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ARTICLE TYPE

A study on the chiral inversion of mandelic acid in humans

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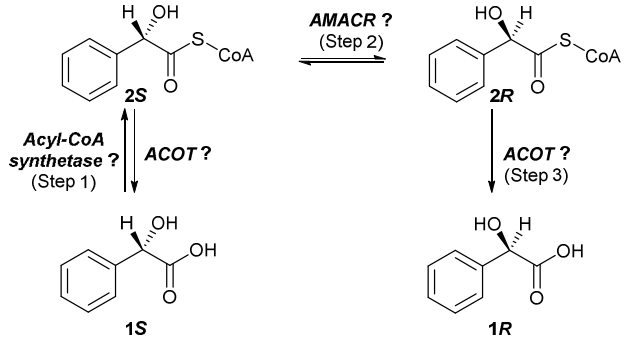
Mandelic acid is a chiral metabolite of the industrial pollutant styrene and is used in chemical skin peels, as a urinary antiseptic and as a component of other medicines. In humans, *S*-mandelic acid undergoes rapid chiral inversion to *R*-mandelic acid by an undefined pathway but it has been proposed to proceed *via* the acyl-CoA esters, *S*- and *R*-2-hydroxy-2-phenylacetyl-CoA, in an analogous pathway to that for Ibuprofen. This study investigates chiral inversion of mandelic acid using purified human recombinant enzymes known to be involved in the Ibuprofen chiral inversion pathway. Both *S*- and *R*-2-hydroxy-2-phenylacetyl-CoA were hydrolysed to mandelic acid by human acyl-CoA thioesterase-1 and -2 (ACOT1 and ACOT2), consistent with a possible role in the chiral inversion pathway. However, human α-methylacyl-CoA racemase (AMACR; P504S) was not able to catalyse exchange of the α-proton of *S*- and *R*-2-hydroxy-2-phenylacetyl-CoA, a requirement for chiral inversion. Both *S*- and *R*-2-phenylpropanoyl-CoA were epimerised by AMACR, showing that it is the presence of the hydroxy- group that prevents epimerisation of *R*- and *S*-2-hydroxy-2-phenylacetyl-CoAs. The results show that it is unlikely that 2-hydroxy-2-phenylacetyl-CoA is an intermediate in the chiral inversion of mandelic acid, and that the chiral inversion of mandelic acid is *via* a different pathway to that of Ibuprofen and related drugs.

Introduction

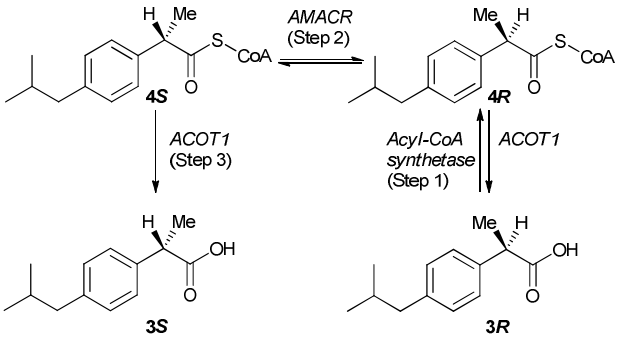
Mandelic acid (2-hydroxy-2-phenylacetic acid **1**) is a common chiral α-hydroxyacid produced from styrene, a hazardous monomer used in the plastics industry.[1, 2] During its metabolism, styrene is converted to phenyloxirane (styrene epoxide) by CYP2E1, the majority of which is ring opened to 1-phenylethane-1,2-diol (styrene glycol) by epoxide hydrolase. The latter is oxidised to mandelic acid and 2-oxo-2-phenylacetic acid (phenylglyoxylic acid).[1, 3-5] Both 1-phenylethane-1,2-diol and mandelic acid **1** are excreted in the urine and monitoring of their levels is used to assess environmental exposure to styrene,[6, 7] whilst 2-oxo-2-phenylacetic acid has been shown to induce striatal-motor toxicity in rats.[8] Mandelic acid **1** is also used pharmaceutically in α-hydroxyacid chemical skin peels for the treatment of acne, scarring and hyperpigmentation[9] and in urinary antiseptics and bladder irrigants.[10] It is also a component of or used in the manufacture of anti-viral agents,[10-12] contraceptives[13] and cyclic peptides.[14] Thioesters of mandelic acid have been investigated as inhibitors of glyoxylase 1 for the treatment of cancer[15] and glycosides containing mandelic acid have also been shown to possess anti-tumour activity.[16]

Mandelic acid **1** has long been known to undergo chiral inversion during its metabolism. In bacteria, bi-directional chiral inversion is catalysed by the Mg^{2+}-dependent mandelate racemase to produce a racemic mixture from either enantiomer.[17-19] In mammals, mandelic acid **1** undergoes uni-directional chiral inversion of the *S*-enantiomer to form the *R*-enantiomer.[3, 4, 6] Gao *et al.*[6] proposed that chiral inversion of *S*-mandelic acid **1*S*** occurred by a three step pathway (Scheme 1), analogous to that for Ibuprofen and other 2-arylpropanoic acid (2-APA) drugs,[20-22] consisting of: formation of *S*-2-hydroxy-2-phenylacetyl-CoA **2*S*** by an acyl-CoA synthetase (Step 1); epimerisation to *R*-2-hydroxy-2-phenylacetyl-CoA **2*R*** by α-methylacyl-CoA racemase (AMACR) *via* a deprotonated (enolate) intermediate (Step 2); and hydrolysis of *R*-2-hydroxy-2-phenylacetyl-CoA **2*R*** to *R*-mandelic acid **1*R*** by an acyl-CoA thioesterase (ACOT) (Step 3). This pathway[6] was proposed based on the structural similarities between mandelic acid **1** and Ibuprofen **3**, in that both compounds possess an aromatic side-chain and a chiral centre adjacent to the carboxylic acid. *S*-2-Hydroxy-2-phenylacetyl-CoA **2*S*** was rapidly hydrolysed and *R*-mandelic acid **1*R*** formed in rat liver cell extracts,[6] but conversion of **2*S*** to **2*R*** was not proven.



Scheme 1 The proposed chiral inversion pathway for mandelic acid **1**. ACOT, acyl-CoA thioesterase; AMACR, α-methylacyl-CoA racemase.

The chiral inversion pathway of Ibuprofen **3*R*** and other 2-APA drugs (Scheme 2) is well-established.[20-22] Only *R*-Ibuprofen **3*R*** is converted into *R*-Ibuprofenoyl-CoA **4*R***, by long-chain fatty acyl-CoA synthetase (Step 1),[23-25] which undergoes *R*- to *S*- chiral inversion.[20] Chiral inversion of *R*-Ibuprofenoyl-CoA **4*R*** is catalysed by α-methylacyl-CoA racemase (AMACR) (Step 2),[20, 21, 26, 27] whilst hydrolysis of the *S*-Ibuprofenoyl-CoA product **4*S*** is probably catalysed by acyl-CoA thioesterase-1 (ACOT1) (Step 3).[22] The pathway necessitates transport of **4*R*** from the cytosol into mitochondria or peroxisomes where AMACR is localised,[28-30] followed by export of **4*S*** to the cytosol for hydrolysis.[20, 22]



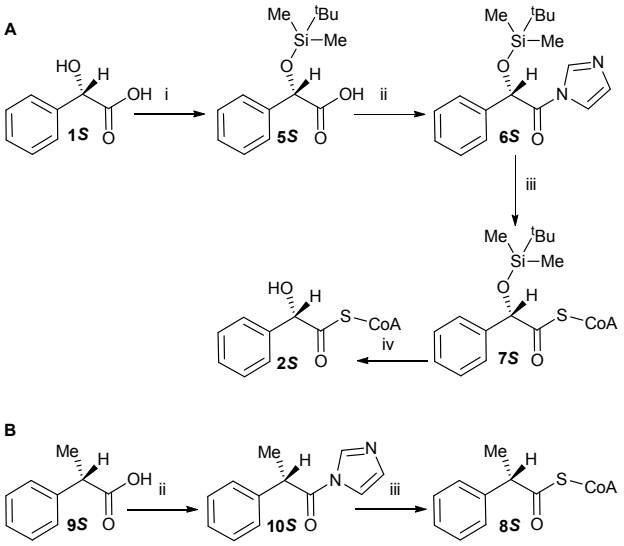
Scheme 2 The chiral inversion pathway of Ibuprofen **3** and other 2-APA drugs. ACOT, acyl-thioesterase; AMACR, α-methylacyl-CoA racemase.

This paper reports an *in vitro* study on the chiral inversion of mandelic acid **1** using recombinant human AMACR and ACOT enzymes to determine if chiral inversion can occur by the same pathway as for Ibuprofen **3**. The results show that 2-hydroxy-2-phenylacetyl-CoA **2** is not a substrate for AMACR, and hence chiral inversion of mandelic acid **1** occurs by a different pathway to Ibuprofen **3**.

Results and Discussion

Chiral inversion of mandelic acid **1** is from the *S*-enantiomer to the *R*-enantiomer (Scheme 1).[4, 6] This uni-directional chiral inversion of mandelic acid from **1*S*** to **1*R*** in mammalian cells implies a multi-step chiral inversion pathway in which either activation of *S*-mandelic acid **1*S*** or production of *R*-mandelic acid **1*R*** is stereospecific. That of Ibuprofen **3** and related drugs is from the *R*-enantiomer to the *S*-enantiomer (Scheme 2),[20] *i.e.* the stereochemical direction of chiral inversion is opposite for the two pathways. The direction of chiral inversion for Ibuprofen **3** is known to be determined by the requirement of long-chain fatty acyl-CoA synthetase for substrates with *R*- configuration.[23-25]

A number of potential acyl-CoA substrates were required in order to test whether mandelic acid **1** chiral inversion was mediated by the Ibuprofen **3** pathway. Gao *et al*.[6] synthesised racemic 2-hydroxy-2-phenylacetyl-CoA esters **2** by reaction of phenylglyoxal with CoA-SH followed by treatment with 2,6-dimethylpyridine.[15] In this study a stereoselective synthesis of **2** was developed (Scheme 3A) as the efficiency of conversion of ACOT substrates is known to be influenced by the epimeric configuration at the 2-position.[22] Direct reaction of mandelic acid **1*R*** or **1*S*** with carbonyldiimidazole did not yield the expected product, and therefore a direct synthesis of **2*R*** and **2*S*** was not possible. The hydroxy groups of mandelic acid **1*R*** or **1*S*** were therefore protected using TBDMS-Cl in a known procedure to give the chiral TBDMS ethers **5*R*** and **5*S***.[14] These were treated with carbonyldiimidazole to give the activated acids **6*R*** and **6*S***. Treatment of **6*R*** and **6*S*** with CoA-SH followed by deprotection with KF gave the desired epimeric acyl-CoA esters **2*R*** and **2*S***. Acidification of the TBDMS-protected acyl-CoA esters **7*R*** and **7*S*** was necessary to prevent base-catalysed hydrolysis of the acyl-CoA ester upon treatment with KF. *R*- and *S*-2-phenylpropanoyl-CoAs **8*R*** and **8*S*** were also synthesised (Scheme 3B) in order to compare the influence of 2-hydroxy *vs*. 2-methyl groups. Direct reaction of 2-phenylpropanoic acid **9*R*** and **9*S*** with carbonyldiimidazole[21] gave the activated acids **10*R*** and **10*S***, which were treated with CoA-SH to give **8*R*** and **8*S***. Myristoyl-CoA **11** was synthesised as a known substrate of ACOT1 and ACOT2,[22] and ±-Fenoprofenoyl-CoA as a known substrate of AMACR.[21]



Scheme 3 Synthesis of substrates. A. *S*-2-Hydroxy-2-phenylacetyl-CoA **2*S***; B. *S*-2-Phenylpropanoyl-CoA **8*S***. *Reagents and conditions:* i. TBDMS-Cl, imidazole, THF, 4°C, 1 hour, then 1.0 M NaOH $_{aq.}$, 105 mins; ii. Carbonyldiimidazole, dichloromethane, room temp., 45 mins; iii. CoA-SH, tri-lithium salt, THF/aq. 0.1 M $NaHCO_3$ (1:1), room temp, >16 h; iv. HCl to pH ~3.5, KF, 18 h. *R*-2-Hydroxy-2-phenylacetyl-CoA **2*R*** and *R*-2-phenylpropanoyl-CoA **8*R*** were synthesised by identical routes from *R*-mandelic acid **1*R*** and *R*-2-phenylpropanoic acid **9*R***, respectively.

Conversion of these acyl-CoA substrates by selected enzymes involved in the Ibuprofen chiral inversion pathway was then investigated. AMACR is known to catalyse epimerisation of 2-APA-CoA substrates, and is localised in mitochondria and peroxisomes[28-30] and was selected for study. ACOT1 is localised in the cytosol[31] and is thought to be the primary enzyme responsible for hydrolysis of 2-APA-CoA esters to their corresponding acids.[22] ACOT2 is located in mitochondria[31] and has been implicated in toxicities of 2-APA drugs, so was also selected for study. Long-chain fatty acyl-CoA synthetase (Ibuprofenoyl-CoA synthetase[24]) was not selected for study as its stereochemical requirements[23-25] are inconsistent with the direction of mandelic acid **1** chiral inversion.[3, 4, 6]

Selected acyl-CoA esters were tested as substrates with ACOT1 and ACOT2. Both enzymes were active with their known substrate, myristoyl-CoA **11**.[22, 32] The 2-hydroxy-2-phenylacetyl-CoA (**2*S*** and **2*R***) and 2-phenylpropanoyl-CoA (**8*S***

and **8*R***) esters were then tested as substrates. All of these acyl-CoA esters were hydrolysed to their corresponding acids and CoA-SH, as judged by the increasing absorbance at 412 nm due to reaction of CoA-SH with DTNB. No hydrolysis above background was observed in negative controls containing heat-inactivated enzyme, thus showing that the reaction was enzyme-catalysed. These results are consistent with the observation that both **2*S*** and **2*R*** were hydrolysed in rat liver cell extracts.[6]

Kinetic parameters for each of the substrates were then determined (Table 1 and 2). Substrates **11**, **2*S*** and **8*S*** showed substrate inhibition, whilst **2*R*** and **8*R*** showed standard Michaelis-Menten behaviour. The 2-hydroxy-2-phenylacetyl-CoA (**2*S*** and **2*R***) and *R*-2-phenylpropanoyl-CoA (**8*R***) esters were modest substrates for ACOT1 and ACOT2 compared to myristoyl-CoA **11**, as judged by k_{cat}/K_m values. 2-Hydroxy-2-phenylacetyl-CoA **2*S*** was converted somewhat more efficiently than **2*R*** by both ACOT1 and ACOT2, as judged by k_{cat}/K_m values. Kinetic plots show that data for **8*S*** fitted the substrate inhibition model (Supplementary Information) for both enzymes, but yielded unreasonable kinetic parameters.

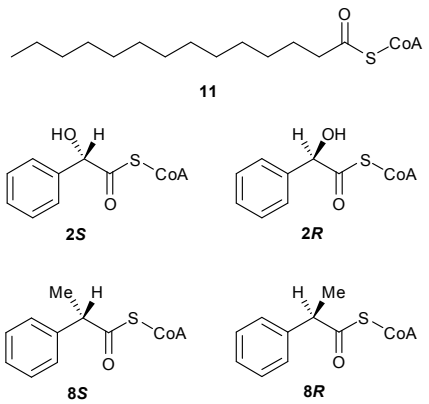


Table 1 Kinetic parameters ± SE for ACOT1. K_i values are reported for those substrates showing uncompetitive substrate inhibition.

Substrate	K_m (μM)	V_{max} (nmol.min^{-1}.mg^{-1})	K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
11	84 ± 170	68 ± 119	28 ± 69	0.054	646
2*S*	156 ± 138	75 ± 56	40 ± 38	0.060	386
2*R*	62 ± 18	12 ± 1.5	None	0.010	159
8*S*	>>10,000	>>10,000	0.11	-	-
8*R*	22 ± 5.7	5.0 ± 0.4	None	0.004	179

Table 2 Kinetic parameters ± SE for ACOT2. K_i values are reported for those substrates showing uncompetitive substrate inhibition.

Substrate	K_m (μM)	V_{max} (nmol.min^{-1}.mg^{-1})	K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
11	29 ± 11	15 ± 10	152 ± 205	0.027	1703
2*S*	172 ± 199	103 ± 104	27 ± 32	0.094	549
2*R*	131 ± 37	20 ± 3	None	0.018	142
8*S*	>>10,000	>>10,000	0.11	-	-
8*R*	5.3 ± 0.3	27 ± 5	None	0.005	178

2-Hydroxy-2-phenylacetyl-CoA (**2*S*** and **2*R***) and 2-phenylpropanoyl-CoA (**8*S*** and **8*R***) esters were then tested as substrates for purified human recombinant AMACR by assaying for exchange of the α-proton with deuterium.[21, 26] Under the reaction conditions the best known AMACR substrate, ±-Fenoprofenoyl-CoA,[21] showed >95% conversion (as measured by conversion of the 2-methyl group signal from a doublet into a single peak, a 1:1:1 triplet J = ~1 Hz, by ^{1}H NMR spectroscopy). Assays for **8*S*** and **8*R***, conducted in parallel under identical conditions, resulted in ~60% conversion of *S*-2-phenylpropanoyl-CoA **8*S***, and ~40% conversion of *R*-2-phenylpropanoyl-CoA **8*R***. Exchange was not observed in negative controls containing heat-inactivated enzyme. Exchange of the α-proton is an obligatory step in the epimerisation reaction catalysed by AMACR,[20, 21, 26] and hence it is highly likely that chiral inversion of **8*S*** and **8*R*** also occurred.

Assays of **2*S*** and **2*R*** were also carried out with AMACR under identical conditions. As expected, control experiments showed that exchange of the α-proton of ±-Fenoprofenoyl-CoA occurred with live enzyme but not heat-inactivated enzyme. The α-proton singlets of **2*S*** and **2*R*** at *ca.* 5.3 p.p.m. in the ^{1}H NMR spectra were unchanged when assays containing active enzyme and heat-inactivated controls were compared (Figure 1). Integration of the adenosine CH proton at 6.0 p.p.m. and the mandelic acid α-proton at *ca.* 5.3 p.p.m. showed they had a constant near 1:1 ratio, with no reduction of signal upon incubation with active AMACR. A reduction in the intensity of the α-proton signal of **2*R*** and **2*S*** would be expected if significant exchange for deuterium had occurred. This result demonstrates that *R*- and *S*- 2-hydroxy-2-phenylacetyl-CoA **2*R*** and **2*S*** do not undergo significant α-proton exchange. Since removal of the α-proton is an obligatory first step in the AMACR epimerisation reaction,[20, 21, 26] it can be concluded that **2*R*** and **2*S*** are not epimerised.

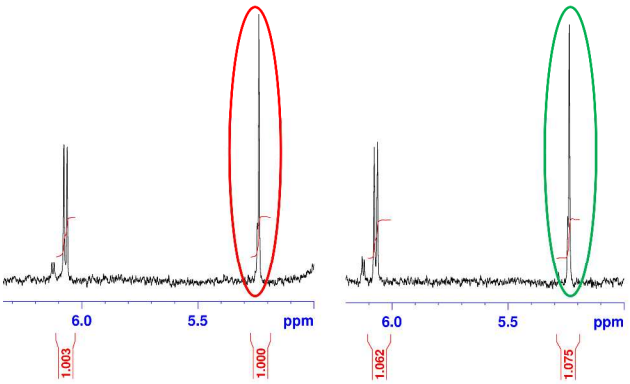


Figure 1 ^{1}H NMR spectra of **2*R*** incubated with AMACR, showing α-proton at *ca.* 5.3 p.p.m. Left panel, heat-inactivated enzyme; Right panel, live enzyme, showing no reduction in signal.

The failure of **2*R*** and **2*S*** to be epimerised by AMACR is not due to them having insufficiently hydrophobic phenyl side-chains, as evidenced by the significant levels of α-proton exchange with the 2-phenylpropanoyl-CoA substrates, **8*S*** and **8*R***. The substrates **2*R*** and **2*S*** differ from **8*R*** and **8*S*** only in having 2-hydroxy or 2-methyl substituents, respectively. The

hydroxyl and methyl substituents are of similar size but are hydrophilic (hydroxy group) and hydrophobic (methyl group), respectively. No X-ray crystal structure for human AMACR has been reported, but structures for the highly similar *M. tuberculosis* homologue have been reported.[33-35] In this enzyme the substrate methyl side-chain is accommodated in a hydrophobic pocket, comprising of methylene groups from the side-chains of His-126, Asp-127, Asn-152, Asp-156, and Leu-217, Tyr-224 and Ile-240 of the second subunit[35] (residue numbers refer to MCR). These residues are identical in human AMACR 1A, corresponding to His-122, Asp-123, Asn-148, Asp-152, Leu-213, Tyr-220, Ile-236 (Supplementary Figure S1). Thus, it appears that **2*R*** and **2*S*** will be substantially excluded from the enzyme as a result of the hydrophilic 2-hydroxy group been unable to occupy the hydrophobic methyl group binding pocket.

An alternative possibility is that the presence of the 2-hydroxy group inhibits formation of the enolate intermediate[20, 21, 26] in the reaction catalysed by AMACR. The *p*Ka of mandelic acid **1** has been measured as 22 (for formation of the enol),[36] compared to a *p*Ka value of ~21 for the α-proton of acyl-CoA esters.[37] The α-proton of 2-hydroxy-2-phenylacetyl-CoAs **2*R*** and **2*S*** are likely to be more acidic than in mandelic acid **1**, suggesting that an enolate could be formed upon binding. This again supports the idea that lack of metabolism of **2*R*** and **2*S*** is due to lack of binding within the AMACR active site.

There are a number of alternative metabolic pathways where **2*S*** and **2*R*** could undergo chiral inversion. One possibility is that chiral inversion of 2-hydroxy-2-phenylacetyl-CoA substrates **2*R*** and **2*S*** is mediated by an alternative 2-methylacyl-CoA racemase/epimerase. The only other enzyme of this type in humans is 2-methylmalonyl-CoA epimerase, which catalyses a chiral inversion step during the metabolism of propionyl-CoA to succinyl-CoA. 2-Methylmalonyl-CoA epimerase utilises a catalytic metal ion[17, 38, 39] in a similar fashion to bacterial mandelate racemase.[17-19] However, the relative positions of the carboxylate ligand in methylmalonyl-CoA and the hydroxyl group in 2-hydroxy-2-phenylacetyl-CoA **2** are different. Citrate, an analogue of the 2-methylmalonyl-CoA substrate, ligates to the active site metal ion of methylmalonyl-CoA epimerase using two carboxylate ligands and not its carboxylate and hydroxy ligands,[38] showing the importance of the relative positions of these ligating groups. Thus, it is unlikely that 2-hydroxy-2-phenylacetyl-CoA **2** will be able to bind to 2-methylmalonyl-CoA epimerase in a manner which is competent for catalysis.

A second possibility is that **2*S*** is metabolised by derivatisation of the hydroxy group. These derivatives are unlikely to be accommodated within the active site of AMACR, based on structural models of the related MCR enzyme.[33-35] The 2-methyl binding pocket in MCR from *M. tuberculosis* can only accommodate small groups,[34] and it is likely that any modification of **2*S*** (*e.g.* by acetylation) would exclude substrates by steric hindrance. Similarly, these derivatives of **2*S*** are unlikely to be able to bind to 2-methylmalonyl-CoA epimerase in a way which is competent for catalysis since chelation to the active site metal will be further compromised.

A further aspect to be considered is the requirement for acyl-CoA formation in order for the proposed pathway (Scheme 1) to occur. Long-chain fatty acyl-CoA synthetase is known to be specific for *R*-APAs,[23-25] whilst activation of *S*-mandelic acid is required. Furthermore, there is no evidence that long-chain fatty acyl-CoA synthetase is able to activate 2-hydroxy-fatty acids. Thus, the involvement of this specific enzyme in the mandelic acid **1** chiral inversion pathway can be excluded based on these stereochemical and other considerations. However, at least 26 acyl-CoA synthetase enzymes are present in the human genome,[40] so potentially an alternative enzyme may be able to catalyse the required conversion of *S*-mandelic acid **1*S*** to *S*-2-hydroxy-2-phenylacetyl-CoA **2*S***. Conversion of racemic long-chain 2-hydroxyfatty acids to their corresponding acyl-CoA esters by an undefined acyl-CoA synthetase has been demonstrated in rat liver and mouse brain extracts.[41, 42] Activation of mandelic acid **1** has not been specifically studied, but naturally occurring long-chain 2-hydroxyfatty acids substrates are the D-isomer, possessing *R*-stereochemistry. Activation of racemic 2-hydroxyfatty acids has also been observed, suggesting that the enzyme may be non-stereoselective.[41] These observations are inconsistent with the known direction of mandelic acid chiral inversion (*S* to *R*). [3, 4, 6]

Conclusions

Mandelic acid **1** is component of a number of drugs[9, 10] and drugs in development[10-14] and is a marker of environmental exposure to styrene.[1-5] Its chiral inversion pathway is of interest in order to understand the metabolism of these xenobiotics. Although mandelic acid **1** and Ibuprofen **3** have superficially similar structures, the results in this paper demonstrate that they are not metabolised by the same pathway. This conclusion is reached based on AMACR being unable to catalyse α-proton exchange of **2*S*** or **2*R*** and therefore it cannot catalyse the required epimerisation reaction. As a consequence, different enzymes must be involved in the chiral inversion step of the two pathways. It is also unlikely that chiral inversion of **2*S*** or **2*R*** can be performed by 2-methylmalonyl-CoA epimerase or that epimerisation of a derivative with a modified 2-hydroxy group could be performed by either enzyme, and this implies that chiral inversion of mandelic acid **1** probably does not proceed *via* an acyl-CoA intermediate at all (as shown in Scheme 1).

Moreover, this study demonstrates that ACOT1 and ACOT2 can hydrolyse both epimers of 2-hydroxy-2-phenylacetyl-CoA **2**, and hence cannot determine the direction of chiral inversion for the proposed pathway as a whole. Metabolism *via* an acyl-CoA intermediate[6] therefore requires stereospecific conjugation of *S*-mandelic acid with CoA-SH to form *S*-2-hydroxy-2-phenylacetyl-CoA **2*S***. The acyl-CoA synthetases which activate 2-hydroxyfatty acids appear to be either specific for substrates with *R*-configuration or are non-stereospecific.[41] These stereochemical observations are inconsistent with the observed direction of mandelic acid **1** chiral inversion for the pathway as a whole (**2*S*** to **2*R***),[3, 4, 6] and further argue for separate pathways.

Chiral inversion of mandelic acid **1** by stereoselective oxidation and reduction is an obvious alternative pathway which does not involve acyl-CoA intermediates. This possibility has been investigated, and stereoselective oxidation of *S*-mandelic acid **1*S*** by NAD^+-dependent dehydrogenases has been observed.[43] *S*-mandelic acid has also been shown to be stereoselectively oxidised by the kidney isoform, but not the liver

isoform, of rat L-2-hydroxyacid oxidase.[44] Chiral inversion of mandelic acid **1** has been observed in rat liver homogenates,[6] implying that L-2-hydroxyacid oxidase cannot be involved. The oxidation product in this alternative pathway is 2-oxo-2-phenylacetate (phenylglyoxylic acid). Previous studies[4] have shown that the majority of 2-oxo-2-phenylacetate is not reduced *in vivo* to mandelic acid **1** (in rats), with only around 1% of the total substrate been converted. Moreover, the reduction reaction appears not to be completely stereoselective, with a reported ratio of products of *ca.* 9:1 (**1*R***: **1*S***). Other studies have reported that metabolism of *S*-mandelic acid **1*S*** is stimulated by NADPH,[45] implying that reduction could take place under some circumstances. The exact pathway for mandelic acid chiral inversion therefore remains unclear.

Experimental

General Experimental

All chemicals were obtained from the Sigma-Aldrich Chemical Co. or Fisher Scientific Ltd and were used without further purification, unless otherwise noted. Reagents were of analytical grade or equivalent (synthesis) or biochemical grade. Oasis HLB cartridges were obtained from Waters Corporation. Myristoyl-CoA **11** and ±-Fenoprofenoyl-CoA were synthesised as previously described.[21, 22] Reactions were performed at ambient temperature, unless otherwise stated. Solvents were evaporated under reduced pressure. NMR spectra were recorded on Bruker Avance III 400.04 MHz or 500.13 MHz spectrometers. Chemical shifts are reported to the nearest 0.01 p.p.m. and coupling constants (*J* values) are reported to 0.1 Hz. Multiplicities of peaks are described as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra were obtained using VG7070E and Bruker microTOF spectrometers in the ES+ mode at the University of Bath Mass Spectrometry Service. Solutions in organic solvents were dried over anhydrous magnesium sulfate and evaporated under reduced pressure. Aqueous solutions were prepared in 18.2 Mega-Ω.cm^{-1} Nanopure water and pH adjusted with HCl or NaOH solutions as appropriate. Construction of expression plasmids for human AMACR[26] and ACOT1 and ACOT2[22] have been previously described. *E. coli* BL21 (DE3) pLysS and Rosetta2 (DE3) expression strains were obtained from Novagen.

Synthesis of *S*-mandelic acid O-TBDMS ether 5*S*[14]

TBDMS-Cl (331 mg, 2.2 mmol, 2.2 eq.) and imidazole (163 mg, 2.4 mmol, 2.4 eq.) were added to a solution of *S*-mandelic acid **1*S*** (152 mg, 1.0 mmol) in anhydrous THF (3 mL) cooled to 4℃ and the reaction mixture was stirred for 1 h. The reaction was allowed to warm to room temperature and stirred for a further 3 h before filtration and concentration *in vacuo*. NaOH (1.0 M, 3 mL) was added and the mixture stirred for 105 minutes before dilution with 3 mL water. The reaction was extracted with diethyl ether (2 x 5 mL), and the reaction mixture acidified to pH ~3.5 with 1.0 M citric acid buffer, pH 3.5 and extracted with diethyl ether (3 x 5 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated to give **5*S*** as a colourless solid (230 mg, 86 %): ^{1}H NMR (400.04 MHz, $CDCl_3$)[14] δ 9.30 (br s, 1H), 7.49-7.28 (m, 5H), 5.22 (s, 1H), 0.92 (s, 9H), 0.12 (s, 3H), 0.00 (s, 3H).

Synthesis of *R*-mandelic acid O-TBDMS ether 5*R*

The title compound was synthesised from **1*R*** (152 mg, 1.0 mmol) by the same method to give **5*R*** (232 mg, 87 %). NMR data was identical to **5*S***.

Synthesis of *S*-2-hydroxy-2-phenylacetyl-CoA 2*S*

5*S* (58 mg, 0.22 mmol) was dissolved in anhydrous dichloromethane (2 mL) and stirred with carbonyldiimidazole (71 mg, 0.44 mmol, 2.0 eq.) for 45 minutes. The reaction was extracted with water (5 x 5 mL), dried over $MgSO_4$, filtered and concentrated to dryness. The residue was dissolved in THF (2 mL) and aq. $NaHCO_3$ (0.1 M, 2 mL) and stirred with reduced coenzyme A, tri-lithium salt (20 mg, 0.025 mmol) for >16 h. The reaction mixture was diluted with water (1 mL), acidified to pH ~3.5 with 1 M HCl and stirred with anhydrous KF (27 mg, 0.46 mmol) for 18 h. The mixture was extracted with diethyl ether (5 x 5 mL) and freeze-dried. The product **2*S*** was purified by solid-phase extraction using an Oasis HLB cartridge. After loading the cartridge was washed with water (3 mL) and eluted with water/acetonitrile (4:1, 7 mL). The elution fraction was concentrated *in vacuo* and freeze-dried to give **2*S*** as a colourless solid (4.3 mg): ^{1}H NMR (500.13 MHz, D_2O) δ 8.45 (s, 1H), 8.15 (s, 1H), 7.41-7.24 (m, 5H), 6.07 (d, 1H, *J*= 7.0 Hz), 5.24 (s, 1H), 4.20-4.08 (m, 2H), 3.94-3.87 (m, 2H), 3.82 (dd, 1H, *J*= 9.5, 4.2 Hz), 3.44 (dd, 1H, *J*= 9.5, 4.3 Hz), 3.30-3.10 (m, 4H), 3.06-2.85 (m, 2H), 2.20-2.07 (m, 2H), 0.75 (s, 3H), 0.63 (s, 3H); HRMS (EI) Calcd. for $C_{29}H_{40}N_7O_{18}P_3SNa$: 922.1267, Found: 922.1240.

Synthesis of *R*-2-hydroxy-2-phenylacetyl-CoA 2*R*

The title compound was synthesised from **5*R*** (40 mg, 0.15 mmol) by the same method to give **2*R*** (3.0 mg). NMR and other data were identical to **2*S***.

Synthesis of *S*-2-phenylpropanoyl-CoA 8*S*

9*S* (30 mg, 0.20 mmol) was dissolved in dichloromethane (2 mL) and stirred with carbonyldiimidazole (65 mg, 0.40 mmol) for 45 minutes. The reaction was extracted with water (5 x 5 mL), dried over $MgSO_4$, filtered and concentrated to dryness. The residue was dissolved in THF (2 mL) and aq. $NaHCO_3$ (0.1 M, 2 mL) and stirred with coenzyme A, tri-lithium salt (16 mg, 0.02 mmol) for >16 h. The reaction mixture was diluted with water (1 mL) and acidified to pH ~3.5 with 1 M HCl. The mixture was extracted with diethyl ether (5 x 5 mL) and freeze-dried. The product was purified by solid-phase extraction using an Oasis HLB cartridge. After loading, the cartridge was washed with water (3 mL) and eluted with water/acetonitrile (1:1, 7 mL). The elution fraction was concentrated *in vacuo* and freeze-dried to give **8*S*** as a colourless solid (2.0 mg): ^{1}H NMR (500.13 MHz, D_2O) δ 8.58 (s, 1H), 8.31 (s, 1H), 7.30-7.16 (m, 5H), 6.11 (d, 1H, *J*= 5.5 Hz), 4.22-4.10 (m, 1H), 4.00-3.89 (m, 2H), 3.77 (dd, 1H, *J*= 9.5 4.5 Hz), 3.49 (dd, 1H, *J*= 9.5 3.9 Hz), 3.30-3.13 (m, 4H), 2.99-2.79 (m, 2H), 2.20-2.07 (m, 2H), 1.38 (d, 3H, *J*= 7.2 Hz), 0.84 (s, 3H), 0.69 (s, 3H); HRMS (EI) Calcd. for $C_{30}H_{43}N_7O_{17}P_3S$: 898.1654, Found: 898.1640.

Synthesis of *R*-2-phenylpropanoyl-CoA 8*R*

In similar fashion to the preparation of **8*S***, **8*R*** was obtained from

the reaction of **9*R*** (30 mg, 0.20 mmol) and coenzyme A, trilithium salt (16 mg, 0.02 mmol) in THF (2 mL) and aq. $NaHCO_3$ (2 mL, 0.1 M) to give **8*R*** as a colourless solid (2.7 mg). NMR and other data were identical to **8*S***.

ACOT assays

Human ACOT1 and ACOT2 were expressed as recombinant His-tag proteins in *E. coli* BL21 (DE3) pLysS.[22] Cells (~2 g) were lysed using Bugbuster (Novagen) in the presence of Benzonase and enzyme purified by metal-chelate chromatography. Purified enzyme was exchanged into 20 mM HEPES-NaOH, pH 7.27 and protein concentrations determined by UV-visible spectrometry. Protein purity was *ca.* 95 – 98 % by SDS-PAGE analyses.

Assays contained 0.109 mg (2.27 nmol) of ACOT1 or 0.099 mg (1.80 nmol) of ACOT2. Reactions were carried out at pH 7.27,[22] as this minimises spontaneous hydrolysis of DTNB.[46] The following substrates (Tables 1 and 2) were used in the assays: Myristoyl-CoA **11** (known substrate for ACOT enzymes[22, 32]); *S*-2-hydroxy-2-phenylacetyl-CoA **2*S***; *R*-2-hydroxy-2-phenylacetyl-CoA **2*R***; *S*-2-phenylpropanoyl-CoA **8*S***; and *R*-2-phenylpropanoyl-CoA **8*R***. Stock concentrations of acyl-CoA esters were determined using ^{1}H NMR.[21] Solutions of acyl-CoA esters were diluted in HEPES-NaOH, pH 7.27 except for myristoyl-CoA **11** where buffer was supplemented with BSA as previously described.[22] This was required to reduce substrate inhibition due to the formation of micelles. Kinetic analyses of **11** with ACOT1 and ACOT2 used substrate concentrations of 2 – 60 μM. All other kinetic analyses used concentrations of 5 – 200 μM. Assays were initiated by addition of enzyme (50 μL) to substrate: DTNB (2 x stock solution) and the reaction monitored for up to 15 minutes. Rates at each substrate concentration were measured using three dependent repeats. Reaction rates were obtained by plotting changes in absorbance for the linear progress curve with Excel. Activities in nmol.min.$^{-1}$mg^{-1} were calculated assuming ε_{412} = 14.15 mM^{-1} cm^{-1} at 25 ºC.[46, 47]

Data was analysed using SigmaPlot 11 and enzyme kinetics module 1.3, fitting to the Michaelis-Menten equation with and without uncompetitive substrate inhibition. The correct model was chosen based on convergence of fitting and visual inspection of plots. Kinetic plots for all substrates are available in Supplementary Information. K_m, K_i and V_{max} values are reported in Tables 1 and 2 as mean values ± SE. K_i values are reported for those substrates showing substrate inhibition.

AMACR assays

Human AMACR was expressed in *E. coli* Rosetta2 (DE3) grown at 37 °C and 220 r.p.m. until an O.D.$_{600}$ = ~1.5 was reached. Cultures were cooled to 22 °C, induced with 0.25 mM IPTG and incubated overnight under the same conditions.[21] Cells (~2 g) were lysed using the 'one shot' (Constant Systems) in ~30 mL NaH_2PO_4-NaOH, 300 mM NaCl and 10 mM imidazole pH 7.2, supplemented with 1 mM PMSF, 1 mM benzamidine-HCl, and 250 u Benzonase (Novagen) and stirred with N-lauroyl-sarcosine [1.5% (w/v)] at 4 °C for 1 hour. The sample was centrifuged (Beckmann JA-14 rotor, 10,000 r.p.m., 15,300 *g*, 10 minutes), filtered through a 0.45 μ filter, and purified by metal-chelate chromatography as previously described.[26] Purified enzyme was dialysed into 20 mM NaH_2PO_4-NaOH, pH 7.4 and stored at -80 °C. Protein concentration was determined by UV-visible spectroscopy.[21] Protein purity of pooled fractions was *ca.* 95 – 98 % by SDS-PAGE analyses.

Assays were conducted in 50 mM NaH_2PO_4-NaOH buffer, pH 7.4 containing *ca.* 85% (v/v) ^2H_2O, 100 μM acyl-CoA substrate and 3.5 μM enzyme as previously described,[21, 26, 27] with negative controls containing heat-inactivated enzyme. ±-Fenoprofenoyl-CoA, an Ibuprofenoyl-CoA analogue and the best AMACR substrate reported to date,[21] was used as a positive control in all assays. Stock concentrations of acyl-CoA esters were determined using ^{1}H NMR.[21] Assays were initiated by addition of enzyme to the substrate in buffer, which were incubated at 30 °C for 1 hour. After this time samples were heated at 50 °C for 10 minutes to inactivate the enzyme and analysed by ^{1}H NMR (500.13 MHz). Conversion of 2-phenylpropanoyl-CoA substrates **8*S*** and **8*R*** was quantified by conversion of the 2-Me doublet at *ca.* 1.0 p.p.m. into a single peak, a 1:1:1 triplet with J = ~1 Hz, as previously described for other substrates.[21, 26] Conversion of 2-hydroxy-2-phenylacetyl-CoA **2*S*** and **2*R*** was monitored by reduction of the α-proton singlet at *ca.* 5.3 p.p.m. due to deuterium incorporation.

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Notes and references

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†Electronic Supplementary Information (ESI) available: Substrate NMR and mass spectra data, NMR spectra of substrates incubated with AMACR and kinetic plots of substrates of ACOT enzymes. See DOI: 10.1039/b000000x/

‡Abbreviations used: ACOT, acyl-CoA thioesterase; AMACR, α-methylacyl-CoA racemase (a.k.a. P504S); 2-APAs, 2-arylpropanoic acids ('profens'); BSA, bovine serum albumin; ^{t}Bu, *tert*-butyl; CoA-SH, coenzyme A (reduced form); CYP2E1, cytochrome P450 2E1; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid); *E. coli*, *Escherichia* coli; ESI-TOF, electrospray ionisation-time-of-flight; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; k_{cat}, 1st order rate constant for conversion of substrate to product; k_{cat}/K_m, selectivity constant; K_i, inhibitor constant; K_m, Michaelis constant; Mandelic acid, 2-hydroxy-2-phenylacetic acid; O.D.$_{600}$, optical density at 600 nm; NAD$^+$, nicotinamide adenine dinucleotide (oxidised form) NMR, nuclear magnetic resonance; PMSF, phenylmethylsulfonyl fluoride; p.p.m., parts per million; r.p.m., revolutions per minute; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE, standard error; TBDMS-Cl, *tert*-Butyldimethylsilyl chloride; THF, tetrahydrofuran. V_{max}, maximum rate of enzyme reaction.

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