Supplementary Information

The perils of rational design – unexpected irreversible elimination of fluoride from 3-fluoro-2-methylacyl-CoA esters catalysed by α-methylacyl-CoA racemase (AMACR; P504S)

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Abbreviations used: AcOH, acetic (ethanoic) acid; CDI, N,N'-carbonyldiimidazole; CoA, coenzyme A; DAST, diethylaminosulfur trifluoride; DBQ, 2,6-dichloro-1,4-benzoquinone; DCM, dichloromethane; ESI-TOF, electrospray ionisation-time-of-flight; HRMS (ES), High resolution mass spectrometry (electrospray); IPTG, isopropyl-β-*D*-thiogalactopyranoside; Pe, petroleum ether; PMSF, phenylmethylsulfonyl fluoride; THF, tetrahydrofuran; p.p.m., parts per million.

Sources of materials: All reactions that require anhydrous conditions were performed under an argon atmosphere. Anhydrous and general grade solvents were purchased from the Sigma-Aldrich Chemical Co. and used without further purification unless otherwise noted. Oasis HLB cartridges were obtained from Waters Corporation. Biochemical grade reagents were purchased from the Sigma-Aldrich Chemical Co. or Fisher Scientific Ltd. Water for aqueous solutions was obtained from a Nanopure Diamond system and was of 18.2 $M\Omega.cm^{-1}$ quality. The Rosetta2 (DE3) expression strain was obtained from Novagen. Metal-chelate chromatography columns were from GE Healthcare. Construction of the expression plasmid for human AMACR has been previously described.[1] Fenoprofenoyl-CoA was synthesised as previously described.[2]

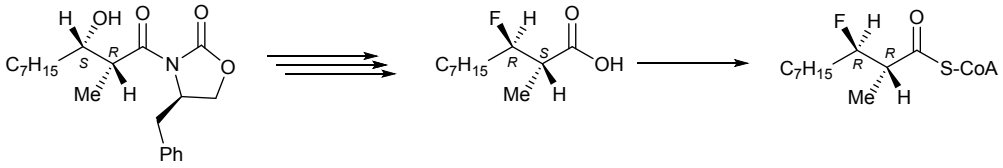
General experimental: Solvents were removed using Büchi rotary evaporators. Thin layer chromatography was performed on Merck silica aluminium plates 60 (F254) and UV light, potassium permanganate or phosphomolibdic acid were used for visualization. Column chromatography was performed using Fisher silica gel (particle size 35-70 micron). Purifications of acyl-CoA esters were performed by solid phase extraction using Oasis HLB 6cc (200 mg) extraction cartridges. Phosphate buffer was prepared from monobasic and dibasic potassium phosphates at the required proportion for 0.1 M pH 7.0 buffer. Optical rotations were recorded on an Optical Activity AA-10 Automatic polarimeter instrument. IR spectra were recorded on Perkin-Elmer RXI FTIR spectrometer instrument. NMR spectra were recorded on Bruker Avance III 400.04 MHz or 500.13 MHz spectrometers in D_2O or $CDCl_3$ and solvent was used as an internal standard. Shifts are given in ppm and *J* values reported to 0.1 Hz. Multiplicities of peaks are described as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Stock concentrations of acyl-CoA esters for assays were determined using ^1H NMR.[2] Mass spectra were recorded by ESI-TOF at the University of Bath Mass Spectrometry Service. High resolution mass spectra were recorded in ES mode. Optical rotations are reported in 10^{-1} deg cm^2 g^{-1}. Aqueous solutions for biological experiments were prepared in 18.2 $M\Omega.cm^{-1}$ Nanopure water and pH-adjusted with aq. HCl or NaOH as appropriate. Syntheses were carried out at ambient temperature, unless otherwise specified.

Assignment of stereochemical configurations

Configurations of chiral centres and double bonds are assigned based on the rules set forth by Cahn, Ingold and Prelog in 1966.[3] Priorities of ligands in this paper are assigned in the following order:

1) Atomic number (mass) of atoms;
2) Atomic number (mass) of neighbouring atoms;
3) Presence of double and triple bonds.

In 3-fluoro-2-methylcarboxylic acids and their derivatives a change in the priority order at carbon 2 occurs upon substitution of fluorine for the hydroxy group. In the aldol products the carboxylate amide or ester takes priority over the side-chain substituent, whilst the reverse is true in the fluorine-containing compounds (rule 2). A second change in priority order occurs upon going from the carboxylic acid to the acyl-CoA ester. This is because fluorine takes priority over oxygen and nitrogen, whilst sulfur takes priority over fluorine in the acyl-CoA ester (according to rule 2) (**Scheme S1**). Hence the relative orientation of ligands around carbon-2 does not change in this latter reaction but the stereochemical assignment changes due to changes in ligand priority.



Scheme S1: Assignment of stereochemistry of compounds

Syn- and *anti*- designation of 3-fluoro-2-methyldecanoyl-CoAs refer to the relative orientations of the methyl group and fluorine atom, whilst *syn*- and *anti*- elimination refers to the relative orientation of the α-proton and fluorine.

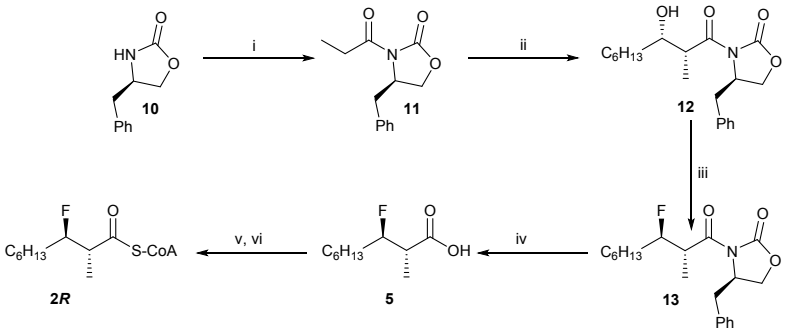
Description of the AMACR reaction and its role in metabolic pathways

AMACR catalyses an *in vitro* reaction in which either an *R*- or *S*-2-methylacyl-CoA ester is converted into a near 1:1 mixture of the C-2 epimers.[1] The reaction differs from that of 2-methylmalony-CoA epimerase,[4,5] in which the product has the opposite configuration at the methyl centre to the substrate, *i.e.* the product is only one epimer. For convenience, the AMACR catalysed reaction is described as 'racemisation' to reflect the fact that both 2-methylacyl-CoA epimers are formed in the reaction.

In vivo metabolism of 2-methyl fatty acids (as their corresponding acyl-CoA esters) occurs with a net *R*- to *S*- conversion of the chiral centre with the methyl group (C-2). This is because the *S*-2-methylacyl-CoA ester is removed by β-oxidation, whilst the *R*-2-methylacyl-CoA ester is not a substrate for the branched-chain acyl-CoA oxidase.[6-9] In the metabolism of Ibuprofen™ and related 2-APA drugs, the *R*-2-APA is specifically converted into the *R*-2-APA-CoA,[10-15] which is acted on by AMACR. Hydrolysis of the 'racemic' (2*R*/*S*) product results in a mixture of *R*- and *S*-2-APA drug, but the *R*-2-APA undergoes further cycles of metabolism. Since the *S*-2-APA is not recycled in this way, a net *R*- to *S*- conversion occurs. This three enzyme metabolic pathway is generally referred to in the literature (reviewed in[16]) as 'chiral inversion'.

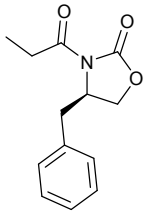
Synthesis of *anti*-3-fluoro-2-methyldecanoyl-CoA (2*R*)

anti-3-Fluoro-2-methyldecanoyl-CoA **2*R*** was synthesised by modification of the route to *anti*-3-fluoro-2-methyldecanoic acid reported by Carnell *et al.*,[17] using octanal in place of tetradecanal. Conversion of the acid to **2*R*** was achieved using carbonyldiimidazole as previously reported.[2]



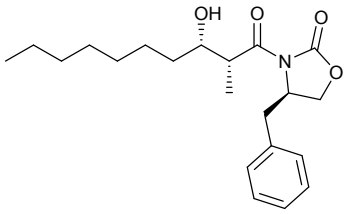
Scheme S2: Synthesis of *anti*-3-fluoro-2-methyldecanoyl-CoA 2***R*.** Reagents and conditions: i. n-BuLi, THF, propanoyl chloride, -78°C, 99%; ii. Bu_2BOTf, Pr^i_2NEt, octanal, DCM, -78°C, 99%; iii. DAST, DCM, -78°C, 64%; iv. LiOH, H_2O_2, H_2O/THF, 0°C, 61%; v. CDI, DCM, rt; vi. CoA-SH-Li^+_3, 0.1 M $NaHCO_{3\ aq.}$ /THF (1:1).

(*R*)-4-Benzyl-3-propanoyloxazolidin-2-one (11)



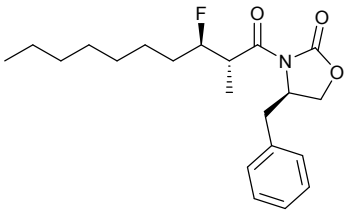
R-Evans' auxiliary **10** (4.056 g, 23 mmol) in anhydrous THF (60 mL) was cooled to -78°C, then *n*-BuLi (1.6 M, 14.3 mL, 23 mmol) in THF was added dropwise and the resulting mixture was stirred at -78°C for 30 min. A solution of propanoyl chloride (2.0 mL, 23 mmol) in anhydrous THF (20 mL) was added dropwise and the reaction mixture was stirred at -78°C for 30 min and then allowed to reach ambient temperature over a period of 1 h. The reaction mixture was quenched by slow addition of saturated aqueous NH_4Cl (80 mL) and extracted with DCM (2 × 100 mL). The combined organic extracts were washed with saturated aq. $NaHCO_3$ and brine. Drying ($MgSO_4$), filtration, evaporation and column chromatography (Pe / EtOAc 4:1) to give **11** (5.31 g, 99 %) as a colourless solid. mp 44-46°C, lit.[18] 43-46°C. ^{1}H NMR (400.04 MHz, $CDCl_3$) δ 7.34-7.15 (5H, m), 4.69-4.61 (1H, m), 4.21-4.12 (2H, m), 3.28 (1H, dd, J = 13.4, 3.3 Hz), 3.04-2.85 (2H, m), 2.76 (1H, dd, J = 13.4, 9.5 Hz), 1.18 (3H, t, J = 7.3 Hz).[19]

(*R*)-4-Benzyl-3-[(2*R*,3*S*)-3-hydroxy-2-methyldecanoyl]oxazolidin-2-one (12)



Dibutylboron triflate in DCM (1.00 M, 1.30 mL, 1.29 mmol) and diisopropylethylamine (0.25 mL, 1.29 mmol) were added to a stirred solution of oxazolidinone **11** (300 mg, 2.14 mmol) in DCM (10 mL) cooled to -78°C and the solution was stirred for 30 min. Octanal (0.15 mL, 0.92 mmol) in DCM (3.0 mL) was added dropwise and the reaction mixture was stirred at -78°C for 30 min, then allowed to reach ambient temperature. The reaction was quenched by slow addition of phosphate buffer (0.1 M, pH = 7, 10 mL). The organic layer was then washed with aq. hydrochloric acid (1 M), saturated aq. $NaHCO_3$ and brine. Drying ($MgSO_4$), filtration, evaporation and column chromatography (Pe / EtOAc 10:1) gave **12** (330 mg, 99%) as a colourless oil: R_f=0.25 Pe:Et_2O 1:1. $[\alpha]^{21}_D$ = -59.3°($CHCl_3$, c = 0.54); IR (liquid film, cm^{-1}) 3514, 1783, 1698; ^1H NMR (400.04 MHz, $CDCl_3$) δ 7.36-7.17 (5H, m), 4.75-4.65 (1H, m), 4.26-4.16 (2H, m), 3.98-3.89 (1H, m), 3.76 (1H, dq *J* = 7.0, 2.7 Hz), 3.25 (1H, dd, *J* = 13.4, 3.3 Hz), 2.84-2.74 (2H, m), 1.35-1.22 (12H, m), 1.25 (3H, d, *J* = 7.0 Hz), 0.87 (3H, t, *J* = 7.0 Hz); ^{13}C NMR (100.59 MHz) δ 177.42, 152.98, 135.03, 129.36, 128.88, 127.33, 71,48, 67.85, 66.09, 55.04, 42.13, 37.72, 33.88, 31.74, 29.47, 25.95, 22.56, 14.00, 10.40; HRMS (ES) ([M + Na]$^+$) Calcd. for $C_{21}H_{31}NNaO_4$: 384.2151, Found: 384.2198.

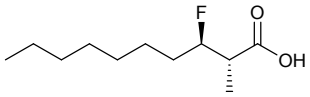
(*R*)-4-Benzyl-3-[(2*S*,3*R*)-3-fluoro-2-methyldecanoyl]oxazolidin-2-one (13)



Compound **12** (156 mg, 0.43 mmol) in anhydrous DCM (3.0 mL) was cooled to -78°C, then DAST (57 µL, 0.43 mmol) in anhydrous DCM (2.0 mL) was added dropwise to the reaction mixture. The mixture was, stirred at -78°C for 2 h, then allowed to reach ambient temperature. The reaction mixture was quenched by slow addition of water (5.0 mL). The organic layer was washed with saturated aq. $NaHCO_3$ and brine. Drying ($MgSO_4$), filtration, evaporation and column chromatography (Pe / EtOAc 10:1) gave **13** (101 mg, 64%) as a colourless oil: R_f=0.66 Pe:EtOAc 5:1. $[\alpha]^{21}_D$ = -42.0°($CHCl_3$, c=0.87). IR (liquid film, cm^{-1}): 1781, 1670, 1498. ^1H NMR (400.04

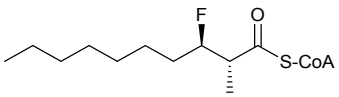
MHz, $CDCl_3$): δ 7.37-7.16 (5H, m), 4.87-4.64 (2H, m), 4.24-4.07 (3H, m), 3.26 (1H, dd, *J* = 13.4, 3.3 Hz), 2.87-2.75 (1H, m), 1.55-1.22 (12H, m), 1.18 (3H, d, *J* = 7.0 Hz), 0.88 (3H, t, *J* = 6.8 Hz). ^{13}C NMR (125.76 MHz) δ 174.31 (d, *J*= 3.0 Hz), 153.04, 135.15, 129.38, 128.86, 127.30, 95.51, 94.15, 66.11, 55.32, 41.95 (d, *J*= 21.2 Hz), 37.78, 31.97 (d, *J*= 21.0 Hz), 31.70, 29.18 (d, *J*= 27.0 Hz), 24.48 (d, *J*= 2.8 Hz), 22.55, 14.01, 13.54 (d, *J*= 8.3 Hz). ^{19}F NMR (470.52 MHz) δ -179.69. HRMS (ES) ($[M + Na]^+$) Calcd. for $C_{21}H_{30}FNNaO_3$: 386.2107, Found: 386.2096.

(2*S*,3*R*)-3-Fluoro-2-methyldecanoic acid (5)



Oxazolidinone **13** (100 mg, 0.28 mmol) in THF (3.0 mL) was cooled to 0°C, then 30% (v/v) aq. H_2O_2 (0.14 mL, 1.7 mmol) and LiOH (13 mg, 0.55 mmol) were added and the reaction mixture was stirred for 12 h. The reaction mixture was quenched by addition of saturated aq. Na_2SO_3 (3.0 mL) and was extracted with DCM. The organic phase was washed with water and brine. Drying ($MgSO_4$), filtration, evaporation and column chromatography (Pe / EtOAc 5:1) gave **5** (34 mg, 61%) as a colourless solid. mp 63-65°C; $[\alpha]^{21}_D$ = +0.74° ($CHCl_3$, c = 0.35); ^1H NMR (400.04 MHz, $CDCl_3$) δ 10.56 (1H, br s), 4.78-4.57 (1H, m), 2.85-2.71 (1H, m), 1.45-1.18 (12H, m), 1.19 (3H, d, *J* = 7.2 Hz), 0.87 (3H, t, *J* = 7.0 Hz). ^{13}C NMR (125.76 MHz) δ 179.93 (d, *J*= 5.6 Hz), 94.28 (d, *J*= 172.1 Hz), 44.36 (d, *J*= 22.1 Hz), 31.74 (d, *J*= 21.2 Hz), 31.74, 29.30, 29.12, 24.82 (d, *J*= 2.8 Hz), 22.61, 14.06, 12.56 (d, *J*= 6.6 Hz); ^{19}F NMR (470.52 MHz) δ -181.96. HRMS (ES) ($[M - H^+]^-$) Calcd. for $C_{11}H_{20}FO_2$: 203.1447, Found: 203.1447.

(2*R*,3*R*)-3-Fluoro-2-methyldecanoyl-CoA (2*R*)

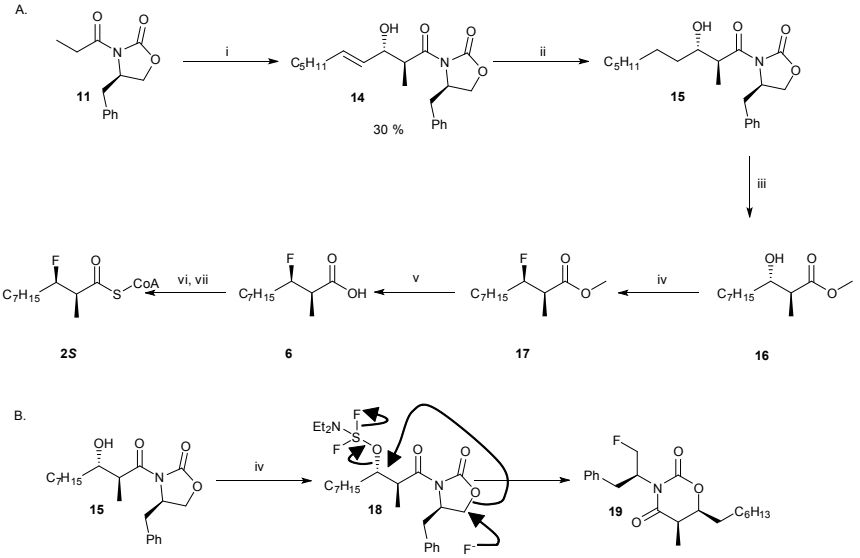


Acid **5** (10 mg, 0.05 mmol) in anhydrous DCM (1.0 mL) was treated with N,N'-carbonyldiimidazole (14 mg, 0.09 mmol) in one portion and the mixture was stirred for 1 h. The mixture was washed with water (5 × 2 mL) and brine. Drying ($MgSO_4$), filtration and evaporation gave the crude imidazolide. This material was dissolved in THF (1.0 mL) and CoA-Li_3 (17 mg, 0.02 mmol) was added, followed by aq. $NaHCO_3$ (0.1 M, 1.0 mL) and the mixture was stirred for 18 h.

The solution was acidified to pH ~3 with aq. HCl (1 M) and the solvents were partly removed under reduced pressure. Water (2.0 mL) was added and the mixture was washed with EtOAc (5 × 3 mL). Solid-phase extraction of the aqueous layer gave **2*R*** (6.0 mg) as white powder: ^{1}H NMR (500.13 MHz, D_2O) δ 8.47 (1H, s), 8.18 (1H, s), 6.07 (1H, d, *J* = 7.1 Hz), 4.18-4.11 (1H, m), 3.92 (1H, s), 3.71-3.68 (1H, s), 3.57 (1H, d, *J* = 4.6 Hz), 3.55 (1H, d, *J* = 4.6 Hz), 3.49-3.43 (2H, m), 3.37-3.33 (1H, m), 3.28-3.23 (1H, m), 3.17 (2H, t, *J* = 6.5 Hz), 2.99-2.91 (2H, m), 2.61 (2H, t, *J* = 6.5 Hz), 2.32 (2H, t, *J* = 6.5 Hz), 1.25-1.09 (10H, m), 1.04 (3H, d, *J* = 7.1 Hz), 0.79 (3H, s), 0.75 (3H, t, *J* = 6.9 Hz), 0.67 (3H, s); ^{19}F NMR (470.52 MHz) δ -181.08; HRMS (ES) ([M - H^+]$^-$) Calcd. for $C_{32}H_{54}FN_7SO_{17}P_3$: 952.2494, Found: 952.2528.

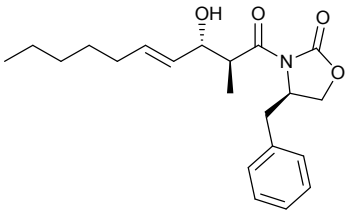
Synthesis of *syn*-3-fluoro-2-methyldecanoyl-CoA (2*S*)

syn-3-Fluoro-2-methyldecanoyl-CoA **2*S*** was synthesised (**Scheme S3a**) by condensation of 2-octenal with propanoyl-Evans' auxiliary **11** to give the unsaturated aldol product **14**. Hydrogenation over Pd/C gave the desired saturated compound **15**. The Evan's auxiliary was exchanged for the methyl ester **16**, which was converted to the fluorinated compound **17** and deprotected under acid conditions to give **6**. Conversion to the acyl-CoA ester **2*S*** was accomplished using the general literature procedure.[2] Direct treatment of **15** with DAST resulted in an unexpected rearrangement (**Scheme S3b**) *via* intermediate **18** to give product **19**. This is believed to result from steric hindrance of the α-methyl group to the nucleophilic attack of the fluoride on the derivatised hydroxy group. Instead fluoride performs a nucleophilic attack on the oxazolidin-2-one ring.



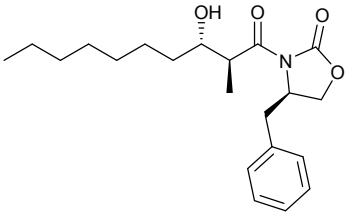
Scheme S3: a) Synthesis of *syn*-3-fluoro-2-methyldecanoyl-CoA **2*S*.** Reagents and conditions: i. Bu_2BOTf, Pr^i_2Net, 2-octenal, Et_2O, -78°C, 30%; ii. H_2, Pd/C, MeOH, 87%; iii. NaOMe, MeOH, 0°C, 56%; iv. DAST, DCM, -78°C, 41%; v. aq. HCl, AcOH, 100°C, 50%; vi. CDI, DCM, rt; vii. CoA-SH-Li^+_3, 0.1 M $NaHCO_{3\ aq.}$ / THF (1:1); **b)** Unexpected rearrangement of **15** upon treatment with DAST. Reagents and conditions: iv. DAST, DCM, -78°C, 99%.

(*R*)-4-Benzyl-3-[(2*S*,3*S*,*E*)-3-hydroxy-2-methyldec-4-enoyl]oxazolidin-2-one (14)



A solution of dibutylboron triflate in DCM (1.0 M, 1.7 mL, 1.71 mmol) and diisopropylethylamine (0.17 mL, 0.99 mmol) were added to **11** (200 mg, 0.86 mmol) in Et_2O (5.0 mL) at 0 ˚C and the solution was stirred for 30 min. The reaction mixture was cooled to -78°C and 2-octenal (0.16 mL, 1.1 mmol) in Et_2O (1.0 mL) was added dropwise. The reaction mixture was stirred at -78°C for 30 min and then allowed to reach ambient temperature. The reaction was quenched by slow addition of aq. phosphate buffer (0.1 M, pH = 7, 7 mL) and the organic layer was washed with aq. HCl (1.0 M), saturated aq. $NaHCO_3$ and brine. Drying ($MgSO_4$), filtration, evaporation and column chromatography (Pe / EtOAc 10:1) gave **14** (92 mg, 30%) as colourless oil: R_f=0.37 Pe:EtOAc 5:1. $[\alpha]^{21}_D$ = +49.8 ($CHCl_3$, c = 0.58). IR (liquid film, cm^{-1}): 3498, 1780, 1698; ^1H NMR (400.04 MHz, $CDCl_3$) δ 7.37-7.20 (5H, m), 5.83-5.69 (1H, m), 5.56-5.46 (1H, m), 4.74-4.64 (1H, m), 4.27-4.12 (3H, m), 3.98-3.89 (1H, m), 3.30 (1H, dd, *J* = 13.5, 3.4 Hz), 2.83-2.72 (1H, m), 2.51 (1H, d, *J* = 7.0 Hz), 2.10-2.01 (2H, m), 1.44-1.23 (6H, m), 1.16 (3H, d, *J* = 7.1 Hz), 0.87 (3H, t, *J* = 7.0 Hz); ^{13}C NMR (125.76 MHz) δ 176.02, 153.35, 135.17, 133.74, 129.34, 128.91, 128.73, 127.32, 73.24, 66.07, 55.35, 42.75, 37.98. 32.18, 31.33, 28.73, 22.42, 13.93, 11.22. HRMS (ES) ($[M + Na^+]^+$) Calcd. for $C_{21}H_{31}NNaO_4$: 382.1994, Found: 382.2054.

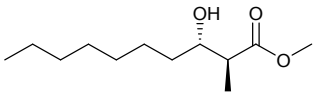
(*R*)-4-Benzyl-3-[(2*S*,3*S*)-3-hydroxy-2-methyldecanoyl]oxazolidin-2-one (15)



Aldol **14** (150 mg, 0.42 mmol) in EtOAc (10 mL) was stirred vigorously under hydrogen in the presence of 10 % Pd/C (10 mass%, 15 mg) for 1 d. The mixture was filtered (Celite) and the solvent was evaporated. Column chromatography (Pe / EtOAc 5:1) gave **15** (132 mg, 87 %) as a colourless oil: R_f=0.37 Pe:EtOAc 5:1. $[\alpha]^{21}_D$ = -40.0°($CHCl_3$, c = 0.5). IR (liquid film, cm^{-1}) 3524, 1780, 1698; ^1H NMR (400.04 MHz, $CDCl_3$) δ 7.38-7.19 (5H, m), 4.74-4.63 (1H, m), 4.25-4.14 (2H, m), 3.94-3.84 (1H, m), 3.78-3.66 (1H, m), 3.33 (1H, dd, *J* = 13.4, 3.3 Hz), 2.81-2.72 (1H, m), 2.51 (1H,

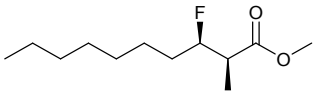
d, *J* = 8.6 Hz), 1.65-1.23 (12H, m), 1.21 (3H, d, *J* = 6.8 Hz), 0.88 (3H, t, *J* = 7.0 Hz). ^{13}C NMR (100.59 MHz) δ 176.84, 153.49, 135.21, 129.38, 128.89, 127.28, 74.61, 65.98, 55.48, 43.22, 37.82, 34.95, 31.75, 29.48, 29.18, 25.40, 22.57, 14.55, 14.01; HRMS (ES) ([M + Na^+]$^+$) Calcd. for $C_{21}H_{31}NNaO_4$: 384.2151, Found: 384.2198.

(2*S*,3*S*)-Methyl 3-hydroxy-2-methyldecanoate (16)



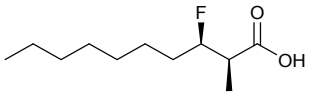
Sodium (48 mg, 2.1 mmol) was stirred with anhydrous MeOH (15 mL) until dissolved then cooled to 0 ℃. Aldol **15** (470 mg, 1.30 mmol) in anhydrous MeOH (5.0 mL) was added and the mixture was stirred at 0 ℃ for 15 min. The reaction mixture was quenched by slow addition of aq. phosphate buffer (0.1 M, pH = 7, 20 mL) and extracted with DCM (4 × 20 mL). The combined organic layers were washed with brine and dried ($MgSO_4$). Filtration, evaporation and column chromatography (Pe / EtOAc 5:1) gave **16** (158 mg, 56%) as a colourless oil. R_f=0.67 Pe:EtOAc 5:1. $[\alpha]^{21}_D$ = +5.9° ($CHCl_3$, c = 1.0); ^{1}H NMR (400.04 MHz, $CDCl_3$) δ 3.70 (3H, s), 3.65 (1H, br s), 2.58-2.44 (2H, m), 1.60-1.22 (12H, m), 1.20 (3H, d, *J* = 7.2 Hz), 0.87 (3H, t, *J* = 7.0 Hz). ^{13}C NMR (100.59 MHz) δ 176.39, 73.32, 51.60, 45.11, 34.71, 31.73, 29.45, 29.16, 25.44, 22.56, 14.26, 13.99. HRMS (ES) ([M + Na^+]$^+$) Calcd. for $C_{12}H_{24}NaO_3$: 239.1623, Found: 239.1634.

(2*R*,3*R*)-Methyl 3-fluoro-2-methyldecanoate (17)



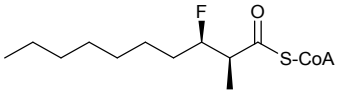
Compound **17** was prepared from **16** (54 mg, 0.25 mmol), following the same procedure as for **13**. The product was purified by column chromatography (Pe / EtOAc 20:1) to give **17** (42 mg, 78%) as a colourless oil: R_f=0.41 Pe:EtOAc 20:1. $[\alpha]^{21}_D$ = +9.1° ($CHCl_3$, c = 0.55).^{1}H NMR (400.04 MHz, $CDCl_3$): δ 4.80-4.59 (1H, m), 3.70 (3H, s), 2.71-2.55 (1H, m), 1.59-1.20 (12H, m), 1.23 (3H, d, *J* = 7.1 Hz), 0.87 (3H, t, *J* = 7.0 Hz). ^{13}C NMR (125.76 MHz) δ 174.02 (d, *J*= 7.8 Hz), 94.06 (d, *J*= 173.7 Hz), 51.87, 44.19 (d, *J*= 22.8 Hz), 32.91 (d, *J*= 21.0 Hz), 31.73, 29.27, 29.11, 25.23 (d, *J*= 3.8 Hz), 22.61, 14.06, 11.59 (d, *J*= 5.4 Hz). ^{19}F NMR (470.52 MHz) δ -189.85.

(2*R*,3*R*)-3-Fluoro-2-methyldecanoic acid (6)



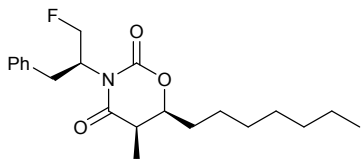
Methyl ester **17** (19 mg, 0.09 mmol) in acetic acid (1.0 mL) and aq. HCl (12 M, 1.0 mL) was heated at 100°C for 4 h. The mixture was cooled to ambient temperature and the solvents were evaporated under reduced pressure. The residue, in DCM, was washed with water (twice) and brine. Drying ($MgSO_4$), filtration, evaporation and column chromatography (Pe / EtOAc 2:1) gave **6** (9 mg, 50%) as a colourless oil; R_f=0.61 Pe:EtOAc 2:1; $[\alpha]^{21}_D$ = +7.5° ($CHCl_3$, c = 0.4); ^{1}H NMR (400.04 MHz, $CDCl_3$) δ 10.38 (1H, br s), 4.86-4.64 (1H, m), 2.73-2.59 (1H, m), 1.53-1.19 (15H, m), 0.88 (3H, t, *J* = 6.9 Hz); ^{19}F NMR (470.52 MHz) δ -190.19. HRMS (ES) ([M - H^+]$^-$) Calcd. for $C_{11}H_{20}FO_2$: 203.1447, Found: 203.1452.

(2*S*,3*R*)-3-Fluoro-2-methyldecanoyl-CoA (2*S*)



Compound **2*S*** was prepared from **6** (7 mg), following the same procedure as for compound **2*R*** to give 2 mg of **2*S***. ^{1}H NMR (500.13 MHz, D_2O) δ 8.71 (1H, s), 8.48 (1H, s), 6.30-6.20 (1H, m), 4.67-4.58 (1H, m), 4.36-4.24 (2H, m), 4.11-4.02 (1H, m), 3.96-3.85 (1H, m), 3.70-3.57 (1H, m), 3.55-3.34 (2H, m), 3.15-2.99 (2H, m), 2.59-2.40 (2H, m), 2.31-2.17 (1H, m), 1.84 (2H, s), 1.50-1.16 (10H, m), 0.97 (3H, d, *J* = 6.5 Hz), 0.86 (3H, s), 0.84 (3H, s); ^{19}F NMR (470.52) δ -187.20.

(5*R*,6*S*)-3-[(*S*)-1-Fluoro-3-phenylpropan-2-yl]-6-heptyl-5-methyl-1,3-oxazinane-2,4-dione (19)

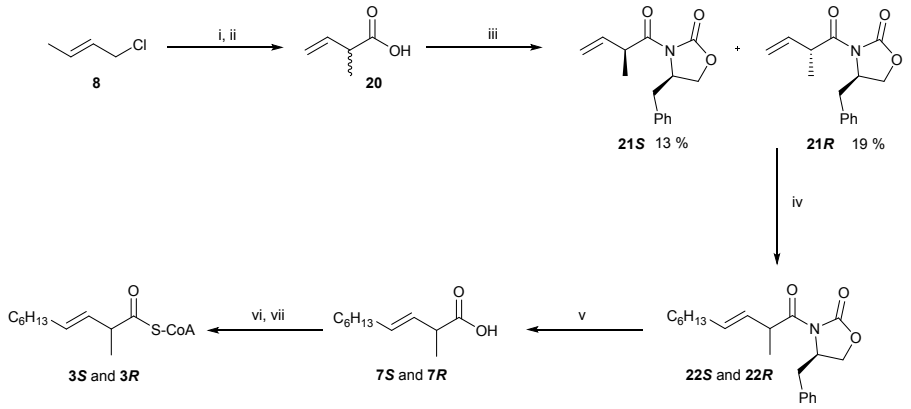


Compound **19** was prepared following the synthesis of **13** from **15** (115 mg, 0.32 mmol). Product was purified by column chromatography (Pe:EtOAc 20:1) to give 116 mg (99 %) of **19** as a colourless oil. R_f=0.72 Pe:EtOAc 5:1. $[\alpha]^{21}_D$ = +66.7° ($CHCl_3$, c = 0.87). IR (neat, cm^{-1}): 1754.82, 1704.78. ^{1}H NMR (500.13 MHz, $CDCl_3$): δ 7.29-7.14 (5H, m), 5.49-5.31 (1H, m), 4.92 (1H, dt, *J* =

48.4, 9.1 Hz), 4.59 (1H, ddd, *J* = 45.4, 9.4, 5.1 Hz), 4.24-4.12 (1H, m), 3.21 (1H, dd, *J* = 13.6, 11.1 Hz), 2.99 (1H, dd, *J* = 13.6, 6.2 Hz), 2.61 (1H, dq, *J* = 7.2, 3.9 Hz), 1.40-1.14 (12H, m), 0.87 (3H, t, *J* = 7.1 Hz), 0.82 (3H, d, *J* = 7.2 Hz). ^{13}C NMR (125.76 MHz) δ 172.53, 150.57, 136.27, 129.19, 128.44, 126.80, 81.79 (d, *J* = 171.1 Hz), 77.03, 53.66 (d, *J* = 17.7 Hz), 39.15, 33.44 (d, *J* = 5.9 Hz), 31.61, 29.13, 28.93 (d, *J* = 4.7 Hz), 24.96, 23.77, 22.51, 13.98, 9.10. ^{19}F NMR (470.59 MHz) δ -221.77. HRMS (ES) ([M + Na]$^+$) Calcd. for $C_{21}H_{30}FNNaO_3$: 386.2102, Found: 386.2102.

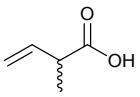
Synthesis of *S*- and *R*- *E*-2-methyldec-3-enoyl-CoA esters (3*S*) and (3*R*)

Synthesis of *S*- and *R*-*E*-2-methyldec-3-enoyl-CoA esters **3*S*** and **3*R*** was accomplished by reaction of the Grignard reagent from crotyl chloride **8** with CO_2 to form the key unsaturated intermediate **20**. Derivatisation with *R*-Evan's auxiliary **10** allowed separation of the diastereoisomers **21*S*** and **21*R***, which reacted with oct-1-ene in a metathesis reaction to give **22*S*** and **22*R***. Deprotection afforded the required acids **7*S*** and **7*R***, which were esterified with CoA-SH as previously described.[2]



Scheme S4: Synthesis of *E*-2-methyldec-2-enoyl-CoA esters **3*S*** and **3*R***. *Reagents and conditions:* i. Mg, I_2, THF, reflux; ii. CO_2, -78°C, H_3O^+, 55%; iii. n-BuLi, $(COCl)_2$, *R*-Evan's auxiliary **10**, THF, -78°C, 13% (**21*S***) and 19% (**21*R***); iv. 1-Octene, Hoveyda-Grubbs 2nd Generation cat., DBQ, DCM, reflux, 80% (**22*S***) and 65% (**22*R***); v. LiOH, H_2O_2, H_2O/THF (1:1), room temperature, 98% (**7*S***) and 99% (**7*R***); vi. CDI, DCM, rt; vii. CoA-SH-Li$^+_3$, 0.1M $NaHCO_{3\ aq.}$ / THF (1:1).

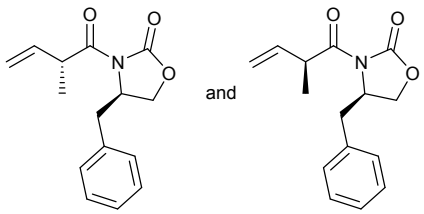
(±)-2-Methylbut-3-enoic acid (20)



Under strictly dry conditions, Mg turnings (275 mg, 11.3 mmol) were stirred with iodine (2 mg) in anhydrous THF (10 mL) for 30 min. Crotyl chloride **8** (2 drops) (*E*-1-chlorobut-2-ene) was added

to initiate reflux and the remaining **8** (1.00 mL, 10.3 mmol) was added dropwise, maintaining gentle reflux. The reaction mixture was stirred at ambient temperature for 30 min, then cooled to -78°C. CO_2 from dry ice was passed through concentrated sulfuric acid and bubbled through the mixture for 30 min. The reaction mixture was then allowed to reach ambient temperature under a flow of CO_2. The reaction mixture was basified to pH 11 with aq. NaOH (4 M) and was washed with Et_2O (3 × 30 mL). The aqueous layer was separated and acidified to pH 2 with aq. HCl (1 M) and extracted with Et_2O (2 × 30 mL). The combined organic layers were washed with brine and dried ($MgSO_4$). The solution was filtered and the solvent was evaporated to give **20** (560 mg, 55%) as a colourless liquid: ^{1}H NMR (400.04 MHz, $CDCl_3$) δ 11.01 (br s, 1H), 5.93 (1H, ddd, *J*= 17.4, 10.3, 7.4 Hz), 5.21-5.12 (1H, m), 3.23-3.12 (m, 1H), 1.30 (d, 3H, *J* = 7.1 Hz).

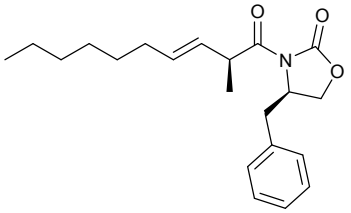
(*R*)-4-Benzyl-3-[(*R*)-2-methylbut-3-enoyl]oxazolidin-2-one (21*R*) and (*R*)-4-benzyl-3-[(*S*)-2-methylbut-3-enoyl]oxazolidin-2-one (21*S*)



Oxalyl chloride (1.60 mL, 18.7 mmol) was added dropwise to **20** (0.940 g, 9.40 mmol) in $CHCl_3$ (15 mL) at 0°C. The reaction mixture was stirred at ambient temperature for 3 h. Evaporation of the solvent and excess reagent gave the corresponding acid chloride. n-BuLi (8.4 mL, 1.6 M, 13 mmol) in THF was added dropwise to **10** (1.165 g, 9.40 mmol) in anhydrous THF (20 mL) at -78°C and the mixture was stirred for 30 min. The acid chloride in anhydrous THF (5.0 mL) was added dropwise. The reaction mixture was stirred at -78°C for 1 h and was then allowed to reach ambient temperature. The reaction mixture was quenched by slow addition of saturated aq. NH_4Cl (25 mL) and was extracted with DCM (2 × 50 mL). The combined organic layers were washed with saturated aq. $NaHCO_3$ and brine. Drying ($MgSO_4$), filtration, evaporation and column chromatography (Pe / EtOAc 10:1) gave **21*R*** (467 mg, 19%) as a colourless oil. $[\alpha]^{21}_D$ = -84.4° ($CHCl_3$, c = 1.2), lit.[20] -87.3°. ^{1}H NMR (400.04 MHz, $CDCl_3$) δ 7.37-7.16 (5H, m), 5.98 (1H, ddd, *J*= 7.7, 10.3, 17.4 Hz), 5.19 (1H, ddd, *J*= 1.2, 1.2, 17.4 Hz), 5.13 (1H, ddd, *J*= 1.0, 1.2, 10.3 Hz), 4.70-4.61 (1H, m), 4.51-4.41 (1H, m), 4.22-4.14 (2H, m), 3.28 (1H, dd, *J* = 3.3, 13.4 Hz), 2.78 (1H, dd, *J* = 9.6, 13.4 Hz), 1.34 (d, 3H, *J* = 6.9 Hz).

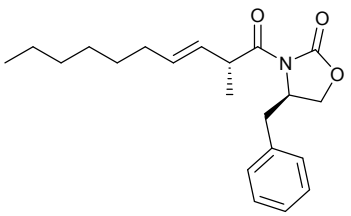
Further elution gave **21*S*** (311 mg, 13%) as a white solid: mp 69-71°C, lit.[20] 72-74°C; $[\alpha]^{21}_D$ = -32.8° ($CHCl_3$, c = 0.43), lit.[20] -28.9°. ^{1}H NMR (400.04 MHz, $CDCl_3$) δ 7.36-7.17 (5H, m), 6.02 (1H, ddd, *J* = 7.6, 10.3, 17.5 Hz), 5.26 (1H, ddd, *J* = 1.1, 1.2, 17.5 Hz), 5.19 (1H, ddd, *J* = 1.0, 1.1, 10.3 Hz), 4.73-4.65 (1H, m), 4.53-4.43 (1H, m), 4.24-4.12 (2H, m), 3.25 (1H, dd, *J* = 3.3, 13.4 Hz), 2.74 (1H, dd, *J* = 9.5, 13.4 Hz), 1.31 (3H, d, *J* = 6.9 Hz).

(*R*)-4-Benzyl-3-[(*S*,*E*)-2-methyldec-3-enoyl]oxazolidin-2-one (22*S*)



Grubbs' catalyst **(**Hoveyda-Grubbs Catalyst, 2nd Generation) (37 mg, 0.06 mmol) and DBQ (21 mg, 0.12 mmol) were stirred in anhydrous DCM (3.0 mL) for 5 min, then **21*S*** (305 mg, 1.18 mmol) and octene (0.37 mL, 2.35 mmol) in anhydrous DCM (2.0 mL) were added. The reaction mixture was stirred at reflux for 40 h. Evaporation and column chromatography (Pe / EtOAc 20:1) gave **22*S*** (324 mg, 80%) as a colourless oil: R_f=0.57 Pe:EtOAc 5:1. $[\alpha]^{21}_D$ = -23.5° ($CHCl_3$, c = 1.53); IR (liquid film, cm^{-1}) 1782, 1700; ^{1}H NMR (400.04 MHz, $CDCl_3$) δ 7.34-7.16 (5H, m), 5.74-5.53 (2H, m), 4.73-4.64 (1H, m), 4.49-4.38 (1H, m), 4.22-4.10 (2H, m), 3.22 (1H, dd, *J* = 13.4, 3.4 Hz), 2.78-2.69 (1H, m), 2.08-2.00 (2H, m), 1.41-1.23 (11H, m), 0.87 (3H, t, *J* = 7.0 Hz); ^{13}C NMR (100.59 MHz) δ 175.21, 152.91, 135.21, 133.32, 129.42, 128.83, 128.37, 127.24, 65.81, 55.09, 40.45, 37.68, 32.49, 31.62, 29.10, 28.72, 22.55, 17.23, 13.99. HRMS (ES) ($[M + Na^+]^+$) Calcd. for $C_{21}H_{29}NNaO_3$: 366.2040, Found: 366.2077.

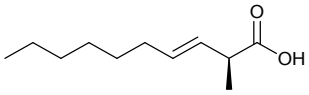
(*R*)-4-Benzyl-3-[(*R*,*E*)-2-methyldec-3-enoyl]oxazolidin-2-one (22*R*)



Compound **22*R*** was prepared from **21*R*** (460 mg, 1.77 mmol), following the same procedure as for compound **22*S***. The product was purified by column chromatography (Pe / EtOAc 20:1) to give **22*R*** (396 mg, 65 %) as a colourless oil: R_f=0.70 Pe:EtOAc 5:1. $[\alpha]^{21}_D$ = -85.3° ($CHCl_3$, c = 1.98); IR (liquid film, cm^{-1}) 2957, 2927, 2872, 2856, 1783, 1699, 1455, 1382, 1355, 1210, 1104, 973, 703;

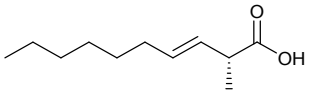
^{1}H NMR (400.04 MHz, $CDCl_3$) δ 7.35-7.17 (5H, m), 5.65-5.50 (2H, m), 4.68-4.58 (1H, m), 4.45-4.36 (1H, m), 4.17-4.12 (2H, m), 3.27 (1H, dd, *J* = 13.3, 3.2 Hz), 2.82-2.73 (1H, m), 2.04-1.95 (2H, m), 1.39-1.20 (11H, m), 0.87 (3H, t, *J* = 7.0 Hz). ^{13}C NMR (100.59 MHz) δ 175.39, 152.88, 135.32, 133.10, 129.37, 128.86, 128.41, 127.26, 65.94, 55.48, 40.72, 37.92, 32.41, 31.65, 29.02, 28.68, 22.52, 18.74, 15.06. HRMS (ES) ([M + Na^+]$^+$) Calcd. for $C_{21}H_{29}NNaO_3$: 366.2040, Found: 366.2063.

(2*S*,*E*)-2-Methyldec-3-enoic acid (7*S*)



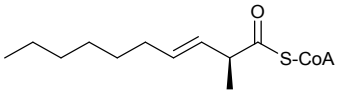
Compound **7*S*** was prepared from oxazolidinone **22*S*** (200 mg, 0.58 mmol), following the same procedure as for compound **5**. The product was purified by column chromatography (Pe / EtOAc 5:1) to give **7*S*** (106 mg, 99 %) as a colourless oil: R_f=0.45 Pe:EtOAc 5:1. $[\alpha]^{21}_D$ = +32.0° ($CHCl_3$, c = 0.77) (lit.[21] $[\alpha]^{20}_D$ = +42.2° (DCM, c = 1.0); ^{1}H NMR (400.04 MHz, $CDCl_3$) δ 11.08 (1H, br s), 5.64-5.44 (2H, m), 3.11 (1H, dq, *J*= 7.1, 7.0 Hz), 2.06-1.94 (2H, m), 1.41-1.20 (11H, m), 0.88 (3H, t, *J* = 6.9 Hz); ^{13}C NMR (100.59 MHz) δ 181.35, 132.92, 127.93, 42.61, 32.32, 31.59, 29.02, 28.70, 22.51, 17.18, 13.96.

(2*R*,*E*)-2-Methyldec-3-enoic acid (7*R*)



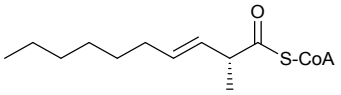
Compound **7*R*** was prepared from oxazolidinone **22*R*** (200 mg, 0.58 mmol), following the same procedure as for compound **5**. The product was purified by column chromatography (Pe / EtOAc 5:1) to give **7*R*** **(**105 mg, 98 %) as a colourless oil: R_f=0.45 Pe:EtOAc 5:1. $[\alpha]^{21}_D$ = -45.9° ($CHCl_3$, c = 0.43) ^{1}H NMR (500.13 MHz, $CDCl_3$) δ 11.02 (1H, br s), 5.57-5.39 (2H, m), 3.05 (1H, dq, *J*= 7.1, 7.0 Hz), 1.98-1.90 (2H, m), 1.34-1.14 (11H, m), 0.81 (3H, t, *J* = 6.9 Hz). ^{13}C NMR (125.76 MHz) δ 181.55, 133.04, 127.95, 42.72, 32.43, 31.69, 29.10, 28.80, 22.62, 17.28, 14.10. HRMS (ES) ([M - H^+]$^-$) Calcd. for $C_{11}H_{19}O_2$: 183.1385, Found: 183.1551.

(2*S*,*E*)-2-Methyldec-3-enoyl-CoA (3*S*)



Compound **3*S*** was prepared from **7*S*** (15 mg), following the same procedure as for compound **2*R*** to give 4 mg of **3*S***. ^{1}H NMR (500.13 MHz, D_2O): δ 8.47 (1H, s), 8.18 (1H, s), 6.08 (1H, d, *J* = 7.1 Hz), 4.70-4.67 (1H, m), 4.20-4.08 (2H, m), 3.93 (1H, s), 3.77-3.70 (1H, m), 3.62 (1H, s), 3.48-3.42 (1H, m), 3.39-3.31 (2H, m), 3.27-3.20 (2H, m), 2.96-2.82 (1H, m), 2.32 (2H, t, *J* = 6.8 Hz), 1.88 (1H, q, *J* = 6.9 Hz), 1.26-1.04 (11H, m), 0.80 (3H, s), 0.73 (3H, t, *J* = 6.5 Hz), 0.68 (3H, s).

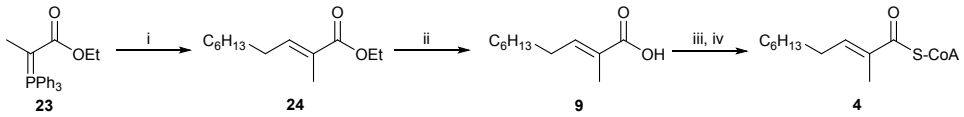
(2*R*,*E*)-2-Methyldec-3-enoyl-CoA (3*R*)



Compound **3*R*** was prepared from **7*R*** (15 mg), following the same procedure as for compound **2*R*** to give 6 mg of **3*R***. ^{1}H NMR (500.13 MHz, D_2O): δ 8.47 (1H, s), 8.18 (1H, s), 6.07 (1H, d, *J* = 7.1 Hz), 4.71-4.67 (1H, m), 4.20-4.08 (2H, m), 3.92 (1H, s), 3.78-3.71 (1H, m), 3.62 (1H, s), 3.48-3.42 (1H, m), 3.38-3.31 (2H, m), 3.27-3.20 (2H, m), 2.96-2.82 (1H, m), 2.32 (2H, t, *J* = 6.8 Hz), 1.88 (1H, q, *J* = 6.9 Hz), 1.31-1.03 (11H, m), 0.80 (3H, s), 0.74 (3H, t, *J* = 6.5 Hz), 0.68 (3H, s).

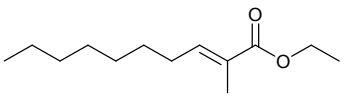
Synthesis of *E*-2-methyldec-2-enoyl-CoA (4)

Ylide **23** was prepared using the method of Baktharaman *et al.*[22] and the desired unsaturated ester **24** prepared by Wittig reaction between ylide **23** and octanal. Ester **24** was hydrolysed to the acid **9** under basic conditions, which was converted to **4** by the literature procedure.[2]



Scheme S5: Synthesis of *E*-2-methyldec-2-enoyl-CoA **4**. *Reagents and conditions:* i. Octanal, DCM, 0 ℃, 77%; ii. KOH, EtOH/H_2O (2:1), rt, 97%; iii. CDI, DCM, rt; iv. CoA-SH-Li^+_3, 0.1 M $NaHCO_{3\ aq.}$ / THF (1:1).

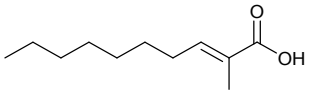
Ethyl (*E*)-2-Methyldec-2-enoate (24)[23]



Octanal (2.62 mL, 16.8 mmol) in anhydrous DCM (20 mL) was added dropwise to **23** (6.09 g, 16.8 mmol) in anhydrous DCM (50 mL) at 0°C and the reaction mixture was allowed to reach ambient temperature over 2 h. Evaporation and column chromatography (Pe / EtOAc 50:1) gave **24** (2.74 g,

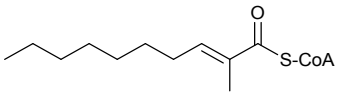
77 %) as a colourless oil: ^{1}H NMR (400.04 MHz, $CDCl_3$) δ 6.75 (1H, tq, *J* = 7.5, 1.5 Hz), 4.17 (2H, q, *J* = 7.1 Hz), 2.19-2.11 (2H, m), 1.83-1.80 (3H, m), 1.49-1.37 (2H, m), 1.34-1.21 (11H, m), 0.90-0.83 (3H, m).

(*E*)-2-Methyldec-2-enoic acid (9)[24]



Ethyl ester **24** (442 mg, 2.1 mmol) in EtOH (10 mL) was stirred with KOH (467 mg, 8.34 mmol) in H_2O (5.0 mL) for 20 h. The reaction mixture was acidified to pH ~3 with aq. HCl (3 M) and the solvents were partly evaporated under reduced pressure. DCM was added to the residue and the organic layer was washed with water and brine. Drying ($MgSO_4$), filtration, evaporation and column chromatography (DCM / MeOH 100:1) gave **9** (374 mg, 97%) as a colourless oil: ^{1}H NMR (400.04 MHz, $CDCl_3$) δ 11.70 (1H, br s), 6.91 (1H, tq, *J* = 7.5, 1.4 Hz), 2.23-2.15 (2H, m), 1.84-1.81 (3H, m), 1.51-1.39 (2H, m), 1.36-1.20 (8H, m), 0.93-0.83 (3H, m).

(*E*)-2-Methyldec-2-enoyl-CoA (4)



Compound **4** was prepared from **9** (10 mg), following the same procedure as for compound **2*R*** to give 3 mg of **4**. ^{1}H NMR (500.13 MHz, D_2O): δ 8.62 (1H, s), 8.37 (1H, s), 6.75 (1H, dt, *J* = 7.8, 1.0 Hz), 6.15 (1H, d, *J* = 6.0 Hz), 4.56 (1H, s), 4.18 (2H, s), 4.12-3.95 (2H, m), 3.84-3.75 (1H, m), 3.57-3.48 (1H, m), 3.37 (2H, t, *J* = 6.4 Hz), 3.28 (2H, t, *J* = 6.4 Hz), 2.95 (2H, t, *J* = 6.5 Hz), 2.36 (2H, t, *J* = 6.9 Hz), 2.18-2.11 (2H, m), 1.74 (3H, s), 1.40-1.05 (10H, m), 0.87 (3H, s), 0.76 (3H, t, *J* = 6.9 Hz), 0.70 (3H, s).

AMACR assays[1,2]

Human AMACR was expressed in *E. coli* Rosetta2 (DE3) at 22 °C overnight shaking at 220 r.p.m., inducting with 0.25 mM IPTG. Cells (~2 g) were lysed using the 'one shot' in ~30 mL 20 mM NaH_2PO_4-NaOH, 300 mM NaCl, 10 mM imidazole, pH 7.2 supplemented with 1 mM PMSF and 250 u benzonase (Novagen) and stirred with N-lauroyl-sarcosine at 4 °C for 1 hour. Following centrifugation, enzyme was purified by metal-chelate chromatography, dialysed into 10 mM NaH_2PO_4-NaOH, pH 7.4 and stored at -80 °C. Protein purity of pooled fractions was *ca.* 95 – 98 % by SDS-PAGE analyses. Protein concentrations were quantified using UV-visible absorbance[2] at

280 nm (ε_{280} = 35785 M^{-1} cm^{-1}) and assumed a molecular mass of 47146.8 Da. for the His-tag protein.[1]

Assays were conducted in 50 mM NaH_2PO_4-NaOH, pH 7.4 containing *ca.* 85% ^2H_2O and 100 μM acyl-CoA substrate as previously described, with negative controls contain heat-inactivated enzyme. ±-Fenoprofenoyl-CoA or *S*-2-methyldecanoyl-CoA with wild-type enzyme were used as positive controls.[1,2] Assays were quenched by heating to 50 °C for 10 minutes before ^1H NMR analysis (500.13 MHz). Conversion of substrates was quantified by conversion of the 2-Me doublet at *ca.* 1.0 p.p.m. into a singlet at *ca.* 1.75 p.p.m., and were corrected for non-enzymatic conversion in heat-inactivated negative controls.[1,2] Kinetic assays contained 1.1 μg (0.023 nmol) protein per assay. Parameters were obtained using SigmaPlot 12. Data was analysed with the Direct Linear Plot[25,26] and non-linear fitting of data to the Michaelis-Menten equation. Error bars are ± SE.

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Time course of AMACR catalysed elimination reaction

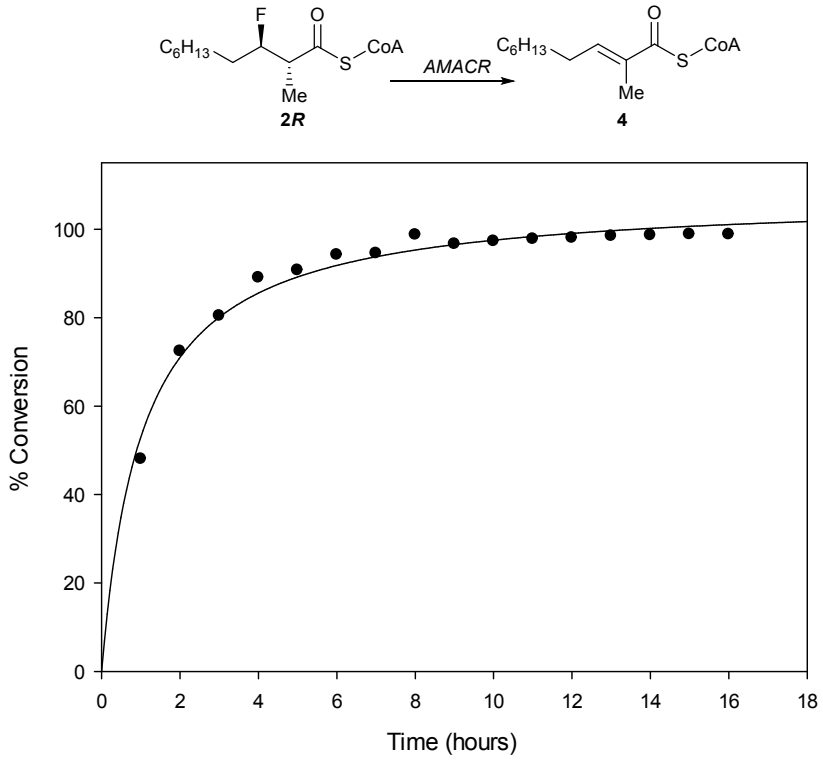
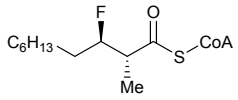
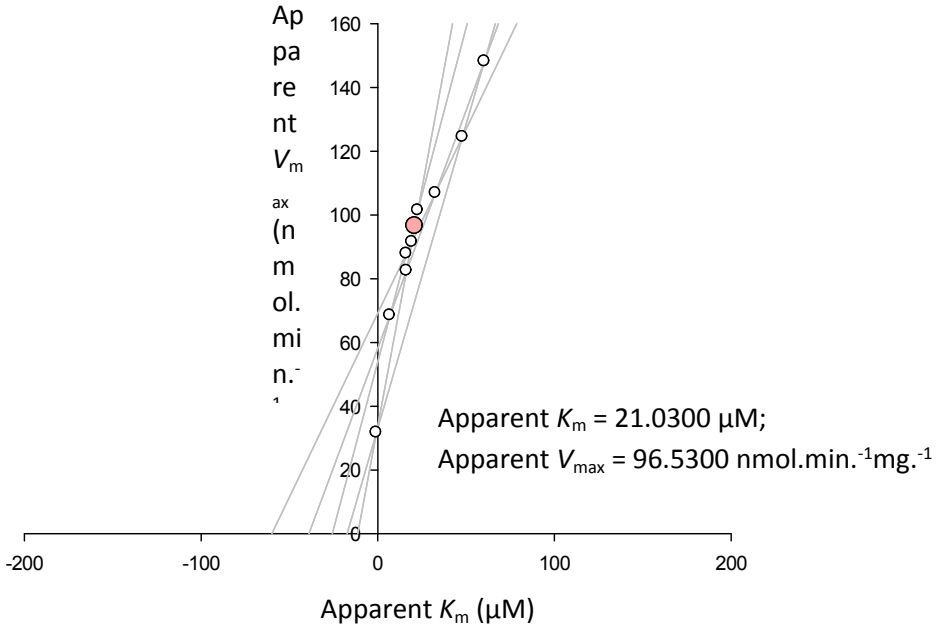


Figure S1: Time course of the AMACR catalysed elimination reaction. The reaction was carried out as described in the experimental using 100 μM substrate and 2.5 μM enzyme. % conversion was calculated based on integration of the peaks at *ca.* 1.1 ppm (**2*R*** methyl group) and *ca.* 1.75 ppm (**4** methyl group). A negative control containing heat-inactivated enzyme showed <5% conversion at all time points.

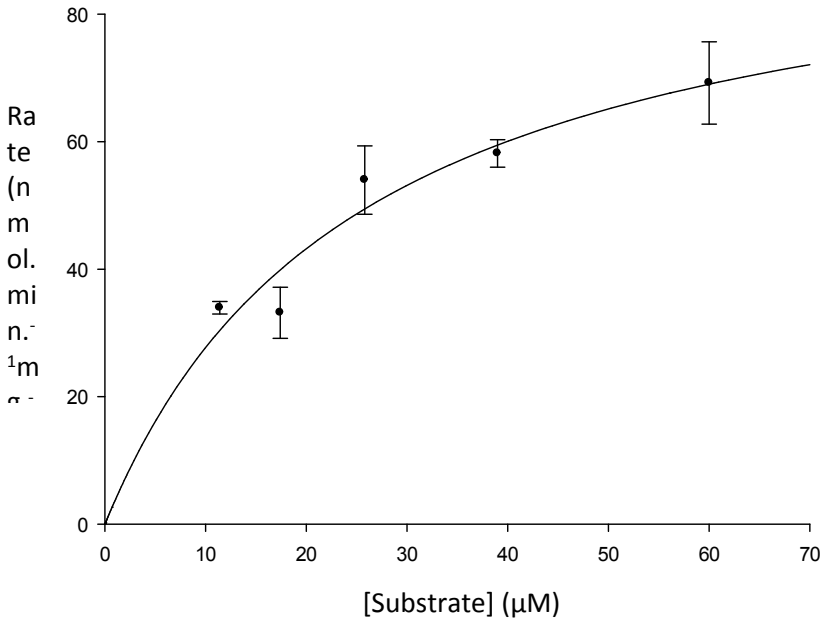
Kinetic analysis of *anti*-(3*R*,2*R*)-3-Fluoro-2-methyldecanoyl-CoA **2*R***



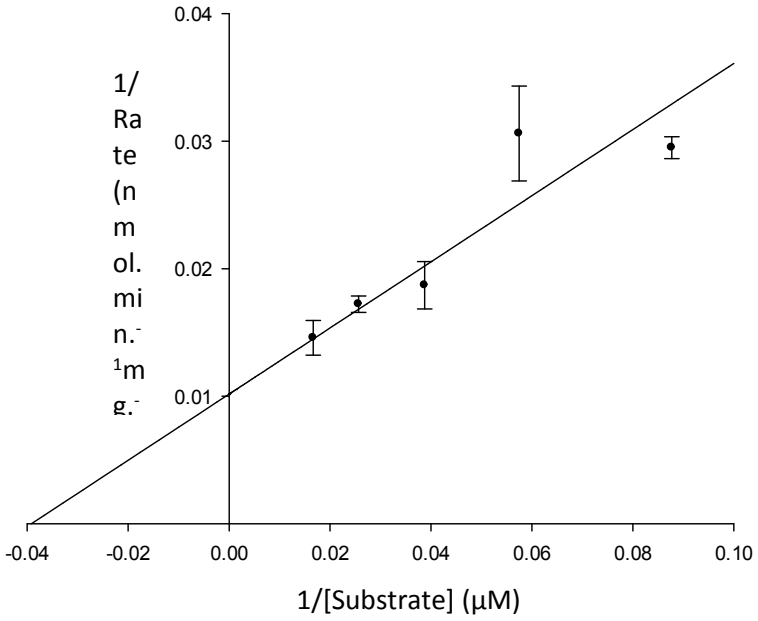
Direct Linear Plot



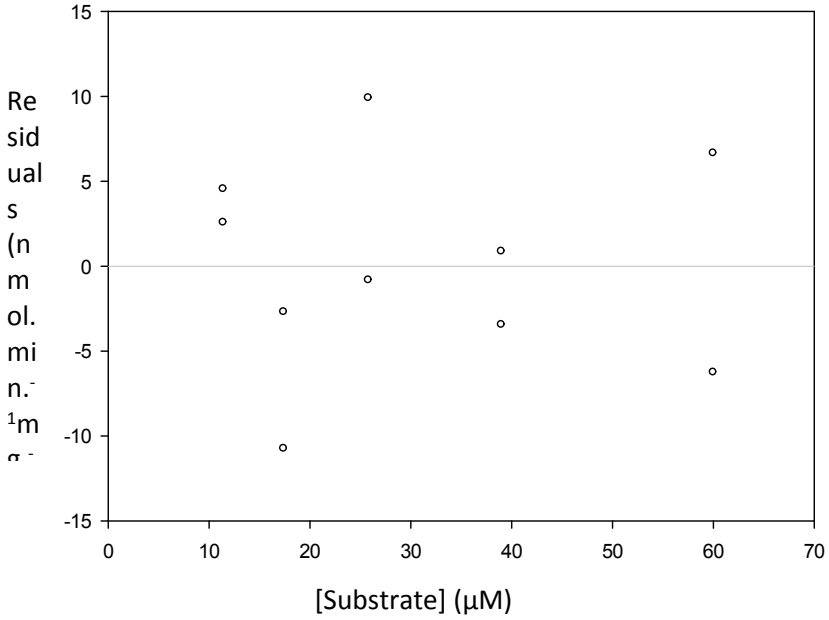
Michaelis-Menten



Lineweaver-Burk



Residuals



Enzyme Kinetics Nonlinear Fit Results

Notebook1
08/08/2013 12:04:26
Michaelis-Menten
Number of Replicates: 2

Parameters

	Value	±Std. Error	95% Conf. Interval			
V_{max}	98.3751	14.1390	65.7699	to	130.9804	nmol.min.$^{-1}$mg.$^{-1}$
K_m	25.5130	8.3049	6.3615	to	44.6645	µM

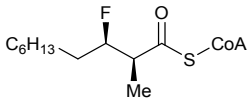
Goodness of Fit

Degrees of Freedom	8
AICc	45.403
R^2	0.841
Sum of Squares	344.768
Sy.x	6.565
Runs Test p Value	0.500

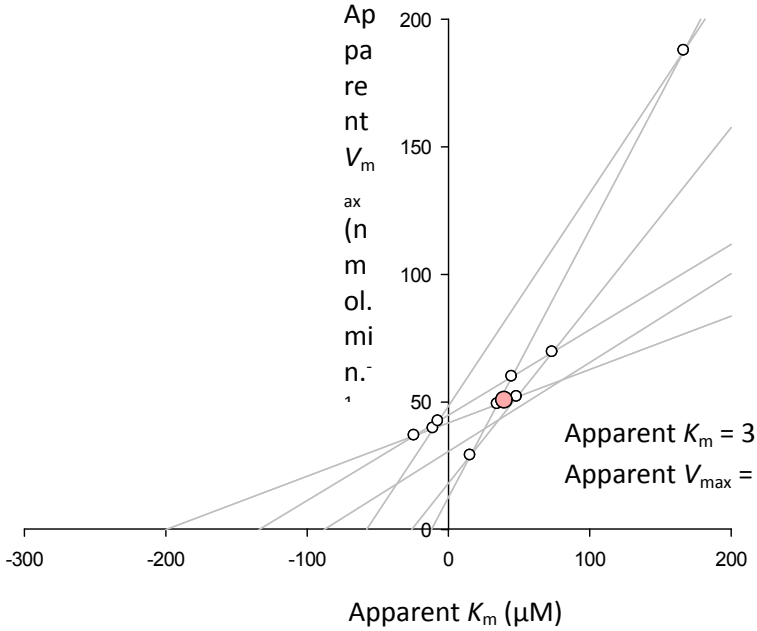
Data

Number of x values	5
Number of replicates	2
Total number of values	10
Number of missing values	0

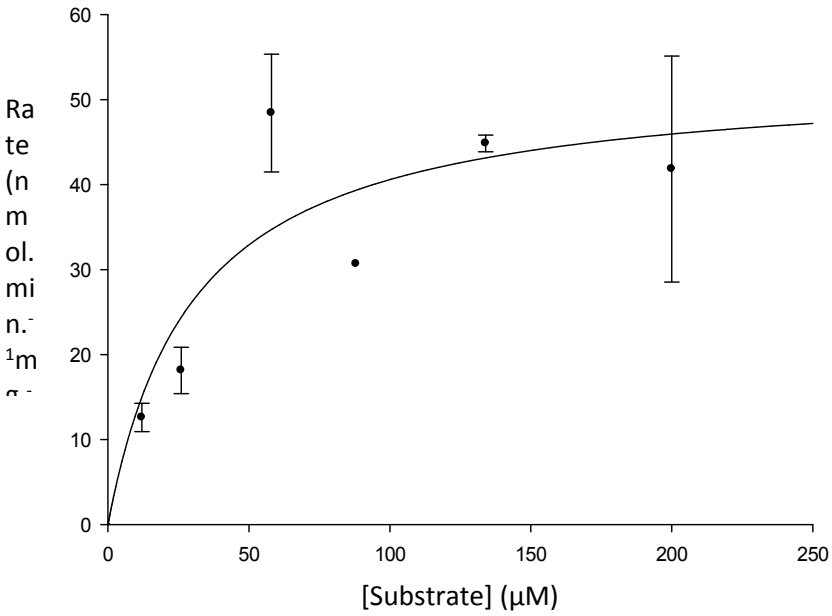
Kinetic analysis of s*yn*-(3*R*,2*S*)-3-Fluoro-2-methyldecanoyl-CoA **2*S***



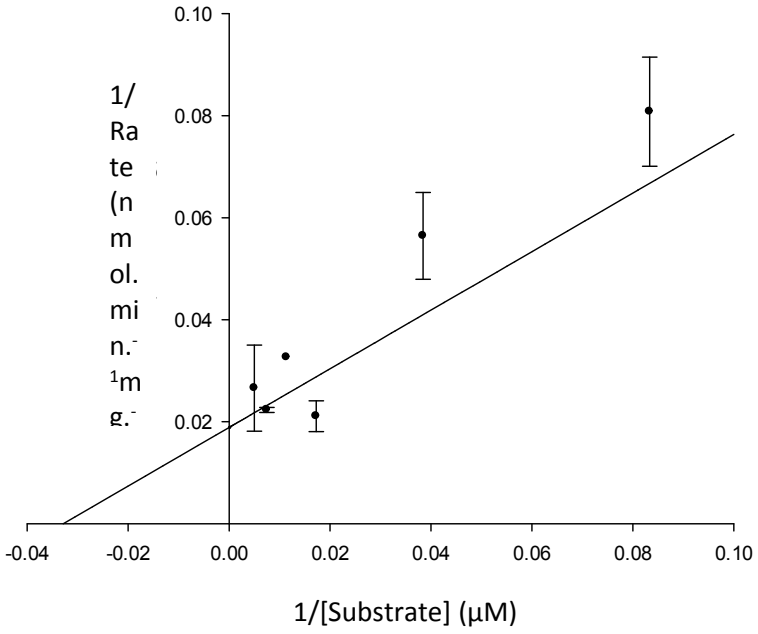
Direct Linear Plot



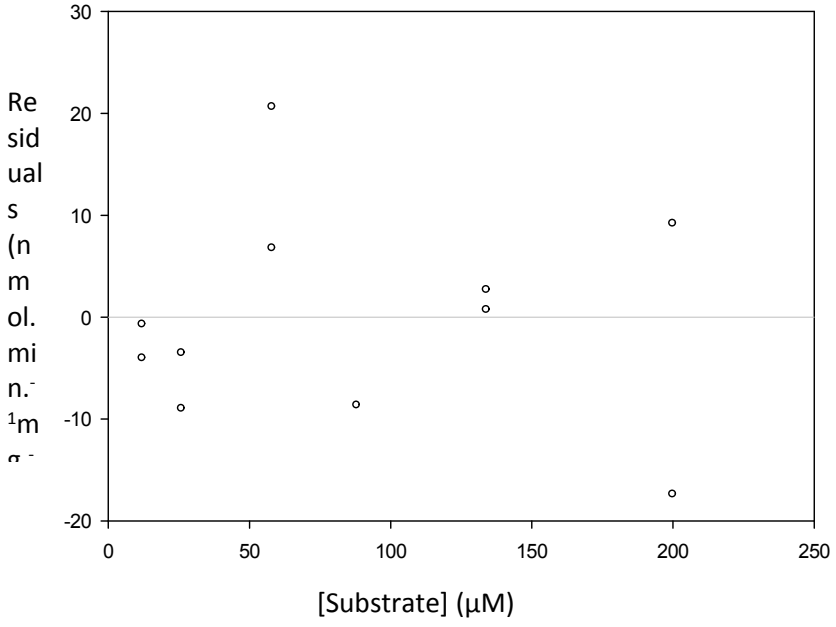
Michaelis-Menten



Lineweaver-Burk



Residuals



Enzyme Kinetics Nonlinear Fit Results

Notebook2
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Michaelis-Menten
Number of Replicates: 2

Parameters

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K_m	30.4107	19.1990	-13.0214	to	73.8428	μM

Goodness of Fit

Degrees of Freedom	9
AICc	59.593
R^2	0.605
Sum of Squares	1,051.835
Sy.x	10.811
Runs Test p Value	0.385

Data

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Number of replicates	2
Total number of values	11
Number of missing values	1

Unsaturated 2-methylacyl-CoA esters ***3R*** and ***3S*** as substrates for AMACR

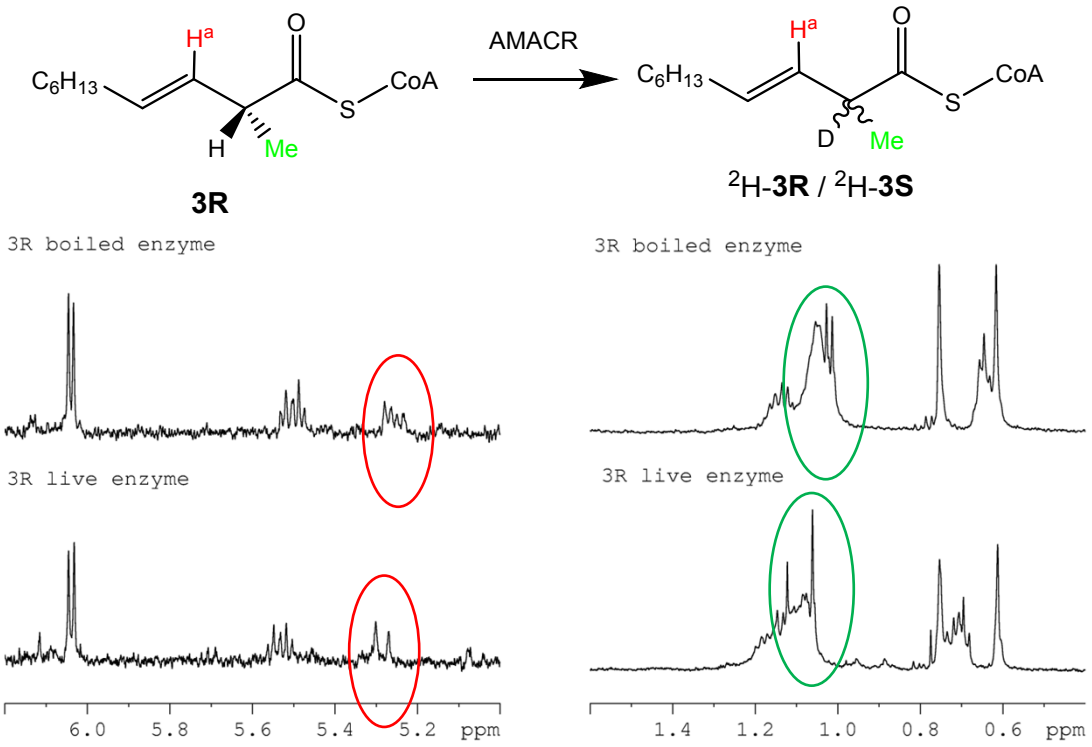

Figure S1. ***3R*** shows exchange of the α-proton in with live AMACR in ^2H_2O. This results in the doublet of the methyl group at 1.04 ppm becoming a singlet (outlined in green) and the concomitant conversion of the doublet of doublets for H^a at 5.29 ppm into a doublet (outlined in red). As exchange is an obligatory step in chiral inversion, it is highly likely that racemization also occurs. An identical reaction occurred upon incubation of ***3S*** with AMACR under identical conditions (data not shown).