Development of Pyridine-Based Inhibitors for the Human Vaccinia-Related Kinases 1 and 2

Ricardo Serafim, Fernando de Souza Gama, Caio dos Reis, Stanley Vasconcelos, André Santiago, Jessica Takarada, Luiz Dutra, Fulvia Di Pillo, Hatylas Azevedo, Alessandra Mascarello, Jonathan Elkins, Katlin Massirer, Opher Gileadi, Cristiano Guimarães, Rafael Counago

Submitted date: 01/03/2019 • Posted date: 04/03/2019
Licence: CC BY 4.0

Citation information: Serafim, Ricardo; de Souza Gama, Fernando; dos Reis, Caio; Vasconcelos, Stanley; Santiago, André; Takarada, Jessica; et al. (2019): Development of Pyridine-Based Inhibitors for the Human Vaccinia-Related Kinases 1 and 2. ChemRxiv. Preprint.

Vaccinia-related kinases 1 and 2 (VRK1 and VRK2) are human Ser/Thr protein kinases associated with increased cell division and neurological disorders. Nevertheless, the cellular functions of these proteins are not fully understood. Despite their therapeutic potential, there are no inhibitors available for VRK1 or VRK2. We report here the discovery and elaboration of an aminopyridine scaffold as a basis for VRK1 and VRK2 inhibitors. The most potent compounds displayed $K_D$ values of 190 nM and 401 nM for VRK1 and VRK2, respectively. Differences in compound binding mode and substituent preferences between the two VRKs were identified by the series structure-activity relationship combined with the crystallographic analysis of key compounds. We expect that our results will serve as a starting point for the design of specific and potent inhibitors against each of the two VRKs based on a pyridine scaffold.
Development of Pyridine-Based Inhibitors for the Human Vaccinia-Related Kinases 1 and 2

Ricardo A. M. Serafim, 1,2,# Fernando H. de Souza Gama, 3,# Caio V. dos Reis, 1,2 Stanley N. S. Vasconcelos, 1,2 André da Silva Santiago, 1,2 Jéssica E. Takarada, 1,2 Luiz A. Dutra, 1,2 Fúlvia Di Pillo, 4 Hatylas Azevedo, 3 Alessandra Mascarello, 3 Jonathan M. Elkins, 2,5 Katlin B. Massirer, 1,2 Opher Gileadi, 5 Cristiano R. W. Guimarães, 3,* Rafael M. Couñago 1,2,*

1 Centro de Química Medicinal (CQMED), Centro de Biologia Molecular e Engenharia Genética (CBMEG), Universidade Estadual de Campinas (UNICAMP), Campinas, SP, 13083-875, Brazil
2 Structural Genomics Consortium, Departamento de Genética e Evolução, Instituto de Biologia, UNICAMP, Campinas, SP, 13083-886, Brazil
3 Aché Laboratórios Farmacêuticos S.A., Guarulhos, SP, Brazil
4 PhD Program in Genetics and Molecular Biology (PGBM), UNICAMP, Brazil
5 Structural Genomics Consortium, Nuffield Department of Medicine, University of Oxford, Oxford OX3 7DQ, UK.

Vaccinia-related kinases, aminopyridine, difluorophenol, kinase inhibitors, structure-based compound development.

ABSTRACT: Vaccinia-related kinases 1 and 2 (VRK1 and VRK2) are human Ser/Thr protein kinases associated with increased cell division and neurological disorders. Nevertheless, the cellular functions of these proteins are not fully understood. Despite their therapeutic potential, there are no inhibitors available for VRK1 or VRK2. We report here the discovery and elaboration of an aminopyridine scaffold as a basis for VRK1 and VRK2 inhibitors. The most potent compounds displayed IC50 values of 190 nM and 401 nM for VRK1 and VRK2, respectively. Differences in compound binding mode and substituent preferences between the two VRKs were identified by the series structure-activity relationship combined with the crystallographic analysis of key compounds. We expect that our results will serve as a starting point for the design of specific and potent inhibitors against each of the two VRKs based on a pyridine scaffold.

The human genome encodes three vaccinia-related kinases (VRK1, VRK2, and VRK3).1 VRK3 has a degraded ATP-binding site and is thought to be a pseudo-kinase,2 while VRK1 and VRK2 have active kinase domains and have been associated with various cellular processes, including cell replication, chromatin remodeling, and response to DNA damage.3–5 VRK2 has two isoforms that share identical kinase domains but locate to distinct cellular compartments: endoplasmic reticulum (VRK2A); and cytoplasm and nucleus (VRK2B).1,6 VRK1 can be found in both nucleus and cytoplasm.7 Genetic studies have associated VRK1 and VRK2 with a number of human diseases and conditions. VRK2 is associated with neurological disorders, such as schizophrenia, multiple sclerosis, major depressive disorder, and epilepsy.7–11 VRK1 overexpression is associated with increased cell division and poor prognosis in a number of cancers.12,13 Despite these advances, our understanding of the cellular roles of human VRKs and their therapeutic potential is limited. Currently, there are no potent or specific small molecule inhibitors of the VRKs.

Previously, our analysis of the Published Kinase Inhibitor Set (PKIS)14 revealed that VRK1 shared a similar activity profile to a small number of kinases, including members of the kinase family Ste20, such as MINK1 and TNIK.15 The Ste20 kinase family also includes MAP4K4, whose phosphate-binding loop (P-loop) can adopt an unusual conformation in which this conserved structural motif folds over the ligand. It has been suggested that this folded P-loop conformation might be targeted to obtain potent and selective kinase inhibitors.16 Co-crystal structures of VRK1 and VRK2 bound to the p90 RSK (ribosomal S6 kinase) inhibitor BI-D1870 (1 - Figure 1A) revealed that both kinases can also adopt the folded P-loop conformation, further suggesting that MAP4K4 and these two VRKs have similar topography and structural features in their binding sites.15 These observations motivated us to search for MAP4K4 chemical matter known to induce a folded P-loop conformation and that could be used as starting points to develop potent and selective inhibitors against VRK1 and VRK2.

The aminopyridine (2 - Figure 1A) is a well-described molecular scaffold that has been shown to accommodate the folded P-loop conformation in MAP4K4.17 Additionally, this scaffold is small and versatile, allowing the attachment of different substituents on both sides of the aminopyridine core by a short synthetic route. The co-crystal structures of VRK1 and VRK2 bound to 1 (BI-D1870) also revealed that the ligand difluorophenol moiety facilitated favorable polar interactions to catalytically-important residues within the VRK ATP-binding site.15
Thus, our design strategy to develop new small molecule inhibitors for VRK1 and VRK2 was to hybridize the aminopyridine core with the difluorophenol group.

Figure 1: (A) Chemical structure of 1 (BI-D1870) and the MAP4K4 inhibitor 2. (B) Synthetic route to aminopyridine derivatives.

Synthesis of the aminopyridine derivatives was performed using a rapid and efficient two-step Suzuki-Miyaura coupling (Figure 1B) with yields ranging between 18% and 89%. Each step was performed by a microwave-assisted reaction in one hour. Details of the procedure are available in the Supporting Information.

To test our hybridization hypothesis, we prepared prototype compound 5, bearing difluorophenol moieties in both 3- and 5-positions of the 2-aminopyridine core (Figure 2A). We employed a thermal-shift assay (Differential Scanning Fluorimetry, DSF) to identify aminopyridine analogues that could bind to the full-length VRK1 (residues Met1-Lys396, VRK1-FL) and the kinase domain of VRK2 (residues Pro14-His335, VRK2-KD). DSF is a robust method to estimate binding affinities and has been used successfully to triage large compound libraries. For these experiments, we considered positive hits those compounds inducing changes in VRK1-FL and VRK2-KD melting temperatures, ΔTm, larger than 2.0 °C. DSF analysis indicated that 5 induced thermal shifts above the hit threshold of 2 °C for both VRK1 and VRK2 (Figure 2 B.C).

Encouraged by these results, we prepared a first series of 2-aminopyridine analogues to explore the relevance of the difluorophenol to the thermal stabilization of the two VRKs (Table 1). For both proteins, simultaneously replacing the difluorophenol moiety with phenol groups in R1 and R2 resulted in a drop of the observed ΔTm, suggesting that increasing the phenol acidic character or altering the electronic properties of the aromatic ring is important for binding. VRK1-FL seemed to tolerate the substitution of a difluorophenol by a phenol on either R1 or R2 position equally well, whereas maintaining a difluorophenol group in R1 proved to be more important for VRK2-KD (8 and 7). Nevertheless, removal of the HB (hydrogen bond) capacity in R2, while maintaining the difluorophenol group in R1, still resulted in a ΔTm drop for both VRKs (9). We attempted to improve compound binding by replacing the difluorophenol in R1 with a variety of groups of different sizes, polarities and electronic properties (Supplementary Table S1), but none of these changes resulted in an improved ΔTm over the difluorophenol moiety.

We next explored the ability of various phenyl derivatives in R2 to optimize the compound binding profile, while maintaining a difluorophenol group in R1 (Table 1). Our results confirmed that polar groups in R2 are important for binding the VRKs and indicated that, overall, these groups were better tolerated in the meta position (10 vs. 11; 12 vs. 13; 14 vs. 15 and 16), although the addition of a carboxylic group in this position was detrimental to binding (17). For VRK2-KD, the best compound had a para-sulfonamide group on the R2 phenyl ring (18), although a benzoazole fused ring (19) was also tolerated in this position. VRK1-FL tolerated both of these groups in R2 equally well. Nevertheless, for VRK1, we could not find a R2 substituent that improved on the difluorophenol moiety (5). For VRK2-KD, further attempts to modify the difluorophenol in R1 while maintaining the sulfonamide group in R2 were unsuccessful (20, 21 and 22).

To better understand the molecular basis for ligand binding, we obtained co-crystals of VRK1-KD (residues Arg3-Glu364) bound to 5 and VRK2-KD bound to 18 (Figure 3). Both structures were solved by molecular replacement (Supplementary Table S2). VRK1-KD crystals had four protein molecules per asymmetric unit, whereas VRK2-KD crystallized with two copies per asymmetric unit. As observed before, the ligand-bound VRK1-KD and VRK2-KD adopted an active conformation, in which conserved residues from structurally-conserved elements of the kinase domain (Glu83 and Lys71 within cA and the ATP-binding site, respectively - VRK1 numbering) are brought into close proximity and the side chain of Asp197 (VRK1 numbering) from the conserved DFG motif (DYG in the VRKs) points towards the ATP-binding site (Figure 3 and Supplementary Figure S1A,B).

Figure 2: Structure and DSF results for prototype compound 5. (A) Prototype compound 5. (B, C) Shown are thermal melting curves in the presence of DMSO (vehicle) or 5 for VRK1-FL (B) and VRK2-KD (C). ΔTm values for 5 are indicated. Values shown represent the mean (± standard deviation) of three independent measurements.
In both crystal structures, electron density for the compounds was of good quality and revealed the ligands bound to the protein ATP-binding site, as expected. For VRK2-KD crystals, the ligand could be built in both protein molecules within the asymmetric unit and adopted identical binding modes. In this pose, the compound 2-amino moiety pointed towards the end of the protein ATP-binding site. The 2-amino group and the pyridine N atom of 18 facilitated one hydrogen bond each to the carbonyl and amide groups of VRK2-KD hinge residues Glu122 and Leu124, respectively. In VRK1-KD crystals, the ligand could be observed in three out of the four protein molecules in the asymmetric unit and, surprisingly, was found in two different poses. The first of these was identical to the one observed for LDSM88 bound to VRK2-KD. In the second binding mode, the 2-amino group of 5 pointed towards the solvent and, together with the pyridine N, facilitated HBs with main chain carbonyl and amide, respectively, from VRK1-KD hinge residue Phe134. The co-structures helped us rationalize the relevance of the difluorophenol moiety for binding. Regardless of the compound binding pose, this group facilitated an HB network with polar side chains from a conserved and catalytic-important lysine (Lys71 in VRK1 or Lys61 in VRK2). Interestingly, the difluorophenol group participating in these contacts displayed distinct dihedral angles to the 2-amino core depending on its attachment position: ~45° in R1 and ~9° in R2. In VRK1-KD, these different orientations of the difluorophenol group are accompanied by a corresponding movement of the side chain from residue Met131, which occupies the gatekeeper position in this protein. Consequently, the difluorophenol group fits tightly between the αC and the gatekeeper residue in both poses. These observations might explain why we could not find substituents that improved binding over the difluorophenol group, showing its pharmacophoric feature.

The VRK2-KD co-structure also revealed that the 18 sulfonamide group pointed away from the protein ATP-binding site and was completely solvent-exposed. A similar observation was made for the difluorophenol group in 5 that did not interact with VRK1-KD αC (Supplementary Figure S1D,F). Our DSF results also indicated that placement of polar groups in the meta-position resulted in slight increases of ΔTm, especially for VRK2-KD (10 vs. 11, for example). At this position, polar groups from the ligand might be able to engage polar groups from VRK2-KD P-loop.

The two binding modes observed for 5 in VRK1 suggested that the 2-amino moiety has no binding preference for each of the hinge carbonyl groups it can interact with (Figures 3A and 3B). This led us to hypothesize that these two interactions were either equally productive or equally weak in the binding process. To address these hypotheses, we synthesized the following analogues: (i) 23, with two amino groups that could interact with both hinge carbonyl groups simultaneously; (ii) 24, with a 2-amino and a space-filling 6-methyl group; (iii) 25, with the 2-amino group removed; and (iv) 26 with the 2-amino group substituted by a 2-methyl group (Table 2). The last two compounds were synthesized to eliminate the desolvation penalty of the 2-amino group.

For VRK2-KD, replacing the 2-amino with a methyl group (26) greatly reduced the observed ΔTm, suggesting that the HB interaction with the hinge carbonyl group is actually productive for VRK2. However, for VRK1-FL, 26 and 5 were equipotent, supporting the hypothesis that the 2-amino moiety contributes little to the binding of 5 to VRK1. This can be further rationalized by the comparison between compounds RS-117 and RS-119 against VRK1 and VRK2. The pair is equipotent against VRK1, as both derivatives would be able to accommodate the difluorophenol group in the back of the binding site by flipping the pose. For VRK2, RS-117 is more potent than RS-119 as only the former would be able to keep the HB interactions with the Glu122 hinge carbonyl and Lys61 catalytic Lys residue, similar to the ones displayed in Figure 3C. VRK1-FL also seemed to be more tolerant to the removal of the 2-amino group than VRK2-KD (25). Those differences between VRK1 and
VRK2 were unexpected as both proteins display highly similar ATP-binding sites. Thus, both our DSF results and crystal structures suggest that VRK1, but not VRK2, is capable of binding equally well to the 2-amino pyridine core in two different conformations. The increase in ΔTm observed for compound 26 (2-methyl pyridine core) over 25 (pyridine core) might arise from favorable electron-donating properties or conformational effects conferred to the pyridine ring system by the presence of an adjacent methyl group. Alternatively, the lack of a substituent group in the 2-position may incur a free energy penalty associated with inability to fill in a cavity in the protein binding pocket. The 2-amino pyridine moiety is a common feature of many kinase inhibitors, and often plays a role in binding to the kinase hinge region (Supplementary Figure S2). We expect that adding a methyl group in the 2-position of the pyridine core will offer an opportunity to increase the selectivity of future VRK1 inhibitors.

Finally, our co-structure revealed that 18 did not induce the expected folded P-loop conformation in VRK2-KD (Supplementary Figure S1A,C). The P-loop is a conserved (GxGxF/YG motif) but flexible region of the kinase domain. Indeed, in our VRK2-KD co-crystal structure, poor electron density maps for this region prevented us building P-loop residues Gly38 and Gly39 into the final protein model. By contrast, the P-loop of VRK1 bound to 5 was found to be folded over the ligand, regardless of the ligand binding pose. The folded P-loop conformation is often stabilized by the interaction of the aromatic residue within this motif (Phe48 in VRK1) and appropriate groups from the ligand. In VRK1-KD, P-loop residue Phe48 is accommodated by the ligand three ring system. Such interaction cannot be facilitated by the equivalent P-loop residue in VRK2-KD (Phe40). A structural alignment between the two VRK structures revealed that the position of the two conserved aromatic residues in the protein P-loop are substantially different, with Phe40 in VRK2 much closer to the protein αC than its structural equivalent in VRK1 (Supplementary Figure S1C).

To confirm binding and estimate compound potency, we employed Isothermal Titration Calorimetry (ITC). Our ITC results indicated that the top compounds by DSF (26 for VRK1-FL and 18, 19 and 13 for VRK2-KD) had Kd values in the sub-micromolar range. The most potent compound for VRK1-FL, 26, had a Kd of 190 nM, whereas for VRK2-KD, 18 had a Kd of 401 nM (Figure 4). For both proteins, compound binding was mostly enthalpy-driven (|ΔH| > |TΔS|), although binding of 18 to VRK2-KD did have a more pronounced entropic contribution than the one observed for compounds 19 and 13 (Supplementary Table S3). At this point, we do not have an explanation for the observed discrepancy between DSF and ITC results for compound 19.

Table 1 - DSF results for 2-amino pyridine derivatives

| #  | R1     | R2     | DSF ΔTm (°C) | VRK1 | VRK2 |
|----|--------|--------|--------------|------|------|
| 6  | HO     | F      | 0.7 ± 0.1    | 1.0 ± 0.1 |
| 7  | F      | OH     | 2.2 ± 0.2    | 1.3 ± 0.1 |
| 8  | F      | OH     | 2.3 ± 0.0    | 2.3 ± 0.1 |
| 9  | F      | CF3    | 0.7 ± 0.2    | 0.7 ± 0.1 |
| 10 | F      | COF    | 1.1 ± 0.3    | 2.3 ± 0.3 |
| 11 | F      | CF3    | 0.2 ± 0.3    | 1.2 ± 0.2 |
| 12 | F      | O      | 1.0 ± 0.1    | 2.0 ± 0.2 |
| 13 | F      | O      | 1.9 ± 0.1    | 2.6 ± 0.1 |
| 14 | F      | CN     | 2.0 ± 0.2    | 2.3 ± 0.2 |
| 15 | F      | CN     | 1.1 ± 0.2    | 1.7 ± 0.1 |
| 16 | F      | CN     | 0.5 ± 0.4    | 1.7 ± 0.1 |
| 17 | F      | COOH   | 1.1 ± 0.1    | 1.1 ± 0.2 |
| 18 | F      | SO3H   | 2.1 ± 0.7    | 3.2 ± 0.0 |
| 19 | F      | NO2    | 2.2 ± 0.2    | 3.0 ± 0.0 |
| 20 | F      | SO3H   | -0.1 ± 0.4   | 0.8 ± 0.1 |
| 21 | F      | SO3H   | 0.1 ± 0.0    | 0.7 ± 0.0 |
| 22 | F      | SO3H   | -0.2 ± 0.0   | 0.3 ± 0.1 |

Table 2 - DSF results for 3,5-disubstituted pyridine derivatives

| #  | DSF ΔTm (°C) | VRK1 | VRK2 |
|----|--------------|------|------|
| 23 | -0.9 ± 0.5   | 0.1 ± 0.2 |
| 24 | -0.1 ± 0.6   | 0.5 ± 0.1 |
| 25 | 1.6 ± 0.4    | -0.4 ± 0.1 |
| 26 | 3.5 ± 0.3    | 1.1 ± 0.2 |

Finally, to ascertain that 18 and 26 represented tractable molecules for development into chemical probes suitable for biological studies, we wanted to test that they were not overtly toxic to human cells. Thus, we performed cell viability assays based on the metabolic conversion of a tetrazolium dye (MTT,
see supplementary information) to its insoluble formazan by HeLa cells. Our results indicated that there was no noticeable effect on cell viability after a 24-hour treatment with 3.2 µM of 18 and only a slight decrease with 3.2 µM of 26; and above 60% of cells remained viable after the compound concentration was raised to 12.5 µM of 18 and 25 µM of 26 (Supplementary Figure S3).

In conclusion, we report here the use of the pyridine scaffold for the development of inhibitors against VRK1 and VRK2. The most potent compounds 26 and 18 displayed $K_D$ values of 190 and 401 nM against VRK1 and VRK2, respectively. Both compounds were not toxic to mammalian cells (up to 3.2 µM). Crystallographic analysis of VRK1 and VRK2 bound to our pyridine analogues highlighted the importance of difluorophenol for binding, and suggested design strategies for the development of specific inhibitors for each of the VRKs. We believe the pyridine scaffold to be a promising candidate for developing potent and selective inhibitors for VRK1 and VRK2. Future optimization will take advantage of the scaffold low molecular weight and its rapid and efficient two-step synthetic route.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. The Supporting Information file contains: Supplementary Tables S1, S2, and S3; Supplementary Figures S1, S2 and S3; and Supplementary Methods.

Accession Codes

The atomic coordinates and crystallographic structures factors for VRK1-KD bound to 5 and for VRK2-KD bound to 18 have been deposited in the Protein Data Bank (www.rcsb.org) with accession codes: 6BU6 and 6NCG, respectively.

AUTHOR INFORMATION

Corresponding Author

*E-mail: rafael.counago@unicamp.br; cristiano.guimaraes@ache.com.br.

ORCID

Ricardo A. M. Serafim: 0000-0003-0614-1798
Fernando H. de Souza Gama: 0000-0002-3745-1046
Stanley N. S. Vasconcelos: 0000-0001-7289-5753

Caio V. dos Reis: 0000-0001-6271-7523
André da Silva Santiago: 0000-0002-3973-3370
Jessica E. Takarada: 0000-0001-7311-8665
Luiz A. Dutra: 0000-0002-3316-8526
Fulvia Di Pillo: 0000-0002-3496-0574
Hatylas Azevedo: 0000-0001-9233-0696
Alessandra Mascarello: 0000-0001-7672-4070
Jonathan M. Elkins: 0000-0003-2858-8929
Katlin B. Massirer: 0000-0001-6390-2560
Opher Gileadi: 0000-0001-6886-898X
Cristiano R. W. Guimarães: 0000-0003-4062-9965
Rafael M. Couñago: 0000-0003-1847-5090

Author Contributions

R.A.M.S., F.H.S.G., S.N.S.V., H.A., A.M. and C.R.W.G. designed all molecules. R.A.M.S., F.H.S.G. and S.N.S.V. synthesized and characterized all molecules. C.V.R. and R.M.C. designed and performed crystallization experiments, x-ray diffraction data collection, and protein structure determination and analysis. A.S.S. designed, performed and analyzed ITC experiments for VRK2-KD, J.E.T. designed, performed, and analyzed ITC experiments for VRK1-FL. C.V.R., A.S.S., and L.A.D. designed, performed, and analyzed DSF experiments. F.D.P. designed, performed and analyzed cell assays. H.A., A.M., J.M.E., K.B.M., O.G., C.R.W.G. and R.M.C. coordinated the project. R.A.M.S. and R.M.C. wrote the manuscript. All authors revised the manuscript. All authors have given approval to the final version of the manuscript. # These authors contributed equally.

Funding Sources

This work was supported by the Brazilian agencies FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) (2013/50724-5 and 2014/5087-0), Embrapii (Empresa Brasileira de Pesquisa e Inovação Industrial), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) (465651/2014-3), the SGC is a registered charity (number 1097737) that receives funds from AbbVie, Bayer Pharma AG, Boehringer Ingelheim, Canada Foundation for Innovation, Eshelman Institute for Innovation, Genome Canada, Innovative Medicines Initiative (EU/FP11) [ULTRA-DD grant no. 115766], Janssen, Merck KGaA Darmstadt Germany, MSD, Novartis Pharma AG, Ontario Ministry of Economic Development and Innovation, Pfizer, Takeda, and Wellcome [106169/Z/F14/Z]. RAMS and SNSV received FAPESP postdoctoral fellowships (2016/25320-6 and...
Abbreviations

VRK, Vaccinia-related kinase; KD, kinase domain; FL, full-length; HB, hydrogen bond; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5- Dimphenyltetrazolium Bromide.

References

(1) Nichols, R. J.; Traktman, P. Characterization of Three Paralogous Members of the Mammalian Vaccinia Related Kinase Family. J. Biol. Chem. 2004, 279 (9), 7934.

(2) Scheeff, E. D.; Eswaran, J.; Bunkoczi, G.; Knapp, S.; Manning, G. Structure of the Pseudokinase VRK3 Reveals a Degraded Catalytic Site, a Highly Conserved Kinase Fold, and a Putative Regulatory Binding Site. Structure 2009, 17 (1), 128.

(3) Kang, T.-H.; Park, D.-Y.; Choi, Y. H.; Kim, K.-J.; Yoon, H. S.; Kim, K.-T. Mitotic Histone H3 Phosphorylation by Vaccinia-Related Kinase 1 in Mammalian Cells. Mol. Cell. Biol. 2007, 27 (24), 8533.

(4) Molitor, T. P.; Traktman, P. Depletion of the Protein Kinase VRK1 Disrupts Nuclear Envelope Morphology and Leads to BAF Retention on Mitotic Chromosomes. Mol. Biol. Cell 2014, 25 (6), 891.

(5) Monsalve, D. M.; Merced, T.; Fernández, I. F.; Blanco, S.; Vázquez-Cedeira, M.; Lazo, P. A. Human VRK2 Modulates Apoptosis by Interaction with Bcl-XL and Regulation of BAX Gene Expression. Cell Death Dis. 2013, 4 (2), e153.

(6) Blanco, S.; Khimcakova, L.; Vega, F. M.; Lazo, P. A. The Subcellular Localization of Vaccinia-Related Kinase-2 (VRK2) Isoforms Determines Their Different Effect on P53 Stability in Tumour Cell Lines. FEBS J. 2006, 273 (11), 2487.

(7) Li, M.; Wang, Y.; Zheng, X. Bin; Ikeda, M.; Iwata, N.; Luo, X. J.; Chong, S. A.; Lee, J.; Rietisch, M.; Zhang, F.; et al. Meta-Analysis and Brain Imaging Data Support the Involvement of VRK2 (Rs2312147) in Schizophrenia Susceptibility. Schizophr. Res. 2012, 142 (1-3), 200.

(8) Chang, H.; Zhang, C.; Xiao, X.; Pu, X.; Liu, Z.; Wu, L.; Li, M. Further Evidence of VRK2 Rs2312147 Associated with Schizophrenia. World J. Biol. Psychiatry 2016, 17 (6), 457.

(9) Azimi, T.; Ghafoori-Fard, S.; Davood Omrani, M.; Mazdeh, M.; Arsang-Jang, S.; Sayad, A.; Taheri, M. Vaccinia Related Kinase 2 (VRK2) Expression in Neurological Disorders: Schizophrenia, Epilepsy and Multiple Sclerosis. Mult. Scler. Relat. Disord. 2018, 19, 15.

(10) Li, M.; Yue, W. VRK2, a Candidate Gene for Psychiatric and Neurological Disorders. Mol. Neuropsychiatry 2018, 4 (3), 119.

(11) Jeong, Y.-H.; Choi, J.-H.; Lee, D.; Kim, S.; Kim, K.-T. Vaccinia-Related Kinase 2 Modulates Role of Dysbindin by Regulating Protein Stability. J. Neurochem. 2018, 147 (5), 609.

(12) Huang, W.; Cui, X.; Chen, Y.; Shao, M.; Shao, X.; Shen, Y.; Liu, Q.; Wu, M.; Liu, J.; Ni, W.; et al. High VRK1 Expression Contributes to Cell Proliferation and Survival in Hepatocellular Carcinoma. Pathol. Res. Pract. 2016, 212 (3), 171.

(13) Ben, Z.; Gong, L.; Qiu, Y. High Expression of VRK1 Is Related to Poor Prognosis in Glioma. Pathol. Res. Pract. 2018, 214 (1), 112.

(14) Elkins, J. M.; Fedele, V.; Szklarz, M.; Abdul Azeez, K. R.; Salah, E.; Mikolajczyk, J.; Romanov, S.; Sepetov, N.; Huang, X. P.; Roth, B. L.; et al. Comprehensive Characterization of the Published Kinase Inhibitor Set. Nat. Biotechnol. 2016, 34 (1), 95.

(15) Coutiño, R. M.; Allerston, C. K.; Savitsky, A.; Azavedo, H.; Godoi, P. H.; Wells, C. I.; Mascarello, A.; de Souza Gama, F. H.; Massitter, K. B.; Zuercher, W. J.; et al. Structural Characterization of Human Vaccinia-Related Kinases (VRK) Bound to Small-Molecule Inhibitors Identifies Different P-Loop Conformations. Sci. Rep. 2017, 7 (1), 7501.

(16) Guimarães, C. R. W.; Rai, B. K.; Munchhof, M. J.; Liu, S.; Wang, J.; Bhattacharya, S. K.; Buckbinder, L. Understanding the Impact of the P-Loop Conformation on Kinase Selectivity. J. Chem. Inf. Model. 2011, 51 (6), 1199.

(17) Ammirati, M.; Bagley, S. W.; Bhattacharya, S. K.; Buckbinder, L.; Carlo, A. A.; Conrad, R.; Cortes, C.; Dow, R. L.; Dowling, M. S.; El-Kattan, A.; et al. Discovery of an in Vivo Tool to Establish Proof-of-Concept for MAP4K4-Based Antidiabetic Treatment. ACS Med. Chem. Lett. 2015, 6 (11), 1128.

(18) Fedorov, O.; Niesen, F. H.; Knapp, S. Kinase Inhibitor Selectivity Profiling Using Differential Scanning Fluorimetry. Methods Mol. Biol. 2012, 795, 109.

(19) Fedorov, O.; Marsden, B.; Pogacic, V.; Rollos, P.; Muller, S.; Bullock, A. N.; Schwaller, J.; Sundstrom, M.; Knapp, S. A Systematic Interaction Map of Validated Kinase Inhibitors with Ser/Thr Kinases. Proc. Natl. Acad. Sci. 2007, 104 (51), 20523.

(20) Qiu, J.; Stevenson, S. H.; O’Beirne, J. M.; Silverman, R. B. 2,6-Difluorophenol as a Biosostere of a Carboxylic Acid: Biosiosteric Analogues of Gamma-Aminobutyric Acid. J. Med. Chem. 1999, 42 (2), 329.

(21) Jain, R.; Mathur, M.; Lan, J.; Costales, A.; Atallah, G.; Ramurthy, S.; Subramanian, S.; Setti, L.; Feucht, P.; Warne, B.; et al. Discovery of Potent and Selective RSK Inhibitors as Biological Probes. J. Med. Chem. 2015, 58 (17), 6766.

(22) Barreiro, E. J.; Kummerle, A. E.; Fraga, C. A. M. The Methylation Effect in Medicinal Chemistry. Chem. Rev. 2011, 111 (9), 5215.

(23) Leung, C. S.; Leung, S. S. F.; Tirado-Rives, J.; Jorgensen, W. L. Methy1 Effects on Protein-Ligand Binding. J. Med. Chem. 2012, 55 (9), 4489.

(24) Xing, L.; Klug-Mceod, J.; Rai, B.; Lunney, E. A. Kinase Hinge Binding Scaffolds and Their Hydrogen Bond Patterns. Bioorganic Med. Chem. 2015, 23 (19), 6520.
Development of Pyridine-Based Inhibitors for the Human Vaccinia-Related Kinases 1 and 2

Ricardo A. M. Serafim, Fernando H. de Souza Gama, Caio V. dos Reis, Stanley N. S. Vasconcelos, André da Silva Santiago, Jéssica E. Takarada, Luiz A. Dutra, Fúlvia Di Pillo, Hatylas Azevedo, Alessandra Mascarello, Jonathan M. Elkins, Katlin B. Massirer, Opher Gileadi, Cristiano R. W. Guimarães, Rafael M. Couñago

1 Centro de Química Medicinal (CQMED), Centro de Biologia Molecular e Engenharia Genética (CBMEG), Universidade Estadual de Campinas (UNICAMP), Campinas, SP, 13083-875, Brazil
2 Structural Genomics Consortium, Departamento de Genética e Evolução, Instituto de Biologia, UNICAMP, Campinas, SP, 13083-886, Brazil
3 Aché Laboratórios Farmacêuticos S.A., Guarulhos, SP, Brazil
4 PhD Program in Genetics and Molecular Biology (PGBM), UNICAMP, Brazil
5 Structural Genomics Consortium, Nuffield Department of Medicine, University of Oxford, Oxford OX3 7DQ, UK.

Supplementary Information

Contents
Cloning, Expression, Purification and Crystallization ................................................................. S3
Structure Solution and Refinement ........................................................................................................... S4
Additional structural analysis .................................................................................................................. S5
Observed binding modes for 3,5-substituted 2-aminopyridines on the PDB ........................................... S6
Thermal-shift Assay (Differential Scanning fluorimetry – DSF) ............................................................. S7
Isothermal calorimetry measurements (ITC) ......................................................................................... S8
Cell viability analysis by MTT assay ..................................................................................................... S9
Chemistry – experimental section and analytical data ......................................................................... S10
**Supplementary Table S1: VRK1 and VRK2 ΔTm (°C) values for all the 44 aminopyridine derivatives.**

| #  | R1          | R2          | DSF ΔTm (°C) VRK1 | VRK2 |
|----|-------------|-------------|------------------|------|
| 5  | HO-F        | HO-CH₂-F   | 3.2 ± 0.3        | 2.2 ± 0.2 |
| 6  | HO-F        | HO-CH₂-F   | 0.7 ± 0.1        | 1.0 ± 0.1 |
| 7  | HO-F        | HO-CH₂-F   | 2.2 ± 0.2        | 1.3 ± 0.1 |
| 8  | HO-F        | HO-CH₂-F   | 2.3 ± 0.0        | 2.3 ± 0.1 |
| 9  | HO-F        | HO-CH₂-F   | 0.7 ± 0.2        | 0.7 ± 0.1 |
| 10 | HO-F        | HO-CH₂-F   | 1.1 ± 0.3        | 2.3 ± 0.3 |
| 11 | HO-F        | HO-CH₂-F   | 0.2 ± 0.3        | 1.2 ± 0.2 |
| 12 | HO-F        | HO-CH₂-F   | 1.0 ± 0.1        | 2.0 ± 0.2 |
| 13 | HO-F        | HO-CH₂-F   | 1.9 ± 0.1        | 2.6 ± 0.1 |
| 14 | HO-F        | HO-CH₂-F   | 2.0 ± 0.2        | 2.3 ± 0.2 |
| 15 | HO-F        | HO-CH₂-F   | 1.1 ± 0.2        | 1.7 ± 0.1 |
| 16 | HO-F        | HO-CH₂-F   | 0.5 ± 0.4        | 1.7 ± 0.1 |
| 17 | HO-F        | HO-CH₂-F   | 1.1 ± 0.1        | 1.1 ± 0.2 |
| 18 | HO-F        | HO-CH₂-F   | 2.1 ± 0.7        | 3.2 ± 0.0 |
| 19 | HO-F        | HO-CH₂-F   | 2.2 ± 0.2        | 3.0 ± 0.0 |
| 20 | HO-F        | HO-CH₂-F   | -0.1 ± 0.4       | 0.8 ± 0.1 |
| 21 | HO-F        | HO-CH₂-F   | 0.1 ± 0.0        | 0.7 ± 0.0 |
| 22 | HO-F        | HO-CH₂-F   | -0.2 ± 0.0       | 0.3 ± 0.1 |
| 23 | HO-F        | HO-CH₂-F   | 1.1 ± 0.1        | 2.1 ± 0.1 |
| 24 | HO-F        | HO-CH₂-F   | 0.9 ± 0.2        | 1.1 ± 0.1 |
| 25 | HO-F        | HO-CH₂-F   | 1.4 ± 0.1        | 1.1 ± 0.1 |
| 26 | HO-F        | HO-CH₂-F   | 1.4 ± 0.2        | 1.0 ± 0.4 |
| 27 | HO-F        | HO-CH₂-F   | 1.1 ± 0.1        | 2.1 ± 0.1 |
| 28 | HO-F        | HO-CH₂-F   | 0.9 ± 0.2        | 1.1 ± 0.1 |
| 29 | HO-F        | HO-CH₂-F   | 1.4 ± 0.1        | 1.1 ± 0.1 |
| 30 | HO-F        | HO-CH₂-F   | 1.4 ± 0.2        | 1.0 ± 0.4 |
| 31 | HO-F        | HO-CH₂-F   | 0.9 ± 0.2        | 1.0 ± 0.1 |
| 32 | HO-F        | HO-CH₂-F   | 0.8 ± 0.3        | 1.0 ± 0.1 |
| 33 | HO-F        | HO-CH₂-F   | -0.1 ± 0.7       | 0.6 ± 0.2 |
| 34 | HO-F        | HO-CH₂-F   | 1.2 ± 0.4        | 0.5 ± 0.3 |
| 35 | HO-F        | HO-CH₂-F   | -0.3 ± 0.5       | 0.5 ± 0.2 |
| 36 | HO-F        | HO-CH₂-F   | 0.0 ± 0.1        | 0.4 ± 0.1 |
| 37 | HO-F        | HO-CH₂-F   | 0.9 ± 0.2        | 0.4 ± 0.1 |
| 38 | HO-F        | HO-CH₂-F   | 0.8 ± 0.0        | 0.4 ± 0.2 |
| 39 | HO-F        | HO-CH₂-F   | 0.6 ± 0.4        | 0.3 ± 0.1 |
| 40 | HO-F        | HO-CH₂-F   | 0.5 ± 0.0        | 0.3 ± 0.1 |
| 41 | HO-F        | HO-CH₂-F   | 0.0 ± 0.1        | 0.2 ± 0.1 |
| 42 | HO-F        | HO-CH₂-F   | 0.5 ± 0.1        | 0.2 ± 0.0 |
| 43 | HO-F        | HO-CH₂-F   | 0.5 ± 0.1        | 0.1 ± 0.1 |
| 44 | HO-F        | HO-CH₂-F   | -0.4 ± 0.4       | 0.1 ± 0.1 |
| 45 | HO-F        | HO-CH₂-F   | 0.2 ± 0.4        | 0.1 ± 0.0 |
| 46 | HO-F        | HO-CH₂-F   | 0.3 ± 0.0        | 0.0 ± 0.0 |
| 47 | HO-F        | HO-CH₂-F   | 0.3 ± 0.2        | 0.0 ± 0.2 |
| 48 | HO-F        | HO-CH₂-F   | 0.5 ± 0.4        | -0.1 ± 0.4 |
| 49 | HO-F        | HO-CH₂-F   | -0.9 ± 0.5       | 0.1 ± 0.2 |
| 50 | HO-F        | HO-CH₂-F   | -0.1 ± 0.6       | 0.5 ± 0.1 |
| 51 | HO-F        | HO-CH₂-F   | 1.6 ± 0.4        | -0.4 ± 0.1 |
| 52 | HO-F        | HO-CH₂-F   | 3.5 ± 0.3        | 1.1 ± 0.2 |
Cloning, expression and purification of VRK1-KD (residues 3-364) and VRK2-KD (residues 14-335) have been reported before. To improve VRK1-KD crystallizability, four clusters of surface entropy reduction mutations (SER) were engineered into this protein: K34A/K35A/E36A; E212A/K214A/E215A; E292A/K293A/K295A and K359A/K360A. The full-length VRK1 (residues 1-396) was cloned into vector pNIC28-Bsa4 using a ligation independent cloning strategy. Our cloning strategy introduced a tobacco etch virus (TEV) protease-cleavable, N-terminal 6xHis tag. For protein production, BL21(DE3)-R3 cells, including a plasmid expressing lambda phosphatase, were cultivated in TB medium (supplemented with 50 μg.ml−1 kanamycin, 35 μg.ml−1 chloramphenicol) at 37 °C until OD600 reached ~3 and then cooled to 18 °C for 1 hour. Isopropyl 1-thio-D-galactopyranoside (IPTG) was added to 0.1 mM, and growth continued at 18 °C overnight. Cells were collected by centrifugation and pellets suspended in 2x lysis buffer (lysis buffer is 50 mM HEPES buffer, pH 7.5, 0.5 M NaCl, 10 mM imidazole, 0.5 mM tris(2-carboxyethyl)phosphine [TCEP], Protease Inhibitors Cocktail Set VII - Calbiochem, 1/1000 dilution) prior to flash-freezing in liquid nitrogen. After thawing, cells were lysed by sonication on ice. Proteins were purified using Ni-Sepharose resin (GE Healthcare) and eluted stepwise in binding buffer with 300 mM imidazole. Removal of hexahistidine tags was performed at 4 °C overnight using recombinant TEV protease while dialyzing against excess gel filtration buffer (25 mM HEPES, 500 mM NaCl, 0.5 mM TCEP, 5% [v/v] glycerol). Proteins were further purified by reverse affinity in Ni-Sepharose followed by gel filtration (Superdex 200 16/60, GE Healthcare). Protein in gel filtration buffer was concentrated to 14 mg.ml−1 (VRK1-KD) or 20 mg.ml−1 (VRK2-KD) using 30 kDa MWCO centrifugal concentrators (Millipore) at 4 °C. Compounds in 100% DMSO were added to protein solutions at 3-fold molar excess and incubated on ice for approximately 30 minutes. This mixture was centrifuged at 14,000 rpm for 10 minutes at 4 °C prior to setting up 150-nl volume sitting drop at three ratios of protein-inhibitor complex to reservoir solution (2:1, 1:1, or 1:2). Crystallization experiments were performed at 20 °C. Crystals were cryoprotected in reservoir solution supplemented with 20–25% glycerol before flash-freezing in liquid nitrogen for data collection. Diffraction data were collected at 100 K at the Advanced Photon Source 24ID-C (APS, Chicago), for VRK2, and at the Advanced Photon Source 24ID-E (APS, Chicago), for VRK1. The best-diffracting crystals grew under the conditions described in Table S1. Crystal optimization used Newman’s buffer system.
Structure Solution and Refinement

Diffraction data were integrated using XDS\(^7\) and scaled using AIMLESS from the CCP4 software suite\(^8\). Molecular replacement (MR) for VRK1-KD bound to 5 was performed with Phaser\(^9\) using VRK1-KD bound to 1 (BI-D1870) (PDB ID 5UVF)\(^10\). VRK2-KD bound to 1 (BI-1870) (PDB ID 5UU1)\(^10\) was used as MR search model for the VRK2-KD bound to 18 dataset. Automated refinement was performed in PHENIX.\(^11\) Coot\(^12\) was used for manual model building and refinement. Structure validation was performed using MolProbity.\(^13\) Structure factors and coordinates have been deposited in the PDB (see Supplementary Table S2).

### Supplementary Table S2 - Crystallographic data

| Data collection   | VRK2/18          | VRK1/5          |
|-------------------|------------------|-----------------|
| X-ray source      | APS 24-ID-C      | APS 24-ID-E     |
| Wavelength (Å)    | 0.979100         | 0.979180        |
| Spatial group     | P 21 21 21       | P 21 21 21      |
| Unit cell (Å)     | 54.53, 67.62, 171.35 | 92.22, 96.57, 193.06 |
| Molecules / asymmetric unit | 2 | 4 |
| Resolution range (Å) | 29.10-2.45    | 19.98-1.80      |
| High resolution range (Å) | 2.55-2.45     | 1.83-1.80      |
| Unique reflections | 24,095 (2,679) | 159,713 (7,810) |
| R\(_{merge}\)     | 0.137 (1.773)   | 0.129 (2.177)  |
| R\(_p.i.m\)       | 0.061 (0.824)   | 0.052 (0.882)  |
| Mean I/σ(I)       | 10.8 (1.2)      | 13.9 (1.5)     |
| Mean CC(1/2)      | 0.998 (0.707)   | 0.999 (0.698)  |
| Completeness (%)  | 99.9 (99.9)     | 99.9 (100.0)   |
| Redundancy        | 10.6 (10.3)     | 13.6 (13.6)    |

### Refinement

| Resolution (Å)    | 29.10-2.45      | 19.98-1.80    |
| RMS bond (Å)      | 0.0084          | 0.012         |
| RMS angle (°)     | 1.994           | 1.497         |
| Average B factor (Å\(^2\)) | 65.06    | 33.22        |
| R\(_{work}\) / R\(_{free}\) (%) | 22.0 / 29.7 | 18.40 / 21.25 |
| N° of atoms (protein/solvent/ligand) | 4,466/136/52 | 9,904/1,173/112 |

### Ramachandran statistics (%)

| Favored / Allowed / Outliers | 98/2/0 | 98/2/0 |
| PDB ID                      | 6NCG   | 6BU6   |

### Crystallization conditions

- **VRK2/18**: 27.5% PEG3350; 200 mM Lithium Sulfate; 0.1 M Buffer system SBG, pH 6.0
- **VRK1/5**: 22% PEG3350; 200 mM Lithium Sulfate; 0.1M Buffer system SBG, pH 7.0
**Supplementary Figure S1**: (A-B) Overall structures of VRK1-KD and VRK2-KD bound to 2-aminopyridine compounds 5 and 18, respectively. (C) Differences in the position of the conserved aromatic residue within VRK1-KD (white) and VRK2-KD (yellow) - Phe48 and Phe40, respectively. (D-E) Molecular surface representation of VRK1-KD bound to 5. (F) Molecular surface representation of VRK1-KD bound to 18.
Supplementary Figure S2: Observed binding modes for 3,5-substituted 2-aminopyridines to human Ser/Thr kinases in the Protein Data Bank. (A) MAP4K4 bound to a 3,5-substituted 2-aminopyridine inhibitor represents the most frequently-observed binding mode for this chemical core. (B) Alternative binding mode for compounds containing a 3,5-substituted 2-aminopyridine core to human Ser/Thr kinases.
VRK1-FL and VRK2-KD proteins were screened against an in-house collection of 44 aminopyridine compounds (Table S2). DSF measurements were made in a 384-well plate. Each well contained 20 μL of 2 μM kinase in 20 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol and the Applied Biosystems Protein Thermal Shift dye at the recommended dilution of 1:1000.

Compounds were solubilized in 100% DMSO to 10 mM final concentration and 100 nL was transferred using a pin transfer device (CyBio) to 10 μM final concentration and 0.1% DMSO. Plates were sealed using optically clear films and transferred to a QuantStudio 6 qPCR instrument (Applied Biosystems). The fluorescence intensity was measured during a temperature gradient from 25 to 95 °C at a constant rate of 0.05 °C/s and protein melting temperatures were calculated based on a Boltzmann function fitting to experimental data, as implemented in the Protein Thermal Shift Software (Applied Biosystems). Protein in 0.1% DMSO was used as a reference. Compounds that caused a shift in protein melting temperature ($\Delta T_m$) of 2 °C or higher compared to the reference were considered positive hits.
Isothermal calorimetry measurements (ITC)

ITC measurements were made using a MicroCal Auto-iTC200 (Malvern, UK), set to 20 °C, 1,000 rpm stirring, 1.5 µL injections, and 180 s between each injection. For all measurements VRK1-FL and VRK2-KD were dialysed overnight against gel filtration buffer, and the dialysis buffer was used to dilute inhibitors 26, 18, 19, and 13. VRK1-FL and VRK2-KD were titrated into a solution containing the inhibitor at one tenth of the protein concentration. The concentrations used for each measurement were: 25 μM 26 and 250 μM VRK1, 24.7 μM 18 and 247 μM VRK2A, 22.7 μM 19 and 227 μM VRK2A, 23.97 μM 13 and 239.7 μM VRK2A. ITC data was analysed with NITPIC, and SEDPHAT; figures were made using GUSSI.14

Supplementary Table S3: Isothermal calorimetry measurements data

| Protein | Inhibitor | ΔG (kJ/mol) | ΔH (kJ/mol) | TAS (kJ/mol) | K_D (nM) |
|---------|-----------|-------------|-------------|--------------|----------|
| VRK1    | 26        | -37,7       | -55,6       | -17,9        | 190      |
| VRK2    | 18        | -35.9       | -24.9       | 11.0         | 401      |
| VRK2    | 19        | -33.8       | -39.9       | -6.2         | 968      |
| VRK2    | 13        | -35.5       | -33.4       | 2.1          | 468      |
Cell viability analysis by MTT assay

The effect of the compounds 26 and 18 on cellular proliferation was assessed by a 3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay, using standard protocol. MTT is a water-soluble tetrazolium salt which is converted to an insoluble purple within the mitochondria. The formazan product is impermeable to the cell membranes and it accumulates in healthy cells. Its concentration can be estimated by optical density. HeLa cells were seeded in a 96-well plate and incubated during 12h with Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum in a CO2 incubator. The medium was removed and the treatment medium was added with different concentrations of 26, 18, or Staurosporine (control). After 24h of treatment, 10 μL of MTT solution (2.5 mg/mL PBS, pH 7.2) was added to each well and incubated at 37 °C for 4h in the dark. The supernatant was removed and 100 μL of DMSO was added into each well to dissolve the formazan crystals. The absorbance was measured at 570 nm and results were expressed as the mean of three biological replicates as a percentage of control (non-treated cells as 100% viability and 5% DMSO cells as 0% viability).

Supplementary Figure S3: Cell viability of compounds 26, 18 and Staurosporine (used as a control). Values shown represent the mean (± standard deviation) of three independent measurements.
All reactions were carried out under a nitrogen atmosphere; all final compounds were characterized by $^1$H NMR, $^{13}$C NMR and electrospray ionization-mass spectrometry (ESI-MS). NMR spectra were recorded on a Bruker 400 MHz or 500 MHz spectrometer. All $^1$H NMR experiments are reported in δ units, parts per million (ppm), and were measured relative to the solvent signals (DMSO) (2.50 ppm). All $^{13}$C NMR spectra are reported in ppm relative to DMSO (39.52 ppm). Sample was submitted for MS analysis to control the exact mass of the compound synthetized. 1μl of the sample (diluted for a final concentration of 0.1 μg/μl in 100% acetonitrile) was analyzed by reverse phase HPLC-ESI-MS using an Acquity H-class HPLC system (Waters Corp. Milford, MA, USA) which is directly connected to the XEVO G2 Sx Q-ToF (Waters) to determine the intact mass of the small compound. The HPLC is equipped with C18 column (ACQUITY UPLC Protein BEH C18 Column, 1.7 μm, 2.1 mm X 50 mm, Waters) for small molecule separation kept at 45 °C. The mobile phase solvent A was 0.1% Formic acid (FA) in water, and solvent B was 0.1% FA in 100% Acetonitrile (ACN). The sample were loaded at a flow rate of 0.5 μl/min, and eluted form C18 column at a flow rate of 400 μl/min with one linear gradient step: one from 3 to 90% solvent B over 2.5 min. The column was regenerated by washing at 100% solvent B for 1.5 min and re-equilibrated at 1% solvent B for 3 min. Exact mass analysis was performed in positive ion electrospray in resolution mode. For internal calibration, the locksspray properties were the following: scan time was fixed at 0.5 seconds, with a mass window of 0.5 Da around Leu-enkephalin (556.2771 Da). The ToF-MS acquisition ranged from 50 Da to 2,000 Da with a scan time fixed at 0.5 second. The cone voltage on the ESI source was fixed at 40 V. MS Raw data was analyzed using MassLynx software developed by Waters. The purity of all final compounds was found to be >95%. Palladium catalyst, boronic acids and cesium carbonate were purchased from commercial source and used as received.
**General procedure for Suzuki-Miyaura coupling**

![Reaction Scheme](image)

A vial equipped with a magnetic stirrer bar, was charged with Cs₂CO₃ (244 mg, 0.75 mmol), Pd(dppf)Cl₂ (22 mg, 10 mol%), boronic acid (0.34 mmol), 3-iodoaminopyridine (90 mg, 0.3 mmol) and ethanol/water 5:1 (2 mL). The reaction mixture was heated at 85 °C under microwave and nitrogen atmosphere for 1 hour (TLC was used to monitor the reaction). After completion, the reaction mixture was filtered through a pad of celite with ethyl acetate as the washing solvent. The product was purified by silica flash chromatography and characterized by ¹H NMR, ¹³C NMR and HRMS. We used the same procedure for both steps.

**4,4’-(2-aminopyridine-3,5-diyl)bis(2,6-difluorophenol)**

The compound 5 was isolated in 51% yield. ¹H NMR (500 MHz, DMSO) δ 10.27 (s, 1H), 10.13 (s, 1H), 8.27 (d, J = 2.4 Hz, 1H), 7.62 (d, J = 2.4 Hz, 1H), 7.45 – 7.35 (m, 2H), 7.25 – 7.16 (m, 2H), 5.90 (s, 2H). ¹³C NMR (126 MHz, DMSO) δ 155.94, 153.72 (d, J = 7.7 Hz), 153.35 (d, J = 7.5 Hz), 151.80 (d, J = 7.7 Hz), 151.42 (d, J = 7.7 Hz), 147.88, 144.85, 135.41, 133.00 (t, J = 16.1 Hz), 132.14 (t, J = 16.3 Hz), 128.62 (t, J = 8.6 Hz), 128.15 (t, J = 8.7 Hz), 123.09, 118.30, 112.30 (dd, J = 15.8, 6.5 Hz), 108.84 (dd, J = 16.2, 6.5 Hz). HRMS (ES): calcd for C₁₇H₁₀F₄N₂O₂ [M+H]⁺: 351.0757. Found: 351.0758.

**4,4’-(2-aminopyridine-3,5-diyl)diphenol**

The compound 6 was isolated in 71% yield. ¹H NMR (500 MHz, DMSO) δ 9.57 (s, 1H), 9.45 (s, 1H), 8.16 (s, 1H), 7.46 (d, J = 1.8 Hz, 1H), 7.42 (d, J = 8.4 Hz, 2H), 7.33 (d, J = 8.4 Hz, 2H), 6.88 (d, J = 8.4 Hz, 2H), 6.82 (d, J = 8.4 Hz, 2H), 5.51 (s, 2H). ¹³C NMR (126 MHz, DMSO) δ 156.89, 156.44, 155.38, 143.56, 134.95, 129.75, 128.79, 128.60, 126.84, 125.82, 120.70, 115.79. HRMS (ES): calcd for C₁₇H₁₄N₂O₂ [M+H]⁺: 279.1134. Found: 279.1195.
The compound 7 was isolated in 76% yield. \(^{1}H\) NMR (500 MHz, DMSO) \(\delta\) 10.09 (s, 1H), 9.56 (s, 1H), 8.22 (d, \(J = 2.4\) Hz, 1H), 7.54 (d, \(J = 2.3\) Hz, 1H), 7.34 (dd, \(J = 14.8, 9.1\) Hz, 4H), 6.86 (d, \(J = 8.5\) Hz, 2H), 5.65 (s, 2H). \(^{13}C\) NMR (126 MHz, DMSO) \(\delta\) 156.88, 156.08, 153.70 (d, \(J = 7.8\) Hz), 151.78 (d, \(J = 7.8\) Hz), 143.99, 134.93, 132.05 (t, \(J = 16.3\) Hz), 129.76, 128.86 (t, \(J = 8.8\) Hz), 128.23, 120.51, 115.67, 108.77 (dd, \(J = 16.1, 6.3\) Hz). HRMS (ES): calcd for C\(_{17}\)H\(_{12}\)F\(_2\)N\(_2\)O\(_2\) \([M+H]^+\): 315.0945. Found: 315.0957.

The compound 8 was isolated in 75% yield. \(^{1}H\) NMR (500 MHz, DMSO) \(\delta\) 10.26 (s, 1H), 9.40 (s, 1H), 8.17 (d, \(J = 2.3\) Hz, 1H), 7.51 (d, \(J = 2.3\) Hz, 1H), 7.43 (d, \(J = 8.6\) Hz, 2H), 6.80 (d, \(J = 8.6\) Hz, 2H), 5.72 (s, 2H). \(^{13}C\) NMR (126 MHz, DMSO) \(\delta\) 156.42, 155.18, 153.33 (d, \(J = 7.5\) Hz), 151.41 (d, \(J = 7.7\) Hz), 144.38, 135.25, 132.91 (t, \(J = 16.1\) Hz), 128.49, 126.83, 125.55, 118.37, 115.66, 112.21 (dd, \(J = 15.9, 6.3\) Hz). HRMS (ES): calcd for C\(_{17}\)H\(_{12}\)F\(_2\)N\(_2\)O\(_2\) \([M+H]^+\): 315.0945. Found: 315.0920.

The compound 9 was isolated in 38% yield. \(^{1}H\) NMR (400 MHz, DMSO) \(\delta\) 10.31 (s, 1H), 8.37 (d, \(J = 2.4\) Hz, 1H), 7.71 (d, \(J = 2.4\) Hz, 1H), 7.47 (dd, \(J = 9.4, 2.1\) Hz, 2H), 7.26 – 7.17 (m, 2H), 7.09 (tt, \(J = 9.2, 2.1\) Hz, 1H), 6.07 (s, 2H). \(^{13}C\) NMR (126 MHz, DMSO) \(\delta\) 163.92 (d, \(J = 14.0\) Hz), 161.97 (d, \(J = 14.0\) Hz), 156.74, 153.34 (d, \(J = 8.0\) Hz), 151.41 (d, \(J = 7.7\) Hz), 145.58, 142.61 – 140.09 (m), 135.78, 133.59 – 131.66 (m), 127.92 (d, \(J = 8.7\) Hz), 122.47, 118.28, 112.32 (dd, \(J = 15.9, 6.4\) Hz), 108.36 (dd, \(J = 19.9, 6.1\) Hz), 101.45 (t, \(J = 26.2\) Hz). HRMS (ES): calcd for C\(_{17}\)H\(_{10}\)F\(_4\)N\(_2\)O \([M+H]^+\): 335.0808. Found: 335.0824.

The compound 10 was isolated in 89% yield. \(^{1}H\) NMR (500 MHz, DMSO) \(\delta\) 10.29 (s, 1H), 8.36 (d, \(J = 2.4\) Hz, 1H), 8.01 – 7.95 (m, 2H), 7.73 (d, \(J = 2.4\) Hz, 1H), 7.63 (dd, \(J = 8.1, 5.2\) Hz, 2H), 7.26 – 7.19 (m, 2H), 6.01 (s, 2H). \(^{13}C\) NMR (126 MHz, DMSO) \(\delta\) 156.49, 153.35 (d, \(J = 7.8\) Hz), 151.42 (d, \(J = 7.7\) Hz), 145.53, 138.84, 135.95, 133.04 (t, \(J = 16.2\) Hz), 130.14, 129.87 (d, \(J = 5.2\) Hz), 129.54 (t, \(J = 15.6\) Hz), 128.04 (t, \(J = 8.7\) Hz), 127.57, 125.40, 123.44, 123.23, 122.94 (d, \(J = 3.8\) Hz), 122.04.
(d, J = 3.8 Hz), 121.06, 118.45, 112.35 (dd, J = 15.8, 6.4 Hz). HRMS (ES): calcd for C\textsubscript{18}H\textsubscript{11}F\textsubscript{5}N\textsubscript{2}O [M+H]\textsuperscript{+}: 367.0870. Found: 367.0944.

4-(2-amino-5-(4-(trifluoromethyl)phenyl)pyridin-3-yl)-2,6-difluorophenol
The compound 11 was isolated in 62% yield. \textsuperscript{1}H NMR (500 MHz, DMSO) δ 10.30 (s, 1H), 8.37 (d, J = 2.4 Hz, 1H), 7.89 (d, J = 8.2 Hz, 2H), 7.73 (d, J = 8.3 Hz, 2H), 7.71 (d, J = 2.4 Hz, 1H), 7.25 – 7.17 (m, 2H), 6.06 (s, 2H). \textsuperscript{13}C NMR (126 MHz, DMSO) δ 156.66, 153.35 (d, J = 7.7 Hz), 151.43 (d, J = 7.8 Hz), 145.66, 141.76, 135.90, 133.07 (t, J = 16.3 Hz), 127.99 (t, J = 8.6 Hz), 126.86, 126.61, 126.09, 125.59 (dd, J = 7.2, 3.4 Hz), 123.33 (d, J = 14.3 Hz), 118.48, 112.30 (dd, J = 15.9, 6.4 Hz). HRMS (ES): calcd for C\textsubscript{18}H\textsubscript{11}F\textsubscript{5}N\textsubscript{2}O \textsuperscript{2}[M+H]\textsuperscript{+}: 367.0870. Found: 367.0905.

4-(6-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)-N-cyclopropylbenzenesulfonamide
The compound 12 was isolated in 61% yield. \textsuperscript{1}H NMR (500 MHz, DMSO) δ 10.29 (s, 1H), 8.40 (d, J = 2.4 Hz, 1H), 7.90 (dd, J = 11.0, 5.6 Hz, 3H), 7.81 (d, J = 8.6 Hz, 2H), 7.73 (d, J = 2.4 Hz, 1H), 7.26 – 7.18 (m, 2H), 6.08 (s, 2H), 2.12 (ddd, J = 9.8, 6.7, 3.4 Hz, 1H), 0.49 (td, J = 7.0, 4.9 Hz, 2H), 0.43 – 0.38 (m, 2H). \textsuperscript{13}C NMR (126 MHz, DMSO) δ 156.67, 153.35 (d, J = 7.8 Hz), 145.72, 141.56, 137.85, 135.90, 133.06 (t, J = 16.2 Hz), 128.17 – 127.83 (m), 127.35, 125.82, 123.17, 118.48, 112.30 (dd, J = 15.8, 6.5 Hz), 24.09, 5.10. HRMS (ES): calcd for C\textsubscript{20}H\textsubscript{17}F\textsubscript{2}N\textsubscript{3}O\textsubscript{3}S \textsuperscript{3}[M+H]\textsuperscript{+}: 418.1037. Found: 418.1056.

3-(6-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)-N-cyclopropylbenzenesulfonamide
The compound 13 was isolated in 66% yield. \textsuperscript{1}H NMR (500 MHz, DMSO) δ 10.29 (s, 1H), 8.33 (d, J = 2.4 Hz, 1H), 8.01 (t, J = 1.6 Hz, 1H), 7.96 (d, J = 7.8 Hz, 1H), 7.88 (d, J = 2.7 Hz, 1H), 7.72 (d, J = 8.0 Hz, 1H), 7.64 (dd, J = 13.0, 5.1 Hz, 2H), 7.25 – 7.17 (m, 2H), 6.03 (s, 2H), 2.14 – 2.08 (m, 1H), 0.48 (td, J = 7.0, 5.0 Hz, 2H), 0.42 – 0.38 (m, 2H). \textsuperscript{13}C NMR (126 MHz, DMSO) δ 156.56, 153.36 (d, J = 7.6 Hz), 151.44 (d, J = 7.7 Hz), 145.35, 140.92, 138.66, 135.71, 133.10 (t, J = 16.1 Hz), 129.70, 129.48, 127.97 (t, J = 8.7 Hz), 124.64, 123.30 (d, J = 19.5 Hz), 118.59, 112.31 (dd, J = 15.8, 6.4 Hz), 24.20, 5.09. HRMS (ES): calcd for C\textsubscript{20}H\textsubscript{17}F\textsubscript{2}N\textsubscript{3}O\textsubscript{3}S \textsuperscript{3}[M+H]\textsuperscript{+}: 418.1037. Found: 418.1056.
**3-(6-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)-2-fluorobenzonitrile**

The compound 14 was isolated in 27% yield. $^1$H NMR (500 MHz, DMSO) $\delta$ 10.30 (s, 1H), 8.21 (t, $J = 2.1$ Hz, 1H), 7.97 (td, $J = 7.9, 1.6$ Hz, 1H), 7.85 (ddd, $J = 7.6, 5.9, 1.6$ Hz, 1H), 7.58 (s, 1H), 7.46 (t, $J = 7.8$ Hz, 1H), 7.23 – 7.14 (m, 2H), 6.13 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 160.42, 158.37, 156.66, 153.36 (d, $J = 7.9$ Hz), 151.43 (d, $J = 8.3$ Hz), 147.16 (d, $J = 3.9$ Hz), 137.53, 135.52 (d, $J = 3.8$ Hz), 133.34 – 132.92 (m), 132.01, 127.71 (d, $J = 8.2$ Hz), 126.91 (d, $J = 12.1$ Hz), 125.84 (d, $J = 3.5$ Hz), 117.87, 114.25, 112.25 (dd, $J = 16.1, 6.2$ Hz), 101.01. HRMS (ES): calcd for C$_{18}$H$_{10}$F$_3$N$_3$O $[M+H]^+$: 342.0854. Found: 342.0868.

**4-(6-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)benzonitrile**

The compound 15 was isolated in 63% yield. $^1$H NMR (500 MHz, DMSO) $\delta$ 10.29 (s, 1H), 8.40 (d, $J = 2.4$ Hz, 1H), 7.89 (d, $J = 8.6$ Hz, 2H), 7.84 (d, $J = 8.6$ Hz, 2H), 7.73 (d, $J = 2.4$ Hz, 1H), 7.25 – 7.17 (m, 2H), 6.12 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 156.85, 153.34 (d, $J = 7.8$ Hz), 151.42 (d, $J = 7.7$ Hz), 145.87, 142.32, 135.86, 133.09 (t, $J = 16.1$ Hz), 132.70, 127.88 (t, $J = 8.6$ Hz), 126.08, 122.86, 119.07, 118.47, 112.31 (dd, $J = 15.8, 6.5$ Hz), 108.65. HRMS (ES): calcd for C$_{18}$H$_{11}$F$_2$N$_3$O $[M+H]^+$: 324.0948. Found: 324.0960.

**3-(6-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)benzonitrile**

The compound 16 was isolated in 64% yield. $^1$H NMR (500 MHz, DMSO) $\delta$ 10.28 (s, 1H), 8.37 (d, $J = 2.4$ Hz, 1H), 8.19 (t, $J = 1.5$ Hz, 1H), 8.04 – 8.01 (m, 1H), 7.72 (ddd, $J = 7.6, 4.9, 1.8$ Hz, 2H), 7.60 (t, $J = 7.8$ Hz, 1H), 7.26 – 7.19 (m, 2H), 6.03 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 156.56, 153.34 (d, $J = 7.7$ Hz), 151.42 (d, $J = 7.5$ Hz), 145.50, 138.90, 135.86, 133.04 (t, $J = 16.2$ Hz), 130.15, 129.94 (d, $J = 8.3$ Hz), 129.05, 128.00 (t, $J = 8.8$ Hz), 122.81, 118.93, 118.41, 112.31 (dd, $J = 15.9, 6.5$ Hz), 112.01. HRMS (ES): calcd for C$_{18}$H$_{11}$F$_2$N$_3$O $[M+H]^+$: 324.0948. Found: 324.0960.

**3-(6-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)benzoic acid**

The compound 17 was isolated in 25% yield. $^1$H NMR (500 MHz, DMSO) $\delta$ 8.31 (d, $J = 2.3$ Hz, 1H), 8.14 (s, 1H), 7.87 (dd, $J = 12.8, 7.8$ Hz, 2H), 7.65 (d, $J = 2.3$ Hz, 1H), 7.53 (t, $J = 7.7$ Hz, 1H), 7.27 – 7.18 (m, 2H), 5.95 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 156.24, 153.41, 151.50 (t, $J = 9.9$ Hz), 118.93, 118.41, 129.05, 128.00 (t, $J = 8.8$ Hz), 122.81, 119.07, 129.05, 128.00 (t, $J = 8.8$ Hz), 122.81, 118.93, 118.41, 112.31 (dd, $J = 15.9, 6.5$ Hz), 112.01. HRMS (ES): calcd for C$_{18}$H$_{11}$F$_2$N$_3$O $[M+H]^+$: 324.0948. Found: 324.0960.
The compound 18 was isolated in 63% yield. $^1$H NMR (500 MHz, DMSO) $\delta$ 10.32 (s, 1H), 8.37 (d, $J = 2.4$ Hz, 1H), 7.86 (d, $J = 8.7$ Hz, 2H), 7.82 (d, $J = 8.7$ Hz, 2H), 7.70 (d, $J = 2.4$ Hz, 1H), 7.34 (s, 2H), 7.21 (d, $J = 7.9$ Hz, 2H), 6.05 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 156.61, 153.39 (d, $J = 7.7$ Hz), 151.46 (d, $J = 7.8$ Hz), 145.64, 141.85, 140.96, 135.87, 133.16 (t, $J = 14.6$ Hz), 127.93 (t, $J = 8.9$ Hz), 126.22, 125.70, 123.39, 118.50, 112.31 (dd, $J = 15.8, 6.5$ Hz). HRMS (ES): calcd for C$_{18}$H$_{12}$F$_2$N$_2$O$_3$ [M+H]$^+$: 343.0894. Found: 343.0868.

4-(6-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)benzenesulfonamide

The compound 19 was isolated in 64% yield. $^1$H NMR (500 MHz, DMSO) $\delta$ 10.30 (s, 1H), 9.35 (s, 1H), 8.48 (d, $J = 1.4$ Hz, 1H), 8.43 (d, $J = 2.4$ Hz, 1H), 8.09 (d, $J = 8.5$ Hz, 1H), 7.85 (dd, $J = 15.8, 6.5$ Hz). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 156.09, 155.73, 153.36 (d, $J = 7.7$ Hz), 151.82, 151.44 (d, $J = 7.7$ Hz), 145.49, 136.07, 135.27, 134.58, 133.02 (t, $J = 16.0$ Hz), 128.20 (t, $J = 8.5$ Hz), 124.40 (d, $J = 7.7$ Hz), 123.13, 119.01, 118.49, 112.29 (dd, $J = 15.8, 6.5$ Hz). HRMS (ES): calcd for C$_{18}$H$_{11}$F$_2$N$_3$OS [M+H]$^+$: 356.0669. Found: 356.0696.

4-(2-amino-5-(benzo[d]thiazol-6-yl)pyridin-3-yl)-2,6-difluorophenol

The compound 20 was isolated in 70% yield. $^1$H NMR (500 MHz, DMSO) $\delta$ 8.35 (d, $J = 2.4$ Hz, 1H), 7.83 (q, $J = 8.7$ Hz, 4H), 7.64 (d, $J = 2.4$ Hz, 1H), 7.33 (s, 2H), 7.02 (d, $J = 1.9$ Hz, 1H), 6.99 (dd, $J = 8.3, 2.0$ Hz, 1H), 6.96 (d, $J = 8.2$ Hz, 1H), 5.87 (s, 2H), 4.28 (s, 4H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 156.65, 145.15, 143.60, 143.00, 141.80, 141.09, 135.56, 130.75, 126.24, 125.68, 123.50, 121.48, 120.04, 117.49, 117.29, 64.10. HRMS (ES): calcd for C$_{19}$H$_{17}$F$_2$N$_3$OS [M+H]$^+$: 384.1018. Found: 384.1028.

4-(6-amino-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)pyridin-3-yl)benzenesulfonamide

The compound 21 was isolated in 52% yield. $^1$H NMR (500 MHz, DMSO) $\delta$ 8.43 (d, $J = 2.4$ Hz, 1H), 7.91 (d, $J = 8.4$ Hz, 2H), 7.87 (d, $J = 8.6$ Hz, 2H), 7.83 (d, $J = 8.6$ Hz, 2H), 7.75 (d, $J = 7.0$ Hz, 3H), 7.39 (s, 4H), 6.09 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 156.46, 146.22, 143.03, 141.93, 141.30, 140.87, 136.22, 129.19, 126.23 (d, $J = 5.7$ Hz), 125.74, 123.58, 119.01. HRSM (ES): calcd for C$_{17}$H$_{16}$N$_2$O$_5$S [M+H]$^+$: 405.0691. Found: 405.0706.
4-(6-amino-5-(4-nitrophenyl)pyridin-3-yl)benzenesulfonamide
The compound 22 was isolated in 33% yield. $^1$H NMR (500 MHz, DMSO) δ 8.46 (d, $J = 2.4$ Hz, 1H), 8.32 (d, $J = 8.8$ Hz, 2H), 7.93 – 7.82 (m, 6H), 7.81 (d, $J = 2.4$ Hz, 1H), 7.35 (s, 2H), 6.21 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) δ 156.41, 146.82, 146.52, 145.05, 141.99, 140.73, 136.23, 131.48 (d, $J = 9.7$ Hz), 130.15, 128.76 (d, $J = 11.8$ Hz), 126.25, 125.76, 123.99, 123.53, 118.24. HRMS (ES): calcd for C$_{17}$H$_{14}$N$_4$O$_4$S [M+H]$^+$: 371.0814. Found: 371.0828.

4,4'-(2,6-diaminopyridine-3,5-diyl)bis(2,6-difluorophenol)
The compound 23 was isolated in 24% yield. $^1$H NMR (500 MHz, DMSO) δ 7.11 – 6.89 (m, 4H), 5.78 (d, $J = 8.0$ Hz, 1H), 5.55 (s, 2H), 5.17 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) δ 158.22, 154.96, 153.28 (d, $J = 7.9$ Hz), 151.36 (d, $J = 7.9$ Hz), 139.16, 131.41 (t, $J = 16.1$ Hz), 130.13 (t, $J = 8.8$ Hz), 111.43 (dd, $J = 15.6, 6.2$ Hz), 106.07, 97.11. HRMS (ES): calcd for C$_{17}$H$_{11}$F$_4$N$_3$O$_2$ [M+H]$^+$: 366.0866. Found: 366.0875.

4,4'-(2-amino-6-methylpyridine-3,5-diyl)bis(2,6-difluorophenol)
The compound 24 was isolated in 55% yield. $^1$H NMR (500 MHz, DMSO) δ 10.18 (s, 2H), 7.17 (s, 1H), 7.15 (d, $J = 8.1$ Hz, 2H), 7.07 (d, $J = 9.2$ Hz, 2H), 5.78 (s, 2H), 2.29 (s, 3H). $^{13}$C NMR (126 MHz, DMSO) δ 154.92, 153.33 (d, $J = 7.7$ Hz), 152.84 (d, $J = 7.5$ Hz), 152.46, 151.41 (d, $J = 7.5$ Hz), 151.00 (d, $J = 7.8$ Hz), 138.99, 132.75 (t, $J = 16.1$ Hz), 132.15 (t, $J = 16.2$ Hz), 130.47 (t, $J = 8.7$ Hz), 128.18 (t, $J = 8.6$ Hz), 123.53, 115.99, 112.57 (dd, $J = 15.6, 6.2$ Hz), 112.09 (dd, $J = 15.8, 6.4$ Hz), 22.48. HRMS (ES): calcd for C$_{18}$H$_{12}$F$_4$N$_2$O$_2$ [M+H]$^+$: 365.0913. Found: 365.0948.

4,4'-(pyridine-3,5-diyl)bis(2,6-difluorophenol)
The compound 25 was isolated in 60% yield. $^1$H NMR (500 MHz, DMSO) δ 10.47 (s, 1H), 10.34 (s, 1H), 8.86 (s, 1H), 8.11 (s, 2H), 7.69 (d, $J = 8.4$ Hz, 2H), 7.36 (d, $J = 7.8$ Hz, 2H). $^{13}$C NMR (126 MHz, DMSO) δ 153.68 (d, $J = 7.6$ Hz), 152.84 (d, $J = 5.8$ Hz), 151.76 (d, $J = 7.7$ Hz), 150.91 (d, $J = 5.9$ Hz), 146.11, 135.41 (t, $J = 16.2$ Hz), 133.90 (t, $J = 16.3$ Hz), 133.37, 131.13, 127.20 (t, $J = 8.7$ Hz), 124.09 (dd, $J = 14.1, 7.7$ Hz), 116.83 (dd, $J = 13.8, 4.9$ Hz), 110.61 (dd, $J = 16.3, 6.7$ Hz). HRMS (ES): calcd for C$_{17}$H$_9$F$_3$NO$_2$ [M+H]$^+$: 336.0648. Found: 336.0656.
4,4'-(2-methylpyridine-3,5-diyl)bis(2,6-difluorophenol)

The compound 26 was isolated in 45% yield. $^1$H NMR (500 MHz, DMSO) δ 10.41 (s, 2H), 8.76 (d, $J = 2.3$ Hz, 1H), 7.87 (d, $J = 2.3$ Hz, 1H), 7.57 (d, $J = 9.8$ Hz, 2H), 7.25 (d, $J = 9.2$ Hz, 2H), 2.48 (s, 3H). $^{13}$C NMR (126 MHz, DMSO) δ 154.09, 153.70 (d, $J = 7.5$ Hz), 152.98 (d, $J = 7.7$ Hz), 151.78 (d, $J = 7.8$ Hz), 151.06 (d, $J = 7.5$ Hz), 145.35, 134.64, 134.17, 133.90 – 133.04 (m), 131.08, 129.35 (t, $J = 8.8$ Hz), 127.17 (t, $J = 8.9$ Hz), 112.94 (dd, $J = 15.9, 6.5$ Hz), 110.19 (dd, $J = 16.4, 6.5$ Hz), 22.95. HRMS (ES): calcd for C$_{18}$H$_{11}$F$_4$NO$_2$ [M+H]$^+$: 350.0804. Found: 350.0833.

4-(2-amino-5-(3,5-difluoro-4-methoxy-2-methylphenyl)pyridin-3-yl)-2,6-difluorophenol

The compound 27 was isolated in 63% yield. $^1$H NMR (400 MHz, DMSO) δ 10.32 (s, 1H), 7.93 (d, $J = 2.3$ Hz, 1H), 7.22 – 7.14 (m, 2H), 7.11 (dd, $J = 11.9, 1.9$ Hz, 1H), 5.93 (s, 2H), 3.92 (s, 3H), 2.15 (d, $J = 2.3$ Hz, 3H). $^{13}$C NMR (126 MHz, DMSO) δ 155.87, 154.80, 153.75 (d, $J = 6.9$ Hz), 153.36 (d, $J = 7.5$ Hz), 151.81 (d, $J = 6.9$ Hz), 151.43 (d, $J = 7.7$ Hz), 147.09, 138.04, 134.76 – 134.21 (m), 133.99 (dd, $J = 8.6, 5.2$ Hz), 133.08 (d, $J = 10.5$ Hz), 127.90 (dd, $J = 9.0, 6.0$ Hz), 123.44, 119.49 (dd, $J = 14.7, 3.0$ Hz), 117.87, 112.74 (dd, $J = 19.0, 2.2$ Hz), 112.23 (dd, $J = 15.9, 6.4$ Hz), 61.78, 12.03. HRMS (ES): calcd for C$_{19}$H$_{14}$F$_4$N$_2$O$_2$ [M+H]$^+$: 379.1070. Found: 379.1077.

4-(6-amino-5-(benzo[d]thiazol-6-yl)pyridin-3-yl)-2,6-difluorophenol

The compound 28 was isolated in 25% yield. $^1$H NMR (500 MHz, DMSO) δ 10.11 (s, 1H), 9.43 (s, 1H), 8.32 (d, $J = 2.5$ Hz, 2H), 8.16 (d, $J = 8.4$ Hz, 1H), 7.74 – 7.66 (m, 2H), 7.46 – 7.37 (m, 2H), 5.90 (s, 2H). $^{13}$C NMR (101 MHz, DMSO) δ 156.93, 156.59, 154.48, 152.80, 145.45, 136.22, 135.75, 134.74, 132.60, 129.13, 127.61, 123.64, 123.17, 120.17, 109.28 (dd, $J = 15.5, 7.7$ Hz). HRMS (ES): calcd for C$_{18}$H$_{11}$F$_2$N$_2$OS [M+H]$^+$: 356.0669. Found: 356.0658.

4-(2-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)-3-fluorobenzamide

The compound 29 was isolated in 62% yield. $^1$H NMR (400 MHz, DMSO) δ 10.11 (s, 1H), 8.35 (d, $J = 2.4$ Hz, 1H), 8.12 (s, 1H), 7.85 – 7.75 (m, 2H), 7.65 (d, $J = 2.3$ Hz, 1H), 7.54 (t, $J = 7.7$ Hz, 2H), 7.42 – 7.32 (m, 2H), 5.86 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) δ 166.31, 160.31, 158.35, 156.21, 153.74 (d, $J = 7.7$ Hz), 151.82 (d, $J = 7.7$ Hz), 145.90, 136.23, 135.81 (d, $J = 6.7$ Hz), 132.71 – 131.75 (m), 131.64, 128.44 (t, $J = 8.8$ Hz), 128.04 (d, $J = 16.4$ Hz), 123.87 (d, $J = 2.6$ Hz), 122.42, 115.17, 114.98, 113.73, 108.77 (dd, $J = 16.2, 6.5$ Hz). HRMS (ES): calcd for C$_{18}$H$_{12}$F$_3$N$_2$O$_2$ [M+H]$^+$: 360.0960. Found: 360.0981.
The compound 30 was isolated in 61% yield. $^1$H NMR (400 MHz, DMSO) $\delta$ 10.14 (s, 1H), 8.31 (d, J = 2.4 Hz, 1H), 7.86 (s, 1H), 7.64 (dd, J = 5.7, 3.2 Hz, 3H), 7.57 – 7.51 (m, 2H), 7.46 – 7.36 (m, 2H), 5.90 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 167.95, 155.80, 153.72 (d, J = 7.8 Hz), 151.80 (d, J = 7.8 Hz), 145.50, 140.34, 135.66 (d, J = 11.2 Hz), 132.23 (t, J = 16.3 Hz), 130.31, 129.74, 129.17, 128.50 (t, J = 8.8 Hz), 127.21, 123.31, 118.23, 108.93 (dd, J = 16.2, 6.6 Hz). HRMS (ES): calcd for C$_{18}$H$_{12}$ClF$_2$N$_3$O$_2$ [M+H]$^+$: 376.0664. Found: 376.0664.

4-(6-amino-5-(4-(hydroxymethyl)phenyl)pyridin-3-yl)-2,6-difluorophenol
The compound 31 was isolated in 18% yield. $^1$H NMR (400 MHz, DMSO) $\delta$ 10.13 (s, 1H), 8.27 (d, J = 2.4 Hz, 1H), 7.59 (d, J = 2.4 Hz, 1H), 7.49 (d, J = 8.1 Hz, 2H), 7.42 (d, J = 8.2 Hz, 2H), 7.37 (d, J = 9.9 Hz, 2H), 5.72 (s, 2H), 5.25 (s, 1H), 4.56 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 155.94, 153.71 (d, J = 7.7 Hz), 151.79 (d, J = 7.8 Hz), 144.59, 141.82, 136.14, 135.27, 132.14 (t, J = 16.4 Hz), 128.72, 128.34, 126.93, 123.29, 120.25, 108.82 (dd, J = 16.1, 6.5 Hz), 62.64. HRMS (ES): calcd for C$_{18}$H$_{14}$F$_2$N$_2$O$_2$ [M+H]$^+$: 329.1102. Found: 329.1117.

4-(6-amino-5-(3-hydroxyphenyl)pyridin-3-yl)-2,6-difluorophenol
The compound 32 was isolated in 64% yield. $^1$H NMR (400 MHz, DMSO) $\delta$ 10.11 (s, 1H), 9.56 (s, 1H), 8.26 (d, J = 2.4 Hz, 1H), 7.57 (d, J = 2.4 Hz, 1H), 7.42 – 7.32 (m, 2H), 7.27 (t, J = 7.8 Hz, 1H), 6.91 (dd, J = 7.8, 4.9 Hz, 2H), 6.78 (dd, J = 8.0, 2.1 Hz, 1H), 5.70 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 157.73, 155.83, 153.75 (d, J = 7.8 Hz), 151.83 (d, J = 7.8 Hz), 144.62, 139.09, 135.01, 132.21 (t, J = 16.4 Hz), 130.01, 128.66 (t, J = 8.7 Hz), 123.19, 120.45, 119.24, 115.41, 114.59, 108.81 (dd, J = 16.1, 6.6 Hz). HRMS (ES): calcd for C$_{18}$H$_{13}$F$_2$N$_2$O$_2$ [M+H]$^+$: 315.0945. Found: 315.0959.

5-(2-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)isoindolin-1-one
The compound 33 was isolated in 63% yield. $^1$H NMR (400 MHz, DMSO) $\delta$ 10.15 (s, 1H), 8.58 (s, 1H), 8.32 (d, J = 2.4 Hz, 1H), 7.77 – 7.71 (m, 2H), 7.67 (d, J = 2.4 Hz, 1H), 7.62 (d, J = 8.8 Hz, 1H), 7.44 – 7.35 (m, 2H), 5.90 (s, 2H), 4.42 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 169.78, 155.92, 153.75 (d, J = 7.6 Hz), 151.84 (d, J = 7.6 Hz), 145.23, 144.79, 141.02, 135.60, 132.25 (t, J = 16.2 Hz), 131.68, 128.50 (d, J = 13.0 Hz), 124.08, 123.24, 119.69, 108.85 (dd, J = 16.2, 6.6 Hz), 45.02. HRMS (ES): calcd for C$_{19}$H$_{13}$F$_2$N$_2$O$_2$ [M+H]$^+$: 354.1054. Found: 354.1035.
The compound 34 was isolated in 19% yield. \(^{1}\)H NMR (400 MHz, DMSO) \(\delta\) 13.13 (s, 1H), 10.13 (s, 1H), 8.28 (d, \(J = 2.4\) Hz, 1H), 8.12 (s, 1H), 7.90 (s, 1H), 7.67 – 7.59 (m, 2H), 7.49 (dd, \(J = 8.6, 1.2\) Hz, 1H), 7.44 – 7.34 (m, 2H), 5.74 (s, 2H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 156.31, 153.73 (d, \(J = 7.7\) Hz), 151.82 (d, \(J = 7.8\) Hz), 144.33, 139.27, 135.54, 133.81, 132.13 (t, \(J = 16.4\) Hz), 129.90, 128.79 (t, \(J = 8.6\) Hz), 127.12, 123.31 (d, \(J = 14.3\) Hz), 120.92, 120.60, 110.51, 108.79 (dd, \(J = 16.1, 6.4\) Hz). HRMS (ES): calcd for C\(_{18}\)H\(_{12}\)F\(_2\)N\(_4\)O \([\text{M+H}]^+\): 339.1057. Found: 339.1034.

The compound 35 was isolated in 61% yield. \(^{1}\)H NMR (400 MHz, DMSO) \(\delta\) 10.15 (s, 1H), 8.32 (d, \(J = 2.4\) Hz, 1H), 7.77 (t, \(J = 8.0\) Hz, 1H), 7.67 (d, \(J = 2.3\) Hz, 3H), 7.50 – 7.44 (m, 2H), 5.96 (s, 2H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 164.89, 160.72, 158.73, 155.78, 153.76 (d, \(J = 7.8\) Hz), 151.84 (d, \(J = 7.7\) Hz), 145.61, 142.54 (d, \(J = 8.7\) Hz), 135.66, 132.29 (t, \(J = 16.4\) Hz), 130.90 (d, \(J = 3.0\) Hz), 128.48 (t, \(J = 8.7\) Hz), 124.69, 123.27, 122.14 (d, \(J = 13.8\) Hz), 118.22, 116.53, 116.34, 108.93 (dd, \(J = 16.2, 6.5\) Hz). HRMS (ES): calcd for C\(_{18}\)H\(_{12}\)F\(_3\)N\(_3\)O\(_2\) \([\text{M+H}]^+\): 360.0960. Found: 360.0981.

The compound 36 was isolated in 26% yield. \(^{1}\)H NMR (500 MHz, DMSO) \(\delta\) 9.44 (s, 1H), 8.42 (d, \(J = 2.4\) Hz, 1H), 8.32 (d, \(J = 8.8\) Hz, 2H), 8.06 (dd, \(J = 6.7, 2.5\) Hz, 1H), 7.86 (d, \(J = 8.8\) Hz, 2H), 7.77 (d, \(J = 2.4\) Hz, 1H), 7.63 (dd, \(J = 7.1, 4.9\) Hz, 2H), 6.26 (s, 2H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 156.35, 155.97, 153.81, 147.07, 146.56, 144.86, 136.88, 133.13, 132.33, 130.05, 126.99, 124.92, 124.35, 124.08, 121.75, 118.13. HRMS (ES): calcd for C\(_{18}\)H\(_{12}\)N\(_4\)O\(_2\)S \([\text{M+H}]^+\): 349.0759. Found: 349.0769.

The compound 37 was isolated in 68% yield. \(^{1}\)H NMR (400 MHz, DMSO) \(\delta\) 10.13 (s, 1H), 8.31 (d, \(J = 2.4\) Hz, 1H), 7.99 (d, \(J = 8.3\) Hz, 2H), 7.64 (dd, \(J = 12.7, 5.3\) Hz, 3H), 7.44 – 7.35 (m, 3H), 5.86 (s, 2H). \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 167.51, 155.87, 153.74 (d, \(J = 7.6\) Hz), 151.83 (d, \(J = 7.7\) Hz), 145.19, 140.84, 135.52, 133.05, 132.21 (t, \(J = 16.3\) Hz), 128.54, 128.09, 123.30, 119.49, 108.89 (dd, \(J = 16.1, 6.6\) Hz). HRMS (ES): calcd for C\(_{18}\)H\(_{13}\)F\(_2\)N\(_3\)O \([\text{M+H}]^+\): 342.1054. Found: 342.1024.
The compound 38 was isolated in 63% yield. $^1$H NMR (400 MHz, DMSO) $\delta$ 10.14 (s, 1H), 8.31 (d, $J = 2.4$ Hz, 1H), 7.98 (s, 1H), 7.86 (d, $J = 7.8$ Hz, 1H), 7.67 (t, $J = 5.3$ Hz, 2H), 7.55 (t, $J = 7.7$ Hz, 1H), 7.44 – 7.34 (m, 3H), 5.84 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 167.93, 155.95, 153.77 (d, $J = 7.6$ Hz), 151.85 (d, $J = 7.6$ Hz), 145.01, 137.86, 135.63, 135.05, 132.24 (t, $J = 16.5$ Hz), 131.44, 128.90, 128.61 (t, $J = 8.6$ Hz), 127.71, 126.72, 123.25, 119.74, 108.84 (dd, $J = 16.2$, 6.5 Hz). HRMS (ES): calcd for C$_{18}$H$_{13}$F$_2$N$_3$O$_2$ [M+H]$^+$: 342.1054. Found: 342.1062.

The compound 39 was isolated in 60% yield. $^1$H NMR (400 MHz, DMSO) $\delta$ 10.21 (s, 2H), 8.30 (d, $J = 2.4$ Hz, 1H), 7.64 (d, $J = 2.4$ Hz, 1H), 7.45 – 7.36 (m, 2H), 7.24 (s, 1H), 7.18 (s, 1H), 7.04 (s, 1H), 5.86 (s, 2H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 158.20, 155.84, 153.73 (d, $J = 7.6$ Hz), 151.81 (d, $J = 7.6$ Hz), 145.28, 140.38, 135.42, 132.23 (t, $J = 16.1$ Hz), 131.15, 130.90, 130.65, 130.40, 128.48 (t, $J = 8.7$ Hz), 125.18, 123.08 (d, $J = 17.8$ Hz), 120.84, 119.57, 118.94, 115.88 (d, $J = 3.8$ Hz), 110.83 (d, $J = 3.5$ Hz), 108.88 (dd, $J = 16.2$, 6.6 Hz). HRMS (ES): calcd for C$_{18}$H$_{11}$F$_5$N$_2$O$_2$ [M+H]$^+$: 383.0819. Found: 383.0841.

The compound 40 was isolated in 26% yield. $^1$H NMR (400 MHz, DMSO) $\delta$ 10.19 (s, 1H), 8.32 (d, $J = 2.2$ Hz, 1H), 7.90 (s, 1H), 7.68 (d, $J = 2.2$ Hz, 1H), 7.59 (dd, $J = 26.9$, 8.9 Hz, 2H), 7.46 – 7.36 (m, 2H), 5.94 (s, 3H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 166.85, 163.21, 161.27, 155.86, 153.73 (d, $J = 8.0$ Hz), 151.82 (d, $J = 7.7$ Hz), 145.42, 140.21 (d, $J = 6.1$ Hz), 135.59, 132.21 (t, $J = 16.3$ Hz), 128.45 (t, $J = 9.0$ Hz), 125.62, 123.12, 118.47, 114.66, 114.48, 108.85 (dd, $J = 16.1$, 6.6 Hz). HRMS (ES): calcd for C$_{28}$H$_{24}$F$_3$N$_3$O$_3$S [M+H]$^+$: 361.0800. Found: 361.0814.

The compound 41 was isolated in 77% yield. $^1$H NMR (500 MHz, DMSO) $\delta$ 8.36 (s, 1H), 7.84 (s, 1H), 7.47 (d, $J = 7.8$ Hz, 2H), 7.34 (s, 2H), 7.06 (d, $J = 7.8$ Hz, 2H), 5.85 (s, 2H), 3.81 (s, 3H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 158.73, 156.74, 145.07, 141.81, 141.13, 135.59, 131.78, 129.83, 128.93, 127.61, 126.26, 125.62 (d, $J = 14.8$ Hz), 123.59, 120.30, 114.41, 55.19. HRMS (ES): calcd for C$_{18}$H$_{17}$N$_3$O$_3$S [M+H]$^+$: 356.1069. Found: 356.1057.
The compound 42 was isolated in 39% yield. 

1H NMR (400 MHz, DMSO) δ 10.11 (s, 1H), 8.29 (d, J = 2.0 Hz, 1H), 8.05 (dd, J = 2.3 Hz, 1H), 7.64 (d, J = 1.9 Hz, 1H), 7.42 (ddd, J = 17.1, 9.7, 6.0 Hz, 3H), 7.32 (dd, J = 10.2, 8.7 Hz, 1H), 5.78 (s, 2H). 

13C NMR (126 MHz, DMSO) δ 156.07, 153.73 (d, J = 7.7 Hz), 151.81 (d, J = 7.7 Hz), 144.75, 135.28, 133.69, 132.13 (t, J = 16.4 Hz), 130.08, 129.17 (d, J = 7.9 Hz), 123.19, 120.75 (d, J = 9.2 Hz), 116.77, 116.59, 108.81 (dd, J = 16.2, 6.4 Hz).

HRMS (ES): calcd for C18H11F3N6O [M+H]+: 385.1025. Found: 385.1016.

The compound 43 was isolated in 58% yield. 

1H NMR (400 MHz, DMSO) δ 10.11 (s, 1H), 9.39 (s, 1H), 8.47 (d, J = 5.9 Hz, 1H), 8.43 (d, J = 2.5 Hz, 1H), 8.19 (dd, J = 7.6, 1.0 Hz, 1H), 7.82 – 7.73 (m, 2H), 7.69 (d, J = 2.4 Hz, 1H), 7.44 – 7.34 (m, 3H), 5.63 (s, 2H). 

13C NMR (126 MHz, DMSO) δ 156.59, 153.75 (d, J = 7.6 Hz), 152.84, 151.83 (d, J = 7.8 Hz), 145.66, 143.25, 136.40, 134.37, 133.60, 132.36 – 131.82 (m), 128.71 (d, J = 21.4 Hz), 128.52 (d, J = 8.7 Hz), 127.83, 127.56, 122.52, 118.20, 117.24, 108.77 (dd, J = 16.2, 6.4 Hz). HRMS (ES): calcd for C20H13F2N3O [M+H]+: 350.1105. Found: 350.1095.

The compound 44 was isolated in 42% yield. 

1H NMR (400 MHz, DMSO) δ 10.15 (s, 1H), 8.57 (d, J = 2.4 Hz, 1H), 8.38 (d, J = 2.4 Hz, 1H), 8.33 (d, J = 2.4 Hz, 1H), 7.70 (d, J = 2.4 Hz, 1H), 7.44 – 7.35 (m, 2H), 6.06 (s, 2H), 4.05 (s, 3H). 

13C NMR (126 MHz, DMSO) δ 162.57, 156.41, 153.75 (d, J = 7.9 Hz), 152.17 – 151.54 (m), 145.59, 144.34, 135.94, 132.27 (t, J = 14.3 Hz), 128.32 (t, J = 8.6 Hz), 127.42, 122.97, 115.35, 115.03, 108.74 (dd, J = 16.2, 6.5 Hz), 95.57, 54.58. HRMS (ES): calcd for C18H12F2N4O2 [M+H]+: 355.1007. Found: 355.1017.

The compound 45 was isolated in 77% yield. 

1H NMR (400 MHz, DMSO) δ 10.15 (s, 1H), 8.33 (t, J = 4.9 Hz, 1H), 8.30 (d, J = 2.4 Hz, 1H), 7.72 (dd, J = 6.9, 2.3 Hz, 1H), 7.67 – 7.61 (m, 2H), 7.43 – 7.33 (m, 3H), 5.92 (s, 2H), 4.77 (s, 1H), 3.52 (s, 2H), 3.35 (d, J = 5.9 Hz, 2H). 

13C NMR (126 MHz, DMSO) δ 163.76, 159.61, 157.62, 155.95, 153.75 (d, J = 7.7 Hz), 151.83 (d, J = 7.6 Hz), 145.12,
135.59, 133.99, 132.71 (d, J = 8.6 Hz), 132.20 (t, J = 16.5 Hz), 130.11, 128.60 (t, J = 8.8 Hz), 124.41 (d, J = 14.3 Hz), 123.16, 118.66, 116.84, 116.66, 109.34 – 108.61 (m), 59.66, 42.09. HRMS (ES): calcd for C20H16F3N3O3 [M+H]+: 404.1222. Found: 404.1231.

5-(2-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)-2-methoxybenzoic acid
The compound 46 was isolated in 31% yield. 1H NMR (400 MHz, DMSO) δ 8.24 (d, J = 2.1 Hz, 1H), 7.55 (s, 2H), 7.47 (d, J = 7.1 Hz, 1H), 7.36 (d, J = 9.4 Hz, 2H), 7.10 (d, J = 8.7 Hz, 1H), 5.68 (s, 2H), 3.81 (s, 3H). 13C NMR (126 MHz, DMSO) δ 169.07, 156.97, 156.49, 154.30 (d, J = 7.8 Hz), 152.38 (d, J = 7.9 Hz), 144.83, 135.52, 131.37, 130.22, 129.73, 129.02 – 128.69 (m), 123.82, 120.33, 113.10, 109.24 (dd, J = 16.2, 6.1 Hz), 56.20. HRMS (ES): calcd for C19H14F2N2O4 [M+H]+: 373.1000. Found: 373.0995.

5-(2-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)-2-fluorobenzonitrile
The compound 47 was isolated in 59% yield. 1H NMR (400 MHz, DMSO) δ 10.17 (s, 1H), 8.33 (d, J = 2.4 Hz, 1H), 8.06 (dd, J = 6.3, 2.3 Hz, 1H), 7.92 (ddd, J = 8.5, 5.3, 2.3 Hz, 1H), 7.68 (d, J = 2.4 Hz, 1H), 7.61 (t, J = 9.1 Hz, 1H), 7.45 – 7.36 (m, 2H), 6.01 (s, 2H). 13C NMR (126 MHz, DMSO) δ 162.71, 160.68, 155.98, 153.75 (d, J = 7.7 Hz), 151.83 (d, J = 7.8 Hz), 145.56, 136.67 (d, J = 8.6 Hz), 135.82, 135.40 (d, J = 3.4 Hz), 134.37, 132.27 (t, J = 16.2 Hz), 128.37 (t, J = 8.8 Hz), 123.09, 117.34, 116.91 (d, J = 19.5 Hz), 114.11, 108.81 (dd, J = 16.2, 6.4 Hz), 100.63 (d, J = 15.4 Hz). HRMS (ES): calcd for C18H10F3N3O [M+H]+: 342.0854. Found: 342.0873.

3-(2-amino-5-(3,5-difluoro-4-hydroxyphenyl)pyridin-3-yl)-N-cyclopropylbenzamide
The compound 48 was isolated in 49% yield. 1H NMR (400 MHz, DMSO) δ 10.14 (s, 1H), 8.48 (d, J = 4.1 Hz, 1H), 8.31 (d, J = 2.4 Hz, 1H), 7.91 (s, 1H), 7.82 (d, J = 7.8 Hz, 1H), 7.68 – 7.61 (m, 2H), 7.55 (t, J = 7.7 Hz, 1H), 7.43 – 7.33 (m, 2H), 5.83 (s, 2H), 2.90 – 2.81 (m, 1H), 0.70 (td, J = 7.0, 4.8 Hz, 2H), 0.61 – 0.52 (m, 2H). 13C NMR (126 MHz, DMSO) δ 167.39, 155.92, 153.73 (d, J = 7.6 Hz), 151.81 (d, J = 7.7 Hz), 145.02, 137.80, 135.54, 135.06, 132.16 (t, J = 16.2 Hz), 131.35, 128.87, 128.62 (t, J = 8.7 Hz), 127.29, 126.46, 123.16, 119.70, 108.82 (dd, J = 16.1, 6.6 Hz), 23.06, 5.76. HRMS (ES): calcd for C21H17F2N3O2 [M+H]+: 382.1367. Found: 382.1393.
| Cmpd ID | Structure | HRMS (ES) |
|---------|-----------|-----------|
| 4a      | ![Structure 4a](image) | calcd for C$_{11}$H$_9$BrN$_2$O $\text{[M+H]}^+$: 264.9977. Found: 264.9987. |
| 4b      | ![Structure 4b](image) | calcd for C$_{12}$H$_{10}$BrN$_2$O$_2$ $\text{[M+H]}^+$: 292.9926. Found: 292.0097. |
| 4c      | ![Structure 4c](image) | calcd for C$_{12}$H$_9$BrN$_4$O $\text{[M+H]}^+$: 305.0038. Found: 305.0034. |
| 4d      | ![Structure 4d](image) | calcd for C$_{14}$H$_{13}$BrF$_3$O$_2$ $\text{[M+H]}^+$: 354.0253. Found: 354.0300. |
| 4e      | ![Structure 4e](image) | calcd for C$_{12}$H$_{10}$BrN$_3$O $\text{[M+H]}^+$: 292.0085. Found: 292.0097. |
| 4f      | ![Structure 4f](image) | calcd for C$_{12}$H$_8$BrF$_2$N$_2$O $\text{[M+H]}^+$: 310.9831. Found: 310.9834. |
| 4g      | ![Structure 4g](image) | calcd for C$_{12}$H$_9$BrF$_3$N$_2$O $\text{[M+H]}^+$: 309.9991. Found: 310.0025. |
| 4h      | ![Structure 4h](image) | calcd for C$_{12}$H$_{11}$BrN$_2$O $\text{[M+H]}^+$: 279.0133. Found: 279.0151. |
| 4i      | ![Structure 4i](image) | calcd for C$_{14}$H$_{10}$Br$_3$N $\text{[M+H]}^+$: 300.0136. Found: 300.0165. |
| 4j      | ![Structure 4j](image) | calcd for C$_{12}$H$_8$Br$_3$N$_3$O$_4$ $\text{[M+H]}^+$: 337.9776. Found: 337.9777. |
| 4k      | ![Structure 4k](image) | calcd for C$_{12}$H$_9$Br$_4$N $\text{[M+H]}^+$: 289.0089. Found: 289.0134. |
calcd for C_{12}H_{16}BrN_{3}O
[M+H]^+: 292.0085.
Found: 292.0097.

4m

4n

calcd for C_{12}H_{10}BrF_{3}N_{2}O
[M+H]^+: 332.9850.
Found: 332.9922.

4o

calcd for C_{12}H_{9}BrFN_{3}O
[M+H]^+: 291.9886.
Found: 291.9922.

4p

calcd for C_{12}H_{9}BrClN_{3}O
[M+H]^+: 325.9696.
Found: 325.9676.

4q

calcd for C_{13}H_{10}BrN_{3}O
[M+H]^+: 309.9991.
Found: 309.9989.

4r

calcd for C_{13}H_{10}BrN_{3}O
[M+H]^+: 304.0085.
Found: 304.0070.

4s

calcd for C_{13}H_{11}BrN_{2}O_{3}
[M+H]^+: 291.1986.
Found: 291.1986.

4t

calcd for C_{15}H_{14}BrN_{3}O
[M+H]^+: 323.0031.
Found: 323.0036.

4u

calcd for C_{15}H_{14}BrN_{3}O
[M+H]^+: 323.0031.
Found: 323.0036.

4v

calcd for C_{15}H_{14}BrN_{3}O
[M+H]^+: 323.0031.
Found: 323.0036.

4w

calcd for C_{15}H_{14}BrN_{3}O
[M+H]^+: 323.0031.
Found: 323.0036.
$^1$H NMR (500 MHz, DMSO-$d_6$) 5.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 5.
$^1$H NMR (500 MHz, DMSO-$d_6$) 6.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 6.
$^1$H NMR (500 MHz, DMSO-$d_6$) 7.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 7.
$^1$H NMR (500 MHz, DMSO-$d_6$) 8.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 8.
$^1$H NMR (400 MHz, DMSO-$d_6$) 9.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 9.
$^{1}H$ NMR (500 MHz, DMSO-$d_6$) 10.

$^{13}C$ NMR (126 MHz, DMSO-$d_6$) 10.
$^1$H NMR (500 MHz, DMSO-$d_6$) 11.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 11.
$^1$H NMR (500 MHz, DMSO-$d_6$) 12.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 12.
$^1$H NMR (500 MHz, DMSO-$d_6$) 13.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 13.
$^1$H NMR (500 MHz, DMSO-$d_6$) 14.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 14.
$^1$H NMR (500 MHz, DMSO-$d_6$) 15.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 15.
$^1$H NMR (500 MHz, DMSO-$d_6$) 16.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 16.
\[ ^1\text{H NMR (500 MHz, DMSO-}\text{d}_6\text{)} \ 17. \]

\[ ^13\text{C NMR (126 MHz, DMSO-}\text{d}_6\text{)} \ 17. \]
$^1$H NMR (500 MHz, DMSO-$d_6$) 18.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 18.
$^1$H NMR (500 MHz, DMSO-$d_6$) 19.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 19.
$^{1}$H NMR (500 MHz, DMSO-$d_6$) 20.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 20.
$^1$H NMR (500 MHz, DMSO-$d_6$) 21.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 21.
$^1$H NMR (500 MHz, DMSO-$d_6$) 22.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 22.
$^1$H NMR (500 MHz, DMSO-$d_6$) 23.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 23.
$^{1}H$ NMR (500 MHz, DMSO-$d_{6}$) 24.

$^{13}C$ NMR (126 MHz, DMSO-$d_{6}$) 24.
\( ^1 \text{H NMR} \) (500 MHz, DMSO-\(d_6\)) 25.

\( ^{13} \text{C NMR} \) (126 MHz, DMSO-\(d_6\)) 25.
$^1$H NMR (500 MHz, DMSO-$d_6$) 26.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 26.
$^1$H NMR (400 MHz, DMSO-$d_6$) 27.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 27.
$^{1}H$ NMR (500 MHz, DMSO-$d_{6}$) 28.

$^{13}C$ NMR (126 MHz, DMSO-$d_{6}$) 28.
$^1$H NMR (400 MHz, DMSO-$d_6$) 29.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 29.
$^1$H NMR (400 MHz, DMSO-$d_6$) 30.

$^1$C NMR (126 MHz, DMSO-$d_6$) 30.
^{1}H NMR (400 MHz, DMSO-d$_{6}$) 31.

^{13}C NMR (126 MHz, DMSO-d$_{6}$) 31.
$^1$H NMR (400 MHz, DMSO-$d_6$) 32.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 32.
$^1$H NMR (400 MHz, DMSO-$d_6$) 33.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 33.
$^{1}H$ NMR (400 MHz, DMSO-$d_6$) 34.

$^{13}C$ NMR (126 MHz, DMSO-$d_6$) 34.
\(^{1}\)H NMR (400 MHz, DMSO-d\(_6\)) 35.

\(^{13}\)C NMR (126 MHz, DMSO-d\(_6\)) 35.
$^1$H NMR (500 MHz, DMSO-$d_6$) 36.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 36.
$^1$H NMR (400 MHz, DMSO-$d_6$) 37.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 37.
\(^{1}H\) NMR (400 MHz, DMSO-\(d_6\)) 38.

\(^{13}C\) NMR (126 MHz, DMSO-\(d_6\)) 38.
$^1$H NMR (400 MHz, DMSO-$d_6$) 39.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 39.
$^1$H NMR (400 MHz, DMSO-$d_6$) 40.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 40.
$^{1}$H NMR (500 MHz, DMSO-$d_6$) 41.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 41.
$^1$H NMR (400 MHz, DMSO-$d_6$) 42.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 42.
$^1$H NMR (400 MHz, DMSO-$d_6$) 43.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 43.
$\text{H NMR (400 MHz, DMSO-$d_6$) 44.}$

$\text{C NMR (126 MHz, DMSO-$d_6$) 44.}$
$\textbf{^1H NMR (400 MHz, DMSO-}d_6\textbf{) 45.}$

$\textbf{^13C NMR (126 MHz, DMSO-d}_6\textbf{) 45.}$
$^{1}$H NMR (400 MHz, DMSO-$d_6$) 46.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 46.
$^1$H NMR (400 MHz, DMSO-$d_6$) 47.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 47.
$^1$H NMR (400 MHz, DMSO-$d_6$) 48.

$^{13}$C NMR (126 MHz, DMSO-$d_6$) 48.
