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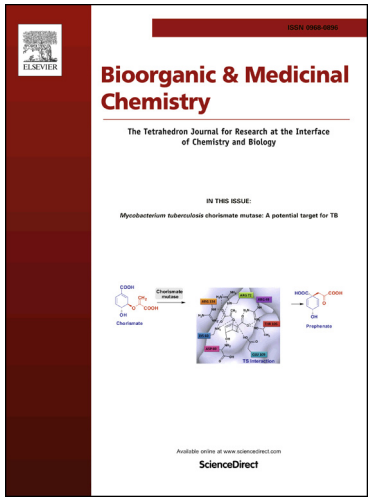

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Design and Synthesis of Pyrazolo[3,4-d]Pyrimidines: Nitric Oxide Releasing Compounds Targeting Hepatocellular Carcinoma

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Abstract

A new series of pyrazolo[3,4-d]pyrimidines tethered with nitric oxide (NO) producing functionality was designed and synthesized. Sulforhodamine B (SRB) protein assay revealed that NO releasing moiety in the synthesized compounds significantly decreased the cell growth more than the des-NO analogues. Compounds **7C** and **7G** possessing N-*para*-substituted phenyl group, released the highest NO concentration of 4.6% and 4.7% respectively. Anti-proliferative activity of synthesized compounds on HepG2 cell line identified compounds **7h, 7p, 14a** and **14b** as the most cytotoxic compounds in the series of IC_{50} = 3, 5, 3 and 5 µM, respectively, compared to erlotinib as a reference drug (IC_{50} = 25 µM). Flow cytometry studies revealed that **7h** arrested the cells in G0/G1 phase of cell cycle while **7p** arrested the cells in S phase. Moreover, docking study of the synthesized compounds on EGFR (PDB code: 1M17) and cytotoxicity study indicated that N-1 phenyl *para* substitution, pyrazole C-3 alkyl substitution and tethering the nitrate moiety through butyl group had a significant impact on the activity.

Keywords

pyrazolo[3,4-d]pyrimidines - Nitric oxide - Hepatocellular carcinoma-EGFR

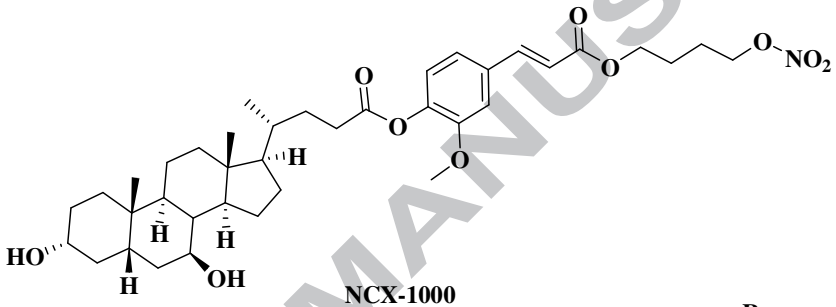
1. Introduction

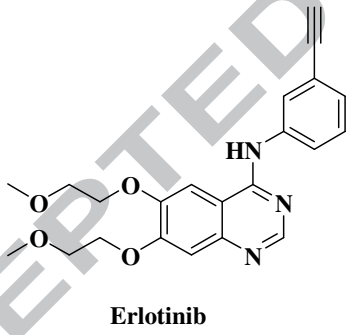
Hepatocellular carcinoma (HCC) is the second cause of cancer-related deaths worldwide since it has high incidence to mortality ratio (1.07).[1] Currently used drugs for

treatment of liver cancer showed severe side effects and do not overcome cancer drug resistance.[2] Cancer resistance to chemotherapeutic agents is a common reason in patient mortality and poor/early prognosis of cancer.[3] Due to this problem and severe side effects of chemotherapeutic agents, *de novo* design and synthesis of new chemotherapeutic agents is a hot topic in cancer research.

Nitric oxide (NO) is an endogenous short-lived free radical obtained from conversion of L-arginine to L-citroline,[4] which is mediated by nitric oxide synthase (NOS).[5] NO producing compounds have a wide biological function such as improving cardiovascular activities,[4, 6] and cerebral circulation disorders,[6] peripheral ischemia revascularization,[4] limb reperfusion and salvage,[4] and also potent as anti-inflammatory,[4, 7-8] analgesic,[8] antipyretic,[8] anti-platelet,[8] antibacterial[9-10] and anti-cancer agents.[4, 8, 11-13] In addition, NO exerts gastrointestinal safety[8] and hepatoprotective properties.[14] NO is known to reverse chemotherapy resistance in colon cancer cells, it was reported that NO inhibited the MRP-3 efflux pump by nitrating the tyrosine and thereby led to inversion of drug resistance.[15-17] Hence, it is likely that NO can be used in conjunction with traditional cancer drugs that have become ineffective due to such modes of efflux. Furthermore, NO is considered as a novel potential therapeutic agent in patients with refractory cancer by sensitizing tumor cells to chemotherapy, radiotherapy or immunotherapy.[18-20] It is noteworthy to mention that, NCX-1000 (**Fig. 1**) is the first hepatoprotective NO donor drug, however, it has not met the endpoint in phase 2 clinical trials.[14, 21]

Epidermal growth factor receptor protein tyrosine kinase (EGFR) is highly expressed in HCC and plays a central hepatoprotective and pro-regenerative role in the liver.[22-23] Treatment of HCC cells with EGFR-specific tyrosine kinase inhibitors such as erlotinib (Tarceva®) (**Fig.1**), neutralizing antibodies cetuximab (Erbitux®) or cyclin-dependent kinase inhibitors (CDKI) such as roscovitine (Seliciclib®) to induce cell cycle arrest and apoptosis and increases chemo sensitivity. Hence, EGFR inhibition has long been an attractive target for anticancer drugs,[23] many efforts have been directed at developing anticancer agents that can interfere with EGFR activity, such as monoclonal antibodies and small-molecule inhibitors.[22]





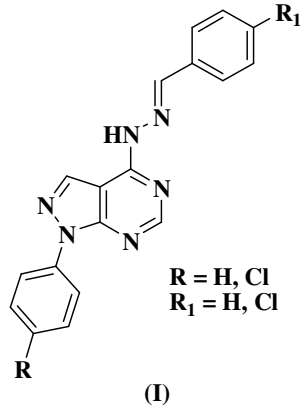


Figure 1: Structures of hepatoprotective[14, 21] and antitumor drugs.[24-25]

Moreover, pyrazolo[3,4-d]pyrimidine derivatives have a considerable chemical and pharmacological importance such as anti-inflammatory,[26-27] tuberculostatic,[26] antiviral,[26-27] antimicrobial,[26-28] and anticancer agents (Compound **I**, **Fig. 1**).[26-29] They were also found to exhibit variable degrees of anticancer activities against HCC,[26, 28] colon carcinoma,[28] cervical carcinoma,[28] skin carcinoma[27] and breast adenocarcinoma[26] by inhibiting different types of enzymes such as EGFR-TK,[28-29] CDK,[28-29] Src and Abl

tyrosine kinase,[29] glycogen synthase kinase-3,[28-29] and B-Raf kinase.[28] There are so many documented cancer resistance to current chemotherapeutic agents including erlotinib.[30] In this regard, further efforts are required to develop NO based strategies for cancer prevention and treatment.

In this context, we envisioned that a novel drug candidate of hybrid structure from two important pharmcophores, the NO donating group and the pyrazolo[3,4-d]pyrimidines moiety will be fruitful to hit cancer cells especially HCC cell line and to overcome the cancer resistance to chemotherapy. To achieve this, we designed a series of novel analogs possessing pyrazolo[3,4-d]pyrimidines linked through alkyl bridge to an organic nitrate ester functionality.

2. Results and Discussion

2.1. Molecular docking study

A library of pyrazolo[3,4-d]pyrimidine/nitrate ester hybrids was designed and energy minimized using MMFF94 force field calculations. The catalytic domain of EGFR was obtained from protein data bank (PDB code: 1M17)[31] and was prepared for docking using Open Eye® software. Open Eye Omega application was used to generate different conformations of each ligand. Docking was conducted using Fred and the data was visualized by Veda application. This software package generates consensus scoring which is a filtering processes to obtain virtual binding affinity, the lower consensus score, the better binding affinity of the ligands towards the receptor. This study revealed that compounds **7h**, **7p** and **14a**, **b** showed better consensus score than the lead drug erlotinib, **Table 1**. The standard erlotinib showed a hydrogen bond (1.71 Å) towards ATP-binding site of EGFR coming from pyrimidine-N2 with Met:769:A (**Fig. 2**). This docking mode similar to cocrystalized docking pose with receptor.[31] However compounds **7h, 7p** and **14a,b** showed a hydrophobic-hydrophobic interaction towards the ATP binding site as well as a hydrogen bond between pyrazole-N2 and Lys:721:A with bond length 2.02, 2.33, 2.28 and 2.40 Å, respectively (**Fig. 3** and **4**). Furthermore, Compound **7a** showed a hydrogen bonding towards ATP-binding site of EGFR with the

same amino acid similar to the standard erlotinib. This hydrogen bond comes from anilinic NH with Met:769:A (2.07 Å).

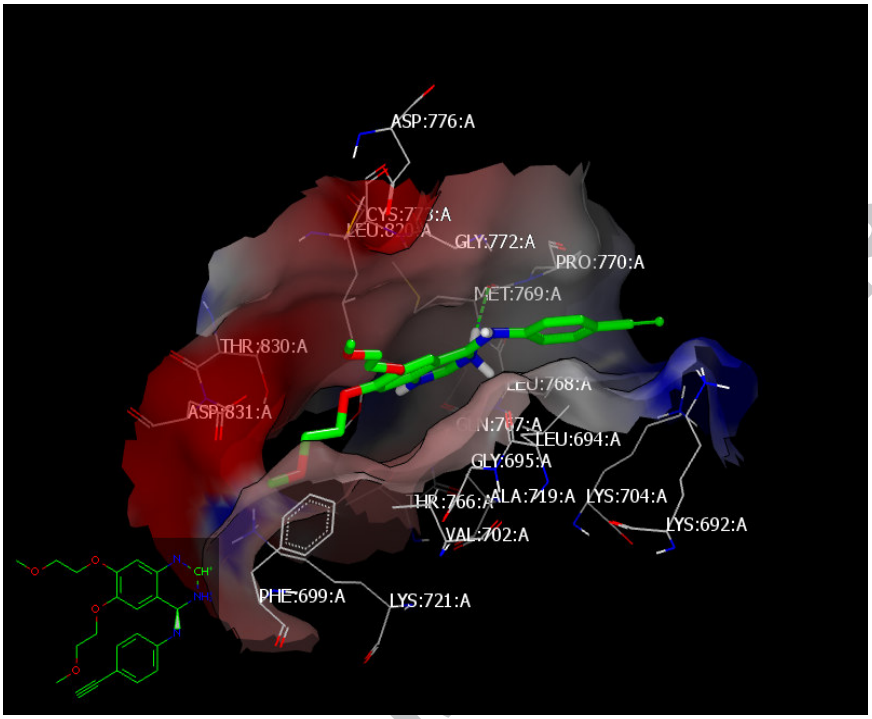


Figure 2: Visual representation for **erlotinib** docked with PDB: 1M17.The dashed lines showing a hydrogen bond towards ATP-binding site of EGFR coming from pyrimidine-N2 with Met:769:A.

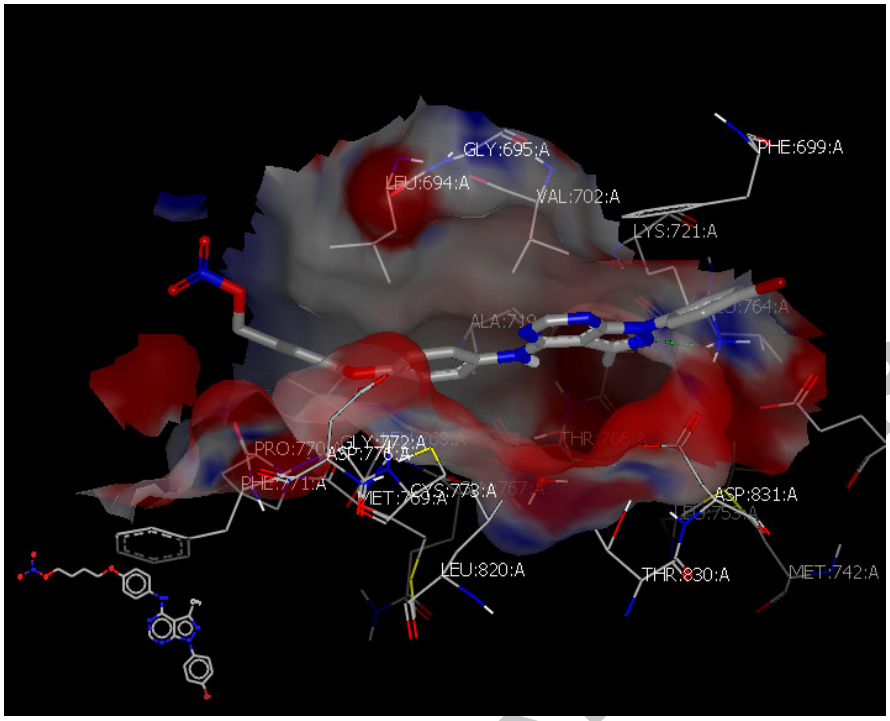


Figure 3: Visual representation for **7h** docked with PDB: 1M17**,** the dashed lines showing the hydrogen bonding towards ATP-binding site of EGFR between pyrazolo N-2 and Lys:721:A.

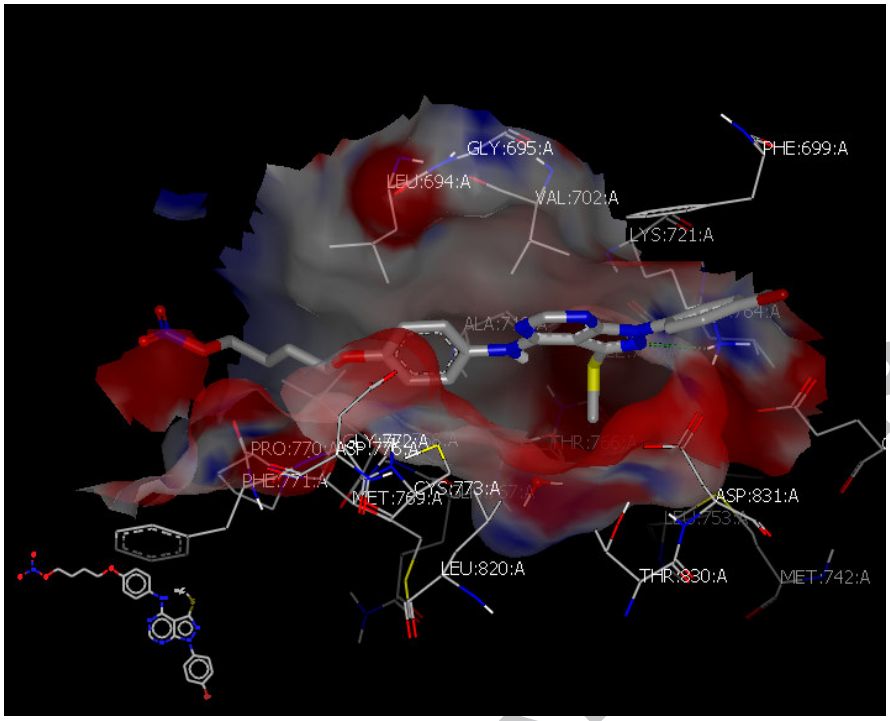


Figure 4: Visual representation for **14a** docked with PDB: 1M17 showing the hydrophobic-hydrophobic interaction towards the ATP binding site and the dashed lines showing the hydrogen bonding between pyrazolo N-2 and Lys:721:A.

Table 1: Molecular modeling consensus scores, IC_{50} and NO release (%) of pyrazolo[3,4-d]pyrimidine/nitrate ester hybrids **7a-p, 14a,b** and **erlotinib** as a reference drug.

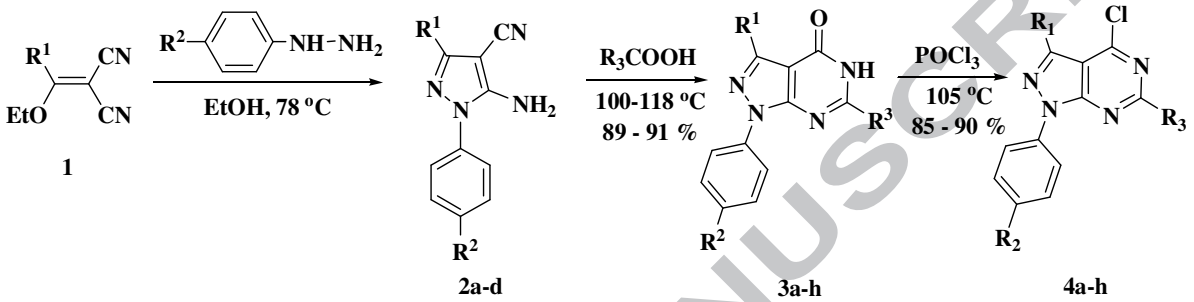
Compound	Consensus score	IC_{50} (µM)	NO release (%)
7a	60	>100	2.3
7b	66	>100	2.1
7c	65	20	4.6
7d	43	25	3.1
7e	56	25	1.5
7f	40	>100	2.6
7g	56	10	4.7
7h	16	3	3.7
7i	53	>100	2.6
7j	43	>100	2.2
7k	52	10	3.8
7l	37	10	1.3
7m	36	25	1.3
7n	30	20	4.1
7o	36	10	4.2
7p	17	5	2.2
14a	14	3	0.7
14b	14	5	3.6
Erlotinib	20	25	-
GTN*	-	-	10.1[32]

* glycerol trinitrate

2.2. Chemistry

The main precursors **2a-d** were synthesized as outlined in Scheme 1 through Michael addition of phenylhydrazine derivatives to the specific alkylidene **1**.[27, 29, 33-38] The generated amino cyanopyrazole **2a-d** were subjected to intramolecular condensations through refluxing in formic acid or acetic acid to afford pyrazolo[3,4-d]pyrimidines-4-ones **3a-h**. The resulting pyrazolo[3,4-d]pyrimidines-4-ones **3a-h** were refluxed in phosphorus oxychloride to give 4-chloropyrazolopyrimidine derivatives **4a-h**, Scheme 1.[33, 39]

Refluxing 4-chloropyrazolopyrimidine derivatives **4a-h** with *p*-aminophenol using triethylamine and sodium iodide as catalysts gave **5a-h**. O-alkylation of anilino derivatives **5a-h** with dibromoalkane named 1,3-dibromopropane and 1,4-dibromobutane in K_2CO_3/CH_3CN under reflux generated the corresponding bromo-alkyl derivatives **6a-h**. The target nitrates **7a-b** were prepared successfully by heating the alkyl bromides **6a-h** with $AgNO_3$ in dry acetonitrile, Scheme 2.

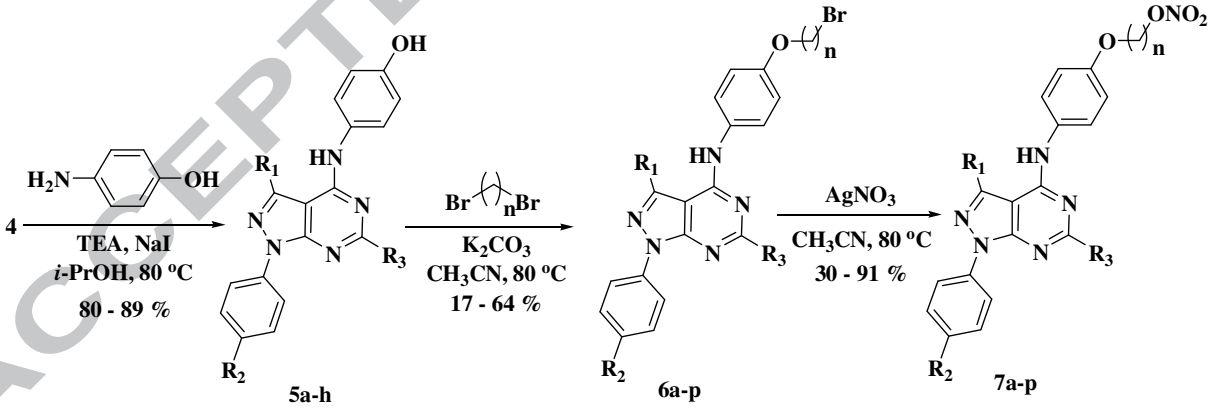


1a, $R_1 = H$
1b, $R_1 = CH_3$

2a, $R_1 = H$, $R_2 = H$
2b, $R_1 = CH_3$, $R_2 = H$
2c, $R_1 =$ H, $R_2 =$ Br
2d, $R_1 = CH_3$, $R_2 = Br$

3a, 4a, $R_1 = H$, $R_2 = H$, $R_3 = H$
3b, 4b, $R_1 = H$, $R_2 = Br$, $R_3 = H$
3c, 4c, $R_1 = CH_3$, $R_2 = H$, $R_3 = H$
3d, 4d, $R_1 = CH_3$, $R_2 = Br$, $R_3 = H$
3e, 4e, $R_1 = H$, $R_2 = H$, $R_3 = CH_3$
3f, 4f, $R_1 = H$, $R_2 = Br$, $R_3 = CH_3$
3g, 4g, $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$
3h, 4h, $R_1 = CH_3$, $R_2 = Br$, $R_3 = CH_3$

Scheme 1. Synthesis of 4-chloropyrazolo[3,4-d]pyrimidine intermediates.



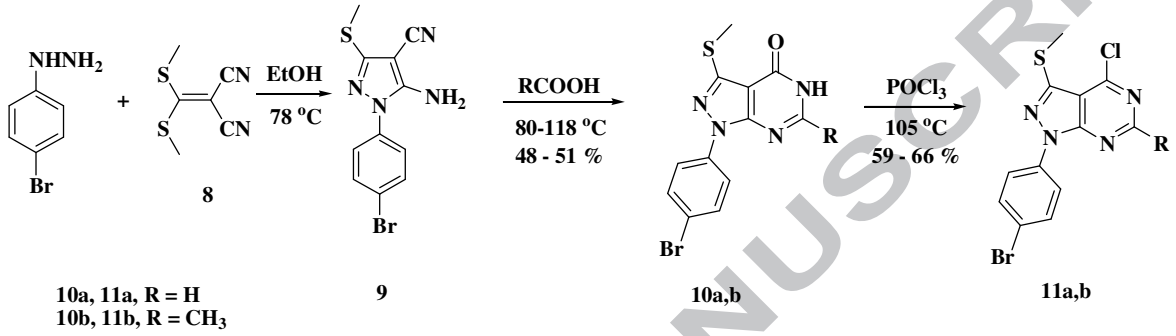
5a, $R_1 = H$, $R_2 = H$, $R_3 = H$
5b, $R_1 = H$, $R_2 = Br$, $R_3 = H$
5c, $R_1 = CH_3$, $R_2 = H$, $R_3 = H$
5d, $R_1 = CH_3$, $R_2 = Br$, $R_3 = H$
5e, $R_1 = H$, $R_2 = H$, $R_3 = CH_3$
5f, $R_1 = H$, $R_2 = Br$, $R_3 = CH_3$
5g, $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$
5h, $R_1 = CH_3$, $R_2 = Br$, $R_3 = CH_3$

6a, 7a, $R_1 = H$, $R_2 = H$, $R_3 = H$, $n = 3$
6b, 7b, $R_1 = H$, $R_2 = H$, $R_3 = H$, $n = 4$
6c, 7c, $R_1 = H$, $R_2 = Br$, $R_3 = H$, $n = 3$
6d, 7d, $R_1 = H$, $R_2 = Br$, $R_3 = H$, $n = 4$
6e, 7e, $R_1 = CH_3$, $R_2 = H$, $R_3 = H$, $n = 3$
6f, 7f, $R_1 = CH_3$, $R_2 = H$, $R_3 = H$, $n = 4$
6g, 7g, $R_1 = CH_3$, $R_2 = Br$, $R_3 = H$, $n = 3$
6h, 7h, $R_1 = CH_3$, $R_2 = Br$, $R_3 = H$, $n = 4$

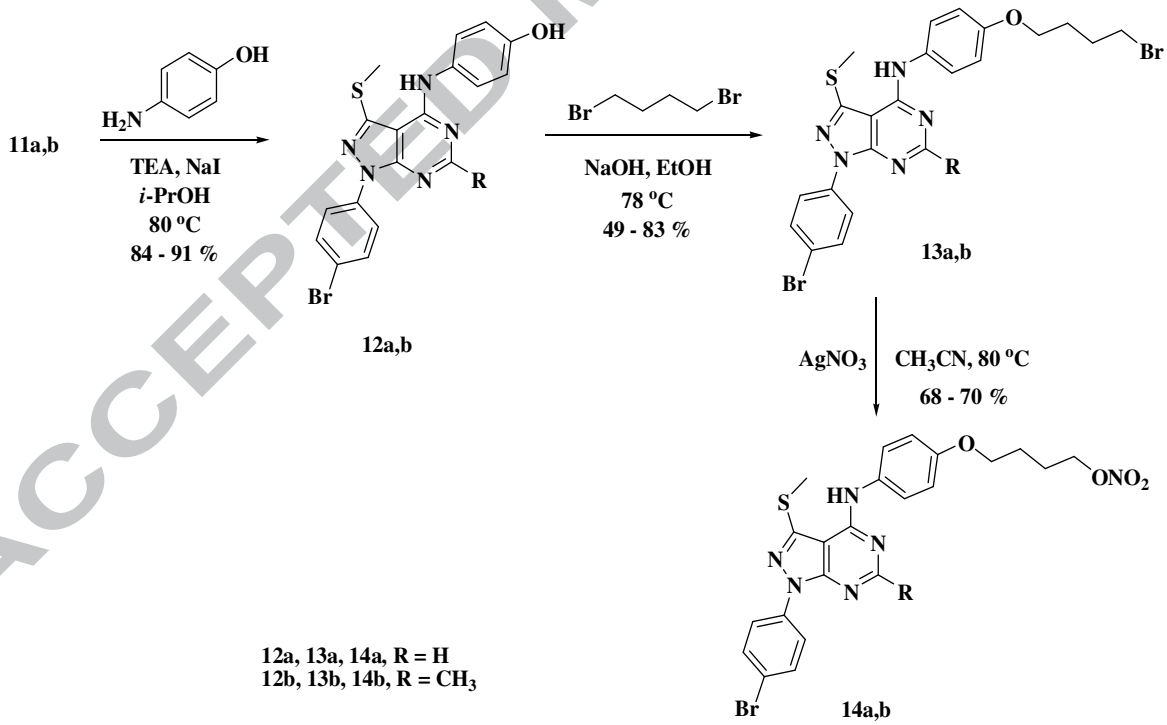
6i, 7i, $R_1 = H$, $R_2 = H$, $R_3 = CH_3$, $n = 3$
6j, 7j, $R_1 = H$, $R_2 = H$, $R_3 = CH_3$, $n = 4$
6k, 7k, $R_1 = H$, $R_2 = Br$, $R_3 = CH_3$, $n = 3$
6l, 7l, $R_1 = H$, $R_2 = Br$, $R_3 = CH_3$, $n = 4$
6m, 7m, $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$, $n = 3$
6n, 7n, $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$, $n = 4$
6o, 7o, $R_1 = CH_3$, $R_2 = Br$, $R_3 = CH_3$, $n = 3$
6p, 7p, $R_1 = CH_3$, $R_2 = Br$, $R_3 = CH_3$, $n = 4$

Scheme 2. Synthesis of NO releasing pyrazolo[3,4-d]pyrimidines.

In order to prepare the designed analogs which contain SMe tethering pyrazole moiety, the departure point starts with preparing the alkylidene compound **8**,[40] Scheme 3. Having in hand compound **8**, compounds **14a-b** were obtained in high yield, Scheme 4, by following similar procedures explained before in schemes 1, and 2.

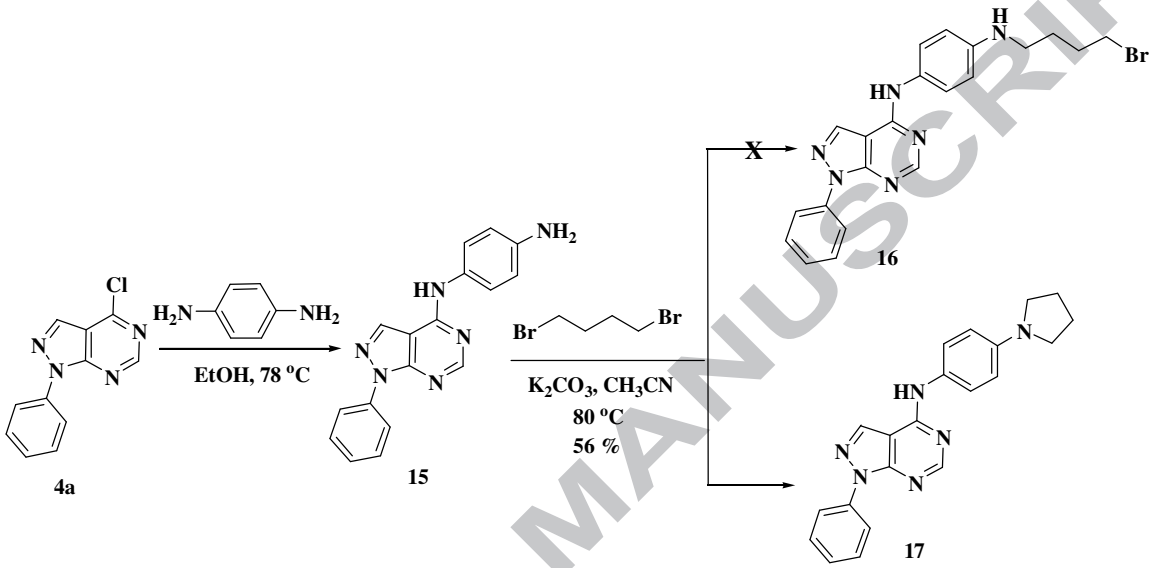


Scheme 3. Synthesis of 3-methylthio 4-chloropyrazolo[3,4-d]pyrimidine intermediates.



Scheme4. Synthesis of NO releasing 3-methylthio pyrazolo[3,4-d]pyrimidines.

Moreover, to explore the bioisosteric effect of changing the oxygen atom in *p*-aminophenol by nitrogen atom using *p*-phenelynediamin, compounds **16** was designed. Compound **4** was coupled with *p*-phenylene diamine and compound **15** was obtained in good yield. However, cyclization reaction occurred between 1,4-dibromobutane and anilino derivative **15** affording pyrrolidine compound **17** (Scheme 5).



Scheme 5. Synthesis of compound **17**

2.3. Measurement of nitric oxide release

To evaluate the thiol-induced nitric oxide generation, final compounds **7a-h** and **14a,b** were subjected to Griess colorimetric method.[41-42] The amount of NO released from the tested compounds, was measured relative to NO released from standard sodium nitrite solution and calculated as percentage of NO released (**Table 1**). The results of measurement of NO release revealed that compounds **7c** and **7g,** which contain N-1 *para*-subsitituted phenyl group released the highest amount of NO among this group (4.6 and 4.7%, respectively). The results suggested that these compounds were able to generate nitrite at pH 1 which confirms the suggested gastrointestinal safety of NO.[43]

2.4. Cytotoxicity screening studies

2.4.1. NCI 60 cell lines screening studies

Eight compounds, **6a, 6e, 6i, 6m, 7a, 7e, 7i** and **7m**, were selected by National Cancer Institute (NCI) according to the protocol of the Drug Evaluation program at National Cancer Institute,[44] for *in vitro* anticancer screening. *In vitro* study using one dose anticancer assay was conducted in full NCI 60 cell lines derived from nine tumor subpanels, including leukemia, melanoma, lung, colon, CNS, ovarian, renal, prostate and breast cancer cell lines. The selected compounds were added at a single concentration (10^{-5} M) and the culture was incubated for 48 h. End point determinations were made with a protein binding dye sulforhodamine B (SRB). Cytotoxicity of each compound were reported as a mean of the percent growth of the treated cells when compared to the untreated control cells, as depicted, in **Table 2.**

Cytotoxicity data (**Table 2**) revealed that the tested compounds showed a weak cell growth inhibition against almost 60 cancer cell lines except compounds **6a**, **7a** exhibited moderate cell growth inhibition against colon cancer cell line HCT-15 (cell growth promotion 59.19%, inhibition 40.81%) and (cell growth promotion 57.54%, inhibition 42.46%), respectively. Also, compounds **6e** and **7e** showed moderate cell growth inhibition against non-small cell lung cancer cell line NCI-H522 (cell growth promotion 45.63%, inhibition 54.37) and (cell growth promotion 57.65%, inhibition 42.35%), respectively. Moreover, compounds **7e** and **7m** record a moderate cell growth inhibition against renal cancer cell line UO-31 (cell growth promotion 40.74%, inhibition 59.26%) and A498 (cell growth promotion 57.57%, inhibition 42.43%), respectively. The moderate cytotoxicity of compounds **7a, 7e, 7i,** and **7m** could be attributed to the NO releasing activity of these compounds.

2.4.2. *In vitro* antitumor against HepG2 Cell line

All target compounds (**7a-p** & **14a,b**) were screened for their cytotoxicity activity against HepG2, IC_{50} was calculated with regard to saline control group and potency was calculated with regard to percentage of antiproliferative activities of erlotinib and tested compounds, as depicted, in **Table 1**.

Our SAR study showed that thirteen of the tested compounds have high to moderate anti-tumor activity towards liver cell line (HepG2) with IC_{50} values ranges

from 3-25 μM in comparison to erlotinib as a reference drug (IC_{50} = 25 μM). Compounds with pyrazolopyrimidines linked with methyl or methylthio group at C-3 of pyrazole ring, *p*-bromophenyl at pyrazole N-1, and nitrooxybutyl group (**7h, 14a;** IC_{50} = 3μM and **7p, 14b;** IC_{50} = 5μM) have the highest activity among the tested compounds as well as the reference drug erlotinib. Also, compounds that don`t have pyrazole C-3 methyl (**7l;** IC_{50} = 10μM) or have unsubstituted phenyl at pyrazole N-1 (**7n;** IC_{50} = 10μM) or tethered to nitrooxy function through shorter alkyl group like propyl (**7g, 7o;** IC_{50} = 10μM) were also more active than erlotinib while compounds **7c-e, 7m** and **7n** with IC_{50} values ranging from 20-25 μM in comparison to erlotinib. Furthermore, because of N-1-unsubstituted phenyl and endowing the pyrazolopyrimidine core with nitric oxide releasing moiety through short alkyl chain (propyl), compounds **7a, 7b, 7f, 7i** and **7j** are the least active among the series. Consequently, increasing lipophilicity at N-1 and substitution at C-3 as well as tethering the nitroxy moiety through long alkyl butyl chain may improve the hydrophobic hydrophobic interaction towards the ATP binding site of EGFR and hydrogen bonding formation and thus increase the ability of the ligands to be more active.

2.4.3. Cell Cycle Arrest

Flow cytometric analyses of compounds **7h** and **7p** were conducted as they are the most active compounds among the series with IC_{50} = 3, 5 μM, respectively. Cell cycle parameters were compared for untreated HepG2 cells that had been incubated for 48 h with **7h** (3μM) or **7p** (5μM), with vehicle DMSO as control. Cell cycle analysis performed by FACS analysis revealed that compounds **7h** and **7p** cause accumulation of cells in G0/G1 and S phase of the cell cycle, respectively. As shown in **Figure 5**, compound **7h** exhibited an increase in the fraction of cells in the G0/G1 phase (91.70% compared to 64.21% for the control) and a decrease in the proportion of cells in the S phase (1.88% compared to 15.90%) and the G2/M phase (4.59% compared to 19.89%). On the other hand, compound **7p** (**Fig. 6)** showed an increase in the fraction of cells in the S phase (34.28% compared to 15.90% in sample control) and a decrease in the proportion of cells in the G2/M phase (0.98% compared to 19.89%). It is concluded

from these results that **7h** is a strong inhibitor of cell cycle progression at the G0/G1 phase while **7p** is a strong inhibitor at the S phase.

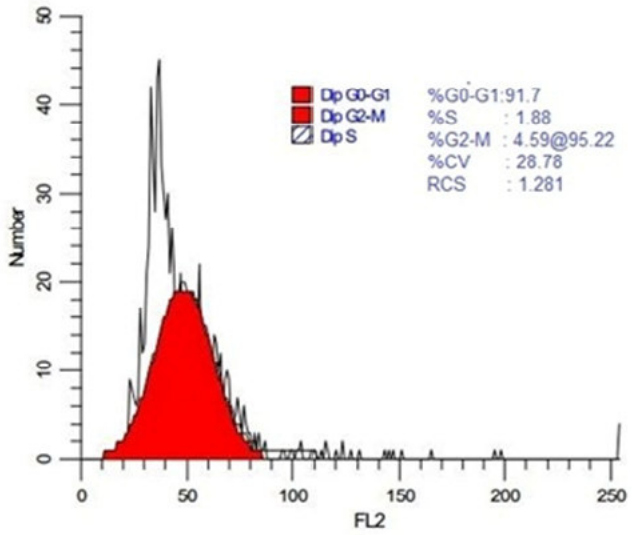


Figure 5. Flow cytometric analysis of cell cycle parameters. HepG2 liver cancer cells were incubated for 48 h in the presence of 3 μM (IC_{50}) of **7h**.

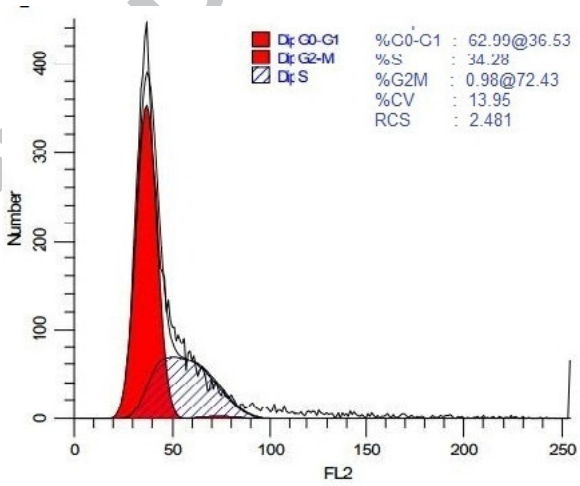


Figure 6. Flow cytometric analysis of cell cycle parameters. HepG2 liver cancer cells were incubated for 48 h in the presence of 5 μM (IC_{50}) of **7p**.

3. Conclusion

Design and synthesis of pyrazolo[3,4-d]pyrimidine derivatives with NO releasing properties have been achieved in an efficient manner. Molecular modeling as well as *in vitro* cytotoxic studies of the target compounds against HepG2 cell lines revealed that compounds **7h, 7p, 14a** and **14b** are more potent as anti-tumor agents than the reference drug (erlotinib) through inducing G0/G1 or S cell cycle arrest. Also, increasing lipophilicity of compounds and installing methyl or methyllthio groups on pyrazole moiety as well as endowing with nitroxybutyl side chain played a crucial role in cytotoxic activity. Finally, the novel synthesized NO-donating hybrids represent a significant strategy against cancer.

4. Experimental procedures

4.1. Chemistry

^{1}H and ^{13}C NMR spectra were acquired on a Bruker AVANCE and Varian Unity INOVA-400 MHz NMR spectrometer, in DMSO using TMS (δ = 0 ppm) as the internal standard for ^{1}H NMR and DMSO-d_6 (δ = 39.51 ppm) for ^{13}C NMR, with the reporting of coupling constants in Hz and the signal multiplicities are reported as singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), doublet of triplets (dt), multiplet (m), or broad (br). HRMS data was obtained using Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Pump). TLC analysis was performed using pre-coated silica gel 60 F_{254} (Merck) sheets. Products were purified via column chromatography using silica gel 150-250 μm (60-120 mesh). TLC plates were visualized by ultraviolet at 254 nm. All reagents and solvents were obtained from commercial suppliers and used as received. All chemical reactions requiring anhydrous conditions were performed with an oven-dried glassware.

4.1.1. Substituted 4-amino-4-cyanopyrazoles 2a-d, pyrazolo[3,4-d]pyrimidinones 3a-c & 3e-g, 4-chloro derivatives 4a-c & 4e-g, 4-anilino derivatives 5a-e & 5g, 2-(bis(methylthio)methylene)malononitrile 8 and 5-amino-1-(4-bromophenyl)-3-(methylthio)-1*H*-pyrazole-4-carbonitrile 9 were prepared according to the reported procedure.[27, 29, 33-38]

4.1.2. General procedure for preparation of 1-aryl-3-alkyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-ones (3a-d, 10a)

A suspension of the appropriate intermediates **2a-d** or **9** (0.01 mol) in 85% formic acid (40 mL) was heated under reflux for 10 h. The reaction mixture was cooled and the separated precipitate was filtered, washed with water, dried and recrystallized from formic acid to afford desired compounds **3a-d** or **10a**.[33, 45-46]

4.1.2.1 1-(4-Bromophenyl)-3-methyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (3d)

White solid; yield 89%; mp 344-345 ^{o}C; IR (KBr, cm^{-1}): 3437 (NH), 1686 (CO), 1584 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.68 (s, 3H, 6-CH_3), 7.73 (d, 2H, J = 8.4 Hz, Ar-H), 8.04 (d, 2H, J = 8.4 Hz, Ar-H), 8.17 (s, 1H, 6-H), 12.39 (s, NH, exchangeable); Anal. Calcd. for $C_{12}H_9BrN_4O$ (305.13): C, 47.24; H, 2.97; N, 18.36. Found: C, 47.17; H, 2.68; N, 18.11.

4.1.2.2. 1-(4-Bromophenyl)-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (10a)

White solid; yield 51%; mp 284-286 ^{o}C; IR (KBr, cm^{-1}): 3350 (NH), 1685 (CO), 1585 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.63 (s, 3H, SCH_3), 7.75 (d, 2H, J = 8.8 Hz, Ar-H), 8.05 (d, 2H, J = 8.8 Hz, Ar-H), 8.21 (s, 1H, 6-H), 12.52 (s, 1H, NH, exchangeable); Anal. Calcd. for $C_{12}H_9BrN_4OS$ (337.20): C, 42.74; H, 2.69; N, 16.62; Found: C, 42.90; H, 2.74; N, 16.80.

4.1.3. General procedure for preparation of 1-Aryl-3,6-dialkyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-ones (3e-h, 10b)

A suspension of the appropriate derivatives **2a-d** or **9** (0.001 mol) was dissolved in glacial acetic acid (3 mL) then $POCl_3$ (0.2 mL) was added quickly. The reaction mixture was refluxed for 2 h (the reaction system was carefully observed by TLC). After cooling the mixture, ice-water was added and the formed precipitate was filtered, washed with water, dried and recrystallized from formic acid to give compounds **3e-h** or **10b.**[39]

4.1.3.1. 1-(4-Bromophenyl)-3,6-dimethyl-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (3h)

White solid; yield 91%; mp 344-346 ^{o}C; IR (KBr, cm^{-1}): 3429 (NH), 1683 (CO), 1607 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.27 (s, 3H, 6-CH_3), 2.35 (s, 3H, 3-CH_3), 7.66 (d, 2H, J = 8.0 Hz , Ar-*H*), 7.96 (d, 2H, J = 8.0 Hz, Ar-*H*), 12.55 (s, 1H, NH); Anal. Calcd. for $C_{13}H_{11}BrN_4O$ (319.16): C, 48.92; H, 3.47; N, 17.55. Found: C, 49.24; H, 3.27; N, 17.80.

4.1.3.2. 1-(4-bromophenyl)-6-methyl-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (10b)

White solid; yield 48%; mp 340-342 ^{o}C; IR (KBr, cm^{-1}): 3420 (NH), 1678 (CO), 1581 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.40 (s, 3H, 6-CH_3), 2.61 (s, 3H, SCH_3), 7.74 (d, 2H, J = 8.8 Hz, Ar-*H*), 8.06 (d, 2H, J = 8.8 Hz, Ar-*H*), 12.38 (s, 1H, NH, exchangeable); Anal. Calcd. for $C_{13}H_{11}BrN_4OS$ (351.22): C, 44.46; H, 3.16; N, 15.95; Found: C, 44.69; H, 3.21; N, 16.13.

4.1.4. General procedure for preparation of 1-Aryl-4-chloro-1*H*-pyrazolo[3,4-d]pyrimidines (4a-h, 11a,b)

A suspension of the appropriate derivatives **3a-h** or **10a,b** (0.001 mol) in phosphorous oxychloride (8 mL) was heated under reflux for 12 h. The reaction mixture was cooled and poured onto ice-cooled water. The formed precipitate was filtered, washed with water, dried and recrystallized from n-hexane to afford the desired pure compounds **4a-h** or **11a,b**.[33]

4.1.4.1. 1-(4-Bromophenyl)-4-chloro-3-methyl-1*H*-pyrazolo[3,4-d]pyrimidine (4d)

White solid; yield 90%; mp 170-172 ^{o}C; IR (KBr, cm^{-1}): 1574 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.74 (s, 3H, 3-CH_3), 7.80 (d, 2H, J = 8.8 Hz, Ar-*H*), 8.14 (d, 2H, J = 8.8 Hz, Ar-*H*), 8.95 (s, 1H, 6-H); Anal. Calcd. for $C_{12}H_8BrClN_4$ (323.58): C, 44.54; H, 2.49; N, 17.31; Found: C, 44.70; H, 2.24; N, 17,45.

4.1.4.2. 1-(4-Bromophenyl)-4-chloro-3,6-dimethyl-1*H*-pyrazolo[3,4-d]pyrimidine (4h)

White solid; yield 89%; mp 178-180 ^{o}C; IR (KBr, cm^{-1}): 1599 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.71 (s, 3H, 6-CH_3), 2.74 (s, 3H, 3-CH_3), 7.79 (d, 2H, J = 8.8 Hz, Ar-*H*), 8.15 (d, 2H, J = 8.8 Hz, Ar-*H*); Anal. Calcd. for $C_{13}H_{10}BrClN_4$ (337.60): C, 46.25; H, 2.99; N, 16.60. Found: C, 46.50; H, 2.72; N, 16.64.

4.1.4.3. 1-(4-bromophenyl)-4-chloro-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidine (11a)

White solid; yield 66%; mp 160-162 ^{o}C; IR (KBr, cm^{-1}): 1573 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.76 (s, 3H, SCH_3), 7.81 (d, 2H, J = 8.8 Hz, Ar-*H*), 8.16 (d, 2H, J = 8.8 Hz, Ar-*H*), 8.95 (s, 1H, 6-H); Anal. Calcd. for $C_{12}H_8BrClN_4S$ (355.64): C, 40.53; H, 2.27; N, 15.75. Found: C, 40.78; H, 2.25; N, 15.98.

4.1.4.4. 1-(4-bromophenyl)-4-chloro-6-methyl-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidine (11b)

White solid; yield 59%; mp 164-165 ^{o}C; IR (KBr, cm^{-1}): 1577 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.73 (s, 3H, 6-CH_3), 2.74 (s, 3H, SCH_3), 7.80 (d, 2H, J = 7.2 Hz, Ar-*H*), 8.15 (d, 2H, J = 7.2 Hz, Ar-*H*); Anal. Calcd. for $C_{13}H_{10}BrClN_4S$ (369.67): C, 42.24; H, 2.73; N, 15.16. Found: C, 42.48; H, 2.71; N, 15.38.

4.1.5. General procedure for preparation of 4-((1-Aryl-3,6-(un)substituted-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenols (5a-h, 12a,b)

A mixture of appropriate derivatives **4a-h** or **11a-b** (10 mmol), 4-aminophenol (1.09 g, 10 mmol), sodium iodide (0.21 g, 1.4 mmol) and triethyl amine (0.84 mL, 10 mmol) in isopropyl alcohol (15 mL) was heated under reflux for 2 h. After cooling, the reaction mixture was diluted with water, and the formed precipitate was collected by filtration, washed with water, and crystallized from ethanol – water to afford required compounds **5a-h, 12a,b.**[47]

4.1.5.1. 4-((1-(4-Bromophenyl)-6-methyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenol (5f)

White solid; yield 80%; mp 299-300 ^{o}C; IR (KBr, cm^{-1}): 3422-3189 (br, OH & NH) and 1580 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.52 (s, 3H, CH_3), 6.82 (d, 2H, J = 8.0 Hz, Ar-*H*); 7.57 (d, 2H, J = 8.0 Hz, Ar-*H*); 7.74 (d, 2H, J = 8.0 Hz, Ar-*H*), 8.22 (d, 2H, J = 8.0 Hz, Ar-*H*); 8.49 (s, 1H, 3-H); 9.39 (bs, 1H, OH, exchangeable); 9.91 (s, 1H, NH, exchangeable); Anal. Calcd. for $C_{18}H_{14}BrN_5O$ (396.24): C, 54.56; H, 3.56; N, 17.67; Found: C, 54.40; H, 3.28; N, 17.80.

4.1.5.2. 4-((1-(4-Bromophenyl)-3,6-dimethyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenol (5h)

White solid; yield 89%; mp 226-227 ^{o}C; IR (KBr, cm^{-1}): 3431-3275 (br, OH & NH) and 1581 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.45 (s, 3H, 3-CH_3), 2.67 (s, 3H, 6-CH_3), 6.79 (d, 2H, J = 8.0 Hz, Ar-*H*); 7.42 (d, 2H, J = 8.0 Hz, Ar-*H*); 7.71 (d, 2H, J = 8.4 Hz, Ar-*H*), 8.21 (d, 2H, J = 8.4 Hz, Ar-*H*); 8.56 (s, 1H, NH, exchangeable); 9.38 (s, 1H, OH, exchangeable). Anal. Calcd. for $C_{19}H_{16}BrN_5O$ (410.27): C, 55.62; H, 3.93; N, 17.07; Found: C, 55.95; H, 3.72; N, 16.81.

4.1.5.3. 4-((1-(4-bromophenyl)-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenol (12a)

Light brown solid; yield 84%; mp 206-207 ^{o}C; IR (KBr, cm^{-1}): 3367 (NH), 3228 (OH) and 1620 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.73 (s, 3H, SCH_3), 6.79 (d, 2H, J = 8.8 Hz, Ar-*H*); 7.41 (d, 2H, J = 8.8 Hz, Ar-*H*); 7.75 (d, 2H, J = 8.8 Hz, Ar-*H*), 8.21 (d, 2H, J = 8.8 Hz, Ar-*H*); 8.42 (s, 1H, 6-H); 8.45 (s, 1H, NH, exchangeable); 9.41 (s, 1H, OH, exchangeable); Anal. Calcd. for $C_{18}H_{14}BrN_5OS$ (428.31): C, 50.48; H, 3.29; N, 16.35; Found: C, 50.64; H, 3.32; N, 16.52.

4.1.5.4. 4-((1-(4-bromophenyl)-6-methyl-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenol (12b)

White solid; yield 91%; mp 216-218 ^{o}C; IR (KBr, cm^{-1}): 3344 (OH & NH) and 1612 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.49 (s, 3H, 6-CH_3), 2.71 (s, 3H,

SC*H*$_3$), 6.79 (d, 2H, *J* = 8.8 Hz, Ar-*H*); 7.46 (d, 2H, *J* = 8.4 Hz, Ar-*H*); 7.75 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 8.21 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.31 (s, 1H, NH, exchangeable), 9.38 (s, 1H, OH, exchangeable); Anal. Calcd. for $C_{19}H_{16}BrN_5OS$ (442.33): C, 51.59; H, 3.65; N, 15.83; Found: C, 51.82; H, 3.69; N, 16.04.

4.1.6. General procedure for preparation of 1-Aryl-N-(4-(bromoalkoxy)phenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6a-p)

To a solution of the appropriate compounds **5a-h** (0.001 mol) in acetonitrile (15 mL), dihaloalkane (0.010 mol), and anhydrous potassium carbonate (0.276 g, 0.002 mol) were added and the reaction mixture was heated under reflux for 10 h. After removal of acetonitrile under vacuo, dichloromethane and water were added to the reaction mixture and the organic layer was washed with water, dried over Na_2SO_4 and the solvent was evaporated under reduced pressure to give a crude product which was purified by flash chromatography on silica gel (AcOEt : n-hexane; 1:9) .[48-50]

4.1.6.1. N-(4-(3-Bromopropoxy)phenyl)-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6a)

White solid; yield 50%; mp 158-159 ^{o}C; IR (KBr, cm^{-1}): 3437 (NH), 1583 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.26 (p, 2H, *J* = 6.3 Hz, $OCH_2CH_2CH_2Br$), 3.69 (t, 2H, *J* = 6.5 Hz, $OCH_2CH_2CH_2Br$), 4.10 (t, 2H, *J* = 6.0 Hz, $OCH_2CH_2CH_2Br$), 7.02 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.36 (t, 1H, *J* = 8.0 Hz, Ar-*H*), 7.56 (t, 2H, *J* = 7.8 Hz, Ar-*H*), 7.70 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.20 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.47 (s, 1H, 6-H), 10.11 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 31.3, 31.9, 65.5, 114.7, 120.8, 126.3, 129.2, 133.7, 138.8, 142.0, 153.0, 155.0, 156.2; Anal Calcd. for $C_{20}H_{18}BrN_5O$ (424.29): C, 56.62; H, 4.28; N, 16.51. Found: C,56.32; H,4.33; N,16.50.

4.1.6.2. N-(4-(4-Bromobutoxy)phenyl)-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6b)

White solid; yield 64%; mp 164-165 ^{o}C; IR (KBr, cm^{-1}): 3426 (NH), 1582 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.91–1.80 (m, 2H, $OCH_2CH_2CH_2CH_2Br$); 2.05–1.93(m, 2H, $OCH_2CH_2CH_2CH_2Br$); 3.63 (t, 2H, *J* = 6.6 Hz, $OCH_2CH_2CH_2CH_2Br$);

4.03 (t, 2H, *J* = 6.3 Hz, OC*H*$_2$CH$_2$CH$_2$CH$_2$Br); 6.00 (d, 2H, *J* = 8.0 Hz, Ar-*H*); 7.36 (t, 1H, *J* = 7.6 Hz, Ar-*H*), 7.57 (t, 2H, *J* = 8.0 Hz, Ar-*H*), 7.68 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.20 (d, 2H, *J* = 8.0 Hz, Ar-*H*); 8.47 (s, 1H, 6-H); 8.50 (s, 1H, 3-H); 10.10 (s, 1H, NH); Anal Calcd. for $C_{21}H_{20}BrN_5O$ (438.32): C, 57.54; H, 4.60; N, 15.98, Found: C, 57.88; H, 4.67; N, 15.76.

4.1.6.3. 1-(4-Bromophenyl)-N-(4-(3-bromopropoxy)phenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6c)

White solid; yield 50%; mp 198-199 ^{o}C; IR (KBr, cm^{-1}): 3436 (NH), 1588 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.25-2.29 (m, 2H, OCH$_2$C*H*$_2$CH$_2$Br), 3.69 (t, 2H, *J* = 6.45 Hz, OCH$_2$CH$_2$C*H*$_2$Br), 4.11 (t, 2H, *J* = 6.0 Hz, OC*H*$_2$CH$_2$CH$_2$Br), 7.02 (d, 2H, *J* = 9.0 Hz, Ar-*H*), 7.68 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 7.76 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 8.23 (d, 2H, *J* = 9.0 Hz, Ar-*H*), 8.49 (s, 2H, 6-H & 3-H), 10.13 (s, NH, exchangeable); Anal Calcd. for $C_{20}H_{17}Br_2N_5O$ (503.19): C, 47.74; H, 3.41; N, 13.92; Found: C, 47.49; H, 3.47; N, 13.93.

4.1.6.4. N-(4-(4-Bromobutoxy)phenyl)-1-(4-bromophenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6d)

White solid; yield 48%; mp 202-203 ^{o}C; IR (KBr, cm^{-1}): 3434 (NH), 1583 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.83-1.88 (m, 2H, OCH$_2$CH$_2$C*H*$_2$CH$_2$Br), 1.97-1.99 (m, 2H, OCH$_2$C*H*$_2$CH$_2$CH$_2$Br), 3.63 (t, 2H, *J* = 6.6 Hz, OCH$_2$CH$_2$C*H*$_2$Br), 4.04 (t, 2H, *J* = 6.0 Hz, OC*H*$_2$CH$_2$CH$_2$Br), 7.00 (d, 2H, *J* = 8.1 Hz, Ar-*H*), 7.67 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 7.77 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 8.22 (d, 2H, *J* = 8.1 Hz, Ar-*H*), 8.49 (s, 2H, 6-H & 3-H), 10.12 (s, NH, D$_2$O exchangeable); Anal Calcd. for $C_{21}H_{19}Br_2N_5O$ (517.22): C, 48.77; H, 3.70; N, 13.54; Found: C, 49.03; H, 3.84; N, 13.32.

4.1.6.5. N-(4-(3-Bromopropoxy)phenyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6e)

White solid; yield 36%; mp 110-111 ^{o}C; IR (KBr, cm^{-1}): 3410 (NH), 1603 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.26 (p, 2H, *J* = 6.3 Hz, OCH$_2$C*H*$_2$CH$_2$Br), 2.76 (s, 3H, C*H*$_3$), 3.69 (t, 2H, *J* = 6.5 Hz, OCH$_2$CH$_2$C*H*$_2$Br), 4.10 (t, 2H, *J* = 6.0 Hz, 2H,

OC*H*$_2$CH$_2$CH$_2$Br), 6.99 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.31 (t, 1H, *J* = 7.3 Hz, Ar-*H*), 7.51-7.56 (m, 4H, Ar-*H*), 8.18 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.36 (s, 1H, 6-H), 8.73 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 14.8, 31.3, 31.9, 65.5, 101.1, 114.3, 120.5, 125.6, 125.8, 129.1, 131.5, 138.7, 142.4, 154.1, 155.4, 155.8, 155.9; DEPT-^{13}C NMR (DMSO-d_6 δ ppm) 14.8, 31.3 (inverted), 31.9 (inverted), 65.5 (inverted), 114.3, 120.5, 125.6, 125.8, 129.1, 155.9. HRESI-MS m/z calcd for $C_{21}H_{20}BrN_5O$: 438.0924 $[M+H]^+$, 440.0904 $[M+H+2]^+$, found: 438.0914 $[M+H]^+$.

4.1.6.6. N-(4-(4-Bromobutoxy)phenyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6f)

White solid; yield 53%; mp126-127 ^{o}C; IR (KBr, cm^{-1}): 3434 (NH), 1611 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.81-1.86 (m, 2H, OCH$_2$CH$_2$C*H*$_2$CH$_2$Br), 1.94-2.01 (m, 2H, OCH$_2$C*H*$_2$CH$_2$CH$_2$Br), 2.75 (s, 3H, C*H*$_3$), 3.61 (t, 2H, *J* = 6.6 Hz, OCH$_2$CH$_2$CH$_2$C*H*$_2$Br), 4.01 (t, 2H, *J* = 6.3 Hz, OC*H*$_2$CH$_2$CH$_2$CH$_2$Br), 6.96 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.31 (t, 1H, *J* = 8.0 Hz, Ar-*H*), 7.50-7.55 (m, 4H, Ar-*H*), 8.18 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.36 (s, 1H, 6-H), 8.71 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 15.3, 27.9, 29.6, 35.3, 67.2, 101.6, 114.7, 120.9, 125.9, 126.2, 129.5, 131.7, 139.2, 142.8, 154.5, 156.1, 156.3, 156.4; HRESI-MS m/z calcd for $C_{22}H_{23}BrN_5O$: 452.1080 $[M+H]^+$, found: 452.1067.

4.1.6.7. 1-(4-Bromophenyl)-N-(4-(3-bromopropoxy)phenyl)-3-methyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6g)

White solid; yield 23%; mp 176-177 ^{o}C; IR (KBr, cm^{-1}): 3420 (NH), 1608 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.24-2.30 (m, 2H, OCH$_2$C*H*$_2$CH$_2$Br), 2.75 (s, 3H, C*H*$_3$), 3.70 (t, 2H, *J* = 6.4 Hz, OCH$_2$CH$_2$C*H*$_2$Br), 4.11 (t, 2H, *J* = 5.8 Hz, OC*H*$_2$CH$_2$CH$_2$Br), 7.00 (d, 2H, *J* = 8.8 Hz, 2H, Ar-*H*), 7.54 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 7.73 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 8.21 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.38 (s, 1H, 6-H), 8.79 (s, NH); Anal Calcd. for $C_{21}H_{19}Br_2N_5O$ (517.22): C, 48.77; H, 3.70; N, 13.54; Found: C, 48.94; H, 3.36; N, 13.72.

4.1.6.8. N-(4-(4-Bromobutoxy)phenyl)-1-(4-bromophenyl)-3-methyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6h)

White solid; yield 38%; mp 174-175 °C; IR (KBr, cm^{-1}): 3435 (NH), 1609 (C=N); ^1H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.82-1.89 (m, 2H, -$OCH_2CH_2CH_2CH_2Br$), 1.96-2.03 (m, 2H, -$OCH_2CH_2CH_2CH_2Br$), 2.75 (s, 3H, CH_3), 3.63 (t, 2H, J = 6.6 Hz, $OCH_2CH_2CH_2Br$), 4.02 (t, 2H, J = 6.2 Hz, $OCH_2CH_2CH_2Br$), 6.97 (d, 2H, J = 8.8 Hz, Ar-*H*), 7.52 (d, 2H, J = 8.4 Hz, Ar-*H*), 7.72 (d, 2H, J = 8.4 Hz, Ar-*H*), 8.21 (d, 2H, J = 8.8 Hz, Ar-*H*), 8.38 (s, 1H, 6-H), 8.78 (s, 1H, NH); Anal Calcd. for $C_{22}H_{21}Br_2N_5O$ (531.24): C, 49.74; H, 3.98; N, 13.18; Found: C, 50.00; H, 4.11; N, 12.88.

4.1.6.9. N-(4-(3-Bromopropoxy)phenyl)-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6i)

White solid; yield 17%; mp 209-210 °C; IR (KBr, cm^{-1}): 3432 (NH), 1583 (C=N); ^1H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.23-2.29 (m, 2H, $OCH_2CH_2CH_2Br$), 2.53 (s, 3H, CH_3), 3.69 (t, 2H, J = 6.0 Hz, $OCH_2CH_2CH_2Br$), 4.10 (t, 2H, J = 5.9 Hz, $OCH_2CH_2CH_2Br$), 7.01 (d, 2H, J = 8.0 Hz, C_6H_4), 7.34 (t, 1H, J=7.4 Hz, C_6H_5), 7.56 (t, 2H, J = 8.0 Hz, C_6H_5), 7.72 (s, 2H, Ar-H), 8.21 (d, 2H, J = 8.0 Hz, Ar-*H*), 8.40 (s, 1H, C3-H); 9.97 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 26.3, 31.3, 31.9, 65.5, 109.5, 114.7, 120.7, 126.1, 126.6, 129.1, 129.6, 133.6, 134.1, 138.9, 139.4, 154.2, 165.5; Anal Calcd. for $C_{21}H_{20}BrN_5O$ (438.32): C, 57.54; H, 4.60; N, 15.98; Found: C, 57.33; H, 4.47; N, 15.81.

4.1.6.10. N-(4-(4-Bromobutoxy)phenyl)-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6j)

White solid; yield 39%; mp 196-197 °C; IR (KBr, cm^{-1}): 3436 (NH), 1581 (C=N); ^1H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.83-1.88 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 1.95-2.02 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 2.53 (s, 3H, CH_3), 3.62 (t, 2H, J = 6.6 Hz, $OCH_2CH_2CH_2CH_2Br$), 4.02 (t, 2H, J = 6.2 Hz, $OCH_2CH_2CH_2CH_2Br$), 6.99 (d, 2H, J = 8.0 Hz, Ar-*H*), 7.34 (t, 1H, J = 8.0 Hz, Ar-*H*), 7.55 (t, 2H, J = 7.9 Hz, Ar-*H*), 7.70 (s, 2H, Ar-*H*), 8.21 (d, 2H, J = 8.0 Hz, Ar-*H*), 8.40 (s, 1H, 3-H), 9.95 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 26.3, 27.4, 29.1, 34.9, 66.8, 114.6, 120.7, 126.1, 129.1, 133.6, 138.9, 143.1, 143.4, 149.6, 151.9, 152.5, 155.5, 156.8, 165.9; Anal Calcd.

for $C_{22}H_{22}BrN_5O$ (452.35): C, 58.41; H, 4.90; N, 15.48; Found: C, 58.70; H, 4.61; N, 15.22.

4.1.6.11. 1-(4-bromophenyl)-N-(4-(3-bromopropoxy)phenyl)-6-methyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6k)

White solid; yield 37%; mp 202-203 ^{o}C; IR (KBr, cm^{-1}): 3429 (NH), 1581 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.24-2.30 (m, , 2H, $OCH_2CH_2CH_2Br$), 2.54 (s, 3H, CH_3), 3.70 (t, 2H, J = 6.4 Hz, $OCH_2CH_2CH_2Br$), 4.11 (t, 2H, J = 6.0 Hz, $OCH_2CH_2CH_2Br$), 7.02 (d, 2H, J = 8.4 Hz, Ar-*H*), 7.75 (dd, 4H, J = 8.2 Hz, Ar-*H*), 8.23 (d, 2H, J = 8.4 Hz, Ar-*H*), 8.40 (s, 1H, 3-H), 10.03 (s, 1H, NH); Anal Calcd. for $C_{21}H_{19}Br_2N_5O$ (517.22): C, 48.77; H, 3.70; N, 13.54; Found: C, 49.10; H, 4.01; N, 13.61.

4.1.6.12. N-(4-(4-Bromobutoxy)phenyl)-1-(4-bromophenyl)-6-methyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6l)

White solid; yield 38%; mp 174-175 ^{o}C; IR (KBr, cm^{-1}): 3431 (NH), 1580 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.82-1.89 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 1.96-2.03 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 2.54 (s, 3H, CH_3), 3.63 (t, 2H, J = 6.6 Hz, $OCH_2CH_2CH_2Br$), 4.03 (t, 2H, J = 6.2 Hz, $OCH_2CH_2CH_2Br$), 7.00 (d, 2H, J = 8.8 Hz, Ar-*H*), 7.76 (dd, 4H, J = 8.4 Hz, Ar-*H*), 8.23 (d, 2H, J = 8.8 Hz, Ar-*H*), 8.50 (s, 1H, 3-H), 10.01 (s, 1H, NH, exchangeable); Anal Calcd. for $C_{22}H_{21}BrN_5O$ (531.24): C, 49.74; H, 3.98; N, 13.18; Found: C, 51.02; H, 4.02; N, 13.48.

4.1.6.13. N-(4-(3-Bromopropoxy)phenyl)-3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6m)

White solid; yield 35%; mp 138-139 ^{o}C; IR (KBr, cm^{-1}): 3445 (NH), 1614 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.23-2.30 (m, 2H, $OCH_2CH_2CH_2Br$), 2.46 (s, 3H, 6-CH_3), 2.71 (s, 3H, 3-CH_3), 3.69 (t, 2H, J = 6.6 Hz, $OCH_2CH_2CH_2Br$), 4.10 (t, 2H, J = 6.0 Hz, $OCH_2CH_2CH_2Br$), 6.98 (d, 2H, J = 8.0 Hz, Ar-*H*), 7.30 (t, 1H, J = 8.0 Hz, Ar-*H*), 7.52 (t, 2H, J = 8.0 Hz, Ar-*H*), 7.60 (d, 2H, J = 9.0 Hz, Ar-*H*), 8.19 (d, 2H, J = 8.0 Hz, Ar-*H*), 8.57 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 14.8, 26.2, 31.3, 31.9, 65.5, 99.3, 114.3, 120.5, 125.0, 125.6, 129.0, 131.7, 138.9, 142.2, 155.2,

155.2, 165.1; Anal Calcd. for $C_{22}H_{22}BrN_5O$ (452.35): C, 58.41; H, 4.90; N, 15.48; Found: C, 58.75; H, 5.21; N, 15.36.

4.1.6.14. N-(4-(4-Bromobutoxy)phenyl)-3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6n)

White solid; yield 36%; mp 138-139 ^{o}C; IR (KBr, cm^{-1}): 3446 (NH), 1617 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.82-1.89 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 1.95-2.02 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 2.47 (s, 3H, 6-CH_3), 2.69 (s, 3H, 3-CH_3), 3.62 (t, 2H, J = 6.6 Hz, $OCH_2CH_2CH_2Br$), 4.03 (t, 2H, J = 6.2 Hz, $OCH_2CH_2CH_2Br$), 6.98 (d, 2H, J = 8.0 Hz, Ar-*H*), 7.31 (t, 1H, J = 8.0 Hz, Ar-*H*), 7.51-7.59 (m, 4H, Ar-*H*), 8.16 (d, 2H, J = 8.0 Hz, Ar-*H*), 8.79 (bs, 1H, NH);^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 15.3, 28.0, 29.6, 33.6, 67.4, 101.0, 110.2, 115.2, 121.4, 121.6, 124.1, 126.4, 129.2, 129.3, 131.1, 138.0, 155.0, 155.1; Anal Calcd. for $C_{23}H_{24}BrN_5O$ (466.37): C, 59.23; H, 5.19; N, 15.02; Found: C, 59.26; H, 5.27; N, 15.17.

4.1.6.15. 1-(4-Bromophenyl)-N-(4-(3-bromopropoxy)phenyl)-3,6-dimethyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6o)

White solid; yield 32%; mp 138-139 ^{o}C; IR (KBr, cm^{-1}): 3443 (NH), 1608 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.24-2.30 (m, 2H, $OCH_2CH_2CH_2Br$), 2.47 (s, 3H, 6-CH_3), 2.71 (s, 3H, 3-CH_3), 3.70 (t, 2H, J = 6.6 Hz, $OCH_2CH_2CH_2Br$), 4.10 (t, 2H, J = 5.8 Hz, $OCH_2CH_2CH_2Br$), 6.99 (d, 2H, J = 8.4 Hz, Ar-*H*), 7.59 (d, 2H, J = 8.8 Hz, Ar-*H*), 7.72 (d, 2H, J = 8.8 Hz, Ar-*H*), 8.21 (d, 2H, J = 8.4 Hz, Ar-*H*), 8.62 (s, 1H, NH); Anal Calcd. for $C_{22}H_{21}Br_2N_5O$ (531.24): C, 49.74; H, 3.98; N, 13.18; Found: C, 49.86; H, 4.05; N, 13.32.

4.1.6.16. N-(4-(4-Bromobutoxy)phenyl)-1-(4-bromophenyl)-3,6-dimethyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (6p)

White solid; yield 30%; mp 148-147 ^{o}C; IR (KBr, cm^{-1}): 3438 (NH), 1614 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.82-1.89 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 1.96-2.03 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 2.46 (s, 3H, 6-CH_3), 2.70 (s, 3H, 3-CH_3), 3.63 (t, 2H, J = 6.6 Hz, $OCH_2CH_2CH_2CH_2Br$), 4.02 (t, 2H, J = 6.2 Hz, $OCH_2CH_2CH_2CH_2Br$),

6.97 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 7.57 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.72 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.21 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 8.61 (s, 1H, NH); Anal Calcd. for $C_{23}H_{23}Br_2N_5O$ (545.27): C, 50.66; H, 4.25; N, 12.84; Found: C, 50.36; H, 4.19; N, 13.06.

4.1.7. General procedure for preparation of N-(4-(4-bromobutoxy)phenyl)-1-(4-bromophenyl)-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (13a,b)

To a solution of the appropriate compounds **12a,b** (0.001 mol) in absolute ethanol (15 mL), 1,4-dibromobutane (1.079 g, 0.005 mol), and sodium hydroxide (0.040 g, 0.001 mol) were added and the reaction mixture was heated under reflux for 15 min. then another 0.001 mol of sodium hydroxide was added and the reflux was continued to another 30 min. until the starting material was consumed. The reaction mixture was cooled, filtered and washed with ethanol (3x5 mL) and n-hexane (3x5 mL). The isolated product was then ercrystallized from acetonitrile and further purification was achieved by flash chromatography on silica gel (benzene).[48-50]

4.1.7.1. N-(4-(4-bromobutoxy)phenyl)-1-(4-bromophenyl)-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (13a)

White solid; yield 83%; mp 154-155 ^{o}C; IR (KBr, cm^{-1}): 3387 (NH), 1612 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.84-1.87 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 1.97-2.01 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 2.75 (s, 3H, SCH_3), 3.63 (t, 2H, *J* = 6.6 Hz, $OCH_2CH_2CH_2CH_2Br$), 4.03 (t, 2H, *J* = 6.4 Hz, $OCH_2CH_2CH_2CH_2Br$), 6.97 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.55 (d, 2H, *J* = 9.2 Hz, Ar-*H*), 7.75 (d, 2H, *J* = 9.2 Hz, Ar-*H*), 8.20 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.44 (s, 1H, 6-H), 8.55 (s, 1H, NH); Anal Calcd. for $C_{22}H_{21}Br_2N_5OS$ (563.31): C, 46.91; H, 3.76; N, 12.43; Found: C, 47.08; H, 3.80; N, 12.67.

4.1.7.2. N-(4-(4-bromobutoxy)phenyl)-1-(4-bromophenyl)-6-methyl-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (13b)

White solid; yield 49%; mp 148-149 ^{o}C; IR (KBr, cm^{-1}): 3336 (NH), 1620 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.82-1.89 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 1.94-2.02 (m, 2H, $OCH_2CH_2CH_2CH_2Br$), 2.51 (s, 3H, 6-CH_3), 2.73 (s, 3H, SCH_3), 3.63

(t, 2H, *J* = 6.6 Hz, $OCH_2CH_2CH_2CH_2Br$), 4.03 (t, 2H, *J* = 6.2 Hz, $OCH_2CH_2CH_2CH_2Br$), 6.97 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.60 (d, 2H, *J* = 9.2 Hz, Ar-*H*), 7.76 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.21 (d, 2H, *J* = 9.2 Hz, Ar-*H*), 8.41 (s, 1H, NH, exchangeable); Anal Calcd. for $C_{23}H_{23}Br_2N_5OS$ (577.33): C, 47.85; H, 4.02; N, 12.13; Found: C, 48.01; H, 4.08; N, 12.40.

4.1.8. General procedure for preparation of 4-(1-aryl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)alkyl nitrate (7a-p, 14a,b)

A solution of the appropriate bromoalkyl derivatives **6a-p** or **13a,b** (0.001 mol) in acetonitrile (5 mL) was treated portion wise with a solution of $AgNO_3$ (0.339 g, 0.002 mol) in acetonitrile (5 mL) and the resulting mixture was heated under reflux for 8 h. the reaction mixture was then filtered, evaporated to dryness to yield the crude product which was purified by flash chromatography on silica gel (n-hexane/AcOEt 9:1 for **7a-p** and benzene for **14a,b**).[20, 50-51]

4.1.8.1. 3-(4-((1-Phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)propyl nitrate (7a)

White solid; yield 50%; mp 176-177 °C; IR (KBr, cm^{-1}): 3431(NH), 1638 (C=N); ^1H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.16 (p, 2H, *J* = 6.3 Hz, $OCH_2CH_2CH_2ONO_2$), 4.09 (t, 2H, *J* = 6.1 Hz, $OCH_2CH_2CH_2ONO_2$), 4.71 (t, 2H, *J* = 6.4 Hz, $OCH_2CH_2CH_2ONO_2$), 7.00 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.36 (t, 1H, *J* = 7.4 Hz, Ar-*H*), 7.56 (t, 2H, *J* = 7.8 Hz, Ar-*H*), 7.70 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.20 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.47 (s, 1H, 6-H), 8.50 (s, 1H, 3-H), 10.11 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 26.3, 64.2, 71.0, 102.5, 115.1, 121.2, 126.7, 129.6, 132.3, 134.2, 139.2, 145.7, 153.5, 156.4, 156.6, 158.5; HRESI-MS m/z calcd for $[M+H]^+$ $C_{20}H_{19}N_6O_4$: 407.1462, found: 407.1448.

4.1.8.2. 4-(4-((1-Phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)butyl nitrate (7b)

Yellow solid; yield 81%; mp 171-172 °C; IR (KBr, cm^{-1}): 3432 (NH), 1620 (C=N); ^1H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.80–1.87 (m, 4H, $OCH_2CH_2CH_2CH_2ONO_2$),

4.03 (t, 2H, *J* = 6.0 Hz, OC*H*$_2$CH$_2$CH$_2$CH$_2$ONO$_2$), 4.61 (t, 2H, *J* = 6.0 Hz, OCH$_2$CH$_2$CH$_2$C*H*$_2$ONO$_2$), 6.99 (d, 2H, *J* = 8.0 Hz, Ar-*H*); 7.37 (t, 1H, *J* = 8.0 Hz, Ar-*H*), 7.56 (t, 2H, *J* = 8.0 Hz, Ar-*H*), 7.68 (s, 2H, Ar-*H*), 8.20 (d, 2H, *J* = 8.0 Hz, Ar-*H*); 8.47 (s, 1H, 6-H), 8.50 (s, 1H, 3-H); 10.11 (s, 1H, NH); HRESI-MS m/z calcd for $[M+H]^+$ $C_{21}H_{21}N_6O_4$: 421.1619, found: 421.1604.

4.1.8.3. 3-(4-((1-(4-Bromophenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)propyl nitrate (7c)

White solid; yield 68%; mp 200-201 ^oC; IR (KBr, cm^{-1}): 3435 (NH), 1632 (C=N); ^1H NMR (300 MHz, DMSO-d_6, δ = ppm) δ = 2.14-2.18 (m, 2H, OCH$_2$C*H*$_2$CH$_2$ONO$_2$), 4.09 (t, 2H, *J* = 6.0 Hz, OC*H*$_2$CH$_2$CH$_2$ONO$_2$), 4.71 (t, 2H, *J* = 6.3 Hz, OCH$_2$CH$_2$C*H*$_2$ONO$_2$), 7.00 (d, 2H, *J* = 9.0 Hz, Ar-*H*), 7.68 (d, 2H, *J* = 9.0 Hz, Ar-*H*), 7.75 (d, 2H, *J* = 9.0 Hz, Ar-*H*), 8.22 (d, 2H, *J* = 9.0 Hz, Ar-*H*), 8.48 (s, 2H, 6-H & 3-H), 10.12 (s, 1H, NH, exchangeable); Anal Calcd. For $C_{20}H_{17}BrN_6O_4$ (485.29): C, 49.50; H, 3.53; N, 17.32; Found: C, 49.17; H, 3.56; N, 17.26;

4.1.8.4. 4-(4-((1-(4-Bromophenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)butyl nitrate (7d)

White solid; yield 30%; mp 176-177 ^oC; IR (KBr, cm^{-1}): 3427 (NH), 1622 (C=N); ^1H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.82-1.86 (m, 4H, OCH$_2$C*H*$_2$C*H*$_2$CH$_2$ONO$_2$), 4.03 (t, 2H, *J* = 5.6 Hz, OC*H*$_2$CH$_2$CH$_2$ONO$_2$), 4.61 (t, 2H, *J* = 5.6 Hz, OCH$_2$CH$_2$C*H*$_2$ONO$_2$), 7.00 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.56-7.73 (m, 2H, Ar-*H*), 7.77 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.23 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.49 (s, 1H, 6-H), 8.55 (s, 1H, 3-H), 10.16 (s, 1H, NH); Anal Calcd. for $C_{21}H_{19}BrN_6O_4$ (499.32): C, 50.51; H, 3.84; N, 16.83; Found: C, 50.38; H, 3.54; N, 17.08.

4.1.8.5. 3-(4-((3-Methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)propyl nitrate (7e)

Yellow solid; yield 76%; mp 130-131 ^oC; IR (KBr, cm^{-1}): 3418 (NH), 1618 (C=N); ^1H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.16 (p, 2H, *J* = 6.3 Hz, OCH$_2$C*H*$_2$CH$_2$ONO$_2$), 2.76 (s, 3H, 3-C*H*$_3$), 4.09 (t, 2H, *J* = 6.1 Hz, OC*H*$_2$CH$_2$CH$_2$ONO$_2$), 4.71 (t, 2H, *J* = 6.4

Hz, $OCH_2CH_2CH_2ONO_2$), 6.98 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.31 (t, 1H, *J* = 7.4 Hz, Ar-*H*), 7.51-7.57 (m, 4H, Ar-*H*), 8.18 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.36 (s, 1H, 6-H), 8.73 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 14.8, 26.3, 64.2, 71.0, 101.1, 114.3, 120.5, 125.6, 125.8, 129.1, 131.5, 138.7, 142.4, 154.1, 155.3, 155.8, 155.9; HRESI-MS m/z calcd for $[M+H]^+$ $C_{21}H_{21}N_6O_4$: 421.1619, found: 421.1613.

4.1.8.6. 4-(4-((3-Methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)butyl nitrate (7f)

Yellow solid; yield 81%; mp 144-145 °C; IR (KBr, cm^{-1}): 3398 (NH), 1626 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.79-1.87 (m, 4H, $OCH_2CH_2CH_2CH_2ONO_2$), 2.75 (s, 3H, 3-CH_3), 4.02 (t, 2H, *J* = 6.0 Hz, $OCH_2CH_2CH_2CH_2ONO_2$), 4.60 (t, 2H, *J* = 6.0 Hz, $OCH_2CH_2CH_2CH_2ONO_2$), 6.96 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.31 (t, 1H, *J* = 8.0 Hz, Ar-*H*), 7.51-7.55 (m, 4H, Ar-*H*), 8.18 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.36 (s, 1H, 6-H), 8.72 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 14.8, 23.0, 25.0, 67.0, 73.6, 101.1, 114.2, 120.5, 125.6, 125.8, 129.1, 131.3, 138.7, 142.4, 154.1, 155.6, 155.8, 156.0; HRESI-MS m/z calcd for $[M+H]^+$ $C_{22}H_{23}N_6O_4$: 435.1775, found: 435.1767.

4.1.8.7. 3-(4-((1-(4-Bromophenyl)-3-methyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)propyl nitrate (7g)

Orange solid; yield 50%; mp 202-203 °C; IR (KBr, cm^{-1}): 3443 (NH), 1622 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.15-2.18 (m, 2H, $OCH_2CH_2CH_2ONO_2$), 2.76 (s, 3H, 3-CH_3), 4.09 (t, 2H, *J* = 6.2 Hz, $OCH_2CH_2CH_2ONO_2$), 4.72 (t, 2H, *J* = 6.4 Hz, $OCH_2CH_2CH_2ONO_2$), 6.99 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.54 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.73 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 8.21 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 8.39 (s, 1H, 6-H), 8.80 (s, 1H, NH); Anal Calcd. for $C_{21}H_{19}BrN_6O_4$ (499.32): C, 50.51; H, 3.84; N, 16.83; Found: C, 50.23; H, 3.88; N, 16.75.

4.1.8.8. 4-(4-((1-(4-Bromophenyl)-3-methyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)butyl nitrate (7h)

Yellow solid; yield 91%; mp 194-195 °C; IR (KBr, cm^{-1}): 3404 (NH), 1618 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.79-1.90 (m, 4H, $OCH_2CH_2CH_2CH_2ONO_2$),

2.75 (s, 3H, 3-C*H*$_3$), 4.03 (t, 2H, *J* = 5.4 Hz, OC*H*$_2$CH$_2$CH$_2$ONO$_2$), 4.61 (t, 2H, *J* = 5.8 Hz, OCH$_2$CH$_2$C*H*$_2$ONO$_2$), 6.97 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.52 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.73 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.21 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.38 (s, 1H, 6-H), 8.78 (s, 1H, NH); Anal Calcd. for $C_{22}H_{21}BrN_6O_4$ (513.34): C, 51.47; H, 4.12; N, 16.37; Found: C, 51.24; H, 3.81; N, 16.60.

4.1.8.9. 3-(4-((6-Methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)propyl nitrate (7i)

White solid; yield 49%; mp 180-181 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.16 (p, 2H, *J* = 6.3 Hz, OCH$_2$C*H*$_2$CH$_2$ONO$_2$), 2.53 (s, 3H, 6-C*H*$_3$), 4.09 (t, 2H, *J* = 6.1 Hz, OC*H*$_2$CH$_2$CH$_2$ONO$_2$), 4.71 (t, 2H, *J* = 6.4 Hz, OCH$_2$CH$_2$C*H*$_2$ONO$_2$), 7.00 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.34 (t, 1H, *J* = 8.0 Hz, Ar-*H*), 7.55 (t, 2H, *J* = 8.0 Hz, Ar-*H*), 7.72 (s, 2H, Ar-*H*), 8.20 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.40 (s, 1H, 3-H), 9.97 (s, 1H, NH); HRESI-MS m/z calcd for $[M+H]^+$ $C_{21}H_{21}N_6O_4$: 421.1619, found: 421.1605.

4.1.8.10. 4-(4-((6-Methyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)butyl nitrate (7j)

White solid; yield 79%; mp 160-161 °C; IR (KBr, cm^{-1}): 3436 (NH), 1627 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.80-1.87 (m, 4H, OCH$_2$C*H*$_2$C*H*$_2$CH$_2$ONO$_2$), 2.53 (s, 3H, 6-C*H*$_3$), 4.03 (t, 2H, *J* = 5.7 Hz, OC*H*$_2$CH$_2$CH$_2$CH$_2$ONO$_2$), 4.61 (t, 2H, *J* = 6.0 Hz, OCH$_2$CH$_2$CH$_2$C*H*$_2$ONO$_2$), 6.99 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.34 (t, 1H, *J* = 8.0 Hz, Ar-*H*), 7.55 (t, 2H, *J* = 8.0 Hz, Ar-*H*), 7.71 (s, 2H, Ar-*H*), 8.20 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.40 (s, 1H, 3-H), 9.96 (s, 1H, NH); HRESI-MS m/z calcd for $[M+H]^+$ $C_{22}H_{23}N_6O_4$: 435.1775, found: 435.1761.

4.1.8.11. 3-(4-((1-(4-Bromophenyl)-6-methyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)propyl nitrate (7k)

White solid; yield 86%; mp 202-203 °C; IR (KBr, cm^{-1}): 3423 (NH), 1581 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.14-2.20 (m, 2H, OCH$_2$C*H*$_2$CH$_2$ONO$_2$), 2.54 (s, 3H, 6-C*H*$_3$), 4.09 (t, 2H, *J* = 6.0 Hz, OC*H*$_2$CH$_2$CH$_2$ONO$_2$), 4.71 (t, 2H, *J* = 6.4 Hz, OCH$_2$CH$_2$C*H*$_2$ONO$_2$), 7.01 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.75 (dd, 4H, *J* = 8.8 Hz, Ar-*H*),

8.22 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.50 (bs, 1H, 3-H), 10.02 (s, 1H, NH); Anal Calcd. for $C_{21}H_{19}BrN_6O_4$ (499.32): C, 50.51; H, 3.84; N, 16.83. Found: C, 50.69; H, 4.02; N, 16.94.

4.1.8.12. 4-(4-((1-(4-Bromophenyl)-6-methyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)butyl nitrate (7l)

White solid; yield 77%; mp 214-215 °C; IR (KBr, cm^{-1}): 3423 (NH), 1628 (C=N); ^1H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.78-1.88 (m, 4H, $OCH_2CH_2CH_2CH_2ONO_2$), 2.54 (s, 3H, 6-CH_3), 4.03 (t, 2H, *J* = 5.2 Hz, $OCH_2CH_2CH_2ONO_2$), 4.61 (t, 2H, *J* = 5.4 Hz, $OCH_2CH_2CH_2ONO_2$), 6.99 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.76 (dd, 4H, *J* = 8.8 Hz, Ar-*H*), 8.22 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.50 (bs, 1H, 3-H), 10.02 (s, 1H, NH); Anal Calcd. for $C_{22}H_{21}BrN_6O_4$ (513.34): C, 51.47; H, 4.12; N, 16.37; Found: C, 51.60; H, 4.18; N, 16.30.

4.1.8.13. 3-(4-((3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)propyl nitrate (7m)

Orange solid; yield 85%; mp 112-113 °C; IR (KBr, cm^{-1}): 3441 (NH), 1622 (C=N); ^1H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.13-2.19 (m, 2H, $OCH_2CH_2CH_2ONO_2$), 2.46 (s, 3H, 6-CH_3), 2.71 (s, 3H, 3-CH_3), 4.09 (t, 2H, *J* = 6.0 Hz, $OCH_2CH_2CH_2ONO_2$), 4.71 (t, 2H, *J* = 6.4 Hz, $OCH_2CH_2CH_2ONO_2$), 6.97 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.30 (t, 1H, *J* = 8.0 Hz, Ar-*H*), 7.52 (t, 2H, *J* = 7.4 Hz, Ar-*H*), 7.60 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.19 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.57 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 14.78, 26.22, 26.34, 64.15, 71.03, 99.26, 114.23, 120.42, 124.93, 125.59, 129.02, 131.84, 138.91, 142.14, 155.03, 155.23, 155.33, 165.24; HRESI-MS m/z calcd for $[M+H]^+$ $C_{22}H_{23}N_6O_4$: 435.1775, found: 435.1761.

4.1.8.14. 4-(4-((3,6-Dimethyl-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)butyl nitrate (7n)

Yellow solid; yield 71%; mp 134-135 °C; ^1H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.79-1.87 (m, 4H, $OCH_2CH_2CH_2CH_2ONO_2$), 2.46 (s, 3H, 6-CH_3), 2.71 (s, 3H, 3-CH_3), 4.03 (t, 2H, *J*=5.7 Hz, OCH_2CH_2 $CH_2CH_2ONO_2$), 4.61 (t, 2H, *J* = 6.0 Hz, OCH_2CH_2 $CH_2CH_2ONO_2$), 6.96 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.30 (t, 1H, *J* = 7.3 Hz, Ar-*H*), 7.52 (t,

2H, *J* = 7.8 Hz, Ar-*H*), 7.59 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.19 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 8.57 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6, δ = ppm) δ = 14.8, 23.0,25.0, 26.2, 66.9, 73.6, 99.3, 114.2, 120.4, 124.9, 125.6, 129.0, 131.6, 138.9, 142.1, 155.2, 155.3, 155.4, 165.2; HRESI-MS m/z calcd for $[M+H]^+$ $C_{23}H_{25}N_6O_4$: 449.1932, found: 449.1917.

4.1.8.15. 3-(4-((1-(4-Bromophenyl)-3,6-dimethyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)propyl nitrate (7o)

Yellow solid; yield 88%; mp 143-144 ^{o}C; IR (KBr, cm^{-1}): 3428 (NH), 1611 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 2.13-2.19 (m, 2H, $OCH_2CH_2CH_2ONO_2$), 2.46 (s, 3H, 6-CH_3), 2.70 (s, 3H, 3-CH_3), 4.09 (t, 2H, *J* = 6.0 Hz, $OCH_2CH_2CH_2ONO_2$), 4.72 (t, 2H, *J* = 6.4 Hz, $OCH_2CH_2CH_2ONO_2$), 6.98 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.59 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 7.72 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 8.21 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.62 (s, 1H, NH); Anal Calcd. for $C_{22}H_{21}BrN_6O_4$ (513.34): C, 51.47; H, 4.12; N, 16.37; Found: C, 51.80; H, 3.92; N, 16.15.

4.1.8.16. 4-(4-((1-(4-Bromophenyl)-3,6-dimethyl-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)butyl nitrate (7p)

Orange solid; yield 90%; mp 128-129 ^{o}C; IR (KBr, cm^{-1}): 3437 (NH), 1623 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.79-1.88 (m, 4H, $OCH_2CH_2CH_2CH_2ONO_2$), 2.46 (s, 3H, 6-CH_3), 2.70 (s, 3H, 3-CH_3), 4.02 (t, 2H, *J* = 5.4 Hz, $OCH_2CH_2CH_2ONO_2$), 4.61 (t, 2H, *J* = 5.8 Hz, $OCH_2CH_2CH_2ONO_2$), 6.96 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.57 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.71 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.20 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.60 (s, 1H, NH); Anal Calcd. for $C_{23}H_{23}BrN_6O_4$ (527.37): C, 52.38; H, 4.40; N, 15.94; Found: C, 52.57; H, 4.45; N, 15.84.

4.1.8.17. 4-(4-((1-(4-bromophenyl)-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)butyl nitrate (14a)

Yellowish white solid; yield 70%; mp 152-153 ^{o}C; IR (KBr, cm^{-1}): 3367 (NH), 1635 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.80-1.87 (m, 4H, $OCH_2CH_2CH_2CH_2ONO_2$), 2.75 (s, 3H, SCH_3), 4.03 (t, 2H, *J* = 5.8 Hz, $OCH_2CH_2CH_2ONO_2$), 4.61 (t, 2H, *J* = 6.2 Hz, $OCH_2CH_2CH_2ONO_2$), 6.97 (d, 2H, *J* = 8.8

Hz, Ar-*H*), 7.55 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.77 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.21 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.45 (s, 1H, 6-H), 8.59 (s, 1H, NH, exchangeable); Anal Calcd. for $C_{22}H_{21}BrN_6O_4S$ (545.41): C, 48.45; H, 3.88; N, 15.41; Found: C, 48.72; H, 3.85; N, 15.72.

4.1.8.18. 4-(4-((1-(4-bromophenyl)-6-methyl-3-(methylthio)-1*H*-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)butyl nitrate (14b)

light yellow solid; yield 68%; mp 130-131 ^{o}C; IR (KBr, cm^{-1}): 3363 (NH), 1620 (C=N); ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.80-1.85 (m, 4H, $OCH_2CH_2CH_2CH_2ONO_2$), 2.49 (s, 3H, 3-CH_3), 2.72 (s, 3H, SCH_3), 4.02 (t, 2H, *J* = 5.6 Hz, $OCH_2CH_2CH_2ONO_2$), 4.61 (t, 2H, *J* = 6.2 Hz, $OCH_2CH_2CH_2ONO_2$), 6.96 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.59 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.74 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.20 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 8.39 (s, 1H, NH, exchangeable); Anal Calcd. for $C_{23}H_{23}BrN_6O_4S$ (559.44): C, 49.38; H, 4.14; N, 15.02; Found: C, 49.62; H, 4.21; N, 15.23.

4.1.9. General procedure for preparation of 1-Phenyl-N-(4-(pyrrolidin-1-yl)phenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (17)

To a solution of **15** (0.302 mol) in acetonitrile (15 mL), 1,4-dibromobutane (1.08 g, 0.005 mol), and anhydrous potassium carbonate (0.276 g, 0.002 mol) were added and the reaction mixture was heated under reflux for 10 h (the reaction was monitored using TLC until the starting materials is consumed in the reaction). To the reaction mixture, dichloromethane and water were added and the organic layer was washed with water, dried over Na_2SO_4 and the solvent was evaporated under reduced pressure to give a crude product which was recrystallized from acetonitrile – water to yield **17**.[48-50]

Yellowish white solid; yield 56%; mp 342-343 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6, δ = ppm) δ = 1.95-1.98 (m, 4H, $-CH_2CH_2CH_2CH_2-$), 3.23-3.27 (m, 4H, $-CH_2CH_2CH_2CH_2-$), 6.59 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 7.35 (t, 1H, *J* = 7.5 Hz, Ar-*H*), 7.52-7.58 (m, 4H, Ar-*H*), 8.19 (d, 2H, *J* = 8.4 Hz, Ar-*H*), 8.41 (s, 1H, Ar-*H*), 9.92 (s, 1H, NH, exchangeable); Anal Calcd. for $C_{21}H_{20}N_6$ (356.42): C, 70.77; H, 5.66; N, 23.58; Found: C, 70.94; H, 5.74; N, 23.81.

4.2. Nitric oxide release measurement

A solution of the appropriate compound (20 µL) in dimethylsulfoxide (DMSO) was added to 2 mL of 1:1 v/v mixture either of 50 mM phosphate buffer (pH 7.4) or of an HCl solution (pH 1) with MeOH, containing $5x10^{-4}$ ML-cysteine. The final concentration of drug was 10^{-4} M. After 1 h at 37 C°, 1 mL of the reaction mixture was treated with 250 µL of Griess reagent [sulfanilamide (4 g), N-naphthylethylenediamine dihydrochloride (0.2 g), 85% phosphoric acid (10 mL) in distilled water (final volume: 100 mL)]. After 10 min at room temperature, the absorbance was measured at 540 nm. Sodium nitrite standard solutions (10–80 nmol/mL) were used to construct the calibration curve. The results were expressed as the percentage of NO released (n= 2) relative to a theoretical maximum release of 1 mol NO/mol of test compound.[20, 52-53]

4.3. *In vitro* Cytotoxicity assay

4.3.1. NCI 60 cell lines

The methodology of the NCI anticancer screening has been described in detail elsewhere (http://www.dtp.nci.nih.gov).[44] Briefly, primary anticancer assay was performed at approximately sixty human tumor cell lines panel derived from nine neoplastic diseases, in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda. Tested compounds were added to the culture at a single concentration (10^{-5} M) and the cultures were incubated for 48 h. End point determinations were made with a protein binding dye, sulforhodamine B (SRB). Results for each tested compound were reported as the percent of growth of the treated cells when compared to the untreated control cells. The percentage growth was evaluated spectrophotometrically versus controls not treated with test agents. The cytotoxic and/or growth inhibitory effects of the most active selected compound were tested in vitro against the full panel of about 60 human tumor cell lines at 10-fold dilutions of five concentrations ranging from 10^{-4} to 10^{-8} M. A 48-h continuous drug exposure protocol was followed and an SRB protein assay was used to estimate cell viability or growth. Using the seven absorbance measurements [time zero (Tz), control growth in the absence of drug (C), and test growth in the presence of drug at the five concentration

levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as: [(Ti - TZ)/(C - TZ)] x100 for concentrations for which Ti > Tz, and [(Ti - TZ)/TZ] x100 for concentrations for which Ti < Tz.

4.3.2. HepG2 cell line

Potential cytotoxicity assay of the newly synthesized compounds, were evaluated in the South Dakota state University, Faculty of Science, Chemistry Department, USA. HepG2 cells seeded for 24 h in 96-well plates[54] were exposed to different concentrations of compounds. Five-fold serial dilution of compounds was carried out in the plate for 5 consecutive wells (final volume 100 mL) and incubated for 24 h using erlotinib as a reference drug. Negative (no cells, NC) and positive (no test chemicals, PC) controls were also considered for each plate to avoid interference of spectrophotometer reading from test chemicals.[55]

$$\text{Viability}(\%) = \frac{A_{\text{Sample}} - A_{\text{NC}}}{A_{\text{PC}} - A_{\text{NC}}} \cdot 100$$

4.3.3. DNA-flow cytometry analysis

HepG2 cells at a density of $3x10^5$ cells were exposed to 3 and 5 µM (IC50) of compounds 7h and 7p, respectively, or to DMSO (0.002%), as a control for 48 h. The cells were detached by trypsinization, washed in ice-cold PBS, harvested by centrifugation and then fixed in ice cold 70% ethanol. Subsequently, cells were washed in PBS and then stained with propidium iodide (50 µg/mL), RNase A (0.1 mg/mL) and 0.05% Triton X-100 (Sigma-Aldrich) for 40 min at 37 ^{o}C. Cell cycle distribution was determined using a FACSort flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) and analyzed using Cell Quest software (Becton Dickinson).[56]

4.4. Docking studies

This was done using OpenEye molecular Modeling software. A virtual library of structurally modified pyrazolo[3,4-d]pyrimidine derivatives were energy minimized using MMFF94 force field, followed by generation of multi-conformers using OMEGA

application. The whole energy minimized library will be docked along with the prepared EGFR (PDB ID: 1M17) using FRED application to generate a physical property (ΔG) reflecting the predicted energy profile of ligand-receptor complex. Vida application can be employed as a visualization tool to show the potential binding interactions of the ligands to the receptor of interest.[57-60]

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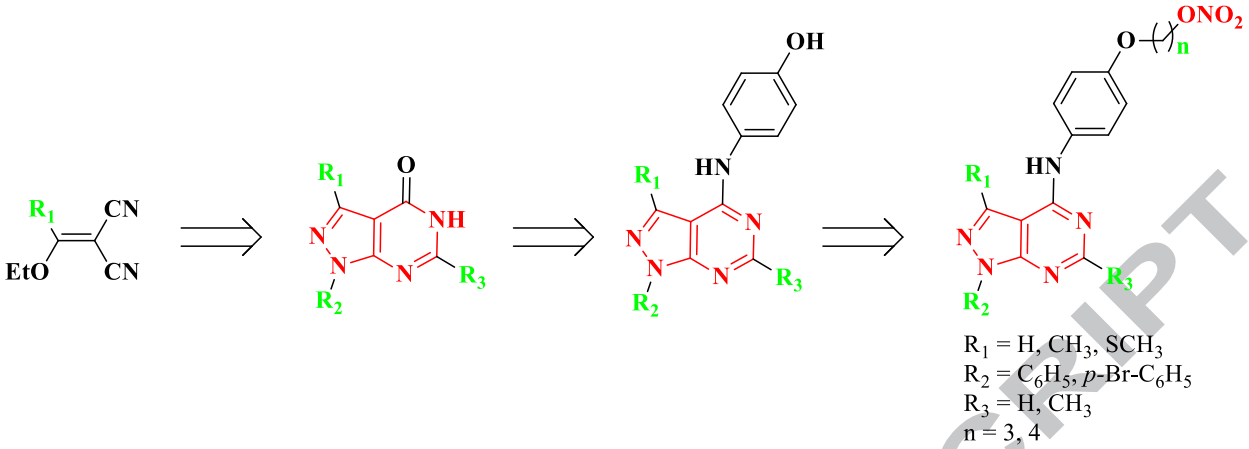

R_1 = H, CH_3, SCH_3
R_2 = C_6H_5, *p*-Br-C_6H_5
R_3 = H, CH_3
n = 3, 4