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BIOISOSTERIC REPLACEMENT IN THE SEARCH FOR ANTIMICROBIAL AGENTS: DESIGN, SYNTHESIS AND ACTIVITY OF NOVEL 6-(1*H*-BENZIMIDAZOL-2-YL)-1-ALKYL-3,5-DIMETHYLTHIENO[2,3-d]PYRIMIDINE-2,4(1*H*,3*H*)-DIONE DERIVATIVES

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The aim. *To apply the concept of bioisosterism for the structural optimization of benzimidazole–thieno[2,3-d] pyrimidine hybrids aimed at developing effective antibacterial agents as potential inhibitors of the bacterial enzyme TrmD.*

Materials and methods. *Organic synthesis methods; structure confirmation by ^{1}H, ^{13}C, and HMBC NMR spectroscopy, LC-MS, and elemental analysis. Molecular docking was performed using AutoDock Vina, AutoDockTools 1.5.6, and Discovery Studio Client. Antimicrobial activity was evaluated using the agar diffusion method, and the impact on biofilm formation/disruption was assessed using the crystal violet assay.*

Results and discussion. *The bioisosteric modification involved oxidation of the thione group in 3,5-dimethyl-4-oxo-2-thioxothieno[2,3-d]pyrimidine-6-carboxylate. The resulting 2,4-dioxothieno[2,3-d]pyrimidine-6-carboxylic acid was activated with 1,1'-carbonyldiimidazole to form a benzimidazole fragment in a one-pot procedure. Alkylation of the obtained hybrid with chloroacetamides led to regioselective products confirmed by HMBC. All synthesized compounds demonstrated significant antimicrobial activity against both Gram-positive and Gram-negative test strains. Compound 5c with a 4-ethoxyphenyl substituent processed the highest activity, including effectiveness against clinical strains of S. aureus and P. aeruginosa. Compound 5c also demonstrated notable biofilm disruption capacity against S. aureus, E. coli, P. aeruginosa, and C. albicans. Molecular docking to the TrmD bacterial enzyme confirmed the formation of a hydrogen bond between the 2-oxo group and Glu121.*

Conclusions. *An efficient method was developed for the synthesis of a novel series of 2-[6-(1H-benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2H)-yl]-N-arylacetamides. Bioisosteric hybrids showed enhanced antimicrobial properties and improved binding affinity to bacterial TrmD. Compound 5c demonstrated high activity against both Gram-positive and Gram-negative strains, including clinical isolates, as well as the ability to disrupt biofilms, highlighting its potential as a promising lead for further development*

Keywords: *thienpyrimidine, benzimidazole, alkylation, antimicrobial activity, docking*

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1. Introduction

Compounds bearing the thieno[2,3-d]pyrimidine core are considered highly promising biologically active agents and have served for many years as scaffolds for drug development [1]. Interest in these derivatives is largely driven by their structural resemblance to purine bases, their synthetic accessibility, and the wide potential for structural modification – particularly at positions 2, 4, 5, and 6 – to enhance target selectivity, solubility, or pharmacokinetic properties. The strategy of modifying structures with already established bioactivity in order to improve their properties remains one of the central approaches in medicinal and pharmaceutical chemistry. This concept is well demonstrated in the development of thienopyrimidine-based anticancer agents targeting various tumor cell lines [2–6]. A recent example of successful structure-based optimization is the FDA-approved drug Relugolix (TAK-385), a gonadotropin-releasing hormone (GnRH) receptor antagonist, which emerged from the optimization of a large library of analogues, including Sufugolix (Fig. 1) [7, 8]. Structural optimization at the 5- and 3-positions of the thieno[2,3-d]pyrimidine-2,4-dione was carried out to reduce cytochrome P450 inhibition, improve in vivo GnRH antagonistic activity, and enhance oral bioavailability [9].

A large cluster of research has been devoted to the investigation of the antimicrobial activity of thieno[2,3-d]pyrimidine derivatives. Among these, several compounds have demonstrated notable activity against protozoa [10–12], viruses [13–15], fungi [16, 17], and a

wide range of Gram-positive and Gram-negative bacteria [18–21], including *Mycobacterium tuberculosis* [22]. An illustrative example of successful structure-based optimization leading to improved antibacterial activity and selectivity involves the replacement of a terminal 4-aminopiperidine moiety with an *n*-octyl group at position 5 of the thieno[2,3-d]pyrimidine core. This modification enhanced conformational compatibility with the active site of bacterial tRNA-(N^1G^{37}) methyltransferase (TrmD), a validated target involved in protein synthesis fidelity (Fig. 1) [23, 24].

The broad spectrum of antimicrobial properties of thieno[2,3-d]pyrimidines makes this group of compounds very promising for the development of drug candidates. Particularly in the context of increasing microbial resistance to treatment due to the improper use of antibacterial agents in medicine, veterinary practice, and agriculture, this has given rise to the so-called 'silent pandemic,' which by 2050 could surpass other leading causes of death [20]. The urgent need for the discovery of new therapeutic agents with mechanisms of action different from the existing ones is one of the key ways to tackle bacterial resistance [21, 22, 25].

The aim of the research. The concept of bioisosterism [26–28] was applied to optimize previously studied thieno[2,3-d]pyrimidine derivatives and design more potent TrmD inhibitors as prospective antibacterial agents with a novel mechanism of action. The structural modification involved replacing the thione group at position 2 of the thieno[2,3-d]pyrimidine core with a carbonyl group and relocating the acetamide moiety to the nitrogen atom at position 1.

2. Planning (methodology) of research

Our previous work on the design of thieno[2,3-d] pyrimidine derivatives as antimicrobial agents served as the foundation for the current study [29–31]. The promising potential of hybrid molecules combining benzimidazole and thieno[2,3-d]pyrimidine scaffolds was previously demonstrated [29], and the elucidated mechanism of antimicrobial action prompted us to explore bioisosteric replacement as a strategy to improve binding within the active site of bacterial TrmD, thereby enhancing inhibitory potency (Fig. 2).

Bioisosteric substitution is one of the most effective approaches to structural modification, allowing the retention or enhancement of biological activity while reducing toxicity, increasing metabolic stability, or improving pharmacokinetic profiles [32].

In the course of designing the target compounds, a bioisosteric replacement of the acetamide fragment was performed by relocating it from the thione group of the analogues to the adjacent heterocyclic nitrogen at position 1 of the thieno[2,3-d]pyrimidine system. Additionally, the sulfur atom at position 2 was substituted with an oxygen atom (Fig. 1). This bioisosteric replacement enables fine-tuning of several ADMET-related parameters of the designed compounds compared to their thione analogues. In particular, it allows for a reduction in molecular weight, molar refractivity, and topological polar surface area (TPSA), as well as the adjustment of lipophilicity to more favorable ranges. Additionally, the introduction of a carbonyl group at position 2 of the thieno[2,3-d]pyrimidine ring may facilitate the formation of additional hydrogen bonds within the active site of the bacterial enzyme TrmD, thereby enhancing binding affinity [33].

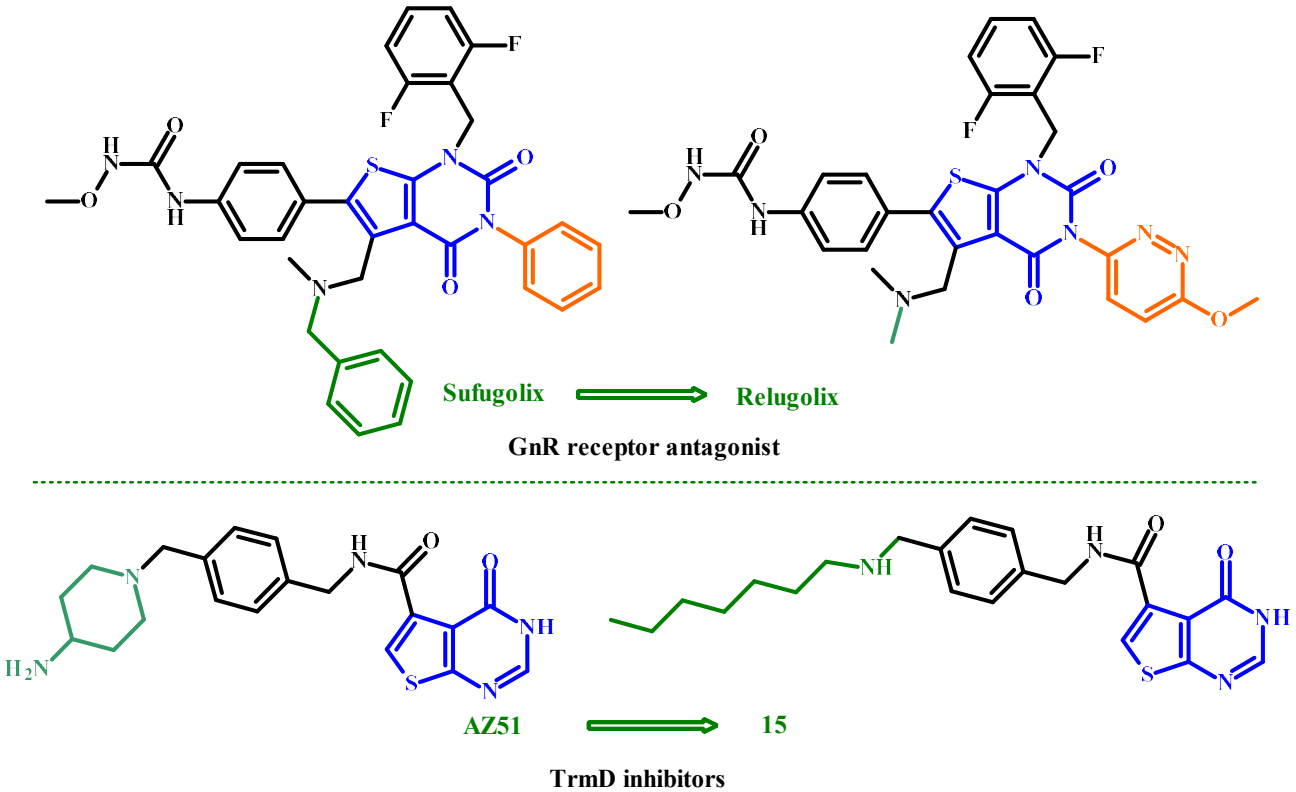


Fig. 1. Examples of structural modification of thieno[2,3-d]pyrimidine derivatives

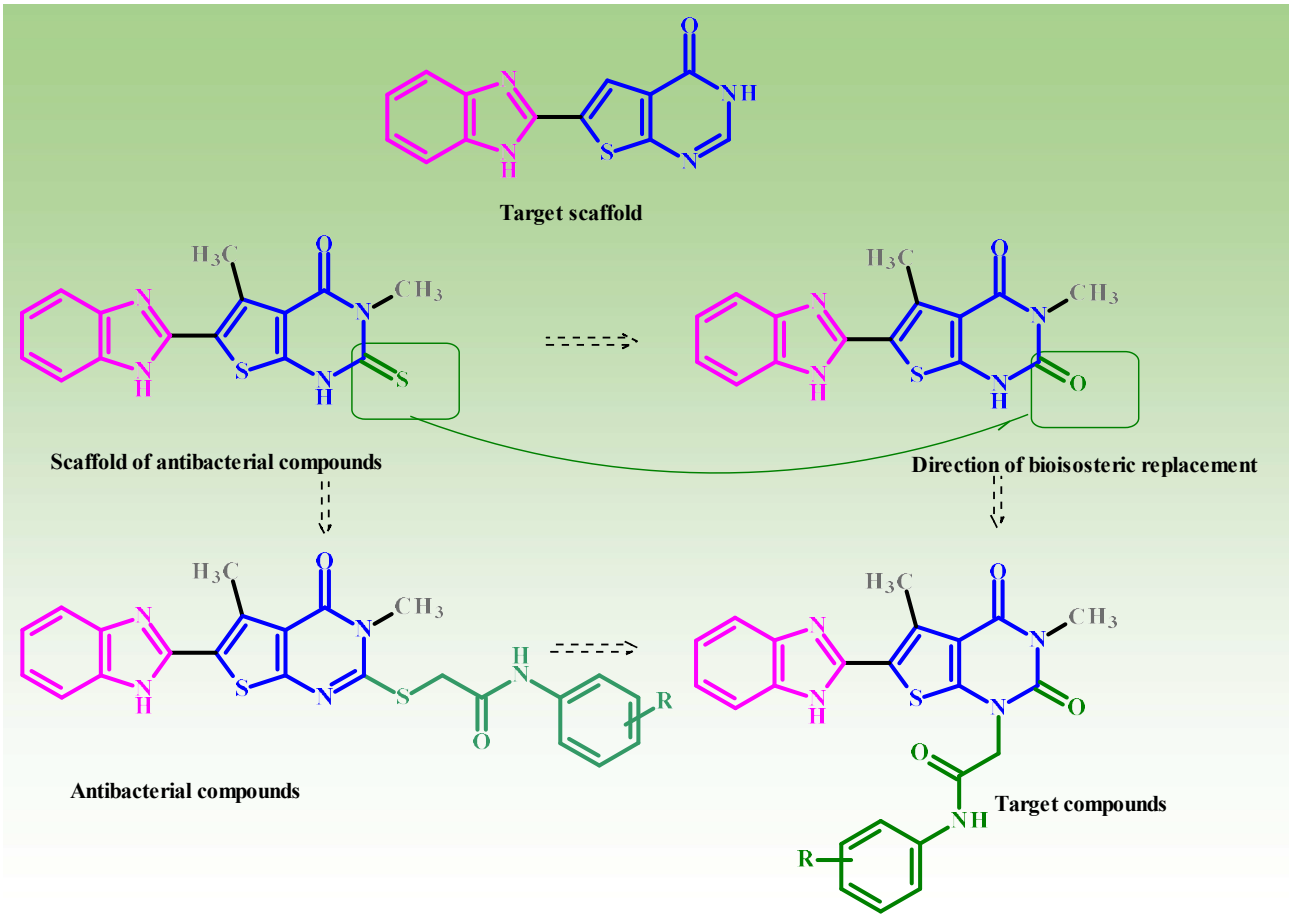


Fig. 2. Design strategy for target compounds with antimicrobial activity

The next stages of the study involved the development of efficient synthetic routes for the target bioisosteres, specifically the 2-[6-(1*H*-benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2H)-yl]-N-arylacetamides, along with comprehensive confirmation of their structures and chemical individuality.

A screening study of all synthesized bioisosteric molecules was planned in order to identify the most promising candidates for further evaluation against clinically isolated microbial strains and to assess their potential to inhibit or disrupt bacterial biofilms, which are a major factor contributing to microbial resistance to antimicrobial therapy.

3. Materials and methods

3. 1. Chemistry

All the convenient solvents and commercially available reagents, including chromatography grades, were used with no additional purification; for additional purification of dimethylformamide, molecular sieves 4Å were applied for 72 hours. For the structural analysis by NMR ^{1}H and ^{13}C, the devices Bruker 170 Avance 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei) and Varian Mercury-400 (400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei) were used with reference to tetramethylsilane. The solvents for NMR studies were deuterated (DMSO-d_6). HMBC correlation spectrum was measured with Bruker 170 Avance 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei). LC-MS spectra were obtained on Shimadzu 10-AV LC, Gilson-215, equipped with an autosampler, with the following detectors: UV (215 and 254 nm), electrospray ionization mass spectrometer (API 150EX), and ELS detectors. For the chromatographic part of the device, a Luna-C18 column, Phenomenex, 5 cm × 2 mm, was used. Euro Vector EA-3000 CHNS apparatus was used for elemental analyses. Kofler hot-bench device was used for measurements of the melting points.

The starting ethyl 3,5-dimethyl-4-oxo-2-thioxo-1,2,3,4-tetrahydrothieno[2,3-d]pyrimidine-6-carboxylate **1** was obtained by the previously reported methods [34].

Ethyl 3,5-dimethyl-2,4-dioxo-1,2,3,4-tetrahydrothieno[2,3-d]pyrimidine-6-carboxylate **(2).**

To 7 g (0.0246 mol) of ethyl 3,5-dimethyl-4-oxo-2-thioxo-1,2,3,4-tetrahydrothieno[2,3-d]pyrimidine-6-carboxylate **1** 3.0 g (0.074 mol) of sodium hydroxide and 22 ml (0.22 mol) of 30% hydrogen peroxide solution were added at 5°C. The mixture was stirred for 72 hours and then acidified with H_3PO_4. The precipitate formed was filtered off and washed with plenty of water. The solid was dried at 65°C. Yield 6.6 g, 82%. M.p. 243–245°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.24 (2H, *q*, *J* = 7.0 Hz, CH_2), 3.16 (3H, *s*, CH_3), 2.71 (3H, *s*, CH_3),

1.27 (3H, *t*, *J* = 7.0 Hz, CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.1, 159.5, 154.1, 150.6, 144.9, 116.2, 114.0, 61.2, 27.1, 14.7, 14.5. LC-MS, m/z: 269 $[MH]^+$. Anal. Calcd. for $C_{11}H_{12}N_2O_4S$: C, 49.25; H, 4.51; N, 10.44. Found: C, 49.34; H, 4.58; N, 10.50.

3,5-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydrothieno[2,3-d]pyrimidine-6-carboxylic acid **(3).**

To 6 g (0.0223 mol) of ethyl 3,5-dimethyl-2,4-dioxo-1,2,3,4-tetrahydrothieno[2,3-d]pyrimidine-6-carboxylate **2** 20 ml of water and 2.7 g (0.067 mol) of sodium hydroxide were added, and the reaction mixture was heated at 70–80°C for 15 hours. Then the solution was acidified with 7.7 ml of orthophosphoric acid (85%), and the precipitate formed was filtered off and washed with plenty of water. The resulted acid **3** was dried at 50°C for 72 hours. Yield 3.9 g, 73%. M.p. > 300°C. ^1H NMR (500 MHz, DMSO-d_6) δ 3.14 (3H, *s*, CH_3); 2.65 (3H, *s*, CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.8, 159.6, 153.5, 150.5, 143.9, 118.1, 114.1, 27.1, 14.5. LC-MS, m/z: 241 $[MH]^+$. Anal. Calcd. for $C_9H_8N_2O_4S$: C, 45.00; H, 3.36; N, 11.66. Found: C, 45.07; H, 3.44; N, 11.68.

6-(1H-Benzimidazol-2-yl)-3,5-dimethylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione **(4).**

To 3.0 g (0.0125 mol) of 3,5-dimethyl-2,4-dioxo-1,2,3,4-tetrahydrothieno[2,3-d]pyrimidine-6-carboxylic acid **3** in 25 ml of dry dimethylformamide 2.19 g (0.0135 mol) of 1,1'-carbonyldiimidazole was added. The reaction mixture was heated at 50°C till the end of carbon dioxide release and then additionally for 15 minutes. Then 1.46 g (0.0135 mol) of ortho-phenylenediamine was added to the reaction mixture and it was additionally heated at 120°C for 3 hours. Then the cool reaction mixture was quenched with 30 ml of cold water, and the precipitate formed was filtered off, washed with water and dried. Yield 2.1 g, 55%. M.p. > 300°C. ^1H NMR (500 MHz, DMSO-d_6) δ 12.47 (1H, *s*, NH), 7.56 (2H, *m*, Ar-H), 7.19 (2H, *m*, Ar-H), 3.18 (3H, *s*, CH_3), 2.78 (3H, *s*, CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.8, 152.3, 150.7, 146.4, 134.7, 118.1, 114.1, 27.2, 15.0. LC-MS, m/z: 313 $[MH]^+$. Anal. Calcd. for $C_{15}H_{12}N_4O_2S$: C, 57.68; H, 3.87; N, 17.94. Found: C, 57.78; H, 3.90; N, 18.05.

General method for preparation of 2-[6-(1h-benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2H)-yl]-N-arylacetamides **5.**

To 0.5 g (0.0016 mol) of 6-(1*H*-benzimidazol-2-yl)-3,5-dimethylthieno[2,3-d]pyrimidine-2,4(1*H*,3*H*)-dione 4 0.22 g (0.0016 mol) of potassium carbonate, 0.0016 mol of corresponding 2-chloro-*N*-arylacetamide and 5 ml of dimethylformamide were added. The reaction mixture was heated at 50°C for 10 hours and then quenched with water (15 ml). The precipitate formed was filtered off and washed with water-ethanol mixture.

2-[6-(1h-benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2H)-yl]-N-phenylacetamide **5a.**

^1H NMR (500 MHz, DMSO-d_6) δ 12.56 (1H, *s*, NH), 10.49 (1H, *d*, NH), 7.68 – 7.51 (4H, *m*, ArH), 7.34 (2H, *dt*, *J* = 8.1, 4.6 Hz, ArH), 7.31 – 7.15 (2H, *m*, ArH), 7.10 (1H, *d*, *J* = 7.8 Hz, ArH), 4.86 (2H, *s*, CH_2), 3.29 (3H, *s*, CH_3), 2.85 (3H, *s*, CH_3). ^{13}C NMR (101 MHz, DMSO) δ 164.4, 158.9, 155.0, 150.6, 145.9, 143.4, 138.8, 135.5, 135.3, 129.3, 124.2, 123.1, 122.5, 119.7, 119.3, 118.8, 114.3, 112.0, 50.9, 28.3, 15.2. LC-MS, m/z: 446 $[MH]^+$. Anal. Calcd. for $C_{23}H_{19}N_5O_3S$: C, 62.01; H, 4.30; N, 15.72. Found: C, 62.08; H, 4.38; N, 15.79.

2-[6-(1H-Benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2H)-yl]-N-(4-methylphenyl)acetamide **5b.**

^1H NMR (500 MHz, DMSO-d_6) δ 12.51 (1H, *s*, NH), 10.40 (1H, *s*, NH), 7.56 (2H, *br.s*, ArH), 7.48 (2H, *d*, *J* = 8.0 Hz, ArH), 7.20 (2H, *d*, *J* = 6.5 Hz, ArH), 7.13 (2H, *d*, *J* = 8.0 Hz, ArH), 4.83 (2H, *s*, CH_2), 3.26 (3H, *s*, CH_3), 2.84 (3H, *s*, CH_3), 2.25 (3H, *s*, CH_3). ^{13}C NMR (101 MHz, DMSO) δ 164.1, 158.9, 155.0, 150.5, 145.9, 136.3, 135.3, 133.2, 129.7, 123.1, 119.7, 119.2, 118.8, 114.3, 112.0, 50.8, 28.3, 20.9, 15.2. LC-MS, m/z: 460 $[MH]^+$. Anal. Calcd. for $C_{24}H_{21}N_5O_3S$: C, 62.73; H, 4.61; N, 15.24. Found: C, 62.79; H, 4.69; N, 15.33.

2-[6-(1H-Benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2H)-yl]-N-(4-ethoxyphenyl)acetamide **5c.**

Yield 81%. M.p. > 300°C. ^1H NMR (500 MHz, DMSO-d_6) δ 12.53 (1H, *s*, NH), 10.34 (1H, *s*, NH), 7.56 (2H, *br.s*, Ar-H), 7.49 (2H, *d*, *J* = 8.5 Hz, Ar-H); 7.21 (2H, *m*, Ar-H); 6.88 (2H, *d*, *J* = 8.5 Hz, Ar-H); 4.82 (2H, *s*, CH_2), 3.97 (2H, *q*, *J* = 6.8 Hz, CH_2), 3.27 (3H, *s*, CH_3), 2.84 (3H, *s*, CH_3), 1.30 (3H, *t*, *J* = 6.8 Hz, CH_3). ^{13}C ЯМР, δ: 163.9, 158.9, 155.3, 154.9, 150.5, 145.9, 135.3, 131.8, 122.7, 121.2, 119.2, 114.9, 114.3, 63.5, 50.7, 28.3, 15.2, 15.1. LC-MS, m/z: 490 $[MH]^+$. Anal. Calcd. for $C_{25}H_{23}N_5O_4S$: C, 61.34; H, 4.74; N, 14.31. Found: C, 61.46; H, 4.80; N, 14.37.

2-[6-(1H-Benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2H)-yl]-N-(3,4-dimethoxyphenyl)acetamide **5d.**

^1H NMR (500 MHz, DMSO-d_6) δ 12.47 (1H, *s*, NH), 10.27 (1H, *s*, NH), 7.57 (2H, *s*, Ar-H), 7.33 (1H, *s*, Ar-H), 7.29–7.14 (2H, *m*, Ar-H), 7.07 (1H, *d*, *J* = 8.7 Hz, Ar-H), 6.92 (1H, *d*, *J* = 8.6 Hz, Ar-H), 4.83 (*s*, 2H, CH_2), 3.74 (*s*, 6H, OCH_3), 3.30 (3H, *s*, CH_3), 2.86 (3H, *s*, CH_3).^{13}C NMR (126 MHz, DMSO-d_6) δ 163.9, 158.8, 155.0, 150.5, 149.0, 145.9, 145.5, 135.3, 132.4, 119.2, 114.3, 112.5, 111.5, 104.6, 56.1, 55.7, 50.7, 28.3, 15.2. LC-MS, m/z: 506 $[MH]^+$. Anal. Calcd. for $C_{25}H_{23}N_5O_5S$: C, 59.40; H, 4.59; N, 13.85. Found: C, 59.47; H, 4.65; N, 13.95.

2-[6-(1H-Benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2H)-yl]-N-(3-chloro-4-fluorophenyl)acetamide **5e.**

^1H NMR (500 MHz, DMSO-d_6) δ 12.53 (1H, s, NH), 10.72 (1H s, NH), 7.99–7.78 (1H, *m*, Ar-H), 7.68–7.55 (2H, *m*, Ar-H), 7.49 (1H, *t*, *J* = 5.9 Hz, Ar-H), 7.40 (1H, *td*, *J* = 9.2, 3.2 Hz, Ar-H), 7.20 (2H, *dt*, *J* = 6.8, 3.5 Hz, Ar-H), 4.85 (2H, *s*, CH_2), 3.27 (3H, *s*, CH_3), 2.84 (3H, *s*, CH_3). ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.8, 158.8, 154.9, 154.8, 152.8, 150.5, 145.9, 136.0, 135.3, 121.1, 120.0, 120.0, 119.8, 119.7, 119.3, 117.6, 117.5, 114.3, 50.9, 28.3, 15.2. LC-MS, m/z: 498 $[MH]^+$. Anal. Calcd. for $C_{23}H_{17}ClFN_5O_3S$: C, 55.48; H, 3.44; N, 14.06. Found: C, 55.49; H, 3.49; N, 14.08.

2-[6-(1H-Benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2H)-yl]-N-[3-(trifluoromethyl)phenyl]acetamide **5f.**

^{1}H NMR (500 MHz, DMSO-d_6) δ 12.55 (s, 1H, NH), 10.87 (*s*, 1H, NH), 8.20 – 8.02 (1H, *m*, Ar-H), 7.78 (1H, *d*, *J* = 8.1 Hz, Ar-H), 7.60 (2H, *m*, Ar-H), 7.54 (1H, *d*, *J* = 7.8 Hz, Ar-H), 7.46 (1H, *d*, *J* = 7.9 Hz, Ar-H), 7.26–7.17 (2H, *m*, Ar-H), 4.89 (2H, *s*, CH_2), 3.29 (3H, *s*, CH_3), 2.85 (3H, *s*, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.2, 158.8, 154.9, 150.5, 145.9, 143.4, 139.5, 135.5, 135.3, 130.6, 130.1, 129.9, 125.5, 123.3, 123.2, 123.1, 122.4, 120.6, 119.3, 118.8, 115.7, 114.3, 112.0, 51.0, 28.3, 15.2. LC-MS, m/z: 514 $[MH]^+$. Anal. Calcd. for $C_{24}H_{18}F_3N_5O_3S$: C, 56.14; H, 3.53; N, 13.64. Found: C, 56.18; H, 3.60; N, 13.68.

2-[6-(1H-Benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2H)-yl]-N-(3,5-dimethoxyphenyl)acetamide **5g.**

^{1}H NMR (500 MHz, DMSO-d_6) δ 12.54 (1H, *s*, NH), 10.48 (1H, *s*, NH), 7.57 (2H, *s*, Ar-H), 7.21 (2H, *dt*, *J* = 6.8, 3.2 Hz, Ar-H), 6.84 (2H, *t*, *J* = 2.9 Hz, Ar-H), 6.26 (1H, *d*, *J* = 3.2 Hz, Ar-H), 4.84 (2H, *s*, CH_2), 3.71 (*s*, 6H, OCH_3, Ar-H), 3.28 (*s*, 3H, CH_3), 2.85 (*s*, 3H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.5, 161.0, 158.8, 154.9, 150.5, 145.9, 140.5, 135.3, 119.3, 114.3, 97.8, 96.3, 55.5, 50.9, 28.3, 15.2. LC-MS, m/z: 506 $[MH]^+$. Anal. Calcd. for $C_{25}H_{23}N_5O_5S$: C, 59.40; H, 4.59; N, 13.85. Found: C, 59.47; H, 4.59; N, 13.93.

3. 2. Antimicrobial activity study

Agar well diffusion method.

According to the WHO recommendations [35, 36] reference strains from the American Type Culture Collection (ATCC) were used as test organisms, including the Gram-positive *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and the Gram-negative strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* NCTC 5055, as well as the yeast *Candida albicans* ATCC 885-653.

Clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida albicans* were used as test strains.

The purity of each microbial culture was confirmed based on characteristic morphological, staining (tintorial), cultural, and biochemical properties. Mueller-Hinton agar (Sigma-Aldrich, St. Louis, MO, USA) and Saburo agar (for fungi) (Sigma-Aldrich, St. Louis, MO, USA) were used, and Petri dishes were incubated at 36 ± 1°C for 24 h for bacteria and at 25°C for 24–48 h for fungi. Bacterial concentration was 10^7 CFU/ml (determined by McFarland standard). Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was used as controls. Pure DMSO was used as a solvent due to the poor solubility of the test compound in diluting DMSO. The screening compounds as well as the standards were introduced to the wells as a DMSO solution in concentrations of 100 μg/ml; the open wells were filled with 0.3 ml of the solution. The level of antimicrobial activity of the compound was assessed based on the diameter of the microbial growth inhibition zone around the well containing the tested agent. The activity was evaluated using the following scale: inhibition zone diameter < 14–15 mm – resistant strain; 15–18 mm – weakly sensitive strain; > 18 mm – sensitive strain. The obtained data were analyzed using methods of variational statistics. A significance level of $p \leq 0.05$ was considered statistically meaningful.

Crystal violet assay for biofilm quantification.

The study of the effect of the compound on the biofilms formation was performed using the reference strains of *S. aureus* ATCC 25923*, E. coli* ATCC 25922*, K. pneumoniae* NCTC 5055*, P. aeruginosa* ATCC 9027*, B. subtilis* ATCC 6633 and *C. albicans* ATCC 885-653, which were seeded on the nutrient agar and incubated for 24 hours at 37°*C* for bacteria and 25°*C* for the fungal strain. The agar culture was washed by adding 1 ml of isotonic sodium chloride solution to prepare the suspension, and the turbidity was adjusted such that it contained approximately 10^9 microbial cells/ml.

The study of biofilm formation inhibitory activity was performed similarly to the previously reported procedure [37]. Biofilms were formed in plastic Petri dishes of 90 mm diameter with the sterile cover glass placed on the bottom. Then, 9 ml of the 18-hour strain culture (diluted 1:100) was added in such a way that the suspension was evenly distributed at the bottom of the dish. To determine the control parameters, sterile nutrient medium was added to several dishes. The Petri dishes were placed to a thermostat for 24 hours (37°C). The next day, the contents of the Petri dishes was carefully removed so as not to destroy the formed biofilm, 9 ml of distilled water and 0.9 ml of a 1% alcohol solution of crystal violet were added, and the mixture was left at room temperature for 45 minutes. Then the formed solution was carefully removed, and the dishes were washed three times with distilled water, and the cover-glass was dried. The optical density of the resulting liquid was measured on a spectrophotometer at a wavelength of 545 nm.

To determine the MIC of the test samples of substance on biofilms (preventing biofilm formation), the compound solution was added to the wells on the 1st day at a concentration of 10 mg/ml and titrated. The plates were incubated for 24 hours at 37°C for bacterial strains and at 25°C for a fungal strain. To determine the control parameters, sterile nutrient medium was added to several dishes. The next day, the contents of the Petri dishes were carefully removed so as not to destroy the formed biofilm, 9 ml of distilled water and 0.9 ml of a 1% alcohol solution of Crystal violet were added, and the mixture was left at room temperature for 45 minutes. Then the formed solution was carefully removed, and the dishes were washed three times with distilled water, and the cover-glass was dried. The optical density of the resulting liquid was measured on a spectrophotometer at a wavelength of 545 nm.

To determine the per cent of inhibition for the test samples of substances on biofilms (preventing biofilm formation), the compound solution was added to the wells on the 2nd day at a concentration of 10 mg/ml and

titrated. The plates were incubated for 24 hours at 37°C for bacterial strains and at 25°C for a fungal strain. To determine the control parameters, sterile nutrient medium was added to several dishes. The next day, the contents of the Petri dishes were carefully removed so as not to destroy the formed biofilm. 9 ml of distilled water and 0.9 ml of a 1% alcohol solution of Crystal violet were added, and the mixture was left at room temperature for 45 minutes. Then the formed solution was carefully removed, and the dishes were washed three times with distilled water, and the cover-glass was dried. The optical density of the resulting liquid was measured on a spectrophotometer (SPECORD 200, Germany) at a wavelength of 545 nm.

$$\% \text{ inhibition} = \frac{(\text{Control optical density} - \text{Test optical density})}{\text{Control optical density}} \times 100.$$

The obtained data was analyzed using Student'-Fischer's algorithm.

3. 3. Docking studies

For the docking studies, we used the same set of computer programs as previously reported [29]; namely AutoDockTools and AutoDock Vina, BIOVIA Draw 2019, Chem3D software, and Discovery Studio 2021. The macromolecule of the enzyme tRNA (Guanine37-N^1)-methyltransferase in its conformation in crystal with the native ligand was downloaded from Protein Data Bank; the ID of the structure is 5ZHN (PDB, 2022). All the docking parameters, validation techniques for the algorithm, and RSMD values were reported previously by us [29].

4. Results

Thieno[2,3-d]pyrimidine-2,4(1H,3H)-diones are commonly synthesized via cyclization of ureas derived from 2-aminothiophene-3-carboxylates under basic conditions [38, 39]. However, the preparation of such ureas typically requires the use of isocyanates as starting materials, which are known for their toxicity and limited stability. To circumvent these limitations, an alternative approach based on oxidation of the corresponding thione group was considered [40]. This strategy was particularly advantageous, as efficient and well-documented procedures for the synthesis of the starting thione derivatives were already available [29, 34].

As the starting compound, the readily available ethyl 3,5-dimethyl-4-oxo-2-thioxothieno[2,3-d]pyrimidine-6-carboxylate **1** was selected. Upon oxidation with hydrogen peroxide under alkaline conditions, the corresponding 2-oxo derivative **2** was obtained (Fig. 3).

Despite the use of alkaline conditions for the oxidation step, ester **2** was obtained in high yield, which is likely attributed to its low solubility in water, in contrast to the starting thione **1**, which is readily soluble in aqueous alkali. We suggest that the limited aqueous solubility of compound **2** at ambient temperature reduces its susceptibility to hydrolysis. Hydrolysis to the corresponding acid **3** occurred only upon heating at 70–80 °C and required approximately 15 hours for complete conversion. The isolated acid **3** was subsequently activated using 1,1′-carbonyldiimidazole (CDI) as a coupling agent. This activation enabled the efficient synthesis of 6-(1*H*-benzimidazol-2-yl)-3,5-dimethylthieno[2,3-d]pyrimidine-2,4(1*H*,3*H*)-dione with satisfactory yield and high purity.

The obtained heterocyclic hybrid **4** was subsequently subjected to alkylation with a series of 2-chloro-N-phenylacetamides, resulting in the formation of monoalkylated products (Fig. 3).

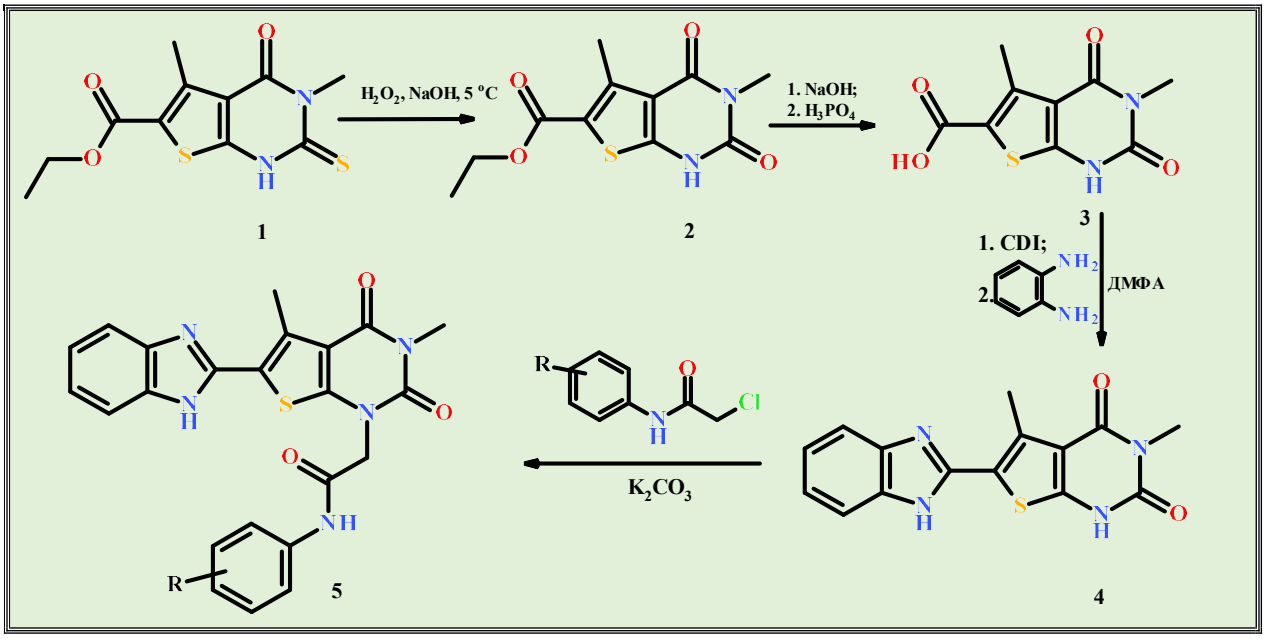


Fig. 3. Synthesis of target acetamides derivatives of 6-(1*H*-benzimidazol-2-yl)-3,5-dimethylthieno[2,3-d]pyrimidine-2,4(1*H*,3*H*)-dione (**5**)

Molecule **4** contains at least three potential nucleophilic centers susceptible to attack by electrophilic agents such as chloroacetamides: the NH groups of the benzimidazole and pyrimidine-2,4-dione moieties, as well as the oxygen atom at position 2 of the thienopyrimidine ring, assuming its imidol tautomeric form. These three reactive sites suggest the possibility of alkylation leading to products with structures 5, 6, or 7 (Fig. 4). Therefore, the structure of the alkylation product was initially uncertain and required clarification.

The most straightforward method for distinguishing among possible alkylation isomers was NMR spectroscopy of the product, specifically analysis of the ^{13}C and ^{1}H nuclei. In the alkylated products, the methylene group resonated in the region of 50.7–51.0 ppm in the ^{13}C NMR spectra, while the corresponding ^{1}H NMR signals appeared at 4.82–4.89 ppm. These chemical shifts are more consistent with methylene groups attached to a nitrogen atom rather than to an oxygen atom.

To further elucidate the site of alkylation, an HMBC experiment was performed for the product of the reaction between compound **4** and 2-chloro-N-(4-methylphenyl)acetamide – **5b**. No HMBC correlations were observed between any benzimidazole protons and the acetamide fragment, supporting the regioselective alkylation at the pyrimidine nitrogen (Fig. 5).

The results of antimicrobial screening using the agar well diffusion method are presented in Table 1. As reference drugs, antibiotics from different classes were used, including streptomycin, gentamicin phosphate, and cefepime. All synthesized derivatives (compounds **4**, **5a–e**) exhibited high antimicrobial activity: compounds **5a** and **5b** demonstrated activity comparable to that of streptomycin and gentamicin, while compounds **4** and **5c–e** showed even higher efficacy, though slightly lower than that of cefepime.

The compounds that demonstrated superior activity against reference strains were further evaluated for their ability to inhibit clinically isolated strains, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida albicans*. These microorganisms are commonly encountered in hospital settings, often in drug-resistant forms. The results are summarized in Table 2.

Table 1

Antimicrobial activity of the synthesised compounds

Compound	Mean diameter of growth inhibition zone in mm, number of repeated experiment $n = 6$*					
	Test strains					
	Gram-positive		Gram-negative			Fungi
	S. aureus ATCC 25923	*B. subtilis* ATCC 6633	*E. coli ATCC* 25922	*K. pneumoniae* NCTC 5055	*P. aeruginosa ATCC* 9027	C. albicans *ATCC* 885-653
4	33.8 ± 0.45	16.6 ± 0.9	35.8 ± 1.3	27.6 ± 0.5	36.0 ± 0.71	26.6 ± 0.6
5a	18.6 ± 0.9	17.4 ± 0.9	19.0 ± 1.2	23.8 ± 0.8	23.8 ± 1.09	27.4 ± 0.10
5b	19.6 ± 0.9	18.4 ± 0.9	20.0 ± 1.2	23.8 ± 0.7	23.8 ± 1.09	27.4 ± 0.10
5c	33.8 ± 0.45	35.8 ± 1.3	20.0 ± 0.7	27.6 ± 0.5	36.0 ± 0.71	26.6 ± 0.6
5d	27.0 ± 0.7	16.8 ± 0.8	20.2 ± 0.8	24.6 ± 0.9	30.4 ± 1.14	27.0 ± 0.7
5e	28.4 ± 0.54	20.0 ± 0.7	25.2 ± 0.4	17.0 ± 0.7	20.4 ± 0.5	23.0 ± 0.7
5f	33.2 ± 1.09	18.0 ± 0.7	35.6 ± 0.5	26.6 ± 0.9	30.6 ± 0.8	19.4 ± 0.5
5g	33.2 ± 1.09	28.4 ± 0.54	20.0 ± 0.7	25.6 ± 0.5	31.6 ± 0.8	26.6 ± 0.6
DMSO (control)	11.6 ± 0.4	15.9 ± 0.4	16.7 ± 0.8	18.3 ± 0.7	13.7 ± 0.7	16.9 ± 0.3
Streptomycin	27.2 ± 0.8	24.3 ± 0.9	24.6 ± 0.6	23.1 ± 0.4	28.2 ± 0.6	growth
Gentamicin phosphate	26.2 ± 0.3	28.1 ± 0.7	25.8 ± 0.6	21.3 ± 0.7	27.1 ± 0.2	growth
Cefepime	36.7 ± 0.5	40. 2 ± 0.8	38.4 ± 0.6	50.2 ± 0.9	36.7 ± 07	growth

Note: * – $p = 0.95$.

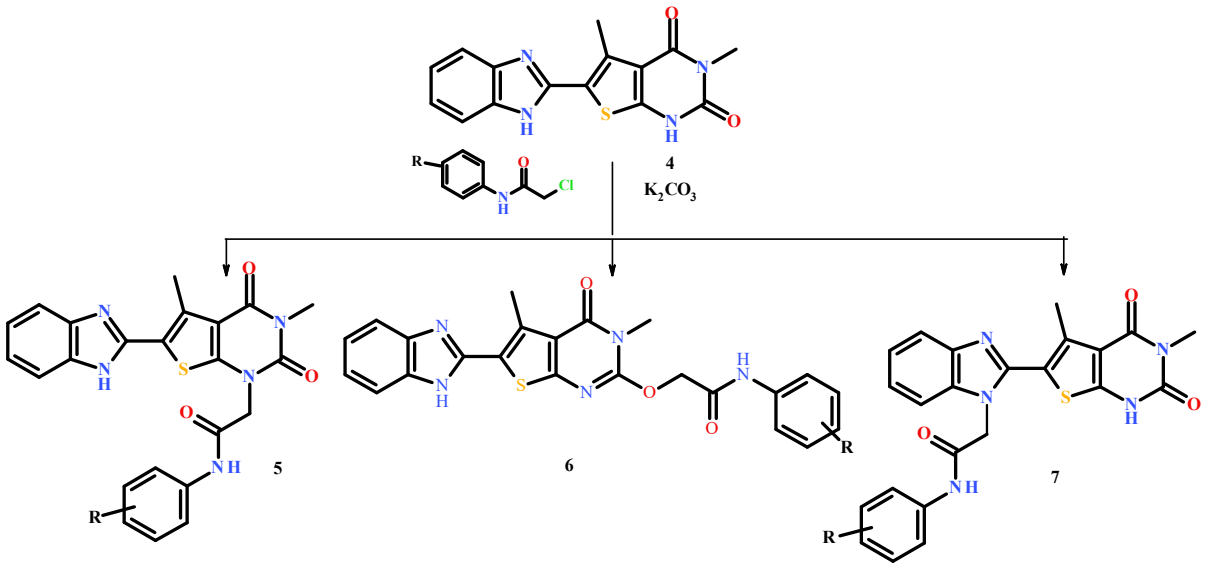


Fig. 4. Possible alkylation pathways of the target hybrid 6-(1H-benzimidazol-2-yl)-3,5-dimethyl-1H-thieno[2,3-d] pyrimidine-2,4-dione

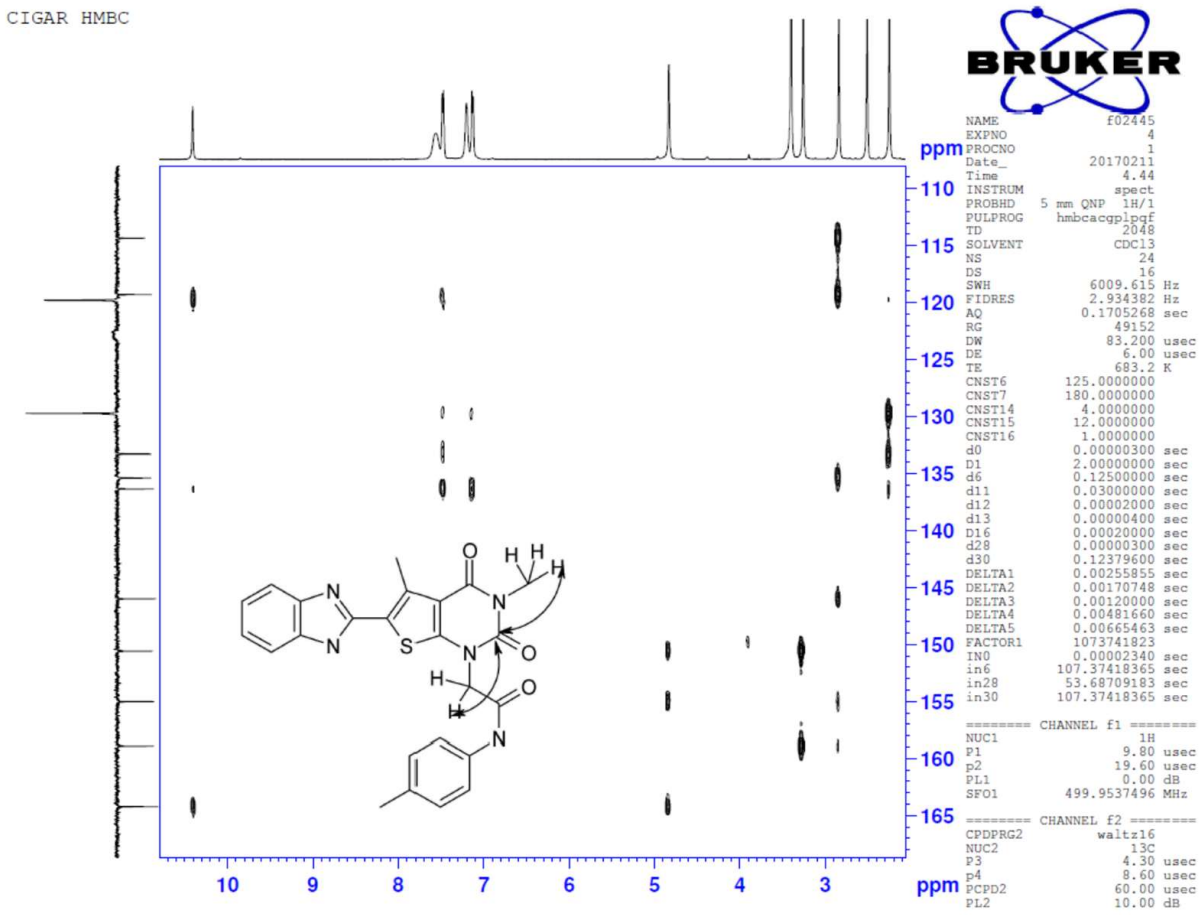


Fig. 5. HMBC spectrum of 2-[6–(1*H*–benzimidazol–2–yl)–3,5–dimethyl–2,4–dioxo–3,4–dihydrothieno[2,3-d] pyrimidin-1(2*H*)-yl]-*N*-(4-methylphenyl)acetamide **5b**

Table 2

The antimicrobial activity of the tested compound against clinical strains ($M \pm m$)

Compound	Mean diameter of growth inhibition zone in mm, number of repeated experiment $n = 6$*				
	Clinical strains				
	S. aureus	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*C. albicans*
4	At the control level	18.6 ± 0.2	15.4 ± 0.5	22.3 ± 0.5	28.1 ± 0.9
5c	20.0 ± 0.7	18.6 ± 0.2	At the control level	23.3 ± 0.6	27.0 ± 0.7
5f	At the control level	At the control level	10.6 ± 0.9	At the control level	19.4 ± 0.5
5g	At the control level	At the control level	At the control level	22.5 ± 0.7	27.4 ± 0.10
DMSO (control)	10.7 ± 0.9	17.1 ± 0.2	16.0 ± 0.4	20.3 ± 0.7	27.2 ± 0.4
Streptomycin	28.2 ± 0.6	24.1 ± 0.6	24.3 ± 0.9	23.1 ± 0.4	growth
Gentamicin phosphate	26.4 ± 0.3	24.5 ± 0.6	28 ± 0.6	20 ± 0.6	growth
Cefepime	35.7 ± 0.8	38.3 ± 0.8	40 ± 0.6	50 ± 0.6	growth

According to the results of this study, the compound bearing a 4-ethoxyphenyl substituent in the phenyl radical (compound **5c**) exhibited the highest antimicrobial activity, particularly against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

As a next step, the ability of derivative **5c** to inhibit the formation and promote the disruption of bacterial biofilms formed by *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans* – typical biofilm-forming pathogens – was investigated. The results are presented in Table 3.

Compound **5c** did not exhibit inhibitory activity against biofilm formation, but demonstrated a pronounced ability to disrupt preformed biofilms. Upon addition of the compound to cultures of *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans*, a statistically significant reduction in established biofilms was observed, with disruption levels reaching 28.2%, 42.7%, 36.6%, and 41.4%, respectively.

To predict the potential mechanism of action and investigate the role of bioisosteric fragment substitution in conformational positioning, molecular docking was performed in the active site of TrmD isolated from *P. aeruginosa*. The results were compared with those of the native inhibitor and a previously described bioisosteric analogue [24, 29].

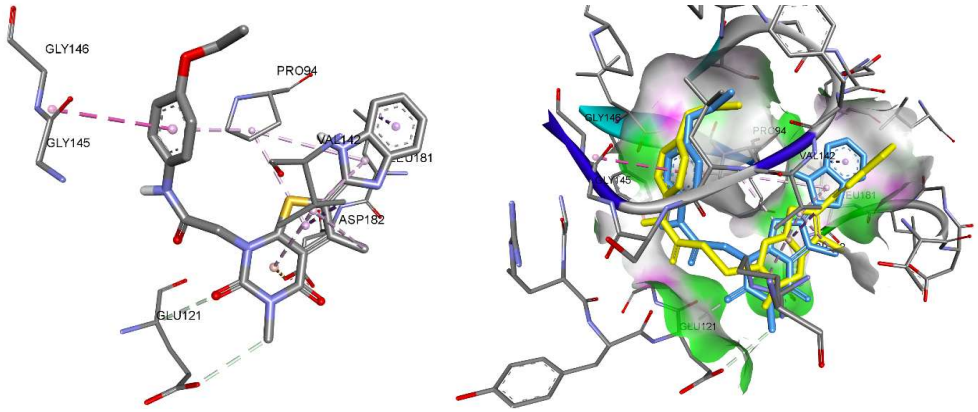
The docking score of compound **5c** was –9.5 kcal/mol, compared to –9.3 kcal/mol for the bioisosteric analogue and –8.2 kcal/mol for the native inhibitor [29]. The high binding affinity of **5c** is attributed to the number and nature of its interactions with key amino acid residues (Fig. 6, *a*), including hydrogen bonding with the polar glutamic acid (Glu121), and hydrophobic contacts with aspartic acid (Asp182), leucine (Leu181), valine (Val142), glycine (Gly145, Gly146), and proline (Pro94) – all of which form part of the TrmD active site. A visualization of the superimposed conformational alignment with the bioisosteric analogue is shown in Fig. 6, *b*.

Table 3

Transmission of the nutrient medium at the exposure of test samples of the studied compound 5c to bacterial biofilms (%, $M \pm m$)

Test samples, mg/ml	Test strains of microorganisms			
	S. aureus		*E. coli*	
	After 1 day	After 2 days	After 1 day	After 2 days
Control	74.1 ± 0.29	49.0 ± 0.28	74.1 ± 0.13	49.0 ± 0.25
5c	77.9 ± 0.09***	55.9± 0.39***	89.6 ± 0.09***	51.3 ± 0.39***
–	*P. aeruginosa*		*C.albicans*	
	after 1 day	after 2 days	after 1 day	after 2 days
Control	74.1 ± 0.29	49.0 ± 0.28	74.1± 0.29	49.0± 0.28
5c	86.2 ± 0.07***	54.6 ± 0.06***	87.3 ± 0.05***	51.2 ± 0.03***

Note: ** – $p < 0.01$; *** – $p < 0.001$ *compared with the control.*



a *b*

Fig. 6. 3D visualization: *a* – interaction of **5c** with amino acids of the active site of TrmD inhibitors; *b* – conformations of both the bioisosteric analogue (yellow structure) and the ligand **5c** (blue structure)

5. Discussion

The synthetic strategy employed in this study demonstrates a practical and selective approach to obtaining *N*-substituted thieno[2,3-d]pyrimidine–benzimidazole hybrids through regioselective alkylation. The choice of oxidative transformation as an alternative to traditional urea cyclization circumvents the use of toxic isocyanates and leverages established methods for preparing thione intermediates, thus enhancing both safety and accessibility of the synthetic route [38, 40].

A key outcome of this work is the unambiguous confirmation of alkylation at the N(1) position of the thienopyrimidine ring. This selectivity is particularly noteworthy given the presence of multiple nucleophilic centers in the hybrid scaffold. In the ^{1}H NMR spectrum (using compounds **4** and **5b** as examples), the methylene protons appeared at 4.83 ppm and exhibited three-bond correlations with carbon signals at 164.1, 155.0, and 150.5 ppm. The signal at 164.1 ppm also showed a correlation with the acetamide NH proton, supporting its assignment as the acetamide carbonyl carbon. The N-methyl group at position 3 of the thieno[2,3-d]pyrimidine ring appeared at 3.26 ppm and displayed HMBC cross-peaks with carbon signals at 150.5 and 158.9 ppm. These correlations enabled the assignment of the 150.5 ppm signal to the C(2) atom of the thienopyrimidine ring and the 158.9 ppm signal to the C(4) carbonyl. Taken together, the HMBC data for the alkylated product clearly indicate that alkylation occurred at the N(1) position of the thieno[2,3-d] pyrimidine ring.

Therefore, the proposed convenient synthetic procedures enabled the efficient preparation of the target 2-[6-(1*H*-benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2*H*)-yl]-N-arylacetamides in high yield and purity, and can be considered highly effective and well-suited for scale-up synthesis. The confirmed regioselective alkylation at the N(1) position of the thieno[2,3-d] pyrimidine ring lays a conceptual foundation for the design of next analogues with enhanced pharmacological potential.

Comparison of the antimicrobial screening results obtained via agar diffusion for the synthesized derivatives **5a–e** with those of their bioisosteric analogues demonstrated that the replacement of the sulfur atom with oxygen, along with the relocation of the acetamide fragment from position 2 to position 1 of the pyrimidine ring, had a favorable impact on biological activity [29]. All compounds exhibited enhanced antimicrobial efficacy against both Gram-positive and Gram-negative bacteria, as well as fungi. Among the acetamide derivatives, the highest activity was observed for compounds **5c**, **5f**, and **5g**, which contained a 4-ethoxy group, a 3-trifluoromethyl group, and a 2,5-dimethoxy substitution pattern on the phenyl ring, respectively. These findings suggest a positive influence of electron-withdrawing substituents in the phenyl moiety on

antimicrobial activity. Compound **5c** demonstrated the most promising activity profile across all tested microbial strains, particularly against *S. aureus, B. subtilis, E. coli,* and *P. aeruginosa*. Specifically, **5c** exceeded the antimicrobial effect of the reference drug streptomycin by 1.3-, 1.5-, 1.4-, and 1.5-fold against the strains, respectively. Compared to gentamicin, compound **5c** showed 1.3- and 1.27-fold higher activity against *S. aureus* and *B. subtilis*, and a 1.32-fold increase against *P. aeruginosa*. While its activity against *P. aeruginosa* was comparable to that of the cephalosporin antibiotic cefepime, it was slightly lower against the other tested strains.

The unsubstituted benzimidazole–thienopyrimidine scaffold **4** demonstrated notable antimicrobial activity, exhibiting growth inhibition against S. *aureus, E. coli, P. aeruginosa,* and *C. albicans*. It is worth noting that the activity of the 2-oxo derivative **4** was significantly higher than that of its structural precursor, the corresponding 2-thioxo analogue (6-(1*H*-benzimidazol-2-yl)-3,5-dimethyl-2-thioxo-1*H*-thieno[2,3-d]pyrimidin-4-one) [29], further supporting the rationale for bioisosteric replacement of the sulfur atom with oxygen.

Assessment of antimicrobial activity against clinically isolated strains provides a more relevant and practical indication of the potential therapeutic efficacy of novel compounds. The selected strains included both Gram-positive and Gram-negative bacteria, as well as opportunistic fungal pathogens. Clinical isolates frequently exhibit higher resistance to conventional antimicrobial agents compared to reference strains, making them critically important targets in the evaluation of new drug candidates. Among the tested compounds, derivative **5c** demonstrated the highest activity against *P. aeruginosa* and *S. aureus* clinical isolates, although it remained less potent than the reference drugs.

The investigation of the compound's ability to interfere with biofilm formation and promote biofilm disruption is particularly significant, given the central role of biofilms in chronic infections, antimicrobial resistance, and the limited efficacy of traditional therapies. In the study of compound **5c**, no statistically significant inhibition of initial biofilm formation was observed. However, in the 48-hour biofilm disruption assay, the addition of the test sample to pre-formed biofilms in Petri dishes resulted in measurable reductions in biofilm density, as indicated by changes in light transmission. The recorded disruption rates were 28.2% for *S. aureus*, 42.7% for *E. coli*, 36.6% for *P. aeruginosa*, and 41.4% for *C. albicans*, with all results statistically significant ($p < 0.001$).

Analysis of the conformational arrangement within the active site of the TrmD enzyme revealed the formation of a strong hydrogen bond (2.5 Å) between the carbonyl group at position 2 of the pyrimidine ring in compound **5c** and the glutamic acid residue Glu121. In contrast, the sulfur atom in the corresponding bioisosteric analogue did not participate in ligand stabilization (Fig. 6, *b*) [29]. Further comparison of the conformations of the two bioisosteric compounds demonstrated that ligand **5c** adopts a more favorable and stable orientation within the active site, involving exclusively those amino acid residues responsible for anchoring the native TrmD inhibitor. The overall findings support the rationale for the applied structural modification strategy, which contributed to the enhanced antimicrobial activity of the synthesized benzimidazole–thiazolopyrimidine hybrids as potential bacterial TrmD inhibitors.

Practical relevance. The results of this study have practical significance in the context of the global problem of antimicrobial resistance. The synthesized benzimidazole–thienopyrimidine hybrids demonstrated pronounced antibacterial activity, while compound **5c** showed the ability to disrupt preformed biofilms of *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans*, which is highly relevant for overcoming biofilm-associated resistance. The obtained results emphasize the potential of the designed compounds as "lead structures" for the development of new antibacterial agents with a novel mechanism of action and prospects for practical application in the treatment of resistant infections.

Study limitations. The antimicrobial activity of the synthesized compounds was assessed primarily using *in vitro* agar diffusion assays, which may not fully reflect their pharmacodynamic behavior in complex biological environments. The lack of MIC/MBC (minimum inhibitory/bactericidal concentration) determination limits the quantitative comparison of potency among analogues. Only a limited number of structural analogues were explored, and further diversification of substituents might help establish a more robust structure–activity relationship and guide rational optimization of the scaffold.

Prospects for further research. The obtained results provide a solid foundation for further in-depth *in vitro* and *in vivo* investigations of the hit compound against antibiotic-resistant microbial strains, as well as for elucidating its mechanism of antimicrobial action. Additionally, further structural optimization may be pursued to enhance its biological activity.

6. Conclusions

In this study, a novel series of 2-[6-(1*H*-benzimidazol-2-yl)-3,5-dimethyl-2,4-dioxo-3,4-dihydrothieno[2,3-d]pyrimidin-1(2*H*)-yl]-*N*-arylacetamides was designed and synthesized through a rational approach involving bioisosteric replacement. The modification of the thione group to an oxo group, along with relocation of the acetamide moiety to the N(1) position of the pyrimidine ring, significantly improved the ADMET properties and predicted binding affinity to the bacterial enzyme tRNA-(m1G37) methyltransferase (TrmD). The structure of the alkylation products was unambiguously confirmed by NMR and HMBC spectroscopy, revealing regioselective alkylation at the N(1) position of the thienopyrimidine core. Molecular docking results further supported these findings, showing favorable interactions with key amino acid residues in the enzyme's active site and highlighting the essential role of the carbonyl group at position 2 in stabilizing the ligand–target complex.

Antimicrobial screening revealed that all synthesized compounds exhibited strong activity against both Gram-positive and Gram-negative reference strains, with compound **5c** displaying the highest and broadest spec-

trum of activity. Comparison with thione analogues confirmed the effectiveness of the applied bioisosteric modification. Further testing against clinically isolated resistant strains confirmed that compound **5c** retained high activity, particularly against *S. aureus* and *P. aeruginosa*, although slightly less potent than cefepime.

Importantly, although compound **5c** did not inhibit biofilm formation, it demonstrated pronounced biofilm-disrupting capacity, reducing preformed biofilms of *S. aureus, E. coli, P. aeruginosa,* and *C. albicans* by up to 42.7%.

Overall, the findings support the therapeutic potential of the benzimidazole–thienopyrimidine hybrid scaffold, particularly compound **5c**, as a promising lead candidate for the development of new antibacterial agents targeting resistant pathogens and biofilm-associated infections.

Conflict of interests

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

The manuscript has no associated data.

Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies when creating the current work.

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