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Tetrahydropyrido[4,3-d] pyrimidines as a new active scaffold for human topoisomerase II inhibitors

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Human topoisomerase II (topoII) is a well-known and validated target for cancer treatment. We previously reported a first set of 6-amino-tetrahydroquinazoline derivatives as novel human topoII inhibitors. Here, we report on the expansion and this molecular scaffold and present 17 additional analogs centered on the tetrahydropyrido[4,3-d]pyrimidine heterocycle. Some of these compounds exhibit promising topoII inhibitory and antiproliferative activities. Compound 24 (ARN21929) shows good in vitro potency, with an IC_{50} of 4.5 ± 1.0 μM, excellent kinetic and thermodynamic solubility, and good metabolic stability. Our results indicate that this new chemical class of topoII inhibitors deserves further exploration and represents a promising starting point for developing novel and potentially safer topoII-targeted anticancer drugs.

Keywords Topoisomerase II inhibitors, Cancer treatment, Tetrahydropyridopyrimidines derivatives.

The biological function of human topoisomerase II (topoII) enzymes is to modulate DNA topology and regulate vital processes like DNA replication, transcription, recombination, and chromosome segregation[1–5]. TopoII is also a validated target in the fight against cancer[1,6,7] and several drugs targeting topoII are in clinical use[1,2]. Specifically, valuable examples of these anticancer drugs are represented by daunorubicin, doxorubicin, etoposide and teniposide. All these molecules are tetracyclic ring structures with a sugar moiety belonging to two main chemical classes: anthracyclines and epipodophyllotoxins (Fig. 1A). Notably, they all act as topoII poisons by trapping the covalent enzyme/DNA cleavage complex formed during DNA topology modifications[6–8]. As a consequence, topoII generates double-stranded breaks in the genome, which activate apoptotic mechanisms and result in cancer cell death. Although effective, this strategy is also responsible for severe unwanted side effects, including the development of myelosuppression, cardiotoxicity (notably with anthracyclines like doxorubicin), and the risk of therapy-related secondary leukemia in patients[6–10] representing a medical issue that has renewed the interest in the drug discovery community to seek new and safer topoII-targeted drugs[4,11–13].

In this context, our group has previously reported tetrahydroquinazoline-based small molecules that are potent topoII inhibitors and do not act as topoII poisons[14–22]. Notably, these compounds chemically differ from topoII poison, as they present a central bicyclic core substituted with different types of (hetero)aryl groups on C2 and C4 positions (Fig. 1A). Building on these findings, here we outline the development of new 17 (tetrahydro) pyridopyrimidine derivatives, describing the structure-activity relationship (SAR) study that elucidated the chemical features essential for the activity (Fig. 1B). Some of them are potent topoII inhibitors, have promising antiproliferative activity against five different human cancer cells and display favorable in vitro pharmacokinetic properties. These findings highlight the potential of these promising derivatives for developing novel anticancer agents.

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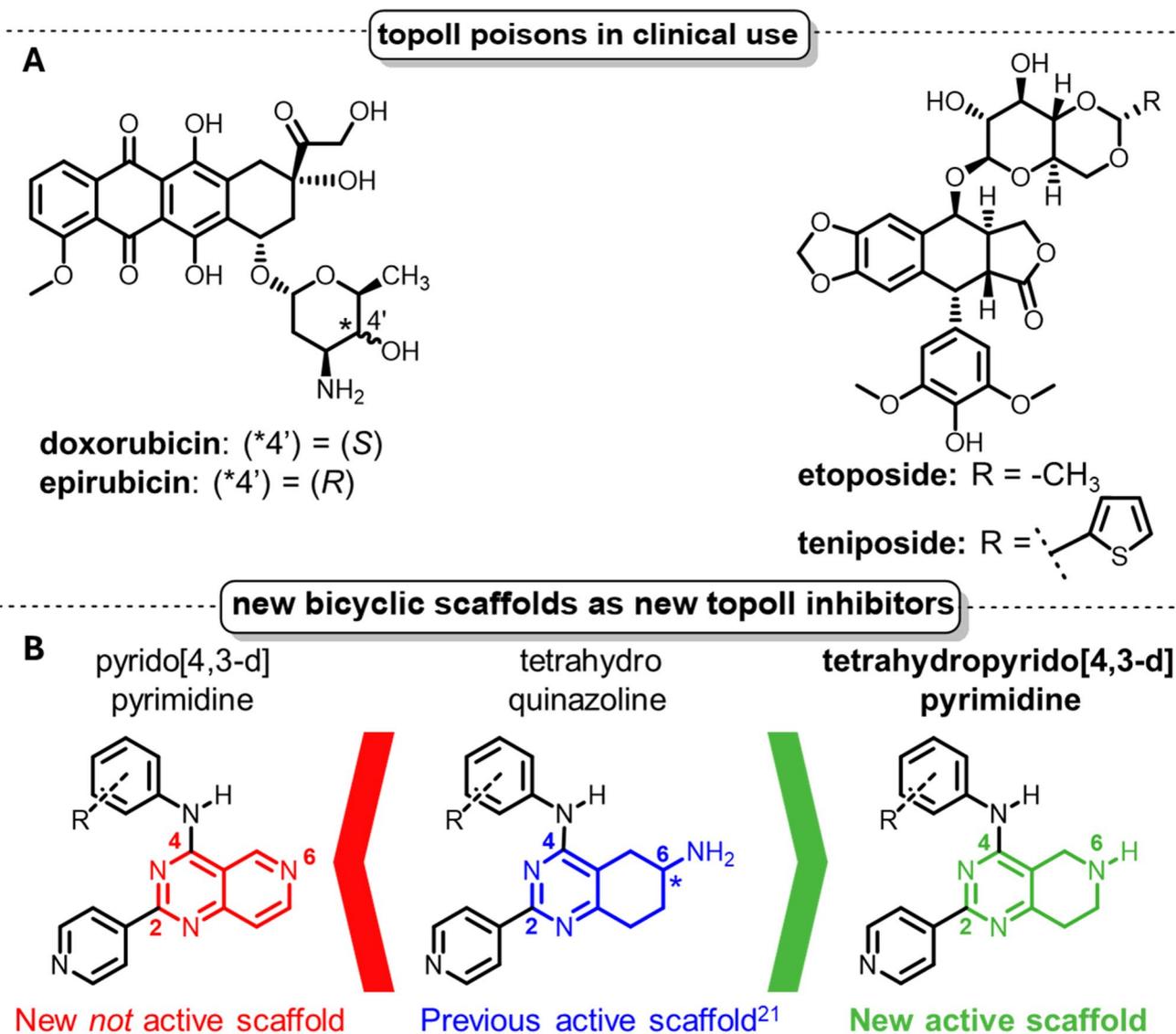
Fig. 1. (**A**) Examples of topoII poison inhibitors in clinical use; (**B**) Scaffolds explored for developing new topoII inhibitors[21].

Results and discussion

Exploring the structure-activity relationship of new topoii inhibitors toward ARN21934

In our previous work, we developed a class of 14 tetrahydroquinazoline derivatives that allowed the identification of the low micromolar selective topoIIα inhibitor ARN21934 (**1**, IC$_{50}$ = 2 ± 0.5 μM, Table 1) starting from the hit compound **2** (IC$_{50}$ = 160 ± 20 μM, Table 1)[21,22]. Indeed the compounds also exhibited a favorable drug-like profile both in vivo and in vitro. In particular, the chemical structure of ARN21934 features a central bicyclic core consisting of a partially saturated quinazoline bearing a pyridine, an aniline, and an amino group in positions 2, 4, and 6, respectively (Fig. 1B).

In the present work, we aim to develop new topoisomerase inhibitors with a modified core heterocycle. During the previous SAR study, the unsubstituted tetrahydroquinazoline **3** (Table 1) confirmed that the presence of the exocyclic amino group in position 6 was essential for topoII inhibition. However, its stereochemistry did not influence the inhibitory activity, as shown by enantiopure compounds **4** and **5** (Table 1)[21]. Therefore, we designed two new scaffolds (Fig. 1B), shifting the nitrogen of the amino group in the endocyclic position and removing the chirality (Fig. 1). First, we replaced tetrahydroquinazoline with tetrahydropyrido[4,3-d] pyrimidine core as in compound **6**. Thus, we mainly explored (i) pyridines with different positions of nitrogen and unsubstituted phenyl rings on C2, and (ii) anilines on position C4 with different substituents embedding electron-donating or electron-withdrawing groups in ortho, meta, and para positions, especially focusing on the alkylamino, methoxy, hydroxy, methyl and fluorine groups.

At this point, we also hypothesized that the substitution pattern in this new tetrahydropyridopyrimidine scaffold could establish interactions that favor topoII inhibition compared to the previously partially saturated quinazoline structures[21]. Therefore, we started a structure-activity relationship study (SAR) focusing our efforts

ARN21934 (1)**	2**	3**	4**	5**	6
2 ± 0.5 μM	160 ± 20 μM	not active	160 ± 20 μM	160 ± 20 μM	not active
ARN19661 (7)	8	9	10	11	12
25 ± 5 μM	not active	not active	not active	not active	not active
13	14	15	16	17	18
not active	not active	not active	not active	not active	not active
19	20	21	22	23	ARN21929 (24)
not active	120 ± 10 μM	not active	160 ± 20 μM	140 ± 20 μM	4.5 ± 1 μM

Table 1. Topoisomerase IIα inhibition*. *Values shown correspond to the $IC_{50} \pm$ standard deviation; ** Compounds initially reported by Ortega et al.[21].
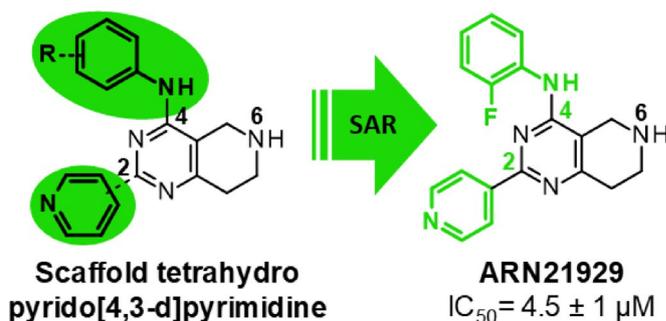

Fig. 2. SAR campaign to identify ARN21929 (**24**).

on two points of modification: the aniline substituent on position 4 and the (hetero)aryl group on position 2 of the bicyclic core (Fig. 2; Table 1). First, we masked the free nitrogen of the aniline group on position 2 with a methyl group, synthesizing compound **9** (Table 1). As observed in previous studies, this modification was detrimental and resulted in a loss of activity against topoII, suggesting this NH group's key hydrogen bond role[21]. With the second set of derivatives **10–12**, we explored the importance of the 4-pyridyl substituent present in compound **7**, replacing it with a phenyl (compound **10**) or changing the anchor point of the pyridyl substituent into positions 3 and 2 (compounds **11** and **12**, respectively). Differently from the previous tetrahydroquinazoline-based inhibitors[21] no one of the modifications was tolerated, and compounds **10–12** were inactive. After confirming that the 4-pyridyl heteroaromatic group was the best substituent in position 2 of the new tetrahydropyridopyrimidine scaffold and the essential role of the free nitrogen of aniline in position 4, we evaluated the decoration of the latter with analogs **13–24**. First, introducing a naked phenyl group, as in compound **13**, resulted in a loss of activity. Then, we explored groups in *para-* and *meta-* positions with different electronic and steric hindrance properties, such as fluorine (**14**), methyl (**15** and **16**), hydroxyl (**17** and **18**), methoxyl (**19** and **20**), monomethylamino (**21** and **22**), and dimethylamino (**23**) groups. This set of

analogs' behavior differed from the series described in our previous work[21]. In our new tetrahydropyrido[4,3-d] pyrimidine inhibitors, none of the substituents explored in the aniline *para-* position successfully increased inhibitory activity against topoII. Only substituents containing methylated oxygen or nitrogen in the aniline *meta-* position, as in compounds **20**, **22**, and **23**, restored the activity of the starting hit, displaying an IC_{50} of 120, 160, and 140 µM, respectively. From these results, we speculated that most likely due to steric hindrance factors, a fluorine atom was the best decoration for the aniline substituent. At this point, we moved it into *ortho-* position, resulting in the single-digit micromolar topoII inhibitor **24** (ARN21929, IC_{50} 4.5 ± 1.0 µM), which was five-fold more potent than compound **7** and with comparable activity of the previous lead ARN21934. In addition to its minimal steric impact, fluorine in *ortho* position of ARN21929 may alter the basicity of the adjacent aniline nitrogen and heterocycle core, potentially contributing to the improved binding profile and activity observed in these derivatives.

We also evaluated the antiproliferative effect of the active compounds **7**, **20–24** in a small panel of cancer cells (Table 2). Indeed, derivatives **1**, **7**, and **20** exhibited good or moderate antiproliferative activity in the low micromolar range against melanoma cell line (A375), breast (MCF7), endometrial (HeLa), lung (A549), and androgen-independent prostate (DU145) cancer cell lines. On the other hand, despite its promising inhibitory activity, ARN21929 showed $IC_{50} > 100$ µM against the same cancer cell lines. This limited cellular response may be associated with low cell permeability of ARN21929 compared to other analogues. Nonetheless, we note that ARN19661 (compound **7**), which differs from ARN21929 by the position of a single fluorine atom, exhibited antiproliferative activity similar to the previous lead compound ARN21934, with IC_{50} values in the 20–30 µM range. This promising result indicates that even minimal chemical modifications to this tetrahydroquinazoline-based scaffold can improve the overall activity.

Finally, we performed preliminary in vitro studies to assess the drug-likeness of ARN21929. This compound presented optimal kinetic (> 250 µM) and thermodynamic (596 µM) solubility. In terms of its metabolic stability, ARN21929 was stable in mouse plasma ($t_{1/2} > 120$ min) while showing moderate half-life in mouse liver microsomes ($t_{1/2} = 30$ min). Thus, despite the suboptimal antiproliferative activity, ARN21929 represents a viable and promising starting point for further exploration to develop a novel class of drug-like Topo II inhibitors with improved potency and efficiency.

Molecular modeling

The binding mode of novel human topoisomerase II (TopoII) inhibitors based on a tetrahydropyrido[4,3-d] pyrimidine molecular scaffold was investigated through molecular docking[23]. To assess alternative and functionally relevant inhibitory mechanisms, docking simulations were conducted considering three distinct binding sites. The first site, corresponding to the DNA cleavage region, was modeled using PDB entry 5GWK, which represents the drug-stabilized cleavage complex bound to etoposide[24]. The second and third sites explored the ATP-competitive mechanism at the N-terminal ATP-binding pocket, using PDB entry 1ZXM[8,25]. In TopoII, the catalytic site contains one Mg^{2+} ion at the ATP-binding site, which is critical for stabilizing ATP or ADP within the pocket. Therefore, docking simulations were performed both in the presence and absence of the Mg^{2+} cation to account for the transiently inactive form of the enzyme[21].

Docking results indicate that ARN21929 exhibits well-defined binding modes across all three target sites, each characterized by distinct interaction patterns. At the DNA cleavage site, the positively charged NH_2 group of ARN21929 forms a cation–π interaction with G'_{+3} and a hydrogen bond with its oxygen atom. The fluorine substituent interacts with the amino group of C_{-1}, while van der Waals interactions with residues such as Met762, Met766, Gly642 and Arg487 further stabilize this binding mode. Additionally, the pyridine nitrogen in the *para* position adopts a geometrically favorable orientation that enables hydrogen bonding with the hydroxyl group at C_{-1}, contributing to the overall stabilization of the ligand within the binding site (Fig. 3A). At the ATP-binding site of TopoII in the presence of Mg^{2+}, the pyridyl group of ARN21929 forms a cation–π interaction with the metal ion and a hydrogen bond with Gly164, resembling the binding mode observed for the previous lead ARN21934 in the same site. Further stabilizing interactions include (i) one hydrogen bond between the positively charged NH_2 group of the tetrahydropyrido[4,3-d]pyrimidine scaffold and the side chain of Asn95; (ii) one hydrogen bond between a nitrogen atom in the pyrimidine ring and the side chain of Asn150; (iii) one hydrogen bond between the fluorine substituent and the side chain of Ile125; and (iv) and the interaction between the fluorine-substituted aniline ring and the side chain of Asn120. Additionally, favorable van der Waals interactions occur with surrounding binding pocket residues (Fig. 3B). In the absence of the Mg^{2+} cation, ARN21929 engages multiple stabilizing interactions within the ATP-binding site. These include (i) one hydrogen

Cell line	A549	DU145	MCF7	HeLa	A375
ARN21934 (**1**)	17.1 ± 8.7	11.5 ± 1.3	15.8 ± 1.3	38.2 ± 9.1	12.6 ± 0.7
ARN19661 (**7**)	23.7 ± 0.5	32.9 ± 4.2	33.4 ± 3.8	32.3 ± 1.5	29.6 ± 0.7
20	35.3 ± 10.2	25.0 ± 7.3	30.1 ± 16.5	23.2 ± 9.6	37.2 ± 8.7
22	61.9 ± 9.0	45.2 ± 30.7	> 100	> 100	35.2 ± 1.0
23	40.8 ± 3.3	42.5 ± 1.8	46.4 ± 12.3	32.4 ± 1.9	88.8 ± 2.3
ARN21929 (**24**)	> 100	–	> 100	> 100	> 100
Etoposide	2.4 ± 2.0	0.5 ± 0.2	8.9 ± 1.6	1.2 ± 0.4	0.5 ± 0.1

Table 2. Cell proliferation results in different cell lines of selected topoii inhibitors*. *Values shown correspond to the IC_{50} (µM) ± standard deviation.
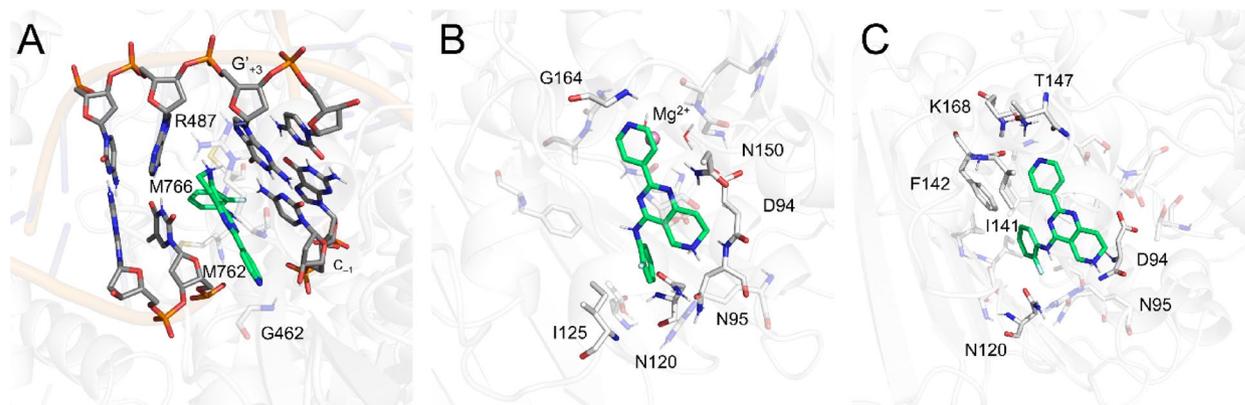
Fig. 3. Binding modes from docking calculations for ARN21929 at the DNA cleavage and ATP sites of TopoII. (**A**) Binding pose of ARN21929 within the DNA-cleaved complex. (**B**) Binding pose of ARN21929 within the ATP-binding site in the presence of Mg^{2+}. (**C**) Binding pose of ARN21929 within the ATP-binding site in the absence of Mg^{2+}. The protein and DNA (present only in A) are shown in semi-transparent cartoon representation, while the nucleotide bases and ligand are displayed in licorice representation. Residues involved in van der Waals interactions are shown in semi-transparent licorice representation. Details on the interactions of each compound in the pockets are reported in the main text.

bond between the pyrimidine nitrogen and the side chain of Lys168; (ii) interactions between the pyrimidine ring and the backbone carbonyl oxygens of Thr147 and Ile141; (iii) a π–π stacking interaction with the aromatic ring of Phe142; (iv) interactions between the fluorine-substituted aniline and the side-chain oxygen of Asn120; (v) one hydrogen bond between the positively charged NH_2 group and the backbone oxygen of Asp94; (vi) and one additional hydrogen bond between the fluorine atom and the side chain amino group of Asn120. The binding is further stabilized by van der Waals interactions with nearby residues (Fig. 3C).

Overall, our docking studies at both the DNA cleavage and ATP-binding sites indicate that ARN21929 may inhibit TopoII by engaging distinct residues at different sites, suggesting a multifaceted mechanism of action (*additional docking details in the Supporting Information*).

Chemistry

The synthetic strategy presented in Scheme 1.a of Fig. 4 allowed obtaining most of the tested compounds (**6–7**, **10–11** and **13–24**) through a five-step procedure. After forming the pyrimidone-like bicyclic heterocycle (intermediate **A**), positions 2 and 4 were activated by chlorination after refluxing in neat phosphorous oxychloride obtaining intermediate **B** with good yield of 71% after two steps. Then, position 4 was selectively functionalized with corresponding aniline derivatives taking advantage of its major reactivity towards aromatic nucleophilic substitution or palladium catalyzed reactions. Thus, we afforded intermediates **C.1-C.14** (Table 3) from moderate to good yields. Then, 4-pyridyl, 3-pyridyl and phenyl substituents were introduced in position 2 of intermediates **D.1-D.16** (Table 3) employing microwave-assisted Suzuki coupling. Final benzyl group removal under reductive conditions in the presence of Pd(OH)₂-C as catalyst and ammonium formate as hydrogen source gave final desired compounds **6**, **7**, **10**, **11**, **13–24**. Compounds **9** and **12** were synthesized with an alternative procedure (Fig. 4, Scheme 1.b). First, the bicyclic heterocycle of **F.1** and **F.2** was assembled directly functionalized in position 2, by reacting methyl 1-benzyl-4-oxo-piperidine-3-carboxylate with the corresponding pyridine-carboxamidine. In the second step, position 4 of intermediate of type **F** was activated after chlorination under reflux in neat phosphorous oxychloride, giving compounds of type **G** with 74% yield. Afterwards, suitable anilines were introduced via microwave-assisted Buchwald reaction affording **H1** and **H2** with 37% and 60% yield respectively. Finally, benzyl group was removed under reductive conditions equivalent to the ones employed in previous Scheme 1.a of Fig. 4. The synthesis of compound **8** was approached with a similar strategy as depicted in Scheme 1.c of Fig. 4. Initially, the fully aromatic bicyclic core was formed by reaction of 4-aminopyridine-3-carboxylic acid and pyridine-4-carboxamidine yielding intermediate **J** functionalized in position 2 with 47% yield. In this case, the activation of position 4 (intermediate **K**) was carried on by refluxing in the presence of POCl₃ and PCl₅ to finally get compound **8** using a microwave-assisted Buchwald coupling reaction.

Conclusions

In summary, we have evolved our partially saturated quinazoline topoII inhibitors into a new class based on a tetrahydropyridopyrimidine scaffold that, notably, is not chiral. These results provide new insight into the key chemical features crucial for the inhibitory activity against topoII and the drug-like profiles of this chemical scaffold. Compounds ARN19661 (**7**) and ARN21929 (**24**) act as potent topoII inhibitors. Focused SAR studies showed that only small substituents in the 4-aniline group were partially tolerated. Indeed, introducing a fluorine atom in *ortho-* position significantly increased topoII inhibition. Among the new 17 tetrahydropyridopyrimidine derivatives, compounds ARN19661 (**7**), **20**, **22**, and **23** inhibit cell proliferation in the low-moderate micromolar range on five cancer cell lines. Moreover, ARN21929 (**24**) showed high kinetic and thermodynamic solubility
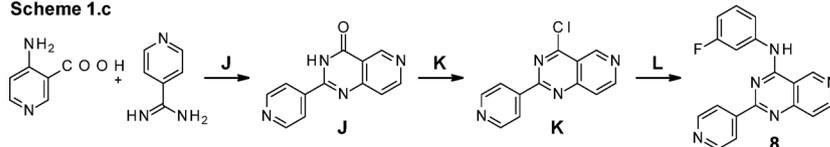
Scheme 1.a
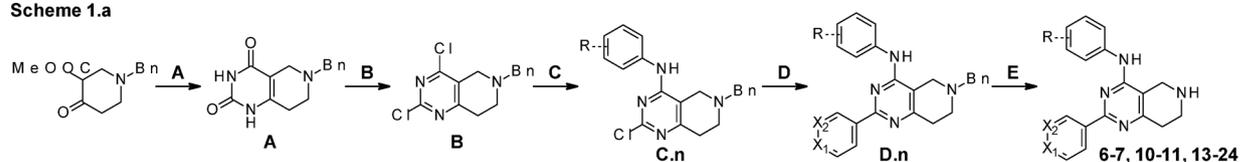
A. Urea, MeONa,EtOH, N_2, reflux, 5h, yield 88 %. **B.** $POCl_3$, N_2, reflux, 5h, yield 81 %. **C.** Aniline, DIPEA, iPrOH, N_2, microwave, 100 °C, 5-16 h or $Pd(OAc)_2$, *rac*-BINAP, aniline, Cs_2CO_3, microwave, 80 °C, 1,4-dioxane, yield 40-83 %. **D.** Boronic acid, $PdCl_2$(dppf).DCM, K_2CO_3, 1,4-dioxane, Ar, microwave, 100 °C, 2 h, yield 45-86 %. **E.** $HCOONH_4$, $Pd(OH)_2$-C, MeOH, Ar, reflux,4 h, yield 20-81 %.

Scheme 1.b
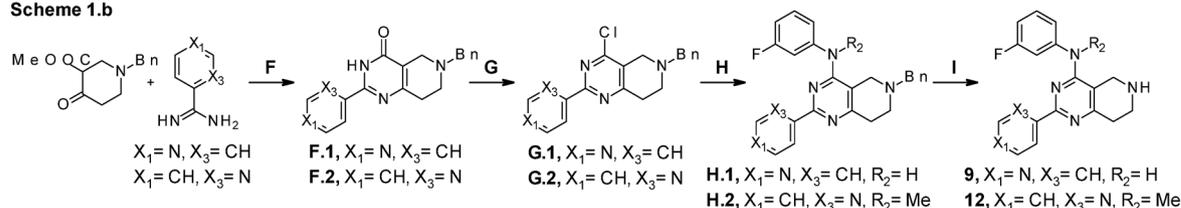
F. MeONa,EtOH, reflux, 5 h, yield 69 % (F.1 and F.2), . **G.** $POCl_3$, N_2, reflux, 5h, yield 74 % (G.1) 70 % (G.2). **H.** Substituted aniline, *rac*-BINAP, $Pd(OAc)_2$, Cs_2CO_3,1,4-Dioxane,100 °C, microwave, yield 37 % (H.1), 60 % (H.2). **I.** $HCOONH_4$, $Pd(OH)_2$-C, MeOH, Ar, reflux, yield 25 % (6), 47 % (9).

Scheme 1.c

J. NMP, Ar, 160 °C, 72 h, yield 47 %. **K.** PCl_5, $POCl_3$, N_2, reflux, 5h, yield 99 %. **L.** *m*-fluoroaniline, *rac*-BINAP, $Pd(OAc)_2$, Cs_2CO_3, 1,4-Dioxane,100 °C, microwave, yield 50 %.

Fig. 4. Scheme of synthetic routes for the obtainment of final compounds.

R	Intermediate	R	X_1	X_2	Intermediate
p-NMe$_2$	**C.1**	*p*-NMe$_2$	N	CH	**D.1**
m-F	**C.2**	*m*-F	N	CH	**D.2**
H	**C.3**	*m*-F	CH	CH	**D.3**
p-F	**C.4**	*m*-F	CH	N	**D.4**
p-Me	**C.5**	H	N	CH	**D.5**
m-Me	**C.6**	*p*-F	N	CH	**D.6**
p-OH	**C.7**	*p*-Me	N	CH	**D.7**
m-OH	**C.8**	*m*-Me	N	CH	**D.8**
p-MeO	**C.9**	*p*-OH	N	CH	**D.9**
m-MeO	**C.10**	*m*-OH	N	CH	**D.10**
p-NMeBoc	**C.11**	*p*-MeO	N	CH	**D.11**
m-NMeBoc	**C.12**	*m*-MeO	N	CH	**D.12**
m-NMe$_2$	**C.13**	*p*-NMeBoc	N	CH	**D.13**
o-F	**C.14**	*m*-NMeBoc	N	CH	**D.14**
		m- NMe$_2$	N	CH	**D.15**
		o-F	N	CH	**D.16**

Table 3. Intermediate compounds of type **C** and **D** of scheme 1.a of Fig. 4.

and favorable in vitro metabolic parameters, thus further supporting this new chemical class as a promising starting point for further studies to develop novel therapeutic agents that may lack the development of secondary leukemias associated with topoII poisons.

Methods

Chemistry

Chemistry general considerations

All the commercial available reagents and solvents were used as purchased from vendors without further purification. Dry solvents were purchased from Sigma-Aldrich. Automated column chromatography purifications were done using a Teledyne ISCO apparatus (CombiFlash® Rf) with pre-packed silica gel columns of different sizes (from 4 g up to 120 g) and mixtures of increasing polarity of cyclohexane and ethyl acetate (EtOAc), cyclohexane and tert-butylmethyl eter (TBME) or dicloromethane (DCM) and methanol (MeOH). NMR experiments were run on a Bruker Avance III 400 system (400.13 MHz for ^{1}H, and 100.62 MHz for ^{13}C), equipped with a BBI probe and Z-gradients. Spectra were acquired at 300 K, using deuterated dimethyl sulfoxide (DMSO–d_6), deuterated methanol (CD$_3$OD) or deuterated chloroform (CDCl$_3$) as solvents. For ^{1}H-NMR, data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = double of doublets, t = triplet, q = quartet, m = multiplet), coupling constants (Hz) and integration. UPLC-MS analyses were run on a Waters ACQUITY UPLC-MS system consisting of a single quadrupole detector (SQD) mass spectrometer equipped with an electrospray ionization interface (ESI) and a photodiode array detector (PDA) from Waters Inc. (Milford, MA, USA). Electrospray ionization in positive and negative modes was applied in the mass scan range 110 – 650 Da. The PDA range was 210–400 nm. The analyses were performed on an ACQUITY UPLC BEH C$_{18}$ column (50 × 2.1mmID, particle size 1.7 μm) with a VanGuard BEH C$_{18}$ pre-column (5 × 2.1 mmID, particle size 1.7 μm). The mobile phase was 10 mM NH$_4$OAc in H$_2$O at pH 5 adjusted with AcOH (A) and 10 mM NH$_4$OAc in MeCN–H$_2$O (95:5) at pH 5.0 (B). Two types of gradients were applied depending on the polarity of the compounds: gradient 1 (5–95% mobile phase B in 2.5 min) or gradient 2 (50–100% mobile phase B in 2.5 min). A quality control (QC) by UPLC-MS (UV at 215 nm) was performed for all compounds before testing (*see supporting information*). A 10 mM stock solution of the compound was prepared in DMSO–d_6, diluted 20-fold with MeCN – H$_2$O (1:1), and analyzed on a Waters ACQUITY UPLC-MS system as defined above. The analyses were run on an ACQUITY UPLC BEH C$_{18}$ column (100 × 2.1 mmID, particle size 1.7 μm) with a VanGuard BEH C$_{18}$ pre-column (5 × 2.1 mm ID, particle size 1.7 μm). The mobile phase was 10 mM NH$_4$OAc in H$_2$O at pH 5 adjusted with AcOH (A) and 10 mM NH$_4$OAc in MeCN – H$_2$O (95:5) at pH 5 (B) with 0.5 mL/min as the flow rate. A linear gradient was applied: 10% B for 0.2 min, 10 – 90% B in 6.0 min, 90 – 100% in 0.1 min, 100% B for 0.7 min.

High-resolution mass spectrometry (HRMS) for accurate mass measurements was performed on a Sciex TripleTOF High-resolution LC-MS using a Waters UPLC ACQUITY chromatographic system (from Waters Inc., Milford, MA, USA) coupled to a TripleTOF 5600 + Mass Spectrometer (from Sciex, Warrington, UK) equipped with a DuoSpray Ion source. The analyses were run on an ACQUITY UPLC BEH C$_{18}$ column (50 × 2.1 mmID, particle size 1.7 μm), using H$_2$O + 0.1% HCOOH (A) and MeCN + 0.1% HCOOH as mobile phase.

All key compounds showed ≥ 95% UV purity at 215 nm as determined by UPLC-MS analysis. (See supporting information for synthetic details).

General procedure 1. Reactions B scheme 1.a and G scheme 1.b (Fig. 4). Pyrimidone fused ring clorination

A suspension of corresponding pyrimidone fused ring (1 mmol) in POCl$_3$ (1.5 ml) was stirred at 120 °C under N$_2$ atmosphere until total solution was observed (around 4 h). POCl$_3$ was then evaporated at low pressure, resulting residue solved in dichloromethane (3 ml), poured onto ice cold NaHCO$_3$ saturated solution (18 ml), aqueous pH adjusted to 7–8 with NaHCO$_3$ (no gas evolution observed after addition), organic layer separated, dried over Na$_2$SO$_4$ and concentrated to dryness at low pressure. Resulting solid normal phase chromatography purification finally yielded titled compound.

General procedure 2. Reactions C scheme 1.a. And L scheme 1.c (Fig. 4). Substituted aniline introduction

A suspension of chlorinated pyrimidine fused ring (1 mmol), corresponding aniline (1.1 mmol) and diisopropylethylamine (5 mmol) in 2-propanol (2 ml) was stirred in a CEM® microwave apparatus at 100– 160 °C (depending of corresponding aniline) until reaction completion or no crude evolution was observed. Then reaction crude was concentrated to dryness at low pressure, solved in dichloromethane (20 ml), extracted with NaHCO$_3$ saturated solution (20 ml), dried over Na$_2$SO$_4$ and concentrated to dryness at low pressure. Final normal phase purification yielded title compound.

General procedure 3. Reaction D scheme 1.a (Fig. 4). Suzuki coupling reaction

A suspension of compound obtained from general method C (1 mmol), corresponding boronic acid (1.2 mmol), PdCl$_2$(dppf) dichloromethane complex (0.1 mmol) and K2CO3 2 M solution (2 mmol) in 1,4-dioxane (10 ml) was stirred in a CEM ® microwave apparatus at 120 °C for 2 h. Resulting crude was portioned between dichloromethane (25 ml), NaHCO3 saturated solution (25 ml), the organic layer dried over Na$_2$SO$_4$ and concentrated to dryness at low pressure. Final normal phase purification yielded title compound.

General procedure 4. Reactions E scheme 1.a and I scheme 1.b (Fig. 4). Benzyl group removal

Under N$_2$ atmosphere, a suspension of compound to be deprotected (1 mmol), ammonium formate (4 mmol), Pd(OH)$_2$/C (20% of starting material weight) was stirred at reflux temperature until reaction completion. Catalyst was filtered off with trough a celite coarse patch and resulting filtrate concentrated to dryness at low pressure. Final normal phase purification yielded title compound.

Synthesis of compound 6. N4,N4-dimethyl-N1-[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl] benzene-1,4-diamine

Step 1. Synthesis of 6-benzyl-1,5,7,8-tetrahydropyrido[4,3-d]pyrimidine-2,4-dione (intermediate A). A suspension of methyl 1-benzyl-4-oxo-3-piperidinecarboxylate hydrochloride (5 g, 16.7 mmol) in ethanol (76 ml), urea

(5.077 g, 83.7 mmol) and sodium methoxide (4.110 g, 75.3 mmol) was stirred at reflux temperature for 16 h. Afterwards the reaction crude was concentrated to dryness at low pressure, resulting solid triturated in water (20 ml), ice cooled, pH adjusted to 8–9 with concentrated HCl and filtrated. Resulting solid was then rinsed with methanol (20 ml) and diethyl ether (20 ml) yielding title compound (3.778 g, yield 88%). Rt = 1.30 min (gradient 1); MS (ESI) m/z: 258.1 [M+H]$^+$, [M+H]$^+$ calculated for $C_{14}H_{16}N_3O_2$: 258.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.90 (s, 1 H), 10.75 (s, 1 H), 7.39–7.29 (m, 4 H), 7.29–7.21 (m, 1 H), 3.62 (s, 2 H), 3.00 (d, J = 1.8 Hz, 2 H), 2.62 (t, J = 5.7 Hz, 2 H), 2.46–2.38 (m, 2 H).

*Step 2. Synthesis of 6-benzyl-2,4-dichloro-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidine (intermediate **B**).* Title compound was obtained using intermediate A (4.130 g, 16.05 mmol) following the general procedure 1 previously described. Final normal phase purification (cyclohexane/AcOEt from 95/5 to 75/25) afforded pure title compound (3.825 g, yield 81%). Rt = 2.53 min (gradient 2); MS (ESI) m/z: 294.0/296.0/298.0 [M+H]$^+$, [M+H]$^+$ calculated for $C_{14}H_{14}Cl_2N_3$: 294.0/296.0/298.0 *(see supporting information for MS spectra of intermediate B).* ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.50–7.31 (m, 5 H), 3.87 (s, 2 H), 3.72 (s, 2 H), 3.08 (s, 2 H), 2.91 (s, 2 H).

*Step 3. N1-(6-benzyl-2-chloro-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl)-N4,N4-dimethyl-benzene-1,4-diamine (Intermediate **C.1**).* Title compound was obtained using intermediate B (200 mg, 0.68 mmol) and 4-dimethylaminoaniline (107.2 mg, 0.75 mmol) following the general procedure 2 previously described at 120 °C for 16 h. Final normal phase purification (cyclohexane/AcOEt from 70/30 to 50/50) afforded pure title compound (214 mg, yield 80%). Rt = 1.38 min (gradient 2); MS (ESI) m/z: 393.2/395.2 [M+H]$^+$, [M+H]$^+$ calculated for $C_{22}H_{24}ClN_5$: 393.2/395.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57 (s, 1 H), 7.42–7.31 (m, 4 H), 7.30–7.26 (m, 1 H), 7.26–7.22 (m, 2 H), 6.76–6.66 (m, 2 H), 3.74 (s, 2 H), 3.45 (s, 2 H), 2.88 (s, 6 H), 2.77–2.60 (m, 4 H).

*Step 4. Synthesis of N4-[6-benzyl-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl]-N1,N1-dimethyl-benzene-1,4-diamine (intermediate **D.1**).* Title compound was obtained using intermediate C.1 (210 mg, 0.53 mmol) and pyridine-4-boronic acid (87.4 mg, 0.64 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 40/60 to 10/90) afforded pure title compound (137.8 mg, yield 61%). Rt = 1.47 min (gradient 2); MS (ESI) m/z: 437.3 [M+H]$^+$, [M+H]$^+$ calculated for $C_{27}H_{28}N_6$: 437.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70–8.61 (m, 2 H), 8.35 (s, 1 H), 8.12–8.03 (m, 2 H), 7.51–7.44 (m, 2 H), 7.44–7.39 (m, 2 H), 7.39–7.33 (m, 2 H), 7.33–7.25 (m, 1 H), 6.83–6.72 (m, 2 H), 3.78 (s, 2 H), 3.57 (s, 2 H), 2.90 (s, 6 H), 2.85–2.79 (m, 2 H), 2.79–2.72 (m, 2 H).

*Step 5. Synthesis of N4,N4-dimethyl-N1-[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]benzene-1,4-diamine (compound **6**).* Title compound was obtained using intermediate **D.1** (135 mg, 0.31 mmol) following the general procedure 4 previously described for 5 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 95/5 to 60/40) afforded pure title compound (80.3 mg, yield 75%). Rt = 1.55 min (gradient 1); MS (ESI) m/z: 347.2 [M+H]$^+$, [M+H]$^+$ calculated for $C_{20}H_{23}N_6$: 347.2. HRMS (ESI) m/z: 347.1979 calcd for $C_{20}H_{23}N_6$ [M+H]+; found m/z: 347.1984. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78–8.56 (m, 2 H), 8.22 (s, 1 H), 8.17–8.00 (m, 2 H), 7.64–7.39 (m, 2 H), 6.87–6.70 (m, 2 H), 3.75 (s, 2 H), 3.01 (t, J = 5.8 Hz, 2 H), 2.90 (s, 6 H), 2.73–2.64 (t, J = 5.8 Hz, 2 H), 2.60 (s, 1 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.0 (Cq), 157.5 (Cq), 157.1 (Cq), 150.1 (CH), 147.1 (C), 145.5 (C), 128.9 (C), 123.7 (CH), 121.3 (CH), 113.1 (C), 112.4 (CH), 42.6 (CH$_2$), 42.4 (CH$_2$), 40.5 (CH$_3$), 32.0 (CH$_2$). QC purity (UV at 215 nm) 99.5%.

Synthesis of compound 7. N-(3-fluorophenyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
*Step 1. Synthesis of 6-benzyl-2-chloro-N-(3-fluorophenyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (Intermediate **C.2**).* Title compound was obtained using intermediate **B** (140 mg, 0.48 mmol) and 3-fluoroaniline (0.06 ml, 0.57 mmol) following the general procedure 2 previously described at 100 °C for 72 h. Final normal phase purification (cyclohexane/TBME from 90/100 to 50/50) afforded pure title compound (73 mg, yield 42%). Rt = 2.85 min (gradient 2); MS (ESI) m/z: 369.1/371.1 [M+H]$^+$, [M+H]$^+$ calculated for $C_{20}H_{18}ClFN_4$: 369.1/371.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.86 (s, 1 H), 7.61–7.45 (m, 1 H), 7.44–7.31 (m, 7 H), 7.31–7.23 (m, 1 H), 6.99–6.88 (m, 1 H), 3.76 (s, 2 H), 3.54 (s, 2 H), 2.72 (s, 4 H).

*Step 2. Synthesis of 6-benzyl-N-(3-fluorophenyl)-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate **D.2**).* Title compound was obtained using intermediate **C.2** (74 mg, 0.20 mmol) and pyridine-4-boronic acid (32.9 mg, 0.24 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 50/50 to 20/80) afforded pure title compound (65.2 mg, yield 79%). Rt = 2.96 min (gradient 2); MS (ESI) m/z: 412.2 [M+H]$^+$, [M+H]$^+$ calculated for $C_{25}H_{23}FN_5$: 412.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70 (dd, J = 4.4, 1.7 Hz, 3 H), 8.17–8.04 (m, 2 H), 7.67 (dt, J = 12.0, 2.3 Hz, 1 H), 7.56 (ddd, J = 8.3, 2.0, 0.9 Hz, 1 H), 7.45–7.39 (m, 3 H), 7.39–7.34 (m, 2 H), 7.32–7.25 (m, 1 H), 6.91 (tdd, J = 8.5, 2.6, 0.9 Hz, 1 H), 3.80 (s, 2 H), 3.65 (s, 2 H), 2.87 (t, J = 5.8 Hz, 2 H), 2.78 (t, J = 6.1 Hz, 2 H).

*Step 3. Synthesis of N-(3-fluorophenyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound **7**).* Title compound was obtained using intermediate **D.2** (60.0 mg, 0.14 mmol) following the general procedure 4 previously described for 4 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 95/5 to 60/40) afforded pure title compound (30.2 mg, yield 52%). Rt = 1.68 min (gradient 1); MS (ESI) m/z: 322.1 [M+H]$^+$, [M+H]$^+$ calculated for $C_{18}H_{17}FN_5$: 322.1. HRMS (ESI) m/z: 322.1463 calcd for $C_{18}H_{17}FN_5$ [M+H]+; found m/z: 322.1468. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70 (td, J = 4.2, 1.6 Hz, 2 H), 8.57 (d, J = 5.3 Hz, 1 H), 8.10 (td, J = 4.6, 1.6 Hz, 2 H), 7.74 (dt, J = 12.1, 2.3 Hz, 1 H), 7.62 (ddd, J = 8.3, 2.0, 0.9 Hz, 1 H), 7.50–7.31 (m, 1 H), 6.90 (tt, J = 8.4, 2.4 Hz, 1 H), 3.82 (d, J = 3.6 Hz, 2 H), 3.02 (td, J = 5.9, 2.6 Hz, 2 H), 2.74 (q, J = 5.4 Hz, 2 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.4 (Cq), 162.0 (Cq, d, JCF = 241.3 Hz), 157.5 (Cq), 156.62 (Cq), 150.2 (CH), 145.13 (Cq), 141.50 (Cq, d, JCF = 11.1 Hz), 129.83 (CH, d, JCF = 9.5 Hz), 121.23 (CH), 117.21 (CH, d, JCF = 2.4 Hz), 114.34 (C), 109.14 (CH, d, JCF = 20.8 Hz), 108.12 (CH, d, JCF = 26.2 Hz), 42.60 (CH2), 42.25 (CH2), 32.07 (CH2). QC purity (UV at 215 nm) 99%.

Synthesis of compound 8. N-(3-fluorophenyl)-2-(4-pyridyl)pyrido[4,3-d]pyrimidin-4-amine
*Step 1. Synthesis of 2-(4-pyridyl)-3 H-pyrido[4,3-d]pyrimidin-4-one (intermediate **J**).*

A mixture of 4-Amino-nicotinic acid (137 mg, 0.96 mmol) and pyridine-4-carboximidamide (582.8 mg, 4.81 mmol) in 1-metil-2-pirrolidone (1 ml) was stirred under Ar atmosphere at 160 °C for 72 h. The reaction crude was poured in water (10 ml), the resulting solid filtered off and the filtrate concentrated at low pressure. Final normal phase purification (DCM/DCM: MeOH 4:1 from 95/5 to 75/25) yielded pure title compound (101 mg, yield 47%). Rt = 1.10 min (gradient 1); MS (ESI) m/z: 225.4 $[M+H]^+$, $[M+H]^+$ calculated for $C_{12}H_9N_4O$: 225.1. ^1H NMR (400 MHz, DMSO-d_6) δ 13.10 (s, 1 H), 9.33 (s, 1 H), 8.88 (d, J = 5.6 Hz, 1 H), 8.82 (d, J = 5.1 Hz, 2 H), 8.10 (d, J = 5.2 Hz, 2 H), 7.68 (d, J = 5.6 Hz, 1 H).

*Step 2. Synthesis of 4-chloro-2-(4-pyridyl)pyrido[4,3-d]pyrimidine (intermediate **K**).*

A mixture of intermediate **J** (85 mg, 0.38 mmol), PCl5 (88.6 mg, 0.42 mmol) and POCl$_3$ (1.1 ml, 11.37 mmol) was stirred under N$_2$ at 120 °C for 16 h. The reaction crude was then concentrated to dryness at low pressure, portioned between DCM (5 ml) and cold NaHCO$_3$ saturated solution (15 ml), the organic layer dried over Na$_2$SO$_4$ and concentrated to dryness at low pressure yielding pure title compound (92 mg, yield 99%). Rt = 1.74 min (gradient 1); MS (ESI) m/z: 243.3/245.3 $[M+H]^+$, $[M+H]^+$ calculated for $C_{12}H_8ClN_4$: 243.1/245.1. ^1H NMR (400 MHz, Acetone-d_6) δ 9.72 (d, J = 0.9 Hz, 1 H), 9.10 (d, J = 5.9 Hz, 1 H), 8.92–8.80 (m, 2 H), 8.49–8.40 (m, 2 H), 8.05 (dd, J = 5.9, 0.9 Hz, 1 H).

*Step 3. N-(3-fluorophenyl)-2-(4-pyridyl)pyrido[4,3-d]pyrimidin-4-amine (compound **8**).*

Title compound was obtained using intermediate K (92 mg, 0.38 mmol) and 3-fluoroaniline (0.045 ml, 0.45 mmol) following the general procedure 2 previously described. Final normal phase purification (DCM/DCM: MeOH 4:1 from 100:0 to 60:40) yielded pure titled compound (61 mg, yield 50%). Rt = 1.93 min (gradient 1); MS (ESI) m/z: 318.4 $[M+H]^+$, $[M+H]^+$ calculated for $C_{18}H_{13}FN_5$: 318.1. ^1H NMR (400 MHz, DMSO-d_6) δ 10.51 (s, 1 H), 9.92–9.80 (m, 1 H), 8.91–8.81 (m, 1 H), 8.81–8.71 (m, 2 H), 8.22 (dq, J = 4.9, 1.6 Hz, 2 H), 7.93–7.86 (m, 1 H), 7.80–7.71 (m, 1 H), 7.53 (tdd, J = 8.2, 6.7, 1.3 Hz, 1 H), 7.06 (td, J = 8.5, 2.6 Hz, 1 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.6 (Cq, d, JCF = 276.1 Hz), 153.71 (Cq), 151.1 (CH), 150.4 (CH), 148.3 (CH), 144.6 (Cq), 140.1 (CH, d, JCF = 10.9 Hz), 130.2 (CH, d, JCF = 9.4 Hz), 121.9 (CH), 120.7 (CH), 118.3 (CH, d, JCF = 2.6 Hz), 111.0 (CH, d, JCF = 21.2 Hz), 110.4 (Cq), 109.4 (CH, d, JCF = 25.7 Hz). QC purity (UV at 215 nm) > 99.5%.

Synthesis of compound 9. N-(3-fluorophenyl)-N-methyl-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
*Step 1. Synthesis of 6-benzyl-2-(4-pyridyl)-3,5,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-one (intermediate **F.1**).*

A suspension of methyl 1-benzyl-4-oxo-3-piperidinecarboxylate hydrochloride (250 mg, 0.84 mmol), pyridine-4-carboximidamide hydrochloride (680 mg, 4.18 mmol) and sodium methoxide (251 mg, 4.60 mmol) in ethanol (3.8 ml) was stirred at reflux temperature for 16 h. Afterwards, the reaction crude was concentrated to dryness at low pressure, triturated in water (40 ml) and filtered. Resulting solid was then rinsed with methanol (0.5 ml). Final normal phase purification, (DCM/DCM: MeOH 4:1 from 90/10 to 70/30) afforded pure title compound (185 mg, yield 69%). Rt = 1.47 min (gradient 1); MS (ESI) m/z: 319.1 $[M+H]^+$, $[M+H]^+$ calculated for $C_{19}H_{18}N_4O$: 319.1. ^1H NMR (400 MHz, DMSO-d_6) δ 12.82 (s, 1 H), 8.77–8.73 (m, 2 H), 8.03–7.98 (m, 2 H), 7.40–7.34 (m, 4 H), 7.32–7.24 (m, 1 H), 3.70 (s, 2 H), 3.27 (s, 2 H), 2.74 (s, 4 H).

*Step 2. Synthesis of 6-benzyl-4-chloro-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidine (intermediate **G.1**).*

Title compound was obtained using Intermediate **F.1** (180.0 mg, 0.57 mmol) following the general procedure 2 previously described. Normal phase purification (DCM/DCM: MeOH 4:1 from 100/0 to 80/20) afforded pure title compound (145 mg, yield 74%). Rt = 2.63 min (gradient 1); MS (ESI) m/z: 337/339 $[M+H]^+$, $[M+H]^+$ calculated for $C_{19}H_{18}ClN_4$: 337/339. ^1H NMR (400 MHz, DMSO-d_6) δ 8.79–8.73 (m, 2 H), 8.20–8.14 (m, 2 H), 7.46–7.34 (m, 4 H), 7.34 (s, 1 H), 3.81 (s, 2 H), 3.67 (s, 2 H), 3.04 (t, J = 5.8 Hz, 2 H), 2.88 (d, J = 5.9 Hz, 2 H).

*Step 3. Synthesis of 6-benzyl-2-(4-pyridyl)-3,5,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-one (intermediate **H.1**).*

A mixture of Pd(OAc)$_2$ (9.7 mg, 0.04 mmol) and rac-BINAP (26.8 mg, 0.04 mmol) in 1,4-dioxane (1.4 ml) was stirred under Ar flushing for 10 min. Then were stepwise added a solution of corresponding intermediate G.1 (142 mg, 0.42 mmol) in 1,4-dioxane (0.7 ml), 3-fluoro-N-methylaniline (65.3 mg, 0.51 mmol) and Cs$_2$CO$_3$ (194.3 mg, 0.59 mmol). The reaction mixture was stirred in a CEM* microwave apparatus at 100 °C for 3 h, filtrated through a celite coarse patch, rinsed with DCM and concentrated to dryness at low pressure. Final normal phase purification (cHexane/AcOEt from 60/40 to 40/60) yielded title compound (66 mg, yield 37%). Rt = 1.71 min (gradient 2); MS (ESI) m/z: 426.5 $[M+H]^+$, $[M+H]^+$ calculated for $C_{26}H_{25}FN_5$: 426.2. ^1H NMR (400 MHz, DMSO-d_6) δ 8.76–8.69 (m, 2 H), 8.28–8.22 (m, 2 H), 7.44–7.32 (m, 1 H), 7.32–7.20 (m, 3 H), 7.09 (tdd, J = 8.5, 2.5, 0.8 Hz, 1 H), 7.05–6.96 (m, 3 H), 6.89 (ddd, J = 8.0, 2.1, 0.9 Hz, 1 H), 3.49 (s, 3 H), 3.40 (s, 2 H), 2.90 (t, J = 6.0 Hz, 2 H), 2.72–2.63 (m, 2 H), 2.59 (s, 2 H).

*Step 4. Synthesis of N-(3-fluorophenyl)-N-methyl-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound **9**).*

Title compound was obtained using intermediate H.1 (63.0 mg, 0.15 mmol) following the general procedure 4 previously described for 6 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 100/0 to 85/15) afforded pure title compound (12.8 mg, yield 25%). Rt = 1.56 min (gradient 1); MS (ESI) m/z: 336.4 $[M+H]^+$, $[M+H]^+$ calculated for $C_{19}H_{19}FN_5$: 336.2. HRMS (ESI) m/z: 336.1619 calcd for $C_{19}H_{19}FN_5$ $[M+H]^+$; found m/z: 336.1625. ^1H NMR (400 MHz, DMSO- d_6) δ 8.76–8.70 (m, 2 H), 8.27–8.22 (m, 2 H), 7.39 (td, J = 8.2, 6.8 Hz, 1 H), 7.12 (dt, J = 10.8, 2.3 Hz, 1 H), 7.03 (tdd, J = 8.5, 2.6, 0.8 Hz, 1 H), 6.95 (ddd, J = 8.1, 2.1, 0.9 Hz, 1 H), 3.54 (s, 3 H), 3.32 (s, 3 H), 2.95 (s, 2 H), 2.89 (t, J = 6.1 Hz, 2 H), 2.77 (t, J = 6.0 Hz, 2 H), 2.30 (s, 1 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.56 (Cq), 162.61 (Cq, d, JCF = 244.0 Hz), 160.60 (Cq), 158.02 (Cq), 150.27 (CH), 148.40 (Cq, d, JCF = 9.8 Hz), 144.89 (Cq), 130.84 (CH, d, JCF = 9.5 Hz), 121.42 (CH), 120.18 (CH, d,

JCF = 2.6 Hz), 118.67 (Cq), 111.65 (CH, d, JCF = 20.9 Hz), 111.33 (CH, d, JCF = 23.1 Hz), 45.29 (CH$_2$), 42.49 (CH$_2$), 40.81 (CH$_3$), 32.14 (CH$_2$). QC purity (UV at 215 nm) 96%.

Synthesis of compound 10. N-(3-fluorophenyl)-2-phenyl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
*Step 1. Synthesis of 6-benzyl-N-(3-fluorophenyl)-2-phenyl-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate **D.3**).*

Title compound was obtained using intermediate **C.2** (125 mg, 0.34 mmol) and phenyl boronic acid (51.1 mg, 0.41 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 100/0 to 80/20) afforded pure title compound (108.0 mg, yield 78%). Rt = 2.22 min (gradient 2); MS (ESI) m/z: 411.3 [M + H]$^+$, [M + H]$^+$ calculated for C$_{26}$H$_{24}$FN$_4$: 411.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.58 (s, 1 H), 8.32–8.23 (m, 2 H), 7.74 (dt, J = 12.2, 2.3 Hz, 1 H), 7.57 (ddd, J = 8.2, 2.0, 0.9 Hz, 1 H), 7.51–7.45 (m, 3 H), 7.44–7.33 (m, 6 H), 7.32–7.24 (m, 1 H), 6.88 (tdd, J = 8.5, 2.6, 0.9 Hz, 1 H), 3.79 (s, 2 H), 3.64 (s, 2 H), 2.84 (t, J = 5.7 Hz, 2 H), 2.77 (t, J = 5.7 Hz, 2 H).

*Step 2. Synthesis of N-(3-fluorophenyl)-2-phenyl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound **10**).*

Title compound was obtained using intermediate **D.3** (105 mg, 0.26 mmol) following the general procedure 4 previously described for 3 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (12.8 mg, yield 25%). Rt = 1.98 min (gradient 1); MS (ESI) m/z: 321.2 [M + H]$^+$, [M + H]$^+$ calculated for C$_{19}$H$_{18}$FN$_4$: 321.1. HRMS (ESI) m/z: 321.1510 calcd for C$_{19}$H$_{18}$FN$_4$: [M + H]+; found m/z: 321.1517. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.45 (s, 1 H), 8.34–8.22 (m, 2 H), 7.81 (dt, J = 12.2, 2.3 Hz, 1 H), 7.62 (ddd, J = 8.3, 2.1, 0.9 Hz, 1 H), 7.55–7.43 (m, 3 H), 7.39 (td, J = 8.2, 6.9 Hz, 1 H), 6.87 (td, 1 H), 3.81 (s, 2 H), 3.02 (t, J = 5.8 Hz, 2 H), 2.72 (t, J = 5.8 Hz, 2 H), 2.61 (s, 1 H). 13 C NMR (151 MHz, DMSO-d_6) δ 162.28 (C), 162.04 (CF, d, JCF = 240.1 Hz), 159.33 (C), 156.49 (C), 141.86 (C, d, JCF = 11.1 Hz), 138.02 (C), 130.06 (CH), 129.83 (CH, d, JCF = 9.5 Hz), 128.45 (CH), 127.28 (CH), 116.93 (d, JCF = 1.9 Hz), 112.89 (C), 108.81 (CH, d, JCF = 21.7 Hz), 107.83 (CH, d, JCF = 26.4 Hz), 42.57 (CH2), 42.41 (CH2), 32.20 (CH2). QC purity (UV at 215 nm) 99%.

Synthesis of compound 11. N-(3-fluorophenyl)-2-(3-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
*Step 1. Synthesis of 6-benzyl-N-(3-fluorophenyl)-2-(3-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate **D.4**).*

Title compound was obtained using intermediate **C.2** (125 mg, 0.34 mmol) and pyridine-3-boronic acid (55.5 mg, 0.41 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 70/30 to 50/50) afforded pure title compound (115.8 mg, yield 83%). Rt = 1.54 min (gradient 2); MS (ESI) m/z: 412.3 [M + H]$^+$, [M + H]$^+$ calculated for C$_{25}$H$_{22}$FN$_5$: 412.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.37 (dd, J = 2.2, 0.8 Hz, 1 H), 8.72–8.61 (m, 2 H), 8.51 (dt, J = 8.0, 1.9 Hz, 1 H), 7.70 (dt, J = 12.1, 2.3 Hz, 1 H), 7.55 (ddd, J = 8.3, 2.0, 0.9 Hz, 1 H), 7.51 (ddd, J = 8.0, 4.8, 0.9 Hz, 1 H), 7.46–7.33 (m, 5 H), 7.32–7.25 (m, 1 H), 6.90 (tdd, J = 8.5, 2.6, 0.9 Hz, 1 H), 3.80 (s, 2 H), 3.64 (s, 2 H), 2.86 (t, J = 5.8 Hz, 2 H), 2.78 (t, J = 5.6 Hz, 2 H).

*Step 2. N-(3-fluorophenyl)-2-(3-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound **11**).*

Title compound was obtained using intermediate **D.4** (110 mg, 0.27 mmol) following the general procedure 4 previously described for 6 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (55.8 mg, yield 65%). Rt = 1.54 min (gradient 1); MS (ESI) m/z: 322.2 [M + H]$^+$, [M + H]$^+$ calculated for C$_{18}$H$_{17}$FN$_5$: 322.1. HRMS (ESI) m/z: 322.1463 calcd for C$_{18}$H$_{17}$FN$_5$ [M + H]$^+$; found m/z: 322.1468. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.39 (dd, J = 2.2, 0.9 Hz, 1 H), 8.65 (dd, J = 4.8, 1.7 Hz, 1 H), 8.59–8.49 (m, 2 H), 7.76 (dt, J = 12.1, 2.3 Hz, 1 H), 7.61 (ddd, J = 8.3, 2.1, 0.9 Hz, 1 H), 7.51 (ddd, J = 8.0, 4.8, 0.9 Hz, 1 H), 7.40 (td, J = 8.3, 6.9 Hz, 1 H), 6.89 (tdd, J = 8.5, 2.6, 0.9 Hz, 1 H), 3.82 (s, 2 H), 3.03 (t, J = 5.8 Hz, 2 H), 2.74 (t, J = 5.8 Hz, 2 H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 162.4 (Cq), 162.0 (Cq, d, JCF = 240.1 Hz), 157.7 (CH), 156.5 (CH), 150.7 (Cq), 148.6 (Cq, d, JCF = 11.1 Hz), 141.6 (CH, d, J = 11.0 Hz), 134.6 (CH), 133.3 (C), 129.9 (CH, d, J = 9.1 Hz), 123.6 (CH), 117.2 (C), 113.5 (C), 109.1 (CH, d, J = 21.4 Hz), 108.1 (CH, d, J = 26.3 Hz), 42.5 (CH$_2$), 42.3 (CH$_2$), 32.1 (CH$_2$). QC purity (UV at 215 nm) 97%.

Synthesis of compound 12. N-(3-fluorophenyl)-2-(2-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
*Step 1. Synthesis of 6-benzyl-2-(2-pyridyl)-3,5,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-one (intermediate **F.2**).*

A suspension of methyl 1-benzyl-4-oxo-3-piperidinecarboxylate hydrochloride (250 mg, 0.84 mmol), Pyridine-2-carboximidamideydrochloride (680 mg, 4.18 mmol) and sodium methoxide (251 mg, 4.60 mmol) in ethanol (3.8 ml) was stirred at reflux temperature for 6 h. Afterwards, the reaction crude was concentrated to dryness at low pressure, triturated in water (40 ml) and filtered. Resulting solid was then rinsed with methanol (0.5 ml). Final normal phase purification, (DCM/DCM: MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (185 mg, yield 69%). Rt = 1.81 min (gradient 1); MS (ESI) m/z: 319.1 [M + H]$^+$, [M + H]$^+$ calculated for C$_{19}$H$_{19}$N$_4$O: 319.1. 1 H NMR (400 MHz, DMSO- d_6) δ 11.95 (s, 1 H), 8.72 (dt, J = 4.8, 1.4 Hz, 1 H), 8.28 (dt, J = 7.9, 1.1 Hz, 1 H), 8.02 (td, J = 7.8, 1.8 Hz, 1 H), 7.62 (ddd, J = 7.6, 4.8, 1.2 Hz, 1 H), 7.33–7.26 (m, 1 H), 3.70 (s, 2 H), 3.28 (s, 2 H), 2.74 (s, 4 H).

*Step 2. Synthesis of 6-benzyl-4-chloro-2-(2-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidine (intermediate **G.2**).*

Title compound was obtained using Intermediate **F.2** (180.0 mg, 0.57 mmol) following the general procedure 2 previously described. Normal phase purification (DCM/DCM: MeOH 4:1 from 90/10 to 0/100) afforded pure title compound (133 mg, yield 70%). Rt = 2.34 min (gradient 1); MS (ESI) m/z: 337.1/339.1 [M + H]$^+$, [M + H]$^+$ calculated for C$_{19}$H$_{18}$ClN$_4$: 337.1/339.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.75 (ddd, J = 4.7, 1.8, 0.9 Hz, 1 H),

8.33 (dt, J = 8.0, 1.1 Hz, 1 H), 7.98 (td, J = 7.8, 1.8 Hz, 1 H), 7.55 (ddd, J = 7.6, 4.7, 1.2 Hz, 1 H), 7.44–7.35 (m, 4 H), 7.35–7.26 (m, 1 H), 3.81 (s, 2 H), 3.66 (s, 2 H), 3.03 (t, J = 5.8 Hz, 2 H), 2.87 (t, J = 5.8 Hz, 2 H).

Step 3. Synthesis of 6-benzyl-N-(3-fluorophenyl)-2-(2-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate H.2).

A mixture of $Pd(OAc)_2$ (8.8 mg, 0.04 mmol) and rac-BINAP (24.5 mg, 0.04 mmol) in 1,4-dioxane (1.2 ml) was stirred under Ar flushing for 10 min. Then were stepwise added a solution of corresponding intermediate **G.2** (130 mg, 0.39 mmol) in 1,4-dioxane (0.7 ml), m-fluoroaniline (0.046 ml, 0.46 mmol) and Cs_2CO_3 (177.8 mg, 0.54 mmol). The reaction mixture was stirred in a CEM° microwave apparatus at 100 °C for 3.5 h, filtrated through a celite coarse patch, rinsed with DCM and concentrated to dryness at low pressure. Final normal phase purification (cHexane/AcOEt from 85/15 to 0/100) yielded title compound (95 mg, yield 60%). Rt = 1.18 min (gradient 2); MS (ESI) m/z: 412.4 [M + H]$^+$, [M + H]$^+$ calculated for $C_{25}H_{23}FN_5$: 412.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.71 (ddd, J = 4.8, 1.8, 0.9 Hz, 1 H), 8.61 (s, 1 H), 8.23 (dt, J = 7.9, 1.1 Hz, 1 H), 8.01 (dt, J = 12.5, 2.3 Hz, 1 H), 7.92 (td, J = 7.7, 1.8 Hz, 1 H), 7.63 (ddd, J = 8.3, 2.1, 0.9 Hz, 1 H), 7.46 (ddd, J = 7.6, 4.7, 1.2 Hz, 1 H), 7.44–7.39 (m, 2 H), 7.36 (dt, J = 9.4, 7.7 Hz, 3 H), 7.31–7.25 (m, 1 H), 6.85 (tdd, J = 8.4, 2.6, 0.9 Hz, 1 H), 3.80 (s, 2 H), 3.67 (s, 2 H), 2.85 (t, J = 5.7 Hz, 2 H), 2.78 (t, J = 5.7 Hz, 2 H).

Step 4. Synthesis of N-(3-fluorophenyl)-2-(2-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound 12).

Title compound was obtained using intermediate **H.2** (92.0 mg, 0.22 mmol) following the general procedure 4 previously described for 6 h. Final normal phase purification (DCM/DCM: NH₃ 1 N MeOH 4:1 from 50/50 to 30/70) afforded pure title compound (34.0 mg, yield 47%). Rt = 1.55 min (gradient 1); MS (ESI) m/z: 322.4 [M + H]$^+$, [M + H]$^+$ calculated for $C_{18}H_{17}FN_5$: 322.1. HRMS (ESI) m/z: 322.1463 calcd for $C_{18}H_{17}FN_5$ [M + H]$^+$; found m/z: 322.1475. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.72 (ddd, J = 4.7, 1.9, 0.9 Hz, 1 H), 8.59 (s, 1 H), 8.24 (dt, J = 7.9, 1.1 Hz, 1 H), 8.07 (dt, J = 12.5, 2.3 Hz, 1 H), 7.93 (td, J = 7.7, 1.8 Hz, 1 H), 7.78–7.61 (m, 1 H), 7.47 (ddd, J = 7.5, 4.7, 1.2 Hz, 1 H), 7.36 (td, J = 8.3, 7.0 Hz, 1 H), 6.85 (tdd, J = 8.4, 2.5, 0.9 Hz, 1 H), 3.93 (s, 2 H), 3.13 (t, J = 5.8 Hz, 2 H), 2.81 (t, J = 5.9 Hz, 2 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.1 (Cq, d, JCF = 240.0 Hz), 161.8 (Cq), 159.55 (Cq), 156.6 (Cq), 155.28 (C), 149.5 (CH), 141.9 (C, d, JCF = 11.3 Hz), 136.8 (CH), 129.7 (CH, d, JCF = 9.7 Hz), 124.5 (CH), 122.87 (CH), 116.7 (CH d, JCF = 12.0 Hz), 112.6 (C), 108.7 (CH, d, JCF = 20.3 Hz), 107.8 (CH, d, JCF = 26.5 Hz), 42.1 (CH₂), 41.9 (CH₂), 31.4 (CH₂). QC purity (UV at 215 nm) 99%.

Synthesis of compound 13. N-phenyl-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
Step 1. Synthesis of 6-benzyl-2-chloro-N-phenyl-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate C.3).

Title compound was obtained using intermediate **B** (200 mg, 0.68 mmol) and aniline (0.07 ml, 0.75 mmol) following the general procedure 2 previously described at 120 °C for 16 h. Final normal phase purification (cyclohexane/AcOEt from 95:5 to 60:40) afforded pure title compound (160 mg, yield 62%). Rt = 1.43 min (gradient 2); MS (ESI) m/z: 351.1/353.1 [M + H]$^+$, [M + H]$^+$ calculated for $C_{20}H_{20}ClN_4$: 351.1/353.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.76 (s, 1 H), 7.57–7.48 (m, 2 H), 7.43–7.32 (m, 6 H), 7.32–7.24 (m, 1 H), 7.17–7.06 (m, 1 H), 3.76 (s, 2 H), 3.53 (s, 2 H), 2.72 (s, 4 H).

Step 2. Synthesis of 6-benzyl-N-phenyl-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (D.5).

Title compound was obtained using intermediate **C.3** (155.0 mg, 0.44 mmol) and Pyridine-4-boronic acid (72.4 mg, 0.53 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 50/50 to 30/70) afforded pure title compound (106.0 mg, yield 61%). Rt = 1.51 min (gradient 2); MS (ESI) m/z: 394.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{25}H_{24}N_5$: 394.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.71–8.65 (m, 2 H), 8.57 (s, 1 H), 8.13–8.06 (m, 2 H), 7.74–7.65 (m, 2 H), 7.47–7.32 (m, 6 H), 7.32–7.25 (m, 1 H), 7.16–7.05 (m, 1 H), 3.80 (s, 2 H), 3.64 (s, 2 H), 2.84 (t, J = 5.5 Hz, 2 H), 2.78 (t, J = 5.4 Hz, 2 H).

Step 3. Synthesis of N-phenyl-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound 13).

Title compound was obtained using intermediate **D.5** (105.0 mg, 0.27 mmol) following the general procedure 4 previously described for 5 h. Final normal phase purification (DCM/DCM: NH₃ 1 N MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (65.6 mg, yield 81%). Rt = 1.43 min (gradient 1); MS (ESI) m/z: 304.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{18}H_{18}N_5$: 304.1. HRMS (ESI) m/z: 304.1557 calcd for $C_{18}H_{18}N_5$ [M + H]$^+$; found m/z: 304.1565. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.75–8.60 (m, 2 H), 8.43 (s, 1 H), 8.18–8.05 (m, 2 H), 7.80–7.71 (m, 2 H), 7.45–7.35 (m, 2 H), 7.10 (tt, J = 7.3, 1.2 Hz, 1 H), 3.81 (s, 2 H), 3.03 (t, J = 5.8 Hz, 2 H), 2.73 (t, J = 5.8 Hz, 2 H), 2.62 (s, 1 H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 162.0 (Cq), 157.5 (Cq), 156.9 (Cq), 150.2 (CH), 145.3 (Cq), 139.5 (Cq), 128.4 (CH), 123.1 (CH), 122.0 (CH), 121.3 (CH), 113.9 (Cq), 42.7 (CH₂), 42.3 (CH₂), 32.1 (CH₂). QC purity (UV at 215 nm) > 99.5%.

Synthesis of compound 14. N-(4-fluorophenyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
Step 1. Synthesis of 6-benzyl-2-chloro-N-(4-fluorophenyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate C.4).

Title compound was obtained using intermediate **B** (225 mg, 0.76 mmol) and p-fluoroaniline (0.082 ml, 0.84 mmol) following the general procedure 2 previously described at 120 °C for 65 h. Final normal phase purification (cyclohexane/AcOEt from 95/5 to 75/25) afforded pure title compound (234 mg, yield 83%). Rt = 1.38 min (gradient 2); MS (ESI) m/z: 369.2/371.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{20}H_{19}ClFN_4$: 369.1/371.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.79 (s, 1 H), 7.59–7.47 (m, 2 H), 7.44–7.32 (m, 4 H), 7.32–7.24 (m, 1 H), 7.23–7.13 (m, 2 H), 3.75 (s, 2 H), 3.50 (s, 2 H), 2.71 (d, J = 2.7 Hz, 4 H).

Step 2. Synthesis of 6-benzyl-N-(4-fluorophenyl)-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate D.6).

Title compound was obtained using intermediate **C.4** (230 mg, 0.62 mmol) and Pyridine-4-boronic acid (102.2 mg, 0.75 mmol) following the general procedure 3 previously described. Final normal phase purification

(cyclohexane/AcOEt from 70/30 to 60/40) afforded pure title compound (170.0 mg, yield 70%). Rt = 1.48 min (gradient 2); MS (ESI) m/z: 412.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{25}H_{23}FN_5$: 412.2. ^{1}H NMR (400 MHz, DMSO- d_6) δ 8.71–8.64 (m, 2 H), 8.62 (s, 1 H), 8.11–8.04 (m, 2 H), 7.76–7.62 (m, 2 H), 7.48–7.39 (m, 2 H), 7.37 (dd, J = 8.4, 6.7 Hz, 2 H), 7.33–7.26 (m, 1 H), 7.25–7.18 (m, 2 H), 3.79 (s, 2 H), 3.61 (s, 2 H), 2.84 (t, J = 5.5 Hz, 2 H), 2.78 (t, J = 5.3 Hz, 2 H).

Step 3. Synthesis of N-(4-fluorophenyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound 14).

Title compound was obtained using intermediate **D.6** (176 mg, 0.43 mmol) following the general procedure reaction 4 previously described for 5 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (72.9 mg, yield 53%). Rt = 1.51 min (gradient 1); MS (ESI) m/z: 322.1 [M + H]$^+$, [M + H]$^+$ calculated for $C_{18}H_{17}FN_5$: 322.1. HRMS (ESI) m/z: 322.1463 calcd for $C_{18}H_{18}N_5$ [M + H]$^+$; found m/z: 322.1465. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.74–8.63 (m, 2 H), 8.50 (s, 1 H), 8.13–8.05 (m, 2 H), 7.79–7.68 (m, 2 H), 7.28–7.18 (m, 2 H), 3.79 (s, 2 H), 3.02 (t, J = 5.8 Hz, 2 H), 2.72 (t, J = 5.8 Hz, 2 H), 2.63 (s, 1 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.9 (Cq), 158.1 (Cq, d, JCF = 240.2 Hz), 156.9 (Cq), 150.17 (CH), 145.2 (Cq), 135.8 (Cq, d, JCF = 3.1 Hz), 124.0 (CH, d, JCF = 8.0 Hz), 121.3 (CH), 114.9 (CH, d, JCF = 22.2 Hz), 113.7 (Cq), 42.61 (CH$_2$), 42.3 (CH$_2$), 32.0 (CH$_2$). QC purity (UV at 215 nm) 99%.

Synthesis of compound 15. N-(p-tolyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
Step 1. Synthesis of 6-benzyl-2-chloro-N-(p-tolyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate C.5).

Title compound was obtained using intermediate **B** (200 mg, 0.68 mmol) and p-toluidine (81 mg, 0.75 mmol) following the general procedure 2 previously described at 100 °C for 16 h. Final normal phase purification (cyclohexane/AcOEt from 95:5 to 70:30) afforded pure title compound (235.6 mg, yield 95%). Rt = 1.55 min (gradient 2); MS (ESI) m/z: 364.1/366.1 [M + H]$^+$, [M + H]$^+$ calculated for $C_{21}H_{22}ClN_4^+$: 364.1/366.1. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.43–7.28 (m, 7 H), 7.14 (d, J = 8.1 Hz, 2 H), 3.82 (s, 2 H), 3.48 (s, 2 H), 2.94–2.80 (m, 4 H), 2.33 (s, 3 H).

Step 2. Synthesis of 6-benzyl-N-(p-tolyl)-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate D.7).

Title compound was obtained using intermediate **C.5** (235 mg, 0.64 mmol) and Pyridine-4-boronic acid (114.7 mg, 0.77 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 80/20 to 50/50) afforded pure title compound (170 mg, yield 65%). Rt = 1.61 min (gradient 2); MS (ESI) m/z: 408.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{26}H_{26}N_5$: 408.5. ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.78–8.61 (m, 2 H), 8.26–8.11 (m, 2 H), 7.54–7.46 (m, 2 H), 7.45–7.34 (m, 4 H), 7.34–7.28 (m, 1 H), 7.20 (d, J = 8.2 Hz, 2 H), 6.08 (s, 1 H), 3.82 (s, 2 H), 3.51 (s, 2 H), 3.00 (t, J = 5.8 Hz, 2 H), 2.90 (t, J = 5.7 Hz, 2 H), 2.37 (s, 3 H).

Step 3. Synthesis of N-(p-tolyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound 15).

Title compound was obtained using intermediate **D.7** (170 mg, 0.42 mmol) following the general procedure 4 previously described for 5 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 95/5 to 20/80) afforded pure title compound (29.7 mg, yield 22%). Rt = 1.57 min (gradient 1); MS (ESI) m/z: 318.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{19}H_{20}N_5$: 318.2. HRMS (ESI) m/z: 318.1713 calcd for $C_{19}H_{20}N_5$ [M + H]$^+$; found m/z: 318.1725. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.72–8.63 (m, 2 H), 8.42 (s, 1 H), 8.12–8.07 (m, 2 H), 7.64–7.58 (m, 2 H), 7.20 (d, J = 8.1 Hz, 2 H), 3.82 (s, 2 H), 3.05 (t, J = 5.8 Hz, 2 H), 2.74 (t, J = 5.7 Hz, 2 H), 2.31 (s, 3 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.51 (Cq), 157.6 (Cq), 157.0 (Cq), 150.2 (CH), 145.3 (Cq), 136.8 (Cq), 132.10 (Cq), 128.80 (CH), 122.16 (CH), 121.29 (CH), 113.15 (Cq), 42.36 (CH$_2$), 42.1 (CH$_2$), 31.7 (CH$_2$), 20.5 (CH$_3$). QC purity (UV at 215 nm) > 99.5%.

Synthesis of compound 16. N-(m-tolyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
Step 1. Synthesis of 6-benzyl-2-chloro-N-(m-tolyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate C.6).

Title compound was obtained using intermediate **B** (200 mg, 0.68 mmol) and m-toluidine (0.083 ml, 0.75 mmol) following the general procedure 2 previously described at 100 °C for 4 h. Final normal phase purification (cyclohexane/TBME from 100/0 to 70/30) afforded pure title compound (129 mg, yield 52%). Rt = 1.64 min (gradient 2); MS (ESI) m/z: 365.2/367.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{21}H_{22}ClN_4$: 365.9/367.9.

Step 2. Synthesis of 6-benzyl-N-(m-tolyl)-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate D.8).

Title compound was obtained using intermediate **C.6** (126 mg, 0.35 mmol) and pyridine-4-boronic acid (56.6 mg, 0.41 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 60/40 to 20/80) afforded pure title compound (66.0 mg, yield 47%). Rt = 0.79 min (gradient 2); MS (ESI) m/z: 408.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{26}H_{26}N_5$: 408.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.73–8.65 (m, 2 H), 8.50 (s, 1 H), 8.15–8.04 (m, 2 H), 7.56–7.50 (m, 2 H), 7.45–7.39 (m, 2 H), 7.37 (dd, J = 8.4, 6.7 Hz, 2 H), 7.32–7.23 (m, 2 H), 6.96–6.89 (m, 1 H), 3.79 (s, 2 H), 3.62 (s, 2 H), 2.84 (t, J = 5.6 Hz, 2 H), 2.78 (t, J = 5.3 Hz, 2 H), 2.34 (s, 3 H).

Step 3. Synthesis of N-(m-tolyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound 16).

Title compound was obtained using intermediate **D.9** (58 mg, 0.14 mmol) following the general procedure 4 previously described for 6 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 95/5 to 55/45) afforded pure title compound (15.4 mg, yield 34%). Rt = 1.56 min (gradient 1); MS (ESI) m/z: 318.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{19}H_{20}N_5$: 318.2. HRMS (ESI) m/z: 318.1713 calcd for $C_{19}H_{20}N_5$ [M + H]$^+$;

found m/z: 318.1718. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.70–8.65 (m, 2 H), 8.38 (s, 1 H), 8.18–8.05 (m, 2 H), 7.66–7.52 (m, 2 H), 7.27 (t, J = 7.7 Hz, 1 H), 6.92 (dq, J = 7.6, 0.9 Hz, 1 H), 3.80 (d, J = 1.4 Hz, 2 H), 3.32 (s, 2 H), 3.02 (t, J = 5.8 Hz, 2 H), 2.72 (s, 2 H), 2.35 (s, 3 H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 161.9 (Cq), 157.4 (Cq), 156.9 (Cq), 150.2 (CH), 145.3 (Cq), 139.4 (Cq), 137.4 (Cq), 128.2 (CH), 123.7 (CH), 122.5 (CH), 121.3 (CH), 119.1 (CH), 113.9 (Cq), 42.6 (CH$_2$), 42.3 (CH$_2$), 32.1 (CH$_2$), 21.2 (CH$_3$). QC purity (UV at 215 nm) 95%.

Synthesis of compound 17. 4-[[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]amino]phenol
*Step 1. Synthesis of 4-[(6-benzyl-2-chloro-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl)amino]phenol (intermediate **C.7**).*

Title compound was obtained using intermediate **B** (200 mg, 0.68 mmol) and *p*-aminophenol (82.4 mg, 0.75 mmol) following general procedure 2 previously described at 100 °C for 16 h. Final normal phase purification (cyclohexane/AcOEt from 85/15 to 50/50) afforded pure title compound (149 mg, yield 60%). Rt = 0.74 min (gradient 2); MS (ESI) m/z: 366.1/368.1 [M + H]$^+$, [M + H]$^+$ calculated for C$_{20}$H$_{20}$ClN$_4$O: 366.1/368.1. ^{1}H NMR (400 MHz DMSO-d_6) δ 9.32 (s, 1 H), 8.57 (s, 1 H), 7.46–7.31 (m, 4 H), 7.30–7.24 (m, 1 H), 7.23–7.12 (m, 2 H), 6.81–6.65 (m, 2 H), 3.74 (s, 2 H), 3.45 (s, 2 H), 2.78–2.59 (m, 4 H).

*Step 2. 4-[[6-benzyl-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl]amino]phenol (intermediate **D.9**).*

Title compound was obtained using intermediate **C.7** (148 mg, 0.40 mmol) and Pyridine-4-boronic acid (72.1 mg, 0.49 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 60/40 to 30/70) afforded pure title compound (75 mg, yield 46%). Rt = 0.77 min (gradient 2); MS (ESI) m/z: 410.2 [M + H]$^+$, [M + H]$^+$ calculated for C$_{25}$H$_{24}$N$_5$O: 410.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.27 (s, 1 H), 8.69–8.61 (m, 2 H), 8.37 (s, 1 H), 8.09–8.02 (m, 2 H), 7.44–7.32 (m, 6 H), 7.31–7.25 (m, 1 H), 6.78 (d, J = 8.8 Hz, 2 H), 3.77 (s, 2 H), 3.56 (s, 2 H), 2.84–2.71 (m, 4 H).

*Step 3. 4-[[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]amino]phenol (compound **17**).*

Title compound was obtained using intermediate **D.9** (75.0 mg, 0.18 mmol) following the general procedure 4 previously described for 5 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 M MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (26.9 mg, yield 47%). Rt = 1.32 min (gradient 1); MS (ESI) m/z: 320.1 [M + H]$^+$, [M + H]$^+$ calculated for C$_{18}$H$_{18}$N$_5$O: 320.1. HRMS (ESI) m/z: 320.1506 calcd for C$_{18}$H$_{18}$N$_5$O [M + H]$^+$; found m/z: 320.1508. ^{1}H NMR (400 MHz, DMSO- d_6) δ 8.71–8.61 (m, 2 H), 8.31 (s, 1 H), 8.10–8.00 (m, 2 H), 7.56–7.33 (m, 2 H), 6.89–6.69 (m, 2 H), 3.77 (s, 2 H), 3.03 (t, J = 5.8 Hz, 2 H), 2.71 (t, J = 5.8 Hz, 2 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.1 (Cq), 157.5 (Cq), 157.2 (Cq), 153.7 (Cq), 150.1 (CH), 145.4 (Cq), 130.6 (Cq), 124.4 (CH), 121.3 (CH), 114.9 (CH), 112.73 (C), 42.4 (CH$_2$), 42.2 (CH$_2$), 31.75 (CH$_2$). QC purity (UV at 215 nm) 96%.

Synthesis of compound 18. 3-[[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]amino]phenol
*Step 1. Synthesis of 3-[(6-benzyl-2-chloro-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl)amino]phenol (intermediate **C.8**).*

Title compound was obtained using intermediate **B** (200 mg, 0.68 mmol) and 3-aminophenol (83.3 mg, 0.75 mmol) following general procedure 2 previously described at 120 °C for 24 h. Final normal phase purification (cyclohexane/AcOEt from 80/20 to 60/40) afforded pure title compound (107 mg, yield 43%). Rt = 0.82 min (gradient 2); MS (ESI) m/z: 366.1/368.1 [M + H]$^+$, [M + H]$^+$ calculated for C$_{20}$H$_{20}$ClN$_4$O: 366.1/368.1. ^{1}H NMR (400 MHz DMSO-d_6) δ 9.41 (s, 1 H), 8.60 (s, 1 H), 7.42–7.32 (m, 4 H), 7.31–7.22 (m, 1 H), 7.11 (t, J = 8.0 Hz, 1 H), 7.00 (t, J = 2.2 Hz, 1 H), 6.95 (ddd, J = 8.0, 2.1, 1.0 Hz, 1 H), 6.52 (ddd, J = 8.1, 2.4, 1.0 Hz, 1 H), 3.75 (s, 2 H), 3.50 (s, 2 H), 2.70 (s, 4 H).

*Step 2. Synthesis of 3-[[6-benzyl-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl]amino]phenol (intermediate **D.10**).*

Title compound was obtained using intermediate **C.8** (100 mg, 0.27 mmol) and Pyridine-4-boronic acid (44.7 mg, 0.33 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 70:30 to 10:90) afforded pure title compound (90 mg, yield 81%). Rt = 0.86 min (gradient 2); MS (ESI) m/z: 410.2 [M + H]$^+$, [M + H]$^+$ calculated for C$_{25}$H$_{24}$N$_5$O: 410.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.38 (s, 1 H), 8.73–8.63 (m, 2 H), 8.41 (s, 1 H), 8.17–8.08 (m, 2 H), 7.45–7.39 (m, 2 H), 7.36 (dd, J = 8.4, 6.7 Hz, 2 H), 7.31–7.25 (m, 1 H), 7.22 (t, J = 2.0 Hz, 1 H), 7.19–7.12 (m, 2 H), 6.51 (dt, J = 7.0, 2.3 Hz, 1 H), 3.79 (s, 2 H), 3.62 (s, 2 H), 2.84 (d, J = 5.6 Hz, 2 H), 2.78 (d, J = 5.4 Hz, 2 H).

*Step 3. Synthesis of 3-[[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]amino]phenol (compound **18**).*

Title compound was obtained using intermediate **D.10** (85 mg, 0.21 mmol) following the general procedure 4 previously described for 5 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 M MeOH 4:1 from 95/5 to 40/60) afforded pure title compound (42.4 mg, yield 64%). Rt = 1.20 min (gradient 1); MS (ESI) m/z: 320.1 [M + H]$^+$, [M + H]$^+$ calculated for C$_{18}$H$_{18}$N$_5$O: 320.1. HRMS (ESI) m/z: 320.1506 calcd for C$_{18}$H$_{18}$N$_5$O [M + H]$^+$; found m/z: 320.1509. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.42 (s, 1 H), 8.80–8.62 (m, 2 H), 8.33 (s, 1 H), 8.19–8.08 (m, 2 H), 7.28 (t, J = 2.1 Hz, 1 H), 7.22–7.09 (m, 2 H), 6.51 (dt, J = 7.2, 2.0 Hz, 1 H), 3.82 (s, 2 H), 3.05 (t, J = 5.8 Hz, 2 H), 2.74 (t, J = 5.8 Hz, 2 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.7 (Cq), 157.6 (Cq), 157.4 (Cq), 156.9 (Cq), 150.2 (CH), 145.3 (Cq), 140.5 (Cq), 129.0 (CH), 121.4 (CH), 113.4 (Cq), 112.7 (CH), 110.3 (CH), 109.0 (CH), 42.4 (CH$_2$), 42.1 (CH$_2$), 31.7 (CH$_2$). QC purity (UV at 215 nm) 97%.

Synthesis of compound 19. N-(4-methoxyphenyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
*Step 1. Synthesis of 6-benzyl-2-chloro-N-(4-methoxyphenyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate **C.9**).*

Title compound was obtained using intermediate **B** (200 mg, 0.68 mmol) and *p*-methoxyaniline (93.0 mg, 0.75 mmol) following general procedure 2 previously described at 120 °C for 15 h. Final normal phase purification (cyclohexane/AcOEt from 90/10 to 70/30) afforded pure title compound (204 mg, yield 79%). Rt = 1.24 min (gradient 2); MS (ESI) m/z: 380.1/382.1 [M + H]⁺, [M + H]⁺ calculated for $C_{21}H_{22}ClN_4O$: 380.1/382.1. ¹H NMR (400 MHz DMSO-d_6) δ 8.67 (s, 1 H), 7.46–7.32 (m, 6 H), 7.32–7.23 (m, 1 H), 6.98–6.87 (m, 2 H), 3.75 (bs, 5 H), 3.47 (s, 2 H), 2.74–2.65 (m, 4 H).

Step 2. Synthesis of 6-benzyl-N-(4-methoxyphenyl)-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate D.11).

Title compound was obtained using intermediate **C.9** (200 mg, 0.53 mmol) and Pyridine-4-boronic acid (86.1 mg, 0.63 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 60/40 to 10/90) afforded pure title compound (171 mg, yield 77%). Rt = 1.32 min (gradient 2); MS (ESI) m/z: 424.3 [M + H]⁺, [M + H]+ calculated for $C_{26}H_{26}N_5O+$: 424.2. 1 H NMR (400 MHz, DMSO-d_6) δ 8.73–8.62 (m, 2 H), 8.47 (s, 1 H), 8.15–8.04 (m, 2 H), 7.63–7.50 (m, 2 H), 7.47–7.39 (m, 2 H), 7.37 (dd, J = 8.4, 6.7 Hz, 2 H), 7.31–7.23 (m, 1 H), 7.03–6.91 (m, 2 H), 3.78 (s, 2 H), 3.77 (s, 3 H), 3.59 (s, 2 H), 2.83 (t, J = 5.7 Hz, 2 H), 2.77 (t, J = 5.4 Hz, 2 H).

Step 3. Synthesis of N-(4-methoxyphenyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound 19).

Title compound was obtained using intermediate **D.11** (166 mg, 0.39 mmol) following the general procedure 4 previously described for 6 h. Final normal phase purification (DCM/DCM: NH₃ 1 M MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (73.2 mg, yield 56%). Rt = 1.46 min (gradient 1); MS (ESI) m/z: 334.2 [M + H]⁺, [M + H]⁺ calculated for $C_{19}H_{20}N_5O$: 334.2. HRMS (ESI) m/z: 334.1662 calcd for $C_{19}H_{20}N_5O$ [M + H]⁺; found m/z: 334.1666. ¹H NMR (400 MHz, DMSO-d_6) δ 8.84–8.59 (m, 2 H), 8.36 (s, 1 H), 8.18–8.04 (m, 2 H), 7.69–7.56 (m, 2 H), 7.09–6.93 (m, 2 H), 3.79 (s, 3 H), 3.78 (s, 2 H), 3.03 (t, J = 5.7 Hz, 2 H), 2.71 (t, J = 5.8 Hz, 2 H), 2.62 (s, 1 H). ¹³C NMR (101 MHz, DMSO-d_6) δ 161.4 (Cq), 157.5 (Cq), 157.1 (Cq), 155.4 (Cq), 150.1 (CH), 145.4 (Cq), 132.3 (Cq), 123.9 (CH), 121.3 (CH), 113.6 (CH), 113.4 (Cq), 55.2 (CH₃), 42.6 (CH₂), 42.4 (CH₂), 32.0 (CH₂). QC purity (UV at 215 nm) > 99.5%.

Synthesis of compound 20. N-(3-fluorophenyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
Step 1. Synthesis of 6-benzyl-2-chloro-N-(3-methoxyphenyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate C.10).

Title compound was obtained using compound **B** (150 mg, 0.51 mmol) and *m*-methoxyaniline (0.07 ml, 0.56 mmol) following the general procedure 2 previously described at 100 °C for 16 h. Final normal phase purification (cyclohexane/AcOEt from 95/5 to 60/40) afforded pure title compound (150 mg, yield 58%). Rt = 1.39 min (gradient 2); MS (ESI) m/z: 381.2/383.2 [M + H]⁺, [M + H]⁺ calculated for $C_{21}H_{22}ClN_4O$: 381.1/383.1. ¹H NMR (400 MHz, DMSO-d_6) δ 8.70 (s, 1 H), 7.43–7.32 (m, 4 H), 7.30–7.25 (m, 1 H), 7.24 (s, 1 H), 7.24–7.20 (m, 1 H), 7.15 (ddd, J = 8.0, 2.0, 1.0 Hz, 1 H), 6.69 (ddd, J = 8.2, 2.6, 1.0 Hz, 1 H), 3.76 (s, 2 H), 3.74 (s, 3 H), 3.52 (s, 2 H), 2.71 (s, 4 H).

Step 2. Synthesis of 6-benzyl-N-(3-methoxyphenyl)-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate D.12).

Title compound was obtained using compound **C.10** (145 mg, 0.38 mmol) and Pyridine-4-boronic acid (62.4 mg, 0.46 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 50/50 to 30/70) afforded pure title compound (130 mg, yield 81%). Rt = 1.52 min (gradient 2); MS (ESI) m/z: 424.3 [M + H]⁺, [M + H]⁺ calculated for $C_{26}H_{26}N_5O$: 424.2. ¹H NMR (400 MHz, DMSO-d_6) δ 8.74–8.64 (m, 2 H), 8.52 (s, 1 H), 8.28–7.98 (m, 2 H), 7.45–7.25 (m, 8 H), 6.67 (ddd, J = 7.9, 2.5, 1.2 Hz, 1 H), 3.79 (s, 2 H), 3.78 (s, 3 H), 3.64 (s, 2 H), 2.85 (t, J = 5.8 Hz, 2 H), 2.78 (t, J = 5.6 Hz, 2 H).

Step 3. N-(3-methoxyphenyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound 20).

Title compound was obtained using intermediate **D.12** (125.0 mg, 0.30 mmol) following the general procedure 4 previously described for 4 h. Final normal phase purification (DCM/DCM: NH₃ 1 N MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (71.8 mg, yield 73%). Rt = 1.47 min (gradient 1); MS (ESI) m/z: 334.2 [M + H]⁺, [M + H]⁺ calculated for $C_{19}H_{20}N_5O$: 334.2. HRMS (ESI) m/z: 334.1662 calcd for $C_{19}H_{20}N_5O$ [M + H]⁺; found m/z: 334.1666. ¹H NMR (400 MHz, DMSO-d_6) δ 8.74–8.66 (m, 2 H), 8.39 (s, 1 H), 8.17–8.09 (m, 2 H), 7.47 (t, J = 2.2 Hz, 1 H), 7.39 (ddd, J = 8.1, 2.0, 1.0 Hz, 1 H), 7.28 (t, J = 8.1 Hz, 1 H), 6.67 (ddd, J = 8.2, 2.6, 0.9 Hz, 1 H), 3.82 (s, 2 H), 3.78 (s, 3 H), 3.31 (s, 1 H), 3.03 (t, J = 5.8 Hz, 2 H), 2.73 (t, J = 5.8 Hz, 2 H). ¹³C NMR (151 MHz, DMSO-d_6) δ 162.1 (Cq), 159.3 (Cq), 157.5 (Cq), 156.8 (Cq), 150.2 (CH), 145.3 (Cq), 140.8 (Cq), 129.10 (CH), 121.3 (CH), 114.1 (Cq), 113.9 (CH), 108.8 (CH), 107.1 (CH), 55.0 (CH₃), 42.6 (CH₂), 42.3 (CH₂), 32.1 (CH₂). QC purity (UV at 215 nm) 96%.

Synthesis of compound 21. N1-methyl-N4-[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]benzene-1,4-diamine
Step 1. Synthesis of tert-butyl N-[4-[(6-benzyl-2-chloro-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl)amino]phenyl]-N-methyl-carbamate (intermediate C.11).

Title compound was obtained using intermediate **B** (235 mg, 0.79 mmol) and 4-(N-tert-Butoxycarbonyl-N-methylamino)aniline (193 mg, 0.87 mmol) following the general procedure 2 previously described at 120 °C for 17 h. Final normal phase purification (cyclohexane/AcOEt from 70/30 to 50/50) afforded pure title compound (243 mg, yield 64%). Rt = 1.82 min (gradient 2); MS (ESI) m/z: x [M + H]⁺, [M + H]⁺ calculated for $C_{26}H_{31}ClN_5O_2$: 480.0/482.0.

Step 2. Synthesis of tert-butyl N-[4-[[6-benzyl-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl]amino]phenyl]-N-methyl-carbamate (intermediate D.13).

Title compound was obtained using intermediate **C.11** (215 mg, 0.45 mmol) and Pyridine-4-boronic acid (73.4 mg, 0.54 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 85/15 to 50/50) afforded pure title compound (178 mg, yield 76%). Rt = 1.82 min (gradient 2); MS (ESI) m/z: 523.3 [M + H]$^+$, [M + H]$^+$ calculated for C$_{31}$H$_{35}$N$_6$O$_2$: 523.3. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.73–8.61 (m, 2 H), 8.57 (s, 1 H), 8.14–8.06 (m, 2 H), 7.71–7.61 (m, 2 H), 7.45–7.39 (m, 2 H), 7.39–7.32 (m, 2 H), 7.32–7.23 (m, 3 H), 3.79 (s, 2 H), 3.63 (s, 2 H), 3.20 (s, 3 H), 2.84 (d, J = 5.5 Hz, 2 H), 2.78 (t, J = 5.6 Hz, 2 H), 1.42 (s, 9 H).

Step 3. Synthesis of N1-methyl-N4-[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]benzene-1,4-diamine (compound 21).

Title compound was obtained solving intermediate **D.13** (175 mg, 0.33 mmol) in 1,4-dioxane (0.8 ml), a solution of HCl (4 M) in 1,4-dioxane (0.8 ml) was dropwise added and the reaction mixture stirred at room temperature for 1 h, then the reaction crude was concentrated to dryness at low pressure, the resulting crude portioned between AcOEt (20 ml) and NaOH 0.1 M (20 ml), the organic layer dried over Na$_2$SO$_4$ and concentrated to dryness at low pressure. Resulting crude solid (140 mg, 0.33 mmol) was treated following the general procedure 4 previously described for 6 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (70.2 mg, yield 64%). Rt = 1.24 min (gradient 1); MS (ESI) m/z: 333.2 [M + H]$^+$, [M + H]$^+$ calculated for C$_{19}$H$_{21}$N$_6$: 333.4. HRMS (ESI) m/z: 333.1822 calcd for C$_{19}$H$_{21}$N$_6$ [M + H]$^+$; found m/z: 333.1823. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.69–8.57 (m, 2 H), 8.15 (s, 1 H), 8.08–8.02 (m, 2 H), 7.45–7.32 (m, 2 H), 6.61–6.53 (m, 2 H), 5.47 (q, J = 5.2 Hz, 1 H), 3.73 (s, 2 H), 3.00 (t, J = 5.8 Hz, 2 H), 2.69 (m, 5 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.9 (Cq), 157.5 (Cq), 157.3 (Cq), 150.0 (CH), 146.6 (Cq), 145.6 (Cq), 128.0 (Cq), 124.3 (CH), 121.3 (CH), 112.9 (Cq), 111.3 (CH), 42.6 (CH$_2$), 42.4 (CH$_2$), 32.0 (CH$_2$), 30.1 (CH$_3$). QC purity (UV at 215 nm) 99%.

Synthesis of compound 22. N3-methyl-N1-[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]benzene-1,3-diamine

Step 1. Synthesis of tert-butyl N-[3-[(6-benzyl-2-chloro-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl)amino]phenyl]-N-methyl-carbamate (intermediate C.12).

Title compound was obtained using intermediate **B** (200 mg, 0.68 mmol) and 3-(N-tert-Butoxycarbonyl-N-methylamino)aniline (175.0 mg, 0.75 mmol) following the general procedure 2 previously described at 100 °C for 16 h. Final normal phase purification (cyclohexane/AcOEt from 85:15 to 65:35) afforded titled compound. Rt = 1.80 min (gradient 2); MS (ESI) m/z: 480.0/482.0 [M + H]+, [M + H]+ calculated for C$_{26}$H$_{31}$ClN$_5$O$_2$+: 480.2/482.0. 1 H NMR (400 MHz, DMSO-d_6) δ 8.74 (s, 1 H), 7.50 (t, J = 2.1 Hz, 1 H), 7.43–7.24 (m, 8 H), 7.02 (ddd, J = 7.9, 2.2, 1.0 Hz, 1 H), 3.76 (s, 2 H), 3.53 (s, 2 H), 3.18 (s, 3 H), 2.72 (s, 4 H), 1.40 (s, 9 H).

Step 2. Synthesis of tert-butyl N-[3-[[6-benzyl-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl]amino]phenyl]-N-methyl-carbamate (intermediate D.14).

Title compound was obtained using intermediate **C.12** (92 mg, 0.19 mmol) and Pyridine-4-boronic acid (31.4 mg, 0.23 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 85/15 to 30/70) afforded pure title compound (86 mg, yield 86%). Rt = 1.87 min (gradient 2); MS (ESI) m/z: 523.3 [M + H]$^+$, [M + H]$^+$ calculated for C$_{31}$H$_{35}$N$_6$O$_2$: 523.3. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70–8.64 (m, 2 H), 8.60 (s, 1 H), 8.16–8.10 (m, 2 H), 7.65 (t, J = 2.1 Hz, 1 H), 7.60–7.54 (m, 1 H), 7.46–7.40 (m, 2 H), 7.40–7.32 (m, 3 H), 7.32–7.25 (m, 1 H), 7.00 (ddd, J = 8.0, 2.2, 1.0 Hz, 1 H), 3.80 (s, 2 H), 3.64 (s, 2 H), 3.22 (s, 3 H), 2.85 (d, J = 5.6 Hz, 2 H), 2.78 (t, J = 5.5 Hz, 2 H), 1.40 (d, J = 1.1 Hz, 9 H).

Step 3. N3-methyl-N1-[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]benzene-1,3-diamine (compound 22).

Title compound was obtained solving intermediate **D.14** (95.0 mg, 0.19 mmol) in 1,4-dioxane (0.4 ml), a solution of HCl (4 M) in 1,4-dioxane (0.4 ml) was dropwise added and the reaction mixture stirred at room temperature for 3 h, then the reaction crude was concentrated to dryness at low pressure, the resulting crude portioned between DCM (20 ml) and NaOH 0.1 M (20 ml), the organic layer dried over Na$_2$SO$_4$ and concentrated to dryness at low pressure. Resulting crude (66 mg, 0.16 mmol) was treated following the general procedure 4 previously described for 4 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (16.1 mg, yield 31%). Rt = 1.36 min (gradient 1); MS (ESI) m/z: 333.3 [M + H]$^+$, [M + H]$^+$ calculated for C$_{19}$H$_{21}$N$_6$: 333.2. HRMS (ESI) m/z: 333.1822 calcd for C$_{19}$H$_{21}$N$_6$ [M + H]$^+$; found m/z: 333.1827. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81–8.56 (m, 2 H), 8.18 (s, 1 H), 8.16–8.11 (m, 2 H), 7.11–7.00 (m, 2 H), 6.95 (ddd, J = 8.0, 2.1, 1.0 Hz, 1 H), 6.30 (ddd, J = 8.1, 2.3, 1.0 Hz, 1 H), 5.66 (q, J = 5.0 Hz, 1 H), 3.79 (s, 2 H), 3.02 (t, J = 5.8 Hz, 2 H), 2.71 (t, J = 5.6 Hz, 5 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.7 (Cq), 157.6 (Cq), 157.1 (Cq), 150.2 (CH), 145.5 (Cq), 140.4 (Cq), 128.7 (CH), 121.5 (CH), 109.6 (CH), 107.6 (CH), 104.9 (CH), 99.6 (C), 42.7 (CH$_2$), 42.4 (CH$_2$), 32.1 (CH$_2$), 30.0 (CH$_3$). QC purity (UV at 215 nm) 99%.

Synthesis of compound 23. N3,N3-dimethyl-N1-[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]benzene-1,3-diamine

Step 1. Synthesis of N1-(6-benzyl-2-chloro-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl)-N3,N3-dimethyl-benzene-1,3-diamine (intermediate C.13).

Title compound was obtained using intermediate **B** (250 mg, 0.85 mmol) and N,N-Dimethyl-1,3-phenylenediamine dihydrochloride (197.4 mg, 0.93 mmol) following the general procedure 2 previously described at 100 °C for 16 h. Final normal phase purification (cyclohexane/AcOEt from 80/20 to 60/40) afforded pure title compound (227.6 mg, yield 68%). Rt = 1.12 min (gradient 2); MS (ESI) m/z: 394.2/396.2 [M + H]$^+$, [M + H]$^+$ calculated for C$_{22}$H$_{24}$ClN$_5$: 394.2/396.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.58 (s, 1 H), 7.43–7.31 (m, 4 H), 7.31–7.24 (m, 1 H), 7.13 (t, J = 8.1 Hz, 1 H), 6.95 (t, J = 2.2 Hz, 1 H), 6.88 (ddd, J = 8.0, 2.0, 0.8 Hz, 1 H), 6.50 (ddd, J = 8.4, 2.5, 0.9 Hz, 1 H), 3.75 (s, 2 H), 3.51 (s, 2 H), 2.88 (s, 6 H), 2.70 (d, J = 2.4 Hz, 4 H).

Step 2. Synthesis of N1-[6-benzyl-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-yl]-N3,N3-dimethyl-benzene-1,3-diamine (intermediate D.15).

Title compound was obtained using compound **C.13** (220 mg, 0.56 mmol) and Pyridine-4-boronic acid (91.5 mg, 0.67 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 70/30 to 20/80) afforded pure title compound (185 mg, yield 76%). Rt = 1.43 min (gradient 2); MS (ESI) m/z: 437.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{27}H_{28}N_6$: 437.2. ^{1}H NMR (400 MHz, DMSO- d_6): δ 8.70–8.64 (m, 2 H), 8.36 (s, 1 H), 8.17–8.09 (m, 2 H), 7.48–7.39 (m, 2 H), 7.39–7.33 (m, 2 H), 7.31–7.25 (m, 1 H), 7.23–7.13 (m, 2 H), 7.06 (ddd, J = 7.9, 2.0, 0.9 Hz, 1 H), 6.49 (ddd, J = 8.3, 2.6, 0.9 Hz, 1 H), 3.79 (s, 2 H), 3.63 (s, 2 H), 2.92 (s, 6 H), 2.84 (t, J = 5.2 Hz, 2 H), 2.77 (t, J = 5.4 Hz, 2 H).

Step 3. N3,N3-dimethyl-N1-[2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]benzene-1,3-diamine (compound 23).

Title compound was obtained using intermediate **D.15** (180.0 mg, 0.41 mmol) following the general procedure 4 previously described for 4 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 N MeOH 4:1 from 70/30 to 20/80) afforded pure title compound (62.8 mg, yield 44%). Rt = 1.55 min (gradient 1); MS (ESI) m/z: 347.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{20}H_{23}N_6$ [M + H]$^+$: 347.2. HRMS (ESI) m/z: 347.1979 calcd for $C_{20}H_{23}N_6$ [M + H]$^+$; found m/z: 347.1978. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.71–8.65 (m, 2 H), 8.22 (s, 1 H), 8.17–8.10 (m, 2 H), 7.25–7.13 (m, 2 H), 7.12 (dt, J = 8.2, 1.3 Hz, 1 H), 6.48 (ddd, J = 8.1, 2.5, 1.1 Hz, 1 H), 3.80 (s, 2 H), 3.02 (t, J = 5.8 Hz, 2 H), 2.92 (s, 6 H), 2.72 (t, J = 5.8 Hz, 2 H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 161.8 (Cq), 157.4 (Cq), 157.0 (Cq), 150.7 (CH), 150.1 (Cq), 145.4 (Cq), 140.2 (Cq), 128.7 (CH), 121.3 (CH), 113.9 (Cq), 110.2 (CH), 107.7 (CH), 106.0 (CH), 42.7 (CH$_2$), 42.3 (CH$_2$), 40.3 (CH$_3$), 32.1 (CH$_2$). QC purity (UV at 215 nm) > 99.5%.

Synthesis of compound 24. N-(2-fluorophenyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine
Step 1. Synthesis of 6-benzyl-2-chloro-N-(2-fluorophenyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate C.14).

A mixture of Pd(OAc)$_2$ (7.8 mg, 0.03 mmol) and rac-BINAP (21.6 mg, 0.03 mmol) in 1,4-dioxane (3.4 ml) was stirred under Ar flushing for 10 min. Then were stepwise added intermediate $_B$ (200 mg, 0.68 mmol), *o*-fluoroaniline (0.066 ml, 0.68 mmol) and Cs$_2$CO$_3$ (268.5 mmol, 0.82 mmol). The reaction mixture was stirred in a CEM* microwave apparatus at 80 °C for 1.5 h, filtrated through a celite coarse patch, rinsed with DCM and concentrated to dryness at low pressure. Final normal phase purification (cyclohexane/AcOEt from 95/5 to 75/25) yielded a mixture where titled compound was the majority one. Resulting crude (205 mg) was used in the next step without any further purification process. Rt = 1.25 min (gradient 2, 77% purity); MS (ESI) m/z: 367.1/369.1 [M + H]$^+$, [M + H]$^+$ calculated for $C_{20}H_{19}ClFN_4$: 367.1/369.1.

Step 2. 6-benzyl-N-(2-fluorophenyl)-2-(4-pyridyl)-7,8-dihydro-5 H-pyrido[4,3-d]pyrimidin-4-amine (intermediate D.16).

Title compound was obtained using crude **C.14** (205 mg, 0.56 mmol estimated) and Pyridine-4-boronic acid (91.1 mg, 0.67 mmol) following the general procedure 3 previously described. Final normal phase purification (cyclohexane/AcOEt from 85/15 to 45/55) afforded pure title compound (149 mg, average yield 65% from previous step). Rt = 1.37 min (gradient 2); MS (ESI) m/z: 412.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{25}H_{23}FN_5$: 412.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67–8.55 (m, 3 H), 8.00–7.88 (m, 2 H), 7.50 (td, J = 7.8, 1.8 Hz, 1 H), 7.45–7.40 (m, 2 H), 7.40–7.34 (m, 2 H), 7.34–7.20 (m, 4 H), 3.79 (s, 2 H), 3.60 (s, 2 H), 2.86 (t, J = 5.7 Hz, 2 H), 2.81 (d, J = 5.0 Hz, 2 H).

Step 3. N-(2-fluorophenyl)-2-(4-pyridyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine (compound 24).

Title compound was obtained using intermediate **D.16** (140.0 mg, 0.34 mmol) following the general procedure 4 previously described for 6 h. Final normal phase purification (DCM/DCM: NH$_3$ 1 M MeOH 4:1 from 95/5 to 75/25) afforded pure title compound (72.2 mg, yield 66%). Rt = 0.33 min (gradient 2); MS (ESI) m/z: 322.2 [M + H]$^+$, [M + H]$^+$ calculated for $C_{18}H_{17}FN_5$: 322.1. HRMS (ESI) m/z: 322.1463 calcd for $C_{18}H_{17}FN_5$ [M + H]$^+$; found m/z: 322.1476. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67–8.58 (m, 2 H), 8.53 (s, 1 H), 8.00–7.90 (m, 2 H), 7.54 (td, J = 7.8, 2.0 Hz, 1 H), 7.38–7.20 (m, 3 H), 3.85 (s, 2 H), 3.09 (t, J = 5.8 Hz, 2 H), 2.76 (t, J = 5.8 Hz, 2 H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 161.7 (Cq), 157.6 (Cq), 157.3 (Cq), 156.7 (Cq, d, JCF = 246.8 Hz), 150.1 (CH), 145.1 (Cq), 127.6 (CH, d, JCF = 1.7 Hz), 126.7 (CH, d, JCF = 8.3 Hz), 126.6 (Cq), 124.2 (CH, d, JCF = 3.5 Hz), 121.2 (CH), 115.7 (CH, d, JCF = 20.2 Hz), 112.9 (Cq), 42.3 (CH$_2$), 42.2 (CH$_2$), 31.6 (CH$_2$). QC purity (UV at 215 nm) > 99.5%.

In vitro biological activity
Cell viability assay
Human cancer cell lines A549 (lung adenocarcinoma, ATCC CCL-185), DU-145 (androgen-independent prostate cancer, ATCC HTB-81), MCF7 (breast adenocarcinoma, ATCC HTB-22) and HeLa (cervical carcinoma, ATCC CCL-2) were obtained from ATCC. Human melanoma cell lines A375 (88113005) were obtained from European Collection of Authenticated Cell Cultures (ECACC).

Cells were routinely grown in minimal essential medium containing Eagle's salts and L-glutamine (A549, DU-145, MCF7, and HeLa) or Dulbecco's Modified Minimal Essential Medium (A375) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5% CO2 at 37 °C. To assess the antiproliferative activity of the compounds, cells were seeded at a density of 2500 cells/well (HeLa), 5000 cells/well (A549, DU145, and A375) or 10,000 cells/well (MCF7) in 96-well plates, and cell viability was measured using the MTT assay. Values are reported as the mean ± SD of two independent experiments.

Topoisomerase Inhibition
0.125 µg of pBR322 (Inspiralis) were incubated with increasing concentrations (0.5–200 µM) of tested compounds for 1 h at 37 °C in the presence/absence of 1 U of human topoisomerase IIα (Inspiralis) in the required buffer

(1X). Reaction products were resolved on a 1% agarose gel prepared in 1X TAE (10 mM Tris 1 mM EDTA, 0.1% acetic acid, pH 8.0). After the electrophoretic run (5 V/cm for about 90 min) the DNA bands were visualized by ethidium bromide staining, photographed and quantified using a Geliance 2000 apparatus.

In vitro DMPK evaluation

In vitro metabolic stability

10 mM DMSO stock solution of test compound was pre-incubated at 37˚C for 15 min with mouse liver microsomes in 0.1 M Tris-HCl buffer (pH 7.4) with 10% DMSO. The final concentration was 4.6 µM (0.1% DMSO). After pre-incubation, the co-factors (NADPH, G6P, G6PDH and $MgCl_2$ pre-dissolved in 0.1 M Tris-HCl) were added to the incubation mixture and the incubation was continued at 37˚C for 1 h. At each time point (0, 5, 15, 30, 60 min), 30 µL of incubation mixture was diluted with 200 µL cold MeCN spiked with 200 nM of an appropriate internal standard, followed by centrifugation at 3.270 x g for 15 min. The supernatant was further diluted (1:1) with H_2O for analysis. A reference incubation mixture (microsomes *without* cofactors) was prepared for each test compound and analyzed at t = 0 and 60 min in order to verify the compounds stability in the matrix. The two time points were diluted as for the time points of the incubation mixture above. The concentration of test compound was quantified by LC-MS/MS on a Waters ACQUITY UPLC-MS/MS system consisting of a triple quadrupole detector (TQD) mass spectrometer equipped with an electrospray ionization interface (ESI) and a photodiode array detector (PDA) from Waters Inc. (Milford, MA, USA). The analyses were run on an ACQUITY UPLC BEH C_{18} (50 × 2.1 mmID, particle size 1.7 µm) with a VanGuard BEH C_{18} pre-column (5 × 2.1 mmID, particle size 1.7 µm) at 40 °C, using 0.1% HCOOH in H_2O (A) and 0.1% HCOOH in MeCN (B) as mobile phase. Electrospray ionization was applied in positive mode. Compound-dependent parameters as MRM transitions and collision energy were developed for each compound. The percentage of test compound remaining at each time point relative to t = 0 was calculated by the response factor on the basis of the internal standard peak area. The percentage of test compound versus time was plotted and fitted by GraphPad Prism (GraphPad Software, Version 5 for Windows, CA, USA, www.graphpad.com) to estimate the compounds half-life ($t_{1/2}$) which was reported as mean value along with the standard deviation ($n = 3$).

In vitro plasma stability

10 mM DMSO stock solution of test compound was diluted 50-fold with DMSO-H_2O (1:1) and incubated at 37˚C for 2 h with mouse plasma added 5% DMSO (pre-heated at 37˚C for 10 min). The final concentration was 2 µM (0.5% DMSO). At each time point (0, 5, 15, 30, 60, 120 min), 50 µL of incubation mixture was diluted with 200 µL cold MeCN spiked with 200 nM of internal standard, followed by centrifugation at 3.500 x g for 20 min. The supernatant was further diluted (1:1) with H_2O for analysis. The concentration of test compound was quantified by LC-MS/MS on a Waters ACQUITY UPLC-MS/MS system as defined above. The response factors, calculated on the basis of the internal standard peak area, were plotted over time. When possible, response vs. time profiles were fitted with Prism (GraphPad Software, Inc., USA) to estimate compounds half-life in plasma.

Aqueous kinetic solubility

The aqueous kinetic solubility was determined from a 10 mM DMSO stock solution of test compound in Phosphate Buffered Saline (PBS) at pH 7.4. The study was performed by incubation of an aliquot of 10 mM DMSO stock solution in PBS (pH 7.4) at a target concentration of 250 µM resulting in a final concentration of 2.5% DMSO. The incubation was carried out under shaking at 25 °C for 24 h followed by centrifugation at 21.100 x g for 30 min. The supernatant was further diluted (4:1) in MeCN and analyzed by LC-MS for the quantification of dissolved compound by UV at 215 nm. The analyses were performed on a Waters ACQUITY UPLC-MS system consisting of a single quadrupole detector (SQD) mass spectrometer equipped with an electrospray ionization interface (ESI) and a photodiode array detector (PDA) from Waters Inc. (Milford, MA, USA). The analyses were run on an ACQUITY UPLC BEH C_{18} column (50 × 2.1 mmID, particle size 1.7 µm) with a VanGuard BEH C_{18} pre-column (5 × 2.1 mmID, particle size 1.7 µm), using 10 mM NH_4OAc in H_2O at pH 5 adjusted with AcOH (A) and 10 mM NH_4OAc in Me-H_2O (95:5) at pH 5 (B) as mobile phase. The aqueous kinetic solubility (in µM) was calculated by dividing the peak area of dissolved test compound (supernatant) and test compound in the reference (250 µM of test compound in MeCN) and multiply by the target concentration and dilution factor.

Thermodynamic solubility

The thermodynamic solubility was determined by addition of Phosphate Buffered Saline (PBS) at pH 7.4 to an excess of solid test compound. The study was performed by incubation of an aliquot of 2.5 mg of test compound in 500 µL of PBS at pH 7.4. The suspension was shaken at 300 rpm for 24 h at 25 °C. At the end of the incubation, the saturated solution was filtered. The filtrate was further diluted with MeCN and then analyzed by LC-MS for the quantification of dissolved compound (in µM) by UV at a specific wavelength (λmax of the test compound). The analyses were performed on a Waters ACQUITY UPLC-MS system as defined above. The test compound was quantified towards a calibration curve in MeCN-H_2O (1:1).

Molecular Docking

Molecular docking was performed at three distinct binding sites: the DNA cleavage site and the ATP-binding pocket at the N-terminal domain of TopoII, in the presence and absence of the metal cofactor Mg^{2+}. For the DNA cleavage site, the structure from PDB entry 5GWK (resolution 3.15 Å)[24] was used, while for exploring the ATP-competitive mechanism, PDB entry 1ZXM (resolution 1.87 Å)[25] was employed. The structures were refined using the Protein Preparation Wizard workflow in Maestro (Release 2021-3)[26]. Specifically, missing side chains and loops were completed with Prime[27], hydrogen atoms were added, and charges and protonation states were assigned by titrating the protein at physiological pH. Steric clashes were minimized through a few refinement

steps until the RMSD of non-hydrogen atoms reached 0.30 Å using the OPLS3 force field[28]. The compounds were prepared using LigPrep software within Maestro. First, hydrogens were added, and ionization states at physiological pH were generated. Then, tautomers and all stereochemical isomers were created. A cubic grid box of $26 \times 26 \times 26$ Å³ was centered on etoposide and the native ATP ligand. Finally, molecular docking was performed using Glide[29–31] with the Extra Precision (XP) scoring function, rewarding the planarity of conjugated π-groups, sampling nitrogen inversions, adding an energy term to account for aromatic hydrogen bonds, and generating a maximum of 15 poses. All other parameters were set to their default values.

Data availability

Data is provided within the manuscript or supplementary information files. The data that support the findings of this study are available from the corresponding author, M.D.V.

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Author contributions

J.A.O. and M.B. designed and synthesized the compounds; J.M.A. and M.N. performed the viability assay; M.A.L.S. performed molecular modeling studies; S.M.B. and A.A. carried out DMPK properties analysis and QC; C.S. and M.D.V conceived the study, supervised the work and wrote the manuscript with the help of N.B and J.A.O. All authors read and approved the manuscript.

Declarations

Competing interests

The authors declare the following competing financial interest(s): M.D.V., J.A.O, J.M.A, and C.S. are coinventors on patent related to this work (WO2018/114700Al). All other authors declare no competing interests.

Additional information

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