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ORIGINAL ARTICLE

In-vitro Antibacterial and Antifungal Screening of Newly Synthesized Trifluoromethylated N-Heterocyclic ketenimines and 1-aza butadiene Derivatives

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KEYWORDS

Antibacterial activity;
Antifungal screening;
Trifluoromethylated ketenimines;
Trifluoromethylated 1-aza butadienes;
Disk Diffusion Agar test;
Minimum inhibitory

ABSTRACT: In our previous work, some trifluoromethylated ketenimines and 1-aza butadienes derivatives from N-H heterocyclic compounds were synthesized, and their characteristics were confirmed by their spectroscopic data as introduced. In this research, the antifungal and antibacterial effects of newly synthesized compounds were evaluated. The antibacterial activity of compounds was studied against *Staphylococcus aureus ATCC25923*, *Escherichia coli ATCC25922*, and *Pseudomonas aeruginosa ATCC9027*. Furthermore, their antifungal activity was monitored against *Candida albicans* ATCC10231. Disk Diffusion Agar test and minimum inhibitory concentration (MIC) methods were used as per the National Committee for Clinical Laboratory Standards (NCCLS) recommendations. The results showed that the ketenimines derivatives from 2-pyridinone and 4- quinazolinone have considerable antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* standard strains. Trifluoromethylated ketenimines and 1-aza butadiene derivatives have no antibacterial effect on P. aeruginosa strain. Moreover, the trimethylated ketenimines and 1-aza butadienes derivated from 4-quinazolinone showed acceptable antifungal activity against *Candida albicans*.

INTRODUCTION

Cross-infections are one of the most important medical problems in developed and developing countries [1]. The tools to determine the bacteria sensitivity to antibiotics are the disc diffusion method and minimum inhibitory concentration (MIC), commonly used in laboratories which have higher accuracy and better cost effect rather than the antibiotic release method.

Since some of these bacteria are non-fastidious, they can be easily left in the surrounding environment and transmitted to susceptible patients. The high resistance of these bacteria to antimicrobial agents including antibiotics, complicates the treatment of their infections and put them into a major medical issue.

It seems that bacteria survivability in the environment and transferability via different means play a major role in the

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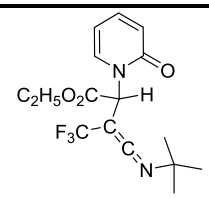
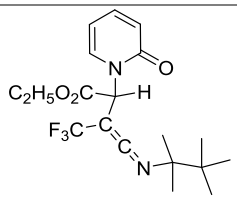
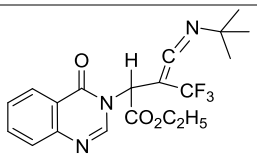
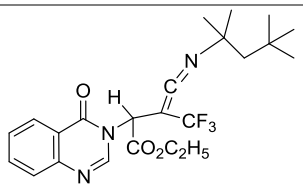
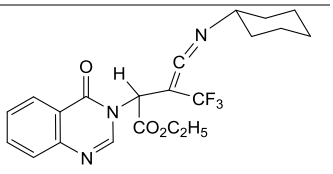
prevalence of these infections. Many studies were conducted to investigate the issue via the phenotypic and genotypic methods. In the course of treatment, it is crucial to determine antibiotic resistance before treatment and avoid usage of unnecessary antibiotics to prevent the emergence of resistant strains [2-6].

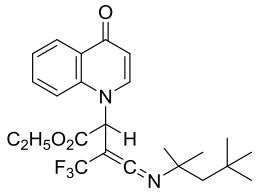
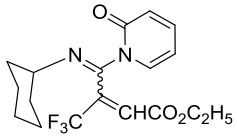
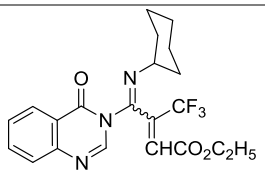
Synthetic antibiotics, these days, are widely used to cope with antibiotic resistance. The quinazolinone and pyridinone derivatives are among a large and important family of products with broad biological activities. In general, they showed useful therapeutic and pharmacological properties such as anti-inflammatory, anticonvulsant, antifungal, anti-HIV, antihypertensive, antitumor, analgesic, and antimalarial activity [7-17].

On the other hand, organofluorine compounds with triflurormethyl (CF3) group have remarkable applications in the agrochemicals and pharmaceuticals due to their functionality, and numerous properties. The trifluoromethyl functionality is often used in medicinal chemistry to impart substantial changes in physiochemical, pharmacological properties and they showed various biological activities [18-21].

Recently, we have introduced a synthesis method for trifluoromethylated ketenimines and 1-aza butadienes from various NH-heterocyclic compounds which their structures were confirmed by instrumental analysis and spectroscopic technics. [22]. In this work, the effects of derivatives (**1-8**) were examined on gram-positive and gram-negative bacteria and fungi. The names and the structures of experimental compounds are given in Table 1.

Table 1. The names and the structures of compounds **1-8**

Entry	Name	Structure
1	Ethyl 2-((tert-butylimino)methylene)-4,4,4-trifluoro-3-(2-oxopyridin-1(2H)-yl)butanoate (**1**)	
2	Ethyl 4,4,4-trifluoro-3-(2-oxopyridin-1(2H)-yl)-2-(((2,3,3-trimethylbutan-2-yl)imino)methylene)butanoate (**2**)	
3	Ethyl 2-((tert-butylimino)methylene)-4,4,4-trifluoro-3-(4-oxoquinazolin-3(4H)-yl)butanoate (**3**)	
4	Ethyl 4,4,4-trifluoro-3-(4-oxoquinazolin-3(4H)-yl)-2-(((2,4,4-trimethylpentan-2-yl)imino)methylene)butanoate (**4**)	
5	ethyl 4-(cyclohexylimino)-2-(4-oxoquinazolin-3(4H)-yl)-3-(trifluoromethyl)but-3-enoate (**5**)	

6	Ethyl 4,4,4-trifluoro-3-(4-oxoquinolin-1(4H)-yl)-2-(((2,4,4-trimethylpentan-2-yl)imino)methylene)butanoate (**6**)	
7	Ethyl 3-((cyclohexylimino)(2-oxopyridin-1(2H)-yl)methyl)-4,4,4-trifluorobut-2-enoate (**7**)	
8	Ethyl 3-((cyclohexylimino)(4-oxoquinazolin-3(4H)-yl)methyl)-4,4,4-trifluorobut-2-enoate (**8**)	

MATERIALS AND METHODS

Standard strains, *Staphylococcus aureus ATCC25923*, *Escherichia coli ATCC25922*, *Pseudomonas aeruginosa ATCC9027* and *Candida albicans ATCC10231* were provided from Sarv Saadat Laboratory, Saadat Abad, Tehran, Iran, and used as test microorganisms in this study.

Determination of Minimum Inhibitory Concentration (MIC) by microdilution method

The basis of this method is the same as the Macro-dilution method, with the difference that instead of a test tube, the microtiter plates (96-well) were piled up with a well. According to standard protocols, the minimum inhibitory concentration (MIC) was assessed for the test solutions by the broth microdilution method. The assay details such as type of culture media and incubation time and temperature for Candida albicans were different from bacterial strains [23-27].

In brief, 100µL of prepared RPMI 1640 (Gibco) for *Candida albicans* and Muller-Hinton Broth (Merck 1.05437. 0500) for the Bacteria were dispensing to a microtiter plates (96-well) containing 10µL of 6.25, 12.5, 25, 50, and 100µg/mL of newly synthesized fluorine compounds solution (**1-8**) were transferred to each well and afterward, 20 µl of bacterial and fungal inocula (1.5×10^8 cfu/mL) was added to each well except for Media control. The experiments were done in triplicate to prevent any possible bias (the final concentration of microorganisms per well was McFarland standard 5×10^5 cfu/mL). Each well was equipped with Shaker's Reader Plate and mixed for 2minutes. Then, the light absorption was read by using a plate reader at 630 nm. Plates were incubated at 35°C for 24 hours for bacteria and at 30°C for 48 hours for *Candida albicans*, after completing incubation, the cloudiness or lack of opacity in the wells was readily observed and absorbed by a plate reader at the above-mentioned wavelength. For positive control, Amphotericin 5 mg/mL, Gentamycin 40ppm, and Cephalexin 500ppm were used for Fungi, Gram negative bacteria, and Gram-positive bacteria, respectively, and sterile normal saline as negative control [23-27].

Agar Well diffusion method

Agar Well diffusion method is mostly like the standard Kirby–Bauer method (disk diffusion) and used to measure the inhibition zone and the anti-microbial property of synthesized fluorine compounds (**1-8**). All compounds were dissolved in 10% dimethyl sulfoxide (DMSO). For this purpose, a 0.5 Mac-Farland opacity suspension in sterile normal saline were prepared from an overnight culture of test microorganisms, then by sterile cotton swabs from the

suspensions, uniformly inoculated on the surface of Muller-Hinton agar mediums and on the surface of SDA for *Candida albicans*. Then, in each plate, 5 wells by sterile pipet were created. Wells were filled by the dilutions of 100 µg/mL of newly synthesized fluorine compounds solution (**1-8**). The experiments were done in triplicate. All plates then were incubated at 37°C for 24 hours. The antimicrobial activity was evaluated by measuring the zone of growth inhibition of bacteria and fungi and 10% DMSO as a negative control [23-27]. Moreover, Antibiotic disks (Positive controls) were used according to CLSI 2016 guidelines. We used appropriate antibiotic discs as Positive controls for each microorganism to compare, for fungi Amphotericin disc and for bacteria based on CLSI 2016. These antibiotics were listed in Table 2.

Table 2. Antibiotics used for each bacterium according to CLSI 2016 instructions

Bacteria	Antibiotics
E.coli	Amikacine (30µg), Nalidixic acid (30µg), Ciprofloxacin (5µg), Gentamycin **(**10µg**)**
S.aureus	Penicilin (10 units), Oxacilin (30µg), Gentamycin (10µg), Tetracycline (300µg), Nitrofurantoin (300µg)
P.aeruginosa	Amikacine (30µg), Imipenem (10µg), Meropenem (10µg), Tobramycin (10µg)

RESULTS AND DISCUSSION

Minimum inhibitory concentration (**MIC**) of compounds (**1-8**) were determined against gram-positive and gram-negative bacteria including *Staphylococcus aureus ATCC25923*, *Escherichia coli ATCC25922, Pseudomonas aeruginosa ATCC9027,* and *Candida albicans ATCC10231.* Tables 3 contain the test results of the MIC.

Table 3. Minimum inhibitory concentration (**MIC**) of selected compounds

Strains	MIC of compounds **(1-8)**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	Concentration µg/ml							
E.coli	6.25	>100	6.25	25	>100	>100	12.5	100
S.aureus	6.25	6.25	6.25	50	6.25	100	12.5	100
P.aeruginosa	>100	>100	>100	>100	>100	100	>100	>100
C. albicans	50	12.5	6.25	100	25	>100	25	100

The minimum inhibitory concentration of Gentamycin for *E.coli* and *P.aeruginosa* was 5.0µg/ml, and the Minimum inhibitory concentration of Cephalexin for *S.aureus* was 6.25 µg/ml. Minimum inhibitory concentration of Amphotericin for *C. albicans* was 6.25µg/ml.

Antibacterial and antifungal activities of compounds (**1-8**) were tested by measuring the zone inhibition by disc diffusion method, and several antibiotics, according to Table 4 were tested as a control.

The significant zone inhibition diameter was obtained in the disc diffusion method. In the case of 50 µg/ml and 100 µg/mL solution of compound 7, the zone inhibition diameter in E.coli was 17.3 mm and 19.6 mm, compared with Gentamycin (10µg). According to CLSI 2016, compound **7** has a better zone inhibition diameter rather than Rifampin (5 µg), Polymyxin B (300 unit), colistin (10 µg), Ampicillin (10 µg), Cephalothin (30 µg), and Trospectomycin (30 µg) antibiotics. Therefore, compound **7** can replace the number of antibiotics use in *E.coli* infections after exerting side effects.

The solution of compounds **1**, **2**, **3**, **5**, and **7**, (100 µg/ml each) showed 20.3, 34.3, 33.3, 26.3, and 23.3 mm zone inhibition diameter in *S.aureus* respectively that can compare with Penicillin (10 units), Oxacillin (30µg), Gentamycin (10µg), Tetracycline (300µg), and Nitrofurantoin (300µg). Therefore, according to CLSI 2016, these compounds have a better zone inhibition diameter than Linezolid (30 µg), Methicillin (5 µg), oxacillin (1 µg), Norfloxacin (10 µg), and the other antibiotic which is listed in CLSI 2016. Therefore, these compounds have the

potential to be considered as a replacement for the number of antibiotics used in *S.aureus* infections after exerting side effects.

The result did not show any significant zone inhibition diameter in *Pseudomonas aeruginosa*, although the impressive zone inhibition diameter from the solution of compounds **2**, **3**, and **5** were obtained in *C. albicans*. The highest zone inhibition diameter was measured for compound **3** (100µg/mL) with 30mm. The inhibition zone of the Amphotericin disc as a positive control was 17mm. According to the results obtained by both methods, nearly all selected compounds showed significant activity against gram-positive bacteria *Staphylococcus aureus*, and some of them showed activity against Gram-negative bacteria *Escherichia coli*. None of them displayed any activity against *Pseudomonas aeruginosa*. The effect of synthesized compounds on the gram-positive bacteria *Staphylococcus aureus* is higher than *E.coli*, since the wall structures of gram-negative bacteria contain purine pores, and these purines are non-specific hydrophilic pores; therefore, they allow to pass the low-molecular hydrophilic compounds up to 10 KD [23].

1-Azabutadiene **7** exhibited the highest activity at MIC = 12.5 µg/ml against *S.aureus* and *E.coli*. Other ketenimines derivatives **1, 2, 3, 5** showed the highest activity against *S.aureus* at the same level of MIC (6.25 µg/ml). Furthermore, compounds **1** and **3** exhibited the highest activity against *E.coli* at MIC = 6.25µg/ml, whereas compounds **6, 8** showed no activity against *S.aureus, E.coli,* and *P.aeruginosa*. Consequently, the antifungal activities of compounds (**1-8**) were investigated against *Candida albicans.* Nearly all compounds except **4**, **6**, **8** presented reasonable activity against antifungal, and compound **3** had the highest activity with MIC= 6.25 µg/ml. The antimicrobial assay has revealed changes in these heterocyclic nuclei have a considerable effect on anti-bacterial and anti-fungal properties. The effect of synthesized compounds on *Staphylococcus aureus* is higher than *E.coli* since the rate of growth and proliferation of this bacterium is higher and fast-growing bacteria are more susceptible to antimicrobial agents. Prokaryotic bacterial cells are also more susceptible to antimicrobial agents due to their lower reproductive rate and growth cycle of eukaryotic cells than prokaryotic cells [28].

Table 4. Inhibition zones mean (mm) of the antibacterial and antifungal activity by Agar well diffusion assay

Compound	Concentration (µg/ml)	Inhibition zones(mm)			
		E.coli	***S.aureus***	***P.aeruginosa***	***C. albicans***
1	6.25	11.6	11.6	-	10.3
	12.50	12.6	13.3	-	12.6
	25.00	14.3	15.6	-	14.3
	50.00	15.6	19.0	-	16.6
	100.00	16.6	23.3	-	18.0
2	6.25	-	12.6	-	11.6
	12.50	-	14.6	-	13.3
	25.00	-	16.0	-	16.6
	50.00	-	18.6	-	20.0
	100.00	-	33.3	-	25.0
3	6.25	11.6	24.0	-	18.3
	12.50	12.6	24.6	-	20.3
	25.00	14.3	25.3	-	23.3
	50.00	15.6	27.3	-	25.6
	100.00	16.6	34.3	-	30.0
4	6.25	-	-	-	-
	12.50	-	-	-	-
	25.00	-	-	-	-
	50.00	10.6	9.0	-	-
	100.00	13.3	12.6	-	10.0

5	6.25	-	15.3	-	11.3
	12.50	-	19.6	-	13.6
	25.00	-	22.3	-	16.3
	50.00	-	24.6	-	18.3
	100.00	-	26.3	-	20.0
6	6.25	-	-	-	-
	12.50	-	-	-	-
	25.00	-	-	-	-
	50.00	-	-	-	-
	100.00	-	10.3	-	10.0
7	6.25	-	-	-	-
	12.50	13.3	15.3	-	-
	25.00	15.3	16.6	-	-
	50.00	17.3	18.6	-	11.3
	100.00	19.6	20.3	-	13.0
8	6.25	-	-	-	-
	12.50	-	-	-	-
	25.00	-	-	-	-
	50.00	-	-	-	-
	100.00	9.0	10.3	-	9.0

CONCLUSIONS

In summary, antibacterial and antifungal activities of some synthesized trifluoro methylated ketenimines (**1-6**) and 1-azabutadienes (**7, 8**) were evaluated by measuring the zone inhibition diameter by the disc diffusion method and minimum inhibitory concentration (MIC). Most of these new fluorinated compounds have antibacterial and anti-fungal properties so that they can be used after further testing and determination of toxicity and sensitivity tests in the synthesis of a new generation of synthetic antibiotics. Additional work for evaluation of the antimalarial and antihypertensive effects and, investigation of cytotoxicity of our products is in progress. Also, Future work may also include using aryl isocyanides and different pharmacophoric heterocyclic precursors.

Conflict of interests

The author declares that there is no conflict of interest.

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