Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Information

A novel colorimetric assay for AMACR utilizing the elimination of 2,4-dinitrophenolate

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Abbreviations used: AMACR, α-methylacyl-CoA racemase (P504S); CDI, carbonyldiimidazole; CoA-SH, reduced coenzyme A; DCM, dichloromethane; DMSO, dimethylsulfoxide; HRMS, high resolution mass spectrometry; PE, petroleum ether; SDM, standard deviation of the mean; SPE, solid phase extraction; THF, tetrahydrofuran.

Characterization of reaction and optimization of assay conditions

Assay conditions were optimized in half-volume 96-well plates in 100 µL of aq. 50 mM NaH_2PO_4-NaOH, pH 7.4. Enzyme (25 µL; 4 x stock solution) and additives (25 µL, 4 x stock solution) or buffer (positive control) were incubated together at room temperature. The assay was initiated by addition of substrate **6** (50 µL, 80 µM; 2 x stock solution) and the reaction was followed for at least 8 minutes at 354 nm. Reaction rates were determined by plotting change in absorbance against time in the linear phase, and rates in $nmol.min^{-1}$ or $nmol.min^{-1}.mg^{-1}$ were calculated as described in experimental methods.

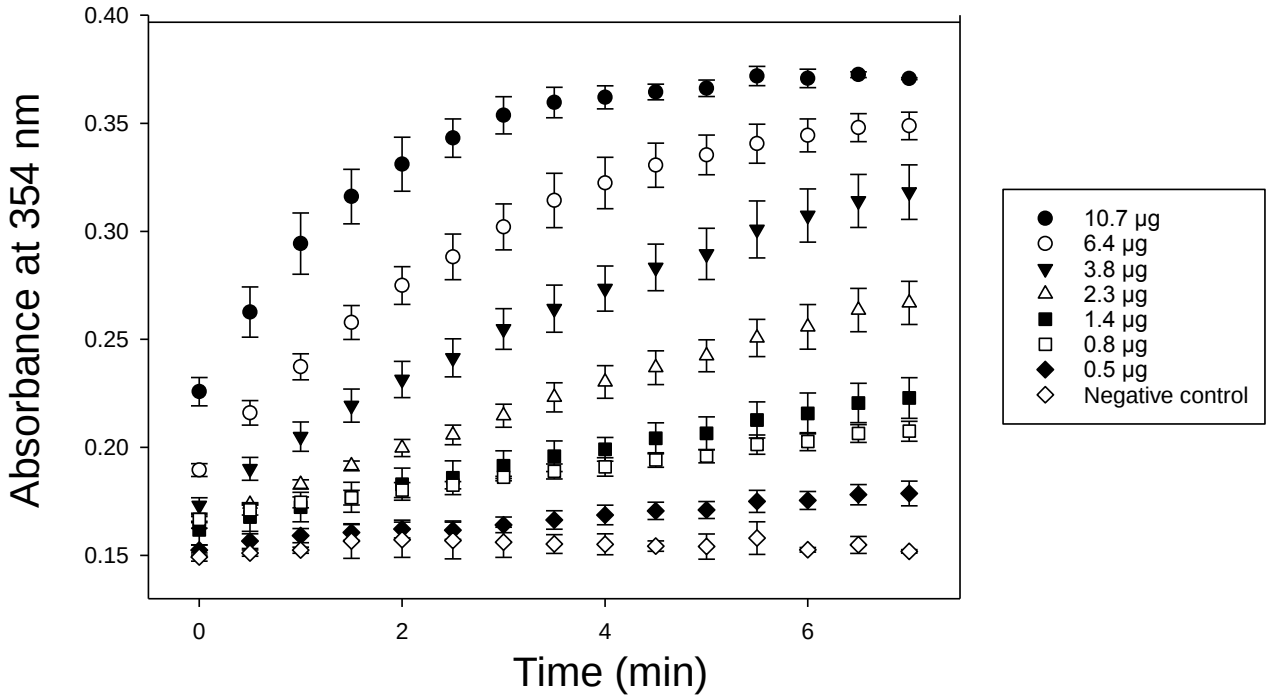


Figure S1: Reaction time course with different amounts of enzyme followed at 354 nm. Reaction was performed by mixing enzyme stock solution containing different amounts of enzyme and buffer, before adding 2 x substrate stock. The amount of enzyme per well is given in the legend. Negative control is buffer in place of active enzyme. Data are means ± SDM (n = 3).

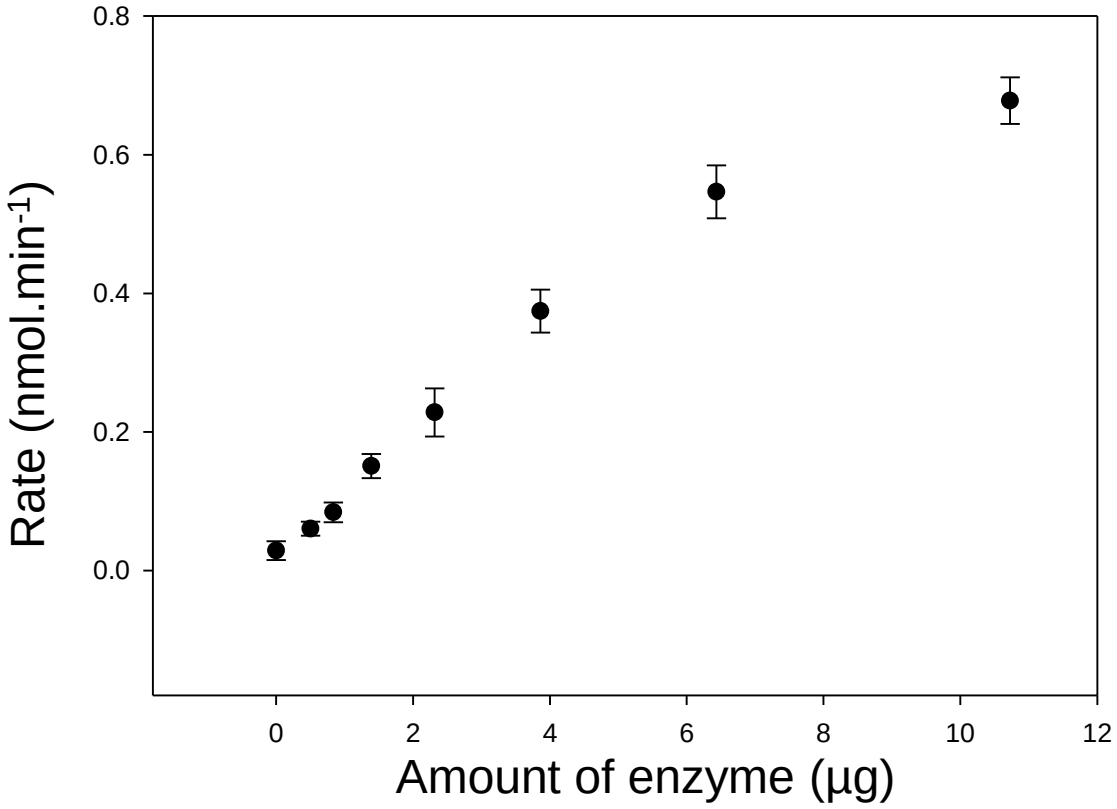


Figure S2: Reaction rate as a function of amount of recombinant human AMACR 1A. Rates were derived by plotting the change of absorbance over a 2 minute time-course. Data are means ± SDM (n = 3).

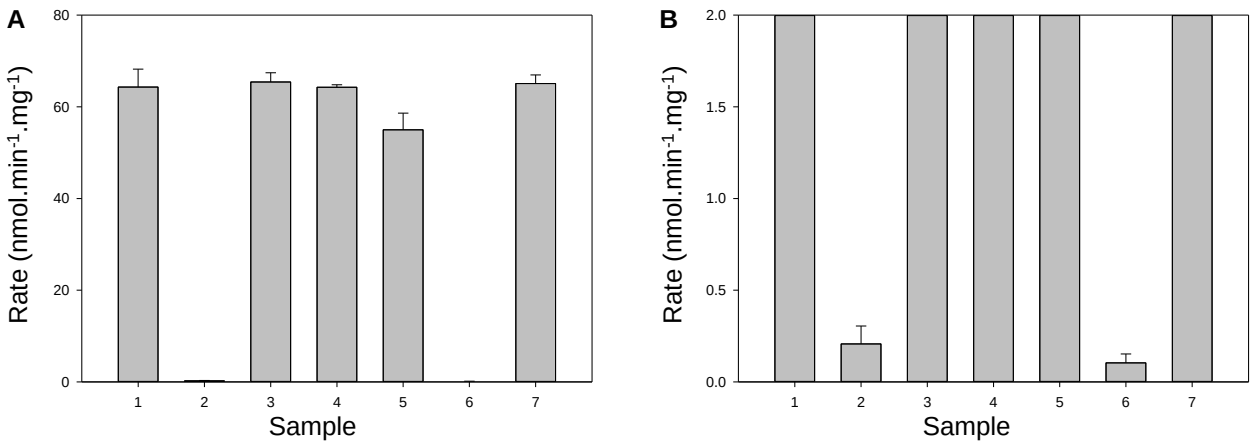


Fig. S3. Optimization of assay conditions. Assays were performed in a final volume of 100 µL in 50 mM NaH_2PO_4-NaOH, pH 7.4, 2 µM recombinant AMACR and 40 µM substrate **6** at 30ºC under the following conditions: 1) Positive control; 2) Negative control (heat-inactivated enzyme); 3) 5 mM 2-mercaptoethanol; 4) 1 mM dithiothreitol; 5) 0.1% (v/v) Triton X-100 (~1.69 mM); 6) 1.5% (w/v) *N*-lauroyl-sarcosine; 7) 0.02 $mg.mL^{-1}$ bovine serum albumin. Activities are means ± SDM (n = 3 repeats). A. Activities in the presence of various agents; B. Expansion of Y-axis of A showing 1.5% (w/v) *N*-lauroyl-sarcosine (sample 6) reduces enzyme activity to a similar level to heat-inactivated enzyme negative control (sample 2).

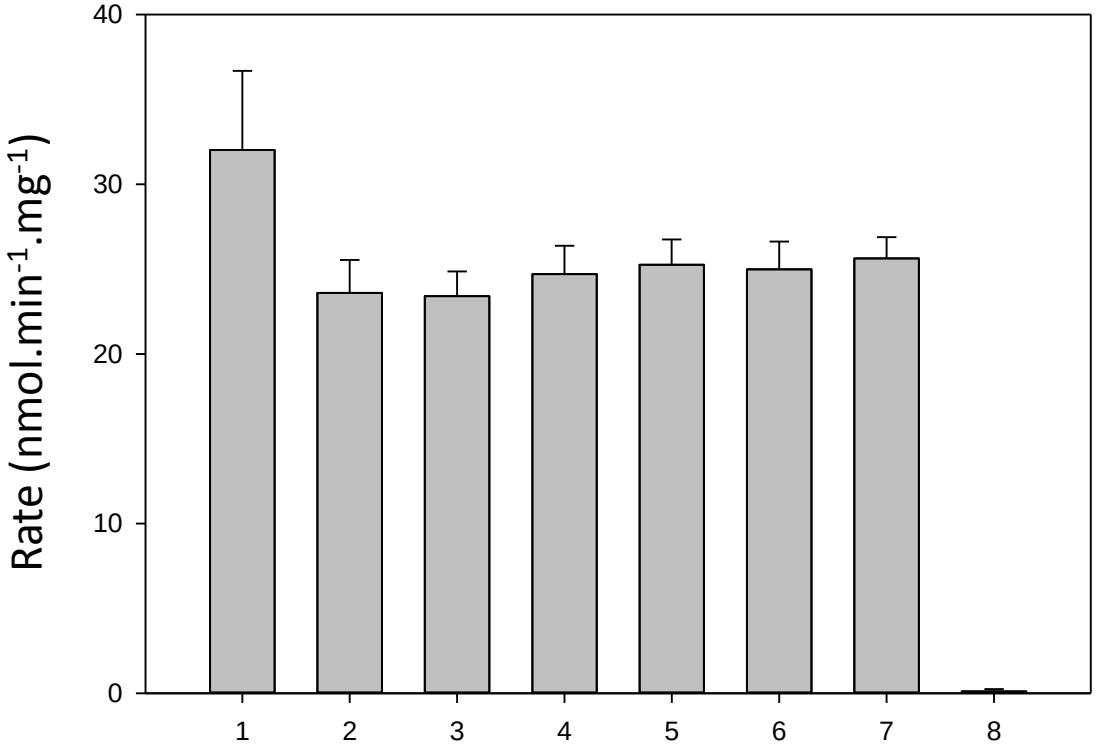


Fig S4: Effect of different Triton X-100 concentrations (v/v) on enzyme activity. 1. Positive control (active enzyme); 2. 0.25%; 3. 0.125%; 4. 0.0625%; 5. 0.03125%; 6. 0.015625%; 7. 0.0078125%; 8. negative control (heat-inactivated enzyme). Activities are means ± SDM (n = 3 repeats).

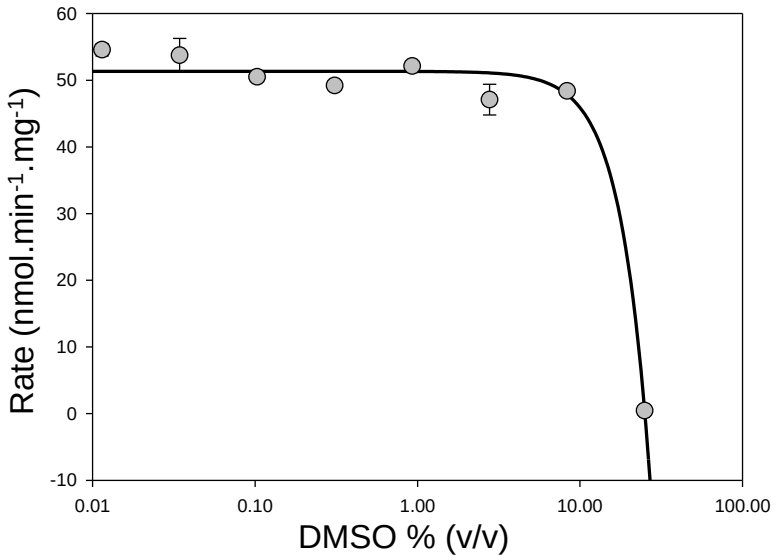


Fig. S5. Effect of DMSO on activity. Assays were performed in half-volume plates in a final volume of 100 µL in 50 mM aq. NaH_2PO_4-NaOH, pH 7.4, 2 µM total recombinant AMACR protein and 40 µM substrate **6** at 30ºC in the presence of variable DMSO concentrations [as % (v/v)]. Activities are means ± SDM (n = 3 repeats).

Experimental

Sources of materials

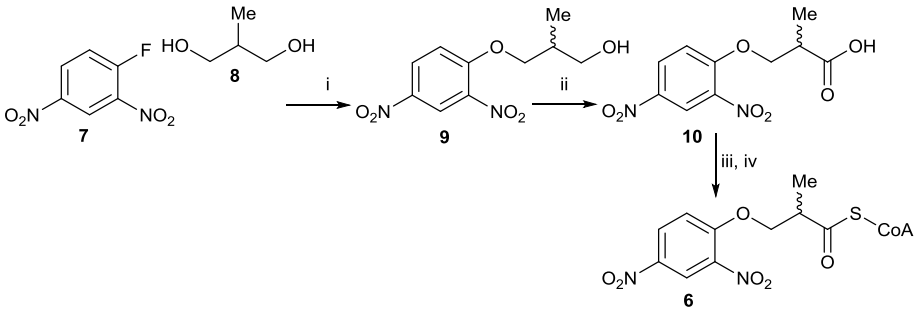
Chemicals were purchased from the Sigma-Aldrich Chemical Co. or Fisher Scientific Ltd., unless otherwise stated and were used without further purification. Reduced coenzyme A, *tri*-lithium salt was purchased from Calbiochem. *N*-Dodecyl-*N*-methylcarbamoyl-CoA **1**[1] and ibuprofenoyl-CoA **2**[2] were synthesized as previously described. Ebselen **12** and Ebselen oxide **13** were purchased from Cayman Chemical.

General Experimental Procedures

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 sodium phosphate and NaOH at the required proportion for aq. 50 mM pH 7.4 buffer. Citric acid buffer was prepared from citric acid and NaOH at the required proportion for 0.8 M pH 4.0 buffer. The pH of aqueous solutions was measured using a Corning 240 pH meter and Corning general purpose combination electrode. The pH meter was calibrated using Fisher Chemicals standard buffer solutions (pH 4.0 - phthalate, 7.0 - phosphate, and 10.0 - borate) at either pH 7.0 and 10.0 or 7.0 and 4.0. Calibration and measurements were carried out at ambient room temperature. 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, DMSO d-6 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 NMR signals 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 ^{1}H NMR. Mass spectra were recorded by ESI TOF at the University of Bath Mass Spectrometry Service. High resolution mass spectra were recorded in ES mode. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. Syntheses were carried out at ambient temperature, unless otherwise specified. Solutions in organic solvents were dried over anhydrous magnesium sulfate and evaporated under reduced pressure. Aqueous solutions for biological experiments were prepared in Nanopure water of 18.2 $M\Omega.cm^{-1}$ quality and were pH-adjusted with aq. HCl or NaOH.

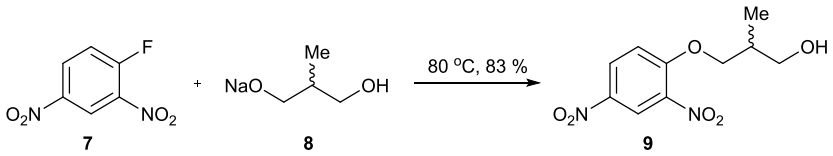
Synthesis of 2*R*,*S*-3-(2,4-dinitrophenoxy)-2-methylpropanoyl-CoA substrate 6

Racemic substrate **6** was synthesized by reaction of 1-fluoro-2,4-dinitrobenzene **7** (Sanger's reagent) with 2-methylpropan-1,3-diol **8** under basic conditions. Treatment of the alcohol product **9** with CrO_3 and H_2SO_4 resulted in the desired acid **10**. This was coupled with CoA-SH using our published procedure[1-4] to give **6**.



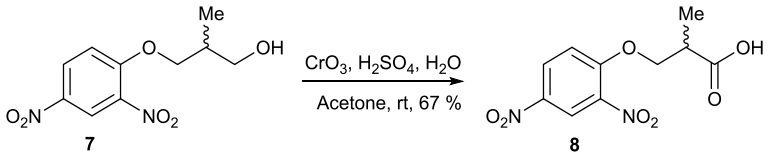
Scheme S1| Synthesis of substrate 6. *Reagents & conditions*: i. Na metal, 83%; ii. CrO_3, conc. H_2SO_4, acetone, 67%; iii. Carbonyldiimidazole, CH_2Cl_2; iv. CoA-SH $(Li^+)_3$, 0.1 M $NaHCO_{3\ aq.}$ / THF (1:1).

Synthesis of 2*R*,*S*-3-(2,4-dinitrophenoxy)-2-methylpropan-1-ol **9**



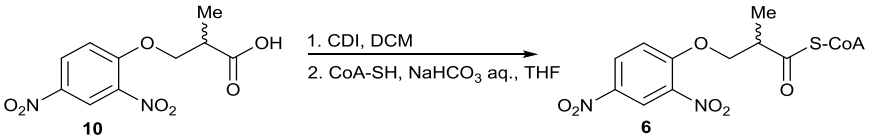
Sodium (141 mg, 6.12 mmol, 1.0 eq.) was added to 2-methyl-1,3-propanediol **8** (2.538 g, 28.16 mmol, 4.6 eq.) and the mixture was stirred for 6 h at ambient temperature. 1-Fluoro-2,4-dinitrobenzene **7** (1.252 g, 6.73 mmol, 1.1 eq.) was added and the reaction mixture was stirred at 80 °C for 2 h and then at ambient temperature overnight. The reaction mixture was extracted with Et_2O (3 × 20 mL) and the combined organic layers were washed with ice-cold water (10 mL), dried ($MgSO_4$) and filtered. The solvent was evaporated. The residue was purified by column chromatography (PE:EtOAc 1:1) to give **9** (1.30 g, 83 %) as a yellow solid: mp 62-64 °C; ^{1}H NMR (400.04 MHz, $CDCl_3$): δ 8.78 (1 H, d, *J* = 2.7 Hz), 8.43 (1 H, dd, *J* = 9.3, 2.7 Hz), 7.24 (d, *J* = 9.3 Hz), 4.29-4.19 (2 H, m), 3.83-3.64 (2 H, m), 2.36-2.21 (1 H, m), 1.82-1.71 (1 H, m), 1.09 (3 H, d, *J* = 7.0 Hz); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ 156.9, 139.9, 138.5, 129.3, 122.1, 114.3, 72.8, 64.4, 35.4, 13.4; IR (KBr disc): 3320, 3240 cm^{-1}; HRMS (m/z): $[M + Na]^+$ calcd. for $C_{10}H_{12}N_2NaO_6$, 279.0593; found, 279.0569; UV-visible spectrum (EtOH) 294 nm (ε = 13.9 mM^{-1} cm^{-1}).

Synthesis of 2*R*,*S*-3-(2,4-dinitrophenoxy)-2-methylpropanoic acid **10**



H_2O (1.4 mL) and conc. H_2SO_4 (0.65 mL) was added to a round-bottom flask containing CrO_3 (756 mg, 7.56 mmol, 1.9 eq.). The mixture was stirred at ambient temperature for 5 min, then **9** (1.02 g, 3.98 mmol, 1.0 eq.) in acetone (18 mL) was added dropwise over 1.5 h. The reaction mixture was stirred for 2 h. Propan-2-ol (0.3 mL) was added and the reaction mixture was stirred overnight. The reaction mixture was filtered (Celite). The Celite was washed with CH_2Cl_2 (20 mL) and the combined filtrates were washed with water (2 × 20 mL). The combined organic layers were washed with brine (20 mL), dried ($MgSO_4$) and filtered. The solvent was evaporated. The residue was purified by column chromatography (PE:EtOAc 1:1) to give **10** (726 mg, 67 %) as an ivory solid: mp 141-143 °C; ^{1}H NMR (500.13 MHz, $(CD_3)_2SO$): δ 12.54 (1 H, s), 8.77 (1 H, d, *J* = 2.8 Hz), 8.51 (1 H, dd, *J* = 9.3, 2.8 Hz), 7.65 (1 H, d, *J* = 9.3 Hz), 4.47-4.38 (2 H, m), 2.95-2.86 (1 H, m), 1.21 (3 H, d, *J* = 7.2 Hz); ^{13}C NMR (125.77 MHz, $(CD_3)_2SO$): δ 174.9, 156.1, 140.2, 139.0, 129.8, 121.6, 116.2, 72.4, 39.0, 13.8; IR (KBr disc): 3084, 1697 cm^{-1}; HRMS (m/z): $[M + Na]^+$ calcd. for $C_{10}H_{10}N_2NaO_7$, 293.0386; found, 293.0358; [M - H]- calcd. for $C_{10}H_9N_2O_7$, 269.0410; found, 269.0410; UV-visible spectrum (EtOH) 294 nm (ε = 12.1 mM^{-1} cm^{-1}).

Synthesis of 2*R*,*S*-3-(2,4-dinitrophenoxy)-2-methylpropanoyl-CoA **6**



Acid **10** (100 mg, 0.37 mmol, 1.0 eq.) was dissolved in dry CH_2Cl_2 (7.0 mL), then *N*,*N*'-carbonyldiimidazole (120 mg, 0.74 mmol, 2.0 eq.) was added and the reaction mixture was stirred at ambient temperature for 1 h. The reaction mixture was washed with H_2O (5 × 7 mL), dried over $MgSO_4$ and filtered. The solvent was evaporated. The residue was dissolved in THF (7 mL), then CoA-SH tri-lithium salt (145 mg, 0.19 mmol, 0.5 eq.) in aq. $NaHCO_3$ (7 mL, 0.1 M) was added and the reaction mixture was stirred at ambient temperature overnight. The reaction mixture was acidified to *ca.* pH 3 with aq. HCl (1.0 M) and the THF was evaporated. The resulting solution was washed with EtOAc (5 × 7 mL) and the crude product was purified by SPE to give **6** (100 mg) as a colourless solid: ^{1}H NMR (500.13 MHz, D_2O): δ 8.74-8.70 (1 H, m), 8.60-8.54 (1 H, m), 8.41 (1 H, dd, *J* = 9.4,

2.8 Hz), 8.35 (1 H, s), 7.36 (1 H, d, *J* = 9.4 Hz), 6.10 (1 H, d, *J* = 5.3 Hz), 4.43-4.33 (1 H, m), 3.79 (1 H, dd, *J* = 9.7, 4.8 Hz), 3.52 (1 H, dd, *J* = 9.7, 4.4 Hz), 3.34 (2 H, t, *J* = 6.6 Hz), 3.30-3.24 (4 H, m), 2.98 (2 H, t, *J* = 6.4 Hz), 2.33 (2 H, t, *J* = 6.6 Hz), 1.21 (3 H, d, *J* = 7.0 Hz, CH3), 0.86 (3 H, s), 0.73 (3 H, s); HRMS (m/z): $[M - H]^{2-}$ calcd. for $C_{31}H_{42}N_9O_{22}P_3S$, 508.5689; found, 508.5703; UV-visible spectrum (H_2O) 259 nm (ε = 15.97 mM^{-1} cm^{-1}), 298 nm (ε = 7.74 mM^{-1} cm^{-1}).

Enzyme assays

Human recombinant AMACR 1A was expressed from the pET30 Ek/LIC plasmid[5] in Rosetta2 (DE3 cells) using Lennox LB media supplemented with kanamycin sulfate (30 $\mu g.mL^{-1}$), chloramphenicol (32 $\mu g.mL^{-1}$) and auto-induction express system 1 (Novagen). Cultures (0.5 L) were grown at 28 ºC and 220 r.p.m. overnight. Enzyme was purified by metal-chelate chromatography as previously described.[1,3,4]

Conversion of **6** (100 µM) by AMACR was determined in ^1H NMR assays as previously described.[1-5] Negative controls contained heat-inactivated enzyme or buffer in place of enzyme. Colorimetric assays were performed in 200 µL final volume in a 96-well plate. The buffer was 50 mM NaH_2PO_4-NaOH, pH 7.4. Recombinant human AMACR 1A (*ca.* 8 µg) was incubated with buffer and any additives (100 µL) for 10 minutes. The assay was initiated by addition of 2 x stock solution of **6** in buffer (80 µM; 100 µL) and the absorbance was monitored at 354 nm for up to 15 minutes at 30ºC using a BMG Labtech FLUOstar Omega plate reader with Omega software. Rates in $\Delta Absorbance.min^{-1}$ were determined using Excel and converted to $nmol.min.^{-1}mg^{-1}$ using the 2,4-dinitrophenolate **5** extinction coefficient (15,300 M^{-1} cm^{-1})[6] with the path-length (0.588 cm) determined by the plate-reader.

Kinetic parameters for **6** were determined in half-volume 96 well plates in 100 µL final volume. Final concentrations of **6** were 9.4, 14.2, 21.4, 32, 48, 72, 108 and 162 µM and ~2 µM total AMACR protein was used. Comparison of k_{cat}/K_m values for ibuprofenoyl-CoA **2** used as a substrate for recombinant human AMACR from *E. coli*[2,5] and HEK cells[7] suggest the concentration of active enzyme in these assays is ~1 nM. Kinetic parameters for substrate and inhibitors were determined using SigmaPlot 13 and median values derived from the Direct Linear Plot[8,9] are reported in the paper. Kinetic plots are reported in this Supplementary Information (Figs. S7C and S8C). The reported rates are those calculated using total protein in the sample. The limit of detection (LOD) is calculated assuming a threshold rate of twice that observed with negative controls containing heat-inactivated enzyme, approximately 3 standard deviations difference.

Characterization of inhibitors

Dose response curves were used to determine IC_{50} values for inhibitors. Enzyme (4 x stock, 150 µL) and inhibitor at the appropriate concentration (4 x stock, 150 µL) were incubated together in 96 well plates at ambient room temperature for 10 minutes. The sample was divided into three repeats of 100 µL before addition of substrate (3 x 100 µL; 40 µM in the assay) and monitoring the reaction as above. Final concentrations of inhibitor in the assay were 100, 33.3, 11.1, 3.7, 1.23, 0.411, 0.137 and 0.045 µM in most cases. Positive controls contained enzyme in buffer and substrate **6** and negative controls buffer and substrate. In some cases 2-3% (v/v) DMSO was included in assays; no significant change in enzyme activity was observed with DMSO concentrations of up to 8% (v/v) (See optimization of assay conditions above). In some cases half-volume 96 well plates were used; identical IC_{50} values were obtained for standard inhibitors using both types of microtitre plate. IC_{50} values were determined using reaction rate, with the data fitted to a 4-parameter programme using SigmaPlot 13 using inhibitor concentration (in µM).

Values of K_i were determined using variable substrate **6** concentrations (same as above) in the presence or absence of 247 nM **2**[10] or 0.418 nM **1**,[7] with rates in the presence of inhibitor compared to no inhibitor controls. Inhibition mode was assigned based on ranking of solutions and visual inspection of plots. Covalent inhibition by **13** was characterized by Kitz-Wilson analysis[11] with inhibitor **13** at 0, 0.9, 1.3, 3, 6.7 and 10 µM incubated with enzyme. Residual activity was determined by dilution of enzyme with 40 µM substrate in buffer after 0, 2, 4, 6, 8 and 10 minutes. Rates were determined by plotting absorbance at 354 nm against time in Excel. First-order rate constants were obtained by plotting ln (activity/initial activity) *vs*. time in SigmaPlot 13. The second-order rate constant ± SE was determined by plotting the derived first-order rate constants ± SE against the concentration of **13** incubated with enzyme (before dilution and addition of substrate in buffer) and fitting of a straight line in SigmaPlot 13.

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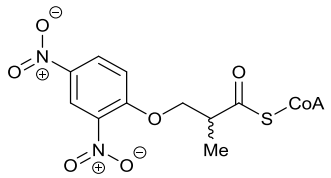
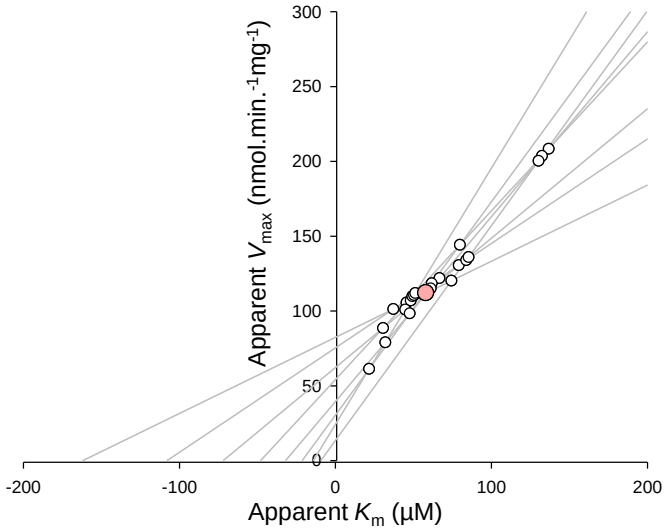
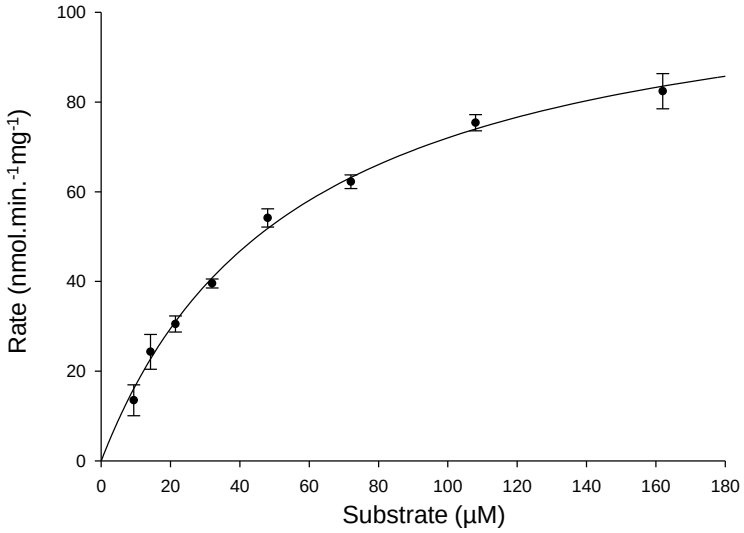
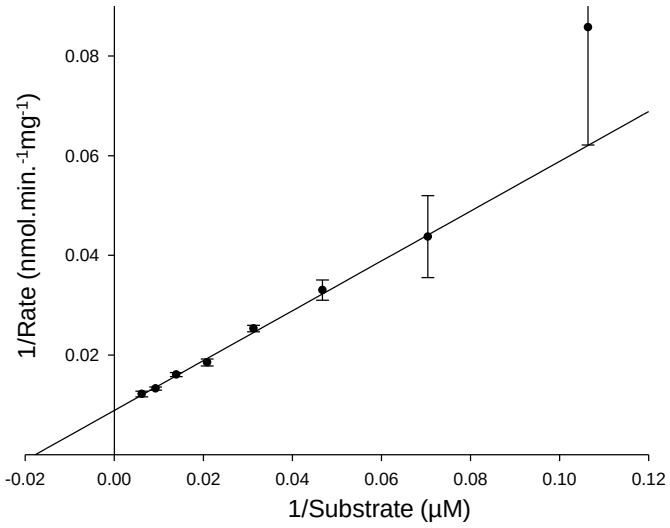
Fig. S6: Kinetic Plots for substrate 6 with recombinant human AMACR 1A

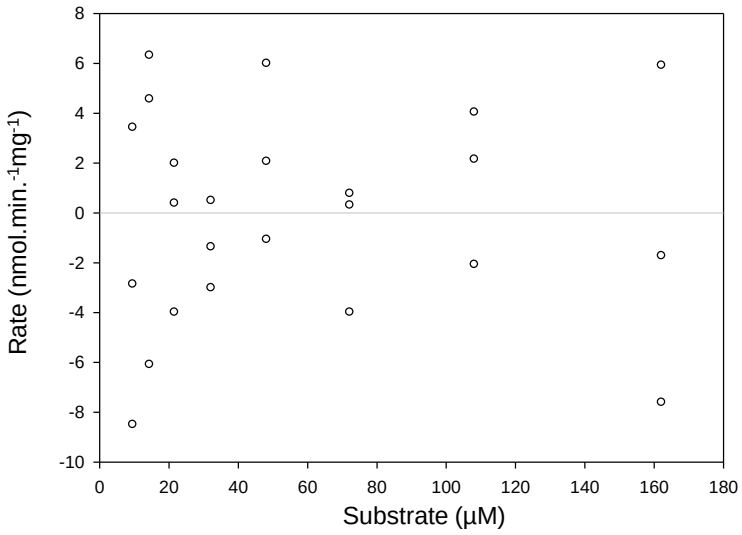




K_m = 57.89 µM; V_{max} = 112.2 nmol.min.$^{-1}$mg^{-1}







Enzyme Kinetics Nonlinear Fit Results

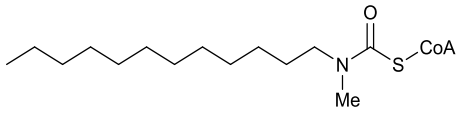
Parameters

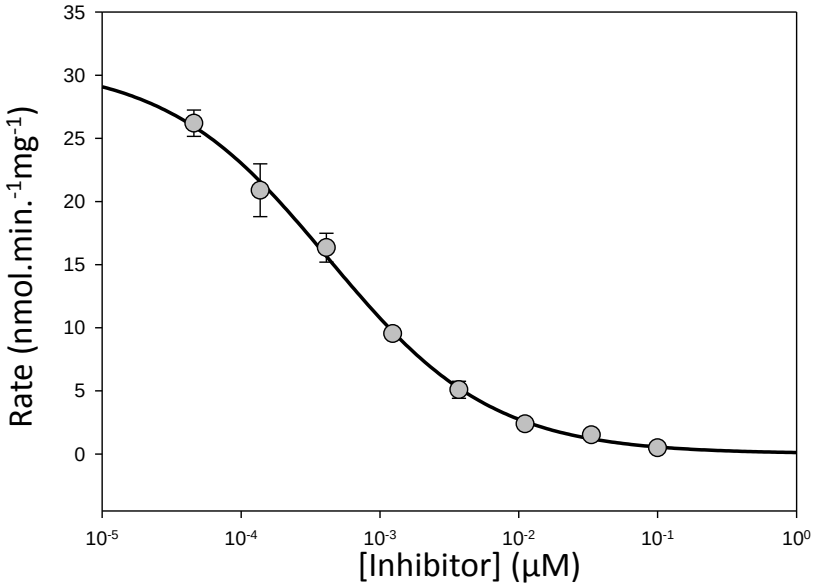
	Value	±Std. Error	95% Conf. Interval		
V_{max} (nmol.min.$^{-1}$mg^{-1})	112.5595	5.2162	101.7416	to	123.3774
K_m (µM)	56.2603	5.9297	43.9625	to	68.5580

Goodness of Fit

Degrees of Freedom	22
AICc	74.733
R^2	0.970
Sum of Squares	400.193
Sy.x	4.265
Runs Test p Value	0.350

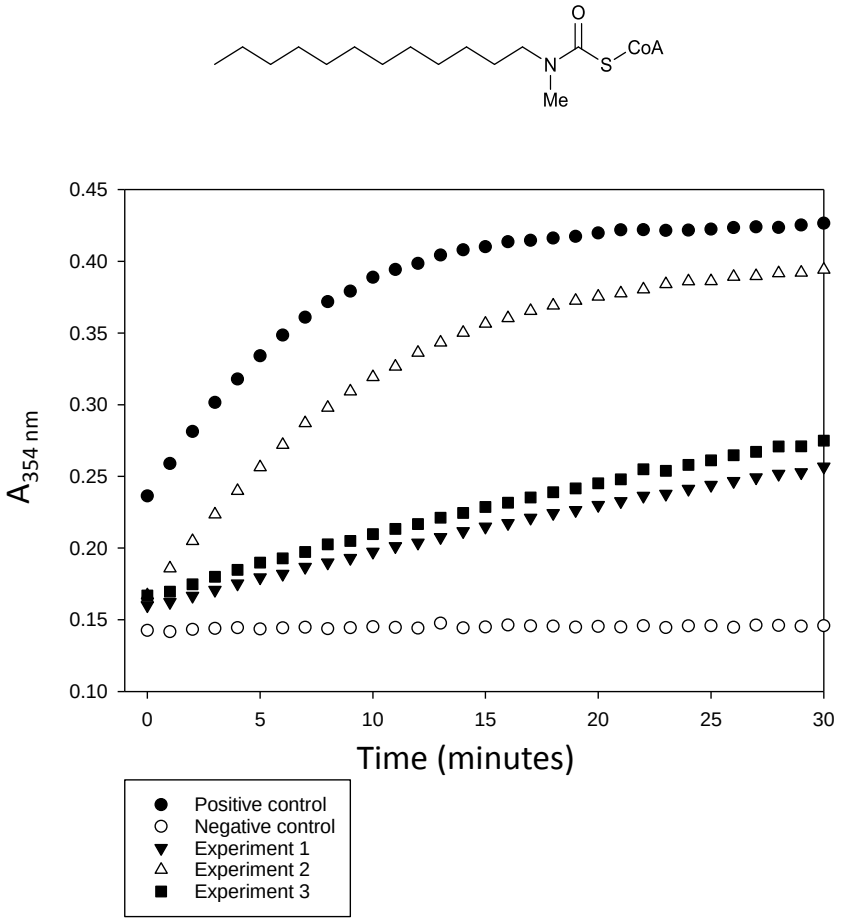
Fig. S7A: Dose-response curve with *N*-dodecyl-*N*-methylcarbamoyl-CoA 1





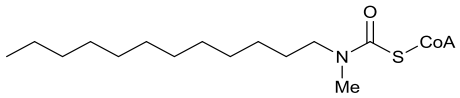
	Coefficient	Std. Error	t	P
Min (nmol.min^{-1}.mg^{-1})	0.0235	0.6158	0.0381	0.9700
Max (nmol.min^{-1}.mg^{-1})	30.8953	2.1498	14.3715	<0.0001
IC_{50} (µM)	0.0004	9.0499 x10^{-5}	4.7218	0.0001
Hillslope	-0.7398	0.0913	-8.1010	<0.0001

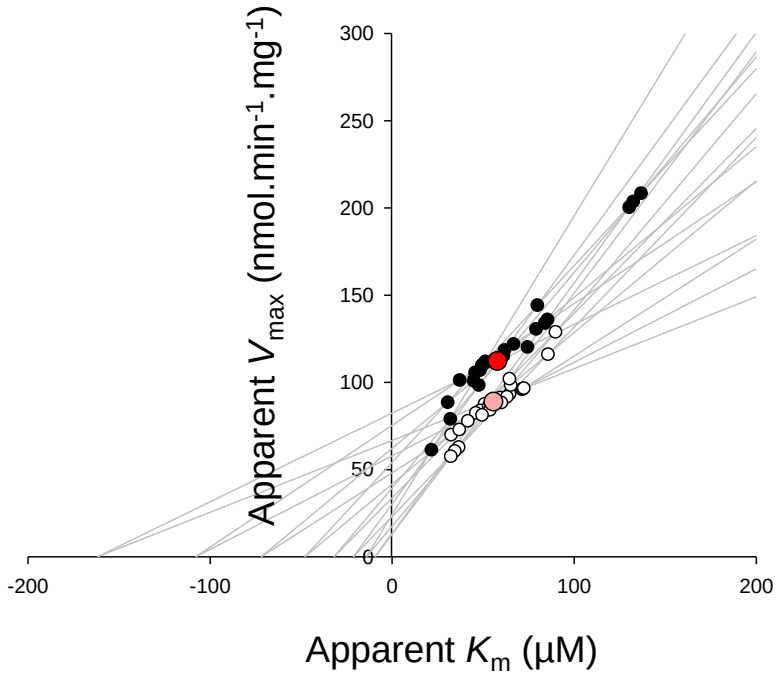
Fig. S7B: Reversibility experiment with *N*-dodecyl-*N*-methylcarbamoyl-CoA 1

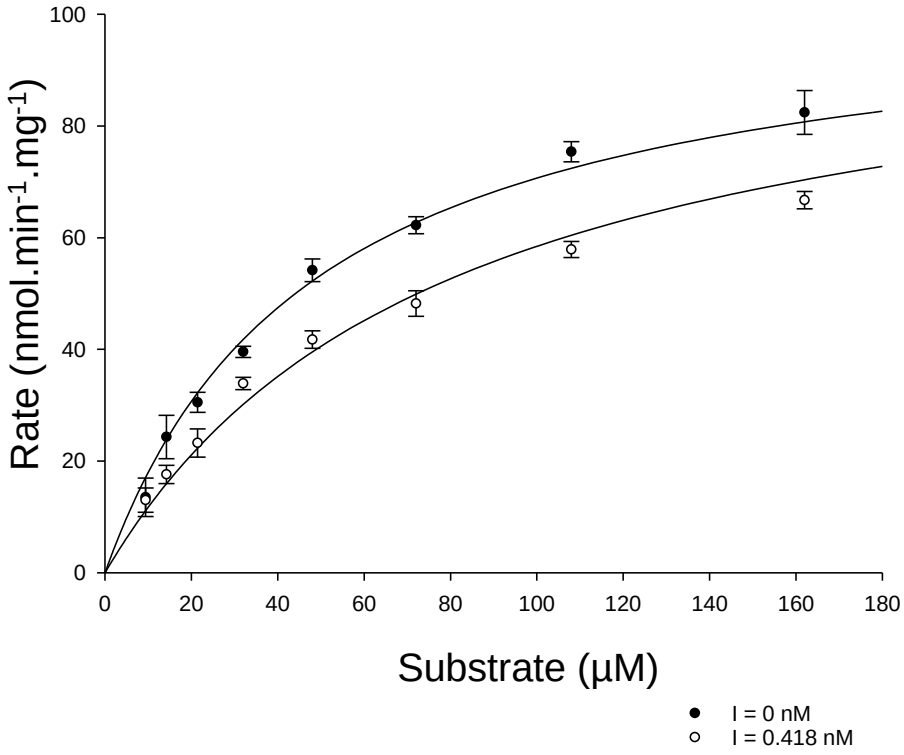


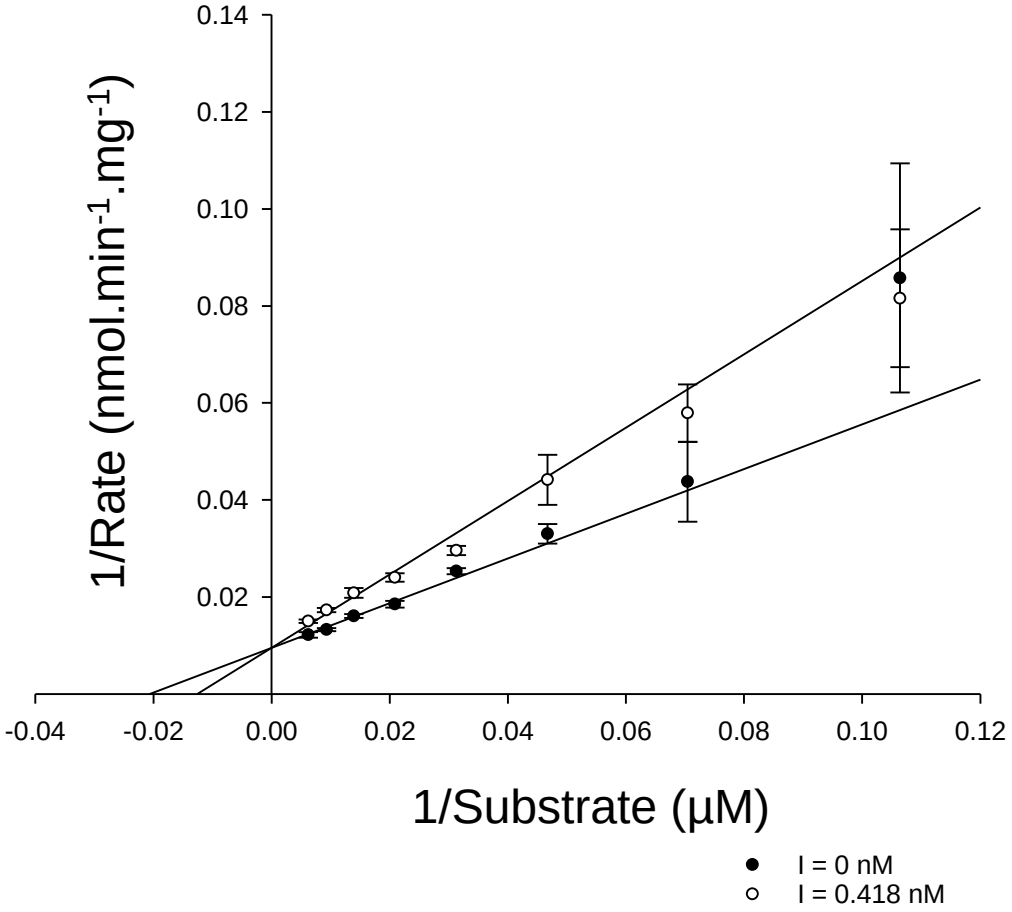
Positive control, enzyme only; Negative control, no enzyme; *N*-Dodecyl-*N*-methylcarbamoyl-CoA **1** was incubated with enzyme at a final concentration of 0.013 µM. Experiment 1 with 10 minute pre-incubation period before adding substrate; Experiment 2, concentrated enzyme pre-incubated as before then diluted 1:50 fold with buffer before adding substrate; Experiment 3, as for experiment 1 but no pre-incubation of enzyme and inhibitor. Results show *N*-Dodecyl-*N*-methylcarbamoyl-CoA **1** is a reversible inhibitor with rapid binding and dissociation.

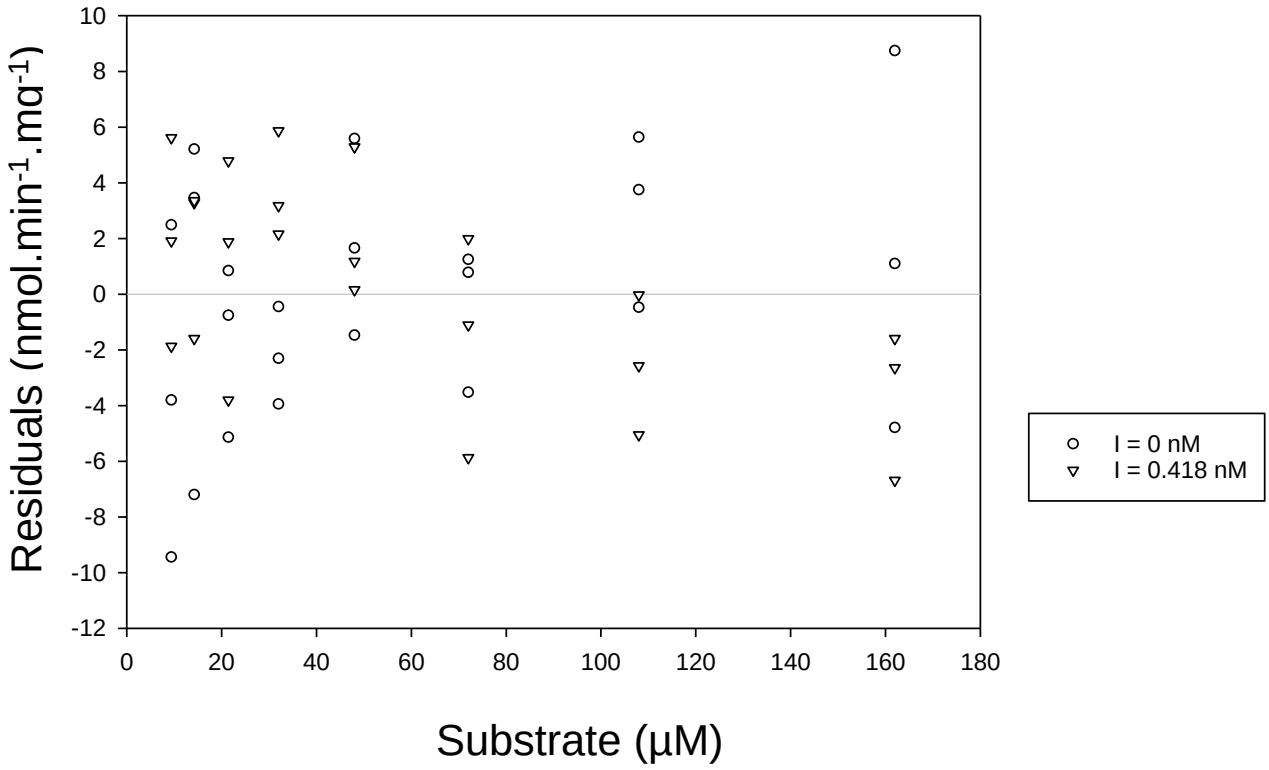
Fig. S7C: Inhibition assays with *N*-dodecyl-*N*-methylcarbamoyl-CoA 1 to determine K_i values








0.14
0.12
0.10
0.08
0.06
0.04
0.02
1/Rate (nmol.min-1.mg-1)
-0.04
-0.02
0.00
0.02
0.04
0.06
0.08
0.10
0.12
1/Substrate (µM)
I = 0 nM
I = 0.418 nM



18/11/2015 12:25:31
Competitive (Full)
Number of Replicates: 3

Parameters

	Value	±Std. Error	95% Conf. Interval		
V_{max} nmol.min^{-1}mg^{-1}	104.8233	3.7185	97.3337	to	112.3128
K_m (µM)	48.2955	4.2260	39.7837	to	56.8073
K_i (nM)	0.6515	9.352e-2	0.4631	to	0.8398

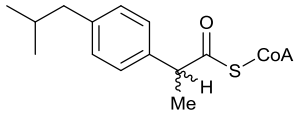
Goodness of Fit

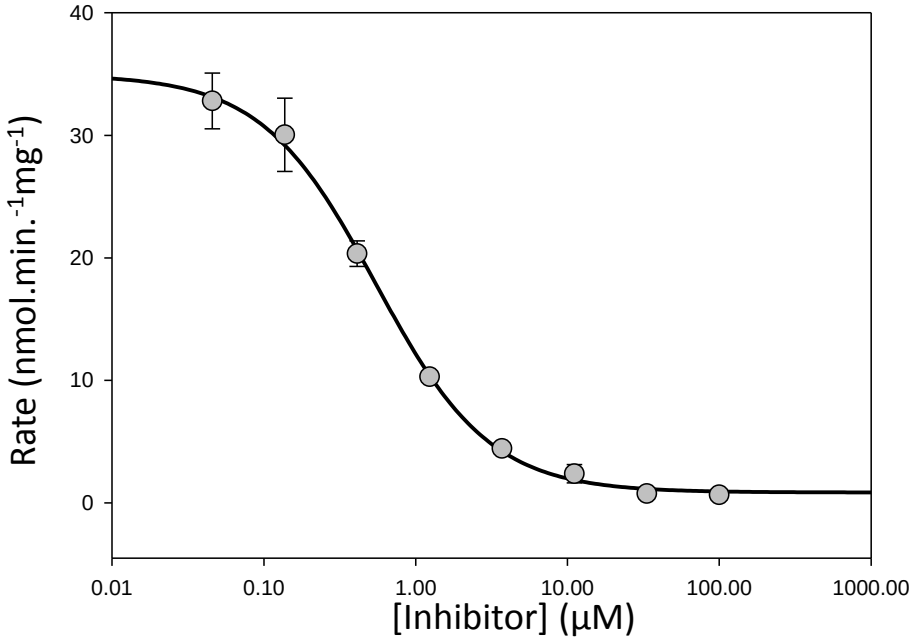
Degrees of Freedom	45
AICc	141.219
R^2	0.966
Sum of Squares	755.372
Sy.x	4.097
Runs Test p Value	0.501

Data

Number of x values	16
Number of replicates	3
Total number of values	48
Number of missing values	0

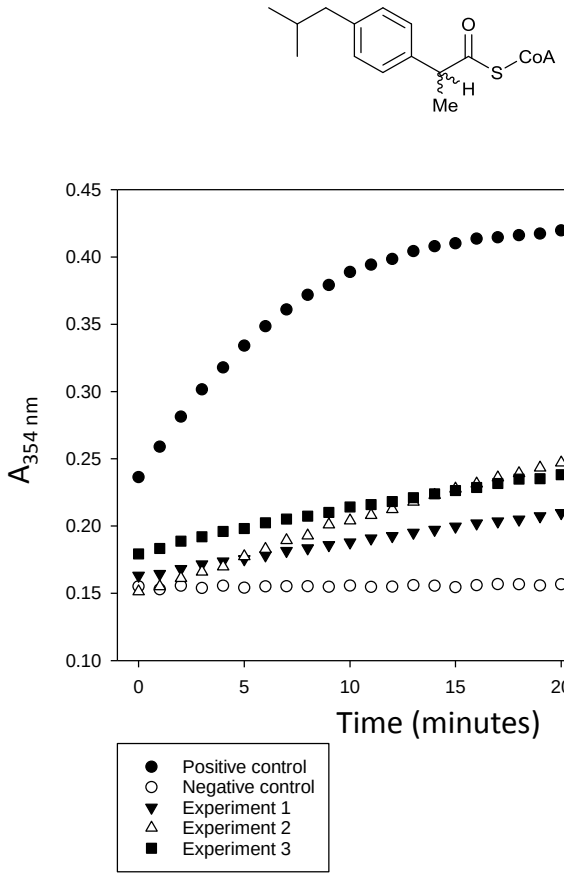
Fig. S8A: Dose-response curve with ibuprofenoyl-CoA 2





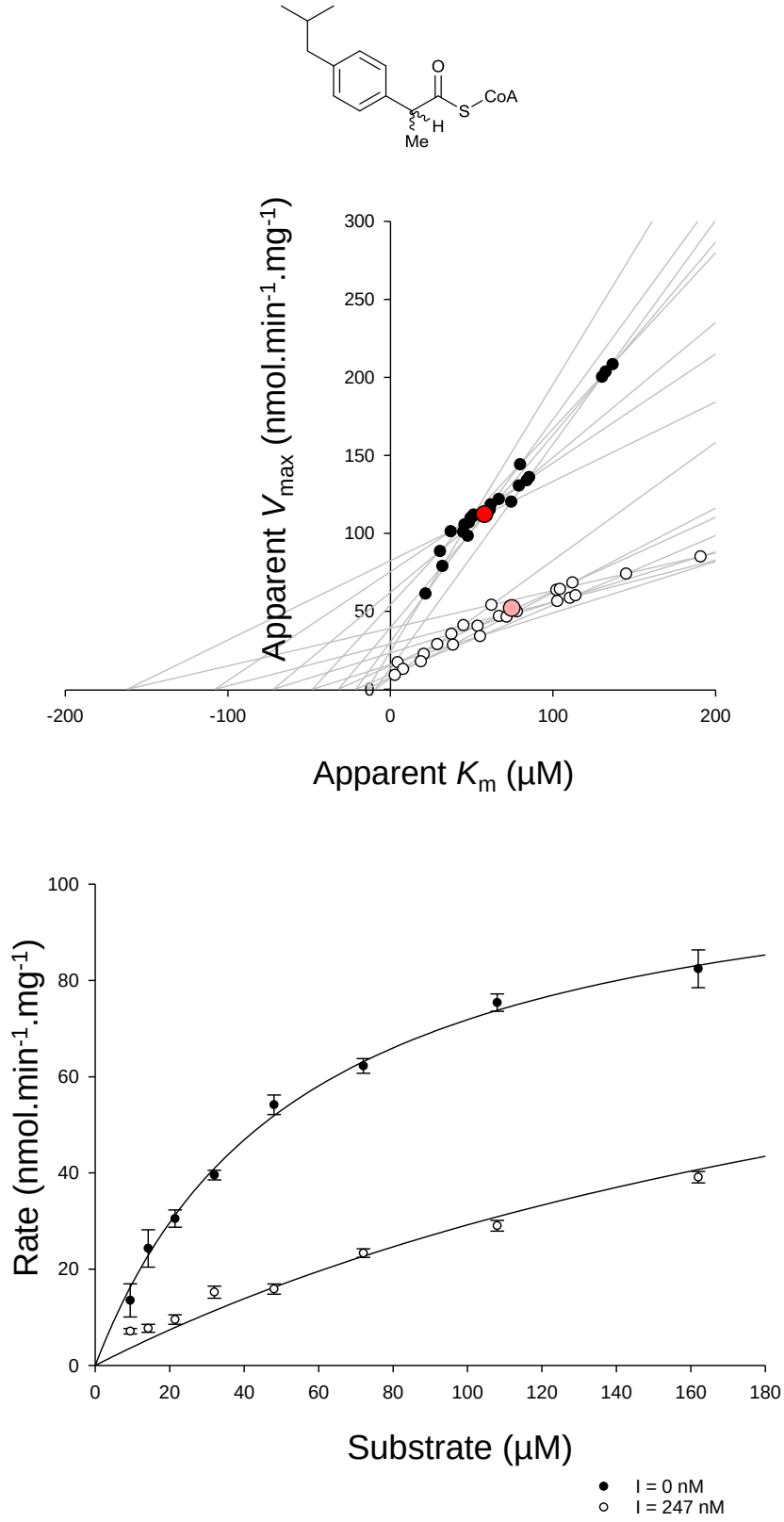
	Coefficient	Std. Error	t	P
Min (nmol.min^{-1}.mg^{-1})	0.8541	0.5273	1.6197	0.1227
Max (nmol.min^{-1}.mg^{-1})	34.9661	1.1702	29.8810	<0.0001
IC_{50} (µM)	0.5446	0.0512	10.6312	<0.0001
Hillslope	-1.1553	0.1144	-10.1022	<0.0001

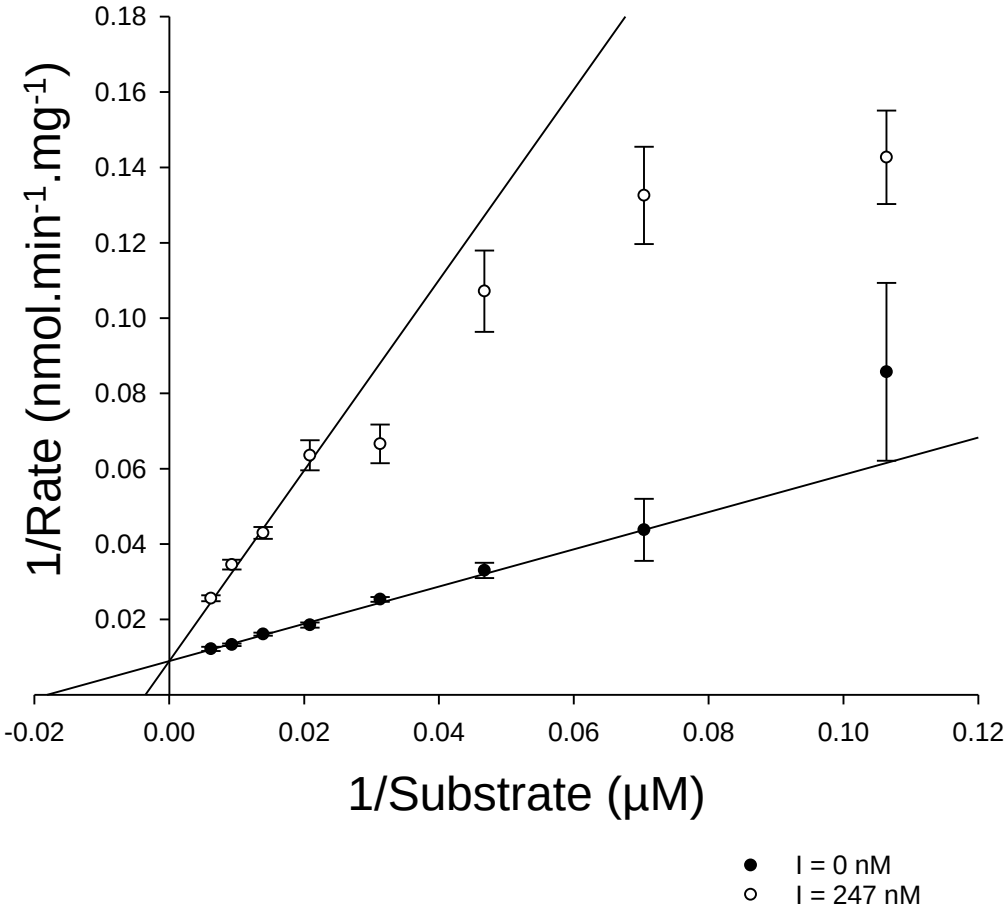
Fig. S8B: Reversibility experiment with ibuprofenoyl-CoA 2

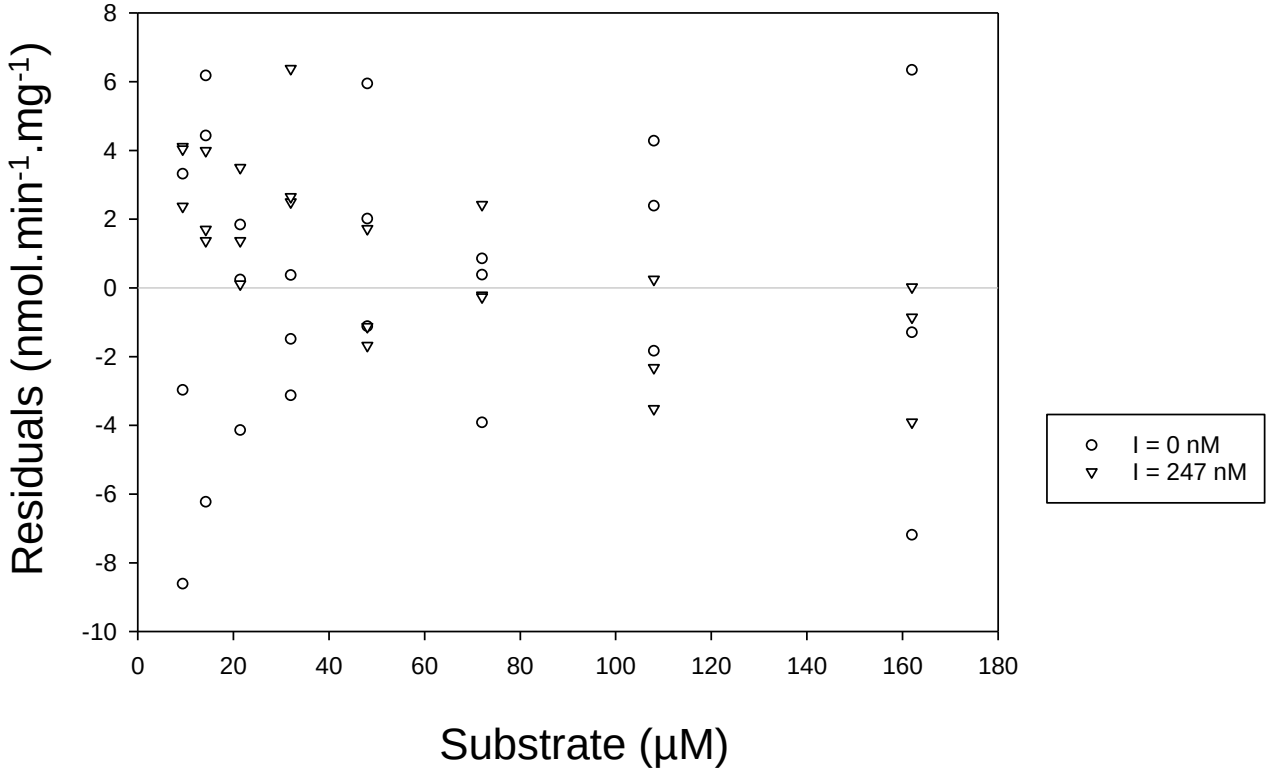


Positive control, enzyme only; Negative control, no enzyme; ibuprofenoyl-CoA **2** was incubated with enzyme at a final concentration of 7.95 µM. Experiment 1 with 10 minute pre-incubation period before adding substrate; Experiment 2, concentrated enzyme pre-incubated as before then diluted 1:50 fold with buffer before adding substrate; Experiment 3, as for experiment 1 but no pre-incubation of enzyme and inhibitor. Results show ibuprofenoyl-CoA **2** is a reversible inhibitor with rapid binding and dissociation.

Fig. S8C: Inhibition assays with ibuprofenoyl-CoA 2 to determine K_i values




0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
1/Rate (nmol.min-1.mg-1)
-0.02
0.00
0.02
0.04
0.06
0.08
0.10
0.12
1/Substrate (µM)
I = 0 nM
I = 247 nM


8
6
4
2
0
-2
-4
-6
-8
-10
Residuals (nmol.min-1.mg-1)
0
20
40
60
80
100
120
140
160
180
Substrate (µM)
I = 0 nM
I = 247 nM

Enzyme Kinetics Nonlinear Fit Results

Notebook3
18/11/2015 12:05:19
Competitive (Full)
Number of Replicates: 3

Parameters

	Value	±Std. Error	95% Conf. Interval		
V_{max} nmol.min^{-1}mg^{-1}	111.3886	4.2499	102.8288	to	119.9484
K_m (µM)	55.0249	4.8176	45.3216	to	64.7281
K_i (nM)	60.0019	4.7833	50.3676	to	69.6362

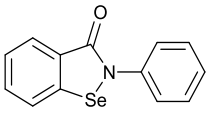
Goodness of Fit

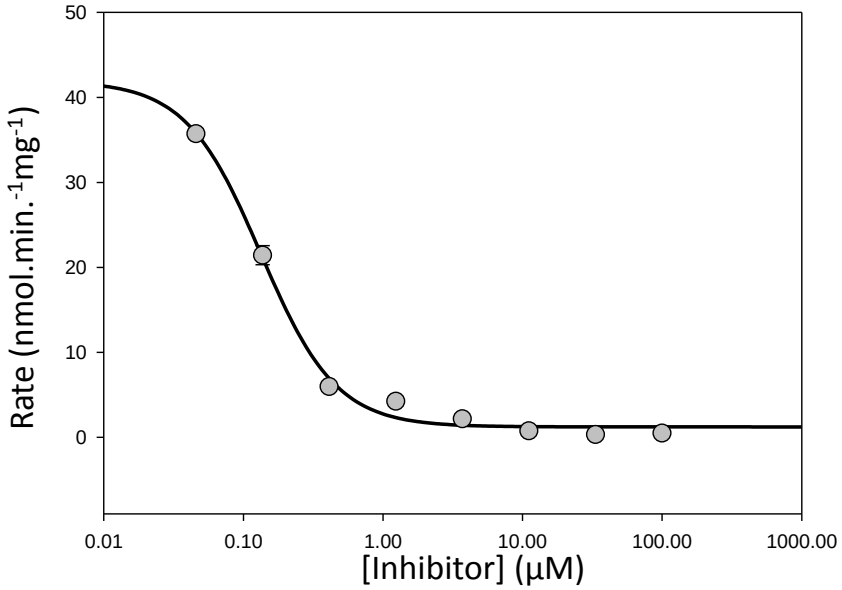
Degrees of Freedom	45
AICc	128.180
R^2	0.978
Sum of Squares	575.688
Sy.x	3.577
Runs Test p Value	0.501

Data

Number of x values	16
Number of replicates	3
Total number of values	48

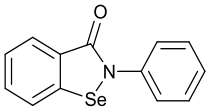
Fig. S9A: Dose-response curve with Ebselen 12

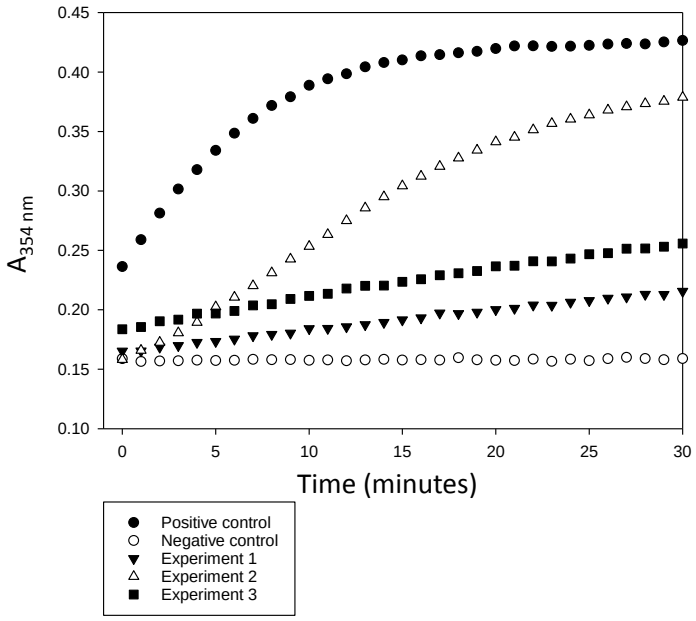




	Coefficient	Std. Error	t	P
Min (nmol.min^{-1}.mg^{-1})	1.2246	0.3378	3.6249	0.0018
Max (nmol.min^{-1}.mg^{-1})	41.9511	2.2918	18.3045	<0.0001
IC_{50} (µM)	0.1335	0.0128	10.4262	<0.0001
Hillslope	-1.6119	0.1688	-9.5499	<0.0001

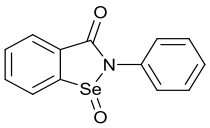
Fig. S9B: Reversibility experiment with Ebselen 12

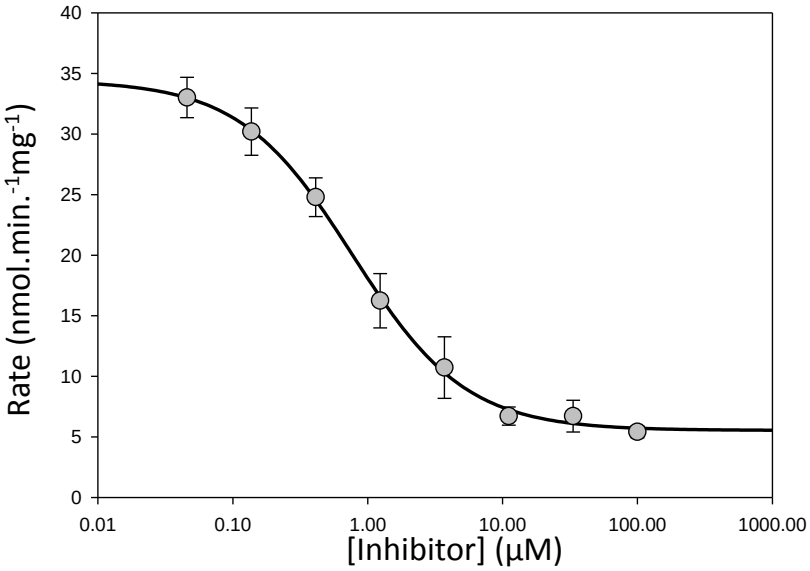




Positive control, enzyme only; Negative control, no enzyme; Ebselen **12** was incubated with enzyme at a final concentration of 0.822 µM. Experiment 1 with 10 minute pre-incubation period before adding substrate; Experiment 2, concentrated enzyme pre-incubated as before then diluted 1:50 fold with buffer before adding substrate; Experiment 3, as for experiment 1 but no pre-incubation of enzyme and inhibitor. Results show that inhibition by Ebselen **12** increases with incubation time and is slowly reversible (a lag phase is observed before activity is restored). This behaviour is consistent with inactivation of the enzyme by covalent modification.

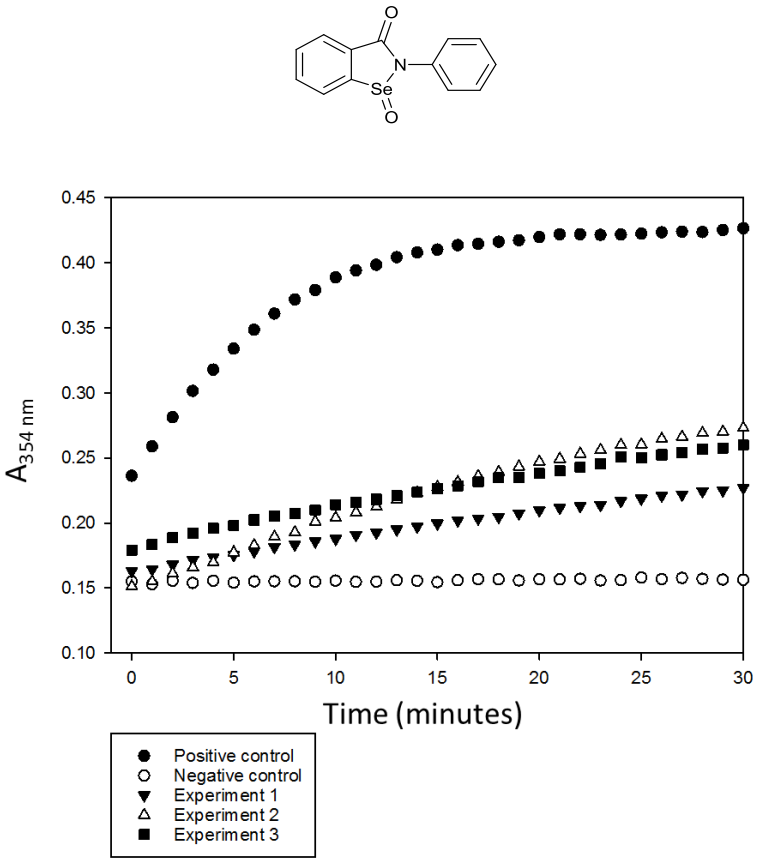
Fig. S10A: Dose-response curve with Ebselen Oxide 13





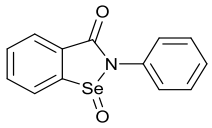
	Coefficient	Std. Error	t	P
Min (nmol.min^{-1}.mg^{-1})	5.5346	0.7365	7.5148	<0.0001
Max (nmol.min^{-1}.mg^{-1})	34.4487	1.4448	23.8425	<0.0001
IC_{50} (µM)	0.7742	0.1164	6.6500	<0.0001
Hillslope	-1.0373	0.1505	-6.8914	<0.0001

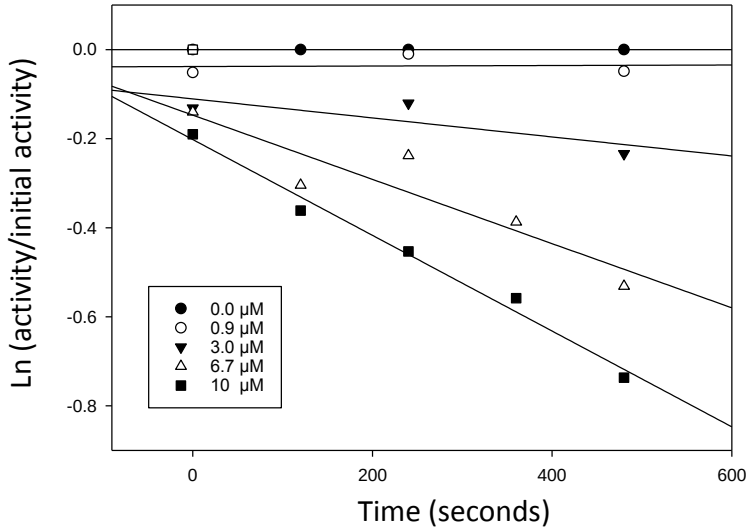
Fig. S10B: Reversibility experiment with Ebselen Oxide 13



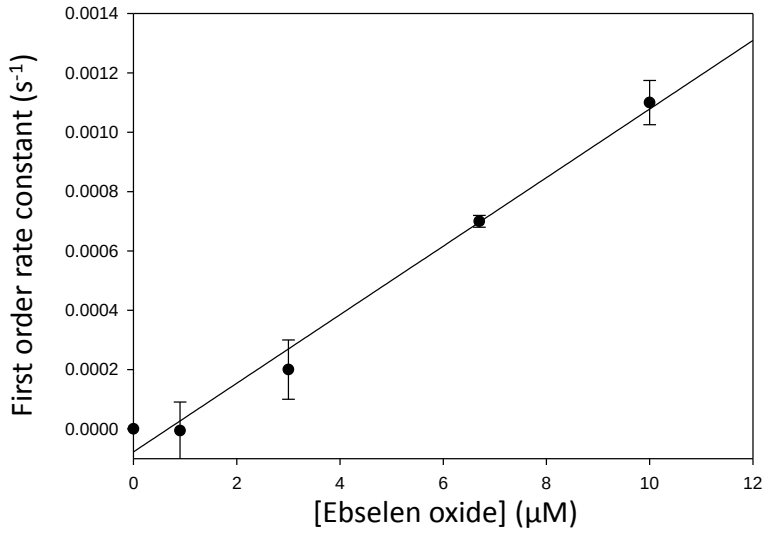
Positive control, enzyme only; Negative control, no enzyme; Ebselen Oxide **13** was incubated with enzyme at a final concentration of 17.5 µM. Experiment 1 with 10 minute pre-incubation period before adding substrate; Experiment 2, concentrated enzyme pre-incubated as before then diluted 1:50 fold with buffer before adding substrate; Experiment 3, as for experiment 1 but no pre-incubation of enzyme and inhibitor. Results show that inhibition by Ebselen Oxide **13** increases with incubation time and is not fully reversible, consistent with inactivation of the enzyme by covalent modification.

Fig. S10C: Ebselen Oxide 13 acting as an irreversible inhibitor



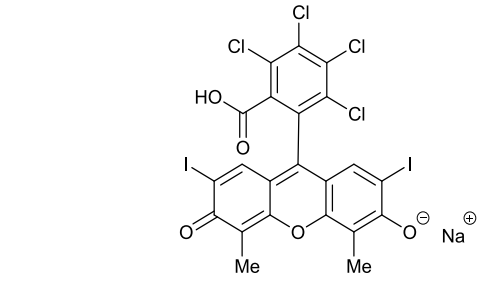


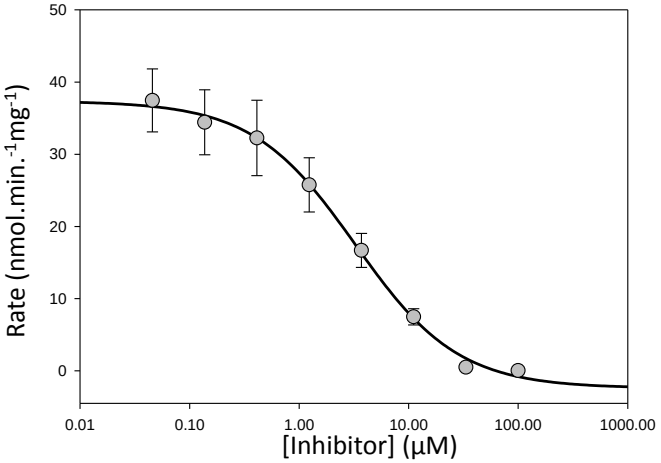
Time dependent inactivation of recombinant AMACR by Ebselen Oxide **13**. Concentrations of Ebselen Oxide **13** (inset) are given for the pre-incubation phase before dilution of the enzyme with substrate **6** in buffer to determine residual activity.



Dependence of inhibition on Ebselen Oxide **13** concentration. The first order rate constant (s^{-1}) derived from above were plotted *vs.* Ebselen Oxide **13** concentration in the pre-incubation phase before dilution for assay to determine residual activity. Data are mean ± standard error.

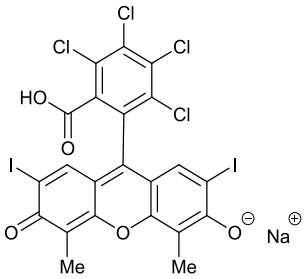
Fig. S11A: Dose-response curve with Rose Bengal 14

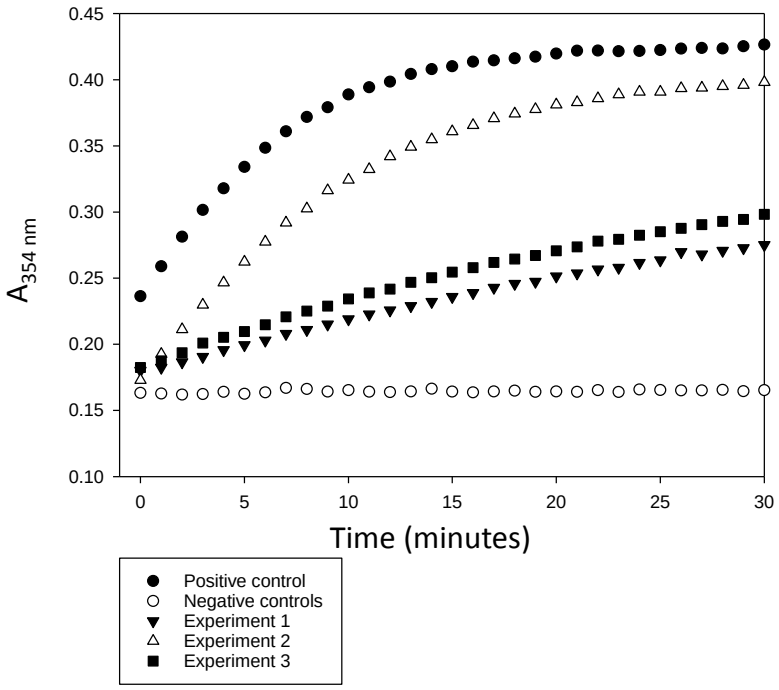




	Coefficient	Std. Error	t	P
Min (nmol.min^{-1}.mg^{-1})	-2.4077	2.5237	-0.9540	0.3515
Max (nmol.min^{-1}.mg^{-1})	37.3666	1.8881	19.7904	<0.0001
IC_{50} (µM)	3.2587	0.7157	4.5531	0.0002
Hillslope	-0.9267	0.1956	-4.7370	0.0001

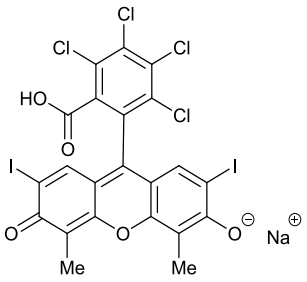
Fig. S11B: Reversibility experiment with Rose Bengal 14

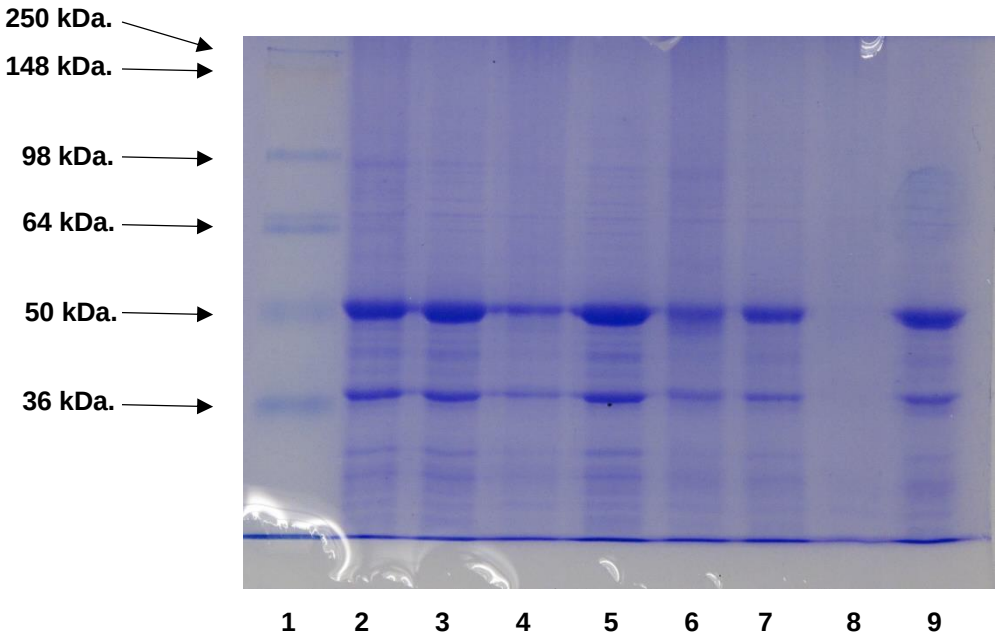




Positive control, enzyme only; Negative control, no enzyme; Rose Bengal **14** was incubated with enzyme at a final concentration of 32 µM. Experiment 1 with 10 minute pre-incubation period before adding substrate; Experiment 2, concentrated enzyme pre-incubated as before then diluted 1:50 fold with buffer before adding substrate; Experiment 3, as for experiment 1 but no pre-incubation of enzyme and inhibitor. Results show Rose Bengal **14** is a reversible inhibitor under these conditions. Prolonged incubation of enzyme with Rose Bengal **14** results in complete degradation of protein (see next page).

Fig. S11C: Photolytic degradation of recombinant AMACR 1A in the presence of Rose Bengal 14





Recombinant AMACR (~9 mg mL^{-1}; 18 µL) was incubated with DMSO (2 µL) or Rose Bengal **14** in DMSO (37 µM, 10 x stock, 2 µL) under the specified conditions and analysed using 10% SDS-PAGE running in Tris-glycine-SDS buffer. Samples incubated in the dark were contained within a 1.5 mL Eppendorf tube; samples incubated in the light were contained within a half-volume 96 well plate and exposed to laboratory light. Samples: 1). SeeBlue Plus2 molecular weight markers (Invitrogen); 2) Rose Bengal, 10 min, dark; 3) DMSO, 10 min, dark; 4). Rose Bengal, 10 min, light; 5). DMSO, 10 min, light; 6). Rose Bengal, 70 min, dark; 7. DMSO, 70 min, dark; 8. Rose Bengal, 70 min, light; DMSO, 70 min, light. Note that condition 4 is the same incubation time as the pre-incubation used in IC_{50} determinations, and the Rose Bengal **14** concentration is about the IC_{50} value (~3.5 µM) in the reaction mixture. Rose Bengal is known to degrade proteins by production of reactive oxygen species.[12-15]