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Stable Analogues of OSB-AMP: Potent Inhibitors of MenE, the *o*-Succinylbenzoate-CoA Synthetase from Bacterial Menaquinone Biosynthesis

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Dedicated to the memory of our colleague and mentor, Prof. David Y. Gin (1967–2011).

MenE, the *o*-succinylbenzoate (OSB)-CoA synthetase from bacterial menaquinone biosynthesis, is a promising new antibacterial target. Sulfonyladenosine analogues of the cognate reaction intermediate, OSB-AMP, have been developed as inhibitors of the MenE enzymes from *Mycobacterium tuberculosis* (mtMenE), *Staphylococcus aureus* (saMenE) and *Escherichia coli* (ecMenE). Both a free carboxylate and a ketone moiety on the OSB side chain are required for potent inhibitory activity. OSB-AMS (**4**) is a competitive inhibitor of mtMenE with respect to ATP ($K_i = 5.4 \pm 0.1$ nM) and a noncompetitive inhibitor with respect to OSB ($K_i = 11.2 \pm 0.9$ nM). These data are consistent with a Bi Uni Uni Bi Ping-Pong kinetic mechanism for these enzymes. In addition, OSB-AMS inhibits saMenE with $K_i^{app} = 22 \pm 8$ nM and ecMenE with $K_i^{OSB} = 128 \pm 5$ nM. Putative active-site residues, Arg222, which may interact with the OSB aromatic carboxylate, and Ser302, which may bind the OSB ketone oxygen, have been identified through computational docking of OSB-AMP with the unliganded crystal structure of saMenE. A pH-dependent interconversion of the free keto acid and lactol forms of the inhibitors is also described, along with implications for inhibitor design.

Introduction

New antibiotics are urgently needed to combat the growing threat of drug-resistant bacterial infections.[1–6] To address this need, antibiotics that act by novel mechanisms must be developed. In this vein, bacterial menaquinone biosynthesis has emerged as a promising new antibacterial target.[7–9] Menaquinone is a lipid-soluble electron carrier that is used in the electron transport chain of cellular respiration (Scheme 1).[10–12] While humans and some bacteria use the alternative redox cofactor ubiquinone, *Mycobacterium tuberculosis*, most Gram-positive bacteria, including *Staphylococcus aureus*, and some Gram-negative bacteria rely solely on menaquinone. Although menaquinone is also used in the mammalian blood clotting cascade, humans lack the de novo biosynthetic pathway,[13] and obtain it from diet or intestinal bacteria. Thus, menaquinone biosynthesis inhibitors should be highly selective for bacteria over the human host.

Menaquinone is biosynthesized from chorismate by a series of at least eight enzymes (Scheme 1).[14–16] The fifth of these is the acyl-CoA synthetase MenE, which carries out a two-step process involving initial activation of *o*-succinylbenzoate (OSB) by adenylation to form a tightly-bound OSB-adenosine monophosphate (AMP) intermediate, followed by attack of CoA to form a thioester product.[17–19] MenE is essential in *M. tuberculosis*,[20] and designed inhibitors of another menaquinone biosynthesis enzyme, MenA, have potent activity against multidrug-resistant *M. tuberculosis* and Gram-positive bacteria.[21] In that vein, however, a human homologue of MenA that converts plant phylloquinone to menaquinone has been identified recently.[22] Menaquinone is also essential in *E. coli*,[23] and mutations in biosynthesis genes are associated with a slow growth phenotype in *S. aureus*.[24–26] In addition to the classical pathway, an alternative (futalosine) biosynthetic pathway has been identified in certain bacteria,[27–29] but the two pathways appear to be mutually exclusive in distribution, and there is no evidence for the presence of this alternative pathway in *M. tuberculosis* or *S. aureus*.[27,29] Thus, MenE is an attractive target for blocking menaquinone biosynthesis as a new antibacterial strategy. Further, since nonreplicating *M. tuberculosis* must respire, inhibitors may also be active against latent tuberculosis infections, which affect an estimated one-third of the global population.[3]

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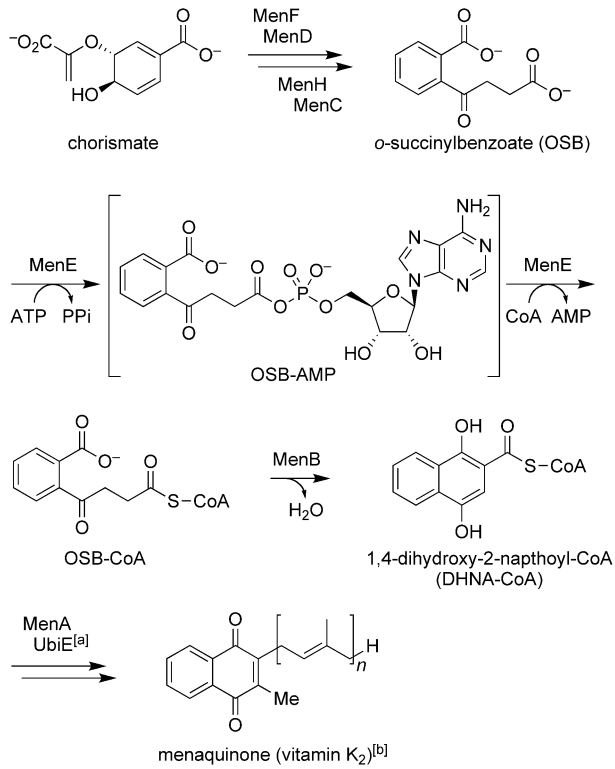


Scheme 1. The *o*-succinylbenzoate (OSB)-CoA synthetase MenE catalyzes two half-reactions involving a tightly-bound OSB-AMP intermediate during menaquinone biosynthesis. [a] A third, as yet unidentified enzyme is thought to be involved in the conversion of 1,4-dihydroxy-2-napthoyl (DHNA)-CoA to menaquinone. [b] $n = 4–13$; $n = 9$ in *M. tuberculosis*; $n = 8$ in *S. aureus*, *E. coli*.

Acyl-CoA synthetases belong to the acyl-CoA synthetase, nonribosomal peptide synthetase adenylation domain, firefly luciferase (ANL) family of adenylate-forming enzymes, which share the same overall fold.[30] This family is, in turn, part of a larger mechanistic superfamily of enzymes that catalyze adenylation of carboxylic acid substrates and subsequent coupling to sulfur, oxygen, or nitrogen nucleophiles. This superfamily includes class I and class II aminoacyl-tRNA synthetases,[31,32] E1-activating enzymes,[33–35] N-type ATP pyrophosphatases,[36–38] and recently discovered amide ligases.[39,40]

A variety of inhibitors of this mechanistic superfamily have been reported previously, most of which are designed to mimic the acyl-AMP intermediate.[41] In particular, acyl sulfonyladenosines, pioneered by Ishida[42] and inspired by sulfamoyladenosine natural products such as nucleocidin and ascamycin,[43–46] have been investigated extensively as aminoacyl-tRNA synthetase inhibitors.[47–50] Such inhibitors have now been applied widely to other enzymes in this mechanistic superfamily, including members of the ANL family,[51–62] E1-activating enzymes,[63–65] asparagine synthetase,[66] and pantothenate synthetase.[67] In addition, electrophilic vinyl sulfonamide inhibitors have been designed to trap the incoming nucleophile in the second half-reaction catalyzed by these enzymes,[63,64,68] leveraging design strategies originally developed to target cysteine proteases.[69,70]

Our laboratories recently used these inhibitor design strategies to develop several sulfonyladenosine-type inhibitors of the acyl-CoA synthetase MenE (Scheme 2).[71] Two of these inhibitors mimic the cognate OSB-AMP reaction intermediate by replacing the reactive phosphate moiety with stable sulfamate (**1**) or sulfamide (**2**) moieties. The third inhibitor is designed to trap the incoming CoA thiol nucleophile with a vinyl sulfonamide electrophile (**3**).

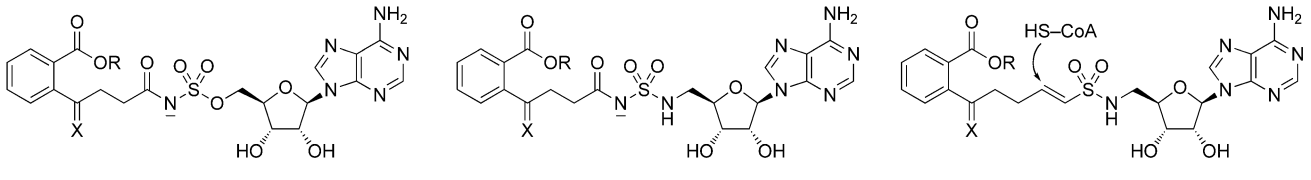
In these inhibitors, the aromatic carboxylate moiety of OSB was masked as the corresponding methyl ester. Concurrently, Mesecar and co-workers reported a related MenE inhibitor in which the aromatic carboxylate was replaced with a trifluoromethyl group.[72] While both of these modifications were tolerated, the resulting inhibitors exhibited relatively modest, low to mid-μM potency.

To evaluate the importance of the aromatic carboxylate to MenE recognition, we sought to evaluate the corresponding carboxylate analogues of our sulfonyladenosine-type inhibitors. We report herein the synthesis and evaluation of these inhibitors and several related analogues, the discovery of a pH-dependent equilibrium between isomeric forms, and the implications for the future design of inhibitors that target MenE and menaquinone biosynthesis.

Results and Discussion

Synthesis of OSB-AMP analogues

The carboxylate-containing inhibitors **4** (OSB-AMS), **5** (OSB-AMSN), and **6** (OSB-AVSN; Scheme 2) were readily synthesized by selective saponification of the methyl esters in **1**–**3**, respec-



1, MeOSB-AMS (R = Me, X = O)
4, OSB-AMS (R = H, X = O)
7, MeOCPB-AMS (R = Me, X = H,H)
10, OCPB-AMS (R = H, X = H,H)

2, MeOSB-AMSN (R = Me, X = O)
5, OSB-AMSN (R = H, X = O)
8, MeOCPB-AMSN (R = Me, X = H,H)
11, OCPB-AMSN (R = H, X = H,H)

3, MeOSB-AVSN (R = Me, X = O)
6, OSB-AVSN (R = H, X = O)
9, MeOCPB-AVSN (R = Me, X = H,H)
12, OCPB-AVSN (R = H, X = H,H)

Scheme 2. MenE inhibitors designed to mimic the OSB-AMP intermediate (AMS, AMSN) or to trap the CoA thiol nucleophile (AVSN). (MeOSB = methyl *o*-succinylbenzoate; MeOCPB = methyl *o*-[3-carboxypropyl]benzoate; OSB = *o*-succinylbenzoate; OCPB = *o*-[3-carboxypropyl]benzoate).

tively (NaOH, MeOH, RT), which were prepared as previously described[71] (for full details see the Supporting Information).

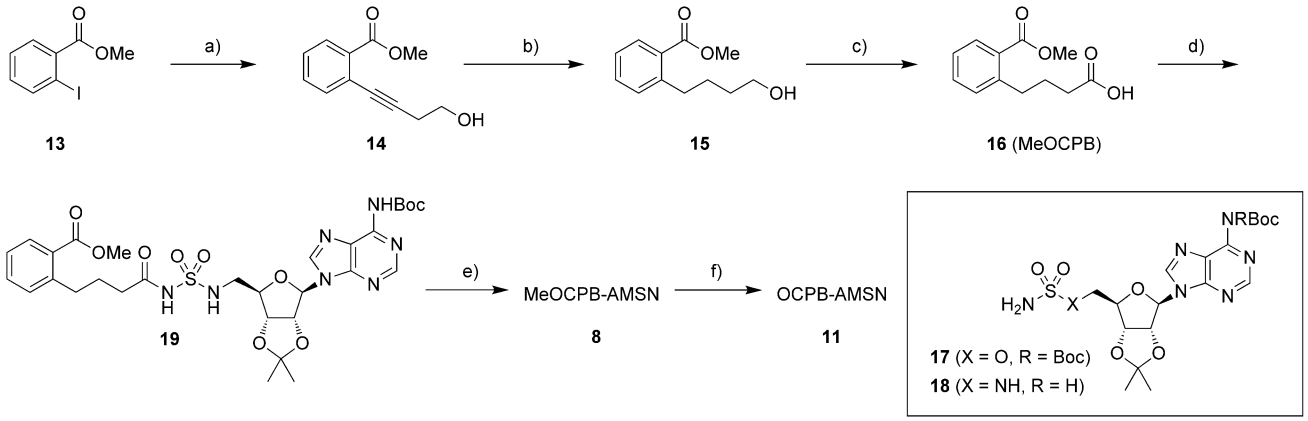
In our initial report, we investigated the importance of the OSB ketone moiety by replacing it with a methylene group ($X = CH_2$, not shown).[71] None of these compounds inhibited MenE when tested at up to 200 μM concentration, and it is possible that this substitution either disrupts a hydrogen-bonding interaction or introduces steric conflicts in the active site. Thus, to investigate further the role of the ketone functionality, we also sought to synthesize the corresponding des-keto analogues **7**–**12** (X = H,H).

The requisite *o*-(carboxypropyl)benzoate (OCPB) side chain **16** was synthesized from iodobenzoate **13** by Sonogashira cross-coupling,[73] alkyne hydrogenation, and Jones oxidation[74] (Scheme 3). Coupling to protected 5′-*O*-sulfamoyladenosine **17**[59] was successful, but after deprotection with TFA, the final acyl sulfamate product **7** (MeOCPB-AMS) proved to be hydrolytically unstable at the acyl sulfamate moiety and could not be purified sufficiently. Thus, **7** and the corresponding free carboxylate **10** (OCPB-AMS) were not pursued further. In contrast, coupling of **16** to the related sulfamide **18**[71] and deprotection afforded the acyl sulfamide **8** (MeOCPB-AMSN), which proved to be stable and could also be converted to the free carboxylate **11** (OCPB-AMSN) by selective hydrolysis of the methyl ester.

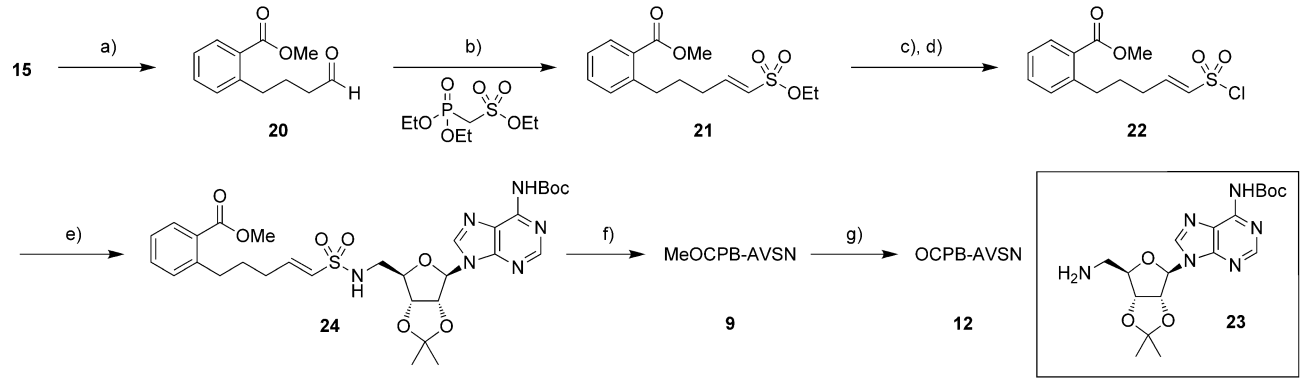
To access the corresponding vinyl sulfonamide analogues, alcohol **15** underwent Dess–Martin oxidation to aldehyde **20**,[75,76] followed by Horner–Wadsworth–Emmons reaction with a diethylphosphorylmethanesulfonate reagent developed by Ghosez and co-workers to afford the vinyl sulfonate **21** (Scheme 4).[77,78] Selective cleavage of the sulfonate ester followed by chlorination provided vinyl sulfonylchloride **22**, which was used without further purification and coupled to protected 5′-aminodeoxyadenosine **23**.[55,71] Deprotection with TFA afforded methyl ester **9** (MeOCPB-AVSN), and saponification of the ester yielded free carboxylate **12** (OCPB-AVSN).

Inhibition of MenE

With these compounds in hand, we tested their inhibitory activities against MenE from *M. tuberculosis* (mtMenE), *S. aureus* (saMenE), and *E. coli* (ecMenE) using coupled assays with



Scheme 3. Synthesis of *o*-(carboxypropyl)benzoate (OCPB) sidechain and des-keto inhibitors **8** and **11**. Reagents and conditions: a) 3-butyn-1-ol, $Pd(PPh_3)_4$, CuI, Et_3N, 55 °C, 24 h, 95%; b) H_2, Pd/C, CH_3OH, RT, 24 h, 89%; c) CrO_3, H_2SO_4, acetone, H_2O, RT, 3 h, 92%; d) **18**, EDC, DMAP, CH_2Cl_2, RT, 2 h, 34%; e) TFA, H_2O, RT, 24 h, 73%; f) 1 N NaOH, CH_3OH, RT, 1.5 h, 22%.



Scheme 4. Synthesis of vinyl sulfonyl chloride sidechain and des-keto inhibitors **9** and **12**. Reagents and conditions: a) Dess–Martin periodinane, CH_2Cl_2, RT, 2 h, 79%; b) *n*-BuLi, THF, -78 °C, 2.5 h, 42%; c) Bu_4NI, acetone, 56 °C, 24 h; d) $SOCl_2$, PPh_3, CH_2Cl_2, RT, 5 h; e) **23**, 2,6-lutidine, CH_2Cl_2, RT, 5 h, 38%; f) TFA, H_2O, RT, 24 h, 73%; g) 1 N NaOH, CH_3OH, RT, 1.5 h, 34%.

MenB, the next downstream enzyme in the menaquinone biosynthesis pathway (Scheme 1).[8,71,79] This coupled assay is based on that described earlier for evaluating the inhibition of MenB, except that the concentrations of MenE and MenB are adjusted to ensure that the MenE-catalyzed reaction is rate-limiting. Assays for saMenE and mtMenE utilized *M. tuberculosis* MenB (mtMenB) as the coupling enzyme, while ecMenE was assayed with *E. coli* MenB (ecMenB). ecMenE, ecMenB, and mtMenB were expressed and purified as described previously,[8,79] while saMenE and mtMenE were cloned and expressed with N-terminal His_6 tags in *E. coli* (BL21) cells, then purified to homogeneity using nickel-affinity chromatography (see the Supporting Information for full details). Reactions were initiated by adding MenE (final concentration 50–100 nM) to a solution containing MenB (5–10 μM), ATP (240 μM), CoA (240 μM), OSB (120–240 μM) and inhibitor (0–200 μM). Formation of DHNA-CoA was monitored at 392 nm, and IC_{50} values were determined by fitting the initial velocity data to the standard dose response equation (Table 1).[71]

We were gratified to find that the free carboxylate analogues **4**–**6** proved to be excellent inhibitors of all three enzymes (Table 1), and were more potent than the original methyl ester analogues **1**–**3**. Notably, the sulfamate **4** (OSB-AMS) was the most potent inhibitor in the carboxylate series for all three enzymes, in contrast to the mixed structure-activity relationship trends observed for the methyl ester series.[71] Further, while neither of the des-keto methyl ester analogues **8** or **9** exhibited appreciable activity, the corresponding des-keto carboxylates **11** and **12** were moderate inhibitors. Thus, deletion of the OSB ketone functionality appears to result in decreased inhibitory activity by approximately 2–3 orders of magnitude (**5** vs. **11**, **6** vs. **12**).

The IC_{50} values for the inhibition of *M. tuberculosis* mtMenE, saMenE, and ecMenE by **4** (OSB-AMS) are within a factor of 2–3 of the enzyme concentrations used in the assay, thus meeting the experimental criterion for tight-binding inhibitors.[80] To provide additional information on the mechanism of enzyme inhibition, K_i^{app} values were determined using the Morrison equation[81,82] as a function of substrate concentration to provide the absolute K_i values for enzyme inhibition. Sulfamate **4** was found to be a competitive inhibitor of mtMenE with respect to ATP ($K_i = 5.4 \pm 0.1$ nM) and a noncompetitive inhibitor with respect to OSB ($K_i = 11.2 \pm 0.9$ nM). These data are consistent with the knowledge that mtMenE follows a Bi Uni Uni Bi Ping-Pong kinetic mechanism in which the addition of ATP and OSB is ordered, with ATP binding first (data not shown). A similar experiment demonstrated that **4** is a competitive inhibitor of ecMenE with respect to OSB with a K_i value of 128 ± 5 nM), consistent with the knowledge that OSB binds first to ecMenE (data not shown). Such a mechanism is consistent with studies on other ligase enzymes.[72,83,84] Although the dependence of K_i^{app} on substrate concentration was not determined for the inhibition of saMenE by **4**, fitting the IC_{50} data to the Morrison equation gave a value for K_i^{app} of 22 ± 8 nM.

Table 1. Inhibition of the MenE enzymes from *M. tuberculosis*, *S. aureus*, *and E. coli*.[a]

Inhibitor	IC_{50} [μM] of MenE from *M. tuberculosis*	*S. aureus*	*E. coli*
1, MeOSB-AMS	14.2 ± 3.3	24.6 ± 3.5	38.0 ± 3.0[b]
2, MeOSB-AMSN	23.5 ± 1.0	>200	34.1 ± 2.8[b]
3, MeOSB-AVSN	117 ± 12	45.7 ± 2.8	5.7 ± 0.7[b]
4, OSB-AMS	0.049 ± 0.007[c]	0.060 ± 0.005[d]	0.21 ± 0.16[e]
5, OSB-AMSN	0.20 ± 0.02	0.24 ± 0.01	0.63 ± 0.14
6, OSB-AVSN	0.16 ± 0.05	0.33 ± 0.05	0.57 ± 0.06
8, MeOCPB-AMSN	>200	>200	>200
9, MeOCPB-AVSN	>200	>200	>200
11, OCPB-AMSN	101 ± 14	85 ± 17	n.d.[f]
12, OCPB-AVSN	106 ± 10	54.4 ± 2.3	31.6 ± 5.5

[a] Assays were performed with mtMenE (50 nM), saMenE (100 nM), or ecMenE (100 nM). [b] Data from ref. [71]. [c] Competitive inhibitor with respect to ATP ($K_i = 5.4 \pm 0.1$ nM) and noncompetitive inhibitor with respect to OSB ($K_i = 11.2 \pm 0.9$ nM). [d] $K_i^{app} = 22 \pm 8$ nM. [e] Competitive inhibitor with respect to OSB ($K_i = 128 \pm 5$ nM). [f] n.d. = not determined.

Active site recognition of OSB-AMP and MenE inhibitors

The increased potency of the aromatic carboxylate analogues **4–6** compared to all previously reported MenE inhibitors suggests that the OSB carboxylate functionality may be recognized specifically by one or more basic side chains in the active site. While cocrystal structures of MenE with substrates or inhibitors have not yet been reported, a crystal structure of the unliganded form of saMenE (PDB ID: 3IPL) has been deposited in the Protein Data Bank by the New York Structural Genomics Research Center.[87] We identified the putative active site in saMenE by comparison to two other acyl-CoA synthetases that have been crystallized with their cognate acyl-AMP intermediates bound (Figure 1).[85,86] This binding site is also conserved across other members of the ANL family.[30,88–93] Upon examination of residues within 12 Å of the center of this binding pocket, we identified a basic residue, Arg222, that may interact with the aromatic carboxylate of OSB (Figure S1 A and B in the Supporting Information). Notably, this residue was not readily identified in sequence alignments guided by the other acyl-CoA synthetase structures, because it lies one extra turn toward the C terminus of helix B3/4 compared to binding pocket residues in the previously determined structures (data not shown).

To evaluate this hypothesis, AutoDock 3.05[94] was used to generate an enzyme–ligand complex of OSB-AMP bound to saMenE. Importantly, the OSB carboxylate is within 5 Å of Arg222 in this docked structure. Similarly, when OSB alone is docked into the active site, it places the aromatic carboxylate within 3 Å of Arg222 (Figure S1 C and D). In addition, Ser302 is within 3 Å of the OSB ketone oxygen in both docked structures, suggesting a possible hydrogen-bonding interaction.

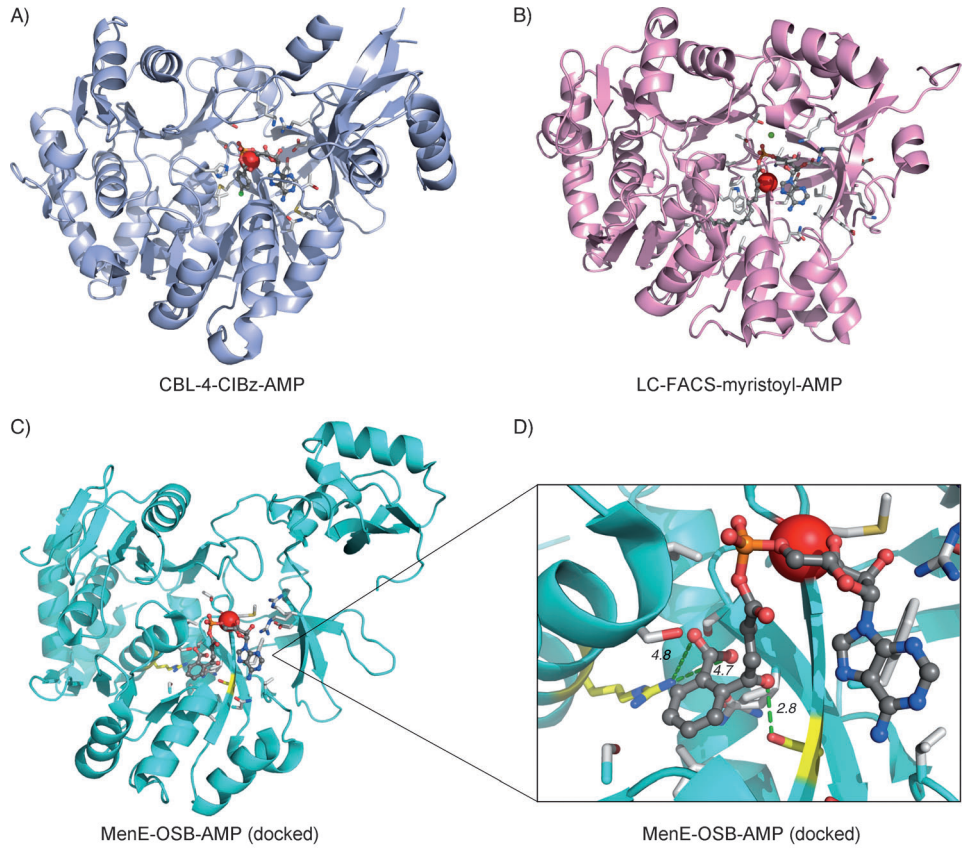


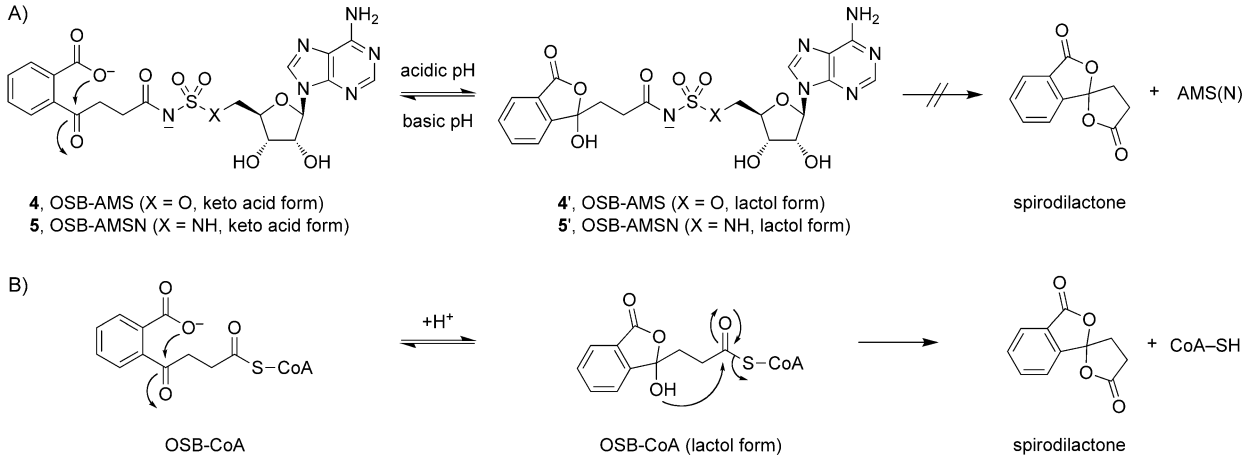
Figure 1. Active sites of acyl-CoA synthetases. A) 4-Chlorobenzoate-CoA synthetase (CBL) with 4-chlorobenzoyl-AMP intermediate and substrate binding residues (PDB: 3CW8).[85] B) Long-chain fatty acyl-CoA synthetase (LC-FACS) with myristoyl-AMP intermediate and substrate binding residues (portions of structure not shown for clarity) (PDB: 1V26).[86] C) *S. aureus* MenE unliganded form with OSB-AMP docked in the putative active site (loop residues 280–284 not shown for clarity) (PDB ID: 3IPL).[87] D) Arg222 and Ser302 (yellow) and putative interactions with OSB aromatic carboxylate (4.7, 4.8 Å) and OSB ketone oxygen (2.8 Å), respectively (loop residues 280–284 not shown for clarity). Side chains within 4 Å of ligands (white) and C terminus of helix B3/4 (red sphere) are shown in each case.

Keto acid–lactol equilibrium in MenE inhibitors

Interestingly, during the synthesis of carboxylates **4**–**6**, we noted distinct NMR spectra after purification by normal-phase silica flash chromatography (MeOH/EtOAc) compared to purification by reversed-phase HPLC (CH_3CN/H_2O/0.1% TFA). We determined that the former corresponded to the free keto acid form of the OSB side chain while the latter corresponded to the lactol form (Scheme 5 and the Supporting Information). These isomeric forms could be interconverted reversibly in CD_3OD by treatment with Na_2CO_3 (keto acid) or TFA (lactol). This raised the possibility that either isomeric form could be the active pharmacophore in these inhibitors.

Subsequently, we evaluated the pH-dependence of the equilibrium by ^{1}H NMR analysis of **5** (OSB-AMSN) in D_2O (Figure 2). Most notably, the ketone α-protons were observed at ≈3.2 ppm at pD ≥ 5.70 (keto acid form) and at ≈2.45 ppm at pD 0.89 (lactol form). The intermediate chemical shifts observed at intermediate pD values (1.32–4.32) are presumed to arise from rapid equilibration of the two isomers on the NMR timescale. An additional upfield shift of both the ketone α-protons and acyl sulfamide

This is consistent with the decreased inhibitory activity observed for both the des-keto analogues **8**, **9**, **11**, and **12** herein and the previously described methylene analogues.[71]



Scheme 5. A) Lactol formation in OSB-AMS (**4**,**4'**) and OSB-AMSN (**5**,**5'**). B) Decomposition of OSB-CoA to form a spirodilactone.

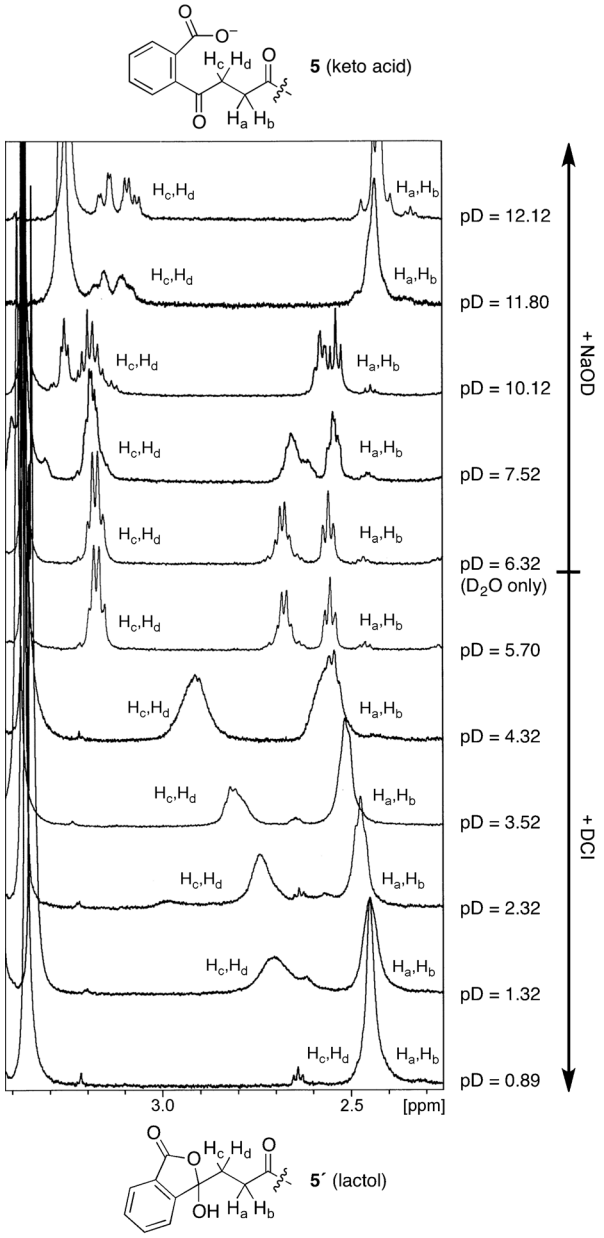


Figure 2. pH dependence of keto acid–lactol equilibrium in OSB-AMSN (**5**), as determined by ^{1}H NMR in D_2O. The isomeric forms (**5**,**5′**) are presumed to be in equilibrium on the NMR timescale between pD 4.32–1.32, based on the intermediate chemical shift of OSB side chain protons H_c,H_d. The additional upfield shift of both H_a,H_b and H_c,H_d above pD 10.12 is presumed to be due to deprotonation of the adenosine 5′-nitrogen.

α-protons above pD 10.12 may be attributed to deprotonation of the adenosine 5′-nitrogen (not shown).

Thus, it is possible that the OSB aromatic carboxylate is important for potent MenE inhibition because the lactol form, formed in solution or in the enzyme active site, is actually the active pharmacophore. While the presence and orientation of Arg222 in the unliganded structure certainly suggest that the keto acid form is the likely pharmacophore, the importance of the lactol form cannot yet be ruled out, since hydrogen-bonding of the ketone oxygen to Ser302 may favor the lactol form. Indeed, the dramatically decreased inhibitory activity of the des-keto analogues **11** and **12** is consistent with either scenario, since lactol formation is precluded in these structures.

Finally, it is interesting to note that the native MenE reaction product, OSB-CoA, is known to undergo spontaneous decomposition through the formation of a spirodilactone (Scheme 5).[17,95] In contrast, **4** (OSB-AMS) and **5** (OSB-AMSN) can also undergo the first step in this pathway to generate the lactol forms **4′** and **5′**, but do not proceed further to spirodilactone formation via displacement of the sulfonyladenosine motif. Thus, even if the lactol form proves not to be relevant to MenE binding, inhibitors based on this structure may have utility as prodrugs in which the negatively charged carboxylate is masked in the lactol form.

Conclusions

We have compared the inhibitory activities of several series of sulfonyladenosine-based MenE inhibitors designed to probe structure-activity relationships in the OSB motif. In these inhibitors, both the free carboxylate and the ketone moiety on the OSB side chain are required for potent inhibition. Analysis of the crystal structure of unliganded saMenE and docking of OSB-AMP into this structure suggest that Arg222 may interact with the OSB carboxylate and Ser302 may bind the OSB ketone, providing a rationale for the increased potency of these inhibitors compared to previously described analogues. The discovery of a stable, isomeric lactol form of these inhibitors may have important implications for the design of additional inhibitors and prodrug variants in the future.

Experimental Section

See the Supporting Information for complete experimental procedures and spectral data for all new compounds.

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Keywords: adenylation · antibiotics · docking · drug design · inhibitors

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