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Methyl-Branched Fatty Acids, Inhibitors of Enoyl-ACP Reductase with Antibacterial Activity from *Streptomyces* sp. A251

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Bacterial enoyl-ACP reductase (FabI) has been demonstrated to be a novel antibacterial target. In the course of our screening for FabI inhibitors, we isolated two methyl-branched fatty acids from *Streptomyces* sp. A251. They were identified as 14-methyl-9(*Z*)-pentadecenoic acid and 15-methyl-9(*Z*)-hexadecenoic acid by MS and NMR spectral data. These compounds inhibited *Staphylococcus aureus* FabI with IC_{50} values of 16.0 and 16.3 μM, respectively, but did not affect FabK, an enoyl-ACP reductase of *Streptococcus pneumonia,* at 100 μM. Consistent with their selective inhibition for FabI, they blocked intracellular fatty acid synthesis as well as the growth of *S. aureus*, but did not inhibit the growth of *S. pneumonia*. Additionally, these compounds showed reduced antibacterial activity against *fabI*-overexpressing *S. aureus*, compared with the wild-type strain. These results demonstrate that the methyl-branched fatty acids show antibacterial activity by inhibiting FabI *in vivo*.

Keywords: Methyl-branched fatty acid, *Staphylococcus aureus*, enoyl-ACP reductase, antibacterial activity

With the emergence of vancomycin-resistant *S. aureus* (VRSA) in 2002, there is an urgent need for new antibiotics [9, 15]. One approach to combating antibiotic resistance is to identify and develop new drugs with novel mechanisms of action. Microbial genomics has led to the discovery of new antibacterial targets for antibacterial drug discovery [12]. Bacterial fatty acid synthesis (FAS) is one of the new genomics-derived antibacterial targets [1, 7, 24]. Enoyl-ACP reductase catalyzes the final and rate-limiting step of the chain elongation process in FAS. There are three isoforms, FabI, FabK, and FabL, in enoyl-ACP reductase. FabI is widely distributed in most bacteria, whereas FabK is present in a few important pathogens such as *S. pneumonia*, *Enterococcus faecalis*, *Clostridium acetobutylicum*, and *Pseudomonas aeruginosa*. *E. faecalis* and *P. aeruginosa* were known to contain FabI as well as FabK. Indeed, the antibacterial target of triclosan, the broad-spectrum biocide in a wide range of consumer goods, has been determined to be FabI [6, 11]. Therefore, inhibitors of enoyl-ACP reductase may be interesting lead compounds for developing effective antibacterial drugs.

Microorganisms produce many kinds of antibiotics that function in an antagonistic capacity in nature, where they have much competition, importantly, antibacterial targets of many antibiotics have not been elucidated [17]. However, FAS inhibitors have not been isolated much from species of microbial origin. Only two FabI inhibitors, cephalochromin and vinaxanthone, have been reported from fungal strains [26, 27]. A few compounds inhibiting other FAS components have been known. Platensimycin, with a potent antibacterial activity, isolated from *Streptomyces platensis*, has been known to target the elongation condensing enzyme FabF [22]. Cerulenin and thiolactomycin, isolated from *Cephalosporium caerulens* and *Nocardia* sp., respectively, selectively inhibit FabF/B and FabH, the condensation enzymes [1].

In the course of the screening program for enoyl-ACP reductase inhibitors from microbial sources, two methyl-branched fatty acids were isolated from *Streptomyces* sp. A251. The inhibitors were identified as 14-methyl-9(*Z*)-pentadecenoic acid (MPDA) [3] and 15-methyl-9(*Z*)-hexadecenoic acid (MHDA) [4] by chemical modification and MS and NMR spectral data (Fig. 1). The antibacterial action of these methyl-branched fatty acids was demonstrated to be mediated by inhibition of FabI. To our knowledge, it is the first time that MPDA has been isolated from natural sources, and its antibacterial activity has not been reported before. MHDA has been isolated from the sponge *Callyspongia fallax* and *Chondrosia remifromis*, but its biological activity has also not been reported before.

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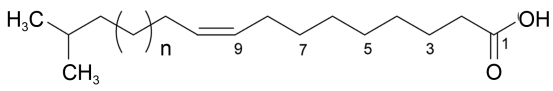

MPDA, n=1
MHDA, n=2

Fig. 1. Chemical structures of MHDA and MPDA.

Materials and Methods

Bacterial Strains and Materials

The bacterial strains used in the antibacterial assays were obtained from the Culture Collection of Antimicrobial Resistant Microbes of Korea and the Korean Collection for Type Cultures. Triclosan, erythromycin, oxacillin, norfloxacin, and chloramphenicol were purchased from Sigma. [1-^{14}C]Acetate (57 μCi/μmol) and L-[U-^{14}C]leucine (306 μCi/μmol) were purchased from Amersham.

Isolation of MPDA and MHDA

MPDA and MHDA were isolated from the fermention broth of an actinomycetal strain, A251. The strain A251 was isolated from a soil sample collected near Gongju-City, Chungcheongnam-Do, Korea. The strain was identified as a *Streptomyces* species on the basis of 16S rDNA sequence. Fermentation was carried out in 1-l Erlenmeyer flasks containing soluble starch 1%, glucose 2%, soybean meal 2.5%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.025%, and $CaCO_3$ 0.2% (adjusted to pH 7.2 before sterilization). The culture supernatant obtained from the culture broth (3 l) was extracted with ethyl acetate. The ethyl acetate extract was subjected to silica gel (56×200 mm, Merck Art No. 7734.9025) column chromatography, followed by elution with $CHCl_3$-MeOH (100:1). Then the active fractions were pooled and concentrated *in vacuo*. The resultant residue was applied to a Sephadex LH-20 column and then eluted with $CHCl_3$-MeOH (1:1). The active fraction dissolved in MeOH was further purified on a reverse-phase HPLC column (YMC C_{18}, 20×250 mm) eluted with CH_3 OH-H_2O (90:10) containing 0.01% trifluoroacetic acid to afford compounds **1** (3.9 mg) and **2** (4.3 mg) with retention times of 27.5 and 34.9 min, respectively. Based on MS and NMR spectral data, compounds **1** and **2** were identified to be 14-methyl-pentadecenoic acid and 15-methyl-hexadecenoic acid, respectively. To determine the double-bond position in compounds **1** and **2**, dimethyl disulfide (DMDS) derivatization was performed [2]. Compound **1** (1.1 mg) was methylated with ethereal diazomethane at room temperature for 1 h [26] and the methyl ester was derivatized with DMDS according to the method of Dunkelblum *et al*. [5]. The EI mass spectrum of the resultant DMDS adduct showed two substantial fragments ions at *m/z* 145 and 217. The third peak was observed at *m/z* 185 owing to the loss of methanol from the *m/z* 217 (Fig. 2). These data indicated the double-bond position at C-9 in compound **1**. The double-bond position of compound **2** was determined in a similar way to be C-9. Thus, the chemical structures of compounds **1** and **2** were identified as 14-methyl-9(*Z*)-pentadecenoic acid [3] and 15-methyl-9(*Z*)-hexadecenoic acid [4], respectively, as follows: 14-methyl-9(*Z*)-pentadecenoic acid: ESI−MS: *m/z* 253 [M-H]$^-$; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 5.36 (2H, m, H-9, H-10), 2.36 (2H, t, *J*=7.2 Hz, H-2), 2.01 (4H, m, H-8, H-11), 1.64 (2H, m, H-3), 1.54 (1H, m, H-14), 1.32−1.37 (10H, m, CH_2×5), 1.19 (2H, m, H-13), 0.88 (6H, d, *J*= 6.6 Hz, H-15, CH_3-16); ^{13}C NMR ($CDCl_3$, 75 MHz)

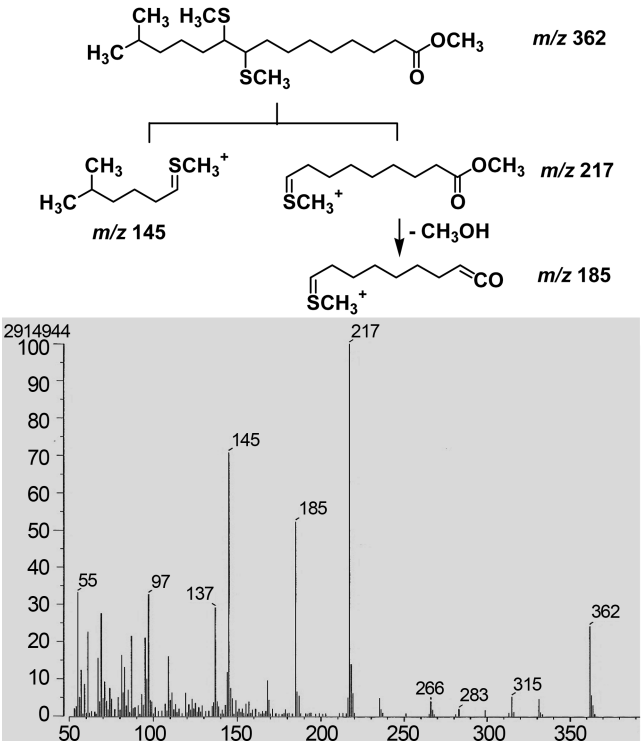

Fig. 2. Mass spectrum of DMDS adducts of MHDA.

δ 178.2 (s, C-1), 130.2 (d), 129.9 (d), 38.9 (t, C-13), 33.9 (t, C-2), 29.4 (t), 29.3 (t), 29.2 (t), 28.1 (d, C-14), 27.7 (t), 27.6 (t), 27.4 (t, C-8, C-11), 24.9 (t, C-3), 22.8 (q, C-15, CH_3-16); 15-methyl-9(*Z*)-hexadecenoic acid: ESI−MS: *m/z* 267 [M-H]$^-$; ^{1}H NMR (300 MHz, $CDCl_3$): ^{1}H NMR ($CDCl_3$, 600 MHz) δ 5.35 (2H, m, H-9, H-10), 2.35 (2H, t, *J*=7.5 Hz, H-2), 2.01 (4H, m, H-8, H-11), 1.64 (2H, m, H-3), 1.53 (1H, m, H-15), 1.32-1.36 (12H, m, CH_2×6), 1.18 (2H, m, H-14), 0.87 (6H, d, *J*=6.6 Hz, H-16, CH_3-17); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 178.7 (s, C-1), 130.0 (d), 129.7 (d), 38.9 (t, C-14), 33.8 (t, C-2), 29.2 (t), 29.1 (t), 29.0 (t), 28.0 (d, C-15), 27.2 (t), 27.1(t), 27.0 (t, C-8, C-11), 24.6 (t, C-3), 22.6 (q, C-16, CH_3-17).

FabI, FabG, and FabK Assays

S. aureus FabI and FabG and *S. pneumoniae* FabK assays were performed as described previously [20, 25, 26]. Assays were carried out in half-area, 96-well microtiter plates. Compounds were evaluated in 100-μl assay mixtures containing components specific for each enzyme (see below). Reduction of the *trans*-2-octenoyl *N*-acetylcysteamine (t-o-NAC thioester) substrate analog was measured spectrophotometrically by following the utilization of NADH or NADPH at 340 nm at 30°C for the linear period of the assay. *S. aureus* FabI assays contained 50 mM sodium acetate, pH 6.5, 400 μM t-o-NAC thioester, 200 μM NADPH, and 150 nM *S. aureus* FabI. The rate of decrease in the amount of NADPH in each reaction well was measured by a microtiter ELISA reader using SOFTmax PRO software (Molecular Devices, CA, U.S.A.). The inhibitory activity was calculated by the following formula: % of inhibition=100×[1−(rate in the presence of compound/rate in the untreated control)]. IC_{50} values were calculated by fitting the data to a sigmoid equation. An equal volume of dimethyl sulfoxide solvent was used for the untreated control. FabK assays

contained 100 mM sodium acetate, pH 6.5, 2% glycerol, 200 mM NH_4Cl, 50 μM t-o-NAC thioester, 200 μM NADH, and 150 nM *S. pneumoniae* FabK. FabG assays contained 100 mM sodium phosphate, pH 7.4, 50 μM acetoacetyl-CoA, 200 μM NADPH, and 40 nM *S. aureus* FabG.

Determination of Minimum Inhibitory Concentrations (MICs)

Whole-cell antimicrobial activity was determined by broth microdilution as described previously [27]. The test strains were grown to the mid-log phase in Mueller–Hinton broth and diluted 1,000-fold in the same medium. Cells (10^5/ml) were inoculated into Mueller–Hinton broth and dispensed at 0.2 ml/well into a 96-well microtiter plate. MICs were determined in triplicate by serial 2-fold dilutions of the test compounds. The MIC was defined as the concentration of a test compound that completely inhibited cell growth during a 24 h incubation at 30°C. Bacterial growth was determined by measuring the absorption at 650 nm using a microtiter ELISA reader.

Measurement of Inhibition of Macromolecular Biosynthesis

The effect of FabI inhibitors on the incorporation of [1-^{14}C]acetate and L-[U-^{14}C]leucine in *S. aureus* was measured as described previously [27]. *S. aureus* was grown to the mid-log phase in LB medium. Each 1-ml culture was treated with drugs for 10 min. An equal volume of DMSO solvent was added to the untreated control. For [1-^{14}C]acetate incorporation, 2 μCi of [1-^{14}C]acetate was then added to the cultures and incubated at 37°C for 1 h in a shaker. After being harvested by centrifugation, the cell pellets were washed twice with PBS. The total cellular lipids were then extracted with chloroform–methanol–water. The incorporated radioactivity in the chloroform phase was measured by scintillation counting. For L-[U-^{14}C]leucine incorporation, 0.6 μCi of L-[U-^{14}C]leucine was added to the cultures and incubated at 37°C for 1 h in a shaker. The incorporation was terminated by the addition of 10% (w/v) TCA and cooling on ice for 20 min. The precipitated material was collected on Whatman GF/C glass microfiber filters, washed with TCA and ethanol, dried, and counted in a scintillation counter. Total counts incorporated at 1 h of incubation without inhibitors ranged from >6,000 for [^{14}C]leucine to >10,000 for [^{14}C]acetate.

Overexpression Assay

The *fabI*-overexpression assay was performed as described previously [27]. *S. aureus* RN4220, *S. aureus* RN4220 (pE194), and *S. aureus* RN4220 (pE194-*fabI*) were used for the overexpression assay. The MICs of FabI inhibitors for these three strains were determined. An increase in the MIC for the *fabI*-overexpressing strain relative to that for the wild type indicates that FabI is the mode of antibacterial action.

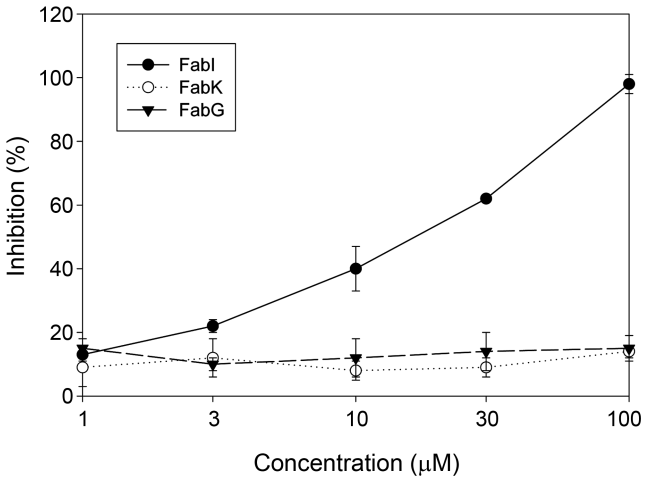

Fig. 3. Inhibitory activity of MHDA on FabI and FabG of *S. aureus* and FabK of *S. pneumoniae*.
The values were represented as the means ± SD in triplicates obtained from two independent experiments.

RESULTS

Identification of MPDA and MHDA as the Selective Inhibitors of FabI

In the screening of FabI inhibitors from the microbial extracts library, two FabI-inhibitory compounds were isolated from *Streptomyces* sp. A251. Their structures were determined to be MHDA and MPDA, respectively [3, 4]. MHDA has been isolated from the sponge *Callyspongia fallax*, but MPDA was isolated from natural sources for the first time in this study.

MHDA inhibited *S. aureus* FabI dose-dependently with an IC_{50} of 16.3 μM (Fig. 3). MPDA also exhibited similar FabI-inhibitory activity (Table 1). To assess whether they selectively inhibit FabI, their effects on FabG, the other reductase of bacterial FAS catalyzing the reduction of β-ketoacyl-ACP to β-hydroxyacyl-ACP, and FabK, an enoyl-ACP reductase of *S. pneumoniae*, were examined. MPDA and MHDA did not inhibit *S. aureus* FabG and *S. pneumoniae* FabK, even at 100 μM (Fig. 3 and Table 1). These data show that MPDA and MHDA selectively inhibit *S. aureus* FabI in the enzyme assay.

Table 1. Comparison of inhibitory effects of MPDA and MHDA on Fab enzymes with those on bacterial viability and macromolecular biosynthesis.

Compounds	IC_{50} (μM)			MIC (μg/ml)		IC_{50} (μM)[d]	
	FabI[a]	FabG[b]	FabK[c]	*S. aureus*	*S. pneumoniae*	[^{14}C]Acetate	[^{14}C]Leucine
MPDA	16.0	>100	>100	32	>128	15.1	>100
MHDA	16.3	>100	>100	32	>128	15.6	>100
Triclosan	0.66	>100	>100	0.01	>128	0.04	>0.1

[a,b]*S. aureus*; [c]*S. pneumoniae*; [d]incorporation in *S. aureus*.

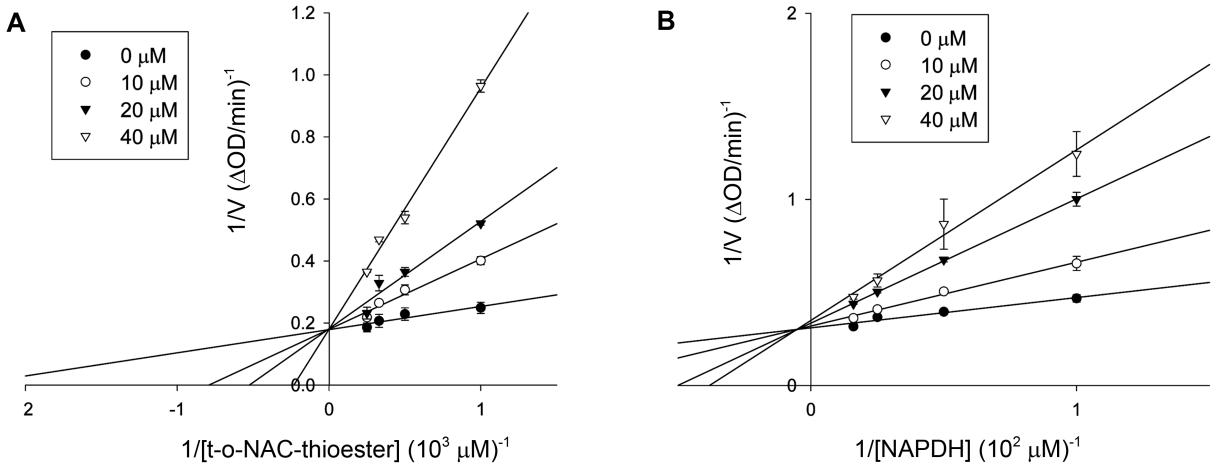

Fig. 4. The mechanism of inhibition of *S. aureus* FabI by MHDA with respect to t-o-NAC thioester (**A**) and NADPH (**B**).
The reciprocals of the initial reaction and substrate (**A**) and cofactor (**B**) concentrations are plotted. The values were represented as the mean ± SD in triplicates obtained from two independent experiments.

Mode of Inhibition of FabI

The inhibition pattern of FabI by MHDA with respect to the substrate and the cofactor was examined with a Lineweaver–Burk plot. The inhibition of *S. aureus* FabI by MHDA was competitive with respect to the substrate t-o-NAC thioester, with a K_i value of 2.8 μM (Fig. 4A). In contrast, MHDA exhibited mixed inhibition with respect to the cofactor, NADPH (Fig. 4B).

Antibacterial Activitiy

MPDA and MHDA were tested for their antibacterial activities. Both MPDA and MHDA showed antibacterial activity with an MIC of 32 μg/ml against Gram-positive bacteria such as *S. aureus* and *S. epidermidis* that have the FabI. However, neither MPDA nor MHDA exhibited antibacterial activity against *S. pneumoniae* with the FabK isoform or against *P. aeruginosa* and *E. faecalis* with the FabI and FabK isoforms, even at 128 μg/ml, as expected. These results indicate a good correlation between enzyme inhibitory activity and antibacterial activity (Table 1).

Inhibition of Cellular Fatty Acid Synthesis

To assess whether the inhibitors inhibit cellular fatty acid synthesis, we determined whether they inhibited the incorporation of [1-^{14}C]acetate into membrane fatty acids *in vivo*. Both MPDA and MHDA indeed blocked fatty acid synthesis *in vivo* compared with the untreated cells, with IC_{50} values of 15.1 and 15.6 μM, respectively (Table 1). The inhibitory potencies of MPDA and MHDA on [1-^{14}C]acetate incorporation were similar with FabI-inhibitory activity. In contrast, the incorporation of leucine into proteins was not inhibited by MPDA, and MHDA, even at 100 μM, whereas the protein synthesis inhibitor chloramphenicol as control inhibited incorporation (data not shown).

Effects on *fabI*-Overexpressing *S. aureus*

An increase in the MIC for the *fabI*-overexpressing strain relative to that for the wild type is indicative of FabI being the mode of antibacterial action [19]. To assess whether overexpression of *fabI* shifts the MICs of MPDA and MHDA for *S. aureus*, their antibacterial activities for the strain that overexpresses *fabI* were investigated. MPDA and MHDA for the strain overexpressing *fabI*, *S. aureus* RN4220 (pE194-*fabI*), were 4-fold increased compared with that for the wild-type strain *S. aureus* RN4220, or the vector-containing strain *S. aureus* RN4220 (pE194) (Table 2). As a positive control, triclosan, the known FabI inhibitor, increased the MIC for the *fabI*-overexpressing strain,

Table 2. Reduced susceptibility of *fabI*-overexpressing *S. aureus* to MPDA and MHDA.

	MIC (μg/ml)		
	S. aureus RN4220	*S. aureus* RN4220 (pE194)	*S. aureus* RN4220 (pE194-*fabI*)
MPDA	32	32	128
MHDA	32	32	128
Triclosan	0.01	0.01	1
Oxacillin	0.25	0.25	0.25
Erythromycin	0.5	64	64
Norfloxacin	1	1	1

whereas erythromycin, the selection marker for the vector pE194, increased the MICs for both the *fabI*-overexpressing strain and the vector-containing strain, indicating that the engineered constructs were functioning as expected. As negative-control compounds, norfloxacin and oxacillin with different modes of action did not change the MICs of the three strains. Thus, these results indicate that MPDA and MHDA inhibit the growth of *S. aureus* through inhibition of FabI.

DISCUSSION

In the course of screening for inhibitors of *S. aureus* FabI from microbial sources, we have isolated two methyl-branched fatty acids, MPDA and MHDA, from *Streptomyces* sp. MPDA has been chemically synthesized [4], but was isolated from natural sources for the first time in this study. MPDA was reported to inhibit the human placenta DNA topoisomerase I that has evolved as a key cellular target for the development of anticancer drugs [21]. MHDA has been isolated from the sponge *Callyspongia fallax* and *Chondrosia remifromis*, but its biological activity has not been reported [2, 3].

The FabI reaction has a compulsory ordered mechanism with the nucleotide cofactors, NADH or NADPH, as the first substrates [18]. MPDA and MHDA could bind to the free enzyme, the enzyme–substrate complex, or both to prevent catalysis. In the first case, the inhibition pattern with respect to the cofactor would be competitive; in the second, the inhibition pattern would be noncompetitive; and in the third case, mixed-type inhibition would be observed. The inhibition of *S. aureus* FabI by MHDA was competitive with respect to the substrate. This suggests that MHDA binds to the enzyme–NADPH complex to prevent the binding of the substrate.

Several synthetic FabI inhibitors including 1,4-disubstituted imidazoles [8], thiopyridines [10], aminopyridines [13], and naphthyridinones [14, 16] have been reported. As natural FabI inhibitors, cephalochromin [26], vinaxanthone [27], EGCG [23], and flavonoids [21] are known. Structurally, MPDA and MHDA are different from these known FabI inhibitors. EGCG and flavonoids have been known to inhibit several targets such as FabG, FabZ, and FabI [21, 23]. In addition, EGCG and flavonoids don't show a direct correlation of FabI-inhibition with antibacterial activity [23]. In contrast, MPDA and MHDA did not inhibit *S. aureus* FabG and *S. pneumonia* FabK, even at 100 μM, and showed the inhibition specific to FabI.

In summary, methyl-branched fatty acids, 14-methyl-9(*Z*)-pentadecenoic acid and 15-methyl-9(*Z*)-hexadecenoic acid, selectively inhibited *S. aureus* FabI and also showed antibacterial activity against *S. aureus*. The antibacterial target of the methyl-branched fatty acids is validated to be FabI. These results may be helpful for the chemical design of new FabI inhibitors for tackling infections caused by multidrug-resistant pathogens.

Acknowledgments

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