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Synthesis and biological evaluation of some new 7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazines as antimicrobial agents

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C H R O N I C L E

Article history:
Received June 20, 2021
Received in revised form
July 18, 2021
Accepted September 20, 2021
Available online
September 20, 2021

Keywords:
Synthesis
Heterocycles
Pyrazole
7H-[1,2,4]Triazolo[3,4-b][1,3,4]thiadiazines
Antimicrobial activity

A B S T R A C T

A series of some new pyrazole-substituted 7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines was synthesized in this study. The structures of target substances were confirmed by using ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry and elemental analysis. The synthesized compounds have been evaluated for antimicrobial activity against five bacterial strains (*Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Staphylococcus aureus*) and two fungal strains (*Candida albicans and Cryptococcus neoformans*). The antimicrobial screening studies of synthesized substances established that 2 of 12 compounds show pronounced antibacterial activity against the strain *Staphylococcus aureus*.

1. Introduction

Microbial infections are becoming an increasingly serious and complex problem for human health around the world. For centuries they have monopolized the prevailing factors of death and disability of millions of humans and are presently plaguing and even ravaging populations worldwide each year, far surpassing the impact of wars.[1] Antimicrobial drugs have caused a dramatic change not only of the treatment of infectious diseases but of a fate of mankind. Antimicrobial chemotherapy made remarkable advances, resulting in the overly optimistic view that infectious diseases would be conquered in the near future. However, in reality, emerging and reemerging infectious diseases have left us facing a counter-charge from infections. The increasing resistance to the current antimicrobial treatment has resulted in crucial need for the discovery and development of novel entity for the infectious treatment with different modes of action that could target both sensitive and resistant strains.[2] There is now an urgent need to develop new agents targeting higher to unexploited bacterial targets and machineries in order to maintain the ability of modern medicine to treat bacterial infections.

One of the promising methods for solving the resistance problem is screening of potential antimicrobial agents among new classes of chemical compounds.[3] The analogs of nitrogen-based heterocycles are an extremely important class of compounds that are widely used in medicinal chemistry. These heterocyclic rings are common structural units in many natural or synthetic agents and approximately 60% of FDA-approved drugs contain a nitrogen heterocycle in their structure. Moreover, the base pairs of DNA and RNA (guanine, cytosine, adenine, uracil and thymine) are also made up of N-

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doi: 10.5267/j.ccl.2021.009.003

heterocyclic compounds, namely purines, pyrimidines, etc. These nitrogen-containing heterocyclic molecules with distinct characteristics and applications have gained prominence in the rapidly expanding fields of organic and medicinal chemistry and the pharmaceutical industry.[4] In recent years, there has been a growing interest in condensed nitrogen-containing heterocyclic systems, as many of them exhibit different types of biological activity [5]. Triazolothiadiazines are among the little-studied representatives of this class of compounds.[6] They exhibit anticancer,[7-9] anti-proliferative [10,11] and antimicrobial activities.[12-14] In view of the facts mentioned above the development of novel triazolo[3,4-*b*][1,3,4]thiadiazines synthesis methods and their biological activity evaluation is an urgent task today.

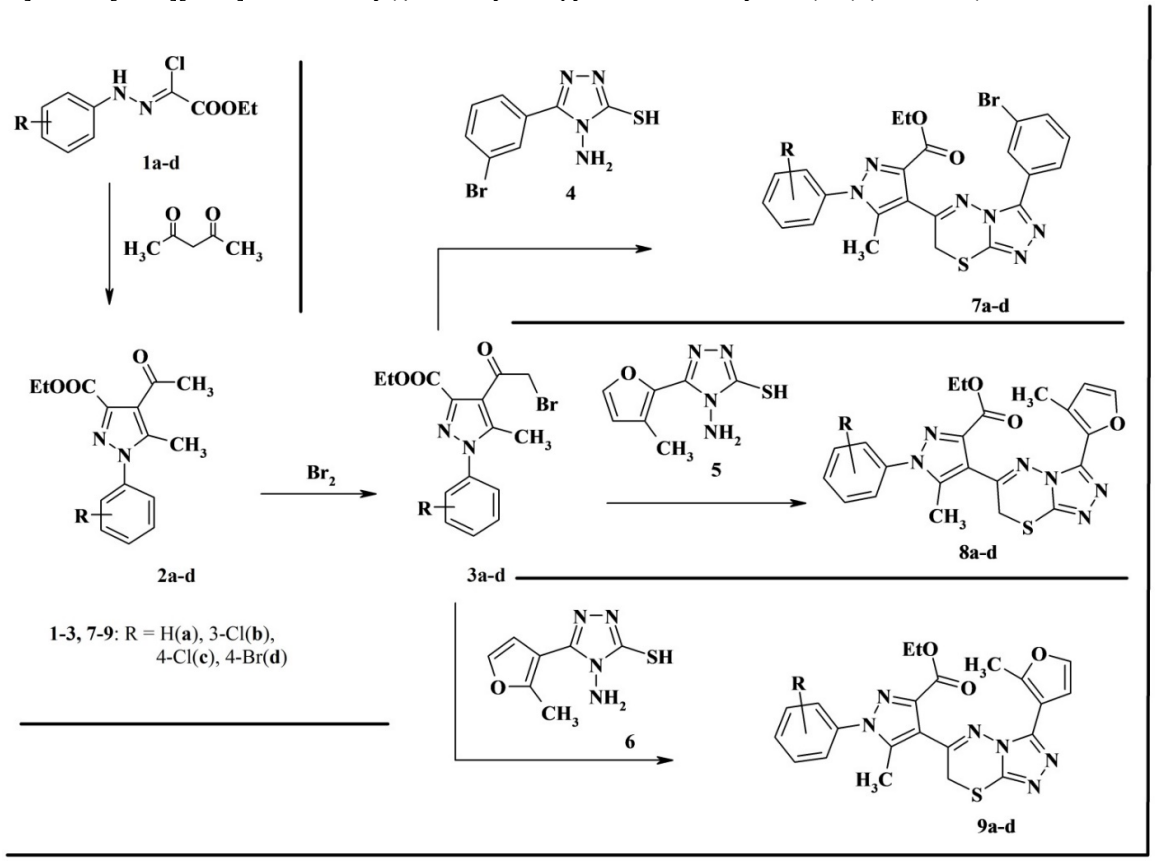
The present work is devoted to the synthesis of a series of novel pyrazole-substituted 7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4] thiadiazines for further pharmacological screening as antimicrobial activity.

2. Results and Discussion

2.1 Chemistry

In view of continuation of our research work to find new effective biologically active nitrogen-containing heterocycles,[15-28] this study focuses on the synthesis and study of antimicrobial activity of new pyrazole-substituted 7*H*-[1,2,4]triazolo[3,4-*b*][1, 3,4]thiadiazines. The starting substances in the synthetic part of this study were ethyl 1-aryl-4-(bromoacetyl)-5-methyl-1*H*-pyrazole-3-carboxylates (**3**). These substances were obtained by a known method[29] as a result of ethyl (2*Z*)-chloro(phenylhydrazone) acetates **1a-d** interaction with acetylacetone, followed by the obtained compound bromination **2a-d**. The corresponding transformation, which proceeds in acetic acid, allowed the target to obtain bromoketones **3a-d** (**Scheme**).

Another curious class of organic compounds that we used for the synthesis of target substances were 4-amino-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones (**4-6**). These derivatives are promising reagents in the construction of biologically active heterocycles.[7] When these binucleophiles interact with α-haloketones, derivatives of [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine are formed. Given the above, the next stage of our work was to carry out the interaction of ethyl 1-aryl-4-(bromoacethyl)-5-methyl-1*H*-pyrazole-3-carboxylates (**3**) with 4-amino-5-aryl(getaryl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones (**4-6**). Our studies have shown that this conversion occurs in ethanol with the formation of 1,3,4-thiadiazole ring. As a result of the presented cyclization were obtained the corresponding ethyl 1-aryl-4-{3-aryl(getaryl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl)}-5-methyl-1*H*-pyrazole-3-carboxylates (**7-9**) (**Scheme 1**).



Scheme. 1.

The structures of the obtained compounds were confirmed by ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry and elemental analysis. All these new compounds gave spectroscopic data in accordance with the proposed structures. The ^{1}H NMR spectra of all compounds show the proton signals of ethoxy groups in basic scaffolds as triplets and quartets observed in the range of 1.41–1.43 and 4.45–4.47 ppm. The endocyclic CH_2 group of all compounds observed as singlets at 3.94-4.03 ppm which proved the formation of 1,3,4-thiadiazole ring. The protons signals due to the furan moiety in compounds **8** and **9** were recorded as characteristic broad singlets, dublets and multiplets observed in the range of 6.42-7.62 ppm. In turn, the signal of the methyl group of the furan ring of the corresponding compounds is presented as a singlet in the range of 2.46-2.68 ppm. The signal of the methyl group at position 5 of pyrazole ring in all compounds appears as a singlet at 2.39-2.45 ppm. Aromatic radicals can be observed as a system of singlets, doublets and multiplets in the range of 7.30-8.25 ppm.

2.2 Antimicrobial activity

Infectious diseases have been creating problems for mankind since centuries. Bacterial, fungal and viral infections have monopolized the dominant factors of death and disability of millions of humans. Despite the availability of a number of antimicrobial agents the main matter of concern in the treatment of specified infections is the limited number of efficacious drugs. A need for new antimicrobial agents is justified as more microorganisms develop resistance to the present drugs available in the market. The antimicrobial study was performed by CO-ADD (The Community for Antimicrobial Drug Discovery), funded by the Wellcome Trust (UK) and The University of Queensland Australia.[30, 31] Evaluation of all synthesized compounds for their antimicrobial activity against five pathogenic bacteria, methicillin-resistant *Staphylococcus aureus (ATCC 43300)* as Gram-positive bacteria, *Escherichia coli (ATCC 25922), Klebsiella pneumonia (ATCC 700603), Acinetobacter baumannii (ATCC 19606)* and *Pseudomonas aeruginosa (ATCC 27853)* as Gram-negative bacteria and antifungal activity against two pathogenic fungal strains *Candida albicans (ATCC 90028)* and *Cryptococcus neoformans var. Grubii (H99; ATCC 208821)* (Table 1). It was revealed that the obtained compounds possess a diverse effect on microorganisms. A pronounced antimicrobial effect was observed in compounds **8b** (GI 88.5%) and **8c** (GI 93.6%) against strain *Staphylococcus aureus (ATCC 43300)*. Compounds **9b** and **9c** demonstrated slightly lower antimicrobial properties to strain *Staphylococcus aureus (ATCC 43300)*, but the results of this activity allow us to conclude that the search for antimicrobial agents among this class of compounds is promising. All other studied substances have shown moderate or low activity against the bacterial or fungal strains used. A structure-activity relationship study revealed that the presence of furane substituents on the position 5 of triazolo ring of basic scaffold was beneficial for the antimicrobial activity. A feature of the most active compounds structure is the presence of a methyl group on the position 3 of furane ring. Also, the research results reveal that the effect of compounds with halogen-containing substituents phenyl ring is required to enhance the biological activity.

Table 1. Antibacterial and antifungal activities of synthesized compounds.

Compound	Sa	Ec	Kp	Ab	Pa	Ca	Cn
7a	33.3	6.9	8.4	-10.5	9.4	22.3	18.7
7b	21.8	-3.8	-16.8	7.9	8. 6	42.5	18.4
7c	23.1	14.5	-10.1	6.8	-8.2	31.5	17.4
7d	33.1	23.4	- 9.4	4.5	-7.7	12.5	9.5
8a	18.5	1 5.7	-7.5	-5.7	9.8	21.7	12. 2
8b	88.5	9.8	10.1	- 8.5	-7.7	41.4	32.4
8c	93.6	1 4.5	-1 1.7	5.4	13.2	55.6	31.8
8d	31.4	12.5	2 6.2	5.9	-0.7	-1. 5	-8.7
9a	21.7	-0.9	15.4	9.7	6.6	21.7	14.9
9b	55.6	12.4	-11.9	7.7	15.3	45.5	17.3
9c	65.1	0.9	7.8	9.5	11.7	65.4	35.6
9d	24.5	7.8	9.9	13.5	15.8	21.8	12.5

Sa – *Staphylococcus aureus* (MRSA) ATCC 43300; **Ec** - *Escherichia coli* ATCC 25922; **Kp** - *Klebsiella pneumoniae* ATCC 700603; **Ab** - *Acinetobacter baumannii* ATCC 19606; **Pa** - *Pseudomonas aeruginosa* ATCC 27853; **Ca** – *Candida albicans* ATCC 90028; **Cn** - *Cryptococcus neoformans* H99 ATCC 208821.

Compounds **8b** and **8c** were selected for the second phase of the study. The Minimal inhibitory concentrations (MIC μg/mL) measurements were performed using Ceftriaxone as a reference drug for antibacterial activity respectively. The safety margin for the active compounds toward human cells was determined through cytotoxicity against human embryonic kidney cell line and hemolysis of human red blood cells. Executed experiment confirmed the antibacterial activity of these compounds against the *Staphylococcus aureus (ATCC 43300).* The results are shown in **Table 2**.

Table 2. The Minimal inhibitory concentrations (MIC μg/mL) measurements for compounds with significant microbial growth inhibition (**8b** and **8c**) using Ceftriaxone and Amphotericin.

Compound	*MIC, μg/mL*	*CC50, μg/mL*	*HC10, μg/mL*
	Staphylococcus aureus (ATCC 43300)	**Hek**	**RBC 0**
8b	32	>32	>32
8c	16	>32	>32
Ceftriaxone	32	NT	NT

Hek – HEK293, human embryonic kidney cells ATCC CRL-1573; RBC – human red blood cells; NT not tested.

3. Conclusions

In summary, we presented an efficient synthetic approach to a synthesis of some new triazolothiadiazines for their antimicrobial activity evaluation. We have shown that the proposed synthetic protocols provided the possibility to design pyrazole-substituted 7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines diversity with a considerable chemical novelty. The antimicrobial screening studies of synthesized compounds revealed that 2 of 12 newly tested substances possess high antibacterial activity against the strain *Staphylococcus aureus (ATCC 43300)*. Further optimization of the structure to improve biological activity is currently in progress.

4. Experimental

4.1 Chemistry

All chemicals were of analytical grade and commercially available. When performing the synthetic part of the work, the reagents of the company Merck (Germany) and Sigma-Aldrich (USA) were used. All reagents and solvents were used without further purification and drying. All the melting points were determined in an open capillary and are uncorrected. NMR spectra were recorded on a Varian Mercury 400 (Agilent Technologies, San Francisco, USA) instrument with TMS or deuterated solvent as an internal reference. Mass spectra were run using Agilent 1100 series LC/MSD (Agilent Technologies, San Francisco, USA) with an API–ES/APCI ionization mode. Elemental analysis was performed on an Elementar Vario L cube instrument (Elementar Analysen systeme GmbH, Hanau, Germany). Satisfactory elemental analyses were obtained for new compounds (C±0.17, H±0.21, N±0.19).

4.1.1 General procedure for the synthesis of 1-aryl-4-{3-aryl(getaryl)-7H-[1,2,4]triazolo[3,4-b] [1,3,4]thiadiazin-6-yl)}-5-methyl-1H-pyrazole-3-carboxylates **(7-9).** To a solution of bromoketone **3** (1 mmol) in 10 ml of ethanol was added 4-amino-5-(3-bromophenyl)-4*H*-1,2,4-triazole-3-thiol (**4**) or 4-amino-5-(3-methylfuran-2-yl)-4*H*-1,2,4-triazol-3-thiol (**5**) or 4-amino-5-(2-methylfuran-3-yl)-4*H*-1,2,4-triazole-3-thiol (**6**) (1 mmol). The mixture was boiled for 4 hours. After cooling, 20 ml of water and 2% ammonia solution were added to pH ~ 8. Obtained precipitate was filtered off and recrystallized from ethanol.

4.1.2 Ethyl 4-{3-(3-bromophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-6-yl)}-5-methyl-1-phenyl-1H-pyrazole-3-carboxylate **(7a).** White solid; Yield: 78 %; mp 206 ºC; ^{1}H NMR (400 MHz, DMSO) δ 1.41 (t, *J* =7.0 Hz, 3H, $CH_2C\mathit{H_3}$), 2.41 (s, 3H, CH_3), 3.98 (s, 2H, CH_2S), 4.45 (q, *J* = 7.0 Hz, 2H, CH_2O), 7.33 (t, *J* = 7.9 Hz, 1H, Ar), 7.43–7.60 (m, 6H, Ar), 8.02 (d, *J* = 7.9 Hz, 1H, Ar), 8.24 (s, 1H, Ar); ^{13}C NMR, δ, ppm: 12.2, 14.3, 27.5, 61.9, 118.0, 122.4, 125.8 (2C), 126.7, 127.9, 129.5 (2C), 129.6, 130.1, 130.7, 133.1, 138.1, 141.6, 142.1, 143.4, 151.1, 151.4, 162.0. ESI-MS: m/z 524 $[M+H]^+$; anal. calcd. for $C_{23}H_{19}BrN_6O_2S$: C 52.78; H 3.66; N 16.06. found: C 52.93; H 3.57; N 16.18.

4.1.3 Ethyl 4-{3-(3-bromophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-6-yl)}-5-methyl-1-(3-chlorophenyl)-1H-pyrazole-3-carboxylate **(7b).** Yellow solid; Yield: 85 %; mp 196 ºC; ^{1}H NMR (200 MHz, DMSO) δ 1.43 (t, *J* = 7.1 Hz, 3H, $CH_2\mathit{CH_3}$), 2.45 (s, 3H, CH_3), 4.00 (s, 2H, CH_2S), 4.47 (q, *J* = 7.1 Hz, 2H, CH_2O), 7.30–7.63 (m, 6H, Ar), 8.03 (d, *J* = 7.0 Hz, 1H, Ar), 8.25 (s, 1H, Ar); ^{13}C NMR, δ, ppm: 11.9, 14.1, 27.3, 61.5, 115.8, 117.5, 118.2, 122.4, 126.3, 126.5, 127.9, 131.5, 131.8, 132.0, 132.9, 135.0, 140.2, 141.1, 147.2, 151.2, 151.5, 161.9, 164.5.; ESI-MS: m/z 558 $[M+H]^+$; anal. calcd. for $C_{23}H_{18}BrClN_6O_2S$: C 49.52; H 3.25; N 15.07; found: C 49.39; H 3.40; N 14.93.

4.1.4 Ethyl 4-{3-(3-bromophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-6-yl)}-5-methyl-1-(4-chlorophenyl)-1H-pyrazole-3-carboxylate **(7c).** Yellow solid; Yield: 71 %; mp 203 ºC; ^{1}H NMR (200 MHz, DMSO) δ 1.43 (t, *J* = 7.2 Hz, 3H, $CH_2C\mathit{H_3}$), 2.42 (s, 3H, CH_3), 3.98 (s, 2H, CH_2S), 4.47 (q, *J* = 7.2 Hz, 2H, CH_2O), 7.34 (t, *J* = 7.9 Hz, 1H, Ar), 7.39–7.63 (m, 5H, Ar), 8.03 (d, *J* = 7.4 Hz, 1H, Ar), 8.24 (s, 1H, Ar); ^{13}C NMR, δ, ppm: 12.2, 14.4, 27.6, 62.1, 118.3, 122.4, 126.7, 127.0 (2C), 127.9, 129.7 (2C), 130.1, 130.7, 133.1, 135.6, 136.6, 141.9, 142.1, 143.3, 151.12, 151.14, 161.8; ESI-MS: m/z 558 $[M+H]^+$; anal. calcd. for $C_{23}H_{18}BrClN_6O_2S$: C 49.52; H 3.25; N 15.07; found: C 49.67; H 3.16; N 15.19.

4.1.5 Ethyl 1-(4-bromophenyl)-4-{3-(3-bromophenyl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-6-yl)}-5-methyl-1H-pyrazole-3-carboxylate **(7d).** White solid; Yield: 89 %; mp 208 ºC; ^{1}H NMR (200 MHz, DMSO) δ 1.43 (t, *J* = 7.0 Hz, 3H, $CH_2\mathit{CH_3}$), 2.44 (s, 3H, CH_3), 4.03 (s, 2H, CH_2S), 4.47 (q, *J* = 7.0 Hz, 2H, CH_2O), 7.32–7.55 (m, 3H, Ar), 7.60 (d, *J* = 7.3

Hz, 1H, Ar), 7.69 (d, *J* = 7.8 Hz, 2H, Ar), 8.04 (bs, 1H, Ar), 8.25 (s, 1H, Ar); ^{13}C NMR, δ, ppm: 12.0, 14.1, 28.2, 61.8, 118.5, 122.2, 126.5, 127.4 (2C), 128.1, 129.3 (2C), 130.3, 130.7, 133.0, 135.8, 138.2, 142.3, 142.5, 143.9, 150.8, 151.0, 161.6; ESI-MS: m/z 603 $[M+H]^+$; anal. calcd. for $C_{23}H_{18}Br_2N_6O_2S$: C 45.87; H 3.01; N 13.95; found: C 46.08; H 3.14; N 13.77.

4.1.6 Ethyl 4-{3-(3-methylfuran-2-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-6-yl)}-5-methyl-1-phenyl-1H-pyrazole-3-carboxylate **(8a).** White solid; Yield: 74 %; mp 167 ºC; ^{1}H NMR (200 MHz, DMSO) δ 1.42 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 2.39 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 3.97 (s, 2H, CH_2S), 4.46 (q, *J* = 7.1 Hz, 2H, CH_2O), 6.42 (d, *J* = 1.6 Hz, 1H, 4-H-furane), 7.40–7.62 (m, 6H, Ph, 5-H-furane); ^{13}C NMR, δ, ppm: 11.1, 11.8, 14.3, 27.6, 61.8, 114.6, 117.9, 124.9, 125.8 (2C), 129.4 (2C), 129.5, 136.9, 138.2, 141.5, 142.2, 142.4, 143.6, 146.2, 150.9, 161.6; ESI-MS: m/z 449 $[M+H]^+$; anal. calcd. for $C_{22}H_{20}N_6O_3S$: C 58.92; H 4.49; N 18.74; found: C 58.71; H 4.56; N 18.56.

4.1.7 Ethyl 4-{3-(3-methylfuran-2-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-6-yl)}-5-methyl-1-(3-chlorophenyl)-1H-pyrazole-3-carboxylate **(8b).** Yellow solid; Yield: 84 %; mp 183 ºC; ^{1}H NMR (200 MHz, DMSO) δ 1.42 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 2.40 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 3.96 (s, 2H, CH_2S), 4.47 (q, *J* = 7.1 Hz, 2H, CH_2O), 6.44 (d, *J* = 1.6 Hz, 1H, 4-H-furane), 7.35–7.55 (m, 5H, Ar, 5-H-furane); ^{13}C NMR, δ, ppm: 11.0, 11.6, 14.1, 26.9, 60.9, 105.0, 114,9, 118.0, 120.3, 125.3, 129.8, 131.6, 136.7, 138.6, 141.3, 142.0, 142.3, 143.6, 146.8, 151.0, 162.0, 164.3; ESI-MS: m/z 483 $[M+H]^+$; anal. calcd. for $C_{22}H_{19}ClN_6O_3S$: C 54.71; H 3.97; N 17.40; found: C 54.48; H 3.86; N 17.62.

4.1.8 Ethyl 5-methyl-1-(4-chlorophenyl)-4-{3-(3-methylfuran-2-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-6-yl)}-1H-pyrazole-3-carboxylate **(8c).** Yellow solid; Yield: 84 %; mp 147 ºC; ^{1}H NMR (200 MHz, DMSO) δ 1.42 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 2.40 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 3.96 (s, 2H, CH_2S), 4.46 (q, *J* = 7.2 Hz, 2H, CH_2O), 6.43 (d, *J* = 1.6 Hz, 1H, 4-H-furane), 7.43 (d, *J* = 9.0 Hz, 2H, Ar), 7.45 (bs, 1H, 5-H-furane), 7.52 (d, *J* = 9.0 Hz, 2H, Ar); ^{13}C NMR, δ, ppm: 11.1, 11.8, 14.3, 27.6, 62.0, 114.6, 118.2, 125.1, 127.0 (2C), 129.6 (2C), 135.5, 136.7, 136.9, 138.3, 142.2, 142.4, 143.6, 146.2, 150.8, 161.8; ESI-MS: m/z 483 $[M+H]^+$; anal. calcd. for $C_{22}H_{19}ClN_6O_3S$: C 54.71; H 3.97; N 17.40; found C 54.53; H 4.09; N 17.16.

4.1.9 Ethyl 1-(4-bromophenyl)-4-{3-(3-methylfuran-2-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine-6-yl)-5-methyl-1H-pyrazole-3-carboxylate **(8d).** White solid; Yield: 73 %; mp 178 ºC; ^{1}H NMR (200 MHz, DMSO) δ 1.42 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 2.40 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 3.96 (s, 2H, CH_2S), 4.46 (q, *J* = 7.1 Hz, 2H, CH_2O), 6.43 (bs, 1H, 4-H-furane), 7.37 (d, *J* = 7.6 Hz, 2H, Ar), 7.46 (bs, 1H, 5-H-furane), 7.68 (d, *J* = 7.6 Hz, 2H, Ar); ^{13}C NMR, δ, ppm: 11.0, 11.6, 14.1, 27.8, 61.9, 114.3, 118.2, 125.4, 127.1 (2C), 130.2 (2C), 135.5, 137.2, 137.5, 138.8, 142.4, 142.7, 144.0, 146.7, 151.0, 162.0; ESI-MS: m/z 528 $[M+H]^+$; anal. calcd. for $C_{22}H_{19}BrN_6O_3S$: C 50.10; H 3.63; N 15.94; found C 49.89; H 3.75; N 15.78.

4.1.10 Ethyl 4-{3-(2-methylfuran-3-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-6-yl)}-5-methyl-1-(3-chlorophenyl)-1H-pyrazole-3-carboxylate **(9a).** White solid; Yield: 75 %; mp 162 ºC; ^{1}H NMR (400 MHz, DMSO) δ 1.42 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 2.40 (s, 3H, CH_3), 2.68 (s, 3H, CH_3), 3.95 (s, 2H, CH_2S), 4.46 (q, *J* = 7.1 Hz, 2H, CH_20), 6.87 (d, *J* = 1.9 Hz, 1H, furane), 7.33 (d, *J* = 1.9 Hz, 1H, furane), 7.46–7.58 (m, 5H, Ph); ^{13}C NMR, δ, ppm: 12.0, 13.8, 14.3, 27.7, 61.8, 107.0, 109.9, 118.3, 125.7 (2C), 129.4 (2C), 129.5, 138.2, 140.6, 141.2, 141.5, 141.9, 148.3, 150.4, 154.4, 162.0; ESI-MS: m/z 449 $[M+H]^+$; anal. calcd. for $C_{22}H_{20}N_6O_3S$: C 58.92; H 4.49; N 18.74; found C 58.71; H 4.56; N 18.59.

4.1.11 Ethyl 4-{3-(2-methylfuran-3-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-6-yl)}-5-methyl-1-(3-chlorophenyl)-1H-pyrazole-3-carboxylate **(9b).** Yellow solid; Yield: 83 %; mp 158 ºC; ^{1}H NMR (200 MHz, DMSO) δ 1.42 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 2.42 (s, 3H, CH_3), 2.68 (s, 3H, CH_3), 3.95 (s, 2H, CH_2S), 4.46 (q, *J* = 7.1 Hz, 2H, CH_20), 6.86 (d, *J* = 1.7 Hz, 1H, 4-H-furane), 7.34 (d, *J* = 1.7 Hz, 1H, 5-H-furane), 7.36–7.55 (m, 4H, Ar); ^{13}C NMR, δ, ppm: 11.9, 13.7, 14.1, 27.4, 61.0, 109.5, 115.7, 117.6, 118.0, 125.1, 126.4, 131.8, 135.0, 140.2, 141.1, 142.0, 147.2, 150.4, 151.1, 154.5, 162.0, 164.6; ESI-MS: m/z 483 $[M+H]^+$; anal. calcd. for $C_{22}H_{19}ClN_6O_3S$: C 54.71; H 3.97; N 17.40; found C 54.53; H 4.09; N 17.26.

4.1.12 Ethyl 4-{3-(2-methylfuran-3-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-6-yl)}-5-methyl-1-(4-chlorophenyl)-1H-pyrazole-3-carboxylate **(9c).** Yellow solid; Yield: 75 %; mp 178 ºC; ^{1}H NMR (200 MHz, DMSO) δ 1.42 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 2.40 (s, 3H, CH_3), 2.67 (s, 3H, CH_3), 3.94 (s, 2H, CH_2S), 4.45 (q, *J* = 7.1 Hz, 2H, CH_20), 6.85 (bs, 1H, 4-H-furane), 7.27–7.60 (m, 5H, Ar, 5-H-furane); ^{13}C NMR, δ, ppm: 11.8, 13.8, 14.3, 27.7, 61.9, 96.9, 107.0, 109.9, 118.6, 126.9 (2C), 129.6 (2C), 135.5, 136.7, 140.6, 141.1, 141.9, 148.3, 150.2, 154.4, 161.7. ESI-MS: m/z 483 $[M+H]^+$; anal. calcd. for $C_{22}H_{19}ClN_6O_3S$: C 54.71; H 3.97; N 17.40; found C 54.95; H 4.18; N 17.02.

4.1.13 Ethyl 1-(4-bromophenyl)-4-{3-(2-methylfuran-3-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine-6-yl)-5-methyl-1H-pyrazole-3-carboxylate **(9d).** White solid; Yield: 72 %; mp 196 ºC; ^{1}H NMR (200 MHz, DMSO) δ 1.42 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 2.40 (s, 3H, CH_3), 2.68 (s, 3H, CH_3), 3.95 (s, 2H, CH_2S), 4.45 (q, *J* = 6.8 Hz, 2H, CH_20), 6.86 (bs, 1H, 4-H-furane), 7.33 (bs, 1H, 5-H-furane), 7.38 (d, *J* = 8.2 Hz, 2H, Ar), 7.68 (d, *J* = 8.2 Hz, 2H, Ar); ^{13}C NMR, δ, ppm: 11.9, 13.5, 14.1, 27.8, 61.4, 98.0, 107.6, 110.4, 119.2, 127.2 (2C), 130.4 (2C), 136.0, 137.5, 140.8, 141.3, 142.2, 148.6, 150.5, 154.5,

162.0. ESI-MS: m/z 528 $[M+H]^+$; anal. calcd. for $C_{22}H_{19}BrN_6O_3S$: C 50.10; H 3.63; N 15.94; found C 50.26; H 3.78; N 15.81.

4.2 Pharmacological/biological assays

4.2.1 Compound preparation. Initially, the tests were carried out at a single compound concentrations of 32 μg/mL in duplicates, to identify any actives. Further, a hit confirmation of the active compounds by a dose response test, using 8 concentrations at 1:2 dilution, in duplicates, to determine the minimum inhibitory concentration (MIC) against bacteria and yeasts, CC50 (concentration at 50% cytotoxicity) against mammalian cells and HC10 (concentration at which 10% hemolysis is induced) against human red blood cells was performed. All substances were dissolved in DMSO to form a stock concentration of 10 mg/mL. Aliquots were diluted in water and 5 μL, dispensed into empty 384-well plates in duplicates for each strain and cell assayed. As soon as cells were added to the plates, this gave a final compound concentration of 32 μg/mL, or in case of a serial dilution assay compound concentrations from 32 to 0.25 μg/mL, in both cases with a maximum DMSO concentration of 0.3%.

4.2.2 Antimicrobial assays. The compounds have been investigated for activity against 4 Gram-negative bacteria (*Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Klebsiella pneumoniae ATCC 700603, Acinetobacter baumannii ATCC 19606*), 1 Gram-positive bacteria (*Staphylococcus aureus ATCC 43300 MRSA*), and 2 yeasts (*Candida albicans ATCC 90028, Cryptococcus neoformans H99 ATCC 208821*), and were carried out by the Community for Open Antimicrobial Drug Discovery (CO-ADD).

All bacteria overnight culturation in Cation-adjusted Mueller Hinton broth (CAMHB) at 37 °C was performed. The resultant mid-log phase cultures were added to each well of the compound containing plates (384-well non-binding surface plates - Corning 3640), giving a cell density of 5 ´ 105 CFU/mL (colony forming units/mL). All plates were covered and incubated at 37 °C for 18 h without shaking. Bacterial growth inhibition was determined by measuring absorbance at 600 nm (OD600) using a Tecan M1000 Pro monochromator plate reader. Yeast strains were cultured at 30 °C on Yeast Extract-Peptone Dextrose (YPD) agar for 3 days. A yeast suspension of 1´ 106 to 5 ´ 106 CFU/mL (as determined by OD530) was prepared from five colonies. These stock suspensions were diluted to a final concentration of 2.5 103 CFU/mL with Yeast Nitrogen Base (YNB) broth, and, subsequently 45 μL of the yeast suspension was added to each well of the compound containing plates (384-well non-binding surface plates - Corning 3640). Plates were covered and incubated at 35 °C for 24 h without shaking. *C. albicans* growth inhibition was determined measuring absorbance at 530 nm (OD530), while *C. neoformans* growth inhibition was determined measuring the absorbance difference between 600 and 570 nm (OD600-570), after the addition of resazurin (0.001% final concentration) and incubation for additional 2 h at 35 °C. The absorbance was measured by means of Biotek Synergy HTX plate reader. Growth inhibition was evaluated as percentage between untreated cells (positive growth control) and media only (negative growth control). Compounds with ≥80% growth inhibition were selected as actives in the initial screening, and minimal inhibitory concentration (MIC) was determined following EUCAST recommendations. 80% growth inhibition were used as threshold for full inhibition.

4.2.3 Cytotoxicity assay. HEK293 (human embryonic kidney) ATCC CRL-1573 cells were counted manually in a Neubauer haemocytometer and then plated in 384-well tissue culture treated plates (Corning 3712) containing the compounds to give a density of 5000 cells/well in a final volume of 50 μL. DMEM supplemented with 10% FBS was used as growth media and the cells were incubated at 37 °C together with the compounds for 20 h in 5% CO_2. Cytotoxicity (or cell viability) was determined by fluorescence, ex: 560/10 nm, em: 590/10 nm (F560/590), after addition of 5 μL of 25 μg/mL resazurin (2.3 μg/mL final concentration) and after incubation at 37 °C for further 3 h in 5% CO_2. Tecan M1000 Pro monochromator plate reader was used for the fluorescence intensity measurement, using automatic gain calculation. CC50 were calculated by means of curve fitting the inhibition values vs. log (concentration) using a sigmoidal dose-response function, with variable fitting values for bottom, top and slope.

4.2.4 Hemolysis assay. Human whole blood was washed three times with 3 volumes of 0.9% NaCl and then resuspended in same to a concentration of 0.5 ´ 108 cells/mL, as determined by manual cell count in a Neubauer haemocytometer with further addition of washed cells to the 384-well compound containing polystyrene plates (Corning 3657) for a final volume of 50 μL. The plates were incubated for 1 h at 37 °C after a 10 min shake on a plate shaker. The next step was centrifugation of plates at 1000g for 10 min to pellet cells and debris, 25 μL of the supernatant was then transferred to a polystyrene 384-well assay plate (Corning 3680). Haemolysis was defined by the supernatant absorbance at 405 mm (OD405) measurement using a Tecan M1000 Pro monochromator plate reader. HC_{10} (concentration causing 10% haemolysis) were established by curve fitting the inhibition values vs. log (concentration) using a sigmoidal dose-response function with variable fitting values for top, bottom and slope. The use of human blood (sourced from the Australian Red Cross Blood Service) for haemolysis trials was approved by The University of Queensland Institutional Human Research Ethics Committee, Approval Number 2014000031.

Acknowledgements

This work was financially supported by the National Research Foundation of Ukraine (project 2020.01/0166).

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