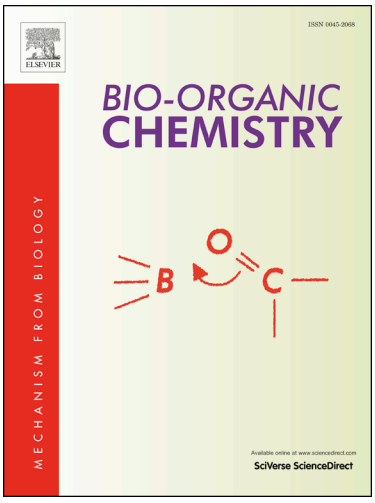

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Synthesis, anticancer evaluation, and molecular docking studies of some novel 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines as cyclin dependent kinase 2 (CDK2) inhibitors

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Abstract

A novel series of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines (**7**-**43**) bearing various anilines at C-4 position and thiophenethyl or thiopentane moieties at C-6 position have been designed and synthesized by molecular hybridization approach. All the synthesized compounds were evaluated for *in vitro* CDK2/cyclin E and Abl kinase inhibitory activity as well as anti-proliferative activity against K-562 (chronic myelogeneous leukemia), and MCF-7 (breast adenocarcinoma) cell lines. The structure-activity relationship (SAR) studies revealed that compounds with thiophenethyl group at C-6 with mono-substituted anilines at C-4 exhibited better CDK2 inhibitory activity compared to alkyl group (thiopentane) at C-6 and di-substituted anilines at C-4 of the scaffold. In particular, compounds having 2-chloro, 3-nitro and 4-methylthio aniline groups at C-4 displayed significant enzymatic inhibitory activity against CDK2 with single digit micro molar IC_{50} values. The *in silico* molecular docking studies suggested possible binding orientation and the binding energies were in agreement with the observed SAR as well as experimental results. In addition, some of the synthesized compounds indicated anti-proliferative effects against K-562 and MCF-7 cancer cell lines with IC_{50} values in a micro molar range. Thus, the synthesized compounds could be considered as new anticancer hits for further lead optimization.

Key words: Pyrazolo[3,4-*d*]pyrimidine; cyclin dependent kinase inhibitor; Anti-proliferative activity; Molecular docking; GLIDE.

1. Introduction

Cancer is an enormous global health burden, affecting almost every region and socio-economic level. It is the second leading cause of death worldwide, accounted for 8.8 million deaths in 2015 and nearly 1 in 6 of all global deaths.[1] The new cancer cases are expected to increase to 15 million per year by 2020, according to the World Health Organization (WHO), unless further precautionary measures are followed.[2] Wide efforts are being carried out in order to discover new treatment approaches as well as to improve prevention and molecular diagnostic systems.[3,4] It is becoming noteworthy to investigate new druggable molecular targets, identify and develop their modulators as novel drugs for the treatment of cancer. Amongst others, protein kinases have

become an important group of drug targets and number of kinase inhibitors in clinical development is rapidly increasing.[5]

Cyclin-dependent kinases (CDKs) are a group of serine/threonine kinases comprising 20 members, of which some are linked with regulation of cell-cycle progression by phosphorylating proteins involved in cell division. For example, formation of active complex composed of CDK2 and cyclin E enables pRb phosphorylation, activation of transcription factor E2F which initiates S phase of the cell cycle.[6] CDK2 then also associates with cyclin A, governing continuous DNA replication and properly programed deactivation of E2F. Deregulations of CDKs or cyclins, as well as the loss of endogenous inhibitory proteins, result in abrogation of cell cycle control, which is connected with development of tumors. Thus CDKs are considered important targets for anticancer drugs.[7,8] The lack of clarity as to which CDK is the most suitable drug target followed by poor selectivity hindered the clinical development of specific CDK inhibitors.[9]

Initially, the importance of CDK2 as a drug target for cancer therapy was in question since CDK2 knockdown investigations failed to block cell proliferation in a number of tumour cell lines,[10] and by mouse knockout investigations where the animals were viable.[11,12] Current investigations employing a chemical genetic method in which CDK2 countenance was maintained, but enzymatic activity was inhibited, affords interesting sign that CDK2 is a valid anticancer drug target.[6] Up to date many CDK2 inhibitors have been developed and some of them (including roscovitine, CYC065, dinaciclib, AT7519, milciclib) undergo clinical evaluation.[13,14] CDK2 inhibitors are also anticipated to have efficacy in combinations with other drugs or where synthetic lethality can be recognized.[15] Recent study proved that a combination of phosphatidylinositol-3-kinase and CDK2 inhibitors induced apoptosis in malignant glioma xenografts via synthetic-lethal interaction.[16] CDK2 was proved as a therapeutic target in BRCA-deficient cancers,[17] ovarian cancer.[18]

Fused pyrimidines have received a great deal of attention due to their immense role as active pharmacophores.[19] The pyrazole annulated on the pyrimidine scaffold leads to pyrazolopyrimidine, which can be looked upon as the bio-isostere of purine.[20] The pharmacological significance of purine nucleus is well established. Oxypurinol and its congeners allopurinol and thiopurinol (Tisopurine) which contains pyrazolo[3,4-*d*]pyrimidine scaffold

inhibit xanthine oxidase enzyme and interfere with the biosynthesis of uric acid, further allopurinol acting as a causative agent for gout.[21] Ibrutinib/PCI-32765 was recently approved by the US FDA for the treatment of mantle cell lymphoma, chronic lymphocytic leukemia and Waldenstrom's macroglobulinemia diseases (**Figure 1**).[22]

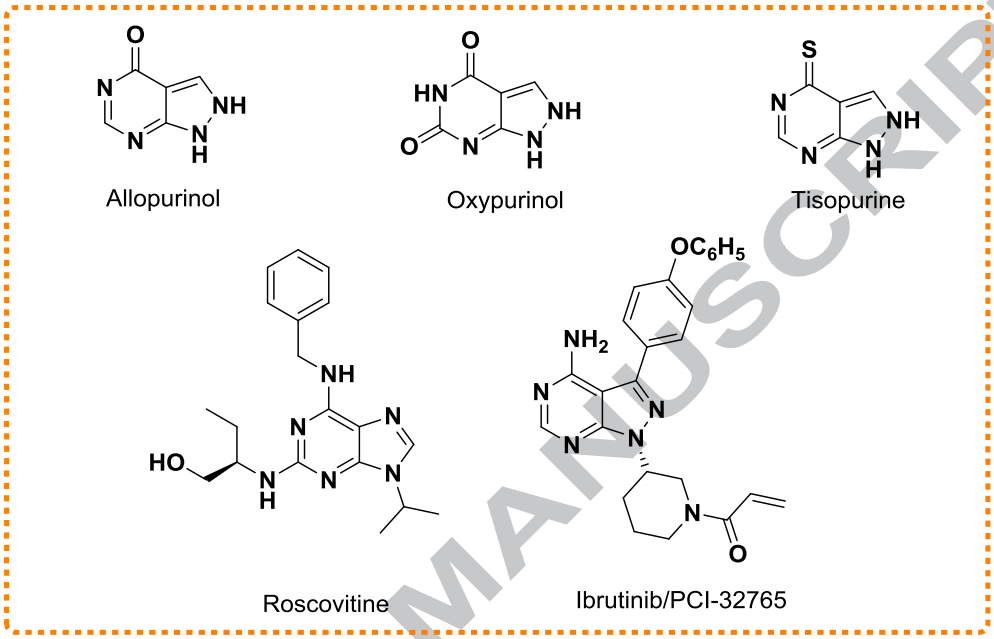


Figure 1. Structures of active drugs containing fused pyrimidine.

Pyrazolo[3,4-*d*]pyrimidine is also reported to encompass biological potential as anticancer, antiviral, antimicrobial, herbicidal, CNS agents (phosphodiesterase 9 and benzodiazepine receptors), anti-inflammatory and cardiovascular activities.[23] **Figure 2** depicts several reported analogs of pyrazolo[3,4-*d*]pyrimidine with anti-cancer activities related to inhibition of various protein kinases.[24-29]

Based on the above mentioned facts and in continuation of our research work on anticancer drug discovery, we envisaged to further exploit the pyrazolo[3,4-*d*]pyrimidine scaffold to synthesize novel CDK2 inhibitors.[30,31] Thus a series of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine was synthesized with a design strategy: a) bioisosteric replacement of purine nucleus, b) incorporation of more lipophilic aromatic amines (C-4) and aliphatic or aromatic groups (C-6). These derivatives were also evaluated against Abl kinase and two cancer cell lines K-562 (chronic myelogeneous leukemia) and MCF-7 (breast adenocarcinoma). Further, *in silico* molecular docking studies were performed to calculate the binding energies and orientations of

these compounds with respect to the active site of CDK-2 protein. The computational results were in agreement with our experimental observations.

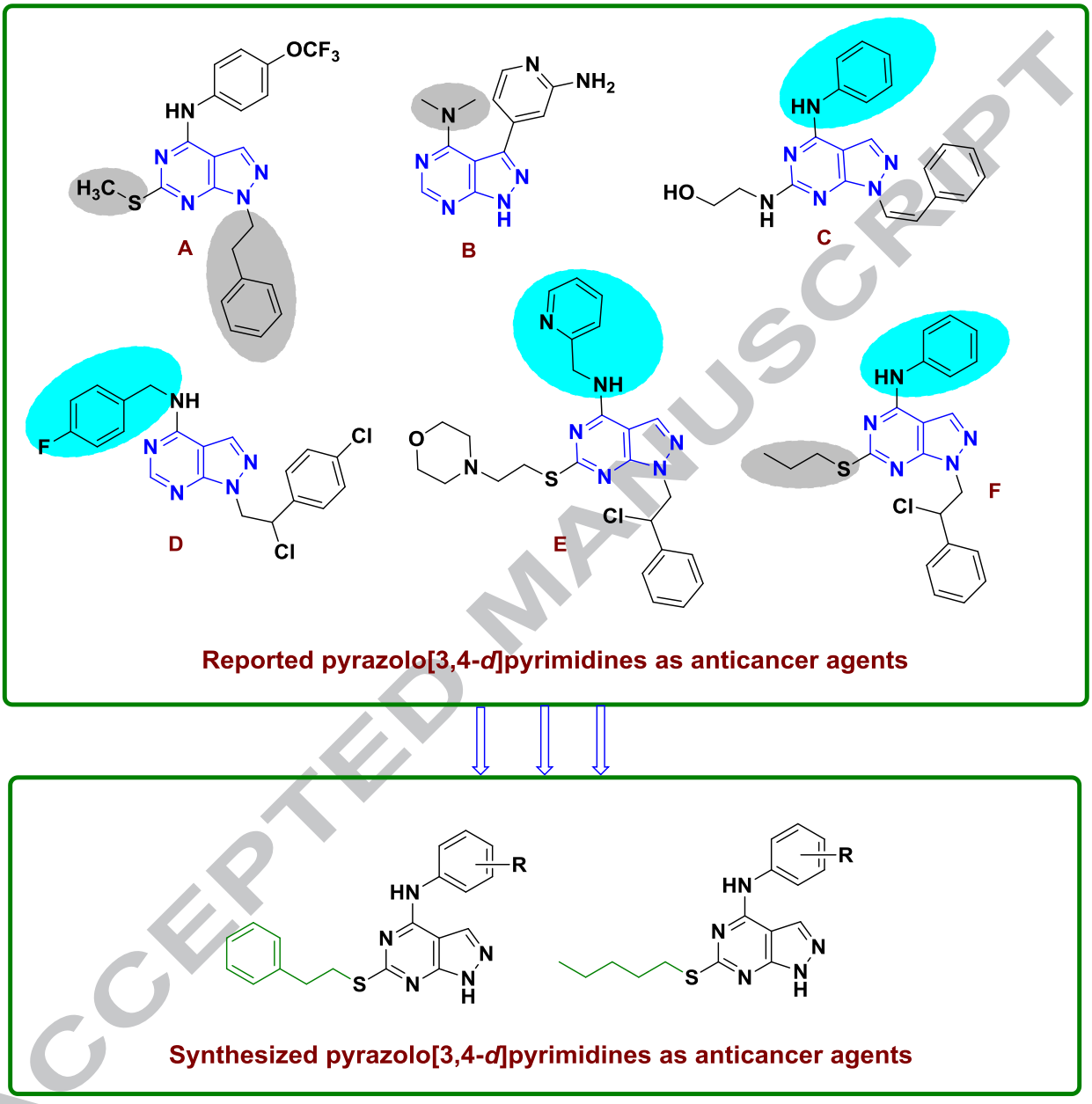


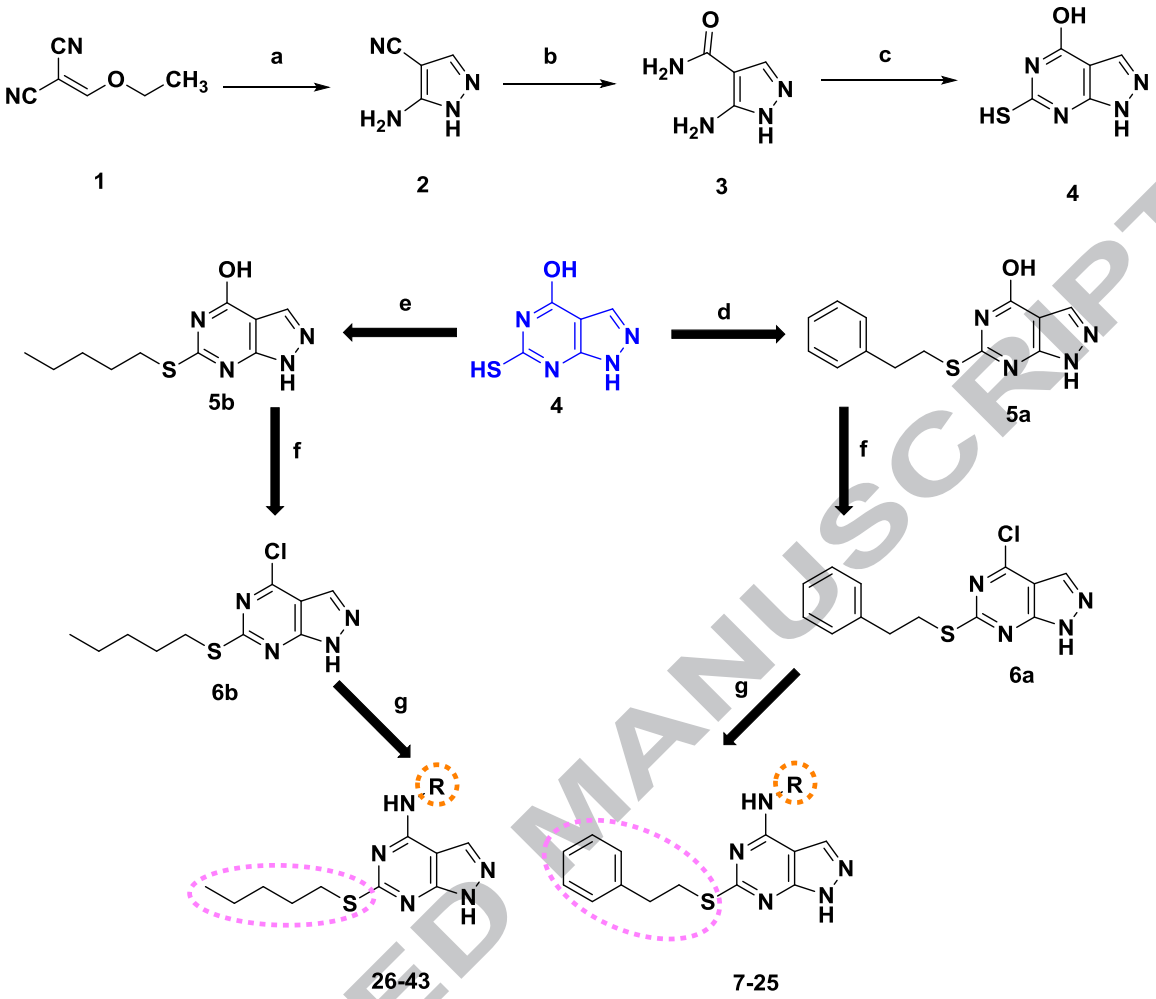
Figure 2. Known derivatives of pyrazolo[3,4-*d*]pyrimidine and their anticancer activities along with the designed molecules. **A**: (K_{i50} against Src, AblT315I = 0.056, 0.01 µM);[24] **B**: (IC_{50} against CDK9 = 17 nM);[25] **C**: (IC_{50} against CDK2 = 0.5µM);[26] **D**: (K_{i50} against Abl = 80 nM);[27] **E**: (K_{i50} against cSrc, Abl = 0.21± 0.02, 0.15±0.02 µM);[28] **F**: (IC_{50} against Src = 1.2 ± 0.4 µM).[29]

2. Results and discussion

2.1. Chemistry

The synthesis of novel series of pyrazolo[3,4-*d*]pyrimidine hybrids was achieved through an efficient and versatile synthetic route as depicted **Scheme 1**. On the pyrazolopyrimidine scaffold, chloro group was introduced at C-4 as a good leaving group, which acted as a most reactive site for different nucleophiles, while thiophenethyl or thiopentane groups were introduced at C-6 position. With a view to prepare the target hybrid molecules (**7-43**), the key intermediates 4-chloro-6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6a**), 4-chloro-6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6b**) were synthesized from ethoxymethylene malononitrile (**1**) in the sequence as illustrated in **Scheme 1**.

From the earlier reported literature methods compound **4** was successfully synthesized.[32] Further under microwave irradiation alkylation of compound **4** was achieved by reacting with 2-chloroethyl benzene in presence of anhydrous potassium carbonate in *N*,*N*-dimethylformamide (DMF) to obtain 6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**5a**). Similarly reacting **4** with 1-bromopentane in 1M NaOH solution at 70 °C resulted in 6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (**5b**) in moderate yield. Chlorination of **5a** and **5b** was easily achieved with Vilsmeier complex ($POCl_3$:DMF) to obtain the halogenated key intermediates 4-chloro-6-(phenethylthio/pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine that are **6a** and **6b**. As displayed in **Scheme 1**, the synthesis of the final hybrid compounds (**7-43**) in good yields (75-95%) was achieved by nucleophilic substitution with various primary amines at C-4. The IR, ^{1}H and ^{13}C NMR spectroscopic data of all the novel compounds were in agreement with the predicted structures, which were further validated by the HR-MS data (Supporting information).



Scheme 1: Synthetic exploration for the preparation of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine analogues (**7-43**).

Reagents and conditions: (a) hydrazine hydrate, ethanol, 80 °C, 3h, 92%; (b) Conc. H_2SO_4, NH_4OH, H_2O, 50 °C, 5h, 90%; (c) potassium ethyl xanthogenate, DMF, 120 °C, 6h, 82%; (d) 2-chloroethyl benzene, K_2CO_3, DMF, 70 °C, microwave, 20 min, 76%; (e) 1-bromopentane, NaOH, H_2O, glacial acetic acid, 50 °C, 5h, RT, overnight, 80%; (f) $POCl_3$, DMF, 80 °C, 2h, 85%; (g) R-NH_2, EtOH, 80 °C, 2h, 75-95%.

The ^{1}H NMR of compound **4** exhibited very distinct singlet signals resonating at δ 13.61, 13.03, 11.86 and 8.42 ppm, which were attributed to the N-H, S-H, O-H and Ar-H protons of the pyrazole ring. Thus, indicating the formation of the fused pyrazolopyrimidine by ring annulation

of 5-amino-1*H*-pyrazole-4-carboxamide (**3**) with potassium ethyl xanthogenate. For compounds **5a** and **5b**, the distinctive methylene signals (Ph-C**H**$_2$-C**H**$_2$-S- and -S-C**H**$_2$-C**H**$_2$-C**H**$_2$-C**H**$_2$-) appeared around δ 3.43-3.39, 3.01-2.97 ppm and δ 3.17-2.87, 1.73-1.43, 1.41-1.14 respectively, while the methyl peak for **5b** was observed at δ 0.86-0.80 ppm. In particular, the disappearance of a distinct singlet signal at around δ 13.03 ppm for mercapto (-S**H**) group evidently indicated the successful alkylation of pyrimidine scaffold. Whereas the most characteristic singlet, doublet and triplet signals at δ 7.32-7.20 ppm were attributed to the aromatic protons (C$_6$**H**$_5$-CH$_2$-CH$_2$-S-) at C-6 of the pyrimidine ring. Furthermore, the absence of characteristic singlet signal at around δ 12.22-12.28 ppm for (-O**H**) at C-4 of pyrimidine ring confirmed the formation of halogenated (-**Cl**) key intermediates 4-chloro-6-(phenethylthio/pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6a** and **6b**). These findings corroborated with their respective ^{13}C NMR, where the methylene carbon peaks at δ 34.61, 31.08 ppm (C$_6$H$_5$-**C**H$_2$-**C**H$_2$-) and δ 30.61-30.37, 30.16-29.69, 29.22-28.11, 21.78-21.73 ppm (-S-**C**H$_2$-**C**H$_2$-**C**H$_2$-**C**H$_2$-) were assigned to **5a** and **5b**. The single methyl peak (**5b)** appeared around 13.90-13.83 ppm while the prominent aromatic signals (**5a** and **5b**) resonated around δ 167.89-109.79 ppm.

The IR spectra of the title compounds **7-43** displayed a reasonably sturdy and characteristic bands around 2921-3033 cm^{-1}, 1270-1300 cm^{-1} accounting for N-H and C-S stretching respectively, while the most prominent band of C-N appearing around 1180-1184 cm^{-1} indicated the formation of final hybrid molecules. Further, the prominent ^{1}H NMR signals of the title compounds **7-43** resonated around δ 13.39-10.28 ppm (exocyclic-N-H), δ 9.94-7.75 ppm (ring-N-H) and 10.02-8.19 ppm (C-3 aromatic proton) while, the singlet or multiplet aromatic proton peaks appeared around δ 8.47-6.84 ppm. For compounds **7-25** the methylene protons (-S-C**H**$_2$-C**H**$_2$-Ph) were observed around δ 3.43-2.81 ppm while for compounds **26-43** the signals (-S-C**H**$_2$-C**H**$_2$-C**H**$_2$-C**H**$_2$-) appeared around δ 3.10-1.20 ppm. The methyl protons for **26-43** resonated around δ 0.87-0.80 ppm. Further the methylthio (-Ph-SC**H**$_3$), methyl (Br-Ph-C**H**$_3$**)**, methoxy (Ph-OC**H**$_3$) and ethynyl-**H** protons resonated around δ 2.47-2.44 ppm, δ 2.30-2.18 ppm, δ 3.77-3.71 ppm and 4.18 ppm respectively. The ^{13}C NMR spectra further confirmed the structures of the title compounds. The characteristic carbon signals C-6, C-4 and C-3 of the pyrimidine ring were observed around δ 167.41-166.40, 155.73-153.11 and 132.88-130.56 ppm, while the various aromatic/heteroaromatic carbons resonated between δ 153.62ppm and 98.12 ppm. Furthermore, the prominent carbon signals observed around δ 55.66-55.61 ppm δ 21.78-

17.74 ppm and 30.17-15.31 ppm were attributed to the methoxy (-O**C**H_3), methyl and thiomethyl carbons respectively. Further, for compounds **7-25** the methylene carbon peaks (-S-**C**H_2-**C**H_2-Ph) were observed around δ 35.51-31.08 ppm while for compounds **26-43** the signals (-S-**C**H_2-**C**H_2-**C**H_2-**C**H_2-) appeared around δ 30.62-21.73 ppm and the methyl peak resonated around δ 13.89-13.83 ppm. In addition, the HR-MS spectra of the title compounds (**7-43)** displayed accurate molecular ion peaks, which were in agreement with their expected molecular weights (supporting information).

2.2. In vitro evaluation of CDK2 and Abl kinase inhibition

All the final compounds were evaluated for CDK2/cyclin E kinase inhibition and the IC_{50} values of various *in vitro* anticancer profiles of the final compounds are summarized in **Table 1**. Abl kinase inhibition was evaluated as a counter screen, to get a preliminary information about selectivity.

Table 1. Anticancer, CDK2 and Abl kinase inhibitory evaluation of novel 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine derivatives.

Code	**R**	**IC_{50} (μM)[a]**			
		CDK2/Cyclin E	**Abl**	**K-562**	**MCF-7**
7	Ph	>12.5	>12.5	19.9	19.2
8	2-ClPh	7.8	>12.5	>6.25	>6.25
9	3-ClPh	13.3	>25	>25	24.0
10	4-ClPh	>25	>25	>25	>25
11	3-NO_2Ph	5.1	>25	24.6	24.3
12	4-NO_2Ph	>12.5	>12.5	>6.25	>6.25
13	2-BrPh	>12.5	>12.5	>6.25	>6.25
14	3-BrPh	>25	>25	27.4	23.9
15	4-BrPh	>25	>25	>50	>50
16	2,4-$(CH_3)_2$Ph	>12.5	>12.5	>6.25	>6.25
17	SCH_3Ph	>25	>25	>6.25	>6.25
18	4-Cl-3-CF_3Ph	13.4	>25	>6.25	>6.25

19	4-Br-3-CF_3Ph	>25	>25	>6.25	>6.25
20	4-Cl-3-NO_2Ph	>25	>25	>6.25	>6.25
21	4-F-2-CH_3Ph	>12.5	>12.5	>6.25	>6.25
22	3-Br-4-CH_3Ph	>25	>25	>6.25	>6.25
23	2-Cl-4-FPh	>12.5	>12.5	>12.5	>12.5
24	3,4-(OCH_3)Ph	15.9	>12.5	>6.25	>6.25
25	4-(ethynyl)Ph	>25	>25	>12.5	>12.5
26	Ph	>50	>50	>100	>100
27	2-ClPh	8.7	>12.5	>25	>25
28	3-ClPh	>25	>25	>12.5	>12.5
29	4-ClPh	>12.5	>12.5	>6.25	>6.25
30	3-NO_2Ph	17.7	>25	>12.5	>12.5
31	4-NO_2Ph	>25	>25	>6.25	>6.25
32	2-BrPh	>25	>25	>12.5	>12.5
33	3-BrPh	>25	>25	>6.25	>6.25
34	4-BrPh	>25	>25	>6.25	>6.25
35	2,4-(CH_3)$_2$Ph	>25	>25	>12.5	>12.5
36	SCH_3Ph	8.8	>25	>6.25	>6.25
37	4-Cl-3-CF_3Ph	>25	>25	>12.5	>12.5
38	4-Br-3-CF_3Ph	>25	>25	>6.25	>6.25
39	4-Cl-3-NO_2Ph	>25	>25	>12.5	>12.5
40	4-F-2-CH_3Ph	>12.5	>12.5	>12.5	>12.5
41	3-Br-4-CH_3Ph	>25	>25	>6.25	>6.25
42	2-Cl-4-FPh	>12.5	>12.5	>6.25	>6.25
43	3,4-(OCH_3)Ph	>12.5	>12.5	>6.25	>6.25
	Roscovitine	0.1	>100	42	11
	Imatinib	>100	0.2	0.5	>10

[a] IC_{50} values were determined in triplicate in the range of 0.05 to 100 µM. IC_{50} value indicates concentration (µM) that inhibits activity of the tested enzyme to 50%. For cytotoxic assays, IC_{50} means the concentration (µM) that inhibits the growth of 50% of cells during a three-day cultivation

Some synthesized compounds displayed an activity in the single digit micro molar range against CDK2/cyclin E. Fascinatingly, it was observed that some compounds containing thiophenethyl group at C-6 displayed prominent anticancer activity (**7**, **9**, **11**, **14**) as compared to compounds **26-43** having thiopentane group. It was also observed that incorporation of phenyl/substituted phenyl groups at C-4 of the pyrazolo[3,4-*d*]pyrimidine nucleus was essential for CDK2 activity. From the tested thiophenethyl series the highest CDK2 inhibitory activity was recorded for compounds **11** (IC_{50} = 5.1 µM) and **8** (IC_{50} = 7.8 µM) with 3-nitroaniline and 2-chloroaniline group at C-4 respectively. Notable significant inhibition was also observed for compounds bearing 3-chloroaniline (**9**; IC_{50} = 13.3 µM), 4-chloro-3-trifluoromethylaniline (**18**; IC_{50} = 13.4 µM) and 3,4-dimethoxyaniline (**24**; IC_{50} = 15.9 µM). For the remaining compounds of this thiophenethyl series, IC_{50} values could not be measured due to a solubility limit (IC_{50} value >12.5 or >25 µM).

Similarly, some thiopentane derivatives (series **26-43**) also exhibited a reasonable activity profile with IC_{50} starting from 8.7 µM. From this series, compounds with 2-chloroaniline (**27**) and 4-methylthioaniline (**36**) showed the best activity with IC_{50} 8.7 µM and 8.8 µM, respectively; this was followed by 3-nitroaniline derivative (**30**) with IC_{50} 17.7 µM. The remaining compounds in this series were less soluble and their IC_{50} were not achieved. In addition, all compounds (**7-43**) were also evaluated against Abl kinase but none of the compounds showed any inhibition in the assayed concentration range, confirming reasonable selectivity towards CDK2 over unrelated Abl.

2.3. Anti-proliferative activity against K-562 and MCF-7 cell lines

All the newly synthesized 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidine analogues **7-43** were further evaluated for their *in vitro* anti-proliferative activity against K-562 (chronic myelogeneous leukemia) and MCF-7 (breast adenocarcinoma) cell lines. Several compounds are displaying appreciable activity with measurable IC_{50} values against the two cell lines, such as compounds **7** (IC_{50} = 19.9, 19.2 µM), **9** (IC_{50} = >25, 24 µM), **11** (IC_{50} = 24.6, 24.3 µM), and **14** (IC_{50} = 27.4, 23.9 µM). The remaining compounds were not active in the tested concentration range.

2.4. Structure-activity relationship (SAR) studies

In general, a careful observation of the structure-activity relationship (SAR) indicated that the CDK inhibitory activity was considerably affected by the nature of various substituents present at C-4 (aromatic ring) and C-6 positions on pyrazolo[3,4-*d*]pyrimidine scaffold. From the two series of compounds (phenethyl and pentane at C6), the phenethyl series gave most number of active compounds (8, 9, 11, 18 and 24), while in pentane series only three molecules (27, 30 and 36) were active. Further analysis of both the series revealed that compounds 8, 9, 11, 27, 30 and 36 with mono substituted phenyl ring (2-Cl-Ph, 3-Cl-Ph, 3-NO_2-Ph, and 4-SCH_3-Ph groups) at C-4 showed better activity than compounds with disubstituted phenyl groups except for compounds **18** and **24** (4Cl-3CF_3-Ph and 3,4-OCH_3-Ph). Compound 11 followed by 8, 9, 27, 30 and 36 presented highest inhibitory activity.

Further, no specific activity was observed for all the compounds against Abl kinase (IC_{50} = >12.5 to >50.0). However, compounds 7, 11 and 14, displayed specific anti-proliferative activity against K-562 (IC50 = 19.9, 24.6 and 27.4) and MCF-7 (IC50 = 19.2, 24.3 and 23.9), indicating that mono-substitutions at C4 with phenethyl at C6 was favorable. In summary, compound 11 was most active, however due to low solubility profile for most of the compounds a conclusive SAR could not be derived. **Figure 3** provides a brief overview of the SAR.

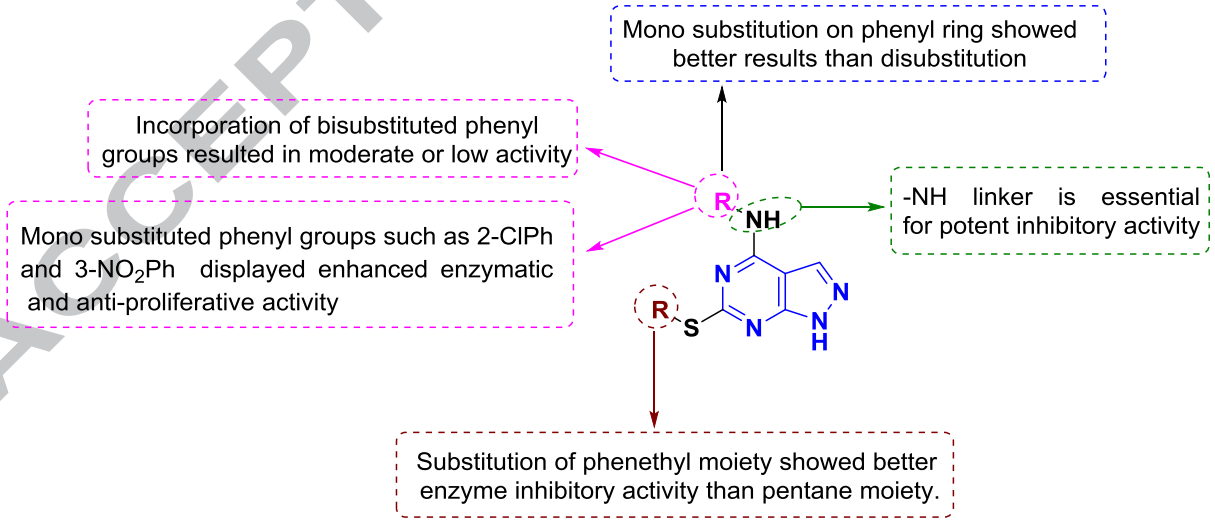


Figure 3. SAR study of 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines as potent anticancer agents.

2.5. Molecular docking study

Computational and bioinformatics tools have become essential part for the design and development of therapeutically effective novel chemical entity.[33] Dinaciclib, a pyrazolo[1,5-*a*]pyrimidine compound, displayed higher binding affinity towards CDKs.[34] Hence, to further understand and substantiate our observed experimental data, molecular docking simulation was performed for the synthesized compounds with the target CDK2 protein.

To validate the docking protocols and to reproduce the reported orientation of R-roscovitine in the predefined binding site of CDK2 (PDB ID: 2A4L), docking studies were performed using Glide program of Schrodinger-Maestro 11.2. From the docking results, the pose of *R*-roscovitine obtained revealed similar molecular interactions as reported.[35] The docked complex presented characteristic hydrogen bonding (H-bond) interactions with crucial residues of the active-site, such as Leu83 interacted with roscovitine by forming a strong [C=O with benzylamino NH (1.91 Å)] and a weak [(C=O with ring nitrogen (2.39 Å)] H-bond. Similarly, the residue Asp86 interacted with OH group of roscovitine via a strong H-bond (1.67 Å), whereas Lys89 exhibited π–cation interaction with phenyl ring of the ligand. **Figure 4** represents the reproduced 3D molecular interactions of the docked pose, validating the docking protocols.

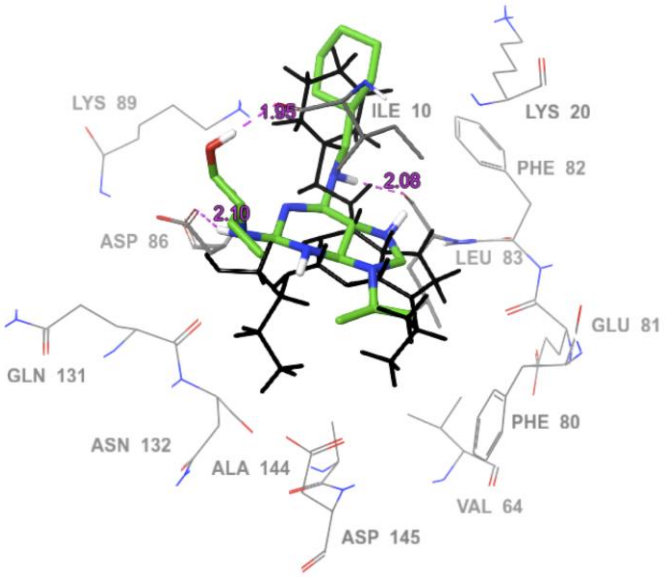


Figure 4: Reported pose of Roscovitine (black thin tube model) and docked pose (thick tube model) into the active-site showing similar interactions (docking validation). Magenta coloured

dashed lines indicate hydrogen bonding, while grey-coloured thin tube amino acids are considered as crucial residues.

The docking experimental data with our synthesized compounds revealed that they fit well into the binding-site and display favourable interactions with the crucial amino acid residues. Interestingly, two nitrogen atoms of pyrazole ring of the best active compound **11** (IC_{50} = 5.1 µM) displayed strong H bond interactions with NH of Leu83 (2.37 Å) and C=O of Glu81 (2.02 Å) respectively, signifying these H bond interactions were crucial for the CDK-2 inhibition. The nitro group of the ligand was in close proximity to Lys89 presenting weak vdW interaction, whereas the acidic and basic amino acid residues (Lys129, Glu131, Asn132 and Asp145) surrounded thiophenethyl group. The exocyclic NH and the side chain sulphur displayed no characteristic interactions with the protein (**Figure 5a**). In the case of moderately active compound **27**, the ring NH displayed H bond interaction with Leu83 (1.69 Å), while the exocyclic NH presented weak H bond interaction (2.59 Å) with Asp86. The side chain was anchored within the catalytic residues Phe80 and Asp145, whereas the pocket consisting of Val18, Lys33, and Asp145 residues surrounded chlorophenyl ring. While in the case of the least active compound **30**, the orientation of the docked pose was observed to be different with respect to best and the moderately active compounds (**11** and **27**). The nitro group showed weak H bond interactions with the basic residues (Lys33 and Asn 132), which could have contributed to the altered pose of the ligand. Furthermore the aliphatic side chain occupied the hydrophobic region (Val64, Phe80, Leu134, and Ala144) resulting in H-bond interactions of the ring and exocyclic nitrogen with different residues of the active site (Leu83 and Ile10). **Figure 5** presents the different molecular interactions of **11**, **27** and **30** with the active site residues of CDK-2 protein (PDB ID: 2A4L).

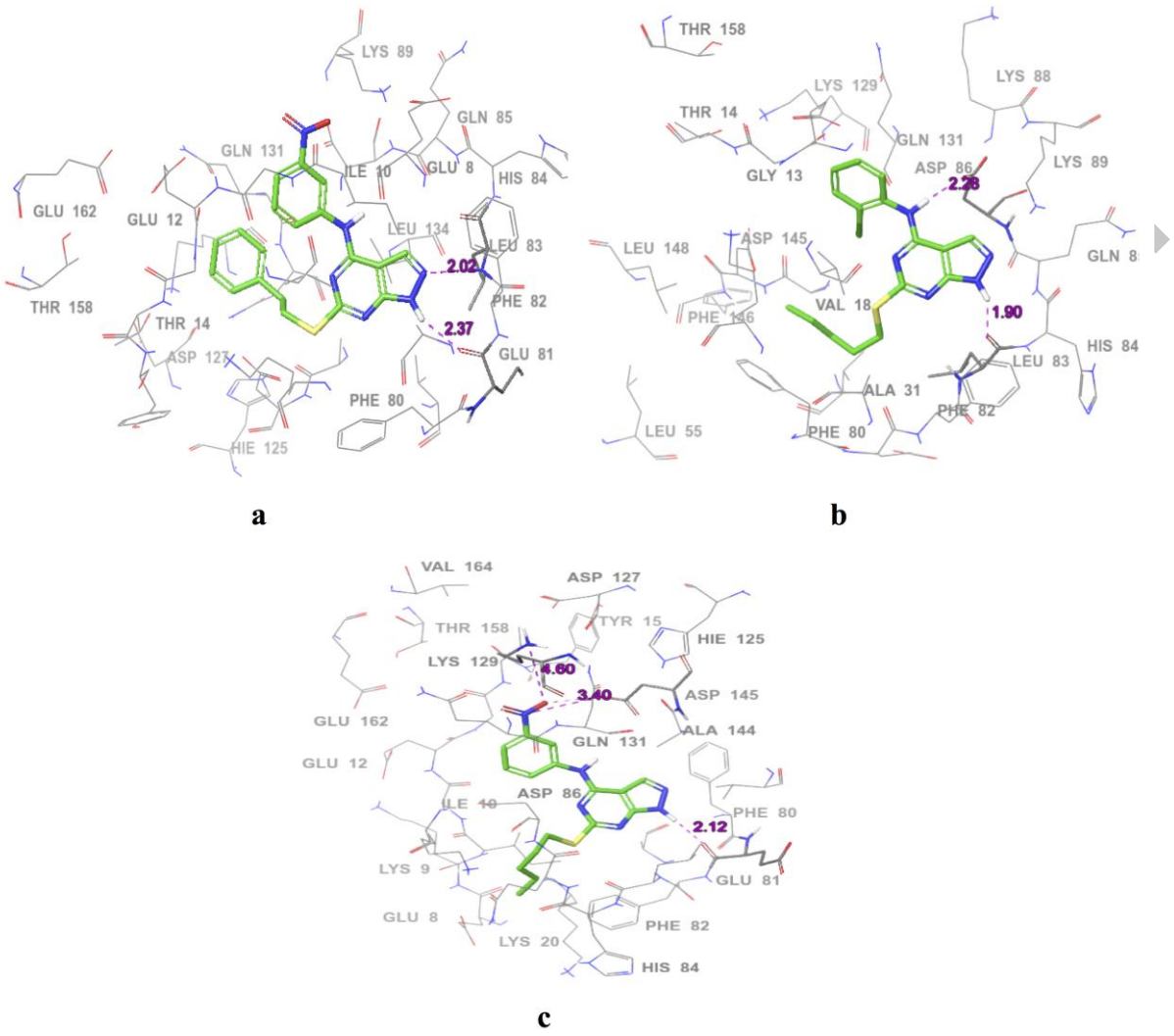


Figure 5. Molecular interactions of a) best active compound **11** b) moderately active compound **27** c) less active compound **30** into the binding site of CDK2/cyclin E protein. Nonpolar hydrogens were hidden for clarity and pink dashed lines indicate H bond.

3. Conclusion

In summary, we have successfully synthesized and characterized a new series of pyrazolo[3,4-*d*]pyrimidine derivatives **7-43** in good yields. The key intermediates 4-chloro-6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6a**), 4-chloro-6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**6b**) allowed us to generate a library of condensed pyrimidines. All synthesized compounds were evaluated for their *in vitro* enzymatic inhibitory activity against CDK2/cyclin E and four

compounds (**8**, **11**, **27**, and **36**) were significantly active with IC_{50} values ranging from 5.1 µM to 8.8 µM. From the SAR, it was clear that the presence of thiophenethyl group at C-6 with mono-substituted anilines at C-4 of the pyrazolo[3,4-*d*]pyrimidine nucleus was essential for anticancer activity. In addition, the binding energies of the best active compounds were in agreement with the experimental data and supported the SAR studies. However, the moderate to poor activity observed for the majority of the compounds could be attributed to the solubility limit and this is currently being addressed in our laboratory. Thus, these preliminary research findings can further guide the researchers at large in developing novel pyrazolo[3,4-*d*]pyrimidine based CDK2 inhibitors as potential anticancer agents.

4. Experimental Section

All the chemicals used in this research work were purchased from Sigma-Aldrich and Merck Millipore, South Africa. All the solvents, except those of laboratory-reagent grade, were dried and purified when necessary according to previously published methods. The progress of the reactions was monitored by thin-layer chromatography (TLC) on pre-coated silica gel plates procured from E. Merck and Co. (Darmstadt, Germany) using 36% ethyl acetate in *n*-hexane as the mobile phase and iodine vapor as the visualizing agent. Purification of crude compounds were performed by crystallization using appropriate solvent and by flash column chromatography using 100-200 mesh silica gel with Methanol (MeOH) and DCM as solvents. The melting points of the synthesized compounds were determined using a Thermo Fisher Scientific (IA9000, UK) digital melting point apparatus and are uncorrected. The IR spectra were recorded on a Bruker Alpha FT-IR spectrometer (Billerica, MA, USA) using the ATR technique. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 and 600 MHz (Bruker, Rheinstetten/Karlsruhe, Germany) spectrometers using $CDCl_3$ and DMSO-d_6. The chemical shifts are reported in δ ppm units with respect to TMS as an internal standard. HR-MS was recorded on an Autospec mass spectrometer with electron impact at 70 eV.

*4.1. Synthesis of 6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-ol (**5a**)*

To a stirred solution of 6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (compound **4**, 0.3g, 0.00179 mol) in *N*,*N*-dimethyl formamide (2 mL), K_2CO_3 (0.247 g, 0.0017 9 mol) was added and stirred at room temperature for 10 min. To this constantly stirred reaction mass, 2-chloroethyl

benzene (0.28 mL, 0.002 mol) was slowly added dropwise and heated at 80 °C for 20 min. in a microwave reactor at 150 psi. After completion of reaction (monitored on TLC), the reaction mixture was poured in ice cold water and extracted with dichloromethane (DCM). The extracted organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure to obtain crude product (dark brown viscous liquid) which was further purified by flash silica column [MeOH/ DCM, 10:90] to afford the desired compound **5a** as light brown solid. Yield: 76%; mp 210-212 °C; FTIR (ATR, cm^{-1}) ν_{max}: 3022 (NH Str.), 2920 (Ar-H Str. of Pyr.), 1671 (C=O Str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.59 (s, 1H, NH), 12.22 (s, 1H, OH), 8.03 (s, 1H, ArH), 7.31 (t, *J* = 2.52 Hz, 4H, ArH), 7.25-7.20 (m, 1H, ArH), 3.41 (t, *J* = 5.04 Hz, 2H, C$\underline{H}_2$), 2.99 (t, *J* = 7.56 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 158.20, 139.93, 128.73, 128.62, 128.41, 126.41, 102.89, 34.61 (C$\underline{H}_2$), 31.08 (C$\underline{H}_2$) ppm.

*4.2. Synthesis of 6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-ol (**5b**)*

To a stirred solution of 6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (compound **4**, 1g, 0.005 mol) in 1M NaOH solution (12 mL), 1-bromopentane (1.48 mL, 0.011 mol) was added dropwise and heated at 70 °C for 6h and later slowly brought to RT and continued stirring for overnight. After completion of reaction (monitored on TLC), glacial acetic acid was added dropwise to yield the crude solid, which further washed with petroleum ether and purified by flash silica column [MeOH/DCM, 05:95] to afford the desired compound **5b** as yellow solid. Yield: 80%; mp 201-203 °C; FTIR (ATR, cm^{-1}) ν_{max}: 3180 (NH Str.), 2953 (Ar-H Str.), 1678 (C=O Str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.54 (s, 1H, NH), 12.28 (s, 1H, OH), 7.93 (s, 1H, ArH), 3.15 (t, *J* = 7.26 Hz, 2H, C$\underline{H}_2$), 1.70-1.63 (m, 2H, C$\underline{H}_2$), 1.39-1.26 (m, 4H, (C$\underline{H}_2$)$_2$), 0.86 (t, *J* = 7.08 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 159.49, 157.75, 135.24, 30.37 (C$\underline{H}_2$), 29.69 (C$\underline{H}_2$), 28.24 (C$\underline{H}_2$), 21.66 (C$\underline{H}_2$), 13.84 (C$\underline{H}_3$) ppm.

*4.3. Synthesis of 4-chloro-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidine(**6a**)*

Vilsmeier-Haack reagent was freshly prepared by the careful addition of $POCl_3$ (3.75 mL, 0.041 mol) in DMF (0.85 mL, 0.011 mol) at 0 °C with constant stirring. To this reaction mixture (maintained at 0 °C), added 6-(phenethylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (5a, 1g, 0.003 mol) and stirred initially for 30 min and later slowly brought to RT and continued stirring for another 30 min. Finally, the reaction mixture was allowed to reflux at 80 °C for 2 h until the TLC

showed full consumption of starting material. The reaction mixture was then poured on ice cold water and neutralized with 10% NaOH solution. Thus, the obtained precipitate was filtered under suction and further purified by flash silica column [MeOH/DCM, 05:95] to afford the desired compound **6a**, as light yellow solid. Yield: 85%; mp 218-220 °C; FTIR (ATR, cm^{-1}) ν_{max}: 3208 (NH Str.), 2927 (Ar-H Str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 14.24 (s, 1H, NH), 8.30 (s, 1H, ArH), 7.31 (t, *J* = 2.48 Hz, 4H, ArH), 7.25-7.19 (m, 1H, ArH), 3.41 (t, *J* = 7.66 Hz, 2H, C$\underline{H}_2$), 3.01 (t, *J* = 7.62 Hz, 2H, C$\underline{H}_2$) ppm;^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.89, 140.07, 128.73, 128.63, 128.39, 126.36, 109.79, 34.58 (C$\underline{H}_2$), 31.93 (C$\underline{H}_2$) ppm.

*4.4. Synthesis of 4-chloro-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidine (**6b**)*

Vilsmeier-Haack reagent was freshly prepared by the careful addition of $POCl_3$ (4.29 mL, 0.047 mol) in DMF (0.97 mL, 0.012 mol) at 0 °C with constant stirring. To this reaction mixture (maintained at 0 °C) was added 6-(pentylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol (5b, 1g, 0.004 mol) and stirred initially for 30 min and later slowly brought to RT and continued stirring for another 30 min. Finally, the reaction mixture was allowed to reflux at 80 °C for 2 h until the TLC showed full consumption of starting material. The reaction mixture was then poured in ice cold water and neutralized with 10% NaOH solution. Thus, the generated precipitate was filtered under suction and further purified by flash silica column [MeOH/DCM, 05:95] to afford the desired compound **6b** as light yellow solid. Yield: 85%; mp 194-196 °C; FTIR (ATR, cm^{-1})ν_{max}: 3095 (NH Str.), 2956 (Ar-H Str.), 1602 , 1556, 1465; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 14.19 (s, 1H, NH), 8.28 (s, 1H, ArH), 3.16 (t, *J* = 7.32 Hz, 2H, C$\underline{H}_2$), 1.73-1.66 (m, 2H, C$\underline{H}_2$), 1.42-1.27 (m, 4H, (C$\underline{H}_2$)$_2$), 0.87 (t, *J* = 7.16 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 168.23, 109.70, 30.42 (C$\underline{H}_2$), 30.38 (C$\underline{H}_2$), 28.11 (C$\underline{H}_2$), 21.66 (C$\underline{H}_2$), 13.84 (C$\underline{H}_3$) ppm.

*4.5. General procedure for the synthesis of 6-(phenethylthio/pentylthio)-N-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amines (**7-43**)*

To a well stirred solution of compound **6a** or **6b** (0.2g, 1Eq.) in absolute ethanol (10 mL), the appropriately substituted anilines (1.1 Eq) was added and the reaction mixture was refluxed for 2-3 h until TLC showed full consumption of starting materials. The excess of solvent was evaporated under reduced pressure to yield the crude solids, which were further purified by recrystallization with methanol to afford the desired title compounds (**7-43**).

4.5.1. *6-(phenethylthio)-N-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**7**)*

White solid; yield: 90%; mp 226-228 °C; FTIR (ATR, cm^{-1})ν_{max}: 3363, 3119, 2918, 1621, 1567, 1491, 1470, 1301, 1183; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.44 (s, 1H, NH), 10.05 (s, 1H, ArH), 8.17 (s, 1H, NH), 7.77 (d, *J* = 7.92 Hz, 2H, ArH), 7.35-7.19 (m, 7H, ArH), 7.10 (t, *J* = 7.34 Hz, 1H, ArH), 3.36 (t, *J* = 5.14 Hz, 2H, C$\underline{H}_2$), 2.97 (t, *J* = 5.14, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.40, 155.51, 153.48, 140.38, 138.90, 128.69, 128.52, 128.31, 126.20, 123.59, 121.34, 98.41, 35.30 (C$\underline{H}_2$), 31.31 (C$\underline{H}_2$) ppm; HRMS (ESI) for $C_{19}H_{16}N_5S$, $[M+H]^+$ calcd: 346.1126, found: 346.1126.

4.5.2. *N-(2-chlorophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**8**)*

White solid; yield: 92%; mp 268-270 °C; FTIR (ATR, cm^{-1})ν_{max}: 3055, 2918, 2718, 1626, 1560, 1379, 1269, 1184; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.47 (s, 1H, NH), 7.98 (s, 1H, NH), 7.62-7.58 (m, 2H, ArH), 7.41-7.08 (m, 8H, ArH), 3.20 (t, *J* = 7.68 Hz, 2H, C$\underline{H}_2$), 2.83 (t, *J* = 7.56 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.95, 155.03, 140.21, 134.86, 130.46, 129.95, 129.39, 128.49, 128.29, 127.81, 126.20, 98.12, 35.33 (C$\underline{H}_2$), 31.37 (C$\underline{H}_2$) ppm.

4.5.3. *N-(3-chlorophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**9**)*

White solid; yield: 85%; mp 216-218 °C; FTIR (ATR, cm^{-1})ν_{max}: 3311, 3210, 3132, 3023, 2917, 1635, 1558, 1473, 1388, 1309, 1176; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.46 (s, 1H, NH), 8.35 (s, 1H, NH), 8.08 (s, 1H, ArH), 7.73 (dd, *J* = 8.08, 1.04 Hz, 1H, ArH), 7.36-7.12 (m, 8H, ArH), 3.39 (t, *J* = 7.62 Hz, 2H, C$\underline{H}_2$), 2.98 (t, *J* = 7.62 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.27, 155.19, 153.30, 140.47, 140.24, 132.99, 132.48, 130.28, 128.57, 128.32, 126.25, 123.05, 120.55, 119.31, 35.06 (C$\underline{H}_2$), 31.52 (C$\underline{H}_2$) ppm; HRMS (ESI) for $C_{19}H_{15}N_5SCl$, $[M+H]^+$ calcd: 380.0733, found: 380.0737.

4.5.4. *N-(4-chlorophenyl)-6-(3-phenylpropyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**10**)*

White solid; yield: 85%; mp 286-288 °C; FTIR (ATR, cm^{-1})ν_{max}: 3052, 2911, 2725, 1627, 1566, 1487, 1383, 1276, 1184, 1080, 1015; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.81 (s, 1H, NH), 8.49 (s, 1H, NH), 7.84 (d, *J* = 8.80 Hz, 2H, ArH), 7.35-7.18 (m, 8H, ArH), 3.37 (t, *J* = 7.74 Hz, 2H, C$\underline{H}_2$), 2.95 (t, *J* = 7.74 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6):δ 166.99, 154.47,

153.53, 140.20, 137.55, 132.23, 128.56, 128.51, 128.31, 127.63, 126.26, 123.16, 98.76, 35.21 ($\underline{C}H_2$), 31.38 ($\underline{C}H_2$) ppm.

4.5.5. *N-(3-nitrophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**11**)*

Yellow solid; yield: 81%; mp 249-251°C; FTIR (ATR, cm^{-1})ν_{max}: 3378, 3100, 3030, 2828, 1629, 1566, 1525, 1478, 1429, 1349, 1250, 1109; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.63 (s, 1H, NH), 8.91 (t, *J* = 2.12 Hz, 1H, ArH), 8.34 (s, 1H, NH), 8.22 (dd, *J* = 8.06, 1.62 Hz, 1H, ArH), 7.91 (dd, *J* = 8.14, 1.98 Hz, 1H, ArH), 7.61 (t, *J* = 8.18 Hz, 1H, ArH), 7.28-7.17 (m, 6H, ArH), 3.41 (t, *J* = 7.62 Hz, 2H, $C\underline{H}_2$), 2.97 (t, *J* = 7.60 Hz, 2H, $C\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.38, 155.45, 153.12, 147.90, 140.35, 140.23, 132.53, 130.00, 128.47, 128.29, 126.46, 126.22, 117.51, 114.85, 98.72, 35.03 ($\underline{C}H_2$), 31.44 ($\underline{C}H_2$) ppm.

4.5.6. *N-(4-nitrophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**12**)*

White solid; yield: 88%; mp 265-267 °C; FTIR (ATR, cm^{-1})ν_{max}: 3033, 2902, 2820, 2752, 1627, 1570, 1515, 1338, 1274, 1183, 1110; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.06 (s, 1H, NH), 9.94 (s, 1H, NH), 8.54 (s, 1H, ArH), 8.17 (s, 4H, ArH), 7.29-7.19 (m, 5H, ArH), 3.41 (t, *J* = 7.70 Hz, 2H, $C\underline{H}_2$), 3.00 (t, *J* = 7.68 Hz, 2H, $C\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.34, 155.39, 153.01, 145.64, 141.83, 140.27, 132.88, 128.51, 128.32, 126.29, 124.72, 120.14, 99.19, 35.08 ($\underline{C}H_2$), 31.39 ($\underline{C}H_2$) ppm; HRMS (ESI) for $C_{19}H_{15}N_6O_2S$, $[M+H]^+$ calcd: 391.0974, found: 391.0977.

4.5.7. *N-(2-bromophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**13**)*

White solid; yield: 85%; mp 266-268 °C; FTIR (ATR, cm^{-1})ν_{max}: 3052, 2921, 2709, 1625, 1585, 1564, 1547, 1507, 1415, 1380, 1268, 1185; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.57 (s, 1H, NH), 7.96 (s, 1H, NH), 7.76 (dd, *J* = 8.0, 1.0 Hz, 1H, ArH), 7.57 (dd, *J* = 7.80, 1.52 Hz, 1H, ArH), 7.44 (dd, *J* = 7.62, 1.16 Hz, 1H, ArH), 7.32-7.24 (m, 4H, ArH), 7.18 (t, *J* = 7.28 Hz, 1H, ArH), 7.08 (d, *J* = 7.28 Hz, 2H, ArH), 3.19 (t, *J* = 7.72 Hz, 2H, $C\underline{H}_2$), 2.82 (t, *J* = 7.68 Hz, 2H, $C\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.85, 155.15, 140.18, 136.35, 133.12, 131.87, 129.79, 128.88, 128.50, 128.29, 126.20, 35.32 ($\underline{C}H_2$), 31.39 ($\underline{C}H_2$) ppm.

4.5.8. *N-(3-bromophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**14**)*

White solid; yield: 81%; mp 265-267 °C; FTIR (ATR, cm^{-1})ν_{max}: 3053, 2914, 2724, 1625, 1558, 1507, 1472, 1379, 1273, 1184; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.61 (s, 1H, NH), 8.42 (s, 1H, NH), 8.23 (s, 1H, ArH), 7.80 (t, *J* = 2.66 Hz, 1H, ArH), 7.29-7.20 (m, 8H, ArH), 3.39 (t, *J* = 7.58 Hz, 2H, C$\underline{H}_2$), 2.98 (t, *J* = 7.56 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.16, 153.39, 140.52, 140.17, 132.46, 130.56, 128.59, 128.33, 126.26, 126.09, 123.55, 121.41, 119.85, 98.75, 35.02 (C$\underline{H}_2$), 31.56 (C$\underline{H}_2$) ppm; HRMS (ESI) for $C_{19}H_{15}N_5SBr$, $[M+H]^+$ calcd: 424.0224, found: 424.0232.

4.5.9. *N-(4-bromophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**15**)*

White solid; yield: 78%; mp 284-286 °C; FTIR (ATR, cm^{-1})ν_{max}: 3100, 3025, 2917, 1613, 1579, 1495, 1473, 1304, 1225, 1180; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.84 (s, 1H, NH), 8.51 (s, 1H, NH), 7.79 (d, *J* = 8.76 Hz, 2H, ArH), 7.46 (d, *J* = 8.80 Hz, 2H, ArH), 7.30-7.18 (m, 6H, ArH), 3.37 (t, *J* = 5.16 Hz, 2H, CH_2), 2.95 (t, *J* = 7.74 Hz, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.98, 153.50, 140.20, 137.97, 132.22, 131.47, 128.51, 128.33, 126.27, 123.52, 115.74, 98.81, 35.21 (C$\underline{H}_2$), 31.38 (C$\underline{H}_2$) ppm.

4.6.0. *N-(2,4-dimethylphenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**16**)*

White solid; yield: 76%; mp 238-240 °C; FTIR (ATR, cm^{-1})ν_{max}: 3100, 3025, 2917, 1613, 1579, 1495, 1473, 1335, 1304, 1225, 1180; ^{1}H-NMR (400 MHz, DMSO-d_6) δ:13.29 (s, 1H, NH), 9.66 (s, 1H, NH), 7.29-7.04 (m, 9H, ArH), 3.22 (s, 2H, C$\underline{H}_2$), 2.88 (s, 2H, C$\underline{H}_2$), 2.31 (s, 3H, C$\underline{H}_3$), 2.14 (s, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.40, 155.73, 140.50, 133.89, 132.55, 131.23, 128.51, 128.25, 127.04, 126.13, 35.51 (C$\underline{H}_2$), 31.28 (C$\underline{H}_2$), 20.64 (C$\underline{H}_3$), 17.74 (C$\underline{H}_3$) ppm; HRMS (ESI) for $C_{21}H_{20}N_5S$, $[M+H]^+$ calcd: 374.1447, found: 374.1439.

4.6.1. *N-(4-(methylthio)phenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**17**)*

White solid; yield: 90%; mp 262-264 °C; FTIR (ATR, cm^{-1})ν_{max}: 3054, 2921, 2809, 2752, 1625, 1583, 1586, 1488, 1417, 1385, 1274, 1243, 1184; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.50 (s, 1H, NH), 8.36 (s, 1H, NH), 7.74 (d, *J* = 8.52 Hz, 2H, ArH), 7.31-7.19 (m, 8H, ArH), 3.37 (t, *J* = 7.80 Hz, 2H, C$\underline{H}_2$), 2.96 (t, *J* = 7.76 Hz, 2H, C$\underline{H}_2$), 2.44 (s, 3H, SC$\underline{H}_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.07, 154.78, 153.49, 140.28, 135.91, 132.20, 128.51, 128.31, 126.70, 126.24,

122.23, 98.58, 35.31 ($\underline{C}H_2$), 31.31 ($\underline{C}H_2$), 15.31 ($\underline{C}H_3$) ppm; HRMS (ESI) for $C_{20}H_{18}N_5S_2$, $[M+H]^+$ calcd: 392.1000, found: 392.1004.

4.6.2. *N-(4-chloro-3-(trifluoromethyl)phenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**18**)*

White solid; yield: 89%; mp 273-275 °C; FTIR (ATR, cm^{-1})ν_{max}: 3033, 2920, 2808, 2752, 1628, 1566, 1507, 1498, 1399, 1315, 1274, 1171, 1153, 1107, 1037; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.93 (s, 1H, NH), 8.49 (d, *J* = 2.52 Hz, 1H, ArH), 8.47 (s, 1H, NH), 8.18 (dd, *J* = 8.82, 2.50 Hz, 1H, ArH), 7.61 (d, *J* = 8.80 Hz, 1H, ArH), 7.29-7.20 (m, 6H, ArH), 3.37 (t, *J* = 7.68 Hz, 2H, $C\underline{H}_2$), 2.96 (t, *J* = 7.66 Hz, 2H, $C\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.21, 155.12, 153.17, 140.21, 138.63, 132.61, 131.89, 126.76, 126.46, 125.52, 124.15, 123.86, 121.44, 119.62, 119.57, 98.83, 35.03 ($\underline{C}H_2$), 31.40 ($\underline{C}H_2$) ppm; HRMS (ESI) for $C_{20}H_{15}N_5F_3SCl$, $[M+H]^+$ calcd: 449.0689, found: 448.0611.

4.6.3. *N-(4-bromo-3-(trifluoromethyl)phenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**19**)*

White solid; yield: 86%; mp 273-275 °C; FTIR (ATR, cm^{-1})ν_{max}: 3033, 2905, 2809, 2752, 1628, 1562, 1507, 1473, 1381, 1315, 1273, 1182, 1131, 1097; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.71 (s, 1H, NH), 8.48 (s, 1H, NH), 8.37 (s, 1H, ArH), 8.08 (t, *J* =6.48 Hz, 1H, ArH), 7.76 (t, *J* = 7.44 Hz, 1H, ArH), 7.29-7.20 (m, 6H, ArH), 3.37 (t, *J* =7.66 Hz, 2H, $C\underline{H}_2$), 2.96 (t, *J* =7.54 Hz, 2H, $C\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.27, 153.11, 140.23, 139.12, 135.27, 132.61, 128.50, 128.32, 128.26, 126.26, 125.49, 124.25, 121.53, 119.82, 35.04 ($\underline{C}H_2$), 31.38 ($\underline{C}H_2$) ppm; HRMS (ESI) for $C_{20}H_{15}N_5F_3SBr$, $[M+H]^+$ calcd: 493.0184, found: 492.0105.

4.6.4. *N-(4-chloro-3-nitrophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**20**)*

White solid; yield: 75%; mp 265-267 °C; FTIR (ATR, cm^{-1})ν_{max}: 3023, 2899, 2827, 2759, 1628, 1557, 1531, 1494, 1432, 1376, 1338, 1275, 1182, 1048; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.95 (s, 1H, NH), 8.75 (s, 1H, ArH), 8.43 (s, 1H, NH), 8.16 (d, *J* = 8.84 Hz, 1H, ArH), 7.68 (dd, *J* = 8.82, 1.66 Hz, 1H, ArH), 7.29-7.18 (m, 6H, ArH), 3.40 (t, *J* = 7.58 Hz, 2H, $C\underline{H}_2$), 2.97 (t, *J* = 7.56 Hz, 2H, $C\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.28, 155.29, 153.02, 147.02,

140.21, 139.17, 132.71, 131.82, 128.50, 128.30, 126.25, 125.57, 118.35, 117.18, 98.82, 34.98 ($\underline{C}H_2$), 31.45 ($\underline{C}H_2$) ppm.

4.6.5. *N-(4-fluoro-2-methylphenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine* ***(21)***

White solid; yield: 80 %; mp 268-270 °C; FTIR (ATR, cm^{-1})ν_{max}: 3103, 3026, 3000, 2934, 1593, 1580, 1492, 1313, 1303, 1210, 1180, 1144; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.34 (s, 1H, NH), 9.72 (s, 1H, NH), 7.39 (q, *J* = 4.76 Hz, 1H, ArH), 7.29-7.04 (m, 8H, ArH), 3.20 (s, 2H, $C\underline{H}_2$), 2.87 (d, *J* = 6.92 Hz, 2H, $C\underline{H}_2$), 2.19 (s, 3H, $C\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.41, 161.66, 155.71, 140.44, 132.79, 132.48, 128.47, 128.24, 126.15, 117.12, 116.89, 113.21, 113.00, 35.50 ($\underline{C}H_2$), 31.28 ($\underline{C}H_2$), 17.90 ($\underline{C}H_3$) ppm; HRMS (ESI) for $C_{20}H_{17}N_5FS$, $[M+H]^+$ calcd: 378.1191, found: 378.1189.

4.6.6. *N-(3-bromo-4-methylphenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine* ***(22)***

White solid; yield: 85%; mp 279-281 °C; FTIR (ATR, cm^{-1})ν_{max}: 3056, 2923, 2745, 1626, 1583, 1550, 1487, 1383, 1270, 1184, 1145, 1038; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.67 (s, 1H, NH), 8.43 (s, 1H, NH), 8.19 (d, *J* = 1.84 Hz, 1H, ArH), 7.68 (dd, *J* = 8.24, 2.00 Hz, 1H, ArH), 7.28-7.18 (m, 7H, ArH), 3.38 (t, *J* = 7.66 Hz, 2H, $C\underline{H}_2$), 2.96 (t, *J* = 7.62 Hz, 2H, $C\underline{H}_2$), 2.30 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.06, 154.62, 153.45, 140.16, 137.83, 130.85, 128.58, 128.30, 126.25, 124.55, 123.64, 120.54, 119.29, 35.07 ($\underline{C}H_2$), 31.56 ($\underline{C}H_2$), 21.78 ($\underline{C}H_3$) ppm; HRMS (ESI) for $C_{20}H_{18}N_5SBr$, $[M+H]^+$ calcd: 439.0466, found: 438.0388.

4.6.7. *N-(2-chloro-4-fluorophenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine* ***(23)***

White solid; yield: 83 %; mp 282-284 °C; FTIR (ATR, cm^{-1})ν_{max}: 3037, 2907, 2724, 1629, 1569, 1488, 1380, 1269, 1256, 1196, 1180; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.28 (s, 1H, NH), 8.08 (s, 1H, NH), 7.65-7.57 (m, 2H, ArH), 7.28-7.17 (m, 5H, ArH), 7.09 (d, *J* = 10.24 Hz, 2H, ArH), 3.18 (t, *J* = 7.76 Hz, 2H, $C\underline{H}_2$), 2.82 (d, *J* = 5.80 Hz, 2H, $C\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 155.10, 140.17, 131.55, 130.92, 130.82, 128.41, 128.26, 126.21, 117.24, 116.98, 115.09, 114.87, 35.36 ($\underline{C}H_2$), 31.32 ($\underline{C}H_2$) ppm.

4.6.8. *N-(3,4-dimethoxyphenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine* ***(24)***

Light yellow solid; yield: 78%; mp 265-267 °C; FTIR (ATR, cm^{-1})ν_{max}: 3063, 2930, 2716, 1629, 1584, 1505, 1457, 1388, 1259, 1232, 1204, 1184, 1129; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.97 (s, 1H, NH), 8.63 (s, 1H, NH), 7.39-7.14 (m, 8H, ArH), 6.85 (d, *J* = 8.40 Hz, 1H, ArH), 3.73 (s, 3H, OC$\underline{H}_3$), 3.71 (s, 3H, OC$\underline{H}_3$), 3.38 (t, *J* = 7.80 Hz, 2H, C$\underline{H}_2$), 2.93 (t, *J* = 7.74 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.40, 148.58, 140.05, 131.11, 128.52, 128.30, 126.29, 111.69, 55.66 (O-$\underline{C}H_3$), 55.51 (O-$\underline{C}H_3$), 35.19 ($\underline{C}H_2$), 31.46 ($\underline{C}H_2$) ppm.

4.6.9. *N-(3-ethynylphenyl)-6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**25**)*

Light yellow solid; yield: 78%; mp 254-256 °C; FTIR (ATR, cm^{-1})ν_{max}: 3230, 3065, 2914, 2750, 1627, 1571, 1543, 1509, 1381, 1257, 1167, 1051; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.82 (s, 1H, NH), 8.51 (s, 1H, NH), 8.03 (s, 1H, Ar$\underline{H}$), 7.86 (d, *J* = 4.54 Hz, 1H, ArH), 7.35-7.18 (m, 8H, ArH), 4.18 (s, 1H, Ethynyl-$\underline{H}$), 3.39 (t, *J* = 7.54 Hz, 2H, C$\underline{H}_2$), 2.97 (t, *J* = 7.52 Hz, 2H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.99, 153.62, 140.13, 138.92, 129.13, 127.04, 126.27, 124.41, 122.09, 122.04, 98.80, 83.30, 80.75, 35.04 ($\underline{C}H_2$), 31.56 ($\underline{C}H_2$) ppm.

4.7.0. *6-(pentylthio)-N-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**26**)*

White solid; yield: 86%; mp 202-204 °C; FTIR (ATR, cm^{-1})ν_{max}: 3079, 2924, 1624, 1568, 1540, 1507, 1496, 1309, 1185; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.39 (s, 1H, NH), 10.02 (s, 1H, ArH), 8.15 (s, 1H, NH), 7.78 (d, *J*=7.88 Hz, 2H, ArH), 7.38 (t, *J* = 7.92 Hz, 2H, ArH), 7.12 (t, *J* = 7.32 Hz, 1H, ArH), 3.09 (t, *J* = 7.38 Hz, 2H, C$\underline{H}_2$), 1.66 (m, 2H, C$\underline{H}_2$), 1.25-1.38 (m, 4H, (C$\underline{H}_2$)$_2$), 0.85 (t, *J* = 7.16 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.73, 155.51, 153.39, 138.96, 128.64, 123.53, 121.22, 98.34, 30.57 ($\underline{C}H_2$), 29.97 ($\underline{C}H_2$), 28.97 ($\underline{C}H_2$), 21.75 ($\underline{C}H_2$), 13.85 ($\underline{C}H_3$) ppm; HRMS (ESI) for $C_{16}H_{18}N_5S$, $[M+H]^+$ calcd: 312.1284, found: 312.1283.

4.7.1. *N-(2-chlorophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**27**)*

White solid; yield: 75%; mp 231-233 °C; FTIR (ATR, cm^{-1})ν_{max}: 3054, 2924, 2719, 1624, 1571, 1383, 1273, 1185, 1057; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.48 (s, 1H, NH), 7.99 (S, 1H, NH), 7.57-7.61 (m, 2H, ArH), 7.35-7.44 (m, 2H, ArH), 2.90 (t, *J* = 7.44 Hz, 2H, C$\underline{H}_2$), 1.46-1.53 (m, 2H, C$\underline{H}_2$), 1.14-1.22 (m, 4H, (C$\underline{H}_2$)$_2$), 0.82 (t, *J* = 6.86 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100

MHz, DMSO-d_6) δ: 167.35, 154.87, 134.96, 132.05, 129.84, 129.43, 128.19, 127.70, 97.95, 30.50 ($\underline{C}H_2$), 29.99 ($\underline{C}H_2$), 29.11 ($\underline{C}H_2$), 21.74($\underline{C}H_2$), 13.87 ($\underline{C}H_3$) ppm.

4.7.2. *N-(3-chlorophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**28**)*

White solid; yield: 92%; mp 224-226 °C; FTIR (ATR, cm^{-1})ν_{max}: 3099, 2928, 1623, 1558, 1541, 1473, 1386, 1304, 1180; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.35 (s, 1H, NH), 8.30 (s, 1H, NH), 8.12 (s, 1H, ArH), 7.70 (d, *J* = 8.28 Hz, 1H, ArH), 7.39 (t, *J* = 8.10 Hz, 1H, ArH), 7.15 (dd, *J* = 7.90, 1.70 Hz, 1H, ArH), 3.12 (t, *J* = 7.32 Hz, 2H, $C\underline{H}_2$), 1.63-1.70 (m, 2H, $C\underline{H}_2$), 1.21-1.41 (m, 4H ($C\underline{H}_2$)$_2$), 0.84 (t, *J* = 7.16 Hz, 3H, $C\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.64, 155.27, 153.21, 140.53, 132.96, 132.43, 130.28, 123.00, 120.47, 119.19, 98.62, 30.51 ($\underline{C}H_2$), 30.14 ($\underline{C}H_2$), 28.75 ($\underline{C}H_2$), 21.76 ($\underline{C}H_2$), 13.87 ($\underline{C}H_3$) ppm.

4.7.3. *N-(4-chlorophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**29**)*

White solid; yield: 88%; mp 253-255 °C; FTIR (ATR, cm^{-1})ν_{max}: 3056, 2923, 2717, 1625, 1567, 1541, 1384, 1267, 1183, 1080, 1013; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.58 (s, 1H, NH), 8.39 (s, 1H, NH), 7.85 (d, *J* = 8.88 Hz, 2H, ArH), 7.43 (d, *J* = 8.88 Hz, 2H, ArH), 3.09 (t, *J* = 7.38 Hz, 2H, $C\underline{H}_2$), 1.61-1.68 (m, 2H, $C\underline{H}_2$), 1.22-1.37 (m, 4H, ($C\underline{H}_2$)$_2$), 0.84 (t, *J* = 7.16 Hz, 3H, $C\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.45, 153.38, 137.76, 132.27, 128.51, 127.40, 122.95, 98.60, 30.58 ($\underline{C}H_2$), 30.12 ($\underline{C}H_2$), 28.88 ($\underline{C}H_2$), 21.76 ($\underline{C}H_2$), 13.87 ($\underline{C}H_3$) ppm.

4.7.4. *N-(3-nitrophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**30**)*

Brown solid; yield: 84%; mp 210-212 °C; FTIR (ATR, cm^{-1})ν_{max}: 3080, 2928, 2757, 1625, 1558, 1522, 1348, 1299, 1175, 1072; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.70 (s, 1H, NH), 8.96 (t, *J* = 2.14 Hz, 1H, ArH), 8.37 (s, 1H, ArH), 8.20-8.23 (m, 1H, ArH), 7.92-7.94 (m, 1H, ArH), 7.66 (t, *J* = 8.22 Hz, 1H, ArH), 3.15 (t, *J* = 7.30 Hz, 2H, $C\underline{H}_2$), 1.62-1.69 (m, 2H, $C\underline{H}_2$), 1.21-1.38 (m, 4H, ($C\underline{H}_2$)$_2$), 0.82 (t, *J* = 7.20 Hz, 3H, $C\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.72, 155.34, 153.14, 147.93, 140.40, 132.55, 130.00, 126.49, 117.56, 114.85, 98.72, 30.49 ($\underline{C}H_2$), 30.16 ($\underline{C}H_2$), 28.57 ($\underline{C}H_2$), 21.74 ($\underline{C}H_2$), 13.86 ($\underline{C}H_3$) ppm.

4.7.5. *N-(4-nitrophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**31**)*

Yellow solid; yield: 93%; mp 268-270 °C; FTIR (ATR, cm^{-1})ν_{max}: 3108, 2923, 1626, 1568, 1508, 1336, 1297, 1250, 1182, 1111; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.66 (s, 1H, NH), 8.33 (s, 1H, NH), 8.27 (d, *J* = 9.24 Hz, 2H, ArH), 8.13 (d, *J* = 9.24 Hz, 2H, ArH), 3.14 (t, *J* = 7.32 Hz, 2H, C$\underline{H}_2$), 1.66-1.73 (m, 2H, C$\underline{H}_2$), 1.25-1.43 (m, 4H, (C$\underline{H}_2$)$_2$), 0.85 (t, *J* = 7.18 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.77, 155.64, 152.77, 145.70, 141.75, 132.57, 124.79, 119.87, 99.00, 30.60 (C$\underline{H}_2$), 30.12 (C$\underline{H}_2$), 28.73 (C$\underline{H}_2$), 21.77 (C$\underline{H}_2$), 13.86 (C$\underline{H}_3$) ppm.

4.7.6. *N-(2-bromophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**32**)*

White solid; yield: 81%; mp 255-257 °C; FTIR (ATR, cm^{-1})ν_{max}: 3052, 2924, 2725, 1626, 1570, 1542, 1507, 1379, 1275, 1184, 1044; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.53 (s, 1H, NH), 7.96 (s, 1H, NH), 7.75-7.78 (m, 1H, ArH), 7.53-7.56 (m, 1H, ArH), 7.45-7.49 (m, 1H, ArH), 7.29-7.33 (m, 1H, ArH), 2.90 (t, *J* = 7.42 Hz, 2H, C$\underline{H}_2$), 1.45-1.53 (m, 2H, C$\underline{H}_2$), 1.14-1.22 (m, 4H, (C$\underline{H}_2$)$_2$, 0.82 (t, *J* = 6.98 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.15, 155.06, 136.37, 133.01, 131.81, 129.82, 128.80, 128.40, 121.69, 97.97, 30.49 (C$\underline{H}_2$), 30.06 (C$\underline{H}_2$), 29.15 (C$\underline{H}_2$), 21.75 (C$\underline{H}_2$), 13.88 (C$\underline{H}_3$) ppm.

4.7.7. *N-(3-bromophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**33**)*

Brown solid; yield: 76%; mp 232-234 °C; FTIR (ATR, cm^{-1})ν_{max}: 3099, 2927, 1622, 1557, 1472, 1298, 1178, 1063; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.41 (s, 1H, NH), 8.32 (s, 1H, NH), 8.26 (t, *J* = 1.78 Hz, 1H, ArH), 7.75 (d, *J* = 8.04 Hz, 1H, ArH), 7.27-7.35 (m, 2H, ArH), 3.12 (t, *J* = 7.28 Hz, 2H, C$\underline{H}_2$), 1.63-1.71 (m, 2H, C$\underline{H}_2$), 1.23-1.41 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, *J* = 7.22 Hz, 3H, C$\underline{H}_2$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.59, 155.19, 153.18, 140.63, 132.41, 130.56, 125.88, 123.32, 121.39, 119.57, 98.61, 30.49 (C$\underline{H}_2$), 30.13 (C$\underline{H}_2$), 28.70 (C$\underline{H}_2$), 21.75 (C$\underline{H}_2$), 13.86 (C$\underline{H}_3$) ppm.

4.7.8. *N-(4-bromophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**34**)*

White solid; yield: 90%; mp 256-258 °C; FTIR (ATR, cm^{-1})ν_{max}: 3056, 2924, 2718, 1626, 1559, 1474, 1383, 1269, 1183, 1065; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.53 (s, 1H, NH), 8.36 (s, 1H, NH), 7.80 (d, *J* = 8.64 Hz, 2H, ArH), 7.55 (d, *J* = 8.80 Hz, 2H, ArH), 3.09 (t, *J* = 7.36 Hz, 2H, C$\underline{H}_2$), 1.61-1.68 (m, 2H, C$\underline{H}_2$), 1.22-1.37 (m, 4H, (C$\underline{H}_2$)$_2$), 0.85 (t, *J* = 7.08 Hz, 3H, C$\underline{H}_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.48, 153.34, 138.22, 132.27, 131.41, 123.25, 98.61, 30.59

($\underline{C}H_2$), 30.12 ($\underline{C}H_2$), 28.89 ($\underline{C}H_2$), 21.76 ($\underline{C}H_2$), 13.89 ($\underline{C}H_3$) ppm; HRMS (ESI) for $C_{16}H_{17}N_5SBr$, $[M+H]^+$ calcd: 390.0383, found: 390.0388.

4.7.9. *N-(2,4-dimethylphenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**35**)*

Brown solid; yield: 90%; mp 256-258 °C; FTIR (ATR, cm^{-1})ν_{max}: 3099, 2922, 1614, 1578, 1473, 1315, 1223, 1180; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.24 (s, 1H, NH), 9.62 (s, 1H, ArH), 7.17 (t, *J* = 9.64 Hz, 2H, ArH), 7.06 (d, *J* = 7.68 Hz, 1H, ArH), 2.95 (s, 2H, $C\underline{H}_2$), 2.31 (s, 3H, $C\underline{H}_3$), 2.13 (s, 3H, $C\underline{H}_3$), 1.56 (s, 2H, $C\underline{H}_2$), 1.25 (s, 4H, $(C\underline{H}_2)_2$), 0.84 (t, *J* = 6.68 Hz, 3H, $C\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.72, 155.74, 133.93, 132.51, 131.16, 127.51, 127.00, 30.58 ($\underline{C}H_2$), 29.80 ($\underline{C}H_2$), 29.10 ($\underline{C}H_3$), 21.76 ($\underline{C}H_2$), 20.62 ($\underline{C}H_3$), 17.72 ($\underline{C}H_2$), 13.90 ($\underline{C}H_3$) ppm; HRMS (ESI) for $C_{18}H_{22}N_5S$, $[M+H]^+$ calcd: 340.1601, found: 340.1596.

4.8.0. *N-(4-(methylthio)phenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**36**)*

Brown solid; yield: 77%; mp 242-244 °C; FTIR (ATR, cm^{-1})ν_{max}: 3064, 2923, 2724, 1624, 1558, 1520, 1507, 1386, 1185, 1083; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.65 (s, 1H, NH), 8.42 (d, *J* = 9.84, 1H, NH), 7.75 (d, *J* = 8.60 Hz, 2H, ArH), 7.29 (d, *J* = 8.64 Hz, 2H, ArH), 3.09 (t, *J* = 7.42 Hz, 2H, $C\underline{H}_2$), 2.47 (s, 3H, $SC\underline{H}_3$), 1.60-1.68 (m, 2H, $C\underline{H}_2$), 1.21-1.36 (m, 4H, $(C\underline{H}_2)_2$), 0.84 (t, *J* = 7.12 Hz, 3H, $C\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.25, 153.57, 135.82, 131.96, 126.74, 122.41, 98.61, 30.61 ($\underline{C}H_2$), 30.17 ($\underline{C}H_2$), 28.98 ($\underline{C}H_2$), 21.77 ($\underline{C}H_2$), 15.38 ($\underline{C}H_3$), 13.90 ($\underline{C}H_3$) ppm.

4.8.1. *N-(4-chloro-3-(trifluoromethyl)phenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**37**)*

White solid; yield: 83%; mp 258-260 °C; FTIR (ATR, cm^{-1})ν_{max}: 3101, 2927, 1628, 1567, 1478, 1437, 1385, 1321, 1256, 1176, 1132; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.60 (s, 1H, NH), 8.51 (d, *J* = 2.52 Hz, 1H, ArH), 8.32 (s, 1H, ArH), 8.10 (dd, *J* = 8.80, 2.52 Hz, 1H, ArH), 7.71 (d, *J* = 8.80 Hz, 1H, ArH), 3.10 (t, *J* = 7.34 Hz, 2H, $C\underline{H}_2$), 1.61-1.68 (m, 2H, $C\underline{H}_2$), 1.21-1.38 (m, 4H, $(C\underline{H}_2)_2$), 0.84 (t, *J* = 7.18 Hz, 3H, $C\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.67, 155.41, 152.99, 138.72, 132.49, 131.94, 126.45, 125.33, 123.73, 119.47, 98.66, 30.48 ($\underline{C}H_2$), 30.01 ($\underline{C}H_2$), 28.57 ($\underline{C}H_2$), 21.73 ($\underline{C}H_2$), 13.83 ($\underline{C}H_3$) ppm.

4.8.2. *N-(4-bromo-3-(trifluoromethyl)phenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**38**)*

Brown solid; yield: 79%; mp 257-259 °C; FTIR (ATR, cm^{-1})ν_{max}: 3099, 2926, 1633, 1559, 1541, 1474, 1436, 1257, 1145, 1097; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.62 (s, 1H, NH), 8.51 (d, *J* = 2.52 Hz, 1H, ArH), 8.34 (s, 1H, NH), 8.03 (dd, *J* = 8.74, 2.50 Hz, 1H, ArH), 7.86 (d, *J* = 8.72 Hz, 1H, ArH), 3.10 (t, *J* = 7.32 Hz, 2H, C$\underline{H}_2$), 1.61-1.68 (m, 2H, C$\underline{H}_2$), 1.22-1.38 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, *J* = 7.14 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.64, 155.37, 152.98, 139.16, 135.28, 132.50, 128.54, 125.39, 124.29, 119.73, 111.31, 98.70, 30.48 (C$\underline{H}_2$), 30.00 (C$\underline{H}_2$), 28.56 (C$\underline{H}_2$), 21.73 (C$\underline{H}_2$), 13.83 (C$\underline{H}_3$) ppm; HRMS (ESI) for $C_{17}H_{16}N_5F_3SBr$, $[M+H]^+$ calcd: 458.0258, found: 458.0262.

4.8.3. *N-(4-chloro-3-nitrophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**39**)*

Yellow solid; yield: 91%; mp 251-253 °C; FTIR (ATR, cm^{-1})ν_{max}: 3098, 2925, 1626, 1558, 1540, 1473, 1339, 1297, 1265, 1182; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.73 (s, 1H, NH), 8.78 (d, *J* = 2.52 Hz, 1H, ArH), 8.33 (s, 1H, ArH), 8.08 (dd, *J* = 8.92, 2.56 Hz, 1H, ArH), 7.76 (d, *J* = 8.88 Hz, 1H, ArH), 3.12 (t, *J* = 7.26 Hz, 2H, C$\underline{H}_2$), 1.61-1.68 (m, 2H, C$\underline{H}_2$), 1.20-1.39 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, *J* = 7.14 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.70, 155.45, 152.90, 147.12, 139.23, 132.54, 131.83, 125.33, 118.20, 116.94, 98.71, 30.48 (C$\underline{H}_2$), 30.14 (C$\underline{H}_2$), 28.50 (C$\underline{H}_2$), 21.76 (C$\underline{H}_2$), 13.86 (C$\underline{H}_3$) ppm.

4.8.4. *N-(4-fluoro-2-methylphenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**40**)*

White solid; yield: 90%; mp 261-263 °C; FTIR (ATR, cm^{-1})ν_{max}: 3100, 2953, 1577, 1490, 1206, 1181; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.29 (s, 1H, NH), 9.69 (s, 1H, ArH), 7.33-7.36 (m, 1H, ArH), 7.20 (t, *J* = 4.76 Hz, 1H, ArH), 7.01-7.11 (m, 1H, ArH), 2.93 (s, 2H, C$\underline{H}_2$), 2.18 (s, 3H, C$\underline{H}_3$), 1.54 (s, 2H, C$\underline{H}_2$), 1.22 (s, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, *J* = 6.96 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.72, 155.80, 132.83, 132.39, 129.41, 117.01, 116.79, 113.15, 112.90, 30.59 (C$\underline{H}_2$), 29.84(C$\underline{H}_2$), 29.13 (C$\underline{H}_3$), 21.76 (C$\underline{H}_2$), 17.87 (C$\underline{H}_2$), 13.87 (C$\underline{H}_3$) ppm.

4.8.5. *N-(3-bromo-4-methylphenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**41**)*

Brown solid; yield: 86%; mp 237-239 °C; FTIR (ATR, cm^{-1})ν_{max}: 3099, 2923, 1623, 1556, 1474, 1395, 1301, 1178; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.63 (s, 1H, NH), 8.30 (s, 1H, NH), 8.23

(d, *J* = 1.84 Hz, 1H, ArH), 7.65 (dd, *J* = 8.28, 2.08 Hz, 1H, ArH), 7.34 (d, *J* = 8.32 Hz, 1H, ArH), 3.12 (t, *J* = 7.28 Hz, 2H, C$\underline{H}_2$), 1.62-1.70 (m, 2H, C$\underline{H}_2$), 1.22-1.40 (m, 4H, (C$\underline{H}_2$)$_2$), 0.84 (t, *J* = 7.22 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.54, 153.23, 138.05, 132.30, 131.98, 130.82, 124.29, 123.61, 120.19, 98.52, 30.50 (C$\underline{H}_2$), 30.13 (C$\underline{H}_2$), 28.72 (C$\underline{H}_2$), 21.75(C$\underline{H}_2$), 21.73(C$\underline{H}_3$), 13.86 (C$\underline{H}_3$) ppm; HRMS (ESI) for $C_{17}H_{19}N_5FS$, $[M+H]^+$ calcd: 344.1348, found: 344.1345.

4.8.6. *N-(2-chloro-4-fluorophenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**42**)*

White solid; yield: 80%; mp 247-249 °C; FTIR (ATR, cm^{-1})ν_{max}: 3054, 2925, 2716, 1625, 1573, 1488, 1382, 1257, 1181; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.44 (s, 1H, NH), 8.04 (s, 1H, ArH), 7.59-7.62 (m, 2H, ArH), 7.29-7.34 (m, 1H, ArH), 2.89 (t, *J* = 7.42 Hz, 2H, C$\underline{H}_2$), 1.45-1.52 (m, 2H, C$\underline{H}_2$), 1.16-1.22 (m, 4H, (C$\underline{H}_2$)$_2$), 0.82 (t, *J* = 6.88 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.20, 161.54, 159.08, 155.10, 131.86, 131.01, 117.13, 116.87, 115.01, 114.78, 98.01, 30.59 (C$\underline{H}_2$), 30.09 (C$\underline{H}_2$), 29.19 (C$\underline{H}_2$), 21.76 (C$\underline{H}_2$), 13.86 (C$\underline{H}_3$) ppm.

4.8.7. *N-(3,4-dimethoxyphenyl)-6-(pentylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**43**)*

Brown solid; yield: 78%; mp 247-249 °C; FTIR (ATR, cm^{-1})ν_{max}: 3055, 2951, 2725, 1625, 1507, 1260, 1233, 1026; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.52 (s, 1H, NH), 8.43 (s, 1H, NH), 7.40 (s, 1H, ArH), 7.28 (s, 1H, ArH), 6.97 (d, *J* = 8.72 Hz, 1H, ArH), 3.76 (d, *J* = 2.12 Hz, 6H, (OC$\underline{H}_3$)$_2$), 3.10 (t, *J* = 7.38 Hz, 2H, C$\underline{H}_2$), 1.59-1.64 (m, 2H, C$\underline{H}_2$), 1.20-1.34 (m, 4H, (C$\underline{H}_2$)$_2$), 0.83 (t, *J* = 7.08 Hz, 3H, C$\underline{H}_3$) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 166.72, 153.97, 148.59, 131.38, 114.58, 111.74, 107.58, 98.57, 55.73 (O$\underline{C}$H$_3$), 55.59 (O$\underline{C}$H$_3$), 30.50 (C$\underline{H}_2$), 30.28 (C$\underline{H}_2$), 28.81 (C$\underline{H}_2$), 21.72 (C$\underline{H}_2$), 13.83 C$\underline{H}_3$) ppm; HRMS (ESI) for $C_{18}H_{22}N_5O_2S$, $[M+H]^+$ calcd: 372.1496, found: 372.1494.

5. *Biological activity*

5.1. *CDK2 and Abl kinase inhibition assays*

CDK2/cyclin E and Abl kinases were produced in Sf9 insect cells via baculoviral infection and purified on a NiNTA column. The kinase reactions were assayed with suitable substrates (1 mg/mL histone H1 for CDK2 and 500 μM peptide GGEAIYAAPFKK for Abl) in the presence of 15 or 10 μM ATP for CDK2 and Abl, respectively, 0.05 μCi [γ-^{33}P]ATP, and the test

compound in a final volume of 10 µL, all in a reaction buffer (60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 2.5 µg / 50 µl $PEG_{20.000}$). The reactions were stopped by adding 5 µL of 3% aq. H_3PO_4. Aliquots were spotted onto P-81 phosphocellulose (Whatman), washed 3× with 0.5% aq. H_3PO_4 and finally air-dried. Kinase inhibition was quantified using a FLA-7000 digital image analyzer. The concentration of the test compounds required to reduce the kinase activity by 50 % was determined from dose-response curves and recorded as their IC_{50}.

5.2. *Anti-proliferative evaluation for K-562 and MCF-7 cell lines*

The tumor cells (purchased from the American Type Culture Collection) were grown in DMEM medium (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum and L-glutamine (0.3 g/L) and were maintained at 37°C in a humidified atmosphere with 5% CO_2. For anticancer cytotoxicity estimations, 10^4 cells were seeded into each well of a 96-well plate, allowed to stabilize for 20 h, and the test inhibitors were then added at different concentrations (ranging from 0.1 to 100 µM or to a solubility limit) in triplicate. Three days after addition of the inhibitors, calcein AM solution (Molecular Probes) was added. One hour later, fluorescence of cells was quantified using a Fluoroskan Ascent (Labsystems) reader and cytotoxic effective concentrations were calculated and expressed as IC_{50} values from dose-response curves. Roscovitine and imatinib were used as reference drugs.

6. *Molecular docking simulation*

Molecular docking experiments were performed using Glide software package[36] implemented in Schrodinger Suite (2017-2) (Schrödinger, Inc., USA)[37] running on Intel CORE i7 based hpZ230 workstation with the Microsoft Windows 10 OS. In this protocol, the protein was kept rigid, while the ligands were allowed to be flexible throughout the docking simulation.

6.1. *Protein preparation*

The starting X-ray solved protein crystal structure of cyclin dependent kinase-2 bound with *R*-roscovitine was retrieved from protein data bank (PDB) bearing ID 2A4L.[35] The protein was prepared by automatic preparation by Protein Preparation Wizard of Glide employing the Optimized Potentials for Liquid Simulations 3 (OPLS3) forcefield. During the pre-processing stage, crystallographic water molecules were removed and added missing hydrogens to the protein structure corresponding to pH 7.0 was achieved. The protein metal ions and cofactors

were viewed and removed from the protein structure. The tool neutralized the side chains that are not close to the binding cavity and do not participate in salt bridges. The pre-processed protein structure was refined initially by optimizing the sample-water orientation followed by restrained minimization of co-crystallized complex using OPLS3, which reorients side chain hydroxyl groups and alleviates potential steric clashes. Thus, the complex obtained was minimized until it reaches the convergent of heavy atom to RMSD 0.3 Å and taken finally in .mae format.

6.2. *Grid file generation*

Receptor grid generation protocol of Maestro 11.2 was used to define the binding-site of the protein (2A4L) for docking simulation by excluding any co-crystallized metals, co-factors, water molecules all of which may have crystallized during experimental crystallization of the CDK-2 protein. A grid box was generated around the centroid of the cognate ligand (*R*-roscovitine) specifying the size for the docking ligands (20 Å) with default settings.

6.3. *Ligand preparation*

The structures of the synthesized ligands and standard *R*-roscovitine were sketched using built panel of Maestro and taken in .mae format. LigPrep is a utility of Schrodinger software suit that combines tools for generating 3D structures from 1D (Smiles) and 2D (SDF) representation, searching for tautomers, steric isomers and perform a geometry minimization of the ligands. By employing Ligprep protocol, all the ligands were prepared using OPLS3 with default settings and the output file was saved in maegz format automatically.

6.4. *Docking simulation*

For precision and accuracy of the docking protocols, the co-crystallized ligand was extracted from the crystal structure of 2A4L and re-docked using Glide docking algorithm (Schrodinger Inc) in its extra precision (XP) mode with default settings without applying any constraints. A good agreement of the obtained pose of docked *R*-roscovitine with cognate ligand indicated the reliability of the selected docking parameters for docking of the synthesized ligands. Hence, by specifying the ligands against the receptor grid, molecular docking was performed using default settings in Glide XP mode.

6.5. *Binding mode analysis*

The protein-ligand complexes were analysed to investigate various types of interactions by utilizing XP visualizer protocol. For the best-scored ligands, the 2D and 3D plots of molecular

ligand-receptor interactions were analysed for hydrogen bond, halogen bond, salt bridges, π-π stacking, and π-cation interactions.

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Conflict of Interest:

Authors hereby declare that there are no financial/commercial conflicts of interest.

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Synthesis, anticancer evaluation, and molecular docking studies of some novel 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines as cyclin dependent kinase 2 (CDK2) inhibitors

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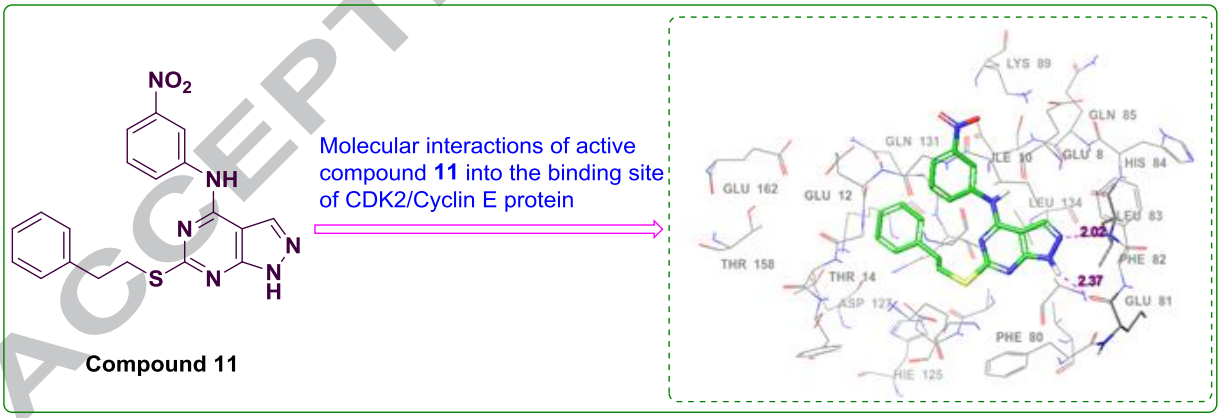
Graphical Abstract



Highlights

1. A new class of pyrazolo[3,4-*d*]pyrimidine analogues were synthesized and characterized.
2. All newly synthesized compounds were screened for their anticancer activity.
3. Compounds **8**, **9**, **11**, **18**, **24**, **27**, **30** and **36** exhibited significant CDK2 inhibitory activity.
4. The binding energies of the best active compounds were in agreement with the experimental data.
5. The structure-activity relationship was discussed.