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Synthesis of Labeled 1-Amino-2-methylenecyclopropane-1-carboxylic Acid, an Inactivator of 1-Aminocyclopropane-1-carboxylate Deaminase

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1-Amino-2-methylenecyclopropane-1-carboxylic acid (2-methylene-ACC) is an irreversible inhibitor for a bacterial enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which catalyzes the conversion of ACC to α-ketobutyrate and ammonia. The inactivation has been proposed to proceed with the ring scission induced by an addition of an enzyme nucleophile, resulting in the formation of a reactive turnover product that then traps an active-site residue. To gain further insight into this unique enzymatic reaction, the tritiated 2-methylene-ACC was prepared and incubated with ACC deaminase to locate and identify the entrapped amino acid residue. The synthesis of this radiolabeled compound and the results of its incubation with ACC deaminase are reported in this paper.

Introduction

1-Aminocyclopropanecarboxylic acid (ACC, **1**) is a naturally occurring amino acid that has been shown to be the precursor of ethylene, an essential phytohormone that regulates seed germination, senescence, fruit ripening, wound healing, and many additional plant growth processes.[1] The conversion of ACC to ethylene in plants is catalyzed by ACC oxidase,[2] which is a mononuclear non-heme iron enzyme characterized by a 2-His-1-carboxylate iron binding motif.[3] However, ACC can also be converted to α-ketobutyrate (**2**) and ammonia (Scheme 1) by a bacterial enzyme originally isolated from a soil bacterium, *Pseudomonas* sp. strain ACP, when ACC is the sole nitrogen source for the growth of this bacterium.[4] This enzyme, ACC deaminase, is a special pyridoxal 5′-phosphate (PLP)-dependent catalyst with a unique capacity to break the cyclopropane ring of ACC. Since ACC does not bear an abstractable α-H and the carboxyl group is retained in the product, the ring opening cannot involve the formation of an α-carbanionic intermediate, which is the common initiating event in the catalysis of PLP-dependent enzymes.[5] Thus, ACC deaminase must adapt an unusual mechanism to accomplish this transformation.

It has been shown that the α-anion equivalent of the vinylglycyl-PLP aldimine (**3**) is a key intermediate in ACC fragmentation[6] and that the regiochemistry of ring cleavage is between the α-C and the pro-*S* β-CH_2 group.[7] More recent experiments using 1-amino-2-methylenecyclopropane-1-carboxylic acid (2-methylene-ACC, **4**) as a mechanistic probe showed that the ring opening of ACC catalyzed by ACC deaminase likely proceeds with the addition of an active-site nucleophile to the pro-*S* β-CH_2 group followed by β-proton abstraction, resulting in ring scission, as shown in Scheme 1.[8] This conclusion was drawn on the basis of the fact that compound **4** is an irreversible inhibitor for ACC deaminase and 2-oxo-3-methyl-3-butenoic acid (**5**) is the sole turnover product of this incubation. The inactivation exhibited a k_{inact} of 1.25 h^{-1} and a K_I of 3.3 mM, with a partition ratio of 8250. Analysis of the turnover product isolated from the incubation of ACC deaminase with **4** having a dideuterated exocyclic methylene moiety further revealed that addition to C-3 instead of C-2′ is the preferred mode of nucleophilic attack in the active site of ACC deaminase. These observations are most consistent with an inactivation mechanism illustrated in Scheme 2 and thus provide compelling evidence for ring cleavage of ACC as a nucleophilic addition initiated event.

The proposed covalent catalysis for ACC deaminase has no precedence in coenzyme B_6-dependent enzymes.[9] To gain further insight into this unique enzymatic

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(1) (a) Yang, S. F.; Hoffman, N. E. *Annu. Rev. Plant Physiol.* **1984**, *35*, 155−189. (b) Bleecker, A. B. *Trends Plant Sci.* **1999**, *4*, 269−274. (c) Pirrung, M. C. *Acc. Chem. Res.* **1999**, *32*, 711−718.

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(3) Hegg, E. L.; Que, L., Jr. *Eur. J. Biochem.* **1997**, *250*, 625−629.

(4) (a) Honma, M.; Shimomura, T. *Agric. Biol. Chem.* **1978**, *42*, 1825−1831. (b) Sheehy, R. E.; Honma, M.; Yamadam, M.; Sasaki, T.; Martineau, B.; Hiat, W. R. *J. Bacteriol.* **1991**, *173*, 5260−5265.

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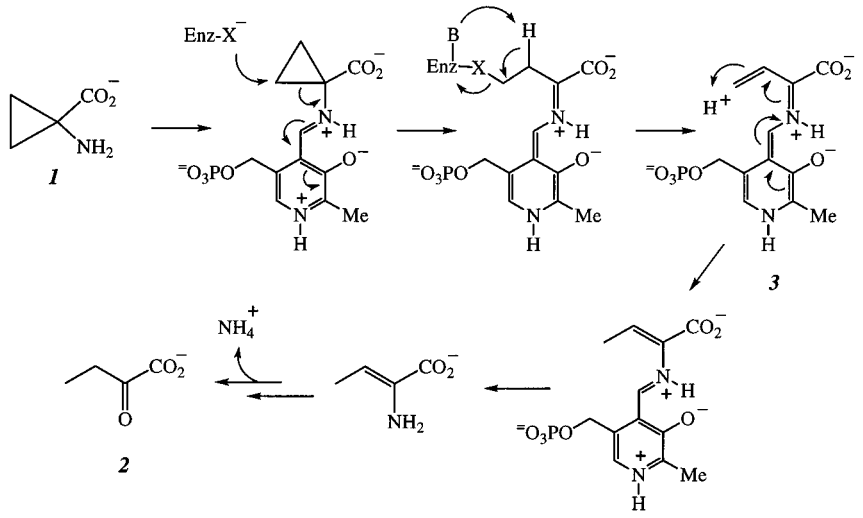
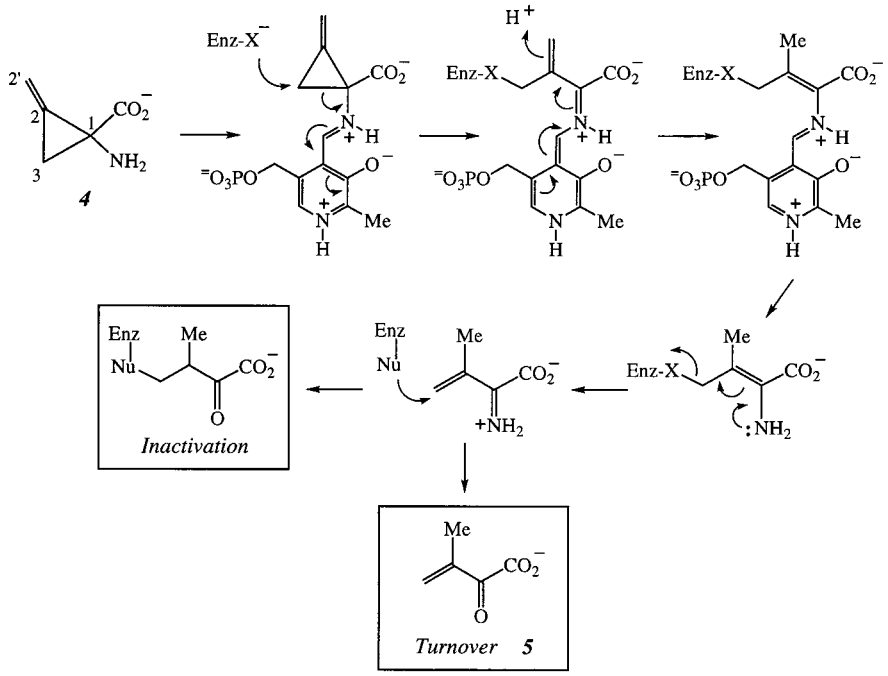
Scheme 1



Scheme 2



reaction, we decided to locate and identify the entrapped amino acid residue using tritiated 2-methylene-ACC (**6**) as the inhibitor. Whether the labeled active-site residue is responsible for the initial nucleophilic attack will then be examined by site-directed mutagenesis. Here, we report our synthesis of the radiolabled 2-methylene-ACC (**6**) along with the outcome of its incubation with ACC deaminase. The mechanistic implications of these results will also be discussed.

Results and Discussion

An efficient synthesis of **4**, in which the exocyclic methylene group was introduced by a zinc-mediated elimination of a 1,2-dibromide, has been developed in our early study of ACC deaminase.[8] However, this scheme is not suitable for making radioisotopically labeled 2-methylene ACC, since the basic carbon skeleton of **4** and all the necessary functional groups were assembled in the first step of the synthesis, leaving little room of manipulation for introducing the tritium labeling at a later stage. For obvious reasons, dealing with radioactive material from the very beginning of a multistep synthetic route is not a method of choice. However, because the α-methylene protons of a methenecyclopropane are known to be base labile,[10] a possible solution is to subject a methylenecyclopropane intermediate such as **13**, the proximal precursor of **4**, to undergo a solvent hydrogen exchange under basic conditions allowing tritium be incorporated at the C-3 position. Unfortunately, the cyclopropane ring of **13** was decomposed upon treatment with either LDA or *t*-BuOK, rendering the deprotonation−reprotonation strategy impractical. Therefore, we decided to develop a new synthesis of 2-methylene-ACC that allows for introduction of the radioisotope in a later step.

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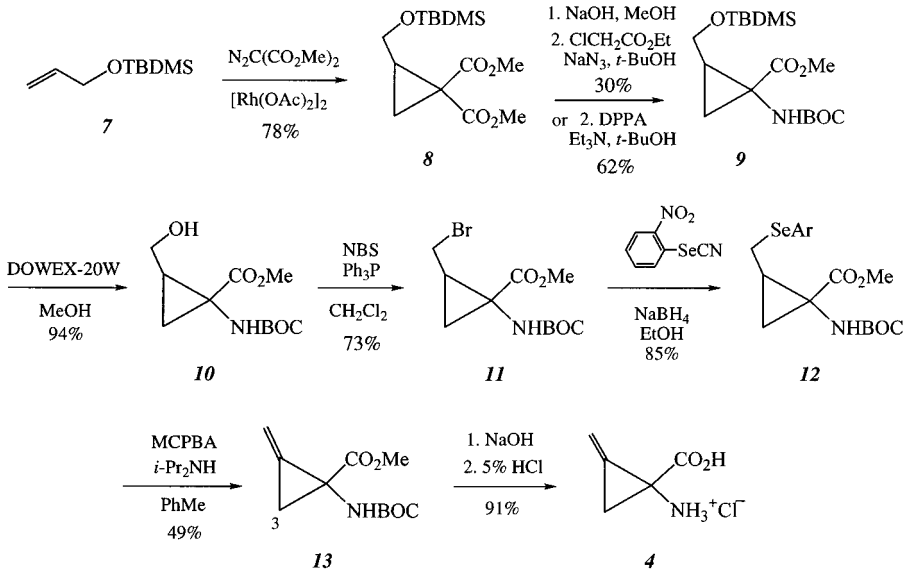
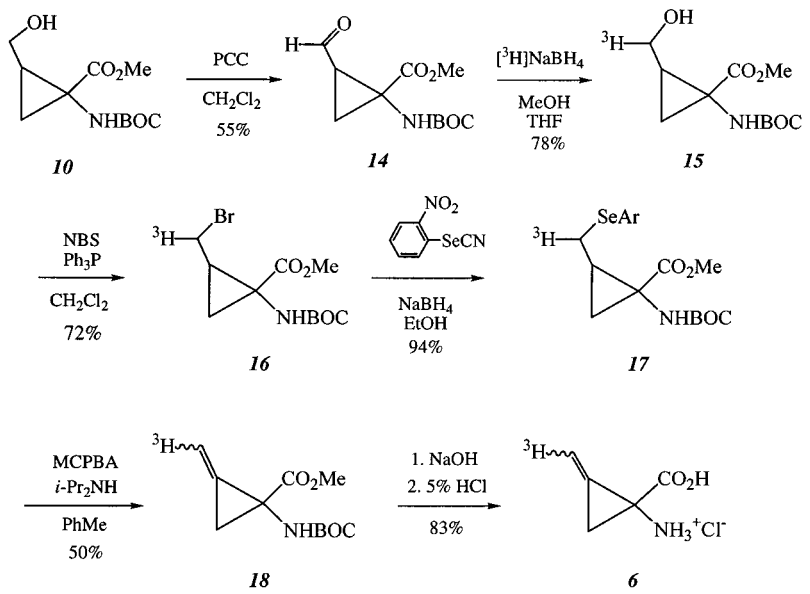
Scheme 3



Scheme 4



Chemical Synthesis. In this new synthesis, the *exo*-methylene-substituted cyclopropane ring was to be constructed using the selenoxide elimination chemistry (see Scheme 3).[11] Once the 2-hydroxymethylcyclopropane derivative (**10**), the precursor for the selenoxide, is made, the tritium may be incorporated at the hydroxymethylene carbon (C-2′) of this precursor through an oxidation−reduction sequence (see Scheme 4). It is worth mentioning that the selenoxide elimination chemistry has been demonstrated to be useful in preparing a variety of methylenecyclopropane derivatives.[12] However, examples in which this method failed to produce the desired products are also known. A recent example was reported by Taguchi et al., who had successfully synthesized 2-(2,2-difluoro-3-methylenecyclopropyl)glycine derivatives using the selenoxide elimination strategy, but were unable to make the corresponding nonfluorinated compounds based on the same approach due to the facile formation of the cyclopropylmethyl cation prior to methylenation.[12c] Thus, the first task of this synthesis was to test whether the exomethylene moiety could be generated by this method to give 2-methylene-ACC (**4**).

As depicted in Scheme 3, the new synthesis was initiated by a ruthenium acetate catalyzed [2 + 1] addition of diazomalonate to **7** to construct the cyclopropane skeleton. The resulting compound **8** was converted via two different methods to an acyl azide intermediate that, upon refluxing with toluene and *t*-BuOH for 7 h, led to the formation of the *tert*-butoxycarbonyl (BOC)-protected cyclopropylamine **9** via a Curtius rearrangement. The *tert*-butyldimethylsilyl (TBDMS) protecting group was removed by using DOWEX-20W acidic resin, and the exposed primary hydroxyl group was converted to the corresponding bromide **11** upon treatment with *N*-bromosuccinimide (NBS) and triphenylphosphine. While

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(12) (a) Natsuko, K.; Yasufumi, O. *Tetrahedron Lett.* **1985**, *26*, 83−84. (b) Taguchi, T.; Kurishita, M.; Shibuya, A.; Aso, K. *Tetrahedron* **1997**, *53*, 9497−9508. (c) Taguchi, T.; Kurishita, M.; Shibuya, A. *J. Fluorine Chem.* **1999**, *97*, 157−159.

selenides may be prepared directly from the corresponding alcohol in the presence of tributyl phosphine,[13] treatment of alcohol **10** with 2-nitrophenyl selenocyanate under these conditions failed to produce the desired selenide **12**. Fortunately, the nucleophilic substitution of bromide **11** with 2-nitrophenylselenolate gave selenide **12** in good yield. Subsequent oxidation followed by refluxing in toluene resulted in the formation of the exocyclic double bond in **13**. Removal of the protecting groups was achieved using NaOH and then 10% aqueous HCl to give 2-methylene-ACC (**4**), isolated as a hydrochloride salt.

Having successfully demonstrated the feasibility of using selenomethyl moiety in **12** as a masked *exo*-methylene group in the synthesis of **4**, the stage was set up for the actual preparation of **6** carrying a tritium label at C-2′. As illustrated in Scheme 4, the unlabeled alcohol **10** was converted to its tritium-labeled form by a sequence of oxidation of **10** with pyridinium chlorochromate (PCC) followed by reduction of the resulting aldehyde **14** with [^{3}H]$NaBH_4$. The subsequent transformations of **15** to **6** were accomplished by the same set of reactions used in the preparation of unlabeled **4**. The specific radioactivity of **6** was determined to be 3.90 mCi/mmol.

Biological Studies. Excess tritium-labeled **6** was incubated with ACC deaminase for 12 h to ensure complete inactivation of the enzyme. The noncovalently bound **6** was first removed by a HiTrap desalting column, and the pooled protein fractions were subjected to further dialysis. Since the enzyme activity was not recovered after extensive dialysis, the inactivation is clearly irreversible. As expected, the inactivated enzyme was radiolabeled due to covalent modification of the protein by 2-methylene-ACC. The concentration of the dialyzed protein solution was determined by Bradford method, and the radioactivity of the inactivated enzyme was analyzed by scintillation counting. To our great surprise, more than five equivalents (5.49 ± 0.35) of **6** per enzyme monomer were found in the inactivated ACC deaminase. Evidently, at least five amino acid residues of this enzyme had been modified during the inactivation.

Mechanistic Implications. It should be noted that cyclopropanes can act as electrophiles when electron-withdrawing substituents are attached.[9a,14] Since both the carboxyl and the PLP-aldimine groups can stabilize an anion, the cyclopropane ring of ACC is expected to be fairly electrophilic in the active site of ACC deaminase due to double activation by these two groups.[15] The previously reported protection from inhibition in the presence of excess substrate clearly demonstrated that the effect of **4**/**6** on ACC deaminase is most likely active site directed.[8] However, the observed stoichiometry indicated that covalent modification at loci beyond the catalytic site could also have occurred. Because the nucleophilic addition to rupture the electrophilic cyclopropyl ring of ACC requires the assistance of PLP, the observed modification of nonactive-site residues may result from the reaction with the turnover product **5** or its equivalents during the incubation. Whether the loss of enzymatic activity was due to the simple entrapment of an active-site nucleophile or a combined effect of modifications at multiple sites to change the protein conformation must await further experiments that are currently being pursued. It is worth mentioning that crystal structure of ACC deaminase from yeast strain *Hansenula saturnus* has recently been determined to 2.0 Å resolution.[16] Future studies of the inactivation as well as catalytic mechanisms of the *Pseudomonas* enzyme will be guided by the structural information deduced from the yeast enzyme, as their translated amino acid sequences are 60% identical.

Experimental Section

All chemicals were products of Aldrich Co. (Milwaukee, WI), unless otherwise specified. NMR spectra (^{1}H at 300 MHz and ^{13}C at 75 MHz) were recorded with a Varian U-300 Spectrometer in $CDCl_3$, unless otherwise specified in the text. Chemical shifts (δ in ppm) are given relative to those for Me_4Si with coupling constants in hertz (Hz). High-resolution mass spectroscopy was performed on a Finnigan Mat 95 (for HR-CI and HR-EI), a VG Analytical 7070E-HF (for FAB), or a Finnigan FT/MS 2001 (for ESI) instrument. Flash chromatography was performed on Lagand Chemical silica gel (230−400 mesh) by elution with the specified solvents. Analytical thin-layer chromatography (TLC) was carried out on Polygram Sil G/UV_{254} plates (0.25 mm). TLC spots were visualized by heating the plate previously stained with a solution of phosphomolybdic acid (3% in EtOH). Dry THF was distilled over sodium and benzophenone. The tritium-labeled compounds were not submitted for NMR and mass spectroscopic analysis due to the possibility of radioactive contamination. Radioactivity was measured by liquid scintillation counting on a Beckman LS 3801 counter using Ecoscint A biodegradable scintillation solution from National Diagnostics (Manville, NJ) as the solvent.

Dimethyl 2-(*tert*-Butyldimethylsilyloxy)methylcyclopropane-1,1-dicarboxylate (8). To a green solution of *tert*-butyldimethylsilylallyl oxide (**7**) (12.4 g, 72 mmol) and [Rh(OAc)$_2$]$_2$ (0.18 g, 0.8 mmol) was added dimethyl diazomalonate[17] (13.4 g, 79 mmol) at a rate of 0.35 mL/h via a syringe pump at 50 °C. The mixture was stirred for an additional 2 h at the same temperature. The reaction was cooled to room temperature, and the entire crude material was chromatographed on silica gel (eluent: hexanes/ethyl acetate, 95:5−3:1) to give the product **8** (17.1 g, 78% yield) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 3.70 (1H, dd, J = 10.8, 4.8), 3.68 (3H, s), 3.67 (3H, s), 3.61 (1H, dd, J = 10.8, 6.3), 2.09 (1H, m), 1.50 (1H, dd, J = 7.5, 5.5), 1.33 (1H, dd, J = 9.3, 4.5), 0.81 (9H, s), −0.02 (6H, s); ^{13}C NMR ($CDCl_3$) δ 170.7, 168.1, 60.5, 52.6, 52.5, 32.4, 29.7, 25.8, 25.5, 18.2, −5.4, −5.5; HRMS (CI) calcd for $C_{14}H_{27}O_5Si$ $(M + H)^+$ 303.1628, found 303.1636.

Methyl (1*RS*,2*RS*)-2-(*tert*-Butyldimethylsilyloxy)methyl-1-(*N*-*tert*-butoxycarbonyl)aminocyclopropane-1-carboxylate (9).[18] **Method A.** To a solution of compound **8** (17.1 g, 56 mmol) in methanol (110 mL) was added aqueous sodium hydroxide (2.4 g, 60 mmol in 16 mL of H_2O). The resulting solution was stirred for 48 h at room temperature. Methanol

(13) For examples, see (a) Honda, T.; Ishikawa, F. *J. Org. Chem.* **1999**, *64*, 5542−5546. (b) Wipf, P.; Kim, Y.; Goldstein, D. M. *J. Am. Chem. Soc.* **1995**, *117*, 11106−11112.

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(15) A few examples: (a) Haddow, J.; Suckling, C. J.; Wood, H. C. S. *J. Chem. Soc., Perkin Trans. 1* **1989**, 1297−1304. (b) Sun, D.; Hurley, L. H. *Biochemistry* **1992**, *31*, 2822−2829. (c) Silverman, R. B.; Ding, C. Z.; Borrillo, L.; Chang, J. T. *J. Am. Chem. Soc.* **1993**, *115*, 2982−2983. (c) Warpehoski, M. A.; Harper, D. E. *J. Am. Chem. Soc.* **1994**, *116*, 7573−7580. (d) Dakoji, S.; Li, D.; Agnihotri, G.; Zhou, H.-q.; Liu, H.-w. *J. Am. Chem. Soc.* **2001**, *123*, 9749−9759.

(16) Yao, M.; Ose, T.; Sugimoto, H.; Horiuchi, A.; Nakagawa, A.; Wakatsuki, S.; Yokoi, D.; Murakami, T.; Honma, M.; Tanaka, I. *J. Biol. Chem.* **2000**, *275*, 34557−34565.

(17) Wulfman, D. S.; McGibboney, B. G.; Steffen, E. K.; Thinh, N. V.; McDaniel, R. S. Jr.; Peace, B. W. *Tetrahedron* **1976**, *32*, 1257−1265.

(18) Wick, L.; Tamm, C.; Boller, T. *Helv. Chim. Acta* **1995**, *78*, 403−410.

was then removed under reduced pressure. The residue was diluted with water, acidified with 5% HCl, and extracted with ethyl acetate. The combined organic extracts were dried over anhydrous sodium sulfate and concentrated. The crude monoacid was taken into dry THF (100 mL), mixed with *cis*-dicyclohexano-18-crown-6 ether (150 mg, 0.4 mmol) and potassium carbonate (13.8 g, 100 mmol), and stirred for 30 min at room temperature. To this mixture was introduced ethyl chloroacetate (5.9 g, 54 mmol), and stirring was continued for an additional 2 h. The suspension was filtered through Celite, and the filtrate was treated with aqueous sodium azide (3.54 g, 54 mmol in 40 mL of H_2O) for 3 h. The mixture was diluted with H_2O and extracted with ether, and the combined organic solution was dried over anhydrous sodium sulfate. The solvents were removed, and the residue was chromatographed on silica gel (eluent: hexanes/ethyl acetate, 5:1−3:1) to afford the acyl azide intermediate (6.7 g, 45% yield) as a colorless oil. A solution of the acyl azide intermediate (6.6 g, 21 mmol) in dry toluene (20 mL) was refluxed for 1 h under nitrogen followed by the addition of *tert*-butyl alcohol (10 mL). After the mixture was refluxed for another 7 h, the solvents were removed under reduced pressure and the residue was purified by silica gel chromatography (eluent: hexanes/ethyl acetate, 5:1). The desired product **9** (5.0 g, 66% yield) was isolated as a white solid.

Method B. To a solution of compound **8** (7.0 g, 23 mmol) in methanol (50 mL) and water (6.5 mL) was added sodium hydroxide (1.0 g, 24 mmol) at 0 °C. The mixture was stirred for 46 h at room temperature. Methanol was then removed under reduced pressure, and the residue was diluted with H_2O, acidified with 5% HCl, and extracted with ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate. Volatile materials were removed to afford the crude monoacid (6.1 g, 92%). The crude monoacid (5.2 g, 18 mmol) was mixed with diphenylphosphoryl azide (5.4 g, 19 mmol) and triethylamine (2.5 g, 25 mmol) in dry *tert*-butyl alcohol (30 mL), and the resulting mixture was refluxed for 2.5 h under nitrogen. The solvents were removed under reduced pressure, and the residue was loaded on a silica gel column eluting with a mixture of ethyl acetate and hexanes (10−33%) to give the product **9** (4.5 g, 68%) as a white solid.

Spectral analyses of **9**: ^{1}H NMR ($CDCl_3$) δ 5.22 (1H, br s), 3.86 (1H, dd, J = 11.1, 6.0), 3.69 (3H, s), 3.58 (1H, dd, J = 11.1, 8.7), 1.70 (1H, m), 1.61 (1H, m), 1.42 (9H, s), 1.38 (1H, m), 0.84 (9H, s), 0.0 (3H, s), −0.02 (3H, s); ^{13}C NMR ($CDCl_3$) δ 171.7, 155.9, 79.8, 60.6, 52.4, 38.1, 32.8, 29.7, 28.2, 25.8, 21.5, 18.2, −5.3, −5.4; HRMS (CI) calcd for $C_{17}H_{34}NO_5Si$ $(M + H)^+$ 360.2175, found 360.2191.

Methyl (1*RS*,2*RS*)-2-Hydroxymethyl-1-(*N*-*tert*-butoxycarbonyl)aminocyclopropane-1-carboxylate (10).[18] A suspension of the starting material **9** (3.95 g, 11 mmol) and DOWEX-50W resin in methanol (160 mL) was vigorously stirred for 1 h at room temperature. The resin was filtered off, and the filtrate was concentrated under reduced pressure. The brown residue was purified by silica gel chromatography (eluent: hexanes/ethyl acetate, 2:1−1:2) to give the alcohol **10** (2.55 g, 94% yield) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 5.20 (1H, br s), 4.0 (1H, dd, J = 11.4, 4.5), 3.74 (3H, s), 3.73 (1H, m), 2.35 (1H, br s), 1.89 (1H, m), 1.64 (1H, m), 1.44 (9H, s), 1.42 (1H, m); ^{13}C NMR ($CDCl_3$ δ 172.4, 156.4, 80.3, 59.6, 52.6, 37.7, 33.9, 28.2, 21.5; HRMS (CI) calcd for $C_{11}H_{20}NO_5$ $(M + H)^+$ 246.1341, found 246.1350.

Methyl (1*RS*,2*RS*)-2-Bromomethyl-1-(*N*-*tert*-butoxycarbonyl)aminocyclopropane-1-carboxylate (11). To a solution of alcohol **10** (0.95 g, 3.8 mmol) in dry CH_2Cl_2 (8 mL) was added triphenylphosphine (1.2 g, 4.6 mmol) followed by *N*-bromosuccinimide (0.75 g, 3.2 mmol) at −78 °C. The mixture was stirred for 2 h at room temperature. The resulting yellow suspension was diluted with ether, washed with water, and then saturated sodium bicarbonate. The organic solution was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was chromatographed on silica gel (eluent: hexanes/ethyl acetate, 10:1−5:1) to afford the bromide **11** (0.87 g, 73% yield) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 5.27 (1H, br s), 3.73 (3H, s), 3.72 (1H, m), 3.53 (1H, m), 1.97 (1H, m), 1.71 (1H, m), 1.50 (1H, m), 1.41 (9H, s); ^{13}C NMR ($CDCl_3$) δ 171.2, 155.7, 80.3, 52.7, 40.9, 33.1, 29.7, 28.3, 25.6.

Methyl (1*RS*,2*RS*)-2-[(2-Nitrophenyl)selenomethyl]-1-(*N*-*tert*-butoxycarbonyl)aminocyclopropane-1-carboxylate (12). To a solution of 2-nitrophenylselenocyanate (0.73 g, 3.2 mmol) in ethanol (25 mL) was added sodium borohydride (0.13 g, 3.5 mmol) at 0 °C under a nitrogen atmosphere. The mixture was stirred at room temperature until no more gas evolution was discernible. To this solution was added portionwise a solution of bromide **11** (0.9 g, 2.9 mmol) in ethanol (15 mL). The resulting suspension was stirred for 20 h, diluted with CH_2Cl_2, washed with brine, and dried over anhydrous sodium sulfate. The solvents were removed under reduced pressure, and the residue was chromatographed on silica gel (eluent: hexanes/ethyl acetate, 5:1−2:1) to give the selenolate **12** (1.1 g, 85% yield) as a viscous syrup: ^{1}H NMR ($CDCl_3$) δ 8.25 (1H, dd, J = 8.4, 1.2), 7.58 (1H, m), 7.48 (1H, m), 7.30 (1H, m), 5.24 (1H, br s), 3.72 (3H, s), 3.21 (2H, d, J = 6.9), 1.86 (1H, m), 1.66 (1H, m), 1.44 (1H, m), 1.42 (9H, s); ^{13}C NMR ($CDCl_3$) δ 171.7, 155.8, 146.8, 133.8, 133.2, 129.4, 126.4, 125.8, 125.6, 80.3, 52.8, 40.2, 30.0, 28.3, 25.0, 23.8; HRMS (FAB) calcd for $C_{17}H_{22}N_2NaO_6{}^{80}Se$ $(M + Na^+)$ 453.0529, found 453.0535.

Methyl 2-Methylene-1-(*N*-*tert*-butoxycarbonyl)aminocyclopropane-1-carboxylate (13). To a solution of compound **12** (0.22 g, 0.5 mmol) in dry methylene chloride (5 mL) was added 60% *meta*-chloroperbenzonic acid (0.29 g, 1 mmol) at −10 °C. After the mixture was stirred for 1 h at the same temperature, diisopropylamine (1.5 mL, 10 mmol) was introduced, and the reaction was refluxed for 30 min. The resulting mixture was diluted with methylene chloride, washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was dissolved in toluene (5 mL) and refluxed for 30 min. Toluene was then removed under reduced pressure and the residue was chromatographed on silica gel (eluent: hexanes/ethyl acetate, 10:1−3:1). The desired alkene **13** (56 mg, 49% yield) was isolated as a white solid: ^{1}H NMR ($CDCl_3$) δ 5.70 (1H, m), 5.54 (1H, m), 5.26 (1H, br s), 3.67 (3H, s), 2.35 (1H, m), 1.79 (1H, m), 1.43 (9H, s); ^{13}C NMR ($CDCl_3$) δ 171.5, 155.8, 133.3, 105.9, 80.2, 52.5, 35.9, 28.2, 20.9; HRMS (CI) calcd for $C_{11}H_{18}NO_4$ $(M + H)^+$ 228.1236, found 228.1234.

1-Amino-2-methylenecyclopropane-1-carboxylic Acid (4). To a solution of **13** (130 mg, 0.57 mmol) in a mixture of water (2.5 mL) and methanol (10 mL) was added sodium hydroxide (26 mg, 0.65 mmol). The mixture was stirred for 40 h at room temperature and then concentrated. The residue was dissolved in water, neutralized with 5% HCl, and extracted with ethyl acetate. The extracts were dried over anhydrous sodium sulfate and evaporated in vacuo. The residue was dissolved in 5% HCl (5 mL) and stirred for 5 h at room temperature. The aqueous solution was extracted with ethyl acetate and then lyophilized to give the acid **4** (79 mg, 91% yield) as a white solid: ^{1}H NMR (D_2O) δ 5.73 (1H, m), 5.65 (1H, m), 2.20 (1H, m), 1.91 (1H, m); ^{13}C NMR (D_2O) δ 171.3, 127.3, 109.7, 53.6, 17.3; HRMS (CI) calcd for $C_5H_8NO_2$ $(M + H)^+$ 114.0555, found 114.0555.

Methyl (1*RS*,2*RS*)-1-(*N*-*tert*-Butoxycarbonyl)amino-2-formylcyclopropane-1-carboxylate (14). A mixture of alcohol **10** (4.37 g, 17.8 mmol), sodium acetate (2.8 g, 35 mmol), and pyridinium chlorochromate (11.5 g, 52 mmol) was vigorously stirred for 40 min at room temperature. The reaction mixture was then passed through a short silica gel column and eluted with ether. The effluent was concentrated, and the residue was chromatographed on silica gel (eluent: hexanes/ethyl acetate, 3:1−2:1) to give the aldehyde **14** (2.4 g, 55% yield) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 9.30 (1H, d, J = 5.7), 3.70 (3H, s), 2.21 (1H, m), 1.80 (1H, m), 1.38 (9H, s), 1.30 (1H, m); HRMS (CI) calcd for $C_{11}H_{18}NO_5$ $(M + H)^+$ 244.1185, found 244.1193.

Methyl (1*RS*,2*RS*)-1-(*N*-*tert*-Butoxycarbonyl)amino-2-[^{3}H]hydroxymethylcyclopropane-1-carboxylate (15). A solution of aldehyde **14** (2.3 g, 9.4 mmol) in dry THF (15 mL) was pretreated with $NaBH_4$ (20 mg, 0.5 mmol) for 10 min at 0 °C. A small ampule containing [^{3}H]$NaBH_4$ (100 mCi) was

opened carefully in a glovebag. Dry THF (3 mL) was added to the ampule, and the suspension was transferred with a plastic pipet to a flame-dried, 250-mL flask. The ampule was rinsed with a small amount of THF three times, and the rinsing solution was also transferred into the flask. To the [^{3}H]$NaBH_4$ suspension was added the pretreated solution at 0 °C, and the mixture was then stirred at room temperature for 1 h. The mixture was again cooled to 0 °C. To the mixture were added anhydrous methanol (10 mL), dry THF (20 mL), and $NaBH_4$ (90 mg, 2.3 mmol), successively. The mixture was stirred for an additional 20 min, at which time TLC analysis indicated complete reaction. The mixture was diluted with ether and treated with 1 M acetic acid. The organic layer was separated, and the aqueous layer was extracted with ether. The combined organic solution was washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The oily residue was chromatographed on silica gel (eluent: hexanes/ethyl acetate, 2:1−1:2) to give the tritium-labeled alcohol **15** (1.79 g, 78%) as a colorless oil. The specific activity of this sample was determined to be 3.87 mCi/mmol.

Methyl (1*RS*,2*RS*)-2-[^{3}H]-Bromomethyl-1-(*N*-*tert*-butoxycarbonyl)aminocyclopropane-1-carboxylate (16), Methyl (1*RS*,2*RS*)-2-[^{3}H]-[(2-Nitrophenyl)selenomethyl]-1-(*N*-*tert*-butoxycarbonyl)aminocyclopropane-1-carboxylate (17), Methyl 2-[^{3}H]-Methylene-1-(*N*-*tert*-butoxycarbonyl)aminocyclopropane-1-carboxylate (18), and 1-Amino-2-[^{3}H]-methylenecyclopropane-1-carboxylic Acid (6). Preparation of these labeled compounds followed the same procedures used to synthesize their nonlabeled counterparts. The specific radioactivity determined for the final compound **6** (S. A., 3.90 mCi/mmol) is an average of three measurements.

Purification of ACC Deaminase.[8a] The recombinant overexpression *Esherichia coli* strain pQNt23/BL21(DE3)-pLysS, which harbors the ACC deaminase gene, was grown in Terrific Broth medium supplemented with 100 μg/mL of ampicillin at 37 °C to an OD_{600} of 0.6. The culture was cooled to 24 °C, induced with 100 μM of isopropylthiogalactoside (IPTG), and incubated for an additional 6 h at 24 °C. The cells were harvested and ruptured by sonication. ACC deaminase was purified to near homogeneity via a previously reported protocol including ammonium sulfate fractionation (20%-50%), DEAE-Sepharose, ω-aminohexyl-Sepharose, and FPLC MonoQ chromatography. The purified enzyme has a specific activity of 4.2 U/mg, with a ratio of $A_{280}/A_{420} = 4.5$.

Enzyme Assay.[8a] A standard 1 mL assay was performed in 50 mM potassium phosphate buffer (pH 7.5) at 23 °C, containing 4 mM of substrate, 8−20 μg ACC deaminase, 125 μg of L-lactate dehydrogenase, and 0.1 mM of NADH. Loss of NADH absorbance at 340 nm (ϵ 6,220) was monitored. The unit of activity is defined as μmoles of NADH consumption per min.

Determination of Stoichiometry of MACC Modification on ACC Deaminase. ACC deaminase (280 μg, 8.1 nmol) was incubated with labeled 2-methylene-ACC (**6**, 10.3 μmol) in 0.1 M potassium phosphate buffer (255 μL, pH 7.5) for 12 h at room temperature. The entire mixture was loaded on a HiTrap desalting column (Amersham) and eluted with 25 mM Tris•HCl buffer (pH 7.5). The protein-containing fractions were pooled and dialyzed against 25 mM potassium phosphate buffer (pH 7.5) for 15 h at room temperature. The dialyzed solution was subjected to Bradfrod assay to determine the protein concentration, and to scintillation counting to estimate the extent of radioisotope incorporation. The stoichiometric relationship between the of covalently linked inhibitor and the protein monomer was deduced from the ratio of the specific radioactivity of the modified protein to that of the inhibitor.

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