Vinaxanthone, a new FabI inhibitor from *Penicillium* sp.

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**Objectives:** Bacterial enoyl-ACP reductase (FabI) has been validated as a novel antibacterial target for tackling infections caused by multidrug-resistant pathogens. A few FabI inhibitors, however, are known. This study isolated a new FabI inhibitor from *Penicillium* sp.

**Methods:** A screening programme led to the selection of a *Penicillium* sp. producing a strong FabI-inhibitory metabolite. The chemical structure of the isolated FabI inhibitor was elucidated by mass spectrometry and nuclear magnetic resonance spectral data. The antibacterial target of the inhibitor was validated by overexpression assays.

**Results:** The isolated FabI inhibitor was elucidated to be vinaxanthone. It selectively inhibited *Staphylococcus aureus* FabI with an IC₅₀ of 0.9 μM; it did not affect FabK, an enoyl-ACP reductase of *Streptococcus pneumoniae*. Consistent with its inhibition of FabI, the inhibitor prevented intracellular fatty acid synthesis while it did not affect protein biosynthesis. It also prevented the growth of *S. aureus* as well as methicillin-resistant *S. aureus* (MRSA) and quinolone-resistant *S. aureus*. Importantly, fabI-overexpressing *S. aureus* showed reduced susceptibility to the inhibitor compared with the wild-type strain, demonstrating that its antibacterial action is mediated by inhibition of FabI.

**Conclusions:** Vinaxanthone is a new FabI-directed antibacterial of natural origin that could have potential for further development as a new anti-MRSA agent.

**Keywords:** enoyl-ACP reductase, antibacterial, fatty acid synthesis, target validation, *Staphylococcus aureus*

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**Introduction**

The appearance of antibiotic-resistant bacteria has steadily increased to become a serious health problem. With the emergence of vancomycin-resistant *Staphylococcus aureus* in 2002, there is an urgent need for new antibiotics.¹,² One approach to combat antibiotic resistance is to identify new drugs that function through novel mechanisms of action.³ Among genomics-driven targets for antibacterial drug discovery, bacterial fatty acid synthesis (FAS) is an attractive antibacterial target since FAS is organized differently in bacteria and mammals.⁴–⁶ Enoyl-ACP reductase (ENR) catalyses the final and rate-limiting step of the chain elongation process in bacterial FAS. There are three isoforms, FabI, FabK and FabL, of ENR. FabI is highly conserved among most bacteria, while *Streptococcus pneumoniae* contain only FabK, *Enterococcus faecalis* and *Pseudomonas aeruginosa* contain both FabI and FabK and *Bacillus subtilis* contain both FabI and FabL. FabI has been validated as an excellent target for antibacterial drug development because it has been proven as a primary antibacterial target of the broad-spectrum biocide, triclosan.⁷,⁸ Therefore, specific inhibitors of FabI 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, and importantly, antibacterial targets of many antibiotics have not been elucidated.⁹ However, FAS inhibitors of microbial origin have not been studied much. Only one FabI inhibitor, cephalochromin, has been reported from a fungus.¹⁰ A few inhibitors of other FAS components are known. Platensimycin with a potent antibacterial activity produced from *Streptomyces platensis* has been reported to target FabF, the elongation condensing enzyme.¹¹ Cerulenin and thiolactomycin produced from *Cephalosporium caerulescens* and *Nocardia* sp., respectively, selectively inhibit the condensation enzymes FabF/B and FabH.¹²

In the course of our continuous screening programme to find potent FabI inhibitors from microbial metabolites, we found that the fermented extract of *Penicillium* sp. F131, an isolate from Korean soil, potently inhibited FabI of *S. aureus*. Here, we report the isolation of vinaxanthone, its selective inhibition against FabI of *S. aureus* and whole cells of various pathogenic bacteria and its target validation.

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949

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Materials and methods

Bacterial strains

The bacterial strains used in antibacterial activity assays were obtained from the Culture Collection of Antimicrobial Resistant Microbes of Korea (CCARM) and the Korean Collection for Type Cultures (KCTC).

Screening and isolation of vinaxanthone

Over 18000 microbial extracts from actinomycetes and fungi were screened against S. aureus FabI and confirmed through a target-based whole cell assay using fabI-overexpressing S. aureus leading to the identification of vinaxanthone from a fungal strain, F131. Vinaxanthone was isolated from the fermented whole medium of the fungal strain F131 that was isolated from soil collected from Seolak Mountain, Kangwon-do, Korea. The strain was identified as a Penicillium species on the basis of the internal transcribed spacer sequence. The fermented whole medium (10 L) was extracted with 50% acetone and the extract was concentrated in vacuo to an aqueous solution, which was then extracted with an equal volume of ethyl acetate (EtOAc) three times. The EtOAc extract was subjected to octadecyl saline (ODS) (Merck Art No. 7734.9025) chromatography, followed by stepwise elution with ACN/H2O (10:90, 30:70, 50:50, 100:0) containing 0.01% trifluoroacetic acid (TFA). The active fractions eluted with ACN/H2O (30:70) were pooled and concentrated in vacuo. The resultant residual was applied to a Sephadex LH-20 column and then eluted with MeOH. The active fraction dissolved in MeOH was further purified by reverse-phase HPLC column (20×150 mm, YMC C18) chromatography with a photodiode array detector. The column was eluted with ACN/H2O (25:75) containing 0.01% TFA at a flow rate of 5 mL/min to afford the active compound (19.6 mg) with >99% purity at a retention time of 36 min. The compound was identified as vinaxanthone (C28H16O14, mol. wt 576 kDa) by mass spectrometry and nuclear magnetic resonance (NMR) data as follows: electrospray ionization-mass spectrometry (ESI-MS): m/z 577.5 (M+H)+; 1H-NMR [300 MHz, dimethyl sulphoxide (DMSO)-d6]: 12.75 (2H, brs, 11,9'-OH), 11.45 (2H, brs, 2,6'-OH), 9.44 (2H, brs, 3,7'-OH), 8.53 (1H, s, H-8), 8.17 (1H, s, H-2'), 6.96 (1H, s, H-8'), 6.94 (1H, s, H-4), 2.55 (3H, s, H-13) and 2.53 (3H, s, H-15); 13C-NMR (75 MHz, DMSO-d6): 201.1 (C-12), 199.2 (C-14), 172.9 (C-4'), 172.6 (C-9), 167.4 (C-11), 167.3 (C-9'), 154.2 (C-3), 152.6 (C-2'), 152.1 (C-7'), 152.1 (C-10'), 150.7 (C-8'), 150.3 (C-4), 141.7 (C-6'), 141.1 (C-2), 136.3 (C-7), 133.4 (C-5), 132.6 (C-6), 126.3 (C-8), 120.9 (C-3'), 120.5 (C-8), 119.8 (C-5'), 119.6 (C-1), 112.4 (C-4), 110.0 (C-9'), 102.3 (C-4), 102.4 (C-8'), 32.1 (C-13) and 29.1 (C-15) (Figure 1).

FabI and FabK assay

S. aureus FabI and S. pneumoniae FabK enzymes were cloned, over-expressed and purified as described previously. Assays were carried out in half-area, 96-well microtitre plates. Vinaxanthone was dissolved in DMSO. The compound was evaluated in 100 μL assay mixtures containing components specific for each enzyme (discussed subsequently). Reduction of the trans-2-octenyl N-acetylcysteaminie (t-o-NAC thioester) substrate analogue was measured spectrophotometrically by following the utilization of nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm at 30°C for the linear period of the assay. S. aureus Fab assays contained 50 mM sodium acetate, pH 6.5, 200 μM t-o-NAC thioester, 200 μM NADPH and 150 nM S. aureus

Zheng et al.

Determination of MIC

Whole-cell antimicrobial activity was determined by the broth microdilution, as described previously. The test strains were grown to the 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 and dispensed at 0.2 mL/well into a 96-well microtitre plate. MICs were determined in triplicate by serial 2-fold dilutions of test compounds. The MIC was defined as the concentration of a test compound that completely inhibits cell growth during a 24 h incubation at 30°C. Bacterial growth was determined by measuring the absorption at 650 nm using a microtitre ELISA reader.

Measurement of inhibition of macromolecular biosynthesis

The effect of FabI inhibitors on the incorporation of [1-14C]acetate and [1-14C]leucine in S. aureus was measured as described previously. S. aureus was grown to the mid-log phase in Luria-Bertani medium. Each 1 mL culture was treated with drugs for 10 min. An equal volume of DMSO solvent was added to the untreated control. FabK assays contained 100 mM sodium acetate, pH 6.5, 2% glycerol, 200 mM NADH, 50 μM t-o-NAC thioester, 200 μM NADH and 150 nM S. pneumoniae FabK.

Figure 1. Structure of vinaxanthone.
New FabI inhibitor of natural origin

Overexpression assay

The fabI overexpression assay was performed as described previously. The wild-type fabI gene from S. aureus RN4220 was amplified by PCR and cloned into vector pE194 yielding recombinant plasmid pE194-fabI. Recombinant pE194-fabI was introduced into electrocompetent S. aureus RN4220 via electroporation. 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 is indicative of FabI being the mode of antibacterial action.

Results

Isolation of vinaxanthone as a new FabI inhibitor

The structure of the FabI inhibitor that was isolated from Penicillium sp. F131 was determined to be vinaxanthone. Vinaxanthone has been isolated from Penicillium glabrum and Penicillium vinaceum, but its biological activity has not been extensively reported.

Vinaxanthone inhibited S. aureus FabI dose-dependently with an IC$_{50}$ of 0.9 µM (Figure 2). To see whether vinaxanthone selectively inhibits FabI, its effects on FabK, ENR of S. pneumoniae, were examined. Vinaxanthone had almost no inhibitory activity against S. pneumoniae FabK, with an IC$_{50}$ of over 100 µM (Figure 2).

Mode of inhibition of FabI by vinaxanthone

The pattern of inhibition of FabI by vinaxanthone with respect to the substrate and the cofactor was examined with a Lineweaver–Burk plot. The inhibition of S. aureus FabI by vinaxanthone was mixed with respect to the substrate, t-o-NAC thioester, with a $K_i$ value of 3.1 µM (Figure 3a). In contrast, vinaxanthone exhibited competitive inhibition with respect to the cofactor, NADPH, with a $K_i$ value of 1.0 µM (Figure 3b).

Inhibition of cellular FAS by vinaxanthone

To see whether vinaxanthone inhibits cellular FAS, we determined whether the compound inhibited the incorporation of acetate into membrane fatty acids in vivo. We measured the effect of the compound on the incorporation of [1-14C]acetate into the membrane fatty acids in S. aureus. In agreement with its FabI inhibitory activity, vinaxanthone indeed blocked FAS in vivo compared with the untreated cells with an IC$_{50}$ value of 4.1 µM (Figure 4). Triclosan as a positive control inhibited acetate incorporation (data not shown). In contrast, the incorporation of leucine into proteins was not affected by vinaxanthone even at 100 µM, whereas the protein synthesis inhibitor chloramphenicol as a positive control inhibited leucine incorporation (data not shown).

Antibacterial activity of vinaxanthone

Vinaxanthone exhibited antibacterial activity against Gram-positive bacteria, including S. aureus 503, S. aureus KCTC 1916, S. aureus RN4220, methicillin-resistant S. aureus (MRSA) CCARM 3167, MRSA CCARM 3506, quinolone-resistant...
S. aureus (QRSA) CCARM 3505, QRSA CCARM 3519 and B. subtilis KCTC 1021, with an MIC of 32 mg/L, and B. cereus KCTC 1661, with an MIC of 4 mg/L. Vinaxanthone did not, however, show antibacterial activity against S. pneumoniae KCTC 3932 with the FabK isoform or against E. faecalis KCTC 3511 and P. aeruginosa KCTC 2742 with the Fabf and FabK isoforms, even at 128 mg/L, as expected. These results showed that there was a good correlation between enzyme inhibitory and antibacterial activity of vinaxanthone.

Effect of vinaxanthone 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.14 The antibacterial activity of vinaxanthone on the fabI-overexpressing strain was investigated to see whether overexpression of fabI shifts the MIC of vinaxanthone for S. aureus. The MIC of vinaxanthone for the fabI-overexpressing strain, S. aureus RN4220 (pE194-fabI), was increased 4-fold compared with those for the wild-type strain, S. aureus RN4220, and the vector-containing strain, S. aureus RN4220 (pE194) (Table 1). As a positive control, the triclosan MIC for the fabI-overexpressing strain was increased. 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. Antibiotics with different modes of action, oxacillin and norfloxacin, as negative controls, did not change the MICs for the three strains, indicating that altered expression of fabI does not change the susceptibility of cells to antibiotics in general. Thus, these results clearly indicate that vinaxanthone inhibits the growth of S. aureus through inhibition of the fabI-encoded ENR.

Discussion

In the course of screening for FabI inhibitors from microbial sources, we isolated vinaxanthone from Penicillium sp. F131. Previously, vinaxanthone has been reported as an inhibitor of semaphorin,12 phospholipase C13 and CD4-MHC class II binding.16 The antibacterial activity of vinaxanthone, however, has not been reported yet.

The FabI reaction has a compulsory ordered mechanism with the nucleotide cofactors NADH or NADPH as the first substrates.17 Vinaxanthone 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, mixed-type inhibition would be observed. The inhibition of S. aureus FabI by vinaxanthone was competitive with respect to NADPH. This suggests that vinaxanthone binds to the free enzyme to prevent the binding of NADPH. Cephalochromin,10 triclosan18 and epigallocatechin gallate (EGCG)19 have also been reported to be competitive inhibitors of FabI with respect to the substrate. The inhibition pattern of known FabI inhibitors with respect to the substrate has hardly been reported. Cephalochromin was reported to be a mixed-type inhibitor of FabI for the substrate.10

Several synthetic FabI inhibitors, including 1,4-disubstituted imidazoles,20 aminopyridines,21 naphthyridinones22,23 and thiopyridines,24 have been reported. As natural FabI inhibitors, cephalochromin,10 EGCG19 and flavonoids25 are known. Structurally, vinaxanthone is different from these known FabI inhibitors. EGCG and flavonoids have been known to inhibit several targets such as FabG, FabZ and FabI.19,25 In addition, EGCG and flavonoids do not show a direct correlation of FabI inhibition with antibacterial activity.19 In contrast, vinaxanthone did not inhibit S. aureus FabG,26 the other reductase of bacterial FAS catalysing the reduction of β-hydroxyacyl-ACP to β-hydroxyacyl-ACP, even at 200 μM (data not shown). Although the possibility of vinaxanthone inhibition of FabL cannot be excluded, because the compound is equally potent against B. subtilis and S. aureus, the selectivity of vinaxanthone towards FabL compared with FabG and FabK could provide potential for further development of a new antibacterial agent.

In summary, vinaxanthone is a potent and selective FabI inhibitor of natural origin; it also shows antibacterial activity against Gram-positive multidrug-resistant bacteria such as MRSA and QRSA. Its antibacterial effect is demonstrated to be the inhibition of FabI. Vinaxanthone may serve as a useful lead compound for tackling infections caused by multidrug-resistant pathogens.
New FabI inhibitor of natural origin

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Transparency declarations

None to declare.

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