

MenA Is a Promising Drug Target for Developing Novel Lead Molecules to Combat *Mycobacterium tuberculosis*

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Abstract: Potent inhibitors of MenA (1,4-dihydroxy-2-naphtoate prenyltransferase) in *Mycobacterium tuberculosis* are identified, and are also effective in inhibiting growth of *Mycobacterium tuberculosis* at low concentrations. The MenA inhibitors possess common chemical structural features of (alkylamino)alkoxyphenyl(phenyl)methanones. Significantly, the MenA inhibitors can be synthesized in a few steps with high overall yields. The representative MenA inhibitors are highly effective in killing nonreplicating *Mycobacterium tuberculosis* that is evaluated by using the Wayne low oxygen model. In addition, a series of drug resistant *Mycobacterium* spp. are sensitive to the MenA inhibitors. The results are expected to be of significance in terms of discovering new lead compounds that can be developed into new drugs to combat unmet diseases caused by *Mycobacterium tuberculosis*.

Key Words: New drug target for *Mycobacterium tuberculosis*, menaquinone biosynthesis, MenA, MenA inhibitors, nonreplicating *Mycobacterium tuberculosis*.

I. INTRODUCTION

Mycobacterium tuberculosis (Mtb) causes tuberculosis (TB) and is responsible for nearly two million deaths annually [1]. Estimates indicate that one-third of the world population is infected with latent Mtb. In particular, people who are HIV-AIDS patients are susceptible to TB infection. Moreover, the emergence of multidrug-resistant (MDR) strains of Mtb seriously threatens TB control and prevention efforts [2]. MDR-Mtb is very expensive to treat; the estimates suggest that it may be ten times as expensive as drug-sensitive Mtb, especially considering the fact that patients with MDR need treatment for three years or more. One third of the 42 million people living with HIV/AIDS worldwide are co-infected with Mtb. Approximately 90% of the people living with HIV die within a few months of becoming sick with TB, if they do not receive proper TB treatment. Persons infected with both HIV and Mtb are 30 times more likely to progress to active TB disease. Recent studies have shown that infection with Mtb enhances replication of HIV and may accelerate the progression of HIV infection to AIDS [3]; for example, the risk of HIV-infected patients developing TB is 5-15% per year after an infectious contact [4]. The current recommended approach to TB treatment is the local directly observed treatment strategy (DOTS) [5]. Even where DOTS has been established, if the MDR rate is locally high, first line drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) alone give an unacceptably low cure rate. Clinical responses of MDR-TB patient to first line drug have been poor, and in some cases there is no response at all [6]. Second line drugs (amikacin, cycloserine, ethionamide, kanamycin,

capreomycin, clofazimine, para-aminosalicylic acid, ciprofloxacin, and ofloxacin) are often poorly effective and tolerated [7]. There are significant problems present with respect to treatment of AIDS and TB co-infected patients. Rifampicin and isoniazid (key drugs of the DOTS therapy) interact with the cytochrome P450 3A4 enzyme pathways, one of the enzymes responsible for drug metabolism. In addition, rifampicin strongly interacts with non-nucleoside reverse transcriptase inhibitors and protease inhibitors for HIV infections [8]. Thus, clinicians avoid starting Highly Active Antiretroviral Therapy (HAART), which consists of three or more highly potent anti-HIV drugs (commonly reverse transcriptase inhibitors and protease inhibitors), until the TB infection has been cleared [9].

In connection with the ongoing studies on the development of "novel" antimycobacterial agents, we discovered 1,4-dihydroxy-2-naphtoate prenyltransferase (MenA) inhibitors which also effective in killing Mtb at low concentrations [10]. The purpose of this article is to describe these findings in full, including previously undisclosed molecules and *in vitro* assay data.

II. NEW TB DRUG TARGETS

There is urgent need and significant interest in developing new TB drugs, however, no new class of TB drugs has been developed in the past 40 years [11-15]. Numerous co-crystal structures of bacterial essential enzymes with their inhibitor molecules have been resolved to date. However, rational drug designs based on essential enzymes existing in *Mycobacterium* spp. have never been achieved successfully. It may be due in part to the lack of 1) appropriate library molecules to screen unexploited bacterial target proteins, and 2) understanding of mycobacterial physiology. On the other hand, a medium-throughput screening approach using whole cell resulted in the reinvestigations of several promising

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leads. As result of extensive medicinal chemistry efforts, the clinical trial drugs such as diarylquinoline (R207910, an inhibitor of F1F0 proton pump of ATP synthase), and nitroimidazoles (PA-824 and OPC-67683, their molecular targets remain undefined, but the molecules are active against cell wall lipid biosynthesis) were developed. Due, in large part, to the resurgent efforts of the TB Alliance (The Global Alliance for TB Drug Development) and its public/private partners, numerous compounds have been developed in order to improve current TB-chemotherapies. An excellent comprehensive review of new anti-tuberculosis chemotherapies including the structures, mode of actions, and pharmacokinetics and pharmacodynamics was recently reported [16]. If several of these drug leads become FDA approved anti-TB drugs, the management of drug-resistant TB would be improved. However, many TB drug leads reported are modifications of known antibacterial reagents, and thus their mode of actions remain the same. Ultimate goal of development of the treatment of TB infections is to discover novel antibacterial agents which interfere with novel (or unexploited) bacterial molecular target.

Mycobacteria are obligate aerobes. However, it has been known that tubercle bacilli encounter hypoxic environments in acute disease as well as in latent infection, and the capability of tubercular bacilli to adapt to hypoxic conditions appears to play an important role *in vivo* [17-20]. For example, Mtb is presumed to lie in a nonreplicating (NR) state (dormancy), particularly in the caseous nodules of the lungs where the lesions have little access to oxygen, and can survive for years in the host by entering a dormant state. About 10% of patients with latent TB are reactivated to cause the risk of fatal diseases [17]. Thus, in addition to necessity of drugs for the treatment of MDR-Mtb, the development of drugs that kill Mtb in any state is very important. However, no TB drugs are effective in killing the dormant form of Mtb *in vivo*. Wayne and co-workers established a link between oxygen starvation and TB drug resistance; they demonstrated that upon depletion of oxygen in culture, Mtb terminates growth and develops into a characteristic dormant form [21, 22]. Importantly, it was observed that the dormant form of Mtb was found to be resistant to most of clinically utilized antimycobacterial agents [23].

The lipid-soluble electron carriers (lipoquinones) occupy a central and essential role in electron transport, and thus,

ATP synthesis. The lipoquinones involved in the respiratory chains of bacteria consist of menaquinones and ubiquinones. From the taxonomic studies it is evident that majority of Gram-positive bacteria including *Mycobacterium* spp. utilize only menaquinone in their electron transport systems [24], and menaquinone biosynthesis is essential for survival of non-fermenting Gram-positive bacteria [25-27]. On the other hand, in the electron transport systems Gram-negative organisms such as *E. coli* utilize ubiquinone (CoQ) under aerobic conditions, and menaquinone under anaerobic conditions. Moreover, the electron transport chain in humans does not utilize menaquinone. Clearly, the electron transport chain is central component in the production of ATP and the subsequent growth of bacteria (Fig. 1). It is speculated that although dormant bacilli are considered to have a less active metabolism and less energy reserves, ATP synthesis in oxidative phosphorylation must be active even in the dormant form in Mtb. Thus, inhibition of menaquinone synthesis could have profound effects on maintenance of dormancy in Mtb. This concept may be supported by the several reports that phenothiazines inhibited NADH:menaquinone oxidoreductase, the first enzyme in bacterial respiratory chain, were effective in killing nonreplicating Mtb [28]. We recently demonstrated that 1) inhibition of MenA (1,4-dihydroxy-2-naphthoate prenyltransferase) (highlighted in Fig. 2) showed significant growth inhibitory activities against drug resistant *Mycobacterium* spp. [10], and 2) effectiveness of MenA inhibitors in killing dormant Mtb *in vitro* using the Wayne model [21-23].

III. MenA (1,4-DIHYDROXY-2-NAPHTHATE PRENYLTRANSFERASE)

Menaquinones are the predominant isoprenoid lipoquinones of Gram-positive bacteria, whereas bacteria such as *E. coli* utilize both menaquinone and ubiquinone (benzoquinone ring structure) (*vide supra*). The details of the biosynthesis of menaquinone in *E. coli* have been reviewed [29-31]. In *E. coli* the synthesis of menaquinone is accomplished by seven enzymes (MenA-MenG). These enzymes are encoded by 2 clusters of genes. The men cluster consists of the *menB*, *C*, *D*, *E*, *F* and a separate cluster containing *menA* and *menG* [32]. Menaquinones are identified by the length and chemical structure of the prenyl side chain. The biosynthesis of menaquinone takes place *via* the intersection of two separate pathways. 1,4-Dihydroxy-2-naphthoate is synthesized from

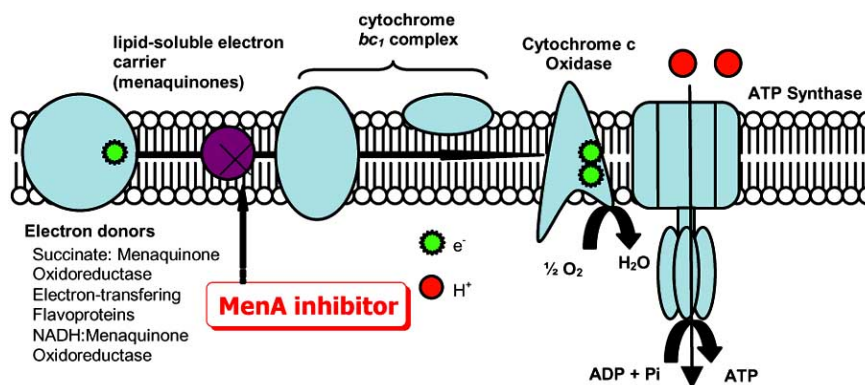


Fig. (1). Proposed schematic electron flow in Mtb.

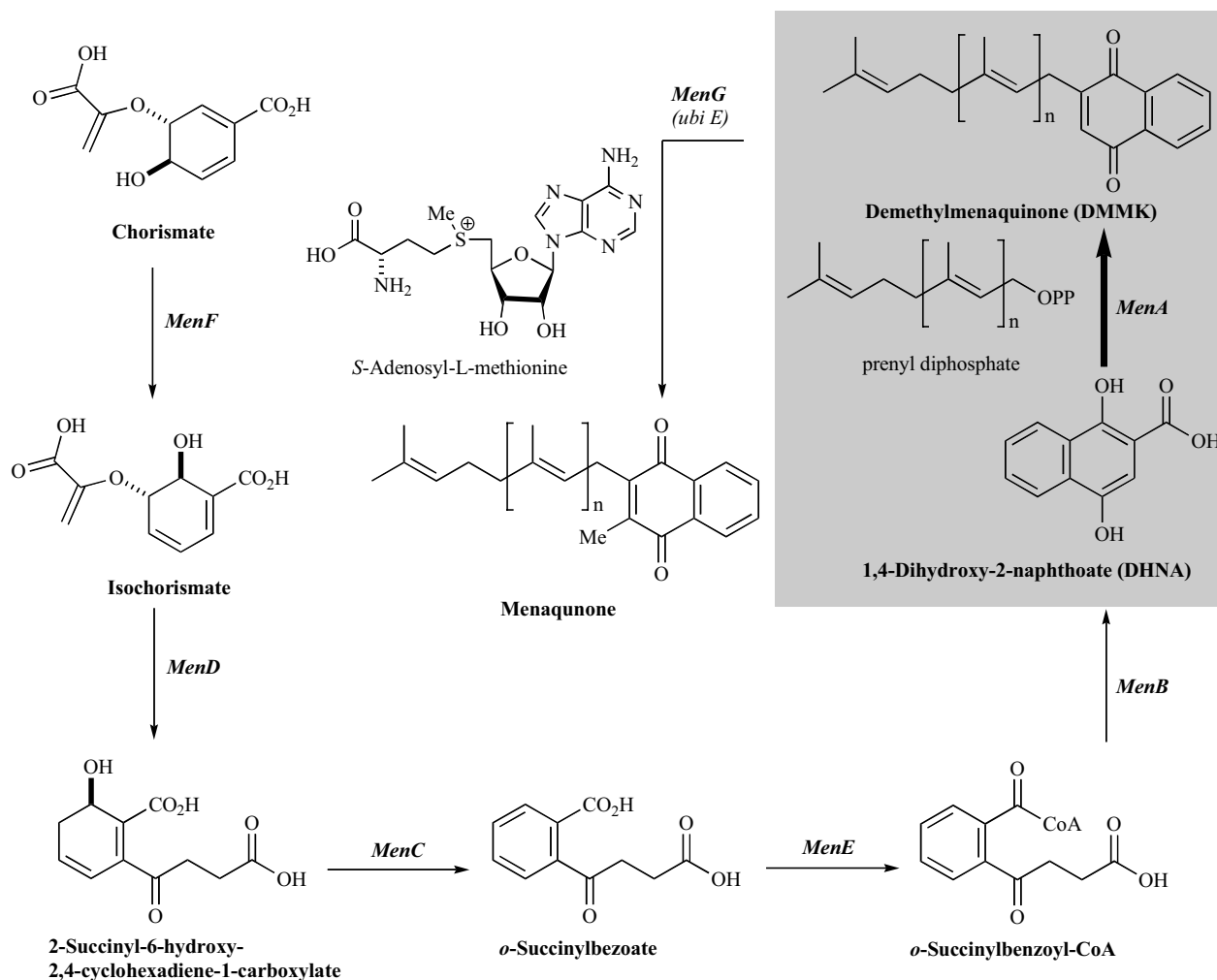


Fig. (2). Menaquinone biosynthesis in *E. coli*.

chorismate catalyzed by MenF, D, C, E, and B. On the other hand, prenyl diphosphate of the appropriate size ($n = 8$ in Mtb, Fig. 2) is biosynthesized by the chain-elongation reaction. The naphthoate ring is then prenylated to form demethylmenaquinone (DMMK). MenG catalyzes methylation of DMMK to form menaquinone. Although menaquinone synthesis has been relatively extensively studied in *E. coli* (due in part to the availability of the *men* mutants), the synthesis of menaquinone in other organisms has received little attention. Menaquinone synthesis in Mtb has been ignored and virtually no information is available about the properties of these enzymes even though they are distinct from the enzymes involved in synthesizing ubiquinone in humans.

Among the menaquinone biosynthesis proteins, MenA is a membrane associated protein that catalyzes prenylation of 1,4-dihydroxy-2-naphthoate (DHNA) to form demethylmenaquinone (DMMK) (highlighted in Fig. 2) [33]. Analysis of the amino acid sequence of MenA was revealed that MenA is likely to have five transmembrane segments, and there are highly conserved Asp which would be located in the inner-plasma membrane as being predicted by using a prediction program (Sosui) [34]. The activity is absolutely dependent on the presence of the divalent cations such as Mg^{2+} . Thus, it

is likely that such divalent cations form ion pairs with Asp residues existing in the catalytic site of MenA. As shown in schematic electron flow (Fig. 1), MenA inhibitors are able to block the electron flow, resulting in the inhibition of ATP generation. We have obtained experimental evidences which support that “MenA is a valid therapeutic target for TB infections”; for examples, 1) all Gram-positive species tested were susceptible to the MenA inhibitors, on the contrary, all Gram-negative species tested were not susceptible to the MenA inhibitors [10], 2) bacterial growth can be rescued by supplementation with vitamin K1 or K2 at concentrations of many fold higher than the MIC of the MenA inhibitors [35], and 3) growth of drug resistant *Mycobacterium* spp. was inhibited by the MenA inhibitors.

IV. MenA INHIBITORS: DESIGN AND SYNTHESIS

There is no detailed biosynthetic study of a formal decarboxylative prenylation of 1,4-dihydroxy-2-naphthoate (DHNA) catalyzed by MenA. We hypothesized that the prenylation of DHNA occurs at the C3-position to produce 3-prenyl-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylate (i in Fig. 3) whose carboxylate would interact with cationic amino acid residues or Mg^{2+} ions directly or indirectly through water molecules(s) in the binding site; this hypothesis was con-

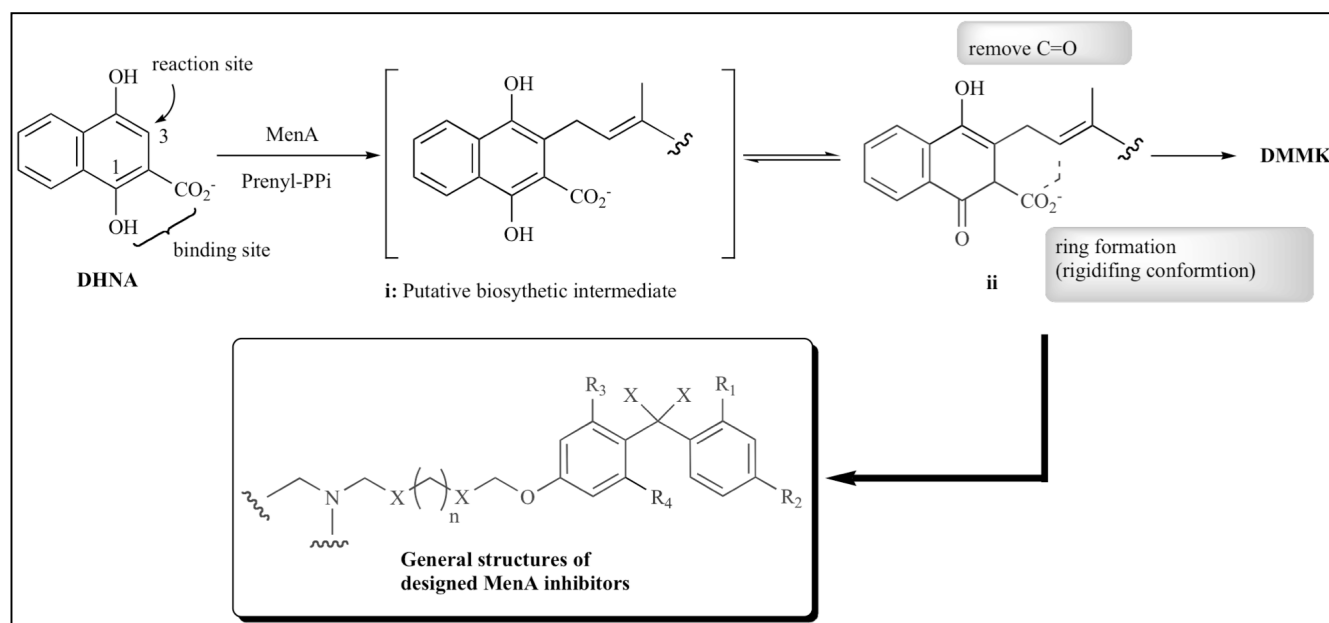


Fig. (3). Design of MenA inhibitors.

cluded based on the observation that 1) the activity of MenA is an absolute requirement for divalent cations, and 2) highly conserved Asps exist in inner-plasma membrane (*vide supra*). The intermediate **i** would be tautomerized to form its keto-form **ii** which concomitantly undergoes decarboxylation to furnish demethylmenaquinone (DMMK). The intermediate **i** should have a stronger affinity against MenA than DMMK, thus, DMMK may readily be dissociated from the MenA catalytic site.

We designed *tertiary* or *secondary* amine or hydrazine containing mimics of **ii** 1) in hope that the amine moiety would interact with Asp residue(s) directly or through the divalent cation(s) in the active site, and 2) in which the chemically unstable 1,4-quinone system is replaced with the hydrophobically substituted-benzophenones (Fig. 3). In this design for MenA inhibitors, the prenyl moiety of DMMK is mimicked by appropriate length of carbon chain or its heteroatom containing analogs.

As illustrated in Scheme 1, the designed DMMK mimics were synthesized in four to six steps in solution or in four to five steps on the polymer-support. The halogen-substituted (4-methoxyphenyl)(phenyl)methanone derivatives **5** were synthesized by using Friedel-Crafts acylations of 2,4-halogenated benzoyl chlorides **1a~e** and anisole derivatives **3a~g** with AlCl_3 in PhNO_2 . Regioselectivities of Friedel-Crafts acylation reactions were vary depending on the structure of anisole derivatives **3a~g**; however, in all cases the *p*-addition products were predominant, and the undesired *o*-addition products were readily separated by chromatography.

Alternatively, the benzophenone derivatives were synthesized *via* Grignard reactions of the morpholin amides **2a~e** with 1-halo-4-methoxybenzene or its derivatives. Although a limited number of Grignard reagents can be prepared by the halogen-metal exchange reactions with $^i\text{PrMgCl}$, these reactions gave rise to the desired benzophenone in over 90% yield without contamination of the over-alkylation product

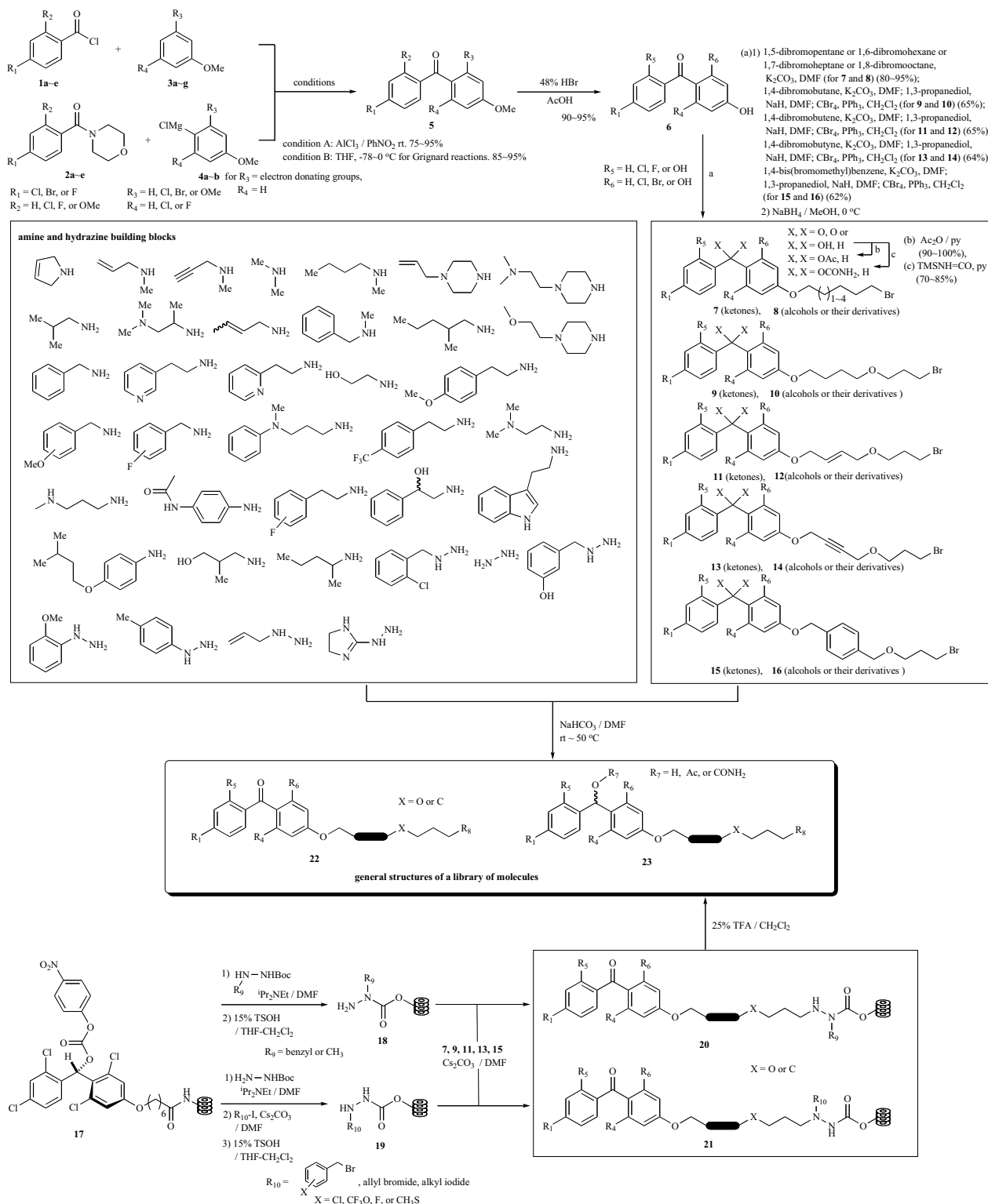
[36]. Demethylation(s) of the halogen-substituted (4-methoxyphenyl)(phenyl)methanone derivatives **5** by the treatment with HBr in aq. AcOH at 130 °C provided the (4-hydroxyphenyl)(phenyl)methanone derivatives **6** in 90~95% yield. The phenolic alcohol at the 4-position was selectively alkylated with the dibromo compounds to yield the desired monobromide in 85~95% yield. The molecules **9**, **11**, **13**, and **15** were synthesized by the additional two steps of alkylation with 1,3-propanediol and subsequent bromination (CBr_4 , PPh_3) reactions. The diphenylmethanol derivatives **8**, **10**, **12**, **14**, and **16** were synthesized by treatment of the corresponding carbonyls with NaBH_4 at 0 °C. The generated alcohols could be further functionalized with acylating reagents or a wide variety of isocyanates. In generation of a library of molecules in Scheme 1, however, acetylations with Ac_2O and carbamations with trimethylisocyanate were performed. Amination reactions of the bromides **7~16** with a wide variety of amines and hydrazine building blocks furnished a library of molecules in solution as pure forms after chromatographic purifications. To efficiently synthesize hydrazine containing molecules we applied the carbamate linker grafted onto LanternTM [37] in which selective alkylation at the N^β position of hydrazine carboxylate resins with the bromide **7**, **9**, **11**, **13**, and **15** provided the 1,2-disubstituted and 1,1-disubstituted hydrazine derivatives after cleavage from the resin **20** and **21** respectively [38]. Thus, a library of diphenylmethanone and diphenylmethanol derivatives was efficiently generated in short steps in solution or on the polymer-support.

V. IN VITRO ASSAYS AGAINST MENA AND MYCOBACTERIUM TUBERCULOSIS

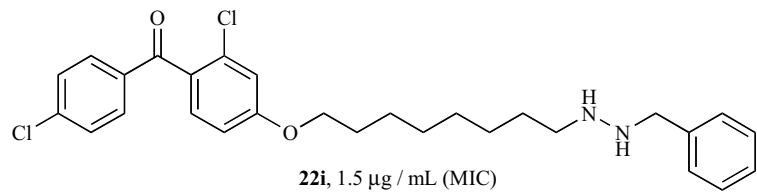
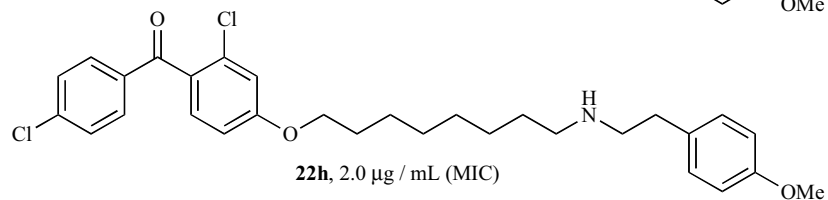
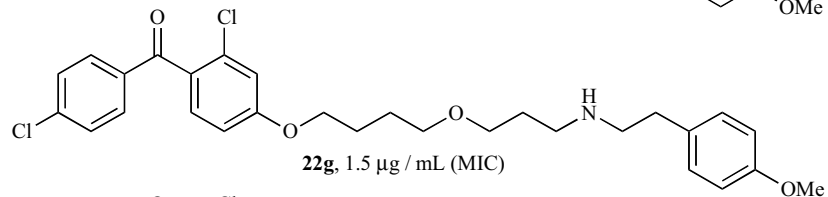
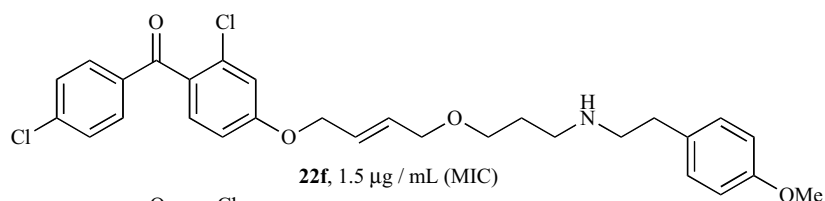
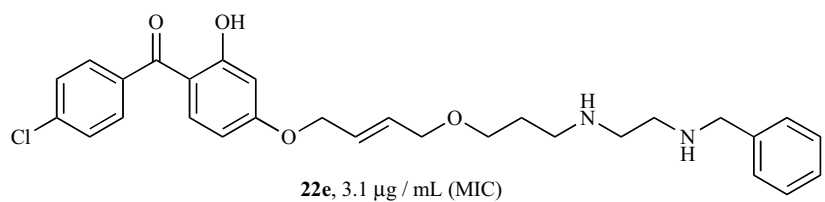
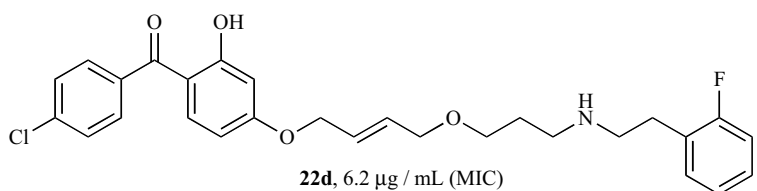
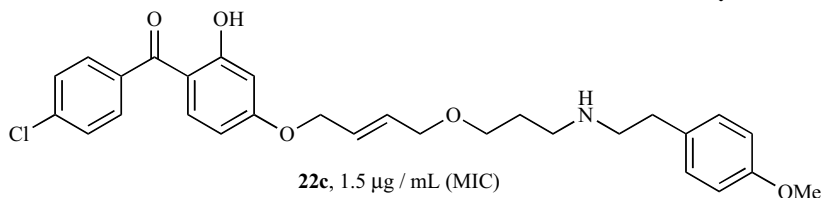
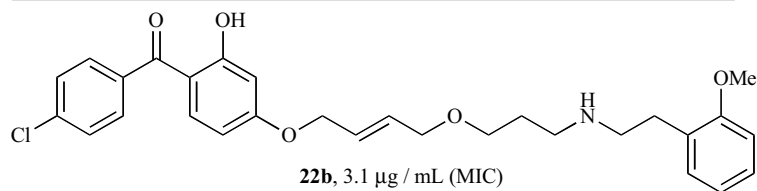
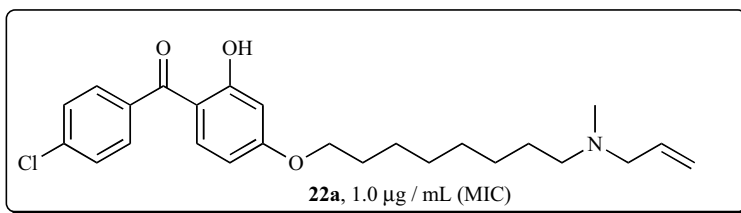
The library of molecules was evaluated in an enzymatic assay *in vitro* (IC_{50}) against *MtbMenA*. In these assays [^3H]-farnesyl diphosphate was utilized in place of nonaprenylphosphate ($n=8$ in Fig. 2). Into the assay mixtures contained 500 μM DHNA, 10 μM [^3H]-farnesyl diphosphate, 5 mM MgCl_2 , 0.1% CHAPS in 100 mM MOPS (pH 8) and an ap-

appropriate amount of membrane protein, a various concentrations of the synthesized molecules (Scheme 1) were added [28]. IC₅₀ values were determined using GraFit version 5.0. A series of molecules exhibited IC₅₀ of less than 10 μ M against MenA and these molecules were evaluated in

mycobacterial growth assay (MIC). Interestingly, in many cases the MIC values were in good agreement with the IC₅₀ values. Representative MenA inhibitors which also exhibited the MIC value of less than 10 μ g/mL were summarized in Fig. (4). In these *in vitro* screening analyses of the library



Scheme 1. Generation of a library of molecules in solution and on polymer-support.



(Fig. 4. Contd....)

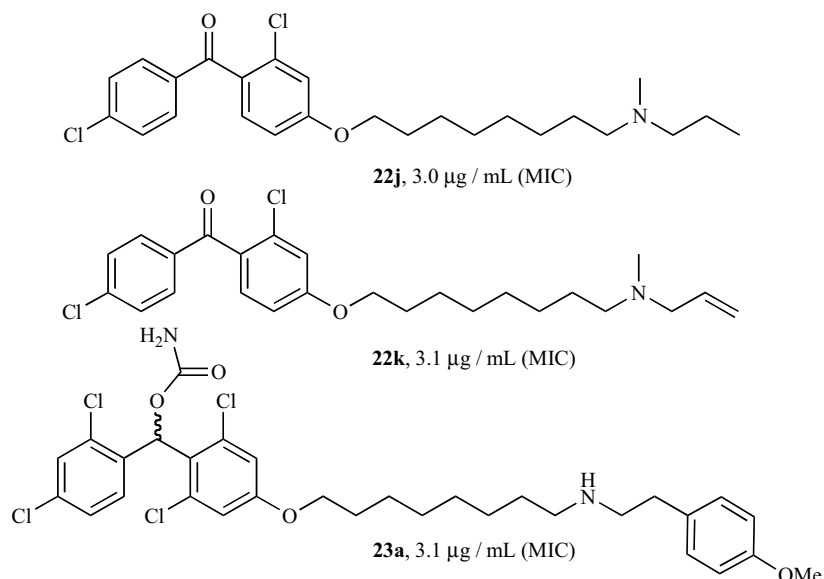


Fig. (4). The active MenA inhibitors possessing mycobactericidal activity (MIC <10 $\mu\text{g/mL}$ against H37Rv strain).

molecules it was revealed that 1) the effective substitution pattern (R_1 , R_4 , R_5 , and R_6) in the benzophenone moiety should be hydrogen for R_4 and R_5 ; the hydrogen atom for the R_4 and R_5 positions showed significantly better activity than other groups such as the OH or Cl or Br group, 2) C5~C7 of sp^3 carbon chain length of the linker (between the phenolic oxygen and terminal nitrogen atom) decreased mycobacterial growth inhibitory activity, on the other hand, the C8 carbon chain length of linker increased the MenA enzyme inhibitory and mycobactericidal activities, 3) the hetero atom such as oxygen can be introduced at the X-position in **22** and **23** without decreasing the activities, 4) the linker moiety can be further diversify by sp^2 or sp carbons (the moiety highlighted

in **22** and **23**), and 5) the allylmethylamine, benzylamine, ethylenediamine, and benzylhydrazine derivatives were the effective terminal amine structures. In general, the reduced forms **23** exhibited lowered MenA inhibitory and mycobactericidal activities. However, *in vitro* activities of the diphenylmethanol derivatives **23** were improved by the modification of R_4 and R_5 groups, and carbamation of the alcohol group as seen in **23a** (Fig. 4). Among the potent MenA inhibitors shown in Fig. (4), **22a**, named allylaminomethanone-A, was resynthesized and determined MICs against a variety of strains of *Mycobacterium* spp. As summarized in Table 1, growth of drug resistant *Mycobacterium* spp. was inhibited by **22a** (entries 2 and 3) at low concentrations.

Table 1. MICs of **22a** and Representative Antimycobacterial Agents (RFP and INH)

Entry	Species and Strain	MIC ($\mu\text{g/mL}$) ^a		
		22a	RFP ^b	INH ^c
1	<i>M.tuberculosis</i> H37Rv	1.0	0.2	0.1
2	<i>M.tuberculosis</i> H37Rv INHr	1.5	0.2	>25
3	<i>M.tuberculosis</i> H37Rv RFP ^r	1.5	>25	0.05
4	<i>M.bovis</i> BCG Tokyo	3.1	0.1	0.1
5	<i>M.avium</i> Flamingo	6.3	3.1	>25
6	<i>M.intracellulare</i> ATCC15984	6.3	3.1	12.5
7	<i>M.aurum</i>	6.3	0.78	6.3
8	<i>M.fortuitum</i> NIHJ1615	12.5	>25	6.3
9	<i>M.smegmatis</i> Takeo	12.5	>25	12.5

^aThe agar dilution method was used. ^bRFP: rifampicin. ^cINH: isoniazid.

VI. ACTIVITY OF MenA INHIBITORS AGAINST LONG-TERM NONREPLICATING *Mycobacterium tuberculosis*

In order to study the dormancy response we evaluated the effectiveness of allylaminomethanone-A (**22a**) against dormant bacteria under low oxygen conditions using the Wayne model [21] with minor modification. An aerobic preculture was diluted 100-fold in tubes and tubes were closed with rubber septa to ensure the anaerobic growth of bacteria. The diluted bacterial suspension was degassed with N₂ and incubated anaerobically. After 24 days (well into nonreplicating phase 2 as defined by the Wayne model), the MenA inhibitor(s) in degassed solution was introduced into dormant phase Mtb and incubated for additional 96 h, after which the bacterial suspension was diluted and plated. Culturable bacteria were then grown aerobically at 37 °C and colonies were counted [39]. As summarized in Fig. (5), at the concentrations of 10 µg/mL **22a** exhibited 320-, 180- and 3-fold more effective in killing nonreplicating bacteria than ethambutol, isoniazid or rifampicin. Significantly, no countable colonies were observed after treatment with **22a** at 50 µg/mL concentrations. It is worth mentioning that **22a** exhibited the most active in killing nonreplicating Mtb *in vitro* among antimycobacterial drugs tested in our laboratories.

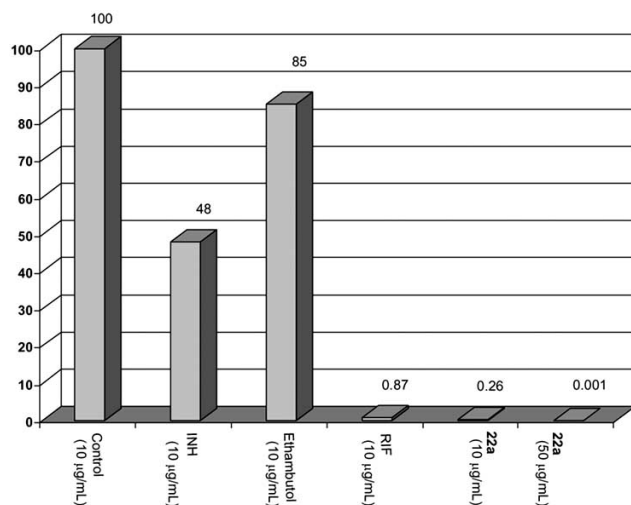


Fig. (5). Bacterial survival after exposure to **22a** and representative antimycobacterial agents.

VII. DISCUSSION AND CONCLUSION

One of ultimate goals of TB drug development is to develop a drug which has also activity against human latent tuberculosis infection. As described above, human utilizes ubiquinone in the electron transport chain, whereas Mtb use only menaquinones. However, it has not been addressed whether menaquinone biosynthetic enzyme genes are expressed in any growth condition in Mtb. The mycobactericidal effect of the representative MenA inhibitor such as **22a** against nonreplicating Mtb strongly suggested that electron transport is important in maintaining bacterial viability under conditions of restricted oxygen. Several data suggested a role for the DosR/DosS/DosT signaling system, which is required

for Mtb genetic response to hypoxia and nitric oxide, in the adaptation of Mtb to conditions that trigger reversible bacterial stasis *in vitro*, and thus the DosR/DosS/DosT signaling system may contribute to latency *in vivo* [40]. MenA inhibitors seem to be able to block the electron flow without inducing a dormancy response in Mtb, suggesting that nonreplicating Mtb will behave as if they are in aerobic conditions. Clearly the electron transport chains are central component in the production of ATP and the subsequent bacterial growth (*vide supra*). It is conceptually very interesting that MenA inhibitors can be developed as indirect ATP synthesis inhibitors. The data described here indicate that lipoquinones would be a unique and new antimycobacterial target.

We previously reported that the other MenA inhibitors inhibited growth of drug-resistant Gram-positive bacteria (i.e. MRSA, VRSA, and linezolid resistant MRSA) at low concentrations, and all Gram-negative bacteria tested were not susceptible to the MenA inhibitors even at high concentrations [10]. These data strongly indicate that MenA is likely to be a valid drug target for not only *Mycobacterium* spp. but also other Gram-positive pathogens. The MenA inhibitors described here can be synthesized cost-efficiently and structural modifications to improve the MenA and mycobacterial growth inhibitory activities can be achieved in a time efficient manner. Our recent studies on pharmacokinetics revealed that the advanced MenA inhibitors (structures not shown) possess excellent oral absorption and high distribution to lung tissue. The results are expected to be of significance in terms of discovering new lead compounds that can be developed into new drugs to combat drug-resistant and nonreplicating *Mycobacterium tuberculosis*. We are currently optimizing the structure of the MenA inhibitor leads to improve mycobactericidal activity *in vivo*. In addition, because combination therapy will remain mandatory to combat *Mycobacterium tuberculosis*, we will establish possible combination of MenA inhibitor with known antimycobactericidal drugs. These data will be reported elsewhere.

EXPERIMENTAL SECTION

General Procedures and Methods

IR absorptions on NaCl plates were run on a Perkin Elmer FT-IR 1600. ¹H NMR spectral data were obtained using Varian 300, 400 MHz instruments. The residual solvent signal was utilized as an internal reference. ¹³C NMR spectral data were obtained using a Varian 100 MHz spectrometer. Chemical shifts were reported in parts per million (ppm) downfield from TMS, using the middle resonance of CDCl₃ (77.0 ppm) as an internal standard. For all NMR spectra, δ values are given in ppm and *J* values in Hz. Mass spectra were obtained at Colorado State University's Central Instrument Facility. Reagents and solvents are commercial grade and were used as supplied. Reaction vessels were flame-dried or oven-dried and cooled under an inert atmosphere when necessary.

Syntheses of MenA Inhibitors, **22** and **23**

Detailed synthesis of **22** through the Friedel-Crafts acylation reactions of **1a–e** and **3a–g** and additional chemical steps were described in reference 10 [10].

Synthesis of 6 via the Grignard Reagents 4b and Subsequent Demethylation

To a stirred solution of **4b** (R_3 =OMe, R_4 =H, 40 mmol), which was obtained by the halogen-metal exchange reaction of 1-iodo-2,4-dimethoxybenzene with $^i\text{PrMgCl}$, in THF (50 mL) was added **2a** (R_1 =Cl, R_2 =H, 33.9 mmol) in THF (20 mL) at -78°C . The reaction mixture was warmed to 0°C over 1h and quenched with aq. NH_4Cl . The water phase was extracted with EtOAc and the combined organic phase was washed with brine, dried over Na_2SO_4 , and evap. *in vacuo*. This was subjected to hydrolysis with 48% HBr (50 mL) in AcOH (25 mL) at 120°C . After 36h, all volatiles were evaporated. Purification by silica gel chromatography (5:1, hexanes:EtOAc) provided (4-chlorophenyl)(2,4-dihydroxyphenyl)methanone (**6**; R_1 , R_4 , R_5 , R_6 = Cl, H, H, OH, 29.0 mmol, 86% overall) as a white powder. Data for (2-chloro-4-hydroxyphenyl)(4-chlorophenyl)methanone: ^1H -NMR (CDCl_3 , 300 MHz): δ 7.52 (d, J = 7.8 Hz, 2H), 7.40 (d, J = 8.1 Hz, 2H), 7.34 (m, 1H), 6.37 (s, 1H), 6.30 (d, J = 8.7 Hz, 1H), 3.99 (bs, 2H); ^{13}C -NMR (CDCl_3 , 100 MHz): 198.9, 166.1, 165.6, 137.9, 136.9, 135.9, 131.5, 130.5, 128.8, 112.5, 108.6, 103.4; IR (neat, cm^{-1}): 3288, 2923, 1629, 1599, 1540; HRMS (FAB) calcd. for $\text{C}_{13}\text{H}_{10}\text{O}_3\text{Cl}$ ($\text{M}+\text{H}^+$) 249.0313, and observed 249.0305.

Synthesis of 7

To a stirred solution of (4-chlorophenyl)(2,4-dihydroxyphenyl)methanone (**6**; R_1 , R_4 , R_5 , R_6 = Cl, H, H, OH, 7.51 mmol) in DMF (20 mL) was added K_2CO_3 (5.2 g, 37.6 mmol) and 1,8-dibromooctane (5.1 g, 18.8 mmol). After 12h, the reaction mixture was quenched with water and extracted with hexanes. The combined extracts were washed with brine, dried over Na_2SO_4 , and concentrated *in vacuo*. Purification by silica gel chromatography (30:1, hexanes:EtOAc) to provide (4-(8-bromooctyloxy)-2-hydroxyphenyl)(4-chlorophenyl)methanone (6.38 mmol, 85 %) as a liquid. Data for (4-(8-bromooctyloxy)-2-hydroxyphenyl)(4-chlorophenyl)methanone (**7**; R_1 , R_4 , R_5 , R_6 = Cl, H, H, OH): ^1H -NMR (CDCl_3 , 300 MHz): δ 12.6 (s, 1H), 7.73 (m, 2H), 7.52 (d, J = 8.4 Hz, 1H), 7.42 (m, 2H), 6.59 (dd, J = 2.4 & 8.7 Hz, 1H), 6.49 (d, J = 2.1 Hz, 1H), 4.06 (t, J = 6.3 Hz, 2H), 3.87 (t, J = 6.3 Hz, 2H), 1.91 (m, 4H), 1.45 (m, 8H); ^{13}C -NMR (CDCl_3 , 75 MHz): 205.6, 163.3, 159.1, 138.0, 132.3, 130.7, 128.1, 121.0, 105.4, 99.5, 68.1, 34.0, 32.7, 29.1, 28.8, 28.7, 28.1, 25.7; LRMS (FAB) $\text{C}_{21}\text{H}_{24}\text{ClO}_3\text{Br}$ found = 438.

Synthesis of 22a

To a stirred solution of (4-(8-bromooctyloxy)-2-hydroxyphenyl)(4-chlorophenyl)methanone (**7**; R_1 , R_4 , R_5 , R_6 = Cl, H, H, OH, 0.046 mmol) in THF (1 mL) were added NaHCO_3 (36.2 mg, 0.46 mmol) and *N*-methylprop-2-en-1-amine (32.6 mg, 0.46 mmol). After 3 days at r.t., the reaction mixture was filtered and all volatiles were evaporated. Purification by PTLC (10:1, hexanes:EtOAc) provided **22a** (0.044 mmol, 95 %) as a white powder. Data for **22a**: ^1H -NMR (300 MHz, CDCl_3): δ 12.20 (s, 1H), 7.66 (d, J = 8.4 Hz, 2H), 7.40 (m, 3H), 6.97 (d, J = 5.4 Hz, 1H), 6.90 (s, 1H), 6.16 (m, 1H), 5.57 (d, J = 10.2 Hz, 2H), 3.82 (t, J = 6.0 Hz, 2H), 3.61 (m, 2H), 3.04 (m, 1H), 2.89 (m, 1H), 2.73 (s, 3H), 1.79 (m, 2H), 1.23 (m, 10H); ^{13}C -NMR (CDCl_3 , 100 MHz): 194.8, 158.9,

139.1, 138.3, 137.9, 130.7(3c), 128.1(2c), 126.1, 125.9(2c), 120.8, 113.8, 68.0, 58.2, 55.2, 39.3, 28.9, 28.8, 28.7, 26.5, 25.4, 23.6; HRMS (FAB) calcd. for $\text{C}_{25}\text{H}_{33}\text{O}_3\text{ClN}$ ($\text{M}+\text{H}^+$) 430.2071, and observed 430.2065. RT: 8.8 min. (Column: Supelco Discovery HS C-18, Solvent: MeOH, FR: 0.5 mL/min).

Synthesis of 11

To a stirred solution of (4-chlorophenyl)(2,4-dihydroxyphenyl)methanone (**6**; R_1 , R_4 , R_5 , R_6 = Cl, H, H, OH, 4.03 mmol) in DMF (20 mL) was added K_2CO_3 (8.1 mmol) and 1,4-dibromobutene (10.1 mmol). After 3h at 0°C , the reaction mixture was quenched with water and extracted with hexanes. The combined extracts were washed with brine, dried over Na_2SO_4 , and concentrated *in vacuo*. Purification by silica gel chromatography (15:1, hexanes:EtOAc) provided *E*-(4-(4-bromobut-2-enyloxy)-2-hydroxyphenyl)(4-chlorophenyl)methanone (3.62 mmol, 90 %) as a liquid. To a stirred solution of 1,3-propanediol (18.1 mmol) in DMF (10 mL) at 0°C was added NaH (60%, 2.1g, ~54.3 mmol). After 10 min., (*E*)-(4-(4-bromobut-2-enyloxy)-2-hydroxyphenyl)(4-chlorophenyl)methanone (3.62 mmol) in DMF (4 mL) was added. The reaction mixture was stirred for 2h and quenched with 0.5 N HCl, and extracted with hexanes. The combined extracts were washed with brine, dried over Na_2SO_4 , and concentrated *in vacuo*. Purification by silica gel chromatography (20:1, hexanes:EtOAc) to provide *E*-(4-(4-chlorophenyl)(2-hydroxy-4-(4-(3-hydroxypropoxy)but-2-enyloxy)phenyl)methanone (1.36 g, 3.44 mmol, 95 %) as a liquid. To a stirred solution of *E*-(4-(4-chlorophenyl)(2-hydroxy-4-(4-(3-hydroxypropoxy)but-2-enyloxy)phenyl)methanone (3.44 mmol) in CH_2Cl_2 (10 mL) was added PPh_3 (4.51 g, 17.2 mmol) and CBr_4 (5.6 g, 17.2 mmol). After 1h at r.t., the reaction mixture was evaporated *in vacuo*. The crude mixture was purified by silica gel chromatography (20:1, hexanes:EtOAc) to provide (*E*)-(4-(4-(3-bromopropoxy)but-2-enyloxy)-2-hydroxyphenyl)(4-chlorophenyl)methanone (3.44 mmol, 100 %) as a liquid. Data for (*E*)-(4-(4-(3-bromopropoxy)but-2-enyloxy)-2-hydroxyphenyl)(4-chlorophenyl)methanone (**11**; R_1 , R_4 , R_5 , R_6 = Cl, H, H, OH): ^1H -NMR (CDCl_3 , 300 MHz): δ 12.53 (s, 1H), 7.60 (m, 2H), 7.47 (m, 3H), 6.52 (d, J = 2.4 Hz, 1H), 6.44 (dd, J = 2.7 & 9.0 Hz, 1H), 5.96 (m, 2H), 4.61 (m, 2H), 4.06 (m, 2H), 3.59 (t, J = 6.0 Hz, 2H), 3.54 (t, J = 6.6 Hz, 2H), 2.14 (m, 2H); ^{13}C -NMR (CDCl_3 , 100 MHz): 198.6, 166.2, 165.2, 137.8, 136.5, 134.9, 131.0, 130.3, 128.6, 126.2, 112.9, 107.9, 101.9, 70.5, 68.1, 67.8, 32.8, 30.6. IR (neat, cm^{-1}): 3208, 2930, 1745, 1635, 1590; LRMS (FAB) $\text{C}_{20}\text{H}_{20}\text{ClO}_4\text{Br}$ found = 438.

Synthesis of 22c

To a stirred solution of *E*-(4-(4-(3-bromopropoxy)but-2-enyloxy)-2-hydroxyphenyl)(4-chlorophenyl)methanone (**11**; R_1 , R_4 , R_5 , R_6 = Cl, H, H, OH, 0.023 mmol) in THF (1 mL) was added NaHCO_3 (0.23 mmol) and 2-(4-methoxyphenyl)ethanamine (0.12 mmol). After 24h at r.t., the reaction mixture was filtered and evaporated. Purification by PTLC (10:1, CHCl_3 : MeOH) to provide **22c** (0.022 mmol, 96 %) as a white powder. Data for **22c**: ^1H -NMR (CDCl_3 , 300 MHz): δ 7.41 (m, 2H), 7.13 (m, 2H), 6.96 (m, 2H), 6.85 (m, 2H), 6.54 (d, J = 9.0 Hz, 1H), 6.72 (d, J = 2.7 Hz, 1H), 6.16 (dd, J = 2.4, 9.0 Hz, 1H), 5.94 (m, 2H), 4.55 (m, 2H), 4.03 (m, 2H)

), 3.72 (s, 3H), 3.58 (t, $J = 6.0$ Hz, 2H), 3.53 (m, 4H), 2.93 (t, $J = 6.6$ Hz, 2H), 2.13 (m, 2H); ^{13}C -NMR (CDCl_3 , 100 MHz): 198.5, 168.6, 162.8, 160.2, 132.6, 130.6, 130.4, 130.3, 130.1, 130.0, 128.9, 128.7, 128.6, 126.8, 115.3, 106.2, 102.5, 70.6, 69.0, 67.7, 55.8, 47.2, 36.3, 35.4, 29.9; IR (neat, cm^{-1}): 3052, 2917, 1601, 1509, 1487; LRMS (FAB) $\text{C}_{29}\text{H}_{32}\text{ClO}_5\text{N}$ found = 497.

Synthesis of other MenA Inhibitors

The other MenA inhibitors were synthesized according to the procedures described above, however, different building blocks were used. The MenA inhibitors possessing hydrazine group were synthesized according to the procedures described in reference 38 [38].

Enzymatic Assay Against MenA

The enzymatic activity was characterized using membrane fractions prepared from *M. tuberculosis* essentially as previously described. *M. tuberculosis* (H37Rv) was grown to mid-log phase in glycerol-alanine-salts medium, washed with saline and harvested by centrifugation. The resulting pellet was irradiated for 18 h at 2,315 Rads/min using a JL Shepard instrument with a ^{137}Cs source. This exposure was calculated to kill 100% of the bacteria but retain 90% of enzyme activity. The washed cell pellet was resuspended in homogenization buffer containing 50 mM MOPS (pH 7.9), 0.25 M sucrose, 10 mM MgCl_2 and 5 mM 2-mercaptoethanol and disrupted by probe sonication on ice with a Sanyo Soni-prep 150 (10 cycles of 60 sec on and 90 sec off). The resulting suspension was centrifuged at 27,000 X g for 15 min. The pellet was discarded and the supernatant was centrifuged at 100,000 X g for 1 h in a Beckman Ti70.1 rotor. The pellet (membranes) was resuspended in homogenization buffer, divided into aliquots and frozen at 70 °C. The protein concentration was estimated using a BCA protein assay kit (Pierce, Rockford, IL). MenA assays were conducted essentially as previously described. Assay mixtures contained 500 μM DHNA, 10 μM [^3H]-farnesyl diphosphate (American Radiolabeled Chemicals, St. Louis, MO), 5 mM MgCl_2 and 0.1% CHAPS in 100 mM MOPS (pH 8) and an appropriate amount of membrane protein. Reactions were stopped by the addition of 1 ml of 0.1 M AcOH in MeOH. The resulting mixture was extracted twice with 3 ml of hexanes and the combined extracts were evaporated to dryness under N_2 and redissolved in CHCl_3 : MeOH (2:1). An aliquot was taken for liquid scintillation counting and the remaining material was subjected to TLC on silica gel plates, which were developed in hexanes : Et_2O (95:5). Radioactive spots on the thin layer plated were located and quantitated using a Bioscan System 200 Imaging Scanner. IC_{50} values were calculated for the inhibitors and compared to the MIC values, in all cases tested there was a good correlation between IC_{50} and MIC.

MIC Determinations Against *M. tuberculosis* H37Rv

We applied colorimetric microtiter plate based method with Alamar blue/visual inspection for the MIC determination with *M. tuberculosis*. The MIC of all compounds synthesized was determined first against H37Rv by the colorimetric microtiter plate based method with Alamar blue/visual inspection. The compounds demonstrated good activ-

ity against H37Rv was tested against MDR-TB strains. The MDR strains are all resistant to isoniazid (INH), streptomycin, rifampicin (RIF), or ethambutol (EMB), and some are resistant to pyrazinamide (PZA) and/or additional second line drugs. Drug susceptibility test methods can vary depending on the size of the inoculum (i.e. density of the cells). We will standardize the size of the inoculum using the frozen stocks with known CFU/mL (approx. 1×10^4). Compounds were tested at 20 μM initially; each microtitre plate has both positive and negative controls and the outside wells are filled with water to help prevent dehydration. If inhibitory activity occurs at 20 μM (this is usually 10 $\mu\text{g}/\text{ml}$ of a compound with approx. 500 MW) in the primary screen, serial dilutions of each compound were prepared, and MICs were determined.

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