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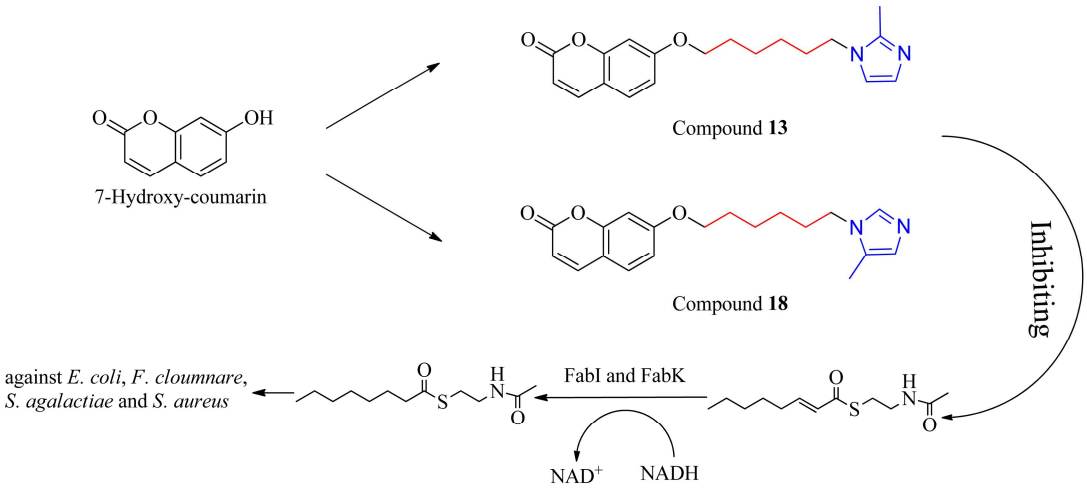

Synthesis and biological evaluation of coumarin derivatives containing imidazole skeleton as potential antibacterial agents

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Abstract

Emergence of multidrug-resistant bacteria causes an urgent need for new generation of antibiotics, which may have a different mechanism of inhibition or killing action from the existing. Here, we report on the design, synthesis, and biological evaluation of thirty-nine coumarin derivatives in order to solve the antibacterial resistance by targeting at the inhibition of biosynthesis pathway of fatty acids. Their antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Flavobacterium cloumnare* are tested and action mechanism against the key enzyme in bacterial fatty acid synthesis pathway are studied. The results show that compounds **13** and **18** have potent and broad spectrum antimicrobial activity. In addition, **9**, **14** and **19** show eminent antimicrobial efficacy toward *S. aureus*, *S. agalactiae*, and *F. cloumnare*. Mechanistically, coumarin derivatives display the antibacterial activity via the control of FabI and FabK function. The structure-activity relationship analysis indicate that the length of linker and imidazole substitute group could significantly influence the antimicrobial activity, as well as the inhibitory activity against FabI and FabK. The structural optimization analysis of coumarin suggest that derivatives **9, 13, 14, 18 and 19** could be a viable way of preventing and controlling bacteria and considered as promising lead compounds for the development of commercial drugs.

Keywords: coumarin derivatives, antibacterial activity, FabI, FabK

1. Introduction

Antibiotic resistance has been a global healthcare problem due to the indiscriminate use of antibiotics and the subsequent creation of bacteria that can survive traditional treatment [1,2]. Namely, antibiotics have dramatically reduced the incidence of many diseases infected by bacteria. In addition, antibiotic resistance has drastically outpaced new antibiotic discovery. For instance, methicillin-resistant *Staphylococcus aureus* is one of the main species of bacteria that cause nosocomial infections in hospitals worldwide [3-5]. It was only two years after the introduction of the methicillin in 1959 that resistant strains of the gram-positive human pathogen *Staphylococcus aureus* emerged. Additionally, many problems remain unresolved due to occasional serious side effects and the appearance of antibiotic-resistant mutant bacteria. Therefore, the search for new drugs effective for the treatment of bacterial infections is of high priority. Focusing on known validated intracellular targets remains a valid approach to identify new drug candidates with novel chemical structures [6]. Interestingly, use of natural products as a screening pool for novel antibiotics has several advantages related to safety, availability, and minimizing the risk of side-effect and addiction [7,8].

Natural products have been used to treat disease for thousands of years and play an increasingly important role in drug discovery and development. In fact, the majority of anti-infectious agents are of natural origin [9,10]. Natural product coumarin, the simplest member of the group of oxygen heterocyclics called benzo-2-pyrones, a secondary metabolite has been reported in many families of plants such as Apiaceae, Asteraceae, Fabiaceae, Rosaceae, Rubiaceae, Rutaceae and Solanaceae [15]. Natural products with a coumarinic moiety have been reported to have multiple biological activities including anticancer, antioxidant, antiinflammatory and antiviral [10-17]. In addition, some investigators have focused on the antimicrobial activity of coumarins [18-20]. For example, Wang et al.

has found that coumarin derivatives showed the antibacterial activity by inhibiting the key enzyme of biosynthesis pathway of fatty acids (FAS) [21].

One of the strategies adopted to control bacterial pathogens is to target the FAS-II, in which the highly conserved enoyl-acyl carrier protein reductase plays a key role [22]. Most eukaryotes contain type I FAS, where all the catalytic domains reside on one polypeptide. Prokaryotes and plants contain the type II or the dissociated FAS-II system, where each enzyme is found on a separate polypeptide. Enzyme catalyzes the last step in the bacterial FAS-II cycle, the reduction of α, β-unsaturated fatty acids esterified to the acyl carrier protein (ACP). The FAS-II pathway consists of some distinct and highly conserved enzymes: β-hydroxacyl-ACP-dehydratase (FabA, FabZ), β-ketoacyl-ACP-reductase (FabG), and enoyl-ACP-reductase (FabI, FabK, FabL) [23,24]. For most bacterias, FabI and FabK are responsible for the concluding reduction step of each elongation cycle. They represent a key physiological regulator of fatty acid biosynthesis, and they have been carried out to be the important drug targets for the development of novel antibacterials [25,26]. This has led to the pursuit of specific FabI and FabK inhibitors as novel antibacterial agents which would not be expected to interfere with comparable human biochemical processes. However, the information about the relationships between the chemical structures and activity of inhibiting FAS-II enzymes is currently limited.

The exploration of new heterocycles that can accommodate potency to multiple biological targets remains an intriguing scientific endeavour. In recent years, many hybrid molecules with coumarin based ring systems have been synthesized utilizing novel synthetic methodologies. Some coumarin derivatives conjugated with nitrogen-containing heterocyclic moieties, such as triazole and pyridine, were synthesized and proved to possess antibacterial bioactivity [27,28]. Thus, hybridizing the coumarin nucleus with other moieties has afforded new molecules with improved antimicrobial activity

profiles.

In this study, our work are directed toward the coumarin antibiotics and their structural modification. According to the previous studies, we hypothesized 1). Coumarin derivatives showed the antibacterial activity by targeting the FAS-II. 2). The chemical structures of coumarin derivatives influenced the antimicrobial activity. The previous researches promoted us to synthesize new derivatives of coumarins with imidazoles skeleton for the sake of finding new effectively antibacterial agents. Based on these things mentioned above, we synthesized several series of novel coumarins derivatives owning nitrogen-containing heterocyclic with different length of chemical bridges. We studied their antibacterial activities against four strains of bacteria of *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*S. agalactiae*), and *Flavobacterium cloumnare* (*F. cloumnare*). Futhermore, we focused on the expression and purification of the recombinant FabI and FabK protein to investigate the mechanism of the inhibitory activity of coumarins derivatives. This study also helped rationalize activities at the investigated drugs targets, pointing to potential strategies for designing more selective compounds.

2. Results and discussion

2.1 Chemistry

The synthesis of initial compounds 7-hydroxy coumarin and 4-methyl-7-hydroxycoumarin were shown in Scheme 1. The 7-hydroxycoumarin was obtained by heating the solution of 2,4-dihydroxybenzaldehyde and $ClCH_2COOC_2H_5$ together with PH_3^+-$CH_2COOC_2H_5$ in ethanol. The 4-methyl-7-hydroxycoumarin was obtained by heating the solution of resorcinol and ethyl acetoacetate together in sulfuric acid.

Scheme 1

The syntheses of the thirty-nine derivatives are based on well described reactions in previous study [29]. Synthesis of coumarin derivatives **1**-**33** followed the general pathway depicted in Scheme 2-4. Compounds **1-5** were synthesized from 7-hydroxy coumarin by reacting with corresponding α, ω-dibromoalkanes and triethylamine in anhydrous acetone at reflux condition. Compounds **6-33** were synthesized in 60-90% yield by treatment of **1-5** compounds with corresponding amines and anhydrous potassium carbonate in acetonitrile at room temperature. In particularly, the derivative **29** was synthesized in 95% yield by treatment of compound **4** using Gabriel reaction.

Scheme 2

Scheme 3

Scheme 4

Scheme 5 reported the synthetic pathway used to prepare compounds **34**-**39**. To assess the effects of the methyl at the *para*-position of oxygen atom, we changed the initial compound 7-hydroxy coumarin to 4-methyl-7-hydroxy coumarin for synthesizing derivatives **34**-**39**. The structures of a total thirty-nine synthesized compounds were confirmed by ESI-MS, ^{1}H and ^{13}C NMR (see the Supplementary data).

Scheme 5

Antibacterial activity

2.2 MICs

The synthesized compounds were tested for their antibacterial activities against two Gram-negative bacteria strains: *E. coli* and *F. cloumnare* and two Gram-positive bacteria strains: *S.*

aureus and *S. agalactiae*. The MICs (minimum inhibitory concentrations) of the compounds against these bacteria were reported in Table 1 and 2, the activities of reference drugs enrofloxacin and norfloxacin were also included. We sought to characterize the structure activity relationship (SAR) for this class of compounds by introducing substituents on the aromatic ring of hydroxy and investigating those compounds with different length of "*C-linker*". We also considered the role of the imidazole analogues in the other item of the linker. Finally, we studied a limited set of variants by exploring substituents of methyl at the C-4 position.

In line with results reported for **14** were against *F. cloumnare*, *S. agalactiae* and *S. aureus* with 2, 4, and 16 μM MIC values, respectively (Table 1). Compound **14** was equal to the positive control enrofloxacin with MIC values of 2 and 8 μM against *F. cloumnare*, *S. agalactiae*, respectively (Table 1). Consistently and in line with the results obtained with the imidazole and 4-methylimidazole derivatives, compounds **9** and **19**, displayed the potent activities against *F. cloumnare*, *S. agalactiae* and *S. aureus* with MIC values of 4, 8, 16 μM and 4, 4, 8 μM, respectively. Remarkably, derivative **28** which introduced a benzimidazole group at the position of the end of linker which had the length of eight atom, exhibited a dramatic loss in activities against *F. cloumnare*, *S. agalactiae* and *S. aureus* with MIC values of 8, 256, 32 μM. Additionally, introducing other substituent group at the position of the item of linker, as with bromine-bearing (**1**, **2**, **3**, **4**, and **5**) or 2-phenylimidazole bearing (**22**, **23**, and **24**) derivatives, did not show activities at these three strains of bacteria.

Table 1

In light with these results, we proposed a rationale for the chemical features required for a balanced and potent activities at *F. cloumnare*, *S. agalactiae* and *S. aureus*. It was found that the length of the R^1 substituent group ("*linker*") played an important role in the antibacterial activities of

compounds. We therefore turned our attention to derivatives bearing this moiety. Reducing or increasing the length of the linker of **14** to six (**13**) or ten (**15**) methylene units were detrimental for activities at the three targets. As anticipated, the antibacterial activities of compounds **13** and **15** against *F. cloumnare*, *S. agalactiae* and *S. aureus* decreased quite substantially (**13**: 16, 128, 64 μM; **15**: 8, 8, 64 μM;). This pattern was also observed in other two coumarin-imidazole hybrid derivatives. Among compounds **6**-**10** and **16**-**20** the order of antibacterial activities showed the potency of **9** > **10** > **8** > **7** > **6** and **19** > **20** > **18** > **17** > **16** which indicated the activities of compounds against these three bacteria with eight carbon atoms of linker were superior to that of with other length of linker.

Notably, activity at *E. coli* of compounds **1**-**28** behaved different rule from the other three strains. According to the date, compounds with six carbon atoms of linker showed the significantly higher activities against *E. coli* than other length of linker. For instance, in **13**, the efficacy against *E. coli* was 8 μM MIC value. By contrast, the results for **11**, **12**, **14**, and **15** suggested that extending or narrowing the carbon chains would lead to a drop in activity against *E. coli*. Derivative **8** maintained the potent activities with MIC values of 32 μM. In agreement with the behavior of **8**, **13**, and **18**, introducing a six carbon atoms of linker would be the best choice for activity against *E. coli*. On the other hand, when the R_2 position coupled with a 2-phenylimidazole or benzimidazole, the suitable length of linker would be four methylene units. It was found that compounds **22** and **26** were 32, 8 μM MIC values respectively with the highest activities among their own groups.

The effect of imidazole group on potency was apparent throughout our results, with its presence at the R_2 position increasing the potency in every example. According to the results, we suggested that the types of substituent group of R^2 could be crucial to the efficacy against bacteria. We synthesized five compounds with the same linker but different types of heterocyclic nitrogen groups in R^2 substituted

position. Predictably, the analogues displayed huge difference of antibacterial activities. Among compounds **9**, **14**, **19**, **24**, and **28,** the order of antibacterial activities showed the potency of **14** > **19** > **9** > **28**> **24** which indicated the activities of compounds with 2-methylimidazole substitutes were superior to that of with the other four substitutes.

To assess the effects of the types of R^2 substituents, we synthesized and tested compounds **29**-**33** which had same length of linker at R_1 position but different substituent groups at R^2 position against *F. cloumnare* and *S. agalactiae*. As shown in Table 2, only derivative **30** coupled with a triazole maintained the activities against *F. cloumnare* and *S. agalactiae* among these five compounds, with the MIC values of 64 and 32 μM, respectively. The other four derivatives displayed no activities against two bacteria at the concentration of 256 μM.

In addition, we further investigated the effect of methyl at the C-4 position. In light with the results of compounds **34**-**39** against four strains of bacteria, we suggested that introducing a methyl group in the distal ring of the O-biphenyl moiety led to a slight drop in activities. Namely, compound **35** showed anti-*F. cloumnare*, *S. agalactiae* and *S. aureus* activities with the MIC values of 4, 8, 64 μM while **9** which lacked a methyl but had the same length of linker was 4, 8, 16 μM MIC values, respectively. Similarly, derivatives **36** and **37** decreased the activities against *F. cloumnare*, *S. agalactiae* and *S. aureus* to 4, 16, 64 μM and 8, 8, 32 μM, respectively. Interestingly, compound **38** increased the activity at *S. agalactiae* to 32 μM compared to **28** (256 μM). Derivative **39** was 16 μM against *F. cloumnare* while its analogue **30** exhibited the activity with MIC value of 64 μM. Consistently, all six derivatives didn't display the activity against *E. coli* at the concentration of 256 μM.

Table 2

195 *2.3 MBCs*

196 We further investigated the MBC values of all compounds against four bacterials. The results
197 confirmed that the four overarching trends what we had noted previously were also suitable for the date
198 of MBC values. First, the eight methylene chains was the best choice for the coumarin derivatives
199 against the three strains of *F. cloumnare*, *S. agalactiae* and *S. aureus*. Extending or narrowing the
200 length of alkyl chain between the corresponding amine and coumarin led to a significant reduction in
201 activities at the three bacterials. For example, the compounds **8**, **9**, and **10**, with the same R^2 substituent
202 group but different in the length of linker, exhibited a great difference in antibacterial activity.
203 Compound **9** which had a six carbon atoms of linker on the position of R^1 showed activity against *F.*
204 *cloumnare* with the MBC value of 8 μM. Otherwise, the MBC values were increased to 16 or 128 μM,
205 if the length was ten or six carbon atoms.

206 Secondly, we suggested that compounds with six carbon atoms of linker would increase the anti-*E.*
207 *coli* activity notably. Derivatives **8** and **13** were the same MBC values of 64 μM. Most importantly,
208 when the same substitution was coupled with a longer or shorter linker, activity at *E. coli* was
209 intensively affected. As shown in Table 3, compounds **7** and **12** displayed the activities with the MBC
210 values of 256 μM. For another, the same trend noted previously was observed from MBC values of
211 **21**-**28**. Derivatives **22** and **26** which were coupled with the four methylene units exhibited the highest
212 activities with MBC values of 128 and 64 μM, respectively. In addition, there are several investigators
213 demonstrated that the bioactivities were influenced by the carbon linker, such as antibacterial, anti-HIV
214 and anti-parasites [30-32]. Indeed, the length of carbon chain was crecial to the antibacterial activities.

215 Table 3

216 Subsequently, the substituents at R^2 position also occupied an important place in the efficacy of

antibacterial. The coumarin derivatives which coupled with imidazole, 2-methylimidazole, 4-methylimidazole or triazole revealed superiority in against bacteria to the compounds coupled with 2-phenylimidazole, benzimidazole, and so on. In **30**, coupled with a triazole group, activities at *F. cloumnare* and *S. agalactiae* were 128 and 64 μM MBC values respectively while other four derivatives (**29**, **31**, **32**, **33**) did not show efficacy against the two strians of bacteria.

Moreover, imidazole analogues had reported other antibacterial activities which was consistent to our result that it could increase the activity against bacteria [33,34]. Furthermore, its derivatives are of great importance in medicinal chemistry and can be used for the synthesis of myriad heterocyclic compounds with different biological activities such as anti-tumor, antithrombotic, antioxidative [35-38]. These studies indicated that synthesis of coumarin-imidazole hybrid derivatives is a right guidance for further researches. The presence of 2-methyl was the structural feature exerting the more evident effect, as suggested by activity data on **14**.

Lastly, it was also confirmed that introducing a methyl group in the distal ring of the O-biphenyl moiety led to a slight influence in activities by the results of **34**-**39**. Similarly, compound **35** showed anti-*F. cloumnare*, *S. agalactiae* and *S. aureus* activities with the MBC values of 16, 16, 256 μM while **9** which had the same carbon chain was 8, 16, 128 μM MBC values, respectively. Consistently, compounds **36** and **37** decreased the activities against *F. cloumnare*, *S. agalactiae* and *S. aureus* to 8, 16, 256 μM and 16, 32, 256 μM, respectively. Conversely, compound **38** increased the activity at *S. agalactiae* to 64 μM compared to **28** (512 μM). Derivative **39** was 64 μM against *F. cloumnare* while its analogue **30** exhibited the activity with MIC value of 128 μM.

Table 4

2.4 Expression and characterization of recombinant protein

The amplification of the FabI gene (expected size 760 bp) of *E. coli* isolate ATCC 35150 and the FabK gene (expected size 960 bp) of *S. agalactiae* isolate WB1445 were shown in Fig. 1A and Fig. 2A, respectively. The plasmids pET32a-FabI and pET32a-FabK were confirmed by restriction enzyme digestion (Fig. 1B and Fig. 2B) and sequence analysis (data not show). Both results confirmed that recombinant plasmids were successfully constructed. Expression analysis of abundant FabI protein and FabK protein were performed after 4 h of induction with 1 mM IPTG. And SDS-PAGE analysis showed that the molecular size of recombinant protein containing 6-histidine tag were estimated to be approximately 43 kDa and 48 kDa, respectively. (Fig. 1C, Fig. 2C). Western blot analysis using anti-His-tag monoclonal antibody confirmed the authenticity of the FabI and FabK as clear bands at the expected position on polyvinylidene fluoride membrane (Fig. 1D, Fig. 2D).

Figure 1

Figure 2

2.5 Identification of (E)-2-octenoyl-acyl-N-acetylcysteamine (t-O-NAC)

By analyzing the data of ^{1}H NMR (nuclear magnetic resonance) (500.23 MHz, MeOD), ^{13}C NMR (125.78 MHz, MeOD), t-O-NAC was identified and its chemical structure was shown in Scheme 6. In addition, the date of general nuclear magnetic resonance spectrum was shown in supplementary date.

Scheme 6

2.6 E. coli FabI and *S. agalactiae* FabK inhibitory activity

In order to investigate the structural requirements involving the pyridoindole core, we have chose 10 derivatives with a substituent of diverse functionalities to rationalize the FabI and FabK inhibitory activities of these compounds in terms of structural modifications. The IC_{50} values of these ten

compounds which showed superior antibacterial activities to inhibit the recombinant FabI and FabK enzyme were shown in Table 5. All derivatives showed the activities of inhibitory FabI and FabK to varying degrees. In **13**, activity at FabI was 1.20 μM IC_{50} value which was the highest inhibitory efficacy. Similarly, in line with result reported for **14**, exhibited the best inhibitory FabK activity with IC_{50} value of 1.13 μM. Conversely, compounds **10** and **12**, displayed the lowest activities inhibitory FabI and FabK, respectively. In light with these results, we suggested a rationale for the chemical features required for potent inhibitory FabI and FabK activities. When the coumarin derivatives coupled with a six methylene units, it would show the balanced perferable inhibitory efficacy of the two enzymes. Interestingly, extending the length of alkyl chain led a change in the balance of inhibitory two proteins. When the length of linker was more than six carbon atoms, derivatives showed the better inhibitory FabK activity than FabI. In comparison, narrowing the length of chain made a converse result. Namely, when the length of alkyl chain was less than six methylene units, activity of derivatives would be led a enhancement at FabI but a drop at FabK.

Table 5

The results of FabI and FabK inhibitory activity of the test compounds were corresponding to the structure-activity relationships of their antibacterial activities. It demonstrated that the potent antibacterial activities of the synthetic compounds were probably correlated to their FabI and FabK inhibitory activities.

3. Conclusions

The coumarin derivatives were structurally designed by employing a molecular hybridization of the coumarin group with the heterocyclic ring imidazole. Thirty-nine coumarin derivatives were synthesized and their antibacterial activity was evaluated against *E. coli*, *F. cloumnare*, *S. aureus* and *S.*

agalactiae. Moreover, the inhibitory activity was evaluated against FabI and FabK. The most promising compounds showed both antibacterial and inhibitory activity and, in particular, the compounds **9**, **13**, **14, 18** and **19**, also exhibited remarkable activity against *F. cloumnare*, *S. aureus* and *S. agalactiae*. The ensuing structure-activity relationships study predictably identified antimicrobial activity represented by the R^1 and R^2 substituent group. Eight carbon atoms length of linker (R^1 substitute position) and imidazole could significantly increase the antimicrobial activity. In addition, six carbon atoms length of linker could be the suitable choice for the inhibitory activity against FabI and FabK. Furthermore, derivative **14**, the most potent compound, showed broad range activity in all in vitro experiments and might represent a good starting point for optimizing the structure to develop more active antibacterials.

In conclusion, the results obtained in this work highlight imidazole substituted coumarin as very promising antibacterial scaffolds acting through enoyl-acyl carrier protein reductases inhibition. This study suggests that the development of more potent enoyl-acyl carrier protein reductases inhibitors could lead to novel broad-spectrum antibacterial drug candidates.

4. Experimental section

4.1 Bacterial strain and growth conditions

Bacterial reference strains of *E. coli* ATCC 35150 and *S. aureus* ATCC 29213 were kindly provided by Prof. Jin-you Duan in college of Chemistry & Pharmacy, Northwest A&F University, Yangling, Shaanxi, China. Bacterial reference strain of *S. agalactiae* WB1445 was kindly provided by Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, Guangdong, China. Bacterial reference strain of *F. cloumnare* FC-G1 was preserved in our laboratory

with 20% glycerol at -80 °C. For experimental work, *E. coli* and *S. aureus* were cultured in Broth medium (BM) at 28 °C and grown overnight with constant shaking. Brain heart infusion (BHI) broth and Shieh broth were chosed for culturing *S. agalactiae* and *F. cloumnare*, respectively.

4.2 Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) of the synthetic compounds were tested against two Gram-negative bacterial strains: *E. coli* and *F. cloumnare*, two Gram-positive bacterial strains: *S. aureus* and *S. agalactiae*, using method recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2009, 2012). In vitro activities of the compounds were tested in Nutrient broth (NB) for bacteria by the two-fold serial dilution method. Seeded broth (broth containing microbial spores) was prepared in NB from 24 h old bacterial cultures on corresponding broth at 28 ± 1 °C. The bacterial suspension was adjusted with sterile saline to a concentration of 1×10^7-2×10^7 cfu/mL and diluted with corresponding broth to a concentration of 1×10^5-2×10^5 cfu/mL. The tested compounds and reference drugs were prepared by two-fold serial dilution to obtain the required concentrations of 256.00, 128.00, 64.00, 32.00, 16.00, 8.00, 4.00, 2.00, 1.00, 0.50 μM and even lower concentration. The tubes were incubated in BOD incubators at 28 ± 1 °C for bacteria. The MICs were recorded by visual observations after 24 h (for bacteria) of incubation. Enrofloxacin and norfloxacin were used as standards for bacteria.

4.3 Minimal bactericidal concentration (MBC)

The method used and described below is an amended version of the procedure described in the BSAC Guide to Sensitivity Testing and can be adapted for determining the minimal bactericidal concentration (MBC) of coumarin derivatives by substituting IsoSensitest broth (ISA;Oxoid) with

corresponding broth. After MIC determination of the compounds tested, an aliquot of 100 µl from all tubes in which no visible bacterial growth was observed were seeded in Nutrient Agar (NA) plates not supplemented with coumarin derivatives. The plates were then incubated for overnight at 28 ± 1 °C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the NA plates.

4.4 Expression and purification of the recombinant FabI and FabK protein

Primers were designed according to the FabI sequence of *E. coli* ATCC 35150 (Genebank accession: YP_006119687). The FabI gene amplification was carried out using genomic DNA of *E. coli* as template and two oligonucleotide primers (Forward: 5'-AGGAGATATACCATGGGTTTTCTTTCCGGTAAGCGCATTCTG -3'; Reverse: 5'-CGCCGAGCTCGAATTCTTATTTCAGTTCGAGTTCGTTCATTGC -3') which introduced *BamH I* and *Hind III* restriction enzyme sites, respectively. The PCR products were ligated with the cloning vector pMD19T (Takara, Dalian, China) to generate the pMD19T-FabI, which was confirmed by sequence analysis (Sangon, Shanghai, China).

Similarly, primers were designed according to the FabK sequence of *S. agalactiae* WB1445 (Genbank accession: NC_004116.1). The FabK gene amplification was carried out using genomic DNA of *S. agalactiae* as template and two oligonucleotide primers (Forward: 5'-ATCGCCATGGTTGATCGGTACTCACTCTTCTTC -3'; Reverse: 5'-ATCGGAATTCTTATAAATCTGACCAGCGGC-3') which introduced *BamH I* and *Hind III* restriction enzyme sites, respectively. The PCR products were ligated with the cloning vector pMD19T (Takara, Dalian, China) to generate the pMD19T-FabK, which was confirmed by sequence analysis (Sangon, Shanghai, China).

346 The FabI (FabK) gene excised from pMD19T-FabI (pMD19T-FabK) by digestion with *BamH*
347 *I*/*Hind III* restriction enzyme (Takara, Dalian, China) was inserted into expression plasmid pET32a (+)
348 (Novagen, Madison, USA) to generate pET32a-FabI (pET32a-FabK). The resulting recombinant
349 plasmid was transformed into *E. coli* BL21 (DE3) (Novagen, Madison, USA), and the recombinant
350 constructs pET32a-FabI (pET32a-FabK) were sequenced by Sangon Biological Company (Shanghai,
351 China). The positive recombinant *E. coli* was grown in Luria bertani (LB) broth with ampicillin (100
352 mg/mL) at 37 °C with shaking, and the expression of recombinant FabI (Fabk) protein was induced by
353 1 mM isopropyl-b-D-thiogalacto-pyranoside (IPTG; SigmaeAldrich Trading Co., Ltd, Shanghai,
354 China). The obtained fusion proteins were determined by sodium dodecyl sulfate-polyacrylamide gel
355 electrophoresis (SDS-PAGE) and visualized after staining with Coommassie brilliant blue R-250
356 (Sigma, USA). Then the recombinant proteins were assessed by western blotting analysis using diluted
357 anti-His-tag monoclonal antibody (1:2000, Abcam, Cambridge, MA, USA) and HRP-conjugated goat
358 anti-mouse IgG (1:2000, Beijing CoWin Biotech Corp., Beijing, China) as antibodies. The result was
359 visualized by staining with DAB (3,3-diamonobenzidine tetrahydrochloride) horseradish peroxidase
360 color development kit (Qiagen, Hilden, Germany).

361 Owning to the hexa-histidine tag at the N-terminus, the recombinant proteins from the constructs
362 were purified using nickelnitrilotriacetic acid (Ni-NTA) column (Qiagen, Shanghai, China) under
363 native conditions as recommended by the manufacturer. After affinity chromatography, the fractions
364 were pooled and dialysed using urea gradient dialysis method. The protein concentration was
365 determined by Micro BCA Protein Assay Kit (Beijing CoWin Biotech Corp., Beijing, China).

366 *4.5 The procedure for the synthesis of (E)-2-octenoyl-acyl-N-acetylcysteamine (t-O-NAC)*

367 To a solution of *(E)*-2-octenoic acid (569 mg, 4 mmol) in dichloromethane (20 mL) was added N,

N-diisopropylcarbodiimide (528 mg, 4mmol) and 4-dimethylaminopyridine (200 mg, 2mmol) at room temperature. After stirring at room temperature for 10 min, *N*-Acetylcysteamine (300 mg, 3 mmol) was added to the reaction mixture, and whole was stirred under room temperature for 24 h. The precipitate was filtered off and washed with dichloromethane (3×40 mL). The solvent was evaporated under reduced pressure, and the residue was treated with water (30 mL) and extracted with ethyl acetate (3×40 mL). The organic layer were combined, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified via silica gel column chromatography with mixed petroleum ether and ethyl acetate (3:1, v/v) as eluent.

4.6 E. coli FabI and *S. agalactiae* FabK inhibitory activity

The measurements of *E. coli* FabI inhibitory activity were performed on an Infinit eM200 pro plate reader (Tecan, Switzerland) in 100 μL of 0.1 M PBS-DTT PH 7.5. The compounds were dissolved in 0.1 M PBS-DTT with a series of concentration as 128, 64, 32, 16, 8, 4, 2 and 1 μM, respectively, then tested in the presence of 20 μL (100 μg/mL) FabI and 10 μL (4 mM) *t*-O-NAC. The reaction was started by addition of 20 μL (2 mM**)** NADH. The reaction mixture was read spectrophotometrically for 1 min by follow the oxidation of NADH to NAD^+ at 340 nm. IC_{50} values were estimated from graphically plotted dose–response curves.

The measurements of *S. agalactiae* FabK inhibitory activity were performed on an Infinit eM200 pro plate reader (Tecan, Switzerland) in 100 μL of 0.1 M ADA-DTT PH 6.5. The compounds were dissolved in 0.1 M ADA-DTT with a series of concentration as 128, 64, 32, 16, 8, 4, 2 and 1 μM, respectively, then tested in the presence of 20 μL (150 μg/mL) FabK and 10 μL (4 mM) *t*-O-NAC. The reaction was started by addition of 20 μL (2 mM**)** NADH. The reaction mixture was read spectrophotometrically for 1 min by follow the oxidation of NADH to NAD^+ at 340 nm. IC_{50} values

were estimated from graphically plotted dose–response curves.

Notes

The authors declare no competing financial interest.

Acknowledgement

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Figure captions

Figure 1. Analysis of FabI expression. (A) PCR amplification of FabI: lane M, DNA marker; lane 1, FabI. (B) Analysis of recombinant plasmid: lane M, DNA marker; lane 1, double enzymes digested pET32a-FabI with *BamH I* and *Hind III*.(C) SDS-PAGE analysis of expressed pET32a-FabI: lane M, Standard protein marker; lane 1, Soluble lysates from uninduced cells containing recombinant pET32a-FabI; lane 2, Insoluble lysates from uninduced cells containing recombinant pET32a-FabI; lane 3, Soluble lysates from induced cells containing recombinant pET32a-FabI; lane 4, Insoluble lysates from induced cells containing recombinant pET32a-FabI. (D) Western blotting analysis of purified FabI using anti-his-tag monoclonal antibody.

Figure 2. Analysis of FabK expression. (A) PCR amplification of FabK: lane M, DNA marker; lane 1, FabK. (B) Analysis of recombinant plasmid: lane M, DNA marker; lane 1, double enzymes digested pET32a-FabK with *BamH I* and *Hind III*.(C) SDS-PAGE analysis of expressed pET32a-FabI: lane M, Standard protein marker; lane 1, Empty pET32a control (uninduced by IPTG); lane 2, Insoluble lysates from induced cells containing recombinant pET32a-FabK; lane 3, Soluble lysates from induced cells containing recombinant pET32a-FabK; lane 4, Insoluble lysates from uninduced cells containing recombinant pET32a-FabK; lane 5, Soluble lysates from uninduced cells containing recombinant pET32a-FabK. (D) Western blotting analysis of purified FabK using anti-his-tag monoclonal antibody.

Scheme 1. Synthetic route of initial compounds 7-hydroxycoumarin and 4-methyl-7-hydroxycoumarin. Reagents and conditions: (a) $ClCH_2COOC_2H_5$, Ph_3^+-$CH_2COOC_2H_5$, CH_3CH_2OH, 80 °C, 2 h; (b) Ethyl acetoacetate, H_2SO_4, ice bath, 16 h.

Scheme 2. Synthetic route of coumarin derivatives **1**-**28**. Reagents and conditions: (c) alkyl dibromide, K_2CO_3, dry acetone, reflux, 20-24 h; (d) imidazole / 2-methylimidazole / 4-methylimidazole / 2-phenylimidazole / benzimidazole, K_2CO_3, CH_3CN, r.t., 20-24 h.

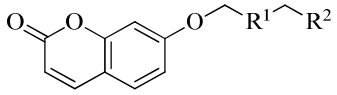
Scheme 3. Synthetic route of coumarin derivatives **29**. Reagents and conditions: (e) Phthalimide potassium salt, DMF, 100 °C, 20-24 h; (f) $NH_2NH_2 \cdot H_2O$, CH_3CH_2OH, 80 °C, 6-8 h.

Scheme 4. Synthetic route of coumarin derivatives **30**-**33**. Reagents and conditions: (c) 1,6-dibromohexane, K_2CO_3, dry acetone, reflux, 20-24 h; (d) triazole / piperazine / parazole / piperidine, K_2CO_3, CH_3CN, r.t., 20-24 h.

Scheme 5. Synthetic route of coumarin derivatives **34**-**39**. Reagents and conditions: (c) 1,6-dibromohexane, K_2CO_3, dry acetone, reflux, 20-24 h; (d) imidazole / 2-methylimidazole / 4-methylimidazole / benzimidazole / triazole, K_2CO_3, CH_3CN, r.t., 20-24 h.

Scheme 6. Synthetic route of (*E*)-2-octenoyl-acyl-N-acetylcysteamine. Reagents and conditions: (g) *N*-Acetylcysteamine, DMAP, DIC, CH_2Cl_2, r.t., 24 h.

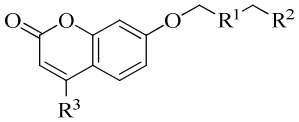
Table 1. MIC values of coumarin derivatives **1**-**28**.



Compound	Substituent group		MIC (μM)			
	R^1	R^2	Gram-negative		Gram-positive	
			F.cloumnare	*E. coli*	*S.agalactiae*	*S. aureus*
1	-		>256.00	>256.00	>256.00	>256.00
2	$-C_2H_4-$		>256.00	>256.00	>256.00	>256.00
3	$-C_4H_8-$	-Br	>256.00	>256.00	>256.00	>256.00
4	$-C_6H_{12}-$		>256.00	>256.00	>256.00	>256.00
5	$-C_8H_{16}-$		>256.00	>256.00	>256.00	>256.00
6	-		>256.00	128.00	>256.00	>256.00
7	$-C_2H_4-$		>256.00	128.00	>256.00	64.00
8	$-C_4H_8-$		64.00	32.00	>256.00	64.00
9	$-C_6H_{12}-$		4.00	>256.00	8.00	16.00
10	$-C_8H_{16}-$		16.00	>256.00	8.00	64.00
11	-		>256.00	64.00	>256.00	>256.00
12	$-C_2H_4-$		>256.00	64.00	>256.00	256.00
13	$-C_4H_8-$		16.00	8.00	128.00	64.00
14	$-C_6H_{12}-$		2.00	>256.00	4.00	16.00
15	$-C_8H_{16}-$		8.00	>256.00	8.00	64.00
16	-		>256.00	16.00	>256.00	>256.00
17	$-C_2H_4-$		>256.00	16.00	>256.00	>256.00
18	$-C_4H_8-$		32.00	16.00	128.00	32.00
19	$-C_6H_{12}-$		4.00	>256.00	4.00	8.00
20	$-C_8H_{16}-$		16.00	>256.00	8.00	32.00
21	-		>256.00	128.00	>256.00	>256.00
22	$-C_2H_4-$		>256.00	32.00	>256.00	>256.00
23	$-C_4H_8-$		>256.00	>256.00	>256.00	>256.00
24	$-C_6H_{12}-$		>256.00	>256.00	>256.00	>256.00
25	-		>256.00	128.00	>256.00	64.00
26	$-C_2H_4-$		>256.00	8.00	>256.00	32.00
27	$-C_4H_8-$		32.00	>256.00	32.00	32.00
28	$-C_6H_{12}-$		8.00	>256.00	256.00	32.00
Enrofloxacin			2.00	1.00	8.00	2.00
Norfloxacin			32.00	1.00	64.00	32.00

"-" Means none carbon atom.

Table 2. MIC values of coumarin derivatives **29**-**39** .



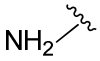
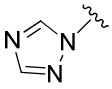
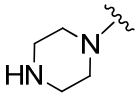
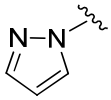
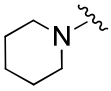
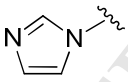
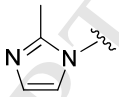
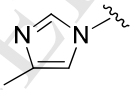
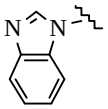
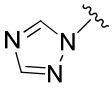
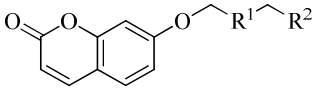
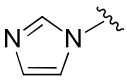
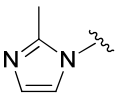
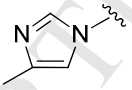
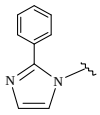
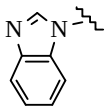
Compound	Substituent group			MIC (μM)			
				Gram-negative		Gram-positive	
	R^1	R^2	R^3	*F.cloumnare*	*E. coli*	*S.agalactiae*	*S. aureus*
29				>256.00		>256.00	
30				64.00		32.00	
31	-C_6H_{12}-		H	>256.00		>256.00	
32				>256.00		>256.00	
33				>256.00		>256.00	
34	-C_6H_{12}-	-Br	CH_3	>256.00	>256.00	>256.00	>256.00
35				4.00	>256.00	8.00	64.00
36				4.00	>256.00	16.00	64.00
37	-C_6H_{12}-		CH_3	8.00	>256.00	8.00	32.00
38				16.00	>256.00	16.00	32.00
39				32.00	64.00	32.00	32.00
Enrofloxacin				2.00	1.00	8.00	2.00
Norfloxacin				32.00	1.00	64.00	32.00

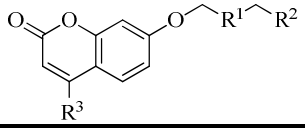
Table 3. MBC values of coumarin derivatives **1**-**28**.



Compound	Substituent group		MBC (μM)			
	R^1	R^2	Gram-negative		Gram-positive	
			F.cloumnare	*E. coli*	*S.agalactiae*	*S. aureus*
1	-		>256.00	>256.00	>256.00	>256.00
2	$-C_2H_4-$		>256.00	>256.00	>256.00	>256.00
3	$-C_4H_8-$	-Br	>256.00	>256.00	>256.00	>256.00
4	$-C_6H_{12}-$		>256.00	>256.00	>256.00	>256.00
5	$-C_8H_{16}-$		>256.00	>256.00	>256.00	>256.00
6	-		>256.00	256.00	>256.00	>256.00
7	$-C_2H_4-$		>256.00	256.00	>256.00	256.00
8	$-C_4H_8-$		256.00	64.00	>256.00	64.00
9	$-C_6H_{12}-$		8.00	>256.00	16.00	128.00
10	$-C_8H_{16}-$		32.00	>256.00	16.00	128.00
11	-		>256.00	256.00	>256.00	>256.00
12	$-C_2H_4-$		>256.00	256.00	>256.00	256.00
13	$-C_4H_8-$		32.00	64.00	256.00	256.00
14	$-C_6H_{12}-$		8.00	>256.00	16.00	128.00
15	$-C_8H_{16}-$		16.00	>256.00	16.00	128.00
16	-		>256.00	64.00	>256.00	>256.00
17	$-C_2H_4-$		>256.00	32.00	>256.00	>256.00
18	$-C_4H_8-$		128.00	64.00	256.00	128.00
19	$-C_6H_{12}-$		8.00	>256.00	16.00	64.00
20	$-C_8H_{16}-$		16.00	>256.00	16.00	128.00
21	-		>256.00	512.00	>256.00	>256.00
22	$-C_2H_4-$		>256.00	128.00	>256.00	>256.00
23	$-C_4H_8-$		>256.00	>256.00	>256.00	>256.00
24	$-C_6H_{12}-$		>256.00	>256.00	>256.00	>256.00
25	-		>256.00	256.00	>256.00	256.00
26	$-C_2H_4-$		>256.00	64.00	>256.00	256.00
27	$-C_4H_8-$		128.00	>256.00	128.00	256.00
28	$-C_6H_{12}-$		32.00	>256.00	512.00	128.00
Enrofloxacin			2.00	2.00	16.00	4.00
Norfloxacin			128.00	2.00	128.00	64.00

"-" Means none carbon atom.

Table 4. MBC values of coumarin derivatives **29**-**39**.



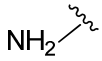
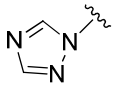
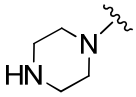
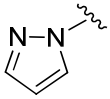
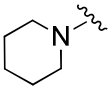
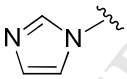
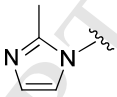
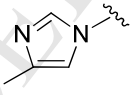
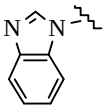
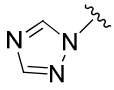
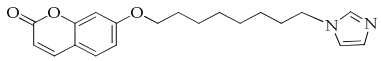
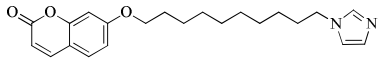
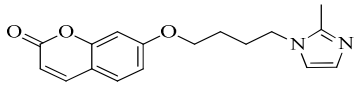
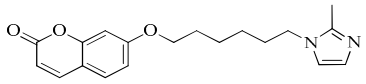
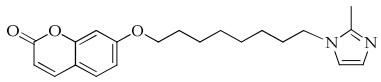
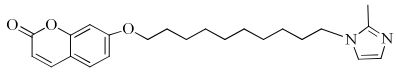
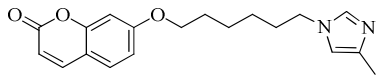
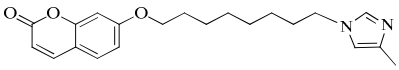
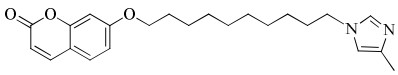
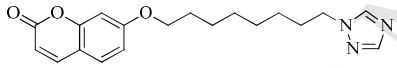
Compound	Substituent group			MBC (μM)			
	R^1	R^2	R^3	Gram-negative		Gram-positive	
				F.cloumnare	*E. coli*	*S.agalactiae*	*S. aureus*
29				>256.00		>256.00	
30				128.00		64.00	
31	-C_6H_{12}-		H	>256.00		>256.00	
32				>256.00		>256.00	
33				>256.00		>256.00	
34	-C_6H_{12}-	-Br	CH_3	>256.00	>256.00	>256.00	>256.00
35				16.00	>256.00	16.00	256.00
36				8.00	>256.00	16.00	256.00
37	-C_6H_{12}-		CH_3	16.00	>256.00	32.00	256.00
38				64.00	>256.00	64.00	256.00
39				64.00	128.00	64.00	256.00
Enrofloxacin				2.00	2.00	16.00	4.00
Norfloxacin				128.00	2.00	128.00	64.00

Table 5. The IC_{50} value of FabI and FabK enzyme catalytic activity inhibited by coumarin compounds.

Compound	Structure	FabI IC_{50} (μM)	FabK IC_{50} (μM)
9		158.12	2.74
10		>256	5.03
12		4.46	>256
13		1.20	3.44
14		111.55	1.13
15		107.70	4.21
18		1.35	3.59
19		116.53	1.75
20		114.84	4.37
30		79.53	7.68

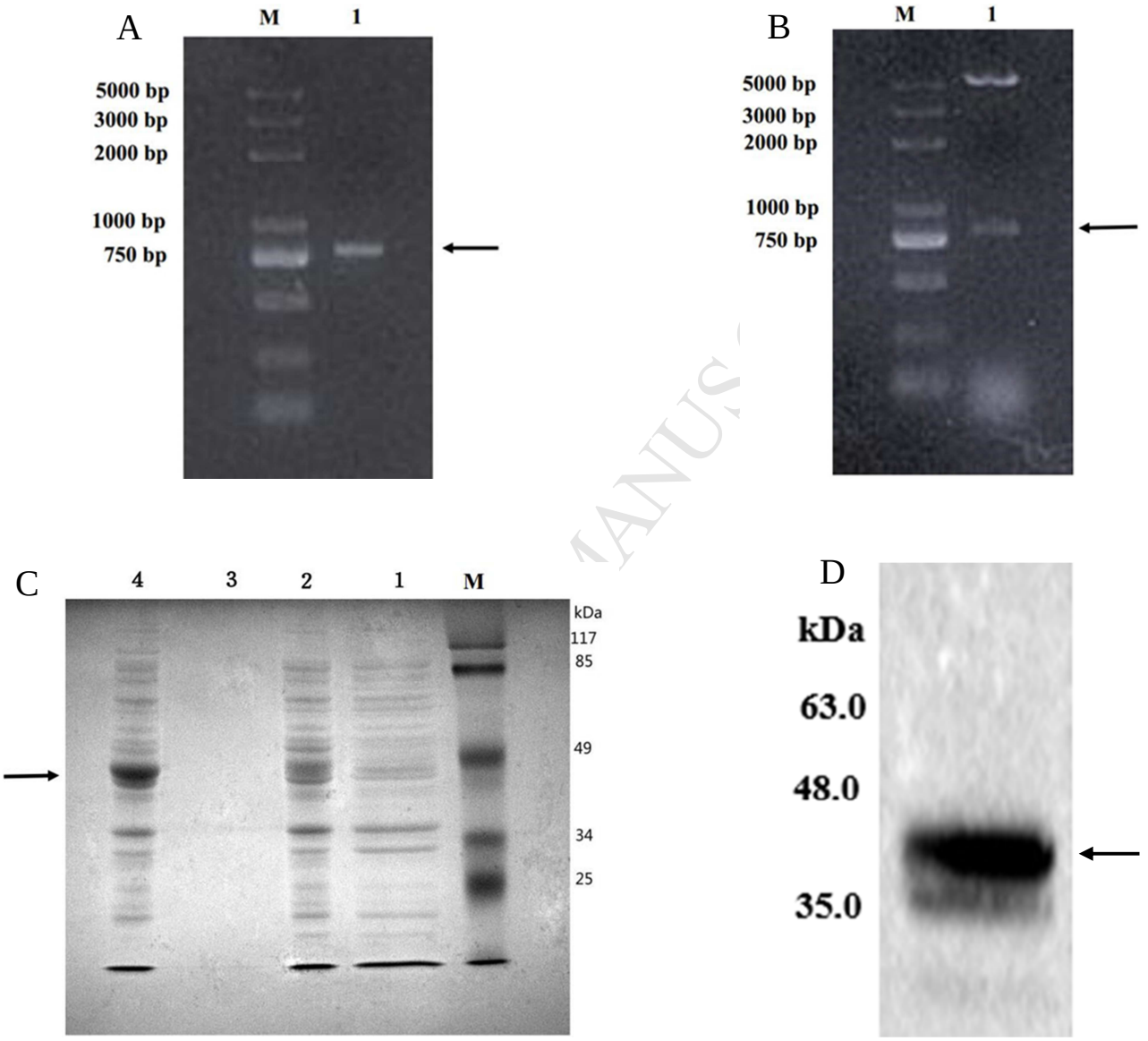

A
M
1
5000 bp
3000 bp
2000 bp
1000 bp
750 bp
B
M
1
5000 bp
3000 bp
2000 bp
1000 bp
750 bp
C
4
3
2
1
M
kDa
117
85
49
34
25
D
kDa
63.0
48.0
35.0

Fig. 1

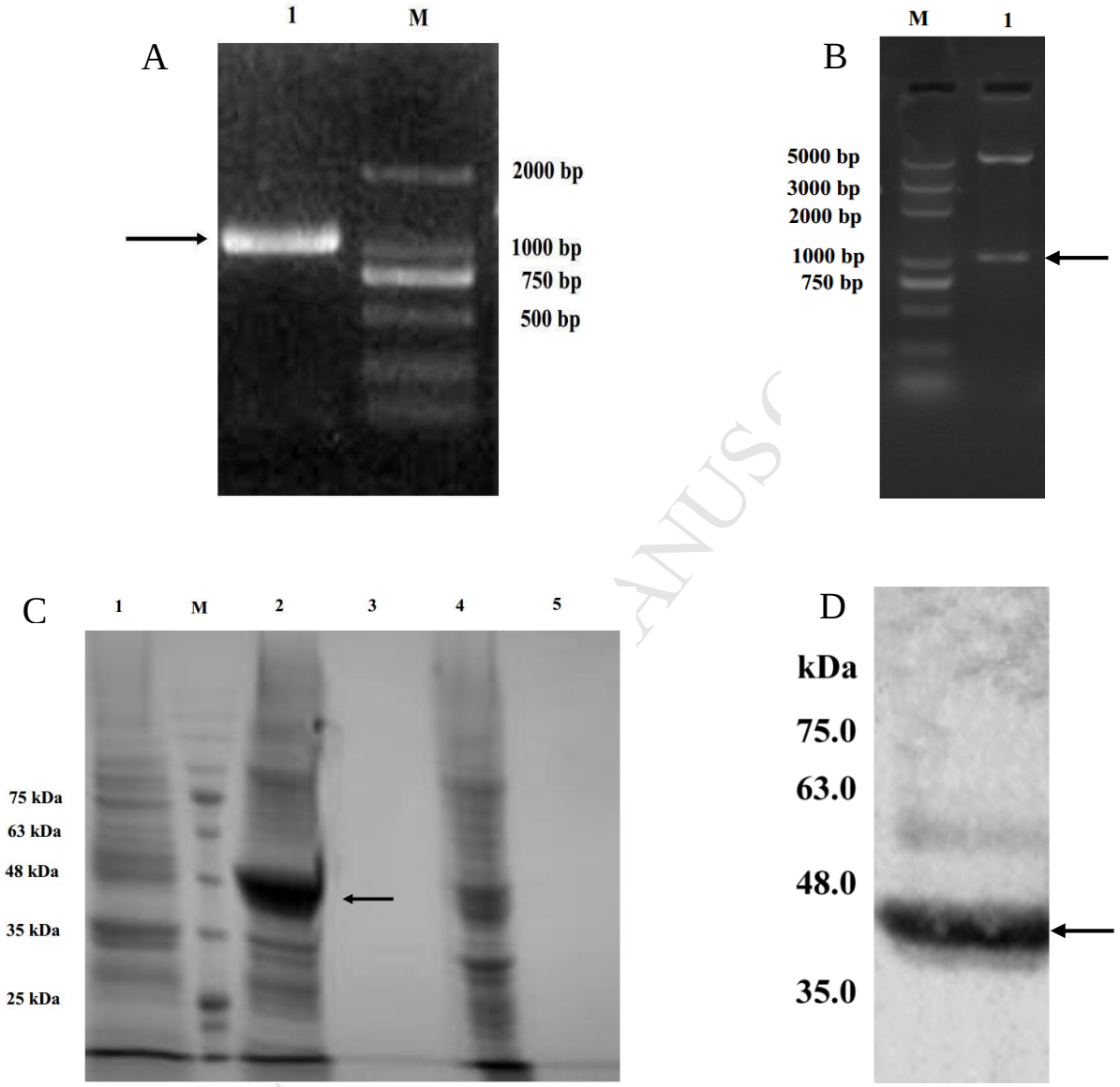
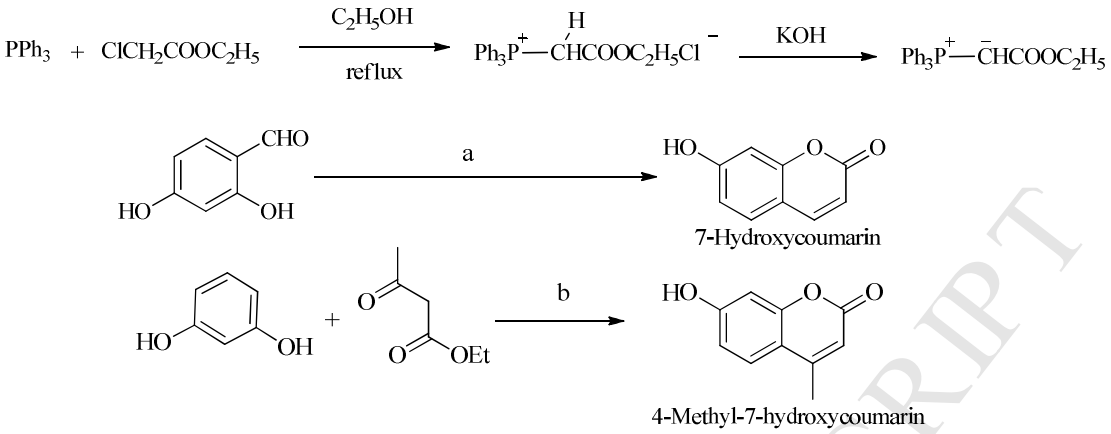
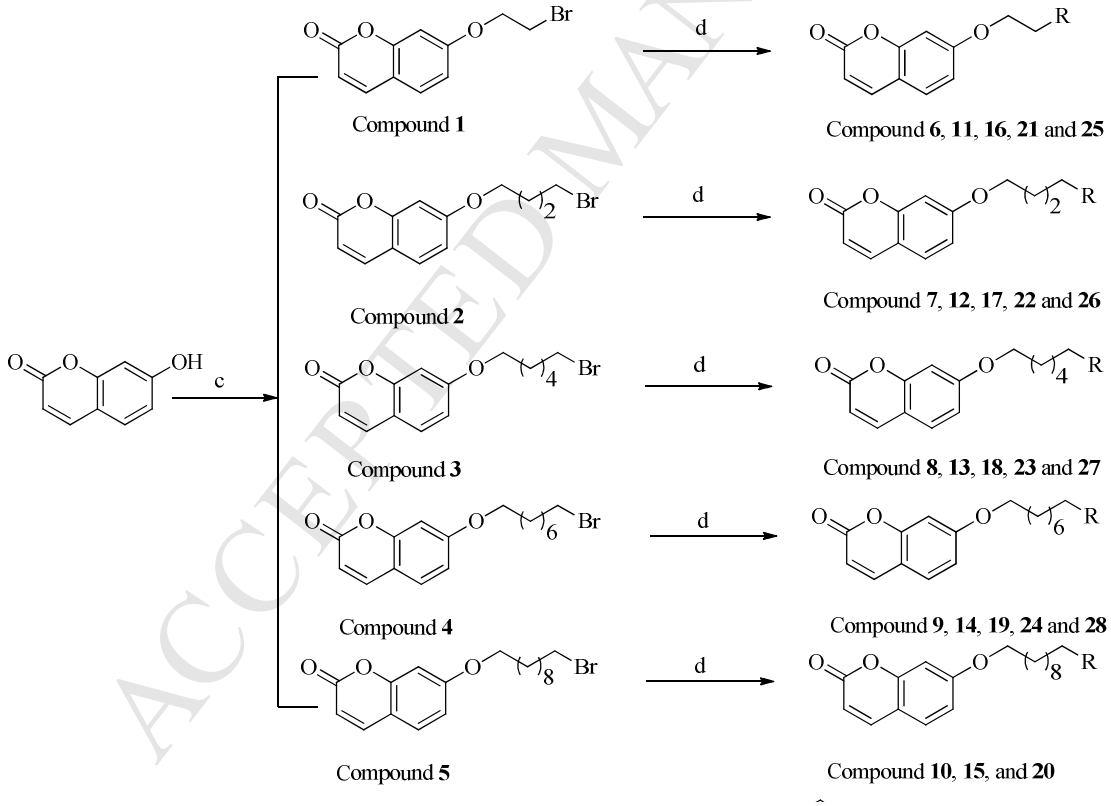


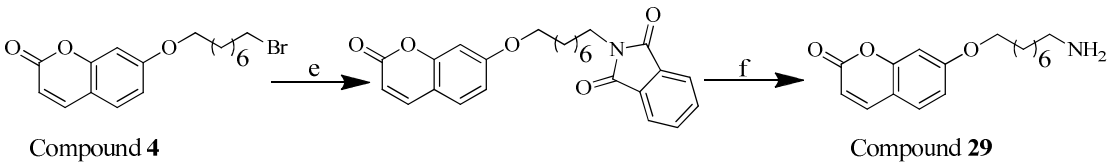
Fig. 2



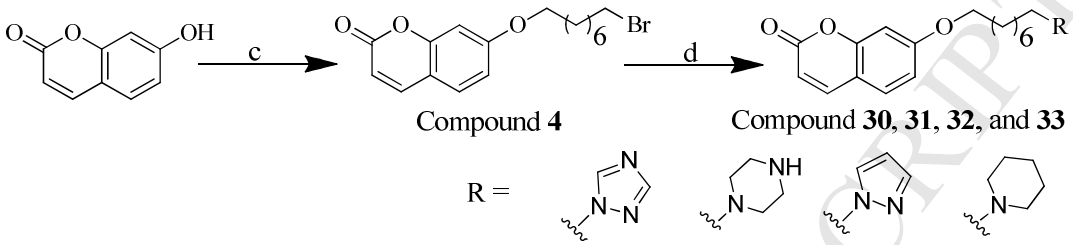
Scheme 1



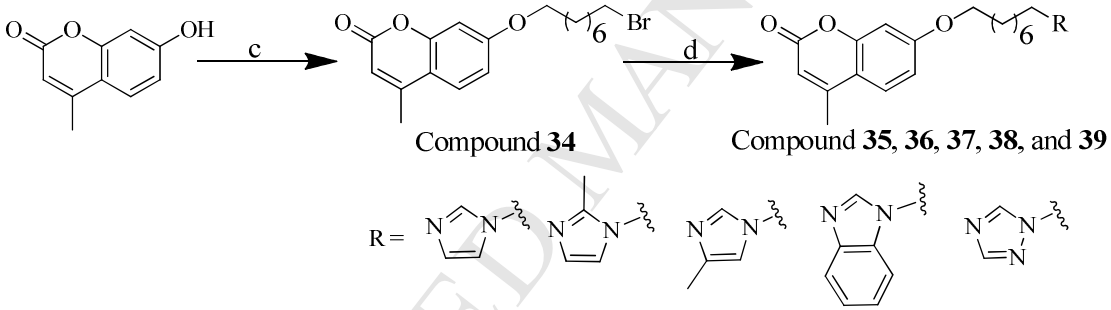
Scheme 2



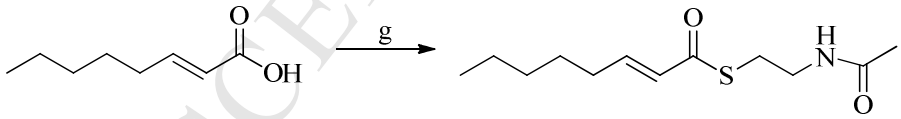
Scheme 3



Scheme 4



Scheme 5



Scheme 6

Highlights

Novel coumarin-imidazoles with antibacterial properties were synthesized.

Coumarin derivatives showed the antibacterial activity by inhibiting the FabI and FabK.

Structure-activity relationship showed the importance of the alkyl linker and imidazole.