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Crystal structure and activities of three biscoumarin derivatives against *Staphylococcus aureus*

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HIGHLIGHTS

- Three new biscoumarin derivatives were successfully synthesized.
- Their structures were verified by single crystal X-ray crystallography.
- The antibacterial activities of the three compounds were further investigated.

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ABSTRACT

Three new biscoumarin derivatives, namely, 3,3′-[(4-nitrophenyl)methylene]bis(4-hydroxy-2*H*-chromen-2-one) (NBH), 3,3′-[(4-methoxyphenyl)methylene]bis(4-hydroxy-2*H*-chromen-2-one) (MBH) and 3,3′-[(4-chloromethylphenyl)methylene]bis(4-hydroxy-2*H*-chromen-2-one) (CBH) were successfully synthesized and their structures were verified by single crystal X-ray crystallography. In their structures, there are two intramolecular H-bonds and the corresponding H-bond energies were calculated by DFT method. The antibacterial activities of NBH, MBH and CBH in vitro against drug-sensitive *Staphylococcus aureus* (ATCC 29213) and methicillin-resistant *S. aureus* (isolated MRSA strains) were further investigated.

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most important pathogens that leads to various illnesses in humans, including wound infections, pneumonia, sepsis, and toxic shock [1–3]. Methicillin-resistant *S. aureus* (MRSA) is the cause of most antibiotic resistant healthcare associated infections, because it spreads rapidly and cause illness more severe, which further leads to mortality and morbidity rates of patients affected by MRSA are high [4,5], and the related proportion of such cases increased significantly [6,7]. In addition, the emergence of MRSA with decreased susceptibility to vancomycin, which is customarily used as the most effective antibiotic to treat for MRSA, induced to the urgent necessity of developing new antimicrobials.

Coumarin (2*H*-chromen-2-one) derivatives, containing aromatic δ-lactones system, are an important class of heterocycles that have attracted significant importance in the field of organic and natural product chemistry [8–11]. For example, biscoumarin and its derivatives have received considerable attention in the past few years for their versatile biological and medical activities because of the ease of fine-tuning the aromatic ring with different substituents on leading to multiple chemical modifications and activities such as antioxidant, anti-inflammatory, antibacterial and anticancer [12–14]. Recognizing the considerable importance of the compounds, the researchers focused on the synthesis of biscoumarin derivatives [15].

In this study, we successfully synthesized three novel biscoumarin derivatives, namely, 3,3′-[(4-nitrophenyl)methylene]bis(4-hydroxy-2*H*-chromen-2-one) (NBH), 3,3′-[(4-methoxyphenyl)methylene]bis(4-hydroxy-2*H*-chromen-2-one) (MBH) and 3,3′-[(4-chloromethylphenyl)methylene]bis(4-hydroxy-2*H*-chromen-2-one) (CBH) (Fig. 1), tested their anti-bacterial activities on drug-sensitive and drug-resistant *S. aureus* in vitro, and then

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calculated their total HB energies by density functional theory (DFT) method.

Experimental

Apparatus and materials

IR spectra were measured using a Brucker Equinox-55 spectrophotometer. ^{1}H NMR spectra, ^{13}C NMR spectra and mass spectra were tested using the Varian Inova-400 spectrometer, Bruker Avance III spectrometer and micrOTOF-Q II mass spectrometer, respectively. The melting points were determined on a XT-4 micro melting apparatus.

S. aureus strain (ATCC 29213) and isolated MRSA strains (1-3) were obtained from Chinese National Center for Surveillance of Antimicrobial Resistance and Xijing Hospital (Xi'an, China), respectively. Antibiotics including levofloxacin, ceftazidime, ceftriaxone, gentamicin and piperacillin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals and solvents were of analytical grade.

General procedure for preparation of biscoumarin derivatives NBH, MBH and CBH

NBH, MBH and CBH were prepared from a reported procedure [16]. In a 100 mL round bottom flask, a mixture of 4-nitrobenzaldehyde (4-methoxybenzaldehyde or 4-chloromethylbenzaldehyde) (10 mmol) and 4-hydroxycoumarin (20 mmol) were placed over a magnetic stirrer and the contents were stirred. To this stirred mixture, a few drops of piperidine were added. The reaction mixture was heated at 90 °C for 3–5 h and the progress was monitored by TLC using hexane–chloroform–ethyl acetate mixture (1:1:1) as eluent. After completion of the reaction, the reaction mixture was allowed to cool in room temperature (25 °C) until precipitation occurred. The precipitates were filtered and then washed with ethanol to get pure products.

3,3′-[(4-Nitrophenyl)methylene]bis(4-hydroxy-2*H*-chromen-2-one) (NBH): Yield: 60%. mp 251–252 °C. ν_{max}(KBr): 1658, 1616, 1564, 1521, 1348, 1109, 763 cm^{-1}. ^{1}H NMR ($CDCl_3$): 11.57(s, 1H), 11.38(s, 1H), 8.18–8.20(d, J = 8.8 Hz, 2H), 8.00–8.10(q, J = 7.6 Hz, J = 7.6 Hz, 2H), 7.67–7.69(t, 2H), 7.40–7.44(t, 6H), 6.12(s, 1H). ^{13}C NMR (CD_3Cl) δ: 169.1, 167.0, 166.4, 164.9, 152.6, 152.3, 146.9, 143.4, 133.4, 127.6, 125.2, 125.2, 124.5, 124.5, 123.9, 116.8, 116.8, 116.7, 116.2, 104.8, 103.3, 36.5. HRMS (ESI$^+$): m/z: calcd for $C_{25}H_{15}NO_8$: 480.0690 [M + Na$^+$]; found: 480.0489.

3,3′-[(4-Methoxyphenyl)methylene]bis(4-hydroxy-2*H*-chromen-2-one) (MBH): Yield: 66%. mp 269–270 °C. ν_{max}(KBr): 1670, 1604, 1560, 1510, 1350, 1256, 1093, 769 cm^{-1}. ^{1}H NMR ($CDCl_3$): 11.51(s, 1H), 11.30(s, 1H), 7.99–8.08(q, J = 6.8 Hz, J = 6.8 Hz, 2H), 7.61–7.65(t, 2H), 7.40–7.42(d, J = 8.0 Hz, 4H), 7.12–7.14(d, J = 8.4 Hz, 2H), 6.84–6.87(d, J = 8.8 Hz, 2H), 6.05(s, 1H), 3.80(s, 3H). ^{13}C NMR (CD_3Cl) δ: 158.4, 132.8, 127.6, 126.9, 124.9, 124.4, 116.6, 114.0, 55.3, 35.5. HRMS (ESI$^+$): m/z: calcd for $C_{26}H_{18}O_7$: 465.0945 [M + Na$^+$]; found: 465.0967.

3,3′-[(4-Chloromethylphenyl)methylene]bis(4-hydroxy-2*H*-chromen-2-one) (CBH): Yield: 63%. mp 254–255 °C. ν_{max}(KBr): 1666, 1616, 1567, 1354, 1095, 908, 767 cm^{-1}. ^{1}H NMR ($CDCl_3$): 11.54(s, 1H), 11.32(s, 1H), 8.00–8.09(q, J = 6.4 Hz, J = 6.4 Hz, 2H), 7.63–7.66(m, 2H), 7.35–7.44(m, 6H), 7.22–7.23(d, J = 6.0 Hz, 2H), 6.08(s, 1H), 4.59(s, 2H). ^{13}C NMR (CD_3Cl) δ: 169.2, 166.9, 165.9, 164.6, 152.5, 152.3, 136.1, 135.7, 133.0, 128.9, 128.0, 126.9, 126.5, 125.0, 124.4, 116.9, 116.7, 116.4, 105.4, 103.8, 45.8, 36.3. HRMS (ESI$^+$): m/z: calcd for $C_{26}H_{17}ClO_6$: 483.0606 [M + Na$^+$]; found: 483.0601.

X-ray crystallography

For compounds NBH, MBH and CBH, three white crystals with approximate dimensions of 0.22 × 0.20 × 0.16 mm^3, 0.20 × 0.20 × 0.12 mm^3, and 0.20 × 0.18 × 0.10 mm^3 were selected for data collection respectively. The X-ray diffraction data were collected on a Bruker SMART APEX II CCD diffractometer equipped with a graphite monochromated Mo Ka radiation (λ = 0.71073 A) by using ω–2θ scan technique at room temperature. The structures were solved by direct methods (SHELXS-97) and refined using the full-matrix leastsquares method on F^2 with anisotropic thermal parameters for all non-hydrogen atoms using SHELXL-97 [17]. The hydrogen atoms were placed in calculated positions and refined using the riding model. The crystal data, details concerning data collection and structure refinement are given in Table 1. Molecular illustrations were prepared using the XP package.

Quantum chemical calculations

Theoretical studies based on density functional theory (DFT) calculations using the Gaussian 09 package [18–20]. In order to obtain precise results that are in good agreement with experimental results, three level of theories have been carried out, they are B3LYP/6-31G*, B3LYP/6-31 + G** and B3LYP/6-311G*, respectively [21–23].

Bacterial susceptibility testing

The minimum inhibitory concentration (MIC) values were determined according to the previously reported method [24], *S. aureus* strains were grown in 100 μL of nutrient Mueller–Hinton (MH) broth in the concentration of 5 × 10^5 CFU/mL, then 100 μL of MH medium containing the NBH, MBH or CBH (0.12 – 256 μg/mL in serial two-fold dilutions) was added to the wells of plates. After they were incubated at 37 °C for 20 h, the lowest concentration of compound without visible bacterial growth in the wells was taken as the MIC value. The experiments were done independently five times, using duplicate samples each time.

Bacterial growth rate assay

The effect of NBH, MBH and CBH to the growth rate of *S. aureus* and MRSA were determined as reported method [25], *S. aureus* and MRSA were cultured in the 150 μL MH broth each well using automated Bioscreen C system (Lab systems Helsinki, Finland), then 150 μL coumarin derivatives solution were added to culture medium at 32 or 128 μg/mL in 35 °C, then the culture medium were shaken for 1 min. As the optical density of each sample, OD600 was obtained in regular intervals of 10 min for 20 h at a wavelength of 600 nm to estimate the concentration of bacterial.

Cytotoxicity assay

For NBH, MBH and CBH, the cytotoxicity with respect to the human umbilical vein endothelial cells (HUVECs) were investigated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Firstly, HUVECs cells (5 × 10^3/well) were grown for 24 h at 37 °C to confluence in 96-well plates in DMEM media with 20% fetal bovine serum, then treated with NBH, MBH or CBH at different concentrations (6.25, 12.5, 25, 50 and 100 μg/mL) for 24 h. After that, 0.5% MTT solution was added into the cultured HUVECs and incubated for 4 h. Finally, the supernatant was removed and 150 μL DMSO was added to each well, the absorbance was read at 490 nm.

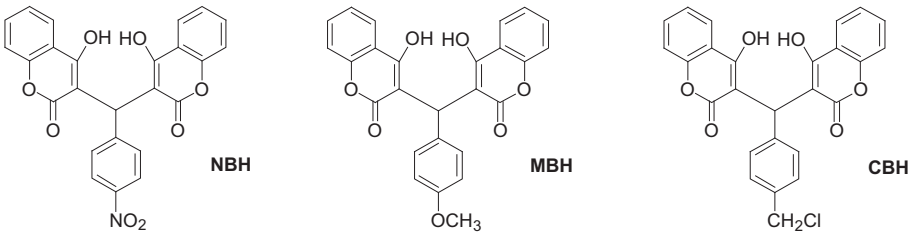

Fig. 1. Chemical structures of NBH, MBH and CBH.

Table 1
Crystal data and structure refinement for NBH, MBH and CBH.

	NBH	MBH	CBH
Formula	$C_{25}H_{15}NO_8$	$C_{26}H_{18}O_7$	$C_{26}H_{17}ClO_6$
Mr	457.38	442.40	460.85
Crystal system	Orthorhombic	Monoclinic	Monoclinic
Space group	$Pna2_1$	$P2_1/c$	$P2_1/c$
a/Å	13.906(3)	9.9800(12)	13.788 (4)
b/Å	14.159(4)	10.4058(14)	10.233 (3)
c/Å	10.294(3)	20.042(2)	15.439 (4)
α/°	90	90	90
β/°	90	94.693(10)	95.633(4)
γ/°	90	90	90
V/Å^3	2026.7(9)	2074.4(4)	2167.9(10)
Z	4	4	4
D_{calc}/g cm^{-3}	1.499	1.417	1.412
μ(Mo Kα)/mm^{-1}	0.114	0.104	0.218
θ range/°	2.05 to 27.90	2.04 to 25.02	1.3 to 27.9
Reflections collected	20617	17150	17272
No. unique data [*R*(int)]	4769[0.0432]	3658[0.0591]	3827[0.0317]
No. data with $I \geqslant 2\sigma(I)$	4127	3151	3250
Goodness-of-fit on F^2	1.073	1.132	1.117
R_1	0.0380	0.0690	0.0658
ωR_2 (all data)	0.0699	0.1978	0.1982

Results

Crystal structure description

The molecular geometry and the atom labeling scheme of NBH, MBH and CBH are presented in Fig. 2. Most of the bond distances and bond angles are of the expected values, and in good accordance with the corresponding values obtained in case of the related bis-coumarin derivatives [26]. According to X-ray diffraction structural determination under mild conditions, it can be seen that Michael addition reaction took place between coumarin C (2 and 11) and aldehyde C (10). As a result, a new compound was obtained.

In the crystal structures of NBH (MBH and CBH), the C10—C20 distance of 1.532 (1.552 and 1.535) Å is longer than an unstrained C(sp^3)—C(Ar) bond, which indicates significant electron delocalization in the benzene ring system. In addition, two 4-hydroxycoumarin moieties are linked through a methylene bridge, wherein one hydrogen atoms has been replaced with a phenyl ring including *p*-nitro, *p*-methoxy and *p*-chloromethyl groups, respectively. Two 4-hydroxycoumarin residues are arranged in a position that permits the formation of two classical intramolecular hydrogen bonds between a hydroxyl group of one coumarin fragment and a lacton carbonyl group of another coumarin fragment helping stabilize the whole structure. The corresponding values of intramolecular hydrogen bonds are listed in Table 2.

Quantum chemical calculations

Geometric parameters of NBH, MBH and CBH

For NBH, MBH and CBH, the fully optimized structures with atomic numbering are shown in Fig. 3; experimental and calculated parameters of the selected bond lengths and bond angles are presented in Table 3.

Under three different basis sets, the calculated geometric parameters of NBH, MBH, and CBH are very close and agree with the experimental findings. The maximum deviation of the selected bond lengths between theoretical and experimental data are (NBH) 0.01363, 0.01142, 0.01243; (MBH) 0.02248, 0.0203, 0.02151; and (CBH) 0.03752, 0.03553, 0.03646, respectively. The maximal deviation of the selected bond angles between theoretical and experimental data are (NBH) 4.202, 4.876, 5.3; (MBH) 1.495, 2.194, 1.672; and (CBH) 2.511, 3.318, 3.663, respectively.

B3LYP/6-31G* exhibited sufficient agreement with experimental data and lower computational cost, so further theoretical study was performed at this level.

Hydrogen bonds energies in NBH, MBH, and CBH

We only used NBH as an example to estimate single and total HB energies. The global minimum structure is stabilized by two HBs (NBH); two higher energy structures is stabilized by one HB (NBH1 and NBH2) respectively. The corresponding values are listed in Table 4. The O_6—$H_6\cdots O_1$ HB energy was calculated to be −50.85593 kJ/mol by the equation $E(O_6-H_6\cdots O_1) = ER^{coor}_{NBH} - E^{coor}_{NBH1}$, from the energy difference between NBH and NBH1, where NBH1 is a global minimum structure with O_3—$H_3\cdots O_4$ HB. Similarly, the O_3—$H_3\cdots O_4$ HB energy was calculated to be −62.16659 kJ/mol from the energy difference between NBH and NBH2 by the equation $E(O_3-H_3\cdots O_4) = ER^{coor}_{NBH} - E^{coor}_{NBH2}$, in which NBH2 was obtained from the global minimum structure NBH, but H_3 was rotated around the C_3—O_3 bond until O_3—$H_3\cdots O_4$ HB rupture occurred [27,28]. We can see that O_3—$H_3\cdots O_4$ HB is stronger than O_6—$H_6\cdots O_1$ HB, which is consistent with the fact that the distance of O_3—O_4 (2.594 Å) is shorter than that of O_6—O_1 (2.610···Å). The total HB energy was calculated to be −113.02252 kJ/mol by the equation $2E^{coor}_{NBH} - (E^{coor}_{NBH1} - E^{coor}_{NBH2})$. Similar to NBH, the O_3—$H_3\cdots O_4$ HB energy for MBH and CBH is also stronger than O_6—$H_6\cdots O_1$ HB energy [$d(O_3$—$O_4) < d(O_1$—$O_6)$]. Their total HB energies are −119.12156 and −115.9132 kJ mol^{-1}, respectively.

Electron density and Laplacian in NBH, MBH and CBH

For NBH, MBH, and CBH, the electron density ($\boldsymbol{\rho_b}$) and the corresponding Laplacian ($\nabla^2\boldsymbol{\rho_b}$) at bond critical points are calculated, which are presented in Table 5. From Table 5 we can see that both O···H bondings have low $\boldsymbol{\rho_b}$ and positive $\nabla^2\boldsymbol{\rho_b}$ values, and values in the upper exocyclic ring are higher than those in the lower exocyclic ring. The results indicate that the electrostatic character of the O···H bonding in the upper exocyclic ring is stronger than that in the lower exocyclic ring [29], which is consistent with the fact that the stronger HB strength in the upper exocyclic ring.

Minimal inhibitory concentration (MIC) assay

The antibacterial activities of NBH, MBH and CBH in vitro were systematically studied by drug-sensitive *S. aureus* and

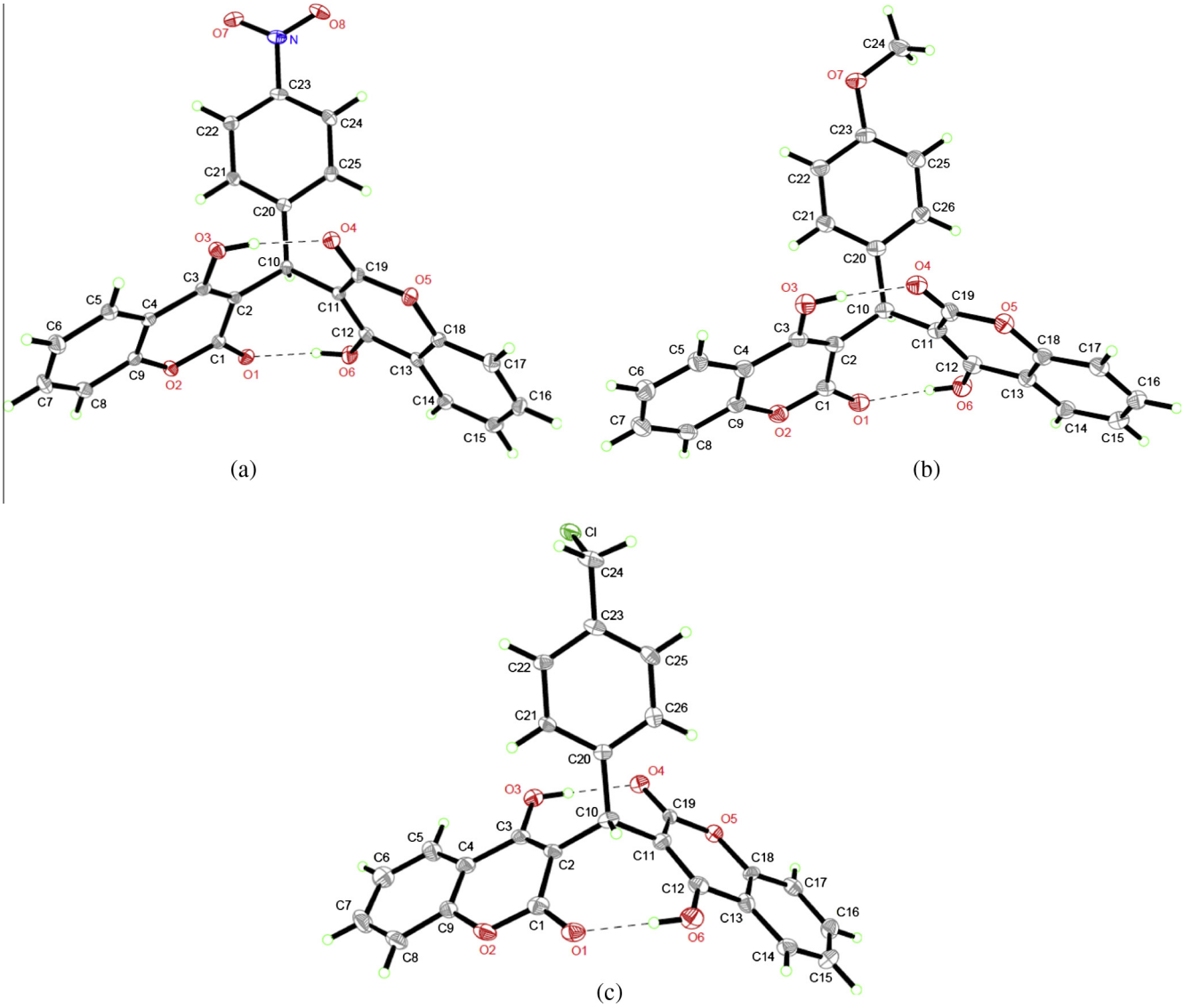

Fig. 2. Crystal structure of the compound NBH (a), MBH (b) and CBH (c).

Table 2
Intramolecular hydrogen bond length (Å) and angles (°) for NBH, MBH and CBH.

D—H···A	D—H/Å	H···A/Å	D···A/Å	∠(D—H···A)/°
NBH				
O_6—H_6···O_1	0.839	1.775	2.610 (17)	172.89
O_3—H_3···O_4	0.840	1.769	2.594 (16)	166.94
MBH				
O_6—H_6···O_1	0.841	1.847	2.686 (8)	176.18
O_3—H_3···O_4	0.840	1.778	2.612 (7)	172.32
CBH				
O_6—H_6···O_1	0.840	1.778	2.618 (3)	179.13
O_3—H_3···O_4	0.840	1.766	2.604 (3)	174.73

methicillin-resistant *S. aureus*. Because of the liposolubility of the three compounds, they were dissolved into the solution with 1% Dimethyl sulfoxide (DMSO) at final concentration. As shown in Table 6, the MIC values of NBH ranged from 16 μg/mL to 32 μg/mL, exerting the best bactericidal activities against nearly all kinds of *S. aureus* investigated. By contrast, MBH and CBH exerted weaker bactericidal effects against *S. aureus* with their MIC values exceed 64 μg/mL for drug-susceptive *S. aureus* and the MRSA strains. Compared with NBH, MBH and CBH, the MIC values of reference antibiotics against *S. aureus* (ATCC 29213) were lower less than 8 μg/mL, but were higher against MRSA strains at varying level.

Bacterial growth inhibition

In order to explore the relationship between the activity and time of NBH?MBH and CBH, we further investigated their growth inhibitory and bactericidal effects by determine the growth rate of *S. aureus* in liquid medium containing the three compounds.

As shown in Fig. 4, NBH significantly inhibited the growth of the drug-sensitive *S. aureus* ATCC 29213 at 32 μg/mL and inhibitied this pathogen completely at 128 μg/mL. NBH also effectively inhibited the growth of the three drug-resistant *S aureus* strains at 32 μg/mL and inhibited the growth of these pathogens significantly at 128 μg/mL (Figs. 5–7). Similar to the results of the MIC values, the other two compounds hardly showed any inhibitory activities on the bacterials at 32 or 128 μg/mL (Figs. 4–7). *S. aureus* growth in MH broth without any compounds, which was used as the control sample, could not inhibit the growth rate significantly.

The analysis of bacterial growth inhibition showed that aside from exerting antibacterial activities on *S. aureus,* NBH also

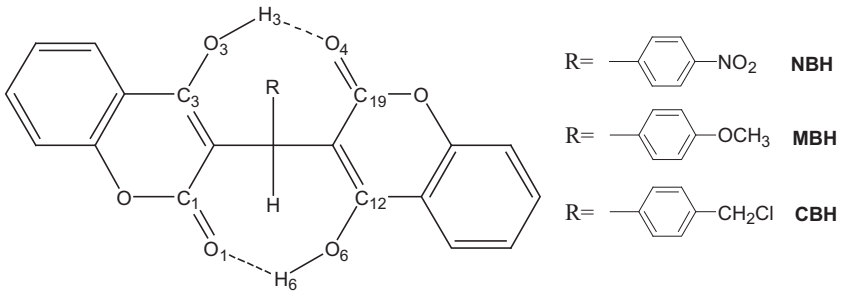

Fig. 3. Schematic presentation of NBH, MBH and CBH.

Table 3
Experimental and calculated parameters of the bond lengths and bond angles of NBH, MBH and CBH.

Name definition	NBH				MBH				CBH			
	X-ray	6-31G*	6-31+G**	6-311G*	X-ray	6-31G*	6-31+G**	6-311G*	X-ray	6-31G*	6-31+G**	6-311G*
$R(C_1—O_1)$	1.227	1.23374	1.23636	1.22650	1.2338	1.23359	1.23628	1.22633	1.2285	1.23376	1.23635	1.22649
$R(C_1—O_2)$	1.3572	1.37083	1.36862	1.36963	1.3509	1.37338	1.37120	1.37241	1.3349	1.37242	1.37043	1.37136
$R(C_{19}—O_4)$	1.2255	1.23071	1.23383	1.22324	1.2249	1.22906	1.23215	1.22155	1.1975	1.22949	1.23250	1.22197
$R(C_{19}—O_5)$	1.3628	1.37362	1.37096	1.37292	1.3726	1.37754	1.37498	1.37715	1.3581	1.37585	1.37347	1.37541
$R(C_2—C_{10})$	1.5186	1.52648	1.52639	1.52607	1.5179	1.52766	1.52743	1.52715	1.5088	1.52691	1.52684	1.52650
$R(C_{10}—C_{11})$	1.5212	1.52504	1.52439	1.52411	1.5285	1.52590	1.52522	1.52495	1.5308	1.52534	1.52466	1.52433
$R(C_{10}—C_{20})$	1.5323	1.53806	1.53812	1.53696	1.5518	1.53948	1.53995	1.53851	1.5348	1.53935	1.53959	1.53813
$A(C_1—O_2—C_9)$	121.083	121.676	121.707	121.716	120.600	121.647	121.672	121.678	120.433	121.647	121.680	121.684
$A(O_1—C_1—O_2)$	115.859	115.801	115.988	116.044	115.487	115.550	115.750	115.801	116.283	115.648	115.826	115.892
$A(C_{18}—O_5—C_{19})$	120.557	121.870	121.861	121.888	120.956	121.930	121.928	121.954	122.889	121.896	121.894	121.915
$A(O_4—C_{19}—O_5)$	115.736	115.755	115.928	116.026	114.538	115.468	115.632	115.739	117.461	115.633	115.787	115.907
$A(C_2—C_{10}—C_{20})$	115.364	115.881	116.083	115.988	115.056	115.568	115.702	115.628	114.532	115.705	115.847	115.763
$A(C_2—C_{10}—C_{11})$	111.630	112.652	112.832	112.757	113.740	112.245	112.456	112.339	112.852	112.468	112.617	112.539
$A(C_{11}—C_{10}—C_{20})$	115.335	114.536	114.625	114.611	113.297	114.759	114.912	114.846	114.678	114.751	114.912	114.883
$D(C_1—C_2—C_{10}—C_{20})$	135.129	133.247	132.465	133.010	135.723	134.406	133.529	134.312	134.707	133.449	132.837	133.396
$D(C_1—C_2—C_{10}—C_{11})$	90.65	92.117	92.345	92.007	91.234	91.385	91.660	91.183	91.632	91.971	92.020	91.683
$D(C_{12}—C_{11}—C_{10}—C_{20})$	136.073	131.871	131.197	130.773	131.739	131.245	130.501	130.067	134.178	131.667	130.860	130.515
$D(C_{12}—C_{11}—C_{10}—C_2)$	89.692	92.858	92.934	93.600	94.384	94.157	94.314	95.055	92.231	93.300	93.560	94.149

Table 4
Single and total HB energies in NBH, MBH and CBH.

System	Total electronic energies[a,b]	$E(O_6—H_6\cdots O_1)$	$E(O_3—H_3\cdots O_4)$	E(total HB)[c]
NBH	−1617.827809			−113.02252
NBH1	−1617.808439	−50.85593		
NBH2	−1617.804131		−62.16659	
MBH	−1527.817795			−119.12156
MBH1	−1527.797835	−52.40498		
MBH2	−1527.792384		−66.71658	
CBH	−1912.220395			−115.9132
CBH1	−1912.200641	−51.86413		
CBH2	−1912.196000		−64.04907	

[a] ZP corrected;
[b] hartree;
[c] kJ/mol.

inhibited the growth rate of both antibiotic susceptive and resistant bacterial strains.

In Vitro Toxicity measurement

To further explore the safety for the possible development, we investigated the cytotoxicity of NBH, MBH and CBH to cultured HUVECs in vitro. Compared with the group, there was no significant difference on cell viability in 200 μg/mL NBH, MBH or CBH treated group ($P > 0.05$), and the IC_{50} to the HUVECs was 796.24, 755.38 and 648.17 μg/mL for NBH, MBH and CBH, respectively (Fig. 8). These results implied that these compounds had much less toxicity to mammalian cells, and had a relatively less toxicity for potential clinical applications.

Table 5
Electron density ($\boldsymbol{\rho_b}$) and Laplacian ($\nabla^2\boldsymbol{\rho_b}$) in NBH, MBH and CBH.

Bond	NBH		MBH		CBH	
	ρ_b	$\nabla^2\rho_b$	ρ_b	$\nabla^2\rho_b$	ρ_b	$\nabla^2\rho_b$
Upper exocyclic ring						
$O_3—H_3$	0.30352	−1.546053	0.304622	−1.562589	0.303716	−1.551914
$C_3—O_3(—H_3)$	0.315425	−0.337381	0.315551	−0.335714	0.315882	−0.334815
$C_{19}═O_4$	0.399455	−0.094182	0.400664	−0.076412	0.400403	−0.0823166
$O_4\cdots H_3$	0.049313	0.154738	0.047717	0.150724	0.048612	0.1531226
Lower exocyclic ring						
$O_6—H_6$	0.30639	−1.57103	0.306789	−1.575185	0.306658	−1.573283
$C_{12}—O_6(—H_6)$	0.31154	−0.351326	0.310623	−0.356691	0.310841	−0.353791
$C_1═O_1$	0.397246	−0.120685	0.397213	−0.116372	0.397169	−0.119333
$O_1\cdots H_6$	0.0446953	0.138151	0.044462	0.137486	0.044526	0.137514

Table 6
MIC values of NBH, MBH, CBH and antibiotics.

Compounds/Drugs	MIC (μg/mL)			
	S. aureas	MRSA	MRSA 2	MRSA 3
NBH	16–32	16–32	16–32	16–32
MBH	128–256	64–28	64–128	>256
CBH	128–256	128–256	128–256	>256
Levofloxacin	<0.125 (S)	8 (R)	8 (R)	8 (R)
Ceftazidime	8 (S)	>256 (R)	256 (R)	64 (R)
Ceftriaxone	4 (S)	>256 (R)	256 (R)	32 (R)
Gentamicin	0.5 (S)	64 (R)	32 (R)	0.5 (S)
Piperacillin	4 (S)	>256 (R)	>256 (R)	8 (S)

S means drug susceptibility, *R* means drug resistance.

Discussion

To get ahead of the problem on resistance, we must look for first-in-class antibacterials and new targets. Anti-bacterial activity and drug resistance of bacteria have a close relationship with the structure of antibiotics, hence the discovery of antibiotics with novel structures and the development of drugs against MRSA are highly active fields. The number of new antibacterials reaching clinical practice has significantly reduced in the last 20 years. Linezolid and daptomycin are the only two new antibiotics whose structures are different compared with previous antibiotics, and daptomycin bacterial resistance has not been solved since the development of the drug in 2003 [30]. Biscoumarins are widely distributed in nature and exhibit a broad pharmacological profile. No antibiotic that has a similar structure as biscoumarin derivatives is clinically being used for infection treatment. In this work, three novel biscoumarin derivatives were demonstrated to be capable of inhibit the growth of MRSA using MICs and bacterial growth inhibitory rate measurement. However, compared with NBH, MBH and CBH exerted almost no effect on any strain of *S. aureus*.

Our results also showed that there are two asymmetrical intramolecular H-bonds in the three compounds, which is considered as an important factor for the biological activity [31]. The molecular structures of the three compounds were also calculated by DFT method and the results were in agreement with the experimental data. In addition, the total HB stabilization energies in NBH, MBH, and CBH were estimated to be −113.02252, −119.12156, and −115.9132 kJ/mol, respectively. These values

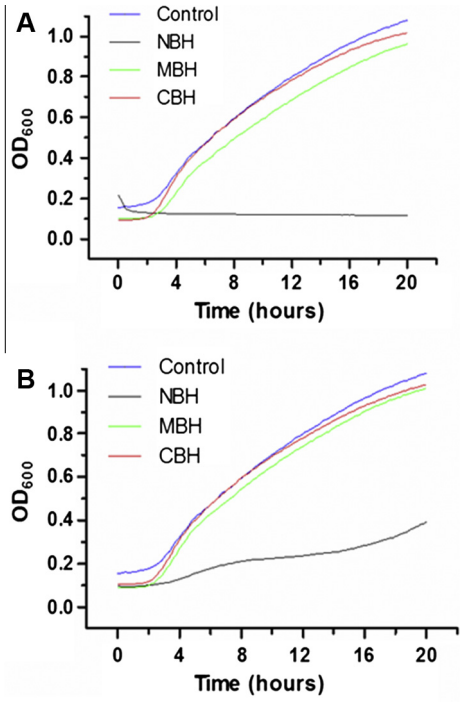

Fig. 4. The time-kill curves with two different concentrations (A: 128 μg/mL; B: 32 μg/mL) of NBH, MBH or CBH on *S. aureus* ATCC 29213.

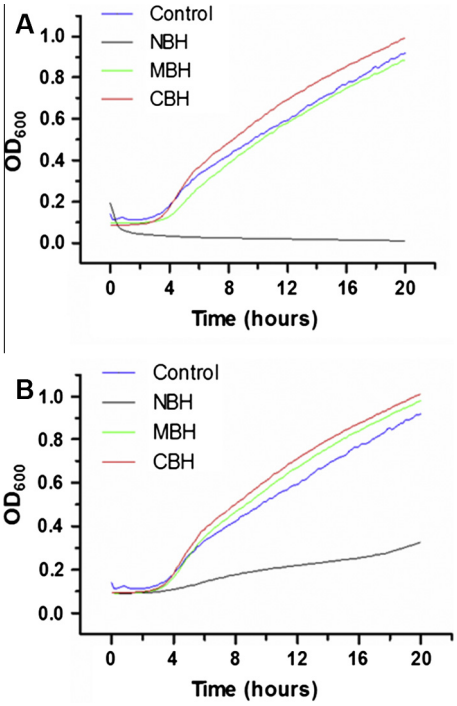

Fig. 5. The time-kill curves with two different concentrations (A: 128 μg/mL; B: 32 μg/mL) of NBH, MBH and CBH on MRSA strain1.

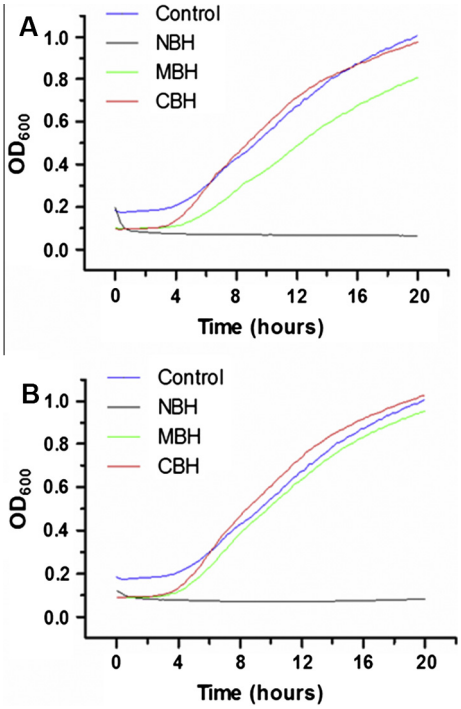

Fig. 6. The time-kill curves with two different concentrations (A: 128 μg/mL; B: 32 μg/mL) of NBH, MBH and CBH on MRSA strain 2.

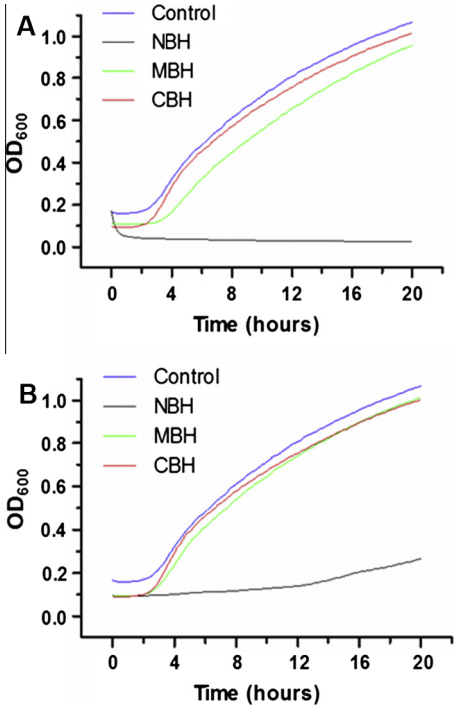

Fig. 7. The time-kill curves with two different concentrations (A: 128 μg/mL; B: 32 μg/mL) of NBH, MBH and CBH on MRSA strain 3.

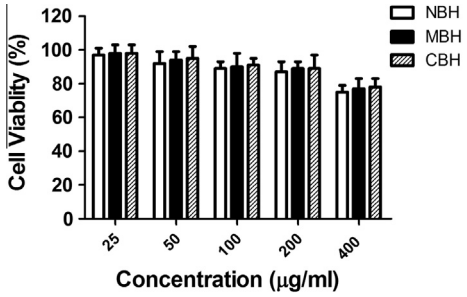

Fig. 8. Cytotoxicity measurement of NBH, MBH and CBH on the HUVECs in vitro.

suggest that the most potent antibacterial activity in NBH was consistent with its weaker HB strengths.

Conclusion

Our results showed that compared with MBH and CBH, the novel biscoumarin derivative NBH had the most potent anti-bacterial efficiency. Intramolecular HB strength is related to the stability of chemical structure, which further affects the binding affinity between molecules and target protein. The activity of NBH is possibly due to its weakest HB strengths, which is effected by the strong electron-withdrawing nitro group.

Supplementary material

CCDC 872212, 888353 and 843711 of compounds NBH, MBH and CBH contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Center, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033).

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