Bioorganic Chemistry 88 (2019) 102934

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Synthesis and molecular docking study of some 3,4-dihydrothieno[2,3-*d*] pyrimidine derivatives as potential antimicrobial agents

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ARTICLE INFO

Keywords:
Synthesis
Thienopyrimidines
Pyrazole
Antibacterial activity
Antifungal activity
Docking

ABSTRACT

In continuation of our research program aiming at developing new potent antimicrobial agents, new series of substituted 3,4-dihydrothieno[2,3-*d*]pyrimidines was synthesized. The newly synthesized compounds were preliminary tested for their *in vitro* activity against six bacterial and three fungal strains using the agar diffusion technique. The results revealed that compounds **7**, **8a**, **10b**, **10d** and **11b** exhibited half the potency of levofloxacine against the Gram-negative bacterium, *Pseudomonas aeruginosa*, while compounds **5a**, **8b**, **10c** and **12** displayed half the potency of levofloxacine against *Proteus Vulgaris*. Whereas, compounds **7**, **10b**, **10d** and **11b** showed half the activity of ampicillin against the Gram-positive bacterium, *B. subtilis*. Most of the compounds showed high antifungal potency. Compounds **3**, **6**, **7**, **9b**, **10a**, **11a**, **11b**, **15** and **16** exhibited double the potency of clotrimazole against *A. fumigatus*. While compounds **3**, **4**, **5a**, **5b**, **9b**, **10a**, **10b**, **10c**, **13**, **15**, **16** and **18** displayed double the activity of clotrimazole against *R. oryzae*. Molecular docking studies of the active compounds with the active site of the B. anthracis DHPS, showed good scoring for various interactions with the active site of the enzyme compared to the co-crystallized ligand.

1. Introduction

Treatment of infectious diseases remains a worldwide problem because of the increasing multi-drug resistant caused by human pathogenic microbes. Despite of the wide range of chemotherapeutics and antibiotics available for medical use, the emerging resistance to them has created a substantial need for new classes of antimicrobials. An essential approach to overcome the problem of drug resistance is to design new compounds with diverse mechanisms of action to reduce cross resistance with available drugs [1].

Thienopyrimidines are of great importance because of their remarkable biological activities including antimicrobial [2–7], antiviral [8,9] and anticancer [10–12] activities. Among these thienopyrimidine derivatives, compounds **A** and **B** (Fig. 1) showed significant antibacterial and antimycobacterial activity when tested *in-vitro* [13]. Whereas, the thieno[2,3-*d*]pyrimidine derivative **C** (Fig. 1) displayed antibacterial activity close to ampicillin against *B. subtilis* and antifungal activity half that of fluconazole against *C. albicans* [14]. Recently, we reported a new series of thienopyrimidines of which compound **D** (Fig. 1) exhibited significant antimicrobial activity against *p. aeruginosa* [15].

Furthermore, the wide range of antimicrobial activity displayed by heterocyclic rings including pyrazoles [16–20], triazoles [17,21,22] pyrroles [23], indoles [24], isoindoles [25] were well recognized. On the other hand, Schiff's bases are considered to be among the most significant group of compounds in medicinal chemistry due to their structural variety, preparative accessibility and wide biological profile particularly antimicrobial activity [26].

Dihydropteroate synthase enzyme is an important antifolate target of many drugs and, has been studied in considerable details in our previous research projects [27,28]. It was reported that compounds targeting this enzyme should include a hydrophobic and planner moiety with hydrogen-bonding potential at the periphery with 1–3 atoms spacers between both components [29].

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https://doi.org/10.1016/j.bioorg.2019.102934
Received 7 January 2019; Received in revised form 11 March 2019; Accepted 30 March 2019
Available online 16 April 2019
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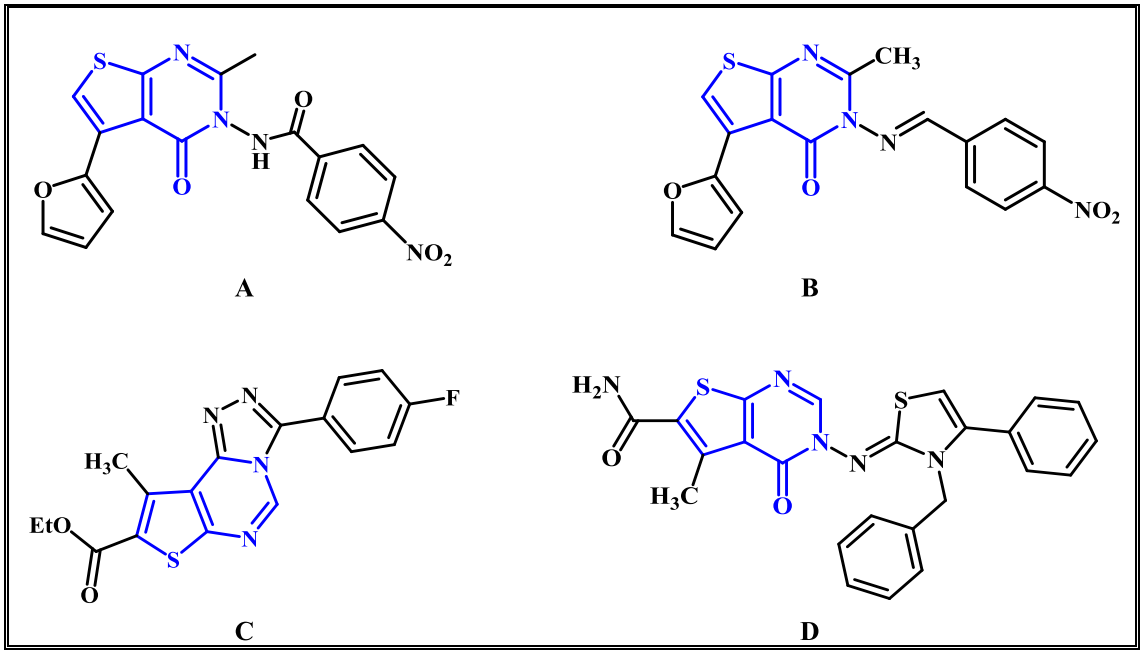


Fig. 1. Thienopyrimidine derivatives having promising antimicrobial activity.

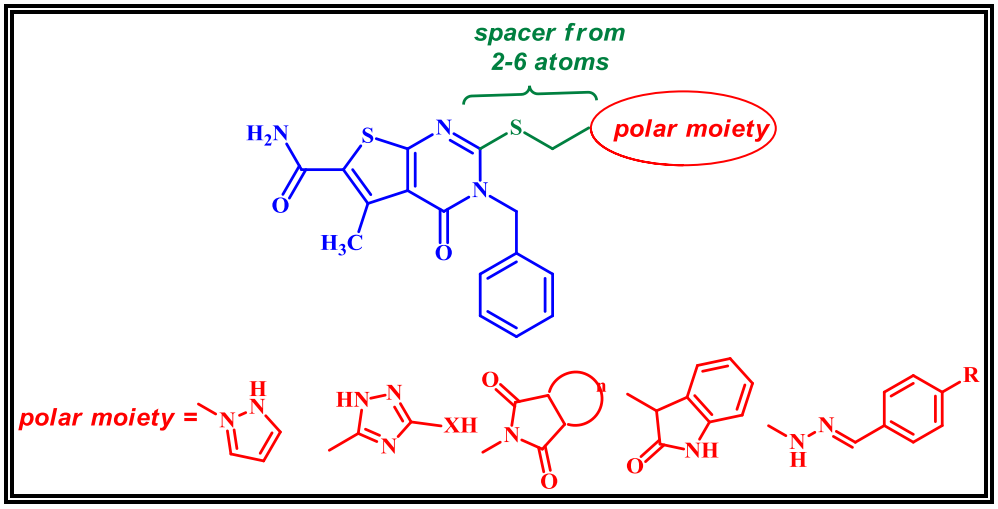


Fig. 2. Rational design of the target antimicrobial thieno[2,3-*d*]pyrimidine-6-Carboxamide derivatives.

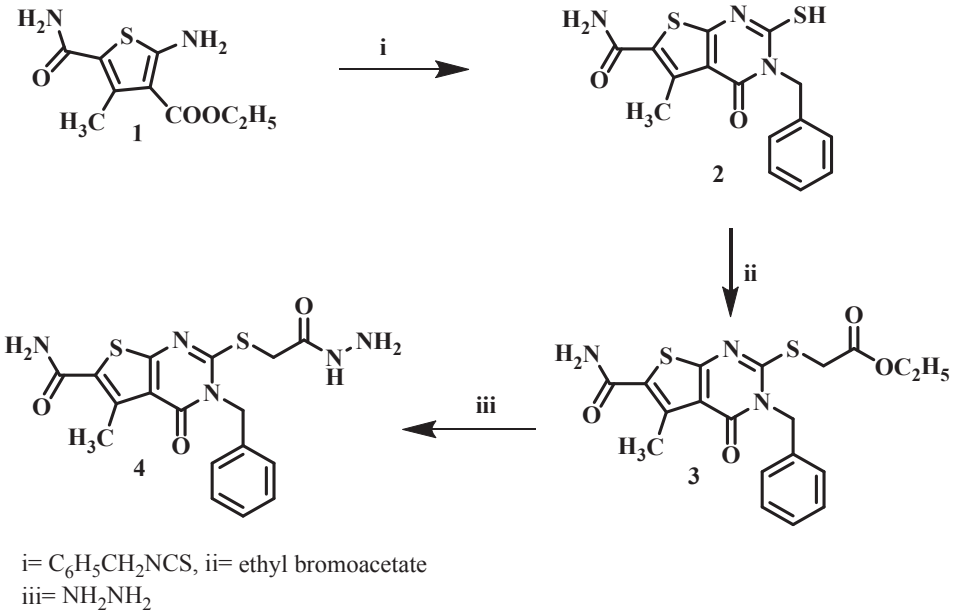
It is well documented that combination of two antimicrobial pharmacophores on the same scaffold is a well-established strategy for the synthesis of more potent drugs [30]. Motivated by these facts and as a continuation of our research program directed towards the discovery of novel antimicrobial agents, we report herein the synthesis and antimicrobial investigation of new hybrid molecules incorporating thienopyrimidine-6-carboxamide attached to various substituted heterocyclic and aryl moieties at position 2 through different atoms spacers (Fig. 2). The atom spacer ranges from 2 to 6 atoms in order to investigate their impact on the anticipated antimicrobial activity. Docking studies of the most active compounds into the active sites of the target enzyme DHPS have been carried out in order to gain an insight into the interaction and binding profiles of these compounds with the receptor binding sites.

2. Results and discussion

2.1. Chemistry

The synthetic procedures adopted to obtain the target compounds are illustrated in Schemes 1–3. In Scheme 1 the key intermediate 3-benzyl-2-mercapto-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide **2** was prepared according to a previously reported procedure [31]. Refluxing **2** with ethyl bromoacetate in the presence of anhydrous potassium carbonate in dry acetone yielded the corresponding ethyl acetate ester **3**. IR spectrum for compound **3** showed absorption band at 1734 cm^{-1} due to C=O of the ester moiety. Its ^1H NMR showed a triplet at 1.82 and a quartet at 4.12 ppm assigned for the ethyl ester group. Refluxing **3** with hydrazine hydrate 99% in methanol afforded the corresponding acid hydrazide **4.** ^1H NMR spectrum for compound **4** lacked the triplet and quartet signals for the ethyl ester protons and showed two D_2O exchangeable singlets assigned for the hydrazide NH_2 and NH protons at 4.34 and 9.34 ppm respectively.

Scheme 2 represents cyclocondensation of the acid hydrazide **4** with methyl 2,4-dioxo-4-(4-substituted phenyl) butyrate in ethanol containing drops of acetic acid to afford the pyrazole derivatives **5a** and **5b**. Their ^1H NMR spectra lacked the upfield D_2O exchangeable singlets assigned for the two NH protons of the hydrazide moiety and showed a singlet at 3.83 ppm assigned for $COOCH_3$ group, besides a singlet at



Scheme 1. Synthesis of compounds **1–4**.

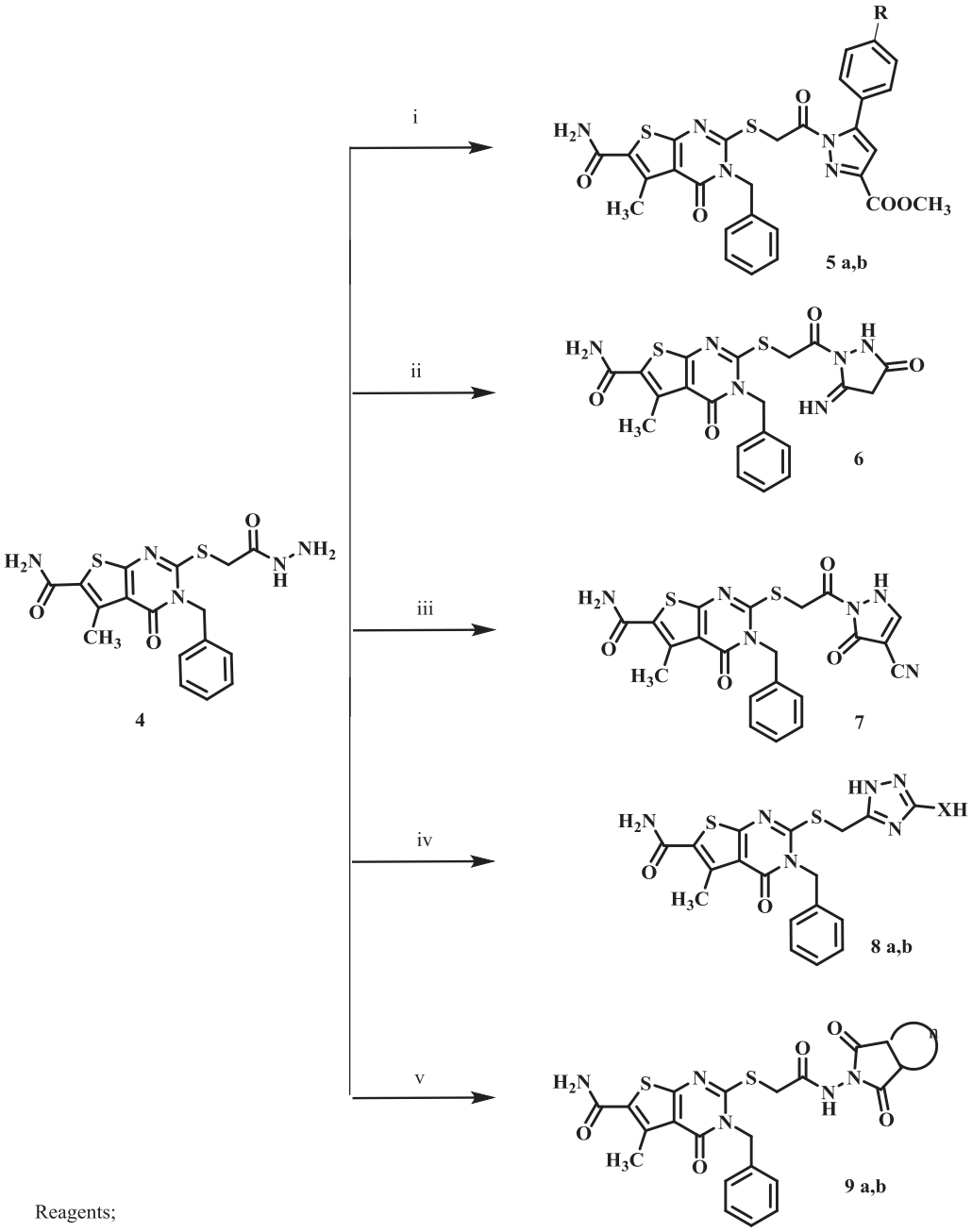
7.12 ppm attributed for pyrazolyl-C_4-H. In addition, compound **5b** showed a singlet at 1.91 ppm assigned for CH_3 protons of p-tolyl moiety. Refluxing acid hydrazide **4** with ethyl cyanoacetate or ethyl ethoxymethylene cyanoacetate in acetic acid gave the pyrazole derivatives **6** and **7** respectively. ^{1}H NMR spectrum of **6** showed two D_2O exchangeable singlets at 9.91 and 10.40 ppm of =NH and pyrazolidine-NH, respectively and a singlet at 1.85 ppm corresponding to C_4- pyrazolidinyl protons. IR spectrum of **7** showed absorption band at 2355 cm^{-1} due to CN group and its ^{1}H NMR spectrum showed a D_2O exchangeable singlet at 10.36 ppm assigned for NH and a singlet at 8.00 ppm integrated for pyrazolyl-C_5-H. Fusion of acid hydrazide **4** with urea or thiourea produces the corresponding triazole derivatives **8a** and **8b**. ^{1}H NMR spectra of **8a** and **8b** showed two D_2O exchangeable singlets at 9.78 and 9.56 ppm assigned for triazolo NH, besides two D_2O exchangeable singlets at 11 and 10.36 ppm assigned for OH and SH at position 3 of the triazole moiety. Refluxing acid hydrazide **4** with succinic anhydride or phthalic anhydride in acetic acid resulted in the formation of 2,5-dioxopyrrolidin-1-ylamino or 1,3-dioxoisoindolin-2-ylamino derivatives **9a** and **9b.** ^{1}H NMR spectrum of **9a** showed a multiplet at 2.36–2.38 ppm due to pyrrolidine $C_{3,4}$ methylene groups. ^{1}H NMR spectrum of **9b** revealed a multiplet at 7.93–7.95 ppm integrated for isoindole-$C_{4,5,6,7}$-H.

Scheme 3 illustrates the condensation of the acid hydrazide **4** with appropriate aryl aldehydes and pyrazole carbaldehydes in glacial acetic acid to yield the corresponding hydrazones **10a–d** and **11a–c** respectively. ^{1}H NMR spectra of **10a–d** and **11a–c** lacked the upfield deuterium exchangeable singlet assigned for the NH_2 protons of the hydrazide moiety. ^{1}H NMR spectra of **10a–d** showed the =CH group as two singlets at (7.78–8.18) and (7.99–8.27) ppm each integrated for half proton indicating mixture of trans and cis isomers, respectively. The −NH−N= proton was observed as two D_2O exchangeable singlets at (11.50–11.75) and (11.70–11.92) ppm each integrated for half proton. ^{1}H NMR spectra of **11a–c** exhibited two singlets at (8.15–8.17) and (8.29–8.31) ppm each assigned for half proton of =CH indicating mixture of trans and cis isomers respectively, two D_2O exchangeable singlets assigned for −NH−N= proton at (11.40–11.51) and (11.60–11.71) ppm each integrated for half proton, a singlet at 8.95–8.97 ppm attributed to pyrazole C_5-H. Reaction of acid hydrazide **4** with isatin in glacial acetic acid afforded **12**. The −NH−N= proton and isatin NH proton were observed as two D_2O exchangeable singlets at 11.30 and 12.50 ppm respectively. On the other hand, condensation of **4** with diethyl malonate gave the 3-oxopropanoate ester **13**. ^{1}H NMR spectrum of **13** showed triplet and quartet at 1.12 and 4.03 ppm assigned for ethyl ester group. Trial to cyclize **13** to the pyrazolidine-3,5-dione derivative**14** using sodium ethoxide was unsuccessful and resulted in the production of **15** instead. ^{1}H NMR spectrum of **15** lacked the signals due to $\underline{CH_2}COOC_2H_5$, S−$CH_2$ and 2 NH protons and showed a triplet and a quartet at 1.28 and 4.41 ppm due to the ethoxy group. Moreover, refluxing **4** with benzoyl chloride in pyridine gave the corresponding benzoyl hydrazino derivative **16**. ^{1}H NMR spectrum of **16** showed a D_2O exchangeable singlet at 10.42 ppm assigned for two NH protons. Attempt to cyclize **16** to pyrazolone derivative **17** in alkaline medium following the procedure reported by Abd Alla et al. [32] was unsuccessful and resulted in formation of compound **18.** ^{1}H NMR spectrum of **18** lacked the upfield D_2O exchangeable singlet assigned for the two NH protons of the hydrazine moiety and the singlet at 4.15 ppm assigned for S−CH_2 protons and showed D_2O exchangeable singlet at 12.44 ppm assigned for OH proton (details in experimental section).

2.2. Biological evaluation

2.2.1. Antimicrobial screening

All the newly synthesized compounds were evaluated for their *in-vitro* antibacterial activity against *Staphylococcus aureus* (RCMB 0100183), *Bacillus subtilis* (RCMB 0100162) and *Staphylococcus epidermidis* (RCMB 0100183) as examples of Gram-positive bacteria in addition to *Pseudomonas aeruginosa* (RCMB 0100243), *Proteus vulgaris* (RCMB 010085) and *Escherichia coli* (RCMB 010052) as examples of Gram-negative bacteria. They were also investigated for their *in-vitro* antifungal potential against *Aspergillus fumigatus, Candida albicans* and *Rhizopus oryzae*. Measurement of their inhibition zones (IZ) using the cup-diffusion technique while twofold serial dilution method was carried out to determine their minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) [33]. Ampicillin and levofloxacin was utilized as reference standard antibacterials whereas clotrimazole was used as antifungal reference. Dimethylsulfoxide (DMSO) was used as blank and showed no antimicrobial activity. Figs. 3–5 represent the inhibition zone diameters, which are attributed to the examined original concentration (5 mg/mL) as a preliminary test. In addition, IZs (nm), MICs (μg/mL), MBCs and MFCs are shown in Tables 1–3.

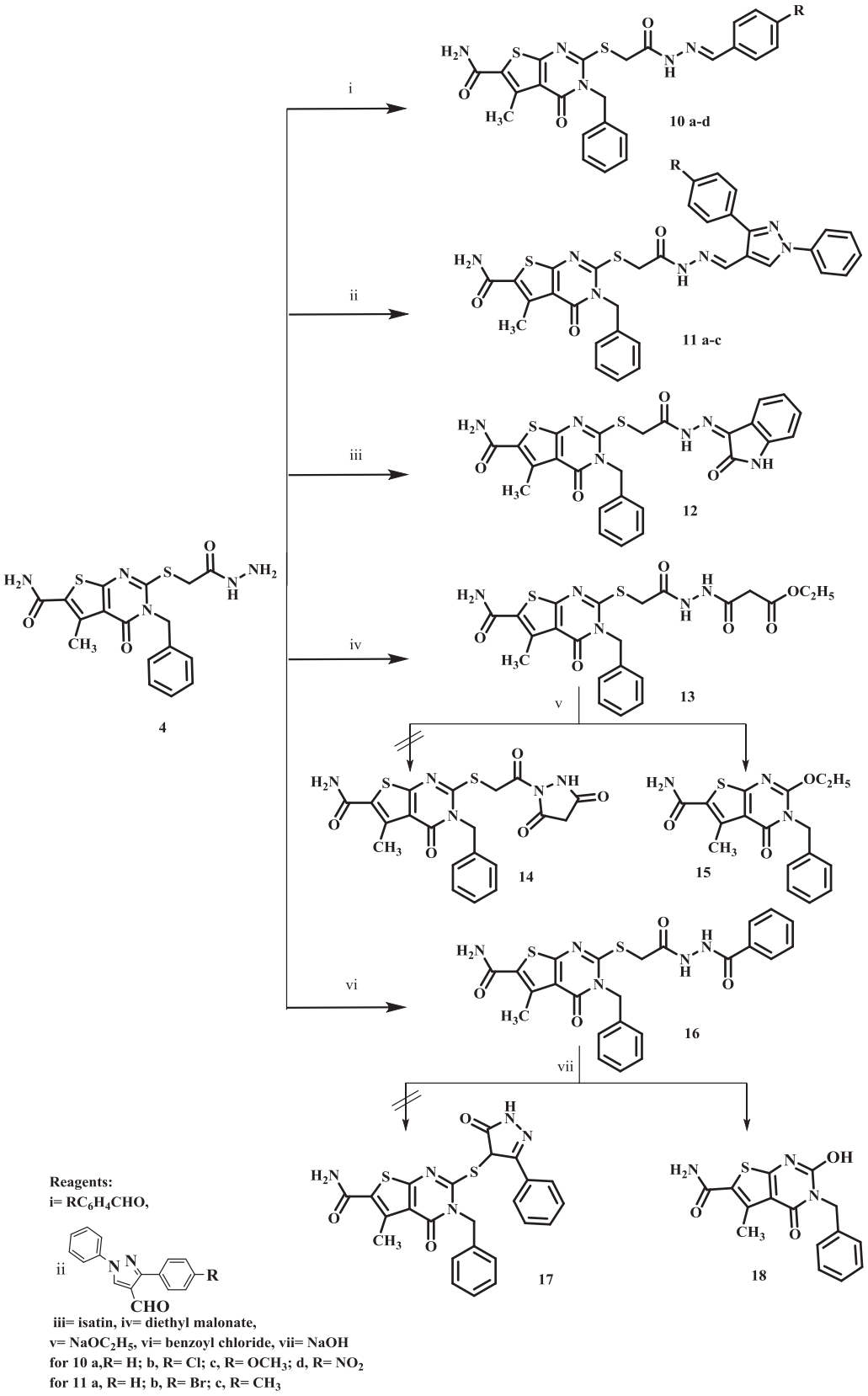


Reagents;
i= $RC_6H_4COCH_2COCOOCH_3$, ii= ethyl cyanoacetate, iii= ethoxymethylene cyanoacetate
iv= urea or thiourea, v= succinic or phthalic anhydride
For 5, a, R= H; b, R= CH_3
For 8, a, X= O; b, X= S
For 9, a, n= 0; b, n= C_4H_4

Scheme 2. Synthesis of compounds **5–9**.

Results in Table 2 revealed that, compounds **4**, **5a** and **11a** showed moderate inhibitory effect (MIC = 25 μg/mL) against *S. aureus* compared to ampicillin (MIC = 6.25 μg/mL). Whereas, compounds **5b**, **9a**, **10d** and **13** exhibited moderate activity (MIC = 50 μg/mL) against *S. epidermidis* compared to the reference (MIC = 12.5 μg/mL). On the other hand, compounds **7, 10b, 10d** and **11b** showed half the potency of ampicillin against *B. subtilis* (MIC = 25 μg/mL vs 12.5 μg/mL). Concerning the activity against Gram-negative bacteria, results revealed that some of the tested compounds exhibited considerable activity against *P. aeruginosa* and *P. vulgaris*. In this context, compounds **7, 8a 10b, 10d** and **11b** exhibited half the potency of levofloxacin against *P. aeruginosa* (MIC, 25 μg/mL vs 12.5 μg/mL), whereas, **5a**, **8b**, **10c** and **12** showed half the activity of levofloxacin against *P. vulgaris* with MIC = 25 μg/mL and 12.5 μg/mL respectively. However, only compounds **4** and **5a** displayed mild activity (MIC = 25 μg/ml) against E. coli compared to Levofloxacin (MIC = 6.25 μg/ml).

Referring to the MIC and MBC limits imitative from the latest National Committee on Clinical Laboratory Standards (NCCLS), the test compounds are classified whether bactericidal or bacteriostatic to the test organism. If the MBC = MIC, the test compound is considered bactericidal while if MBC > MIC the test compound is considered bacteriostatic. Consequently, compounds **4, 5a, 6** and **10a** were



Scheme 3. Synthesis of compounds **10–18**.

bactericidal against *P. aeruginosa* while other compounds were bacteriostatic (Table 2).

Concerning the antifungal activity, most of the tested compounds exhibited broad spectrum activity. Compounds **4, 6** and **10a** displayed half the activity (MIC = 25 μg/ml) of the reference clotrimazole (MIC = 12.5 μg/mL) against *C. Albicans* Whereas, compounds **3**, **6**, **7**, **9b**, **10a**, **11a**, **11b**, **15** and **16** displayed double the activity of clotrimazole against *A. Fumigatus* with MIC = 50 μg/mL. On the other

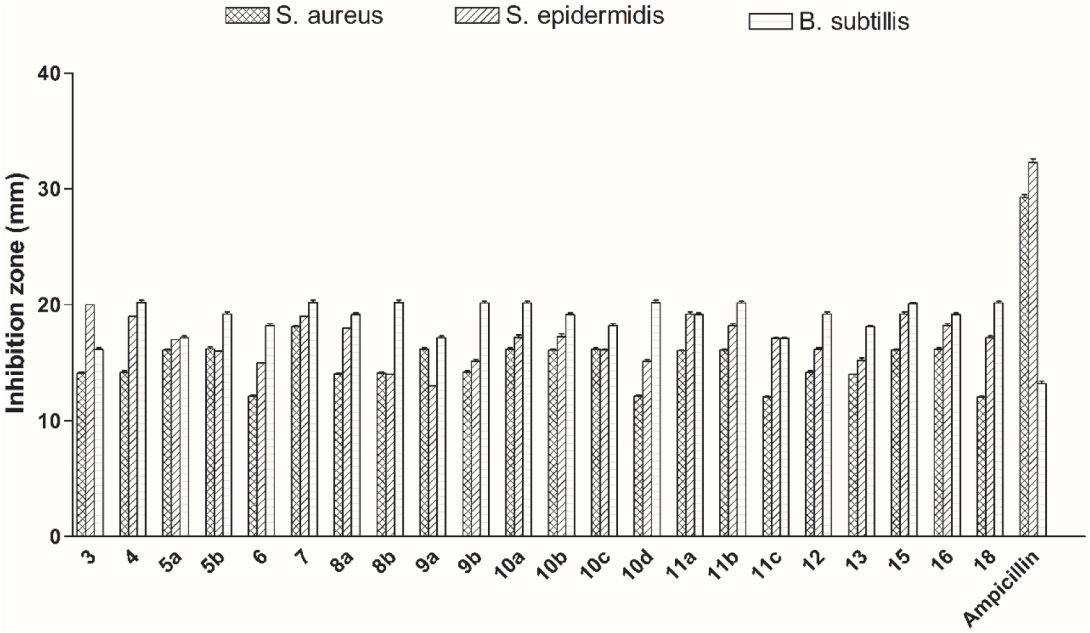


Fig. 3. Prelimenary *in-vitro* antibacterial activity of compounds **3–18** against Gram positive bacteria.

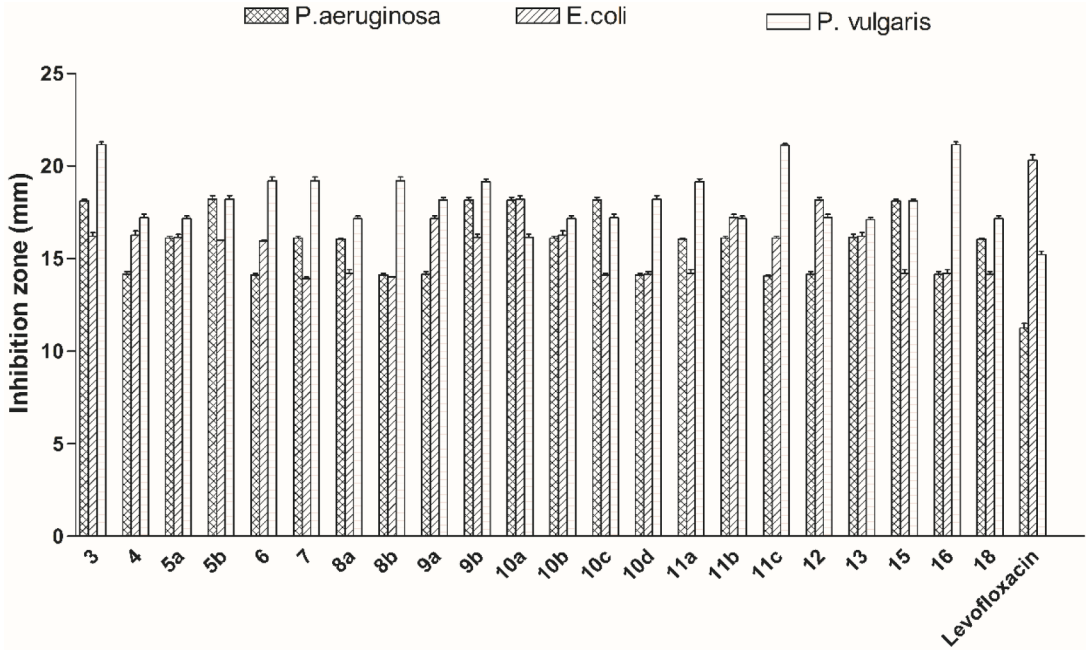


Fig. 4. Prelimenary *in-vitro* antibacterial activity of compounds **3–18** against Gram negative bacteria.

hand, compounds **4**, **5a**, **5b**, **8a**, **8b**, **9a**, **10b**, **10c**, **10d**, **11c**, **12**, **13** and **18** were equipotent to clotrimazole against *A. fumigatus*. Regarding the activity against *R. oryzae* Compounds **3**, **4**, **5a**, **5b**, **9b**, **10a**, **10b**, **10c**, **13**, **15**, **16** and **18** were two times more potent (MIC = 50 μg/mL) than clotrimazole (MIC = 100 μg/mL), while compounds **6**, **7**, **8a**, **8b**, **9a**, **10d**, **11a**, **11b**, **11c** and **12** were equipotent to clotrimazole against *R. oryzae* (*Table 3*).

A deep insight into the structure of the synthesized compounds and their antimicrobial activity revealed that the pyrazole-3-carboxylate derivatives **5a, 5b** and 5-imino-3-oxopyrazolidine **6** showed insignificant activity against *P. aeruginosa.* However, introduction of 4-cyano-5-oxopyrazoline in compound 7 increased the activity to be half that of levofloxacin. On the other hand, the pyrazole carboxylate **5a** showed half the potency of levofloxacin against *P. vulgaris.* Replacement of the pyrazole carboxylate moiety in **5a** with 4-cyano-5-oxopyrazoline in compound **7** abolished the activity against *P. vulgaris.* Regarding the triazole derivatives **8a, b** data showed that the 5-hydroxy triazole derivative **8a** with 2 atoms spacer between the triazole moiety and the thienopyrimidine ring displayed half the levofloxacin activity against *P. aeruginosa*, whereas its 5-mercapto analogue **8b** showed half the activity of the reference against *P. vulgaris*. On the other hand, the 1,3-dioxoisoindolin-2-ylamino analogue **9b** exhibited half the activity of levofloxacin against *P. aeruginosa*. Whereas, the 2,5-dioxopyrrolidin-1-ylamino derivative **9a** showed weak antibacterial activity against *P. aeruginosa.* Out of the hydrazones **10a-d,** compounds **10b** (R = Cl) and **10d** (R = NO_2) showed half the levofloxacin activity against *P. aeruginosa,* while the hydrazone **10c** (R = OCH3) displayed half the activity of levofloxacin against P. vulgaris. Among the pyrazole hydrazones

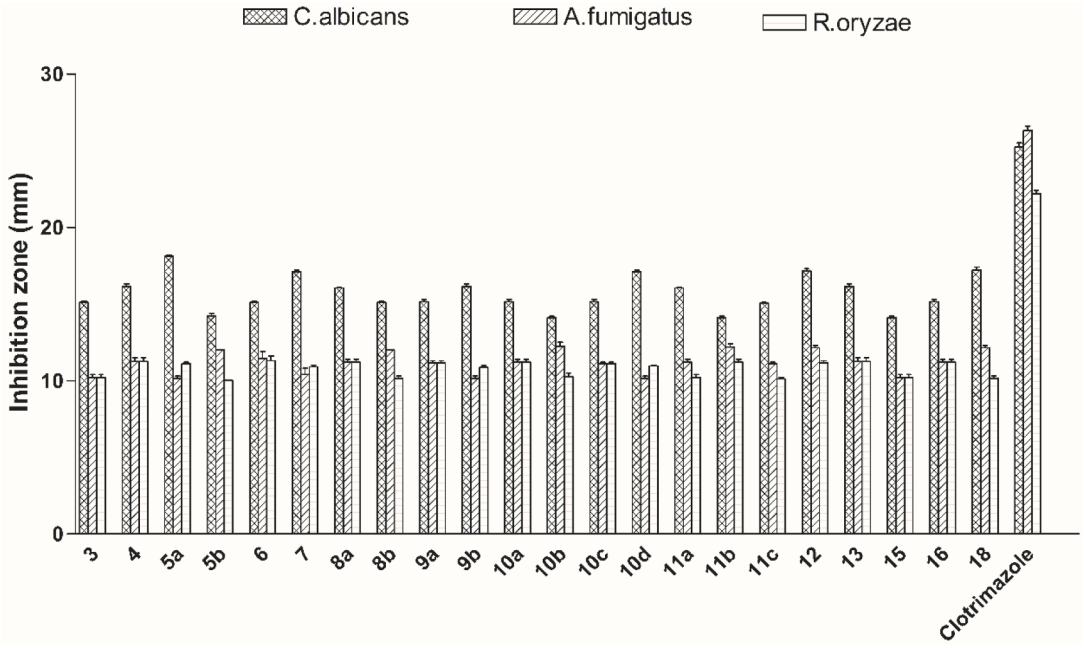


Fig. 5. Prelimenary *in-vitro* antifungal activity of compounds **3–18** against fungi.

Table 1
The inhibition zones (IZ) in mm diameter of the synthesized compounds **3–18** in mm.

Compound Number	Gram positive organisms			Gram negative organisms			Fungi		
	S. aureus	*S. epidermidis*	*B. subtillis*	*P. aeruginosa*	*E. coli*	*P. vulgaris*	*C. albicans*	*A. fumigatus*	*R. oryzae*
3	14	20	16	18	16	21	15	10	10
4	14	19	20	14	16	17	16	11	11
5a	16	17	17	16	16	17	18	10	11
5b	16	16	19	18	16	18	14	12	10
6	12	15	18	14	16	19	15	11	11
7	18	19	20	16	14	19	17	10	11
8a	14	18	19	16	14	17	16	11	11
8b	14	14	20	14	14	19	15	12	10
9a	16	13	17	14	17	18	15	11	11
9b	14	15	20	18	16	19	16	10	11
10a	16	17	20	18	18	16	15	11	11
10b	16	17	19	16	16	17	14	12	10
10c	16	16	18	18	14	17	15	11	11
10d	12	15	20	14	14	18	17	10	11
11a	16	19	19	16	14	19	16	11	10
11b	16	18	20	16	17	17	14	12	11
11c	12	17	17	14	16	21	15	11	10
12	14	16	19	14	18	17	17	12	11
13	14	15	18	16	16	17	16	11	11
15	16	19	20	18	14	18	14	10	10
16	16	18	19	14	14	21	15	11	11
18	12	17	20	16	14	17	17	12	10
Ampicillin	29	32	13	_	_	_	_	_	_
Levofloxacin	_	_	_	11	20	15	_	_	_
Clotrimazol	_	_	_	_	_	_	25	26	22

11a-c, compound **11b** (R = Br) having 6 atoms spacer displayed half the activity of levofloxacin against *P. aeruginosa* Moreover, the indoline hydrazone **12** displayed considerable activity against *P. vulgaris*.

In conclusion, antibacterial screening revealed that most of the tested compounds demonstrated considerable inhibitory effects on the growth of Gram-negative and Gram-positive bacterial strains. Generally, most of the studied thienopyrimidines revealed better activity against the Gram- negative rather than Gram-positive bacterial strains. The 4-cyano-5-oxopyrazoline derivative **7** and the hydrazones **10b**, **10d** and **11b** emerged as the most active compounds in this study against *B. subtilis* and *P. aeruginosa*. However, the pyrazole carboxylate **5a**, the mercapto triazole **8b**, and the hydrazones **10c** and **12** displayed the highest activity among the tested compounds against *P. vulgaris*.

Concerning antifungal activity, compounds (**3**, **6**, **7**, **9b**, **10a**, **11a**, **11b**, **15** and **16)** were two times more potent than clotrimazole against *A. fumigatus*. While, compounds **3**, **4**, **5a**, **5b**, **9b**, **10a**, **10b**, **10c**, **13**, **15**, **16** and **18** were two times more potent than clotrimazole against *R. oryzae*. Whereas, for *C. albicans* the pyrazolo derivative **6** and Schiff's base **10a** showed half the potency of clotrimazole.

2.3. Molecular docking studies

Dihydropteroate synthase (DHPS) is an enzyme of the folate biosynthesis pathway which catalyzes the formation of 7,8-

Table 2
Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the synthesized compounds **3–18** in μg/mL.

Compound Number	Gram positive organisms						Gram negative organisms					
	S. aureus		*S. epidermidis*		*B. subtillis*		*P. aeruginosa*		*E. coli*		*P. vulgaris*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
3	50	50	100	100	50	50	100	100	100	100	100	200
4	25	50	100	150	100	100	50	50	25	50	100	100
5a	25	50	200	200	50	50	50	50	25	50	25	50
5b	50	50	50	100	50	50	50	100	50	100	100	100
6	50	50	100	150	50	50	50	50	50	50	100	100
7	100	100	100	100	25	50	25	50	100	100	100	200
8a	100	100	100	150	50	50	25	50	100	100	100	100
8b	100	200	200	200	100	100	100	100	50	100	25	50
9a	100	200	50	100	100	100	100	100	50	100	100	100
9b	50	100	100	150	100	100	50	100	50	50	100	100
10a	50	50	100	100	50	50	50	50	50	50	100	200
10b	100	100	100	150	25	50	25	50	100	100	100	100
10c	100	200	200	200	100	100	100	100	50	100	25	50
10d	50	50	50	100	25	50	25	50	50	50	100	100
11a	25	50	100	150	50	50	50	100	50	100	100	100
11b	50	50	100	100	25	50	25	50	50	50	100	200
11c	50	100	100	150	100	100	50	100	50	50	100	100
12	100	200	200	200	100	100	100	100	50	100	25	50
13	100	200	50	100	100	100	100	100	50	100	100	100
15	50	50	100	150	50	50	50	100	50	100	100	100
16	100	200	100	100	100	100	100	100	50	100	100	200
18	50	100	100	150	100	100	50	100	50	50	100	100
Ampicillin	6.25	–	12.5	–	12.5	–		–	–	–		_
Levofloxacin		_		_		_	12.5	–	6.25	–	12.5	–

_ Not tested.

Table 3
Antifungal minimal inhibitory concentrations (MIC, μg/mL) and minimal Fungicidal concentrations (MFC, μg/mL) of the synthesized compounds **3–18** in μg/mL.

Compound Number	Fungi					
	C. albicans		*A. fumigatus*		*R. oryzae*	
	MIC	MFC	MIC	MFC	MIC	MFC
3	100	100	50	50	50	50
4	25	50	100	100	50	50
5a	50	50	100	100	50	50
5b	50	50	100	100	50	50
6	25	50	50	50	100	100
7	100	100	50	50	100	100
8a	100	100	100	100	100	100
8b	100	100	100	100	100	100
9a	100	100	100	100	100	100
9b	100	100	50	50	50	50
10a	25	50	50	50	50	50
10b	100	100	100	100	50	50
10c	100	100	100	100	50	50
10d	50	50	100	100	100	100
11a	50	100	50	50	100	100
11b	50	50	50	50	100	100
11c	100	100	100	100	100	100
12	100	100	100	100	100	100
13	100	100	100	100	50	50
15	50	50	50	50	50	50
16	100	100	50	50	50	50
18	100	100	100	100	50	50
Clotrimazol	12.5	_	100	_	100	_

dihydropteroate from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate and *para*-aminobenzoic acid.

DHPS is the long-standing target of the sulfonamide class of antibiotics that compete with pABA. In the wake of sulfa drug resistance, targeting the structurally rigid (and more conserved) pterin site has been proposed as an alternate strategy to inhibit DHPS in wild-type and sulfa drug resistant strains. In BaDHPS, Asp101, Asn120, Asp184, Lys220, and a structural water molecule provide a hydrogen bond donor/acceptor constellation that recognizes the pterin ring. Arg254 at the "base" of the pocket provides a stacking platform for the pterin ring and, together with His265 and Asn27, also provides an anion binding pocket for the β-phosphate of the substrate [34].

Molecular docking studies of our active newly synthesized compounds altogether with (7-Amino-1-methyl-4,5-dioxo-1,4,5,6-tetrahydro-pyrimido(4,5-c)pyridazin-3-yl)-acetic acid into B. anthracis DHPS active site (PDB ID code: 4DAI). (Fig. 6) were performed using Molecular Operating Environment (MOE-Dock 2016.08) software [35].

Validation of docking protocol was performed by re-docking the ligand into active site and it showed similar binding interactions compared to that of co-crystallized ligand with RMSD < 2. All the docked compounds were well anchored inside the active site and showed hydrogen bonds with amino acid residues Lys 220 and Asp 101. In compound **7** the interactions showed hydrogen bonding between N_1 of the pyrazole ring and Asp 101, carbonyl of amide side chain at position 6 and Ser221, carbonyl oxygen of the pyrazole ring with Lys 220. In addition hydrophobic interactions were displayed with the thiophene ring and Lys 220 (Fig. 7). In compound **10b** three hydrogen bonding were observed between imine nitrogen of the hydrazone andAsp101, N_1 of the pyrimidine and Arg 254, Sulfanyl group on position 2 of pyrimidine ring and Lys 220 (Fig. 8). Molecular modeling studies of our target compound **10d** indicated that carbonyl oxygen of carbonyl methylsulfanyl side chain and nitrogen of the 6-carboxamide contribute hydrogen bonding interaction with Lys 220 and Asp 101 respectively (Fig. 9). Compound **11b** showed 2 hydrogen bonding interactions between nitrogen of the 6-carboxamide and Asp 101, N_2 of pyrazole ring and Lys 220, beside hydrophobic interactions with Lys 220 (Fig. 10).

The molecular modeling docking scores, amino acid interactions and the hydrogen bond lengths of all tested compounds were summarized in Table 4.

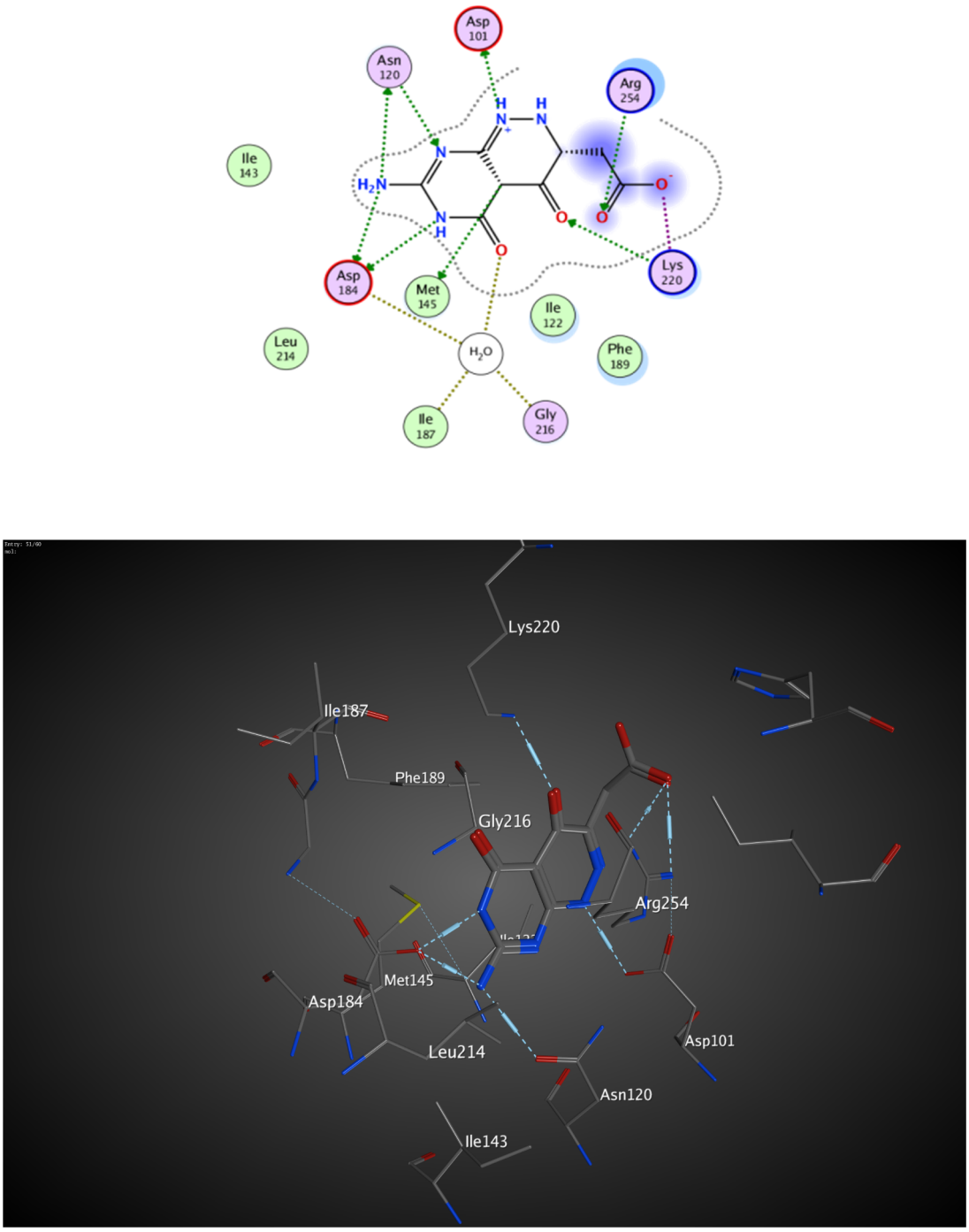
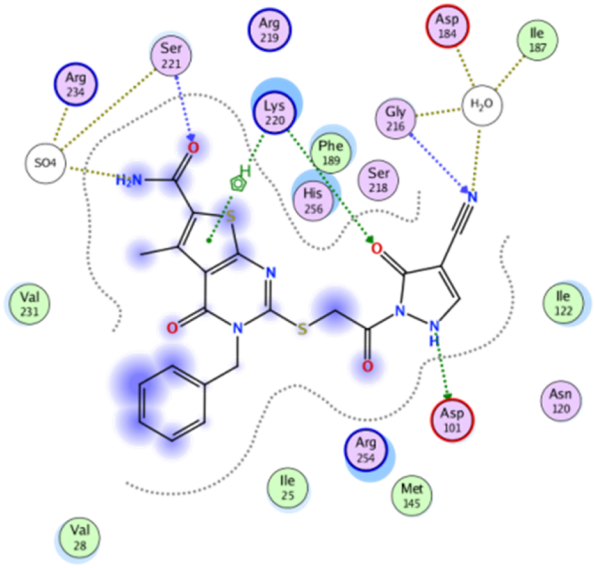


Fig. 6. 2D and 3D View from a molecular modeling study of the minimum-energy structure of the complex of redocked ligand in DHPS (PDB ID: 4DAI). Viewed using Molecular Operating Environment (MOE) module.

3. Conclusion

Inconclusion, the objective of the present study was to design, synthesize and investigate the antimicrobial activities of some new 3,4-dihydrothieno[2,3-*d*] pyrimidine derivatives linked to various bioactive heterocyclic and aryl moieties at position-2 through different atoms spacers. The new compounds were evaluated for their antimicrobial activity to study the effect of such structural modification on the biological activity. The results revealed that compounds **7, 8a 10b, 10d** and **11b** displayed half the potency of levofloxacin against *P. aeruginosa*. In addition, compounds **5a**, **8b**, **10c** and **12** exhibited half the potency of levofloxacin against *P. vulgaris*. Whereas, for *B. subtilis* compounds **7, 10b, 10d** and **11b** showed half the activity of the reference. Additionally, most of the newly synthesized compounds showed significant antifungal activity. Docking study for compounds **7, 10b, 10d** and **11b** that proved to have the highest antibacterial activity showed comparable energy scores and binding interactions to that observed with co-crystallized ligand (7-Amino-1-methyl-4,5-dioxo-1,4,5,6-tetrahydro-pyrimido(4,5-c)pyridazin-3-yl)-acetic acid into B. anthracis DHPS active site (PDB ID code: 4DAI). Based on previously mentioned results, it is apparent that substituted thieno[2,3-*d*]pyrimidines could be considered as possible promising leads that deserve more structural modification to achieve new potent antimicrobials.



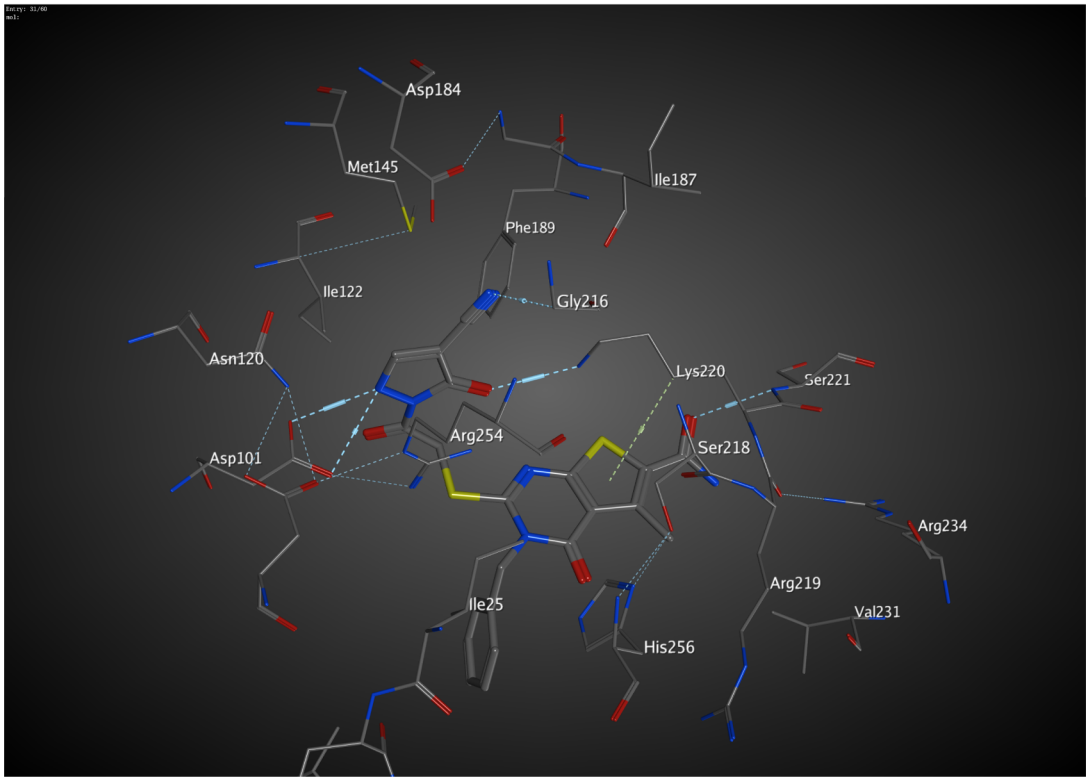


Fig. 7. 2D and 3D View from a molecular modeling study of the minimum-energy structure of the complex of 7 docked in DHPS (PDB ID code: 4DAI). Viewed using Molecular Operating Environment (MOE) module.

4. Material and methods

4.1. Chemistry

Melting points were determined in open-glass capillaries on a Gallen–Kamp melting point apparatus and are uncorrected. The IR spectra (KBr) were recorded on a Perkin-Elmer 1430 spectrophotometer. The ^{1}H NMR spectra were determined on a Brucker Avance spectrometer (300 MHz) at the Central laboratory, Faculty of Science, University of Cairo using tetramethylsilane (TMS) as the internal standard and DMSO-d_6 as the solvent. The chemical shifts are given in ppm δ values (s, singlet; d, doublet; t, triplet and m, multiplet). ^{13}C NMR spectra were determined on Brucker Avance spectrometer (75 MHz) at the Central laboratory, Faculty of Science, University of Cairo using TMS as internal standard. Electron impact mass spectra (EIMS) were run on a gas chromatograph/mass spectrometer Shimadzu GCMS/QP-2010 plus (70 eV) at the micro analytical center, Faculty of Science, University of Cairo. Microanalyses were performed at the regional center for mycology and biotechnology, University of Al-Azhar. The results of the microanalysis were within ± 0.4% of the calculated values. Follow up the reactions and checking the homogeneity of the compounds were made by ascending TLC run on silica gel G (Merck 60) coated glass plates. The spots were visualized, by exposure to iodine vapour or UV-Lamp at λ 254 nm for few seconds.

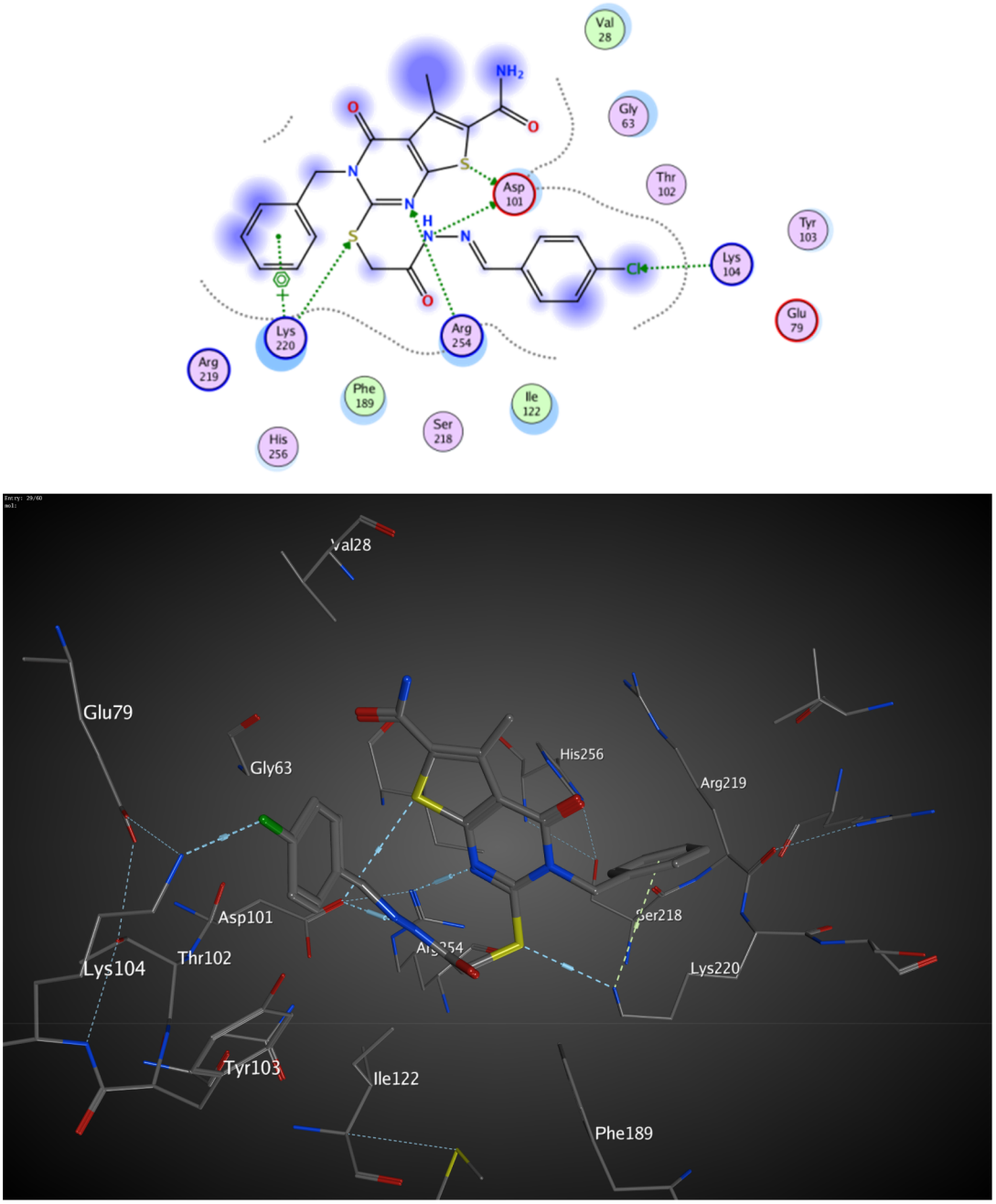


Fig. 8. 2D and 3D View from a molecular modeling study of the minimum-energy structure of the complex of 10b docked in DHPS (PDB ID code: 4DAI). Viewed using Molecular Operating Environment (MOE) module.

The synthetic strategies adopted for the synthesis of the intermediate and final compounds are depicted in Schemes 1–3. The key intermediate **2** was synthesized in our labs as described [31].

4.1.1. Ethyl 2-(3-benzyl-6-carbamoyl-5-methyl-4-oxo-3,4-dihydrothieno [2,3-d] pyrimidin-2-ylthio)acetate ***(3)***

A mixture of **2** (10 mmol, 3.31 g), ethyl bromoacetate (10 mmol, 1.67 g, 1.1 ml) and anhydrous potassium carbonate (15 mmol, 2 g) in dry acetone (10 ml) was heated under reflux for 3–4 h. The reaction mixture was cooled, poured onto crushed ice, the precipitate formed was filtered, washed with water, dried and crystallized from ethanol; yield: 64%, m.p. 206–209 °C. IR (cm^{-1}):3395, 3175 (NH_2); 1734 (C=O ester), 1668 (C=O amide); 1608 (C=N); 1511 (C=C); 1230 (C–S–C); 1154, 1111 (C–O–C).^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 1.82 (t, J = 7.0 Hz, 3H, $COOCH_2\underline{CH_3}$); 2.68 (s, 3H, CH_3); 4.06 (s, 2H, S–CH_2); 4.12 (q, J = 7.0 Hz, 2H, $COO\underline{CH_2}CH_3$); 5.30 (s, 2H, N–CH_2); 7.25–7.36 (m, 5H, phenyl-H); 7.65 (s, 2H, $CONH_2$, D_2O exchangeable). MS (EI) m/z (%): 418 [$M^{+\cdot}$+1] (19.24); 417 [$M^{+\cdot}$] (19.83). Anal. Calcd. for ($C_{19}H_{19}N_3O_4S_2$) (417.50): C, 54.66; H,4.59; N, 10.06. Found: C, 54.82; H,4.74; N,10.13.

4.1.2. 3-Benzyl-2-(2-hydrazinyl-2-oxoethylthio)-5-methyl-4-oxo-3,4-dihydrothieno [2,3-d]pyrimidine-6-carboxamide ***(4)***

A mixture of **3** (1 mmol, 0.42 g), hydrazine hydrate 99% (6 mmol, 0.3 g, 0.3 ml) in 15 ml methanol was heated under reflux for 7 h during which a precipitate was formed, filtered while hot, washed with ethanol: water 1:1, dried and crystallized from DMF/ water; yield: 83%, m.p. 262–265 °C. IR (cm^{-1}): 3475, 3323, 3259 (NH_2, NH); 1676 (C=O); 1591 (C=N); 1515 (C=C); 1236 (C–S–C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm):2.71 (s, 3H, CH_3); 3.94 (s, 2H, S–CH_2); 4.34 (s, 2H,

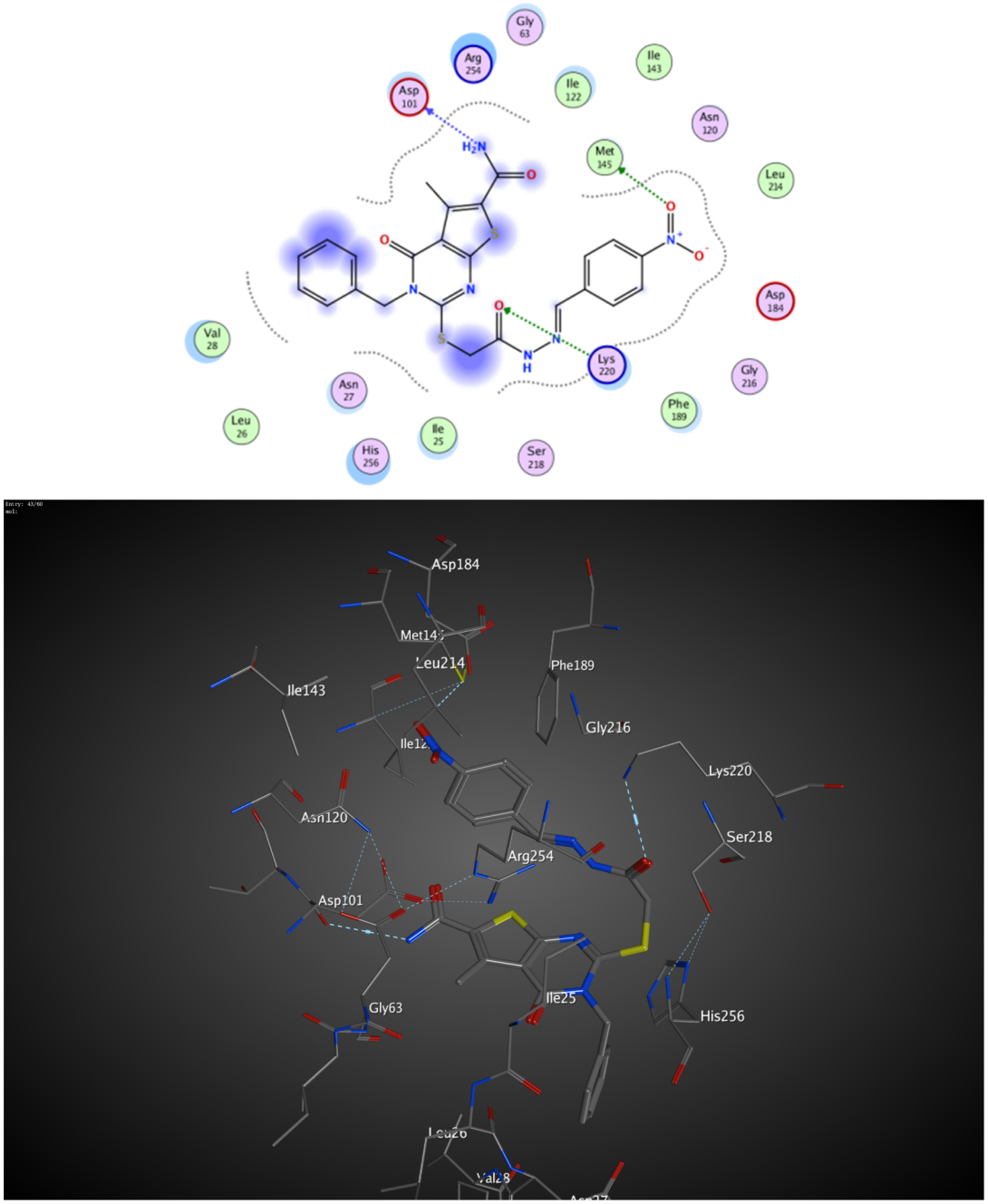


Fig. 9. 2D and 3D View from a molecular modeling study of the minimum-energy structure of the complex of 10d docked in DHPS (PDB ID code: 4DAI). Viewed using Molecular Operating Environment (MOE) module.

NH-NH_2, D_2O exchangeable); 5.32 (s, 2H, N—CH_2); 7.24–7.38 (m, 5H, phenyl-H); 7.60 (s, 2H, $CONH_2$, D_2O exchangeable); 9.34 (s, 1H, NH—NH_2, D_2O exchangeable). MS (EI) *m/z* (%): 404 [$M^{+\cdot}$+1] (54.55); 403 [$M^{+\cdot}$] (69.09). Anal. Calcd. for ($C_{17}H_{17}N_5O_3S_2$) (403.48) C, 50.61; H, 4.25; N, 17.36. Found: C, 50.47; H, 4.32; N, 17.51.

4.1.3. Methyl 1-[2-(3-benzyl-6-carbamoyl-5-methyl-4-oxo-3,4-dihydrothieno [2,3-d]pyrimidin-2-ylthio)acetyl]-5-(4-substituted phenyl)-1H-pyrazole-3-carboxylates ***(5a,b)***

A mixture of acid hydrazide **4** (1 mmol, 0.4 g) and methyl 2,4-dioxo-4-(4-substituted phenyl) butyrate (1 mmol) in absolute ethanol (10 ml) and 0.5 ml glacial acetic was heated under reflux for 5–7 h. The reaction mixture was evaporated to dryness under vacuum; the residue was triturated with cold ethanol and the precipitate obtained was filtered, washed with cold ethanol, dried and crystallized from ethanol/water.

4.1.3.1. Methyl 1-[2-(3-benzyl-6-carbamoyl-5-methyl-4-oxo-3,4-dihydrothieno [2,3-d]pyrimidin-2-ylthio)acetyl]-5-phenyl-1H-pyrazole-3-carboxylates ***(5a)***. Yield: 55%, m.p. 152–154 °C. IR (cm^{-1}): 3345, 3216 (NH_2); 1720 (C=O ester); 1665 (C=O amide); 1590 (C=N); 1513 (C=C); 1215 (C—S—C); 1168 (C—O—C). ^1H NMR (300 MHz, DMSO-d_6) (δ ppm): 2.71 (s, 3H, CH_3); 3.83 (s, 3H, $COOCH_3$); 4.21 (s, 2H, S—CH_2); 5.33 (s, 2H, N—CH_2); 7.12 (s, 1H, pyrazolyl-C_4-H); 7.25–7.29 (m, 5H, phenyl-H); 7.31–7.38 (m, 5H, phenyl-H); 7.61 (s, 2H, $CONH_2$, D_2O exchangeable).MS (EI) *m/z* (%): 574 [$M^{+\cdot}$+1] (14.84); 573 [$M^{+\cdot}$] (28.02). Anal. Calcd. for ($C_{28}H_{23}N_5O_5S_2$) (573.64): C, 58.63; H, 4.04; N, 12.21. Found: C, 58.75; H, 3.98; N, 12.40.

4.1.3.2. Methyl 1-[2-(3-benzyl-6-carbamoyl-5-methyl-4-oxo-3,4-dihydrothieno [2,3-d]pyrimidin-2-ylthio)acetyl]-5-(4- tolyl)-1H-pyrazole-3-carboxylates ***(5b)***. Yield: 60%, m.p. 183–186 °C. IR (cm^{-1}): 3344, 3187 (NH_2); 1723 (C=O

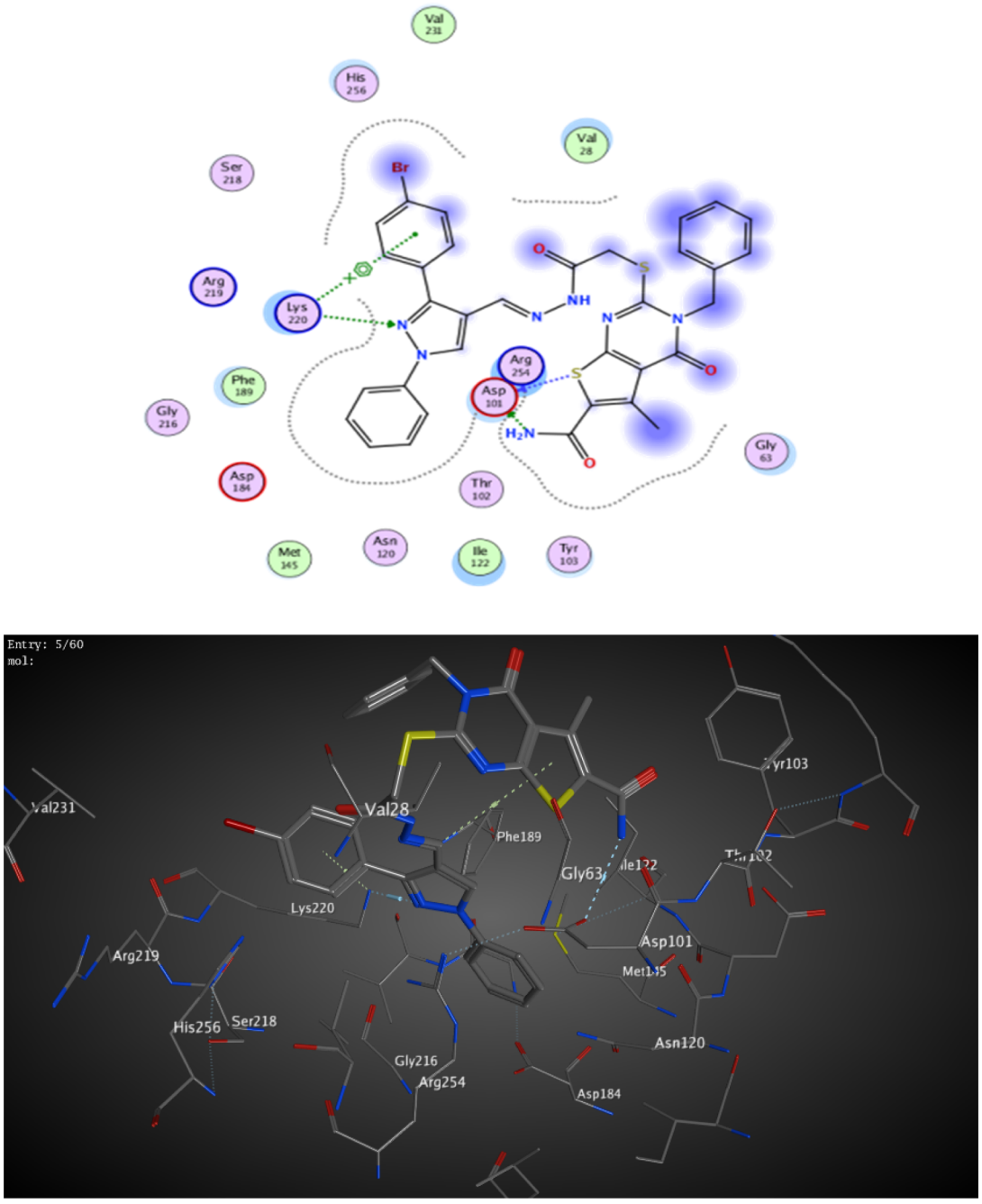


Fig. 10. 2D and 3D View from a molecular modeling study of the minimum-energy structure of the complex of 11b docked in DHPS (PDB ID code: 4DAI). Viewed using Molecular Operating Environment (MOE) module.

ester); 1684 (C═O amide); 1602 (C═N); 1515 (C═C); 1210 (C–S–C); 1160 (C–O–C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 1.91 (s, 3H, CH_3); 2.70 (s, 3H, CH_3); 3.83 (s, 3H, $COOCH_3$); 4.05 (s, 2H, S–CH_2); 5.31 (s, 2H, N–CH_2); 7.12 (s, 1H, pyrazolyl-C_4-H); 7.25–7.31 (m, 5H, phenyl-H); 7.32 (d, J = 7.2 Hz, 2H, 4-tolyl-$C_{3,5}$-H); 7.34(d, J = 7.2 Hz, 2H, 4-tolyl-$C_{2,6}$-H); 7.60 (s, 2H, $CONH_2$, D_2O exchangeable). Anal. Calcd. for ($C_{29}H_{25}N_5O_5S_2$) (587.67): C, 59.27; H, 4.29; N, 11.92. Found: C, 59.41; H, 4.32; N, 12.11.

4.1.4. 3-Benzyl-2-[2-(5-imino-3-oxopyrazolidin-1-yl)-2-oxoethylthio]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide ***(6)***

A mixture of acid hydrazide **4** (1 mmol, 0.4 g) and ethyl cyanoacetate (1 mmol, 0.11 g, 0.11 ml) in glacial acetic acid (5 ml) was heated under reflux for 7 h. The reaction mixture was evaporated to dryness and the residue was triturated with cold ethanol and the precipitate obtained was filtered, washed with cold ethanol, dried and crystallized from ethanol; yield: 66%, m.p. 280–282 °C. IR (cm^{-1}): 3368, 3315, 3207 (NH_2, NH); 1677 (C═O); 1610 (C═N); 1511 (C═C); 1224 (C–S–C). ^{1}H NMR (300 MHz,DMSO-d_6) (δ ppm): 1.85 (s, 2H, CH_2, pyrazolyl-C_4-H); 2.69 (s, 3H, CH_3); 4.71 (s, 2H, S–CH_2); 5.30 (s, 2H, N–CH_2); 7.23–7.35 (m, 5H, phenyl-H); 7.65 (s, 2H, $CONH_2$, D_2O exchangeable); 9.91 (s, 1H, ═NH, D_2O exchangeable); 10.40 (s, 1H, NH, D_2O exchangeable). MS(EI) m/z (%): 471 [$M^{+\cdot}$ + 1] (10.06); 470 [$M^{+\cdot}$] (16.25). Anal. Calcd. for ($C_{20}H_{18}N_6O_4S_2$) (470.52): C, 51.05; H, 3.86; N, 17.86. Found: C, 51.13; H, 3.92; N, 17.99.

Table 4
Molecular modeling results for the tested compounds and re-docked ligand during docking in the DHPS enzyme.

Compound ID	E-score	No. of hydrogen bonds	Hydrogen bonding residues	Distance A°	H-bond E-score Kcal/mol
7	−6.987	3	Lys 220	3	−11.1
			Asp 101	2.98	−9.4
			Ser 221	2.98	−2.2
10b	−6.2197	3	Lys 220	3.22	−2
			Asp 101	3.06	−6.1
			Arg 254	3.02	−4.8
10d	−7.3156	2	Lys 220	3.16	−2.1
			Asp 101	3.17	−1.3
11b	−7.443	2	Lys 220	3.11	−4.3
			Asp 101	2.93	−1.4
Ligand	−5.5490	6	Lys 220	2.69	−6.5
			Asp 101	2.96	−8.7
			Arg 254	3.02	−3.6
			Asp 184	2.85	−5.4
			Asn 120	2.9	−3
				2.85	−5.8

*4.1.5. 3-Benzyl-2-[2-(4-cyano-5-oxo-2,5-dihydro-1H-pyrazol-1-yl)-2-oxoethylthio]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide **(7)***

A mixture of acid hydrazide **4** (1 mmol, 0.4 g) and ethyl ethoxymethylene cyanoacetate (1 mmol, 0.17 g) in glacial acetic acid (5 ml) was heated under reflux for 7 h. The reaction mixture was evaporated to dryness under vacuum and the residue was triturated with cold ethanol, the precipitate obtained was filtered, washed with cold ethanol, dried and crystallized from acetic acid; yield: 70%, m.p. > 300 °C. IR (cm^{-1}): 3459, 3204 (NH_2, NH); 2355 (C≡N); 1672 (C=O); 1607 (C=N); 1518 (C=C); 1213 (C−S−C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 2.73 (s, 3H, CH_3); 4.05 (s, 2H, S−CH_2); 5.31 (s, 2H, N−CH_2); 7.24–7.36 (m, 5H, phenyl-H); 7.59 (s, 2H, $CONH_2$, D_2O exchangeable); 8.00 (s, 1H, pyrazolyl-C_5-H); 10.36 (s, 1H, NH, D_2O exchangeable). MS (EI) *m/z* (%): 481 [$M^{+\cdot}$+1] (42.46); 480 [$M^{+\cdot}$] (37.99). Anal. Calcd. for ($C_{21}H_{16}N_6O_4S_2$) (480.52):C, 2.49; H, 3.36; N, 17.49. Found; C, 52.64; H, 3.43; N, 17.68.

*4.1.6. 3-Benzyl-2-[(3-hydroxy-1H-1,2,4-triazol-5-yl)methylthio]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamides **(8a,b)***

A mixture of acid hydrazide **4** (1 mmol, 0.4 g) and urea or thiourea (1 mmol) was refluxed in acetic acid for 8 h. The solid obtained was filtered, dried and crystallized from DMF/ethanol.

*4.1.6.1. 3-Benzyl-2-[(3-hydroxy or mercapto-1H-1,2,4-triazol-5-yl) methylthio]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamides **(8a)***. Yield: 73%, m.p. 278–281 °C. IR (cm^{-1}): 3504 (OH); 3345, 3320, 3216 (NH_2, NH); 1650, 1634 (C=O amide); 1600 (C=N); 1512 (C=C); 1205 (C−S−C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 2.72 (s, 3H, CH_3); 4.05 (s, 2H, S−CH_2); 5.33 (s, 2H, N−CH_2); 7.25–7.38 (m, 5H, phenyl-H); 7.6 (s, 2H, $CONH_2$, D_2O exchangeable); 9.78 (s, 1H, NH, D_2O exchangeable); 11 (s, 1H, OH, D_2O exchangeable). Anal. Calcd. for ($C_{18}H_{16}N_6O_3S_2$) (428.49): C, 50.45; H, 3.76; N, 19.61. Found: C, 50.61; H, 3.80; N, 19.74.

*4.1.6.2. 3-Benzyl-2-[(3-mercapto-1H-1,2,4-triazol-5-yl)methylthio]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamides **(8b)***. Yield: 80%, m.p. < 300 °C. IR (cm^{-1}): 3340, 3300, 3205 (NH_2, NH); 2560 (SH); 1675 (C=O amide); 1601 (C=N); 1515 (C=C); 1211 (C−S−C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 2.71 (s, 3H, CH_3); 4.05 (s, 2H, S−CH_2); 5.32 (s, 2H, N−CH_2); 7.25–7.37 (m, 5H, phenyl-H); 7.60 (s, 2H, $CONH_2$, D_2O exchangeable); 9.56 (s, 1H, NH, D_2O exchangeable); 10.36 (s, 1H, SH, D_2O exchangeable). MS(EI) *m/z* (%): 445 [$M^{+\cdot}$+1] (77.45); 444 [$M^{+\cdot}$] (74.51). Anal. Calcd. for ($C_{18}H_{16}N_6O_2S_3$): C, 48.63; H, 3.63; N, 18.90. Found: C, 48.82; H, 3.61; N, 18.89.

*4.1.7. 3-Benzyl-2-[2-(2,5-dioxopyrrolidin-1-ylamino or 1,3-dioxoisoindolin-2-ylamino)-2-oxoethylthio]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d] pyrimidine-6-carboxamides **(9a,b)***

A mixture of acid hydrazide **4** (1 mmol, 0.4 g) and succinic anhydride or phthalic anhydride (1 mmol) in glacial acetic acid (5 ml) was refluxed for 4 h, evaporated to dryness under vacuum. The residue was triturated with ethanol and the precipitate obtained was filtered, washed with ethanol, dried and crystallized from acetic acid.

*4.1.7.1. 3-Benzyl-2-[2-(2,5-dioxopyrrolidin-1-ylamino)-2-oxoethylthio]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d] pyrimidine-6-carboxamide **(9a)***. Yield: 54%, m.p. 252–255 °C. IR (cm^{-1}): 3410, 3228, 3191 (NH_2, NH); 1705, 1660 (C=O); 1604 (C=N) and 1532 (C=C); 1205 (C−S−C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 2.36–2.38 (m, 4H, pyrrolidine-$C_{3,4}$- CH_2); 2.70 (s, 3H, CH_3); 4.05 (s, 2H, S−CH_2); 5.32 (s, 2H, N−CH_2); 7.27–7.33 (m, 5H, phenyl-H); 7.63 (s, 2H, $CONH_2$, D_2O exchangeable); 9.95, 10.21 (2 s, 1H, CONHN, D_2O exchangeable). ^{13}C NMR (75 MHz, DMSO-d_6) (δ ppm):14.72, 27.92, 28.70, 34.29, 46.62, 118.95, 126.67, 127.45, 127.55, 128.59, 135.16, 136.25, 159.24, 162.38, 163.59, 165.04, 169.76, 173.48. Anal. Calcd. for ($C_{21}H_{19}N_5O_5S_2$) (485.54): C, 51.95; H, 3.94; N, 14.42. Found: C, 52.12; H, 4.04; N, 14.71.

*4.1.7.2. 3-benzyl-2-[(2-((1,3-dioxooctahydro-2H-isoindol-2-yl)amino)-2-oxoethyl)thio]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide **(9b)***. Yield: 62%, m.p. 251–253 °C. IR (cm^{-1}): 3464, 3253, 3202 (NH_2, NH); 1789, 1737, 1694, 1640 (C=O); 1576 (C=N); 1512 (C=C) and 1209 (C−S−C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 2.71 (s, 3H, CH_3); 4.25 (s, 2H, S−CH_2); 5.33 (s, 2H, N−CH_2); 7.29–7.32 (m, 5H, phenyl-H); 7.6 (s, 2H, $CONH_2$, D_2O exchangeable);7.93–7.95 (m, 4H, isoindole-$C_{4,5,6,7}$-H); 11.00 (s, 1H, CONHN, D_2O exchangeable). MS(EI) *m/z* (%): 534 [$M^{+\cdot}$+1] (19.42); 533 [$M^{+\cdot}$] (38.83). Anal. Calcd. for ($C_{25}H_{19}N_5O_5S_2$) (533.58): C, 56.27; H, 3.59; N, 13.13. Found: C, 56.33; H, 3.64; N, 13.27.

*4.1.8. 3-Benzyl-2-{2-[2-(arylidene)hydrazinyl]-2-oxoethylthio}-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamides **(10a-d)***

A mixture of acid hydrazide **4** (1 mmol, 0.4 g) and the appropriate aryl aldehyde (1.1 mmol) in glacial acetic acid (5 ml) was heated under reflux for 6–8 h during which the product precipitated. The reaction mixture was cooled to room temperature and the product was filtered, washed with ethanol and crystallized from acetic acid.

*4.1.8.1. (E)-3-benzyl-2-{2-[2-(benzylidenehydrazinyl)-2-oxoethyl]thio}-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide **(10a)***. Yield: 88%, m.p. 263–266 °C. IR (cm^{-1}): 3337, 3168 (NH_2, NH); 1671 (C=O); 1602 (C=N); 1508 (C=C); 1218 (C−S−C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 2.68 (s, 3H, CH_3); 4.15 (s, 1H, S−CH_2), 5.31 (s, 2H, N−CH_2); 7.24–7.38 (m, 5H, phenyl-H); 7.42 (m, 1H, phenyl C_4-H), 7.5 (m, 2H, phenyl $C_{3,5}$-H), 7.6 (s, 2H, $CONH_2$, D_2O exchangeable);7.78 (s, $^1/_2$H, =CH trans); 7.99 (s, $^1/_2$H, =CH cis); 11.5 (s, $^1/_2$H, NH trans, D_2O exchangeable); 11.7 (s, $^1/_2$H, NH cis, D_2O exchangeable). Anal. Calcd. for ($C_{24}H_{21}N_5O_3S_2$) (491.59): C, 58.64; H, 4.31; N, 14.25. Found: C, 58.84; H, 4.39; N, 14.37.

*4.1.8.2. (E)-3-benzyl-2-{2-[2-(4-chlorobenzylidene)hydrazinyl)-2-oxoethyl]thio}-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide **(10b)***. Yield: 76%, m.p. 238–241 °C. IR (cm^{-1}): 3363, 3129 (NH_2, NH); 1660 (C=O); 1588 (C=N); 1532 (C=C); 1211 (C−S−C); 684 (C−Cl). ^{1}H NMR (300 MHz,DMSO-d_6) (δ ppm): 2.68 (s, 3H, CH_3); 4.2 (s, 2H, S−CH_2), 5.35 (s, 2H, N−CH_2); 7.25–7.36 (m, 5H, phenyl-H); 7.48 (d, J = 7.2 Hz, 2H, p-chlorophenyl $C_{2,6}$-H); 7.62 (s, 2H, $CONH_2$, D_2O exchangeable);7.72 (d, J = 7.2 Hz, 2H, p-chlorophenyl $C_{3,5}$-H); 8.02 (s, $^1/_2$H, =CH trans); 8.19 (s, $^1/_2$H, =CH cis); 11.75 (s,

$^1/_2$H, NH trans, D_2O exchangeable); 11.91 (s, $^1/_2$H, NH cis, D_2O exchangeable). MS (EI) *m/z* (%) 527 [$M^{+\cdot}+2$] (2.25); 526 [$M^{+\cdot}+1$] (76.4); 525 [$M^{+\cdot}$] (6.74). Anal. Calcd. for ($C_{24}H_{20}ClN_5O_3S_2$) (526.03): C, 54.80; H, 3.83; N, 13.31. Found: C, 54.88; H, 3.81; N, 13.63.

4.1.8.3. (E)-3-benzyl-2-{2-[2-(4-methoxybenzylidene)hydrazinyl)-2-oxoethyl]thio}-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide ***(10c)***. Yield: 80%, m.p. 251–253 °C. IR (cm^{-1}): 3327, 3173 (NH_2, NH); 1677 (C═O); 1603 (C═N); 1511 (C═C); 1166 (C−S−C); 1028 (C−O−C). ^{1}H NMR (300 MHz,DMSO-d_6) (δ ppm): 2.69 (s, 3H, CH_3); 3.80 (s, 3H, OCH_3); 4.14 (s, 2H, S−CH_2), 5.34 (s, 2H, N−CH_2); 7.13 (d, J = 8.7 Hz, 2H, p-methoxyphenyl $C_{3,5}$-H); 7.26–7.33 (m, 5H, phenyl-H); 7.62 (s, 2H, $CONH_2$, D_2O exchangeable); 7.76 (d, J = 8.7 Hz, 2H, p-methoxyphenyl $C_{2,6}$-H); 8.16 (s, $^1/_2$H, ═CH trans); 8.27 (s, $^1/_2$H, ═CH cis); 11.73 (s, $^1/_2$H, NH trans, D_2O exchangeable); 11.92 (s, $^1/_2$H, NH cis, D_2O exchangeable). Anal. Calcd. for ($C_{25}H_{23}N_5O_4S_2$) (521.61): C, 57.57; H, 4.44; N, 13.43. Found: C, 57.66; H, 4.51; N, 13.58.

4.1.8.4. (E)-3-benzyl-5-methyl-2-{2-[2-(4-nitrobenzylidene)hydrazinyl)-2-oxoethyl]thio}-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide ***(10d)***. Yield: 75%, m.p. 271–273 °C. IR (cm^{-1}): 3349, 3183 (NH_2, NH); 1690 (C═O); 1585 (C═N); 1512 (C═C); 1477 (N−O); 1221 (C−S−C). ^{1}H NMR (300 MHz,DMSO-d_6) (δ ppm): 2.69 (s, 3H, CH_3); 4.14, 4.56 (2 s, 2H, S−CH_2), 5.34 (s, 2H, N−CH_2); 7.25–7.36 (m, 5H, phenyl-H); 7.62 (s, 2H, $CONH_2$, D_2O exchangeable); 7.97 (d, J = 8.7 Hz, 2H, p-nitrophenyl $C_{2,6}$-H); 8.03 (s, $^1/_2$H, ═CH trans); 8.17 (s, $^1/_2$H, ═CH cis); 8.24–8.28 (m, 2H, p-nitrophenyl $C_{3,5}$-H); 11.96 (s, $^1/_2$H, NH trans, D_2O exchangeable); 12.12 (s, $^1/_2$H, NH cis, D_2O exchangeable). ^{13}C NMR (75 MHz, DMSO-d_6) (δ ppm): 14.70, 46.72, 67.72, 123.99, 126.74, 127.45, 127.78, 128.02, 128.57, 128.61, 135.18, 140.30, 157.96, 159.41, 163.49 and 168.69. Anal. Calcd. for ($C_{24}H_{20}N_6O_5S_2$) (536.58): C, 53.72; H, 3.76; N, 15.66. Found: C, 53.88; H, 3.80; N, 15.79.

4.1.9. 3-Benzyl-2-{2-[2- ((3-aryl-1-phenyl-1H-pyrazol-4-yl)methylene) hydrazinyl]-2-oxoethylthio}-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d] pyrimidine-6-carboxamides ***(11a-c)***

A mixture of acid hydrazide **4** (1 mmol, 0.4 g) and the appropriate pyrazole aldehyde (1.1 mmol) in glacial acetic acid (5 ml) was refluxed for 4 h during which precipitation of the crude product occurred. The reaction mixture was then cooled to room temperature and the product was filtered, washed with ethanol, dried and crystallized from acetic acid.

4.1.9.1. (E)-3-benzyl-2-{2-[2-((1,3-diphenyl-1H-pyrazol-4-yl)methylene) hydrazinyl]-2-oxoethylthio}-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d] pyrimidine-6-carboxamide ***(11a)***. Yield: 78%, m.p. 269–271 °C. IR (cm^{-1})**:** 3373, 3217 (NH_2, NH); 1677 (C═O); 1594 (C═N); 1515 (C═C); 1210 (C−S−C). ^{1}H NMR (300 MHz,DMSO-d_6) (δ ppm): 2.70 (s, 3H, CH_3); 4.41 (s, 2H, S−CH_2), 5.35 (s, 2H, N−CH_2); 7.30–7.94 (m, 15H, 3 phenyl-H); 7.75 (s, 2H, $CONH_2$, D_2O exchangeable); 8.17 (s, $^1/_2$H, ═CH trans); 8.30 (s, $^1/_2$H, ═CH cis) 8.97 (s, 1H, pyrazolyl-C_5-H); 11.40 (s, $^1/_2$H, NH trans, D_2O exchangeable); 11.60 (s, $^1/_2$H, NH cis, D_2O exchangeable). MS (EI) *m/z* (%): 634 [$M^{+\cdot}+1$] (19.82); 633 [$M^{+\cdot}$] (11.85). Anal. Calcd. for $C_{33}H_{27}N_7O_3S_2$ (633.74): C, 62.5645; H, 4.29; N,15.47. Found: C, 62.66; H, 4.38; N, 15.81.

4.1.9.2. (E)-3-benzyl-2-{2-[2-((3-(4-bromophenyl)-1-phenyl-1H-pyrazol-4-yl)methylene)hydrazinyl]-2-oxoethylthio}-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide ***(11b)***. Yield: 84%, m.p. 278–280 °C. IR (cm^{-1})**:**3311, 3178 (NH_2, NH); 1681 (C═O); 1595 (C═N); 1509 (C═C); 1223 (C−S−C); 728 (C-Br). ^{1}H NMR (300 MHz,DMSO-d_6) (δ ppm): 2.70 (s, 3H, CH_3); 4.06, 4.37(2 s, 2H, S−CH_2), 5.35(s, 2H, N−CH_2); 7.29–7.95 (m, 14H, 3 phenyl-H); 7.73(s, 2H, $CONH_2$, D_2O exchangeable,);8.15 (s, $^1/_2$H, ═CH trans); 8.29 (s, $^1/_2$H, ═CH cis) 8.97 (s, 1H, pyrazolyl-C_5-H); 11.51 (s, $^1/_2$H, NH trans, D_2O exchangeable); 11.71 (s, $^1/_2$H, NH cis, D_2O exchangeable). Anal. Calcd. for ($C_{33}H_{26}BrN_7O_3S_2$) (712.64): C, 55.62; H, 3.68; N, 11.21. Found: C, 55.71; H, 3.72; N, 11.56.

4.1.9.3. (E)-3-benzyl-2-{2-[2-((1-phenyl-3-(p-tolyl)-1H-pyrazol-4-yl) methylene)hydrazinyl]-2-oxoethylthio}-5-methyl-4-oxo–3,4-dihydrothieno [2,3-d]pyrimidine-6-carboxamide ***(11c)***. Yield: 90%, m.p. 285–287 °C. IR (cm^{-1})**:** 3302, 3249 (NH_2, NH); 1671 (C═O); 1602 (C═N); 1507 (C═C); 1230 (C−S−C). ^{1}H NMR (300 MHz,DMSO-d_6)(δ ppm): 2.31 (s, 3H, CH_3 p-tolyl); 2.71 (s, 3H, CH_3); 4.07, 4.42 (2 s, 2H, S−CH_2), 5.35 (s, 2H, N−CH_2); 7.26–8.01(m, 16H, 3 phenyl-H and NH_2); 8.15 (s, $^1/_2$H, ═CH trans); 8.31 (s, $^1/_2$H, ═CH cis); 8.95 (s, 1H, pyrazolyl-C_5-H); 11.49 (s, $^1/_2$H, NH trans, D_2O exchangeable); 11.68 (s, $^1/_2$H, NH cis, D_2O exchangeable). ^{13}C NMR (75 MHz, DMSO-d_6) (δ ppm): 19.97, 26.07, 40.71, 51.92, 121.92, 123.83, 124.011, 124.10, 131.99, 133.57, 133.82, 134.27, 134.80, 140.41, 140.48, 141.66, 143.16, 144.23, 156.42, 157.14, 163.23, 164.71, 165.01, 167.60, 167.71, 168.78, 168.82 and 172.92. Anal. Calcd. for ($C_{34}H_{29}N_7O_3S_2$) (647.77): C, 63.04; H,4.51; N, 15.14. Found: C, 63.12; H, 4.47; N, 15.23.

4.1.10. 3-Benzyl-5-methyl-4-oxo-2-{2-oxo-2-[2-(2-oxoindolin-3-ylidene) hydrazinyl]ethylthio}-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide ***(12)***

A mixture of acid hydrazide **4** (1 mmol, 0.4 g) and isatin(1 mmol, 0.147 g) in glacial acetic acid (5 ml) was heated under reflux for 5 h during which precipitation of the product occurred. The reaction mixture was then cooled to room temperature and the product was filtered, washed with ethanol and crystallized from acetic acid; yield: 70%, m.p. 248–250 °C. IR (cm^{-1})**:** 3345, 3190 (NH_2, NH); 1687 (C═O); 1583 (C═N); 1510 (C═C); 1206 (C−S−C).^{1}H NMR (300 MHz,DMSO-d_6) (δ ppm):2.75 (s, 3H, CH_3); 4.22 (s, 2H, S−CH_2); 5.35 (s, 2H, N−CH_2); 6.89–7.41 (m, 9H, Ar-H); 7.56 (s, 2H, $CONH_2$, D_2O exchangeable); 11.30 (s, 1H, −NH−N═, D_2O exchangeable); 12.50 (s, 1H, NH isatin, D_2O exchangeable). MS (EI) *m/z* (%): 533 [$M^{+\cdot}+1$] (45.76); 532 [$M^{+\cdot}$] (64.41). Anal. Calcd. for ($C_{25}H_{20}N_6O_4S_2$) (532.59) C, 56.38; H, 3.79; N, 15.78. Found: C, 56.51; H, 3.82; N, 15.94.

4.1.11. Ethyl 3-{2-[2-(3-benzyl-6-carbamoyl-5-methyl-4-oxo-3,4-dihydrothieno [2,3-d] pyrimidin-2-ylthio)acetyl]hydrazinyl}-3-oxopropanoate ***(13)***

A mixture of acid hydrazide **4** (1 mmol, 0.4 g) and diethyl malonate (5 ml) was heated at 150oC on oil bath for 8 h. The reaction mixture was triturated with ether, the precipitate obtained was filtered, washed with ether, dried and crystallized from ethanol/ water; yield: 64%, m.p. > 300 °C. IR (cm^{-1}): 3432, 3300, 3180 (NH_2, NH); 1730 (C═O ester); 1670 (C═O amide); 1615 (C═N); 1510 (C═C); 1219 (C−S−C); 1072 (C−O−C). ^{1}H NMR (300 MHz,DMSO-d_6) (δ ppm): 1.12 (t, J = 6.9 Hz, 3H, $COOCH_2\underline{CH_3}$); 2.67 (s, 3H, CH_3); 3.20 (s, 2H, $\underline{CH_2}COOC_2H_5$); 4.02 (s, 2H, S−$CH_2$); 4.03 (q, J = 6.9 Hz, 2H, $COO\underline{CH_2}CH_3$); 5.28 (s, 2H, N−CH_2); 7.23–7.31 (m, 5H, phenyl-H); 7.60 (s, 2H, $CONH_2$, D_2O exchangeable); 10.21 (s, 1H, NH, D_2O exchangeable); 10.36 (s, 1H, NH, D_2O exchangeable). MS (EI) *m/z* (%): 518 [$M^{+\cdot}+1$] (18.84); 517 [$M^{+\cdot}$] (18.26). Anal. Calcd. for ($C_{22}H_{23}N_5O_6S_2$) (517.58) C,51.05; H, 4.48; N,13.53. Found: C, 51.23; H,4.60; N, 13.76.

4.1.12. 3-Benzyl-2-ethoxy-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d] pyrimidine-6-carboxamide ***(15)***

A mixture of **13** (1 mmol, 0.52 g) and sodium ethoxide (1 mmol, 0.023 g sodium in 5 ml absolute ethanol) was heated under reflux for 7 h. The reaction mixture was evaporated to dryness under vacuum. The residue triturated with cold ethanol and the precipitate obtained was filtered, washed with cold ethanol, dried and crystallized from ethanol; yield: 45%, m.p. 245–247 °C. IR (cm^{-1}): 3374, 3275, 3220 (NH_2, NH); 1687 (C═O); 1620 (C═N); 1514 (C═C); 1213 (C−S−C); 1150 (C−O−C). ^{1}H NMR (300 MHz,DMSO-d_6) (δ ppm): 1.28 (t, J = 6.8 Hz, 3H, O-$CH_2\underline{CH_3}$); 2.71 (s, 3H, CH_3); 4.41 (q, J = 6.8 Hz, 2H,

O−CH_2); 5.13 (s, 2H, N−CH_2); 7.22–7.35 (m, 5H, phenyl-H); 7.58 (s, 2H, $CONH_2$, D_2O exchangeable). ^{13}C NMR (75 MHz,DMSO-d_6) (δ ppm):13. 81; 14.59; 43.71; 65.42; 117.68; 125.76; 127.36; 127.51; 128.43; 136.51; 136.57; 154.43; 158.49; 163.14; 163.66. MS (EI) *m/z* (%): 344 [$M^{+\cdot}$+1] (2.90); 343 [$M^{+\cdot}$] (37.79). Anal. Calcd. for $C_{17}H_{17}N_3O_3S$ (343.4): C, 59.46; H, 4.99; N, 12.24. Found: C, 59.68; H, 5.12; N, 12.51.

4.1.13. 2-[2-(2-Benzoylhydrazinyl)-2-oxoethylthio]-3-benzyl-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide ***(16)***

A mixture of acid hydrazide **4** (1 mmol, 0.4 g) and benzoyl chloride (2 mmol, 0.28 g, 0.23 ml) in pyridine (5 ml) was heated under reflux for 4 h. The reaction mixture was cooled, poured onto crushed ice and acidified with dil. HCl. The precipitate was filtered, dried and crystallized from ethanol; yield: 65%, m.p. 276–278 °C. IR (cm^{-1}): 3370, 3295, 3200 (NH_2, NH); 1658 (C═O); 1616 (C═N); 1510 (C═C); 1166 (C−S−C). ^{1}H NMR (300 MHz,DMSO-d_6) (δ ppm): 2.71 (s, 3H, CH_3); 4.15 (s, 2H, S−CH_2); 5.34 (s, 2H, N−CH_2); 7.23–7.38 (m, 5H, phenyl-H); 7.45–7.57 (m, 3H, phenyl-$C_{3,4,5}$-H); 7.62 (s, 2H, $CONH_2$, D_2O exchangeable); 7.85 (d, J = 7.2 Hz, 2H, phenyl-$C_{2,6}$-H); 10.42 (s, 2H, 2NH, D_2O exchangeable). MS(EI) *m/z* (%): 508 [$M^{+\cdot}$+1] (18.84); 507 [$M^{+\cdot}$] (23.29). Anal. Calcd. for ($C_{24}H_{21}N_5O_4S_2$) (507.58): C, 56.79; H, 4.17; N, 13.80. Found: C, 56.84; H, 4.20; N, 13.87.

4.1.14. 3-Benzyl-2-hydroxy-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxamide ***(18)***

To a solution of 5 ml sodium hydroxide 10%, **16** (1 mmol, 0.5 g) was added. The reaction mixture was refluxed for 5 h. After cooling, the reaction mixture was acidified with 2 N HCl, the precipitated solid was filered, dried and crystallized from ethanol; yield: 70%, m.p. > 300 °C**.** IR (cm^{-1})**:** 3506 (OH); 3368, 3315, 3207 (NH_2, NH); 1677 (C═O); 1610 (C═N); 1511 (C═C); 1210 (C−S−C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 2.64 (s, 3H, CH_3); 5.01 (s, 2H, N−CH_2); 7.22–7.34 (m, 5H, phenyl-H); 7.47 (s, 2H, $CONH_2$, D_2O exchangeable); 12.44 (s, 1H, OH, D_2O exchangeable). ^{13}C NMR (75 MHz, DMSO-d_6) **(**δ ppm): 14.34; 42.86; 113.46; 123.27; 126.98; 127.38; 128.25; 137.26; 137.30; 150.13; 151.98; 159.17; 163.43. MS(EI) *m/z* (%): 316 [$M^{+\cdot}$+1] (32.33); 315 [$M^{+\cdot}$] (70.4). Anal. Calcd. for ($C_{15}H_{13}N_3O_3S$) (315.35): C,57.13; H, 4.16; N, 13.33. Found: C, 57.15; H. 4.17; N, 13.42.

4.2. Biological screening

4.2.1. Antimicrobial evaluation

4.2.1.1. Inhibition-zone measurements. Whatman filter papers were prepared as discs of standard size (6.0 mm diameter). For sterilization; they were kept into 1.0 Oz screw capped wide mouthed containers. These containers are kept at 150 °C in a hot air oven. Then, the sterilized standard filter paper discs impregnated with a test compound solution in DMF (100 ml, 5 mg/mL) were then placed on nutrient agar plate seeded in triplicates with the appropriate test organism. Standard concentrations of 106 CFU/mL (Colony Formation Units/mL) and 104 CFU/mL were utilized for antimicrobial assay [36]. Pyrex glass Petri dishes (9 cm in diameter) have been used and two discs of filter paper have been inoculated on each plate. The test organisms were*Staphylococcus epidermidis* (RCMB 0100183), *Staphylococcus aureus* (RCMB 0100183) and *Bacillus subtilis* (RCMB 0100162) as examples of Gram-positive bacteria while for Gram-negative bacteria*; Proteus vulgaris* (RCMB 010085), *Escherichia coli* (RCMB 010052), and *Pseudomonas aeruginosa* (RCMB 0100243) were used. *In-vitro* antifungal potential was also evaluated against *Candida albicans, Rhizopus oryzae and Aspergillus fumigatus.* Ampicillin and levofloxacin were used as standard antibacterial agents; while clotrimazole was used as standard antifungal agent. The solvent DMF was used as control at the same aforementioned concentration with no visible change in bacterial growth. Incubation of the plates took place at 37 °C for 24 h for both bacteria and fungi. The mean inhibition zone in a range of environmental and clinically pathogenic microorganisms was measured in mm ± standard deviation. The resulting inhibition zones are recorded in Table 1. Compounds with growth inhibition zones (> 10 mm) using the twofold serial dilution technique, were further assessed for their minimal inhibitory concentrations (MICs) [37].

4.2.1.2. Minimal inhibitory concentration (MIC) measurement. For the determination of antibacterial and antifungal activity. The microdilution susceptibility test in MüllereHinton Broth (Oxoid) and Subouraud Liquid Medium (Oxoid) were used, respectively [38]. Tested compounds, ampicillin, levofloxacin and clotrimazole were used to prepare stock solutions in DMF at concentrations 1000 μg/mL. Each stock solution has been diluted with standard method broth (Difco) to prepare serial twofold dilutions in the range of were prepared using 200, 100, 50, 25, 12.5, 6.25 and 3.125 μg/mL of the broth containing approximately 106 CFU/mL of test bacteria was added to each well of 96-well microtiter plate. Incubation of the sealed microplates at 37 °C was continued for 24 h for both antibacterial activity and for antifungal activity in a humid chamber. At the end of the incubation period, the lowest concentrations of the substance that inhibited the growth of the tested organisms judged by the absence of visible turbidity were the minimum inhibitory concentrations (MICs). Control experiments with DMF and uninoculated media were done under the same conditions parallel to the test compounds.

4.2.1.3. Minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) measurement. MIC tests were further introduced to measure the MBC and MFC as follows: A loop-full from the tube that show no visible growth (MIC) was spread over a quarter of Müller–Hinton agar plate [39]. The plates were then examined for growth after 18 h of incubation. still, the tube containing the lowest concentration of the test compound that was no ableto produce growth on subculture plates was judged to contain the MBC or MFC of that compound for the particular test organism, According to the MIC and MBC or MFC limits derived from the latest National Committee on Clinical Laboratory Standards (NCCLS), it can be decided whether the test compound is bacteriostatic or bactericidal to the test organism. If MBC > MIC, the test compound is considered bacteriostaticbutif the MBC = MIC, the test compound is counted as bactericidal (Table 2).

4.3. Molecular docking studies

Molecular docking procedures were performed on the most active compounds **7, 10b, 10d** and **11b** using Molecular Operating Environment (Moe-Dock 2016.08) software [35]. The structures of the previously mentioned compounds were constructed using the builder button. In addition, these compounds were prepared for docking experiment by subjecting their structures to energy minimization using the default MMFF94x force field in MOE program. Co-crystal structures of *B. anthracis* DHPS with (7-Amino-4,5-dioxo-1,4,5,6-tetrahydro-pyrimido(4,5-*c*)pyridazin-3-yl)-acetic acid (PDB ID code: **4DAI**) was downloaded from the protein data bank and used as a template for the target enzyme. The active site of DHPS was generated using the MOE-Alpha site finder, and then ligands were docked within this active site using the MOE Dock. MOE was also used to calculate the best score between the ligands and the active site interactions. Ten conformers of the ligand were retained with the highest and best score by default which shows the best ligand active site interactions.

Validation of docking protocol was performed by re-docking the ligand into active site and it showed similar binding interactions compared to that of co-crystallized ligand with root mean square deviation RMSD < 2.

Declaration of interest

The authors declared no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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