Supporting Information

Coupled Methyl-Group Epimerization and Reduction by Polyketide Synthase Ketoreductase Domains. Ketoreductase-Catalyzed Equilibrium Isotope Exchange

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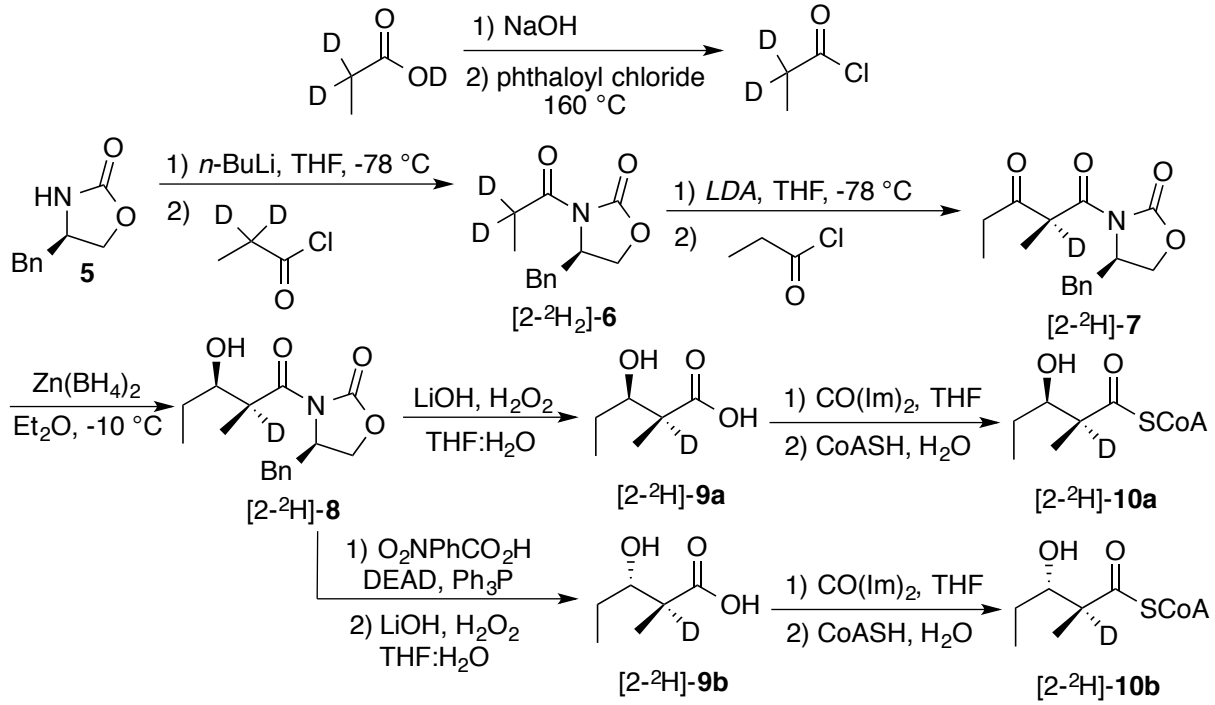
Materials

Isopropylthio-β-D-galactopyranoside (IPTG) was purchased from Invitrogen. All other chemical reagents were purchased from Sigma-Aldrich and utilized without further purification. Ni-NTA affinity resin was purchased from Qiagen. Amicon Ultra Centrifugal Filter Units (Amicon Ultra-15, 30,000 MWCO) were purchased from Millipore. Recombinant EryACP6, EryKR1, RifKR7, NysKR1, RifKR7, EryKR6, TylKR1, and Sfp were each expressed and purified as previously described.[1-7] Reference standards of methyl (2*S*,3*R*)-2-methyl-3-hydroxypentanoate (**4a**), methyl (2*S*,3*S*)-2-methyl-3-hydroxypentanoate (**4b**), methyl (2*R*,3*S*)-2-methyl-3-hydroxypentanoate (**4c**), and methyl (2*R*,3*R*)-2-methyl-3-hydroxypentanoate (**4d**), prepared as previously described,[3] were used as standards for chiral GC-MS analysis and for comparison with the corresponding [2-^{2}H]-2-methyl-3-hydroxypentanoates.

Methods. General methods were as previously described.[3,8] ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance III HD Ascend 600 MHz spectrometer. Optical rotations were measured using a Jasco P-1010 polarimeter. Chiral GC-MS analysis was performed on a GC–MS Hewlett-Packard 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. A Thermo LXQ equipped with Surveyor HPLC system and an Agilent Zorbax C18 column (50 × 2.1 mm, 3.5 μm) was used to analyze diketide-CoA compounds 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. Growth media and conditions used for *E. coli* and standard methods for handling *E. coli in vivo* and *in vitro* were those described previously, unless otherwise noted.[8] All DNA manipulations were performed following standard procedures.[8] All proteins were handled at 4 °C unless otherwise stated. Protein concentrations were determined according to the method of Bradford[9] using Hewlett Packard 8452A Diode Array or Thermo Evolution Array UV/Vis spectrophotometers with bovine serum albumin as the standard. SDS-PAGE gels were imaged and analyzed with a Bio-Rad ChemiDoc MP System.

Synthesis of deuterated diastereomers of [2-^{2}H]-2-methyl-3-hydroxypentanoic acid (9) and [2-^{2}H]-2-methyl-3-hydroxypentanoyl-CoA (10). Diasteromerically pure *syn*-[2-^{2}H]-(2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid (9a) and *syn*-[2-^{2}H]-(2*R*,3*S*)-2-methyl-3-hydroxypentanoic acid (9c) were synthesized by acylation of the chiral imide enolates followed by stereospecific reduction of the derived 2-methyl-3-ketopentanoyl oxazolidinones, as previously described for unlabeled *syn*-2-methyl-3-hydroxypentanoic acids.[10-13] The *anti*-[2-^{2}H]-(2*S*,3*S*)-2-methyl-3-hydroxypentanoic acid (9b) and *anti*-[2-^{2}H]-(2*R*,3*R*)-2-methyl-3-hydroxypentanoic acid (9d) diastereomers, identical to reference compounds prepared as previously described by the di-*n*-butylborontriflate method,[3] were prepared by Mitsunobu inversion of the 3-hydroxyl group in each of the corresponding synthetic intermediates, [2-^{2}H]-8 and [2-^{2}H]-14.



Scheme S1. Synthesis of [2-^{2}H]-(2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid (**9a**), [2-^{2}H]-(2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-CoA (**10a**), [2-^{2}H]-(2*S*,3*S*)-2-methyl-3-hydroxypentanoic acid (**9b**), and [2-^{2}H]-(2*S*,3*S*)-2-methyl-3-hydroxypentanoyl-CoA (**10b**).

[2-^{2}H$_2$]-Propionyl-(4′*R*-benzyl)-*N*-oxazolidinone ([2-^{2}H$_2$]-6). To a stirred solution of **5** (500 mg, 2.82 mmol) in anhydrous THF (10.0 mL) at -78 °C was added dropwise *n*-BuLi (1.6 M solution in heptane, 1.8 mL, 2.88 mmol). The reaction mixture was stirred for 10 min and [2-^{2}H$_2$]-propionyl chloride (2.8 mmol), prepared as previously described,[13] was

added. After 10 min the cooling bath was removed and the reaction was allowed to warm to 0 °C over 30 min, then quenched with saturated aq. NH_4Cl. The THF was removed by rotary evaporation and the mixture was extracted with CH_2Cl_2. The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo. Flash silica gel chromatography yielded 629 mg (94 %) of [2-$^2H_2$]-**6**. $[\alpha]_D$ -76.0° (c 0.9, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$) δ 1.18 (s, 3H), 2.79 (dd, *J* = 6, 12 Hz, 1H), 3.32 (dd, *J* = 6, 12 *Hz*, 1H), 4.19-4.24 (m, 2H), 4.68-4.71 (m, 1H), 7.24-7.38 (m, 5H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 8.2, 37.9, 55.2, 66.2, 127.4, 128.9, 129.4, 135.3, 153.5, 174.1. The ^{13}C NMR spectrum matched that of unlabeled **6**[13] except for the absence of the peak at δ 29.0 corresponding to the deuterium-substituted C-2. Integration of the ^1H NMR spectrum indicated d_2 ~100%.

[2-^{2}H]-7. To a stirred solution of [2-$^2H_2$]-**6** (200 mg, 0.85 mmol) in anhydrous THF (4.0 mL) at -78 °C was added dropwise LDA (2.0 M solution in heptanes, 0.5 mL, 1 mmol) and the solution was stirred for 2.5 h. After addition of propionyl chloride, the reaction was further stirred for 30 min, then quenched with saturated aq. NH_4Cl/D_2O and extracted with CH_2Cl_2. GC-MS analysis confirmed the formation of [2-^{2}H]-**7** containing small amounts of recovered **6**.

Zinc borohydride: To a stirred suspension of $NaBH_4$ (760 mg, 20 mmol) in anhydrous ether (10.0 mL) was added $ZnCl_2$ (1.0 M in ether, 10.0 mL, 10 mmol). The mixture was stirred at rt for 24 h, the residue was separated, and the homogenous solution was stored at 4 °C.

[2-^{2}H]-8. To a stirred solution of [2-^{2}H]-**7** (150 mg, 0.52 mmol) in anhydrous ether at -20 °C was added zinc borohydride (0.76 mmol) and the mixture was stirred for 45 min under N_2 atmosphere. The reaction mixture was then poured into ice-cold water and stirred for 1 h. The organic layer was separated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude mixture was then purified using flash chromatography to give 80 mg of [2-^{2}H]-**8** (80%), as well as 50 mg of recovered starting material. $[\alpha]_D$= -40.5° (c 0.77, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$) δ 1.03 (t, *J* = 9.0 Hz, 3 H), 1.21 (s, 3H), 1.59-1.64 (m, 2H), 2.71 (b, 1H, OH), 2.78-2.82 (m, 1H), 3.32 (dd, *J* = 6, 12 Hz, 1H),

3.92 (m, 1H), 4.21-4.24 (m, 2H), 4.70-4.74 (m, 1H), 7.24-7.38 (m, 5H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 10.0, 10.5, 26.8, 38.0, 55.3, 66.2, 73.2, 127.4, 129.0, 129.4, 135.2, 153.2, 177.2. The ^{13}C spectrum matched that of unlabeled **8**[13] except for the absence of the peak at δ 41.8 corresponding to the deuterated C-2. Integration of the ^{1}H NMR spectrum indicated d_1 ~100%.

(2S,3*R*)-[2-^{2}H]-2-Methyl-3-hydroxypentanoic acid (9a). To a stirred suspension of [2-^{2}H]-**8** (142 mg, 0.48 mmol) in 2.0 mL of 4:1 THF:H_2O was added 20 mg of LiOH and 55 μL of 30% H_2O_2. The reaction mixture was stirred for 2 h at rt. The excess H_2O_2 was quenched by addition of Na_2SO_3. The reaction mixture was extracted with ethyl acetate to remove any neutral organic impurities, then acidified to pH 5.0 and extracted with ethyl acetate. Finally the aqueous layer was acidified to pH 2.0 and extracted with ethyl acetate to give 52 mg of acid [2-^{2}H]-**9a** (82%). Chiral GC-MS analysis of the derived methyl ester, (2*S*,3*R*)-[2-^{2}H]-**4a**, prepared by derivatization with $TMSCHN_2$, showed that the synthetic sample consisted of a single diastereomer. [2-^{2}H]-**9a**, $[\alpha]_D$ -2.6° (c 2.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.01 (t, *J* = 6 Hz, 3H), 1.21 (s, 3H), 1.50-1.57 (m, 2H), 3.86-3.91 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 10.3, 10.34, 26.6, 73.2, 180.2. The ^{13}C spectrum matched that of unlabeled **9a**[13] except for the absence of the peak at δ 43.7 corresponding to the deuterated C-2. Integration of the ^{1}H NMR spectrum indicated d_1 ~100%.

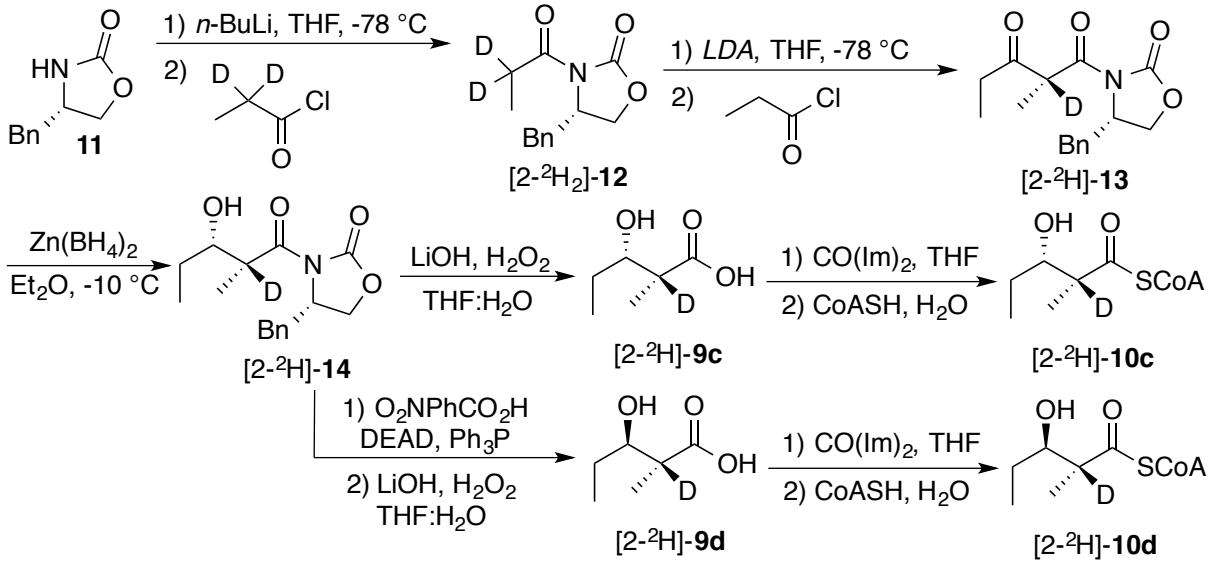
(2S,3*R*)-[2-^{2}H]-2-Methyl-3-hydroxypentanoyl-CoA (**10a**)**.** To a stirred solution of acid **[2-^{2}H]-9a** (10 mg, 0.07 mmol) in anhydrous THF was added 1,1'-carbonyldiimidazole (22.7 mg, 0.14 mmol, 2.0 eq) at 0 °C and the solution was stirred for 1 h. To this reaction mixture was added CoASH (10 mg, 13 μmol) dissolved in H_2O (1.0 mL) and the solution was stirred at room temperature (rt) for an additional 4 h. The organic solvent was removed by rotary evaporation and the aqueous phase was extracted with ether to remove organic byproducts. The CoA thioester product [2-^{2}H]-**10a** was purified by HPLC (Dynamax) using a Phenomenex Gemini semi-preparative C18 column, (150 × 10 mm, 10 μm) equilibrated with 5% CH_3CN/H_2O. The sample was eluted with a linear gradient from 5% to 80% of CH_3CN/H_2O. Each peak was collected separately, CH_3CN was removed by rotary evaporation and the water was removed by lyophilization. Each

fraction was separately analyzed by HPLC-ESI(+)-MS using an Agilent Zorbax C18 column (50 × 2.1 mm, 3.5 μm) and a linear gradient from 5% to 65% of CH_3CN/H_2O. (2S,3*R*)-[2-^{2}H]-2-methyl-3-hydroxypentanoyl-CoA (**10a**). Yield, 6 mg; ESI(+)-MS $[M+H]^+$ observed *m/z* 883.4, calculated for [2-^{2}H]-**10a**-H^+ *m/z* 883.2; ^{1}H NMR (D_2O, 600 MHz) δ 0.65 (s, 3H), 0.88 (s, 3H), 0.88 (m, 3 H), 0.96 (s, 3H), 1.46 (m, 2H), 2.48 (m, 2H), 2.64 (m, 1H), 2.88 (m,1H), 3.23-3.50 (m, 4H), 3.57 (m, 1H), 3.57 (m, 2H), 3.88 (m, 2H), 4.08 (m, 1H), 4.40 (m, 2H), 4.72 (m, 1H), 6.10 (m, 1H), 7.31 (m, 1NH), 8.27 (s, 1H), 8.53 (s, 1H).

(2*S*,3*S*)-[2-^{2}H]-2-Methyl-3-hydroxypentanoic acid (9b). A solution of diethylazodicarboxylate (DEAD, 40% in toluene, 3.2 mL, 7.0 mmol) in benzene (5.0 mL) was added to (2*S*,3*R*)-[2-^{2}H]-**8** (290 mg, 1.0 mmol), triphenylphosphine (1.8 g, 7.0 mmol), and *p*-nitrobenzoic acid (1.2 g, 7.0 mmol) in benzene (10.0 mL) at rt (rt). The resulting solution was stirred for 24 h at rt. The solvent was removed under vacuum and the slurry was filtered over silica gel. The silica gel was washed with excess diethyl ether and the combined organic filtrate was concentrated to 20.0 mL, then mixed with 20.0 mL of hexane and the resulting mixture was stirred overnight to precipitate PPh_3O as a white solid. The clarified filtrate was concentrated and purified by SiO_2 column chromatography to afford 310 mg of the (3*S*)-*p*-nitrobenzoate ester. $[\alpha]_D$ -2.6° (c 2.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.98 (t, *J* = 9.0 Hz, 3 H), 1.21 (s, 3H), 1.79-1.84 (m, 1H), 2.08-2.32 (m, 1H), 3.08-3.11 (m, 1H), 4.09-4.17 (m, 1H), 4.19-4.28 (m, 2H), 4.62-4.66 (m, 1H), 5.55 (m, 1H), 7.07-7.11 (m, 2H), 7.26-7.29 (m, 3H), 7.80-7.91 (m, 1H), 8.24-8.37 (m, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 8.9, 13.8, 24.3, 37.7, 55.4, 65.2, 65.9, 123.6, 127.4, 128.9, 129.2, 130.7, 135.6, 139.9, 151.4, 153.1, 163.8, 167.2, 1734.3. The ^{13}C spectrum corresponded to that of unlabeled material except for the absence of the deuterium-substituted peak for C-2 (41.6 ppm). ESI(+)-HRMS of unlabeled *p*-nitrobenzoate ester, $[M+H]^+$ *m/z* 441.1656, calcd for $C_{23}H_{24}N_2O_7$, $[M+H]^+$ *m/z* 441.1656. The (3*S*)-*p*-nitrobenzoate ester (230 mg, 0.50 mmol) was dissolved in 4:l THF:water (8 mL) at 0 °C to which was added 320 μL of 30% aq H_2O_2 and 120 mg of LiOH. The mixture was stirred at 0 °C for 1 h and then for 12 h at rt. A solution of aq. $Na_2S_20_3$ (2.0 mL) was added and the mixture was washed with CH_2Cl_2 (4 x 5 mL). The aqueous layer was acidified with 6 M HCl to pH 3.0 and extracted with ethyl acetate to

remove *p*-nitrobenzoic acid, then further acidified to ca. pH 1 with 6 M HCl and extracted with CH_2Cl_2 (4 x 5 mL). The combined CH_2Cl_2 extracts were dried over Na_2SO_4 and concentrated to yield 40 mg of acid (2*S*,3*S*)-[2-^{2}H]-**9b**. Chiral GC-MS of the derived methyl ester (2*S*,3*S*)-[2-^{2}H]-**4b,** showed the presence of 10% (2*S*,3*R*)-[2-^{2}H]-**4a** (Figure S1). (2*S*,3*S*)-[2-^{2}H]-**9b**, $[\alpha]_D$ 17.9° (c 0.9, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.01 (t, *J* = 6 Hz, 3H), 1.23 (s, 3H), 1.50-1.59 (m, 2H), 3.88-3.91 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 10.3, 10.4, 26.5, 73.1, 180.5. The ^{13}C spectrum matched that of unlabeled **9b**[13] except for the absence of the peak at δ 43.7 corresponding to the deuterated C-2. Integration of the ^{1}H NMR spectrum indicated d_1 ~100%.

(2*S*,3*S*)-[2-^{2}H]-2-Methyl-3-hydroxypentanoyl-CoA (**10b**)**.** To a stirred solution of acid [2-^{2}H]-**9b** (15 mg, 10 mmol) in anhydrous THF was added 1,1'-carbonyldiimidazole (25 mg, 0.15 mmol) and the solution was stirred for 1 h. To this mixture was added CoASH (10 mg, 13 μmol) dissolved in H_2O (1.0 mL) and the reaction was stirred at rt for an additional 4 h. The (2*S*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoyl-CoA **(**[2-^{2}H]-**10b**) (3 mg) was isolated, purified by HPLC, and then analyzed by LC-ESI(+)-MS as described above for [2-^{2}H]-**10a**. ESI(+)-MS $[M+H]^+$ observed *m/z* 883.4, calculated for [2-^{2}H]-**10b**-H^+ *m/z* 883.2.



Scheme S2. Synthesis of [2-^{2}H]-(2*R*,3*S*)-2-methyl-3-hydroxypentanoic acid (**9c**), [2-^{2}H]-(2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-CoA (**10c**), [2-^{2}H]-(2*R*,3R)-2-methyl-3-hydroxypentanoic acid (**9d**), and [2-^{2}H]-(2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-CoA (**10d**).

[2-$^2H_2$]-Propionyl-(4′*S*-benzyl)-*N*-oxazolidinone ([2.$^2H_2$]-12). [2.$^2H_2$]-**12** was prepared from (4′*S*-benzyl)-*N*-oxazolidinone (**11**) and [2-$^2H_2$]-propionyl chloride in the same manner as described for [2.$^2H_2$]-**6**. $[\alpha]_D$ -66.08° (c 1.25, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$) δ 1.21(s, 3H), 2.79 (dd, *J* = 6, 12 Hz, 1H), 3.31 (dd, *J* = 6, 12 Hz, 1H), 4.17-4.23 (m, 2H), 4.67-4.71 (m, 1H), 7.23-7.37 (m, 5H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 8.1, 37.9, 55.1, 66.2, 127.3, 128.9, 129.4, 135.4, 153.5, 174.1. The ^{13}C spectrum matched that of unlabeled **12**[13] except for the absence of the peak at δ 29.0 corresponding to the deuterium-substituted C-2. Integration of the ^1H NMR spectrum indicated d_2 ~100%.

(2*R*,3*S*)-[2-^2H]-14. (2*R*,3*S*)-[2-^2H]-**14** was synthesized by $Zn(BH_4)_2$ reduction of the corresponding 3-ketoimide [2-^2H]-**13**, as described for the synthesis of (2*S*,3R)-[2-^2H]**-8**. $[\alpha]_D$ +34.9° (c 1.35, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$) δ 1.03 (t, *J* = 9.0 Hz, 3 H), 1.21 (s, 3H), 1.59-1.64 (m, 2H), 2.71 (b, 1H, OH), 2.78-2.82 (m, 1H), 3.33 (dd, *J* = 6, 12 Hz, 1H), 3.93 (m, 1H), 4.21-4.25 (m, 2H), 4.70-4.74 (m, 1H), 7.24-7.38 (m, 5H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 10.0, 10.4, 26.8, 38.1, 55.3, 66.2, 73.2, 127.4, 129.0, 129.4, 135.2, 153.2, 177.1. The ^{13}C spectrum matched that of unlabeled **14**[13] except for the absence of the peak at δ 41.8 corresponding to the deuterated C-2. Integration of the ^1H NMR spectrum indicated d_1 ~100%.

(2*R*,3*S*)-[2-^2H]-2-Methyl-3-hydroxypentanoic acid (9c). (2*R*,3*S*)-[2-^2H]-**9c** was prepared by LiOH/H_2O_2 hydrolyis of (2*R*,3*S*)-[2-^2H]-**14,** as described for the synthesis of (2*S*,3*R*)-[2-^2H]-**9a** $[\alpha]_D$ -6.49° (c 1.6, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$) δ 1.02 (t, *J* = 6 Hz, 3H), 1.24 (s, 3H), 1.53-1.56 (m, 2H), 3.88-3.90 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 10.2, 10.4, 26.6, 73.2, 181.3. The ^{13}C spectrum matched that of unlabeled **9c**[13] except for the absence of the peak at δ 43.7 corresponding to the deuterated C-2. Integration of the ^1H NMR spectrum indicated d_1 ~100%.

(2*R*,3*S*)-[2-^2H]-2-Methyl-3-hydroxypentanoyl-CoA (**10c**)**.** (2*R*,3*S*)-[2-^2H]-**10c** was prepared from (2*R*,3*S*)-[2-^2H]-**9c** in the same manner as described for [2-^2H]-**10a.** (2R,3*S*)-[2-^2H]-2-methyl-3-hydroxypentanoyl-CoA (**10c**): ESI(+)-MS $[M+H]^+$ observed *m/z* 883.6, calculated for [2-^2H]-**10c**-H^+ *m/z* 883.2.

(2*R*,3*R*)-[2-^2H]-2-Methyl-3-hydroxypentanoic acid (9d). (2*R*,3R)-[2-^2H]-**9d** was prepared from (2*R*,3*S*)-[2-^2H]-**14** by the same Mitsunobu inversion/hydrolysis procedure

described for **(2*S*,3*S*)-[2-^{2}H]-9b.** Chiral GC-MS of the derived methyl ester (2*R*,3*R*)-[2-^{2}H]-**4d,** confirmed the diastereomeric purity of (2*R*,3*R*)-[2-^{2}H]-**9d** (Figure S2). (2*R*,3*R*)-[2-^{2}H]-**9d** $[\alpha]_D$ -26.5° (c 1.4, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.97-1.02 (m, 3H), 1.23 (s, 3H), 1.48-1.55 (m, 2H), 3.86-3.89 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 10.2, 10.4, 26.6, 73.1, 181.0.

(2*R*,3*R*)-[2-^{2}H]-2-Methyl-3-hydroxypentanoyl-CoA (**10d).** (2*R*,3*R*)-[2-^{2}H]-**10d** was prepared in the same manner as described for [2-^{2}H]-**10b.** (2R,3*R*)-[2-^{2}H]-2-methyl-3-hydroxypentanoyl-CoA (**10d**): ESI(+)-MS $[M+H]^+$ observed *m/z* 883.4, calculated for [2-^{2}H]-**10d**-H^+ *m/z* 883.2.

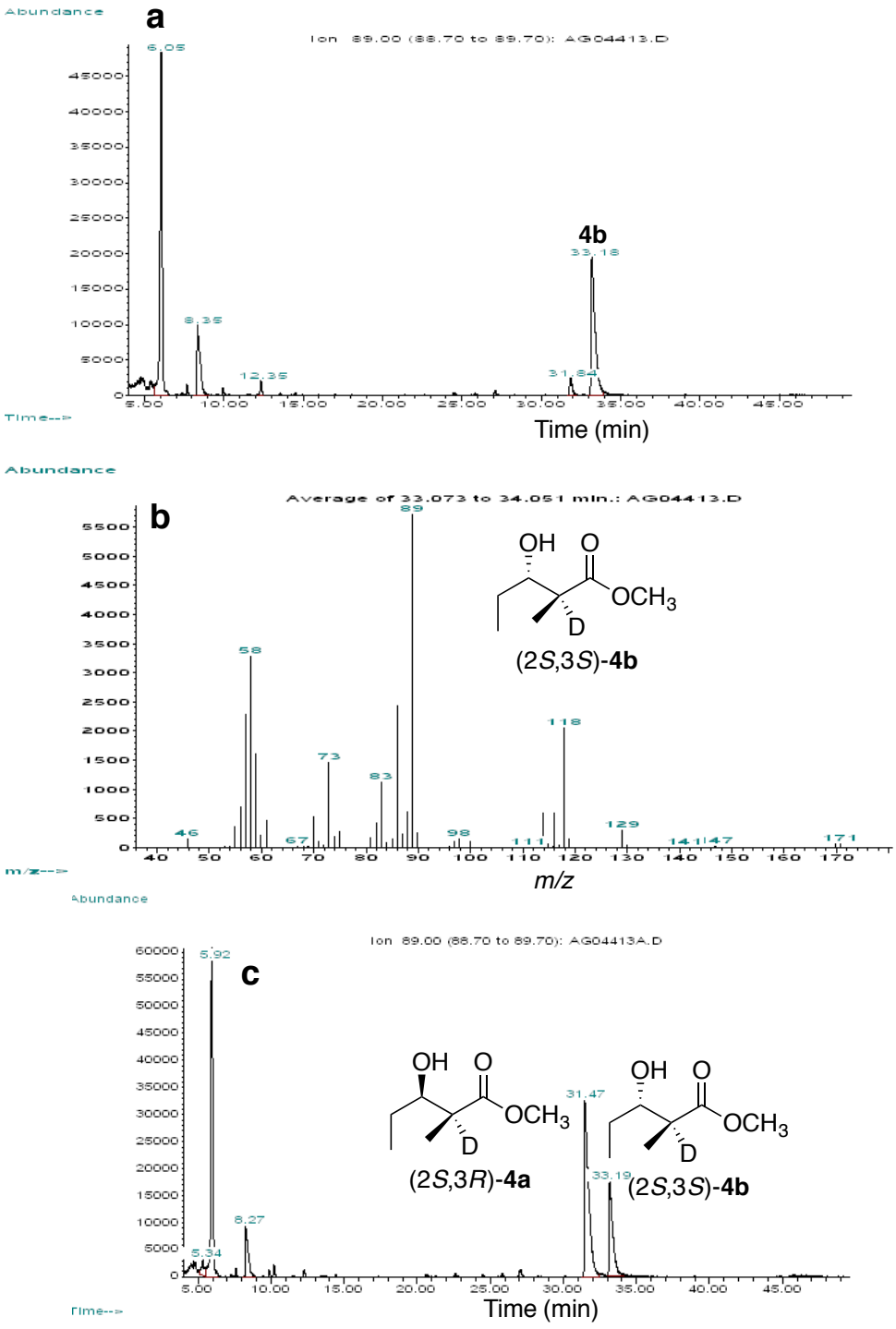


Figure S1. Chiral GC-MS analysis of methyl (2*S*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoate (**4b**). Varian CP-Chirasil-DEX CB capillary column, temperature program of (1) initial temp 50 °C for 2 min, (2) increase at rate of 1 °C/min up to 90 °C, and then (3) 20 °C/min to final temp of 200 °C. a. Methyl (2*S*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoate (**4b**), XIC, *m/z* 89. b. (2*S*,3*S*)-[2-^{2}H]-**4b**, TIC. c. (2*S*,3*S*)-[2-^{2}H]-**4b** plus added (2*S*,3*R*)-[2-^{2}H]-**4a**, XIC *m/z* 89.

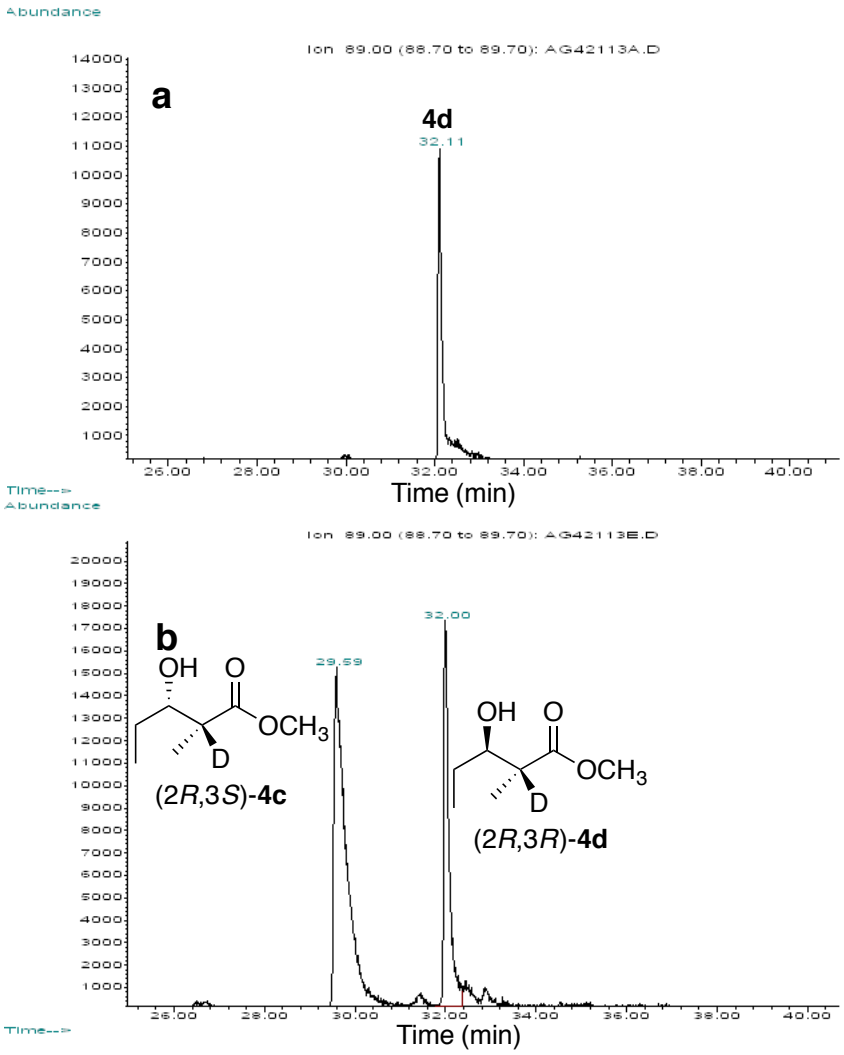


Figure S2. Chiral GC-MS analysis of methyl (2*R*,3*R*)-[2-^{2}H]-2-methyl-3-hydroxypentanoate (**4d**). Column and conditions were the same as for analysis of **4b**. a. Methyl (2*R*,3*R*)-[2-^{2}H]-2-methyl-3-hydroxypentanoate (**4d**), XIC, *m/z* 89. b. (2*R*,3*R*)-[2-^{2}H]-**4d** plus added (2*R*,3*S*)-[2-^{2}H]-**4c**, XIC *m/z* 89.

Chemoenzymatic synthesis of ACP-bound diketide thioesters, [2-^{2}H]-2-methyl-3-hydroxypentanoyl-EryACP6 (1a-1d). Each ACP-bound diketide thioester was prepared by incubation of the appropriate [2-^{2}H]-2-methyl-3-hydroxypentanoyl-CoA thioester **10a**-**10d** with *apo*-EryACP6 and Sfp. In a typical procedure, (2*R*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoyl-CoA (**10c**, 30.0 μL of 7.3 mM soln, final conc 1.1 mM), *apo*-EryACP6 (12.5 μL of 8 mM soln, final conc 500 μM), Sfp (2.8 μL of 2.12 mM soln, final conc 30 μM), $MgCl_2$ (4.0 μL of 500 mM soln, final conc 10 mM), and DTT (5.0 μL of 50 mM soln, final conc 1.25 mM) were incubated in 50 mM phosphate buffer (pH 7.2) (tot

vol 200 μL) for 45 min at 37 °C. HPLC-ESI(+)-MS-MS analysis used a Phenomenex Jupiter C4 column (150 mm × 2 mm, 5.0 μm) equilibrated with 30% CH_3CN/H_2O (0.1% formic acid). The sample was eluted with a linear gradient from 30% to 100% CH_3CN/H_2O (0.1% formic acid) with a flow rate of 200 μL/min]. The ESI(+)-MS confirmed the formation of [2-^{2}H]-2-methyl-3-hydroxypentanoyl-EryACP6 (**1c**), observed *m/z* 11746.7, predicted *m/z* 11746.4; *N*-gluconyl-**1c**,[14] observed $[M+178]^+$ *m/z* 11924.7, predicted 11924.4 (Figure S3). Collision-induced fragmentation (CID) of the $[M]^{13+}$ ion, *m/z* 904.6 in the ESI-MS spectrum, gave a ppant ejection fragment[15,16] *m/z* 376.31, predicted for [2-^{2}H]-**3**, *m/z* 376.2 (Figure S4).

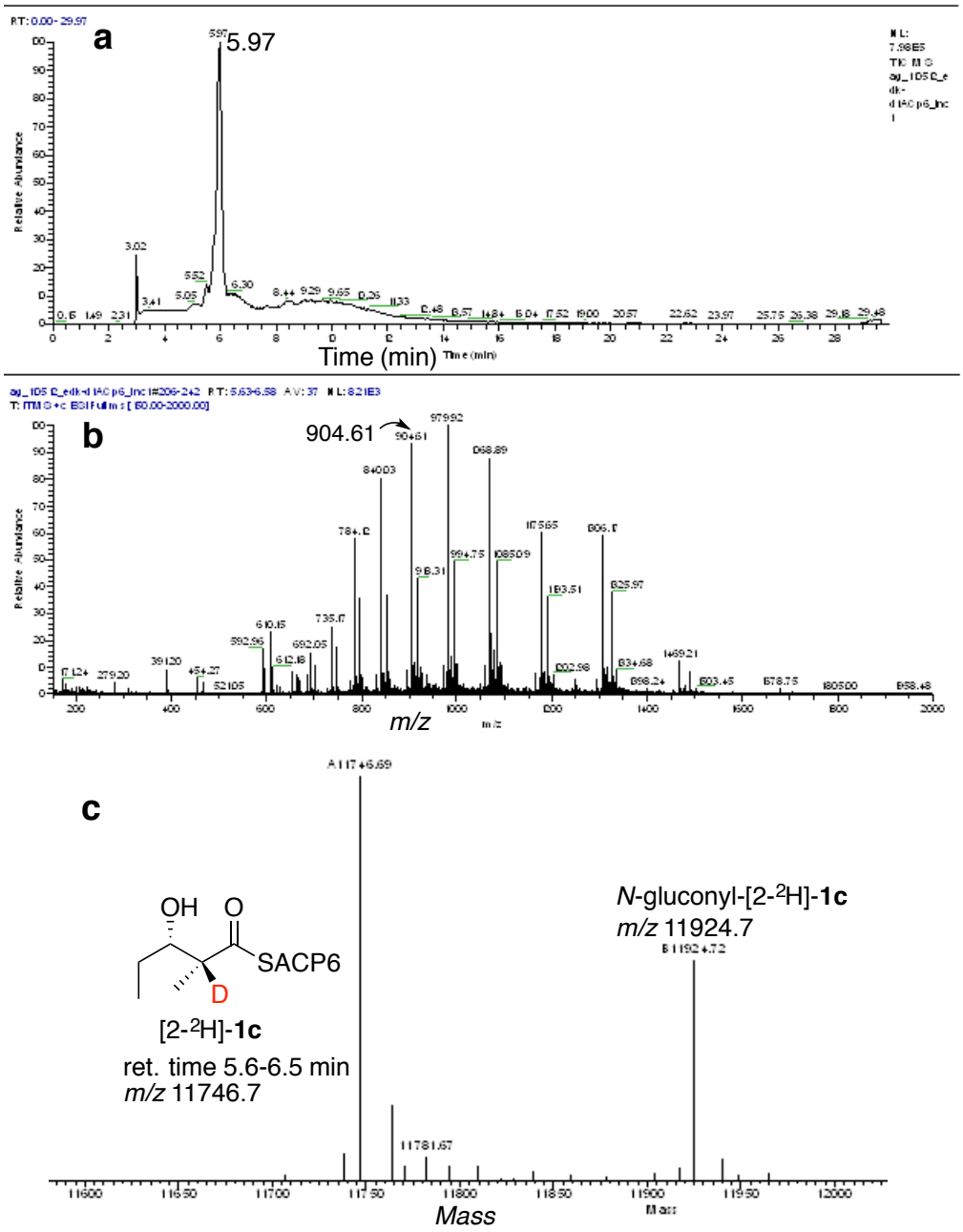


Figure S3. LC-ESI(+)-MS analysis of (2*R*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoyl-EryACP6 (**1c**). a. LC-MS. b. ESI(+)-MS of peak at 5.97 min. c. calc full mass of [2-^{2}H]-**1c**, *m/z* 11746.7. Peak at *m/z* 11924.7 [M+178] corresponds to *N*-gluconoyl-[2-^{2}H]-**1c**.

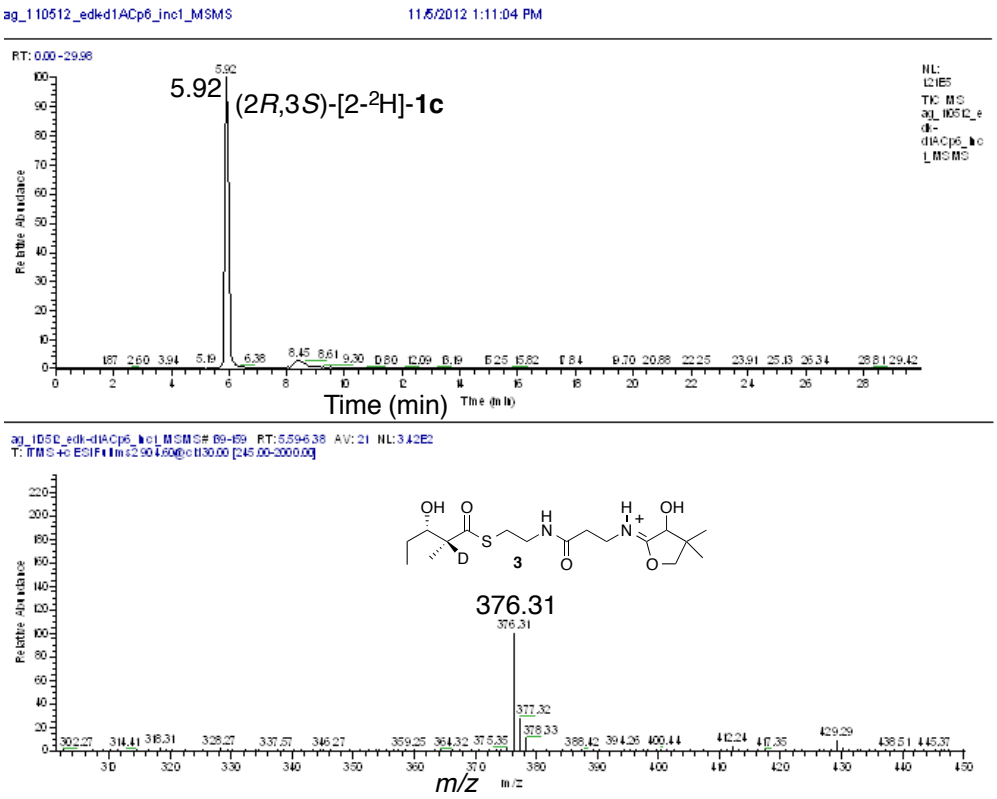


Figure S4. LC-ESI(+)-MS-MS analysis of (2*R*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoyl-EryACP6 (**1c**). CID ppant ejection fragment **3** from *m/z* 904.6 ion.

Equilibrium isotope exchange assay. Incubation of [2-^{2}H]-2-methyl-3-hydroxypentanoyl-ACP (1a-1d) with KR domains and catalytic NADP$^+$. For each incubation, [2-^{2}H]-2-methyl-3-hydroxypentanoyl-ACP (**1a-1d**, 1.0 equiv) was incubated with catalytic NADP$^+$ (0.05 equiv) and the appropriate KR domain (0.25 equiv). Thus [2-^{2}H]-2-methyl-3-hydroxypentanoyl-ACP (**1a-1d**, 90 μL of 500 μM soln, final conc 300 μM), EryKR1 (5.6 μL of 2.0 mM soln, final conc 75 μM), and NADP$^+$ (1.5 μL of 1.5 mM soln, final conc 1.5 μM) were incubated in 50 mM phosphate buffer (pH 7.2) (tot vol 150 μL) at room temp. Samples were withdrawn at periodic intervals up to 60 min and analyzed directly by LC-ESI(+)-MS-MS (Table S1,Figures S5-S9).

Table S1. Equilibrium isotope exchange assay. Time-dependent washout of deuterium from [2-^{2}H]-2-methyl-3-hydroxypentanoyl-ACP **1a**-**1d** by incubation with KR domains and catalytic $NADP^+$.[a]

Time (min)	0	2	5	10	15	20	30	40	50	60
(2*S*,3*R*)-[2-^{2}H]-**1a** + EryKR1										
a. % D washout	0	2	4	5	7	14	22	28	35	56
b. % D washout	0	4	4	4	9	17	24	27	32	54
c. % D washout	0	2	4	4	5	13	20	30	37	54
Avg %D washout (a/b/c)	**0**	**3**	**4**	**4**	**7**	**15**	**22**	**28**	**35**	**55**
(2*S*,3*S*)-[2-^{2}H]-**1b** + RifKR7										
d. % D washout	0	0	9	9	17	24	27	35	48	54
e. % D washout	0	4	9	11	18	23	26	37	47	52
Avg %D washout (d/e)	**0**	**2**	**9**	**10**	**18**	**24**	**27**	**36**	**48**	**53**
(2*S*,3*S*)-[2-^{2}H]-**1b** + NysKR1										
f. % D washout	0	4	7	21	23	25	35	41	44	46
g. % D washout	0	5	5	18	20	24	35	38	41	49
Avg %D washout (f/g)	**0**	**5**	**6**	**20**	**22**	**25**	**35**	**40**	**43**	**48**
(2*R*,3*S*)-[2-^{2}H]-**1c** + EryKR6										
h. % D washout	0	0	0	0	1	4	1	1	1	3
i. % D washout	0	0	0	0	1	1	1	1	3	5
Avg %D washout (h/i)	**0**	**0**	**0**	**0**	**1**	**3**	**1**	**1**	**2**	**4**
(2*R*,3*R*)-[2-^{2}H]-**1d** + TylKR1										
j. % D washout	0	0	0	0	2	2	5	5	6	8
k. % D washout	0	0	0	0	0	4	4	7	6	7
Avg %D washout (j/k)	**0**	**0**	**0**	**0**	**1**	**3**	**5**	**6**	**6**	**8**

a. The measured intensity of the *m/z* 376 peak for the 2-methyl-3-hydroxypentanoyl-pantetheinate ejection fragment **3** was corrected for the contribution of natural abundance ^{13}C from the d_0 species, *m/z* 375.

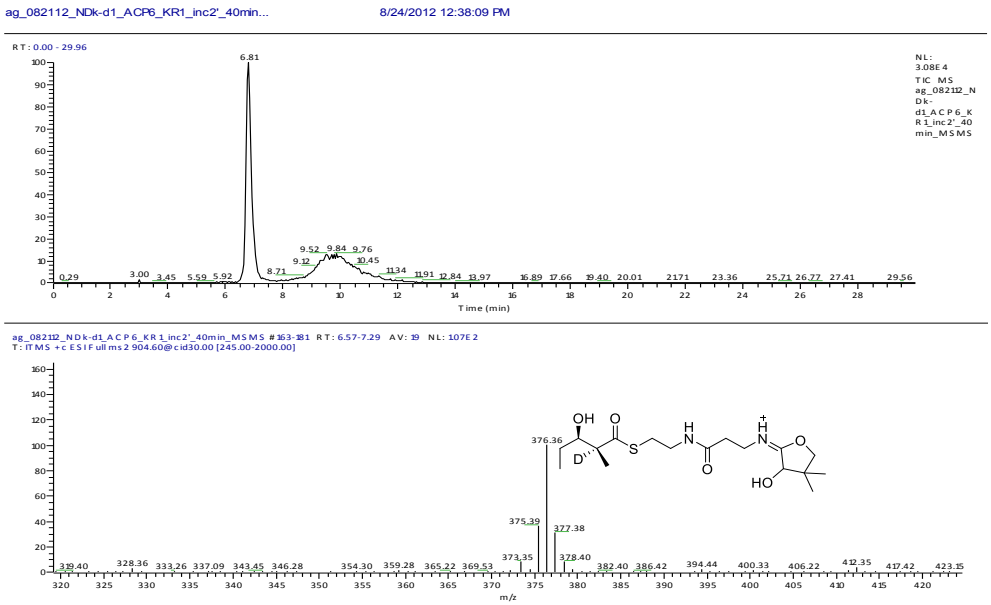


Figure S5. LC-ESI(+)-MS-MS analysis of 40 min incubation of (2*S*,3*R*)-[2-^{2}H]-**1a** with EryKR1 and catalytic NADP$^+$. a) LC-MS (TIC). b) MS-MS, pantetheinate ejection fragment **3** from peak with ret time 6.57-7.29 min, *m/z* 375.4, 376.4.

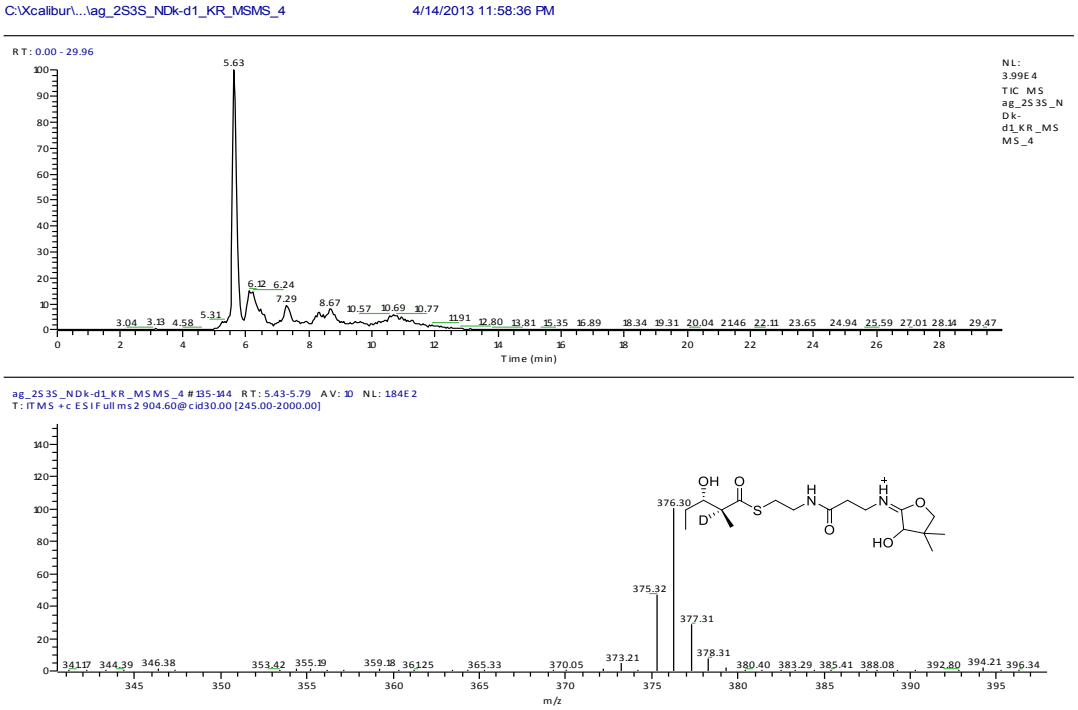


Figure S6. LC-ESI(+)-MS-MS analysis of 40 min incubation of (2*S*,3*S*)-[2-^{2}H]-**1b** with RifKR7 and catalytic NADP$^+$. a) LC-MS (TIC). b) MS-MS, pantetheinate ejection fragment **3** from peak with ret time 5.43-5.79 min, *m/z* 375.3, 376.3.

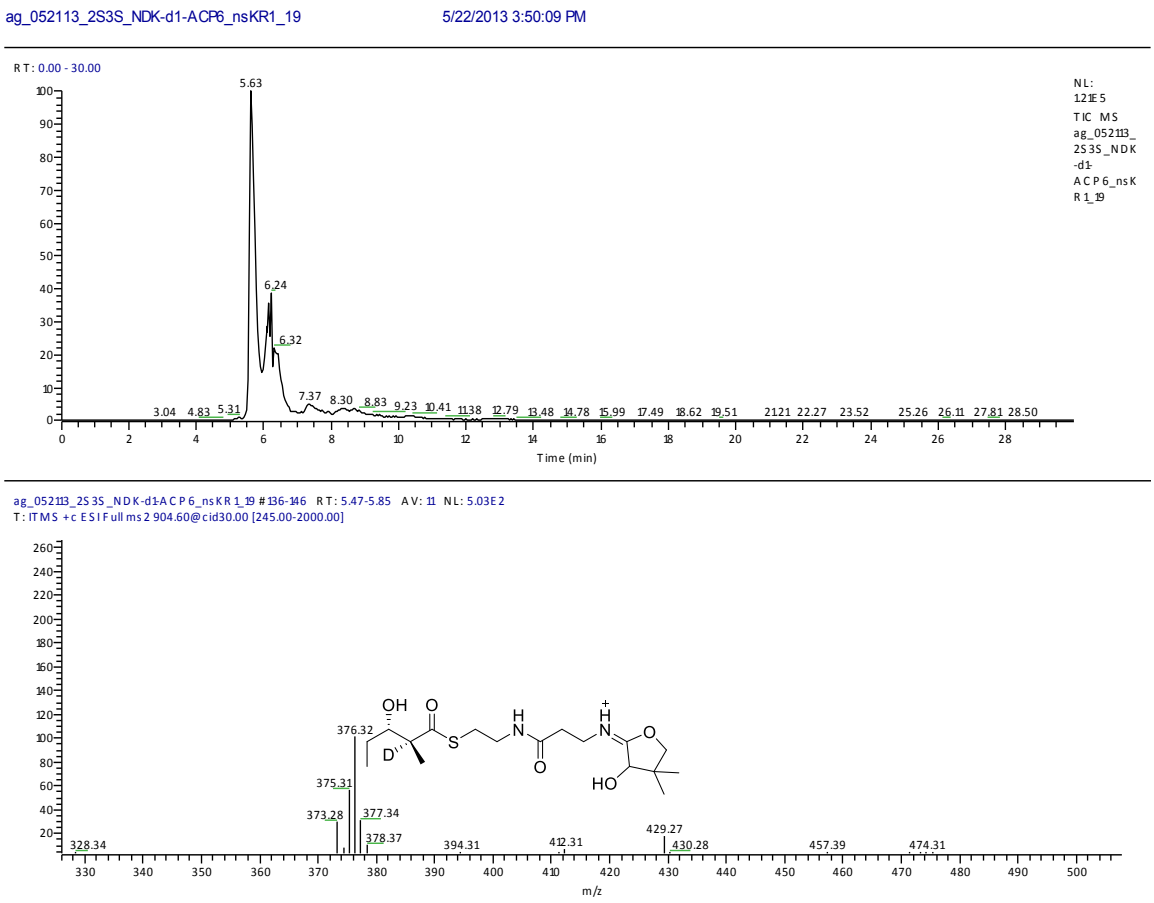


Figure S7. LC-ESI(+)-MS-MS analysis of 40 min incubation of (2*S*,3*S*)-[2-^{2}H]-**1b** with NysKR1 and catalytic NADP$^+$. a) LC-MS (TIC). b) MS-MS, pantetheinate ejection fragment **3** from peak with ret time 5.47-5.85 min, *m/z* 375., 376.3.

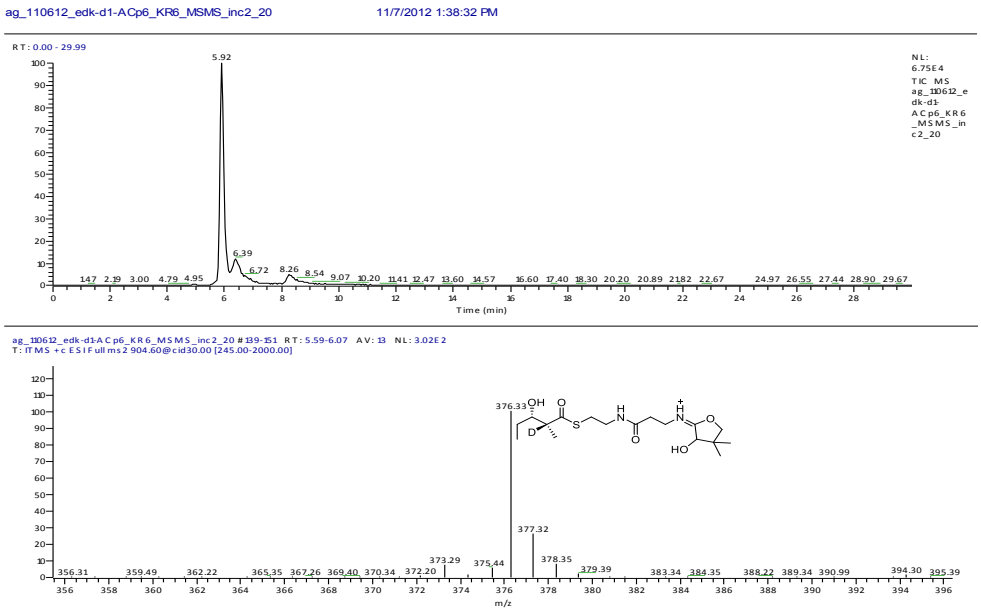


Figure S8. LC-ESI(+)-MS-MS analysis of 40 min incubation of (2*R*,3*S*)-[2-^{2}H]-**1c** with EryKR6 and catalytic NADP$^+$. a) LC-MS (TIC). b) MS-MS, pantetheinate ejection fragment **3** from peak with ret time 5.59-6.07 min, *m/z* 375.4, 376.3.

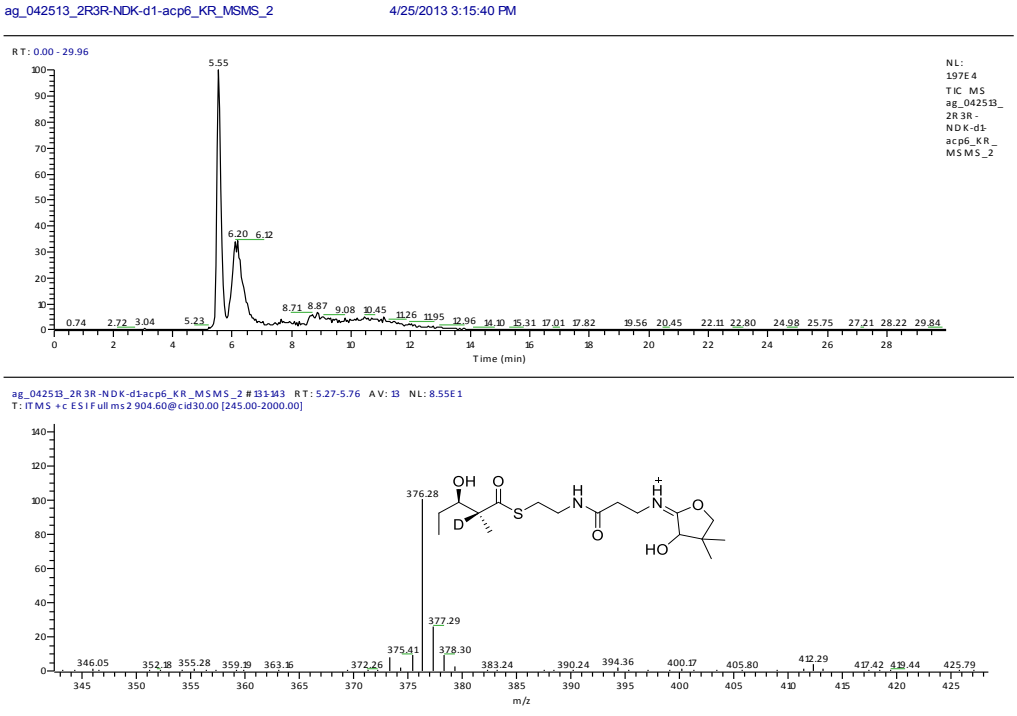


Figure S9. LC-ESI(+)-MS-MS analysis of 40 min incubation of (2*R*,3*R*)-[2-^{2}H]-**1d** with TylKR1 and catalytic NADP$^+$. a) LC-MS (TIC). b) MS-MS, pantetheinate ejection fragment **3** from peak with ret time 5.27-5.76 min, *m/z* 375.4, 376.3.

Equilibrium isotope exchange assay. Control experiments.

1. **-NADP$^+$**: (2*S*,3*R*)-[2-^{2}H]-2-Methyl-3-hydroxypentanoyl-ACP (**1a**, 1.0 equiv) was incubated with EryKR1 domain (0.25 equiv) in the absence of added NADP$^+$. LC-ESI(+)-MS-MS after 40 min showed 9% washout of deuterium, plus minor amounts of 2-methyl-3-ketopentanoyl-pant ejection fragment (*m/z* 373.3).

2. **-EryKR1**: (2*S*,3*R*)-[2-^{2}H]-2-Methyl-3-hydroxypentanoyl-ACP (**1a**, 1.0 equiv) was incubated with NADP$^+$ (0.05 equiv) in the absence of EryKR1 domain. LC-ESI(+)-MS-MS after 30 min showed no washout of deuterium.

3. **Extended incubation time:** (2*S*,3*R*)-[2-^{2}H]-2-Methyl-3-hydroxypentanoyl-ACP (**1a**, 1.0 equiv) was incubated with EryKR1 domain (0.25 equiv) and NADP$^+$ (0.05 equiv) for extended times. LC-ESI(+)-MS-MS after 12 and 24 min shows 61% and 78% washout of deuterium, respectively.

4. **Chiral GC-MS analysis.** (2*S*,3*R*)-[2-^{2}H]-2-Methyl-3-hydroxypentanoyl-ACP (**1a**) (600 μM) was incubated with EryKR1 (150 μM) and catalytic NADP$^+$ (60 μM) in 50 mM phosphate buffer (pH 7.2) (tot vol 500 μL) at room temp for 15 min. The reaction was quenched and the 2-methyl-3-hydroxypentanoic acid was hydrolytically released from the ACP thioester by addition of 200 μL of 0.5 M NaOH, followed by incubation at 65 °C for 20 min. The reaction mixture was acidified with 1 M HCl to pH ~2 and extracted with ethyl acetate. After evaporation of the solvent, the residue was dissolved in 100 μL of methanol, followed by addition of TMS-CHN_2. The derived methyl ester **4a** was analyzed by chiral GC-MS as described above (Figure S10). Direct comparison with authentic standards of **4a**-**4d** confirmed that the recovered methyl ester was exclusively (2*S*,3*R*)-**4a**. Similar incubations of (2*S*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoyl-ACP (**1b**) with RifKR7 and with NysKR1 confirmed that the recovered diketide methyl esters were exclusively (2*S*,3*S*)-**4b** (Figure S11).

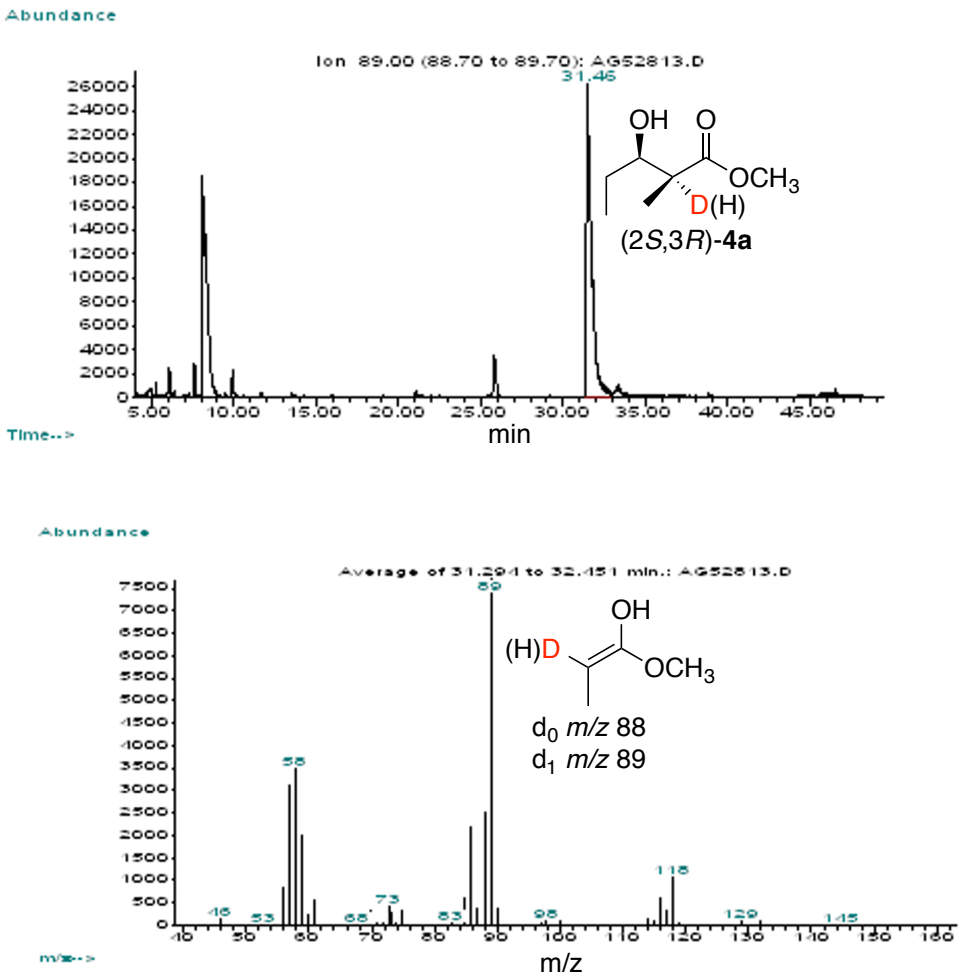


Figure S10. Chiral GC-MS analysis of methyl (2*S*,3*R*)-[2-^{2}H]-2-methyl-3-hydroxypentanoate (**4a**) from 15 min equilibrium isotope exchange of [2-^{2}H]-**1a** with EryKR1, hydrolysis, and methylation. Column and conditions were the same as described in Figure S1. a. Methyl (2S,3*R*)-[2-^{2}H]-2-methyl-3-hydroxypentanoate (**4a**), XIC, *m/z* 89. b. MS of **4a**.

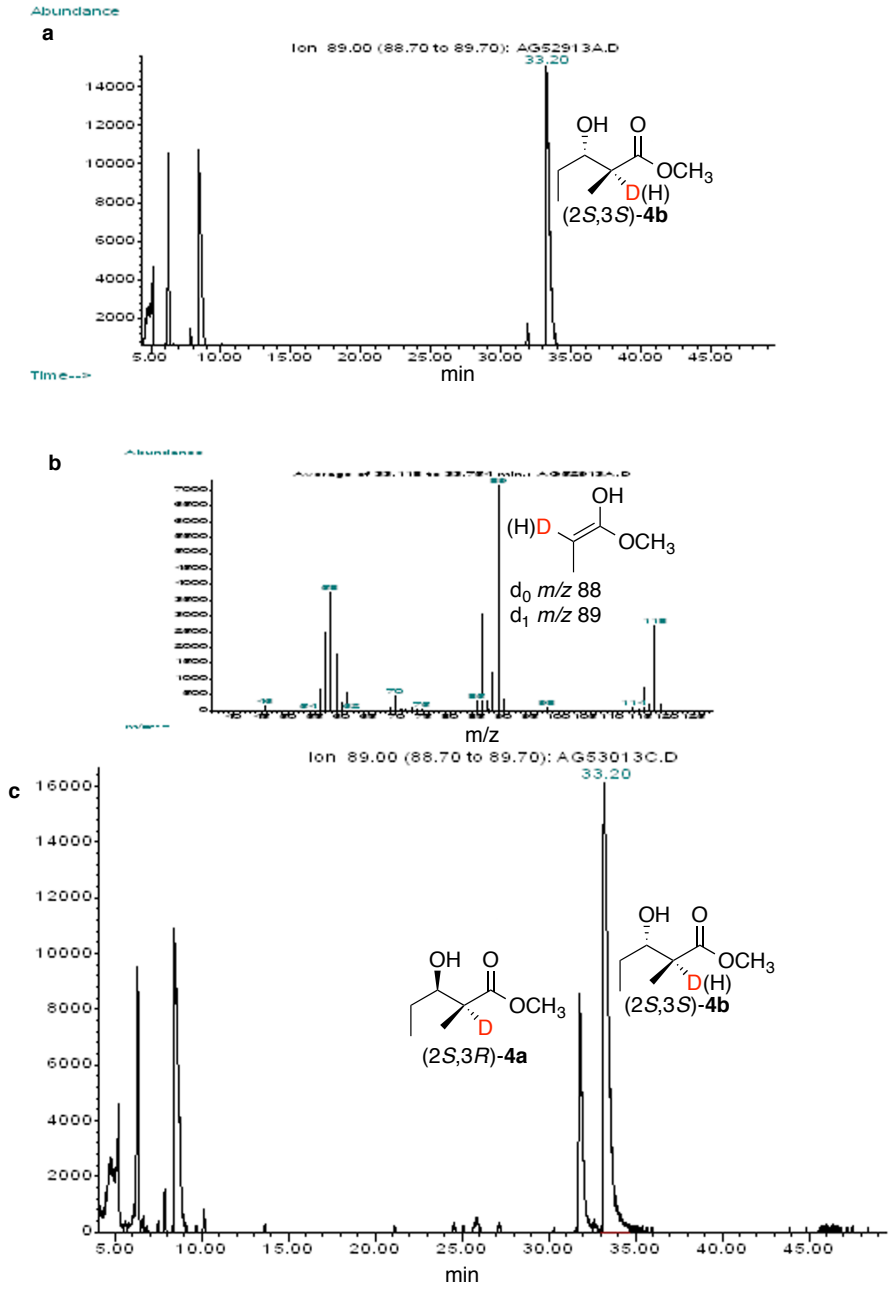


Figure S11. Chiral GC-MS analysis of methyl (2*S*,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoate (**4b**) from 15 min equilibrium isotope exchange of [2-^{2}H]-**1b** with RifKR7, hydrolysis, and methylation. Column and conditions were the same as described in Figure S1. a. Methyl (2S,3*S*)-[2-^{2}H]-2-methyl-3-hydroxypentanoate (**4b**), XIC, *m/z* 89. b. MS of **4b**. c. GC-MS of **4b** plus added **4a**.

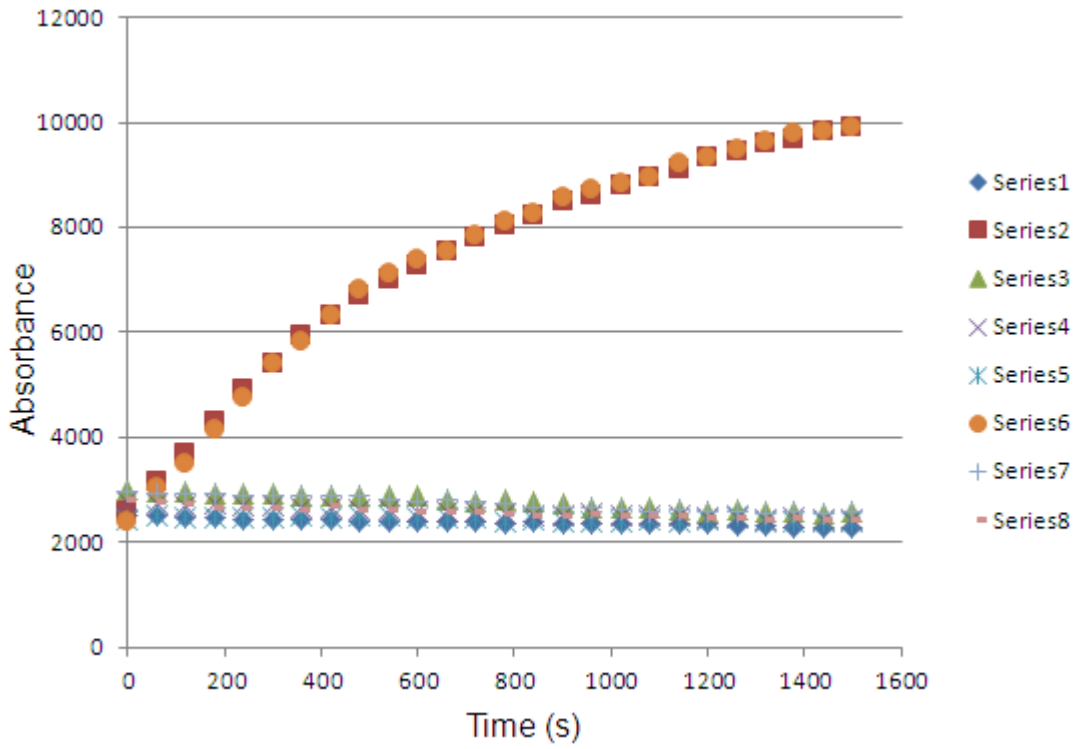


Figure S12. Kinetics of KR-catalyzed oxidation of diketide-SNAC diastereomers. (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (5.0 mM) or (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SNAC (5.0 mM) was incubated at room temperature with either EryKR1 (100 μM) or EryKR6 (100 μM) plus $NADP^+$ (10 μM) in 96-well microtitre plates and the formation of NADPH was monitored at 340 nm. Series 1, (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC and EryKR1; Series 2, (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC, EryKR1, and $NADP^+$; Series 3, (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC; Series 4, $NADP^+$; Series 5, (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SNAC, EryKR1, and $NADP^+$; Series 6, (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SNAC, EryKR6, and $NADP^+$; Series 7, (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SNAC plus EryKR6; Series 8, (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SNAC. KR-catalyzed oxidation only takes place when each KR domain is paired with the cognate diastereomer of its reduced substrate in the presence of $NADP^+$. Note also that the rates of the EryKR1- and EryKR6-catalyzed oxidations are identical.

The kinetics of KR-catalyzed oxidation of the corresponding ACP-bound substrates was also determined using a microplate reader to continuously monitor the fluorescence of NADPH at 432 nm. EryKR1 (200 μM), $NADP^+$ (100 μM), (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP (**1a**) (0 – 300 μM): k_{cat} 0.07 s^{-1}, K_m (**1a**) 122±15 μM; k_{cat}/K_m 0.58 $mM^{-1}s^{-1}$. EryKR6 (200 μM), $NADP^+$ (100 μM), (2*R*,3S)-2-methyl-3-hydroxypentanoyl-ACP (**1c**) (0 – 300 μM): k_{cat} 0.073 s^{-1}, K_m (**1c**) 127±19 μM; k_{cat}/K_m 0.57 $mM^{-1}s^{-1}$.

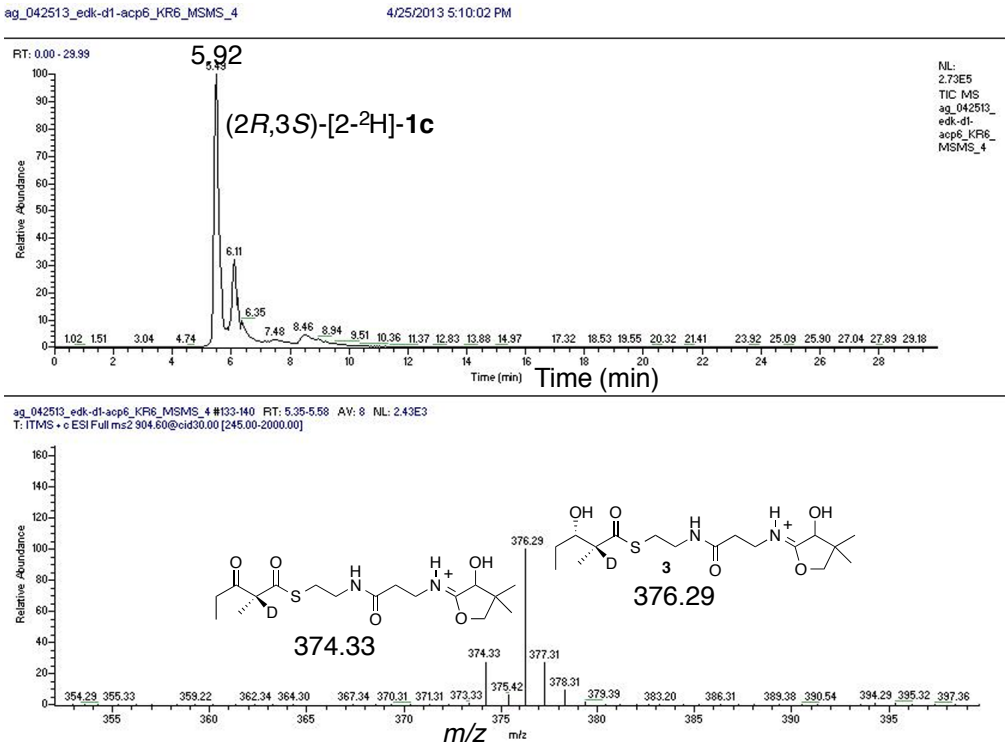


Figure S13. LC-ESI(+)-MS-MS analysis of 30 min incubation of (2*R*,3*S*)-[2-^{2}H]-**1c** (1.0 eq) with EryKR6 (0.25 eq) and NADP$^+$ (0.1 eq). a) LC-MS (TIC). b) MS-MS, pantetheinate ejection fragments from primary LC peak with ret time 5.35-5.58 min, *m/z* 374.3 ([2-^{2}H]-2-methyl-3-ketopentanoyl-pantetheinate; *m/z* 376.3 ([2-^{2}H]-2-methyl-3-hydroxypentanoyl-pantetheinate,

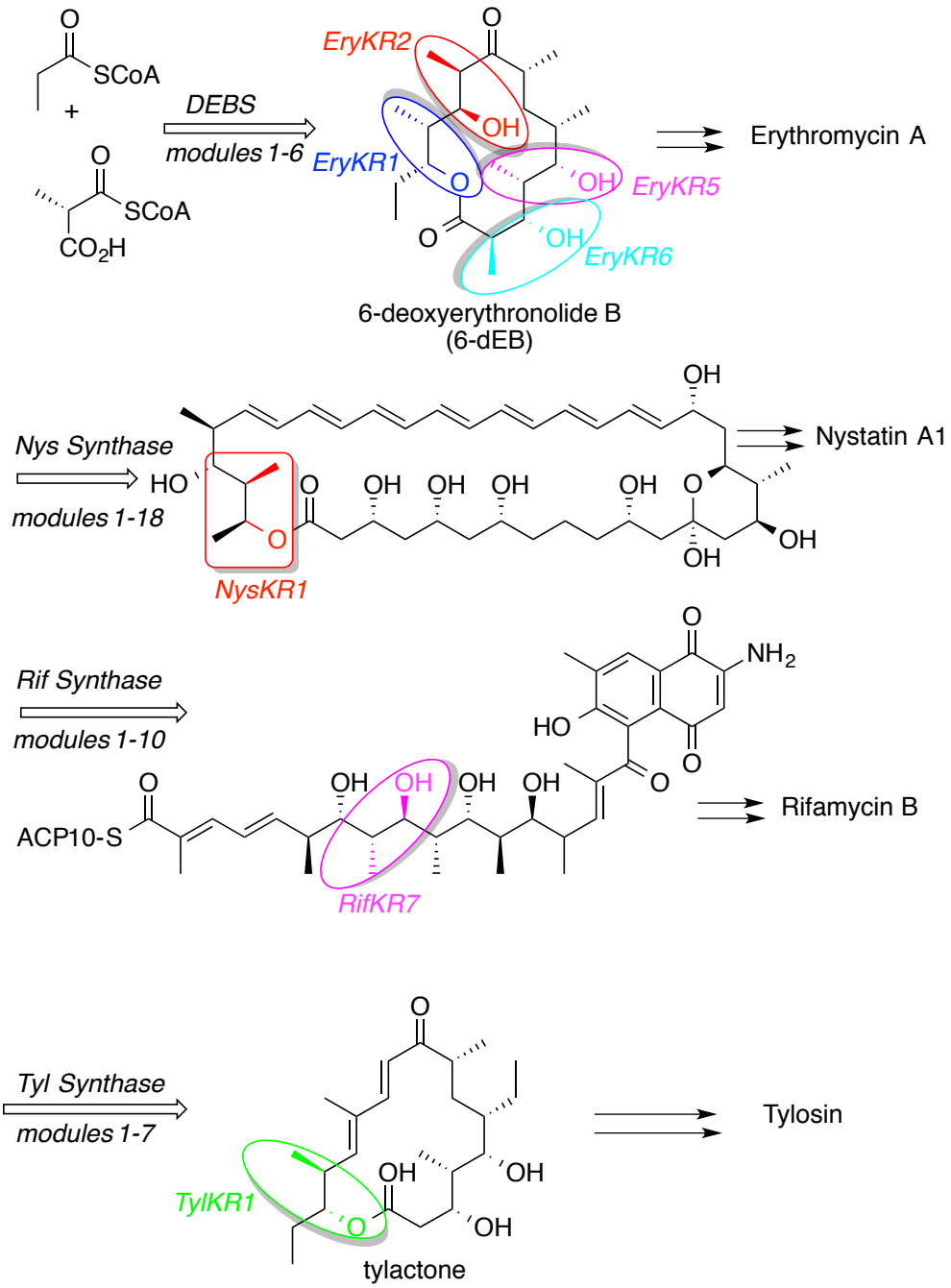


Figure S14. Structures of typical polyketides, showing stereochemical features introduced by representative KR domains. Note that in the rifamycin synthase, RifKR5 has (2*R*,3*S*)-2-methyl-3-hydroxyacyl specificity, RifKR6 has epimerizing (2*S*,3*R*)-2-methyl-3-hydroxyacyl specificity, RifKR7 has epimerizing (2*S*,3*S*)-2-methyl-3-hydroxyacyl specificity, and RifKR8 has (2*R*,3*R*)-2-methyl-3-hydroxyacyl specificity. RifKR10, which provides the substrate for dehydration by RifDH10, also has epimerizing (2*S*,3*S*)-2-methyl-3-hydroxyacyl specificity.

Supplemental References

(1) Chen, A. Y.; Cane, D. E.; Khosla, C. *Chem. Biol.* **2007**, *14*, 784-792.

(2) Chen, A. Y.; Schnarr, N. A.; Kim, C. Y.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **2006**, *128*, 3067-3074.

(3) Valenzano, C. R.; Lawson, R. J.; Chen, A. Y.; Khosla, C.; Cane, D. E. *J. Am. Chem. Soc.* **2009**, *131*, 18501-18511.

(4) You, Y. O.; Khosla, C.; Cane, D. E. *J. Am. Chem. Soc.* **2013**, *135*, 7406-7409.

(5) Castonguay, R.; He, W.; Chen, A. Y.; Khosla, C.; Cane, D. E. *J. Am. Chem. Soc.* **2007**, *129*, 13758-13769.

(6) Castonguay, R.; Valenzano, C. R.; Chen, A. Y.; Keatinge-Clay, A.; Khosla, C.; Cane, D. E. *J. Am. Chem. Soc.* **2008**, *130*, 11598-11599.

(7) Keatinge-Clay, A. T. *Chem. Biol.* **2007**, *14*, 898-908.

(8) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, A Laboratory Manual, Second Edition*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

(9) Bradford, M. *Anal. Biochem.* **1976**, *72*, 248-254.

(10) Evans, D. A.; Ennis, M. D.; Le, T.; Mandel, N.; Mandel, G. *J. Am. Chem. Soc.* **1984**, *106*, 1154-1156.

(11) Evans, D. A.; Britton, T. C.; Ellman, J. A. *Tetrahedron Lett.* **1987**, *28*, 6141-6144.

(12) Cane, D. E.; Ott, W. R. *J. Am. Chem. Soc.* **1988**, *110*, 4840-4841.

(13) Cane, D. E.; Tan, W.; Ott, W. R. *J. Am. Chem. Soc.* **1993**, *115*, 527-535.

(14) Geoghegan, K. F.; Dixon, H. B.; Rosner, P. J.; Hoth, L. R.; Lanzetti, A. J.; Borzilleri, K. A.; Marr, E. S.; Pezzullo, L. H.; Martin, L. B.; LeMotte, P. K.; McColl, A. S.; Kamath, A. V.; Stroh, J. G. *Anal. Biochem.* **1999**, *267*, 169-184.

(15) Dorrestein, P. C.; Bumpus, S. B.; Calderone, C. T.; Garneau-Tsodikova, S.; Aron, Z. D.; Straight, P. D.; Kolter, R.; Walsh, C. T.; Kelleher, N. L. *Biochemistry* **2006**, *45*, 12756-12766.

(16) Meluzzi, D.; Zheng, W. H.; Hensler, M.; Nizet, V.; Dorrestein, P. C. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3107-3111.