Spiropentylacetyl-CoA, A Mechanism-Based Inactivator of Acyl-CoA Dehydrogenases

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Received October 31, 1997

Abstract: Acyl-CoA dehydrogenases (ACDs) are FAD-dependent enzymes that catalyze the conversion of an appropriate fatty acyl-CoA thioester substrate to the corresponding *trans*-α,*β*-enoyl-CoA product. Early studies have shown that the dehydrogenation is stereospecific and is initiated by the abstraction of the *pro-R* α-H, followed by the transfer of the *pro-R* *β*-H, as a hydride equivalent, to the bound FAD. However, recent studies of the inactivation of ACDs by a metabolite of hypoglycin A, (methylenecyclopropyl)acetyl-CoA (MCPA-CoA), led to an alternative mechanism in which the reducing equivalent is delivered from the initially formed α-anion to the bound FAD via a single electron transfer process. To further explore the observed mechanistic discrepancy, we have reexamined the inhibitory properties of a closely related MCPA-CoA analogue, spiropentylacetyl-CoA (SPA-CoA), which was previously reported as a tight-binding inhibitor for ACDs. In contrast to early results, our data showed that SPA-CoA is a mechanism-based inhibitor for pig kidney medium-chain acyl-CoA dehydrogenase (MCAD) and *Megasphaera elsdenii* short-chain acyl-CoA dehydrogenase (SCAD) and that the inactivation is time-dependent, active-site-directed, and irreversible. More importantly, both (*R*)- and (*S*)-SPA-CoA could effectively inactivate MCAD, and the resulting inhibitor−FAD adducts appear to have one of the three-membered rings of the spiropentyl moiety cleaved. Since the inactivation is nonstereospecific with respect to C_β−C bond scission, the ring opening of SPA-CoA leading to enzyme inactivation is likely initiated by a spiropentylcarbinyl radical. Such a radical-induced ring fragmentation is expected to be extremely facile and may bypass the chiral discrimination normally imposed by the enzyme. Thus, these results are consistent with our early notion that MCAD is capable of mediating one-electron redox chemistry. Interestingly, it was also found that (*R*)-SPA-CoA is an irreversible inhibitor for SCAD, while the *S*-epimer is only a competitive inhibitor for the same enzyme. The selective inhibition exhibited by these compounds against two closely related dehydrogenases is likely a consequence of the distinct steric and electronic demands imposed by the active sites of MCAD and SCAD. Such information is important for the design of novel class-selective inhibitors to control and/or regulate fatty acid metabolism.

Acyl-CoA dehydrogenases (ACDs) are FAD-dependent enzymes that catalyze the conversion of the fatty acyl-CoA substrate (**1**) to the corresponding *trans*-α,*β*-enoyl-CoA product (**3**), with concomitant reduction of the flavin coenzyme.[1] There exists six distinct subclasses of ACDs involved in fatty acid metabolism, short-chain (SCAD), medium-chain (MCAD), long-chain (LCAD), very long chain (VLCAD), isovaleryl-CoA (IVD), and short/branched chain acyl-CoA dehydrogenases (SBCAD).[2] While each of these enzymes displays a preferential substrate specificity, they all adopt a common mechanism in which the initial step has been established to be the abstraction of the *pro-R* α-H by a conserved glutamate in the active site.[3] This is followed by the removal of a *β*-H from the nascent carbanion intermediate **2**, and the stereochemistry of this step has been shown to be *pro-R* specific as well.[4] Since the desaturation is effectively a two-electron redox reaction, relaying the reducing equivalents from intermediate **2** to the active-site-bound flavin is commonly believed to be a direct hydride transfer process (eq 1).[1,3] However, oxidation of the carbanion

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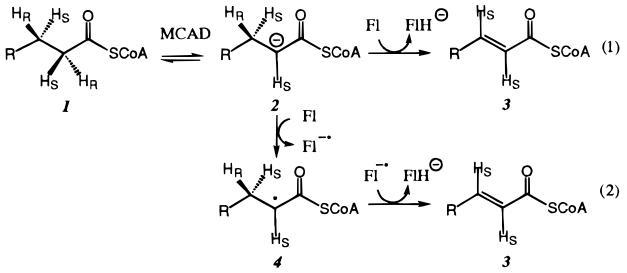
S0002-7863(97)03763-3 CCC: $15.00
Published on Web 02/24/1998

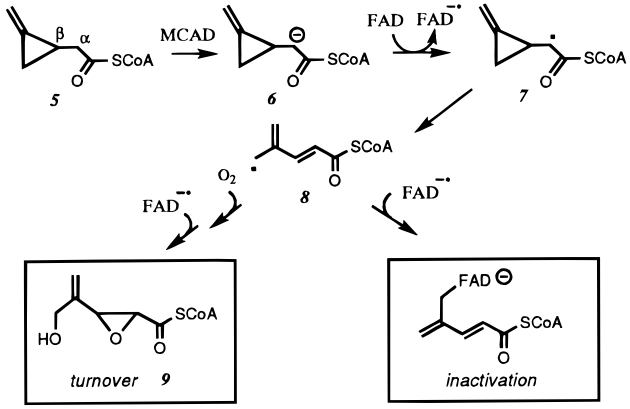
2 via a single electron transfer route involving the formation of a transient radical species (**4**) and a flavin semiquinone radical is also possible (eq 2). The latter hypothesis has gained support from recent inactivation studies described below.[5]



Over the last two decades, a wide variety of mechanism-based inhibitors have been reported for this important class of enzymes.[6] These inhibitors can be divided into two groups depending on whether the enzyme itself or the FAD coenzyme is the target for their actions. For example, the 2- and 3-alkynoyl thioester derivatives have been shown to inactivate the acyl-CoA dehydrogenases via trapping of the active-site base, glutamate.[6,7] The inactivation mechanism involves the abstraction of the α- or γ-H of the inhibitor by the active-site base, glutamate, to generate a reactive 2,3-allenic CoA intermediate, which is suitably poised to trap the same glutamate (Glu-367 in bacterial SCAD and Glu-376 in pig liver MCAD). On the contrary, (methylenecyclopropyl)acetyl-CoA (MCPA-CoA, **5**), a metabolite of hypoglycin A, inactivates both MCAD and SCAD via covalent modification of FAD (Scheme 1),[5,8] and both enantiomers of MCPA-CoA have been shown to be effective inhibitors.[5a,b,d] The lack of stereospecificity of bond rupture at C_β of MCPA-CoA (**5**) is interesting and strongly implies that this ring-opening step leading to inactivation is likely a spontaneous event induced by a cyclopropylcarbinyl radical (**7**).[5] The identification of **9** as the major turnover product, when the incubation was conducted aerobically, further sustains the intermediacy of a ring-opened radical **8** (Scheme 1).[5e,g] Thus, these results may be extrapolated to support the notion that dehydrogenases are capable of mediating one-electron oxidation−reduction.

In a recent investigation of disordered fatty acid metabolism in MCPA-treated rats, Tserng et al. came across a synthetic byproduct which inhibits MCAD without affecting amino acid

Scheme 1



metabolism.[9] This synthetic byproduct was later found to be spiropentylacetic acid (SPA), and its inhibitory activity was manifested both in vivo (with SPA) and in vitro (with SPA-CoA, **16**) in a rat model. An early study had shown that SPA itself is stable to alkaline hydrolysis with no detectable ring opening,[10] which led Tserng et al. to conclude that SPA-CoA may simply act as a tight-binding inhibitor.[9] While this postulation is plausible, it should be noted that SPA-CoA shares a highly strained ring skeleton with MCPA-CoA (**5**), and more bulky derivatives such as 3-indolepropionyl-CoA and *trans*-3-indoleacryloyl-CoA are known substrates of acyl-CoA dehydrogenase.[11] Thus, it is perplexing why SPA-CoA (**16**) can fit into the active site and yet not be processed by this enzyme. In an attempt to clarify this ambiguity, we have reexamined the inhibitory properties of SPA-CoA against two acyl-CoA dehydrogenases: pig liver MCAD[12] and a recombinant *Megasphaera elsdenii* SCAD.[3f] A detailed mechanistic inquiry to address the time dependency, stereospecificity/stereoselectivity, and the nature of this inactivation was performed with pure enzymes. Reported herein are our results and the mechanistic implications.

Results and Discussions

Preparation of Spiropentylacetyl-CoA (16). The precursor for the synthesis of spiropentylacetyl-CoA (**16**, SPA-CoA) is (methylenecyclopropyl)acetic acid (**13**, MCPA), which is derived from cyclopropanation of 3-buten-1-ol (**10**) by 1,1-dichloroethane in the presence of *n*-butyllithium.[13] The resulting product **11** was subjected to base-induced elimination, deprotection, and oxidation in sequence to give **13**. Conversion of **13** to SPA (**15**) was achieved by the treatment with diazomethane and lead tetraacetate to give **14**, followed by alkaline hydrolysis of the methyl ester (Scheme 2).[14] Condensation of SPA with isobutyl chloroformate and coupling to coenzyme A[5g,15] in aqueous THF solution (pH 8−8.5) afforded the desired spiropentylacetyl-CoA (**16**). This compound was later purified by a HPLC Partisil-C_{18} column, and the overall yield from MCPA (**13**) was 50%.

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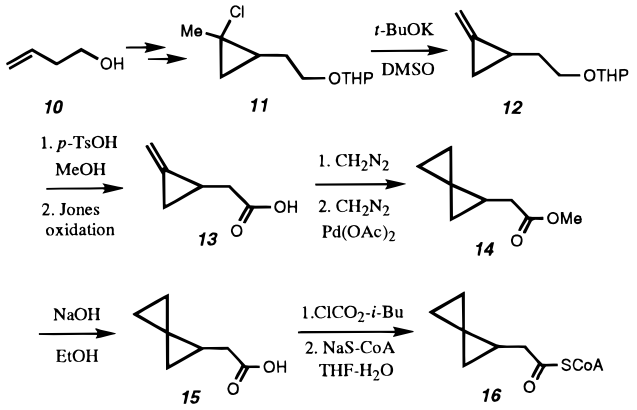
Scheme 2



Studies of Activity of Spiropentylacetyl-CoA on MCAD and SCAD. The effect of SPA-CoA (**16**) on the catalytic activity of MCAD was investigated by the incubation of MCAD with 10 mol equiv of SPA-CoA under aerobic conditions at 25 °C. As shown in Figure 1, time-dependent loss of activity of MCAD was noted during the incubation. It was also found that bleaching of the flavin chromophore (λ_{max} 450 nm) ensued concurrently with the loss of enzyme activity (Figure 1). Since the activity and the bleached flavin chromophore of the inactivated enzyme remained unchanged after prolonged dialysis, the inactivation is clearly irreversible and most likely involves covalent modification of the FAD coenzyme. Similar results were also observed when SCAD was treated with SPA-CoA. Again, time-dependent irreversible inactivation of the enzyme occurred with concomitant bleaching of the active-site flavin coenzyme.

Denaturation of Inactivated Enzyme. MCAD that had been inactivated with SPA-CoA was denatured by mixing vigorously with four volumes of methanol at 25 °C. This treatment released the noncovalently bound FAD from the active site of MCAD into the buffer solution. The suspension was centrifuged, and the protein precipitate was washed with 50 mM potassium phosphate buffer, pH 7.6. The supernatant and the washings were pooled, and the methanol was evaporated by a steady stream of nitrogen. While the reduced flavin in free solution is known to be rapidly reoxidized upon exposure to air, the spectrum of the combined supernatant showed no indication of any oxidized flavin chromophore. This observation provided additional evidence substantiating the formation of covalently modified FAD−inhibitor adducts as the cause of inactivation. Similar results were also observed when SCAD was exposed to SPA-CoA. Thus, an analogous mode of inactivation is proposed for the latter case as well.

Treatment of Reduced Flavin Adducts with Ferricenium Hexafluorophosphate. It has been shown that ferricenium salt ($Fc^+PF_6^-$), a strong oxidant, can be used as an alternate electron acceptor to effectively reoxidize substrate-reduced acyl-CoA dehydrogenases.[7c,16] To assess the effect of ferricenium salt on MCAD which has been inactivated by SPA-CoA, three portions of $FcPF_6$ (5 mol equiv each) were added in succession to the sample solution and the change of the electronic spectrum was monitored. Not surprisingly, neither MCAD reactivation nor FAD reoxidation occurred. The inability of ferricenium salt to reoxidize the modified flavin was also noted when MCAD was pretreated with MCPA-CoA (**5**), an inhibitor which is known to form covalent adduct(s) with FAD.[5,8] These findings lent further credence supporting that FAD of MCAD is covalently modified by SPA-CoA. The above experiments were also performed with SCAD, and a similar conclusion was reached.

Determination of Stoichiometry of Inactivation of MCAD and SCAD by SPA-CoA. The stoichiometry of the inactivation between SPA-CoA and acyl-CoA dehydrogenases was determined using [α-^{3}H]SPA-CoA (**18**), which was prepared by the reactions delineated in Scheme 3. The synthesis involved treatment of compound **14** with lithium diisopropylamide followed by quenching with ^{3}HOH. The resulting product **17** was hydrolyzed in alkaline solution and then coupled with coenzyme A to afford [α-^{3}H]SPA-CoA (**18**) in 52% overall yield with a specific activity of 0.32 mCi/mmol. The reaction conditions were first tested using ^2H_2O in the quenching step; the isotope incorporation was found to be regioselective at the α-C, and the extent of incorporation was ≥90%. Ten molar equivalents of the labeled SPA-CoA (**18**) was incubated with MCAD (40 nmol), and the inactivated enzyme was exhaustively dialyzed as previously described. Appropriate controls without inactivator and/or with boiled enzyme were also prepared. Our results established a 1:1 stoichiometry between SPA-CoA and inactivated MCAD (per subunit). It should be noted that calculations to determine the stoichiometry of inactivation with racemic **18** were calibrated to account for 50% loss of the radiolabels prior to covalent modification of the enzyme. When an analogous experiment was performed with SCAD, a similar 1:1 ratio was also found between inactivated SCAD and inhibitor. Such a stoichiometric ratio (1:1) is consistent with an inactivation mechanism involving covalent modification of the active site flavin coenzyme.

Analysis of MCAD Inactivated by [α-^{3}H]SPA-CoA. MCAD was incubated with 0.5 equiv of [α-^{3}H]SPA-CoA (**18**) in 50 mM potassium phosphate buffer (pH 7.6) at room temperature for 30 min. The reaction was quenched with active charcoal (10% solution),[5d] and the resulting suspension was mixed vigorously and then centrifuged to precipitate the charcoal. Interestingly, approximately 52% of the total radioactivity was found in the supernatant. Since only substoichiometric amounts of inhibitor are used, and the CoA derivatives and protein molecules are readily adsorbed by charcoal, the majority of radioactivity detected in the supernatant must result from enzyme-mediated solvent exchange of [α-^{3}H]SPA-CoA. Thus, an α-proton abstraction by the active-site base, Glu-376, leading to its exchange with the solvent water, must be a requisite step of SPA-CoA-mediated inactivation.

In a separate experiment, the incubation, carried out under identical conditions, was quenched with 90% methanol instead of charcoal. The denatured protein was precipitated, washed free of inhibitor, and submitted for scintillation counting. Since only about 1% of the total radioactivity was detected in the protein precipitate, the inactivation path leading to modification of the protein itself is insignificant. It should be pointed out that all of these readings had been calibrated against controls prepared in parallel with boiled enzyme. Hence, the above findings provided direct evidence corroborating that inactivation is likely induced by an α-proton abstraction, analogous to the first catalytic step, and the principal target of SPA-CoA is the flavin cofactor, not the enzyme itself.

Studies of Stereospecificity of Inactivation of MCAD and SCAD by SPA-CoA. The mechanism emerged from the above results is similar to that proposed for the inactivation of MCAD and SCAD by MCPA-CoA (**5**) (Scheme 1). To test whether the ring cleavage is a stereospecific process, we have prepared

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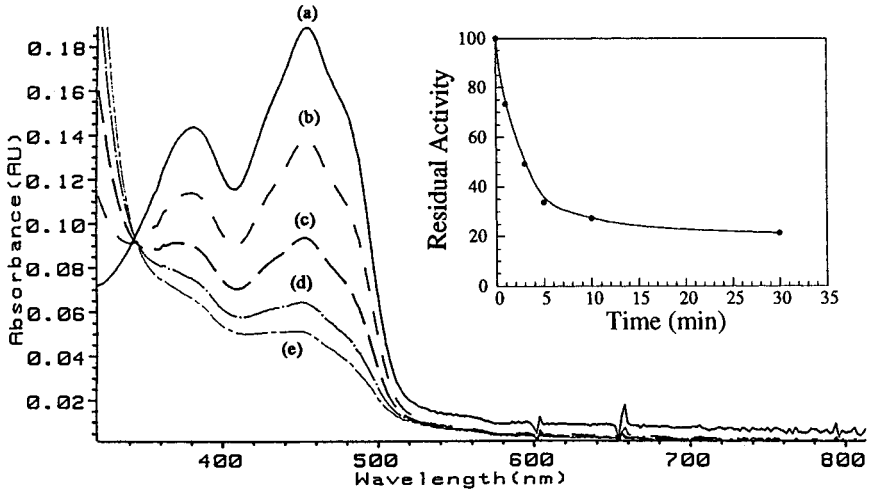
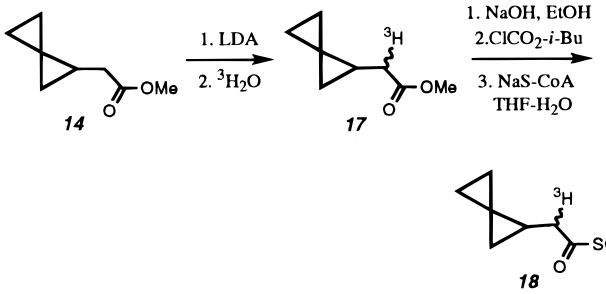
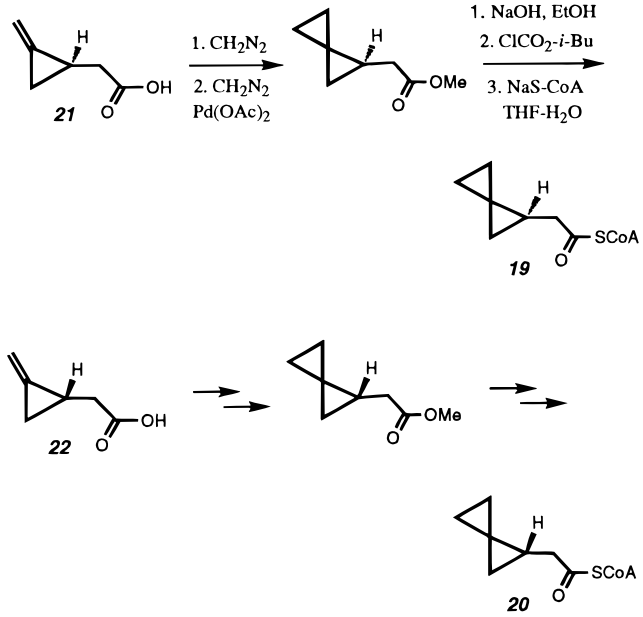


Figure 1. Effect of SPA-CoA on the spectrum of MCAD. MCAD, 13.0 mM in 0.4 mL of 50 mM potassium phosphate buffer, pH 7.6, at 25 °C, was incubated with 10 equiv of SPA-CoA (**16**) under aerobic conditions. Intermediate spectra were recorded after incubation for (a) 0 min, (b) 2 min, (c) 5 min, (d) 10 min, and (e) 30 min, respectively. The inset shows the effect of SPA-CoA on the activity of MCAD under the same conditions.

Scheme 3



Scheme 4



(*R*)- and (*S*)-SPA-CoA (**19** and **20**, respectively). The key intermediates to these two enantiomers were (*R*)- and (*S*)-MCPA (**21** and **22**, respectively), and their synthesis followed a reported procedure.[17] Subsequent conversion of MCPA to SPA-CoA was achieved through a series of reactions adapted from those delineated in Scheme 2, involving methylation of MCPA as a methyl ester, cyclopropanation of the exocyclic methylene group, hydrolysis of the ester group, and coupling of the resultant carboxylic acids with coenzyme A (Scheme 4). The effect of these SPA-CoA isomers on the catalytic activity of MCAD was analyzed by a method analogous to the successive titration reported by Wenz et al.[8e] As shown in Figure 2, a plot of the residual activity observed under aerobic conditions versus total equivalents of SPA-CoA added led to a partition ratio of approximately 2 for both (*R*)- and (*S*)-SPA-CoA. Since the partition ratio defines the number of latent inhibitor molecules converted to product relative to each turnover leading to enzyme inactivation, the equivalent ratios strongly suggested that the inactivation by either (*R*)- or (*S*)-SPA-CoA follows the same chemical course. Thus, the C_β bond cleavage must be nonstereospecific since both enantiomers of SPA-CoA are competent irreversible inactivators for MCAD.

Analogous experiments were also performed with SCAD, and a partition ratio of 30 was estimated for (*R*)-SPA-CoA (**19**), which is an irreversible inactivator for SCAD. However, to our surprise, the activity of SCAD was only slightly affected upon treatment with excess (*S*)-SPA-CoA (**20**, 300 equiv) and the absorbance of FAD was unchanged during incubation. Furthermore, no turnover products could be detected in the reaction mixture, and no α-H exchange was noted when (*S*)-SPA-CoA was recovered from the enzyme incubation in deuterated buffer (pD 7.2). In fact, (*S*)-SPA-CoA exhibited a competitive inhibitory effect against MCPA-CoA (**5**) with SCAD, as judged by the 69% reduction in the rate of inactivation of SCAD when (*S*)-SPA-CoA was coincubated with **5**. These findings implicate that (*S*)-SPA-CoA can bind to the active site of SCAD, albeit in a nonproductive manner. It is likely that the steric/spatial demands of the relatively bulky spiropentyl moiety may have obtruded the active-site base, Glu-367, of SCAD to effectively abstract the α-H. Thus, the fact that (*S*)-SPA-CoA is an irreversible inhibitor for MCAD but only a weak competitive inhibitor for SCAD makes it a new class-selective inhibitor for this important family of enzymes.

Kinetic Studies of Inhibition of MCAD and SCAD by (*R*)- and (*S*)-SPA-CoA. The competence of each enantiomer of SPA-CoA to inactivate MCAD and SCAD was further studied by kinetic analysis. As mentioned above, incubation of SCAD with (*R*)-SPA-CoA (**19**) resulted in time-dependent loss of

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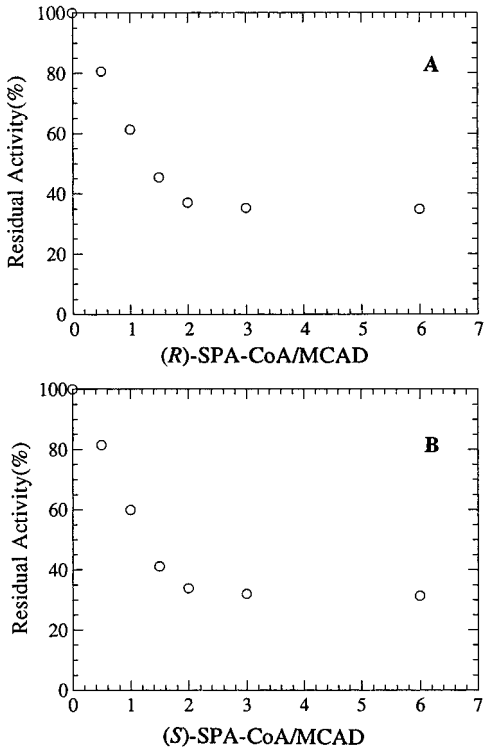


Figure 2. Effect of SPA-CoA on the catalytic activity of MCAD. A series of samples containing MCAD (13.0 mM) and appropriate amounts of SPA-CoA in 0.4 mL of 50 mM potassium phosphate buffer (pH 7.6) were prepared. The incubation was carried out aerobically at 25 °C for 1 h, and the residual activity of each sample was then assayed. These figures show the percentage of residual activity versus the ratio of SPA-CoA to enzyme: (A) (*R*)-SPA-CoA (**19**) and (B) (*S*)-SPA-CoA (**20**).

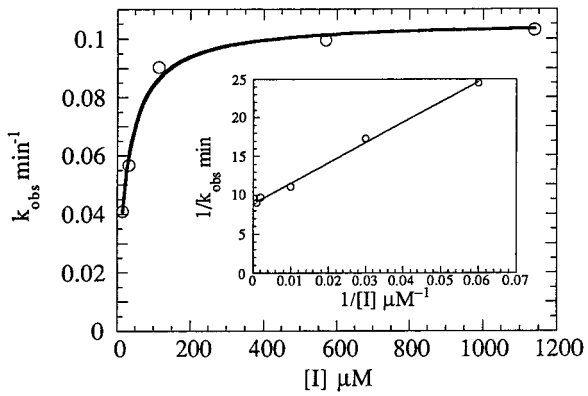


Figure 3. Effect of (*R*)-SPA-CoA on the catalytic activity of SCAD. The enzyme (9.0 mM) was incubated aerobically with (*R*)-SPA-CoA (**19**) in 0.4 mL of 50 mM potassium phosphate buffer, pH 7.6, at 25 °C. The observed inactivation rates (k_{obs}) at different inhibitor concentrations (16.0, 31.9, 114, 570, and 1140 mM) were recorded and plotted against inhibitor concentration. The inset shows the double-reciprocal plot of k_{obs} versus (*R*)-SPA-CoA concentration.

enzyme activity. The loss of activity follows saturation kinetics and also exhibits dependence on inhibitor concentration. The apparent dissociation constant (K_I) and the inactivation rate constant (k_{inact}) were deduced from the double-reciprocal plots of first-order rate constants (k_{obs}) versus values of inhibitor concentration. As shown in Figure 3, the K_I and k_{inact} were determined to be 25.5 mM and 0.11 min^{-1}, respectively, from this analysis. Since bleaching of the FAD chromophore accompanied time-dependent loss of enzymic activity, the extent of inhibition of MCAD by (*R*)- and (*S*)-SPA-CoA was followed

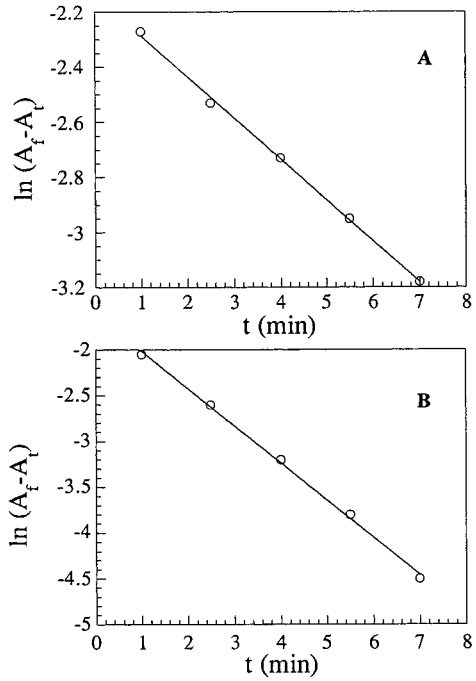


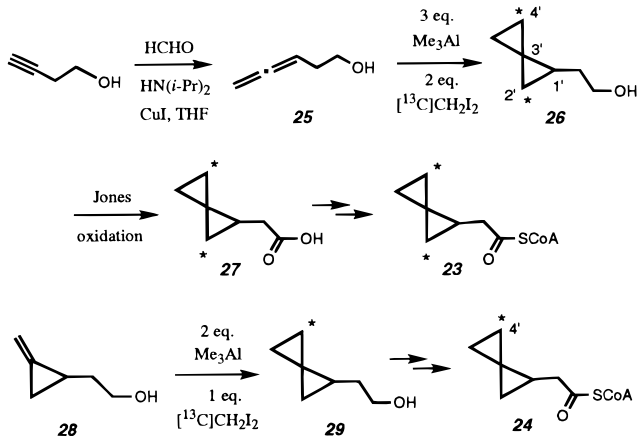
Figure 4. Effect of (*R*)- and (*S*)-SPA-CoA on the spectrum of MCAD monitored at 450 nm. MCAD, 13.0 mM in 0.4 mL of 50 mM potassium phosphate buffer, pH 7.6, was incubated under aerobic conditions at 25 °C with 20 equiv of inhibitor: (A) (*R*)-SPA-CoA (**19**) and (B) (*S*)-SPA-CoA (**20**). A_t is the absorbance at a given time during the incubation, and A_f is the absorbance at the end of the inactivation. The slope of the semilogarithmic plot of the changes of the absorbance ($A_f - A_t$) versus the incubation time gave the pseudo-first-order rate constants.

spectrophotometrically at 450 nm. Shown in Figure 4, A_t represents the absorbance at a given time during the incubation, while the absorbance at the end of the inactivation is designated as A_f. The slope of a semilogarithmic plot of the changes of the absorbance ($A_f - A_t$) versus the incubation time gave the observed inactivation rate constants (k_{obs}) of 0.15 and 0.41 min^{-1} for (*R*)- and (*S*)-SPA-CoA (0.26 mM), respectively, under the assay conditions.[18] Although the partition ratios for the inactivation of MCAD are the same for both isomers, the inactivation caused by (*S*)-SPA-CoA (**20**) is about 2.7 times faster than the observed rate for the *R*-enantiomer (**19**). Given the steric bulk of the spiropentyl group, it is conceivable that the alignment of the C_α−H bond toward the active-site base is different for the *R*- and *S*-isomers. Such an aberrance in bond orientation may have hampered the abstraction of the C_α proton by the active site base and contributed to the disparity of the inactivation rate. It is noteworthy that the rate of inactivation of MCAD by SPA-CoA is about 2.5−7 times slower than that by MCPA-CoA.[17c] Thus, MCPA-CoA is a more effective inactivator than SPA-CoA.

Characterization of the Covalent Adduct(s) between MCAD and SPA-CoA. While deprotonation of an α-H and formation of a covalent adduct with flavin as the initial and final episodes of SPA-CoA-mediated inactivation are established by the above experiments, the detailed reaction course and the nature of the reactive intermediate(s) involved remain elusive. Since both SPA-CoA and MCPA-CoA possess a highly strained

(18) Attempts to determine k_{inact} and K_I for the inactivation of MCAD by **19** or **20** based on a similar approach shown in Figure 3 proved to be problematic. The inactivation follows first-order kinetics only at high inhibitor concentration but deviates from the first-order kinetics at low inhibitor concentration. The amount of inhibitor (260 mM) used in the incubations shown in Figure 4 was at the saturation conditions.

Scheme 5



cyclic skeleton, it is conceivable that the inactivation by SPA-CoA, similar to that caused by MCPA-CoA, also involves formation of a ring-opened reactive intermediate which couples with FAD. Thus, structural characterization of the resultant inhibitor−FAD adduct(s) may provide significant insights into the inactivation mechanism. To facilitate the structural assignment, we have chemically synthesized the doubly labeled (C_4' and C_2', **23**) and the singly labeled (C_4', **24**) [^{13}C]SPA-CoA. As shown in Scheme 5, cyclopropanation of allenic alcohol **25** using 2 equiv of [^{13}C]CH_2I_2 and 3 equiv of trimethylaluminum gave **26** in good yield.[19] The [2′,4′-$^{13}C_2$]spiropentyl alcohol **26** was oxidized to the corresponding acid (**27**), which was later coupled to coenzyme A. The crude product was purified by HPLC to give **23** in 29% overall yield. The monolabeled SPA-CoA (**24**) was obtained by starting from methylenecyclopropyl alcohol (**28**), which was treated with 1 equiv of [^{13}C]CH_2I_2 and 2 equiv of trimethylaluminum to give **29** in excellent yield. The spiropentyl alcohol **29** was then oxidized with Jones reagent, and the resultant acid was coupled to coenzyme A. Purification by HPLC gave **24** in 60% overall yield.

The incubation was conducted anaerobically with MCAD (0.47 mmol) using 5 equiv of [^{13}C]SPA-CoA in 50 mM potassium phosphate buffer (pH 7.6) at 25 °C, and the progress of inactivation was monitored at A_{450}. After 1 h, most of the unreacted SPA-CoA was removed by ultrafiltration. The resulting mixture was concentrated and subjected to ^{13}C−^{1}H-detected HMQC (heteronuclear multiple quantum coherence) experiments. The spectra obtained from these incubations all consisted of an array of resonances within the range of 1−3.2 ppm in the ^{1}H dimension with cross-peaks between 15 and 45 ppm in the ^{13}C dimension (Figure 5). Evidently, more than one ^{13}C-enriched FAD adduct was generated in the incubation. Since the chemical shifts of these new ^{1}H and ^{13}C signals are distinct from those of an intact spiropentane structure, ring cleavage of SPA-CoA must have occurred during inactivation. Furthermore, the absence of any signal downfield from 3.2 and 45 ppm in the ^{1}H and ^{13}C dimensions, respectively, clearly indicated that none of the covalent adducts contain an olefinic carbon as part of the appended chain to FAD.

Mechanisms of Inactivation of MCAD and SCAD by SPA-CoA. On the basis of the inactivation mechanism of MCAD by MCPA-CoA and the experimental results presented herein, likely pathways for irreversible inactivation of acyl-CoA dehydrogenase by SPA-CoA (**16**) can now be proposed. As shown in Scheme 6, one possibility is that the reaction advances via C_α−H deprotonation, followed by an anion (**30**)-induced ring

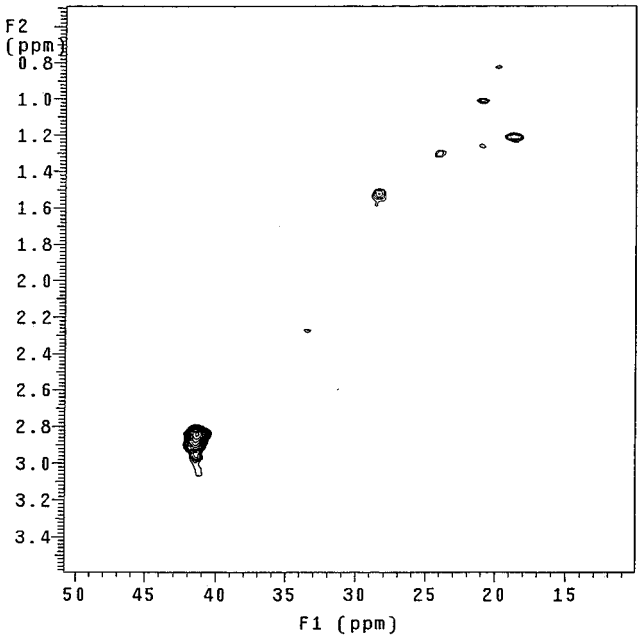


Figure 5. ^{13}C−^{1}H detected HMQC spectrum (500 MHz, $^{2}H_2O$) showing the resonances of the covalent adducts between MCAD and [2′,4′-$^{13}C_2$]spiropentylacetyl-CoA (**23**). Number of transients = 64; temperature = 25 °C; accumulation time = 10 h.

fragmentation of one or both rings of the spiropentyl moiety to give ring-opened intermediates (**31**−**33**), which can then covalently modify the FAD coenzyme (route A and/or B). However, the fact that the ring skeleton of methyl spiropentylacetate, a model for SPA-CoA, is stable under basic conditions[10] infers that the relief of ring strains by the α-anion-induced ring scission is insufficient to compensate for the required activation energy.[9] Furthermore, the lack of resonance stabilization of the ring-opened products (**31**−**33**) also renders the ring cleavage thermodynamically less favored. Interestingly, our finding that both (*R*)- and (*S*)-SPA-CoA are effective inhibitors for MCAD clearly demonstrated that C_β bond cleavage leading to ring fragmentation is nonstereospecific. Since in normal catalysis, the C_β−H bond rupture step is *pro-R*-specific (eqs 1 and 2),[4a,b] the lack of stereospecificity found in this inactivation study strongly suggests that the ring opening of SPA-CoA is likely a spontaneous event, which is not directly enzyme-catalyzed. Hence, analogous to the inactivation mechanism proposed for MCPA-CoA, a one-electron oxidation of the anion intermediate **30** to give a transient α-spiropentyl radical **34** is an appealing alternative (Scheme 6, route C).

In view of the great ring strain associated with spiropentane (63.5 kcal/mol),[20] the spiropentylcarbinyl (SPC) radical (**34**), once formed, should undergo ring opening more rapidly than the cyclopropylcarbinyl (CPC) radical, which has a ring strain of 27.6 kcal/mol[20] and fragments at a rate of 10^8 s^{-1} at 25 °C.[21] It has been shown that incorporation of the CPC moiety into a more strained system,[22] and/or the addition of multiple methyl

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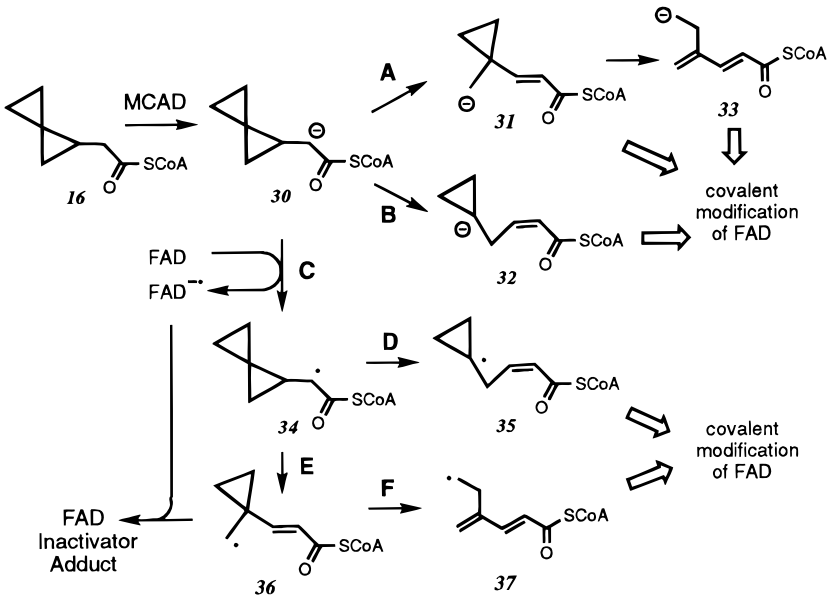
(21) (a) Griller, D.; Ingold, K. U. *Acc. Chem. Res.* **1980**, *13*, 317. (b) Nonhebel, D. C. *Chem. Soc. Rev.* **1993**, 347. (c) Newcomb, M. *Tetrahedron* **1993**, *49*, 1151.

(22) (a) Newcomb, M.; Manek, M. B.; Glenn, A. G. *J. Am. Chem. Soc.* **1991**, *113*, 949. (b) Bowry, V. W.; Lusztyk, J.; Ingold, K. U. *J. Am. Chem. Soc.* **1991**, *113*, 5687. (c) Eaton, P. E.; Yip, Y. C. *J. Am. Chem. Soc.* **1991**, *113*, 7692. (d) Choi, S.-Y.; Eaton, P. E.; Newcomb, M.; Yip, Y. C. *J. Am. Chem. Soc.* **1992**, *114*, 6326.

Scheme 6



groups[22b] or phenyl groups[23] to stabilize the product from a CPC ring opening, could accelerate the radical rearrangement rate constants by 1−3 orders of magnitude. In fact, a recent study of the ring opening of (2-methylenecyclopropyl)carbinyl radical (analogue of **7**), led to a rate constant of $(3-4) \times 10^9$ s^{-1} at 5 °C, which at 25 °C was estimated to be $(6-7) \times 10^9$ s^{-1}.[24] A proportionate rate acceleration for SPC radical is expected on the premise that the ring strains of methylenecyclopropyl system (**5**) and spiropentyl skeleton (**16**) are at least comparable.[25] Studies by Newcomb et al. on secondary ethoxycarbonyl-substituted radicals showed that these radicals fragment as fast or faster than their alkyl radical counterparts due to transition state polarization effects.[26] Since **34** is a secondary ester-substituted radical, its ring-opening rate may also be further enhanced by transition-state polarization effects. Thus, the nonstereospecificity of ring scission observed for SPA-CoA is consistent with a radical mechanism that may be considered to be spontaneous and consequently may bypass the chiral discrimination normally imposed by the enzyme.

As depicted in Scheme 6, rupture of the spiropentyl system (**34**) could generate either a tertiary cyclopropyl (**35**) or a primary cyclopropylcarbinyl radical (**36**) (routes D and E, respectively). Consecutive ring opening of **36** could afford an acyclic radical intermediate **37** (route F).[27] The one-electron-reduced flavin semiquinone could be intercepted by any and/or all of these transient radical species leading to irreversible, covalent modification of the cofactor and ultimately enzyme inactivation. On the other hand, electron rebound from the flavin semiquinone to reduce these inhibitor radicals prior to their covalent coupling with the oxidized flavin coenzyme is also feasible. It should be pointed out that there are at least two common alkylation sites on FAD, the N_5 and C_{4a} on the isoalloxazine ring, both of which are receptive to covalent bond formation with the putative reactive intermediate(s).[8d,e,28] Thus, a blending of these possibilities furnishes a series of structural candidates for the inhibitor−FAD adduct(s) (Scheme 7).[29] Limited by the availability and affordability of the labeled precursors, only two ^{13}C-labeled SPA-CoA were synthesized (see Scheme 5), and the HMQC NMR spectrum of the modified enzyme is shown in Figure 5. The complexity of the spectrum indicates the presence of more than one species which are likely formed by joining FAD and the ring-opened intermediates in various combinations (Scheme 7). While the ^{13}C peak at 42 ppm may be ascribed to a N-linked carbon shown in **38**, an unambiguous identification of this species and the assignment of other signals in Figure 5 must await more spectral analysis and comparison with appropriate model compounds. However, the most valuable information revealed in Figure 5 is the lack of any resonance in the downfield region. Since none of the labeled carbons become sp^2 hybridized in the adducts, secondary ring opening appears to be unlikely. Thus, compounds **39** and **40** may be eliminated as possible structures.

(23) (a) Castellino, A. G.; Bruice, T. C. *J. Am. Chem. Soc.* **1988**, *110*, 7512. (b) Newcomb, M.; Manek, M. B. *J. Am. Chem. Soc.* **1990**, *112*, 9662. (c) Newcomb, M.; Johnson, C. C.; Manek, M. B.; Varick, T. R. *J. Am. Chem. Soc.* **1992**, *114*, 10915.

(24) Horner, J. H.; Johnson, C. C.; Lai, M.-t.; Liu, H.-w.; Martin-Esker, A. A.; Newcomb. M.; Oh, E. *Bioorg. Med. Chem. Lett*. **1994**, *4*, 2693.

(25) The ring strain of methylenecyclopropane has been estimated to be 40.8 kcal/mol.[20] It should be noted that the apparently greater ring strain associated with the spiropentyl system is misleading since it contains one more cyclopropyl ring than the methylenecyclopropane case. Interestingly, recent calculations showed that the major source of the "strain" resulting from the introduction of a trigonal center into cyclopropane is not the increase in angle strain but the loss of a very strong cyclopropane C−H bond (Johnson, W. T. G.; Borden, W. T. *J. Am. Chem. Soc*. **1997**, *119*, 5930).

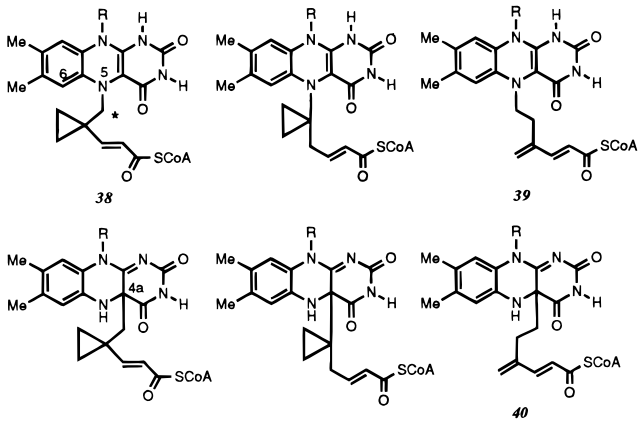
(26) Newcomb, M.; Horner, J. H.; Emanuel, C. J. *J. Am. Chem. Soc*. **1997**, *119*, 7147.

(27) Cyclopropylcarbinyl radicals (such as **34**) readily undergo β-scission because the SOMO can assume an eclipsed conformation with respect to a β,γ bond. On the contrary, cyclopropyl radicals (such as **35**) cannot attain such a conformation without the development of great strain, and therefore, β-scission is hampered due to a much higher activation energy (Kennedy, A. J.; Walton, J. C.; Ingold, K. U. *J. Chem. Soc., Perkin Trans. 2* **1982**, 751).

(28) (a) Olson, S. T.; Massey, V.; Ghisla, S.; Whitfield, C. D. *Biochemistry* **1979**, *18*, 4724. (b) Ghisla, S.; Olson, S. T.; Massey, V.; Lhoste, J. M. *Biochemistry* **1979**, *18*, 4733. (c) Ghisla, S.; Ogata, H.; Massey, V.; Schonbrunn, A.; Abeles, R. H.; Walsh, C. T. *Biochemistry* **1976**, *15*, 1791. (d) Schonbrunn, A.; Abeles, R. H.; Walsh, C. T.; Ghisla, S.; Ogata, H.; Massey, V. *Biochemistry* **1976**, *15*, 1798. (e) Zeller, H. D.; Ghisla, S. In *Flavins and Flavoproteins*; Edmondson, D. E., McCormick, D. B., Eds.; Walter de Gruyter: Berlin, 1987; p 161.

(29) The structures listed in Scheme 7 for the inhibitor−enzyme adducts are by no means a complete set of all possible variations. For example, a fully oxidized C-6-substituted flavin has also been proposed as the structure of a minor component isolated from the incubation of MCPA-CoA (**5**) with MCAD (Zeller, H. D.; Ghisla, S. In *Flavins and Flavoproteins*; Edmondson, D. E., McCormick, D. B., Eds.; Walter de Gruyter: Berlin, 1987; p 161).

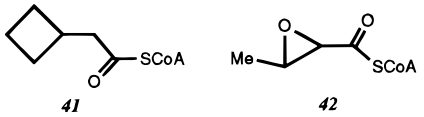
Scheme 7



The inference that no secondary ring cleavage occurs is in agreement with a radical mechanism, since the rate of the ring cleavage of **36** is expected to be significantly retarded comparing to that of the first ring opening due to the decrease of ring strain. This prediction is also consistent with the slow ring-opening rate observed by Bowry et al. on the β-(methylcyclopropyl)-carbinyl radical, which is a related mimic of **36**.[22b] More importantly, a rapid electron rebound from flavin semiquinone may result in quenching of radical **36** to generate the corresponding anion **31** and thus prevent further ring cleavage.

Summary and Conclusion

In summary, we have extended our inquiry to understand the mechanistic intricacies of an important class of enzymes, acyl-CoA dehydrogenases, by conducting detailed mechanistic investigations with an unnatural inhibitor, SPA-CoA (**16**). In contrast to an early report, this compound was found to be a mechanism-based inactivator for pig kidney MCAD and a recombinant SCAD originally derived from *M. elsdenii*. Using tritium-labeled SPA-CoA (**18**) in the incubation revealed the formation of an irreversible and covalent linkage between SPA-CoA and the FAD cofactor of MCAD and SCAD. The tritium washout results provided direct evidence that C_α proton abstraction is a requisite step of inactivation. On the basis of the HMQC analysis with ^{13}C-labeled SPA-CoA samples (**23** and **24**), the single-ring-opened radicals (**36** and perhaps **35**) are postulated as the reactive intermediates responsible for FAD adducts (Schemes 6 and 7).[30] SPA-CoA in enantiomerically pure forms (**19** and **20**) were synthesized, and both isomers were shown to be competent inactivators for MCAD with nearly identical partition ratios ($k_{cat}/k_{inact} \approx 2$). Clearly, the inactivation is nonstereospecific with respect to the ring cleavage. Since the C_β−H cleavage of the *trans* dehydrogenation step in normal catalysis is *pro-R* specific, the lack of stereospecificity of C_β−C bond rupture for SPA-CoA strongly suggests that this ring-opening step leading to enzyme inactivation is likely a spontaneous event induced by a spiropentylcarbinyl radical (**34**). These results provided further evidence supporting that MCAD is capable of mediating one-electron redox chemistry. Similar studies were also performed with SCAD. Interestingly, the (*R*)-SPA-CoA (**19**) was found to be a mechanism-based irreversible inhibitor and the (*S*)-SPA-CoA (**20**) was only a competitive inhibitor of SCAD. Recently, cyclobutylacetyl-CoA (**41**, CBA-CoA) has been shown to be a substrate for MCAD, but an inhibitor for SCAD by forming a covalent adduct with its flavin coenzyme.[31] Another related example is (*E*)-2,3-epoxybutyryl-CoA (**42**), which is an irreversible inactivator for SCAD but a



competitive inhibitor for MCAD.[15] The selectivity exhibited by these compounds on inhibition against two closely related dehydrogenases is likely a consequence of the distinct steric and electronic demands imposed by the active sites of MCAD and SCAD. Further investigation 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 Beckman LS 3801 counter using Ecoscint A biodegradable scintillation solution from National Diagnostics (Manville, NJ). Analytical GC was performed on an HP 5890 instrument, using a cross-linked phenylmethyl silicone gum fused silica capillary column (0.53 mm × 30 m). High-performance liquid chromatography (HPLC) 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 Varian U-300 or U-500 spectrometer. Chemical shifts are reported on the δ scale relative to the 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). TLC spots were visualized either with UV light or by dipping the plates 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 after 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 (THF) was dried over sodium and benzophenone; methylene chloride, dimethyl sulfoxide (DMSO), and dimethylformamide (DMF) were dried over calcium hydride; pyridine and triethylamine were dried over KOH. It should be noted that the tritium-labeled compounds reported in this paper were not submitted for NMR and/or mass spectroscopic analysis due to the possibility of radioactive contamination. However, satisfactory analytical results were obtained with unlabeled and/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 *M. elsdenii* SCAD gene (pWTSCADT$_{7-7}$).[3f] The medium-chain acyl-CoA dehydrogenase (MCAD), from pig kidney, was purified according to the procedure of Thorpe.[12] Concentration of the holoenzyme was determined spectrophotometrically on the basis of 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.

(30) Ab initio calculations were carried out to compute the energies of primary cyclopropylcarbinyl and tertiary cyclopropyl radicals using an unrestricted Hartree−Fock level of theory with 6-31G* basis set (UHF/6-3/G*). The results suggested that the energy difference is about 5 kcal/mol, favoring the primary cyclopropylcarbinyl radical. These energy differences reflect the bond dissociation energies (BDEs) of primary alkyl C−H versus the tertiary cyclopropyl C−H bonds (Borden, W. T. Personal communication).

(31) Shin, I.; Li, D.; Becker, D. F.; Stankovich, M. T.; Liu, H.-w. *J. Am. Chem. Soc.* **1994**, *116*, 8843.

Enzyme Assay. The enzyme activity was determined by a published procedure using phenazine methosulfate (PMS) or phenazine ethosulfate (PES) as an external 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,12]

Determination of Partition Ratio. A series of sample solutions each containing 13.0 mM MCAD (or 9.0 mM SCAD) and an appropriate amount of **19** or **20** were prepared in 400 mL of 50 mM potassium phosphate buffer, pH 7.6, to give $[I]_0/[E]_0$ ratios of 1.0−100. These samples were stirred gently at 25 °C for 1 h. Subsequently, an aliquot of 50 mL was taken from each sample and was added to the assay cocktail. The activity was measured immediately. The control experiments were carried out in the same manner without an inactivator. The partition ratio was deduced by plotting the residual activity versus $[I]_0/[E]_0$.

Kinetic Analysis of Inactivation. In a typical inactivation experiment, an appropriate amount of inactivator (**16**, **19**, or **20**) was added to the enzyme solution (MCAD, 13.0 mM; SCAD, 9.0 mM) in 400 mL of 50 mM potassium phosphate buffer, pH 7.6, at 25 °C. At various time intervals, aliquots (50 mL) were removed and diluted with assay cocktail (0.7 mL) and the residual enzyme activity was determined. For the "protection" experiment, the enzyme (SCAD 14.4 mM; MCAD 13.0 mM) was co-incubated with MCPA-CoA (**5**, 27.0 mM) and compound **16** (46.6 mM) at 25 °C. The rate of flavin chromophore bleaching was monitored at 450 nm. Meanwhile, the residual enzyme activity was also determined over time as described above.

Tritium Washout Study. The tritium-labeled inactivator (**18,** 20 nmol, 0.32 mCi/mmol) was incubated with enzyme (SCAD or MCAD, 40.0 nmol) in 500 mL of 50 mM potassium phosphate buffer (pH 7.6) at 25 °C. A 100-mL aliquot of the reaction mixture was removed at time zero and counted for radioactivity. After a period of 60 min, the incubation was quenched by addition of activated charcoal (10% solution), and the resulting suspension was mixed vigorously on a vortex mixer for 1 min. The suspension was centrifuged, and the supernatant was removed and analyzed for radioactivity by a scintillation counter. These readings were calibrated against controls prepared in parallel with boiled enzyme (100 °C, 10 min).

Determination of Reversibility of Inactivation and Stoichiometry of Tritium Incorporation. An appropriate amount of enzyme (SCAD or MCAD) was incubated with SPA-CoA (**16**) and/or the labeled SPA-CoA (**18**) (10 equiv) for an extended period until no further inactivation ensued. The incubation mixture was dialyzed against 50 mM potassium phosphate buffer, pH 7.6, at 4 °C for 2 days with 10 buffer changes. The residual enzyme activity of each of the incubation mixtures was determined before and after dialysis. Further, aliquots (50 mL) of the "labeled-incubation" mix (with **18**) were removed at various time intervals and submitted for scintillation counting. The readings were calibrated against a control prepared in parallel with boiled enzyme. In addition, a portion of the inactivated enzyme, devoid of unbound inactivator **18**, was treated with 90% MeOH to denature the protein. After centrifugation, the radioactivity in the supernatant and in the precipitate was determined by a scintillation counter.

Treatment of Reduced Flavin Adducts with Ferricenium Hexafluorophosphate ($FcPF_6$). The ferricenium salt ($FcPF_6$) was prepared according to a literature procedure.[7d] In a typical experiment, enzyme (MCAD, 5 nmol) was incubated aerobically with SPA-CoA (**16**, 25 nmol) until no further inactivation (monitored by FAD bleaching and enzyme activity) occurred. At which time, $FcPF_6$ (15 equiv) was added in three portions; the reoxidation of the flavin chromophore was monitored at 450 nm and the remaining enzyme activity before and after ferricenium treatment was also determined. A control experiment was run in parallel in which the SPA-CoA was substituted by the natural substrate, octanoyl-CoA (10 nmol), and/or MCPA-CoA (**5**, 25 nmol).

Two-Dimensional $^1H\{^{13}C\}$ HMQC Analysis. The ^{13}C-labeled inhibitors **23** and **24** (5 mol equiv) were separately incubated with MCAD (20 mg, 0.47 mmol) in 50 mM potassium phosphate buffer (pH 7.6) at room temperature. The reaction was incubated for 1 h to ensure complete inactivation and then ultrafiltered through an Amicon YM-10 filter to remove unreacted SPA-CoA and any possible turnover products. The resulting mixture was concentrated to 500 mL, transferred into a NMR tube, and subjected to $^{13}C-^1H$-detected HMQC analysis on a Varian 500 spectrometer.

2-[2-(1-Chloro-1-methylcycloprop-2-yl)ethoxy]tetrahydropyran (11). To a chilled solution (0 °C) of 3,4-dihydro-2*H*-pyran (23.5 g, 279 mmol) in anhydrous ether (150 mL) were added *p*-toluenesulfonic acid (0.1 g) and 3-buten-1-ol (**10**; 5 g, 69 mmol). The resulting mixture was stirred at room temperature for 5 h and was then quenched by the addition of concentrated ammonium hydroxide (1 mL) and methanol (10 mL). The solvent was evaporated in vacuo, and ether was added to the residue. The precipitated ammonium *p*-toluenesulfate was filtered, the filtrate was concentrated, and the crude product was purified by flash column chromatography (0−5% ethyl acetate in hexanes) to give 2-(3-butenyloxy)tetrahydropyran in nearly quantitative yield (10.8 g): ^1H NMR ($CDCl_3$) δ 5.83 (1H, m, ═CH−C), 5.08 (1H, dd, J = 17.2, 1.8, ═CH_2), 5.02 (1H, m, ═CH_2), 4.59 (1H, m, 2-H of THP), 3.79 (2H, m, CH_2O)*, 3.46 (2H, m, CH_2O of THP)*, 2.34 (2H, m, CH_2CH═CH_2), 1.80−1.50 (6H, m, Hs of THP); ^{13}C NMR ($CDCl_3$) δ 135.3 (═CH), 116.3 (CH_2═), 98.8 (C-2 of THP), 66.8 (side chain CH_2O), 62.3 (CH_2O of THP), 34.2 (*C*H_2CH═CH_2), 30.7, 25.5, 19.6 (Cs of THP).

A total of 10.8 g (69 mmol) of 2-(3-butenyloxy)tetrahydropyran and 8.4 g (85 mmol) of 1,1-dichloroethane were mixed in 50 mL of anhydrous ether. The solution was cooled to between −30 and −40 °C and maintained at that temperature throughout the 3.5-h addition of 48 mL of 1.6 M *n*-butyllithium in hexane. After the addition was complete, the cooling bath was removed and the solution was stirred for 12 h at 20 °C. The reaction was quenched by the addition of 20 mL of water, and all the salts present in the flask were dissolved within 15−20 min. The organic layer was collected and concentrated. The residual light yellow oil was purified by flash column chromatography (5% ethyl acetate in hexanes) to give **11** in 61% yield (9.2 g): ^1H NMR ($CDCl_3$) δ 4.60 (1H, m, 2-H of THP), 3.84 (2H, m, CH_2O), 3.50 (2H, m, CH_2O of THP), 1.92−1.53 (10H, m), 1.59 (3H, s, CH_3), 0.92 (1H, m); ^{13}C NMR ($CDCl_3$) δ 99.1 (C-2 of THP), 67.1 (CH_2O), 62.4 (CH_2O of THP), 45.6, 31.2, 30.0, 25.7, 23.0, 22.9, 22.0, 19.8.

2-[2-(Methylenecycloprop-2-yl)ethoxy]tetrahydropyran (12). Compound **11** (9.2 g, 42 mmol) was added dropwise over a period of 30 min to an anhydrous dimethyl sulfoxide solution (15 mL) containing 5.1 g (46 mmol) of potassium *tert*-butoxide. After stirring at 70 °C for 7 h, the reaction was cooled slowly to ambient temperature and then poured into ice water. The aqueous solution was extracted with ether (3 × 50 mL), and the pooled ether extracts were washed with brine, dried, filtered, and concentrated in vacuo. The crude residue was purified by flash column chromatography (5% ethyl acetate in hexanes) to give **12** in 83% yield (6.3 g): ^1H NMR ($CDCl_3$) δ 5.43, 5.41 (1H each, d, J = 1.2, ═CH_2), 4.60 (1H, t, J = 3.2, 2-H of THP), 3.80 (2H, m, CH_2O), 3.48 (2H, m, CH_2O of THP), 1.84−1.51 (9H, m), 1.24 (1H, m), 0.78 (1H, m); ^{13}C NMR ($CDCl_3$) δ 103.6 (═CH_2), 97.8 (C-2 of THP), 69.5 (CH_2O), 61.7 (CH_2O of THP), 30.2, 25.0, 19.1, 15.0, 8.3.

(Methylenecyclopropyl)acetic Acid (13). Compound **12** (6.3 g, 34.6 mmol) and *p*-toluenesulfonic acid monohydrate (1.9 g, 0.01 mol) were dissolved in 200 mL of methanol. This mixture was stirred at room temperature overnight. Methanol was removed in vacuo, and the residue was diluted with water. The aqueous layer was extracted with ether, and the combined ether layers were dried, filtered, and concentrated to give a colorless oil in 60% yield (1 g): ^1H NMR ($CDCl_3$) δ 5.42 (1H, s, ═CH_2), 5.37 (1H, s, ═CH_2), 3.72 (2H, t, J = 6.3, CH_2OH), 1.68−1.46 (3H, m, CH_2CH_2OH and 1-*H*), 1.26 (1H, m, 3-H), 0.78 (1H, m, 3-H); ^{13}C NMR ($CDCl_3$) δ 136.1 (═CH_2), 103.0 (CH_2═*C*), 62.5 (CH_2OH), 36.0 (*C*H_2CH_2OH), 12.6 (C-1), 9.2 (C-3).

(Methylenecyclopropyl)ethanol (1 g, 10 mmol) obtained above was dissolved in 30 mL of acetone. Jones reagent was added dropwise at 0 °C until the color of the reaction mixture remained red brown. The resulting solution was stirred for 1 h. The excess oxidizing reagent was quenched with 2-propanol. The reaction solution was then diluted with water followed by repeated extraction with ether. The combined ether layers were extracted with 10% NaOH solution (3 × 50 mL), and the combined aqueous phases were acidified to pH < 2 with 6 N HCl. The resulting solution was re-extracted with three 50-mL portions of ether, and the pooled organic phases were dried and concentrated in

vacuo to give **13** as a colorless oil in 70% yield (0.8 g): ^{1}H NMR ($CDCl_3$) δ 5.50, 5.41 (1H each, s, $=CH_2$), 2.37 (2H, d, $J = 7.2$, α-H), 1.70 (1H, m, 1-H), 1.37 (1H, m, 3-H), 0.88 (1H, m, 3-H); ^{13}C NMR ($CDCl_3$) δ 179.1 (C=O), 134.1 (C-2), 104.4 ($=CH_2$), 37.7 (CH_2CO_2H), 10.9 (C-1), 9.5 (C-3).

Methyl Spiropentylacetate (14). To a solution of **13** (200 mg, 1.8 mmol) in ether (10 mL) was added a solution of diazomethane (prepared from 2.5 g of diazald in 25 mL of ether) dropwise at 0 °C. The reaction was stirred for 10 min, followed by the addition of an equal amount of diazomethane in ether and 20 mg of $Pd(OAc)_2$. After the mixture was stirred for an additional 2 h, the resulting suspension was filtered through a Celite plug, concentrated, and purified by flash chromatography (2% ethyl ether in pentane) to give product **14** in 72% yield (181 mg): ^{1}H NMR ($CDCl_3$) δ 3.66 (3H, s, OCH_3), 2.38, 2.25 (1H each, dd, $J = 16.1$, 6,8, side chain CH_2), 1.38 (1H, m), 0.87 (1H, m), 0.79−0.72 (3H, m), 0.64 (1H, m), 0.55 (1H, m); ^{13}C NMR ($CDCl_3$) δ 173.8 (C=O), 51.5 (OCH_3), 37.5 (CH_2CO), 14.5, 13.3, 12.1, 6.1, 3.5.

Spiropentylacetic Acid (15). To a solution of methyl spiropentylacetate (180 mg, 1.3 mmol) in a mixture of 20 mL of ethanol and water (v/v = 4:1) was added sodium hydroxide (52 mg, 1.3 mmol). After refluxing for 10 min, the solution was acidified with 2 N HCl. The mixture was then extracted with methylene chloride, and the combined organic extracts were dried over magnesium sulfate. Subsequent filtration and concentration gave spiropentylacetic acid in 91% yield (149 mg): ^{1}H NMR ($CDCl_3$) δ 10.62 (1H, br s, COOH), 2.45, 2.30 (1H each, dd, $J = 16.4$, 6.8, CH_2CO), 1.38 (1H, m), 1.05 (1H, dd, $J = 7.7$, 4.4), 0.87−0.66 (4H, m), 0.58 (1H, t, $J = 4.4$); ^{13}C NMR ($CDCl_3$) δ 180.2 (C=O), 37.9 (CH_2CO), 14.8, 13.3, 12.3, 6.4, 3.8.

Spiropentylacetyl-CoA (16). Compound **16** was prepared from **15** in 76% yield (43 mg) according to a well-documented mixed anhydride procedure: ^{1}H NMR (^2H_2O) δ 8.59, 8.29 (1H each, s, adenine H's), 6.22 (1H, d, $J = 6.1$, ribose anomeric H), 4.91 (1H, buried under ^{2}HOH peak), 4.66, 4.31 (1H each, s, ribose H's), 4.32 (2H, s, ribose CH_2O), 4.09 (1H, s), 3.91 (1H, dd, $J = 9.7$, 4.7), 3.62 (1H, dd, $J = 9.7$, 4.6), 3.51 (2H, t, $J = 6.2$), 3.38 (2H, t, $J = 6.2$), 3.03 (2H, t, $J = 6.2$), 2.71, (1H, dd, $J = 16.4$, 6.5, CH_2CO of SPA), 2.50 (1H, dd, $J = 16.4$, 7.6, CH_2CO of SPA), 2.49 (2H, t, $J = 6.4$), 1.37 (1H, m), 1.05 (1H, dd, $J = 7.7$, 4.2), 0.96, 0.82 (3H each, s, Me's), 0.92−0.80 (2H, m), 0.76 (1H, m), 0.70 (1H, m), 0.62 (1H, t, $J = 4.2$); high-resolution FAB-MS calcd for $C_{28}H_{45}N_7O_{17}P_3S$ $(M + 1)^+$ 876.1806, found 876.1844.

Methyl [α-^{3}H]Spiropentylacetate (17). To a chilled (−78 °C) lithium diisopropylamide solution (3.2 mmol, prepared from 1.3 mL of *n*-butyllithium (2.5 M in hexane) and 0.32 g of diisopropylamine in THF) was added dropwise a solution of methyl spiropentylacetate (**14**, 306 mg, 2.14 mmol in 50 mL of THF). The resulting mixture was gradually warmed to 0 °C and stirred for 2 h. The reaction was cooled again to −78 °C and slowly mixed with 20 mL of ^3H_2O (S.A. 1.8 mCi/mmol). The reaction mixture was stirred at 0 °C for 1 h and quenched with ice-cold saturated ammonium chloride. After being stirred for another 10 min, the mixture was extracted with ether and the combined organic extracts were dried, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (2% ethyl ether in pentane) to afford product **17** in 95% yield (0.28 g). In a separate experiment, the reaction was quenched with ^2H_2O and the extent of isotope incorporation at α-C was estimated to be >90% on the basis of NMR integration.

[α-^{3}H]Spiropentylacetyl-CoA (18). By adapting a similar procedure used for the synthesis of spiropentylacetic acid (**15**) from methyl spiropentylacetate (**14**), the labeled analogue was obtained from methyl [α-^{3}H]spiropentylacetate (**17**) in 76% (0.13 g). The [α-^{3}H]spiropentylacetic acid was later coupled with coenzyme A to give the title compound in 72% yield (41 mg), with a specific activity of 0.32 mCi/mmol.

(*R*)- and (*S*)-Spiropentylacetyl-CoA (19 and 20). (*R*)- and (*S*)-Spiropentylacetyl-CoA (**19** and **20**) were obtained from the corresponding (methylenecyclopropyl)acetic acids (**21** and **22**, respectively) by following the procedure used for the synthesis of spiropentylacetyl-CoA (**16**) from (methylenecyclopropyl)acetic acid (**13**). Preparations of **21** and **22** have already been reported.[17]

3,4-Pentadien-1-ol (25). To a round-bottom flask equipped with a condenser fitted with a $CaCl_2$ drying tube were added 3-butyn-1-ol (10 g, 140 mmol), diisopropylamine (29 g, 286 mmol), paraformaldehyde (11 g, 360 mmol), and 275 mL of dry THF. The mixture was stirred vigorously as the copper(I) iodide (14 g, 73 mmol) was added in small portions. The reaction mixture was heated at a gentle reflux with stirring overnight. The resulting brown suspension was cooled to room temperature, filtered through a Celite plug and concentrated under reduced pressure. The dark brown residue was diluted with water and acidified with 3 N HCl. The aqueous layer was extracted with ether, and the combined ethereal layers were washed with water and brine, dried, filtered, and concentrated in vacuo to give a yellow-orange oil. The crude product was purified by flash chromatography (5% ethyl ether in pentane) to give the title compound **25** in 56% yield (6.7 g): ^{1}H NMR ($CDCl_3$) δ 5.09 (1H, m, $CH_2=C=CH-$), 4.68 (2H, m, $CH_2=C=CH-$), 3.45 (2H, td, $J = 7.0$, 7.0, CH_2OH), 2.23 (2H, m, CH_2CH_2OH), 1.66 (1H, br s, OH); ^{13}C NMR ($CDCl_3$) δ 86.2 ($CH_2=C=CH-$), 74.9 ($CH_2=C=CH-$), 61.6 (CH_2OH), 31.3 (CH_2CH_2OH).

[2′,4′-$^{13}C_2$](2-Hydroxyethyl)spiropentane (26). To a solution of **25** (48 mg, 0.57 mmol) and $[^{13}C]CH_2I_2$ (0.4 g, 1.43 mmol) in 5 mL of methylene chloride was added dropwise trimethylaluminum (1.45 mL, 2.85 mmol, 3.0 M in hexanes) over 10 min at 0 °C under nitrogen. The first equivalent should be added very slowly due to the exothermic formation of aluminum alkoxide. After the clear reaction mixture was stirred at room temperature for 16 h, it was cooled to 0 °C. The reaction was quenched with 5 mL of 1 N NaOH, and the resulting mixture was stirred vigorously for an additional 1 h. The organic layer was separated, and the aqueous layer was extracted with methylene chloride (2 × 5 mL). The combined organic layers were washed with water, dried, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (10:1 to 4:1 hexanes/ethyl acetate) to give the desired product **26** in 85% yield (54 mg): ^{1}H NMR ($CDCl_3$) δ 3.67 (2H, t, $J = 6.9$, CH_2OH), 1.75−0.63 (7H, m), 1.10 (1H, m), 0.47 (1H, m). Since the product is a mixture of diastereomers, ^{13}C NMR of **26** displays three signals at δ 12.05, 6.12, and 3.67 in a ratio of about 2:1:1. ^{13}C NMR ($CDCl_3$) of the unlabeled species: δ 62.9 (CH_2OH), 35.8 (CH_2CH_2OH), 22.3, 14.1, 12.1, 6.1, 3.7.

[2′,4′-$^{13}C_2$]Spiropentylacetic Acid (27). Compound **26** (54 mg, 0.48 mmol) was oxidized by Jones reagent (see the synthesis of **13** for procedure) to give the corresponding acid **27** in 71% yield (0.43 mg): ^{13}C NMR ($CDCl_3$) δ 12.1, 6.13, 3.50 (mixture of diastereomers).

[2′,4′-$^{13}C_2$]Spiropentylacetyl-CoA (23). Acid **27** (20 mg, 0.16 mmol) was coupled with coenzyme A (50 mg, 65.1 mmol) to give the final product **23** in 85% yield (48 mg): ^{13}C NMR (D_2O) δ 13.9, 7.71, 5.43 (mixture of diastereomers); high-resolution FAB-MS calcd for $^{13}C_2C_{26}H_{45}N_7O_{17}P_3S$ $(M + H)^+$ 878.1873, found 878.1858.

[4′-^{13}C](2-Hydroxyethyl)spiropentane (29). Synthesis of compound **29** was achieved by a procedure similar to that used to make compound **26**. In this experiment, 2.1 equiv of trimethylaluminum (0.54 mL, 2.0 M solution in hexanes) and 1.1 equiv of $[^{13}C]CH_2I_2$ (0.15 g, 0.56 mmol) were used for the cyclopropanation of alcohol **28** (50 mg, 51 mmol, an intermediate generated during the synthesis of **13** from **12**). The desired product was obtained in 82% yield (47 mg): ^{13}C NMR ($CDCl_3$) δ 6.17, 3.67 (mixture of diastereomers).

[4′-^{13}C]Spiropentylacetyl-CoA (24). Compound **24** was prepared in 73% combined yield (44 mg) from the corresponding acid which in turn was derived from **29**. ^{13}C NMR (D_2O) of the intermediate acid: δ 6.14, 3.50. ^{13}C NMR of **24**: δ 5.19, 2.89 (mixture of diastereomers). FAB-MS calcd for $^{13}C_1C_{27}H_{45}N_7O_{17}P_3S$ $(M + H)^+$: 877.2. Found: 877.2

Acknowledgment. We thank Dr. Vikram Roongta for providing technical assistance in obtaining the HMQC spectra and Professor Marian Stankovich for the generous gift of *E. coli* (pWTSCADT$_{7-7}$)-BL21(DE3)pLys strain. We owe a special debt of gratitude to Professor Weston Borden for sharing with us his computation results. This work was supported by a National Institutes of Health grant (GM40541). I.S. is a recipient of the Stanwood Johnston Memorial Fellowship from the University of Minnesota.

JA9737634