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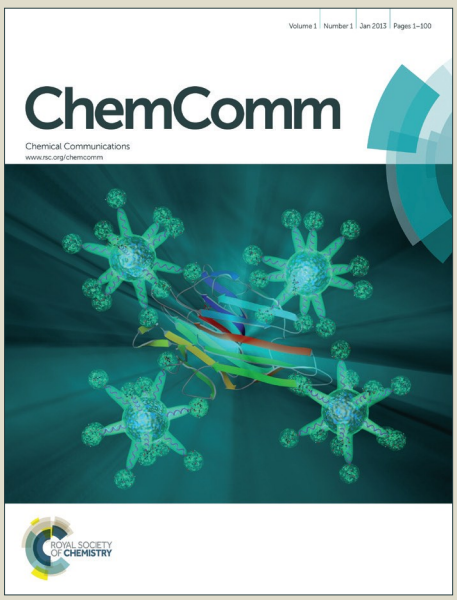

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Rational Design and Synthesis of Substrate-Product Analogue Inhibitors of α-Methylacyl-coenzyme A Racemase from *Mycobacterium tuberculosis*†

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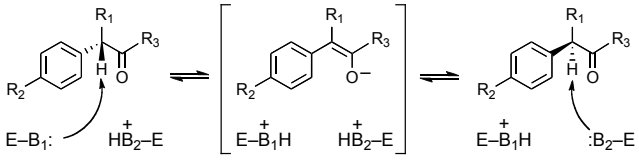
2,2-Bis(4-*i*-butylphenyl)propanoyl-CoA and 2,2-bis(4-*t*-butylphenyl)propanoyl-CoA are rationally designed, *gem*-disubstituted substrate-product analogues that competitively inhibit α-methylacyl-coenzyme A racemase from *Mycobacterium tuberculosis* with K_i values of 16.9 ± 0.6 and 21 ± 4 μM, respectively, exceeding the enzyme's affinity for the substrate by approximately 5-fold.

With the exception of utilizing compounds that mimic the electronic and/or geometric characteristics of the substrate(s) or product(s), or the intermediate(s) or transition state(s) formed during catalysis,[1-3] there are few general strategies for designing enzyme inhibitors. Our observation that mandelate racemase (MR, **Scheme 1**) is inhibited by benzilate[4-6] suggested a new strategy for designing inhibitors of racemases and epimerases. This strategy utilizes substrate-product analogues that incorporate structural features of both the substrate and the product of an enzyme-catalyzed racemization or epimerization reaction. However, such analogues proved to be only modest inhibitors of glutamate, serine, and proline racemases, which have compact active sites.[7,8] That the inhibition of these enzymes was not as effective as in the case of MR suggested that this inhibitor design strategy requires enzymes with capacious and plastic active sites. To test this hypothesis, we rationally designed substrate-product analogue inhibitors of α-methylacyl-coenzyme A racemase from *Mycobacterium tuberculosis* (EC 5.1.99.4, *Mt*MCR), employing *gem*-disubstitution on the carbon-2 stereocenter of a substrate by the group undergoing motion (i.e., the acyl groups of *Mt*MCR substrates) during epimerization. Herein, we report that the novel, *gem*-disubstituted, substrate-product analogues **5** (**Fig. 1** and **Scheme 2**) competitively inhibit *Mt*MCR with binding affinities commensurate with the affinities of inhibitors identified for the human homologue (*vide infra*), thereby demonstrating the utility of our inhibitor design strategy.

Scheme 1. Similar reactions catalyzed by MR and *Mt*MCR



enzyme	substrate	R_1	R_2	R_3	B_1	B_2
MR	mandelate	OH	H	O^-	Lys 166	His 297
*Mt*MCR	ibuprofenoyl-CoA	CH_3	*i*-Bu	SCoA	Asp 156	His 126

gem-Disubstituted inhibitors are reminiscent of bisubstrate analogue inhibitors. Such analogues combine structural features of the two substrates required by a target enzyme,[1,9] and thereby often yield enhanced specificity and binding, the latter arising because of entropic effects.[10] Similarly, substrate-product analogues for racemases and epimerases combine structural attributes of two ligands and enhanced binding affinity is anticipated due to the additional binding determinants, provided that the additional steric bulk is tolerated.

The X-ray crystal structure of MR complexed with the substrate analogue (*S*)-atrolactate revealed that the carboxylate, hydroxyl group, and *ipso* carbon of the phenyl ring of the substrate bind in the same plane (**Fig. 1A**),[11] so that the enantiomers of mandelate are likely bound in a mirror-image orientation (**Fig. 1B**).[12,13] X-ray crystallographic studies[4] and site-directed mutagenesis experiments[5] suggest that the phenyl ring moves between two sub-sites (i.e., *R*- and *S*-pockets, **Fig. 1B**) during catalysis. MR is competitively inhibited by benzilate (K_i = 0.6 mM, *cf.* K_S = 1.0 mM; **Fig. 1C**),[14] indicating that the active site can accommodate the two phenyl rings of this substrate-product analogue. Thus *gem*-disubstituted inhibitors such as benzilate may be designed to present binding determinants simultaneously to the *R*- and *S*-pockets. Indeed, we subsequently showed that 3,3,3-trifluoro-2-hydroxy-2-(trifluoromethyl)propanoate is a highly potent substrate-product analogue inhibitor of MR based on the substrate 3,3,3-trifluorolactate (K_i = 27 μM).[15,16]

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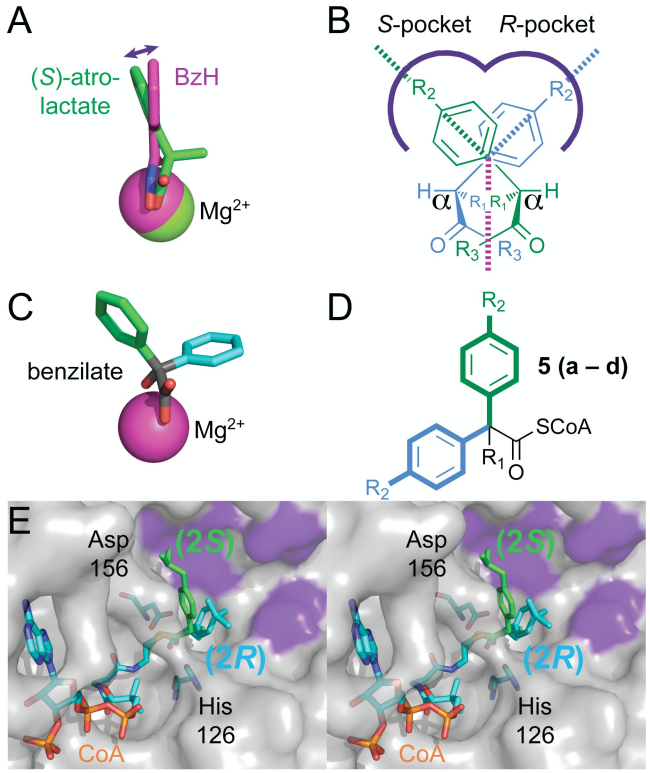


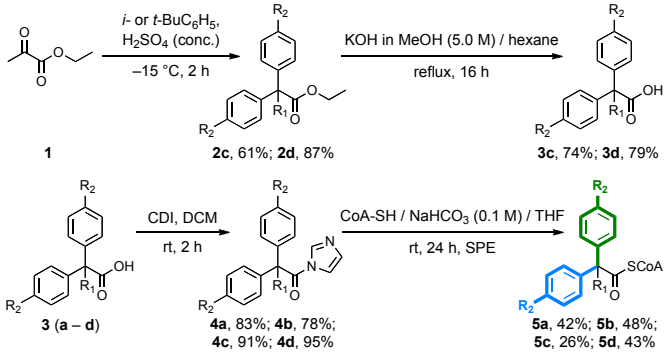
Fig. 1. Binding of ligands to MR and *Mt*MCR. (A) Superposition of the substrate analogue (*S*)-atrolactate (PDB 1MDR)[11] and the intermediate analogue BzH (PDB 3UXK)[4] bound at the active site of MR. The active site Mg^{2+} is shown in space-filling representation. (B) Mirror-image binding of substrate enantiomers (superposed) results in three substituents on the α-carbon lying in the same plane (magenta dotted line) with the α-hydrogens being antipodal.[12,13] Aryl rings attached to the α-carbon atom project linearly to define the *R*- and *S*-pockets of MR for binding the phenyl ring of *R*- and *S*-mandelate (where R_1 = OH, R_2 = H, and R_3 = O^-), respectively. (C) Benzilate with its two phenyl groups binding at the active site of the C92S/C264S/K166C triple mutant of MR.[15] (D) *gem*-Diaryl substrate-product analogue inhibitors of *Mt*MCR. (E) Stereoview showing the superposition of the substrates (2*S*)- and (2*R*)-ibuprofenoyl-CoA bound at the active site of *Mt*MCR (PDB 2GCE).[17] The CoA moiety and α-CH_3 remain fixed, while the 2-aryl moiety moves over a Met-rich surface (purple) during catalysis.

To test our hypothesis that enzymes with capacious active sites might be more amenable to inhibition by substrate-product analogues, we applied our inhibitor design strategy to *Mt*MCR. *Mt*MCR is a cofactor-independent enzyme that catalyzes the 1,1-proton transfer reaction[17,18] which effects the reversible epimerization of the CoA-thioesters derived from a variety of (2*R*)- and (2*S*)-methyl branched fatty acids,[19] a number of C27 bile acid intermediates,[20] and 2-arylpropionic acids (e.g., ibuprofen, **Scheme 1**).[21,22] The *Mt*MCR-catalyzed epimerization of ibuprofenoyl-CoA is very similar to the racemization of mandelate catalyzed by MR (**Scheme 1**). Studies using 2-^{2}H-labeled or 2-^{3}H-labeled substrates[17-19,22-24] and X-ray crystallographic studies on *Mt*MCR indicate that the enzyme is a two-base epimerase and epimerization proceeds via an enol/enolate intermediate.[17,23,25] Structural studies conducted by Wierenga and co-workers[23] on *Mt*MCR complexed with (*R*,*S*)-ibuprofenoyl-CoA, (2*S*)-methylmyristoyl-CoA, and (2*R*)-methylmyristoyl-CoA revealed that the nonpolar acyl group moves through a Met-rich hydrophobic pocket during catalysis (**Fig. 1E**), while the CoA and α-CH_3 groups remain bound at their respective binding sites.[17] Substrate-product analogues could therefore be designed that are *gem*-disubstituted with the hydrophobic group undergoing motion.

Our synthesis of such a substrate-product analogue inhibitor (**5**) is outlined in **Scheme 2** (also see ESI). For the *para*-substituted species (R_2 = *i*-butyl or *t*-butyl), *gem*-disubstitution was accomplished using the general synthesis of 2,2-diarylpropanoates described by Patai and Dayagi.[26] Ethyl pyruvate (**1**) was reacted with either *i*- or *t*-butylbenzene in concentrated sulfuric acid at –15 °C to afford **2c** and **2d**, respectively, after purification using silica gel chromatography. Vigorous stirring was required since the immiscible mixture of *i*- or *t*-butylbenzene and the ethyl pyruvate/sulfuric acid solution became quite viscous as the reaction proceeded. Conversion to **3c** and **3d** was achieved by hydrolysis using 5 M KOH in hexanes/MeOH. Finally, thioesters **5a** – **d** were prepared by activating **3a** – **d** with CDI to form **4a** – **d** in dry DCM, followed by reaction with the trilithium salt of CoA in an aqueous bicarbonate solution.[18] The resulting product was purified using semi-preparative solid-phase extraction to afford thioesters **5a** – **d** in yields of 26 – 48%.

Using a continuous circular dichroism (CD)-based assay and (2*S*)-ibuprofenoyl-CoA as the substrate,[27] we examined the inhibitory effect of **3a** – **d** and **5a** – **d** on *Mt*MCR activity. Recombinant *Mt*MCR was overproduced as a fusion protein bearing an N-terminal hexahistidine tag and purified using nickel ion affinity chromatography (see ESI). Thioesters **5a** – **d** are competitive inhibitors of *Mt*MCR (**Table 1**), with **5c** and **5d** being the most effective inhibitors (**Fig. 2**) and binding with affinities ~5-fold greater than that exhibited for the substrate (2*S*)-ibuprofenoyl-CoA (assuming $K_m \approx K_S$). Clearly, *Mt*MCR can simultaneously accommodate the two aryl groups of **5a** – **d**. The geminal phenyl groups of **5a** and **5b** afforded a binding affinity that was weaker than that observed for the substrate by ~3-fold. The similar binding affinity for these two inhibitors suggests that the α-CH_3 group in **5b** does not contribute significantly to the binding affinity, perhaps because of the less efficient binding of the phenyl groups relative to larger hydrophobic acyl groups. However, Lloyd and co-workers found that (*S*)-2-methyldecanoyl-CoA and decanoyl-CoA exhibited K_m values of 614 and 225 μM, respectively, also suggesting the methyl group does not contribute to ground

Scheme 2. Synthesis of the *gem*-diaryl substrate-product analogues.



a, R_1 = R_2 = H; **b**, R_1 = CH_3, R_2 = H; **c**, R_1 = CH_3, R_2 = *i*-butyl; **d**, R_1 = CH_3, R_2 = *t*-butyl

state binding by human α-methylacyl-CoA racemase (AMACR).[28] The addition of the *i*- or *t*-butyl groups in **5c** or **5d**, respectively, enhanced the inhibition ~20-fold, consistent with the presence of additional hydrophobic interactions within the large hydrophobic cavity that comprises the binding sites for the acyl moiety of substrate epimers.[17] Comparison of the IC_{50} values for the inhibition of *Mt*MCR by CoA and the free acids **3a** – **d** (**Table 1**) reveals that the presence of both the acyl and CoA moieties in the inhibitor are required for better binding. The surprising exception is **3c**, which was a reasonably effective inhibitor, binding with an affinity similar to that of the substrate. This observation suggests that inhibitors might be developed that either lack the CoA moiety or have only part of the CoA structure.

Since **5a** bears an α-proton similar to the substrate, *Mt*MCR could potentially catalyze exchange of this proton with deuterium from solvent D_2O. Using a concentration of *Mt*MCR exceeding that typically employed under assay conditions by at least 1000-fold, and monitoring the signals arising from the α-proton and phenyl protons of **5a** by ^{1}H NMR spectroscopy, no *Mt*MCR-catalyzed exchange was observed over 24 h (see ESI). Thus, the bound orientation of **5a** places the α-proton in a location where it is not amenable to exchange. Such a binding orientation is consistent with the two phenyl rings occupying the binding sites for the 4-*i*-butylphenyl groups of the enantiomers of ibuprofenoyl-CoA (**Fig. 1E**) with the α-proton located in the binding pocket for the α-methyl group where it is not accessible to the Brønsted acid-base catalysts.

*Mt*MCR shares a similar mechanism and substrate specificity with its homologue human AMACR (43% amino acid sequence identity with *Mt*MCR).[29] Elevated levels of AMACR have been associated with a variety of cancers,[31] including prostate cancer (PCa)[32,33] for which the enzyme serves as a biomarker.[33-35] Knockdown of AMACR gene expression suppresses the growth of several PCa cell lines suggesting that AMACR may be a therapeutic target for PCa.[29,36-39] Several efforts to develop inhibitors of AMACR have been reported. Analogues of the natural substrate branched chain α-methylacyl-CoA thioesters possessing one or more β-fluorine atoms were synthesized as potential suicide substrates.[40] These compounds, however, did not covalently modify the enzyme as anticipated but acted as competitive inhibitors with K_i values of 0.9 – 20 μM (K_m/K_i = 27–1.2). Morgenroth *et al*.[41] synthesized 2-methylenacyl-CoA thioesters as AMACR binding ligands; e.g., (13*E*/12)-13-iodo-2-methylentridec-12-enoyl-CoA acted as a competitive inhibitor with a K_i value of 19 μM (K_m/K_i = 1.5). One of the most potent inhibitors is *N*-dodecyl-*N*-methylcarbamoyl-CoA with a K_i value of 98 nM ($K_m/K_i \approx 969$).[42] Because these inhibitors closely resemble the structure of the substrates, one might anticipate that they would lack specificity. A high-throughput screen of a 5,000-compound library for inhibitors of AMACR activity led to the identification of several compounds that were not structural mimics of the substrates but inhibited AMACR with IC_{50} values between 0.8 and 85 μM ($K_m/K_i \approx 107$ & 1.0, respectively).[29] Some of these compounds were irreversible inactivators of AMACR.

In summary, we have demonstrated that utilizing *gem*-disubstituted substrate-product analogues furnishes an

TABLE 1. Inhibition of *Mt*MCR

compound	inhibition results	
	IC_{50}, μM[a]	K_i, μM[b]
coenzyme A	1.2 (± 0.2) × 10^3	nd[c]
(*S*)-ibuprofen[d]	39 (± 5) × 10^3	nd
(2*S*)-ibuprofenoyl-CoA	–	106 ± 15 (K_m)
diphenylacetic acid (**3a**)[d]	> 6.3 × 10^3[e]	nd
diphenylacetyl-CoA (**5a**)	1.1 (± 0.3) × 10^3	358 ± 39
2,2-diphenylpropanoic acid (**3b**)[d]	42 (± 5) × 10^3	nd
2,2-diphenylpropanoyl-CoA (**5b**)	610 ± 73	343 ± 72
2,2-bis(4-*i*-butylphenyl)-propanoic acid (**3c**)[d]	316 ± 37	102 ± 9
2,2-bis(4-*i*-butylphenyl)-propanoyl-CoA (**5c**)	39.5 ± 0.9	16.9 ± 0.6
2,2-bis(4-*t*-butylphenyl)-propanoic acid (**3d**)[d]	–[f]	nd
2,2-bis(4-*t*-butylphenyl)-propanoyl-CoA (**5d**)	44 ± 8	21 ± 4

[a]IC_{50} values determined with [(2*S*)-ibuprofenoyl-CoA] = 100 μM and [*Mt*MCR] = 0.18 nM. [b]Values reported are the averages of three independent trials and the errors are the standard deviations. [c]Not determined. [d]Determined in the presence of 20% DMSO. [e]Value estimated assuming competitive inhibition with only 24 (± 3)% inhibition observed and [**3a**] = 20 mM. [f]Only 15 (± 0.5)% inhibition observed at [**3d**] = 480 μM; however, it is likely that weak inhibition arises from solubility problems (see **Fig. 50S** in the ESI).

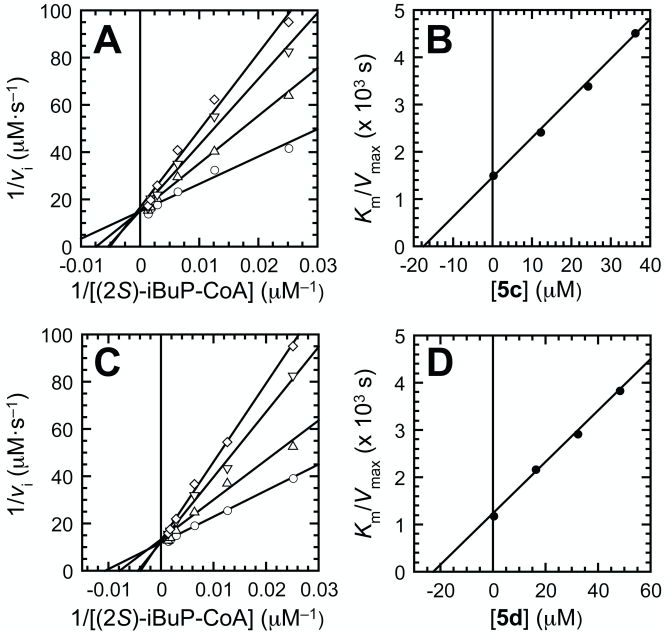


Fig 2. Inhibition of *Mt*MCR by **5c** and **5d**. (A) and (C) Representative Lineweaver-Burk plots showing the competitive inhibition of *Mt*MCR by **5c** and **5d**, respectively. Concentrations (μM): **5c**: 0 (○), 12 (△), 24 (▽), 36 (◇); **5d**: 0 (○), 16 (△), 32 (▽), 48 (◇); (2*S*)-ibuprofenoyl-CoA: 40, 80, 160, 360, 600, and 800; and *Mt*MCR: 0.18 nM (7.5 ng/mL). (B) and (D) Representative plots of the apparent K_m/V_{max} values (determined from direct fits of Eqn. 1S to initial velocity data (i.e., Michaelis-Menten plots; see **Figs. 56S** and **57S** in the ESI)) vs. concentrations of **5c** and **5d**, respectively.[30] K_i values (determined in triplicate) are given in **Table 1**.

effective strategy for designing inhibitors of racemases and epimerases with capacious active sites. This rational approach offers an alternative to the well-established strategy of using β-halo compounds[43,44] to generate suicide substrates of racemases and epimerases catalyzing formation of enol/enolate intermediates, or to the strategies of designing an inhibitor based on the structure of the substrate(s), product(s), intermediate(s), or transition state(s) of enzyme-catalyzed reactions. The inhibitors described in the present work may furnish tools for studying cholesterol ester metabolism in *M. tuberculosis*,[45] or lead compounds for the development of AMACR inhibitors directed against PCa. The additional acyl-like chain on such *gem*-disubstituted inhibitors may endow the inhibitor with specificity, obviating its recognition by other fatty acyl-CoA-metabolizing enzymes. Of course, *in vivo* studies will require structural alterations and prodrug strategies to circumvent hydrolysis of the thioester and the lack of cell permeability arising from the CoA moiety, respectively.[46,47] Alternatively, the reasonable binding affinity exhibited by *gem*-disubstituted diaryl acids such as **3c** may offer a means of overcoming the need for incorporation of the CoA moiety.

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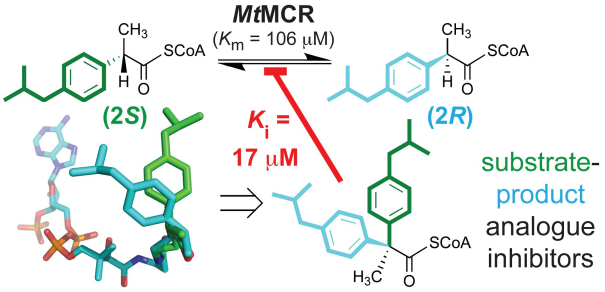
Table of Contents Graphic



gem-Disubstituted substrate-product analogues competitively inhibit α-methylacyl-coenzyme A racemase from *Mycobacterium tuberculosis*, binding with affinities exceeding that of the substrate by ~5-fold.