CHAPTER SIX

Analysis of enzyme reactions using NMR techniques: A case study with α-methylacyl-CoA racemase (AMACR)

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Methods in Enzymology, Volume 690
ISSN 0076-6879, https://doi.org/10.1016/bs.mie.2023.07.005

Abstract

α-Methylacyl-CoA racemase (AMACR; P504S) catalyzes the conversion of *R*-2-methylacyl-CoA esters into their corresponding *S*-2-methylacyl-CoA epimers enabling their degradation by β-oxidation. The enzyme also catalyzes the key epimerization reaction in the pharmacological activation pathway of ibuprofen and related drugs. AMACR protein levels and enzymatic activity are increased in prostate cancer, and the enzyme is a recognized drug target. Key to the development of novel treatments based on AMACR inhibition is the development of functional assays. Synthesis of substrates and purification of recombinant human AMACR are described. Incubation of *R*- or *S*-2-methylacyl-CoA esters with AMACR in vitro resulted in formation of epimers (at a near 1–1 ratio at equilibrium) via removal of their α-protons to form an enolate intermediate followed by reprotonation. Conversion can be conveniently followed by incubation in buffer containing $^{2}H_2O$ followed by ^{1}H NMR analysis to monitor conversion of the α-methyl doublet to a single peak upon deuterium incorporation. Incubation of 2-methylacyl-CoA esters containing leaving groups results in an elimination reaction, which was also characterized by ^{1}H NMR. The synthesis of substrates, including a double labeled substrate for mechanistic studies, and subsequent analysis is also described.

Abbreviations

AMACR 1A	α-methylacyl-CoA racemase, splice variant 1A.
CDI	carbonyl diimidazole.
CoA-SH	coenzyme A (reduced sulfhydryl form).
DAST	Diethylamino sulfur trifluoride.
DCM	dichloromethane.
DMF	Dimethylformamide.
DMSO-d_6	deuterated dimethyl sulfoxide.
ERETIC	Electronic Reference To access In-vivo Concentrations.
FID	free induction decay.
HPLC	high-performance liquid chromatography.
Me	methyl.
NMR	nuclear magnetic resonance.
NOE	nuclear Overhauser effect.
rpm	revolutions per minute.
SDS	sodium dodecyl sulfate.
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis.
T_1	resonance intensity relaxation time.
THF	tetrahydrofuran.

1. Introduction

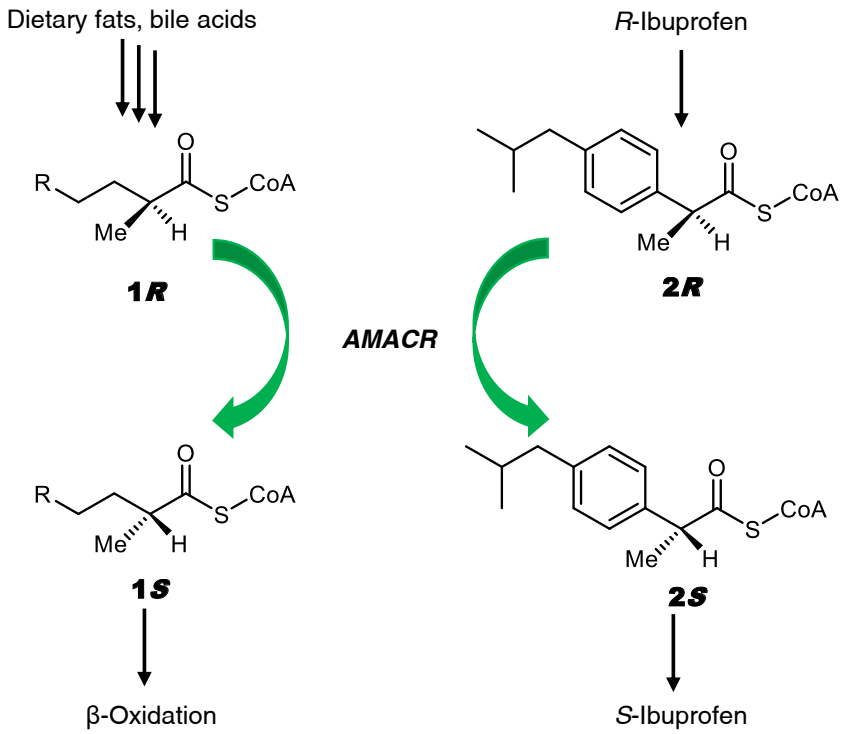
Central to the study of enzymes as drug targets is the ability to measure the conversion of substrates to products using a variety of

different assays. Assays based on absorbance, fluorescence and luminescence are typically used when the accurate measurement of enzymatic reaction rates is required (Brooke, Ghoshray, Ibrahim, & Lloyd, 2023). Racemases and epimerases, which catalyze changes in the stereochemical configuration of substrates, can also be assayed by circular dichroism and similar methods (Bearne & Hayden, 2023; Lloyd et al., 2021). These assays are often continuous, allowing multiple readings during the time-course which facilitates the accurate measurement of rates (Brooke et al., 2023; Olp, Kalous, & Smith, 2020; Petrova et al., 2019; Qu et al., 2013; Yevglevskis et al., 2017). Discontinuous assays can also be used, where the enzymatic reaction is quenched at one or more time-points before analysis. If the enzymatic reaction does not generate a change in the absorbance, fluorescence, or luminescence signal, then depletion of substrate or formation of product can be quantified using radiochemical methods (Kumar-Sinha et al., 2004; Wilson et al., 2011), chromatographic methods such as HPLC (Kershaw et al., 2001; Mukherji et al., 2001; Mukherji, Kershaw, Schofield, Wierzbicki, & Lloyd, 2002; Shieh & Chen, 1993), or mass spectrometry (De Boer, Lingeman, Niessen, & Irth, 2007; Greis, 2007; Rathore et al., 2010). These methods can be labor-intensive, but this disadvantage is offset by their relatively high sensitivity and the potential to separate substrate or products from other components within the enzymatic reaction mixture which may otherwise interfere with the analysis. Despite their many advantages, the above assays convey limited information about the chemical changes which are taking place during the reaction and these methods are susceptible to interference to various degrees.

Nuclear magnetic resonance (NMR) is a well-established method for the characterization of chemical reactions and has been extensively used to study enzymatic reactions. NMR assays can be used to measure kinetic parameters (Exnowitz, Meyer, & Hackl, 2012), but in the past many studies have been used to delineate metabolic pathways and confirm reaction products (Baldwin et al., 1990; Baldwin et al., 1991; Baldwin, Lloyd, et al., 1993; Lloyd et al., 1999), identify intermediates (Baldwin et al., 1990; Baldwin et al., 1991), probe the substrate or reaction selectivity of the enzyme (Baldwin et al., 1992, 1997; Baldwin, Lee, et al., 1993; Dubus et al., 2001; Lloyd et al., 2004; Shibata, Lloyd, Baldwin, & Schofield, 1996), or provide detailed mechanistic information about the enzyme (Baldwin et al., 1990; Baldwin et al., 1991; Baldwin & Bradley,

1990; Baldwin, Lloyd, et al., 1993; Darley et al., 2009a, 2009b; Woodman et al., 2011). Under the assay conditions the concentration of substrates and products are generally far higher than that of the enzyme, and this facilitates the investigation because substrate and product signals are not obscured by those from the enzyme. Commonly, ^{1}H NMR is used in these studies, but there are variety of other nuclei which can be used including ^{2}H (Dios et al., 2023), ^{13}C (Darley et al., 2009a, 2009b; Dios et al., 2023; Shieh, Gou, Liu, Chen, & Chen, 1993), ^{15}N (Hall, Cuellar-Baena, Denisov, & Kirik, 2013), ^{19}F (Dalvit, Ardini, et al., 2003; Dalvit, Fagerness, Hadden, Sarver, & Stockman, 2003) and ^{31}P (Eicher, Snoep, & Rohwer, 2012), amongst others. ^{19}F NMR is particularly appealing as the nuclei has high sensitivity (comparable to ^{1}H), ^{19}F has high (100%) natural abundance, and has particular importance in drug discovery given the high prevalence of fluorine within drug molecules but the low abundance elsewhere (Dalvit, Ardini, et al., 2003; Dalvit, Fagerness, et al., 2003; Johnson, Shu, Zhuo, & Meanwell, 2020; Kirk, 2006; Lloyd, 2020).

The enzyme α-methylacyl-CoA racemase (AMACR) [also known as ibuprofenoyl-CoA epimerase (Reichel, Brugger, Bang, Geisslinger, & Brune, 1997; Schmitz, Helander, Hiltunen, & Conzelmann, 1997)] catalyzes a key change in stereochemical configuration in branched chain fatty acid degradation and the pharmacological activation of ibuprofen and similar drugs (Lloyd, Darley, Wierzbicki, & Threadgill, 2008; Lloyd et al., 2013; Lloyd et al., 2021) (Scheme 1). In vitro, AMACR catalyzes the conversion of *R*- or *S*-2-methylacyl-CoA esters to a near 1 to 1 epimeric mixture (Darley et al., 2009a, 2009b; Woodman et al., 2011). In humans the enzyme exists as several mRNA spliced variants (Mubiru, Valente, & Troyer, 2005; Ouyang et al., 2011), with AMACR 1A known to be active (Darley et al., 2009a, 2009b; Woodman et al., 2011) and AMACR $1A_{del}$ predicted to also catalyze changes in stereochemical conversion (Lloyd et al., 2013). Deficiency of AMACR 1A protein in humans is associated with neurological disorders (Ferdinandusse et al., 2000), whilst increased AMACR 1A protein and activity is reported in prostate and other cancers and it is a novel drug target for prostate cancer [reviewed in (Lloyd et al., 2008; Lloyd et al., 2013; Lloyd et al., 2021)]. In this chapter we describe various NMR assays for measuring the enzymatic activity of recombinant AMACR 1A.



Scheme 1 Role of AMACR in branched-chain fatty acid degradation and pharmacological activation of ibuprofen and similar drugs. β-Oxidation requires *S*-2-methylacyl-CoA esters **1*S***, but *R*-2-methylacyl-CoA esters **1*R*** are produced from dietary fatty acids or by oxidation of cholesterol (bile acids); R = aliphatic side-chain or cholesterol derivative. *R*-Ibuprofen (inactive) is stereoselectively converted into *S*-ibuprofen via their corresponding acyl-CoA esters **2*R*** and **2*S*** by a unidirectional pathway (because only *R*-ibuprofen can be converted to the corresponding acyl-CoA ester) (Lloyd et al., 2008; Lloyd et al., 2013; Lloyd et al., 2021; Mukherji et al., 2003).

2. Purification of recombinant human AMACR 1A

2.1 Key resources table

Reagent or Resource	Source	Identifier
Bacterial and virus strains		
Rosetta2 (DE3) competent cells or glycerol freeze	Novagen	71400
Chemicals, peptides, and recombinant proteins		

Acetone	Merck	270725-1L
Overnight Express Autoinduction System 1	Novagen	71300-3
Bacteriological agar	Thermo Fisher Scientific	11479632
Benzamidine-HCl	Merck	434760
Benzonase®	Merck	E1014
$CaCl_2$	Merck	C5670
Chloramphenicol	Thermo Fisher Scientific	10368030
Ethanol	Merck	1009832511
HCl [~35% (w/v) aq. solution]	Merck	087617. AK
Imidazole	Thermo Fisher Scientific	15895158
Kanamycin sulfate	Thermo Fisher Scientific	10031553
Lennox LB media	Thermo Fisher Scientific	11313249
N-Lauroylsarcosine, sodium salt	Merck	L9150-100G
NaCl	Thermo Fisher Scientific	10316943
NaH_2PO_4	Thermo Fisher Scientific	10133153
NaOH	Thermo Fisher Scientific	10192863
PMSF	Merck	PMSF-RO
See Blue™ Plus2 pre-stained protein standards (for SDS-PAGE analysis)	Thermo Fisher Scientific	LC5925
Recombinant DNA		
AMACR 1A cDNA in pET30 EK/LIC plasmid	In-house	Darley et al. (2009a)

2.2 Materials and equipment

All solutions are aqueous unless otherwise noted and should be made up in 18.2 MΩ/cm Milli-Q water (Millipore) or equivalent. Solutions used to transform or grow cells should be sterilized by autoclaving or filtration (using a 0.22 μm filter) before use if not purchased sterile. Consumables used to transform or grow cells should be sterilized by autoclaving for 15 min at 121 °C and 0.1 MPa (15 pounds per square inch, PSI) or bought sterile. Equipment and solutions for cell harvesting and subsequent steps do not need to be sterile.

- pH meter and calibration solutions at pH 4.0, 7.0 and 10.0.
- 5 M and 1 M aq. NaOH.
- ~10 M and ~1 M HCl [35% (w/v) purchased stock and a 1 in 10 dilution in water].
- Lennox Luria-Bertani media (autoclaved).
- Lennox LB media containing 1% (w/v) agarose (autoclaved).
- 30 mg/mL aq. kanamycin sulfate (filter sterilized) (1000× stock).
- 34 mg/mL chloramphenicol in ethanol (filter sterilized) (1000× stock).
- 50 mM aq. $CaCl_2$ (filter sterilized).
- Lennox LB media (1 or 2 × 1 L final volume in a 2 L baffled shaker flask. The media (20 g) will need to be dissolved in 929 mL of water. Addition of auto-induction solutions 1, 2 and 3 will give a final volume of 1 L).
- Additional 1 × Lennox LB media (e.g., 3 × 100 mL) for competent cell production and starter cultures.
- Lennox LB agar plates (1 for each sample to be transformed).
- 200 mM PMSF dissolved in acetone (propan-2-one).
- 200 mM benzamidine-HCl dissolved in water.
- Start buffer: 20 mM NaH_2PO_4–NaOH, 300 mM NaCl, 10 mM imidazole, pH 7.2.
- Elution buffer: Start buffer supplemented with 300 mM imidazole. The buffer will need to be titrated to pH 7.2 with HCl.
- 20% (w/v) aq. *N*-lauroylsarcosine, pH adjusted to ~7.2 as necessary.
- Dialysis buffer: 20 mM NaH_2PO_4–NaOH, pH 7.2.
- Beckman centrifuge with JA 10 rotor.
- 500 mL centrifuge tubes.
- 25 mL pipettes [Thermo Fisher Scientific 11839660].
- 10 μL, 20 μL, 200 μL and 1 mL Gilson pipettes, and corresponding clear, yellow, and blue tips.

- 1.5 mL Eppendorf tubes.
- 5 mL tubes.
- 50 mL Falcon tubes.
- Pasture pipettes.
- Constant Systems "One Shot" cell disruptor.
- Solutions required to perform SDS-PAGE analysis.
- Bench-top centrifuge capable of taking 1.5 mL Eppendorf tubes.
- Shaking cell incubator suitable for growing *E. coli* cells at 37 and 28 °C.
- HisTrap FF Column (5 mL) (Merck, GE17-5255-01).
- Dialysis tubing.

Alternatives:

Reagents should be of bioreagent or equivalent grade. Any suitable centrifuges, metal-chelate chromatography columns, equipment of similar specification, and SDS-PAGE molecular weight markers may be used.

2.3 Step-by-step method details

2.3.1 Transformation of plasmid and growth of recombinant E. coli cells

The transformed plasmid encodes for human AMACR 1A with an N-terminal His-tag (Darley et al., 2009a). The protein can be purified using metal-chelate chromatography using Ni-containing columns (Darley et al., 2009a; Yevglevskis, Bowskill, et al., 2014; Yevglevskis et al., 2017) (Fig. 1).

Timing: Day 1.

1. Using aseptic technique, prepare competent Rosetta2 (DE3) cells from a glycerol freeze (Mojanaga, Acharya, & Lloyd, 2023), growing at 37 °C with shaking at ~200 rpm. This host strain is relatively slow growing so a 300 μL glycerol freeze inoculum is recommended. Chloramphenicol (34 μg/mL) should be included in any cultures as this ensures retention of the plasmid in the host strain, which is required for optimal protein expression. Alternatively, use commercial competent cells.
2. Prepare LB agar supplemented with 30 μg/mL kanamycin sulfate and 34 μg/mL chloramphenicol and pour ~25 mL into each of the required plates (Mojanaga et al., 2023).
3. Transform 1–2 μL of plasmid (~0.75–1.5 μg) into competent cells using the high-efficiency 5-minute method (Mojanaga et al., 2023; Pope & Kent, 1996). Incubate the cells at 37 °C overnight.

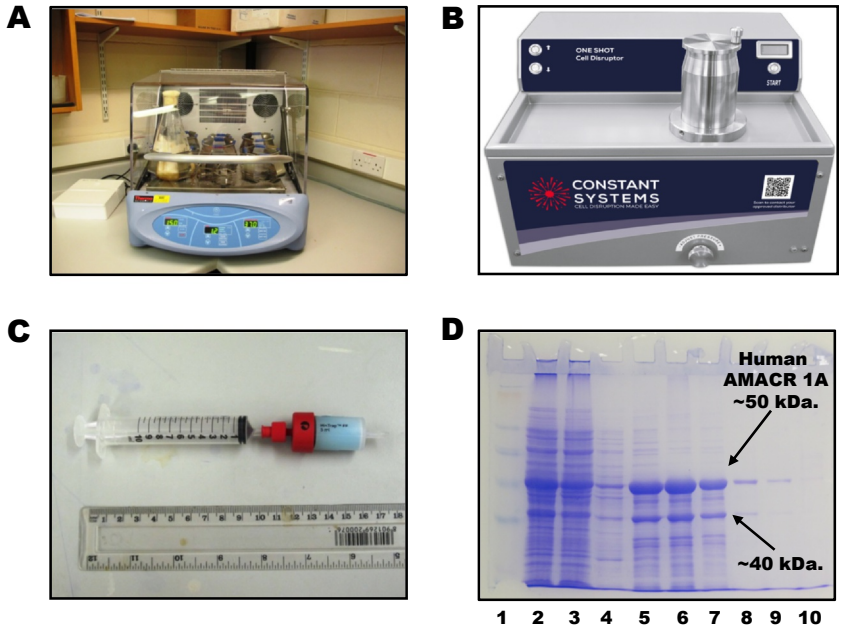


Fig. 1 Purification of recombinant AMACR protein using metal-chelate chromatography. (A) Shaker flask containing transformed *E. coli* grown in an incubator/shaker. (B) The Constant Systems "One-Shot" used to lyse cells and produce a cell extract. Photo used with permission of Constant Systems. (C) A 5 mL HisTrap column and syringe used to purify the crude extract. (D) SDS-PAGE analysis. Lanes are 1. SeeBlue™ Plus2 prestained protein standards (Thermo Fisher Scientific); 2. Crude extract; 3. Flow-through and column wash; 4. "40 mM imidazole wash"; 5–10, elution fractions 1–6 from column. The eluted AMACR protein is indicated with the arrow. Note the highlighted band for AMACR 1A at ~50 kDa. and the presumed proteolytic fragment at ~40 kDa.

Timing: Day 2

4. Using aseptic technique, in the morning pick single colonies into white screw-capped tubes containing 5 mL LB media supplemented with 30 μg/mL kanamycin sulfate and 34 μg/mL chloramphenicol. Grow at 37 °C with shaking at ~170–220 rpm throughout the day. Use this culture to inoculate the required number of starter cultures in a 50 mL Falcon tube containing the same media (20 mL) and grow under the same conditions overnight.

Timing: Day 3

5. Using aseptic technique, add Express auto-induction media stock solutions to the LB media stock solution (to give a final volume of 1 L). Supplement this media with kanamycin sulfate and chloramphenicol and add the starter culture (20 mL). Incubate at 28 °C with shaking as above until the following morning (~24 h) (Fig. 1A). Note: a short incubation of 60–90 min at 37 °C may be used to ensure sufficient cell density and hence induction. The temperature should be adjusted to 28 °C after this initial phase.
6. Harvest the cells using centrifugation using a Beckman JA-10 rotor at 10,000 rpm, 14,300 × *g*, 30 min at 4 °C. Cell culture should be placed in 500 mL centrifuge bottles that need to be paired with identical weight. Discard the residual media (and dispose of as contaminated biological waste) and transfer the pellet into a plastic bag, press into a thin sheet, and store at −80 °C until required. 2 L of culture media should give ~ 5 g of cells.

Note: The method can be paused at this point as the subsequent protein purification will take several days.

2.3.2 Purification of recombinant human AMACR using metal-chelate chromatography

Timing: Day 4.

1. Thaw ~2 g of cells in a 50- or 100-mL glass beaker and resuspend cells in 30 mL of start buffer supplemented with 1 mM PMSF, 1 mM benzamidine-HCl, and 250 units of Benzonase®. Cells may need to be resuspended using a Pasteur pipette by carefully pumping up and down several times. Lyse the cells using the "One Shot" (Mojanaga et al., 2023) (Fig. 1B) and centrifuge the sample in a 50 mL Falcon tube using a Beckman centrifuge and JA-14 rotor at 10,000 rpm, 15,300 × *g*, 10–20 min at 4 °C to remove bubbles. Resuspend the pellet using a Pasteur pipette and transfer the sample back to the original beaker. Stir as slowly as possible in the cold room and add *N*-lauroylsarcosine stock solution dropwise to a final concentration of 1.5% (w/v) and continue to stir for 1 h. Centrifuge as above to produce the crude extract.
2. Wash the HisTrap column (Fig. 1C) with 30 mL water using a syringe and a connecting flow adaptor. Equilibrate the column with 30 mL start buffer supplemented with 0.2 mM PMSF and 0.2 mM benzamidine-

HCl. The flowrate should be such that the liquid drips at a moderate rate and does not form a constant stream. Add PMSF and benzamidine-HCl to the elution buffer, and form "wash buffer" containing 40 mM imidazole-HCl by mixing 1 mL of elution buffer with 9 mL start buffer.

3. Filter the crude extract through a 0.2 μm filter and retain ~50 μL for SDS-PAGE analyses. Load the remaining crude extract onto the equilibrated column using the syringe, collecting the flow-through in a beaker. The flowrate should be such that liquid "slow drips" from the column. When all the crude extract is loaded, wash the column with 10 mL of start buffer supplemented with protease inhibitors. Keep the crude extract and flow-through samples on ice.
4. Wash the column with 5 mL "Wash buffer" (prepared in step 2), collecting the eluent in a 5 mL tube, keeping the sample on ice. This removes loosely bound protein contaminants from the column. The flowrate should be the same as that used when equilibrating the column.
5. Elute the column using "elution buffer" supplemented with protease inhibitors, collecting 6 × 5 mL fractions, keeping the samples on ice. The flowrate should be the same as in the above step.
6. Prepare samples of the crude extract, flow-through, "wash" and elution fractions for SDS-PAGE analysis as described (Mojanaga et al., 2023). Use 20 μL of each sample; the crude extract and flow-through samples will require preparation by trichloroacetic acid (TCA) precipitation. Load molecular weight markers and samples onto 12.5% gels and analyze by SDS-PAGE. We use Tris/glycine/SDS running buffer (Ahn, Yim, Choi, & Yun, 2001), but other similar running systems (Mojanaga et al., 2023) will work just as well. Stain the gels using Coomassie Brilliant Blue R-250 stain (Mojanaga et al., 2023).
7. Select the required elution fractions (generally #1 to #3) (Fig. 1D). Pool the required fractions and dialyze against 3 × ~650 mL of dialysis buffer at 4 °C for a minimum of 3 h a time (we generally perform the first dialysis overnight). Finally, determine the protein concentration by UV–visible spectroscopy using ε_{280} = 35,785 per M/cm (Woodman et al., 2011) and a molecular weight of 47,146.8 Da. (Darley et al., 2009a). Store aliquoted samples at −80 °C, freezing the sample rapidly in dry ice or liquid nitrogen [to minimize changes in pH during the freezing process (Pikal-Cleland, Rodriguez-Hornedo, Amidon, & Carpenter, 2000)].
8. Wash the column with water (30 mL) followed by 20% (v/v) ethanol (30 mL) to clean and store the column. Use the end adapters to seal the column and store at 4 °C.

2.4 Expected outcomes, advantages and limitations

The above protocol enables the production of AMACR which is suitable for use in enzyme assays. The above protocol for transforming and growing cells (Yevglevskis et al., 2017) is optimized for both amount of enzyme produced and ease of production, but we have successfully used *E. coli* BL21 (DE3) as a host cell with induction using isopropyl-β-D-thiogalactopyranoside (IPTG) (Darley et al., 2009a), and Rosetta2 (DE3) with IPTG induction (Yevglevskis, Bowskill, et al., 2014). The optimized protocol uses growth at 28 °C, which is a compromise between reducing temperature to maximize soluble protein expression and ensuring there is sufficient growth for induction to occur [at lower temperatures the glucose present in the auto-induction media is not sufficiently depleted for the host cell to switch over to lactose as a carbon source, which is required for induction (Studier, 2005)].

The constructed plasmid containing the cDNA for human AMACR 1A encodes for a protein with a N-terminal His-tag (Darley et al., 2009a), allowing purification in one step. The described protocol makes use of column purification using a syringe, but a chromatography system could be used if desired. Solubilization of AMACR in the crude extract requires the use of relatively harsh detergent conditions (milder detergents such as 0.25% (v/v) Triton X-100 do not efficiently solubilize the enzyme). This extraction is a compromise to maximize yield at the expense of high enzymatic activity. Dialysis of the purified protein is required to remove residual buffer components such as imidazole-HCl, which will interfere in subsequent NMR assays if still present [because the concentration will be so much greater than the substrate even after ~100-fold dilution of the enzyme into the assay mixture (Section 4.3.1, point 1)]. Buffer exchange can also be performed using gel filtration chromatography, but the volumes are relatively large, and the protein solution will need to be concentrated before exchange can be performed. This protocol should finally yield 10–15 mL of enzyme stock, at ~2.8 mg/mL at 80–85% purity (Fig. 1D). The relatively intense band at ~40 kDa. always occurs at a constant ratio to the band of human AMACR 1A at ~50 kDa. and is thought to be a proteolytic degradation product with a truncated C-terminus.

2.5 Optimization and troubleshooting

The above protocols are optimized, and few control experiments are required. Rosetta2 (DE3) glycerol stocks should be periodically renewed to

ensure high levels of protein expression. Optimization of protein loading may be required if a different staining method is used during SDS-PAGE analysis (Mojanaga et al., 2023).

2.6 Safety considerations and standards

Eye protection and lab coats should be worn. Gloves should be used where there is a risk that hands may encounter toxic, carcinogenic, or hazardous materials such as PMSF and reagents used during SDS-PAGE. A dust mask should be used when handling irritant powders such as SDS. SDS-PAGE requires the use of high voltages, and equipment should always be switched off and unplugged before assembling or disassembling. Care should be exercised when using the hot block when preparing samples for SDS-PAGE analysis, to avoid burns and to prevent exposure to volatile chemicals such as 2-mercaptoethanol. Centrifugation samples must be properly balanced to within 0.1 g such that each pair of tubes have equal weight; single tubes should be balanced against a blank containing water. Avoid over-filling the centrifuge tubes as this can result in leakage of liquid with consequent unbalancing of samples and damage to the centrifuge. Cryogenic materials such as dry ice and liquid nitrogen should be used in a well-ventilated area, and a facemask and insulated gloves should be worn when handling liquid nitrogen. Chemical waste should be disposed of through approved routes, and biological waste should be decontaminated by autoclaving or other approved methods. Risk assessments should be completed according to institutional guidelines before any experimental work is undertaken. Good Laboratory Practice should be followed. As the experiment involves use of Genetically Modified Organisms (GMOs), the worker and project may need to be registered.

3. Synthesis and quantification of acyl-CoA substrates

3.1 Key resources table

Reagent or Resource	Source	Identifier
Chemicals, Peptides, and Recombinant Proteins		
Acetone	Merck	270725
Acetonitrile	Merck	34851
Ammonium bicarbonate	Merck	11213
Ammonium chloride	Merck	213330

1-Bromooctane	Merck	152951
Carbonyl diimidazole (CDI)	Merck	802301
Celite	Merck	64843
C^2HCl_3	Merck	151823
CrO_3	Merck	236470
Dibutylboron trifluoromethanesulfonate solution (1.0 M in DCM)	Merck	261475
Dichloromethane (DCM)	Merck	270997
Diethyl [2-^{13}C]-malonate	Merck	281859
Diethylamino sulfur trifluoride (DAST)	Merck	2325253
Diethyl ether	Merck	309966
Diisopropylethylamine	Merck	387649
Dimethylformamide (DMF)	Merck	227056
Dry ice	BOC	None
Ethyl acetate (ethyl ethanoate)	Merck	319902
Evans auxiliary (both *R*- and *S*-)	Merck	294640 (S) 300977 (R)
1-Fluoro-2,4-dinitrobenzene	Merck	42085
^2H_2O (deuterated water)	Merck	151882
Hydrochloric acid (HCl)	Merck	087617. AK
Hydrochloric acid (HCl)–^{2}HCl (35% in ^2H_2O)	Merck	543047
Hydrogen peroxide [30% (w/v) aqueous solution]	Merck	216763
Iodomethane	Merck	67692
Lithium hydroxide hydrate	Merck	254274
Magnesium sulfate (anhydrous)	Merck	746452
2-Methyl-1,3-propanediol	Merck	375721
n-Butyllithium (1.6 M in hexanes)	Merck	186171
n-Butyllithium (2.5 M in hexanes)	Merck	230707

Octanal	Merck	8.06901
Oxalyl chloride	Merck	221015
Petroleum ether (40–60)	Merck	32299-M
Phosphate buffer (0.1 M)	Merck	76847
Potassium carbonate	Merck	209619
Propionyl chloride	Merck	P51559
Reduced coenzyme A, tri-lithium salt	Merck	234101
Silica gel	Merck	1.07754
Sodium bis(trimethylsilyl)amide (1.0 M in THF)	Merck	245585
Sodium chloride (NaCl)	Thermo Fisher Scientific	10316943
Sodium hydride (60% suspension in oil)	Merck	452912
Sodium hydrogen carbonate ($NaHCO_3$)	Merck	S6014
Sodium metal	Thermo Scientific Chemicals	11341428
Sodium sulfite	Merck	239321
Tetrahydrofuran (THF)	Merck	401757

3.2 Materials and equipment

Aqueous solutions should be made up in 18.2 MΩ/cm Milli-Q water (Millipore) or equivalent.

- Brine (saturated aq. NaCl).
- 0.1 M aq. $NaHCO_3$.
- 1 M aq. HCl.
- 1 M aq. K_2CO_3.
- Saturated aq. NH_4Cl.
- Saturated aq. NH_4HCO_3.
- 0.1 M Sodium phosphate buffer.
- Büchi rotary evaporator.
- Freeze-dryer.
- Reversed phase solid-phase extraction (SPE) cartridges [Waters Sep-Pak Vac 3cc (500 mg) C18 cartridges].

Alternatives:

Any ACS quality reagents should work. DCM must be anhydrous, otherwise the CDI will decompose. Acidification of reaction mixtures containing synthesized acyl-CoA esters can be successfully performed using Dowex X50 (acid form) instead of HCl (Darley et al., 2009a; Woodman et al., 2011).

3.3 Step-by-step method details

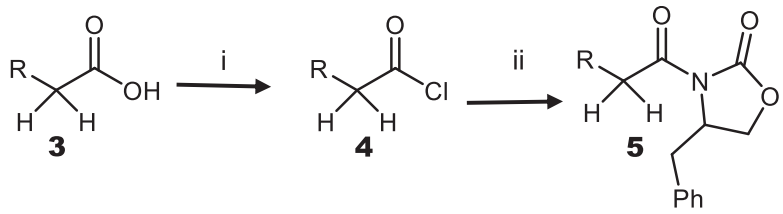
3.3.1 General synthetic procedures

Syntheses were carried out at ambient temperature, unless otherwise specified. Solutions in organic solvents were dried with $MgSO_4$. Thin layer chromatography was performed on Merck silica aluminum plates 60 (F254) and visualized with UV light, potassium permanganate or phosphomolybdic acid. Column chromatography was performed using Fisher silica gel (particle size 35–70 μm). NMR spectra were recorded at 22 °C at 400.04 or 500.13 MHz (^{1}H) and 100.59 or 125.76 MHz (^{13}C) on Bruker Avance III NMR spectrometers (Material and Chemical Characterisation Facility (MC^2)) in $^{2}H_2O$, DMSO-D_6 or C^2HCl_3. 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. ^{1}H NMR spectra recorded for known compounds matched data reported in the literature unless otherwise stated. Acyl-CoA esters were characterized by ^{1}H NMR and HRMS. Aqueous solutions were prepared in Nanopure water of 18.2 MΩ/cm quality and were pH-adjusted where necessary with aq. HCl or NaOH. 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 Thermo Fisher Scientific 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. Phosphate buffer was prepared from monobasic sodium phosphate and NaOH at the required proportions. The following General Methods were used in the described syntheses.

3.3.1.1 General Method A: Acid coupling to evans auxiliary

Coupling of the acid to an Evan's auxiliary is performed by activation of the chosen acid **3** to the corresponding acid chloride **4**. The Evan's auxiliary is deprotonated and acylated using the acid **4** to give the Evan's protected product **5** (Scheme 2).

1. Weigh out 2–5 mmol of acid **3** and place in a round bottom flask at room temperature. Dissolve in dry DCM (typically 10–20 mL) and stir at medium speed.



Scheme 2 Acid **3** activation as acid chloride **4** and coupling to Evan's auxiliary to produce **5**. R = variable side-chain. *Reagents and conditions:* (i) oxalyl chloride, DMF, DCM; (ii) *R*- or *S*-4-benzyloxazolidin-2-one, *n*-butyl lithium, THF −78 °C.

2. Add oxalyl chloride (1.5–2 equivalents). **CAUTION: Oxalyl chloride is a toxic liquid and care should be taken to work in a fume hood with appropriate Personal Protective Equipment** . Add DMF (1 drop)—this acts to catalyze the reaction.
3. After 1 h remove all the volatile solvents using a rotary evaporator. Take care that the vapors are vented into a fume hood.
4. Dissolve the residue into dry tetrahydrofuran (THF, 10 mL).
5. Into a second round-bottom flask weigh out the chosen Evans' auxiliary (0.9 equivalents) and dissolve in dry THF (20 mL). Cool this solution to −78 °C using a dry ice/acetone bath. To the cooled solution add *n*-butyl lithium (2.5 M in hexanes, 1 equivalent) over the course of 15 min. **Care is needed with *n*-butyl lithium as it reacts vigorously with water and can lead to fires.**
6. Keeping the reaction at −78 °C, slowly add the acid chloride solution (dropwise from a syringe) over 10 min. Allow the reaction to warm to room temperature over 1 h and then leave to stir for a further 1 h.
7. Add saturated aq. NH_4Cl (10 mL) to the reaction, dropwise at first and then once no further reaction is seen, more swiftly.
8. Extract the mixture with DCM three times. To do this use a separating funnel of around 50 mL capacity. Add DCM (20 mL), shake the separating funnel with inversion, remembering to release any pressure build up by opening the tap once inverted. On settling the lower layer will be the DCM and should be collected into a clean conical flask. Repeat this twice more and combine the DCM layers.
9. The combined organic layers should be washed with aq. K_2CO_3 (10 mL, 1 M) and brine (10 mL). To do this add the washing solution, mix, and allow to settle and then collect the organic layer again.

10. Dry the organic solution using solid portions of anhydrous magnesium sulfate. To do this add sufficient magnesium sulfate that when swirled at least some of the drying agent produces a 'snowstorm' effect—being able to freely move in the vessel, not all clumping together.
11. Filter the dried solution through filter paper and then remove all the volatile solvents using a rotary evaporator to afford a crude product.
12. If needed the crude product **5** can be purified using silica gel column chromatography.

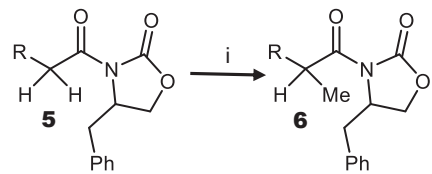
3.3.1.2 General Method B: Methylation

Stereoselective methylation can be performed by deprotonation of the Evan's protected acid **5** followed by treatment with methyl iodide to give the stereoselective methylated product **6** (Scheme 3).

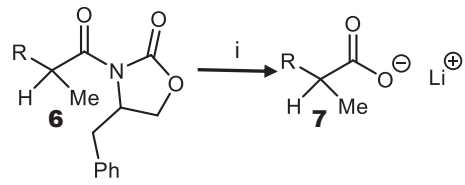
1. Place a solution of sodium bis(trimethylsilyl)amide (1.0 M in THF, 3.3 mmol) into a round bottom flask fitted with a suba seal and cool to −78 °C using a dry ice/acetone bath.
2. To the cooled solution add a solution of the Evans' auxiliary protected acid **5** (also in THF, 3 mmol in 10 mL).
3. Allow to stir for 1 h at this temperature.
4. Add iodomethane (15 mmol) to THF (10 mL) and cool the resulting solution to −78 °C.
5. Add the iodomethane solution to the first solution over 15 min and allow the reaction to stir for a further 3 h at this temperature and then allow the reaction to reach room temperature.
6. The reaction was then worked up following the steps 7–12 from General Method A.

3.3.1.3 General Method C: Evan's auxiliary cleavage

Cleavage of the acyl group from the protected Evan's auxiliary **6** gives the corresponding acid **7** (Scheme 4).



Scheme 3 Methylation of Evan's auxiliary protected acid **5** to give **6**. R = variable side-chain. *Reagents and conditions:* (i) $NaN(SiMe_3)_2$, MeI, THF. The *R*-Evan's auxiliary gives *R*-2-methylacyl products, and the *S*-Evan's auxiliary gives *S*-2-methylacyl products.



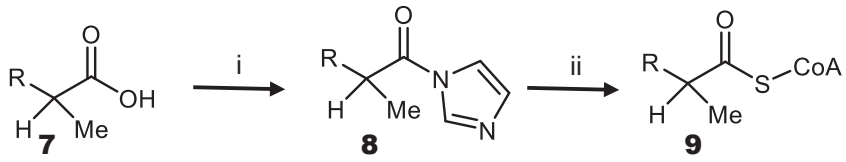
Scheme 4 Alkali hydrolysis of methylated product **6** to give deprotected acid **7**. R = variable side-chain. *Reagents and conditions:* (i) H_2O_2, LiOH, THF/H_2O.

1. Dissolve the methylated product **6** (typically 1–2 mmol) in a mixture of THF (30 mL) and water (10 mL).
2. Cool the solution in an ice bath to 0 °C.
3. To this cooled solution add aq. hydrogen peroxide (30%, 0.75 mL per mmol of methylated product) and lithium hydroxide hydrate solution (2 equivalents).
4. Stir the resulting solution for 3 h, ensuring that the temperature remains at 0 °C.
5. Quench the reaction by adding aq. sodium sulfite (1.5 M, 13.5 mL, 20 mmol).
6. Acidify the mixture to pH 1 by adding HCl (1.0 M)—check by spotting a small sample of liquid onto pH paper using e.g., a TLC spotter.
7. Extract the resulting solution three times using DCM.
8. Dry the combined organic layer with magnesium sulfate, filter and remove the volatile solvents using a rotary evaporator to give the crude product.
9. If needed the product can be purified using silica gel column chromatography.

3.3.1.4 General Method D: Synthesis of acyl-CoA esters

The acid **7** is activated as the corresponding imidazolide **8**. This is purified and reacted with reduced CoA to give the required acyl-CoA ester **9** (Scheme 5).

1. Weigh out 0.1 mmol of acid **7** (approximately 25–37 mg of material, depending on the structure) and place in a small round bottomed flask, e.g., 10 mL at room temperature. Dissolve in dry DCM (approximately 1.5–2 mL) and stir at a medium speed.
2. Weigh out 0.2 mmol of carbonyl diimidazole (CDI, 2 equivalents, 32 mg) and dissolve in 1–1.5 mL of dry DCM (total volume = 3 mL). Add the resulting solution dropwise to the solution prepared in step 1 and continue to stir for 1 h after which a milky white solution will be obtained (due to precipitation of imidazole product).



Scheme 5 Synthesis of coenzyme A ester **9** from acid **7** via imidazolide intermediate **8**. R = variable side-chain. *Reagents and conditions:* (i) carbonyl diimidazole, DCM; (ii) CoA-SH, 0.1 M $NaHCO_3$ aq./THF (1:1).

3. Transfer the reaction mixture to a separating funnel, using a second stirrer bar to catch the one in the reaction mixture. Wash the mixture with 2 mL of water while shaking. When shaking the separating funnel remember to invert and carefully open the tap to release pressure at regular intervals, as DCM is extremely volatile. Place the separating funnel with tap downwards and allow the layer to separate. PRO-TIP: if the layers do not separate properly, add a few drops of brine to the solutions. Separate the layers and place the original bottom (DCM) layer back in the separating funnel. Repeat this procedure another 4 times or until a clear bottom layer is obtained (all the imidazole product is removed). Finally, wash the bottom layer with 2 mL of brine.
4. Transfer the lower (DCM) layer containing the acyl-imidazolide product **8** to a conical flask. Ensure that no visible upper aqueous layer is transferred. If necessary, transfer most of the DCM layer, then add a small amount of additional DCM (no more than 1 mL) to the residual aqueous layer and separate. Leave a small amount of lower layer behind in the separating funnel to ensure no carry-over into the conical flask. Add anhydrous $MgSO_4$ to the conical flask followed by gentle swirling. Add further $MgSO_4$ until separated fine particles are obtained (if clumps are obtained, this means that the solution is still wet).
5. Filter the dried DCM layer using a glass funnel and filter paper into a 25 mL round bottom or pear-shaped flask. Remove the DCM under reduced pressure using a Büchi rotary evaporator. The recommended vacuum can be found within the manual.
6. Redissolve the acyl-imidazolide **8** in 3 mL of THF and stir at room temperature. Weigh out 31 mg (0.04 mmol, 0.4 equivalents of acyl-imidazolide) of reduced CoA dissolved in 0.1 M aq. $NaHCO_3$ (3 mL) and add to the solution. Continue to stir overnight (approximately 18 h).
7. Switch the freeze-dryer on and allow it to temperature equilibrate and establish the vacuum at least one hour before needed (in Step 12).

8. Acidify the resulting mixture from step 6 to pH ~3 by adding 1 M aq. HCl dropwise using a 200 μL Gilson pipette and yellow tip, testing the *p*H as required by spotting a small volume onto pH paper. The reaction will "fizz" due to the formation of CO_2 from the $NaHCO_3$ present in the mixture.
9. Remove the stirrer bar from the reaction mixture using a second stirrer bar and remove the THF under reduced pressure using a Büchi rotary evaporator. The recommended vacuum can be found within the manual. This will leave the aqueous residue behind.
10. Add water (2 mL) and transfer the mixture into a separating funnel. Wash with ethyl acetate (5 × 3 mL) as described in step 3 to remove residual carboxylic acid (resulting from hydrolysis of excess acyl-imidazolide). The lower (aqueous) layer is the one that should contain the desired acyl-CoA product.
11. Ensure that there is no visible upper (ethyl acetate layer) in the sample. If there is, reseparate using the separating funnel, then place the sample onto dry ice to freeze. Alternatively, use liquid nitrogen to freeze the sample. **CAUTION: Liquid nitrogen splashes cause severe burns. Always use a lab coat, facemask, and cryogenic gloves when handling**.
12. Place the flask containing the frozen sample on the freeze-dryer and allow to dry overnight. A white powder will usually be obtained (unless the original acid was colored). PRO-TIP: Ensure that the sample is completely frozen before placing it on the freeze-dryer "tree" (therefore it is important to remove all organic solvents before attempting to freeze-dry, as these will depress the melting point). Keep the sample on dry ice beforehand. The sample will appear off-white or gray if not properly frozen. Once the flask is on the freeze-dryer "tree" wrap the flask in Saran Wrap (Cling Film).
13. Condition the Sep-Pak column by washing with acetonitrile (4 mL) and water (4 mL). Use a N_2 or compressed air line to push the solvent through the column, **but do not allow the column to run dry**.
14. Redissolve the crude acyl-CoA mixture is ~4 mL of water and load it onto the column, again using pressure to push the sample onto the column. Successively wash the column with 4 mL of water followed by 10%, 25%, 40% and 50% (v/v) acetonitrile in water, collecting each elution fraction in a separate round-bottom or pear-shaped flask. Note that each column can purify around 200 mg of acyl-CoA ester, so if you are expecting more divide the sample into batches and use more than one column.

15. Carefully remove the acetonitrile from each sample which contains it, using a Büchi rotary evaporator. Acyl-CoA esters are labile, so keep the water bath at 20 °C or as close to this temperature as possible. Then freeze-dry each individual sample as described in steps 11 and 12 above.
16. Redissolve the fractions in ~1 mL of $^{2}H_2O$. Vortex-mix the samples for 5 min to remove any micelles, and centrifuge for 2 min at 13,400 rpm in a bench-top centrifuge to remove bubbles. Immediately transfer the samples into NMR tubes and analyze by ^{1}H NMR to determine which contain the desired product. Recover the NMR samples and pool the required fractions together and freeze-dry (ideally in a 10 mL pear-shaped flask to maximize recovery). This will typically be the fractions eluted from the SPE step at 25% and 40% (v/v) acetonitrile in water, but less lipophilic acyl-CoA esters may be found in the 10% (v/v) acetonitrile fraction. Highly lipophilic acyl-CoA esters may elute in the 50% (v/v) acetonitrile fraction.
17. Redissolve the sample in 1 mL H_2O (or $^{2}H_2O$ if required) and transfer to a 1 mL Eppendorf tube. Vortex-mix the sample for 5 min to remove any micelles and centrifuge in a bench-top centrifuge at 13,600 rpm for 2 min to remove any bubbles. Mix 50 μL sample with 500 μL of $^{2}H_2O$ and vortex-mix the sample for 2 min followed by centrifugation for 1–2 min.
18. Determine the concentration by ^{1}H NMR (see Section 4.5.1). We also analyze each sample by high-resolution negative ion electrospray mass spectrometry (ESI MS).

The method described here is using carbonyl diimidazole, which we have found to be the most reliable and can be used on different scales. The described method (Yevglevskis et al., 2019) yields between 3.5 and 7.0 mg of acyl-CoA, sufficient for substrate and inhibitor testing. The synthesis also works on a larger scale (Yevglevskis et al., 2017). We have also successfully used an alternative procedure using ethyl chloroformate (Darley et al., 2009a). We have also synthesized acyl-CoA esters from various commercially available acids including ibuprofen and analogs (Woodman et al., 2011) and mandelic acid (Yevglevskis, Bowskill, et al., 2014) using the same procedure.

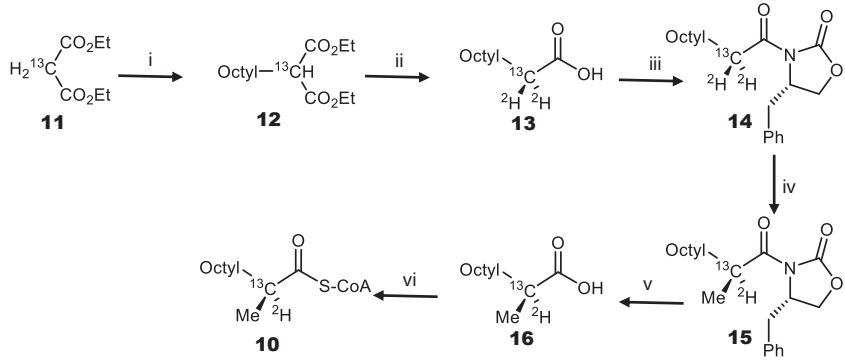
3.3.2 Synthesis of S-2-[^{13}C]-2-[^{2}H]-2-methyldecanoyl-CoA 10

S-2-methyldecanoic acid was synthesized from decanoic acid by General Methods A, B and C as described above. Formation of the acyl-CoA ester was achieved using the ethyl chloroformate method (Darley et al., 2009a). For the double labeled compound **10**, ^{13}C-Diethylmalonate **11** was

converted to the alkylated intermediate **12** and then to the doubly labeled acid **13**. This was converted to the corresponding doubly labeled *S*-2-methyldecanoyl-CoA **10** via intermediates **14** to **16** by an extension of the method for the unlabeled compound (Darley et al., 2009a) (Scheme 6).

3.3.2.1 Synthesis of ethyl [2-^{13}C]-2-ethoxycarbonyldecanoate 12

1. Place sodium hydride (60% in oil, 1.00 g, 6.25 mmol) into a round bottom flask (50 mL). Add dry dimethylformamide (DMF, 20 mL) and add a magnetic stirrer bar.
2. To this solution add diethyl [2-^{13}C]-malonate **11** (1.00 g, 6.25 mmol) and stir the resulting solution for 30 min. The reaction should be sealed with a suba seal (rubber septum), and an argon balloon fitted over the top.
3. Add 1-bromooctane (1.6 g, 8.2 mmol) and stir the reaction for a further 16 h.
4. Add saturated aqueous ammonium chloride (10 mL). Extract the resulting mixture twice with ethyl acetate (20 mL) (using a separating funnel).
5. Wash the combined extracts with aqueous HCl (1 M, 10 mL) and water (three times, each of 10 mL).
6. Dry the organic layer using solid portions of $MgSO_4$—add sufficient that at least some of the solid remains free flowing on swirling the conical flask.
7. Filter the solution and remove all the volatile solvents from the filtrate using a rotary evaporator to give the crude product.
8. The product **12** can be purified using silica gel column chromatography.



Scheme 6 Synthesis of *S*-2-[^{13}C]-2-[^{2}H]-methyldecanoyl-CoA **10**. *Reagents and conditions:* (i) NaH, 1-bromooctane, DMF, 59%; (ii) 35% aq. ^{2}HCl, 160 °C, 97%; (iii) oxalyl chloride, DCM, DMF; (iv) *R*-4-benzyloxazolidin-2-one, *n*-butyl lithium, THF −78 °C; (v) $NaN(SiMe_3)_2$, MeI, THF, −78 °C, 68%; (vi) LiOH, H_2O_2, 0 °C, 91%; (vii) ethyl chloroformate, CoA-SH, THF (Darley et al., 2009a, 2009b).

3.3.2.2 Synthesis of 2-[^{13}C]-2-[$^{2}H_2$]-decanoic acid 13

1. Suspend **12** (950 mg, 3.5 mmol) in ^{2}HCl/$^{2}H_2O$ (35%, 10 mL) in a 25 mL round bottom flask.
2. Attach a reflux condenser to the flask and heat with stirring at 160 °C for 48 h. Make sure that the reaction does not lose solvent during the heating process. If any is lost, add sufficient $^{2}H_2O$ to make up the volume.
3. Allow the mixture to cool to room temperature and the extract three times with ethyl acetate (10 mL), using a separating funnel and collecting the organic layer each time.
4. Dry the combined organic layers using solid portions of $MgSO_4$—add sufficient that at least some of the solid remains free flowing on swirling the conical flask. Filter the solution and remove all the volatile solvents from the filtrate using a rotary evaporator to give the crude product.
5. The product **13** can be purified using silica gel column chromatography.

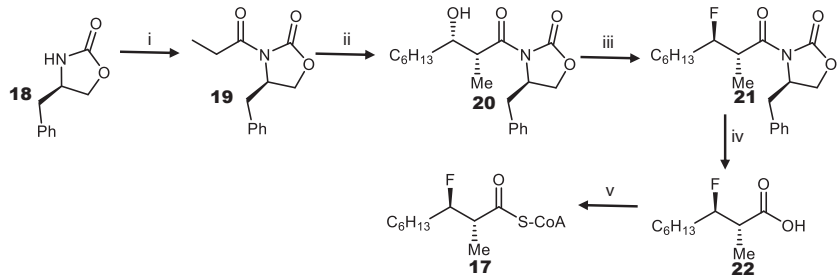
Synthesis of **14**, **15** and **16** was achieved by General Methods A, B and C. **10** was produced from **16** by the ethyl chloroformate method (Darley et al., 2009a).

3.3.3 Synthesis of Anti-(2R,3R)-3-fluoro-2-methyldecanoyl-CoA (17)

Anti-3-fluoro-2-methyldecanoyl-CoA **17** was synthesized by coupling the appropriate Evan's auxiliary **18** with propanoic acid to give **19** followed a mixed aldol/Claisen condensation to give **20**. Treatment with diethylamino sulfur trifluoride (DAST) gave **21** with inversion of stereochemistry. This was deprotected to acid **22** by General Method C and converted to the acyl-CoA **17** by General Method D (Yevglevskis, Lee, Threadgill, Woodman, & Lloyd, 2014) (Scheme 7).

3.3.3.1 Synthesis of (*R*)-4-benzyl-3-propanoyloxazolidin-2-one 19

1. Place *R*-Evans' auxiliary **18** (4.056 g, 23 mmol) in a round bottom flask. Add THF (60 mL) and cool to −78 °C using a dry ice/acetone bath.
2. Add n-BuLi (1.6 M, 14.3 mL, 23 mmol) in dry THF and stir the resulting mixture at −78 °C for 30 min
3. Dissolve propanoyl chloride (2.0 mL, 23 mmol) in anhydrous THF (20 mL) and then add this dropwise to the reaction mixture. Stir the resulting mixture for 30 min at this temperature and then allow to reach room temperature over 1 h.



Scheme 7 Synthesis of *anti*-3-fluoro-2-methyldecanoyl-CoA **17**. *Reagents and conditions*: (i) *n*-Butyl lithium, 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 tri-lithium salt, 0.1 M $NaHCO_3$ aq./THF (1:1) (Yevglevskis, Lee, et al., 2014).

4. Quench the reaction by slow addition of saturated aq. ammonium chloride (80 mL).
5. Using a separating funnel extract the mixture with DCM (100 mL) twice.
6. Wash the combined organic extracts with saturated aq. NH_4HCO_3 and then brine.
7. Dry the organics using solid portions of $MgSO_4$—the solution is dry when further solid portions are found to be free flowing on swirling the mixture in a conical flask.
8. Filter the mixture and remove the volatile organics from the filtrate using a rotary evaporator.
9. The crude product **19** can be purified using silica gel column chromatography with a mobile phase of petroleum ether/ethyl acetate (4:1).

3.3.3.2 Synthesis of (*R*)-4-benzyl-3-[(2*R*,3*S*)-3-hydroxy-2-methyldecanoyl] oxazolidin-2-one 20

1. Place the product **19** (300 mg, 2.14 mmol) in a round bottom flask and dissolve in DCM (10 mL). Cool this solution of −78 °C using a dry ice/acetone bath.
2. Add dibutylboron triflate in DCM (1.0 M, 1.30 mL, 1.29 mmol) and diisopropylethylamine (0.25 mL, 1.29 mmol).
3. Stir the resulting mixture for 30 min
4. Add a solution of octanal (0.15 mL, 0.92 mmol) in DCM (3 mL) and then stir the mixture for a further 30 min, then allow the reaction to reach room temperature.

5. Quench the reaction by slowly adding aq. phosphate buffer (0.1 M, pH = 7.0, 10 mL), prepared as described in Section 3.3.1.
6. Separate the organic layer and wash it sequentially using a separating funnel with hydrochloric acid (1 M), saturated aq. NH_4HCO_3 and brine.
7. Dry the organics using solid portions of $MgSO_4$—the solution is dry when further solid portions are found to be free flowing on swirling the mixture in a conical flask.
8. Filter the mixture and remove the volatile organics from the filtrate using a rotary evaporator.
9. The crude product **20** can be purified using silica gel column chromatography with a mobile phase of petroleum ether/ethyl acetate (10:1).

3.3.3.3 Synthesis of (*R*)-4-benzyl-3-[(2*S*,3*R*)-3-fluoro-2-methyldecanoyl]oxazolidin-2-one 21

1. Place **20** (156 mg, 0.43 mmol) in a round bottom flask and then add DCM (3.0 mL) and cool the solution to −78 °C using a dry ice/acetone bath.
2. Add diethylamino sulfur trifluoride (DAST, 57 μL, 0.43 mmol) as a solution in DCM (2.0 mL).
3. Stir the reaction at this temperature for 2 h, then allow it to reach room temperature.
4. Quench the reaction by slow addition of water (5.0 mL).
5. Separate the organic layer using a separating funnel and wash sequentially with saturated ammonium bicarbonate and brine.
6. Dry the organics using solid portions of $MgSO_4$—the solution is dry when further solid portions are found to be free flowing on swirling the mixture in a conical flask.
7. Filter the mixture and remove the volatile organics from the filtrate using a rotary evaporator.
8. The crude product **21** can be purified using silica gel column chromatography with a mobile phase of petroleum ether/ethyl acetate (5:1).

3.3.3.4 Synthesis of (2*S*,3*R*)-3-fluoro-2-methyldecanoic acid 22

The Evans auxiliary-protected product **21** can be cleaved using General Method C.

3.3.3.5 Synthesis of (2*R*,3*R*)-3-fluoro-2-methyldecanoyl-CoA 17

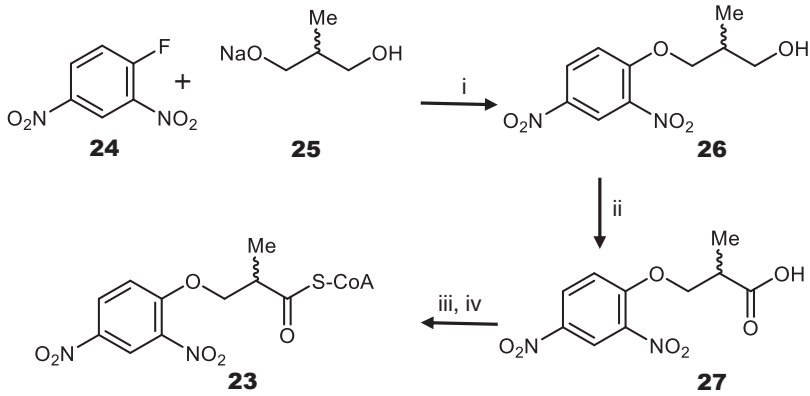
The acyl-CoA ester can be prepared from **22** following General Method D.

3.3.4 Synthesis of colorimetric substrate 23

Synthesis of colorimetric substrate **23** (Scheme 8) was performed by coupling Sanger's reagent **24** with 2-methylpropan-1,3-diol **25** to give **26**. The alcohol **26** was oxidized to the corresponding carboxylic acid **27**, which then converted to the acyl-CoA ester **23** using General Method D (Yevglevskis et al., 2017).

3.3.4.1 Synthesis of 2*R*,*S*-3-(2,4-dinitrophenoxy)-2-methylpropan-1-ol 26

1. Add 2-methyl-1,3-propanediol **25** (2.538 g, 28.2 mmol, 4.6 eq.) to a round bottom flask fitted with a suba seal (rubber septum). To this add sodium metal (141 mg, 6.12 mmol, 1.0 eq.). **Sodium is stored under oil and should be handled extremely carefully as it reacts violently with water.** Excess oil can be removed by wiping with tissue and small portions of sodium can be carved with a knife to expose clean surfaces free of oxidation. Excess sodium can be disposed of using isopropanol.
2. Stir the reaction for 6 h (or until the metal has fully dissolved).
3. Add 1-fluoro-2,4-dinitrobenzene (Sanger's reagent, **24**) (1.252 g, 6.73 mmol, 1.1 eq.) and stir the reaction mixture at 80 °C for 2 h and then at room temperature for a further 16 h.
4. The reaction mixture is extracted with diethyl ether (20 mL) three times, using a separating funnel.
5. The combined organic layers are washed with ice-cold water (10 mL).



Scheme 8 Synthesis of colorimetric substrate **23**. *Reagents and conditions*: (i) Na metal, 83%; (ii) CrO_3, conc. H_2SO_4, acetone, 67%; (iii) CDI, DCM; (iv) CoA-SH tri-lithium salt, 0.1 M $NaHCO_3$ aq./THF (1:1) (Yevglevskis et al., 2017).

6. Dry the organics using solid portions of $MgSO_4$—the solution is dry when further solid portions are found to be free flowing on swirling the mixture in a conical flask.
7. Filter the mixture and remove the volatile organics from the filtrate using a rotary evaporator.
8. The crude product **26** can be purified using silica gel column chromatography with a mobile phase of petroleum ether/ethyl acetate (1:1).

3.3.4.2 Synthesis of 2*R*,*S*-3-(2,4-dinitrophenoxy)-2-methylpropanoic acid 27

1. Add **26** (1.02 g, 3.98 mmol, 1.0 eq.) to a round bottom flask and dissolve in acetone (18 mL).
2. In a separate flask mix water (1.4 mL) and concentrated H_2SO_4 (0.65 mL). **Take care with concentrated H_2SO_4 as this is extremely corrosive—suitable Personal Protective Equipment should be used.**
3. To this add CrO_3 (756 mg, 7.56 mmol, 1.9 eq.).
4. Stir this mixture for 5 min
5. Add the CrO_3 solution dropwise over 1.5 h to the solution of **26**.
6. Stir the resulting mixture for 2 h then add isopropanol (0.3 mL).
7. Leave the reaction to stir for a further 16 h.
8. Filter the reaction mixture through celite—use a sintered filter funnel with a layer of celite (around 1 cm deep).
9. Wash the celite with DCM (20 mL).
10. Combine the filtrates and using a separating funnel wash them with water (two portions of 20 mL).
11. Wash the combined organic layers with brine.
12. Dry the organics using solid portions of $MgSO_4$—the solution is dry when further solid portions are found to be free flowing on swirling the mixture in a conical flask.
13. Filter the mixture and remove the volatile organics from the filtrate using a rotary evaporator.
14. The crude product can be purified using silica gel column chromatography with a mobile phase of petroleum ether/ethyl acetate (1:1).

3.3.4.3 Synthesis of 2*R*,*S*-3-(2,4-dinitrophenoxy)-2-methylpropanoyl-CoA 23

The acyl-CoA ester **23** can be prepared using General Method D (Section 3.3.1.4).

3.4 Expected outcomes, advantages and limitations

3.4.1 Synthesis of precursor acids

The reported methods give access to a wide range of precursor acids suitable for the generation of AMACR substrates (Darley et al., 2009a; Yevglevskis et al., 2017; Yevglevskis, Lee, et al., 2014), including labeled compounds. Extension of this methodology provide inhibitor precursors (Yevglevskis, Bowskill, et al., 2014; Maksims Yevglevskis et al., 2018; Yevglevskis et al., 2019) and could be further extended to provide precursors to novel inhibitors. When synthesizing labeled compounds, it is recommended that any novel route is tested using non-labeled starting materials to ensure that it works.

3.4.2 Synthesis of acyl-CoA esters (General Method D)

General Method D gives reliable synthesis of acyl-CoA esters, which we have used to synthesize a wide range of substrates and inhibitors for AMACR and other enzymes (Kershaw et al., 2001; Woodman et al., 2011; Yevglevskis, Bowskill, et al., 2014; Yevglevskis et al., 2017; Yevglevskis, Lee, et al., 2014; Yevglevskis et al., 2019). The method even works for precursor acids which have strongly electron-withdrawing substituents within the side-chains (which will decrease the nucleophilicity of the precursor carboxylic acid group) with reasonable synthetic yields (Yevglevskis et al., 2019). The method works on different scales, but we have found that the reported scale works well for routine synthesis of acyl-CoA esters for testing using synthesized or commercially-available carboxylic acids.

The synthetic method uses a 2-fold molar excess of carbonyl diimidazole (CDI) compared to precursor acid to effect quantitative conversion of the precursor acid to the corresponding imidazolide. The excess CDI will also react with any traces of water within the solvent, ensuring that the reaction conditions are dry. We also use a 2.5-fold excess of imidazolide intermediate (Scheme 5, **8**) compared to reduced CoA to ensure maximum formation of the acyl-CoA. The two-step coupling protocol maximizes coupling efficiency and enables the presence of the imidazolide intermediate to be checked by ^{1}H NMR when using acids which may be difficult to couple; this also allows the integrity of the CDI to be easily checked (which will hydrolyze is the presence of moisture) using an acid which is known to be coupled. Key to the success of the method is the quality of the CDI and CoA. The latter should be stored tightly sealed at −80 °C to minimize oxidation of the reduced sulfhydryl form to the disulfide form. The use of reversed-phase solid-phase extraction allows facile purification of the product acyl-CoA whilst removing the imidazole product and any salts from the sample.

We routinely characterize our synthetic acyl-CoA esters using ^{1}H NMR (and ^{19}F NMR where fluorine is present) and high-resolution electrospray mass spectrometry. Sample concentrations are generally too low to use ^{13}C NMR unless the acyl-CoA is isotopically labeled. This is a higher level of characterization used by many workers, who only use mass spectrometry as an analytical method. If an additional test of purity is required, we recommend use of analytical reversed-phase HPLC (Carnell et al., 2007; Carnell et al., 2013). We routinely determine the concentration of our acyl-CoA product using ^{1}H NMR as described in Section 4.5, but other methods are available e.g., use of a known concentration of internal standard within the NMR sample and comparison of integral sizes (Pal, Easton, Yaphe, & Bearne, 2018; Pal, Khanal, Marko, Thirumalairajan, & Bearne, 2016). We did not use absorbance at 260 nm as reported by some previous workers (Carnell et al., 2007) since aromatic side-chains within the acyl moiety will also absorb at this wavelength (and hence an accurate extinction coefficient would not be determined). There are also colorimetric methods for quantifying acyl-CoA esters based on their reaction with hydroxylamine, but 3-ketoacyl-CoA esters are resistant to treatment (Stadtman, 1957). The released CoA-SH can be quantified using DTNB (Ellman's reagent) (Eyer et al., 2003; Riddles, Blakeley, & Zerner, 1979), but this is known to give a small false positive color reaction even at pH 7.27 which gives the lowest background absorbance (Riddles et al., 1979). There are other colorimetric assays which can be used (Stadtman, 1957), but these each have their own disadvantages.

3.5 Optimization and troubleshooting

These experiments are robust and optimized, and little further optimization is needed. Failure of General Method D is almost always due to exposure of the CDI to moisture, which readily hydrolyzes to imidazole. This problem is most easily diagnosed using a control reaction with an acid known to efficiently produce the corresponding imidazolide. It is important to check the different fractions eluting from the SPE column as the ones containing the acyl-CoA product will be determined by its lipophilicity. The acetonitrile concentrations used to elute the SPE column may need to be adjusted if the acyl-CoA product is likely to be particularly hydrophilic or lipophilic. Computational methods exist for computing log*P* values. A small amount [$\sim$ 1% (v/v)] of acetonitrile may be required in the equilibration and aqueous wash buffers to prevent hydrophobic collapse of the reversed-phase media (which may result in sub-optimal purification of hydrophilic acyl-CoA esters).

3.6 Safety considerations and standards

Eye protection and a lab coat should be worn at all times whenever working with solvents, acids, bases, vacuums (including when using the Büchi rotary evaporator or freeze-dryer), or high pressures. Appropriate Personal Protective Equipment (PPE) including gloves, should be worn where there is a risk that hands may encounter toxic, carcinogen, or hazardous chemicals (for example, carbonyl diimidazole is a very good electrophile and acylating agent). **CAUTION: Extreme care should be taken when using both sodium metal and *n*-BuLi. Sodium metal reacts violently with water and can cause fires where flammable solvents are present—keep flammable solvents away from the reaction, use the minimum quantity of sodium metal possible and have material available to quench any fire if it occurs. For this either a fire blanket or bucket of dry sand would be the best options. Similar precautions are suitable for the use of *n*-BuLi.**

Dry ice and liquid nitrogen should be used in well-ventilated areas (otherwise there is a risk of suffocation). **Liquid nitrogen (and dry ice/acetone) will cause severe burns. A facemask and cryogenic gloves should be worn when handling these materials.** Insulated cryogenic tongs may be used for delicate work when gloves are impractical. The freeze-dryer operates at high vacuums and flasks placed on the "tree" should be carefully inspected for "star cracks" before use, clipped onto the freeze dryer, and covered in Saran Wrap (Cling Film). Chemical waste should be disposed of through approved routes. Waste halogenated solvents such as chloroform and DCM should be disposed of separately from non-halogenated solvents (THF and ethyl acetate). Risk assessments should be completed according to institutional guidelines before any experimental work is undertaken. Good Laboratory Practice and all local rules should be followed.

4. NMR assays of recombinant human AMACR 1A

4.1 Key resources table

Reagent or Resource	Source	Identifier
Chemicals, Peptides, and Recombinant Proteins		
Acyl-CoA esters	Various	See details in Section 3
$^{2}H_2O$	Merck	151882
HCl [~35% (w/v) aq. solution]	Merck	087617.AK

NaH_2PO_4	Thermo Fisher Scientific	10133153
NaOH	Thermo Fisher Scientific	10192863
Recombinant AMACR 1A	In-house	Section 2
$CHCl_3$	Merck	650498
$C2HCl_3$	Merck	151823
N, *N*'-dicyclohexylcarbodiimide	Merck	D80002
S-1-phenylethylamine	Merck	8070470010

4.2 Materials and equipment

All solutions are aqueous unless otherwise noted and should be made up in 18.2 MΩ/cm Milli-Q water (Millipore) or equivalent.

- pH meter and calibration solutions at pH 4.0, 7.0 and 10.0.
- 5 M and 1 M aq. NaOH.
- ~10 M and ~1 M HCl [35% (w/v) purchased stock and a 1 in 10 dilution in water].
- 500 mM NaH_2PO_4–NaOH, pH 7.4 (we generally make up ~100 mL, aliquot into several small batches and autoclave to keep sterile and prevent microbial growth. Stocks are stable for several weeks at room temperature if unopened or if re-autoclaved soon after opening). **NB: Do not store this stock solution at 4 °C as the phosphate will precipitate.**
- Aqueous stock solutions of acyl-CoA esters which may include the following: *R*- or *S*-2-methyldecanoyl-CoA (Darley et al., 2009a); straight-chain acyl-CoA esters (Sattar et al., 2010); Ibuprofenoyl-CoA and related acyl-CoA esters (Woodman et al., 2011); 3-Fluoro-2-methylacyl-CoA esters (Yevglevskis et al., 2016; Yevglevskis, Lee, et al., 2014); 2-aryl-thiopropanoyl-CoA esters (Yevglevskis et al., 2019); colorimetric substrate (Petrova et al., 2019; Yevglevskis et al., 2017); and assorted other acyl-CoA esters (Yevglevskis, Bowskill, et al., 2014; Maksims Yevglevskis et al., 2018). Stocks should be stored at −80 °C.
- 10 μL, 20 μL, 200 μL and 1 mL Gilson pipettes, and corresponding clear, yellow, and blue tips.
- 1.5 mL Eppendorf tubes.
- 5 mm NMR tubes.
- Bench-top centrifuge capable of taking 1.5 mL Eppendorf tubes.

- Vortex mixer.
- Water baths set at 30 and 50 °C.

Alternatives:

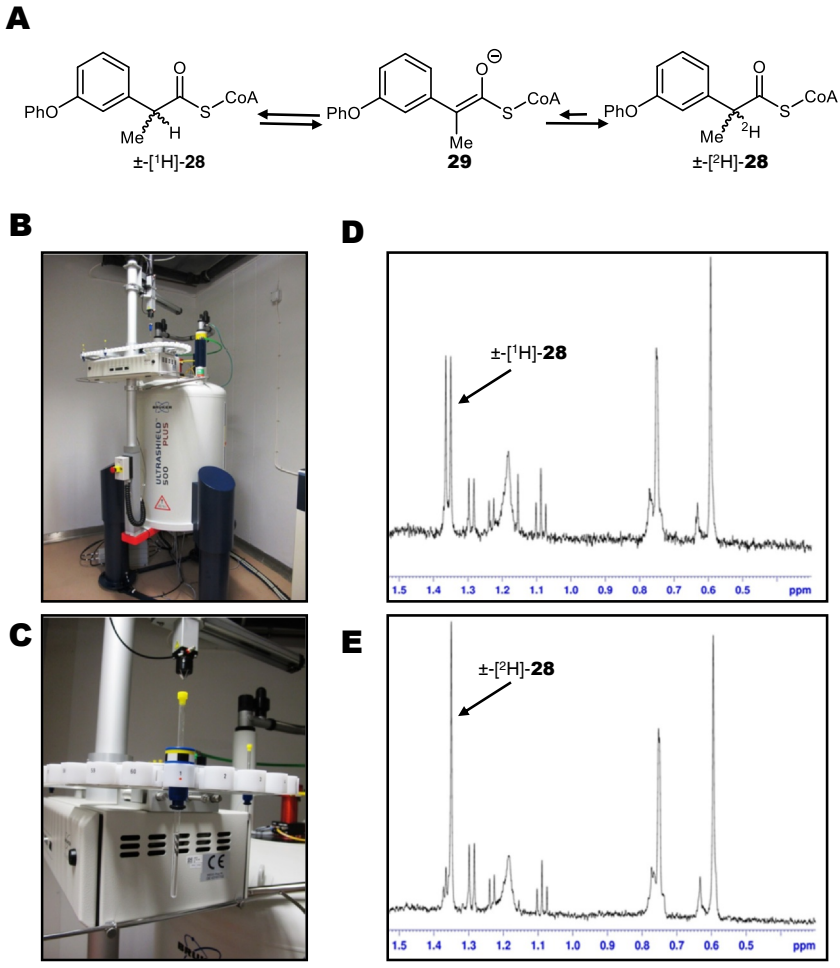
ACS or similar quality reagents are required. Equipment with similar specifications may be used.

4.3 Step-by-step method details

4.3.1 ^{2}H-exchange assay for acyl-CoA ester substrates

Exchange of the α-proton or protons of an acyl-CoA ester is a surrogate for measuring change in stereochemical configuration of a 2-methylacyl-CoA substrate such as fenoprofenoyl-CoA ± -[^{1}H]-**28** (Scheme 9). Deprotonation to give an enolate intermediate **29** is an obligatory pre-requisite (Darley et al., 2009a). Re-deuteration gives product ± -[^{2}H]-**28**. The assay works with a 1 to 1 mixture of epimers at carbon-2 (as shown in Scheme 9) or with single epimers (in which case a change of stereochemical configuration will occur) (Darley et al., 2009a; Woodman et al., 2011). The extent of ^{2}H-exchange is about twice that of the change in stereochemical configuration (Darley et al., 2009a) for single epimers, as the deprotonated intermediate is re-deuterated to give a near 1 to 1 mixture of epimeric products (Darley et al., 2009a; Woodman et al., 2011), differing in the stereochemical configuration at carbon-2 (Darley et al., 2009a). Exchange of α-protons of straight-chain acyl-CoA esters can also occur, without any change in stereochemical configuration (Sattar et al., 2010).

1. Calculate the required volumes of stock reagents to make up the assay mixture (550 μL final volume): 500 mM Phosphate buffer, pH 7.4 (55 μL); $^{2}H_2O$ (478 μL); Fenoprofenoyl-CoA ± -[^{1}H]-**28** substrate (4.49 mM stock, 12.2 μL); Enzyme (7.2 mg/mL, 4.6 μL). Note that if including organic drug molecules in the assays, solutions will be in DMSO-D_6 in which case the volume of $^{2}H_2O$ will need to be reduced accordingly and a DMSO-only control may also be required. We have previously demonstrated that up to 10% (v/v) DMSO has little effect on AMACR enzymatic activity, as measured by the colorimetric assay (Yevglevskis et al., 2017).
2. Equilibrate two water baths at 30 °C (for the assay) and 50 °C (to quench the reaction), respectively.
3. Thaw the enzyme and substrate stock solutions (kept at −80 °C) either in your hands or using water in a beaker at ~35 °C (just warm to the touch) with gentle periodic mixing. Rapid freezing and thawing of



Scheme 9 The NMR deuterium assay illustrated using fenoprofenoyl-CoA ± -[^{1}H]-**28** as substrate. (A) Reaction catalyzed by AMACR showing enolate intermediate **29**. (B) The Bruker Avance III NMR spectrometer. (C) Samples in the NMR tube waiting to be loaded into the spectrometer. (D) NMR spectra of negative control with heat-inactivated enzyme, showing doublet at ~1.35 ppm. (E) NMR spectra of positive control with active enzyme, showing single peak (triplet with J = ~1 Hz) at ~1.35 ppm due to presence of α-^{2}H. *Reproduced from* Yevglevskis, Bowskill, et al. (2014) with permission from the Royal Society of *Chemistry*.

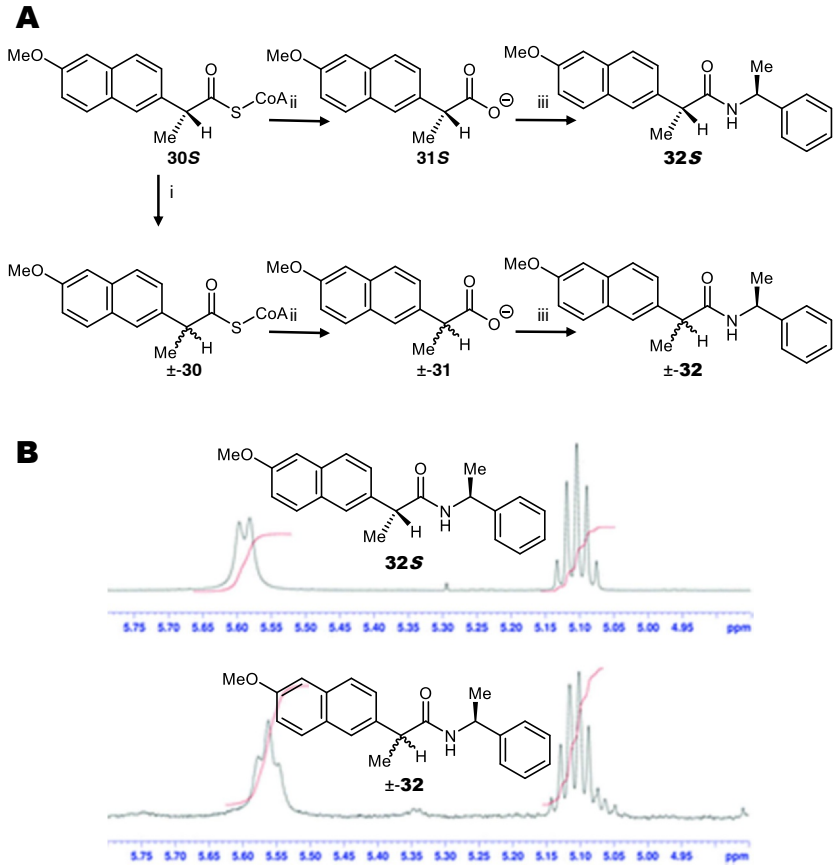
buffers in the enzyme stocks limits changes in pH (Pikal-Cleland et al., 2000), which otherwise could denature the enzyme. Keep the enzyme in an ice/water bath and the substrate at ambient room temperature once thawed.

4. Prepare negative control samples (inactivated enzyme) by heating enzyme stock solution in a 1.5 mL Eppendorf tube at 95 °C for 10 min in a hot block. Briefly centrifuge the sample in a bench-top centrifuge and place sample in the ice/water bath until use. Note that negative controls will be required for each different acyl-CoA substrate or substrate concentration. PRO-TIP: add water to the hot block well (if safe to do so) to ensure efficient heat transfer to the sample.
5. Pipette buffer and $^{2}H_2O$ into the required number of 1.5 mL Eppendorf tubes and close the lids.
6. Vigorously vortex-mix the substrate stock solution for 5 min, followed by centrifugation at 13,600 rpm in a bench-top centrifuge for 2 min to remove bubbles. This is essential because acyl-CoA esters are amphipathic and often form micelles with a variable propensity.
7. Open the lid of each individual Eppendorf tube, and pipette acyl-CoA stock solution into each solution and close the lid. Do this individually for each tube to minimize deuterium-exchange with atmospheric moisture. Vortex-mix each Eppendorf tube containing assay mixture for two minutes followed by one to two minutes of centrifugation to remove micelles. This should be done immediately prior to initiating the assay by adding enzyme.
8. Pipette enzyme solution (assays) or heat-inactivated enzyme (negative controls) onto the inside of the lid of the Eppendorf tube. Carefully close the lids and place the Eppendorf tubes into a bench-top centrifuge and initiate the assay by centrifuging at 13,600 rpm for 15 s. Place the tubes into an Eppendorf tube float and transfer into the 30 °C water bath as quickly as possible. Alternatively, immediately place the assay into a 5 mm NMR tube for time-course analysis.
9. After 1 h transfer the Eppendorf float and tubes to the 50 °C water bath for 10 min followed by centrifuging the tubes for 15 s (to bring down all the liquid into the bottom of the tube). **Note that samples may be stored at −80 °C overnight at this point. If doing so, thaw the samples using warm water as described in step 3 and vortex-mix the samples as described in step 7 (otherwise any micelles will interfere with subsequent NMR analysis)**. Transfer each sample into a 5 mm NMR tube using a glass pipette ready for analysis.

4.3.2 Analysis of the stereochemical outcome of the reaction of 2-methylacyl-CoA esters with AMACR

These techniques can be used to determine the stereochemical configuration of the α-carbon [bearing the methyl group in the 2-methylacyl-CoA esters (Darley et al., 2009a; Woodman et al., 2011; Yevglevskis, Bowskill, et al., 2014)]. This can be used to measure equilibrium constant for the enzymatic reaction. Following incubation of the substrate (*S*-naproxenoyl-CoA **30*S***, Scheme 10A) with AMACR to give **±-30**, the products are hydrolyzed to **±-31** and derivatized to **±-32**. Negative controls using heat-inactivated enzyme give **31*S*** and **32*S*** as no change in stereochemical configuration occurs (Scheme 10A). Experiments to determine the ratio of deuterium wash-in and the change in stereochemical configuration (Darley et al., 2009a) are performed in a similar manner, except that the assay is performed in presence of ^2H_2O and substrate concentrations will typically be 100 μM.

1. To measure the equilibrium constant, pool samples to be treated (containing ~10 mg in non-deuterated reaction mixture in ~20 mL total volume) together in a 50 mL Falcon tube. Add ~3.8 mg of recombinant AMACR and incubate at 30 °C overnight (~16 h). Perform a negative control under the same conditions lacking enzyme or containing heat-inactivated enzyme.
2. Add 1 mL of 10 M aq. NaOH and incubate at 30 °C for 30 min [the half-life of acyl-CoA esters is reported to be 1–2 min in 0.1 *N* NaOH (Stadtman, 1957)]. This hydrolyzes the acyl-CoA esters (Darley et al., 2009a; Woodman et al., 2011).
3. Acidify the reaction mixture with aq. 10 M or 1 M HCl to around pH 4, checking the pH by spotting a small volume of mixture onto pH paper. Transfer the acidified mixture into a separating funnel and extract with 3 × 5 mL of $CHCl_3$. After each extraction carefully separate the lower (organic) layer from the upper (aqueous) layer.
4. Dry the pooled organic layer, filter, and remove the solvent under vacuum as described in Section 3.3.14 Step 5. The suggested vacuum will be specified in the manual for the Büchi rotary evaporator. Redissolve the residue in 600 μL of C^2HCl_3, transfer the sample into a 5 mm NMR tube, and quantify the acid using ^1H NMR (see Section 4.5.1.)
5. Transfer the sample to a small round-bottom flask and add 1.5 mol equivalents of *S*-1-phenylethylamine followed by 1.5 mol equivalents of *N*, *N*'-dicyclohexylcarbodiimide in dry C^2HCl_3. Allow the reaction to proceed for at least 30 min, filter if required, and analyze by ^1H NMR.



Scheme 10 Determination of stereochemical configuration of substrates following incubation with AMACR. (A) Assay and chemical derivatization of products, as illustrated with *S*-naproxenoyl-CoA **30*S*** and heat-inactivated (top) and active enzyme (bottom). *Reagents and conditions*: (i) active AMACR, phosphate buffer; (ii) ~0.5M aq. NaOH; (iii) *N*, *N*′-dicyclohexylcarbodiimide, *S*-1-phenylethylamine, C_2HCl_3; (B) ^{1}H NMR analysis of naproxenoyl *S*-1-phenylethylamides **32**. The spectra show the signals for the α-proton of *S*-1-phenylethylamide and the NH of the amide linkage. Top: Without exposure to enzyme; Bottom: Following exposure to active AMACR 1A. The signals appear as two superimposed doublets at 5.55 ppm and two superimposed quintets at 5.10 ppm. *Reproduced from Woodman et al. (2011) with permission from the Royal Society of Chemistry.*

4.4 Expected outcomes, advantages and limitations

We have reported protocols for assays of several different AMACR substrates using NMR techniques. A key technique is the ^{2}H-exchange assay, as this allows facile evaluation of a wide range of compounds as AMACR substrates (or evaluation of the potential for inhibitor α-proton exchange). This is marked contrast to radiochemical assays (Bhaumik et al., 2007; Savolainen et al., 2005), where separate synthesis of each radiolabeled substrate is required. A key advantage of the NMR assay is that it allows product characterization, and this led to the recognition that AMACR could catalyze elimination reactions (Yevglevskis, Lee, et al., 2014) as well as the known reaction resulting in the change in stereochemical configuration (Darley et al., 2009a; Woodman et al., 2011).

The physiological reaction catalyzed by AMACR is relatively simple as the epimeric substrate and product have identical ^{1}H NMR spectra (because the chiral centers within the CoA moiety are remote from the center undergoing the change in stereochemical configuration). Hence the developed assay uses direct analysis of unpurified reaction mixtures. We chose to use phosphate buffer within the assay because this is silent within ^{1}H (and ^{19}F) NMR spectra, but other deuterated buffers could be used. The pH value of phosphate buffer decreases with increasing temperature (Stevens, 1992), but these changes will be less than 0.1 pH units even on going from ambient room temperature to 50 °C. On the other hand, inclusion of $^{2}H_2O$ will increase the pH value [by ~0.5 pH units for fully deuterated solvent (Schowen, 2007), less when only part deuteration of solvent is used, as in this case]. AMACR is known to have relatively broad pH optimum with high activity between ~5.5 and 8.5 (Schmitz, Albers, Fingerhut, & Conzelmann, 1995; Schmitz, Fingerhut, & Conzelmann, 1994; Shieh & Chen, 1993), and the "pH" of the reaction will be within this range during the assay. The technique can be used to estimate kinetic parameters for the substrate (Darley et al., 2009a, 2009b; Woodman et al., 2011), providing that initial rates are used. Individual (not averaged) rate measurements at different substrate concentrations should be used in this determination, and data can be plotted using standard methods (Brooke et al., 2023).

The main draw-back of this technique is the operation of a solvent kinetic isotope effect (Pirali, Serafini, Cargnin, & Genazzani, 2019; Watt, 2010) lowering measured rates. The magnitude of this effect will be dependent on the extent of substrate conversion and the proportion of $^{2}H_2O$ present in the assay mixture. On the other hand, incorporation of

deuterium into product will dampen the reverse reaction, favoring the conversion of ^{1}H-containing substrate. Again, the magnitude of this effect will be dependent on the extent of conversion, as the backwards reaction becomes more likely as the ^{2}H-labeled product becomes more prevalent in the reaction mixture. The presence of $^{2}H_2O$ in the reaction mixture will have less impact on reaction rates for eliminating substrates (Yevglevskis et al., 2017; Yevglevskis, Lee, et al., 2014), as these reactions are irreversible, and deuterium is not incorporated into the product.

An alternative strategy is to conduct the assay in $^{1}H_2O$-based buffers followed by freeze-drying of the reaction mixture and redissolving the residue in $^{2}H_2O$ and NMR analysis (Baldwin et al., 1990; Baldwin et al., 1991; Baldwin et al., 1992, 1997; Baldwin & Bradley, 1990; Baldwin, Lee, et al., 1993; Baldwin, Lloyd, et al., 1993; Lloyd et al., 1999). These assays require the use of a volatile buffer (typically NH_4HCO_3) and allow incubation on a larger scale. These assays are more labor-intensive but avoid the issue of kinetic isotope effects, allow analysis of more complex reaction mixtures, and furnish isolated compounds for characterization and further experiments. In some experiments, products decompose upon freeze-drying in the presence of NH_4HCO_3 and mild acidification of the crude reaction mixture is required (Baldwin et al., 1990; Baldwin et al., 1991; Baldwin et al., 1992, 1997).

4.5 Quantification and data analysis methods

4.5.1 Data collection and referencing

Sensitivity is the biggest limiting factor in utilizing NMR for enzyme studies, and generally the highest sensitivity equipment should be used. Spectrometers operating at 500 MHz for ^{1}H are routine and are suitable for this. Signal to noise is critical when investigating samples at low concentrations and this is aided by use of the highest sensitivity probes, where possible. For instance, many institutions will have NMR probes set up for biological samples that produce intrinsically high signal to noise for ^{1}H observation. Other factors are important—high quality NMR tubes should be chosen (certainly they should be specified for use at 500 MHz and above). Shimming of samples is crucial to ensure good line-shape, and modern spectrometers will have the ability to use automatic gradient shimming, either on deuterium signals from deuterated solvents, or on proton signals, for instance where experiments are conducted in either pure $^{1}H_2O$, or 90% $^{1}H_2O$/10% $^{2}H_2O$.

One issue of importance is the quantification of the CoA esters. As noted above, previous works have made use of a variety of methods,

including using internal standards in NMR (Pal et al., 2016; Pal et al., 2018) and the UV absorbance of solutions (based on the aromatic part of the CoA molecule) (Carnell et al., 2007; Carnell et al., 2013) and other colorimetric methods (Eyer et al., 2003; Riddles et al., 1979; Stadtman, 1957). However, measuring the absorbance of the adenosine moiety is not suitable when there may be other chromophores in the CoA ester under study, e.g., ibuprofenoyl-CoA, as these will alter the extinction coefficient. As a result, NMR methods of quantification are useful. This area has been reviewed in several comprehensive papers with extensive examples (Pauli, Godecke, Jaki, & Lankin, 2012; Pauli, Jaki, & Lankin, 2005; Simmler, Napolitano, McAlpine, Chen, & Pauli, 2014).

Broadly there are two main ways to quantify an NMR sample.

1) Use of a standard of fixed and known concentration in the sample.
2) Use of electronic methods [e.g., an electronic reference signal external to the sample (Claridge, 2016)].

For the first method, using internal standards, two variants are possible. In the first, the reference material is introduced to the sample under test. This may be problematic in enzyme assays as there is the danger of interference with the enzyme activity. Standards may be introduced after the enzymatic reaction has finished. Alternatively, an NMR sample tube with a capillary insert can be used. This can take a solution in the same solvent as the sample, or it can be a different solvent. For instance, if an assay is being conducted in $^{1}H_2O$, the capillary insert could have $^{2}H_2O$ and an appropriate internal standard (IS). Suitable internal standards are required to be soluble in the solvent of choice and able to give NMR signals in regions of the NMR spectrum where no other signals will occur (Rundlof et al., 2010). For aqueous samples a range of standards exist including maleic acid (*cis*-butenedioic acid) or its sodium salt, fumaric acid (*trans*-butenedioic acid) or its sodium salt, sodium acetate (sodium ethanoate), and d_4-trimethylsilylpropionic acid, sodium salt. The last is notable as it bears a trimethylsilyl-group, equivalent to the use of tetramethylsilane in organic solvents, producing a singlet peak set at 0 ppm. Other requirements are important for these standards—notably that they are solids that are not hygroscopic and can be accurately weighed. It is also important to take account of the purity of the standard.

Two variants of electronic methods are commonly used. In one, known as ERETIC, (Electronic Reference To access In-vivo Concentrations), a

small electronic radio frequency (rf) signal is generated and added to the free induction decay (FID) (Akoka, Barantin, & Trierweiler, 1999; Barantin, LePape, & Akoka, 1997). On processing this provides an additional line that can be used for calibration (after suitable calibration against an accurate standard itself). Other approaches make use of recording the NMR spectrum of an accurately prepared sample and then using the same experiment, with identical parameters, for the unknown sample. Simple comparison can be used with reasonable accuracy.

Although it is widely believed that an NMR resonance is proportional to the relative number of nuclei giving rise to the signal, this is only valid under well-defined experimental conditions, and routine ^{1}H experiments, for instance, may only be accurate to within 10–20%. The other well-known belief is that ^{13}C spectra should not and cannot be integrated. This is certainly true for routine acquisition conditions, but with appropriate care it is possible to meaningful concentration data of carbon (and indeed other) nuclei.

The use of multiple pulses to acquire data requires that the perturbation of the spins of the nuclei is allowed to fully relax between pulses. This entails recycling times of at least $5 \times T_1$ of the slowest relaxing nuclei. If this condition is met, then 90-degree pulse angles may be used, maximizing the signal per transient. If smaller tip angles are used, such as 30 degrees, then shorter delays may be used. To ensure this, one could measure all the T_1 times for the nuclei, however in practice previous knowledge is usually available on related compounds.

One further source of intensity distortion in heteronuclear spectra recorded with broadband proton decoupling is the influence of Nuclear Overhauser Effect (NOE) arising from saturation of nearby protons. Since the chemical environment at each heteroatom nuclei will differ, different levels of NOE will be experienced. To counter this data can be acquired with measures taken to suppress the NOE, while still retaining the benefits of proton decoupling (minimizing signal overlap and giving optimum signal to noise). Combining the lack of NOE and the need to pulse slowly means that quantitative measurements on such nuclei can require extended time periods.

4.5.2 Data processing

Once data has been collected, further steps during processing can enhance results. This includes the application of exponential functions to broaden lines (typically for hetero nuclei) and zero-filling to assist in definition of

the line-shape. Spectra must also be correctly phased as deviations from pure absorption-mode will reduce integrated intensities. Integration is also important and correct choice of start and finish points is significant. Ideally an integral should cover 10–20 Hz either side of the peak in ^{1}H spectra. However, this is not always possible due to the proximity of other resonances. Carbon satellites can be troublesome as they represent around 1% of the total signal and overlap from larger signals may be significant.

One approach that may be used is direct line-shape fitting, where resonances are decomposed into clusters of singlet peaks (based on either Lorentzian or Gaussian shape). This has applicability in removing overlapping signals and may be of use, notably with proton–deuterium exchange experiments (see below).

4.5.3 NMR assays with double labeled substrates

Routine observation of ^{13}C nuclei in substrates at concentrations suitable for enzyme assays is challenging. This is due to several reasons including the smaller gyromagnetic ratio (roughly a quarter of that of ^{1}H) and the fact that ^{12}C is not an NMR nucleus (it has a spin quantum number of zero) and makes up nearly 99% of the carbon in naturally occurring samples. To some extent this issue can be offset by using enriched substrates, as is common in protein NMR studies. In double labeled experiments, deuterium is also present. While the ^{13}C spectra are recorded with ^{1}H decoupling, reducing carbon signals to singlets by suppressing the proton coupling, any carbon nuclei attached to one or more deuterium atoms will show carbon–deuterium coupling (as ^{2}H has a spin quantum number of one, each ^{2}H splits the carbon signal into three lines).

In experiments conducted with double labeled substrates in $^{1}H_2O$, the loss of deuterium produces two effects, Firstly the coupling from the ^{2}H atom is lost, and the carbon resonance will now appear as a singlet (assuming only one ^{2}H atom was originally present). Secondly it is common that replacement of ^{2}H with ^{1}H (or vice versa) results in a small change in the chemical shift for the carbon. Thus, the new peak for the now protonated substrate occurs at 47.55 ppm, whereas the deuterated starting material shows a shift of 47.11 ppm (Darley et al., 2009a). This allows for easy integration of the peaks (assuming all criteria for quantification have been met), and this allows the course of the reaction to be followed.

Following the loss of the α-proton in unlabeled substrates in $^{2}H_2O$ using ^{1}H NMR is slightly more complicated. Here, the methyl signal may be used. Initially the methyl is seen as a doublet at ~1.36 ppm (or ~1.0 ppm

for substrates with alkyl side-chains), arising from $^{3}J_{HH}$ coupling (of around 6.8 Hz). Replacement of the proton with a deuteron changes the methyl signal to a slightly broadened singlet (the $^{3}J_{HD}$ coupling is too small to show splitting patterns) (Scheme 9). There is also a small up-field shift in the methyl signal (note this is smaller than the change observed in ^{13}C spectra as the deuterium atom is not directly attached). The result is that the new singlet overlays the right hand (up-field) half of the doublet of the starting material. To extract useful integration data from this experiment, the assumption is made that the left hand (down-field) half of the integral will be matched by the right hand (up-field half) and can thus be subtracted to give the integration of the product.

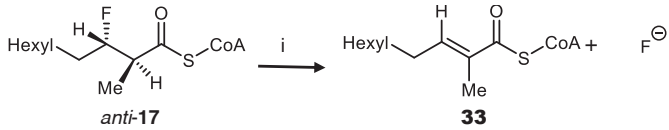
4.5.4 NMR assays with eliminating substrates

The initial design of the fluorinated substrate **17** was to allow for direct observation via ^{19}F NMR of epimerization and proton/deuterium exchange, as the fluorine resonance would shift on epimerization and show different coupling with loss of the α-proton. However, the substrate **17** underwent a fluoride elimination reaction to produce an unsaturated compound instead. Although not the intended outcome this allowed the enzyme catalyzed reaction to be followed very clearly, as the initial doublet at 1.1 ppm declines with a new methyl signal appearing at 1.8 ppm (Scheme 11). In addition, a new peak at 6.15 ppm for the alkene product **33** also appeared (Yevglevskis et al., 2016; Yevglevskis, Lee, et al., 2014). This allowed the quantification of inhibitor activity by ^{1}H NMR analysis (Yevglevskis et al., 2016).

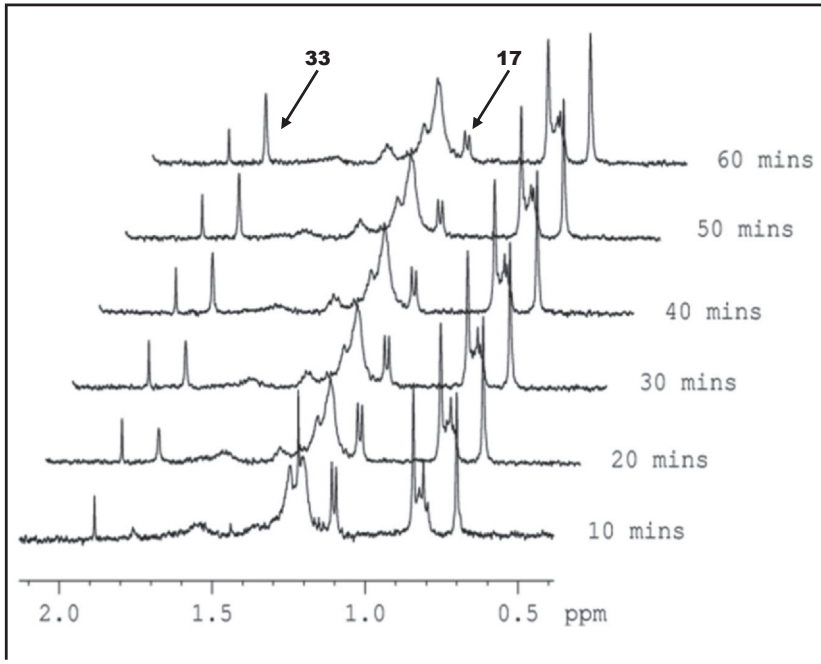
Following the realization that elimination reactions could be induced by AMACR, we set out to prepare a colorimetric substrate **23**, by eliminating 2,4-dinitrophenolate **34** to give alkene product **35** (Scheme 12). This being successful obviated the need for following the assay by NMR, although similar changes to the ^{1}H spectrum can be observed, as an unsaturated product is produced (Yevglevskis et al., 2017).

4.6 Optimization and troubleshooting

These experiments are robust and optimized, and little further optimization is needed. Well-calibrated pipettes and good pipetting technique are key to generating good data. Positive controls containing enzyme and known substrates and negative controls containing heat-inactivated enzyme should always be used.



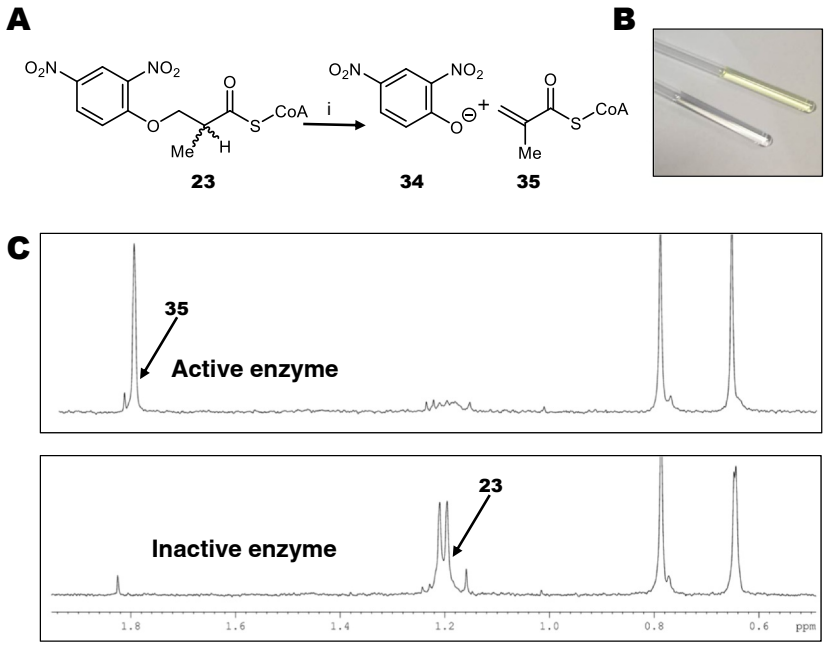
anti-**17** **33**



Scheme 11 Elimination of fluoride in substrate **17** by AMACR to give unsaturated product **33**. The reaction can be followed by the disappearance of the substrate methyl doublet at ~1.1 ppm and the appearance of the product methyl singlet at ~1.8 ppm. *Reagents and conditions*: Active AMACR, phosphate buffer, ~85% (v/v) $^{2}H_2O$. *Reproduced from* Yevglevskis, Lee, et al. (2014) *with permission from the Royal Society of Chemistry.*

4.7 Safety considerations and standards

Eye protection and a lab coat should be always worn, and appropriate Personal Protective Equipment (PPE) including gloves, should be worn where there is a risk that hands may come encounter toxic, carcinogen, or hazardous chemicals. Chemical waste should be disposed of through approved routes (including for halogenated solvents), and biological waste should be decontaminated by autoclaving or other approved methods. Care should be exercised when using a hot block to heat-inactivate enzyme for use in negative controls. Risk assessments should be completed



Scheme 12 Reaction of colorimetric substrate with AMACR. (A) Elimination of substrate **23** to give 2,4-dinitrophenolate **34** and unsaturated product **35**. *Reagents and conditions:* (i) active AMACR, phosphate buffer, ~85% (v/v) $^{2}H_2O$; (B) NMR samples showing negative control with heat-inactivated enzyme (colorless) and positive control with active enzyme (yellow). (C) ^{1}H NMR spectra for samples shown in B showing the methyl doublet for 1 at ~1.2 ppm and product methyl singlet at ~1.8 ppm. *Reproduced from Yevglevskis et al. (2017) with permission from the Royal Society of Chemistry.*

according to institutional guidelines before any experimental work is undertaken. Good Laboratory Practice should be followed. Appropriate caution should be exercised during NMR experiments due to the potential for high magnetic fields.

Acknowledgments

This work was supported at various times by EU grant QLG3-CT-2002–00696 (Refsum's disease: Diagnosis, Pathology and Treatment), Cancer Research UK, Prostate Cancer UK (grants S10–03 and PG14–009) and Biochemical Society Biochemical Summer Vacation Studentships. The authors gratefully acknowledge the Material and Chemical Characterisation Facility (MC^2) at the University of Bath (doi.org/10.15125/mx6j-3r54) for technical support and assistance in this work. Parts of this work have been reproduced with permission from Royal Society of Chemistry publications. We thank Constant Systems, Daventry UK for providing the photo of the "one Shot" shown in Fig. 1B. The authors are part of the Cancer Research @ Bath (CR@B) network.

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