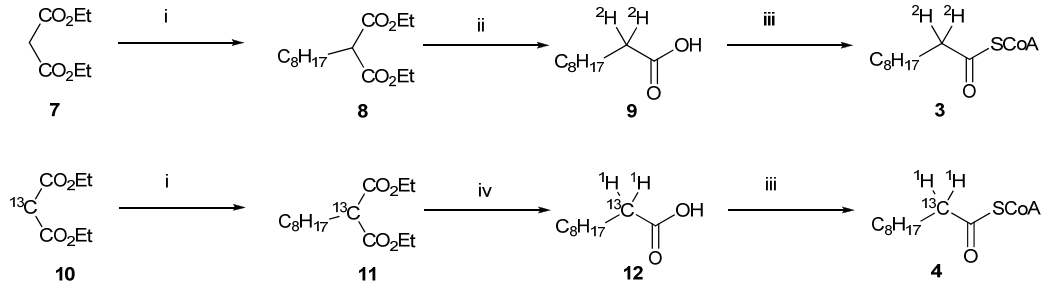
Synthesis of substrates

2,2-[$^2H_2$]-Decanoyl-CoA (**3**) was synthesised using an extension of the method previously described by us.[1] Thus, diethyl malonate **7** (Scheme 1) was deprotonated and the resulting anion alkylated with 1-bromooctane to give the mono-substituted malonate **8**. Prolonged treatment with boiling concentrated ^{2}HCl in ^2H_2O exchanged the α-proton with deuterium, hydrolysed the ethyl esters and caused decarboxylation of the intermediate 2-^{2}H-2-octylmalonic acid to give the required di-deuterated decanoic acid **9**. This was then coupled with CoA using ethyl chloroformate by our previously reported method[1] to provide the corresponding CoA ester **3**.

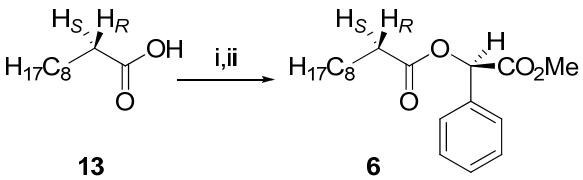
The required ^{13}C-decanoyl-CoA substrate **4** was synthesised by analogy to the method for the dideuterio compound **3** above. Thus, reaction of the anion derived from diethyl 2-[^{13}C]-malonate **10** with 1-bromooctane gave **11**. Hydrolysis of the esters and decarboxylation with boiling aq. ^{1}HCl, furnished the labeled acid **12** in 54% overall yield. Coupling with CoA gave the desired substrate **4** (Scheme 1).



Scheme 1. Syntheses of 2,2-[$^2H_2$]-decanoyl CoA **3** and of 2-[^{13}C]-decanoyl CoA **4**. *Reagents and yields*: i, NaH, 1-bromooctane, DMF, 52% (**8**), 59% (**11**)[1]; ii, 35% ^{2}HCl / ^2H_2O, reflux, 8 d, 51%; iii, EtOCOCl, Et_3N, CoASH[1]; iv, 35% ^{1}HCl / ^1H_2O, reflux, 90%.

Stereochemical analysis of reaction products

Decanoyl-CoA **2** was incubated with active enzyme until *ca.* 60% exchange of the α-protons with deuterium had occurred. Following quenching of the reaction, the CoA ester was hydrolysed under alkaline conditions. The reaction mixture was then acidified and the decanoic acid isotopomeric mixture **13** was extracted into dichloromethane. The carboxylic acids were converted into their acid chlorides by treatment with oxalyl chloride; these acid chlorides were then used to acylate the free OH group of *R*-2-mandelate methyl ester to form the diastereomeric decanoyl esters **6**.



Scheme 2. Synthesis and structure of methyl *O*-decanoyl-*R*-mandelate **6**. *Reagents and yields*: i, $(COCl)_2$, DMF, CH_2Cl_2; ii, methyl *R*-mandelate, Et_3N, DMAP, CH_2Cl_2, 81% over 2 steps.

Experimental

General: All chemicals were obtained from the Sigma-Aldrich Chemical Co. or Fisher Ltd. and were used without further purification, unless otherwise specified. Experiments were conducted at ambient temperature, unless noted otherwise. Organic extracts were dried over $MgSO_4$ and filtered. Solvents were evaporated under reduced pressure. Melting points are uncorrected. NMR spectra were obtained on JEOL Eclipse Delta (270 MHz), Varian Mercury (400 MHz) or Varian Inova (600 MHz) spectrometers, in $CDCl_3$ solution, except where noted. Coupling constants (*J*) are reported in Hz to the nearest 0.5 Hz. Decanoyl-CoA, octanoyl-CoA, butanoyl-CoA and acetyl-CoA were purchased as their Li_3 salts from Larodan Fine Chemicals AB, Malmö, Sweden. 2-Methylpropanoyl-CoA, pentanoyl-CoA, hexanoyl-CoA, heptanoyl-CoA, labeled decanoyl-CoA and *S*-2-methyldecanoyl-CoA esters were synthesised from their corresponding acids by the reported method.[1] Recombinant human AMACR 1A was purified as described.[1]

Ethyl 2-ethoxycarbonyldecanoate (8). Diethyl malonate **7** (2.00 g, 12.5 mmol) was treated with NaH (275 mg, 6.87 mmol) and 1-bromooctane (1.20 g, 6.25 mmol) in dry DMF (50 mL), as previously described for the 2-[^{13}C] isotopomer,[1] to give **8** (890 mg, 52%) as a colourless oil: δ_H 0.72 (3 H, t, *J* = 7.0, 10-CH_3), 1.00-1.25 (18 H, m, 6 × CH_2 + 2 × CH_3), 1.76 (2 H, m, 3-CH_2), 3.17 (1 H, t, *J* = 7.4, 2-CH); 4.06 (4 H, q, *J* = 7.0, 2 × OCH_2).

2-[$^2H_2$]-Decanoic acid (9). Diester **8** (890 mg, 3.27 mmol) was treated with 35% ^2HCl / ^2H_2O (10 mL) at reflux for 8 d. Work-up as for the 2-[$^2H_2$]-2-[^{13}C] isotopomer[1] gave **9** (289 mg, 51%) as a colourless oil: δ_H 0.86 (3 H, t, *J* = 6.6, 10-CH_3), 1.20-1.40 (12 H, m, 6 × CH_2), 1.60 (2 H, m, 3-H_2); MS (ES^-) *m/z* 174.1544 (M – H) ($^{13}C_1{}^{12}C_9{}^2H_2{}^1H_{17}{}^{16}O_2$ requires 174.1545), 173.1510 (100%; M – H) ($^{12}C_{10}{}^2H_2{}^1H_{17}{}^{16}O_2$ requires 173.1511).

2-[^{13}C]-Decanoic acid (12). Ethyl 2-[^{13}C]-2-ethoxycarbonyldecanoate **11**[1] (1.00 g, 3.7 mmol) was treated with aq. HCl, as for the synthesis of the 2-[$^2H_2$]-2-[^{13}C] isotopomer,[1] to give **12** (570 mg, 90%) as a colourless oil: ^{1}H NMR δ_H 0.87 (3 H, t, *J* 6.8, 10-CH_3), 1.27 (12 H, m, 6 × CH_2), 1.60 (2 H, m, 3-H_2), 2.40 (2 H, dt, *J* = 135.2, 7.4 Hz, 2-$^{13}CH_2$); ^{13}C NMR δ_C 14.08, 22.64, 24.66 (d, *J* = 33.8, 3-C), 29.04 (4-C), 29.25 (d, *J* = 33.8, 5-C), 29.37 (C-6), 31.84, 33.89 (d, *J* = 55.2, 2-C for 1,2-$^{13}C_2$ isotopomer), 33.90 (enhanced, 2-C), 33.90 (d, *J* = 34.5, 2-C for 2,3-$^{13}C_2$ isotopomer), 179.28 (d, *J* = 55.2, 1-C); MS (ES^-) *m/z* 174.1476 (0.7%; M – H) ($^{12}C_7{}^{13}C_3H_{19}O_2$ requires 174.1486), 173.1453 (8.8%; M – H) ($^{12}C_8{}^{13}C_2H_{19}O_2$ requires 173.1453), 172.1419 (100%; M – H) ($^{12}C_9{}^{13}C_1H_{19}O_2$ requires 172.1419).

Methyl *R*-2-(decanoyloxy)phenylacetate (6). Decanoic acid (400 mg, 2.32 mmol) was treated with oxalyl chloride (590 mg, 4.64 mmol) and DMF (20 μL) in CH_2Cl_2 (5.0 mL). The evaporation residue, in CH_2Cl_2 (5.0 mL) was stirred with methyl *R*-2-hydroxyphenylacetate (385 mg, 2.32 mmol), Et_3N (470 mg, 4.64 mmol) and 4-dimethylaminopyridine (1.0 mg) under N_2 for 16 h. Washing (aq. citric acid, brine), drying ($MgSO_4$) and evaporation gave **6** (625 mg, 81%) as a pale yellow oil: δ_H 0.85 (3 H, t, J = 6.8, decanoyl 10-CH_3), 1.23-1.34 (12 H, m, 6 × CH_2), 1.63-1.68 (2 H, m, decanoyl 3-H_2); 2.38-2.47 (2 H, m, decanoyl 2-H_2); 3.70 (3 H, s, OMe); 5.90 (1 H, s, $CHCO_2Me$); 7.23-7.45 (5 H, m, Ph-H_5). Irradiation at δ 1.65 resolved the peaks at δ 2.28-2.37 into two doublets at δ 2.30 and δ 2.37, with ^2J = 15 Hz.

Enzyme assays: Assays were conducted in a final volume of 0.7 mL in buffer as previously described,[1] using purified recombinant AMACR 1A (54 μg, 1.15 nmol). Incubations were at 30°C and the reactions were terminated at the required time by heating at 50°C for 10 min. Incubations of acyl-CoAs used the following substrates (**Fig. 3** of main paper): decanoyl-CoA **2**, octanoyl-CoA, heptanoyl-CoA, hexanoyl-CoA, pentanoyl-CoA, butanoyl-CoA, acetyl-CoA and 2-methylpropanoyl-CoA were performed in buffer containing ^2H_2O and were analysed directly by ^1H NMR at 400 or 600 MHz. Incubations of 2-[^{13}C]-decanoyl-CoA **4** were performed in buffer containing ^2H_2O and were analysed directly by ^{13}C NMR at 100.6 MHz. Incubations of 2,2'-[$^2H_2$]-decanoyl-CoA **3** were performed in buffer containing ^1H_2O and were analysed by ^1H NMR; ^2H_2O to *ca.* 10% (v/v) was added following termination of the reaction before analysis. All data is based on two replicants of each substrate concentration and substrate conversions were corrected for non-enzymatic exchange levels in negative controls. Concentrations of acyl-CoA esters were determined by UV-visible spectroscopy, using ε_{260} = 16 mM^{-1} cm^{-1}.[2]

Kinetic analyses used six different concentrations of substrate which were incubated for 1 h (*S*-2-methyldecanoyl-CoA **1*S***) or 4 h (decanoyl-CoA **2**). Data was corrected for non-enzymatic exchange levels in negative controls, and analysed using SigmaPlot 10.0.1 with enzyme kinetics module 1.3 and parameters estimated using the direct linear plot.[3, 4] The kinetic parameter k_{cat} was calculated assuming a molecular mass of 47146.8 Da for AMACR.[1] Parameters are also calculated using the Michaelis-Menten plot and are report ± standard error.

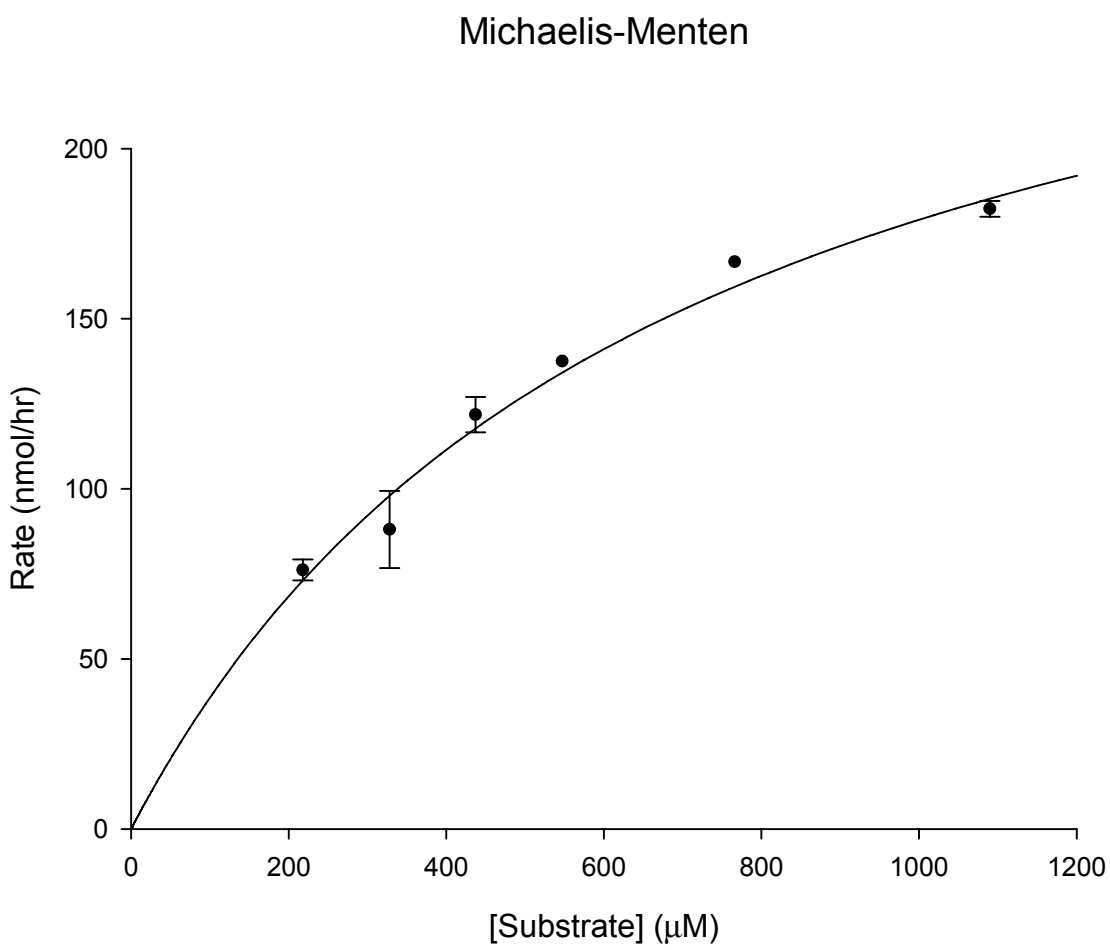
Determination of configuration of deuterated enzymatic products. Decanoyl-CoA **2** was exchanged using AMACR to *ca.* 60% conversion as described above. The products were hydrolysed (NaOH) and acidified[1] and the exchanged acids **13** were extracted (CH_2Cl_2) and dried ($MgSO_4$). The evaporation residue (*ca*. 500 μg, 2.90 μmol) was dissolved in CH_2Cl_2 (25 μL) and

treated with oxalyl chloride (2.45 μL, 29 μmol) and DMF (0.5 μL) for 6 h under N_2. The evaporation residue, in $CDCl_3$ (90 μL), was treated with 4-dimethylaminopyridine (DMAP) (90 μg, 700 nmol), methyl *R*-2-hydroxyphenylacetate (methyl *R*-mandelate) (480 μg, 2.90 μmol) and Et_3N (800 nL, 5.8 μmol) under N_2 for 16 h. The mixture was washed (aq. citric acid), dried ($MgSO_4$) and diluted to 700 μL with $CDCl_3$ for 600 MHz NMR analysis.[5] The signal at δ 1.65 (3-CH_2) was irradiated during NMR analysis to decouple and simplify the signals in the α-CH_2 region.

References

1. D. Darley, D. S. Butler, S. J. Prideaux, T. W. Thornton, A. D. Wilson, T. J. Woodman, M. D. Threadgill and M. D. Lloyd, *Org. Biomol. Chem.*, 2009, **7**, 543-552.
2. I. Rudik and C. Thorpe, *Arch. Biochem. Biophys.*, 2001, **392**, 341-348.
3. A. Cornish-Bowden and R. Eisenthal, *Biochem. J.*, 1974, **139**, 721-730.
4. R. Eisenthal and A. Cornish-Bowden, *Biochem. J.*, 1974, **139**, 715-720.
5. D. Parker, *J. Chem. Soc.-Perkin Trans. 2*, 1983, 83-88.

Enzyme kinetic data for *S*-2-methyldecanoyl-CoA 1*S*



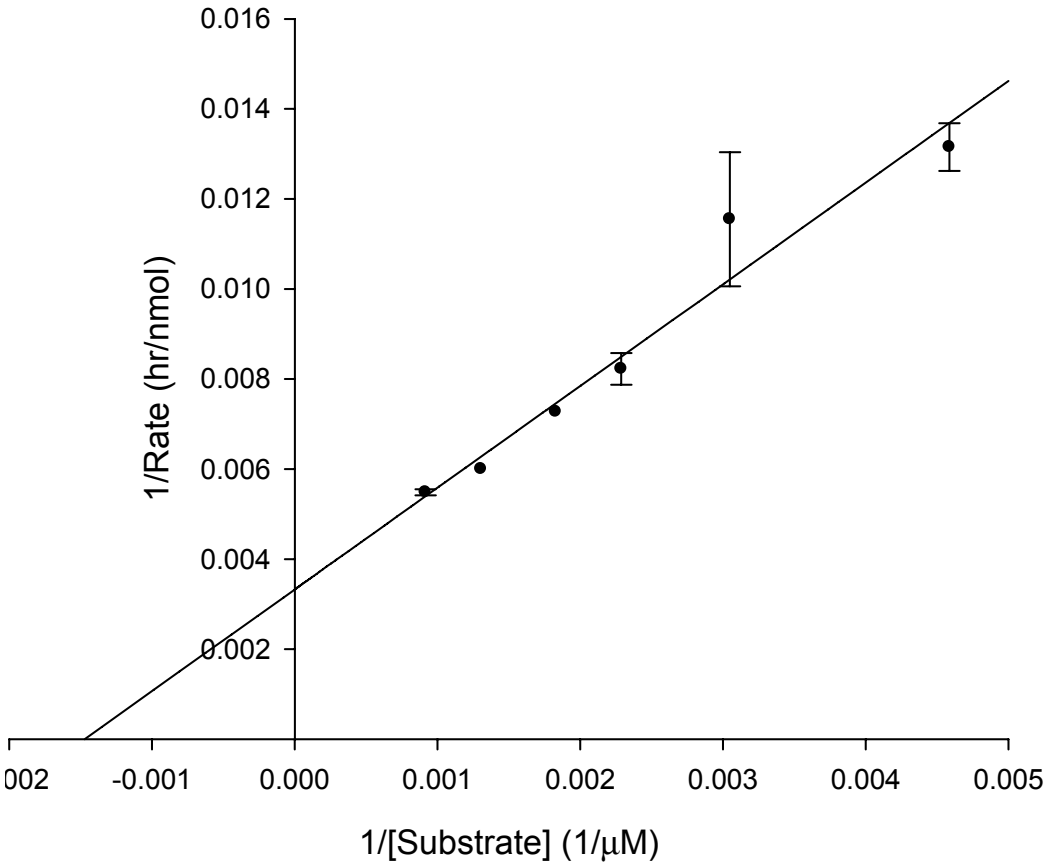
Apparent K_m = 680 ± 120 μM

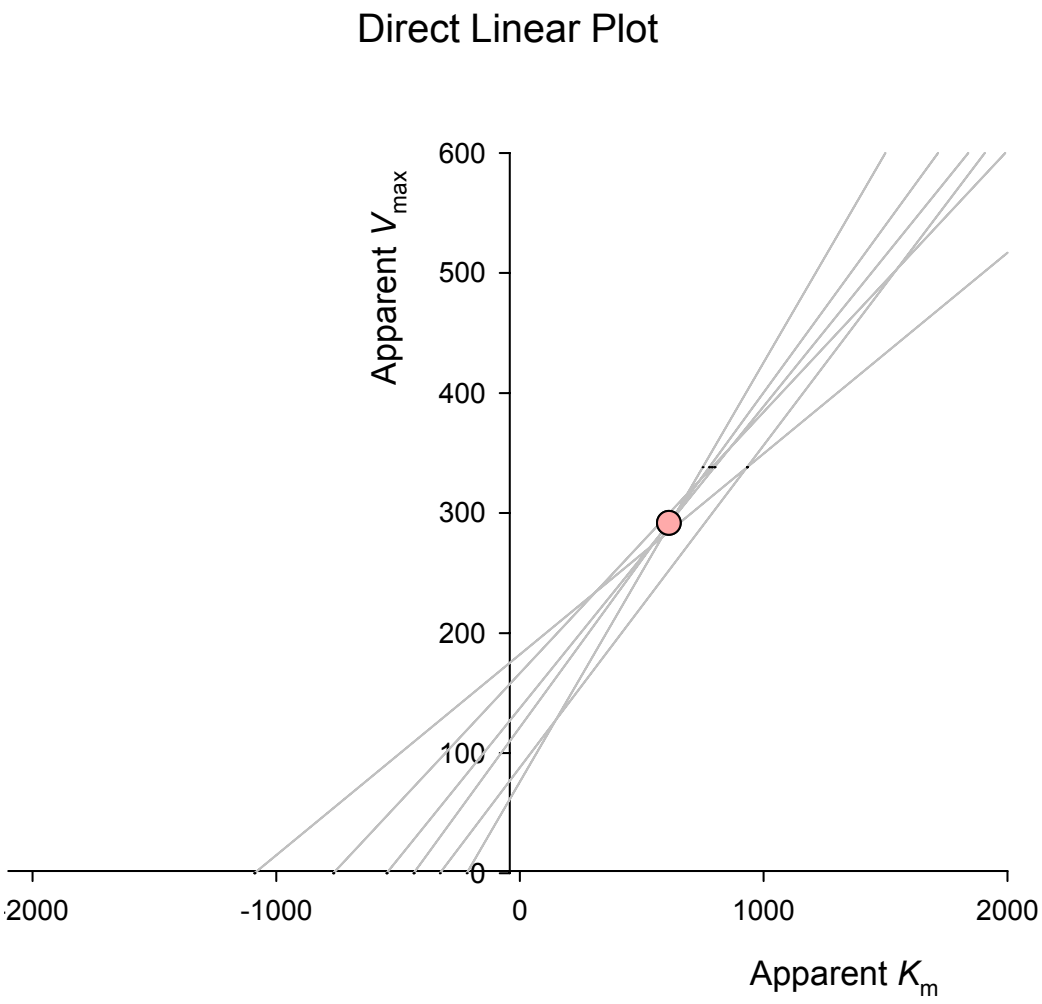
Apparent V_{max} = 301 ± 28 nmol/hr = 5.01 ± 0.46 nmol/min = 91.98 ± 8.45 nmol/min/mg

Apparent k_{cat} = 72.48 ± 6.66 x 10^{-3} s^{-1}

Apparent k_{cat}/K_m = 106.58 M^{-1} s^{-1}

0.054 mg (1.15 nmoles) enzyme used per assay.


Lineweaver-Burk
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
1/Rate (hr/nmol)
002
-0.001
0.000
0.001
0.002
0.003
0.004
0.005
1/[Substrate] (1/μM)



Figures based on whole data set

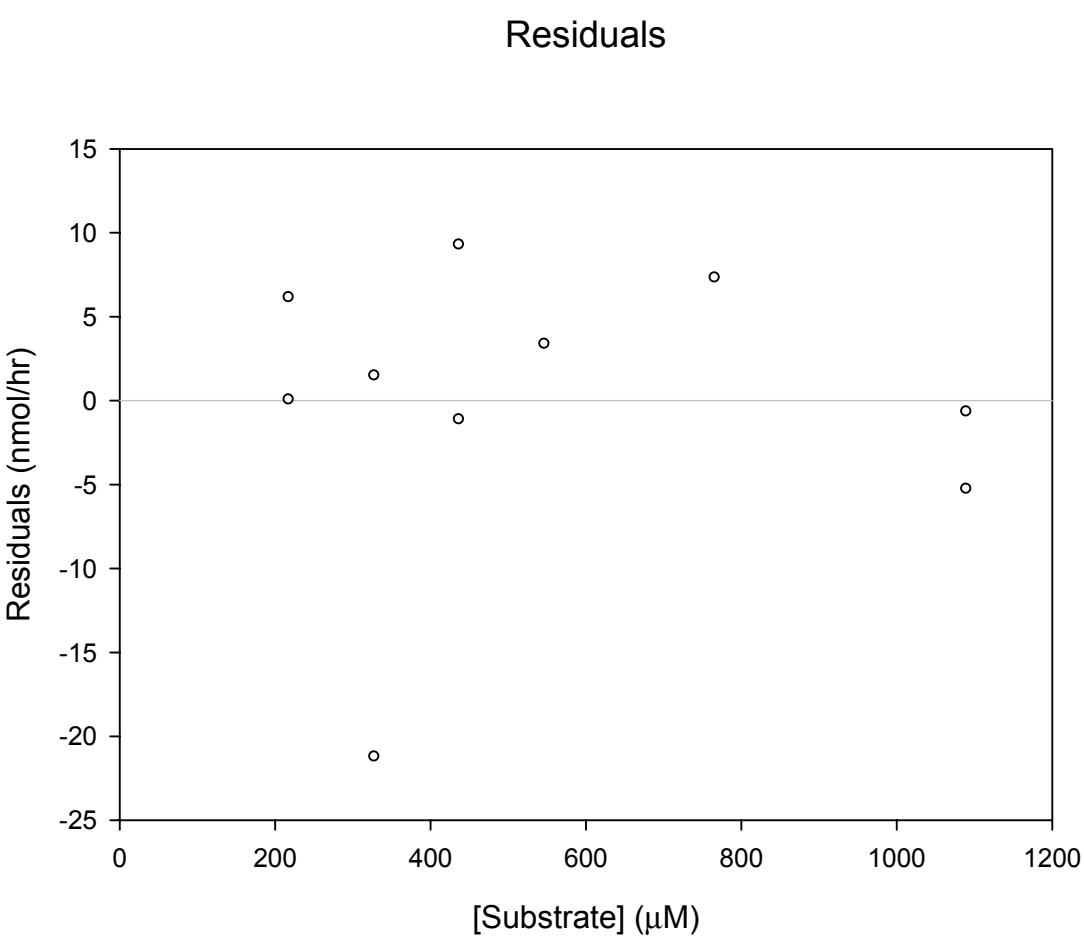
Apparent K_m = 615 μM

Apparent V_{max} = 290.9 nmol/hr = 4.84 nmol/min = 88.96 nmol/min/mg

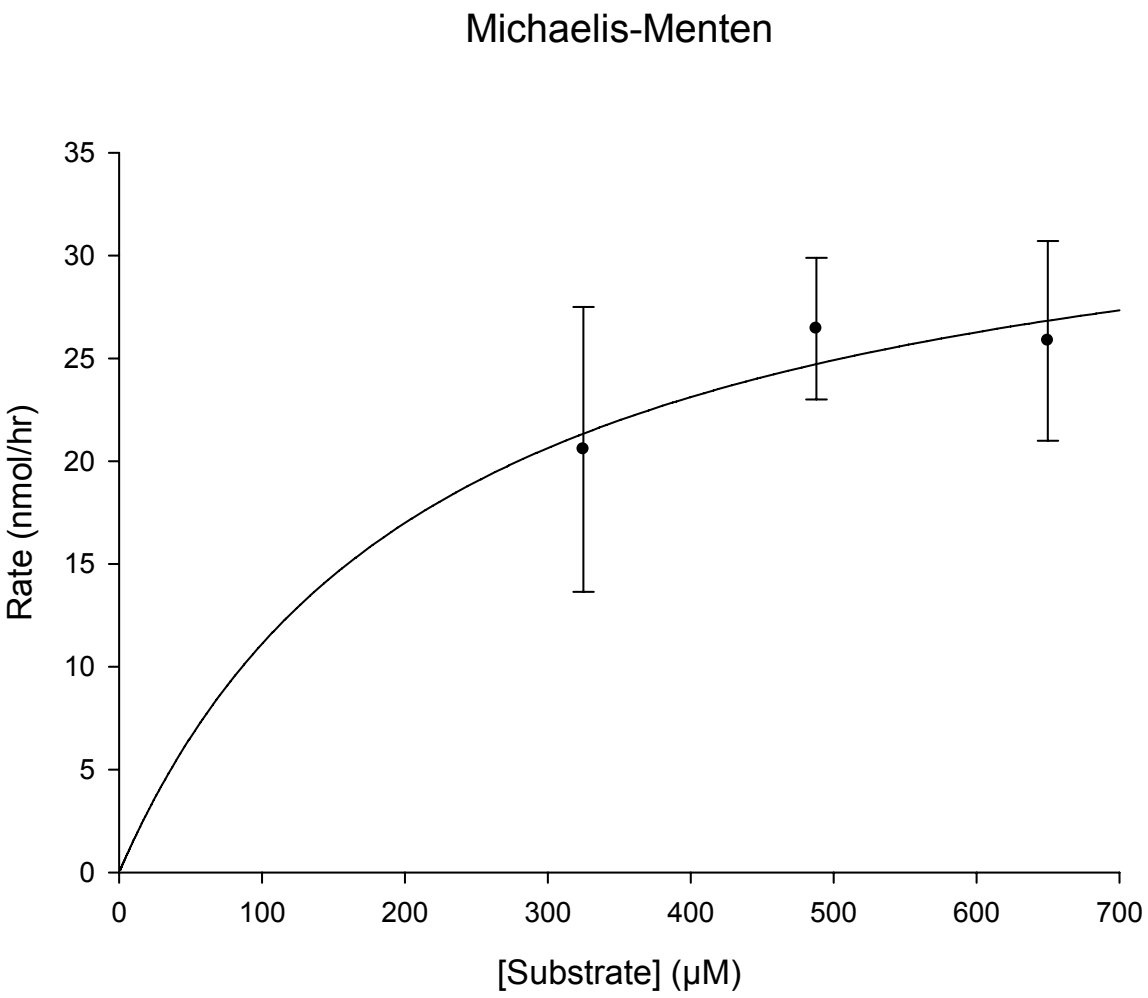
Apparent k_{cat} = 70.1 x 10^{-3} s^{-1}

Apparent k_{cat}/K_m = 114 M^{-1} s^{-1}

0.054 mg (1.15 nmoles) enzyme used per assay.


Residuals
15
10
5
0
-5
-10
-15
-20
-25
Residuals (nmol/hr)
0
200
400
600
800
1000
1200
[Substrate] (μM)

Decanoyl-CoA 2, 325-650 μM substrate



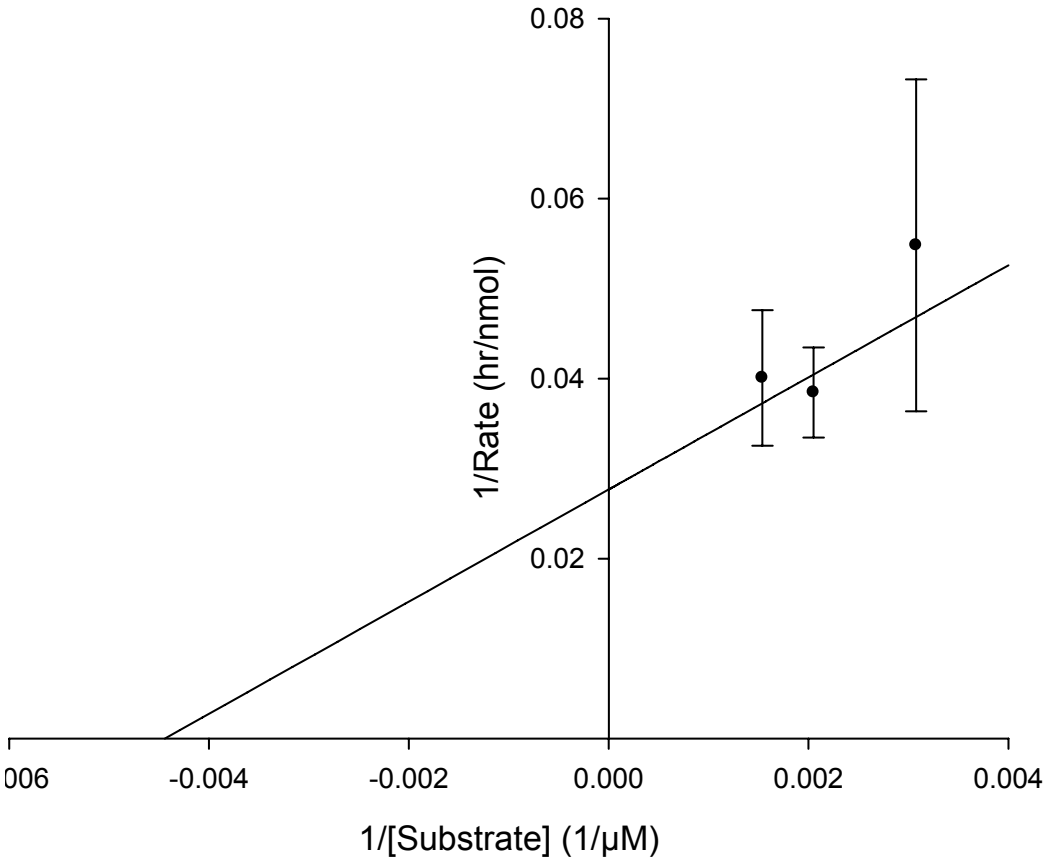
Apparent K_m = 225 ± 415 μM

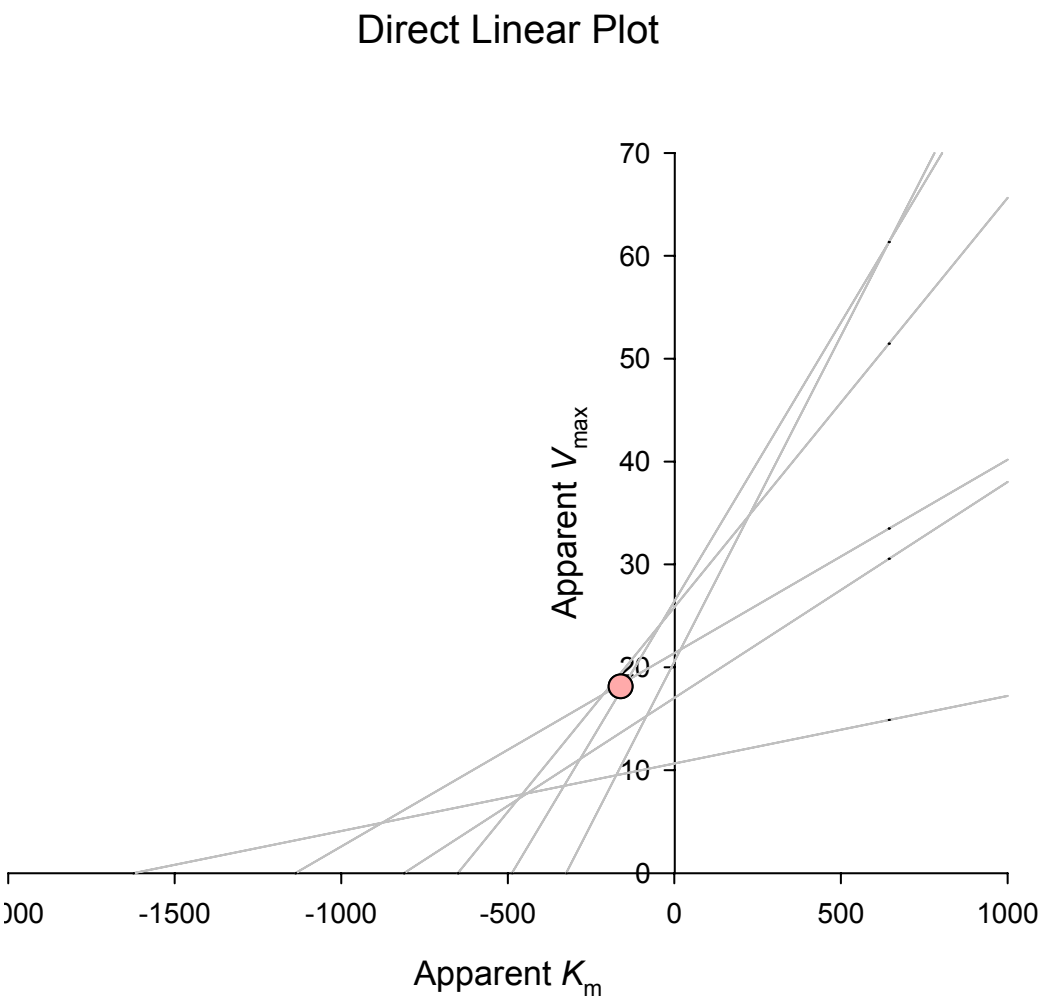
Apparent V_{max} = 36.12 ± 21.45 nmol/hr = 0.601 ± 0.356 nmol/min = 10.63 ± 6.31 nmol/min/mg

Apparent k_{cat} = 8.71 ± 5.17 x 10^{-3} s^{-1}

Apparent k_{cat}/K_m = 38.71 M^{-1} s^{-1}

0.054 mg (1.15 nmoles) enzyme used per assay.


Lineweaver-Burk
0.08
0.06
0.04
0.02
1/Rate (hr/nmol)
006
-0.004
-0.002
0.000
0.002
0.004
1/[Substrate] (1/μM)



Plot above is for whole data set, 325-1625 µM

Estimates of Km and Vmax based on rates obtained at 325, 488 and 650 µM substrate only.

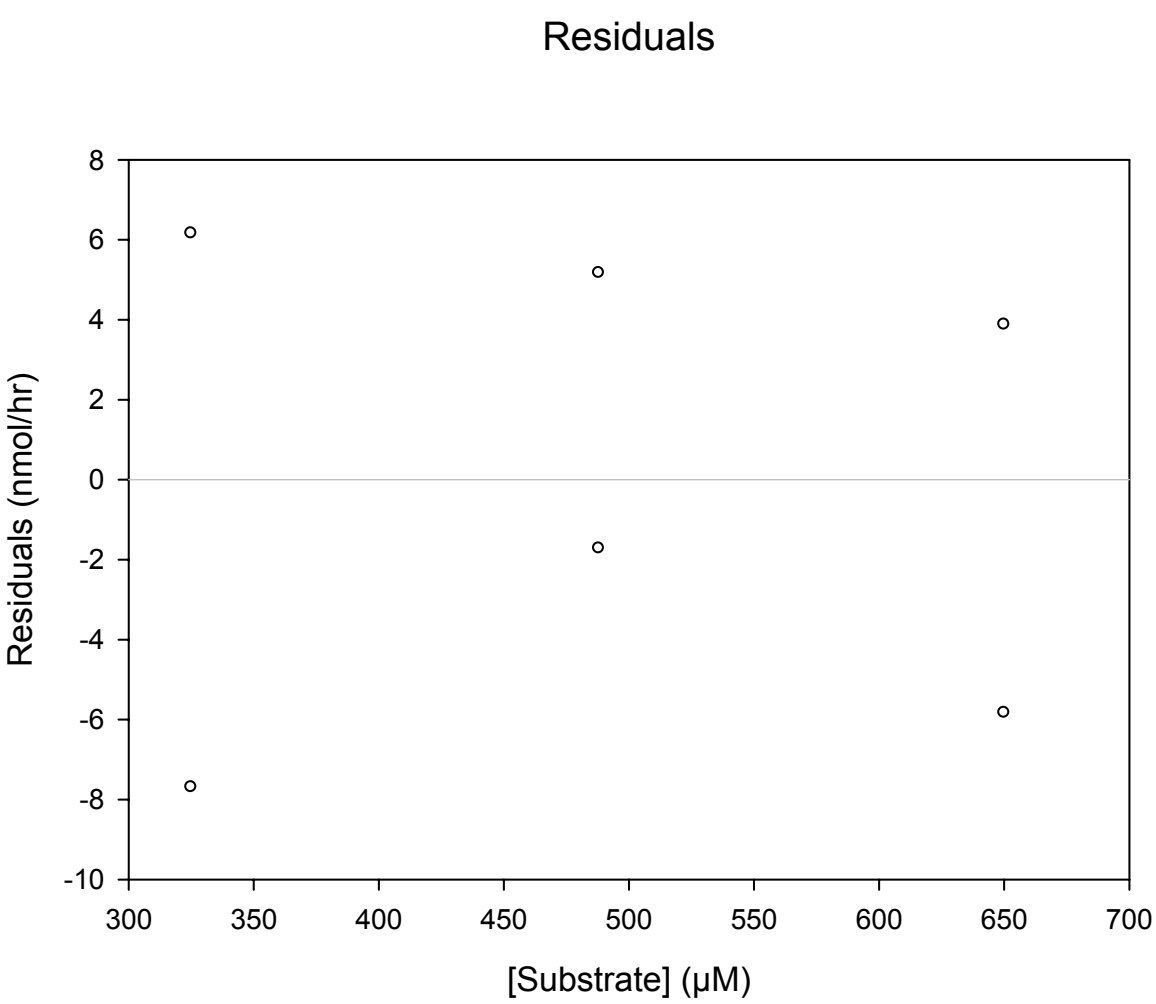
Apparent K_m = 224 µM

Apparent V_{max} = 34.78 nmol/hr = 0.579 nmol/min = 10.63 nmol/min/mg

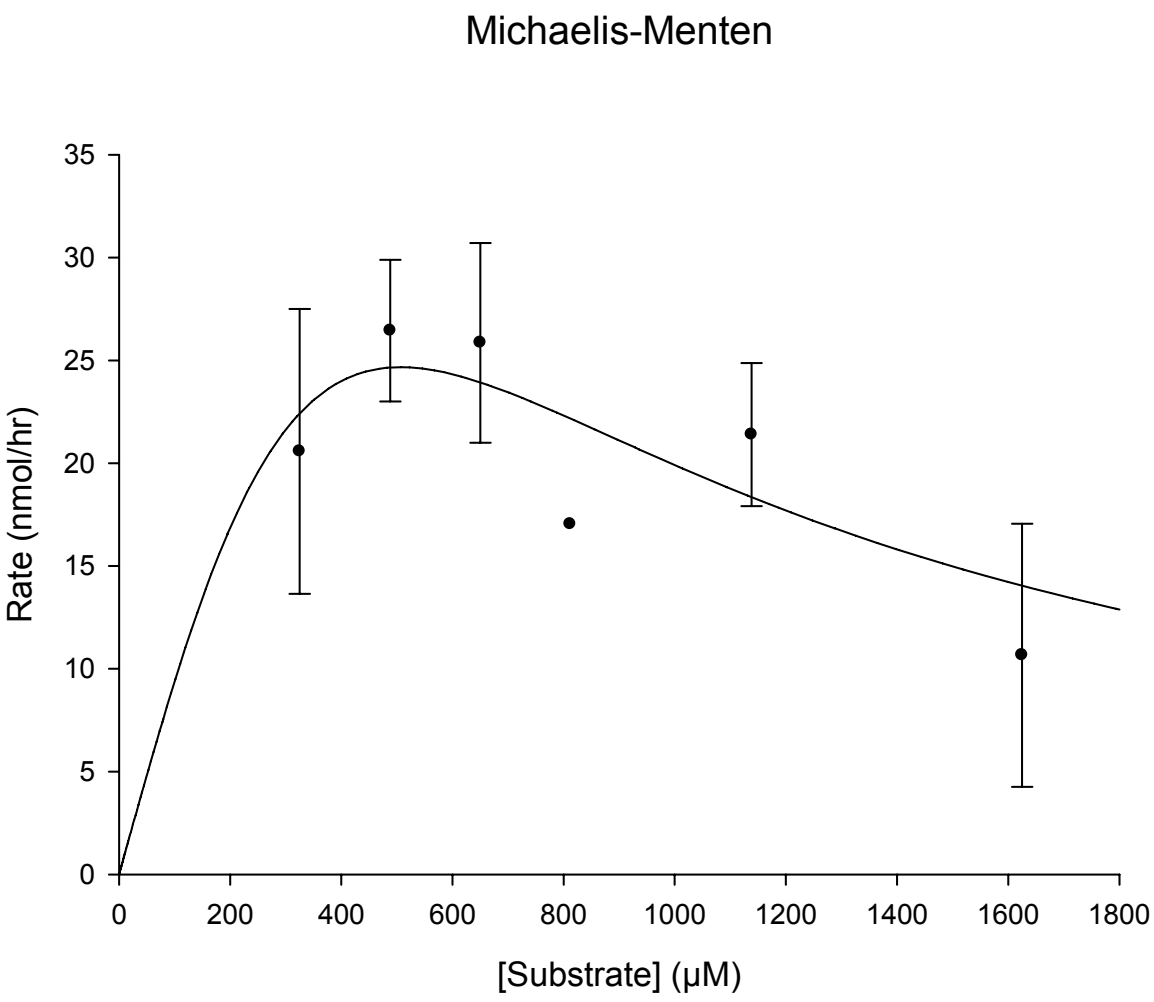
Apparent k_{cat} = 8.39 x 10^{-3} s^{-1}

Apparent k_{cat}/K_m = 37.46 M^{-1} s^{-1}

0.054 mg (1.15 nmoles) enzyme used per assay.


Residuals
Residuals (nmol/hr)
8
6
4
2
0
-2
-4
-6
-8
-10
300
350
400
450
500
550
600
650
700
[Substrate] (μM)

Decanoyl-CoA, 325 – 1625 μM substrate showing non-competitive substrate inhibition.



Vmax = 12520 nmol/hr
Km = 128400 μM
Ki = 2 μM

Equation used to fit data was for non-competitive substrate inhibition:

$$v = V_{max}\,[S]/K_m + [S] + [S]^2/K_i$$

Where v = rate, V_{max} = maximum rate, K_m = Michaelis constant, [S] = substrate concentration, and K_i = inhibition constant. Clearly the obtained values for kinetic parameters are unreasonable despite graphical evidence for non-competitive substrate inhibition (above). Inspection of direct linear plot shows deviation from Michaelis-Menten kinetics when considering all substrate concentrations.