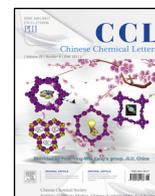




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Original article

## Synthesis and biological evaluation of dihydrotriazine derivatives as potential antibacterial agents

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### ABSTRACT

A series of 1,4-dihydro-1,3,5-triazine derivatives were designed and synthesized and their antibacterial and antifungal activities were evaluated. Most of the synthesized compounds showed potent inhibition of several Gram-positive bacterial strains (including multidrug-resistant clinical isolates) and Gram-negative bacterial strains, with minimum inhibitory concentrations (MICs) in the range of 2.1–181.2  $\mu\text{mol/L}$ . Compounds **7a** and **7c** presented the most potent inhibitory activities against Gram-positive bacteria (e.g., *Staphylococcus aureus* 4220), Gram-negative bacteria (e.g., *Escherichia coli* 1924), and the fungus *Candida albicans* 7535, with MICs of 2.1 or 4.1  $\mu\text{mol/L}$ . Especially, compound **7a** was the most potent, with an MIC of 2.1  $\mu\text{mol/L}$  against four multidrug-resistant, Gram-positive bacterial strains. The cytotoxic activity of the compound **7a**, **7c** and **7f** was assessed in HepG2 cells, and the results suggest that 1,4-dihydro-1,3,5-triazine derivatives bearing a 6-benzylloxynaphthalen moiety are interesting scaffolds for the development of novel antibacterial agents.

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## 1. Introduction

The management of bacterial infections has become increasingly difficult with the emergence of antimicrobial resistance. In particular, the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are causing significant problems worldwide [1]. Methicillin-resistant *S. aureus* (MRSA) is a major pathogen causing both hospital- and community-acquired infections. Among these organisms, MRSA alone accounts for nearly one-half of the deaths attributed to antibiotic-resistant infections. In 2013, the Centers for Disease Control and Prevention reported that more than 11,000 people died from

MRSA-related infections in the United States alone [2]. Therefore, there is an urgent need for the development of new antibacterial agents with divergent and unique structures and mechanisms of action that differ from those of existing antimicrobial agents [3].

Medicinal chemistry studies have shown that a 1,3,5-triazine scaffold is an outstanding pharmacophore, and many molecules with this scaffold have been reported as antibacterial agents. 1,2-Dihydro-1,3,5-triazine (Baker triazines) compounds are by far the best known, with several studies exploring their potential as inhibitors of eukaryotic dihydrofolate reductase (DHFR), an important enzyme in the *de novo* pathway of purine and thymidine synthesis, and small molecules targeting this enzyme have demonstrated utility as potential antibiotics [4,5]. Feng *et al.* reported that the compound *N*<sup>2</sup>-methyl-6-(5-methylisoxazol-3-ylloxy)-*N*<sup>4</sup>-(4-(trifluoromethyl)phenyl)-1,3,5-triazine-2,4-diamine displayed an antimicrobial effect as high as 97.7% at a concentration of 200  $\mu\text{g/mL}$  [6]. Singga *et al.* found that triazine derivatives inhibited some Gram-positive and Gram-negative bacteria by inhibiting cell membrane growth [7].

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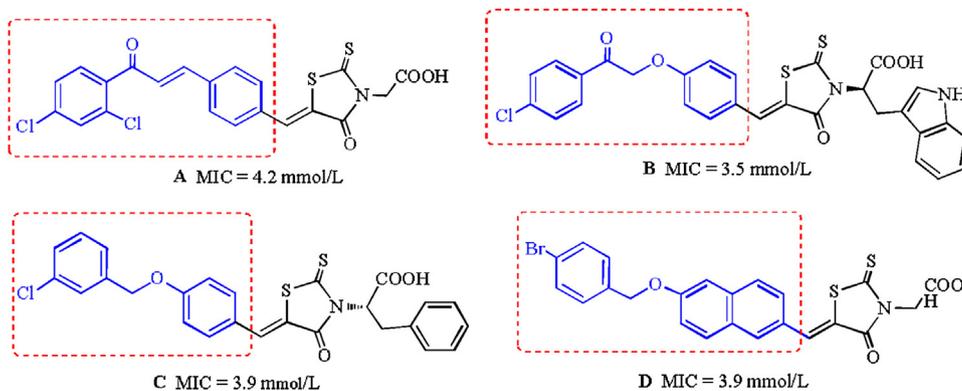


Fig. 1. Previously reported antibacterial compounds.

In our previous work, we found that several rhodamine derivatives bearing chalcone [8], (2-oxo-2-phenylethoxy)benzylidene [9], and (benzyloxy)benzylidene [10] moieties (Fig. 1) showed moderate to strong activities against several Gram-positive bacterial strains, including multidrug-resistant clinical isolates. Unfortunately, compounds belonging to this series did not show any bacteriostatic activity against Gram-negative bacteria. As a part of our ongoing studies toward the development of novel antibacterial agents, here we designed and synthesized a series of 1,4-dihydro-1,3,5-triazine derivatives using a hybrid strategy, in which the rhodamine moiety was replaced by a dihydrogen triazine scaffold. Thus, a series of 26 novel 1,4-dihydro-1,3,5-triazine derivatives (Fig. 2) were synthesized and evaluated for their antibacterial activities *in vitro*. The substituents on the phenyl

ring were changed simultaneously to investigate their contribution to the biological activity.

## 2. Results and discussion

The *in vitro* antibacterial activities of the synthesized compounds were evaluated using a 96-well microtiter plate and a serial dilution method to obtain the minimum inhibitory concentrations (MICs) for different strains (including multidrug-resistant clinical isolates). Two Gram-positive strain (*S. aureus* RN 4220 and *S. Mutans* KTCT 3289), five Gram-negative strains (*Escherichia coli* KCTC 1924, *E. coli* CCARM 1356, *P. aeruginosa* 2742, *P. aeruginosa* 2004, and *Salmonella enterica* serovar Typhimurium 2421) and one fungus (*Candida albicans* 7535) were used for the biological assay. Gatifloxacin, moxifloxacin, norfloxacin, and oxacillin were used as

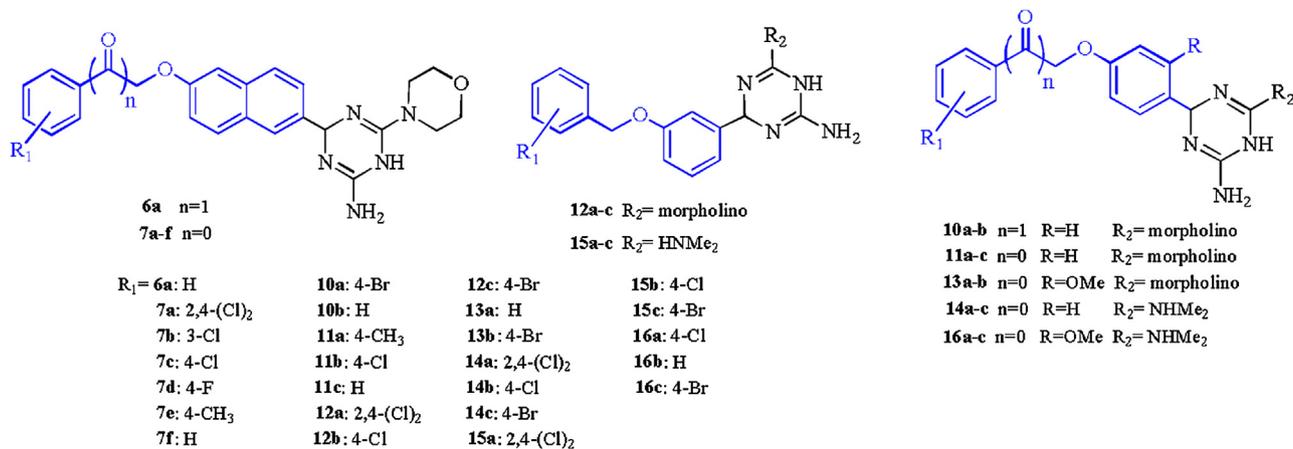


Fig. 2. Synthesized target compounds.

**Table 1**Inhibitory activity (MIC,  $\mu\text{mol/L}$ ) of compounds **6a**, **7a–f**, **10a–b**, **11a–c**, **12a–c**, **13a–b**, **14a–c**, **15a–c** and **16a–c** against various bacteria.

Compound	R <sub>1</sub>	Gram-positive strains		Gram-negative strain			Fungus		
		<i>S. aureus</i>	<i>S. Mutans</i>	<i>E. Coli</i>		<i>P. aeruginosa</i>	<i>S. typhimurium</i>	<i>C. albicans</i>	
		4220 <sup>a</sup>	3289 <sup>b</sup>	1924 <sup>c</sup>	1356 <sup>d</sup>	2742 <sup>e</sup>	2004 <sup>f</sup>	2421 <sup>g</sup>	7535 <sup>h</sup>
<b>6a</b>	H	72.2	>144.4	72.2	>144.4	>144.4	>144.4	>144.4	72.2
<b>7a</b>	2,4-(Cl) <sub>2</sub>	2.1	2.1	2.1	8.3	16.5	16.5	8.3	4.1
<b>7b</b>	3-Cl	4.5	8.9	4.5	35.6	71.2	35.6	35.6	8.9
<b>7c</b>	4-Cl	2.2	8.9	4.5	35.6	71.2	35.6	35.6	4.5
<b>7d</b>	4-F	18.5	18.5	9.2	147.7	>147.7	147.7	147.7	9.2
<b>7e</b>	4-CH <sub>3</sub>	4.7	18.6	9.3	37.3	74.6	74.6	74.6	37.3
<b>7f</b>	H	38.5	38.5	9.6	154.1	>154.1	154.1	154.1	9.6
<b>10a</b>	4-Br	67.9	>135.6	135.6	>135.6	>135.6	>135.6	>135.6	135.6
<b>10b</b>	H	>162.8	>162.8	>162.8	>162.8	>162.8	>162.8	>162.8	>162.8
<b>11a</b>	4-CH <sub>3</sub>	21.1	168.8	42.2	>168.8	>168.8	>168.8	>168.8	42.2
<b>11b</b>	4-Cl	20	80.2	40.1	>160.3	>160.3	>160.3	>160.3	40.1
<b>11c</b>	H	175.3	>175.3	175.3	>175.3	>175.3	>175.3	>175.3	>175.3
<b>12a</b>	2,4-(Cl) <sub>2</sub>	9.2	36.9	18.5	147.8	>147.8	73.9	147.8	18.5
<b>12b</b>	4-Cl	20	80.2	40.1	>160.3	>160.3	160.3	>160.3	40.1
<b>12c</b>	4-Br	18.1	72.2	36.1	>144.4	>144.4	144.4	>144.4	36.1
<b>13a</b>	H	>161.9	>161.9	>161.9	>161.9	>161.9	>161.9	>161.9	>161.9
<b>13b</b>	4-Br	33.8	135.3	33.8	>135.3	>135.3	>135.3	>135.3	>135.3
<b>14a</b>	2,4-(Cl) <sub>2</sub>	10.2	20.5	2.6	40.9	163.6	163.6	81.8	20.5
<b>14b</b>	4-Cl	22.4	44.8	22.4	>179.2	>179.2	>179.2	179.2	44.8
<b>14c</b>	4-Br	19.9	39.9	10	79.8	>159.6	>159.6	79.8	39.9
<b>15a</b>	2,4-(Cl) <sub>2</sub>	10.2	40.9	5.1	81.8	163.6	81.8	81.8	20.5
<b>15b</b>	4-Cl	89.6	89.6	44.8	>179.2	>179.2	179.2	179.2	89.6
<b>15c</b>	4-Br	39.9	79.8	19.9	159.6	>159.6	159.6	79.8	39.9
<b>16a</b>	4-Cl	82.7	82.7	41.3	>165.3	>165.3	>165.3	>165.3	165.3
<b>16b</b>	H	181.2	>181.2	181.2	>181.2	>181.2	>181.2	>181.2	>181.2
<b>16c</b>	4-Br	37.1	148.5	74.2	>148.5	>148.5	>148.5	>148.5	148.5
Gatifloxacin		0.7	0.7	5.3	42.6	2.7	5.3	1.3	1.3
Moxifloxacin		0.6	0.6	5.0	318.9	2.5	5.0	1.2	1.2
Fluconazole		n.d	n.d	n.d	n.d	n.d	n.d	n.d	3.3
Itraconazole		n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.09

n.d.: Not determined.

<sup>a</sup> *Staphylococcus aureus* RN 4220 (*S. aureus* RN 4220 is a genotype of *S. aureus*. KCTC, Korean Collection for Type Cultures; CCARM, Culture Collection of Antimicrobial Resistant Microbes).<sup>b</sup> *Streptococcus mutans* 3289.<sup>c</sup> *Escherichia coli* KCTC 1924.<sup>d</sup> *Escherichia coli* CCARM 1356.<sup>e</sup> *Pseudomonas aeruginosa* 2742.<sup>f</sup> *Pseudomonas aeruginosa* 2004.<sup>g</sup> *Salmonella enterica* serovar Typhimurium 2421.<sup>h</sup> *Candida albicans* 7535.

positive controls for antibacterial activity, and fluconazole and itraconazole were used as positive controls for antifungal activity.

The *in vitro* antibacterial and antifungal activities of the synthesized compounds are shown in Table 1. Most of the synthesized compounds showed potent inhibitory activities against the different bacteria and one fungus, with MICs ranging from 2.1 to 181.2  $\mu\text{mol/L}$ . Compounds **7a** and **7c** exhibited the highest activity of all the compounds synthesized against *S. aureus* 4220, with an MIC of 2.1  $\mu\text{mol/L}$ . Against the Gram-negative *E. coli* 1924, compounds **7a**, **7b**, **7c**, **14a**, and **15a** were equipotent or more potent than the positive controls gatifloxacin and moxifloxacin (MIC = 5.0  $\mu\text{mol/L}$ ), with an MIC of 2.1  $\mu\text{mol/L}$ . The potency of compound **7a**, with an MIC of 8.3  $\mu\text{mol/L}$ , was 5-fold greater than that of the positive control gatifloxacin (MIC = 42.6  $\mu\text{mol/L}$ ). The potency of compounds **7b**, **7c**, **7e**, and **14a**, with an MIC of 35.6  $\mu\text{mol/L}$  against *E. coli* 1356, was 9-fold greater than that of the positive control moxifloxacin (MIC = 318.9  $\mu\text{mol/L}$ ). However, for the *P. aeruginosa* 2742 and 2004 strains, only compound **7a** exhibited good activity, with an MIC of 16.5  $\mu\text{mol/L}$ . Against *C. albicans* 7535, compounds **7a** and **7c** displayed the strongest

potency of all the compounds synthesized, with an MIC of 4.1  $\mu\text{mol/L}$ , which was slightly lower than that of fluconazole (MIC = 3.3  $\mu\text{mol/L}$ ). Previously, we reported that rhodamine derivatives bearing benzyloxy benzaldehyde or benzyloxy naphthaldehyde moieties did not show any activity against the Gram-negative bacteria *E. coli* and *P. aeruginosa* [11], but dihydro-1,3,5-triazine derivatives displayed moderate to high levels of inhibition against these strains, indicating that the dihydro-1,3,5-triazine scaffold is critical for drug potency. A comparison of the potency of compounds **6a** and **7a–f** revealed that the introduction of an additional ketone moiety reduced the antimicrobial activity. The introduction of some halogen groups on the phenyl ring generally increased the antimicrobial activity relative to that of the non-substituted derivatives. Notably, the position of the Cl substituent on the phenyl ring affected the potency, with activity in the order of 2,4-(Cl)<sub>2</sub> > 4-Cl  $\geq$  3-Cl. Isosteric replacement with a morpholine or dimethylamine group at the *N*-position of the dihydro-1,3,5-triazine ring generally did not affect the antimicrobial activity. It is noteworthy that compounds bearing a 2,4-Cl<sub>2</sub> substituted phenyl ring showed excellent antimicrobial activities. Therefore, these

**Table 2**  
Inhibitory activity (MIC,  $\mu\text{mol/L}$ ) of compounds **6a**, **7a–f**, **10a–b**, **11a–c**, **12a–c**, **13a–b**, **14a–c**, **15a–c** and **16a–c** against clinical isolates of multidrug-resistant Gram-positive strains.

Compound	R <sub>1</sub>	MRSA		QRSA	
		3167 <sup>a</sup>	3506 <sup>b</sup>	3505 <sup>c</sup>	3519 <sup>d</sup>
<b>6a</b>	H	72.2	144.4	144.4	72.2
<b>7a</b>	2,4-(Cl) <sub>2</sub>	2.1	2.1	2.1	2.1
<b>7b</b>	3-Cl	2.2	4.5	8.9	4.5
<b>7c</b>	4-Cl	2.2	4.5	8.9	8.9
<b>7d</b>	4-F	4.6	9.2	18.6	4.6
<b>7e</b>	4-CH <sub>3</sub>	4.7	4.7	9.3	9.3
<b>7f</b>	H	9.6	19.3	19.3	9.6
<b>10a</b>	4-Br	67.9	>135.6	>135.6	135.6
<b>10b</b>	H	>162.8	>162.8	>162.8	>162.8
<b>11a</b>	4-CH <sub>3</sub>	42.2	42.2	168.8	42.2
<b>11b</b>	4-Cl	20.0	80.2	80.2	40.1
<b>11c</b>	H	87.6	>175.3	>175.3	>175.3
<b>12a</b>	2,4-(Cl) <sub>2</sub>	9.2	18.5	18.5	9.2
<b>12b</b>	4-Cl	20.0	40.1	80.2	40.1
<b>12c</b>	4-Br	18.1	36.1	72.2	36.1
<b>13a</b>	H	>161.9	>161.9	>161.9	>161.9
<b>13b</b>	4-Br	33.8	67.6	135.3	67.6
<b>14a</b>	2,4-(Cl) <sub>2</sub>	10.2	20.5	20.5	5.1
<b>14b</b>	4-Cl	22.4	89.6	89.6	44.8
<b>14c</b>	4-Br	10.0	79.8	79.8	39.9
<b>15a</b>	2,4-(Cl) <sub>2</sub>	5.1	40.9	40.9	20.4
<b>15b</b>	4-Cl	44.8	179.2	179.2	89.6
<b>15c</b>	4-Br	19.9	10.0	79.8	39.9
<b>16a</b>	4-Cl	82.7	41.3	165.3	82.7
<b>16b</b>	H	>181.2	>181.2	>181.2	>181.2
<b>16c</b>	4-Br	37.1	37.1	148.5	74.2
Gatifloxacin		5.3	5.3	21.2	10.6
Moxifloxacin		2.5	2.5	10.0	10.0
Norfloxacin		25.0	12.5	>200.4	>200.4
Oxacillin		>159.4	>159.4	2.5	2.5

<sup>a</sup> Methicillin-resistant *S. aureus* CCARM 3167.<sup>b</sup> Methicillin-resistant *S. aureus* CCARM 3506.<sup>c</sup> Quinolone-resistant *S. aureus* CCARM 3505.<sup>d</sup> Quinolone-resistant *S. aureus* CCARM 3519.

results provide further evidence that the 2,4-Cl<sub>2</sub> substituted benzene ring plays a critical role in the antimicrobial activity of these compounds, which is consistent with the results obtained for a previously reported series of rhodamine and aminoguanidine derivatives [12,13].

As indicated in Table 2, the inhibitory activities of all the synthesized compounds were tested against the clinical isolates of several different multidrug-resistant, Gram-positive bacterial strains (MRSA CCARM 3167 and 3506, and quinolone-resistant *S. aureus* (QRSA) CCARM 3505 and 3519). Compound **7a** exhibited the

most potent activity against the MRSA and QRSA strains, with an MIC of 2.1  $\mu\text{mol/L}$ , making it equipotent or more potent than gatifloxacin and norfloxacin (MICs of 8 and 4  $\mu\text{g/mL}$  against MRSA 3167 and 3506, respectively). In general, against multidrug-resistant strains, series **7** compounds were much more potent than the compounds in the other series. Compounds **7a**, **7b**, **7d**, and **14a** exhibited 2 to 4-fold greater activities, with MICs of 2.1–4.1  $\mu\text{mol/L}$ , than gatifloxacin and moxifloxacin (MICs = 10.0–21.2  $\mu\text{mol/L}$ ), and the activity of compound **7a** was equal to that of oxacillin (MIC = 2.5  $\mu\text{mol/L}$ ).

To see whether the antibacterial activity of the synthesized compounds were selectively toxic, we evaluated the cytotoxicity of compound **7a**, **7c** and **7f** using a standard technique. As shown in Table 3, compound **7f** exhibited weaker activity than **7c** against the different bacteria, in spite of its slight greater cytotoxicity than **7c**, comparably indicating that the promising antibacterial activity of these compounds may not be due to their cytotoxicity, but some unknown mechanism of action.

To rationalize the observed antibacterial activity and understand the possible mechanism of action of these compounds, a libdocking investigation was undertaken. The crystal structure data (*S. aureus* DHFR) were obtained from the protein data bank (PDB ID: 3fra) [14,15]. Enzyme structures were checked for missing atoms, bonds and contacts. Hydrogen atoms were added to the enzyme structure. Water molecules and bound ligands were manually deleted. Preferred coordination modes of **7a** and **7f** with dihydrofolate reductase (DHFR) protein are presented in Fig. 3. Fragment B of **7f** is bound into the active site, in which the benzyl group shows H-bond interaction with Val 31 and Ala 7. Fragment A of **7a** is bound into the active site where the morpholino group shows H-bond interaction with Phe 92, Leu 5, and Val 31, comparably indicated its more potent activity than that of **7f**. The preliminary docking results imply that compounds **7a** and **7f** possibly display their antibacterial activity through the interaction with DHFR protein by targeting residues of the active cavities of DHFR.

### 3. Conclusions

Based on our previous work, we synthesized a series of 1,4-dihydro-1,3,5-triazine derivatives and evaluated their antibacterial activities against several Gram-positive and Gram-negative bacteria, as well as a fungus (*C. albicans*). Some of these compounds exhibited high inhibitory activities against the Gram-positive and Gram-negative bacteria, including several multidrug-resistant clinical isolates. Compound **7a** showed the most potent antimicrobial activity (MIC = 2.1  $\mu\text{mol/L}$ ) against selected MRSA and QRSA strains. Preliminary docking study showed that these compounds have a good interaction with the active cavities of DHFR, possibly exhibit their potency via inhibiting DHFR. Further study examining the mechanisms of action of these compounds is currently underway in our laboratory.

### 4. Experimental

The synthesis of the target compounds is presented in Scheme 1. A series of intermediates (**4**, **5**, **8**, and **9**) was obtained by reacting hydroxybenzaldehyde or vanillin with the appropriate substituted benzyl chlorides or 2-bromo-1-phenylethanones [16]. Then, the intermediates were reacted with moroxydine hydrochloride or metformin hydrochloride in acetic acid to generate the target compounds. The structures of the synthesized compounds were confirmed by <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, and mass spectroscopy (see Supporting information).

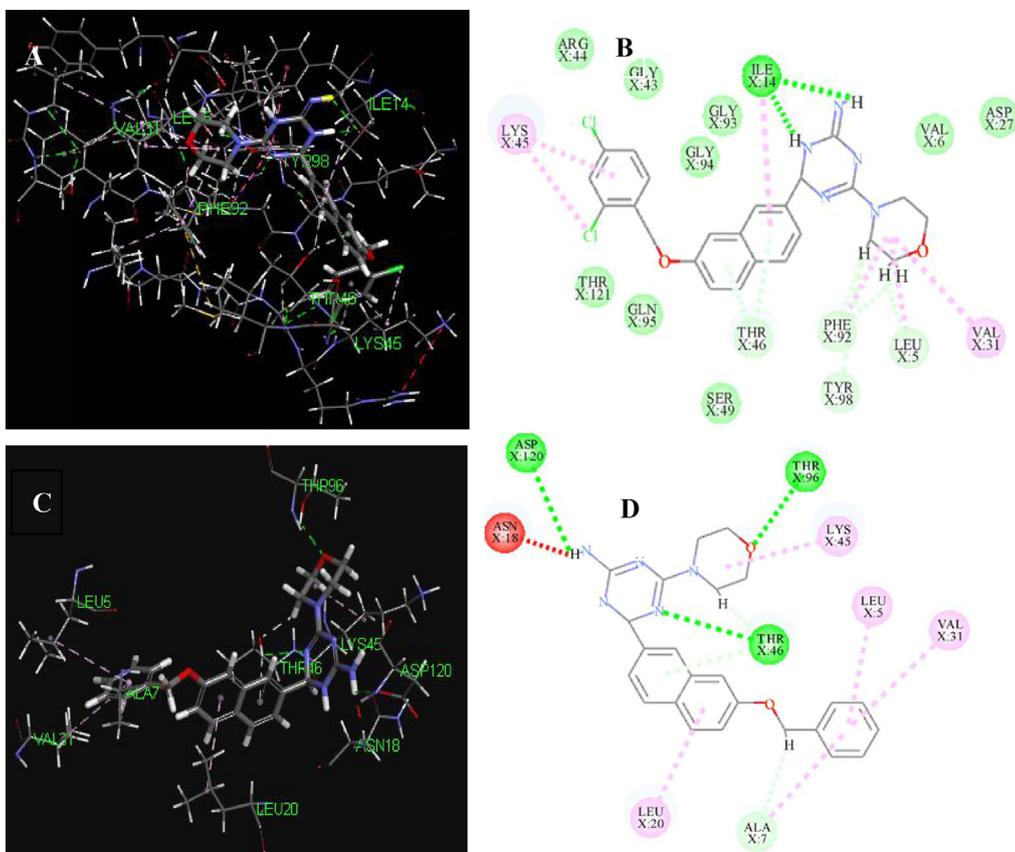
**Table 3**  
Antibacterial activity and cytotoxicity for **7a**, **7c** and **7f**.

	Test organisms	<b>7a</b>	<b>7c</b>	<b>7f</b>
MIC ( $\mu\text{mol/L}$ )	<i>S. aureus</i> 4220	2.1	2.2	38.5
	MRSA 3167	2.1	2.2	9.6
IC <sub>50</sub> <sup>a</sup> ( $\mu\text{mol/L}$ )	HepG2 <sup>b</sup>	34.3	46.7	51.1

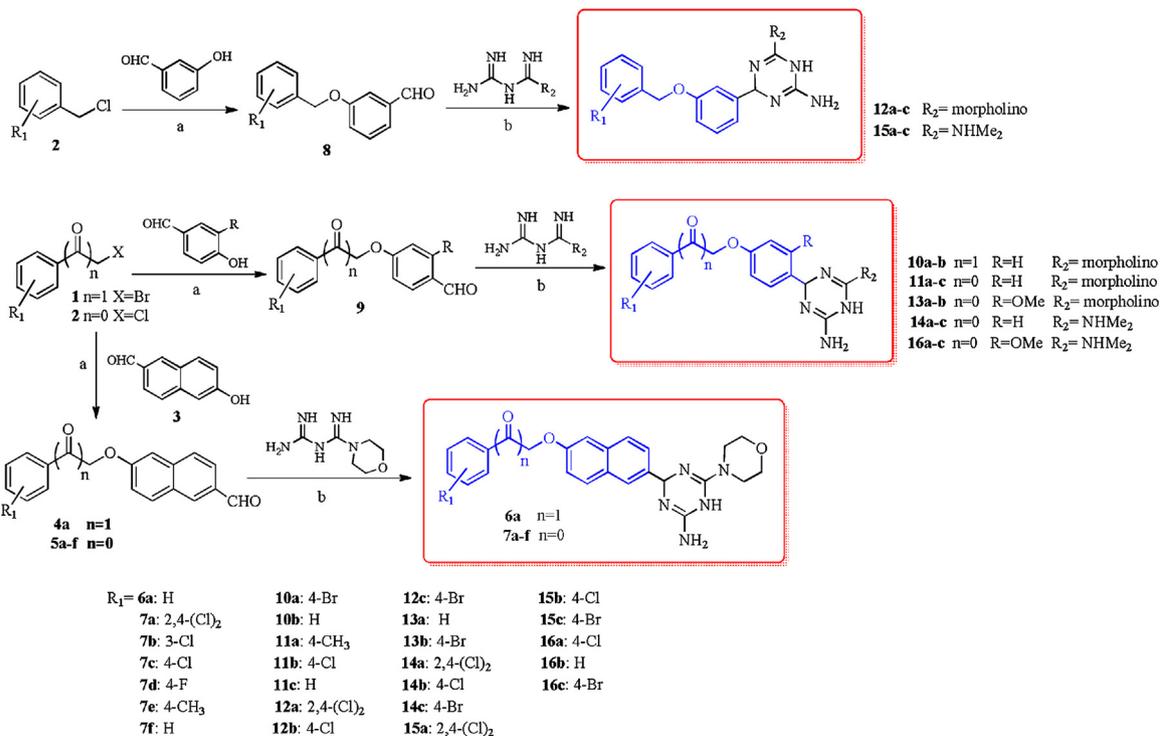
<sup>a</sup>IC<sub>50</sub> is the concentration required to inhibit the cell growth by 50%.

Data represent the average of three independent experiments running in triplicate. Variation was generally between 5–10%.

<sup>b</sup>Human hepatic cells.



**Fig. 3.** Docking result of compound **7a** and **7f**. (A) Key residues in binding site surrounding **7a**. (B) 2D molecular docking modeling of compound **7a** with 3fra. (C) Key residues in binding site surrounding **7f**. (D) 2D molecular docking modeling of compound **7f** with 3fra.



**Scheme 1.** Synthetic scheme for the synthesis of compounds **6a**, **7a–f**, **10a–b**, **11a–c**, **12a–c**, **13a–b**, **14a–c**, **15a–c** and **16a–c**. Reagents and conditions (a) K<sub>2</sub>CO<sub>3</sub>, DMF, 60 °C (b) AcOH, 120 °C, 4–8 h.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ccllet.2017.05.022>.

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