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Studies of 1-Amino-2,2-difluorocyclopropane-1-carboxylic Acid: Mechanism of Decomposition and Inhibition of 1-Aminocyclopropane-1-carboxylic Acid Deaminase

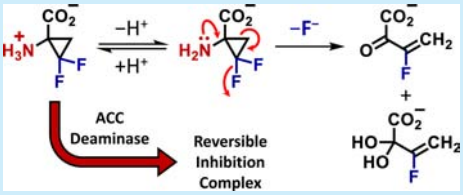
Cheng-Hao Liu,[†,⊥] Shao-An Wang,[†,⊥] Mark W. Ruszczycky,[‡] Huawei Chen,[§,∥] Keqiang Li,[§] Kazuo Murakami,[§] and Hung-wen Liu*[,†,‡,§]

[†]Department of Chemistry and [‡]Division of Medicinal Chemistry, College of Pharmacy, University of Texas at Austin, Austin, Texas 78712, United States

[§]Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455, United States

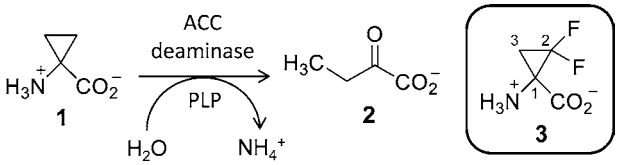
Supporting Information

ABSTRACT: 1-Amino-2,2-difluorocyclopropane-1-carboxylic acid (DFACC) is of interest in the study of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase due to the increased reactivity of its cyclopropyl functionality. It is shown that DFACC is unstable under near-physiological conditions where it primarily decomposes via specific-base catalysis to 3-fluoro-2-oxobut-3-enoic acid with a rate constant of 0.18 ± 0.01 min^{-1}. Upon incubation with ACC deaminase, DFACC is found to be a slow-dissociating inhibitor of ACC deaminase with submicromolar affinity.



1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase is a pyridoxal-5′-phosphate (PLP)-dependent enzyme that catalyzes the decomposition of ACC (**1**) to α-ketobutyrate (**2**) and ammonia (see Scheme 1).[1] It has been identified in a wide range of bacteria and fungi.[1] The substrate of ACC deaminase is also the immediate biosynthetic precursor to ethylene, which is produced in plants via oxidation of ACC.[2−4] While ethylene is an essential plant hormone that regulates fruit ripening, seed germination, and leaf senescence,[4,5] it can also be detrimental causing senescence, chlorosis, and abscission when produced in excessive quantities under conditions of stress.[4−6] The potential use of ACC deaminase to mitigate the deleterious effects of ethylene production has thus made it a focus of enzymological research to better understand its atypical mechanism of action.[6b,7]

Scheme 1. Reaction Catalyzed by ACC Deaminase (1 → 2) and the Structure of DFACC (3)



The opening of the cyclopropane ring of ACC catalyzed by ACC deaminase is essentially a redox-neutral deamination reaction. The chemistry of ACC deaminase thus stands in contrast to that of typical PLP-dependent transaminases which instead couple the oxidative deamination of amino acids to the reductive amination of β-keto acids. The mechanism by which ACC is both deaminated and linearized by ACC deaminase along with the role played by PLP in this process remains an unresolved and intriguing question for which a number of possibilities have been proposed.[7]

It is well documented that species containing a *gem*-difluorocyclopropane are susceptible to nucleophilic attack at both the CH_2 and CF_2 centers of the cyclopropane ring.[8−11] Likewise, alkyl-substituted *gem*-difluorocyclopropanes have also been proposed to undergo 1,4-elimination reactions whereby fluoride is eliminated concomitant with ring opening.[12−14] Hence, the *gem*-difluoro analog of ACC, 1-amino-2,2-difluorocyclopropane-1-carboxylic acid (DFACC, **3**), has been recognized for its potential to provide additional insight into the mechanism of ACC deaminase on account of the greatly enhanced reactivity of the *gem*-difluorocyclopropane moiety.[14−16]

Little is known, however, about the impact of introducing a basic amino functionality at the α-carbon of *gem*-difluorocyclopropyl amino acids. Such a modification may promote ring opening in aqueous systems at near-neutral pH. This would have consequences for the use of DFACC in both mechanistic studies of ACC deaminase and as a potential ligand[15] for biological receptors. Herein we report the synthesis of racemic DFACC and show that it undergoes a pH-dependent ring-opening reaction that may involve a carbanionic intermediate. Interestingly, despite its instability, DFACC is still effective at reversibly inhibiting ACC deaminase with a K_i in the submicromolar range.

The chemical synthesis of *rac*-DFACC began with silylation of 2-(4′-methoxyphenyl)-2-propen-1-ol (**4**) and subsequent

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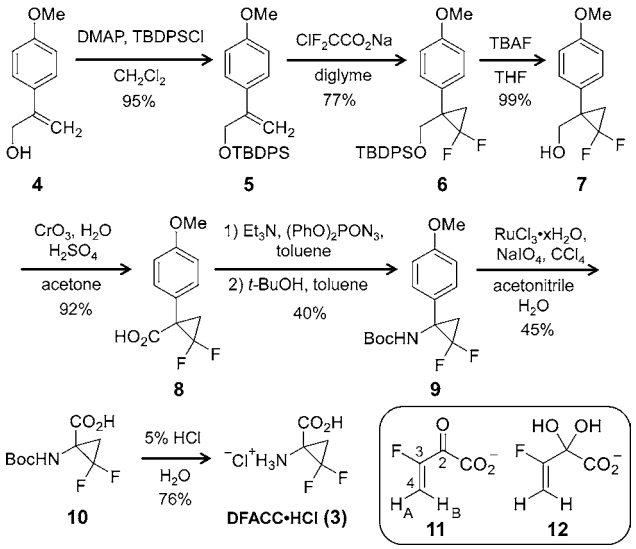
cyclization using difluorocarbene to generate the difluoro-substituted cyclopropane **6** (see Scheme 2). The silyl protecting

Scheme 2. Chemical Synthesis of *rac*-DFACC (3)



group of **6** was removed, and the resulting alcohol (**7**) was oxidized to afford the carboxylic acid **8**. A Curtius rearrangement of **8** was then performed to produce the *Boc*-protected amine **9**. Next, the 4′-methoxyphenyl functionality of **9** was oxidatively cleaved using ruthenium tetroxide generated *in situ* to yield **10**. After deprotection of the *Boc* group of **10**, the crude product was purified using DOWEX 50WX8 cation exchange resin to afford DFACC (**3**) as the HCl salt.

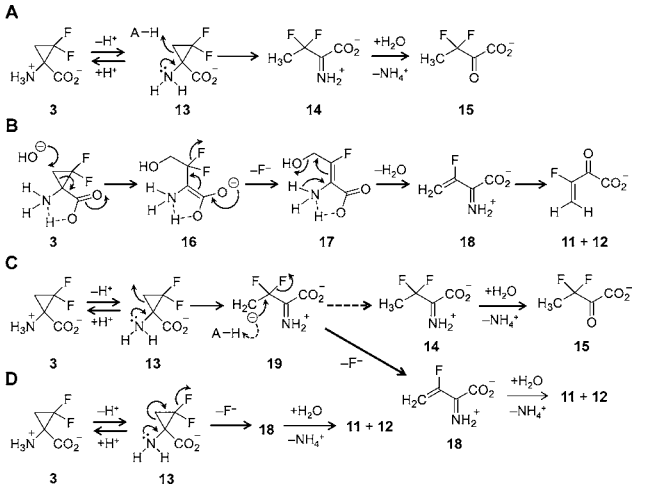
Despite its stability as a HCl salt, when DFACC is incubated in D_2O at neutral pD, it undergoes decomposition with a half-life of ∼4 min (see below). This results in the production of two new species along with release of a free fluoride ion. ^{1}H NMR of the major (ca. 75%) product demonstrated an *ABX* spin system (*X* = F) with the *AB* protons appearing in the 5.65−5.85 ppm region and a J_{AB} coupling constant of 4.8 Hz (see Figure S3.3). The ^{19}F NMR likewise exhibited a doublet of doublets splitting pattern with J_{AF} = 15.0 Hz and J_{BF} = 46.0 Hz (see Figure S3.3). The magnitudes of J_{AF} and J_{BF} are most consistent with three-bond couplings between the fluorine and both the *A* and *B* protons rather than two-bond couplings, which can exceed 80 Hz.[17−19] The ^{13}C NMR of the major product also revealed the presence of two carbonyl carbons in the 165−195 ppm region and thus implicated an α-keto acid (see Supporting Information (SI)). Moreover, electrospray ionization mass spectrometry (ESI-MS) of the product mixture exhibited a signal at 117.00 *m*/*z* (negative ion mode) consistent with a composition of $C_4H_2FO_3^-$ (calculated mass: 116.9993 Da). The major decomposition product was thus assigned as 3-fluoro-2-oxobut-3-enoic acid (**11**; see Scheme 2).

The NMR spectra of the minor decomposition product (ca. 25%) showed features very similar to those of **11**. Again an *ABX* system was observed (*X* = F); however, the two *AB* quartets are found shifted upfield to the 4.70−4.95 ppm region. Likewise, the C2 ^{13}C resonance, which in the case of **11** is observed as a doublet at 189.4 ppm and split by the fluorine at C3, is replaced by a doublet ^{13}C resonance at 91.4 ppm (see SI). This implies that the C2 carbonyl seen in **11** is absent and led to the assignment of the second product as the corresponding hydrate (**12**). The assignments for the two decomposition products were also consistent with additional measurements made using various two-dimensional NMR techniques (^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC), which are provided in the SI.

In addition to **11** and its hydrate (**12**), a new compound which makes up roughly 10% of the total product distribution (see SI) was also found when the reaction was run in H_2O. This new product was identified as 3,3-difluoro-2-oxobutyric acid (**15**) by spectroscopic analysis (see Figure S3.5). Notably, **15** was not produced at a detectable level in the reactions run in D_2O, and there was no evidence for deuterium incorporation at C4 of either **11** or its hydrate in D_2O.

Based on these observations, four mechanisms describing the decomposition of DFACC in aqueous solution were considered (see Scheme 3). The first of these (**A**), which can account for

Scheme 3. Hypotheses for the Decomposition of DFACC (3)



the formation of **15** in buffered H_2O, involves ring opening of the conjugate base of DFACC (**13**) whereby a solvent-derived proton is transferred to the C3-carbon (**13** → **14**). The resulting intermediate would then undergo hydrolysis to produce the stable species **15**. Mechanism **B** in Scheme 3 involves formation of a halohydrin intermediate (**16**). This mechanism begins with nucleophilic attack by hydroxide at C3 and is reminiscent of the reaction of 1-acetyl-2,2-difluoro-3-phenylcyclopropane with phenylthiolate[8,9] and the addition of bromide to (2,2-difluorocyclopropyl)-phenylketone in *N*-pentylpyridinium bromide.[10] While the α-amino group of DFACC is not directly involved in the ring-opening step of mechanism **B** (i.e., **3** → **16**), it is important for the conversion of **17** to **18**/**11**.

Alternatively, the amino functionality could play a direct role in the DFACC ring opening as shown in mechanisms **C** and **D** of Scheme 3. Both of these mechanisms, like **A**, also involve an initial rapid acid−base equilibrium; however, in the case of **C**, the proposed ring opening results in a transient carbanion intermediate (**19**) that can undergo either protonation (**19**→ **14**) or elimination (**19** → **18**). In contrast, mechanism **D** involves a one-step (i.e., (intra-D_N)D_N) elimination to produce **18** directly from **13**. Neither mechanism **B** nor **D**, however, can explain the coproduction of **15** in H_2O−solvent systems and would thus require the participation of a second, competing process such as **13** → **14** in mechanism **A**.

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To examine these hypotheses, the role of the α-amino group in the decomposition of DFACC was investigated more closely. *N*-Acetyl *rac*-DFACC was prepared from **9** (see SI) and found to be stable at all pH values considered. This result demonstrates the importance of a basic α-amino group for the observed decomposition of DFACC. The pH-dependence of the DFACC decomposition rate was then determined by following the disappearance of DFACC at different pH's using ^{19}F NMR. Triplicate reactions were run in 1.0 M succinate or phosphate (H_2O) buffers at pH values ranging from 4.5 to 8.0 and a constant ionic strength of 3 M. DFACC was observed to decay via a first-order process with a pH-dependent rate constant (see Section S4 in the SI). This plot demonstrates that the decomposition of DFACC is slowed at low pH and reaches a plateau under alkaline conditions indicating base catalysis.

In order to obtain a measure of the apparent pK_a and maximum first-order rate constant, k, for decomposition of DFACC, the data in Figure S4.3 were fit using eq 1,

$$\log_{10} k_{app} = \log_{10}[10^{-pK_a}/(10^{-pK_a} + 10^{-pH})] + \log_{10} k \quad (1)$$

This provided values of 5.88 ± 0.06 for the pK_a and 0.18 ± 0.01 min^{-1} for k. The observed pK_a is too low to correspond to the second ionization constant of phosphoric acid, which is 6.8 at an ionic strength of 3 M.[20] However, this pK_a is consistent with those for the α-amino group of β,β-difluoroalanine and β,β-difluorophenylalanine, which have pK_a values roughly 2 units lower than their nonfluorinated counterparts.[21] Therefore, given the pK_a of 8.15 for ACC,[22] the corresponding pK_a for DFACC is expected be ca. 6.0. This result suggests that decomposition of DFACC involves specific-base catalysis where the conjugate base of DFACC (i.e., **13**) is the reactive species that formally undergoes ring opening. This conclusion argues against formation of a halohydrin intermediate as shown in mechanism **B**. The above result along with the observation that decomposition of **3**/**13** is unaffected by the addition of a weak acid such as phenol (see SI) also casts doubt on the signifcance of pathway **A**, which involves general-acid catalysis.

Both mechanisms **C** and **D** in Scheme 3, however, are consistent with these results. The (intra-D_N)D_N ring opening of mechanism **D** is analogous to an E1cB version of the 1,4-elimination reactions that have been proposed for the dehydrohalogenation of substituted *gem*-difluorocyclopropanes[12−14] and is consistent with the propensity of cyclopropanes to behave more as conjugated π-systems rather than sp^3-hybridized carbocycles.[23] However, mechanism **D** would require a competing process such as **A** in order to explain **15** and the product distributions in H_2O versus D_2O. In contrast, mechanism **C** can account for the formation of all observed products. More importantly, it possesses a product-determining step (i.e., **19** → **18**/**14**) that would be susceptible to a normal solvent deuterium kinetic isotope effect and thus explain the formation of **15** in H_2O as opposed to D_2O buffers.

While mechanism **C** involves the intermediacy of a β-fluorocarbanion (i.e., $RCF_2CH_2^-$), this species could be stabilized through negative hyperconjugation.[24] Though a competing α-fluorinated carbanion (i.e., $RCH_2CF_2^-$) could likewise be stabilized inductively, its stability may be offset by orbital repulsion between the lone pairs on the α-carbanion and the adjacent fluorines,[25−27] resulting in preferential formation of the β-fluorocarbanion **19**. Taken together, the available evidence seems to be most consistent with mechanism **C**.

In order to evaluate the potential of DFACC as a mechanistic probe of ACC deaminase, we first checked to see whether it would interact with the enzyme. The activity of ACC deaminase in the presence of DFACC (**3**) was thus assayed by following the deamination of ACC to α-ketobutyrate (**2**) at pH 7.0 using ^{1}H NMR spectroscopy as shown in Figure 1. Each

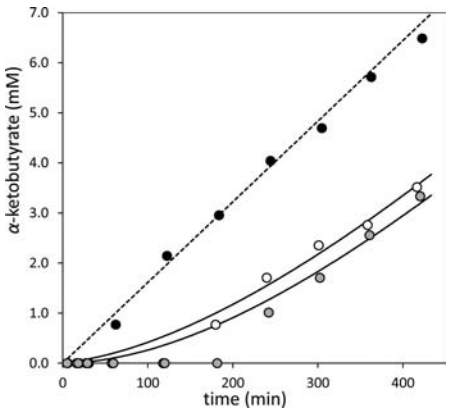

Figure 1. Inhibition of ACC deaminase with *rac*-DFACC (**3**). Reactions were run at rt with 0.5 μM enzyme in 500 μL of 100 mM NaPi buffer (pH 7.0) with 20 mM ACC and 0 (black), 5 (white), or 50 μM (gray) *rac*-DFACC. Reactions were followed by ^{1}H NMR (see SI). The uninhibited time course was fit to a straight line to obtain the rate v_0 (broken line), while the inhibition time courses were fit simultaneously using eqs 2 and 3. Fits exclude points where product is undetectable except at t = 0 min.

time trace exhibits a lag period during which the concentration of **2** increases to detectable levels. In the absence of *rac*-DFACC, this period is less than 1 h. In the presence of 5 and 50 μM DFACC, however, this period is extended to at least 2 and 3 h, respectively. Following the lag, each reaction reaches a roughly constant rate (ca. 16 μM/min with no DFACC) for the remainder of the observation period. This is consistent with a <10% drop in enzyme saturation assuming a Michaelis constant of 4 mM[7g] and indicated an enzyme specific activity of ∼1 μmol·min^{-1}mg^{-1}. Furthermore, when fresh ACC was added to each reaction after 48 h, by which time all of the original ACC had been consumed, equivalent rates of turnover were observed in each of the three reactions. These observations revealed that DFACC is a reversible instead of an irreversible inhibitor for ACC deaminase. Furthermore, no evidence of inhibition was observed when ACC deaminase was incubated in the presence of DFACC following its decomposition (see Figure S7.3 in the SI). This indicates that the decomposition product **11** does not react with the enzyme despite being a potential Michael acceptor and that DFACC itself is responsible for the inhibition.

The rapid decomposition of DFACC in comparison to the lag times suggested a simplified kinetic model from which estimates of the DFACC dissociation constant K_i and dissociation rate constant k_d could be made using the progress curves in Figure 1. This model assumes that a binding equilibrium between DFACC and enzyme is rapidly established such that the initial fraction f_0 of enzyme being inhibited is given by

$$f_0 = x_0K_M/[K_i(K_M + s_0) + x_0K_M] \quad (2)$$

where x_0 and s_0 are the initial concentrations of DFACC and ACC, respectively, and K_M is the Michaelis constant (4 mM). If

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dissociation of the enzyme−inhibitor complex is slow versus DFACC decomposition, then the observed time course of product formation will be given by

$$p(t) = v_0 t - v_0 f_0 [1 - \exp(-k_d t)]/k_d \quad (3)$$

where v_0 is ∼16 μM/min (see SI for derivation). When eqs 3/2 were fit simultaneously to both inhibition time courses in Figure 1, values of K_i = 120 ± 40 nM and k_d = 0.20 ± 0.01 h^{-1} were obtained. It should be emphasized that the true value of K_i may be significantly smaller if there is no rapid equilibration between the enzyme and inhibitor. The kinetic parameter estimates are nevertheless consistent with the conclusion that *rac*-DFACC is a reversible, slow-dissociating inhibitor of ACC deaminase that binds with submicromolar affinity.

■ ASSOCIATED CONTENT

Supporting Information

Details regarding the synthetic procedures, enzyme assays, kinetic analyses, and spectroscopic characterization of all compounds discussed. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01570.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: h.w.liu@mail.utexas.edu.

Present Address

[‖]Oncology iMed, AstraZeneca, Waltham, MA.

Author Contributions

[⊥]C.-H.L. and S.-A.W. contributed equally.

Notes

The authors declare no competing financial interest.

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