Supporting Information

Stereospecificity of the Dehydratase Domain of the Erythromycin Polyketide Synthase

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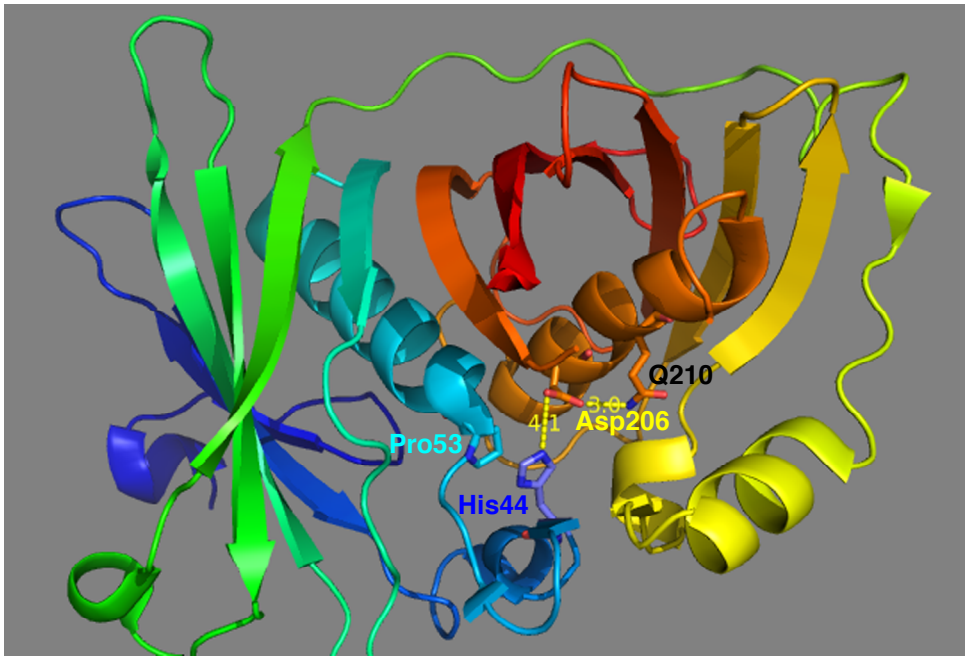


Figure S1. DEBS DH4, showing double hotdog fold with catalytic dyad His44, stacked over Pro53, and Asp206, H-bonded to ε–NH_2 of Gln210 (PDB ID: 3EL6).[1]

Materials and methods. All chemical reagents as well as authentic (2*E)*-2-methylpentenoic acid were purchased from Sigma-Aldrich and utilized without further purification. Recombinant Sfp, DEBS [KS6][AT6], ACP6, KR1, and KR6[2-4] and TYLS KR1[5] were each expressed and purified as previously described. The expression, purification, and stereochemical characterization of RIFS KR7 following the previously described combinatorial incubation and GC-MS analysis protocols[6,7] will be described separately.[8] Recombinant DEBS DH4 was expressed and purified as previously described.[1] *anti*-(2*R*,3*R*)-2-Methyl-3-hydroxypentanoic acid (**2a**), (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-*N*-acetylcysteamine (**2b-SNAC**) thioester, and propionyl-*N*-acetylcysteamine thioester (**4**) were each prepared as described.[9-11] A Bio-Rad FX-Pro Plus Molecular Imager was utilized for radio-TLC analysis. GC-MS analysis was performed on a GC–MS Hewlett-Packard Series 2 GC-MSD, 70 eV EI in positive ion mode. A Thermo LXQ was utilized for HPLC-MS analysis in positive ion mode.

Synthesis of authentic product standards

(2*Z*)-2-Methyl-2-pentenoic acid (5). Addition of bromine to (2*E*)-2-methyl-2-pentenoic acid (**3**) followed by elimination of HBr gave (*2Z*)-*2*-methyl-pentenoic acid (**5**) as confirmed by ^{1}H and ^{13}C NMR analysis, in agreement with the literature data.[12] GC-MS analysis (Varian CP-Chirasil-DEX CB, 25 m × 0.32 mm; temperature program of initial temp 65 °C for 1 min, increase at rate of 0.5 °C/min up to 100 °C then 20°C/min to final temp. of 200 °C) resolved the *E* and *Z* isomers of 2-methyl-2-pentenoic acid.

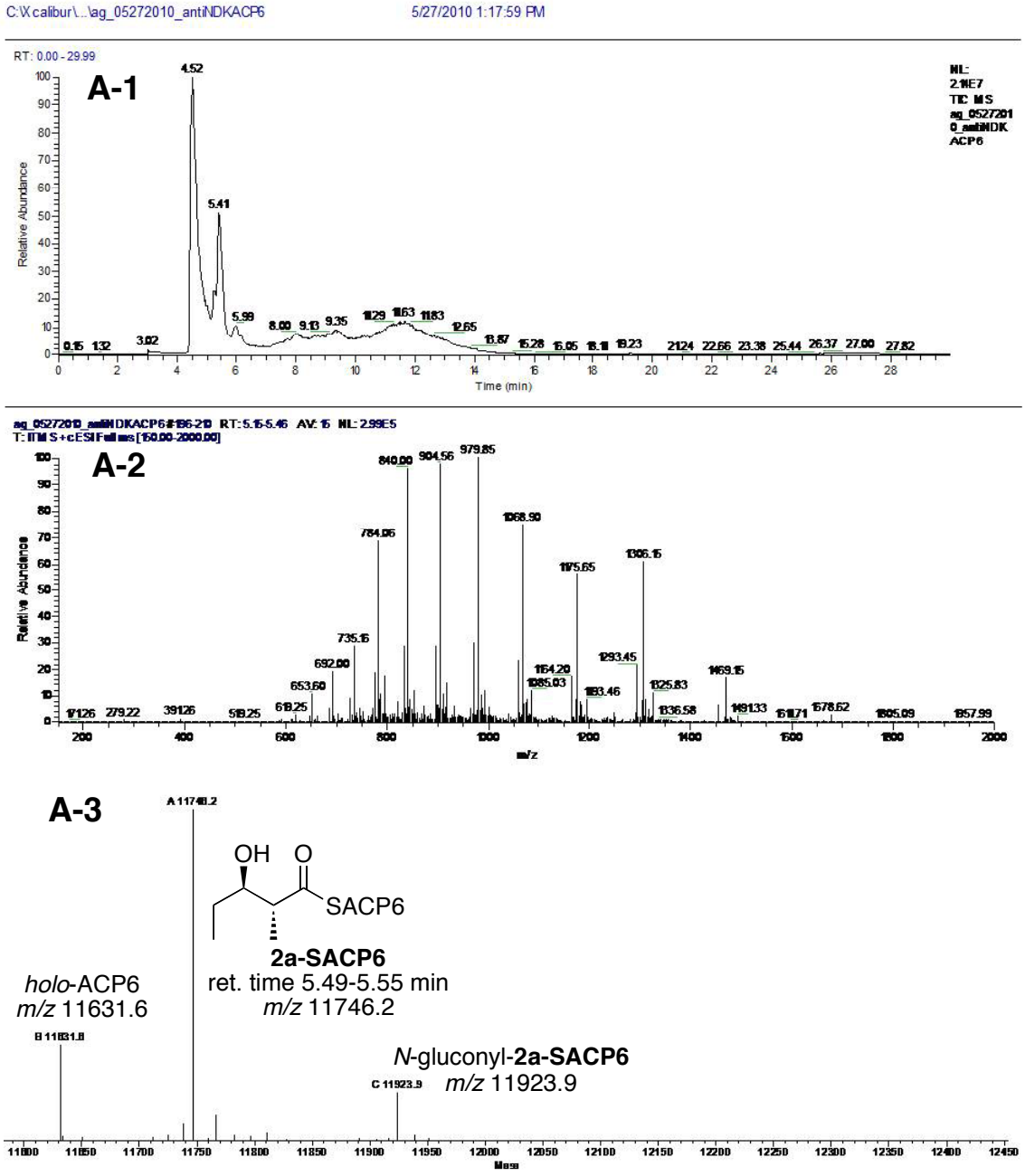
(2*E*,4*R*,5*R*)-2,4-Dimethyl-5-hydroxy-2-heptenoic acid (7). The authentic acid **7** was prepared following the published synthetic route.[11] The derived methyl ester **7-Me** was analyzed by HPLC-MS and by GC-MS (Varian CP-Chirasil-DEX CB, 25 m × 0.32 mm; temperature program of initial temp 50 °C for 1 min, increase at rate of 15°C/min up to 200 °C) and by HPLC-MS.

Chemoenzymatic synthesis of ACP-bound substrates

(2*R*,3*R*)-2-Methyl-3-hydroxypentanoyl-CoA (2a-SCoA). To 13 mg (0.1 mmol) of (2*R*,3*R*)-2-methy-3-hydroxypentanoic acid (**2a**) in 1 mL THF under nitrogen was added carbonyldiimidazole (25

mg, 0.15 mmol, 1.5 equiv.) The mixture was stirred for 1 h and then a solution of HSCoA (10 mg, 13 μmol, 0.1 equiv) in water (1 mL) was added. The reaction was followed by HPLC (Phenomenex Gemini C18 column, 150 × 4.6 mm, UV monitoring λ 254 nm) and after 1 h the reaction was complete. The organic solvent was removed by rotary evaporation and the aqueous phase was extracted with ether to remove organic byproducts. The product was purified by HPLC using a Phenomenex Gemini semi-preparative C18 column, 150 × 10 mm, 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, the 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 2.1×50 mm and a linear gradient from 5% to 65% of CH_3CN/H_2O. (2*R*,3*R*)-2-Methyl-3-hydroxypentanoyl-CoA (**2a-SCoA**): Yield, 4 mg; calculated mass *m/z* 881.7; observed *m/z* 882; ^{1}H NMR (D_2O, 400 MHz) δ 0.65 (s, 3H), 0.68 (s, 3H), 0.74 (m, 3 H), 0.96 (m, 3H), 1.02 (m, 1H), 1.33 (m, 1H) 2.28 (m, 2H), 2.64 (m, 2H), 2.88 (m, 2H), 3.19 (m, 3H), 3.42 (m, 1H), 3.65m, 2H), 3.88 (m, 2H), 4.08(m, 1H), 4.40 (m, 1H), 4.72 (m, 2H), 6.10 (m, 1H), 7.31 (m, 1NH), 8.27 (s, 1H), 8.53 (s, 1H).[13]

(2*R*,3*R*)-2-Methyl-3-hydroxypentanoyl-ACP (2a-SACP6). A mixture of 120 μM *apo*-ACP6, 30 μM Sfp, 10 mM $MgCl_2$, 2.5 mM DTT, and 400 μM (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-CoA (**2a-SCoA**) in a total volume of 80 μL of 50 mM phosphate buffer (pH 7.2) was incubated for 30 min at 37 °C. LC-ESI(+)-MS^3 analysis confirmed the formation of predominately (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP (**2a-SACP6**) (ret. time 5.42-5.70 min; *m/z* 11746.2 (observed); predicted *m/z* 11745.4) accompanied by a small amount of *holo*-ACP (*m/z* 11631.6) and the *N*-gluconyl-derivative[14] of **2a-SACP6** (*m/z* 11923.9 [M+178] (Figure S2A). The collision-induced (CID) ppant ejection[15,16] of the *m/z* 904.56 ion [M^{13+}] gave the predicted fragment ion *m/z* 375.33 corresponding to **2a-pant** (Figure S2B). CID of the *m/z* 375.33 ion gave a typical MS^3 pant-fragmentation pattern[15,16] (Figure S2C).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(Figure S2A)

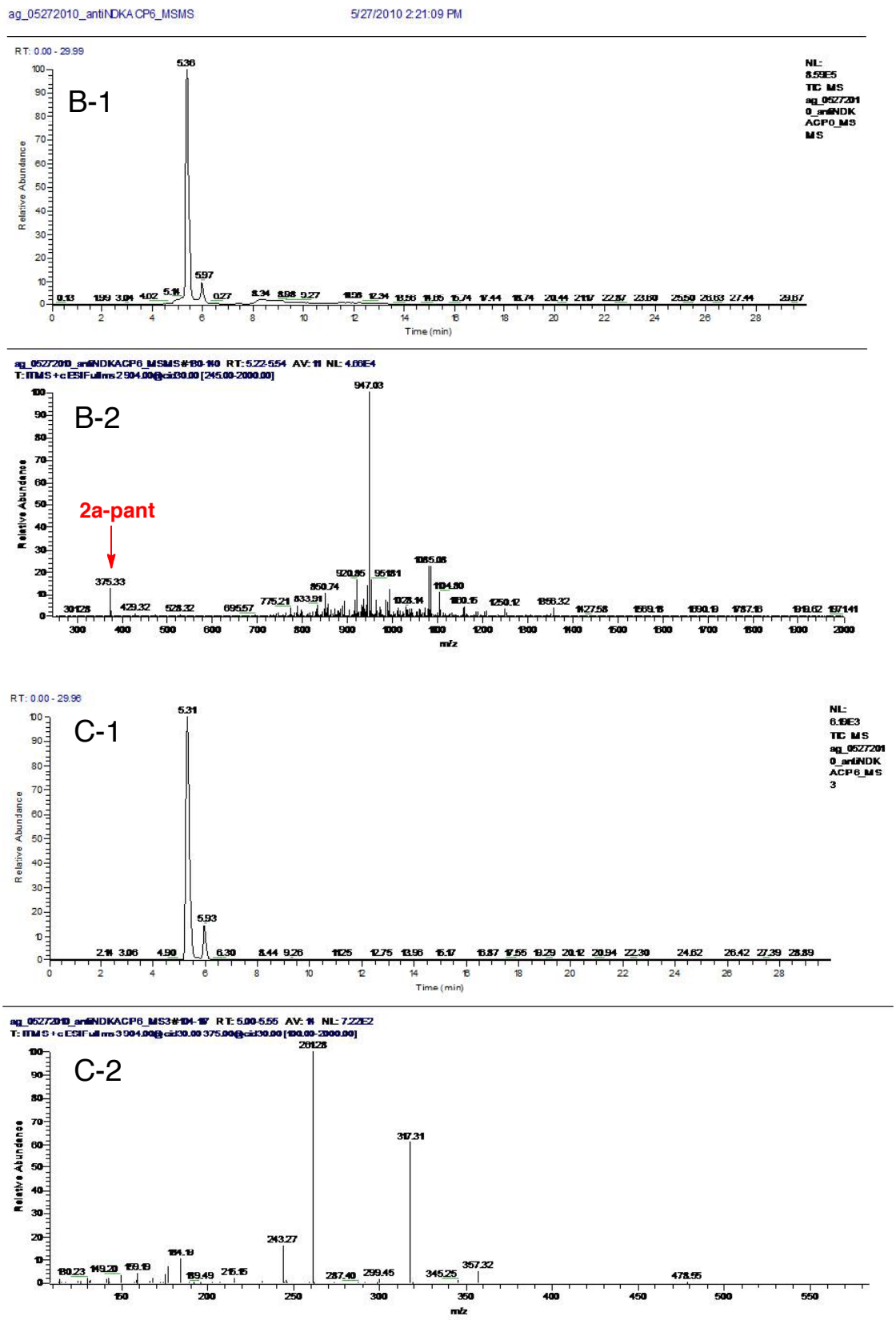
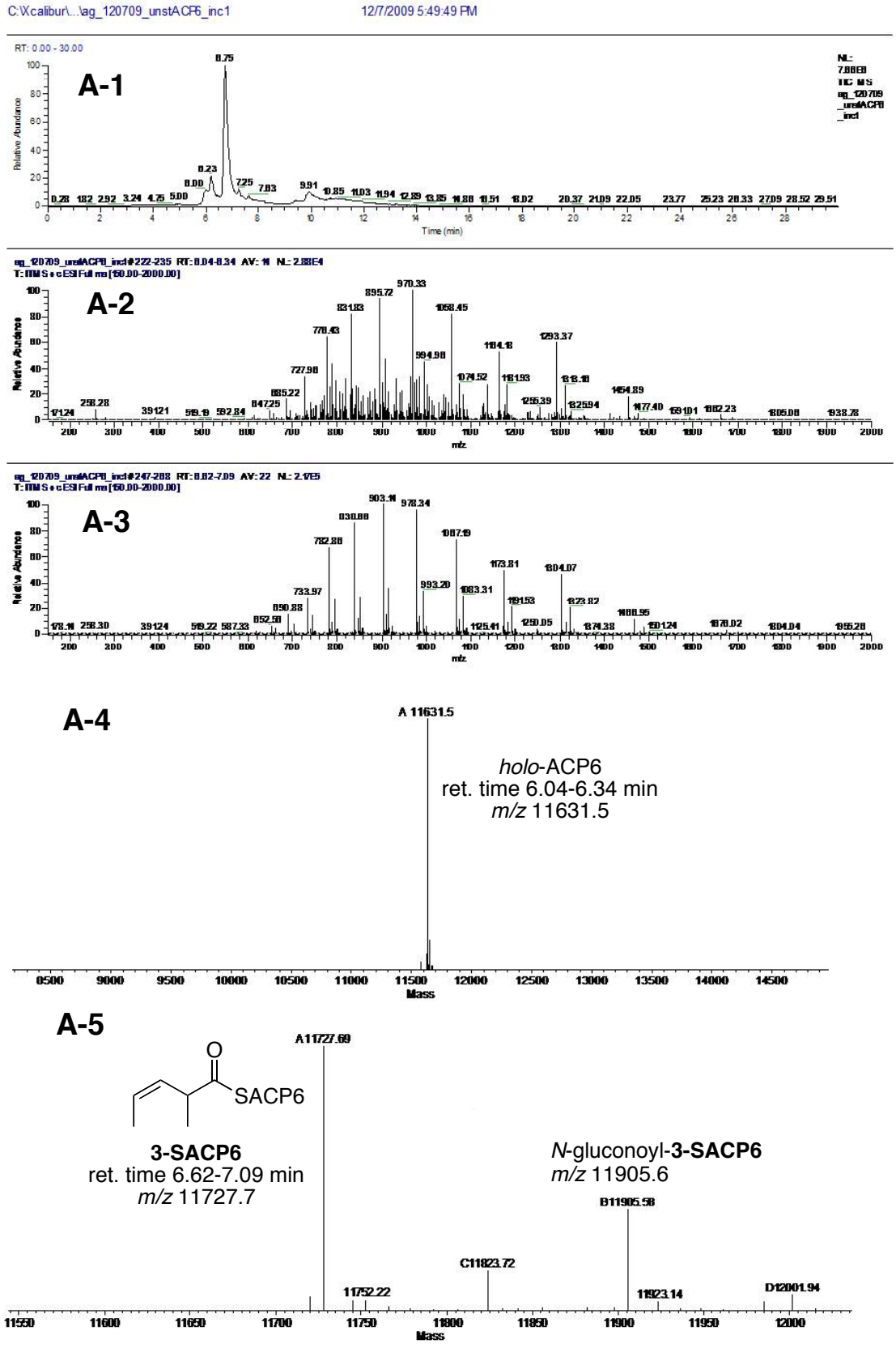


Figure S2. LC-ESI(+)-MS^3 analysis of *anti*-(2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP6 (**2a-SACP6**). A. LC-MS. Panel A-1, LC-MS; Panel A-2, LC-ESI(+)-MS; Panel A-3, calculated full mass of **2a-SACP6**, *m/z* 11746.2. Peak at *m/z* 11923.9 [M+178] corresponds to *N*-gluconoyl-**2a-SACP6**. B. LC-MS-MS. PPant ejection on *m/z* 904.56 peak [M^{13+}] to give **2a-pant** (*m/z* 375.33). C. LC-MS-MS-MS, CID of **2a-pant**, *m/z* 375.33.

(2*E*)-2-Methyl-2-pentenoyl-CoA (3-SCoA). To 2-methyl-2-pentenoic acid (15 mg, 0.13 mmol, 1 eq) in dry CH_3Cl_2 (1.0 mL) at 0 °C was added triethylamine (50 μL, 0.35 mmol 2.7 eq) followed by ClCOOEt (32 μL, 0.32 mmol, 2.5 eq) and the reaction mixture was stirred for 2 h. The solvent was then removed under vacuum and the residue was suspended in THF. The insoluble salts were removed by centrifugation. The mixed anhydride so formed was added to an aqueous solution ($NaHCO_3$ in H_2O, pH 8.0) of CoASH (15.0 mg, 0.02 mmol, 0.15 eq), and the mixture stirred further for 3 h. The reaction mixture was analyzed by LC-MS which showed formation of the desired unsaturated diketide acyl-CoA **3-SCoA** with no trace of free HSCoA. The organic solvent was evaporated and the crude acyl-CoA was purified by HPLC using a Phenomenex Gemini semi-preparative C18 column, 150 × 10 mm, 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, the 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 2.1 × 50 mm and a linear gradient from 5% to 65% of CH_3CN/H_2O. The yield of the purified **3-SCoA** was 6 mg (0.070 mmol, 35% with respect to starting CoASH). Observed *m/z* 864.1, calculated *m/z* 863.1; ^{1}H NMR (D_2O, 400 MHz) δ 0.76 (s, 3H), 0.89 (s, 3H), 1.01 (m, 3H), 1.79 (m, 2H), 2.21 (s, 3H), 2.43 (m, 2H), 3.00 (m, 2H), 3.34 (m, 2H), 3.45-3.57 (m, 2H), 3.83 (m, 1H), 4.03 (m, 2H), 4.25 (m, 3H), 4.75-4.92 (m, 2H), 6.16 (m, 1H), 6.77 (m, 1H), 8.25 (s, 1H), 8.55 (s, 1H).

(2*E*)-2-Methyl-2-pentenoyl-ACP thioester (3-ACP). A mixture of 120 μM *apo*-ACP6, 30 μM Sfp, 10 mM $MgCl_2$, 2.5 mM DTT, and 400 μM (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-CoA (**2a-SCoA**) in a total volume of 80 μL of 50 mM phosphate buffer (pH 7.2) was incubated for 30 min at 37 °C. LC-ESI(+)-MS^3 analysis confirmed the formation of predominately (2*E*)-2-methyl-2-pentenoyl-ACP (**3-SACP6**) (ret. time 6.62-7.09 min; *m/z* 11727.7 (observed); predicted *m/z* 11726.9) accompanied by a small amount of *holo*-ACP (ret. time 6.04-6.34 min; *m/z* 11631.5) (Figure S3).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(Figure S3A)

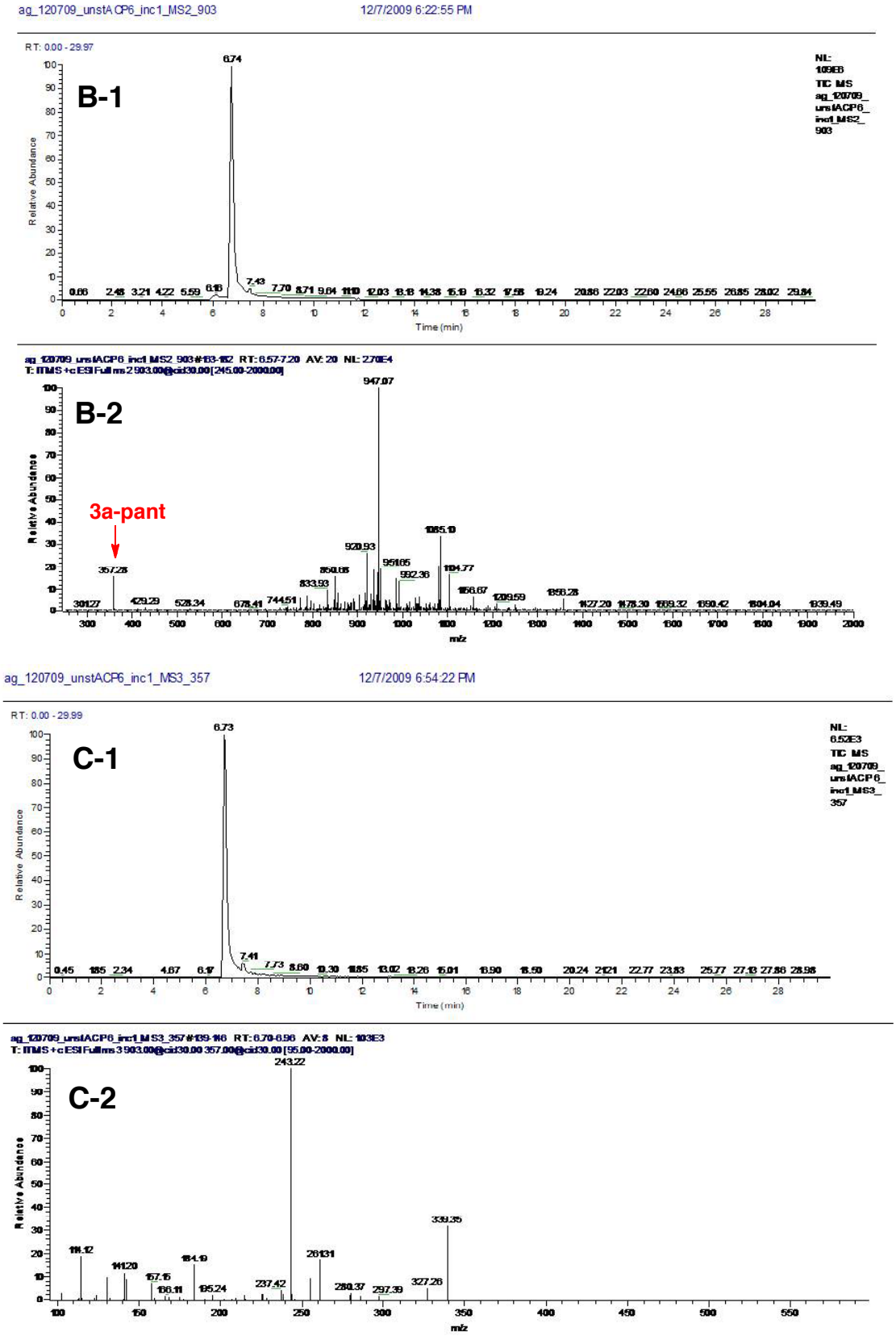
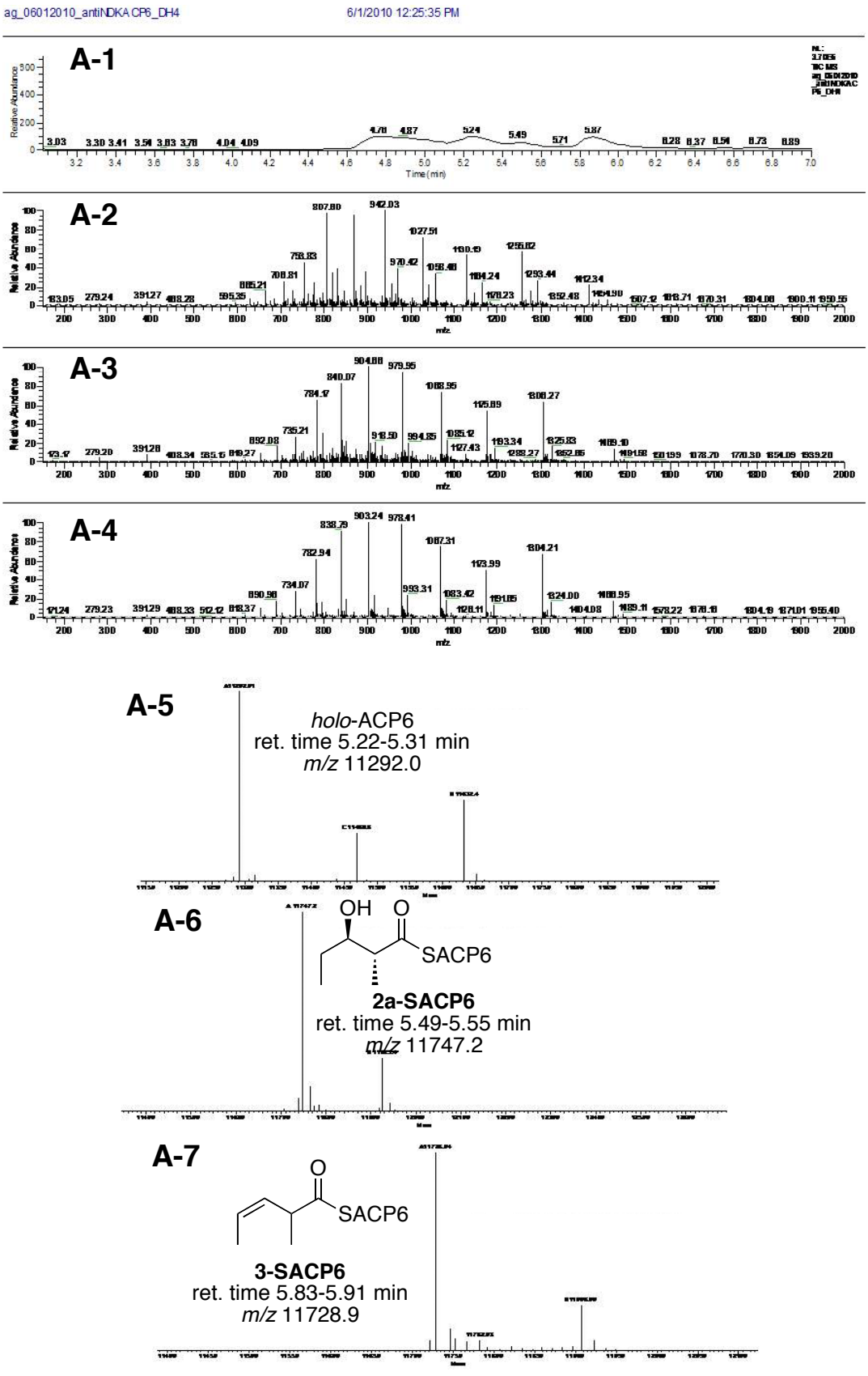


Figure S3. LC-ESI(+)-MS^3 analysis of (2E)-2-methyl-2-pentenoyl-ACP (**3-SACP6**). A. LC-MS. Panel A-1, LC-MS; Panel A-2, ESI(+)-MS of component ret. time 6.04-6.34 min; Panel A-3, ESI(+)-MS of component ret. time 6.62-7.09; Panel A-4, calculated full mass for *holo*-ACP6; Panel A-5, calculated full mass for **3a-SACP6**. Peak at *m/z* 11905.6 [M+178] corresponds to *N*-gluconyl-**3-SACP6**. B. LC-

MS-MS. PPant ejection on *m/z* 903.14 peak [M^{13+}] to give **3-pant** (*m/z* 357.28). C. LC-MS-MS-MS, CID of **3-pant**, *m/z* 357.28.

Incubation of (2*R*,3*R*)-2-Methyl-3-hydroxypentanoyl-ACP (2a-SACP6) with DH4: A mixture of 105 μM **2a-SACP** and 210 μM DH4 in a total volume of 40 μL of 50 mM phosphate buffer (pH 7.2) was incubated for 1 h at 25 °C. LC-ESI(+)-MS^3 analysis confirmed the formation of mixture of (2*E*)-2-methyl-2-pentenoyl-ACP (**3-SACP**) (ret. time 5.83-5.91 min; *m/z* 11728.9 (observed); predicted *m/z* 11726.4), accompanied by residual (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP (**2a-SACP6**) (ret. time 5.49-5.55 min; *m/z* 11747.2 (observed); predicted *m/z* 11745.4) (Figure S4). The LC-MSMS and LC-MSMSMS fragmentation patterns obtained for **2a-SACP6** and **3-SACP6** are in close agreement with the mass fragmentation patterns for the individual chemo-enzymatically prepared samples of each compound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A-6
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O
SACP6
2a-SACP6
ret. time 5.49-5.55 min
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m/z 11728.9

(Figure S4A)

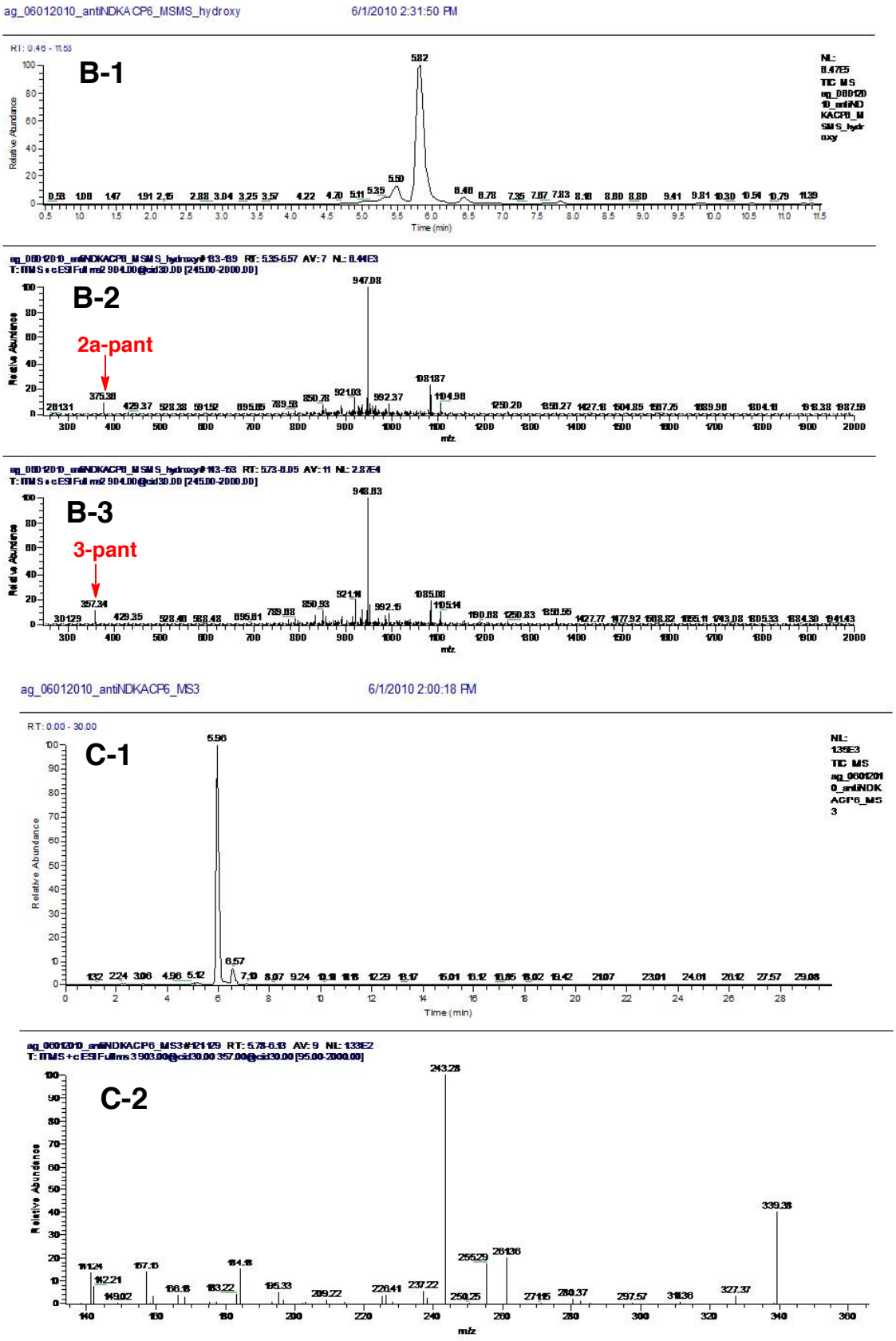
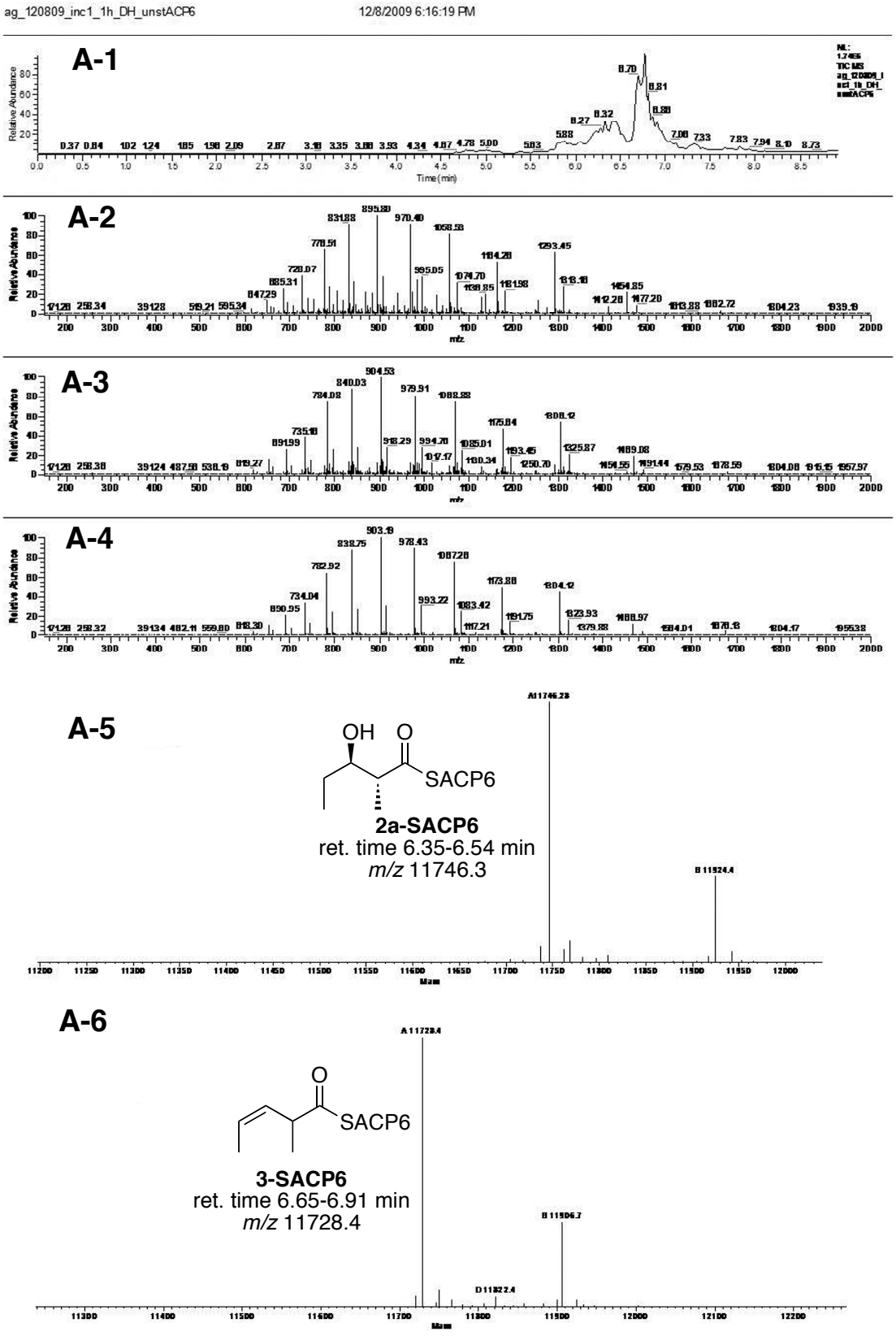


Figure S4. LC-ESI(+)-MS^3 analysis of incubation of **2a-SACP** with DH4. A. LC-MS, Panel A-1, LC-MS; Panels A-2 and A-5, *holo*-ACP6; Panels A-3 and A-6, **2a-**SACP6; Panels A-4 and A-7, **3-**SACP6. B**.** LC-MS-MS. PPant ejection products **2a-pant** (Panel B-2) and **3-pant** (Panel B-3). C. LC-MS-MS-MS on **3-pant**, *m/z* 357.

Incubation of (2*E*)-2-Methyl-2-pentenoyl-ACP (3-ACP6) with DH4: A mixture of 105 μM **3-SACP6** and 210 μM DH4 in a total volume of 40 μL of 50 mM of phosphate buffer (pH 7.2) was incubated for 1 h at 25 °C. LC-ESI(+)-MS^3 analysis confirmed the formation of mixture of (2*E*)-2-methyl-2-pentenoyl-ACP (**3-SACP**) (ret. time 6.65-6.91 min; *m*/*z* 11728.4 (observed); predicted *m*/*z* 11726.4), accompanied by (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP (**2a-SACP6**) (ret. time 6.35-6.54 min; *m*/*z* 11746.3 (observed); predicted *m*/*z* 11745.4), thereby confirming that DH4 can also catalyze the reverse hydration reaction (Figure S5). The LC-MSMS and LC-MSMSMS fragmentation patterns obtained for **2a-SACP6** and **3-SACP6** are in close agreement with the mass fragmentation patterns for the individual chemo-enzymatically prepared samples of each compound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O
SACP6
2a-SACP6
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Mass

(Figure S5A)

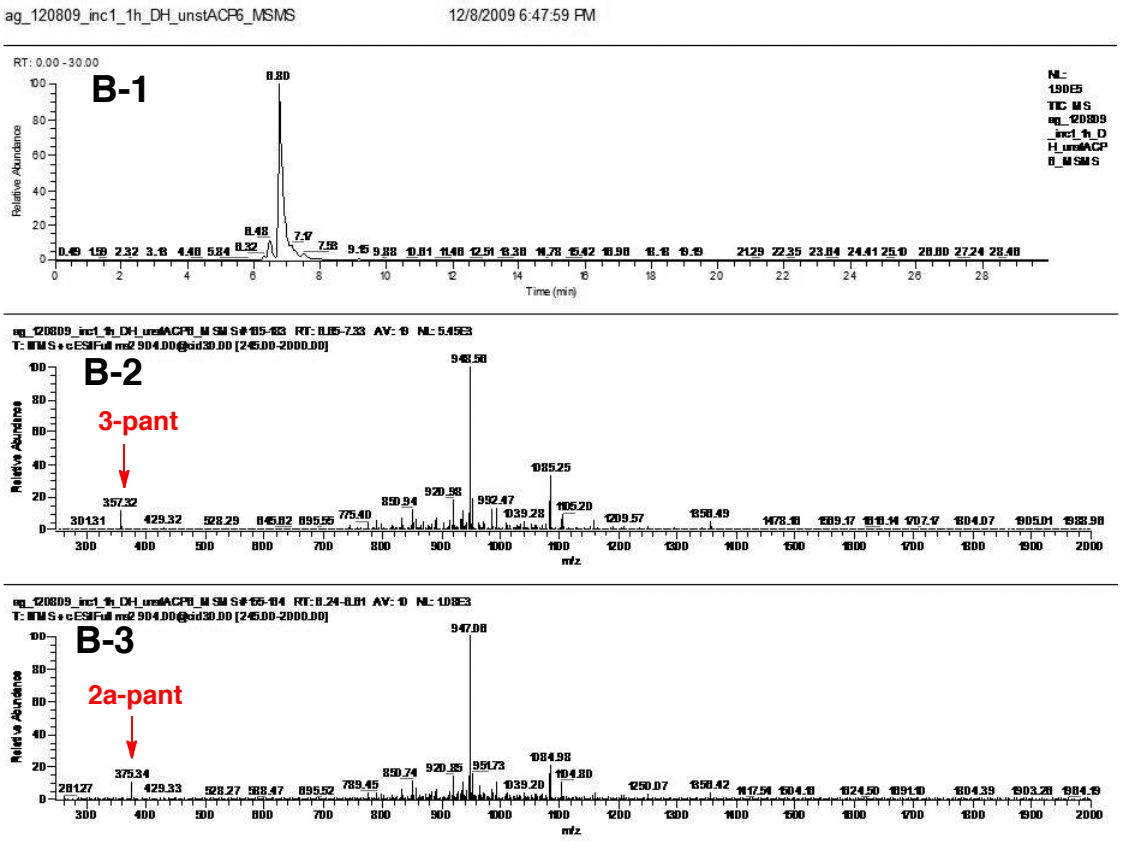

Figure S5. LC-ESI(+)-MS^2 analysis of incubation of **3-SACP6** with DH4. A. LC-MS, Panel A-1, LC-MS; Panels A-2, *holo*-ACP6; Panels A-3 and A-5, **2a-**SACP6; Panels A-4 and A-6, **3-**SACP6. B**.** LC-MS-MS. PPant ejection products **3-pant** (Panel B-2) and **2a-pant** (Panel B-3).

Assay of DEBS DH4 activity and substrate specificity by TLC-phosphorimaging. In a total volume of 50 μL of phosphate buffer (100 mM, pH 7.2) DEBS [KS6][AT6] (40 μM) was preincubated with propionyl-SNAC (**4**) (5 mM added as solution in DMSO) or (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**2b-SNAC**) (5 mM added as solution in DMSO) at 25 °C. After 1 h ACP6 (200 μM), TYLS KR1 (300 μM) and DEBS DH4 in presence of NADPH (2 mM) and DL-[2-14C]-methylmalonyl-CoA (300 μM) were added and the mixture was incubated for 1 h. Alternatively DEBS DH4 (300 μM) was added to the mixture after 1 h and the incubation was continued for an additional hour. The enzymatic reaction was quenched by addition of NaOH (20 μL, 0.5 M solution in water) and the reaction mixture was incubated for 20 min at 65 °C to facilitate enzymatic product release from ACP6. Aqueous HCl (20 μL, 1 M) was then added followed by ethyl acetate extraction (3 × 100 μL). The organic solvent was removed by Speed-Vac and the crude organic extract was resuspended in 20 μL of ethyl acetate and analyzed by TLC (silica gel; EtOAc/CH_2Cl_2 9:1, 0.1% AcOH). Radio-TLC

results were visualized by phosphorimaging. Formation of the expected products was confirmed by comparison with (2*E*)-2-methyl-2-pentenoic acid and (2*E*,4*R*,5*R*)-2,4-dimethyl-5-hydroxy-2-heptenoic acid standards (Figure S6).

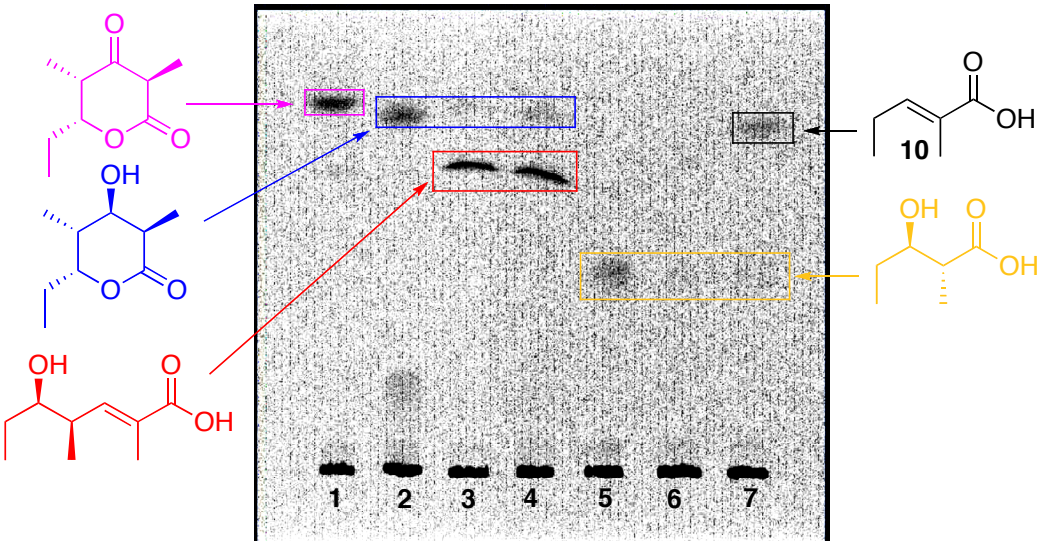

Figure S6. Radio-TLC phosphorimaging of incubation of DEBS [KS6][AT6], ACP6, TYLS KR1 and DEBS DH4 with [2-^{14}C]-2-methylmalonyl-CoA and (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**2b-SNAC**) (Lane 1-4) or propionyl-SNAC (**4**) (Lane 5-7). **Lane 1.** Without TYLS KR1 and DEBS DH4; **Lane 2**. TYLS KR1, without DEBS DH4; **Lane 3**. Coincubated with TYLS KR1 and DEBS DH4 for 1 h; **Lane 4.** Incubated with TYLS KR1 for 1 h, then DEBS DH4 was added and incubation continued for 1 h; **Lane 5.** Without TYLS KR1 and DEBS DH4; **Lane 6.** Coincubated with TYLS KR1 and DEBS DH4 for 1 h; **Lane 7.** Incubated for 1 h with TYLS KR1 incubated for 1 h, then DEBS DH4 was added and incubation continued for 1 h.

A similar experimental procedure was used to test the substrate specificity of DEBS DH4 toward *syn*-(2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP, *syn*-(2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP, and *anti*-(2*S*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP that were generated *in situ* with DEBS KR1, DEBS KR6, and RIFS KR7, respectively. DEBS [KS6][AT6] was incubated with (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**4b-SNAC**) or propionyl-SNAC (**6**) under the conditions described above. After 1 h, ACP6 and DL-[2-^{14}C]-2-methylmalonyl-CoA and either DEBS KR1, DEBS KR6, or RIFS KR7 (data not shown) were added in the presence of NADPH. The incubation was continued for 1 h, after which DEBS DH4 (300 μM) was added and the mixture was incubated for another 1 h. The reaction was quenched and the ethyl acetate extracts were analyzed by radio-TLC phosphorimaging, in all cases confirming the lack of D44-catalyzed dehydration (Figure S7).

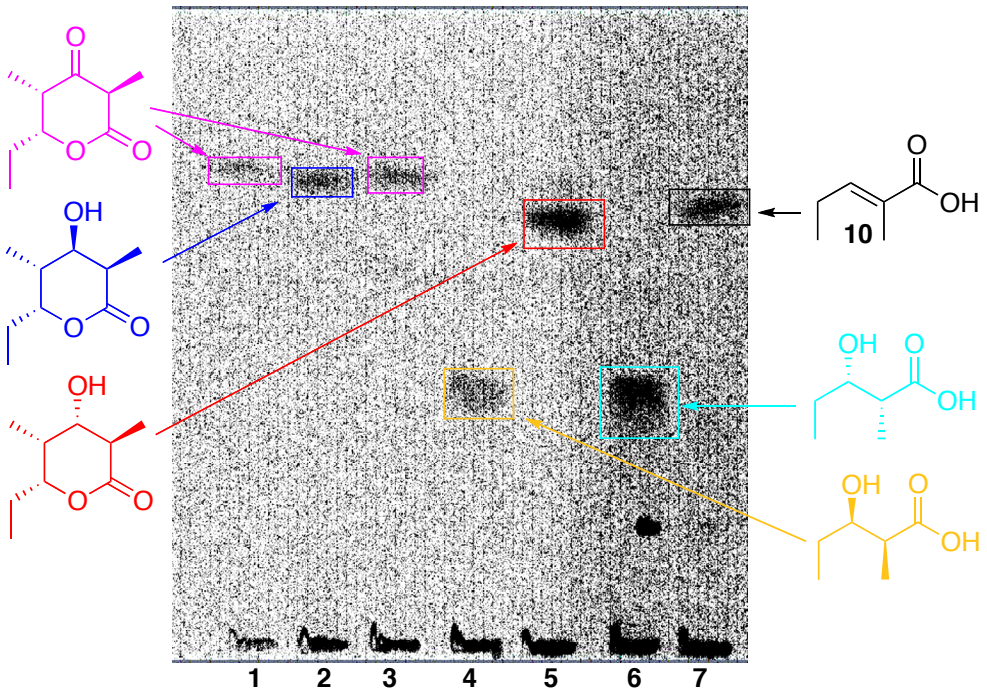

Figure S7. Radio-TLC phosphorimaging of incubation of DEBS [KS6][AT6], ACP6, recombinant KR domain and DEBS DH4 with [2-^{14}C]-2-methylmalonyl-CoA and (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**2b-SNAC**) (Lane 1-3, 5) or propionyl-SNAC (**4**) (Lane 4, 6, 7). **Lane 1.** No KR domain and no DEBS DH4; **Lane 2**. TYLS KR1 and no DEBS DH4; **lane 3.** DEBS KR1 and DEBS DH4 (DEBS KR1 does not reduce triketide substrates); **lane 4.** DEBS KR1 and DEBS DH4; **lane 5.** DEBS KR6 and DEBS DH4; **lane 6.** DEBS KR6 and DEBS DH4; **lane 7.** Positive control, TYLS KR1 and DEBS DH4.

GC-MS and HPLC-MS assay of DEBS DH4 reaction stereospecificity. The DH4 assay was based on the previously described protocol for the assay of ketoreductase stereospecificity.[6,7,17] DEBS [KS6][AT6] (40 μM) was preincubated with either propionyl-SNAC (**4**) (5 mM added as solution in DMSO) or (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC ((**2b-SNAC**) (5 mM added as solution in DMSO) in phosphate buffer (500 μL, 100 mM, pH 7.2) for 1 h at 25 °C. After 1 h ACP6 (200 μM), TYLS KR1 (300 μM), NADPH (2 mM) and methylmalonyl-CoA (300 μM) were added and the mixure was incubated for 1 h. DEBS DH4 (300 μM) was then added and the incubation was continured for 1 h. Basic hydrolysis (200 μL of 0.5 M aq. NaOH) was carried out for 20 min at 65 °C followed by acidification with 200 μL of 1 M HCl. The enzymatic products were isolated by extraction of the reaction mixture with ethyl acetate (3 × 700 μL) followed by evaporation of the solvent. For the reaction primed with propionyl-SNAC, the crude products were resuspended in 100 μL EtOAc and analyzed by GC-MS, with direct comparison with synthetic (2*E*)-2-methyl-2-pentenoic acid. (Figure S8).

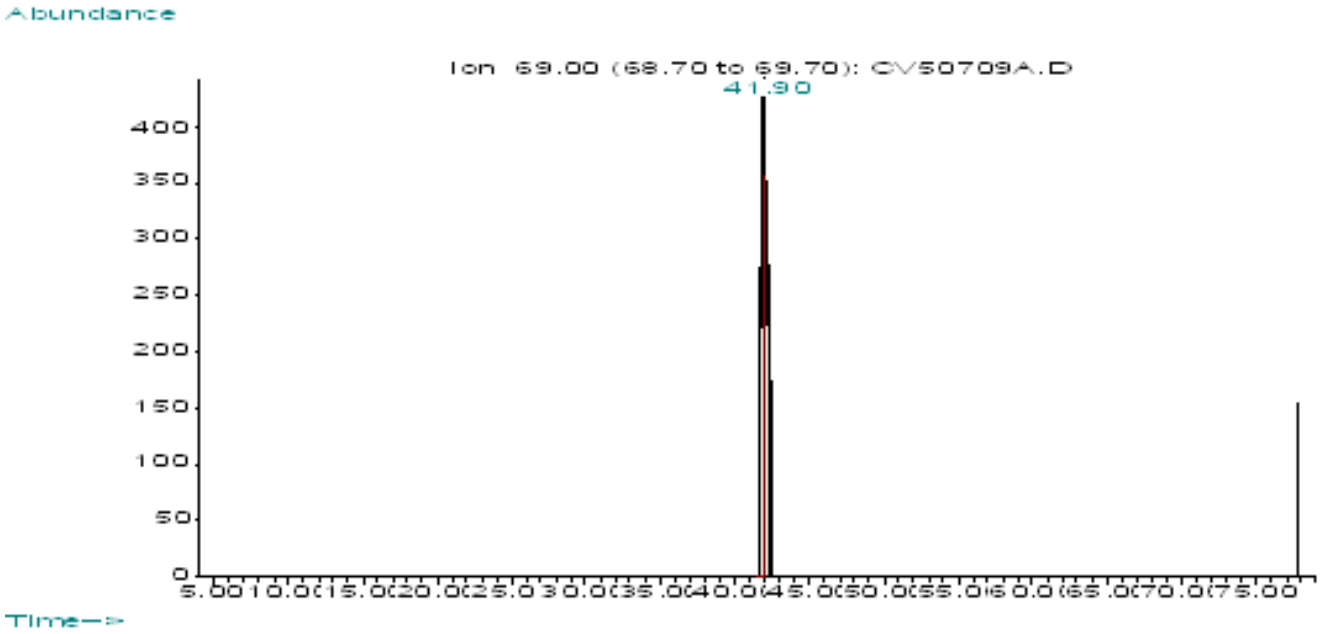

Figure S8. GC-EI-MS (XIC at *m*/*z* 69, base peak) of (2*E*)-2-methyl-2-pentenoic acid (**10**) (ret. time 41.90 min) produced by incubation of propionyl-SNAC with DEBS [KS6][AT6], DEBS ACP6, TYLS KR1 and DEBS DH4 in the presence of methylmalonyl-CoA and NADPH.

For the enzymatic incubation with (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**2b-SNAC**), the concentrated organic extract was dissolved in 100 μL of MeOH and treated with 20 μL of trimethylsilyldiazomethane (2 M in hexane) at 25 °C for 5 min to generate the corresponding methyl ester **7-Me** and the mixture was analyzed by GC-MS (Figure S9) and LC-MS (Figure S10), including direct comparison with synthetic **7-Me**.

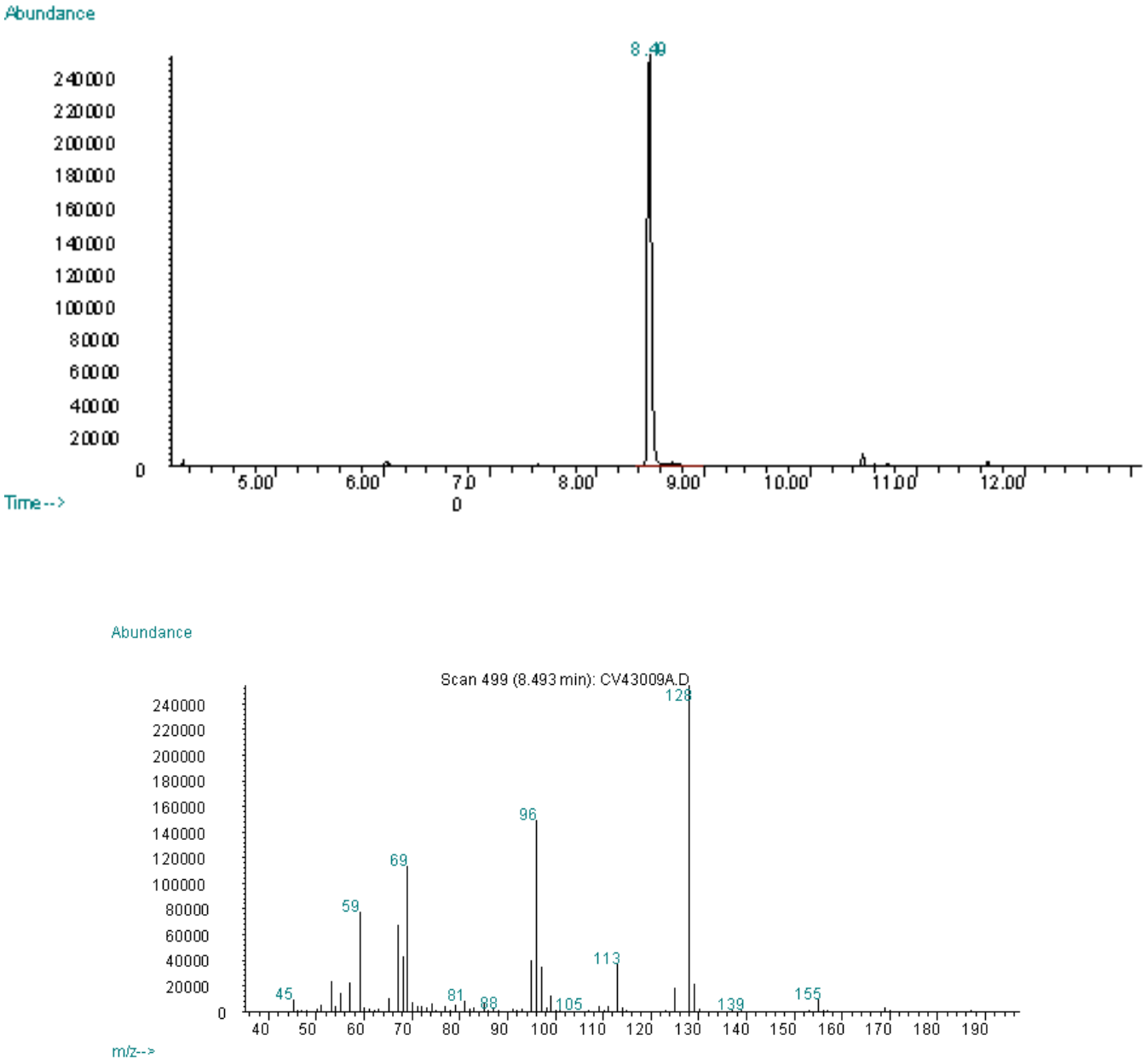

Figure S9. GC-EI-MS (XIC at *m/z* 128, base peak) of (2*E*,4*R*,5*R*)-2,4-dimethyl-5-hydroxy-2-heptenoic acid methyl ester (**7-Me**) (ret. time 8.49 min) produced by incubation of (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**2b-SNAC**) with DEBS [KS6][AT6], DEBS ACP6, TYLSKR1 and DEBS DH4 in the presence of methylmalonyl-CoA and NADPH.

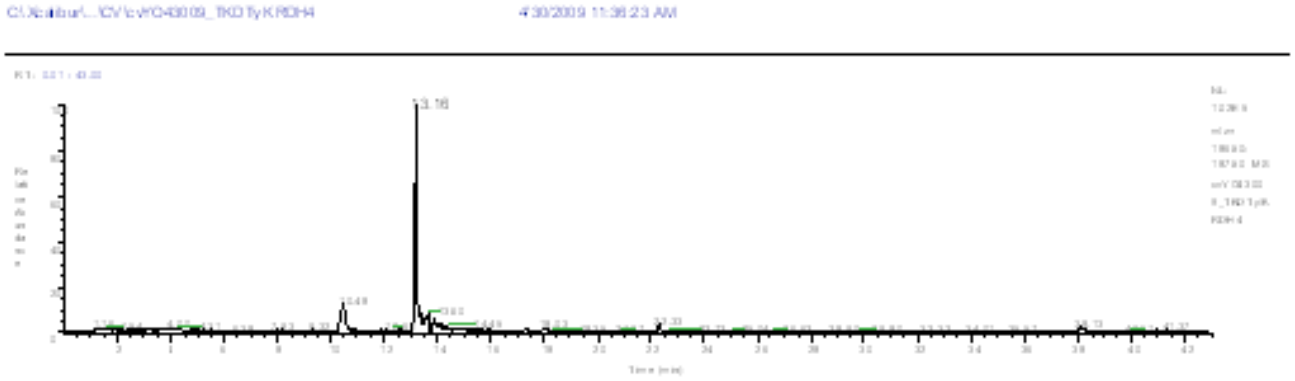

Figure S10 HPLC-MS (XIC at *m/z* 187, [M+1]) of (2*E*,4*R*,5*R*)-2,4-dimethyl-5-hydroxy-2-heptenoic acid methyl ester (**7-Me**, ret. time 13.16 min) produced by incubation of (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC (**2b-SNAC**) with DEBS [KS6][AT6], DEBS ACP6, TYLS KR1 and DEBS DH4 in the presence of methylmalonyl-CoA and NADPH. Agilent Eclipse XDB-C18 (3.5 μm × 150 mm) reverse phase column pre-equilibrated with 95 % water + 0.1 % formic acid and 5% acetonitrile; compounds were eluted with a linear gradient from 5%-100% acetonitrile over 30 min.

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