Stereospecific (Methylenecyclopropyl)acetyl-CoA Inactivation of General Acyl-CoA Dehydrogenase from Pig Kidney

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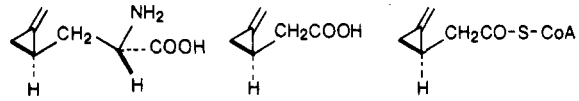
An efficient synthesis of racemic methylenecyclopropaneacetic acid has been developed. A mixture of the corresponding diastereomeric coenzyme A esters, one of which is the metabolite of the nonprotein amino acid hypoglycin from *Blighia sapida* directly responsible for its hypoglycemic potency, reacts stereospecifically with general acyl-CoA dehydrogenase from pig kidney to irreversibly inactivate the enzyme.

The amino acid hypoglycin A found in unripe ackee fruit (*Blighia sapida*) which causes Jamaican Vomiting Sickness[2-4] is (+)-α-amino-2-methylenecyclopropanepropionic acid. It is metabolized by rats through transamination to methylenecyclopropanepyruvic acid followed by oxidative decarboxylation to (*R*)-2-methylenecyclopropaneacetic acid (MCPA).[5-7] The coenzyme A thioester of MCPA irreversibly inactivates pig kidney general acyl-CoA dehydrogenase;[8] MCPA-CoA may thus be a "suicide" substrate directly responsible for hypoglycin toxicity.



hypoglycin A MCPA MCPA-CoA

Extensive experimental work over the past 35 years directed toward elucidating hypoglycin's activity and molecular mechanism of action has invariably depended upon limited supplies of naturally derived material. An interest in methylenecyclopropane chemistry[9-11] has led us to begin work on the molecular mechanism of this inactivation process, addressing initially this severe and long-standing impediment to detailed mechanistic investigations. The obstruction posed by a limited availability of MCPA from natural hypoglycin has now been circumvented through development of a convenient synthesis of racemic methylenecyclopropaneacetic acid and the corresponding diastereomeric coenzyme A thioesters.

Results and Discussion

Racemic methylenecyclopropaneacetic acid was prepared through an efficient four-step sequence of reactions (Scheme I). Addition of the carbene derived from 1,1-dichloroethane and *n*-butyllithium in hexanes[12] to 1, the tetrahydropyranyl (THP) ether of 3-buten-1-ol,[13] gave 93% of distilled cyclopropane **2**, based on a 56% recovery of starting material **1** (44% conversion). Small-scale runs gave similar yields and higher conversions, but less product. Dehydrochlorination with potassium *tert*-butoxide in dimethyl sulfoxide afforded 87% of distilled methylenecyclopropane **3**, which was hydrolyzed to 2-(methylenecycloprop-2-yl)ethanol (**4**). The oxidation of this primary alcohol with Jones reagent in acetone gave carboxylic acid **5** in 81% yield.

Scheme I

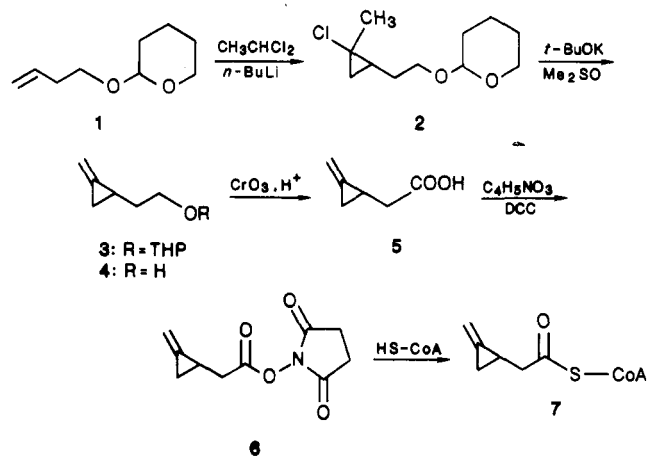


Table I. Absorbance at 446 nm of General Acyl-CoA Dehydrogenase with Progressive Inactivation by MCPA-CoA

[MCPA-CoA]/[GAD][a]	A_{446}	[MCPA-CoA]/[GAD][a]	A_{446}
0	0.358	3.2	0.221
1.2	0.310	4.1	0.189
2.2	0.262	5.1	0.162

[a] Initial concentration of enzyme: 13.3 μM.

The mixture of diastereomeric coenzyme A thiol esters was made by condensing **5** with *N*-hydroxysuccinimide[14] followed by reaction of the active ester **6** with coenzyme A in aqueous tetrahydrofuran (100%, based on coenzyme A as determined by the hydroxylamine procedure).[15]

Inactivation of the general acyl-CoA dehydrogenase from pig kidneys (E.C. 1.3.99.3) by naturally derived MCPA-CoA has been investigated.[8] Hypoglycin isolated from

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ackee fruit was converted to methylenecyclopropaneacetic acid with L-amino acid oxidase and hydrogen peroxide, the coenzyme A ester was prepared, and this ester was shown to irreversibly inactivate the flavin-dependent enzyme. Successive aliquots of MCPA-CoA proportionally diminished both the absorption intensity of the riboflavin chromophore at 446 nm and the dehydrogenase activity of the enzyme.[8]

The present work extends those findings. at 25 °C, the mixture of diastereomeric coenzyme A esters **7** inactivates the GAD enzyme (Table I).

Quantitative comparisons with the earlier work can be made by using the relation [MCPA-CoA] = 0.5 [7]. A plot of flavin absorbance at 446 nm observed[8] under aerobic conditions vs. equivalents of MCPA-CoA added had an initial slope of -4×10^{-2}; our data (Table I) give a slope of -4×10^{-2} for this same dependence. Thus the stereoisomer of MCPA-CoA, an epimer at cyclopropane-C1, shows no significant influence on the inactivation of enzyme by MCPA-CoA itself, and the inhibition is stereospecific.

From the data in Table I may be derived a plot of percent remaining enzymic activity, $100(A - A_\infty)/(A_0 - A_\infty)$, where $A_\infty = A_0(23.5 - 13.3)/23.5$, against the MCPA-CoA/GAD ratio: it is linear with a slope of −19%/equiv ($r = 0.996$). Thus the estimated partitioning ratio between turnover and inactivation for the MCPA-CoA inhibitor is 4.

The most immediately practical consequence of this new result, an experimentally based assurance that coenzyme A esters from racemic methylenecyclopropaneacetic acid are suitable for investigations of the MCPA-CoA inactivation of general acyl-CoA dehydrogenase from pig kidneys, is that large-scale studies based on synthetic **7** applied to structure determinations for covalently bound inhibitor–enzyme FAD reaction products, or mechanistic work with isotopically labeled versions of **7**, may be undertaken without need for an optical resolution. The synthetic route of Scheme I may be employed to provide methylenecyclopropaneacetic acid labeled specifically at any carbon with carbon-13 or carbon-14 or with deuterium in the exocyclic methylene unit or in place of any other hydrogen. While other syntheses of racemic methylenecyclopropaneacetic acid have been recorded,[16] they have not been particularly efficient and have not been utilized for enzyme inhibition studies.

The issue of stereospecificity in the inactivation of GAD by MCPA-CoA has not been raised previously; rather, the stereochemical characteristics of hypoglycin and of enzymatically derived methylenecyclopropaneacetic acid, as well as of the inactivation process, have been largely ignored. The demonstrated stereochemical correlation between hypoglycin and (+)-(*S*)-3-methylpentanoic acid,[7] which implies *R* stereochemistry at Cl of the 2-methylenecyclopropane moiety of hypoglycin, was inadvertently reported[6] as evidence for the *S* configuration—a mistake unamended since 1968. Nor has the natural form of MCPA-CoA been explicitly remarked as having the *R* stereochemistry in earlier work. Structural formulas as well as *R,S* designations for hypoglycin A (hypoglycin) and hypoglycin B in a 1985 volume of Beilstein[17] sustain this error.

There is scant precedent for stereospecific reactions of branched fatty acids with flavin-dependent dehydrogenases; other examples will surely follow and may play a useful role in ongoing efforts to elucidate the chemistry of the MCPA-CoA/GAD inactivation process[18] and of uninhibited α,β-dehydrogenations of fatty acid coenzyme A esters by flavin enzymes.[19,20]

Experimental Section

Unless otherwise noted, preparative reactions were conducted under a nitrogen atmosphere and in glassware that had been flame-dried and cooled under a nitrogen stream. ^{1}H NMR spectra were recorded on either a Varian XL-100, a Nicolet NT 360-MHz, or a Nicolet QE 300-MHz instrument and were referenced to tetramethylsilane (Me_4Si) at 0.0 ppm and/or chloroform at 7.26 ppm. ^{13}C NMR spectra were recorded on a QE 300-MHz instrument and referenced to the middle signal of the triplet due to $CDCl_3$ at 77 ppm. Infrared spectra were recorded on a Beckman IR 4242 spectrophotometer. Ultraviolet spectra were obtained with Beckman DU-7 or Hewlett-Packard 8450-A spectrophotometers. Reagents and solvents were of reagent grade and used as supplied unless otherwise indicated. E + R Microanalytical Laboratory, Corona, NY, provided the elemental analysis.

2-[2-(1-Chloro-1-methylcycloprop-2-yl)ethoxy]tetrahydro-2*H*-pyran (2). To a 500-mL three-necked round-bottom flask was added 20.4 g (0.13 mol) of 2-(3-butenyloxy)tetrahydro-2*H*-pyran (**1**), 15.8 g (0.16 mol) of 1,1-dichloroethane, and 20 mL of ether freshly distilled from sodium benzophenone ketyl. The solution was cooled to between −30 and −40 °C and rigorously maintained at that temperature throughout the 3.5-h addition of 90 mL of 1.6 M *n*-butyllithium in hexanes. After the addition was complete, the cooling bath was removed, and the solution was stirred at 25 °C for 12 h. Workup began with the addition of 20 mL of distilled water; after 15–20 min, all the salts present in the flask had dissolved and the water layer was separated. The organic solution was concentrated by rotary evaporation, and the remaining light yellow liquid was distilled carefully to contain foaming to give 11.49 g of olefin **1** (56% recovery) as forerun and 11.79 g (93% yield, based on 44% conversion) of cyclopropane **2**: bp 50–55 °C (0.05 Torr); ^{1}H NMR ($CDCl_3$) δ 4.65 (s, 1 H), 3.82 (m, 2 H), 1.5–2.0 (m, 11 H, singlet at 1.6 ppm), 1.90 (m, 2 H), 0.7 (m, 1 H); ^{13}C NMR ($CDCl_3$) signals at 91.9, 70.0, 66.8, 45.2, 44.5, 43.9, 41.6, 41.1, 39.7, 39.0, 37.5 ppm, with indications of diastereomers.

2-[2-(Methylenecycloprop-2-yl)ethoxy]tetrahydro-2*H*-pyran (3). Into a 100-mL three-necked round-bottom flask with a magnetic stir bar were placed 30 mL of dimethyl sulfoxide, freshly distilled from calcium hydride, and 14.4 g (0.13 mol) of potassium *tert*-butoxide. The flask was fitted with a thermometer and an addition funnel, and the solution was heated to 70 °C. To the base was added 26.5 g (0.12 mol) of 2-[2-(1-chloro-1-methylcycloprop-2-yl)ethoxy]tetrahydro-2*H*-pyran dropwise over 30 min. The reaction was left to stir at 70 °C for 6.5 h and then cooled and poured into ice water. The aqueous solution was extracted with ether, and the ether solution was washed with brine, dried over magnesium sulfate, decanted, and concentrated by rotary evaporation. The residue was distilled through a 10-cm Vigreux column to give 19.02 g (87% yield) of **3**: bp 61–64 °C (0.35 Torr); ^{1}H NMR ($CDCl_3$) δ 5.42 (d, 2 H), 4.6 (s, 1 H), 3.7–4.0 (m, 2 H) 3.4–3.6 (m, 2 H), 1.1–2.0 (m, 10 H), 0.7–0.9 (m, 1 H).

2-(Methylenecycloprop-2-yl)ethanol (4). To a 1-L round-bottom flask were added 19.02 g (0.11 mol) of protected alcohol **3**, 5.71 g of *p*-toluenesulfonic acid monohydrate (0.03 mol), and 500 mL of methanol. This mixture was stirred at room temperature for 24 h; 5.6 g of potassium carbonate was added, and stirring was continued for another hour while most of the base dissolved. Methanol was distilled at atmospheric pressure, and water was added to the residue. The aqueous phase was extracted with ether, the ether was removed by rotary evaporation, and the residue was distilled under water aspirator vacuum at 45–57 °C

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to yield 5.71 g of the alcohol (58% yield): ^{1}H NMR ($CDCl_3$) δ 5.41 (d, 2 H), 3.75 (t, 2 H), 1.3–1.8 (m, 4 H), 1.28 (t, 1 H), 0.91 (m, 1 H); ^{13}C NMR ($CDCl_3$) signals at 136, 103, 62, 36, 13, 9 ppm.

Methylenecyclopropaneacetic Acid (5). Into a 500-mL three-necked round-bottom flask equipped with a low-temperature thermometer, a mechanical stirrer, a nitrogen atmosphere, and an addition funnel were placed 2.21 g (22.5 mmol) of 2-(methylenecycloprop-2-yl)ethanol and 300 mL of acetone (reagent grade, from freshly opened bottle). This solution was cooled to −20 °C and 16.5 mL of Jones reagent was added over 30 min. During the addition the temperature was allowed to rise to −5 °C and was maintained at that temperature for 6 h. The product mixture was then decanted into a single-necked round-bottom flask holding a magnetic stir bar, and 1–2 mL of 2-propanol was added to cause the orange solution to turn blue-green. A solid also formed, from which the liquid was decanted into a separatory funnel containing water and ether. About 2 mL of concentrated HCl was added, and the aqueous layer was extracted several times with ether. The ether was then extracted with 10% aqueous sodium hydroxide, the basic aqueous solution was acidified with concentrated HCl, and the acidic aqueous solution was extracted several times with ether. The ethereal solution was dried over magnesium sulfate, decanted, and concentrated by rotary evaporation to yield 2.06 g (82% yield) of acid **5** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 10.52 (s, 1 H), 5.50 (d, 2 H) 2.40 (d, 2 H), 1.6–1.9 (m, 1 H), 1.2–1.5 (m, 1 H), 0.8–1.0 (m, 1 H). Anal. Calcd for $C_6H_8O_2$: C, 64.27; H, 7.19. Found: C, 64.44; H, 7.33.

***N*-[(2-Methylenecyclopropyl)acetoxy]succinimide (6).** To 65 mL of ethyl acetate was added 0.99 g (8.5 mmol) of *N*-hydroxysuccinimide (Aldrich), and the solution was clarified by filtration. To 30 mL of ethyl acetate was added 0.935 g (8.3 mmol) of methylenecyclopropaneacetic acid, and this solution was added to the *N*-hydroxysuccinimide solution. To 70 mL of ethyl acetate was added 1.73 g of dicyclohexylcarbodiimide (DCC); the resulting slightly cloudy solution was filtered and added to the acid/succinimide solution. The reaction mixture was swirled a little, left undisturbed for 16 h, and filtered to remove the dicyclohexylurea. Ethyl acetate was removed from the filtrate by rotary evaporation. Residual solvent was removed under high vacuum before the off-white residue was placed in the freezer. Within 24 h the residue had completely solidified. The yield of crude **6** was 1.47 g (84% yield).

Coenzyme A Esters of Racemic Methylenecyclopropaneacetic Acid (7). Dissolved oxygen was removed from 3 mL of doubly distilled water by three freeze/high-vacuum/freeze cycles followed by bubbling nitrogen through the liquid for 30 min. To the water was added 50 mg of coenzyme A (Sigma), which dissolved immediately and gave a positive indication for a free thiol group according to the nitroprusside test.[21] To about 6 mL of tetrahydrofuran (THF), freshly distilled from sodium and benzophenone ketyl, was added 0.69 g of the crude succinimide **6**. While a nitrogen atmosphere was maintained, 0.168 g of sodium bicarbonate was added to the coenzyme A solution. After the base had dissolved, about 0.7 mL of the succinimide/THF solution was added to the basic coenzyme A solution with the nitrogen gas providing agitation. In about 1 min, the nitroprusside test was negative until the spot was dipped into a methanol/potassium hydroxide solution, after which the spot became bright red. The coenzyme A ester solution was made slightly acidic with 10% perchloric acid. The solvents were removed at room temperature by rotary evaporation until a clear slightly viscous liquid remained. This solution still tested negative for free thiols by the nitroprusside test, but tested positive when the spot was hydrolyzed.

The concentration of the thioester was determined by following the hydroxylamine procedure;[20] 2.7 mL of a solution with a concentration of 24 mM (100% yield based on coenzyme A) was obtained.

Enzyme. General acyl-CoA dehydrogenase (GAD; E.C. 1.3.99.3) was isolated from kidneys obtained from freshly slaughtered pigs and was purified by following, and in some details adapting, protocols described by Thorpe and co-workers.[21-23] As forewarned by Thorpe, the effectiveness of hydroxyapetite columns proved erratic. In the present work the wash from the hydroxyapetite column was purified through the use of a Sephacryl-200 sizing column.

The concentration cm^{-1},[21] total flavin in the enzyme solution used for the inactivation experiments, determined by measuring absorbance at 446 nm and applying the extinction coefficient ϵ_{446} = 15.4 mM^{-1} cm^{-1},[21] was found to be 23.5 μM. The concentration of active enzyme in this solution was determined by an established activity assay[21] (0.5 mL buffer, 0.05 mL each of 2,6-dichlorophenolindophenol, phenazine methosulfate, and octanoyl-CoA solutions, and 0.005 mL of the enzyme solution) to be 13.3 μM.

Inactivation Followed by Ultraviolet Spectroscopy. To 500 μL of a solution of enzyme (23.5 μM, 13.3 μM active enzyme) in 0.1 M potassium phosphate buffer at pH 7.6 was added at 9 °C in succession five 10-μL aliquots of **7**, 0.325 mM; spectral changes were inconveniently slow, the solution was brought to 25 °C, and all further additions of inhibitor (40-μL aliquots, spectra recorded after 10 min) were done at this temperature. The data obtained are summarized in Table I and Figure 1 (in supplementary material): progressive additions of **7** led to progressive reductions in A_{446}.

Inactivation Followed by Enzyme Activity Assays. Dilution of a solution of enzyme with successive aliquots of **7** was followed by monitoring A_{446} and measuring enzymic activity 10 min or more after each addition of **7**. The results confirmed the proportionality between reduction in A_{446} and dehydrogenase activity noted previously.[21]

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Supplementary Material Available: Figure of absorbance vs. wavelength for inactivation of general acyl-CoA dehydrogenase by thiol ester **7** (1 page). Ordering information is given on any current masthead page.

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