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Novel 4-methylthienopyrimidines as antimicrobial agents: synthesis, docking study and *in vitro* evaluation

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ABSTRACT

Compounds with thieno[2,3-d]pyrimidine core modified with amide group at position five of the heterocyclic system were reported as ligands to bacterial TrmD, which is an enzyme responsible for protein synthesis in bacterial cells and its blockage leads to the death of bacteria or makes them less resistant to antibiotic therapy. The great problem of antibiotic resistance, especially of Gram-negative bacteria like *Pseudomonas aeruginosa,* forced us to design and study the antimicrobial properties of novel thieno[2,3-d]pyrimidine with amide function at position six as possible bacterial TrmD inhibitors. For the synthesis of the target derivatives with an aromatic pyrimidine cycle and a methyl group at position four, the Suzuki reaction was used. The previously unknown key intermediate 4,5-dimethylthieno[2,3-d] pyrimidine-6-carboxylic acid was further transformed into various novel derivatives of 4-methylthieno[2,3-d] pyrimidines by interaction with amines. The antimicrobial activity screening results show that benzyl amides of 4,5-dimethylthienopyrimidines were the most active, especially against *Bacillus subtilis* and *P. aeruginosa*. The docking studies also revealed that benzylamides showed the best binding parameters to the selective inhibitors' active site of tRNA (Guanine37-N^1)-methyltransferase, an enzyme isolated from *P. aeruginosa*.

INTRODUCTION

Resistance of bacterial strains to the existing antibiotics causes many problems in the therapy of diseases of bacterial aetiology (Ventola, 2015); the situation can be highly dangerous in the case of such pathogens as *Pseudomonas aeruginosa* (Khan *et al*., 2020; Pang *et al*., 2019; Yakout and Abdelwahab, 2022). It is known that the composition of antibiotics (Jones *et al*., 2022; Olsson *et al*., 2020) or their protection from deactivating enzymes using corresponding inhibitors (Brown and Wobst, 2022) is the possible way to solve this problem. The selection of more stable molecules in the existing groups of antibiotics is also a promising strategy to improve the therapy; Plazomicin (Wagenlehner *et al*., 2019), dalbavancin (Cercenado, 2017), oritavancin (Mitra *et al*., 2015), and meropenem (El-Gamal, 2011) serve as good examples of such molecules.

One more strategy of antimicrobial drug development, with some problems and limitations (Tommasi *et al*., 2015), is the development of inhibitors of bacterial protein targets, which could even form a new class of antibiotic or antibacterial agents. One of these attractive targets is bacterial TrmD, which is crucially different from its ortholog of eukaryotes and archaea (Goto-Ito *et al*., 2017). Blockage of this enzyme leads to +1 frameshifting at the ribosomal translation stage of protein synthesis (Gamper *et al*., 2015). The disruption of protein synthesis in its turn negatively influences the production of bacterial membrane proteins, such as efflux drugs pumps (Masuda *et al*., 2019), which leads to the misfunctioning of the outer membrane of Gram-negative bacterial cells (Zhang *et al*., 2013), can favorize the accumulation of antibiotics and increases their activity (Richter *et al*., 2017).

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Small molecules draw the attention of researchers as candidates for bacterial TrmD inhibitors. Among the most promising heterocyclic systems which are present in the structure of highly active inhibitors, the assemblies of pyrazole with indole were reported (Whitehouse *et al*., 2019). Compounds with pyrazole heterocyclic pattern modified with 3-indolyl or 2-thienylcaboxamide substituents were identified as leaders in HTS assays aiming at the development of TrmD *P. aeruginosa* inhibitors (Zhong *et al*., 2019a). The derivatives of thieno[2,3-d] pyrimidine are one the effective classes of the well-known TrmD inhibitors (Hill *et al*., 2013), which allowed to disclose differences in inhibitors' binding mechanisms between Gram-negative tRNA (Guanine37-N^1)-methyltransferases from mycobacterial and also their Gram-positive counterparts. The study of thienopyrimidine derivatives complexes with different TrmDs also helped to get a deeper insight into the molecular mechanism of their action (Zhong *et al*., 2019b). One of the newest results concerning libraries potential of thieno[2,3-d] pyrimidines as TrmD inhibitors is the study that revealed the derivative of 3,5,6,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-d] pyrimidine modified at position three with 3-indolylthioacetamide substituent as the most active compound (Malasala *et al*., 2022). Besides, the activity of thienopyrimidines as TrmD inhibitors has also been reported (Badiger *et al*., 2015; Triloknadh *et al*., 2021). It is also interesting that thieno[2,3-d]pyrimidin-6-carboxylic acids derivatives are inhibitors of N-acetyltransferases of bacterial polysaccharides, which showed nanomolar inhibitory activity and were active against *Campylobacter jejuni* PglD (De Schutter *et al*., 2017).

MATERIALS AND METHODS

Chemistry

All the convenient solvents and commercially available reagents including chromatography grades we 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 or $CDCl_3$). 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 chromatographical part of the device 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.

Ethyl 4-chloro-5-methylthieno[2,3-d]pyrimidine-6-carboxylate (1)

It was prepared according to the previously reported method (Triloknadh *et al*., 2018) and chromatographically purified. Eluent $CHCl_3$:iPrOH (97:3).

Ethyl 4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxylate (2)

To the three-neck round-bottomed flask (1 l) 50 g (0.195 mol) of ethyl 4-chloro-5-methylthieno[2,3-d]pyrimidine-6-carboxylate (**1**), 17.5 g (0.29 mol) of methylboronic acid, 3.11 g (0.0043 mol) of $Pd(dppf)Cl_2 \cdot CH_2Cl_2$, and 44.3 g (0.76 mol) of potassium fluoride were placed under the argon atmosphere. The amount of 1,4-dioxane taken was enough for the free stirring of the reaction mixture. The reaction mixture was heated in the silicone bath at 90°C for 24 hours. The reaction progress was monitored by TLC in the system ethyl acetate-hexane 1:2. After the full conversion of the starting chloride the solvent was evaporated (reduced pressure) and the crude mixture of organic products was dissolved in a minimal amount of ethyl acetate. The product was isolated chromatographically using the pad of silica and ethyl acetate-hexane (1:2) as the eluent. The main product was collected in the second fraction. Yield 38%, yellowish crystals; mp 113°C–114°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.33 (t, 3H, J = 7.1 Hz, CH_3), 2.88–2.98 (m, 6H, $2CH_3$), 4.35 (q, 2H, J = 7.1 Hz, CH_2), and 8.96 (s, 1H, CH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.5, 16.3, 24.8, 62.1, 125.5, 130.4, 140.8, 155.2, 162.4, 165.9, and 166.9. LC-MS, *m*/*z*: 237 $[M+H]^+$. Anal. Calcd. for $C_{11}H_{12}N_2O_2S$: C, 55.91; H, 5.12; N, 11.86. Found: C, 56.04; H, 5.17; N, 11.93.

4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxylic Acid (3)

To a conical flask (100 ml) 5 g (0.021 mol) of ethyl ester of 4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxylic acid (**2**) and 2,5 g (0.063 mol) sodium hydroxide were placed. Water (20 ml) was added to the reaction mixture and the content of the flask was gradually heated to boiling. The reaction mixture in a flask was boiled for 3 hours and then cooled and acidified with 85% orthophosphoric acid. Then the formed precipitate of compound **3** was drained. Plenty of water was used to remove inorganic admixtures by washing the filter cake. The precipitate was dried to constant mass at 60°C. Yield: 53%, a white powder; mp > 300°C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.88–2.92 (m, 6H, $2CH_3$), 8.91 (s, 1H, CH), and 13.57 (br. s, 1H, COOH); ^{13}C NMR (125 MHz, DMSO-d_6): δ 15.5, 24.2, 126.9, 130.1, 139.0, 154.4, 163.6, 164.9, and 166.3. LC-MS, *m*/*z*: 209 $[M+H]^+$. Anal. Calcd. for $C_9H_8N_2O_2S$: C, 51.91; H, 3.87; N, 13.45. Found: C, 52.10; H, 3.89; N, 13.59.

General method of 4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxamides synthesis

To 0.15 g (0.73 mmol) of 4,5-dimethylthieno[2,3-*d*] pyrimidine-6-carboxylic acid (**3**), 1,1′-carbonyldiimidazole (CDI) 0.13 g (0.8 mmol) and 1.5 ml of anhydrous DMF were added. The reaction mixture was heated with vigorous stirring (70°C–80°C) then 0.0008 moles of the corresponding amine was added and the temperature was adjusted to 100°C and stirred more for 4–5 hours. After the mixture was cooled and 20 ml of water was added, the precipitate formed was drained. An analytical sample was dried to constant mass (ethanol was used as a solvent for crystallization).

N-benzyl-4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxamide (4a)

Yield 65%, a white powder; mp 155°C–156°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.75 (s, 3H, CH_3), 2.91 (s, 3H, CH_3), 4.49 (d, 1H, J = 5.7 Hz, CH_2), 7.23–7.30 (m, 1H, H Ar), 7.31–7.40 (m, 4H, H Ar), 8.92 (s, 1H, CH), and 9.10 (m, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6): δ 16.6, 24.5, 43.4, 127.4, 127.7, 128.8, 130.2, 131.4, 132.8, 139.4, 154.2, 162.6, 164.5, and 166.4. LC-MS, *m*/*z*: 298 $[M+H]^+$. Anal. Calcd. for $C_{16}H_{15}N_3OS$: C, 64.62; H, 5.08; N, 14.13. Found: C, 64.70; H, 5.10; N, 14.21.

4,5-dimethyl-N-(4-methylbenzyl)thieno[2,3-d]pyrimidine-6-carboxamide (4b)

Yield 53%, a white powder; mp 176°C–177°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.30 (s, 3H, CH_3), 2.75 (s, 3H, CH_3), 2.91 (s, 3H, CH_3), 4.45 (d, 1H, *J* = 5.6 Hz, CH_2), 7.16 (d, 1H, *J* = 7.2 Hz, H Ar), 7.25 (d, 1H, *J* = 7.2 Hz, H Ar), 8.86 (br s, 1H, NH), and 8.90 (s, 1H, CH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 16.6, 21.1, 24.5, 43.2, 127.8, 129.4, 130.2, 131.5, 132.7, 136.4, 136.5, 154.2, 162.6, 164.4, and 166.4. LC-MS, *m*/*z*: 312 $[M+H]^+$. Anal. Calcd. for $C_{17}H_{17}N_3OS$: C, 65.57; H, 5.50; N, 13.49. Found: C, 65.59; H, 5.57; N, 13.57.

N-(4-chlorobenzyl)-4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxamide (4c)

Yield 92%, a white powder; mp 200°C–201°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.76 (s, 3H, CH_3), 2.92 (s, 3H, CH_3), 4.48 (d, 1H, *J* = 5.6 Hz, CH_2), 7.31–7.45 (m, 4H, H Ar), 8.91 (s, 1H, CH), and 8.98 (br. s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6): δ 16.1, 24.0, 42.3, 128.0, 128.3, 128.7, 129.2, 129.7, 130.7, 131.5, 132.5, 138.0, 153.8, 162.2, 164.0, and 165.9. LC-MS, *m*/*z*: 332 $[M+H]^+$. Anal. Calcd. for $C_{16}H_{14}ClN_3OS$: C, 57.92; H, 4.25; N, 12.66. Found: C, 57.99; H, 4.36; N, 12.73.

N-(4-fluorobenzyl)-4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxamide (4d)

Yield 68%, a white powder; mp 175°C–176°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.75 (s, 3H, CH_3), 2.91 (s, 3H, CH_3), 4.48 (d, 1H, *J* = 6.5 Hz, CH_2), 7.15 (t, 2H, *J* = 8.3 Hz, H Ar), 7.41 (m, 2H, H Ar), 8.90 (s, 1H, H-4), and 8.97 (br. s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 16.6, 24.5, 42.8, 115.4, 115.7, 129.8, 129.8, 130.2, 131.3, 132.9, 135.6, 135.6, 154.2, 160.5, 162.6, 162.9, 164.4, and 166.4. LC-MS, *m*/*z*: 316 $[M+H]^+$. Anal. Calcd. for $C_{16}H_{14}FN_3OS$: C, 60.94; H, 4.47; N, 13.32. Found: C, 61.11; H, 4.53; N, 13.34.

N-(4-methoxybenzyl)-4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxamide (4e)

Yield 59%, a white powder; mp 159°C–160°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.74 (s, 3H, CH_3), 2.91 (s, 3H, CH_3), 3.75 (s, 3H, OCH_3), 4.43 (d, 1H, *J* = 6.5 Hz, CH_2), 6.92 (d, 1H, *J* = 8.3 Hz, H Ar), 7.29 (d, 1H, *J* = 8.3 Hz, H Ar), 8.89 (br s, 1H, NH), and 8.90 (s, 1H, CH); ^{13}C NMR (125 MHz, DMSO-d_6): δ 16.1, 24.0, 42.4, 55.0, 113.7, 128.7, 129.7, 130.9, 131.1, 132.1, 153.7, 158.3, 162.0, 163.9, and 165.9. LC-MS, *m*/*z*: 328 $[M+H]^+$. Anal. Calcd for $C_{17}H_{17}N_3O_2S$: C, 62.37; H, 5.23; N, 12.83. Found: C, 62.48; H, 5.31; N, 12.85.

(4,5-dimethylthieno[2,3-d]pyrimidin-6-yl)(morpholin-4-yl) methanone (4f)

Yield 43%, a white crystalline powder; mp 195°C–196°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.55 (s, 3H, CH_3), 2.90 (s, 3H, CH_3), 3.19–3.69 (m, 8H, H morpholine), and 8.90 (s, 1H, CH); ^{13}C NMR (125 MHz, DMSO-d_6): δ 16.4, 24.1, 66.5, 129.3, 129.7, 129.8, 153.8, 162.6, 163.9, 167.2, and 168.7. LC-MS, *m*/*z*: 278 $[M+H]^+$. Anal. Calcd for $C_{13}H_{15}N_3O_2S$: C, 56.30; H, 5.45; N, 15.15. Found: C, 56.38; H, 5.46; N, 15.23.

6-(1H-benzimidazol-2-yl)-4,5-dimethylthieno[2,3-d] pyrimidine (5)

To 3.0 g (0.0144 mol) of 4,5-dimethylthieno[2,3-*d*] pyrimidine-6-carboxylic acid (**3**) 2.53 g (0.0156 mol) of 1,1'-carbonyldiimidazole and 25 ml of anhydrous DMF were added. The reaction mixture was stirred and heated at 70°C–80°C before the addition of 1.7 g (0.0156 mol) of *o*-phenylenediamine and the heating continued at 130°C–140°C for 3–5 hours. After cooling to the reaction mixture 100 ml of purified water was added and the precipitate formed was filtered off and washed with plenty of water. An analytical sample was purified by boiling in ethanol. Yield 62%, a beige powder; mp 239°C–240°C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.94 (s, 3H, CH_3), 2.96 (s, 3H, CH_3), 7.25 (br. s, 2H, H Ar), 7.64 (br. s, 2H, H Ar), 8.90 (s, 1H, CH), and 12.91 (br. s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 16.8, 24.6, 126.9, 131.0, 131.3, 146.0, 153.8, 163.8, and 167.1. LC-MS, *m*/*z*: 281 $[M+H]^+$. Anal. Calcd. for $C_{15}H_{12}N_4S$: C, 64.26; H, 4.31; N, 19.98. Found: C, 64.29; H, 4.39; N, 20.06.

General method of N-Aryl-2-[2-(4,5-dimethylthieno[2,3-d] pyrimidin-6-yl)-1H-benzimidazol-1-yl]acetamides (6) synthesis via alkylation of 6-(1H-benzimidazol-2-yl)-4,5-dimethylthieno[2,3-d]pyrimidine (5)

To 0.28 g (0.001 mol) of 6-(1*H*-benzimidazol-2-yl)-4,5-dimethylthieno[2,3-*d*]pyrimidine (**5**) 0.14 g (0.001 mol), 0.001 mol of corresponding alkylating agent and 3 ml of dried dimethylformamide were added, and after the content of the flask was heated at 120°C for 5 hours, then chilled reaction mixture was quenched with water and the precipitate formed was drained and dried at raised temperature (65°C). All of compound **6** were additionally purified by boiling in ethanol.

2-[2-(4,5-dimethylthieno[2,3-d]pyrimidin-6-yl)-1H-benzimidazol-1-yl]-N-phenylacetamide (6a)

Yield 78%, a white powder; Mp >300°C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.58 (s, 3H, CH_3), 2.92 (s, 3H, CH_3), 5.13 (s, 2H, CH_2), 7.04 (t, 1H, *J* = 6.5 Hz, H Ar), 7.24–7.41 (m, 4H, H Ar), 7.48 (d, 2H, *J* = 7.1 Hz, H Ar), 7.61 (d, 1H, *J* = 7.1 Hz, H Ar), 7.78 (d, 1H, *J* = 7.1 Hz, H Ar), 8.95 (s, 1H, CH), and 10.34 (br. s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6): δ 16.7, 24.2, 47.8, 111.5, 119.6, 119.9, 120.2, 123.0, 123.9, 124.1, 124.5, 129.3, 129.7, 134.1, 136.4, 138.7, 143.0, 146.7, 153.9, 164.1, 165.4, and 167.9. LC-MS, *m*/*z*: 414 $[M+H]^+$. Anal. Calcd for $C_{23}H_{19}N_5OS$: C, 66.81; H, 4.63; N, 16.94. Found: C, 66.96; H, 4.65; N, 17.01.

N-(4-methylphenyl)-2-[2-(4,5-dimethylthieno[2,3-d]pyrimidin-6-yl)-1H-benzimidazol-1-yl]acetamide (6b)

Yield 68%, a white powder; Mp >300°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H, CH_3), 2.58 (s, 3H, CH_3), 2.93 (s, 3H, CH_3), 5.10 (s, 2H, CH_2), 7.08 (d, 2H, *J* = 7.8 Hz, H Ar), 7.29–7.40 (m, 4H, H Ar), 7.62 (d, 1H, *J* = 6.8 Hz, H Ar), 7.78 (d, 1H, *J* = 6.8 Hz, H Ar), 8.96 (s, 1H, CH), and 10.26 (br. s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6): δ 16.7, 20.8, 24.2, 47.7, 111.5, 119.6, 119.9, 123.0, 123.8, 124.6 129.6, 129.7, 133.1, 134.1, 136.2, 136.4, 143.0, 146.7, 153.9, 164.1, 165.1, and 167.9. LC-MS, *m*/*z*: 428 $[M+H]^+$. Anal. Calcd for $C_{24}H_{21}N_5OS$: C, 67.43; H, 4.95; N, 16.38. Found: C, 67.54; H, 5.06; N, 16.48.

N-(3,5-dimethoxyphenyl)-2-[2-(4,5-dimethylthieno[2,3-d] pyrimidin-6-yl)-1H-benzimidazol-1-yl]acetamide (6c)

Yield 81% yield, a white powder; mp 282°C–283°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.58 (s, 3H, CH_3), 2.93 (s, 3H, CH_3), 3.67 (s, 6H, 2 OCH_3), 5.10 (s, 2H, CH_2), 6.22 (s, 1H, H Ar), 6.70 (s, 2H, H Ar), 7.29–7.41 (m, 2H, H Ar), 7.62 (d, 1H, *J* = 7.6 Hz, H Ar), 7.78 (d, 1H, *J* = 7.6 Hz, H Ar), 8.96 (s, 1H,

CH), and 10.31 (br. s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 16.7, 24.3, 47.8, 55.5, 96.2, 97.9, 111.5, 119.9, 123.0, 123.9, 124.5, 129.8, 134.1, 136.5, 140.4, 143.0, 146.7, 154.0, 161.0, 164.2, 165.5, and 167.9. LC-MS, *m/z*: 474 $[M+H]^+$. Anal. Calcd for $C_{25}H_{23}N_5O_3S$: C, 63.41; H, 4.90; N, 14.79. Found: C, 63.51; H, 4.97; N, 14.88.

Microbiology

The antimicrobial and antifungal activities of the obtained substances were estimated according to the recommendation of WHO and the national standard of Ukraine (Coyle, 2005; Nekrasova, 2007) using *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633 as two Gram-positive bacteria and the set of three Gram-negative strains, which were *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *Proteus vulgaris* ATCC 4636 strains. The fungal strain *Candida albicans* ATCC 885/653 was used for antifungal activity studies. For the "agar well" diffusion method the microbial load was 10^7 CFU in 1 ml of the nutrient media and it was determined according to the MacFarland standard (McFarland, 1907). According to the national recommendations (Ministry of Public Health of Ukraine, 2001), each microorganism culture of 18–24-hour growth was used for the test. For antimicrobial activity studies, Muller–Hinton (HIMedia Laboratories Pvt. Ltd., India) agar was used, while Sabouraud agar (HIMedia Laboratories Pvt. Ltd., India) was used to study fungi. The administration of each tested sample including standards (Streptomycin) was performed in form of dimethylsulfoxide solutions with 100 µg/ml concentrations in the form of 0.3-ml aliquots (Magaldi *et al*., 2004). The activity of every tested compound sample was estimated three times experimentally. The assessment of antibacterial activity against each microorganism was performed according to the measured value of growth inhibition zones.

The activity of the compounds was assessed by the following criteria (Coyle, 2005; Nekrasova, 2007): absence of the growth inhibition zone or its diameter less than 10 mm, very low sensitivity of a microorganism to the tested compound in this concentration; the diameter near 10–15 mm, low sensitivity of a microorganism to the tested compound in this concentration; the diameter near 15–25 mm, good sensitivity of a microorganism to the tested compound in this concentration; the diameter exceeded 25 mm, high sensitivity of a microorganism to the tested compound in this concentration.

Docking studies

For the docking studies, we used the same set of computer programs as previously reported (Severina *et al*., 2020; Vlasov *et al*., 2021); namely AutoDockTools 1.5.6rc3 and AutoDock Vina, BIOVIA Draw 2019, Chem3D software, and Discovery Studio V17.2.0.16349. 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). The docking procedure obeyed all the conventional algorithms (Torres *et al*., 2016). The output formats of the structures were transformed to a PDBQT format. All the docking parameters, validation techniques for the algorithm, and RSMD values were reported previously by us (Vlasov *et al*., 2021).

RESULTS AND DISCUSSION

Although some compounds with 4-O-alkylthieno[2,3-d]pyrimidine core were reported to be inactive as TrmD inhibitors, the most active compound **I** with oxo group at position four has been reported in the same article (Zhong *et al*., 2019b) and 4-N-alkyl derivatives showed good activity as inhibitors to the acetyltransferase of bacterial amino-sugar **II** (De Schutter *et al*., 2017). Antimicrobial properties were also revealed earlier for thieno[2,3-d]pyrimidine-4-carboxamides, which are also the compounds with aromatic pyrimidine ring, and notably benzylamide **III** was the most active against *P. aeruginosa* (Vlasova *et al*., 2019). The problem of the aromatic pyrimidine ring to antibacterial activity remained unclear. We found it interesting to obtain the derivatives with a small alkyl substituent at position four of thienopyrimidine attached to the heterocyclic system without any additional atoms linker O or N atoms.

For the further preparation of attractive antimicrobial substances, we additionally considered two possible main ways of modifying the carboxylic function at position six of the thienopyrimidine cycle: one of them is the preparation of amides and the other one is the use of heterocyclization methods which showed their effectiveness for construction of thieno[2,3-d]pyrimidines bearing 6-heteryl substituent with antimicrobial properties (Hill *et al*., 2013; Vlasov *et al*., 2021; Zhong *et al*., 2019b).

The examples of 4-alkylthieno[2,3-d]pyrimidines are quite rare, although recently some 4-methylthieno[2,3-d]pyrimidines with different heterocycles at position six were reported as ones with antiproliferative dose-dependent activity according to NCI-H460 xenograft model and their safety profile was found acceptable (Lin *et al*., 2018). The building blocks for these structures were obtained by reaction of 4-chlorothieno[2,3-d]pyrimidine with methylboronic acid. We found this approach promising and decided to test an opportunity of synthesis of 4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxylic acid as a perfect building block for the synthesis of amides and the construction of benzimidazole substituent at position six of the core heterocyclic system.

The first preparation of 4-alkyl derivatives of thieno[2,3-d]pyrimidine was reported in 1971 (Manhas and

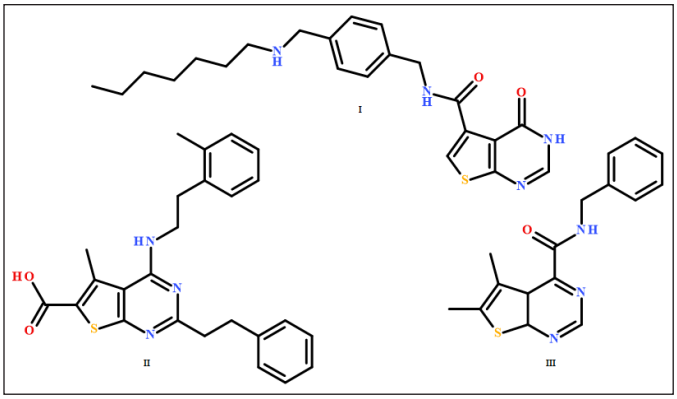

Figure 1. The derivatives of thieno[2,3-d]pyrimidine with antibacterial properties.

Sharma, 1971). The authors used 4-chloride of 2-phenyl-5,6,7,8-tetrahydro[1]-benzothieno[2,3-d]pyrimidine as the starting compound for the preparation of 4-methylsulphonylmethyl-2-phenyl-5,6,7,8-tetrahydro[1]-benzothieno[2,3-d]pyrimidine, which was further reduced with aluminum amalgam in aqueous tetrahydrofuran. The example of incidental preparation of 4-alkyl thieno[2,3-d]pyrimidine occurred during the experimental selection of metalation reagent (Therkelsen *et al*., 2004). Preparation of 4-methyl derivatives by cyclization of 4-chloro-5-(trimethylsilylethynyl)pyrimidines with the addition of sodium hydrosulfide was also suggested (Sakamoto *et al*., 1986). Preparation of 4-alkylthieno[2,3-d]pyrimidines by Suzuki coupling was first reported for the preparation of 4-butyl derivative as the result of the interaction of $Pd(PPh_3)_4$-mediated reaction of the corresponding chloride with butylboronic acid (Fernandez-Mato *et al*., 2011); similar transformations for methylboronic acid were reported recently (Lin *et al*., 2018).

In view of the high effectiveness of methylation in the reaction of different aryl and hetaryl halides in reaction with methylboronic acid (Falb *et al*., 2017; Karroum *et al*., 2018), we studied the Pd-catalyzed reaction of previously reported ethyl 4-chloro-5-methylthieno[2,3-d]pyrimidine-6-carboxylate (Triloknadh *et al*., 2018) with methylboronic acid (Fig. 2). It is interesting that the reaction appeared very sensitive to the quality of the starting chloride. For the crude chloride in the reaction with boronic acid, we saw the traces of the by-product with quasimolecular ion peak 459 (m/z), which probably resulted from Pd-catalyzed side N-arylation reaction of the chloride **1** with its oxo-derivative, which might have been as admixture. Therefore, for the scaling up of the reaction, the starting chloride **1** was purified chromatographically.

The reaction of pure compound 1 with methylboronic acid catalyzed by $PdCl_2$ dppf allowed the preparation of 4-methyl derivative **2** in satisfactory yield (Fig. 2). At the next step, the ester 2 was hydrolyzed in alkaline conditions to give acid 3 with an excellent yield. Synthesis of amides 4 was performed by reaction of the acid **3** with the series of primary benzyl amines and morpholine in DMF, which was promoted by CDI. All of the amides **4** were obtained with good yields 43%–92%. In the ^{1}H NMR spectra of the compounds **4** two signals of methyl groups at positions 4 and 5 of thienopyrimidine are observed at 2.54–2.75 and 2.90–2.92 ppm; the position of the down-field signal is not much influenced by the amide substituent which confirms that this is the signal of CH_3 group at more distant form carboxamide substituent position four. For all of the compound **4**, the signal of a proton of thieno[2,3-*d*]pyrimidine at position two is observed as a singlet at 8.90–8.98 ppm. For benzylamides **4a-4e** the doublet signals of the methylene group are observed at 4.43–4.49 ppm while the broaden signal of NH is obviously seen at 8.86–9.10 ppm; the signals of aromatic protons are observed at 6.92–7.40 ppm. For morpholyl amide **4f** the signals of methylene groups are at 3.19–3.69 ppm. ^{13}C NMR spectra of the synthesized derivatives **4** have signals of both CH_3 groups of thieno[2,3-*d*]pyrimidine at 16.1–16.6 ppm and 24.0–24.6 ppm.

We also studied the reaction of acid **3** in conditions similar to amides **4** synthesis using o-phenylenediamine as an amine. It was found that the reaction at 120°C–130°C during 2–3 hours results in the product of benzimidazole ring closure with the formation of 6-(1*H*-benzimidazol-2-yl)-4,5-dimethylthieno[2,3-*d*] pyrimidine **5**. In the ^{1}H NMR spectrum of the derivative 6, the signals of thienopyrimidine CH3 groups are observed as two singlets at 2.94 та 2.96 ppm; the proton at position two of the core heterocyclic system shows its signal at 8.90 ppm. The signals

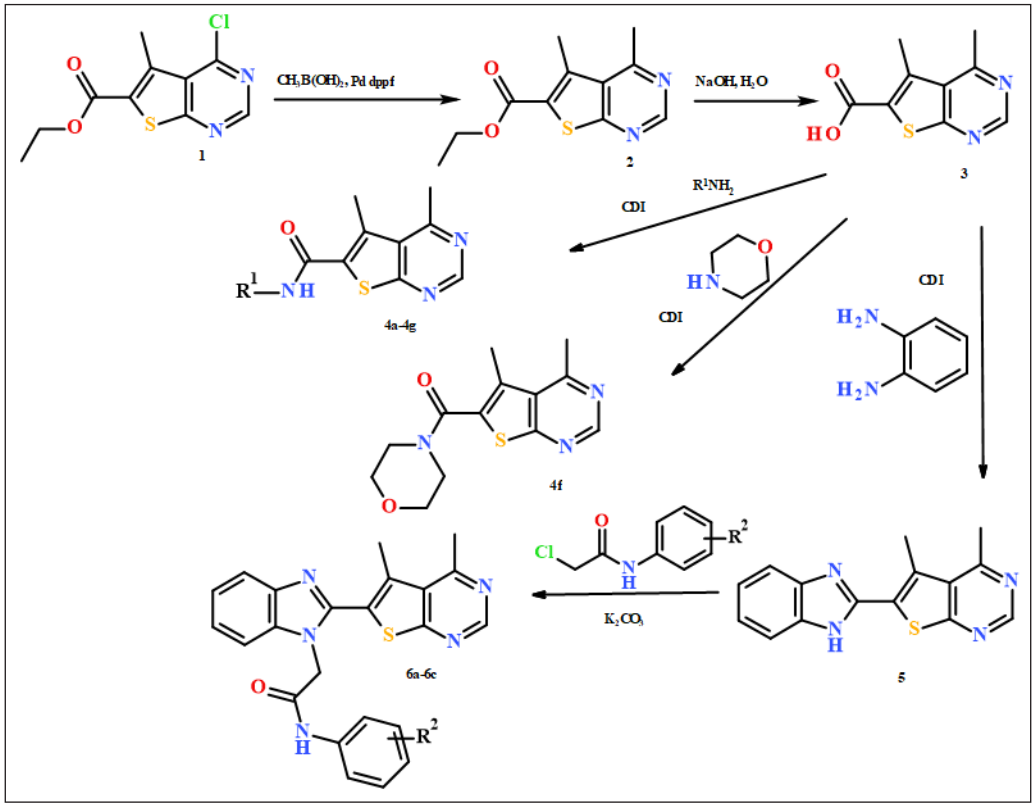

Figure 2. Preparation of 4-methylthieno[2,3-d]pyrimidine derivatives **4a-f, 5,** and **6a-c.**

of the benzimidazole system in the spectrum are observed as two groups at 7.25 (br. s, 2H, H Ar) and 7.64 (br. s, 2H, H Ar), while the signal of benzimidazole NH is at 12.91 ppm.

Compound **5** with its benzimidazole fragment was found suitable for alkylation with chloroacetamides. The alkylation was performed in dimethylformamide using potassium carbonate as the base. 2-[2-(4,5-dimethylthieno[2,3-*d*]pyrimidin-6-yl)-1*H*-benzimidazol-1-yl]-*N*-arylacetamides **6a-6c** were isolated with good yields and crystallized from ethanol. ^{1}H NMR spectra of the compounds 6 in comparison with the compound **5** have additional signals of protons of the CH_2 group of acetamide in the region 5.10–5.13 ppm, while the signals of NH groups are observed in the region 10.26–10.34 ppm. The ^{13}C NMR spectra of **6a-6c** differ from the spectrum of **5** by additional signals of CH_2 at 47.7–47.8 ppm and C=O signals at 165.1–165.5 ppm.

The results of antimicrobial activity screening, which was performed by agar "well" method for all of the synthesized compounds, showed that all of the compounds with the fragment of 4-methylthieno[2,3-d]pyrimidine were active against most bacteria and *Candida* fungi (Table 1). Among the Gram-positive bacteria *B. subtilis ATCC 6633* was the most sensitive; the compounds also most effectively inhibited the growth of *P. aeruginosa ATCC 27853* among the Gram-negative strains. Ester **2** has been found more active than the corresponding acid **3**. The introduction of the benzylamide fragment also increased the activity of the compounds **4c**, **4d**, and **4e** correspondingly 4-Cl, 4-F, and 4-OCH_3 against *B. subtilis* and *P. aeruginosa*. Amide **4f** with morpholine fragment did not display high antimicrobial activity regardless of its better solubility. The results of the antimicrobial activity study also showed the introduction of a benzimidazole ring instead of a carboxyl group. The antimicrobial activity of the compounds synthesized was found to be a little less than the activity of the reference drug Streptomycin, which was selected as the reference drug which is an aminoglycoside antibiotic with a wide spectrum of activity against both Gram-positive and Gram-negative bacteria; it is also active against *Mycobacterium tuberculosis*; this drug is also widely used in clinics (Ahmed *et al*., 2013; Jansen *et al*., 2016; Tereshko *et al*., 2003). Unfortunately, not of the TrmD inhibitors, for now, has been approved for medical use.

To suggest the possible mechanism of antibacterial activity for all the compounds which were studied for the antimicrobial activity we performed the docking study to the active site of TrmD selective inhibitors. The TrmD which was isolated from *P. aeruginosa* (Zhong *et al*., 2019b) was used as a protein model. The validation of docking methodology and RMSD calculation of the reference ligand pose to the native ligand were reported earlier (Vlasov *et al*., 2021, 2022). According to the docking studies, the obtained compounds showed different affinity to the active site TrmD inhibitor (Table 2).

The lowest level of affinity was predicted for both ester **2** and acid **3** (−7.4 kcal/mole against −8.2 kcal/mole affinity for the native ligand). Transformation of the carboxyl group to the benzimidazole cycle improves the binding energy for derivative 5 (−8.0 kcal/mole). Alkylation of the benzimidazole cycle increases the affinity; the scoring function for arylacetamides **6a-c** reaches −8.7 kcal/mole value. The affinity results were predicted for amides **4**, especially benzylamides **4a-e**, which showed the values of scoring function from −9.3 to −9.9 kcal/mole. The calculated scoring function values are in good correlation with *in vitro* experimental results.

Detailed analysis of the interaction of the ligands with amino acids of the active site shows many hydrophobic interactions. Among them, there was the interaction of methyl group at position four of thieno[2,3-d]pyrimidine with the pyrrolidine cycle of proline Pro94, which can be a sign of the existence of stable ligand-enzyme conformations. All the studied ligands interact with the residues of glutamic acid Gly145, 146, and/or glutamine Gln95, which were proven to be the site of TrmD cofactor S-adenosylmethionine (SAM) binding. This determines the deep immersion of 4-methylthieno[2,3-d]pyrimidines in the cavity of the active site competing with SAM and thus inhibiting bacterial TrmDs like the one isolated from *P. aeruginosa*. As to the results of the docking, the best parameters of antibacterial activity via inhibition of TrmD were shown by benzyl amides **4a-e** (Fig. 3).

Table 1. Antimicrobial activity screening results for 4-methylthieno[2,3-d]pyrimidine derivatives **2**,**3**, **4a-f**, **5**, **6a-c**.

Substances*	Diameters (mm) of growth inhibition zone, number of test repetitions n = 3					
	Gram positive bacteria		Gram negative bacteria			*Fungus*
	Staphylococcus aureus ATCC 25923	*Bacillus subtilis ATCC 6633*	*Escherichia coli ATCC 25922*	*Proteus vulgaris ATCC 4636*	*Pseudomonas aeruginosa ATCC 27853*	*Candida albicans ATCC 653/885*
2	16, 17, 17	25, 26, 25	17, 17, 17	15, 15, 16	23, 24,23	23, 23, 23
3	15, 15, 16	20, 20, 20	15, 15, 16	16, 15, 16	22, 21, 21	18, 18, 17
4a	17, 18, 17	21, 21, 21	17, 17, 17	17, 17, 17	20, 20, 20	22, 21, 21
4b	20, 20, 21	21, 22, 22	18, 18, 19	16, 17, 16	22, 20, 21	22, 21, 21
4c	20, 21, 20	23, 24, 23	18, 18, 18	16, 16, 17	24, 23, 24	23, 22, 22
4d	20, 21, 21	24, 25, 25	20, 20, 20	18, 18, 18	24, 23, 23	23, 22, 22
4e	20, 20, 21	26, 25, 26	17, 17, 18	14, 15, 15	23, 23, 25	23, 22, 22
4f	17, 17, 18	20, 21, 21	17, 17, 17	16, 16, 16	22, 22, 21	18, 17, 18
5	20, 20, 20	26, 27, 26	20, 19, 19	17, 16, 17	25, 23, 23	22, 23, 23
6a	20, 20, 21	26, 25, 25	18, 17, 17	17, 17, 17	24, 24, 24	23, 23, 24
6b	20, 20, 20	27, 27, 26	20, 21, 19	17, 17, 17	22, 23, 24	24, 23,2 4
6c	21, 21, 20	26, 26, 26	20, 20, 21	17, 18, 16	22, 22, 23	23, 22, 24
Streptomycin	30, 30, 30	27, 28, 28	25, 25, 25	26, 24, 24	26, 26, 25	**

*The concentration of the compounds and the comparison drug streptomycin 100 µg/mL. ** Lack of antifungal activity.

Table 2. The resuls of the docking studies of 4-methylthieno[2,3-d]pyrimidine derivatives **2**, **3**, **4a-f**, **5**, **6a-c** to the active site of selective TrmD *P. aeruginosa* inhibito rs.

Ligand	Binding energy kcal/mol	Hydrophobic interaction	Hydrogen interaction	Other interaction
Native ligand	−8.2	Tyr141, Ser93(2)#, Pro94 (4), Pro149(2), Ile138, Leu143, Gly 145, Gly146	Leu143, Gln95, Glu121, Gly139, Asp182	–
2	−7.4	Ser93(2), Pro94(2), Gly145, Gly146, Ile138(2), Leu143(3), Pro94(3), Val142*, Leu143, Tyr141, Pro149(2)	Ile138, Trp136*	–
3	−7.4	Ser93 (2), Pro94(5), Gly145, Gly146, ile138(2), leu143(3), val142*, tyr141, pro149(2)	Ile138, Trp136*	–
4a	−9.6	Pro94(5), Val142*, Leu143, Gly145, Pro149	Tyr91*, Gly122*, Leu92	–
4b	−9.9	Gly145, Gly146, Pro94(6), Val142*, Ile138, Leu143(2),Tyr141, Pro149	Tyr91*, Gly122*, Leu92	–
4c	−9.8	Gly145, Gly146, Pro94(5), Val142*, Ile138, Leu143(2), Tyr141, Pro149	Tyr91*, Gly122*, Leu92	–
4d	−9.9	Pro94(5), Val142, Leu143, Pro149, Gly145	Tyr91*, Ile138, Gly139*, Gly122*, Leu92	Gly139, Tyr141Halogen Fluorine
4e	−9.3	Gly145, Gly146, Pro94(5), Val142(2)*, Leu143, Pro149	Tyr91*, Gly118, Leu92, Ser137*, Gly139*	
4f	−8.6	Ser93(2), Pro94(7), Val142*, Leu143(2), Pro149(2), Ile138	Ser137*, Ile138, Gly145, Trp136, Tyr120	
5	−8.0	Val142(2), Pro94(3), Gly145	Arg159*, Leu143	Asp159* Pi-Cation
6a	−8.6	Val142(3)*, Pro94(2), Ile25* Leu143, Gln95(2)	Arg159*, Thr177*, Leu143	Asp159* (2) Pi-Cation
6b	−8.3	Pro94(5), Val142(3)*	Gln95, Leu180*	Arg159*Pi-Cation, Asp182 Pi-Anion, His185*Pi-Sulfur
6c	−8.7	Val142(4)*, Pro94(5), Leu143, Pro149	Thr177(2)*, Gln95(2), Pro94	Arg159* Pi-Cation, Asp182(2) Pi-Anion

The number of bonds is given in brackets
* The amino acids which do not interact with native ligand in the experiment

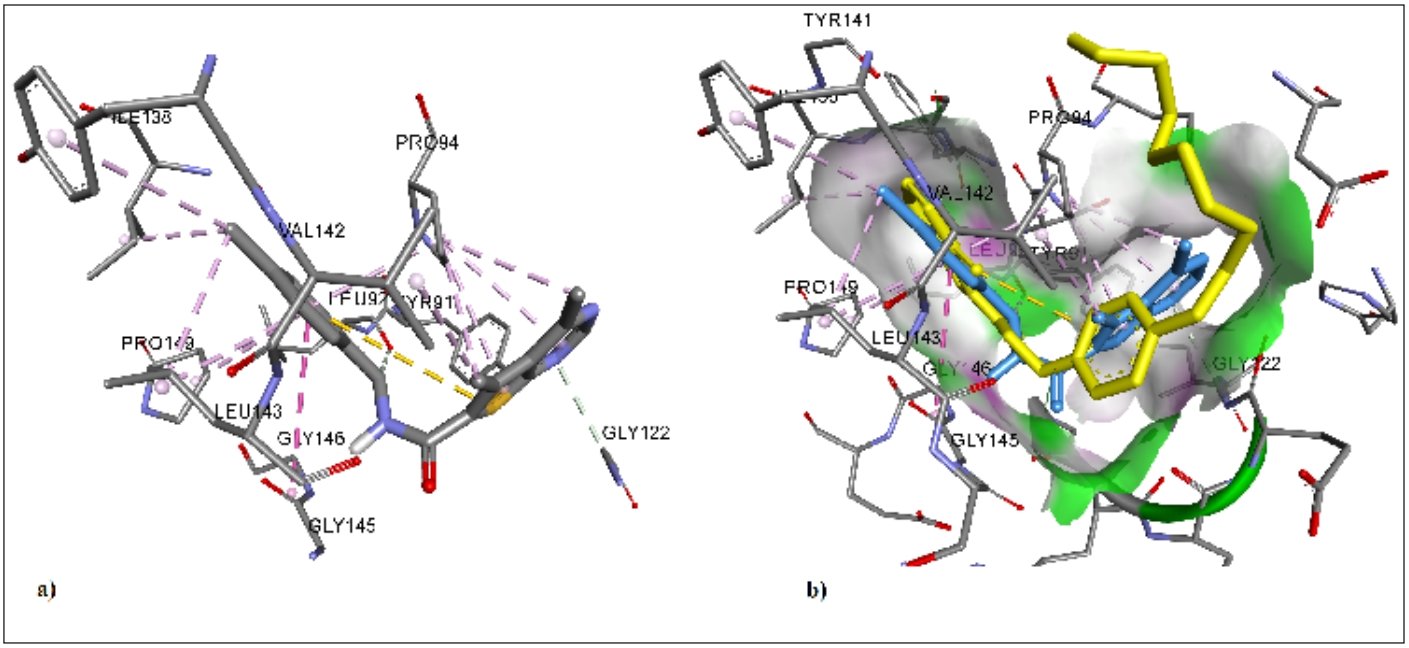

Figure 3. 3D visualization of the ligand **4b** and the amino acids of the active site of selective TrmD inhibitors (a) conformation with reference inhibitor (yellow structure) (Zong *et al*., 2019) in the active site of *P. aeruginosa* TrmD.

CONCLUSION

As the result of modification of position four of thieno[2,3-d] pyrimidine using Suzuki reaction of methyl boronic acid and readily available ethyl 4-chloro-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylate the ester and 4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxylic acid resulted from hydrolysis of the ester were obtained. Modification of 4,5-dimethylthieno[2,3-d]pyrimidine-6-carboxylic acid gave the series of amides and the derivative with benzimidazole fused system at position six. All of the synthesized compounds showed antimicrobial properties, especially against the strains of *B. subtilis* and *P. aeruginosa*. The most promising antimicrobial properties were determined for benzyl amides of 4,5-dimethylthieno[2,3-d] pyrimidine-6-carboxylic acid. The docking studies of the obtained compounds to the active site of selective TrmD inhibitors revealed that benzyl amides also had the best binding parameters to the tRNA (Guanine37-N^1)-methyltransferase of *P. aeruginosa*. The docking studies confirmed the suggested mechanism of antibacterial action as *in silico* results are in correlation with the *in vitro* experiment.

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AUTHOR CONTRIBUTIONS

Concept and design were contributed by S.V.V., V.A.G., O.D.V., and H.I.S.; data acquisition and data analysis were contributed by K.Y.K., V.S.V, P.E.S., H.I.S., O.D.V., O.V.B., and S.V.V.; preparation and submission of the manuscript were done by S.V.V., H.I.S., K.Y.K., and O.V.B.; supervision and final approval were contributed by V.A.G., S.V.V., H.I.S., and O.V.B. All the authors have made a significant contribution to the content of the submitted manuscript; they adjusted it and agree with its publication.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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