VOLUME 115, NUMBER 5
MARCH 10, 1993

JOURNAL
OF THE
AMERICAN
CHEMICAL
SOCIETY

Inactivation of Medium-Chain Acyl-CoA Dehydrogenase by a Metabolite of Hypoglycin: Characterization of the Major Turnover Product and Evidence Suggesting an Alternative Flavin Modification Pathway

Ming-tain Lai, Ding Li, Eugene Oh, and Hung-wen Liu*

Contribution from the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received September 18, 1992*

Abstract: Medium-chain acyl-CoA dehydrogenase (MCAD) is a FAD-dependent enzyme that catalyzes the first step of the fatty acid oxidation cycle. When MCAD is exposed to (methylenecyclopropyl)acetyl-CoA (MCPA-CoA), a metabolite of hypoglycin A and the causative agent of Jamaican vomiting sickness, time-dependent inactivation follows with concomitant bleaching of the active-site FAD. Earlier studies have led to the postulation that the inactivation may involve a spontaneous ring fragmentation induced by a transient α-cyclopropyl radical, and thus suggest a one-electron oxidation pathway. In an effort to find more evidence for the proposed mechanism, we have isolated and characterized the major turnover product, a CoA ester consisting of a disubstituted terminal olefin, an epoxide, and a hydroxymethyl group, from the aerobic incubation mixture of MCPA-CoA and MCAD. Formation of this product may be initiated by trapping the acyclic radical intermediate with O_2 to form a transient peroxy radical which, upon receiving one electron from flavin semiquinone followed by an intramolecular epoxidation, gives rise to the observed turnover product. The identification of such a highly oxygenated species as the major turnover product strongly sustains the intermediacy of a ring-opened radical, and as such, the departure from the expected inactivation may directly result from trapping of this radical intermediate by O_2. This contention was subsequently substantiated by observing that the partition ratio is nearly 0 under anaerobic incubation. Interestingly, further investigation of the anaerobic inhibition resulted in the discovery of a minor inactivation pathway involving covalent modification of flavin at a locus other than the isoalloxazine ring. Although the chemical nature of the new inhibitor–coenzyme adduct(s) has yet to be elucidated, a structure having MCPA-CoA linked to the N(10) ribityl side chain is appealing. The mechanistic insights derived from this study provide compelling evidence supporting our early notion that inactivation of MCAD by MCPA-CoA is likely to proceed through a radical mechanism.

Medium-chain acyl-CoA dehydrogenase (MCAD), also known as the general acyl-CoA dehydrogenase (GAD), is a flavin adenine dinucleotide (FAD) dependent enzyme which catalyzes the oxidation of medium-chain fatty acyl-CoA substrates to the corresponding *trans*-α,β-enoyl-CoA products with concomitant reduction of the active-site flavin in the first step of the fatty acid oxidation cycle.[1] This cycle is an important source of ATP in biological systems, as it supplies up to 90% of the ATP in cardiac muscle.[2] In addition to the essential role MCAD plays in the β-oxidation of fatty acids, the catalytic pathway this enzyme follows is mechanistically intriguing, since the desaturation step involves the cleavage of two kinetically stable C–H bonds. Evidence has accumulated supporting a C_α proton abstraction as the initial step leading to dehydrogenation with bond cleavage at both C_α and C_β being *pro-R* stereospecific.[3] When this enzyme is

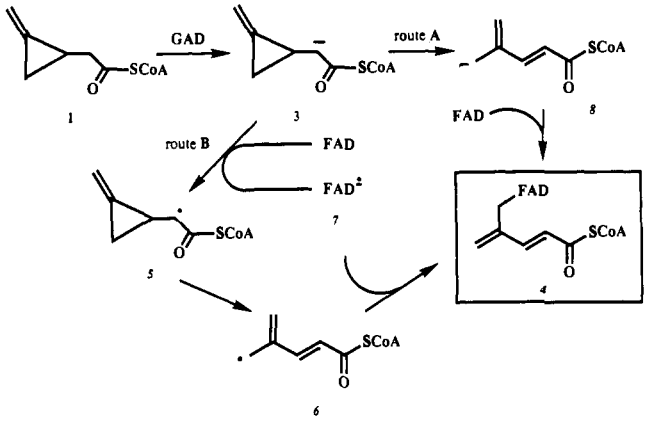
(1) (a) Thorpe, C.; Matthews, R. G.; Williams, C. H. *Biochemistry* **1979**, *18*, 331. (b) Ghisla, S.; Thorpe, C.; Massey, V. *Biochemistry* **1984**, *23*, 3154. (c) Pohl, B.; Raichle, T.; Ghisla, S. *Eur. J. Biochem.* **1986**, *160*, 109. (d) Ghisla, S. In *Flavins and Flavoproteins*; Brays, R. C., Engel, P. C., Mayhen, S. G., Eds.; Walter de Gruyter: Berlin, 1984; p 385 and references cited therein.

(2) Bremer, J.; Osmundsen, H. In *Fatty Acid Metabolism and Its Regulation*; Numa, S., Ed.; Elsevier Science Publisher: New York, 1984; p 141.

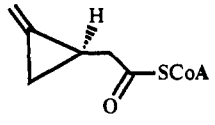
(3) (a) Biellmann, J. F.; Hirth, C. G. *FEBS Lett.* **1970**, *9*, 55, 335. (b) Bucklers, L.; Umani-Ronchi, A. S.; Rêtey, J.; Arigoni, D. *Experientia* **1970**, *26*, 931.

0002-7863/93/1515-1619$04.00/0

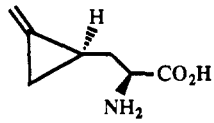
Scheme I



exposed to (methylenecyclopropyl)acetyl-CoA (MCPA-CoA, **1**), a metabolite of hypoglycin A (**2**) that causes Jamaican vomiting sickness,[4] time-dependent inactivation ensues with concomitant bleaching of the active-site FAD.[5] Analogous to the normal



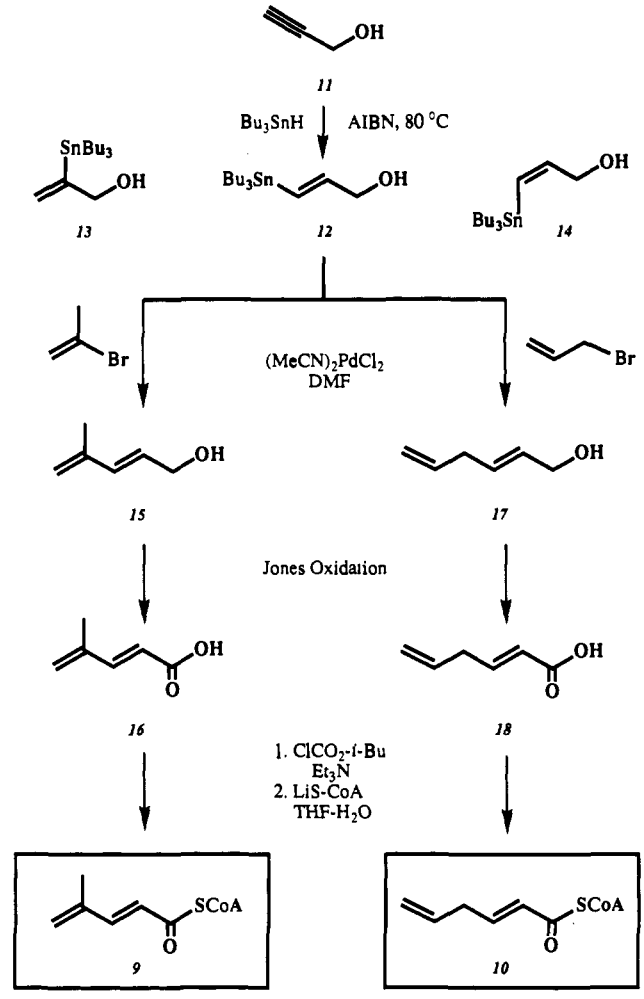
(1*R*)-MCPA-CoA (*1*)



HYPOGLYCIN A (*2*)

catalysis, the mechanism of this inhibition is widely believed to advance via a C_α deprotonation, (**3**), followed by ring fragmentation and covalent modification of the flavin coenzyme (**4**) (Scheme I, route A).[5] Such direct anion-induced ring opening leading to inhibition is mechanistically lucid; however, this inactivation proceeds in a nonstereospecific manner in contrast to the *pro-R* stereospecificity for the productive route, since the two C-1 epimers of MCPA-CoA are both effective inhibitors.[6] That the partition ratios of the (1*R*)- and (1*S*)-MCPA-CoA are nearly identical lends further credence to this conclusion.[6b,d] Since the rearrangement of an α-cyclopropyl radical to the straight-chain alkyl radical is extremely rapid,[7] such nonstereospecific inactivation could be envisaged as a spontaneous ring fragmentation induced by a transient α-cyclopropyl radical **5**, with subsequent modification of FAD resulting from the recombination of the nascent acyclic radical **6** with the flavin semiquinone **7**. This explanation supports a one-electron oxidation pathway (Scheme I, route B) as opposed to the anion-induced ring cleavage mechanism (Scheme I, route A).[8] Although the nonstereospecificity of C_β bond

Scheme II



cleavage of MCPA-CoA may be a consequence of opposite binding preferences of the two C-1 epimers at either side of the flavin ring in a mirror-image orientation, studies of the inactivation of MCAD by (1*R*)- and (1*S*)-MCPA-CoA bearing a stereospecific tritium label at C_α have provided direct and revealing evidence that the two diastereomers of MCPA-CoA bind to the same locus in the active site of MCAD.[6d] A recent study dealing with the α- and β-deuterium kinetic isotope effects on this inactivation has also dismissed the interconversion between (1*R*)- and (1*S*)-MCPA-CoA by way of (2-methylenecyclopropylidene)acetyl-CoA as a possible cause for the lack of stereospecificity of ring cleavage of MCPA-CoA.[9] As the partition ratio defines the number of latent inhibitor molecules converted to product relative to each turnover leading to enzyme inactivation,[10] the ratio of ca. 3 found for this inactivation clearly indicates that MCAD is capable of turning over the latent inhibitor, prior to being inactivated. In view of the fact that this action of MCAD on the inhibitor is expected to occur via a course similar to that of inactivation, these mechanistic ambiguities of whether route A or B is the more likely inactivation pathway may thus be resolved if the turnover product(s) can be isolated and characterized. Recently we have reported the structural elucidation of a major turnover product from the enzymatic incubation of MCPA-CoA under aerobic conditions.[11] Presented in this paper are the detailed accounts on the isolation and structural determination of this turnover product and a comparison of the results obtained under aerobic and anaerobic incubation. Interestingly, analysis of the inactivation

(4) (a) Tanaka, K. In *Handbook of Clinical Neurology*; Vinken, P. J., Bruyn, G. W., Eds.; Elsevier-North Holland: Amsterdam, 1979; Vol. 37, Chapter 17, p 511. (b) Stuart, K. L. In *Hypoglycin*; Kean, E. A., Ed.; Academic Press: New York, 1975; p 39. (c) Osmundsen, H. *Trends Pharmacol. Sci.* **1986**, *7*, 186. (d) Tanaka, K.; Ikeda, Y. In *Fatty Acid Oxidation: Clinical, Biochemical, and Molecular Aspects*; Tanaka, K., Coates, P. M., Eds.; Alan R. Liss, Inc.: New York, 1990; p 167.

(5) (a) Osmundsen, H.; Sherratt, H. S. A. *FEBS Lett.* **1975**, *55*, 81. (b) Kean, E. A. *Biochem. Biophys. Acta* **1976**, *422*, 81. (c) Abeles, R. H. In *Enzyme Activated Irreversible Inhibitors*; Seiler, N., Jung, M. J., Koch-Weser, J., Eds.; Elsevier: Amsterdam, 1978; p 1. (d) Ghisla, S.; Wenz, A.; Thorpe, C. In *Enzyme Inhibitors*; Brodbeck, U., Ed.; Verlag Chemie: Weinheim, Germany, 1980; p 43. (e) Wenz, A.; Thorpe, C.; Ghisla, S. *J. Biol. Chem.* **1981**, *256*, 9809. (f) Ghisla, S.; Melde, K.; Zeller, H. D.; Boschert, W. In *Fatty Acid Oxidation: Clinical, Biochemical, and Molecular Aspects*; Tanaka, K.; Coates, P. M., Eds.; Alan R. Liss, Inc.: New York, 1990; p 185.

(6) (a) Lenn, N. D.; Shih, Y.; Stankovich, M. T.; Liu, H.-w. *J. Am. Chem. Soc.* **1989**, *111*, 3065. (b) Lai, M.-t.; Liu, H.-w. *J. Am. Chem. Soc.* **1990**, *112*, 4034. (c) Baldwin, J. E.; Ostrander, R. L.; Simon, C. D.; Widdison, W. C. *J. Am. Chem. Soc.* **1990**, *112*, 2021. (d) Lai, M.-t.; Liu, L.-d.; Liu, H.-w. *J. Am. Chem. Soc.* **1991**, *113*, 7388.

(7) (a) Griller, D.; Ingold, K. U. *Acc. Chem. Res.* **1980**, *13*, 317. (b) Stubbe, J. *Biochemistry* **1988**, *27*, 3893 and references cited therein. (c) Newcomb, M.; Glenn, A. G. *J. Am. Chem. Soc.* **1989**, *111*, 275.

(8) An alternate pathway has lately been put forward in which the inactivation is ascribed to the formation of a tightly bound complex between FAD and the inhibitor, instead of formation of the covalent adduct **4**: Tserng, K.-Y.; Jin, S.-J.; Hoppel, C. L. *Biochemistry* **1991**, *30*, 10755.

(9) Baldwin, J. E.; Widdison, W. C. *J. Am. Chem. Soc.* **1992**, *114*, 2245.

(10) Silverman, R. B. In *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press: Boca Raton, FL, 1988; Vol. 1, p 5.

(11) Lai, M.-t.; Liu, H.-w. *J. Am. Chem. Soc.* **1992**, *114*, 3160.

under anaerobic conditions led to the discovery of a new class of flavin-inhibitor adduct. These findings appear to support our early conclusion that MCAD is capable of mediating one-electron oxidation–reduction.

Results and Discussion

Preparation of Thioenoate Standards. Despite the ambiguity in the detailed mechanism of this inactivation, there is little doubt that covalent modification of the flavin coenzyme in the active site of MCAD is the major inactivation pathway and is triggered by ring fragmentation of the highly strained MCPA-CoA. Depending on the mode of the ring cleavage (Scheme I, route A or B), a conjugated δ-anion **8** or the corresponding acyclic radical **6** is likely to be generated as the nascent reactive intermediate which upon coupling with FAD leads to inactivation. Since MCPA-CoA, with its latent group unmasked, can partition to turning over and to inactivation, the ring-cleavage step may be the branching point, after which the aforementioned transient ring-opened species (**6** or **8**) may be processed to complete a catalytic cycle and be released as a product in competition with the inactivation. Thus, it is highly probable that the turnover products are the protonated acyclic thioenoate derivatives. In an attempt to test this hypothesis, we have chemically synthesized two of the possible ring-opened thioenate products (**9** and **10**, derived from allylic and vinylic bond cleavage, respectively) to be used as HPLC and NMR standards.

As depicted in Scheme II, the key intermediate, 3-(tri-*n*-butylstannyl)-2-propen-1-ol (**12**), was synthesized from propargyl alcohol (**11**) in the presence of 1.3 molar equiv of tributyltin hydride and a catalytic amount of azobis(isobutyronitrile) (AIBN).[12] It is essential that slight excess tin hydride is used in this hydrostannylation reaction, since excess stannane induces isomerization of the initially formed *Z* isomer to the desired *E* isomer,[12] which was readily separable from the mixture of **13** and **14** by flash chromatography. Cross coupling of **12** with 2-bromopropene catalyzed by bis(acetonitrile)dichloropalladium ($Pd(MeCN)_2Cl_2$) produced conjugated allyl alcohol **15**,[13] which upon treatment with Jones reagent gave **16** in 70% yield. Condensation of **16** with isobutyl chloroformate followed by coupling to coenzyme A in aqueous THF solution (pH 8–8.5)[6d,14] afforded the desired (*E*)-thioenoate **9**. The corresponding linear isomer **10** was prepared by coupling of allyl bromide with the same vinyltin precursor **12** via an identical sequence. The crude products were purified on a HPLC Partisil-C_{18} column (4.5 × 250 mm) eluted with 30% methanol in 50 mM potassium phosphate buffer, pH 5.3.[15] After removal of methanol in vacuo, the desired fractions were desalted by reversed-phase chromatography[15] and then lyophilized. Interestingly, both **9** and **10** exhibited similar retention times (ca. 25 min) under the HPLC purification conditions.

Enzymatic Incubation and Product Isolation. The inactivation was performed under aerobic conditions by mixing the purified enzyme (0.6 μmol)[16] with 3 molar equiv of chemically synthesized MCPA-CoA[6a] in 50 mM potassium phosophate buffer (pH 7.6) at room temperature. The incubation was continued for 15 min to ensure complete inactivation, and the resulting mixture, without further treatment, was loaded in batches onto an HPLC Partisil-C_{18} column (4.5 × 250 mm) and was eluted with 30% methanol in 50 mM potassium phosphate buffer (pH 5.3). The elution profile is shown in Figure 1. To our great surprise, the thioenoates **9** and **10**, which should be eluted at approximately 25 min, could not be detected. Instead, the major constituent in the incubation mixture was found in the peak with a retention time of 4.3 min which was absent in the control that was concurrently prepared with boiled enzyme. Except for two small humps at 8.3 and 10.8 min, other peaks having retention times of 3.9, 5.5, and 12.5 min

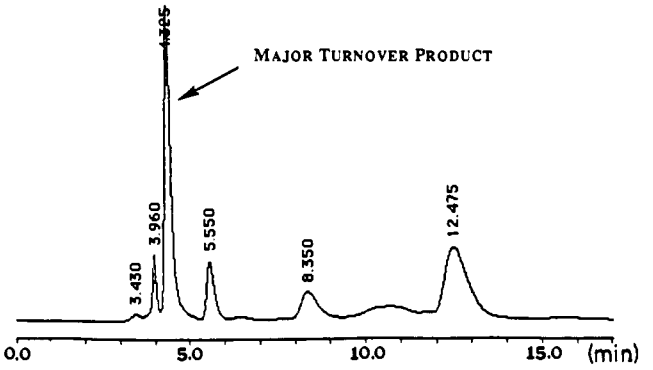


Figure 1. HPLC separation of the mixture resulting from the incubation of MCAD (0.6 μmol) with chemically synthesized MCPA-CoA (1.8 μmol) in 50 mM potassium phosphate buffer (pH 7.6) under aerobic conditions. The elution conditions and the assignment of the peaks are described in the text.

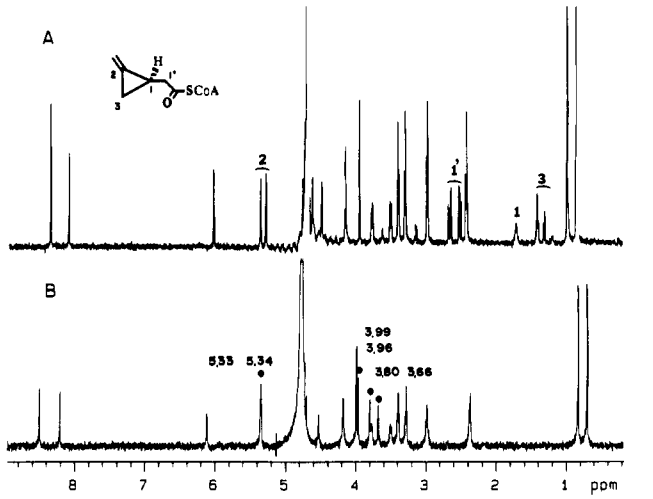


Figure 2. ^1H NMR (500 MHz, ^2H_2O) spectra of (1*R*)-MCPA-CoA (A) and the isolated major turnover product **19** (B).

were identified as coenzyme A, FAD, and unreacted MCPA-CoA, respectively. These two unknown species may be protein derivatives since they could be depleted by filtering the sample through a Microcon-10 microconcentrator (Amicon). Even if the two unknown species were derived from MCPA-CoA, integration of these peaks revealed that the major peak accounts for more than 60% of the overall turnover products. After removal of methanol in vacuo, the pooled fractions of the major peak were repurified by HPLC, desalted by reversed-phase chromatography (eluting with water and then methanol),[15] and then concentrated.

Characterization of the Major Turnover Product. The presence of the CoA moiety in the major turnover product was first suggested by the electronic spectrum of this compound, which exhibits an absorption maximum at 260 nm, analogous to that of coenzyme A. This speculation was substantiated by ^1H-NMR studies of this product, measured with a sample which was repeatedly dissolved in ^2H_2O and lyophilized prior to analysis. As shown in Figure 2B, its ^1H NMR spectrum displayed four sets of resonances, at δ 5.35 and 5.34 (1 H each), 3.99 and 3.96 (1 H each), 3.80 (1 H), and 3.68 (1 H), in addition to those of the coenzyme A. While this data clearly attested the retention of CoA as a structural entity in this compound, the disappearance of the characteristic resonances at δ 1.62, 1.27, and 1.06 for the cyclopropyl hydrogens (Figure 2A), and the signals at δ 2.57 and 2.42 for the side-chain methylene hydrogens, definitely indicated the destruction of the cyclopropyl group of the original MCPA-CoA skeleton. The chemical shifts of those new peaks observed for the product strongly suggested the retention of a terminal methylene moiety and the presence of a highly oxygenated framework in its structure. Although no coupling was found in the COSY analysis, a TOCSY experiment[17] with a mixing time

(12) Jung, M. E.; Light, L. A. *Tetrahedron Lett.* **1982**, 3851.
(13) Stille, J. K.; Groh, B. L. *J. Am. Chem. Soc.* **1987**, *109*, 813.
(14) Belcher, M. *Methods Enzymol.* **1981**, *72*, 404.
(15) Eberhard, A. *Chromatogram* **1987**, *8*, 10.
(16) Thorpe, C. *Methods Enzymol.* **1981**, *71*, 366.

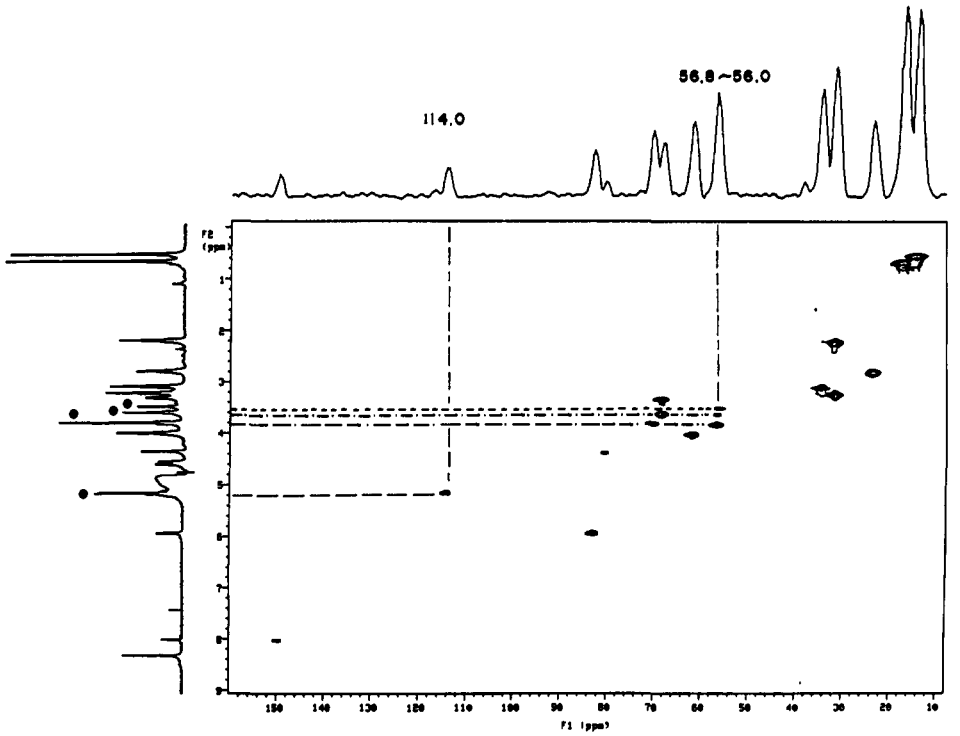
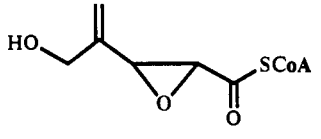


Figure 3. ^{13}C–^{1}H-detected HMQC spectrum of the isolated major turnover product **19**.

of 80 ms clearly revealed the correlation between the resonances of δ 5.35/5.34 and 3.99/3.96, as well as δ 3.80 and 3.68. Despite many attempts, direct MS measurement failed to detect the molecular ion of this compound. The major peaks of its high-resolution FABMS at 866.0141 and 887.9980 are clusters of the (CoA + Na^+ + $2K^+$) and (CoA + $2Na^+$ + $2K^+$) charge complexes, calculated for $C_{21}H_{34}N_7O_{16}P_3SK_2Na$ (866.0167) and $C_{21}H_{33}N_7O_{16}P_3SK_2Na_2$ (887.9987), respectively.

In order to gather further information to assist structural elucidation, ^{13}C NMR analyses were also attempted. However, owing to the scarcity of the sample, an indirect recording appeared to be more propitious. The chemical shifts of the carbon signals were measured by a ^{13}C–^{1}H-detected HMQC (heteronuclear multiple quantum coherence) experiment,[17] in which four pertinent peaks appearing at δ 114.0, 56.8, 56.4, and 56.0 were found to be the corresponding carbons bearing the δ 5.35/5.34, 3.99/3.96, 3.80, and 3.68 protons (Figure 3). Integration of the results cited above led to a structure of a CoA ester (**19**) for this major turnover product. The assigned structure is consistent with data obtained



19

from GC–MS analysis where the turnover product (**19**) was hydrolyzed, reduced with borane, and subsequently acetylated to afford the expected diacetate, which gave the expected molecular ion peak at $(M + 1)^+$ 215 (isobutane as the carrier gas) or $(M + 18)^+$ 232 (ammonia as the carrier gas).

Synthesis and Spectral Comparison of the Methyl Ester of 19. In order to confirm our structural assignment and to determine the relative configuration of the oxirane moiety in the proposed structure of this major turnover product, the corresponding (*E*)- and (*Z*)-methyl esters (**20** and **21**, respectively) were synthesized by the steps delineated in Scheme III. The initial hydrostannylation of methyl propiolate followed an analogous procedure used in the preparation of **12**, giving compounds **22** and **23** in a 1:1 ratio. Meanwhile, a palladium-catalyzed hydrostannylation of propargyl alcohol followed by iodination yielded 3-iodo-2-propen-1-ol (**24**) and the desired 2-iodo-2-propen-I-ol (**25**) in equal quantities.[12] After separation and derivatization, the protected 2-iodoallyl product **26** was coupled to vinylstannane **22** in a palladium-mediated cross-linking reaction giving (*E*)-methyl 4-[(*tert*-butyldimethylsiloxy)methyl]-2,4-pentadienoate (**27**) with retention of the double-bond geometry present in the coupling precursors.[13] Regioselective epoxidation converting **27** to **29** was accomplished by a procedure using *tert*-butyl peroxide and methyllithium.[18] The silyl protecting group of **29** was then removed by treatment with fluoride to afford the desired model compound **20**. Preparation of the corresponding *Z* isomer **21** was effected by an identical sequence, except for the addition of 1 equiv of acetic acid in the last deprotection step to prevent lactonization of the final product.

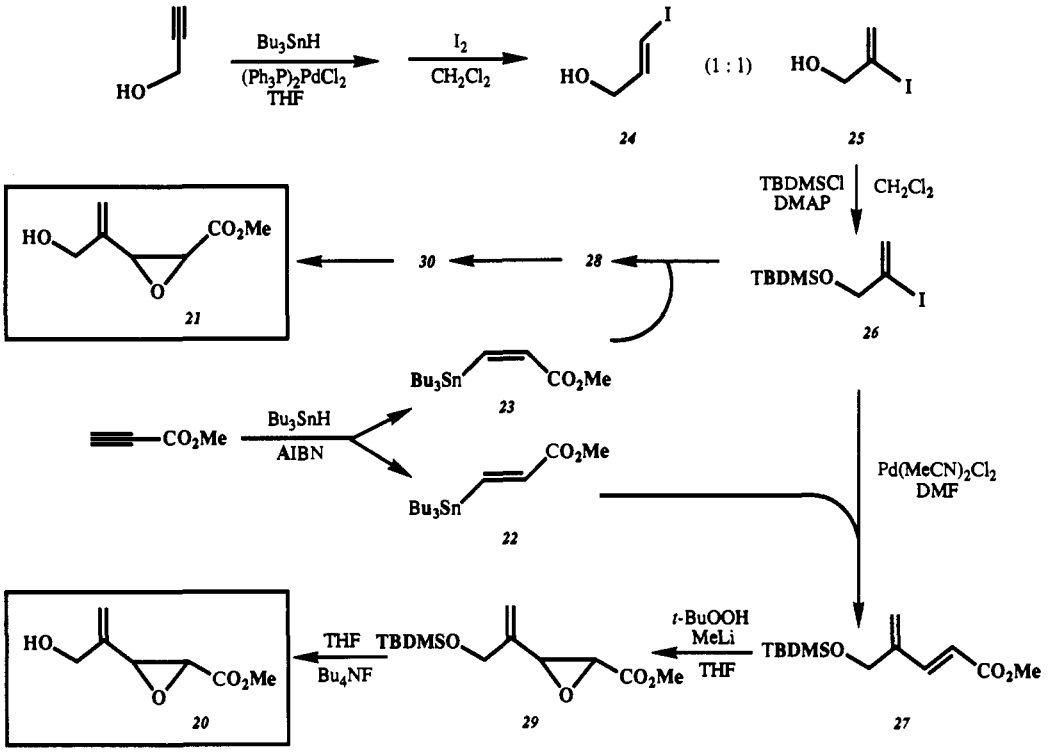
As can be seen in Figure 4, the chemical shifts and the splitting patterns of the NMR signals, especially the epoxy proton peaks of **19**, are closely related to those of **20**, but are quite distinct from those of **21**. Thus, the structure of the major turnover product is as proposed with the α,β-epoxy moiety in a trans geometry. The minor mismatching of the chemical shifts between the resonance of **19** and those of the methyl ester standard **20** may be attributed to the structural variation of the ester moieties and to the different solvents used for spectral measurements. Consistent with this assignment are the similar coupling constants of the epoxy proton of the trans methyl ester **20** and the turnover product (Figure 5).

The identification of **19** as the major turnover product of this inactivation strongly supports the intermediacy of ring-opened radical **6** which, after recombination with the flavin semiquinone, leads to covalent modification of FAD (Scheme I, route B). Since the reactions of carbon radicals with molecular oxygen are well documented, particularly those of cyclopropylcarbinyl and hom-

(17) For a good review of COSY, TOCSY, and HMQC experiments, see: Martin, G. E.; Zektzer, A. S. In *Two-Dimensional NMR Methods for Establishing Molecular Connectivity*; VCH Publishers: New York, 1988. The fact that no correlation was found by the COSY experiment may be attributed to the weak coupling between peaks.

(18) Clark, C.; Hermans, P.; Meth-Cohn, O.; Moore, C.; Taljaard, H. C.; van Vuuren, G. *J. Chem. Soc., Chem. Commun.* **1986**, 1378.

Scheme III



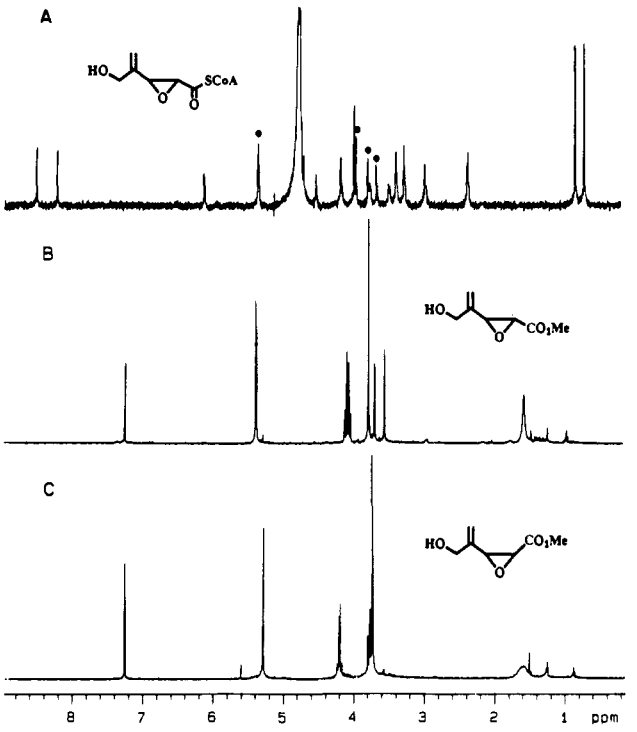


Figure 4. ^{1}H NMR (500 MHz, $CDCl_3$) spectra of the isolated major turnover product **19** (A) and the corresponding chemically synthesized (*E*)- and (*Z*)-methyl esters, **20** and **21** (B and C, respectively).

oallylic species,[19] the inactivation derailment may be envisioned as trapping the acyclic radical **6** with O_2 to form transient peroxy radical **31**, which upon reduction by one-electron transfer from the active-site-bound flavin semiquinone gives rise to peroxy anion

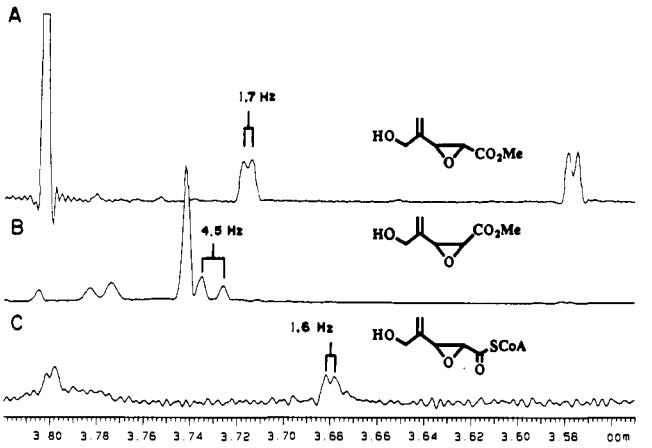


Figure 5. ^{1}H NMR (500 MHz, $CDCl_3$) spectra showing the epoxyl proton resonances of the isolated major turnover product **19** (A) and the corresponding chemically synthesized (*Z*)- and (*E*)-methyl esters, **21** and **20** (B and C, respectively).

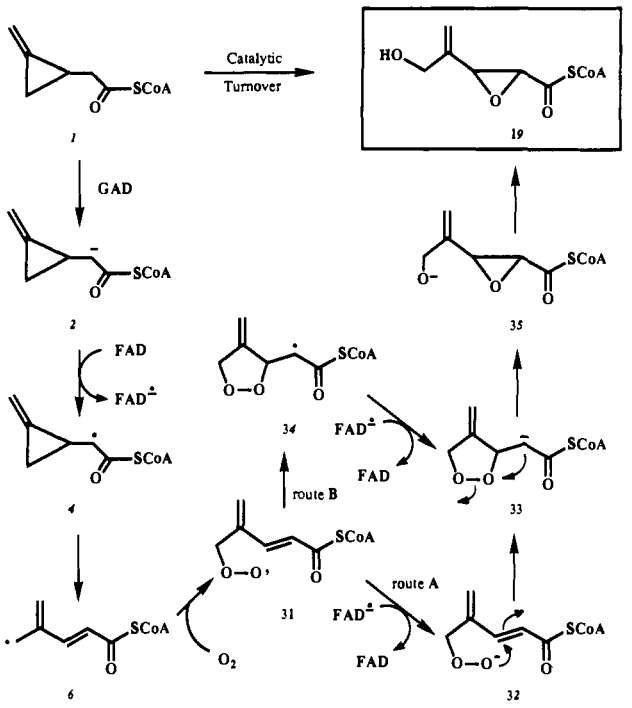
22. Because the partition ratio of this inactivation is approximately 3, reaction with oxygen instead of coupling with the flavin coenzyme is clearly a more facile process for the ring-opened radical intermediate **6**. As depicted in Scheme IV, this reroute culminates in an intramolecular epoxidation[20] converting **32** via 1,2-dioxolanylcarbinyl anion **33** to the observed turnover product **19**. An alternate mechanism could involve an intramolecular cyclization of **31** to produce 1,2-dioxolanylcarbinyl radical **34** and then a one-electron reduction to give the corresponding anion **33**.[21] The mechanistic insights derived from this study provide highly convincing evidence sustaining our early notion that inactivation of MCAD by MCPA-CoA is likely to proceed through a radical mechanism. These results may also be extrapolated to suggest that MCAD is capable of mediating one-electron oxidation–reduction.

(19) (a) Nakamura, E.; Inubushi, T.; Aoki, S.; Machii, D. *J. Am. Chem. Soc.* **1991**, *113*, 8980. (b) Beckwith, A. L. J. *Tetrahedron* **1981**, *37*, 3073. (c) Porter, N. A. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1980; Vol. IV, p 261. (d) Beckwith, A. L. J.; Ingold, K. U. In *Rearrangements in Ground and Excited States*; de Mayo, P., Ed.; Academic Press: New York, 1980.

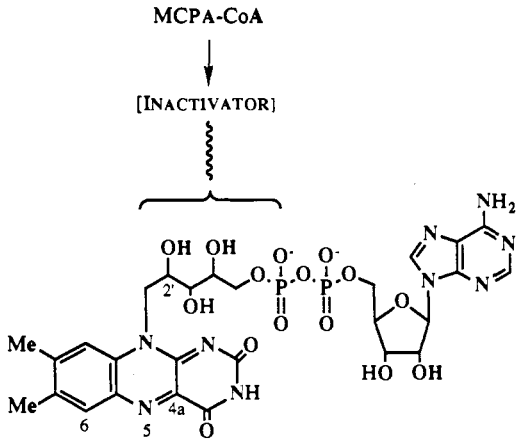
(20) (a) Rontani, J.-F. *Tetrahedron Lett.* **1991**, 6551. (b) Dowd, P.; Ham, S. W. *J. Am. Chem. Soc.* **1991**, *113*, 9403.

(21) (a) Feldman, K. S.; Simpson, R. E. *J. Am. Chem. Soc.* **1989**, *111*, 4878. (b) Feldman, K. S.; Kraebel, C. M. *J. Org. Chem.* **1992**, *57*, 4574.

Scheme IV



Incubation of MCPA-CoA with MCAD under Anaerobic Conditions. As pointed our earlier, the partition ratio of this inactivation under aerobic conditions has been determined to be greater than 3.[6b] The outcomes of the aforementioned analysis clearly revealed that the deviation from inactivation is primarily due to the trapping of the transient radical intermediate **6** by oxygen to form the turnover product **19**. It is therefore anticipated that,



in the absence of oxygen, all of the reactive radical species **6** would couple with the nascent flavin semiquinone in the active site to form covalent coenzyme–inhibitor adducts leading to enzyme inactivation. The partition ratio of inactivation under anaerobic conditions is thus expected to be 0. As part of a logical extension of the mechanistic studies of this inactivation, we have carried out incubations by mixing the purified enzyme (5.6 nmol) with 7 molar equiv of MCPA-CoA in 50 mM potassium phosphate buffer (pH 7.6) at room temperature in the absence of O_2. Following the procedure described earlier, the incubation was continued for 15 min, and the resulting mixture, after being filtered through a Microcon-10 microconcentrator, was purified by an HPLC Partisil-C_{18} column (4.5 × 250 mm) eluted with 30% methanol in 50 mM potassium phosphate buffer (pH 5.3). Interestingly, only two species could be detected by HPLC, which were later identified as the unreacted MCPA-CoA and the coenzyme A itself. As anticipated, the major turnover product **19** found in the aerobic incubation was missing. This observation is pivotal since it clearly substantiates our early speculation that

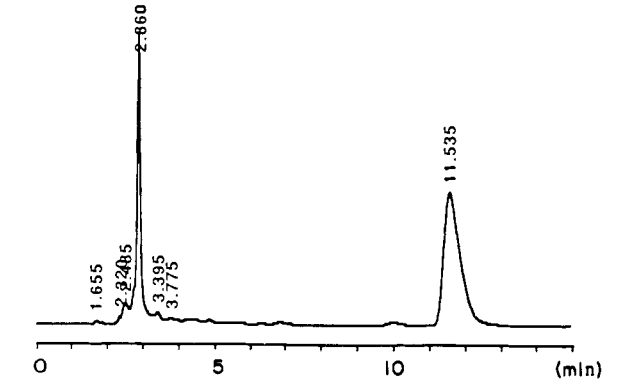


Figure 6. HPLC separation of the mixture resulting from the incubation of MCAD (5.6 nmol) with chemically synthesized MCPA-CoA (39.2 nmol) in 50 mM potassium phosphate buffer (pH 7.6) under anaerobic conditions. The elution conditions and the assignment of the peaks are described in the text.

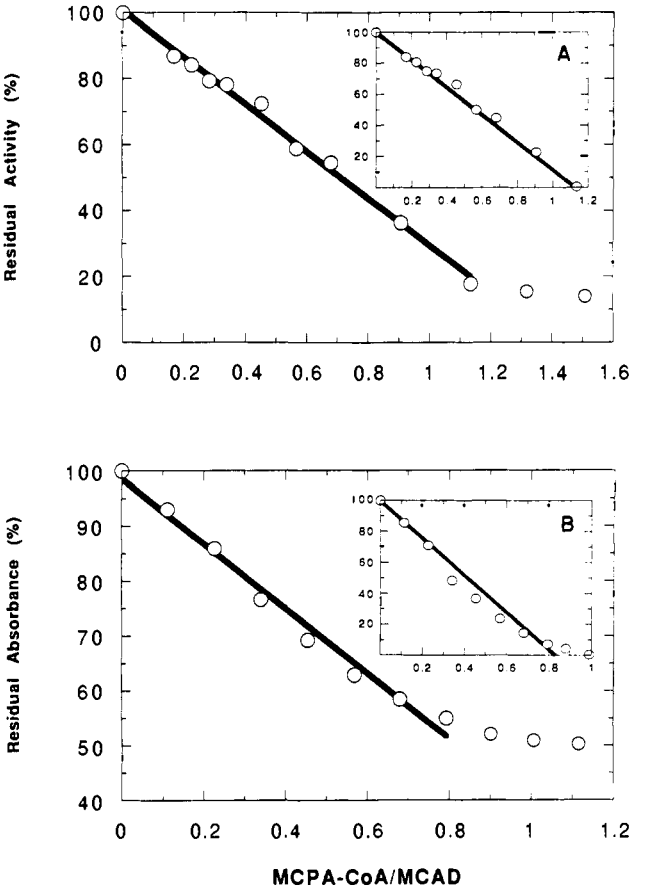


Figure 7. Determination of the partition ratio of MCAD inactivation by MCPA-CoA under anaerobic conditions by monitoring (A) the residual activity and (B) the chromophoric bleaching at 446 nm. The insets are the corrected plots. The experimental details are described in the text and the Experimental Section.

depletion of O_2 would forestall its coupling with the key intermediate **6** and, consequently, the formation of **19**. As shown in Figure 6, trace amounts of several unknown species having retention times akin to that of coenzyme A are discernible; however, their quantities are minuscule, and thus the formation of any turnover product is not obvious. On the basis of these findings, the partition ratio of this inactivation under anaerobic conditions is practically 0. It should be noted that since the incubation sample was analyzed without prior denaturation of the protein, possible still-enzyme-bound flavin–inhibitor adducts were removed by ultrafiltration.

More precise analyses were performed by monitoring the effect of MCPA-CoA on the catalytic activity of MCAD as well as the

bleaching of the flavin chromophore under anaerobic conditions. As shown in Figure 7, plots of the residual enzyme activity or the residual absorbance at A_{446} versus the total equivalents of MCPA-CoA used in the incubation gave linear lines which, after extrapolation, intercept the x-axis at 1.4 and 1.6, respectively. Since the inactivated enzyme retained above-base-line absorption at 446 nm even in the presence of excess inhibitor with prolonged incubation, a correction was made in which the final absorbance subtracted from the observed absorbance was plotted against the amount of inhibitor utilized in the incubation. The good linear correlation, as shown in the inset of Figure 7B, clearly disclosed that bleaching of the enzyme chromophore to the maximal level only requires approximately 1 equiv of MCPA-CoA, which translates to the observed partition ratio of 0. Identical results were also obtained when the variations of the residual activity, calibrated against the final background activity at the end of the inactivation, were plotted versus the concentration of the inhibitor (Figure 7A). These findings unequivocally demonstrated that under anaerobic conditions the inactivation, manifesting its optimal efficiency, exhibits an ideal 1:1 stoichiometry between the inhibitor and the target enzyme.

Formation of Flavin–Inhibitor Adducts under Anaerobic Conditions. There is little doubt that modification of the flavin coenzyme is the major inactivation pathway; yet, it was noted earlier that, under conditions giving complete inactivation of the enzyme with MCPA-CoA, the chromophoric absorption of FAD at 446 nm was only partially bleached.[5e,6,9] Since covalent modification of the enzyme-bound FAD, at either C_6 or C_{4a}–N^5 as previously suggested,[5f] would change this flavin chromophore, the residual absorption at 446 nm had been ascribed to the covalent adducts. However, this assumption conflicts with the experimental fact that complete inactivation with excess MCPA-CoA led to different extents of chromophore bleaching depending on whether the incubation was conducted aerobically or anaerobically (Figure 8). Similar observations had also been noted by other investigators in this field.[5e,9] The apparent inequality of the absorbance at 446 nm of the completely inactivated enzymes derived from aerobic and anaerobic incubations strongly suggested the presence of other inactivation pathways involving no alteration of the isoalloxazine chromophore. Furthermore, since the extents of inactivation estimated spectrophotometrically are less than those determined through enzyme assays and the bleaching of the flavin chromophore levels off first while small loss of enzyme activity continues,[6a] all of these phenomena so far mentioned may be attributed to a minor component of inactivation through alkylation of the enzyme itself.[5d,e,6a] This scenario may involve the coupling of the acyclic radical **6** either with FAD to generate the inhibitor–coenzyme adducts as previously surmised or with an amino acid residue in the active site of MCAD leading to enzyme inactivation. In order to test this contention, 0.5 molar equiv of (1*S*,α*S*)-[α-^{3}H]-MCPA-CoA, available from earlier work,[6d] was incubated with MCAD (5.6 nmol) under anaerobic conditions. The reaction was quenched with active charcoal (10% suspension), and after centrifugation, the radioactivity of the supernatant was measured by scintillation counting. Although MCAD-mediated deprotonation of MCPA-CoA had been determined to be *pro-R* stereospecific,[6d] in accordance with the previous results, 25% radioactivity was detected in the supernatant, resulting from α-proton abstraction by an active-site nucleophile. The observed tritium wash out may be credited to the contaminated 1*S*,α*R* species in the sample, which was only 84% diastereometrically pure,[6d] and to the less than ideal binding of the 1*S* isomer in the active site, which could additionally be a minor factor contributing to the modest stereochemical stringency in removal of the α-proton.[22] An identical incubation was repeated except that the reaction was quenched with 90% methanol to denature the enzyme. After centrifugation, the protein precipitate was subjected to scintillation counting. Since less than 1% of radioactivity was detected in the denatured protein, this result clearly indicated that modification of protein itself is not a significant inactivation process.[23] Consequently, as shown in Table I, the remaining 74% of radioactivity must have been trapped by the activated charcoal. In light of the fact that the partition ratio is 0 under anaerobic conditions and only 0.5 equiv of tritium-labeled MCPA-CoA was used in the incubation, all of the inactivators should participate in covalent flavin adduct formation, since the possibility of protein modification has already been debunked by previous experiments. However, a close examination of Figure 7 revealed that only 30% of the flavin chromophore at 446 nm was quenched upon the addition of 0.5 equiv of inhibitor under anaerobic conditions. It is therefore apparent that only a fraction (0.3 equiv) of the added inhibitor actually modified the coenzyme in a manner causing the bleaching of the A_{446} absorption. If one assumes a direct correlation between modification of the flavin coenzyme and the bleaching of its chromophore, the labeling level in flavin adduct should not exceed 44% [74% × (0.3/0.5)] of the total radioactivity. Since the observed level of tritium incorporation into the flavin–inhibitor adduct of 74% surpassed the calculated upper limit (44%), this strongly suggested that the other 0.2 equiv of MCPA-CoA modified FAD at a locus other than the isoalloxazine moiety. This conclusion is consistent with an earlier report that addition of dithionite to the completely inactivated enzyme led to further decline of the 446-nm absorbance,[5e] which may now be interpreted as reduction of the covalently modified and catalytically inept, albeit chromophorically intact, FAD.

In an effort directed to gain more evidence to support the above premise, MCAD (5.6 nmol) was incubated with 7 molar equiv of MCPA-CoA under aerobic and/or anaerobic conditions, and the inactivated enzyme was denatured with 90% methanol. The unmodified FAD released was quantified by using an HPLC Partisil-C_{18} column (4.5 × 250 mm) eluted with 30% methanol in 50 mM potassium phosphate buffer (pH 5.3) and was compared with the amount of FAD released from a control sample which had not been exposed to MCPA-CoA. Recalling that the 446-nm chromophore of the completely inactivated enzyme was bleached to nearly 70% and 60% under aerobic and anaerobic conditions, respectively (Figure 8), it was intriguing to discover that only trace amounts, instead of 30% and 40%, of intact FAD were detected from the corresponding denatured samples. A reference exper-

Table I. Residual Radioactivity Retained in the Resulting Fractions of the Denatured MCAD after Incubation with (1*S*,α*S*)-[α-^{3}H]MCPA-CoA[a]

fraction	radioactivity (%)
supernatant	25.2
protein precipitate	0.7
flavin-inactivator adducts	74.1[b]

[a] The iterative procedures used are described in the Experimental Section. [b] Percentage of tritium trapped by the activated charcoal.

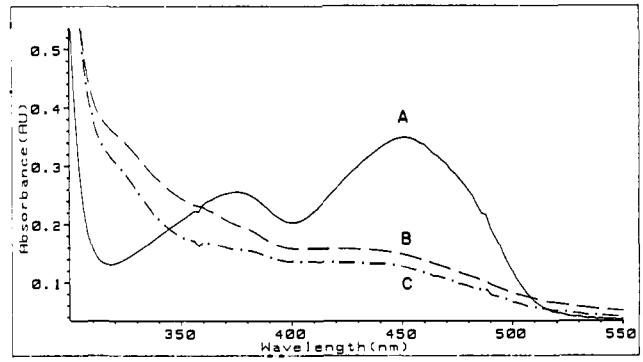


Figure 8. Electronic absorption of MCAD (300–500 nm) before and after inactivation by MCPA-CoA: (A) native MCAD, (B) after anaerobic inactivation, and (C) after aerobic inactivation. The incubation conditions are described in the Experimental Section.

(22) Lai, M.-t.; Oh, E.; Liu, H.-w. *Biomed. Lett.* **1992**, *2*, 1423.

(23) It should be noted that incubations with excess (7 molar equiv) tritium-labeled MCPA-CoA led to a similar finding. Analogous results were also found when ^{14}C-labeled MCPA-CoA was used as the inhibitor: Zeller, H.; Ghisla, S. In *Flavins and Flavoproteins*; Edmondson, D. E., McCormick, D. B., Eds.; Walter de Gruyter: Berlin, 1987; p 161.

iment was also performed by denaturing an aliquot of fresh MCAD solution, whose concentration had been adjusted to give an absorbance at 446 nm identical with that of the completely inactivated enzyme. After correlation with the original control, the amount of FAD released from the reference sample was found to be at least 20% more than that liberated from the aerobically incubated mixture. An even greater difference of 30% was found between the anaerobically inactivated sample and the corresponding reference specimen. These results strongly indicated that the residual absorption at 446 nm of the completely inactivated enzyme is not due to the intact flavin coenzyme, but is instead a property of the modified FAD with an unaltered isoalloxazine moiety. This conclusion is in accordance with the aforementioned tritium incorporation experiment and once again attested that modification of flavin coenzyme by MCPA-CoA could occur not only on the isoalloxazine ring but also on other parts of the cofactor, leaving its chromophore unchanged. Although derivatization of the isoalloxazine skeleton of FAD is still the predominant inactivation pathway, the current results revealed that alternative modification routes are possible, the increasing significance of which becomes apparent especially under anaerobic conditions. Evidently, the effective concentration of the reactive intermediate **6** is low in the presence of oxygen due to its facile conversion to turnover product; however, in the absence of oxygen, more **6** is available for the modification of FAD.

Conclusion. The results summarized in this paper provide a detailed account of the mechanistics studies of the inactivation of MCAD by MCPA-CoA, the causative agent of Jamaican vomiting sickness. Earlier studies have shown that this inactivation is nonstereospecific and a spontaneous ring fragmentation induced by a transient cyclopropyl radical **4** may have been the cause. Thus, a one-electron oxidation pathway involving the formation of ring-opened radical **6**, which subsequently modifies the flavin coenzyme, had been proposed as the inactivation mechanism. Since more than 4 molar equiv of MCPA-CoA are required to completely inactivate the enzyme under aerobic conditions, the identification of a highly oxygenated species (**19**) as the major turnover product strongly supports the intermediacy of the acyclic radical **6**, and as such the departure from the expected inactivation may result from trapping of this radical intermediate **6** by O_2. Interestingly, unlike the flavin oxidases which react directly with oxygen, reoxidation of the two-electron-reduced MCAD by O_2 is kinetically lethargic, especially for the ligand-bound enzyme complex.[24] Its rapid reoxidation in vivo requires the assistance of two electron-transfer flavoproteins (ETF) which funnel electrons from the reduced coenzyme to the mitochondrial respiratory chain.[25] However, suppression of the reactivity of FAD in MCAD toward O_2 is not due to eradication of oxygen from the active site, but is instead due to the desolvation of the active site upon substrate binding, which leads to the destabilization of the superoxide anion generated during flavin reoxidation.[24] Thus, despite the low reactivity exhibited by the target enzyme toward oxygen, enough oxygen must still be accessible in the active site to couple with the radical intermediate **6**. This contention is substantiated by the current studies showing that the partition ratio of this inactivation is nearly 0 under anaerobic conditions. It is evident that in the absence of O_2 all radicals **6** generated in the active site of MCAD are prevalently reacted with the flavin coenzyme, leading to inactivation; hence a stoichiometry of 1:1 was observed.

Further studies of the anaerobic inhibition, especially the analysis of the unusually high absorbance at 446 nm of the inactivated enzyme, led to the discovery of a minor inactivation pathway involving covalent modification of flavin at a locus other than the isoalloxazine ring. Although the chemical nature of the new inhibitor–coenzyme adduct(s) has yet to be elucidated, a structure having MCPA-CoA linked to the N(10) ribityl side chain is appealing. This speculation is supported by the structural information derived from the highly homologous pig liver enzyme, in which a strong hydrogen bond (~2.9 Å) between the carbonyl of the thioester substrate and the ribityl 2′-OH of the FAD was observed.[26] This 2′-OH on the flavin ribityl side chain is essential for substrate binding and may also be crucial for catalysis, since MCAD reconstituted with 2′-deoxy-FAD is devoid of any activity.[27] In fact, a new type of flavin–inhibitor skeleton linked via an ether linkage has been previously proposed for a compund isolated from the incubation of MCAD and MCPA-CoA.[28] If this new inhibitor–coenzyme adduct is indeed connected by an ether linkage, and further considering the fact that ribityl-OH may not be particularly nucleophilic for a Michael addition, the coupling step could likely be a radical-initiated process. It is hoped that eventual elucidation of the structure of the adduct(s) will provide further insights into the mechanism of action of the acyl-CoA dehydrogenases.

Experimental Section

Melting points were determined with a Mel-Temp apparatus and are uncorrected. Mass spectra were obtained with a VG 7070E-HF spectrometer. ^1H and ^{13}C NMR spectra were recorded on an IBM NR/200 or NR/300 or a Varian 500 spectrometer. Chemical shifts are reported in ppm on the δ scale relative to internal standard (tetramethylsilane, sodium 2,2-dimethyl-2-silapentane-5-sulfonate, or appropriate solvent peaks) with coupling constants given in hertz. NMR assignments labeled with an asterisk (*) may be interchangeable. HPLC analysis and/or separation were conducted with a Hewlett-Packard 1090A instrument equipped with an HP3392 integrator. Flash 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 vanillin/ethanol/H_2SO_4 (1:98:1) or phosphomolybdic acid (7% EtOH solution) and then heating. The drying agent used in the routine workup was anhydrous magnesium sulfate. Radioactivity was measured by liquid scintillation counting, and Ecoscint A (National Diagnostics, Manville, NJ) was used as scintillation cocktail. Solvents, unless otherwise specified, were reagent grade and distilled once prior to use.

Enzyme. Medium-chain acyl-CoA dehydrogenase (MCAD), isolated from pig kidneys, was purified to homogeneity according to the procedure of Thorpe et al.[16] with the addition of octyl-Sepharose column chromatography to aid in the removal of contaminating enolyl hydratase activity.[29] The overall yield of a typical purification was 200–300 nmol of purified MCAD/kg of kidney cortex. The concentration of the holoenzyme was determined spectrophotometrically with a molar absorptivity of 15.4 mM^{-1} cm^{-1} at 446 nm for oxidized MCAD.[16] The chromatographic, electrophoretic, and spectral properties of this protein are identical with those cited in the literature.

Enzyme Assay. The enzyme activity was determined by using phenazine methosulfate (PMS) as the electron carrier to mediate the transfer of reducing equivalents from octanoyl-CoA to 2,6-dichlorophenolindophenol (DCPIP).[16] A standard 0.7-mL assay was performed, as described before,[16] in 50 mM potassium phosphate buffer (pH 7.6) at 25 °C containing 33 μM octanoyl-CoA, 30 μM DCPIP, 1.4 mM PMS, and 0.3 mM EDTA. The reaction was initiated by the addition of an appropriate amount of MCAD. Loss of DCPIP absorbance at 600 nm, before and after the addition of MCAD, was monitored.

Determination of Partition Ratios under Anaerobic Conditions. A series of identical samples containing MCAD (5.6 nmol) in 200 μL of 50 mM potassium phosphate buffer (pH 7.6) were degassed and replenished with dry argon via a Schlenk inert atmosphere system. This cycle was repeated at least 10 times over a period of 1 h. To each of these samples was added, via a gastight syringe, an anaerobic solution of MCPA-CoA of known concentration. Since part of the sample solution was evaporated during this manipulation, the volume of the resulting

(24) Wang, R.; Thorpe, C. *Biochemistry* **1991**, *30*, 7895.

(25) (a) Crane, F. L.; Beinert, H. *J. Biol. Chem.* **1956**, *218*, 717. (b) Ruzicka, F. J.; Beinert, H. *J. Biol. Chem.* **1977**, 252, 8440. (c) Gorelick, R. J.; Schopfer, L.; Ballou, D. P.; Massey, V.; Thorpe, C. *Biochemistry* **1985**, *24*, 6830. (d) Lenn, N. D.; Stankovich, M. T.; Liu, H.-w. *Biochemistry* **1990**, *29*, 3709.

(26) Kim, J. P.; Wang, M.; Djordjevic, S.; Paschke, R. In *New Developments in Fatty Acid Oxidation*; Coates, P. M.; Tanaka, K., Eds.; Wiley-Liss: New York, 1992; p 111.

(27) Ghisla, S., Engst, S.; Moll, M.; Bross, P.; Strauss, A. W.; Kim, J. P. In *New Developments in Fatty Acid Oxidation*; Coates, P. M., Tanaka, K., Eds.; Wiley-Liss: New York, 1992; p 127.

(28) Zeller, H.; Ghisla, S In *Flavins and Flavoproteins*; Curti, B.; Ronchi, S., Zanetti, G., Eds.; Walter de Gruyter, Berlin, 1990; p 315.

(29) Lau, S. M.; Powell, P.; Buettner, H.; Ghisla, S.; Thorpe, C. *Biochemistry* **1986**, *25*, 4184.

mixture was adjusted to 200 μL with oxygen-free water. The incubation of each sample was continued for 15 min and stopped by dilution of the incubation mixture (aliquots of 20 μL) in 0.7 mL of the standard assay system containing 33 μM octanoyl-CoA, 30 μM DCPIP, 1.4 mM PMS, and 0.3 mM EDTA in 50 mM potassium phosphate buffer (pH 7.6), thereby allowing the determination of the residual enzyme activity. Each reading is the average of at least seven measurements. In order to monitor the bleaching of the chromophore at 446 nm, the incubation was scaled up to 16.7 nmol of MCAD along with the appropriate amount of MCPA-CoA in 600 μL of buffer. After incubation for 15 min at 25 °C the absorbance at 446 nm of each sample was recorded.

Incubation of MCAD with [α-^{3}H]MCPA-CoA. The tritium-labeled (1*S*,α*S*)-MCPA-CoA (2.8 nmol, specific activity 7.3 mCi/mmol) was incubated with MCAD (5.6 nmol) in 200 μL of 50 mM potassium phosphate buffer (pH 7.6) at 25 °C for 15 min under anaerobic conditions. The reaction was quenched with active charcoal (10% solution), and the resulting suspension was mixed vigorously on a vortex mixer for 1 min and centrifuged to precipitate the charcoal. The supernatant (100 μL) was then removed and analyzed by scintillation counting. An identical incubation was performed except that the reaction was quenched with 1.8 mL of methanol. The denatured protein, after centrifugation, was collected and subjected to scintillation counting. The supernatant was concentrated in vacuo to remove methanol and then diluted with water to 1 mL prior to the addition of 100 mg of activated charcoal. After mixing and centrifugation to precipitate the charcoal, aliquots of the supernatant (100 μL) were analyzed by scintillation counting. These readings were calibrated against controls prepared in parallel with boiled enzyme.

Spectral Analysis of Incubation of MCAD with MCPA-CoA. A mixture of MCAD (13.6 nmol) and 7 molar equiv of MCPA-CoA was incubated in 600 μL of 50 mM potassium phosphate buffer (pH 7.6) at 25 °C for 15 min. The electronic absorption of this reaction mixture was recorded at the end of the incubation to determine the extent of flavin bleaching. The anaerobic analysis was performed on a sample of identical composition following the incubation procedure described above.

Analysis of FAD Released from Inactivated MCAD. A mixture of MCAD (5.6 nmol) and 7 molar equiv of MCPA-CoA was incubated in 200 μL of 50 mM potassium phosphate buffer (pH 7.6) at 25 °C for 15 min. The reaction was quenched by adding 1.8 mL of methanol, and the mixture was then centrifuged to remove the denatured proteins. The supernatant was concentrated under reduced pressure, and the aqueous residue was analyzed by using an HPLC Partisil-C_{18} column (4.5 × 250 mm) eluted (1 mL/min) with 30% methanol in 50 mM potassium phosphate buffer (pH 5.3). The result was calibrated against a blank which contained no inhibitor. An identical incubation was also carried out under anaerobic conditions.

Preparation of Standards. (*E*)-3-(Tributylstannyl)-2-propen-1-ol (12). To a mixture of tributyltin hydride (22.5 g, 77.2 mmol) and propargyl alcohol (**11**, 5 g, 59.4 mmol) under argon was added a catalytic amount of azobis(isobutyronitrile) (AIBN). After being stirred for 2 h at 80 °C, the reaction mixture was directly applied to a silica gel column and purified by flash chromatography (1% ethyl acetate in hexane) to give pure **12** in 76% yield: ^{1}H NMR ($CDCl_3$) δ 6.66 (1 H, dt, *J* = 17.8, 6.0 Hz, 2-H), 6.16 (1 H, d, *J* = 17.8 Hz, 3-H), 4.14 (2 H, br t, *J* = 6.0 Hz, 1-H), 1.48 (6 H, m), 1.31 (6 H, m), 0.93 (6 H, m, $SnCH_2$), and 0.92 (9 H, t, *J* = 6.3 Hz, Me) (the latter four resonances are signals of the Bu_3Sn group); ^{13}C NMR ($CDCl_3$) δ 147.1 (C-2)*, 128.3 (C-3)*, 66.3 (C-1), 29.1, 27.3, 13.7, 9.4 (C's of Bu_3Sn group). The byproduct of this reaction was a mixture of **13** and **14**, which showed similar chromatographic behaviors and were not separable by use of an open column.

(*E*)-4-Methyl-2,4-pentadien-1-ol (15) and (*E*)-2,5-Hexadien-1-ol (17). Stannyl alcohol **12** (1.08 g, 3.1 mmol) and 2-bromopropene (0.4 g, 3.3 mmol) were added to a suspension of $Pd(CH_3CN)_2Cl_2$ (40 mg, 0.15 mmol) in dry DMF (10 mL) under argon at room temperature. The reaction mixture was stirred overnight, and the reaction was then quenched by 10% NH_4OH (10 mL). After being stirred for an additional 10 min, the resulting mixture was extracted with ether, and the combined organic extracts were dried over anhydrous magnesium sulfate. The solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography (10% ethyl acetate in hexane) to afford **15** in 25% yield. Synthesis of compound **17** from **12** (2.0 g, 5.8 mmol) and allyl bromide (0.7 g, 6.2 mmol) was effected by the same procedure in 77% yield. Compound **15**: ^{1}H NMR ($CDCl_3$) δ 6.30 (1 H, d, *J* = 15.8 Hz, 3-H), 5.77 (1 H, dt, *J* = 15.8, 5.9 Hz, 2-H), 4.95 (2 H, s, $=CH_2$), 4.17 (2 H, d, *J* = 5.9 Hz, 1-H), 2.33 (1 H, br s, OH), 1.82 (3 H, s, Me); ^{13}C NMR ($CDCl_3$) δ 141.4 (C-4), 133.6 (C-2), 128.8 (C-3), 116.4 (C-5), 60.3 (C-1), 18.5 (Me). Compound **17**: ^{1}H NMR ($CDCl_3$) δ 5.85–5.57 (3 H, m, 2,3,5-H), 5.01 (1 H, dd, *J* = 13.1, 1.5 Hz, 6-H), 4.97 (1 H, m, 6-H), 4.05 (2 H, d, *J* = 4.4 Hz, 1-H), 2.76 (2 H, dd, *J* = 5.8, 5.1 Hz, 4-H), 2.22 (1 H, br s, OH); ^{13}C NMR ($CDCl_3$) δ 136.3 (C-5), 130.3 (C-3)*, 130.1 (C-2)*, 115.5 (C-6), 63.4 (C-1), 36.3 (C-4).

(*E*)-4-Methyl-2,4-pentadienoic Acid (16) and (*E*)-2,5-Hexadienoic Acid (18). To a solution of **15** (50 mg, 0.5 mmol) in 10 mL of acetone was added Jones reagent that was prepared by mixing chromium oxide (26.72 g) with concentrated sulfuric acid (23 mL) followed by water dilution to a final volume of 100 mL. Addition of Jones reagent was continued until the red color persisted for at least 1 min. The resulting mixture was stirred at room temperature for 30 min to ensure the completion of reaction. The excess oxidizing reagent was quenched with 2-propanol. The solution was then filtered to remove the green precipitate and concentrated under reduced pressure. The crude product was purified by flash chromatography (10% ethyl acetate in hexane) to give **16** in 89% yield. Compound **17** (0.4 g, 4.1 mmol) was converted to **18** by an identical procedure in 92% yield. Compound **16**: ^{1}H NMR ($CDCl_3$) δ 7.44 (1 H, d, *J* = 15.7 Hz, 3-H), 5.87 (1 H, d, *J* = 15.7 Hz, 2-H), 5.40 (2 H, br s, $=CH_2$), 1.90 (3 H, s, Me); ^{13}C NMR ($CDCl_3$) δ 172.6 (C=O), 149.3 (C-3), 140.5 (C-5), 125.3 (C-2), 118.0 (C-4), 18.0 (Me). Compound **18**: ^{1}H NMR ($CDCl_3$) δ 9.23 (1 H, br s, OH), 7.07 (1 H, dt, *J* = 15.6, 6.6 Hz, 3-H), 5.89 (1 H, d, *J* = 15.6 Hz, 2-H), 5.74 (1 H, m, 5-H), 5.13 (1 H, s, 6-H), 5.08 (1 H, dd, *J* = 6.8, 1.5 Hz, 6-H), 2.98 (2 H, m; 4-H); ^{13}C NMR ($CDCl_3$) δ 171.9 (C=O), 149.2 (C-3), 133.5 (C-5), 121.6 (C-2), 117.5 (C-6), 36.1 (C-4).

(*E*)-4-Methyl-2,4-pentadienoyl-CoA (9) and (*E*)-2,5-Hexadienoyl-CoA (10). To a solution of compound **16** (30 mg, 0.27 mmol) in methylene chloride (5 mL) was added triethylamine (38 μL, 0.27 mmol) under an argon atmosphere. After the mixture was stirred for 10 min, isobutyl chloroformate (34.7 μL, 0.27 mmol) was added dropwise at 0 °C. The reaction mixture was agitated vigorously; during mixing, fuming was noted. After the mixture was stirred at room temperature for 1 h, the solvent was removed under reduced pressure, and the remaining mixed anhydride was redissolved in THF (5 mL) 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 deoxygenated by repeated freeze and thaw cycles under high vacuum. The solution was adjusted to pH 8.0 by adding 1 N NaOH. To this CoA solution was added, via a cannula, the mixed-anhydride solution under a positive argon pressure. The pH of the resulting mixture was readjusted to 8.0, and stirring was continued for an additional 10 min. The pH value of the solution was then changed to 5.0–5.5 by adding diluted perchloric acid. The organic solvent (THF) was evaporated under reduced pressure, and the remaining aqueous solution was extracted twice with ether to remove any residual organic soluble materials. The aqueous solution was then lyophilized. The crude product was chromatographed on a preparative HPLC Partisil-C_{18} column (4.5 × 250 mm, 5 μm) and eluted with 30% methanol in 50 mM potassium phosphate buffer (pH 5.3, flow rate 1 mL/min, monitoring wavelength 260 nm). Under these conditions, the retention time of the CoA derivative was found to be ca. 25 min. The acyl-CoA-containing fractions were negative in the nitroprusside test[30] and had an A_{223}/A_{256} ratio of 0.45–0.5. These fractions 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 with water (1 mL/min). The eluting solvent was changed 20 min later to methanol to wash out compound **9**. The organic solvent was removed under reduced pressure, and the aqueous solvent was lyophilized to give pure **9** as a white powder. The overall yield was 38%. Acid **18** was converted to **10** by an identical procedure in 35% yield. Compound **9**: ^{1}H NMR (^2H_2O) δ 8.51, 8.23 (1 H each, s, adenine H), *7.46* (1 H, d, *J* = 16.0 Hz, 3-H), 6.14 (1 H; d, *J* = 6.3 Hz ribose anomeric H), *5.80* (1 H, d, *J* = 16.0 Hz, 2-H), *5.41* (2 H, br s, $=CH_2$), 4.80–4.63 (2 H, m, ribose H), 4.55 (1 H, s, ribose H), 4.19 (2 H, br s, ribose CH_2O), 3.98 (2 H, s), 3.77 (1 H, m), 3.51 (1 H, m), 3.41 (2 H, t, *J* = 6.6 Hz), 3.35 (2 H, t, *J* = 6.4 Hz), 2.95 (2 H, t, *J* = 6.4 Hz), 2.30 (2 H, t, *J* = 6.6 Hz), *1.92* (3 H, s, 4-Me), 0.81, 0.67 (3 H each, s, Me). Compound **10**: ^{1}H NMR (^2H_2O) δ 8.51, 8.24 (1 H each, s, adenine H), *7.10* (1 H, dt, *J* = 15.5, 6.5 Hz, 3-H), 6.07 (1 H, d, *J* = 6.0 Hz, ribose anomeric H), *5.85* (1 H, d, *J* = 15.5 Hz, 2-H), *5.70* (1 H, m, 5-H), *5.16* (1 H, s, 6-H), *5.00* (1 H, br d, *J* = 6.6 Hz, 6-H), 4.74 (2 H, m, ribose H), 4.47 (1 H, br s, ribose H), 4.12 (2 H, br s, ribose CH_2O), 3.90 (1 H, s), 3.89 (1 H, d, *J* = 6.5 Hz), 3.77 (1 H, m), 3.50 (1 H, m), 3.34 (2 H, t, *J* = 6.6 Hz), 3.28 (2 H, t, *J* = 6.5 Hz), *2.95* (2 H, m, 4-H), 2.86 (2 H, t, *J* = 6.4 Hz), 2.32 (2 H, t, *J* = 6.6 Hz), 0.79, 0.66 (3 H each, s, Me). The measured sample was repeatedly dissolved in ^2H_2O and lyophilized prior to ^{1}H NMR analysis. The chemical shifts of the acyl signals are shown in italics.

(*E*)-Methyl 3-(Tributylstannyl)acrylate (22) and (*Z*)-Methyl 3-(Tributylstannyl)acrylate (23). To a mixture of tributyltin hydride (18.1 g, 62 mmol) and methyl propiolate (5 g, 59.4 mmol) was added a cata-

(30) Stadtman, E. R. *Methods Enzymol.* **1957**, *3*, 931.

lytic amount of AIBN under argon at 60 °C. After being stirred for 2 h, the mixture was purified by flash chromatography (0.5% ethyl acetate in hexane) to give pure **22** and **23** (ca. 1:1) in 76% combined yield. Compound **22**: ^{1}H NMR ($CDCl_3$) δ 7.73 (1 H, dd, J = 19.4, 1.7 Hz, 2-H), 6.29 (1 H, dd, J = 19.4, 1.7 Hz, 3-H), 3.73 (3 H, s, OMe), 1.48 (6 H, m), 1.29 (6 H, m), 0.95 (6 H, t, J = 8.1; $SnCH_2$), 0.87 (9 H, t, J = 7.3 Hz, Me) (the latter four resonances are signals of the Bu_3Sn group); ^{13}C NMR ($CDCl_3$) δ 165.2 (C=O), 152.8 (C-3), 136.0 (C-2), 51.5 (OMe), 29.0, 27.2, 13.6, 9.6 (C's of Bu_3Sn group). Compound **23**: ^{1}H NMR ($CDCl_3$) δ 7.16 (1 H, d, J = 13.0 Hz, 2-H), 6.73 (1 H, d, J = 13.0 Hz, 3-H), 3.74 (3 H, s, OMe), 1.47 (6 H, m), 1.28 (6 H, m), 0.96 (6 H, t, J = 8.3 Hz, $SnCH_2$), 0.87 (9 H, t, J = 7.2 Hz, Me) (the latter four resonances are signals of the Bu_3Sn group); ^{13}C NMR ($CDCl_3$) δ 168.0 (C=O), 157.4 (C-3), 135.0 (C-2), 51.5 (OMe), 29.1, 27.3, 13.7, 11.0 (C's of Bu_3Sn group).

(*E*)-3-Iodo-2-propen-1-ol (24) and (*Z*)-2-Iodo-2-propen-1-ol (25). To a THF solution (5 mL) of propargyl alcohol (**11**, 5 g, 89.3 mmol) containing $Pd(PPh_3)_2Cl_2$ (80 mg, 0.1 mmol) was added tributyltin hydride (27.3 g, 93.8 mmol) dropwise over a period of 30 min at 0 °C. The resulting mixture was stirred for an additional 10 min and then filtered. The solvent was removed under reduced pressure to give a mixture of tin isomers. To a methylene chloride solution of this mixture was added I_2 (22.7 g, 89.3 mmol). The resulting mixture was stirred at room temperature overnight. Saturated Na_2SO_3 solution was added to quench the excess I_2, and the aqueous phase was extracted with methylene chloride. The combined organic extracts were washed with brine, dried, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (0.5% ethyl acetate in hexane) to afford desired **24** and **25** (1:1) in 49% combined yield. Compound **24**: ^{1}H NMR ($CDCl_3$) δ 6.63 (1 H, dt, J = 14.5, 5.4 Hz, 2-H), 6.33 (1 H, m, 3-H), 4.00 (2 H, d, J = 5.4 Hz, 1-H), 3.03 (1 H, br s, OH); ^{13}C NMR ($CDCl_3$) δ 144.5 (C-2), 139.7 (C-3), 64.7 (C-1). Compound **25**: ^{1}H NMR ($CDCl_3$) δ 6.37 (1 H, dd, J = 1.7, 1.6 Hz, 3-H), 5.84 (1 H, dd, J = 1.6, 1.4 Hz, 3-H), 4.15 (2 H, br s, 1-H), 2.15 (1 H, br s, OH); ^{13}C NMR ($CDCl_3$) δ 124.4 (C-3), 110.3 (C-2), 70.9 (C-1).

1-(*tert*-Butyldimethylsiloxy)-2-iodopropene (26). To a solution of **25** (1.2 g, 6.5 mmol) in methylene chloride (50 mL) were added (dimethylamino)pyridine (0.8 g, 6.5 mmol) and *tert*-butyldimethylsilyl chloride (1.1 g, 7.3 mmol) in sequence at 0 °C. After being stirred for 3 h, the solution was quenched with water and then extracted with methylene chloride. The combined organic extracts were dried, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (2% ethyl acetate in hexane) to give the desired product in 95% yield: ^{1}H NMR ($CDCl_3$) δ 6.41 (1 H, dd, J = 1.7, 1.6 Hz, 3-H), 5.80 (1 H, d, J = 1.6 Hz, 3-H), 4.16 (2 H, d, J = 1.7 Hz, 1-H), 0.91 (9 H, s, C-Me), 0.08 (6 H, s, Si-Me); ^{13}C NMR ($CDCl_3$) δ 122.9 (C-3), 109.7 (C-2), 71.1 (C-1), 25.8 (C-Me), 18.3 (Me-*C*), -5.3 (Si-Me).

(*E*)-Methyl 4-[(*tert*-Butyldimethylsiloxy)methyl]-2,4-pentadienoate (27) and (*Z*)-Methyl 4-[(*tert*-Butyldimethylsiloxy)methyl]-2,4-pentadienoate (28). To a suspension of $Pd(CH_3CN)_2Cl_2$ (32 mg, 0.12 mmol) in dry DMF (10 mL) was added vinyl iodide **26** (1.4 g, 4.0 mmol), followed by the addition of **22** (0.8 g, 2.7 mmol) under argon at room temperature. After the mixture was stirred overnight, the reaction was quenched with 10% ammonium hydroxide solution (10 mL) and mixed for an additional 10 min. The resulting mixture was extracted with ether, and the combined organic extracts were dried over anhydrous magnesium sulfate. The solvent was evaporated under reduced pressure, and the residue was partially separated by flash chromatography (2% ethyl acetate in hexane). The crude product was resubjected to flash chromatography and eluted with 0.5% ethyl acetate in hexane to give pure **27** in 57% yield, with 35% recovery yield of the starting material. Preparation of **28** was effected via an identical procedure by coupling **26** (1.7 g, 5.0 mmol) and **23** (1.0 g, 3.3 mmol) in the presence of palladium catalyst. The yield was 41%, with 40% of the starting material recovered. Compound **27**: ^{1}H NMR ($CDCl_3$) δ 7.33 (1 H, d, J = 16.2 Hz, 3-H), 5.88 (1 H, d, J = 16.2 Hz, 2-H), 5.65, 5.47 (1 H each, d, J = 1.7 Hz, $=CH_2$), 4.32 (2 H, br s, OCH_2), 3.75 (3 H, s, OMe), 0.92 (9 H, s, C-Me), 0.08 (6 H, s, Si-Me); ^{13}C NMR ($CDCl_3$) δ 167.2 (C=O), 143.8 (C-4)*, 143.1 (C-3)*, 122.0 (C-5), 117.2 (C-2), 62.0 (OCH_2), 51.4 (OMe), 25.6 (C-Me), 18.1 (Me-*C*), -5.6 (Si-Me). Compound **28**: ^{1}H NMR ($CDCl_3$) δ 6.44 (1 H, d, J = 12.6 Hz, 3-H), 5.77 (1 H, d, J = 12.6 Hz, 2-H), 5.52, 5.33 (1 H each, d, J = 1.3 Hz, $=CH_2$), 4.34 (2 H, s, OCH_2), 3.69 (3 H, s, OMe), 0.90 (9 H, s, C-Me), 0.06 (6 H, s, Si-Me); ^{13}C NMR ($CDCl_3$) δ 166.3 (C=O), 143.9 (C-4), 140.7 (C-3), 119.5 (C-2), 117.7 (C-5), 64.1 (OCH_2), 51.1 (OMe), 25.6 (C-Me), 18.0 (Me-*C*), -5.7 (Si-Me).

(*E*)-Methyl 4-[(*tert*-Butyldimethylsiloxy)methyl]-2,3-epoxy-4-pentenoate (29) and (*Z*)-Methyl 4-[(*tert*-Butyldimethylsiloxy)methyl]-2,3-epoxy-4-pentenoate (30). To a solution of *t*-BuOOH (2.1 mL, 3 M in 2,2,4-trimethylpentane) in THF (10 mL) was added methyllithium (3.0 mL, 1.4 M in ether) immediately followed by **27** (0.11 g, 0.4 mmol) under argon at -78 °C. The resulting mixture was warmed up to room temperature and stirred overnight. Saturated ammonium chloride solution was added to quench the reaction, and the mixture was then extracted with ether. The combined organic extracts were dried, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (2% ethyl acetate in hexane) to give the desired product **29** in 37% yield. Compound **30** was prepared from **28** (0.9 g, 3.5 mmol) by an identical procedure in 30% yield. Compound **29**: ^{1}H NMR (CD_2Cl_2) δ 5.24 (2 H, br s, $=CH_2$), 4.04 (2 H, s, OCH_2), 3.68 (3 H, s, OMe), 3.53 (1 H, d, J = 1.9 Hz, 2-H), 3.39 (1 H, d, J = 1.9 Hz, 3-H), 0.86 (9 H, s, C-Me), 0.02 (6 H, s, Si-Me); ^{13}C NMR (CD_2Cl_2) 169.4 (C=O), 143.3 (C-4), 114.8 (C-5), 62.8 (OCH_2), 58.0 (C-2)*, 54.5 (C-3)*, 52.8 (OMe), 25.9 (C-Me), 18.5 (Me-*C*), -5.3 (Si-Me). Compound **30**: ^{1}H NMR ($CDCl_3$) δ 5.23, 5.21 (1 H each, s, $=CH_2$), 4.18 (2 H, s, OCH_2), 3.72-3.66 (2 H, m, buried under OMe peak, 2- and 3-H), 3.69 (3 H, s, OMe), 0.90 (9 H, s, C-Me), 0.06 (6 H, s, Si-Me); ^{13}C NMR ($CDCl_3$) δ 167.8 (C=O), 140.1 (C-4), 113.2 (C-5), 64.2 (OCH_2), 56.3 (C-2)*, 55.0 (C-3)*, 52.3 (OMe), 26.0 (C-Me), 18.5 (Me-*C*), -5.3 (Si-Me).

(*E*)-Methyl 2,3-Epoxy-4-(hydroxymethyl)-4-pentenoate (20) and (*Z*)-Methyl 2,3-Epoxy-4-(hydroxymethyl)-4-pentenoate (21). To a solution of **30** (30 mg, 0.1 mmol) in THF (3 mL) were added acetic acid (6 μL) and tetrabutylammonium fluoride (110 μL, 1 M in THF) in sequence at room temperature. After being stirred for 2 h, the solution was concentrated in vacuo. The residue was purified by flash chromatography (20% ethyl acetate in hexane) to give the desired product **21** in 38% yield. Compound **21**: ^{1}H NMR ($CDCl_3$) δ 5.29 (2 H, s, $=CH_2$), 4.22, 4.20 (1 H each, d, J = 12.0 Hz, OCH_2), 3.78, 3.73 (1 H each, d, J = 4.5 Hz, 2- and 3-H), 3.74 (3 H, s, OMe); ^{13}C NMR ($CDCl_3$) δ 168.0 (C=O) 140.4 (C-4), 115.0 (C-5), 64.0 (OCH_2), 56.3 (C-2)*, 54.8 (C-3)*, 52.3 (OMe). The byproduct of this reaction is the corresponding lactone: ^{1}H NMR ($CDCl_3$) δ 5.46, 5.45 (1 H each, d, J = 1.3 Hz, $=CH_2$), 4.98, 4.46 (1 H each, d, J = 12.4 Hz, OCH_2), 3.95 (1 H, d, J = 4.0 Hz, 3-H)*, 3.76 (1 H, d, J = 4.0 Hz, 2-H)*; ^{13}C NMR ($CDCl_3$) δ 168.0 (C=O) 142.2 (C-4), 118.1 (C-5), 68.4 (OCH_2), 55.6 (C-2)*, 53.5 (C-3)*. The *E* isomer **20** was synthesized from **29** in 52% yield by a similar procedure, except that the addition of acetic acid was unnecessary and the reaction was complete in 15 min. Compound **20**: ^{1}H NMR ($CDCl_3$) δ 5.39, 5.38 (1 H each, s, $=CH_2$), 4.12, 4.06 (1 H each, d, J = 13.0 Hz, OCH_2), 3.79 (3 H, s, OMe), 3.72, 3.57 (1 H each, d, J = 1.7 Hz, 2- and 3-H); ^{13}C NMR ($CDCl_3$) δ 168.9 (C=O), 142.2 (C-4), 117.4 (C-5), 62.2 (OCH_2), 58.0 (C-2)*, 53.7 (C-3)*, 52.6 (OMe).

Acknowledgment. We thank Dr. Vikram Roongta for his assistance with the NMR measurements and Ms. Julie A. Robertson for her technical help on the preparation of the thioenoate standards. This work was supported by a National Institutes of Health grant (GM 40541). H.-w.L. also thanks the National Institutes of Health for a Research Career Development Award (GM 00559).