

Antimicrobial and Anti-pathogenic Activity of New Naphtho [1,2,4] Triazol-Thiadiazin Derivatives

Maryam Kouhkan^{1*}, Miri Mahmoody², Jabbar Khalafy³, Ali Souldozi², Mahsa Mohammadlou³, Nazila Khorram Maslak³

¹Young Researchers and Elite Club, Urmia Branch, Islamic Azad university, Urmia, Iran

²Faculty of Science, Urmia Branch, Islamic Azad University, Urmia, Iran

³Department of Chemistry, Faculty of Science, Urmia University, Urmia 57154, Iran

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*Corresponding author:

Maryam Kouhkan,

Email: kouhkanmaryam@gmail.com

Abstract

Background: Antimicrobials are one of the extremely important categories of drugs for the treatment, control, and prevention of microbial diseases, but the development of drug resistance against clinically used antibacterial agents has increased the demand for the design and synthesis of new drugs. We have previously synthesized new series of 10-substituted-5H-naphtho[1,2-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazin derivatives (4a-4f). In this study, we evaluated the antimicrobial activity of these derivatives against some pathogenic microorganisms.

Methods: The reaction of 2-bromo-1,4-naphthoquinone with 4-amino-5-aryl-4H-1,2,4-triazole-3-thiols in ethanol at 50 °C gave the corresponding 2-[(4-amino-5-aryl-4H-1,2,4-triazole-3-yl)thio]naphthalene-1,4-diones. Moreover, their treatment with EtOH/HCl under reflux conditions produced 10-substituted-5H-naphtho[1,2-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-5-ones through intramolecular cyclization. The well agar diffusion and agar dilution methods were used during the preliminary evaluation of antimicrobial activity for the determination of inhibition zone (IZ) and minimum inhibitory concentration (MIC).

Results: Seven tetracyclic heterocyclic ring systems were produced under reflux conditions. The structures of all the products were identified by their FT-IR, ¹H, and ¹³C NMR spectral data and by elemental analysis. The results revealed that the antibacterial activity of compounds 4a, 4b, 4c, and 4d are higher than that of the others, and compounds 4d, 4a, 4e, and 4f exerted the greatest effect on fungal samples.

Conclusion: All synthesized compounds exhibited promising antibacterial and antifungal activity. In this study, compounds 4a-4g exhibited highly potent antimicrobial activity and acceptable selectivity index against Staphylococcal and Candida infections.

Keywords: Antibacterial activity, Antifungal activity, Antifungal, Triazol, Thiadiazine



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Introduction

Resistance is the ability of a bacteria against the inhibitory effect of antibacterial agents on the proliferation and growth of bacteria. Through the indiscriminate use of antibiotics, resistant microorganisms have emerged during recent decades and caused numerous problems in the treatment of infections induced by these microorganisms. Therefore, an attempt at producing drugs to deal with these outstanding threats to global health is an essential need. To this end, one strategy is employing a combination of different active parts in one molecule. In this strategy, each drug moiety is designed to bind independently to different biological targets and accumulate at both target sites synchronously (1-3). It was found that 1, 2,

4-triazole and naphthalene derivatives exhibit a wide variety of pharmacological and biological activities such as antibacterial (4,5), antimycobacterial (6,7), anti-inflammatory (8), antifungal (9,10), antineoplastics (10), anticancer (11), and antiviral (12) activities. Thiadiazines are also efficient antibacterial and antifungal compounds (12), and their use in the treatment of the bacteria *Helicobacter pylori* and their role as reverse transcriptase inhibitors of the human immunodeficiency virus have been reported (13). On the other hand, the studies conducted on a large number of p-naphthol derivatives for antibacterial and antifungal activities indicated that these compounds exhibit preferential activity against gram-positive bacteria and human pathogenic fungi. In fact, in



the present study, the synthesis of three-ring heterocycles has been designed in such a way that all three heterocycles have antimicrobial power and can exert their synergistic effect by being present in one molecule at the same time. As mentioned in the previous study, 10-substituted-5H-naphtho[1,2-e][1,2,4] triazolo[3,4-b][1,3,4] thiadiazin-5-ones derivatives were synthesized in the presence of different substitutions at the 5-triazole position as follows:

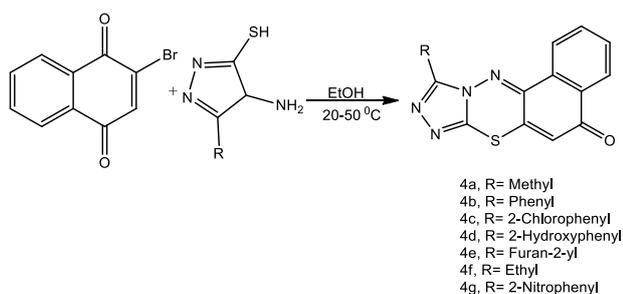
4a: 10-methyl -5H-naphtho[1,2-e] [1,2,4] triazolo [3,4-b][1,3,4] thiadiazin-5-ones **4b:** 10-phenyl -5H-naphtho [1,2-e] [1,2,4] triazolo[3,4-b] [1,3,4] thiadiazin-5-ones **4c:** 10-chlorophenyl -5H-naphtho[1,2-e] [1,2,4] triazolo [3,4-b][1,3,4] thiadiazin-5-ones **4d:** 10-hydroxyphenyl -5H-naphtho[1,2-e] [1,2,4] triazolo[3,4-b] [1,3,4] thiadiazin-5-ones **4e:** 10-furan-2-yl -5H-naphtho [1,2-e] [1,2,4] triazolo [3,4-b][1,3,4] thiadiazin-5-ones **4f:** 10-ethyl -5H-naphtho[1,2-e] [1,2,4] triazolo[3,4-b] [1,3,4] thiadiazin-5-ones **4g:**10-nitrophenyl -5H-naphtho [1,2-e][1,2,4] triazolo[3,4-b] [1,3,4] thiadiazin-5-ones.

Since the synthesis and evaluation of antimicrobial activity is an important part of our research program (14-16), in this study, we aimed to evaluate the antibacterial and antifungal activity of these derivatives against some microorganisms that cause the largest number of infections and, in some cases, have exhibited resistance to currently available antibiotics such as Methicillin-resistant *Staphylococcus*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, *Candida tropicalis*, *Candida krusei*, and *Candida glabrata*.

Materials and Methods

Preparation of Naphtho [1,2,4]triazol-Thiadiazin

General procedure for the synthesis of 10-substituted-5H-naphtho[1,2-e][1,2,4] triazolo[3,4-b][1,3,4] thiadiazin-5-ones (4a-4g) was as follows: Concentrated HCl (4-5 drops) was added to the solution of 2-[(4-amino-5-aryl (alkyl)-4H-1,2,4-triazol-3-yl)thio]naphthalene-1,4-dione (1 mmol) in EtOH (20 mL). Then, the mixture was refluxed for 4 hours and left to cool at room temperature, and the resulting precipitate was filtered and washed with cold EtOH and dried to give products 4a-4g as orange needles in 56-82% yields, as depicted in Scheme 1 (14).



Scheme 1. Synthesis of 10-Substituted-5H-Naphtho[1,2-e][1,2,4]Triazolo [3,4-b][1,3,4]Thiadiazin-5-Ones (4a-4g)

Antibacterial Screening

The antibacterial activity of synthesized compounds was tested against gram-positive and gram-negative bacteria, including *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, and Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 700698. These chosen strains were proceeded to screen the antibacterial activity because they are commonly isolated pathogens from hospitalized patients with intestinal ailments and blood and skin infections (17). All microorganisms were obtained from the Urmia University of Medical Sciences. The standardization of each bacterial inoculum was done following the Clinical and Laboratory Standards Institute (CLSI). Briefly, Mueller-Hinton agar (MHA) medium was used to prepare MHA agar plates which were inoculated with the bacterial suspension (10^6 CFU/mL) uniformly onto the surface of agar plates, and wells of uniform diameter were punched on the solidifying agar media by the sterile borer. Test compounds were then dissolved in 99.5% dimethyl sulfoxide (DMSO) to obtain 1 mg/mL stock solutions. Afterward, 100 μ L of each of these solutions were dispensed into the labeled wells (18-20). Cefprozime and ciprofloxacin (Sigma, Aldrich, US) were used with the same method as standard drugs, and DMSO was used as solvent control. Next, Petri plates were transferred to the incubator for 18 hours at 35°C, and the final results were evaluated by measuring the average diameter of the inhibition zone around each well. Experiments were performed in triplicate.

Agar Dilution Method

The agar dilution method was performed according to the procedures described by the CLSI (21). Stock solutions of antimicrobial compounds were prepared in DMSO, added to molten MHA (Becton Dickinson, Franklin Lakes, MD, USA) at 50 °C, and then poured into Petri plates to provide two-fold dilutions ranging from 7.8 to 1000 μ g /mL. Bacterial inoculums were prepared from a 24-hour culture on MHA. Bacterial suspensions were applied to agar plates using a multipoint inoculator to give a final concentration of 1.5×10^5 CFU per spot (22). The agar plates were then incubated at 35 °C for 18 hours and were evaluated visually. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial agent observed in complete inhibition of visible growth or the growth of less than five colonies per spot (23).

Antifungal Activity

Agar Dilution Assay

In vitro antifungal activity of 10-substituted-5H-naphtho[1,2-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-5-ones was evaluated against standardized clinically important fungi, including *C. tropicalis* (PTCC 5028), *C. albicans* (ATCC 10239), *C. glabrata* (IFRC 1627), and *C. krusei* (ATCC 6258). These yeasts were chosen because they are major human fungal pathogens that cause mucosal deep tissue infection, they may cause systemic

infection in immune-compromised situations due to their great adaptability to different host niches (24). All fungi were obtained from the Shiraz University of Medical Sciences. The antifungal activity of triazole derivatives was determined using the agar-well diffusion method (25). Then, 100 μL of each fungal suspension with a cell density of 1.5×10^8 CFU/mL was applied on the surface of the pre-sterilized and autoclaved Sabouraud Dextrose Agar (SDA) Petri dishes and spread using a shaped glass spreader. Wells with 6 mm of diameter were made at the center of each of the SDA Petri plates with the help of a sterilized cork borer. Next, the wells were filled with triazole derivatives as prepared above with three replications for each treatment, and the zone of inhibition was calculated based on the mean values. Fluconazole (1 mg/mL) and DMSO were used as positive and negative controls, respectively. All Petri dishes, including triazoles and controls, were allowed to be diffused and incubated at 35 °C for 48 hours. The antifungal activity of derivatives was determined after incubation.

In the next step, the MICs were determined by the Agar dilution method according to the procedures described by the CLSI (26). Two-fold serial dilutions of the compounds and reference drug (fluconazole) were prepared in SDA to obtain the required concentrations of 25-1000 $\mu\text{g/mL}$. The fungal inoculates were adjusted to 0.5 McFarland standard (1.5×10^8 CFU/mL) (27), and the suspensions were then diluted to give 10^6 CFU/mL per spot. Petri dishes were spot inoculated with 10 μL of each prepared fungal suspension (10^6 CFU/spot) and incubated at 25 °C for 48 hours. At the end of the incubation period, MIC was determined, which is the lowest concentration of the test compound that resulted in no visible growth on the plate. To get the minimum fungicidal concentration (MFC), a loopful was taken from the MIC tubes and streaked on SDA plates. The growth was observed after incubation at 37 °C for 48 hours, and the lowest concentration was recorded as MFC which exhibited no growth (28).

Statistical Analysis

Statistical analysis was run using Microsoft Excel 2010 based on three independent experiments, and the results were expressed as mean.

Results

Antibacterial Screening

All newly synthesized compounds (4a-4g) were evaluated for their in vitro antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus*, *S. pyogenes*, and MRSA using the agar well diffusion and dilution methods. Ceftizoxime and ciprofloxacin were used as reference standards. The antibacterial results of novel series of 10-substituted-5H-naphtho[1,2e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-5-ones are summarized in Table 1 and Figure 1.

All tested compounds (4a-4g) inhibited the growth of *S. aureus* and MRSA, but compounds 4a, 4b, 4c, and 4d were more active than the others. The growth of *E. coli* and *P.*

Table 1. Minimum Inhibitory Concentrations ($\mu\text{g/mL}$) and Inhibition Zones (1 mg/mL) of Synthesized Compounds against Tested Bacteria

Compounds	MIC(IZ)			
	<i>Staphylococcus aureus</i>	Methicillin-resistant <i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
4a	7.8 (25)	15.6 (17)	250 (10)	500 (NA)
4b	7.8 (25)	6.6 (17)	250 (10)	500 (NA)
4c	7.8 (25)	15.6 (17)	250 (10)	500 (NA)
4d	7.8 (25)	15.6 (17)	250 (10)	500 (NA)
4e	250 (15)	250 (15)	250 (10)	500 (NA)
4f	250 (25)	250 (10)	250 (10)	500 (NA)
4g	125 (26)	500 (11)	250 (10)	500 (NA)
Ceftizoxime	125 (25)	125 (11)	100 (16)	(14) 500
Ciprofloxacin	0.07 (40)	0.07 (40)	(45) 15	1.5 (42)

Note. MIC: Minimum inhibitory concentration; IZ: Inhibition zone; NA: No activity.

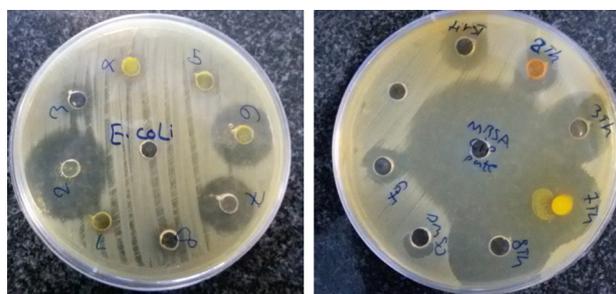


Figure 1. Inhibition Zone of Compounds against *Escherichia coli* and *Methicillin-resistant Staphylococcus aureus* in 1mg/mL Concentration

aeruginosa was also inhibited by the compounds at the range of 250 $\mu\text{g/mL}$ concentration. Furthermore, some of the compounds showed the same or higher antibacterial activity with ceftizoxime; however, they exhibited poor activity compared to ciprofloxacin. Additionally, the MBC of compounds was two or three-fold higher than that of the corresponding MIC results.

Antifungal Screening

The antifungal potential of tested compounds was evaluated according to their zone of inhibition and minimum inhibition concentration against tested fungal pathogens. The results were compared with the activity of fluconazole and are summarized in Table 2 and Figure 2.

The results revealed that all the synthesized compounds have potent antifungal activity against tested pathogenic fungi. Compound 4d showed maximum inhibition zone in *Candida albicans*, *Candida tropicalis*, *Candida krusei*, and *Candida glabrata* with a diameter of 25 ± 0.64 mm. Moreover, MIC was recorded as the lowest concentration of a compound that inhibits the growth of the tested microorganisms. Compared with the MIC values with the standard fluconazole (MIC=1000 $\mu\text{g/mL}$), some compounds exhibited the highest potential in vitro antifungal activity against all evaluated fungi, especially compounds 4d (MIC=50 to 100 $\mu\text{g/mL}$) and 4a, 4e, 4f

Table 2. Minimum Inhibitory Concentrations ($\mu\text{g}/\text{mL}$) and Inhibition Zones (1 mg/mL) of Synthesized Compounds against Tested Fungi

Compounds	MIC (IZ)			
	<i>Candida tropicalis</i>	<i>Candida albicans</i>	<i>Candida krusei</i>	<i>Candida glabrata</i>
4a	400 (13)	200 (16)	200 (16)	200 (12)
4b	400 (13)	800 (16)	400 (13)	400 (12)
4c	800 (17)	400 (25)	400 (13)	400 (15)
4d	100 (25)	100 (25)	50 (25)	100 (17)
4e	400 (15)	200 (25)	200 (16)	200 (16)
4f	400 (25)	400 (18)	200 (16)	200 (18)
4g	400 (15)	800 (25)	200 (16)	800 (12)
Fluconazole	200 (15)	100 (17)	200 (16)	200 (14)

Note. MIC: Minimum inhibitory concentration; IZ: Inhibition zone.

(MIC=200 to 400 $\mu\text{g}/\text{mL}$). Additionally, compounds 4b and 4c (MIC=400 to 800 $\mu\text{g}/\text{mL}$) demonstrated acceptable antifungal activity, and the MFC of compounds was the same or two-fold higher than that of the corresponding MIC results.

Discussion

All synthesized polycyclic heterocycles were tested for their antimicrobial activities, and MICs were recorded as a minimum concentration of compounds that inhibit the growth of tested microorganisms. The obtained results revealed that our new tetracyclic heterocyclic ring system (4a-4g) has good antimicrobial activity, but the highest inhibitory activity was observed against Gram-positive bacteria which can be explained by the presence of lipophilic moieties in triazolo scaffold. The widest spectrum of antibacterial activity was exerted by 4a, 4b, 4c, and 4d, suggesting that the phenyl ring, which contains an electron-withdrawing or electron-releasing substituent at the C-2 position of the [1,2,4] triazole ring exerts a good antibacterial effect against *S. aureus* and *MRSA*. These observations are similar to the results reported by Kumar et al (29). Interestingly, the most potent antibacterial compound in the present study belongs to the C-2 Hydroxyphenyl compound. As evidenced by inhibition zone sizes, the antibacterial activity of compounds against Gram-positive was somewhat stronger than that of *ceftizoxime*, while they displayed moderate antimicrobial activity against the Gram-negative bacterial strain. In addition, the antifungal screening results revealed that compounds are active against the fungal strains used in the study due to the presence of triazole thiadiazin moiety in **10** naphtha, which increased the antimicrobial activity. Moreover, compounds 4a, 4d, 4e, and 4f had better antifungal activity against tested fungal strains which is comparable to fluconazole, but significant activity was observed for compound 4d that was more potent than the control drug (fluconazole) against standard strains such as *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. glabrata*. Additionally, the structural and antimicrobial similarity between our synthesized compounds and *Fluconazole*

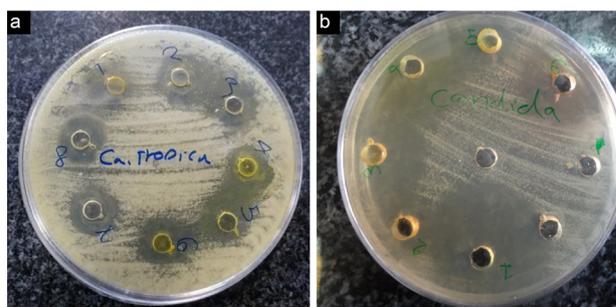


Figure 2. Inhibition Zone of Compounds against *Candida tropicalis* (a) and *Candida albicans* (b) in 1 mg/mL Concentration

suggested that the mechanism of the action of the new compounds may be similar to that of fluconazole. Furthermore, this compound may be a good alternative for treating and controlling infections caused by *MRSA*, which is an extremely significant public health concern. In case further studies establish the safety and efficiency of these compounds, they may be represented as an excellent prototype with anti-*MRSA* activity in the development of new drugs for medical use.

In sum, some novel 10-substituted-5Hnaphtho[1,2-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-5-ones (4a-4g) were synthesized, and their antimicrobial activities were investigated. The majority of the compounds were found to possess interesting antibacterial and antifungal activities against the tested microbial strains when compared to the standard drugs (i.e., ceftizoxime and fluconazole). The antimicrobial activities of the compounds were improved by structural modifications in the naphtho thiadiazin group attached to the triazole ring. Thus, these active compounds could be extremely good candidates for further antimicrobial research. Our previous study synthesized 3-substituted 5H [1,2,4]triazolo[3',4':2,3][1,3,4]thiadiazino[5,6-b]quinoxalines and investigated their antimicrobial activities (30). This molecule bears the greatest structural resemblance to the molecule studied in the present work, and the basic difference between these two structures is a substitution. In the previous study, the [1,3,4]thiadiazin ring was connected to the two rings of quinoxaline and [1,2,4]triazole, while in this study, [1,3,4]thiadiazin ring is located between the two rings of naphthol and [1,2,4]triazole. Comparing the antimicrobial power of the previous study and the current study yields very interesting results. By comparing these two studies, it can be found that the presence of naphthol substitution near the thiadiazole ring significantly increases the antimicrobial power of this molecule. More interestingly, by attaching different substitutions to the 2-triazole position, we can still notice high antimicrobial power, especially against gram-positive bacteria and fungi. Therefore, it can be concluded that in this molecule and generally similar molecules with this structure, the presence of the naphthol moiety adjacent to the thiadiazole ring significantly increases the antimicrobial power and exerts a synergistic effect. The result obtained in this research can play a key role in the synthesis of

tricyclic, [1,2,4] triazole-[1,3,4] thiadiazin derivatives with high antimicrobial activity and double synergistic effect.

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Authors' Contribution

Conceptualization: Maryam Kouhkan.

Data curation: Maryam Kouhkan, Miri Mahmoody, Jabbar Khalafy.

Formal analysis: Maryam Kouhkan, Miri Mahmoody, Jabbar Khalafy.

Funding acquisition: Maryam Kouhkan, Ali Souldozi.

Investigation: Maryam Kouhkan, Miri Mahmoody, Jabbar Khalafy.

Methodology: Maryam Kouhkan.

Project administration: Maryam Kouhkan.

Validation: Maryam Kouhkan, Miri Mahmoody, Jabbar Khalafy, Ali Souldozi, Mahsa Mohammadlou, Nazila Khorram Maslak.

Visualization: Maryam Kouhkan, Miri Mahmoody, Jabbar Khalafy.

Resources: Maryam Kouhkan, Miri Mahmoody, Jabbar Khalafy.

Supervision: Maryam Kouhkan, Miri Mahmoody, Jabbar Khalafy.

Writing—original draft: Maryam Kouhkan, Miri Mahmoody, Jabbar Khalafy, Ali Souldozi.

Writing—review & editing: Maryam Kouhkan, Miri Mahmoody, Jabbar Khalafy, Ali Souldozi Babatunde Akinde.

Competing Interests

There is no conflict of interests.

Ethical Approval

This work did not require ethical approval under the research governance guidelines operating at the time of the research.

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