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Design, synthesis and bioactivity studies of novel triazolopyrimidinone compounds

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ABSTRACT: This study was aimed to develop novel compounds to combat antimicrobial resistance, which is one of the biggest threats to global health. For this purpose, compounds bearing triazolopyrimidinone ring and N-(methylnaphthalene)piperazine (**NMP**) hybrids were designed and synthesized. Ten new compounds were synthesized and after proving their chemical structures were tested for antimicrobial activity using disk diffusion and microdilution method against Gram-negative bacterial strains (*Escherichia coli* and *Pseudomonas aeruginosa*), Gram-positive bacterial strains (*Staphylococcus aureus* and *Enterococcus faecalis*) and fungal strains (*Candida albicans* and *Candida parapsilosis*). Anti-biofilm activity and ethidium bromide accumulation assay results were also determined for the selected compounds. Among the tested compounds, hybrid compound **H5** showed promising activity against *E. faecalis* with 16-fold potency compared to its precursor, **TP5**. Additionally, it has statistically significant inhibition of biofilm production at 10 µg/ml dose against *E. coli* and *P. aeruginosa* and a decreasing effect on the relative accumulation of ethidium bromide in *P. aeruginosa* at 100 µg/ml dose (85.07%) after 30 min. 2,5-disubstitued[1,2,4]triazolo[1,5-a]pyrimidinone heterocyclic core structure and its antimicrobial activity are reported to the literature for the first time in this study.

KEYWORDS: Antimicrobial resistance; triazolopyrimidinone; *N*-(methylnaphthalene)piperazine; microwave-assisted synthesis; ethidium bromide accumulation; anti-biofilm activity.

1. INTRODUCTION

Due to the fact that antimicrobial resistance impacts effectiveness of the treatment negatively against various infections caused by bacteria, fungi etc., has become an important risk threatening public health [1]. World Health Organization (WHO) has declared a list of pathogens in order of priority to guide discovery and development of novel antibiotics which are aimed to be effective against tuberculosis, Gram-negative (e.g. *Pseudomonas aeruginosa*, *Enterobacteriaceae*) and Gram-positive (e.g. *Enterococcus faecium, Staphylococcus aureus*) pathogens those have over medium priority [2]. The United Nations Interagency Coordination Group (UN IACG) calls on to increase investment and innovation in quality-assured novel compounds as new antimicrobial agents [3].

Hybridization of active fragments is a common strategy in the field of medicinal chemistry on drug development studies, including the discovery of novel antimicrobial agents [4-6]. Among fused 1,2,4-triazole and pyrimidine derivatives, [1,2,4]triazolo[1,5-*a*]pyrimidine structure have been recently reported in the literature possessing different bioactivities [7-14]. As a matter of fact, moderate antimicrobial effects of [1,2,4]triazolo[1,5-*a*]pyrimidinonecarbonitrile derivatives against *E. coli, S. aureus, Aspergillus flavus* and *Candida albicans* were reported [15]. On the other hand, efflux pump inhibitory activity of **NMP** was reported for the first time in 2005 and studied extensively since then [16-19]. Therefore, **NMP** has been widely used as a fragment combined with several antibiotics in novel hybrid molecules [20, 21]. Taken together, our interest focused on triazolopyrimidinone structures and their hybrids with **NMP** moiety (Figure 1).

In this study, we synthesized six different 2-substitued-3*H*-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-one derivatives (**TP1**-**TP6**) and their **NMP** hybrids (**H1**-**H6**) as potential antimicrobial agents. In order to evaluate structure-activity relationship, aliphatic chain / heterocyclic ring / non-substituted phenyl ring / *p*-electron withdrawing group substituted phenyl ring / *p*-electron donating group substituted phenyl ring substituents were chosen for design.

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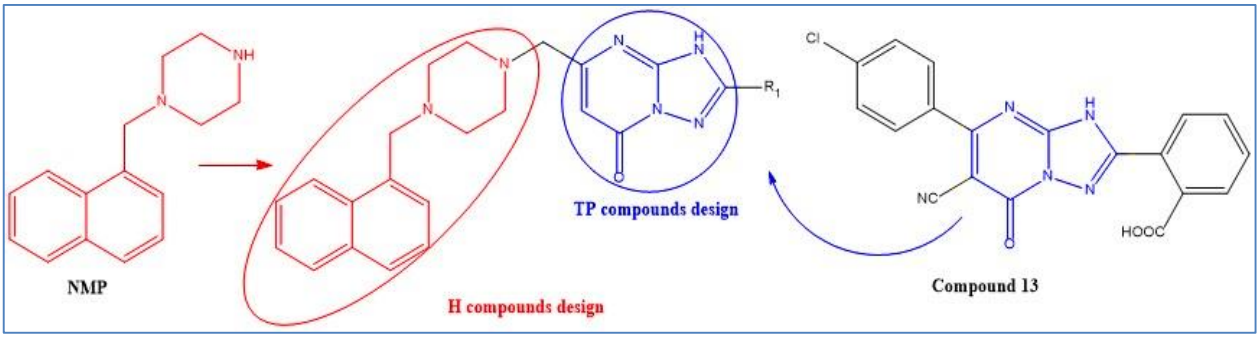

Figure 1. Design strategy of the synthesized compounds; [1,2,4]triazolo[1,5-a]pyrimidin-7-one derivative (compound 13, [15]) and NMP [17] hybridization.

The antimicrobial effect properties of the obtained compounds were determined by using disk diffusion and microdilution methods against Gram-negative bacteria (*P. aeruginosa* and *E. coli*), Gram-positive bacteria (*E. faecalis* and *S. aureus*) and fungi (*C. albicans* and *C. parapsilosis*). Certain compounds were selected based on their antimicrobial effect properties for evaluating their efflux pump inhibitory properties by using ethidium bromide (EtBr) accumulation assay and also for determining anti-biofilm activity on *E. coli* and *P. aeruginosa* strains.

2. RESULTS and DISCUSSION

2.1. Chemistry

Target molecules (**TP1-TP6** and **H1-H6**) were synthesized according to the methods indicated in the literature with slight changes [22-27]. 5-substituted-3-aminotriazole structure was synthesized in two-step except 3-amino-5-(methylthio)-4*H*-1,2,4-triazole. In the first step, aminoguanidine bicarbonate and substituted acyl chlorides were stirred in toluene at room temperature to give amide derivatives. Then, amide-derived compounds were heated in water (MW, 3-15 minutes) for cyclization [22]. **TP1-TP6** compounds obtained by the reaction of ethyl 4-chloroacetoacetate with 5-substituted-3-aminotriazole compounds in acetic acid (MW, 20 min, 180°C) [23]. (Figure 2).

The absence of carbonyl absorption bands in FT-IR spectra confirmed the structure of 5-substituted-3-aminotriazole derivatives and melting point values were in accordance with the reference [24]. The structure of **TP1-TP6** compounds were verified by the spectroscopic methods reported in the materials and methods section. Considering ^{1}H-NMR spectrum of the compounds, one singlet between δ 6.11 – 6.21 ppm with the integration of one hydrogen atom confirmed the enone structure while one singlet between δ 4.60 – 4.70 ppm with the integration of two hydrogen atoms indicated the methylene group adjacent to the chlorine atom. On the other hand, hydrogen atoms belonging to the substituents were observed in the expected regions and integrations. Chemical shifts of carbon atoms were also detected at their predicted ppm values in the ^{13}C-NMR spectra. The melting point and spectral data of the compounds, which have already been reported, are in accordance with the data in the literature [25,26]. The title compounds except for **TP1** and **TP4** are original and were reported with their full spectra data in this study.

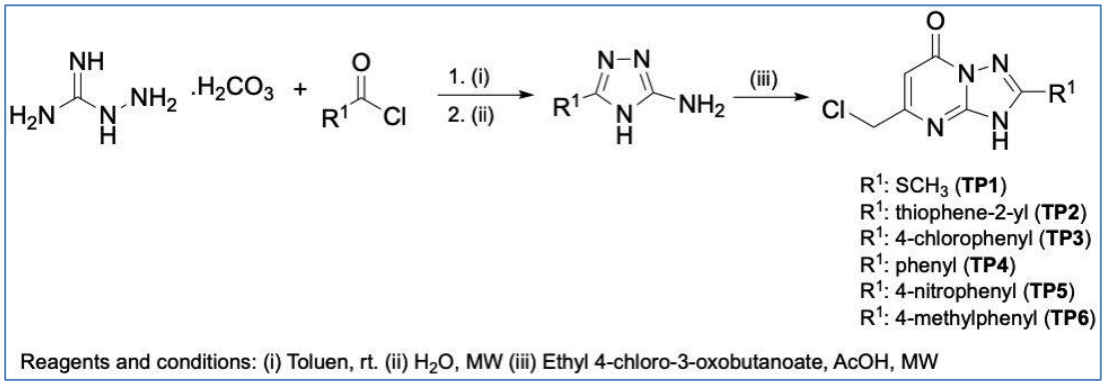

Figure 2. Synthesis pathway of **TP1-TP6** compounds.

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NMP (1-(naphthalen-1-ylmethyl)piperazine) was synthesized by the reaction of 1-chloromethylnaphthalene and piperazine in THF [28]. The chemical shifts, integration values and coupling constants from the ^{1}H-NMR spectra confirmed the **NMP** structure. The molecular ion peak was detected at its calculated *m/z* value. The obtained spectral data are compatible with the literature.

In the last step of synthesis, **TP1-TP6** compounds and **NMP** were reacted in DMF in the presence of Cs_2CO_3 under microwave conditions to give 2-substituted-5-((4-(naphthalen-1-ylmethyl)piperazin-1-yl)methyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7(3*H*)-one (**H1-H6**) derivatives (Figure 3).

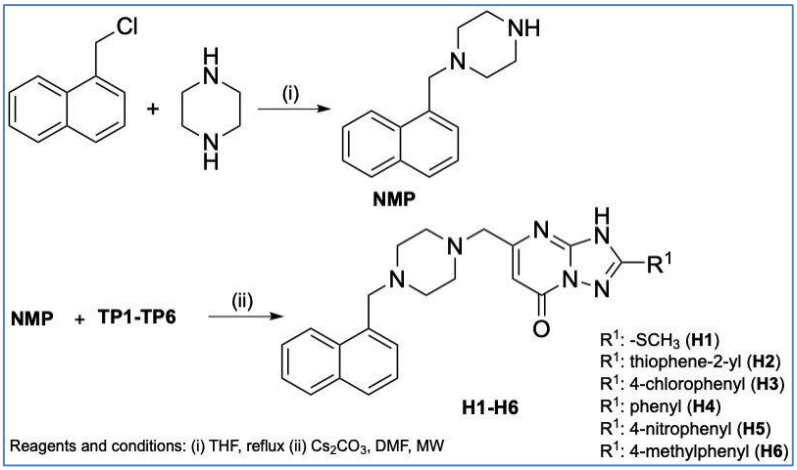

Figure 3. Synthesis pathway of **H1-H6** compounds.

The most prominent peaks in the range of 1618-1639 cm^{-1} in FT-IR spectrum belong to the C=O stretching bands of the amide functional group [29]. The proton adjacent to the carbonyl group was observed between δ 6.11-6.21 ppm for **TP1-TP6** compounds while a slight chemical shift was noted towards high field namely δ 5.67-5.82 ppm for **H1-H6** compounds. Exchangeable proton on the nitrogen of the heterocyclic ring was not detected in DMSO-*d6*. Other aromatic and aliphatic proton signals were observed in the expected chemical shift with the expected splitting patterns in accordance with the target structure. The molecular ion peaks are in full agreement with the calculated *m/z* values in MS spectra. The HRMS data of the compounds are within the range of acceptable limits. Taken together, it is concluded that the targeted structures were obtained.

2.2. Antimicrobial activity studies

2.2.1. Disk diffusion test

Mean inhibition zone diameters determined by disk diffusion test against bacterial and fungal strains were demonstrated in Table 1. The amount of active substances in the disks were 100 µg/disk. Quality control ranges of gentamicin and fluconazole were in the limit values according to the EUCAST criteria. DMSO was found to be ineffective against bacteria and fungi in studied concentrations.

According to the disk diffusion test, substances did not show inhibitory effects against fungi (*C. albicans* and *C. parapsilosis*) and *P. aeruginosa* strain. Best activity against *E. coli* was shown by **TP1** and **H1**; against *S. aureus* shown by **TP5** and **H5** and against *E. faecalis* **TP2**, **TP5**, **TP6**, **H1**, **H2**, **H4**, **H5** and **H6**.

It might be concluded that hybridization increased the activity of the compounds. For instance, it was seen that the **TP5** compound had no activity while the **H5** compound (**TP5-NMP** hybrid) has shown moderate activity against *E. coli* and a slight improvement in the activity was observed against *S. aureus*.

2.2.2. Microdilution method

Minimum inhibitory concentration (MIC) values (µg/ml) of the active substances against bacteria and fungi were shown in Table 2. Compounds were tested between 2048 µg/ml - 1 µg/ml concentration in these experiments. According to the results of the microdilution method, it was determined that the MIC of the active compounds against *E. coli*, one of the Gram-negative bacterial strains, was found to be 1024 µg/ml and higher. As inhibition of growth was not observed in the disk diffusion test against Gram-negative strain *P. aeruginosa*, it was determined that MIC values of active compounds (**H1**, **H4** and **H5**) were determined as 512 µg/ml and higher according to the results of microdilution method. All active compounds were found to have

inhibitory effects against *S. aureus* at the studied concentrations. **H3** was found to be the most effective compound against *S. aureus* (MIC: 64 µg/ml). It was observed MIC values of the active compounds were 32 µg/ml and higher against *E. faecalis*. Active compounds were found to be ineffective against *C. albicans* and *C. parapsilosis* in the studied concentrations. DMSO was found to be ineffective against bacteria and fungi in the studied concentrations. Quality control ranges of gentamicin were in the limit values according to the EUCAST criteria.

The results of the microdilution study are in accordance with the disk diffusion study; hybridization with **NMP** has generally increased the activity of the compounds.

Unlike other compounds, hybridization of **TP2** decreased the *P. aeruginosa* and *E. coli* inhibitory activity. Compound **H5**'s inhibitory activity on *E. faecalis* was increased 16-fold and **H3**, **H4**, **H6** inhibitory activity on *E. faecalis* was increased 8-fold by hybridization.

Table 1. Mean inhibition zone diameter and standard deviation values (mm) of the compounds against bacteria and fungi.

Compounds	Strains					
	E. coli	*P. aeruginosa*	*S. aureus*	*E. faecalis*	*C. albicans*	*C. parapsilosis*
TP1	8.67±0.58	-	7.00±0.00	8.66±0.00	-	-
TP2	-	-	-	9.00±0.00	-	-
TP3	-	-	-	8.00±0.00	-	-
TP4	-	-	-	8.00±0.00	-	-
TP5	-	-	7.67±0.58	9.00±0.00	-	-
TP6	-	-	-	9.00±0.00	-	-
H1	9.00±0.00	-	7.67±0.58	9.00±0.00	-	-
H2	-	-	-	9.00±0.00	-	-
H3	-	-	-	8.66±0.00	-	-
H4	-	-	-	9.00±0.00	-	-
H5	7.67±0.58	-	8.00±0.00	9.00±0.00	-	-
H6	-	-	-	9.00±0.00	-	-
GEN	24.00±0.00	20.00±0.00	25.00±0.00	25.00±0.00	nt	nt
FLU	nt	nt	nt	nt	26.00±0.00	27.00±0.00

(-): no inhibition zone, GEN: Gentamicin, FLU: Fluconazole, nt: not tested.

Table 2. MIC values (µg/ml) of the compounds against bacteria and fungi.

Compounds	Strains					
	E. coli	*P. aeruginosa*	*S. aureus*	*E. faecalis*	*C. albicans*	*C. parapsilosis*
TP1	1024	1024	1024	128	>2048	>2048
TP2	1024	1024	1024	128	>2048	>2048
TP3	1024	512	256	256	>2048	>2048
TP4	2048	1024	512	256	>2048	>2048
TP5	2048	2048	512	512	>2048	>2048
TP6	1024	1024	512	256	>2048	>2048
H1	1024	512	512	64	>2048	>2048
H2	2048	2048	512	64	>2048	>2048
H3	1024	1024	64	32	>2048	>2048
H4	1024	512	512	32	>2048	>2048
H5	1024	512	128	32	>2048	>2048
H6	1024	1024	256	32	>2048	>2048
GEN	0.25	0.5	0.5	4	nt	nt
FLU	nt	nt	nt	nt	1	1

GEN: Gentamicin, FLU: Fluconazole, (nt): not tested

2.2.3. Anti-biofilm activity experiments

The examination on antibiofilm effects the compounds were carried out with Gram-negative strains in the presence of the compounds at different concentrations (10 µg/ml, 100 µg/ml and 1000 µg/ml). Inhibition and induction of biofilm production levels of *P. aeruginosa* and *E. coli* in the presence of the compounds were demonstrated in Table 3. To evaluate the effect of used hybridization strategy on the antibiofilm activity **TP1**, **TP4** and **TP5** were studied with their hybrids. **H1** (10 µg/ml, 100 µg/ml), **TP1** (100 µg/ml), **H4** (10 µg/ml, 100 µg/ml), **TP4** (10 µg/ml, 100 µg/ml), **H5** (10 µg/ml) and **TP5** (10 µg/ml, 100 µg/ml) inhibited the biofilm production levels of *P. aeruginosa* and *E. coli* strains.

According to the antibiofilm activity test results, hybridization didn't have any effect on the biofilm production. All tested compounds, except **TP1** on *P. aeruginosa*, inhibited biofilm production of *E. coli* and *P. aeruginosa* at 10 µg/ml dose.

Table 3. Effects of the compounds on biofilm production of *P. aeruginosa* and *E. coli*.

Compounds		Strains		Compounds		Strains	
		E. coli	***P. aeruginosa***			***E. coli***	***P. aeruginosa***
TP1	**10 µg/ml**	↓*	↑	**H1**	**10 µg/ml**	↓*	↓
	100 µg/ml	↓*	↓		**100 µg/ml**	↓*	↓
	1000 µg/ml	↓*	↑		**1000 µg/ml**	-	↑*
TP4	**10 µg/ml**	↓*	↓	**H4**	**10 µg/ml**	↓*	↓
	100 µg/ml	↓*	↓*		**100 µg/ml**	↓*	↓
	1000 µg/ml	↑*	↑		**1000 µg/ml**	-	↑*
TP5	**10 µg/ml**	↓*	↓*	**H5**	**10 µg/ml**	↓*	↓*
	100 µg/ml	↓*	↓		**100 µg/ml**	↑*	↓
	1000 µg/ml	-	↓		**1000 µg/ml**	-	↓

(-): no effect (↓): inhibition of biofilm production (↑): induction of biofilm production (*): statistically significant

2.2.4. Ethidium bromide (EtBr) accumulation assay

Due to the fact that, NMP has efflux pump inhibitory properties in Gram-negative bacteria, newly synthesized hybrids' effect on efflux pump has been tested. In order to clarify the possible mechanisms of action of the selected compounds that were the most potent compounds in microdilutation assay against *E. coli* and *P. aureginosa*, EtBr accumulation assay was carried out. Selected compounds (**H1**, **H4** and **H5**) efficacy at different concentrations (400 µg/ml, 200 µg/ml and 100 µg/ml) on EtBr accumulation by *E. coli* and *P. aeruginosa* was tested and the data were presented in figures (Figure S52, Figure S53) Compound **H5'** on EtBr accumulation by *E. coli* presented in Figure 4.

According to the EtBr assay, it was observed that the presence of **H4** and **H5** compounds at 400 µg/ml concentrations did not change the relative accumulation of EtBr by *E. coli* between 0-30 min incubation. These results showed that efflux pumps of *E. coli* were inhibited by the compounds. The relative accumulation values of EtBr in *E. coli* were found to be 93.56%, 93.19% and 97.76% in the presence of **H1** (100 µg/ml), **H4** (200 µg/ml) and **H5** (200 µg/ml), respectively. In *P. aeruginosa*, the relative accumulation values of EtBr were found to be 96.37% and 91.77% in the presence of 400 µg/ml and 200 µg/ml of **H5**, respectively.

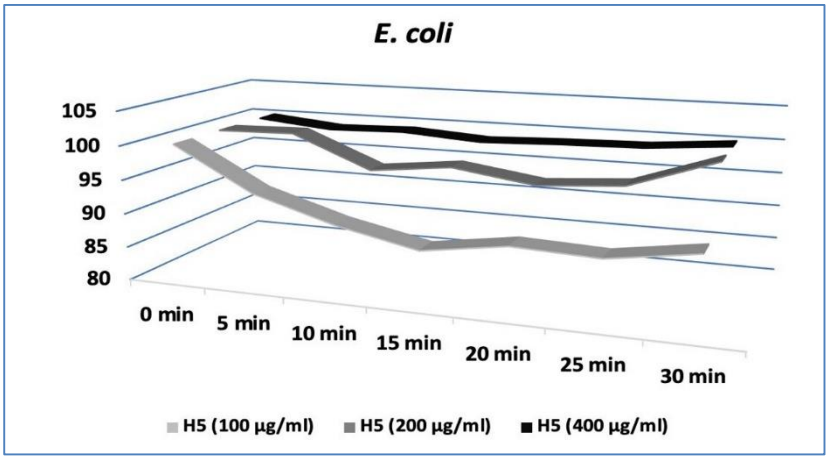

Figure 4. Effects of the compound **H5** on EtBr accumulation by *E. coli*.

3. CONCLUSION

Our results suggest that the compounds **H1-H6** have antimicrobial properties against both Gram-positive and Gram-negative bacteria. Hybridization has generally increased activity in both methods namely, disk diffusion and microdilution test. Moreover, the compounds may have effect on efflux pumps of the bacteria according to the results of EtBr accumulation assay. We also demonstrated that the compounds altered the biofilm production of the strains in a dose-dependent manner. **H5** was found to be the most promising compound in this study.

In conclusion, tested compounds may have potential for the treatment of infections, and further experiments such as molecular studies on the expression of efflux pump and biofilm related genes are necessary to investigate the mechanism of action of the compounds.

4. MATERIALS AND METHODS

4.1. Chemistry

All reagents, solvents and building blocks were obtained from Sigma Chemical Co. (USA), Merck (Germany), Tokyo Chemical Industry C. LTD. (Japan), Alfa Aesar / Acros Organics (Thermo Fisher Scientific) and Carlo Erba Reagents (Italy). All solvents used in this study have high purity grade. Thin layer chromatography (TLC) was carried out on silica gel coated aluminum sheets with F254 indicator at room temparature. Petroleum ether:ethyl acetate (10:2) and methanol:dichloromethane:ethyl acetate:n-hexane (2:2:4:4) mobile phases were used for **TP** series and **H** series, respectively. Microwave (MW) irradiation synthesis of the compounds was conducted on Milestone MicroSYNTH (Milestone S.r.l., Italy). Melting points were determined on Stuart SMP 30 (Staffordshire, ST15 OSA, United Kingdom) and are uncorrected. Infrared spectra were run on a Perkin Elmer Spectrum 100 FT-IR (Perkin Elmer Inc., MA) equipped with a Universal ATR Sampling Accessory and the frequencies were expressed in cm^{-1}. NMR spectra were recorded on Varian AS 400 Mercury Plus NMR (Varian Inc., Palo Alto, CA, USA) spectrometer. Chemical shifts were reported in parts per million (ppm) and the coupling constants (*J*) were expressed in Hertz (Hz) without internal standard. Splitting patterns were appointed as *s, br. s, d, t* and *m* (singlet, broad singlet, dublet, triplet and multiplet; respectively). Thermo MSQ Plus LC/MS (Thermo Scientific Inc., USA) was used for obtaining Mass spectra (ESI-MS). HR-MS were determined on Agilent 6200 Series TOF and 6500 Series Q-TOF LC/MS System with ESI (+) ionization and determined results are within ±0.4 % of calculated values.

Synthesis of 5-substituted-3-amino-1,2,4-triazole

Aminoguanidine bicarbonate (12 mmol) and substituted / non substituted acyl chloride (10 mmol) in 20 ml toluene were stirred for 2h at ice bath and then for 12h-14h at rt. The formed precipitate was filtered off, washed with solvent and dried. The precipitate was dissolved in water, pH was adjusted to 10-11 with Na_2CO_3, then the formed precipitate was filtered off and dried. In the last step, compounds were refluxed in water (MW, 100 W, 3-15 minutes) for cyclization.

Synthesis of 1-[(Naphtalen-1-yl)methyl]piperazine (NMP)

Piperazine (6 mol) was suspended in 75 ml THF and refluxed until piperazine dissolved. Following dissolution, 1 mol of 1-chloromethylnaftyl was added to the reaction flask. The reaction was refluxed (6h), cooled down, filtered off and washed with THF and ethyl acetate. The filtrate basified (pH> 12) with KOH in 5% brine / water mixture and extracted with dichloromethane and ethyl acetate, respectively. The organic phases were combined, dried with Na_2SO_4, concentrated and purified by column chromatography (4:1 ethyl acetate:methanol) to give **NMP**.

Off-white solid; yield, 78 %; m.p., 67 °C, ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 8.23 (d, J = 8.0 Hz, 1H), 7.84 (d, *J* = 8.0 Hz, 1H), 7.80 (t, *J* = 8.0 Hz, 1H), 7.51 (m, 2H), 7.41 (m, 2H), 3.81 (s, 2H), 2.6 (br. s, 4H), 2.4 (br. s, 4H). ESI-MS *m /z* 227.30 $[M+H]^+$ (100)

General synthesis of TP1-TP6

5-Substituted-3-amino-1,2,4-triazole (10 mmol) and ethyl 4-chloroacetoacetate (20 mmol) in 12-18 ml acetic acid (MW, 20 min, 180°C). The formed solid was filtered off, washed and dried to yield **TP1-TP6**. Compounds **TP1-TP6** (3*H*-2-substituted-5-chloromethyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-7-one) used in the next step with no further purification and crude yield of the compounds is between 45-65 %.

3*H*-5-(Chloromethyl)-2-(metylthio)-[1,2,4]triazolo[1,5-*a*]pyrimidine-7-one (TP1)

White solid; yield, 54 %; m.p., 128°C (crys. from MeOH) (reported 226-230°C (crys. from DMF) [25]), ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 6.11 (s, 1H), 4,60 (s, 2H), 2.55 (s, 3H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 164.1, 155.2, 151.7, 149.3, 100.3, 41.3, 13.9 ppm; HRMS *m/z*: 230.0029 (Calcd. For $C_7H_7ClN_4OS$ 230.0016).

3*H*-5-(Chloromethyl)-2-(thiophene-2-yl)-[1,2,4]triazolo[1,5-*a*]pyrimidine-7-one (TP2)

Beige solid; yield, 47 %; m.p. 136 °C; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 7.76 (m, 2H), 7.16 (m, 1H), 6.17 (s, 1H), 4,70 (s, 2H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 158.5, 155.9, 151.7, 149.9, 132.9, 129.7, 128.7, 128.6, 100.5, 41.5 ppm; HRMS *m/z*: 266.0029 (Calcd. for $C_{10}H_7ClN_4OS$ 266.0021).

3*H*-5-(Chloromethyl)-2-(4-chlorophenyl)-[1,2,4]triazolo[1,5-*a*]pyrimidine-7-one (TP3)

Beige solid; yield, 65 %; m.p. 102°C; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 8.08 (m, 2H), 7.57 (m, 2H), 6.18 (s, 1H), 4,68 (s, 2H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 160.8, 156.1, 151.9, 150.1, 135.7, 129.5 (2C), 129.1, 128.9, 100.4, 41.5 ppm; HRMS *m/z*: 294.0075 (Calcd. for $C_{12}H_8Cl_2N$ O 294.0067).

3*H*-5-(Chloromethyl)-2-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-7-one (TP4)

Yellowish solid; yield, 47 %; m.p. 154°C; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 8.09 (m, 2H), 7.53 (m, 3H), 6.18 (s, 1H), 4,67 (s, 2H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 156.4, 156.2, 151.8, 150.1, 130.9, 129.3 (2C), 127.1 (2C), 100.4, 41.7 ppm.

3*H*-5-(Chloromethyl)-2-(4-nitrophenyl)-[1,2,4]triazolo[1,5-*a*]pyrimidine-7-one (TP5)

Yellow solid; yield, 58 %; m.p. 121°C; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 8.30 (m, 4H), 6.21 (s, 1H), 4,69 (s, 2H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 159.8, 156.0, 152.0, 150.1, 148.9, 136.2, 128.2 (2C), 124.6 (2C), 100.3, 41.3 ppm; HRMS *m/z*: 305.0316 (Calcd. for $C_{12}H_8ClN_5O_3$ 305.0309).

3*H*-5-(Chloromethyl)-2-(4-methylphenyl)-[1,2,4]triazolo[1,5-*a*]pyrimidine-7-one (TP6)

Beige solid; yield, 42 %; m.p. 142 °C; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 7.97 (m, 2H), 7.32 (m, 2H), 6.18 (s, 1H), 4.66 (s, 2H), 2.34 (s, 3H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 161.3, 157.1, 156.1, 151.7, 140.4, 140.8, 129.8 (2C), 127.1 (2C), 100.3, 41.7, 21.5 ppm; HRMS *m/z*: 274.0621 (Calcd. for $C_{13}H_{11}ClN_4O$ 274.0612)

General synthesis of H1-H6

TP1-TP6 (1 mmol) and NMP (2 mmol) were stirred in DMF (10-16 ml) in the presence of Cs_2CO_3 (1.5 mmol) using MW irradiation (150 W, 15-30 min, 95°C). The solid (excess of Cs_2CO_3) was filtered off and the filtrate was concentrate and then purified by column chromatography (10:2 chloroform:methanol). The compounds were recrystallized from methanol or acetone.

3*H*-2-(Methylthio)-5-((4-(naphthalen-1-ylmethyl)piperazin-1-yl)methyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-one (H1)

Yellowish crystal; yield, 32 %; m.p. 195°C (from methanol); IR (ATR) ν_{max} 1634, 1532, 1300, 969, 791, 771 cm^{-1}; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 8.23 (d, J = 8.0 Hz, 1H), 7.91-7.83 (m, 2H), 7.55-7.41 (m, 4H), 5.75 (s, 1H), 3.95 (s, 2H), 3.66 (s, 2H), 2.74 (br. s, 4H), 2.63 (br. s, 4H), 2.54 (s, 3H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 163.1, 156.2, 155.4, 152.5, 141.9, 133.9, 132.4, 128.7(2C), 128.5, 128.2, 126.3, 126.2, 125.6, 125.1, 97.9, 59.9, 58.9(2C), 52.3, 51.6, 13.8 ppm; ESI-MS *m /z* 421.58 $[M+H]^+$ (3), 141.26 $[C_{11}H_9]^+$ (100); HRMS *m/z*: 421.1809 (Calcd. for $C_{22}H_{24}N_6OS$ 421.1810).

3*H*-5-((4-(Naphthalen-1-ylmethyl)piperazin-1-yl)methyl)-2-(thiophen-2-yl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-one (H2)

Beige crystal; yield, 32 %; m.p., 204°C (from methanol); IR (ATR) ν_{max} 2803, 1633, 1537, 1509, 1132, 1001, 773 cm^{-1}; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 8.25 (d, *J* = 8.0 Hz, 1H), 7.90-7.79 (m, 2H), 7.62-7.61 (m, 1H), 7.55 – 7.40 (m, 5H), 7.13-7.11 (m, 1H), 5.68 (s, 1H), 3.86 (s, 2H), 3.33 (br. s, 4H), 3.27 (s, 2H), 2.44 (br. s, 4H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 162.8, 158.4, 157.0, 136.7, 133.9 (2C), 132.5, 128.6, 128.1, 128.1 (2C), 127.7, 127.2, 126.2 (3C), 125.6, 125.2, 94.8, 64.2, 64.2, 60.9, 60.9, 53.4 (2C) ppm; ESI-MS *m /z* 457.33 $[M+H]^+$ (75), 141.32 $[C_{11}H_9]$+ (100); HRMS *m/z:* 457.1806 (Calcd. for $C_{25}H_{24}N_6OS$ 457.1810).

3*H*-2-(4-Chlorophenyl)-5-((4-(naphthalen-1-ylmethyl)piperazin-1-yl)methyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-one (H3)

Beige crystal; yield, 38 %; m.p. 213°C (from methanol); IR (ATR) ν_{max} 2808, 1637, 1540, 1517, 1415, 1011, 774 cm^{-1}; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 8.25 (d, *J* = 8.0 Hz, 1H), 8.11-8.07 (m, 2H), 7.89-7.80 (m, 2H), 7.54-7.40 (m, 6H), 5.67 (s, 1H), 3.86 (s, 2H), 3.31 (br. s, 4H), 3.27 (s, 2H), 2.44 (br. s, 4H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 162.3, 159.8, 159.5, 158.6, 134.5, 133.9, 133.8, 132.5, 132.2, 129.0, 128.6, 128.4, 128.1, 127.7, 126.2, 126.1 125.6, 125.2, 94.6, 64.4, 60.9, 53.4 ppm. ESI-MS *m /z* 487.29 $[M+H+2]^+$ (9), 485.29 $[M+H]^+$ (27), 141.30 $[C_{11}H_9]^+$ (100); HRMS *m/z* 485.1850 (Calcd. for $C_{27}H_{25}ClN_6O$ 485.1856).

3*H*-5-((4-(Naphthalen-1-ylmethyl)piperazin-1-yl)methyl)-2-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-one (H4)

Yellowish crystal; yield, 45 %; m.p. 188°C (from methanol); IR (ATR) ν_{max} 2806, 1639, 1543, 1444, 1137, 1001, 788 cm^{-1}; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 8.26 (d, *J* = 8.0 Hz, 1H), 8.11-8.09 (m, 2H), 7.90-7.80 (m, 2H), 7.54 – 7.35 (m, 7H), 5.68 (s, 1H), 3.86 (s, 2H), 3.34 (br. s, 4H), 3.28 (s, 2H), 2.44 (br. s, 4H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 162.1, 160.4, 159.7, 158.7, 134.5, 133.9 (2C), 133.3, 132.5, 129.2, 128.8 (2C), 128.6, 128.1, 127.7, 126.7 (2C), 126.2, 126.1, 125.6, 125.2, 94.5, 64.4, 60.9 (2C), 53.5 (2C) ppm. ESI-MS *m/z* 451.39 $[M+H]^+$ (30), 141.29 $[C_{11}H_9]^+$ (100); HRMS *m/z* 451.2240 (Calcd. for $C_{27}H_{26}N_6O$ 451.2246).

3*H*-5-((4-(Naphthalen-1-ylmethyl)piperazin-1-yl)methyl)-2-(4-nitrophenyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-one (H5)

Yellow crystal; yield, 42 %; m.p. 235°C (from methanol); IR (ATR) ν_{max} 2809, 1639, 1537, 1515, 1341, 791 cm^{-1}; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 8.34 (s, 4H), 8.26 (d, *J* = 8.0 Hz, 1H), 7.91-7.84 (m, 2H), 7.55 – 7.42 (m, 4H), 5.82 (s, 1H), 3.99 (s, 2H), 3.81 (br. s, 2H), 2.87 (br. s, 4H), 2.72 (br. s, 4H) ppm. ESI-MS *m/z* 496.31 $[M+H]^+$ (24), 141.32 $[C_{11}H_9]^+$ (100); HRMS *m/z* 496.2095 (Calcd. for $C_{27}H_{25}N_7O_3$ 496.2097).

3*H*-5-((4-(Naphthalen-1-ylmethyl)piperazin-1-yl)methyl)-2-(*p*-tolyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-one (H6)

Beige crystal; yield, 44 %; m.p. 199°C (from methanol); IR (ATR) ν_{max} 2807, 1618, 1540, 1508, 1415, 1135, 1001, 773 cm^{-1}; ^{1}H-NMR (DMSO-*d6*, 400 MHz) δ 8.26 (m, 1H), 7.98 (m, 2H), 7.91-7.79 (m, 2H), 7.54 – 7.40 (m, 4H), 7.23 (m, 2H), 5.66 (s, 1H), 3.86 (s, 2H), 3.30 (br. s, 4H), 3.26 (s, 2H), 2.46 (br. s, 4H), 2.32 (s, 3H) ppm; ^{13}C-NMR (DMSO-*d6*, 100 MHz) δ 162.1, 160.6, 159.7, 158.8, 138.6, 134.5, 133.9 (2C), 132.5, 130.6, 129.4 (2C), 128.6,

128.1, 127.7, 126.7, 126.2 (2C), 126.1, 125.6, 125.2, 94.5, 64.4, 60.9 (2C), 53.5, 53.4, 21.4 ppm. ESI-MS *m/z* 465.40 $[M+H]^+$ (25), 141.28 $[C_{11}H_9]^+$ (100); HRMS *m/z* 465.2393 (Calcd. for $C_{28}H_{28}N_6O$ 465.2402).

4.2. Antimicrobial activity

4.2.1. Strains and growth media

American type culture collection (ATCC) strains were used in antimicrobial activity experiments. Gram-negative bacterial strains (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) and Gram-positive bacterial strains (*Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212) were grown on Mueller–Hinton Agar (MHA) (Merck, Germany); and fungal strains (*Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019) were grown on Sabouraud dextrose agar (SDA, Merck, Germany) at 37°C for 24 h before experiments. All microorganisms were stored in brain-heart infusion broth (Merck, Germany) including 10% glycerine at -80°C.

4.2.2. Disk diffusion test

Inhibition zone diameters were determined by disk diffusion test [30]. Bacterial and fungal strains were grown at 37°C for 24 h, and microbial suspensions were prepared using fresh colonies and physiological saline. Microbial suspensions were adjusted to 0.5 McFarland turbidity with a densitometer device (Biosan, Latvia). MHA and SDA plates were inoculated with suspensions using sterile cotton swabs. Plates were air dried and sterile blank disks (6 mm in diameter) (Oxoid, UK) were placed on the surface of the plates. Each solution (10 µl) including compounds (100 µg/disk) was added to the sterile disks, and the plates were incubated at 37°C for 16-20 h. Each sample was tested in triplicate. After incubation period, inhibition zone diameters were measured, and mean inhibition zone diameters ± standard deviation values were reported. Gentamicin (Sigma) and fluconazole (Sigma) were used as reference agents for antimicrobial activity, and quality control ranges were evaluated according to the EUCAST criteria (Breakpoint tables for interpretation of MICs and zone diameters Version 8.0 valid from 2018-01-01; method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts, EUCAST Defin. Doc. E.Def 7.3.1, 2017).

4.2.3. Microdilution method

Minimum inhibitory concentration (MIC) values of the compounds were determined by broth microdilution method in 96-well microplates [30]. Bacterial and fungal strains were grown at 37°C for 24 h. Microbial suspensions were prepared using colonies and physiological saline. Suspensions were adjusted to 0.5 McFarland turbidity and diluted 100-fold. Cation adjusted Mueller-Hinton broth (Merck, Germany) (50 µL) was added to the wells for bacteria and 50 µL RPMI medium (Sigma, Germany), buffered with 3-(N-morpholino) propanesulfonic acid was added to the wells for yeasts. Each compound (50 µL) was added into the first wells and 1/2 serial dilutions were performed. Microbial suspensions (50 µL) were added to the wells and incubated at 37°C for 24 h. Each sample was tested in triplicate and the MIC values were defined as the lowest concentrations that inhibited the growth of bacteria and fungi. Ciprofloxacin (Sigma) and fluconazole was used as reference agents and quality control ranges were evaluated according to the EUCAST.

4.2.4. Anti-biofilm activity experiments

Anti-biofilm effects of the compounds were investigated by spectrophotometric method using crystal violet staining in microplates [31]. Bacterial strains were grown on MHA at 37°C for 24 h. Tryptic soy broth (TSB) (Merck) (160 µL) containing 2.5% glucose was added to the wells. 20 µL of each compound and bacterial suspensions (0.5 McFarland) were added into the wells of 96-well microplates. After the incubation period at 37°C for 24 h, the wells were aspirated and washed three times with 200 µL phosphate buffered saline. Then, microplates were air dried, and bacteria that adhered to the surface of the wells were fixed with 200 µL methanol for 15 min. After fixation process, methanol was removed and the wells were air-dried. 200 µL 0.1% crystal violet solution was added to the wells for 5 min at room temperature. Crystal violet solution was removed the plates were washed with tap water, and the plates were air dried. 200 µL ethanol (95%) was added to the wells for 15 min, and the contents of the wells were transferred to sterile microplates. Spectrophotometric measurements were performed using microplate reader (Varioskan Flash, Thermo-Scientific, USA) at 570 nm. Optical density (OD) values of negative and positive control groups were evaluated. All tests were carried out in triplicate and statistical analyses were performed.

4.2.5. Ethidium bromide (EtBr) accumulation assay

Ethidium bromide accumulation assay was performed to investigate the inhibition of efflux pumps in bacteria by modification of fluorometric method using EtBr [32]. Bacteria were grown on MHA plates at 37°C for 24h. Fresh colonies were taken with sterile swabs and suspended in MHB. Bacterial suspensions were incubated with orbital shaking (80 rpm) (MaxQ 6000, Thermo-Scientific) until the cell concentrations reached to McFarland 5 turbidity. Bacterial cells were centrifuged at 9000 rpm for 5 min and resuspended in 500 µl MHB containing ethidium bromide (10 µg/ml) and active compounds (2-fold dilutions, 100, 200 and 400 µg/ml concentrations). After incubation, bacterial cells were centrifuged and re-suspended in 1 ml MHB. 200 µl suspensions were transferred into the wells of 96-well microplates (black color, F-bottom, Thermo-Scientific), and fluorescence of the samples was monitored for 30 min (5 min of intervals) using microplate reader. The excitation wavelengths - emission wavelengths were 520 - 600 nm, respectively. All tests were carried out in triplicate.

Statistical analyses

Statistical analyses were performed by the GraphPad program using t-test ($p < 0.05$ values were determined as significant).

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Appendix A. Supplementary Material

Supplementary material related to this article can be accessed at https://dx.doi.org/10.29228/jrp.121.

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