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Investigation of Menaquinone Biosynthetic Pathway in *Mycobacterium*

Tuberculosis: Catalytic Mechanism and Inhibition Studies of MenB and MenE

A Dissertation Presented

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Huaning Zhang

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Abstract of the Dissertation

Investigation of Menaquinone Biosynthesis Pathway in *Mycobacterium*

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The lipid soluble redox cofactor menaquinone is an essential component of the mycobacterial respiratory chain. Selective inhibitors of menaquinone biosynthesis are thus promising lead compounds for the treatment of latent tuberculosis (TB) infections. Menaquinone is synthesized from chorismate by the action of at least 8 enzymes including MenB which catalyzes an intramolecular Dieckmann condensation to form the naphthalenoid aromatic compound 1,4-dihydroxynaphthoyl-CoA from o-succinylbenzoyl-CoA (OSB-CoA), and MenE, an acyl-CoA synthetase, which converts OSB to OSB-CoA.

Currently we are comparing and contrasting the mechanism of the *M. tuberculosis* MenB enzyme with that of MenB from *E. coli* as well as with other members of the crotonase superfamily including BadI, which catalyzes a retroDieckmann reaction. Crystallographic and kinetic data indicate that

conserved catalytic residues play similar roles in the reactions catalyzed by MenB and BadI. However, the studies also suggest differences in the mechanisms of the reactions catalyzed by the *M. tuberculosis* and *E. coli* MenB enzymes and they might utilize the different substrates for catalysis.

In our studies, several inhibitors, targeting MenB, have been selected and optimized from the initial high throughput screening. The most potent compound against MenB exhibits excellent inhibition *in vitro* with an IC_{50} value of 262 nM. On the other hand, a series of mechanism-based inhibitors of MenE have been designed and synthesized. The most potent compound inhibits MenE with an IC_{50} value of 5.7 μM. These studies will help us to validate that the enzymes in menaquinone biosynthetic pathway are targets for the development of novel microbial chemotherapeutics.

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2-KC-CoA	2-ketohexanecarboxyl-CoA
3-BP CoA	3-benzoylpropionic CoA
AA-CoA	acetoacetyl CoA
AIDS	acquired immunodeficiency syndrome
AMP	adenosine monophosphate
ATP	adenosine triphosphate
B. sutbtilis	*Bacillus subtilis*
BCE	before the common era
BP	before present
calcd	calculated
Carb	carboxymethylproline synthase
CBD	4-chlorobenzoyl-CoA dehalogenase
CD	circular dichroism
CDI	N, N'-carbonyldiimidazole
CoA	coenzyme A
conc.	concentrated
COSY	correlation spectroscopY
crotonase	enoyl-CoA hydratase
DCI	$\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase
DHNA	dihydroxynaphthoic acid
DNA	deoxyribonucleic acid

E. coli	*Escherichia coli*
ECI	$\Delta^{3,2}$-enoyl-CoA isomerase
ecMenB	MenB from *E. coli*
ecMenE	MenE from *E. coli*
FAD	flavin adenine dinucleotide (oxidized form)
FADH2+	flavin adenine dinucleotide (reduced form)
HICH	3-hydroxyisobutyryl-CoA hydrolase
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple bond correlation
IPTG	isopropyl-β-d-thiogalactopyranoside
ITC	isothermal titration calorimetry
LAM	lipoarabinomannans
LC-FACS	long chain acyl-CoA synthetases
LM	lipomannans
M. phlei	*Mycobacterium phlei*
M. tuberculosis	*Mycobacterium tuberculosis*
MDR-TB	multidrug-resistant TB
MenB	1, 4-Dihydroxy-2-naphthoyl-CoA synthase
MenE	OSB-CoA synthase
MGCH	3(*S*)-methylglutaconyl-CoA hydratase
MK	menaquinone
MMCD	methylmalonyl-CoA decarboxylase
mtMenB	MenB from *M. tuberculosis*

mtMenE	MenE from *M. tuberculosis*
NAD+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NAP	nitroimidazopyran
NRP	nonreplicating persistence phase
OCH	6-oxocamphor hydrolase
OCPB-CoA	o-(3-carboxypropyl)-benzoic CoA
OSB	o-succinylbenzoic acid
PAS	*p*-aminosalicylic acid
PPi	pyrophosphate
R. palustris	*Rhodopseudomonas palustris*
S. aureus	*Staphylococcus aureus*
S. coelicolor A3(2)	*Streptomyces coelicolor A3(2)*
SAM	*S*-adenosylmethionine
saMenB	MenB from *S. aureus*
SAR	structure activity relationship
SCVs	small-colony variants
SEPHCHC	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate
SHCHC	2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate
TB	tuberculosis
THDP	thiamin diphosphate
THF	tetrahydrofuran

TPP	thiamine pyrophosphate
WHO	World Health Organization
XDR-TB	extensively drug-resistant TB

CHAPTER 1: TUBERCULOSIS AND THE MENAQUINONE BIOSYNTHETIC PATHWAY

The newest statistics from the World Health Organization (WHO) report 2008 showed that there were an estimated 9.2 million new active cases of tuberculosis (TB) in 2006, of which 709, 000 (8%) were HIV-positive. In addition the disease claims nearly 1.7 million lives annually, of which 0.2 million are among HIV-positive people *(1)*. There were an estimated 0.5 million cases of multidrug-resistant TB (MDR-TB) in 2006 (*1*). The stark reality, largely overlooked, is that among infectious diseases, TB has been and still is a major global health problem and the leading cause of death (*2, 3*). The major problems in the treatment and control of TB include the role of this disease as a major opportunistic pathogen in patients with HIV-AIDS and the emergence of MDR-TB.

Overview of tuberculosis (TB)

TB is a common and often deadly contagious disease caused by mycobacteria, mainly *Mycobacterium tuberculosis* (*4*). Other mycobacteria such as *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti*, and *Mycobacterium microti* also cause mycobacterial infection, but these species are less common.

The earliest unambiguous detection of *M. tuberculosis* is in the remains of bison dated 17, 000 years before present (BP) (*5*). Skeletal remains showed that prehistoric humans (4000 BCE) had TB, and tubercular decay has been found in the spines of mummies from 3000-2400 BCE (*6*). The answer of whether TB

originated in cattle and then transferred to humans, or diverged from a common ancestor infecting a different species, is currently unclear (*7*).

M. tuberculosis, is an aerobic rod-shaped bacillus that divides every 16 to 20 hours, an extremely slow rate compared with other bacteria such as *Escherichia coli* (*8*). Since *M. tuberculosis* has a cell wall but lacks a phospholipid outer membrane, it is classified as a Gram-positive bacterium. However, *M. tuberculosis* does not retain the crystal violet stain or stains very weakly by Gram staining due to its high lipid and mycolic acid content of its cell wall (*9*), and so is classified as an acid-fast bacterium (*10*). This unique waxy cell wall makes it hydrophobic and resistant to oral fluids.

Although *M. tuberculosis* can only replicate and grow within a host organism, it can survive in the dry state for weeks as an endospore in the environment and withstand weak disinfectants (*11*).

TB is transmitted through the air when people who have the disease cough, sneeze, or spit. A single sneeze can release up to 40,000 droplets (0.5 to 5 µm in diameter of each droplet) (*12*). Each one of these droplets may transmit the disease, since the infectious dose of TB is very low and the inhalation of just a single bacterium can cause a new infection (*13*). Transition can only occur from people with active not latent TB (*4*). People of close contact (prolonged, frequent, or intense contact) with patients are at particularly high risk of being infected, with an estimated 22% infection rate.

The distribution of TB is not uniform across the globe with about 80% of the population in many Asian and African countries testing positive in tuberculin tests, while only 5-10% of the US population testing positive (*1*).

About 90% of those infected with *M. tuberculosis* have asymptomatic, latent TB infection, with only a 10% lifetime chance that a latent infection will progress to TB disease (*4*). However, if untreated, the death rate for these active TB cases is more than 50% (*14*).

Current diagnosis, prevention and treatment of TB

TB usually attacks the lungs (75% of active cases are pulmonary TB) but can also affect the central nervous system, the lymphatic system, the circulatory system, the genitourinary system, the gastrointestinal system, bones, joints, and even the skin. The typical symptoms of TB include a chronic cough with blood-tinged sputum, fever, night sweats, and weight loss (*15*). Infection of other organs causes a wide range of symptoms. The diagnosis relies on radiology (commonly chest X-rays), a tuberculin skin test, blood tests, as well as microscopic examination and microbiological culture of bodily fluids (*16*). Prevention relies on screening programs and vaccination, usually with Bacillus Calmette-Guérin (BCG) vaccine (*17, 18*).

TB treatment is difficult and requires long courses of multiple antibiotics. Five current first-line drugs for treating TB are isoniazid, rifampicin, ethambutol, pyrazinamide, and streptomycin (**Figure 1.1**).
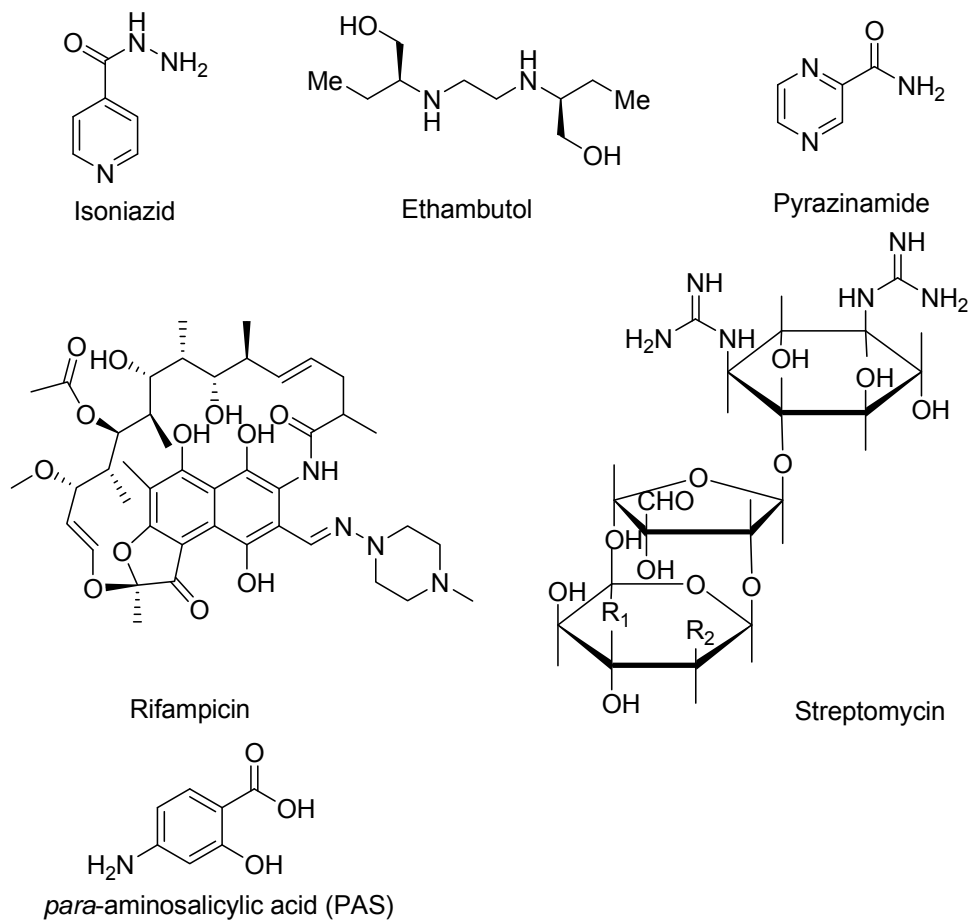
Figure1.1: Current five first-line anti-TB drugs and *p*-aminosalicylic acid (PAS)

Second-line drugs (SLDs) include six classes of antibiotics (**Table 1.1**). A drug may be classed as second-line instead of first-line for one of three possible reasons: it may be less effective than the first-line drugs (e.g., *p*-aminosalicylic acid (PAS) (**Figure 1.1**)); or, it may cause a range of serious side-effects including hepatitis, depression and hallucinations (e.g., cycloserine); or it may be unavailable in many developing countries (e.g., fluoroquinolones).

Table 1.1: Six classes of second-line drugs

Type	Examples
Aminoglycosides	Amikacin (AK) and kanamycin
Polypeptides	Capreomycin, viomycin and enviomycin
Fluoroquinolones	Ciprofloxacin (CIP), levofloxacin and moxifloxacin (MXF)
Thioamides	Ethionamide and prothionamide
Cycloserine	
p-Aminosalicylic acid (PAS)	

Soon after the discovery of the anti-TB activity of streptomycin in 1944, it became apparent that *M. tuberculosis* was capable of rapidly developing drug resistance. It was then found that PAS could be used in combination with streptomycin to prevent, or delay, streptomycin resistance (*19-21*). With the discovery of isoniazid in 1951, it was found that it was much more powerful in combinations with streptomycin and PAS than when used alone and the first combination chemotherapy regimens (included daily isoniazid, streptomycin and high dose of PAS) were standardized with long duration of 18-24 months (*22-24*). The introduction of rifampicin in 1966 shortened the course of treatment (*25-27*). Eventually, the 6 months short-course chemotherapy, with combinations of rifampicin, isoniazid and pyrazinamide and ethambutol or streptomycin became standard. These drugs are administered for the first two months of treatment followed by four months of treatment with isnoiazid and rifampicin alone (*15*). The

initial two month treatment destroys bacteria in all growth stages, whereas in the four month continuation phase rifampicin kills any residual dormant bacilli and isoniazid kills any rifampicin-resistant mutants that commence replication (*28*). For latent TB, the standard treatment is six to nine months of isoniazid alone.

Mechanisms of action of anti-TB drugs

The main issue in anti-TB drug discovery is to develop compounds that have a new mode of action and the potential to shorten the treatment of TB to less than 2 months. Indeed, long-term therapies increase the chances of treatment failure, TB relapse, and emergence of multidrug-resistant (MDR) *M. tuberculosis* strains (*29*).

Hence there are two main objectives in the treatment of TB: to kill actively metabolizing bacilli in the lung cavities and to destroy less actively replicating and near-dormant bacilli in acidic and oxygen-free lesions that may otherwise cause a relapse of the disease (*28*). The determination of the biochemical processes targeted by anti-TB drugs is still undergoing and has been reviewed (*30-33*). The reality is that there are still many anti-TB drugs that have been used for decades with little or no knowledge of their mechanism of action. Therefore understanding the mechanisms of current anti-TB drugs can lead to the identification of already validated biological targets of *M. tuberculosis*. These targets can then be used for the search of better inhibitors starting with the use of modern high-throughput screening of chemical libraries. **Table 1.2** shows several known targets of anti-TB drugs including first-line, second-line and some of the new drugs.

Table 1.2: Mode of action of anti-TB drugs

Anti-TB drugs	Mechanisms of action
Isoniazid, pyrazinamide, ethionamide, prothionamide, thiacetazone, and PA-824 or OPC-67683	Fatty acid biosynthesis inhibitors
Ethambutol, *d*-cycloserine and amoxicillin, clofazimine	Arabinogalactam and peptidoglycan biosynthesis inhibitors
Streptomycin, kanamycin, amikacin, capreomycin, clarithromycin, and linezolid	Inhibitors of protein synthesis
Rifampin, rifapentin, and fluoroquinolones	Inhibitors of DNA-based processes
p-Aminosalicylic acid	Inhibitors of dihydrofolate reductase or siderophore biosynthesis
TMC 207	Inhibitors of the proton pump $F_0F_1H^+ATPase$

Multi-drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB)

Multidrug-resistant TB (MDR-TB) is TB that is resistant to at least two of the major first-line bactericidal drugs, isoniazid and rifampicin. With widespread use of rifampicin-containing regimens, resistance to multiple drugs, notably against isoniazid and rifampicin, appeared. In the US, 0.5% of new cases and 3.0% of recurrent cases were resistant to both isoniazid and rifampicin in 1982 but this

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proportion increased to 3.1% and 6.9% by 1991(*34*) and similar data have been reported worldwide (*35*). Hence, the growth of MDR-TB prevalence has become the major threat that the TB epidemic poses today.

The genes coding for multi-drug, membrane proteins that recognize different toxic compounds and pump them out of bacterial cells, have been identified in mycobacteria, and *M. tuberculosis*. However, they do not seem to play a major role in the emergence of MDR strains (*36-38*). To the contrary, multi-drug resistance is thought to be the consequence of stepwise accumulation of random mutations in the chromosome selected under the environmental pressure of chemotherapy (*39*).

In this regard, it was observed that short-course chemotherapy regimens including four or five drugs (rifampicin, isoniazid, pyrazinamide and ethambutol or streptomycin) were still effective in the presence of resistance to isoniazid alone and longer course protocols could still treat patients with rifampicin mono-resistance (*40*). In contrast, with the resistance to isoniazid and rifampicin combined i.e., MDR-TB, the course of treatment needs prolongation from the standard 6 months to 18–24 months and the cure rate decreases from nearly 100% to less than, or equal to, 60% (*34*).

Extensively drug resistant TB (XDR-TB) is a relatively rare type of MDR-TB and defined as TB which is resistant to isoniazid and rifampicin, plus resistant to any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin). It has emerged from the mismanagement of MDR-TB and once created, can spread from one person to another.

Worldwide prevalence of XDR-TB is estimated to be 6.6% in all the studied countries among multidrug-resistant *M. tuberculosis* strains (*41*).

Because XDR-TB is resistant to first-line and second-line drugs, treatment options for patients are much more limited. XDR-TB is of special concern for persons with HIV infection or other conditions that can weaken the immune system. These persons are more likely to develop TB disease once they are infected, and also have a higher risk of death once they develop TB.

Persistence and latency of TB

Progression from TB infection to TB disease occurs when *M. tuberculosis* overcomes the immune system and begins to replicate. In primary TB disease, this occurs soon after infection (*4*). However, in the majority of cases, a latent infection occurs that shows no obvious symptoms (*4*). These dormant bacilli can reproduce active TB in 2-23% per lifetime of these latent cases, often many years after infection (*42*). The risk of reactivation increases when the immune system is suppressed. For instance, in TB patients co-infected with HIV, the risk of reactivation increases to 10% per year (*14*).

While the biology and mechanism of *M. tuberculosis* latency is not fully understood, theories on how *M. tuberculosis* enters into the host system and subverts host immune responses in favor of survival, growth and persistence of mycobacteria in macrophages have been put forward.

TB infection begins when the mycobacteria reach the pulmonary alveoli, where they invade and replicate within the endosomes of alveolar macrophages

(*4, 43*). The primary site of infection in the lungs is called the Ghon focus, and is generally located in either the upper part of the lower lobe, or the lower part of the upper lobe (*4*). Bacteria are transported by dendritic cells through the bloodstream to other tissues and organs where secondary TB lesions can develop in other parts of the lung (particularly the apex of the upper lobes), peripheral lymph nodes, kidneys, brain, and bone (*4, 44*). All parts of the body can be affected by the disease, though it rarely affects the heart, skeletal muscles, pancreas and thyroid (*45*).

TB is classified as one of the granulomatous inflammatory conditions. Macrophages, T lymphocytes, B lymphocytes and fibroblasts are among the cells that aggregate to form a granuloma, with lymphocytes surrounding the infected macrophages. The granuloma functions not only to prevent dissemination of the mycobacteria, but also provides a local environment for communication of cells in the immune system. Within the granuloma, T lymphocytes (CD4$^+$) secrete cytokines such as interferon gamma, which activates macrophages to destroy the bacteria with which they are infected (*46*). T lymphocytes (CD8$^+$) can also directly kill infected cells (*43*). However, bacteria are not always eliminated within the granuloma, but can become dormant, resulting in a latent infection (*4*).

The entry and persistence of *M. tuberculosis* within the host immune system is attributed to lipoarabinomannans (LAM) and their precursors lipomannans (LM), two predominant glycolipids of *M. tuberculosis* cell wall (*47*).

LM are recognized by TLR2, one of pattern-recognizing receptors, and induce pro-inflammatory activation of macrophages against *M. tuberculosis* infection.

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However, the TLR2-dependent activation of macrophages and dendritic cells favors the direction of adaptive immune responses toward the Th2-type responses, which results in intracellular survival of *M. tuberculosis* (*47*) (**Figure 1.2**).
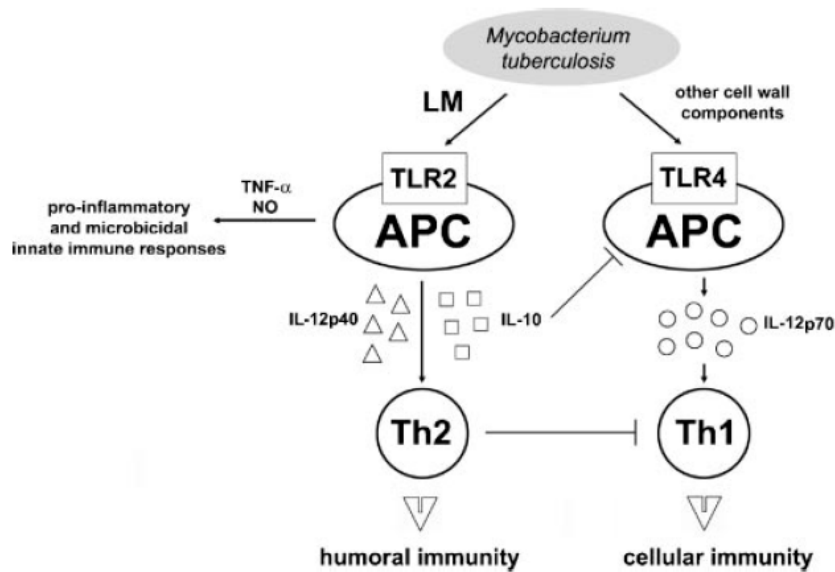
Figure 1.2: Dual role of TLR2 in immunity against *M. tuberculosis*. Activation of TLR2 by LM, most likely by its tri-acylated forms, leads to pro-inflammatory innate immune responses. On the other hand, TLR2-mediated activation of APC skews adaptive immune responses toward those of Th2-type, e.g., by blocking TLR4-mediated production of IL-12, and boosts humoral immunity not harmful to *M. tuberculosis*, an intracellular pathogen (*47*).

At the same time, LAM mimic endogenous host components through the modification of LAM with oligomannosides upon binding to macrophage and dendritic cell receptors, thus evoking immunosupression and evading from the host defense mechanisms (*47*).

Wayne and Hayes (*48*) have conducted pioneering studies of the dormant state of *M. tuberculosis*. In their famous *in vitro* Wayne model of persistence, *M. tuberculosis* cultures are subjected to self-generated oxygen depletion in sealed containers. Growth under such conditions leads to a physiologically well defined anaerobic non-replicating synchronized state of the bacilli. When the oxygen tension in the sealed tubes is reduced to 0.06%, *M. tuberculosis* enters in a non-replicating persistence (NRP) phase where it can survive for extended period without a significant drop in viability (*48*). Synchronized replication can be relapsed upon reintroduction of oxygen (*48, 49*). Their work also suggested that *in vitro*-grown non-replicating tubercle bacilli have a reduced susceptibility to the cidal activity of TB drugs (*48*). This physiological state of the bacillus is being referred to as "drug tolerant" or "phenotypically drug resistant" (*50-52*).

However, the poor understanding of the mechanisms used by *M. tuberculosis* to persist in the total absence of visible growth is still one of the major obstacles in finding and developing drugs that are active against non-replicating bacilli.

Drug targets in persistent and latent *M. tuberculosis* infection

The non-replicating persistence phenotype of *M. tuberculosis* (NRP-MTB) is assumed to be responsible for the maintenance of latent infection and the requirement of long treatment duration for active TB due to the reduced drug susceptibility (*48, 53*).

The latent or non-replicating persistent *M. tuberculosis* are resistant to all conventional anti-TB drugs but they become sensitive to metronidazole (**Figure**

1.3) *in vitro* (*54*). Nitroimidazopyran (NAP) drugs such as PA-824 (**Figure 1.3**) were reported to inhibit the synthesis of protein and cell wall lipid (*55*). In contrast to current anti-TB drugs, nitroimidazopyrans exhibited bactericidal activity against both replicating and static *M. tuberculosis*. Lead compound PA-824 inhibits a more terminal step than isoniazid, namely the oxidation of hydroxymycolates to ketomycolates, a lipid class making up the mycobacterial pseudo-outer membrane and over one-third of the dry weight of *M. tuberculosis* (*56*). In addition, PA-824 demonstrates potential bactericidal activity against multidrug-resistant (*55*).
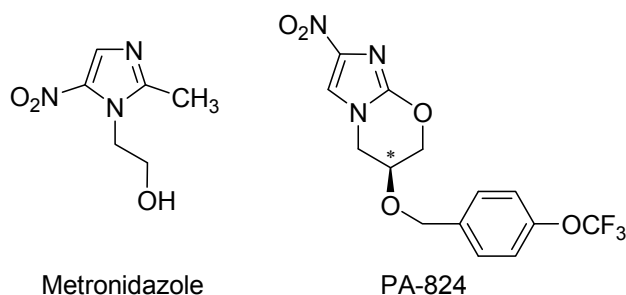
Figure 1.3: Structure of metronidazole and PA-824

Significant progress in this area was recently made by Boshof *et al*. who utilized groupings of DNA microarray profiles to analyze the transcriptional response of *M. tuberculosis* to drugs and growth-inhibitory conditions (*57*). These profiles accurately clustered mechanisms of action of several known drugs and successfully predicted novel mechanisms for previously unknown drugs. Given the critical role of oxygen in the generation of cellular energy and bacterial long-term survival, oxidative phosphorylation is a central component in the production of adenosine triphosphate (ATP) and the subsequent growth and pathogenesis

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of *M. tuberculosis*. Cellular respiration, and more specifically the type II NADH menaquinone oxidoreductase, then was identified as the target of a novel class of phenothiazines (**Figure 1.4**) (*58*).
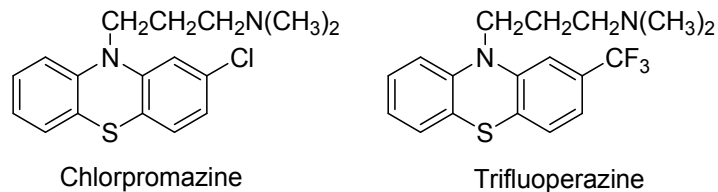
Figure 1.4: Structure of phenothiazines: chlorpromazine and trifluoperazine

Menaquinone biosynthesis in respiratory chain

In the respiratory chain, which is also called the electron transport chain, electrons flow from electron donors such as NADH and $FADH_2$ (succinate) to downstream electron acceptors. In aerobic respiration the electron acceptor is oxygen, while in anaerobic respiration a variety of molecules such as nitrate, dimethyl sulfoxide, fumarate and sulfate, can act as electron acceptors. At the same time, protons are pumped across the membrane to generate a proton gradient that is then used to produce ATP, the main energy intermediate in living organisms. **Figure 1.5** shows the proposed pathway of aerobic electron flow in mycobacteria.

Quinones including ubiquinones and menaquinones are lipid-soluble molecules that shuttle electrons and protons between the membrane-bound protein complexes in the electron transport chain (**Figure 1.6**). The major structural difference within these classes lies on different length of the repeated iosoprenoid units.
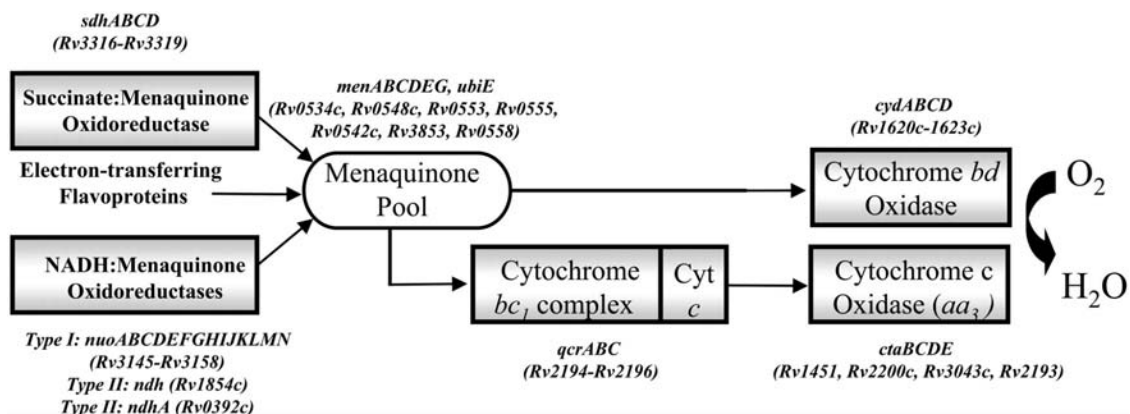
Figure 1.5: Proposed pathway of aerobic electron flow in mycobacteria. Complexes are shown in boxes, with corresponding gene names and GenBank accession numbers given outside of the boxes (*58*).
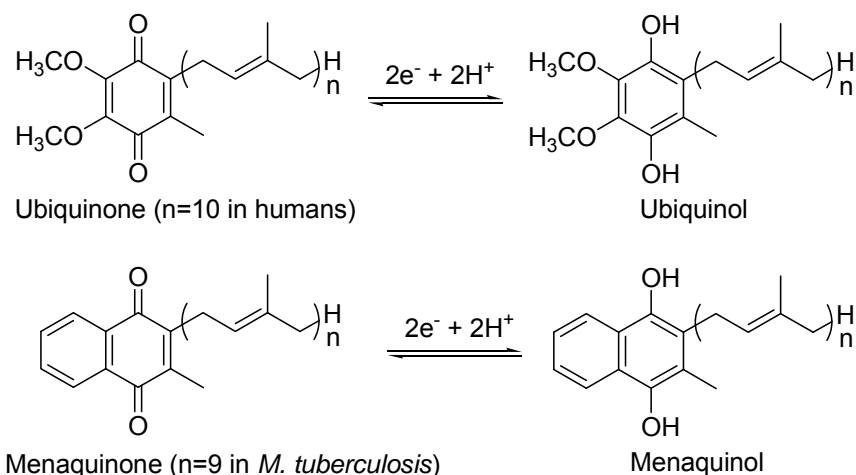
Figure 1.6: Oxidized and reduced forms of ubiquinone and menaquinone.

The quinones in mammals are ubiquinone (Coenzyme Q) with 10 isoprene units (**Table 1.2**) (*59*). They are located in the inner mitochondrial membrane and participate in electron transport. By contrast, prokaryotes employ either the ubiquinone or menaquinone (MK) in the electron transport system. The specific quinones employed also differ in the chain length of the isoprene chain (**Table**

1.3). The enzymes responsible for electron transport are located in membranes, or associated with structures related to membranes or membranes fragments.

Table 1.3: Quinones used in the electron transport

Mammals	Ubiquinone (Coenzyme Q)	CoQ-10 (n=10)
Prokaryotes	Menaquinone (MK) and ubiquinone	*E. Coli* : CoQ-8 (n=8) – aerobic MK-8 (n=8) – anaerobic *Bacillus subtilis*: MK-7 (n=7) *M. tuberculosis*: MK-9 (n=9)

n: units of the isoprene chain

Eukaryotes and aerobic Gram negative bacteria use only ubiquinones in the electron transport. Facultative anaerobic bacteria, such as *E. coli*, use ubiquinones in aerobic conditions and menaquinones in anaerobic conditions (*59, 60*). Most Gram positive bacteria and anaerobic bacteria, e.g. *M. tuberculosis* (MK-9) and *Bacillus subtilis* (MK-7) use only menaquinones (*60*).

Menaquinone (Vitamin K$_2$) biosynthetic pathway is absent in humans and might be an attractive target for anti-TB drug discovery

Although latent *M. tuberculosis* are not replicating, they must presumably respire in order to survive. Compounds that target components of the mycobacterial respiratory chain thus have the potential to sterilize latent TB infections. Menaquinone is an essential component of the electron transport chain in *M. tuberculosis*. Hence the menaquinone biosynthetic pathway might be

an intriguing target for the development of antimycobacterial drugs against both replicating as well as non-replicating bacteria populations (*61*).

Menaquinone in the form of viatamin K_2 plays an important role in blood clotting for humans because it is involved as a cofactor in γ-carboxylation of glutamic acid residues in thrombin (*62*). However, humans lack the biosynthetic pathway for menaquinone and therefore this compound must be obtained in the diet or from intestinal bacteria. It is possible that the menaquinone biosynthesis pathway may be a novel drug target against TB for the following reasons, although humans require menaquinone (vitamin K_2): first, menaquinone is the sole quinone in *M. tuberculosis*; and second, this pathway is absent in humans: human obtain menaquinone either in their diet or from intestinal bacteria. The ideal hypothetical TB therapeutic would be one that inhibited menaquinone biosynthesis in *M. tuberculosis* but not in the intestinal bacteria that supply menaquinone for vitamin K_2.

Menaquinone biosynthetic pathway in *E. coli*

The biosynthesis of menaquinone has been most heavily studied in *E. coli* (*63*) and to a lesser extent in *B. subtilis* (*64-66*) and *Mycobacterium phlei* (*67-76*). The first six enzymes in the E. coli pathway are encoded by an operon (**Figure 1.7**), and the proposed biosynthetic pathway in *E. coli* is shown in **Figure 1.8**.
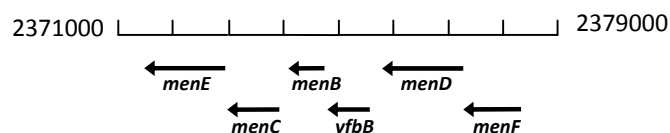
Figure 1.7: The *men* operon in *E. coli*
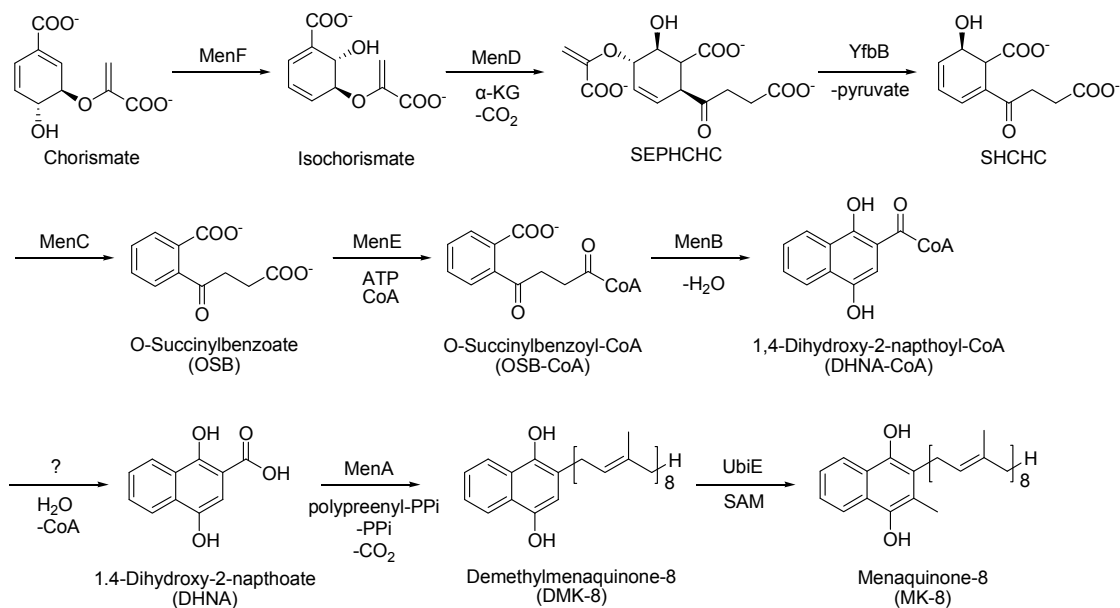
Figure 1.8: Menaquinone biosynthetic pathway in *E. coli*

Chorismate, derived from the shikimate pathway, is converted into isochorismate catalyzed by MenF, an isochorismate synthase (*77*). The condensation of isochorismate with α-ketoglutarate is catalyzed by MenD, and requires thiamine pyrophosphate (TPP) to form 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate (SEPHCHC) (*78, 79*). The transformation of SEPHCHC to 2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate (SHCHC) requires an additional enzyme, YfbB (*80, 81*). SHCHC is dehydrated to the aromatic compound O-succinyl benzoic acid (OSB) by MenC (*82*). The activation of OSB to aliphatic mono-CoA thioester (OSB-CoA), catalyzed by MenE, requires the cofactor CoA and ATP (*83*). MenB catalyzes the cyclization reaction to form the naphthalenoid aromatic compound (DHNA-CoA) (*84*). After the DHNA-CoA is hydrolyzed to dihydroxynaphthoic acid (DHNA), MenA catalyzes the attachment of the prenyl side chain with the loss of a carboxyl

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group(*85*). The last step of the pathway is methylation, catalyzed by UbiE, an *S*-adenosylmethionine (SAM)-dependent methyl transferase (*86*).

Menaquinone biosynthesis in *M. tuberculosis*

The *M. tuberculosis* genome contains homologs of all the *E. coli* men genes (**Table 1.4**) except the MenF and YfbB homologs. In *E. coli*, the key enzymes of the menaquinone pathway are organized into a distinct operon, while in *M. tuberculosis*, homologs of *men*A, *men*B, *men*C, *men*D, *men*E, and *men*H are clustered in one region of the genome (**Figure 1.9**).

Table 1.4: Homologs of the *E. coli* menaquinone biosynthetic enzymes in *M. tuberculosis*

E. coli enzymes	Homologs in *M. tuberculosis*
ecMenD (P17109. 556 aa.) SEPHCHC synthase	Rv0555 (mtMenD); 556 aa. 30% identical / 43% similar over 503 aa.
ecMenC (P29208. 320 aa.) OSB synthase	Rv0553 (mtMenC); 321 aa. 29% identical / 46% similar over 181 aa.
ecMenE (P37353. 451 aa.) OSB-CoA synthase	Rv0542c (mtMenE); 451 aa. 29% identical / 47% similar over 275 aa.
ecMenB (P27290. 285 aa.) DHNA synthase	Rv0548c (mtMenB); 285 aa. 48% identical / 62% similar over 285 aa.
ecMenA (P32166. 308 aa.) DHNA prenyl transferase	Rv0534c (mtMenA); 292 aa. 36% identical / 51% similar over 271 aa.
ecUbiE (P0A887. 251 aa.) DMK methyl transferase	Rv0558 (mtMenH); 234 aa. 43% identical / 56% similar over 226 aa.
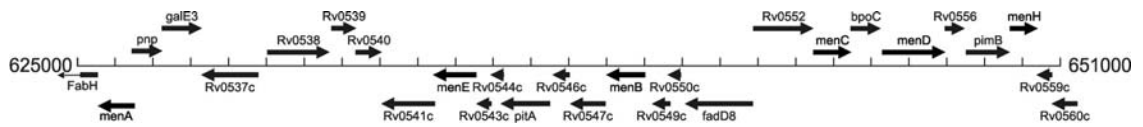
Figure1.9: Clustering of *men* gene homologs in *M. tuberculosis*

The alternative biosynthetic pathway is not present in *M. tuberculosis*

Menaquinone is an obligatory component of the electron-transfer pathway in some bacteria. However, there is no trace of *men* gene orthologs in the genome of *Streptomyces coelicolor A3(2)* (*87-89*), even though it produces menaquinones. Similarly, some pathogens that synthesize menaquinone, including *Helicobacter pylori* and *Campylobacter jejuni*, have also been reported to lack *men* gene homologs (*90-93*). Recent studies support the existence of an alternative biosynthetic pathway, the futalosine pathway, in *S. coelicolor A3(2)* (**Figure 1.10**) (*94, 95*). However, there is no evidence for the presence of this alternate pathway in *M. tuberculosis*.
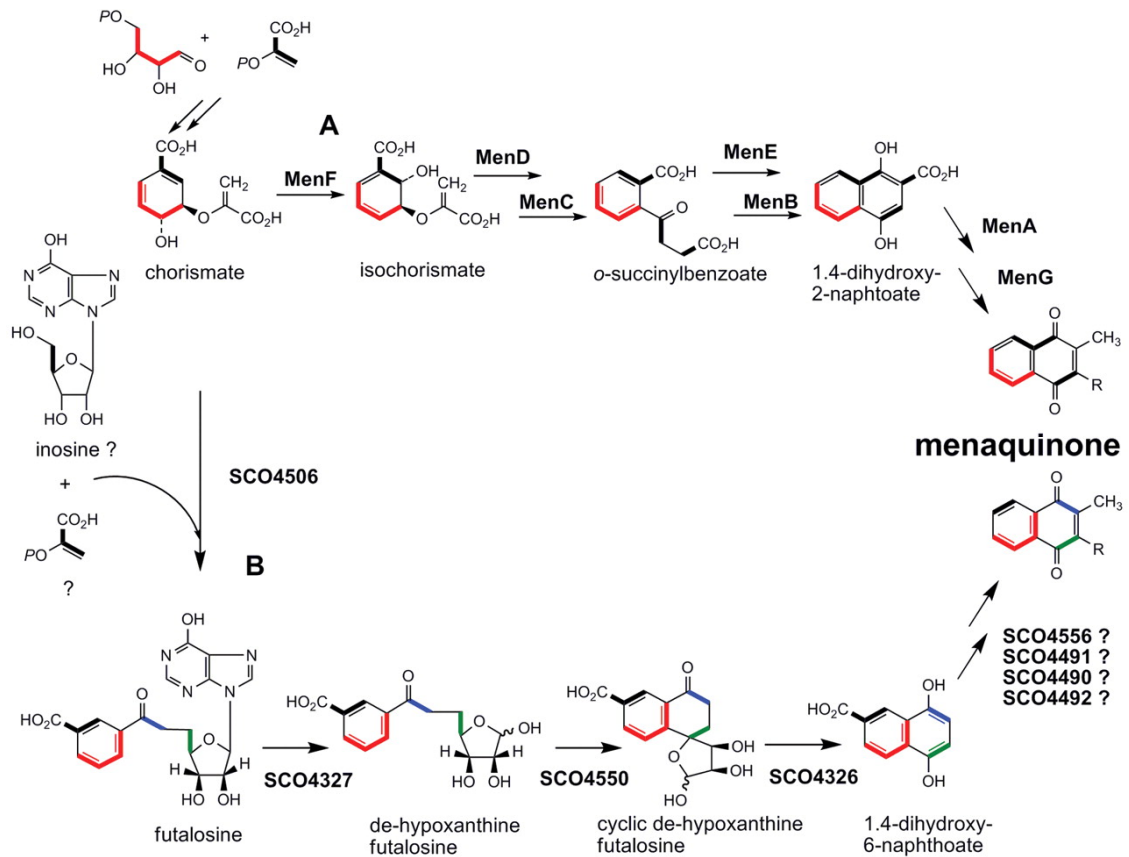
Figure 1.10: Menaquinone biosynthetic pathway in *S. coelicolor A3(2)*. (A) Classical pathway in *E. coli*. Red and black bold lines show carbons originated from erythrose-4-phosphate and phosphoenolpyruvate, respectively. (B) Alternative pathway. Green and blue bold lines indicate two carbon units derived from C-5 and C-6 of glucose via different metabolic pathways. Based on the annotation of the open reading frames of *S. coelicolor A3(2),* SCO4491 (prenylation), SCO4556 (methylation), SCO4490 (decarboxylation), and SCO4492 (decarboxylation) (modified from (*94*)).

Overview of my research

My research is mainly focused on the central portion in the menaquinone biosynthetic pathway in *M. tuberculosis*. The long term goal of our research is to completely understand the enzymes from the menaquinone biosynthetic pathway, to design and develop potent inhibitors targeting this pathway, and to validate this pathway as a target for the development of novel microbial chemotherapeutics.

MenB catalyzes the formation of a carbon-carbon bond through an intramolecular Claisen/Dieckmann condensation. In chapter 2, mechanistic studies of the reaction catalyzed by MenB will focus on comparing the mechanism of the *M. tuberculosis* MenB (mtMenB) enzyme with that of MenB from *E. coli* (ecMenB). We will address the following questions: what are the substrates for both enzymes, what is the functional role of D185 mtMenB which is not conserved in ecMenB, and can MenB catalyze the hydrolysis of DHNA-CoA?

In chapter 3, we will continue to discuss the mechanism of MenB reaction by contrasting with BadI, a member of the crotonase superfamily which catalyzes a retroDieckmann reaction. mtMenB shares 35% sequence identity with orthologs of BadI. The proposed catalytic roles for the residues in the active site of MenB are complementary to the roles proposed for the active-site residues of BadI. However, the usage of different stereochemistry by MenB and BadI provides us new understanding of MenB reaction mechanism.

The SAR studies on MenB inhibitors are based on the two types of leads (2-amino-4-oxo-phenylbutanoic acids and benzoxazinones) identified from high throughput screening, which will be discussed in chapter 4. In chapter 5, a series of 5′-O-(N-acylsulfamoyl) adenosines (acyl-AMS) and related compounds which inhibit MenE by mimicking tightly-bound OSB-AMP intermediates will be introduced. Information obtained from these studies will help us to evaluate the importance of MenB or MenE in menaquinone biosynthesis, and to design better inhibitors for the development of novel microbial antibiotics.

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CHAPTER 2: MECHANISM OF REACTION CATALYZED BY 1, 4-DIHYDROXY-2-NAPHTHOYL-COA SYNTHASE FROM *M. TUBERCULOSIS* (MTMENB)

Background

Introduction of crotonase superfamily

1, 4-Dihydroxy-2-naphthoyl-CoA synthase (MenB) belongs to the crotonase superfamily. Members of this family catalyze mechanistically diverse reactions and share sequence identities ranging from 15 to 45%. It has been proposed that the crotonase superfamily has evolved by divergent evolution from a common ancestor: the oxyanion hole has been retained for the stabilization of enolate anion intermediates, while the new residues have been selected to alter the substrate specificity and the catalyzed chemistry (*1-4*). The oxyanion hole is composed of two halves in which an amide proton forms a hydrogen bond with oxyanion intermediate. The first half is formed by sequence motif FXXGXD, with the second-to-last residue in the sequence contributing its amide proton. The second half is formed by the sequence motif GXG (normally GGG), with the second residue contributing its amide (**Figure 2.1**).

The diversity of the catalytic residues correlates with the diversity of the reactions they catalyze (**Figure 2.1 and 2.2**). Many enzymes in this superfamily catalyze the hydration of 2-enoyl-CoA thioesters and the isomerization of double bonds. Other reactions catalyzed by crotonase superfamily members include decarboxylation, thioester hydrolysis, dehalogenation, Dieckmann condensation, reverse Dieckmann condensation and reverse aldol condensation. The

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1 crotonase rat-----------------------------GANFQYIITEKKGKNSSVGLIQLNRPKALNA 31
2 MGCH Human     -----------------------SSEMKTEDELRVRHLEEENRGIVVLGINRAYGKNS 35
3 BadI R.pal     ----------------------------MQFEDLIY--EIRNGVAWIIINRPDKMNA 27
4 MenB M.tub     VVAPAGEQGRSSTALSDNPFDAKAWRLVDGFDDLTDITYHRHVDDATVRVAFNRPEVRNA 60
5 MMCD E.coli    ------------------------------MSYQYVNVVTINKVAVIEFNYGRKLNA 27
6 DCI rat        -----------------------------AYESIQVTSAQKHVLHVQLNRPEKRNA 27
7 CarB P.car     ------------------------------------MVFEENSDEVRVITLDHPNKHNP 23
8 CBD Ps.sp.     -------------------------------MYEAIGHRVEDGVAEITIKLPRHRNA 26
9 FHL P.flu      -------------------------MSTYEGRWKTVKVEIEDGIAFVILNRPEKRNA 32
10 HICH Human    --------------------------MTDAAEEVLLGKKGCTGVITLNRPKFLNA 29
11 OCH R.sp.     --------------------MKQLATPFQEYSQKYENIRLERDGGVLLVTVHTEGKSLV 39
12 ECI Yeast     --------------------------MSQEIRQNEKISYRIEGPFFIIHLINPDNLNA 32


                                                  ◆
1 crotonase     LCNGLIEELNQALETFEEDPAVGAIVLTG------GEK-AFAAGADIKEMQNRTFQDC-- 82
2 MGCH Human    LSKNLIKMLSKAVDALKSDKKVRTIIIRS------EVPGIFCAGADLKERAKMSSSEVGP 89
3 BadI R.pal    FRGTTCDELIKALYKAGYDKDVGAIVLAG------AGDRAFCTGGDQSTHDGN---YDGR 78
4 MenB M.tub    FRPHTVDELYRVLDHARMSPDVGVVLLTGNGPSPKDGGWAFCSGGDQRIRGRSGYQYASG 120
5 MMCD E.coli   LSKVFIDDLMQALSDLN-RPEIRCIILRAP-----SGSKVFSAGHDIHELPSGGRDPLS- 80
6 DCI rat       MNRAFWRELVECFQKISKDSDCRAVVVSG-------AGKMFTSGIDLMDMASDILQPPGD 80
7 CarB P.car    FSRTLETSVKDALARANADDSVRAVVVYG------GAERSFSAGGDFNEVKQLSRSEDIE 77
8 CBD Ps.sp.    LSVKAMQEVTDALNRAEEDDSVGAVMITG-------AEDAFCAGFYLREIPLDKGVAGVR 79
9 FHL P.flu     MSPTLNREMIDVLETLEQDPAAGVLVLTG-------AGEAWTAGMDLKEYFREVDAGPEI 85
10 HICH Human   LTLNMIRQIYPQLKKWEQDPETFLIIIKG------AGGKAFCAGGDIRVISEAEKAKQKI 83
11 OCH R.sp.    WTSTAHDELAYCFHDIACDRENKVVILTG-------TGPSFCNEIDFTSFNLGTPHDWDE 92
12 ECI Yeast    LEGEDYIYLGELLELADRNRDVYFTIIQS-------SGRFFSSGADFKGIAKAQGDDTNK 85


                                                  ◆
1 crotonase     -----------YSGKFLSHWDHITRIKKPVIAAVNGYALGGGCELAMMCDIIYAG-EKAQ 130
2 MGCH Human    -----------FVSKIRAVINDIANLPVPTIAAIDGLALGGGLELALACDIRVAA-SSAK 137
3 BadI R.pal    GTVG---------LPMEELHTAIRDVPKPVIARVQGYAIGGGNVLATICDLTICS-EKAI 128
4 MenB M.tub    DTADTVDVARAGRLHILEVQRLIRFMPKVVICLVNGWAAGGGHSLHVVCDLTLASREYAR 180
5 MMCD E.coli   -----------YDDPLRQITRMIQKFPKPIISMVEGSVWGGAFEMIMSSDLIIAA-STST 128
6 DCI rat       DVARIAWYLRDLISRYQKTFTVIEKCPKPVIAAIHGGCIGGGVDLISACDIRYCT-QDAF 139
7 CarB P.car    E----------WIDRVIDLYQAVLNVNKPTIAAVDGYAIGMGFQFALMFDQRLMA-STAN 126
8 CBD Ps.sp.    DHFR------IAALWWHQMIHKIIRVKRPVLAAINGVAAGGGLGISLASDMAICA-DSAK 132
9 FHL P.flu     LQEK------IRREASQWQWKLLRMYAKPTIAMVNGWCFGGGFSPLVACDLAICA-DEAT 138
10 HICH Human   AP--------VFFREEYMLNNAVGSCQKPYVALIHGITMGGGVGLSVHGQFRVAT-EKCL 134
11 OCH R.sp.    IIFE-----------GQRLLNNLLSIEVPVIAAVNG-PVTNAPEIPVMSDIVLAAESATF 140
12 ECI Yeast    YPSETSKWVSNFVARNVYVTDAFIKHSKVLICCLNGPAIGLSAALVALCDIVYSINDKVY 145
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 1 crotonase    FGQPEILLGTIPGAGGTQRLTRAVG-KSLAMEMVLTGDRISAQDAKQAGLVSKIFP----  185
 2 MGCH Human   MGLVETKLAIIPGGGGTQRLPRAIG-MSLAKELIFSARVLDGKEAKAVGLISHVLEQNQE  196
 3 BadI R.pal   FGQVGPKMGSVDPGYGTAFLARVVG-EKKAREIWYMCKRYSGKEAEAMGLANLCVP----  183
 4 MenB M.tub   FKQTDADVGSFDGGYGSAYLARQVG-QKFAREIFFLGRTYTAEQMHQMGAVNAVAE----  235
 5 MMCD E.coli  FSMTPVNLGVPYNLVGIHNLTRDAG-FHIVKELIFTASPITAQRALAVGILNHVVE----  183
 6 DCI rat      FQVKEVDVGLAADVGTLQRLPKVIGNRSLVNELTFTARKMMADEALDSGLVSRVFP---D  196
 7 CarB P.car   FVMPELKHGIG-CSVGAAILGFTHG-FSTMQEIIYQCQSLDAPRCVDYRLVNQVVES---  181
 8 CBD Ps.sp.   FVCAWHTIGIGNDTATSYSLARIVG-MRRAMELMLTNRTLYPEEAKDWGLVSRVYP----  187
 9 FHL P.flu    FGLSEINWGIPPGNLVSKAMADTVG-HRQSLYYIMTGKTFGGQKAAEMGLVNESVP----  193
10 HICH Human   FAMPETAIGLFPDVGGGYFLPRLQG--KLGYFLALTGFRLKGRDVYRAGIATHFVDS-EK  191
11 OCH R.sp.    QDGPHFPSGIVPGDGAHVVWPHVLG-SNRGRYFLLTGQELDARTALDYGAVNEVLS----  195
12 ECI Yeast    LLYPFANLGLITEGGTTVSLPLKFG-TNTTYECLMFNKPFKYDIMCENGFISKNFNMP--  202


 1 crotonase    VETLVEEAIQCAEKIANNSKIIVAMAKESVNAAFEMTLTEGNKLE--KKLFYSTFATDDR  243
 2 MGCH Human   GDAAYRKALDLAREFLPQGPVAMRVAKLAINQGMEVDLVTGLAIE--EACYAQTIPTKDR  254
 3 BadI R.pal   HDELDAEVQKWGEELCERSPTALAIAKRSFN-MDTAHQAGIAGMG--MYALKLYYDTDES  240
 4 MenB M.tub   HAELETVGLQWAAEINAKSPQAQRMLKFAFN-LLDDGLVGQQLFA--GEATRLAYMTDEA  292
 5 MMCD E.coli  VEELEDFTLQMAHHISEKAPLAIAVIKEELRVLGEAHTMNSDEFERIQGMRRAVYDSEDY  243
 6 DCI rat      KDVMLNAAFALAADISSKSP--VAVQGSKINLIYSRDHSVDESLDYMATWNMSMLQTQDI  254
 7 CarB P.car   -SALLDAAITQAHVMASYPASAFINTKRAVNKPFIHLLEQTRDAS--KAVHKAAFQARDA  238
 8 CBD Ps.sp.   KDEFREVAWKVARELAAAPTHLQVMAKERFHAGWMQPVEECTEFE--IQNVIASVTHPHF  245
 9 FHL P.flu    LAQLREVTIELARNLLEKNPVVLRAAKHGFKRCRELTWEQNEDYLYAKLDQSRLLDTEGG  253
10 HICH Human   LAMLEEDLLALKSPSKENIASVLENYHTESKIDRDKSFILEEHMDKINSCFSANTVEEII  251
11 OCH R.sp.    EQELLPRAWELARGIAEKPLLARRYARKVLTRQLRRVMEADLSLGLAHEALAAIDLGMES  255
12 ECI Yeast    SSNAEAFNAKVLEELREKVKGLYLPSCLGMKKLLKSNHIDAFNKANSVEVNESLKYWVDG  262
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Figure 2.1: Sequence alignment of characterized crotonase superfamily members.
Residues of the oxyanion hole are in blue, with the hydrogen bond donating residues
marked with diamonds. Characterized catalytic residues are in red; putative and
uncharacterized active-site residues are in gray. The cyan residues mark the start of the
C-terminal domain in structurally characterized enzymes.
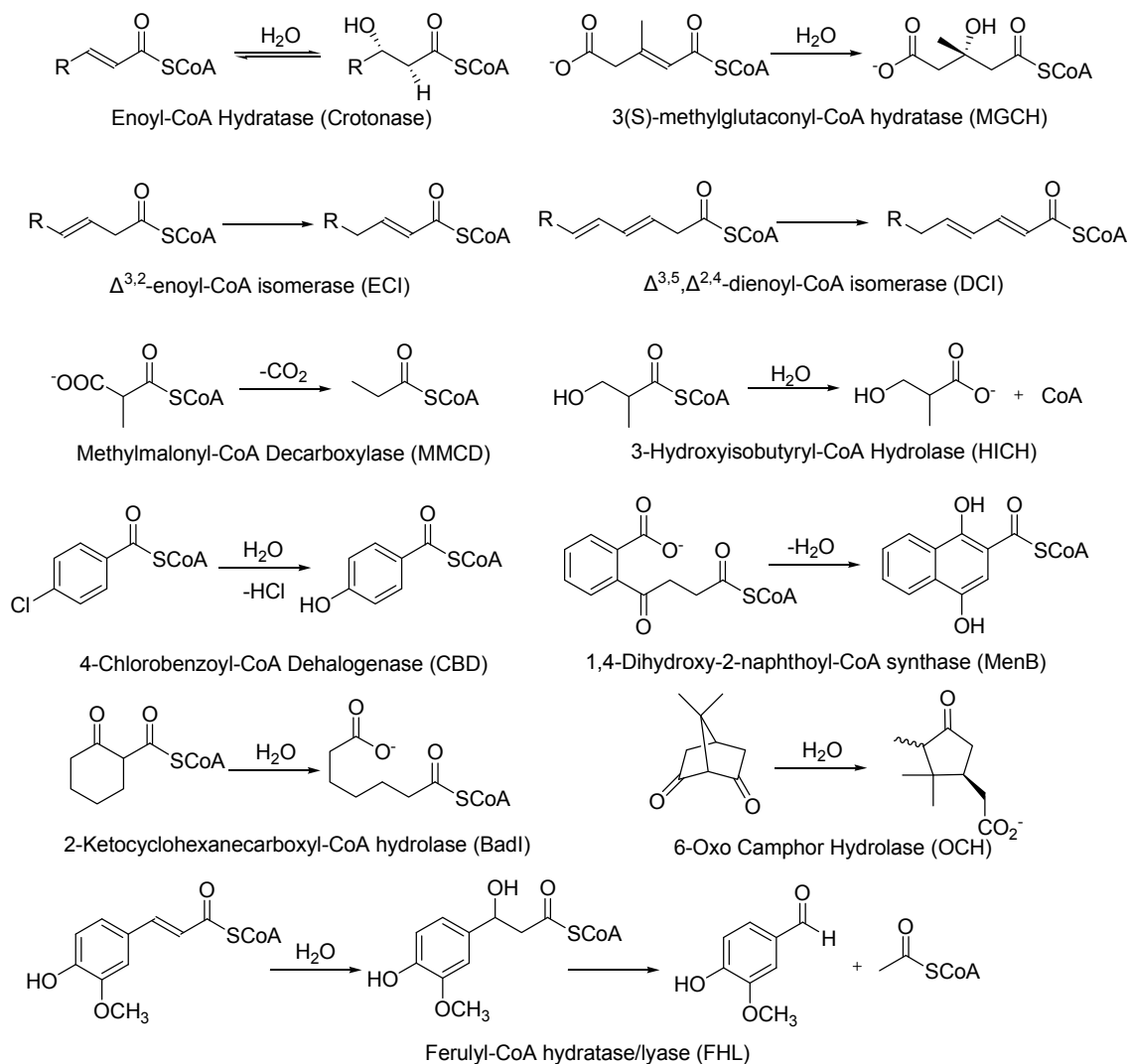
Figure 2.2: Reactions catalyzed by members of the crotonase superfamily. Various reactions include hydration (enoyl-CoA hydratase (*5-7*) and MGCH (*8*)), isomerization (ECI (*9*) and DCI (*10*)), decarboxylation (MMCD (*11*)), thioester hydrolysis (HICH (*12*)), dehalogenation (CBD (*13*)), Dieckmann condensation (MenB (*14*)), reverse Dieckmann condensation (OCH (*15*) and BadI (*16*)) and reverse aldol condensation (FHL (*17*)).

conserved oxyanion hole stabilizes oxyanion intermediate, while the divergent catalytic residues involve in substrate binding and catalysis of various reactions.

Since superfamily members may be evolved from the same ancestor, they share a common structural scaffold. The crotonase fold is a right-handed spiral composed of a core of β-sheets surrounded by α-helices. Eleven crystal structures have been solved and published: enoyl-CoA hydratase (crotonase) from rat (*18*), 3(*S*)-methylglutaconyl-CoA hydratase (MGCH) from human (*19*), 4-chlorobenzoyl-CoA dehalogenase (CBD) from *Pseudomonas sp.* (*13*), $\Delta^{3,5}, \Delta^{2,4}$-dienoyl-CoA isomerase (DCI) from rat (*20*), methylmalonyl-CoA decarboxylase (MMCD) from *E. coli* (*11*), $\Delta^{3,2}$-enoyl-CoA isomerase (ECI) from *Saccharomyces cerevisiae* (Yeast) (*9*), 6-oxocamphor hydrolase (OCH) from *Rhodococcus sp.* (*21*), MenB from *M. tuberculosis* (*14*) carboxymethylproline synthase (Carb) from *Pectobacterium Carotovora* (*22*), MenB from *S. aureus* (*23*), and 3-hydroxyisobutyryl-CoA hydrolase (HICH) from human (*24*). All these structures contain this particular arrangement of secondary structures (**Figure 2.3**). Most of crotonase members form hexamers (dimers of trimers). The active site is located at the outer edge of the trimer, near the trimer-trimer interface. The active substrate molecule is buried inside the protein, while the CoA portion threads out of the active site, occupying a cleft in the enzyme where it makes many contacts with water.

Crotonase superfamily members can be categorized into three sub-groups according to the position of their C-terminus (**Figure 2.3**). In first sub-group

42

-CoA
tase

MCH   ECI   MMCD

OCH   CarB
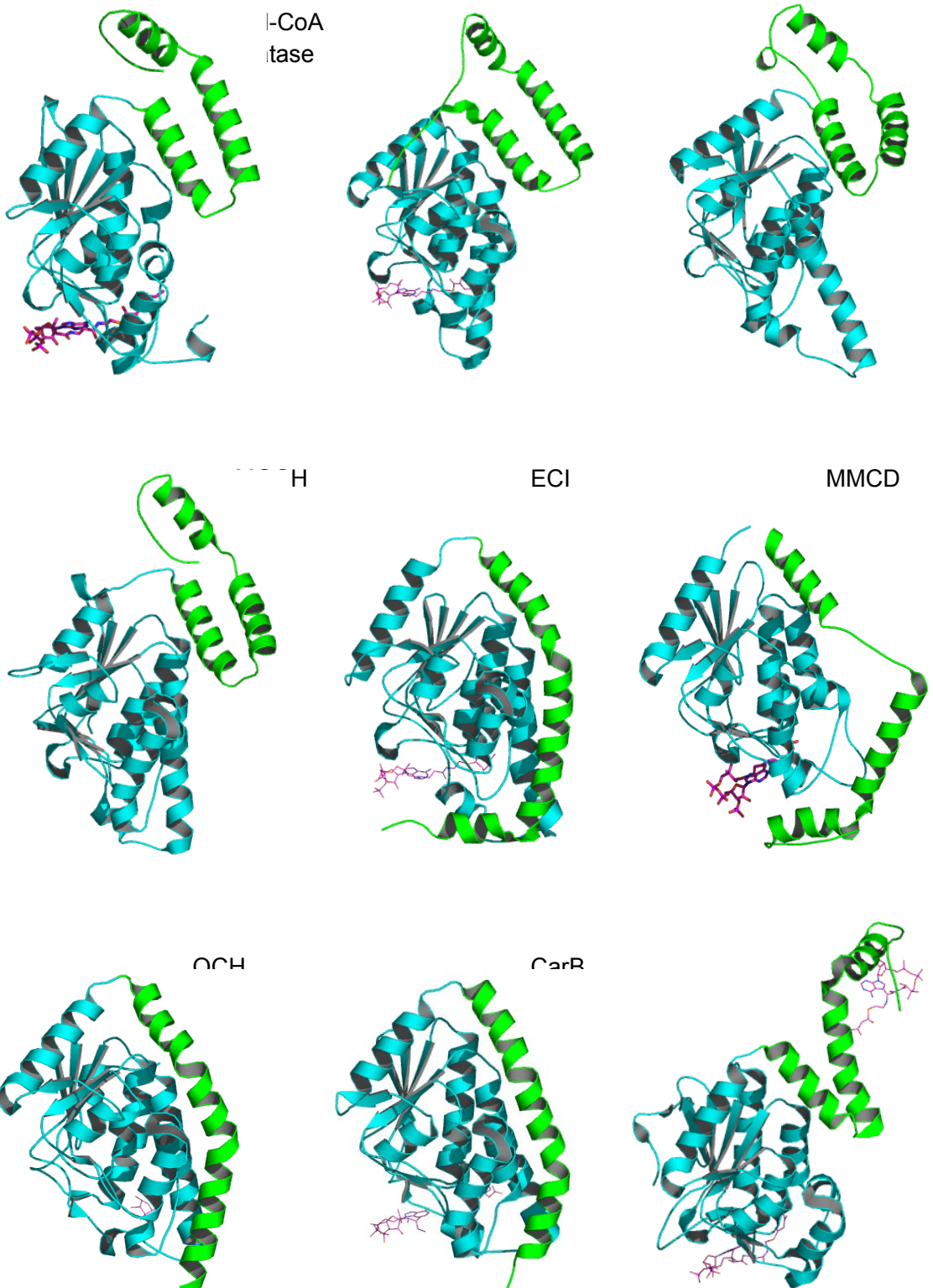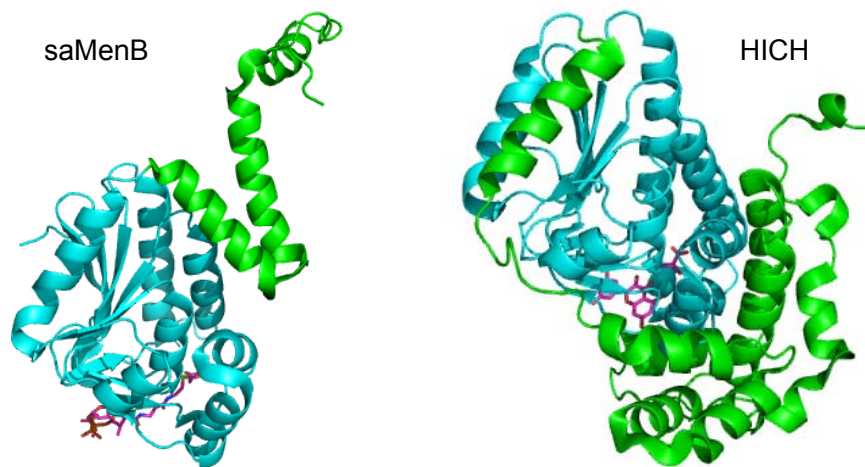
Figure 2.3: Structures of enzymes from the crotonase superfamily. The α, β core is in blue; the divergent C-terminal domain is in green; bound substrate analogs are in pink.

including enoyl-CoA hydratase, CBD, DCI and MGCH, the C-terminus protrudes away from the monomer and interacts with the active site of a neighboring monomer within the trimer. Second group includes ECI, MMCD, OCH and CarB where their C-terminus folds back to interact with active site of the same monomer. Human HICH has an extremely long C-terminus and can be categorized into second group. In third group, mtMenB and saMenB, the C-terminus crosses the trimer-trimer interface and interacts with the active site of a monomer from the opposing trimer.

Crystal structures of mtMenB and saMenB Bound with Acetoacetyl CoA

The crystal structures of mtMenB and saMenB in complex with acetoacetyl CoA (AcAc-CoA) (**Figure 2.4**) have been published by our group (*14*) and Ulaganathan (*23*) respectively.

44
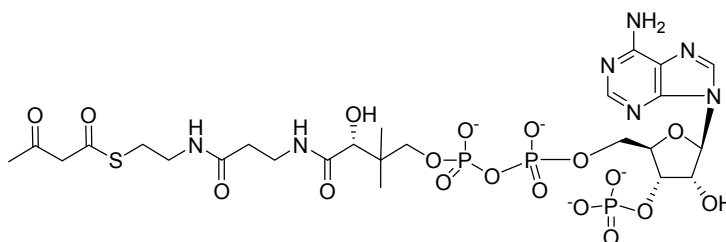
Figure 2.4: Structure of acetoacetyl CoA (AcAc-CoA)

Both mtMenB and saMenB proteins are hexamers (dimer of trimers). **Figure 2.5** shows the crystal structures of mtMenB. Unlike most of the members in crotonase superfamily, the C-terminus of MenB crosses the trimer-trimer interface and interacts with the active site of a monomer from the opposing trimer (**Figure 2.5A**). The active site residues are located on the outer edge of trimer, near the trimer-trimer interface. The acetoacetyl portion of the substrate analogue is buried within a cleft in the enzyme, while the CoA portion threads out of the active site making enough contacts with solvent molecules on the surface (**Figure 2.5B**). The thioester carbonyl forms hydrogen bonding with the backbone amide protons of the residues of oxyanion hole (Gly 105 and Gly 161). Catalytic residues D185, S190, D192 (mtMenB numbering) from one monomer and Y287 (mtMenB numbering) from the C-terminus of another monomer are positioned in the active site (**Figure 2.5B**).
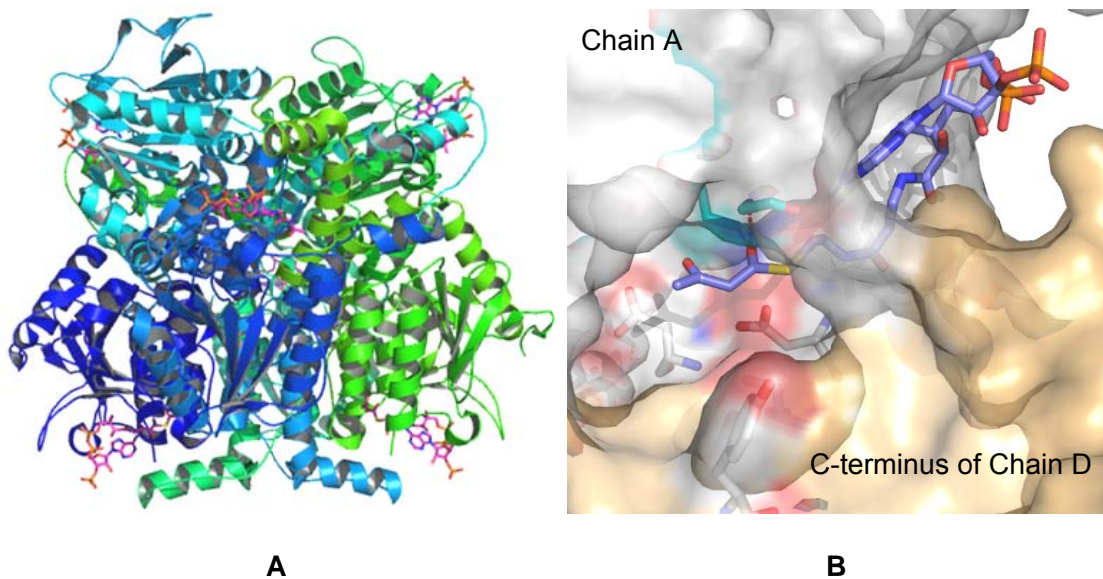
Figure 2.5: Crystal structures of mtMenB bound with AcAc-CoA. Figure A shows that mtMenB is a hexamer and Figure B shows the active site of mtMenB.

saMenB is associated with bacterial growth of small-colony variants (SCVs) of *S. aureus*. SCVs of *S. aureus* are associated with persistent infections and may be selectively enriched during antibiotic therapy. *S. aureus* SCV phenotype isolates were characterized by reduced susceptibility to gentamicin, reduced hemolytic activity, slow growth, and menadione auxotrophy. Sequencing of the genes involved in menadione auxotrophy revealed mutations in *menb* in all three strains with the SCV phenotype (*25*). The mutations included (i) a 9-bp deletion from nucleotides 55 to 63 which corresponds to the amino acid sequence of F19Y20G21, (ii) a frameshift mutation that resulted in a premature stop codon at position 230, and (iii) a point mutation that caused the amino acid substitution Gly233 to Val233 (*25*).

Based on the structure of saMenB (**Figure 2.6**), the bulky mutation of G233V may affect the folding at trimer-trimer interface in the C-terminus, thus impact the whole structure. The truncation of the C-terminus after residue 230 not only removes the conserved catalytic residue Y246, but also destroys the trimer-trimer interface contact.
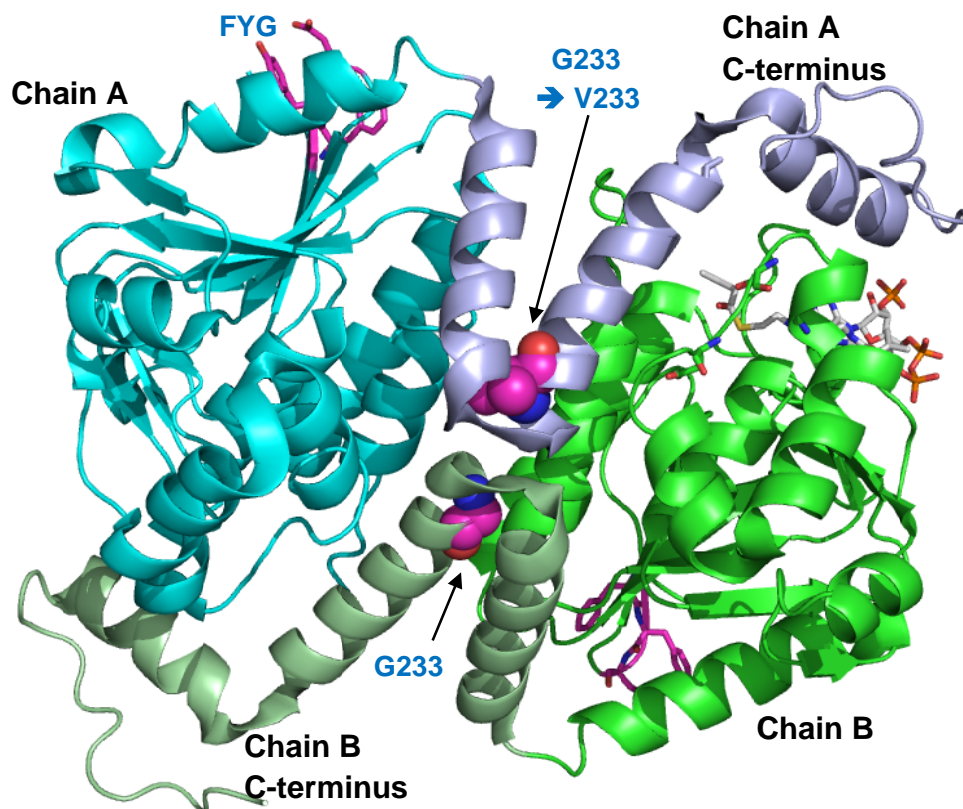
Figure 2.6: Structure of saMenB showing G233V mutation may alter the C-terminus folding. The G233V mutation was simulated by using PyMOL.

The active site residues of mtMenB and saMenB can be superimposible (**Figure 2.7**). The major difference lies on that the D185 mtMenB is not conserved in saMenB where there is a residue G144. The different directions the

Tyr residues point are probably because that AcAc-CoA lacks the OSB moiety for fixing the positions of the active site residues.
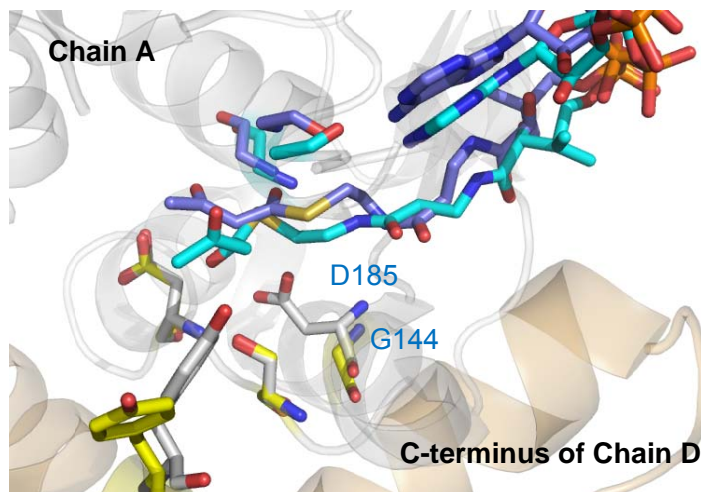
Figure 2.7: Structures of mtMenB and saMenB. The active site residues can be superimposible. The active site residues of mtMenB are colored in white while that of saMenB are shown in yellow.

MenB catalyzes Dieckmann condensation reaction

MenB catalyzes the formation of a carbon-carbon bond through an intramolecular Claisen/Dieckmann condensation (*26, 27*) (**Figure 2.8**). In the reaction, the aliphatic OSB carboxylate is activated by the formation of a CoA thioester which results in an acidification of the α-protons. Subsequently, the thioester is deprotonated to form an enolate intermediate and a β-keto ester is generated through the nucleophilic attack of the enolate anion on the second OSB carboxylate. The enzyme catalyzes the reaction by stabilizing the enolate anion and providing a good leaving group for the carboxylate (*28*). The driving force of this ring closure reaction would probably also be supplied by the

aromatization process. A usual Dieckmann type reaction requires activation at two carboxyl groups whereas in this case we have shown that only one carboxyl group is activated. Ser190, Asp192 and Tyr287 are conserved in mtMenB. Asp185 is not conserved in all MenBs such as MenB from *E. coli* and from *S. aureus*.

OSB-CoA   DHNA-CoA
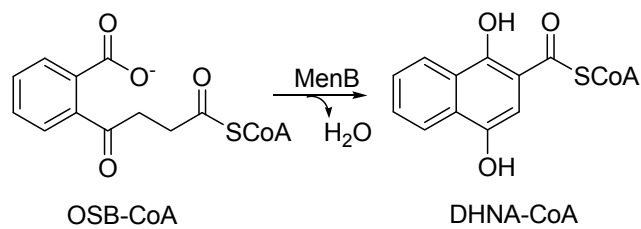
Figure 2.8: Reaction catalyzed by MenB

In this chapter, we will focus on the MenB reaction by comparing the mechanism of the *M. tuberculosis* MenB enzyme with that of MenB from *E. coli*. The following questions will be addressed:

1. Do mtMenB and ecMenB utilize the same mechanism and the same substrate for catalytic reaction?

2. D185 mtMenB is crucial for reaction. However, there is no homolog in ecMenB and saMenB. What is the functional role of D185 for catalysis?

3. The gene encoding for the enzyme which catalyzes the hydrolysis of DHNA-CoA to DHNA has not been found. Are the hydrolysis and instability of DHNA-CoA relevant to MenB?

Materials and methods

Reagents and general methods

The *E. coli* strains XL1-Blue, BL21 (DE3), BL21 CodonPlus, *Pfu Turbo* DNA polymerase were obtained from Stratagene. DpnI was ordered from Biolab Inc. T4 DNA ligase, and restriction enzymes (NdeI, XhoI, BamHI) were purchased from Invitrogen. High pure plasmid isolation kit (Roche) was used for minipreps of DNA plasmids. Qiaquick PCR purification kit (Qiagen) was used for purification of DNA products from agarose gels, restriction digest and PCRs. Oligonucleotides (primers) were ordered from IDT[®]. The thrombin, streptavidin-agarose, pET15b and pET23b vectors were purchased from Novagen. His-Bind resin and Ni-NTA His-Bind resin were obtained from Invitrogen. DE52 resin was from Whatman and Q Sepharose resin was bought from Pharmacia Biotech. Centriplus units were from Millipore. QuikChange site-directed mutagenesis kit was obtained from Stratagene[®] (Stratagene, La Jolla, CA).

The extinction coefficients and masses of proteins were calculated based on the amino acid sequences using ProtParam program (http://us.expasy.org/tools /protparam.html).

All other chemical reagents were purchased from Sigma-Aldrich or Fisher.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian NMR spectrometer with tetramethylsilane as an internal standard at 25 ºC. Data are reported as follows: chemical shift in ppm (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, and multiplet), and integration. Mass spectra were obtained on an Agilent Technologies MS instrument with ionization voltages of 50 eV. Column

chromatography was performed with SiO_2 (Silicyclo SiliaFlash 60 (230-400 mesh)).

Expression and purification of wild-type MenB from M. tuberculosis (mtMenB)

The *menb* gene *Rv0548c* (945 bp), used for the overexpression and purification of MenB, encoding for a putative 1,4-dihydroxynaphthoyl-CoA synthase from *M. tuberculosis*, was previously isolated by PCR from genomic DNA, cloned into the pET-15b plasmid (Novagen) and placed in frame with an N-terminal His-tag sequence, by Yuguo Feng. Protein expression was performed using *E. coli* CodonPlus cells. Transformed cells were grown in 800 mL of LB media containing 0.2 mg/mL ampicillin and induction was achieved using 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) overnight at 25 ºC. Cells were harvested by centrifugation at 5,000 rpm for 20 min at 4 ºC, resuspended in 30 mL of His-binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9) and lysed by 3 passages through a French Press cell (1,000 psi). Cell debris was removed by centrifugation at 33,000 rpm for 90 min at 4 ºC. MenB was purified using His affinity chromatography: the supernatant was loaded to a column containing 3 mL of His-bind resin (Novagen), charged with 9 mL of charge buffer (Ni^{2+}). The column was washed with 20 mL of His-binding buffer and 20 mL of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9). MenB was eluted using a gradient of 20 mL elute buffer (0.5 M imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9). Fractions containing MenB were collected and the imidazole removed by chromatography on G-25 resin using 20 mM NaH_2PO_4, 0.1

M NaCl at pH 7.0, as storing buffer. The concentration of MenB was determined by measuring the absorption at 280 nm using an extinction coefficient of 41,370 $M^{-1}cm^{-1}$ calculated from the primary sequence. The protein was assessed by SDS-PAGE as a dominant band of 37 kDa. The enzyme was concentrated by using Centricon-30 (Amicon) and stored at -80 °C.

Cloning, expression and purification of mutant mtMenB

mtMenB D185G and D185E mutant plasmids were prepared using QuickChange site-directed mutagenesis with the following primers:

mtMenB D185G primers:

Forward: 5' CGCTTCAAGCAGACCGGGGCCGACGTCGGCAGC 3'

Reverse: 5' GCTGCCGACGTCGGCCCCGGTCTGCTTGAAGCG 3'

mtMenB D185E primers:

Forward: 5' CTTCAAGCAGACCGAGGCCGACGTCGGCAG 3'

Reverse: 5' CTGCCGACGTCGGCCTCGGTCTGCTTGAAG 3'

mtMenB mutant of D185N, S190A, D192N and Y287F were prepared by Yuguo Feng. The procedure for the expression and purification of mutant mtMenB was used as described for wild-type mtMenB.

Cloning, expression and purification of wild-type MenB from E. coli (ecMenB)

The *menb* gene *b2262* (858 bp) was amplified from crude *E. coli* cell by PCR and cloned into the pET-15b plasmid (Novagen) by using the oligonucleotides (primers) forward: (5') GGAGGAATTCCATATGATGATTTATCCTGATGA (3')

and reverse: (5') CGCGGATCCTTATTACGGATTCCGTTTGAA (3'). Use of the *NdeI* and *BamHI* restriction sites placed the *menb* gene in-frame with an N-terminal Hig-tag sequence. Protein expression was performed using BL21 (DE3) cells. Transformed cells were grown in 800 mL of LB media containing 0.2 mg/mL ampicillin and induction was achieved using 1 mM IPTG overnight at 25 ºC. Cells were harvested by centrifugation at 5,000 rpm for 20 min at 4 ºC, resuspended in 30 mL of Ni-NTA Bind Buffer (10 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0) and lysed by 3 passages through a French Press cell (1,000 psi). Cell debris was removed by centrifugation at 33,000 rpm for 90 min at 4 ºC. MenB was purified using His affinity chromatography: the supernatant was loaded to a column containing 3 mL of Ni-NTA His-Bind resins (Novagen). The column was washed with 20 mL of Ni-NTA Bind Buffer and 20 mL of wash buffer (20 mM imidazole, 300 mM NaCl, 20 mM sodium phosphate buffer, pH 8.0). MenB was eluted using a gradient of 20 mL elute buffer (250 mM imidazole, 300 mM NaCl, 20 mM sodium phosphate buffer, pH 8.0). Fractions containing MenB were collected and the imidazole removed by chromatography on G-25 resin using 20 mM NaH_2PO_4, 0.1 M NaCl at pH 7.0, as storing buffer. N-terminal His-tag was cleavage by thrombin overnight at room temperature. With the His-tag, *ec*MenB was not stable. The concentration of MenB was determined by measuring the absorption at 280 nm using an extinction coefficient of 36,040 $M^{-1}cm^{-1}$ calculated from the primary sequence. The protein was assessed by SDS-PAGE as a dominant band of 31 kDa. The enzyme was concentrated by using Centricon-10 (Amicon) and stored at -80 ºC.

Cloning and expression of mutant ecMenB

ecMenB G156D was prepared using QuickChange site-directed mutagenesis with the following primers:

Forward: 5' GCCATCTTCGGTCAGACTGACCCGAAAGTCGGT 3'

Reverse: 5' GGAGGAACCGACTTTCGGGTCAGTCTGACCGAA 3'

The procedure for the expression and purification of mutant ecMenB was used as described for wild-type ecMenB.

Cloning, expression and purification of wild-type MenB from S. aureus (saMenB)

The *menb* gene *SAOUHSC_00985* (822 bp) was amplified from *S. aureus*_NCTC8325 genomic DNA by PCR and cloned into the pET-15b plasmid (Novagen) by using the oligonucleotides (primers) forward: (5') GGAATTCCATATGATGACTAACAGACAATGGGAAAC (3') and reverse: (5') CGGGATCCTTATGGGAATTTAGGGAATTGA (3'). Use of the *NdeI* and *BamHI* restriction sites placed the *menb* gene in-frame with an N-terminal Hig-tag sequence. Protein expression was performed using BL21 (DE3) cells. Transformed cells were grown in 800 mL of LB media containing 0.2 mg/mL ampicillin and induction was achieved using 0.5 mM IPTG overnight at 25 ºC. Cells were harvested by centrifugation at 5,000 rpm for 20 min at 4 ºC, resuspended in 30 mL of Ni-NTA Bind Buffer (10 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0) and lysed by 3 passages through a French Press cell (1,000 psi). Cell debris was removed by centrifugation at 33,000 rpm for 90 min at 4 ºC. saMenB was purified using His affinity

chromatography: the supernatant was loaded to a column containing 3 mL of Ni-NTA His-Bind resins (Novagen). The column was washed with 20 mL of Ni-NTA Bind Buffer and 20 mL of wash buffer (20 mM imidazole, 300 mM NaCl, 20 mM sodium phosphate buffer, pH 8.0). saMenB was eluted using a gradient of 20 mL elute buffer (250 mM imidazole, 300 mM NaCl, 20 mM sodium phosphate buffer, pH 8.0). Fractions containing saMenB were collected and the imidazole removed by chromatography on G-25 resin using 20 mM NaH_2PO_4, 0.1 M NaCl at pH 7.5, as storing buffer. saMenB was assessed by SDS-PAGE as the dominant band at 30 kDa. N-terminal Hig-tag was cleaved by thrombin overnight at room temperature. With the Hig-tag or without Hig-tag, saMenB was not stable. The concentration of saMenB was determined by measuring the absorption at 280 nm using an extinction coefficient of 31,390 $M^{-1}cm^{-1}$ calculated from the primary sequence. The enzyme was concentrated by using Centricon-10 (Amicon) and stored at -80 ºC.

Cloning, expression and purification of mutant saMenB

saMenB G144D was prepared using QuickChange site-directed mutagenesis with the following primers:

Forward: 5' GCTATTTTTGGACAAACTGATCCTAAAGTAGGTTCATTTG 3'

Reverse: 5' CAAATGAACCTACTTTAGGATCAGTTTGTCCAAAAATAGC 3'

The procedure for the expression and purification of mutant saMenB was used as described for wild-type saMenB.

Cloning, expression and purification of Wild-type YfbB from E. coli

The *ybfB* gene *b2263* (759 bp) was amplified from crude *E. coli* cell by PCR and cloned into the pET-15b plasmid (Novagen) by using the oligonucleotides (primers) forward: (5') GGAATTCCATATGATCCTGCACGCGCAGGC (3') and reverse: (5') GCCCTAGGTCAGAAACGCAAGATCTGCGCC (3'). Use of the *NdeI* and *BamHI* restriction sites placed the *yfbB* gene in-frame with an N-terminal Hig-tag sequence. Protein expression was performed using BL21 (DE3) cells. Transformed cells were grown in 800 mL of LB media containing 0.2 mg/mL ampicillin and induction was achieved using 1 mM IPTG overnight at 25 ºC. Cells were harvested by centrifugation at 5,000 rpm for 20 min at 4 ºC, resuspended in 30 mL of His-binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9) and lysed by 3 passages through a French Press cell (1,000 psi). Cell debris was removed by centrifugation at 33,000 rpm for 90 min at 4 ºC. YfbB was purified using His affinity chromatography: the supernatant was loaded to a column containing 3 mL of His-bind resin (Novagen), charged with 9 mL of charge buffer (Ni^{2+}). The column was washed with 20 mL of His-binding buffer and 20 mL of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9). YfbB was eluted using a gradient of 20 mL elute buffer (0.5 M imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9). Fractions containing YfbB were collected and the imidazole removed by chromatography on G-25 resin using 20 mM NaH_2PO_4, 0.1 M NaCl at pH 7.0, as storing buffer. The concentration of YfbB was determined by measuring the absorption at 280 nm using an extinction coefficient of 37,650 $M^{-1}cm^{-1}$ calculated from the primary sequence. The protein was

assessed by SDS-PAGE as a dominant band of 27 kDa. The enzyme was concentrated by using Centricon-10 (Amicon) and stored at -80 ºC.

Coupled assay of MenB reaction

OSB-CoA, the substrate for MenB, is unstable and decomposes rapidly to spirodilactone. The half-life of degradation is 15 min at 30 ºC and more than 2 hours at 0 ºC (*27*). Therefore, we used a coupled assay with *E. coli* MenE, the preceding enzyme in the menaquinone biosynthesis pathway, which synthesizes OSB-CoA *in situ*, to assay the MenB reaction (**Figure 2.9**). All coupled reactions were performed in 20 mM NaH_2PO_4 pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$ and an excess of *E. coli* MenE. Formation of DHNA-CoA was monitored on a CARY-300 spectrophotometer at 25 °C by following the increase in absorption at 392 nm using an extinction coefficient of 4, 000 $M^{-1}cm^{-1}$.
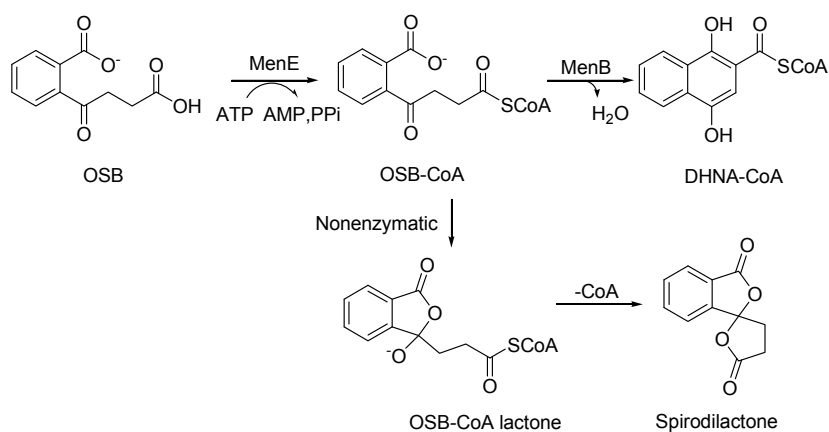
Figure 2.9: The formation of the naphthoic ring catalyzed by MenB and the

spirodilactone in a non-enzymatic reaction

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Pre-incubation of the MenB coupled assay

The pre-incubation of MenE, ATP, CoA and OSB was performed in 20 mM NaH$_2$PO$_4$ pH 7.0, 150 mM NaCl, 1 mM MgCl$_2$ and an excess of *E. coli* MenE for 3 min to allow complete conversion of OSB to OSB-CoA. MenB reaction was initiated by the addition of MenB. Formation of DHNA-CoA was monitored on a CARY-300 spectrophotometer at 25 °C by following the increase in absorption at 392 nm using an extinction coefficient of 4, 000 M^{-1}cm^{-1}.

Ellman assay for detecting thioesterase activity

Ellman Assay was used to detect the free sulphydryl groups using Ellman's reagent (5, 5'-Dithio-bis (2-nitrobenzoic acid) (DTNB)) (*29*). The thioesterase activities of MenB and YfbB were monitored at 410 nm in the presence of excess Ellman's reagent.

Kinetic data analysis

Determining K$_m$ and *k$_{cat}$*: V$_{max}$ and K$_m$ values were obtained by fitting all the data to the Michaelis-Menten equation using GraFit 4.0, $v = \dfrac{V_{max}[S]}{K_m + [S]}$. *k$_{cat}$* values were obtained using the relationship between *k$_{cat}$* and V$_{max}$: $V_{max} = k_{cat} * [E]$.

Purification of chorismate from E. coli cells

Chorismate was purified from an *E. coli* KA12 strain that had been engineered by constructing mutant strain of *E. coli* which lacks chorismate mutase activity (*30*). Chorismate was subsequently purified using the procedure

described by Grisostomi C. etc. (*30*). 15 mL of *E. coli* KA12 cell culture was grown from a single colony at 37 ºC. 800 mL of growth medium A (1.6 g casamino acids, 1.6 g yeast extract, 32.8 mg L-tryptophan, 18 mL 50 × Vogel & Bonner salts) in 4 L Erlenmeyer flask was autoclaved, added with glucose to 0.16% (w/v), and then inoculated with 15 mL preculture. The culture was grown at 30 ºC for 6 hours to reach OD_{260} = 1.9-2.1 and then centrifuged at 5000 g for 15 min. The cells were resuspended in nonsterile 500 mL of accumulation medium B (6.4 g Na_2HPO_4, 0.68 g kH_2PO_4, 9 g glucose, 1.35 g NH_4Cl, 10.15 mg $MgCl_2 \cdot 6H_2O$, 1 mg L-tryptophan). The suspension was shaken at 30 ºC for 16 hours allowing for production of chorismate and its secretion into the medium. After removing the cells centrifugation, the supernatant was collected, brought to pH 9.0 and transferred to an ion-exchange column (BioRad AG1-X8, 200-400 mesh, Dowex 1-Cl; 6 × 2.4 cm). The column was washed with 80 mL water and chorismate was eluted as the ammonium salt with 1 M NH_4Cl (pH8.5). The chorismate-containing fractions were collected by monitoring the absorbance at 274 nm, acidified with concentrated HCl to pH 1.5, and extracted with dichloromethane (3 × 35 mL) to remove most of the phenylpyruvate and then extracted with ethyl acetate (4 × 25 mL). The water was dried over Na_2SO_4 and ethyl acetate was removed under the vacuum. The crude chorismate was yellow oil, which was stored at -80 ºC.

Crude chorismate was purified by semi-preparative HPLC (Vydac C18 column, 10 μm particle diameter, 10 mm i.d., 250 mm length). A linear gradient (0-10 min 0% acetonitrile, 10-20 to 30% acetonitrile in 5% acetic acid water) was applied. The absorbance of the eluent was monitored at 274 nm. The chorismate

fraction was eluted from 3-6 min. The peak of interest was collected over several runs, frozen, and lyophilized. Around 100 mg of chorismate was obtained. ESI-MS [M+Na$^+$]: calcd 249.03 ($C_{10}H_{10}Na^+$); found 249.0.

Enzymatic synthesis of o-succinylbenzoic acid (OSB) from chorismate

4.4 mM Chorismate, 5 mM glutamate, 5 mM NAD$^+$, 50 μM THDP, 1 μM MenF, 1 μM MenB, 0.5 μM MenC, 1 μM YfbB and 20 unit *L*-glutamic dehydrogenase were incubated in phosphate buffer (50 mM NaH$_2$PO$_4$, 2 mM MgSO$_4$, pH 7.8) at 37 ºC for 4 hours. The product was purified by semi-preparative HPLC (Vydac C18 column, 10 μm particle diameter, 10 mm i.d., 250 mm length). A linear gradient (0-10 minuest 0% acetonitrile, 10-20 to 30% acetonitrile in 5% acetic acid water) was applied. The absorbance of the eluent was monitored at 254 nm. The OSB faction was eluted at 13 min. ESI-MS [M-H$^-$]: calcd 221.05 ($C_{11}H_9O_5^-$); found 221.1.

Synthesis of o-succinylbenzoic acid (OSB)

40.0 g of succinic acid (0.34 mol) was heated in a three-neck round bottom flask to about 200 ºC until melting. Then 30.0 g of phthalic anhydride (0.2 mol) and 16.4 g of sodium acetate (0.2 mol) were added and mixed. The reaction mixture was refluxed at the temperature of 200-220 ºC for 30-40 min until CO$_2$ and water evolution ceased. The reaction mixture was extracted with boiling water four times (4 × 100 mL). 13.2 g of crude spirodilactone was precipitated from the boiling water when it was cooled down. 10.3 g (0.05 M) of pure

spirodilactone was obtained after recrystallization with MeOH (25% of yield). 10.3 g (0.05 M) of spirodilactone was then refluxed in 500 mL of 1.5 M NaOH solution for 30 min. The cooled solution was changed to pH 1-2 with HCl and extracted with 100 mL ethyl acetate for three times. Ethyl acetate was removed under the vacuum and 10.5 g (0.05 mol) of OSB was obtained as white powders (95% of yield) (**Figure 2.10**). ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 7.85-7.62 (4H, m), 2.61(2H, t), 2.49 (2H, t) ppm (**Figure 2.11**). ^{13}C NMR (400 MHz, $(CD_3)_2CO$): ESI-MS δ 172.78, 166.93, 133.56, 129.83, 126.89, 125.49, 123.11, 34.42, 29.10 ppm (**Figure 2.12**). ESI-MS [M-H$^-$]: calcd 221.05 ($C_{11}H_9O_5^-$); found 221.1.
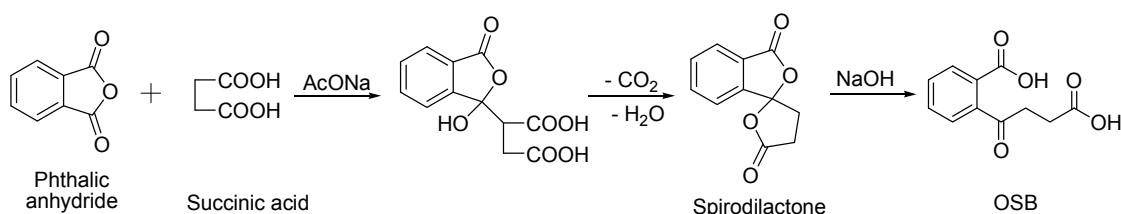
Figure 2.10: Synthesis of o-succinylbenzoic acid (OSB)
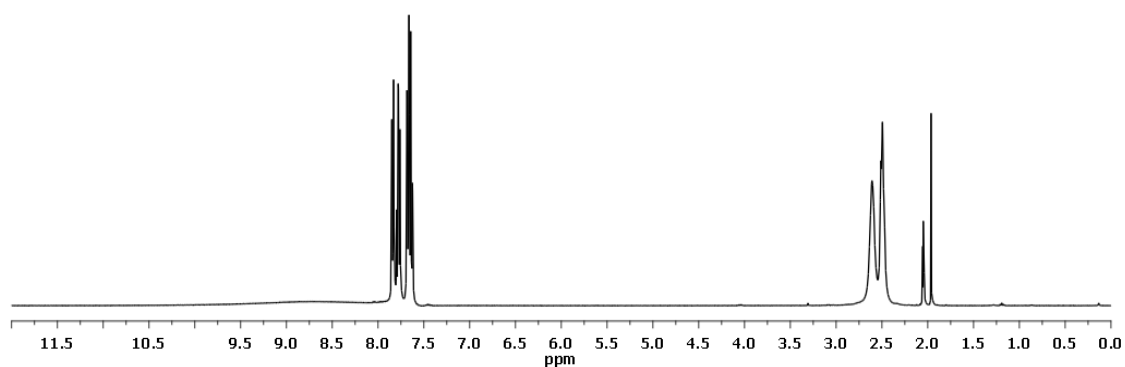
Figure 2.11: ^{1}H NMR spectrum ($(CD_3)_2CO$) of OSB, 12-0 ppm
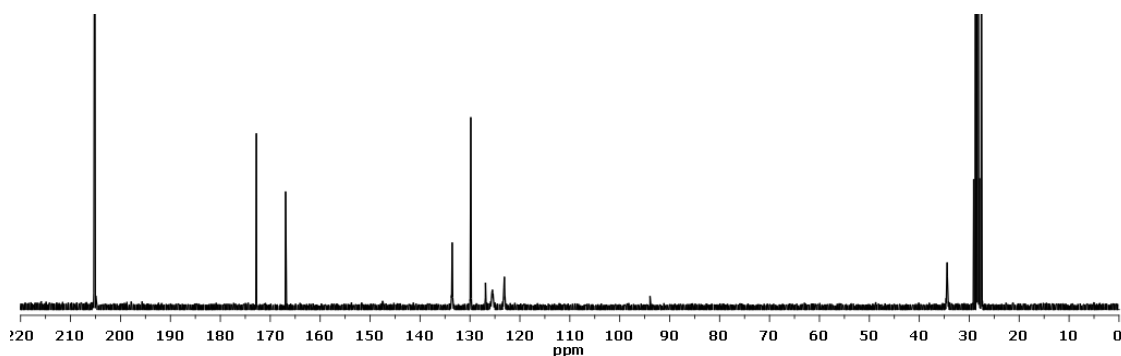
Figure 2.12: ^{13}C NMR spectrum ($(CD_3)_2CO$) of OSB, 220-0 ppm

Synthesis of N-[2'-(3-carbozypropionyl) benzoyl] imidazole

N-[2'-(3-carbozypropionyl) benzoyl] imidazole synthesis was refered to Rainer Kolkmann method (*31*). In short, 222 mg of OSB (1 mmol) and 356 mg of N, N'-carbonyldiimidazole (CDI) (2.2 mmol) were mixed in 8 mL of anhydrous THF at 35 °C under the protection of N_2. 120 µL of acetic acid was added after 5 hours and the reaction mixture was kept at 35 °C for another 30 min. THF and water were removed under the vacuum. The yellow oil was redissolved in 3 mL of THF and left at -20 °C for crystallization. The recovered crystals were washed with chilled water and 100 mg of product (0.36 mmol, two step yield is 36%) was obtained as white crystals after drying (**Figure 2.13**). ^1H NMR (400 MHz, $(CD_3)_2SO$): δ 12.32 (1H, broad s), 7.98-7.69 (5H, m), 7.27(1H, d), 6.97(1H, d), 3.07-2.87(2H, m), 2.08-1.89 (2H, m) ppm (**Figure 2.14**). ESI-MS [M-H$^-$]: calcd 221.05 ($C_{14}H_{11}N_2O_4$$^-$); found 221.1.

Figure 2.13: Synthesis of N-[2'-(3-carbozypropionyl) benzoyl] imidazole

Figure 2.14: ^{1}H NMR spectrum ((CD$_3$)$_2$SO) of N-[2'-(3-carbozypropionyl) benzoyl] imidazole, 13-0 ppm

Synthesis of methyl ester OSB

100 mg N-[2'-(3-carbozypropionyl) benzoyl] imidazole (0.36 mmol) and 41 mg sodium methoxide (0.72 mmol) were incubated in 5 mL of methanol overnight at room temperature. The mixture was washed with ethyl acetate, changed pH to 2 with HCl. After extraction with ethyl acetate, removal of the organic solvent under the vacuum gave 25 mg (0.11 mmol) of the product as a white powder (the yield was 30%) (**Figure 2.15**). ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.84-7.52 (4H, m), 3.85 (3H, s), 3.11 (2H, t), 2.58 (2H, t) ppm (**Figure 2.16**). ^{13}C NMR (400 MHz, CD$_3$OD): ESI-MS δ 206.02, 176.55, 169.18, 143.97, 133.64, 131.57, 130.98, 130.27, 128.05, 38.54, 29.37 ppm (**Figure 2.17)**. ESI-MS [M-H$^-$]: calcd 235.07 (C$_{12}$H$_{11}$O$_5{}^-$); found 235.1.

Figure 2.15: Synthesis of methyl ester OSB

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Figure 2.16: ^{1}H NMR spectrum (CD$_3$OD) of methyl ester OSB, 10-2 ppm.

Figure 2.17: ^{13}C NMR spectrum (CD$_3$OD) of methyl ester OSB, 220-0 ppm

HMBC Spectrum of methyl ester of OSB

A two-dimensional HMBC (Heteronuclear Multiple Bond Correlation) experiment was performed to confirm regioselective incorporation of the methyl group to the aromatic carboxylic acid. In HMBC, cross peaks are between protons and carbons that are two or three bonds away (J = 2-15 Hz), while direct one-bond cross-peaks are suppressed. In **Figure 2.18**, the characteristic methyl group's protons at 3.85 ppm show bond connectivity with the aromatic ketone carbon at 169.18 ppm. In addition, two adjacent methylene groups' protons (δ 3.11 and 2.58 ppm) show connectivity with the ketone and carboxylate carbonly carbons (δ 206.02 and 176.55 ppm) forming a square.

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Figure 2.18: HMBC spectrum (500 MHz, CD$_3$OD) of methyl ester OSB

Synthesis of methylamine OSB

52.5 mg N-[2'-(3-carbozypropionyl) benzoyl] imidazole (0.19 mmol) was dissolved 1 mL of methylamine in THF (2M, 2 mmol). 1 mL each of THF and H$_2$O were added, and the reaction mixture was stirred overnight at room temperature. The mixture was then washed with ethyl acetate. The pH of the solution was adjusted to 2 by addition of conc. HCl and the mixture was extracted with ethyl acetate. 30 mg (0.13 mmol) light yellow powder was obtained (the yield was 68%) (**Figure 2.19**). ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.74-7.52 (4H, m), 2.96 (3H, s), 2.43 (2H, m), 1.85 (1H, m), 1.72 (1H, m) ppm (**Figure 2.20**). ESI-MS [M-H$^-$]: calcd 234.08 (C$_{12}$H$_{12}$NO$_4^-$); found 234.0.

Figure 2.19: Synthesis of methylamine OSB

Figure 2.20: ^{1}H NMR spectrum (CD$_3$OD) of methylamine OSB, 9-0 ppm

Synthesis of dimethylamine OSB

The same procedure was used for the synthesis of dimethylamine OSB as had been used for the synthesis of methylamine OSB except that dimethylamine replaced methylamine. Starting with 52.5 mg N-[2'-(3-carbozypropionyl) benzoyl] imidazole (0.19 mmol) and 250 μL of 40% dimethylamine (2 mmol), 20 mg (0.08 mmol) of yellow powder was obtained (the yield was 50%) (**Figure 2.21**). ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.10-7.32 (4H, m), 3.32 (2H, t), 3.10 (3H, s), 2.77 (3H, s), 2.70 (2H, t) ppm (**Figure 2.22**). ESI-MS [M-H$^-$]: calcd 248.10 C$_{13}$H$_{14}$NO$_4^-$); found 248.0.

Figure 2.21: Synthesis of dimethylamine OSB

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Figure 2.22: ^{1}H NMR spectrum (CD$_3$OD) of dimethylamine OSB, 9-0 ppm

Standard procedure for the synthesis of acyl-CoA thioester

Acyl-CoA thioesters were prepared *via* activation of the acid with ethylchloroformate (**Figure 2.23**) (*32*). The acid (1 mmol) was dissolved in 20 mL of anhydrous tetrahydrofuran (THF), with stirring and external cooling by an ice bath. Dry triethylamine (1 mmol) was added, followed by dropwise addition of ethylchloroformate (1 mmol). After an additional hour of stirring in the ice bath, the precipitated triethyl ammonium chloride was removed by gravity filtration, and the filtrate was subsequently transferred to a dry addition funnel.

Figure 2.23: Synthesis of CoA thioester

CoA (40 µmol) was dissolved in 10 mL of water, and the pH was adjusted to 7 with 0.1 M NaOH. The solution of activated acid was added dropwise over 30 min to the CoA solution, while maintaining pH 7 by adding 0.1 M NaOH dropwise as necessary. After addition was complete, the reaction was allowed to stir

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another four hours. The reaction mixture was acidified to pH 1-2 using conc. HCl and extracted with ethyl acetate. The water layer was frozen in liquid nitrogen and lyophilized.

The product was purified from the lyophilized powder by semi-preparative HPLC (Vydac C18 column, 10 μm particle diameter, 10 mm i.d., 250 mm length). A linear gradient (0 to 40% acetonitrile, 40 min) was applied. The absorbance of the eluent was monitored at 260 nm. The peak of interest was collected over several runs, frozen, and lyophilized. The purified product was redissolved in water and quantitated by measuring the absorbance at 260 nm (ε_{260} for CoA: 1.69×10^4 $M^{-1}cm^{-1}$).

Synthesis of 3-benzoylpropionic CoA (3-BP CoA)

Starting with 3-benzoylpropionic acid, the standard procedure for the synthesis of coenzyme A thioester was used as described on page 63. ^{1}H NMR (600 MHz, D_2O) spectrum (**Figure 2.24**) was assigned in **Table 2.1**. ESI-MS [M-H$^-$]: calcd 926.17 ($C_{31}H_{43}N_7O_{18}P_3S^-$); found 926.1.

Figure 2.24: ^{1}H NMR spectrum (D_2O) of 3-BP CoA, 10-0 ppm

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Table 2.1: Chemical shifts of the protons of 3-benzoylpropionic CoA (3-BP CoA)

¹H	δ (ppm)	¹H	δ (ppm)
2a	2.95, t, 2H	4'	4.50, d, 1H
3a	3.32, t, 2H	5'	4.17, d, 1H
6a, 10a	7.92, t, 2H	1''	3.74. 3.44, s, 2H
7a, 9a	7.50, t, 2H	3''	3.92, s, 1H
8a	7.55, m, 1H	5''	3.32, t, 2H
2	8.11, s, 1H	6''	2.30, t, 2H
8	8.42, s, 1H	8''	3.25, t, 2H
1'	6.05, d, 1H	9''	2.95, t, 2H
2'	4.72, dd, 1H	10''	0.87, s, 3H
3'	4.68, dd, 1H	11''	0.73, s, 3H

COSY and HMBC of 3-BP CoA

The assignment of ¹H NMR spectrum was also based on the two-dimensional COSY and HMBC analysis. In the two-dimensional COSY (COrrelation SpectroscopY) experiments, magnetization is transferred by scalar coupling. Protons that are more than three chemical bonds apart give no cross signal because the ^4J coupling constants are close to 0. Therefore, only signals of protons which are two or three bonds apart are visible in a COSY spectrum. The

COSY spectrum (**Figure 2.25**) of 3-BP CoA showed three pairs of cross peaks which represent the interaction between the 2a and 3a (δ 2.95 and 3.32 ppm), 5'' and 6'' (δ 3.32 and 2.30 ppm), and 8'' and 9'' (δ 3.25 and 2.95 ppm) methylene groups.

In the HMBC spectrum of 3-BP CoA (**Figure 2.26**), 4a-carbon (220 ppm) has the cross peaks with 2a-, 3a- and 5a-protons. 4''-carbon (δ 177.5 ppm) corresponds with 3''- and 5''-protons. 7''-carbon (δ 177.6 ppm) talks with 6''- and 8'' protons.

Figure 2.25: COSY spectrum of 3-BP CoA (600 HMz, D$_2$O)

Figure 2.26: HMBC spectrum of 3-BP CoA (600 HMz, D_2O)

Synthesis of o-(3-carboxypropyl)-benzoic CoA (OCPB-CoA)

OCPB-CoA was synthesized using MenE from *E. coli*. ATP, CoA, OCPB and MenE were incubated for 3 hours at room temperature in phosphate buffer (20 mM NaH_2PO_4, 0.1 M NaCl, 1 mM $MgCl_2$ at pH 7.0). The reaction mixture was purified by semi-preparative HPLC (Vydac C18 column, 10 μm particle, 10 mm i.d., 250 mm length). A linear gradient (0-40% acetonitrile in 20 mM ammonia acetate, 40 min) was applied. OCPB-CoA was collected, frozen and lyophilized. ^{1}H NMR (600 MHz, D_2O) spectrum of OCPB-CoA was showed in **Figure 2.27**.

The assignment for OCPB-CoA was given in **Table 2.2**. ESI-MS [M-H⁻]: calcd 956.18 ($C_{32}H_{45}N_7O_{19}P_3S^-$); found 956.2.

Figure 2.27: ¹H NMR spectrum (D₂O) of OCPB-CoA, 10-0 ppm

Table 2.2: Chemical shifts of the protons of OCPB-CoA

¹H	δ (ppm)	¹H	δ (ppm)
2a	2.44, t, 2H	4'	4.03, d, 1H
3a	1.92, m, 2H	5'	4.26, d, 2H
4a	2.79, t, 2H	3"	4.62, s, 1H
6a-8a	7.20-7.40, m, 3H	5"	3.33, t, 2H
2	8.28, s, 1H	6"	2.61, t, 2H
8	8.69, s, 1H	8"	3.46, t, 2H
1'	6.20, d, 1H	9"	2.99, t, 2H
2'	3.86, dd, 1H	10"	0.91, s, 3H
3'	3.57, dd, 1H	11"	0.77, s, 3H

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Synthesis of methyl ester OSB-CoA

24 mg (0.1 mmol) of OSB methyl ester was reacted with 19 mg CDI (0.12 mmol) in 10 mL of anhydrous THF for 2 hours at 35 °C under nitrogen. CoA (40 µmol) was dissolved in 10 mL of water, and the pH was adjusted to 7 with 0.1 M NaOH. THF solution was added dropwise over 30 min to the CoA solution, while maintaining pH 7 by adding 0.1 M NaOH dropwise as necessary. After addition was complete, the reaction was allowed to stir another three hours (**Figure 2.28**). The reaction was acidified to pH 1-2 using 1M HCl and extracted with ethyl acetate. The water layer was frozen in liquid nitrogen and lyophilized. The product was purified from the lyophilized powder by semi-preparative HPLC (Vydac C18 column, 10 µm particle diameter, 10 mm i.d., 250 mm length). A linear gradient (1 to 40% acetonitrile, 40 min) was applied. The absorbance of the eluent was monitored at 260 nm. Methyl ester OSB-CoA was eluted at 18 min and collected over several runs, frozen and lyophilized. ^{1}H NMR (600 MHz, D$_2$O) spectrum of methyl ester OSB-CoA was shown in **Figure 2.29**. The assignment for methyl ester OSB-CoA was given in **Table 2.3**. ESI-MS [M-H$^-$]: calcd 984.17 (C$_{33}$H$_{45}$N$_7$O$_{20}$P$_3$S$^-$); found 984.1.

Figure 2.28: Synthesis of methyl ester OSB-CoA

Figure 2.29: ^{1}H NMR spectrum (D$_2$O) of methyl ester OSB-CoA, 10-0 ppm

Table 2.3: Chemical shifts of the protons of methyl ester OSB-CoA

^{1}H	δ (ppm)	^{1}H	δ (ppm)
2a	2.95, t, 2H	4'	(4.50) 4.15, d, 1H
3a	3.26, t, 2H	5'	(4.17) 3.91, d, 1H
6a-10a	7.58-7.31, m, 4H	1''	3.72. 3.45, s, 2H
12a	3.79, s, 3H	3''	3.92, s, 1H
2	8.11, s, 1H	5''	3.34, t, 2H
8	8.44, s, 1H	6''	2.32, t, 2H
1'	6.04, d, 1H	8''	3.21, t, 2H
2'	(4.72) 4.48, dd, 1H	9''	2.95, t, 2H
3'	(4.68) 4.15, dd, 1H	10'',11''	0.77, 0.64,s, 6H

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Synthesis of methylamine OSB-CoA and dimethylamine OSB-CoA

Starting with methylamine OSB and dimethylamine OSB, the standard procedure on page 67 was used to synthesize the methylamine and dimethylamine derivatives of OSB-CoA. Methylamine OSB-CoA was not stable due to the dilactone degradation. Dimethylamine OSB-CoA was stable with ESI-MS [M-H$^-$]: calcd 998.20 ($C_{34}H_{48}N_8O_{19}P_3S^-$); found 997.1.

Synthesis of dimethoxy DHNA-CoA

Starting with dimethoxy DHNA, the standard procedure on page 67 was used to synthesize dimethoxy DHNA-CoA. ESI-MS [M-H]: calcd 980.17 ($C_{35}H_{47}N_7O_{19}P_3S^-$); found 980.1.

Circular dichroism (CD) spectra of wild-type mtMenB and its mutants

The far-UV circular dichroism (CD) spectra of the wild-type mtMenB protein and its mutant variants were measured at a protein concentration of 20 µM in 20 mM NaH$_2$PO4, 150 mM NaCl, pH 7.0 buffer at 25 °C by using an AVIV 62 DS spectrometer equipped with a Peltier temperature control unit. The far-UV CD spectra of the wild-type BadI protein and its mutant variants were similarly recorded at a protein concentration of 20 µM in 20 mM Tris-HCl, 150 mM NaCl, pH 7.9 buffer at 25 °C.

CD spectroscopy of different mutants indicated mtMenB mutants retained very similar overall folding as wild-type protein, suggesting that mutagenesis had not altered the overall structure of the protein and that the reduced activity of the

mutant enzymes was not a consequence of a major change in protein structure (**Figure 2.30**).

Figure 2.30: CD spectra of wild-type and mutant mtMenB

Isothermal titration calorimetry (ITC) binding experiment of mtMenB

Isothermal titration calorimetry (ITC) was used to analyze the interactions of mtMenB and ligands, by measuring the stepwise change of ΔH° during the titration of the ligand into the protein solution. The interaction of mtMenB with 3 BP-CoA, OCPB-CoA, acetoacetyl CoA, methyl ester OSB-CoA, dimethylamine OSB-CoA and dimethoxy DHNA-CoA was monitored. Data was analyzed by applying the one site model to the mtMenB-ligand interaction and using nonlinear least squares curve fitting. ITC experiments were performed on a MicroCal VP-ITC unit with a cell volume of 1.445 mL. All the experiments were conducted in 20 mM NaH_2PO_4, 0.1 M NaCl buffer (pH 7.0) at 25 °C. The concentration of mtMenB was 100 mM and the ligand concentration varied from 1 mM to 2 mM depending on its binding affinity for the enzyme. All solutions were degassed

under the vacuum prior to use. The first data point was routinely deleted before curve fitting to eliminate the effect of diffusion at the protein/ligand interface at the titration syringe tip during the pre-titration equilibration period. Nonlinear least-squares curve fitting was performed using Origin v 5.0 using a standard one-site model supplied by MicroCal.

Alpha-proton exchange reactions catalyzed by mtMenB

Wild-type mtMenB (100 μM) and its mutants were incubated with 2 mM of methyl ester OSB-CoA, 3-BP CoA and OCPB-CoA in a NMR tube, respectively. Proton-deuterium exchange of α-proton of the ligand was monitored by NMR (600 HMz) spectroscopy at 1h, 3 h, 1 day, 3 days, and 1 week. Prior to the incubation, mtMenB was transferred into D_2O phosphate buffer (150 mM NaCl, 20 mM NaH_2PO_4 in D_2O at pD of 7.0). The ligands separated from HPLC were lyophilized with D_2O 3 times.

Observing the degradation of DHNA in D_2O

DHNA was incubated in D_2O pD 10.0 in order to observe its degradation product. Without incubation, ^1H NMR (400 MHz, D_2O) of DHNA: δ 7.87-7.30 (4H, m), 6.89 (1H, s) ppm; ^{13}C NMR (400 MHz, D_2O) of DHNA: δ 176.18, 151.76, 143.25, 127.84, 125.83, 122.58, 122.49, 110.50, 107.54 ppm. After 2 hour incubation, ^1H NMR (400 MHz, D_2O) of DHNA degradation product: δ 7.67-7.11 (4H, m) ppm; ^{13}C NMR (400 MHz, D_2O) of DHNA degradation product: δ 176.11, 151.97, 142.67, 127.59, 125.80, 124.74, 122.55, 121.17, 110.34 ppm (**Figure**

2.31). ESI-MS [M-H$^-$]: calcd 218.02 (C$_{11}$H$_5$O$_5$$^-$); found 217.0.
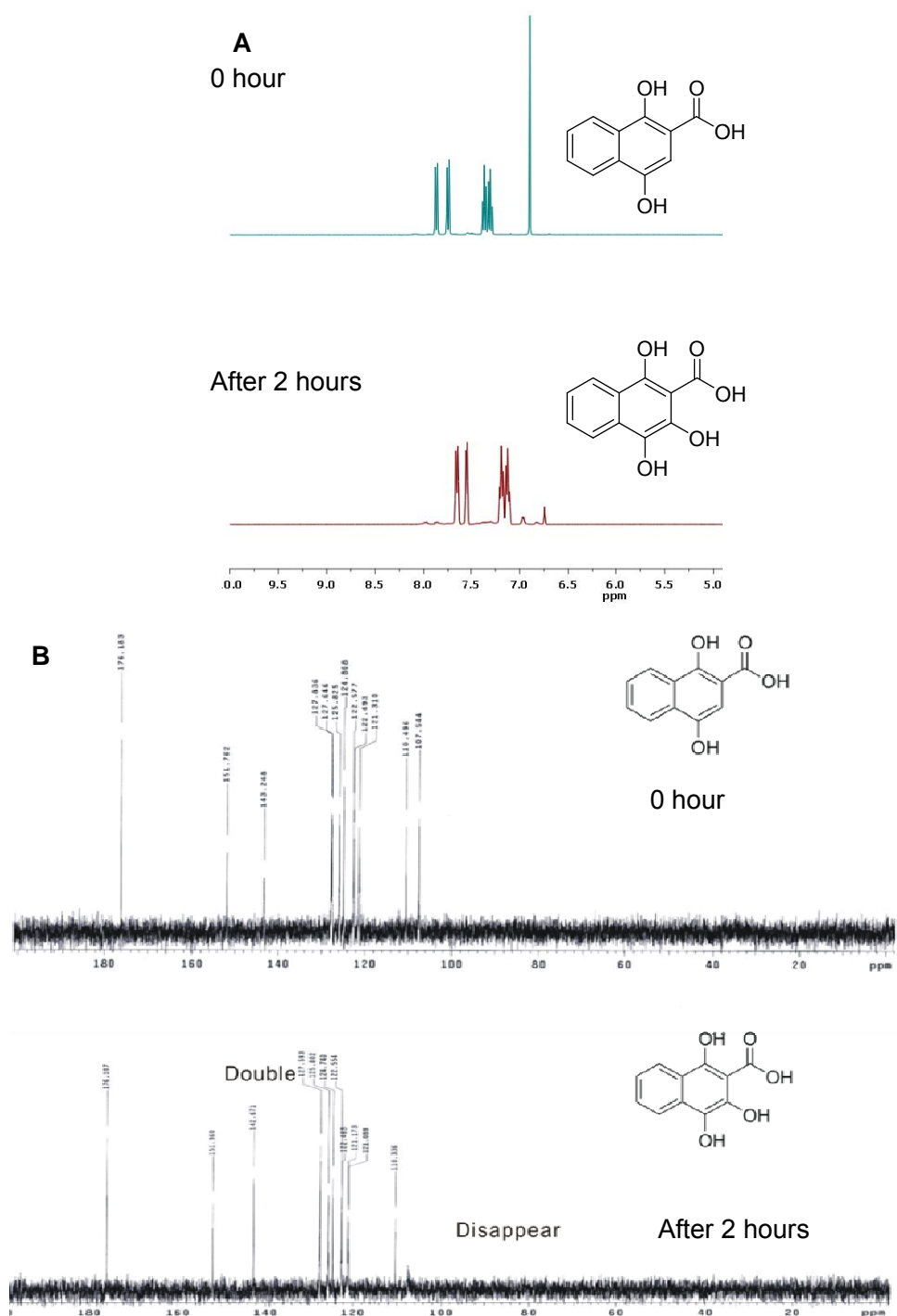
Figure 2.31: Degradation of DHNA. Figure A: ^{1}H NMR spectra (D$_2$O, pD 14.0) of DHNA.
Figure B: ^{13}C NMR spectra (D$_2$O, pD 14.0) of DHNA.

X-Ray crystallography of mtMenB bound with dimethoxy DHNA-CoA

mtMenB along with dimethoxy DHNA-CoA were submitted to Dr. James J. Truglio and Prof. Caroline Kisker at the Rudolf Virchow Center, DFG Research Center for Experimental Biomedicine (Germany). The structures were solved with dimethoxy DHNA-CoA bound in the active site.

Structural analysis

The structural data was analyzed using PyMOL (DeLano Scientific LLC.). Superpositions of enzymes structure were generated by aligning the residues of the oxyanion hole. Protein secondary structure analysis was obtained from website (http://www.rcsb.org/pdb/home/home.do) based on method mentioned in reference (*33*).

Results and discussion

Expression and catalytic activity of MenBs (mtMenB, ecMenB and saMenB)

The MenB enzymes from *M. tuberculosis*, *E. coli* and *S. aureus* (mtMenB, ecMenB and saMenB) were expressed with an N-terminal His-tag and purified by affinity chromatography as previously described (**Figure 2.32**).

12% SDS-PAGE gel of mtMenB (37 kDa)

1. Whole cells
2. Flow through fraction
3. Binding buffer fraction
4. Wash buffer fraction
5. Elute buffer fraction
6. Ladder
7-9. G25 column fraction

12% SDS-PAGE gel of ecMenB (31 kDa)

1. Flow through fraction
2. Binding buffer fraction
3. Wash buffer fraction
4. Ladder
5. Elute buffer fraction
6-8. G25 column fraction

12% SDS-PAGE gel of saMenB (30 kDa)

1. Pellet
2. Flow through fraction
3. Binding buffer fraction
4. Wash buffer fraction
5-7. Elute buffer fraction
8-10. G25 column fraction
11. Ladder

Figure 2.32: SDS-PAGE gels of mtMenB, ecMenB and saMenB

Because His-tagged ecMenB and saMenB precipitated readily, N-terminal His-tags were cleavage by thrombin after purification. Non-His-tagged ecMenB was then stable and its catalytic activity wasn't affected after the cleavage of the N-terminal His-tag. However, the stability of non-His-tagged saMenB wasn't improved, and both saMenBs showed extremely low catalytic activity which was only observed at 10 μM enzyme concentration. The kinetic parameters for the MenB enzymes are shown in **Table 2.4**. Surprisingly, given the slow growth rate of *M. tuberculosis*, k_{cat} for mtMenB was significantly larger than for ecMenB and saMenB.

Table 2.4: Kinetic parameter for wild-type mtMenB, ecMenB and saMenB

Enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1}·μM^{-1})
mtMenB	27.7 ± 0.9	22.4 ± 2.1	1.2 ± 0.2
ecMenB	3.7 ± 0.1	25.9 ± 3.3	0.14 ± 0.03
saMenB	Showed very low activity at 10 μM saMenB		

Binding and alpha-proton exchange studies of substrate analogues of mtMenB

A series of MenB substrate analogues have been synthesized to investigate the mechanism of the reaction catalyzed by MenB (**Figure 2.33**). The ITC data suggests that changes to the structure of the OSB acyl group only have a modest effect on the affinity of the ligands for the enzyme with the exception of the dimethylamide OSB-CoA analogue (**Table 2.5**). Based on the ITC data, it is suggested that the aromatic carboxyl group in the OSB portion and entire CoA

molecule are essential for binding. The interaction between the CoA thioester and oxyanion-hole residues contributes the major binding capacity for substrate. It has been shown that both CoA and AcAc-CoA (**Figure 2.4**) have good binding affinity with the protein although they lack the OSB framework. The aromatic carboxyl group is also important for binding since methyl ester OSB-CoA (K_d = 11.5 µM) has a better binding affinity then 3-BP CoA (K_d = 33.0 µM). The fact of the poor binding affinity of 3-BP CoA which is even worse than CoA and AcAc-CoA indicates that the aromatic ketone in the OSB portion might not be the key factor for binding. Dimethylamide OSB-CoA cannot bind into the active site, suggesting that the space for fitting the aromatic carboxyl group into the active site is crucial.
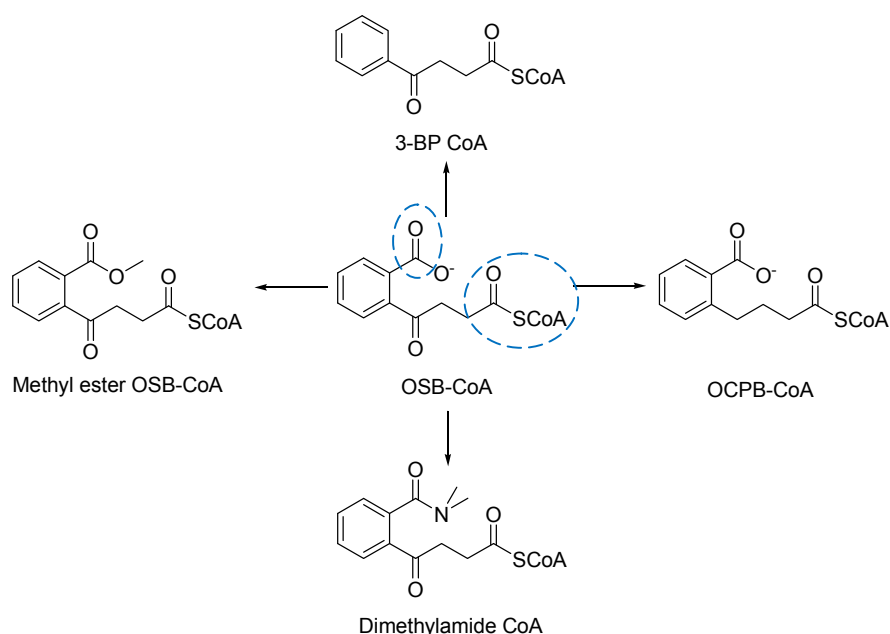
Figure 2.33: Structures of MenB substrate analogues. Both aromatic carboxyl group and CoA thioester are crucial for binding.

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Table 2.5: Binding affinity of mtMenB with substrate analogues (by ITC)

Substrate analogue	K_d (µM)
CoA	24.2 ± 1.4
AcAc-CoA	21.0 ± 1.2
Methyl ester OSB-CoA	11.5 ± 1.2
OCPB-CoA	16.6 ± 1.5
3-BP CoA	33.0 ± 1.6
Dimethylamide OSB-CoA	No binding at 4 mM of ligand

Based on the mechanism of Dieckmann condensation which is proposed in reference (*14*), the proton at the α-carbon of CoA thioester will be abstracted to form an oxyanion before the nucleophilic attack occurs (**Figure 2.34**). Therefore, a proton-deuterium exchange at this position could happen if the substrate analogues are perfectly fitted into the active site. 2 mM of methyl ester OSB-CoA, 3-BP CoA and OCPB-CoA were incubated with wild-type mtMenB (100 µM) and its mutants in D_2O, respectively, at different length of period. Unfortunately, no α-proton exchange was observed in the experiment. One reasonable explanation is that the conformation of the active site of MenB doesn't adapt to the deprotonation upon binding with the above substrate analogues. It was suggested neither of these substrate analogues correctly position into the active site and prepare to abstract the proton of α-carbon of thioester.
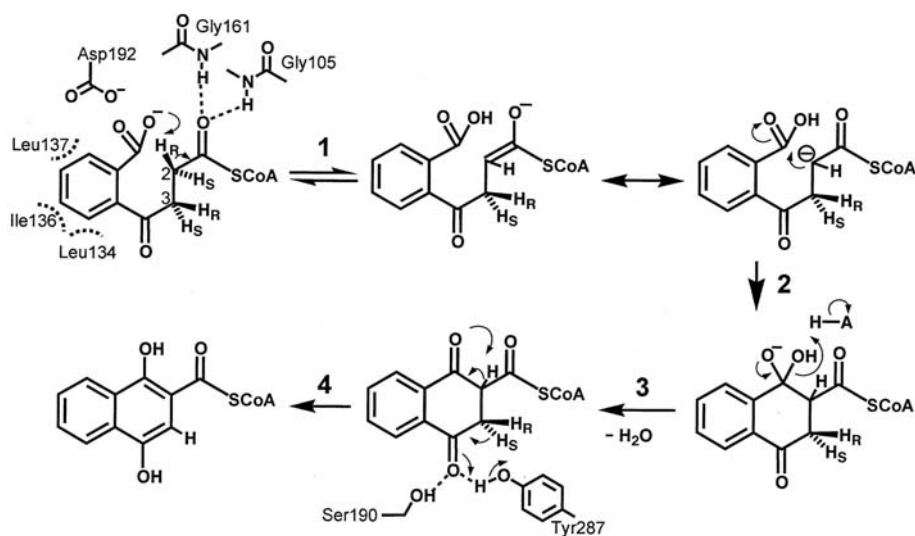
Figure 2.34: Proposed mechanism of reaction catalyzed by MenB

If the leaving group in the proposed mechanism is water, then methanol should work as a better leaving group. In this regard, we designed substrate analogue, methyl ester OSB-CoA, assuming it might be a good substrate for MenB since it contains a better leaving group. However, it was shown that methyl ester OSB-CoA is not a substrate for MenB. Therefore, it can be deduced that the free carboxyl group is essential for the reaction. The lack of activity of OSB methyl ester led us to reconsider the decomposition of OSB-CoA and its relationship to the enzyme catalyzed reaction.

We also tried to use the OCPB-CoA and 3-BP CoA for co-crystallization with mtMenB. Unfortunately, the experiments were not successful.

Lactone OSB-CoA might be the substrate for mtMenB

The substrate for MenB, OSB-CoA, is unstable and decomposes to spirodilactone in solution as shown in **Figure 2.35.** The decomposition might

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undergo a lactone intermediate and therefore the equilibrium of OSB-CoA and lactone OSB-CoA might exist in solution before the release of the CoA molecule (**Figure 2.35**).
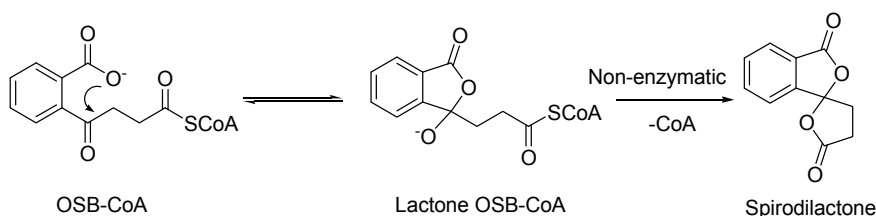
Figure 2.35: The degradation of OSB-CoA to spirodilactone

The study of the equilibrium between OSB and lactone OSB (**Figure 2.36**) was instead used to examine the equilibrium between OSB-CoA and lactone OSB-CoA since the existence of the molecule CoA won't affect the equilibrium. To examine the formation of a lactone from OSB, NMR spectra of OSB were acquired under a variety of conditions. It was found that in D_2O at pD 14.0 the major species was OSB. However under acidic conditions or in CD_3CN (0.025% DCl) the lactone of OSB was formed which was characterized by a new resonance in the NMR spectrum at 112 ppm (**Figure 2.37**). Therefore it can be concluded that basic conditions favor OSB and OSB-CoA while acidic conditions stabilize the lactone formation.
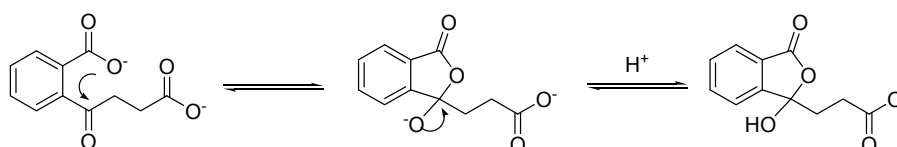
Figure 2.36: The equilibrium of OSB and lactone OSB
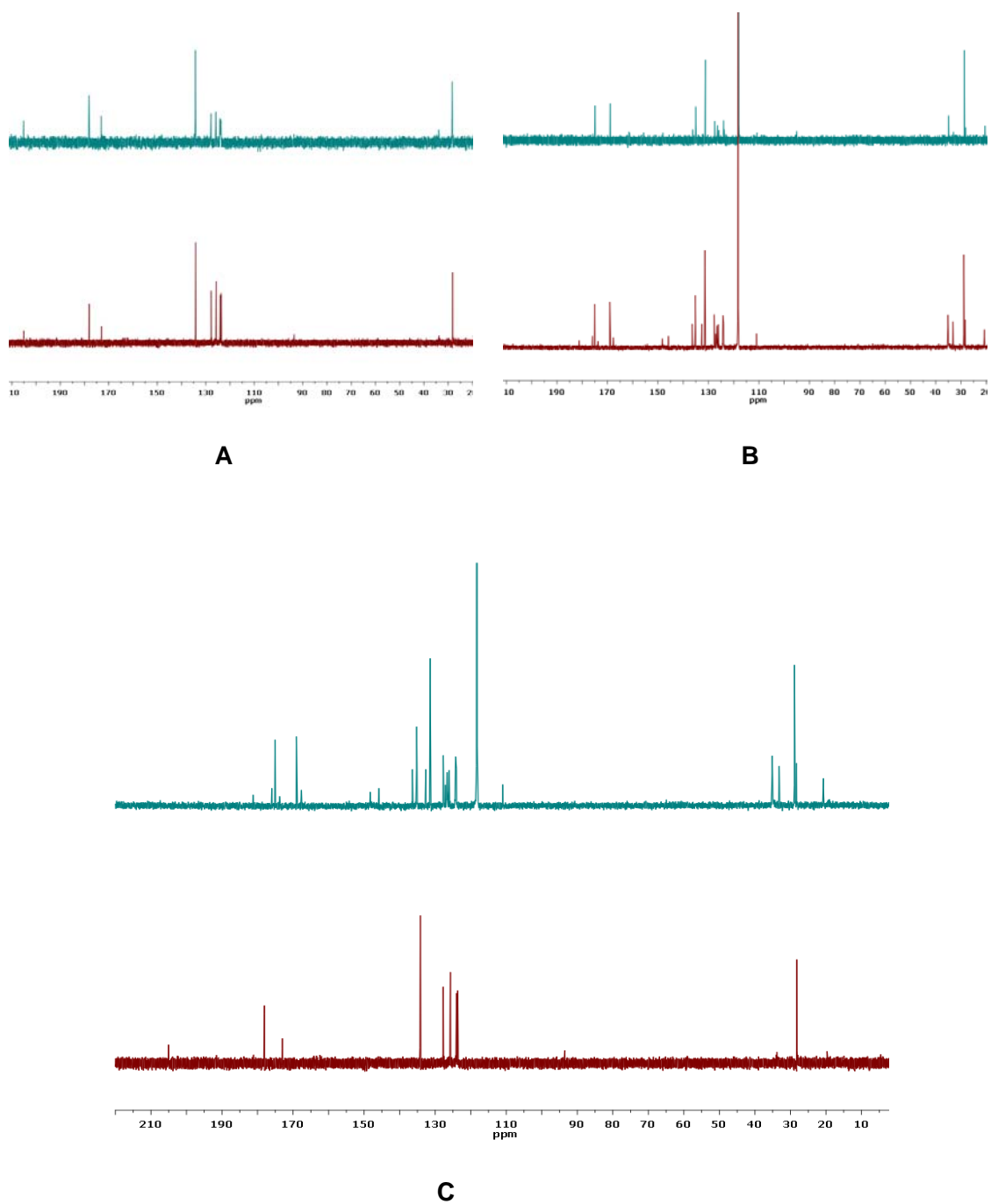
Figure 2.37: ^{13}C NMR spectra of OSB and lactone OSB. Figure A: OSB in D$_2$O at pD 14.0; Figure B: OSB in CD$_3$CN (0.025% DCl); C: OSB in D$_2$O at pD 14.0 and CD$_3$CN (0.025% DCl) with 2 hour incubation.

In order to directly observe lactone formation we speculated that NMR spectroscopy could be used to monitor lactone formation in situ. This required synthesis of OSB labeled with ^{13}C at the keto carbonyl. A synthetic route for the labeled OSB using ^{13}C glutamate is shown in **Figure 2.38**. However the overall yield of OSB was only about 5-10%. Consequently, the biosynthesis of OSB needs further optimization before labeled starting material can be committed.
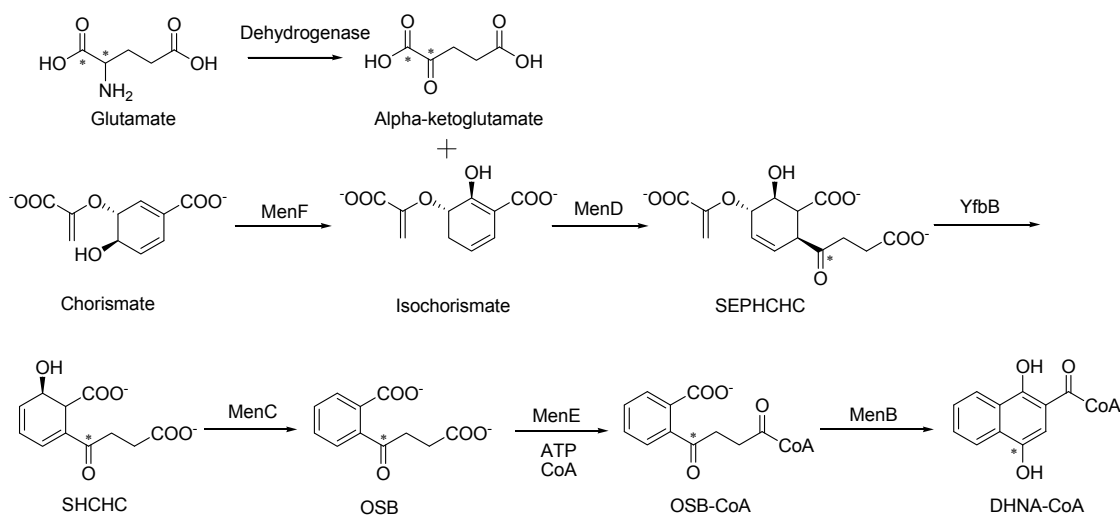
Figure 2.38: Synthesis of ^{13}C labeled OSB-CoA from chorismate

Direct binding experiments and NMR alpha-proton exchange studies together with enzyme kinetics indicate that the aromatic carboxylate in OSB-CoA is essential for reactivity. In addition, methyl ester OSB-CoA and OCPB-CoA are not the substrates for mtMenB, indicating the requirement for the aromatic carboxyl group. Since OSB and OSB-CoA react in solution to form a lactone, we hypothesized that this could be the actual substrate for mtMenB. This possibility is attractive since the lactone carbonyl is more reactive than a free carboxylate.

In order to explore the possibility that the OSB-CoA lactone is the substrate for mtMenB, pre-incubation experiments were performed with 4 µM of ecMenE. Since OSB-CoA will yield spirodilactone with a half-life of 15 min at 30 ºC and more than 2 hours at 0 ºC (*27*), more than 90% of substrates don't convert to spirodilactone and are in the equilibrium between OSB-CoA and lactone OSB-CoA based within 3 min's pre-incubation at 25 ºC (**Figure 2.35**).

Pre-incubation for wild-type mtMenB reaction showed different kinetic results supporting the lactone intermediate (**Table 2.6**). Without pre-incubation, the values of k_{cat} and K_m values were 27.7 ± 0.9 min^{-1} and 22.4 ± 2.1 µM respectively. With pre-incubation, the k_{cat} value didn't change too much since the ring closure step is the rate-limiting step. The K_m value, however, decreased about 20 fold indicating mtMenB has a better binding affinity with lactone OSB-CoA than with OSB-CoA.

In contrast, pre-incubation for wild-type ecMenB reaction relatively increased the K_m value for OSB-CoA, suggesting that ecMenB has a better binding affinity with OSB-CoA than with lactone OSB-CoA (**Table 2.6**).

Table 2.6: Different kinetic data of MenB reaction with/without

pre-incubation

	k_{cat} (min^{-1})	K_m (µM)	k_{cat}/K_m (min^{-1}µM^{-1})
Wild-type mtMenB			
Pre-incubation	24.6 ± 0.4	1.3 ± 0.1	18.9 ± 1.9
No incubation	27.7 ± 0.9	22.4 ± 2.1	1.2 ± 0.2
Wild-type ecMenB			
Pre-incubation	3.9 ± 0.2	40.4 ± 4.1	0.09 ± 0.01
No incubation	3.7 ± 0.1	25.9 ± 3.3	0.14 ± 0.03
D185E mtMenB			
Pre-incubation	0.1 ± 0.01	3.7± 0.3	0.035 ± 0.005
No incubation	0.14 ± 0.01	4.8 ± 0.3	0.029 ± 0.004

The different pre-incubation results of wild-type mtMenB and wild-type ecMenB reactions suggest that they may utilize lactone OSB-CoA and OSB-CoA as a substrate, respectively. Since the lactone formation activates the carboxylate of OSB-CoA, why would ecMenB favor the substrate of lactone OSB-CoA? Based on the sequence assignment of mtMenB and ecMenB (**Figure 2.39**), the major difference of proposed catalytic residues in the active site of mtMenB and ecMenB is that D185 mtMenB is not conserved in ecMenB. Consequently it was anticipated that D185 would play a key role in the catalytic mechanism of mtMenB.

```
mtMenB    VVAPAGEQGRSSTALSDNPFDAKAWRLVDGFDDLTDITYHRHVDDATVRVAFNRPEVRNA 60
ecMenB    MIYPDEAMLYAPVEWHD---------CSEGFEDIR----YEKSTDGIAKITINRPQVRNA 47
saMenB    ---------MTNRQWET---------LRE-YDEIK----YEFY-EGIAKVTINRPEVRNA 36
                        :                  : ::::     :.   :. .:::::***:****


mtMenB    FRPHTVDELYRVLDHARMSPDVGVVLLTGNGPSPKDGGWAFCSGGDQRIRG-RSGYQYAS 119
ecMenB    FRPLTVKEMIQALADARYDDNIGVIILTG------AGDKAFCSGGDQKVRGDYGGYKDDS 101
saMenB    FTPKTVAEMIDAFSRARDDQNVSVIVLTG------EGDLAFCSGGDQKKRG-HGGYVGED 89
          * * ** *:   .:   ** . ::.*::***        *. ********: **  .**   .


mtMenB    GDTADTVDVARAGRLHILEVQRLIRFMPKVVICLVNGWAAGGGHSLHVVCDLTLASREYA 179
ecMenB    ----------GVHHLNVLDFQRQIRTCPKPVVAMVAGYSIGGGHVLHMMCDLTIAA-DNA 150
saMenB    ----------QIPRLNVLDLQRLIRIIPKPVIAMVKGYAVGGGNVLNVVCDLTIAA-DNA 138
                        :*::*:.** **   ** *:..:* *:: ***: *:::****:*: : *


mtMenB    RFKQTDADVGSFDGGYGSAYLARQVGQKFAREIFFLGRTYTAEQMHQMGAVNAVAEHAEL 239
ecMenB    IFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQALDMGLVNTVVPLADL 210
saMenB    IFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQEALDMGLVNTVVPLEKV 198
           * **...*****.*:*:.*:** **:* ****::* * * *::   :** **:*.   .:


mtMenB    ETVGLQWAAEINAKSPQAQRMLKFAFNLLDDGLVGQQLFAGEATRLAYMTDEAVEGRDAF 299
ecMenB    EKETVRWCREMLQNSPMALRCLKAALNADCDGQAGLQELAGNATMLFYMTEEGQEGRNAF 270
saMenB    EDETVQWCKEIMKHSPTALRFLKAAMNADTDGLAGLQQMAGDATLLYYTTDEAKEGRDAF 258
          *    ::*. *:  :** * * ** *:*    ** .* * :**:** * * *:*. ***:**


mtMenB    LQKRPPDWSPFPRYF 314
ecMenB    NQKRQPDFSKFKRNP 285
saMenB    KEKRDPDFDQFPKFP 273
           :** **:. * :
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Figure 2.39: Sequence alignment of mtMenB, ecMenB and saMenB. mtMenB and ecMenB share 46% identity; mtMenB and saMenB share 50% identity; and ecMenB and saMenB share 62% identity. Residues of the oxyanion hole are in blue; conserved catalytic residues are in red; and unconserved active-site residues are in gray. The cyan residues mark the start of the C-terminal domain in mtMenB and saMenB whose structures have been characterized.

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Catalytic functional role of D185 mtMenB

Although the oxyanion hole residues together with S190, D192 and Y287 (mtMenB numbering) are conserved in all MenB enzymes, D185 in mtMenB is replaced with a glycine in ecMenB and saMenB (**Figure 2.39**). Site-directed mutagenesis was used to replace each of these residues and to examine their role in the overall reaction (**Table 2.7**). D185N, D185G, D192N and Y287F mtMenB enzymes showed no activity at the concentration of 3 μM of enzyme, while D185E and S190A mutants have k_{cat}/K_m value reduced 40 and 600-fold respectively compared to the wild-type of mtMenB.

Table 2.7: Kinetic parameter mutant mtMenB, ecMenB and saMenB

Enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1}·μM^{-1})
wt mtMenB	27.7 ± 0.9	22.4 ± 2.1	1.2 ± 0.2
D185E	0.14 ± 0.01	4.8 ± 0.3	0.029 ± 0.004
S190A	0.10 ± 0.01	40.2 ± 4.5	0.002 ± 0.001
D185N, D185G, D192N, and Y287F	No activity at 3 μM mtMenB		
wt ecMenB	3.7 ± 0.1	25.9 ± 3.3	0.14 ± 0.03
G156D	No activity at 3 μM ecMenB		
wt saMenB	Showed very low activity at 10 μM saMenB		
G144D	No activity at 10 μM saMenB		

Although D185 mtMenB is not conserved in all MenBs such as ecMenB and saMenB in which they all use a glycine, it is conserved as the E164 in enoyl-CoA hydratase from rat which involves in the addition/abstraction of α-proton (*34-39*) and shares the same stereochemistry of pro-2*R* proton as mtMenB (*40*). Enoyl-CoA hydratase, the prototypical member of the crotonase superfamily, specifically catalyzes the *syn*-hydration of *trans*-2-enoyl-CoA thioesters to the corresponding 3-*(S)*-hydroxyacyl-CoA thioesters (**Figure 2.40**) (*41, 42*). The equilibrium of the enoyl-CoA hydratase-catalyzed reaction favors the hydration by a factor of 3.5 at 25 °C (*43*).

trans-2-enoyl-CoA   3-(*S*)-hydroxyacyl-CoA
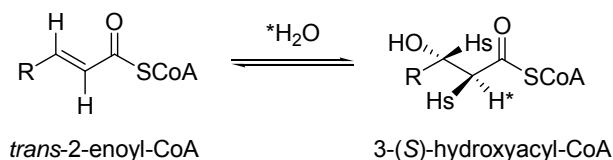
Figure 2.40: *S*-specific reaction catalyzed by enoyl-CoA hydratase

D185 mtMenB and E164 enoyl-CoA hydratase are located in similar positions in the active site (**Figure 2.41**A). Therefore, D185 may facilitate the reaction by abstracting the pro-2*R* proton. From the crystal structure of mtMenB bound with AcAc-CoA, the distance between the D185 and pro-2*R* proton is 3.87 Å (**Figure 2.41**B), which could be even shorter if the lactone OSB-CoA is bound. This is comparable with the distance between E164 enoyl-CoA hydratase and pro-2*R* proton of its ligand, which is 3.03 Å (**Figure 2.41**C).

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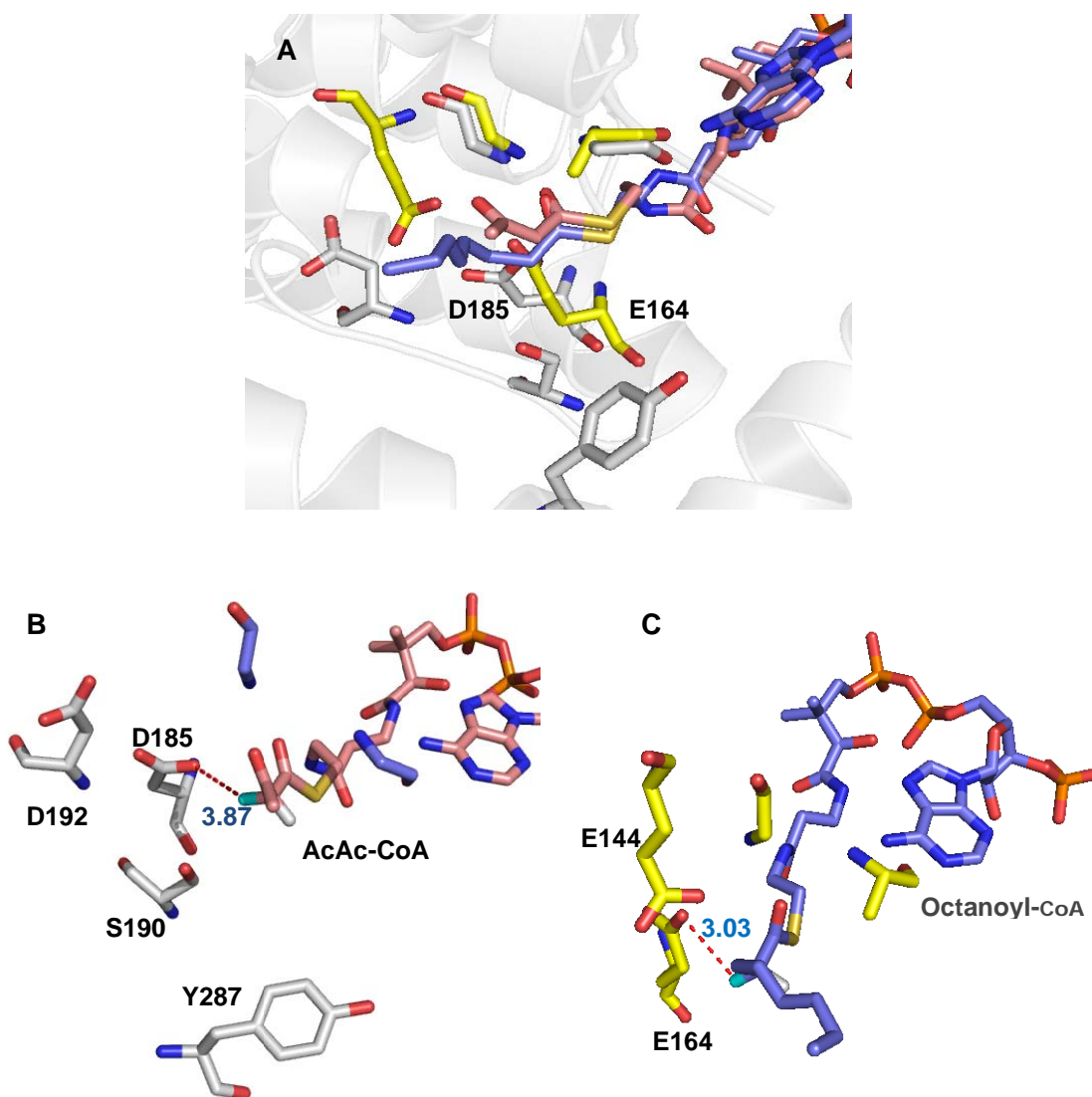
Figure 2.41: Crystal structures of mtMenB and enoyl-CoA hydratase. Figure A shows that D185 mtMenB is in the similar position as E164 enoyl-CoA hydratase. Figure B shows that D185 is close to the pro-2*R* proton of AcAc-CoA. Figure C shows that E164 is close to the pro-2*R* proton of octanoyl-CoA.

D185 clearly plays a critical role in the mechanism of the mtMenB reaction. Replacement of this residue with a glycine causes a complete loss of activity, while even subtle changes such as the replacement of D185 with a glutamate cause a dramatic decrease in activity (**Table 2.7**). The sensitivity of the reaction rate to changes in D185 mirrors that observed for replacements of the two glutamates in the active site of enoyl-CoA hydratase. For example, both the E164D and E164Q enoyl-CoA hydratase mutants have dramatically reduced activity (1000-330,000 folds) (*44*). This sensitivity to mutagenesis reflects the critical positioning of the carboxylate side chain in the active site.

However, homologue of D185 mtMenB is a G156 in ecMenB, which indicates that mtMenB and ecMenB might utilize different mechanism for catalysis. In one hand, D185 mtMenB might be involved in abstracting the pro-2R proton while ecMenB must use other base for the abstraction. On the other hand, this aspartic acid residue might contribute to the selectively of the substrate between OSB-CoA and lactone OSB-CoA. Two mutations of D185N and D185G resulted in the total loss of mtMenB activity and K_m values of two mutants couldn't be estimated accordingly. Although the replacement of D185 with an aspartate caused a 150-fold decrease in activity, D185E reduced the K_m value for OSB-CoA, indicating the loss of D185 mtMenB increased the binding affinity for OSB-CoA compared to the value observed for the wild-type enzyme (**Table 2.7**).

The sequence alignment (**Figure 2.42**) of around 300 MenBs from different organisms indicate that whether D or G is conserved is a result of evolvement.

Figure 2.43 shows that G is conserved in all MenBs from organisms close to *E. coli* while D is conserved in all MenBs from organisms close to *M. tuberculosis.*

```
eco_b2262_menB__ECK2256__JW225       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecj_JW2257_menB__dihydroxynaph       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecc_c2805_menB__naphthoate_syn       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
eci_UTI89_C2545_menB__naphthoa       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecp_ECP_2306_naphthoate_syntha       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecv_APECO1_4299_menB__naphthoa       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecl_EcolC_1386_naphthoate_synt       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecd_ECDH10B_2423_menB__dihydro       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecx_EcHS_A2408_menB__naphthoat       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecw_EcE24377A_2558_menB__napht       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecy_ECSE_2522_dihydroxynaphtho       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecm_EcSMS35_2417_menB__naphtho       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
sfv_SFV_2333_menB__naphthoate_       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
sfx_S2475_menB__naphthoate_syn       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
sfl_SF2341_menB__naphthoate_sy       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecf_ECH74115_3404_menB__naphth       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
sbc_SbBS512_E2641_menB__naphth       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
sbo_SBO_2299_menB__naphthoate_       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ssn_SSON_2323_menB__naphthoate       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ecs_ECs3150_naphthoate_synthas       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
ece_Z3522_menB__naphthoate_syn       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
sdy_SDY_2458_menB__naphthoate_       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
seg_SG2336_menB__naphthoate_sy       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
set_SEN2289_menB__naphthoate_s       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
sed_SeD_A2651_menB__naphthoate       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
see_SNSL254_A2492_menB__naphth       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
sek_SSPA0520_naphthoate_syntha       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
seh_SeHA_C2547_menB__naphthoat       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
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sea_SeAg_B2443_menB__naphthoat       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
sew_SeSA_A2535_menB__naphthoat       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
spq_SPAB_00674_naphthoate_synt       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
spt_SPA0556_menB__naphthoate_s       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
stt_t0556_menB__naphthoate_syn       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
sty_STY2537_menB__naphthoate_s       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
stm_STM2307_menB__naphthoate_s       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
sec_SC2307_menB__naphthoate_sy       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
ses_SARI_00592_naphthoate_synt       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
cko_CKO_00528_naphthoate_synth       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
esa_ESA_00951_naphthoate_synth       AAEN-AVFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
ent_Ent638_2812_naphthoate_syn       AAEN-AVFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYNAQE
kpe_KPK_1489_menB__naphthoate_       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
kpn_KPN_02660_menB__naphthoate       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAQQ
eca_ECA1213_menB__naphthoate_s       AAEN-AIFGQTGPRVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAAQ
spe_Spro_3281_naphthoate_synth       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAAA
ypb_YPTS_2652_naphthoate_synth       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
ypy_YPK_1591_naphthoate_syntha       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
ypg_YpAngola_A1783_menB__napht       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
ypi_YpsIP31758_1484_menB__naph       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
ypp_YPDSF_1935_naphthoate_synt       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
ypn_YPN_2120_naphthoate_syntha       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
ypa_YPA_2017_naphthoate_syntha       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
yps_YPTB2558_menB__naphthoate_       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
ypm_YP_2336_menB__naphthoate_s       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
ypk_y1662_menB__naphthoate_syn       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
ype_YPO2525_menB__naphthoate_s       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
yen_YE1377_menB__naphthoate_sy       AADN-AIFGQTGPKVGSFDGGWGAAYMARIVGQKKAREIWFLCRQYDAKQ
plu_plu3071_menB__naphthoate_s       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKQ
pmr_PMI1743_menB__naphthoate_s       AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYNAQE
hsm_HSM_1612_naphthoate_syntha       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYNAQE
hso_HS_0562_menB__naphthoate_s       AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYNAQE
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hiq_CGSHiGG_08420_naphthoate_s     AAEN-AIFGQTGPKVGSFDGGWGASYMARLVGQKKAREIWFLCRQYNAQE
hin_HI0968_menB__naphthoate_sy     AAEN-AIFGQTGPKVGSFDGGWGASYMARLVGQKKAREIWFLCRQYNAQE
hip_CGSHiEE_07120_naphthoate_s     AAEN-AIFGQTGPKVGSFDGGWGASYMARLVGQKKAREIWFLCRQYNAQE
hit_NTHI1141_menB__naphthoate_     AAEN-AIFGQTGPKVGSFDGGWGASYMARLVGQKKAREIWFLCRQYNAQE
pmu_PM1096_menB__naphthoate_sy     AADN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRQYDAKE
apa_APP7_1910_naphthoate_synth     AADN-AKFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRMYDAQE
apj_APJL_1860_menB__naphthoate     AADN-AKFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRMYDAQE
apl_APL_1824_menB__naphthoate_     AADN-AKFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRMYDAQE
hdu_HD1925_menB__naphthoate_sy     AAEN-AKFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRMYDAQE
msu_MS1792_menB__naphthoate_sy     AADN-AKFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRMYDAKE
asu_Asuc_0650_naphthoate_synth     AADN-AKFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRMYDAKE
aha_AHA_0529_menB__naphthoate_     AADN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRMYDAKQ
asa_ASA_3735_menB__naphthoate_     AADN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRMYDAQQ
vco_VC0395_A1559_menB__naphtho     AAEN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRFYNAQE
vch_VC1973_naphthoate_synthase     AAEN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRFYNAQE
vfm_VFMJ11_1794_menB__naphthoa     AAEN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRFYDAQE
vfi_VF_1669_menB__dihydroxynap     AAEN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRFYDAQE
vvu_VV1_3170_naphthoate_syntha     AAEN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRFYDAQE
vvy_VV1118_naphthoate_synthase     AAEN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRFYDAQE
vha_VIBHAR_01431_naphthoate_sy     AADN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRFYNAQE
vpa_VP0931_naphthoate_synthase     AADN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRFYNAQE
pin_Ping_0350_naphthoate_synth     AADN-AQFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWFLCRMYDAQE
hha_Hhal_1129_naphthoate_synth     AAEN-ARFGQTGPRVGSFDGGFGASYMASVVGQKKAREIWFLCRQYDAQE
cph_Cpha266_2091_naphthoate_sy     AADN-AIFGQTGPKVGSFDGGWGASYMARLVGQKKAREIWYLCRQYNAAD
cli_Clim_2050_naphthoate_synth     AAEN-AVFGQTGPKVGSFDGGWGASYMARLVGQKKAREIWYLCRQYNAAE
cch_Cag_1719_naphthoate_syntha     AAEN-AVFGQTGPRVGSFDGGWGASYMARLVGQKKAREIWFLCRQYNAAE
cte_CT1846_menB__naphthoate_sy     AAEN-ARFGQTGPRVGSFDGGWGASYMARLVGQKKAREIWYLCRQYNAQE
cpc_Cpar_0357_naphthoate_synth     AAEN-ARFGQTGPRVGSFDGGWGASYMARLVGQKKAREIWYLCRQYNAQE
plt_Plut_0328_naphthoate_synth     AAEN-ARFGQTGPRVGSFDGGWGASYMARLVGQKKAREIWYLCRQYTAQE
pvi_Cvib_0394_naphthoate_synth     AAEN-AIFGQTGPKVGSFDGGWGASYMARLVGQKKAREIWYLCRQYNAQE
pph_Ppha_2433_naphthoate_synth     AADN-AIFGQTGPKVGSFDGGWGASYMARLVGQKKAREIWFLCRQYNASE
cpb_Cphamn1_2077_naphthoate_sy     AAEN-AVFGQTGPKVGSFDGGWGASYMARLVGQKKAREIWYLCRQYNAQE
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paa_Paes_1880_naphthoate_synth      AAEN-AIFGQTGPKVGSFDGGWGASYMARIVGQKKAREIWYLCRQYNAQE
cts_Ctha_1987_naphthoate_synth      AAEN-AIFGQTGPKVGSFDGGYGASYMARLVGQKKAREIWYLCRQYNAQQ
amu_Amuc_0351_naphthoate_synth      AADN-AKFGQTGPKVGSFDGGLGSSYLARIVGQKKAREIWYLCRQYDARQ
pcu_pc1064_menB__naphthoate_sy      AANN-ARFGQVGPKVGSFDGGLGSSYLARIVGQKKAREIWYLCRQYDAKE
ppp_PHYPADRAFT_141103_hypothet      AADN-AVFGQTGPKVGSFDAGYGCSMMARLVGQKKAREMWFLAKFYSAEE
osa_4327859_Os01g0662700__hypo      AADN-AIFGQTGPKVGSFDAGYGTSIMSRLVGPKKAREMWFLSRFYTADE
ath_AT1G60550_naphthoate_synth      AADN-AIFGQTGPKVGSFDAGYGSSIMSRLVGPKKAREMWFMTRFYTASE
cre_CHLREDRAFT_112040_MEN2__na      AADN-AIFGQTGPKVGSFDAGYGSTHMARLVGQKKAREMWFLARLYDARE
olu_OSTLU_40076_predicted_prot      AADN-AVFGQTGPKVGSFDAGYGSTHMARLIGQKKAREMWFLARLYNASD
rxy_Rxyl_2893_naphthoate_synth      AAEN-AIFGQVGPRVGSFDGGYGASVLTQLVGPKRAKEIWFLCRQYTARE
dar_Daro_1616_naphthoate_synth      AADN-ARFGQTGPRVGSFDAGLGAGLMARTIGLKRAKEVWLLCRQYDATT
ava_Ava_0166_naphthoate_syntha      AADN-AIFGQTGPKVGSFDGGFGASYLARIVGQKKAREIWFLCRQYDAQQ
ana_all2347_naphthoate_synthas      AADN-AIFGQTGPKVGSFDGGFGASYLARIVGQKKAREIWFLCRQYDAQQ
npu_Npun_R3968_naphthoate_synt      AADN-AIFGQTGPKVGSFDGGFGASYLARIVGQKKAREIWFLCRQYDAQQ
cyt_cce_0831_menB__naphthoate_      AADN-AVFGQTGPKVGSFDGGFGASYLARIIGQKKAREIWFLCRQYNAEQ
ter_Tery_4088_naphthoate_synth      AADN-AIFGQTGPKVGSFDGGFGASYLARIVGQKKAREIWFLCRQYTAEQ
syn_sll1127_menB__1_4-dihydrox      AADN-AIFGQTGPKVGSFDGGFGSSYLARIVGQKKAREIWYLCRQYSAQE
mar_MAE_45860_menB__naphthoate      AADN-AIFGQTGPKVGSFDGGFGSSYLARVVGQKKAREIWFLCRQYNAQQ
amr_AM1_5258_menB__naphthoate_      AADN-AIFGQTGPKVGSFDGGFGASYLARVVGQKKAREIWFLCRQYDAQA
cyb_CYB_0565_menB__naphthoate_      AADN-AIFGQTGPRVGSFDGGFGAAYLARVVGQKKAREIWFLCRQYTAAQ
cya_CYA_0530_menB__naphthoate_      AADN-AIFGQTGPRVGSFDGGFGASYLARVVGQKKAREIWFLCRQYTAAQ
tel_tll2458_menB__naphthoate_s      AAEN-AIFGQTGPKVGSFDAGFGASYLARIVGQKKAREIWFLCRQYTAQE
ote_Oter_3116_naphthoate_synth      AADN-AIFGQVGPKMGSFDGGFGSSYLARLVGQKKAREIWFLCRQYNAQQ
syf_Synpcc7942_0597_naphthoate      AADN-AVFGQTGPKVGSFDGGFGASYLARLVGQKKAREIWFLCRQYGAKE
syc_syc0926_d_menB__naphthoate      AADN-AVFGQTGPKVGSFDGGFGASYLARLVGQKKAREIWFLCRQYGAKE
syp_SYNPCC7002_A0268_menB__nap      AADN-AIFGQTGPKVGSFDGGFGASYMARIVGQKKAREIWFLCRQYDAQQ
pmb_A9601_06641_menB__naphthoa      ASEN-AIFGQTGPRVGSFDAGFGSSYLARLVGQRKAKEIWFLCRKYNSEE
pmg_P9301_06341_menB__naphthoa      AAEN-AIFGQTGPRVGSFDAGFGSSYLARLVGQRKAKEIWFLCRKYNSKE
pmh_P9215_06901_naphthoate_syn      ASEN-AIFGQTGPRVGSFDAGFGSSYLARLVGQRKAKEIWFLCRKYNSKE
pmi_PMT9312_0608_naphthoate_sy      ASEN-AIFGQTGPRVGSFDAGFGSSYLARLVGQRKAKEIWFLCRKYNSKE
pmc_P9515_06731_menB__naphthoa      ASEN-AIFGQTGPRVGSFDAGFGSSYLARLVGQRKAKEIWFLCRKYNSKE
pmm_PMM0608_menB__naphthoate_s      ASEN-AIFGQTGPRVGSFDAGFGSSYLARLVGQRKAKEIWFLCRKYNSKE
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pme_NATL1_06641_menB__naphthoa      AADN-AMFGQTGPKVGSFDAGFGSSYLARVVGQKKAREIWFLCRKYGAKE
pmn_PMN2A_0044_naphthoate_synt      AADN-AIFGQTGPKVGSFDAGFGSSYLARVVGQKKAREIWFLCRKYGAKE
syw_SYNW0998_menB__naphthoate_      AADN-AVFGQTGPKVGSFDGGFGAGYLARVVGQRKAREIWFLCRRYGADE
syd_Syncc9605_1123_naphthoate_      AADN-AVFGQTGPKVGSFDGGFGAGYLARVVGQRKAREIWFLCRRYGAKE
sye_Syncc9902_1333_naphthoate_      AADN-AMFGQTGPKVGSFDGGFGAGYLARVVGQRKAREIWFLCRRYGAKD
syx_SynWH7803_1022_menB__napht      AAEN-AVFGQTGPRVGSFDGGFGAGYLARVVGQRKAREIWFLCRRYGAEE
syg_sync_1527_menB__naphthoate      AADN-AVFGQTGPRVGSFDGGFGAGYLARVVGQRKAKEIWFLCRQYGAEQ
pmf_P9303_18811_menB__naphthoa      AAEN-AVFGQTGPKVGSFDGGFGAGYLARVVGQRKAREIWFLCRQYGAEE
pmt_PMT0405_menB__naphthoate_s      AAEN-AVFGQTGPKVGSFDGGFGAGYLARVVGQRKAREIWFLCRQYGAEE
pma_Pro1053_menB__naphthoate_s      AADN-AVFGQTGPRVGSFDAGFGAGYLARVVGQRKAREIWFLCRKYGAQE
pmj_P9211_10421_naphthoate_syn      ASDN-AIFGQTGPKVGSFDGGFGAGYLTRVVGQRKAREIWFLCRRYSSEE
syr_SynRCC307_1141_menB__napth      AADN-AQFGQTGPRVGSFDGGYGCAHLARLVGQRKAREIWFLCRRYNAEQ
bat_BAS4748_naphthoate_synthas      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYNAQE
btk_BT9727_4586_menB__naphthoa      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYNAQE
bar_GBAA5109_menB__naphthoate_      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYNAQE
baa_BA_5527_enoyl-CoA_hydratas      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYNAQE
ban_BA5109_menB__naphthoate_sy      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYNAQE
btl_BALH_4419_menB__naphthoate      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYNAQE
bcz_BCZK4608_menB__naphthoate_      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYNAQE
bce_BC4853_naphthoate_synthase      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYSAQE
bca_BCE_5013_menB__naphthoate_      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYNAQE
bwe_BcerKBAB4_4695_naphthoate_      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYSAQE
bcy_Bcer98_3491_naphthoate_syn      AADN-AVFGQTGPKVGSFDGGYGAGYLARMVGHKKAREIWYLCRQYNAQE
gka_GK2873_naphthoate_synthase      AADN-AIFGQTGPKVGSFDGGYGAGYLARIVGHKKAREIWYLCRQYTAQE
gtn_GTNG_2771_naphthoate_synth      AADN-AIFGQTGPKVGSFDGGYGAGYLARIVGHKKAREIWYLCRQYNAQE
afl_Aflv_0383_menB__dihydroxyn      AADN-AIFGQTGPKVGSFDGGYGAGYLARIVGHKKAREIWYLCRQYNAQE
bld_BLi03220_menB__naphthoate_      AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
bli_BL02406_menB__naphthoate_s      AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
bay_RBAM_027780_naphthoate_syn      AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
bsu_BSU30800_menB__naphthoate_      AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
bpu_BPUM_2716_menB__naphthoate      AADN-AVFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
oih_OB2323_naphthoate_synthase      AADN-AIFGQTGPKVGSFDAGYGAGYLARLVGHKRAREIWYLCRQYNADE
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sas_SAS0981_naphthoate_synthas    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sam_MW0929_menB__naphthoate_sy    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sax_USA300HOU_0993_menB__napht    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sae_NWMN_0915_memB__naphthoate    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sao_SAOUHSC_00985_naphthoate_s    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
saa_SAUSA300_0948_menB__naphth    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sac_SACOL1054_menB__naphthoate    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sar_SAR1019_menB__naphthoate_s    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sah_SaurJH1_1127_naphthoate_sy    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
saw_SAHV_1038_menB__naphthoate    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
saj_SaurJH9_1104_naphthoate_sy    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sav_SAV1045_menB__naphthoate_s    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sau_SA0898_menB__naphthoate_sy    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sab_SAB0912_menB__naphthoate_s    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sha_SH1917_menB__naphthoate_sy    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
sep_SE0746_naphthoate_synthase    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
ser_SERP0632_menB__naphthoate_    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
ssp_SSP1746_naphthoate_synthas    AADN-AIFGQTGPKVGSFDAGYGSGYLARIVGHKKAREIWYLCRQYNAQE
lsp_Bsph_4275_naphthoate_synth    AADN-ARFGQTGPKVGSFDAGYGSGYLARIIGHKKAREIWYLCRQYDAQQ
esi_Exig_0625_naphthoate_synth    AADN-AKFGQTGPKVGSFDAGYGSGYLARVVGHKKAREIWYLCRQYDAQQ
llm_llmg_1831_menB__naphthoate    ASEN-AKFGQTGPRVGSFDAGYGAGLLAAMVGQKKAREIWFLCRQYTAKE
llc_LACR_0771_naphthoate_synth    ASEN-AKFGQTGPRVGSFDAGYGAGLLAAMVGQKKAREIWFLCRQYTAKE
lla_L0171_menB__naphthoate_syn    ASEN-AKFGQTGPRVGSFDAGYGAGLLAAMVGQKKAREIWFLCRQYTAQE
dsy_DSY0520_menB__naphthoate_s    ASEN-ARFGQTGPRVGSFDAGYGAGYLARIVGHKKAREIWYLCRQYTAQE
lmf_LMOf2365_1697_menB__naphth    AADN-AKFGQTGPNVGSFDAGYGSGYLARVIGHKKAKEVWFMCRQYTADE
lmo_lmo1673_menB__naphthoate_s    AADN-AKFGQTGPNVGSFDAGYGSGYLARVIGHKKAKEVWFMCRQYTADE
lin_lin1781_naphthoate_synthas    AADN-AKFGQTGPNVGSFDAGYGSGYLARVIGHKKAKEVWFMCRQYTADE
lwe_lwe1691_menB__naphthoate_s    AADN-AKFGQTGPNVGSFDAGYGSGYLARVIGHKKAKEVWFMCRQYTADE
lfe_LAF_0969_naphthoate_syntha    AADN-AMFGQTGPKVGSFDAGYGSGYLARVIGHKRAKEVWFLNHFYTADE
lsl_LSL_0174_menB__naphthoate_    AADN-AMFGQTGPKVGSFDAGYGSGYLARVIGHKRAKEVWFLNHFYTAQE
lrf_LAR_0833_dihydroxynapthoic    AADN-AKFGQTGPKVGSFDAGYGSGYLARVIGHKRAKEVWFLNHFYSAEE
lre_Lreu_0887_naphthoate_synth    AADN-AKFGQTGPKVGSFDAGYGSGYLARVIGHKRAKEVWFLNHFYSAEE
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lbr_LVIS_0066_naphthoate_synth      AADN-AQFGQTGPKVGSFDAGYGSGYLARVIGHKRAKEVWFLNHFYSAEE
ooe_OEOE_0279_naphthoate_synth      AADN-AKFGQTGPKVGSFDAGYGSGYLARVIGHKRAKEVWFLNHWYTADE
lci_LCK_01696_menB__dihydroxyn      AADN-AKFGQTGPKVGSFDAGYGSGYLARVVGHKRAKEVWFLNHVYSADE
lme_LEUM_0016_naphthoate_synth      AADN-AKFGQTGPMVGSFDAGYGSGYLARVIGHKRAKEVWFLNHFYTAEE
efa_EF0445_menB__naphthoate_sy      AAEN-AKFGQTGPNVGSFDGGYGSGYLARVIGHKKAKEVWFMCKQYSAQE
cau_Caur_0094_naphthoate_synth      AADN-AIFGQTGPKVGSFDGGYGSNLLARMVGDKKAREIWYLCRQYNAQQ
tfu_Tfu_1409_naphthoate_syntha      AADN-ARFGQTGPKVGSFDGGYGSWLLAQTVGLKKAREIWYLCRQYTAQE
pgi_PG1523_menB__naphthoate_sy      AAEH-ARFGQTGPKVGSFDGGFGSSYLARCVGQKKTREIWFLCRQYTAAE
dps_DP0252_naphthoate_synthase      ASEN-AKFGQTGPKVGSFDAGFGSSYLARQVGQKKAREIWFLCDQYSAQE
bfr_BF1318_naphthoate_synthase      ASEN-AIFGQTGPRVGSFDAGFGSSYLARVVGQKKAREIWFLCRKYNAQE
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bth_BT_4702_naphthoate_synthas      ASEN-AIFGQTGPRVGSFDAGFGASYLARVVGQKKAREIWFLCRKYNAQE
pdi_BDI_1137_naphthoate_syntha      ASEN-AIFGQTGPRVGSFDAGFGSSYLARVVGQKKAREIWFLCRKYNAQE
bvu_BVU_2417_naphthoate_syntha      ASEN-AIFGQTGPKVGSFDAGFGSSYLARIVGQKKAREIWFLCRQYSAQE
lip_LI0807_menB__naphthoate_sy      ASEN-AIFGQTGPRVGSFDAGLGSSYLARIVGQKKVREIWFLCRQYTAQE
pna_Pnap_2124_naphthoate_synth      CSDK-AQFGQVGPKMGSVDPGYGTAFLARVVGEKKAREIWYLNRRYSGAE
lch_Lcho_1186_2-ketocyclohexan      ASEK-AIFGQVGPKMGSVDPGYGTAFLARVVGEKKAREIWYLNRRYSGAE
vei_Veis_4105_enoyl-CoA_hydrat      CSDK-AQFGQVGPKMGSVDPGYGTAFLARVVGEKKAREIWYLNRRYGGAE
rpa_RPA0653_badI__2-ketocycloh      CSEK-AIFGQVGPKMGSVDPGYGTAFLARVVGEKKAREIWYMCKRYSGKE
rpt_Rpal_0720_2-ketocyclohexan      CSEK-AIFGQVGPKMGSVDPGYGTAFLARVVGEKKAREIWYMCRRYSGKE
rpe_RPE_0612_naphthoate_syntha      CSEK-AIFGQVGPKMGSVDPGYGTAFLARVVGEKKAREIWYMCRRYSGKE
rpc_RPC_1033_naphthoate_syntha      CSEK-AIFGQVGPKMGSVDPGYGTAFLARVVGEKKAREIWYMCRRYSGKQ
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pla_Plav_1770_naphthoate_synth      AGES-AIFGQVGPKMGSVDPGFGTAYLARVVGEKKAREIWYMCRRYPARE
reu_Reut_C6107_1_4-dihydroxy-2      ASDT-AIFGQVGPKVGSVDPGFGTAFLSRVVGEKKAREIWFLCRRYPAKQ
bch_Bcen2424_6400_naphthoate_s      ASEA-AQLGQVGPRVGSVDPGFGTALLARVLGEKRAREVWFLCRRYTARE
bcn_Bcen_1429_naphthoate_synth      ASEA-AQLGQVGPRVGSVDPGFGTALLARVLGEKRAREVWFLCRRYTARE
bcm_Bcenmc03_6839_enoyl-CoA_hy      ASET-AIFGQVGPKVGSVDPGFGTAYLARIIGEKRAREIWYLCRKYSAKE
eba_ebA1954_badI__2-ketocycloh      ASEK-AQLGQAGPRVGSVDPGFGTALLARVVGEKKAREIWYLCRRYTAQE
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bxe_Bxe_C1035_1_4-dihydroxy-2-     AKES-AIFRQVGPMVGSFDAGYGTWYLEDLVGKKRAKEIWYCNQKITARE
rca_Rcas_4218_naphthoate_synth     AADN-AIFGQTGPIVGSFDAGFGSSYLAAIVGQKKAREIWYLCRQYNAQQ
rrs_RoseRS_0024_naphthoate_syn     AADN-AIFGQTGPIVGSFDAGFGASYLAAIVGQKKAREIWYLCRQYNAQQ
mtf_TBFG_10559_naphthoate_synt     ASREYARFKQTDADVGSFDGGYGSAYLARQVGQKFAREIFFLGRTYTAEQ
mra_MRA_0555_menB__naphthoate_     ASREYARFKQTDADVGSFDGGYGSAYLARQVGQKFAREIFFLGRTYTAEQ
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mle_ML2263_menB__naphthoate_sy     ASREHARFKQTDADVGSFDGGYGSAYLARQIGQKFAREIFLLGRAYTAEQ
mmi_MMAR_0895_menB__naphthoate     ASRQHARFKQTDADVGSFDGGYGSAYLARQVGQKFAREIFFLGRPYTAEQ
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mpa_MAP4044c_menB__naphthoate_     ASREHARFKQTDADVGSFDGGYGSAYLARQVGQKFAREIFFLGREYTAEQ
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mgi_Mflv_0025_naphthoate_synth     ASREHARFKQTDADVGSFDGGYGSAYLARQAGQKFAREIFFLGRPYTAEQ
mva_Mvan_0947_naphthoate_synth     ASREHARFKQTDADVGSFDGGYGSAYLASQVGQKFAREIFFLGRAYTAEQ
mjl_Mjls_0729_naphthoate_synth     ASRQHARFKQTDADVGSFDGGFGSAYLARQTGQKFAREIFFLGRAYDAET
mkm_Mkms_0749_naphthoate_synth     ASRQHARFKQTDADVGSFDGGFGSAYLARQTGQKFAREIFFLGRAYDAET
mmc_Mmcs_0735_naphthoate_synth     ASRQHARFKQTDADVGSFDGGFGSAYLARQTGQKFAREIFFLGRAYDAET
msm_MSMEG_1075_menB__naphthoat     ASREHARFKQTDADVGSFDGGFGSAYLARQTGQKFAREIFFLGRAYDAQT
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sen_SACE_6914_menB__naphthoate     ASAEHARFKQTDADVGSFDGGFGSAYLARQVGQKFAREIFFLGRPYTAEQ
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cjk_jk1870_menB__naphthoate_sy     ASREHAKFKQTDADVGSFDAGYGSAYLAKQVGQKFAREIFFLGEAIDAET
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cgl_NCgl0446_cgl0463__naphthoa     ASRQEARFKQTDADVGSFDAGYGSAYLAKMVGQKNAREIFFLGRTYDAER
cgt_cgR_0566_naphthoate_syntha     ASRQEARFKQTDADVGSFDAGYGSAYLAKMVGQKNAREIFFLGRTYDAER
cef_CE0475_naphthoate_synthase     ASRQEARFKQTDADVGSFDAGYGSAYLAKMVGQKFAREIFFLGRTYSAED
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cdi_DIP0421_menB__naphthoate_s    ASREHAKFKQTDADVGSFDAGYGSAYLAKMVGQKFAREIFFLGRTYDAET
art_Arth_3283_1_4-dihydroxy-2-    ASRQHGKFKQTDATVGSFDAGYGSALLARQIGQKAAREIFFLAREYSAED
aau_AAur_3275_menB__naphthoate    ASREHGKFKQTDATVGSFDAGYGSALLARQIGQKAAREIFFLAREYSADD
rsa_RSal33209_2700_naphthoate_    ASKEHGKFKQTDATVGSFDAGYGSALLARQIGQKRAREIFFLAREYSADD
krh_KRH_04970_menB__naphthoate    ASAEHGKFKQTDATVGSFDAGYGSALLARQVGQKFAREIFFLADEYSAED
cms_CMS_2584_menB__naphthoate_    ASAEHGRFKQTDADVGSFDGGYGSAYFARQVGQKAAREVFFLAEEHSAQR
cmi_CMM_0576_menB__naphthoate_    ASAEHGRFKQTDADVGSFDGGYGSAYFARQVGQKAAREVFFLAEEHSAQR
lxx_Lxx01440_menB__naphthoate_    ASEEHGRFKQTDADVGSFDAGYGSAYFARQVGQKFGREVFFLAREYSARR
pac_PPA0907_naphthoate_synthas    ASREYARFKQVDANVGSFDAGYGSALLARQIGDKRAREIFFLAETYDAEQ
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bba_Bd3492_menB__naphthoate_sy    ASQEHAIFKQTDPDVASFDSGYGSAYLARMVGQKRAREIFFLGRNYSAQE
sat_SYN_02400_naphthoate_synth    ASKEHAVFKQTDPDVASFDSGYGSAYLAKHIGQKRAREIFFLGLDYSAQD
swd_Swoo_4886_naphthoate_synth    ASKEHAIFKQTDPDVASFDSGYGSAYLAKMIGQKRAREIFFLGFNYSADE
sse_Ssed_4478_naphthoate_synth    ASKEHAIFKQTDPDVGSFDSGYGSAYLAKMVGQKRAREIFFLGFNYSADE
spl_Spea_4225_naphthoate_synth    ASKEHAVFKQTDPDVGSFDSGYGSAYLAKMIGQKRAREIFFLGFNYTAEE
shl_Shal_4273_naphthoate_synth    ASKEHAVFKQTDPDVGSFDSGYGSAYLAKMIGQKRAREIFFLGFNYSAEE
slo_Shew_3814_naphthoate_synth    ASKEHAVFKQTDPDVGSFDSGYGSAYLAKMIGQKRAREIFFLGFNYSADE
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saz_Sama_3635_naphthoate_synth    ASKEHAVFKQTDPDVASFDSGYGSAYLAKMVGQKRAREIFFLGFNYSADE
sbn_Sbal195_4500_naphthoate_sy    ASKEHAIFKQTDPDVASFDSGYGSAYLAKMIGQKRAREIFFCGFNYSADE
sbm_Shew185_4358_naphthoate_sy    ASKEHAIFKQTDPDVASFDSGYGSAYLAKMIGQKRAREIFFCGFNYSADE
sbl_Sbal_4340_naphthoate_synth    ASKEHAIFKQTDPDVASFDSGYGSAYLAKMIGQKRAREIFFCGFNYSADE
shn_Shewana3_4123_naphthoate_s    ASKEHAVFKQTDPDVASFDSGYGSAYLAKMIGQKRAREIFFCGFNYSADE
she_Shewmr4_3918_naphthoate_sy    ASKEHAVFKQTDPDVASFDSGYGSAYLAKMIGQKRAREIFFCGFNYSADE
shm_Shewmr7_4010_naphthoate_sy    ASKEHAVFKQTDPDVASFDSGYGSAYLAKMIGQKRAREIFFCGFNYSADE
son_SO_4739_menB__naphthoate_s    ASKEHAIFKQTDPDVASFDSGYGSAYLAKMIGQKRAREIFFCGFNYSADE
spc_Sputcn32_3920_naphthoate_s    ASKEHAIFKQTDPDVASFDSGYGSAYLAKMIGQKRAREIFFCGFNYSADE
shw_Sputw3181_4046_naphthoate_    ASKEHAIFKQTDPDVASFDSGYGSAYLAKMIGQKRAREIFFCGFNYSADE
ppr_PBPRB1048_putative_naphtho    ASKDMLFSNRQILMSHHLIQATVRLTLPR---------------------
hsl_OE2561R_menB__naphthoate_s    ASREHAKFKQTDPDVASFDGGFGSAYLAKQVGQKTAREIFFLGKTYDAEA
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hma_rrnAC0841_ech1__naphthoate      ASEEHAKFLQTDPDVGSFDGGFGSAYLARQVGQKKAREVFFLGKTYDAAE

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fjo_Fjoh_2784_naphthoate_synth      ASKEHAIFKQTDADVTSFDGGYGSAYLAKMVGQKKAREIFFLGRNYSAQE

chu_CHU_1897_menB__1_4-dihydro      ASKEHAIFKQTDADVTSFDGGYGSAYLAKMVGQKKAREIFFLGRNYSAQE
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Figure 2.42: Part of sequence alignment of MenBs from different organisms
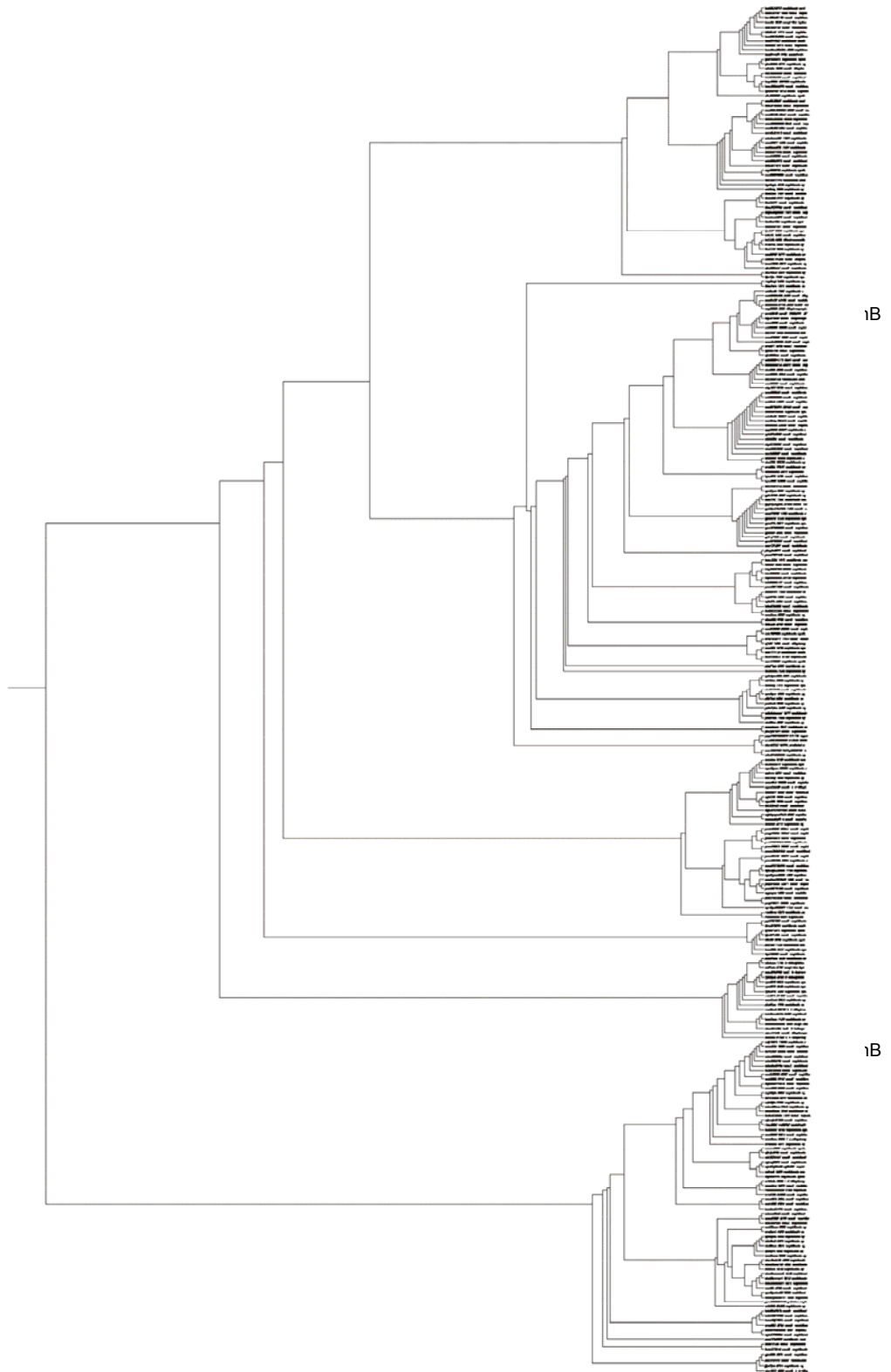
Figure 2.43: The phylogenetic tree of MenBs from different organisms

mtMenB and ecMenB might utilize difference mechanism for catalysis

Based on the special function role of D185 residue, a new mechanism of reaction catalyzed by mtMenB is proposed in **Figure 2.44** in which D185 abstracts alpha proton and is related to lactone substrate. mtMenB utilizes the lactone OSB-CoA as a substrate in which the carboxylate is activated. Catalytic residue D185 may function as a base and facilitate the water to abstract the pro-2R proton. Carbon-carbon bond is formed through the nucleophilic attack. Then residues of S190, D192 and Y287 are involved in removing water molecule and another pro-3S proton after the ring closure.
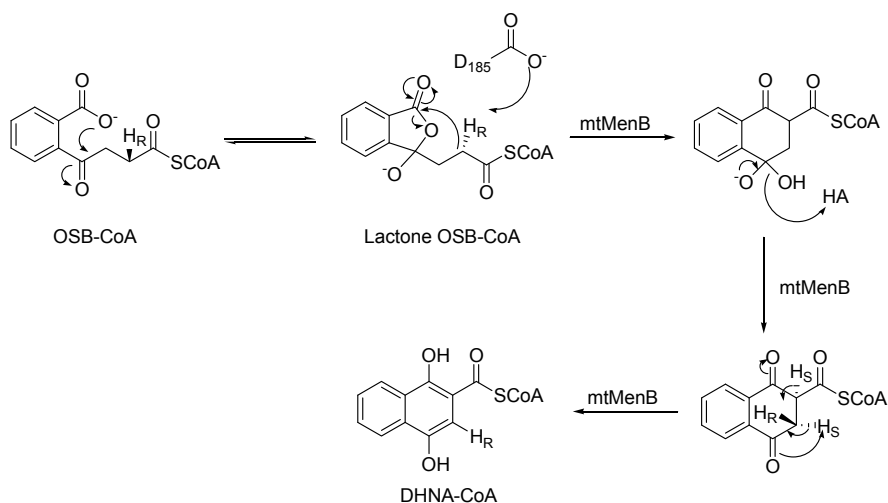
Figure 2.44: Proposed mechanism of the reaction catalyzed by mtMenB

In contrast, the homolog in ecMenB is G156 which cannot abstract the pro-2R proton. Pre-incubation experiment indicated that the substrate for ecMenB is the OSB-CoA not lactone OSB-CoA. On the other hand, methyl ester OSB-CoA is also not the substrate of ecMenB suggests the carboxyl group may be involved in deprotonating the pro-2R proton in a way as what has been proposed in our

group's previous publication (**Figure 2.45**). The conserved catalytic residues of S119, D121 and Y216 ecMenB play similar functional role for enzymatic catalysis.
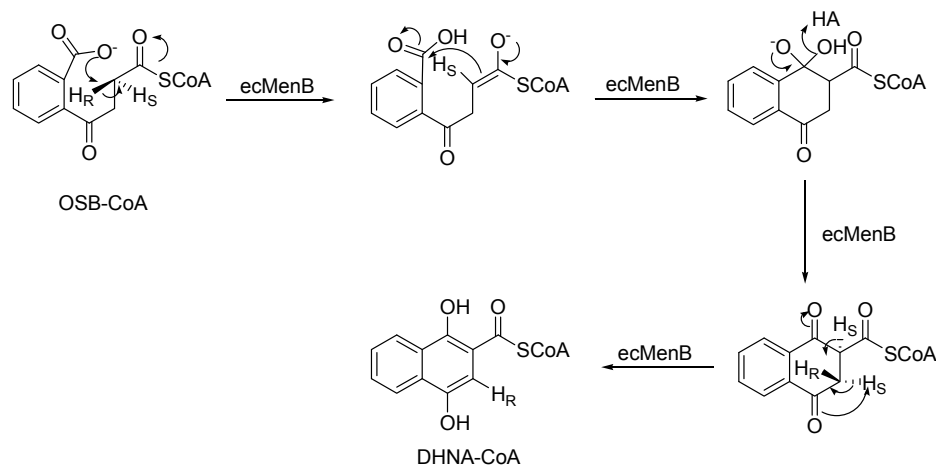
Figure 2.45: Proposed mechanism of the reaction catalyzed by ecMenB

YfbB and MenB cannot catalyze the hydrolysis of DHNA-CoA

The enzyme which catalyzes the hydrolysis of DHNA-CoA to DHNA is still unknown (**Figure 2.46**). It was initially suggested that product of the MenB reaction was DHNA. Later, the ORF *yfbB* in *E. coli* was thought to encode a hydrolase (*45*). There is no homolog of *yfbB* in *M. tuberculosis*.
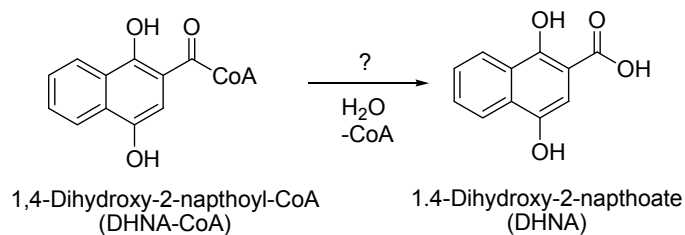
Figure 2.46: Hydrolysis of DHNA-CoA to DHNA

We cloned and expressed YfbB with an N-terminal His-tag from *E. coli*. The YfbB enzyme from *E. coli* was purified by affinity chromatography as a pure band

at 27 kDa (**Figure 2.47**). However, the purified YfbB was unable to catalyze the hydrolysis of DHNA-CoA. Recently, YfbB was characterized as a SHCHC synthase that catalyzes the conversion of SEPHCHC into SHCHC, an earlier step in the menaquinone biosynthetic pathway in *E. coli* (*46*) (**Figure 2.48**). Clearly, YfbB is not the right enzyme to catalyze the hydrolysis of DHNA-CoA.
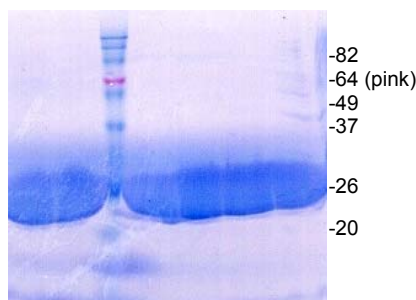
Figure 2.47: SDS-PAGE gel of YfbB showes it has a molecular weight of 27 kDa
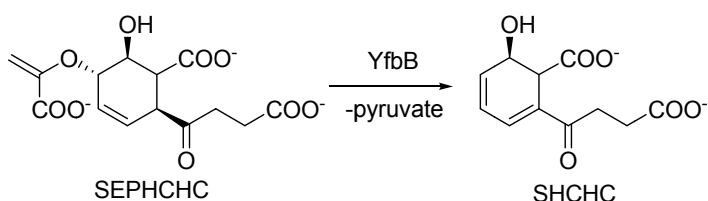
Figure 2.48: YfbB from *E. coli* catalyzes the conversion from SEPHCHC to SHCHC

In our attempts to characterize the product of the MenB reaction, we observed that DHNA-CoA was unstable in solution with the formation of a new peak at 340 nm, and that the decomposition of DHNA-CoA accelerated at basic pH values (**Figure 2.49**). At pH 7.0 which is the pH we use for MenB coupled assay, the DHNA-CoA is relatively stable. Since there is no gene coding for hydrolysis near the *men* cluster in *E. coli*, we therefore questioned that whether

the degradation of MenB product was due to the hydrolysis of DHNA-CoA and whether MenB can also catalyze this hydrolysis at higher pH.

MenB 800 nM, pH 7.0 for 30 min   MenB 800 nM, pH 7.5, for 30 min

MenB 800 nM, pH 8.0, for 30 min   MenB 800 nM, pH 8.5, for 30 min
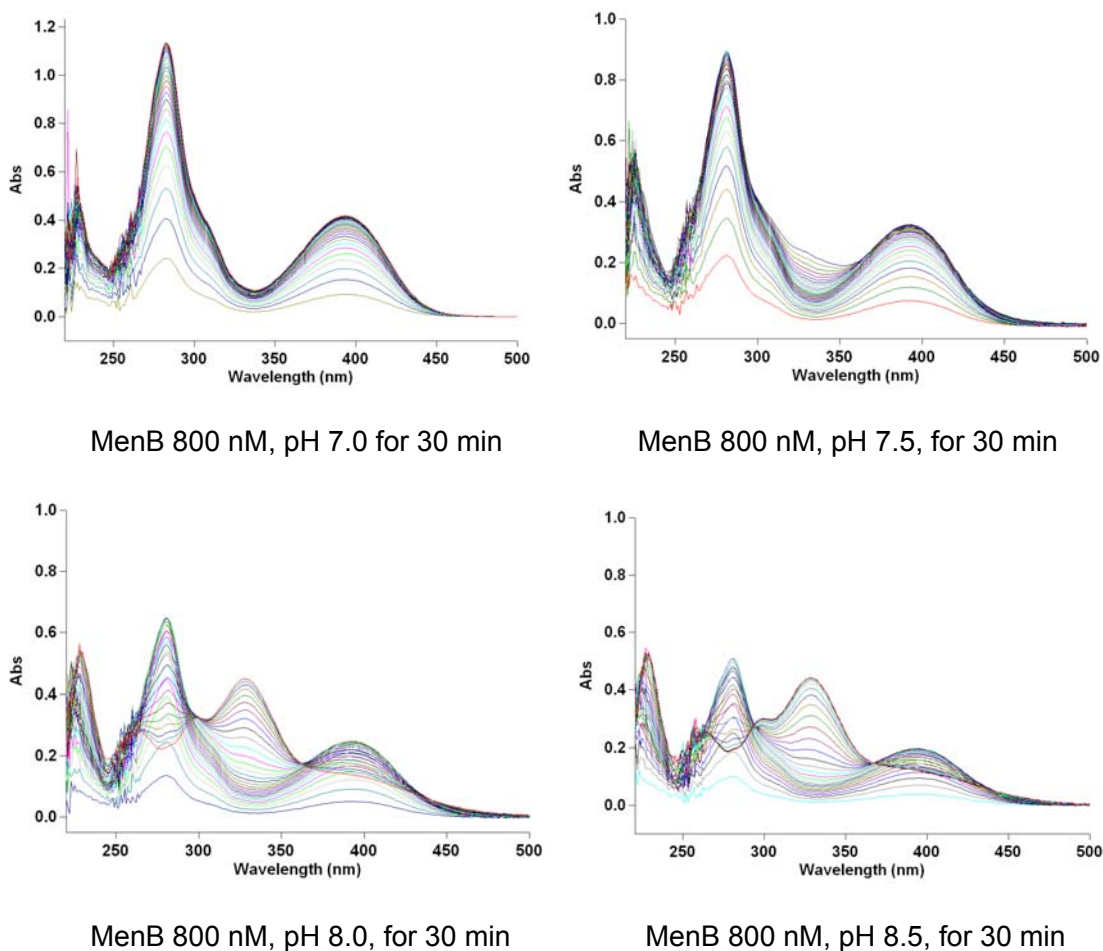
Figure 2.49: UV scan monitoring the degradation of DHNA-CoA at different pH conditions

Therefore, OSB, ATP, CoA, MenE and MenB were incubated at pH 7.0, pH 7.5, pH 8.0 and pH 8.5 for 30 min respectively. LC-MS analysis of the different reaction mixtures was performed to determine whether MenB can catalyze the hydrolysis of DHNA-CoA. However, DHNA was not detected in the reaction mixtures at different pH conditions, and the degradation product has the

molecular weight of 967.1 (DHNA-CoA has the molecular weight of 953.1). Based on the molecular weight of 967.1, the degradation reaction is proposed in **Figure 2.50**. Removing the MenB by HPLC didn't block the degradation of DHNA-CoA, which indicates that MenB cannot catalyze the hydrolysis of DHNA-CoA, and DHNA-CoA is intrinsically unstable in basic condition and its degradation is not related to MenB.

MW 953.1   MW 951.1   MW 969.1   MW 967.1
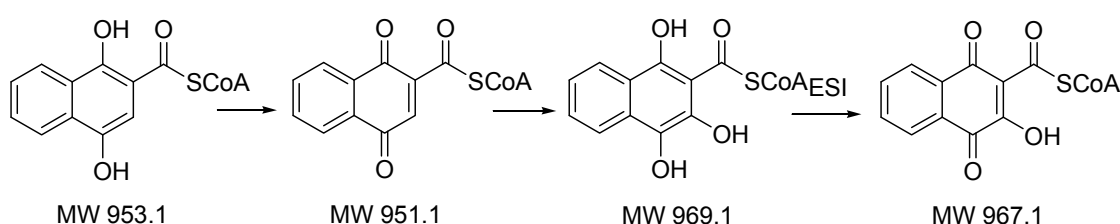
Figure 2.50: Proposed degradation reaction of DHNA-CoA in solution

In order to confirm the structure of the degradation product of DHNA-CoA, the NMR spectroscopy of DHNA was analyzed assuming the CoA portion won't affect the degradation reaction. DHNA was incubated it in D_2O at pD 10.0 for 2 hours to accelerate the degradation. ^1H NMR spectrum and ^{13}C NMR spectrum confirmed that DHNA converted to structure **2** by oxidation and water addition in solution. However, ESI-MS spectra indicated that DHNA degradation product has the molecuar weight of 218.1 which coresponds to the structure **3**, suggesting a further oxidation occured during the process of ionization (**Figure 2.51**).

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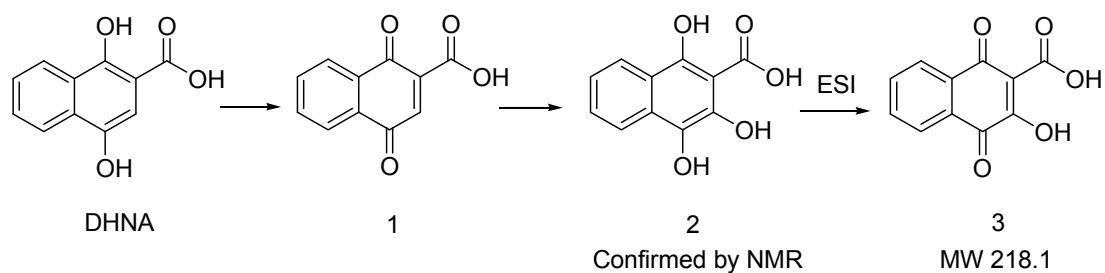
Figure 2.51: Degradation reaction of DHNA in solution

Crystal structure of mtMenB in complex with dimethoxy DHNA-CoA

The crystal structures of mtMenB and saMenB in complex with AcAc-CoA have been published by our group (14) and Ulaganathan (23) respectively. However AcAc-CoA lacks many of the structural features either of the substrate or product (**Figure 2.52**).
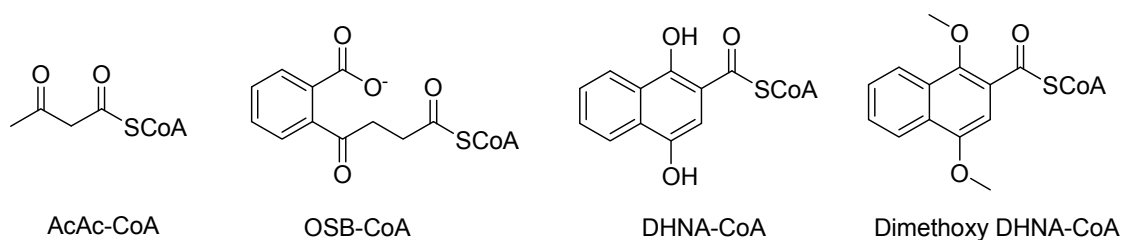
Figure 2.52: Structures of AcAc-CoA, OSB-CoA, DHNA-CoA and dimethoxy DHNA-CoA

We know that the product of MenB is not stable due to its oxidative degradation. We designed a stable compound by replacing the hydroxyl groups in the naphthalene moiety with methoxy groups. Dimethoxy DHNA-CoA is a stable product analogue and can be synthesized and purified by HPLC. It binds with mtMenB with a K_d value of 13.7 µM (by ITC). In addition, this product

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analogue is a competitive inhibitor of mtMenB with a K_i value of 157.2 ± 16.1 µM (**Figure 2.53**).
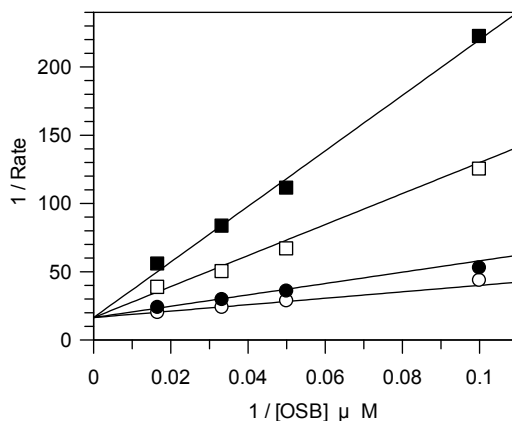
Figure 2.53: Inhibition of mtMenB with dimethoxy DHNA-CoA. A lineweaver-Burk plot of the inhibition of mtMenB by dimethoxy DHNA-CoA in the absence of inhibitor (○), in the presence of 120 µM of dimethoxy DHNA-CoA(●), 600 µM of dimethoxy DHNA-CoA (□) or 1200 µM of dimethoxy DHNA-CoA (■).

We successfully obtained the 2.5 Å structure of dimethoxy DHNA-CoA bound to mtMenB. Interestingly, although the CoA molecule is bound in the same position as that in the AcAc-CoA structure, the dimethoxy DHNA moiety occupies a hydrophobic pocket adjacent to the active site (**Figure 2.54**). In this structure the catalytic tyrosine Y287 has rotated toward the active site in order to accommodate the dimethoxy acyl group, while the side chain of R296 has also moved. Although this structure does not provide any additional information on the location of active site residues when the substrate or product are bound in the active site, the discovery of a hydrophobic binding pocket adjacent to the active site may be useful for future inhibitor discovery efforts.

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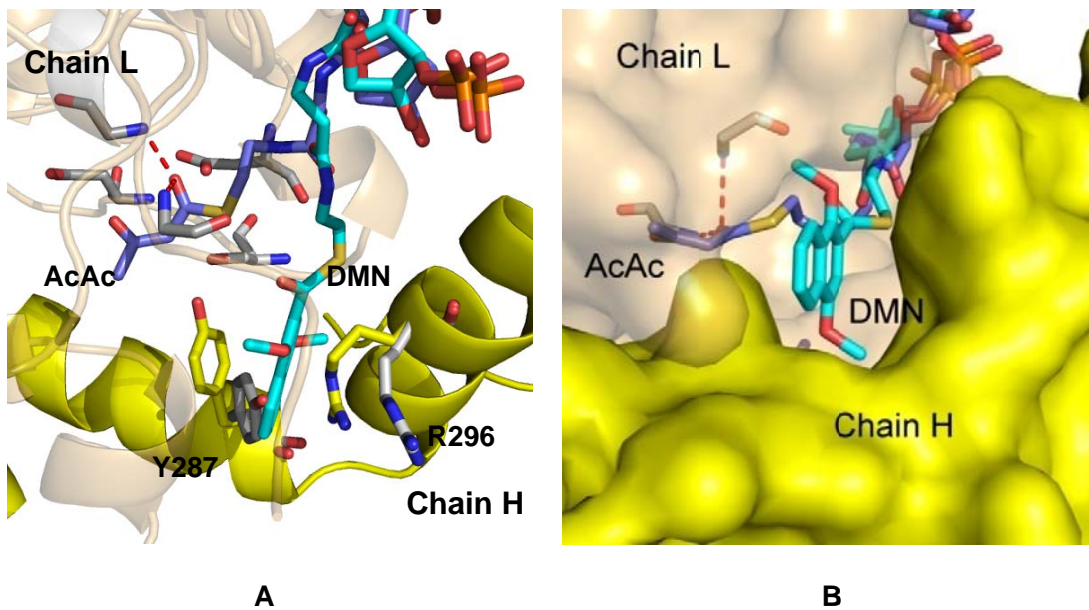
Figure 2.54: Crystal structure of mtMenB bound with dimethoxy DHNA-CoA. The active site residues of mtMenB bound with AcAc-CoA are colored in white and the position-changed residues of mtMenb bound with dimethoxy DHNA-CoA (DMN) are shown in yellow.

Conclusions

MenB, a crotonase superfamily member, catalyzes the formation of a carbon-carbon bond through an intramolecular Claisen/Dieckmann condensation. Catalytic residues of Ser190, Asp192 and Tyr287 (mtMenB numbering) are conserved in all MenBs, while the residue of Asp185 (mtMenB numbering) is not conserved in all MenBs such as MenB from *E. coli* and from *S. aureus*.

mtMenB, ecMenB and saMenB were cloned and expressed. Except saMenB, both mtMenB and ecMenB showed good catalytic activities with the k_{cat}/K_m value of 1.2 ± 0.2 min$^{-1}\cdot\mu$M^{-1} and 0.14 ± 0.03 min$^{-1}\cdot\mu$M^{-1} respectively. Hence, the mechanism of MenB reaction catalyzed by enzyme from *M. tuberculosis* was mainly compared with the reaction catalyzed by MenB from *E. coli*.

A series of substrate analogues were synthesized to examine the mechanism of MenB reaction. Direct binding experiments and NMR alpha-proton exchange studies together with enzyme kinetics indicate that the aromatic carboxylate in OSB-CoA is essential for reactivity. Methyl ester OSB-CoA is not substrate for both mtMenB and ecMenB, indicating the free carboxyl group is essential for the reaction. The lack of activity of OSB methyl ester led us to reconsider the decomposition of OSB-CoA and its relationship to the enzyme catalyzed reaction.

The different pre-incubation results of mtMenB and ecMenB reactions suggest that they may utilize lactone OSB-CoA and OSB-CoA as a substrate, respectively. This means mtMenB and ecMenB might utilize difference mechanism for catalysis. mtMenB utilizes the lactone OSB-CoA as a substrate in which the carboxylate is activated. The catalytic residue D185 may function as a

base and facilitate the water to abstract the pro-2R proton. In contrast, ecMenB accepts the OSB-CoA as a substrate, and the carboxyl group of OSB-CoA may be involved in deprotonating the pro-2R proton in a way as what has been proposed in our group's previous publication.

It was initially suggested that MenB can also catalyze the hydrolysis of DHNA-CoA. Later, the ORF *yfbB* in *E. coli* was thought to encode a hydrolase. Our experiments confirmed that both MenB and YfbB cannot catalyze the hydrolysis reaction. The instability of DHNA-CoA in solution is not relevant to MenB.

Dimethoxy DHNA-CoA is a stable product analogue of MenB. In the crystal structure of mtMenB in complex with dimethoxy DHNA-CoA, the dimethoxy DHNA moiety occupies a hydrophobic pocket adjacent to the active site. Although this structure does not provide any additional information about the active site, the discovery of a hydrophobic binding pocket adjacent to the active site may be useful for future inhibitor discovery efforts.

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CHAPTER 3: MENB AND BADI CATALYZE DIECKMANN CONDENSATION AND REVERSE DIECKMANN CONDENSATION

Background

In the crotonase superfamily, there are two enzymes which catalyze similar but totally reverse reactions. The carbon-carbon bond formation (Dieckmann condensation) catalyzed by MenB and carbon-carbon bond hydrolysis (reverse Dieckmann condensation) catalyzed by BadI (2-ketocyclohexanecarboxyl-CoA hydrolase) represent unique and rare reactions among enzymatic catalysts in general (**Figure 3.1**). mtMenB shares 35% sequence identity with orthologs of BadI. The proposed catalytic roles for the residues in the active site of MenB are possibly complementary to the roles proposed for the active-site residues of BadI.
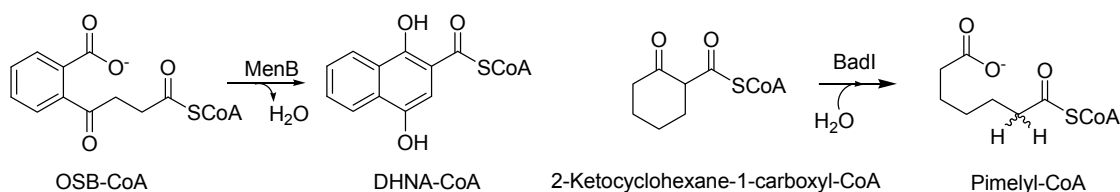
Figure 3.1: Reactions catalyzed by MenB and BadI

Both of the reactions are essentially irreversible. The MenB-catalyzed ring-forming reaction is rendered irreversible by the formation of an aromatic system. The BadI-catalyzed ring-cleaving reaction is not reversible because the product is ionized upon its release from the active site. Obviously, MenB and BadI share an intriguing evolutionary relationship. These two enzymes are likely to share similar active site configurations, and their active-site residues might serve

similar catalytic functions. Therefore it might be interesting to study BadI in order to further understand the mechanism of the MenB reaction.

BadI catalyzes the hydrolysis of 2-ketocyclohexanecarboxyl-CoA to pimelyl-CoA by a reverse Dieckmann condensation. This reaction is the last step in the anaerobic degradation pathway of benzoate in *Rhodopseudomonas palustris* which results in the cleavage of the six-membered ring (**Figure 3.2**) (*1*). The mechanism of this reaction is the reverse of the mechanism of the MenB-catalyzed reaction. The catalytic residues found in MenB are similarly conserved in BadI as Ser138, Asp140 and Tyr235 (**Figure 2.1**).
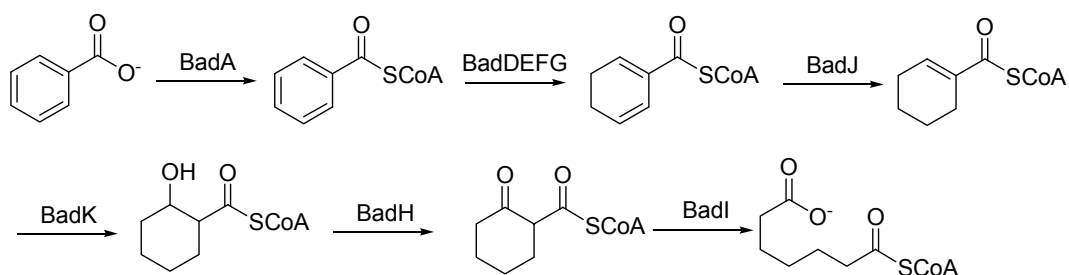
Figure 3.2: Pathway for anaerobic benzoate degradation in *R. palustris*

The detailed mechanism of reaction catalyzed by mtMenB and ecMenB has been discussed in chapter 2. In this chapter, we will discuss the BadI reaction and then focus on comparing these two reverse reactions catalyzed by MenB and BadI.

Materials and methods

Expression and purification of wild-type BadI from R. palustris

The gene encoding BadI (*badI*) was cloned in pET17b vector and sequenced by Ellen D. Eberhard in the laboratory of Prof. John A. Gerlt at University of Illinois at Urbana-Champaign. The gene was cloned in frame with *NdeI* and *XhoI* restrictions sites and without a hexahistidine tag. Protein was optimally expressed from transformed *E. coli* BL21 (DE3) cells. BL21 cells (100 µl) were transformed with 1 µL of mini-prepped plasmid by heat shock. The transformants were resuspended in 700 µL of LB, 200 µL of which were plated on LB-Amp plate. The plate was incubated at 37 ºC for overnight. A 15 mL starter culture (LB-Amp) was inoculated from a single colony and shaken at 37 ºC for approximately 8 hours. 800 mL LB-Amp media was inoculated with 1 mL of starter culture, and shaken at 37 ºC overnight.

The cells were collected by centrifugation at 5,000 rpm for 20 min at 4 ºC, and resuspended in 30 mL of 20 mM Tris-HCl, pH 7.9. The cells were lysed by sonication (Sonicator 3000, Misonix) at level 5, alternating a 5 second pulse with a 10 second rest, for a total pulse time of 10 min. The cell debris was removed by centrifugation at 33,000 rpm for 90 min at 4 ºC. The supernatant liquid was loaded onto a pre-equilibrated anion exchange column (DE52 resin, Whatman 2.6 cm × 70 cm). The column was washed with 300 mL of 20 mM Tris-HCl, pH 7.9. A linear gradient (800 mL, 0 to 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) was applied while collecting 8 mL fractions and monitoring the eluent at 280 nM. The column was then washed with 1 M NaCl and re-equilibrated in the starting buffer.

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BadI was eluted in approximately fractions 27-40, appearing as the dominant band at 28 kDa as assessed by SDS-PAGE. The BadI-containing fractions were collected and concentrated to 20 mL in a Millipore stirred cell, using a 10,000 MWCO Millipore ultrafiltration membrane. The solution was then dialyzed at 4 °C against 20 mM Tris-HCl, pH 7.9, to remove salt.

The protein sample was further purified by strong anion exchange chromatography (Q sepharose, Pharmacia Biotech, 5 mm × 100 mm) at 4 °C. The protein was loaded in 20 mM Tris-HCl, pH 7.9. The column was washed with 15 mL of 20 mM Tris-HCl, pH 7.9. A linear gradient (400 mL, 0 to 0.35 M NaCl, 20 mM Tris-HCl, pH 7.9) was applied while 6 mL fractions were collected. BadI was eluted in fraction 40-70, appearing as the dominant band at 28 kDa assessed by SDS-PAGE. The concentration of BadI was determined by measuring the absorption at 280 nm using an extinction coefficient of 32,790 $M^{-1}cm^{-1}$ calculated from the primary sequence. The BadI containing fractions were collected, concentrated and stored at -80 °C. The non-His-tagged protein purified by this procedure was submitted for X-ray crystallography.

Expression and purification of mutant BadI

BadI mutants of S138A, D140N and Y235F were prepared by Ellen D. Eberhard in the laboratory of Prof. John A. Gerlt at University of Illinois at Urbana-Champaign. The procedure for the expression and purification of mutant BadI was used as described for wild-type BadI.

CD spectra of wild-type BadI and its Mutants

The far-UV CD spectra of the wild-type BadI protein and its mutant variants were measured at a protein concentration of 20 μM in 20 mM Tris-HCl, 150 mM NaCl, pH 7.9 buffer at 25 ºC by using an AVIV 62 DS spectrometer equipped with a Peltier temperature control unit. CD spectra were obtained for all the mutant enzymes, and the resulting spectra were identical to that of the wild-type enzyme (**Figure 3.3**), suggesting that the mutagenesis did not altered the overall structure of the protein and the decrease of the activity is not due to the unfolding of the protein.
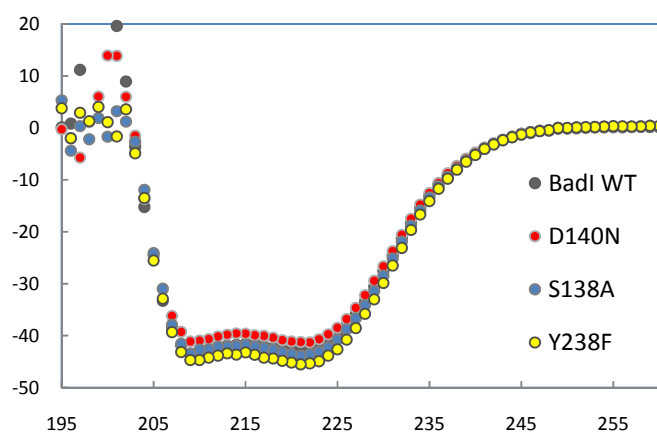
Figure 3.3: CD spectra of wild-type and mutant BadI

Assay of BadI reaction

2-Ketohexanecarboxyl-CoA gives an absorbance peak at 314 nm when incubated in the presence of Mg^{2+} at pH 8 (**Figure 3.4**), which is due to the formation of an Mg^{2+}-enolate complex similar to that formed by acetoacetyl-CoA in the presence of Mg^{2+} ions at pH 8 (*2, 3*). BadI activity was assayed by monitoring the decrease of absorbance at 314 nm. The assay depends on the

loss of absorbance of an Mg^{2+}-enolate complex that occurs when the alicyclic ring of pimelyl-CoA is cleaved (**Figure 3.5**). BadI reaction performed in 20 mM Tris-HCl, 150 mM NaCl, and 100 mM $MgCl_2$, pH 8.0 buffer. Activity was calculated by using an extinction coefficient of 1,210 $M^{-1}cm^{-1}$ for 2-ketohexanecarboxyl-CoA.
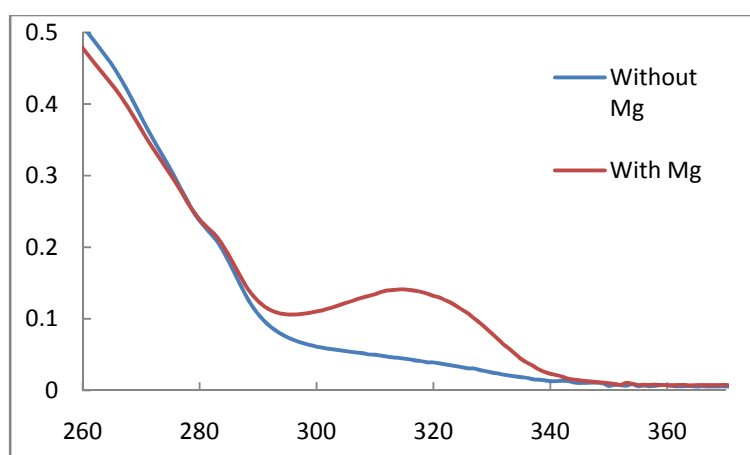
Figure 3.4: Formation of an Mg^{2+}–enolate complex of 2-ketohexanecarboxyl-CoA

2-Ketocyclohexane-1-carboxyl-CoA   Pimelyl-CoA
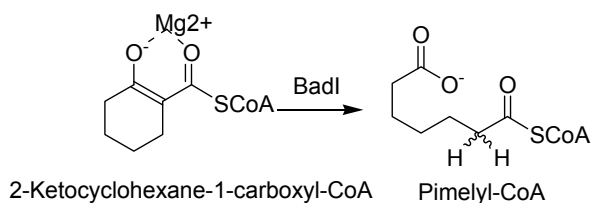
Figure 3.5: Assay of BadI reaction based on the disappearance of Mg^{2+}-enolate complex

Magnesium dependence of BadI reaction

In order to characterize the magnesium dependency for BadI reaction, the reaction catalyzed by BadI was incubated in 20 mM Tris-HCl, 150 mM NaCl, and 100 mM $MgCl_2$, pH 8.0 buffer and magnesium free buffer. Reaction mixtures

were characterized by HPLC (Phenomenex C18 analytical column, 5 µm particle diameter, 4.6 mm i.d., 250 mm length). A linear gradient (0 to 40% acetonitrile, 40 min) was applied. Pimely-CoA was eluted at 11 min. When there was no magnesium in the reaction buffer, the same product was formed and eluted from HPLC at 11 min (**Figure 3.6**).
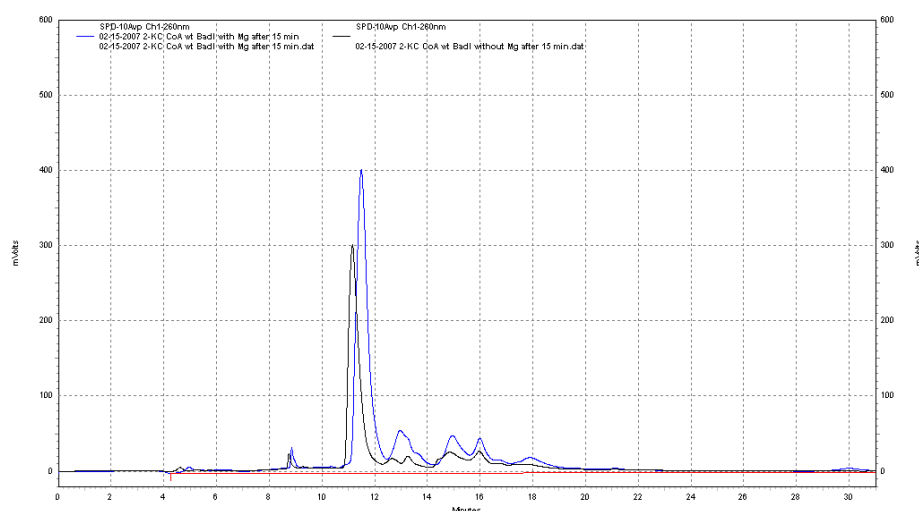
Figure 3.6: BadI reaction is not magnesium dependent. Without the incubation with magnesium, the same product was formed characterized by HPLC at 11 min.

Synthesis of 2-ketohexanecarboxyl-CoA

Ethyl 2-cyclohexanone-carboxylate (25 mmol) was hydrolyzed with 1 M NaOH in 50 mL of H_2O overnight at room temperature. After acidifying to pH 1-2 using 1 M HCl, 2-cyclohexanone-carboxylic acid (21 mmol) was precipitated from the cold water. The acid (1mmol) was dissolved in 20 mL of tetrahydrofuran, with stirring and external cooling by an ice bath. Dry triethylamine (2 mmol) was added, followed by dropwise addition of ethylchloroformate (2 mmol). After an additional hour of stirring in the ice bath, the precipitated triethyl ammonium

chloride was removed by gravity filtration. CoA (40 μmol) was dissolved in 10 mL of water, and the pH was adjusted to 7 with 0.1 M NaOH. The solution of activated acid was added dropwise over 30 min to the CoA solution, while maintaining pH at 7 by adding 0.1 M NaOH dropwise as necessary. After addition was complete, the reaction was allowed to stir another four hours. The reaction was acidified to pH 1-2 using 1 M HCl and extracted with ethyl acetate. The pH of the water layer was then raised to 10-11 with 5 M NaOH, kept at this pH for 1 h and then brought back to pH of 7 (**Figure 3.7**). The solution was frozen in liquid nitrogen and lyophilized. The product was purified from the lyophilized powder by semi-preparative HPLC (Vydac C18 column, 10 μm particle diameter, 10 mm i.d., 250 mm length). A linear gradient (0 to 40% acetonitrile, 40 min) was applied. The absorbance of the eluent was monitored at 260 nm. The peak of interest was collected over several runs, frozen and lyophilized. ESI-MS [M-H$^-$]: calcd 890.17 ($C_{28}H_{43}N_7O_{18}P_3S^-$); found 890.0.
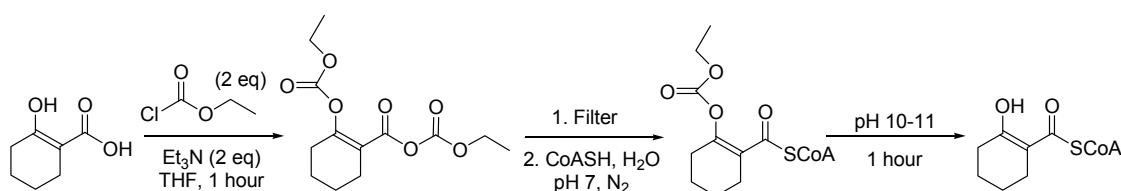
Figure 3.7: Synthesis of 2-ketohexanecarboxyl-CoA

Synthesis of cyclohexenecarboxyl-CoA

Starting with cyclohexenecarboxylic acid (Aldrich), the standard procedure on page 67 was used to synthesize cyclohexenecarboxy-CoA. ESI-MS [M-H$^-$]: calcd 874.17 ($C_{28}H_{43}N_7O_{17}P_3S^-$); found 874.0.

X-Ray crystallography of BadI

   BadI along with cyclohexenecarboxy-CoA were submitted to Dr. Margaret Luk-Paszyc, Dr. Kolappan Subramaniapillai and Prof. Caroline Kisker at the Rudolf Virchow Center, DFG Research Center for Experimental Biomedicine (Germany). The structures were solved in *apo*- form and with cyclohexenecarboxyl-CoA bound in the active site.

Results and discussion

BadI catalyzes reverse Dieckmann condensation

BadI belongs to crotonase superfamily. It catalyzes the reverse Dieckmann Condensation which is reverse to the reaction catalyzed by MenB. The BadI from *R. palustris* was expressed without His-tag and purified by anion exchange chromatography as previously described in materials and methods section. The pure BadI protein was shown on SDS-PAGE as 28 kDa band (**Figure 3.8**).
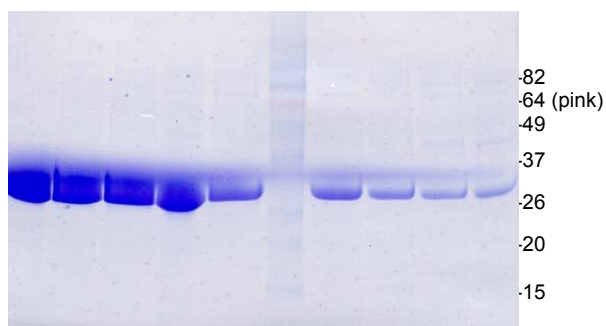
Figure 3.8: SDS-PAGE gel of BadI after DE52 and Q sepharose columns

The reaction catalyzed by BadI is not magnesium dependent as described in **Figure 3.6**. Two possible enantiomers of the substrate for BadI, *S*-2-ketohexanecarboxyl-CoA (*S*-KC-CoA) and *R*--2-ketohexanecarboxyl-CoA (*R*-KC-CoA) can interconvert *via* enolization (**Figure 3.9**). It has been reported that BadI can catalyze the hydrolysis of *S*-KC-CoA, but not *R*-KC-CoA (*4*). BadI can be assayed by monitoring the loss in absorbance due to the formation of a magnesium enolate with the substrate. However, complication in assaying the enzyme is that the substrate can exist as two enantiomers that are in equilibrium. Therefore an accurate measure of the concentration of the substrate is

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impossible. The apparent the k_{cat}/K_m value of wild-type BadI is 11.9 ± 1.6 min^{-1} µM^{-1} (k_{cat} and K_m values are 3395.9 ± 125.2 min^{-1} and 285.4 ± 32.4 µM).
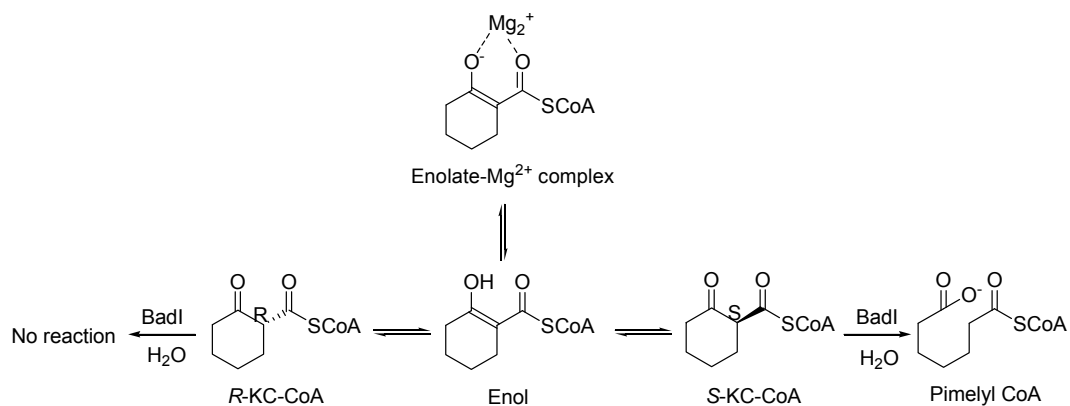
Figure 3.9: Keto enol tautomerism of BadI substrates and interconversion of the S and R diastereomers

S138, D140 and Y235 in BadI are conserved in MenB (**Figure 2.1**). The catalytic activities of BadI mutants of Y235 F, D140N and S138A were measured. Since the mutagenesis caused the K_m values of the mutants to increase to even bigger values, measurement of the apparent k_{cat}/K_m values of BadI mutants relied on the plot of the initial rate *vs.* substrate concentration when [S] << K_m ($\dfrac{k_{cat}}{K_m} = \dfrac{V_i}{[S] \times [E]}$) (**Figure 3.10**). Y235 F, D140N and S138A catalyzed the reactions at rates which were 176, 313 and 4677 fold reduced, respectively, compared to wild-type BadI (**Table 3.1**). Therefore, the residues probably work together as a triad, with Ser 138 playing a central role.

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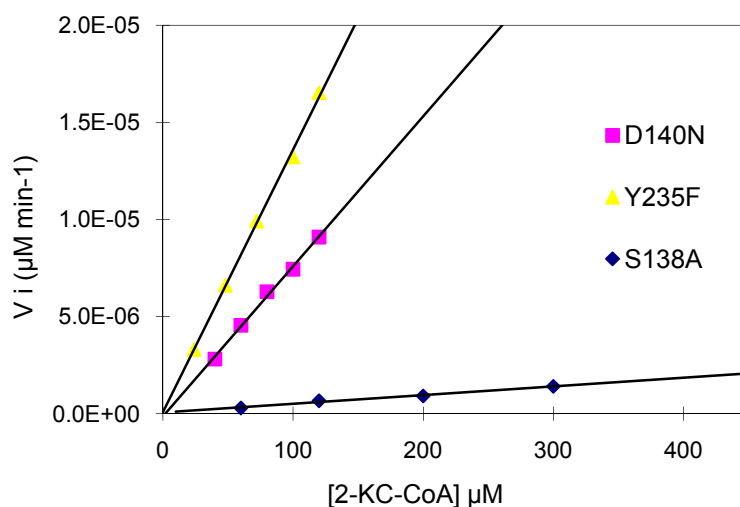
Figure 3.10: Apparent k_{cat}/K$_m$ values of BadI mutants. Each mutant concentration was 2 µM.

Table 3.1: Apparent k_{cat}/K$_m$ values of BadI and its mutants

Enzyme	k_{cat}/K$_{m\ app}$ (min^{-1}·µM^{-1})	Ratio
Wild-type BadI	11.9	1
S138A	0.0025	1/4677
D140N	0.038	1/313
Y235F	0.067	1/176

The proposed mechanism of the reaction catalyzed by BadI is based on our mutagenic data and stereochemistry published in reference (*4*) (**Figure 3.11**). D140 may function as a base to activate the water molecular to attack the β-carbonyl of the substrate. The subsequent tetrahedral intermediate forms an enolate which is stabilized by oxyanion hole residues. S138 may direct the pro-2*S* proton to the enolate to yield the product.

136
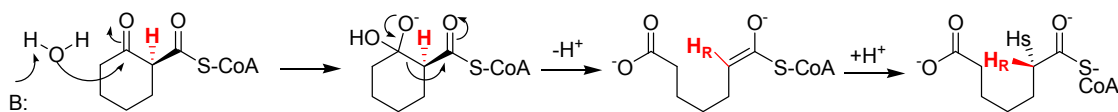
Figure 3.11: Proposed mechanism of BadI reaction

Comparison of crystal structures of mtMenB and BadI

The *apo-* structure of BadI and the structure of BadI with cyclohexenecarboxyl-CoA (**Figure 3.12**) bound in the active site were solved by Dr. Margaret Luk-Paszyc, Dr. Kolappan Subramaniapillai and Prof. Caroline Kisker at the Rudolf Virchow Center, DFG Research Center for Experimental Biomedicine (Germany). Comparison of the active sites structure when the ligand is bound to that of the unliganded active site revealed minimal structural differences, indicating that the active site is rigid and that the binding of the substrate analogue would not be expected to induce any major structural perturbations in the protein.
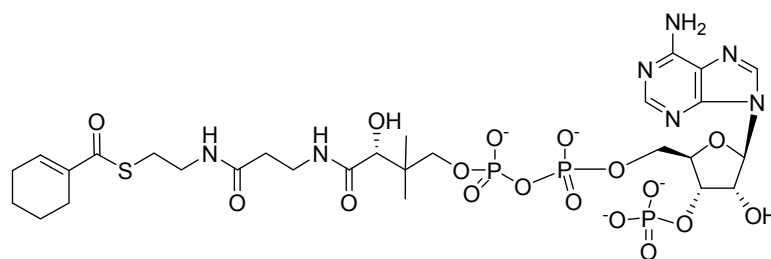
Figure 3.12: Structure of cyclohexenecarboxyl-CoA

Like many of the previously characterized members in the crotonase superfamily, BadI is hexamer (dimer of trimers). The overall fold displays a right hand spiral with a core composed of β-sheets surrounded by α-helices. In

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contrast to MenB, the C-terminus in each BadI monomer folds back to contribute residues to the active site in the same monomer. Consequently, the active site of each monomer contains residues only from that monomer (**Figure 3.13**).
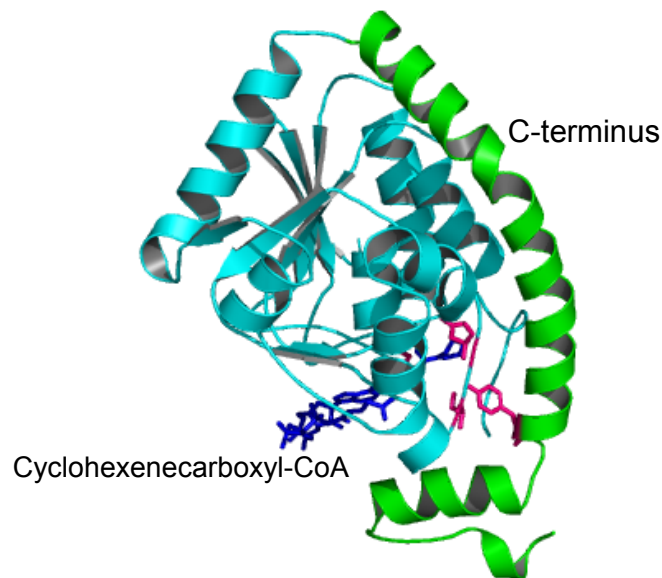
Figure 3.13: Structure of BadI. The C-terminal α-helices involved in subunit interactions are colored in green. The side chains of the active site residues are displayed in pink. The substrate analogue, cyclohexenecarboxyl-CoA, is colored in blue.

The active site residues are located on the outer edge of the trimer, near the trimer-trimer interface. The cyclohexene portion of the substrate analogue is buried within a cleft in the enzyme, while the CoA portion threads out of the active site making enough contacts with water molecules on the surface. The thioester carbonyl forms hydrogen bonding interaction with the backbone amide protons of the residues of the oxyanion hole (Gly 66 and Gly 110). Catalytic residues S138, D140 and Y235 occur within 5 Å of the bound substrate analogue and are positioned on the same side of the molecule (**Figure 3.14**).
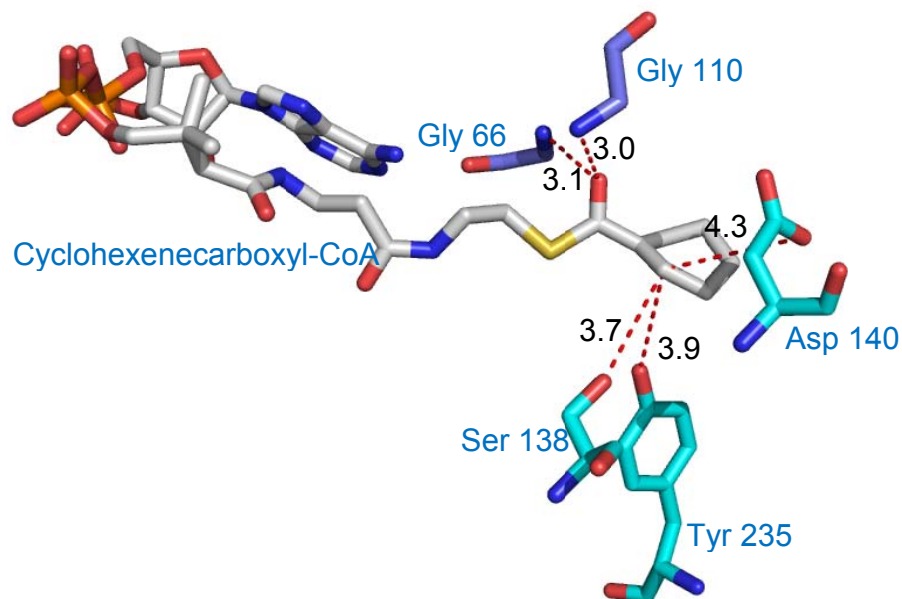
Figure 3.14: Active site of BadI. The distance between catalytic residues and oxyanion-hole residues to substrate analogue are in Angstroms.

Because the substrate analogue lacks an oxygen substituent on the β-carbon as occurs in the true substrate, it presumably lacks a major hydrogen-bonding contact. Therefore, the cyclohexene portion of the molecule may not be bound in a manner that represents the binding mode of the actual reaction.

mtMenB and BadI share 35% sequence identity. In both cases, the α-helices of the C-terminus provide the major contacts between the subunits of the hexamer. In BadI, this region folds along the subunit and involves in the active site of the same subunit. In contrast, mtMenB's C-terminus folds back, crosses the trimer-trimer interface, and forms part of the active site of the opposing monomer. Remarkably, even with this structural difference, the active sites of mtMenB and BadI are superimposable (**Figure 3.15** A and B). mtMenB S190 and

D192 are in the same position of BadI S138 and D140, while Tyr287 in mtMenB and Tyr235 points to the different direction. The position of BadI Y235 may represent the actual binding mode in the MenB active site since since cyclohexenecarboxy-CoA is a better mimic of the true BadI substrate than AcAc-CoA is of the MenB substrate. D185 mtMenB is not conserved in the BadI structure in which it is a glycine instead.

In both structures, the active site residues are all positioned on one side of the substrate analogues, which is similar to the active site of enoyl-CoA hydratase in that the catalytic residues all occur on the same side of the substrate with respect to the plane of the thioester (**Figure 3.15** C and D). The same architecture in the active site indicates that MenB, BadI and enoyl-CoA hydratase may share the comparable configuration of the reaction intermediates.
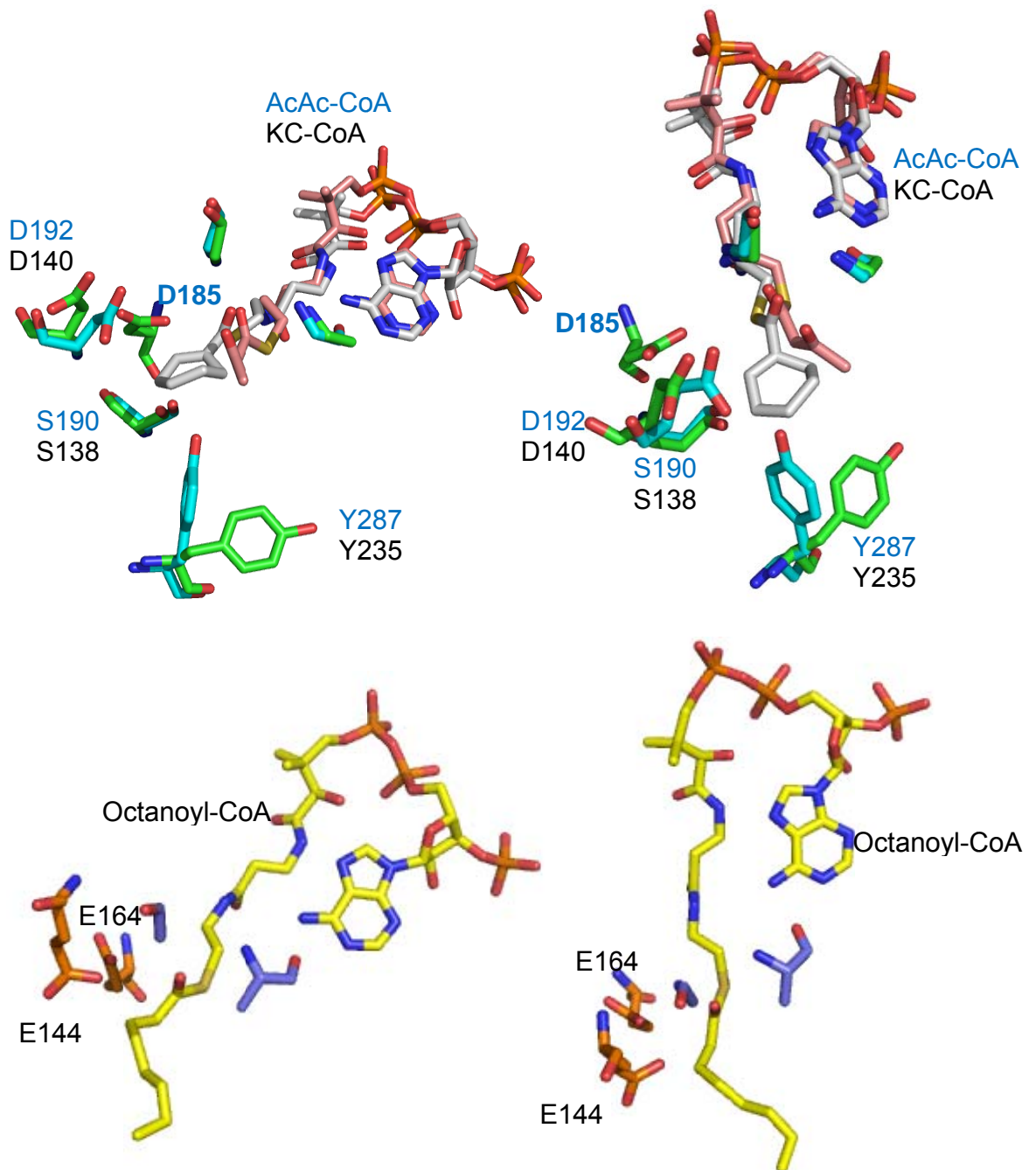
Figure 3.15: Crystal structures of mtMenB, BadI and enoyl-CoA hydratase. Figure A and B: superimposition of the active site of mtMenB and BadI (different view). MenB active-site residues are labeled in blue and BadI active-site residues are labeled in black. Figure C and D: The active site of enoyl-CoA hydratase (different view). All catalytic residues are positioned at the same side.

Stereochemistry of reactions catalyzed by MenB and BadI

The reactions catalyzed by MenB and BadI are the mechanistic reverse of each other. The stereochemical courses of the reaction catalyzed by these two enzymes were elucidated. In contrast, the stereo-specificities of the analogous steps, enolate formation in the reaction catalyzed by MenB and enolate protonation in the reaction catalyzed by BadI, are divergent. MenB catalyzes the abstraction of the pro-2*R* proton in the initiation of the reaction (*5*), while BadI catalyzes the addition of the pro-2*S* proton in the termination step (*4*). The divergent stereo-specificities are likely due to the active-site architecture.

The pro-2*R* stereochemistry has also been found in the reaction catalyzed by enoyl-CoA hydratase. X-ray crystallography and site-directed mutagenesis of enoyl-CoA hydratase have demonstrated that the two glutamate residues, Glu-144 and Glu-164, form part of a catalytic diad and act in concert to facilitate the *syn* addition/elimination reaction (*6-18*) (**Figure 3.16**). E164 in enoyl-CoA hydratase, which is involved in the addition/abstraction of the pro-2*R* proton, is structurally homologous to D185 in mtMenB (**Figure 2.41**).

Z-Enolation
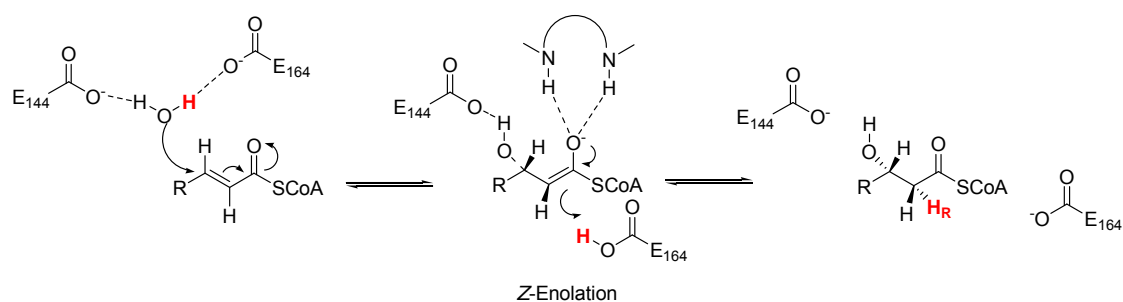
Figure 3.16: Mechanism of the reaction catalyzed by rat mitochondrial enoyl-CoA hydratase

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The active sites of MenB, BadI and enoyl-CoA hydratase are similar in that the catalytic residues are arranged on the same side of the substrate, with respect to the plane of the thioester (**Figure 3.15**). The thioester moieties of the bound substrate analogues share similar positions in the active sites of all structurally characterized members in the crotonase superfamily. The oxygen atom of the thioester is bound in the conserved oxyanion hole, forming hydrogen bonds with two amide hydrogens of the peptide backbone. The sulfur atom of the thioester is fixed in its position by the coenzyme A portion of the molecule. Since the oxygen and the sulfur are anchored in the same positions, it can be assumed that the plane of the thioester does not rotate during catalysis within the active site. Therefore, the enolate, which is stabilized by the oxyanion hole, will also maintain the same plane.

However the stereochemistry of MenB, BadI and enoyl-CoA hydratase are different (**Figure 3.17**), thus substrate must orient differently with the active site residues. mtMenB and enoyl-CoA hydratase share the same pro-2R stereochemistry. Like enoyl-CoA hydratase, the substrate of mtMenB presents the pro-2R proton to D185, resulting in the Z-enolate configuration of the intermediate. However, in ecMenB which lacks D185, we propose that the substrate carboxyl abstracts the pro-2R proton, thus leading to an enolate intermediate with the E configuration. In the case of BadI, the substrate binds in a conformation similar to that required for the ecMenB. However in the case of BadI, enzyme group abstracts the pro-2S proton.
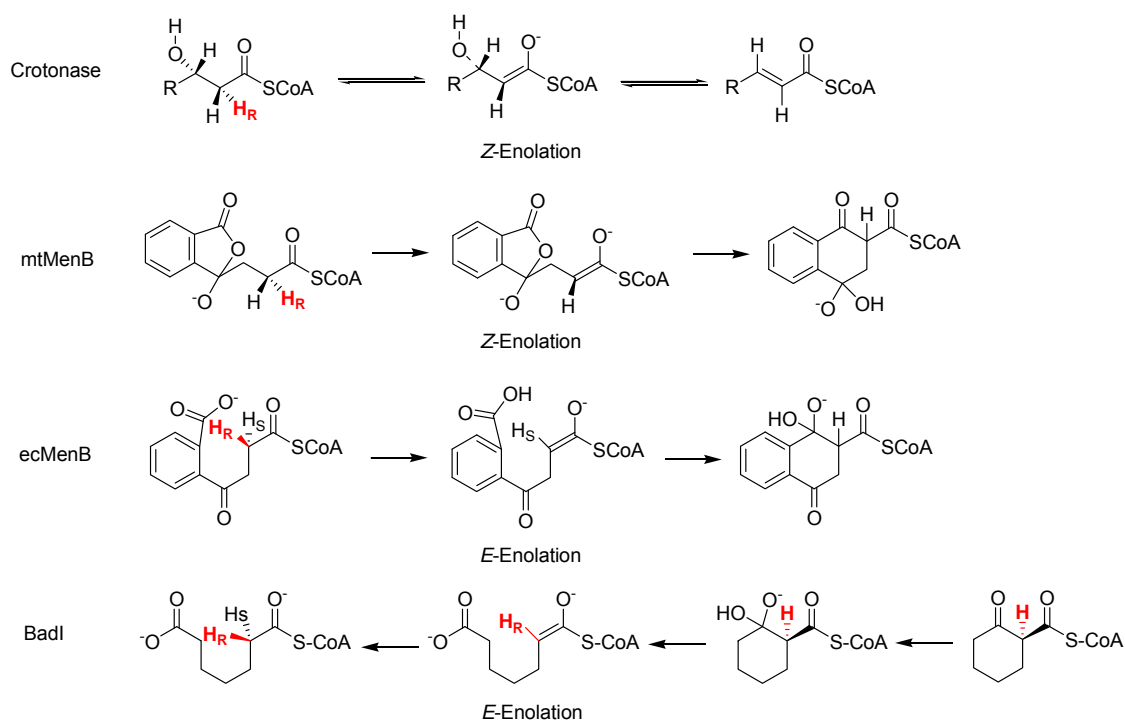
Figure 3.17: The divergent stereo-specificities of the reactions catalyzed by enoyl-CoA hydratase, mtMenB, ecMenB, and BadI. Pro-2*R* proton is abstracted in enoyl-CoA hydratase and MenB reaction while it is retained in the α-carbon in BadI reaction.

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Conclusions

MenB and BadI belong to the crotonase superfamily and catalyze similar but totally reverse reactions. MenB catalyzes carbon-carbon bond formation (Dieckmann condensation) while BadI catalyzes the carbon-carbon bond hydrolysis (reverse Dieckmann condensation). Therefore it is interesting to compare the both enzymes.

The mutagenic data indicates that the conserved residues of S138, D140 and Y235 are important for the reaction, with Ser 138 playing a central role. The crystal structure of BadI in complex with cyclohexenecarboxyl-CoA also suggests that these three catalytic residues occur within 5 Å of the bound substrate analogue.

In crystal structures of MenB and BadI, the active site residues are all positioned on one side of the substrate analogues with respect to the plane of the thioester, which is similar to the active site of enoyl-CoA hydratase. However the stereochemistry of MenB, BadI and enoyl-CoA hydratase are different. mtMenB and enoyl-CoA hydratase share the pro-2*R* proton stereochemistry. In mtMenB, the substrate presents the pro-2*R* proton to D185, resulting in Z-enolate configuration of the intermediate. However, in ecMenB which lacks D185, we propose that the substrate carboxyl abstracts the pro-2*R* proton, thus leading to an enolate intermediate with the *E* configuration. In the case of BadI, the substrate binds in a conformation similar to that required for the ecMenB, but the pro-2*S* proton is added to the reaction.

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CHAPTER 4: INHIBITION STUDIES OF MTMENB

Introduction

The central goal of our project is based on the mechanistic understanding of bacterial menaquinone biosynthesis, to design and synthesize potent inhibitors against menaquinone biosynthetic enzymes and to validate menaquinone biosynthesis as a target for the development of novel anti-TB chemotherapeutics. In this chapter, we will discuss our inhibition studies of mtMenB which is in the early stage of the drug development process (**Figure 4.1**) including exploration and lead selection. Further steps will be continued in our group.

Exploration
- Biological target identification, validation and characterization
- Chemical libraries
- High throughput screening

Lead Selection
- *In vitro* enzyme assays
- Initial *in vitro* SAR
- Property screens
- Initial synthetic enhancement

Lead optimization
- *In vivo* assay SAR and *in vivo* PK and metabolism
- Selectivity assays
- X-ray & NMR binding studies, computational modeling
- Custom property studies for SPR

Development Selection
- *In vitro* & *in vivo* toxicology
- Formulation
- In depth property characterization
- clinical candidate advancement

Figure 4.1: Stages of drug discovery process

Materials and methods

Pilot screening to establish assay conditions for high throughput screening

Pilot screens were performed to identify the most appropriate conditions for a high-throughput screen aimed at identifying lead MenB inhibitors. The MenE/MenB coupled assay was performed under the range of substrate concentration from 200 µM to 300 µM, and reaction time from 20 min to 60 min.

High throughput screening of potent inhibitors of mtMenB

High throughput screening was carried out to select small molecules that inhibit mtMenB activity at the ICCB-Longwood Screening Facility at Harvard Medical School. Assays were carried out in 384-well plates at 25 °C. Each well contained of 40 µL of mixture that included 250 µM of OSB, ATP and CoA, 0.15 µM of mtMenB, 3 µM of *E. coli* MenE and ~ 23 µM of screening compound in 20 mM NaH_2PO_4, 1 mM $MgCl_2$, 150 mM NaCl buffer (pH 7.0). OSB, ATP, CoA and mtMenB were mixed and then screening compounds were added by pin transfer. The reaction was initiated by adding *E. coli* MenE. After 60 min the extent of the reaction we assessed by measuring the absorbance of each reaction at 405 nm (since this the closest filter to 392 nm) using an *Envision* plate reader. The mtMenB mutant Y287F, which is inactive, was used as a positive control. The wells which were added with DMSO instead of screening compound were used as a negative control.

Pyrophosphate release assay

Pyrophosphate (PPi) is released in the process of reaction catalyzed by MenE. PPi is determined in this procedure according to the following coupled reactions. Two moles of NADH are oxidized to NAD^+ per mole of pyrophosphate consumed. The reaction is monitored on a CARY-300 spectrophotometer at 25 °C at 340 nm using an extinction coefficient of 6, 220 $M^{-1}cm^{-1}$.

$$PP_i + \text{D-Fructose-6-phosphate} \xrightarrow{\substack{\text{Fructose-6-phosphate} \\ \text{kinase}}} \text{D-Fructose-1,6-diphosphate} + P_i$$

$$\text{D-Fructose-1,6-diphosphate} \xrightarrow{\text{Aldolase}} \text{D-Glyceraldehyde-3-phosphate} + \text{Dihydroxyacetone phosphate}$$

$$\text{D-Glyceraldehyde-3-phosphate} \xrightarrow{\substack{\text{Triosephosphate} \\ \text{isomerase}}} \text{Dihydroxyacetone phosphate}$$

$$2 \text{ Dihydroxyacetone phosphate} + 2 \text{ NADH} + 2 \text{ H}^+ \xrightarrow{\substack{\text{Glycerophosphate} \\ \text{dehydrogenase}}} 2 \text{ glycerol-3-phosphate} + 2 \text{ NAD}^+$$
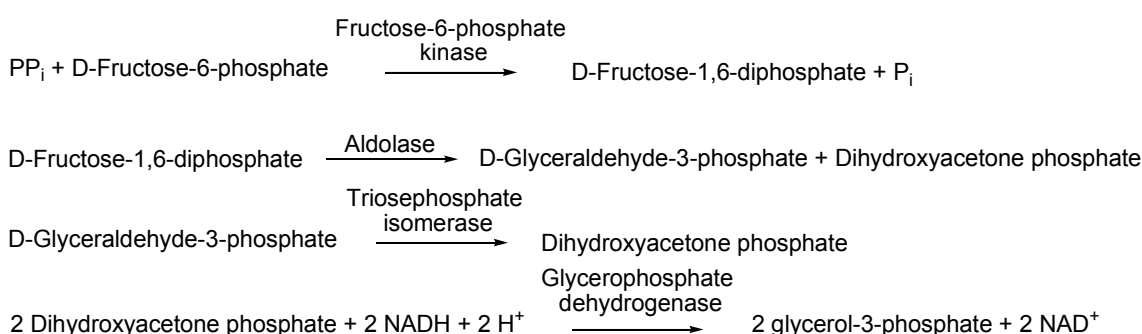
Fluorescence titration of mtMenB with hexachlorophene

Equilibrium fluorescence titrations were performed at 25 °C in a Fluorolog-3-21 fluorimeter by making microliter additions of the ligand to a solution of mtMenB (5 uM). The excitation wavelength was 290 nm and the emission wavelength was 343 nm. A control experiment was performed following the same procedure but omitting enzyme from the cuvette. The data were fit to equation (1) to obtain K_d.

$$\Delta F = \Delta F_{max} \left(\frac{K_d + [E] + [L] - \sqrt{K_d + [E] + [L]^2 - 4[L][E]}}{2[E]} \right) \quad (1)$$

Assay for inhibition of mtMenB

Reactions were performed in 20 mM NaH_2PO_4 pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$ and were initiated by adding MenE (final concentration 4 µM) to a solution containing MenB (200 nM), ATP (120 µM), CoA (120 µM), OSB (30 µM) and inhibitor (0–200 µM). The formation of DHNA-CoA was monitored at 392 nm and initial velocities were determined using an extinction coefficient of 4,000 $M^{-1}cm^{-1}$. IC_{50} values were calculated by fitting the initial velocity data (v_i) obtained at different inhibitor concentrations ([I]) to equation (2) using Grafit 4.0.

$$v_i = \frac{100\%}{1 + [I]/IC_{50}} \quad (2)$$

The type of inhibition was determined by Lineweaver-Burk plot analysis. K_i values for competitive inhibition were determined by fitting the data to equation (3) while equation (4) was used for non-competitive inhibition using Grafit 4.0.

$$v_i = \frac{V_{max} \times [S]}{K_m\left(1 + {[I]}/{K_i}\right) + [S]} \quad (3)$$

$$v_i = \frac{V_{max} \times [S]}{([S] + K_m)\left(1 + {[I]}/{K_i}\right)} \quad (4)$$

Determination of M. tuberculosis antimicrobial activity

MIC_{90} data for *M. tuberculosis* were acquired by Susan E. Knudson in the Department of Bioagricultural Sciences and Pest Management at Colorado State University.

Determination of B. subtilis antimicrobial activity

Whole-cell antimicrobial activity was determined by a broth microdilution procedure. Different inhibitors were dissolved in DMSO and serially diluted into VY medium (Veal-Yeast medium, ATCC medium) on a 96-well plate. The final test concentrations ranged from 300 μM to 0.01 μM of inhibitors. 10 μL of overnight grown cells of *B. subtilis* were added into each well. Inoculated plates were incubated at 37 °C for 24 hours. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of compound that inhibited visible growth as estimated by OD_{600} reading using a 96-well plate reader.

Supplement experiment with DHNA by agar overlay method

Liquid growth experiments with DHNA in LB and VY media were unsuccessful. This is due to the instability of DHNA in aqueous aerated media which has been discussed in Chapter 2. Therefore, soft agar overlay procedures were used for supplement by DHNA (*1*). Approximately 5 μg amounts of DHNA were spotted on blank paper disks placed on soft agar overlays with different concentration of inhibitors, and enhancement of growth around the disks was monitored after 24 hours of growth.

Supplement experiment with menadione and menaquinone-7 (MK-7)

Different concentration of menadione and menaquinone-7 (MK-7), from 10 μM to 300 μM was added to medium wells which contained different

concentration of inhibitors, ranging from 300 µM to 0.01 µM. The 96-well plates were incubated at 37 °C for 24 hours and read at OD_{600}.

Results and discussion

Pilot screening of mtMenB and hexachlorophene

Around 3,000 compounds were analyzed in a pilot screen to optimize the screening conditions. Z' factor is a simple statistical parameter for use in evaluation and validation of high throughput screening assays (*2*). The number of Z' factor was calculated based on the formula based on Equation (*5*). An optimal assay has a Z' score of greater than 0.7 and we generally achieved Z scores in the range from 0.6 to 0.7 during the pilot as we increased the reaction time to 60 minutes (**Table 4.1**).

$$Z' = 1 - \frac{(3SD_+ + 3SD_-)}{|Ave_+ - Ave_-|} \quad (5)$$

SD $_+$ = positive control standard deviation   Ave $_+$ = positive control average

SD $_-$ = negative control standard deviation   Ave $_-$ = negative control average

Table 4.1: Z' score of different reaction time

Reaction time	SD$_+$	SD$_-$	Ave$_+$	Ave$_-$	Z'
20 min	0.0084	0.0086	0.052	0.143	0.44
30 min	0.0080	0.0083	0.052	0.157	0.54
40 min	0.0077	0.0081	0.052	0.166	0.60
60 min	0.0073	0.0079	0.052	0.173	0.63

The best positive result from the pilot screening was hexachlorophene (**Figure 4.2**), which is an antimicrobial compound, being most active against Gram-positive bacteria (*3*). Frederick has proposed that hexachlorophene appears to inhibit respiration by interfering with membrane-bound components of the electron transport chain (*4*). Since menaquinone is an essential component of

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the electron transport chain, we propose that hexachlorophene could act by inhibiting MenB, thereby decreasing the amount of menaquinone in the membrane.
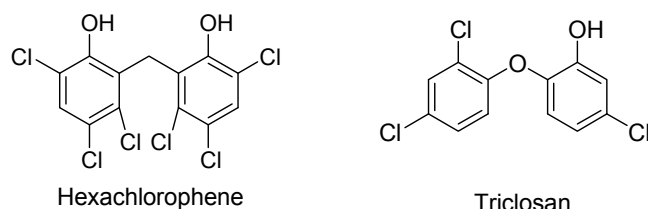
Figure 4.2: The structures of hexachlorophene and triclosan

In our *in vitro* enzymatic kinetic assay, hexachlorophene is a competitive inhibitor of mtMenB with an IC_{50} value of 9.3 ± 0.9 µM, K_i value of 7.4 ± 0.8 µM and K_d value of 17.2 ± 0.5 µM (**Figure 4.3**). The MIC_{90} value of hexachlorophene against *M. tuberculosis* is < 0.78 µg/mL. Triclosan, an antibacterial chemical mainly targeting the FabI in bacteria fatty acid biosynthesis, has the similar structure of hexachlorophene. However, it showed very poor inhibition (more than 200 µM) against mtMenB.

A

B

C
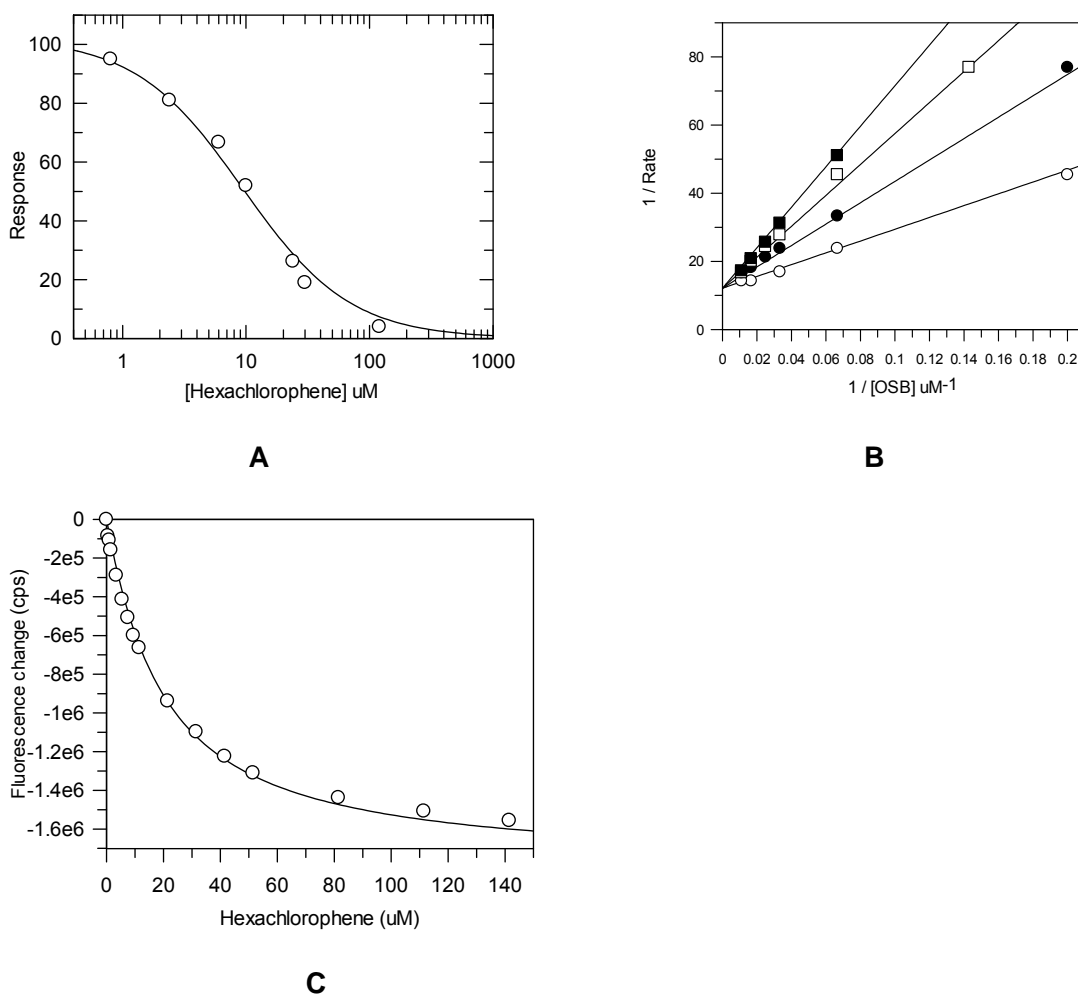
Figure 4.3: Inhibition and binding data of hexachlorophene of mtMenB. Figure A: IC$_{50}$ of hexachlorophene versus mtMenB. B) Lineweaver-Burk plot. The inhibition hexachlorophene against mtMenB in the absence of inhibitor (○), in the presence of 6 μM of hexachlorophene(●), 12 μM of hexachlorophene (□) or 18 μM of hexachlorophene (■). C) Fluorescence titration of mtMenB with hexachlorophene. Excitation wavelength is 290 nm, and emission wavelength is 343 nm.

High throughput screening of mtMenB

We screened around 100,000 small molecular compounds from the library of Known Bioactives (Pilot screening) and Commercial Compounds (**Table 4.2**) at the ICCB-Longwood Screening Facility at Harvard Medical School (June, September and October 2007).

Table 4.2: Libraries of screened compounds

	Library Name	Number of Compounds	Plate Numbers	Number of Plates
Known Bioactive	Biomol ICCB Known Bioactives2-Hign Conc.	480	1791-1792	2
	Biomol ICCB Known Bioactives1-Medium Conc.	480	1361-1362	2
	Ninds Custom Collection 2	1,040	1920-1923	4
	Prestwick 1 Collection	1,120	1568-1571	4
Commercial Compounds	Asinex 1	12,378	1671-1706	36
	ChemBridge 3	19,560	1577-1606	30
	ChemDiv 4	14,677	1607-1648	42
	Enamine 2	26,576	1715-1790	76
	Life Chemicals 1	3,893	1649-1660	12
	Maybridge 5	3,212	1661-1670	10
	ChemDiv3	16,544	1473-1519	47
	ChemDiv 5	1,249	1709-1712	4
	MixCommercial 5	268	1520	1
	Maybridge 4	4,576	1521-1533	13
	Biomol-TimTec 1	8,518	1534-1558	25

Microsoft Excel spreadsheet was used to organize and analyze screening data. Cut-offs for defining the potency of inhibitors were calculated as follows. We classified hits into three groups: strong hits are those compounds that inhibited the reaction greater than 70%, (**Figure 4.4**), medium hits are 50% < inhibition < 70% (**Figure 4.5**) and weak hits are 30% < inhibition < 50% (**Figure 4.6**).

$$30\% \text{ of inhibition:} N_{ave} - (N_{ave} - P_{ave}) \times 30\%$$

$$50\% \text{ of inhibition: } N_{ave} - (N_{ave} - P_{ave}) \times 50\%$$

$$70\% \text{ of inhibition: } N_{ave} - (N_{ave} - P_{ave}) \times 70\%$$

N_{ave}: negative control average based on the absorbance of negative wells in each plate;

P_{ave}: positive control average based on the absorbance of positive wells in each plate.
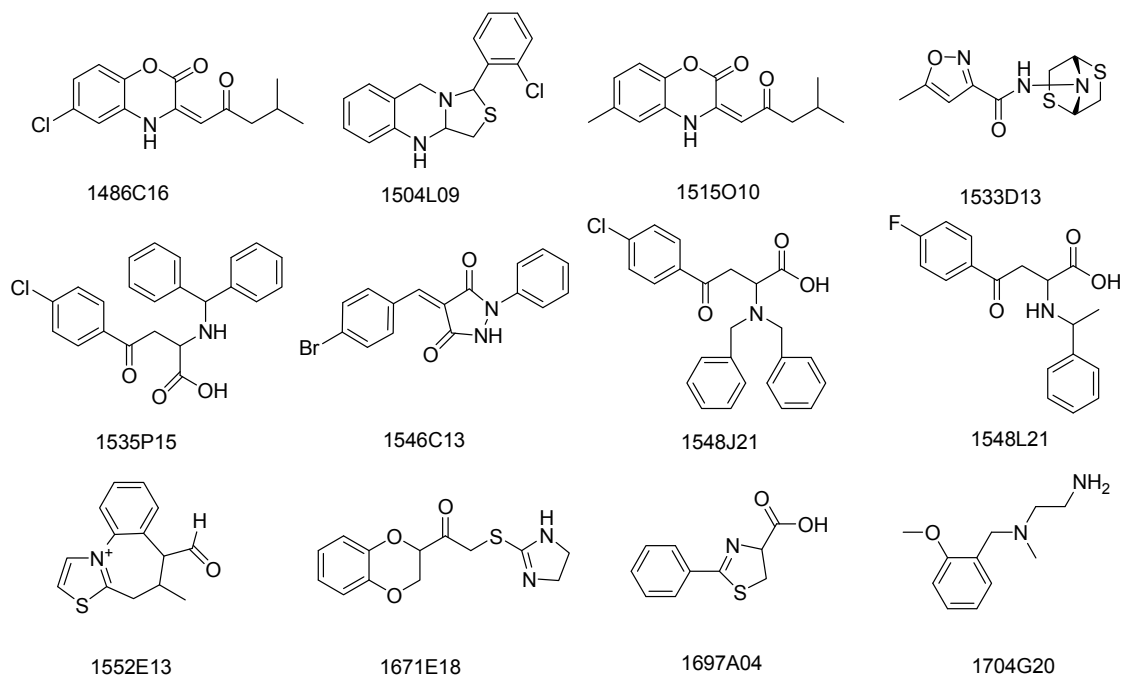
Figure 4.4: High throughput screening hits with strong inhibition

1482I15   1538N21   1540P05   1570I08   1577B21

1578I09   1615I20   1649F03   1670O07   1671O16

1701M18   1755P21   1727B01   1772J21   1922O06
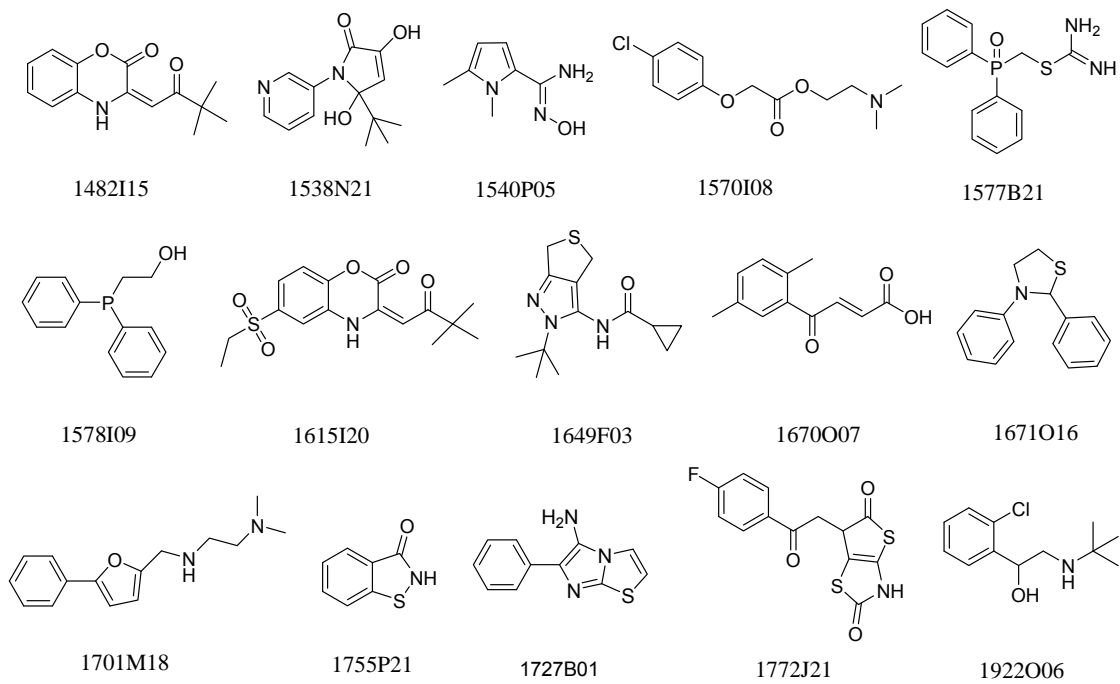
Figure 4.5: High throughput screening hits with medium inhibition

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1504B09   1528L17   1535J17   1535F17   1536L17

1537L14   1547C04   1550L21   1554A01   1557E17

1579A13   1579C15   1580A14   1580A16   1598A13

1598C22   1665E01   1668B11   1672A08   1673C02

1674C08   1674C15   1674D14   1675A14   1678A14

1678C10   1679A04   1679A15   1680B14   1680C01

1702A08   1704I20   1757E12   1765F09   1789K04
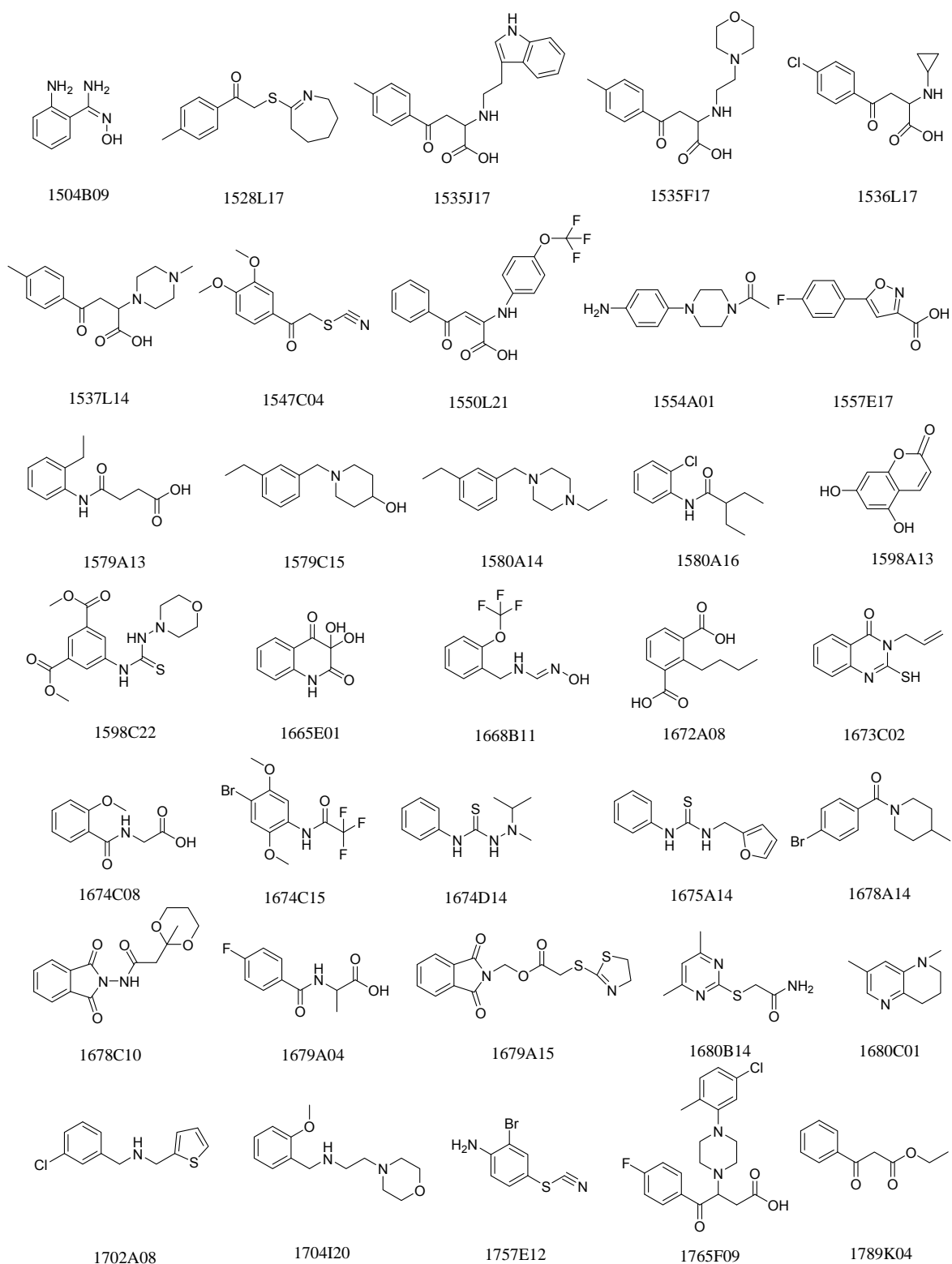
Figure 4.6: The structures of high throughput screening hits with weak inhibition
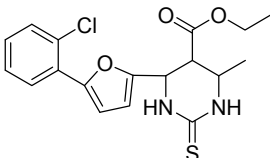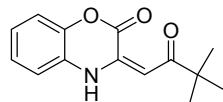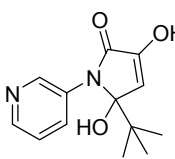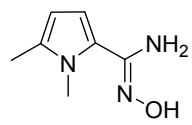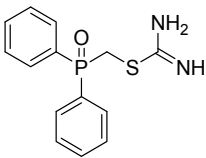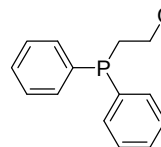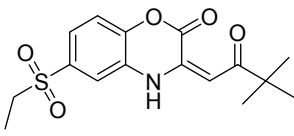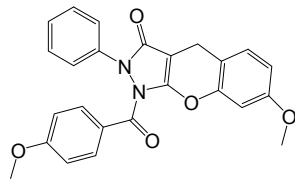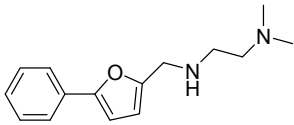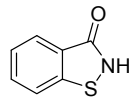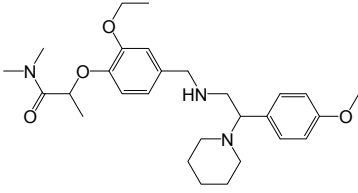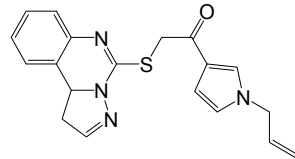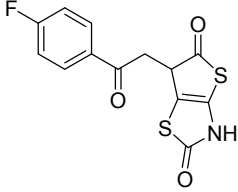
Second screens of "cherry picks"

After completing the primary screen, we analyzed the structures of "positives" and selected some compounds for second screening called "cherry picks". The requested compounds included all the "strong" hits, and the "medium" hits with reasonable structures. 1 µl of each selected screening positive compound (~5 µg) was provided and the second screening was performed in our lab (**Table 4.3** and **Table 4.4**). For those compounds we are interested from the "cherry picks", the inhibition was measured in the presence and absence of 0.1% Triton X-100 according to the Shoichet's method (*5*); molecules that inhibit only in the absence of detergent are considered likely promiscuous aggregators.

Table 4.3: Inhibition data of second screens for strong hits (inhibitor concentration is 12 μM)

ID No.	Structure	Activity (%)	ID No.	Structure	Activity (%)
1486 C16		79.4	1504 L09		74.1
1515 O10		64.7	1533 D13		78.2
1535 P15		23.5	1546 C13		67.6
1548 J21		5.0	1548 L21		64.7
1552 E13		82.3	1670 O07		52.9
1671 E18		64.7	1697 A04		82.3
1704 G20		94.1	1727 B01		100

Table 4.4: Inhibition data of second screens for medium hits (inhibitor concentration is 37.5 µM)

ID No.	Structure	Activity (%)	ID No.	Structure	Activity (%)
1474 D06		18.9	1482 I15		61.1
1538 N21		66.7	1540 P05		66.7
1577 B21		66.7	1578 I09		75.0
1615 I20		16.7	1622 I10		66.7
1701 M18		66.7	1755 P21		72.2
1759 E18		61.1	1759 I10		55.6
1772 J21		44.4			

From the results of "cherry picks", it can be noted that a series of positive compounds, such as 1548 L21, have the backbone of OSB moiety of MenB substrate. The inhibition activities of the "substrate-like" molecules were tested against ecMenE by using PPi release assay and MenE/MenB assay using limited concentration of mtMenB. However, there was no inhibition observed at the concentration of inhibitor of 200 µM, suggesting that this compound class primarily targets MenB in the coupled assay. In contrast, compounds such as 1486 C16 are reminiscent of the DHNA MenB product. Therefore, our subsequent SAR studies have primarily focused on the structure of "substrate-like" 2-amino-4-oxo-phenylbutanoic acids and "product-like" benzoxazinones (**Figure 4.7**).
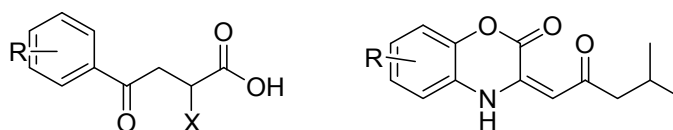
Figure 4.7: Structure of 2-amino-4-oxo-phenylbutanoic acids and benzoxazinones

In vitro enzymatic inhibition assay of "substrate-like" inhibitors
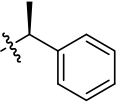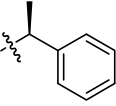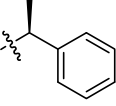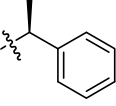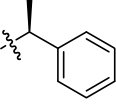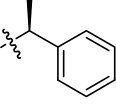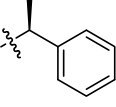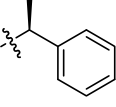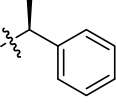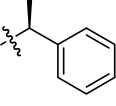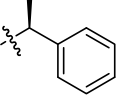
Our current SAR studies are primarily based on the structure of "substrate-like" 2-amino-4-oxo-phenylbutanoic acids. We have designed a series of molecules which contain the OSB moiety with the different substitutions in the aromatic ring and with different substitutions at the α-carbon. These compounds were synthesized by Xiaokai Li in our group. The inhibition of different inhibitors was measured by ecMenE/mtMenB coupled assay which is described in the Materials and Methods section. The substrate and mtMenB were fixed as 30 µM

and 200 nM, respectively. The enzyme inhibition data are summarized in **Table 4.5**.
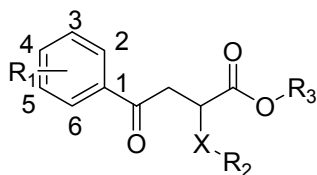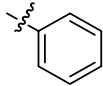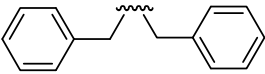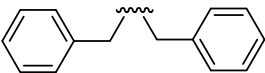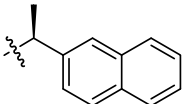
Table 4.5: Inhibition of "substrate-like" inhibitors

Compound	R_1	X	R_2	R_3	IC_{50} (µM)	MIC_{90} M. tb (µg/mL)
1	H	NH		H	112.1 ± 10.7	7.81
2	4-F	NH		H	13.2 ± 0.75	8.46
3	4-Cl	NH		H	8.54 ± 0.80	7.52
4	4-Br	NH		H	105.4 ± 15.0	9.67
5	4-NO$_2$	NH		H	>200	194.66
6	4-OMe	NH		H	>200	8.41
7	2-F	NH		H	8.70 ± 0.80	24.34
8	2-Cl	NH		H	8.50 ± 0.80	5.94

Compound	R_1	X	R_2	R_3	IC_{50} (µM)	MIC_{90} *M. tb* (µg/mL)
9	2-Br	NH	CH(CH₃)Ph	H	0.600 ± 0.07	12.80
10	2-I	NH	CH(CH₃)Ph	H	0.630 ± 0.034	6.25
11	2-NO$_2$	NH	CH(CH₃)Ph	H	2.1 ± 0.2	9.57
12	2-OMe	NH	CH(CH₃)Ph	H	>200	11.07
13	2-CF$_3$	NH	CH(CH₃)Ph	H	2.10 ± 0.19	11.70
14	2-COOMe	NH	CH(CH₃)Ph	H	>200	>300
15	3-Cl	NH	CH(CH₃)Ph	H	>200	13.95
16	3-NO$_2$	NH	CH(CH₃)Ph	H	>200	228.67
17	2,4-diF	NH	CH(CH₃)Ph	H	1.40 ± 0.18	7.11
18	2-Cl, 4-F	NH	CH(CH₃)Ph	H	1.10 ± 0.08	12.50
19	2-Br, 4-F	NH	CH(CH₃)Ph	H	0.430 ± 0.032	12.50

Compound	R_1	X	R_2	R_3	IC_{50} (μM)	MIC$_{90}$ *M. tb* (μg/mL)
20	2-CF$_3$, 4-F	NH	(1-phenylethyl)	H	0.823 ± 0.094	7.39
21	2,4-diCl	NH	(1-phenylethyl)	H	0.262 ± 0.027	20.30
22	2,6-diCl	NH	(1-phenylethyl)	H	7.11 ± 0.11	0.89
23	4-Cl	NH	(1-phenylethyl)	H	41.9 ± 3.4	10.37
24	4-Cl	NH	(benzyl)	H	17.2 ± 2.7	28.89
25	4-Cl	NH	(4-pyridylmethyl)	H	>200	9.85
26	4-Cl	NH	(diphenylmethyl)	H	18.1 ± 2.2	14.04
27	4-Cl	NH	(1-cyclohexylethyl)	H	3.25 ± 0.33	12.30
28	4-Cl	NH	(cyclohexyl)	H	10.0 ± 0.9	16.19
29	4-Cl	NH	(cyclooctyl)	H	7.25 ± 0.60	699.85
30	4-Cl	NH	n-octyl	H	15.1 ± 1.6	12.17
31(*6*)	4-Cl	S	(1-phenylethyl)	H	>200	51.89

Compound	R_1	X	R_2	R_3	IC_{50} (µM)	MIC_{90} *M. tb* (µg/mL)
32(*7*)	4-Cl	CH_2		H	>200	45.41
33	4-Cl	N		H	2.2 ± 0.2	NA
34	4-Cl	N		CH_3	NA	< 0.78
35	4-Cl	NH		H	25.1 ± 4.0	NA

One conclusion that can be drawn from the above data is that the addition of a bulky substituent (R_1) at either the 2 or 4 position of the aromatic ring, or the addition of substitution at 3-position of the phenyl group resulted in significant reduction in enzymatic inhibition. In addition, while electron withdrawing groups increased the potency of inhibition, electron donating groups reduced the affinity of the compounds for MenB. The 2, 4-dichloro substituted compound **21** is the best inhibitor so far indentified with an IC_{50} value of 260 nM. Therefore, it can be concluded that two factors determine the inhibition activity for the aromatic substitutions: the size and electronic effect of the groups at the 2- and/or 4-position.

In addition to the importance of electron withdrawing substituents on the aromatic ring, the SAR data revealed two other important structural features for MenB inhibition: an amino group at position X and a hydrophobic R_2 group. Replacement of X with sulfur (compound **31**) or carbon (compound **32**) caused a significant reduction in inhibitory activity, while lower IC_{50} values were observed

when R_2 was a (S)-1-phenylethyl (such as compound **21**), a (S)-1-cyclohexylethyl (compound **27**) or diphenylmethyl (compound **33**). In addition, a series of OSB analogues lack the corresponding R_2 substituents and don't show inhibition against mtMenB (**Figure 4.8**). Although structures of the inhibitors bound to MenB are not currently available, analysis of existing structural data suggests that the R_2 substituent may occupy the hydrophobic pocket revealed in the dimethoxy DHNA-CoA structure (**Figure 2.52**).
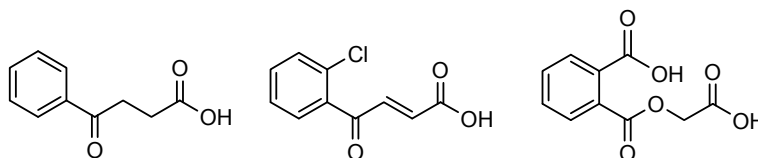
Figure 4.8: Structures of compounds showed poor inhibition against mtMenB
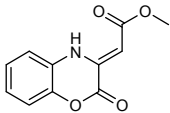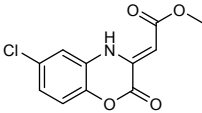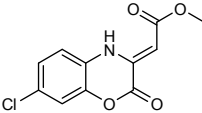
Compound **8** is a competitive inhibitor of mtMenB with a K_i value of 8.1 ± 0.6 µM (**Figure 4.9**). It can be deduced that the "substrate-like" inhibitors occupy the active site and compete with substrate for the active site.
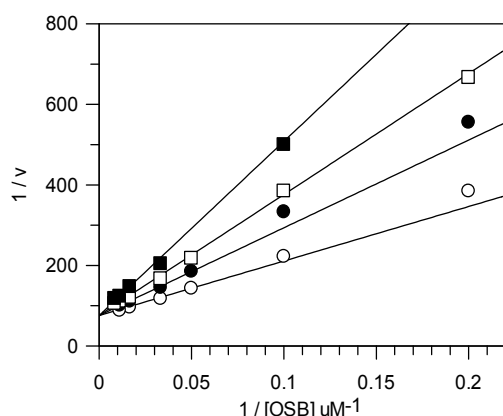
Figure 4.9: Compound 8 is a competitive inhibitor. The inhibition of compound 8 against mtMenB in the absence of inhibitor (○), in the presence of 5 µM of compound 8 (●), 10 µM of compound 8 (□) or 18 µM of compound 8 (■).

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In vitro enzymatic inhibition assay of "product-like" inhibitors

The SAR studies on "product-like" benzoxazinone inhibitors are less well advanced. Compounds 35-37 were synthesized and their IC_{50} values were measured (**Table 4.6**).

Table 4.6: Inhibition of "product-like" inhibitors

Compound	Structure	IC_{50} (μM)	MIC_{90} *M. tb* (μg/mL)
36		10.1 ± 0.9	<0.78
37		23.1 ± 1.0	NA
38		46.2 ± 2.9	NA

Unlike "substrate-like" inhibitors, compound **36** ("product-like") is a non-competitive inhibitor with a K_i value of 14.1 ± 0.6 μM (**Figure 4.10**). Non-competitive inhibition occurs when the inhibitor binds at a site away from the substrate binding site, causing a reduction in the catalytic rate. A non-competitive inhibitor could be a promiscuous inhibitor. However, the inhibition was not affected in the presence or absence of 0.1% Triton X-100, indicating that the inhibition of compound **36** is not due to the promiscuous aggregation. Further SAR studies as well as binding assays will be conducted.

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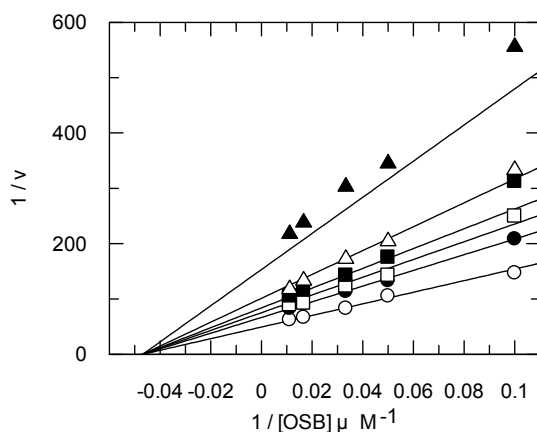
Figure 4.10: Compound 36 is a non-competitive inhibitor. The inhibition of compound **36** against mtMenB in the absence of inhibitor (○), in the presence of 5 µM of compound **36** (●), 7.5 µM of compound **36** (□), 10 µM of compound **36** (■), 15 µM of compound **36** (△) or 30 µM of compound **36** (▲).

MK-7 can partially rescue the growth of B. subtilis

In order to validate whether MenB and menaquinone biosynthesis are essential for bacterium growth, the supplement experiments with DHNA, menadione and MK-7 were performed. These experiments were performed with *B. subtilis* instead of *M. tuberculosis* since this organism can be grown in normal lab conditions (BSL-1) and MenB from *B. subtilis* and mtMenB share 50.6% identity over 265 residues overlap. *B. subtilis* is a fast growing Gram positive bacterium that also requires menaquinone for survival. The menaquinone biosynthetic pathway of *B. subtilis* is the same as in *M. tuberculosis* except that *M. tuberculosis* uses MK-9 while *B. subtilis* uses MK-7 for electron transportation.

The experiments using DHNA and menadione were unsuccessful since DHNA was unstable in solution and menadione indeed inhibited the growth of the

bacterium. However, supplement with MK-7 rescued the growth of *B. subtilis* (**Figure 4.11**). **Figure 4.12** showed that compound **8** killed its growth at the concentration of <200 µM (60 µg/mL). While the supplement of MK-7 partially overcome the inhibition, which indicates that menaquinone biosynthesis is required for bacterial survival.
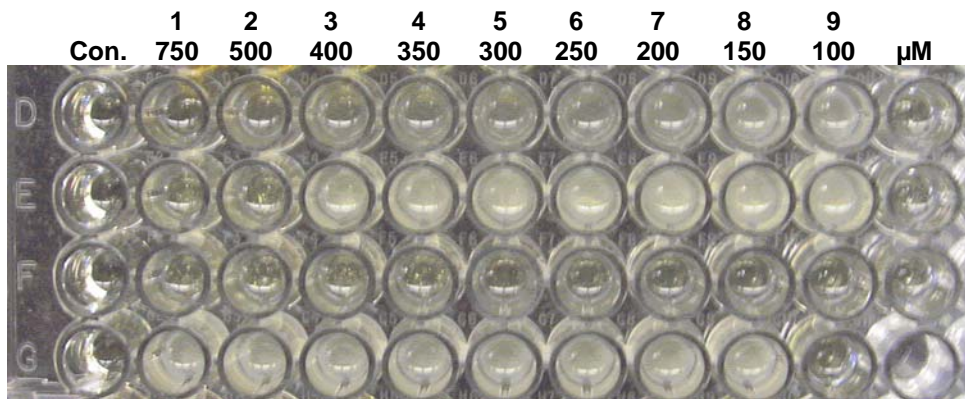
Figure 4.11: Supplement experiment with MK-7. Lane D1-D9 contained compound 8 (concentration from 750-100 µM). Lane E1-E9 contained same concentration of compound 8 and 200 µM of MK-7 each. Lane F1-F8 were negative control (no cells grew) and lance G1-G8 were positive controls (cells grew).
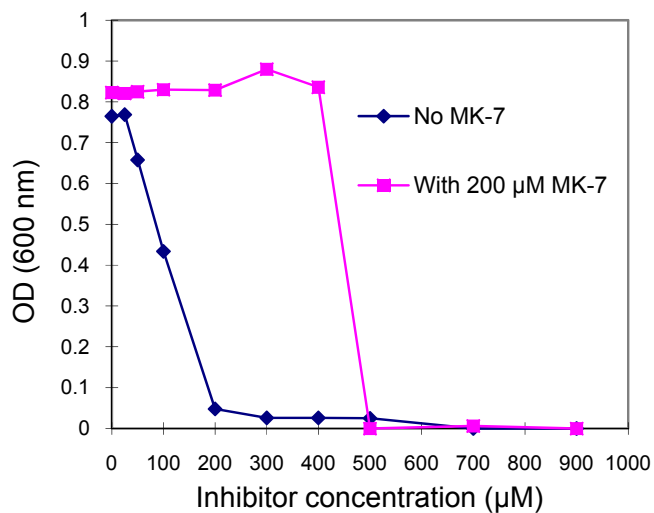
Figure 4.12: MK-7 partially overcomed the inhibition of compound 8

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In vitro antibacterial activity of inhibitors against M. tuberculosis

The MIC_{90} values of different inhibitors against *M. tuberculosis* were measured by Susan E. Knudson in the Department of Microbiology, Immunology and Pathology, Colorado State University (**Table 4.5 and Table 4.6**). Basically, IC_{50} and MIC_{90} had a reasonable linear correlation with each other, which indicates these groups of compounds target mtMenB. However, it is important to note that several compounds have very good MIC_{90} values but have poor IC_{50} values, suggesting that mtMenB might not be the only target.

Interestingly, the methyl ester compound **34** has extremely good MIC_{90} value of < 0.78 μg/mL. Although we don't have the MIC_{90} value of its acid compound **33**, it can be deduced by comparing with other compounds that the methyl ester improves the antibacterial activity probably because it increases the molecule hydrophobicity and facilitates transport across the mycobacterial membrane*.*

Conclusions

Two types of lead inhibitors of mtMenB including "substrate-like" 2-amino-4-oxo-phenylbutanoic acids and "product-like" benzoxazinones were identified from high throughput screening.

Our initial SAR studies on 2-amino-4-oxo-phenylbutanoic acids suggest that the introduction of electron withdrawing substituents on the aromatic ring (R_1) is essential for inhibition. In addition, two other important structural features for MenB inhibition include an amino group at position X and a hydrophobic R_2 group. The best inhibitor (compound **21**) indentified so far has an IC_{50} value of 260 nM. The "substrate-like" inhibitors are competitive inhibitors of mtMenB.

In contrast, the SAR studies on "product-like" inhibitors are less well advanced. Benzoxazinone compound **36** is a non-competitive inhibitor with an IC_{50} value of 10 µM and a K_i value of 14 µM.

Although we do not have structures of these inhibitors bound to mtMenB, the structure of the product analogue dimethoxy DHNA-CoA bound to mtMenB provides a clue concerning how the compounds may bind to the enzyme. The hydrophobic pocket might provide a potential location for the R_2 group of 2-amino-4-oxo-phenylbutanoic acids and non-competitive inhibitors to bind.

The MIC_{90} values of different inhibitors against *M. tuberculosis* are basically correlated to their IC_{50} values, indicating MenB is the target for these two types of inhibitors. The inhibition can be partially overcome by the supplement with MK-7, which indicates that menaquinone biosynthesis is required for bacterial survival.

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CHAPTER 5: EXPRESSION AND INHIBITION STUDIES OF MENE

This chapter is based on part of work that has been published in: Mechanism-based inhibitors of MenE, an acyl-CoA synthetase involved in bacterial menaquinone biosynthesis. Lu X, Zhang H, Tonge PJ, Tan DS, Bioorg Med Chem Lett. 2008 Nov 15; 18(22):5963-6. Epub 2008 Aug 12.

Background

OSB-CoA synthase (MenE), an acyl-CoA synthetase, catalyzes a magnesium-dependent multisubstrate reaction, resulting in the conversion of OSB to OSB-CoA *via* a two-step process involving the initial ATP-dependent adenylation of OSB to form a reactive OSB-AMP intermediate, followed by thioesterification with CoA to form OSB-CoA (**Figure 5.1**) (*1, 2*). The mutagenesis technology using transposon site hybridization (TraSH) identified *mene* gene is essential in *M. tuberculosis* (*3*).
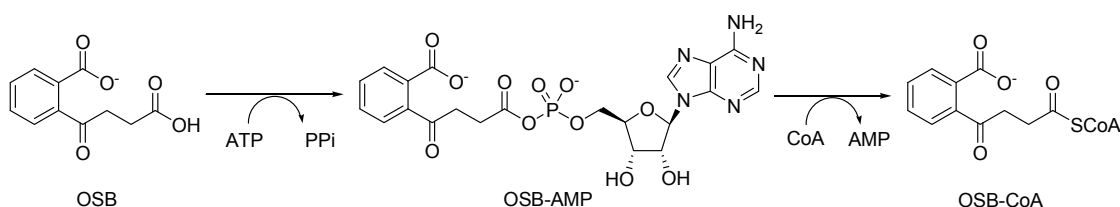
Figure 5.1: MenE reaction

The MenE reaction is analogous to that catalyzed by the long chain acyl-CoA synthetases (LC-FACS). Similarly, the synthesis of fatty acyl-CoA includes two discrete steps: 1) the formation of a fatty acyl-AMP molecule as a stable

intermediate and 2) the formation of a fatty acyl-CoA molecule as the final product (**Figure 5.2**) (*4, 5*).

fatty acid + ATP → fatty acyl-AMP + PPi

fatty acyl-AMP + CoA → fatty acyl-CoA + AMP
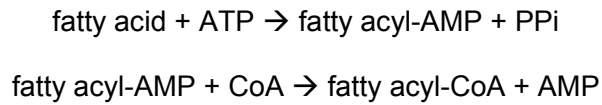
Figure 5.2: Long chain fatty acyl-CoA synthetases reaction

The molecular mechanism of LC-FACS is proposed to be compatible with the Bi Uni Uni Bi Ping-Pong based on the three high resolution structures of *Thermus thermophilus* HB8 LC-FACS (ttLC-FACS) (*5*) and extensive kinetic studies of the rat enzyme (*6*). The reaction scheme is summarized as follows (**Figure 5.3**). The binding of ATP triggers the closed conformation and opening of the W234 gate of the fatty acid-binding tunnel (**Figure 5.3** A and B). After the fatty acid molecule binds and the fatty acyl-AMP intermediate is formed, the pyrophosphate molecule leaves (**Figure 5.3** C). A CoA molecule then binds and the final product fatty acyl-CoA is formed (**Figure 5.3** C and D). Finally, the fatty acyl-CoA followed by the AMP leave after opening of the C-terminal domain (**Figure 5.3** E) (*5, 7*).
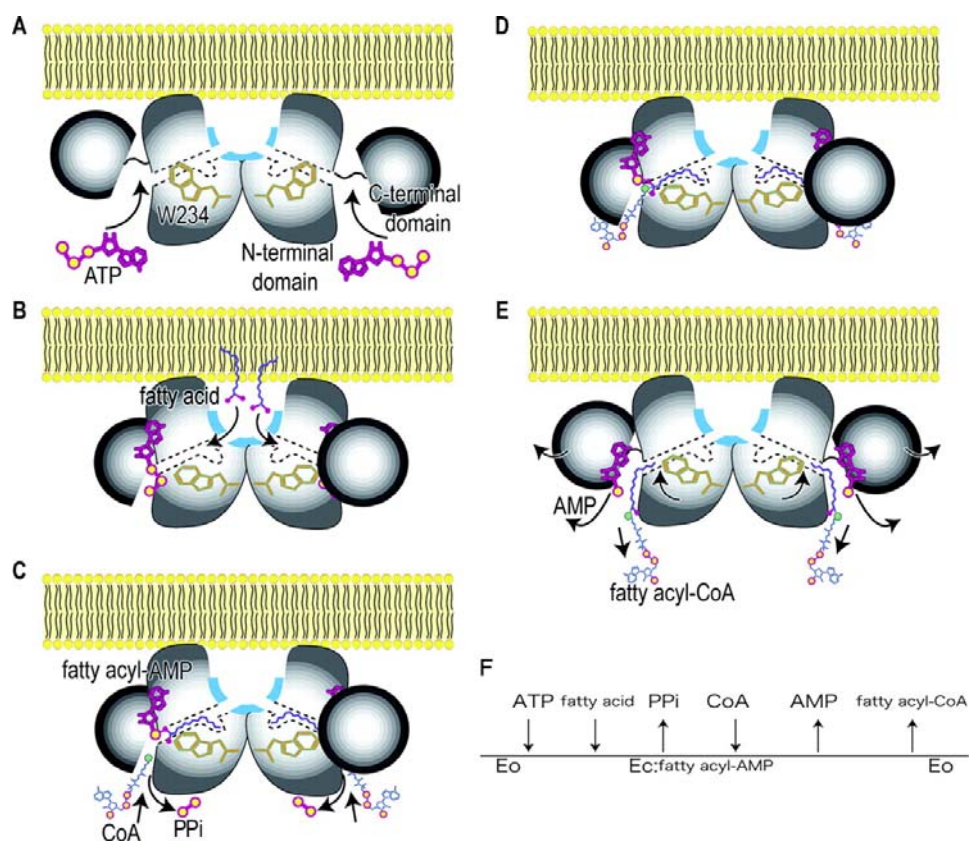
Figure 5.3: The schematic mechanism for the catalysis by ttLC-FACS (taken from reference (5)).

Acyl-CoA synthetases belong to a superfamily of structurally and mechanistically related adenylate-forming enzymes, and the crystal structures of several superfamily members have been solved (*5, 8-10*). Analogous adenylation reactions are also catalyzed by structurally unrelated aminoacyl-tRNA synthetases (*11*).

A series of 5′-*O*-(*N*-acylsulfamoyl) adenosines (acyl-AMS) and related compounds were reported to inhibit such adenylate-forming enzymes by mimicking the cognate, tightly bound acyl-AMP intermediates (*12-22*) (**Table 5.1**).

These molecules were inspired by a class of sulfamoyladenosine natural products that includes nucleocidin and ascamycin (*23, 24*).
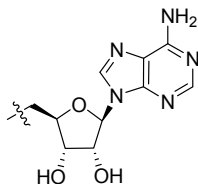
Table 5.1: Representative inhibitors of aryl acid adenylation enzymes

Inhibitor	Enzyme	K_i^{app}/nm	Organism
	YbtE	0.3–1.1	*Y. pestis*
	MbtA	5.1–6.6	*M. tuberculosis*
	MbtA	3.7–3.8	*M. tuberculosis*
	DhbE	85	*B. subtilis*
	EntE	9	*E. coli*
	AsbC	250 (IC$_{50}$ value)	*B. anthracis*

In this chapter, we will discuss our efforts to obtain the soluble mtMenE using the different expression hosts including *M. smegmatis and E. coli*. Although mtMenE has proven difficult to obtain, ecMenE expresses very well in *E. coli.* In addition, a series of mechanism-based inhibitors which mimic the tightly-bound

OSB-AMP intermediates were designed and their inhibition against MenE enzyme will be discussed.

Materials and methods

Expression and purification of ecMenE

The *mene* gene *b2260* (1356 bp), used for the overexpression and purification of ecMenE was previously isolated by PCR from genomic DNA, cloned into the pET-15b plasmid (Novagen) and placed in frame with an N-terminal His-tag sequence, by Yuguo Feng. Protein expression was performed using *E. coli* BL21 (DE3) cells. Transformed cells were grown in 800 mL of LB media containing 0.2 mg/mL ampicillin and induction was achieved using 1 mM IPTG overnight at 25 ºC. Cells were harvested by centrifugation at 5,000 rpm for 20 min at 4 ºC, resuspended in 30 mL of His-binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9) and lysed by 3 passages through a French Press cell (1,000 psi). Cell debris was removed by centrifugation at 33,000 rpm for 90 min at 4 ºC. MenB was purified using His affinity chromatography: the supernatant was loaded to a column containing 3 mL of His-bind resin (Novagen), charged with 9 mL of charge buffer (Ni^{2+}). The column was washed with 20 mL of His-binding buffer and 20 mL of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9). ecMenB was eluted using a gradient of 20 mL elute buffer (0.5 M imidazole, 0.5 M NaCl, 20 mM Tris HCl, pH 7.9). Fractions containing ecMenE were collected and the imidazole removed by chromatography on G-25 resin using 20 mM NaH_2PO_4, 0.1 M NaCl at pH 7.0, as storing buffer. The concentration of ecMenE was determined by measuring the absorption at 280 nm using an extinction coefficient of 104,770 $M^{-1}cm^{-1}$ calculated from the primary

sequence. The enzyme was concentrated by using Centricon-30 (Amicon) and stored at -80 ºC.

Cloning and expression of mtMenE in M. smegmatis cells

The *mene* gene *Rv0542c* (1089 bp) was amplified from *M. tuberculosis* genomic DNA by PCR and cloned into the pVVAP, pVV16 and pJAM2 plasmid by using the following oligonucleotides (primers):

Vector	Primer sequence (from 5' to 3' sequence)	Restriction site
pVVAP and pVV16	F: GGAATTCCATATGGTGCTGGGTGGCAGCGACCC	NdeI
	R: CCCAAGCTTCTATTGATCGGCTTCACCGGCGAA	HindIII
pJAM2	F: CGGGATCCGTGCTGGGTGGCAGCGACCCGGAC	BamHI
	R: GCTCTAGACTATTGATCGGCTTCACCGGCGAA	XbaI

The above plasmids were transformed into *M. smegmatis* competent cell by electroporation (2.5 kV). The transformants were resuspended in 900 µL of 7H9 medium, 300 µL of which were plated on a 7H10-Kan plate. The plate was incubated at 35.5 ºC for 3 to 4 days. A 15 mL rich culture (7H9 medium with 0.2% glycerol, 1.5 mL OADC and 5 µL Tween 80) with kanamycin (30 µg/mL) was inoculated from a single colony and shaken at 35.5 ºC for approximately 36 hours. 800 mL 7H9 medium was inoculated with 1 mL of rich culture, and shaken at 35.5 ºC for 24 hours or longer until the OD_{600} value reached around 0.4. 0.4% acetamide was added to cells harboring the pVVAP and pJAM2, and the cells were incubated at 35.5 ºC or 25 ºC for an additional 24 hours (there is no

induction promoter in pVV16 vector). Cells were harvested by centrifugation at 5,000 rpm for 20 min at 4 ºC.

Cloning and expression of mtMenE in E. coli cells

The *mene* gene *Rv0542c* (1089 bp) was amplified from *M. tuberculosis* genomic DNA by PCR and cloned into the pET23b and pET43b (N terminus and C terminus His-tag) plasmids by using the following oligonucleotides (primers). The gene was also cloned into pET15b plasmid by Hua Xu in our group.

Vector	Primer sequence (from 5' to 3' sequence)	Restriction site
pET23b	F: GGAATTCCATATGATGCTGGGTGGCAGCGACCC	NdeI
	R: CCGCTCGAGTTGATCGGCTTCACCGGCGAACCG	XhoI
pET43b N His-tag	F: TTCCCCCGGGATGCTGGGTGGCAGCGAC	SmaI
	R: CCGCTCGAGCTATTGATCGGCTTCACCGGCGAA	XhoI
pET43b C His-tag	F: GACTAGTATGCTGGGTGGCAGCGACCCGGCATT	BamHI
	R: CCGCTCGAGTTGATCGGCTTCACCGGCGAACCG	XhoI

Four plasmids were transformed into *E. coli* Rosette2 (DE3) (Novagen) competent cells by heat shock. Transformed cells were grown in 800 mL of LB medium containing 0.2 mg/mL ampicillin and induction was achieved using 0.5 mM IPTG overnight at 25 ºC. Cells were harvested by centrifugation at 5,000 rpm for 20 min at 4 ºC.

E. coli rare codon mutagenesis

In order to improve the expression of heterologous *M. tuberculosis* MenE in *E. coli* cell, a series of mutagenesis were performed to mutant the rare *E. coli* codons to inherent codons. The primers are listed in **Table 5.2**.

Table 5.2: Primer sequences for *E. coli* rare codon mutagenesis

		Primer (from 5' to 3' sequence)
1	Forward	GCCGACGACCGTCTCGGTCAGCGTGTGGTC
	Reverse	GACCACACGCTGACCGAGACGGTCGTGGGC
2	Forward	ACCGCCGCGCCGCGTGAGCTGCATGTCGTG
	Reverse	CACGACATGCAGCTCACGCGGCGCGGCGGT
3	Forward	GTGAACGTGCTGCCGCGTCGCGGCATCGGC
	Reverse	GCCGATGCCGCGACGCGGCAGCACGTTCAC
4	Forward	TTACCGAACGCGATCAAGCGTTTGGGTTCTGGC
	Reverse	GCCAGAACCCAAACGCTTGATCGCGTTCGGTAA
5	Forward	TTGGGTTCTGGCCGTCGTTACACGTCGCTG
	Reverse	CAGCGACGTGTAACGACGGCCAGAACCCAA
6	Forward	CTCGACGGGGTCCGTCTGCGTGTGCTGGCCGGC
	Reverse	GCCGGCCAGCACACGCAGACGGACCCCGTCGAG
7	Forward	TTGCTGGGCGCTTTGCGTGTGGGCGAGCAGATT
	Reverse	AATCTGCTCGCCCACACGCAAAGCGCCCAGCAA
8	Forward	GGCGGGCCGGCCCCGCGTCCGATCCTGGACGCC
	Reverse	GGCGTCCAGGATCGGACGCGGGGCCGGCCCGCC
9	Forward	GGTGGTTGCGACCTTCGCGACGCACGCGTACAG
	Reverse	GACATGCGCACGCAGCGCTTCCAGCGTTGGTGG

Primer (from 5' to 3' sequence)		
10	Forward	GAATTACCCAACGCGATCAAGAGATTGGGTTCTG
	Reverse	CAGAACCCAATCTCTTGATCGCGTTGGGTAATTC
11	Forward	AGAAGCGCTGCGTGCGCATGTCGCGCGCAC
	Reverse	GTGCGCGCGACATG CGCACG CAGCGCTTCT
12	Forward	ACCACCAACGCTGGAA GCG CTG CGT GCGCA
	Reverse	TGCGCACGCAGCGCTTC CAGCGTTGGTGGT
13	Forward	ACGCGATAAAGCGTTTGGGTTCTGGCCGGCGATAC
	Reverse	GTATCGCCGGCCAGAACCCAAACGCTTTATCGCGT

Expression and purification of rare codon mutant of mtMenE in E. coli cells

After 13 steps of rare codon mutagenesis, the plasmid was transformed into *E. coli* Rosette2 (DE3) (Novagen) competent cells by heat shock. Transformed cells were grown in 800 mL of LB medium containing 0.2 mg/mL ampicillin and 5% glycerol and induction was achieved using 0.5 mM IPTG overnight at 16 ºC. Cells were harvested by centrifugation at 5,000 rpm for 20 min at 4 ºC, resuspended in 30 mL of Ni-NTA Bind Buffer (10 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0) and lysed by 3 passages through a French Press cell (1,000 psi). Cell debris was removed by centrifugation at 33,000 rpm for 90 min at 4 ºC. MenB was purified using His affinity chromatography: the supernatant was loaded to a column containing 3 mL of Ni-NTA His-Bind resins (Novagen). The column was washed with 20 mL of Ni-NTA Bind Buffer and 20 mL of wash buffer (20 mM imidazole, 300 mM NaCl, 20 mM sodium phosphate buffer, pH 8.0). mtMenE was eluted using a gradient of 20 mL elute buffer (250 mM

imidazole, 300 mM NaCl, 20 mM sodium phosphate buffer, pH 8.0). The concentration of MenB was determined by measuring the absorption at 280 nm using an extinction coefficient of 17,900 $M^{-1}cm^{-1}$ calculated from the primary sequence. SDS-PAGE gel showed not a pure band as 37 kDa.

Coupled assay of MenE reaction

The coupled reactions were performed in 20 mM NaH_2PO_4 pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$ buffer. 20 nM of ecMenE was incubated with ATP (120 μM), CoA (120 μM), OSB (0-120 μM) and excess MenB (4 μM). The formation of DHNA-CoA was monitored at 392 nm and initial velocities were determined using an extinction coefficient of 4,000 $M^{-1}cm^{-1}$. V_{max} and K_m values were obtained by fitting all the data to the Michaelis-Menten equation (1) using GraFit 4.0. k_{cat} values were obtained using the equation (2) .

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

$$V_{max} = k_{cat} \times [E] \quad (2)$$

Assay for inhibition of ecMenE

Reactions were performed in 20 mM NaH_2PO_4 pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$ buffer. 20 nM of ecMenE was incubated with inhibitor (0–200 μM), ATP (120 μM) and CoA (120 μM) for 10 minutes prior to the addition of MenB (4 μM) and OSB (30 μM). The formation of DHNA-CoA was monitored at 392 nm and initial velocities were determined using an extinction coefficient of 4,000 $M^{-1}cm^{-1}$.

IC$_{50}$ values were calculated by fitting the initial velocity data (v_i) obtained at different inhibitor concentrations ([I]) to equation (3) using Grafit 4.0.

$$v_i = \frac{100\%}{1 + [I]/IC_{50}} \quad (3)$$

Results and discussion

Expression of ecMenE and enzymatic activity

ecMenB was expressed and purified as pure protein band as 52 kDa (**Figure 5.4**). Compared to mtMenE whose molecular weight is 37 kDa, ecMenE is much larger. They share 29.8% identity in 275 residues overlap. Instead of using PPi release assay which only represents the first step of MenE reaction, ecMenE/mtMenB coupled assay was employed to determine the enzymatic activity. The k_{cat} and K_m value are 140.0 ± 2.5 and 4.9 ± 0.3, respectively, for ecMenE when the concentration of ATP and CoA were fixed.

12% SDS-PAGE gel of ecMenE (52 kDa)

1. Flow through fraction
2. Binding buffer fraction
3. Wash buffer fraction
4-5. Elute buffer fraction
6. Ladder
7-10. G25 column fraction
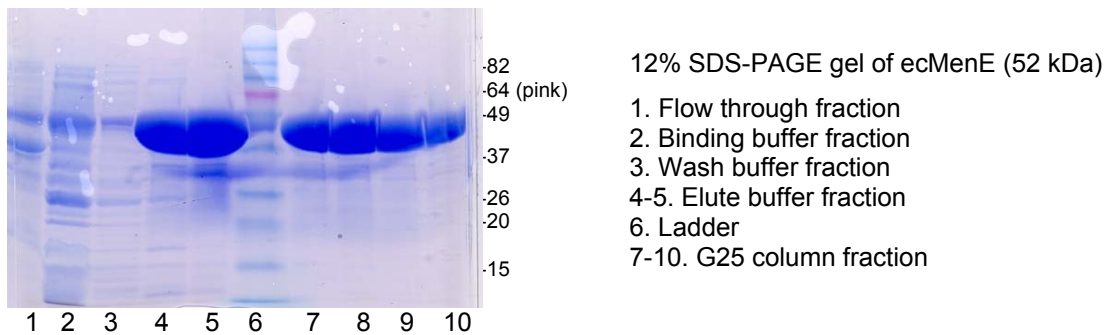
Figure 5.4: SDS-PAGE gel of ecMenE

Expression of mtMenE

We have encountered an enormous difficulty during the process of expressing mtMenE in heterologous systems. In *M. smegmatis* cells, there was no expression of the protein by using pVVAP, pVV16 and pJAM2 expression vectors. Similarly, the expression in *E. coli* pET15b, pET23b and pET43b (designed N- and C-terminus His-tag) all failed. We speculated that the lack of success at expressing mtMenE in *E. coli* might be due to the presence of a large number of rare codons (35 rare codons among 362 residues) (**Figure 5.5**).

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GTG CTG GGT GGC AGC GAC CCG GCA TTG GTC GCG GTG CCC ACC CAG CAT

GAG TCC TTG CTG GGC GCT TTG CGA GTG GGC GAG CAG ATT GAC GAC GAC

GTC GCC CTG GTA GTG ACG ACG TCA GGA ACC ACG GGA CCG CCC AAG GGC

GCC ATG TTG ACC GCG GCG GCC TTG ACC GCC AGC GCC TCG GCC GCC CAC

GAC CGG CTC GGC GGA CCG GGC AGC TGG CTG TTG GCT GTG CCG CCG TAT

CAC ATC GCC GGG CTG GCG GTG CTG GTG CGC AGC GTG ATC GCC GGA TCA

GTT CCT GTC GAA CTG AAC GTC TCC GCG GGA TTC GAT GTC ACC GAA TTA

CCC AAC GCG ATA AAG AGA TTG GGT TCT GGC CGG CGA TAC ACG TCG CTG

GTC GCC GCA CAG TTG GCC AAG GCA CTT ACC GAC CCG GCG GCC ACG GCC

GCG CTG GCC GAA TTG GAC GCG GTG CTG ATC GGC GGC GGG CCG GCC CCC

CGG CCC ATC CTG GAC GCC GCG GCC GCC GCC GGC ATC ACG GTG GTG CGC

ACC TAC GGC ATG AGC GAG ACC TCG GGC GGC TGT GTC TAC GAC GGC GTT

CCG CTC GAC GGG GTC CGG CTG AGG GTG CTG GCC GGC GGC CGC ATA GCT

ATC GGC GGT GCG ACC CTG GCC AAG GGC TAT CGC AAC CCG GTC TCG CCC

GAT CCG TTC GCC GAG CCA GGC TGG TTT CAC ACC GAC GAC CTT GGC GCC

CTT GAA TCG GGT GAT TCG GGT GTG CTG ACC GTG CTG GGC CGA GCC GAC

GAA GCG ATC AGC ACG GGC GGA TTC ACC GTG CTG CCG CAG CCA GTG GAG

GCC GCA CTG GGC ACC CAC CCT GCG GTG CGT GAC TGC GCG GTT TTT GGA

CTT GCC GAC GAC CGA CTC GGT CAG CGA GTG GTC GCC GCG ATT GTG GTC

GGC GAC GGA TGC CCA CCA CCA ACG CTA GAA GCG CTG CGG GCG CAT GTC

GCG CGC ACC CTG GAC GTC ACC GCC GCG CCC CGA GAG CTA CAT GTC GTG

AAC GTG CTA CCG CGA CGC GGC ATC GGC AAG GTG GAC CGG GCA GCG TTG

GTG CGC CGG TTC GCC GGT GAA GCC GAT CAA TAG

Figure 5.5: Rare *E. coli* codons in *M. tuberculosis mene* DNA sequence. Red = rare Arg codons; green = rare Leu codon; blue = rare Ile codon; orange = rare Pro codon; and pink = rare Gly codon. Residues with yellow background are mutation sites.

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Most amino acids are encoded by more than one codon, and *E. coli,* and indeed all cells, uses a specific subset of the 61 available amino acid codons for the production of most mRNA molecules (*25, 26*). So-called major codons are those that occur in highly expressed genes, whereas the minor or rare codons tend to be in genes expressed at a low level. When the mRNA of heterologous target genes is overexpressed in *E. coli*, differences in codon usage can disturb translation due to the demand for one or more tRNAs that may be rare or lacking in the population (*27-29*). Approaches normally used to overcome this problem include targeted mutagenesis to remove rare codons or the addition of rare codon tRNAs in specific cell lines. The commercial available Rosetta2 (DE3) host strain (Novagen) which is compatible with pET vectors, is designed to enhance the production of rare codon tRNAs. However, the expression by using Rosetta2 competent cells was unsuccessful again.

Therefore, 14 rare *E. coli* codon sites among 35 rare codon sites were mutated into major codon (**Figure 5.5**). The mutation plasmid was expressed in Rosetta2 (DE3) cells. Although the expression level has been greatly improved, most of the expressed mtMenE protein was found in the pellet (**Figure 5.6** lane 1-3). Two methods including lowering the induction temperature and adding glycerol were employed to improve the solubility of mtMenE protein. It can be noted that a small portion of the protein was soluble when 4% of glycerol was added and 16 °C was used for induction (**Figure 5.6** lane 4-11). However, SDS-PAGE gel showed that mtMenE was not pure with a distinct lower band around 20 kDa after purification (**Figure 5.7**). The lower band could be the truncated

mtMenE. The purified fraction showed the MenE activity. The detailed kinetic analysis was not conducted due to the impurity. Therefore, the solubility problem should be addressed in the future.

1. Whole cell before induction
2. Pellet after induction
3. Supernatant after induction
4. 16°C induction supernatant
5. 16°C induction pellet
6. 16°C induction, 4% glycerol, supernatant

7. 16°C induction with 4% glycerol pellet
8. 25 °C induction supernatant
9. 25 °C induction pellet
10. 25 °C induction, 4% glycerol, supernatant
11. 25 °C induction, 4% glycerol, pellet
12. Ladder
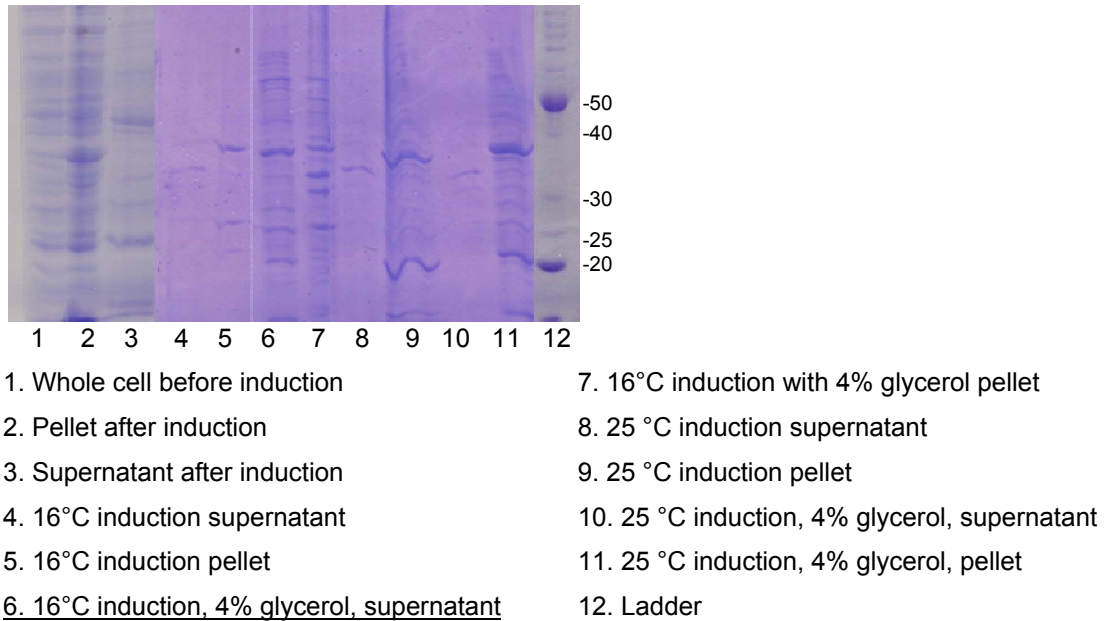
Figure 5.6: Expression of mtMenE after rare codon mutations. Sample in lane 4-11 were pre-treated with 5 µL of His-Bind resin and loaded with the resin, so they basically represented proteins with His-tag.

SDS-PAGE gel

1. Flow through fraction
2. Binding buffer fraction
3. Wash buffer fraction
4-5. Elute buffer fraction
6. Ladder
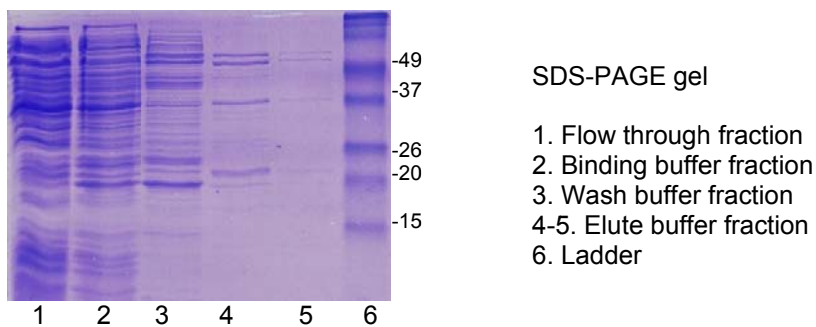
Figure 5.7: The SDS-PAGE gel showed mtMenE was not pure after purification.

Recently, our collaborator, Professor Kisker's lab, completed the total synthesis of *mene* DNA without *E. coli* codon bias and they successfully obtained the good expression and soluble protein, indicating the expression problem in *E. coli* is due the high frequency of rare codons in the mtMenE gene.

Design of mechanism-based inhibitors of MenE

MenE converts OSB to OSB-CoA *via* a two-step process involving initial ATP-dependent adenylation of OSB to form a reactive OSB-AMP intermediate, followed by thioesterification with CoA to form OSB-CoA (**Figure 5.1**). Based on the structure of intermediate, 5′-*O*-(*N*-acylsulfamoyl) adenosines (acyl-AMS) and related compounds were designed to inhibit MenE by mimicking tightly-bound OSB-AMP intermediates. To avoid potential pharmacological liabilities, we replaced aromatic carboxylate of OSB with a neutral methyl ester, since this carboxylate is not directly involved in the reaction mechanism (*30, 31*) and we have found that the aromatic methyl ester OSB-CoA (MeOSB-CoA) is more stable than OSB-CoA which has been discussed in chapter 2. Thus, we envisioned that MeOSB-AMS (**1**) or its sulfamide analog MeOSB-AMSN (**2**) might be effective inhibitors of MenE and menaquinone biosynthesis (**Figure 5.8**).

We also considered that the corresponding vinyl sulfonamide MeOSB-AVSN (**3**) might inhibit MenE through covalent binding to the incoming CoA thiol nucleophile during the second half-reaction (**Figure 5.9**), forming a mimic of the tetrahedral intermediate. Michael acceptors have been used extensively to inhibit cysteine proteases (*32*), and also to target protein thiol nucleophiles in

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1, MeOSB-AMS (X = O)
4 (X = CH₂)

2, MeOSB-AMSN (X=O)
5 (X = CH₂)

3, MeOSB-AVSN (X = O)
6 (X = CH₂)
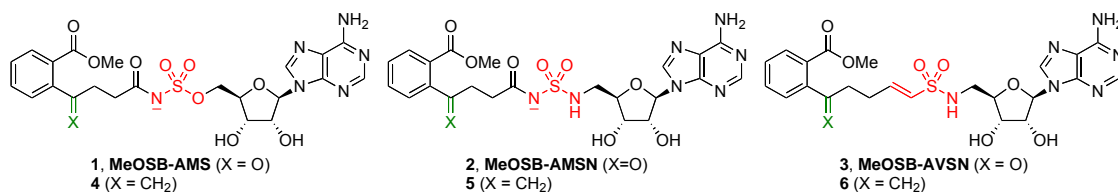
Figure 5.8: Structures of designed inhibitors of MenE. The sulfamate (**1**, **4**) and sulfamide (**2**, **5**) functionalities (red) are designed to mimic the phosphate group in the cognate OSB-AMP reaction intermediate. The vinyl sulfonamide moiety (**3**, **6**) is designed to trap the incoming CoA nucleophile covalently. The corresponding *exo*-methylene analogs (**4–6**) are designed to probe the importance of the aromatic ketone functionality (green) for binding.

polyketide and non-ribosomal peptide synthetases (*33, 34*). Based on studies of Roush and coworkers on the inherent reactivity of various sulfonyl-based Michael acceptors (*35*), we selected the vinyl sulfonamide moiety to provide the requisite balance of reactivity and selectivity to bind CoA in the MenE active site without reacting promiscuously with other nucleophiles.
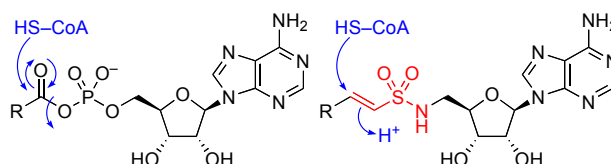
Figure 5.9: Mechanism of covalent inhibition. (*left*) The CoA thiol nucleophile attacks the carbonyl group in the acyl-AMP intermediate during the second half-reaction catalyzed by acyl-CoA synthetases. (*right*) A vinyl sulfonamide Michael acceptor is appropriately positioned to trap the incoming nucleophile and form a covalent adduct.

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Inhibition of ecMenE

Since we were unable to reliably express mtMenE, IC_{50} values were determined against ecMenE (**Table 5.3**). We were gratified to find that both the sulfamate MeOSB-AMS (**1**) and sulfamide MeOSB-AMSN (**2**) were effective inhibitors of MenE (**Table 5.3**). Moreover, the vinyl sulfonamide analog MeOSB-AVSN (**3**) was proved to be the most potent inhibitor, with an IC_{50} value of 5.7 ± 0.7 μM; kinetic analysis indicated that this compound is a slow-binding inhibitor, suggesting a conformational change during binding. In contrast, none of the corresponding *exo*-methylene analogs (**4**–**6**) inhibited the enzyme at up to 200 μM concentration. No inhibition was observed when assays were performed using a limiting concentration of MenB (200 nM) in the presence of excess MenE (5 μM), indicating that the compounds do no inhibit MenB.

Table 5.3: Inhibition of ecMenE by designed inhibitors 1–6

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	38.0 ± 3.0	**4**	>200
2	34.1 ± 2.8	**5**	>200
3	5.7 ± 0.7	**6**	>200

It is interesting to note that the vinyl sulfonamide analog MeOSB-AVSN (**3**) is the most potent inhibitor of MenE. In contrast to the sulfamate and sulfamide analogs **1** and **2**, this compound lacks the carbonyl and adjacent heteroatom of the acyl phosphate group in OSB-AMP, which may be involved in hydrogen bonding interactions, based on the cocrystal structure of a related fatty acyl-CoA synthetase with myristoyl-AMP. These results also contrast with the relative

potencies of related inhibitors which are nM inhibitors of the NRPS salicylate adenylation enzyme MbtA (**Table 5.1**).   This may be due to the structure differences between the methyl ester OSB-AMP intermediate analogues and OSB-AMP. Although the aromatic carboxyl group in OSB is not directly involved in the formation of OSB-CoA, we note that the OSB methyl ester (**Figure 5.10**) is not a substrate for MenE. Thus removal of the methyl ester functionality in compounds **1**-**3** may significantly improve their affinity for MenE. Our results also suggest that the OSB ketone group is required for inhibition, as shown by the complete lack of activity in *exo*-methylene analogs **4**–**6**. Therefore, modified compound **1-3** could show better inhibition potency if the methyl group is removed.
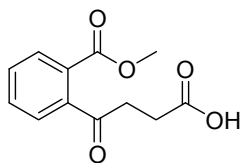
Figure 5.10: The structure of OSB methyl ester

Conclusions

OSB-CoA synthase (MenE), an acyl-CoA synthetase, catalyzes a magnesium-dependent multisubstrate reaction, resulting in the conversion of OSB to OSB-CoA *via* a two-step process involving the initial ATP-dependent adenylation of OSB to form a reactive OSB-AMP intermediate, followed by thioesterification with CoA to form OSB-CoA. Based on the structure of intermediate, 5´-*O*-(*N*-acylsulfamoyl) adenosines (acyl-AMS) and related compounds were designed to inhibit MenE by mimicking tightly-bound OSB-AMP intermediates.

The mtMenE enzyme has proven difficult to be expressed heterologously even using *M. smegmatis* as an expression host. We started to obtain small amounts of active enzyme having optimized the first 14 codons in the gene for expression in *E. coli*. The rare codon mutagenesis improved the expression level of mtMenE in the *E. coli* host; however, the protein was not very soluble. In contrast, ecMenE expresses very well in *E. coli*.

Since we were unable to reliably express mtMenE, IC_{50} values were determined against ecMenE. Both the sulfamate MeOSB-AMS (**1**) and sulfamide MeOSB-AMSN (**2**) were effective inhibitors of MenE. Moreover, the vinyl sulfonamide analog MeOSB-AVSN (**3**) was proved to be the most potent inhibitor, with an IC_{50} value of 5 μM. Although the aromatic carboxyl group in OSB is not directly involved in the formation of OSB-CoA, we note that the OSB methyl ester is not a substrate for MenE. Therefore we propose that removal of the methyl ester functionality in compounds **1**-**3** could show better inhibition potency.

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