Supporting Information

Structure and stereospecificity of the dehydratase domain from the terminal module of the rifamycin polyketide synthase

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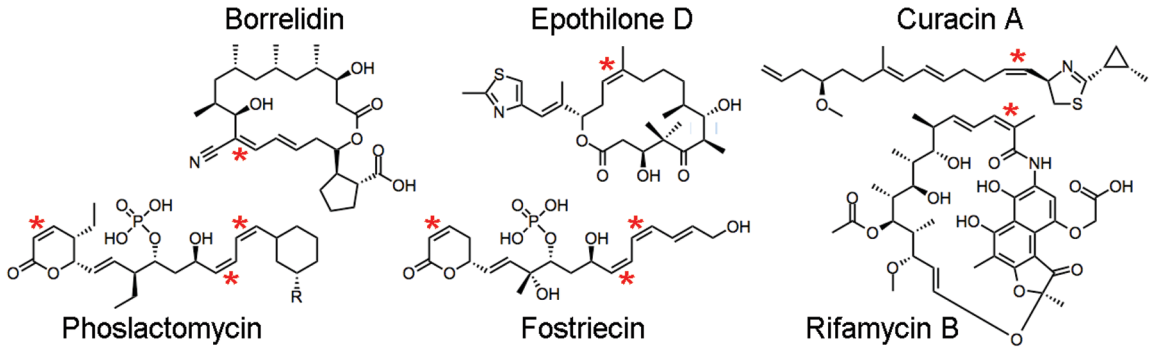


Figure S1. Polyketides with *cis* double bonds

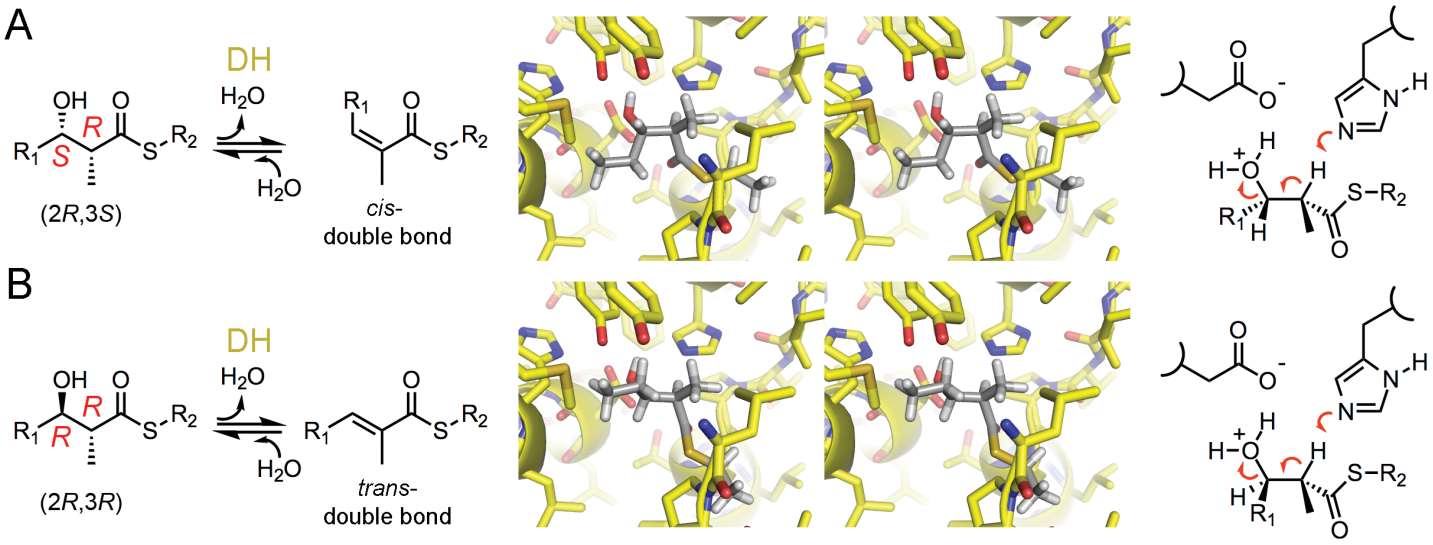


Figure S2. Dehydration of (2*R*,3*S*)- and (2*R*,3*R*)-2-methyl-3-hydroxyacyl thioesters. A) The *syn* dehydration of a (2*R*,3*S*) intermediate would result in a *cis*-enoyl thioester. The stereodiagram shows how the C2 proton and the C3 hydroxyl group of a (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl intermediate would be aligned within the active site of RifDH10 for the *syn* elimination reaction. B) The *syn* dehydration of a (2*R*,3*R*) intermediate would result in a *trans*-enoyl thioester. The stereodiagram shows how the C2 proton and the C3 hydroxyl group of a (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl intermediate would be aligned in the active site of RifDH10 for the *syn* elimination reaction.

Domain boundaries and design of recombinant RifDH10 and RifACP10

Figure S3. Rifamycin PKS partial module 9 and module 10. Rifamycin polyketide synthase [*Amycolatopsis mediterranei* U32] Sequence ID: ref|YP_003762843.1|Length: 3413

ADTASDAVSLGLAGADHPLLGAVVQLPQSDGLVFTSRLSLRSHPWLADHAVRDVVIVPGT
GLVELAVRAGDEAGCPVLDELVIEAPLVVPRRGGVRVQVALGGPADDGSRTVDVFSLRED
ADSWLRHATGVLVPENRPRGTAAFDFAAWPPPEAKPVDLTGAYDVLADVGYGYGPTFRAV
RAVWRRGSGNTTETFAEIALPEDARAEAGRFGIHPALLDAALHSTMVSAAADTESYGDEV
RLPFAWNGLRLHAAGASVLRVRVAKPERDSLSLEAVDESGGLVVTLDSLVGRPVSNDQLT
TAAG

ATGGCGGACACGGCAAGCGACGCAGTAAGCCTCGGCCTGGCAGGTGCGGATCACCCGCTGCTGGGTGCAGTCGTGCAATTG
CCGCAGAGCGATGGCCTGGTCTTTACGTCCCGCTTGAGCCTGCGCTCCCATCCGTGGTTGGCGGATCACGCGGTTCGCGAC
GTTGTTATTGTGCCGGGCACTGGTCTGGTTGAACTGGCCGTCCGTGCTGGCGACGAAGCCGGCTGCCCGGTGCTGGATGAG
CTGGTGATCGAAGCGCCGCTGGTCGTCCCGCGTCGTGGCGGTGTGCGTGTTCAAGTCGCACTGGGTGGTCCGGCGGATGAC
GGTTCGCGCACCGTTGACGTCTTTAGCCTGCGTGAGGACGCCGATAGCTGGCTGCGTCACGCTACCGGTGTGCTGGTTCCA
GAGAATCGTCCGCGTGGTACCGCTGCCTTCGATTTTGCGGCGTGGCCTCCGCCGGAAGCGAAGCCGGTCGACCTGACGGGC
GCATACGATGTTTTGGCGGACGTTGGTTACGGTTATGGCCCGACGTTCCGCGCAGTGCGTGCCGTGTGGCGTCGCGGCTCC
GGTAACACCACCGAAACCTTTGCGGAGATCGCGCTGCCGGAGGACGCGCGTGCGGAGGCAGGCCGTTTCGGTATTCATCCG
GCACTGCTGGATGCCGCGCTGCATAGCACCATGGTCAGCGCCGCTGCGGATACCGAGAGCTATGGCGATGAAGTTCGTCTG
CCGTTCGCATGGAATGGTTTGCGCCTGCACGCGGCTGGTGCGAGCGTCCTGCGTGTGCGCGTTGCCAAACCAGAACGCGAT
AGCCTGAGCCTGGAGGCGGTTGACGAGTCTGGTGGCTTGGTTGTGACGCTGGACTCTCTGGTGGGTCGTCCTGTGAGCAAC
GACCAGCTGACCACTGCAGCGGGT
N-terminus: CATATG (NdeI site)
C-terminus: TAACTCGAG (Stop codon/XhoI)

Figure S4. RifDH10 amino acid sequence**.** The synthetic gene encoding RifDH10 domain was subcloned in the pET-28a vector and the recombinant protein was expressed with a N-terminal His_6-tag in *E.coli* BL21 (DE3). Protein expression and purification procedures are described below.

LAGLEPAERGQVLLELVRAQVAGVLGYRAAHQVDPDQGLFEIGFDSLTAIELRNRLRART
ERKISPGVVFDHPTPALLAAHLNELL

ATGCTGGCCGGCCTGGAACCTGCAGAACGCGGTCAAGTCCTGCTGGAGCTGGTGCGTGCGCAGGTTGCGGGCGTGCTGGGC
TACCGTGCTGCCCATCAGGTTGACCCGGATCAAGGTCTGTTCGAGATCGGTTTTGACAGCCTGACGGCGATCGAGCTGCGC
AACCGTTTGCGTGCGCGCACCGAACGTAAGATTAGCCCGGGTGTCGTTTTCGATCACCCGACTCCGGCGTTGCTGGCAGCA
CACTTGAATGAGCTGCTG
N-terminus: CATATG (NdeI site)

Figure S5. RifACP10 amino acid sequence**.** The synthetic gene encoding RifACP10 was initially ligated into the NdeI/XhoI sites of pET-28a. Since the resultant recombinant RifACP10 was obtained only as insoluble inclusion bodies when expressed in *E.coli* BL21 (DE3), the corresponding RifACP10-NusA protein was constructed.

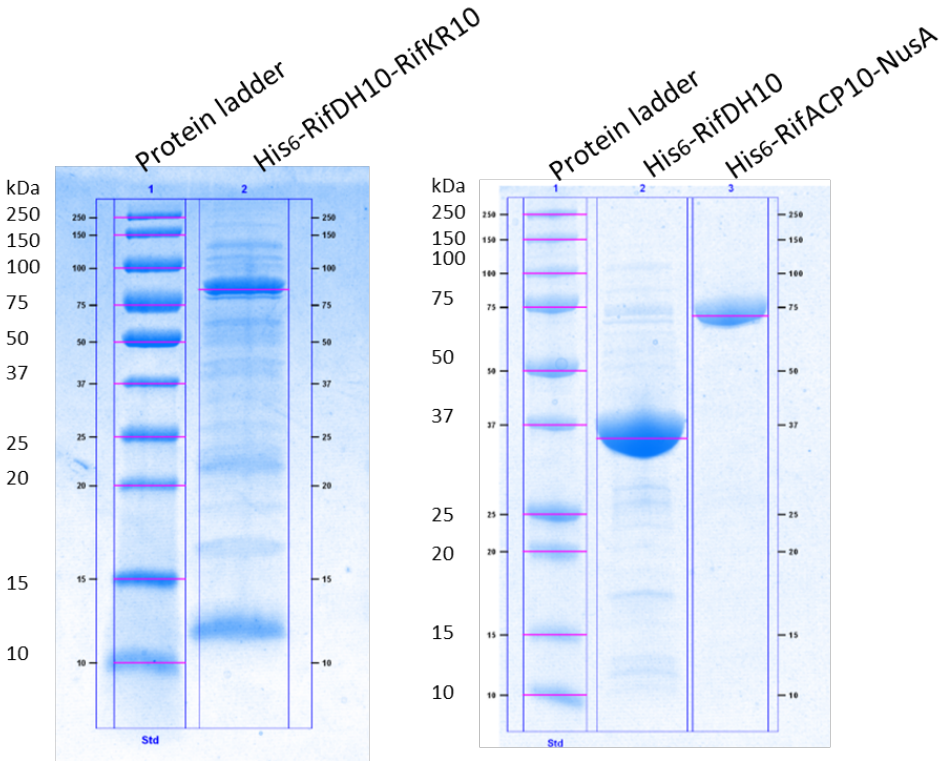


Figure S6. SDS-PAGE of recombinant RifDH10-KR10, RifDH10 and RifACP10-NusA

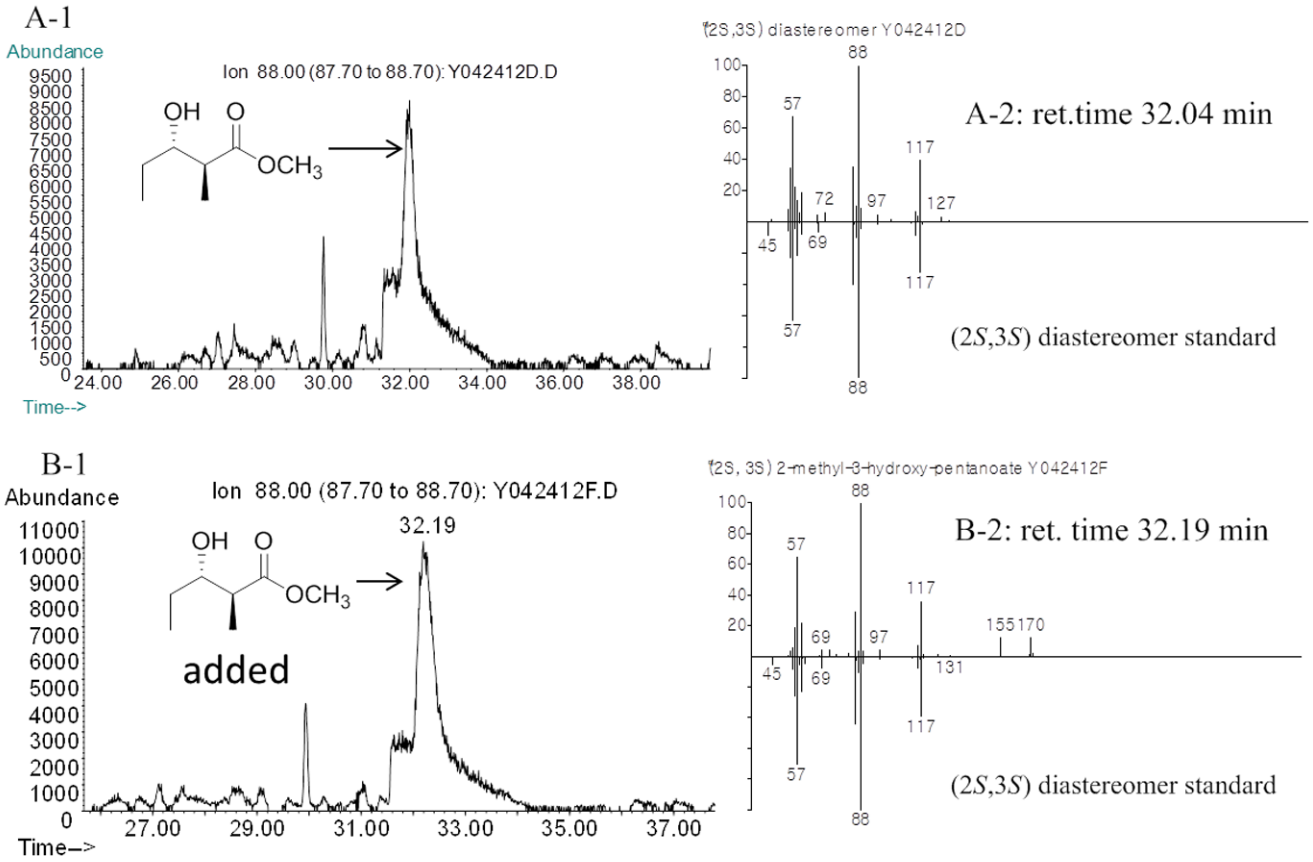


Figure S7. Chiral GC-MS analysis of the incubation of (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10-NusA with RifKR7 in the absence of RifDH10 (Method 4). **A**: Methyl (2*S*,3*S*)-2-methyl-3-hydroxypentanoate (**4a**) from RifKR7-catalyzed reduction of (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10-NusA and **B**: Co-injection of (2*S*, 3*S*)-**4a** with A. **A-1 and B-1**: Extracted ion current (XIC) at *m/z* 88 (base peak). **A-2 and B-2**: Mass spectra of selected peak corresponding to (2*S*,3*S*)-**4a** upper half, observed spectra, lower half, inverted mass spectra of reference standard.

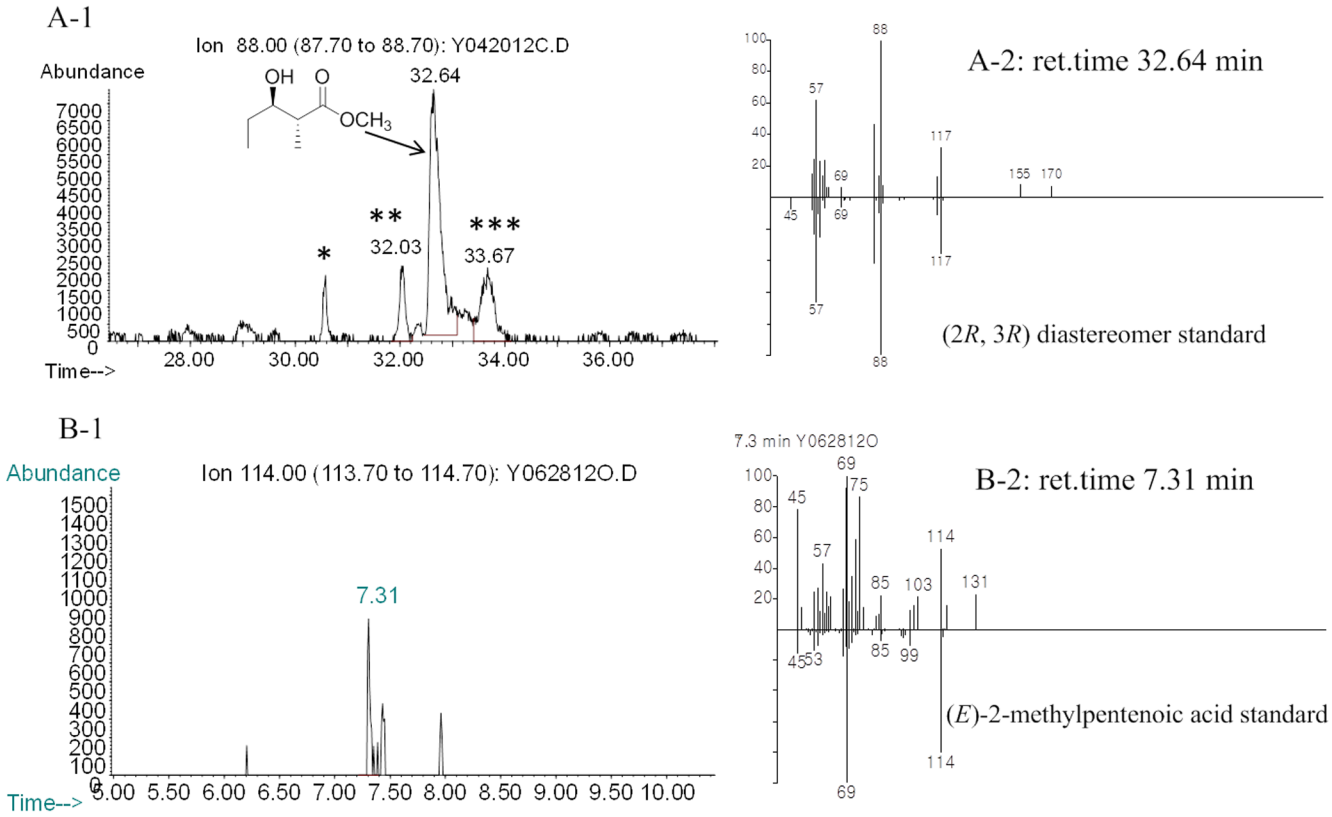


Figure S8. Chiral GC-MS analysis of the incubation of (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10-NusA with TylKR1 and RifDH10 (Methods 2 and 4). **A**: Methyl 2-methyl-3-hydroxypentanoates from TylKR1-catalyzed reduction of (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10-NusA and **B**: Minor peak corresponding to (*E*)-2-methyl-2-pentenoic acid (**3**) generated by RifDH10-coupled dehydration of A. **A-1**: Extracted ion current (XIC) at *m/z* 88 (base peak). The major diastereomer observed was (2*R*,3*R*)-**4b**. The minor diastereomers are marked with asterisks as (2*R*,3*S*)-**4c** (*), (2*S*,3*R*)-**4d** (**) and (2*S*,3*S*)-**4a** (***). **A-2**: mass spectra of selected peak corresponding to (2*R*,3*R*)-**4b**, upper half, observed spectrum, lower half, inverted mass spectrum of reference standard. **B-1**: Extracted ion current (XIC) at *m/z* 114 (base peak). **B-2**: Mass spectrum of selected peak, upper half, observed spectrum, lower half, inverted mass spectra of reference standard. (Note both the lower intensity and lower purity of the ret. time 7.31 min peak containing **3** plus contaminants, compared to the yield and purity of **3** derived from an incubation carried out in the presence of RifKR7 (cf Figure 3)

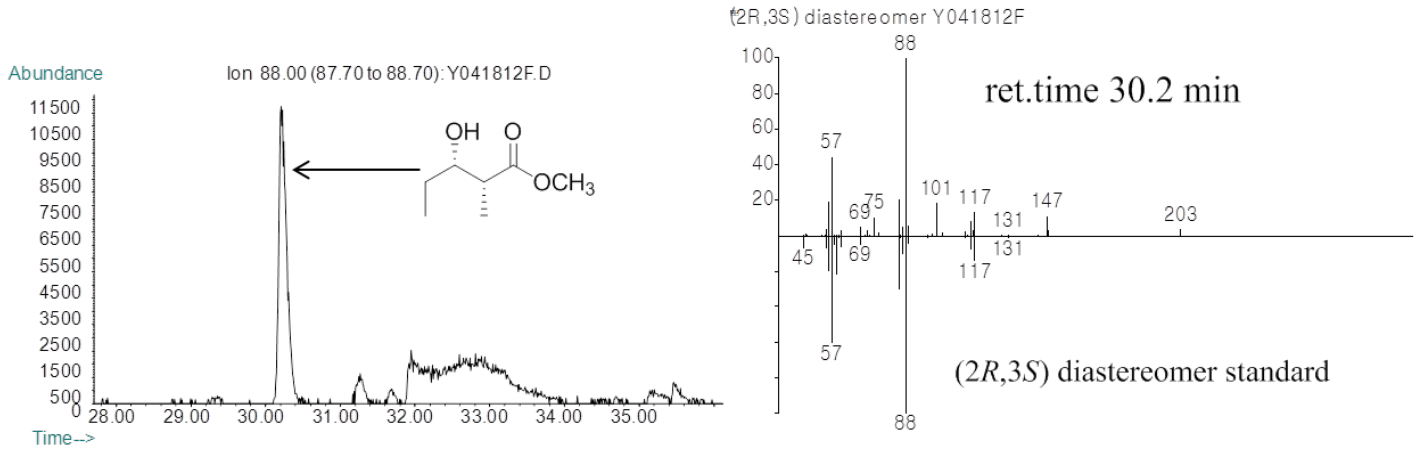


Figure S9. Chiral GC-MS analysis of the incubation of (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10-NusA with EryKR6 and RifDH10 (Method 4). **Left panel**: Extracted ion current (XIC) at *m/z* 88 (base peak). **Right panel**, mass spectra of a selected peak corresponding to (2*R*,3*S*)-**4c**, upper half, observed spectrum, lower half, inverted mass spectrum of reference standard. No dehydration product **3** was observed.

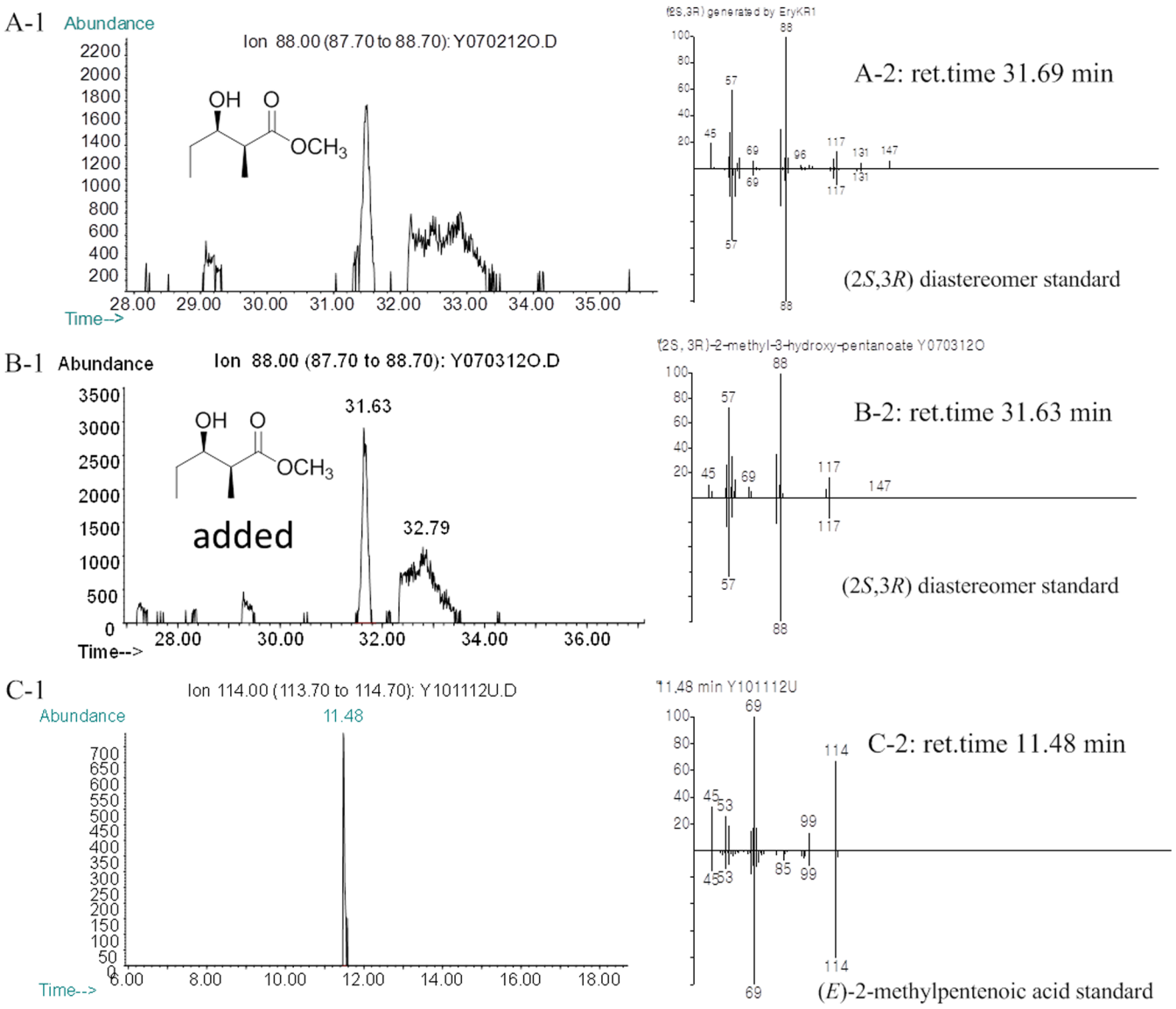


Figure S10. Chiral GC-MS analysis of the incubation of (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10-NusA with EryKR1 and RifDH10. **A**: Methyl 2-methyl-3-hydroxypentanoates from EryKR1-catalyzed reduction of (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10-NusA (Method 4) and **B**: Co-injection of (2*S*,3*R*)-**4d** diastereomer with A. Envelope at 32.79 min contains minor amounts of (2*S*,3*S*)-**4a. C**: Minor amount of **3** generated by RifDH10-coupled reaction of A, due to presence of (2*S*,3*S*)-**1a** in reduced RifACP10-bound diketide. (Method 1) **A-1 and B-1**: Extracted ion current (XIC) at *m/z* 88 (base peak). **A-2** and **B-2**: mass spectra of selected peak corresponding to (2*S*,3*R*)-**4d**, upper half, observed spectrum, lower half, inverted mass spectrum of reference standard. **C-1**: Extracted ion current (XIC) at *m/z* 114 (base peak). **C-2**: mass spectrum of selected peak, upper half, observed spectrum, lower half, inverted mass spectrum of reference standard **3**.

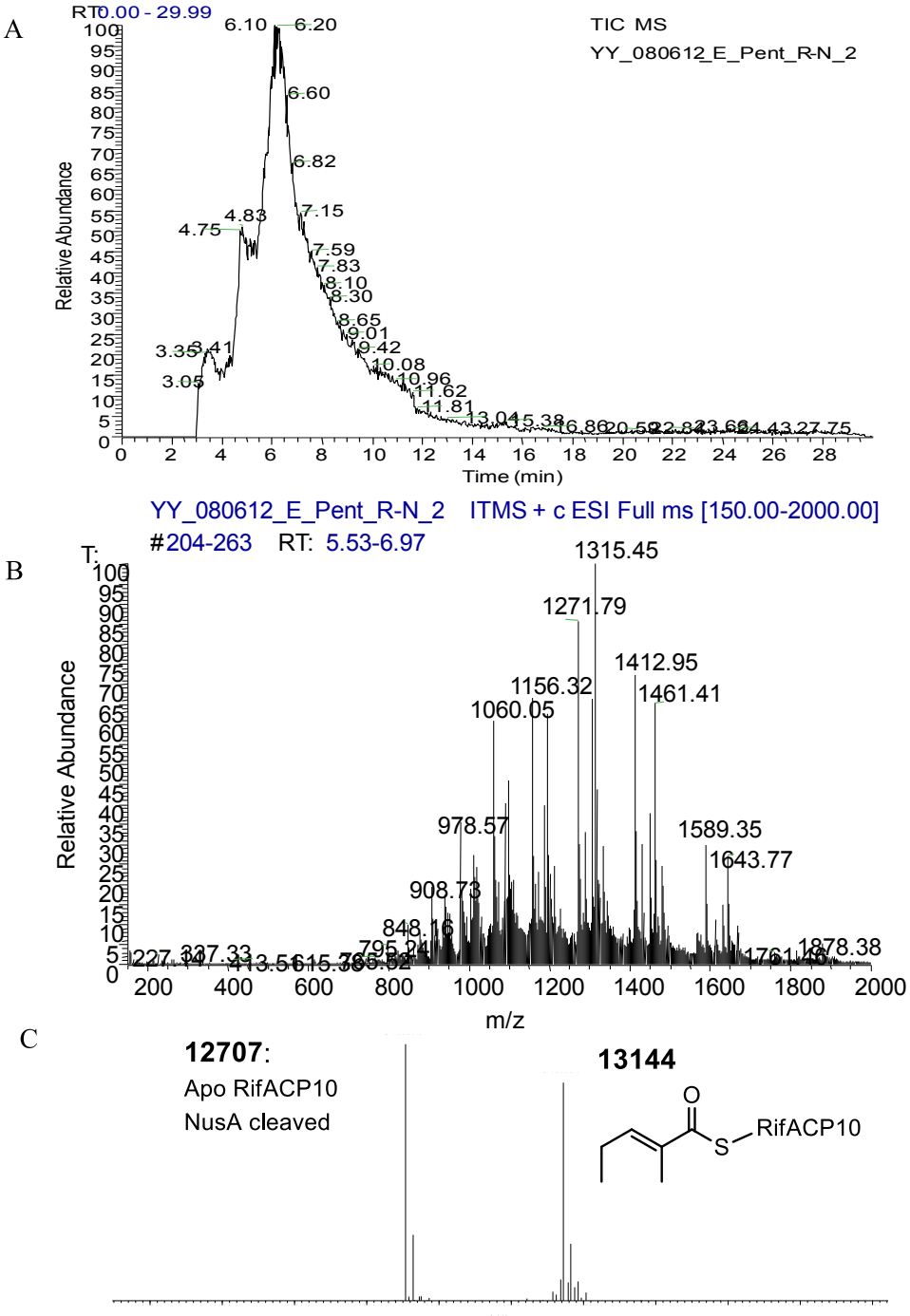
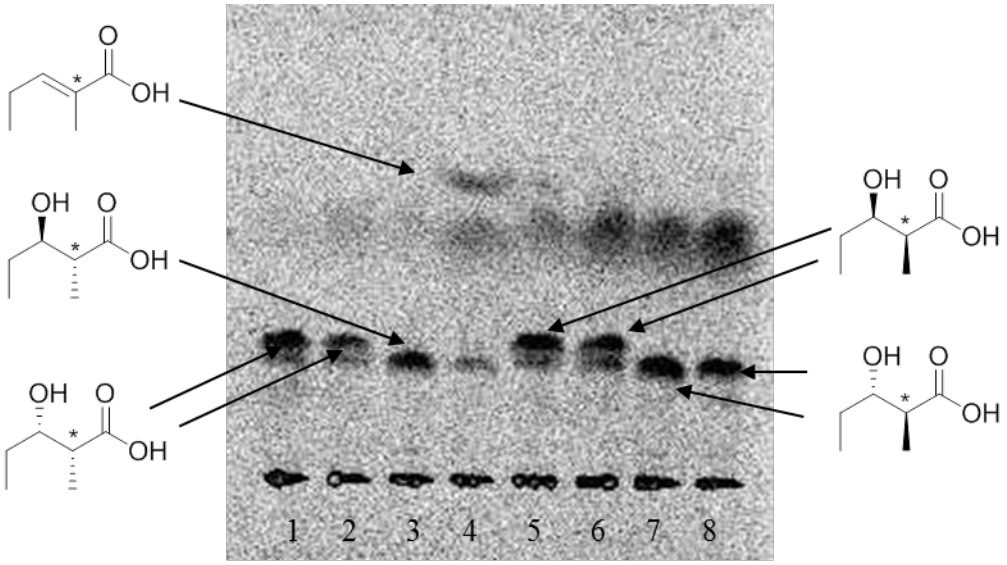


Figure S11. LC-ESI(+)-MS analysis of (*E*)-2-methyl-2-pentenoyl-RifACP10 generated by incubation of (*E*)-2-methylpentenoyl-CoA with Sfp and *apo*-RifACP10-NusA, followed by proteolytic cleavage of the NusA fragment with HRV 3C protease. **A**. LC-MS; **B**. LC-ESI(+)-MS; **C**. calculated full mass of (*E*)-2-methylpentenoyl-RifACP10 (theoretical $[M+H]^+$ 13143.9 Da). *apo*-RifACP10 was also observed (theoretical mass $[M+H]^+$ 12706.7 Da).



	EryKR6	TylKR1	EryKR1	RifKR7	RifDH10
1	+	-	-	-	-
2	+	-	-	-	+
3	-	+	-	-	-
4	-	+	-	-	+
5	-	-	+	-	-
6	-	-	+	-	+
7	-	-	-	+	-
8	-	-	-	+	+

Figure S12. TLC-phosphorimaging of diketide acid products from combinatorial enzyme reactions involving propionyl-SNAC, Ery[KS6][AT6], EryACP6, RifDH10, [^{14}C]-methylmalonyl-CoA, NADPH, and varying KR domains. Only Expt 4 with TylKR1 and RifDH10 produced 2-methyl-2-pentenoic acid. Note that the achiral TLC assay cannot distinguish *syn*-(2*S*,3*R*)- from the enantiomeric *syn*-(2*R*,3*S*)-2-methyl-3-hydroxypentanoic acid nor *anti*-(2*S*,3*S*)- from the enantiomeric *anti*-(2*R*,3*R*)-2-methyl-3-hydroxypentanoic acid.

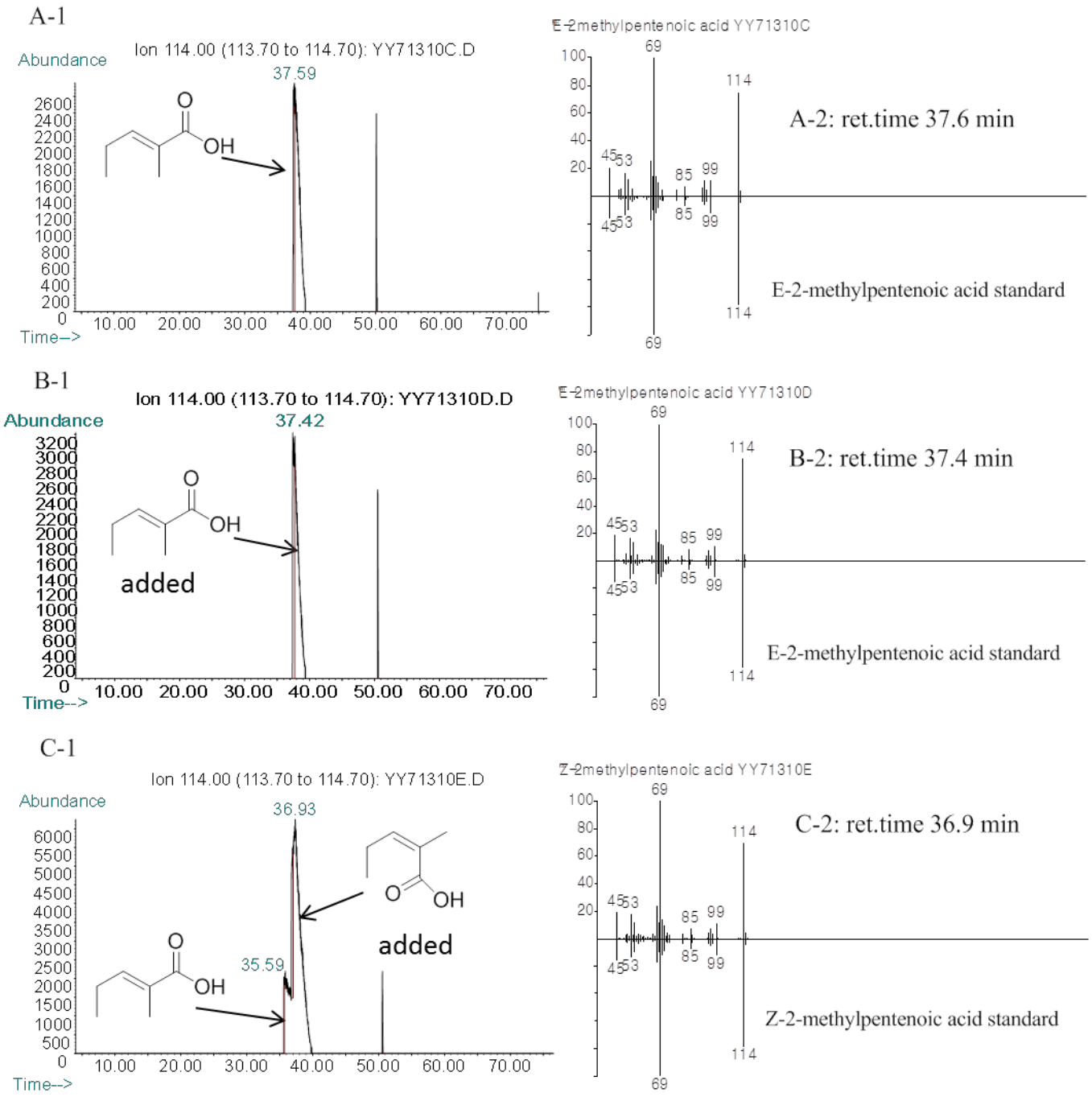


Figure S13. GC-MS analysis of the incubation of *in situ*-generated (2*R*)-2-methyl-3-ketopentanoyl-EryACP6 with TylKR1, NADPH, and RifDH10 (Method 3). **Left panels**: Extracted ion current (XIC) at *m/z* 114 (base peak). **Right panels**, mass spectra of selected peaks corresponding to unsaturated diketide acid **3**, upper half, observed peaks, lower half, mass spectrum of reference standard **3**. **A**. Reaction prouct. **B**. A plus authentic (*E*)-**3**. **C**. A plus authentic (*Z*)-2-methyl-2-pentenoic acid.

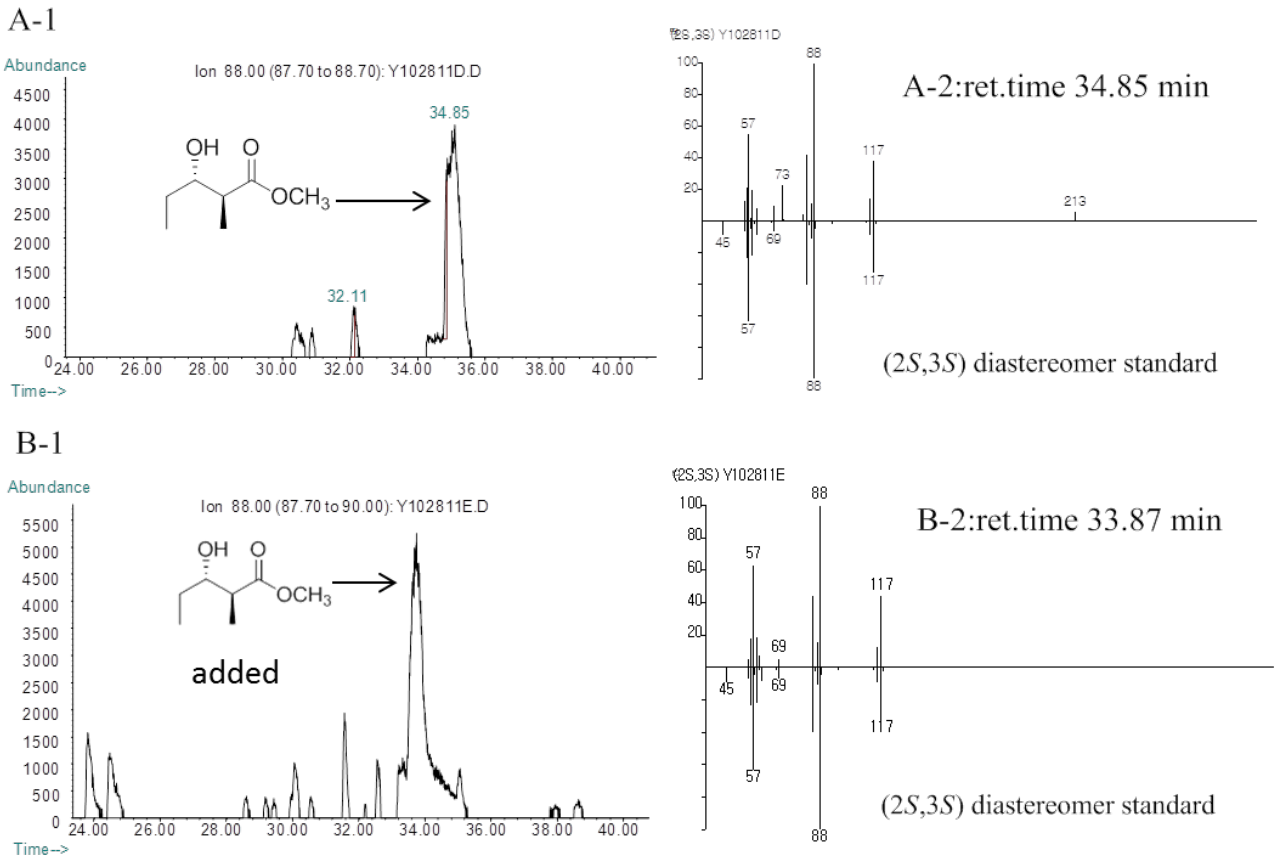


Figure S14. Chiral GC-MS analysis of the incubation of *in situ*-generated (*2R*)-2-methyl-3-ketopentanoyl-EryACP6 with RifDH10-KR10 (Method 4). Formation of methyl (2*S*,3*S*)-2-methyl-3-hydroxypentanoate. **Left panels**: Extracted ion current (XIC) at *m/z* 88 (base peak). **Right panels**, mass spectra of selected peaks corresponding to **4a**, upper half, observed spectrum, lower half, inverted mass spectrum of reference standards. **A** (2*S*,3*S*)-**4a** generated by RifDH10-KR10. **B**. A plus (2*S*,3*S*)-**4a** generated by enzymatic incubation by RifKR7 alone. No dehydration products such as **3** could be detected.

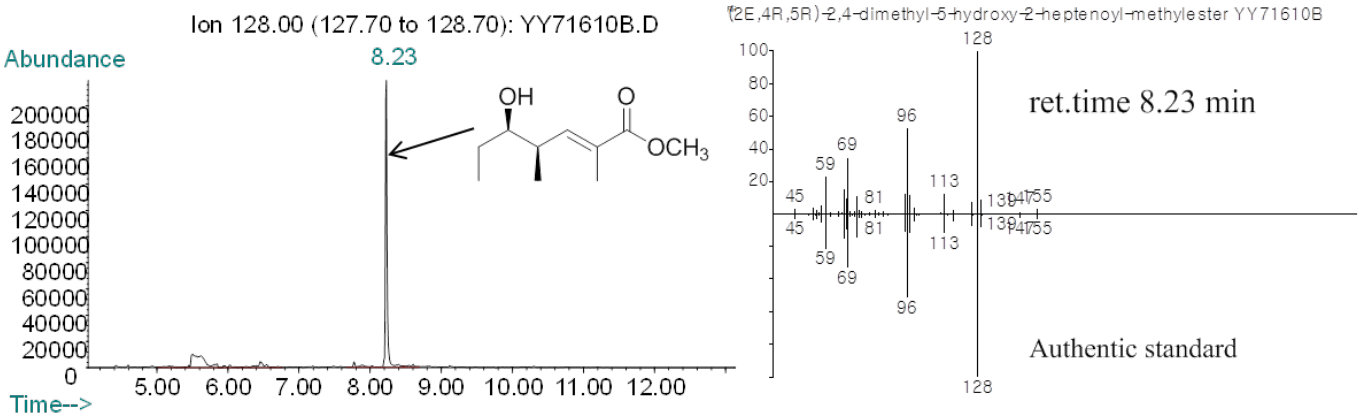


Figure S15. Chiral GC-MS analysis of the incubation of *in situ*-generated (2*R 4S 5R*)-2,4-dimethyl-3-keto-5-hydroxyheptanoyl-EryACP6 with TylKR1 and RifDH10. Reaction produces (2*E,4R,5R*)-2,4-dimethyl-5-hydroxy-2-heptenoyl methyl ester resulting from dehydration of the intermediate (2*R*,3*R,4S,5R*)-2,4-dimethyl-3,5-dihydroxyheptanoyl-EryACP6, hydrolysis, and methylation. **Left panel**: Extracted ion current (XIC) at *m/z* 128 (base peak for (2*E,4R,5R*)-2,4-dimethyl-5-hydroxy-2-heptenoyl methyl ester). **Right panel:** mass spectrum of selected peak corresponding to the unsaturated triketide acid methyl ester, upper half, observed peak, lower half, mass spectrum of reference standard.

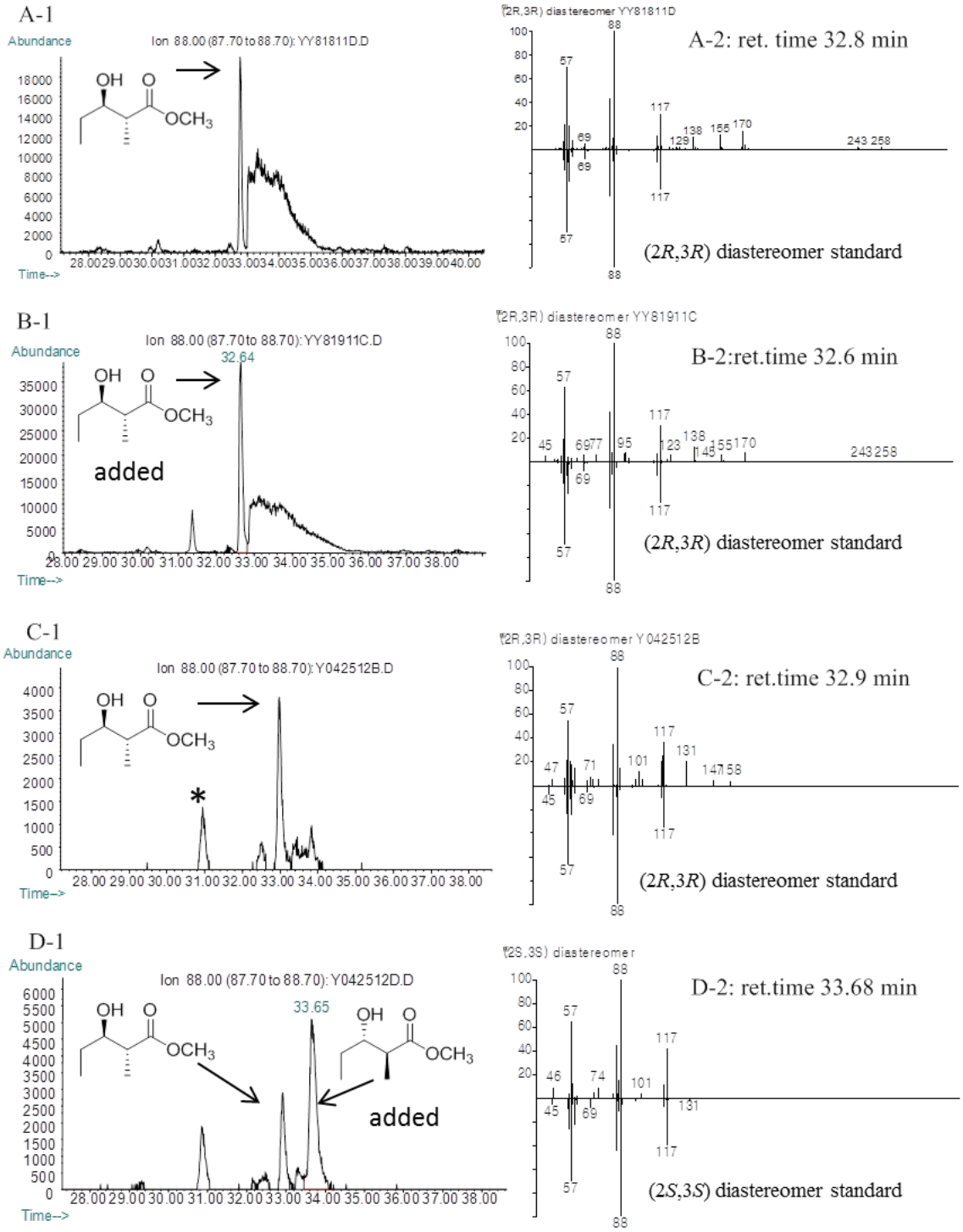


Figure S16. Chiral GC-MS analysis of the incubation of (*E*)-2-methylpentenoyl-EryACP6 with RifDH10 (Method 4). **Left panels**: Extracted ion current (XIC) at *m/z* 88 (base peak). **Right panels**, mass spectra of selected peaks corresponding to diastereomers, upper half, observed spectrum, lower half, inverted mass spectrum of reference standard. **A** and **C**. (2*R*,3*R*)-**4b** produced by RifDH10. **B.** A plus (2*R*,3*R*)-**4b** standard. **D**. C plus (2*S*,3*S*)-**4a** standard.

Synthesis of acyl-*S*-NAC and acyl-*S*-pantetheine thioesters

NMR and LC-MS characterization of *S*-pantetheine esters. ^{1}H NMR analysis of *S*-pantetheine esters was performed on a Varian Mercury 400 MHz instrument. LC-MS was performed on an Agilent Technologies 1200 Series HPLC with a Gemini C_{18} column (5 □m, 2 × 50 mm; Phenomenex) coupled to an Agilent Technologies 6130 quadrupole mass spectrometer system equipped with an electrospray-ionization source. A 5–95% B gradient over 12 min at a flow rate of 0.7 mL/min was run in which the mobile phases were (A) H_2O with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid.

N-Acetylcysteamine (NAC), Meldrum's acid derivatives, the methylketene dimer, (2*RS*)-2-methyl-3-ketobutanoyl-*S*-NAC, (2*RS*)-2-methyl-3-ketopentanoyl-*S*-NAC, and (3*R*)-3-hydroxypentanoyl-*S*-NAC were synthesized as previously reported,[1] as was *trans*-2-butenoyl-*S*-pantetheine.[2]

(2*RS*)-2-Methyl-3-ketopentanoyl-*S*-pantetheine. Sodium borohydride (80 mg, 16 eq.) was added to 94 mg D-pantethine (1 eq.) dissolved in 5 mL 80:20 MeOH:0.25 M $NaHCO_3$ (aq.) over 20 min at 22 °C. After 1 h, the reaction was quenched by adding glacial acetic acid dropwise until bubbling ceased and then was buffered with 250 mM HEPES (pH 7.5). 11.2 mg (1.4 eq.) of methylketene dimer was added to the solution at 22 °C. After 1 h, the reaction was evaporated and salts were removed using a plug of silica gel (2% MeOH:dichloromethane). *(2RS)-2-Methyl-3-ketopentanoyl-S-pantetheine*: ^{1}H NMR (400 MHz, D_2O) δ 0.71 (s, 3H), 0.74 (s, 3H), 0.81-0.88 (t, 3H, *J* = 8 Hz), 1.20-1.25 (d, 3H, *J* = 8 Hz), 2.28-2.33 (m, 2H) 2.51-2.58 (q, 2H, J = 8 Hz), 2.90-2.97 (t, 2H, *J* = 7.2 Hz), 3.20-3.26 (m, 2H), 3.30-3.40 (m, 2H), 3.40-3.45 (m, 2H), 3.81 (s, 1H), 4.00-4.05 (q, 1H, *J* = 8 Hz). ESI-MS expected mass: 391.5, observed mass: 391.2.

(2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-*S*-pantetheine, (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-*S*-pantetheine, (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-*S*-pantetheine, and (2*S*,3*S*)-2-methyl-3-hydroxypentanoyl-*S*-pantetheine. Each of the stereoisomers of 2-methyl-3-hydroxypentanoyl-*S*-pantetheine was prepared from the incubation of (2*RS*)-2-methyl-3-ketopentanoyl-*S*-pantetheine with various recombinant KR domains that have been shown to stereospecifically reduce the corresponding NAC-bound compounds (TylKR1, AmpKR2, EryKR1, and AmpKR1, respectively).[1] Reduction reactions were carried out in 10% (v/v) glycerol, 150 mM HEPES (pH 7.5), 100 mM NaCl, 200 mM D-glucose, 500 μM $NADP^+$, 10 μM KR, 1 μM GDH (cloned from *Bacillus subtilis*), and 2-10 mM (2*RS*)-2-methyl-3-ketopentanoyl-*S*-pantetheine in a total volume of 200 μL for 24 h at 22 °C. The

reaction was monitored by TLC, and the reduction product was purified by silica gel chromatography with 1:1 ethyl acetate:acetone.

***trans*-2-Methyl-*2*-butenoyl-*S*-pantetheine (11).** D-pantethine (1 mL of 0.5 M aq. soln) was reduced to D-pantetheine by addition of 75 mg DTT (1 eq.) and stirring for 1 h at 22 °C. Water was removed by vacuum to leave a clear, viscous liquid that was resuspended in 10 mL DCM. *trans*-2-Methyl-2-butenoic acid (100 mg, 2.1 eq.) (TCI Chemicals), 150 mg EDC (2.0 eq.) (Alfa Aesar), and 15 mg DMAP (0.25 eq.) (Acros Organics) were dissolved in 2 mL DCM and added to the pantetheine solution. The reaction was stirred at 0 °C for 1 h and 22 °C for an additional hour. All solvent was removed by vacuum, leaving a clear, viscous liquid that was purified by silica gel chromatography with 1:1 ethyl acetate:acetone. *trans-2-Methyl-2-butenoyl-S-pantetheine (**11**)*: ^{1}H NMR (400 MHz, D_2O) δ 0.71 (s, 3H), 0.74 (s, 3H), 1.66-1.70 (m, 6H), 2.29 (t, 3H, *J* = 6.5 Hz), 2.91 (t, 3H, *J* = 6.5 Hz), 3.20-3.36 (m, 8H), 3.81 (s, 1H), 6.80 (qq, 1H, *J*1 = 1.0 Hz, *J*2 = 6.6 Hz).

3*R*-Hydroxybutanoyl-*S*-pantetheine. RifDH10 (30 μM) was incubated with 10 mM *trans*-2-butenoyl-*S*-pantetheine in a 1 mL solution containing 150 mM NaCl, 10% (v/v) glycerol, 150 mM HEPES pH 7.5. After 24 h, the reaction was injected on a C_{18} reversed-phase HPLC column (100% water with 0.1% TFA to 100% MeOH with 0.1% TFA, 30 min), and the peak corresponding to the hydrated species was collected and concentrated under vacuum. *3R-Hydroxybutanoyl-S-pantetheine*: ^{1}H NMR (400 MHz, D_2O) δ 0.71 (s, 3H), 0.74 (s, 3H), 1.05 (d, 3H, *J* = 6.4 Hz), 2.29 (t, 3H, *J* = 6.5 Hz), 2.63 (d, 2H, *J* = 6.4 Hz), 2.89 (td, 2H, *J*1 = 2.2 Hz, *J*2 = 6.3 Hz), 3.21 (m, 3H), 3.32 (m, 3H) 3.46 (d, 1H, *J* = 4.4 Hz), 3.48 (d, 1H, *J* = 4.4 Hz), 3.81 (s, 1H), 4.09 (h, 1H, *J* = 6.5 Hz).

(*2R*,3*R*)-2-Methyl-3-hydroxybutanoyl-*S*-pantetheine (12b). Prepared in the same manner as 3*R*-hydroxybutanoyl-*S*-pantetheine, substituting *trans*-2-methyl-*2*-butenoyl-*S*-pantetheine for *trans*-2-butenoyl-*S*-pantetheine. *(2R,3R)-2-Methyl-3-hydroxybutanoyl-S-pantetheine (**12b**):* ^{1}H NMR (400 MHz, D_2O) δ 0.71 (s, 3H), 0.74 (s, 3H), 0.96 (d, 3H, *J* = 7.1 Hz), 1.04 (d, 3H, 6.3 Hz), 2.29 (t, 3H, *J* = 6.5 Hz), 2.63 (dd, 3H, *J*1 = 1.2 Hz, *J*2 = 8.1 Hz), 3.20-3.36 (m, 8H), 3.81 (s, 1H).

***cis*-2-Methyl-*2*-butenoyl-*S*-pantetheine (13).** Prepared similarly to *trans*-2-butenoyl-*S*-pantetheine, substituting 100 mg of *cis*-2-methyl-2-butenoic acid (TCI Chemicals) for *trans*-2-methyl-2-butenoic acid. *cis-2-Methyl-2-butenoyl-S-pantetheine (**13**)*: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (s, 3H), 1.06 (s, 3H), 1.28 (t, 3H, *J* = 7.1 Hz), 1.96 (dq, 3H, *J*1 = 1.7 Hz, *J*2 = 7.3 Hz), 2.02 (t, 3H, *J* = 2.0 Hz), 2.44 (t, 2H, *J* = 6.3 Hz), 3.11 (q, 2H, *J* = 6.7 Hz), 3.49-3.65 (m, 4H), 4.01 (d, 1H, *J* = 4.8 Hz), 4.14 (q, 3H, *J* = 7.4 Hz), 5.93 (qq, 1H, *J*1 = 2.4 Hz, *J*2 = 7.8 Hz).

***trans*,*trans*-2,4-Hexadienoyl-*S*-pantetheine.** *trans*,*trans*-2,4-Hexadienoic acid (1.0 g, Alfa Aesar) was stirred in 5 mL oxalyl chloride at 22 □C under a flow of nitrogen gas. After the oxalyl chloride had evaporated, reduced D-pantetheine (see synthesis of *trans*-2-methyl-*2*-butenoyl-*S*-pantetheine for D-pantethine reduction protocol) dissolved in 8.5 mL DCM and 1.5 mL TEA was added to the activated acid, and set to stir at 0 °C for 1 h, followed by quenching with 2 mL methanol. The reaction was washed with brine and all solvent was removed under vacuum, leaving a viscous liquid that was purified by silica gel chromatography with 1:1 ethyl acetate:acetone. *trans,trans-2,4-Hexadienoyl-S-pantetheine*: ^{1}H NMR (400 MHz, D_2O) δ 0.71 (s, 3H), 0.74 (s, 3H), 1.12 (t, 1H, *J* = 7.3 Hz), 1.71 (d, 2H, 6.6), 2.29 (t, 3H, *J* = 6.5 Hz), 2.96 (t, 3H, *J* = 6.5), 3.20-3.36 (m, 8H), 3.81 (s, 1H), 6.16 (m, 3H), 7.14 (dd, 1H, *J* = 10.6 Hz).

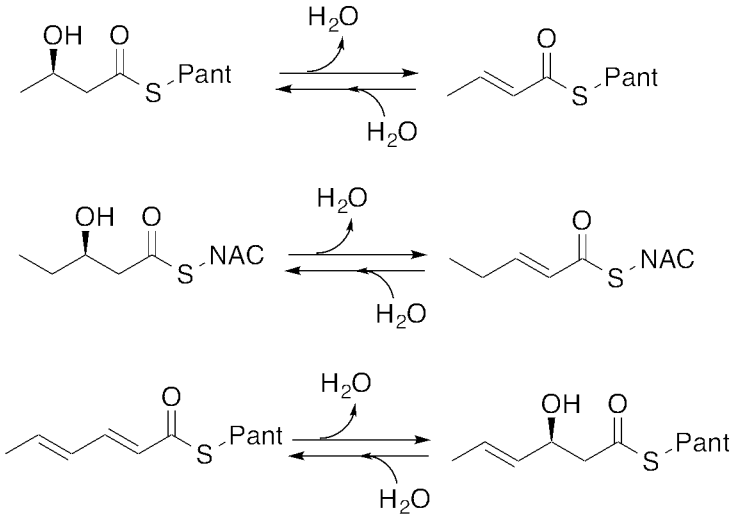


Figure S17. RifDH10-catalyzed dehydration/hydration of acyl thioester analogues.

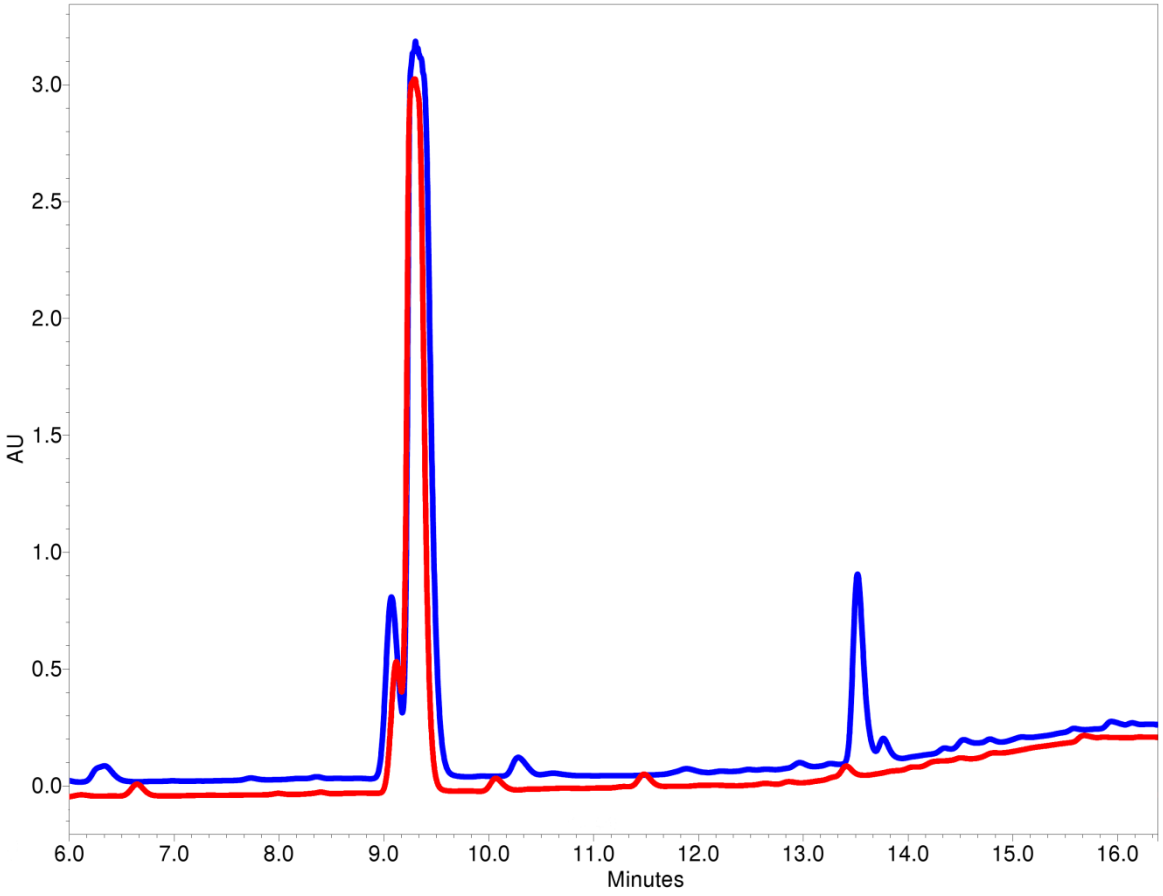


Figure S18. Dehydration of (3*R*)-3-hydroxybutanoyl-*S*-NAC. Reversed-phase HPLC analysis of a control (red, no enzyme) and the RifDH10-catalyzed dehydration (blue) of (3*R*)-3-hydroxybutanoyl-*S*-NAC (9.2 min), with product eluting at 13.5 min. Product peak was collected and confirmed with LC/MS: expected mass 187.3; observed mass: 187.0.

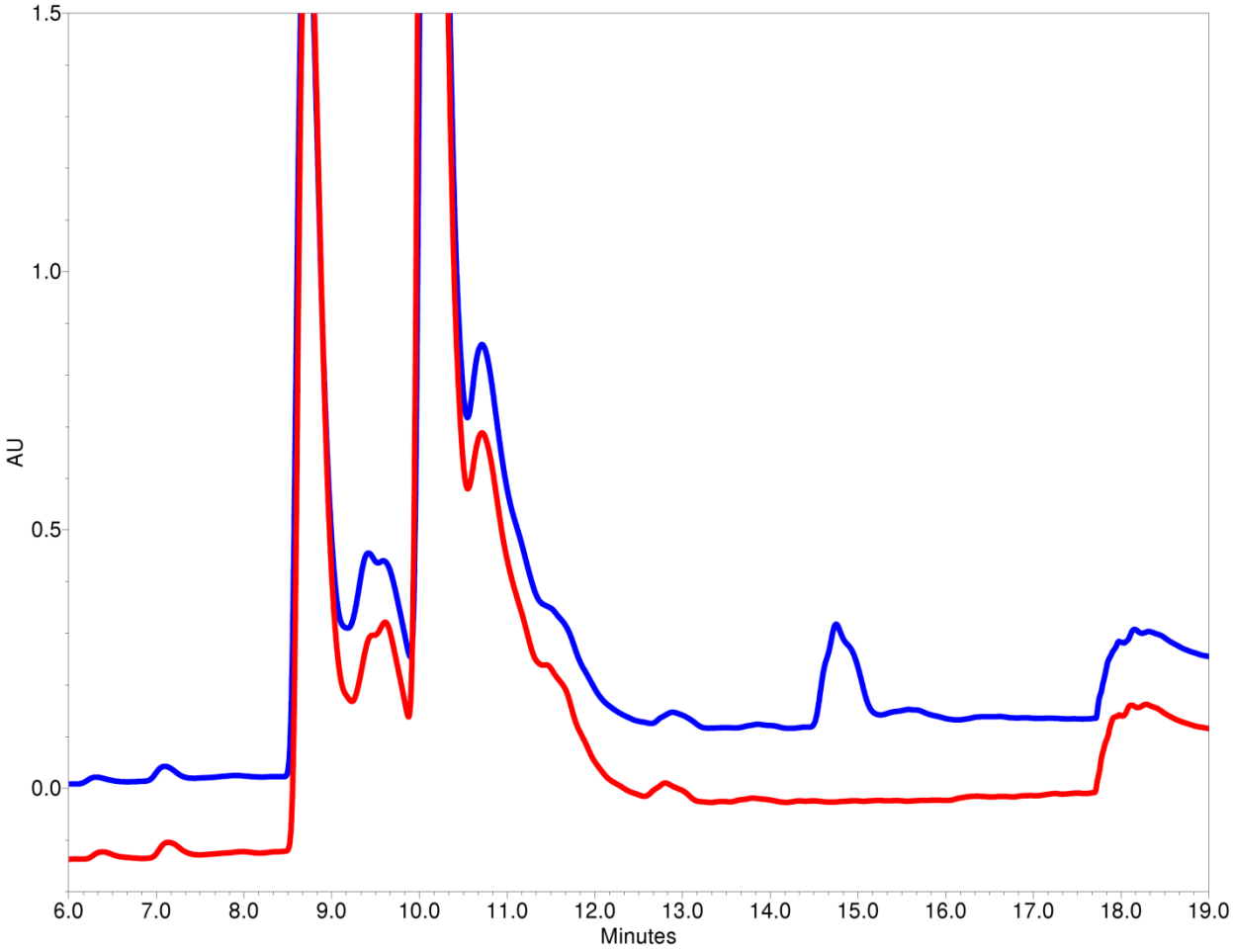


Figure S19. Dehydration of (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-*S*-NAC. Reversed-phase HPLC analysis of a control (red, no enzyme) and the RifDH10 catalyzed dehydration (blue) of (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-*S*-NAC (10.2 min), with product eluting at 14.8 min. The significant peak at 8.9 min is residual (2*RS*)-2-methyl-3-oxopentanoyl-*S*-NAC which was not reduced by the preceding TylKR1 catalyzed reduction. Product peak was collected and confirmed with LC/MS: expected mass 215.3; observed mass: 216.0.

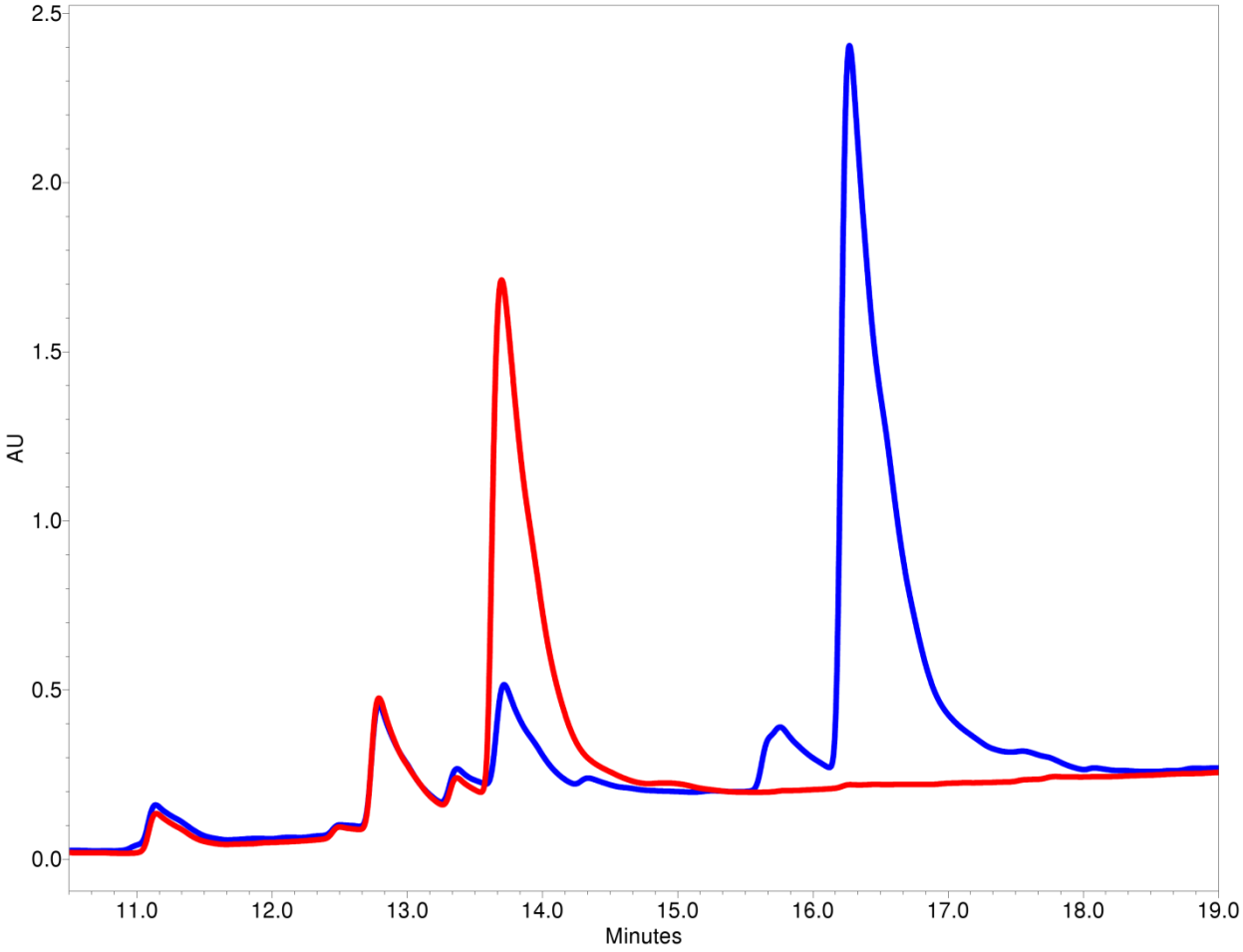


Figure S20. Dehydration of (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-*S*-pantetheine**.** Reversed-phase HPLC analysis of a control (red, no enzyme) and the RifDH10-catalyzed dehydration (blue) of (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-*S*-pantetheine (13.8 min), with product eluting at 16.4 min. Product peak was collected and confirmed with LC/MS: expected mass 374.5; observed mass: 374.0.

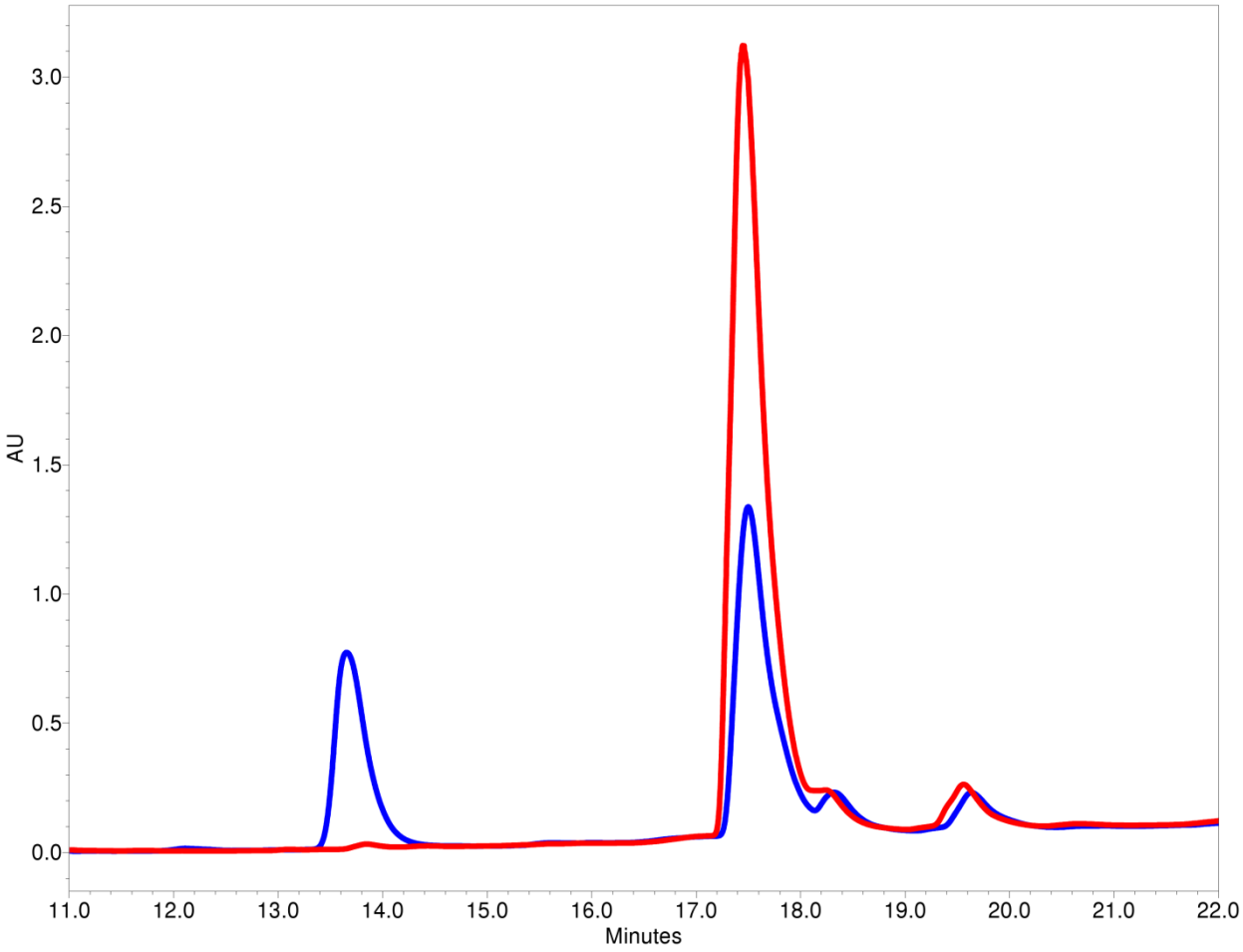


Figure S21. Hydration of (*E*)-2-butenoyl-*S*-pantetheine**.** Reversed-phase HPLC analysis of a control (red, no enzyme) and the RifDH10-catalyzed hydration (blue) of (*E*)-2-butenoyl-*S*-pantetheine (17.6 min), with product eluting at 13.8 min. Product peak was collected and confirmed with LC/MS: expected mass 364.5; observed mass: 365.0.

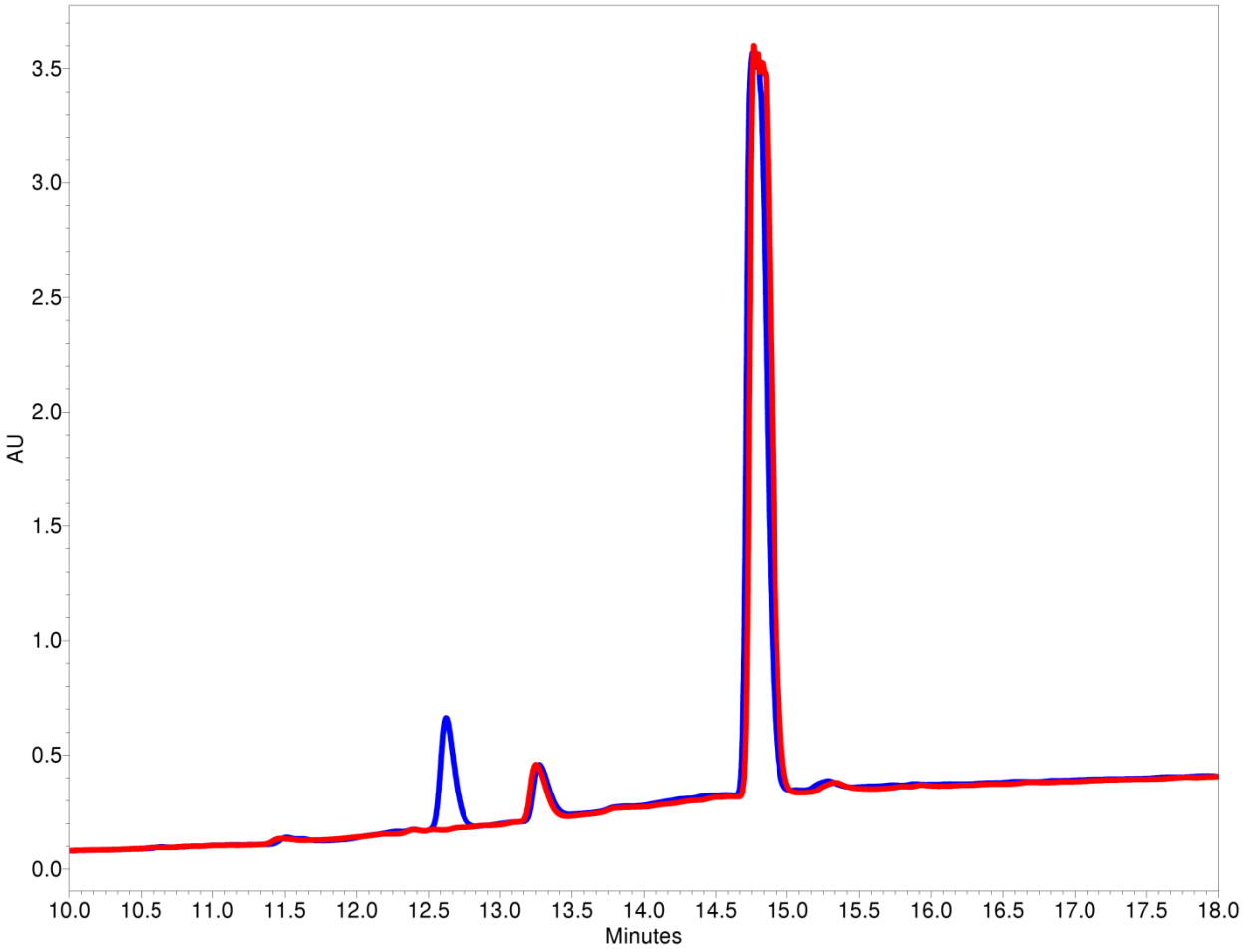


Figure S22. Hydration of (*E*)-2-methyl-2-butenoyl-*S*-pantetheine. Reversed-phase HPLC analysis of a control (red, no enzyme) and the RifDH10-catalyzed hydration (blue) of (*E*)-2-methyl-2-butenoyl-*S*-pantetheine (14.8 min), with product eluting at 12.6 min. Product peak was collected and confirmed with LC/MS: expected mass 378.5; observed mass: 378.0.

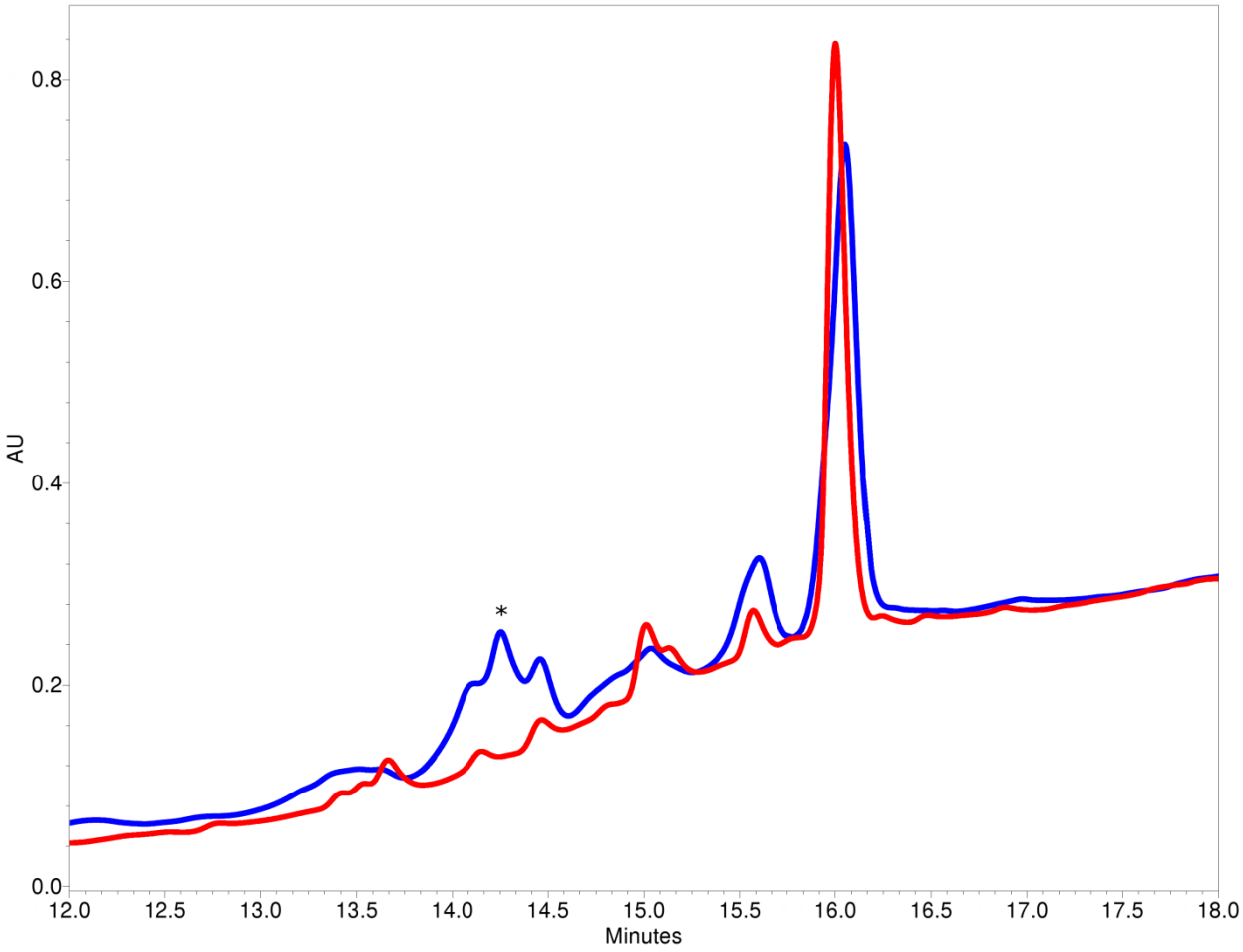


Figure S23. Hydration of (*E,E*)-2,4-hexadienoyl-*S*-pantetheine. Reversed-phase HPLC analysis of a control (red, no enzyme) and the RifDH10 catalyzed hydration (blue) of (*E,E*)-2,4-hexadienoyl-*S*-pantetheine (16.1 min), with product eluting at 14.3 min. The hydrated product has been highlighted with an asterisk due to adjacent contaminating peaks, which are also present in the control reaction. Product peak was collected and confirmed with LC/MS: expected mass 390.5; observed mass: 391.0.

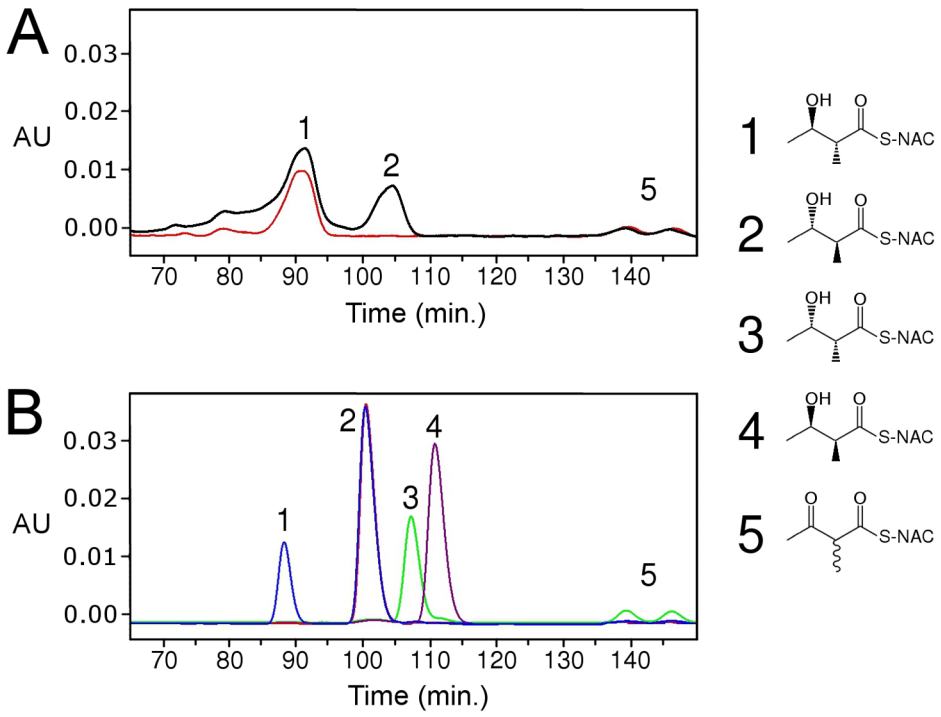


Figure S24. Stereochemistry of hydrated product from RifDH10-catalyzed hydration of (*E*)-2-methyl-2-butenoyl-*S*-pantetheine. (*E*)-2-Methyl-2-butenoyl-*S*-pantetheine was incubated with RifDH10, and the resultant hydrated product was purified by reverse-phase HPLC. The acyl group was subsequently transferred to *S*-NAC, and subjected to chiral HPLC analysis. (A) Chiral HPLC trace of the product of the RifDH10 catalyzed hydration of (*E*)-2-methyl-2-butenoyl-*S*-pantetheine (red). The run was repeated after being spiked with authentic (2*S*,3*S*)-2-methyl-3-hydroxy-butanoyl-*S*-NAC (black trace). (B) A series of authentic standards analyzed on the same chiral column used to determine retention times of each of the stereoisomers of 2-methyl-3-hydroxy-butanoyl-*S*-NAC.

Supplemental References.

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2. Zheng, J., Gay, D. C., Demeler, B., White, M. A., and Keatinge-Clay, A. T. (2012) Divergence of multimodular polyketide synthases revealed by a didomain structure, Nat. Chem. Biol. 8, 615-621.