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STUDIES OF THE INACTIVATION OF GENERAL ACYL-COA DEHYDROGENASE BY (1*R*)- AND (1*S*)-(METHYLENECYCLOPROPYL)ACETYL-COA

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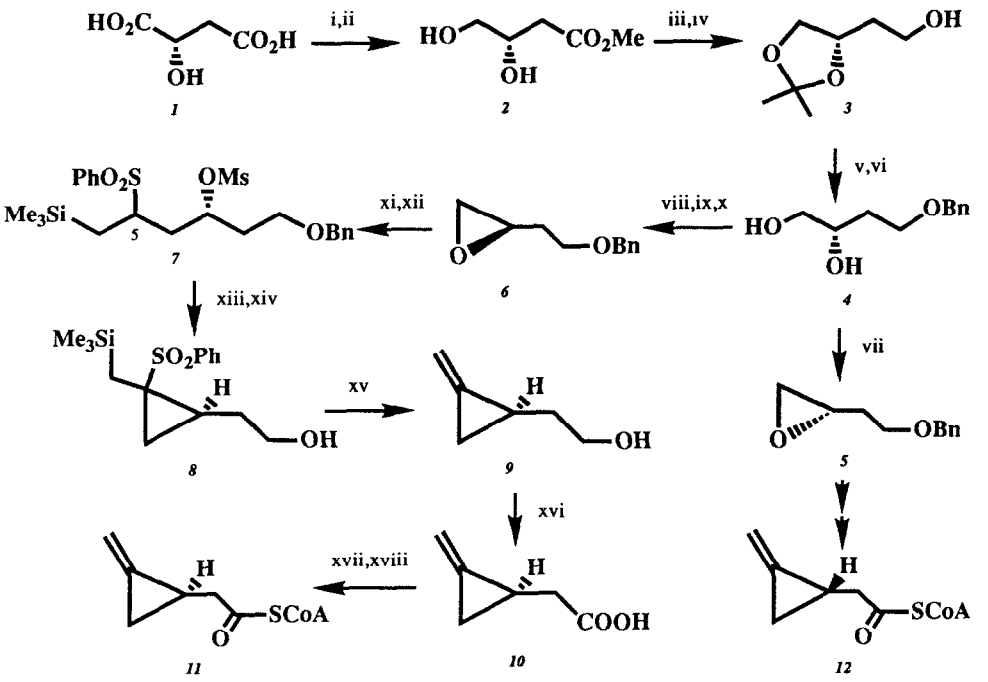
ABSTRACT: Studies of the inactivation of general acyl-CoA dehydrogenase by the title compounds under saturation conditions showed that the association of the 1*S* isomer is weaker than that of the 1*R* isomer.

(1*R*)-(Methylenecyclopropyl)acetyl-CoA (MCPA-CoA), which is derived *in vivo* from hypoglycin, has been identified as the causative agent of Jamaican vomiting sickness.[1] The site at which hypoglycin toxicity occurs has been established to be the flavin containing short- and medium-chain acyl-CoA dehydrogenases.[2] Since acyl-CoA dehydrogenases catalyze the first step of β-oxidation, converting a fatty acyl thioester substrate to the corresponding α,β-enolyl-CoA product,[3] inhibition of these enzymes results in the accumulation of short chain fatty acyl-CoAs and the suppression of acetyl-CoA/NADH production, both of which contribute to the induction of hypoglycemia.[1] The molecular basis of this inactivation is believed to proceed with an initial C_α anion formation, followed by ring fragmentation, and then covalent modification of the flavin coenzyme.[3] Although there is little doubt that the methylenecyclopropyl group embodied in this molecule plays a pivotal role in the actions of hypoglycin, the actual mode of the ring cleavage is still disputable. A direct anion-induced pathway has been generally accepted as the mechanism for the ring-opening step;[2] however, this process may also be envisaged as occurring via a transient α-cyclopropyl radical intermediate. Because the rearrangement of an α-cyclopropyl radical to the straight-chain alkyl radical is extremely rapid,[4] one would expect that the ring cleavage would be a spontaneous and, consequently, nonstereospecific process in a one-electron oxidation mechanism. This speculation was lately substantiated since both (1*R*)- and (1*S*)-MCPA-CoA are found to be effective inhibitors.[5] In view of the fact that removal of the β-H during normal catalysis has been reported to be pro-*R* stereospecific,[3] the apparent lack of stereospecificity of C_β bond cleavage of MCPA-CoA strongly suggested a radical-initiated mechanism for the ring opening step. However, this rationale was derogated by the discovery of the different rates of the inactivation caused by these two isomers, with the 1*R* isomer as the more reactive inhibitor.[5,6] Although the rate discrepancy may be a result of a rate-limiting enzyme-catalyzed isomerization of the 1*S* to the 1*R* isomer by way of (2-methylenecyclopropylidene)acetyl-CoA, this possibility was recently excluded based on the lack of kinetic isotope effect of [1-^{2}H]MCPA-CoA.[7] In an attempt to clarify this mechanistic ambiguity we have carried out a kinetic analysis of this inhibition under saturation conditions. Reported in this paper are the results of this study and their implications on the mechanism of this inactivation.

The (1*R*)- and (1*S*)-MCPA-CoA (**11** and **12**, respectively) used in this experiment were prepared in enantiomerically pure form from a single and readily available chiral synthon, (*S*)-(-)-malic acid (**1**), by a modified procedure of Kabat and Wicha.[8] As depicted in Scheme I, the construction of the methylenecyclopropane entity followed a strategy developed earlier by Hsiao and Hannick.[9] A similar sequence has also been used, starting with (*R*)-(+)-glycidol, in our recent synthesis of (1*R*)-MCPA-CoA;[10] however, the latter procedure requires further and ungainly manipulation to add an extra carbon to the side chain of the methylenecyclopropane intermediate. The enantiomeric purity of the key intermeidates **5** and **6**, as revealed by ^{1}H-NMR analysis in the

SCHEME I

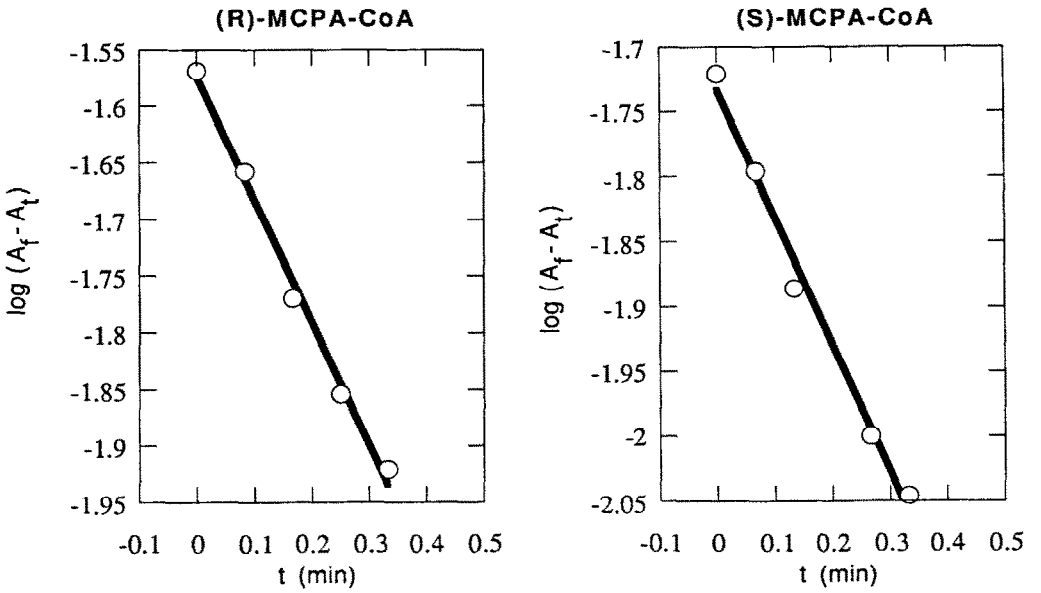


i, AcCl, MeOH, room temp., 12 h (100%); ii, BH_3, Me_2S, $NaBH_4$, THF, r.t., 1 h (83%);[11] iii, TsOH, Me_2CO, benzene, molecular sieves 4 Å, reflux, 12 h (85%); iv, LAH, ether, r.t., 1 h (86%); v, $PhCH_2Br$, NaH, THF, r.t., 12 h (72%); vi, TsOH, MeOH, r.t., 30 min (93%); vii, Ph_3P, diethyl azodicarboxylate (DEAD), benzene, reflux, 12 h (63%);[12] viii, $Bu^t(Ph)_2SiCl$, DMAP, CH_2Cl_2, r.t., 3 h (97%); ix, CH_3SO_2Cl, Et_3N, CH_2Cl_2, r.t., 12 h (98%); x, $(Bu^n)_4NF$, THF, r.t., 2 h (56%);[13] xi, $PhSO_2(CH_2)_2TMS$, Bu^nLi, ether, -78 °C to r.t., 12 h (92%); xii, CH_3SO_2Cl, Et_3N, CH_2Cl_2, r.t., 12 h (95%); xiii, $LiN(Pr^i)_2$ (LDA), THF, -78 °C to r.t., 12 h (70%); xiv, BF_3 etherate, EtSH, CH_2Cl_2 (82%); xv, $(Bu^n)_4NF$, THF; r.t., 30 min (92%); xvi, Jones oxidation, r.t., 30 min (76%); xvii, $ClCO_2Bu^i$, Et_3N, CH_2Cl_2, r.t., 1 h; xviii, NaSCoA, THF-H_2O (pH 8), 10 min (38%, 2 steps).

presence of lanthanide shifting $Eu(hfc)_3$, was found to be greater than 99%. Since compound **10** gave a nearly identical optical rotation to that of (1*R*)-MCPA prepared previously by chemical resolution,[5] the key cyclopropanation step is clearly stereospecific and proceeds with inversion of configuration.

The effect of each of the MCPA-CoA isomer on the catalytic activity of general acyl-CoA dehydrogenase (GAD) was analyzed by the incubation of 87.4 μM of each isomer with 4.03 μM of GAD (20:1, inhibitor to enzyme ratio) in a total volume of 1 mL of potassium phosphate buffer (pH 7.6) at room temperature. As anticipated, time dependent inactivation occurred with concomitant bleaching of the active-site FAD.[2] The inhibition was monitored spectrophotometrically at 446 nm, the changes of which directly reflects the extent of

enzyme inactivation. While A_t represents the absorbance at a given time during the incubation, the absorbance at the end of the inactivation is designated A_f. As depicted in the above figures, a semi-logarithmic plot of the changes of the absorbance at 446 nm (A_f-A_t) under aerobic conditions versus the incubation time gave the k_{inact} of 1.73 x 10^{-2} and 1.63 x 10^{-2} sec^{-1} for the (1*R*)- and (1*S*)-MCPA-CoA, respectively.[14] Previous results revealed that the inactivation caused by the 1*R* isomer was 1.57 times faster than that observed for the 1*S* isomer when the experiment was performed using a ratio of 7:1 of inhibitor to enzyme.[5c] The rate difference found under these conditions may be ascribed to the distinction of binding of the two epimers to GAD. The present results may imply that the association of the (1*S*)-MCPA-CoA-GAD complex is weaker than that of the other isomer, therefore, the system must be fully saturated with the inhibitor in order to achieve similar rate of inactivation. Furthermore, a 1.3 kinetic isotope effect had been observed for the α-proton abstraction from the 1*S* isomer, whereas the naturally occurring 1*R* isomer did not display this characteristic.[5c,15] Since α-proton abstraction has been shown to be partially rate limiting in the acyl-CoA catalysis, this reaction could be very sensitive to the steric environment around the C_α of the inhibitor in the active site. Thus, the kinetic isotope effect also suggests a less than ideal binding of the 1*S* isomer and could additionally be a minor component contributing to the rate difference.



In light of the fact that the partition ratios of the inactivation of GAD caused by (1*R*)- and (1*S*)-MCPA-CoA are 3.4 and 4.0, respectively,[5b] the results of nearly identical inactivation rate constants found for these two isomers under saturation conditions further support our early notion that inactivation by either (1*R*)- or (1*S*)-MCPA-CoA follows the same chemical course. Such a lack of stereospecificity of bond rupture at β-C of MCPA-CoA in the active-site of GAD strongly suggests that the ring-opening step leading to inactivation is likely to be a spontaneous event, induced by an α-cyclopropyl radical. Since the rearrangement of an α-cyclopropyl radical to the corresponding ring-opened acyclic radical is an extremely facile process, the ring cleavage may elude the chiral

discrimination imposed by the enzyme. The intermediacy of such an α-cyclopropyl radical was recently substantiated by the isolation of the major turnover product, a CoA ester consisting of a disubstituted terminal olefin, an epoxide moiety, and a hydroxymethyl group from the incubation mixture of MCPA-CoA and GAD.[16] Formation of this product may be initiated by trapping the acyclic radical intermediate with O_2 to form a transient peroxy radical which, upon receiving one electron from flavin semiquinone followed by an intramolecular epoxidation, gives rise to the observed turnover product. Thus, inactivation of GAD by MCPA-CoA, the causative agent of Jamaican vomiting sickness, is likely to proceed through a radical mechanism.

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