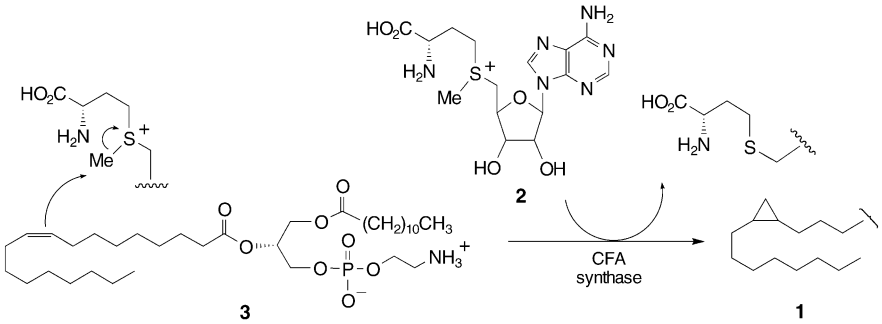
Cyclopropane Fatty Acid Synthase from *Escherichia coli*: Enzyme Purification and Inhibition by Vinylfluorine and Epoxide-Containing Substrate Analogues

Erich J. Molitor,[b,c] Beth M. Paschal,[a, b] and Hung-wen Liu*[a, b]

Dedicated to Professor Dulio Arigoni on the occasion of his 75th birthday.

Cyclopropane fatty acids (CFAs) found in the lipids of many eubacteria are known to play diverse roles in a variety of cellular events. A precursor of CFAs is a phospholipid, such as 3-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE), which contains an unsaturated fatty acyl chain. Cyclopropanation at the $\Delta^{9,10}$-oleoyl acyl chain is catalyzed by CFA synthase by using *S*-adenosyl-L-methionine as the methylene donor. Several mechanisms for the ring-formation step have been postulated, but there is little experimental evidence to distinguish among these proposals. To study this intriguing reaction, we have developed a convenient purification method that allows the isolation of significant quantities of CFA synthase of high purity and good activity. Two phospholipid derivatives, PFOPE, which contains a 9-/10-vinylfluorooleoyl chain, and PEOPE, a 9,10-epoxyoleoyl-containing compound, were designed to probe for contending carbocationic or enzyme nucleophile/anionic mechanisms. It was found that PFOPE is not a substrate but an inhibitor for CFA synthase. This observation is consistent with a carbocation mechanism in which the fluorine substitution at the vinyl position deactivates the olefin and also renders a carbocation intermediate/transition state unstable. The fact that PEOPE functions solely as a reversible inhibitor argues against the direct involvement of a nucleophile in CFA formation and lends credence to a carbocation mechanism. The convenient enzyme preparation and the inhibition studies described herein will certainly aid in further elucidation of the reaction catalyzed by CFA synthase.

Cyclopropane fatty acids (CFAs, **1**) are known to occur in the lipids of many eubacteria,[1] including *Mycobacterium tuberculosis*, which is the causative agent of tuberculosis.[2] These unusual fatty acids have been postulated to play diverse roles in a variety of cellular events including protection of bacteria from environmental stress, alteration of membrane structural integrity, resistance to macrophage-mediated oxidative damage, and overall virulence.[3] The biosyntheses of CFAs are catalyzed by CFA synthase with *S*-adenosyl-L-methionine (AdoMet, **2**) as the methylene donor.[3a, 4] The substrate is a phospholipid containing an unsaturated fatty acyl (UFA) chain (**3**) whose double bond is positioned 9 – 11 carbon units from the ester linkage of the phospholipid backbone (Scheme 1).[4] Hence the catalysis must occur at the interface of the membrane and the surrounding aqueous environment. Our interest in this enzyme is motivated not only by the intriguing chemistry and enzymology involved, but also by the enzyme's potential as a target for antibacterial strategies as there is no apparent human homologue.



Scheme 1.

The gene (*cfa*) encoding *E. coli* CFA synthase has been cloned,[3a] heterologously expressed,[4d] and the corresponding protein has been purified by using a phospholipid-floatation method.[4d] Poor yields resulting from the purification process, low purity caused by phospholipid contamination, and weak activity after removal of the phospholipids,[5] pressed us to develop an improved methodology to obtain CFA synthase for our in vitro studies. Accordingly, the *cfa* gene was PCR amplified from *E. coli* genomic DNA, cloned into a pET28b(+) vector, and expressed in *E. coli* BL21(DE3) cells. The *N*-His_6-tagged CFA synthase was purified by Ni-NTA chromatography in the presence of sorbitan monolaurate to yield 97 mg protein per liter of culture with an activity of 188 U per mg of protein (1 U = 1 pmol of product per min at 37 °C).[6] The formation of enzyme aggregates partially accounts for poor activity. After numerous futile attempts to optimize a combination of detergents, buffers, and phospholipids to boost enzyme activity, it was discovered that protein aggregation could be minimized by the addition of 80 μM bovine serum albumin (BSA) in the purification buffer.[7] The CFA synthase purified under these conditions exhibits an activity of 19800 U mg^{-1}, which corresponds to nearly two orders of magnitude improvement over previous results. Thus,

[a] *Prof. H.-w. Liu, B. M. Paschal*
Division of Medicinal Chemistry, College of Pharmacy
Department of Chemistry and Biochemistry
University of Texas, Austin, Texas 78712 (USA)
Fax: (+1) 512-471-2746
E-mail: h.w.liu@mail.utexas.edu

[b] *Prof. H.-w. Liu, Dr. E. J. Molitor, B. M. Paschal*
Department of Chemistry, University of Minnesota
Minneapolis, Minnesota 55455 (USA)

[c] *Dr. E. J. Molitor*
Current address:
Dowpharma, The Dow Chemical Company
Midland, MI, 48667 (USA)

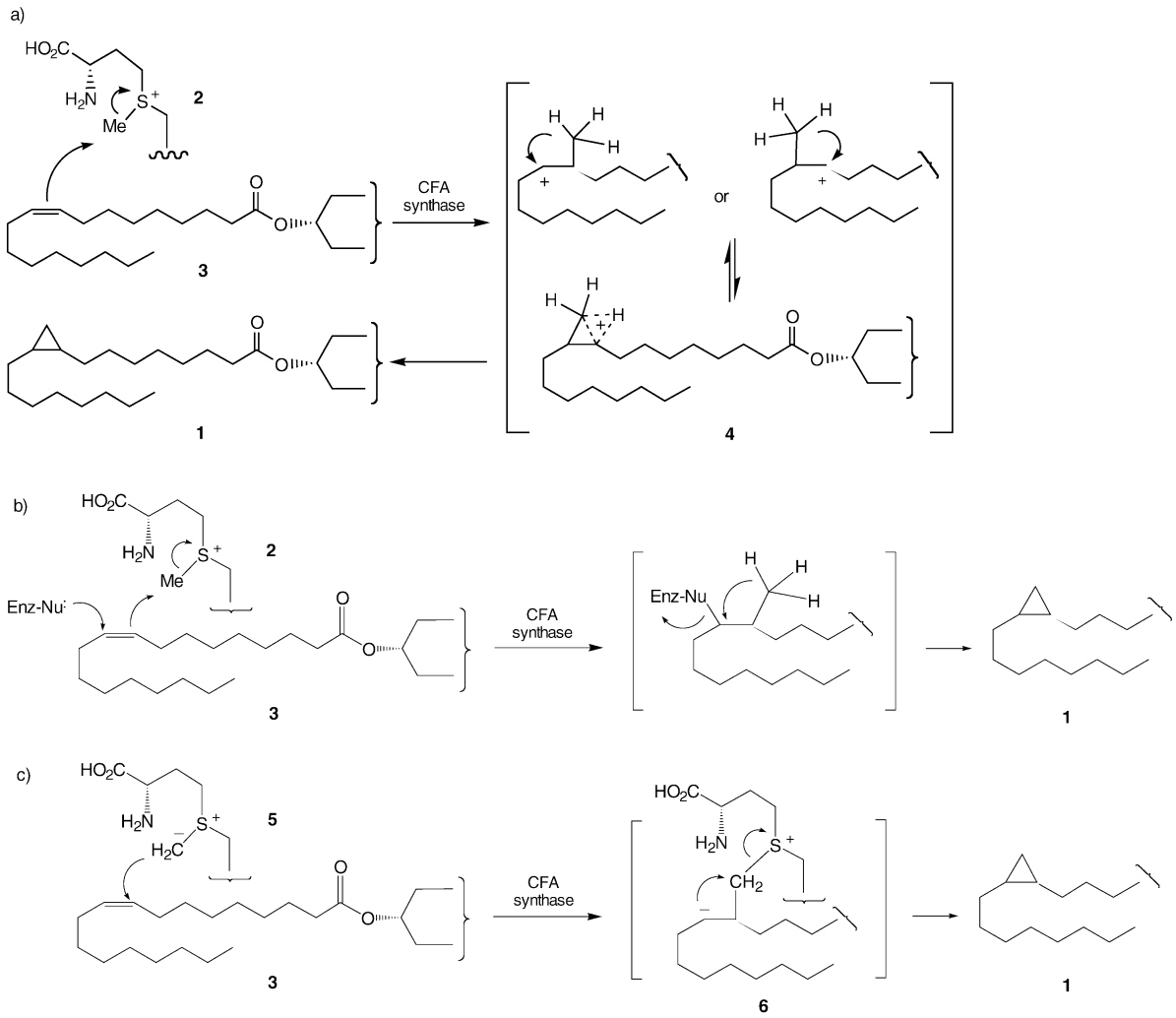
  DOI: 10.1002/cbic.200300767

for the first time, significant quantities of phospholipid-free CFA synthase of high purity and good activity are readily accessible.

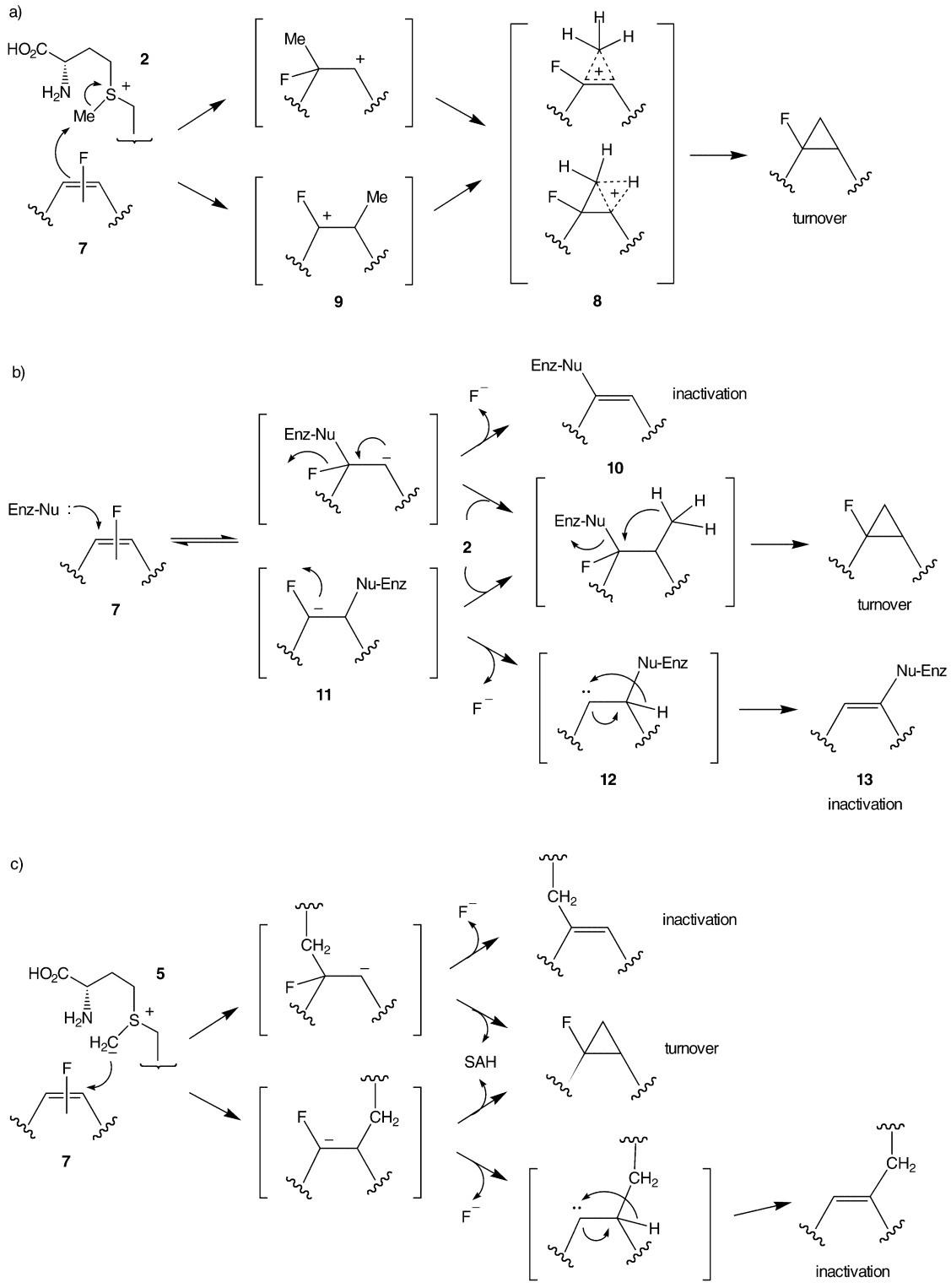
At this point, the stage was set for exploring the mechanism of CFA synthase. Several mechanisms had been proposed. Most often cited is one that involves the formation of a corner-centered or a nonclassical delocalized carbocation intermediate (**4**) in a direct nucleophilic attack of AdoMet by the olefinic moiety of the substrate (Scheme 2a).[8] However, how this intermediate is formed and subsequently processed is not obvious because neither the olefin nor the proton being removed in the ring-closure step is chemically activated. Two alternatives have also been proposed. As shown in Scheme 2b, the reaction could be initiated by the attack of an active site nucleophile, which would subsequently be expelled in the ring-closure step.[9] A route involving transfer of the methylene group from a sulfur ylide (**5**), or its equivalent, to **3** has also been postulated (Scheme 2c).[10] In these two cases, the transition state has a more carbanionic character (such as in **6**).[11] To obtain evidence for ring formation involving either a carbocation or a carbanion intermediate/transition state, two derivatives of the natural substrate, 3-palmitoyl-2-oleoyl-phosphatidyl ethanolamine (POPE, **3**), were synthesized as mechanistic probes.

Compound **7**, 3-palmitoyl-2-(9-/10-fluorooleoyl)phosphatidyl ethanolamine (PFOPE), containing a vinylfluorinated olefin, is expected to markedly decrease the stability of an electron-deficient transition state (**4**/**8**);[12] this would result in either a reduced rate or a complete lack of turnover of the fluorinated species (Scheme 3, mechanism a).[13] If mechanism b is operative, incubation with **7** may lead to enzyme inactivation by either a β-elimination of the fluoride ion to give **10** or an α-elimination of the fluoride ion followed by a 1,2-H shift to give **13**,[14] depending on the regiospecificity of the initial nucleophilic attack. Similar outcome can also be envisioned for mechanism c. In contrast, epoxide-containing compound **14** (3-palmitoyl-2-(9,10-epoxyoleoyl)phosphatidylethanolamine—PEOPE), which was prepared by oxidation of the double bond of POPE (**3**) with *m*-chloroperbenzoic acid (Scheme 4), is designed to probe a mechanism with significant carbanion character. The electrophilic nature of an epoxide makes it a suitable reagent to trap an enzyme-active-site nucleophile. Compound **7** was prepared as a mixture of 9- and 10-fluorooleoyl derivatives as depicted in Scheme 5.

Upon incubation of **7** (a mixture of **7a**/**7b**) and **14** with CFA synthase, it was found that neither compound is a substrate for

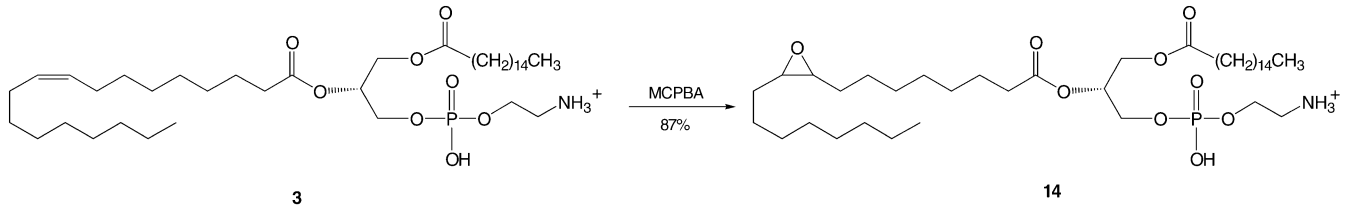


Scheme 2.

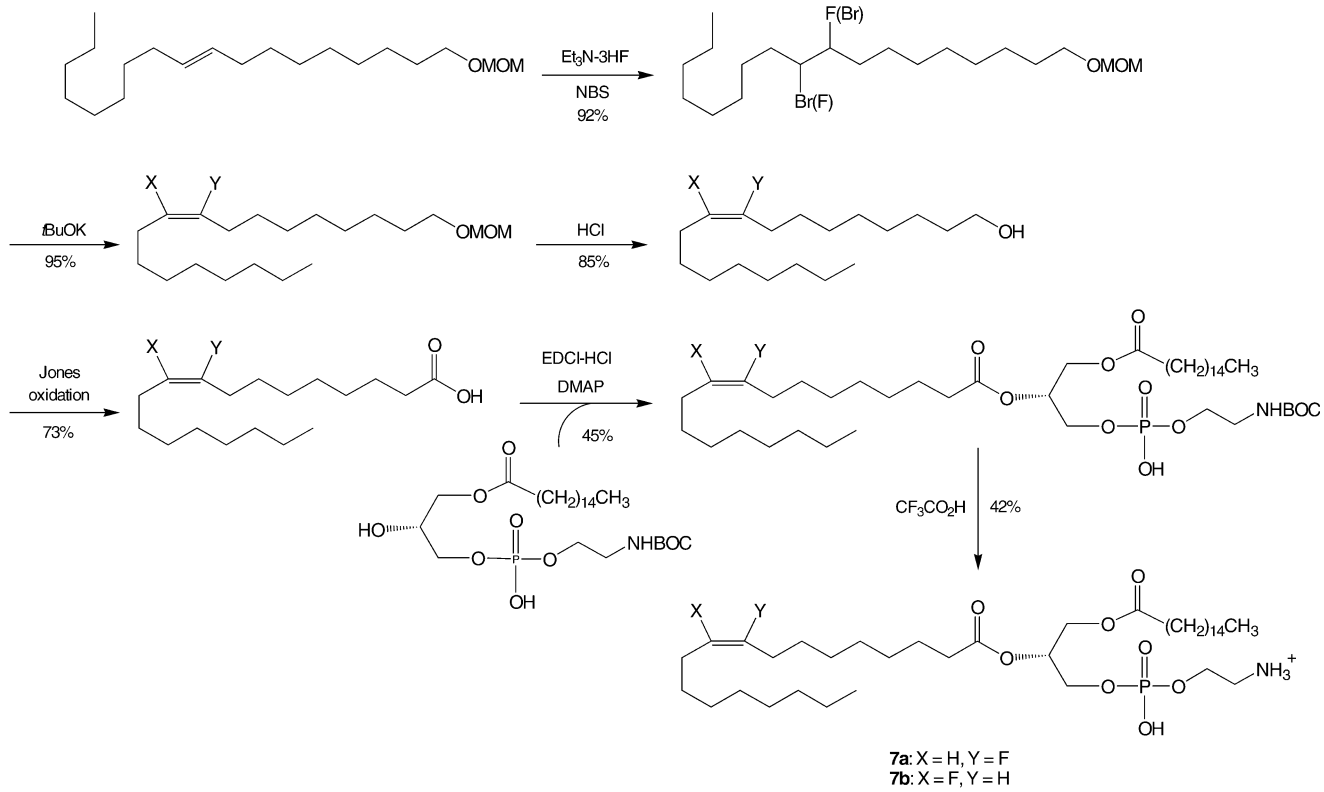


Scheme 3.

the enzyme. Instead, both are reversible inhibitors against the natural substrate (**3**).[15] Coincubation with **7** led to a modest (30 – 70%) reduction of the enzyme activity, while greater than 97% reduction was observed with **14** under the same conditions. It should be noted that oleic acids fluorinated at C-7 and C-12 are both substrates for the synthase, albeit with reduced turnover efficiency (32% and 5%, respectively) when incubated with cell-free extracts of *Lactobacillus plantarum*.[16] The observation that compound **7** cannot be processed by CFA synthase clearly indicates that the fluorine substitution at the vinyl position can more effectively deactivate the olefin and destabilize the carbocation intermediate/transition state. This finding is consistent with a carbocation mechanism. The failure of both **7** and **14** to form a covalent adduct with the enzyme argues



Scheme 4.



Scheme 5.

against the direct involvement of an enzyme nucleophile or a sulfur ylide in CFA formation,[17] and lends further credence to a carbocation mechanism.[18] The fact that compound **14** is an excellent inhibitor suggests that the epoxy oxygen in **14** may interact favorably with one or more active-site residues allowing **14** to compete more efficiently than the substrate for the enzyme active site.

In summary, our study has led to an efficient enzyme-purification protocol, identified the first phospholipid inhibitor for CFA synthase, and provided further insight into the mechanism of this intriguing cyclopropanation reaction. The convenient enzyme preparation and the inhibition studies described here will, no doubt, aid in further elucidation of the catalysis by CFA synthase.

Acknowledgements

This work is supported in part by the National Institutes of Health grant GM40541. E.J.M. and B.M.P. were trainees of a NIGMS Biotechnology Training Grant (2T32 GM08347) and a NIGMS Chemistry Biology Interface Training Grant (5T32 GM08700), respectively.

Keywords: biosynthesis · cyclopropanation · cyclopropane fatty acids · enzyme catalysis · inhibitors

[1] a) H. Goldfine, *Curr. Top. Membr. Transp.* **1982**, *17*, 1 – 43; b) B. S. Moore, H. G. Floss in *Comprehesive Natural Product Chemistry, Vol. 1* (Eds.: D. Barton, K. Nakanishi, O. Meth-Cohn), Pergamon, New York, **1999**, pp. 61 – 82; c) J. Salaün in *Topics in Current Chemistry Vol. 207* (Ed.: A. de Meijere) Springer, Berlin, **2000**, pp. 1 – 67.

[2] a) P. C. Hopwell in *Tuberculosis: Pathogenesis, Protection and Control* (Ed.: B. R. Bloom), American Society for Microbiology, Washington, DC, **1994**, pp. 25 – 46; b) K. M. George, Y. Yuan, D. R. Sherman, C. E. Barry III, *J. Biol. Chem.* **1995**, *270*, 27 292 – 27 298.

[3] a) D. W. Grogan, J. E. Cronan, Jr., *J. Bacteriol.* **1984**, *158*, 286 – 295; b) E. J. Dufourc, I. C. P. Smith, H. C. Jarrell, *Biochemistry* **1984**, *23*, 2300 – 2323; c) D. W. Grogan, J. E. Cronan, Jr., *J. Bacteriol.* **1986**, *166*, 872 – 877; d) M. Monteoliva-Sanchez, A. Ramos-Cormenzana, N. J. Russell, *J. Gen. Microbiol.* **1993**, *139*, 1877 – 1884; e) A.-Y. Wang, J. E. Cronan, *Mol. Microbiol.*

1994, *11*, 1009 – 1017; f) M. S. Glickman, J. S. Cox, W. R. Jacobs, Jr., *Mol. Cell* **2000**, *5*, 717 – 727.

[4] a) J. E. Cronan, Jr., R. Reed, F. R. Taylor, M. B. Jackson, *J. Bacte*riol. **1979**, *138*, 118 – 121; b) F. R. Taylor, J. E. Cronan, Jr., *Biochemistry* **1979**, *18*, 3292 – 3300; c) F. R. Taylor, D. W. Grogan, J. E. Cronan, Jr., *Methods Enzymol*. **1981**, *71*, 133 – 139; d) A.-Y. Wang, D. W. Grogan, J. E. Cronan, Jr., *Biochemistry* **1992**, *31*, 11 020 – 11 028; e) D. W. Grogan, J. E. Cronan, Jr., *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 429 – 441.

[5] For example, the *E. coli* enzyme is extremely labile in the absence of substrate and/or product-like lipids,[4d] having a half-life of 16 h at 4 °C, and only a few minutes at 37 °C.[3a, 4c]

[6] The in vitro assay was conducted by adding CFA synthase, lipid vesicles (consisting of 33% **3** and 66% 1,2-dipalmitoyl-phosphatidyl glycerol), and adenosyl homocysteine hydrolase (SAHase), to 72 μg mL^{-1}, 1.5 mg mL^{-1}, and 0.96 U mL^{-1} in potassium phosphate (50 mM), pH 7.5. Reactions were initiated by adding [^{3}H]AdoMet (0.5 mM, 15 μCi μmol^{-1}) and carried out at 37 °C. Aliquots (60 μL) were removed at 1 min intervals and quenched on filter papers, which were successively washed with 0.62 N and 0.31 N trichloroacetic acid and water, followed by scintillation counting.

[7] Purification was carried out with Ni-NTA resin (Qiagen) incorporating 5 mg mL^{-1} BSA in all buffers. Yields were 110 mg L^{-1}. The isolated CFA synthase exhibits no absorption above 300 nm and contains no redox-active metal ions.

[8] a) E. Lederer, *Q. Rev. Chem. Soc*. **1969**, *23*, 453 – 481; b) J. H. Law, *Acc. Chem. Res*. **1971**, *4*, 199 – 203; c) P. H. Buist, D. B. MacLean, *Can. J. Chem*. **1981**, *59*, 828 – 838; d) Y. Yuan, C. E. Barry III, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 12 828 – 12 833.

[9] The fact that *E. coli* CFA synthase is susceptible to sulfhydryl reagents[4d] implies that the nucleophile is an active-site cysteine residue.

[10] T. Cohen, G. Herman, T. M. Chapman, D. Kuhn, *J. Am. Chem. Soc*. **1974**, *96*, 5627 – 5628.

[11] While the isolated double bond in **3** is clearly not activated, interaction with a proximal positively charged amino acid residue may provide the necessary activation in the transition state and the stabilization of the resulting carbanion intermediate **6**. Thus, an open approach is taken, as nothing is completely implausible prior to being experimentally disproved.

[12] a) B. J. Baker, R. G. Kerr in *Topics in Current Chemistry, Vol. 167*, Springer, Berlin, **1993**, pp. 1 – 32; b) J. T. Welch, S. Eswarakrishnan, *Fluorine in Bioorganic Chemistry*, Wiley, New York, **1991**.

[13] While a fluorine substituent at the α-position to a cation center (such as in **9**) can stabilize it by back donation, this effect is expected to be overridden by the overall deactivation of the double bond.

[14] Formation of **11** is not favored due to the repulsion between the electron pair of the anionic center and those of the fluorine. Such a repulsive interaction can be alleviated by α-elimination of the fluoride ion to give a carbene intermediate **12**. The subsequent 1,2-H shift is well documented in carbene chemistry and is expected to be extremely facile (J. D. Evenseck, K. N. Houk, *J. Phys. Chem*. **1990**, *94*, 5518).

[15] Competitive inhibition assays were carried out by incubating 2.0 mg mL^{-1} lipid vesicles (15% **7** or **14**, 30% **3**, 55% DPPG and 1,2-dipalmitoyl-phosphatidyl ethanolamine (DPPE), the latter two for solubility and head-group ratio uniformity), CFA synthase (0.15 mg mL^{-1}), SAHase (0.5 U mL^{-1}) in buffer and initiated with [^{3}H]AdoMet (0.4 mM, 15 μCi μmol^{-1}). Reactions were carried out for 20 min at 37 °C.

[16] P. H. Buist, R. A. Pon, *J. Org. Chem*. **1990**, *55*, 6240 – 6241. Inductive destabilization of either a corner-centered or nonclassical carbocation transition state is believed to be the cause of reduced turnover 7- and 12-fluoro olefins by CFA synthase.

[17] This result also concurs well with the fact that CFA synthase contains no metal, as sulfur ylide (**5**, or a metallo-carbenoid species) formation, shown in Scheme 2c, is expected to be metal-ion mediated.

[18] A few examples in which the intermediacy of a carbocation species has been proposed for a cyclopropanation reaction are the reaction catalyzed by squalene synthase (B. S. Blagg, M. B. Jarstfer, D. H. Rogers, C. D. Poulter, *J. Am. Chem. Soc*. **2002**, *124*, 8846 – 8853), chrysanthemyl diphosphate synthase (S. B. Rivera, B. D. Swedlund, G. J. King, R. N. Bell, C. E. Hussey, D. M. Shattuck-Eidens, W. M. Wrobel, G. D. Peiser, C. D. Poulter, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 4373 – 4378), and a catalytic antibody (T. Li, K. D. Janda, R. A. Lerner, *Nature* **1996**, *379*, 326 – 327).

