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Atromentin and Leucomelone, the First Inhibitors Specific to Enoyl-ACP Reductase (FabK) of *Streptococcus pneumoniae*

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Abstract Two potent inhibitors of FabK, the enoyl-acyl carrier protein (ACP) reductase of *Streptococcus pneumoniae*, were isolated from the solid state fermentation of an unidentified fungus F010248. Their structures were identified to be atromentin and leucomelone by various spectral analysis. Atromentin and leucomelone inhibited the FabK with IC_{50} values of 0.24 and 1.57 μM, respectively, while did not inhibit FabI, the enoyl-ACP reductase of either *Escherichia coli* or *Staphylococcus aureus*, even at 200 μM. Atromentin and leucomelone are the first inhibitors specific to the enoyl-ACP reductase (FabK) of *Streptococcus pneumoniae*.

Keywords atromentin, leucomelone, terphenyl, enoyl-ACP reductase, FabK, *Streptococcus pneumoniae*

Fatty acid biosynthesis in bacteria is essential to the production of a number of lipid-containing components including the cell membrane. The bacterial fatty acid system (FAS II) utilizes discrete monofunctional enzymes that operate in conjunction with acyl carrier protein (ACP)-associated substrates. Mammalian fatty acid synthase (FAS I) differs from FAS II in that lipid biosynthesis is mediated by a single multifunctional enzyme-ACP complex. The differences in prokaryote and eukaryote fatty acid biosynthesis offer an attractive opportunity for selective FASII inhibition which is a potential strategy for the development of antibacterial agents [1~3]. Bacterial enoyl-ACP reductase that catalyzes the final and rate-limiting step in the type II FAS has been validated as a novel target for antibacterial drug development. Indeed, the antibacterial target of triclosan [4], the broad spectrum biocide in a wide range of consumer goods, and isoniazid [5], used in the treatment of tuberculosis for 50 years, have been determined to be the FabI. There are three isoforms, FabI, FabK, and FabL, in enoyl-ACP reductase. FabI is widely distributed in most of bacteria but, FabK is present in few important pathogens such as *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Clostridium acetobutylicum* and *Pseudomonas aeruginosa*. *E. faecalis* and *P. aeruginosa* were known to contain FabI as well as FabK. Therefore, FabK inhibitors will be promising as an antibacterial drug specific to the important respiratory pathogen *S. pneumonia* and required for enoyl reductase therapy to be effective on *E. faecalis* and *P. aeruginosa* [6~8]. Although there have been a number of reports on the designing and development of FabI inhibitors [9~11], little has been studied for FabK inhibitors. In the course of the screening program for FabK inhibitors from microbial resources, two terphenyl type compounds were isolated from the solid state fermentation of an identified fungus F010248. They are identified as atromentin (**1**) and leucomelone (**2**) by spectral analysis. We report here the isolation and FabK-specific inhibitory activity of **1** and **2** (Fig. 1).

The fungal stain F010248 was isolated from a soil sample that was collected in a corn field around Kongju-city, Chungchongnam-do, Korea. Fermentation was carried out in a liquid culture medium containing 2% glucose, 0.2% yeast extract, 0.5% peptone, 0.05% $MgSO_4$, and 0.1% KH_2PO_4 (pH 5.7 before sterilization). A piece of the strain F010248 from a mature plate culture was inoculated into a 500-ml Erlenmeyer flask containing 80 ml of the above sterile seed liquid medium and cultured on a rotary shaker

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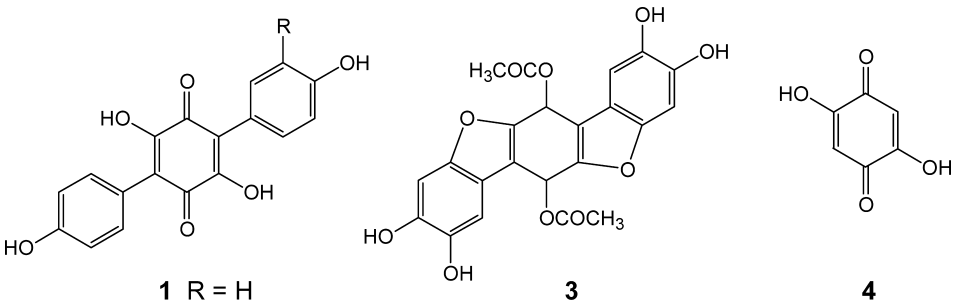

Fig. 1 The structures of atromentin (**1**), leucomelone (**2**) and related compounds.

(150 rpm) at 28°C for 3 days. For the production of active compounds, 5 ml of the seed culture were transferred into 500 ml Erlenmeyer flasks (54 flasks) containing 80 g of the bran medium, and cultivated for 7 days at 28°C. The solid state culture was extracted with acetone, and the extract was concentrated *in vacuo* to an aqueous solution. The aqueous solution was adjusted to the pH 3, which was then extracted three times with an equal volume of EtOAc. The EtOAc extract was concentrated *in vacuo* to dryness. The crude extract was subjected to ODS (YMC s-150 μm) column chromatography, followed by stepwise elution with MeOH : H_2O (10 : 90, 30 : 70, 50 : 50, 70 : 30, 100 : 0). The active fractions eluted with both MeOH : H_2O (30 : 70) and MeOH : H_2O (50 : 50) were pooled and concentrated *in vacuo*. The residue was applied again to Sephadex LH-20 column and then eluted with MeOH. The active fractions were pooled and concentrated *in vacuo*. The residue dissolved in MeOH was further purified by HPLC RP 18 reverse phase HPLC column (20×250 mm, YMC C_{18}) chromatography with a photodiode array detector. The active substances or inhibitors eluted with MeOH : H_2O (50 : 50) containing 0.01% trifluoroacetic acid at a flow rate of 3 ml/minute to afford **1** (9.7 mg) and **2** (11.4 mg) at retention times of 26.8 and 18.2 minutes, respectively, as black powders. The structures of **1** and **2** were identified to be atromentin [12, 13] and leucomelone [14], respectively, by comparing their spectroscopic data such as UV, mass, ^{1}H-NMR and ^{13}C-NMR data with the values previously reported.

FabK assays were carried out in 96-well microtitre plates by modifying the FabI assay as previously reported [3]. Compounds were evaluated in 100 μl assay mixtures containing components specific for each enzyme (see below). Reduction of the *trans*-2-octenoyl-*N*-acetylcysteamine thioester (t-o-NAC) substrate analog was measured spectrophotometrically by following the UV adsorption of NADH at 340 nm at 30°C for the linear period of the assay. FabK assays contained 100 mM sodium acetate, pH 6.5, 2.0% glycerol, 200 mM NH_4Cl, 50 μM t-o-NAC, 200 μM NADH, and 150 nM *S. pneumoniae* FabK. The rate of decrease in the amount of NADH in each reaction well was measured by a microtiter ELISA reader using SOFTmax PRO software (Molecular Devices, California, USA). 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. For FabI assays, *S. aureus* FabI assay contained 50 mM sodium acetate, pH 6.5, 400 μM t-o-NAC, 200 μM NADPH, and 150 nM *Staphylococcus aureus* FabI. The *Escherichia coli* FabI assay contained 50 mM sodium phosphate, pH 7.5, 200 μM t-o-NAC, 200 μM NADH and 150 nM *E. coli* FabI. The inhibitory activity was determined in the same methods as for *S. pneumoniae* FabK, as described above.

As shown in Fig. 2, **1** and **2** showed inhibitory activity on *S. pneumoniae* FabK in a dose-dependant fashion with IC_{50} values of 0.24 and 1.57 μM, respectively, while their structurally-related compounds, polyozellin (**3**) we previously isolated [15] and commercially available 2,5-dihydroxy-1,4-benzoquinone (**4**), did not inhibit the FabK even at 100 μM. It suggests that both the 2,5-dihydroxy-1,4-benzoquinone and the phenol moieties could be important for their activity. To elucidate the mechanism of inhibition by **2** on the FabK, a lineweaver-burk plot analysis was conducted (Fig. 3). **2** inhibited *S. pneumoniae* FabK in a mixed manner with the substrate t-o-NAC-thioester. The K_i and K_m values for *S. pneumoniae* FabK were 4.1×10^{-7} M and 1.9×10^{-4} M, respectively.

To evaluate whether **1** and **2** are specific to FabK, their inhibitory activities on either *E. coli* or *S. aureus* FabI was tested (Table 1). **1** and **2**, however, did not show any inhibitory activity on *E. coli* or *S. aureus* FabI even at 100 μM while triclosan, a well-known inhibitor of FabI, as a positive control strongly inhibited on *E. coli* or *S. aureus*

FabI with IC_{50} (μM) values of 0.44 and 1.39, respectively. This indicates that **1** and **2** have a specific inhibitory effect on the FabK isoform of enoyl-ACP reductase.

Atromentin and leucomelone did not show antibacterial activity up to 100 μg/ml against *S. pneumoniae*, *P. aeruginosa* and *E. faecalis*. Some polyphenol compounds have been reported to be pumped out in bacteria [16]. For example, phenolic compounds such as rhein, gossypol, or resveratrol have been reported to be extruded by a multidrug resistance (MDR) pump in *P. aeruginosa*, *E. coli*, or *Salmonella enterica*, since their antibacterial activity was uncovered by the treatment with MDR inhibitors, MC_{207110} or INF_{271}, or in an MDR mutant. Also, some cathechin gallates or flavanone-3-ols such as fustin and taxifolin were reported not to exhibit antibacterial activity against *E. coli* while other flavonols such as fisetin and quercetin showed antibacterial activity, although all of them inhibited *E. coli* FabI [11]. AG205, a potent FabK-inhibitory, has been

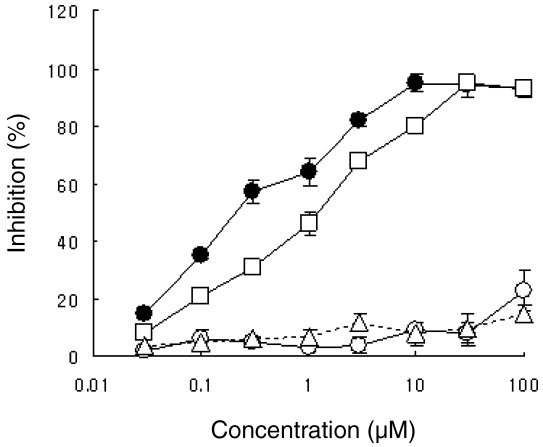

Fig. 2 Inhibitory effects of compounds **1**~**4** on *Streptococcus pneumoniae* FabK.

The values were represented as the mean±S.D. of experiments performed in triplicate. ●, **1**; □, **2**; ○, **3**; △, **4**.

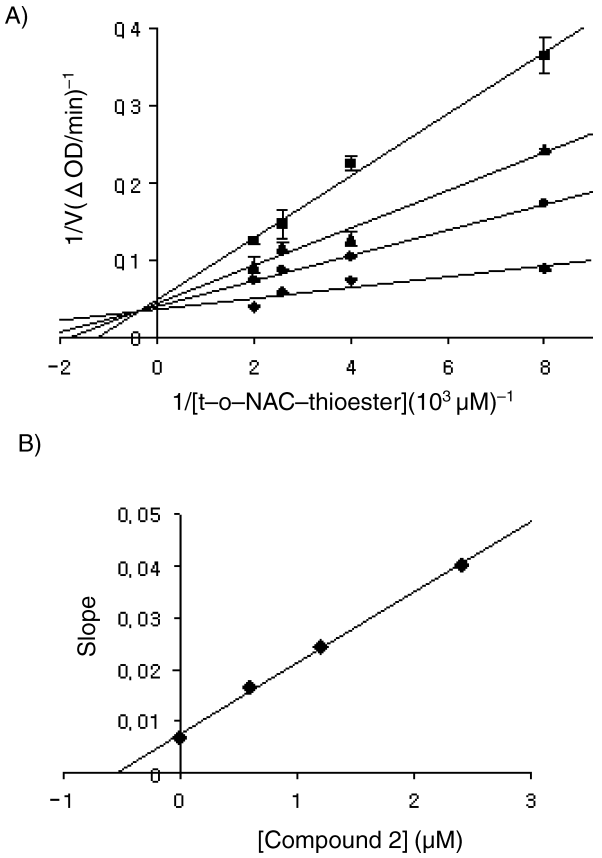

Fig. 3 The mechanism of FabK inhibition by compound **2** respective to t-o-NAC thioester (A), and K_i determination of compound **2** (B).

(A) The reciprocals of the initial reaction and substrate concentration are plotted. The values were represented as the mean±S.D. of experiments performed in triplicate. (B) The slope values of the lines from graph A are plotted versus the inhibitor concentration, affording a line obtained by linear regression. The intercept point of this line with the *x*-axis gives an approximate K_i value of 0.41 μM for compound **2**. ◆, 0 μM; ●, 0.6 μM; ▲, 1.2 μM; ■, 2.4 μM.

Table 1 Inhibitory activity of **1**, **2** and related compounds on *Streptococcus pneumoniae* FabK, *Escherichia coli* FabI, and *Staphylococcus aureus* FabI

Compounds	IC_{50} (μM)		
	FabK (*S. pneumoniae*)	FabI (*E. coli*)	FabI (*S. aureus*)
1	0.24	>200	>200
2	1.57	>200	>200
3	>200	>200	>200
4	>200	>200	>200
Triclosan	>200	0.44	1.39

reported to show reduced activity against some *S. pneumoniae* isolates, which was assumed to be due to an efflux pump [17]. Taken together with these recent studies, our results suggest that the loss of antibacterial effect of atromentin and leucomelone on *S. pneumoniae* is presumably due to the efflux effect of cell wall.

Atromentin which belong to the mushroom pigments and *p*-terphenyl compounds was first described in 1878 by Thörner [18] and reviewed in 2006 by Liu [19]. The structure of atromentin isolated from a lignicolous mushroom *Paxillus panuoides* and other mushrooms has been spectroscopically elucidated [12, 13] and chemically synthesized [20]. Atromentin has been reported to have anticoagulant activity [21]. Leucomelone was first reported as a pigment component of the edible black mushroom *Polyporus leucomelas* [22] and has been chemically synthesized [14]. However, no biological activity of leucomelone has been reported. The production of atromentin and leucomelone by a soil derived filamentous fungus is reported for the first time in this study.

Few compounds such as indole naphthyridinones, AG205, and AE 848 have been reported to inhibit *S. pneumoniae* FabK [17, 23]. The indole naphthyridinone-based compound, however, was a non-selective inhibitor since it strongly inhibited the FabI of *S. aureus* and *E. coli* as well. Inhibitory effects of AG205 and AE 848 against FabI have been not reported yet. As far as we know, atromentin and leucomelone are the first FabK-specific inhibitors.

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