Synthesis and use of isotope-labelled substrates for a mechanistic study on human α-methylacyl-CoA racemase 1A (AMACR; P504S)†‡

Daniel J. Darley,[a] Danica S. Butler,[b] Samuel J. Prideaux,[a] Thomas W. Thornton,[a] Abigail D. Wilson,[a] Timothy J. Woodman,[a] Michael D. Threadgill[a] and Matthew D. Lloyd*[a]

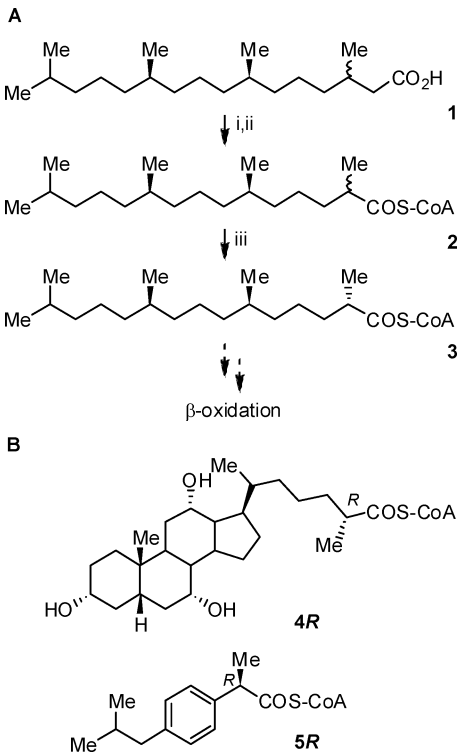
Received 3rd September 2008, Accepted 27th October 2008
First published as an Advance Article on the web 28th November 2008
DOI: 10.1039/b815396e

α-Methylacyl-CoA racemase (AMACR) is an important enzyme for the metabolism of branched-chain lipids and drugs. The enzyme is over-expressed in prostate and other cancers. AMACR 1A, the major splice variant, was purified from recombinant *E. coli* cells as a His-tag protein. Purified enzyme catalysed chiral inversion of both *S*- and *R*-2-methyldecanoyl-CoA, with an equilibrium constant of 1.09 ± 0.14 (2*S*/2*R*). Reactions with ^{2}H-labelled substrate showed that loss of the α-proton was a prerequisite for chiral inversion. Reactions conducted in ^2H_2O indicated that reprotonation was not stereospecific. These results are the first mechanistic study on any recombinant mammalian α-methylacyl-CoA racemase.

Introduction

Branched-chain fatty acids are important components of the human diet and are used as drugs, *e.g.* ibuprofen.[1,2] Phytanic acid (3*R*/*S*,7*R*,11*R*,15-tetramethylhexadecanoic acid) (Scheme 1A, **1**), a 3-methyl branched-chain fatty acid, is derived from chlorophyll A and is abundant in red meat and dairy products. The presence of the 3-methyl group prevents β-oxidation of the CoA ester. Instead, phytanic acid **1** is initially metabolised as the CoA ester in peroxisomes by a preliminary four-step α-oxidation pathway to give pristanic acid (2*R*/*S*,6*R*,10*R*,14-tetramethylpentadecanoic acid), which is converted into pristanoyl-CoA (Scheme 1A, **2**). The stereochemical configurations of phytanic acid **1** are retained, so a diastereomeric mixture of 2*R*- and 2*S*-pristanoyl CoA **2** is produced in the early steps. This homochiral compound is then subject to β-oxidation, initially in peroxisomes and subsequently in mitochondria for chain-shortened derivatives.[1,2] Only substrates with *S*-configuration at the 2-position can be metabolised by peroxisomal β-oxidation[3,4] but 2-methyl-fatty acids with 2*R* configuration or their precursors are common in the diet (*e.g.* phytanic **1**, pristanic acid **2** and derivatives) or are produced endogenously, *e.g.* bile acid precursors. Thus chiral inversion of *R*-2-methylacyl-CoAs is required before β-oxidation (*e.g.* Scheme 1, **2**, **3**).

α-Methylacyl-CoA racemase (AMACR; E.C. 5.1.99.4) catalyses inversion of configuration at the 2-position of fatty acyl-CoA esters from 2*R* to 2*S* and controls entry of metabolites into peroxisomal β-oxidation.[2] The enzyme sequence contains an N-terminal mitochondrial-targetting signal and a C-terminal peroxisomal-targetting signal-1 (PTS-1) variant[5] and is localised in both organelles.[6] The enzyme can catalyse chiral inversion of 2-methyl fatty acyl-CoA substrates with diverse structures, including pristanoyl-CoA **2** and its chain-shortened derivatives, bile acid precursors (*e.g.* dihydroxycholestanoyl-CoA, **4**) and ibuprofenoyl-CoA **5** (Scheme 1B).[2] Chiral conversion of 2*R*-ibuprofenoyl-CoA **5*R*** in the racemic mixture into its 2*S*-epimer **5*S*** is required for full pharmacological activity, since 2*R*-ibuprofen is inactive



Scheme 1 A) Role of AMACR in the degradation of branched-chain fatty acids. i) α-Oxidation pathway; ii) acyl-CoA synthetase; iii) AMACR. B) Structures of other substrates of AMACR, dihydroxycholestanoyl-CoA **4** and ibuprofenyl-CoA **5**.

[a]*Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, United Kingdom. E-mail: M.D.Lloyd@bath.ac.uk*
[b]*Chemistry Research Laboratory, Mansfield Road, University of Oxford, Oxford, OX1 3TA, United Kingdom*

† Abbreviations used: AMACR, α-methylacyl-CoA racemase; BSA, bovine serum albumin; CoA, coenzyme A; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; THF, tetrahydrofuran.

‡ Electronic supplementary information (ESI) available: NMR spectra and kinetic plots. See DOI: 10.1039/b815396e

as a cyclo-oxygenase (COX) inhibitor.[7] Patients suffering from deficiency of AMACR accumulate 2*R*-methyl branched-chain fatty and bile acids,[4,5,8] indicating that AMACR activity is essential for metabolism of these lipids. Increased levels of AMACR are associated with various cancers, and protein levels have been widely used as a biomarker for prostate cancer.[2]

Despite the recent interest in AMACR as a biomarker for cancer, little biochemical work has been performed with this enzyme, with only one study on native human AMACR.[9] This study reported that both *R*- and *S*-2-methyltetradecanoyl-CoA were substrates but no equilibrium constant was determined. Release of tritium from labelled substrates was also reported,[9–12] which is consistent with a deprotonated intermediate. Subsequently, a number of alternative splice variant forms of AMACR were identified in prostate cancer tissues.[13] It is therefore not clear whether the previous study[9] utilised a single AMACR isoform or multiple isoforms. Site-directed mutagenesis and X-ray crystallographic studies have been reported on the homologous enzyme (MCR) from *M. tuberculosis*.[10,11] These results are consistent with a deprotonated intermediate, with His-122/Glu-237 acting as a base to deprotonate the substrate and Asp-152 acting as an acid or vice versa, depending on the stereochemistry of the substrate (numbers refer to human AMACR 1A). The authors proposed that the stereochemistry of the product depends on the conformation of the 2-methylacyl-CoA sidechain and the catalytic mechanism of MCR is formally a 1,1-proton transfer.[11] These active site catalytic residues are conserved in human AMACR 1A.

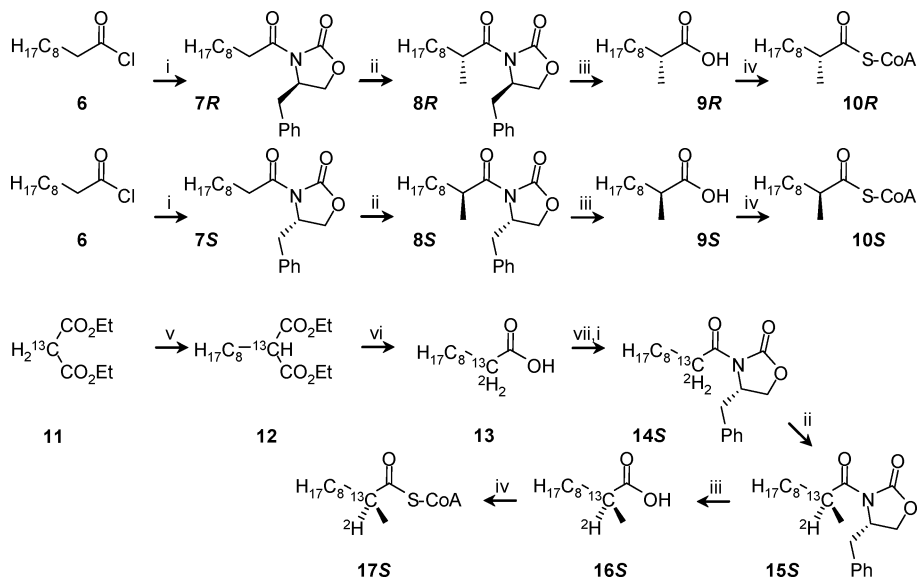
AMACR is an important cancer marker but little is known about its biochemical role. In this paper, we report the expression of human AMACR 1A, the major isoform, and biochemical and mechanistic studies on the recombinant enzyme. Key questions in this study were: 1) Could human AMACR 1A catalyse chiral inversion in both directions or only one (in the metabolic pathway, activity is used to convert *R*-2-methylacyl-CoA esters to *S*-2-methylacyl-CoA esters)? 2) What is the value of the equilibrium constant for the AMACR-catalysed reaction? 3) What is the stereochemical course of the deprotonation and reprotonation steps?

Results and discussion

Synthesis of substrates

Mammalian AMACR enzymes are able to catalyse the chiral inversion of a range of substrates. 2-Methyldecanoyl-CoA **10** was chosen as a substrate for this study, since it is structurally one of the simplest available. This compound also closely mimics the structure of pristanoyl-CoA **3** and both stereoisomers are synthetically accessible. Previous studies on native human AMACR[9] and recombinant MCR (a *Mycobacterium tuberculosis* homologue)[10,11] showed that 2-methyltetradecanoyl-CoA was a substrate for both enzymes. Synthesis of *R*-2-methyldecanoic acid **9*R*** by enzymatic resolution with lipases has been reported[14,15] but we took a chemical approach to their syntheses. Prolinol has been employed as a chiral auxiliary in the syntheses of the enantiomers of **9** by α-methylation of *N*-decanoyl-*L*-prolinol and α-octylation of *N*-propanoyl-*L*-prolinol[16] but the routes were not efficient. Both stereoisomers of 2-methyldecanoyl-CoA **10** were required for our study in order to determine whether human AMACR 1A could catalyse chiral inversion in only one or in both directions.

Scheme 2 shows our synthetic route to the two stereoisomers of 2-methyldecanoyl-CoA, **10*S*** and **10*R*.** Our enantioselective approach used a modification of that previously reported for the enantiomers of 2-methyldodecanoic acid,[17] *i.e.* stereoselective methylation of enolates derived from *N*-acyl chiral auxiliaries. Treatment of the anions derived from the enantiomeric 4-benzyloxazolidin-2-one Evans' auxiliaries with decanoyl chloride afforded the 3-decanoyloxazolidinones **7*R*** and **7*S***. Formation



Scheme 2 Syntheses of 2-methyldecanoyl-CoA enantiomers **10*R*** and **10*S*** and synthesis of 2*S*-2-[^{13}C]-2-[^{2}H]-methyldecanoyl-CoA **17*S***. *Reagents and conditions*: i, *R*-4-benzyloxazolidin-2-one or *S*-4-benzyloxazolidin-2-one, BuLi, THF –78 °C; ii, NaN(SiMe$_3$)$_2$, MeI, THF, –78 °C, 68%; iii, LiOH, H_2O_2, 0 °C, 91%; iv, ethyl chloroformate, CoA-SH, THF. v, NaH, 1-bromooctane, DMF, 59%; vi, 35% aq. ^{2}HCl, 160 °C, 97%; vii, oxalyl chloride, CH_2Cl_2, DMF.

of the enolates with sodium hexamethyldisilazide, followed by methylation with iodomethane at low temperature, gave the 2-methyldecanoyl compounds **8*R*** and **8*S*.** In both cases, the diastereomeric excess was >97.5%, as shown by the ^{1}H NMR spectra. The oxazolidinone auxiliary was cleaved with lithium hydroperoxide to afford the desired enantiomeric 2-methyl acids **9*R*** and **9*S*** in 51% overall yield. Following extensive optimisation of coupling conditions, production of the substrate CoA esters **10*S*** and **10*R*** was routinely achieved on 10–20 mg scales through the use of ethyl chloroformate to activate the acid as its mixed anhydride. CoA esters were usually prepared immediately before use in enzymatic assays.

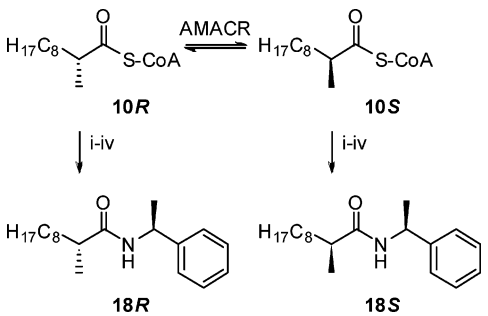
Synthesis of the *S*-2-[^{13}C]-2-[^{2}H] isotopomer **17*S*** was required for the NMR mechanistic studies (Scheme 2B), as formation of the enolate (with loss of ^{2}H) would be followed by protonation with ^{1}H from solvent water during the enzyme mechanism. The enzyme-catalysed exchange of ^{2}H with ^{1}H from the solvent could be monitored directly by ^{13}C NMR spectroscopy of the reaction mixture, with the ^{13}C signal from the starting **17*S*** appearing as a triplet through $^1J_{C\text{-}D}$ coupling and the corresponding signal from the **10*R*** or **10*S*** product appearing as a singlet owing to the broadband ^{1}H-decoupling during the NMR acquisition. The ^{13}C label was introduced as the simple starting material diethyl 2-[^{13}C]-malonate **11**. Deprotonation and alkylation of the intermediate enolate with 1-iodooctane furnished diethyl 2-octyl-2-[^{13}C]-malonate **12** in *ca.* 60% yield after optimisation of the reaction conditions, especially the solvent and the molar ratios of the reactants. Prolonged treatment with boiling 35% deuterium chloride in ^2H_2O hydrolysed the two esters, triggered monodecarboxylation (incorporating one deuterium from solvent) and caused exchange of the other α-hydrogen (incorporating the second deuterium) to give the 2-[^{13}C]-2-[$^2H_2$]-decanoic acid **13**. This was converted into its acid chloride and used to acylate the anion of the *S*-Evans' auxiliary to afford the isotopically labelled acyloxazolidinone **14*S***. From here, treatment with strong base removed one of the deuteriums and stereoselective reaction of the anion with iodomethane led to *S*-2-[^{13}C]-2-[^{2}H]-**15*S*** in good yield. The 2-methyldecanoic acid **16*S*** was produced by removal of the Evans' auxillary and coupled with CoA-SH to provide the required isotopically labelled and enantiomerically pure CoA ester **17*S*.** Hydrolytic removal of the auxiliary did not result in exchange of label, thus indicating no loss of chirality on conversion of **15*S*** to **16*S***.

Mechanistic studies on recombinant human AMACR

AMACR and related enzymes are proposed to catalyse their reactions by a deprotonation/reprotonation mechanism *via* an enol, enolate or a carbanion intermediate.[2] However, a carbanion intermediate is much less likely, as it would be tetrahedral and thus not facilitate inversion of configuration. Consistent with this mechanism is the loss of a deuterium from the α-position of the substrates upon incubation with native human AMACR[9] (2-methyltetradecanoyl-CoA substrate), native rat AMACR[12] (pristanoyl-CoA **3** and dihydroxycholestanoyl-CoA **4** substrates), and *M. tuberculosis* MCR[10,11] (2-methyltetradecanoyl-CoA substrate). The X-ray crystal structures of MCR with ligands bound[11] are consistent with this mechanism. For some acyl-CoA reactions, a ketene intermediate has also been proposed[18] and this intermediate is also consistent with the observed loss of deuterium from substrates.

Recombinant human AMACR 1A was required for this study. The cDNA was sub-cloned into the pET30 Ek/LIC vector and transformed into recombinant *E. coli* cells. An over-expressed band at ~45 KDa was observed at *ca.* 20% total protein consistent with the predicted size of 47 KDa, which was absent from untransformed cells. This band could be conveniently purified to *ca.* 98% purity (by SDS-PAGE analyses) on a *ca.* 20 mg scale using affinity chromatography.

Initially, we investigated whether AMACR 1A could catalyse chiral inversion of both **10*R*** and **10*S*** substrates. Following incubation of both enantiomers of substrate with active enzyme, the reaction was stopped with NaOH and the acid products were derivatised with *S*-1-phenylethylamine (Scheme 3). Analysis by ^{1}H NMR spectroscopy showed, by examination of the peaks at δ 1.13 and δ 1.11 (2-CH_3 groups of **18*S*** and **18*R*** *N*-(*S*-1-phenylethylamide) derivatives), that chiral inversion had occurred in both cases, thus showing that AMACR 1A was a true racemase (see Supplementary Information for spectra). No chiral inversion was observed in the absence of active enzyme. Incubation of substrates for extended time periods resulted in establishment of an equilibrium with a value of 1.09 ± 0.14 (2*S*/2*R*) from either **10*S*** or **10*R***. These results contrast with those observed for AMACR purified from rat liver cytosol and mitochondria [~0.66 (2*S*/2*R*)] using ibuprofenoyl-CoA (Scheme 1, **5**) as substrate.[19] Our *in vitro* studies show that, at equilibrium, the enzyme-catalysed reaction produces near-equal concentrations of both substrate enantiomers. The β-oxidation pathway removes only the 2*S*-enantiomer, so that net flux through the enzyme is 2*R* to 2*S* in cells.



Scheme 3 Assay of AMACR activity by chiral derivatisation. *Reagents*: i, NaOH; ii, HCl; iii, oxalyl chloride, DMF (cat.). CH_3Cl_2; iv, *S*-l-phenylethylamine.

Rates for conversion at different substrate concentrations of **10*S*** and **10*R*** were measured by determining the extent of chiral inversion after 60 min by NMR analyses of the derivatives **18*S*** and **18*R***. Kinetic parameters were obtained by direct fitting of these rates to the Michaelis–Menten curve (see Supplementary Information for kinetic values and derived plots). Kinetic parameters were determined using the direct linear plot. This plot determines median values and the obtained parameters are less sensitive to outlier points and makes no assumption about the distribution of errors. Estimates of kinetic parameters are therefore often considered to be more accurate.[20,21] For **10*S*** the following apparent kinetic parameters were determined: $K_m = 1.2$ mM; $V_{max} = 77.5$ μmol min^{-1} mg^{-1}; $k_{cat} = 60.9$ s^{-1}; $k_{cat}/K_m = 50750$ s^{-1} M^{-1}. For **10*R***, the following apparent kinetic parameters were

determined: K_m = 1.2 mM; V_{max} = 68.4 μmol min^{-1} mg^{-1}; k_{cat} = 53.8 s^{-1}; k_{cat}/K_m = 44800 s^{-1} M^{-1}. The theoretical equilibrium constant can be calculated from the ratio of k_{cat}/K_m values to be 1.13 : 1 (**10*S*/10*R***), consistent with the observed value of 1.09 ± 0.14 (**10*S*/10*R***).

We then carried out a series of experiments using labelled substrates or reactions in ^2H_2O in order to investigate the mechanism of recombinant AMACR 1A. When enzyme-catalysed racemisation of unlabelled substrate **10*S*** or **10*R*** was conducted in ^2H_2O, the ^{1}H NMR signal arising from the 2-proton of the substrate (δ 2.60) disappeared and the doublet associated with the 2-methyl group (δ 1.00) changed to a singlet in a time-dependent manner (Fig. 1). Since such spectral changes did not occur in the absence of enzyme, these observations indicate that proton–deuterium exchange of the α-proton must occur during catalysis.

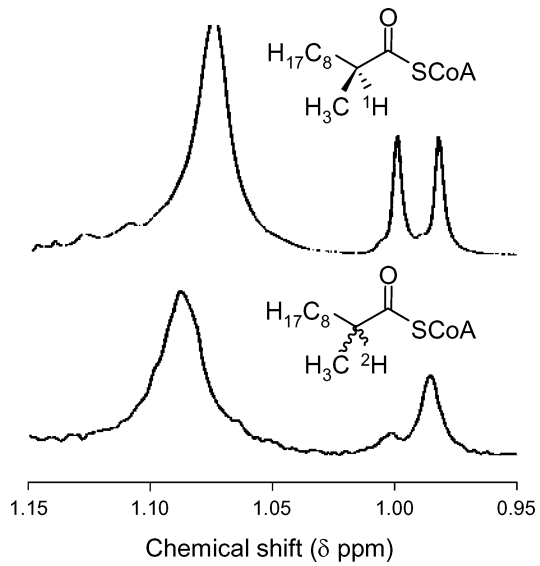


Fig. 1 ^{1}H NMR spectrum (400 MHz) showing change in methyl group coupling upon AMACR-catalysed α-proton exchange.

In some experiments, aliquots of reaction mixture in ^2H_2O were taken after different extents of conversion, quenched with NaOH and the resulting acids converted to their *S*-1-phenylethyl amides **18*R*** and **18*S*.** This experiment allows investigation of the reprotonation of the catalytic intermediate. Careful analysis of this diastereomeric mixture by ^{1}H NMR revealed a direct correlation between the extent of chiral inversion and the degree of deuterium exchange in both **10*S*** and **10*R*** (Fig. 2A and B, respectively). The rate of deuterium exchange (7.5 ± 3.0 nmol min^{-1} for **10*S***; 8.0 ± 1.5 nmol min^{-1} for **10*R***) was greater than chiral inversion (3.3 ± 1.6 nmol min^{-1} for **10*S***; 4.2 ± 0.8 nmol min^{-1} for **10*R***) for both substrates. This implies that in a significant proportion of the deprotonation events to form the intermediate, reprotonation with deuterium occurred without chiral inversion. Based on the ratios of the observed rates, it appears that *ca.* 1.9 to 2.2 deprotonation events occur for each chiral inversion. This implies the formation of a planar (enol/enolate) intermediate followed by a non-stereospecific addition of $^2H^+$. The observed equilibrium constant of 1.09 ± 0.14 also suggests a mechanism in which a planar intermediate is reprotonated in a non-stereospecific manner. At longer times (higher levels of conversion), significant amounts

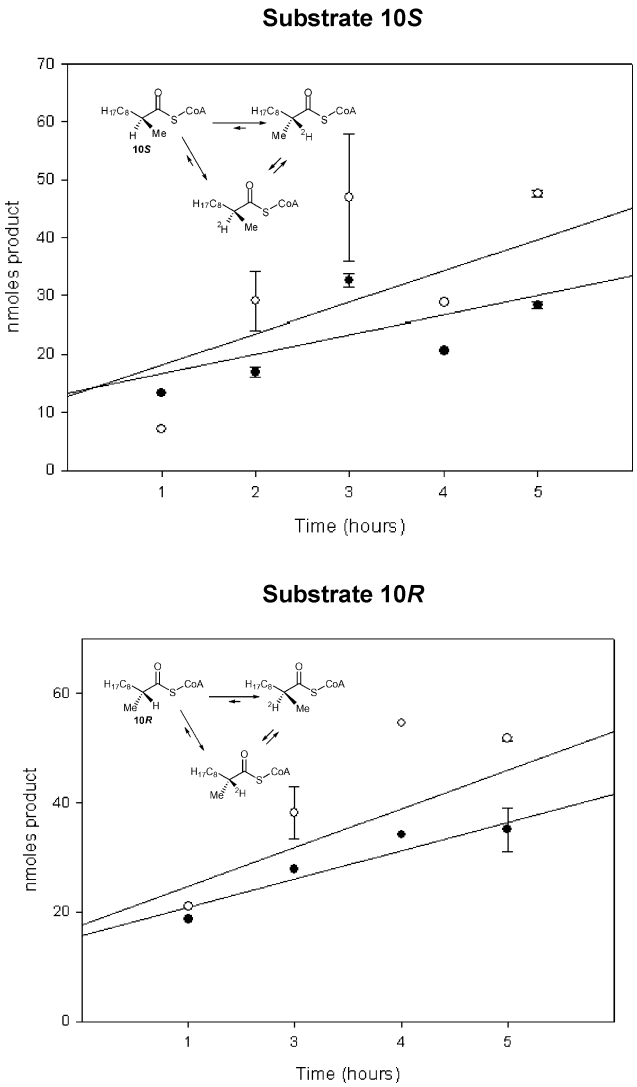


Fig. 2 Deuterium exchange and chiral inversion of *S*- and *R*-2-methyldecanoyl-CoA by AMACR in deuterated solvent, as measured by stopped assay. 2*S*-Substrate **10*S*** (top); B. 2***R***-substrate **10*R*** (bottom). Chiral inversion (closed circles); deuterium exchange (open circles). Note that reactions contained *ca.* 10% ^1H_2O (corresponding to *ca.* 20 nmol product) and a small amount of the other stereoisomer and hence lines do not intercept at the origin.

of deuteriated **10** will be present in the reaction mixture as a product, and it is a potential substrate for enzyme-catalysed chiral inversion. However, the enzyme may convert substrate having 2-^{1}H in preference to substrate with 2-^{2}H, owing to a kinetic isotope effect. These results are entirely consistent with crystallographic data on MCR (the *M. tuberculosis* homologue),[11] which shows that, during the chiral inversion reaction, the positions of the CoA ester moiety and methyl group are fixed and the side-chain moves over a hydrophobic surface at the entrance of the active site, thus allowing accommodation of the hydrogen in the either of the two stereochemical configurations.

The enzyme-catalysed deuterium–proton exchange was also investigated in ^1H_2O through the use of doubly labelled *S*-2-methyldecanoyl-CoA **17*S*** bearing both ^{13}C and ^{2}H at the α-position. This allows study of the deprotonation event in the

catalytic cycle. The proton-decoupled ^{13}C NMR spectrum of the substrate exhibited a triplet ($\delta = 47.11$, $^{1}J_{C\text{-}D} = 20$ Hz) arising from the ^{13}C α-carbon coupling to ^{2}H (spin = 1). Incubation with AMACR 1A gave rise to the formation of a singlet signal in the ^{1}H-decoupled ^{13}C NMR at δ 47.55, corresponding to the ^{13}C with an attached ^{1}H (Fig. 3A). The ^{13}C NMR resonance of the newly formed singlet was shifted down-field by about 0.44 p.p.m., compared to the central peak of the initial triplet signal, due to an $^{1}H/^{2}H$ isotope effect. This shift allowed the two signals to be resolved and their relative intensities to be quantified by integration of the spectra (Fig. 3B). The nOe enhancement effect in the ^{13}C spectrum resulting from the substitution of ^{1}H for ^{2}H upon reaction was determined to be *ca.* 1.45 (see experimental for details). The reaction was significantly slower than expected due to a kinetic isotope effect, implying that dedeuteration is the rate limiting step in this substrate. The reaction was reversible, since the triplet signal in the ^{13}C NMR spectra reappeared after removal of the water by lyophilisation and addition of $^{2}H_2O$ and further enzyme. Hydrolysis and derivatisation of the product as described above showed that a *ca.* 1 : 1 equilibrium between the 2*R*- and the 2*S*-isomers of the 2*RS*-2-[^{13}C]-methyldecanoyl-CoA was established (Fig. 3C), consistent with the equilibrium constant measured for the unlabelled substrates **10*S*** and **10*R***.

Conclusions

Considering the importance of AMACR in cancer, it is surprising that few studies have been performed on the mechanism and characterisation of this important enzyme, with most of the few studies taking place on native enzyme.[9,12,19,22] The only reports on recombinant enzyme have been on MCR,[10,11] the homologue from *M. tuberculosis.* Preliminary characterisation of rat recombinant AMACR (a.k.a. 'ibuprofenoyl-CoA epimerase') has also been reported.[7,23]

Our report is the first mechanistic characterisation of any recombinant human AMACR. Our NMR assays using labelled substrate or solvent provide convenient methods for investigating the mechanism and characterising substrate deprotonation, reprotonation and chiral inversion by this important enzyme. We have demonstrated that AMACR 1A is able to catalyse chiral inversion of both enantiomers of this particular substrate. Our results are consistent with a 1,1-proton transfer mechanism[11] in which the 2-methylacyl-CoA substrate undergoes deprotonation to form a planar enol/enolate intermediate (Fig. 4A and B). Evidence for the planar nature of the intermediate comes from the *cis* relationship of the 2-methyl group and thioester carbonyl group in the MCR structure.[11] There is also the formal possibility that a ketene intermediate (Fig. 4C) may be involved as this would also result in deuterium exchange. However there is no literature precedent for the latter intermediate in reactions catalysed by a racemase.

Our results demonstrate that the deprotonation of either substrate enantiomer is followed by non-stereospecific reprotonation of the planar intermediate, hence giving an *in vitro* equilibrium constant close to one. Reactions in the presence of $^{2}H_2O$ show incorporation of deuterium into both substrate and product. Examination of the MCR crystal shows that the catalytic residues His-122/Glu-237 and Asp-152 (numbering refers to human AMACR 1A) are at the interface of the protein dimer and close

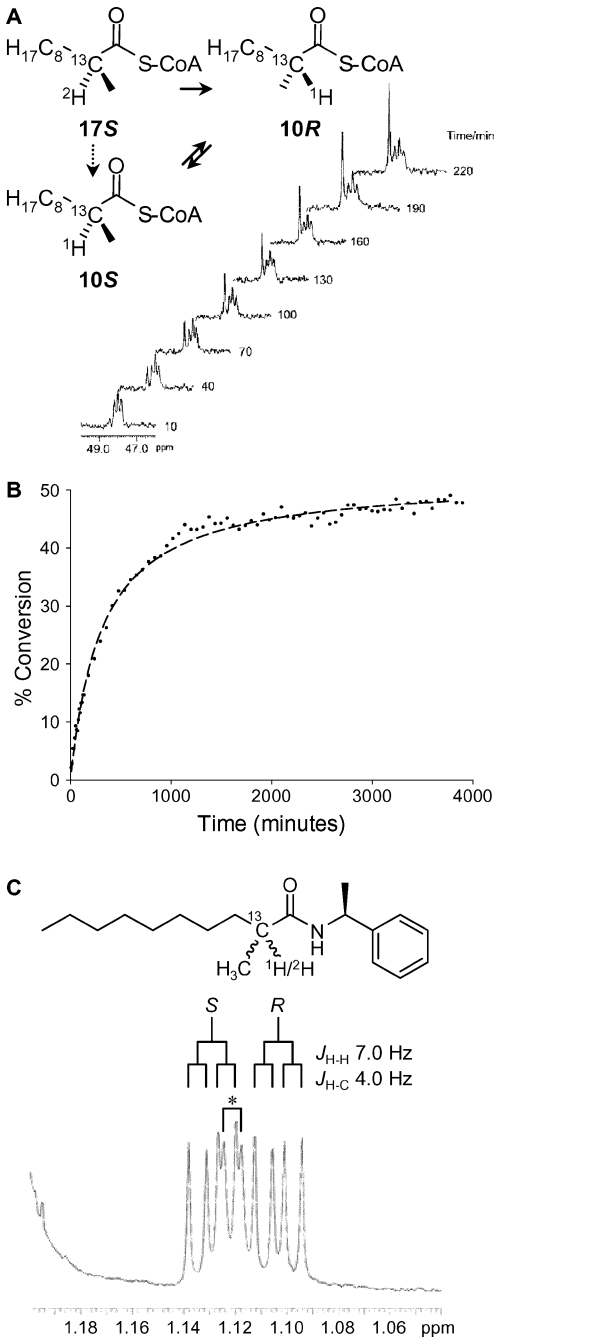


Fig. 3 Conversion of 2*S*-2-[^{13}C]-2-[^{2}H]-methyldecanoyl-CoA **17*S*** by AMACR. **A**. stack-plot of part ^{13}C NMR spectra showing disappearance of substrate triplet at δ 47.11 and appearance of product singlet at δ 47.55 (selected time points shown). Reactions catalysed by AMACR with the various substrates shown on the left-hand side. The dotted arrow indicates a minor pathway. **B**. Graph of appearance of product with time. Data are corrected for a nOe enhancement of the product by a factor of 1.448 and are fitted to a single parameter rectangular hyperbola, $f = a^{*}x/(b + x)$ with $a = 52.0 \pm 0.3$ and $b = 311.5 \pm 8.9$; **C**. $H_3CHCC{=}O$ region of ^{1}H NMR spectrum of a diastereomeric mixture of 2-methyldecanoic acid *N*-(*S*-1-phenylethyl)amides **18**. 2*S*-2-[^{13}C]-2-[^{2}H]-methyldecanoyl-CoA **17*S*** was incubated with AMACR 1A in H_2O, leading to ^{1}H–^{2}H exchange and racemisation. Derivatisation with *S*-1-phenylethylamine gave **18**. The two sets of double doublets arise from coupling of the CH_3 with ^{13}C and with the 'washed in' ^{1}H. The asterisk indicates the CH_3 doublet from **18** arising from unreacted deuteriated **17*S***.

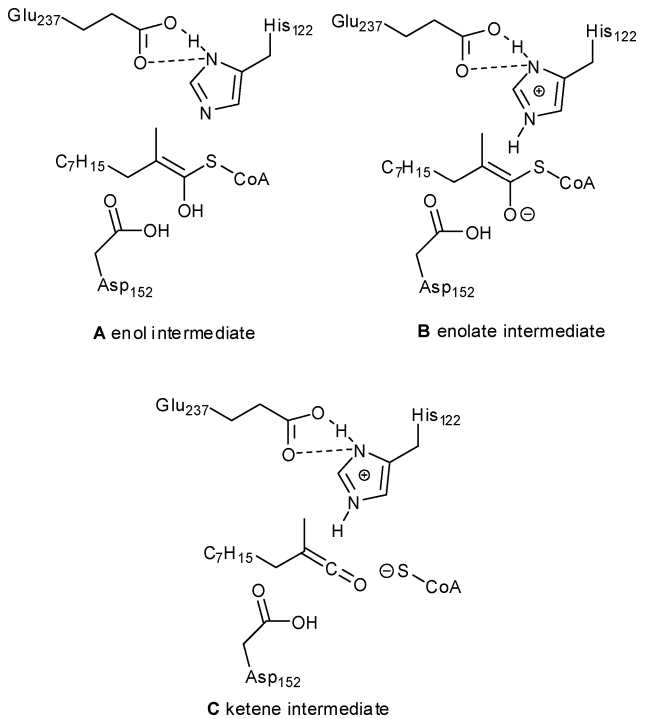


Fig. 4 Proposed catalytic intermediates in the AMACR 1A-catalysed racemisation reaction.

to the surface of the protein, with ordered waters within the active site within H-bonding distance of catalytic residues.[11] The catalytic residues are in a two-base arrangement,[24,25] either side of the substrate.[11] The substrate sidechain projects outside the active site and interacts with a hydrophobic region in order to accommodate the conformational changes upon chiral inversion. Our deuterium exchange data suggests that either the catalytic bases can exchange deuterium during the catalytic cycle (or between product release and binding of new substrate) or that reprotonation is from bulk solvent, as suggested for MCR.[11] The levels of product deuteration by AMACR are higher than observed for some classical two-base racemases (*e.g.* mandelate racemase[24]), in which less than one deuterium atom is incorporated per chiral inversion. In classical two-base mechanism racemases the active sites are locked away from bulk solvent and deuterium incorporation into the product can be different for the chiral inversion in different directions.[24] Very little deuterium is usually incorporated into residual substrate in classical two-base racemases, *e.g.* mandelate racemase[24] and proline racemase.[25] Despite the catalytic residues being in a two-base arrangement, AMACR incorporates deuterium at the same levels into both substrate and product, regardless of the direction of chiral inversion. This behaviour more closely resembles a classical one-base racemase mechanism in this respect.[24]

AMACR has been widely proposed as a marker for prostate and other cancers, with more than two hundred and eighty reports documenting over-production of AMACR in prostate,[26,27] breast,[28] colon,[29] renal[30] and other cancer cells.[31] The majority of reports have focussed on prostate cancer,[27,32,33] since the levels of over-expression are high (up to nine-fold higher than in non-cancerous cells[27]) and are consistently observed.[33] AMACR (a.k.a. P504S) is now widely used as a biomarker for prostate cancer.[33] High dietary phytanic acid levels directly correlate with increased expression and activity of AMACR[34] and elevated prostate cancer risk.[35] Sequence polymorphisms of AMACR, *e.g.* M9V, have been documented that increase risk of prostate[36] and colon[37] cancer. A number of questions remain about AMACR and the pathological link between phytanic acid **1** and cancer. Prostate cancer cells over-produce several other splice variants of AMACR,[13] in addition to AMACR 1A. Similarly, the M9V polymorphism is associated with increased risk of prostate[36] and colon cancer.[37] It is not clear exactly what role these cancer-associated splice variants and polymorphisms play, but our results will facilitate study of these important issues.

Experimental

General methods

All chemicals were obtained from the Sigma-Aldrich Chemical Co. or Fisher Ltd. and were used without further purification unless otherwise stated. Experiments were conducted at ambient temperature, unless noted otherwise. Tetrahydrofuran was freshly distilled from Na/benzophenone ketyl. Petroleum ether refers to the fraction boiling between 40–60 °C. The brine was saturated. The chromatographic stationary phase was silica gel. Organic extracts were dried over $MgSO_4$ and filtered. Solvents were evaporated under reduced pressure. Melting points are uncorrected. NMR spectra were obtained on a JEOL Eclipse EX spectrometer at 270 MHz or Varian EX 400 spectrometer at 400 (^{1}H), 61.41 (^{2}H) or 100.6 (^{13}C) MHz, on solutions in deuteriochloroform, except where noted. Spectra are referenced to tetramethylsilane in deuteriochloroform or the residual water peak (^2H_2O). Coupling constants (J) are reported in Hz. Molecular biology reagents were obtained from New England BioLabs, Stratagene, Promega or Novagen. The AMACR 1A gene in the pGEM-T vector was obtained from Professor R. J. A. Wanders, University of Amsterdam, The Netherlands. Solutions for molecular biology were sterilised by autoclaving or filtration. Plastic-ware was sterilised by autoclaving or was purchased sterile directly from the manufacturer. Buffer solutions were made using 18 MΩ Milli-Q water (Millipore) and pH adjusted at ambient room temperature using pH 7.0 and 4.0 or 10.0 standards. Protein columns were obtained from GE Healthcare. ^{13}C- and ^{2}H-isotopomers contained >98% ^{13}C and ^{2}H, respectively, as demonstrated by mass spectrometry.

R-4-Benzyl-3-decanoyloxazolidin-2-one (7*R*)[38]

R-4-Benzyloxazolidin-2-one (1.0 g, 5.6 mmol), in dry tetrahydrofuran (20 mL), was cooled to −78 °C. n-Butyl lithium (2.5 M in hexanes, 2.3 mL, 5.7 mmol) was added drop wise during 15 min, followed by decanoyl chloride **6** (1.18 g, 6.2 mmol). The solution was allowed to warm to 0 °C during 1 h and stirred for a further 1 h. Saturated aqueous ammonium chloride (10 mL) was added and the mixture was extracted thrice with dichloromethane. The combined organic layers were washed with aqueous potassium carbonate (1.0 M) and brine (10 mL) and were dried. Evaporation and chromatography (ethyl acetate/petroleum ether 1 : 9) gave **7*R*** (1.17 g, 62%) as a colourless oil that crystallised on standing to a colourless solid: mp 37–39 °C; $[\alpha]_{20}^{D}$ −60.6 (*c* 1.0, dichloromethane); δ_H 0.87 (3 H, t, *J* 6.7, CH_3), 1.26 (12 H, m, 6 × CH_2), 1.68 (2 H, t, *J* 7.3, decanoyl 3-H_2), 2.74 (1 H, dd, *J* 13.4, 9.6,

C*H*HPh), 2.90 (2 H, m, decanoyl 2-H_2), 3.28 (1 H, dd, *J* 13.4, 3.2, CH*H*Ph), 4.16 (2 H, m, 5-H_2), 4.65 (1 H, m, 4-H), 7.30 (5 H, m, Ph-H_5); δ_C 14.45, 22.70, 24.31 (decanoyl 3-C), 29.17, 29.31, 29.44, 29.48, 31.91, 35.57 (decanoyl 2-C), 37.97, 55.19, 66.17, 127.36, 128.97, 129.45, 135.38, 153.49, 173.45 (decanoyl 1-C).

_S_-4-Benzyl-3-decanoyloxazolidin-2-one (7*S*)

Treatment of *R*-4-benzyloxazolidin-2-one with BuLi and **6**, as for the synthesis of **7*R***, gave **7*S*** (1.72 g, 93%) (mp 37–39 °C). $[\alpha]_{20}^{D}$ +63.7 (*c* 1.0, dichloromethane).

3-(*R*-2-Methyldecanoyl)-4*R*-4-benzyloxazolidin-2-one (8*R*)

Sodium bis(trimethylsilyl)amide (1.0 M in tetrahydrofuran, 3.3 mL, 3.3 mmol) was cooled to −78 °C and **7*R*** (1.0 g, 3.0 mmol) in tetrahydrofuran at 0 °C was added dropwise. The mixture was stirred for 1 h at −78 °C. Iodomethane (0.9 mL, 15 mmol) in tetrahydrofuran (0.5 mL) at −78 °C was added and the solution was stirred for a further 3 h. The reaction was quenched with saturated aqueous ammonium chloride (10 mL). The mixture was extracted thrice with dichloromethane. The combined organic layers were washed with aqueous sodium sulfite (1.0 M) and dried. Evaporation and chromatography (ethyl acetate/petroleum ether, 1 : 9) gave **8*R*** (650 mg, 63%) as a colourless oil: $[\alpha]_{20}^{D}$ −73.5 (*c* 1.0, dichloromethane); δ_H 0.86 (3 H, t, *J* 6.7, decanoyl 10-H_3), 1.20 (3 H, d, *J* 6.9, CH_3CH), 1.24 (m, 12H, 6 × CH_2), 1.40 (1 H, m, decanoyl 3-H), 1.71 (1 H, m, decanoyl 3-H), 2.75 (1 H, dd, *J* 13.2, 9.5, C*H*HPh), 3.26 (1 H, dd, *J* 13.4, 3.2, CH*H*Ph), 3.68 (1 H, m, CHC=O), 4.11 (2 H, m, 5-H_2), 4.66 (1 H, m, 4-H), 7.30 (5 H, m, Ph-H_5); δ_C 14.09, 17.35 (decanoyl 2-CH_3), 22.64, 27.25, 29.24, 29.46, 29.65, 31.85, 33.46 (decanoyl 3-C), 37.71 (decanoyl 2-C), 37.90, 55.36, 65.99, 127.32, 128.92, 129.44, 135.35, 153.05, 177.37.

3-(*S*-2-Methyldecanoyl)-4*S*-4-benzyloxazolidin-2-one (8*S*)

Treatment of **7*S*** with sodium bis(trimethylsilyl)amide and iodomethane, as for the synthesis of **8*R***, gave **8*S*** (68%). $[\alpha]_{20}^{D}$ +76.6 (*c* 1.0, dichloromethane).

***R*-2-Methyldecanoic acid (9*R*)**

Compound **8*R*** (650 mg, 1.9 mmol), in tetrahydrofuran (27 mL) and water (9 mL), was stirred with aqueous hydrogen peroxide (30%, 1.4 mL) and lithium hydroxide hydrate (150 mg, 3.8 mmol) at 0 °C for 3 h. The reaction was quenched by addition of aqueous sodium sulfite (1.5 M, 13 mL, 20 mmol). The mixture was acidified with aqueous HCl (1.0 M) to pH 1 and extracted thrice with dichloromethane. Drying, evaporation and chromatography (ethyl acetate/petroleum ether 2 : 1) gave **9*R*** (320 mg, 91%) as a colourless oil: $[\alpha]_D^{20}$ −16.3 (*c* 1.0, methanol) (lit. $[\alpha]_D^{20}$ −15.79 (neat)[14]; $[\alpha]_D$ = −15.4 (*c* 0.84, methanol)[15]); δ_H 0.86 (3 H, t, *J* 6.7, 10-H_3), 1.16 (3 H, d, *J* 6.9, 2-CH_3), 1.24 (13 H, m, 6 × CH_2 + 3-H), 1.65 (1 H, m, 3-H), 2.43 (1 H, m, 2-H); δ_C 14.09, 16.80, 22.65, 27.13, 29.25, 29.42, 29.50, 31.85, 33.50, 39.38, 183.49.

***S*-2-Methyldecanoic acid (9*S*)**

Compound **8*S*** was hydrolysed with lithium hydroxide and hydrogen peroxide, as for the synthesis of **9*R***, to give **9*S*** (0.26 g, quant): $[\alpha]_D^{20}$ +15.8 (*c* 1.0, methanol).

Ethyl [2-^{13}C]-2-ethoxycarbonyldecanoate (12)

Sodium hydride (60% in oil, 1.00 g, 6.25 mmol) was suspended in dimethylformamide (20 mL) and diethyl [2-^{13}C]-malonate **11** (1.00 g, 6.25 mmol) was added. The mixture was stirred for 30 min before 1-bromooctane (1.6 g, 8.2 mmol) was added and the mixture was stirred for 16 h. Saturated aqueous ammonium chloride (10 mL) was added and the mixture was extracted twice with ethyl acetate. The combined extracts were washed once with aqueous HCl (1.0 M) and thrice with water and dried. Evaporation and chromatography (ethyl acetate/petroleum ether, 1 : 19) gave **12** (1.00 g, 59%) as a colourless oil: δ_H 0.87 (3 H, t, *J* 6.8, 10-H_3), 1.27 (18 H, m, 2 × CH_2CH_3 and 6 × CH_2), 1.87 (2 H, m, 3-H_2), 3.30 (1 H, dt, *J* 131.6, 7.5, 2-H), 4.81 (4 H, q, *J* 6.8, 2 × OCH_2); δ_C 14.01, 22.57, 27.25, 28.7 (d, *J* 28.7, 3-C), 29.11, 29.13, 29.17, 29.21, 31.75, 52.02 (2-C), 61.16, 169.55 (d, *J* 56.7, 1-C).

2-[^{13}C]-2-[$^2H_2$]-Decanoic acid (13)

A suspension of **12** (950 mg, 3.5 mmol) in ^{2}HCl/^2H_2O (35%, 10 mL) was stirred at 160 °C for 48 h. The cooled mixture was extracted thrice with ethyl acetate. The combined extracts were dried. Evaporation gave a colourless oil, which crystallised to give **13** (600 mg, 97%) as a colourless solid: mp ~25 °C [lit.[39] mp 29–31 °C for unlabelled material]; δ_H 0.87 (3 H, t, *J* 6.8, 10-CH_3), 1.27 (12 H, m, 6 × CH_2), 1.60 (2 H, m, 3-CH_2); δ_C 14.07, 22.64, 24.51 (d, *J* 33.8, 3-C), 28.98, 29.20, 29.23, 29.37, 31.84, 33.47 (qn, *J* 19.2, 2-C), 180.34 (d, *J* 45.4, 1-C); δ_D 2.33 (d, *J* 19.6, 2-$^2H_2$).

4-Benzyl-3-(2-[^{13}C]-2,2-[$^2H_2$]-decanoyl)oxazolidin-2-one (14*S*)

Compound **13** (500 mg, 2.8 mmol) was stirred in dichloromethane (10 mL) with dimethylformamide (1 drop) and oxalyl chloride (500 mg, 3.9 mmol) for 20 min. Evaporation gave crude 2-[^{13}C]-[$^2H_2$]-decanoyl chloride. This material was treated with Evans' auxiliary, as for the synthesis of **7*R***, to give **14*S*** (720 mg, 79%) as a colourless oil: δ_H 0.90 (3 H, m, decanoyl 10-CH_3), 1.31 (12 H, m, 6 × CH_2), 1.68 (2 H, m, 3-CH_2), 2.76 (1 H, dd, *J* 13.3, 9.6, C*H*HPh), 3.30 (1 H, dd, *J* 13.3, 3.4, CH*H*Ph), 4.18 (2 H, m, 5-H_2), 4.67 (1 H, m, 4-H), 7.46 (5 H, m, Ph-H_5); δ_C 14.11, 22.66, 24.14 (d, *J* 34.5, decanoyl 3-C), 29.07, 29.27, 29.41, 29.43, 31.86, 34.9 (qn, *J* 19.2, decanoyl 2-C), 37.90, 55.12, 66.12, 127.32, 128.93, 129.41, 135.30, 153.45 (oxazolidinone 2-C), 173.49 (d, *J* 49.1, decanoyl 1-C); δ_D 2.87 (d, *J* 19.2, decanoyl 2-$^2H_2$).

4-Benzyl-3-(*S*-2-methyl-2-[^{13}C]-2-[^{2}H]-decanoyl)-4*S*-oxazolidin-2-one (15*S*)

Compound **14*S*** (700 mg, 2.1 mmol) was treated with iodomethane, as for the synthesis of **8*R***, to give **15*S*** (600 mg, 81%) as a colourless oil: $[\alpha]_{20}^{D}$ +59.2 (*c* 0.3, dichloromethane); δ_H 0.88 (3 H, t, *J* 6.7, decanoyl 10-H_3), 1.18 (3 H, d, *J* 4.0, decanoyl 2-CH_3), 1.31 (14 H, m, 7 × CH_2), 2.75 (1 H, dd, *J* 13.3, 9.4, C*H*HPh), 3.26 (1 H, dd, *J* 13.2, 3.2, CH*H*Ph), 4.16 (2 H, m, 5-H_2), 4.67 (1 H, m, 4-H), 7.27 (5 H, m, Ph-H_5); δ_C 14.08, 17.22 (d, *J* 33.8, decanoyl 2-CH_3), 22.63, 27.21, 29.23, 29.44, 29.62, 29.66, 31.84, 33.32 (d, *J* 34.5, decanoyl 3-C), 37.31 (t, *J* 20.7, decanoyl 2-C), 55.32, 65.98, 127.30, 128.90, 129.43, 135.33, 153.05, 177.36 (d, *J* 48.3, decanoyl 1-C); δ_D 3.69 (d, *J* 20.7, decanoyl 2-^{2}H).

S-2-Methyl-2-[^{13}C]-2-[^{2}H]-decanoic acid (16*S*)

Compound **15*S*** (500 mg, 1.4 mmol) was treated with hydrogen peroxide and lithium hydroxide, as for the synthesis of **9*R***, to give **16*S*** (260 mg, quant.) as a colourless oil: $[\alpha]_{20}^{D}$ +59.2 (*c* 0.3, dichloromethane); δ_H 0.87 (3 H, t, *J* 7.0, 10-H_3), 1.25 (12 H, m, 6 × CH_2), 1.16 (3 H, d, *J* 4.0, 2-CH_3), 1.42 (1 H, m, 3-H), 1.65 (1 H, m, 3-H); δ_C 14.08, 17.22 (d, *J* 33.8, 2-CH_3), 22.63, 27.21, 29.23, 29.44, 29.62, 29.66, 31.84, 33.32 (d, *J* 34.5, 3-C), 37.31 (t, *J* 20.7, 2-C), 55.32, 65.98, 127.30, 128.90, 129.43, 135.33, 153.05, 177.36 (d, *J* 48.3, 1-C); δ_D 2.43 (d, *J* 19.2, 2-^{2}H).

S-2-Methyl-*N*-(*S*-1-phenylethyl)decanamide (18*S*)

S-2-Methyldecanoic acid **9*S*** (9.3 mg, 0.05 mmol), in dichloromethane (0.5 mL), was stirred with dimethylformamide (1 μL) and oxalyl chloride (20 μL) for 20 min. The solvent and excess reagents were removed under a stream of nitrogen. The residue, in dichloromethane (0.5 mL), was stirred with *S*-1-phenylethylamine (20 μL) for 20 min. The mixture was washed with aqueous HCl (1 M, 3 × 0.5 mL). Evaporation gave **18*S*** (13.8 mg, 93%) as a colourless gum: δ_H 0.86 (3 H, t, *J* 7.0, decanoyl 10-H_3), 1.13 (3H, d, *J* 6.8, decanoyl 2-CH_3), 1.24 (14H, m, 7 × CH_2), 1.52 (3 H, d, *J* 7.2, PhCHC*H*$_3$), 2.12 (1 H, m, decanoyl 2-H), 5.18 (1 H, m, NHC*H*), 5.71 (1 H, br d, *J* 6.7, NH), 7.35–7.40 (5 H, m, Ph-H_5).

R-2-Methyl-*N*-(*S*-1-phenylethyl)decanamide (18*R*)

Compound **9*R*** was treated with oxalyl chloride and *S*-1-phenylethylamine, as for the synthesis of **18*S***, to give **18*R*** (14.1 mg, 95%) as a colourless gum: δ_H 0.86 (3 H, t, *J* 7.0, decanoyl 10-H_3), 1.11 (3 H, d, *J* 6.4, decanoyl 2-CH_3), 1.29 (14 H, m, 7 × CH_2), 1.52 (3 H, d, *J* 6.8, PhCHC*H*$_3$), 2.12 (1 H, m, decanoyl 2-H), 5.18 (1 H, m, NHC*H*), 5.71 (1 H, br d, *J* 6.7, NH), 7.35–7.40 (5 H, m, Ph-H_5).

Synthesis of CoA esters[40]

In an Eppendorf cup, 2-methyldecanoic acid (4.7 mg, 4.7 μL, 25 μmol), in dry tetrahydrofuran (0.5 mL), was shaken with triethylamine (3.0 mg, 3.0 μL, 30 μmol) and ethyl chloroformate (3.2 mg, 2.8 μL, 30 μmol) for 30 min. The solvent and unreacted reagent were removed under a stream of nitrogen. The residue was suspended in tetrahydrofuran (0.7 mL) and centrifuged (2 min × 13000 r.p.m.). The supernatant solution was added to CoA-SH (8.2 mg, 10 μmol) in aqueous sodium hydrogen carbonate (100 mM, 0.5 mL). The mixture was shaken for 15 min then extracted with ethyl acetate (3 × 0.5 mL). The aqueous layer was acidified to pH 3 through the addition of small portions of Dowex X50 (H^+ form) and washed with ethyl acetate (0.5 mL). The solvent was removed under vacuum and the residue was taken into aqueous NaH_2PO_4–NaOH (50 mM, 1 mL) to give the CoA ester (~10 mM). The final concentration was determined by UV-visible spectroscopy ($\varepsilon_{260} = 16.0\ mM^{-1}\ cm^{-1}$).[41] For **10*R*** and **10*S*** δ_H (D_2O) 0.65 (3 H, s, CoA(CH_3)), 0.74 (3 H, t, *J* 6.9, CH_2CH_3), 0.78 (3 H, s, CoA(CH_3)), 1.01 (1 H, d, *J* 6.6, C*H*CH_3), 1.1 (12 H, m, 6 × CH_2), 1.31 (1 H, m, C*H*HCH), 1.48 (1H, m, CH*H*CH), 2.33 (2 H, t, *J* 6.9, 2H, CoA(CH_2)), 2.60 (1H, m, C*H*CH_3), 2.90 (2H, m, CoA(SCH_2)), 3.25 (2H, m, CoA(CH_2), 3.35 (2H, m, CoA(CH_2)), 3.45 (1H, m, CoA(CH)), 3.74 (1H, m, CoA(CH)), 3.93 (1H, s, CoA(CH)), 4.14 (2H, m, CoA(CH_2)), 4.48 (1H, s, CoA(CH)), 6.08 (1 H, d, *J* 6.6, CoA(OCHN)), 8.18 (1 H, s, CoA(ArH)), 8.47 (1H, s, CoA(ArH)).

Expression and purification of recombinant AMACR

PCR reactions using KOD polymerase were used to amplify AMACR 1A from the cDNA cloned into the pGEM-T vector[5] with forward (GACGACGACAAGATGGCACTGCAGGGCA TC) and reverse (GAGGAGGAGCCCGGTTAGAGACTAGC TTTTAC) primers. Reaction mixtures contained the following: sterile water (30 μL); buffer (5 μL; 20 mM Tris-HCl, pH 7.5); dNTPs (5 μL; 0.2 mM); $MgSO_4$ (2 μL; 1.5 mM); template (1 μL; 100 ng); primers (2 × 3 μL; 0.4 μM); polymerase (1 μL; 1 U). The following programme was used: 95 °C, 2 min; 30 cycles of 95 °C, 20 s; 52 °C, 30 s; 70 °C, 1 min; followed by 72 °C, 10 min and 4 °C thereafter. The progress of the reaction was analysed by 1% (w/v) agarose gel electrophoresis and the PCR product was purified (Qiagen kit). Following treatment of the PCR product with T4 DNA polymerase in the presence of dATP to generate vector-compatible overhangs, the PCR product was added to the pET30 Ek/LIC vector (Novagen) and the annealing reaction was allowed to proceed at 22 °C for 5 min. The annealed vector and insert mixture was transformed into Novablue competent *E. coli* cells (Novagen) and colonies were screened by colony PCR. DNA sequencing confirmed that the sequence of isolated clones was identical to AMACR 1A (Swissprot Q9UHK6) with no polymorphisms.

Recombinant AMACR 1A was expressed at 37 °C in *E. coli* BL21 (DE3) cells using Lennox LB media supplemented with kanamycin sulfate (30 μg mL^{-1}). At OD_{600} = 0.7, cells were transferred to 30 °C and 150 rpm and, after 10 min, induced with 0.25 mM IPTG. Cells were harvested 3 h post-induction by centrifugation (Beckman JA-10 rotor, 14300 × *g*, 30 min, 4 °C) and stored at −80 °C. Protein purification was carried out at 4 °C, unless otherwise stated. The cell pellet (2.85 g) was resuspended in NaH_2PO_4–NaOH (20 mM), NaCl (300 mM), imidazole (10 mM), pH 7.2, phenylmethylsulfonyl fluoride (PMSF, 1 mM) (30 mL) and lysed with a 'one-shot' system (Constant Systems) at 21 lb in^{-2} (145 KPa). Aqueous *N*-lauroylsarcosine [20% (w/v), 2.0 mL] was added and the solution was stirred for 1 h. The extract was centrifuged (Beckman JA-14 rotor, 9632 × *g*, 10 min, 4 °C) and polyethyleneimine [to 0.1% (v/v)] was added to the extract with stirring. After 30 min, the extract was centrifuged as before. The crude extract was loaded onto a 5 mL His-Trap FF column equilibrated in the above buffer with PMSF (0.2 mM), washed with the same buffer (10 mL) and eluted with imidazole-HCl (300 mM), pH 7.2 in buffer. Fractions (12 × 1.5 mL) were analysed by SDS-PAGE using a 10% running gel. Fractions #2–9 were pooled, exchanged into 10 mM Tris-HCl, pH 7.5, *N*-lauroylsarcosine [(0.5% (v/v)] and concentrated to 22 mg mL^{-1} (Bradford analysis, BSA standard[42]). Using this method, recombinant AMACR 1A was routinely purified to *ca.* 98% (as judged by SDS-PAGE analyses).

NMR studies of the AMACR-catalysed reaction

Unlabelled 2*S*- or 2*R*-substrate **10*R*** or **10*S*** (1.0 mM) was incubated with AMACR 1A (0.3 mg mL^{-1}) in 50 mM NaH_2PO_4–NaOH, pH 7.2 in a total volume of 0.7 mL at *ca.* 20 °C. Reactions

monitoring proton exchange were performed in the same assay mixture in the presence of *ca.* 90% ^2H_2O. At the required time the reaction was quenched with 10 M NaOH, and the extent of proton exchange was measured by integration of the peaks at $\delta = 1.16$ p.p.m. (α-methyl group) and $\delta = 2.43$ (α-proton) in the ^{1}H NMR spectrum. The acid products were extracted and converted to their *N*-(*S*-1-phenylethyl) amides, and deuterium exchange and chiral inversion was measured by integration of the peaks at $\delta = 1.13$ and $\delta = 1.11$. Kinetic parameters were determined using six concentrations of substrate for incubations of 60 min and rates calculated from % conversions determined by ^{1}H NMR. Data were fitted to the Michaelis–Menten equation using SigmaPlot 10.0.1 with enzyme kinetic module 1.3 (Systat) and the direct linear plot.[20,21] k_{cat} values were calculated assuming a MW for His-tag AMACR 1A of 47146.8 Da (protparam; http://www.expasy.ch/tools/protparam.html).

S-2-[^{13}C]-[^{2}H]-Substrate **17*S*** (1.0 mM) was incubated with AMACR 1A (0.3 mg mL^{-1}) in 50 mM NaH_2PO_4–NaOH, pH 7.2 in a total volume of 0.7 mL at ~20 °C and monitored by ^{13}C NMR, integrating the triplet at $\delta = 47.11$ (substrate) and the singlet at δ 47.55 (^{1}H product) to determine the extent of conversion (after correcting for nOe enhancement, see below). Data was fitted to a two parameter single rectangular hyperbola, $f = a*x/(b + x)$.

To determine a value for any nOe enhancement of the ^{13}C NMR signal, *S*-2-[^{13}C]-2-[^{2}H]-methyldecanoyl-CoA **17*S*** was incubated with AMACR 1A until the integrals for the singlet and triplet signals (at δ 47.55 and δ 47.11) reached the ratio of 1 : 1. The reaction was then quenched and the CoA esters were hydrolysed with aq. NaOH (10 M, NaOH, 20 min) then acidified (HCl) and the acids were extracted into $CDCl_3$. Comparing the integrals of the ^{1}H NMR signals arising from the α-methyl group (CH_3; δ 1.16) and the α-proton (CH; δ 2.43) showed that the α-carbon carried 35% ^{1}H. The nOe enhancement of the singlet peak in the ^{13}C NMR spectra was thus calculated to be ~1.45.

Acknowledgements

We thank Professor R. J. A. Wanders and Dr. S. Ferdinandusse (Academic Medical Centre, University of Amsterdam, The Netherlands) for AMACR 1A cDNA. We thank one of the referees for highlighting a hitherto unappreciated aspect of this work. This work was supported by EU grant QLG3-CT-2002-00696 (Refsum's disease: Diagnosis, Pathology and Treatment) and by Cancer Research UK. Parts of this work were performed by SJP, TWT and ADW as an undergraduate project for the MPharm degree (University of Bath, U.K.).

Notes and references

1 A. S. Wierzbicki, M. D. Lloyd, C. J. Schofield, M. D. Feher and F. B. Gibberd, *J. Neurochem.*, 2002, **80**, 727–735; M. Mukherji, C. J. Schofield, A. S. Wierzbicki, G. A. Jansen, R. J. A. Wanders and M. D. Lloyd, *Prog. Lipid Res.*, 2003, **42**, 359–376.
2 M. D. Lloyd, D. J. Darley, A. S. Wierzbicki and M. D. Threadgill, *FEBS J.*, 2008, **275**, 1089–1102.
3 W. Schmitz and E. Conzelmann, *Euro. J. Biochem.*, 1997, **244**, 434–440.
4 S. Ferdinandusse, H. Rusch, A. E. M. van Lint, G. Dacremont, R. J. A. Wanders and P. Vreken, *J. Lipid Res.*, 2002, **43**, 438–444.
5 S. Ferdinandusse, S. Denis, P. T. Clayton, A. Graham, J. E. Rees, J. T. Allen, B. N. McLean, A. Y. Brown, P. Vreken, H. R. Waterham and R. J. A. Wanders, *Nat. Genet.*, 2000, **24**, 188–191.
6 L. Amery, M. Fransen, K. De Nys, G. P. Mannaerts and P. P. Van Veldhoven, *J. Lipid Res.*, 2000, **41**, 1752–1759; S. Ferdinandusse, S. Denis, L. Ijlst, G. Dacremont, H. R. Waterham and R. J. A. Wanders, *J. Lipid Res.*, 2000, **41**, 1890–1896; T. J. Kotti, K. Savolainen, H. M. Helander, A. Yagi, D. K. Novikov, N. Kalkkinen, E. Conzelmann, J. K. Hiltunen and W. Schmitz, *J. Biol. Chem.*, 2000, **275**, 20887–20895.
7 C. Reichel, H. Bang, K. Brune, G. Geisslinger and S. Menzel, *Biochem. Pharmacol.*, 1995, **50**, 1803–1806.
8 S. Ferdinandusse, H. Overmars, S. Denis, H. R. Waterham, R. J. A. Wanders and P. Vreken, *J. Lipid Res.*, 2001, **42**, 137–141.
9 W. Schmitz, C. Albers, R. Fingerhut and E. Conzelmann, *Euro. J. Biochem.*, 1995, **231**, 815–822.
10 K. Savolainen, P. Bhaumik, W. Schmitz, T. J. Kotti, E. Conzelmann, R. K. Wierenga and J. K. Hiltunen, *J. Biol. Chem.*, 2005, **280**, 12611–12620.
11 P. Bhaumik, W. Schmitz, A. Hassinen, J. K. Hiltunen, E. Conzelmann and R. K. Wierenga, *J. Mol. Biol.*, 2007, **367**, 1145–1161.
12 W. Schmitz, R. Fingerhut and E. Conzelmann, *Euro. J. Biochem.*, 1994, **222**, 313–323.
13 G. L. Shen-Ong, Y. Feng and D. A. Troyer, *Cancer Res.*, 2003, **63**, 3296–3301; J. N. Mubiru, G. L. Shen-Ong, A. J. Valente and D. A. Troyer, *Gene*, 2004, **327**, 89–98; J. N. Mubiru, A. J. Valente and D. A. Troyer, *Prostate*, 2005, **65**, 117–123.
14 P. Berglund, M. Holmquist, E. Hedenstrom, K. Hult and H. E. Hogberg, *Tetrahedron-Asymmetry*, 1993, **4**, 1869–1878.
15 L. M. Hutchins, L. Hunter, N. Ehya, M. D. Gibbs, P. L. Bergquist and C. A. Hutton, *Tetrahedron-Asymmetry*, 2004, **15**, 2975–2980.
16 P. E. Sonnet and R. R. Heath, *J. Org. Chem.*, 1980, **45**, 3137–3139.
17 K. Morigaki, S. Dallavalle, P. Walde, S. Colonna and P. L. Luisi, *J. Am. Chem. Soc.*, 1997, **119**, 292–301.
18 N. F. Yaggi and K. T. Douglas, *J. Am. Chem. Soc.*, 1977, **99**, 4844–4845.
19 W. R. Shieh and C. S. Chen, *J. Biol. Chem.*, 1993, **268**, 3487–3493.
20 A. Cornish-Bowden and R. Eisenthal, *Biochem. J.*, 1974, **139**, 721–730.
21 R. Eisenthal and A. Cornish-Bowden, *Biochem. J.*, 1974, **139**, 715–720.
22 C. S. Chen, T. L. Chen and W. R. Shieh, *Biochim. Biophys. Acta*, 1990, **1033**, 1–6; C. S. Chen, W. R. Shieh, P. H. Lu, S. Harriman and C. Y. Chen, *Biochim. Biophys. Acta*, 1991, **1078**, 411–417; W. R. Shieh, D. M. Gou, Y. C. Liu, C. S. Chen and C. Y. Chen, *Anal. Biochem.*, 1993, **212**, 143–149.
23 C. Reichel, R. Brugger, H. Bang, G. Geisslinger and K. Brune, *Mol. Pharmacol.*, 1997, **51**, 576–582.
24 V. M. Powers, C. W. Koo, G. L. Kenyon, J. A. Gerlt and J. W. Kozarich, *Biochemistry*, 1991, **30**, 9255–9263.
25 G. J. Cardinal and R. H. Abeles, *Biochemistry*, 1968, **7**, 3970–3978.
26 Z. Jiang, B. A. Woda, K. L. Rock, Y. D. Xu, L. Savas, A. Khan, G. Pihan, F. Cai, J. S. Babcook, P. Rathanaswami, S. G. Reed, J. C. Xu and G. R. Fanger, *Am. J. Surg. Path.*, 2001, **25**, 1397–1404.
27 J. Luo, S. Zha, W. R. Gage, T. A. Dunn, J. L. Hicks, C. J. Bennett, C. N. Ewing, E. A. Platz, S. Ferdinandusse, R. J. Wanders, J. M. Trent, W. B. Isaacs and A. M. De Marzo, *Cancer Res.*, 2002, **62**, 2220–2226.
28 A. K. Witkiewicz, S. Varambally, R. L. Shen, R. Mehra, M. S. Sabel, D. Ghosh, A. M. Chinnaiyan, M. A. Rubin and C. G. Kleer, *Cancer Epidemiology Biomarkers & Prevention*, 2005, **14**, 1418–1423.
29 Z. Jiang, G. R. Fanger, B. F. Banner, B. A. Woda, P. Algate, K. Dresser, J. C. Xu, S. G. Reed, K. L. Rock and P. G. Chu, *Cancer Detect. Prev.*, 2003, **27**, 422–426.
30 Y.-T. Chen, J. J. Tu, J. Kao, X. K. Zhou and M. Mazumdar, *Clin. Cancer Res.*, 2005, **11**, 6558–6566.
31 P. T. Went, G. Sauter, M. Oberholzer and L. Bubendorf, *Pathology*, 2006, **38**, 426–432.
32 S. Zha, S. Ferdinandusse, S. Denis, R. J. Wanders, C. M. Ewing, J. Luo, A. M. De Marzo and W. B. Isaacs, *Cancer Res.*, 2003, **63**, 7365–7376; S. Zheng, B. L. Chang, S. D. Isaacs, K. E. Wiley, A. R. Turner, G. A. Hawkins, E. R. Bleecker, P. C. Walsh, D. A. Meyers, W. Isaacs and J. Xu, *Am. J. Hum. Genet.*, 2002, **71**, 420; S. Q. L. Zheng, B. L. Chang, D. A. Faith, J. R. Johnson, S. D. Isaacs, G. A. Hawkins, A. Turner, K. E. Wiley, E. R. Bleecker, P. C. Walsh, D. A. Meyers, W. B. Isaacs and J. F. Xu, *Cancer Res.*, 2002, **62**, 6485–6488.
33 T. Thornburg, A. R. Turner, Y. Q. Chen, M. Vitolins, B. Chang and J. Xu, *Future Oncol.*, 2006, **2**, 213–223.
34 J. A. Mobley, I. Leav, P. Zielie, C. Wotkowitz, J. Evans, Y. W. Lam, B. S. L'Esperance, Z. Jiang and S. Ho, *Cancer Epidemiol. Biomarkers*

Prev., 2003, **12**, 775–783; C. Kumar-Sinha, R. B. Shah, B. Laxman, S. A. Tomlins, J. Harwood, W. Schmitz, E. Conzelmann, M. G. Sanda, J. T. Wei, M. A. Rubin and A. M. Chinnaiyan, *Am. J. Pathol.*, 2004, **164**, 787–793.
35 J. F. Xu, T. Thornburg, A. R. Turner, M. Vitolins, D. Case, J. Shadle, L. Hinson, J. L. Sun, W. N. Liu, B. L. Chang, T. S. Adams, S. L. Zheng and F. M. Torti, *Prostate*, 2005, **63**, 209–214.
36 A. M. Levin, K. A. Zuhlke, A. M. Ray, K. A. Cooney and J. A. Douglas, *Prostate*, 2007, **67**, 1507–1513; S. E. Daugherty, Y. Y. Shugart, E. A. Platz, M. D. Fallin, W. B. Isaacs, R. M. Pfeiffer, R. Welch, W. Y. Huang, D. Reding and R. B. Hayes, *Prostate*, 2007, **67**, 1487–1497.
37 S. E. Daugherty, E. A. Platz, Y. Y. Shugart, M. D. Fallin, W. B. Isaacs, N. Chatterjee, R. Welch, W. Y. Huang and R. B. Hayes, *Cancer Epidemiology Biomarkers & Prevention*, 2007, **16**, 1536–1542.
38 D. E. Levy, F. Lapierre, W. S. Liang, W. Q. Ye, C. W. Lange, X. Y. Li, D. Grobelny, M. Casabonne, D. Tyrrell, K. Holme, A. Nadzan and R. E. Galardy, *J. Med. Chem.*, 1998, **41**, 199–223.
39 J. F. Wolfe and C.-L. Mao, *J. Org. Chem.*, 1967, **32**, 3382–3386.
40 J. T. Bernert and H. Sprecher, *J. Biol. Chem.*, 1977, **252**, 6736–6744.
41 J. F. Baker-Malcolm, L. Haeffner-Gormley, L. P. Wang, M. W. Anders and C. Thorpe, *Biochemistry*, 1998, **37**, 1383–1393.
42 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248–254.

