

# Synthesis, *in silico* and *in vitro* antimicrobial activity of *N*-(benzyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamides

Sergiy V. Vlasov<sup>1</sup>, Oleksandr V. Borysov<sup>2,3</sup>, Hanna I. Severina<sup>1</sup>, Vitaliy S. Vlasov<sup>1</sup>, Amjad Ibrahim M. Abu Sharkh<sup>1</sup>, Victoriya A. Georgiyants<sup>1</sup>

<sup>1</sup> National University of Pharmacy of the Ministry of Health of Ukraine, Kharkiv, Ukraine

<sup>2</sup> Institute of Organic Chemistry of the National Academy of Sciences of Ukraine, Kyiv, Ukraine

<sup>3</sup> Enamine Ltd., Kyiv, Ukraine

Corresponding author: Hanna I. Severina (severina.ai@ukr.net)

Received 25 July 2023 ♦ Accepted 14 November 2023 ♦ Published 29 January 2024

**Citation:** Vlasov SV, Borysov OV, Severina HI, Vlasov VS, Abu Sharkh AIM, Georgiyants VA (2024) Synthesis, *in silico* and *in vitro* antimicrobial activity of *N*-(benzyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamides. Pharmacia 71: 1–9. <https://doi.org/10.3897/pharmacia.71.e110013>

## Abstract

According to the recent studies bezylcarboxamide fragment attached to the thiophene ring of thieno[2,3-*d*]pyrimidine is beneficial for antimicrobial activity of the compounds. Therefore we focused our efforts on constructing of the simple molecules such as *N*-(benzyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamides to get deeper insight into their antimicrobial activity. As the optimal procedure for preparation of target compounds we choose 1,1'-carbonyldiimidazole promoted interaction of 5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxylic acid with the series of substituted benzyl amines. The obtained amides showed good activity against the strains of *S. aureus* and *B. subtilis*, which was higher for the derivative without substituents in benzene ring or the compounds with small substituents like methyl or methoxyl groups in the para-position of the benzene ring. Docking studies showed that despite the good values of the scoring functions, the conformational analysis of the ligands' poses in the active site revealed their ability for only partial inhibition of TrmD of *P. aeruginosa*.

## Keywords

thiophene, pyrimidine, amides, coupling, docking, antimicrobials

## Introduction

Thieno[2,3-*d*]pyrimidines are well known due to their antimycobacterial (Malothu et al. 2018; Harrison et al. 2019), antimicrobial (Gill et al. 2017; Triloknadh et al. 2018; Lagardère et al. 2022) and antifungal properties (Shaaban et al. 2019; El-Dash et al. 2021). Theinopyrimidine scaffolds are considered as the privileged among the inhibitors of bacterial tRNA (Guanine37-N<sup>1</sup>)-methyltransferase (TrmD) (Hill et al. 2013; Zhong et al. 2019).

Thieno[2,3-*d*]pyrimidine-6-carboxylic acids and their benzylamides have attracted attention as biologically active compounds for many years, but the number of publications on their activity is relatively small. The authors of the paper (De Schutter et al. 2017) performed a profound study on the search for antimicrobial agents among derivatives of thieno[2,3-*d*]pyrimidine-6-carboxylic acids, which were studied as inhibitors of *N*-acetyltransferases of bacterial sugars. Numerous studies conducted by the researchers used of high-throughput screening

methods to establish the inhibitory concentrations of the reference derivatives and the series of 5-methyl-4-(alkyl-amino)-2-(2-arylethyl)thieno[2,3-*d*]pyrimidine-6-carboxylic acid against *C. jejuni* PglD. Based on the obtained results, the authors (De Schutter et al. 2017) developed and optimized the methods of obtaining more selective inhibitors of the corresponding enzyme. As a result of research, lead compounds **I–III** were found that had both a low molar inhibitory concentration and sufficient metabolic stability (Fig. 1).

As part of research on the development of synthetic methods, the authors (Bogolubsky et al. 2007) suggested preparation of several thieno[2,3-*d*]pyrimidine-6-carboxamides with benzylic substituents. For preparation of amides in this case Mukayama reagent (1.2 equivalent) was used with 2.44 equivalents DIPEA in MeCN; the reaction was performed in the pressure tube at 100 °C. The synthesis of modulators of orphan receptors of retinoic acid have been developed based on *N*-(benzyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamides (Claremon et al. 2017). The obtained compounds **IV** became part of a large study that was focused on development of that synthetic drugs that could treat inflammatory, autoimmune and metabolic disorders (Fig. 2).

In our recent research we revealed the promising antimicrobial activity for benzyl amides of 4,5-dimethylthienopyrimidines **V**, which were active against *B. subtilis* and *P. aeruginosa* and their mechanism of action was possibly due to the inhibition of TrmD (Vlasov et al. 2023). In view of the high activity of 4,5-dimethylthienopyrimidine-6-carboxylic acid benzyl amides we decided to obtain their previously unknown 4-oxo-analogs and test them for antimicrobial activity. Benzyl carboxamide pattern were found to be favourable for antimicrobial activi-

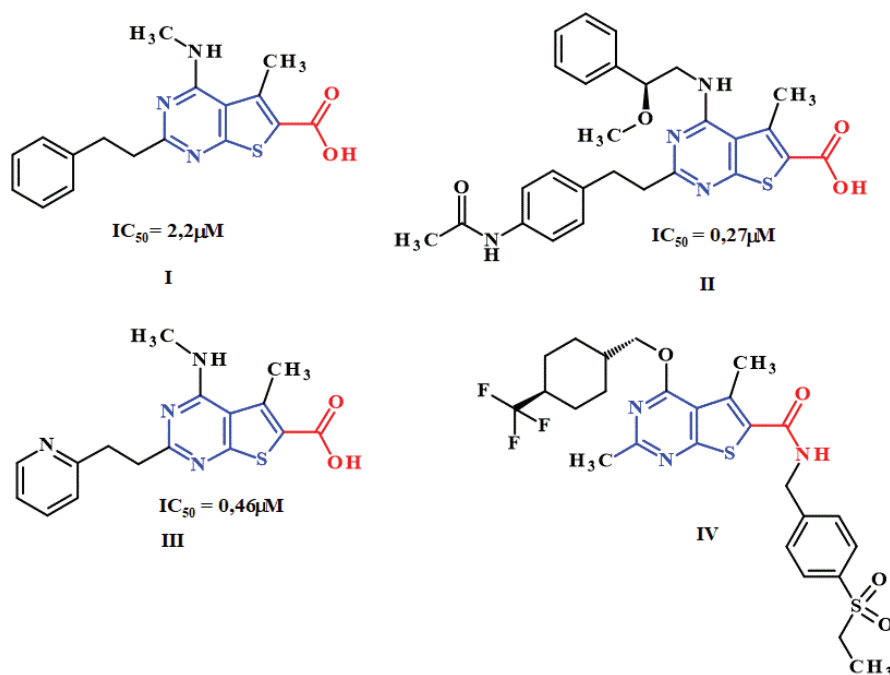
ty of thieno[2,3-*d*]pyrimidines at both position 5 (Zhong et al. 2019) and position 6 (Vlasov et al. 2023). Among thieno[2,3-*d*]pyrimidines with benzyl carboxamide group at position 5 high affinity to TrmD active site together with the high antimicrobial activity *in vitro* was reported for the compounds with oxo group at position 4 of thieno[2,3-*d*]pyrimidine fragment **VIa**. On the other hand, 4-Oalkyl analogues were found to be less active and displayed the binding results mostly at concentration higher than 50 μM (Zhong et al. 2019).

In order to check whether 4-oxo group might have been also beneficial in the structure of thieno[2,3-*d*]pyrimidin-6-carboxamides with benzyl fragments at amide substituent we decided to obtain the series of simply substituted *N*-(benzyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamides and test their antimicrobial activity with parallel docking studies to TrmD. Taking into account the low reactivity of the ester group in the ethyl 5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxylate, which complicated direct interaction with benzylamine, we decided to consider the possibility of using peptide couplings-reagents that allow easy synthesis of amides starting from carboxylic acids (El-Faham and Albericio 2011).

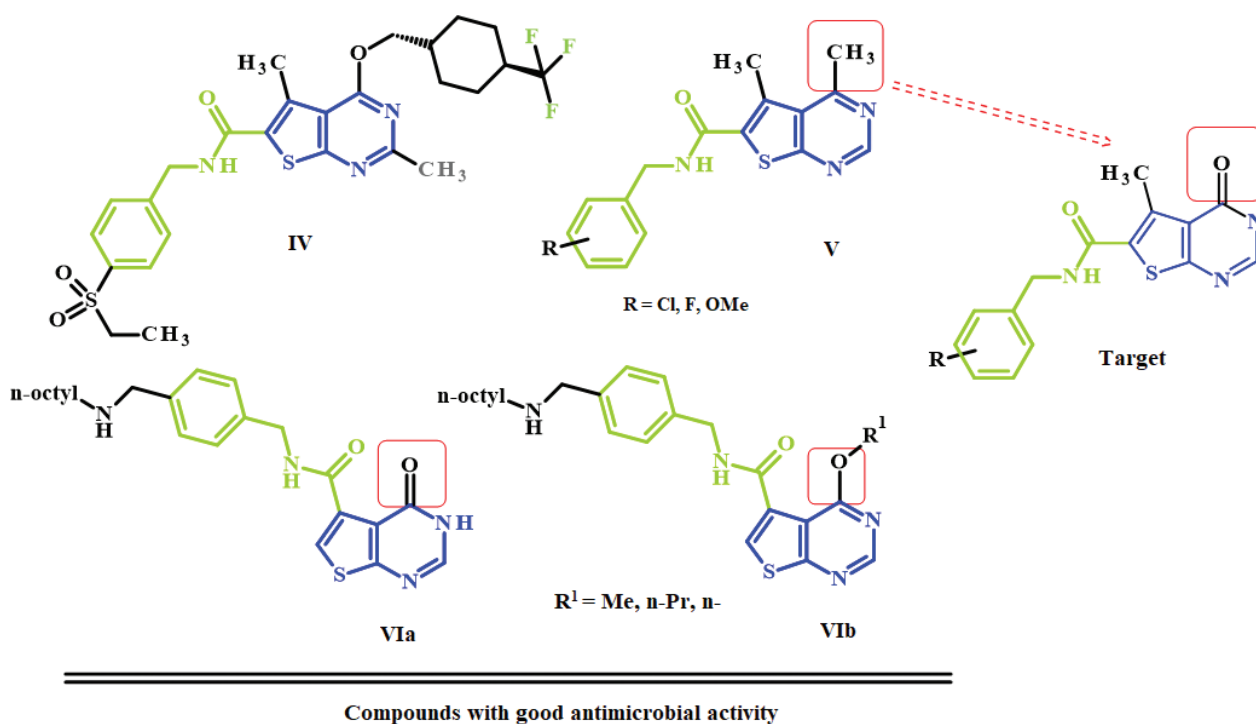
## Materials and methods

### Chemical part

All solvents and reagents were obtained from commercial sources. The melting points were determined in a capillary using an electrothermal IA9100X1 (Bibby Scientific Limited, Staffordshire, UK) digital melting point apparatus. The elemental analyses were performed on



**Figure 1.** The derivatives of thieno[2,3-*d*]pyrimidine-6-carboxylic acids with antimicrobial properties.



**Figure 2.** Design strategy for target thieno[2,3-*d*]pyrimidines with benzyl amides fragment as antimicrobial activity.

a Euro Vector EA-3000 (Eurovector SPA, Redavalle, Italy) microanalyzer and were within 0.4% of the theoretical values.  $^1\text{H}$  NMR spectra for the compounds **5.1**, **5.2**, **5.3** and **5.5** were recorded on Bruker Avance DRX 500 500 MHz spectrometers, respectively;  $^{13}\text{C}$  NMR spectra - on a Bruker Avance DRX 500 spectrometer at 125 MHz, respectively, solvents -  $\text{DMSO-}d_6$ , internal standard TMS ( $^1\text{H}$ ,  $^{13}\text{C}$ ). For the compound **5.4**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were acquired by Varian-600 at 600 MHz and 150 MHz respectively. LC/MS spectra were recorded on Agilent 1100 HPLC instrument equipped with diode matrix and mass detectors (Agilent LC-MSD SL), column Zorbax SB-C18 (4.6 mm  $\times$  15 mm). Atmospheric Pressure Chemical Ionization (APCI) was used in the experiment.

4-Oxo-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid was prepared according to the previously reported procedure (Mohamed et al. 2020) from its ethyl ester (Elmogy et al. 2023).

#### **General method of synthesis of *N*-benzylamides of 4-oxo-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid (5.1-5.5)**

To 0.25 g (0.00118 mol) of 4-oxo-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid and 0.195 g (0.0012 mol) of 1,1'-carbonyldiimidazole 1 ml of anhydrous dimethylformamide was added. The reaction mixture was heated at 50  $^\circ\text{C}$  for 10 minutes until the release of carbon dioxide was complete. Then to 0.0012 mole of the corresponding amine was added to the resulting solution of 4-oxo-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid imidazolide and the reaction flask was tightly closed to protect against moisture. The reaction mixture was

heated at 80  $^\circ\text{C}$  for 3 hours. Then the reaction mixture was cooled and quenched with water to form the crystalline product. The precipitate formed was filtered off and washed with plenty of cold water. The products were additionally crystallized from ethanol.

#### ***N*-Benzyl-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide 5.1.**

Yield – 0.293 g (83%), white crystals. M. p. 224–225  $^\circ\text{C}$ . Anal. Calcd. for  $\text{C}_{15}\text{H}_{13}\text{N}_3\text{O}_2\text{S}$ , % (299.34): C, 60.19; H, 4.38; N, 14.03. Found, C, 60.12; H, 4.45; % N, 14.17.  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$ , ppm: 2.70 (s, 3H,  $\text{CH}_3$ ), 4.43 (d, 2H,  $J = 5.2$  Hz,  $\text{CH}_2$ ), 7.28–7.36 (m, 4H, Ar-H), 8.14 (d, 1H,  $J = 4.8$  Hz, CH), 8.75 (t, 1H,  $J = 4.6$  Hz, NH amide), 12.53 (d, 1H,  $J = 4.6$  Hz, NH pyrimidine).  $^{13}\text{C}$  NMR (125 MHz,  $\text{DMSO-}d_6$ )  $\delta$ , ppm: 14.9; 42.8; 123.5; 126.8; 127.2; 127.6; 128.3; 136.1; 139.2; 147.2; 158.3; 161.9; 163.9. LC-MS  $m/z$  (ES+) 300.0 ( $\text{MH}^+$ ).

#### **5-Methyl-*N*-(4-methylbenzyl)-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide 5.2**

Yield – 0.262 g (71%), white crystals. M. p. 246–247  $^\circ\text{C}$ . Anal. Calcd. for  $\text{C}_{16}\text{H}_{15}\text{N}_3\text{O}_2\text{S}$ , % (313.37): C, 61.32; H, 4.82; N, 13.40. Found, % C, 61.41; H, 4.88; N, 13.51.  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$ , ppm: 2.26 (s, 3H,  $\text{CH}_3$ ), 2.68 (s, 3H,  $\text{CH}_3$ ), 4.37 (d, 2H,  $J = 5.5$  Hz,  $\text{CH}_2$ ), 7.12 (d, 2H,  $J = 7.5$  Hz, ArH), 7.19 (d, 2H,  $J = 7.8$  Hz, ArH), 8.13 (s, 1H, CH), 8.71 (t, 1H,  $J = 5.2$  Hz, NH amide), 12.52 (s, 1H, NHpyrimidine).  $^{13}\text{C}$  NMR (125 MHz,  $\text{DMSO-}d_6$ )  $\delta$ , ppm: 14.9; 20.6; 42.6; 96.8; 118.9; 123.5; 127.3; 128.8; 136.2; 147.2; 149.6; 152.3; 158.3; 163.9. LC-MS  $m/z$  (ES+) 314.0 ( $\text{MH}^+$ ).

### ***N*-(4-methoxybenzyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide 5.3**

Yield – 0.307 g (79%), white crystals. M. p. 209–210 °C. Anal. Calcd. for C<sub>16</sub>H<sub>15</sub>N<sub>3</sub>O<sub>3</sub>S, % (329.37): C, 58.35; H, 4.59; N, 12.75. Found, % C, 58.44; H, 4.60; N, 12.88. <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ, ppm: 2,68 (s, 3H, CH<sub>3</sub>), 3,71 (s, 3H, OCH<sub>3</sub>), 4,35 (d, 2H, *J* = 4,3 Hz, CH<sub>2</sub>), 6,88 (d, 2H, *J* = 8,5 Hz, ArH), 7,24 (d, 2H, *J* = 8,2 Hz, ArH), 8,13 (s, 1H, CH), 8,69 (br. s, 1H, NH amide), 12,52 (s, 1H, NHpyrimidine). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>) δ, ppm: 14.9; 42.3; 55.0; 113.7; 123.5; 127.8; 128.6; 131.1; 136.0; 147.2; 158.2; 158.3; 161.7; 163.9. LC-MS *m/z* (ES+) 330.0 (MH<sup>+</sup>).

### **5-Methyl-4-oxo-*N*-(4-fluorobenzyl)-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide 5.4**

Yield – 0.247 g (66%), white crystals. M. p. 235–236 °C. Anal. Calcd. for C<sub>15</sub>H<sub>12</sub>FN<sub>3</sub>O<sub>2</sub>S, % (317.33): C, 56.77; H, 3.81; N, 13.24. Found, % C, 56.82; H, 3.83; N, 13.25. <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ, ppm: 2.69 (s, 3H, CH<sub>3</sub>), 4.40 (d, 2H, *J* = 4.6 Hz, CH<sub>2</sub>), 7.11–7.18 (m, 2H, Ar-H), 7.31–7.38 (m, 2H, ArH), 8.13 (s, 1H, CH), 8.75 (br. s, 1H, NHamide), 12.52 (br. s, 1H, NHpyrimidine). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>) δ, ppm: 15.42; 42.66; 115.42; 115.55; 124.01; 127.97; 129.71; 129.76; 135.86; 135.88; 136.74; 147.71; 158.81; 160.83; 162.36; 162.44; 164.42. LC-MS *m/z* (ES+) 318.0 (MH<sup>+</sup>).

### **5-Methyl-4-oxo-*N*-(3,4-dichlorobenzyl)-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide 5.5**

Yield – 0.299 g (69%), white crystals. M. p. 255–256 °C. Anal. Calcd. for C<sub>15</sub>H<sub>11</sub>Cl<sub>2</sub>N<sub>3</sub>O<sub>2</sub>S, % (368.23): C, 48.93; H, 3.01; N, 11.41. Found, % C, 49.03; H, 3.12; N, 11.58. <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ, ppm: 2.70 (s, 3H, CH<sub>3</sub>), 4.41 (d, 2H, *J* = 5.2 Hz, CH<sub>2</sub>), 7.31 (d, 1H, *J* = 8,2 Hz, Ar-H), 7.53–7.62 (m, 2H, ArH), 8.14 (s, 1H, CH), 8.78 (br. s, 1H, NHamide), 12.52 (br.s, 1H, NHpyrimidine). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>) δ, ppm: 14.9; 41.9; 123.6; 127.1; 127.7; 129.3; 130.5; 130.8; 136.6; 140.5; 147.3; 158.3; 162.0; 164.0. LC-MS *m/z* (ES+) 370.0 (MH<sup>+</sup>).

## **Microbiological studies**

The study of the antimicrobial activity of the synthesized compounds was carried out at the Laboratory of Biochemistry of Microorganisms and Nutrient Media of the Mechnikov Institute of Microbiology and Immunology of the NAMS of Ukraine (Kharkiv) under the supervision of Candidate of biological sciences, senior researcher T. P. Osolodchenko.

The antimicrobial activity of the obtained compounds was evaluated in accordance with WHO recommendations (Coyle 2005) on test strains of *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Proteus vulgaris* ATCC 4636, *Candida albicans* ATCC 885/653.

For the agar well diffusion method, the microbial load was 10<sup>7</sup> microbial cells per 1 ml of medium and was determined according to the McFarland standard (McFarland 1907). An 18–24-hour culture of microorganisms was used. The microbial suspension was prepared according to the information letter of the Ministry of Health of Ukraine (Kyiv 2001). Muller-Hinton agar was used for the study of bacteria and Sabouraud agar was used for the *Candida albicans* strain. The tested compounds were introduced by into agar “wells” (Magaldi et al. 2004) in the form of a solution in DMSO at a concentration of 100 µg/ml in a volume of 0.3 ml, the comparison compound “Mepepnam” - in the form of a solution in DMSO (100 µg/ml). Antibacterial activity was assessed by measuring growth inhibition zones of the corresponding microorganism, the experiment was repeated three times. Preparation of the microbial suspension of microorganisms was carried out using the Densi-La-Meter device (manufactured by PLI-VA-Lachema, the Czech Republic; wavelength 540 nm).

When assessing the antimicrobial activity of the compounds, the following criteria were used (Magaldi et al. 2004; Nekrasova et al. 2007): the absence of a zone of inhibition of the growth of the microorganism or a zone of inhibition not exceeding 10 mm was considered as the lack of sensitivity of the microorganism to the compound or insufficient concentration of the studied substances; a zone of growth inhibition with a diameter of about 10–15 mm — low sensitivity of the culture of the microorganism to the substance under investigation at this concentration; zone of growth inhibition with a diameter of 15–25 mm — sensitivity of the microorganism to the studied substance; zone of growth retardation, the diameter of which exceeds 25 mm — high sensitivity of microorganisms to the studied substances.

## **Docking studies**

Molecular docking was performed using the AutoDock Vina and AutoDockTools 1.5.6 programs. A macromolecule from the PDB (Protein Data Bank 2023): TrmD *Pseudomonas aeruginosa* PDB ID – 5ZHN was used as a target protein. Construction of a virtual database of candidate structures was carried out using the BIOVIADraw 2021 program and saved in .mol format. The structures were optimized by Chem3D using the MM2 molecular mechanics algorithm, saved in .pdb format and converted to .pdbqt using AutoDockTools-1.5.6. Discovery Studio Visualizer 2021 Client was used to remove the solvent and native ligand from the protein. The prepared macromolecule was saved in .pdb format. Polar hydrogen atoms were added to the protein structure by AutoDockTools-1.5.6 and saved in .pdbqt format. The size of the Grid box and its centre were determined by the native ligand of subunit A: TrmD (PDB ID 5ZHN): *x* = 40.04, *y* = 107.23, *z* = -3.40; size *x* = 18, *y* = 22, *z* = 20. To validate the docking method the native ligand, compound **VIa** – *N*-(4-((octylamino)methyl)-benzyl)-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-5-carboxamide (Zhong et al. 2019), was extracted

and then used for the re-docking process after the number of torsions was set and saved as .pdbqt. AutoDock Vina was used for docking. The calculation of RMSD value was performed with ProFit Results available online. RMSD value between the native and the reference conformation of the compound **VIa** equals 1.952 Å, therefore the study is reliable. Visualization and analysis of the obtained docking results are performed by Discovery Studio 2021 Client.

## Result and discussion

The first step of the synthetic scheme is the hydrolysis of ethyl 5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxylate (Elmongy et al. 2023) in order to convert it into 5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxylic acid (Mohamed et al. 2020). The reaction was carried out by alkaline hydrolysis of the ester under the action of a threefold excess of sodium hydroxide in an aqueous medium at slight boiling. The acid was isolated by acidification an aqueous solution of sodium salt with orthophosphoric acid followed by filtering the product and thoroughly washing it with a large amount of water.

For the coupling of 5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxylic acid with the series of benzylamines we used 1,1'-carbonyldiimidazole which showed its high efficacy in our recent research (Vlasov et al. 2023) (Scheme 1). Despite its age, 1,1'-carbonyldiimidazole is still popular and its advantages include efficiency, sufficient cheapness, and a minimum of by-products formed. Therefore, we decided to apply it and try it as a peptide-coupling reagent for the reaction between benzylamines and 5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxylic acid. An important advantage when planning the synthesis was the variability of solvents, which are suitable for interaction with 1,1'-carbonyldiimidazole. We chose dimethylformamide, which can be easily dehydrated using zeolites.

The synthetic scheme in this case requires a two-stage procedure. At the first stage, a DMF soluble imidazolide is gen-

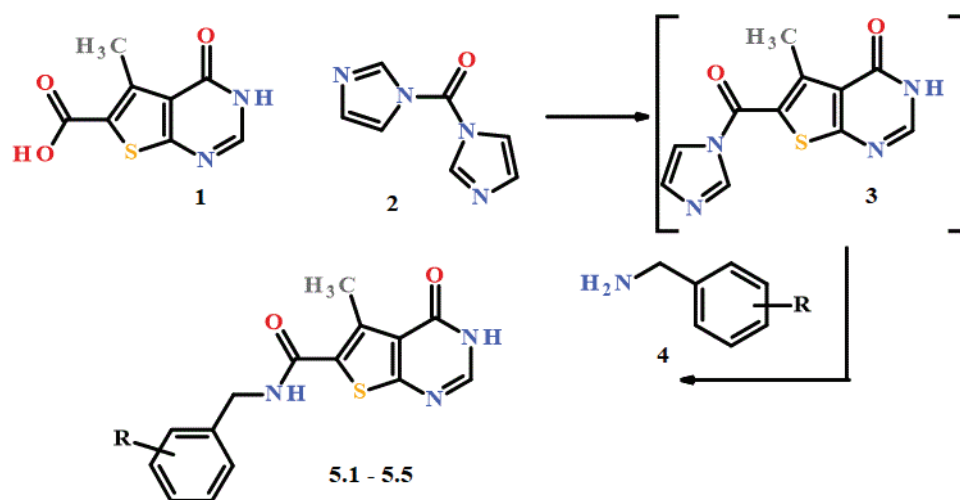
erated by mixing 5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxylic acid with a coupling reagent in dimethylformamide under slight heating, this process usually lasts 10–15 minutes. At the second stage, the corresponding amine is added directly to the same reactor without significant changes in the regime. In this way, both stages of the process can be performed one-pot, which makes the scheme more economical. The procedure does not require high temperature or pressure. Moreover, their isolation is achieved by diluting the reaction mixture with water.

The structure of all obtained target benzylamides of 5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxylic acid **5** was confirmed using a number of modern instrumental methods of analysis.

According to the data of <sup>1</sup>H NMR spectroscopy, all the obtained amides are characterized by signals of amide NH protons in the range of 8.69–8.78 ppm, which has the form of a triplet, indicating its proximity to the methylene group of the benzylic fragment. The signal of the last group has the form of a doublet and is observed in the range of 4.35–4.43 ppm.

All amides have the typical signal of the NH group in position 3 of the dihydrothieno[2,3-*d*]pyrimidine system, which is observed in the range of 12.52–12.53 ppm. and has the appearance of a broaden singlet. Spectral data show that the chemical shift of this signal depends little on the type of the substituent in the amide fragment, which additionally indicates the proper assignment of this signal. The presence of a pyrimidine ring with a free position 2 is evidenced by the presence of a clear CH signal in the range of 8.13–8.14 ppm, which appears as a sharp singlet. The signals of the methyl group of the thiophene nucleus are observed as a singlet in the range of 2.68–2.70 ppm.

The obtained compounds **5** has a clear pattern of signals, which corresponds to the substitution of the aromatic part of the benzyl amide fragment. Thus, in the case of *N*-benzyl-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide **5.1**, the following pattern of signals are observed in the resonance region of aromatic protons 7.28–7.36 (m, 4H, Ar) and 8.14 (d, 1H, *J* = 4.8 Hz,



Scheme 1.

CH). For 5-methyl-*N*-(4-methylbenzyl)-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide **5.2** and *N*-(4-methoxybenzyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide **5.3** the signals typical for AA'BB' systems, such as two groups of doublet signals are observed in at 7.12 (d, 2H, *J* = 7.5 Hz) and 7.19 (d, 2H, *J* = 7.8 Hz) for the methyl derivative **5.2** while for the methoxy derivative **5.3** the signals are at 6.88 (d, 2H, *J* = 8.5 Hz) and 7.24 (d, 2H, *J* = 8.2 Hz) correspondingly.

The CH<sub>3</sub> signal of the substituent is in the spectrum of amide **5.2** is at 2.26 (s, 3H, CH<sub>3</sub>), and the methoxy group signal of the compound **5.3** is at 3.71 (s, 3H, OCH<sub>3</sub>). The presence of such a substituent as a fluorine atom in 5-methyl-4-oxo-*N*-(4-fluorobenzyl)-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide **5.4** contributes to the splitting of the signals of aromatic protons and they appear as a multiplet 7.11–7.18 (m, 2H, Ar-H), 7.31–7.38 (m, 2H, Ar-H). In the spectrum of 3,4-dichlorosubstituted derivative **5.5**, the aromatic proton signals have the following form: 7.31 (d, 1H, *J* = 8.2 Hz, Ar-H), 7.53–7.62 (m, 2H, Ar-H).

In order to evaluate the structure and purity of the compounds, the method of liquid chromatography-mass spectrometry (LC-MS) was used, which allowed us to confirm the purity of the obtained samples and to prove that the molecular weight of the obtained samples corresponds to the suggested structures.

We used the <sup>13</sup>C NMR spectroscopy method, which clearly showed the presence of the appropriate number of carbon atoms for each sample of amides **5** and allowed

us to confirm the presence of fragments that contain groups with carbon atoms as substituents. Thus, signals of CH<sub>3</sub> groups in the thiophene nucleus are observed at 14.9 ppm. The signals of the CH<sub>2</sub> groups is observed in the region 41.9–42.8 ppm and their position depends much on the substituent. For 5-methyl-*N*-(4-methylbenzyl)-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide, the signal of the methyl group at the phenyl ring is observed at 20.6 ppm, and the signal of the methoxy group for *N*-(4-methoxybenzyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamide **5.3** it is present at 55.0 ppm.

The study of antimicrobial activity against standard strains of microorganisms has been carried out for the synthesized *N*-benzylamides of 4-oxo-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid **5**. The study was conducted in comparison with the drug Meropenem, which is a modern and very effective antibiotic. The results of the research are presented in Table 1.

To predict the possible mechanism of antibacterial action of target thienopyrimidine derivatives **5**, molecular docking was carried out in the active site of selective inhibitors of tRNA (Guanine37-*N*<sup>1</sup>)-methyltransferase (TrmD) isolated from *P. aeruginosa* (Zhong et al. 2019). The procedure for re-docking of the native ligand – *N*-(4-((octylamino)methyl)benzyl)-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-5-carboxamide – and its visualization have been performed and described in our previous studies (Vlasov et al. 2021).

The studied thienopyrimidines demonstrated different degrees of affinity to the TrmD inhibitor site (Table 2).

**Table 1.** Antibacterial activity of *N*-(benzyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamides **5.1–5.5**.

Compound/R	Diameters of zones of growth retardation in mm, number of repetitions of the experiment, n = 3					
	<i>Staphylococcus aureus</i> ATCC 25923	<i>Escherichia coli</i> ATCC 25922	<i>Proteus vulgaris</i> ATCC 4636	<i>Pseudomonas aeruginosa</i> ATCC 27853	<i>Bacillus subtilis</i> ATCC 6633	<i>Candida albicans</i> ATCC 653/885
<b>5.1/H</b>	24, 23, 24	23, 23, 24	21, 22, 21	22, 22, 22	24, 24, 24	20, 21, 21
<b>5.2/4-CH<sub>3</sub></b>	22, 23, 23	20, 21, 20	20, 21, 21	21, 21, 22	23, 24, 23	17, 16, 17
<b>5.3/4-OCH<sub>3</sub></b>	23, 22, 22	20, 21, 21	19, 19, 19	20, 20, 20	23, 24, 23	18, 17, 17
<b>5.4/4-F</b>	19, 21, 21	19, 20, 20	19, 20, 19	21, 20, 20	21, 22, 23	18, 18, 18
<b>5.5/3,4-diCl</b>	22, 21, 22	20, 21, 20	18, 19, 19	19, 19, 19	22, 22, 22	17, 19, 18
<b>Mepenam</b>	35, 34, 34	31, 32, 32	30, 30, 31	32, 32, 32	35, 34, 35	15, 14, 15

**Table 2.** Docking results of *N*-substituted benzyl-5-methyl-4-oxo-thieno[2,3-*d*]pyrimidine-6-carboxamide **5.1–5.5** in the active site of the inhibitor TrmD isolated from *P. aeruginosa*.

Ligand	Binding energy kcal/mol	Hydrophobic interaction	Hydrogen interaction	Other interaction
Native ligand	-8.2	Tyr141, Ser93(2) <sup>#</sup> , Pro94 (4), Pro149(2), Ile138, Leu143, Gly145, Gly146	Leu143, Gln95, Glu121, Gly139, Asp182	-
<b>5.1 / H</b>	-9.7	Ser93, Pro94(5), Tyr91*, Leu143, Pro149,	Tyr91*, Asp182	-
<b>5.2 / 4-CH<sub>3</sub></b>	-9.7	Ser93, Pro94(5), Leu92*, Ile138 Pro149(2), Tyr91*, Leu143	Asp182(2), Pro94	-
<b>5.3 / 4-OCH<sub>3</sub></b>	-9.4	Ser93, Pro94(4), Gly145, Gly146, Val142*, Leu143, Pro149	Ile138, Asp182, Gly145, Trp136*	-
<b>5.4 / 4-F</b>	-10.1	Ser93, Pro94(5), Tyr91*, Leu143, Pro149	Tyr91*, Ile138, Ser137*, Asp182	Trp136* Halogen
<b>5.5 / 3,4-diCl</b>	-10.1	Ser93, Pro94(5), Gly145, Gly146, Ile138(2), Leu92*, Pro149(2), Tyr91, Leu143	Gln95, Asp182(2), Pro94, Ser137*	Trp136* Halogen

<sup>#</sup>The number of interactions is indicated in brackets;

\* Amino acids which do not interact with the native ligand.

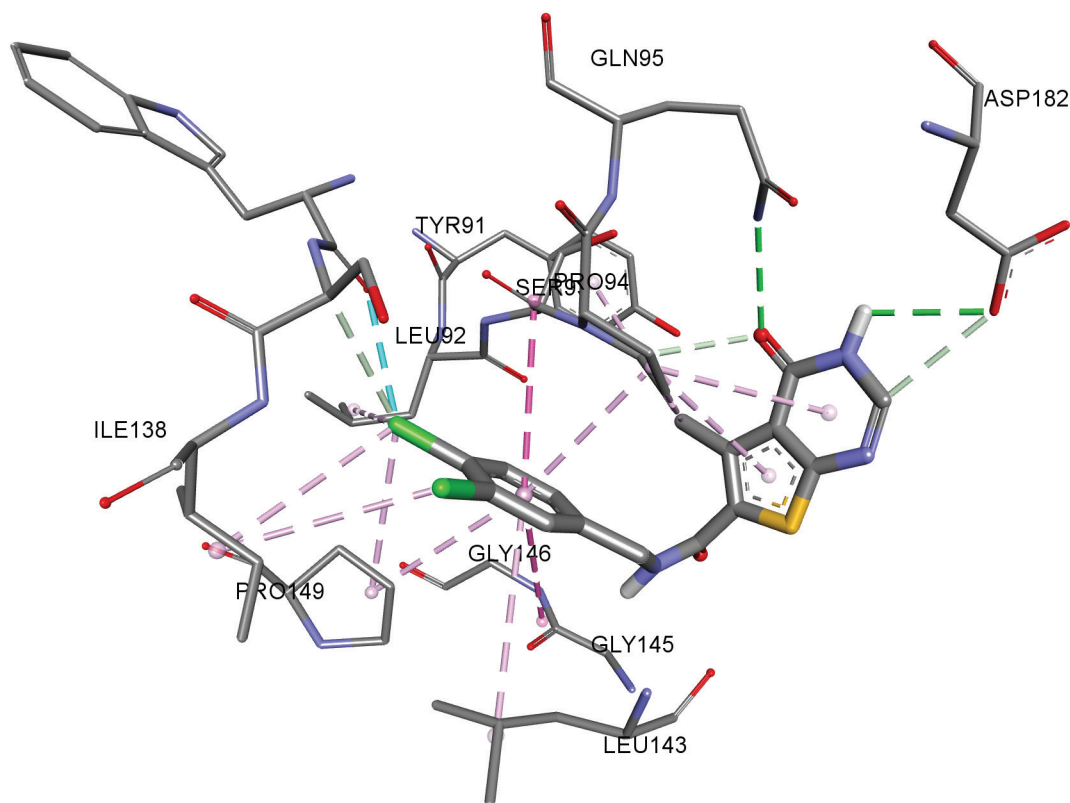
For all studied benzamide derivatives, a high degree of affinity to the site of the TrmD inhibitor was calculated, which exceeds the affinity of the native reference ligand: from -9.7 to -10.1 kcal/mol relative to -8.2 kcal/mol, respectively. The best affinity values were predicted for derivatives 5.4 and 5.5 substituted by halogen in the benzyl radical.

The possibility of forming a stable conformation with a branched network of hydrophobic interactions and additional fixation by hydrogen bonds was recorded (Fig. 3). The tetrahedral network of interactions between the pyrrolidine ring of proline Pro94, the thienopyrimidine ring and the methyl radical in position 5 indicates conformational stability in the active site. Only compounds 5.3 and 5.5 were able to interact with the glutamic acid residues Gly145, 146 and only compound 5.5 form the hydrogen bond with glutamine Gln95; all of these amino acids are important for binding with S-adenosylmethionine (SAM) a co-factor of TrmD. That means that most of the ligands 5 except of 5.3 and 5.5 cannot deeply immerse into the active site and effectively compete with SAM and thus should not be considered as the highly effective ligands of TrmD. In addition, according to experimental data, interaction with tyrosine residues Tyr141 and Tyr120 (Zhong et al. 2019), which are not visualized even in the immediate environment during docking of the studied compounds, is key to the manifestation of the inhibitory effect on TrmD.

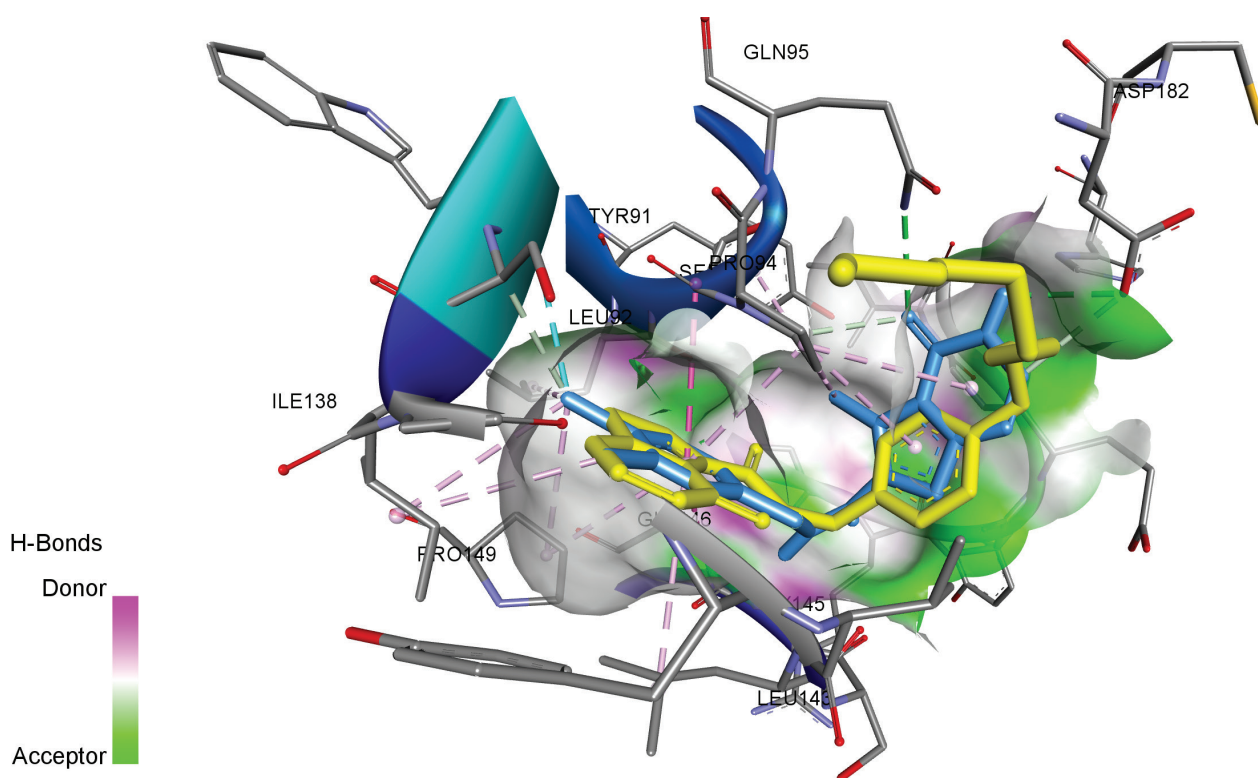
Additional prediction of Halogen Acceptor interactions of chlorine atom (compound 5.5) and fluorine atom (compound 5.4) with the carbonyl group of tryptophan (Trp136) can explain the better values of their scoring

functions (Table 2). On the other hand, tryptophan is not an acid of the active site and its interaction may hardly be the marker for inhibitory properties. The compatible conformation of the reference ligand and 5.5 (Fig. 4) demonstrates the absence of overlap by thienopyrimidine fragments, and immersion into the depth of the hydrophobic pocket occurs with the benzamide, not the thienopyrimidine fragment of compound 5.5.

The suggested procedure for *N*-(benzyl)-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxamides synthesis starting from readily available 4-oxo-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid was found to be highly effective and produced us the target molecules with high yields and high purity. The results of antimicrobial activity screening showed that most amides 5 have high antimicrobial activity against the strain of *Staphylococcus aureus* and *Bacillus subtilis* and their antimicrobial activity against *Pseudomonas aeruginosa* is lesser. Moreover, compounds with an unsubstituted benzene ring or light substituents such as methyl (-CH<sub>3</sub>) or methoxyl (-OCH<sub>3</sub>) groups in the para-position of the benzene nucleus turned out to be the most active in relation to all of the bacterial strains. The expected activity against *Pseudomonas aeruginosa* strain was not as high as the activity of the previously reported 4-thieno[2,3-*d*]pyrimidine analogues (Vlasov et al. 2023). The docking studies revealed that despite the good performance of the scoring functions, the analysis of the conformational placement indicates their ability for moderate inhibition of TrmD isolated from *Pseudomonas aeruginosa*.



**Figure 3.** 3D visualization of the interaction of ligand 5.5 with the amino acid residues of the active site of the TrmD inhibitor of TrmD *P. aeruginosa*.



**Figure 4.** 3D visualization of the compatible conformation the ligand 5.5 (blue molecule) and the reference inhibitor (yellow molecule) (Zhong et al. 2019) in the active site of TrmD *P. aeruginosa*.

## Conclusions

A series of *N*-benzylamides of 4-oxo-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid were synthesized by peptide coupling the starting acid with substituted benzylamines upon its activation with 1,1'-carbonyldiimidazole. The study of the antimicrobial activity of *N*-benzylamides of 4-oxo-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid by the agar-well diffusion method showed their high activity against the strain of *Staphylococcus aureus* and *Bacillus subtilis* and lesser activity against *Pseudomonas aeruginosa*. The compounds with an unsubstituted benzene ring or light substituents such as methyl (-CH<sub>3</sub>) or methoxyl (-OCH<sub>3</sub>) groups in the para-position of the benzene nucleus were most active against both strains. Docking studies to the active site of TrmD inhibitors isolated from *P. aeruginosa* showed that despite the good values of the scoring functions, the conformational analysis indicated the ability for moderate inhibition of TrmD of *P. aeruginosa*.

The suggested methods for synthesis of benzylamides and the docking to TrmD can be applied for

more thieno[2,3-*d*]pyrimidine-6-carboxylic acids as the starting compounds for preparation of the novel series of benzyl amides suitable for the development of new antimicrobials.

## Acknowledgements

The authors acknowledge Enamine Ltd. for the measurement of <sup>1</sup>H, <sup>13</sup>C NMR and LC-MS spectra of the obtained substances. We are grateful to T.P. Osolodchenko (Mechanikov Institute of Microbiology and Immunology of the NAMS of Ukraine, Kharkiv, Ukraine) for her assistance in carrying out antimicrobial research.

The research was funded by the Ministry of Health Care of Ukraine at the expense of the State Budget in the framework # 2301020 "Scientific and scientific-technical activity in the field of health protection" on the topic "Synthesis and study of new thienopyrimidines for the detection of antimicrobial and related types of pharmacological activity" (Order of the Ministry of Health of Ukraine of November 17, 2020 № 2651).

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