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Structure-activity relationships of rationally designed AMACR 1A inhibitors

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ABSTRACT

α-Methylacyl-CoA racemase (AMACR; P504S) is a promising novel drug target for prostate and other cancers. Assaying enzyme activity is difficult due to the reversibility of the 'racemisation' reaction and the difficulties in the separation of epimeric products; consequently few inhibitors have been described and no structure–activity relationship study has been performed. This paper describes the first structure–activity relationship study, in which a series of 23 known and potential rational AMACR inhibitors were evaluated. AMACR was potently inhibited (IC_{50} = 400–750 nM) by ibuprofenoyl-CoA and derivatives. Potency was positively correlated with inhibitor lipophilicity. AMACR was also inhibited by straight-chain and branched-chain acyl-CoA esters, with potency positively correlating with inhibitor lipophilicity. 2-Methyldecanoyl-CoAs were ca. 3-fold more potent inhibitors than decanoyl-CoA, demonstrating the importance of the 2-methyl group for effective inhibition. Elimination substrates and compounds with modified acyl-CoA cores were also investigated, and shown to be potent inhibitors. These results are the first to demonstrate structure–activity relationships of rational AMACR inhibitors and that potency can be predicted by acyl-CoA lipophilicity. The study also demonstrates the utility of the colorimetric assay for thorough inhibitor characterisation.

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1. Introduction

Branched-chain fatty acids (e.g. phytanic acid pristanic acids) are common components of the human diet, and derivatives of such com-pounds are used as drug molecules e.g. ibuprofen [1,2]. Degradation of branched-chain fatty acids occurs as the acyl-CoA ester, and the acyl-CoA oxidases and other enzymes involved in β-oxidation have an ab-solute requirement for S-2-methylacyl-CoAs [3–5]. However, R-2-me-thylacyl-CoAs are produced from dietary and endogenous fatty acids and these cannot be immediately degraded by β-oxidation. The enzyme α-methylacyl-CoA racemase [1,2] (AMACR; P504S; E.C. 5.1.99.4) cat-alyses conversion of R-2-methylacyl-CoAs to a near 1:1 epimeric mix-ture [6,7] by a deprotonation/reprotonation reaction [7,8], probably via an enolate intermediate [9] (this reaction is referred to as "race-misation" [10]). The resulting S-2-methylacyl-CoAs are degraded by β-oxidation whilst the 2R epimers are further processed to the 2S epimers by AMACR [1,2]. AMACR also plays a key role in the in vivo pharma-cological activation of R-ibuprofen by conversion to S-ibuprofen, via the corresponding acyl-CoA esters [1,2,11]. The S-ibuprofen resulting from this pathway exerts its anti-inflammatory effect by inhibiting
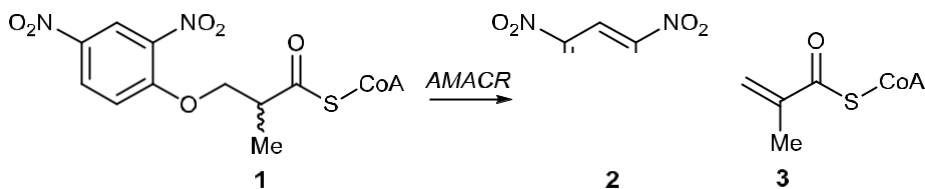
cyclooxygenase-1 and -2 [12].

AMACR protein levels are increased in prostate [13,14] and several other cancers [1,15–18]. Catalytic activity of AMACR is increased by 4-to 10-fold in prostate cancer cells [19,20], with the AMACR 1A splice variant [1,2,21–24] (possessing "racemase" activity [7,10]) showing the most significant increase in expression [19,20]. Reducing AMACR 1A levels using siRNA or shRNA approaches [19,25,26] has been shown to reduce proliferation of prostate cancer cells via a pathway which is synergistic with the use of an androgen receptor antagonist, studies which have validated AMACR 1A as a chemotherapeutic target. Some advanced prostate cancer cell lines revert from castrate-resistant (a.k.a. androgen-independent) growth to androgen-dependent growth upon knockdown of AMACR 1A [26]. Consequently, AMACR has attracted considerable interest as a prostate cancer biomarker [1,2,27] and drug target [25,28–31]. However, the lack of a convenient assay to measure AMACR activity [32,33] has severely hampered the development of AMACR inhibitors as new chemotherapeutic drugs against cancers that over-express AMACR, and consequently only a few rationally designed inhibitors of AMACR [28–30,34] or MCR [31,35] (M. tuberculosis homologue) have been reported. No systematic study of AMACR

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inhibitor SAR has been undertaken [32,33].

Recently, we reported a versatile continuous assay for AMACR based on the utilisation of our novel substrate 1 that can eliminate 2,4-dinitrophenolate 2, which can be monitored by absorbance at 354 nm, and unsaturated product 3 (Scheme 1). This new assay [32] was used to examine the potency of two known acyl-CoA inhibitors (N-dodecyl-N-methylcarbamoyl-CoA 4 [29] and ibuprofenoyl-CoA 5 [6]; Fig. 1) and selected known non-specific protein-modification agents [25]. This paper reports the first systematic examination of SAR for rationally designed acyl-CoA inhibitors of AMACR. Compounds investigated (Fig. 1) include those with aromatic side-chains, (5–11); Straight-chain acyl-CoA esters (12–17); Branched-chain substrates (18–21) and pro-duct 22. Analogues of known inhibitors with modified 2-methylacyl-CoA moieties (4, 23–26) were also examined. The results reveal a correlation between potency and lipophilicity of the inhibitors, con-sistent with observations on MCR inhibitors [35], the homologous en-zyme from M. tuberculosis.

2. Results and discussion

AMACR is a promising novel cancer drug target, but therapeutic development in this field has been slow due to the lack of a robust enzyme assay. Thus, the majority of studies reporting AMACR in-hibitors have largely focussed on rationally designed drugs [28–30,32,33]. In most cases, only one or a few examples of each in-hibitor type has been evaluated, and no systematic SAR study has been performed. Initial SAR studies have been carried out on reversible [31] and irreversible [35] inhibitors of MCR (the M. tuberculosis homologue). In addition, different research groups have used different assays during their studies, making it difficult to compare results directly. In this study, the SAR of rational AMACR inhibitors were explored using a series of acyl-CoA esters (Fig. 1). These included compounds previously tested as substrates (5–11 [6,11]; 12–17 [36]; 18–20, 22 [7,10]). Most of these compounds have not been tested as inhibitors with the ex-ception of 5 [28,32,37,38] and 13, 15–17 (which were previously re-ported to be inactive [37,38]). Compound 21 was included as an epimer of 20, and has not been previously reported as a substrate or inhibitor (although the 3-fluoro-2-methylhexadecanoyl-CoA analogues are po-tent inhibitors [28]). Compound 24 is a synthetic intermediate to 25, and has not been previously tested as a substrate or inhibitor. Com-pounds 22, 25 and 26 are intermediates in the subsequent β-oxidation pathway [39], and have not been previously tested as substrates or inhibitors. Analogues of compounds 23, and 25 with different side-chains have been previously tested as inhibitors of AMACR or MCR [9,30]. Compound 4 was previously reported as the most potent AMACR inhibitor [29,32], and is included as an acyl-CoA core ana-logue.

2.1. Chemical synthesis of acyl-CoA inhibitors

(2S,3S)-3-Fluoro-2-methyldecanoyl-CoA 21 was synthesised by an analogous route to (2R,3R)-3-fluoro-2-methyldecanoyl-CoA 20 [10,28], using an Evans' auxiliary strategy (Scheme 2). Aldol-like reaction of deprotonated 27 with octanal gave the (2S,3R)-3-hydroxy-2-methyl intermediate 28. From here, alcohol 28 was activated and replaced with fluoride with inversion of configuration, using DAST to give 3-fluoro-2-methyl derivative 29. The reaction is thought to go with

inversion of stereochemical configuration (by analogy with the work of Carnell et al. [28]). Removal of the Evans' auxiliary from intermediate 29 provided the carboxylic acid 30 under mild conditions that involved in situ generation of lithium hydroperoxide. Intermediate 30 was sub-sequently converted to the CoA thioester 21 using the standard syn-thetic method with N,N'-carbonyldiimidazole [6,10,11,32,33,40]. This compound was stable in solution in the absence of AMACR, showing that the relative geometry of the α-proton and fluorine atom was syn-(anti- epimers rapidly eliminate fluoride, presumably by an E2 me-chanism [10]).

2-Methylenedecanoyl-CoA 23 was synthesised by an adaptation of the method reported by Morgenroth et al. [30] (Scheme 3). Meldrum's acid 33 was acylated with octanoic acid 32 using DCC activation; the intermediate ketone was reduced to the octyl-Meldrum's acid 31 with sodium triacetoxyborohydride generated in situ. Subsequent reaction of 31 with Eschenmoser's salt gave the 2-methylene ester 34. Base-hy-drolysis furnished the 2-methylene acid 35, which was coupled with CoA-SH by a mixed anhydride approach to give 2-methylenedecanoyl-CoA 23.

2-Methyl-3-oxodecanoyl-CoA 25 was synthesised by the method of Reen et al. [41] from 36 (Scheme 4). The ketone in 37 was protected as the cyclic acetal 36. Hydrolysis of the ester group in 36 gave the cor-responding acid 38, which was then coupled with CoA [6,10,11,32,33,40] to give 24. Acidolysis of the acetal protection pro-vided 25.

2S,3R-3-Hydroxy-2-methyldecanoyl-CoA 26 was synthesised (Scheme 5) from the acyl-Evan's auxiliary 28 (Scheme 2, vide supra) by hydrolysis with lithium hydroperoxide to give acid 39, which was converted to the CoA ester 26 using the standard procedure [6,10,11,32,33,40].

2.2. Evaluation of inhibitors

The selected AMACR inhibitors were evaluated using the colori-metric assay [32]. Incubation of active human AMACR 1A with sub-strate 1 results in production of 2,4-dinitrophenolate 2 and unsaturated product 3 (Scheme 1). Hence, enzyme activity can be determined based on measuring the absorbance of 2 at 354 nm over the assay time course. Inhibitory potency was assessed using dose–response curves to de-termine IC_{50} values (Fig. 1).

As expected, ibuprofenoyl-CoA 5 and its derivatives 6–9 were in-hibitors of the enzyme, with most having IC_{50} values of ca. 500 nM (Fig. 1). Variation of the structure of the side-chain in these inhibitors appeared to make little difference to inhibitory activity (as judged by IC_{50} values), although fenoprofenoyl-CoA 6 appeared to be slightly more potent than the other examples and naproxenoyl-CoA 9 appeared to be slightly less so. All these compounds are known substrates of AMACR [6] and are predicted to behave as competitive inhibitors. Ibuprofenoyl-CoA 5 has been previously confirmed to be a competitive inhibitor of AMACR, with $K_i = 60$ nM [32], consistent with observa-tions of other workers on the human and rat enzymes [28,37,38]. The mandelic acid derivatives R- and S-2-hydroxy-2-phenylacetyl-CoA 10 and 11 were also modest inhibitors (Fig. 1), binding approximately ten times less strongly than compounds 5–9. Compounds 10 and 11 are not substrates of AMACR, since enzyme-catalysed α-proton exchange does not occur [11]. This result with 10 and 11 also demonstrates that in-hibitors can possess a 2-hydroxy- group in addition to the previously
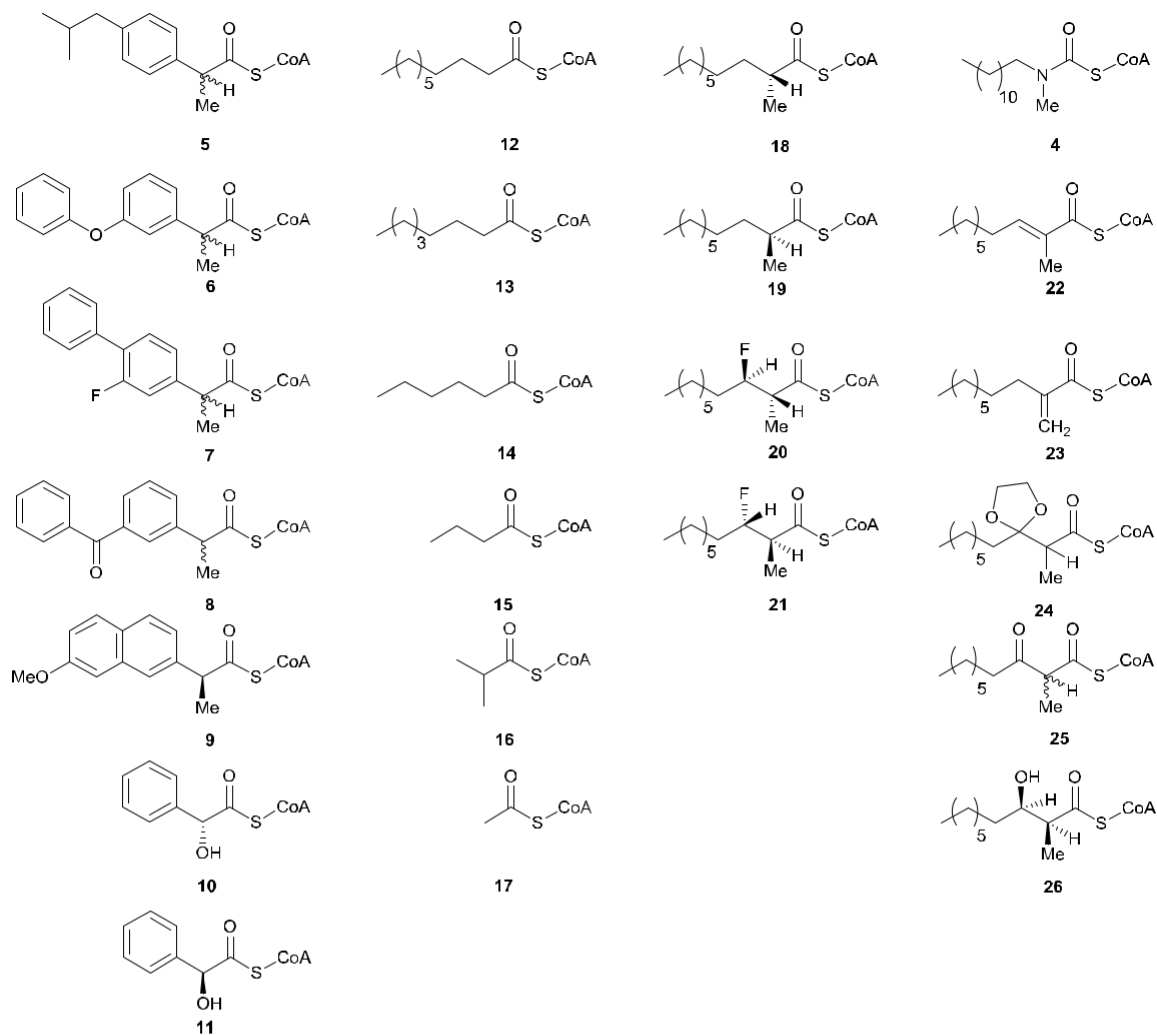
| Compound	IC$_{50}$ (nM)	miLogP*	Previously tested as substrate?	Previously tested as Inhibitor?
4	~0.4	-2.61	No	Yes [29, 32]
5	540	-4.18	Yes [6, 29, 49]	Yes [28, 32, 37, 38]
6	400	-3.77	Yes [6, 11]	Not tested
7	590	-3.62	Yes [6]	Not tested
8	560	-4.07	Yes [6]	Not tested
9	750	-4.25	Yes [6]	Not tested
10	3.8×10^3	-5.46	Not a substrate [11]	Not tested
11	2.3×10^3	-5.46	Not a substrate [11]	Not tested
12	3.1×10^3	-3.63	Yes [36]	Not tested
13	9.6×10^3	-4.50	Yes [36]	Yes – No inhibition [37, 38]
14	1.6×10^4	-4.98	Yes [36]	Not tested
15	$>1.0 \times 10^5$	-5.30	Poor substrate [36]	Yes – No inhibition [37, 38]
16	$>1.0 \times 10^5$	-5.38	Not a substrate [36]	Yes – No inhibition [37, 38]
17	$>1.0 \times 10^5$	-5.55	Not tested	Yes – No inhibition [37, 38]
18	930	-3.39	Yes [7]	Not tested
19	1170	-3.39	Yes [7, 36]	Not tested
20	200	-3.61	Yes [10]	Not tested[†]
21	300	-3.61	Not tested	Not tested[†]
22	180	-3.34	Not a substrate [10]	Yes – No inhibition [37, 38]
23	600	-3.56	Not tested	Not tested[‡]
24	4.1×10^3	-4.02	Not tested	Not tested
25	360	-4.55	Not tested	Not tested[‖]
26	560	-4.43	Not tested	Not tested

Fig. 1. Structures for AMACR inhibitors, as measured by the colorimetric assay [32]. *Calculated miLogP values are for the acyl-CoA ester (http://www. molinspiration.com/cgi-bin/properties). [†](2R,3R)- and (2S,3S)-3-Fluoro-2-methylhexadecanoyl-CoA were previously shown to be AMACR inhibitors [28]. [‡]Several side-chain analogues of 23 reported as inhibitors [30]. [‖]Binding of the 2-methylacetoacetyl-CoA enolate to MCR observed by X-ray crystallography [9]. (See above-mentioned references for further information.)
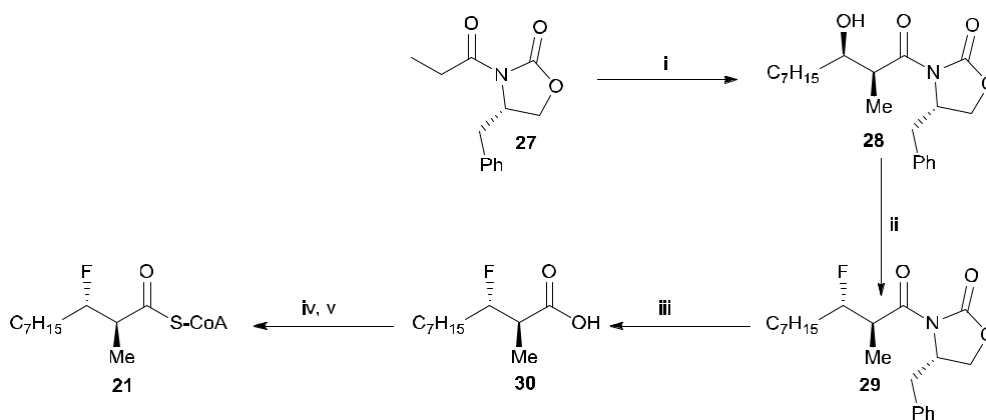
Scheme 2. Synthesis of (2S,3S)-3-fluoro-2-methyldecanoyl-CoA 21. Reagents and conditions: i. Bu$_2$BOTf, Pr$^i_2$NEt, octanal, CH$_2$Cl$_2$, −78 °C, 76%; ii. DAST, CH$_2$Cl$_2$, −78 °C, 35%; iii. LiOH, H$_2$O$_2$, H$_2$O/THF, 0 °C, 92%; iv. CDI, CH$_2$Cl$_2$, rt; v. CoA-SH Li$^+_3$, 0.1 M NaHCO$_3$ aq./THF (1:1).
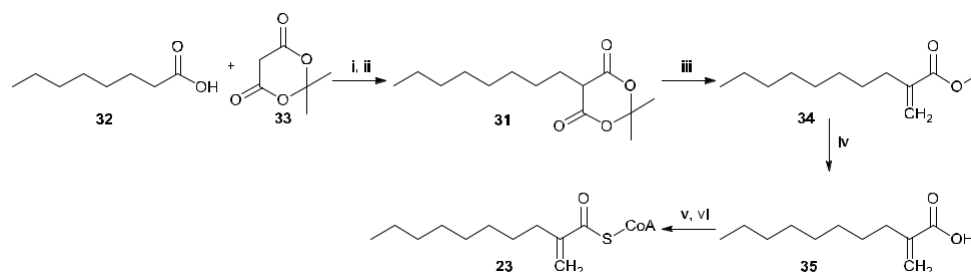
Scheme 3. Synthesis of 2-methylenedecanoyl-CoA 23. Reagents and conditions: i, DCC, DMAP, CH$_2$Cl$_2$; ii, NaBH$_4$, AcOH, 89% over two steps; iii, Me$_2$N$^+$=CH$_2$ I$^-$, MeOH, 74%; iv, NaOH, EtOH, 80%; v, NEt$_3$, EtOCOCl, THF; vi, aq. KHCO$_3$ (2.5% w/v), CoA-SH Li$^+_3$, THF.

reported 2-trifluoromethyl- [28] and 2-chloro- [29] groups in place of the 2-methyl group. A wide range of aromatic inhibitor side-chains can therefore be accommodated by the enzyme, consistent with predictions made based on the MCR crystal structures [8] and biochemical data [6,11].

Acyl-CoA esters 12–17, possessing alkyl side-chains, were also as-sessed as inhibitors (Fig. 1). The potency of inhibition for acyl-CoA esters with side-chains of four carbons or fewer (15–17) is very weak, with low levels of inhibition (15–30%) observed even at very high in-hibitor concentrations (100 μM). Inhibition increased as alkyl chain-length increased. This behaviour is consistent with that of straight-chain acyl-CoA esters acting as substrates [36], where increased levels of α-proton exchange are observed with increasing chain length. Our results showing inhibition of AMACR by straight-chain acyl-CoA esters contrasts with the early observations of Schmitz et al., who reported that these compounds were not inhibitors of the native human and rat enzymes [37,38].

Inclusion of a 2-methyl group on the inhibitor increased potency by about 3-fold (compounds 18 and 19 compared to 12), again consistent with the finding that 2-methylacyl-CoA esters are much more efficient substrates than their straight-chain equivalents [36]. R-2-Methyldeca-noyl-CoA 18 appeared to be a slightly more potent inhibitor than S-2-

methyldecanoyl-CoA 19, and this probably reflects the physiological role of AMACR in the conversion of R-2-methylacyl-CoAs to their S-2-methylacyl-CoA epimers [1,2].

2-Methyldecanoyl-CoA derivatives with more acidic α-protons are better inhibitors than their parent compounds (Fig. 1). R,R-3-Fluoro-2-methyldecanoyl-CoA 20 was about 5-fold more potent than was R-2-methyldecanoyl-CoA 18. A similar trend was observed with S,S-3-fluoro-2-methyldecanoyl-CoA 21 and S-2-methyldecanoyl-CoA 19, al-though both of these compounds were slightly less potent than their R-epimers. The product of the reaction, E-2-methyldec-2-enoyl-CoA 22, is also a potent inhibitor. It is therefore difficult to determine if the ob-served IC$_{50}$ values for 20 or 21 reflect the conversion of these substrates [10], product inhibition by 22 or both. These observations contrast with early studies [37,38], which suggest that 22 was not an inhibitor of AMACR.

2-Methyl-3-oxodecanoyl-CoA 25 was also a good inhibitor of AMACR. The α-proton of this compound is relatively acidic and 25 undergoes rapid non-enzymatic α-proton exchange with solvent via an enolate intermediate. It was therefore not possible to analyse the in-fluence of 2-methyl group stereochemical configuration. The precursor 24 was a much poorer inhibitor than 25, presumably due to reduced acidity of the α-proton. It is also possible that the additional steric bulk
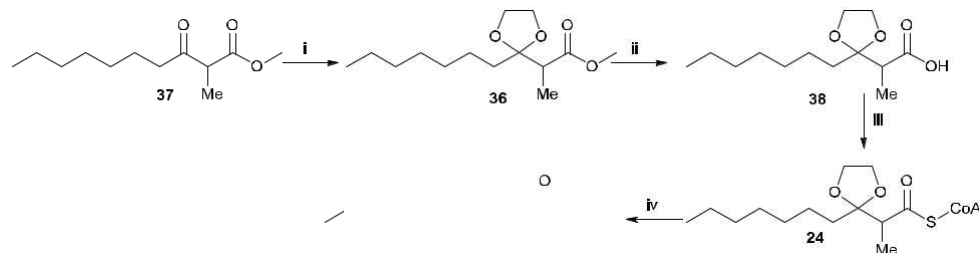
Scheme 4. Synthesis of 2-methyl-3-oxodecanoyl-CoA 25. Reagents and conditions: i, ethane-1,2-diol, TMSCl, CH$_2$Cl$_2$, 82%; ii, aq. NaOH/MeOH, 40%; iii, CDI, CH$_2$Cl$_2$; v, CoA-SH Li$^+_3$, 0.1 M NaHCO$_3$ aq./THF (1:1); iv, aq. HCl, acetone.
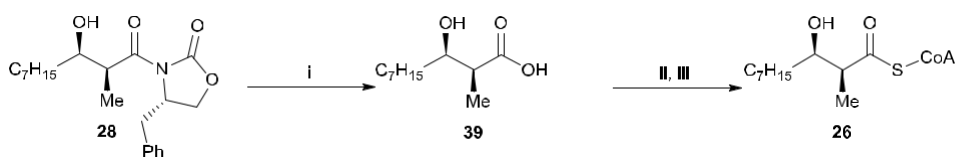
Scheme 5. Synthesis of (2S,3R)-3-hydroxy-2-methyldecanoyl-CoA 26. Reagents and condi-tions: i. NaOH, H_2O_2, rt, quant.; ii. CDI, CH_2Cl_2, rt; iii. CoA-SH Li^+_3, 0.1 M $NaHCO_3$ aq./THF (1:1).
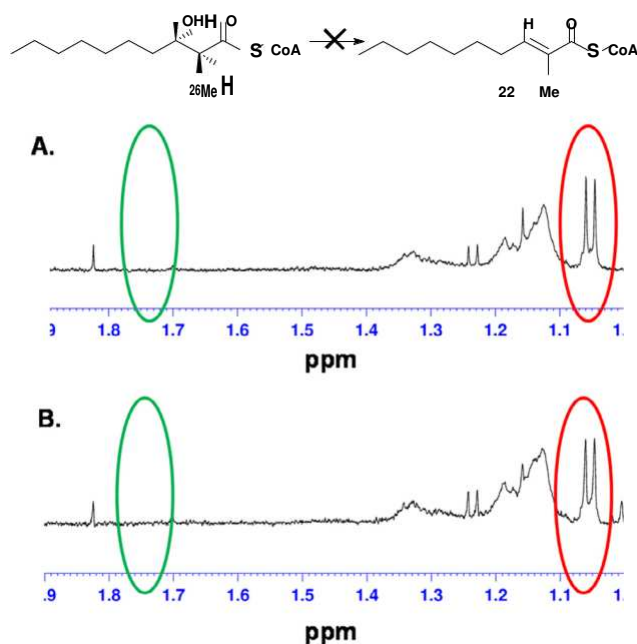

Fig. 2. Incubation of 2S,3R-3-hydroxy-2-methyldecanoyl-CoA 26 with AMACR in buffer and ^2H_2O. A. Heat-inactivated enzyme; B. live enzyme. Red circles highlight doublet for substrate 2-methyl group, showing no exchange of the α-proton had occurred (conversion to a single peak occurs on exchange to α-^{2}H upon 'racemisation' [7,11]). Green circles denote expected position of 2-methyl singlet for the expected unsaturated product 22, showing that no elimination reaction has occurred.

at carbon-3 contributes to the lower potency of 24 compared to 25, as the 5-membered ring of 24 will be twisted out of plane relative to the aliphatic side-chain. However, AMACR is known to be able to accept substrates with diverse side-chain structures [6,28,36–38] and it is notable that ibuprofenoyl-CoA 5 and derivatives 6–9 (which have aromatic rings at the equivalent position) are potent inhibitors (Fig. 1).

2S,3R-3-Hydroxy-2-methyldecanoyl-CoA 26 is also a relatively po-tent inhibitor. Incubation of 26 with active AMACR did not result in an elimination reaction, as judged by the lack of a peak at δ 1.75 ppm [10] from the 2-methyl group of the anticipated product 22 (Fig. 2), prob-ably because hydroxide is a relatively poor leaving group (water pK_a = ~14.0–15.7), compared to fluoride (HF pK_a = 3.2) [42]. Simi-larly, ^{1}H NMR analysis of the reaction products showed that 26 did not undergo α-proton exchange, and hence 26 is not a substrate of AMACR. It is notable that 22, 24 and 26 are intermediates in the branched-chain acyl-CoA β-oxidation pathway. One may speculate that these com-pounds could provide some regulation of AMACR by negative feedback control, and hence control entry of R-2-methylacyl-CoA esters into the β-oxidation pathway. Whether or not this is physiologically significant will depend on rates of flux through the β-oxidation pathway and whether intermediates 22, 24 and 26 are sequestered away from AMACR.

Acyl-CoAs which mimic the planar enolate intermediate are good inhibitors of AMACR (Fig. 1). E-2-Methyldec-2-enoyl-CoA 22 and 2-methylenedecanoyl-CoA 23 bind strongly due to having a planar sp^2-hybridized α-carbon. This result is consistent with a previous study in which 2-methyleneacyl-CoAs were shown to be good competitive

inhibitors [2,30]. The enolate analogue N-dodecyl-N-methylcarbamoyl-CoA 4 is the best rationally designed inhibitor (IC_{50} = 0.4 nM) reported to date [29,32]. Inhibition by 4 is ca. 2000× more potent than by 18 and 19 (Fig. 1). This high potency of 4 appears to largely result from the mimicking of the enolate intermediate by the carbamoyl moiety. It is also notable that the determined IC_{50} value for 4 in this study (0.4 nM) is significantly lower than that previously determined by Carnell et al. (98 nM), when assayed against HEK-derived human AMACR using ibuprofenoyl-CoA 5 as substrate [29]. It appears that the colorimetric assay consistently determines higher levels of compound potency than other assays (e.g. K_i = 60 nM [32] vs. 56 μM [28,38] for an ibuprofe-noyl-CoA epimeric mixture) The reasons for this discrepancy is not entirely clear, but higher apparent potency may be a consequence of using a substrate undergoing an irreversible reaction to measure ac-tivity (and hence avoiding the error introduced by the presence of the reverse reaction). Alternatively, this may be related to the extent of substrate or inhibitor micelle formation under the different assay con-ditions.

The determined IC_{50} value of 4 (0.4 nM) is around half the calcu-lated 'active' enzyme concentration in the assay [32] (based on com-parison of k_{cat}/K_m values for the E. coli and HEK cell derived enzymes [29]). Hence 4 could be behaving as a tight-binding inhibitor. Com-pound 4 behaves as a rapidly reversible competitive inhibitor of AMACR, with a Hill coefficient of ~0.7 [32]. These observations are consistent with the zone A inhibitor behaviour described by Straus and Goldstein [43,44], i.e. the enzyme active site concentration is < 0.1 × the apparent K_i value (0.65 nM [32]). This rapidly reversible inhibition is significantly different behaviour to that observed for similar com-pounds (gem- carbamoyl inhibitors and N-decyl-carbamoyl-CoA) with the highly homologous bacterial enzyme MCR, where time-dependent inactivation was observed [35]. The reasons for this difference in be-haviour are not entirely obvious.

2.3. Influence of the side-chain lipophilicity on inhibitor potency

AMACR is able to catalyse the 'racemisation' of substrates with structurally diverse side-chains [1,2]. The accommodation of these di-verse structures is thought to be a result of non-specific binding of the side-chain by hydrophobic interactions to a methionine-rich surface [8]. Consistent with this, the MCR gem- [31] and gem- carbamate [35] inhibitors show increased potency for compounds with more hydro-phobic alkyl side-chains. Consequently, we were interested to in-vestigate whether inhibitor potency was related to the lipophilicity of the inhibitor side-chain. A plot of determined IC_{50} values vs. calculated LogP values for the acyl-CoA inhibitor (Fig. 3) showed that high LogP values tend to produce low IC_{50} values. Systematic trends were ob-served within those compounds containing aromatic side-chains (5–11), with potency positively correlating with lipophilicity. It is also notable that 10 and 11, possessing a single phenyl group side-chain are significantly less potent than compounds containing more lipophilic side-chains. This consistent behaviour tends to suggest that side-chain lipophilicity is driving potency, with the 2-hydroxy group of 10 and 11 making a smaller contribution. Systematic trends were also observed for inhibitors possessing alkyl side-chains (12–17), showing that lipo-philicity is also an important determinant of potency for this series.

The enolate analogue 4 has a potency increased by ~875-fold compared to that predicted based on LogP values (measured IC_{50} = ~0.4 nM vs. ~350 nM predicted for miLogP = 2.61) (Supplementary Information, Fig. S1), showing the effectiveness of the
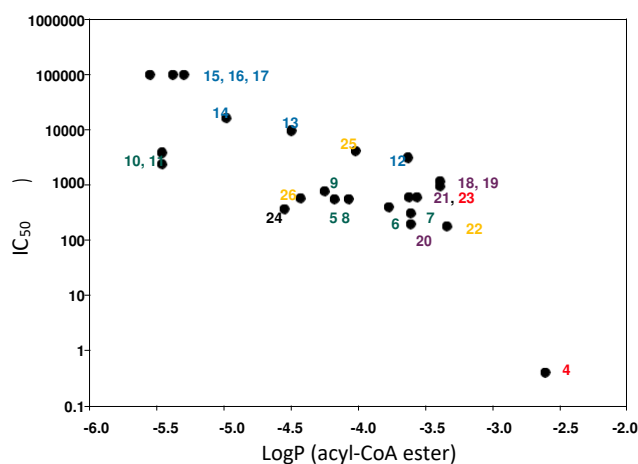
Fig. 3. Correlation of inhibitor potency with lipophilicity (miLogP value; http://www.molinspiration.com/cgi-bin/properties). Compound numbers refer to structures shown in Fig. 1. Compounds with green numbers are those with aromatic side-chains (5–11); Compounds with blue numbers are straight-chain acyl-CoA esters and isobutanoyl-CoA (12–17); Compounds with purple num-bers are 2-methyldecanoyl-CoA and 3-fluoro-2-methyldecanoyl-CoAs (18–21); Compounds with orange numbers are intermediates in the β-oxidation pathway which occurs subsequent to AMACR activity (22, 25 and 26); Compounds with red numbers are inhibitors or analogues of known inhibitors (4 and 23); Compound 24 is a synthetic intermediate to 25.

carbamate moiety in promoting inhibition. Acyl-CoA esters do not comply with Lipinski guidelines and hence AMACR inhibitors are de-livered as their acid pro-drugs [28,30,32,34] which are converted to the acyl-CoA in vivo. Although 4 has very high potency, delivery as the pro-drug will be challenging because carbamates readily decarboxylate to the corresponding amine.

3. Conclusions

This is the first systematic SAR study of rationally designed AMACR inhibitors. The study illustrates that extremely diverse side-chain structures which can be accommodated. A minimal level of side-chain lipophilicity is required for efficient binding. For compounds with aromatic side-chains, a single aromatic ring results in modest inhibition whilst more than one aromatic ring or an aromatic ring with alkyl substituents results in much more potent inhibition. Similarly, a minimum of a 6-carbon alkyl chain appears to be required for reason-ably efficient inhibitor binding, with increased potency resulting from addition of further −CH2− groups. Our results allow investigation of the contribution to potency made by the individual structural elements of these inhibitors.

AMACR has attracted much attention as both a novel drug target and cancer marker since its involvement in prostate cancer was re-ported [14,19]. However, exploitation of this discovery has been ex-tremely limited, largely due to the absence of a suitable assay with which to test inhibitor potency [33]. This study shows that our novel colorimetric assay [32] allows quick and accurate measurement of drug potency and detailed kinetic characterisation of inhibitors. The sys-tematic investigation of novel inhibitor SAR and therapeutic develop-ment is now possible.

4. Materials and methods

4.1. Sources of materials

Chemicals were purchased from the Sigma-Aldrich Chemical Co. or Fisher Scientific Ltd., unless otherwise stated, and were used without further purification. Reduced coenzyme A, tri-lithium salt, was

purchased from Calbiochem. Acyl-CoA esters 4 [33], 5–9 [6], 10 and 11 [11] were synthesised as previously described. Acyl-CoA esters 12–17 were purchased from Larodan Lipids. Substrates 18–20 and product 22 were synthesised as described [7,10]. Human recombinant AMACR 1A was expressed and purified and substrate 1 synthesised as previously described [32].

4.2. General experimental procedures

Thin layer chromatography was performed on Merck silica alumi-nium plates 60 (F254) and UV light, potassium permanganate or phosphomolybdic acid were used for visualisation. Column chromato-graphy was performed using Fisher silica gel (particle size 35–70 µm). Purifications of acyl-CoA esters were performed by solid phase extrac-tion using Oasis HLB 6cc (200 mg) extraction cartridges. Phosphate buffer was prepared from monobasic sodium phosphate and NaOH at the required proportions. Citric acid buffer was prepared from citric acid and NaOH at the required proportion for 0.8 M pH 4.0 buffer. The pH of aqueous solutions was measured using a Corning 240 pH meter and Corning general purpose combination electrode. The pH meter was calibrated using Fisher Chemicals standard buffer solutions (pH 4.0 − phthalate, 7.0 − phosphate, and 10.0 − borate) at either pH 7.0 and 10.0 or 7.0 and 4.0. Calibration and measurements were carried out at ambient room temperature. IR spectra were recorded on Perkin-Elmer RXI FTIR spectrometer instrument. NMR spectra were recorded on Bruker Avance III 400.04 MHz or 500.13 MHz spectrometers in D_2O, $(CD_3)_2SO$ or $CDCl_3$ and the solvent was used as an internal standard. Shifts are given in ppm and J values reported to ± 0.1 Hz. Multiplicities of NMR signals are described as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Stock concentrations of acyl-CoA esters for assays were determined using ^{1}H NMR. Mass spectra were recorded by ESI TOF. High resolution mass spectra were recorded in ES mode. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. Syntheses were carried out at ambient temperature, unless otherwise specified. Solutions in organic solvents were dried over anhydrous $MgSO_4$ and the solvents were evaporated under reduced pressure. Aqueous solutions for biological experiments were prepared in Nanopure water of 18.2 MΩ.cm^{-1} quality and were pH-adjusted with aq. HCl or NaOH.

4.3. (4S)-4-Benzyl-3-[(2S,3R)-3-hydroxy-2-methyldecanoyl]-1,3-oxazolidin-2-one (28)

(S)-(+)-4-Benzyl-3-propanoyl-2-oxazolidinone 27 (3.0 g, 12.9 mmol) in CH_2Cl_2 (30.0 mL) was cooled to -78 °C. Dibutylboron triflate (1.0 M in CH_2Cl_2, 13.0 mL, 12.9 mmol) and Pr^i_2NEt (2.3 mL, 12.9 mmol) were added and the mixture was stirred for 30 min before octanal (1.4 mL, 9.2 mmol) in CH_2Cl_2 (9.0 mL) was added dropwise. The mixture was stirred at -78 °C for further 30 min and then at room temperature for 2 h. aq. Sodium phosphate buffer at pH 7.0 (100 mM, 100 mL) was added slowly to the reaction mixture. The organic layer was washed [aq. HCl (1.0 M), aq. NaHCO3 (saturated), brine] and dried. Column chromatography (Petroleum ether/EtOAc 10:1 → 6:1) gave 28 (2.53 g, 76%) as a colourless oil. [α]$_D^{21}$ +51.4 (c 0.74 in CHCl3); IR ν$_{max}$ 3517 (OH), 1780 (C]O), 1692 (C]O) cm^{-1}; ^{1}H NMR (500.13 MHz; CDCl3) δ$_H$ 7.26−7.07 (5H, m, Ar-H), 4.65−4.55 (1 H, m, 4-H), 4.16−4.05 (2 H, m, 5-H), 3.89−3.80 (1 H, m, 3′-H), 3.68 (1 H, qd J = 7.0, 3.0 Hz, 2′-H), 3.14 (1 H, dd, J = 13.0, 3.0 Hz, CHHAr), 2.92 (1 H, s, OH), 2.70 (1 H, dd, J = 13.0, 9.0 Hz, CHHAr), 1.50−1.10 (15H, m, 6 × CH_2 and CH_3CH), 0.79 (3 H, t, J = 6.5 Hz, 10′-H3); ^{13}C NMR (125.77 MHz, CDCl3) δ$_C$177.23 (1′-C), 152.87 (2-C), 134.92 (Ar-C), 129.24 (Ar-C), 128.73 (Ar-C), 127.18 (Ar-C), 71.35 (3′-C), 65.96 (5-C), 54.91 (4-C), 42.02 (2′-C), 37.53 (CHHAr), 33.77 (CH_2), 31.62 (CH_2), 29.35 (CH_2), 29.05 (CH_2), 25.84 (CH_2), 22.45 (CH_2), 13.90 (CH_3CH), 10.31 (10′-C); ESI-MS m/z 384.2134 [M+Na]$^+$ ($C_{21}H_{31}NNaO_4$ requires 384.2151).

4.4. (4S)-4-Benzyl-3-[(2R,3S)-3-fluoro-2-methyldecanoyl]-1,3-oxazolidin-2-one (29)

(Diethylamino)sulfur trifluoride (0.5 mL, 3.9 mmol) in CH_2Cl_2 (10.0 mL) was added dropwise to **28** (1.4 g, 3.9 mmol) in CH_2Cl_2 (20.0 mL) at −78 °C and the mixture was stirred for 2 h at this tem-perature. It was stirred for a further 2 h at room temperature, before being quenched with water (50 mL). The organic layer was washed (saturated aq. $NaHCO_3$, brine). Column chromatography (petroleum ether/EtOAc 30:1 gave **29** (490 mg, 35%) as a colourless oil. $[\alpha]_D^{21}$ +49.2 (c 0.63 in $CHCl_3$); IR v_{max} 1782 (C]O), 1700 (C]O) cm^{-1}; ^1H NMR (500.13 MHz; $CDCl_3$) δ_H 7.27−7.06 (5H, m, Ar-H), 4.76−4.57 (2H, m, 4-H and 3′-H), 4.15−3.96 (3 H, m, 5-H and 2′-H), 3.17 (1 H, dd, J = 13.5, 3.5 Hz, CHHAr), 2.72 (1 H, dd, J = 13.5, 9.5 Hz, CHHAr), 1.70−1.14 (12H, m, 6 × CH_2), 1.10 (3 H, d, J = 7.0 Hz, CH_3CH), 0.80 (3 H, t, J = 6.5 Hz, 10′-H_3); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C 174.37 (d, J = 2.8 Hz, 1′-C), 153.12 (2-C), 135.25 (Ar-C), 129.47 (Ar-C), 128.93 (Ar-C), 127.37 (Ar-C), 94.89 (d, J = 169.8 Hz, 3′-C), 66.19 (5-C), 55.38 (4-C), 42.03 (d, J = 20.9 Hz, 2′-C), 37.85 (CHHAr), 32.04 (d,

J = 20.9 Hz, 4′-CH_2), 31.79 (CH_2), 29.37 (CH_2), 29.16 (CH_2), 24.57 (d, J = 2.8 Hz, 5′-CH_2), 22.64 (CH_2), 14.10 (10′-C) and 13.62 (d, J = 8.5 Hz, 2′-CH_3); δ_F (470 MHz, $CDCl_3$) −179.67; ESI-MS m/z 386.2126 $[M+Na]^+$ ($C_{21}H_{30}FNNaO_3$ requires 386.2107).

4.5. (2R,3S)-3-Fluoro-2-methyldecanoic acid (30)

aq. H_2O_2 [30% (w/w), 0.7 mL] and LiOH (62 mg, 2.6 mmol) were added to **29** (472 mg, 1.3 mmol) in THF (14 mL) at 0 °C. The mixture was stirred at ambient temperature for 20 h before being quenched with sat. aq. sodium sulfite (14 mL). The THF was evaporated and the mix-ture was acidified with aq. HCl (1.0 M) to pH 1.0 and extracted with CH_2Cl_2 (100 mL). The organic phase was washed with water (75 mL) and brine (75 mL). The combined organic layers were dried and the solvent was evaporated. Column chromatography (petroleum ether/
EtOAc 5:1) gave **30** (245 mg, 92%) as a white solid. mp 64–65 °C; $[\alpha]_D^{21}$ −7.8 (c 0.51 in $CHCl_3$); IR v_{max} 2925 (OH), 1693 (C]O) cm^{-1}; ^1H NMR (400.04 MHz; $CDCl_3$) δ_H 10.89 (1H, br s, OH), 4.58−4.50 (1H, m, 3-H), 2.58−2.52 (1H, m, 2-H), 1.75−1.23 (12H, m, 6 × CH_2), 1.20 (3H, d, J = 7.2 Hz, CH_3CH), 0.88 (3H, t, J = 6.8 Hz, 10-H_3); ^{13}C NMR (100.60 MHz, $CDCl_3$) δ_C 180.02 (d, J = 5.6 Hz, C]O), 94.28 (d, J = 172.2 Hz, 3-C), 44.37 (d, J = 22.0 Hz, 2-C), 31.74 (d, J = 21.1 Hz, 4-CH_2), 31.74 (CH_2), 29.30 (CH_2), 29.12 (CH_2), 24.82 (d, J = 3.0 Hz, 5-CH_2), 22.61 (CH_2), 14.05 (10-C) and 12.56 (d, J = 6.6 Hz, 2-CH_3); ^{19}F NMR (470 MHz, $CDCl_3$) δ_F −181.94; ESI-MS m/z 203.1449 $[M−H]^-$ ($C_{11}H_{20}FO_2$ requires 203.1447).

4.6. (2S,3S)-3-Fluoro-2-methyldecanoyl-CoA (21)

N,N′-Carbonyldiimidazole (48 mg, 0.29 mmol) was added to (2R,3S)-3-fluoro-2-methyldecanoic acid **30** (30.0 mg, 0.15 mmol) in anhydrous CH_2Cl_2 (2 mL) and the mixture was stirred at ambient temperature for 1 h. CH_2Cl_2 (3 mL) was added to the mixture, which was washed with water (5 × 2 mL) and brine (2 mL) and dried. The solvent was evaporated to obtain the crude acyl-imidazole inter-mediate. Aq. $NaHCO_3$ (1.0 mL, 0.10 M) and tri-lithium CoA-SH (17.0 mg, 0.02 mmol) was added to the crude intermediate in THF (1.0 mL) and the mixture was stirred at ambient temperature for 18 h. The THF was evaporated and the residue was acidified to ca. pH 3 by addition of aq. HCl (1.0 M). The mixture was diluted with water (2.0 mL) and washed with EtOAc (3 × 3 mL). The crude aqueous so-lution was freeze-dried and purified with solid-phase extraction to give

21 (13.6 mg) as a white solid. ^1H NMR (500 MHz, D_2O) δ_H 8.63 (1H, s, adenosine CH), 8.38 (1H, s, adenosine CH), 6.16 (1H, d, J = 6.0 Hz, adenosine CH), 4.30−4.10 (2H, m, adenosine CH_2), 3.97 (1H, s, ade-nosine CH), 3.85−3.72 (1H, m, CoA(OCHH)), 3.58−3.47 (1H, m, CoA(OCHH)), 3.39 (2H, t, J = 6.5 Hz, CoA(CH_2)), 3.29 (2H, t,

J = 6.0 Hz, CoA(CH_2)), 3.08−2.90 (3H, m, CoA(SCH_2) and $CHCH_3$), 2.36 (2H, t, J = 6.5 Hz, CoA(CH_2)), 1.68−1.44 (2H, m, CHH and CHH), 1.39−1.12 (10H, m, 5 × CH_2), 1.07 (3H, d, J = 7.0 Hz, CHCH_3), 0.87 (3H, s, CoA(CH_3)), 0.81−0.70 (6H, m, CH_2CH_3 and CoA(CH_3)); ^{19}F NMR (470 MHz) δ_F −181.11; ESI-MS m/z 475.6220 $[M−2H]^{2-}$ ($C_{32}H_{53}FN_7O_{17}P_3S$ requires 475.6208).

4.7. 2,2-Dimethyl-5-octyl-1,3-dioxane-4,6-dione (31)

4-Dimethylaminopyridine (1.296 g, 10.6 mmol), N,N′-dicyclohex-ylcarbodiimide (1.0 M in CH_2Cl_2, 11.1 mL, 11.1 mmol) and octanoic acid **32** (1.6 mL, 10 mmol, 1.0 eq.) were added to Meldrum's acid **33** (1.455 g, 10.1 mmol) in dry CH_2Cl_2 (100 mL). The mixture was stirred at ambient temperature for 40 h. The precipitate was removed by fil-tration and the filtrate was washed with aq. $KHSO_4$ (1.0 M, twice), water and brine, then dried. AcOH (6.0 mL) was added to the filtrate. To this solution, $NaBH_4$ (802 mg, 21.2 mmol) was added in portions during 1 h and the mixture was stirred for an additional 20 h. The evaporation residue was dissolved in Et_2O (100 mL) and washed with water (twice) and brine (100 mL). Drying and evaporation gave **31** (2.30 g, 89%) as a white solid: mp. 64–65 °C (lit. [45] mp. 65–67 °C); ^1H NMR (400.04 MHz, $CDCl_3$): δ_H 3.49 (1H, t, J = 4.9 Hz, dioxane 5-H), 2.14−2.04 (m, 2H, octyl 1-H_2), 1.77 (3H, s, 2-CH_3), 1.75 (3H, s, 2-CH_3), 1.48−1.38 (2H, m, octyl 3-H_2), 1.37−1.19 (10H, m, octyl 4,5,6,7-H_8), 0.86 (3H, t, J = 7.0 Hz, octyl 8-H_3); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ_C 165.6, 104.7, 46.1, 31.8, 29.5, 29.2, 29.1, 28.4, 26.9, 26.6, 26.5, 22.6, 14.1; IR (KBr disc) n_{max} 1752 (C]O) cm^{-1}; ESI-MS m/z 279.1577 $[M +Na]^+$ $C_{14}H_{24}NaO_4$ requires 279.1572; 257.1732 $[M+H]^+$ ($C_{14}H_{25}O_4$ requires 257.1753).

4.8. Methyl 2-methylenedecanoate (34)

Compound **31** (2.10 g, 8.19 mmol) was dissolved in anhydrous MeOH (26 mL). Eschenmoser's salt (3.79 g, 20.5 mmol, 2.5 eq.) was added and the mixture was heated at reflux for 40 h. The solvent was evaporated. The residue, in Et_2O (100 mL), was washed with aq. $KHSO_4$ (1.0 M), water and brine and was dried. Evaporation and column chromatography (petroleum ether/EtOAc 10:1) gave **34** (1.10 g, 74%) as a colourless oil (lit.[46] oil): IR (neat) n_{max} 1725 (C]O) cm^{-1}; ^1H NMR (400.04 MHz, $CDCl_3$): δ_H 6.12−6.10 (1H, m,]CHH), 5.52−5.49 (1H, m,]CHH), 3.73 (3H, s, OCH_3), 2.31−2.23 (2H, m, 3-H_2), 1.49−1.37 (2H, m, 4-H_2), 1.34−1.19 (10H, m, 5,6,7,8,9-H_{10}), 0.86 (3H, t, J = 7.1 Hz, 10-H_3); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ_C 167.8, 140.8, 124.4, 51.7 (two carbons), 31.8, 29.3, 29.2, 29.2, 28.3, 22.6, 14.0.

4.9. 2-Methylenedecanoic acid (35)

Methyl ester **34** (870 mg, 4.39 mmol) was stirred at 50 °C for 2 h with aq. NaOH (1.0 M, 15 mL, 15 mmol) in EtOH (57 mL), then cooled to ambient temperature and acidified to pH ca. 3. The volatile solvents were evaporated. The residue, in Et_2O (50 mL), was washed with water (twice) and brine and was dried. Evaporation and column chromato-graphy (petroleum ether/EtOAc 3:1) gave **35** (650 mg, 80%) as a col-ourless oil (lit. [47] oil): IR (neat) n_{max} 1696 (C]O) cm^{-1}; ^1H NMR (400.04 MHz, $CDCl_3$): δ_H 12.05 (1H, br s), 6.34−6.23 (1H, m,]CHH), 5.70−5.59 (1H, m, =CHH), 2.32−2.25 (2H, m, 3-H_2), 1.54−1.40 (2H, m, 4-H_2), 1.36−1.19 (10H, m, 5,6,7,8,9-H_{10}), 0.87 (3H, t, J = 7.0 Hz, 10-H_3); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ_C 173.0, 140.2, 126.9, 31.8, 31.4, 29.3, 29.2, 29.2, 28.3, 22.6, 14.1; ESI-MS m/z 207.1349 $[M+Na]^+$ $C_{11}H_{20}NaO_2$ requires 207.1361; 183.1396 $[M−H]^-$ ($C_{11}H_{19}O_2$ re-quires 183.1385).

4.10. 2-Methylenedecanoyl-CoA (23)

Ethyl chloroformate (17 μL, 19 mg, 0.18 mmol) was added to **35** (33 mg, 0.18 mmol) and NEt_3 (25 μL, 18 mg, 0.18 mmol) in anhydrous

THF (2.0 mL) and the mixture was stirred at ambient temperature for 1 h. CoA-SH tri-lithium salt [(28 mg, 0.04 mmol) in aq. KHCO$_3$ (2.5%), 2.0 mL] was added and the mixture was stirred at ambient temperature for 16 h. The mixture was acidified to pH ca. 3 with aq. HCl (1.0 M) and the THF was evaporated. The solution was washed with EtOAc (5 × 3 mL) and the crude product was purified by SPE to give 23 (7.0 mg) as a colourless solid: ^{1}H NMR (500.13 MHz, D$_2$O): δ$_H$ 8.47 (1H, s), 8.16 (1H, s), 6.07 (1H, d, J = 7.0 Hz), 5.96 (1H, s), 5.55 (1H, s), 4.19−4.11 (1H, m), 3.75 (1H, dd, J = 9.8, 5.1 Hz), 3.46 (1H, dd, J = 9.8, 4.8 Hz), 3.36 (2H, t, J = 6.5 Hz), 3.31−3.25 (2H, m), 2.95 (2H, t, J = 6.2 Hz), 2.33 (2H, t, J = 6.6 Hz), 2.16 (2H, t, J = 7.3 Hz), 1.30−1.22 (2H, m), 1.18−1.06 (10H, m), 0.79 (3H, s), 0.75 (3H, t, J = 6.8 Hz), 0.65 (3H, s); ESI-MS m/z 465.6152 [M−2H]$^{2−}$ (C$_{32}$H$_{52}$N$_7$O$_{17}$P$_3$S requires 465.6177).

4.11. Methyl 2-(2-heptyl-1,3-dioxolan-2-yl)propanoate (36)

Ethane-1,2-diol (5.27 g, 84.8 mmol) was added to 37 (606 mg, 2.83 mmol) in dry CH$_2$Cl$_2$ (26 mL). Me$_3$SiCl (1.84 g, 2.15 mL, 17.0 mmol) was added and the mixture was stirred at ambient tem-perature for 3 d. Further ethane-1,2-diol (5.27 g) and Me$_3$SiCl (1.84 g) were added and the mixture was stirred for a further 3 d. Water (25 mL) was added and the mixture was extracted with CH$_2$Cl$_2$ (3 × 20 mL). The combined organic layers were washed with water (5 × 70 mL) and brine (70 mL) and were dried. Evaporation and column chromato-graphy (petroleum ether/EtOAc 10:1) gave 36 (600 mg, 82%) as a colourless oil: IR (neat) n$_{max}$ 1740 (C]O) cm$^{−1}$; ^{1}H NMR (400.04 MHz, CDCl$_3$) δ$_H$ 4.04−3.90 (4H, m, OCH$_2$CH$_2$O), 3.67 (3H, s, OCH$_3$), 2.83 (1H, q, J = 7.2 Hz, CHCH$_3$), 1.81−1.62 (2H, m, heptyl 1-H$_2$), 1.42−1.20 (10H, m, heptyl 2,3,4,5,6-H$_{10}$), 1.18 (3H, d, J = 7.2 Hz, propanoate 3-H$_3$), 0.86 (3H, t, J = 7.1 Hz, heptyl 7-H$_3$); ^{13}C NMR (125.77 MHz, CDCl$_3$): δ$_C$ 173.9, 111.3, 65.5, 65.4, 51.7, 46.7, 35.0, 31.7, 29.7, 29.2, 22.8, 22.6, 14.0, 12.5; ESI-MS m/z 281.1761 [M+Na]$^+$ (C$_{14}$H$_{26}$NaO$_4$ requires 281.1729), 259.1883 [M+H]$^+$ (C$_{14}$H$_{27}$O$_4$ requires 259.1909).

4.12. 2-(2-Heptyl-1,3-dioxolan-2-yl)propanoic acid (38)

Aq. NaOH (1.0 M, 6.7 mL, 6.7 mmol) was stirred with 36 (345 mg, 1.34 mmol) in MeOH (30 mL) at ambient temperature for 2 h, then at 65 °C for 2 h. The mixture was cooled to ambient temperature and citric acid buffer (0.8 M, pH 4.0, 15 mL) was added. The mixture was ex-tracted with CH$_2$Cl$_2$ (3 × 30 mL). The combined organic layers were washed with water and brine and dried. Evaporation and column chromatography (petroleum ether/EtOAc 2:1) gave 38 (130 mg, 40%) as a colourless oil: IR (neat) n$_{max}$ 1709 (C]O) cm$^{−1}$; ^{1}H NMR (400.04 MHz, CDCl$_3$): δ$_H$ 10.78 (1H, br s, OH), 4.10−3.94 (4H, m, OCH$_2$CH$_2$O), 2.83 (1H, q, J = 7.2 Hz, CHCH$_3$), 1.80−1.67 (2H, m heptyl 1-H$_2$), 1.43−1.20 (10H, m, heptyl 2,3,4,5,6-H$_{10}$), 1.23 (3H, d, J = 7.2 Hz, CHCH$_3$), 0.87 (3H, t, J = 7.1 Hz, heptyl 7-H$_3$); ^{13}C NMR (125.77 MHz, CDCl$_3$): δ$_C$ 176.9, 111.4, 65.4, 65.4, 46.6, 34.7, 31.7, 29.6, 29.2, 22.8, 22.6, 14.0, 12.2; ESI-MS m/z 267.1549 [M+Na]$^+$ (C$_{13}$H$_{24}$NaO$_4$ requires 267.1572), 245.1729 [M+H]$^+$ (C$_{13}$H$_{25}$O$_4$ re-quires 245.1753).

4.13. 2-(2-Heptyl-1,3-dioxolan-2-yl)propanoyl-CoA (24)

Using the same method as for 21, 24 was prepared from 38 (31 mg, 0.13 mmol, 1.0 eq.) by sequential treatment with N,N'-carbonyldiimi-dazole (41 mg, 0.25 mmol, 2.0 eq.) and CoA-SH tri-lithium salt (29 mg, 0.04 mmol, 0.3 eq.) to give 24 (10 mg) as a colourless solid: ^{1}H NMR (500.13 MHz, D$_2$O) δ$_H$ 8.57 (1H, s), 8.30 (1H, s), 6.13 (1H, d, J = 6.1 Hz), 4.21−4.14 (1H, m), 3.99−3.88 (4H, m), 3.78 (1H, dd, J = 9.6, 4.5 Hz), 3.50 (1H, dd, J = 9.7, 4.5 Hz), 3.39 (2H, t, J = 6.6 Hz), 3.32−3.22 (2H, m), 3.12 (1H, 2 × q, J = 7.0 Hz; both epimers), 2.93 (2H, t, J = 6.2 Hz), 2.36 (2H, t, J = 6.6 Hz), 1.66−1.53 (2H, m), 1.26−1.11 (8H, m), 1.08 (3H, d, J = 7.0 Hz, CHCH$_3$), 0.85 (3H,

s), 0.76 (3H, t, J = 7.1 Hz, CH$_2$CH$_3$), 0.72 (3H, s); ESI-MS m/z 506.6219 [M+Na]$^{2+}$ (C$_{34}$H$_{55}$N$_7$NaO$_{19}$P$_3$S requires 506.6192), 495.6301 [M +H]$^{2+}$ (C$_{34}$H$_{56}$N$_7$O$_{19}$P$_3$S requires 495.6282).

4.14. 2R,S-2-Methyl-3-oxodecanoyl-CoA (25)

Compound 24 (7 mg, 7.1 µmol) was dissolved in water (0.8 mL) and acetone (1.0 mL). Aq. HCl (1.0 M, 0.2 mL) was added and the reaction mixture was stirred overnight. The acetone was evaporated and the residue freeze-dried to give 25 (6 mg) as a colourless solid: ^{1}H NMR (500.13 MHz, D$_2$O): δ$_H$ 8.49 (1H, s), 8.33 (1H, s), 6.10 (1H, d, J = 5.8 Hz), 4.20−4.10 (2H, m), 3.99 (1H, q, J = 7.0 Hz), 3.76 (1H, dd, J = 9.7, 5.0 Hz), 3.38−3.28 (2H, m), 3.22 (2H, t, J = 6.3 Hz), 2.97−2.88 (2H, m), 2.57−2.44 (2H, m), 2.30 (2H, t, J = 6.6 Hz), 1.41−1.32 (2H, m), 1.16 (3H, d, J = 7.0 Hz), 1.12−1.04 (8H, m), 0.80 (3H, s), 0.69 (3H, s), 0.68 (3H, t, J = 7.0 Hz); ESI-MS m/z 473.6174 [M−2H]$^{2−}$ (C$_{32}$H$_{52}$N$_7$O$_{18}$P$_3$S requires 473.6151).

4.15. 2S,3R-3-Hydroxy-2-methyldecanoyl-CoA (26)

2S,3R-3-Hydroxy-2-methyldecanoic acid 39 was synthesised [48] from the Evan's auxiliary-protected acid 28 by hydrolysis with LiOH and H$_2$O$_2$. Following the procedure used for 21, 2S,3R-3-hydroxy-2-methyldecanoyl-CoA 26 was prepared from 2S,3R-3-hydroxy-2-me-thyldecanoic acid 39 (40 mg, 0.20 mmol, 1.0 eq.), CDI (64 mg, 0.40 mmol, 2.0 eq.) and CoA-SH tri-lithium salt (78 mg, 0.10 mmol,
0.5 eq.) to give 26 (12 mg) as a colourless solid: ^{1}H NMR (500.13 MHz, D$_2$O) δ$_H$ 8.48 (1H, s), 8.20 (1H, s), 6.09 (1H, d, J = 6.5 Hz), 4.22−4.10 (1H, m), 3.81−3.70 (1H, m), 3.48 (1H, dd, J = 9.8, 4.8 Hz), 3.37 (2H, t, J = 6.6 Hz), 3.25 (2H, t, J = 6.3 Hz), 2.97−2.87 (2H, m), 2.74−2.68 (1H, m), 2.34 (2H, t, J = 6.5 Hz), 1.39−1.29 (2H, m), 1.21−1.09 (10H, m), 1.06 (3H, d, J = 6.9 Hz, CHCH$_3$), 0.81 (3H, s), 0.74 (3H, t, J = 7.1 Hz, CH$_2$CH$_3$), 0.68 (3H, s); ESI-MS m/z [M−2H]$^{2−}$ 474.6217 (C$_{32}$H$_{54}$N$_7$O$_{18}$P$_3$S requires 474.6229).

4.16. Evaluation of inhibition of AMACR by test compounds

Colorimetric assays were performed as previously described [32]. Dose response curves were used to determine IC$_{50}$ values for inhibitors. Enzyme (4 × stock, 150 µL) and inhibitor at the appropriate con-centration (4 × stock, 150 µL) were incubated together in 96 well plates at ambient room temperature for 10 min. The sample was divided into three repeats of 100 µL before addition of substrate (2 × stock, 3 × 100 µL; final concentration of 40 µM in the assay) and monitored at 354 nm. Each 200 µL assay contained ca. 8 µg of total AMACR protein (0.85 µM, assuming a molecular weight of 47,146.8 Da. with one active site per monomer [7]). Final concentrations of inhibitor in the assay were 100, 33.3, 11.1, 3.7, 1.23, 0.411, 0.137 and 0.045 µM unless otherwise stated. Positive controls contained enzyme and substrate 1 only and negative controls buffer and substrate. Rates in Absorban-ce.min$^{−1}$ were determined using Excel and converted to nmol.-min.$^{−1}$mg$^{−1}$ using the 2,4-dinitrophenoxide 2 extinction coefficient (15,300 M$^{−1}$ cm$^{−1}$) [32] with the path-length (0.588 cm) determined by the plate-reader. IC$_{50}$ values were determined using reaction rate, with the data fitted to a 4-parameter logistic using SigmaPlot 13 using Log$_{10}$ inhibitor concentration (in µM). In some cases 2−3% (v/v) DMSO was included in assays; no significant change in enzyme activity was observed with DMSO concentrations of up to 8% (v/v) [32]. Half-vo-lume 96 well plates were used for some inhibitors; identical IC$_{50}$ values were obtained for standard inhibitors using both types of microtitre plate. IC$_{50}$ values [32] for N-dodecyl-N-methylcarbamoyl-CoA 4 and ibuprofenoyl-CoA 5 were determined contemporaneously with the in-hibitors described in this study, with the same batch of enzyme.

4.17. Computational analysis of potency of inhibition

Lipophilicity of acyl-CoA esters was assessed by calculation of miLogP values using the molecular properties calculator (http://www.molinspiration.com/cgi-bin/properties). miLogP values were calculated using molecular smiles obtained from Chemdraw Professional 15. IC_{50} values (in nM) were plotted against the obtained LogP value using SigmaPlot 13.

Disclosure statement

MJ, GLL, MDT, TJW and MDL are named inventors on patent applications on the use and application of the colorimetric assay. The other authors report no conflicts of interest. The authors alone are re-sponsible for the content and writing of this article.

Appendix A. Supplementary material

Supplementary Information contains dose-response curves and other biological evaluation of inhibitors and spectroscopic character-isation data for compounds. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10. 1016/j.bioorg.2018.04.024.

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