Screening of Flavonoids as Candidate Antibiotics against *Enterococcus faecalis*

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β-Ketoacyl acyl carrier protein synthase (KAS) III, the most divergent member of the condensing enzyme family, is a key catalyst in bacterial fatty acid biosynthesis and, thus, an attractive target for novel antibiotics. Here, we perform docking studies between *Enterococcus faecalis* KAS III (efKAS III) and one flavanone and 11 hydroxyflavanones with hydroxy groups at various positions. The MIC values of these flavanones for *E. faecalis* and vancomycin-resistant *E. faecalis* (*VREF*) were measured, and binding affinities to efKAS III were determined. Naringenin (**9**), eriodictyol (**10**), and taxifolin (**12**), with high-scoring functions and good binding affinities, docked well with efKAS III, resulting in MIC values in the range 128−512 μg/mL. Our results indicate that hydrogen bonds between the 5- and 4′-hydroxy groups and the side-chain of Arg38 and the backbone carbonyl of Phe308 are the key interactions for efKAS III inhibition. These flavanones are good candidate KAS III inhibitors and may be utilized as effective antimicrobials.

Antimicrobial drugs have proven remarkably effective for the control of bacterial infections.[1] However, it is evident that bacterial pathogens are unlikely to capitulate unconditionally to treatment,[1] since many such organisms have evolved numerous defense mechanisms against antimicrobials in recent years. Additionally, more than half of the infections treated in hospitals involve Gram-positive bacteria, such as *Enterococci* and *Staphylococcus aureus*.[2,3] *Enterococci* are part of the normal intestinal flora, but also cause serious infections in animals and humans. Approximately 90% of enterococcal infections are caused by *Enterococcus faecalis*, a Gram-positive bacterium inhabiting the alimentary canals of humans and animals that is capable of causing life-threatening disease in humans, especially in nosocomial (hospital) environments.[4−6] One of the major reasons underlying the continuing survival of these organisms in the hospital environment is their intrinsic resistance to several commonly used antibiotics and, perhaps more importantly, their ability to acquire resistance to all currently available antibiotics, either via mutation or by receipt of foreign genetic material through transfer of plasmids and transposons.[7,8] Novel antibiotics are urgently required to overcome this resistance. This may be effectively achieved through the development of innovative technologies to uncover new leads that may be developed as antibacterial agents.[9−11]

The type II fatty acid synthesis (FAS) pathway essential for bacterial survival has recently emerged as a prime target for newly developed antibiotics.[12−15] In the type II FAS, each reaction is catalyzed by individual enzymes, in contrast to the type I FAS pathway, which involves a single polypeptide chain.[16] β-Ketoacyl acyl carrier protein synthase (KAS) III, which catalyzes the initial condensation reaction between acyl-CoA and malonyl-ACP and is involved in feedback regulation of the biosynthetic pathway via product inhibition, plays a central role in bacterial type II FAS.[17,18] KAS III can be distinguished from the other two bacterial condensing enzymes, KAS I and KAS II, on the basis of selectivity for acyl-CoA over acyl-ACP and a difference in catalytic triad composition (Cys-His-Asn in contrast to Cys-His-His in KAS I and II).[19−21] The essence of type II FAS for bacterial viability, together with major differences between the two FAS types, suggests that selective compounds binding type II FAS may be developed with potential utility as antibacterial drugs. KAS III, a key catalyst in type II FAS, is a particularly promising target for novel antibiotics.[22−25]

The natural polyphenolic flavonoids are common constituents of plants involved in traditional medicine.[26] Many flavonoids display low toxicity in mammals, and some are widely used in the treatment of several diseases.[27] Several flavonoids display antibacterial activity.[28−30] Their structures are diversified by oxidation, alkylation, and glycosylation.[31] Despite extensive examination of the antimicrobial activities of naturally occurring flavonoids, several high-quality investigations have examined the relationship between flavonoid structure and antimicrobial activity.[29] In a previous study, we confirmed that the flavanone naringenin showed effective antimicrobial activity against *E. faecalis* and is a candidate inhibitor of KAS III of the bacterium.[32] In this study, we selected 12 natural and synthetic flavanones for characterization as inhibitors of KAS III using docking studies.[29] We targeted *E. faecalis* KAS III (efKAS III), investigating the interactions between the 12 flavanones and KAS III by MIC tests and molecular modeling methods. Binding of antimicrobial flavanones to efKAS III was subsequently assessed using biophysical screening methods, including STD (saturation transfer difference)-NMR and fluorescence experiments. Since inhibitors of KAS III are good new antimicrobial drug candidates, it is important to elucidate the structure−activity relationships and antibacterial activities of hydroxyflavanones.

Results and Discussion

Flavonoids exert inhibitory effects on various human pathogens, including antibiotic-resistant Gram-positive and -negative bacteria.[21] The 12 flavanones are listed in Table 1. Most flavanones displayed reasonable solubility, whereas compounds **2**−**6** were only partially soluble at high concentrations. As shown in Table 2, antimicrobial activities of these compounds against *E. faecalis* and *VREF* were evaluated by measuring MIC values. Among these, only six flavanones (**3**, **8**, **9**, **10**, **11**, and **12**) have antimicrobial activities in the range 128−512 μg/mL. We measured the MIC of the reference molecule, thiolactomycin (TLM), a known antibiotic inhibitor of ecKAS III,[11] as 256 μg/mL against two bacteria. Considering the MIC of TLM, these six compounds have similar (or good) activities against *E. faecalis* and slightly lower activities against *VREF*. Interestingly, compound **3** exhibited antimicrobial activity (256 μg/mL) despite partial solubility.

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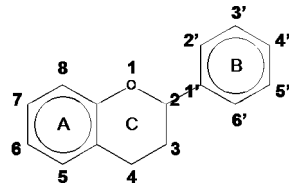
Table 1. Nomenclature of the Flavanones Based on the Position of Their Substituents[a]



		position of substituents							
compound	nomenclature	3	5	6	7	2′	3′	4′	5′
1	flavanone	H	H	H	H	H	H	H	H
2	2′-hydroxyflavanone	H	H	H	H	OH	H	H	H
3	3′- hydroxyflavanone	H	H	H	H	H	OH	H	H
4	4′- hydroxyflavanone	H	H	H	H	H	H	OH	H
5	6- hydroxyflavanone	H	H	OH	H	H	H	H	H
6	7- hydroxyflavanone	H	H	H	OH	H	H	H	H
7	5,7- dihydroxyflavanone (pinocembrin)	H	OH	H	OH	H	H	H	H
8	6,2′-dihydroxyflavanone	H	H	OH	H	OH	H	H	H
9	5,7,4′-trihydroxyflavanone (naringenin)	H	OH	H	OH	H	H	OH	H
10	5,7,3′,4′-tetrahydroxyflavanone (eriodictyol)	H	OH	H	OH	H	OH	OH	H
11	3,7,3′,4′,5′-pentahydroxyflavanone (dihydrobinetin)	OH	H	H	OH	H	OH	OH	OH
12	3,5,7,3′,4′-pentahydroxyflavanone (taxifolin)	OH	OH	H	OH	H	OH	OH	H

[a] Each flavanone present as a racemic mixture.

Table 2. Antimicrobial Activities of Flavanones against *E. faecalis* and *VREF* and Binding Affinity to efKAS III

	MIC (μg/ml)				
compound	*E. faecalis*	*VREF*	LigScore	PLP	binding affinity to efKAS III (M^{-1})
1	nd[a]	nd	2.42	51.52	2.82
2	nd	nd	2.31	45.12	2.87×10^1
3	256	256	2.51	57.01	9.77×10^2
4	nd	nd	2.36	53.39	3.1×10^1
5	nd	nd	2.17	51.63	4.2×10^1
6	nd	nd	2.38	50.45	8.79×10^2
7	>512	>512	2.39	53.62	1.48×10^1
8	256	256	2.25	57.69	6.76×10^2
9	256	512	2.92	57.15	1.61×10^6
10	256	512	2.97	60.88	4.19×10^6
11	256	>512	3.1	58.31	7.94×10^6
12	128	512	3.17	57.93	6.76×10^7

[a] nd: the MIC value was not determined, since each flavanone was not completely dissolved.

The three-dimensional structure of efKAS III has been established by homology modeling, based on the X-ray structure of *Staphylococcus aureus* KAS III (saKAS III) determined in a previous study by our group.[32] To screen for possible inhibitors of efKAS III, we performed a ligand docking study of flavanones with efKAS III.

The docking scores between flavanones and efKAS III are presented in Table 2. Among these, only four compounds (**9**−**12**) docked well in the primary binding site of efKAS III, with high LigScore and PLP values, compared to other flavanones. The LigScore functions consist of three distinct terms that describe van der Waals interactions, polar attraction between ligand and protein, and the desolvation penalty attributed to binding of polar ligand atoms to the protein, and vice versa.[33] The PLP (piecewise linear potential) score represents the sum of all pairwise atomic interactions of the ligand/receptor complex.[34] The docking models of efKAS III and the four flavanones with high LigScore are depicted in Figure 1A. As shown in Figure 1B, in a binding model of **9** and efKAS III, 5- and 4′-hydroxy groups interacted with the backbone carbonyl of Phe308 and the side-chains of Arg38 and Cys113, respectively. Hydrogen bonds between the 5-, 3′-, and 4′-hydroxy groups of flavanones and Phe308, Arg38, and Asn276 of the enzyme were also observed in the case of compounds **10** and **12** (Figure 1C and D). Additionally, the 3-hydroxy group of **12** formed an extra hydrogen bond with the backbone carbonyl of Phe220.

Hydrogen bonds between the 5- and 4′-hydroxy groups and the side-chain of Arg38, and the backbone carbonyl of Phe308, constitute key interactions for efKAS III inhibition. The hydroxy group at the B ring of flavonoids is very important for antimicrobial effects.[27] However, compound **1**, **5**, **6**, and **7** do not possess hydroxy groups at the B ring. Moreover, whereas **2**, **3**, and **8** contain B ring hydroxyl groups, these hydroxy groups are in positions unsuitable to form hydrogen bonds with efKAS III. These compounds also do not have the 5-hydroxy group that forms hydrogen bonds with Arg38. Compound **4** contains a hydroxy group at the 4′-position, as do other antimicrobial flavanones, but does not exhibit antimicrobial activity, possibly because of partial solubility in aqueous buffers.

Representative aromatic regions of the STD-NMR spectra of efKAS III bound to four antimicrobial flavanones (1 mM) are shown in Figure 2. The proton signals in STD-NMR spectra are caused by magnetization transfer from the protein to ligand, providing direct evidence of protein−ligand interactions. Protons of the ligand in closest contact with the protein are more affected than remote protons and yield the most intense signals.[35] In STD-NMR experiments, the exchange of hydroxy protons of flavanone in aqueous solution is severely hampered by fast chemical exchange in conjugation with a short relaxation time.[34] Accordingly, we expected that magnetization transfer from the protein to the hydroxy group would be represented by the closest proton signals. Proton signals are assigned at the top of Figure 2. STD-NMR peak intensities were compared to the most intense peak (6.85 ppm). The ^{1}H NMR spectrum of naringenin in the presence of efKAS III protein is presented in Figure 2A (top and bottom). STD data show that the resonances of H-2′, -3′, -5′, and -6′ at the B ring, as well as H-6 and H-8 at the A ring, display interactions with the protein. In Figures 2B and 2C, compounds **10** and **12** are compared. Compared with **9**, the STD-NMR spectra of **10** display the proton resonances at the same positions, except for a signal of H-3′ arising from substitution of a hydroxy group (Figure 2B). As shown in Figure 2C, in addition to protons

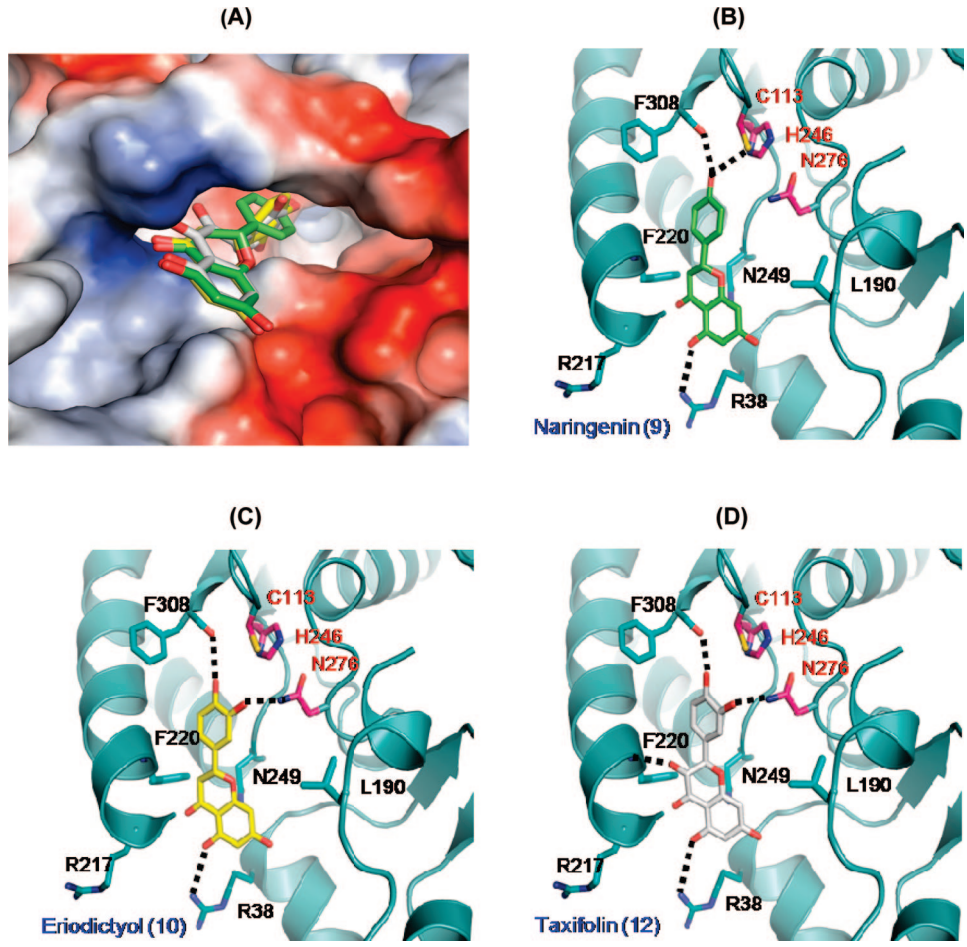


Figure 1. Docking models of the flavonoids and KAS III. (A) Surface model of the substrate binding site of efKAS III. (B) Docking model of compound **9** (naringenin) and efKAS III. (C) Docking model of compound **10** (eriodictyol) and efKAS III. (D) Docking model of compound **12** (taxifolin) and efKAS III. Hydrogen bonds are depicted as dashed lines.

at the B and C rings, compound **12** displays resonances for H-2 and H-3, indicative of interactions between 3-hydroxy groups and the protein. These STD-NMR spectra, showing that resonances of **10** and **12** interact with efKAS III, imply that binding of these three flavanones occurs mainly via hydroxy groups at the B and C rings. However, weak peak intensity was seen with compound **8** (Figure 2D), consistent with results from the ligand docking study. However, we could not obtain STD-NMR spectra of efKAS III binding to compound **11**, because it was insoluble at the high concentrations (1 mM) required for these experiments. Fluorescence measurements further confirmed these findings.

The efKAS III protein contains four tryptophan residues at positions 34, 149, 187, and 314. Among these, Trp34 is in closest proximity to the substrate binding site of efKAS III. Protein fluorescence was decreased upon ligand binding to efKAS III. Fluorescence quenching was used to estimate the binding constant, *K*. The binding (or dissociation) constant, *K*, is defined as [complex]/[free protein][free inhibitor].[37] We successfully analyzed the binding affinities between efKAS III and partially soluble flavanones with hydroxy groups using this method, because fluorescence experiments require lower concentrations of flavanones than does STD-NMR (see legend to Figure 3). Fluorescence titration curves for efKAS III with compounds **12** and **8** are depicted in Figure 3. The fluorescence intensity changed with increasing concentrations of flavanone. These spectral changes were attributed to the formation of protein−flavanone complexes. As shown in Figure 3A, a significant decrease was evident in the quenching of efKAS III tryptophan fluorescence in the presence of compound **12**. The *K* value for **12** binding was 6.76×10^7 M^{-1}. However, only a limited decrease in fluorescence quenching was obtained with **8**, with a *K* value of 6.76×10^2 M^{-1} (Figure 3B). As shown in Table 2, *K* values for binding of the other four antimicrobial flavanones, **3**, **9**, **10**, and **11**, to efKAS III were 9.77×10^2, 1.61×10^6, 4.19×10^6, and 7.94×10^6 M^{-1}, respectively. The binding *K* values for non-antimicrobial flavanones, including the partially soluble compounds, such as **2**, **4**, **5**, and **6**, were less than 10^3 M^{-1}. Compounds **3** and **8** displayed low binding constants for efKAS III, but inhibited bacterial growth at 256 μg/mL. Therefore, these two flavanones may target proteins other than KAS III.

In conclusion, six flavanones, **3**, **8**, **9**, **10**, **11**, and **12**, displayed antimicrobial activity against *E. faecalis* and vancomycin-resistant *E. faecalis* (*VREF*), with MIC values in the range 128−512 μg/mL. The binding constant for TLM was determined to be 7.59×10^3 M^{-1} (data not shown). The flavanones appear to be more potent inhibitors of efKAS III than TLM. Accordingly, more potent inhibitors may be generated by further optimization of flavanones.

Using a ligand docking study to identify possible inhibitors of efKAS III, we identified four compounds (**9**−**12**) as inhibitors that docked well with efKAS III, as a result of intensive hydrogen-bonding networks. STD-NMR experiments on antimicrobial flavanones complexed with efKAS III disclosed that **9**, **10**, and **12** bound efficiently to efKAS III protein. Differences between the spectra, with and without saturation, primarily disclosed resonances belonging to ligand protons bound to efKAS III. Moreover, data obtained from fluorescence quenching experiments implied tight interactions of compound **9** (naringenin), **10** (eriodictyol), and **12** (taxifolin) with efKAS III, with

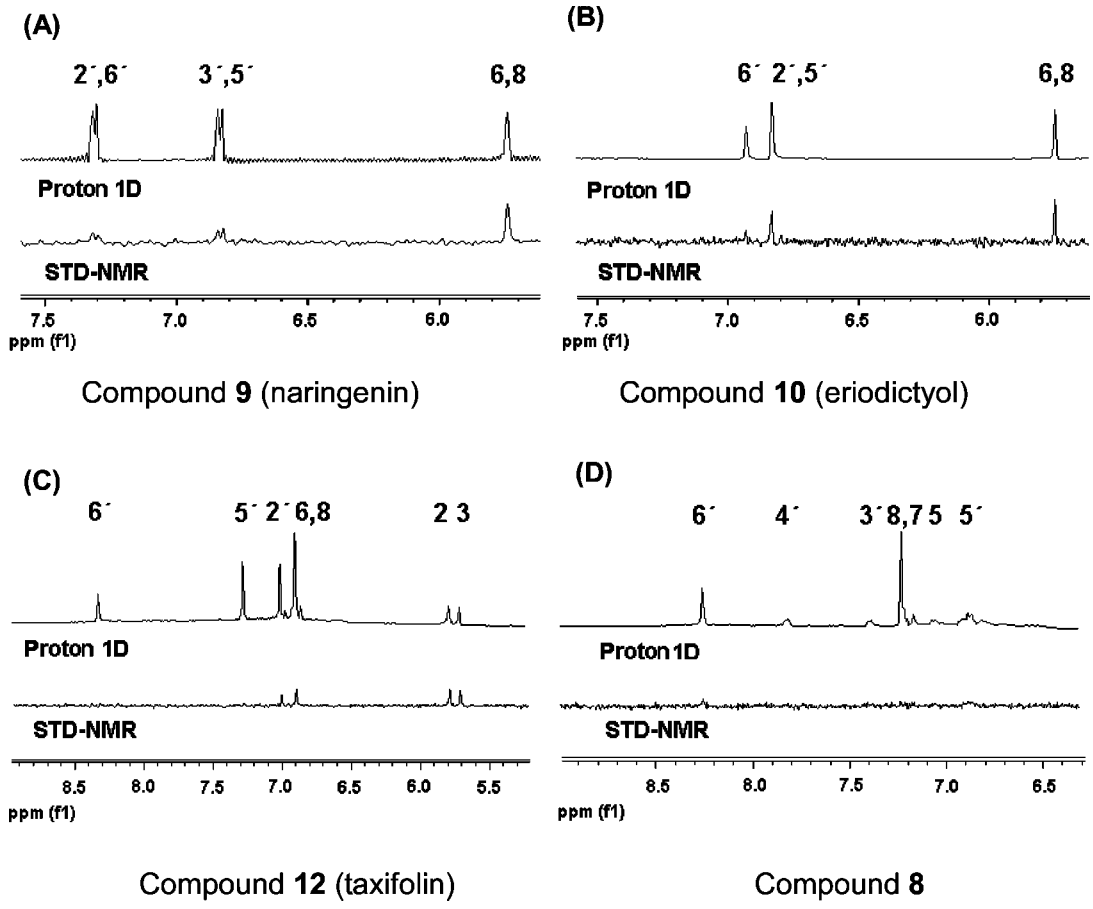


Figure 2. Representative results of the STD-NMR binding assay. (A) ^{1}H NMR spectra of compound **9** and its STD effects. (B) ^{1}H NMR spectra of compound **10** and its STD effects. (C) ^{1}H NMR spectra of compound **12** and its STD effects. (D) ^{1}H NMR spectra of compound **8** and its STD effects.

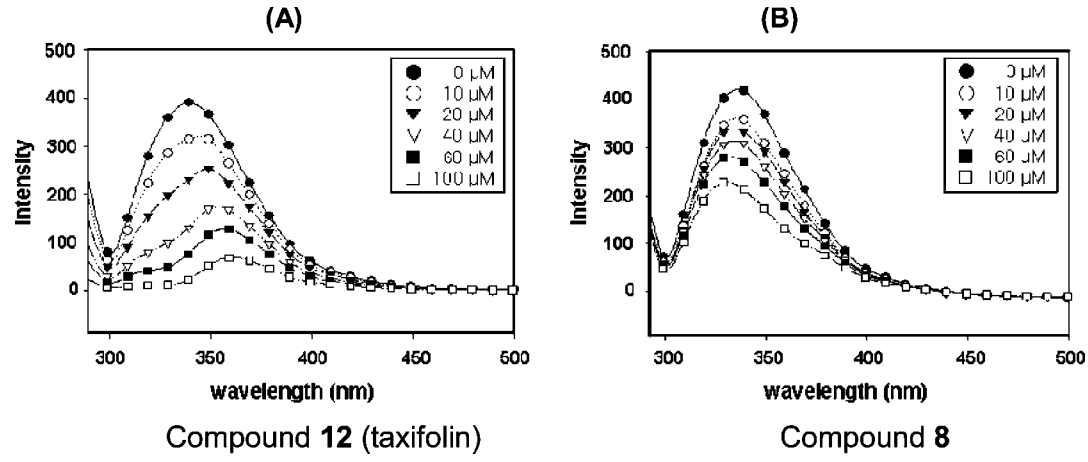


Figure 3. Fluorescence spectra of efKAS III in the presence of different concentrations of flavanones (0, 10, 20, 40, 60, and 100 μM) at pH 7.0. Samples were excited at 290 nm, and emission spectra recorded for light scattering effects from 290 to 600 nm. (A) Fluorescence spectra of an aqueous solution of 10 μM efKAS III in the presence of different concentrations of compound **12**. (B) Fluorescence spectra of an aqueous solution of 10 μM efKAS III in the presence of different concentrations of compound **8**.

binding constants in the micromolar range. However, the other flavanones displayed very low binding affinity to efKAS III. Results from STD-NMR and fluorescence experiments correlated well with ligand docking data. Accordingly, we conclude that these flavanones are good candidate KAS III inhibitors as well as antimicrobial agents. Potent flavanone inhibitors of efKAS III have been successfully screened, but display only modest antibacterial activity, probably owing to the complex nature of drug uptake by cells. Further hit optimization of these flavanones is essential to identify more potent antimicrobial agents.

Experimental Section

Chemicals. Flavanones were purchased from Sigma Chemical Co. and INDOFINE Chemical Company, Inc., and DMSO-d_6 and D_2O from Cambridge Isotope Laboratories (Andover, MA).

Construction of the efKAS III Expression Plasmid. The *fab*H gene encoding β-ketoacyl-ACP synthase III was amplified from *Enterococcus faecalis* V583 genomic DNA. The sense primer, 5′-tcctccaaaatagtcattttcttca-3′, and antisense primer, 5′-tttgaaaggcatggagcaag-3′, were designed on the basis of the sequence of GenBank accession number

29350190. At the 5′-end of each primer, a restriction site (*Nde*I site for the sense primer and *Bam*HI site for the antisense primer) was attached to facilitate cloning. PCR was performed under the following conditions: 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 1 min at 72 °C. The resulting product was sequenced and cloned into the *Nde*I and *Bam*HI sites of the pET-15b vector. The ligation mixture was transformed into *Escherichia coli* DH5α competent cells.

Expression and Purification of efKAS III. The pET-15b/efKAS III plasmid was transformed into the expression host, *E. coli* BL21 (DE3). Transformed cells were grown on Luria−Bertani (LB) agar plates containing 50 μg/mL ampicillin. SDS-PAGE was utilized to verify overexpression of efKAS III with an N-terminal polyhistidine tag. A single colony was used to inoculate 50 mL of LB medium with 50 μg/mL ampicillin and grown overnight in a shaking incubator at 37 °C. Fully grown culture (10 mL) was mixed with 1 L of fresh LB medium with 50 μg/mL ampicillin and grown at 37 °C until an optical density of 1.0 at 600 nm. Protein was induced with 1 mM IPTG, and the culture grown for 5 more hours at 30 °C. Cells were harvested, and the pellet was stored at −80 °C.

All lysis and purification processes were performed at 4 °C. The frozen pellet was resuspended in buffer A containing 50 mM sodium phosphate and 300 mM NaCl and lysed by ultrasonication. The cell lysate was centrifuged for 30 min at 4 °C and 8000 rpm and the supernatant loaded onto a HITrap chelating column (GE) preequilibrated with buffer A. The column was washed with buffer A, and the bound material eluted with a linear gradient of 0 to 600 mM imidazole. The efKAS III-containing fractions were pooled and concentrated with an AmiconUltra (Millipore) by exchanging with buffer B (50 mM sodium phosphate, 100 mM NaCl, pH 8.0). At each stage of the purification process, SDS-PAGE was applied to identify the efKAS III-containing fraction.

MIC Test. The MIC test of flavanone activity against *E. faecalis* was performed using the broth microdilution method.[29] Twelve natural and synthetic flavanones were collected. The 2D structures of hydroxyflavanones are listed in Table 1. *E. faecalis* and *VREF* were grown to the midlog phase in Mueller-Hinton broth and diluted 100-fold in the same medium, respectively. A 20 μL aliquot of the diluted cell suspension (10^6 to 10^7 colony forming units) was applied to inoculate each well of a 96-well plate containing 100 μL of Mueller-Hinton broth with the indicated concentrations of inhibitors. The plate was incubated at 37 °C for 20 h. MIC was defined as the lowest concentration of antibiotic exerting complete inhibition of visible growth, compared to an antibiotic-free control well. Experiments were replicated at least three times to verify the reproducibility of the methodology using the above conditions.

Ligand Docking. Each flavanone was docked to efKAS III using AutoDock.[38] The Lamarckian Genetic Algorithm (LGA) of AutoDock 3.05 was employed for docking experiments. MD simulations on the final docking structure were performed in the canonical ensemble (NVT) at 300 K, and the distance-dependent function of the dielectric constant was used for calculation of the energetic maps in a vacuum system using the InsightII/Discover program. All atoms of the system were considered explicitly, and their interactions computed using the consistent valence force field. A distance cutoff of 10 Å was used for van der Waals interactions and electrostatic interactions. The time step in the MD simulations was 1 fs, and MD simulation was performed for 2 ns. Coordinates were saved every 1 ps. The average structure was calculated for the 2 ns trajectory and submitted to final energy minimization by performing 10 000 steps of the steepest descent method.

NMR Spectroscopy. ^{1}H NMR, ^{13}C NMR, COSY, HMQC, HMBC, and NOESY experiments were performed for resonance assignment of flavanones. The sample concentrations used were approximately 50 mM. For the ^{1}H NMR experiment, 16 transients with 32 K data points were collected for each increment, with a relaxation delay of 1.5 s. The 90° pulse was 6.6 μs with a spectral width of 5000 Hz, and the ^{13}C NMR spectrum was obtained at a spectral width of 22 137 Hz using 64 K data points. HMQC, HMBC, and COSY spectra were acquired with 2048 data points for t_2 and 256 for t_1 increments using the magnitude mode, and NOESY spectra were collected with 2048 data points for t_2 and 256 for t_1 using the TPPI method, i.e., time-proportional phase increments at a mixing time of 800 ms.[39–42] The long-range coupling time for HMBC was 70 ms. All NMR spectra were processed using the NMRPIPE on an SGI workstation.[43]

Saturation transfer difference NMR (STD-NMR) was performed to identify the inhibitors bound to efKAS III at Korea Basic Science Institute. Spectra were collected with and without saturation of the spectral region containing only protein resonances at 298 K.[30,44] In the presence of excess ligand, differences between the spectra primarily disclose resonances belonging to ligand protons bound to protein. 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 a 100 μs delay between each pulse in all STD-NMR experiments. The total duration of the saturation time was set to 2 s.

For STD-NMR experiments, we added 10 μM recombinant efKAS III in 25 mM sodium phosphate buffer with 150 mM NaCl, pH 7.0, and each candidate inhibitor at a protein:ligand molar ratio of 1:100. In total, 1024 scans for each STD-NMR experiment were acquired, and a WATERGATE sequence was used to suppress the water signal. A spin-lock filter (5 kHz strength and 10 ms duration) was applied to suppress the protein background.

Fluorescence Analysis. Experiments were performed at 25 °C on an RF-5301PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). Tryptophan-specific intrinsic fluorescence of efKAS III (10 μM) in sodium phosphate buffer was scanned between 290 and 500 at 290 nm excitation.[45] Each hydroxyflavanone was titrated to a final protein: inhibitor ratio of 1:10. All solutions included similar buffer concentrations. The sample was contained in a 2 mL thermostat cuvette with excitation and emission path lengths of 10 mm. Fluorescence quantum yields of efKAS III and flavanones were determined by tryptophan emission. K_d values were determined by fitting to the following equation:[46]

$$\log\left(\frac{F_0 - F}{F}\right) = \log\frac{1}{K_d} + n\log[\text{inhibitor}]$$

where F_0 and F represent fluorescence intensities of efKAS III at 342 nm in the absence and presence of inhibitor, respectively, while n is the number of inhibitor binding sites on the protein.

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