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ORIGINAL ARTICLE

Design, synthesis and biological evaluation of novel flavone Mannich base derivatives as potential antibacterial agents

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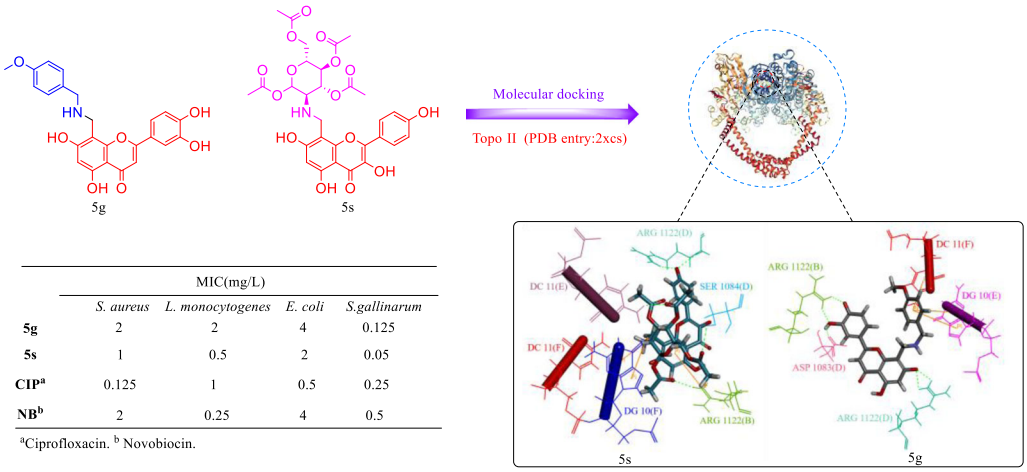
Abstract

A series of novel Mannich base derivatives of flavone containing benzylamine moiety was synthesized using the Mannich reaction. The results of antifungal activity are not ideal, but its antifungal effect has a certain increase compared to flavonoids. After that, four bacteria were used to test antibacterial experiments of these compounds; compound **5g** (MIC = 0.5, 0.125 mg/L) showed significant inhibitory activity against *Staphylococcus aureus* and *Salmonella gallinarum* compared with novobiocin (MIC = 2, 0.25 mg/L). Compound **5s** exhibited broad spectrum antibacterial activity (MIC = 1, 0.5, 2, 0.05 mg/L) against four bacteria. The selected compounds **5g** and **5s** exhibit potent inhibition against Topo II and Topo IV with IC_{50} values (0.25–16 mg/L). Molecular docking model showed that the compounds **5g** and **5s** can bind well to the target by interacting with amino acid residues. It will provide some valuable information for the commercial antibacterial agents.

Graphical Abstract



	MIC(mg/L)			
	S. aureus	*L. monocytogenes*	*E. coli*	*S.gallinarum*
5g	2	2	4	0.125
5s	1	0.5	2	0.05
CIP[a]	0.125	1	0.5	0.25
NB[b]	2	0.25	4	0.5

[a]Ciprofloxacin. [b] Novobiocin.

Keywords Flavonoids · Benzylamine · Mannich · Antibacterial · 3D-QSAR · Molecular docking

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Introduction

Flavonoids are widely found in the plant kingdom and have a wide variety of biological functions [1–5] and medicinal values, including antifungal [6], antibacterial [7], antioxidant [8], anti-aging [9] and anti-tumor [10, 11]. Hence, we tested their antifungal activity and found that orientin and isoorientin have excellent antifungal activities against *Gibberella sanbinetti* at 20 mg/L (supplementary materials). It is regrettable that luteolin was almost inactive. The structural differences between orientin, isoorientin and luteolin are reflected in the 8th or 6th position of ring A, which a very interesting phenomenon and worthy of study (Fig. 1). Although we have found that orientin and isoorientin have excellent antifungal activity, their costly nature has restricted their use as antimicrobial agents. In addition, it has been reported that substitution at the 8th position is superior to substitution at the 6th position of flavone derivatives [12, 13]. Therefore, compounds that give better antimicrobial activity by modification of position 8 of the flavone deserve attention and research.

Benzylamine is widely used in the field of pesticides because of its unique imino structure [14, 15], such as pencycuron, which is widely used in the control of rice sheath blight fungicide. Thus, we introduced the benzylamine structure at the 8th position of various flavones to try to increase their activity using the Mannich reaction. After screening the bioactivities of orientin and isoorientin, we get the inspiration from the orientin and isoorientin, which also introduced a carbohydrate at the 8th position of the flavone using the Mannich reaction to examine the effect on antifungal activity, which method provides a valuable reference for our further study of flavone carbohydrate.

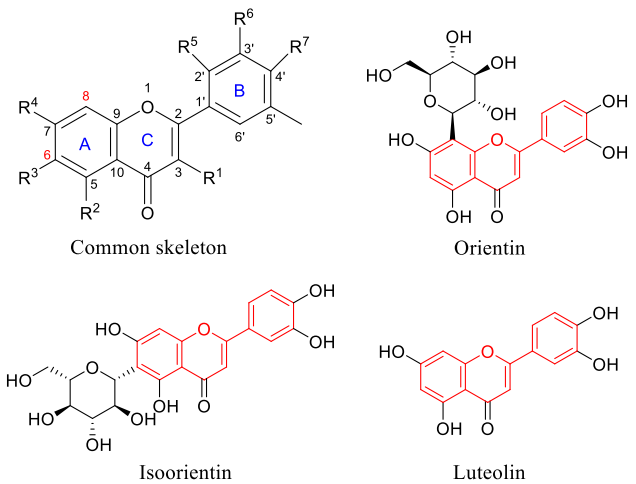


Fig. 1 Skeleton of the flavone scaffold and the structure of orientin, isoorientin and luteolin

A series totaling 19 compounds were synthesized and used for testing antifungal activity against *Gibberella sanbinetti* and *Gaeumannomyces graminis*. After the in vitro antifungal activity experiments were completed, we found that the antifungal activity of these compounds did not achieve the desired effect. So, we envision bacteria may be a good candidate for inhibition experiment of such compounds. Then, all of the compounds were tested for antibacterial activity against two gram-positive bacteria, namely *Staphylococcus aureus* and *Listeria monocytogenes*, and two gram-negative bacteria, namely *Escherichia coli* and *Salmonella gallinarum*. On the other hand, Zhihua Sui et al. [16] and Bernard et al. [17] have reported that flavonoids can be used as inhibitors of topoisomerase. Finally, we selected topoisomerase II (Topo II) as the target for molecular docking and 3D-QSAR to determine critical structural factors responsible for their antibacterial efficacy.

Results and discussion

Chemistry

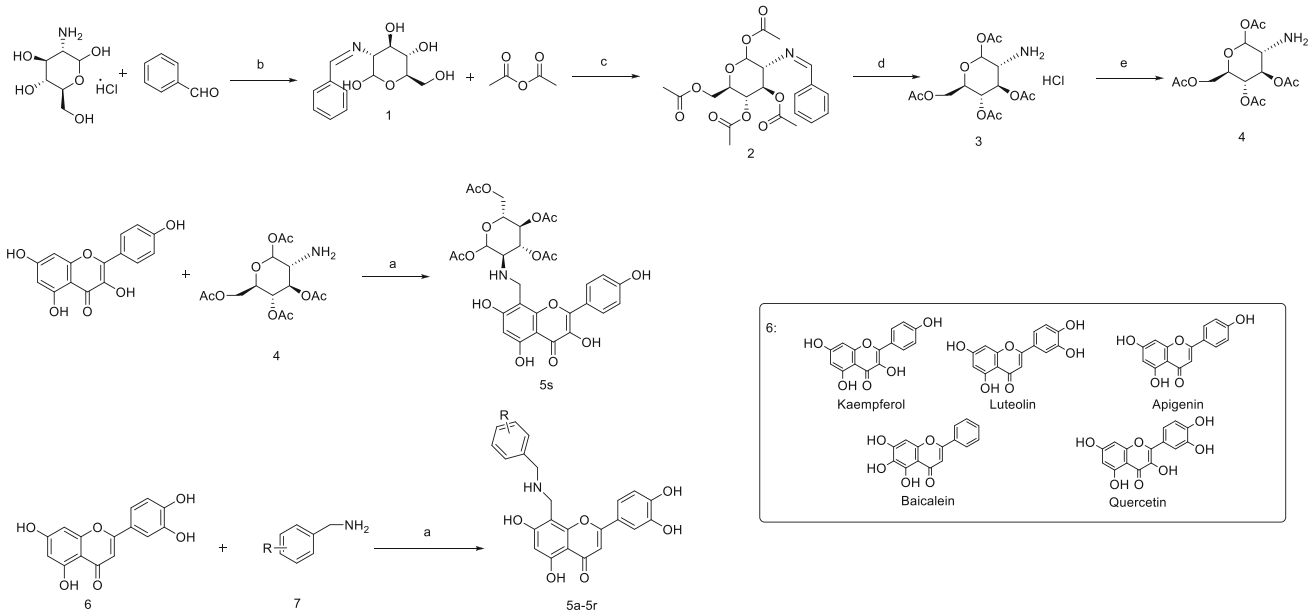
A series of novel Mannich base derivatives of flavone were synthesized, and the general pathway is outlined in Scheme 1. Benzylamine was introduced at the 8th position by reaction of benzylamine with flavone in isopropanol under formaldehyde conditions. The target compounds have not been reported except for **5a** [18] and **5i** [19]. In addition, we have synthesized glucosamine that can react with flavone by protecting and deprotecting glucosamine groups, and innovative structures were synthesized that introduced a glucosamine on the flavone structure using the Mannich reaction. Since the glucosamine compounds cannot directly undergo a Mannich reaction, the glucosamine is first protected by an amino group and then forms an ester with an acid anhydride [20], and finally the deprotected compound can undergo a Mannich reaction with kaempferol.

Biological activity

Antibacterial activity

All newly synthesized compounds were evaluated for in vitro antibacterial activity against two gram-positive bacteria, namely *Staphylococcus aureus* and *Listeria monocytogenes*, and two gram-negative bacteria, namely *E. coli* and *Salmonella gallinarum*. Novobiocin and ciprofloxacin were used as a reference standard. The results of the in vitro antibacterial activity screening of the test compounds are summarized in Table 1.

Most of the derivatives exhibited moderate to potent antibacterial activities, demonstrating the rationality of our



Scheme 1 General synthesis of compounds **5a–5s**. Reagents and conditions: (a) HCHO (aq), isopropanol, 35 °C; (b) NaOH (aq), 0 °C; (c) pyridine, 40 °C, 6 h; (d) HCl, acetone, rt; (e) H_2O, CH_3COONa(aq), rt

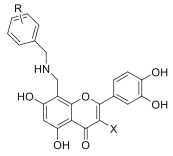
design strategy. Four compounds (**5c**, **5g**, **5q** and **5s**) were found to display improved antibacterial activities against *Staphylococcus aureus* compared with novobiocin (MIC = 2 mg/L), with MIC values ranging from 0.5 to 2 mg/L. Particularly, compound **5g** was determined to be the most potent inhibitor against Staphylococcus aureus, with an MIC value fourfold higher than novobiocin (0.5 mg/L). Three derivatives (**5g**, **5k** and **5s**) exhibited improved antibacterial activity against *Listeria monocytogenes* in comparison with ciprofloxacin (MIC = 1 mg/L), with MIC values ranging from 0.5 to 2 mg/L. Among these, compound **5s** exhibited the most potent activity against Listeria monocytogenes with an MIC value of 0.5 mg/L, which was better than ciprofloxacin (1 mg/L) but worse than novobiocin (0.25 mg/L). Regarding the inhibition of gram-negative bacteria, two derivatives (**5g** and **5s**) were found to show improved antibacterial activities against *E. coli* compared with novobiocin (MIC = 4 mg/L), with MIC values ranging from 2 to 4 mg/L. Two derivatives (**5g** and **5s**) were found to display improved antibacterial activities against *S. gallinarum* compared with ciprofloxacin (MIC = 0.25 mg/L), with MIC values of 0.125 and 0.05 mg/L, respectively. Noticeably, compound **5s**, identified as the most potent inhibitor against *S. gallinarum* with a MIC value at 0.05 mg/L, showed fivefold higher inhibitory activity than novobiocin (0.5 mg/L), and tenfold higher than ciprofloxacin (0.25 mg/L).

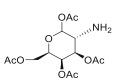
As given in Table 1, when the R position is replaced with a substituent, the antibacterial effect will be improved. When R is a 3-chloro group, the bacteriostatic effect is slightly improved, and when the 4-methyl group is substituted, the bacteriostatic effect is increased by about twice until R is replaced by 4-methoxy and antibacterial effect reached its peak. In addition, from the data, it can be concluded that when the flavonoid skeleton of the compound is luteolin and kaempferol, a very good antibacterial effect is exhibited. Another eye-catching finding was that compound **5s** exhibited a broad spectrum inhibitory effect on all four bacteria, which we envisioned is due to the introduction of carbohydrate structures. In summary, these findings indicate that the antibacterial efficacy of the designed compounds may be attributed to a combination of factors, such as 4-methyl substitution at the R position, variety of flavonoids and introduction of carbohydrate structure.

Inhibitory effects against Topo II and Topo IV

In order to determine the relationships by which the 4-methoxybenzylamine and carbohydrate structures induce their antibacterial activities, the inhibitory activity of selected compounds (**5g** and **5s**) against topoisomerase II and topoisomerase IV isolated from *E. coli* was examined. As given in Table 2, compounds **5s** showed more potent inhibition than **5g** against the two enzymes. On the other hand, there was a good correlation between the MICs and the IC_{50}s (Tables 1, 2), suggesting that inhibition of the Topo II and Topo IV by the 4-methyl and carbohydrate structure suppresses bacterial cell growth. These results indicate that the 4-methyl and carbohydrate structure put a great deal of pressure on the survival of the bacteria.

Table 1 Chemical structure of target compounds and their MIC values against *S. aureus*, *L. monocytogenes*, *E. coli* and *S. gallinarum*



Compd.	Flavone	R	MIC(mg/L)			
			S.a[a]	*L.m*[a]	*E.c*[a]	*S. g*[a]
5a	Baicalein	H	> 128	> 128	> 128	> 128
5b	Baicalein	4-CH_3	32	32	32	> 128
5c	Baicalein	4-OCH_3	1	16	32	16
5d	Baicalein	3-Cl	64	64	32	> 128
5e	Luteolin	H	> 128	64	> 128	64
5f	Luteolin	4-CH_3	64	64	64	64
5g	Luteolin	4-OCH_3	2	2	4	0.125
5h	Luteolin	3-Cl	32	16	16	8
5i	Quercetin	H	64	> 128	> 128	> 128
5j	Quercetin	4-CH_3	64	64	64	32
5k	Quercetin	4-OCH_3	16	2	32	4
5l	Quercetin	3-Cl	64	32	64	32
5m	Apigenin	4-CH_3	> 128	> 128	> 128	> 128
5n	Apigenin	4-OCH_3	32	16	32	16
5o	Kaempferol	H	64	> 128	64	64
5p	Kaempferol	4-CH_3	64	64	32	32
5q	Kaempferol	4-OCH_3	2	16	16	8
5r	Kaempferol	3-Cl	64	16	32	32
5s	Kaempferol		1	0.5	2	0.05
CIP[b]			0.125	1	0.5	0.25
NB[c]			2	0.25	4	0.5

[a]*Staphylococcus aureus* (ATCC-12600); *Listeria monocytogenes* (ATCC-15313); *Escherichia coli* (ATCC-25922); *S. gallinarum* (ATCC-9184)
[b]Ciprofloxacin
[c]Novobiocin

Table 2 Inhibitory effects of selected compounds against topoisomerase II and topoisomerase IV

Compd.	IC_{50}(mg/L)	
	Topo II[a]	Topo IV[b]
5g	0.5	16
5s	0.25	8
NB[c]	0.5	4
CIP[d]	0.5	8

[a]Topoisomerase II supercoiling activity
[b]Topoisomerase IV decatenation activity
[c]Ciprofloxacin
[d]Novobiocin

3D quantitative structure–activity relationships

In this study, 3D-QSAR models for the four different bacterial species were established (see the support information for the models not described here). After observation, we found that the models of the four bacteria are not much different. So, *S. gallinarum* is used as a target and compound **5g** is taken as the example to show the structure–activity relationships. The pMIC values were calculated on the basis of the in vitro activity experiment results, and the linear regression was determined with a genetic function algorithm (GFA) resulting in an r^2 value of 0.959, demonstrating the accuracy and reliability of the model. The 3D-CoMFA maps are shown in Fig. 2. In the electrostatic map shown in Fig. 2a, the red region indicates that the stronger the negative charge of the substituent in this region, the more favorable the activity of the compound, and the blue region indicates that the stronger the positive charge of the substituent in this region, the more favorable the activity of the compound. In the steric map shown in Fig. 2b, the yellow region indicates that the increase in substituents in this region is not conducive to the activity of the compound, while the blue region is the opposite. It can be observed that the electrostatically favorable zone is located in the luteolin structure, while the electrostatically unfavorable zone covers the space around positions 2 and 3 of the benzylamine structure. In the steric contour map, the sterically favored region almost covers the entire structure. We can conclude that the introduction of any other substituents or carbohydrate structure on the flavonoid skeleton can increase its biological activity. The 3D-CoMFA maps may provide valuable clues for further structural optimization.

Molecular docking analysis

In order to further discern the relationship between the structure of the compounds and the inhibition of bacterial activity, all synthesized compounds were molecularly docked using Discover Studio 3.5. The detailed interactions of representative derivative compounds **5g** and **5s** with Topo II (PDB code:2XCS, crystallized from S. aureus) are shown in Fig. 3. In the binding models of compounds **5g** and **5s**, their scaffolds and amino acid residues linked together tightly, suggesting that the pose of **5g** and **5s** into the Topo II-binding site revealed suitable shape complementarity with the binding pocket. As shown in Fig. 3a, Arg 1122(D) forms a hydrogen bond with a hydroxyl group located on the kaempferol structure. Ser 1084(D) and Dc 11(E) form two hydrogen bonds with hydroxyl groups located on the carbohydrate structure. Arg 1122(B) not only established a cation–π interaction with the kaempferol structure, but also formed a hydrogen bond with the carbohydrate structure. The amino group which linked the kaempferol structure to the carbohydrate structure forms cation–π interaction and hydrogen bond with Dg 10(F) and Dc 11(F), respectively. For Fig. 3b, Arg 1122(D) forms a hydrogen bond with a hydroxyl group located on the luteolin structure. Asp 1083(D) and Arg 1122(B) form three hydrogen bonds together with two hydroxyl groups on the luteolin structure. Dc 11(F) and Dg 10(E) established π–π interactions with phenyl ring which belongs to the benzy-

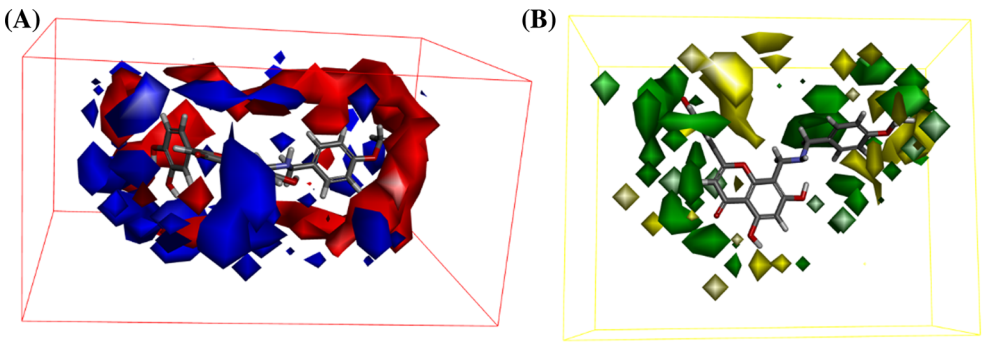


Fig. 2 Electrostatic map (**a**) and steric map (**b**) for the 3D-CoMFA model (for *S. gallinarum*)

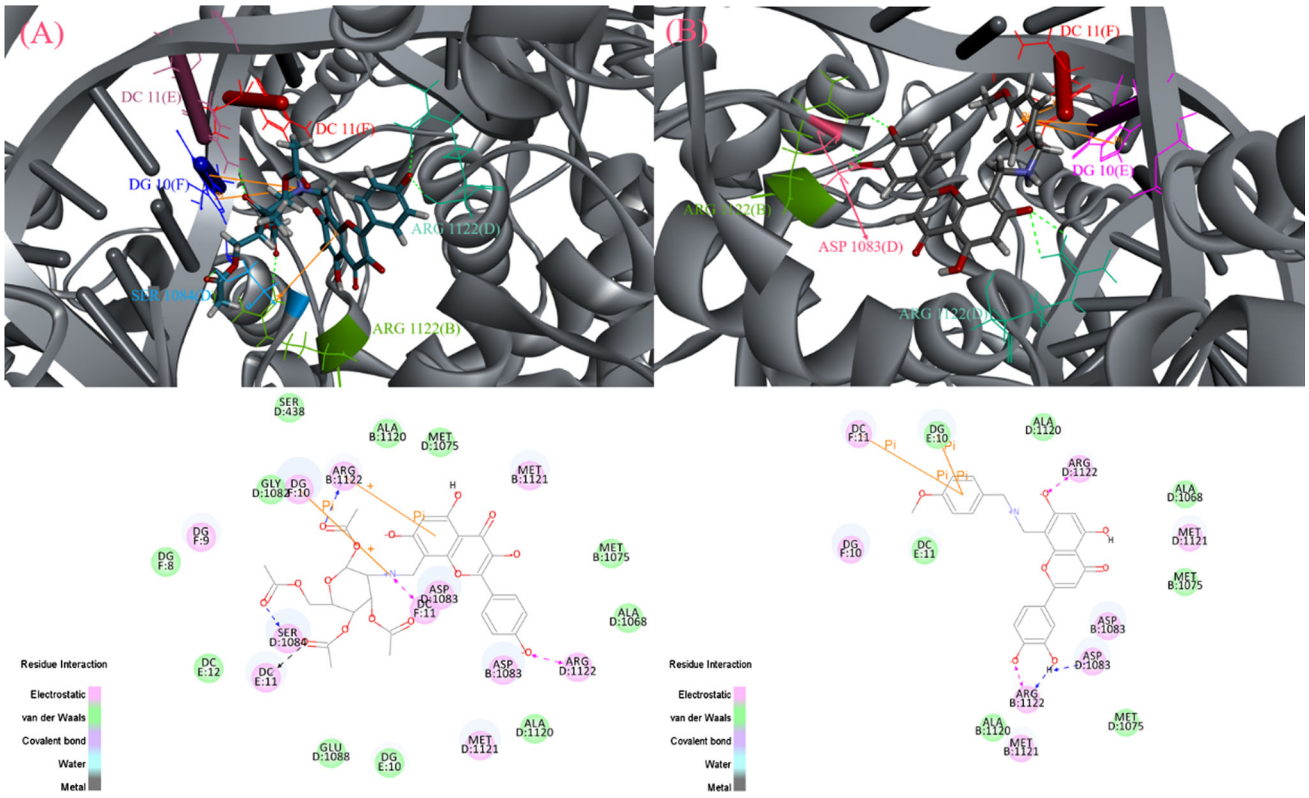


Fig. 3 **a** Binding model of **5s** (3D&2D diagram). **b** Binding model of **5g** (3D&2D diagram). The π interactions are displayed as orange solid line and H-bond is displayed as green dotted line

lamine structure. From the docking results above, we can see that the binding effect of **5s** and amino acid residues is much better than **5g**, which may be the reason why its antibacterial effect is better than **5s**.

Conclusion

A series of novel Mannich base derivatives of flavone was synthesized and evaluated for their antifungal activity against *G. sanbinetti* and *G. gramini*. The result shows that the antifungal effect is not ideal, but it may provide valuable information for the synthesis of the fungicide derivatives of the flavone. Furthermore, the antibacterial activities of the compound test set were determined against *Staphylococcus aureus*, *Listeria monocytogenes*, *E. coli* and *Salmonella gallinarum*, and most exhibited moderate to potent activities. Most surprisingly, **5g** and **5s** exhibited excellent antibacterial activity against four bacteria. SAR analysis and molecular docking simulations show that the promising antibacterial efficacy of our designed compounds can be attributed to a combination of factors, such as 4-methyl substitution at the R position, variety of flavonoids and introduction of carbohydrate structure. We are further modifying the flavone Mannich base derivatives to obtain more effective Topo II inhibitors and looking for valuable information for further structural optimization and development of novel Topo II inhibitors.

Experimental

Chemistry

All chemicals were purchased from Energy, Meryer, and Aladdin Chemicals and were used as received. ^{1}H NMR and ^{13}C NMR spectra were carried out using Agilent DD2 600 Hz spectrometer with DMSO-d6 as the solvent and tetramethylsilane as the internal standard. ESI–MS spectra were carried out on a Mariner System 5304 mass spectrometer. Elemental analyses were performed on a CHN-O-Rapid instrument. Quantitative structure–activity relationship (QSAR) analyses and molecular docking were performed with Discovery Studio3.5.

General procedure for synthesis of target compounds

The flavone (1 mmol) was dissolved in isopropanol (20 mL), 37% formaldehyde (75 μL) was added, and the amine

(1.5 mmol) was added after stirring for 20 min. The mixture was reacted at 35 °C until complete, filtered, washed with isopropanol and then finally dried to give the target compound. The experimental process is monitored by TLC.

Biological assay

Minimum inhibitory concentration (MIC) [21]

The in vitro antibacterial activity for all synthesized compounds was evaluated using the agar dilution method [22, 23]. Twofold serial dilutions of the compounds and reference drug (ciprofloxacin) were prepared in LB broth–agar medium. Compounds (10.0 mg) were dissolved in DMSO (1 mL), and the solution was diluted with water (9 mL). Further progressive double dilution with melted LB broth–agar medium was performed to obtain the required concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.05 mg/L, and the MIC values were calculated separately.

Enzyme inhibitory effects against Topo II and Topo IV

The in vitro enzymatic inhibition assay of the target compounds was performed based upon methods described by Sato et al. [24] and Peng and Marians [25]. First, the *E. coli* suspension is extracted to obtain a crude enzyme solution. After the purification steps, the selected compounds are determined by gel electrophoresis to obtain data of different gradient concentrations, thereby calculating the IC_{50} values.

3D-QSAR analysis

The three-dimensional (3D) quantitative structure–activity relationship was performed with Discovery Studio3.5, using genetic function algorithm (GFA) [26]. In this study, 19 compounds with definite pMIC values were selected as the model dataset. Then, Discovery Studio3.5 was used to optimize the energy of the initial conformation of all compounds. Finally, we run the "Create 3D-QSAR Model" protocol to get the model.

Molecular docking

The crystal structure of bacterial DNA Topo II (PDB code:2XCS) was downloaded from the RCSB Protein Data Bank. In the "Tool Explore," we run the "Clean Protein" protocol to get protein that can be used for docking. Then, we define the active site of the receptor protein. Finally, we draw all the compounds in "Chemdraw" software and import them into Discovery Studio for ligand preparation. The molecular docking procedures were performed by using CDOCKER protocol for receptor–ligand interactions section of Discovery Studio3.5 [27].

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