Studies of Acyl-CoA Dehydrogenase Catalyzed Allylic Isomerization: A One-Base or Two-Base Mechanism?

Srikanth Dakoji, Injae Shin, Donald F. Becker, Marian T. Stankovich, and Hung-wen Liu*

Contribution from the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455

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Abstract: Acyl-CoA dehydrogenases are flavoproteins that catalyze the conversion of a fatty acyl thioester substrate to the corresponding α,β-enoyl-CoA product. It has been well established that a glutamate residue in the active site [e.g., E367 in short-chain acyl-CoA dehydrogenase (SCAD) of *Megasphaera elsdenii*] is responsible for the initial α-proton abstraction. Early studies have also shown that this class of enzymes is capable of catalyzing γ-H abstraction to afford the allylic isomerization between α,β- and β,γ-enone thioesters and/or inactivation by 2- or 3-acetylenic acyl-CoA derivatives. Although a *dual* role has been proposed for the glutamate residue in both α- and γ-deprotonation, the existence of a second active-site basic group to mediate the observed reactions occurring at γ-C remains a feasible mechanism. In an attempt to discern between these two possibilities, we have prepared a few oxirane-containing acyl-CoA derivatives aimed at trapping active-site bases in the vicinity of the α- and/or γ-C. It was found that 2,3-epoxybutyryl-CoA is a new class-selective irreversible inactivator against SCAD; however, the inability of other oxirane-containing probes to react with these enzymes prompted us to tackle this mechanistic problem by directly studying the role of Glu-367 in SCAD-catalyzed 1,3-isomerization. The effect of E367Q mutation on the proficiency of SCAD to mediate the γ-H exchange of crotonoyl-CoA was examined. The capabilities of the wild-type SCAD and its E367Q mutant to catalyze the γ-H abstraction during the inactivation by 2-butynoyl-CoA was also compared. The fact that the mutant protein fails to promote γ-H exchange/abstraction provides strong evidence supporting a one-base mechanism of this enzyme-catalyzed allylic isomerization. Since the catalysis of acyl-CoA dehydrogenases is closely related, such a one-base mechanism is expected to be conserved within this family of enzymes.

Acyl-CoA dehydrogenases are flavoproteins that catalyze the first step of β-oxidation by converting a fatty acyl thioester substrate to the corresponding α,β-enoyl-CoA product.[1] Based on the preferred chain length of the substrates, this class of enzymes can be further divided into four subgroups: very long-chain (VLCAD), long-chain (LCAD), medium-chain (MCAD), and short-chain (SCAD) acyl-CoA dehydrogenase.[2] It has been well established that reactions catalyzed by these enzymes proceed via a carbanion mechanism, in which a glutamate residue (e.g., E367 in SCAD of *Megasphaera elsdenii* and E376 in pig liver MCAD) has been identified as the active-site base responsible for the initial α-proton abstraction.[3]

Early studies have shown that this class of enzymes is also capable of catalyzing allylic isomerization between α,β- and β,γ-unsaturated thioesters.[4] The best known example is the interconversion between vinylacetylpantetheine and crotonoylpantetheine mediated by SCAD from *M. elsdenii* (Figure 1A).[4a] The inactivation of MCAD by 2-octynoyl-CoA, which involves an initial rate-limiting γ-proton abstraction to afford a reactive allene intermediate, is another well-documented case (Figure 1B).[5a] A similar isomerization has also been established as the key step in the inactivation of these enzymes by a 3-acetylenic thioester (Figure 1C).[4a] A more recent example is the inactivation of pig kidney MCAD by 3-methyleneoctanoyl-CoA and 3-methyl-*trans*-2-octenoyl-CoA in which a common anion derived from an allylic isomerization was proposed as the reactive species responsible for flavin modification (Figure 1D).[4b] In all these cases, abstraction of a γ-proton (Figure 1A, B, and D)[4−6] or protonation at the γ-C (Figure 1C)[4a] is a prerequisite for the observed transformation. While it is conceivable that the α- and γ-deprotonations are mediated by two different active-site basic groups, it is equally possible that these two independent events are catalyzed by a single active-site base.

The role of a glutamate residue in α-deprotonation catalyzed by acyl-CoA dehydrogenases has been well defined.[3] When the bidentate nature of a carboxylate group is considered, the involvement of a single glutamate to interact with both α- and

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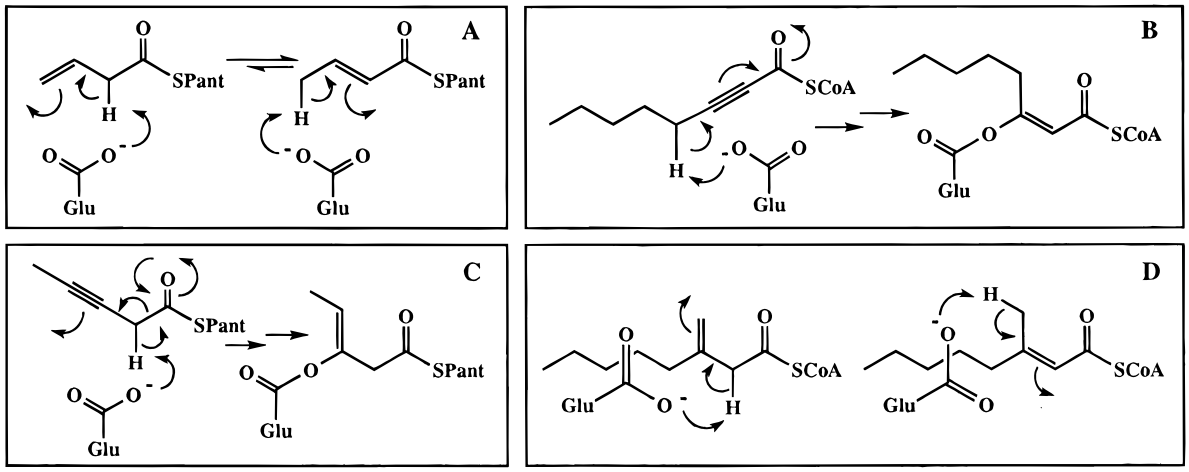


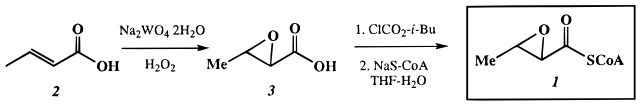
Figure 1. (A) SCAD catalyzed isomerization of (3-butenoyl)pantetheine to (2-butenoyl)pantetheine.[4a] (B) Inactivation of MCAD by 2-octynoyl-CoA.[5a,b] (C) Inactivation of SCAD by (3-pentynoyl)pantetheine;[4a,5c,6b] a β,γ-unconjugated structure was proposed for the inhibitor−enzyme adduct.[4] (D) Inactivation of MCAD by 3-methyleneoctanoyl-CoA and 3-methyl-*trans*-2-octenoyl-CoA.[4b] Pant: pantetheine.

γ-positions during allylic isomerization becomes a particularly plausible hypothesis. However, the issue of whether a two-base or a one-base mechanism is operative in this class of enzymes has never been thoroughly investigated. For example, a glutamate residue was shown to be the target of covalent modification in an earlier study on the inactivation of *M. elsdenii* SCAD by 3-pentynoylpantetheine.[4a] Although this residue is very likely the same catalytic base that abstracts the α-proton, its proposed role in the γ-protonation to furnish the allene intermediate still remains to be confirmed. By using 2-acetylenic acyl-CoA derivatives as mechanism-based inhibitors for pig liver SCAD and pig kidney MCAD, Thorpe and co-workers recently discovered that the residue being modified in each case is the glutamate responsible for α-proton abstraction.[5] The fact that the same glutamate residue is trapped by different mechanism-based inhibitors is intriguing. These results, however, only demonstrate that the catalytically essential glutamate of these dehydrogenases is highly reactive and is in close proximity to the electrophilic center of the allenic intermediate but provide little insight into the issue of the two-base/one-base mechanism.

Interestingly, a mechanistically related enzyme, glutaryl-CoA dehydrogenase, has been shown to catalyze an α,γ-proton shift without substantial exchange with solvent hydrogens.[6b] This result nicely illustrates the involvement of a single monoprotic base, the identity of which is unknown, in deprotonation/reprotonation at both α- and γ-C of glutaryl-CoA. It should be noted that glutaryl-CoA dehydrogenase differs from other flavin-dependent dehydrogenases in that its catalysis involves an additional decarboxylation step. Furthermore, unlike *M. elsdenii* SCAD, which promotes the exchange of both α- and β-hydrogens of the substrate in the absence of an electron acceptor,[7] glutaryl-CoA dehydrogenase catalyzes the exchange only at β-C under similar conditions.[6b] Hence, the conclusion drawn from studies of glutaryl-CoA dehydrogenase may not be applicable to other acyl-CoA dehydrogenases. Clearly, evidence from currently available data cannot conclusively support a one-base mechanism for acyl-CoA dehydrogenases.

In an attempt to discern between these two possibilities (i.e., one-base vs two-base mechanism), we have synthesized a few oxirane-containing acyl-CoA derivatives and have determined their effects on the wild-type SCAD/MCAD and the mutant SCAD (E367Q) whose active-site glutamate (Glu-367) has been replaced by a glutamine.[3f] The design of these acyl-CoA derivatives (**1**, **9**, and **16**) as affinity alkylating agents was based on the inveterate tendency of their oxiranyl moiety to label active-site nucleophiles.[8] In a separate set of experiments, the role of Glu-367 of SCAD in the catalysis of 1,3-isomerization was investigated by comparing the competence of the wild-type and the E367Q mutant enzyme to exchange the γ-proton of crotonoyl-CoA. Furthermore, the consequence of the E367Q mutation of SCAD on its capability of catalyzing allylic isomerization was also examined by incubation of the wild-type and mutant enzymes in turn with 2-butynoyl-CoA—a mechanism-based inhibitor whose mode of action is initiated by a γ-proton abstraction. Reported herein are the results and the mechanistic implications of these studies.

Scheme 1



Results and Discussion

Effect of an α,β-Oxirane-Containing Acyl-CoA Derivative (1) on SCAD and MCAD. Compound **1** was prepared from crotonoic acid (**2**) in three steps as shown in Scheme 1. Epoxidation of **2** to give **3** was achieved by treatment with tungstate/H_2O_2.[9] Condensation of **3** with isobutyl chloroformate followed by coupling with coenzyme A in aqueous THF solution (pH 8−8.5)[10] gave the desired product **1**. The crude product was purified on a HPLC Partisil-C_{18} column using 30% methanol in 50 mM potassium phosphate buffer, pH 5.3, as the eluant.[10b,11] The isolated yield of the last coupling step was 45%.

When compound **1** was incubated with SCAD from *M. elsdenii*,[3f] time-dependent inactivation occurred exhibiting a k_{inact} of ca. 0.03 min^{-1}. Failure to regenerate any catalytic activity

(7) The α-H exchange is due to the reversible deprotonation−reprotonation mediated by E367. However, the exchange of β-H is a result of reversible hydride transfer between the substrate anion and the flavin coenzyme, and it is the reduced flavin that undergoes proton exchange.

(8) A few examples include: (a) Shokat, K.; Uno, T.; Schultz, P. G. *J. Am. Chem. Soc.* **1994**, *116*, 2261. (b) Willson, M.; Lauth, N.; Perie, J.; Callens, M.; Opperdoes, F. R. *Biochemistry* **1994**, *33*, 214. (c) Kim, D. H.; Kim, K. B. *J. Am. Chem. Soc.* **1991**, *113*, 3200. (d) Mühlbacher, M.; Poulter, C. D. *Biochemistry* **1988**, *27*, 7315. (e) Pollack, R. M.; Bantia, S.; Bounds, P. L.; Koffman, B. M. *Biochemistry* **1986**, *25*, 1905. (f) Kiorpes, T. C.; Hoerr, D.; Ho, W.; Weaner, L. E.; Inman, M. G.; Tutwiler, G. F. *J. Biol. Chem.* **1984**, *259*, 9750.

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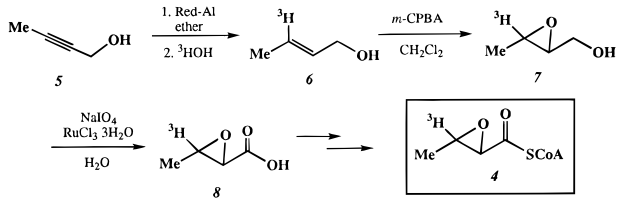
(11) Eberhard, A. *Chromatogram* **1987**, *8*, 10.

Scheme 2



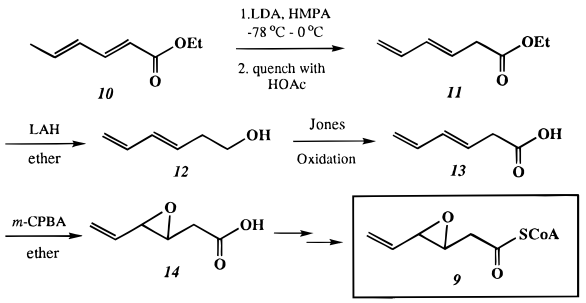
after exhaustive dialysis (2 days in 100 mM potassium phosphate buffer, pH 7.5) and the protection of SCAD from inhibition by acetoacetyl-CoA, a well-known tight binding inhibitor for this enzyme,[12] clearly indicated that the inactivation is irreversible and is active-site directed. Since the flavin chromophore remains unchanged throughout the incubation, loss of activity must involve the modification of the SCAD protein itself. Interestingly, compound **1** is only a competitive inhibitor for the pig kidney MCAD.[13] Such a distinct outcome upon treatment of these two closely related enzymes with a single compound was also noted in an earlier report in which cyclobutanecarbonyl-CoA was found to be a suicide inhibitor for SCAD, but a substrate for MCAD.[14]

To confirm the inactivation observed with SCAD, tritium-labeled inhibitor **4** was also prepared. As depicted in Scheme 2, isotopic labeling was introduced by quenching the Red-Al reduction of 2-butynol (**5**) with [^{3}H]H_2O (100 mCi/mL).[15] The resulting crotyl alcohol (**6**) was converted, via **7**, to 2,3-epoxybutanoic acid (**8**) by treatment with *m*-chloroperbenzoic acid (*m*-CPBA) followed by sodium periodate/ruthenium chloride oxidation.[16] The final steps were analogous to those shown in Scheme 1. The labeled compound **4** was isolated in 24% overall yield with a specific activity of 0.16 mCi/mmol.

As anticipated, when compound **4** (1 molar equiv) was incubated with SCAD from *M. elsdenii* (30 μmol) and quenched with charcoal (10% solution),[17] little tritium release (<7%) was detected in the supernatant after centrifugation. A 1.3:1 stoichiometry between **4** and the modified SCAD was determined after extensive dialysis of the inactivated enzyme (32 molar equiv of **4** was used in this case). When the resulting inactivated SCAD was treated with 90% methanol to denature the protein, less than 1% of radioactivity was detected in the supernatant. These results are all consistent with a pathway in which oxirane **1** irreversibly inactivates SCAD via covalent modification of an active-site nucleophile. The inactivated enzyme was heat-denatured, washed with 0.1 M Tris buffer (pH 7.5), and redissolved in a basic buffer (0.1 M Tris, 6 M guanidine hydrochloride, pH 9.25).[5a] After 12 h of incubation, the solution was treated with 10% charcoal and centrifuged, and the supernatant was submitted to scintillation counting. Interestingly, almost all of the radioactivity was released under these conditions. This finding suggested that the inactivator is bound to SCAD via an ester linkage to an enzyme carboxylate group.

In a separate experiment, the E367Q mutant of SCAD was prepared that exhibited nearly identical physical properties as the wild-type enzyme, albeit presenting virtually no catalytic activity.[3f] Since incubation of **4** with this mutant SCAD followed by dialysis resulted in no tritium labeling, the identity of the reactive nucleophile could thus be assigned as Glu-367. It is also evident that the α,β-oxiranyl moiety is an effective trap for the active-site base responsible for the initial α-deprotonation.

Scheme 3



Effects of a β,γ- and a γ,δ-Oxirane-Containing Acyl-CoA Derivative (9 and 15) on SCAD and MCAD. If the same glutamate (e.g., Glu-367 of *M. elsdenii* SCAD) is capable of interacting with both α- and γ-positions during allylic isomerization, one would anticipate that this glutamate (Glu-367) may also be trapped by a β,γ-oxiranyl-containing thioester such as **9**. Synthesis of compound **9** from ethyl sorbate (**10**) was effected by the reactions delineated in Scheme 3. Deprotonation using a 1:1 mixture of lithium diisopropylamide (LDA) and hexamethylphosphoramide (HMPA) followed by kinetic protonation afforded the unconjugated ester **11**.[18] Subsequent hydride reduction, Jones oxidation, and epoxidation were all straightforward steps providing **14** as the end product. The CoA thioester of **14** was prepared by the same mixed anhydride method described above to give the final product **9**.

Upon incubation with SCAD from *M. elsdenii*, compound **9** was disappointingly found to be only a competitive inhibitor for this enzyme. A similar competitive inhibitory effect was also observed when the pig kidney MCAD was treated with **9**. The inability of **9** to react with the catalytically active glutamate in both enzymes raised a concern about the hypothesized single-base mechanism. Since the amide group of Glu-367 of SCAD in an enzyme−substrate complex is expected to be hydrogen bonded to the carbonyl oxygen of the acyl thioester, which is also hydrogen bonded to the ribityl 2′-OH of the FAD,[19] such structural constraints may have impeded Glu-367 from reaching the C_γ position of the substrate/inhibitor and thus precluded it from reacting with compound **9**. The same argument may also be applied to MCAD, which exhibits similar structural features as SCAD.[3d] However, unlike the α-C of compound **1** that is activated by the neighboring carbonyl group, the γ-C of **9** bearing only an isolated vinyl substituent is less susceptible to nucleophilic attack. Hence, the incompetence of **9** to trap the catalytically active glutamate or other nearby nucleophiles may simply be attributed to the weak electrophilic nature of its oxirane moiety. As a result, the γ,δ-oxiranyl-containing pentenoyl-CoA (**16**) which has a more reactive γ-C emerged as a more appealing active-site nucleophile probe.

The precursor of **16** was readily prepared from 4-pentenoic acid (**17**) as delineated in Scheme 4. Epoxidation of **17** with *m*-CPBA and subsequent coupling of the resulting epoxy acid **18** with coenzyme A led to the product **15** in 72% yield. The γ,δ-epoxide itself (**15**) is not reactive enough to trap the active-

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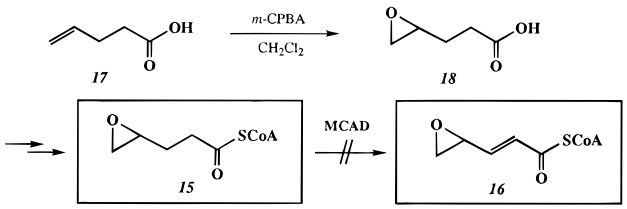
(19) The substrate recognition residues in the active-site of *M. elsdenii* SCAD were identified by crystal structural analysis of the enzyme-acetoacetyl-CoA complex (Djordjevic, S.; Pace, C. P.; Stankovich, M. T.; Kim, J. J. P. *Biochemistry* **1995**, *34*, 2163).

Scheme 4



site nucleophile directly; however, it is expected to be a substrate for acyl-CoA dehydrogenase. As shown in Scheme 4, after enzyme-catalyzed α,β-desaturation, the product **16** is likely to be an effective trap and may react with the active-site base near the γ-C of the substrate/inhibitor.

Interestingly, incubation with the *M. elsdenii* SCAD and the pig kidney MCAD showed that compound **15** is not a substrate but only a competitive inhibitor for both enzymes. For example, the rate of inactivation of MCAD by (methylenecyclopropyl)-acetyl-CoA (MCPA-CoA), a well-known mechanism-based inhibitor for acyl-CoA dehydrogenases,[17,20] was reduced to 64% in the presence of 45 molar equiv of **15**. It should also be noted that no turnover products could be detected from the incubation of **15** (7.3 mM) with MCAD (175 μM) in deuterated potassium phosphate buffer (pD 7.2) at room temperature for 4 h. More importantly, the recovered **15** retained all of its α-hydrogens. The lack of α-H exchange in the retrieved starting material and the absence of any new products in the incubation mixture clearly indicated that compound **15** was not processed by MCAD. Analogous results were also found when SCAD was incubated with compound **9** and/or **15**. It is worth mentioning that the absorption of the flavin chromophore of acyl-CoA dehydrogenases, upon binding with lipophilic acyl-CoA derivatives, is generally well resolved. This change typically includes the appearance of a characteristic vibronic band as a shoulder at 480 nm.[6a,21] It has been suggested that the resolution of the vibrational features of the flavin chromophore results from part of the isoalloxazine ring lying in an environment less conducive to H-bonding.[21c] Interestingly, no such change was observed for MCAD and/or SCAD complexed with either compound **9** and/or **15**. These results indicated that these oxirane derivatives may bind in the active site differently as compared to the normal substrate. Thus, the inability of the active-site glutamate to react with **9** and **15** is likely a result of nonproductive binding and, thus, provides no insight into the one-base and/or two-base mechanism.

Effect of E367Q Mutation on SCAD Catalyzed γ-H Exchange of Crotonoyl-CoA. Since our attempts to distinguish between the one-base and two-base mechanisms by using CoA derivatives bearing oxiranyl moiety as probes to trap the corresponding active-site nucleophiles led to ambiguous results, we decided to take a more direct approach to investigate the role of the active-site glutamate (Glu-367) in SCAD-catalyzed 1,3-isomerization. Our experiment is based on an early observation that incubation of crotonoyl-CoA with SCAD in deuterated buffer without added electron acceptors (such as electron transfer flavoprotein, or artificial electron acceptors) resulted in deuterium incorporation at the γ-C of the recovered crotonoyl-CoA.[4a] If the one-base mechanism is operative, the same active-site base (Glu-367) responsible for α-H abstraction should also be the designated residue catalyzing the observed γ-H exchange. Thus, to address the possible dual role played by Glu-367, the effect of E367Q mutation on the capability of SCAD to mediate the γ-H exchange of crotonoyl-CoA was examined. As expected, incubation of crotonoyl-CoA with the *M. elsdenii* SCAD led to the complete exchange of its γ-H's in deuterated solvent (Figure 2, spectrum B). However, the γ-CH_3 (δ 1.70, d, J = 7.0) of crotonoyl-CoA remained intact when the SCAD(E367Q) mutant protein was used in the incubation (Figure 2, spectrum C). These data strongly support a single-base mechanism.

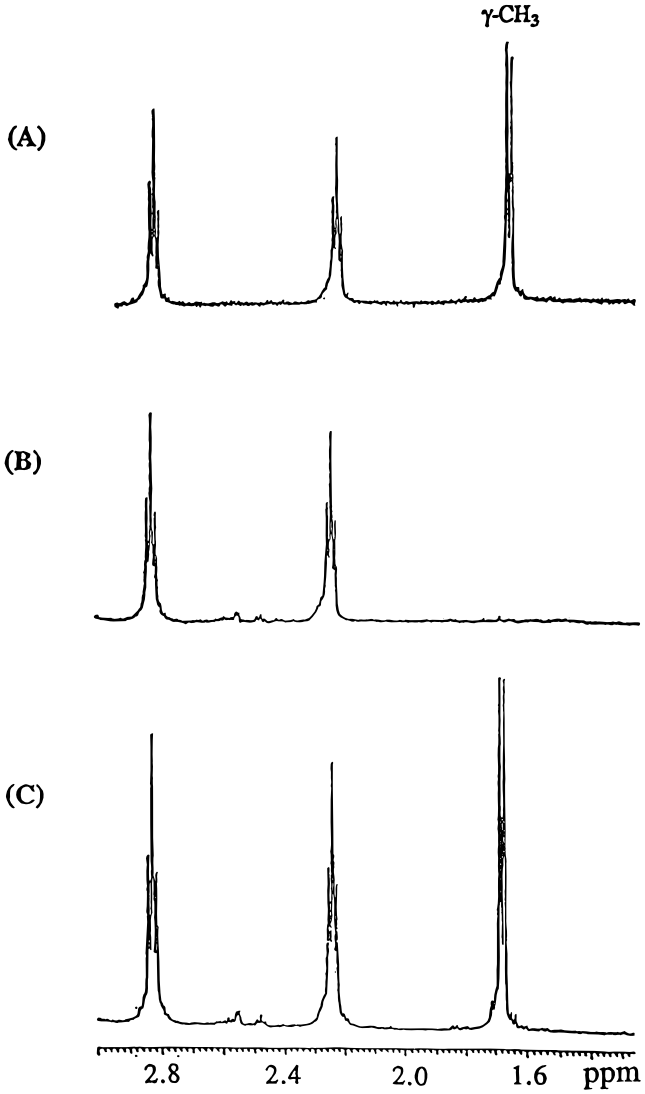


Figure 2. ^{1}H NMR spectra (500 MHz) showing the δ 2.9−1.6 region of crotonoyl-CoA in 50 mM potassium phosphate buffer in ^2H_2O (pD 7.2): (A) control (no enzyme), (B) incubated with SCAD, (C) incubated with SCAD(E367Q) mutant protein. The doublet at δ 1.68 is the resonance of the γ-Me of crotonoyl-CoA. The peaks at δ 2.25 and 2.82 are the resonances of the methylene hydrogens of the CoA moiety.

Effects of 2-Butynoyl-CoA (19) on SCAD and Its E367Q Mutant Protein. In order to gain more evidence to further substantiate the above results, we have also studied the catalytic role of the active-site glutamate during the incubation with 2-alkynoyl-CoA derivatives that are well-known mechanism-based inhibitors for both pig kidney MCAD[5a,b] and pig liver SCAD.[5c] As mentioned earlier, the inactivation is initiated by

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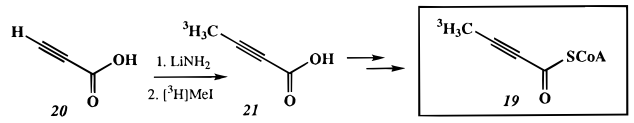
Scheme 5



a rate-limiting γ-deprotonation step to generate a reactive allene intermediate, which eventually traps the active-site glutamate rendering these enzymes inactive. The glutamate being modified in each case is the one responsible for α-H abstraction; however, whether it is also involved in γ-H removal has never been fully established. With the SCAD(E367Q) mutant protein at hand, we can now verify the hypothesized role of Glu-367 of SCAD in both α- and γ-H abstraction. Since the initial γ-H abstraction is a prerequisite step for enzyme inactivation, the synthesis of [4-^{3}H]-2-alkynoyl-CoA was deemed necessary for this experiment. While 2-pentynoyl-CoA was used in the early work to inactivate pig liver SCAD,[5c] the minimal homologue of this class of inhibitors, 2-butynoyl-CoA, was chosen as the probe due to the ready availability of the appropriate labeling reagents.

As shown in Scheme 5, synthesis of [4-^{3}H]-2-butynoyl-CoA (**19**) was accomplished in two steps. The alkylation of the dianion of propiolic acid (**20**) with [^{3}H]CH_3I gave [4-^{3}H]-2-butynoic acid (**21**), which was then coupled with coenzyme A. The final product was purified by HPLC and coeluted as a single peak when mixed with the nonlabeled standard. The specific activity of the radiolabeled material was 1.51 mCi/mmol. As anticipated, butynoyl-CoA is an irreversible inhibitor for *M. elsdenii* SCAD with an observed rate (k_{obs}) of 2.5 s^{-1} in the presence of 55 μM inhibitor. Because the flavin chromophore remained unchanged, the inactivation must result from modification of Glu-367 as determined in the early work using pentynoyl-CoA.[5c] A stoichiometry of 1.2:1 of the inactivator per SCAD monomer was deduced from the incubation with excess **19** followed by extensive dialysis. When the same experiment was repeated with the SCAD(E367Q) mutant enzyme, less than 1% of the total radioactivity was found in the protein. These results lent further evidence indicating that the residue being modified in the active site of SCAD is indeed Glu-367. Interestingly, when **19** was incubated with wild-type SCAD, approximately 41% of the tritium was exchanged with solvent hydrogens. In contrast, only 0.5% of the total radioactivity was released upon incubating with the SCAD(E367Q) mutant enzyme. The fact that the mutant protein fails to promote γ-H exchange of 2-butynoyl-CoA strongly attests that Glu-367 is also responsible for the γ-H abstraction and thus supports a one-base mechanism of this enzyme-catalyzed allylic isomerization. Since the catalysis of acyl-CoA dehydrogenases is closely related, such a one-base mechanism is thus expected to be conserved within this family of enzymes.

Characterization of the Covalent Adduct between SCAD and 2-Butynoyl-CoA. In order to study the nature of the covalent adduct between SCAD and 2-butynoyl-CoA, the 3,4-$^{13}C_2$-labeled inactivator **22** was also prepared (Scheme 6). Coupling of carbon tetrabromide with [1,2-$^{13}C_2$]acetaldehyde (**23**) in the presence of zinc and triphenylphosphine[22] gave [2,3-$^{13}C_2$]-1,1-dibromo-1-propene (**24**). Reaction of **24** with 2.0 equiv of *n*-BuLi, followed by quenching with anhydrous CO_2-(g), and acid workup afforded [3,4-$^{13}C_2$]-2-butynoic acid (**25**), which was later coupled with coenzyme A. The crude product was purified by HPLC to give **22** in 33% overall yield. Two equivalents of **22** was incubated with SCAD from *M. elsdenii* (0.33 μmol) in ^2H_2O at room temperature for 1 h, and the

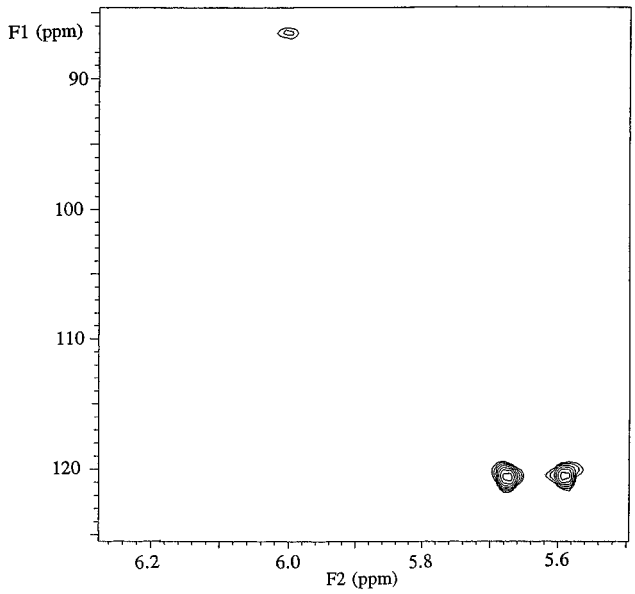
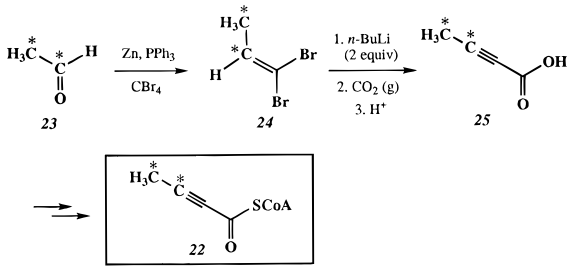


Figure 3. $^{13}C-^1H$-Detected HMQC spectrum (500 MHz, ^2H_2O) showing the resonances of the covalent adduct between SCAD and [3,4-$^{13}C_2$]-2-butynoyl-CoA (**22**). Number of transients = 80; temperature = 25 °C; accumulation time = 12 h.

Scheme 6



incubation mixture was then subjected to the HMQC (heteronuclear multiple quantum coherence) experiment.[23] As illustrated in Figure 3, the HMQC spectrum showed one pertinent carbon signal at δ 120.7, which also displays cross peaks at δ 5.68 and 5.58 in the ^{1}H-dimension. The chemical shift of this methylene carbon falls within the range as that of the terminal olefinic carbon of compound **29**, a byproduct isolated from the mixed anhydride coupling reaction of [3,4-$^{13}C_2$]-2-butynoic acid (**25**) with CoA. Repeating the HMQC experiment with reduced spectral width revealed that this carbon signal is a doublet with a characteristic J_{C4-C3} coupling constant of 77 Hz. Due to the scarcity of the labeled sample, attempts to detect the β-C signal, whose chemical shift would be more informative for structural assignment, were futile. However, the absence of the resonance of an olefinic methine moiety in the HMQC spectrum is indicative of a β-substituted skeleton for the adduct. Furthermore, many examples of enzyme inactivation due to the formation of an allene intermediate during catalysis are known,[24] and the structures of the adduct in a few cases, such as the inactivation of β-hydroxydecanoyl thioester dehydrase by 3-decynoyl-*N*-acetylcysteamine[25] and the 3-oxo-Δ^5-steroid isomerase

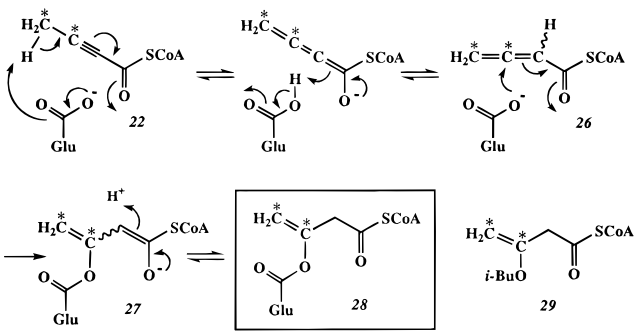
(22) Corey, E. J.; Fuchs, P. L. *Tetrahedron Lett.* **1972**, *36*, 3769.

(23) For a good review of HMQC experiment, see: Martin, G. E.; Zektzer, A. S. *Two-Dimensional NMR Methods for Establishing Molecular Connectivity*; VCH Publishers: New York, 1988.

(24) For general references, see: Silverman, R. B. *Mechanism-based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press: Boca Raton, FL, 1988; Vols. I and II.

(25) (a) Endo, K.; Helmkamp, G. M., Jr.; Block, K. *J. Biol. Chem.* **1970**, *245*, 4293. (b) Schwab, J. M.; Ho, C.-K.; Li, W.-B.; Townsend, C. A.; Salituro, G. M. *J. Am. Chem. Soc.* **1986**, *106*, 5309.

Scheme 7



by acetylenic secosteroid substrate analogues,[26] have been determined. It was found that the inactivator moiety is always linked to the center carbon of the allene intermediate. Our data are no exception, since it is most consistent with a structure of β-oxygenated β,γ-unsaturated butenoyl derivative (**28**). Compound **28** must arise from the attack of Glu-367 at the more electrophilic β-C of the allene intermediate (**26**, Scheme 7). A similar β,γ-unsaturated structure had been proposed for the adduct formed during the inactivation of the *M. elsdenii* SCAD by 3-pentynoylpantetheine (Figure 1C).[4a] Interestingly, the covalent adduct, 3-(N^{im}-histidinyl)-3-decenoyl thioester, generated in the inactivation of β-hydroxydecanoyl thioester dehydrase by 3-decynoyl-*N*-acetylcysteamine, is also a β,γ-unsaturated species.[25b] It was suggested that the 1,2-double bond of the dienolate intermediate (such as **27** in Scheme 7) is orthogonal to the 3,4-double bond, and therefore the C-4 protonation is disfavored.[25b]

Conclusion

A comparison of the capabilities of the wild-type and mutant acyl-CoA dehydrogenases to catalyze the 1,3-isomerization between α,β- and β,γ-enoyl-CoA substrate/inhibitor has provided compelling evidence supporting the theory that a single glutamate residue is responsible for the observed deprotonation/reprotonation at both α- and γ-Cs. While the C_α-C_β bond of the acyl-CoA substrate is juxtaposed between the isoalloxazine ring of FAD and the carboxylate of Glu-367 in SCAD,[19] the alignment of Glu-367 is apparently flexible. The possible flexibility of the carboxylate group combined with its bidentate nature may allow Glu-367 to interact with both the α- and γ-positions.[27] It should be noted that the competence of a single active-site base to effect a 1,3-proton shift has been inferred for a number of enzymes.[28] This glutamate residue in SCAD can be labeled by an oxiranyl-containing acyl-CoA derivative **1**. A relevant precedent is the inactivation of 3-oxo-Δ^5-steroid isomerase by oxiranyl steroid substrates in which Asp-38 was identified as the nucleophile being labeled.[29] Interestingly, extensive studies have firmly established Asp-38 as the active-site general base responsible for 1,3-hydrogen transfer in 3-oxo-Δ^5-steroid isomerase.[30] While compound **1** is an irreversible inactivator for SCAD, it is only a competitive inhibitor for MCAD. Thus, oxirane **1** is a new class-selective inhibitor for acyl-CoA dehydrogenases. The selectivity exhibited by **1** on inhibition against two closely related dehydrogenases (MCAD and SCAD) is reminiscent of that found for cyclobutanecarbonyl-CoA.[14] These results, in conjunction with the known three-dimensional structures of MCAD[3d] and SCAD,[19] may allow us to better define the specific substrate/inhibitor binding characteristics of these enzymes. Such information is important for the design of novel class-selective inhibitors to control and/or regulate fatty acid metabolism.

Experimental Section

General. GC−MS analysis was performed on an HP 5890A gas−liquid chromatograph and a VG 7070E-HF spectrometer. Ultraviolet−visible spectroscopy was recorded on a Shimadzu UV-160 or a Hewlett-Packard 8452A spectrophotometer. Radioactivity was measured by liquid scintillation counting on a Beckmann J2100 counter using Ecoscint A biodegradable scintillation solution from National Diagnostics (Manville, NJ). High-performance liquid chromatography analysis and/or purification were conducted with either a Hewlett-Packard 1090A instrument equipped with an HP3392 integrator or a Beckman 110B instrument. ^{1}H NMR and ^{13}C NMR spectra were recorded on an IBM NR/200 or NR/300 or a Varian U-300 or U-500 spectrometer. Chemical shifts are reported on the δ scale relative to internal standard (tetramethylsilane or appropriate solvent peaks) with coupling constants given in hertz. NMR assignments labeled with an asterisk (*) may be interchangeable. Flash column chromatography was performed on columns of various diameters with J. T. Baker (230−400 mesh) silica gel by elution with the solvents reported. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 G-254 plates (25 mm) and developed with the solvents mentioned. TLC spots were visualized either with UV light or by dipping into the staining solutions of $KMnO_4$ (1%) or vanillin/ethanol/ H_2SO_4 (1:98:1) or phosphomolybdic acid (7% EtOH solution) and then heating. The drying reagent used in the routine workup was anhydrous magnesium sulfate. Solvents, unless otherwise specified, were of analytical reagent grade or the highest quality commercially available. For anhydrous reactions, the solvents were pretreated prior to distillation as follows: tetrahydrofuran was dried over sodium and benzophenone; methylene chloride, dimethyl sulfoxide, and dimethylformamide were dried over calcium hydride; pyridine and triethylamine were dried over KOH. It should be noted that the tritium-containing compounds prepared in this work were not submitted to NMR analysis or exact mass measurements due to the possibility of radioactive contamination. However, satisfactory analytical results were obtained with the corresponding unlabeled or deuterium labeled analogues.

Enzymes. The short-chain acyl-CoA dehydrogenase (SCAD) was purified from an *Escherichia coli* strain BL21(DE3)pLys, which carries and overexpresses the *Megasphaera elsdenii* SCAD gene ($pWTSCADT_7$-7). The expression and purification of this protein has been described.[30] The medium-chain acyl-CoA dehydrogenase (MCAD), from pig kidneys, was purified according to the procedure of Thorpe.[13] The overall yield of a typical purification was 200−300 nmol of purified MCAD/kg of kidney cortex. The chromatographic, electrophoretic, and spectral properties of these purified proteins were identical with those cited in the literature. Concentration of the holoenzyme was determined spectrophotometrically based on a molar absorptivity of 13.9 mM^{-1} cm^{-1} at 446 nm for oxidized SCAD and 15.4 mM^{-1} cm^{-1} at 446 nm for oxidized MCAD.

Enzyme Assay. The enzyme activity was determined by using phenazine methosulfate (PMS) or phenazine ethosulfate (PES) as the electron carrier to mediate the transfer of reducing equivalents from substrate (octanoyl-CoA for MCAD and butyryl-CoA for SCAD) to 2,6-dichlorophenolindophenol (DCPIP).[3f,13] A standard 0.7-mL assay

(26) (a) Penning, T. M.; Talalay, P. *J. Biol. Chem.* **1981**, *256*, 6851. (b) Penning, T. M.; Heller, D. N.; Balasubramanian, T. M.; Fenselau, C. C.; Talalay, P. *J. Biol. Chem.* **1982**, *257*, 12589.

(27) Analysis of the X-ray crystal structure of the SCAD/acetoacetyl-CoA complex revealed a distance of 3.00 Å between the nearest carboxylate oxygen (O1) of Glu-367 and the pro-*R* α-H of the acyl thioester. Interestingly, the distance between the nearest carboxylate oxygen (O2) of Glu-367 and the γ-H of the inhibitor was measured as 2.84 Å.

(28) (a) Schwab, J. M.; Henderson, B. S. *Chem. Rev.* **1990**, *90*, 1203. (b) Creighton, D. J.; Murthy, N. S. R. K. *The Enzymes*; Academic Press: San Diego, 1990; Vol. XIX, p 323.

(29) (a) Bevins, C. L.; Bantia, S.; Pollack, R. M.; Bounds, P. L.; Kayser, R. H. *J. Am. Chem. Soc.* **1984**, *106*, 4957. (b) Bounds, P. L.; Pollack, R. M. *Biochemistry* **1987**, *26*, 2263.

(30) (a) Kuliopulos, A.; Mildvan, A. S.; Shortle, D.; Talalay, P. *Biochemistry* **1989**, *28*, 149. (b) Xue, L.; Talalay, P.; Mildvan, A. S. *Biochemistry*, **1990**, *29*, 7491. (c) Kuliopulos, A.; Mullen, G. P.; Xue, L.; Mildvan, A. S. *Biochemistry* **1991**, *30*, 3169.

was performed in 50 mM potassium phosphate buffer (pH 7.6) at 25 °C, containing 30 μM DCPIP, 1.4 mM PMS (or PES), 0.3 mM EDTA, and 33 μM acyl-CoA substrate. The reaction was initiated by the addition of an appropriate amount (typically 2.5 nmol) of MCAD or SCAD. Loss of DCPIP absorbance at 600 nm (ϵ 22 000 M^{-1} cm^{-1}) before and after the addition of MCAD or SCAD was monitored.

Test for the Reversibility of the Inactivation. A sample containing wild-type SCAD (29.32 μM) and inactivator (**1** or **19**, 1.46 mM) was incubated for 60 min at room temperature to ensure complete inactivation of the enzyme. The inactivated enzyme was dialyzed against 100 mM potassium phosphate buffer (pH 7.6) over two days at 4 °C with seven buffer changes. The residual enzyme activity was determined before and after dialysis. A control experiment was performed under identical conditions without the inactivator.

Kinetic Analysis of Inactivation. In a typical inactivation experiment, an appropriate amount of the inactivator dissolved in water was incubated with the enzyme solution (15−34 μM in 100 mM potassium phosphate buffer, pH 7.6) at 25 °C. At various time intervals, aliquots (50 μL) of the incubation mixture were taken and diluted into the standard assay cocktail (0.7 mL), and the remaining enzyme activity was determined as described above. For the "protection" experiment, the enzyme was preincubated with excess acetoacetyl-CoA for 15 min at room temperature, followed by the addition of inactivator. The residual enzyme activity was monitored over time as described above. A typical example included the treatment of SCAD (0.53 μM) with acetoacetyl-CoA (7.6 μM), followed by the addition of an inactivator (**1** or **19**) to give a final concentration of 20.8 μM.

Tritium Washout Study. The tritium-labeled inactivator (30 μM of **4** or 0.1 mM of **19**) was incubated with enzyme [SCAD or SCAD-(E367Q)] in 500 μL of 100 mM potassium phosphate buffer (pH 7.6) at 25 °C. A 100-μL aliquot of the reaction mixture was removed at time zero and counted for radioactivity. After a period of 60 min, the incubation was quenched with active charcoal (10% solution), and the resulting suspension was mixed vigorously on a vortex mixer for 1 min followed by centrifugation to precipitate the charcoal.[17] The supernatant was removed and analyzed by a scintillation counter. These readings were calibrated against controls prepared in parallel with boiled enzyme (100 °C, 10 min).

Determination of Stoichiometry of Tritium Incorporation. The enzyme (SCAD, 11.0 μM) was incubated with an excess of labeled inactivator (**4** or **19**) for 60 min to ensure complete inactivation of the enzyme. The inactivated enzyme was dialyzed against 100 mM potassium phosphate buffer (pH 7.6) at 4 °C for 2 days with seven buffer changes. An aliquot (50 μL) of the incubation mixture was removed before and after dialysis to count for radioactivity. The reading was calibrated against a control prepared with boiled enzyme. These data allowed the ratio of inactivator per enzyme subunit to be determined. In addition, a portion of the inactivated enzyme, after extensive dialysis, was treated with 90% MeOH to denature the protein. After centrifugation, the percent radioactivity in the supernatant was determined by a scintillation counter.

NMR Analysis of γ-Proton Exchange Mediated by SCAD and SCAD(E367Q). A mixture of the enzyme [SCAD, 59.9 μM; or SCAD-(E367Q), 38.9 μM] and crotonoyl-CoA (4.94 mM) was incubated in 100 mM potassium phosphate buffer prepared with ^2H_2O (pD 7.2) at room temperature for 4 h. The protein was then removed by an Amicon microconcentrator, and the filtrate was subjected to ^{1}H NMR analysis. In order to avoid complications, the original enzyme solution was diluted with 10 mL of the deuterated phosphate buffer and concentrated via ultrafiltration to ensure the complete exchange of all the exchangeable hydrogens of the enzyme. This procedure was repeated twice prior to incubation with crotonoyl-CoA.

General Procedure for Coupling of Acid and Coenzyme A. To a solution of carboxylic acid (0.32 mmol) in 5 mL of methylene chloride was added triethylamine (44.5 μL, 0.32 mmol) under argon. After being stirred for 10 min, isobutyl chloroformate (41.0 μL, 0.32 mmol) was added dropwise at 0 °C, and the stirring was continued at room temperature for 1−3 h. The solvent was then removed under reduced pressure, and the remaining mixed anhydride was re-dissolved in 5 mL of THF to give a cloudy solution. Meanwhile, a solution of coenzyme A was prepared by dissolving the sodium salt of coenzyme A (50 mg, 50 μmol) in distilled water (5 mL) that had been previously deoxygenated by repeated freeze and thaw cycles under high vacuum. The CoA solution was adjusted to pH ~8.0 by adding 1 N NaOH. The mixed anhydride solution was added to the resulting CoA solution. After 1 h, the pH of the solution was adjusted to 5.0−5.5 by adding dilute perchloric acid. The organic solvent (THF) was evaporated *in vacuo*, and the remaining aqueous solution was extracted twice with ether to remove any residual organic materials. The aqueous solution was then lyophilized. The crude product was chromatographed on a preparative HPLC Partisil-C_{18} column (10 × 250 mm, 5 μm) and eluted with 30% methanol in 50 mM potassium phosphate buffer (pH 5.3, flow rate 2.5 mL/min, monitoring wavelength 260 nm). The fractions containing the product were pooled, concentrated under reduced pressure to remove methanol, and then lyophilized. The resulting acyl-CoA product was desalted by loading the sample into the same HPLC C_{18} column and washing it with water (2.5 mL/min). The eluting solvent was changed 25 min later to methanol to wash out the purified product. The organic solvent was removed under reduced pressure, and the aqueous solvent was lyophilized to give the pure CoA derivative as a white powder.

(*E*)-2,3-Epoxybutanoic Acid (3). To a solution of sodium hydroxide (1 g, 25 mmol) in 13 mL of water was added crotonoic acid **2** (4.3 g, 50 mmol). After the solution was warmed to 55 °C, sodium tungstate dihydrate (1.65 g, 50 mmol) was introduced with stirring followed by dropwise addition of 30% hydrogen peroxide (8.1 mL, 70 mmol). The reaction temperature was held at 63−65 °C by cooling with an ice-water bath, and the pH was kept at pH ≥ 4 by the addition of NaOH (30% solution) as needed. After being stirred for 1 h at the same temperature, the mixture was cooled to room temperature and acidified to pH 2.5 with 30% H_2SO_4. The solution was saturated with ammonium chloride and extracted with ether (5 × 50 mL). The combined organic extracts were dried, filtered, and concentrated. The crude product was purified by flash column chromatography (4:1 to 1:1 hexane/EtOAc) to give the desired product **3** in 80% yield. ^{1}H NMR ($CDCl_3$) δ 10.65 (1H, br s, COO*H*), 3.30−3.21 (2H, m, 2,3-H's), 1.41 (3H, d, J = 5.0, Me); ^{13}C NMR ($CDCl_3$) δ 174.0 (C-1), 54.3 (C-2)*, 52.6 (C-3)*, 16.3 (C-4).

(*E*)-2,3-Epoxybutyryl-CoA (1). Acid **3** was coupled with coenzyme A to give the final product **1** in 45% yield. ^{1}H NMR (D_2O, signals of oxiranyl moiety shown in italics) δ 8.75, 8.53 (1H each, s, adenine H's), 6.29 (1H, d, J = 5.4, ribose anomeric H), 4.96 (2H, m), 4.71 (1H, br s, ribose H), 4.39 (2H, br s, $C(Me)_2CH_2O$), 4.13 (1H, s, $HOCHCMe_2$), 3.98, 3.73 (1H each, m, ribose H's), 3.58 (2H, t, J = 6.3), *3.50* (1H, d, J = 2.0, 2-H), 3.45 (2H, m), *3.43* (1H, overlapped, 3-H), 2.71 (2H, t, J = 6.5), 2.58 (2H, t, J = 6.4), *1.48* (3H, d, J = 5.1, 4-Hs), 1.05, 0.93 (3H each, s, 2 × Me): High-resolution FABMS calcd for $C_{25}H_{41}N_7O_{18}P_3S$ $(M + H)^+$ 852.1442; found 852.1471.

***trans*-[3-^{3}H]-2-Butenol (6).** To a solution of 2-butyn-1-ol (**5**, 0.3 g, 4.3 mmol) in 10 mL of anhydrous ether was added dropwise an ether solution (10 mL) of Red-Al (3.87 mL, 8.56 mmol) at −78 °C under argon. The addition was completed in 10 min, the mixture was allowed to warm up to room temperature, and stirring was continued overnight. The resulting mixture was chilled to −78 °C and quenched with 200 μL of ^{3}HOH (specific activity = 100 mCi/mL). After stirring for 20 min at this temperature, an aliquot of 100 μL of H_2O was added to fully quench the reducing agent. The reaction mixture was aged for an additional 10 min, and the temperature was gradually returned to room temperature. The reaction mixture was concentrated *in vacuo* and chromatographed directly on a silica gel column (1:1 ether/pentane) to give **6** as a colorless oil in 82% yield. To ensure the success of the experiment, the reaction conditions were tested/optimized with ^2H_2O prior to the use of ^{3}HOH. Spectral data of the C-3 deuterated sample: ^{1}H NMR ($CDCl_3$) δ 5.60 (1H, m, 2-H), 4.04 (2H, d, J = 5.6, 1-Hs), 1.69 (3H, s, Me), 1.50 (1H, s, OH); ^{13}C NMR ($CDCl_3$) δ 130.1 (C-2), 63.7 (C-1), 17.6 (C-4).

***trans*-[3-^{3}H]-2,3-Epoxybutanol (7).** To a solution of **6** (0.1 g, 1.39 mmol) in 4.0 mL of methylene chloride was added 50−60% *m*-chloroperbenzoic acid (*m*-CPBA, 0.96 g; 2.78 mmol). The solution was stirred for 12 h at room temperature. The crude mixture was chromatographed directly on a silica gel column (1:1 ether/pentane) to afford **7** as a colorless oil in 90% yield. Spectral data of the C-3 deuterated sample obtained from a test experiment: ^{1}H NMR ($CDCl_3$) δ 3.87 (1H, dd, J = 12.5, 2.5, 1-H), 3.59 (1H, dd, J = 12.5, 4.5, 1-H),

2.85 (1H, m, 2-H), 1.30 (3H, s, Me); ^{13}C NMR ($CDCl_3$) δ 61.2 (C-1), 59.0 (C-2), 16.6 (C-4).

***trans*-[3-^{3}H]-2,3-Epoxybutanoic Acid (8).** To a solution of **7** (0.15 g, 1.68 mmol) in 4.0 mL of acetonitrile was added a mixture of $NaIO_4$ (1.5 g, 7.1 mmol), $RuCl_3 \cdot 3H_2O$ (13.6 mg, 51.9 μmol) and water (106 μL, 5.9 mmol) in one portion. The reaction mixture was vigorously stirred at room temperature overnight. The reaction was complete after 12 h, monitored by TLC (1:1 hexane/EtOAc), and the solution turned greenish black over the course of the reaction. The crude mixture was chromatographed directly on a silica gel column (4:1 ether/pentane) to give **8** as a white solid in 74% yield. Spectral data of the C-3 deuterated sample obtained from a test experiment: ^{1}H NMR ($CDCl_3$) δ 3.20 (1H, s, 2-H), 1.39 (3H, s, Me).

***trans*-[3-^{3}H]-2,3-Epoxybutyryl-CoA (4).** Acid **8** was coupled with coenzyme A to furnish the final product **4** as a white solid (specific activity = 0.16 mCi/mmol). The labeled product eluted with an identical retention time as the unlabeled standard **1**: HPLC (Partisil-C_{18} column) t_R = 7.8 min (see General Procedures for separation conditions).

Ethyl (*E*)-3,5-Hexadienoate (11). To a solution of diisopropylamine (12.4 mL, 90 mmol) in 60 mL of dry THF was added a solution of *n*-butyllithium in hexane (37 mL, 90 mmol, 2.5 M) at −10 °C under argon. After the solution was stirred for 30 min, the reaction mixture was cooled to −78 °C. To this solution was added 20 mL of hexamethylphosphoramide (HMPA), and stirring was continued for 20 min. Ethyl sorbate (**10**, 7 g, 50 mmol) in dry THF (20 mL) was then added dropwise at −78 °C. After the addition was complete, the reaction mixture was stirred for an additional 30 min at the same temperature. The resulting dark red solution was poured into a well-stirred solution of 18 mL of acetic acid and 100 mL of ice water. The reaction mixture was extracted with pentane (3 × 50 mL), and the combined extracts were washed with $NaHCO_3$ and water, dried, filtered, and concentrated. The residue (6 g, 86%), which was essentially pure, was used for the next reaction without further purification. ^{1}H NMR ($CDCl_3$) δ 6.32 (1H, dt, J = 16.9, 10.4, 5-H), 6.12 (1H, dd, J = 15.2, 10.4, 4-H), 5.77 (1H, dt, J = 15.2, 7.2, 3-H), 5.14 (1H, d, J = 16.9, 6-H), 5.04 (1H, d, J = 10.1, 6-H), 4.12 (2H, q, J = 7.2, OCH_2), 3.09 (2H, d, J = 7.2, 2-Hs), 1.23 (3H, t, J = 7.2, Me); ^{13}C NMR ($CDCl_3$) δ 171.1 (C=O), 136.1, 133.9, 125.4, 116.5, 60.4 (OCH_2), 37.7 (C-2), 13.8 (Me).

(*E*)-3,5-Hexadien-1-ol (12). To a well-stirred suspension of lithium aluminum hydride (2.0 g, 52.6 mmol) in 100 mL of anhydrous ether was added dropwise a solution of ester **11** (5.4 g, 38.6 mmol) in 5 mL of anhydrous ether at 0 °C. The ice bath was removed, and the mixture was allowed to warm to room temperature. After being stirred for 30 min, the reaction was quenched by sequential addition of 2 mL of water, 2 mL of 15% NaOH, and 6 mL of water. The mixture was filtered, and the filtrate was washed with brine, dried, filtered, and concentrated *in vacuo* to give crude **12**. Without further purification the title compound was subjected to oxidation by the Jones reagent.

(*E*)-3,5-Hexadienoic Acid (13). Alcohol **12** (307 mg, 3.06 mmol) was dissolved in acetone and treated at 0 °C with Jones oxidant (prepared by mixing 26.7 g of chromium trioxide and 23 mL of concentrated sulfuric acid followed by water dilution to a final volume of 100 mL) until the red orange color persisted for at least 1 min. The resulting mixture was stirred at room temperature for 30 min to ensure complete oxidation of **12**. Excess oxidizing reagent was quenched with 2-propanol at 0 °C. The reaction mixture was then diluted with water, and the product was extracted into the organic phase with ether (5 × 50 mL). The combined organic extracts were dried, filtered, and concentrated *in vacuo*. The crude product was purified by flash column chromatography (4:1 ether/pentane) to give the desired acid in 31% yield. ^{1}H NMR ($CDCl_3$) δ 11.6 (1H, bs, COOH), 6.21 (1H, dt, J = 16.5, 10.2, 5-H), 6.15 (1H, dd, J = 15.2, 10.2, 4-H), 5.76 (1H, dt, J = 15.2, 7.1, 3-H), 5.17 (1H, d, J = 16.5, 6-H), 5.07 (1H, d, J = 10.0, 6-H), 3.16 (2H, d, J = 7.1, 2-Hs); ^{13}C NMR ($CDCl_3$) δ 178.1 (C-1), 136.2, 135.0, 124.7, 117.4, 37.5 (C-2).

***trans*-3,4-Epoxy-5-hexenoic Acid (14).** To a solution of acid **13** (1 g, 9 mmol) in 100 mL of methylene chloride was added 57−86% *m*-CPBA (3 g, 22.5 mmol) at room temperature. After being stirred overnight, the reaction mixture was filtered through silica gel and washed with hexane. The filtrate was directly loaded onto the silica gel column and purified by flash chromatography (4:1 to 1:2 hexane/EtOAc) to give the desired product **14** in 20% yield. ^{1}H NMR ($CDCl_3$) δ 5.86 (1H, ddd, J = 17.1, 10.6, 5.3, 5-H), 5.38 (1H, d, J = 17.1, 6-H), 5.28 (1H, d, J = 10.6, 6-H), 4.85−4.83 (1H, m, 4-H), 4.35 (1H, dt, J = 6.2, 3.2, 3-H), 3.15 (1H, br s, COO*H*), 2.77 (1H, dd, J = 17.8, 6.2, 2-H), 2.53 (1H, dd, J = 17.8, 3.2, 2-H); ^{13}C NMR ($CDCl_3$) δ 175.8 (C-1), 132.5 (C-5), 117.9 (C-6), 87.4 (C-4), 71.4 (C-3), 36.4 (C-2).

***trans*-3,4-Epoxy-5-hexenoyl-CoA (9).** Acid **14** was coupled with coenzyme A to give the CoA product **9** in 70% yield. ^{1}H NMR (D_2O, signals of the oxiranyl moiety shown in italics) δ 8.74, 8.51 (1H each, s, adenine H's), 6.27 (1H, d, J = 5.1, ribose anomeric H), *6.01* (1H, ddd, J = 18.5, 11.0, 6.0, 5-H), *5.49* (1H, d, J = 18.5, 6-H), *5.44* (1H, d, J = 11.0, 6-H), *5.05* (1H, m, 4-H), 4.92 (2H, m), 4.66 (1H, s, ribose H), *4.54* (1H, m, 3-H), 4.38 (2H, bs, $C(Me)_2CH_2O$), 4.13 (1H, s, $HOCHC(Me)_2$), 3.96, 3.73 (1H each, m, ribose H), 3.58 (2H, t, J = 6.1), 3.42 (2H, t, J = 6.2), *3.09* (1H, dd, J = 18.2, 6.4, 2-H), 2.70 (2H, t, J = 6.2), *2.67* (1H, dd, J = 18.2, 3.2, 2-H), 2.56 (2H, t, J = 6.2), 1.04, 0.91 (3H each, s, 2 × Me).

4,5-Epoxypentanoic Acid (18). To a solution of the commercially available 4-pentenoic acid **17** (1 g, 10 mmol) in 80 mL of methylene chloride was added 57−86% *m*-CPBA (3.4 g, 20.0 mmol) at room temperature. After being stirred overnight, the solution was directly loaded on the silica gel and purified by flash column chromatography (2:1 to 1:2 hexane/EtOAc) to give the desired product **18** in 90% yield. ^{1}H NMR ($CDCl_3$) δ 4.72−4.60 (1H, m, 4-H), 3.90 (1H, dd, J = 12.3, 2.7, 5-H), 3.64 (1H, dd, J = 12.3, 4.5, 5-H), 2.70−2.45 (2H, m, 2-H's), 2.50−2.32 (2H, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 178.8 (C-1), 80.9 (C-4), 64.1 (C-5), 28.7 (C-2), 23.2 (C-3).

4,5-Epoxypentanoyl-CoA (15). Acid **18** was coupled with coenzyme A to give the CoA product **15** in 80% yield. ^{1}H NMR (D_2O) δ 8.37, 8.04 (1H each, s, adenine H's), 5.98 (1H, d, J = 6.0, ribose anomeric H), 4.88−4.83 (2H, m), 4.42 (1H, br s), 4.10 (2H, br s, $C(Me)_2CH_2O$), 3.85 (1H, s, $HOCHC(Me)_2$), 3.70−3.65 (2H, m), 3.48 (1H, dd, J = 12.6, 5.1, 5-H), 3.40−3.36 (1H, m), 3.16−3.10 (2H, m), 3.25−3.15 (2H, burried under triethylamine·HCl salt), 3.01−2.95 (2H, m), 2.57 (2H, t, J = 6.0), 2.50−2.45 (2H, m, 2-Hs), 2.28 (2H, t, J = 6.0), 2.28−2.10 (1H, m, 3-H), 1.95−1.60 (1H, m, 3-H), 0.72, 0.58 (3H each, s, 2 × Me): High-resolution FABMS calcd for $C_{26}H_{43}N_7O_{18}P_3S$ $(M + H)^+$ 866.1755; found 866.1743.

[4-^{3}H]-2-Butynoic Acid (21). To a solution of anhydrous ammonia (10 mL) at −40 °C was added a small piece of Li metal. The subsequent addition of a few crystals of $Fe(NO_3)_3$ changed the color of the reaction solution from blue to gray, which is indicative of the formation of $LiNH_2$. More Li metal (78.7 mg, 11.4 mmol) was added, and the resulting mixture was vigorously stirred for 20 min. Propiolic acid (**20**, 176 μL, 2.85 mmol) in 3 mL of anhydrous ether was added dropwise to this solution, and stirring was continued for 10 min prior to the addition of $[^3H]CH_3I$ (70 μmol in 0.69 mL of toluene, specific activity = 75 mCi/mmol) in 3 mL of dry ether. Twenty minutes later, a solution of iodomethane (142 μL, 2.28 mmol) in 5 mL of dry ether was added dropwise, and the reaction mixture was stirred at −40 °C for 6 h and then at room temperature for an additional 6 h. After quenching with saturated NH_4Cl solution (20 mL) at 0 °C, the resulting mixture was acidified with concentrated sulfuric acid and stirred for 30 min. The product was extracted into ether (3 × 75 mL), and the combined organic layers were dried and concentrated. The crude product was purified by flash column chromatography on silica gel (1:1 ether/pentane) to provide **21** as a white solid in 46% yield. ^{1}H NMR ($CDCl_3$) of the unlabeled **21**: δ 2.01 (3H, s, Me).

[4-^{3}H]-2-Butynoyl-CoA (19). The labeled acid **21** and the unlabeled 2-butynoic acid were each coupled with coenzyme A to afford the corresponding acyl-CoA product as a white solid in 67% yield. Both unlabeled and labeled products were eluted with identical retention times: HPLC (Partisil-C_{18} column) t_R = 9.4 min. The specific activity of the labeled product = 1.51 mCi/mmol. Spectral data of the unlabeled **19**: ^{1}H NMR (D_2O, signal of the acyl moiety given in italics) δ 8.60, 8.31 (1H each, s, adenine H's), 6.21 (1H, d, J = 6.0, ribose anomeric H), 4.96 (2H, m), 4.63 (1H, s, ribose H), 4.29 (2H, br s, $C(Me)_2CH_2O$), 4.07 (1H, s, $HOCHCMe_2$), 3.90, 3.61 (1H each, m, ribose H), 3.51 (2H, t), 3.39 (2H, m), 3.11 (2H, t, J = 6.5), 2.47 (2H, t, J = 6.4), *2.08* (3H, s, 4-Hs), 0.95, 0.81 (3H each, s, 2 × Me).

[2,3-$^{13}C_2$]-1,1-Dibromo-1-propene (24). To a solution of 100 mL

of dry methylene chloride were added Zn dust (2.96 g, 45.4 mmol), triphenylphosphine (11.9 g, 45.4 mmol), and carbon tetrabromide (15.1 g, 45.4 mmol) at 0 °C. The solution was stirred at room temperature for 30 h. During the course of the reaction, the solution changed colors from brown to green, and finally a pink color persisted. The reaction was cooled to 0 °C, and the reaction flask was fitted with a dry-ice condenser. Neat [1,2-$^{13}C_2$]acetaldehyde (1 g, 22.7 mmol) was added dropwise to the above solution, and the resulting mixture was stirred for an additional 5 h at room temperature. After mixing with 500 mL of pentane, the reaction was let stand for 4 h followed by filtration of the precipitated residue. The extraction procedure was repeated (2 × 300 mL), and the combined organic layers were dried and concentrated *in vacuo*. The desired product was obtained as a clear liquid in 45% yield. ^{1}H NMR ($CDCl_3$) δ 6.43 (1H, dqd, $J_{H-C} = 160.0$, $J_{H-C-C-H} = 6.8$, $J_{H-C-C} = 2.0$, 2-H), 1.68 (3H, dt, $J_{H-C} = 129.0$, $J_{H-C-C-H} = J_{H-C-C} = 6.8$, 3-Hs); ^{13}C NMR ($CDCl_3$) δ 133.5 (d, $J_{C-C} = 43.5$, C-2), 18.5 (d, $J_{C-C} = 43.5$, C-3).

[3,4-$^{13}C_2$]-2-Butynoic Acid (25). To a solution of **24** (0.98 g, 4.9 mmol) in 30 mL of dry THF was added dropwise a solution of 2.5 M *n*-BuLi (10.8 mmol, 4.3 mL) in hexane at −78 °C. The reaction was stirred at −78 °C for 1 h and then at room temperature for an additional 1 h. The temperature of the reaction flask was returned to −40 °C, and a gentle stream of dry CO_2 gas was bubbled into the above solution until the rate of uptake decreased considerably (ca. 8 h). The reaction was quenched by the addition of saturated NH_4Cl solution. The organic solvent was removed *in vacuo*, and the pale yellow residue was redissolved in 15 mL of 1 N NaOH. The resulting mixture was washed with ether (2 × 35 mL), the aqueous layer was acidified with 6 N HCl, and the product was extracted into ether layer (3 × 50 mL). The organic layers were combined, dried, and concentrated *in vacuo*. The desired compound was obtained as a off-white solid in 85% yield. ^{1}H NMR ($CDCl_3$) δ 2.03 (3H, dd, $J_{H-C} = 133.0$, $J_{H-C-C} = 10.8$, 4-Hs); ^{13}C NMR ($CDCl_3$) δ 168.0 (C-1), 88.6 (d, $J_{C-C} = 65.9$, C-3), 3.9 (d, $J_{C-C} = 65.9$, C-4).

[3,4-$^{13}C_2$]-2-Butynoyl-CoA (22). Acid **25** (15.0 mg, 0.18 mmol) was mixed with triethylamine (27.4 μL, 0.20 mmol) and isobutyl chloroformate (23.1 μL, 0.18 mmol) in 5 mL of methylene chloride at 0 °C, and the solution was stirred for 1 h. The remaining protocol was identical to the general coupling procedures except the pH of the reaction was carefully maintained at 7.5. The final product, co-eluted with the authentic sample, was obtained as a white solid in 86% yield. ^{1}H NMR (D_2O, signal of the acyl moiety given in italics) δ 8.56, 8.26 (1H each, s, adenine H's), 6.15 (1H, d, $J = 6.5$, ribose anomeric H), 4.95 (2H, m), 4.61 (1H, br s), 4.27 (2H, br s, $C(Me)_2CH_2O$), 4.04 (1H, s, $HOCHCMe_2$), 3.85 (1H, m), 3.59 (1H, m), 3.47 (2H, m), 3.37 (2H, m), 3.10 (2H, m), 2.45 (2H, m), *2.08* (3H, dd, $J_{H-C} = 133.4$, $J_{H-C-C} = 10.7$, 4-Hs), 0.92, 0.78 (3H each; s, 2 × Me); ^{13}C NMR (D_2O) δ 98.5 (d, $J_{c-c} = 64.3$, C-3), 6.2 (d, $J_{c-c} = 64.3$, C-4). Interestingly, compound **29** was found as the major product when the pH of the coupling reaction was maintained at 10. NMR of **29**: ^{1}H NMR (D_2O) δ 8.53, 8.24 (1H each, s, adenine H's), 6.03 (1H, d, $J = 6.6$, ribose anomeric H), *5.30* (1H, d, $J_{Ha-C} = 161$, 4-H)*, *5.10* (1H, dd, $J_{Hb-C} = 161$, $J_{Hb-C-C} = 3$, 4-H)*, 4.92 (2H, m), 4.57 (1H, br s), 4.21 (2H, br s, $C(Me)_2CH_2O$), 4.00 (1H, s, $HOCHCMe_2$), *3.89* (2H, d, $J = 6.5$, $(Me)_2CHCH_2O$), 3.82 (1H, dd, $J = 10.0$, 5.0, ribose), 3.53 (1H, dd, $J = 10.0$, 5.0), 3.43 (2H, m), 3.35 (2H, m), *2.85* (2H, m, 2-Hs), 2.59 (2H, m), 2.42 (2H, t, $J = 6.6$), *1.88* (1H, m, $(Me)_2CHCH_2O$), *0.87* (9H, d and s overlap, $(CH_3)_2CHCH_2O$ and Me of CoA), 0.73 (3H, s, Me of CoA); ^{13}C NMR (D_2O) δ 135.6 (d, $J = 76$, C-3), 113.2 (d, $J = 76$, C-4).

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