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SYNTHESIS, ANTIBACTERIAL, ANTIBIOFILM EVALUATION AND MOLECULAR DOCKING STUDIES OF 3-METHYL-2-PROPYL-2*H*-[1,2,4]TRIAZOLO[4,3*b*] [1,2,4,6]THIATRIAZINE-1,1-DIOXIDE

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ABSTRACT. In the current study, a simple method for the synthesis of 3-methyl-2-propyl-2*H*-[1,2,4]triazolo[4,3*b*][1,2,4,6]thiatriazine-1,1-dioxide (**2**) was carried out. In the presence of pyridine, a reaction between amidine (**1**) and sulfuryl chloride occurs. FTIR spectroscopy, ^{1}H and ^{13}C NMR, mass spectra, and elemental analysis were utilized in order to verify the structure of a novel synthetic molecule. The antibacterial activities of compound (**2**) were tested against eight pathogenic bacteria and the minimum inhibitory concentration as well as minimum bactericidal concentration were determined. Moreover, the possible antibiofilm effect of compound (**2**) was evaluated. Molecular docking was investigated to determine the interaction between compound (**2**) and eight crystal structures of bacterial and yeast proteins associated with virulence activity and antimicrobial resistance. Our results showed that the new 3-methyl-2-propyl-2*H*-[1,2,4]triazolo[4,3*b*][1,2,4,6]thiatriazine-1,1-dioxide (**2**) compound has a moderate antibacterial activity toward the selected pathogenic bacteria. The obtained MICs varied from 32 to 512 µg/mL being the lowest values attributed to *Staphylococcus epidermidis* ATCC 14990 and *Streptococcus mutans* ATCC 25175 (MIC = 32 µg/mL).We noted also that heterocyclic compound (**2**) may inhibit bacterial biofilm formation at concentration depend manner with a lowest value obtained against *S.mutans* ATCC 25175 (BIC_{50} = 490 µg/mL). Molecular docking showed a promising inhibitory activity of compound **(2)** on TetM-mediated tetracycline resistance (3J25) and *Staphylococcus aureus* gyrase (3G7B) with lower binding energy compared to the other target proteins.

KEY WORDS: Synthesis, Thiatriazine-1,1-dioxide, Antibacterial, Antibiofilm, Molecular docking

INTRODUCTION

Six-membered heterocycles, such as those containing the 1,2,4,6-thiatriazine-1,1-dioxide moiety, play a significant role in a variety of metabolic activities[1, 2]. 1,2,4,6-thiatriazine-1,1-dioxide constitute an important and essential part of heterocyclic chemicals due to their wide usage as pharmaceutical agents. Many thiatriazine-1,1-dioxide derivatives have been discovered to have herbicidal [3], fungicidal [4], antitubercular [5] and anti-HIV activities [6]. Recently, molecular docking was applied to study the potential effect of various heterocyclic derivatives [7, 8]. Hagar and collaborators confirmed the inhibitory effect of heterocyclic drugs (Amodiaquine) on SARS-CoV-2 using computational modeling strategies [9]. More recently, Gondru and collaborators demonstrated the antibacterial, anti-candida and antibiofilm potential of synthesized triazole-thiazole hybrids [10]. This study aimed to synthesize a 3-methyl-2-propyl-2*H*[1,2,4] triazolo[4,3*b*][1,2,4,6]thiatriazine-1,1-dioxide (**2**) compound, then determine its chemical properties, antibacterial and antibiofilm activities. Molecular docking was applied to confirm the

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interaction of the new synthesized compound with eight bacterial and yeast target proteins associated with virulence activity and antimicrobial resistance.

RESULTS AND DISCUSSION

*Synthesis of 3-methyl-2-propyl-2H-[1,2,4]triazolo[4,3b][1,2,4,6]thiatriazine-1,1-dioxide (**2**)*

The starting material, *N*-propyl-*N'*-(4*H*-1,2,4-triazol-3-yl)ethanamidine (**1**), prepared according to our previous study [2], was reacted with sulfuryl chlorideunder reflux conditions to furnish the title compound (**2**) (Figure 1). The structural elucidation of the newly synthesized compound was carried out using Fourier transform infrared (FT-IR), 1H-NMR, 13C-NMR, MS, and elemental analysis data. Compound (**2**)'s FT-IR spectra showed absorption bands for SO_2 at 1345 cm^{-1} and C=N at 1632 cm^{-1}. The ^{1}H NMR spectra of **(2)** revealed complete absence of the NH proton signals of amidine and triazole, whereas these signals from CH_3-CH_2-CH_2-N group were present.

^{13}C NMR spectrum revealed the signal of the different carbons and confirmed the formation of 3-methyl-2-propyl-2*H*-[1,2,4]triazolo[4,3*b*][1,2,4,6]thiatriazine-1,1-dioxide (**2**). The elemental analyses and MS spectra of heterocyclic compound (**2**) were found to be in good agreement with its assigned structure.

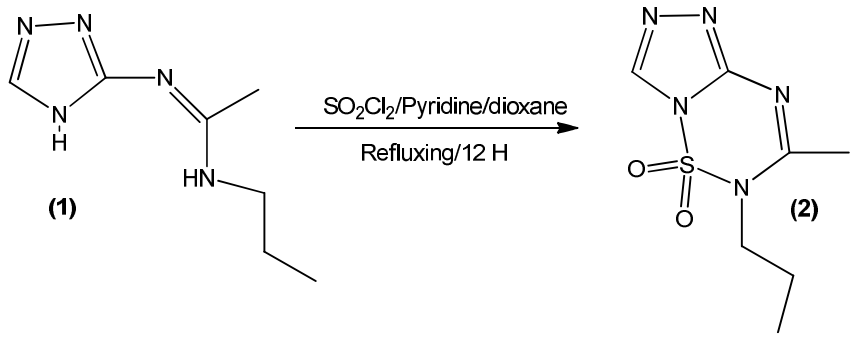

Figure 1. Synthetic route to 3-methyl-2-propyl-2*H*-[1,2,4]triazolo[4,3*b*][1,2,4,6]thiatriazine-1,1-dioxide (**2**).

*Antibacterial activities of the new synthesized compound (**2**)*

The antibacterial [11],antifungal [12] and antiviral [13] activities of 1,2,4-triazoles have been reported in previous researches. More recently, Turukarabettu and collaborators reported the antibacterial activity of S-heterocyclic 1,2,3-triazole derivatives [14].

In this study a moderate antibacterial activity of the new 3-methyl-2-propyl-2*H*-[1,2,4]triazolo[4,3*b*][1,2,4,6]thiatriazine-1,1-dioxide (**2**) against the selected pathogenic bacteria was noticed (Table 1). Compound (**2**) was more active against Gram positive *cocci* compared to Gram positive and Gram-negative bacilli. MICs values vary from 32 µg/mL to 512 µg/mL being the lowest values obtained against *Staphylococcus epidermidis* ATCC 14990 and *Streptococcus mutans* ATCC 25175 (MIC 32 µg/mL). While the MBC were generally started from 128 µg/mL and reach more than 2024 (Table 1). In addition, compound (**2**) may be effective against *Streptococcus salivarius* ATCC 13419 and *Micrococcus luteus* ATCC 10240 (MIC 64 µg/mL). No significant antibacterial activities were observed when compound (**2**) was tested against Gram positive and Gram-negative bacilli (MIC 256 to 512 µg/mL). We also noted that tetracycline was more effective than compound (**2**) (MICs value range from 2 to 64 µg/mL) against all the tested bacterial strains.

Table 1. Antibacterial activity of the synthetized compound (2) against pathogenic bacteria.

	Antibacterial susceptibility			
	[a]TET		New synthetized compound (**2**)	
	[b]MIC (µg/mL)	[c]MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
Streptococcus mutans ATCC 25175	2	4	32	128
Streptococcus oralis ATCC 6249	4	8	512	1024
Streptococcus salivarius ATCC 13419	8	8	64	512
Streptococcus thermophilus ATCC 19258	16	32	128	512
Staphylococcus aureus ATCC 25923	8	32	512	1024
Staphylococcus epidermidis ATCC 14990	8	16	32	128
Micrococcus luteus ATCC 10240	16	32	64	256
Lactobacillus plantarum ATCC 8014	32	64	256	1024
Bacillus subtilis ATCC 6051	64	128	512	1024
Escherichia coli ATCC 25922	32	64	256	2048
Pseudomonas aeruginosa ATCC 27853	64	128	512	>2048

a, Tetracycline; b, Minimum inhibitory concentration; d, Minimum bactericidal concentration.

Biofilm inhibition assay

Biofilm formation of six references strains (Table 2) was determined in 96-well micro titer plates supplemented with compound (**2**) (from 0 to 10240 µg/mL). As showed in Figure 2, the new synthesized compound exhibited a moderate anti-biofilm activity against the selected strains at a concentration dependent manner. The biofilm formation is a considerable virulence factor of bacteria [15]. Biofilm-forming bacteria were more resistant to antimicrobial agents than platonic one[16]. More recently, André and collaborators reported the antibacterial activity of 1,2,3-triazole [17]. In this study, the antibiofilm activity of compound (**2**) against six bacterial strains was tested (Figure 2) and we noticed that BIC_{50} values were greater than that necessary to stop the development of free bacterial cells in suspension (Table 2).

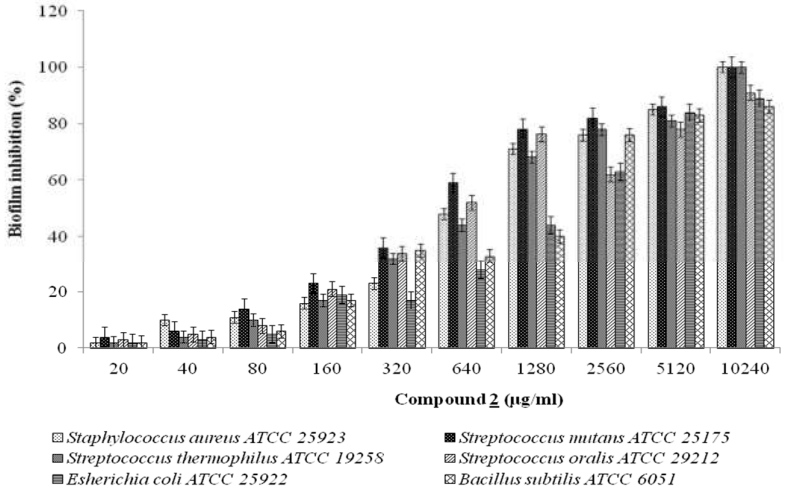

Figure 2. Effect of compound (**2**) on the attachment of the tested bacteria expressed as percentage inhibition evaluated by the XTT assay. The values were the average of three independent determinations. Error bars represent standard deviations.

Table 2. Antibiofilm effect of compound 2against bacterial strains.

Bacterial strains	Inhibition of biofilm (%)	
	[a]BIC_{50} (µg/mL)	[b]BIC_{90} (µg/mL)
Staphylococcus aureus ATCC 25923	680	6800
*Streptococcus mutans*ATCC 25175	490	6670
Streptococcus thermophilus ATCC 19258	770	7700
*Streptococcus oralis*ATCC 29212	600	9850
Escherichia coli ATCC 25922	1620	>10240
Bacillus subtilis ATCC 6051	1580	>10240

[a]Compound **2** minimum biofilm inhibition concentration that showed a 50% biofilm inhibition.
[b]Compound **2** minimum biofilm inhibition concentration that showed a 90% biofilm inhibition.

The lowest BIC_{50} was found with *S. mutans* ATCC 25175 (490 µg/mL). Also, we found that *B. subtilis* ATCC 6051 and *E. coli* ATCC 25922 were less vulnerable to compound (**2**) showing BIC_{50}values of 1580 and 1610 µg/mL, respectively. The highest value of BIC_{90} was observed with *B. subtilis* ATCC 6051 and *E. coli* ATCC 25922 (BIC_{90}>10240 µg/mL).Based on these finding, compound (**2**) may be used as alternatives for inhibiting bacterial biofilms. *Photobacterium ganghwense* subsystem of a set of proteins implemented in biological process.

Table 3. The overall docking scores (S) and root-mean-square deviation (RMSD) of the ligand and the interaction with selected proteins.

Protein receptor (PDB ID)	S (kcal/mol)	RMSD (Å)
TetM-mediated tetracycline resistance (3J25)	-6.4	1.0
Staphylococcus aureus gyrase (3G7B)	-6.0	0.6
Streptococcus mutans TetR/AcrR transcriptional regulator (3MVP)	-5.7	1.0
Escherichia coli penicillin-binding protein (PBP)1b (5HL9)	-5.5	1.7
AcrBMultidrug efflux pump (1T9Y)	-5.3	2.1
Escherichia coli dihydropteroate synthase (1AJ0)	-5.4	2.1
Candida albicans Als3 adhesin from (4LEB)	-5.1	1.3
*Candida albicans s*ecreted aspartic proteinase (2QZW)	-5.7	0.9

Table 4. Hydrogen bonds formed from the interaction of bacterial and yeast target protein and compound (**2**).

PDB ID	Index	Residue	AA	Distance H-A	Distance D-A	Donor angle	Protein donor?	Side chain	Donor atom	Acceptor atom
3J25	1	19A	LEU	3.08	3.56	110.53	√	×	259 [N2+]	10237 [O-]
	2	22A	SER	3.2	3.7	114	√	√	312 [O3]	10235 [O-]
	3	38A	LYS	3.41	3.83	106.93	√	√	540 [N3+]	10235 [O-]
	4	43A	THR	2.95	3.61	126.92	√	√	619 [O3]	10235 [O-]
3G7	1	54B	ASN	2.46	3.37	149.38	√	√	3439 [N2+]	5915 [O-]
B	2	129B	SER	2.97	3.5	115.09	√	√	4239 [O3]	5911 [N2]

Abbreviations: AA = amino acid, H-A = distance between hydrogen and acceptor atoms, and D-A = distance between donor and acceptor atoms.

Molecular docking

Docking of the ligand on TetM-mediated tetracycline resistance (3J25) and *S. aureus* gyrase (3G7B) showed a promising inhibitory activity with better binding energy -6.4, and -6.0 kcal/mol, respectively, compared to other proteins selected in this study (Table 3). We also noted that the binding energies of compound (**2**), *C. albicans Als*3 adhesin (4LEB) and *C. albicans* secreted aspartic proteinase (2QZW) were less than -6.0 kcal/mol (Table 3). As presented in Figure 3, Table 3 and 4, TetM-mediated tetracycline resistance (3J25) residues (LEU19A, SER22A, LYSA,

and THR43A) formed four hydrogen bonds, a hydrophobic interaction with THR40A, an amid-pi stacked bond by THR43, an attractive by LYS38, and a hydrogen bond by THR18. The ligand also showed a good interaction with *S. aureus* gyrase (3G7B) (Figure 4), which is mediated by two hydrogen bonds (formed by ASN54B, and SER129B) and three hydrophobic interactions by GLU58B, ILE86B, and THR173B. The interaction distances, acceptor atoms, donor atoms, and atom donor angles are given in Table 4 and 5.

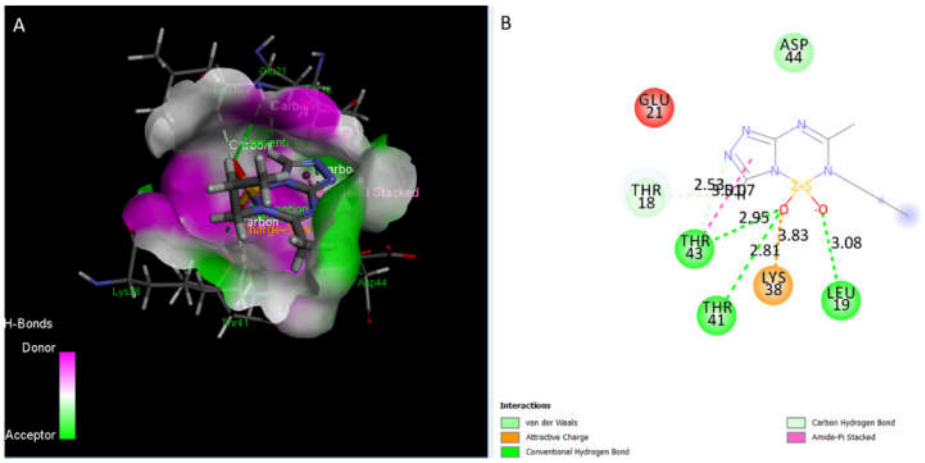

Figure 3. Discovery studio visualization of 3D and 2D of the interaction of TetM-mediated tetracycline resistance (3J25) with compound (**2**).

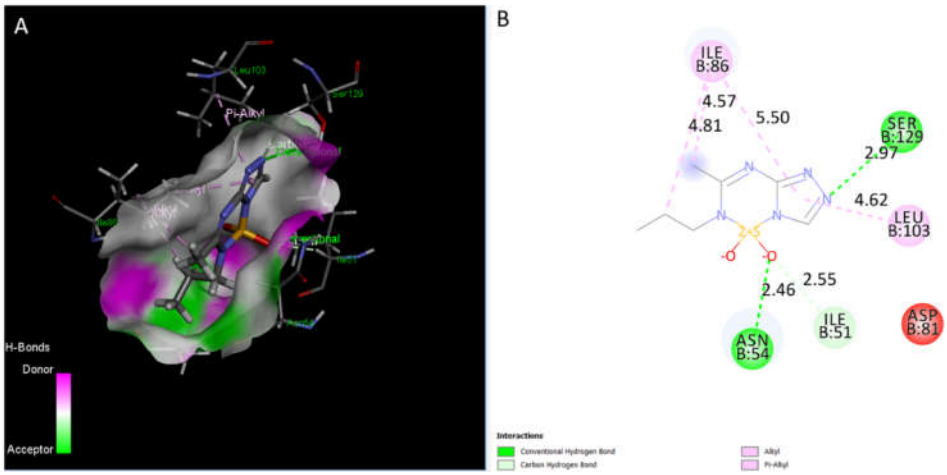

Figure 4. Discovery studio visualization of 3D and 2D of the interaction of Staphylococcus aureus gyrase (3G7B) enzyme and compound (**2**).

Table 5. Hydrophobic Interactions of bacterial and yeast target protein and compound (**2**).

Protein PDB ID	Index	Residue	AA	Distance	Ligand Atom	Protein Atom
TetM-mediated tetracycline resistance (3J25)	1	40A	THR	3.74	10229	566
Staphylococcus aureus gyrase (3G7B)	1	58B	GLU	3.54	5906	3493
	2	86B	ILE	3.88	5903	3923
	3	173B	THR	3.68	5903	4934

EXPERIMENTAL

Instrumentation

An electrothermal 9100 melting point device was used to determine all melting points (mp) (Weiss-Gallenkamp, Loughborough, UK). A Fourier Transform Infrared Spectrometer was used to capture infrared spectra (Nicolet IR200 FT-IR, USA). On a Brüker AC 300 MHz spectrometer (USA), ^{1}H and ^{13}C NMR spectra were obtained in dimethyl sulfoxide-d6 (DMSO-d_6) solutions as the solvents containing TMS. The chemical shifts were expressed as a percentage of TMS (internal reference). A Perkin-Elmer analyzer equipment (series II-CHNS/O, USA) was used to perform the elemental studies. Positive electron spray ionization (ESI) positive MS spectra were collected using a BrükerDaltonics LC-MS spectrometer (USA).

*Synthesis of the triazolothiatriazine-1,1-dioxide (**2**)*

In 40 mL of anhydrous 1,4-dioxane, sulfuryl chloride (670 mg, 5.0 mmol) was added to a mixture in a drop-by-drop technique of amidine (**1**) (835.6 mg, 5.0 mmol) and 10 mmol (0.8 mL) of pyridine. After reflux for 12h and left to cool, the mixture was heated. Under vacuum, the solvent was evaporated then the obtained solid was recrystallized from a mixture of methylene chloride with ethanol (v/v 7:3) to yield 3-methyl-2-propyl-2*H*-[1,2,4]triazolo[4,3*b*][1,2,4,6]thiatriazine-1,1-dioxide (**2**).

*3-Methyl-2-propyl-2H-[1,2,4]triazolo[4,3-b][1,2,4,6]thiatriazine-1,1-dioxide (**2**)*
As beige powder; 48%; mp 184–186°C. ^{1}H NMR (DMSO-d_6)$_{\delta ppm}$: 1.15 (3H, t, J = 8.2, C<u>H</u>$_3$-CH$_2$-CH$_2$-N); 1.75-1.82 (2H, m, CH$_3$-C<u>H</u>$_2$-CH$_2$-N); 2.38 (3H, s, C<u>H</u>$_3$-C(N) = N); 3.40 (2H, t, J = 8.2, CH$_3$-CH$_2$-C<u>H</u>$_2$-N); 8.87 (1H, s, N = C<u>H</u>-N-SO$_2$). ^{13}C (DMSO-d_6)$_{\delta ppm}$: 11.5 (<u>C</u>H$_3$-CH$_2$-CH$_2$-N); 20.5 (CH$_3$-<u>C</u>H$_2$-CH$_2$-N); 23.61 (<u>C</u>H$_3$-C(N) = N); 45.4 (CH$_3$-CH$_2$-<u>C</u>H$_2$-N); 145.2 (N = <u>C</u>H-N-SO$_2$); 157.7 (N = <u>C</u>(N)-N); 163.3 (N = **C**(CH$_3$)-N). IR (FT-RI 200) $_{\nu cm-1}$: 1345 (SO$_2$); 1632 (C = N). ESI-MS: m/z 230 $[M + 1]^+$. Anal. calcd. for $C_7H_{11}N_5O_2S$ (%): C, 36.67; H, 4.84; N, 30.55; S, 13.99. Found, %: C, 36.68; H, 4.85; N, 30.54; S, 14.00.

*Determination of the minimal inhibitory and minimal bactericidal concentrations of the synthetized compound (**2**)*

Using the broth microdilution technique, the minimum inhibitory concentration (MIC) as well as the minimum bactericidal (MBC) concentrations of tetracycline (concentration range from 0 to 512 µg/mL) and the newly synthesized compound (0 to 2048 µg/mL) was evaluated using the broth microdilution method [18].

In total, 11 bacterial strains (seven Gram positive *Cocci*, two Gram positive and two Gram negative *bacilli*) were used in this study (Table 1). The new synthesized compound was twofold serial diluted in Mueller Hinton (MH) Broth in a 96-well plate. After that 10 µL obtained from overnight culture of each tested strain, were added to each well and incubated during 24 h at 37 °C. MIC was considered as the lowest dose of the substance that fully inhibited the observable growth of bacteria.

In order to evaluate MBC values, 10 µL of each well medium was plated on MH plates and kept during 24 h at 37 °C. MBC was considered as the lowest concentration at which 99% of the bacterium cells were destroyed [19].

Effect of triazolothiatriazine-1,1-dioxide (2) on biofilm formation using XTT reduction assay

The newly synthesized compound was examined for its ability to inhibit the production of biofilm by six bacterial strains (Table 2) using XTT reduction assay [20, 21]. A serial dilution of triazolothiatriazine-1,1-dioxide (**2**) from 0 to 10240 µg/mL was added to U bottom 96-well plates supplemented brain heart infusion (BHI) containing 2% glucose (w/v). Then each strain (10 µL, 10^9 cfu/mL) were added to well plates and incubated during 24 h at 37 °C. After that, the adhering cells in biofilms were rinsed three times, and then 100 µL of PBS and 12 µL of sterilized XTT (1 mg/mL)-menadione (1 mM) solution (12.5:1 v/v) were added to each well and kept in the dark at 37 °C during 3 h. In the next step, 100 µL of the solution was moved to a new plate, and the color variation of the solution was determined with Elisa reader (OD 492 nm). Finally, biofilm inhibition (%) was determined according the following equation [(OD growth control-OD sample)/OD growth control] × 100.

The minimum biofilm inhibition concentration BIC_{50} and BIC_{90} were defined as the lowest concentration of compound that demonstrated 50% and 90% of biofilm inhibition, respectively [22].

Molecular docking studies

As shown in Table 3, eight crystal structures of bacterial and fungal proteins associated with virulence activity and antimicrobial resistance were obtained from PDB database [23], they were subjected to water molecules removal, 3D-structure protonation, and energy minimization by Molecular Operating Environment (MOE) suite (demo version 2019; Chemical Computing Group Inc: Montreal, QC, Canada). The compounds with more negative binding energy were further analyzed for interaction with protein residues by Protein-Ligand Interaction Profiler (PLIP) web server [24], and Discovery Studio Visualizer 2020 [25]. The synthetic molecule (Figure 1) was sketched by MOE software and prepared for docking.

CONCLUSION

The new synthesized 3-methyl-2-propyl-2*H*-[1,2,4]triazolo[4,3*b*][1,2,4,6]thiatriazine-1,1-dioxide (**2**) can be seen as alternative chemical product to prevent bacterial growth in suspension and the development of bacterial biofilm formation. Molecular docking supports the inhibitory activity of compound (**2**) on TetM-mediated tetracycline resistance and *S. aureus* gyrase with lower binding energy.

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