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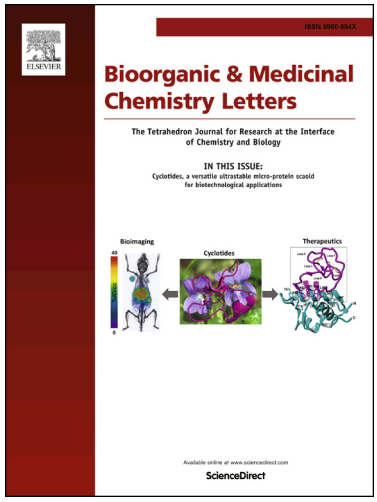

Synthesis and evaluation of the antibacterial activities of aryl substituted dihydrotriazine derivatives

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PII: S0960-894X(18)30230-0
DOI: https://doi.org/10.1016/j.bmcl.2018.03.037
Reference: BMCL 25691

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 20 November 2017
Revised Date: 25 January 2018
Accepted Date: 15 March 2018

Please cite this article as: Zhang, T-Y., Yu, Z-K., Jin, X-J., Li, M-Y., Sun, L-P., Zheng, C-J., Piao, H-R., Synthesis and evaluation of the antibacterial activities of aryl substituted dihydrotriazine derivatives, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.03.037

Synthesis and evaluation of the antibacterial activities of aryl substituted dihydrotriazine derivatives

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Abstract

Five series of dihydrotriazine derivatives containing chalcone (**13a–i**), phenoxy acetophenone (**14a–b**), benzyl benzene (**15a–c**), naphthoxyl acetophenone (**16a–b**) and benzyl naphthalene (**17a–h**) moieties were designed and synthesized. The antibacterial and antifungal activities of these compounds were evaluated against several strains of Gram-positive and Gram-negative bacteria, as well as a single fungus. Compound **17h** was found to be the most potent of all of the compounds tested, with an MIC value of 0.5 μg/mL against several Gram-positive (*Staphylococcus aureus* 4220 and QRSA CCARM 3505) and Gram-negative (*Escherichia coli* 1924) strains of bacteria. However, this compound was inactive against *Pseudomonas aeruginosa* 2742 and *Salmonella typhimurium* 2421, indicating that its antibacterial spectrum is similar to those of the positive controls gatifloxacin and moxifloxacin. The cytotoxic activity of the compound **13i**, **16b** and **17h** was assessed in Human normal liver cells.

***Keywords*:** Antibacterial activitis; Antifungal activities; Cytotoxicity; dihydrotriazine.

Many different types of disease are caused by bacterial infection. In principle, pathogenic bacteria and conditional pathogens can produce toxins and secondary metabolites that can lead to a variety of physical discomforts such as rashes, fever and chills during blood circulation. The discovery of antibiotics and other antimicrobial agents has played a critical role in the fight against bacteria. However, recent increases in drug-resistant bacteria mean that the standard-of-care antibacterial drugs currently used in clinical practice could soon become ineffective, making it difficult to manage these diseases. There is therefore an urgent need for the development of new antibacterial drugs that exert their activity through unique mechanisms of action, enabling them to inhibit the growth of drug-resistant bacteria. The importance of this task is highlighted by the large number government agencies, scientific institutions and clinicians involved in the search for new antimicrobial agents.[1]

1,3,5-triazine derivatives have been reported to exhibit a wide range of interesting biological properties including anticancer, anti-HIV and antimicrobial activities.[2-6] For example, Feng *et al.* reported that N^2-methyl-6-(5-methylisoxazol-3-yloxy)-N^4-(4-(trifluoromethyl)phenyl)-1,3,5-triazine-2,4-diamine displayed an antimicrobial activity of up to 97.7% when it was used at a concentration of around 200 μg/mL.[7] Furthermore, Singga *et al.* reported that the growth some Gram-positive and Gram-negative bacteria could be inhibited by triazine derivatives, which exerted their activity by restricting the growth of the bacterial cell membranes.[8] In our previous work, we reported the identification of a series of 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid derivatives, and demonstrated that all of the compounds belonging to this series showed outstanding bacteriostatic activity against Gram-positive bacteria, as exemplified by compounds A, B, C and D (MIC = 2 μg/mL) (Fig. 1).[9-12] Unfortunately, however, compounds belonging to this series did not show any bacteriostatic activity against Gram-negative bacteria. In this study, we designed and synthesized five novel series of compounds (**13a**–**i**, **14a**–**b**, **15a**–**c**, **16a**–**b**, **17a**–**h**) (Fig. 2) using a hybrid strategy with compounds A–D (Fig. 1) as the lead compounds. Notably, the 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid moiety in these lead compounds was replaced with a dihydrotriazine ring. These compounds

were subsequently evaluated in terms of their antibacterial activities with an aim of identifying a new series of potent antimicrobial agents.

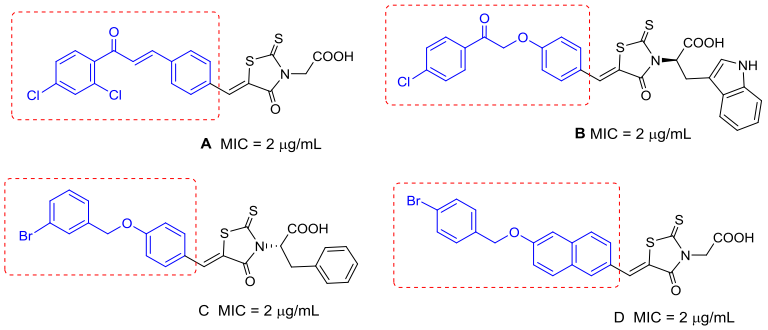
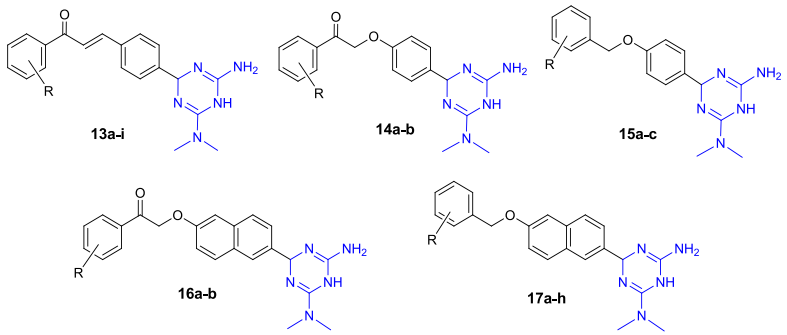


Figure 1. Previously reported antibacterial compounds

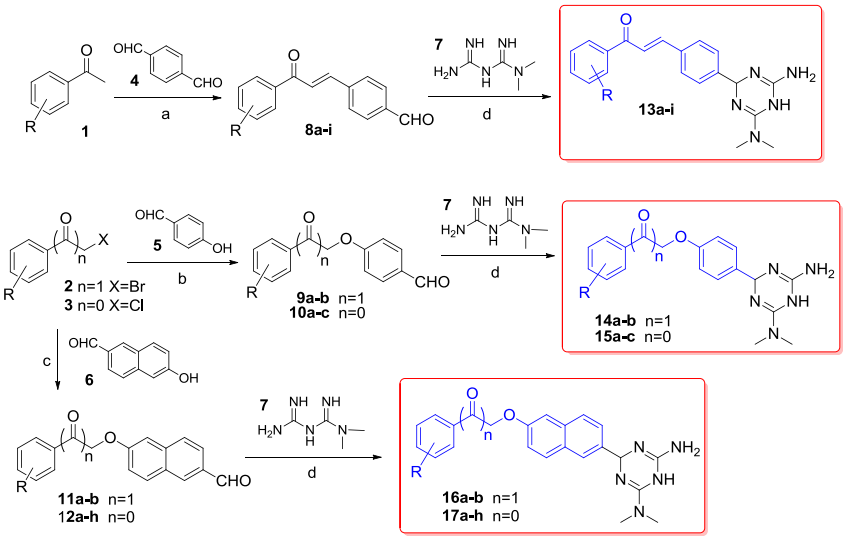


13a: R= H	13i: R= 2,4-$(Cl)_2$	17a: R= H
13b: R= 4-F	14a: R= H	17b: R= 4-CH_3
13c: R= 2-Br	14b: R= 4-Br	17c: R= 2-Cl
13d: R= 4-Br	15a: R= H	17d: R= 3-Cl
13e: R= 2-OCH_3	15b: R= 4-CH_3	17e: R= 4-Cl
13f: R= 3-OCH_3	15c: R= 4-F	17f: R= 2-F
13g: R= 4-CH_3	16a: R= H	17g: R= 4-Br
13h: R= 2,4-$(Cl)_2$	16b: R= 4-Br	17h: R= 2,4-$(Cl)_2$

Figure 2. Designed target compounds

The route used to synthesize the five different series of dihydrotriazine derivatives is shown in Scheme 1. Metformin hydrochloride was prepared by the reaction of dicyandiamide with minocycline hydrochloride according to a previously reported method.[13] Acetophenone was reacted with terephthalaldehyde in the presence of ethylene glycol to afford a series of (*E*)-4-(3-oxo-3-phenylprop-1-en-1-yl)benzaldehydes (**8a–i**). Two 4-(2-oxo-2-phenylethoxy)benzaldehydes (**9a–b**) were prepared by reacting the corresponding substituted 2-bromo-1-phenylethanones (**2**) with 4-hydroxybenzaldehyde in the presence of K_2CO_3. Two 6-(2-oxo-2-phenylethoxy)-2-naphthaldehydes (**11a–b**) were prepared using the same method by reacting **2** with 6-hydroxy-2-naphthaldehyde (**6**). Three

4-(benzyloxy)benzaldehydes (**10a–c**) and eight 6-(benzyloxy)-2-naphthaldehydes (**12a–h**) were obtained by reacting substituted chloromethylbenzene (**3**) with 4-hydroxybenzaldehydes (**5**) and **6**, respectively. The reductive cyclization reactions of the five intermediate series (**8a–i**, **9a–b**, **10a–c**, **11a–b**, **12a–h**) with metformin hydrochloride (**7**) afforded the corresponding novel dihydrotriazine derivatives (**13a–i**, **14a–b**, **15a–c**, **16a–b**, **17a–h**). The structures of the synthesized dihydrotriazine compounds were characterized by ^{1}H NMR, ^{13}C NMR and HRMS analyses. The purity (≥95%) of the compound is verified by the HPLC study performed on Develosil C18 (4.6 mm×250 mm, 5 µm) column using a mixture of solvent 0.1FA acetonitrile/0.1FA water at the flow rate of 1 mL/min and peak detection at 334 nm under UV. [14]



13a: R= H
13b: R= 4-F
13c: R= 2-Br
13d: R= 4-Br
13e: R= 2-OCH_3
13f: R= 3-OCH_3
13g: R= 4-CH_3
13h: R= 2,4-$(Cl)_2$
13i: R= 2,4-$(Cl)_2$
14a: R= H
14b: R= 4-Br
15a: R= H
15b: R= 4-CH_3
15c: R= 4-F
16a: R= H
16b: R= 4-Br
17a: R= H
17b: R= 4-CH_3
17c: R= 2-Cl
17d: R= 3-Cl
17e: R= 4-Cl
17f: R= 2-F
17g: R= 4-Br
17h: R= 2,4-$(Cl)_2$

Scheme 1. Synthetic scheme for the synthesis of compounds **13a-i**, **14a-c**, **15a-c**, **16a-b** and **17a-h**. Reagents and conditions : (a) NaOH, EtOH, 23 ^{o}C, 3-4 h (b) K_2CO_3 , DMF (c) K_2CO_3 , DMF, 60 ^{o}C (d) AcOH, 120 ^{o}C, 4-8h

The *in vitro* antimicrobial and antifungal activities of the synthesized compounds were evaluated against various bacteria, including multidrug-resistant clinical isolates,

as well as one fungus. These screening assays were conducted in 96-well microtiter plates to obtain the minimum inhibitory concentration (MIC) values of the different compounds.[15] Gatifloxacin, moxifloxacin and fluconazole were used as positive controls. The compounds were screened against several Gram-positive strains (*S. aureus* 4220, QRSA CCARM 3505, MRSA CCARM 3167, *S. mutans* 3289) and Gram-negative strains (*Escherichia coli* 1924, *Pseudomonas aeruginosa* 2742, *Salmonella typhimurium* 2421), as well as one fungus (*Candida albicans* 7535), and the results are shown in Table 1. Most of the newly synthesized compounds exhibited potent inhibitory activities against the different bacteria and single fungus tested in the current study with MICs in the range of 0.5 to 64 μg/mL.

Compounds belonging to series **13**, **14**, **15** and **16** showed moderate levels of antimicrobial activity against the different strains of Gram-positive bacteria and fungus, whereas compounds belonging to series **17** exhibited potent activity against the Gram-positive bacteria and fungus with MIC values of 0.5–2 μg/mL (except for **17a**). It is noteworthy that compound **17h** exhibited the greatest activities of all of the compounds prepared in the current study with MIC values in the range of 0.5–1 μg/mL, making its potency comparable to those gatifloxacin and moxifloxacin. Furthermore, compound **17h** was determined to be 8-fold more potent than moxifloxacin (MIC = 4 μg/mL) and 16-fold more potent than Gatifloxacin (MIC = 8 μg/mL) towards QRSA CCARM 3505. In terms of its activity towards the fungus *C. albicans* 7535, compound **17h** displayed the strongest activity of all of the compounds synthesized in the current study with an MIC value of 1 μg/mL, making it equipotent to fluconazole. All of the compounds prepared in this study were found to be inactive against the Gram-negative bacteria *P. aeruginosa* 2742 and *S. typhimurium* 2421, but did display good activity against *E. coli* 1924 with MIC values in the range of 0.5–64 μg/mL. Once again, compounds belonging to series **17** showed the greatest antibacterial activities of all the compounds tested, with MIC values in the range of 0.5–8 μg/mL. Compound **17h**, in particular, exhibited the highest potency with an MIC value of 0.5 μg/mL, making it 4-fold more potent than both of the controls (MIC = 2 μg/mL).

Table 1. Inhibitory activities (MIC[a], μg/mL) of the five different series of compounds against clinical isolates of Gram-positive and Gram-negative bacteria, as well as one fungus.

Compd	R	Gram-positive strains				Fungus	Gram-negative strains		
		S. aureus[b]	QRSA CCARM	MRSA CCARM	*S.mutans*	*C. albicans*	*E.coli*	*P. aeruginosa*	*S. typhinurium*
		4220[b]	3505[c]	3167[d]	3289[e]	7535[f]	1924[g]	2742[h]	2421[i]
13a	H	32	32	32	32	32	64	>64	>64
13b	4-F	32	32	32	16	32	32	>64	>64
13c	2-Br	64	32	32	16	32	64	>64	>64
13d	4-Br	16	8	8	16	16	16	>64	>64
13e	2-OCH_3	64	64	64	64	32	64	>64	>64
13f	3-OCH_3	32	16	16	16	16	16	>64	>64
13g	4-CH_3	32	16	16	16	16	32	>64	>64
13h	2,4-$(CH_3)_2$	32	32	32	32	32	32	>64	>64
13i	2,4-$(Cl)_2$	8	8	8	4	8	8	64	>64
14a	H	>64	>64	>64	>64	>64	>64	>64	>64
14b	4-Br	32	64	32	32	32	64	>64	>64
15a	H	64	32	32	32	32	32	>64	>64
15b	4-CH_3	32	32	32	16	16	16	>64	>64
15c	4-F	64	64	32	64	64	64	>64	>64
16a	H	32	32	32	64	32	64	>64	>64
16b	4-Br	8	4	4	8	16	8	64	64
17a	H	16	8	8	8	8	8	>64	>64
17b	4-CH_3	2	2	2	2	4	2	64	64
17c	2-Cl	2	1	1	2	1	1	64	64
17d	3-Cl	2	1	1	2	1	1	64	64
17e	4-Cl	1	1	1	1	1	1	64	64
17f	2-F	2	4	2	4	4	4	64	64
17g	4-Br	1	1	1	1	1	1	64	64
17h	2,4-$(Cl)_2$	0.5	0.5	1	1	1	0.5	64	64
Gatifloxacin		0.25	8	2	0.25	0.5	2	1	0.5
Moxifloxacin		0.25	4	1	0.25	0.5	2	1	0.5
Fluconazole		nd[j]	nd	nd	nd	1	nd	nd	nd

[a] The antibacterial tests was carried out three times, and the average values were taken as the MICs.
[b] *Staphylococcus aureus* 4220. [c] Quinolone-resistant *Staphylococcus aureus* 3505.
[d] Methicillin-resistant *Staphylococcus aureus* 3167. [e] *Streptococcus mutans* 3289. [f] *Candida albicans* 7535. [g] *Escherichia coli* 1924.
[h] *Pseudomonas aeruginosa* 2742. [i] *Salmonella typhimurium* 2421. [j] nd: Not determined.

Analysis of the structure activity relationships revealed that the inclusion of a dihydrotriazine ring was critical for the antibacterial activity because all of the corresponding aldehyde intermediates were inactive, as reported in our previous

work.[9-12] Compounds belonging to series **13**, **14** and **15** exhibited similar levels of antibacterial activity, indicating that the length of the linker between the two benzene rings had very little effect on the activity of these compounds. In contrast, the inclusion of a naphthalene nucleus, as exemplified by the compounds in series **16** and **17**, resulted in a significant difference in the activity, which indicated that the length of the bridge was critical to the activities of these compounds. Furthermore, the introduction of substituents to the phenyl ring had very little impact on the antimicrobial activity except for the compounds in series **17**, where the introduction of substituents to the benzene ring resulted in an increase in the activity. It is also noteworthy that compound **17h**, bearing a 2,4-dichloro-substituted phenyl ring, showed excellent antimicrobial activities. These results therefore provide further evidence that the inclusion of a 2,4-dichloro-substituted benzene ring is critical to the activity of these compounds, which is consistent with the results obtained for a previously reported series of rhodamine and aminoguanidine derivatives.[16,17]

To determine whether the compounds synthesized in the current study were selectively toxic towards bacteria, we evaluated the cytotoxicities of several representative compounds (**13i**, **16b**, **17h**) using a standard technique.[18] As shown in the Table 2, compound **13i** exhibited weaker activity than **17h** against the different bacteria, in spite of its slight greater cytotoxicity than **17h**, These results therefore suggested that the promising antibacterial activities of these compounds could be attributed to some unknown mechanism of action rather than their general toxicity.[19]

Table 2. Antibacterial activity and cytotoxicity for 13i, 16b and 17h

	Test organisms	13i	16b	17h
MIC (μmol/L)	*S. aureus 4220*	19.27	16.70	1.13
	MRSA 3167	19.27	8.35	2.27
IC_{50}[a] (μmol/L)	L02[b]	45.06	58.70	58.86

[a] IC_{50} is the concentration of compound required to inhibit the growth of the cells by 50%. Values represent the average of three independent experiments running in triplicate. Variation was generally between 5–10%.
[b] Human normal liver cells.

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) [20-22]. 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 1**7a** and 1**7h** with dihydrofolate reductase (DHFR) protein are presented in figure 3. Fragment B of 1**7a** is bound into the active site, in which the benzyl group shows Amide-Pi stacked interaction with Leu 5. Fragment A of 1**7h** is bound into the active site where the dihydrotriazine ring shows H-bond interaction with Phe 92, Leu 5, and Asp 27, comparably indicated its more potent activity than that of 1**7a**. The preliminary docking results imply that compounds 1**7a** and 1**7h** possibly display their antibacterial activity through the interaction with DHFR protein by targeting residues of the active cavities of DHFR.

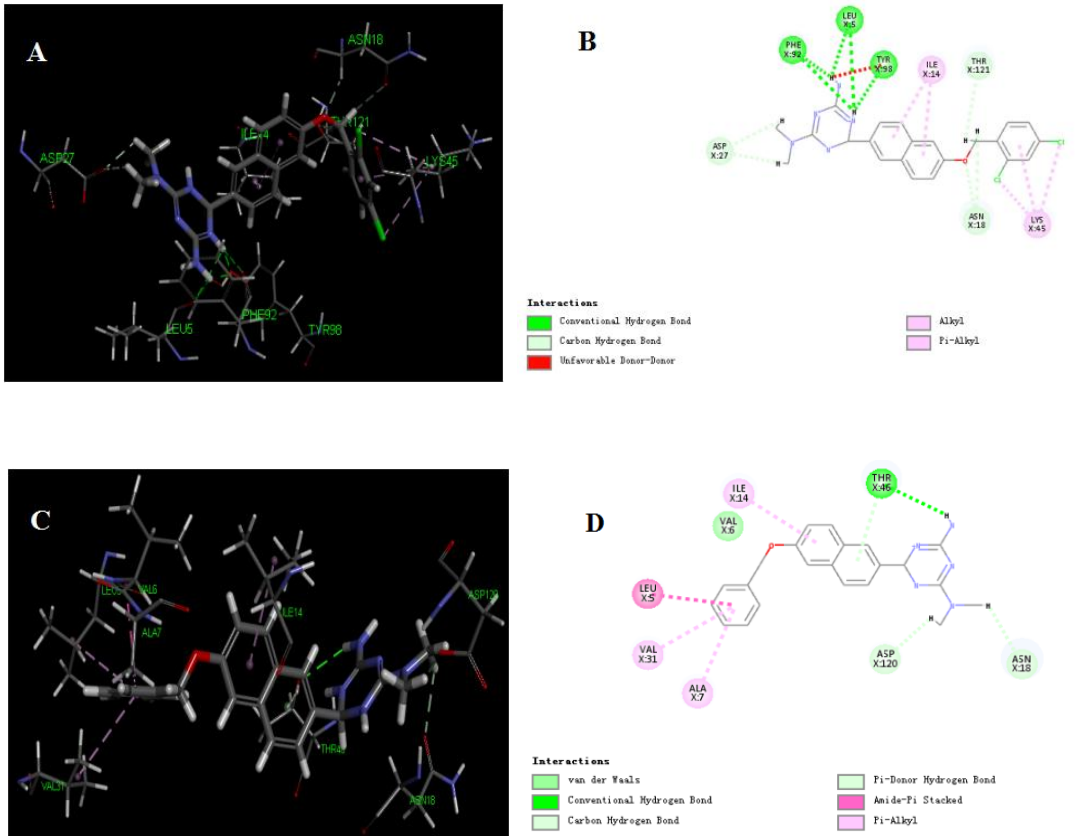


Figure 3. Docking result of compound **17a** and **17h**. (A) Key residues in binding site surrounding **17h**. (B) 2D molecular docking modeling of compound **17h** with 3fra. (C) Key residues in binding site surrounding **17a**. (D) 2D molecular docking modeling of compound **17a** with 3fra.

In order to confirm this phenomenon, compound **17h (**MIC= 0.5 μg/mL**)** was tested for its ability to inhibit DHFR activity *in vitro* enzyme assays (Fig. 4). [23] The test molecule rendered a good inhibitory against the dihydrofolate reductase at 10 μmol/L, comparably indicated its cause 58% decrease than that of the control group. The results imply that compound **17h** exert its antibacterial activity through DHFR inhibition.

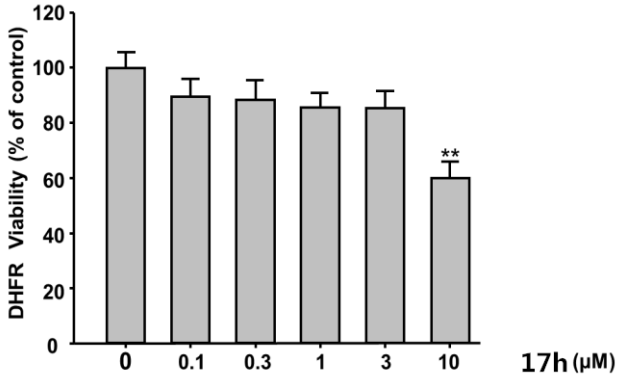


Figure 4. Inhibition of DHFR activities *in vitro*.

In conclusion, five new series of dihydrotriazine derivatives were synthesized using a hybrid strategy and their antibacterial activities were evaluated *in vitro* using standard techniques. The results showed that some of the compounds exhibited pronounced inhibitory activities towards the growth of several strains of Gram-positive bacteria with MIC values in the range of 0.5–64 μg/mL. All five compound series exhibited good antibacterial activities with MIC values in the range of 0.5–64 μg/mL. Notably, all of the compounds in series **17** (except for **17a**) showed strong activity against Gram-positive bacteria and fungus with MIC values of 0.5–2 μg/mL. Compound **17h** exhibited the most potent antibacterial activity of all the compounds in this series, making it comparable to gatifloxacin and moxifloxacin. Furthermore, compound **17h** (MIC = 0.5 μg/mL) was found to be 8- and 16-fold more potent towards QRSA CCARM 3505 than moxifloxacin and gatifloxacin, respectively. This compound also showed excellent activity against *E. coli* 1924 and *C. albicans* 7535 with MIC values of 0.5 and 1 μg/mL, respectively. However, compound **17h** was ineffective against *P. aeruginosa* 2742 and *S. typhimurium* 2421. These results therefore suggest that dihydrotriazine derivatives could potentially be used to develop

potent antibacterial and antifungal agents for the clinical treatment of infectious diseases. Preliminary docking study showed that these compounds have a good interaction with the active cavities of DHFR, possibly exhibit their potency via inhibiting DHFR. In vitro enzyme study implies that compound **17h** exert its antibacterial activity through DHFR inhibition.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (Grant numbers 81260468, 81460524 and 81060257) and the Natural Science Foundation of Jilin Province (No. 20160101218JC).

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14. *Preparation of **17h*****:** Synthesized intermediate compounds (1 mmol) and Metformin hydrochloride (1 mmol) were refluxed in glacial acetic acid (7 mL) at 120 ^{o}C for 4–6 h. The whole processes of the reactions were traced by TLC, then

removed solvent under reduced pressure. The crude products were purified by column chromatography (dichloromethane : methanol = 20 : 1). Yield 82%; m.p. 266-268 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6, ppm) δ 9.04 (s, 2H, NH_2), 7.92 (d, 2H, J = 9.0 Hz, Ar-H), 7.83 (s, 1H, Ar-H), 7.73-7.68 (m, 2H, Ar-H), 7.58-7.48 (m, 4H, Ar-H and NH), 7.30 (dd, 1H, J = 9.0, 3.0 Hz, Ar-H) 5.96 (s, 1H, CH), 5.28 (s, 2H, CH_2), 3.06 (s, 6H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6, ppm) δ 157.85, 156.91, 156.24, 136.48, 134.72, 134.14, 134.07, 133.80, 131.92, 130.28, 129.42, 128.44, 128.14, 128.05, 125.20, 124.93, 119.74, 107.74, 66.84, 62.70, 37.36 (2C). HRMS (MALDI) calcd for $C_{22}H_{21}Cl_2N_5O$: 442.1196, found: 442.1193 (M+1). HPLC purity of 96.94% (retention time = 15.33 min).

15. *Evaluation of anti-bacterial activity in vitro*: The micro-organisms used in the present study were *S. aureus* 4220, *E. Coli* 1942, *S.mutans* 3289, *P. Aeruginosa* 2742, *S. Typhinurium* 2421 and *C. Albicans* 7535. The strains of multidrug-resistant clinical isolates were methicillin-resistant *S. aureus* (MRSA CCARM 3167) and quinolone-resistant *S. aureus* (QRSA CCARM 3505). Clinical isolates were collected from various patients hospitalized in several clinics. Test bacteria were grown to mid-log phase in MuellereHinton broth (MHB) and diluted 1000-fold in the same medium. The bacteria of 10^5 CFU/mL were inoculated into MHB and dispensed at 0.2 mL/well in a 96-well microtiter plate. As positive controls, oxacillin and norfloxacin were used. Test compounds were prepared in DMSO, the final concentration of which did not exceed 0.05%. A two-fold serial dilution technique was used to obtain final concentrations of 64-0.25 mg/mL. The MIC was defined as the concentration of a test compound that completely inhibited bacteria growth during 24 h incubation at 37 ^{o}C. Bacteria growth was determined by measuring the absorption at 650 nm using a microtiter enzyme-linked immunosorbent assay (ELISA) reader. All experiments were carried out three times.

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18. *Cytotoxicity on human cells*: The cytotoxicity test of selected compounds was

measured through the colorimetric MTT assay. Human normal liver cells (L02) suspension in DMEM medium supplemented with 10% FBS and antimycotic was added in 96-well microplates at 1.8×10^4 cells/well. A variety of concentrations of the test compounds (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.625 μM/L) dissolved by distilled 10% DMSO was added to each well. Incubation for 24h at 37℃ under 5% CO_2, 2.5mg/mL of MTT solution was added to each well. Further the plate was incubated for 4h. Then, the medium was removed and the resulting formazan crystals were dissolved with 100 μL DMSO. After shaking 10 min, the optical density was measured at 570 nm using a microtiter ELISA reader. The selected compounds were used as positive control, whereas untreated cells were used as negative controls. The IC_{50} values were defined as the concentrations inhibiting 50% of cell growth. All experiments were performed in triplicate.

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23. *Inhibition of DHFR activities in vitro:* The test of compound **17h** was measured through the ELISA assay. Solid-phase antibody was prepared by coating the microtiter plate wells with purified human dihydrofolate reductase (DHFR) antibody. A variety of concentrations of the test compound (0, 0.1, 0.3, 1, 3, 10 μmol/L) was combined with DHFR. Incubation for 1.5 h at 37℃, HRP labeled was added to become antibody-antigen-enzyme-antibody complex. After washing completely, TMB substrate was added, TMB substrate was becoming blue color at HRP enzyme-catalyzed. Reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450nm. The concentration of DHFR activity was calculated by a standard curve.

Synthesis and evaluation of the antibacterial activities of aryl substituted dihydrotriazine derivatives

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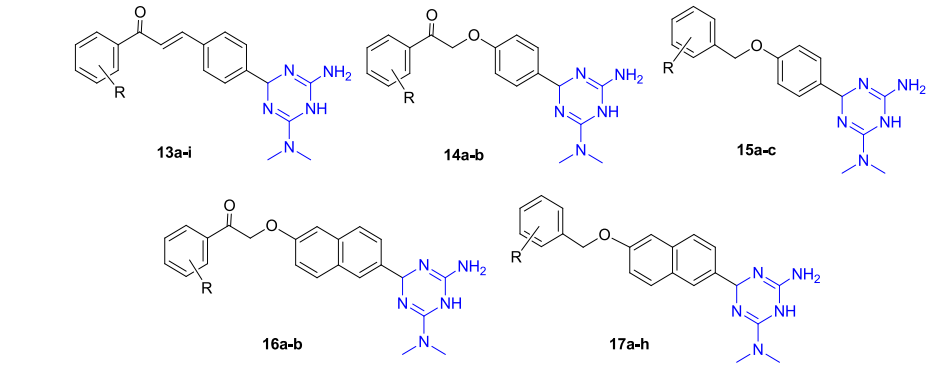
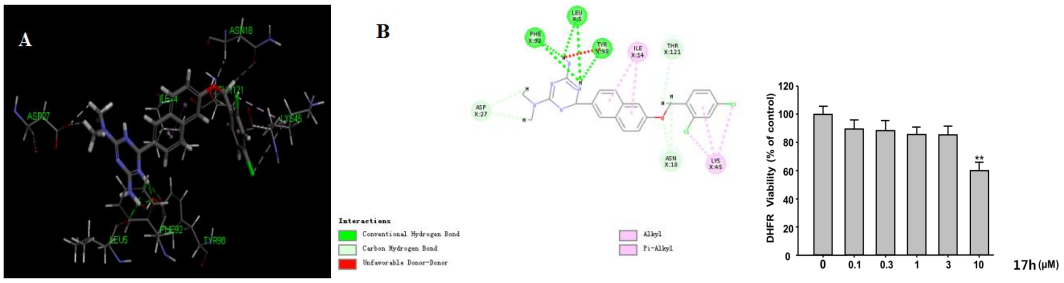


17h MIC 0.5 μg/mL against *Staphylococcus aureus* 4220 and QRSA



Five novel series of dihydrotriazine derivatives were designed, synthesized, and evaluated for their antibacterial and antifungal activity.

Research Highlights

1) Five series of dihydrotriazine derivatives were designed and synthesized.

2) **17h** showed the most potent with MIC values of 0.5 μg/mL against selected strains of bacteria.

3) Cytotoxicity results indicate that these compounds are selectively toxic towards bacteria.