Chalcomoracin and Moracin C, New Inhibitors of *Staphylococcus aureus* Enoyl-Acyl Carrier Protein Reductase from *Morus alba*

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Bacterial enoyl-acyl carrier protein (ACP) reductase has been confirmed as a novel target for antibacterial drug development. In the screening of inhibitors of *Staphylococcus aureus* enoyl-ACP reductase (FabI), we found that a methanol extract of leaves of *Morus alba* L. potently inhibited *S. aureus* FabI as well as growth of *S. aureus*. The active principles were identified as chalcomoracin and moracin C by MS and NMR analysis. Chalcomoracin and moracin C inhibited *S. aureus* FabI with IC_{50} of 5.5 and 83.8 μM, respectively. They also prevented the growth of *S. aureus* with minimum inhibitory concentration (MIC) of 4 and 32 μg/mL, respectively. Consistent with their inhibition against FabI and bacterial growth, they prevented [^{14}C]-acetate incorporation into fatty acid in *S. aureus* while didn't affect protein synthesis. In this study, we reported that chalcomoracin and moracin C, potent antibacterial compounds from *Morus alba*, inhibited FabI and fatty acid synthesis.

Key words chalcomoracin; moracin C; enoyl-acyl carrier protein reductase; *Staphylococcus aureus*; FabI; fatty acid synthesis

Morus alba L. (Moraceae), known as white mulberry, has been widely cultivated in many Asian countries where the leaves were used as food for silkworms. The leaves of the plant has been traditionally used to treat diabetic hyperglycemia.[1] Also, they have hypotensive, diuretic, bacteriostatic and antivirotic properties and have been applied widely in clinic, which has important values to gerontal diseases and delayed consenescence.[2] However, potent antibacterial activity of the leaves of *Morus alba* L. and its components, chalcomoracin and moracin C, have been little known even though they are derived from the long-time edible plant and furthermore their mode of actions have been unknown.[3]

Bacterial fatty acid synthesis (FAS) is an attractive antibacterial target, since FAS is organized differently in bacteria and mammals.[4,5] Fatty acid biosynthesis in bacteria is crucial for the production of a number of lipid-containing components, including the cell membrane, which is essential for growth of bacteria. The bacterial fatty acid system (FAS II) employs discrete monofunctional enzymes which operate in conjunction with acyl carrier protein (ACP)-associated substrates, whereas mammalian fatty acid synthase (FAS I) is mediated by a single multifunctional enzyme-ACP complex. The differences in prokaryote and eukaryote fatty acid biosynthesis provide an attractive opportunity for selective FAS II inhibition, which is a potential strategy for the development of antibacterial agents. Bacterial enoyl-ACP reductase catalyzes the final and rate-limiting step in type II FAS, which is essential for growth of bacteria. In addition, it has been validated as a novel target for the development of antibacterial drugs.[6,7] Four isoforms, FabI, FabK, FabL, and FabV have been detected in enoyl-ACP reductase. FabI is distributed broadly throughout the majority of bacteria including *Staphylococcus aureus*. Indeed, FabI has been identified as the antibacterial target of both triclosan,[8] a broad spectrum biocide used in a wide range of consumer goods, and isoniazid,[9] which has been utilized for 50 years in the treatment of tuberculosis. Therefore, inhibitors of *S. aureus* FabI may prove to be interesting lead compounds for the development of effective antibacterial drugs. Several synthetic[10–14] and natural[15–18] FabI inhibitors have been reported, but since some compounds showed toxicity and their structures are not so diverse for further study, Fab I inhibitors with new scaffold and low toxicity are desired.

Since *S. aureus* is the most common pathogen and especially multidrugs-resistant *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is becoming increasingly prevalent in community-acquired infections,[19] new antibacterial with new mode of action like *S. aureus* FabI inhibitors is urgently needed. In the course of the program for the screening of *S. aureus* FabI inhibitors from plant resources, we found that the methanol extract of normal edible leaves of *M. alba* significantly inhibited *S. aureus* FabI as well as growth of *S. aureus*. Bioassay-guided fractionation of the extract led to the isolation of chalcomoracin[20] (**1**) and moracin C[21] (**2**) (Fig. 1). Moracin C has been found from both UV-treated leaves and fungus-infected shoots of *M. alba*.[21,22] In this study, antibacterial activity of the leaves of *M. alba* and isolation of moracin C from the normal plant were reported for the first time. Importantly, we reported that its antibacterial components **1** and **2** inhibited *S. aureus* FabI and intracellular fatty acid synthesis in *S. aureus*.

MATERIALS AND METHODS

General Experimental Methods NMR spectra were recorded on a Bruker 300 and 500 spectrometer (Korea Basic Science Institute). The electrospray ionization (ESI)-MS data were recorded with a Jeol JMS-HX110/110A mass spectrometer. Column chromatography on silica gel (Kieselgel 60, 70–230 mesh, Merck) and thin-layer chromatography on pre-coated 60 F_{254} silica gels (0.25 mm, Merck) were conducted. All chemicals utilized in the study, including methanol (MeOH), ethyl acetate (EtOAc), chloroform ($CHCl_3$), butanol (BuOH), acetonitrile (ACN), and hexane, were of analytical grade. Norfloxacin, triclosan, and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, U.S.A.).

Materials The dried *M. alba* L. leaves (620 g) were purchased at KyungDong market, Seoul, Korea. A voucher specimen (PBC-210A) has been deposited in the Plant

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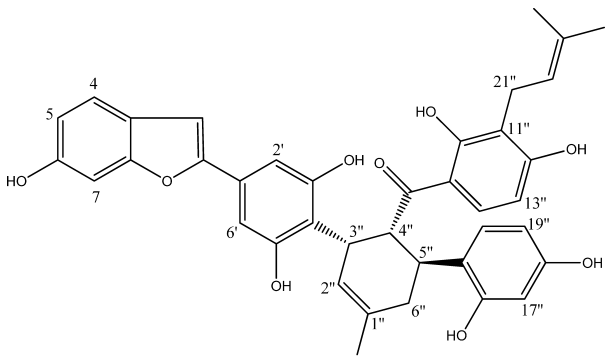

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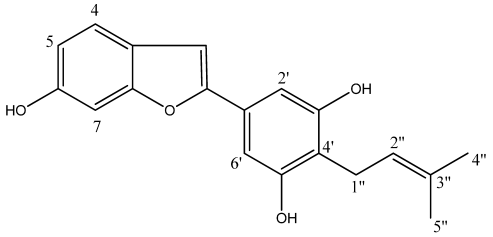

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Fig. 1. The Structures of Chalcomoracin (**1**) and Moracin C (**2**)

Extract Bank, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea.

Extraction and Isolation The powdered sample (620 g) of *M. alba* L. leaves were extracted three times with 6 L of methanol at room temperature. The methanol solution was concentrated *in vacuo*, and the concentrated extracts (77 g) were sequentially partitioned into hexane, EtOAc, BuOH, and water-soluble fractions for FabI assay. The EtOAc fraction (1.7 g) with FabI-inhibition activity was subjected to SiO_2 (Merck Art No. 7734.9025) column chromatography followed by stepwise elution with $CHCl_3$–MeOH (50 : 1, 24 : 1, 8 : 1, 4 : 1, 2 : 1, 1 : 1, 1 : 2), affording seventy-one fractions (Fr. 1–Fr. 71). Two groups of fraction, Fraction I (Fr. 30–34) eluted with $CHCl_3$–MeOH (8 : 1–4 : 1) and Fraction II (Fr. 15–18) eluted with $CHCl_3$–MeOH (24 : 1), exhibited FabI-inhibitory activity. Fraction I were pooled and concentrated *in vacuo*. The residue (64 mg) was applied to thin layer chromatography (TLC) on pre-coated silica gel 60 F_{254} plates (Merck No. 1.05744.0001, Darmstadt, Germany) with chloroform–methanol (8 : 1) to yield fifteen bands (B1–B15) and the FabI inhibitory activity of each was evaluated. The active band (B12, 20 mg) was further purified by TLC on silica gel 60 RP-18 F_{254} plates (Merck No. 1.15389.0001, Darmstadt, Germany) with ACN–water (65 : 35) containing 0.1% TFA to yield **1** (2.3 mg). Also Fraction II (95 mg) were further purified by TLC on pre-coated silica gel 60 F_{254} plates with chloroform–methanol (8 : 1) to yield **2** (10 mg). The structures of **1** and **2** were determined by 1D- and 2D-NMR spectral data. NMR spectra were recorded on a Bruker Biospin Avance 500 and 300 spectrometer (Korea Basic Science Institute).

Compound **1**: $C_{39}H_{36}O_9$, a brown amorphous powder. ^{1}H-NMR (acetone-d_6, 500 MHz) δ: 1.57 (3H, s, H-24″), 1.71 (3H, s, H-25″), 1.94 (3H, s, H-7″), 2.18 (1H, brd, J=17.5 Hz, H-6″), 2.49 (1H, brd, J=17.5 Hz, H-6″), 3.26 (2H, brd, J=7 Hz, H-21″), 3.75 (1H, m, H-5″), 4.10 (1H, brs, H-3″), 4.66 (1H, dd, J=4.0, 4.5 Hz, H-4″), 5.17 (1H, t, J=7 Hz, H-22″), 5.78 (1H, brs, H-2″), 6.30 (1H, dd, J=2.0, 8.5 Hz, H-19″), 6.51 (1H, d, J=9.0 Hz, H-13″), 6.53 (1H, d, J=2.0 Hz, H-17″), 6.77 (2H, s, H-2′,6′), 6.77 (1H, dd, J=2.0, 8.5 Hz, H-5), 6.92 (1H, brs, H-3), 6.93 (1H, d, J=2.0 Hz, H-7), 6.98 (1H, d, J=8.5 Hz, H-20″), 7.35 (1H, d, J=8.5 Hz, H-4), 8.46 (1H, d, J=9.0 Hz, H-14″), ^{13}C-NMR (CD_3OD, 500 MHz) δ: 156.2 (C-2), 101.8 (C-3), 123.0 (C-3a), 121.8 (C-4), 113.0 (C-5), 157.3 (C-6), 98.3 (C-7), 156.9 (C-7a), 133.0 (C-1′), 104.8 (C-2′), 158.9 (C-3′), 117.0 (C-4′), 158.9 (C-5′), 104.8 (C-6′), 133.8 (C-1″), 124.8 (C-2″), 41.9 (C-3″), 48.8 (C-4″), 37.0 (C-5″), 32.8 (C-6″), 23.7 (C-7″), 210.0 (C-8″), 113.5 (C-9″), 164.4 (C-10″), 116.0 (C-11″), 161.9 (C-12″), 108.0 (C-13″), 132.4 (C-14″), 122.5 (C-15″), 155.9 (C-16″), 103.4 (C-17″), 157.3 (C-18″), 107.2 (C-19″), 128.9 (C-20″), 22.3 (C-21″), 123.5 (C-22″), 131.6 (C-23″), 25.8 (C-24″), 17.7 (C-25″), ESI-MS: 671.9 $[M+Na]^+$, 647.8 $[M-H]^-$.

Compound **2**: $C_{19}H_{18}O_4$, a brown amorphous powder. ^{1}H-NMR (CD_3OD, 300 MHz) δ: 1.66 (3H, s, H-4″), 1.78 (3H, s, H-5″), 3.32 (2H, brd, J=6.9 Hz, H-1″), 5.26 (1H, brt, J=6.9 Hz, H-2″), 6.72 (1H, dd, J=2.1, 8.4 Hz, H-5), 6.78 (2H, s, H-2′,6′), 6.82 (1H, d, J=0.9 Hz, H-3), 6.88 (1H, brd, J=2.1 Hz, H-7), 7.32 (1H, d, J=8.4 Hz, H-4), ^{13}C-NMR (CD_3OD, 500 MHz) δ: 156.6 (C-2), 101.3 (C-3), 123.4 (C-3a), 121.9 (C-4), 113.2 (C-5), 157.3 (C-6), 98.6 (C-7), 156.8 (C-7a), 130.4 (C-1′), 103.9 (C-2′), 157.7 (C-3′), 117.0 (C-4′), 157.7 (C-5′), 103.9 (C-6′), 23.4 (C-1″), 124.5 (C-2″), 131.5 (C-3″), 26.2 (C-4″), 18.0 (C-5″), ESI-MS: 311.3 $[M+H]^+$, 309.3 $[M-H]^-$.

Assay of FabI *S. aureus* FabI enzyme was cloned, overexpressed, and purified as previously described.[23] Assays were conducted in half-area, 96-well microtiter plates. The compounds were evaluated in 100 μL assay mixtures containing components specific to each enzyme (see below). The reduction of the *trans*-2-octenoyl *N*-acetylcysteamine (t-o-NAC thioester) substrate analog was spectrophotometrically measured by following the utilization of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm at 30°C over 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 using a microtiter enzyme-linked immunosorbent assay (ELISA) reader using SOFTmax PRO software (Molecular Devices, California, U.S.A.). The inhibitory activity was calculated *via* the following formula: % of inhibition=100×[1−(rate in the presence of compound/rate in the untreated control)]. The 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 controls.

Determination of Antibacterial Susceptibility Antibacterial activity of the compounds against *S. aureus* RN4220[24] and MRSA CCARM 3167 was determined *via* broth microdilution, as previously described.[15] MRSA CCARM3167 was obtained from the Culture Collection of Antimicrobial Resistant Microbes of Korea. The test strains were grown to mid-log phase in Mueller–Hinton broth and diluted 1000-fold in the same medium. Cells (10^5/mL) were inoculated into Mueller–Hinton broth, then dispensed at 0.2 mL/well in 96-well microtiter plates. Minimum inhibitory concentrations (MICs) were determined in triplicate *via* the

serial dilution of test compounds. The MIC was defined as the concentration of a test compound required to completely inhibit cell growth at 30°C. Bacterial growth was determined by measuring the absorption at 650 nm with a microtiter ELISA reader.

Measurement of Inhibition of Macromolecular Biosynthesis The effect of **1** and **2** on the incorporation of [1-^{14}C]acetate and L-[U-^{14}C]leucine in *S. aureus* was measured as previously described.[15] *S. aureus* was grown to mid-log phase in LB medium. Each 1 mL culture was treated for 10 min with drugs. An equal volume of dimethyl sulfoxide (DMSO) solvent was added to the untreated controls. For [1-^{14}C]acetate incorporation, 2 μCi of [1-^{14}C]acetate was subsequently added to the cultures and incubated for 1 h at 37°C in a shaker. After being harvested by centrifugation, the cell pellets were washed twice in phosphate buffered saline (PBS) buffer. The total cellular lipids were then extracted with chloroform–methanol–water. The incorporated radioactivity in the chloroform phase was measured *via* 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 for 1 h at 37°C in a shaker. The incorporation was terminated *via* the addition of 10% (w/v) trichloroacetic acid (TCA) and 20 min of cooling on ice. The precipitated material was collected on Whatman GF/C glass microfiber filters, washed with TCA and ethanol, dried, and counted with a scintillation counter. Total counts incorporated at 1 h of incubation without inhibitors ranged from >6000 for [^{14}C]leucine to >10000 for [^{14}C]acetate.

RESULTS AND DISCUSSION

Our continued screening of plant extracts for FabI inhibitors using a combination of whole-cell and enzyme assays resulted in the identification of the normal leaves of *Morus alba*. Compounds **1** and **2** were isolated from the methanol extract of the normal leaves of *Morus alba via* activity-guided fractionation using EtOAc extraction, SiO_2 column chromatography, and preparative SiO_2 thin layer chromatography from the extract of the *Morus alba* leaves. The ^{1}H- and ^{13}C-NMR data of **1** were similar with those reported for Diels–Alder type adducts, sorocenol H, mongolicin F, and chalocmoracin. The presence of the methylcyclohexane ring of chalcomoracin was determined by the chemical shifts (δ) and coupling constants (Hz) of protons in acetone-d_6; δ 1.94 (3H, s, H-7″), 4.10 (1H, brs, H-3″), 4.66 (1H, t, J=4.5 Hz, H-4″), 3.75 (1H, m, H-5″), 2.49 (1H, brd, J=17.5 Hz, H-6″), and 5.78 (1H, brs, H-2″). The ^{1}H- and ^{13}C-NMR data of **2** revealed structural features of 2-phenylbenzofuran. The spectrum revealed the presence of 3-methyl-2-butenyl (prenyl) group at δ 1.66 (3H, s, H-4″), 1.78 (3H, s, H-5″), 3.32 (2H, brd, J=6.9 Hz, H-1″), and 5.26 (1H, brt, J=6.9 Hz, H-2″). Disposition of the two hydroxyl and prenyl groups was deduced from the two proton singlet at 6.78 (2H, s, H-2′,6′). The above spectral data were compared with those in the literature,[10,11] and compounds **1** and **2** were identified as chalcomoracin and moracin C, respectively.

Compound **1** potently inhibited *S. aureus* FabI in a dose-dependent fashion with an IC_{50} of 5.5 μM, which was fifteen times higher activity than that (83.8 μM) of **2** (Fig. 2). In order to determine whether **1** and **2** inhibit cell growth, their antibacterial activity against the Gram-positive pathogen *S. aureus* including MRSA was evaluated. Compound **1** exhibited potent antibacterial activity against MRSA CCARM3167 with a MIC of 2 μg/mL, which was sixteen times higher activity than that (32 μg/mL) of **2** (Fig. 3). Compounds **1** and **2** also exhibited potent antibacterial activity against *S. aureus* RN4220 with MIC of 4 and 32 μg/mL, respectively (Table 1). This data

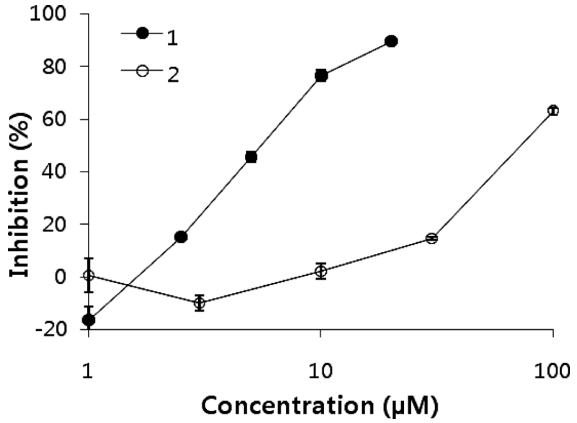

Fig. 2. Inhibitory Activity of **1** and **2** against *S. aureus* FabI

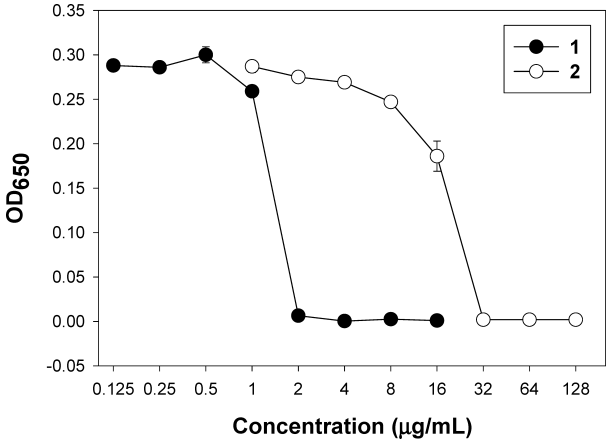

Fig. 3. Antibacterial Activity of **1** and **2** against MRSA CCARM3167

Antibacterial activity was carried out as described in Materials and Methods. The values were represented as the mean±S.D. in triplicates obtained from two independent experiments.

Table 1. Inhibitory Activity of Compounds **1** and **2** against Enoyl-ACP Reductases, Bacterial Viability and Fatty Acid Biosynthesis and Protein Biosynthesis in *S. aureus*

	IC_{50} (μM)	MIC (μg/mL)		IC_{50} (μM)	
	saFabI[a]	*S. aureus*[b]	MRSA[c]	[^{14}C]Acetate	[^{14}C]Leucine
1	5.5	4	2	24.8	157.1
2	83.8	32	32	74.8	188.4

a) *S. aureus* FabI; *b*) *S. aureus* RN4220, *c*) MRSA CCARM3167.

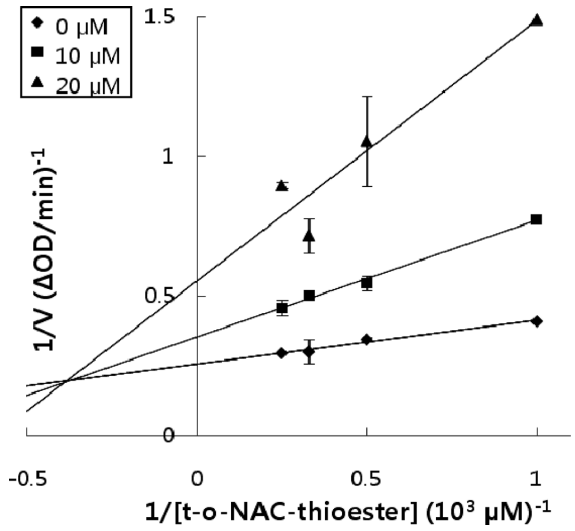

Fig. 4. The Mechanism of Inhibition of *S. aureus* FabI by **1** Respective to t-o-NAC Thioester

The reciprocals of the initial reaction and substrate concentrations are plotted. The values were expressed as the means±S.D. from triplicate repetitions of two independent experiments.

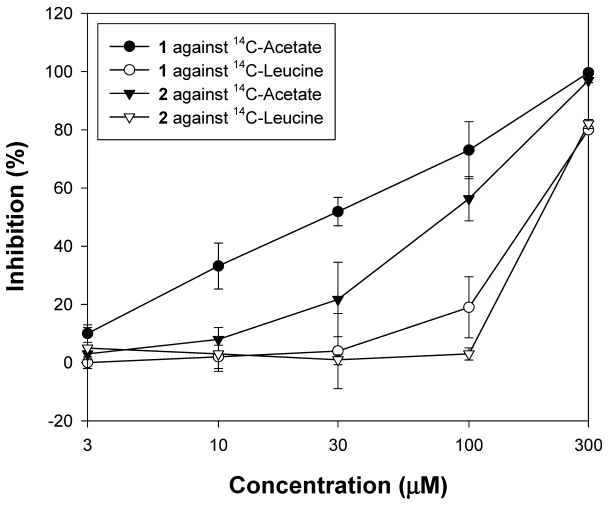

Fig. 5. Effects of **1** and **2** on Fatty Acid Biosynthesis and Protein Biosynthesis in *S. aureus*

Fatty acid biosynthesis and protein biosynthesis assays were carried out as described in Materials and Methods. The values were represented as the mean±S.D. in duplicates obtained from two independent experiments.

reflected a strong correlation between FabI-inhibitory and antibacterial activity of **1** and **2**.

The FabI reaction has a compulsory ordered mechanism with the nucleotide cofactors, NADH or NADPH, as the first substrates.[25] Compound **1** 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 non-competitive; and in the third case, a mixed-type inhibition would be noted. In the Lineweaver–Burk plot analysis (Fig. 4), compound **1** evidenced a mixed-type inhibition against *S. aureus* FabI with respect to the substrate, t-o-NAC-thioester, with a K_i of 2.9 μM. Compound **1** also showed a mixed-type inhibition with NADPH with a K_i of 0.73 μM. This indicates that compound **1** binds to both the free enzyme and the FabI-NADPH complex.

In order to determine whether the antibacterial effect of **1** and **2** are attributable to the inhibition of fatty acid synthesis, we attempted to ascertain whether the compound blocked the incorporation of acetate into membrane fatty acids *in vivo*. We measured the effect of the compound on the incorporation of [1-^{14}C]acetate into the membrane fatty acids in *S. aureus* (Fig. 5). Compounds **1** and **2** did, indeed, inhibit fatty acid synthesis *in vivo* as compared to the untreated cells, with IC_{50} of 24.8 and 74.8 μM, respectively (Table 1). Triclosan, used as a positive control, inhibited acetate incorporation (data not shown). In contrast, the incorporation of leucine into proteins was not influenced by **1** and **2** at 100 μM. Even though compounds **1** and **2** appear to inhibit protein biosynthesis at over 100 μM, the compounds clearly evidenced high specificity for inhibition of fatty acid synthesis.

Several synthetic FabI inhibitors, including 1,4-disubstituted imidazoles,[10] aminopyridines,[11] naphthyridinones,[12,13] and thiopyridines[14] have been reported in previous studies. Cephalochromin,[15] vinaxanthone,[16] epigallocatechin gallate (EGCG),[17] and flavonoids[18] have all been identified as natural FabI inhibitors. Cephalochromin has a naphtho-γ-pyrone skeleton, and vinaxanthone incorporate a chromanyl xanthene skeleton. Thus, chalcomoracin and moracin C having a prenylphenyl benzofuran skeleton are structurally different from the previously reported FabI inhibitors. In addition, chalcomoracin showed the different inhibition of pattern with a mixed-type inhibition against *S. aureus* FabI with respect to the substrate, t-o-NAC-thioester, from cephalochromin and vinaxanthone with a competitive inhibition.

Since chalcomoracin and moracin C are derived from *Morus alba* leaves, which were used as food for silkworms and has been traditionally used to treat diabetic hyperglycemia, they may have potential for the development of a new broad-spectrum antibacterial, as well as an anti-MRSA agent, with very low toxicity.

In conclusion, chalcomoracin strongly inhibited *S. aureus* FabI and also exhibited potent antibacterial activity against *S. aureus* including MRSA. Also, moracin C moderately inhibited *S. aureus* FabI and exhibited antibacterial activity against *S. aureus*. In this study, we reported the inhibitory effects of chalcomoracin and moracin C, antibacterial principles of the leaves of *Morus alba*, on both enoyl-ACP reductase and fatty acid synthesis. These compounds may provide a useful chemical scaffold for the development of a novel broad-spectrum antibacterial, which also functions as an anti-MRSA agent.

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