

Supporting Information

Elucidation of the Cryptic Methyl Group Epimerase Activity of Dehydratase Domains from Modular Polyketide Synthases Using a Tandem Modules Epimerase Assay

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Figure S1. Mega3.0 (<http://www.megasoftware.net>) sequence alignment of active sites for PKS DH domains including DH domains from reductase-inactive PKS modules from polyether PKS and dehydratase-active DH domains. PKS source: Ery, erythromycin; Mon, monensin; Nan, nanchangmycin; Nig, nigericin; Pic, picromycin; Rif, rifamycin.

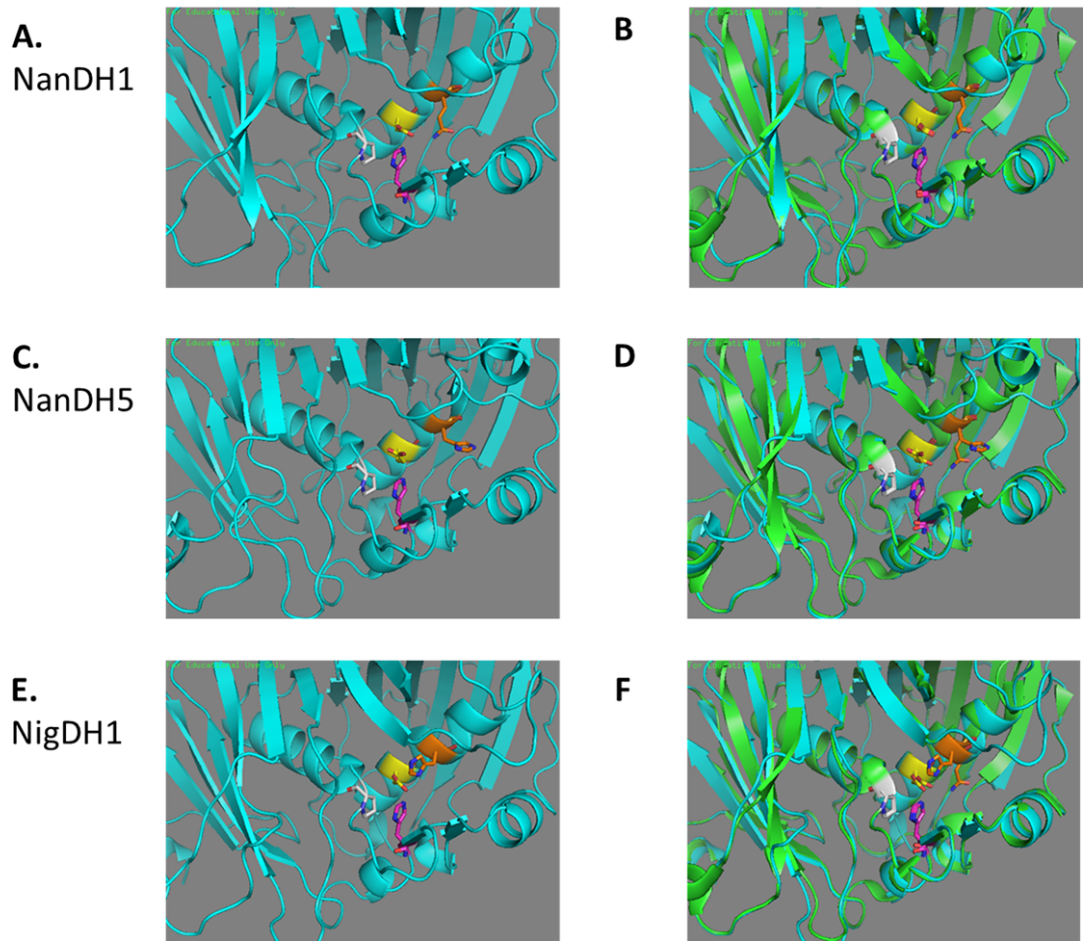


Figure S2. SWISS-MODEL¹ (<https://swissmodel.expasy.org/>) protein structure homology modeling of DH domains, generated using the EryDH4 structure (PDB number: 3EL6) as the template. A. Predicted active site center of NanDH1; B. Protein structure overlap of NanDH1 with EryDH4; C. Predicted active site center of NanDH5; D. Protein structure overlap of NanDH5 with EryDH4; E. Predicted active site center of NigDH1; F. Protein structure overlap of NigDH1 with EryDH4.

Materials. Cosmid 3C5 encoding NanDH1 and NanDH5 from *Streptomyces nanchangensis* has been previously described.² DNA primers were synthesized by Integrated DNA Technologies. Ampicillin, carbenicillin, chloramphenicol, isopropylthio- β -D-galactopyranoside (IPTG), kanamycin and Phusion Flash High-Fidelity PCR Master Mix were purchased from Thermo Scientific. All other chemical reagents purchased from Sigma-Aldrich were of the highest quality available and utilized without further purification. Restriction enzymes and T4 DNA ligase were purchased from NEB and used according to the manufacturer's specifications. Competent *E. coli* 5-alpha, DH10 beta, and BL21(DE3) were purchased from New England Biolabs Inc (NEB). Pre-charged 5 mL HisTrapTM FF columns were purchased from GE Healthcare Life Sciences. Amicon Ultra Centrifugal Filter Units (Amicon Ultra-15, 5K, 10K, 30K, 100K MWCO) were purchased from Millipore. The expression vectors for PicKR3⁰, NanKR1⁰, AmpKR2, EryKR6, and TylKR1, were cloned into *E. coli* BL21(DE3) and protein was expressed and purified using the same protocols used as previously described.³⁻⁶ NANS Module 2+TE was expressed in the *E. coli* BAP1 strain harboring the *sfp* gene for the surfactin phosphopantetheinyl transferase and purified using the previously described protocols.⁷ Nan[KS1][AT1] was expressed in *E. coli* Rosetta 2(DE3) from Novagen as previously described and purified through the immobilized metal ion affinity step to >95% homogeneity.⁴ *holo*-NanACP1 was expressed and purified following the previously described protocols.^{4,7} Recombinant *apo*-EryACP6, and Sfp were each expressed and purified as previously described.^{8,9} EryDH4 and NanDH2 were each expressed and purified as previously described.^{7,8} SDS-PAGE gradient gels (4-20% or 12% acrylamide) and Precision Plus ProteinTM Standards (dual color) were purchased from Bio-Rad. Reference samples of methyl (2*RS*,4*RS*)-2,4-dimethyl-5-ketohexanoate, prepared as previously described,⁷ were used as standards for chiral GC-MS analysis. 2-Methyl-3-ketopentanoyl-SNAC and acetyl-SNAC were synthesized as previously described.¹⁰ The ACP-bound substrate (2*R*,3*S*)-[2-²H]-2-methyl-3-hydroxypentanoyl-EryACP6 ([2-²H]-**6b**) was prepared as previously described.^{11,12} (2*R*,3*S*)-2-Methyl-3-hydroxypentanoyl-EryACP6 (**6b**) and (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-EryACP6 (**6a**) were each prepared as previously described.⁸

Methods. General methods were as previously described.^{9,13} All DNA manipulations were performed following standard procedures.¹³ Plasmid DNA was purified using a NEB Monarch[®] Plasmid Miniprep Kit. DNA sequencing was carried out by Genewiz, South Plainfield, NJ. Synthetic genes,

optimized for expression in *E. coli*, were prepared by DNA 2.0. Growth media and conditions used for *E. coli* strains and standard methods for handling *E. coli* *in vivo* and *in vitro* were those described previously,¹³ unless otherwise noted. All proteins were handled at 4 °C unless otherwise stated. Protein concentrations were determined according to the method of Bradford, using Tecan Microplate Reader with bovine serum albumin as the standard. Protein purity and size was estimated using SDS-PAGE and visualized using Coomassie Blue stain and analyzed with a Bio-Rad ChemiDoc MP System. Protein accurate molecular weight was identified by Agilent 6530 Accurate-Mass Q-TOF LC-MS. A Thermo LXQ equipped with Surveyor HPLC system and a Phenomenex Jupiter C4 column (150 mm × 2 mm, 5.0 μm) was utilized for analysis of diketide-ACP compounds. HPLC-ESI-MS/MS analysis was carried out in positive ion mode for analysis of pantetheinate ejection fragments, as previously described. Chiral GC-MS analysis was carried out on a GC-MS HewlettPackard Series 2 GC-MSD, 70 eV EI in positive ion mode with a Varian CP-Chirasil-DEX CB capillary column, 25 m × 0.32 mm.

Domain boundaries and design of recombinant NigDH1

Figure S3. Nigericin PKS module 0 and 1.

LOCUS ABC84456 2415 aa linear BCT 29-JUN-2007
DEFINITION NigAI [*Streptomyces violaceusniger*].
ACCESSION ABC84456
VERSION ABC84456.1
DBSOURCE accession DQ354110.1

```
1 MDSAAPTRTPQQAEPPIAVVGMACRLPHAPSPSAFWRLLRQGGNAITTLPDDRRRTGTATT
  (.....NIGERICIN PKS LOADING MODULE.....)
961 GLTFKELGFDLSLAATELSERLGAATGLPLTATLTFDHPTPLAVADHLRARTPATGPSAT
1021 PPAATTPAPRDTDEPIAVVAMGCRYPGGVDSPEALWRLVAEGADAIGEFPRDRGWDLAAL
1081 FDPDPERPGTSHAHEGGFLHDASEFDAEFFGISPREALATDPQQRLLLETAWETFERAGI
1141 RSTALRSSPTGVFVGVTSQDYGPRLHEAPKGLDGHLLLTGGTPSVASGRVAFTFGLEGPAV
1201 SVDTACSSSLVAVHLAVRALRQGECTLALAGGVTVMAAPGMFTGFSRQRLAPDGRCKPF
1261 AAAADGTGWGEGVGLLLLERLSDARRDGHRVLAVIRGSAVNQDGASNGLTAPNGPSQQRV
1321 IRQALADARLSPSEVDAVEAHGTGTTLGDPIEAQALLATYGRQRPGDRPLWLGLSKSNIG
1381 HTQAAAGVAGVIKVMAMRHGLLLPATLHIDAPSPHVNWDGGAVRLLTERVEWRRDEHPRR
1441 AGVSSFGISGTNAHLILEQAPEPGPAPSGARTDEGHRVFPWVLSARSAEALRGQARELAA Nig [KS1] [AT1]
1501 RTAADHTAADHTPSPLDVGWSLITTRTAFEHRAVVVGGDRAELTAALQSLAADETHPGVV
1561 GPDVTRSGAAADPGPVLVFPQGSQWAGMGAGLLDASPVFAARVAECERALAPHVDWSLT
1621 DVLRGAEGAAELSRVDVPPVLWAAMVSLAAVWAEYGVRPPAVVGHSQGEIAAAVVAGAL
1681 SLEDGAKVVALRSKALRRLAGGGAMASLALGHERTEELSLGLGDRAAAVVAVVNGPEST
1741 VVSGPPEQVAAAVTACREADERARLIEVNYASHSPQVDEIAHELIELLGGVEPVEVSGSG
1801 VAFYSTVTGGRADVSVLDTGYWVRNLRERVRFAEAIQALLADGHRVFIEASTHPVLTMAM
1861 RESFEHADSGAAAVPTLRRDHGDLAQLTKSVARAFLAGAEVDWSAAFPADPTPCTVDLPT
1921 YAFQRRRYWLDAPGGPGGDPRALGLAAADHPLLGAAVRLADGSGHVLTGRLSPQTHGWLA
1981 DHVVAGVALVPAALMEWALRAADESGCGAVEELALRLPLVMPTTGGRCVQVMVGPPADD
2041 GRRDLAIYSLPEEALRTGGDTDMCHAVGVLPAVPESGATEPPGTWPPPEAELMDTDGF NigDH1
2101 YERIAASGYAYGAAFQGLRAVWRDGADLVADVELPKAAGEPGGFGIHPALLDAALHPTLL
2161 TGHLDSGPEHTGERMWLPFTVSGVSLWAAEATAVRVRLTPGSRPAEGERELRVVADAVG
2221 APVLTIDALVLRPAEADQLRSLNTGRVATAGGSGGVRRRAAAAAGVSSVDWAARLARLS
2281 AVDRYRLLGLVREHAATVLGHTDAEAVHADANFKELGFDSLTAVELRDLAAATGLRLP
2341 AALIFRYPTPEGIAHHLVRLSTDGTAATPTATVPNTSTRLPRQPTDEMSAAQRLESAS NigACP1
2401 ADQVLEFIDNELGVS
```

Figure S3. Nigericin PKS Modules 0 and 1. Domain boundaries and design of recombinant NigDH1.

NigDH1 Amino Acid Sequence. The synthetic gene encoding the NigDH1 domain was subcloned into the corresponding sites of the *Nde*I- and *Xho*I-digested pET-28a vector and the recombinant protein was expressed with an N-terminal His₆-tag in *E. coli* BL21 (DE3).

APGGPGGDPRALGLAAADHPLLGA~~AVRLADGSGHVLTGRLSPQTHGWLADHVVAGVALVPAALMEWAL~~
RAADESGCGAVEELALRLPLVMP~~TTGGRCVQVMVGP~~PADDGRRDLAIYSLPEEALRTGGDTDMCHAVG
VLGPAVPESGATEPPGTWPPPEAELMDTDG~~FYERIAASGYAYGAA~~FQGLRAVWRD~~GADLVADVELPKAA~~

GEPGGFGIHPALLDAALHPTLLTGHLDSGPEHTGERMWLPFTVSGVSLWAAEATAVRVRLTPGSRPAEG
EREELRVVVADAVGAPVLTIDALVLRPAEADQLRSLNTGRVATAGG

(N-terminal *NdeI* site)

CATATGGCACCGGGTGGCCCGGGTGGCGACCCGCGTGCCTGGGTCTGGCGGCGGCGGATCACCCACTG
CTGGGTGCCGAGTCCGTCTGGCGGACGGTAGCGGTTCATGTGCTGACCGGCCGTCTGTCTCCGAAACT
CACGGTTGGCTTGGGACCACGTAGTGGCGGGTGTTCGCTGGTCCCAGGCGGCGAGCACTGATGGAATGG
GCGCTGCGTGCAGCGGACGAGTCCGGCTGTGGTGTGTTGGAAGAACTGGCCCTGCGTTTGCCGCTGGTT
ATGCCGACGACCGGCGGCCGCTGCGTTCAGGTTCATGGTCCGCCCCGCGGCGGATGACGGTCGTCGTGAC
CTGGCAATTTACAGCCTGCCGGAAGAAGCCCTGCGCACCGGTGGCGACACGGATTGGATGTGCCACGCC
GTGGGTGTTCTGGGTCCGGCAGTCCCTGAGAGCGGTGCCACCGAGCCACCTGGTACCTGGCCGCCGCCG
GAAGCGGAACTGATGGATAACCGACGGTTTCTACGAGCGCATTGCAGCGTCCGGTTACGCCTATGGTGCA
GCGTTTCAAGGTCTGCGTGCAGGTGTGGCGGACGGTGCCGATCTGGTGGCCGATGTTGAACTGCCGAAA
GCTGCCGGTGAGCCGGGTGGCTTCGGCATCCATCCGGCACTGCTGGACGCTGCTCTGCATCCGACGCTG
TTGACCGGCCACCTGGATAGCGGTCCGGAGCACACTGGCGAGCGTATGTGGCTGCCGTTACCGTGAGC
GGCGTTTTCGCTGTGGGCAGCCGAGGCCACGGCAGTGCCTGTCCGCCTGACCCAGGTAGCCGTCCGGCA
GAGGGCGAGCGGAGCTGCGTGTGTCGTCGCGGATGCCGTGGGTGCCCGGTTTTGACGATCGATGCT
CTGGTGTTCGTCGCGCGGAAGCGGACCAGCTGCGCAGCCTGAATACCGGCCGTGTCGCGACCGCGGGT
GGCTAACTCGAG (Stop codon and C-terminal *XhoI* site)

Cloning of NanDH1. On the basis of the domain boundaries as previously illustrated,^{2,4} the DNA sequence from Cosmid 3C5 encoding NanDH1 was amplified by PCR using the primers 5'-ATCGTAATCCATATGTGGCTGGCGCCGGACCACGGCCGCGAGGGCCGCACGGCC-3' and 5'-TGATTCGATGAATTCAACCACCCGCGGCCCCGCTCGCCTCGCGCACCTGCCG-3' carrying *NdeI* and *EcoRI* restriction sites, respectively (underlined) and stop codon (bold). The resultant amplicon was ligated into the corresponding sites of pET28a to give the NanDH1 expression vector pXXQ-NanDH1.

Cloning of NanDH5. The DNA sequence from Cosmid 3C5 encoding NanDH5 was amplified by PCR using the primers 5'-ATCGTAATCCATATGGCGGCTGGCCGCGGACCAGCGACGTCAGCGGGCCGG-3' and 5'-TGATTCGATGAATTCACGCGCCGTGGAACGCCGTTCCGCGGCTTTCAGCCAGTCGCCG-3' carrying *NdeI* and *EcoRI* restriction sites, respectively (underlined) and stop codon (bold). The resultant amplicon was ligated into the corresponding sites of pET28a to give the NanDH5 expression vector pXXQ-NanDH5.

General Procedures for Recombinant Protein Expression and Purification. The expression plasmids for NanDH1, NanDH5 and NigDH1 proteins were individually transformed into competent cells of *E. coli* BL21 (DE3) and the resulting single colonies were inoculated into LB media containing 50 mg/L kanamycin and incubated overnight at 37 °C. The 10 mL overnight seed culture was transferred into 500 ml of Super Broth with 50 µg/ml kanamycin in a 2.5 L flask and grown at 37 °C until an OD₆₀₀ of 0.4-0.8. The broth was cooled to 18 °C for 1 h and added 0.2 mM of final concentration IPTG to induce protein expression and the cell culture was continuously grown for an additional 40-48 h at 18 °C. The cells were harvested by centrifugation at 4,200 g for 20 min and the cell pellet was washed by water and dissolved in 35 ml lysis buffer (1 M NaCl, 50 mM phosphate, 50 mM imidazole, pH 7.8) and stored at -80 °C. The cells in lysis buffer from -80 °C freezer were thawed at room temperature, following sonication and removal of cell debris by centrifugation (23 000g for 50 min), the supernatant was passed through a 0.45 µm PVDF membrane filter and loaded on a lysis-buffer-equilibrated, precharged 5 mL HisTrap™ FF column (GE Healthcare). The column was washed with 25 mL lysis buffer and then 25 mL washing buffer (50 mM sodium phosphate, 1 M NaCl, 60 mM imidazole, pH 7.6). Finally, proteins were eluted from the Ni-column by elution buffer (150 mM NaCl, 50 mM phosphate, pH 7.4, 150 mM imidazole). The eluted fractions were collected, concentrated by ultrafiltration with an Amicon filter MWCO 30 K, the buffer was exchanged with exchange buffer (50 mM sodium phosphate, 10% glycerol, 100 mM NaCl, pH 7.2), concentrated, and stored at -80 °C until use. Protein purity was assessed as >90% by 4-20% acrylamide SDS-PAGE and Bio-Rad Image Lab Software, and His₆-tagged fusion proteins were utilized without further modifications. The molecular mass M_D of each protein was verified by Agilent Technologies Q-TOF LC-ESI-MS and matched the predicted values.

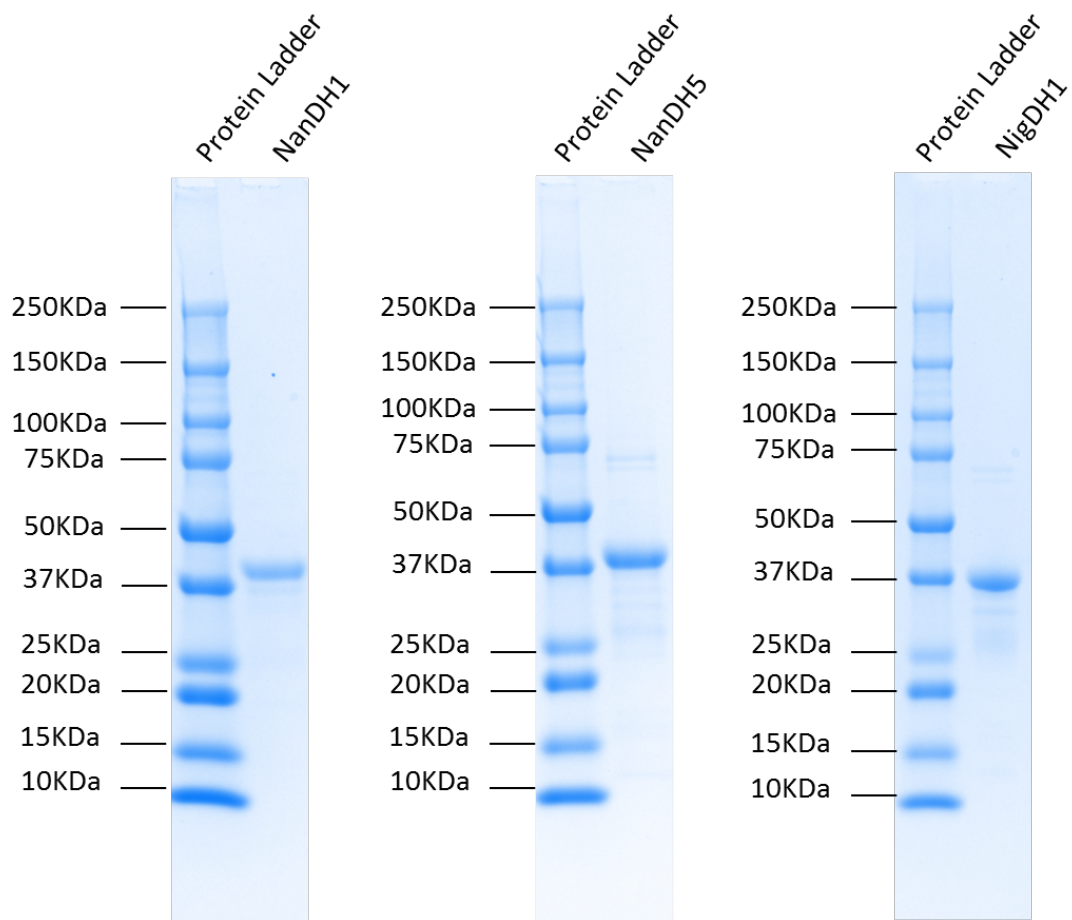


Figure S4. SDS-PAGE analysis of recombinant NanDH1, NanDH5 and NigDH1.

Table S1. Predicted MW and observed ESI-MS MD of recombinant NanDH1, NanDH5 and NigDH1.

Protein	MW (calc, Da)	LC-QTOF (M_D, Da)
NanDH1	36089.27	36089.82
NanDH5	35433.25	35433.09
NigDH1	35093.74	35094.59

Incubation of DH domains with (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (5a). The (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC was obtained by incubation of 20 μ M TylKR1, 10 mM 2-methyl-3-ketopentanoyl-SNAC, 10 mM NADPH in 50 mM NaH₂PO₄ buffer (100 mM NaCl, 2.5 mM TCEP pH 7.2) in a total volume of 500 μ L for 24 h at room temperature. (The incubation mixture also contained \sim 1% glycerol from the protein solutions and DMSO from the SNAC substrate solution.) The reaction mixture was acidified with 100 μ L 1 M HCl and extracted with ethyl acetate (3 \times 600 μ L). After evaporation of the solvent, the concentrated organic extract was dissolved in 650 μ L of 50 mM NaH₂PO₄ buffer (100 mM NaCl, 2.5 mM TCEP pH 7.2), then 200 μ L was transferred into each tube containing 50 μ L of 125 μ M NanDH1, or NanDH5 or NigDH1 and incubated for 2h at room temperature. The reaction mixture was acidified with 100 μ L of 1 M HCl and extracted with ethyl acetate (3 \times 600 μ L). The concentrated organic extract was dissolved in 300 μ L methanol and analyzed by HPLC-MS. HPLC was carried out at a flow rate of 0.2 ml/min at room temperature. Eluent A was 0.1% formic acid in water, and eluent B was 100% acetonitrile. HPLC conditions used were 5%-95% buffer B for 15 min, 95-100% buffer B for another 5 min, 100-5% buffer B for 5 min and then 5% buffer B for 5 min.

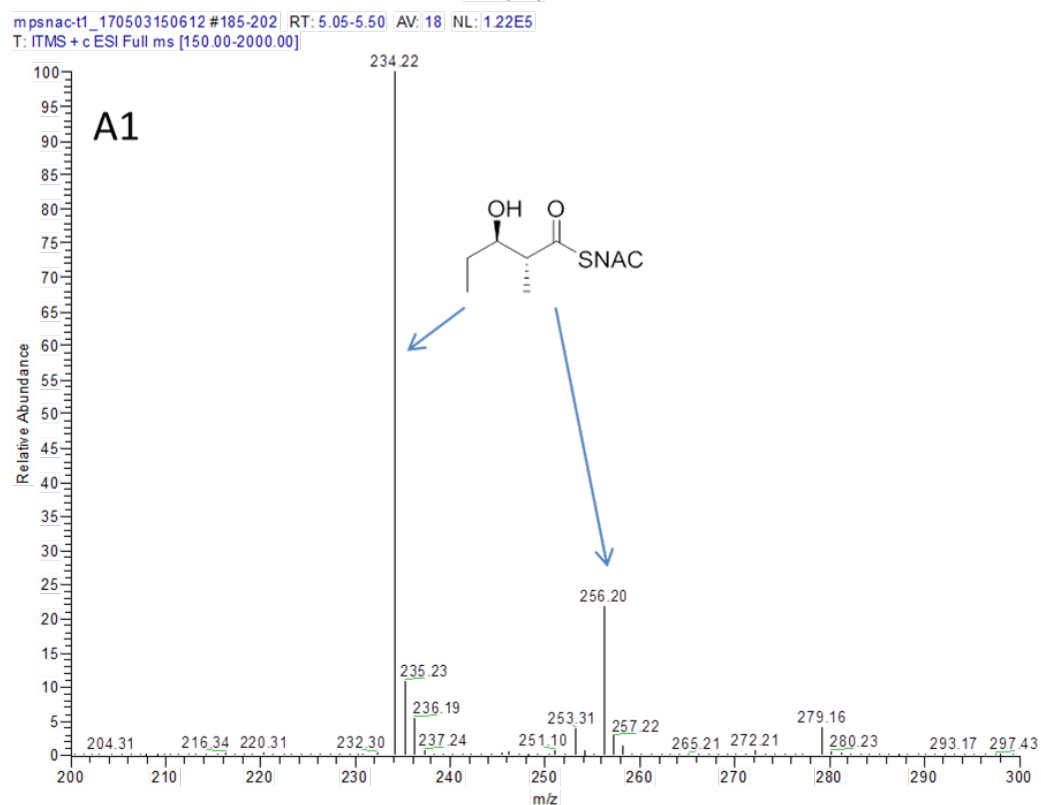
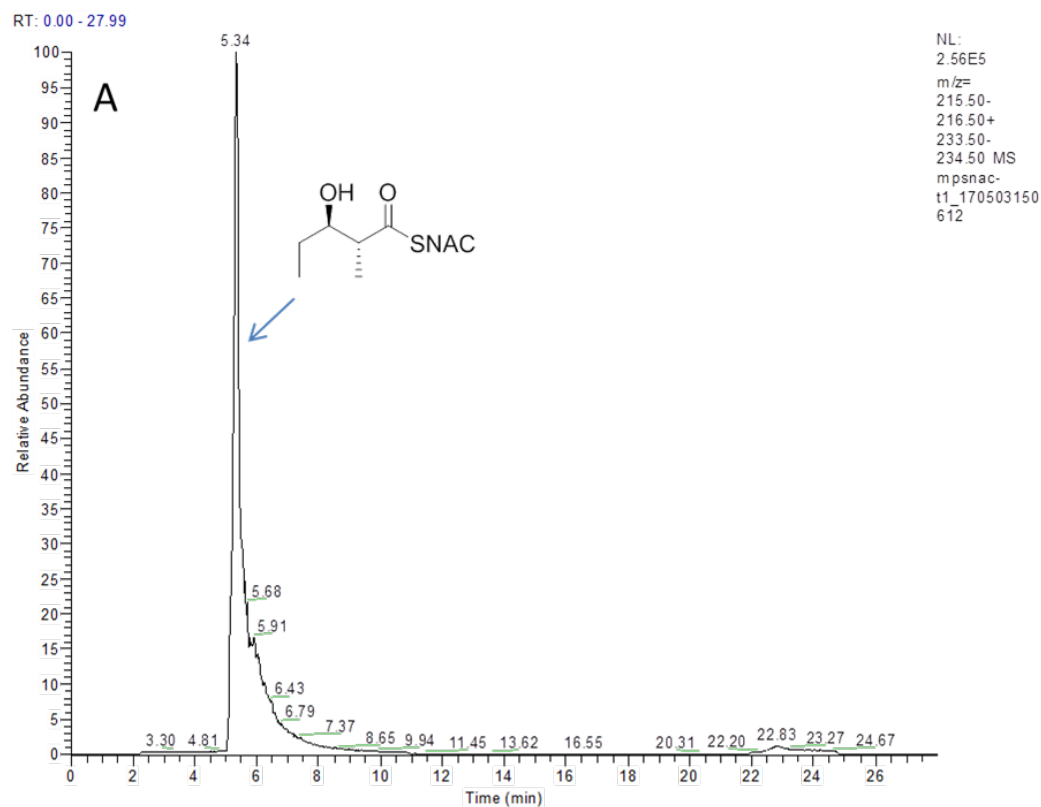


Figure S5. LC-MS analysis of extracted products (*2R,3R*)-2-methyl-3-hydroxypentanoyl-SNAC (**5a**) from incubation of 2-methyl-3-ketopentanoyl-SNAC with TyIKR1 in presence of NADPH. A, (*2R,3R*)-2-methyl-3-hydroxypentanoyl-SNAC (**5a**); A1, MS for **5a** from A.

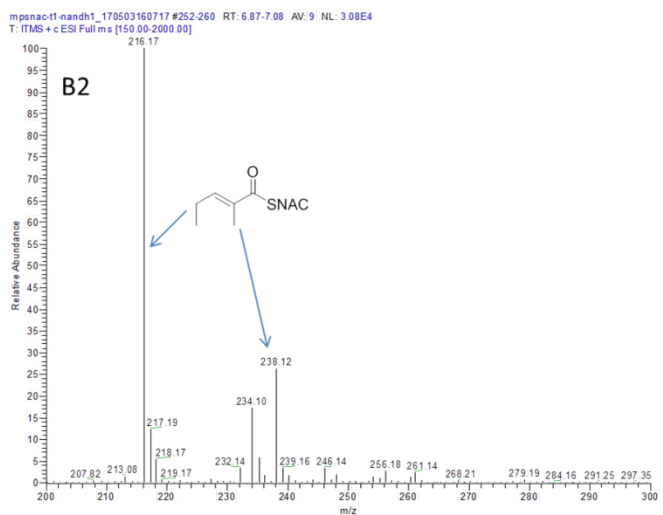
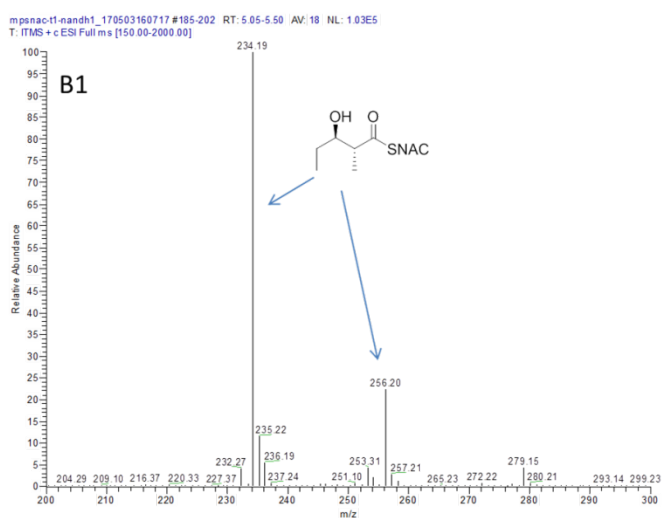
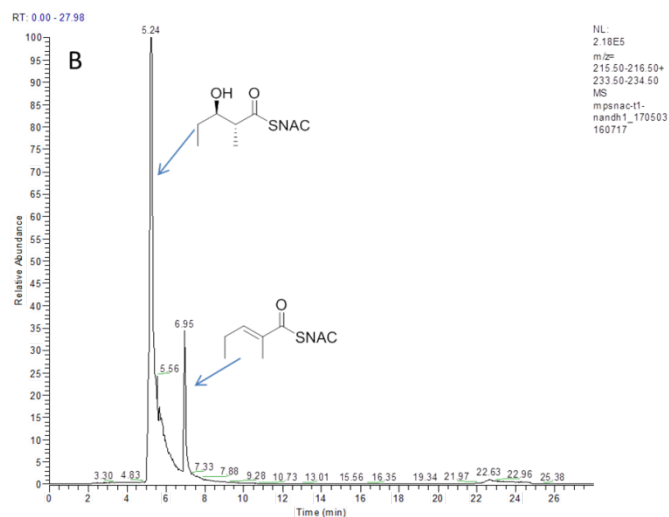


Figure S5 (cont). LC-MS analysis of extracted products from incubation of **5a** with NandH1 domain. B, incubation of **5a** from A with NandH1; B1, MS of **5a** from B; B2, MS of **7** from B.

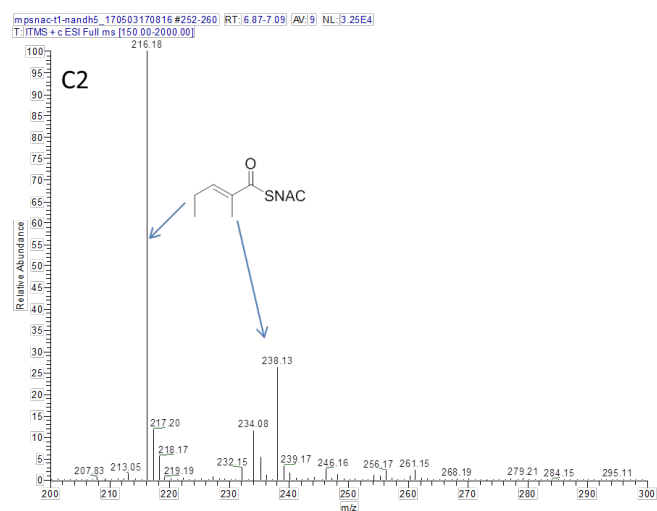
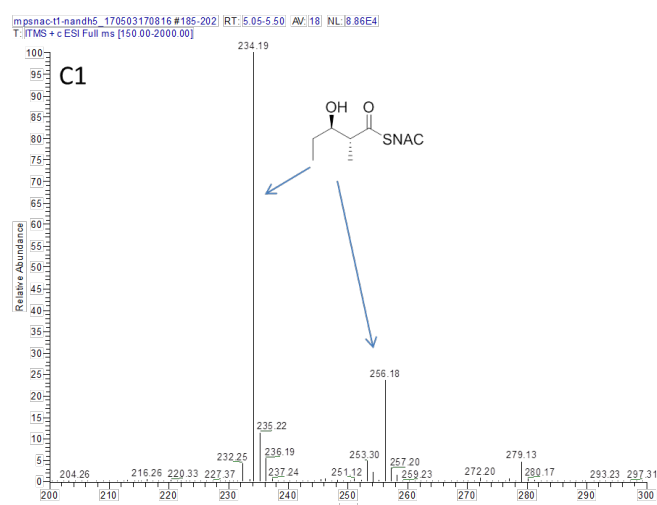
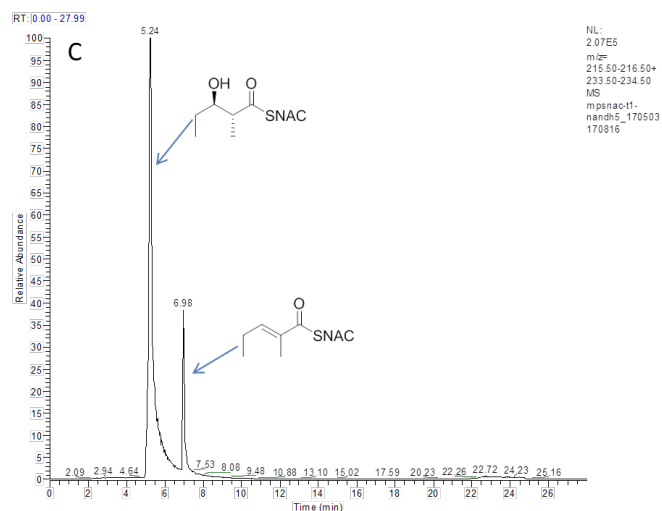


Figure S5 (cont). LC-MS analysis of extracted products from incubation of **5a** with NandH5 domain. C, incubation of **5a** from A with NandH5; C1, MS of **5a** from C; C2, MS of **7** from C.

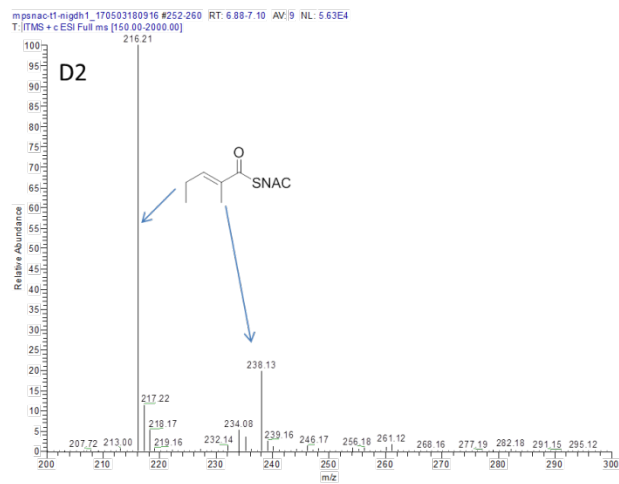
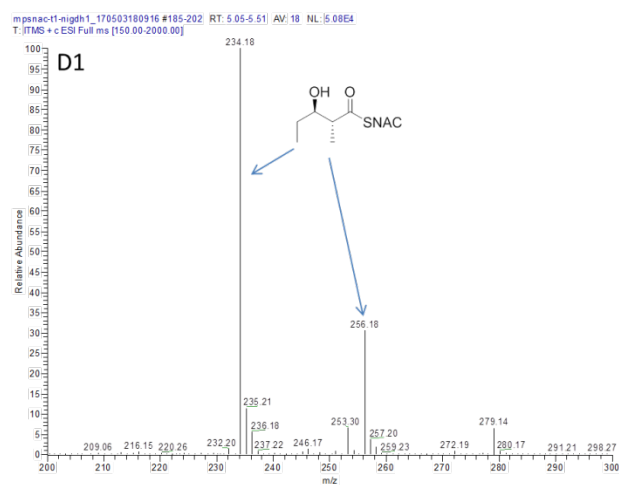
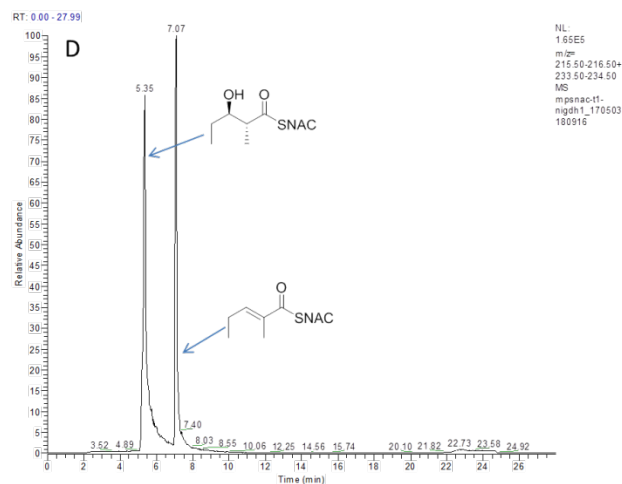


Figure S5 (cont). LC-MS analysis of extracted products from incubation of **5a** with NigDH1 domain. D, incubation of **5a** from A with NigDH1; D1, MS of **5a** from D; D2, MS of **7** from D.

Incubation of DH domains with (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SNAC (5b**).** The incubation and treatment were carried out as described above with incubation of DH domains and (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC, except the TylKR1 domain was replaced by the AmpKR2 domain. There was no dehydrated product detected by HPLC-MS.

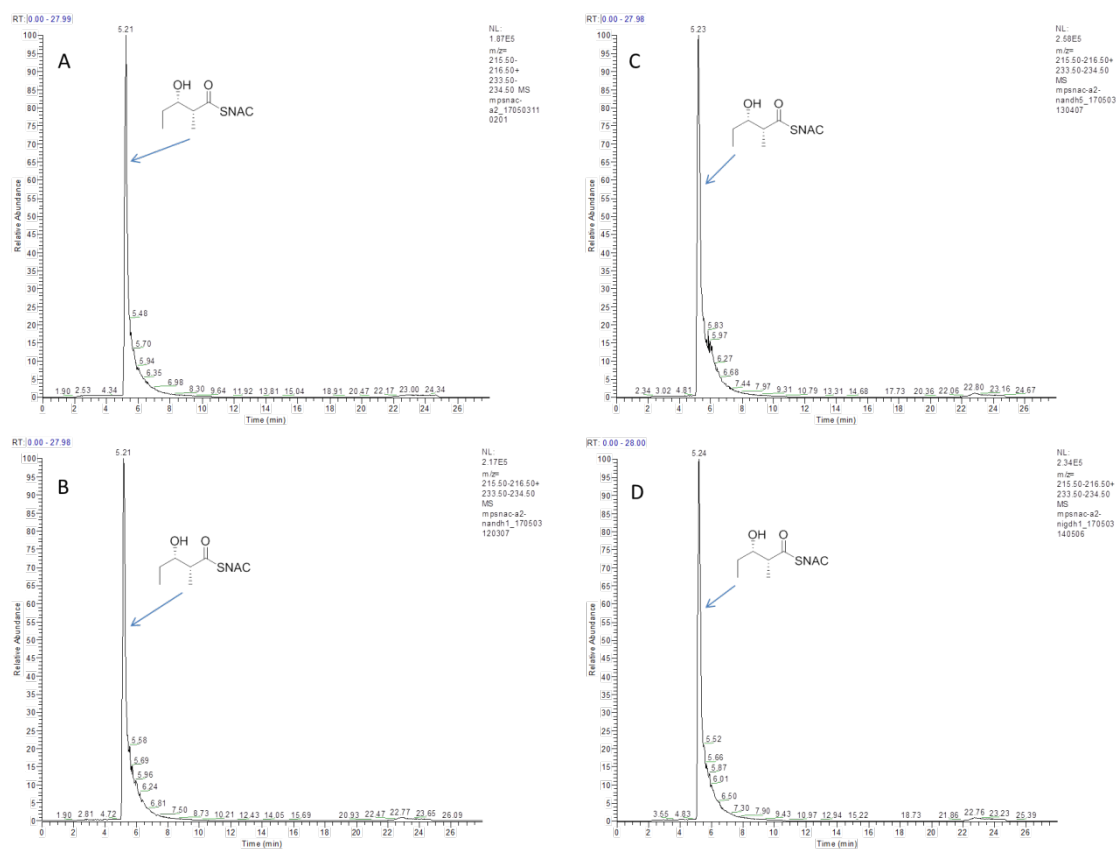


Figure S6. LC-MS analysis of extracted products from incubation of 2-methyl-3-ketopentanoyl-SNAC with AmpKR2, NADPH, and DH domains. A, **5b** from incubation of 2-methyl-3-ketopentanoyl-SNAC, AmpKR2, and NADPH; B, incubation of A with NandH1; C, incubation of A with NandH5; B, incubation of A with NigDH1. No dehydration product was detected.

Incubation of (2*R*,3*R*)-2-Methyl-3-hydroxypentanoyl-ACP (6a-SEryACP6) with DH proteins. (2*R*,3*R*)-2-Methyl-3-hydroxypentanoyl-EryACP6 (**6a**) was prepared as described previously.⁸ In a typical assay, a mixture of 120 μ M *apo*-ACP6, 30 μ M Sfp, 10 mM MgCl₂, and 400 μ M (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-CoA (**6a-CoA**) in a total volume of 200 μ L of 50 mM phosphate buffer (2.5 mM TCEP, pH 7.2) was incubated for 30 min at 37 °C. LC-ESI (+)-MS² analysis confirmed the formation of predominantly (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP (**6a-SEryACP6**). The collision-induced (CID) ppant ejection of the *m/z* 904.46 ion [M¹³⁺] gave the predicted fragment ion *m/z* 375 corresponding to (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-pant (**6a-pant**) (Figure S7). A mixture of 105 μ M (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-SACP6 (**6a-SEryACP6**) and 20 μ M DH protein (NanDH1, NanDH5 or NigDH1) in a total volume of 40 μ L of 50 mM of phosphate buffer (2.5 mM TCEP, pH 7.2) was incubated for 30 min at room temperature. LC-ESI(+)-MS² analysis confirmed the formation of 2-methyl-2-pentenoyl-ACP (**8-SEryACP6**) (fragment ion *m/z* 357 (observed); predicted *m/z* 357), thereby confirming that all DH proteins can catalyze the dehydration reaction of (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-SACP6 (**6a-SEryACP6**) (Figures S8-S10).

Similar protocols were carried out to prove that none of the three DH proteins could catalyze the dehydration reaction of (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP6 (**6b-SEryACP6**) by incubation of chemoenzymatically prepared (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP6 (**6b-SEryACP6**) and each DH domain protein for 1 h at room temperature. No dehydrated product was observed by LC-MS/MS.

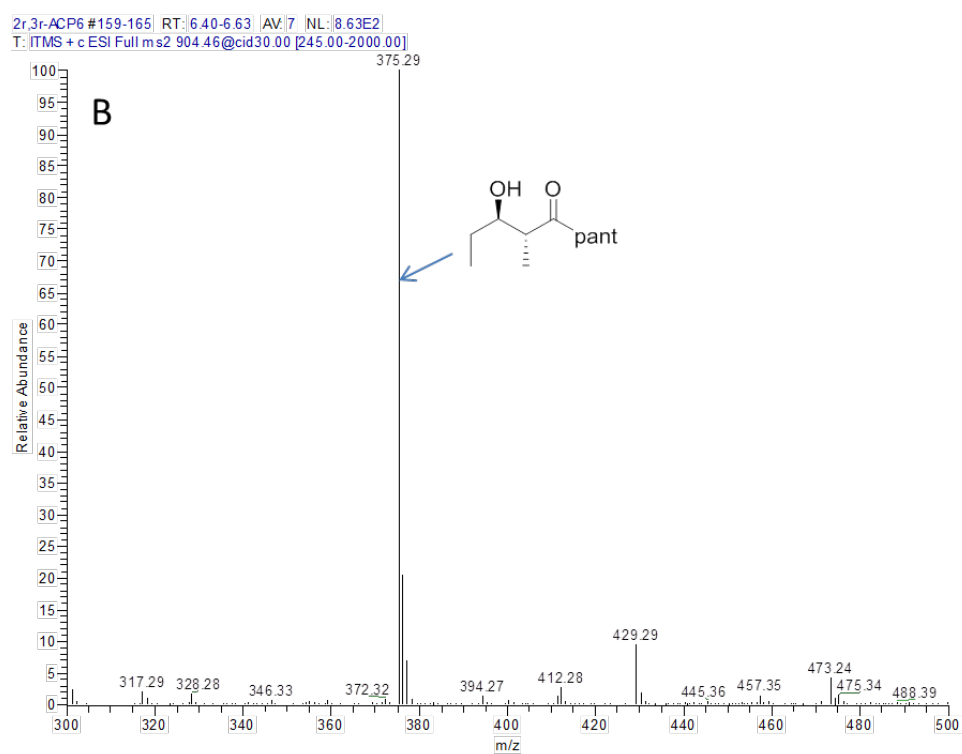
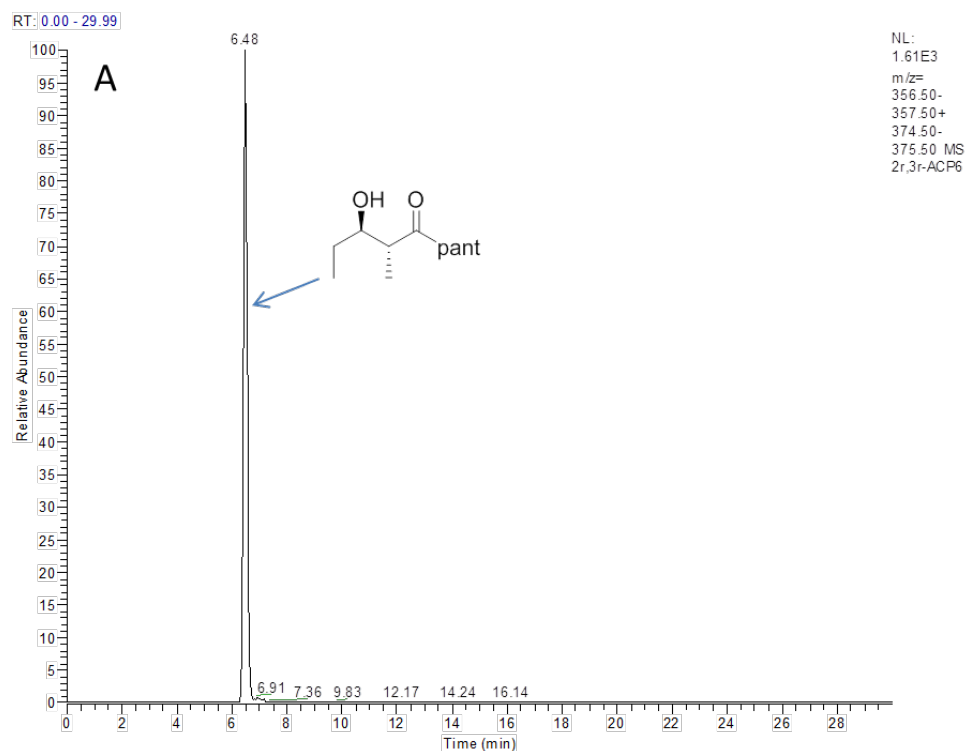


Figure S7. LC-ESI(+)-MS² analysis of (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-EryACP6 (6a-SeryACP6). A. LC-MS-MS. B. PPant ejection on m/z 904.46 peak [M^{13+}] to give 6a-pant (m/z 375.29).

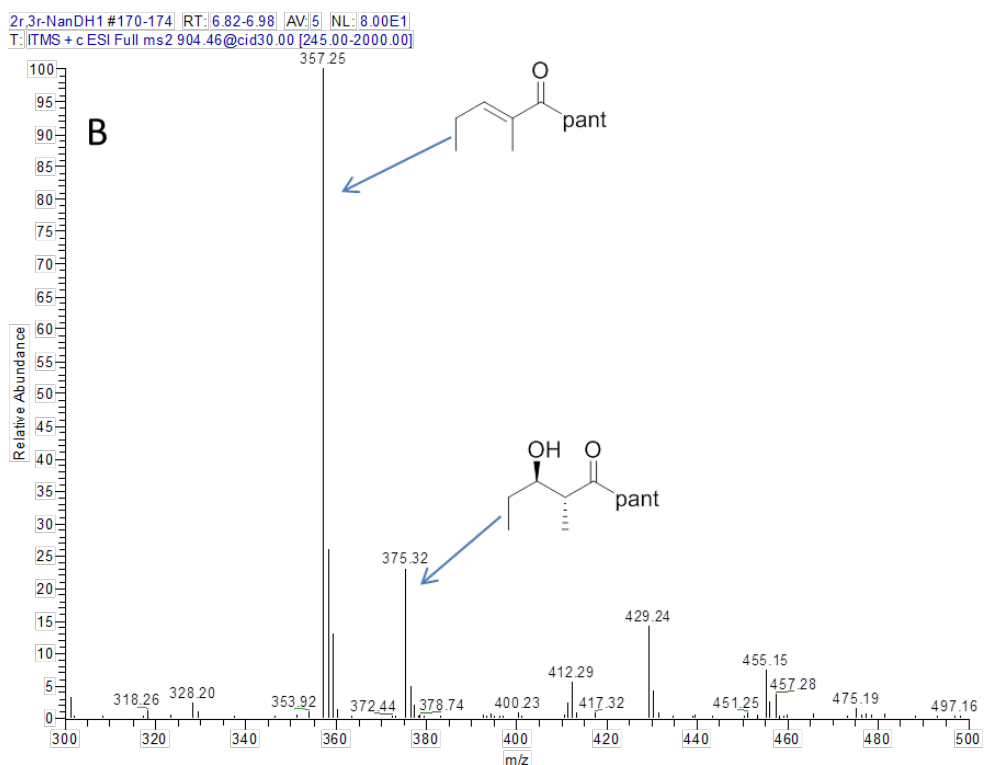
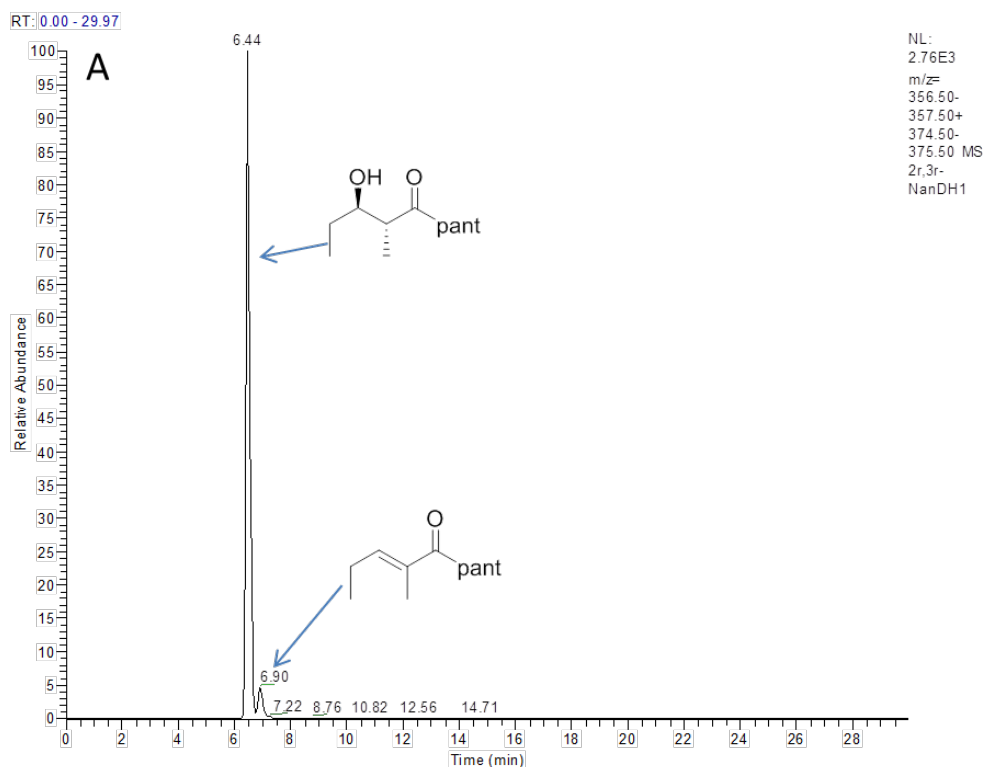


Figure S8. LC-ESI(+)-MS² analysis of 2-methyl-pentenoyl-EryACP6 (**8-SEryACP6**) generated by incubation of (2R,3R)-2-methyl-3-hydroxypentanoyl-EryACP6 (**6a-SEryACP6**) with NanDH1 for 30 min. A. LC-MS-MS. B. PPant ejection on m/z 904.46 peak [M^{13+}] to give 2-methyl-pentenoyl-pant (**8-pant**) (m/z 357.25).

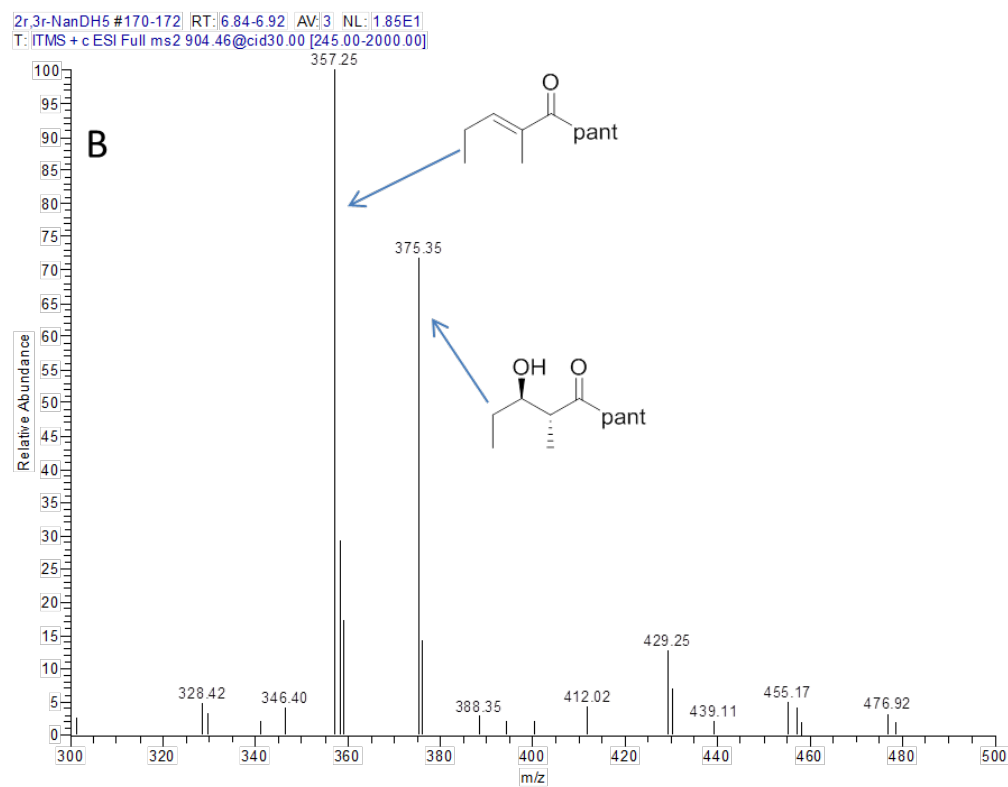
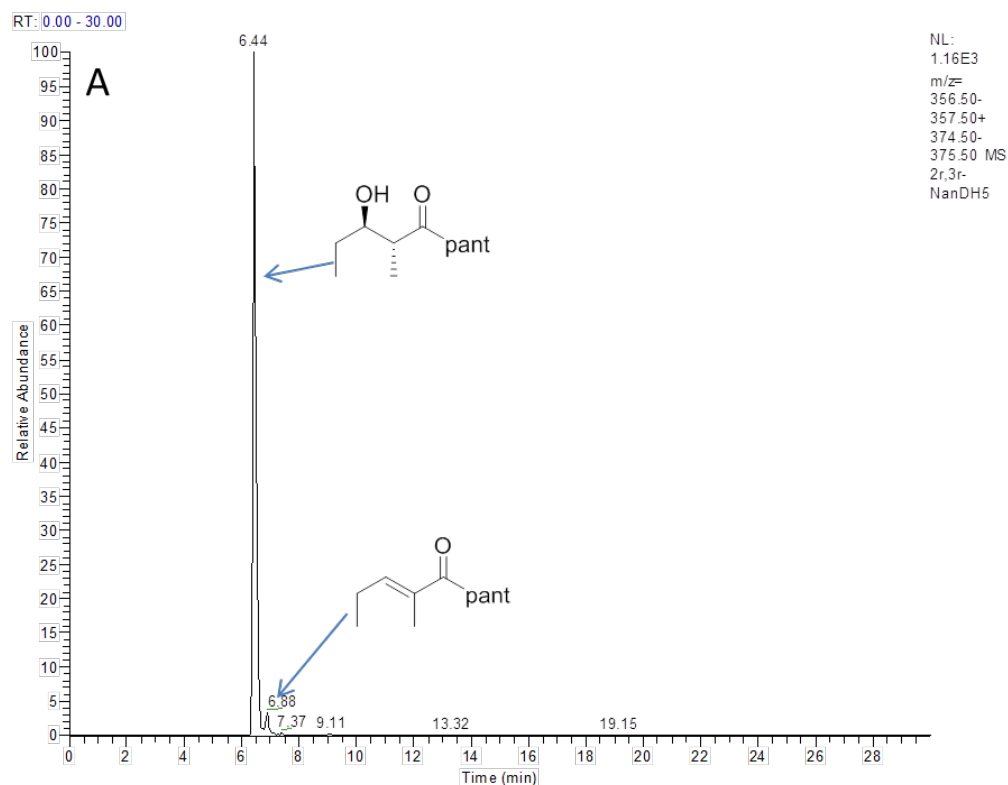


Figure S9. LC-ESI(+)-MS² analysis of 2-methylpentenoyl-EryACP6 (**8-SEryACP6**) generated by incubation of (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-EryACP6 (**6a-SEryACP6**) with NanDH5 for 30 min. A. LC-MS-MS. B. PPant ejection on *m/z* 904.46 peak [*M*¹³⁺] to give 2-methylpentenoyl-pant (**8-pant**) (*m/z* 357.25).

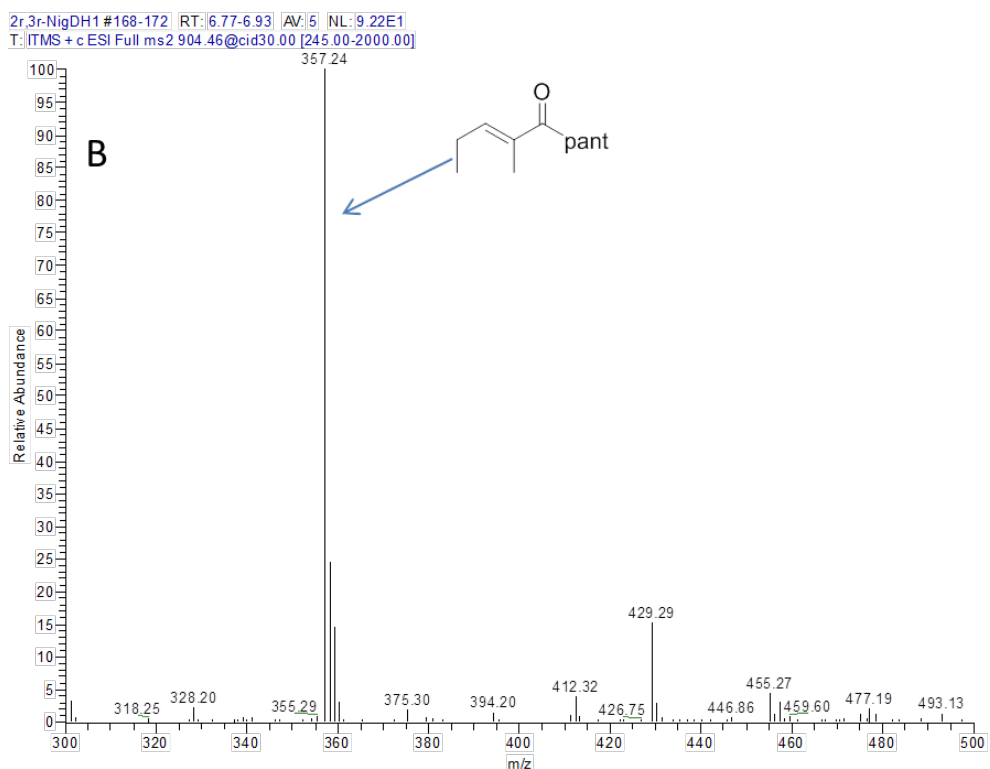
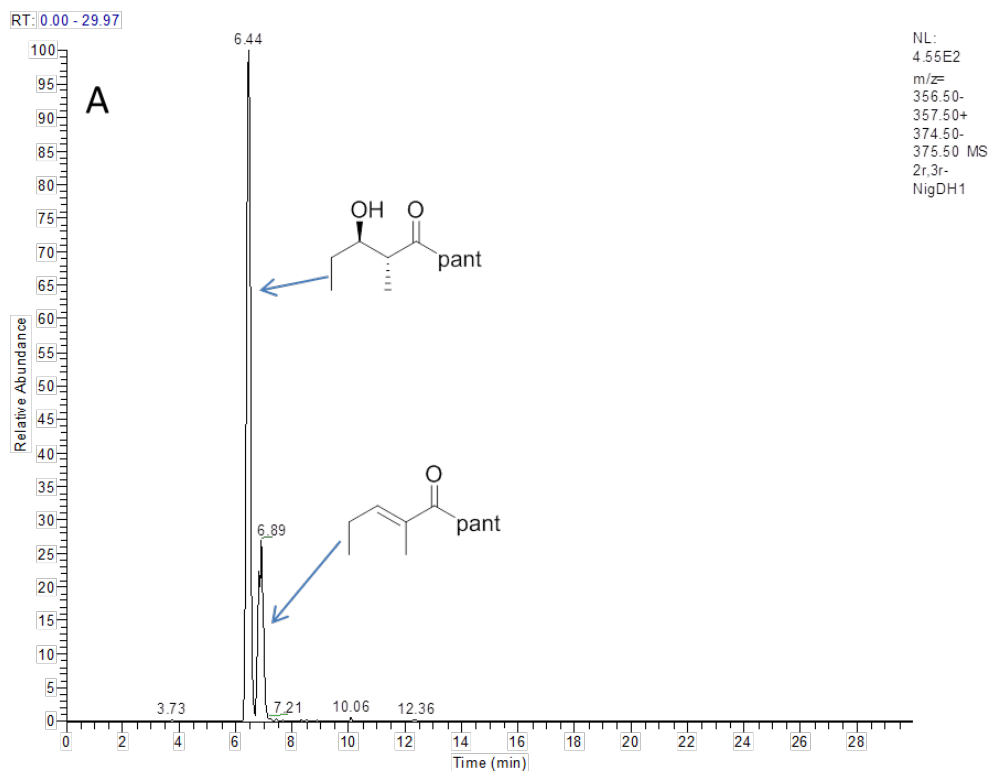


Figure S10. LC-ESI(+)-MS² analysis of 2-methylpentenoyl-EryACP6 (**8-SEryACP6**) generated by incubation of (2R,3R)-2-methyl-3-hydroxypentanoyl-EryACP6 (**6a-SEryACP6**) with NigDH1 for 30 min. A. LC-MS-MS. B. PPant ejection on m/z 904.46 peak [M^{13+}] to give 2-methylpentenoyl-pant (**8-pant**) (m/z 357.24).

Tandem Equilibrium Isotope Exchange Assay of DH Proteins. The Tandem EIX assay was based on the previously described protocol and carried out in duplicate.^{11,12} In a typical assay, chemoenzymatically prepared [2-²H]-(2*R*, 3*S*)-2-methyl-3-hydroxypentanoyl-EryACP6 ([2-²H]-**6b**) (90 μ L of 500 μ M solution, 45 nmol, final concentration 300 μ M), EryKR6 (11.25 nmol, 75 μ M), DH proteins (11.25 nmol, 75 μ M), and NADP⁺ (1.5 μ L of 1.5 mM soln, 2.25 nmol, final concentration 15 μ M) were incubated in 50 mM phosphate buffer (pH 7.2) (total volume 150 μ L) at room temperature. Samples were withdrawn at periodic intervals up to 60 min and frozen in liq N₂, before direct analysis of the pantetheinate ejection fragments by LC-ESI(+)-MS/MS, as previously described.^{11,12}

Table S2. Tandem EIX Assay of NanDH1 and NanDH5 Domains.

DH	time (min)					
	0	10	20	30	45	60
	Deuterium exchange of [2- ² H]- 6b (%) ^a					
EryKR6	0	0	4	4	5	5
NanDH1	0	3	6	13	15	18
NanDH5	0	5	9	14	15	17

^aAverage of two or more measurements ($\pm 2\%$)

Table S3. Tandem EIX Assay of NigDH1 Domains.

DH	time (min)						
	0	10	20	30	40	50	60
	Deuterium exchange of [2- ² H]- 6b (%) ^a						
EryKR6	0	0	4	4	5	6	6
NigDH1	0	3	10	17	20	21	23
NanDH2	0	3	11	16	16	21	26
EryDH4	0	3	11	13	15	17	20

^aAverage of two or more measurements ($\pm 2\%$)

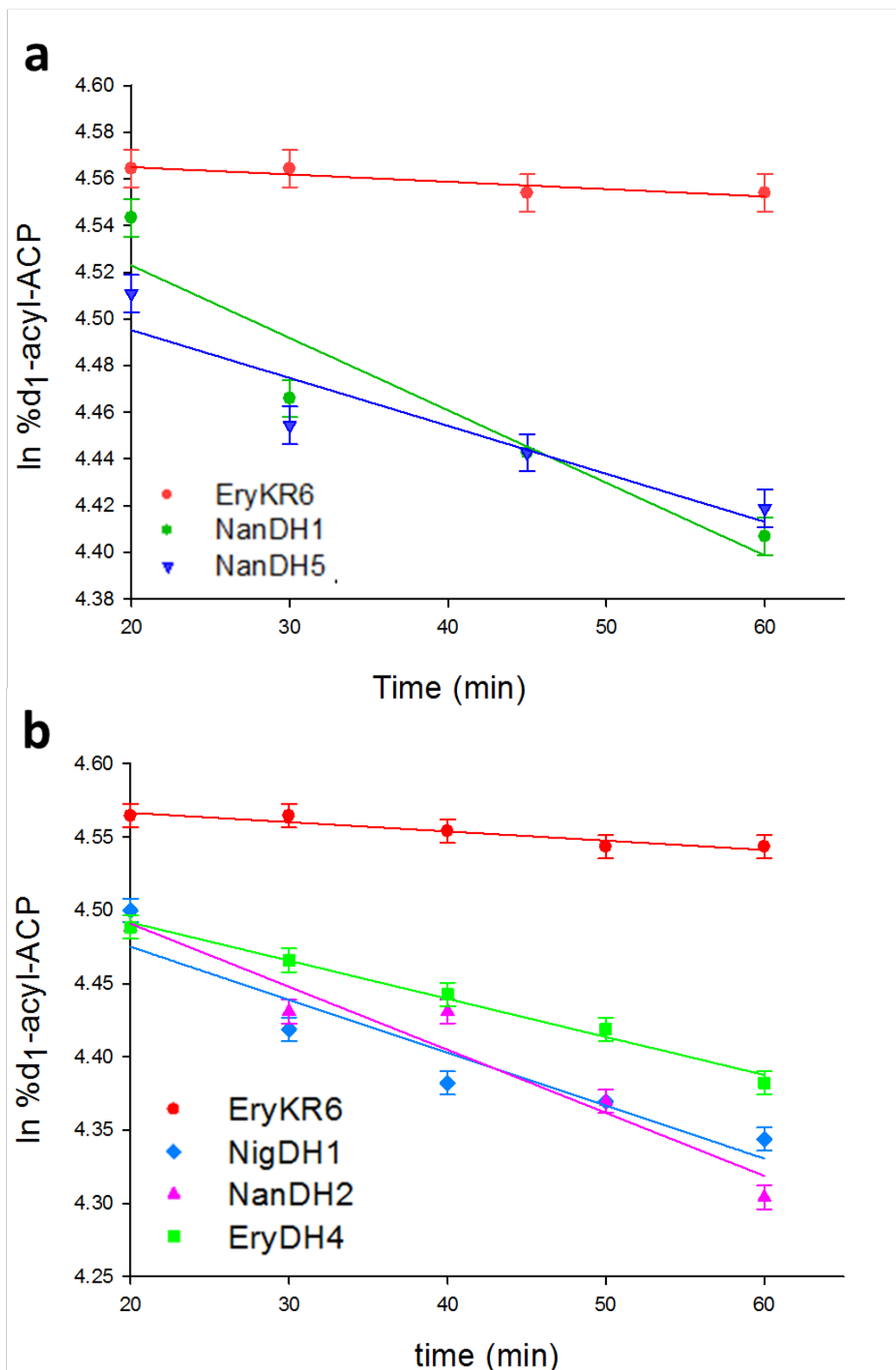


Figure S11. Time-dependent tandem EIX washout of deuterium from $[2-^2\text{H}]$ -(2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-EryACP6 ($[2-^2\text{H}$ -**6b**) by DH domains. Error bars represent standard deviations of duplicate data points.

Incubation of Recombinant KR⁰ and DH Domains with Reconstituted Nan[KS1][AT1] plus *holo*-NanACP1 and NANS Module2+PicTE. The protocol for GC-MS assay of the products of recombinant reconstituted mixtures of PKS components was based on those previously described.^{7,9} In a typical assay, 5 mM acetyl-SNAC was pre-incubated with 40 μ M Nan[KS1][AT1] didomain protein in a total volume of 800 μ L of phosphate buffer (50 mM sodium phosphate, 100 mM NaCl, 2.5 mM TCEP, pH 7.2). After 30 min, 400 μ L of solution containing 600 μ M *holo*-NanACP1, 1.5 mM methylmalonyl-CoA, 30 μ M NANS Module2+PicTE, 15 mM NADPH, and 100 μ L of phosphate buffer or 600 μ M DH or KR⁰ domains were added. The enzyme mixture was incubated at room temperature and 400- μ L samples were withdrawn at periodic intervals for 15 min, 30 min and 1 h. The withdrawn samples immediately were transferred into contained 100 μ L of 1 M HCl and extracted with ethyl acetate (4 \times 600 μ L). The concentrated organic extracts were analyzed by chiral GC-MS, as described below.

Chiral GC-MS Quantitative and Stereochemical Analysis of the Tandem Modules Feeding Chemoenzymatically-Generated (2*S*,4*R*)-2,4-dimethyl-5-ketohexanoate (12) by Tandem Modules Epimerase Assay. The concentrated organic extracts of the above-described enzymatic incubations were dissolved in 200 μ L of methanol and then treated with 5 μ L of TMSCHN₂ (2 M in hexane) for 10 min at room temperature. The derived 2,4-dimethyl-5-ketohexanoic acid methyl ester (**12-Me**) was directly analyzed by chiral GC-MS. GC-MS spectra were recorded on GC-MS Hewlett-Packard Series II GC-MSD instrument, 70 eV EI in positive ion mode, using a Varian CP-Chirasil-DEX CB capillary column (25 m \times 0.32 mm, Agilent Technologies) and a temperature program of (1) initial temp 60 $^{\circ}$ C for 1 min, (2) increase at rate 0.3 $^{\circ}$ C/min up to 75 $^{\circ}$ C for 1 min, (3) 5 $^{\circ}$ C/min up to 100 $^{\circ}$ C for 1 min, and then 20 $^{\circ}$ C/min to final temp 210 $^{\circ}$ C for 5 min. The extracted ion current (XIC) of the chromatogram was analyzed set on the base peak at m/z 88 of the 2,4-dimethyl-5-ketohexanoic acid methyl ester (**12-Me**) with direct comparison to authentic standards, as previously described.⁷

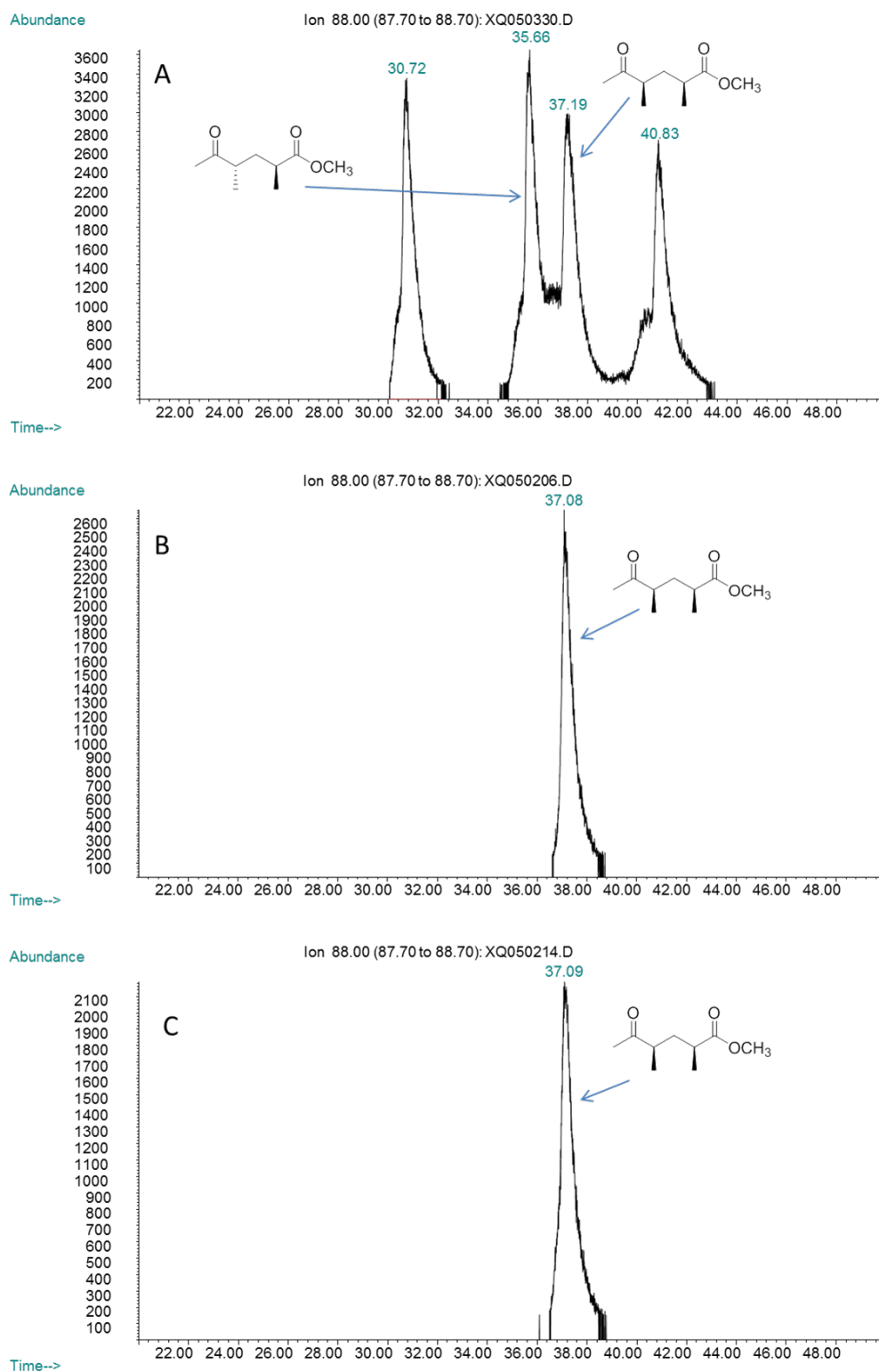


Figure S12. Chiral GC-EI(+)-MS analysis of the (2*S*,4*R*)-2,4-dimethyl-5-ketohexanoic acid methyl ester (**12-Me**) resulting from incubation of NANS Module 2+TE with Nan[KS1][AT1], acetyl-SNAC, *holo*-NanACP1, NADPH, methylmalonyl-CoA and NanKR1⁰ or PicKR3⁰. **12-Me** XIC, *m/z* 88. A. Synthetic mixture of four diastereomers of methyl (2*RS*,4*RS*)-2,4-dimethyl-5-ketohexanoate; B. NanKR1⁰; C. PicKR3⁰.

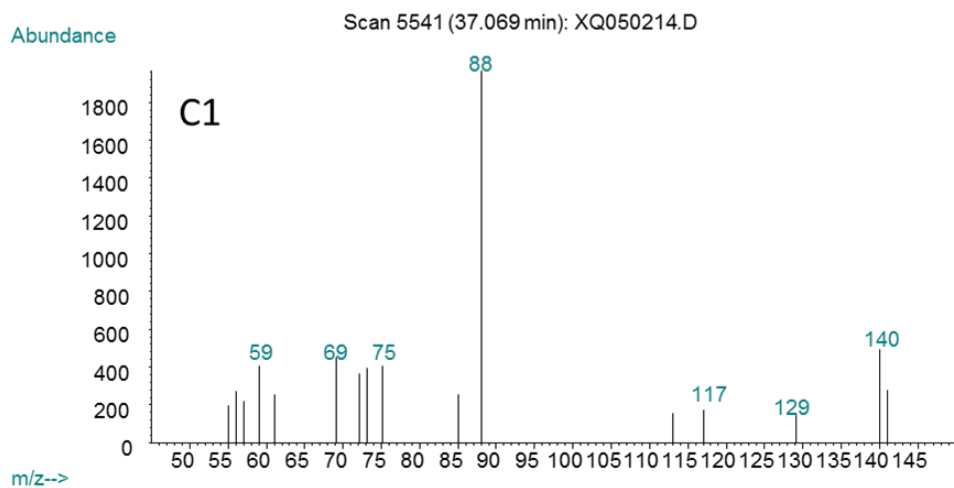
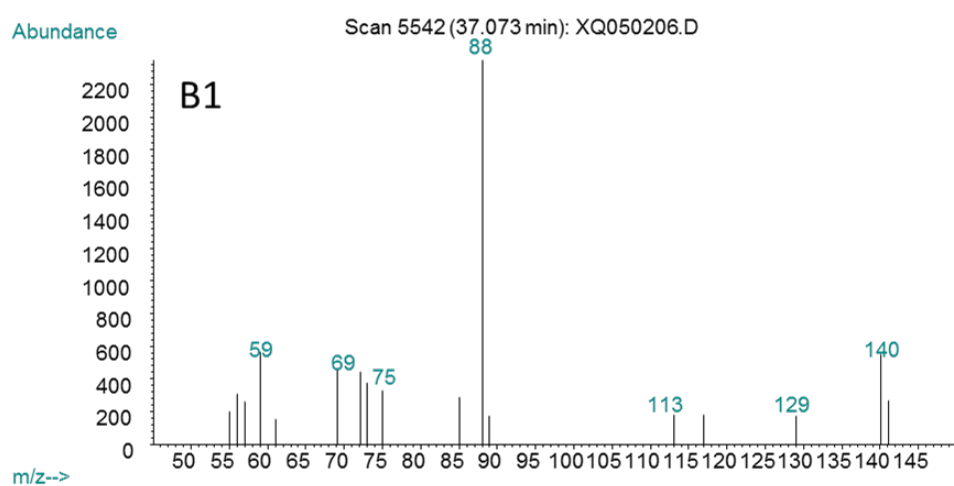
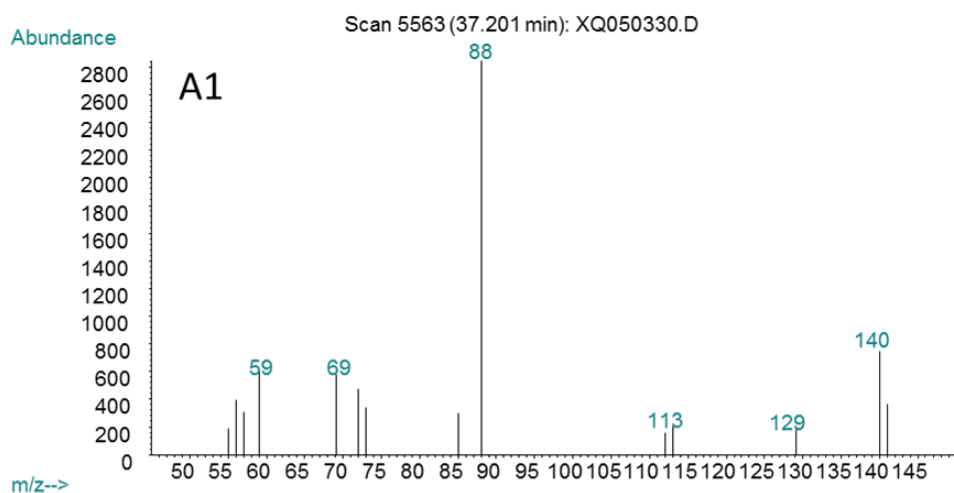


Figure S12 (cont). A1, MS for **12-Me** from A; B1, MS for **12-Me** from B; C1 MS for **12-Me** from C.

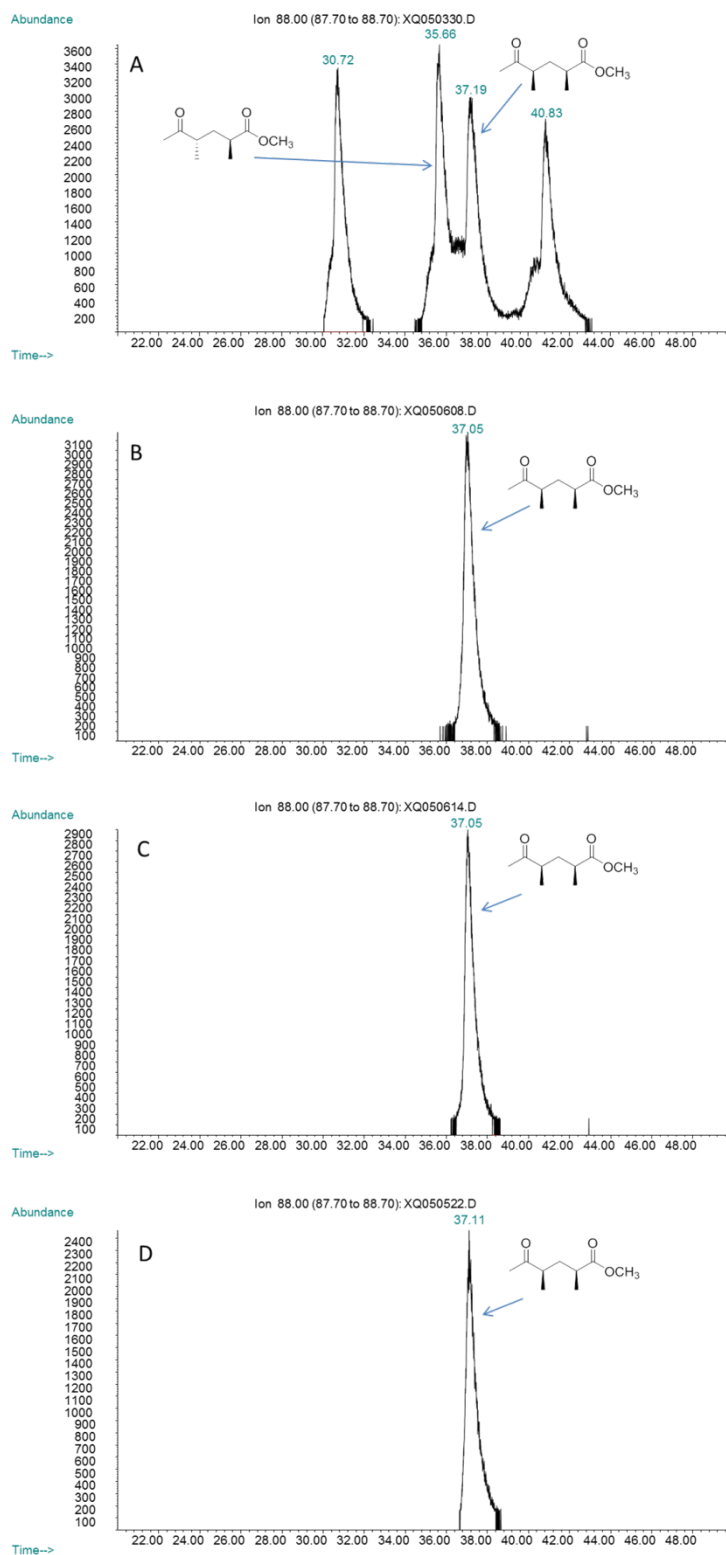


Figure S13. Chiral GC-EI(+)-MS analysis of **12-Me** resulting from incubation of NANS Module 2+TE with Nan[KS1][AT1], acetyl-SNAC, *holo*-NanACP1, NADPH, methylmalonyl-CoA and NanDH1 or NanDH5 or NigDH1. **12-Me**, XIC, m/z 88. A. Synthetic mixture of four diastereomers of methyl (2*RS*,4*RS*)-2,4-dimethyl-5-ketohexanoate; B. NanDH1; C. NanDH5; D. NigDH1.

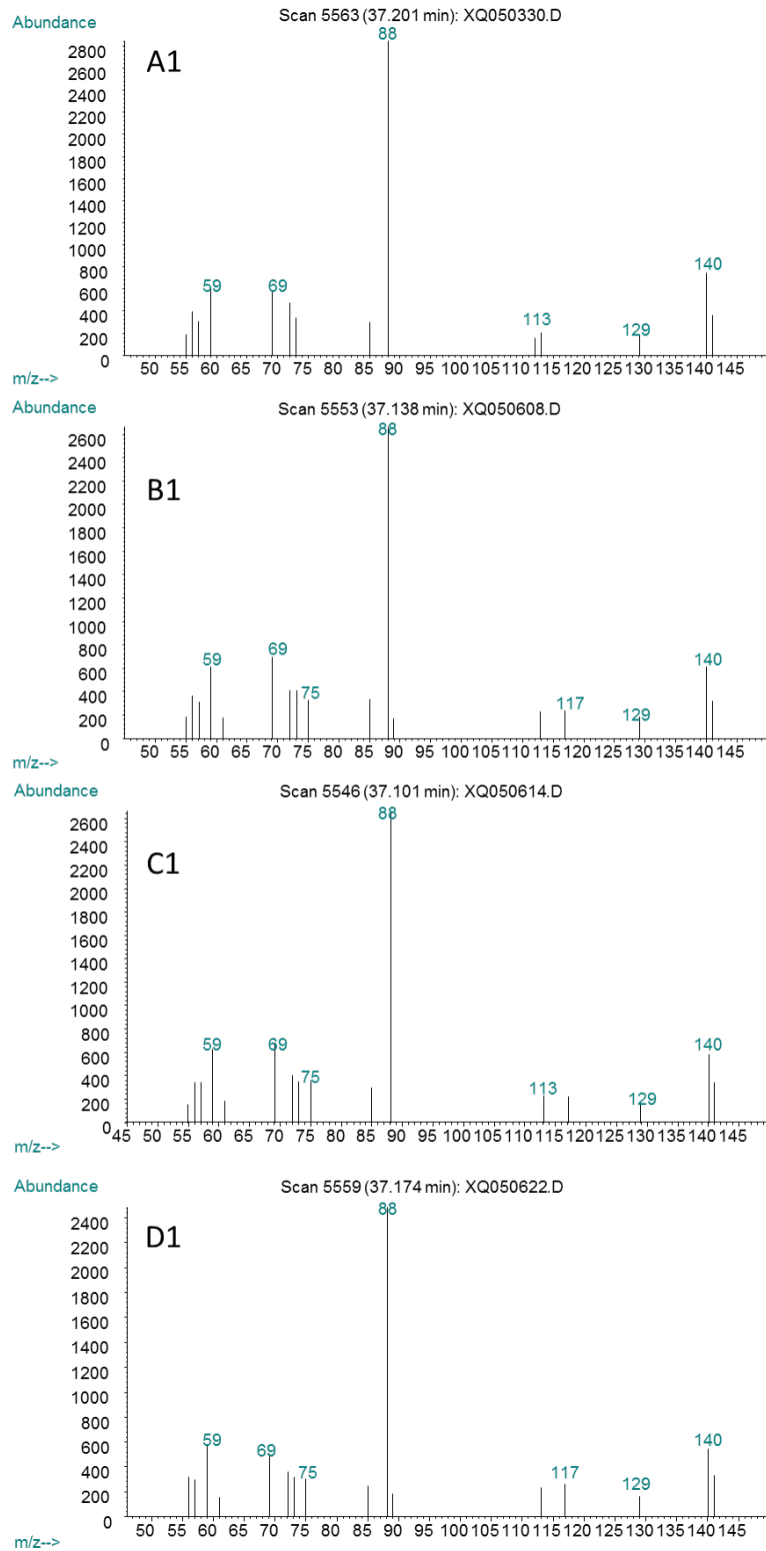


Figure S13 (cont). A1, MS for **12-Me** from A; B1, MS for **12-Me** from B; C1 MS for **12-Me** from C; D1 MS for **12-Me** from D.

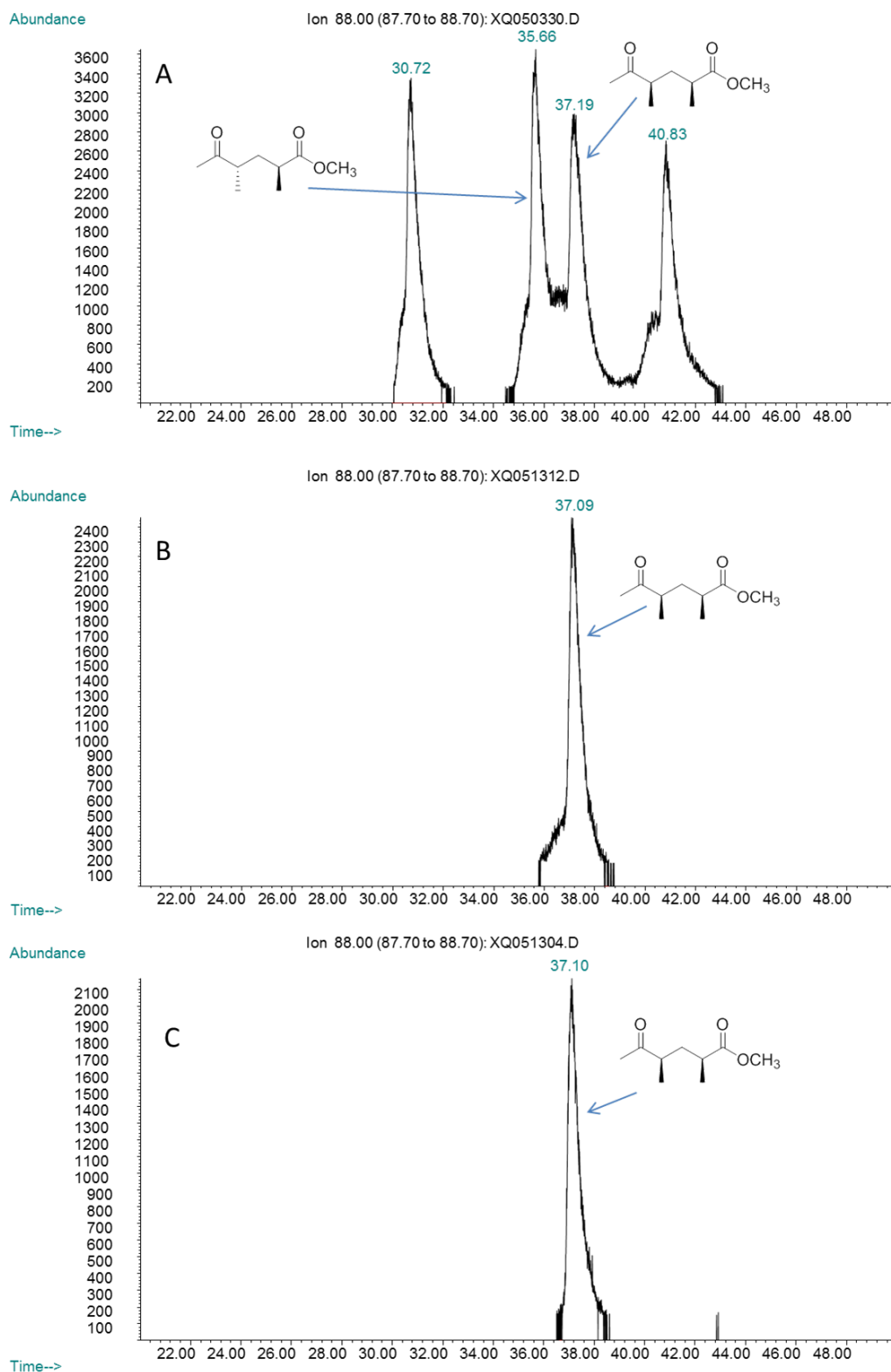


Figure S14. Chiral GC-EI(+)-MS analysis of **12-Me** resulting from incubation of NANS Module 2+TE with Nan[KS1][AT1], acetyl-SNAC, *holo*-NanACP1, NADPH, methylmalonyl-CoA and EryDH4 or NanDH2. **12-Me**, XIC, *m/z* 88. A. Synthetic mixture of four diastereomers of methyl (2*RS*,4*RS*)-2,4-dimethyl-5-ketohexanoate; B. EryDH4; C. NanDH2.

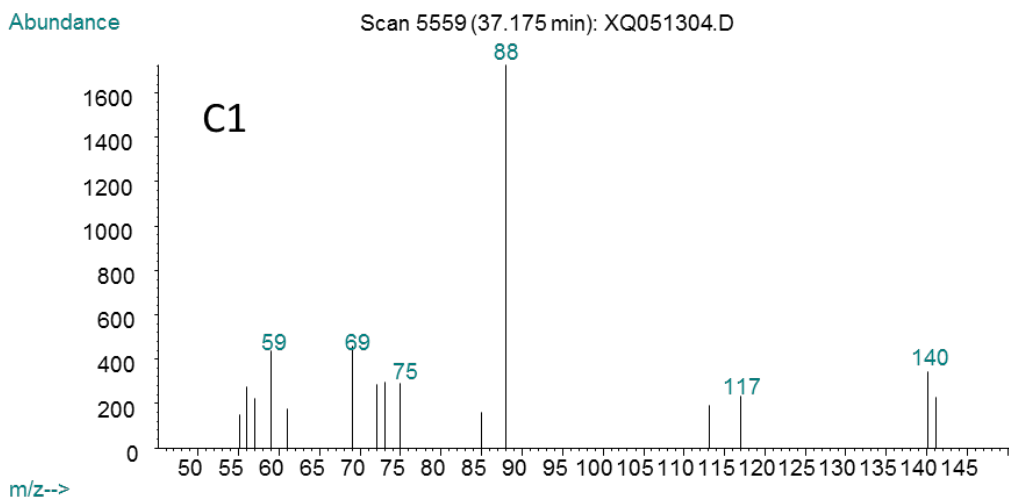
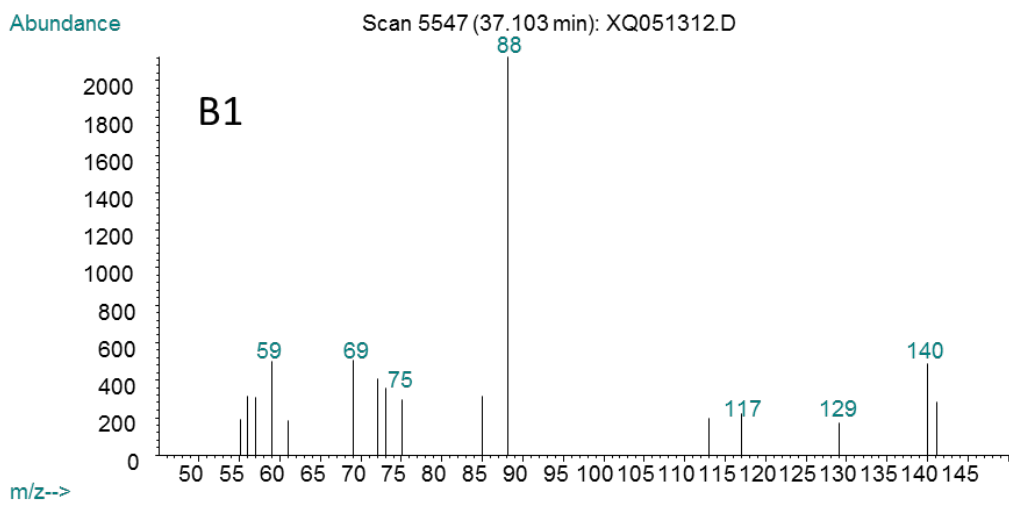
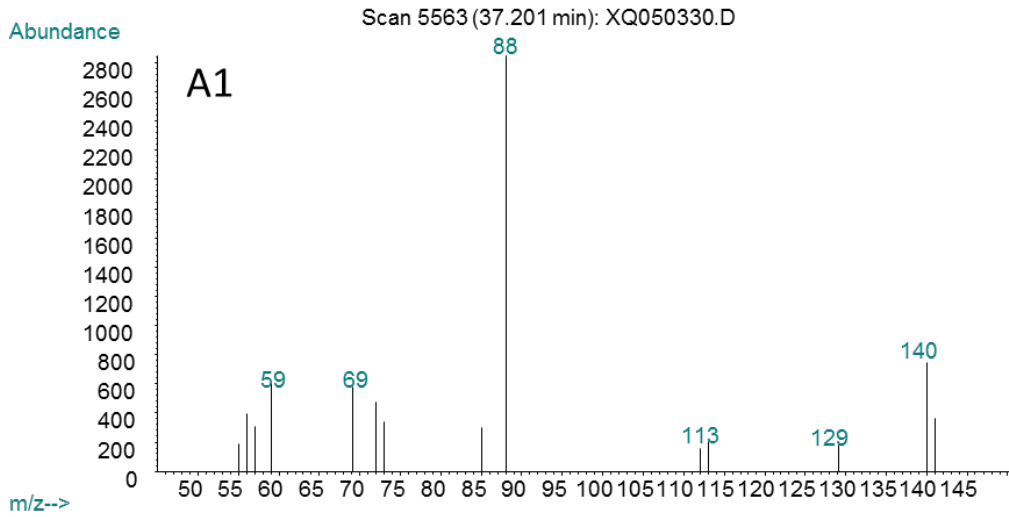


Figure S14 (cont). A1, MS for 12-Me from A; B1, MS for 12-Me from B; C1 MS for 12-Me from C.

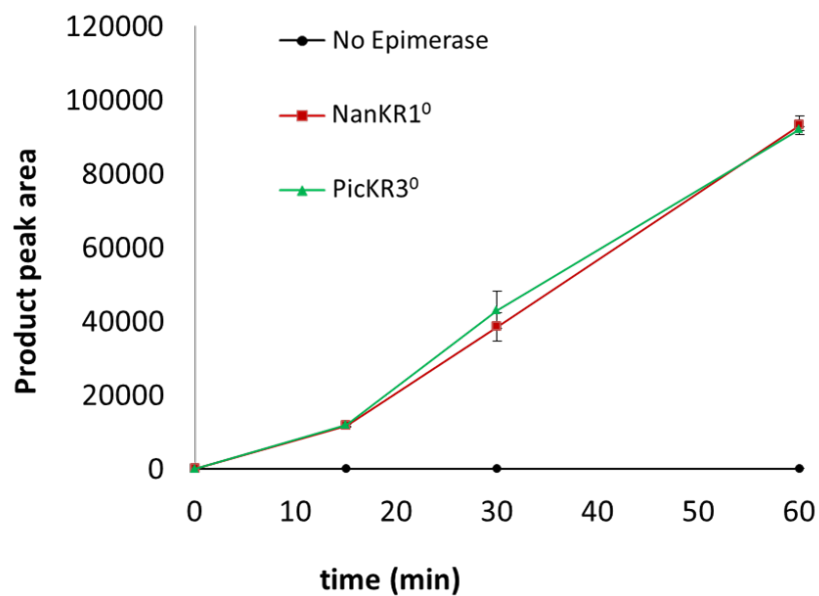


Figure S15. TME assay of time-dependent formation of **12-Me** generated by incubation of NANS Module 2+TE with Nan[KS1][AT1], acetyl-SNAC, *holo*-NanACP1, NADPH, methylmalonyl-CoA and epimerase-active NanKR1⁰ or PicKR3⁰, based on GC-MS quantitation of the derived **12-Me** (XIC, *m/z* 88). Error bars represent standard deviations of duplicate data points.

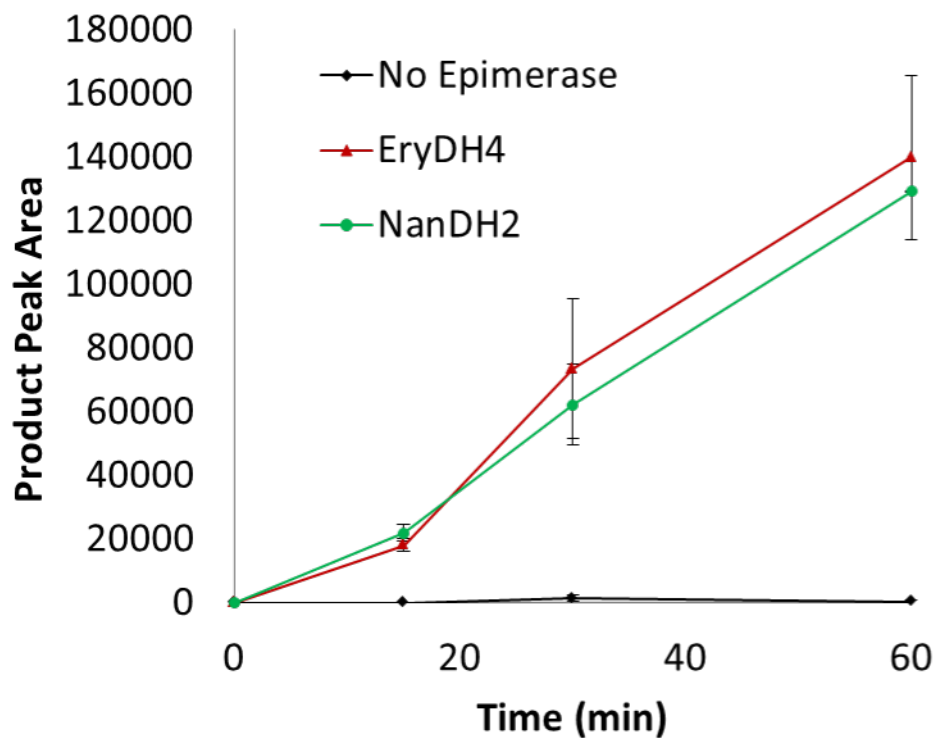


Figure S16. TME assay of time-dependent formation of **12-Me** generated by incubation of NANS Module 2+TE with Nan[KS1][AT1], acetyl-SNAC, *holo*-NanACP1, NADPH, methylmalonyl-CoA and DH domains (EryDH4, NanDH2) based on GC-MS quantitation of the derived **12-Me** (XIC, m/z 88). Error bars represent standard deviations of duplicate data points.

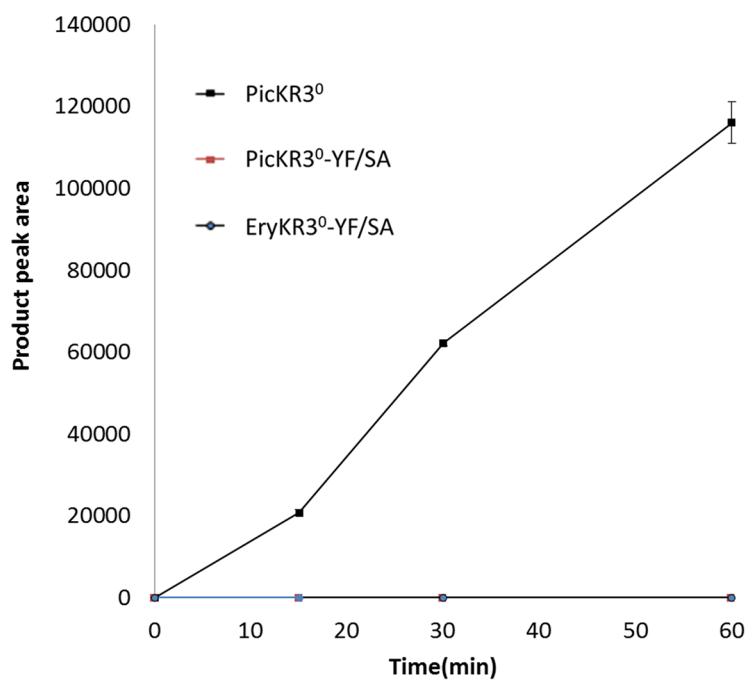


Figure S17. TME assay of time-dependent formation of **12-Me** generated by incubation of NANS Module 2+TE with Nan[KS1][AT1], acetyl-SNAC, *holo*-NanACP1, NADPH, methylmalonyl-CoA and epimerase-active (PicKR3⁰) as well as epimerase-inactive double mutants PicKR3⁰-S385A/Y398F and EryKR3⁰-S349A/Y362F (negative controls). The derived **12-Me** was quantitated by GC-MS (XIC, m/z 88). Error bars represent standard deviations of duplicate data points. Neither double mutant had any detectable epimerase activity.

Supplemental References

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