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Design and synthesis of novel protein kinase CK2 inhibitors on the base of 4-aminothieno[2,3-d]pyrimidines

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Abstract. An extension of our previous research work has resulted in a number of new ATP-competitive CK2 inhibitors that have been identified among 4-aminothieno[2,3-d]pyrimidine derivatives. The most active compounds obtained in the course of the research are 3-(5-p-tolyl-thieno[2,3-d]pyrimidin-4-ylamino)-benzoic acid, **5e** (**NHTP23**, IC_{50} = 0.01 μM), 3-(5-phenyl-thieno[2,3-d]pyrimidin-4-ylamino)-benzoic acid, **5g** (**NHTP25**, IC_{50} = 0.065 μM) and 3-(6-methyl-5-phenyl-thieno[2,3-d]pyrimidin-4-ylamino)-benzoic acid, **5n** (**NHTP33**, IC_{50} = 0.008 μM). Structure–activity relationships of the tested 4-aminothieno[2,3-d]pyrimidine derivatives have been studied and their binding mode with ATP-acceptor site of CK2 has been proposed. A negative effect of intramolecular hydrogen bonding in the compounds' structure is discussed.

Keywords: Protein kinase CK2; Inhibitor; Aminothieno[2,3-d]pyrimidine; Drug design; Docking; Intramolecular hydrogen bond.

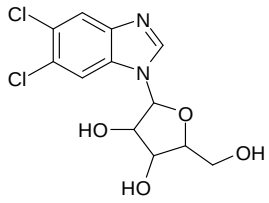
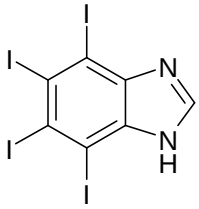
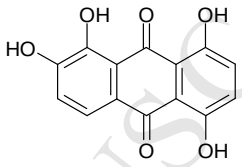
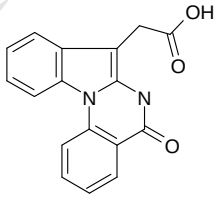
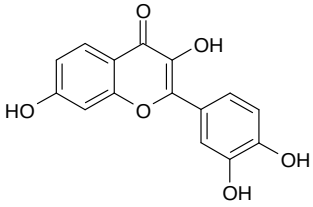
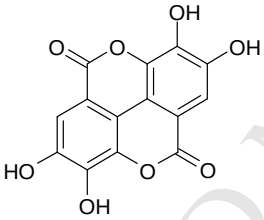
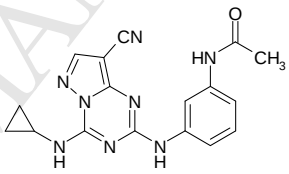
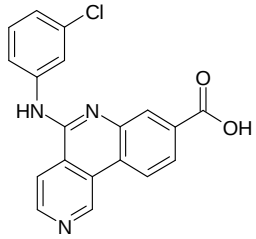
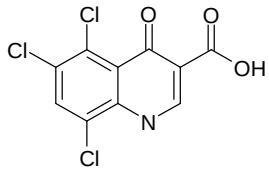
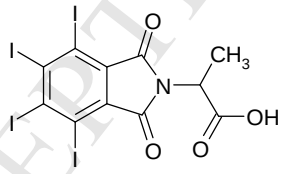
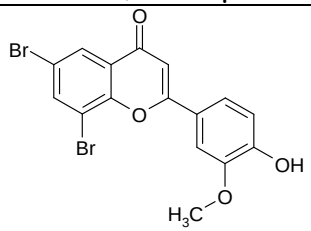
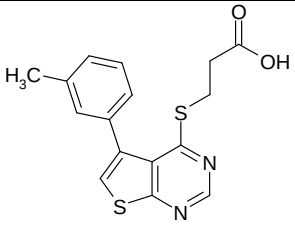
1. Introduction. Protein kinase CK2 is a well-known highly conserved and constitutively active serine/threonine protein kinase that plays an important role in many cellular processes, such as cell cycle progression, apoptosis, cell differentiation and transcription. Overexpression and hyperactivation of this enzyme has been observed in a wide variety of human diseases [1]. It has been proven that CK2 inhibition is critical for treatment and prevention of inflammatory processes, infection and cancer. The history of CK2 as a drug target began with the identification of its inhibitor DRB in 1986 [2]. To date, a significant number of active CK2 inhibitors from different chemical classes have been developed [3-4] (the most famous and active ones are presented in Table 1). However, only one compound (CX-4945 [5], IC_{50}=1 nM) has entered phase II clinical trials so far (ClinicalTrials.gov Identifier: NCT02128282). Therefore, discovery of specific, low toxic and highly active inhibitors of protein kinase CK2 is important for development of novel therapeutic agents.

Taking into consideration the significance of this matter we have been actively searching for CK2 inhibitors during the last decade [6] and identified potent compounds among different chemical classes (Table 1) such as 3-carboxy-4(1*H*)-quinolone derivatives [7], 4,5,6,7-tetrahalogeno-1*H*-isoindole-1,3(2*H*)-diones [8], flavone derivatives [9, 10] and (thieno[2,3-d]pyrimidine-4-ylthio)carboxylic acid derivatives [11]. High CK2 inhibitory activity of the compounds from the last class induced us to study thieno[2,3-d]pyrimidines

thoroughly. In this work, we focus on the synthesis and evaluation of 4-aminothieno[2,3-d]pyrimidine derivatives.

Table 1

Chemical structure and inhibitory activity of well-known inhibitors of protein kinase CK2

			
DRB [2] K_i = 23 μM	TIBI [12] K_i = 0.023 μM	Quinalizarin [13] K_i = 0.06μM	IQA [14] K_i = 0.17 μM
			
Fisetin [15] K_i = 0.17 μM	Ellagic acid [16] K_i = 0.06 μM	Pyrazolotriazine 15a [17] K_i = 0,00026 μM	CX-4945 [5] K_i = 0,00038 μM
			
Quinolone 7 [7] K_i = 0.06 μM	TID 46 [8] K_i = 0.1 μM	FNH79 [10] K_i = 0.0018 μM	Compound 6a [11] K_i = 0.04 μM

2. Experimental section

2.1. Molecular docking

Ligands were prepared for docking with energy minimization and converted into pdbqt format with Vega ZZ [18]. Docking was done in crystal structure of CK2 (PDB ID:

4GRB [19]) using AutoDock 4.2 with default parameters. The crystal structure chosen for the docking is one of the recent human CK2-inhibitor complexes resolved. It has sufficient resolution, (2.15 Å), doesn't contain mistakes or sequence alterations, and the conservative water molecule W1 located near Lys68 in ATP-binding site is preserved. Resulted docking complexes were analysed with AutoDockTools-1.5.6 [20].

2.2. Biology

Compounds were tested using in vitro kinase assay[21]. Each test was done in triplicate in a reaction volume of 30 μL, containing 6 μg of peptide substrate RRRDDDSDDD (New England Biolabs); 10 units of recombinant human CK2 holoenzyme (New England Biolabs); 50 μM ATP and γ-labeled ^{32}P ATP, diluted to specific activity 100 μCi/μM; CK2 buffer (20 mM Tris-HCl, pH 7.5; 50 mM KCl; 10 mM $MgCl_2$) and inhibitor in varying concentrations. Incubation time was 20 min at 30^o C. The reaction was stopped by adding an equal volume of 10 % *o*-phosphoric acid and the reaction mixture was loaded onto 20-mm discs of phosphocellulose paper (Whatman). Disks were washed three times with 1 % *o*-phosphoric acid solution, air-dried at room temperature, and counted by the Cherenkov method in a beta-counter (LKB). As negative control an equal volume of DMSO was added to the reaction mixture. Percent inhibition was calculated as ratio of substrate-incorporated radioactivity in the presence of inhibitor to the radioactivity incorporated in control reactions, i.e. in the absence of inhibitor. Serial dilutions of inhibitor stock solution were used to determine its IC_{50} concentration. The IC_{50} values represent means of triplicate experiments with SEM never exceeding 15%.

Selectivity tests were performed on protein kinases ASK1, JNK3, c-Met, Aurora A, ROCK1, FGFR1 and Tie2 according to the supplier's recommendations (Millipore). Kinase residual activity, determined in the presence of 10 μM of inhibitor is expressed as a percentage of the control without inhibitor. Final concentration of ATP in the experiment was 100 μM.

2.3. Chemistry

All studied 4-aminothieno[2,3-d]pyrimidine derivatives were synthesized starting from the corresponding carbonyl compounds, according to Scheme 1. The first three steps of chemical synthesis were described by our research group previously [22].

Nuclear magnetic resonance spectra were recorded with Varian Mercury VRX-400 spectrometer using DMSO-d_6 as solvent and tetramethylsilane as internal standard. Chemical shift values (δ) are quoted in ppm, and coupling constants (J) in Hz.

*2.3.1. Substituted 2-amino-3-carbethoxythiophene (**a**).* A mixture of ethyl cyanoacetate (107 mL, 1 mol), elemental sulfur (32 g, 1 mol) and the corresponding ketone (1 mol) in ethanol (200 mL) was stirred at room temperature. To this mixture, diethylamine (80 mL) was added dropwise during 12 h with stirring. Then the reaction mixture was left on 14 hours; the precipitate was filtered, washed by aqueous alcohol (1:1) and dried in air. Yield 50-88%.

*2.3.2. Substituted thienopyrimidinones (**b**).* A mixture of formamide (2 mol) and 2-aminothiophene (1 mol) was heated at 160-170^{o} C for 24 h. The hot mixture was diluted

with isopropyl alcohol (80 mL) and cooled. The solid product was collected by filtration and washed with isopropyl alcohol (60 mL) and water (60 mL) and dried at 60° C. Yield 85-90%.

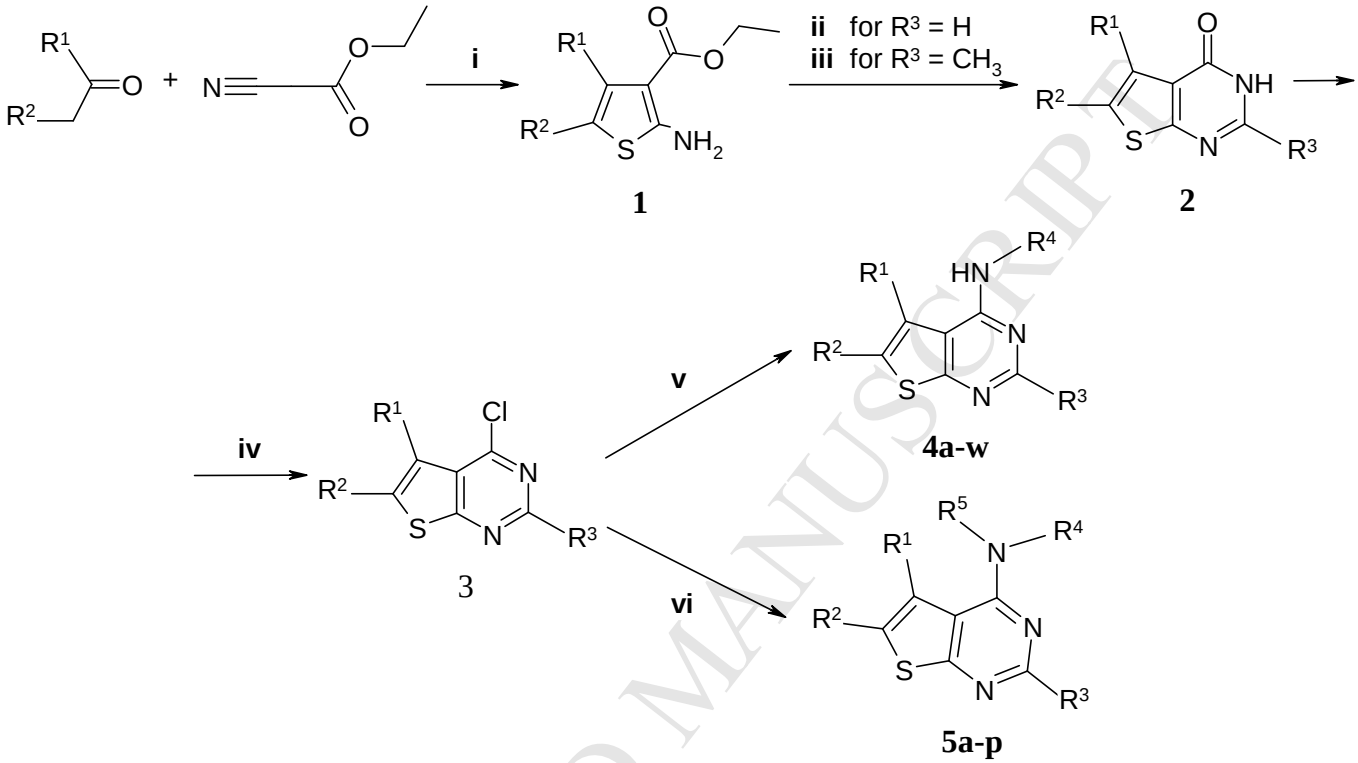
*2.3.3. Substituted thienopyrimidinones (**c**).* Corresponding aminothiophene (0.01 mol) was supplemented with dioxane (30 mL), acetonitrile (0.85 mL) and dioxane (30 mL) saturated with hydrogen chloride. The mixture has been stirred for 12 h at room temperature and neutralized by ammonia. The solid product was filtered, washed with isopropanol alcohol (4 mL) and water (10 mL). Yield 85- 90%.

*2.3.4. Substituted 4-chloro-thieno[2,3-d]pyrimidines (**d**).* A mixture of thienopyrimidinone (0.01 mol), $POCl_3$ (11 mL) and PCl_5 (1.5 g) was quickly heated to 105° C and boiled for 5-6 hours. The solvent was evaporated. Dry product was dissolved in methylene chloride (12 mL) and neutralized by cold solution of NaOH (0.5 N). The organic phase was separated and dried over Na_2SO_4, filtered, and then solvent was evaporated in vacuum. The residue was crystallized from isopropyl alcohol. Yield 80%.

*2.3.5. Substituted 4-aminothieno[2,3-d]pyrimidine (**e**).* A mixture of corresponding amino acid (0.011 mol) dissolved in the minimum amount of water, ethanol (15 mL), triethylamine (0.5 mL) and starting compound (0.01 mol) was heated to 100° C and boiled for 24 h. The solvent was evaporated; the residue was recrystallized from ethanol and water. Yield 30-80%.

*2.3.6. Substituted 4-aminothieno[2,3-d]pyrimidine (**f**).* A mixture of 4-chlorothieno[2,3-d]pyrimidine (0.01 mol), aminobenzoic acid (0.011 mol) and DMF (dimethylformamide) (2 mL) was heated to 150° C and boiled for 6 hours. The precipitate was filtered and washed with acetone (4 mL). To obtain corresponding bases,

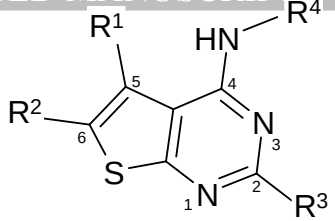
hydrochloride was dissolved in ethanol and supplemented with triethylamine (0.01 mol). Yield 60-80%.

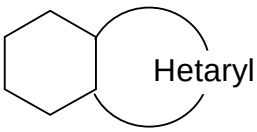
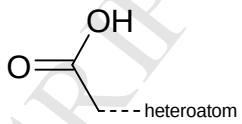
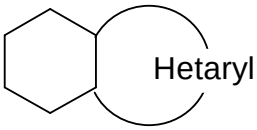
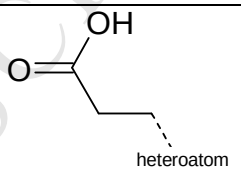
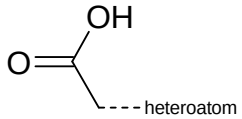
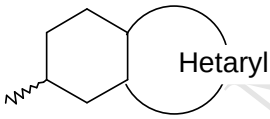
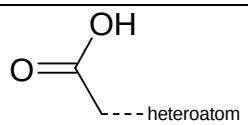
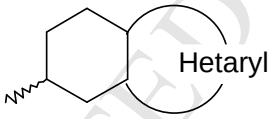
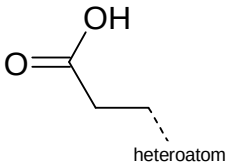
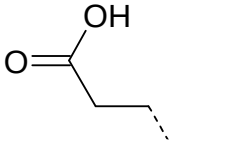
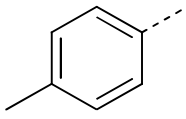
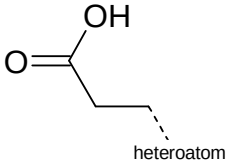
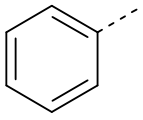
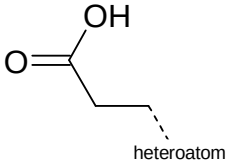
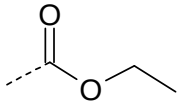
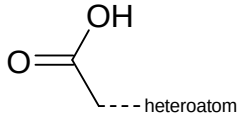


Scheme 1. Scheme of synthesis for compounds **4a-w, 5a-p**. Reagents and conditions: (i) S, $(C_2H_5)_2NH$, C_2H_5OH, r.t., 12 h, 50-88 %; (ii) $HCONH_2$, 160-170^o C, 24 h, 85-90%; (iii) CH_3CN, dioxane, HCl, r.t., 12 h, 85-90 %; (iv) PCl_5, $POCl_3$, 105^o C, 5-6 h, 80 %; (v) R^4NH_2, H_2O, C_2H_5OH, Et_3N, 100^o C, 24 h, 30-80 %; (vi) R^4NH_2, DMF, 150^o C, 6 h, 60-80 %.

Table 2

Structure and inhibitory activity of 4-aminothieno[2,3-d]pyrimidine derivatives prior to chemical optimization



Compound №	R^1	R^2	R^3	R4	IC_{50}, μM
4a			H		>33
4b			H		9.5
4c	CH_3	CH_3	H		>33
4d			H		>33
4e			H		16
4f	CH_3	CH_3	H		>33
4g		H	H		>33
4h		H	H		>33
4i	CH_3		H		>33

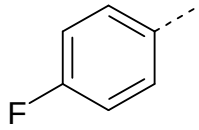
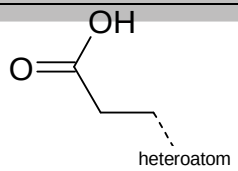
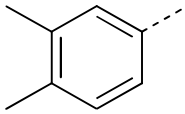
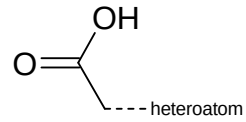
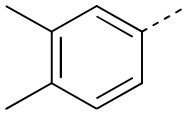
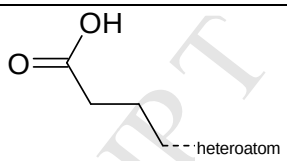
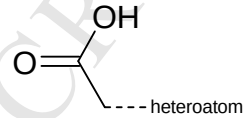
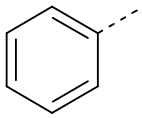
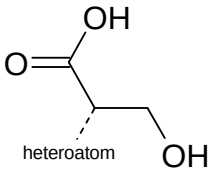
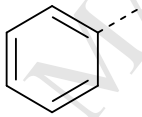
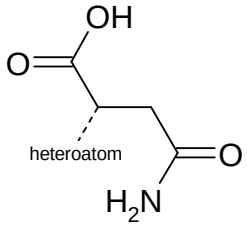
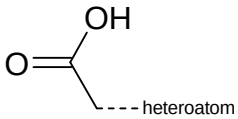
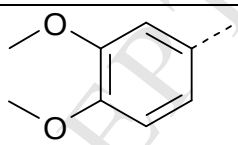
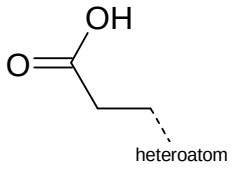
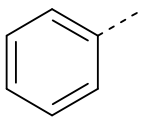
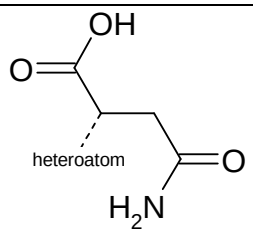
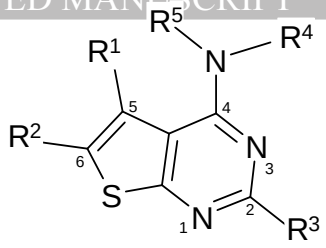
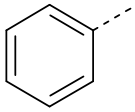
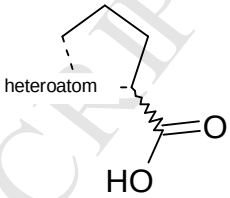
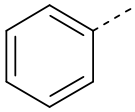
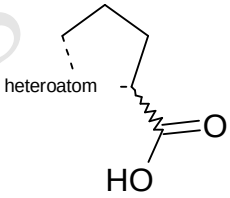
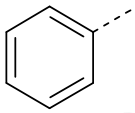
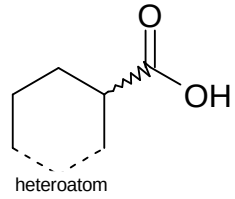
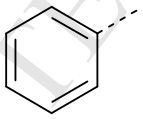
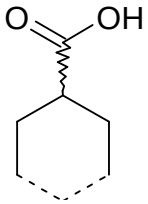
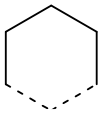
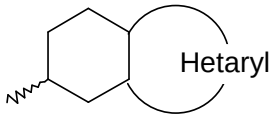
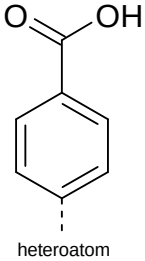
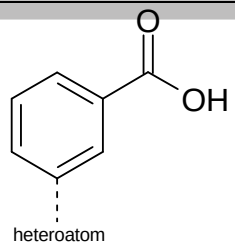
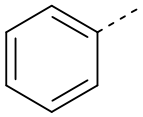
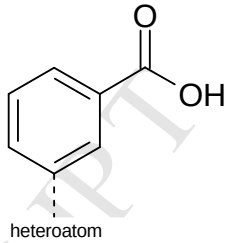
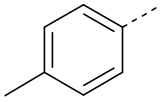
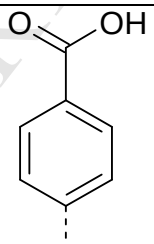
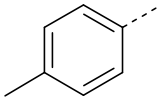
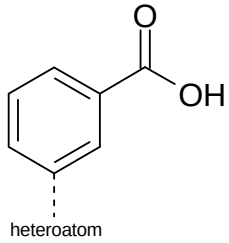
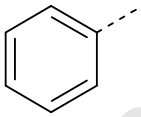
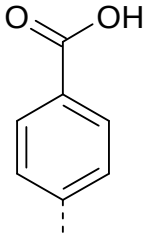
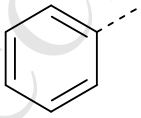
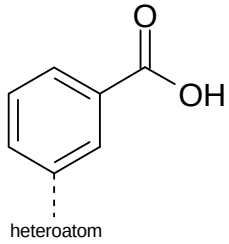
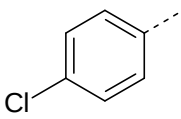
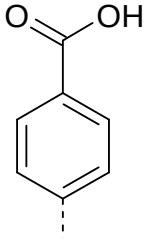
4j		H	H		>33
4k		H	H		>33
4l		H	H		>33
4m	H	CH_3	H		>33
4n	H		H		>33
4o	H		H		>33
4p	H	CH_2-CH_3	H		>33
4q		H	H		>33
4r	H		CH_3		>33

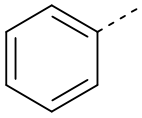
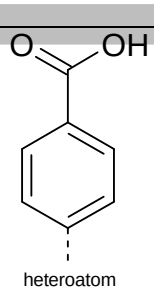
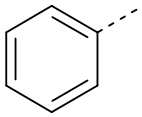
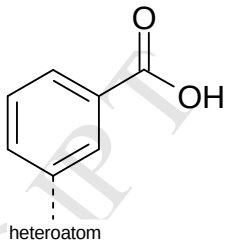
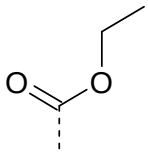
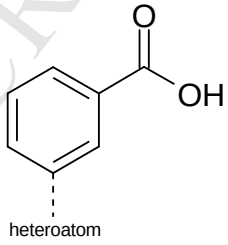
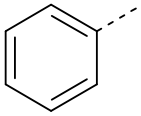
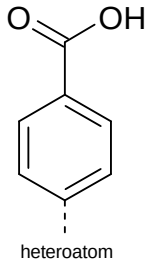
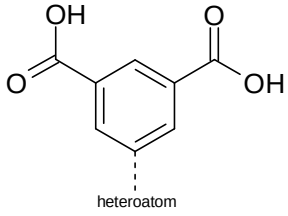
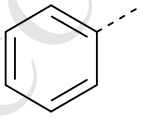
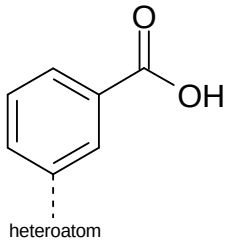
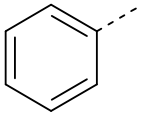
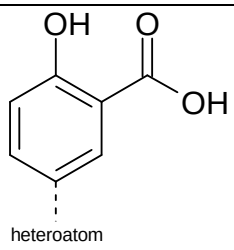
Table 3

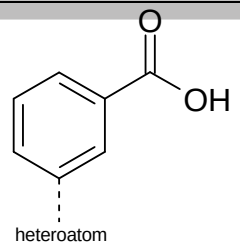
Structure and inhibitory activity of 4-aminothieno[2,3-d]pyrimidine derivatives after chemical optimization



Compound №	R^1	R^2	R^3	R^4	R^5	IC_{50}, μM
4s	H		H			>33
4t	H		CH_3			>33
4u	H		CH_3			>33
4v	H		CH_3			>33
4w	CH_3	COOH	CH_3			>33
5a			H	H		>33

5b	CH_3	CH_3	H	H		3.8
5c	H		H	H		0.26
5d [22]		H	H	H		0.65
5e [22] (NHTP23)		H	H	H		0.01
5f		H	H	H		1.1
5g (NHTP25)		H	H	H		0.065
5h		H	H	H		0.83

5i	H		CH_3	H		>33
5j	H		CH_3	H		1.5
5k [22]	CH_3		H	H		6.5
5l		CH_3	H	H		1.5
5m	H	CH_3	H	H		>33
5n (NHTP33)		CH_3	H	H		0.008
5o		CH_3	H	H		0.31

5p	CH_3	COOH	H	H		>33

*2.3.1. N-(5,6,7,8-Tetrahydro[1]benzothieno[2,3-d]pyrimidin-4-yl)glycine hydrochloride (**4a**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1.82 (4H, s, CH_2), 2.76 (2H, s, CH_2), 2.92 (2H, s, CH_2), 4.11 (2H, d, CH_2), 6.89 (1H, s, NH), 8.25 (1H, s, 2-H), 12.60 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 22.27, 25.24, 26.02, 42.82, 115.92, 126.73, 132.11, 152.84, 156.84, 164.97, 171.98.). Anal. Calc. for $C_{12}H_{14}ClN_3O_2S$: C, 48.08; H, 4.71; S, 10.70. Found: C, 48.04; H, 4.68; S, 10.67.

*2.3.2. N-(5,6,7,8-Tetrahydro[1]benzothieno[2,3-d]pyrimidin-4-yl)-β -alanine hydrochloride (**4b**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1.81 (4H, s, CH_2), 2.42-2.46 (2H, m, CH_2), 2.74 (2H, s, CH_2), 2.91 (2H, s, CH_2), 3.60-3.63 (2H, m, CH_2), 6.88 (1H, s, NH), 8.25 (1H, s, 2-H), 12.64 (bs, COOH).). Anal. Calc. for $C_{13}H_{16}ClN_3O_2S$: C, 49.76; H, 5.14; S, 10.22. Found: C, 49.73; H, 5.13; S, 10.19.

*2.3.3. N-(5,6-Dimethylthieno[2,3-d]pyrimidin-4-yl)glycine hydrochloride (**4c**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.39 (3H, s, CH_3), 2.44 (3H, s, CH_3), 4.12 (2H, s, CH_2), 7.05 (1H, s, NH), 8.24 (1H, s, 2-H), 12.60 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 13.39, 14.40, 42.88, 116.95, 124.61, 128.98, 152.68, 156.88, 164.25, 172.00. Anal. Calc. for $C_{10}H_{12}ClN_3O_2S$: C, 43.88; H, 4.42; S, 11.71. Found: C, 43.86; H, 4.41; S, 11.69.

*2.3.4. N-(7-Methyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4-yl)glycine hydrochloride (**4d**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1.05 (3H, d, CH_3), 1.43-1.48(1H, m, CH), 1.89-1.94 (2H, m, CH_2), 2.33-2.40 (1H, m, CH), 2.82-3.04 (3H, m, CH), 4.10 (2H, d, CH_2), 6.89 (1H, s, NH), 8.24 (1H, s, 2-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 21.38, 25.75, 28.68, 30.32, 33.15, 42.88, 115.78, 126.38, 131.68, 152.87, 156.81, 165.08, 171.98. Anal. Calc. for $C_{13}H_{16}ClN_3O_2S$: C, 49.76; H, 5.14; S, 10.22. Found: C, 49.74; H, 5.13; S, 10.20.

*2.3.5. N-(7-Methyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4-yl)-β-alanine hydrochloride (**4e**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1.03 (3H, d, CH_3), 1.39-

1.45 (1H, m, CH), 1.85-1.90 (2H, m, CH_2), 2.30-2.37 (1H, m, CH), 2.56-2.60 (2H, m, CH_2), 2.78-3.00 (3H, m, CH), 3.67-3.71 (2H, m, CH_2), 6.63 (1H, s, NH), 8.25 (1H, s, 2-H), 12.22 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 21.35, 25.56, 28.64, 30.32, 33.13, 33.78, 36.78, 115.73, 126.38, 131.35, 153.07, 156.92, 164.94, 173.83. Anal. Calc. for $C_{14}H_{18}ClN_3O_2S$: C, 51.29; H, 5.53; S, 9.78. Found: C, 51.28; H, 5.52; S, 9.76.

*2.3.6. N-(5,6-Dimethylthieno[2,3-d]pyrimidin-4-yl)-β-alanine hydrochloride (**4f**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.36 (3H, s, CH_3), 2.40 (3H, s, CH_3), 2.59 (2H, t, CH_2), 3.67-3.71 (2H, m, CH_2), 6.79(1H, s, NH), 8.26 (1H, s, 2-H), 12.33 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 13.35, 14.27, 33.74, 36.82, 116.87, 124.63, 128.61, 152.94, 156.94, 164.09, 173.85. Anal. Calc. for $C_{11}H_{14}ClN_3O_2S$: C, 45.91; H, 4.90; S, 11.14. Found: C, 45.90; H, 4.88; S, 11.12.

*2.3.7. N-[5-(4-Methylphenyl)thieno[2,3-d]pyrimidin-4-yl]-β-alanine hydrochloride (**4g**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.38 (3H, s, CH_3), 2.43-2.45 (2H, m, CH_2), 3.56-3.58 (2H, m, CH_2), 5.65 (1H, s, NH), 7.31 (4H, s, C_6H_4), 7.41 (1H, s, 6-H), 8.41 (1H, s, 2-H), 12.27 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 21.27, 33.55, 36.52, 113.65, 121.06, 129.00, 129.93, 132.74, 134.96, 138.14, 153.89, 157.07, 166.77, 173.47. Anal. Calc. for $C_{16}H_{16}ClN_3O_2S$: C, 54.93; H, 4.61; S, 9.17. Found: C, 54.92; H, 4.59; S, 9.15.

*2.3.8. N-(5-Phenylthieno[2,3-d]pyrimidin-4-yl)-β-alanine hydrochloride (**4h**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.43-2.46 (2H, m, CH_2), 3.56-3.60 (2H, m, CH_2), 5.61 (1H, s, NH), 7.45-7.51 (6H, m, aromatic-H), 8.42 (1H, s, 2-H), 12.29 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 33.51, 36.51, 113.60, 121.43, 128.80, 129.14, 129.37, 134.98, 135.65, 153.93, 157.05, 166.82, 173.48. Anal. Calc. for $C_{15}H_{14}ClN_3O_2S$: C, 53.65; H, 4.20; S, 9.55. Found: C, 53.64; H, 4.18; S, 9.51.

*2.3.9. N-[6-(Ethoxycarbonyl)-5-methylthieno[2,3-d]pyrimidin-4-yl]glycine hydrochloride (**4i**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1.30 (3H, t, J = 7.1 Hz, CH_3, C_2H_5), 2.89 (3H, s, CH_3), 4.15 (2H, d, CH_2),4.29 (2H, q, J = 7.1 Hz, CH_2, C_2H_5), 7.48 (1H, s, NH), 8.39 (1H, s, 2-H), 12.61 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 14.47, 15.89, 43.04, 61.59, 117.00, 139.83, 155.82, 158.54, 162.45, 166.61, 171.52. Anal. Calc. for $C_{12}H_{14}ClN_3O_4S$: C, 43.44; H, 4.25; S, 9.66. Found: C, 43.42; H, 4.24; S, 9.63.

*2.3.10. N-[5-(4-Fluorophenyl)thieno[2,3-d]pyrimidin-4-yl]-β-alanine (**4j**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.43-2.46 (2H, m, CH_2), 3.58-3.61 (2H, m, CH_2), 5.70 (1H, s, NH), 7.31 (2H, t, C_6H_4), 7.48 (3H, s, C_6H_4, 6-H), 8.41 (1H, s, 2-H), 12.32 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 33.53, 36.47, 113.57, 116.11, 116.33, 121.64, 131.36, 131.93, 133.90, 153.95, 157.01, 161.28, 163.72, 166.81, 173.68. Anal. Calc. for $C_{15}H_{12}FN_3O_2S$: C, 56.77; H, 3.81; S, 10.10. Found: C, 56.75; H, 3.80; S, 10.9.

*2.3.11. N-[5-(3,4-Dimethylphenyl)thieno[2,3-d]pyrimidin-4-yl]glycine hydrochloride (**4k**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.32 (6H, s, $2CH_3$), 4.03 (2H, d, CH_2), 5.85 (1H, s, NH), 7.16-7.25 (4H, m, $C_6H_3(CH_3)_2$, 6-H), 8.30 (1H, s, 2-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 19.59, 42.81, 113.69, 121.17, 126.40, 130.18, 130.50, 133.11, 135.12, 136.82, 137.53, 153.72, 156.93, 166.91, 171.49. Anal. Calc. for $C_{16}H_{16}ClN_3O_2S$: C, 54.93; H, 4.61; S, 9.17. Found: C, 54.92; H, 4.60; S, 9.15.

*2.3.12. 4-{[5-(3,4-Dimethylphenyl)thieno[2,3-d]pyrimidin-4-yl]amino}butanoic acid hydrochloride (**4l**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1.62 (2H, t, CH_2), 2.10 (2H, t, CH_2), 2.33 (6H, s, $2CH_3$), 3.42 (2H, t, CH_2), 5.24 (1H, s, NH), 7.19-7.22 (4H, m, $C_6H_3(CH_3)_2$, 6-H), 8.30 (1H, s, 2-H), 11.98 (bs, COOH). Anal. Calc. for $C_{18}H_{20}ClN_3O_2S$: C, 57.21; H, 5.33; S, 8.48. Found: C, 57.20; H, 5.31; S, 8.46.

*2.3.13. N-(6-Methylthieno[2,3-d]pyrimidin-4-yl)glycine (**4m**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ:2.53 (3H, s, CH_3), 4.11 (2H, d, CH_2), 7.27 (1H, s, NH), 8.20 (1H, s, 5-H), 8.27 (1H, s, 2-H),12.63 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 16.33, 42.22, 116.93, 136.63, 153.21, 156.26, 165.39, 172.03. Anal. Calc. for $C_9H_9N_3O_2S$: C, 48.42; H, 4.06; S, 14.36. Found: C, 48.40; H, 4.02; S, 14.32.

*2.3.14. N-(6-Phenylthieno[2,3-d]pyrimidin-4-yl)serine (**4n**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 3.89-3.91 (2H, m, CH_2), 4.80-4.81 (1H, m, CH), 5.16 (bs, OH), 7.37-7.41 (1H, m, C_6H_5), 7.48-7.52 (2H, m, C_6H_5), 7.70-7.72 (2H, m, C_6H_5), 8.04 (1H, d, NH), 8.29 (1H, s, 5-H), 8.34 (1H, s, 2-H), 12.74 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 56.65, 61.57, 116.08, 117.97, 125.98, 128.96, 129.75, 133.53, 138.66, 154.00, 156.77, 165.50, 172.45. Anal. Calc. for $C_{15}H_{13}N_3O_3S$: C, 57.13; H, 4.16; S, 10.17. Found: C, 57.10; H, 4.14; S, 10.15.

*2.3.15. N^2-(6-Phenylthieno[2,3-d]pyrimidin-4-yl)asparagine (**4o**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.67-2.81 (2H, m, CH_2), 5.04-5.08 (1H, m, CH), 6.98 (1H, s, C_6H_5), 7.37-7.51 (4H, m, C_6H_5), 7.69 (2H, d, NH_2), 8.10 (1H, s, 5-H), 8.16 (1H, d, NH), 8.35 (1H, s, 2-H), 12.84 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 37.09, 50.61, 115.66, 117.88, 126.04, 128.99, 129.75, 133.46, 138.82, 154.02, 156.52, 165.51, 171.45, 173.65. Anal. Calc. for $C_{16}H_{14}N_4O_3S$: C, 56.13; H, 4.12; S, 9.37. Found: C, 56.10; H, 4.09; S, 9.35.

*2.3.16. N-(6-Ethylthieno[2,3-d]pyrimidin-4-yl)glycine (**4p**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1,35 (3H, t, J = 7.3 Hz, CH_3, C_2H_5), 2.90 (2H, q, J = 7.3 Hz, CH_2, C_2H_5), 4.10 (2H, d, CH_2), 7.28(1H, s, 5-H), 8.01 (1H, t, NH), 8.19 (1H, s, 2-H), 12.37 (bs, COOH). Anal. Calc. for $C_{10}H_{11}N_3O_2S$: C, 50.62; H, 4.67; S, 13.51. Found: C, 50.59; H, 4.64; S, 13.49.

*2.3.17. N-[5-(3,4-Dimethoxyphenyl)thieno[2,3-d]pyrimidin-4-yl]-β-alanine (**4g**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.42-2.46 (2H, m, CH_2), 3.60-3.63 (2H, m, CH_2), 3.82 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 5.73-5.77 (1H, s, 6-H), 6.90-7.03 (3H, m, $C_6H_3(OCH_3)_2$), 7.24 (1H, s, NH), 8.31 (1H, s, 2-H),12.12 (bs, COOH). Anal. Calc. for $C_{17}H_{17}N_3O_4S$: C, 56.81; H, 4.77; S, 8.92. Found: C, 56.79; H, 4.74; S, 8.90.

*2.3.18. N^2-(2-Methyl-6-phenylthieno[2,3-d]pyrimidin-4-yl)asparagine (**4r**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.45 (3H, s, CH_3), 2.71-2.73 (2H, m, CH_2), 5.02-5.07 (1H, m, CH), 6.82 (1H, s, C_6H_5), 7.32-7.44 (4H, m, C_6H_5), 7.66 (2H, d, NH_2), 7.82 (1H, d, NH), 7.99 (1H, s, 5-H), 12.64 (bs, COOH).). Anal. Calc. for $C_{17}H_{16}N_4O_3S$: C, 57.29; H, 4.53; S, 9.00. Found: C, 57.25; H, 4.51; S, 9.00.

*2.3.19. 1-(6-Penylthieno[2,3-d]pyrimidin-4-yl)proline (**4s**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.14-2.33 (4H, m, H-proline), 4.04-4.16 (2H, m, H-proline), 4.70-4.78 (1H, m, H-proline), 7.34 (1H, t, C_6H_5), 7.43 (2H, t, C_6H_5), 7.72 (2H, d, C_6H_5), 7.85 (1H, s, 5-H), 8.25 (1H, s, 2-H),12.44 (bs, COOH). Anal. Calc. for $C_{17}H_{15}N_3O_2S$: C, 62.75; H, 4.65; S, 9.85. Found: C, 62.73; H, 4.62; S, 9.83.

*2.3.20. 1-(2-Methyl-6-phenylthieno[2,3-d]pyrimidin-4-yl)proline (**4t**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.11-2.31(4H, m, H-proline), 2.43 (3H, s, CH_3), 4.01-4.13 (2H, m, H-proline), 4.71-4.77 (1H, m, H-proline), 7.32 (1H, t, C_6H_5), 7.42 (2H, t, C_6H_5), 7.70 (2H, d,

C_6H_5), 7.79 (1H, s, 5-H), 12.33 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ:). Anal. Calc. for $C_{18}H_{17}N_3O_2S$: C, 63.70; H, 5.05; S, 9.45. Found: C, 63.69; H, 5.01; S, 9.43.

*2.3.21. 1-(2-Methyl-6-phenylthieno[2,3-d]pyrimidin-4-yl)piperidine-3-carboxylic acid (**4u**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1.63-1.69 (1H, m, H-piperidine), 1.83-1.90 (2H, m, H-piperidine), 2.03-2.08 (1H, m, H-piperidine), 2.49 (3H, s, CH_3), 2.60-2.65 (1H, m, H-piperidine), 3.39-3.52 (2H, m, H-piperidine),4.22-4.26 (1H, m, H-piperidine), 4.45-4.48 (1H, m, H-piperidine), 7.34-7.45 (3H, m, C_6H_5), 7.71 (2H, d C_6H_5), 7.81 (1H, s, 5-H), 12.37 (bs, COOH). Anal. Calc. for $C_{19}H_{19}N_3O_2S$: C, 64.57; H, 5.42; S, 9.07. Found: C, 64.55; H, 5.41; S, 9.03.

*2.3.22. 1-(2-Methyl-6-phenylthieno[2,3-d]pyrimidin-4-yl)piperidine-4-carboxylic acid (**4v**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1.69-1.78 (2H, m, H-piperidine),1.98-2.02(2H, m, H-piperidine), 2.47 (3H, s, CH_3), 2.55-2.61(1H, m, H-piperidine), 3.28-3.35 (2H, m, H-piperidine), 4.49-4.52 (2H, m, H-piperidine), 7.32 (1H, t, C_6H_5), 7.41 (2H, t, C_6H_5), 7.68 (1H, s, 5-H), 7.72 (2H, d, C_6H_5), 12.16 (bs, COOH). Anal. Calc. for $C_{19}H_{19}N_3O_2S$: C, 64.57; H, 5.42; S, 9.07. Found: C, 64.54; H, 5.41; S, 9.05.

*2.3.23. 2,5-Dimethyl-4-piperidin-1-ylthieno[2,3-d]pyrimidine-6-carboxylic acid (**4w**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1.64-1.70 (6H, m, H-piperidine), 2.49 (3H, s, CH_3), 2.72 (3H, s, CH_3), 3.36-3.40 (4H, m, H-piperidine), 13.08 (bs, COOH). Anal. Calc. for $C_{14}H_{17}N_3O_2S$: C, 57.71; H, 5.88; S, 11.00. Found: C, 57.69; H, 5.86; S, 11.00.

*2.3.24. 4-[(7-Methyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4-yl)amino]benzoic acid hydrochloride (**5a**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 1.06 (3H, d, CH_3), 1.42-1.48 (1H, m, CH), 1.91-1.94 (2H, m, CH_2), 2.37-2.44 (1H, m, CH), 2.87-2.90 (1H, m, CH), 3.14-3.19 (2H, m, CH_2), 7.79 (2H, d, J = 8.3 Hz, C_6H_4), 7.90 (2H, d, J = 8.3 Hz, C_6H_4), 8.44 (1H, s, NH), 8.46 (1H, s, 2-H), 12.66 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 21.37, 25.24, 28.70, 30.33, 33.33, 117.82, 120.40, 124.79, 126.55, 130.38, 133.70, 144.04, 152.23, 154.45, 166.77, 167.37.). Anal. Calc. for $C_{18}H_{18}ClN_3O_2S$: C, 57.52; H, 4.83; S, 8.53. Found: C, 57.50; H, 4.81; S, 8.52.

*2.3.25. 3-[(5,6-Dimethylthieno[2,3-d]pyrimidin-4-yl)amino]benzoic acid hydrochloride (**5b**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.36 (3H, s, CH_3), 2.40 (3H, s, CH_3), 7.44(1H, t, J = 7.6 Hz, C_6H_4), 7.71 (1H, d, J = 7.6 Hz, C_6H_4), 7.92 (1H, d, J = 7.6

Hz, C_6H_4), 8.20 (1H, s, C_6H_4), 8.44 (1H, s, NH), 8.72 (1H, s, 2-H), 12.68 (bs, COOH). Anal. Calc. for $C_{15}H_{14}ClN_3O_2S$: C, 53.33; H, 4.77; S, 9.49. Found: C, 53.32; H, 4.74; S, 9.46.

*2.3.26. 3-[(6-Phenylthieno[2,3-d]pyrimidin-4-yl)amino]benzoic acid hydrochloride (**5c**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 7.39 (1H, t, J = 7.6 Hz, C_6H_4), 7.51 (3H, t, J = 7.3 Hz, C_6H_5), 7.69 (1H, d, J = 7.6 Hz, C_6H_4), 7.72 (2H, d, J = 7.3 Hz, C_6H_5), 8.27 (1H, d, J = 7.6 Hz, C_6H_4), 8.30 (1H, s, C_6H_4), 8.43 (1H, s, NH), 8.53 (1H, s, 5-H), 9.80 (1H, s, 2-H), 13.03 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 115.64, 118.82, 121.88, 124.19, 125.27, 126.17, 129.24, 129.78, 131.61, 133.33, 139.76, 139.98, 153.61, 154.56, 166.34, 167.33. Anal. Calc. for $C_{19}H_{14}ClN_3O_2S$: C, 59.45; H, 3.68; S, 8.35. Found: C, 59.44; H, 3.66; S, 8.32.

*2.3.27. 4-{[5-(4-Methylphenyl)thieno[2,3-d]pyrimidin-4-yl]amino}benzoic acid hydrochloride (**5d**)* [22].

*2.3.28. 3-{[5-(4-Methylphenyl)thieno[2,3-d]pyrimidin-4-yl]amino}benzoic acid hydrochloride (**5e**)* [22].

*2.3.29. 4-[(5-Phenylthieno[2,3-d]pyrimidin-4-yl)amino]benzoic acid hydrochloride (**5f**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 7.44(2H, d, J = 8.1 Hz, C_6H_4), 7.50 (1H, s, NH), 7.59 (5H, s, C_6H_5), 7.72 (1H, s, 6-H), 7.84 (2H, d, J = 8.1 Hz, C_6H_4), 8.65 (1H, s, 2-H), 12.72 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 115.16, 119.10, 123.31, 125.16, 129.28, 129.43, 129.73, 130.76, 134.43, 135.55, 142.81, 153.27, 154.54, 167.17, 167.83. Anal. Calc. for $C_{19}H_{14}ClN_3O_2S$: C, 59.45; H, 3.68; S, 8.35. Found: C, 59.43; H, 3.66; S, 8.32.

*2.3.30. 3-[(5-Phenylthieno[2,3-d]pyrimidin-4-yl)amino]benzoic acid hydrochloride (**5g**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 7.39-7.42 (2H, m, aromatic-H), 7.58-7.61 (7H, m, aromatic-H), 7.69 (1H, s, NH), 8.02 (1H, s, 6-H), 8.61 (1H, s, 2-H), 13.04 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 114.73, 121.12, 122.97, 124.39, 124.61, 129.13, 129.36, 129.70, 131.86, 134.60, 135.66, 139.02, 153.39, 154.94, 167.40, 167.67. Anal. Calc. for $C_{19}H_{14}ClN_3O_2S$: C, 59.45; H, 3.68; S, 8.35. Found: C, 59.44; H, 3.67; S, 8.34.

*2.3.31. 4-{[5-(4-Chlorophenyl)thieno[2,3-d]pyrimidin-4-yl]amino}benzoic acid hydrochloride (**5h**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 7.56-7.61 (6H, m, aromatic-H),

7.64 (1H, s, NH), 7.74-7.79 (3H, m, aromatic-H), 8.61 (1H, s, 2-H), 12.43 (bs, COOH). Anal. Calc. for $C_{19}H_{13}Cl_2N_3O_2S$: C, 54.56; H, 3.13; S, 7.67. Found: C, 54.55; H, 3.10; S, 7.65.

*2.3.32. 4-[(2-Methyl-6-phenylthieno[2,3-d]pyrimidin-4-yl)amino]benzoic acid hydrochloride (**5i**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.44 (3H, s, CH_3), 7.33-7.45 (3H, m, C_6H_5), 7.62 (2H, d, J = 7.6 Hz, C_6H_5), 7.90 (2H, d, J = 8.3 Hz, C_6H_4), 8.01 (2H, d, J = 8.3 Hz, C_6H_4), 8.18 (1H, s, NH), 9.71 (1H, s, 5-H), 12.64 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 26.13, 115.44, 116.67, 119.92, 124.78, 126.04, 129.03, 129.74, 130.56, 133.44, 138.64, 144.21, 154.13, 162.57, 167.40. Anal. Calc. for $C_{20}H_{16}ClN_3O_2S$: C, 60.37; H, 4.05; S, 8.06. Found: C, 60.35; H, 4.03; S, 8.04.

*2.3.33. 3-[(2-Methyl-6-phenylthieno[2,3-d]pyrimidin-4-yl)amino]benzoic acid hydrochloride (**5j**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.46 (3H, s, CH_3), 7.35-7.48 (4H, m, C_6H_5), 7.60-7.66 (3H, m, C_6H_5, C_6H_4), 8.21 (1H, s, C_6H_4), 8.33 (1H, d, C_6H_4), 8.40 (1H, s, NH), 9.66 (1H, s, 5-H), 12.97 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 26.15, 115.51, 116.37, 121.63, 123.87, 124.87, 126.00, 128.96, 129.19, 129.73, 131.70, 133.53, 138.37, 140.26, 154.39, 162.60, 167.09, 167.71. Anal. Calc. for $C_{20}H_{16}ClN_3O_2S$: C, 60.37; H, 4.05; S, 8.06. Found: C, 60.36; H, 4.02; S, 8.05.

*2.3.34. 3-{[6-(Ethoxycarbonyl)-5-methylthieno[2,3-d]pyrimidin-4-yl]amino}benzoic acid (**5k**)* [22].

*2.3.35. 4-[(6-Methyl-5-phenylthieno[2,3-d]pyrimidin-4-yl)amino]benzoic acid hydrochloride (**5l**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.34 (3H, s, CH_3), 6.93 (1H, s, NH), 7.27 (2H, d, J = 8.3 Hz, C_6H_4), 7.54-7.56 (2H, m, C_6H_5), 7.65-7.68 (3H, m, C_6H_5), 7.80 (2H, d, J = 8.3 Hz, C_6H_4), 8.59 (1H, s, 2-H), 12.67 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 14.13, 116.47, 118.54, 124.95, 129.66, 129.82, 130.68, 130.83, 134.34, 142.64, 152.49, 153.33, 165.06, 167.12. Anal. Calc. for $C_{20}H_{16}ClN_3O_2S$: C, 60.37; H, 4.05; S, 8.06. Found: C, 60.35; H, 4.02; S, 8.04.

*2.3.36. 5-[(6-Methylthieno[2,3-d]pyrimidin-4-yl)amino]isophthalic acid (**5m**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.62 (3H, s, CH_3), 7.57 (1H, s, NH), 8.19 (1H, s, C_6H_3), 8.44 (1H, s, C_6H_3), 8.73 (1H, s, C_6H_3), 8.73 (1H, s, 5-H), 9.63 (1H, s, 2-H). Anal. Calc. for $C_{15}H_{11}N_3O_4S$: C, 54.71; H, 3.37; S, 9.74. Found: C, 54.69; H, 3.35; S, 9.72.

*2.3.37. 3-[(6-Methyl-5-phenylthieno[2,3-d]pyrimidin-4-yl)amino]benzoic acid hydrochloride (**5n**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.33 (3H, s, CH_3), 6.82 (1H, s, aromatic-H), 7.29-7.37 (2H, m, aromatic-H), 7.55-7.64 (6H, m, aromatic-H), 7.93 (1H, s, NH), 8.55 (1H, s, 2-H), 13.04 (bs, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): δ: 14.12, 116.11, 120.35, 123.80, 124.17, 129.43, 129.52, 129.75, 129.90, 130.69, 131.79, 134.05, 134.47, 138.88, 152.66, 153.70, 164.84, 167.28. Anal. Calc. for $C_{20}H_{16}ClN_3O_2S$: C, 60.37; H, 4.05; S, 8.06. Found: C, 60.34; H, 4.03; S, 8.02.

*2.3.38. 2-Hydroxy-5-[(6-methyl-5-phenylthieno[2,3-d]pyrimidin-4-yl)amino]benzoic acid hydrochloride (**5o**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 2.34 (3H, s, CH_3), 6.60 (1H, s, aromatic-H), 6.87 (1H, d, J = 8.8 Hz, aromatic-H), 7.20 (1H, dd, J = 2.7 Hz, J = 8.8 Hz, aromatic-H), 7.54-7.66 (5H, m, C_6H_5), 7.79 (1H, d, NH), 8.49 (1H, s, 2-H). Anal. Calc. for $C_{20}H_{16}ClN_3O_3S$: C, 58.04; H, 3.90; S, 7.75. Found: C, 58.00; H, 3.86; S, 7.73.

*2.3.39. 4-[(3-Carboxyphenyl)amino]-5-methylthieno[2,3-d]pyrimidine-6-carboxylic acid (**5p**)* ^{1}H NMR (400 MHz, DMSO-d_6): δ: 3.08 (3H, s, CH_3), 7. 43 (1H, t, J = 7.8 Hz, aromatic-H), 7.69 (1H, d, J = 7.6 Hz, aromatic-H), 7.93 (1H, d, J = 7.6 Hz, aromatic-H), 8.22 (1H, s, aromatic-H), 8.40 (1H, s, NH), 8.61 (1H, s, 2-H). Anal. Calc. for $C_{15}H_{11}N_3O_4S$: C, 54.71; H, 3.37; S, 9.74. Found: C, 54.69; H, 3.35; S, 9.73.

3. Results and Discussion

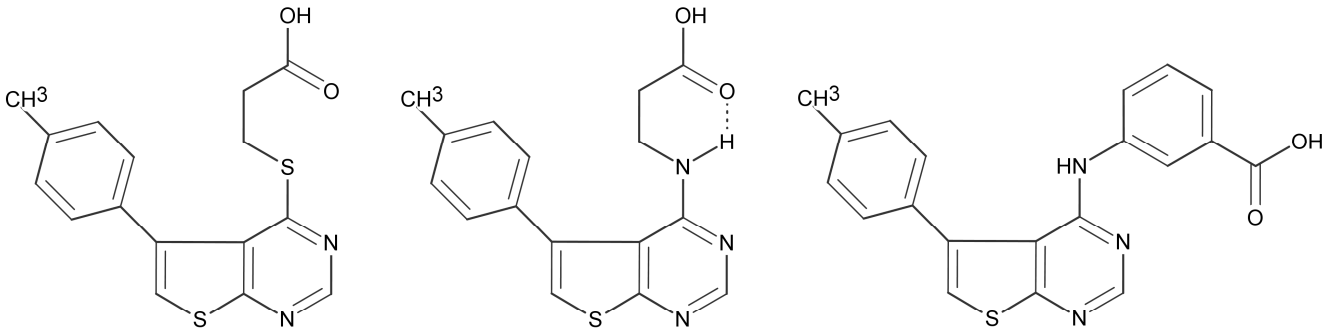
3.1. In vitro activity of the first set of 4-aminothieno[2,3-d]pyrimidine derivatives

The 4-aminothieno[2,3-d]pyrimidines were synthesized as analogs of compounds described previously [11]. Taking into account high potency of the substituted (thieno[2,3-d]pyrimidine-4-ylthio)carboxylic acids, it was expected that the obtained derivatives (Table 2) would also inhibit the protein kinase CK2 activity in submicromolar range. Besides this, new compounds in which atom of Sulfur in fourth position of the thieno[2,3-

d]pyrimidine heterocycle is replaced to Nitrogen should display higher metabolic stability [23] and thus they could be considered as more perspective drug candidates.

Biochemical tests in vitro revealed that only 2 out of 18 new compounds were active in micromolar range. These are **4b** (IC_{50} = 9.5 μM) – 3-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-ilamino)propanoic acid and **4e** (IC_{50} =16 μM) – 3-(7-methyl-5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-ilamino)propanoic acid.

It is interesting that compound **4g** (Fig. 1), which structure differs by only one atom (S is replaced by N) if compare to previously studied compound **6a** [11] (IC_{50} = 0.1 μM) (Fig. 1), showed no activity.



Compound 6a [11]
IC_{50} = 0.1 μM

Compound 4g
IC_{50} > 33 μM

Compound 5e
IC_{50} = 0.01 μM

Fig. 1. Chemical structure of previously described compound **6a** [11] and newly obtained compounds.

3.2. Molecular models of the first set of 4-aminothieno[2,3-d]pyrimidine derivatives bound to CK2 ATP-binding site

When analyzing data obtained in the in vitro tests, we were keen to know why compounds, which are close structural analogs of previously reported CK2 inhibitors, displayed no activity. Trying to provide an explanation for this phenomenon, we investigated molecular complexes of the tested compounds with the CK2 obtained with docking. It turned out that all newly synthesized compounds have a binding mode similar to the one we described previously [11]. The thieno[2,3-d]pyrimidine core contributed to tight ligand fixation in the ATP-binding site making hydrophobic contacts with Val53, Val66, Val116, Met163, Ile174 and hydrogen bond with Val116 of the hinge region. Other hydrogen bonds are formed in the phosphate-binding region between the compounds' carboxylic acid moiety and Lys68 and/or Asp175.

The obtained complexes didn't provide an explanation why the studied compounds are inactive compared to 4-mercaptothieno[2,3-d]pyrimidines. The activity loss might be caused by decreased van der Waals interactions and shortening bond length (C-S: 1.8 Å vs C-N: 1.4 Å) due to the replacement of Sulfur atom to Nitrogen atom. However, in our previous work [11] it was shown that shortening of linker`s length by about 1 Å (in the investigated compounds, the distance between Sulfur atom and Oxygen atom of carboxyl group in acetic acid is about 3.5 Å; the distance between Sulfur atom and Oxygen atom of carboxyl group in propionic acid is about 4.7 Å) led to significant decrease of inhibitory activity but not to a total activity loss. Here, the distance shortening by 0.4 Å (1.8 Å minus 1.4 Å) is much lower than 1 Å, but the activity loss is total. Therefore, there must be other factors contributing to the effect of such activity change. Taking into account a minor difference between these derivatives, most likely the reason of activity loss was an

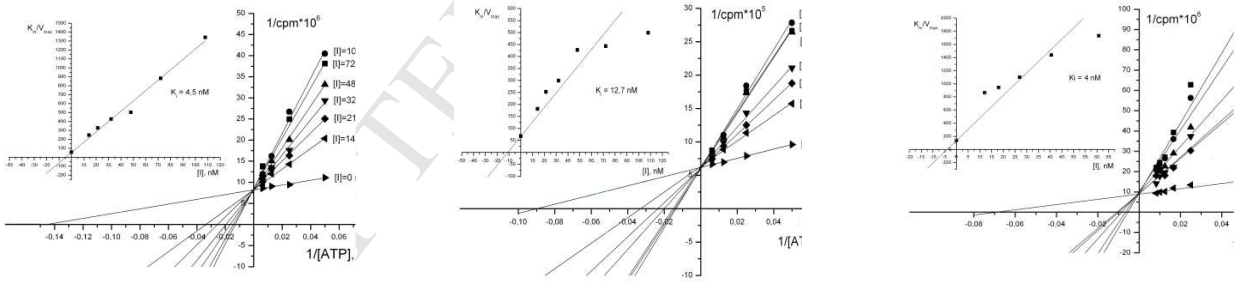
introduction of a hydrogen bond donor. We suppose that the compounds with amino group could form intramolecular hydrogen bond NH···O=C (Fig. 1, compound 4g). It is presumed that this interaction causes 4-aminothieno[2,3-d]pyrimidine pseudo-cyclization resulting in reduction of the ligand's overall length and inability to form high binding affinity complexes with CK2 due to lack of intermolecular hydrogen bonds with Lys68 and Asp175. It should be noted that the molecular docking program we used in this study did not predict the pseudo-cyclization, and during the ligand position and conformation search the carboxylic group switches to intermolecular H-bonds with Lys68 and Asp175, which is more energetically and sterically favourable in terms of the docking algorithm and scoring function. Thus, formation of internal hydrogen bonds in small molecule ligands should be considered at various stages of inhibitor design, and the docking software settings should be adjusted appropriately when possible.

3.3. Inhibitory potency and structure-activity relationships of the second set of 4-aminothieno[2,3-d]pyrimidine derivatives

Based on the results obtained with the first set of molecules, the second set of 4-aminothieno[2,3-d]pyrimidine derivatives with R^4 = carboxyphenyl and 4-piperidine-1-yl-thieno[2,3-d]pyrimidine derivatives was synthesized (Fig. 1, compound 5e). We were guided by the fact that in this case these substituents cannot form any intramolecular hydrogen bonds and thus they are within optimal length in order to be involved into formation of H-bonds with amino acid residues in the depths of the CK2 ATP-binding site.

Structure and activity data for the obtained inhibitors is represented in Table 3. As one can see, about 60% of the newly synthesized compounds inhibit CK2 activity in the range from 0.008 μM to 10 μM. The most active CK2 inhibitors are compounds 3-(5-p-tolyl-thieno[2,3-d]pyrimidin-4-ylamino)benzoic acid, **5e** (**NHTP23**, IC_{50} = 0.01 μM), 3-(5-phenylthieno[2,3-d]pyrimidin-4-ylamino)benzoic acid, **5g** (**NHTP25**, IC_{50} = 0.065 μM) and 3-(6-methyl-5-phenyl-thieno[2,3-d]pyrimidin-4-ylamino)benzoic acid, **5n** (**NHTP33**, IC_{50} = 0.008 μM).

Kinetic studies of **5e**, **5g** and **5n** have shown that the activity of 4-aminothieno[2,3-d]pyrimidine derivatives is a result of competitive binding in ATP-acceptor site of CK2 (Fig. 2). Inhibition constants (K_i) for these compounds are 4.5 nM, 12.7 nM and 4 nM, respectively.



A B C

Fig. 2. Lineweaver–Burk plots of CK2 inhibition by **5e** (A), **5g** (B) and **5n** (C). K_i values are 4.5 nM, 12.7 nM and 4 nM, respectively. Concentration of compounds **5e**, **5g** varied from 0 nM to 108 nM, concentration of compound **5n** varied from 0 nM to 60.75 nM. Enzyme activities were tested as described in Materials and Methods.

As it was expected, the replacement of aliphatic carboxylic acids to aromatic ones in ligands' structure led to increased inhibitory activity. It is demonstrated by compounds **5b** (R^4 = C_6H_4COOH-*m*), **4c** (CH_2COOH) and **4f** (C_2H_4COOH) with IC_{50} values of 3.8 μM, > 33 μM and > 33 μM, respectively, and also by compounds **5k** (R^4 = C_6H_4COOH-*m*) and **4i** (CH_2COOH) with IC_{50} of 6.5 μM and > 33 μM, respectively. The position of carboxyl group of phenyl substituent R^4 is also important. Comparison of the ligands with aromatic carboxylic acids and identical R^1, R^2, R^3 has shown that the most active inhibitors have R^4 = C_6H_4COOH-*m*. For example, the compound **5i** (R^4 = C_6H_4COOH-*p*) is inactive, while **5j** (R^4 = C_6H_4COOH-*m*) has IC_{50} 1.5 μM. The activity of compound **5l** (R^4 = C_6H_4COOH-*p*) is 200 times lower than the one of compound **5n** (R^4 = C_6H_4COOH-*m*) (IC_{50} is 1.5 μM and 0.008 μM, respectively). The activity of compounds **5e** and **5g** with R^4 = C_6H_4COOH-*m* is by an order of magnitude higher if compare to **5d** and **5f** with R^4 = C_6H_4COOH-*p* (IC_{50} values are 0.01 μM, 0.065 μM and 0.65 μM, 1.1 μM, respectively). It should be noted that introduction of additional substituents in phenyl moiety leads to activity loss. For example, compound **5o** which contains an additional hydroxyl group in the carboxyphenyl substituent has IC_{50}= 0.31 μM, whereas compound **5n** with R^4 = C_6H_4COOH-*m* has IC_{50} = 0.008 μM.

Substituents R^1 and R^2 have less effect on the compounds' activity. The R^1 effect is increased in the range C_6H_5 < 4-ClC_6H_4 < 4-$CH_3C_6H_4$ (**5f**, **5h**, **5d**, IC_{50} = 1.1 μM, 0.83 μM and 0.65 μM, respectively and **5g**, **5e** IC_{50} = 0.065 μM and 0.01 μM, respectively). Substituent R^2 affects the activity more than R^1 that can be seen from pairs of compounds **5f-5l**, **5b-5k**, **5g-5n** having IC_{50} values 1.1-1.5 μM, 3.8-6.5 μM and 0.065-0.008 μM, respectively.

All the tested compounds have hydrogen atom or methyl group as R^3 substituent. Presence of methyl group shows negative effect on ligands' activity that is demonstrated by assessment of compounds **5c** and **5j** (IC_{50} is 0.26 μM and 1.5 μM, respectively).

3.4. Selectivity

To check the inhibitors' selectivity towards CK2 the effect of compounds **5e**, **5g** and **5n** was studied on a limited number of protein kinases available in our laboratory immediately. Kinase profiling included four serine/threonine (ASK1, JNK3, Aurora A and ROCK1) and three tyrosine protein kinases (FGFR1, c-Met and Tie2). The aim of this research was to quickly assess if our compounds display wide spectrum protein kinase inhibition effect similar to known inhibitors, for example, staurosporine [24].

The results of the test showed that the compounds at concentration 10 μM did not inhibit activity of these kinases significantly (Table 4) with the exception of Aurora A kinase.

Table 4

Kinase profiling of **5e**, **5g** and **5n**[*]

№	CK2	ASK1	JNK3	FGFR1	c-Met	Aurora A	Tie2	ROCK1
5e	1.14 %	122 %	85,7 %	60,5 %	53,7 %	36,2 %	109 %	105,8 %
5g	1.51 %	122 %	89,5 %	78,9 %	71,5 %	38,9 %	119,7 %	88,6 %
5n	0.91 %	112 %	70,3 %	73,9 %	87,2 %	15,3 %	84,2 %	101,5 %

[*] *kinase residual activity, determined in the presence of 10 μM of inhibitor is expressed as a percentage of the control without inhibitor. Final concentration of ATP in the experiment was 100 μM.*

3.5. Molecular modeling of the second set of 4-aminothieno[2,3-d]pyrimidine derivatives bound to CK2 ATP-binding site

Inspection of docking poses of compounds **4s-w**, **5a-p** (Fig. 3) has shown that 4-aminothieno[2,3-d]pyrimidine derivatives have the same positions in the CK2 ATP-binding site as compounds **4a-r**. A number of hydrophobic contacts between the ligands and amino acid residues Leu45, Val53, Val66, Met163, Ile174 and Phe113 (π-π stacking) have been identified. In addition, it has been observed that the thienopyrimidine core is located in the hinge region of the active site and forms a hydrogen bond with the main chain NH group of Val116. Also, carboxyl group of the ligands is oriented towards phosphate-binding site and forms hydrogen bonds with Asp175 and Lys68. Furthermore, it is connected via water-mediated hydrogen bonds with carboxyl group of Glu81 and main chain NH group of Trp176. Substituents R^1 and R^2 are oriented outward of the ATP-binding site, and form hydrophobic contact with Leu45. Since the substituent R^3 is located close to the hinge region, one should take into account that bulky substituents in this position may create steric hindrance and result in total activity loss of the compounds. We also assume that presence of carboxyphenyl substituents in R^4 could provide tight ligand fixation between Phe113 and Ile174 due to π-π stacking that could explain higher activity

of structurally optimized 4-aminothieno[2,3-d]pyrimidines if compared to 4-mercaptothieno[2,3-d]pyrimidines [11].

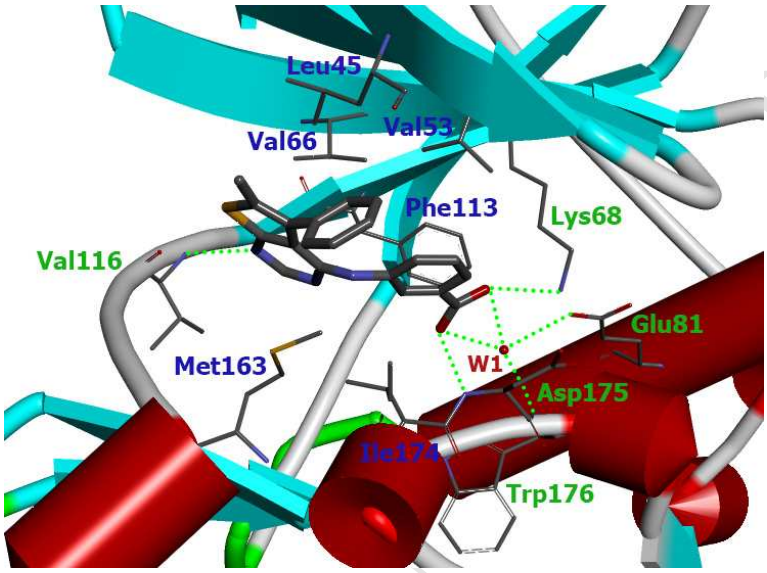


Fig. 3. Binding mode of compound **5n** obtained with molecular docking. Intermolecular hydrogen bonds are indicated with dotted lines. Amino acid residues that participate in hydrophobic interactions have dark blue labels; amino acid residues that form H-bond with the ligand have green labels.

Also, we made superposition of two crystal structures - PDB ID: 4GRB which have been used for docking and PDB ID: 3PE1 [25] complexed with inhibitors 5n and CX-4945, respectively (Fig. 4). Obtained data showed that all active site amino acid side chains in both CK2 crystal structures have very similar positions, and both inhibitors orient their carboxyl acid residues towards Lys68 forming H-bonds with Lys68, Asp175 and Val116. It is observed that thieno[2,3-d]pyrimidine core of 5n and benzo[c][2,6]naphthyridine of CX-4945 partially overlap. This indicates that the

compounds have similar hydrophobic contacts with CK2 ATP-acceptor site residues. Also, inhibitor 5n displays π-π stacking interaction with Phe113 residue, while CX-4945 doesn't.

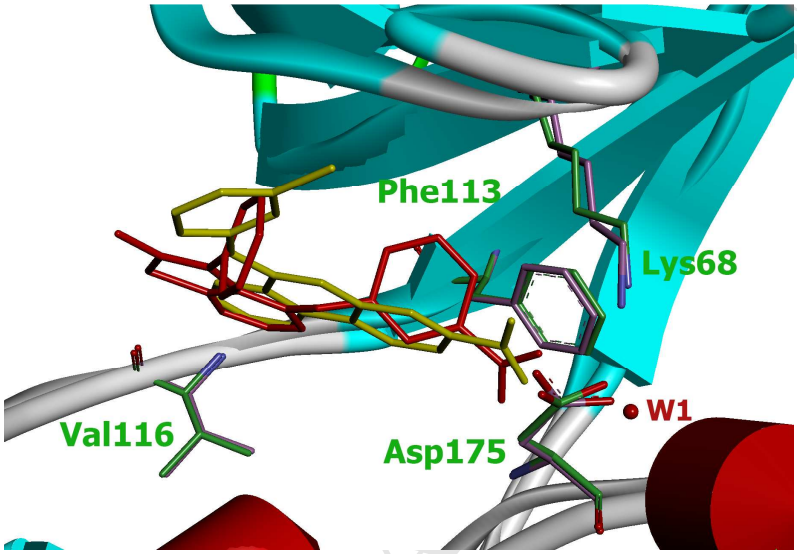


Fig. 4. Superposition of inhibitors 5n (red) and CX-4945 (yellow) complexed with CK2 crystal structures 4GRB and 3PE1.

4. Conclusions

Rational design of biologically valuable compounds requires deep knowledge about structure of both small molecule ligands and corresponding macromolecular target as well as their mode of interaction. In the course of this work, new CK2 inhibitors among 4-aminothieno[2,3-d]pyrimidines have been identified. The foundation of this study is based on our previously published results for substituted (thieno[2,3-d]pyrimidin-4-ylthio)carboxylic acids. During structure-activity relationship analysis, the important role of intramolecular hydrogen bonding has been proposed. Intramolecular hydrogen bonds are abundant in medicinal chemistry, and in most cases they produce positive effect by

stabilizing ligand conformation or interaction network in a binding site of a target protein. Here, the ability of some 4-aminothieno[2,3-d]pyrimidine derivatives to form intramolecular hydrogen bond NH···O=C could be a reason of their inactivation due to prevention of intermolecular hydrogen bonds formation with amino acid residues Asp175 and Lys68. This effect was indirectly confirmed during chemical optimization stage, which allowed us to design new, more active compounds with the chemical structure that prevents formation of intramolecular hydrogen bond. The most active inhibitors obtained (**5e** (**NHTP23**), **5g** (**NHTP25**) and **5n** (**NHTP33**)) are ATP-competitive possessing K_i values 4.5 nM, 12.7 nM and 4 nM, respectively. As shown, there is a necessity to be aware of internal hydrogen bonding phenomenon, and one should take it into account during small molecule screening and lead generation.

Author contribution

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Analysis and interpretation of data: Olga V. Ostrynska, Volodymyr G. Bdzhola, Andriy G. Golub, Nadiia V. Briukhovetska

Drafting of manuscript: Olga V. Ostrynska, Volodymyr G. Bdzhola, Andriy G. Golub, Sergiy M. Yarmoluk

Critical revision: Volodymyr G. Bdzhola, Sergiy M. Yarmoluk

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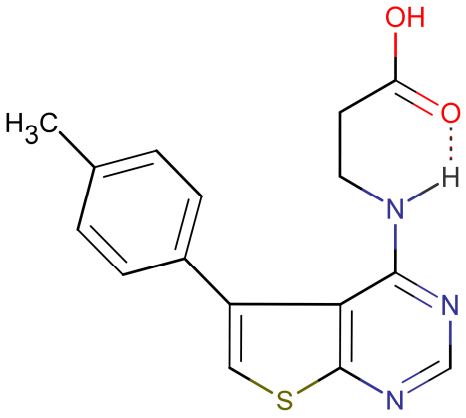
Graphic table of contents



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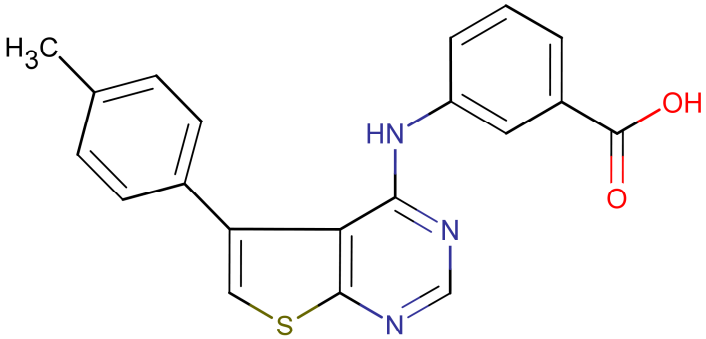

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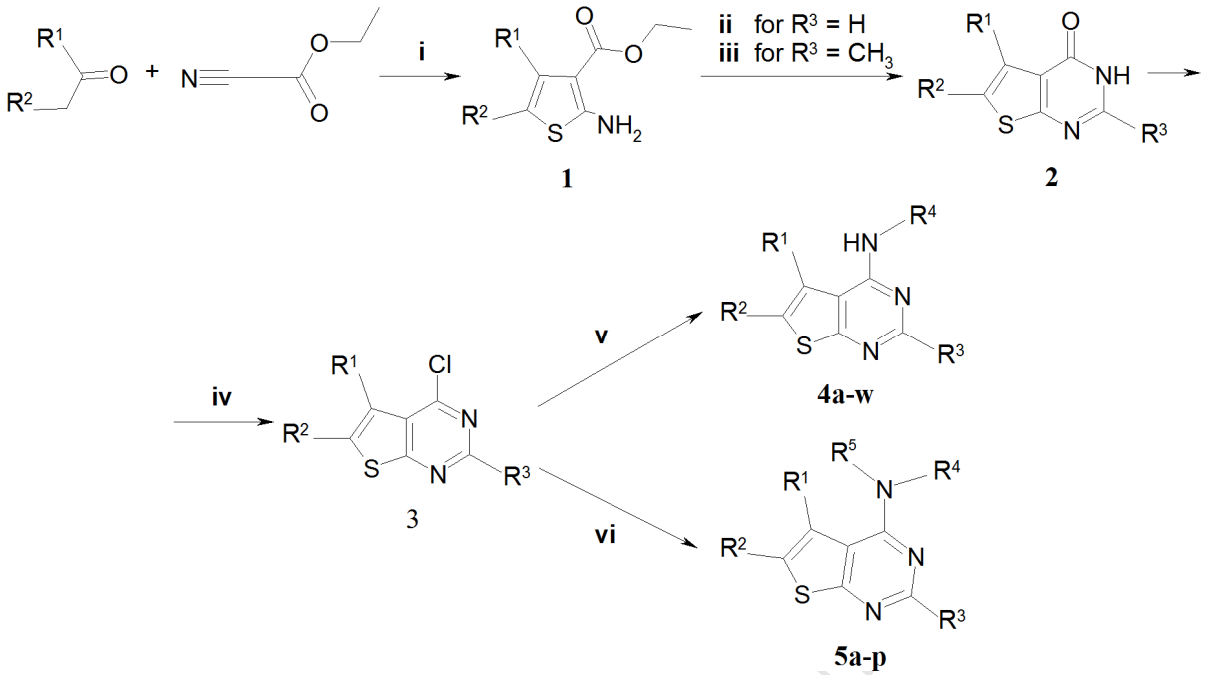

- A new CK2 inhibitors of 4-aminothieno[2,3-d]pyrimidine class have been developed
- Structure–activity relationships of the inhibitors have been studied
- Their binding mode in ATP-acceptor site of CK2 has been proposed
- A negative effect of intramolecular hydrogen bonding is discussed