Ethylene Biosynthesis. 12. Analog Approach to the Active Site Topography of the Ethylene-Forming Enzyme. Novel Hydroxamate Inhibitors

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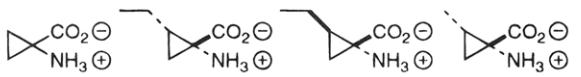
In order to understand both the substrate specificity and active site topography of the ethylene-forming enzyme (EFE), a number of analogs of its substrate, 1-aminocyclopropanecarboxylic acid, have been prepared and studied as inhibitors. Because of the dependence of EFE activity on iron, hydroxamic acids, a functional group known to bind iron tightly, derived from several small carboxylic/amino acids were studied along with the parent amino acids. The activity of these materials was assayed *in vitro* against the purified EFE from apple fruit. The varying potency of the amino acid hydroxamates suggests that they do not act simply by binding to iron and removing it from the enzyme. The order of their potency was consistent with the idea that binding reflects both metal chelation and hydrophobic interactions in the active site. The most potent inhibitor, ACC-hydroxamate, has about 1 μM K_i.

Introduction

The topography of the active site of the ethylene-forming enzyme (EFE), which is involved in the conversion of 1-aminocyclopropanecarboxylic acid (ACC, **1**) to the plant growth hormone ethylene,[2] has been of interest since the initial evidence concerning the existence of the EFE *in vivo* was reported.[3] The first attempts at mapping the active site, by Yang and Ichihara,[4] demonstrated discrimination among the four stereoisomers of 2-ethyl-ACC (coronamic and *allo*-coronamic acids), which serve



1 *allo*-coronamic acid coronamic acid **2**

as substrates for 1-butene production. While ACC itself is achiral, its proteinaceous binding site is chiral, therefore chiral ACC analogues bind diastereomerically and possess distinguishable kinetic constants. Subsequent work has established that the 2-methyl-substituted ACC analogues, substrates for propene production, are better substrates for the EFE[5,6] and that the absolute configuration best accepted by the enzyme is 1*R*,2*S* (**2**).[7] On this basis, the crude active site topography shown in Figure 1 was proposed.[7]

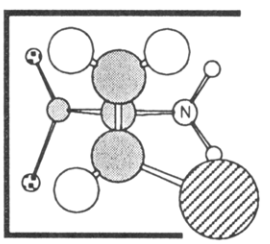


Figure 1. Perspective view of the sterically-limiting active site of the ethylene-forming enzyme inferred by substrate selectivity.

The mechanism by which the EFE selects among the wide variety of other potential amino acid substrates present in plant tissue is of greater physiological significance than the chiral discrimination discussed above. The control point for the biosynthesis of ethylene is *via* the production of ACC by ACC synthase.[7] The EFE is present in at least low amounts in most plant tissues and is greatly amplified during ripening and some stress responses. It therefore seems reasonable that glycine and alanine in particular would be able to reach the active site of a functioning enzyme, since they present less sterically-demanding side chains than the cyclopropane ring of ACC. Furthermore, the non-heme iron class of enzymes of which the EFE is a member is not known for its exquisite selectivity. A fairly wide latitude in substrate specificity has been noted in Baldwin's laboratory[8] with the closely-related isopenicillin-N-synthase enzyme. One question to be raised, then, is the possible metabolic loss of reducing equivalents in concert with dioxygen consumption due to competition by amino acids other than the intended substrate, ACC. It is also clear that potent inhibitors of ethylene biosynthesis could have benefit in the preservation of fruits, vegetables, and flowers. Inhibitors of the EFE might therefore be valuable. The recent purification of the ethylene-forming enzyme by several groups[9] permits the examination of

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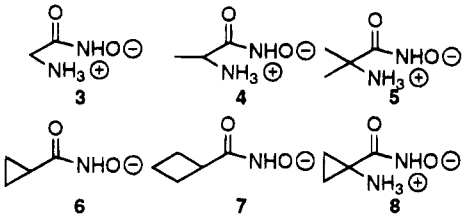
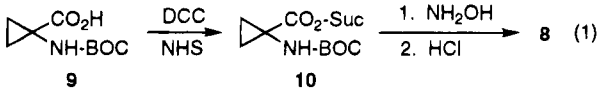
the kinetic properties of alternative substrates and inhibitors *in vitro*. Studies have been conducted here with both natural and unnatural "small" amino acids, cycloalkanecarboxylic acids, and their hydroxamate derivatives. The latter were of particular interest because the EFE requires iron for activity and hydroxamic acids are known to have high affinity for transition metals, generally through bidentate binding.[10]

Results

Three amino acid hydroxamates **3**–**5** were obtained from commercial sources.[11] The small cycloakanehydroxamates **6** and **7** were obtained by hydroxylaminolysis of the corresponding esters. The ACC-hydroxamate **8** was



prepared by the straightforward route described in eq 1: formation of the active ester of the BOC derivative and hydroxylaminolysis. 1-Aminocyclobutanecarboxylic acid (ACBC) was prepared by the Bucherer–Leib method.[12]



Each of these compounds, in addition to the amino acids glycine, alanine, and aminoisobutyric acid (AIB), was tested as an inhibitor of the native, isolated EFE using a conventional Dixon analysis,[13] where the inhibitor concentration is varied at a constant substrate concentration. An example of one such Dixon plot, for **5**, is given in Figure 2. Because of its most potent inhibition of the EFE, compound **8** was also subjected to full kinetic analysis, in which both substrate and inhibitor concentrations are varied, using both a Hanes–Woolf plot[14] and direct computational analysis of the data using the integrated form of the Michaelis–Menten equation.[14] Interestingly, **8** also showed slow turnover by the EFE. Kinetic study of this process showed that **8** has a somewhat higher Michaelis constant and a 7-fold lower k_{cat} than ACC (Chart 1). It has a k_{cat}/K_m of 2.29×10^3 M^{-1} s^{-1} compared to that for ACC of 4.45×10^4 M^{-1} s^{-1},

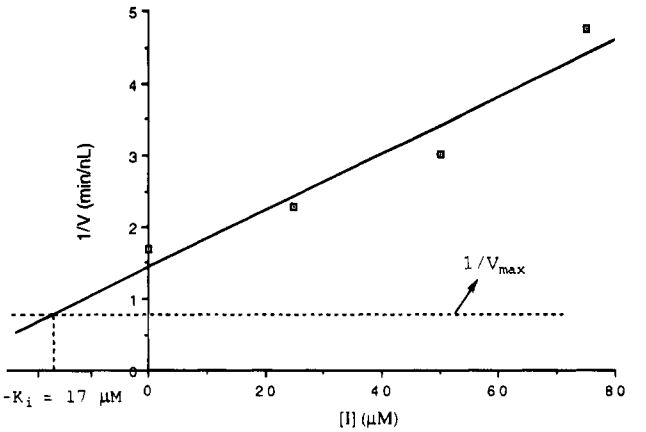


Figure 2. Dixon plot of the inhibition of ethylene-forming enzyme by **5**.

Chart 1

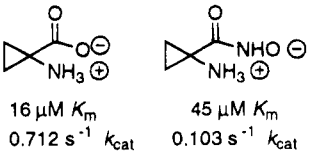
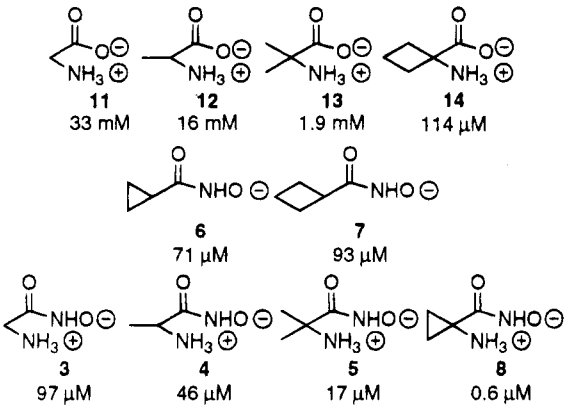


Chart 2



meaning that the EFE prefers ACC over the hydroxamate by a factor of ~20.

As can be seen from the inhibition data collected in Chart 2, rather weak inhibition of the EFE is exhibited by the two naturally-occurring amino acids glycine (**11**) and L-alanine (**12**), both having greater than millimolar K_is. The two unnatural disubstituted amino acids, AIB (**13**) and ACBC (**14**), are more potent, with K_is around a millimolar, which can be attributed to constructive hydrophobic interactions (*vide infra*). Comparable activities are seen in the carboxylic acid hydroxamates **6** and **7**, though they give slightly nonlinear Dixon plots. In the series of amino acid hydroxamates, a consistent decrease in inhibition constant (more potent inhibition) with increasing substitution on the α-carbon is noted. The kinetic analysis of the inhibition of the EFE by **8**, the most potent member of this class, is shown in Figure 3. This Hanes–Woolf plot shows noncompetitive inhibition against ACC (*vide infra*). Replotting the slopes of these lines gives a 182 μM K_i.

Discussion

Glycine and alanine have about a thousand-fold smaller affinity for the EFE than does ACC, which should serve

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(10) Kurzak, B.; Kozlowski, H.; Farkas, E. *Coord. Chem. Rev.* **1992**, *114*, 169–200.

(11) All of the amino acid hydroxamates in this work are depicted as their zwitterions for convenience and consistency, though their ionic states in aqueous solution were not determined. The pK_a of hydroxamic acids (acetohydroxamic) is in the ~9 range (Gerstein, J.; Jecks, W. P. *J. Am. Chem. Soc.* **1964**, *86*, 4655–4663), comparable to that of primary amines. However, the cyclopropane ring in particular decreases the basicity of the nitrogen by "conjugation", with ACC titrating at pH 8.1. The least ambiguity concerning its zwitterionic nature therefore exists for compound **8**.

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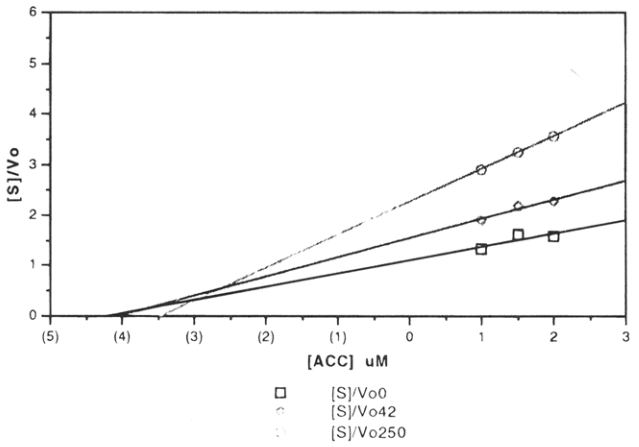
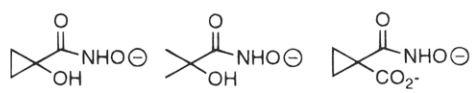


Figure 3. Hanes–Woolf plot of the inhibition of ethylene-forming enzyme by **8**.

to minimize the amount of adventitious oxidation of these substrates *in vivo*. The fact that the series of amino acid hydroxamates shows differential activities and linear Dixon plots demonstrates that they are not merely serving as iron chelators to remove the required metal from the active site. In that instance, all of the hydroxamates should have similar potencies simply based on their similar association constants for iron. The assertion that the activity is active site-directed and not a simply reflection of hydroxamate affinity for iron is further supported by the very weak (K_i > 1 mM) inhibitory potencies of the hydroxy acid hydroxamates **15**–**17** (data not shown). The cycloalkane hydroxamates **6** and **7**, based on their nonlinear Dixon plots, may have bimodal action involving both binding to the iron in the active site with a high affinity and removing it from the enzyme with a lower affinity, similar to **15**–**17**. They also show that the hydroxamate greatly aids in the binding of inhibitors to the EFE: hydroxamates **6** and **7** have affinities superior to the amino acids despite their lack of a key point of recognition (and reaction[15]) between substrate and enzyme: the α-amino group. The results with amino acid hydroxamates support potent binding through the hydroxamate and provide good evidence for a positive hydrophobic interaction between the α-alkyl substituents and the active site of the enzyme.



15 **16** **17**

The most potent inhibitor, ACC-hydroxamate (**8**), is also a substrate and therefore should be competitive with ACC, but the kinetic inhibition pattern observed is noncompetitive. However, this should cause little concern since it is likely a reflection of more general problems in the kinetic study of the EFE. Because the current gas chromatographic assay is noncontinuous, a single Hanes–Woolf line requires many replicates at each point, limiting the number of points per line to a suboptimal three and limiting the number of lines that can be obtained simultaneously with a single enzyme preparation. The potency of the binding of **8** to the enzyme

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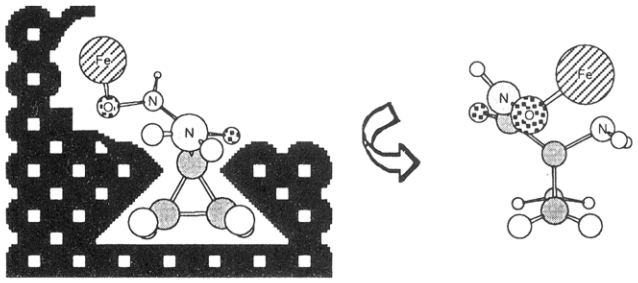

Figure 4. Cartoon representing one conception of the ethylene-forming enzyme active site based on hydroxamate inhibition.

was determined from three different experiments, Dixon inhibition analysis, the Hanes–Woolf multiple line kinetic analysis, and the saturation kinetics of its action as a substrate. The affinities derived from these experiments are 0.6 μM, 182 μM, and 45 μM, respectively. The greater K_i seen in the Hanes–Woolf analysis can be explained by the imprecision in an assay with fewer replicates and is regarded as least representative of the true affinity of **8** for the EFE. The divergence of the Dixon K_i for **8** when ACC is the substrate and the K_m when **8** is the substrate can be ascribed to the expected difference between the inhibitor dissociation constant and the Michaelis constant if the k_{cat} step is fast relative to substrate/inhibitor dissociation.[16] That is, K_m is the same as K_s, the dissociation constant of the enzyme–substrate complex (and, in this case, K_i, the dissociation constant of the enzyme–inhibitor complex), only when the rate of reversal of ES (or EI) formation is much faster than k_{cat}. Thus, **8** is likely a "sticky" substrate, one that is much less likely to dissociate once the ES complex is formed than to proceed through the catalytic mechanism. Therefore, for inhibition by **8**, $K_i = K_s$, but for ACC turnover, $K_m \neq K_s$.

A stylized picture for the binding of **8** to the EFE can be proposed as shown in Figure 4. It includes a hydrophobic site optimized for recognition of the cyclopropane. That metal binding by these inhibitors is important is also implied by our data, but for this to be so the hydroxamate must either bind to the iron in a monodentate mode (pictured) or, to permit bidentate binding, the protein must have a highly convoluted surface to oppose the hydroxamate with iron directly across its active site. A similar mode of binding might apply to the substrate carboxylate. Ligation of the iron by the α-amino nitrogen in the amino acid hydroxamates is also possible (Figure 4, rotated). While useful in increasing the activity of these inhibitors (>100-fold), this possible favorable interaction prompts consideration of a similar mechanism in the catalytic machinery. The EFE might find it advantageous to bind the amino group to be oxidized directly to the redox-active metal for an inner-sphere electron transfer. However, it has been implied[9c] that the iron already bears two or three histidine ligands from the enzyme, so the octahedral coordination geometry of iron would limit the binding of other ligands besides O_2 or the cosubstrate ascorbate.

Semiquantitative evaluation of the inhibition of the EFE by the amino acid hydroxamates **3**, **4**, and **8** shows that when only a single methyl group (alanine) is available for binding, the energetic cost is 2.4 kcal/mol

(16) Walsh, C. *Enzymatic Reaction Mechanisms*; W. H. Freeman: San Francisco, 1979; p 66.

vs the cyclopropane, and when there is no alkyl substituent to occupy the cyclopropane binding site (glycine), the cost is 2.8 kcal/mol. These values likely reflect the energetic cost of creating a void in the EFE that is usually occupied by the cyclopropane ring of the ACC substrate. In a similar vein, Matthews has studied the energetic cost of creating a void in the hydrophobic interior of a protein (T4 lysozyme) using both X-ray crystallography and thermochemistry. His data suggest that each Å^3 of free space results in an energy loss of ~24 cal/mol.[17] Dividing this value into the differential affinity between the natural substrate and glycine (2.8 kcal/mol) suggests that the volume of the cyclopropane binding site in the EFE is 117 Å^3. This value seems reasonable for a cyclopropyl → hydrogen change since voids of up to 150 Å^3 are created in T4 lysozyme by a Leu → Ala mutation that replaces an isopropyl group with a hydrogen.

Conclusion

A novel and potent inhibitor of the ethylene-forming enzyme from apple tissue, aminocyclopropanehydroxamic acid (**8**), has been prepared and studied. It is a competitive inhibitor against ACC as well as a slow substrate, with a micromolar K_i.

Experimental Section

General. Unless otherwise noted, all materials were obtained from commercial suppliers and were used without further purification. THF and dioxane were distilled from sodium/benzophenone under inert atmosphere. Flash column chromatography was performed using silica gel 9385-9 (Merck). TLC plates were silica gel 60, 0.2 mm in thickness. Melting points were determined in capillary tubes and were uncorrected. Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded at 300 and 75 MHz, respectively.

Cyclobutanespiro-5′-hydantoin.[18] To a solution of cyclobutanone (1.27 g, 18 mmol) in 14 mL of ethanol and 12.5 mL of water were added sodium cyanide (1.34 g, 27 mmol) and ammonium carbonate (6.80 g, 71 mmol). The mixture was refluxed for 6 h with stirring. After dilution with water, the cooled mixture was acidified with concd hydrochloric acid. The solution was concentrated by rotary evaporation and a white solid precipitated after cooling on ice for 3 h. The crude product was recrystallized from water to give the pure hydantoin as a white crystal (0.90 g, 35%), mp 224.5–225 °C. ^{1}H NMR (D_2O): δ 1.71–1.85 (m, 2H), 2.24–2.39 (m, 4H).

1-Aminocyclobutane-1-carboxylic Acid. Cyclobutanespiro-5′-hydantoin (0.90 g, 6.4 mmol) was suspended in 3 N sodium hydroxide (20 mL) and heated to reflux for 50 h. The reaction mixture was cooled and acidified to pH 6 with concd hydrochloric acid. Precipitates were removed by filtration. The solvent was removed by rotary evaporation and the resulting solid was dissolved in 1 N HCl and run through a Dowex 50W-X8 column. The crude product from the column was crystallized from water/acetone to give the amino acid as a white, fiberlike solid (0.30 g, 40%), mp 295–296 °C (lit. 290–296 °C, 290 °C).[19] R_f 0.18 (silica gel, water:butanol:acetic acid = 5:4:1, ninhydrin). ^{1}H NMR (D_2O): δ 1.86–1.97 (m, 2H), 2.07–2.17 (m, 2H), 2.32–2.42 (m, 2H). ^{13}C NMR (D_2O): δ_{TSP} 15.58, 30.96, 60.23, 178.61.

1-Cyclopropanecarbohydroxamic Acid (6). To an ice-cooled solution of hydroxylamine hydrochloride (3.48 g, 50 mmol) in water (7 mL) and absolute ethanol (6 mL) was slowly added 10 N NaOH (10 mL) with stirring, and then with continued stirring was added ethyl cyclopropylformate (5.7 g, 50 mmol) over a period of 30 min. The reaction mixture was stirred at room temperature for 8 h and then neutralized to pH 6 with concd hydrochloric acid. The solvent was removed by rotary evaporation. The residue was extracted with hot ethyl acetate (3 × 30 mL) and rotary evaporated. The crude product was recrystallized from ethyl acetate to give the title compound as a white crystal (3.03 g, 60%), mp 124.5–125 °C. ^{1}H NMR (DMSO-d_6): δ 10.51 (broad s, 1H), 8.68 (broad s, 1H), 1.36 (m, 1H), 0.65 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 170.10, 11.09, 5.53. Anal. Calcd. for $C_4H_7NO_2$: C, 47.52; H, 6.98; N, 13.85. Found: C, 47.24; H, 6.91; N, 13.88.

1-Cyclobutanecarbohydroxamic Acid (7). To an ice-cooled solution of hydroxylamine hydrochloride (3.48 g, 50 mmol) in water (7 mL) and absolute ethanol (6 mL) was slowly added 10 N NaOH (10 mL) with stirring, and then with continued stirring was added ethyl cyclobutylformate (6.4 g, 50 mmol) over a period of 30 min. The reaction mixture were stirred at room temperature for 8 h and then neutralized to pH 6 with concd hydrochloric acid. The solvent was removed by rotary evaporation. The residue was extracted with hot ethyl acetate (3 × 30 mL) and rotary evaporated. The crude product was purified by flash chromatography on silica gel (ethyl acetate) to give the title compound as a white crystal (4.60 g, 80%), mp 66.5–67 °C. ^{1}H NMR (DMSO-d_6): δ 1.72–2.00 (m, 4H), 2.11 (m, 2H), 2.85 (m, 1H), 8.64 (broad s, 1H), 10.28 (broad s, 1H). ^{13}C NMR (DMSO-d_6): δ 17.98, 24.36, 35.80, 170.77. Anal. Calcd for $C_5H_9NO_2$: C, 52.16; H, 7.88; N, 12.17. Found: C, 52.27; H, 7.95; N, 12.22.

1-(Boc-Amino)cyclopropane-1-carboxylic Acid (9).[20] A solution of ACC (246 mg, 2.47 mmol) in a mixture of dioxane (5 mL), water (2.5 mL), and 1 N NaOH (2.5 mL) was stirred and cooled in an ice–water bath. Di-*tert*-butyl pyrocarbonate (594 mg, 2.72 mmol) was added, and stirring was continued at room temperature for 10 h. The solution was cooled in an ice–water bath, covered with a layer of ethyl acetate (10 mL), and acidified with 0.1 M H_2SO_4 to pH 2. The aqueous phase was extracted with ethyl acetate (3 × 10 mL), washed with water (2 × 20 mL), dried over anhydrous magnesium sulfate, and rotary evaporated. The title compound was obtained in 75% yield (366 mg), mp 178–178.5 °C. ^{1}H NMR (DMSO-d_6): δ 0.93 (s, 2H), 1.24 (s, 2H), 1.36 (s, 9H), 7.40 (broad s, 1H), 12.30 (broad s, 1H). ^{13}C NMR (DMSO-d_6): δ 16.56, 28.13, 33.15, 77.74, 155.63, 174.41.

Boc-ACC *N*-Hydroxysuccinimide Ester (10). Dicyclohexylcarbodiimide (1.26 g, 6.03 mmol) was added to a solution of **9** (1.10 g, 5.48 mmol) and *N*-hydroxysuccinimide (651 mg, 5.48 mmol) in anhydrous dioxane (60 mL) with cooling. The reaction mixture was allowed to stand in the refrigerator for 24 h. The formed dicyclohexylurea was filtered and washed with dioxane. The filtrate was concentrated *in vacuo* to give a yellow oil which was triturated with ether and filtered to yield **10** as a white crystal (1.47 g, 90%), mp 138.5–139 °C. ^{1}H NMR ($CDCl_3$): δ 1.44 (s, 9H), 1.49 (m, 2H), 1.72 (m, 2H), 2.79 (s, 4H), 5.23 (broad s, 1H). ^{13}C NMR ($CDCl_3$): δ 19.61, 20.56, 25.54, 28.03, 33.91, 168.82. Anal. Calcd for $C_{13}H_{18}N_2O_6$: C, 52.35; H, 6.08; N, 9.39. Found: C, 52.39; H, 6.10; N, 9.30.

Boc-ACC Hydroxamate. To a solution of **10** (782 mg, 2.73 mmol) in dioxane (15 mL) was added hydroxylamine hydrochloride (194 mg, 2.73 mmol) in 10 mL of dimethylformamide to which had been added triethylamine (276 mg, 2.73 mmol). The mixture was stirred overnight, filtered and concentrated *in vacuo*. The residue was extracted with hot ethyl acetate (3 × 10 mL) and rotary-evaporated. The crude product was purified by flash chromatography on silica gel (ethyl acetate) to give the title compound as a white crystal (354 mg, 60%), mp 155–155.5 °C. ^{1}H NMR (DMSO-d_6): δ 0.81 (m, 2H), 1.16 (m, 2H), 1.36 (s, 9H), 7.18 (broad s, 1H), 8.63 (broad s, 1H), 10.42 (broad s, 1H). ^{13}C NMR (DMSO-d_6): δ 16.01, 28.19, 33.46, 78.10, 155.35, 169.15. Anal. Calcd for $C_9H_{16}N_2O_4$: C, 49.99; H, 7.46; N, 12.95. Found: C, 50.08; H, 7.51; N, 12.85. When the preceding step was conducted on a 0.5 g scale and

(17) Baase, W. A.; Eriksson, A. E.; Zhang, X. J.; Heinz, D. W.; Sauer, U.; Blaber, M.; Baldwin, E. P.; Wozniak, J. A.; Matthews, B. W. *Faraday Discuss.* **1992**, *93*, 173–81.

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(20) Wheeler, T. N.; Ray J. A. *Synth. Commun.* **1988**, *18*, 141.

the resulting material used in this step without intermediate purification (filtering the reaction directly into DMF containing hydroxylamine hydrochloride/triethylamine), the overall yield was 75%.

1-Aminocyclopropane-1-carbohydroxamic Acid Hydrochloride (8). An anhydrous HCl/CH_2Cl_2 solution was prepared by adding trimethylsilyl chloride to anhydrous methanol to CH_2Cl_2 at 0 °C under nitrogen. The final concentration of HCl was 1 M. The resulting solution was allowed to stir at 0 °C for 30 min and at room temperature for an additional 30 min. Boc-ACC hydroxamate (500 mg, 2.3 mmol) was added to the anhydrous HCl/CH_2Cl_2 solution (4000 mol%) prepared as above at 0 °C. The reaction mixture was stirred at 0 °C for 15 min. The solution was rotary evaporated and recrystallized from ethanol/water (3:1) to give **8** as a white crystal (350 mg, 100%), mp 166–166.5 °C dec. ^1H NMR (DMSO-d_6): δ 1.26 (s, 2H), 1.29 (s, 2H), 8.71 (broad s, 3H), 9.04 (broad s, 1H), 10.68 (broad s, 1H). ^{13}C NMR (DMSO-d_6): δ 11.69, 33.43, 166.27. Anal. Calcd for $C_4H_9N_2O_2Cl$: C, 31.49; H, 5.95; N, 18.36. Found: C, 31.55; H, 6.00; N, 18.27.

EFE Activity and Inhibition Assay. The EFE was extracted and purified from golden delicious apples using our previously-described protocol through the Butyl-Toyopearl 650M column.[9c] About 0.5–50 μg of protein in 50 μL volume was added to 2.95 mL of activity assay buffer [50 mM Tris-HCl (pH 7.2), 10% (v/v) glycerol, 20 mM $FeSO_4$, 0.1 mM ACC, 10 mM ascorbate, 5 mM $NaHCO_3$] in 25 mL Erlenmeyer flasks. The flasks were sealed with serum caps and incubated for 20 min at 23 °C on a rocker. The headspace (1.0 mL) was analyzed using a Varian 3300 gas chromatograph equipped with a flame ionization detector and an 80% Porapak N/20% Porapak Q column. Ethylene production was quantitated by comparison with a 97.7 ppm ethylene/helium gas mixture (Alltech Associates). A unit was defined as 1 nL min^{-1}. For inhibition studies with Dixon analysis, a substrate concentration of 200 μM and triplicate analysis at each of five inhibitor concentrations was used. For the determination of the competitive inhibition by **8** using multiple lines, the inclusion of bovine serum albumin (2 mg/mL) was necessary to obtain consistent results.

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