.5 mM DTT, 20% (*v/v*) glycerol, pH 7.5) using a Sephadex G-25 column (Sigma-Aldrich, bead size 50-150 μm, 10 mL resin/mL protein solution), then concentrated again before storage. Final protein concentrations before storage were estimated using the $\varepsilon_{280\ nm}$ calculated by ExPASY ProtParam as follows: His_6-KSAT2: $\varepsilon_{280\ nm}$ = 93,390 M^{-1} cm^{-1}, KSAT3-His_6: $\varepsilon_{280\ nm}$ = 92,360 M^{-1} cm^{-1}) KSAT6-His_6: $\varepsilon_{280\ nm}$ = 71,390 M^{-1} cm^{-1}. All proteins were aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C with a final concentration of 10-30 mg/mL.

Purification of $Mod3_{TE}$-His_6, AT^0 $Mod3_{TE}$-His_6, AT^0ACP^0 $Mod3_{TE}$-His_6, and ACP^0 $Mod3_{TE}$-His_6, $Mod6_{TE}$-His_6, and AT^0 $Mod6_{TE}$-His_6. The His-tagged DEBS modules with thioesterase constructs were heterologously expressed in *E. coli* BAP1 as described above. Cleared cell lysates were prepared in Buffer B (200 mM sodium phosphate, 200 mM sodium chloride, 30% (*v/v*) glycerol, 2.5 mM EDTA, 2.5 mM DTT, pH 7.5) as described above, diluted three-fold with Buffer A, and passed over a Ni-NTA agarose column (Qiagen, approximately 1 mL/g cell paste) on an ÄKTApurifier FPLC. The column was washed with Buffer A until the eluate reached an $A_{280\ nm}$ < 0.05. Protein was eluted with Buffer D containing 100 mM imidazole. The eluate was diluted two-fold with Buffer E (50 mM HEPES, 2.5 mM EDTA, 2.5 mM DTT, 20% glycerol, pH 7.5), loaded onto a HiTrap Q HP column (GE Healthcare, 5 mL), and eluted with a linear gradient from 0 to 1 M sodium chloride in Buffer E over 30 column volumes (5 mL/min). Fractions containing the target protein (eluted at ~350 mM sodium chloride) were pooled by $A_{280\ nm}$ and concentrated with an Amicon Ultra 30,000 MWCO centrifugal concentrator. The protein was flash-frozen in liquid nitrogen and stored at -80°C at a final concentration of 20-30 mg/mL, which was estimated using the calculated $\varepsilon_{280\ nm}$ ($Mod3_{TE}$-His_6 constructs: 203,280 $M^{-1}cm^{-1}$; $Mod6_{TE}$-His_6 constructs: 206,260 M^{-1} cm^{-1}).

Purification of His_6-MBP-Mod2 and His_6-MBP-AT^0 Mod2. Cleared lysates were prepared as described for other DEBS modules with the exception buffer A being supplemented with Buffer F (20 mM TRIS-HCl, 300 mM Sodium Chloride, and 1 mM DTT, 20% (*v/v*) glycerol, pH 7.5). The clear lysate was diluted three-fold with Buffer F and loaded by gravity onto Amylose resin (2 mL/g cell paste) into a fritted column and washed with Buffer F until the eluate reached $A_{280\ nm}$ < 0.05. The protein was eluted with Buffer F containing 10 mM maltose and concentrated to ~1 mg/mL using an Amicon Ultra 30,000 MWCO centrifugal concentrator. The protein was then dialyzed overnight against Buffer E containing 50 mM NaCl with TEV protease (1 mg/50 mg protein substrate) to remove the MBP tag. The protein was loaded onto a HiTrap Q HP column and eluted by a linear gradient from 0 to 1 M NaCl in Buffer E over 30 column volumes. Fractions containing

the desired protein were identified by SDS-PAGE (eluting at ~350 mM NaCl), pooled, and concentrated in an Amicon Ultra 30,000 MWCO centrifugal concentrators. Protein aliquots were flash-frozen in liquid nitrogen and stored at -80°C at a final concentration of 8-12 mg/mL, which was estimated using the calculated $\varepsilon_{280\ nm}$ (158,360 $M^{-1}cm^{-1}$).

Fluoromalonate and methylmalonate. Fluoromalonate and methylonate were prepared as described previously (7). Diethylfluoromalonate and diethylmethylmalonate (0.5 mL, 3.2 mmol) were saponified with methanolic sodium hydroxide (2 M, 3.5 mL) in dichloromethane and methanol (9:1 v/v, 32 mL) and the sodium salt isolated by filtration through a Büchner funnel with a fine porosity glass frit (8).

Fluoromalonyl-CoA. Fluoromalonyl-CoA was prepared enzymatically from fluoromalonate and CoA using MatB and ATP as described previously (7). The reaction mixture (10 mL) contained 100 mM sodium phosphate, pH 7.5, phosphoenolpyruvate (5 mM), TCEP (2.5 mM), magnesium chloride (5 mM), fluoromalonate (10 mM), ATP (2.5 mM), pyruvate kinase/lactate dehydrogenase (36 U), myokinase (20 U), CoA (2 mM) and MatB (10 μM). The mixture was incubated at 37°C for 2 h and lyophilized overnight. The residue was dissolved in water (1.6 mL) and acidified to pH ~2 by addition of 70% (v/v) perchloric acid (160 μL). Insoluble material was removed by centrifugation at 18,000 × *g* for 10 min. The supernatant was adjusted to pH 6 by addition of 10 M sodium hydroxide (100 μL) and desalted on an Agilent 1200 HPLC system using a Zorbax Eclipse XDB C-18 column (5 μm, 9.4 × 250 mm, Agilent) with a linear gradient from 0 to 10% methanol over 9 min with 50 mM sodium phosphate, 25 mM trifluoroacetic acid, pH 4.5 as the aqueous mobile phase (3 mL/min). Fractions eluting near the void volume, containing both fluoromalonyl-CoA and CoA, were lyophilized overnight, dissolved in water (1 mL), and purified using a Zorbax Eclipse XDB C-18 column (5 μm, 9.4 × 250 mm) with a linear gradient from 0 to 10% acetonitrile over 30 min with 0.1% formic acid as the aqueous mobile phase (3 mL/min). Fractions were screened by ESI-MS and those containing pure fluoromalonyl-CoA were lyophilized overnight. The fluoromalonyl-CoA solutions were stored at -80°C.

ESI-MS screening method for fluoromalonyl-CoA. Fractions were screened as described previously (7). Preparative HPLC fractions were screened on an Agilent 1260 HPLC system using a Kinetex 50 XB-C18 column (5 μm, 5.0 × 30 mm, Phenomenex) with a linear gradient from 0 to 100% acetonitrile over 3 min with 0.1% formic acid as the aqueous mobile phase (0.8 mL/min). Mass spectra were collected on an Agilent 6130 single quadrupole MS with ESI source, operating in negative and positive ion scan mode (7).

N-acetylcysteamine (SNAC). Cysteamine (2 g, 13 mmol) was added to a 250 mL Erlenmeyer flask containing 100 mL of water. Sodium bicarbonate (4.4 g, 5.2 mmol) and potassium hydroxide (~988 mg, 1 pellet) were added and the solution was left to stir at r.t for 5 min. Acetic anhydride (1.66 mL, 16.5 mmol) was added dropwise over the course of 2 min and the reaction was left stirring at r.t for 1 h. The solution was then adjusted to pH 7 with 6 M hydrochloric acid and the product was extracted with 3 × 200 mL of ethyl acetate. All organic washes were combined and dried with magnesium sulfate and solvent was removed by rotary evaporation to produce a clear oil at >99% yield. **^{1}H-NMR** (400 MHz, $CDCl_3$ = 7.26 ppm)**:** δ 1.36 (t, 1H, 8.5 Hz), 2.00 (s, 3H), 2.62-2.70 (m, 2H), 3.40-3.43 (m, 2H), 6.10 (s, N-H). **^{13}C-NMR** (101 MHz, $CDCl_3$ = 77.36 ppm) δ 23.3, 24.6, 42.5, 170.7.

N-acetylcysteamine thioester of 2-methyl-3-oxopentanoic acid (oxoNDK-SNAC). The protocol was adapted from a previous study (9). 2-methyl-3-oxopentanoic acid ethyl ester (316 mg, 2 mmol) was saponified in 1 M sodium hydroxide at r.t for 2 h. The reaction was acidified to pH 1 and 2-methyl-3-oxopentanoic acid was extracted in 3 × 15 mL ethyl acetate. All organic washes were combined and dried with magnesium sulfate and solvent was removed by rotary evaporation. 2-methyl-3-oxopentanoic acid was then dissolved in 10 mL of dichloromethane in a 25 mL round bottom flask on ice. SNAC (310 mg, 2 mmol) and DMAP (40 mg, 0.3 mmol) where added and the reaction was allowed to stir for 5 min. EDC (422 mg, 2.2 mmol) was added and the reaction was stirred at room temperature for 24 h. The reaction was washed with 2 × 20 mL of saturated ammonium chloride. The aquous fractions were pooled and washed further with 3 × 20 mL ethyl acetate. All organic washes were combined and dried with magnesium sulfate and solvent was removed by rotary evaporation to produce a yellowish oil at 80% yield. **^{1}H-NMR** (400 MHz, $CDCl_3$ = 7.26 ppm)**:** δ 1.06 (t, 3H, 7.2 Hz), 1.38 (d, 3H, 7.1 Hz), 1.96 (s, 3H), 2.47-2.62 (m, 2H), 3.00-3.13 (m, 2H), 3.37-3.49 (m, 2H), 3.79 (q, 1H, 7.0 Hz), 5.89 (s, N-H). **ESI-MS $[M-H]^-$:** calculated for $C_{10}H_{17}NO_3S$, 230.1 *m/z*, found 230.1 *m/z*.

Enzymatic synthesis of the N-acetylcysteamine thioester of (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid (NDK-SNAC) using PIKS KR1. This protocol was adapted from a previous study (10). Oxo-NDK-SNAC was dissolved in DMSO (1 M, 10 mL total volume) and added to a solution containing 300 mM HEPES pH 7.5, 10% glycerol, sodium chloride (100 mM), D-glucose (300 mM), and $NADP^+$ (0.1 mM). The reaction was initiated by the addition of glucose-1-dehydrogenase (20 U/mL) and PIKS KR1 (15 μM) and the reaction was allowed to stir at r.t for 3 h. The reaction was quenched with 1 reaction volume of 5 M sodium chloride (5 mL) and the product was extracted with 3 × 20 mL ethyl acetate. The sample was loaded on a Biotage®SNAP cartridge KP-Sil 25 g column and run on a Isolera One by Biotage with ACI™ using a 0-10%

methanol gradient with DCM as the second mobile phase. The fractions containing the final product were pooled together and rotovapped to dryness to yield the final product as a clear oil at 70% yield. **^{1}H-NMR** (600 MHz, $CDCl_3$ = 7.26 ppm)**:** δ 0.96 (t, 3H, 7.4 Hz), 1.20 (d, 3H, 7.0 Hz), 1.39-1.56 (m, 2H), 1.96 (s, 3H), 2.50 (s, 1H, O-H), 2.73 (qd, 1H, 7.1 and 3.7 Hz), 2.96-3.08 (m, 2H), 3.38-3.50 (m, 2H), 3.83 (dq, 1H, 8.4 and 4.3 Hz), 5.91 (s, 1H, N-H). **^{13}C-NMR** (151 MHz, $CDCl_3$ = 77.36 ppm)**:** δ 10.7, 11.4, 23.5 27.5, 28.9, 39.7, 53.3, 74.0, 170.6, 204.5. **ESI-MS [M-H]$^+$:** calculated for $C_{10}H_{19}NO_3S$, 234.1158 *m/z*, found 234.1157 *m/z*.

N-acetylcystamine thioester of propionic acid (Propionyl-SNAC). Propionic acid (150 mg, 2 mmol) was dissolved in 10 mL of dichloromethane on ice in a 25 mL round-bottom flask. SNAC (310 mg, 2 mmol) and DMAP (40 mg, 0.3 mmol) where added and the reaction was allowed to stir for 5 min. EDC (422 mg, 2.2 mmol) was added and the reaction was left stirring and was allowed to come to room temperature overnight. The reaction was washed with 2x20 mL of water. The water layers were washed further with 3 × 20 mL ethyl acetate. All organic washes were combined and dried with magnesium sulfate. After filtration, solvent was removed by rotary evaporation to produce a yellow oil at 10% yield. **^{1}H-NMR** (600 MHz, $CDCl_3$ = 7.26 ppm)**:** δ 1.14 (t, 3H, 7.7 Hz), 1.91 (s, 3H), 2.55 (q, 2H, 7.5 Hz), 2.98 (t, 2H, 6.6 Hz), 3.37 (q, 2H, 6.4 Hz), 6.21 (s, N-H). **^{13}C-NMR** (226 MHz, $CDCl_3$ = 77.36 ppm) δ 9.9, 23.4, 28.6, 37.7, 39.9, 170.7, 201.0. **ESI-MS [M-H]$^+$:** calculated for $C_7H_{13}NO_2S$, 176.0740 *m/z*, found 176.0739 *m/z*. (1-^{13}C)propionyl-SNAC was synthesized in the using the same protocol as above and was confirmed by ^{13}C NMR and **ESI-MS [M-H]$^+$:** calculated for $C_7H_{13}NO_2S$, 177.0774 *m/z*, found 177.0773 *m/z*.

N-acetylcystamine thioester of [3-^{13}C]-propionic acid (Propionyl-SNAC). [3-^{13}C]-sodium propionate (96 mg, 1 mmol) was dissolved in 10 mL of acetone on ice in a 25 mL round-bottom flask. SNAC (155 mg, 1 mmol) and DMAP (10 mg, 0.1 mmol) were added and the reaction was allowed to stir for 5 min. EDC (230 mg, 1.2 mmol) was added and the reaction was removed from ice and was left stirring at r.t for 24 h. The reaction mixture was partitioned with 30 mL of water and was washed with 2 × 20 mL of DCM. The combined organic washes were washed with 20 mL brine and dried with magnesium sulfate. After filtration, solvent was removed by rotary evaporation to produce a cloudy yellow oil. The oil was dissolved in ethyl acetate and was run over a 100 mL silica column with ethyl acetate as the mobile phase. The fractions containing the desired product were combined and solvent was removed by rotary evaporation. The final product was a yellow oil obtained at 15% yield. **^{1}H-NMR** (400 MHz, $CDCl_3$ = 7.26 ppm)**:** δ 1.01 and 1.33 (dt, 3H, J_{HH} = 7.5 Hz and J_{CH} = 129 Hz), 1.96 (s, 3H), 2.59 (qd, 2H, 7.5 Hz and 4.6 Hz), 3.01 (t, 2H, 6.4 Hz), 3.42 (q, 2H, 6.2 Hz), 5.92 (s, N-H). **^{13}C-NMR** (101 MHz, $CDCl_3$ = 77.36 ppm) δ 9.7,

23.2, 28.4, 37.3 and 37.6 (*Jcc* = 34), 39.7, 170.3, 200.9. **ESI-MS [M-H]$^+$:** calculated for $C_7H_{13}NO_2S$, 177.0774 *m/z*, found 177.0772 *m/z*. For absolute quantification, an ^{1}H NMR (in D_2O with a vanillin standard) was obtained for a 84 mM stock solution of propionyl-SNAC.

[4-^{13}C]-diethylmethylmalonate. The protocol was adapted from a previous study (11). Sodium metal (Na^0, 200 mg, 8.7 mmol) was added to 15 mL of ethanol (on ice, in a 50 mL round bottom flask) over the course of 45 min. After 20 min, diethylmalonate (1.1 g, 6.67 mmol) was added and the sample was removed from the ice. The sample was put under nitrogen and ^{13}C-iodomethane (0.55 mL, 10 mmol) was added. The reaction was left stirring at r.t overnight. The sample was rotovapped to dryness and the precipitate was dissolved in 20 mL of saturated ammonium bicarbonate. The product was extracted with 3 × 40 mL washes of diethyl ether and rotovapped. The sample was loaded on a Biotage® SNAP cartridge KP-Sil 10g column and run on a Biotage Isolera One with ACI™ using a 0-25% ethyl acetate gradient with hexanes as the second mobile phase. The fractions containing the product were screened using an iodine chamber. The fractions containing the final product were pooled together and rotovapped to dryness to yield the final product at 15% yield. **^{1}H-NMR** (400 MHz, $CDCl_3$ = 7.26 ppm)**:** δ 1.20, 1.53 (dd, 3H, 7.4 Hz (J_{HH}), 131 Hz (J_{CH})), 1.23 (t, 6H, 7.16 Hz), 3.32-3.44 (m, 1H), 4.08-4.24 (m, 4H). **^{13}C-NMR** (101 MHz, $CDCl_3$ = 77.36 ppm) δ 13.7, 23.0, 46.23, 46.6, 61.6, 170.4.

Enzymatic synthesis of [4-^{13}C]-methylmalonyl-CoA. [4-^{13}C]-methylmalonate was generated by saponification of [4-^{13}C]-diethylmethylmalonate and used for the enzymatic preparation of [4-^{13}C]-methylmalonyl-CoA as described for fluoromalonyl-CoA. The reaction mixture (10 mL) contained 100 mM sodium phosphate, pH 7.5, phosphoenolpyruvate (5 mM), TCEP (2.5 mM), magnesium chloride (5 mM), [4-^{13}C]-diethylmethylmalonate (7 mM), ATP (2.5 mM), pyruvate kinase/lactate dehydrogenase (36 U), myokinase (20 U), CoA (2 mM) and MatB (10 µM). The mixture was incubated at 37°C for 1 h and lyophilized overnight. The residue was dissolved in water (1.6 mL) and acidified to pH ~2 by addition of 70% (*v/v*) perchloric acid (160 µL). Insoluble material was removed by centrifugation at 18,000 × *g* for 10 min. The supernatant was adjusted to pH 6 by addition of 10 M sodium hydroxide (100 µL) and desalted on an Agilent 1200 HPLC system using a Zorbax Eclipse XDB C-18 column (5 µm, 9.4 × 250 mm, Agilent) with a linear gradient from 0 to 10% acetonitrile over 30 min with 0.1% formic acid as the aqueous mobile phase (3 mL/min). Fractions were screened by ESI-MS and those containing pure [4-^{13}C]-methylmalonyl-CoA were lyophilized overnight. **^{1}H-NMR** (900 MHz, D_2O =4.79 ppm)**:** δ 0.80 (s, 3H), 0.92 (s, 3H), 1.26 and 1.41 (d, 3H, 131 Hz (J_{CH})), 2.40 (t, 2H, 6.7 Hz), 3.01-3.08 (m, 2H), 3.34 (t, 2H, 6.2 Hz), 3.43 (td, 2H, 6.6 Hz and 2.6 Hz), 3.61 (dd, 1H, 9.8 Hz and 4.5 Hz), 3.86 (dd, 1H, 9.7 Hz and 4.5 Hz), 4.0 (s, 1H), 4.23-4.30 (m. 2H), 4.58 (dt, 1H, 2.4 and 2.6 Hz), 4.83-4.85

(m, 1H), 4.85-4.88 (m, 1H), 6.18 (d, 1H, 6.0 Hz), 8.41 (s, 1H), 8.63 (s, 1H). **^{13}C-NMR** (226 MHz, D_2O) δ 16.2, 21.0, 23.5, 31.1, 38.0, 41.0, 41.1, 56.2, 67.8, 74.7, 76.7, 76.8, 76.9, 76.9, 86.2, 90.2, 121.2, 145.2, 147.5, 151.2, 152.5, 176.1, 176.7, 177.5, 202.7. **ESI-MS [M-H]$^+$:** calculated for $C_{25}H_{40}N_7O_{19}P_3S$, 869.1424 *m/z*, found 869.1419 *m/z*.

2-propanone *O*-benzyl oxime. *O*-benzylhydroxylamine hydrochloride (199 mg, 1 mmol) was placed in a 10 mL scintillation vial and was dissolved in 10 mL of 50 mM sodium phosphate at pH 4.5. After 5 min, acetone (730 µL, 10 mmol) was added and the reaction was left stirring at r.t for 72 h. The reaction was washed with 3 × 20 mL DCM, dried with magnesium sulfate, filtered, and solvent was removed by rotatory evaporation resulting in a clear liquid at 90% yield. **^{1}H-NMR** (400 MHz, $CDCl_3$ = 7.26 ppm)**:** δ 1.83 (s, 3H), 1.90 (s, 3H), 5.08, (s, 2H), 7.25-7.41 (m, 5H). **^{13}C-NMR** (101 MHz, $CDCl_3$ = 77.36 ppm) δ 16.1, 22.2, 75.6, 127.9, 128.2, 128.6, 138.6, 155.6. **ESI-MS [M-H]$^+$:** calculated for $C_{10}H_{13}NO$, 164.1070 *m/z*, found 164.1069 *m/z*. For absolute quantification, an ^{1}H NMR spectra (in D_2O with a vanillin standard) was obtained for a 604 mM stock solution of 2-propanone *O*-benzyl oxime in DMSO.

Triketide lactone production using Mod3$_{TE}$, KSAT3, Mod6$_{TE}$, and KSAT constructs. All assay mixtures contained 400 mM sodium phosphate, pH 7.5, phosphoenolpyruvate (50 mM), TCEP (5 mM), magnesium chloride (10 mM), ATP (2.5 mM), pyruvate kinase/lactate dehydrogenase (15 U/mL), myokinase (10 U/mL), methylmalonyl-CoA epimerase (5 µM), CoA (1 mM), MatB (20 µM), methyl- or fluoromalonate (5 mM). The mixture was incubated at 37ºC for 30-45 min and initiated by the addition of NDK-SNAC (5 mM) and DEBS protein (10 µM). When used, DszAT (30 µM) was also added to the reaction mixture. The reaction was incubated at 37°C for 24 h (Mod3) and 16 h (Mod6). 50 µL aliquots were removed and quenched with 70% (*v/v*) perchloric acid (2.5 µL). Samples were centrifuged at 18,000 × *g* for 10 min at r.t to pellet the precipitated protein. The supernatant (50 µL) was removed and flash frozen. Excess salts were removed by centrifugation at 18,000 × *g* for 5 min at r.t. The supernatant was removed and analyzed on a Zorbax Eclipse XDB C-18 column (3.5 µm, 3 × 150 mm, 35ºC, Agilent) using a linear gradient from 0 to 40% acetonitrile over 14 min with 0.1% formic acid as the aqueous mobile phase after an initial hold at 0% acetonitrile for 30 s (0.8 mL/min). Products were monitored using an agilent G1315D diode array detector (TKL, $A_{260\,nm}$; F-TKL, $A_{247\,nm}$). For absolute quantification, standard curve were generated using F-TKL and TKL standards (7).

Initial rate of triketide lactone production using Mod3$_{TE}$ and KSAT3 constructs. All assay mixtures contained 400 mM sodium phosphate, pH 7.5, phosphoenolpyruvate (50 mM), TCEP (5 mM), magnesium chloride (10 mM), ATP (2.5 mM), pyruvate kinase/lactate dehydrogenase (15

U/mL), myokinase (10 U/mL), methylmalonyl-CoA epimerase (5 μM), CoA (1 mM), MatB (20 μM), methyl- or fluoromalonate (5 mM). The mixture was incubated at 37ºC for 30-45 min and initiated by the addition of NDK-SNAC (5 mM) and DEBS protein (10 μM). When used, DszAT (30 μM) was also added to the reaction mixture. The reaction was incubated at 37°C. 50 μL Aliquots were removed over the course of 9 h, quenched, processed and analyzed as described above. For monitoring intial rate of product formation using malonate (5 mM) and AT^0 $Mod3_{TE}$, the same assay condtions as described above were carried out with the following changes. When DszAT (30 μM) was used, aliquots were removed and quenched over the course of 90 min. When DszAT was omitted, aliquots were removed and quenched over the course of 7 h. For quantification, the samples were analyzed on a Titan C-18 column (1.9 μm, 2.1 × 50 mm, r.t, Sigma) using a linear gradient from 0 to 40% acetonitrile over 5 min with 0.1% formic acid as the aqueous mobile phase after an initial hold at 0% acetonitrile for 12 s (0.6 mL/min). H-TKL products were identified using an Agilent 6530 QTOF Accurate Mass Spectrometer in the negative mode. For reactions containing DszAT, H-TKL was quantified with a standard curve of an H-TKL authentic standard using an agilent G1315D diode array detector (H-TKL, $A_{256\ nm}$). For reactions in which DszAT was omitted, samples were quantified in MS negative mode using an H-TKL standard curve.

Triketide lactone production using Mod2 and KSAT2 constructs. All assay mixtures contained 400 mM sodium phosphate, pH 7.5, phosphoenolpyruvate (20 mM), TCEP (5 mM), magnesium chloride (5 mM), ATP (2.5 mM), pyruvate kinase/lactate dehydrogenase (15 U/mL), myokinase (10 U/mL), methylmalonyl-CoA epimerase (5 μM), CoA (0.1 mM or 1 mM), MatB (20 μM), and either methylmalonate or fluoromalonate (5 mM). Reactions were initiated by addition of NDK-SNAC (5 mM) and DEBS protein (10 μM) and incubated at 37°C for 16 h. Aliquots were removed, quenched, processed and analyzed as described above.

Quantification of fluorinated condensation products formed with $Mod3_{TE}$. All assay mixtures contained 400 mM sodium phosphate, pH 7.5, phosphoenolpyruvate (50 mM), TCEP (5 mM), magnesium chloride (10 mM), ATP (2.5 mM), pyruvate kinase/lactate dehydrogenase (15 U/mL), myokinase (10 U/mL), methylmalonyl-CoA epimerase (5 μM), CoA (1 mM), MatB (20 μM), and fluoromalonate (5 mM). The mixture was incubated at 37ºC for 30-45 min and initiated by the addition of NDK-SNAC (5 mM) and DEBS AT^0 $Mod3_{TE}$ (10 μM). When used, DszAT (30 μM) was also added to the reaction mixture at this time. The reaction was incubated at 37°C for 16 h. 20 μL aliquots were removed and quenched with 70% (*v/v*) perchloric acid (1 μL). Samples were centrifuged at 18,000 × *g* for 10 min at r.t to pellet the precipitated protein. The supernatant (20 μL) was removed and flash frozen. Excess salts were removed by centrifugation at 18,000 × *g* for

5 min at r.t. The supernatant was removed and analyzed on a Titan C-18 column (1.9 µm, 2.1 × 50 mm, r.t, Sigma) using a linear gradient from 0 to 40% acetonitrile over 4 min with 0.1% formic acid as the aqueous mobile phase after an initial hold at 0% acetonitrile for 12 s (0.6 mL/min). The triketide lactone was identified using an Agilent 6530 QTOF Accurate Mass Spectrometer in the negative mode. Products were monitored using an agilent G1315D diode array detector (F-TKL, $A_{254\ nm}$). For absolute quantification, standard curves were generated using an F-TKL standard (7). To quantify the terminal ketone and α-fluorocarboxylic acid products formed as a result of triketide thioester hydrolysis and subsequent decarboxylation, 30 µL of the reactions were adjusted to ~pH 4.5 using 0.5 µL of 50% (*v/v*) formic acid solution and combined with 30 µL of *O*-benzylhydroxylamine hydrochloride (200 mM) in 20 mM sodium phosphate, pH 4.5 (12). The reaction was incubated at room temperature for 48 h. Samples were centrifuged at 18,000 × *g* for 10 min at r.t to pellet the precipitated protein. The supernatant (55 µL) was removed and flash frozen. Excess salts were removed by centrifugation at 18,000 × *g* for 5 min at r.t. The supernatant was removed and analyzed on a Titan C-18 column (1.9 µm, 2.1 × 50 mm, r.t., Sigma). The fluoromethyl ketone product was analyzed using a linear gradient from 0 to 45% acetonitrile over 1 min with 5 mM ammonium formate, pH 6.5 as the aqueous mobile phase after an initial hold at 0% acetonitrile for 12 s (0.6 mL/min). This was then followed by a 4 min isocratic phase at 45% acetonitrile (0.6 µL/min). The α-fluorocarboxylic acid product was analyzed using a linear gradient from 0 to 24% acetonitrile over 1 min with 5 mM ammonium formate, pH 6.5 as the aqueous mobile phase after an initial hold at 0% acetonitrile for 12 s (0.6 mL/min). This was then followed by a 3.8 min gradient from 24 to 28% acetonitrile (0.6 µL/min). Products were identified using an Agilent 6530 QTOF Accurate Mass Spectrometer in the positive mode as well as monitored for quantification using an agilent G1315D diode array detector ($A_{256\ nm}$). For quantification, a standard curve was generated with 2-propanone *O*-benzyl oxime using an agilent G1315D diode array detector ($A_{256\ nm}$).

Triketide lactone production using propionyl-SNAC with the Mod2 + Mod3$_{TE}$ mini-PKS system. All reactions contained 400 mM sodium phosphate (pH 7.5), phosphoenolpyruvate (50 mM), TCEP (10 mM), magnesium chloride (5 mM), ATP (2.5 mM), pyruvate kinase (18 U/mL), myokinase (10 U/mL), methylmalonyl-CoA epimerase (5 µM), MatB (20 µM), CoA (0.1 mM), methylmalonate (5 mM, omitted from reaction with both AT^0 constructs) and fluoromalonate (30 mM), propionyl-SNAC (5 mM) and reduced nicotinamide adenine dinucleotide phosphate (NADPH; 5 mM). Reactions were initiated by addition of Mod2 (10 µM), Mod3$_{TE}$ (10 µM), and DszAT (30 µM) and incubated overnight at 37ºC. Aliquots were removed, quenched, and processed as described above. The samples were analyzed on an EclipsePlus C-18 RRHD column (1.8 µm, 2.1 × 50 mm, r.t, Agilent) using a linear gradient from 0 to 40% acetonitrile over 4 min

with 0.1% formic acid as the aqueous mobile phase after an initial hold at 0% acetonitrile for 12 s (0.6 mL/min). The triketide lactone products were identified using an Agilent 6530 QTOF Accurate Mass Spectrometer in the negative mode. Additionally, the samples were subjected to MS/MS analysis using an Agilent 6460 QQQ Mass Spectrometer. Samples were analyzed on a Poroshell 120 SB-Aq column (2.7 μm, 2.1 × 50 mm, r.t, Agilent) using the same gradient and flow rate as described above with a fragmentation voltage of 100 V and collision energies of 15 and 25 V.

Acyl-CoA hydrolysis by ACP^0 $Mod3_{TE}$. Acyl-CoA hydrolysis was determined using a fluorimetric coupled assay (detecting NADH fluorescence at 360 nm) that was adapted from an acyltransferase hydrolysis assay described previously (13). All reactions where composed of three solutions. Solution 1 (25 μL) contained sodium phosphate pH 7.5 (100 mM), α-ketoglutaric acid (1.6 mM), TPP (1.6 mM), NAD^+ (1.6 mM), and α-KGDH (1.6 U/mL). Solution 2 (25 μL) contained methylmalonyl-CoA (5-200 μM). Solution 3 (50 μL) contained sodium phosphate pH 7.5 (50 mM) and $Mod3_{TE}$ ACP^0 (400 nM). Solutions 1 and 2 were combined and the reaction was initiated with the addtion of solution 3. Combination of solutions 1 and 2 prior to intiation of the reaction allowed free-CoA in the acyl-CoA stock to be consumed.

NADH fluorescence was monitored using a Synergy Mx multimode microplate reader (Biotek) containing a High Energy Xenon Flash light source (double grating monochromators with 250-900 nm range). Assays were run in a 96-well microtiter plate (black polystyrene, flat bottom, half area, non-binding surface, Corning). Reactions were run for 10 min at 30ºC with the maximum number of measurements. Kinetic parameters (k_{cat}, K_M) were determined by fitting the data using Microcal Origin to the equation: $v_o = v_{max}[S] / (K_M + [S])$, where v is the initial rate and [S] is the substrate concentration. Data are reported as mean ± s.e. ($n = 3$) with standard error derived from the nonlinear curve fitting. Error bars on graphs represent mean ± s.d. ($n = 3$). Error in k_{cat}/K_M is calculated by propagation of error from the individual kinetic parameters.

Determination of malonyl-ACP occupancy in $Mod3_{TE}$ with DszAT. All assay mixtures contained 50 mM sodium phosphate, pH 7.5, TCEP (5 mM), magnesium chloride (10 mM), methylmalonyl-CoA epimerase (1 μM), and either methylmalonyl-CoA (1 mM), malonyl-CoA (1 mM), or fluoromalonyl-CoA (1 mM). The reaction was initiated by the addition DEBS $Mod3_{TE}$ (1 μM). When used, DszAT (1 μM) was also added to the reaction mixture. The reaction was incubated at 37°C for 20 min, at which point 1.25 μL of trypsin (2.5 μg/μL) was added and the reaction was incubated at 37ºC for another 90 min. The reaction was quenched using liquid N_2 and was stored at -80ºC until analysis. Excess salts were removed by centrifugation at 18,000 × g for

5 min at r.t. The supernatant was removed and analyzed on an AdvanceBio Peptide Map (2.7 μm, 2.1 × 250 mm, 55ºC, Agilent) column using a linear gradient from 0 to 60% acetonitrile (90%) and 0.1% formic acid (10%) over 55 min with 0.1% formic acid as the aqueous mobile phase after an initial hold at 100% 0.1% formic acid for 5 min (0.4 mL/min). Appropriate peptides were identified using an Agilent QTOF 6530 mass spectrometer. Products were characterized using both MS1 (exact mass <2 ppm) and MS2 (phosphopantetheine ejection). The samples were analyzed in positive mode with a fragmentor voltage of 150 and collison energy of 35 V. The parent ions monitored were 683.7 *m/z* (*holo*-ACP, +3), 717.0 *m/z* (methylmalonyl-ACP, +3), 712.3 *m/z* (malonyl-ACP, +3), 718.3 *m/z* (fluoromalonyl-ACP, +3). For percent-occupancy, the MS1 extracted-ion-chromatograms for both the *acyl*-ACP and *holo*-ACP were integrated and calculated as follows:

$$\left(\frac{Acyl-ACP}{Acyl-ACP+Holo-ACP}\right)*100\%$$

Hydrolysis of malonyl-CoA derivatives by DszAT. All assay mixtures contained 50 mM sodium phosphate, pH 7.5, TCEP (5 mM), magnesium chloride (10 mM), methylmalonyl-CoA epimerase (1 μM), and methylmalonyl-CoA (1 mM), malonyl-CoA (1 mM), or fluoromalonyl-CoA (1 mM). The reaction initiated by the addition DszAT (3 μM). The reaction was incubated at 37°C for 10 min and 10 μL aliquots were removed after 10 s, 1 min, 2 min, 3 min, 5 min, and 10 min. The aliquots were quenched by pipetting the 10 μL aliquot into an eppendorf tube containing 1 μL of 70% (*v/v*) perchloric acid. Samples were centrifuged at 18,000 × *g* for 10 min at r.t to pellet the precipitated protein. The supernatant (10 μL) was removed and flash frozen. Excess salts were removed by centrifugation at 18,000 x *g*. Samples containing fluoromalonyl-CoA and malonyl-CoA were analyzed on an EclipsePlusC18 RRDH column (1.8 μm, 2.1 × 50 mm, 27ºC, Agilent) using a linear gradient from 0 to 5% acetonitrile over 4 min with 10 mM ammonium formate (not pH adjusted) as the aqueous mobile phase after an initial hold at 0% acetonitrile for 12 s (0.6 mL/min). Samples containing methylmalonyl-CoA were analyzed on an EclipsePlusC18 RRDH column (1.8 μm, 2.1 × 50 mm, 27ºC, Agilent) using a linear gradient from 0 to 10% acetonitrile over 8 min with 0.1% Formic acid as the aqueous mobile phase after an initial hold at 0% acetonitrile for 12 s (0.6 mL/min). Products were monitored using an Agilent G1315D diode array detector (fluoromalonyl-CoA, methylmalonyl-CoA, malonyl-CoA, and CoASH $A_{260\ nm}$). Given the A_{260} absorbance of free-CoA and the malonyl-CoA derivatives, the CoA released could be calculated using the absorbance percentage free-CoA accounted for between the two compounds.

Monitoring [4-^{13}C]-methylmalonyl-CoA incorporation into AT^0 ACP^0 Mod3$_{TE}$ by ^{13}C-NMR. All assay mixtures contained 100 mM sodium phosphate, pH 7.5, TCEP (5 mM), magnesium chloride (10 mM), methylmalonyl-CoA epimerase (5 μM), and [4-^{13}C]-methylmalonyl-CoA (1 mM). The reaction was initiated by the addition of NDK-SNAC (5 mM) and DEBS Mod3$_{TE}$ or AT^0 ACP^0 Mod3$_{TE}$ (10 μM) to a final reaction volume of 300 μL. The reaction was incubated at 37°C for 18.5 h and the reactions were quenched with 70% (*v/v*) perchloric acid (15 μL). The internal standard, [2-^{13}C]-sodium acetate (final concentration, 2 mM in D_2O), was added to a final volume of 355 μL and the samples were centrifuged at 18,000 × *g* for 10 min at r.t to pellet the precipitated protein. The supernatant (350 μL) was removed and inserted into a Shigemi tube (D_2O) for ^{13}C-NMR. The products were quantified using an external standard solution containing [2-^{13}C]-sodium acetate (2 mM in D_2O), [4-^{13}C]-methylmalonyl-CoA (1 mM), [3-^{13}C]-sodium propionate (1 mM), [4-^{13}C]-methylmalonic acid (1 mM), and [3-^{13}C]-propionyl-SNAC (1 mM) in 100 mM sodium phosphate, pH 7.5 containing TCEP (5 mM) and magnesium chloride (10 mM). TKL and propionyl-CoA formed under these conditions were quantified using additional external standards of [2-^{13}C]-sodium acetate (2 mM in D_2O) and either TKL (1.3 mM) or propionyl-CoA (23 mM) in the same buffer. For solutions containing an unlabeled standard, the ratio between the standard and the internal standard ([2-^{13}C]-sodium acetate) was normalized to the appropriate isotopic ratio. All experimental data were collected on a Bruker Biospin 900 MHz spectrometer. For AT^0 ACP^0 Mod3$_{TE}$, the concentration of TKL was below the limit of detection by this method and was therefore determined using LCMS. Samples were separated by UHPLC on a Titan C-18 column (1.9 μm, 2.1 × 50 mm, r.t, Sigma) using a linear gradient from 0 to 40% acetonitrile over 4 min with 0.1% (v/v) formic acid as the aqueous mobile phase after an initial hold at 0% acetonitrile for 20 s (0.6 mL/min). TKL was identified using an Agilent 6530 QTOF Accurate Mass Spectrometer in negative ion mode and quantified using a calibration curve with a TKL standard.

^{19}F-NMR analysis of reactions with AT^0 Mod3$_{TE}$ and AT^0 ACP^0 Mod3$_{TE}$. All assay mixtures contained 400 mM sodium phosphate, pH 7.5, phosphoenolpyruvate (50 mM), TCEP (5 mM), magnesium chloride (10 mM), ATP (2.5 mM), pyruvate kinase/lactate dehydrogenase (15 U/mL), myokinase (10 U/mL), methylmalonyl-CoA epimerase (5 μM), CoA (1 mM), MatB (20 μM), fluoromalonate (5 mM). The mixture was incubated at 37ºC for 30-45 min and initiated by the addition of NDK-SNAC (5 mM) and DEBS AT^0 Mod3$_{TE}$ (10 μM) or AT^0 ACP^0 Mod3$_{TE}$ (10 μM). When used, DszAT (30 μM) was also added to the reaction mixture (final volume, 500 μL). The reaction was incubated at 37°C for 14 hours and quenched with 70% (*v/v*) perchloric acid (25 μL). The internal standard TFA (8mM in D_2O) was added to a final volume of 600 μL. Samples were centrifuged at 18,000 × *g* for 10 min at r.t to pellet the precipitated protein and the sample was pipetted into an NMR tube. To quantify fluoroacetate formation, a solution containing 400 mM

sodium phosphate, pH 7.5, TCEP (5 mM), magnesium chloride (10 mM), fluoroacetate (7 mM), and the TFA internal standard (8 mM in D_2O) was used. A control experiment was performed containing all of the reagents described above without DEBS and NDK-SNAC. All experimental data was collected on a Bruker AV-600 MHz spectrometer. To determine F-TKL concentrations, a portion of the reaction was removed and analyzed on a Zorbax Eclipse XDB C-18 column (3.5 μm, 3 × 150 mm, 35ºC, Agilent) using a linear gradient from 0 to 40% acetonitrile over 14 min with 0.1% formic acid as the aqueous mobile phase after an initial hold at 0% acetonitrile for 30 s (0.8 mL/min). Products were monitored using an agilent G1315D diode array detector (F-TKL, $A_{247\ nm}$) and compared against an F-TKL standard calibration curve.

Supplementary Results

Table S1. (A) Strains and plasmids used for this study. (B) Oligonucleotides used for gene and plasmid construction. (C) gBlock assembly map for synthetic PIKSKR1 gene.

A. Strains and plasmids

Strain	*Genotype*	*Source*
BL21(de3)	F⁻ *omp*T *gal dcm lon hsd*S_B(r_B⁻ m_B⁻) λ(DE3 [*lac*I *lac*UV5-T7 *gene 1 ind*1 *sam*7 *nin*5])	Novagen
BAP1	F⁻ *omp*T *gal dcm lon hsd*S_B(r_B⁻ m_B⁻) λ(DE3 [*lac*I *lac*UV5-T7 *gene 1 ind*1 *sam*7 *nin*5]) Δ*prpRBCDE* (*sfp* (T7)*, prpE* (T7))]	(1)

Plasmid	*Description*	*Source*
pET16BHis$_{10}$-rpMatB	*His$_{10}$*-rpMatB (T7), *lacI*, Cb^r, ColE1	This study
pET28a-His$_6$-Epi.SCo	*His$_6$-epi.SCo* (T7), *lacI*, Km^r, ColE1	(7)
pET28a-His$_6$-KSAT2	*His$_6$-matB.SCo* (T7), *lacI*, Km^r, ColE1	This study
pET21c-His$_6$-Mod3$_{TE}$ ACP^0	*DEBS$_{Mod3}$+TE ACP^0-His$_6$* (T7), *lacI,* Cb^r, ColE1	This study
pET21c-His$_6$-Mod3$_{TE}$ AT^0 ACP^0	*DEBS$_{Mod3}$+TE AT^0 ACP^0-His$_6$* (T7), *lacI,* Cb^r, ColE1	This study
pET21c-KSAT^0-His$_6$	*DEBS$_{Mod3}$+KSAT/AT^0-His$_6$* (T7), *lacI,* Cb^r, ColE1	This study
pET21c-KS^{0}AT^0-His$_6$	*DEBS$_{Mod3}$+KSAT/KS^{0}AT^0-His$_6$* (T7), *lacI,* Cb^r, ColE1	This study
pET21c-KS^{0}AT-His$_6$	*DEBS$_{Mod3}$+KSAT/KS^0-His$_6$* (T7), *lacI,* Cb^r, ColE1	This study
pET28a-His$_6$-PIKSKR1	*His$_6$-PIKSKR1* (T7), *lacI*, Km^r, ColE1	This study
pFW3	*DszAT.SCe-His$_6$* (T7), *lacI,* Cb^r, ColE1	(3)
pSV272- His$_6$-MBP-DEBS$_{Mod2}$	*His$_6$-MBP-DEBS$_{Mod2}$* (T7), *lacI*, Km^r, ColE1	(7)
pSV272-His$_6$-MBP-DEBS$_{Mod2}$/AT^0	*His$_6$-MBP-DEBS$_{Mod2}$/AT^0* (T7), *lacI*, Km^r, ColE1	(7)
pAYC138	*DEBS$_{Mod6}$+TE/AT^0-His$_6$* (T7), *lacI,* Cb^r, ColE1	(3)
pRSG54	*DEBS$_{Mod6}$+TE-His$_6$* (T7), *lacI,* Cb^r, ColE1	(2)
pAYC11	*DEBS$_{Mod6}$+KSAT* (T7), *lacI,* Cb^r, ColE1	(5)
pRSG34	*DEBS$_{Mod3}$+TE-His$_6$* (T7), *lacI,* Cb^r, ColE1	(2)
pAYC136	*DEBS$_{Mod3}$+TE/AT^0-His$_6$* (T7), *lacI,* Cb^r, ColE1	(3)
pAYC02	*DEBS$_{Mod3}$+KSAT-His$_6$* (T7), *lacI,* Cb^r, ColE1	(5)

B. Oligonucleotide sequences

Name	*Sequence*
PIKS_KR1 F2	aacgttgggcaggtctggttgatctgcctgcccagccggatg
PIKS_KR1 R2	ttgcacgatgtgcacgacgaacctgttccgctgtcagggtatcaac
KSAT2 F1	attatggtctcttatggagccgatcgcgatcgtc
KSAT2 R1	ataatggtctcaaagcccggtagaaccagccgtc
KS^{0}AT^0 F1	cgtccggcgtagaggatcgagatctcgatcccgcgaaattaatacgactcactatagggg
KS^{0}AT^0 R1	cgccaggtgcagcgccaccagcgacgacgatgccgcggtgtcgacgctgatcgacggccc
KS^{0}AT^0 F2	gggccgtcgatcagcgtcgacaccgcggcatcgtcgtcgctggtggcgctgcacctggcg
KS^{0}AT^0 R2	gcgtcctccaacgtgagcgccccggcgacgtgcgcggcggcgatctcgccctgcgcgtgc
Mod3TE ACP0 F1	gggcctggaccgggtcgacgtggtgcagccggtgttgttcgcggtgatggtgtcgctggc
Mod3TE ACP0 R1	ttgcgcagggccatcgcgttgagcgcgtcgagtccgagctcgctgaacgcgcggcgcacg

Mod3TE ACP0 F2	cgtgcgccgcgcgttcagcgagctcggactcgacgcgctcaacgcgatggccctgcgcaa
Mod3TE ACP0 R2	ttgttagcagccggatctcagtggtggtggtggtggtgctcgagtgcggccgcaagcttg

C. gBlock assembly map for synthetic PIKSKR1 gene:

Name	*Sequence*
PIKS-KR1-block1	ttatggtctcttatggcaaccggtgatgattggcgttatcgtattgattggaaacgtctgcctgcagcagaaggtagcgaacgtaccggtctgagcggtcgttg gctggcagttacaccggaagatcatagcgcacaggcagcagcagttctgaccgcactggttgatgccggtgcaaaagttgaagttctgacagccggtgc agatgatgatcgtgaagcactggcagcacgtctgacagcgctgaccacaggtgatggttttaccggtgttgttagcctgctggatggtctggttccgcaggtt gcatgggttcaggccctgggtgatgcaggtattaaagcaccgctgtggtctgttacccagggtgcagttagcgttggtcgtctggatactccggcagatcctg atcgtgcaatgctgtggggtctgggtcgtgttgttgcactggaacatccggaacgttgggcaggtctggttgatctcgaatgagaccatta
PIKS-KR1-block2	Ttatggtctctcgaagcctgcccagccggatgcagcagcactggcccatctggttaccgcactgagcggtgccaccggtgaagatcagattgcaattcgta ccaccggtctgcatgcacgtcgtctggcacgtgcaccgctgcatggtcgtcgtccgacccgtgattggcagccgcatggcaccgttctgattacaggtggta caggtgcactgggtagccatgcagcacgttggatggcacatcatggtgcagaacatctgctgctggttagccgtagcggtgaacaggcaccgggtgcaa cccagctgaccgcagaactgaccgcaagcggtgcccgtgttaccattgcagcatgtgatgttgcagatccgcatgcaatgcgtaccctgctggacgcaatt ccggcagaaacaccgctgacagcagttgttcataccgcaggcgctctggatgatggtattgttgataccctgacagcggaattcctgagaccatta
PIKS-KR1-block3	Attatggtctctttcccaggttcgtcgtgcacatcgtgcaaaagcagttggtgcaagcgttctggatgaactgacacgtgatctggatctggatgcatttgttctgt ttagcagcgttagcagcacactgggtattccgggtcagggtaattatgcaccgcataatgcatatctggacgccctggcagcccgtcgtcgcgcaacaggt cgtagcgcagtgagcgttgcctggggtccgtgggatggtggtggtatggcagccggtgatggtgttgccgaacgtctgcgtaatcatggtgttccgggtatgg acccggaactggcgctggcagcgctggaaagcgcactgggacgtgatgaaaccgcaattaccgttgcagatatcgattgggatcgtttttatctggcatat agcagcggtcgtccgcagccgctggttgaagaactgccggaagttcgtcgtattattgatgcacgtgattaagctttgagaccattat

Figure S1. SDS-PAGE gels of purified proteins. (A) Enzymes used in generation of substrates and loading ACPs (Lane **1**, mmCoA Epi; **2**, rpMatB; **3**, PIKS KR1; **4**, DszAT). (B) KSAT didomains for triketide production **5**, KSAT2; **6**, KSAT6; **7**, KSAT3; **8**, KSAT^{0}3; **9**, KS^{0}AT3; **10**, KS^{0}AT^{0}3). (C) Enzymes used for polyketide production (**11**, Mod2; **12**, AT^0 Mod2; **13**, Mod3$_{TE}$; **14**, AT^0 Mod3$_{TE}$; **15**, AT^0 ACP^0 Mod3$_{TE}$; **16**, ACP^0 Mod3$_{TE}$; **17**, Mod6$_{TE}$; **18**, AT^0 Mod6$_{TE}$).

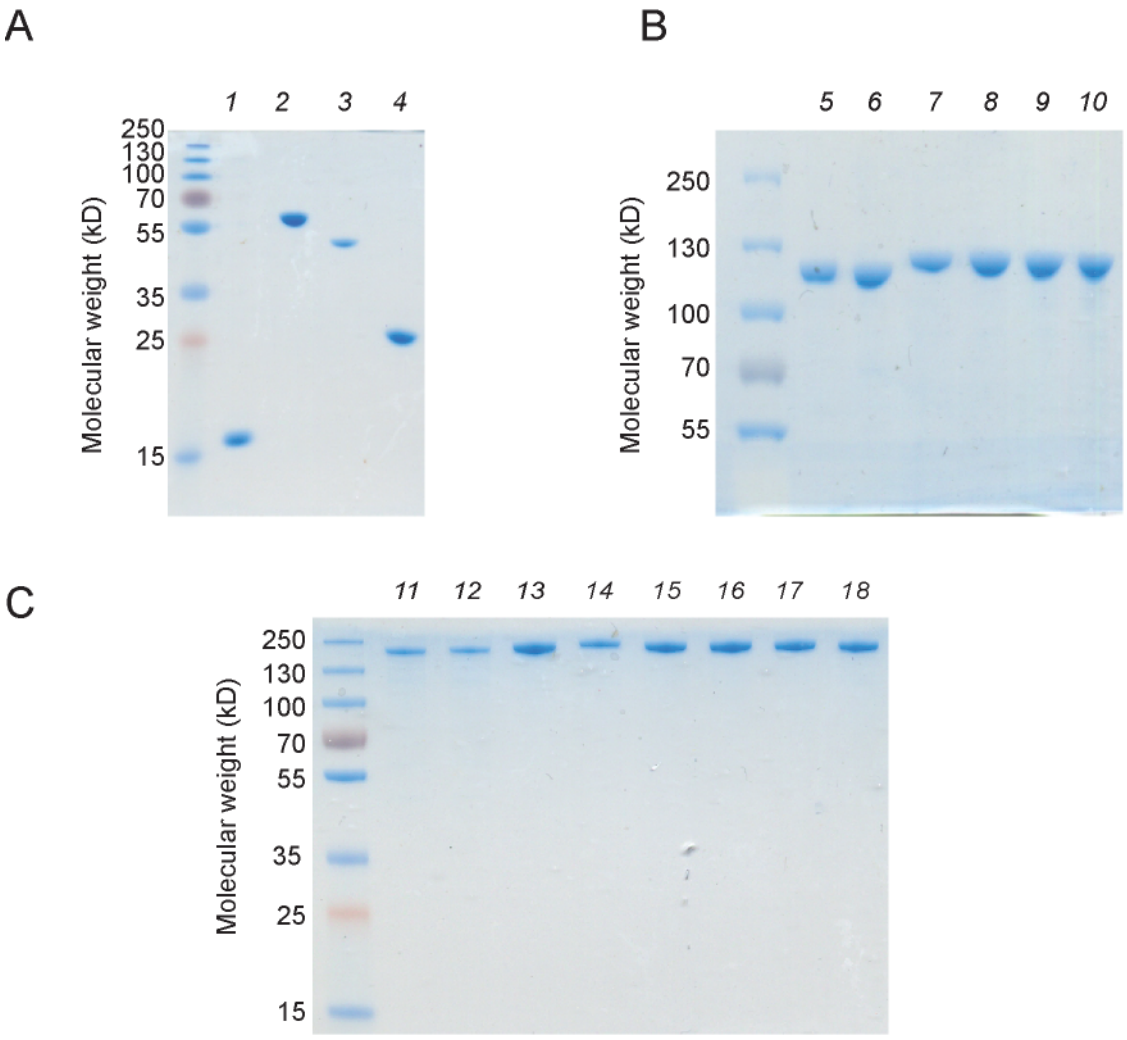


Figure S2. NMR spectra of NDK-SNAC. (A) ^{1}H NMR. (B) ^{13}C NMR.

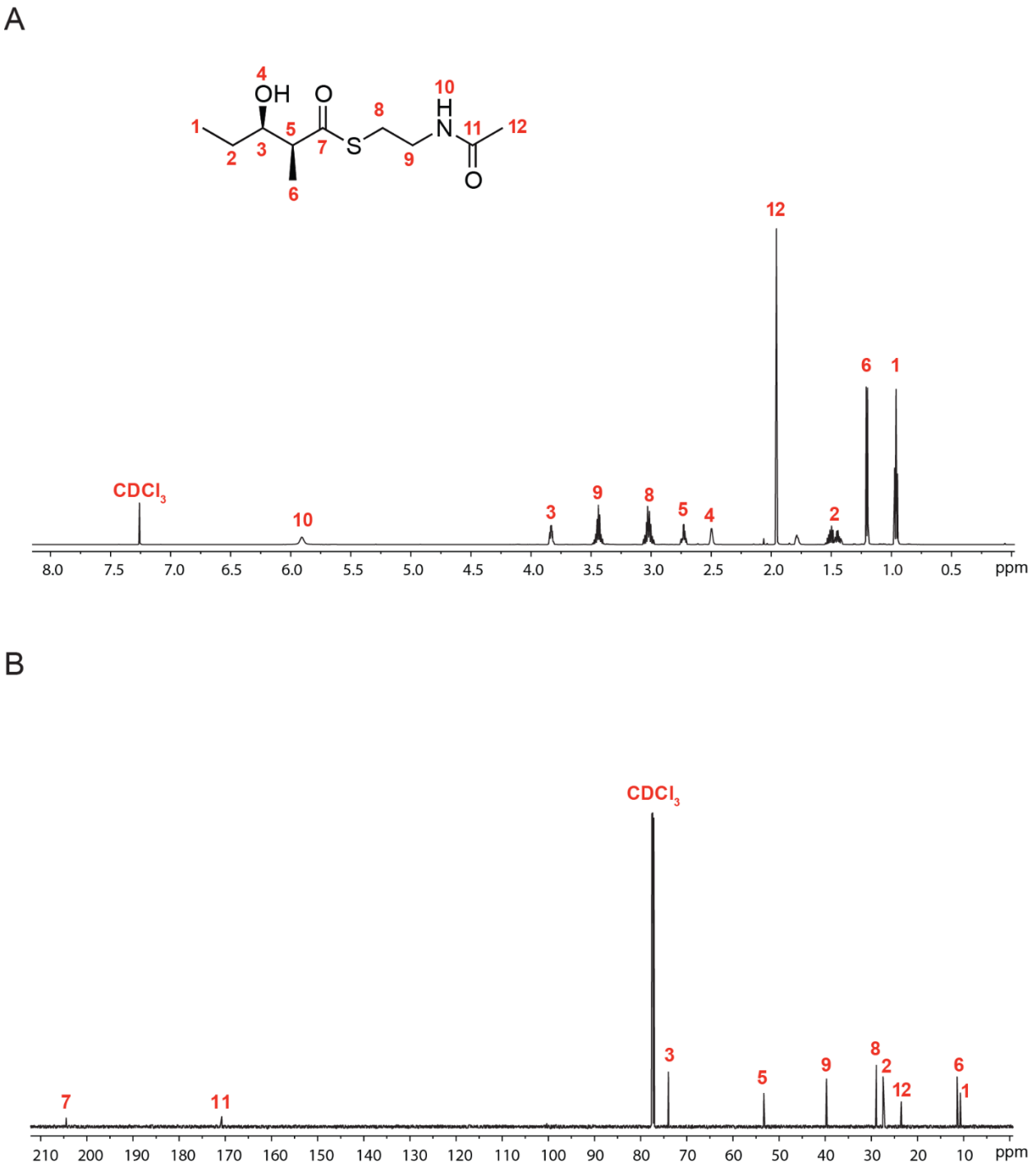


Figure S3. (A) Initial rate of TKL formation with WT Mod3$_{TE}$ (black), AT^0 Mod3$_{TE}$ (grey), ACP^0 Mod3$_{TE}$ (blue), AT^0 ACP^0 Mod3$_{TE}$ (purple), and KSAT3 didomain (red). (B) Close-up of the initial rate of TKL formation for the Mod3$_{TE}$ mutants and truncation products (AT^0 Mod3$_{TE}$ (grey), ACP^0 Mod3$_{TE}$ (blue), AT^0 ACP^0 Mod3$_{TE}$ (purple), and KSAT3 didomain (red)). The rate of product formation is displayed in the manuscript text in Figure 1B. The error bars represent s.d. (*n=3*).

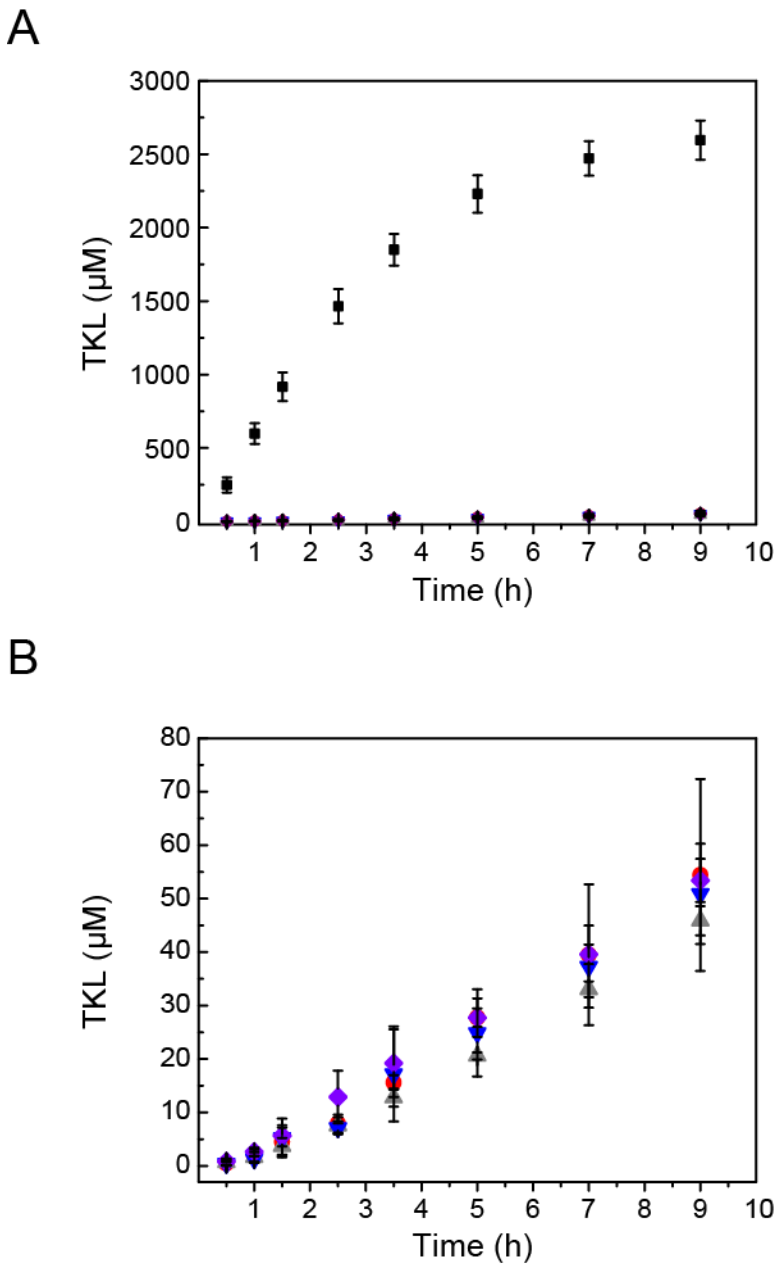


Figure S4. Initial rate of F-TKL formation with Mod3$_{TE}$ WT (black), AT^0 Mod3$_{TE}$ (grey), ACP^0 Mod3$_{TE}$ (blue), AT^0 ACP^0 Mod3$_{TE}$ (purple), and KSAT3 didomain (red). The rate of product formation is displayed in the manuscript text in **Figure 1B**. The error bars represent s.d. (*n=3*).

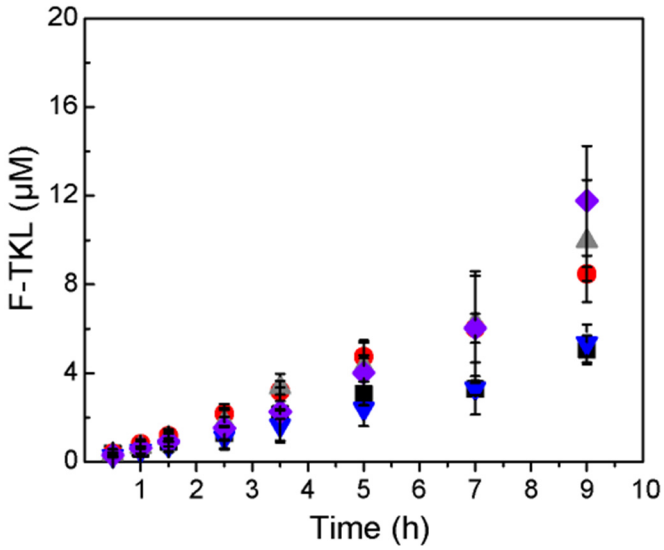


Figure S5. NMR spectra of [4-^{13}C]-methylmalonyl-CoA. (A) ^{1}H-NMR. (B) ^{13}C-NMR. The peak observed at 11.9 ppm on the ^{13}C-spectrum represents the ^{13}C-labeled carbon of propionyl-CoA, a known decomposition product of methylmalonyl-CoA. The integration ratios between this peak and the ^{13}C-labeled peak of the product is 0.005:1. Other quantitative NMR experiments described in this manuscript found that the integration of methylmalonyl-CoA is ~0.5 times the integration of an equivalent concentration of propionyl-CoA, suggesting that the product is of >99.5% purity.

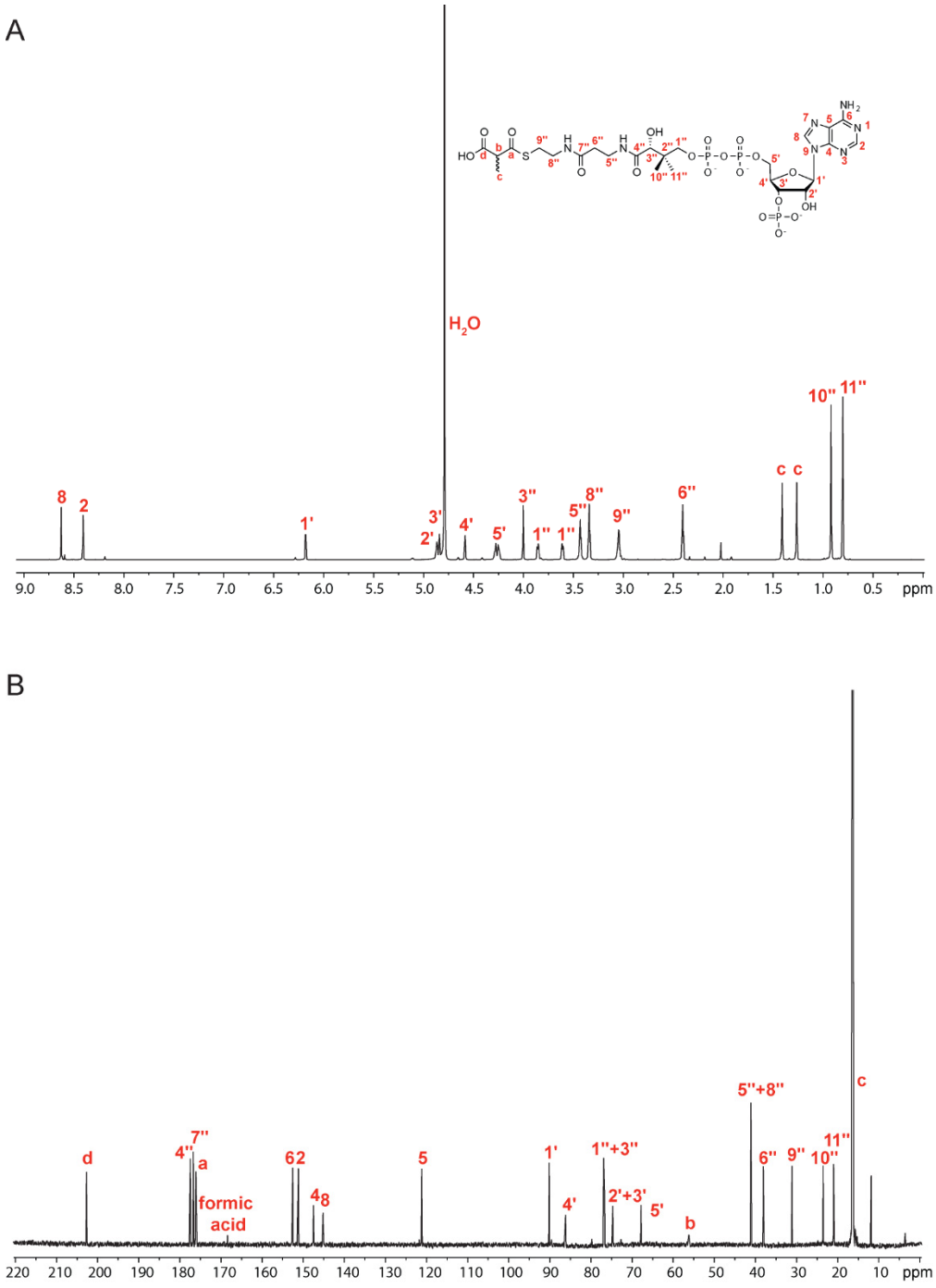
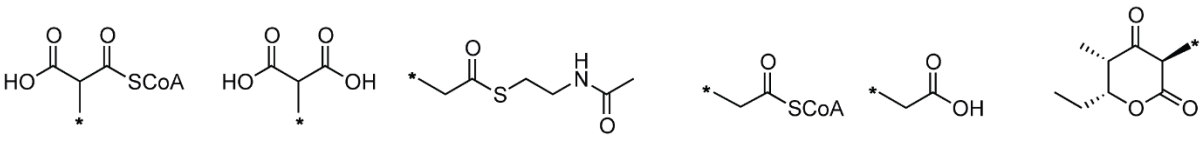


Figure S6. ^{13}C-NMR traces aligning the different reactions of the [4-^{13}C]methylmalonyl-CoA stoichiometry experiment (845 μM of ^{13}C-labeled compounds). The peak at 23.24 ppm represents the [2-^{13}C]sodium acetate standard. The concentration of the various products and substrates are tabulated in the chart below. The values in the chart have been normalized using the "No enzyme control" experiment. Mass balances for all experiments were observed to fall within 109 ± 5% of the expected value. As observed, the propionyl-SNAC and propionyl-CoA peaks can be resolved and integrated. Data are reported in mean ± s.d. (*n=3*).



1 **2** **3** **4** **5** **6**

	Module3$_{TE}$ WT	Module3$_{TE}$ AT^0 ACP^0
Compound	Average ± s.d. (μM)	Average ± s.d. (μM)
1	230 ± 42	536 ± 47
2	290 ± 14	-16 ± 13
3	22 ± 6	83 ± 7
4	20 ± 6	69 ± 8
5	2 ± 5	76 ± 10
6	130 ± 3.4	0.86 ± 0.5

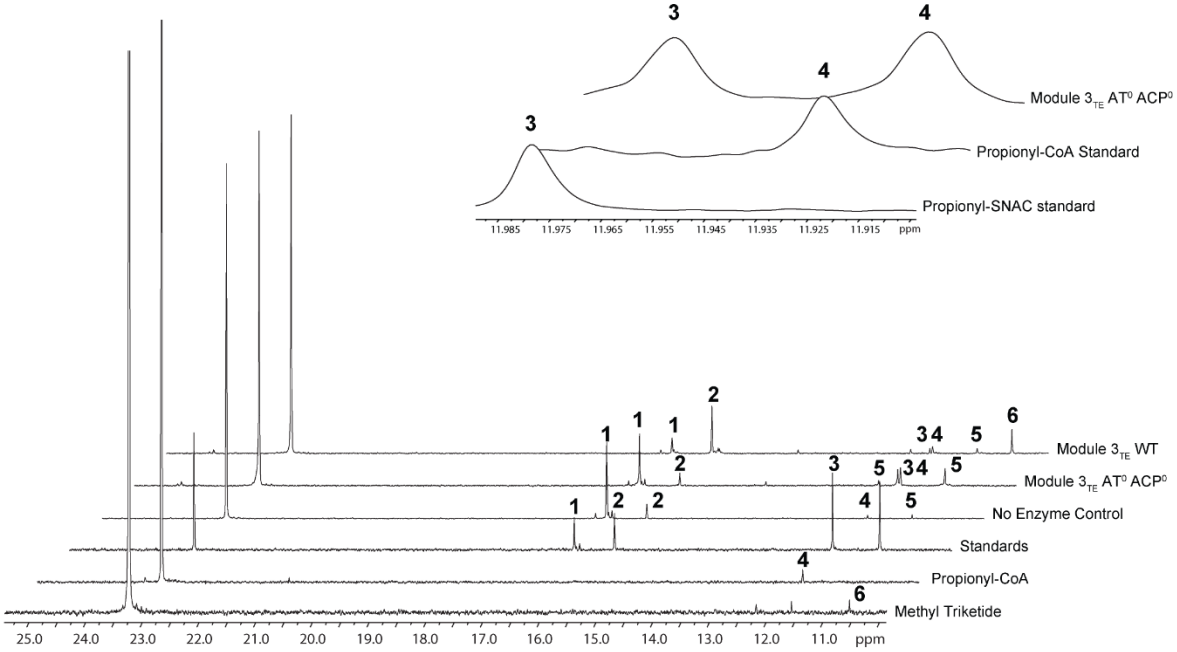


Figure S7. Steady state kinetic analysis of ACP^0 Mod3$_{TE}$ hydrolysis of malonyl-CoA derivatives. CoA release is quantified using NADH fluorescence as described on p. S15. (A) Scheme representing hydrolysis of methylmalonyl-CoA by ACP^0 Mod3$_{TE}$. (B) Michaelis-Menten curve for methylmalonyl-CoA. The error bars on the data points represent s.d. (*n=3*) and the kinetic parameters are reported as the mean ± s.e. determined from non-linear curve fitting.

A

B

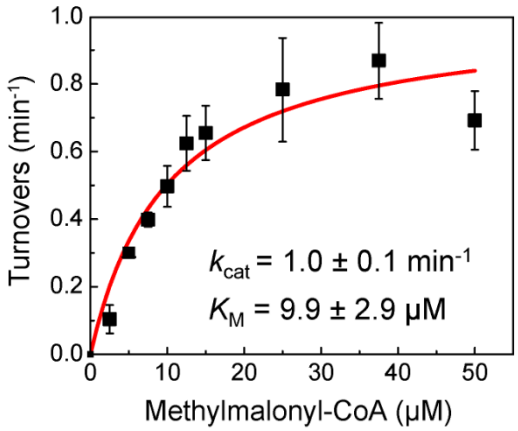


Figure S8. ^{19}F-NMR analysis of AT^0 ACP^0 Mod3$_{TE}$ F-TKL assay. AT^0 ACP^0 Mod3$_{TE}$ (10 µM), NDK-SNAC (5 mM), fluoromalonate (5 mM), and CoA (1 mM) incubated under *in situ* regeneration conditions and analyzed by ^{19}F NMR after 14 h. The control reactions omitted NDK-SNAC and AT^0 ACP^0 Mod3$_{TE}$. Fluoroacetate (**1**) was quantified using a trifluoroacetic acid (**2**) internal standard. F-TKL was quantified using LC-UV (A_{247}) using a standard curve with an authentic sample. The J_{HF} of the fluoroacetate standard is 48.3 Hz and was found to be 48.4 Hz in the fluoroacetate generated in the reaction. Additionally, no fluoroacetate is observed in the control reaction, suggesting that DEBS is responsible for the decarboxylation product.

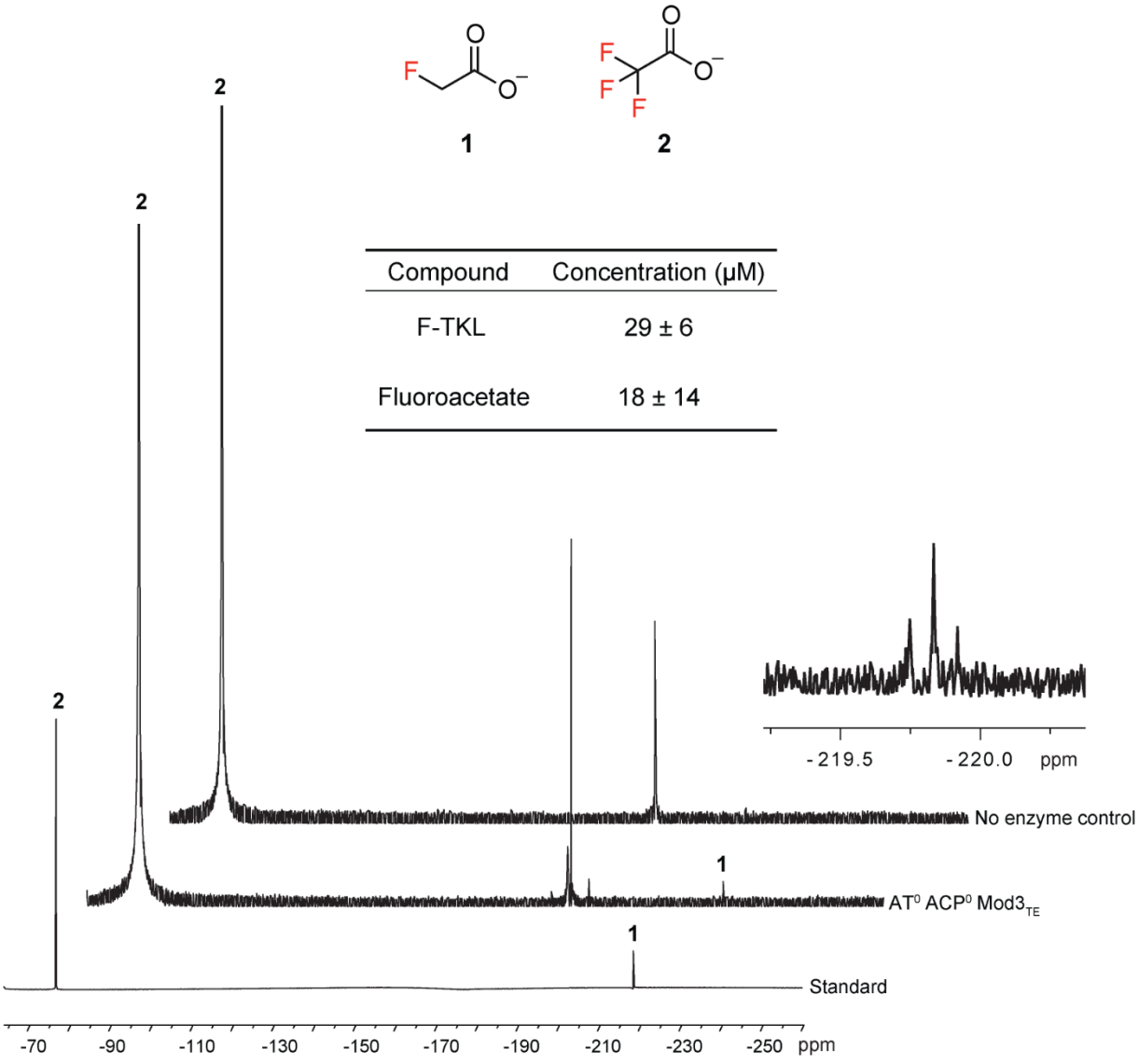


Figure S9. Initial rate of F-TKL formation with AT^0 $Mod3_{TE}$ (black) and AT^0 ACP^0 $Mod3_{TE}$ (red) complemented with DszAT. Data are mean ± s.d. (*n=3*).

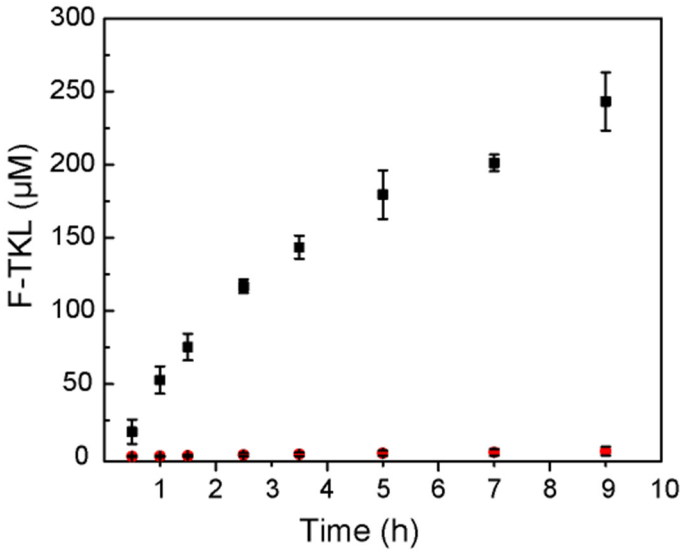


Figure S10. ^{19}F-NMR spectrum of a reaction containing AT^0 Mod3$_{TE}$ (10 μM), DszAT (30 μM), NDK-SNAC (5 mM), fluoromalonate (5 mM), and CoA (1 mM) incubated under *in situ* regeneration conditions for 14 h. No fluoroacetate is observed above the NMR detection limit in this reaction (fluoroacetate appears as a triplet (-220 ppm, J_{HF} = 48.3 Hz), see arrow). This result indicates that complementation of AT^0 Mod3$_{TE}$ with DszAT also restores coupling between fluoromalonyl decarboxylation and C-C bond formation.

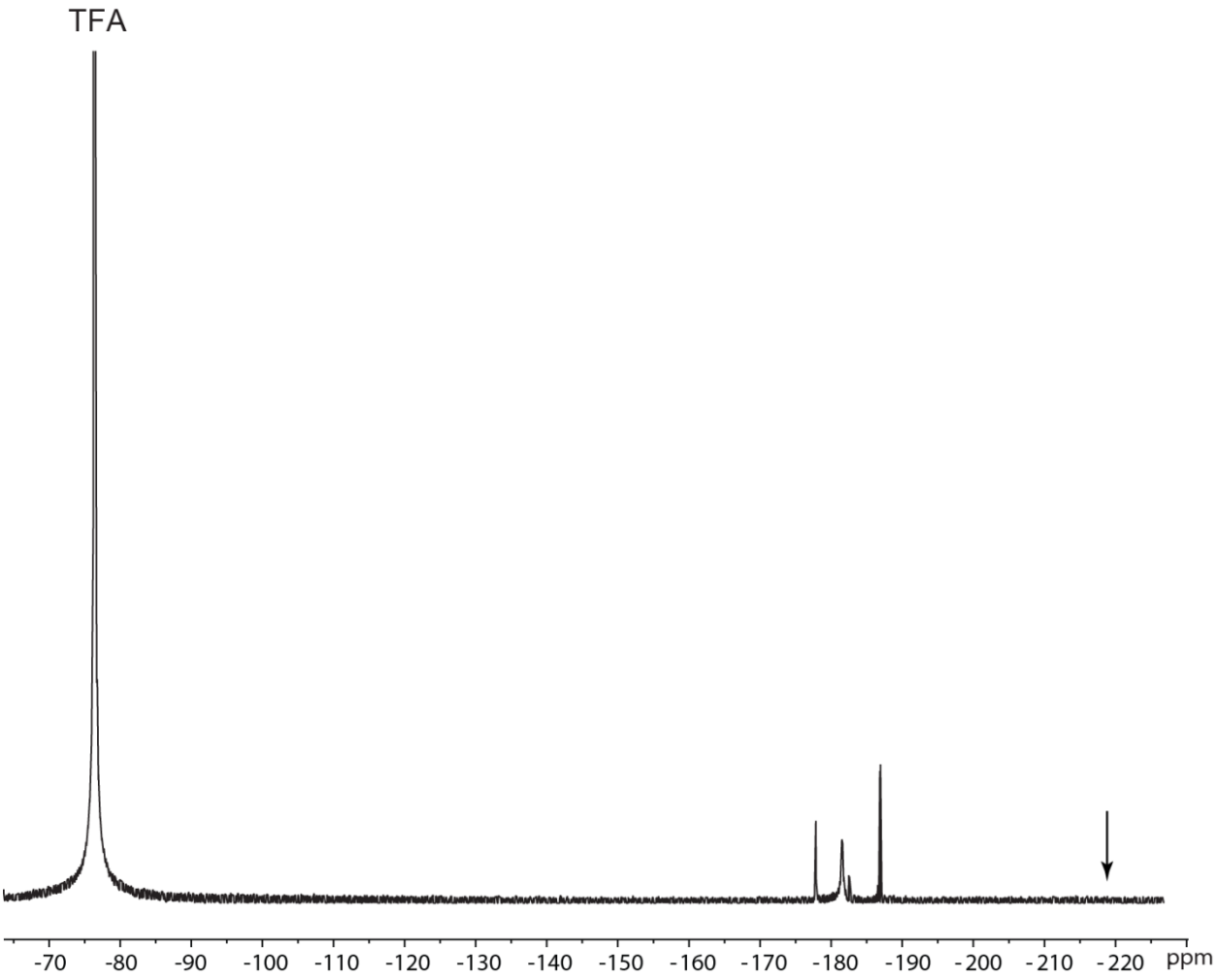
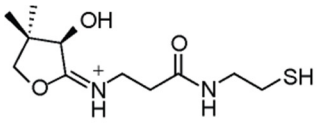
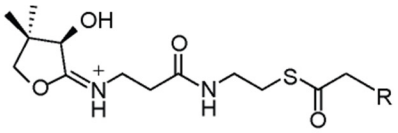


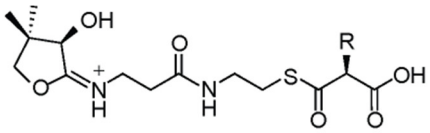
Figure S11. (A) MS/MS characterization of phosphopantetheine ejection fragments of *holo*-ACP, malonyl-ACP, methylmalonyl-ACP, and fluoromalonyl-ACP. The MS/MS spectrum of (B) malonyl-ACP, (C) methylmalonyl-ACP, (D) fluoromalonyl-ACP, (E) *holo*-ACP can be observed as well.



1



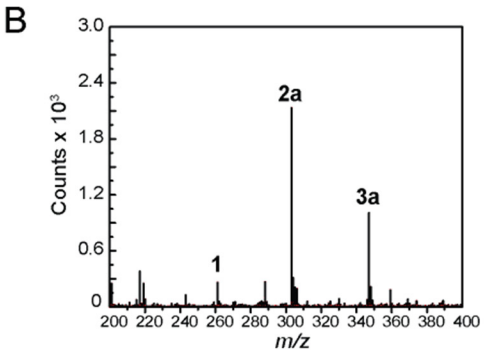
2a R = H
2b R = Me
2c R = F

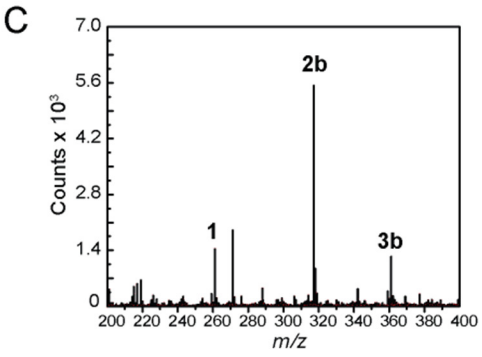


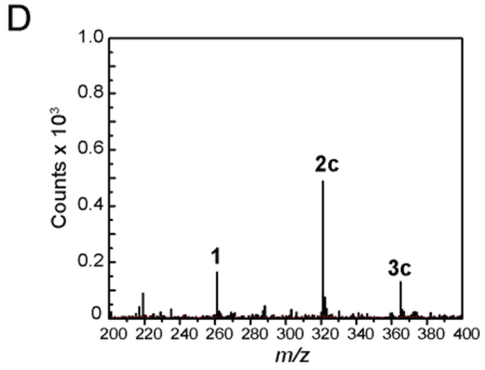
3a R = H
3b R = Me
3c R = F

A

	1 (*m/z*)	2 (*m/z*)	3(*m/z*)
holo	**Obs:** 261.1273 **Exp:** 261.1267	**n/a**	**n/a**
a	**Obs:** 261.1268 **Exp:** 261.1267	**Obs:** 303.1360 **Exp:** 303.1373	**Obs:** 347.1267 **Exp:** 347.1271
b	**Obs:** 261.1263 **Exp:** 261.1267	**Obs:** 317.1519 **Exp:** 317.1530	**Obs:** 361.1421 **Exp:** 361.1428
c	**Obs:** 261.1267 **Exp:** 261.1267	**Obs:** 321.1285 **Exp:** 321.1279	**Obs:** 365.1165 **Exp:** 365.1177







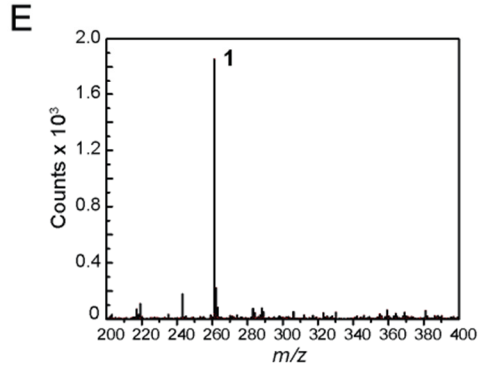


Figure S12. Initial rates of extender unit hydrolysis by DszAT (3 μM). Data are mean ± s.d. (*n*=*3*).

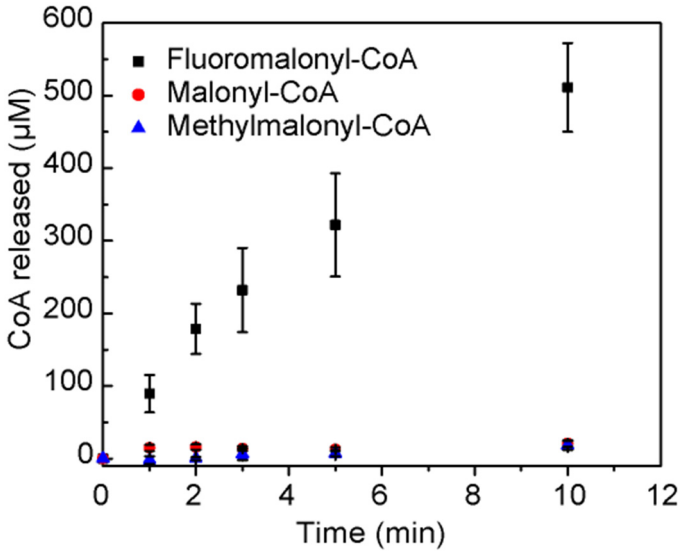


Figure S13. Initial rate of H-TKL formation with AT^0 $Mod3_{TE}$ complemented with DszAT (10.8 ± 0.3 μM min^{-1}, black) and AT^0 $Mod3_{TE}$ (0.006 ± 0.001 μM min^{-1}, red). Data are mean ± s.d. (*n*=*3*).

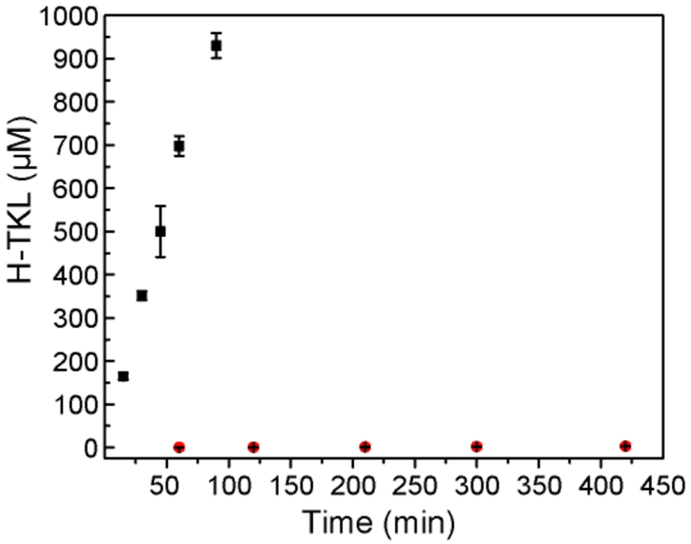


Figure S14. Determining other major products formed in the chain extension reaction catalyzed by AT^0 $Mod3_{TE}$ and DszAT with fluoromalonyl-CoA. (A) Reaction scheme for formation of the α-fluorocarboxylic acid (**1**) and fluoromethyl ketone (**2**). (B) ^{19}F-NMR analysis of the reaction in 12.5% D_2O shows new peaks, one with a chemical shift consistent with a fluoromethyl ketone (**2**) and a triplet splitting pattern resulting from fluorine coupling to the two equivalent hydrogens bound to the ipso carbon (J_{HF} = 47.5 Hz) (14). A second triplet is observed (arrow) and believed to be the result of epimerization of the methyl group. The α-fluoro carboxylic acid precursor (**1**) to **2** was then noted and appears as a doublet (J_{HF} = 49.9 Hz). (C) LC/QTOF analysis of **2** before and **1** and **2** after derivatization with *O*-benzyl hydroxylamine (12). **1** was not retained on the column before derivatization and the EIC is therefore unavailable.

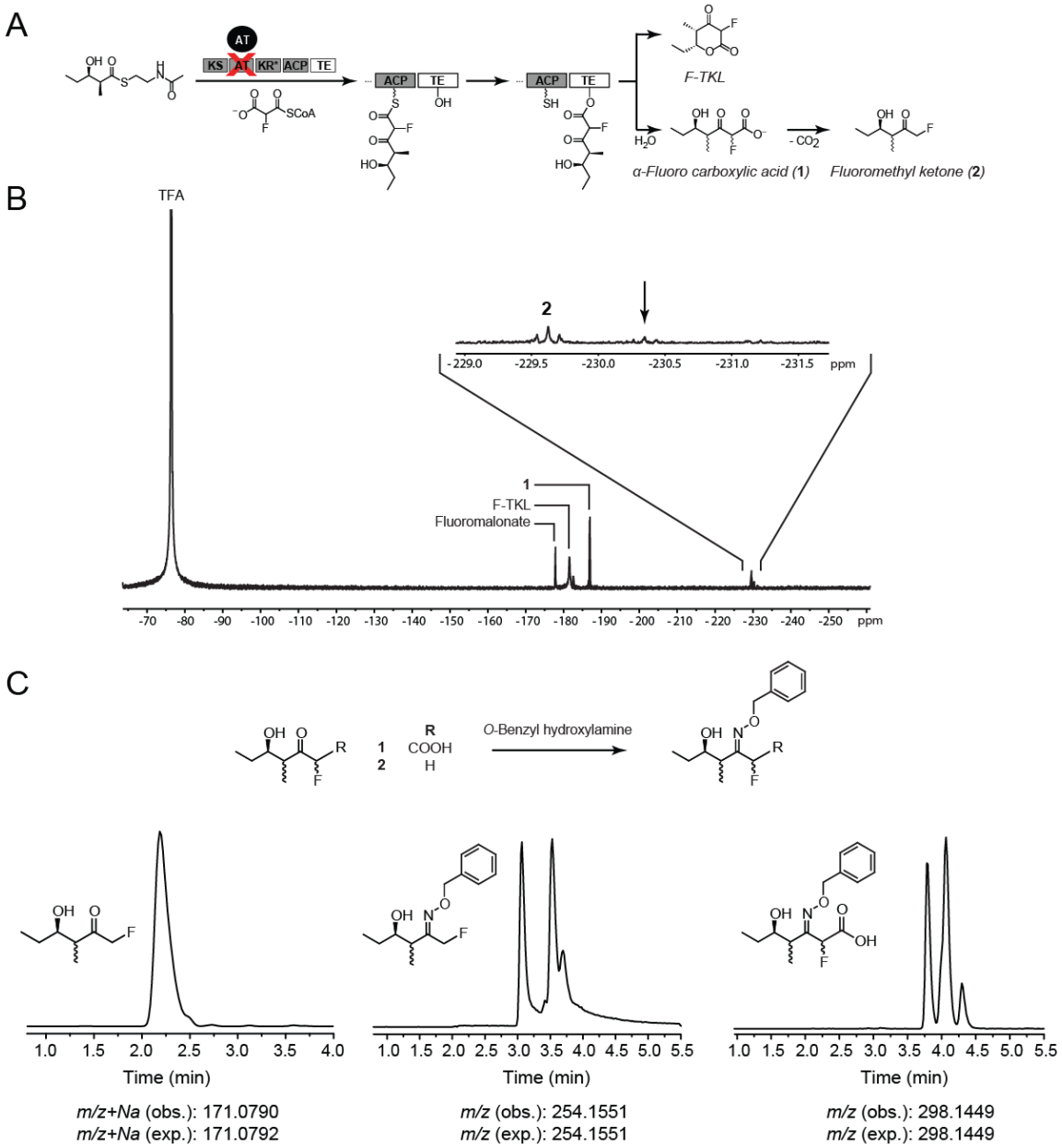
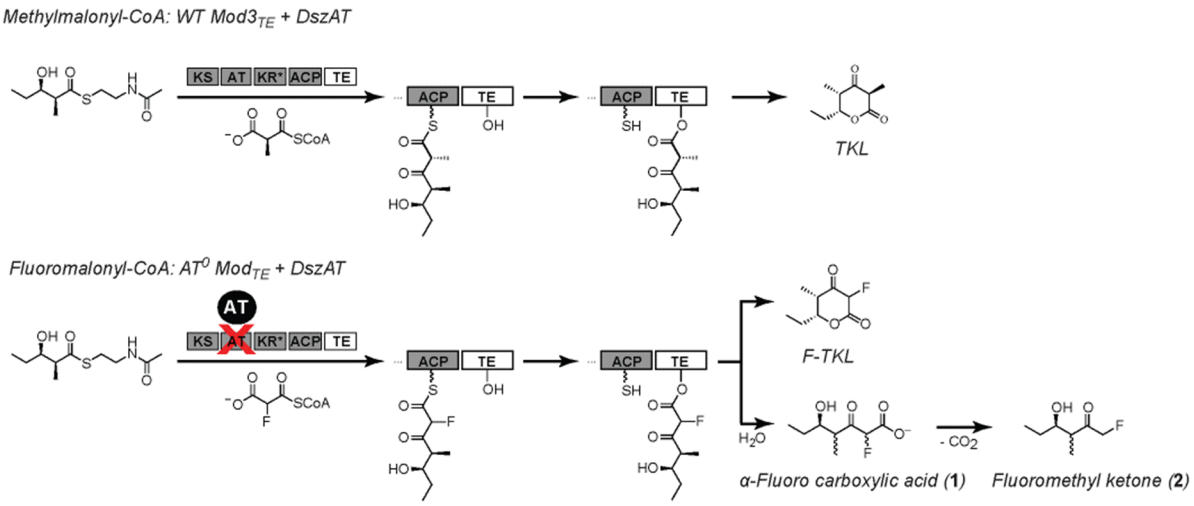
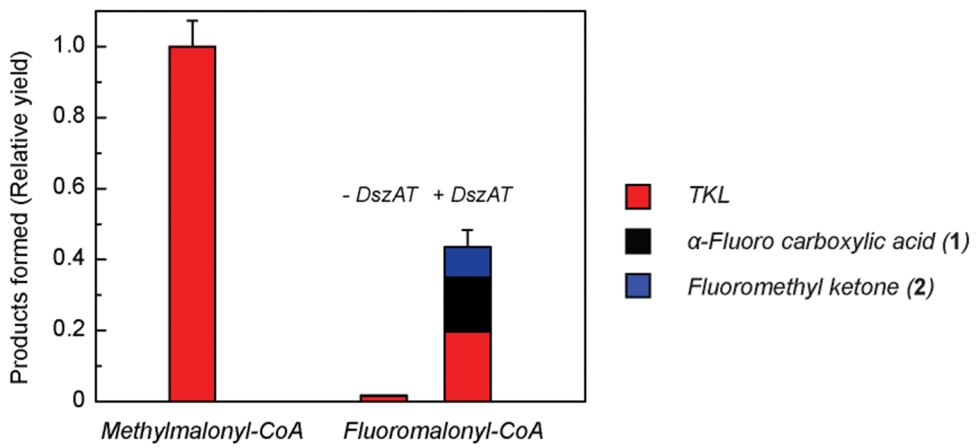


Figure S15. Comparison of C-C bond formation stoichiometry. (A) Reaction pathways for fluoromalonyl- and methylmalonyl-CoA. (B) Normalized bar graph for the relative yields of C-C bond products. Terminal ketones and carboxylic acids were included as a single C-C bond-forming event as they diverge from the F-TKL pathway after fluoromalonyl-CoA is incorporated. Data are represented average ± s.e. (propagated from the s.d. (*n=3*) of the individual products). The fluoromethyl ketone and α-fluorocarboxylic acid were quantified by LC-UV after derivatization with *O*-benxylhydroxylamine. (C) Tabulated data with concentrations of each observed product. Data are average ± s.d. (n.d., not detected; -, not measured)

A



B



C

Construct	DszAT	Extender unit	TKL (μM)	**1** (μM)	**2** (μM)
KS AT ACP	–	R = Me	2600 ± 130	*n.d.*	*n.d.*
KS ~~AT~~ ACP	–	R = F	40.0 ± 0.5	–	3.75 ± 0.5
KS ~~AT~~ ACP	+	R = F	512 ± 27	390 ± 110	226 ± 26

Figure S16. NMR spectra of propionyl-SNAC. (A) ^{1}H-NMR. (B) ^{13}C-NMR.

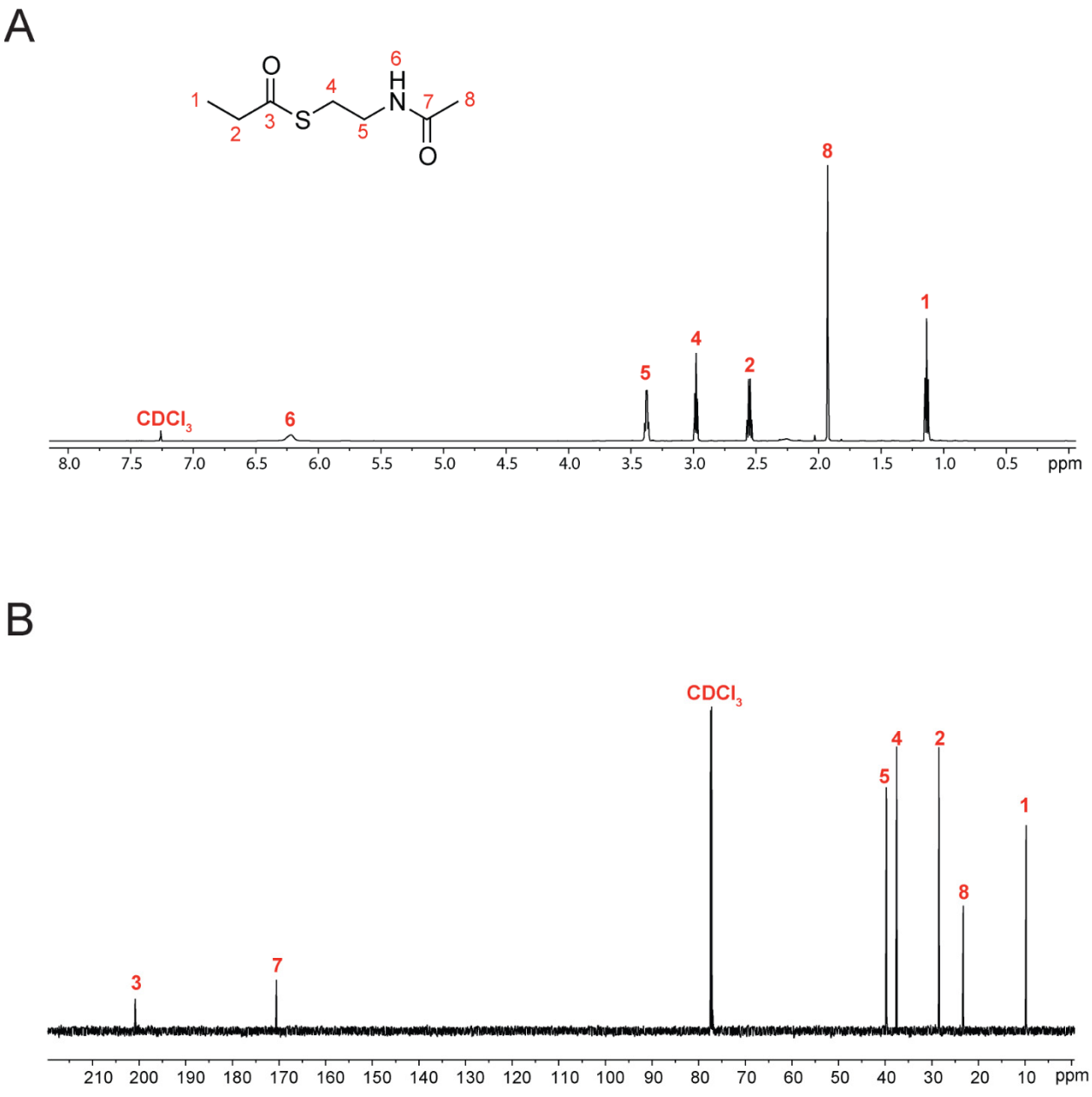


Figure S17. Exact mass spectra for the triketides observed from the mini-PKS. (A) The Me/F product generated using Mod2 + AT^0 $Mod3_{TE}$ and DszAT with unlabled propionyl-SNAC (black) and ^{13}C-labled propionyl-SNAC (red). (B) The F/Me product generated using AT^0 Mod2 + $Mod3_{TE}$ and DszAT with unlabled propionyl-SNAC (black) and ^{13}C-labled propionyl-SNAC (red). (C) The F/F product generated using AT^0 Mod2 + AT^0 $Mod3_{TE}$ and DszAT with unlabled propionyl-SNAC (black) and ^{13}C-labled propionyl-SNAC (red).

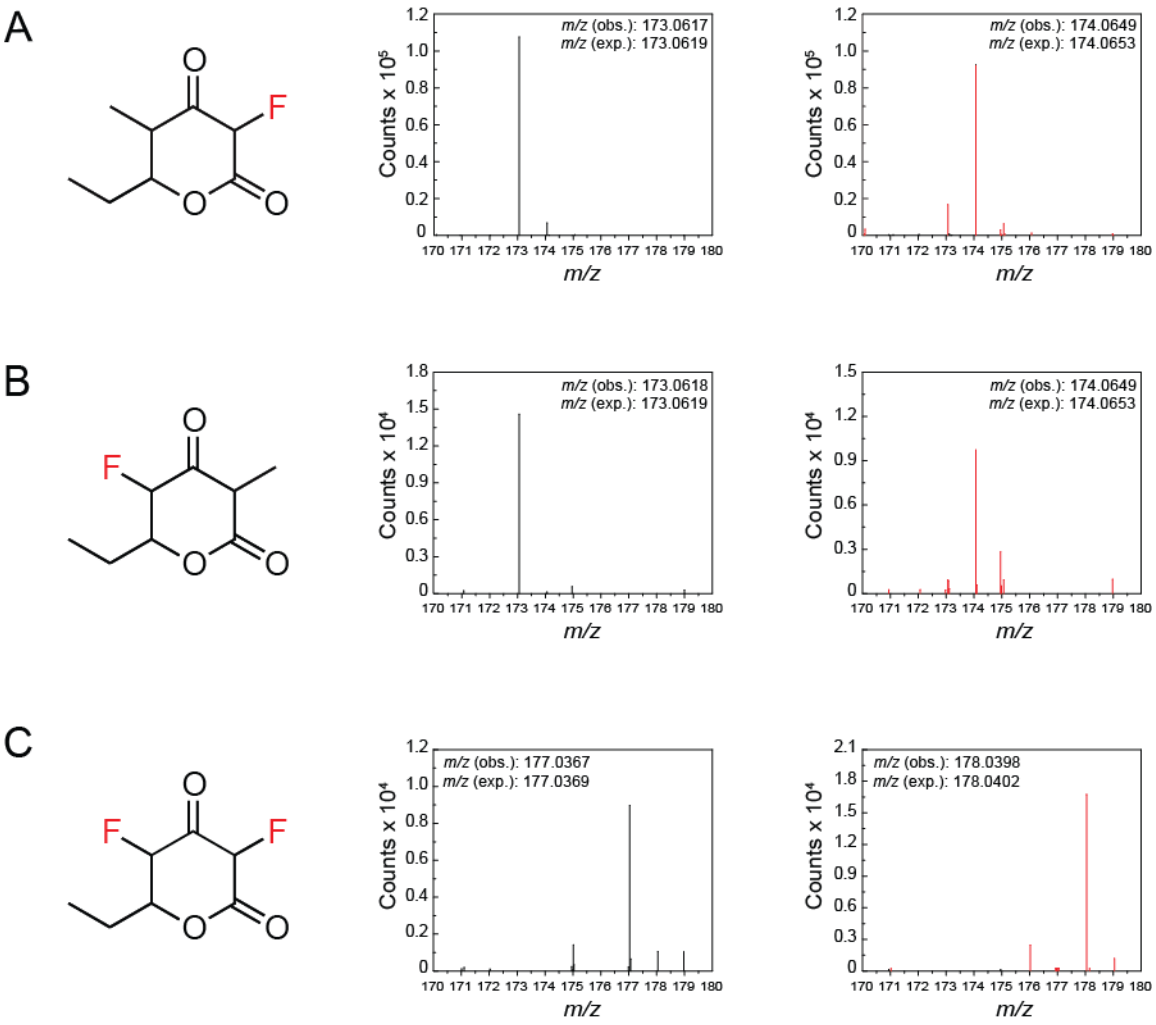
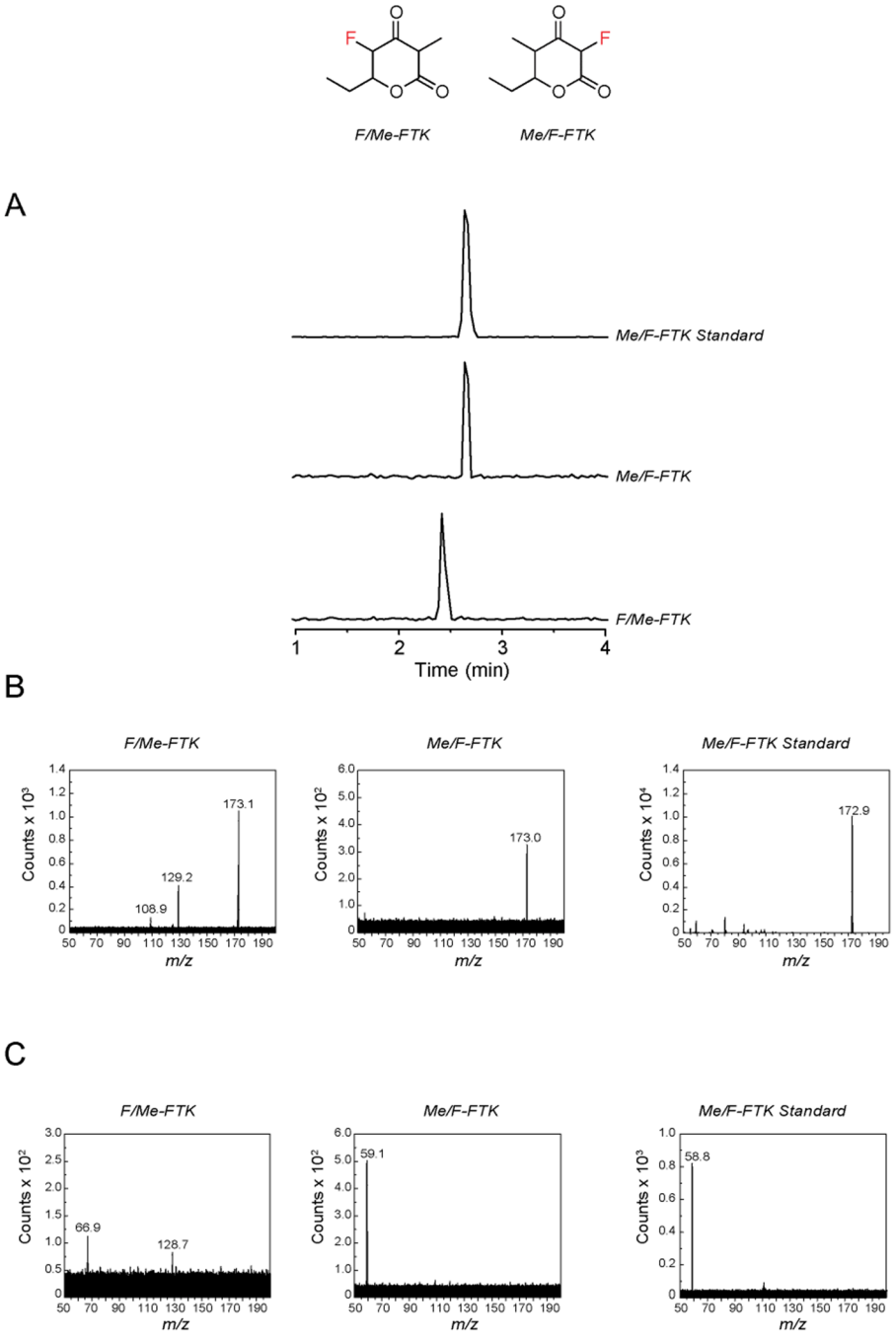


Figure S18. Further characterization of the Me/F and F/Me TKL products. (A) Product ion chromatogram (173.1 *m/z*) for Me/F (Frag. voltage 100 V and collision energy 25 V) and F/Me (Frag. voltage 100 V and collision energy 15 V) reactions as well as an Me/F authentic standard (Frag. voltage 100 V and collision energy 25 V). As shown, the two TKLs have different retention times in this 4 min method. (B) The product ion spectra (Frag. voltage 100 V and collision energy 15 V) for the two TKLs and the chemical standard for the Me/F triketide. (C) The product ion spectra (Frag. voltage 100 V and collision energy 25 V) for the two TKLs and the chemical standard for the Me/F triketide. Note that under these conditions the F/Me and Me/F triketide have different fragmentation patterns.



Literature Cited

1. Pfeifer BA, Admiraal SJ, Gramajo H, Cane DE, & Khosla C (2001) Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*. *Science* 291(5509):1790-1792.
2. Gokhale RS, Tsuji SY, Cane DE, & Khosla C (1999) Dissecting and exploiting intermodular communication in polyketide synthases. *Science* 284(5413):482-485.
3. Wong FT, Chen AY, Cane DE, & Khosla C (2010) Protein-protein recognition between acyltransferases and acyl carrier proteins in multimodular polyketide synthases. *Biochemistry* 49(1):95-102.
4. Gibson DG*, et al.* (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6(5):343-345.
5. Chen AY, Schnarr NA, Kim CY, Cane DE, & Khosla C (2006) Extender unit and acyl carrier protein specificity of ketosynthase domains of the 6-deoxyerythronolide B synthase. *J Am Chem Soc* 128(9):3067-3074.
6. Engler C, Kandzia R, & Marillonnet S (2008) A one pot, one step, precision cloning method with high throughput capability. *PLoS One* 3(11):e3647.
7. Walker MC*, et al.* (2013) Expanding the fluorine chemistry of living systems using engineered polyketide synthase pathways. *Science* 341(6150):1089-1094.
8. Theodorou V, Skobridis K, Tzakos AG, & Ragoussis V (2007) A simple method for the alkaline hydrolysis of esters. *Tetrahedron Letters* 48(46):8230-8233.
9. Bonnett SA, Papireddy K, Higgins S, del Cardayre S, & Reynolds KA (2011) Functional characterization of an NADPH dependent 2-alkyl-3-ketoalkanoic acid reductase involved in olefin biosynthesis in *stenotrophomonas maltophilia*. *Biochemistry* 50(44):9633-9640.
10. Piasecki SK*, et al.* (2011) Employing modular polyketide synthase ketoreductases as biocatalysts in the preparative chemoenzymatic syntheses of diketide chiral building blocks. *Chem Biol* 18(10):1331-1340.
11. Rocha DF, Wouters FC, Machado G, & Marsaioli AJ (2013) First biosynthetic pathway of 1-hepten-3-one in *Iporangaia pustulosa* (Opiliones). *Sci Rep* 3:3156.
12. Palla KS, Witus LS, Mackenzie KJ, Netirojjanakul C, & Francis MB (2015) Optimization and expansion of a site-selective N-methylpyridinium-4-carboxaldehyde-mediated transamination for bacterially expressed proteins. *J Am Chem Soc* 137(3):1123-1129.
13. Dunn BJ, Cane DE, & Khosla C (2013) Mechanism and specificity of an acyltransferase domain from a modular polyketide synthase. *Biochemistry* 52(11):1839-1841.
14. Welch JT & Seper KW (1988) Synthesis, regioselective deprotonation, and stereoselective alkylation of fluoro ketimines. *J Org Chem* 53(13):2991-2999.