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1-Phenyl ethyl substituted tetrahydro-2*H*-1,3,5-thiadiazine-2-thione derivatives as promising antimicrobial agents

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Abstract: Seven derivatives of 1-phenyl ethyl group containing 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-thiones (THTT) were prepared and examined for their antibacterial and antifungal properties by using Microplate Alamar Blue Assay (MABA) and agar tube dilution protocol respectively. *In vitro* antifungal potential was investigated against five human pathogens and compared with the standard drugs amphotericin B and miconazole. *In vitro* antibacterial activity was investigated against four pathogens and compared with the ofloxacin. All compounds exhibited very promising antifungal activities against all tested pathogens. Structure activity relationship showed the importance of the presence of 1-phenyl ethyl substituent at *N*-3 of THTT nucleus for antifungal effects. However, these compounds showed significant antibacterial activity only against *S. aureus*. The compound 6c of the series was found most active compound that displayed promising antifungal potential against all tested pathogens [Growth Inhibition (GI) = 100%], and also showed promising antibacterial potential against *S. aureus* (GI% = 83.49) which is very much closer to the standard ofloxacin (GI% = 88.05). The study may be useful in the development of improved antimicrobial agents.

Keywords: Phenyl ethyl substituted thiadiazine, anti-fungal activity, anti-bacterial activity, miconazole.

INTRODUCTION

Human pathogens are concomitant with a variety of moderate to severe infections especially the current rise of multi-drug resistance in pathogens has made an urgent and alarming call for new and effective antimicrobial agents (Dinarvand and Spain, 2021). Tetrahydro-2*H*-1,3,5-thiadiazine thione (THTT) skeleton is well known for exhibiting pharmacological properties (Arshad *et al*., 2021; Rodríguez *et al*., 2012), including anti-microbial 1 (Sağlam *et al*., 2011), antileishmaniasis 2 (Fidalgo *et al*., 2004) and antiepileptic 3 (Semreen *et al*., 2010) (fig. 1). Compounds containing THTT nucleus are already documented as prodrugs, the biologically inactive derivatives of active drug molecules, which exert their pharmacological effect through isothiocyanates after being hydrolyzed under physiological conditions (Semreen *et al*., 2010; Goksøyr, 1964).

The effects of various substituents at both nitrogen atoms of thiadiazine skeleton on biological potency are already well established (El-Shorbagi, 2000). In a recent report, 1-phenyl ethyl group at THTT nucleus was identified as an important substituent that exhibited improved antimicrobial activities (Ullah *et al*., 2021). With this guideline which owned only one example, we designed and prepared some more analogues with 1-phenyl-ethyl substituent at *N*-3 or at *N*-5 positions of THTT skeleton and studied their comparative anti-microbial effects. Herein, we report comparative anti-bacterial and anti-fungal potential of a small set of seven derivatives of tetrahydro-2*H*-1,3,5-thiadiazine-2-thiones (THTT) containing 1-phenyl ethyl group of type 8 as shown in scheme 1.

MATERIALS AND METHODS

General experimental details

NMR spectroscopy was obtained by using Bruker Avance-500 spectrometer. Chemical shifts (δ) are described in ppm (parts per million). The letter m is used to denote multiplet. Electron ionization (EI) was measured on JEOL (JMS-600H). Shimadzu (FTIR-8900) was used to obtain FTIR spectra. Silica gel plates (60 HF_{254}) were used for thin layer chromatography (TLC). All the reagents (synthetic grade) and solvents (anhydrous) were acquired commercially from Sigma Aldrich.

General procedure for the synthesis of 3,5-disubstituted tetrahydro-2H-1,3,5-thiadiazine-2-thiones (8a-g)

A mixture was prepared containing 10 mmol of alkyl-, or 1-phenyl ethyl amine (4) and 0.561 gm of potassium hydroxide (1 equiv, 10 mmol, 20%) in absolute EtOH (10 mL). 3.6 mL of Carbon disulfide (CS_2, 6 equiv, 60 mmol) was added in small portion to the mixture with continuous

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stirring. At ambient temperature (25°C) stirring kept continued for 3 hours. Dithiocarbamates (5) were produced in the mixture, after that 1.9 mL formalin solution (2.2 equiv, 22 mmol, 35%) was added gradually whereas continuous stirring kept for further 1 hour. The mixture was turned into a clear solution. This clear solution was mixed over 15 minutes to another already prepared solution of 0.75 gm amine 7 (alkyl-, or 1-phenyl ethyl amine, 10 mmol) and 20 mL of phosphate buffer (pH 7.8). Stirred the resultant mixture for 1 hour further and then ice cooled to 0 °C. The mixture was then acidified up to pH 2 (8% HCl). Stirring of 30 minutes resulted precipitation and ppt were filtered and recrystallized by ethyl alcohol. THTT (8a-g) were obtained as white solids, the purified compounds were subjected for characterization and NMR pure samples were biologically screened for their antimicrobial potential. Spectroscopic analysis of the compounds 8a-f was observed very similar to the reported data (Arshad *et al*., 2018) while new THTT derivative 8g data is included herein.

Materials for antifungal assay protocol

Five human fungal pathogens namely *Candida albicans* (ATCC 2091), *Trichphyton rubrum* (clinical isolate), *Aspergillus niger* (ATCC 32611), *Fusarium lini* (ATCC 46066), and *Microsporum canis* (ATCC 11622) were obtained from microbial bank of International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan. Miconazole was purchased from Sigma Aldrich, MO, USA and Amphotericin B from ICN Biomedicals Inc., Ohio, USA; while Sabouraud dextrose agar was purchased from Merck Specialities Private Limited, Goa, India.

Materials for Antibacterial Assay Protocol

Four bacterial pathogens; *Staphylococcus aureus* (ATCC 25923), *Shigella flexenari* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) were obtained from microbial bank of ICCBS, University of Karachi, Pakistan. Oxoid Mueller Hinton Broth was purchased from Thermo Fisher Scientific Inc., Massachusetts, USA while Ofloxacin was purchased from Bio Basic Inc., Markham, Canada.

Antifungal activity protocol

Prepared THTT derivatives 8a-g were inspected for their antifungal activity by using the agar tube dilution protocol (Hostettmann *et al*., 1991; Arfan *et al*., 2010). Initial screening was performed at 400 µg/mL concentration along with final screening at 200 µg/mL for five human pathogens namely *C. albicans T. rubrum A. niger F. lini* and *M. canis*. Results were recorded as percentage of growth inhibition (GI%). For comparison purpose amphotericin B, a standard drug as positive control was used for *A. niger* whereas the standard drug miconazole for *C. albicans*, *T. rubrum*, *F. lini and M. canis*. Sabouraud dextrose agar (SDA) with pH 5.5–5.6 was utilized as media. A piece of 4 mm diameter from a seven days old culture fungus was inoculated in tubes then incubated for a period of 7 days at 27 ± 1°C. Growth was calculated by linear growth measurement in mm and percentage of growth inhibition (GI%) was determined in relation to the negative control using medium supplemented with DMSO. Three replicate testings were performed.

Antibacterial activity protocol

All the synthesized THTT analogues (8a-g) were also investigated for their *in vitro* antibacterial potential against four pathogens; *S. flexenari*, *P. aeruginosa*, *E. coli*, and *S. aureus* by adopting Microplate Alamar Blue Assay (MABA) procedure (Pettit *et al*., 2005). All compounds were used in DMSO as concentration of 50 µg/mL. For comparability, standard drug ofloxacin was also tested in same concentration. Antibacterial potential was recorded as percentage of growth inhibition (GI%). For organism's growth, Mueller Hinton media was utilized. Inoculums were standardized at 0.5 McFarland turbidity index. Incubation of 96 well plates was done for 18 to 20 hours. Alamar Blue Dye was included in all wells and kept in a shaking incubator for about 2 to 3 hours at 80 rpm. The indicative results were recorded at 570 and 600 nm as absorbance values by the ELISA reader and the results are mean of three replicate tests.

STATISTICAL ANALYSIS

All values in tables were expressed as standard error of mean (± SEM) and three (03) replicate tests were performed. Enzyme-linked Immunosorbent Assay (ELISA) reader from Molecular Devices, CA, USA was used for measuring the absorbance at 570 and 600 nm. Data was calculated by using EZ-Fit Enzyme Kinetics by Perrella Scientific Inc. (Massachusetts, USA).

RESULTS

Seven derivatives of tetrahydro-2*H*-1,3,5-thiadiazine-2-thione (8a-f) including a new THTT derivative (8g) were prepared in excellent yields (80-86%) as shown in scheme 1 by following literature protocol (Arshad *et al*., 2018). Diversity in structures of THTTs was achieved successfully by incorporating variety of amines to decorate THTT skeleton.

The obtained derivatives were confirmed through ^{1}H NMR spectroscopy. All compounds showed the characteristic peaks in ^{1}H NMR spectrum, related to the 4-CH_2 and 6-CH_2 of thiadiazine nucleus between the ranges of δ value 4.1 to 4.4 ppm that confirmed the presence of THTT nucleus. Overall, the spectral data of all compounds were observed very similar to the reported data (Arshad *et al*., 2018).

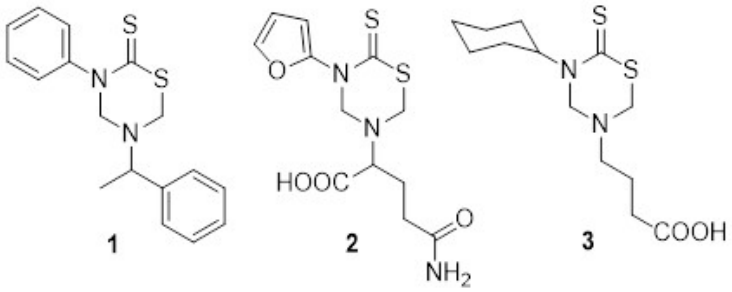
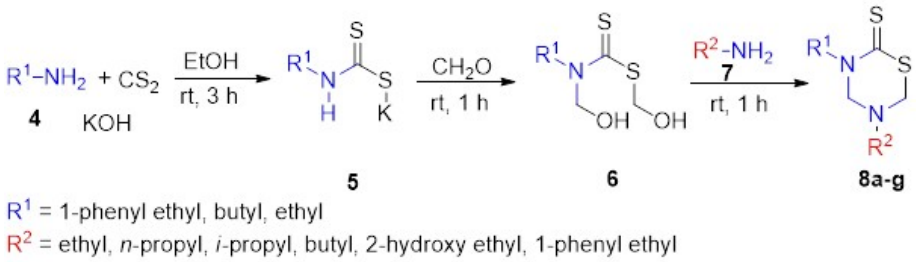


Fig. 1: Biologically active THTT molecules.



Scheme 1: Preparation of 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-2-thione (THTT) derivatives.

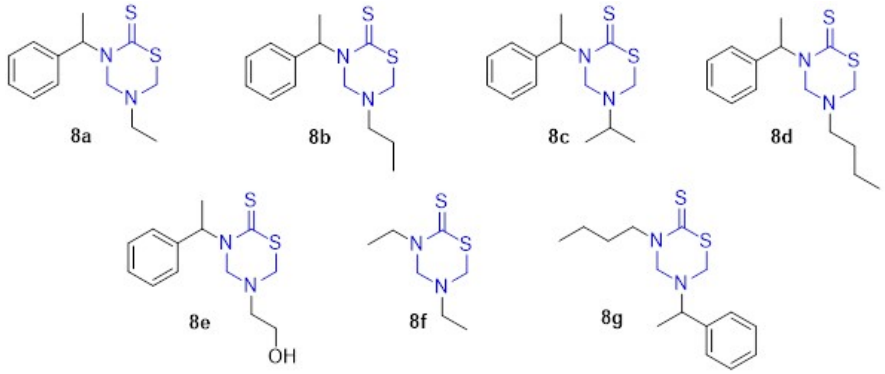


Fig. 2: Disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-2-thione (THTT) derivatives 8a-g.

Characterization of New THTT Derivative (8g)

This study was done to confirm the structure of newly synthesized compound 3-butyl-5-(1-phenylethyl)-1,3,5-thiadiazinane-2-thione (8g). White solid; 2.47 gm (84%); R_f (60% EtOAc/hexane) 0.24; IR (KBr) ν_{max} 2956, 2856, 1465, 1328, 1118, 708 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.85–0.94 (m, 5H), 1.23–1.32 (m, 5H), 1.40–1.48 (m, 2H), 3.79–3.84 (m, 1H), 4.11–4.14 (m, 1H), 4.17–4.21 (m, 1H), 4.40–4.43 (m, 2H), 7.31–7.33 (m, 3H), 7.49–7.51 (m, 1H), 7.67–7.69 (m, 1H); MS (positive EI) *m/z* 294 (32, M), 105 (100, M - 189).

Antifungal Activity

In order to find out antimicrobial potential of THTT analogues 8a-g, we investigated *in vitro* antifungal activity against five human pathogens by adopting the agar tube dilution procedure (Hostettmann *et al*., 1991). Growth was calculated by linear growth measurement in mm and percentage of growth inhibition (GI%) was determined with respect to the negative control and compared with drug assisted positive control. With the same procedure three (03) replicate tests were performed. Interestingly, all tested THTT compounds generally exhibited very inspiring antifungal potential against all tested fungi as shown in table 1.

Antibacterial Activity

All the synthesized THTT analogues (8a-g) were also investigated for *in vitro* antibacterial potential against four bacterial pathogens by using Micro plate Alamar Blue Assay (MABA) procedure (Pettit *et al*., 2005).

Growth was calculated by linear growth measurement in mm and percentage of growth inhibition (GI%) was determined in relation to the negative control. With the same procedure, three (03) replicate tests were performed. In general, all tested compounds found weakly active against all bacterial pathogens as shown in table 2.

Table 1: Results of antifungal activities at 200 µg/mL as % growth inhibition zone (± SEM)[a]

Entry	Compound	Fungi				
		Trichphyton rubrum	*Candida albicans*	*Aspergillus niger*	*Microsporum canis*	*Fusarium lini*
1.	8a	100	100	60.5 ± 1.0	–	100
2.	8b	100	100	80.2 ± 1.5	–	100
3.	8c	100	100	100	100	100
4.	8d	100	100	–	100	70.6 ± 0.8
5.	8e	100	100	–	70.6 ± 0.8	100
6.	8f	50.2 ± 1.5	80.2 ± 1.2	–	50.2 ± 1.5	90.4 ± 0.2
7.	8g	30.2 ± 2.0	40.2 ± 1.6	–	–	–
8.	Miconazole	100	100	–	100	100
9.	Amphotericin B			100		

[a]Result represents as mean of triplicate ± standard error of mean (SEM); testing incubation period 7 days at 27 ± 1 °C. –, No inhibition observed.

Table 2: Results of antibacterial activities at 50 µg/mL[a]

Entry	Compound	Growth inhibition zone (%)			
		Escherichia coli	*Shigella flexenari*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
1.	8a	–	5.76 ± 0.5	33.95 ± 0.7	–
2.	8b	–	12.12 ± 0.7	80.23 ± 0.1	–
3.	8c	–	2.86 ± 0.1	83.49 ± 0.6	–
4.	8d	–	–	24.99 ± 0.9	–
5.	8e	–	–	14.90 ± 0.4	–
6.	8f[b]	–	–	–	–
7.	8g	–	–	28.58 ± 0.5	–
8.	Ofloxacin	83.79 ± 0.8	85.24 ± 0.5	88.05 ± 0.6	82.45 ± 0.7

[a] Result represents as mean of triplicate ± standard error of mean (SEM). –, No inhibition observed. [b] Not tested.

DISCUSSION

The study revealed that the THTT derivatives 8a-e containing 1-phenyl ethyl group attached at *N*-3 (–NCSS) fragment of THTT nucleus (entries 1-5, table 1) exhibited very inspiring effects (generally 100% GI) against all pathogens except for *A. niger* for which these compounds showed reduced or no activity. Gratifyingly, compound 8c (entry 3, table 1) showed promising antifungal potential against all pathogens (GI% = 100). In contrast, analogue 8g with 1-phenyl ethyl (–$CHCH_3Ph$) substituent present at *N*-5 position of THTT nucleus (entry 7, table 1) found almost inactive for all the same tested pathogens. For instance compare compound 8d with 8g, having same substituents but on opposite positions of THTT ring exhibited dissimilar activities, i.e., former is highly active while later showed reduced activity against all the tested pathogens (entry 4 *vs* 7, table 1). Additionally, when compound 8a with 1-phenyl ethyl group (*N*-3 substituent) replaced with ethyl group on THTT nucleus and transformed as compound 8f, also displayed reduced antifungal potential for the same pathogens (entry 1 *vs* 6, table 1). It indicated the effectiveness of substituent 1-phenyl ethyl group on THTT nucleus.

In connection with antibacterial studies, all the tested THTT derivatives showed notable antibacterial activity against *S. aureus* within the range of (GI% = 14.90–83.49 ± 0.9). Likewise antifungal, compound 8c was found the most active compound among all THTT analogues against *S. aureus* with (GI% = 83.49 ± 0.6; entry 3, table 2) which is comparable to the standard drug ofloxacin (GI% = 88.05 ± 0.6, entry 8, table 2). However, a very weak response was observed against *S. flexenari* (GI% = 2.86–12.12 ± 0.7; entries 1-3, table 2) from compounds (8a-8c). It is observed that all the compounds showed no activity against *E. coli* and *P. aeruginosa*.

CONCLUSION

In summary, a small set of 1-phenyl ethyl (–$CHCH_3Ph$) group containing analogues of 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-2-thione (THTT) were prepared and investigated for their *in vitro* antifungal potential against five human pathogens following the standard agar tube dilution procedure and antibacterial potential against four pathogens by using Microplate Alamar Blue Assay (MABA) protocol. Structure activity relationship showed the importance of 1-phenyl ethyl

(–$CHCH_3Ph$) substituent at *N*-3 position of the THTT nucleus. These compounds displayed very significant activity against all tested fungi generally as of 100 percent growth inhibition (GI% = 100). However, they displayed significant antibacterial activity only against *S. aureus* (GI% = 14.90–83.49). It is worthiness that the compound 8c of the series was found the most active compound that displayed promising antifungal potential (GI% = 100) as well as significant antibacterial activity (GI% = 83.49) and therefore could be useful in designing new antimicrobial agents.

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