CHAPTER III

3. Design, Synthesis and Biological evaluation of benzofuran based pyrazolo-pyrimidine derivatives as PI3Kδ selective inhibitors.

Design strategy

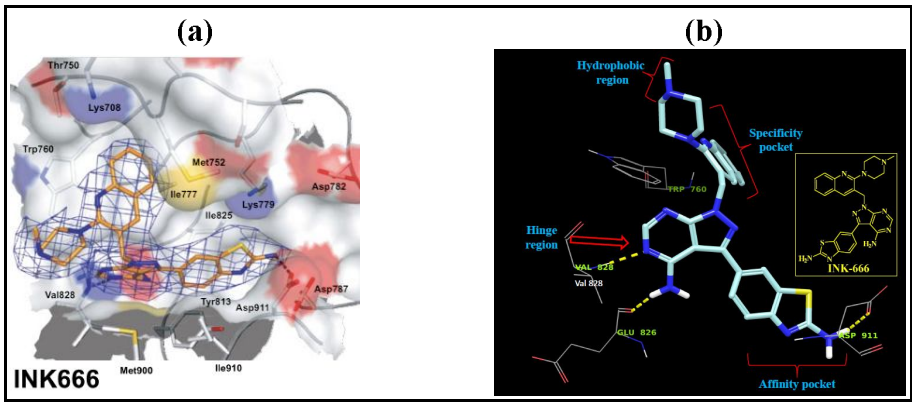


Figure 25: a) INK-666 co-crystal with PI3Kδ enzyme. b) Docking image of INK-666 (PDBID: **2WXK**)

There are many PI3Kδ inhibitors reported in literature, among them we selected Intellikine compounds (INK-666 and INK-654) as reference compound due to their selectivity and potent PI3Kδ inhibitory activity. Alex Bernd et al. has reported the crystal structure of PI3Kδ protein with INK654 and INK666 showing their selective inhibitory properties. In crystal structure of PI3Kδ enzyme, interaction of INK-654 and INK-666 at ATP binding site showed that *N*- methyl piperazine ring is projected towards hydrophobic region which form *H*-bonding with Asp_{832}, **Figure 25** (PDBID: **2WXK**). Pyrazolo pyrimidine ring interacts with Val_{828} and Glu_{826} through hydrogen bond in hinge region. Core quinoline ring sandwiched between Trp_{760} and Met_{752} in specificity pocket and benzthiazole ring projected deeper in the affinity pocket and form effective interaction with Asp_{787} and Asp_{911}. The SAR study of Intellikine

compounds demonstrated potent PI3Kδ inhibition with excellent selectivity in *in vitro*.

Unfortunately, INK-666/654 could not progress to clinic, although reasons are undisclosed, Umbralisib (**Ukoniq)** has structural similarity to INK-666/654 and it adopts propeller shape in PI3Kδ enzyme pocket. Recently it has have been approved (February 2021) for marginal zone lymphoma and follicular lymphoma treatment. In both compounds, pyrazolo pyrimidine ring serve as hinge binder and aryl substitution on pyrazolo pyrimidine ring interacts with affinity region residue. Thus, keeping these two ring systems constant which are essential for PI3K inhibitions, we have designed novel series in which 2,3- disubstituted quinoline ring of INK-666/INK-654 was bioisosterically replaced with benzofuran ring to get novel, potent and selective PI3Kδ inhibitors, **Figure 26.**

Benzofuran has great pharmacological applications, such as anti-inflammatory agents, anti-fungal agents, anti-cancer agents, anti-microbial agents etc. Initially we did *in silico* study using INK-666 crystal structure for benzofuran derivative and found encouraging results with all the key interactions in the hinge region (Val_{282}), specificity pocket (Trp_{760}), affinity pocket (Asp_{911}) and hydrophobic region, **Figure 26**, (PDBID: **2WXK**). Hence, we synthesized nineteen compounds to generate sustainable structure activity relationship (SAR). Hinge binder pyrazolo pyrimidine and benzofuran ring were kept constant, modifications were done on R (N-methyl-ethyl piperazine, morpholine) and R_1 (aryl and heteroaryl derivative). All compounds were evaluated for PI3Kδ inhibitory activity; selected compounds were further tested for PI3K isoform selectivity.

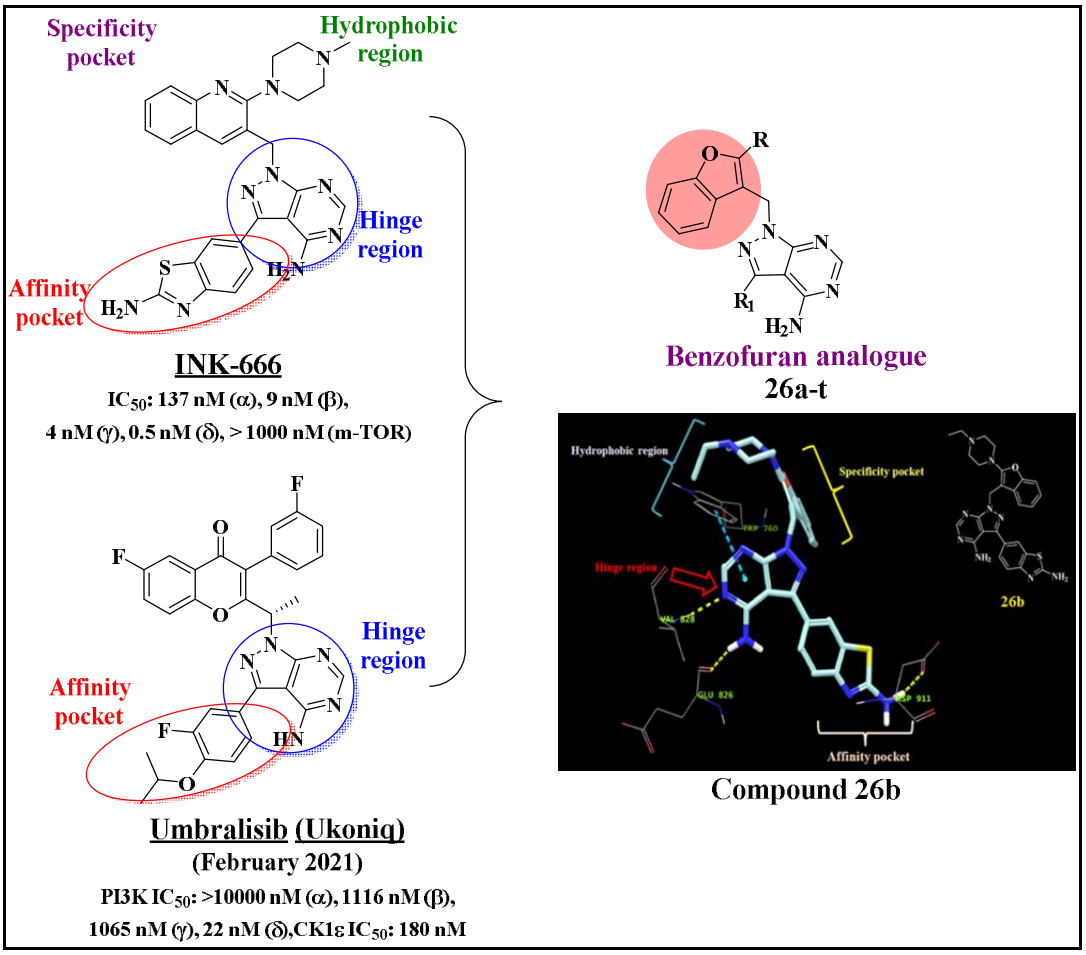


Figure 26: Designing strategy using benzofuran bioisoster (PDBID: **2WXK**)

3.1. Chemistry

3.1.1. Materials and Methods

All reagents used were obtained from Sigma Aldrich and were used without further purification. Solvents were purchased from a commercial source and used after distilling or drying according to the known methods. All the air and/or moisture sensitive reactions were carried out in dry solvents, under the nitrogen atmosphere. Melting points were recorded in open glass capillaries, using a scientific melting point apparatus (Mettler Toledo, Switzerland) and are uncorrected.

The ^{1}H NMR spectra were recorded on a Bruker Avance-400 (400MHz) spectrometer, Switzerland. The chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (tetramethylsilane), either in $CDCl_3$, CD_3OD or DMSO-d_6. Signal multiplicities are represented as s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), bs (broad singlet), and m (multiple). ^{13}C NMR spectra were recorded on Bruker Avance-400 at 100 MHz either in $CDCl_3$, CD_3OD or DMSO-d_6.

Mass spectra (ESI-MS) were obtained on Shimadzu LCMS 2010-A spectrometer, Japan. Elemental analyses were carried out, using a Perkin-Elmer 2400 CHN analyser, UK. UPLC analysis were carried out at λmax 220nm, using column YMC-Triart C18 (100*2.0 mm) on Water acquity UPLC, Europe (Austria).

Progress of the reactions was monitored by TLC, using precoated TLC plates (E. Merck Kiesegel 60 F254, Germany) and the spots were visualized by UV and/or iodine vapours. The chromatographic purification was performed on silica gel (230-400 mesh). Few compounds were directly used for the next step without purification and analysis.

In the next section, we highlighted some examples of bioisosteric replacement which encourages us to design novel PI3Kδ inhibitors by replacing quinoline ring with to benzofuran ring system, importance of benzofuran as a pharmacophore and possible routes for the synthesis of benzofuran.

3.1.2. Benzofuran in biological system

3.1.2.1. Examples of bioisosteric replacement

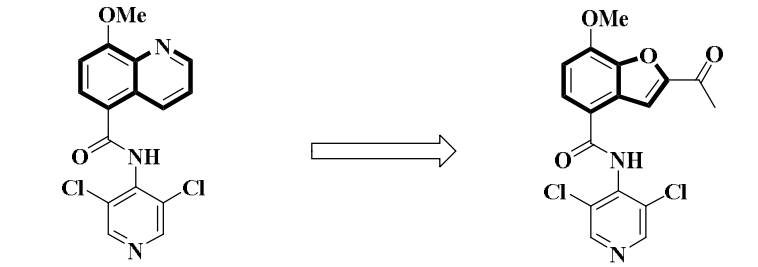
"Bioisoster are chemical substituents or groups with similar physical or chemical properties that have similar biological properties to a chemical compound" **[81]**.

The main purpose of exchanging one substituent with another is to;

- Increased the potency/selectivity of compound
- Reduced toxicity
- Reduced metabolism

We have listed some examples of quinoline ring replacement with benzofuran in brief.

Example1



A (Quinoline analogue)
PDE 4 inhibitors
IC_{50}: 0.17 μM

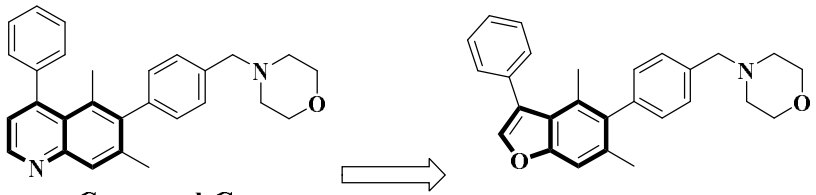
B (Furan analogue)
PDE 4 inhibitors
IC50: 0.001 mM

Figure 27: Benzofuran bioisoster of quinoline (PDE4 inhibitors)

Buckley et al. reported PDE4 (Phosphodiesterase-4) inhibitors for treatment of asthma. Compound **A.** (quinoline analogue) and compound **B.** (benzofuran analogue) were found to be potent PDE4 inhibitors with IC_{50}: 0.17

μM and 0.001 μM respectively. However, replacement of quinoline (compound **A**) with benzofuran (compound **B**) leads to 170 fold increase in potency, **Figure 27 [82-83]**.

Example 2



Compound C
Anti-tubercular
FadD32 inhibitor
MIC_{90}: 0.45 μM

Compound D
Anti-tubercular
FadD32 inhibitor
MIC_{90}: 2.5 μM

Figure 28: Benzofuran bioisoster of quinoline (FadD32 inhibitors)

Fang et al. reported many quinoline based compounds as FadD32 (Fatty acid degradation protein D32) inhibitors for treatment of tuberculosis. Compound **C** showed MIC_{90}: 0.45 μM, whereas compound **D** showed MIC_{90}: 2.5 μM. Thus, in this example there was drop in the *in vitro* potency (5.55 fold) due to other parameter such as solubility, permeability and metabolic stability **[84]**.

From above two examples it became obvious that, quinoline ring can be replaced with benzofuran.

3.1.2.2. Benzofuran as therapeutic agent

Benzofuran heterocycle is present in several synthetic and natural compounds which have various biological uses such as anti-parasitic, immune-suppressive, anti-inflammatory, anti-cancer, anti-viral, antioxidant and anti-fungal, **Figure 29**.

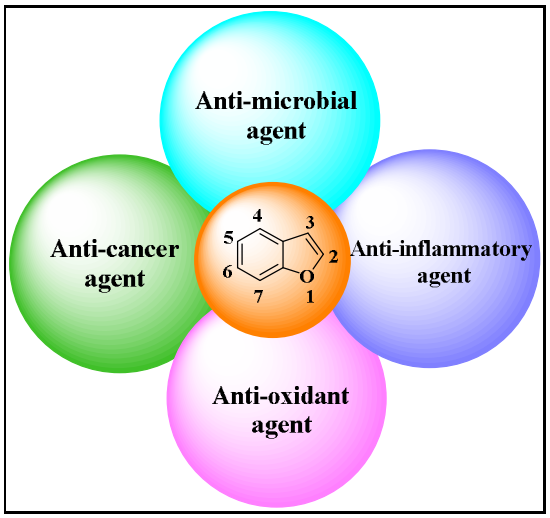
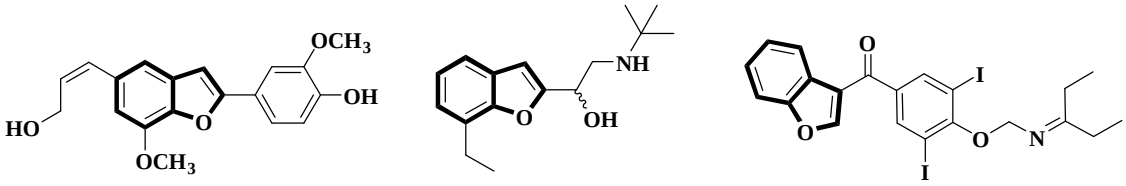


Figure 29: Benzofuran as therapeutic agent

The most well-known and important natural products containing benzofuran ring are Ailanthoidol, Bufuralol and Amiodarone. Ailanthoidol is used as antiviral, anticancer, antiproliferative, antioxidant, anti-inflammatory, antifungal and immuno-suppressive agents, Bufuralol is non-selective adrenoceptor blocking agent and Amiodarone is used as antiarrhythmic agent that mainly used to treat ventricular arrhythmias, **Figure 30**.



Ailanthoidol **Bufuralol** **Amiodarone**

Figure 30: Natural products containing benzofuran ring

There are many benzofuran derivatives used as an inhibition of N-Myristoyltransferase enzyme as anti-fungal agent. Ebiike et al. reported RO-09-4609; one of the best antifungal agent, **Figure 31 [85]**. Griseofulvin is an antifungal drug used to treat various types of dermatophytoses (ringworm).

Benzofuran-3-carbohydrazide derivative showed excellent anti-mycobacterial and anti-fungal activity against *Candida albicans.* Compounds (**E** and **F**) were found to be much potent antifungal agents with MIC value of 8 and 2 mg/mL respectively.

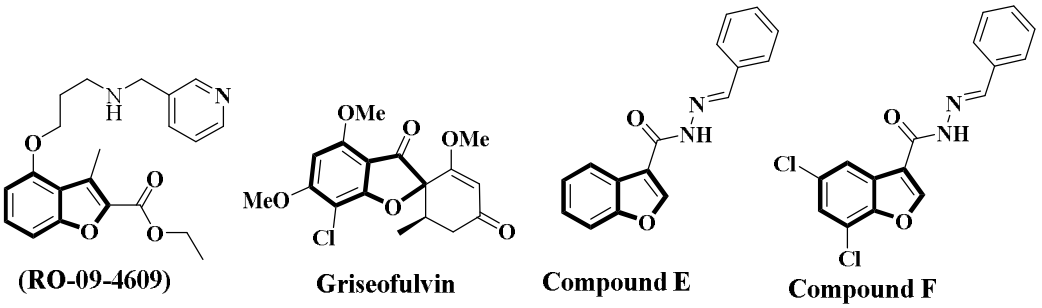


Figure 31: Benzofuran as antiparasitic and antifungal agents

Yadav et al. reported benzofuran derivatives as anti-inflammatory agent, mainly acts as COX (Cyclooxygenase) inhibitors. Among all compounds synthesizes, compound **G** showed excellent anti-inflammatory and COX-2 inhibitory activity with IC_{50} = 4.2μM. Compound **H** and Compound **I** were screened against the pro inflammatory cytokines TNF-α and Interleukin-6 (IL-6). Compounds **H** and **I** showed potent inhibition of pro inflammatory cytokine (% inhibition = 76-100% inhibition at 10μM concentration), **Figure 32 [86]**.

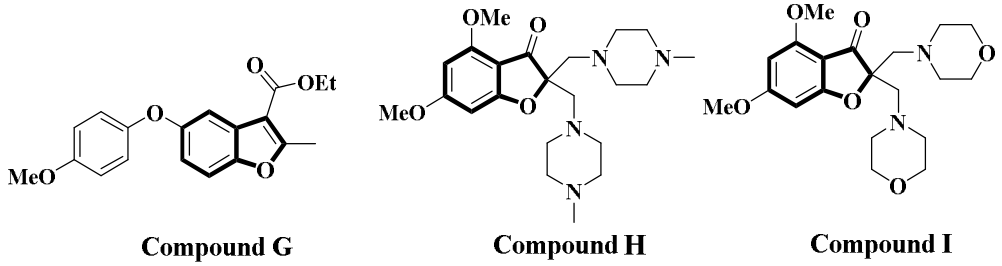


Figure 32: Benzofuran as anti-inflammatory agents

William Sellers et al. recently showed that the extension of dimethylamine and benzyl group of compound **J** by a 4-piperidinopiperidine (compound **K**) ring expressively enriched m-TOR inhibitory activity, **Figure 33 [87-88]**. Antczak et al. developed the benzofuran-4, 5-diones (compound **L**) as selective Human Peptide Deformylase inhibitor. In mouse xenograft model compound H showed excellent tumor suppression **[89]**.

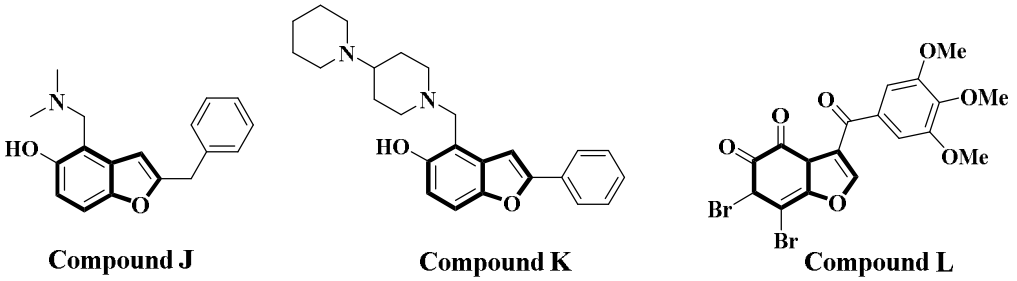


Figure 33: Benzofuran as anti-cancer agent

Compound **M** (67.1%) showed potent antioxidant properties in 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay **[90]**. Similarly Benzofuran-2-carbohydrazide (Compound **N**) and Benzofuran-2-oxadiazole (Compound **O**) derivatives showed potent antioxidant activity in DPPH radical scavenging assay (% inhibition; Compound **N** = 38.5%; Compound **O** = 35.24%), **Figure 34 [91]**.

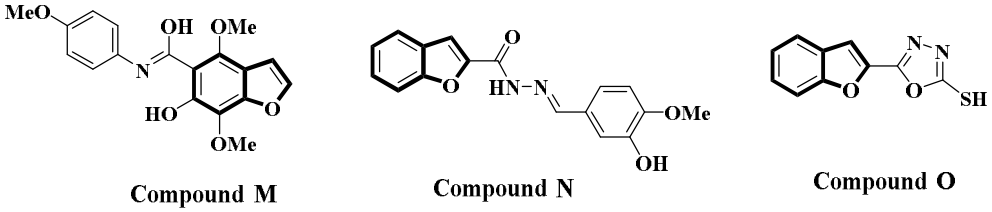


Figure 34: Benzofuran as anti-oxidant

Benzofuran and its derivatives are central pharmacophores and privileged skeleton in medicinal chemistry and have been featured in a number

of clinically used drugs. Benzofuran has diverse role in biological system thus it is considered as key heterocycle in drug designing by many medicinal chemists due to its clinical importance.

3.1.2.3. General method for preparation of benzofuran

Various synthetic routes are depicted in literature for synthesis of benzofuran **[92]**. Some of commonly used methods are listed below.

Perkin synthesized Benzofuran from 2H-chromen-2-one using bromine to give Dibromo-2H-chromen-2-one intermediate, which on hydrolysis using KOH, followed by Perkin rearrangement lead to the formation of benzofuran**, Figure 35 [93]**.

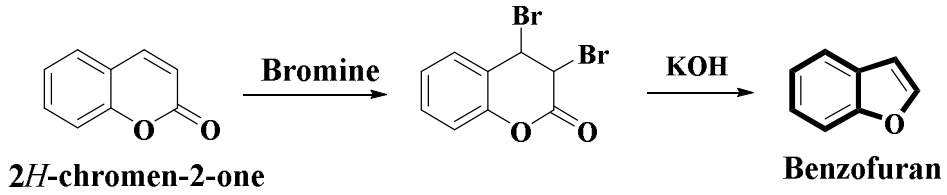


Figure 35: Perkin benzofuran synthesis

In another method, Salicylaldehyde was reacted with chloroacetic acid to form phenoxy acetic acid. This was cyclized using acetic anhydride and sodium acetate in acetic acid to give benzofuran, **Figure 36 [94]**.

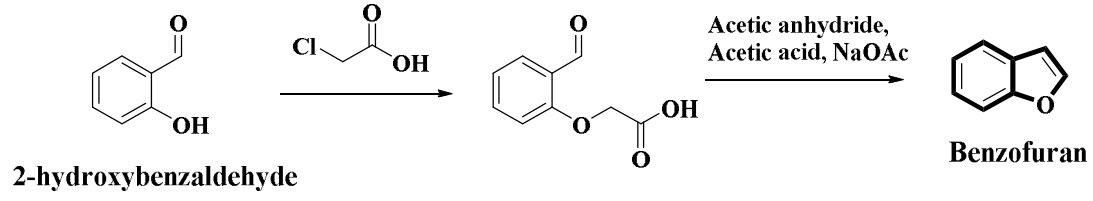
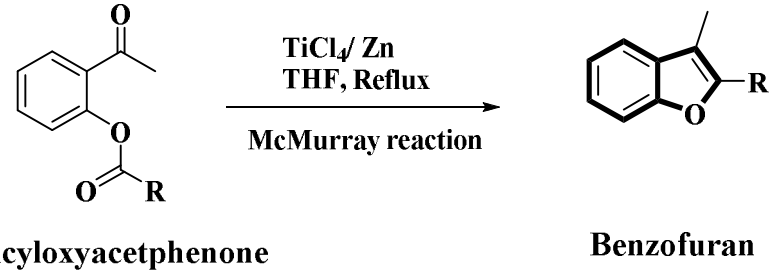


Figure 36: Benzofuran synthesis from salicylaldehyde

Alternately, 2-Acyloxy acetophenone can be subjected for intramolecular cyclization, using "McMurry reaction" to give 2-subistuted benzofuran, **Figure 37 [95]**.



2-Acyloxyacetphenone **Benzofuran**

Figure 37: Benzofuran synthesis through McMurry reaction

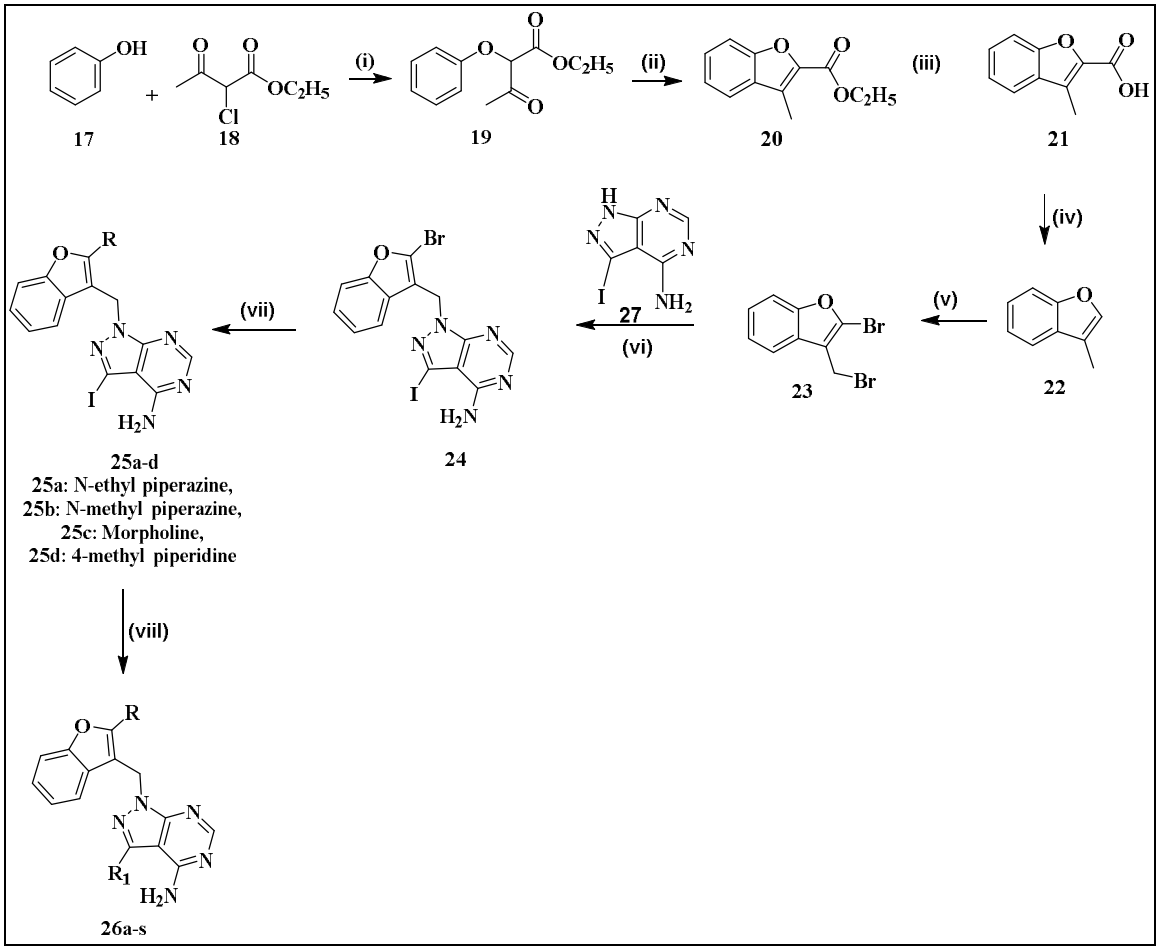
As describe in the designing section, in this chapter we designed benzofuran based PI3Kδ inhibitors by bioisosteric replacement of quinoline ring of INK-666/654. Total 19 compounds were designed and general procedure for the synthesis of **26a-s** is described below.

3.1.3. General procedure for the synthesis of title compounds 26a-s

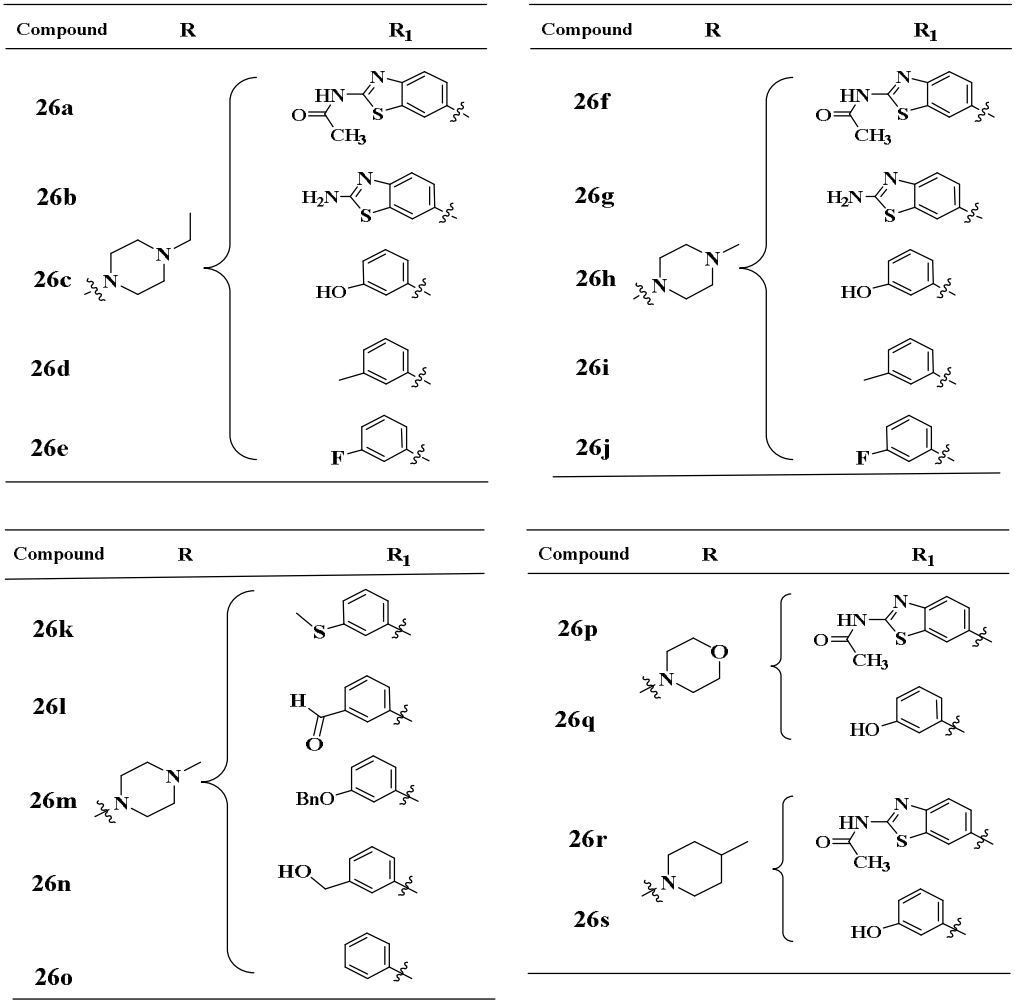
The compounds **26a-s** was synthesized as described in **Scheme 3**. Phenol **17** was reacted with ethyl 2-chloro-3-oxobutanoate to give phenol ether **19** which was cyclized using sulfuric acid by cyclodehydration mechanism into ethyl 3-methylbenzofuran-2-carboxylate **20**. Hydrolysis of ester **20** was carried out in KOH solution to give acid derivative **21**. Thermal decarboxylation of **21** gave 3-methylbenzofuran **22**, Free radical bromination at benzylic methyl was carried out in *N*-Bromosuccinimide (NBS) and catalytic benzoyl peroxide in carbon tetrachloride (CCl_4) to get dibromide benzyl benzofuran **23**, which was reacted with 3-iodo-*1H*-pyrazolo [3,4-*d*] pyrimidin-4-amine **27** to get iodo bromo compound **24**. Bromo compound **24** was reacted with *N*-substituted (R) piperazine (R= methyl or ethyl) by refluxing for 12 h. to get compound **25**. The

Iodo compound **25** was subjected to Suzuki coupling reactions with aryl, heteroaryl boronate ester or boronic acid as a preferred method to furnish compound **26a-s [96-97]**.

Scheme 3: Synthesis of benzofuran pyrazolo pyrimidine compounds **26a-s**



Reagents and conditions:(i) K_2CO_3, Acetone, Reflux, 5 hr; (ii) Conc.H_2SO_4, 0-5 ^oC, 4 hr; (iii) 10% KOH, 26 ^oC, 2 hr; (iv) 280^oC, 2 hr; (v) NBS, CCl_4, Reflux, 5 hr; (vi) t-BuOK, DMF, 26 ^oC, 15 hr; (vii) **R** (Secondary amine), (1,4)-Dioxane, Reflux, 12 hr; (viii) Aryl boronic acid (**R_1**), $PdCl_2$ $(PPh_3)_2$, $KHCO_3$, DMF: H_2O, 90 ^oC, 2 hr.



3.1.3.1. Detailed experimental procedure for the synthesis of benzofuran pyrazolo pyrimidine compounds 26a-s (Scheme 3)

Step I: Preparation of ethyl 3-oxo-2-phenoxybutanoate **19.**

Phenol (70 gm, 745 mmol) was dissolved in acetone (1200 ml). K_2CO_3 (154.21 gm, 1117.5 mmol) was added. Reaction mixture was stirred for 15 min at room temperature and ethyl 2-chloro-3-oxobutanoate (133.88 gm, 819.5 mmol) was slowly added. Reaction mixture was refluxed for 5 h. Reaction was cooled up to room temperature, filtered and washed with cold acetone. Filtrate was concentrated to give crude product, which was purified using column

chromatography using Ethyl acetate: n-Hexane mobile phase to give the title compound ethyl 3-oxo-2-phenoxybutanoate **19** as oil (88.9 g, Yield: 53.6 %). Purity: 96.50%

^{1}H NMR (CDCl-*d_1*, 400 MHz) δ ppm: 1.25-1.56 (m, 3H), 1.98 (s, 1H), 2.37 (s, 3H), 4.17-4.31 (m, 2H), 6.88-6.93 (m, 2H), 6.97-7.06 (m, 1H), 7.07-7.38 (m, 2H). **ESI-MS:** 223.3 $(M+H)^+$. **Purity** (UPLC): 95.50%.

Step II: Preparation of ethyl 3-methylbenzofuran-2-carboxylate **20.**

Ethyl 3-oxo-2-phenoxybutanoate **19 (**63 g, 283.78 mmol) was added in H_2SO_4 (63 ml) was added at -12 ^oC within 1 h. Reaction mixture was stirred for 1 h at room temperature. Ice cold water (100ml) was added to above solution and product was extracted using DCM (150ml). Organic layer is washed with sat. $NaHCO_3$ solution (2 X 50 ml) and concentrated to give ethyl 3-methylbenzofuran-2-carboxylate **20** as colourless oil**.** (38 g, 186.27 mmol, yield: 65 %). Purity= 98.78%

^{1}H NMR ($CDCl_3$-*d_1*, 400 MHz) δ ppm: 1.44 (t, *J* = 7.2 Hz, 3H), 2.59 (s, 3H), 4.45(q, *J* = 7.2 Hz, 2H), 7.02-7.32 (m, 1H), 7.42-7.46 (m, 1H), 7.54 (d, *J = 8 Hz*, 1H), 7.63 (d, *J = 8 Hz*, 1H). **ESI-MS:** 205.3 $(M+H)^+$.

Step III: Preparation of 3-methylbenzofuran-2-carboxylic acid **21.**

Ethyl 3-methylbenzofuran-2-carboxylate **20** (50 gm, 277.7 mmol) was suspended in 10% KOH solution (370 ml). Above suspension was heated up to 80 ^oC for 3h. Reaction mixture was cooled up to 0 ^oC and acidified using conc. HCl. Solid precipitate was filtered and washed with cold water and dried to give 3-methylbenzofuran-2-carboxylic acid **21** as white solid **(**45 gm**,** Yield: 92%). Purity:-99.50%

^{1}H NMR ($CDCl_3$-d_1, 400 MHz) δ ppm: 2.64 (s, 3H), 7.31-7.35 (m, 1H), 7.47-7.51 (m, 1H), 7.56-7.66 (m, 1H), 7.66-7.68 (m, 1H). **ESI-MS:** 174.8 $(M-H)^-$.

Step IV: Preparation of 3-methylbenzofuran **22.**

3-methylbenzofuran-2-carboxylic acid **(**45 gm, 255 mmol) was heated at 280 ^oC for 2 h. Solid melts to liquid to give crude product. The crude solid was extracted using DCM (2850 ml) and washed with Sat. $NaHCO_3$ (50 ml), concentrated to give 3-methylbenzofuran **22** as oil (30 g, Yield: 89%). Purity: 99.53%

^{1}H NMR ($CDCl_3$-d_1, 400 MHz) δ ppm: 2.24 (s, 3H), 7.21-7.30 (m, 2H), 7.39-7.40 (m, 1H), 7.44-7.46 (m, 1H), 7.51-7.54(m, 1H). **ESI-MS:** 133.20 $(M+H)^+$.

Step V: Preparation of 2-bromo-3-(bromomethyl) benzofuran **23.**

3-methylbenzofuran **22 (**14.5 g, 109.8 mmol) was dissolved in CCl_4 (200 ml). N-Bromosuccinimide (NBS) (39.10 gm, 219.6 mmol) and catalytic benzoyl peroxide (0.360 gm) were added to above solution. Reaction mixture was refluxed for 5 h. After completion of reaction the solution was cooled and solid was filtered. Solid was washed with CCl_4 (50 ml), combine filtrate was concentrated to give crude product, which was purified using column chromatography by using 0-50% Ethyl acetate: n-hexane mobile phase as solid 2-bromo-3-(bromomethyl) benzofuran **23** (7 g, Yield: 22%). Purity: 95.19%

^{1}H NMR (DMSO-d_6, 400 MHz) δ ppm: 4.55 (s, 2H), 7.29-7.33 (m, 2H), 7.42-7.47 (m, 1H), 7.61-7.64 (m, 1H). **ESI-MS:** 289.8 $[M]^+$, 291.8 $[M+2]^+$.

Step VI: Preparation of 1-((2-bromobenzofuran-3-yl)methyl)-3-iodo-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine **24.**

3-iodo-1*H*-pyrazolo [3,4-*d*] pyrimidin-4-amine **27** (6.26 gm, 24 mmol) was dissolved in DMF (80 ml), Potassium tert-butoxide (2.95 gm, 26.4 mmol) was added. Reaction mixture was stirred for 30 min at room temperature. 2-bromo-3-(bromomethyl) benzofuran **23** (7 gm**,** 24 mmol) dissolved in DMF (30 ml) was added to above suspension and reaction mixture was stirred for 18 hr at room temperature. Reaction mixture was quenched by adding cold water (250 ml) to give crude product as solid, which was filtered and washed with water. The crude solid was purified by column chromatography using 0-10% methanol: DCM mobile phase to give pure 1-((2-bromobenzofuran-3-yl)methyl)-3-iodo-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine **24** as off white solid (4 g, Yield: 35.46%). Purity: 96.57%

^{1}H NMR (DMSO-d_6, 400 MHz) δ ppm: 5.53 (s, 2H), 7.20-7.22 (m, 1H), 7.27-7.31 (m, 1H), 7.48 (d, *J* = 7.2 Hz, 1H), 7.58 (d, *J* = 8 Hz, 1H), 8.30 (s, 1H). **ESI-MS:** 471.8 $(M+2)^+$.

Step VII: Preparation of **25a-d**.

1-((2-bromobenzofuran-3-yl) methyl)-3-iodo-1H-pyrazolo [3, 4-d] pyrimidin-4-amine (24) (0.9 gm, 1.91 mmol) was suspended in secondary amine (15 ml) reaction mixture was refluxed for 18 h. After completion of reaction the solution was cooled and poured into ice cooled water (50 ml). Solid was filtered and washed with cold water (2 X 25 ml) to give pure products **(25a-d)** (Yield: 60-70%).

3.1.3.2. Spectral data of intermediate 25a-d

1-((2-(4-ethylpiperazin-1-yl)benzofuran-3-yl)methyl)-3-iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine 25a

25a was prepared following the general procedure described in section 3.1.3.1 (Step VII) as a white solid.

^{1}H NMR (DMSO-d_6, 400 MHz) δ ppm: 1.04 (t, J = 7.2 Hz, 3H), 2.36-2.42 (m, 2H), 3.42-3.45 (m, 4H), 5.53 (s, 2H), 7.06-7.08 (m, 2H), 7.32-7.39 (m, 2H), 8.30 (s, 1H), **ESI-MS**: 504.2 $(M+H)^+$. Purity: 97.08%

3-iodo-1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine 25b

25b was prepared following the general procedure described in section 3.1.3.1 (Step VII) as a white solid.

^{1}H NMR (MeOD-d_1, 400 MHz) δ ppm: 2.35 (s, 3H), 2.55-2.57 (m, 4H), 3.39-3.41 (m, 4H), 5.58 (s, 2H), 7.07-7.12 (m, 2H), 7.25-7.27 (m, 1 H), 7.40-7.42 (m, 1H), 8.27 (s, 1H). **ESI-MS**: 490.0 $(M+H)^+$. **Purity** (UPLC): 96.35%.

3-iodo-1-((2-morpholinobenzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (25c)

25c was prepared following the general procedure described in section 3.1.3.1 (Step VII) as a white solid.

^{1}H NMR (DMSO-d_6, 400 MHz) δ ppm: 3.39-3.41 (m, 4H), 3.70-3.85 (m, 4H), 5.54 (s, 2H), 7.09-7.13 (m, 2H), 7.31-7.39 (m, 2H), 8.30 (s, 1H), **ESI-MS**: 477.0 $(M+H)^+$. **Purity** (UPLC): 95.45%.

3-iodo-1-((2-(4-methylpiperidin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine 25d

25d was prepared following the general procedure described in section 3.1.3.1 (Step VII) as a white solid.

^{1}H NMR (DMSO-d_6, 400 MHz) δ ppm: 0.86-0.87 (m, 1H), 0.93-0.96 (m, 3H), 1.18-1.28 (m, 2H), 1.66-1.69 (m, 2H), 3.02-3.08 (m, 2H), 3.71-3.74 (m, 2H), 5.50 (s, 2H), 7.03-7.07 (m, 2H), 7.28-7.39 (m, 2H), 8.29 (s, 1H), **ESI-MS**: 489.1 $(M+H)^+$. **Purity** (UPLC): 96.30%.

Step VIII: Preparation of compound **26a-s**

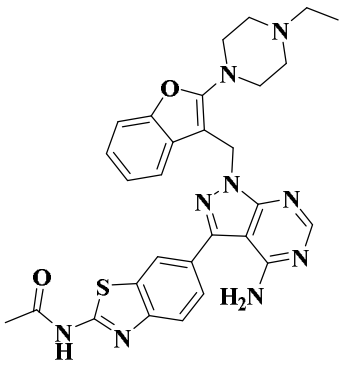
A mixture of (1.00 mmol) of **R** substituted iodo benzofuran (**25)** and (0.01 mmol) bistriphenylphosphine palladium dichloride [$PdCl_2$ $(PPh_3)_2$] in DMF (10 ml) was heated to 80-85 ^oC. To this mixture (1.00 mmol) Substituted boronic acid/ester (dissolved in 9 ml of DMF) and (6.00 mmol) potassium bicarbonate ($KHCO_3$) (dissolved in water 10 ml) were added. The mixture was heated for 2 h at 95 ^oC. The mixture was cooled to 0-5 ^oC; water (50ml) was added and stirred for 1h. The crude product was filtered and washed with water and dried. Crude product was purified using flash chromatography using MeOH: DCM gradient to give the titled compound. (Yield: 65-80 %).

3.1.3.3. Spectral data of final compounds 26a-s

All final compounds were characterised using ^{1}H NMR, ESI-MS, and CHN analysis, melting point are also reported (uncorrected). Most potent compounds **26c** and **26h** were fully characterised using ^{1}H NMR, ^{13}C NMR, ESI-MS and IR. Purity of all compounds was checked using UPLC (ultra-performance liquid chromatography).

N-(6-(4-amino-1-((2-(4-ethylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzo[d]thiazol-2-yl)acetamide (26a)

26a was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

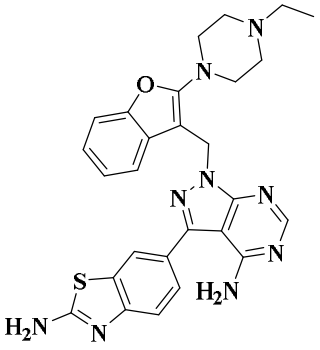


26a

MP: 217-218 ^{o}C, **Yield:** 60%. **^{1}H NMR** (DMSO-d_6, 400 MHz) δ ppm: 1.04 (s, 3H), 2.21 (s, 3H), 2.30-2.40 (m, 2H), 2.35-2.40 (m, 4H), 3.52 (s, 4H), 5.61 (s, 2H), 7.08-7.09 (m, 2H), 7.32-7.34 (d, J = 8 Hz, 1H), 7.49-7.51 (d, J = 8 Hz, 1H), 7.63-7.65 (m, 1H), 7.83-7.85 (d, J = 8 Hz, 1H), 8.19 (s, 1H), 8.39 (s, 1H), 12.43 (1H). **Analysis (CHNS):** Calculated for $C_{29}H_{29}N_9O_2S$: C, 61.36; H, 5.15; N, 22.21; S, 5.65; Found: C, 61.44; H, 5.24; N, 22.31, S; 5.74. **ESI-MS**: 568.1 $(M+H)^+$. **Purity** (UPLC): 98.50%.

6-(4-amino-1-((2-(4-ethylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzo[d]thiazol-2-amine 26b

26b was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

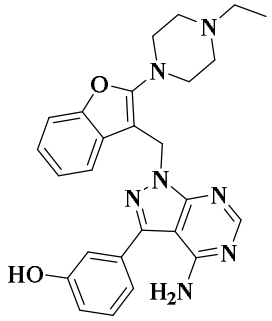


26b

MP: 210-211°C, **Yield:** 62%. **^{1}H NMR** (MeOH-d_1, 400 MHz) δ ppm: 0.98-1.10 (m, 3H), 2.30-2.50 (m, 6H), 3.45-3.70 (m, 4H), 5.59 (s, 2H), 7.00-7.15 (m, 2H), 7.32-7.33 (m, 1H), 7.43-7.50 (m, 3H), 8.20 (s, 1H), 8.34 (s, 1H). **Analysis (CHNS):** Calculated for $C_{27}H_{27}N_9OS$: C, 61.70; H, 5.18; N, 23.98; S, 6.10; Found: C, 61.78; H, 5.25; N, 24.05, S; 6.17. **ESI-MS:** 526.2 $(M+H)^+$. **Purity** (UPLC): 97.90%.

3-(4-amino-1-((2-(4-ethylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenol 26c

26c was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

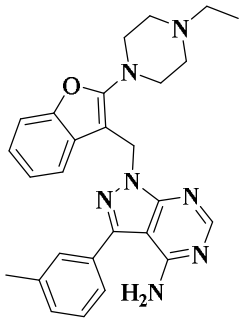


26c

MP: 201-202°C, **Yield:** 50%. **^{1}H NMR** (DMSO-d_6, 400 MHz) δ ppm: 1.33-1.17 (m, 3H), 2.53-2.55 (m, 2H), 2.60-2.70 (m, 4H), 3.42-3.55 (m, 4H), 5.66 (s, 2H), 6.89-6.91 (m, 1H), 7.05-7.13 (m, 4H), 7.26-7.28 (m, 1H), 7.32-7.36 (m, 1H), 7.48-7.50 (m, 1H), 8.33 (s, 1H). **^{13}C NMR** (100 MHz, DMSO-d_6): 12.47, 49.23, 52.15, 52.52, 94.46, 97.51, 110.25, 115.26, 116.27, 118.47, 119.17, 122.42, 123.13, 130.50, 130.80, 134.54, 144.30, 149.67, 154.57, 156.28, 157.97, 158.33, 158.59. **IR (KBr):** 3458, 1624, 1579, 1566, 1458, 1271, 881. **Analysis (CHNS):** Calculated for $C_{26}H_{27}N_7O_2$: C, 66.51; H, 5.80; N, 20.88; Found: C, 66.60; H, 5.90; N, 20.99. **ESI-MS**: 470.2 $(M+H)^+$. **Purity** (UPLC): 97.80%.

1-((2-(4-ethylpiperazin-1-yl)benzofuran-3-yl)methyl)-3-(m-tolyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine 26d

26d was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

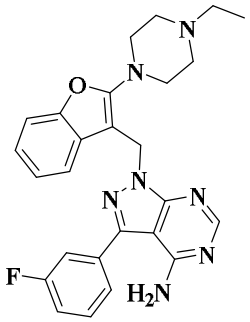


26d

MP: 190-191°C, **Yield:** 55%. **^{1}H NMR** (DMSO-d_6, 400 MHz) δ ppm: 1.04 (t, *J* = 8Hz, 3 H), 2.37-2.40 (m, 5H), 3.50-3.52 (m, 4H), 5.60 (s, 2H), 7.06-7.09 (m, 2H), 7.28-7.34 (m, 2H), 7.41-7.51 (m, 4H), 8.35 (s, 1H). **Analysis (CHNS):** Calculated for $C_{27}H_{29}N_7O$: C, 69.36; H, 6.25; N, 20.97; Found: C, 69.48; H, 6.34; N, 21.02. **ESI-MS:** 468.25 $(M+H)^+$. **Purity** (UPLC): 98.10%.

1-((2-(4-ethylpiperazin-1-yl)benzofuran-3-yl)methyl)-3-(3-fluorophenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine 26e

26e was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

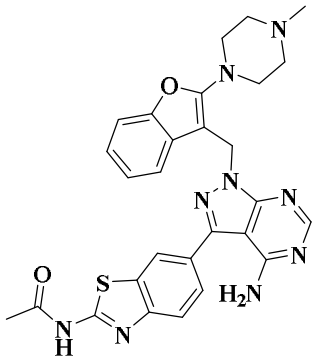


26e

MP: 185-186°C, **Yield:** 53%. **^{1}H NMR** (DMSO-d_6, 400 MHz) δ ppm: 1.02-1.06 (m, 3H), 2.37-2.40 (m, 2H), 3.50-3.53 (m, 4H), 5.61 (s, 2H), 7.04-7.09 (m, 2H), 7.27-7.59 (m, 6H), 8.37 (s, 1H). **Analysis (CHNS):** Calculated for $C_{26}H_{26}FN_7O$: C, 66.23; H, 5.56; F, 4.03; N, 20.79; Found: C, 66.31; H, 5.60; F, 4.08; N, 20.84. **ESI-MS:** 472.25 $(M+H)^+$. **Purity** (UPLC): 98.40%.

N-(6-(4-amino-1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzo[d]thiazol-2-yl)acetamide 26f

26f was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.



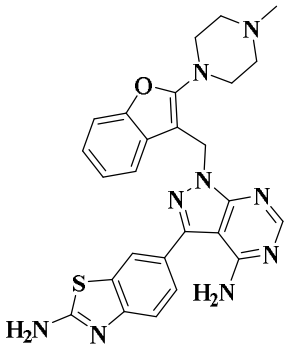
26f

MP: 222-223°C, **Yield:** 57%. **^{1}H NMR** (DMSO-d_6, 400 MHz) δ ppm: 2.21 (s, 3H), 2.23 (s, 3H), 2.35-2.49 (m, 4H), 3.48-3.50 (m, 4H), 5.60 (s, 2H), 7.05-

7.08 (m, 2H), 7.30-7.33 (m, 1H), 7.49-7.51 (m, 1H), 7.62-7.65 (m, 1H) , 7.83 (d, *J* = 8.0 Hz, 1H), 8.17 (d, *J* = 1.6 Hz, 1H), 8.34 (s, 1H), 12.41 (s, 1H). **Analysis (CHNS):** Calculated for $C_{28}H_{27}N_9O_2S$: C, 60.74; H, 4.92; N, 22.77; S, 5.79; Found: C, 60.85; H, 5.02; N, 22.79; S, 5.85. **ESI-MS**: 554.2 $(M+H)^+$. **Purity** (UPLC): 97.65%.

6-(4-amino-1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzo[d]thiazol-2-amine 26g

26g was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

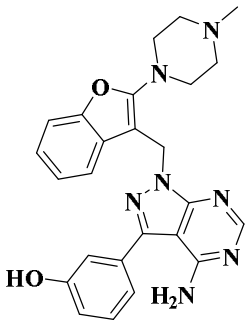


26g

MP: 215-216°C, **Yield:** 42%. **^{1}H NMR** (MeOH-d_1, 400 MHz) δ ppm: 2.43 (s, 3H), 2.45-2.55 (bs, 4H), 3.44-3.70 (m, 4H), 5.60 (s, 2H), 7.05-7.20 (m, 2H), 7.30-7.32 (m, 1H), 7.42-7.48 (m, 3H), 8.21 (s, 1H), 8.33 (s, 1H). **Analysis (CHNS):** Calculated for $C_{26}H_{25}N_9OS$: C, 61.04; H, 4.93; N, 24.64; S, 6.27; Found: C, 61.16; H, 5.00; N, 24.70; S, 6.32. **ESI-MS**: 512.0 $(M+H)^+$. **Purity** (UPLC): 97.85%.

3-(4-amino-1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenol 26h

26h was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

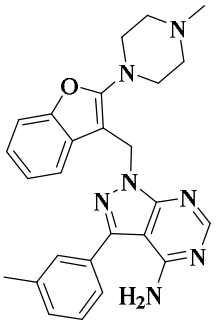


26h

MP: 211-212^{o}C, **Yield:** 54%. (400 MHz, DMSO-d_6):- δ ppm 2.31 (s, 3H), 2.67 (t, J = 1.6 Hz, 4H), 3.52 (t, J = 4.4 Hz, 4H), 5.59 (s, 2H), 6.84-6.86 (m, 1H), 7.01-7.13 (m, 4H), 7.30-7.35 (m, 2H), 7.50-7.52 (m, 1H), 8.36 (s, 1H), 9.71 (s, 1H). **^{13}C NMR** (100 MHz, DMSO-d_6): 40.62, 48.94, 54.60, 94.95, 97.95, 110.30, 115.27, 116.29, 118.58, 119.16, 122.34, 123.14, 130.37, 130.80, 134.54, 134.94, 144.32, 149.73, 154.57, 156.28, 157.88, 158.35, 158.59. **IR (KBr):** 3558, 2924, 1626, 1581, 1447, 1460, 1273, 1004, 740. **Analysis (CHNS):** Calculated for $C_{25}H_{25}N_7O_2$: C, 65.92; H, 5.53; N, 21.52; Found: C, 66.02; H, 5.61; N, 21.60. **ESI-MS**: 456.2 $(M+H)^+$. **Purity** (UPLC): 98.15%.

1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-3-(m-tolyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine 26i

26i was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

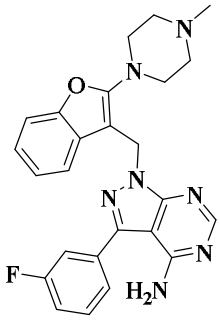


26i

MP: 190-191°C, **Yield:** 51%. **^{1}H NMR** ($CDCl_3$-d_1, 400 MHz) δ ppm: 2.42 (s, 3H), 2.46 (s, 3H), 2.65-2.85 (m, 4H), 3.50-3.60 (m, 4H), 5.43 (s, 2H), 5.66 (s, 2H), 7.11-7.13 (m, 2H), 7.22-7.30 (m, 1H), 7.36-7.45 (m, 4H),7.66-7.67 (m, 1H), 8.44 (s, 1H). **Analysis (CHNS):** Calculated for $C_{26}H_{27}N_7O$: C, 68.85; H, 6.00; N, 21.62; Found: C, 68.98; H, 6.09; N, 21.70. **ESI-MS**: 454.1 $(M+H)^+$. **Purity** (UPLC): 97.34%.

3-(3-fluorophenyl)-1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine 26j

26j was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.



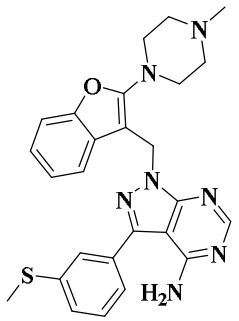
26j

MP: 182-183°C, **Yield:** 55%. **^{1}H NMR** ($CDCl_3$-d_1, 400 MHz) δ ppm: 2.37 (s, 3H), 2.41-2.46 (m, 4H), 3.49-3.61 (m, 4H), 5.44-5.49 (m, 2H), 5.67 (s, 2H),

7.09-7.17 (m, 3H), 7.35-7.52 (m, 4H), 7.64-7.66 (m, 1H), 8.47 (s, 1H). **Analysis (CHNS):** Calculated for $C_{25}H_{24}FN_7O$: C, 65.63; H, 5.29; F, 4.15; N, 21.43; Found: C, 65.80; H, 5.35; F, 4.20: N, 21.50. **ESI-MS**: 458.2 $(M+H)^+$. **Purity** (UPLC): 98.82%.

1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-3-(3-(methylthio)phenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine 26k

26k was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

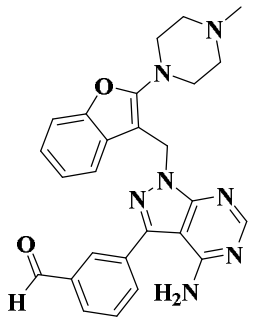


26k

MP: 194-195°C, **Yield:** 48%. **^{1}H NMR** ($CDCl_3$-*d_1*, 400 MHz) δ ppm: 2.36 (s, 3H), 2.47 (s, 3H), 2.75-2.80 (m, 4H), 3.66-3.74 (m, 4H), 5.19 (s, 2H), 5.67 (s, 2H), 7.09-7.11 (m, 2H), 7.22-7.26 (m, 1H), 7.30-7.32 (m, 1H), 7.33-7.35 (m, 2H), 7.41-7.46 (m, 1H), 7.63-7.65 (m, 1H), 8.34 (s, 1H). **Analysis (CHNS):** Calculated for $C_{26}H_{27}N_7OS$: C, 64.31; H, 5.60; N, 20.19; S, 6.60; Found: C, 64.42; H, 5.66; N, 20.28: S, 6.66. **ESI-MS**: 486.2 $(M+H)^+$. **Purity** (UPLC): 98.21%.

3-(4-amino-1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzaldehyde 26l

26l was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

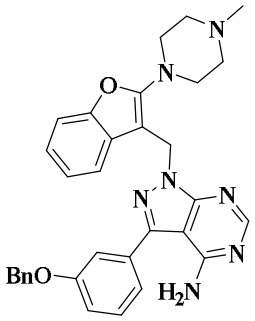


26l

MP: 171-172°C, **Yield:** 61%. **^{1}H NMR** ($CDCl_3$-*d_1*, 400 MHz) δ ppm: 2.51 (s, 3H), 2.82-284 (m, 4H), 3.70-3.74 (m, 4H), 5.37 (s, 2H),5.68 (s, 2H), 7.12-7.14 (m, 2H), 7.27-7.30 (m, 1H), 7.68-7.72 (m, 2H), 7.91-7.98 (m, 2H), 8.17-8.18(m, 1H), 8.48 (s, 1H), 10.09(s, 1H). **Analysis (CHNS):** Calculated for $C_{26}H_{25}N_7O_2$: C, 66.79; H, 5.39; N, 20.97; Found: C, 66.90; H, 5.56; N, 21.10. **ESI-MS**: 468.1 $(M+H)^+$. **Purity** (UPLC): 97.18%.

3-(3-(benzyloxy)phenyl)-1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine 26m

26m was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

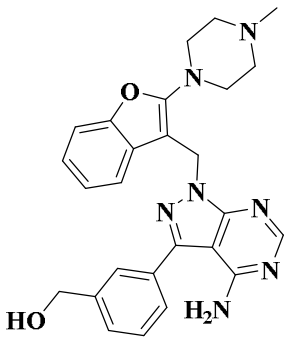


26m

MP: 192-193°C, **Yield:** 55%. **^{1}H NMR** ($CDCl_3$-d_1, 400 MHz) δ ppm: 2.47 (s, 3H), 2.76-2.79 (m, 4H), 3.50-3.90 (m, 4H), 5.13 (s, 2H), 5.32 (s, 2H), 5.65 (s, 2H), 7.06-7.09 (m, 1H), 7.11-7.13 (m, 2H), 7.19-7.20 (m, 2H), 7.32-7.37 (m, 1H), 7.39-7.43 (m, 5H), 7.68-7.69 (m, 1H), 8.42 (s, 1H). **Analysis (CHNS):** Calculated for $C_{32}H_{31}N_7O_2$: C, 70.44; H, 5.73; N, 17.97; Found: C, 70.59; H, 5.88; N, 18.02. **ESI-MS:** 546.1 $(M+H)^+$. **Purity** (UPLC): 98.42%.

(3-(4-amino-1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenyl)methanol 26n

26n was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

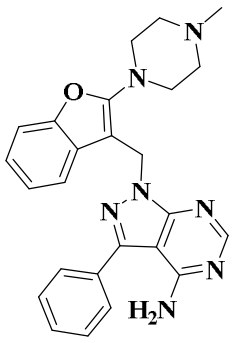


26n

MP: 183-184°C, **Yield:** 47%. **^{1}H NMR** ($CDCl_3$-d_1, 400 MHz) δ ppm: 2.40 (s, 3H), 2.60-2.80 (m, 4H), 3.59-3.62 (m, 4H), 4.77 (s, 2H), 5.41 (s, 2H), 5.67 (s, 2H), 7.09-7.13 (m, 2H), 7.24-7.26 (m, 1H),7.42-7.51 (m, 2H), 7.55-7.70 (m, 3H), 8.45 (s, 1H). **Analysis (CHNS):** Calculated for $C_{26}H_{27}N_7O_2$: C, 66.51; H, 5.80; N, 20.88; Found: C, 66.68; H, 5.86; N, 20.94. **ESI-MS:** 470.2 $(M+H)^+$. **Purity** (UPLC): 97.36%.

1-((2-(4-methylpiperazin-1-yl)benzofuran-3-yl)methyl)-3-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine 26n

26n was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

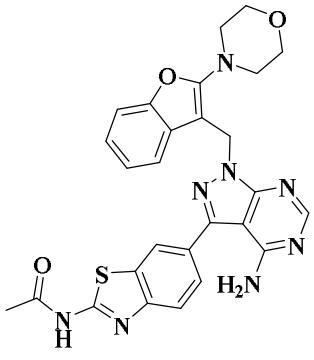


26o

MP: 168-169°C, **Yield:** 55%.**^{1}H NMR** (DMSO-d_6, 400 MHz) δ ppm: 2.23 (s, 3H), 2.40-2.52 (m, 4H), 3.48-3.50 (m, 4H), 5.59 (s, 2H), 7.05-7.08 (m, 2H), 7.30-7.32 (m, 1H), 7.45-7.47 (m, 1H), 7.48-7.62 (m, 5H), 8.34 (s, 1H). **Analysis (CHNS):** Calculated for $C_{25}H_{25}N_7O$: C, 68.32; H, 5.73; N, 22.31; Found: C, 68.49; H, 5.80; N, 22.42. **ESI-MS:** 440.1 $(M+H)^+$. **Purity** (UPLC): 97.02%.

N-(6-(4-amino-1-((2-morpholinobenzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzo[d]thiazol-2-yl)acetamide 26p

26p was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

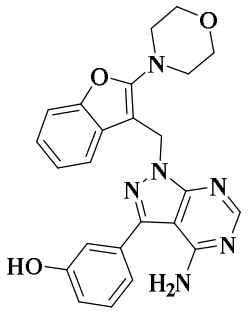


26p

MP: 230-231°C, **Yield:** 56%. **^{1}H NMR** (DMSO-d_6, 400 MHz) δ ppm: 2.18 (s, 3H), 3.47-3.49 (t, J = 8 Hz, 4H), 3.74-3.76 (t, J = 4.4 Hz, 4H), 5.63 (s, 2H), 7.08-7.11 (m, 2H), 7.33-7.35 (m, 1H), 7.52-7.54 (dd, J_1 = 5.2 Hz, J_2 = 2.4 Hz, 1H), 7.63-7.65 (dd, J_1 = 8.4 Hz, J_2 = 1.6 Hz, 1H), 7.83-7.85 (d, J = 8 Hz, 1H), 8.18 (s, 1H), 8.35 (s, 1H), 12.41 (s, 1H). **Analysis (CHNS):** Calculated for $C_{27}H_{24}N_8O_3S$: C, 59.99; H, 4.48; N, 20.73; S, 5.93; Found: C, 60.10; H, 4.56; N, 20.88; S, 5.99. **E SI-MS:** 540.9 $(M+H)^+$. **Purity** (UPLC): 98.38%.

N-(6-(4-amino-1-((2-morpholinobenzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzo[d]thiazol-2-yl)acetamide 26q

26q was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

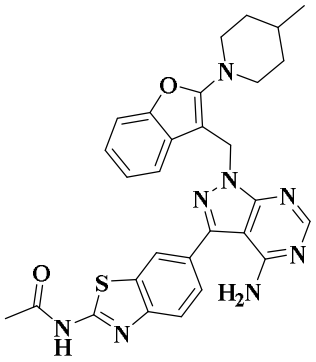


26q

MP: 195-196°C, **Yield:** 50%. **^{1}H NMR** (DMSO-d_6, 400 MHz) δ ppm: 3.46-3.48 (t, J = 4.8 Hz, 4H), 3.73-3.76 (t, J = 4.8 Hz, 4H), 5.61 (s, 2H), 6.83-6.86 (m, 1 H), 7.00-7.11 (m, 4H), 7.30-7.35 (m, 2H), 7.51-7.53 (m, 1H), 8.31 (s, 1H), 9.68 (s, 1H). **Analysis (CHNS):** Calculated for $C_{24}H_{22}N_6O_3$: C, 65.15; H, 5.01; N, 18.99; Found: C, 65.30; H, 5.12; N, 19.05. **ESI-MS:** 442.9 $(M+H)^+$. **Purity** (UPLC): 97.86%.

N-(6-(4-amino-1-((2-(4-methylpiperidin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)benzo[d]thiazol-2-yl)acetamide 26r

26r was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.

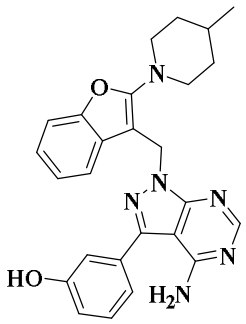


26r

MP: 229-230°C, **Yield:** 55%. **^{1}H NMR** (DMSO-d_6, 400 MHz) δ ppm: 0.93-0.95 (d, J = 8 Hz, 3H), 1.22-1.33 (m, 2H), 1.52-1.59 (m, 1H), 1.67-1.70 (m, 2H), 2.21 (s, 3H), 3.05-3.30 (m, 2H), 3.81-3.85 (d, J = 16 Hz, 2H), 5.59 (s ,2H), 7.05-7.09 (m, 2H), 7.28-7.31 (dd, J_1 = 7.2 Hz, J_2 = 2.4 Hz, 1H), 7.47-7.49 (m, 1H), 7.62-7.65 (dd, J_1 = 8.2 Hz, J_2 = 1.4 Hz, 1H), 7.82-7.84 (d, J = 8 Hz, 1H), 8.17 (s, 1H), 8.35 (s, 1H), 12.41 (s, 1H). **Analysis (CHNS):** Calculated for $C_{29}H_{28}N_8O_2S$: C, 63.03; H, 5.11; N, 20.28; S, 5.80; Found: C, 63.18; H, 5.20; N, 20.34; S, 5.92. **ESI-MS:** 553.0 $(M+H)^+$. **Purity** (UPLC): 98.76%.

3-(4-amino-1-((2-(4-methylpiperidin-1-yl)benzofuran-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenol 26s

26s was prepared following the general procedure described in section 3.1.3.1 (Step VIII) as a white solid.



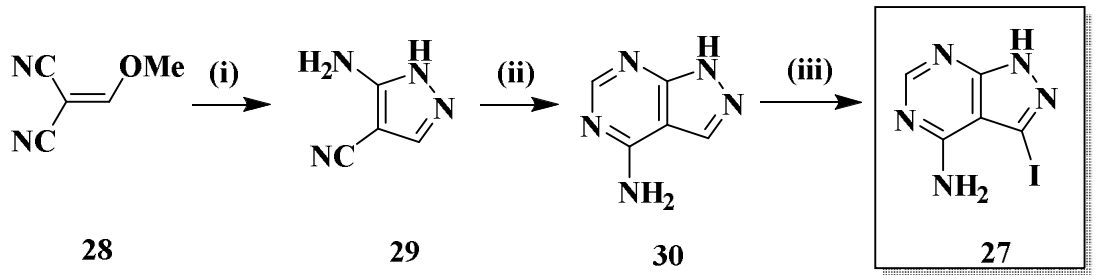
26s

MP: 190-191°C, **Yield:** 54%. **^{1}H NMR** (DMSO-d_6, 400 MHz) δ ppm: 0.83-0.85 (d, *J* = 6.8 Hz, 3H), 1.23-1.33 (m, 2H), 1.55-1.58 (m, 1H), 1.67-1.70 (d, *J* = 12 Hz, 2H), 3.05-3.17 (m, 2H), 3.80-3.83 (d, *J* = 12 Hz, 2H), 5.57 (s, 2H), 6.83-6.85 (d, *J* = 8 Hz, 1H), 7.01-7.09 (m, 4H), 7.29-7.33 (t, *J* = 7.6 Hz, 2H), 7.46-7.48 (t, *J* = 8 Hz, 1H), 8.34 (s, 1H), 9.67 (s, 1H). **Analysis (CHNS):** Calculated for $C_{26}H_{26}N_6O_2$: C, 68.70; H, 5.77; N, 18.49; Found: C, 68.82; H, 5.86; N, 18.56. **ESI-MS:** 454.9 $(M+H)^+$. **Purity** (UPLC): 98.14%.

3.1.4. General procedure for the synthesis of 3-iodo-1H-pyrazolo [3, 4-*d*] pyrimidin-4-amine 27

2-(methoxymethylene) malononitrile **28 (Scheme 4)** reacted with hydrazine hydrate to get 5-amino-1H-pyrazole-4-carbonitrile **29**, which was cyclised using formamide to give 1H-pyrazolo[3,4-d]pyrimidin-4-amine **30**. Free radicle iodination was carried out at 3rd position using *N*-Iodosuccinimide in DMF to get 3-iodo-1*H*-pyrazolo [3, 4-*d*] pyrimidin-4-amine **[98]**.

Scheme 4: Synthetic scheme of 3-iodo-1H-pyrazolo [3, 4-d] pyrimidin-4-amine **(27)**



28 **29** **30** **27**

Reagents and conditions:(i) NH_2-NH_2, 80-85 ^oC, 5 hr; (ii) Formamide, 180-185 ^oC, 4 hr; (iii) NIS, DMF, 80-85 ^oC, 16 hr.

3.1.4.1. Detailed experimental procedure for Synthesis of Iodo 27 compounds (Scheme 4)

Step I: Preparation of 5-amino-1H-pyrazole-4-carbonitrile **29.**

2-(methoxymethylene) malononitrile (10 gm, 93 mmol) **28** was suspended in hydrazine hydrate (5.93 gm, 185 mmol) (99%). Reaction mixture was heated at 80-85 ^oC for 5 h. After completion of reaction the solution was cooled and cold water (50 ml) was added, Solid was filtered. Solid was washed with cold water (50 ml) and suck dried to give 5-amino-1H-pyrazole-4-carbonitrile **29** as light orange solid (8.0 g, 74 mmol, yield: 80 %). Dried product is directly used for next reaction.

Step II: Preparation of 1H-pyrazolo[3,4-d]pyrimidin-4-amine **30.**

5-amino-1H-pyrazole-4-carbonitrile (8.0gm, 74 mmol) **29** was suspended in formamide (80 ml). Reaction mixture was heated at 180-185 ^oC for 4 h. After completion of reaction, reaction mixture was cooled and ice cold water (300ml) was added and stirred for 1 h. Solid was filtered, suck dried to give 1H-pyrazolo[3,4-d]pyrimidin-4-amine **30** as off white solid (8.5 g, Yield: 85%).

^{1}H NMR (DMSO-d_6, 400 MHz) δ ppm: 7.59 (bs, 2H), 8.07 (s, 1H), 8.13 (s, 1H), 13.33 (s , 1H). **HR-MS:** 136.0560 $(M+H)^+$. **Purity** (UPLC): 98.13%.

Step III: Preparation of 3-iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine **27.**

1H-pyrazolo[3,4-d]pyrimidin-4-amine (8.0gm, 59.2 mmol) **30** was suspended in DMF (50 ml). N-Iodosuccinimide (20 gm, 89 mmol) (NIS) was added. Reaction mixture was heated at 80-85 ^oC for 16 h. After completion of reaction, reaction mixture was cooled and ice cold water (200ml) was added and stirred for 1 h. Solid was filtered, suck dried to give 3-iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine **27** as off white solid (11 g, Yield: 71.2 %).

^{1}H NMR (DMSO-d_6, 400 MHz) δ ppm: 8.17 (s, 1H), 13.80 (s, 1H). **ESI-MS:** 261.8 $(M+H)^+$. **Purity** (UPLC): 99.00%.

3.2. Biological evaluation

As described earlier, we synthesised overall nineteen compounds in benzofuran series. All synthesized compounds were assessed for PI3Kδ inhibitory activities in order to establish SAR. Some additional profiling studies (isoform selectivity, CYP, hERG) for selected compounds were carried out. Selected compounds were docked in PI3Kδ ATP binding pocket to see the orientation and interaction in hinge region, affinity pocket, selectivity pocket and hydrophobic region. A brief assay protocol of all biological studies is as follows.

3.2.1. *In-vitro* PI3K inhibitory activity assay

PI3K Kinase activity assay kit from Millipore was used to screen inhibitory activity of test compounds. The assay was carried out using experimental protocol described in the section **2.2.1** in the chapter II.

3.2.2. *In-vitro* CYP inhibition study assay

The assay was carried out using experimental protocol described in the section **2.2.7** in the chapter II.

3.2.3. hERG inhibition (Rb efflux assay)

The assay was carried out using experimental protocol described in the section **2.2.8** in the chapter II.

3.2.4. Docking protocol

Docking protocol was carried out using experimental protocol described in the section **2.2.10** in the chapter 2.

3.3. Result and Discussion

In this section, we summarized results and discussion of benzofuran pyrazolo pyrimidine based PI3Kδ inhibitors:

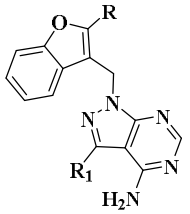
- *In vitro* PI3Kδ inhibitory activity, selectivity and SAR
- *In vitro* CYP inhibition study and hERG liabilities
- Docking studies

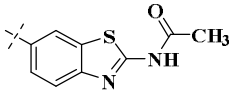
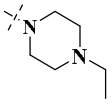
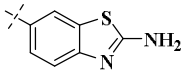
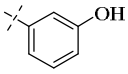
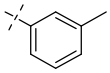
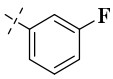
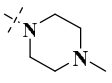
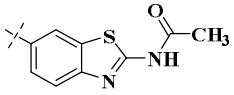
3.3.1. In vitro PI3Kδ inhibitory activity, selectivity and structure activity relationship (SAR)

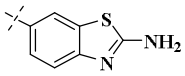
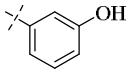
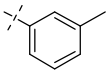
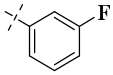
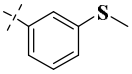
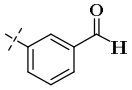
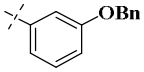
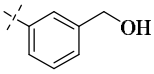
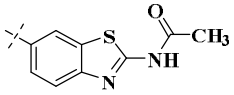
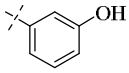
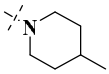
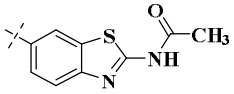
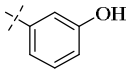
All the synthesized compounds were assessed for their PI3Kδ inhibitory activity; INK-666 was employed as the positive control, **Table 12**. It was found that the majority of substituted benzofuran-3-yl) methyl)-1H-pyrazolo [3,4-*d*] pyrimidin-3-yl) (**26a-s**) analogues displayed varying degree of PI3Kδ inhibitory activity at 100 nM concentration.

The initial set of Compound **26a** to **26j** bearing an *N*-ethyl piperazine substitution (R) on a benzofuran ring showed excellent to moderate PI3Kδ inhibitory activity. Compound **26a** with acylated N-(benzo[d]thiazol-2-yl), compound **26b**, deacylated *N*-(benzo[d]thiazol-2-yl) derivative and compound **26c** showed excellent inhibitory activity with an IC_{50}: 0.60 nM, 0.58 nM and 0.90 nM respectively. Compound **26d** and compound **26e** showed moderate inhibitions (% inhibition; **26d**: 79% inhibition and **26d**: 68% inhibition at 100 nM concentrations).

Table 12: *In vitro* PI3Kδ inhibitory activity data Benzofuran pyrazolo pyrimidine derivatives



Sr. No.	R	R_1	PI3Kδ inhibition (%)[a, b]	PI3Kδ IC_{50} (nM)[c]
26a			**108**	**0.60**
26b			**112**	**0.58**
26c			**99**	**0.90**
26d			79	ND
26e			68	ND
26f			**102**	**0.68**

26g		**100**	**0.60**
26h		**99**	**0.95**
26i		78	10.1
26j		53	ND
26k		52	ND
26l		55	ND
26m		60	8.50
26n		50	ND
26o		45	ND
26p	 	65	7.20
26q		60	7.10
26r	 	60	6.62
26s		62	6.90
	INK-666	**110**	**0.50**

[a]All the data are shown as the mean for at least two experiments. [b]PI3Kδ inhibition at the concentration of 100 nM using PI3K Kinase Activity assay kit from Millipore. [c]The IC_{50} values for PI3Kδ inhibition. ND: not detected.

In second set, compounds with N-methyl piperazine substitution (R) on a benzofuran ring showed moderate to potent PI3K inhibitory activity. Compound **26f**, **26g** and **26h** were found to be most potent (IC_{50}: **26f** = 0.68

nM, **26g** = 0.60 and **26h** = 0.95 nM), whereas compound **26i**, **26j**, **26k**, **26l**, **26m** and **26n** showed moderate inhibitions.

Benzthiazole and 3-hydroxy phenyl substitution (R_1) showed excellent PI3Kδ inhibitory activity. Hence, in the third set, few more compounds were synthesised by keeping benzthiazole and 3-hydroxy phenyl substitution (R_1) uniform, piperazine was replaced with 4-methylpiperidine and morpholine ring. These compounds showed moderate PI3Kδ inhibitory activity (IC_{50}; **26p**: 7.20 nM, **26q**: 7.10 nM, **26r**: 6.62 nM and **26s**: 6.90 nM) Thus, compound **26a**, **26b**, **26c**, **26f**, **26g** and **26h** were found to be comparable with standard compound (INK-666). Selected compounds (**26a**, **26b**, **26c**, **26f**, **26g** and **26h)** were evaluated for PI3K isoform selectivity.

3.3.2. Isoform selectivity study of selected compounds

Based on the above preliminary PI3Kδ inhibitory activity results, most potent compounds **26a**, **26b, 26c, 26f, 26g** and **26h** were evaluated for their selectivity against PI3K isoforms (α, β and γ) and mTOR.

Table 13: Isoform selectivity of compounds against PI3K (α, β, γ, and δ) and mTOR activities

Comp.	Biochemical IC_{50} [nM][a]				
	PI3Kα[b]	PI3Kβ[b]	PI3Kγ[b]	PI3Kδ[b]	mTOR[b] (p70S6K)
26a	320	10	90	0.60	>1000
26b	154	11	4	0.58	>1000
26c	**350**	**28**	**43**	**0.90**	**>1000**
26f	310	12	92	0.68	>1000
26g	150	10	4	0.60	>1000
26h	**350**	**28**	**45**	**0.95**	**>1000**

INK-666	**137**	**9**	**4**	**0.50**	**>1000**
Umbralisib	**>10000**	**1116**	**1065**	**22**	**>1000**

[a]The IC_{50} values are shown as the mean for at least two experiments.[b]PI3K inhibitory activity assay Kit (Millipore) was used to screen the test compounds.

As shown in above **Table 13**, Initial hit **26a, 26b, 26f and 26g** and **10k** showed excellent selectivity against mTOR, over PI3K isoform. Compound **26a** (IC_{50}: 0.60 nM) demonstrated 533, 17, 150 and >1000 fold selectivity over PI3Kα, β, γ and m-TOR respectively. Similar isoform selectivity was observed for compound **26f** (IC_{50}: 0.68 nM) showed 456, 18, 135 and >1000 fold selectivity over PI3Kα, β, γ and m-TOR respectively. Thus compound **26a** and **26f** were borderline selective for PI3Kβ isoform (~17/18 fold). Compound **26b** and **26g** were selective for PI3K α, β isoform and m-TOR, but showed only 7 fold selectivity for PI3Kγ isoform. However, **26c** and **26h** showed exceptional selectivity for PI3Kδ isoform against all other PI3K isoforms and m-TOR (>1000), 389, 31, 48 and 368, 29, 47 fold for PI3Kα, β, γ isoform respectively. In general, it was observed that the potency and selectivity of benzofuran based PI3Kδ inhibitors can be modulated using suitable substituents at R^2 position.

3.3.3. CYP (Cytochrome) inhibition and hERG (human ether-a-go-go-related gene) liabilities for 26c and 26h

Additional profiling studies of **26c** and **26h** were carried out and it was found to be devoid of CYP (<10% CYP inhibition at 10 μM concentration, for CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2C19 and CYP3A4) and hERG liabilities (IC_{50}: > 30 μM).

3.3.4. Molecular modelling study

Molecular docking studies of compound **26b**, **26c, 26g** and **26h** were carried out by using (Glide version 6.7) Schrodinger software to study an interaction of INK666 with PI3Kδ receptor (PDB ID: **2WXK**). **Figure 38** is docking image for compound **26b**, **26c, 26g** and **26h.** The active site was defined to include residues within 10 A^o and the key amino acid residues in binding and specificity pockets are highlighted in respective figures.

As described in designing section, INK-666 and INK-654 interacts effectively with PI3Kδ ATP binging site. Similarly, docking studies illustrate that **26b**, **26c, 26g** and **26h** interacts closely with the key residues of PI3Kδ ATP-binding pockets, pyrazolo pyrimidine ring served as the hinge binder and it forms key hydrogen bonds with Val_{828} and Glu_{826}. All Compounds adopts propeller-shaped conformation, where the benzofuran moiety was found to be sandwiched into the induced hydrophobic specificity pocket between Trp_{760} and Met_{752} in PI3Kδ specificity pocket. In compound **26b** and **26g** benzthiazole ring interacts in affinity region, whereas in compound **26c** and **26h** favours to accommodate 3-hydroxy phenyl ring in the affinity pocket through hydrogen bond with Asp_{911}, N-methyl/ethyl piperazine ring was projected towards hydrophobic region. Thus, through docking it is clear that benzofuran analogue were potent for PI3Kδ inhibiton.

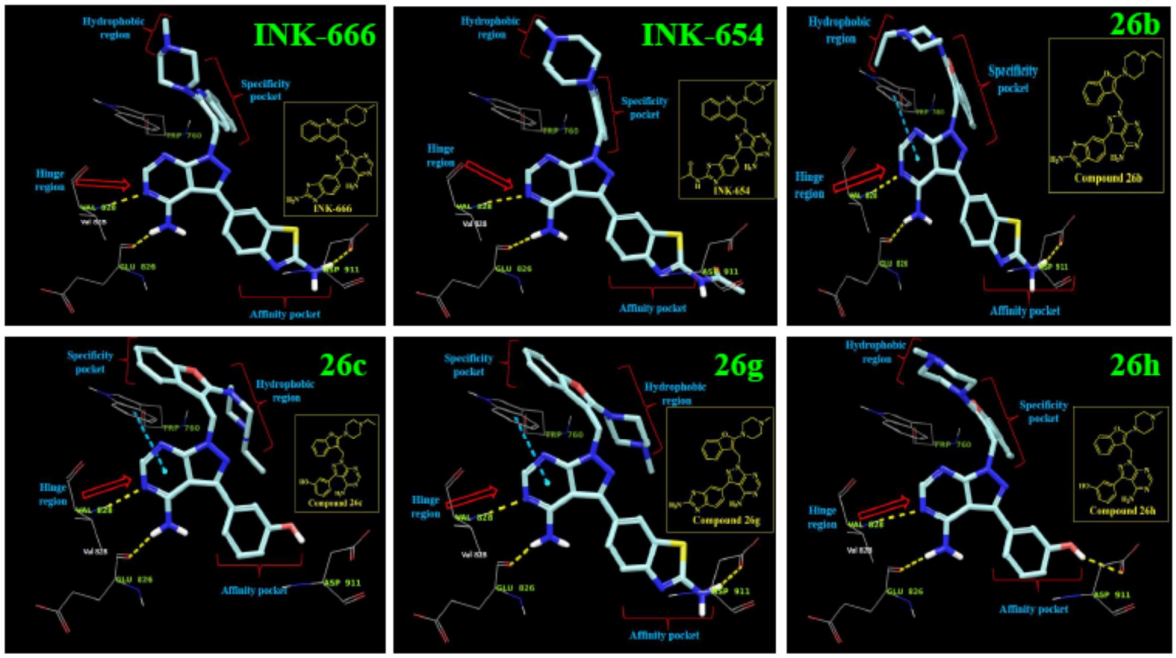


Figure 38: Docking of INK-666, INK-654, Compound **26b**, **26c**, **26g** and **26h** (PDB ID: **2WXK**)

3.4. Conclusion

Based on *in vitro* results, we summarized that among 19 novel compounds tested for PI3Kδ activities, compound **26c** and **26h** were found to be potent and selective PI3Kδ inhibitors over mTOR. *In vitro* results validate our hypothesis of designing benzofuran based novel, potent and selective PI3Kδ inhibitors as a bioisosteric replacement of quinoline ring of INK-666/654 with benzofuran ring system.

Additional profiling studies of compound **26c** and **26h** was carried out and it was found to be devoid of CYP inhibitory activity and showed no hERG liabilities (IC50:>30 µM). Docking results of **26b**, **26c, 26g** and **26h** correlates with its potent *in vitro* PI3Kδ activity.

Further, compound **26c** and **26h** will be subjected for PK profiling in mice followed by efficacy evaluation using xenograft model to check anticancer and CIA model to check anti-inflammatory activity in mice.