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Original article

Functionalized chalcones with basic functionalities have antibacterial activity against drug sensitive *Staphylococcus aureus*

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Abstract

A library of chalcones with basic functionalities were evaluated for antibacterial activity against drug sensitive strains of *Staphylococcus aureus* and *Escherichia coli*. The most active compounds were **2-52** and **2-57** (MIC 6.3 μM *S. aureus*). These compounds had no activity against *E. coli* (MIC > 100 μM). Both compounds were characterized by a ring A that was substituted with 2-hydroxy-4,6-dimethoxy-3-(1-methylpiperidin-4-yl) groups. The phenolic OH and 1-methylpiperidinyl groups were required for activity but the phenolic OH may play a more critical role. While the compounds were comparable to licochalcone A in terms of antibacterial activity, they caused less hemolysis of sheep erythrocytes at high concentrations (100 μM). It was noted that the structural requirements for limiting hemolytic activity were less stringent than those required for antibacterial activity. The present findings suggest that the chalcone framework is an attractive template for optimization to achieve better potency, lower toxicity and a wider spectrum of antibacterial activity.

Keywords: Chalcones; Basic substituents; Antibacterial activity; Structure–activity relationship; Protection against hemolysis

1. Introduction

The rapid development of bacterial strains resistant to antibacterial agents poses a significant threat to global health [1]. In particular, attention has focused on the Gram positive organism *Staphylococcus aureus* because many strains of this organism are now resistant against clinically useful antibiotics like methicillin and vancomycin [2]. The problem is further compounded by the rapid emergence of multidrug resistant organisms. For example, it look only a few years after the introduction of linezolid [3], an oxazolidinone derivative, for clinical use before reports of resistant organisms and clinical failures emerged [4,5]. Given this situation, there is an urgent need to discover and develop new antibacterial agents. Fortunately, several promising compounds have been identified. These include the cyclic lipopeptide daptomycin which has activity against methicillin resistant *S. aureus* (MRSA) and vancomycin-susceptible strains of enterococci [6,7] and several cationic antimicrobial peptides (for example MX-226) that are in later stages of clinical trials [8].

Licochalcone A is a retrochalcone isolated from the roots and rhizomes of *Glycyrrhiza inflata*. It is active against a wide range of Gram positive organisms but not against Gram negative bacteria and eukaryotes [9]. Licochalcone A is also active against MRSA with minimum inhibitory concentrations (MICs) ranging from 18.4 to 47.0 μM depending on the strain of organism [10,11]. A structure–activity relationship (SAR) study showed that of the two phenolic hydroxyl (OH) groups attached to rings A and B of licochalcone A (Fig. 1), the OH on ring A was more important for antibacterial activity [12]. The prenyl side chain on ring B contributed to lipophilicity, and could be replaced by groups with comparable lipophilic character, like *n*-hexyl, without loss of antibacterial activity. Licochalcone A has been used as a lead compound for the design of more potent antibacterial agents based on the chalcone template. In one study, Nielson et al. [13] introduced

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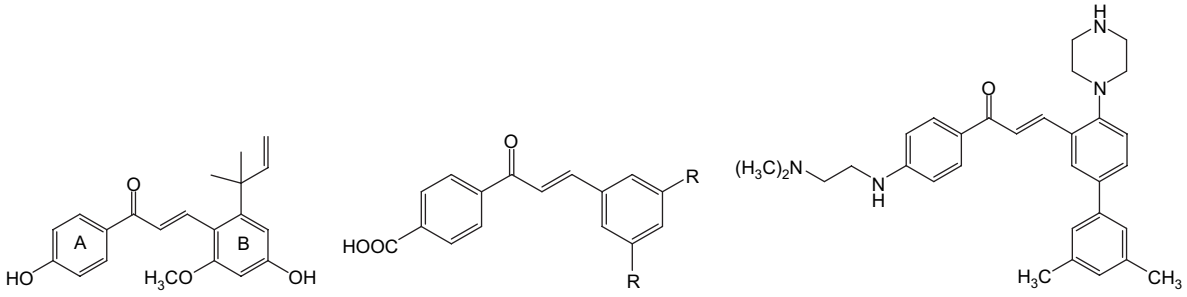


Licochalcone A Compound 1: (a) R = Br (b) R = CF3 [13] Compound 2 [14]

Fig. 1. Structures of Licochalcone A and other chalcones reported to have antibacterial properties [13,14].

carboxyl groups in place of the phenolic OH in an attempt to improve the intrinsically poor solubility of licochalcone A. Compounds **1a** and **1b** were found to be the most active compounds with MIC of 2 μM against *S. aureus* (Fig. 1). The presence of the lipophilic substituents (CF_3, Br) on ring B of these compounds contributed significantly to activity.

In another study, the same authors prepared a library of chalcones with basic substituents [14]. The rationale for introducing basic groups that are protonated at physiological pH is that bacterial membranes are rich in negatively charged phospholipids and thus, would attract positively charged molecules. Following the initial attraction which is mainly electrostatic, the agent would permeate and insert itself into the bacterial membrane to exert its lethal disruptive effects [15]. Other properties of the agent like its shape, size and lipophilicity would determine the extent to which permeation can take place. In that study [14], compound **2** was found to be the most promising compound (MIC 2 μM against a methicillin resistant strain of *S. aureus*) (Fig. 1). Unlike licochalcone A, compound **2** selectively disrupted bacterial membranes and did not cause significant hemolysis of erythrocytes. This selective activity is highly commendable and may be explained by the fact that mammalian erythrocytes have a large proportion of zwitterionic phospholipids and cholesterol, besides negatively charged phospholipids. The positively charged agent is probably attracted to the negatively charged phospholipids found in the outer glycocalix layer of the erythrocyte, following which it is ineffectively partitioned into zwitterionic compartment of the membrane [15]. Thus it is not hemolytic to mammalian erythrocytes.

In view of the potential antibacterial activity of cationic chalcones, we have synthesized a library of chalcones with basic functionalities with the purpose of determining if antibacterial activity is a general property of cationic chalcones or restricted only in members with specific substitution patterns. In our design, heterocyclic rings like piperazine, piperidine and pyridine were introduced as basic functionalities. Aliphatic amino groups were avoided because of their association with reactive metabolites [16]. These compounds were evaluated for antibacterial activity on non-resistant strains of *S. aureus* and *E. coli* and promising candidates were further evaluated for their potential to cause hemolysis of erythrocytes.

2. Chemistry

The structures of the chalcones are given in Table 1. They were organized into 11 groups according to the substitution pattern on ring A. Chalcones in Groups 1, 6, 7 and 8 had two methoxy groups at positions 2 and 4, and a basic heterocyclic ring (1-methyl piperidin-4-yl, 1-ethylpiperidin-4-yl, 1-methylpiperazin-4-yl or 4-[(1′-piperidinyl)piperidin-1-yl] at position 5 of ring A. Starting from Group 1, the inclusion of a phenolic OH group gave Group 2. Omitting the 4-methoxy group from Group 1 gave Group 10 and replacing the 4-methoxy of Group 10 with 4-OH gave Group 11. No basic substituent was present in Groups 5 and 9 and only basic groups were present in Groups 3 and 4. The antibacterial properties of these groups would serve to highlight the importance of the omitted functionalities.

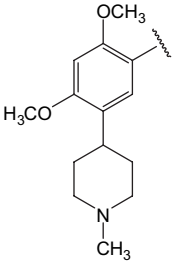
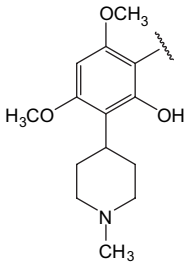
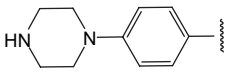
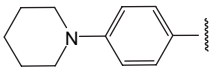
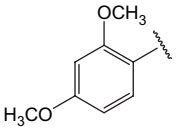
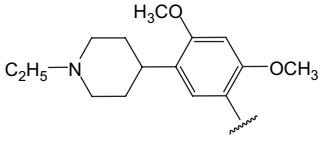
Ring B was substituted with halogens in most cases but we included substituents with different Hansch π and Hammett σ values in Groups 1 and 2. Basic groups were also introduced on ring B either as substituents (dimethylamino, 4-methylpiperazin-1-yl) or in place of the phenyl ring B (pyridine).

The synthesis of the chalcones was described in earlier reports [17,18]. Briefly they were prepared by the Claisen–Schmidt condensation of benzaldehydes and acetophenones substituted with appropriate groups. The benzaldehydes were either purchased or synthesized, as in the case of 4-(4-methylpiperazin-1-yl)benzaldehyde (Scheme 1a). The syntheses of representative acetophenones, 1-(2,4-dimethoxy-5-(4-methylpiperazin-1-yl) phenyl) ethanone (**7-1**) and 1-(5-(1,4′-bipiperidin-1-yl)-2,4-dimethoxyphenyl) ethanone (**8-1**), which were the acetophenones for Groups 7 and 8, are shown in Scheme 1b. The compounds were characterized by ^{1}H NMR and mass spectroscopy, and their purities determined by reversed phase HPLC on two different mobile phases [17,18].

3. Results

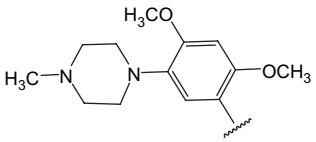
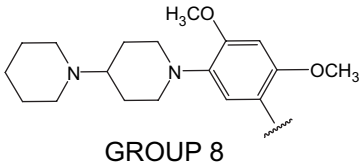
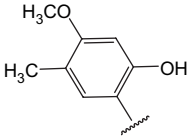
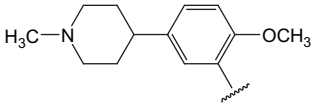
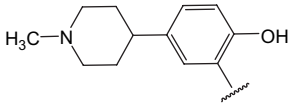
The minimum inhibitory concentrations (MICs) of the chalcones were determined on a methicillin sensitive strain of *S. aureus* (ATCC 13150) and *E. coli* (ATCC 49696) using a microdilution assay method [19]. Licochalcone A and penicillin G were used as positive controls. The results are given in Table 1. The MIC of licochalcone A against *E. coli* was

Table 1
Structures of chalcones and their antibacterial MIC against *S. aureus*

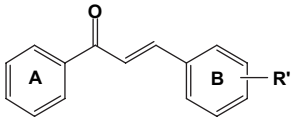
Code	Ring A[a]	R′ on ring B[a]	MIC (μM)[b] *S. aureus*	% Hemolysis[c]
1-5	 GROUP 1	2′-Cl	25	ND
1-6		H	>100	ND
1-7		4′-Cl	>100	ND
1-120		Pyridin-4-yl[d]	>100	ND
1-121		Pyridin-3-yl[d]	>100	ND
1-122		Pyridin-2-yl[d]	>100	ND
1-123		4′-(4-MP)[e]	>100	ND
1-60		2′-CH_3	>100	ND
1-61		4′- CH_3	>100	ND
1-62		2′-CH_3O	>100	ND
1-63		4′-CH_3O	>100	ND
1-64		2′-F	>100	ND
1-65		4′-F	>100	ND
1-66		4′-CF_3	50	ND
2-17	 GROUP 2	2′-Cl	50	ND
2-2		4′-Cl	25	5.5
2-3		H	>100	ND
2-4		2′-F	>100	ND
2-50		4′-F	>100	ND
2-51		2′-CH_3	50	ND
2-52		4′-CH_3	6.25	6.7
2-53		2′-CH_3O	50	ND
2-54		4′-CH_3O	50	ND
2-55		3′-CH_3O	>100	ND
2-56		2′,4′-$(CH_3O)_2$	>100	ND
2-57		3′-Cl	6.25	6.5
2-58		4′-CN	>100	ND
2-59		4′-CF_3	>100	ND
2-124		4′-(4-MP)[d]	>100	ND
2-59a		4′-$(CH_3)_2N$	25	10.3
3-100	 GROUP 3	2′,4′-$(CH_3O)_2$	100	ND
3-101		2′-Cl	>100	ND
3-102		4′-Cl	25	11.6
3-103		H	>100	ND
4-104	 GROUP 4	2′-Cl	>100	ND
4-105		4′-Cl	>100	ND
4-106		H	>100	ND
4-107		2′,4′-$(CH_3O)_2$	>100	ND
5-14	 GROUP 5	4′-Cl	>100	ND
5-15		2′-Cl	>100	ND
5-16		H	>100	ND
5-110		Pyridin-4-yl[d]	>100	ND
5-111		Pyridin-3-yl[d]	>100	ND
5-112		Pyridin-2-yl[d]	>100	ND
5-113		4′-(4-MP)[e]	>100	ND
6-130	 GROUP 6	2′-Cl	>100	ND
6-131		H	>100	ND
6-132		4′-Cl	50	ND
6-133		Pyridin-4-yl[d]	>100	ND
6-134		4′-(4-MP)[e]	>100	ND

(*continued on next page*)

Table 1 (*continued*)

Code	Ring A[a]	R′ on ring B[a]	MIC (μM)[b] *S. aureus*	% Hemolysis[c]
7-140		2′-Cl	>100	ND
7-141		2′-F	>100	ND
7-142		H	>100	ND
7-143		4′-(4-MP)[e]	>100	ND
7-144		Pyridin-4-yl[d]	>100	ND
	GROUP 7			
8-160		2′-Cl	>100	ND
8-161		2′-F	>100	ND
	GROUP 8			
9-150		2′-Cl	>100	ND
9-151		2′-CH_3O	>100	ND
9-152		H	>100	ND
	GROUP 9			
10-8		2′-Cl	>100	ND
10-9		4′-Cl	>100	ND
10-10		H	100	ND
	GROUP 10			
11-11		2′-Cl	25	14.8
	GROUP 11			
	Licochalcone A		6.3	19.9[f]
	Penicillin G		0.1	ND

[a] Rings A and B refer to the rings in the chalcone template:



[b] Minimum inhibitory concentrations (MICs) against *S. aureus* (ATCC 13150).

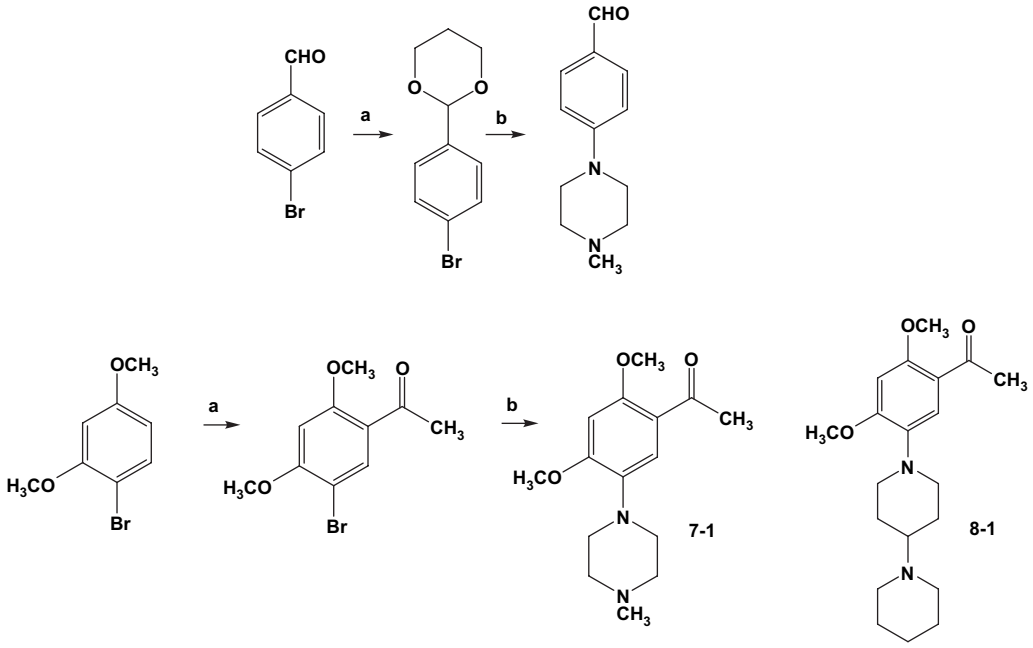
[c] % Hemolysis was determined on sheep red blood cells at a fixed concentration of 100 μM. Readings were not corrected for the solvent (1% v/v DMSO)-induced hemolysis which was found to be 8.6%. Therefore, readings less than 8% indicated absence of hemolysis at 100 μM. ND = Not determined.

[d] Ring B of chalcone is replaced by pyridine.

[e] 4′-(4-MP) = 4′-(4-methylpiperazin-1-yl).

[f] Licochalcone A showed concentration dependent hemolysis: 25 μM: 11.6%, 100 μM: 19.9%, 300 μM: 47.9%.

greater than 100 μM and was not followed up. Licochalcone A was more active against *S. aureus*, with an MIC of 6.3 μM. Like licochalcone A, the chalcones were found to be more active against *S. aureus* than *E. coli*. The MIC of the chalcones against *E. coli* exceeded 100 μM and were not investigated further. Thirteen compounds were found to have MIC ≤ 100 μM against *S. aureus*. Eight of these compounds belonged to Group 2. The most active compounds, 1-(2-hydroxy-4,6-dimethoxy-3-(1-methylpiperidin-4-yl)phenyl-3-(4-methylphenyl) prop-2-en-1-one (**2-52**) and 1-(2-hydroxy-4,6-dimethoxy-3-



Scheme 1. (a) Reagents and conditions: (a) 1,3-propandiol, *p*-TsOH, toluene, reflux 24 h; (b) (i) $Pd_2(dba)_3$ tertiary sodium butoxide, toluene, reflux 10 h, (ii) Aqueous 1 M HCl [18]. (b) Reagents and conditions: (a) boron trifluoroetherate, acetic anhydride, DCM, (b) $Pd_2(dba)_3$, Cs_2CO_3, rac-BINAP, toluene, reflux 10 h, 1-methylpiperazine (to give 7-1) or 1,4′-bipiperidine (to give 8-1) [18].

(1-methylpiperidin-4-yl)phenyl-3-(3-chlorophenyl) prop-2-en-1-one (**2-57**), had MIC values of 6.3 μM, which were comparable to licochalcone A.

Licochalcone A was reported to be a potent membrane active agent and to cause hemolysis of erythrocytes at high concentrations [14,20]. We found that licochalcone A caused concentration dependent hemolysis of sheep erythrocytes, with 11.6% hemolysis observed at 25 μM, 19.9% at 100 μM and 47.9% at 300 μM. Chalcones that had MIC $\leq$ 25 μM were also tested for hemolytic activities on sheep erythrocytes at a fixed concentration of 100 μM. The results showed that these compounds caused 8–15% hemolysis, as compared to 20% hemolysis with licochalcone A tested at the same concentration. However, the hemolytic data in Table 1 were not corrected for solvent (DMSO)-induced hemolysis. Control experiments showed that the solvent (1% DMSO) caused about 8% hemolysis which meant that most of the compounds, in particular the two most active compounds (**2-52**, **2-57**), actually caused negligible hemolysis.

4. Discussion

As mentioned earlier, more than half of the chalcones with MIC $\leq$ 100 μM were from Group 2. In addition, the two most active compounds were also members of this Group. Thus, the substituted ring A in Group 2 appeared to be strongly associated with antibacterial activity. Analysis of the structure–activity relationships pointed to the importance of the phenolic OH and the basic 1-methylpiperidinyl group for activity. This was evident from the following observations:

(i) Ring A in Group 2 had two methoxy groups, a 4-methylpiperidin-1-yl ring and a phenolic OH group. Removal of the phenolic OH group gave the Group 1 compounds which were poorly represented in terms of antibacterial activity. The contrasting MIC values of compounds **2-52** (6.3 μM) and its Group 1 analogue (**1-61**, MIC > 100 μM) serves to underscore the importance of the phenolic OH for activity.

(ii) The contribution of the basic group was highlighted by the observation that no member in Groups 5 and 9 (which had no basic group) showed antibacterial activity. On the other hand, two members in Group 3 which had a piperazine ring attached to ring A, demonstrated antibacterial activity.

(iii) The presence of dimethoxy groups on ring A was a common feature of many Groups but their presence did not contribute significantly to activity. This was seen from compounds **3-100** and **3-102** which were active despite the absence of methoxy groups as well as compound **11-11** (MIC 25 μM) which had a phenolic OH in place of a methoxy substituent.

(iv) As for the relative contributions of the phenolic OH and the basic 1-methylpiperidine ring in Group 2, we propose that their joint presence on ring A was important for activity. It is possible that the phenolic OH plays a more critical role than the basic group. This is because poor or no activity is observed in as many groups with basic moieties (Groups 4, 7 and 8) as those without basic moieties (Groups 5 and 9).

Nielson et al. reported that chalcones with basic groups on both rings A and B showed stronger antibacterial activity than those compounds that had basic groups on either ring A or B [14]. We did not find this to be true for the present series. Compounds **1-123**, **2-124**, **6-134** and **7-143** had basic groups

on both rings A and B and they were devoid of activity. An exception was compound **2-59a** (MIC 25 μM) which had a 4-dimethylamino substituent on ring B. Unfortunately, this substituent was present only in Group 2 and not in other Groups. Thus we could not confirm if its activity was related to the smaller bulk of the dimethylamino group compared to the larger 4-methylpiperazine ring. In any case, the most active compounds (**2-52**, **2-57**) had only one basic group on ring A and lipophilic substituents (chloro, methyl) on ring B.

The clustering of active compounds in Group 2 raises the question as to whether there were unique physicochemical features associated with this group. Various steric, electronic, topological and lipophilic descriptors had been collected for the compounds [18]. It was found that Group 2 was distinguished from the other groups by its large polar volume and H bond donor descriptors. Polar volume was determined by the substituents on ring A. The large polar volumes of the Group 2 compounds were largely due to the phenolic OH and the protonated 4-methylpiperidin-1-yl substituents. The phenolic OH also contributed to the H bond donor properties of this Group. Thus, the unique physicochemical properties of the Group 2 compounds could be traced to key structural entities on ring A.

The importance of lipophilicity for antibacterial activity had been emphasized in other reports [12,13] but we did not find this parameter to be critical for activity. For example, the lipophilicities of compounds **2-52** and **2-57**, measured in terms of ClogP (using ChemDraw Ultra, 10.0 CambridgeSoft, 2005), were 4.4 and 4.6, respectively. They were slightly less lipophilic than licochalcone A (Clog P 4.7) despite having comparable antibacterial activities. ClogP of compound **2** in Fig. 1 was 6.2 and it was reported to be more active than licochalcone A [14]. In addition, several compounds in the library had the same ClogP values as **2-52** and **2-57** but were inactive. Clearly, lipophilicity was not the main factor influencing activity.

An outstanding feature of the present series of chalcones was their low levels of hemolytic activity compared to licochalcone A. Compounds **2-52** and **2-57** caused negligible hemolysis at 100 μM. Chalcones from other Groups which had weaker antibacterial activities were likewise found to cause limited hemolysis. These observations suggested that the structural requirements for limiting hemolytic activity were less stringent that those required for antibacterial activity and may be an attractive feature of this series of compounds.

Finally, no activity was observed for the compounds against *E. coli*, a Gram negative organism. It is widely known that Gram positive and negative organisms have significantly different membrane compositions and architecture [15] which would explain the selective activity of the present compounds against Gram positive *S. aureus*. In this respect, our library of compounds compared less favourably to that reported by Nielson et al. [14] in which equal potency was observed against both Gram positive and negative organisms. This is in spite of several shared elements in that library and our own. On the other hand, it shows that with appropriate modifications of the template, it is possible to extend the scope of antibacterial activity, which in turn emphasizes the versatility and potential of the chalcone framework as a template for antibacterial design.

5. Conclusion

In conclusion, we have identified two basic chalcones **2-52** and **2-57** with comparable antibacterial activities (MIC 6.3 μM, *S. aureus*) to licochalcone A. In addition, both compounds caused limited hemolysis of erythrocytes at 100 μM. SAR showed that the joint presence of the phenolic OH and basic 1-methylpiperidinyl ring was important for activity. The preferred location of the basic heterocycle was ring A and introducing another basic group on ring B resulted in less or no activity. In contrast to the specific requirements for antibacterial activity, several compounds from different Groups which had varying MIC values were found to cause negligible hemolysis of erythrocytes even at 100 μM. In view of these findings, we propose that the Group 2 chalcones are a promising template for optimization of antibacterial activity against Gram positive organisms.

6. Experimental methods

6.1. Materials

Luria–Bertani (LB) broth and licochalcone A were purchased from Merck Pte. Ltd., Singapore. Penicillin G was purchased from Sigma Aldrich Chemical Co, Singapore. Sheep erythrocytes were purchased from Bloxwich Pte Ltd, Singapore. *S. aureus* (ATCC 13150) and *E. coli* (ATCC 49696) were gifts from School of Chemical and Life Sciences, Nanyang Polytechnic, Singapore.

6.2. Determination of minimum inhibitory concentration (MIC)

The method described in the National Committee for Clinical Laboratory Standards [19] was followed. Licochalcone A, penicillin G and test compounds were dissolved in DMSO to give stock solutions of 10 mM and subsequently diluted to the desired concentrations (at least 6) with medium. The maximum DMSO content of the diluted stock solutions was 12.5% v/v in water. Organisms were grown on LB agar in a Petri dish for 24 h. An inoculum was transferred under aseptic conditions into a sterile flask containing LB broth (25 ml) and incubated for 18 h at 35–37 °C, with gentle agitation (150 rpm) on an orbital shaker. Hundred microlitres of the overnight culture was added to LB broth (10 ml) to prepare a 1% culture. The flask was incubated for 3–4 h, 37 °C. At 30 min intervals, 1 ml of the broth was aseptically transferred into a cuvette and its absorbance at 600 nm was read on a spectrophotometer. Normal LB broth was used as blank. The time taken for the bacteria to reach its logarithmic phase of growth was noted. The approximate number of cells (colony forming units, CFU) at the log phase was determined by an equation previously determined by the laboratory (School of Chemical and Life Sciences, Nanyang Polytechnic, Singapore) that related cell count at the log phase for a particular organism to absorbance. Thereafter, the experiment was repeated again and at the time equivalent to the log growth phase, a seeding cell culture of 2.5×10^5 CFU/ml in LB broth was prepared. Ninety microlitres of the seeding cell

culture was dispensed into each well of a 96 well plate, followed by 10 μl of a known concentration of the test compound. Penicillin was used as the positive control. The negative control was 1.25% v/v DMSO in distilled water, which was the highest concentration of DMSO in each well. The processes were carried out under aseptic conditions. The plate was incubated at 37 °C for 20 h after which absorbances were determined on a microtitre plate reader. MIC was the lowest concentration of test compound that was able to inhibit visible growth of the bacteria and was determined in triplicates.

6.3. Determination of hemolytic activity of compounds on sheep erythroctyes

The method reported by Nielsen et al. [14] was followed. Sheep erythrocytes were washed three times in sterile phosphate buffered saline (PBS). Each washing step was carried out by centrifuging the cells at 490 g, 7 min, RT, discarding the supernatant after each wash. The cells were resuspended to give a concentration of 5×10^8 cells/ml PBS. An aliquot (20 μl) of the cell suspension was added to 800 μl of buffer solution containing 100 μM test compound (licochalcone A and chalcones with MIC $\leq$ 25 μM) in 1% v/v DMSO in PBS. Controls were 1% v/v DMSO in PBS and sterile water. The cell suspensions were incubated at 37 °C, 90 min (with shaking), after which the contents were centrifuged (1300 g, 5 min) and absorbances read at 540 nm (with absorbance set at 670 nm for the reference cell). The absorbance readings of the test compounds were expressed as a % of the absorbance of sterile water (equivalent to 100% hemolysis) to give % hemolysis.

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