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Synthesis and evaluation of analogues of *S*-adenosyl-L-methionine, as inhibitors of the *E. coli* cyclopropane fatty acid synthase

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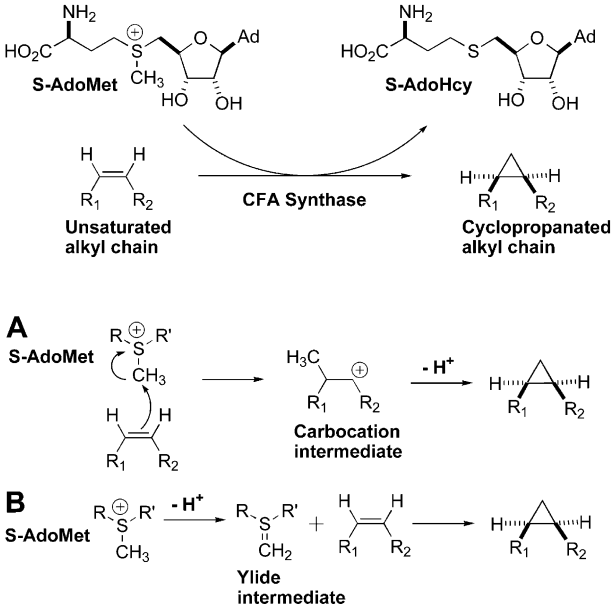
Abstract—Analogues of *S*-adenosyl-L-methionine were synthesized and evaluated as inhibitors of the purified *E. coli* cyclopropane fatty acid synthase, a model for *M. tuberculosis* cyclopropane synthases that are potential targets for antituberculous drugs. Our results show that the presence of the adenosine moiety, in the inhibitor, is required for strong binding, but that the sulfonium charge is less important. The best inhibitors found were *S*-adenosyl-L-homocysteine and its sulfoxides.

The cyclopropyl group is present in numbers of natural products and its unusual properties have always attracted chemists and biochemists.[1] Although Nature has devised numerous ways to biosynthesize the cyclopropane ring,[2] the most fascinating one, remains the direct methylenation of double bonds catalyzed by cyclopropane synthases. *E. coli* cyclopropane fatty acid synthase (CFAS) and its closely related homologues from *M. tuberculosis*, are the best known representatives of this class of enzymes.[3] The reaction proceeds by transfer of a methylene group from the activated methyl group of *S*-adenosyl-L-methionine (*S*-AdoMet) to the (*Z*)-double bond of an unsaturated fatty acid chain, resulting in the formation of a cyclopropane ring on the alkyl chain (Scheme 1). Early in vivo studies[4] showed that two of the three methyl protons of *S*-AdoMet are retained in the product, just as the vinylic protons of the substrate are. The stereochemistry is also retained, that is, the (*Z*)-double bond gives a *cis*-cyclopropane,[5] although *trans*-cyclopropane are also found in *M. tuberculosis* mycolic acids. Even though a mechanism involving a carbocation intermediate is often cited in the literature,[6,3] other reasonable alternatives, such as the so-called ylide mechanism,[7] have been proposed and deserve consideration (Scheme 1). Beside recent crystallographic data[8] supporting the carbocation mechanism, there is no other experimental argument in favor of one or the other mechanism. Attention has recently focused on this class of enzymes because cyclopropanation of mycolic acids in *M. tuberculosis* has been associated with virulence and persistance of the pathogen.[9] Hence, cyclopropane synthases might be good targets for new antituberculous drugs. Tuberculosis remains a major cause of death in the world and there is a real need for new drugs to combat resistant strains of *M. tuberculosis*, against existing antibiotics.



Scheme 1. The reaction catalyzed by CFAS, and the two possible reaction mechanisms: (A) carbocation mechanism; (B) ylide mechanism.

Keywords: Cyclopropane fatty acid synthase; *S*-adenozyl-L-methionine; Inhibitors; *S*-adenozyl-L-homocysteine sulfoxide; Antituberculous drug.
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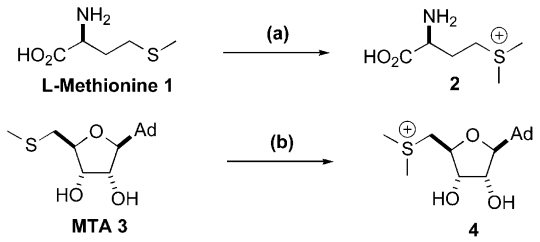


Scheme 2. Synthesis of the sulfonium analogues. Reagents and conditions: (a) ICH_3 (2 equiv), H_2O, 50 °C; (b) ICH_3 (3 equiv), HCOOH, 40 °C.

Table 1. Inhibition constants (K_i) for compounds **1**–**5**

Compd	Inhibition constant K_i μM[a]
1	> 150 10^3 (±7500)
2	140 10^3 (±700)
3	1.5 10^3 (±75)
4	2.5 10^3 (±125)
D,L-**5**	150 (±7)[b]
L-**5**	30 (±7)

[a] Competitive inhibition constants (K_i) were determined from replots of the apparent K_m/V_{max} values versus inhibitor concentration.
[b] Mixture of 2 diastereomers de = 60%.

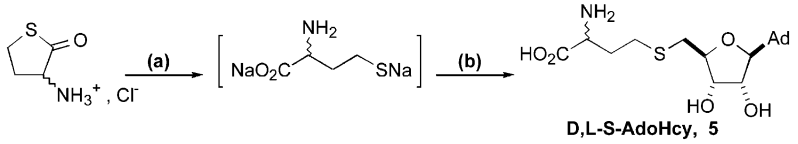
We have been interested in studying CFAS from *E. coli* as a model for *M. tuberculosis* cyclopropane synthases, for which an in vitro assay is still lacking. We have purified and conducted initial mechanistic experiments on this enzyme (to be published elsewhere). We report here the synthesis of diverse *S*-AdoMet analogues and their evaluation as inhibitors, as a starting point for a more comprehensive structure–activity relationship analysis and inhibitor screening.

In a search for a simple in vitro assay for *E. coli* CFAS, as an alternative to the tedious radiochemical assay,[10] we first attempted to replace the expensive and labile substrate, *S*-AdoMet, by a simpler sulfonium. We thus prepared sulfoniums **2**[11] and **4**[12,13] by methylation of the corresponding sulfides (Scheme 2). Because **2** and **4** were not radiolabeled, the enzymatic reaction was monitored by HPLC, by following the transformation of sulfoniums **2** or **4** in their respective sulfides (Nucleosil 100Å C18, 25 mm 4.6 mm, 95:5 (v/v) mixture of aqueous 7 mM acetic acid and methanol, 1.5 mL/min, λ = 230 nm for separation of **2** and methionine, 75:25 (v/v) mixture of aqueous 7 mM acetic acid and methanol, 1.5 mL/min, λ = 254 nm for separation of **4** and 5′-desoxy-5′-methylthioadenosine (MTA), **3**). Unfortunately, neither **2** nor **4** were transformed in sulfide by the enzyme; **2** and **4** are in fact poor competitive inhibitors (see Table 1).

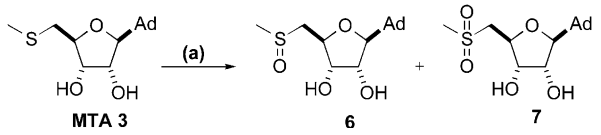
Table 1 summarizes the inhibiting properties of sulfonium and sulfide analogues of *S*-AdoMet.[14] All compounds showed competitive inhibition with respect to *S*-AdoMet. It is clear from Table 1 that the positive charge of the sulfonium is not required for high affinity. On the other hand, the adenosine moiety is necessary for strong binding, probably because the aromatic ring interacts favorably with the active site. Interestingly we found that the physiological product, *S*-adenosyl-L-homocysteine (L-*S*-AdoHcy), with the L-configuration at the amino-acid α-carbon, had a K_i of 30 μM, a value seven times lower than that previously reported,[15] 220 μM. We therefore synthesized the mixture of D,L-*S*-AdoHcy diastereoisomers starting from D,L-homocysteine thiolactone (Scheme 3)[16] to see whether epimerization could explain this discrepancy. D,L-*S*-AdoHcy **5** was obtained as a mixture of diastereomers, 80% D-*S*-AdoHcy and 20% of L-*S*-AdoHcy, as indicated by HPLC (Atlantis dC18 5 μm, 4.6×150 mm, 100% H_2O, 1.3 mL/min, λ = 254 nm). The diastereoisomeric mixture, de = 60%, showed a higher K_i value when compared to L-*S*-AdoHcy, consistent with the fact that the D-isomer is poorly, if at all, recognized by the enzyme.

Another possibility that could explain the observed discrepancy, noted above, could be the presence of a contaminant in our preparation, with a high affinity. Although commercial and synthetic L-*S*-AdoHcy, obtained from L-homocysteine thiolactone,[16] were pure, small amount of sulfoxide could have been formed. This hypothesis was conceivable because sulfides tend to be easily oxidized and the S–O bond could mimic the putative ylide intermediate (Scheme 1), and thus L-*S*-AdoHcy sulfoxides could show a high affinity for the active site. Different sulfoxides and sulfones were thus synthesized to test that hypothesis. However, as discussed below the affinity of L-*S*-AdoHcy sulfoxides cannot explain the difference in K_i for D-*S*-AdoHcy observed in the present study and in the previous report. The origin of the discrepancy remains unclear.

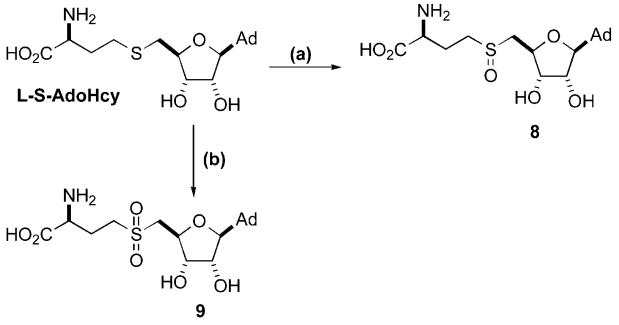
MTA sulfoxides and sulfone, **6**[17] and **7**,[18] were obtained by treatment of **3** with H_2O_2 in aqueous acetic acid,[19] followed by chromatographic separation (Scheme 4). **6** was obtained as a mixture of two unseparable diastereomers (50:50), as shown by ^1H and ^{13}C NMR. Applying the same protocol on L-*S*-AdoHcy[19] (Scheme 5) led to **8**,[20] a 45:55 mixture of diastereomeric sulfoxides, as measured by HPLC. In this case, no trace of sulfone **9** was detected. The two sulfoxides, **8a** and **8b**, were separated by HPLC, on a small scale (Atlantis dC18 5 μm, 4.6×150 mm, 100% H_2O at a flow rate of 1.5 mL/min, λ = 254 nm) to be tested separately. L-*S*-AdoHcy sulfone **9**, was originally obtained by a complex oxidation protocol.[19] Alternatively, we first used the method developed by Balicki,[21] for oxidazing bulky or electron



Scheme 3. Synthesis of D,L-*S*-AdoHcy. Reagents and conditions: (a) NaOH (3 equiv), H_2O; (b) 5′-desoxy-5′-chloroadenosine (0.75 equiv), K_i, reflux.



Scheme 4. Synthesis of sulfoxides and sulfone of MTA. Reagents and conditions: (a) H_2O_2 (2 equiv), CH_3COOH/H_2O 1:1. Separation of **6** and **7**, obtained in a ratio of 3 to 1, was achieved by flash column chromatography (SiO_2, 70% CH_2Cl_2–30% EtOH).



Scheme 5. Synthesis of sulfoxides and sulfone of L-*S*-AdoHcy. Reagents and conditions: (a) H_2O_2 (10 equiv), H_2O; (b) $KMnO_4$ (2.5 equiv), CH_3COOH/H_2O 4/1.

deficient sulfides. The reaction proceeded within 0.5–2 h upon addition of L-*S*-AdoHcy to a suspension of UHP (Urea Hydrogen Peroxide) and trifluoroacetic anhydride in acetonitrile. Unfortunatly, in our case the reaction stopped at the sulfoxide oxidation state. Finally, **9**[22] was obtained by the action of $KMnO_4$ on L-*S*-AdoHcy in acetic acid.[23]

Table 2 summarizes the inhibition data for the sulfoxides and sulfones prepared in this report. All compounds behaved as competitive inhibitors with respect to *S*-AdoMet. A graphical analysis is shown on Figure 1. Oxidation of the sulfides, MTA and *S*-AdoHcy, has various effects on the affinity. While MTA sulfoxides and sulfones are stronger inhibitors than MTA, 5 times and 7 times respectively, indicating a favorable interaction for the S–O dipole, this effect was not observed for the *S*-AdoHcy derivatives. However, the orientation of the S–O bond in *S*-AdoHcy sulfoxides is important since one of the diastereoisomers, **8a**, has a lower K_i than the other stereoisomer **8b**. The affinity of sulfone **9** is comparable to that of sulfoxide **8b**. Altogether, these findings do not support the ylide mechanism (Scheme 1), although they do not disprove it.

Table 2. Inhibition constants (K_i) for compounds **6** to **9**

Compd	Inhibition constant K_i μM[a]
6	500 (±25)[b]
7	220 (±11)
8	50 (±2.5)[b]
8a	30 (±2.5)
8b	150 (±2.5)
9	100 (±5)

[a] Competitive inhibition constants (K_i) were determined from replots of the apparent K_M/V_{max} values versus inhibitor concentration.
[b] Mixture of 2 diastereomers, **6** de = 0%, **8** de = 10%.

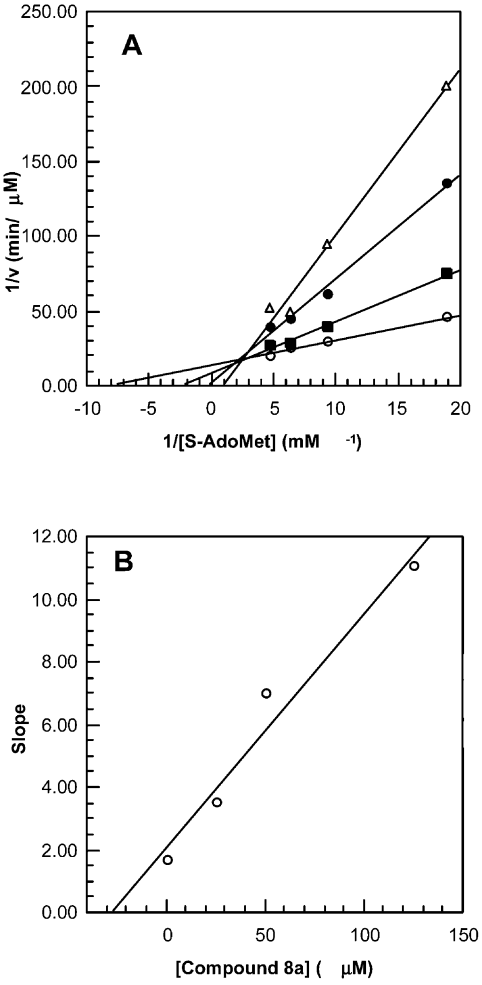


Figure 1. Inhibition of CFA synthase by **8a**. (A) Double reciprocal plot for inhibition by **8a**. Assays were conducted as described in note 14, in the presence of 0 μM (open circle), 25 μM (closed square), 50 μM (closed circle), 125 μM (open triangle) of **8a**; (B) Replot of slope (K_M/V_{max}) versus inhibitor concentration.

In a thorough study, Borchardt et al.[24] synthesized different analogues of *S*-AdoMet as potential inhibitors of methyltransferases (involved in mRNA modification). Interestingly, they found that sinefungine and A9145, also known to inhibit CFAS,[25] were by far the most potent inhibitors, and that the *S*-AdoHcy sulfoxides (unseparated diastereomeric mixture) and sulfones were as good as *S*-AdoHcy or slightly weaker inhibitors. Our results presented here, concerning the inhibition of CFAS, a potential target for antituberculous drugs, are quite similar. One might argue that the *S*-AdoMet binding site of the methyltransferase family, taken as a whole, are similar. This will add another constrain in the development of specific CFAS inhibitors.

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13. Selected data for compound **4**: ^{1}H NMR (D_2O) δ 8.35 (s, 2H, aromatic), 6.15 (d, J = 25.2 Hz, 1H, $C_{1'}H$), 4.95 (m, 1H, $C_{2'}H$), 4.55 (m, 2H, $C_{3'}H$ and $C_{4'}$H), 3.90 (m, 2H, $C_{5'}H$), 2,95 (s, 3H, CH_3), 2.90 (s, 3H, CH_3). ESI [m/z 312 (M^+); {calcd for $C_{12}H_{18}N_5O_3S^+$} 312].
14. The assay mixture (0.1 mL final volume) consisted of the following components: 20 mM potassium phosphate buffer, pH 7.4; 0.1 mg of a phospholipide dispersion (prepared as in reference 10); *S*-[methyl-^{3}H]-adenosyl-L-methionine at a final specific activity of 25 mCi/mmol, variable concentration; inhibitor at variable concentration; and 3 μg of purified CFAS (purified as in reference 10, with modifications). Incubations were carried out for 15 min at 37 °C, and the reaction was stopped by adding 1 mL of Cl_3CCOOH (10% w/v). The entire reaction mixture was filtreted onto a 2.5 cm glass fiber filter disk (Whatman GF/A). The filter disks were washed twice with 1 mL Cl_3COOH (10% w/v) and 3 times with 1 mL of water. After drying, the filter disks were transferred to a scintillation vial, and 3 mL of Optiphase HighSafe (Wallac) scintillation fluid were added and the radioactivity counted (Rackbeta, LKB, Sweeden).
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17. Selected data for compound **6** de = 0%: ^{1}H NMR (D_2O) δ 8.18 (s, 1H, C_8H), 8.05 (s, 1H, C_2H), 6.00 (d, J = 5.2 Hz, 1H, $C_{1'}H$), 4.95 (m, 1H, $C_{2'}H$), 4.47 (m, 2H, $C_{3'}H$ and $C_{4'}H$), 3.45 (m, 2H, $C_{5'}H$), 2.75 (s, 3H, CH_3). ^{13}C NMR (D_2O) δ 154.8 (C6), 152.1 (C2), 148.0 (C4), 139.7 and 139.4 (C8), 118.2 (C5), 87.8 (C1′), 77.9 and 77.3 (C4′), 72.4 (C2′ and C3′), 55.7 and 54.1 (C5′), 37.0 and 36.5 (CH_3). ESI [m/z 314 ($M+H^+$); {calcd for $C_{11}H_{15}N_5O_4S+H^+$} 314].
18. Selected data for compound **7**: ^{1}H NMR (D_2O) δ 8.24 (s, 1H, C_8H), 8.19 (s, 1H, C_2H), 6.02 (d, J = 5.2 Hz, 1H, $C_{1'}H$), 5.15 (m, 1H, $C_{2'}H$), 4.49 (m, 1H, $C_{4'}H$), 4.39 (m, 1H, $C_{3'}H$), 3.75 (m, 2H, $C_{5'}H$), 2.96 (s, 3H, CH_3). ^{13}C NMR (D_2O) δ 155.2 (C6), 152.1 (C2), 148.5 (C4), 140.0 (C8), 112.5 (C5), 88.2 (C1′), 77.8 (C4′), 72.3 and 71.8 (C2′ and C3′), 55.9 (C5′), 41.0 (CH_3).
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20. Selected data for compound **8** de = 10%: ^{1}H NMR (D_2O) δ 8.13 (s, 1H, C_8H), 8.06 (s, 1H, C_2H), 5.95 (s, 1H, $C_{1'}H$), 4.95 (m, 1H, $C_{2'}H$), 4.45 (m, 1H, $C_{4'}H$), 4.39 (m, 1H, $C_{3'}H$), 3.61 (m, 1H, CαH), 3.35 (m, 2H, $C_{5'}H$), 2.95 (m, 2H, CγH), 2.11 (m, 2H, CβH). ^{13}C NMR (D_2O) δ 175.4 (COOH), 155.9 (C6), 153.2 (C2), 149.1 (C4), 140.5 (C8), 119.4 (C5), 88.7 (C1′), 78.8 and 78.3 (C4′), 73.2 (C2′ and C3′), 54.2 and 54.0 (C5′ and Cα), 47.5 (Cγ), 25.1 (Cβ). ESI [m/z 401 ($M+H^+$); {calcd for $C_{14}H_{20}N_6O_6S+H^+$} 401].
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22. Selected data for compound **9**: ^{1}H NMR (D_2O) δ 8.19 (s, 2H, C_8H and C_2H), 6.05 (s, 1H, $C_{1'}H$), 4.95 (m, 1H, $C_{2'}H$), 4.65 (m, 1H, $C_{4'}H$), 4.45 (m, 1H, $C_{3'}H$), 4.02 (m, 1H, CαH), 3.75 (m, 2H, $C_{5'}H$), 3.35 (m, 2H, CγH), 2.27 (m, 2H, CβH). ESI [m/z 417 ($M+H^+$); {calcd for $C_{14}H_{20}N_6O_7S+H^+$} 417].
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