Acyl-CoA thioesterase-1 and -2 catalyse hydrolysis of 2-APA-CoA esters. The results suggest ACOT-1 plays a key role in the chiral inversion of ibuprofen and other 2-APA drugs.
Hydrolysis of ibuprofenoyl-CoA and other 2-APA-CoA esters by human acyl-CoA thioesterases-1 and -2 and their possible role in the chiral inversion of profens.

Xiao Qu, Amanda Allan, Grace Chui, Thomas J. Hutchings, Ping Jiao, Lawrence Johnson, Wai Y. Leung, Portia K. Li, Georgina R. Steel, Andrew S. Thompson, Michael D. Threadgill, Timothy J. Woodman, and Matthew D. Lloyd*

Medicinal Chemistry, Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U. K.

*Author for correspondence: M.D.Lloyd@bath.ac.uk

Keywords: Acyl-CoA thioesterase (ACOT); Ibuprofen; Branched-chain fatty acids; α-Methylacyl-CoA racemase (AMACR; P504S); NSAID.

Abbreviations used: ACOT, acyl-CoA thioesterase; BSA, bovine serum albumin; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid); HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, NSAIDs, non-steroidal anti-inflammatory drugs; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.
Abstract

Ibuprofen and related 2-arylpropanoic acid (2-APA) drugs are often given as a racemic mixture and the $R$-enantiomers undergo activation \textit{in vivo} by metabolic chiral inversion. The chiral inversion pathway consists of conversion of the drug to the coenzyme A ester (by an acyl-CoA synthetase) followed by chiral inversion by $\alpha$-methylacyl-CoA racemase (AMACR; P504S). The enzymes responsible for hydrolysis of the product $S$-2-APA-CoA ester to the active $S$-2-APA drug have not been identified. In this study, conversion of a variety of 2-APA-CoA esters by human acyl-CoA thioesterase-1 and -2 (ACOT-1 and -2) was investigated. Human recombinant ACOT-1 and -2 (ACOT-1 and -2) were both able to efficiently hydrolyse a variety of 2-APA-CoA substrates. Studies with the model substrates $R$- and $S$-2-methylmyristoyl-CoA showed that both enzymes were able to efficiently hydrolyse both of the epimeric substrates with $(2R)$- and $(2S)$- methyl groups. ACOT-1 is located in the cytosol and is able to hydrolyse 2-APA-CoA esters exported from the mitochondria and peroxisomes for inhibition of cyclo-oxygenase-1 and -2 in the endoplasmic reticulum. It is a prime candidate to be the enzyme responsible for the pharmacological action of chiral inverted drugs. ACOT-2 activity may be important in 2-APA toxicity effects and for the regulation of mitochondrial free coenzyme A levels. These results support the idea that 2-APA drugs undergo chiral inversion \textit{via} a common pathway.
1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in both human and veterinary medicine. Important examples of NSAIDs include aspirin, indomethacin, diclofenac and ibuprofen. Ibuprofen is a member of the 2-arylpropanoic acid (2-APA; a.k.a. profens) group of drugs, and it is probably the most widely used non-prescription drug. The accepted pharmacological targets of ibuprofen, cyclo-oxygenases-1 and -2 (COX-1 and -2), are potently inhibited by its S-enantiomer, whilst the R-enantiomer is much less effective [1]. In vivo the R-enantiomer of ibuprofen undergoes rapid uni-directional conversion into the S-enantiomer [2, 3], a metabolic process known as chiral inversion. Consequently, R-ibuprofen is activated as an inhibitor of COX-1 and -2.

The chiral inversion pathway (Figure 1) consists of three phases: 1) Stereoselective conversion of the R-ibuprofen 1R to R-ibuprofenoyl-CoA 2R by long-chain acyl-CoA synthetase [4-6] via an acyl-adenylate intermediate [7]; 2) Conversion of the R-ibuprofenoyl-CoA 2R into a ca. 1:1 mixture of R- and S-ibuprofenoyl-CoA 2R/2S by α-methylacyl-CoA racemase [8-10] (ibuprofenoyl-CoA epimerase [11, 12]); and 3) Hydrolysis of both ibuprofenoyl-CoA epimers 2 [13] to a racemic mixture of ibuprofen 1 by an undefined acyl-CoA thioesterase (ACOT). Product R-ibuprofen 1R is reconverted back to its acyl-CoA ester, whilst S-ibuprofen 1S is not recycled and this accounts for the overall uni-directional R → S chiral inversion of the pathway. Most other 2-APA drugs also undergo in vivo chiral inversion in humans (reviewed in [8]), and α-methylacyl-CoA racemase has been shown to accept several other 2-APA-CoA esters as substrates [9].

Microsomal [14] long-chain fatty acyl-CoA synthetase (ibuprofenoyl-CoA synthetase [4]) produces R-2-APA-CoA esters which are imported into mitochondria and peroxisomes (probably via the acyl-carnitine shuttle [15]), for chiral inversion by α-methylacyl-CoA racemase [16-18]. Cyclo-oxygenase-1 and -2 (a.k.a. prostaglandin E2 synthetase or endoperoxidase) [1, 19] are located in the endoplasmic reticulum [20, 21], implying that S-2-APA-CoA esters must be exported (via a reverse carnitine shuttle [22]) to interact with the target. Hydrolysis of 2-APA-CoA esters within
mitochondria has also been linked to 2-APA toxicity [23, 24]. Humans contain a large number of ACOT enzymes [25-27], with ACOT-1 localised in the cytosol and ACOT-2 localised in mitochondria [27]. This paper reports a study on the possible roles of human ACOT-1 and ACOT-2 in the 2-APA chiral inversion pathway.

2. Materials and Methods

2.1. Sources of materials:
All chemicals were obtained from the Sigma-Aldrich Chemical Co. or Fisher Scientific Ltd and were used without further purification, unless otherwise noted. Aqueous solutions were made in 18.2 Mega-Ω.cm Milli-Q water and pH adjusted with HCl or NaOH solutions as appropriate. KOD polymerase, the pET46 vector system, competent cells, Bugbuster, and Benzonase were obtained from Novagen. IPTG was from Calbiochem. Dpn1 was from New England Biolabs. Primers and protein molecular weight markers were from Invitrogen. Plasmids were obtained from imaGenes GmbH, Germany (http://www.lifesciences.sourcebioscience.com/; ACOT-1: IRCM0512A0824D; Hs.568046; ACOT-2: IRAUp969B0534D; Hs.649479). The syntheses of myristoyl-CoA 3 and R- and S-2-methylmyristoyl-CoA, 4R and 4S, are described in Supplementary Information available from the corresponding author. ±-Fenoprofenoyl-CoA 5, ±-flurbiprofenoyl-CoA 6, ±-ibuprofenoyl-CoA 2, S-ketoprofenoyl-CoA 11 and S-naproxenoyl-CoA 12 were synthesised as previously described [9].

2.3. Sub-cloning of human ACOT-1 and -2:
ACOT-1 was amplified using the following primers: forward, GACGACGACAAGATGGCGGCGACGCTGAT; reverse, GAGGAGAAGCCCGGTTAACACTCTTGTGAGGATTGTCCC. ACOT-2 was amplified using the following primers: forward, GACGACGACAAGATGTCTAACAAGCTTCTTTCTCCCC; reverse, GAGGAGAAGCCCGGTTAACACTCTTGTGAGGATTGTCCC. PCR reactions contained the
following: plasmid template (1 µL; 100 ng); primers (2 x 3 µL; 5 µM each); buffer (5 µL); dNTPs (5 µL); MgSO₄ (2 µL); KOD polymerase (1 µL, 2.5 activity units); and sterile water (30 µL). Reactions were amplified by the following procedure: 94 ºC, 2 min; 35 cycles of: 94 ºC, 15 s; 52 ºC, 30s; 68 ºC, 2 min; followed by 72 ºC, 2 min. The reaction products were treated with dpn1 (2 µL, 40 activity units) for 2 h at 37 ºC to remove template DNA, and the presence of the amplified DNA confirmed by 1% agarose gel electrophoresis. The PCR product was purified using a QIAquick PCR purification kit (Qiagen) and quantified (GeneQuant). The product was inserted into the pET46 vector according to the manufacturers’ instructions and transformed into Giga cells. Single colonies were selected, grown in 5 mL Lennox LB media supplemented with ampicillin (50 µg/mL) and plasmids prepared. The presence of the required insert in the plasmid was confirmed by PCR analysis using 1 µL template in a final volume of 20 µL followed by 1% (w/v) agarose gel electrophoresis. DNA sequencing confirmed the sequence of ACOT-1 was identical to A1L172 (http://www.uniprot.org). The sequence of ACOT-2 was identical to P49753 with the known A454V polymorphism.

2.4. Expression and purification of ACOT enzymes

Expression plasmids were transformed into E. coli BL21 (DE3) pLysS [28] and grown in Lennox LB media supplemented with ampicillin (50 µg/mL) and chloramphenicol (32 µg/mL) at 37 ºC and 190 r.p.m. Starter culture (30 mL) was used to inoculate 1L of the same media, grown under the same conditions until OD₆₀₀ = ~1.5 and induced with 1 mM IPTG. Cells were harvested after 3 hours by centrifugation (JA-10 rotor, 9,000 r.p.m., 14 300 g, 20 min., 4 ºC) and stored at -80 ºC.

Cells (ca. 5 g) were lysed using 30 mL Bugbuster supplemented with 250 u Benzonase at 4 ºC, followed by centrifugation [28]. The crude extract was filtered and loaded onto a 5 mL HisTrap FF Ni²⁺ column equilibrated in 20 mM NaH₂PO₄-NaOH, 300 mM NaCl, 10 mM imidazole, pH 7.2. The column was washed with 10 mL buffer and eluted with 300 mM imidazole-HCl, pH 7.2 in the
same buffer. Fractions (5 mL) were analysed by 10% SDS-PAGE, and those containing ACOTs were pooled and dialysed against 20 mM HEPES-NaOH, pH 7.27 (3 x 650 mL). Protein concentrations were determined using absorbance at 280 nm with parameters calculated with protparam (http://web.expasy.org/protparam/) for the His-tag enzyme: ACOT-1; M_w = 48 009.1 Da., \( \varepsilon_{280} = 48 360 \, \text{M}^{-1} \, \text{cm}^{-1} \); ACOT-2; M_w = 54 978.3 Da., \( \varepsilon_{280} = 55 350 \, \text{M}^{-1} \, \text{cm}^{-1} \).

2.5. ACOT assays

Assays were conducted in 50 mM HEPES-NaOH, pH 7.27 in a final volume of 100 \( \mu \)L at 25 ºC. Rates at each substrate concentration were measured using three dependent repeats. Reactions were carried out at pH 7.27, as this minimizes spontaneous hydrolysis of DTNB [29]. Assay mixtures contained substrate at the required concentration, DTNB (100 \( \mu \)M) and enzyme (50-75 \( \mu \)g). Myristoyl-CoA 3 and R- and S-2-methylmyristoyl-CoA (4R and 4S) were pre-incubated with BSA at a constant ratio [substrate: BSA (100: 2.88 \( \mu \)M)] for 10 minutes before assaying. Assays using 2-APA-CoA substrates did not contain BSA. Reaction rates were obtained by plotting changes in absorbance with Excel. Activities in nmol.min.\(^{-1}\)mg\(^{-1}\) were calculated assuming \( \varepsilon_{412} = 14.15 \, \text{mM}^{-1} \, \text{cm}^{-1} \) at 25 ºC [29, 30], and kinetic parameters obtained using the Direct Linear Plot [31, 32] in SigmaPlot 11 and enzyme kinetics module 1.3. Kinetic plots for all substrates are available in Supplementary Information, available from the corresponding author.

2.6. Structural models of ACOT-2

Models were produced based on the X-ray crystal structure of human ACOT-2 determined without any substrate [32]. The binding pocket was identified based on the proximity to the active site residues Ser-294, Asp-388 and His-422. Once docked, the 2-APA-CoAs were subjected to molecular mechanics and dynamics calculations to establish optimal docking conformations; during these calculations, the enzyme and coenzyme A moiety were restrained to original conformations. The 2-APA-CoA and binding pocket were subjected to molecular dynamics and finally molecular
mechanics calculations to give the final structures. Calculations were performed using the Tripos Associates force fields within the SYBYL-X 2.0 software suite on a dual Intel quad core workstation (Windows 7). Gasteiger-Hückel charges were calculated and used within the complexes.

3. Results

Recombinant human ACOT-1 and ACOT-2 were produced with an N-terminal His-tag sequence and purified by metal-chelate chromatography. The 2-APA-CoA esters which had previously been shown to be substrates for α-methylacyl-CoA racemase [9] were synthesised for testing as substrates (Figure 2). R- and S-2-methylmyristoyl-CoA (4R and 4S) were synthesised by modification of the route for R- and S-2-methyldecanoyl-CoA [33] to investigate the effect of the 2-methyl group epimeric configuration on substrate conversion. Myristoyl-CoA 3 was synthesised as a known good substrate of ACOT-1 and ACOT-2 [28].

The conversion of acyl-CoA esters to their corresponding acids and reduced coenzyme A by ACOT-1 and -2 was assessed using the reported assay with DTNB [28]. All of the tested acyl-CoA esters were efficiently converted to products (Tables 1 and 2). Myristoyl-CoA 3, R-2-methylmyristoyl-CoA 4R and S-2-methylmyristoyl-CoA 4S showed Michaelis-Menten behaviour as substrates for ACOT-1 and ACOT-2. In the case of ACOT-1 (Table 1) a $K_m$ value of 39 µM was determined for myristoyl-CoA 3, compared to 9.3 and 12 µM for 2-methylmyristoyl-CoA 4R and 4S, respectively. Catalytic efficiency (as measured by $k_{cat}/K_m$) was determined to be 151 M$^{-1}$ s$^{-1}$ for myristoyl-CoA 3, 128 M$^{-1}$ s$^{-1}$ for R-2-methylmyristoyl-CoA 4R and 230 M$^{-1}$ s$^{-1}$ for S-2-methylmyristoyl-CoA 4S, showing that the 2S-substrate was somewhat preferred. In the case of ACOT-2 (Table 2), R-2-methylmyristoyl-CoA 4R was converted most efficiently (as judged by $k_{cat}/K_m$), followed by myristoyl-CoA 3 and S-2-methylmyristoyl-CoA 4S. Thus, both ACOT
enzymes show some preference for particular epimeric configurations of the 2-methylmyristoyl-CoA substrate 4.

Hydrolysis of 2-APA-CoA esters to their corresponding acids by ACOT-1 and ACOT-2 was then investigated, and all of the tested 2-APA-CoAs were good substrates (Tables 1 & 2) and proper Michaelis-Menten kinetics were observed. In the case of ACOT-1 (Table 1) most substrates, including ibuprofenoyl-CoA (Figure 3), were converted with similar efficiencies ($k_{cat}/K_m = 140-220 \text{ M}^{-1} \text{s}^{-1}$), except for S-ketoprofenoyl-CoA 7S ($k_{cat}/K_m = 45 \text{ M}^{-1} \text{s}^{-1}$). This shows that ACOT-1 has a broad substrate selectivity. In the case of ACOT-2 (Table 2), the best 2-APA-CoA substrates were ±-fenoprofenoyl-CoA 5 and ±-flurbiprofenoyl-CoA 6 ($k_{cat}/K_m = \text{ca. } 320 \text{ M}^{-1} \text{s}^{-1}$), followed by S-naproxenoyl-CoA 8 ($k_{cat}/K_m = 249 \text{ M}^{-1} \text{s}^{-1}$), S-ketoprofenoyl-CoA 7 ($k_{cat}/K_m = 145 \text{ M}^{-1} \text{s}^{-1}$) and ±-ibuprofenoyl-CoA 2 ($k_{cat}/K_m = 70 \text{ M}^{-1} \text{s}^{-1}$). Thus, ACOT-2 showed a somewhat higher variability in substrate conversion efficiency, with the best substrates been converted somewhat more efficiently than by ACOT-1 ($k_{cat}/K_m = 346 \text{ cf } 230 \text{ s}^{-1} \text{ M}^{-1}$).

The substrate selectivity of ACOT-1 and -2 was further investigated by a substrate docking study (Figure 4) starting from the crystal structure of the ACOT-2 enzyme without any substrate bound [32]. ACOT-1 and ACOT-2 have almost identical core amino acid sequences, and hence are expected to possess similar substrate selectivities. The binding position of the acyl-CoA moiety was established by proximity to Ser-294, His-422 and Asp-388, the catalytic triad which performs the hydrolysis reaction. Two small pockets accommodate the methyl groups of $2R$- and $2S$- substrates, explaining how both epimers of 2-methyl substrates can be hydrolysed and the preference for branched-chain acyl-CoA esters over straight-chain acyl-CoA esters. The aromatic side-chain common to all 2-APA drugs projects into a deep lipophilic pocket, forming transient $\pi$ stacking interactions with the side-chain of Trp-391. This lipophilic pocket also accommodates the various substituents on the aromatic ring in the various 2-APA drugs. The phosphopantetheinyl- side-chain of the CoA moiety is enclosed within a narrow tunnel lined with acidic and basic residues. Several
possible sites were available for the 3-phospho-adenosine moiety, and modelling studies proved inconclusive with multiple binding configurations with similar energies observed.

4. Discussion

Humans contain a large number of ACOT enzymes [25-28, 34], and ACOTs -1, -2, -4 and -8 were identified as potentially been able to hydrolyse substrates possessing 2-methyl groups based on previous work. ACOT-1 was selected for further study since it was potentially able to hydrolyse inverted 2-APA-CoA esters exported from mitochondria and peroxisomes into the cytosol. ACOT-2 was also selected for further study as it is localised in mitochondria, and can potentially hydrolyse 2-APA-CoA esters imported via the acyl-carnitine shuttle and hence potentially contribute to 2-APA-mediated toxicity mechanisms [23]. ACOT-1 and ACOT-2 are both type I enzymes and share very high sequence identity within their core regions [34]; ACOT-2 mainly differs in that it possesses a N-terminal mitochondrial targeting signal which is absent in ACOT-1 (Supplementary Information, Figure S1).

Both ACOT-1 and ACOT-2 show limited discrimination between different chiral configurations of the substrate, as demonstrated by conversion of both R- and S-2-methylmyristoyl-CoA (4R and 4S). The results are consistent with the previous observations that both epimers of ibuprofenoyl-CoA [13] and fenoprofenoyl-CoA [35] can be hydrolysed in the chiral inversion pathway. The broad substrate selectivity of ACOT-1 and ACOT-2 is similar to that observed for α-methylacyl-CoA racemase [9], and is consistent with a common chiral inversion pathway existing for all 2-APA drugs undergoing chiral inversion. The observed uni-directional R→S chiral inversion in vivo is due to stereoselective formation of R-2-APA-CoA esters by long-chain fatty acyl-CoA synthetase [4-6]. R-Flurbiprofen does not undergo chiral inversion in humans, although it does in other mammalian species [36, 37], despite ±-flurbiprofenoyl-CoA 6 been a substrate for both AMACR [9] and ACOTs. Studies on purified long-chain fatty acyl-CoA synthetase [4] have shown that R-
Flurbiprofen is not converted to $R$-flurbiprofenoyl-CoA $6R$. It therefore seems likely that the failure of flurbiprofen to undergo chiral inversion in humans is due to non-formation of the acyl-CoA ester in vivo [8, 9].

The results in this paper show that both ACOT-1 and ACOT-2 possess broad substrate selectivity. Modelling studies (Figure 4) support the roles of the proposed catalytic residues [38] and explain how the enzyme is able to accommodate straight-chain and both epimeric configurations of 2-methyl substrates. Branched-chain substrates are slightly preferred (based on $k_{cat}/K_m$ values), presumably due to accommodation of the 2-methyl group within their binding pockets and this is consistent with other enzymes that metabolise branched-chain acyl-CoA substrates [39]. The π stacking interactions with the side-chain of Trp-391 explains the favourable binding of 2-APA-CoA esters, and the lipophilic binding pocket the tolerance for diverse side-chain structures. The situation is reminiscent of that observed for AMACR [9], the enzyme catalysing the chiral inversion step in the metabolic pathway, in which broad substrate selectivity is achieved by non-specific hydrophobic interactions with a methionine-rich surface. Some 2-APA-CoA substrates are converted much less efficiently by ACOT-1 or ACOT-2 than most substrates, most notably $S$-ketoprofenoyl-CoA $7S$ (ACOT-1) and ±-ibuprofenoyl-CoA $2$ (ACOT-2). The reasons why these substrates are converted less efficiently are unclear.

In summary, 2-APA-CoA esters are good substrates for both ACOT-1 and ACOT-2. These enzymes appear to play a central role in the chiral inversion pathway of 2-APA drugs, catalysing the third step in the pathway. The results also support a common chiral inversion pathway for all 2-APA drugs.

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Figure 1: The chiral inversion pathway of 2-APA drugs, using ibuprofen 1 as an example.

Figure 2: Acyl-CoA esters tested as substrates with human ACOT-1 and -2.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol. min.⁻¹ mg⁻¹)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (s⁻¹ M⁻¹)</th>
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<tr>
<td>Myristoyl-CoA 3</td>
<td>39</td>
<td>7.40</td>
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<tr>
<td>$R$-2-Methylmyristoyl-CoA 4R</td>
<td>9.3</td>
<td>1.48</td>
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<tr>
<td>$S$-2-Methylmyristoyl-CoA 4S</td>
<td>12</td>
<td>3.37</td>
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<td>±-Fenoprofenoyl-CoA 5</td>
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<td>6.89</td>
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<tr>
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<td>5.07</td>
<td>0.0040</td>
<td>218</td>
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<tr>
<td>±-Ibuprofenoyl-CoA 2</td>
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<td>1.63</td>
<td>0.0013</td>
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<tr>
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<td>3.0</td>
<td>0.52</td>
<td>0.0004</td>
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**Table 1:** Kinetic parameters determined for human ACOT-1. Reported values are medians obtained from the Direct Linear Plot.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol. min.$^{-1}$mg$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
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<tr>
<td>Myristoyl-CoA 3</td>
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<td>$S$-2-Methylmyristoyl-CoA 4S</td>
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**Table 2:** Kinetic parameters determined for human ACOT-2. Reported values are medians obtained from the Direct Linear Plot.
Figure 3: Kinetic plots of ibuprofenoyl-CoA hydrolysis catalysed by ACOT-1. A. Direct Linear Plot; B. Michaelis-Menten plot. Error bars are ± standard error (± SE).
Figure 4: S-ibuprofenoyl-CoA bound into the ACOT2 binding pocket [32]. Ser-294 is located in the base of the pocket in close proximity to the carbonyl group of the thioester; His-422 and Asp-388 are located deeper within the enzyme. The exact orientation of the 3-phosphoadenosine moiety could not be established with several possible binding channels available.
Supplementary Information

Hydrolysis of ibuprofenoyl-CoA and other 2-APA-CoA esters by human acyl-CoA thioesterases-1 and -2 and their possible role in the chiral inversion of profens.

Xiao Qu, Amanda Allan, Grace Chui, Thomas J. Hutchings, Ping Jiao, Lawrence Johnson, Wai Y. Leung, Portia K. Li, Georgina R. Steel, Andrew S. Thompson, Michael D. Threadgill and Matthew D. Lloyd*

Medicinal Chemistry, Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U. K.

*Author for correspondence: M.D.Lloyd@bath.ac.uk

Abbreviations used: ACOT, acyl-CoA thioesterase; BSA, bovine serum albumin; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid); HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis;
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<tr>
<td>Kinetic plots for ACOT-2 with 2-APA-CoA substrates</td>
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<td>Figure S1: Sequence alignment of human ACOT-1 and ACOT-2</td>
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<tr>
<td>References</td>
<td>p 58</td>
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</table>
Synthetic route to substrates

R-2-Methylmyristoyl-CoA $4R$ and S-2-methylmyristoyl-CoA $4S$ were synthesized by the same route as for S-2-methyldecanoyl-CoA and R-2-methyldecanoyl-CoA [40] (Scheme S1). Myristoyl-CoA 3 was synthesized by the reaction of myristic acid 9 (tetradecanoic acid) with carbonyl di-imidazole followed by coenzyme A. 2-APA-CoA esters were synthesized as previously described [9].

Scheme S1: Synthesis of substrates: A) Myristoyl-CoA 3; B) R-2-methylmyristoyl-CoA $4R$; C) S-2-methylmyristoyl-CoA $4S$;
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. Coenzyme A tri-lithium salt was purchased from Calbiochem. Reagents were of analytical grade or equivalent (synthesis) or biochemical grade. Sources of biological reagents are reported in the experimental section of the main paper. NMR spectra were recorded on either a JEOL GX 270 (270.05 MHz \(^1\)H; 67.8 MHz \(^{13}\)C) or a JEOL EX 400 (399.65 MHz \(^1\)H; 100.4 MHz \(^{13}\)C; 376.05 MHz) spectrometer. Spectra were referenced to the residual solvent peak, or externally via the solvent lock signal. Coupling constants (J) are reported in Hz to the nearest 0.1 Hz. Mass spectra were obtained using a VG 7070 mass spectrometer. Optical rotations were recorded on an Optical Activity AA-10 Automatic polarimeter with a path-length of 1 decimetre (1 dm). Concentrations (c) are quoted in g/100 mL. Column chromatography was performed using silica gel 60 (0.040-0.063 mm, Merck). Experiments were conducted at ambient temperature, unless otherwise stated. Solutions in organic solvents were dried using anhydrous sodium sulfate and solvents were evaporated under reduced pressure.

Synthesis of 1-(1H-imidazol-1-yl)-tetradecan-1-one (10)

A solution of myristic acid 9 (106 mg, 0.46 mmol) in dichloromethane (10 mL) was treated with solid portions of carbonyl di-imidazole (142 mg, 0.87 mmol) and the resulting reaction mixture was stirred under argon for 1 hour. The reaction mixture was washed with water (4 x 10 mL) and then dried over anhydrous sodium sulfate. Evaporation of the volatile organics gave a colourless solid 10, 126 mg (98.5 %). \(^1\)H-NMR (400.04 MHz, CDCl\(_3\)): \(\delta = 0.88 \text{ (t, J = 6.5 Hz, 3H)}\), 1.22-1.45 (m, 20H), 1.79 (pentet, \(J = 7.5\) Hz, 2H), 2.85 (t, \(J = 7.4\) Hz, 2H), 7.10 (m, 1H), 7.47 (t, \(J = 1.4\) Hz, 1H), 8.16 (s, 1H).
Synthesis of myristoyl-CoA (3)

A solution of 10 (15 mg, 0.054 mmol) and coenzyme A, tri-lithium salt (14 mg, 0.018 mmol) were reacted in THF (1 mL) and aq. NaHCO₃ solution (1 mL, 0.1 M). The reaction was stirred under nitrogen for 16 hours. The reaction was acidified to pH 4.5 with HCl (1M) and then washed repeatedly with ethyl acetate (6 x 5 mL). Freeze-drying of the aqueous layer afforded 3 as a colourless solid (18 mg). ESI-MS m/z calcd. for [M-H]⁻ C₃₃H₆₁N₇O₁₇P₃S: 976.3063; found 976.3016.

Synthesis of S-4-Benzyl-3-tetradecanoyloxazolidin-2-one (11S)

Oxalyl chloride (2.10 g, 3.2 ml, 16.7 mmol) was added slowly to a solution of myristic acid 9 (2.80 g, 12.3 mmol) in chloroform (20 mL) at 0 ºC. The reaction was allowed to warm to room temperature over 1 hour. Evaporation of the volatiles in vacuo afforded the acid chloride as a yellow oil (~3 mL). nBuLi (4.5 mL, 1.6 M, 7.2 mmol) was added dropwise to a solution of S-4-benzyloxazolidinone (1.20 g, 6.7 mmol) in dry THF (20 mL) at -78 ºC, followed by the acid chloride (2 mL). Addition of the acid chloride gave a colourless precipitate, which dissolved as the reaction was warmed to 0 ºC over 1 hour. The reaction was stirred at this temperature for a further hour and then quenched by the addition of a saturated ammonium chloride solution (10 mL). The reaction mixture was extracted with dichloromethane (3 x 25 mL). The combined organics were washed with aqueous K₂CO₃ (20 mL, 1M) and brine (20 mL). Drying over anhydrous sodium sulfate and removal of the volatiles in vacuo gave the crude product as a yellow oil (3.40 g). Purification by column chromatography (petroleum ether/ethyl acetate, 9:1) afforded a colourless oil that solidified on standing to a colourless solid, 1.79 g (69.0 %). ¹H-NMR (400.04 MHz, CDCl₃): δ = 0.88 (t, J = 6.6 Hz, 3H), 1.20-1.44 (m, 20H), 1.69 (quintet, J = 7.2 Hz, 2H), 2.76 (dd, J = 9.6, 13.4 Hz, 1H), 2.93 (m, 2H), 3.30 (dd, J = 3.3, 13.4 Hz, 1H), 4.17 (m, 2H), 4.67 (m, 1H), 7.20 (m, 2H), 7.26-7.36 (m, 3H).
**Synthesis of R-4-Benzyl-3-tetradecanoyloxazolidin-2-one (11R)**

In a parallel synthesis 11R was obtained from myristic acid 9 (2.80 g, 12.3 mmol) and R-4-benzyloxazolidinone (1.20 g, 6.7 mmol) as a colourless oil which solidified on standing to a colourless solid, 2.50 g (96 %). NMR spectra were identical to 11S.

**Synthesis of 3-(S-2-Methyltetradecanoyl)-4-S-4-benzyloxazolidinone (12S)**

A solution of 11S (1.70 g, 4.40 mmol) in dry THF (5 mL) was added dropwise to a cooled (-78 °C) solution of lithium bis(trimethylsilyl)amide (8.8 mL, 1.0 M in THF, 8.8 mmol), under an argon atmosphere. The reaction mixture was stirred for 1 hour at this temperature before methyl iodide (1.4 mL, 3.12 g, 22 mmol) in THF (0.5 mL) at -78 °C was added. The reaction was stirred for a further 3 hours at -78 °C and then allowed to warm to 0 °C. The reaction was quenched with saturated ammonium chloride (15 mL) and then extracted with dichloromethane (3 x 20 mL). The combined organics were washed with aqueous sodium sulfite (1M) and then dried over anhydrous sodium sulfate. Evaporation of the volatile organics afforded an orange oil. Purification by flash chromatography (petroleum ether/ethyl acetate, 20:1) gave 12S as a colourless oil, 560 mg (31.1 %). 1H-NMR (400.04 MHz, CDCl3): δ = 0.88 (t, J = 6.6 Hz, 3H), 1.22 (d, J = 6.8 Hz, 3H), 1.22-1.35 (m, 20H), 1.36-1.47 (m, 1H), 1.73 (m, 1H), 2.76 (dd, J = 9.6, 13.3 Hz, 1H), 3.27 (dd, J = 3.2, 13.4 Hz, 1H), 3.70 (sextet, J = 6.6 Hz, 1H), 4.11-4.22 (m, 2H), 4.67 (m, 1H), 7.21 (m, 2H), 7.24-7.36 (m, 3H).

**Synthesis of 3-(R-2-Methyltetradecanoyl)-4-R-4-benzyloxazolidinone (12R)**

Treatment of 11R (2.5 g, 6.5 mmol) with lithium bis(trimethylsilyl)amide (8.8 mL, 1.0 M in THF, 8.8 mmol) and methyl iodide (4.56 g, 2.0 mL, 32.5 mmol), as for the synthesis of 12S, afforded 12R as a colourless oil, 690 mg (26.4 %). NMR spectra were identical to 12S.
Synthesis of S-2-Methyltetradecanoic acid (13S)

A solution of 12S (550 mg, 1.4 mmol) in THF (25 mL) and water (8 mL) at 0 ºC was treated with hydrogen peroxide (1.2 mL, 30 %) and lithium hydroxide (70 mg, 2.8 mmol). The reaction was maintained at this temperature for 3 hours and then quenched with aqueous sodium sulfite (11 mL, 1.5 M). The reaction mixture was acidified to pH 1 with HCl (1M) and then extracted with dichloromethane (3 x 25 mL). The combined extracts were dried over sodium sulfate and then evaporated in vacuo to give a colourless oil. Purification by flash chromatography (petroleum ether/ethyl acetate, 3:2) gave 13S as a colourless oil, 270 mg (80 %). $^1$H-NMR (400.04 MHz, CDCl$_3$): $\delta$ = 0.88 (t, $J$ = 6.6 Hz, 3H), 1.18 (d, $J$ = 7.0 Hz, 3H), 1.22-1.37 (m, 20H), 1.37-1.48 (m, 1H), 1.63-1.73 (m, 1H), 2.46 (sextet, $J$ = 6.9 Hz, 1H). $^{13}$C-NMR (125. MHz, CDCl$_3$): $\delta$ = 14.10, 16.80, 22.69, 27.15, 29.48, 29.52, 29.61, 29.65, 29.68, 31.93, 39.42, 183.63. $[\alpha]_D^{20}$ = +23.8 ° (c 0.034, methanol).

Synthesis of R-2-Methyltetradecanoic acid (13R)

Treatment of 12R (690 mg, 1.72 mmol) with lithium hydroxide (82 mg, 3.44 mmol) and hydrogen peroxide (1.5 mL, 30 % in H$_2$O) as for the synthesis of 13S, afforded 13R as a colourless oil which solidified on standing to a colourless solid, 280 mg (67.3 %). NMR spectra were identical to 13S. $[\alpha]_D^{20}$ = -23.9 ° (c 0.014, methanol)

Synthesis of S-1-(1H-imidazol-1-yl)-2-methyltetradecan-1-one (14S)

A solution of 13S (100 mg, 0.41 mmol) in dichloromethane (10 mL) was treated with carbonyl diimidazole (133 mg, 0.82 mmol) in dichloromethane as described for the synthesis of 10. Evaporation of the volatile organics gave a colourless oil which solidified on standing to give a colourless solid 14S, 130 mg (92 %). $^1$H-NMR (400.04 MHz, CDCl$_3$): $\delta$ = 0.88 (t, $J$ = 6.5 Hz, 3H),
1.22-1.33 (m, 22H), 1.50-1.61 (m, 1H), 1.80-1.89 (m, 1H), 3.05 (sextet, J = 6.8 Hz, 1H), 7.11 (t, J = 0.8 Hz, 1H), 7.49 (d, J = 1.4 Hz, 1H), 8.17 (s, 1H).

**Synthesis of R-1-(1H-imidazol-1-yl)-2-methyltetradecan-1-one (14R)**

As for the synthesis of 14S, 14R was prepared by treatment of 13R (100 mg, 0.41 mmol) with carbonyl di-imidazole (133 mg, 0.82 mmol) in dichloromethane. 14R was obtained as a colourless oil that solidified on standing to give a colourless solid, 115 mg (96.1 %). NMR spectra were identical to 14S.

**Synthesis of S-2-Methylmyristoyl-CoA (4S)**

A solution of 14S (12 mg, 0.041 mmol) in THF (1 mL) and coenzyme A, tri-lithium salt (11 mg, 0.014 mmol) in THF (1 mL) and aq. NaHCO₃ solution were reacted as described for the synthesis of 3. Freeze-drying of the aqueous layer afforded 4S as a colourless solid (14 mg). ESI-MS m/z calcd. for [M-H]- C₃₆H₆₃N₇O₁₇P₃S: 990.3219; found 990.3177.

**Synthesis of R-2-Methylmyristoyl-CoA (4R)**

In similar fashion to the preparation of 4S, 4R was obtained from the reaction of 14R (15 mg, 0.051 mmol) and coenzyme A, tri-lithium salt (14 mg, 0.018 mmol) in THF (1 mL) and aq. NaHCO₃ solution (1 mL, 0.1 M). Freeze-drying gave 4R as a colourless solid (18 mg). ESI-MS m/z calcd. for [M-H]- C₃₆H₆₃N₇O₁₇P₃S: 990.3219; found 990.3176.
Myristoyl-CoA 3, ACOT-1

\[
\text{Me(C}_10\text{O})\text{S-CoA}
\]

Direct Linear Plot

\[
\text{Apparent } K_m
\]

\[
\text{0 -100 -50 0 50 100}
\]

\[
\text{Apparent } V_{\text{max}}
\]

\[
\text{0 5 10 15 20}
\]
Parameters

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Goodness of Fit

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<th>Value</th>
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Data

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<tr>
<td>Number of replicates</td>
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<tr>
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<td>24</td>
</tr>
<tr>
<td>Number of missing values</td>
<td>0</td>
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</table>
$R$-2-Methylmyristoyl-CoA $4R$, ACOT-1

![Diagram of R-2-Methylmyristoyl-CoA 4R, ACOT-1](image)

**Direct Linear Plot**

- $\text{Apparent } K_m$
  - -100
  - -50
  - 0
  - 50
  - 100

- $\text{Apparent } V_{\text{max}}$
  - 0
  - 1
  - 2
  - 3
  - 4

**Graph:**

- Axes: $\text{Apparent } K_m$ on the x-axis and $\text{Apparent } V_{\text{max}}$ on the y-axis.
- Data points indicate the relationship between $K_m$ and $V_{\text{max}}$.
**Parameters**

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<td>$V_{\text{max}}$</td>
<td>1.3465</td>
<td>0.1023</td>
<td>1.1324 to 1.5606</td>
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<td>$K_m$</td>
<td>7.7008</td>
<td>2.0800</td>
<td>3.3472 to 12.0544</td>
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**Goodness of Fit**

- Degrees of Freedom: 19
- AICc: -65.237
- $R^2$: 0.822
- Sum of Squares: 0.660
- Sy.x: 0.186
- Runs Test p Value: 0.254

**Data**

- Number of x values: 7
- Number of replicates: 3
- Total number of values: 21
- Number of missing values: 0
Lineweaver-Burk

1/[Substrate] 1/(µM)
-0.2 0.0 0.2 0.4 0.6 0.8

1/Rate 1/(nmol.-1min.-1mg-1)
2
4
6
8
10

Residuals

[Substrate] (µM)
0 20 40 60 80 100 120

Residuals (nmol.-1min.-1mg-1)
-0.4
-0.2
0.0
0.2
0.4
0.6

[Substrate] (µM)
S-2-Methylmyristoyl-CoA 4S, ACOT-1

[Chemical structure of S-2-Methylmyristoyl-CoA 4S]

Direct Linear Plot

[Graph showing apparent $K_m$ vs. apparent $V_{max}$]

- Apparent $K_m$:
  - -100
  - -50
  - 0
  - 50
  - 100

- Apparent $V_{max}$:
  - 0
  - 2
  - 4
  - 6
  - 8
  - 10
  - 12
  - 14
**Parameters**

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<td>$V_{max}$</td>
<td>3.4628</td>
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<td>3.0464 to 3.8792</td>
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<td>$K_m$</td>
<td>12.4377</td>
<td>2.2548</td>
<td>7.7615 to 17.1139</td>
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**Goodness of Fit**

- Degrees of Freedom: 22
- AICc: -53.600
- $R^2$: 0.935
- Sum of Squares: 1.905
- Sy.x: 0.294
- Runs Test p Value: 0.050

**Data**

- Number of x values: 8
- Number of replicates: 3
- Total number of values: 24
- Number of missing values: 0
Lineweaver-Burk

Residuals

±-Fenoprofenoyl-CoA 5, ACOT-1
**Parameters**

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<tr>
<th>Parameter</th>
<th>Value</th>
<th>±Std. Error</th>
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<td>$V_{\text{max}}$</td>
<td>12.8319</td>
<td>2.0165</td>
<td>8.6498 to 17.0140</td>
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<tr>
<td>$K_{\text{m}}$</td>
<td>109.0979</td>
<td>27.7494</td>
<td>51.5481 to 166.6477</td>
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**Goodness of Fit**

- Degrees of Freedom: 22
- AICc: -38.315
- $R^2$: 0.964
- Sum of Squares: 3.602
- Sy.x: 0.405
- Runs Test p Value: 0.237

**Data**

- Number of x values: 8
- Number of replicates: 3
- Total number of values: 24
- Number of missing values: 0
±-Flurbiprofenoyl-CoA 6, ACOT-1

Direct Linear Plot
Parameters

<table>
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<th>Value</th>
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<th>95% Conf. Interval</th>
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<td>( V_{\text{max}} )</td>
<td>11.2139</td>
<td>1.6125</td>
<td>7.8697 to 14.5582</td>
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<td>( K_m )</td>
<td>117.8644</td>
<td>26.8142</td>
<td>62.2541 to 173.4747</td>
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Goodness of Fit

- Degrees of Freedom: 22
- AICc: -54.182
- \( R^2 \): 0.972
- Sum of Squares: 1.860
- Sy.x: 0.291
- Runs Test p Value: 0.216

Data

- Number of x values: 8
- Number of replicates: 3
- Total number of values: 24
- Number of missing values: 0

Michaelis-Menten

![Graph of Michaelis-Menten model with substrate concentration (µM) on the x-axis and rate (nmol·min⁻¹·mg⁻¹) on the y-axis.]
±-Ibuprofenoyl-CoA 2, ACOT-1

Direct Linear Plot

Apparent $K_m$
-100 -50 0 50 100

Apparent $V_{max}$
0 2 4 6 8
Enzyme Kinetics Nonlinear Fit Results

Notebook1
13/08/2011 20:04:09
Michaelis-Menten
Number of Replicates: 3

Parameters

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<th>Value</th>
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<td>$V_{max}$</td>
<td>1.5811</td>
<td>0.1008</td>
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<td>$K_m$</td>
<td>5.0993</td>
<td>1.2597</td>
<td>2.4796 to 7.7190</td>
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Goodness of Fit

- Degrees of Freedom: 21
- AICc: -65.326
- $R^2$: 0.849
- Sum of Squares: 0.980
- Sy.x: 0.216
- Runs Test p Value: 0.158

Data

- Number of x values: 8
- Number of replicates: 3
- Total number of values: 23
- Number of missing values: 1
Lineweaver-Burk

Residuals
$S$-Ketoprofenoyl-CoA 7, ACOT-1

\[
\begin{align*}
\text{Direct Linear Plot} \\
\text{Apparent } K_m & -100 -50 0 50 100 \\
\text{Apparent } V_{\text{max}} & 0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
\end{align*}
\]
### Parameters

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<th>±Std. Error</th>
<th>95% Conf. Interval</th>
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<td>1.2309</td>
<td>0.3441</td>
<td>0.5015 to 1.9604</td>
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<tr>
<td>$K_m$</td>
<td>44.7606</td>
<td>25.5387</td>
<td>-9.3801 to 98.9013</td>
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### Goodness of Fit
- Degrees of Freedom: 16
- AICc: -57.669
- $R^2$: 0.664
- Sum of Squares: 0.476
- Sy.x: 0.173
- Runs Test p Value: 0.213

### Data
- Number of x values: 7
- Number of replicates: 3
- Total number of values: 18
- Number of missing values: 3

---

**Michaelis-Menten**

---

![Michaelis-Menten plot](image_url)
Lineweaver-Burk

1/Rate (nmol\cdot min^{-1}\cdot mg^{-1})

1/[Substrate] 1/(µM)

-0.1 0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7

Residuals

Residuals (nmol\cdot min^{-1}\cdot mg^{-1})

[Substrate] (µM)

0 20 40 60 80 100 120

-0.4 -0.2 0.0 0.2 0.4 0.6
5-Naproxenoyl-CoA 8, ACOT-1

\[
\begin{align*}
\text{MeO} & \quad \text{O} \\
\quad & \quad \text{Me} \quad \text{S-CoA}
\end{align*}
\]

Direct Linear Plot

![Direct Linear Plot](image-url)
### Parameters

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<th>Value</th>
<th>±Std. Error</th>
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### Goodness of Fit

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<td>AICc</td>
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<tr>
<td>$R^2$</td>
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<td>Sum of Squares</td>
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<td>Sy.x</td>
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<td>Runs Test p Value</td>
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### Data

- Number of x values: 8
- Number of replicates: 3
- Total number of values: 24
- Number of missing values: 0

---

**Michaelis-Menten**

![Michaelis-Menten graph](image)
Myristoyl-CoA 3, ACOT-2

\[
\text{Me}_{10} - \text{O} \quad \text{S-CoA}
\]

Direct Linear Plot

Apparent $K_m$

-100 -50 0 50 100

Apparent $V_{\text{max}}$

0 5 10 15 20 25 30
Parameters

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<th>Value</th>
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<td>$K_{\text{m}}$</td>
<td>15.4965</td>
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Goodness of Fit

- Degrees of Freedom: 22
- AICc: -70.983
- $R^2$: 0.960
- Sum of Squares: 0.924
- Sy.x: 0.205
- Runs Test p Value: 0.443

Data

- Number of x values: 8
- Number of replicates: 3
- Total number of values: 24
- Number of missing values: 0
$R\text{-}2\text{-}\text{Methylmyristoyl-CoA 4R, ACOT-2}$

![Direct Linear Plot](image-url)

**Direct Linear Plot**

- Apparent $K_m$
- Apparent $V_{max}$
Michaelis-Menten

![Graph](image)

**Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>± Std. Error</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>1.0804</td>
<td>8.920e-2</td>
<td>0.8949 to 1.2659</td>
</tr>
<tr>
<td>$K_m$</td>
<td>2.4503</td>
<td>0.9447</td>
<td>0.4857 to 4.4149</td>
</tr>
</tbody>
</table>

**Goodness of Fit**

- Degrees of Freedom: 21
- AICc: -61.393
- $R^2$: 0.637
- Sum of Squares: 1.162
- Sy.x: 0.235
- Runs Test p Value: 0.447

**Data**

- Number of x values: 8
- Number of replicates: 3
- Total number of values: 23
- Number of missing values: 1
S-2-Methylmyristoyl-CoA 4S, ACOT-2

Direct Linear Plot

Apparent $K_m$
-100 -50 0 50 100

Apparent $V_{max}$
0 10 20 30 40
### Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>±Std. Error</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>5.7688</td>
<td>0.3536</td>
<td>5.0354 to 6.5022</td>
</tr>
<tr>
<td>$K_m$</td>
<td>31.7988</td>
<td>4.6683</td>
<td>22.1171 to 41.4805</td>
</tr>
</tbody>
</table>

### Goodness of Fit

- Degrees of Freedom: 22
- AICc: -56.794
- $R^2$: 0.971
- Sum of Squares: 1.668
- Sy.x: 0.275
- Runs Test p Value: 0.387

### Data

- Number of x values: 8
- Number of replicates: 3
- Total number of values: 24
- Number of missing values: 0
±-Fenoprofenoyl-CoA 5, ACOT-2
Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>± Std. Error</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>9.5307</td>
<td>0.8409</td>
<td>7.7867 to 11.2747</td>
</tr>
<tr>
<td>$K_m$</td>
<td>45.0815</td>
<td>8.5257</td>
<td>27.3998 to 62.7631</td>
</tr>
</tbody>
</table>

Goodness of Fit

- Degrees of Freedom: 22
- AICc: -30.431
- $R^2$: 0.959
- Sum of Squares: 5.003
- Sy.x: 0.477
- Runs Test p Value: 0.350

Data

- Number of x values: 8
- Number of replicates: 3
- Total number of values: 24
- Number of missing values: 0
±-Flurbiprofenoyl-CoA 6, ACOT-2

- Diagram of ±-Flurbiprofenoyl-CoA 6, ACOT-2 with chemical structure.

- Direct Linear Plot graph with axes labeled as Apparent $K_m$ and Apparent $V_{max}$.

- The graph shows a scatter plot with lines of best fit for different data points.
Michaelis-Menten

Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
<th>±Std. Error</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>9.7130</td>
<td>1.1102</td>
<td>7.3319 to 12.0941</td>
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<tr>
<td>$K_m$</td>
<td>30.5008</td>
<td>8.4578</td>
<td>12.3604 to 48.6413</td>
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Goodness of Fit

<table>
<thead>
<tr>
<th>Goodness of Fit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degrees of Freedom</td>
<td>14</td>
</tr>
<tr>
<td>AICc</td>
<td>-4.180</td>
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<tr>
<td>R²</td>
<td>0.933</td>
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<tr>
<td>Sum of Squares</td>
<td>7.474</td>
</tr>
<tr>
<td>Sy.x</td>
<td>0.731</td>
</tr>
<tr>
<td>Runs Test p Value</td>
<td>0.277</td>
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</table>

Data

<table>
<thead>
<tr>
<th>Data</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of x values</td>
<td>8</td>
</tr>
<tr>
<td>Number of replicates</td>
<td>2</td>
</tr>
<tr>
<td>Total number of values</td>
<td>16</td>
</tr>
<tr>
<td>Number of missing values</td>
<td>0</td>
</tr>
</tbody>
</table>
±-Ibuprofenoyl-CoA 2, ACOT-2

Direct Linear Plot

Apparent $K_m$ vs. Apparent $V_{\text{max}}$
Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Std. Error</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>3.2478</td>
<td>0.5254</td>
<td>2.1481 to 4.3475</td>
</tr>
<tr>
<td>$K_m$</td>
<td>33.7370</td>
<td>12.8536</td>
<td>6.8336 to 60.6403</td>
</tr>
</tbody>
</table>

Goodness of Fit

- Degrees of Freedom: 19
- AICc: -34.474
- $R^2$: 0.840
- Sum of Squares: 2.858
- Sy.x: 0.388
- Runs Test p Value: 0.199

Data

- Number of x values: 8
- Number of replicates: 3
- Total number of values: 21
- Number of missing values: 3
S-Ketoprofenoyl-CoA 7, ACOT-2

Direct Linear Plot

Apparent $K_m$ vs. Apparent $V_{max}$
Michaelis-Menten

Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>±Std. Error</th>
<th>95% Conf. Interval</th>
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</thead>
<tbody>
<tr>
<td>$V_{max}$</td>
<td>0.7170</td>
<td>7.780e-2</td>
<td>0.5502 to 0.8839</td>
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<tr>
<td>$K_m$</td>
<td>9.4655</td>
<td>3.4336</td>
<td>2.1011 to 16.8299</td>
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Goodness of Fit

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Degrees of Freedom</td>
<td>14</td>
</tr>
<tr>
<td>AICc</td>
<td>-65.768</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.754</td>
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<tr>
<td>Sum of Squares</td>
<td>0.159</td>
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<tr>
<td>Sy.x</td>
<td>0.107</td>
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<tr>
<td>Runs Test p Value</td>
<td>0.501</td>
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</table>

Data

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of x values</td>
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<tr>
<td>Number of replicates</td>
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</tr>
<tr>
<td>Total number of values</td>
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<tr>
<td>Number of missing values</td>
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5-Naproxenoyl-CoA 8, ACOT-2
Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>±Std. Error</th>
<th>95% Conf. Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>0.5242</td>
<td>3.025e-2</td>
<td>0.4593 to 0.5891</td>
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<tr>
<td>$K_{\text{m}}$</td>
<td>1.8480</td>
<td>0.5116</td>
<td>0.7506 to 2.9453</td>
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</tbody>
</table>

Goodness of Fit

- Degrees of Freedom: 14
- AICc: -79.194
- $R^2$: 0.720
- Sum of Squares: 6.876e-2
- Sy.x: 7.008e-2
- Runs Test p Value: 0.060

Data

- Number of x values: 8
- Number of replicates: 2
- Total number of values: 16
- Number of missing values: 0
**Figure S1:** Primary sequence alignment of human ACOT-1 (A1L172) and ACOT-2 (P49753).

Sequences were obtained from [http://www.uniprot.org/](http://www.uniprot.org/) and aligned with ClusterW ([http://www.ch.embnet.org/software/ClustalW.html](http://www.ch.embnet.org/software/ClustalW.html)) with default parameters. Active site catalytic triad residues are shown in green.