(2 × 5 mL) to remove excess benzaldehyde. The aldol product was purified by preparative HPLC on a Dynamax 20 × 250 mm C18 column, eluting with 0.1% acetic acid/20% methanol at 5 mL/min. The product elution was followed at 254 nm; lyophilization of the product gave 46 mg (94.5%) of 2-amino-4-hydroxy-4-phenylbutanoic acid. The 300-MHz ^{1}H NMR of this product is shown in Figure 1A.

Preparation of Dihydrokynurenines. To a solution of 21 mg of L-kynurenine (Sigma) in 5 mL of H_2O was added 10 mg of $NaBH_4$. The reaction mixture was stirred overnight at room temperature, and the initially pale yellow solution became colorless. The UV spectrum of the solution showed formation of a new peak at 283 nm and disappearance of the peak at 360 nm due to kynurenine. The reaction was then lyophilized, and the solid was dissolved in 5 mL of 0.1% acetic acid. The diastereomers of dihydro-L-kynurenine were separated by preparative HPLC on a Rainin HPLC system, using a 20 × 250 nm C18 column (Dynamax, Rainin) in 0.1% acetic acid at 5 mL/min. The elution of the dihydro-L-kynurenines was detected by following the UV absorbance at 283 nm on an LDC Spectromonitor 3000. The separation of these diastereomers is difficult, and less than 0.5 mg could be separated during each run. The fractions containing the separated diastereomers were pooled and lyophilized to give 7.2 mg of the first peak (4*S* isomer) and 12.4 mg of the second peak (4*R* isomer). The 300-MHz ^{1}H NMR spectra of the diastereomers are shown in Figure 1B and C.

Acknowledgment. This research was supported by a grant from the National Institutes of Health to RSP (GM-42588).

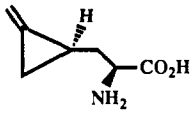
Mechanistic Study on the Inactivation of General Acyl-CoA Dehydrogenase by a Metabolite of Hypoglycin A

Ming-tain Lai, Li-da Liu, and Hung-wen Liu*

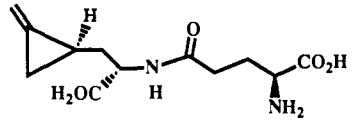
Contribution from the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received March 28, 1991*

Abstract: General acyl-CoA dehydrogenase (GAD) is a flavin-dependent (FAD) enzyme that catalyzes the oxidation of a fatty acyl-CoA to the corresponding α,β-enolyl-CoA. When GAD is exposed to (methylenecyclopropyl)acetyl-CoA (MCPA-CoA), a metabolite of hypoglycin A that is the causative agent of Jamaican vomiting sickness, time-dependent inhibition occurs with concomitant bleaching of the active-site FAD. The inactivation mechanism is generally believed to be initiated by C_α anion formation followed by ring fragmentation and the covalent modification of FAD. However, formation of a cyclopropyl radical intermediate through one-electron oxidation followed by ring opening and then radical recombination to yield a modified FAD is an appealing alternative. As described herein, studies of the inactivation of GAD by (1*S*)- and (1*R*)-MCPA-CoA bearing a stereospecific tritium label at C_α have provided direct evidence suggesting that C_α proton abstraction occurs during inactivation and the two diastereomers of MCPA-CoA bind to the same locus in the active site of GAD. Despite the fact that the inactivations mediated by (1*R*)- and (1*S*)-MCPA-CoA proceed at different rates, the observed partition ratios are almost identical. Using [α,α-$^2H_2$]MCPA-CoA as inhibitors, we have found that the sluggish inactivation observed for (1*S*)-MCPA-CoA is not due to mechanistic rerouting, but is instead a result of the retardation of the initial deprotonation step. Thus, the equivalent partition ratios found in these studies clearly indicate that inactivation by either (1*R*)- or (1*S*)-MCPA-CoA follows the same chemical course. Such a lack of stereospecificity for the bond rupture at C_β of MCPA-CoA in the enzyme active site suggests that the ring-opening step leading to inactivation is likely a spontaneous event. Since the rearrangement of α-cyclopropyl radicals to ring-opened alkyl radicals is extremely rapid, the ring cleavage induced by an α-cyclopropyl radical may bypass the chiral discrimination normally imposed by the enzyme. Thus, the mechanistic insights deduced from this study support our early notion that inactivation of GAD by MCPA-CoA is likely to proceed through a radical mechanism.

Hypoglycin A (**1**), a methylenecyclopropane-containing amino acid, has been isolated together with its γ-glutamyl conjugate hypoglycin B (**2**) from the arillus and seeds of unripe ackee



HYPOGLYCIN A (**1**)



HYPOGLYCIN B (**2**)

(*Blighia sapida*).[1] While ripe ackee fruit serves as a dietary staple in Jamaica, ingestion of hypoglycin from unripe fruit has been recognized as the cause of Jamaican vomiting sickness.[2] The ingested hypoglycin is metabolized in vivo to methylenecyclopropaneacetic acid (MCPA) in two steps, and the subsequent coenzyme A derivative (MCPA-CoA, **3**) is the actual causative agent of Jamaican vomiting sickness.[3] The site at which hypoglycin toxicity occurs has been shown to be flavin-containing acyl-CoA dehydrogenases.[4] Since acyl-CoA dehydrogenase catalyzes the first step of β-oxidation, converting a fatty acyl thioester substrate to the corresponding α,β-enolyl-CoA product,[5]

(1) (a) Hassal, C. H.; Reyle, K. *Biochem. J.* **1955**, *60*, 334. (b) Fowden, L.; Pratt, H. M. *Phytochemistry* **1973**, *12*, 1677.

(2) (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: New York, 1975; p 39. (c) Osmundsen, H. *Trends Pharm. 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.

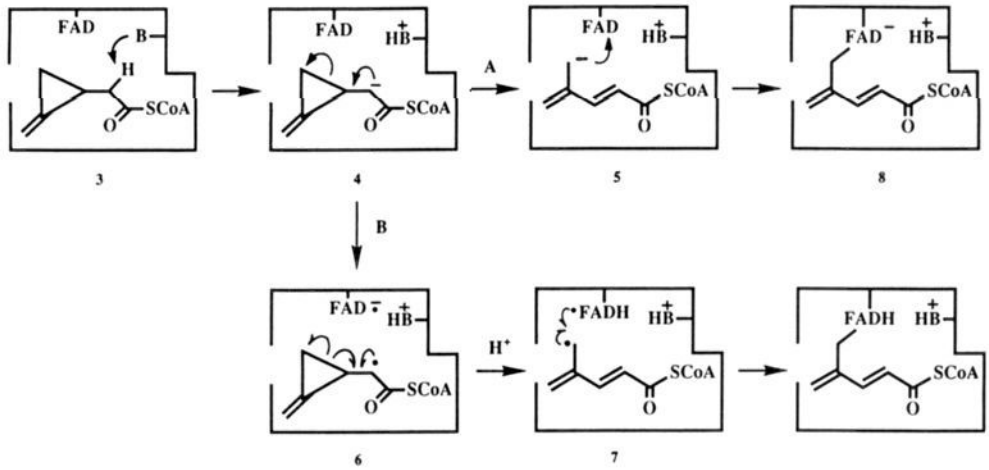
(3) (a) Tanaka, K.; Isselbacher, K. J.; Shih, V. *Science* **1972**, *175*, 69. (b) von Holt, C. *Biochim. Biophys. Acta* **1966**, *125*, 1. (c) von Holt, C.; von Holt, M.; Bohm, H. *Biochim. Biophys. Acta* **1966**, *125*, 11. (d) Black, D. K.; Landor, S. R. *J. Chem. Soc. C* **1968**, 288.

(4) (a) Osmundsen, H.; Sherratt, H. S. A. *FEBS Lett.* **1975**, *55*, 81. (b) Kean, E. A. *Biochim. Biophys. Acta* **1976**, *422*, 81. (c) Abeles, R. H. In *Enzyme Activated Irreversible Inhibitors*; Seiler, N., Jung, M. J., Koch-Weser, J., Eds.; Elsevier-North Holland: Amsterdam, 1978; p 1. (d) Ghisla, S.; Wenz, A.; Thorpe, C. In *Enzyme Inhibitors*; Brodbeck, U., Ed.; Verlag Chim.: 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.

(5) (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*; Bray, R. C., Engel, P. C., Mayhen, S. G., Eds.; Walter de Gruyter & Co.: Berlin, 1984; p 385, and references cited therein.

0002-7863/91/1513-7388$02.50/0

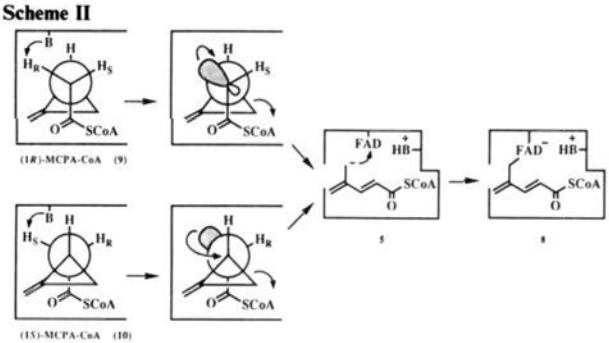
Scheme I



inhibition of this enzyme results in the accumulation of short-chain fatty acyl-CoAs and the suppression of acetyl-CoA/NADH production, both of which contribute to the induction of hypoglycemia.[6] In fact, the overall mortality of Jamaican vomiting sickness victims was over 80% before the introduction of glucose infusion treatment in 1954.

The dehydrogenation catalyzed by general acyl-CoA dehydrogenase (GAD) has been shown to be initiated by a C_α deprotonation, which is synchronous with a C_β hydride expulsion, yielding the α,β-enolyl-CoA in a net trans elimination.[5,7] When GAD is exposed to MCPA-CoA (**3**), time-dependent inactivation ensues with covalent modification of the bound FAD coenzyme, possibly as a C_6-substituted oxidized flavin–adduct or a C_{4a},N^5-dihydroflavin–adduct.[4f] The chemical course of this inhibition is also believed to be initiated by an α-proton abstraction (Scheme I). However, the highly strained ring structure of MCPA-CoA derails the subsequent step, producing a conjugated δ-anion **5** through ring fragmentation (Scheme I, route A). This anion then executes a nucleophilic attack leading to the covalent modification of the flavin coenzyme.[4] The crucial ring cleavage of **4** leading to inactivation has been proposed to be a direct anion-induced process. However, the modification of FAD could also occur through recombination of the flavin semiquinone radical and the acyclic radical **7**, which is derived from **4** via the transient α-cyclopropyl radical **6** through a one-electron-oxidation route (Scheme I, route B). Despite the fact that Jamaican vomiting sickness has been studied for many years, the question of whether the inactivation occurs through radical- or anion-mediated modification has never been fully addressed.

Scheme II



Recently, we have found that this inactivation is nonstereospecific since the partition ratio of the inactivation of GAD caused by racemic MCPA-CoA is identical with that obtained from incubation with naturally derived optically active MCPA-CoA.[8,9] Because the rearrangement of an α-cyclopropyl radical to the straight-chain alkyl radical is an extremely rapid process,[10] such nonstereospecific inactivation may be envisaged as a spontaneous ring fragmentation induced by the transient α-cyclopropyl radical **6**, which supports the mechanism shown in Scheme I, route B. However, study of the three-dimensional structure of GAD from pig liver reveals that its active site has enough room to bind the acyl-CoA substrate at either side (*re* or *si* face) of the flavin ring.[11] Although the naturally occurring acyl-CoA substrate appears to bind to GAD only at the *re* face of the FAD cofactor,[12] it is possible that the inhibitors, (1*R*)- and (1*S*)-MCPA-CoA (**9** and **10**), are bound to the active site of GAD on opposite sides of FAD in a mirror-image orientation. Subsequent deprotonation followed by ring cleavage may follow a stereochemical course identical with the dehydrogenation of the acyl-CoA substrate for both isomers, resulting in bond rupture at C_β with opposite stereospecificity (Scheme II). Thus the apparent lack of the stereospecificity of C_β bond cleavage of MCPA-CoA may be a consequence of opposite binding preferences of (1*R*)- and (1*S*)-MCPA-CoA, not a result of a radical-initiated spontaneous ring fragmentation as

(6) (a) Jelliffe, D. B.; Stuart, K. L. *Br. Med. J.* **1954**, *1*, 175. (b) Soling, H. D.; Willms, B.; Friedrichs, D.; Kleineke, J. *Eur. J. Biochem.* **1968**, *4*, 364. (c) Bressler, R.; Corredor, C. F.; Brendel, K. *Pharmacol. Rev.* **1969**, *21*, 105.

(7) It is noteworthy that the mechanism of an elimination reaction is determined solely by the lifetime of the carbanion: Banait, N. S.; Jencks, W. P. *J. Am. Chem. Soc.* **1990**, *112*, 6950. In support of a concerted (E2) mechanism for this dehydrogenation, the initially formed C_α anion of a straight-chain acyl-CoA substrate has been suggested to be a transient species due to the large difference between the pK_a of the C_α proton (>16) of the substrate and that of the protein base (~4), which is most likely a glutamate residue at position 401.[5d] However, when the transfer of reducing equivalents from the C_α anion to FAD was blocked by using appropriate substrate analogues, enolate formation in these substrates was observed at neutral pH: Powell, P. J.; Thorpe, C. *Biochemistry* **1988**, *27*, 8022. In fact, the apparent pK_a for the C_α proton of a straight-chain acyl-CoA substrate in the active site of GAD has been estimated to be 6.0 in an enzyme-catalyzed solvent hydrogen exchange experiment: Ikeda, Y.; Tanaka, K. In *Fatty Acid Oxidation; Clinical, Biochemical, and Molecular Aspects*; Tanaka, K., Coates, P. M., Eds.; Alan R. Liss, Inc.: New York, 1990; p 107. These findings, together with the results obtained from an electrochemical study of the effects of substrate binding on GAD-mediated electron relay (Lenn, N. D.; Stankovich, M. T.; Liu, H.-w. *Biochemistry*, **1990**, *29*, 3709) and may spectroscopic analyses of the nature of the enzyme–substrate charge-transfer complex, suggest that the C_α anion of a straight-chain acyl-CoA generated in the active site of GAD is thermodynamically stable, at least in the absence of electron acceptors. As long as the lifetime of this carbanion is significant, the overall elimination will be stepwise.

(8) Lenn, N. D.; Shih, Y.; Stankovich, M. T.; Liu, H.-w. *J. Am. Chem. Soc.* **1989**, *111*, 3065.

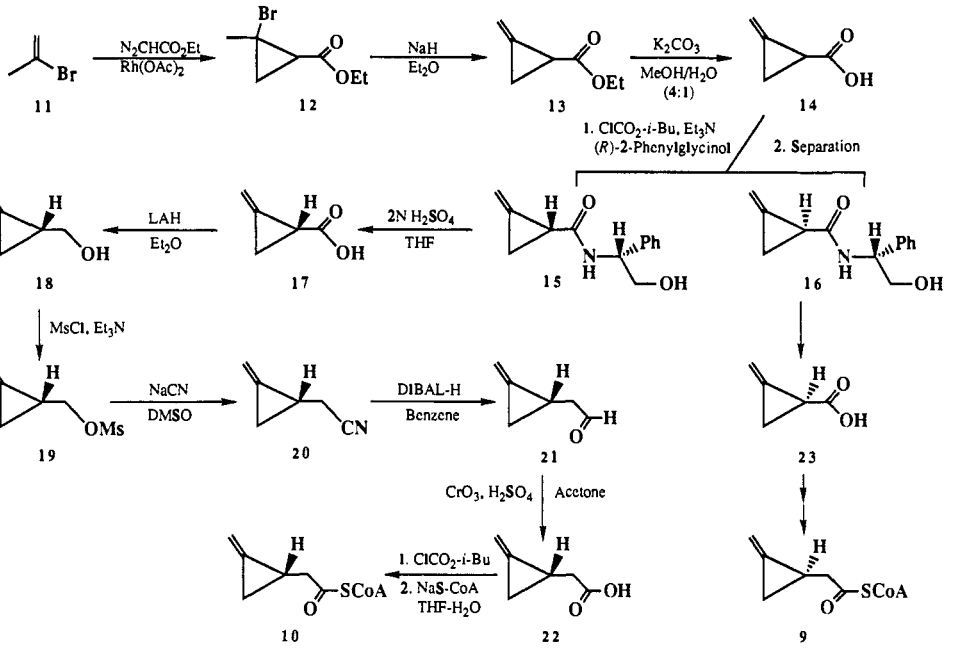
(9) MCPA-CoA used in the original inhibition studies was prepared from hypoglycin via L-amino acid oxidase mediated deamination, H_2O_2-induced decarboxylation, and acyl-CoA synthetase catalyzed thioester formation.[4] However, the opticaly purity of the naturally derived MCPA was estimated to be only 17%.[14]

(10) (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.

(11) Kim, J. P.; Wu, J. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6677.

(12) Manstein, D. J.; Pai, E. F.; Schopfer, L. M.; Massey, V. *Biochemistry* **1986**, *25*, 6807.

Scheme III



had been previously surmised. In a preliminary communication,[13] we described the use of MCPA-CoA enantiomers to confirm the nonstereospecific nature of this inactivation. We report here a full account of the synthesis of MCPA-CoA and its isotopically labeled derivatives in both enantiomerically pure forms, and the inactivation of GAD by these compounds to resolve the aforementioned mechanistic ambiguity. As detailed below, the insights gained from these experiments have allowed us to define the stereochemical course of this inhibition. These findings also support our early notion that GAD is capable of mediating one-electron oxidation–reduction.[8,13]

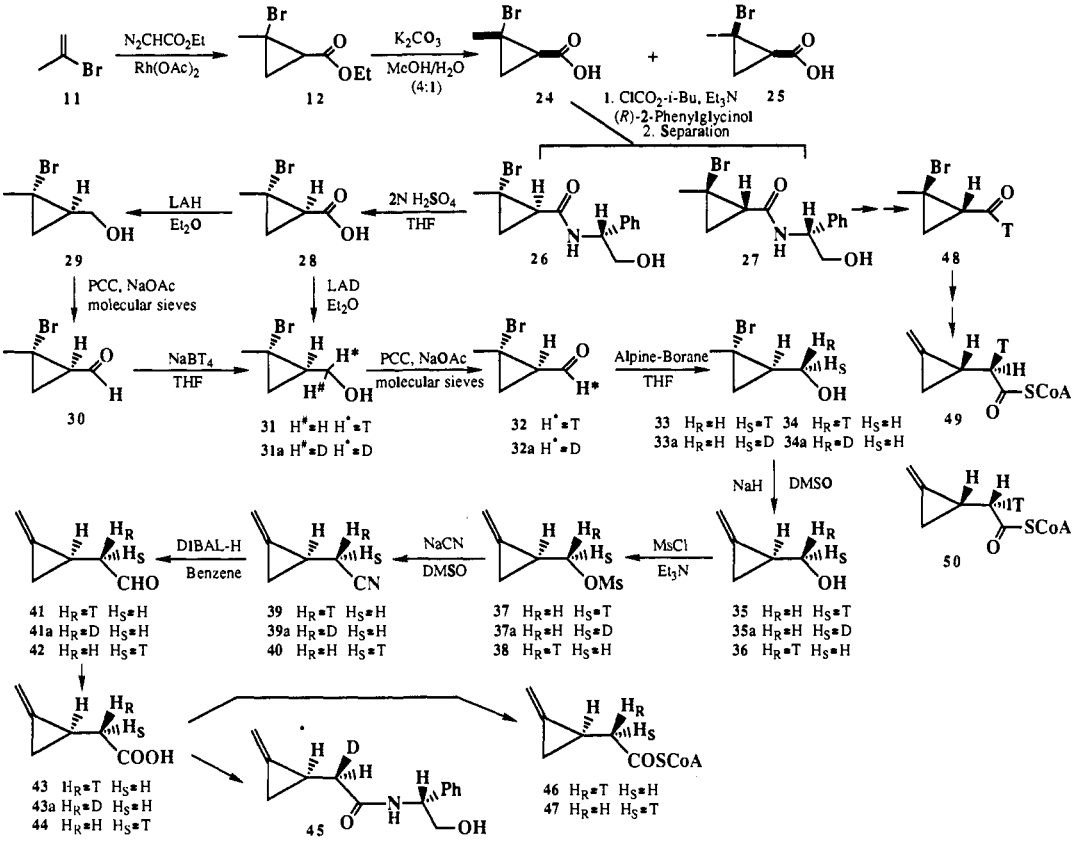
Results and Discussion

Preparation and Incubation of Enantiomerically Pure MCPA-CoA with GAD. Although our early results suggested that the inactivation of GAD by MCPA-CoA appears to be equally effective with both MCPA-CoA epimers, this finding contradicted a recent report in which the C_α antipode of naturally derived MCPA-CoA was found to have no significant effect on the inactivation of GAD.[14] Since both of these disparate conclusions were deduced by comparison of the inactivation efficiency of racemic MCPA-CoA with the values found in the literature obtained with MCPA-CoA derived from a natural source,[4d,e] the conflicting results may be attributed to differences in the conditions and the purities of the materials used in the experiments described in these studies. In an attempt to resolve this stereochemical discrepancy, we have prepared MCPA-CoA in both enantiomerically pure forms. As depicted in Scheme III, synthesis of the enantiomerically pure MCPA-CoA (**9** and **10**) was initiated by a rhodium acetate catalyzed cyclopropanation of 2-bromopropene (**11**) with ethyl diazoacetate.[15] Sodium hydride induced elimination followed by base hydrolysis converted the resulting product **12** to methylenecyclopropanecarboxylate (**14**) in good yield.[16] Derivatization of **14** with (*R*)-2-phenylglycinol led to a diastereomeric mixture (**15** and **16**), which could be readily separated by flash chromatography.[17] Since the relative elution order of diastereomeric amides of this class by liquid adsorption chromatography has been well established[17,18] the retention times of amides **15** and **16** established them as the *R,R* and *S,R* isomers, respectively. The separation of isomers **15** and **16** on an HPLC silica gel column eluted with 10% 2-propanol in methylene chloride showed that their enantiomeric purities were greater than 99%. The resolved amide **15** was converted to (1*R*)-methylenecyclopropanemethanol (**18**) in 77% yield by acid hydrolysis and then hydride reduction. ^{1}H NMR analysis of its Mosher ester showed a greater lanthanide-induced shift of the methoxyl signal than that of the 1*S* isomer derived from **16**.[19] This result confirmed the aforementioned stereochemical assignment of these two epimers. Chain elongation converting **18** to MCPA (**22**) was accomplished in four steps with an overall 36% yield. This acid (**22**) showed an optical rotation of +8.60 ($CHCl_3$, *c* 1.6).[20] Condensation of **22** with isobutyl chloroformate followed by coupling to coenzyme A in aqueous THF solution (pH 8–8.5)[21] afforded the desired (1*S*)-MCPA-CoA (**10**). The corresponding 1*R* epimer (**9**) was prepared from amide **16** via **23** by an identical sequence. The crude MCPA-CoA was purified on a HPLC C_{18} column and eluted with 30% methanol in 50 mM potassium phosphate buffer, pH 5.3.[22] The yield of thioester formation was 40%.

Following the method of Wenz et al.,[4e] the effects of the MCPA-CoA isomers (**9** and **10**) on the catalytic activity of GAD were analyzed by successive titration with aliquots of each isomer. A plot of the residual activity observed under aerobic conditions versus the total equivalents of MCPA-CoA added gave a partition ratio of 3.4 and 4.0 for (1*R*)- and (1*S*)-MCPA-CoA, respectively.[13,23] These results clearly indicate that both diastereomers of MCPA-CoA are competent inhibitors; therefore, the inactivation is nonstereospecific.[24] Such a lack of stereospecificity of bond rupture at C_β of MCPA-CoA in the enzyme active site provided compelling evidence suggesting that the ring-opening

(13) Lai, M.-t.; Liu, H.-w. *J. Am. Chem. Soc.* **1990**, *112*, 4034.
(14) Baldwin, J. E.; Parker, D. W. *J. Org. Chem.* **1987**, *52*, 1475.
(15) Hubert, A. *Synthesis* **1976**, 600.
(16) (a) Newman, M. S.; Merrill, S. *J. Am. Chem. Soc.* **1955**, *77*, 5549. (b) Carbon, J. A.; Martin, W. B.; Swett, L. R. *J. Am. Chem. Soc.* **1958**, *80*, 1002.
(17) (a) Helmchen, G.; Nill, G.; Flockerzi, D.; Youssef, M. S. K. *Angew. Chem., Int. Ed. Engl.* **1979**, *18*, 63. (b) Baek, D.-J.; Daniels, S. B.; Reed, P. E.; Katzenellenbogen, J. A. *J. Org. Chem.* **1989**, *54*, 3963.
(18) (a) Helmchen, G.; Nill, G.; Flockerzi, D.; Schühle, W.; Youssef, M. S. K. *Angew. Chem., Int. Ed. Engl.* **1979**, *18*, 62. (b) Helmchen, G.; Volter, H.; Schühle, W. *Tetrahedron Lett.* **1977**, 1417.
(19) (a) Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* **1973**, *95*, 512. (b) Yasuhara, F.; Yamaguchi, S. *Tetrahedron Lett.* **1977**, 4085.
(20) The optical rotations of acids **22** and the corresponding 1*R* epimer were erroneously reported in our preliminary communication[13] as +0.86 ($CHCl_3$, *c* 1.6) and −0.82 ($CHCl_3$, *c* 1.5), respectively. The correct readings should be +8.60 and −8.16 for these two acids.
(21) Belcher, M. *Methods Enzymol.* **1981**, *72*, 404.
(22) Eberhard, A. *Chromatogram* **1987**, *8*, 10.
(23) A similar result was also obtained for the inactivation by racemic MCPA-CoA in which a partition ratio of 3.4 was observed.
(24) A similar stereochemical investigation has also been reported recently: Baldwin, J. E.; Ostrander, R. L.; Simon, C. D.; Widdison, W. C. *J. Am. Chem. Soc.* **1990**, *112*, 2021.

Scheme IV



step leading to inactivation may be a spontaneous event, induced by an α-cyclopropyl radical **6** (Scheme I, route B). However, as mentioned above, these results are also consistent with the interpretation that (1*R*)- and (1*S*)-MCPA-CoA binds to the active site of GAD in opposite orientations which, after deprotonation, lead to bond rupture at C_β with opposite stereospecificity (Scheme II).

An important aspect distinguishing these two inactivation mechanisms is the stereoselectivity of the initial C_α deprotonation. Assuming such a proton abstraction is mediated by a single active-site nucleophile, inactivation by route B of Scheme I would result in the loss of the same C_α hydrogen from both (1*R*)- and (1*S*)-MCPA-CoA. In contrast, inactivation via the mechanism of Scheme II would predict an opposite stereoselectivity of C_α proton abstraction for the two epimers. In order to discern between these two inactivation mechanisms, enantiomerically pure MCPA-CoAs bearing a stereospecific tritium label at C_α were synthesized, allowing the stereochemical outcome of the deprotonation at C_α to be defined.

Preparation and Incubation of Enantiomerically Pure MCPA-CoA with Tritium Labeling at C_α. Synthesis of tritium labeled (1*R*)- and (1*S*)-MCPA-CoA followed a strategy similar to that used in the preparation of the parent molecules (**9** and **10**) (Scheme IV).[25] The key intermediate, 2-bromo-2-methylcyclopropanecarboxylic acid, was obtained as a mixture of two isomers (**24** and **25**, 2:1) that are readily separable by flash chromatography (5% ethyl acetate/hexane). The syn disposition of the large groups (bromine and carboxyl) in **25** was assigned on the basis of an observed nuclear Overhauser enhancement between 1-H and 2-Me in a ^{1}H NMR NOESY experiment. Since the anti isomer **24** was isolated as the major product, the subsequent synthesis was carried out with **24**. As shown in Scheme IV, derivatization of **24** with (*R*)-2-phenylglycinol gave a diastereomeric mixture of **26** and **27** that could be resolved by flash chromatography (20% ethyl acetate/hexane). According to the elution order, compound **27**, which had a longer retention time, was designated the 1*S*,2*R* isomer, while compound **26**, which eluted before **27** under identical conditions, was designated the 1*R*,2*S* isomer.[17,18,26] The enantiomeric purity of these samples was determined to be greater than 99% by HPLC and confirmed by ^{1}H NMR analysis since **26** and **27** have different chemical shifts for the 2-Me resonance (δ 1.78 and 1.87, respectively). The resolved amide **26** was converted in three steps to aldehyde **30**, which was then reduced by sodium [^{3}H]borohydride. The resulting alcohol **31** (7.5 mCi/mol), upon oxidation with pyridinium chlorochromate (PCC), yielded aldehyde **32** which, as anticipated, retained most of the isotopic labeling (7.3 mCi/mol).[27] Conversion of **32** to the stereospecifically labeled alcohol (**33**/**34**) was accomplished by Alpine-Borane (Aldrich) reduction.[28] When (*R*)-Alpine-Borane was used in the reduction, this well-developed reducing agent converted **32** to **33** with tritium labeling at the α-H_S locus. Replacing the reducing agent with (*S*)-Alpine-Borane afforded **34** as the product, bearing tritium at the α-H_R position. In order to assess the stereospecificity of this reduction, identical reactions were performed on a deuterated sample (**32a**) that was prepared from **28** by lithium aluminum deuteride reduction followed by PCC oxidation. To our delight, the diastereotopic protons of interest (α-H_R and α-H_S) in **33a**/**34a** were well resolved at δ 3.70 and 3.53. The diminishing of the high-field signal (δ 3.53) from isomer **33a** and the low-field signal (δ 3.70) from isomer **34a** permitted an unambiguous assignment of these resonances to the protons at α-H_S and α-H_R, respectively. The stereospecificity of

(25) The key step of this synthesis is a stereospecific reduction by Alpine-Borane. Since Alpine-Borane will also reduce an exocyclic methylene moiety, this functional group was masked as a bromide derivative prior to reduction.

(26) The stereochemical assignments were eventually confirmed by comparing the optical rotations of the MCPAs (**43** and **44**) generated in this synthesis with those of **22** and its 1*R* epimer.

(27) (a) Banerji, K. K. *Bull. Chem. Soc. Jpn.* **1978**, *51*, 2732. (b) Shih, Y.; Yang, D.-y.; Weigel, T. M.; Liu, H.-w. *J. Am. Chem. Soc.* **1990**, *112*, 9652.

(28) (a) Midland, M. M.; Greer, S.; Tramontano, A.; Zderic, S. A. *J. Am. Chem. Soc.* **1979**, *101*, 2352. (b) Midland, M. M.; DcDowell, D. C.; Hatch, R. L.; Tramontano, A. *J. Am. Chem. Soc.* **1980**, *102*, 867. (c) Brown, H. C.; Pai, G. G. *J. Org. Chem.* **1985**, *50*, 1384.

Table I. Stereochemical Analysis of GAD-Mediated α-Proton Abstraction

substrate	% of tritium washout	
	control	sample
(1*S*,α*R*)-[α-^{3}H]MCPA-CoA	0.2	94.6
(1*S*,α*S*)-[α-^{3}H]MCPA-CoA	0.1	26.4
(1*R*,α*R*)-[α-^{3}H]MCPA-CoA	0.08	96.3
(1*R*,α*S*)-[α-^{3}H]MCPA-CoA	0.08	16.5

this reduction was estimated by comparing the integration of these signals to be greater than 94%, translating to an 88% enantiomeric excess. Chain elongation converting **33**/**34** to acids **43**/**44** was effected by the same reaction sequence used in the synthesis of the unlabeled acid **22** (Scheme III). The corresponding deuterated species (**33a**–**43a**) were also prepared so as to monitor the stereospecificity of these reactions. Derivatization of **43a** with (*R*)-2-phenylglycinol gave the amide **45**, whose α-H_R and α-H_S signals are well resolved at δ 2.40 and 2.21, respectively. Judging from the integration of these resonances, the diastereomeric purity of **45** was estimated to be at least 84%. Evidently the cyanide substitution step is fully stereospecific and proceeds with clear inversion. Conversion of (1*R*)-MCPAs (**43** and **44**) to their CoA derivatives (**46** and **47**) followed a sequence identical with that described in the synthesis of **9** and **10**. The corresponding tritiated (1*S*)-MCPAs (**49** and **50**) were synthesized analogously from amide **27**.

Each of the stereospecifically tritiated MCPA-CoAs were incubated with GAD for 15 min, after which the reaction was quenched by activated charcoal (10% suspension). After centrifugation, the radioactivity of the supernatant was measured by scintillation counting. Identical incubations using denatured enzyme were run in parallel to serve as controls. As shown in Table I, the incubations of (1*R*,α*R*)- and (1*S*,α*R*)-[α-^{3}H]-MCPA-CoA (**46** and **49**, respectively) were found to release 4 times as much radioactivity as the corresponding α*S* tritium-labeled MCPA-CoA isomers (**47** and **50**). Clearly, GAD-catalyzed deprotonation of MCPA-CoA stereoselectively removes the α-proton from the *pro-R* position. The *pro-R* stereochemical convergency exhibited by both (1*R*)- and (1*S*)-MCPA-CoA strongly suggests that these diastereomers bind to the active site of GAD at the same locus in the same orientation. On the basis of this observation, the pathway shown in Scheme II may be ruled out as a possible inactivation route.

Preparation and Incubation of Enantiomerically Pure MCPA-CoA with Double-Deuterium Labeling at C_α. The above results clearly indicate that both diastereomers of MCPA-CoA are competent inhibitors and the enzyme inactivations they mediate are closely related, both physically and chemically, as manifested by their binding preference and partition ratios. However, it is worth noting that the inactivation of GAD by (1*R*)- and (1*S*)-MCPA-CoA (**9** and **10**) proceeded at different rates.[13,24] As shown in Figure 1, the inactivation caused by (1*R*)-MCPA-CoA is 1.57 times faster than that observed for the 1*S* isomer under identical conditions. It was suggested that the rate difference may be a result of a rate-limiting enzyme-catalyzed isomerization of **10** to **9** by way of (2-methylenecyclopropylidene)acetyl-CoA.[24] While such an isomerization is conceivable through a ring-opening and reclosure sequence, or via a reduction–oxidation process involving hydride transfer from C-1 to FAD, the driving force for these transformations is not immediately obvious. A more lucid interpretation, requiring no mechanistic rerouting, is that the above rate distinction may simply reflect differences in the accessibility of the MCPA-CoA isomers (**9** and **10**) to the enzyme active-site nucleophile, which is responsible for the C_α proton abstraction from the inhibitor that triggers the subsequent modification of FAD. This contention is appealing since α-proton abstraction is partially rate limiting in the normal catalytic turnover of fatty acyl-CoA by GAD.[5] This deprotonation step is expected to be very sensitive to the steric environment around C_α of **9** and **10** in the enzyme active site.[13] In an effort to gain further insights into this inactivation, we have synthesized (1*S*)-[α,α-$^2H_2$]- and

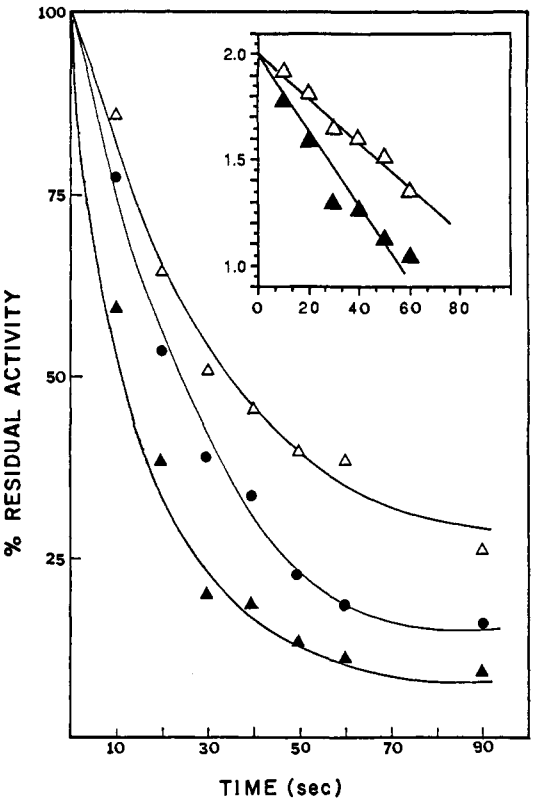
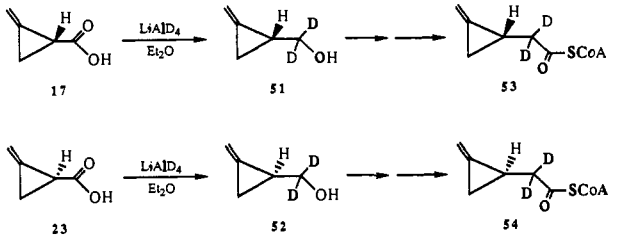


Figure 1. Inactivation of GAD by MCPA-CoA. The incubation conditions are detailed in the Experimental Section. The inset shows a semilogarithmic plot of the percentage of residual activity versus the inactivation time: (▲) (*R*)-MCPA-CoA, (●) racemic MCPA-CoA, and (△) (*S*)-MCPA-CoA.

Scheme V



(1*R*)-[α,α-$^2H_2$]MCPA-CoA (**53** and **54**) according to the reaction sequence shown in Scheme III, except that lithium aluminum deuteride replaced LAH in the reduction of **17** and **23** (Scheme V).

With these α,α-dideuterated MCPA-CoAs in hand, we have measured and compared the inhibition rates of GAD by the labeled and unlabeled inhibitors. As shown in Figure 2, the inhibition rate of (1*R*)-[α,α-$^2H_2$]MCPA-CoA was almost the same as that of unlabeled (1*R*)-MCPA-CoA. However, the inactivation by (1*S*)-MCPA-CoA was more sluggish when the α,α-dideuterated species was used in the incubation. The kinetic isotope effect of 1.3 observed in the later case clearly demonstrates that α-proton abstraction from (1*S*)-MCPA-CoA is partially rate limiting.

Conclusion

Studies of the inactivation of GAD by (1*R*)- and (1*S*)-MCPA-CoA bearing a stereospecific tritium label at C_α (**46**, **47**, **49**, **50**) have provided, for the first time, direct evidence suggesting that C_α proton abstraction occurs during inactivation and the two diastereomers of MCPA-CoA binds to the same locus in the active site of GAD. Careful analysis also revealed that inactivation caused by the incubation of (1*S*)-MCPA-CoA is slightly slower than that by the 1*R* isomer. This observation has raised the concern that inactivation of GAD by the two diastereomeric inhibitors may be mechanistically distinct. Using the dideuterated

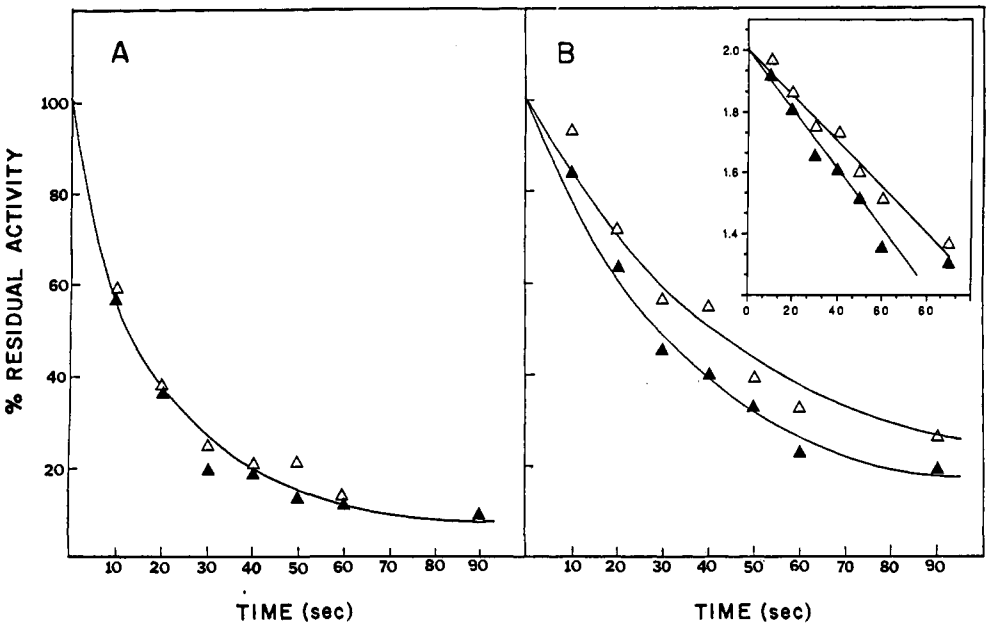


Figure 2. Effects of isotope substitution on the inactivation of GAD by MCPA-CoA. Panel A: (▲) (1*R*)-MCPA-CoA and (△) (1*R*)-[α,α-$^2H_2$]-MCPA-CoA. Panel B: (▲) (1*S*)-MCPA-CoA and (△) (1*S*)-[α,α-$^2H_2$]MCPA-CoA. The inset in Panel B shows a semilogarithmic plot of the percentage of residual activity versus the inactivation time: (▲) (1*S*)-MCPA-CoA and (△) (1*S*)-[α,α-$^2H_2$]MCPA-CoA.

species **53** and **54** as inhibitors, we have found that the sluggish inactivation observed for (1*S*)-MCPA-CoA is not due to mechanistic rerouting, but is instead a result of the retardation of the initial deprotonation step which, in turn, may reflect the less than ideal positioning between the enzyme base and the *pro-R* hydrogen of this isomer in the active site of GAD.[29] It is likely that the steric interaction imposed by the methylenecyclopropyl group of (1*S*)-MCPA-CoA obstructs the nucleophile's abstraction of the C_α proton and consequently slows down the inactivation. Despite the differing rates of (1*R*)- and (1*S*)-MCPA-CoA-mediated GAD inactivation, the observed partition ratios are almost identical. Since the partition ratio defines the number of latent inhibitor molecules converted to product relative to each turnover leading to enzyme inactivation, the equivalent partition ratios found in the present study clearly indicate that the inactivation by either (1*R*)- or (1*S*)-MCPA-CoA follows the same chemical course; therefore, the inactivation must be nonstereospecific. Such a lack of stereospecificity for the bond rupture at C_β of MCPA-CoA in the enzyme active site strongly suggests that the ring-opening step leading to inactivation is likely a spontaneous event, induced by an α-cyclopropyl radical. Since the rearrangement of α-cyclopropyl radicals to ring-opened alkyl radicals is extremely rapid, the ring cleavage may bypass the chiral discrimination normally imposed by the enzyme. Interestingly, a recent report has shown that the C_α anion (**4**) or its enolate, generated by LDA in THF, is kinetically stable and preferentially undergoes a bimolecular reaction in solution, in addition to the unimolecular ring-opening isomerization.[30] If ring cleavage of **3** is indeed an anion-induced process and the resulting allylic anion **5** is the reactive intermediate in the active site that covalently modifies FAD to inactive the enzyme, one would expect that its precursor **4** may be reprotonated and released from the active site or coupled with the nearby flavin coenzyme prior to the kinetically lethargic unimolecular isomerization step.[31] Since the modified cofactor in the inactivated enzyme has been partially characterized as an adduct of the flavin fused solely with a skeleton similar to acyclic anion **5**,[4f] these results appear to be consistent with the interpretation that the initially formed C_α anion (**4**) exists only as a transient intermediate and that ring cleavage is induced by a C_α radical (**6**) via a one-electron-oxidation route.[32] Thus, the mechanistic insights deduced from this study support our early notion that GAD may be capable of mediating one-electron oxidation–reduction and inactivation of GAD by MCPA-CoA proceeds through a radical mechanism.

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 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 * may be interchangable. Flash chromatography was performed in 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. Solvents, unless otherwise specified, were reagent grade and distilled once prior to use. It should be noted that the tritium-containing compounds prepared in this work were not submitted to elemental analysis or exact mass measurement due to the possibility of radioactive contamination. However, satisfactory analytical results were obtained with the corresponding unlabeled or deuterium-labeled analogues.

Enzyme. General acyl-CoA dehydrogenase (GAD), isolated from pig kidneys, was purified to homogeneity according to the procedure of Thorpe et al.[33] with the addition of octyl-Sepharose column chromatography to aid in the removal of contaminating enoyl hydratase activity.[34] The overall yield of a typical purification was 200–300 nmol of purified GAD/kg of kidney cortex. Concentration of the holoenzyme was de-

(29) Interestingly, the K_I's of (1*S*)- and (1*R*)-MCPA-CoA were estimated in a preliminary study to be 1.15 and 2.38 μM, respectively. Thus, GAD appears to have a higher affinity toward the 1*S* isomer than the 1*R* isomer.

(30) Baldwin, J. E.; Ghatlia, N. D.; Parker, D. W. *Bioorg. Chem.* **1990**, *18*, 221.

(31) However, the absence of adduct formation between FAD and the initially formed C_α anion **4** may also be explained by the reversibility of such an addition or the inaccessibility of this carbanion species toward the flavin coenzyme. A thorough analysis of the docking of MCPA-CoA with respect to FAD in the active site of GAD would provide invaluable insights into this question.

(32) It is conceivable that the nonstereospecificity observed for the cleavage of the highly strained methylenecyclopropane ring during the inactivation of GAD by MCPA-CoA is due to the racemization at C_α via enolization of the C_α anion (**4**). Although this possibility cannot be ruled out by our current data, the fact that an inert C–H bond at C_β of a straight-chain acyl-CoA substrate is cleaved in a highly stereospecific fashion renders such a E1cB mechanism less likely.

(33) Thorpe, C. *Methods Enzymol.* **1981**, *71*, 366.

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

termined spectrophotometrically with a molar absorptivity of 15.4 mM^{-1} cm^{-1} at 446 nm for oxidized GAD.[33] 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). A standard 0.7-mL assay was performed, as described before,[33] in 50 mM potassium phosphate buffer (pH 7.6) at 25 °C, containing 33 μM octanoyl-CoA, 30 μM DCPIP, 10 mM PMS, and 60 μM EDTA. The reaction was initiated by the addition of an appropriate amount of GAD. Loss of DCPIP absorbance at 600 nm, before and after the addition of GAD, was monitored. Under these conditions, the purified GAD showed a V_{max} of 4.27 (mmol of DCPIP) (mg of FAD)$^{-1}$ min^{-1}, and a K_m of 2.25 μM.

Substrate Preparation. Ethyl 2-Bromo-2-methylcyclopropanecarboxylate (12). Ethyl diazoacetate (21.7 g, 0.19 mol) was added slowly via an automatic Sage syringe over a period of 2 days to a stirred solution of rhodium(II) acetate (90 mg, 0.2 mmol) in 2-bromopropene (**11**; 50 g, 0.41 mol) at room temperature under an argon atmosphere. The resulting mixture was filtered to remove the precipitated rhodium salts. The filtrate was concentrated and then distilled under reduced pressure [bp 70–80 °C (35 Torr)] to give ethyl 2-bromo-2-methylcyclopropanecarboxylate (**12**) as a colorless oil in 86% yield. Since the product was a mixture of trans and cis isomers, the NMR spectra of **12** showed two sets of overlapping signals: ^{1}H NMR ($CDCl_3$) δ 4.13, 4.09 (2 H each, *q*, *J* = 7.1, OCH_2's), 2.23 (1 H, dd, *J* = 9.3, 6.6, 1-H of trans isomer), 1.80, 1.77 (3 H each, s, 2-Me's), 1.74–1.65 (2 H, m, 1-H, 3-H of cis isomer), 1.52 (1 H, dd, *J* = 9.3, 6.6, 3-H of trans isomer), 1.36 (1 H, t, *J* = 6.6, 3-H of trans isomer), 1.23, 1.22 (3 H each, t, *J* = 7.1, Me's), 1.15 (1 H, dd, *J* = 7.6, 5.2, 3-H of cis isomer); ^{13}C NMR ($CDCl_3$) δ 170.1, 169.2 (C=O's), 61.0, 60.9 (OCH_2's), 33.2, 32.9 (C-2's), 30.7, 29.6 (C-1's), 28.7, 24.2 (C-3's), 23.8, 22.8 (2-Me's), 14.3, 14.2 (Me's).

Ethyl Methylenecyclopropanecarboxylate (13). To a rapidly stirred, refluxing slurry of sodium hydride (6.0 g, 0.25 mol) and **12** (33.1 g, 0.16 mol) in ether (250 mL) under an argon atmosphere was added 1 mL of ethanol. Refluxing with stirring was continued overnight. The resulting mixture was filtered and concentrated under reduced pressure. The residue was then distilled under reduced pressure [bp 55–65 °C (35 Torr)] to give ethyl methylenecyclopropanecarboxylate (**13**) as a colorless oil in 81% yield: ^{1}H NMR ($CDCl_3$) δ 5.44 (2 H, d, *J* = 2.0, $=CH_2$), 4.07 (2 H, q, *J* = 7.2, OCH_2), 2.18 (1 H, m, 1-H), 1.73, 1.55 (1 H each, m, 3-H's), 1.19 (3 H, t, *J* = 7.2, Me); ^{13}C NMR ($CDCl_3$) δ 171.9 (C=O), 130.3 (C-2), 104.4 ($=CH_2$), 60.6 (OCH_2), 18.0 (C-1), 14.1 (C-3), 11.3 (Me).

Methylenecyclopropanecarboxylic Acid (14). To a solution of **13** (5 g, 39.7 mmol) in a mixture of 200 mL of methanol and water (v/v 4:1) was added 20 g of anhydrous potassium carbonate. After being stirred at room temperature for 2 days, the solution was acidified with 2 N HCl. The mixture was then extracted with methylene chloride and the combined organic extracts were dried over anhydrous magnesium sulfate. Subsequent filtration and concentration gave methylenecyclopropanecarboxylic acid (**14**) in 94% yield: ^{1}H NMR ($CDCl_3$) δ 5.52 (2 H, d, *J* = 1.8, $=CH_2$), 2.22 (1 H, m, 1-H), 1.85, 1.68 (1 H each, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 179.0 (C=O), 129.9 (C-2), 105.0 ($=CH_2$), 17.9 (C-1), 12.2 (C-3); high resolution CI-MS (CH_4) calcd for $C_5H_7O_2$ (M + 1)$^+$ 99.0446, found 99.0445.

***N*-[(*R*)-1′-Phenyl-2′-hydroxyethyl]-(1*R*)-methylenecyclopropanecarboxamide (15) and *N*-[(*R*)-1′-Phenyl-2′-hydroxyethyl]-(1*S*)-methylenecyclopropanecarboxamide (16).** To a solution of acid **14** (3.67 g, 37.4 mmol) and triethylamine (5.2 mL, 37.4 mmol) in THF (150 mL) was added isobutyl chloroformate (4.85 mL, 37.4 mmol) at 0 °C. The solution was stirred at room temperature for 1 h followed by the addition of (*R*)-phenylglycinol (5.1 g, 37.4 mmol) in 20 mL of THF. The resulting mixture was kept stirring for an additional 3 h and then filtered. The filtrate was concentrated to give crude amides **15** and **16**. The crude products were purified repeatedly by flash chromatography (30% ethyl acetate/hexane) to separate these diastereomers. The combined yield of purified compounds **15** (2.6 g) and **16** (2.1 g) was 58%. The enantiomeric excess (ee) of each isomer was determined by HPLC equipped with a Spheris ORB silica gel column (1 × 25 cm) that was eluted with 10% 2-propanol in methylene chloride (flow rate 4.0 mL/min, monitoring wavelength 254 nm). The retention times of isomers **15** and **16** under these conditions were 5.9 and 6.8 min, respectively. The enantiomeric purities of both isomers were found to be greater than 99%. **15**: ^{1}H NMR ($CDCl_3$) δ 7.37–7.28 (5 H, m, Ar H's), 6.43 (1 H, br s, NH), 5.54 (2 H, br s, $=CH_2$), 5.01 (1 H, dt, *J* = 12.1, 5.0, 1′-H), 3.82 (2 H, t, *J* = 5.0, 2′-H's), 2.92 (1 H, br s, OH), 2.16 (1 H, m, 1-H), 1.73. 1.58 (1 H each, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 171.7 (C=O), 139.0 (Ar C), 130.6 (C-2), 128.9, 127.9, 126.7 (Ar C's), 105.3 ($=CH_2$), 66.5 (C-2′), 56.1 (C-1′), 20.0 (C-1), 11.2 (C-3). **16**: ^{1}H NMR ($CDCl_3$) δ 7.38–7.27 (5 H, m, Ar H's), 6.33 (1 H, br s, NH), 5.55 (2 H, br s, $=CH_2$), 5.04 (1 H, dd, *J* = 11.5, 5.0, 1′-H), 3.85 (2 H, m, 2′-H's), 2.77 (1 H, br s, OH), 2.17 (1 H; m, 1-H), 1.72, 1.62 (1 H each, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 171.7 (C=O), 139.1 (Ar C), 130.7 (C-2), 128.9, 127.9, 126.7 (Ar C's), 105.2 ($=CH_2$), 66.4 (C-2′), 56.0 (C-1′), 20.0 (C-1), 11.2 (C-3); high-resolution FAB-MS calcd for $C_{13}H_{16}NO_2$ (M + 1)$^+$ 218.1181, found 218.1165.

(1*R*)-Methylenecyclopropanecarboxylic Acid (17). To a solution of **15** (2.3 g, 10.6 mmol) in THF (50 mL) was added equal volume of 4 N sulfuric acid (50 mL). After being refluxed for 4 h, the solution was cooled to room temperature and extracted with ether. The combined organic extracts were dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated in vacuo and the residue was purified by flash chromatography (10% ethyl acetate/hexane). The desired (1*R*)-methylenecyclopropanecarboxylic acid (**17**) was isolated in 95% yield. The NMR spectra of **17** are identical with those obtained for **14**; high-resolution CI-MS (CH_4) calcd for $C_5H_7O_2$ (M + 1)$^+$ 99.0446, found 99.0445.

(1*R*)-Methylenecyclopropanemethanol (18). To a suspension of lithium aluminum hydride (760 mg, 20 mmol) in anhydrous ether (150 mL) was added dropwise an ethereal solution (10 mL) of compound **17** (980 mg, 10 mmol) at 0 °C. The resulting mixture was stirred under argon overnight. The excess lithium aluminum hydride was carefully quenched by water and the resulting white precipitate was dissolved in 1 N NaOH. The milky-white aqueous phase was extracted with ether. The combined organic extracts were dried over anhydrous magnesium sulfate and filtered. The solvent was removed under reduced pressure to give the desired alcohol (**18**) in 81% yield: ^{1}H NMR ($CDCl_3$) δ 5.40, 5.34 (1 H each, d, *J* = 1.2, $=CH_2$), 3.55, 3.38 (1 H each, m, CH_2O), 2.90 (1 H, br s, OH), 1.70 (1 H, m, 1-H), 1.24, 0.89 (1 H each, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 132.2 (C-2), 103.0 ($=CH_2$), 64.2 (CH_2O), 16.9 (C-1), 7.2 (C-3); high-resolution FAB-MS calcd for C_5H_9O (M + 1)$^+$ 85.0653, found 85.0668. In order to confirm the stereochemical assignment, the Mosher ester of **18** was prepared and analyzed by NMR. Specifically, to a methylene chloride (15 mL) solution of α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA; 325 mg, 1.39 mmol), 4-(dimethylamino)pyridine (DMAP; 170 mg, 1.39 mmol), and compound **18** (117 mg, 1.39 mmol) was added 1,3-dicyclohexylcarbodiimide (DCC, 300 mg, 1.45 mmol) at 0 °C under argon. The resulting cloudy mixture was stirred at room temperature for 5 h. The precipitate was filtered and the filtrate was concentrated in vacuo. After purification by flash chromatography (0.5% ethyl acetate in hexane), the desired Mosher ester was isolated in 83% yield: ^{1}H NMR ($CDCl_3$) δ 7.55 (2 H, m, Ar H's), 7.43–7.39 (3 H, m, Ar H's), 5.43, 5.41 (1 H each, d, *J* = 1.1, $=CH_2$), 4.23 (2 H, d, *J* = 7.5, CH_2O), 3.58 (3 H, s, OMe), 1.89 (1 H, m, 1-H), 1.24, 1.07 (1 H each, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 166.6 (C=O), 132.4 (C-2), 131.4, 129.6, 128.4, 127.4 (Ar C's), 105.3 ($=CH_2$), 77.2 (OCF_3), 69.0 (CC=O), 55.5 (OMe), 13.9 (C-1), 8.8 (C-3). The magnitude of $Eu(fod)_3$-induced downfield shift for the methoxy signal of the MTPA ester of **18** is larger than that of the (1*S*)-methylenecyclopropanemethanol.

(1*R*)-(Methylenecyclopropyl)methyl Methanesulfonate (19). To a solution of alcohol **18** (680 mg, 8.1 mmol) in methylene chloride (50 mL) at 0 °C was added triethylamine (1.6 mL, 8.2 mmol). After the solution was stirred for 10 min, methanesulfonyl chloride (184 μL, 8.2 mmol) was added dropwise. The resulting mixture was stirred at room temperature for 1 h. The reaction was quenched with water and then extracted with methylene chloride. The combined organic extracts were washed with 1 N HCl, saturated $NaHCO_3$, and brine in sequence, dried over anhydrous magnesium sulfate, and filtered. The solvent was evaporated under reduced pressure to give the desired mesylate **19** in 92% yield: ^{1}H NMR ($CDCl_3$) δ 5.49, 5.44 (1 H each, d, *J* = 1.1, $=CH_2$), 4.19 (1 H, dd, *J* = 10.6, 6.6, CH_2O), 3.98 (1 H, dd, *J* = 10.6, 8.4, CH_2O), 2.98 (3 H, s, Me), 1.87 (1 H, m, 1-H), 1.39 (1 H, br t, *J* = 9.2, 3-H), 1.07 (1 H, m, 3-H); ^{13}C NMR ($CDCl_3$) δ 130.9 (C-2), 105.9 ($=CH_2$), 73.0 (CH_2O), 37.8 (OMs), 14.4 (C-1), 9.2 (C-3); high-resolution FAB-MS calcd for $C_6H_{11}O_3S$ (M + 1)$^+$ 163.0429, found 163.0437.

(1*S*)-Methylenecyclopropaneacetonitrile (20). To a mixture of 15-crown-5 (2.4 g, 11.1 mmol) and sodium cyanide (544 mg, 11.1 mmol) in anhydrous dimethyl sulfoxide (10 mL) was added dropwise a solution of mesylate (1.2 g, 7.4 mmol) in 3 mL of dimethyl sulfoxide. The reaction mixture was maintained at room temperature overnight. The solution was then mixed with water and extracted with methylene chloride. The combined organic extracts were washed with 1 N HCl, saturated $NaHCO_3$, and brine in sequence. The organic fractions were collected, dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (2% ethyl ether/petroleum ether) to give pure **20** in 62% yield: ^{1}H NMR ($CDCl_3$) δ 5.53, 5.44 (1 H each, d, *J* = 1.2, $=CH_2$), 2.40 (2

H, d, J = 6.5, CH_2CN), 1.67 (1 H, m, 1-H), 1.40 (1 H, br t, J = 9.0, 3-H), 0.96 (1 H, m, 3-H); ^{13}C NMR ($CDCl_3$) δ 132.0 (C-2), 118.4 (CN), 105.5 ($=CH_2$), 20.6 (CH_2CN), 10.6 (C-1), 9.4 (C-3); high-resolution CI-MS (CH_4) calcd for C_6H_8N $(M + 1)^+$ 94.0657, found 94.0660.

(1*S*)-Methylenecyclopropaneacetaldehyde (21). To a solution of **20** (400 mg, 4.3 mmol) in dry benzene (50 mL) was added diisobutylaluminum hydride (4.3 mL, 1 M in hexane) at 0 °C under argon. After being stirred at 0 °C for 3 h, the reaction was quenched with saturated ammonium chloride solution (20 mL). The resulting mixture was stirred for an additional 10 min and then acidified with 2 N HCl. The organic fraction was separated and collected, and the aqueous fraction was extracted with ether. The combined organic extracts were dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo. The desired (1*S*)-methylenecyclopropaneacetaldehyde (**21**) was isolated in 83% yield: ^1H NMR ($CDCl_3$) δ 9.85 (1 H, t, J = 2.0, CHO), 5.50, 5.45 (1 H each, d, J = 1.6, $=CH_2$), 2.45 (2 H, m, CH_2CHO), 1.65 (1 H, m, 1-H), 1.21, 0.89 (1 H each, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 201.5 (C=O), 128.5 (C-2), 104.8 ($=CH_2$), 47.1 (CH_2CHO), 9.7 (C-1)*, 9.1 (C-3)*; high-resolution FAB-MS calcd for C_6H_9O $(M + 1)^+$ 97.0653, found 97.0672.

(1*S*)-Methylenecyclopropaneacetic Acid (22). Compound **21** (300 mg, 3.1 mmol) was dissolved in acetone (20 mL) and treated with 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 till 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 oxidation. 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 organic extracts were dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (10% ethyl acetate/hexane) to give the desired acid **22** acid in 76% yield: $[\alpha]_D$ +8.60 ($CHCl_3$, c 1.6);[18] ^1H NMR ($CDCl_3$) δ 9.64 (1 H, br s, OH), 5.51, 5.42 (1 H each, d, J = 1.6, $=CH_2$), 2.39 (2 H, d, J = 7.2, CH_2COOH), 1.72 (1 H, m, 1-H), 1.38, 0.89 (1 H each, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 178.6 (C=O), 134.1 (C-2), 104.3 ($=CH_2$), 37.7 (CH_2CO_2H), 10.9 (C-1), 9.4 (C-3).

(1*S*)-(Methylenecyclopropyl)acetyl-CoA (10). To a solution of methylenecyclopropaneacetic acid (**22**; 30 mg, 0.27 mmol) in methylene chloride (5 mL) was added triethylamine (38 μL, 0.27 mmol) under an argon atmosphere. After stirring for 10 min, isobutyl chloroformate (34.7 μL, 0.27 mmol) was added dropwise at 0 °C. The reaction was agitated vigorously; during mixing fuming was noted. After stirring 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 MCPA-CoA was chromatographed on a preparative HPLC IBM C_{18} column (10 × 250 mm, 5 μm) and eluted with 30% methanol in 50 mM potassium phosphate buffer (pH 5.3, flow rate 3 mL/min, monitoring wavelength 260 nm).[21] Under these conditions, the retention time of MCPA-CoA was found to be 15 min. The MCPA-CoA-containing fractions were negative in the nitroprusside test[35] 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 IBM C_{18} column and washing with water (3 mL/min). The eluting solvent was changed 20 min later to methanol to wash out MCPA-CoA. The organic solvent was removed under reduced pressure and the aqueous solvent was lyophilized to give pure MCPA-CoA (**10**) as a white powder. The overall yield was 40%. The optical rotation of **10** was measured in 50 mM potassium phosphate buffer (pH 5.3) to be −8.4 (c 0.46);[36] ^1H NMR (^2H_2O) δ 8.51, 8.22 (1 H each, s, adenine H's), 6.10 (1 H, s, ribose anomeric H), *5.41*, *5.33* (1 H each, d, J = 1.8, $=CH_2$), 4.95–4.88 (1 H, buried under ^2HOH peak), 4.68, 4.48 (1 H each, s, ribose H's), 4.14 (2 H, s, ribose CH_2O), 3.93 (2 H, s), 3.79 (1 H, m), 3.46 (1 H, m), 3.35 (2 H, t, J = 11), 3.24 (2 H, t, J = 11), 2.90 (2 H, t, J = 10), *2.57* (1 H, br d, J = 12.0, $CH_2C=O$ of MCPA), *2.42* (1 H, br d, J = 12.0, $CH_2C=O$ of MCPA), 2.33 (2 H, t, J = 10), *1.62* (1 H, m, 1-H), *1.27*, *1.05* (1 H each, m, 3-H's), 0.81, 0.67 (3 H each, s, Me's); the measured sample was repeatedly dissolved in ^2H_2O and lyophilized prior to ^1H NMR analysis. The chemical shifts of the MCPA signals are shown in italic; high-resolution FAB-MS calcd for $C_{27}H_{43}N_7O_{17}P_3S$ $(M + 1)^+$ 862.1649, found 862.1702.

(1*S*)-Methylenecyclopropanecarboxylic Acid (23). To a solution of **16** (2.3 g, 10.6 mmol) in THF (50 mL) was added an equal volume of 4 N sulfuric acid (50 mL). After being refluxed for 4 h, the solution was cooled to room temperature and extracted with ether. The combined organic extracts were dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated in vacuo and the residue was purified by flash chromatography (10% ethyl acetate/hexane). The desired (1*S*)-methylenecyclopropanecarboxylic acid (**23**) was isolated in 95% yield. The NMR spectra of **23** are identical with those obtained for **14** and **17**.

(1*R*)-(Methylenecyclopropyl)acetyl-CoA (9). Following the same reaction sequence used for the synthesis of the 1*S* isomer (**10**) from acid **17**, the 1*R* isomer of MCPA-CoA (**9**) was prepared from acid **23** in 12% overall yield. The NMR spectra of the intermediates and the final product **9** generated in this synthesis are identical with those of their epimeric counterparts described above. The optical rotation of the chain elongation product, (1*R*)-methylenecyclopropaneacetic acid, was determined as $[\alpha]_D$ −8.16 ($CHCl_3$, c 1.5): high-resolution FAB-MS calcd for $C_{27}H_{43}N_7O_{17}P_3S$ $(M + 1)^+$ 862.1649, found 862.1706.

***trans*-2-Bromo-2-methylcyclopropanecarboxlic Acid (24) and *cis*-2-Bromo-2-methylcyclopropanecarboxylic Acid (25).** To a solution of **12** (16 g, 77.3 mmol) in a mixture of 600 mL of methanol and water (v/v 4:1) was added 60 g of anhydrous potassium carbonate. After being stirred at room temperature for 2 days, the solution was acidified with 2 N HCl. The mixture was then extracted with methylene chloride and the combined organic extracts were dried over anhydrous magnesium sulfate. Subsequent filtration and concentration gave a mixture of the trans and cis isomers. These two isomers were separated by flash chromatography (5% ethyl acetate/hexane) to give **24** and **25** in 2:1 ratio. The combined yield of **24** and **25** was 91%. **24**: ^1H NMR ($CDCl_3$) δ 9.68 (1 H, br s, OH), 2.32 (1 H, dd, J = 9.2, 6.6, 1-H), 1.92 (3 H, s, Me), 1.67 (1 H, dd, J = 9.2, 6.6, 3-H), 1.48 (1 H, t, J = 6.6, 3-H); ^{13}C NMR ($CDCl_3$) δ 176.3 (C=O), 33.6 (C-2), 29.3 (C-1), 25.3 (C-3), 23.9 (Me). **25**: ^1H NMR ($CDCl_3$) δ 9.68 (1 H, br s, OH), 1.84 (3 H, s, Me), 1.80 (1 H, dd, J = 9.2, 6.6, 1-H), 1.74 (1 H, dd, J = 9.2, 6.6, 3-H), 1.29 (1 H, t, J = 6.6, 3-H); ^{13}C NMR ($CDCl_3$) δ 175.5 (C=O), 33.2 (C-2), 30.9 (C-1), 28.4 (C-3), 23.8 (Me); ^1H NMR NOESY, irradiation at δ 1.74 caused nuclear Overhauser enhancement at δ 1.29 and irradiation at δ 1.29 led to the enhancement of δ 1.84, 1.80, and 1.74 signals; high-resolution FAB-MS calcd for $C_5H_8BrO_2$ $(M + 1)^+$ 178.9708, found 178.9711.

***N*-[(*R*)-1′-Phenyl-2′-hydroxyethyl]-(1*R*,2*S*)-2-bromo-2-methylcyclopropanecarboxamide (26) and *N*-[(*R*)-1′-Phenyl-2′-hydroxyethyl]-(1*S*,2*R*)-2-bromo-2-methylcyclopropanecarboxamide (27).** Compound **24** (8.3 g, 46.3 mmol) was converted to **26** and **27**, in 85% yield, by a procedure identical with that used for the synthesis of **15** and **16** from **14**. These two diastereomers were separated by flash chromatography eluted with 20% ethyl acetate in hexane. The diastereomeric purities of these compounds were determined by HPLC (Spheris ORB silica gel column, 10% 2-propanol in methylene chloride) to be greater than 99%. **26**: ^1H NMR ($CDCl_3$) δ 7.37–7.30 (5 H, m, Ar H's), 6.49 (1 H, br d, J = 4.7, NH), 5.07 (1 H, dd, J = 11.7, 4.7, 1′-H), 3.91 (2 H, m, 2′-H's), 2.34 (1 H, br s, OH), 2.17 (1 H, dd, J = 8.7, 8.4, 1-H), 1.78 (3 H, s, Me), 1.57–1.51 (2 H, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 168.6 (C=O), 138.8, 129.0, 128.0, 126.6 (Ar C's), 66.5 (C-2′), 56.3 (C-1′), 33.8 (C-2), 31.5 (C-1), 23.8 (Me)*, 22.9 (C-3)*. **27**: ^1H NMR ($CDCl_3$) δ 7.39–7.28 (5 H, m, Ar H's), 6.59 (1 H, br d, J = 11.9, NH), 5.04 (1 H, dt, J = 11.9, 5.0, 1′-H), 3.88 (2 H, br dd, J = 5.1, 5.0, 2′-H's), 2.59 (1 H, br t, J = 5.1, OH), 2.12 (1 H, dd, J = 8.3, 7.5, 1-H), 1.87 (3 H, s, Me), 1.51–1.48 (2 H, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 169.0 (C=O), 138.7, 129.0, 128.1, 126.7 (Ar C's), 66.7 (C-2′), 56.4 (C-1′), 33.7 (C-2), 31.4 (C-1), 23.7 (Me)*, 23.0 (C-3)*. Since the 2-Me signals of **26** and **27** are well resolved (appearing at δ 1.78 and 1.87, respectively), they can be used as internal references to estimate the enantiomeric purity of these samples. Careful analysis of the ^1H NMR spectra of **26** and **27** confirmed that the chiral purities of both compounds are greater than 99%: high-resolution FAB-MS calcd for $C_{13}H_{17}BrNO_2$ $(M + 1)^+$ 298.0443, found 298.0424.

(1*R*,2*S*)-2-Bromo-2-methylcyclopropanecarboxylic Acid (28). Conversion of compound **26** (8.0 g, 26.8 mmol) to **28** was accomplished by

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

(36) It should be noted that the optical rotation of MCPA-CoA is dominated by the contribution from coenzyme A, which gives a rotation of −7.4 (c 1.4) under identical conditions.

a procedure identical with that used for the synthesis of **17** from **15**. The crude product was purified by flash chromatography (10% ethyl acetate/hexane) to give **28** in 88% yield: ^{1}H NMR ($CDCl_3$) δ 9.68 (1 H, br s, OH), 2.32 (1 H, dd, J = 9.2, 6.6, 1-H), 1.92 (3 H, s, Me), 1.67 (1 H, dd, J = 9.2, 6.6, 3-H), 1.48 (1 H, t, J = 6.6, 3-H); ^{13}C NMR ($CDCl_3$) δ 176.3 (C=O), 33.6 (C-2), 29.3 (C-1), 25.3 (C-3), 23.9 (Me); high-resolution FAB-MS calcd for $C_5H_8BrO_2$ $(M + 1)^+$ 178.9708, found 178.9711.

(1*R*,2*S*)-2-Bromo-2-methylcyclopropanemethanol (29). **28** (4.2 g, 23.5 mmol) was converted to **29** by a procedure similar to that described for the synthesis of **18** from **17**. A single product was obtained in nearly quantitative yield: ^{1}H NMR ($CDCl_3$) δ 3.70 (1 H, dd, J = 11.7, 6.4, CH_2O), 3.53 (1 H, dd, J = 11.7, 8.4, CH_2O), 1.80 (3 H, s, Me), 1.79 (1 H, m, 1-H, partially buried under the Me peak, 1.31 (1 H, dd, J = 10.0, 6.4, 3-H), 0.63 (1 H, t, J = 6.4, 3-*H*); ^{13}C NMR ($CDCl_3$) δ 62.4 (CH_2O), 31.8 (C-2), 29.6 (C-1), 24.5 (C-3), 21.2 (Me); high-resolution FAB-MS calcd for $C_5H_{10}BrO$ $(M + 1)^+$ 164.9916, found 164.9911.

(1*R*,2*S*)-2-Bromo-2-methylcyclopropanecarboxaldehyde (30). To a solution of pyridinium chlorochromate (5 g, 23.2 mmol), sodium acetate (4.4 g 53.6 mmol), and powdered 3-Å molecular sieve (3 g) in dry methylene chloride (200 mL) at 0 °C under argon was added **29** (3.5 g, 21.2 mmol), which was dissolved in 10 mL of methylene chloride. After stirring for 1 h at 0 °C and 1 h at room temperature, an equal volume of anhydrous ether was added into the reaction and the resulting mixture was stirred for an additional 30 min. The dark-brown solution was then filtered through silica gel and washed extensively with ether. The combined filtrates were evaporated to give the desired aldehyde in 93% yield: ^{1}H NMR ($CDCl_3$) δ 9.62 (1 H, d, J = 3.5, CHO), 2.59 (1 H, ddd, J = 9.0, 6.5, 3.5, 1-H), 1.86 (3 H, s, Me), 1.71 (1 H, dd, J = 9.0, 6.5, 3-H), 1.65 (1 H, t, J = 6.5, 3-H); ^{13}C NMR ($CDCl_3$) δ 198.0 (C=O), 37.4 (C-2), 35.0 (C-1), 25.4 (C-3), 24.3 (Me); high-resolution FAB-MS calcd for C_5H_6BrO $(M - 1)^+$ 160.9603, found 160.9608.

(1*R*,2*S*)-[α-^{3}H]-2-Bromo-2-methylcyclopropanemethanol (31). An ampule containing sodium [^{3}H]borohydride (100 mCi, SA >9.5 Ci/mmol) was opened in a glovebag under an argon atmosphere. The radioactive reducing agent was dissolved in THF (5 mL) and transferred to a 100-mL round-bottom flask. The vial was rinsed with more THF and this wash was added to a chilled reaction flask at 0 °C. To this solution was added a THF solution (100 mL) of compound **30** (1.6 g, 9.8 mmol) that had been treated with trace amount of sodium borohydride. The resulting mixture was allowed to react at that temperature for 2 h followed by the addition of nonradioactive $NaBH_4$ (370 mg, 9.8 mmol), which was suspended in 5 mL of THF. After being stirred for an additional 2 h at room temperature, the reaction was quenched with acetone, diluted with water, and extracted thoroughly with methylene chloride. The combined organic extracts were dried over anhydrous magnesium sulfate and concentrated. **31** was obtained in 93% yield with a specific activity of 7.5 mCi/mmol.

(1*R*,2*S*)-[α,α-$^2H_2$]-2-Bromo-2-methylcyclopropanemethanol (31a). Following the same procedure used in the synthesis of **29**, **28** (2.1 g, 11.7 mmol) was reduced by lithium aluminum deuteride (936 mg, 23.4 mmol) to give **31a**. The yield was 86%: ^{1}H NMR ($CDCl_3$) δ 3.70 (0.08 H, dd, J = 11.7, 6.4, CH_2O), 3.49 (0.08 H, dd, J = 11.7, 8.4, CH_2O), 1.80 (3 H, s, Me), 1.79 (1 H, m, 1-H, partially buried under the Me peak), 1.31 (1 H, dd, J = 10.0, 6.4, 3-H), 0.63 (1 H, t, J = 6.4, 3-H); ^{13}C NMR ($CDCl_3$) δ 61.6 (residual CH_2O), 31.9 (C-2), 29.3 (C-1), 24.5 (C-3), 21.2 (Me); high-resolution CI-MS (CH_4) calcd for $C_5H_8{}^2H_2BrO$ $(M + 1)^+$ 167.0072, found 167.0085.

(1*R*,2*S*)-[α-^{3}H]-2-Bromo-2-methylcyclopropanecarboxaldehyde (32) and (1*R*,2*S*)-[α-$^2H_1$]-2-Bromo-2-methylcyclopropanecarboxaldehyde (32a). According to the procedure identical with that used for the synthesis of **30** from **29**, **31** (1.5 g, 9.1 mmol) was converted to **32** in 95% yield. The same procedure was also applied to the preparation of **32a**. **32a**: ^{1}H NMR ($CDCl_3$) δ 2.55 (1 H, dd, J = 9.0, 6.5, 1-H), 1.84 (3 H, s, Me), 1.70 (1 H, dd, J = 9.0, 6.5, 3-H), 1.63 (1 H, t, J = 6.5, 3-H); ^{13}C NMR ($CDCl_3$) δ 197.6 (t, residual C=O), 37.2 (C-1), 35.0 (C-2), 25.4 (C-3), 24.3 (Me); high-resolution CI-MS (CH_4) calcd for $C_5H_7{}^2H_1BrO$ $(M + 1)^+$ 163.9837, found 163.9812.

(1*R*,2*S*,α*S*)-[α-^{3}H]-2-Bromo-2-methylcyclopropanemethanol (33), (1*R*,2*S*,α*S*)-[α-$^2H_1$]-2-Bromo-2-methylcyclopropanemethanol (33a), (1*R*,2*S*,α*R*)-[α-^{3}H]-2-Bromo-2-methylcyclopropanemethanol (34), and (1*R*,2*S*,α*R*)-[α-$^2H_1$]-2-Bromo-2-methylcyclopropanemethanol (34a). A solution of compound **32** (700 mg, 4.3 mmol) in THF (5 mL) was added via cannula to a THF solution of (*R*)-Alpine-Borane (0.5 M in THF, 9 mL) at 0 °C under argon. The reaction was kept stirring at the same temperature for 30 min. Enough acetaldehyde to quench the excess reducing reagent was added until the release of hydrogen gas subsided. After stirring for an additional 10 min, the solvent was evaporated via water aspirator. The residual oil was dissolved in anhydrous ether (10 mL), cooled to 0 °C, mixed with ethanolamine, and stirred for 15 min at 0 °C. The resulting white precipitate was removed by filtration and washed with ether. The combined organic filtrates were extracted with brine, dried over anhydrous magnesium sulfate, and concentrated. The crude product was purified by flash chromatography (10% ethyl acetate/hexane followed by 30% ethyl acetate/hexane) to give pure **33** in 76% yield. The same procedure was also used to prepared **33a** from **32a**. **33a**: ^{1}H NMR ($CDCl_3$) δ 3.70 (1 H, br d, J = 6.3, α-H_R), 3.53 (0.06 H, m, α-H_S), 1.81 (3 H, s, Me), 1.79 (1 H, m, 1-H, partially buried under the Me peak), 1.32 (1 H, dd, J = 10.0, 6.5, 3-H), 0.64 (1 H, t, J = 6.5, 3-H); ^{13}C NMR ($CDCl_3$) δ 62.0 (t, CH^2HO), 31.9 (C-2), 29.5 (C-1), 24.5 (C-3), 21.2 (Me). The enantiomeric purity of this sample was determined to be 88% (ee) based on the integrations of the α-H_R and α-H_S signals. When (*S*)-Alpine-Borane was used as the reducing reagent, the same reaction procedure led to the formation of **34** (72% yield) and **34a** (75% yield) from **32** and **32a**, respectively. **34a**: ^{1}H NMR ($CDCl_3$) δ 3.70 (0.05 H; m, α-H_R), 3.52 (1 H, br d, J = 8.4, α-H_S), 1.80 (3 H, s, Me), 1.79 (1 H, m, 1-H, partially buried under the Me peak), 1.32 (1 H, dd, J = 10.0, 6.5, 3-H), 0.64 (1 H, t, J = 6.5, 3-H); ^{13}C NMR ($CDCl_3$) δ 62.1 (t, CH^2HO), 31.8 (C-2), 29.5 (C-1), 24.5 (C-3), 21.2 (Me); the enantiomeric purity of this sample was determined to be 90% (ee) based on the integrations of the α-H_R and α-H_S signals; high-resolution CI-MS CH_4) calcd for $C_5H_9{}^2H_1BrO$ $(M + 1)^+$ 165.9994, found 165.9983.

(1*S*,α*S*)-[α-^{3}H]Methylenecyclopropanemethanol (35), (1*S*,α*S*)-[α-$^2H_1$]Methylenecyclopropanemethanol (35a), and (1*S*,α*R*)-[α-^{3}H]-Methylenecyclopropanemethanol (36). **33** (700 mg, 6.1 mmol) was added to a solution of sodium hydride (200 mg, 8.3 mmol) in dry dimethyl sulfoxide (10 mL) under an argon atmosphere. The solution was heated to 90 °C and a few drops of ethanol were added to initiate the reaction. After being stirred at 90 °C for 1 h, the solution was cooled to room temperature and then diluted with water (10 mL). The resulting mixture was acidified with 1 N HCl and then extracted with methylene chloride. The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure. The desired product **35** was isolated in 87% yield. **35a** was obtained analogously from **33a** in 85% yield: ^{13}C NMR ($CDCl_3$) δ 132.2 (C-2), 103.0 (=CH_2), 64.2 (t, CH^2HO), 16.9 (C-1), 7.2 (C-3).

(1*S*,α*S*)-[α-^{3}H](Methylenecyclopropyl)methyl Methanesulfonate (37), (1*S*,α*S*)-[α-$^2H_1$](Methylenecyclopropyl)methyl Methanesulfonate (37a), and (1*S*,α*R*)-[α-^{3}H](Methylenecyclopropyl)methyl Methanesulfonate (38). Conversion of **35** and **35a** to the corresponding **37** and **37a** was effected by a procedure similar to that used for the synthesis of **19**. The same procedure was also employed to prepare compound **38** from **36**. **37a**: ^{1}H NMR ($CDCl_3$) δ 5.52, 5.49 (1 H each, d, J = 1.1, =CH_2), 4.19 (0.03 H, m, α-H_S), 4.00 (1 H, br d, J = 8.4, α-H_R), 3.02 (3 H, s, Me), 1.89 (1 H, m, 1-H), 1.47 (1 H, br t, J = 9.0, 3-H), 1.07 (1 H, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 130.9 (C-2), 105.9 (=CH_2), 73.0 (t, CH^2HO), 37.9 (OMs), 14.3 (C-1), 9.2 (C-3); high-resolution CI-MS (CH_4) calcd for $C_6H_{10}{}^2H_1SO_3$ $(M + 1)^+$ 164.0507, found 164.0481.

(1*R*,α*R*)-[α-^{3}H]Methylenecyclopropaneacetonitrile (39), (1*R*,α*R*)-[α-$^2H_1$]Methylenecyclopropaneacetonitrile (39a), and (1*R*,α*S*)-[α-^{3}H]-Methylenecyclopropaneacetonitrile (40). **37** and **37a** were converted to **39** and **39a**, respectively, by a procedure that had been used for the synthesis of **20**. **40** was also prepared by the same procedure. **39a**: ^{1}H NMR ($CDCl_3$) δ 5.52, 5.42 (1 H each, d, J = 1.2, =CH_2), 2.39 (1 H, d, J = 6.5, CH^2HCN), 1.67 (1 H, m, 1-H), 1.41 (1 H, m, 3-H), 0.94 (1 H, m, 3-H); ^{13}C NMR ($CDCl_3$) δ 131.8 (C-2), 118.0 (CN), 105.3 (=CH_2), 20.1 (CH^2HCN), 10.3 (C-1), 9.4 (C-3); high-resolution CI-MS (CH_4) calcd for $C_6H_7{}^2H_1N$ $(M + 1)^+$ 95.0735, found 95.0742.

(1*R*,α*R*)-[α-^{3}H]Methylenecyclopropaneacetaldehyde (41), (1*R*,-α*R*)-[α-$^2H_1$]Methylenecyclopropaneacetaldehyde (41a), and (1*R*,α*S*)-[α-^{3}H]Methylenecyclopropaneacetaldehyde (42). According to the procedure used for the synthesis of **21**, compounds **41**, **41a**, and **42** were obtained from the corresponding **39**, **39a**, and **40**. **41a**: ^{1}H NMR ($CDCl_3$) δ 9.81 (1 H, t, J = 2.0, CHO), 5.50, 5.44 (1 H each, d, J = 1.6, =CH_2), 2.42 (1 H, m, CH^2HCHO), 1.68 (1 H, m, 1-H), 1.20, 0.89 (1 H each, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 201.3 (C=O), 128.1 (C-2), 104.6 (=CH_2), 47.4 (CH^2HCHO), 9.5 (C-1)*, 9.1 (C-3)*; high-resolution CI-MS (CH_4) calcd for $C_6H_8{}^2H_1O$ $(M + 1)^+$ 98.0732, found 98.0724.

(1*R*,α*R*)-[α-^{3}H]Methylenecyclopropaneacetic Acid (43), (1*R*,α*R*)-[α-$^2H_1$]Methylenecyclopropaneacetic Acid (43a), and (1*R*,α*S*)-[α-^{3}H]-Methylenecyclopropaneacetic Acid (44). **43**, **43a**, and **44** were obtained from the corresponding aldehydes **41**, **41a**, and **42** by Jones oxidation previously used for the preparation of **22**. **43a**: ^{1}H NMR ($CDCl_3$) δ 9.74 (1 H, br s, OH), 5.51, 5.42 (1 H each, d, J = 1.6, =CH_2), 2.37 (1 H, d, J = 7.2, CH^2HCOOH), 1.72 (1 H, m, 1-H), 1.39, 0.90 (1 H each, m, 3-H's); ^{13}C NMR ($CDCl_3$) δ 178.2 (C=O), 134.0 (C-2), 104.3 (=CH_2), 37.5 (CH^2HCO_2H), 10.9 (C-1), 9.4 (C-3).

N-[(R)-1'-Phenyl-2'-hydroxyethyl]-(1R,αR)-[α-$^2H_1$]methylcyclopropaneacetamide (45). This compound was prepared from **43a** by a procedure identical with that used for the synthesis of **26** and **27** from **24**. The unlabeled species was also prepared to serve as a standard. Unlabeled species: ^1H NMR ($CDCl_3$) δ 7.40–7.29 (5 H, m, Ar H's), 6.55 (1 H, br d, J = 6.7, NH), 5.47, 5.42 (1 H each, d, J = 2.2, $=CH_2$), 5.07 (1 H, dd, J = 11.8, 4.9, 1'-H), 3.88 (2 H, d, J = 4.9, 2'-H), 2.69 (1 H, br s, OH), 2.40 (1 H, dd, J = 15.6, 7.0, α-H_R), 2.21 (1 H, dd, J = 15.6, 7.0, α-H_S), 1.71 (1 H, br t, J = 7.0, 1-H), 1.40 (1 H, br t, J = 7.0, 3-H), 0.90 (1 H, m, 3-H); ^{13}C NMR ($CDCl_3$) δ 172.5 (C=O), 139.2 (Ar C), 134.3 (C-2), 128.8, 127.8, 126.7 (Ar C's), 104.4 ($=CH_2$), 66.2 (C-2'), 55.7 (C-1'), 40.0 (C-α), 11.7 (C-1), 9.6 (C-3). **45**: ^1H NMR ($CDCl_3$) δ 7.36–7.26 (5 H, m, Ar H's), 6.67 (1 H, br d, J = 6.7, NH), 5.47, 5.42 (1 H each, d, J = 2.2, $=CH_2$), 5.04 (1 H, dd, J = 11.8, 4.9, 1'-H), 3.84 (2 H, d, J = 4.9, 2'-H), 2.37 (0.08 H, br d, J = 6.9, α-H_R), 2.20 (1 H, br d, J = 6.9, α-H_S), 1.69 (1 H, m, 1-H), 1.39 (1 H, m, 3-H), 0.89 (1 H, m, 3-H); based on the integration of the α-H_R signal, the enantiomeric purity of this sample is greater than 84% (ee); ^{13}C NMR ($CDCl_3$) δ 172.0 (C=O), 139.2 (Ar C), 134.0 (C-2), 128.8, 127.8, 126.6 (Ar C's), 104.0 ($=CH_2$), 66.5 (C-2'), 55.5 (C-1'), 40.1 (C-α), 11.7 (C-1), 9.6 (C-3); high-resolution FAB-MS calcd for $C_{14}H_{17}{}^2H_1NO_2$ $(M + 1)^+$ 233.1416, found 233.1413.

(1R,αR)-[α-^{3}H](Methylenecyclopropyl)acetyl-CoA (46) and (1R,αS)-[α-^{3}H](Methylenecyclopropyl)acetyl-CoA (47). With the same procedure given for the conversion of **22** to **10**, compounds **46** and **47** were prepared from **43** and **44**, respectively. The specific activity of **46** was 7.1 mCi/mmol and the specific activity of **47** was 7.3 mCi/mmol.

(1S,αR)-[α-^{3}H](Methylenecyclopropyl)acetyl-CoA (49) and (1S,αS)-[α-^{3}H](Methylenecyclopropyl)acetyl-CoA (50). **49** and **50** were prepared from **27** via **48** based on a sequence identical with that used for the syntheses of **46** and **47** from **26**. The NMR spectra of the unlabeled intermediates such as (1S,2R)-2-bromo-2-methylcyclopropanecarboxylic acid, (1S,2R)-2-bromo-2-methylcyclopropanemethanol, and (1S,2R)-2-bromo-2-methylcyclopropanecarboxaldehyde are superimposable with those of **28**, **29**, and **30**, respectively. The specific activity of **49** was 1.3 mCi/mmol and the specific activity of **50** was 2.8 mCi/mmol.

(1R)-[α,α-$^2H_2$]Methylenecyclopropanemethanol (51) and (1S)-[α,α-$^2H_2$]Methylenecyclopropanemethanol (52). To a suspension of lithium aluminum deuteride (760 mg, 20 mmol) in anhydrous ether (150 mL) was added dropwise an ethereal solution (10 mL) of compound **17** (980 mg, 10 mmol) at 0 °C. The resulting mixture was stirred under argon overnight. The excess reducing reagent was carefully quenched by water and the resulting white precipitates were dissolved in 1 N NaOH. The milky-white aqueous phase was extracted with ether. The combined organic extracts were dried over anhydrous magnesium sulfate and filtered. The solvent was removed under reduced pressure to give the desired alcohol (**51**) in 78% yield: ^1H NMR ($CDCl_3$) δ 5.40, 5.35 (1 H each, d, J = 1.2, $=CH_2$), 3.03 (1 H, br s, OH), 1.70 (1 H, br s, 1-H), 1.24, 0.89 (1 H each, m, 3-H's); judging from the integration of the residual α-methylene signals at δ 3.55 and 3.38, the deuterium incorporation was estimated to be greater than 96%; ^{13}C NMR ($CDCl_3$) δ 132.0 (C-2), 103.4 ($=CH_2$), 64.2 (very weak, C^2H_2O), 16.9 (C-1), 7.2 (C-3); high-resolution FAB-MS calcd for $C_5H_7{}^2H_2O$ $(M + 1)^+$ 87.0810, found 87.0821. Compound **52** was similarly prepared from **23** (880 mg, 9 mmol) in 80% yield. The spectra of **52** are identical with those of **51**.

(1S)-[α,α-$^2H_2$](Methylenecyclopropyl)acetyl-CoA (53) and (1R)-[α,α-$^2H_2$](Methylenecyclopropyl)acetyl-CoA (54). The α,α-dideuterated (1S)- and (1R)-MCPA-CoAs were prepared from **51** and **52**, respectively. The same strategy developed for the synthesis of **9** and **10** was followed to make these labeled molecules. The NMR spectra of the deuterated intermediates generated in this series are similar to the spectra obtained for the corresponding unlabeled species, except for the diminishing of the signals of α-methylene hydrogens and the resonance of the α-carbon in NMR.

Inhibition of GAD with MCPA-CoA. A series of identical samples containing GAD (5 nmol) and chemically synthesized MCPA-CoA (35 nmol) in 40 μL of 50 mM potassium phosphate buffer (pH 7.6) were prepared. The incubation of each sample was stopped at different time intervals (10, 20, 30, 40, 60, and 90 s) by dilution of the incubation mixture 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), allowing the determination of the residual enzyme activity.

Incubation of GAD with [α-^{3}H](Methylenecyclopropyl)acetyl-CoA. The tritium-labeled MCPA-CoA (35 nmol) was incubated with GAD (5 nmol) in 100 μL of 50 mM potassium phosphate buffer (pH 7.6) containing 0.3 mM EDTA at 25 °C for 15 min. Activated charcoal (10% solution, 200 μL) was added to the reaction at the end of the incubation. The resulting solution was mixed vigorously on a vortex mixer for 1 min followed by centrifugation to precipitate the charcoal. The supernatant (100 μL) was then removed and analyzed by scintillation counting. These readings were calibrated against controls prepared in parallel with boiled enzyme.

Acknowledgment. This work was supported by National Institutes of Health Grant (GM 35906). H.-w.L. also thanks the National Institutes of Health for a Research Career Development Award (GM 00559).