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Flavonoid Inhibitors of β-Ketoacyl Acyl Carrier Protein Synthase III against Methicillin-Resistant *Staphylococcus aureus*

Jee-Young Lee, Juho Lee, Ki-Woong Jeong, Eunjung Lee, and Yangmee Kim*

Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea
**E-mail: ymkim@konkuk.ac.kr*
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β-Ketoacyl acyl carrier protein synthase III (KAS III) initiates fatty acid synthesis in bacteria and is a key target enzyme to overcome the antibiotic resistance problem. In our previous study, we found flavonoid inhibitors of *Enterococcus faecalis* KAS III and proposed three potent antimicrobial flavonoids against *Enterococcus faecalis* and Vancomycin-resistant *Enterococcus faecalis* with MIC values in the range of 128-512 μg/mL as well as high binding affinities on the order from 10^6 to 10^7 M^{-1}. Using these series of flavonoids, we conducted biological assays as well as docking study to find potent flavonoids inhibitors of *Staphylococcus aureus* KAS III with specificities against *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus*. Here, we propose that naringenin (5,7,4'-trihydroxyflavanone) and eriodictyol (5,7,3',4'-tetrahydroxyflavanone) are potent antimicrobial inhibitors of *Staphylococcus aureus* KAS III with binding affinity of 3.35×10^5 and 2.01×10^5 M^{-1}, respectively. Since Arg38 in efKAS III is replaced with Met36 in saKAS III, this key difference caused one hydrogen bond missing in saKAS III compared with efKAS III, resulting in slight discrepancy in their binding interactions as well as decrease in binding affinities. 4'-OH and 7-OH of these flavonoids participated in hydrogen bonding interactions with backbone carbonyl of Phe298 and Ser152, respectively. In particular, these flavonoids display potent antimicrobial activities against various MRSA strains in the range of 64 to 128 μM with good binding affinities.

Key Words : KAS III, Fatty acid synthesis, Methicillin-resistant *Staphylococcus aureus*, Docking, Flavonoids

Introduction

Fatty acids are a main subject of formation of cell membranes.[1,2] In bacteria, fatty acid synthase (FAS), a multifunctional enzyme complex system, regulates fatty acid synthesis and each enzymes are recognized good therapeutic target of development of antimicrobial drugs.[2] Among these enzymes, β-ketoacyl-acyl carrier protein synthase III (KAS III), which is called as a condensing enzyme, is initiated the fatty acid synthesis and a remarkable target for design of antibiotics.[3,4]

KAS III is a homodimeric form and its size of about 35-50 kDa.[5] It has highly conserved active site with a Cys-His-Asn catalytic triad in various bacteria.[6-8] The three-dimensional structure of KAS III is also highly conserved in various bacteria, and its inhibitors may act as good antibiotics with broad-spectrum activity.

Flavanone is the major flavonoid found in citrus fruits, with phytochemical properties. It is well known that flavonoids (including flavanones) have various biological activities in human diseases, such as anticancer, antioxidant, and antibacterial activity, and display low toxicity in mammals.[9-12] MRSA is a notorious resistant microbe that is related on several difficult to treat infections in humans and therefore the conquest of MRSA is important and urgent need in human life.[13] Therefore the development of natural antibiotics is good chance to overcome the resistance of known antibiotics for bacterial infection, such as MRSA.

In our previous studies, we successfully designed antimicrobial inhibitors of KAS III using various methods of *in silico* screening.[14-17] Especially, we performed a docking study of *Enterococcus faecalis* KAS III (efKAS III) and discovered three antimicrobial flavanone inhibitors of efKAS III.[15] These flavanones also showed antimicrobial activities against *Staphylococcus aureus* (*S. aureus*). Therefore, we assumed that these flavanones can be potent inhibitors of *S. aureus* KAS III (saKAS III) with good antimicrobial activities, too. In this study, we conducted docking study of saKAS III with flavonoids and determined binding affinities using fluorescence quenching analysis as well as saturation-transfer difference NMR (STD-NMR) spectroscopy. As a result, we proposed flavonoid inhibitors of saKAS III with potent antimicrobial activities against *S. aureus* and MRSAs.

Methods

Docking Study. Using the X-ray crystallography structure of saKAS III (1ZOW.pdb and 3IL7.pdb) and we defined the active site of saKAS III, based on the binding model of x-ray complex structure of efKAS III and CoA (3IL4.pdb).[18,19] The various flavonoids were docked to saKAS III using AutoDock.[20] The Lamarckian Genetic Algorithm (LGA) of the Autodock 3.05 was used for docking experiments. Distance-dependent function of the dielectric constant was used for the calculation of the energetic maps and all other parameters were used by default value. We carried out 150

and 250 independent docking processes for each complex.

Antimicrobial Activity. Minimum inhibitory concentrations (MICs) of flavonoids against bacteria were determined using a broth microdilution assay. The clinical isolates of MRSAs (CCARM 3089, CCARM 3090, CCARM 3108 and CCARM 3126) were supplied by the Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women's University in Korea and *S. aureus* [KCTC 1621] was purchased from the Korean Collection for Type Cultures, Korea Research Institute of Bioscience & Biotechnology (Taejon, Korea). Briefly, single colonies of bacteria were inoculated into LB (Luria-Bertani) and cultured overnight at 37 ºC. An aliquot of the culture was transferred to 10 mL fresh LB and incubated for an additional 3-5 h at 37 ºC until mid-logarithmic phase. Two-fold serial dilutions of peptides in 1% peptone were prepared. Diluted peptides (100 mL) were added to 100 ml of cells (2×10^6 CFU/mL) in 96-well microtiter plates and incubated at 37 ºC for 16 h. The lowest concentration of peptide that completely inhibited growth was defined as the MIC.

Expression and Purification of saKAS III. saKAS III were expressed in *E. coli* BL21 (DE3) and purified. The pET-15b/KAS III plasmid was transformed into the expression host. All expression and purification procedures were performed as described previously reports.[14-17] The gene encoding saKAS III was obtained by PCR with a set of primers (5'-gttcaactatgaacgtgggt-3' and 5'-tgttgttttgacatcattaccga-3') and subcloned into *Bam*HI-*Eco*RI sites of the pGEX-4T-1 expression vector (GE Healthcare), resulting in pGEX-saKAS III coding for a glutathione *S*-transferase (GST)-saKAS III fusion protein. The recombinant saKAS III was expressed in *E. coli* (DE3) as a GST fusion protein and purified from the supernatant of disrupted cells by GSTrap column (Amersham Pharmacia Biotech). To remove the GST moiety, the fusion protein was incubated with 1:10 (mg/unit) thrombin in PBS buffer (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) for 12 h at room temperature. Once digestion is complete, GST was removed by a GSTrap column. At each stage of the purification process, SDS-PAGE was applied to identify the saKAS III-containing fraction.

STD-NMR. The protein was saturated on-resonance at −1.0 ppm and off-resonance at 40 ppm, with a cascade of 40 selective Gaussian-shaped pulses of 50 ms duration and 100 ms delay between each pulse in all STD-NMR experiments with 298 K. The total duration of the saturation time was set to 2 s. For STD-NMR experiments, 10 mM recombinant saKAS III in 50 mM sodium phosphate buffer, 100 mM NaCl, pH 8.0, and candidate inhibitors were mixed at a protein:ligand ratio of 1:100. In total, 1024 scans for each experiment were acquired, and a WATERGATE sequence used to suppress the water signal. A spin-lock filter (5 kHz strength and 10 ms duration) was applied to suppress the protein background. All NMR spectra were recorded on Bruker Avance 500 MHz NMR spectrometers at KBSI.

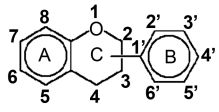
Fluorescence Quenching. We titrated each candidate inhibitor to 10 μM saKAS III protein solution in 50 mM sodium phosphate buffer containing 100 mM NaCl at pH 8.0, with a final protein-to-inhibitor ratio of 1:10. The sample was placed in a 2 mL thermostatted cuvette, with excitation and emission path lengths of 10 nm. Using tryptophan emission, we determined the fluorescence quantum yields of KAS III and the ligand. The detailed methods are provided in our previous article.[15,16]

Results and Discussion

Antimicrobial Activities of Flavonoids. MIC of seven flavonoids were measured against *S. aureus* and four MRSA strains as listed in Table 2. Among these, only five flavanones (1-5) showed antimicrobial activities in the range of 64-256 μg/mL. The reference molecule, thiolactomycin (TLM), a known antibiotic inhibitor of KAS III,[21] showed MIC of 256 μg/mL against MRSAs. Compound **4** and **5** exhibited good antimicrobial activities (64-128 μg/mL) compared with the rest compounds including TLM. Especially, **4** (naringenin) showed good MIC against MRSAs better than *S. aureus*.

To estimate the possibility of five antimicrobial flavonoids as inhibitors of saKAS III, we determined their binding affinities by fluorescence quenching experiments as well as

Table 1. Nomenclature of the flavanones based on the position of their substituents[a]



Compound	Nomenclature	Position of Substituent							
		3	**5**	**6**	**7**	**2'**	**3'**	**4'**	**5'**
1	3'-Hydroxyflavanone	H	H	H	H	H	OH	H	H
2	5,7-Dihydroxyflavanone (Pinocembrine)	H	OH	H	OH	H	H	H	H
3	6,2'-Dihydroxyflavanone	H	H	OH	H	OH	H	H	H
4	5,7,4'-Trihydroxyflavanone (Naringenin)	H	OH	H	OH	H	H	OH	H
5	5,7,3',4'-Tetrahydroxyflavanone (Eriodictyol)	H	OH	H	OH	H	OH	OH	H
6	3,7,3',4',5'-Pentahydroxyflavanone (Dihydrobinetin)	OH	H	H	OH	H	OH	OH	OH
7	3,5,7,3',4'-Pentahydroxyflavanone (Taxifolin)	OH	OH	H	OH	H	OH	OH	H

[a]Each flavanone present in a racemic mixture.

Table 2. Antimicrobial activities of 7 flavanones against *S. aureus* and MRSAs and their binding affinities (K_d) to saKAS III

Compound	MIC (μg/mL)					LigScore	PSA[a]	LogP[b]	K_d (M^{-1})
	S. aureus	MRSA 3089	MRSA 3090	MRSA 3108	MRSA 3126				
TLM	256	256	256	256	128	–	62.6	2.62	7.80×10^4
1	> 256	128	128	128	128	2.06	46.5	2.68	1.48×10^3
2	128	256	256	256	256	1.38	66.8	2.60	7.05×10^2
3	128	128	128	128	128	2.23	66.8	2.62	3.72×10^3
4	64	128	64	64	64	4.71	87.0	2.12	3.35×10^5
5	256	128	64	128	64	4.17	107.2	1.63	2.01×10^5
6	> 256	> 256	> 256	> 256	> 256	3.80	127.4	0.50	1.04×10^6
7	256	> 256	> 256	> 256	> 256	3.97	127.4	0.71	1.41×10^4

[a]Polar surface area (PSA) and [b]LogP are calculated using Molinspiration software (www.molinspiration.com).[22,23]

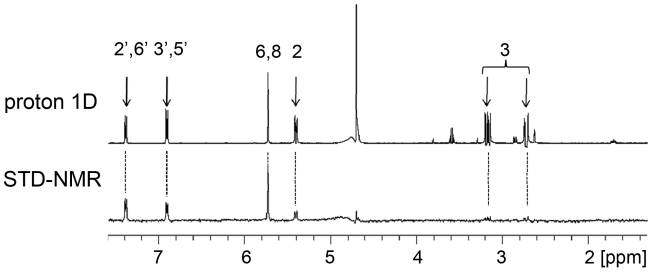


Figure 1. Representative result of the STD-NMR binding assay of compound **4** (Naringenin).

STD-NMR experiments and proposed binding models by docking study.

Binding Affinities of Flavonoids for saKAS III. The difference spectra of seven flavonoids obtained from STD-NMR experiments disclosed resonances belonging to ligand protons bound to saKAS III. Among seven compounds **4**, **5**, and **6** compounds bound to saKAS III with strong intensity. 7 also bound to saKAS III, but showed very weak signal. Our experiments confirmed that the reference molecule, TLM, interacted with the enzyme, as expected. STD-NMR spectra of compound **4** is shown in Figure 1.

Since the chemical group of ligand in strongest contact with protein displays the most intense NMR intensities, STD-NMR may support the site-specific binding information of ligand in active site of protein. In STD-NMR spectra of compound **4**, ^{1}H signals of B-ring (2', 3', 5', and 6') and A-ring (6 and 8) were preserved after saturation transfer with similar intensities, while the ^{1}H signal of C-ring (2 and 3) were almost disappeared compared with ^{1}H spectra. It may guessed that A- and B-ring of compound **4** participated in binding interaction of saKAS III with strong contact, but C-ring does not contribute to bind of compound **4**.

We determined binding constants of these seven compounds and TLM to saKAS III using fluorescence quenching experiments. Fluorescence titration curves of compounds are presented in Figure 2 and the binding constants of compounds are listed in Table 2. The fluorescence intensity was altered with increase of inhibitor concentrations. As shown in Table 2, the K_d values of the strong binding inhibitors, **4**, **5** and **6**, were estimated as 3.35×10^5, 2.01×10^5, and 1.04×10^6 M^{-1}, respectively, while that of the known inhibitor, TLM, was

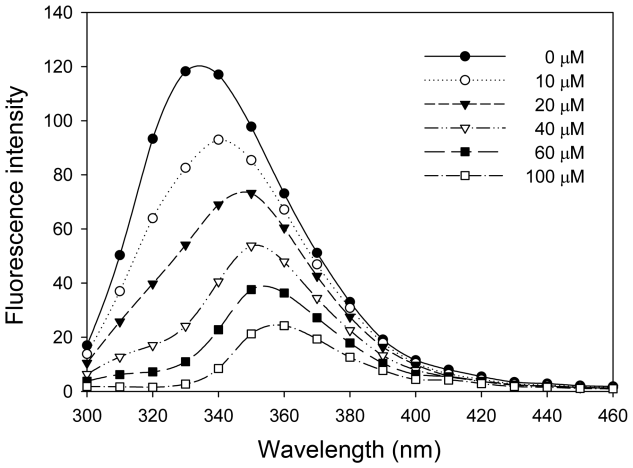


Figure 2. Fluorescence spectra of saKAS III in the presence of compound **4** (Naringenin).

7.80×10^4 M^{-1}. However, **6** did not showed antimicrobial activities against *S. aureus* as well as MRSA strains. The K_d of weak binder, compound **7**, was 1.41×10^4 M^{-1}. Even though compound **1**, **2**, and **3** showed antimicrobial activities against *S. aureus* and four MRSAs, they did not bind to saKAS III. Therefore, their antimicrobial activities may be caused by the inhibition of other target proteins. Based on these results, we propose that **4** (naringenin) and **5** (eriodictyol) with strong binding affinities as well as high antimicrobial activities are potent antimicrobial flavonoid inhibitors of saKAS III.

Docking Results of saKAS III and Flavonoids. To evaluate several biological assays and propose the binding model of flavonoids and saKAS III, we performed docking with all seven flavonoids and saKAS III, and calculated ligand score (LigScore) as listed in Table 2. Four flavonoids (**4**, **5**, **6** and **7**) docked well into active site of saKAS III and showed good LigScores.

Four well docked flavonoids formed two hydrogen bonds with saKAS III. They have 4'-OH and 7-OH and these two hydroxyl groups participate in hydrogen bonding interactions with saKAS III; 4'-OH formed hydrogen bond with backbone oxygen of Phe298 and 7-OH also participated in hydrogen bonding interaction with backbone carboxyl oxygen of Ser152 as shown in Figure 3. This binding model

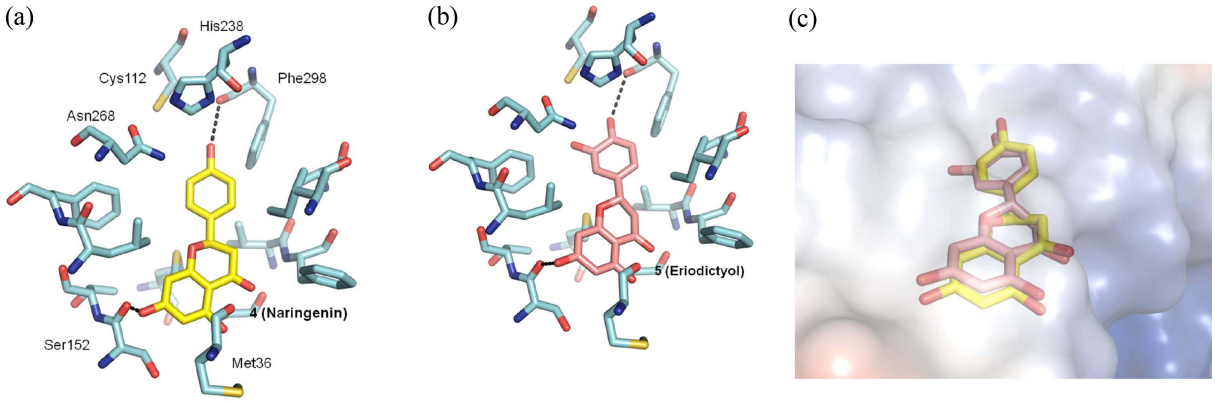


Figure 3. Docking model of Flavonoids and saKAS III. (a) Model of compound **4** and saKAS III. (b) Model of compound **5** and saKAS III. Hydrogen bonds are dictated by black dashed lines. (c) Superimposed model of compound **4** and **5** at active site of saKAS III.

correlates well with the results of STD-NMR of compound **4** and saKAS III. The superimposed docking model of compound **4**-saKAS III and **5**-saKAS III are depicted in Figure 3(c).

Interestingly, compound **6** (dihydrobinetin) and **7** (taxifolin) which did not show antimicrobial activities against MRSAs docked well in saKAS III with good LigScore and formed hydrogen bonding interactions with saKAS III. It may be due to poor permeabilizing abilities of these flavonoids into the bacterial cell membrane. To estimate the cell permeability of compound **6** and **7**, we calculated LogP and polar surface area (PSA) for all compounds as listed in Table 2. These two properties can be used to the predict cell permeability in transport across cell membranes. We already obtained the information about the proper range of these two properties for design of potent KAS III inhibitors related to binding affinity and cell permeability in our previous reports.[16] For example, optimum Log P was in the range of 2-3.5 and optimum PSA was in the range of 55-100 Å^2. In Table 2, while LogP of **6** and **7** is under 2 and PSA is over 100, LogP and PSA of compound **4** and **5** are within the acceptable range of our criterion.

In our previous study of efKAS III and flavanones, we proposed that well docked flavanones formed three hydrogen bonds with active site residues of efKAS III, such as Arg38, Phe220, His246, Asn276, and Phe308.[15] In case of efKAS III, 5- and 4'-hydroxy groups of compound **4** and **5** interacted with the backbone carbonyl of Phe308 which corresponds to Phe298 in saKAS III and side-chains of Arg38 which corresponds to Met36 in saKAS III, respectively. 3'-hydroxy groups of **5** also formed hydrogen bond with Asn276 which corresponds to Asn268 in saKAS III. The superimposed binding model at the active site of efKAS III and saKAS III is depicted in Figure 4. These two bacterial KAS III have sequence similarity over 50% and the similarity of active site residues is over 90%. As shown in Figure 4, only remarkable difference of active site residue appears in which Arg38 in efKAS III is replaced with Met36 in saKAS III. This difference caused one hydrogen bond missing in saKAS III compared with efKAS III. Even though the active site of KAS III is highly conserved in various bacterial strains, the small structural perturbation of active sites of KAS III in different bacteria caused the slight discrepancy in binding interactions. Lackness of one hydrogen bonding interaction in saKAS III compared to efKAS III may cause decrease in binding affinities of flavonoids about one order for saKAS III compared with those for efKAS III.

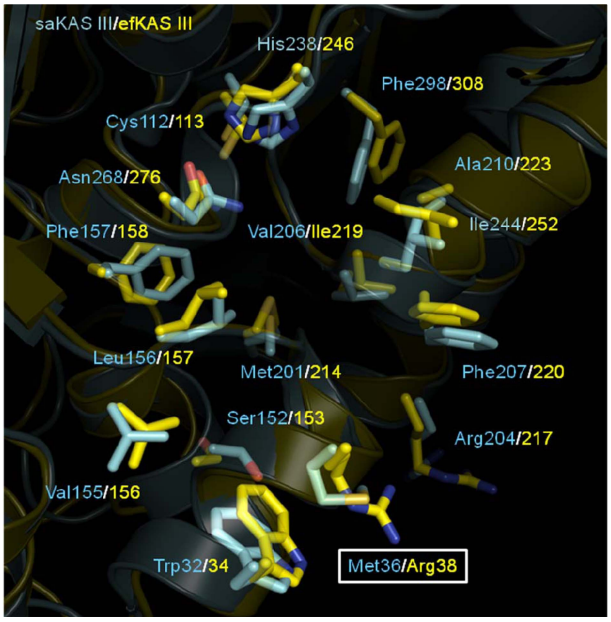


Figure 4. The similarity or difference existed in active site of saKAS III and that of efKAS III.

Based on these results, we propose that two flavanone, **4** (naringenin) and **5** (eriodictyol) are potent antimicrobial inhibitors of saKAS III with good binding affinities as well as high antimicrobial activities. Further optimization will be performed to improve their antimicrobial activities.

Conclusion

In our previous study, we suggested three flavonoids inhibitors of efKAS III with antimicrobial activities against *E. faecalis* and VREF. Based on these series of flavonoids, we tried to find saKAS III inhibitors which displayed

specificities against *S. aureus* and various MRSA strains. From MIC test, we first found five antimicrobial flavonoids against *S. aureus* and four MRSAs with the MIC values in the range of 64-256 μg/mL. Using STD-NMR and fluorescence quenching experiments, we confirmed that two flavonoids, **4** (naringenin) and **5** (eridictyol) with good antimicrobial activities showed good binding affinities to saKAS III, 3.35×10^5 and 2.01×10^5 M^{-1}, respectively. From docking study, we proposed the binding model of these two flavonoids and saKAS III. 4'-OH and 7-OH of flavonoids participated in hydrogen bonding interactions with backbone oxygen of Phe298 and Ser152, respectively. As a result, we can propose that naringenin (5,7,4'-trihydroxyflavanone) and eriodictyol (5,7,3',4'-tetrahydroxyflavanone) are potent antimicrobial inhibitors of saKAS III with good antimicrobial activities against various MRSA strains in the range of 64 to 128 μM.

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