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Second-Generation CD73 Inhibitors Based on a 4,6-Biaryl-2-thiopyridine Scaffold

Rayane Ghoteimi,^[a] Abdennour Braka,^[b] Céline Rodriguez,^[c] Emeline Cros-Perrial,^[c] Valentin Duvauchelle,^[a] Jean-Pierre Uttaro,^[a] Christophe Mathé,^[a] Christine Ménétrier-Caux,^[c] Lars Petter Jordheim,^[c] Laurent Chaloin,^[b] and Suzanne Peyrottes^{*[a]}

Various series of 4,6-biaryl-2-thiopyridine derivatives were synthesized and evaluated as potential ecto-5'-nucleotidase (CD73) inhibitors. Two synthetic routes were explored and the coupling of 4,6-disubstituted 3-cyano-2-chloro-pyridines with selected thiols allowed us to explore the structural diversity. Somehow divergent results were obtained in biological assays on CD73 inhibition using either the purified recombinant protein or cell-based assays, highlighting the difficulty to target protein-protein interface on proteins existing as soluble and membrane-bound forms. Among the 18 new derivatives

obtained, three derivatives incorporating morpholino substituents on the 4,6-biaryl-2-thiopyridine core were shown to be able to reverse the adenosine-mediated immune suppression on human T cells. The higher blockade efficiency was observed for 2-((3-cyano-4,6-bis(4-morpholinophenyl)pyridin-2-yl)thio)-N-(isoxazol-3-yl)acetamide (with total reversion at 100 μ M) and methyl 2-((3-cyano-4,6-bis(4-morpholinophenyl)pyridin-2-yl)thio)acetate (with partial reversion at 10 μ M). Thus, this series of compounds illustrates a new chemotype of CD73 allosteric inhibitors.

Introduction

CD73 is an ecto-5'-nucleotidase attached to the cell surface through a glycosylphosphatidylinositol (GPI) link or present in the plasma in a soluble form.^[1] It is a metalloenzyme that catalyzes the conversion of adenosine 5'-monophosphate (AMP) into adenosine and inorganic phosphate. In the tumor microenvironment where oxygen supply can be very low, it has been demonstrated that CD73 is overexpressed leading to the production and accumulation of a high concentration of extracellular adenosine. Through its binding to A2A receptors, adenosine plays a pivotal role in tumor immune escape promoting its growth, invasion and metastasis.^[2] Furthermore, it has been found that CD73 is overexpressed in various types of cancer.^[3] Due to its critical role in tumor proliferation and immune regulation, CD73 becomes a valuable target in onco-immunology.^[4] Its inhibition has been widely studied in recent

years, leading to clinical trials using either monoclonal antibodies (mAbs) or a small molecule.^[4b,5] When inhibiting CD73 activity using small molecules, most of the studies have been performed targeting the active site with substrate analogs. Historically, AOPCP (5'-(α,β -methylene)diphosphate adenosine) is amongst the first ADP (5'-adenosine diphosphate) analog described as a potent CD73 inhibitor^[6] and since then it has been widely used as starting point to design novel substrate analogs by our team and others.^[5b,7] Several reports also concerned non-nucleotidic CD73 inhibitors,^[7a] which were described as competitive^[5c,8] or uncompetitive^[9] inhibitors.

It is worth noting that the active site is not always the only and best target as undesirable effects may arise due to interactions with other enzymes and receptors binding AMP, or structurally related molecules. Recently, by using a bioinformatic approach, we identified a new binding pocket that is distinct from the active site and located at the protein-protein interface of the CD73 dimer.^[10] Given the presence of a unique allosteric site, this approach may offer a better selectivity against enzymes accepting similar substrates and/or a peculiar mechanism of action.^[11] Amongst the 33 commercially available compounds which were initially evaluated for CD73 enzyme inhibition,^[10] two compounds (Figure 1) exhibited strong inhibitory activities. However, they showed sub-optimal pharmacological properties that limited their potential for further clinical development. As example, their lipophilicity has hampered their evaluation in cell culture experiments due to their precipitation in biological aqueous media. Therefore, it is of great interest to pursue this research in an attempt to find better inhibitors with improved pharmacological properties.

Here, we used an *in-silico* approach for the design and the synthesis of new potential allosteric inhibitors aiming to explore structural diversity starting from the previously identified hit compounds (Figure 1).

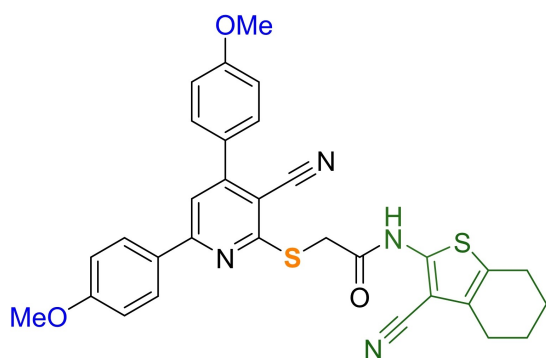
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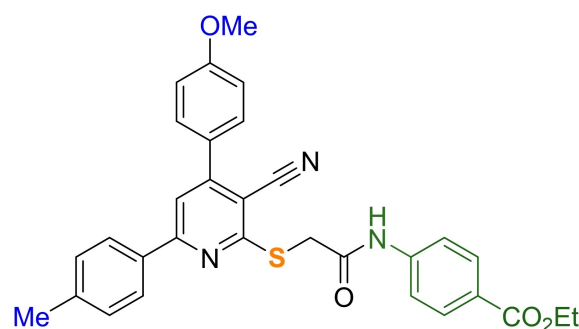
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**RR4**

Inhibition of soluble *h*CD73 at 5 μ M = 73%
Docking score = 93.5
cLog *P* = 6.9

**RR6**

Inhibition of soluble *h*CD73 at 5 μ M = 80%
Docking score = 91.3
cLog *P* = 6.6

Figure 1. Previously identified allosteric CD73-inhibitors.^[10]

Results and Discussion

Preliminary dataset and in silico study

Protein-protein interfaces often present highly apolar areas,^[11a,12] thus requiring the design of hydrophobic compounds that violate Lipinski's rules of 5 (either molecular weight > 500 Da and/or cLog *P* value > 5). Not surprisingly, although **RR4** and **RR6** (Figure 1) showed significant enzymatic inhibition toward soluble *h*CD73, their cLog *P* values remained relatively high (6.6 and 6.9). Interestingly, both compounds were structurally related and incorporated a central 3-cyanopyridine core. This last was substituted at positions 4 and 6 with aryl moieties and functionalized with a side chain connected at position 2 of the 3-cyanopyridine through a sulfur atom. Given the similarity between the two compounds and the consistency of the *in silico* study with the results of the inhibition assays, we decided to develop this series of compounds by designing new analogs of **RR4** and **RR6** with the aim to lower their clog *P* value while keeping high docking scores (DS) and possibly high inhibitory activity.

In light of these observations, we considered a generic structure divided into three parts allowing to explore the structural diversity (Figure 2). Accordingly, we conceived a small virtual library of **RR4** and **RR6** analogs, about 80 derivatives, that could be accessed by the retrosynthetic approach proposed in Scheme 1 and based on the commercial availability of starting materials.

Chemical structures were submitted to virtual screening on CD73 structure by targeting the dimer interface and using a flexible docking to establish a ranking list of most suitable functional groups (according to their DS compared to that of the original hit). With a DS cut-off value of 80, we selected two series of molecules (Table 1) exhibiting DS in the same range as **RR4** and **RR6** (80.3–102.9 vs 93.5 for **RR4** and 91.3 for **RR6**) but with lower cLog *P* values (3.5–5.5 vs 6.6 for **RR6** and 6.9 for

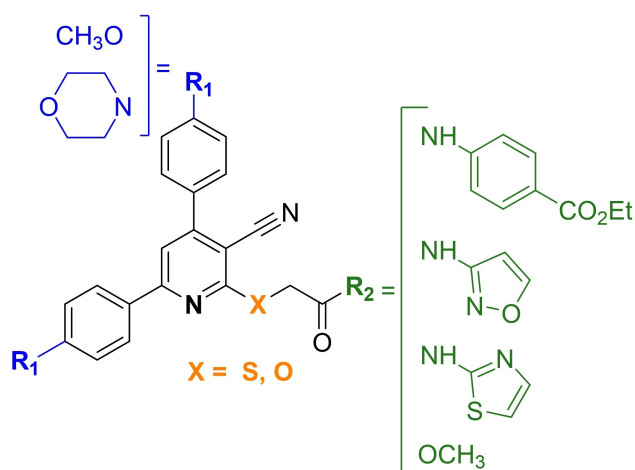
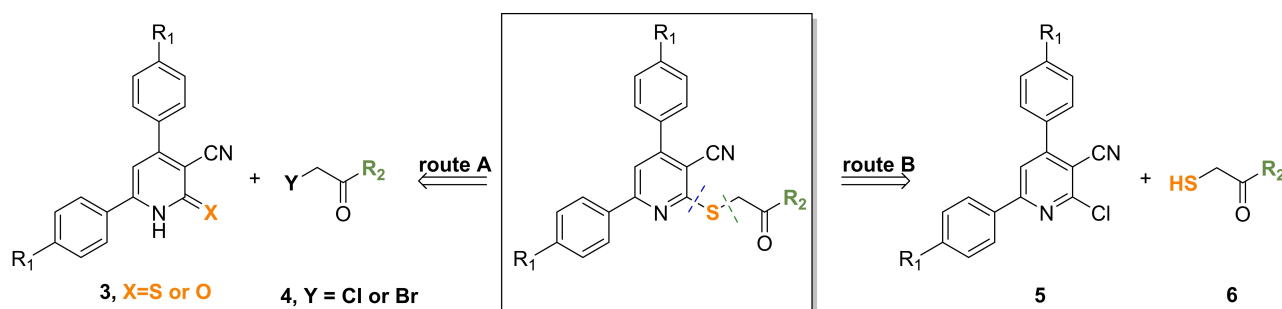


Figure 2. Generic structure of the compounds considered for virtual screening.

RR4). Thus, in the first series, compound **1 a** may be viewed as a combination of **RR4** and **RR6** with a similar DS, and the replacement of the sulfur atom by an oxygen atom (compound **1 aa**) seems to have little impact on both the DS and the cLog *P* values. However, the introduction of a thiazole (**1 c**), an isoxazole (**1 b**) or a methyl ester (**1 d**) as groups gave lower clog *P* values albeit with lower DS.

In the second series, replacing the methoxy group of the aryl moieties by a morpholinyl group (Table 1, series 2) decreased cLog *P* values while keeping high DS (compounds **2 a–c**). Given these preliminary results and the considered synthetic approach (Scheme 1), we embarked on the preparation of these analogs in order to evaluate *in vitro* their potential as CD73 inhibitors and to initiate a structure-activity relationship study.



Scheme 1. Retro-synthetic pathways envisaged for the preparation of the two series of compounds.

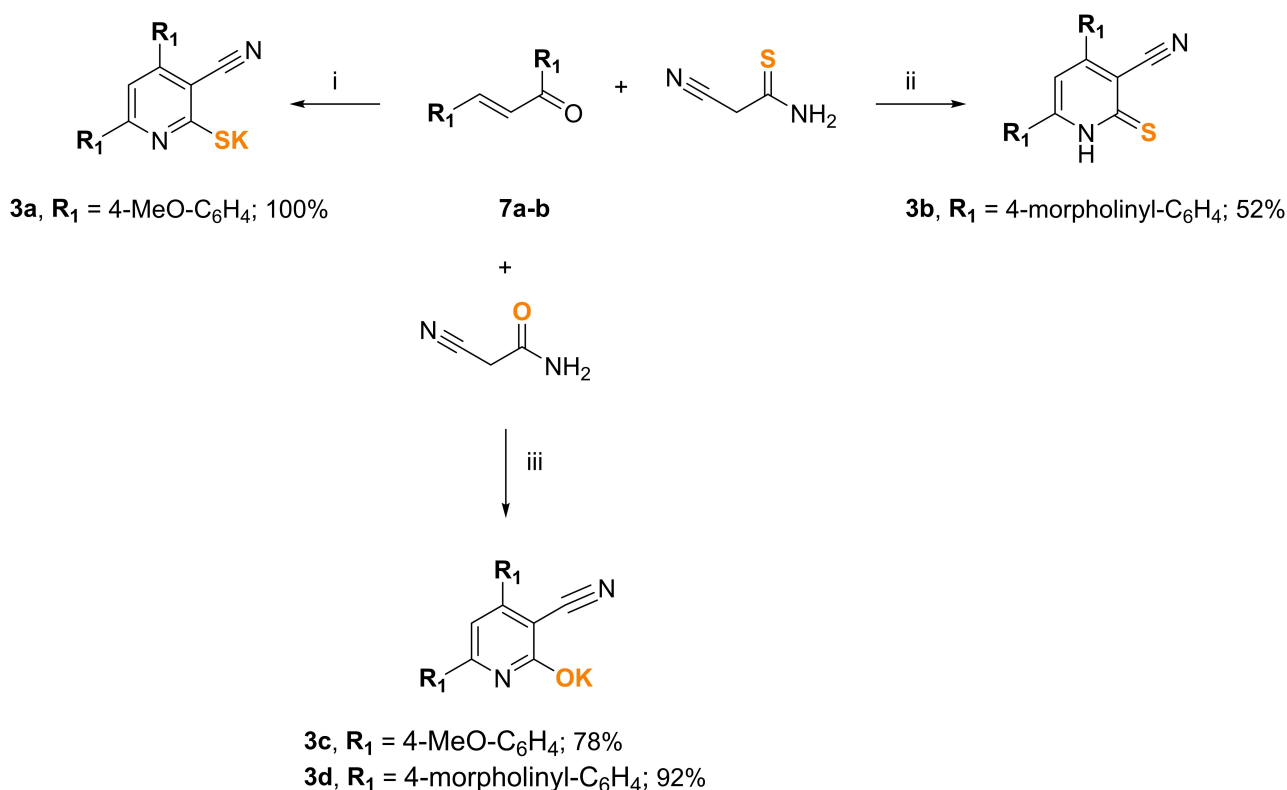
Synthesis

To obtain the desired compounds, we needed a versatile methodology using common key intermediates. Thus, two strategies presented in Scheme 1 were considered. The first one (route A) relies on the alkylation of 4,6-disubstituted 3-cyano-2-thiopyridines or 2-pyridones (3) with various selected halogenated substituents (4), whereas the second strategy (route B) relies on the nucleophilic aromatic substitution of 4,6-disubstituted 3-cyano-2-chloro-pyridines (5) using various selected thiols (6).

When using the first strategy (A), the synthesis of the key intermediates related to 3-cyano-2-thiopyridine (3, X=S, Scheme 1) and to the 3-cyano-2-pyridone (3, X=O, Scheme 1)

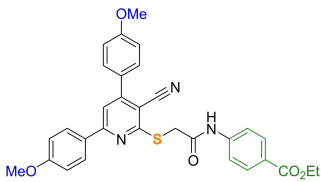
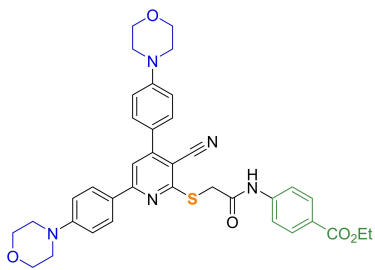
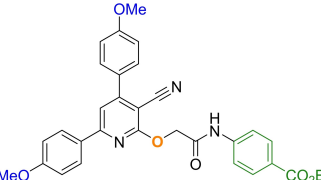
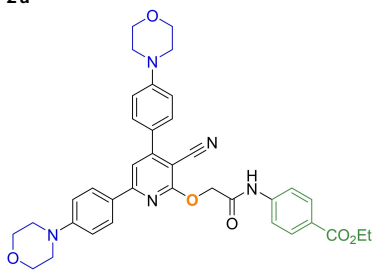
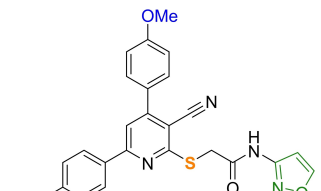
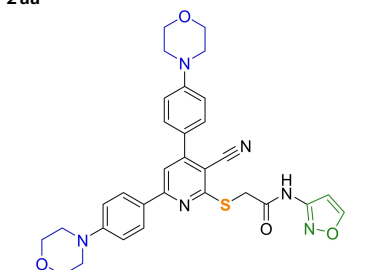
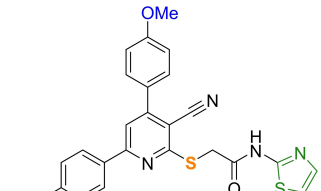
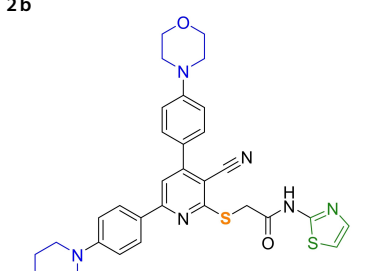
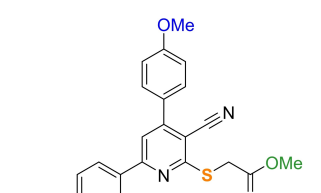
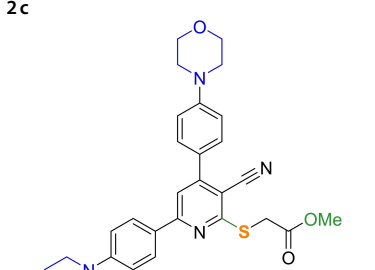
was firstly considered on the basis of the literature data.^[13] Although compounds 3 a–d could be obtained with good yields (Scheme 2) from the corresponding chalcones 7 a–b, these procedures appeared substrate-dependent and the isolation steps were tedious.

Once these starting materials obtained, we focused on the synthesis of the halogenated derivatives (4, Scheme 1). Thus, compounds 4 a–b were obtained in modest to high yields by reacting chloroacetyl chloride with the corresponding amine derivatives, *i.e.* benzocaine and 3-amino-1,2-oxazole, following previously described procedures.^[14] Then, coupling reactions between 3 a–d and 4 a (Scheme 3) were carried out according to literature data,^[15] affording 2 a, 1 aa and 2 aa in low to moderate yields. Unfortunately, compound 1 a was only



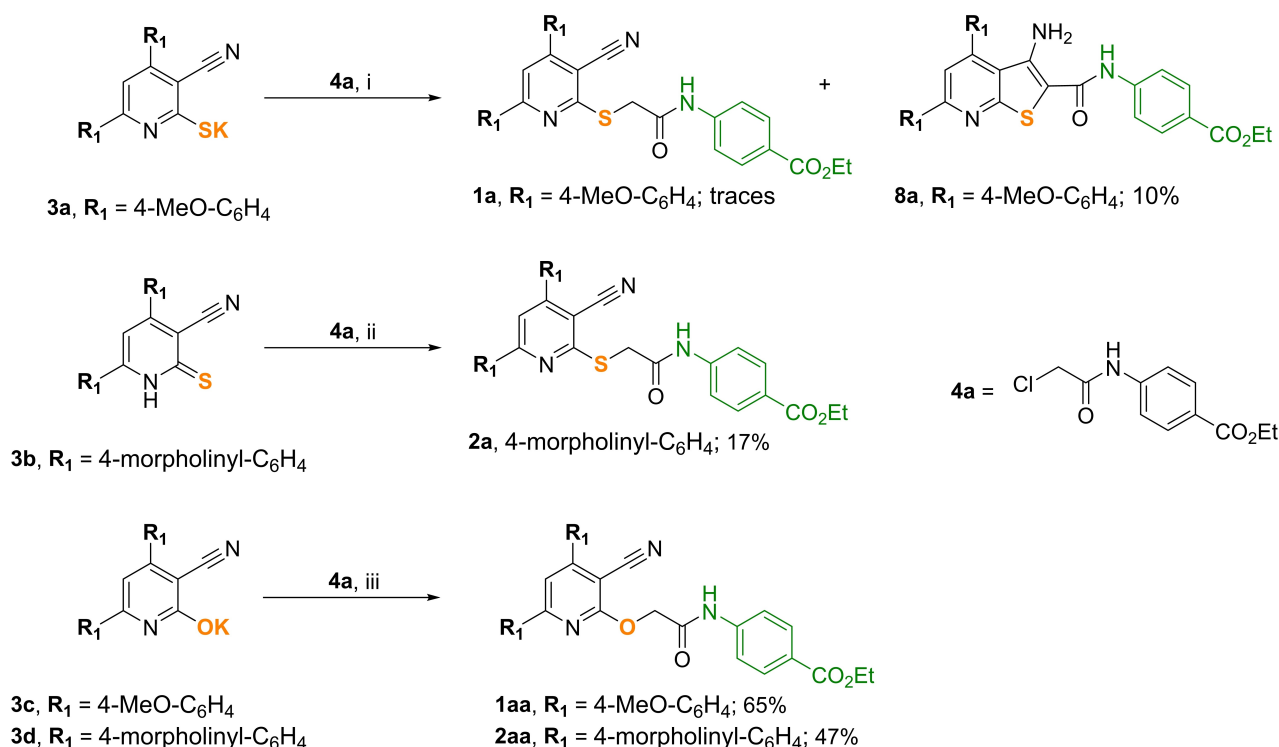
Scheme 2. Synthesis of 4,6-diaryl 3-cyano-2-thiopyridines 3 a–b and 4,6-diaryl 3-cyano-2-pyridones 3 c–d. (i) tBuOK, tBuOH, 35 °C, 18 h; (ii) Piperidine, EtOH, 100 °C, 20 h; (iii) tBuOK, EtOH, 0 °C to rt 15 min, rt, 3 days.

Table 1. Selected analogs on the basis of the *in silico* study.

Series 1 Structure	DS	cLog P	Series 2 Structure	DS	cLog P
 1a	96.2	5.5	 2a	102.9	5.0
 1aa	95.6	5.2	 2aa	107.0	4.6
 1b	86.0	4.0	 2b	93.8	3.5
 1c	87.6	4.9	 2c	95.6	4.3
 1d	80.3	4.0	 2d	83.7	3.5

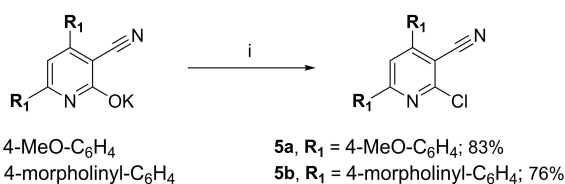
observed by NMR in mixed fractions, and another bicyclic structure was isolated in low yield and identified as compound **8a**. Thus, it is worth noting that given the acidic hydrogens of

the methylene, either structures **1** or **2** could easily evolve into the corresponding fluorescent bicyclic derivatives when using an excess of base or long reaction time.

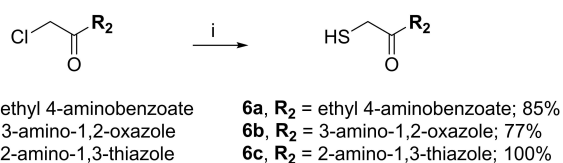


Scheme 3. Alkylation of 4,6-diaryl 3-cyano-2-thiopyridines **3a–b** and crude 4,6-diaryl 3-cyano-2-pyridones **3c–d** with reagent **4a**, (i) $\text{CH}_3\text{CO}_2\text{Na}$ (3 equiv.), DMF, rt, 3 days; (ii) K_2CO_3 (1.1 equiv.), DMF, rt, 30 min.; (iii) K_2CO_3 (4 equiv.), acetone, reflux, overnight.

On the basis of this first set of results concerning route A (procedures to obtain **3a** and **3b** appeared substrate-dependent, their purification was tedious and their alkylation by *N*-chloroacetyl derivatives led to a mixture of compounds difficult to separate by column chromatography), we concluded that route A would not allow us to easily obtain chemical diversity and we decided to consider route B.



Scheme 4. Preparation of 4,6-diaryl 3-cyano-2-chloropyridines **5a** and **5b**, (i) POCl_3 reflux, 3 days.



Scheme 5. Synthesis of thiols **6a–c**, (i) CH_3COSK , DMF, rt, 30 min. for **4a** and **4c**, 1 h for **4b**; then EtONa , EtOH , 30 min. for **6a** and **6c**, 1 h for **6b**.

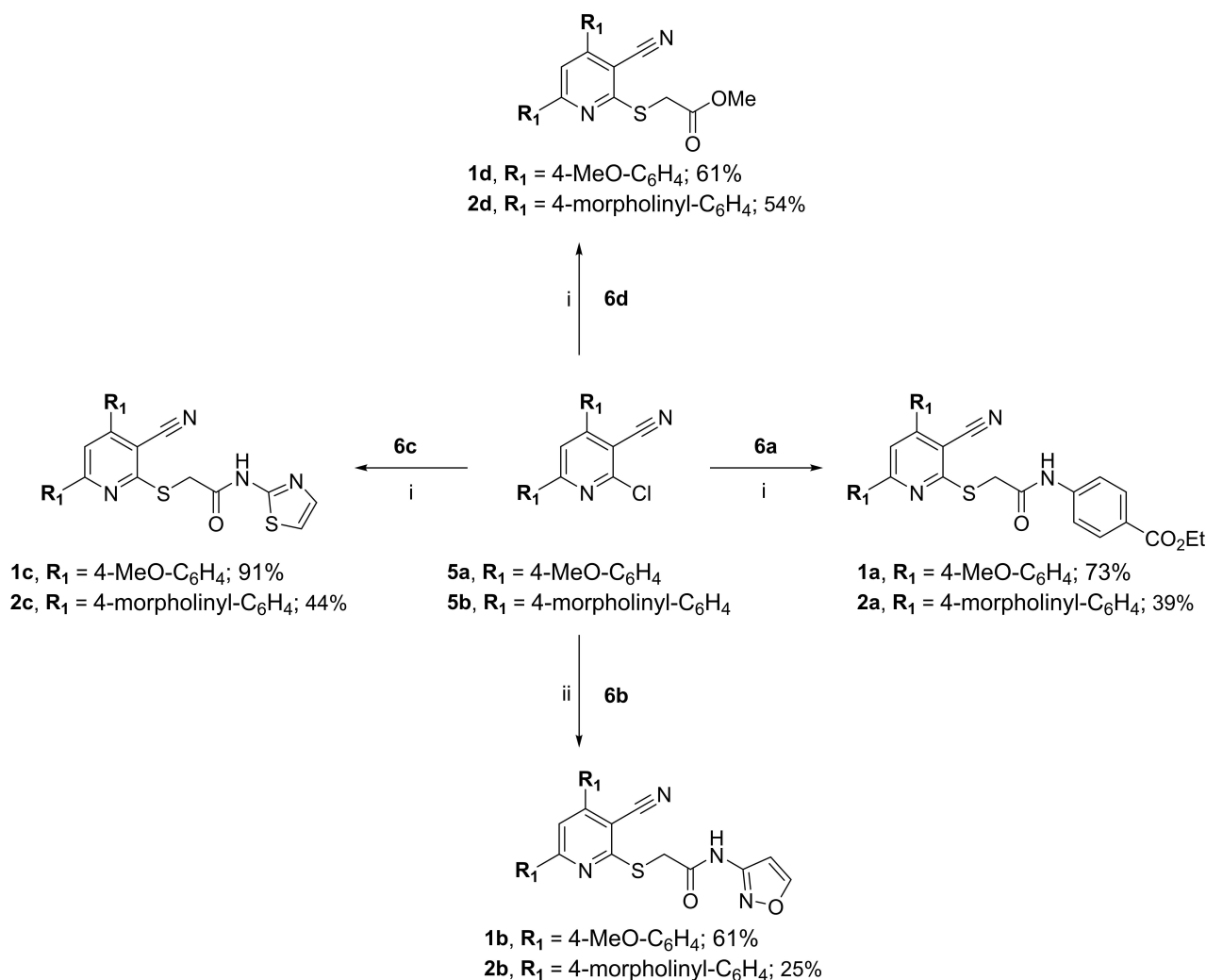
Therefore, we explored the second strategy B leveraging our recent work,^[13b] related to the preparation of the desired 4,6-disubstituted 3-cyano-2-chloropyridines (Scheme 1, 5). Thus, when refluxing the crude 3-cyano-2-pyridone **3c** and **3d** obtained beforehand (Scheme 2) in phosphorus oxychloride, the corresponding 4,6-diaryl 3-cyano-2-chloropyridines **5a** and **5b** (Scheme 4) were isolated in good yields.

In order to obtain the required thiols (Scheme 1, 6), *N*-chloroacetyl derivatives **4a–b** and commercially available **4c** were subsequently treated with potassium thioacetate and sodium ethoxide providing thiols **6a–c** (Scheme 5) in good yields (77–100%) and crude could be used without further purification.

At this stage, all starting materials required for route B were available and the two series of *S*-alkylated 3-cyano-2-thiopyridines were obtained by treating either compound **5a** or **5b** with a small excess (3.6 eq.) of the previously synthesized thiols **6a–c** or the commercially available methyl thioglycolate **6d** (Scheme 6) and no more than 1.6 eq. of potassium carbonate.

In the first series, compounds **1a–d** were obtained with yields ranging from 61 to 91%, while in the second series, compounds **2a–d** were obtained with lower yields (25–54%). One of the advantages of this strategy is that no tedious purification step was needed until the last step where all substituted compounds were purified and only traces of bicyclic derivatives were detected.

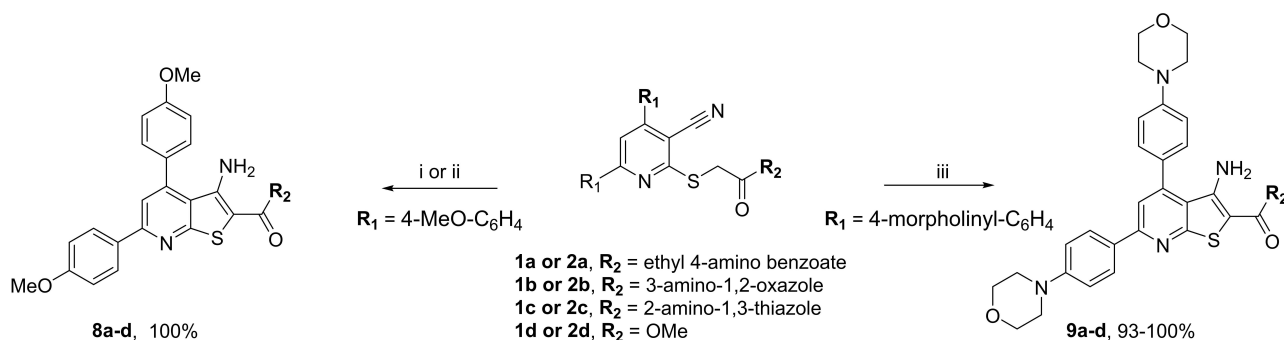
Finally, we took the opportunity to explore and to study a third series of compounds as potential inhibitors of CD73. In



Scheme 6. Synthesis of compounds **1a–d** & **2a–d**, (i) K_2CO_3 (1.6 equiv.), DMF, 1 h, rt or (ii) 50°C .

this last series, compounds **8a–d** and **9a–d** were obtained in quantitative yields by treating the linear structures **1a–c** and **2a–d**, respectively, with 6 equivalents of potassium carbonate and compound **1d** with LiOH (Scheme 7).

Thus, comparing the two routes initially considered, as for compounds **1a** and **2a**, route B was more efficient than route A to prepare 4,6-diaryl 3-cyano-2-thiopyridines. Nevertheless, route A allowed us to access 4,6-diaryl 3-cyano-2-pyridone derivatives **1aa** and **2aa** easily in order to compare the impact



Scheme 7. Synthesis of bicyclic derivatives **8a–d** & **9a–d**, (i) K_2CO_3 (6 equiv.), DMF, rt 16 h for **8a**, rt to 100°C , 16 h for **8b–c**, (ii) LiOH, (1.2 equiv.), DMF, rt, 16 h for **8d**, (iii) K_2CO_3 (6 equiv.), DMF, rt, 16 h for **9a** and **9c**, 50°C , 16 h for **9b** and **9d**.

of an oxygen atom and a sulfur atom towards the inhibition of CD73. By taking advantage of a side-reaction and the formation of bicyclic derivatives (**8a–d** and **9a–d**), we were also able to obtain a third series of compounds. All compounds were fully characterized by ^1H and ^{13}C NMR and mass spectrometry, and then subjected to biological evaluation.

Inhibitory activity of synthesized compounds toward hCD73

The CD73 inhibition assays (Tables 2–4) were performed using both the soluble form of the protein (purified recombinant hCD73) and the membrane-bound form expressed by cultured breast cancer cells (MDA-MB-231). In Tables 2–4, the inhibitory activities are represented as percentage inhibition of the AMPase activity of CD73 and AOPCP was used as positive control ($85 \pm 5\%$ of hCD73 inhibition at $5\ \mu\text{M}$ and $84 \pm 2\%$ of CD73 inhibition at $100\ \mu\text{M}$ in cell-based assay),^[7d] even so its mode of action (competitive inhibitor) is expected to be different for our novel derivatives. In addition, the intrinsic toxicity of the newly synthesized compounds (**1a–d**, **1aa**, **2aa**, **2a–d**, **8a–d** and **9a–d**), by the means of their antiproliferative activity at $100\ \mu\text{M}$, was also assessed using the same cells.

Enzymatic inhibition assays with compounds from series 1 showed that two groups (benzyl-ethoxy or isoxazole), introduced at position 2 of the cyanopyridine, preserved the inhibitory activity of compounds **1a** and **1b** (87% and 78% inhibition at $5\ \mu\text{M}$, respectively, Table 2). Their replacement by a thiazole or a methoxy group was detrimental, leading to a lower activity of compounds **1c** and **1d** (27% and 30% of

inhibition at $5\ \mu\text{M}$, respectively). When replacing the thioacetamide linker by an oxo-acetamide (compound **1aa**), the activity was lost (9% of inhibition) in comparison to **1a**.

The introduction of morpholino groups at positions 4 and 6 is expected to improve aqueous solubility of the compounds. In this respect, series 2 included the corresponding analogues from series 1 and compounds showed slightly reduced inhibitory activities (Table 3), around 70% of inhibition for compounds **2a** and **2b**. As already observed in series 1, the thiazole group (compound **2c**) was found to be detrimental but to a lesser extent than in series 1 for the blockade of CD73 enzymatic activity, and the methoxy-containing compounds in series 2 (**2d**) was completely inactive on the recombinant protein.

The cyclic forms of previous derivatives were also evaluated toward the recombinant purified enzyme (Table 4) and led to moderate inhibition ($42\text{--}57\%$ for derivatives **8a**, **8c**, **8d** and **9b** at $5\ \mu\text{M}$) or an overall loss of efficacy, especially for compounds **8b**, **9a**, **9c** and **9d** ($< 25\%$ of inhibition at $5\ \mu\text{M}$). The worse combination was the introduction of a methoxy group in position 2, with morpholino at positions 4 and 6 within a cyclic form of the molecule (**9d**). Interestingly, the smallest cyclic compound **8d** is still active with 50% of inhibition.

Cancer cells were first used to evaluate intrinsic cytotoxicity of potential CD73-inhibitors. These experiments showed a high variation in intrinsic toxicity, with 5 of 18 compounds (**1a**, **1c**, **2a**, **2b** and **2d**) showing less than 40% survival after 3 days incubation. No obvious correlation to structure was seen. The same cells (expressing CD73) were then used to evaluate the inhibition of membrane-bound CD73. When compounds were

Table 2. Inhibitory activities and cytotoxic activity of compounds from series 1. Values are means of at least three independent experiments \pm SD (recombinant CD73) or \pm SEM (cell-based assays).

Compound	Structure	CD73 inhibition [%]	Cell survival [$100\ \mu\text{M}$]
Series 1		Recombinant CD73 ($5\ \mu\text{M}$)	Cell-based assay ($100\ \mu\text{M}$)
1a		87 ± 3	ND ^[a]
1b		78 ± 0.5	27 ± 7
1c		27 ± 3	22 ± 1
1d		30 ± 5	ND ^[a]
1aa		9 ± 1	32 ± 10

[a] Not determined due to low solubility.

Table 3. Inhibitory activities and cytotoxic activity of compounds from series 2. Values are means of at least three independent experiments \pm SD (recombinant CD73) or \pm SEM (cell-based assays).

Compound	Structure	CD73 inhibition [%]	Cell survival [100 μ M]	
Series 2		Recombinant CD73 (5 μ M)	Cell-based assay (100 μ M)	
2a		70 \pm 1	37 \pm 4	28 \pm 6
2b		68 \pm 3	44 \pm 13	19 \pm 4
2c		59 \pm 4	35 \pm 6	95 \pm 13
2d	OMe	–14 \pm 10	39 \pm 8	26 \pm 8
2aa		41 \pm 7	49 \pm 10	83 \pm 6

assessed at 100 μ M, we observed between 22 and 49% inhibition (except **9a** with 3%). Contrary to assays on recombinant protein, series 2 had a slightly higher efficiency than series 1. We did not observe any correlation with the results obtained with recombinant protein, and the results were very similar between compounds of each series, rendering any SAR study irrelevant.

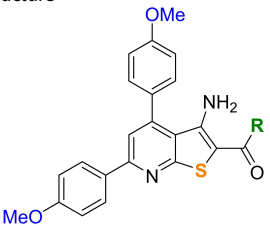
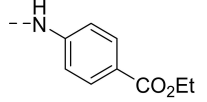
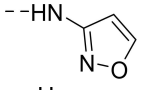
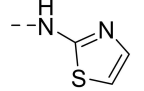

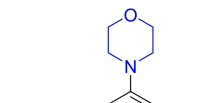
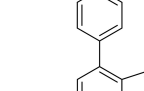
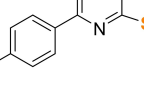

Searching for genetic variants of CD73 in MDA-MB-231 cells, we observed two transcripts as earlier reported in hepatocellular carcinoma cells (confirmed by RT-PCR and sequencing, data not shown).^[16] The short transcript lacks exon 7 inducing the production of a 55 amino-acids shorter protein. We do not know if both proteins are expressed at the cell surface (unsuccessful attempts with Western blots), but we cannot exclude that the presence of the two proteins modifies CD73 activity and sensitivity to allosteric inhibitors. This could explain, at least in part, differences in results between recombinant protein and CD73 expressed by MDA-MB-231 cells.

Molecular modeling

Assuming that the binding site for all derivatives is located at the dimer interface as previously observed for RR4/RR6, docking predictions were performed for few of them in order to understand their differences in activity. The main contacts previously observed in presence of RR4/RR6 were detected with

some variations of distance leading to weaker or stronger interactions between CD73 amino acids and the compounds. The cyano group is always connected to His456 and Tyr484 through hydrogen bonding (2.6 and 2.5 Å, respectively) and the methoxy group on the aryl substituent to Lys547 (Figure 3A). Same binding was obtained with compound **1a** while docking of derivative **2a** resulted in a little shift leading to weaken its connection with His456. Nevertheless, the morpholino group preserved the interaction with Lys547 (Figure 3B). Derivatives carrying an isoxazole group were found in two orientations and **1b** preserved the regular binding while **2b** (morpholino version) was moving away losing its connection with Lys547 (Figure 3C). Same observation was done when comparing compounds **2a** and **2b** (hydrogen bond distance increases up to 4.4 Å, Figure 3D). The replacement of thioacetamide linkage by thioester was detrimental in activity and may be explained by an important movement of the binding orientation for compound **1aa** (Figure 3E). The cyclization which can be an advantage in metabolic stability or to render the molecule more rigid and planar was not detected as an improvement, most probably due to the loss of the strong interaction between cyano group and His456/Tyr484 for derivative **8a** in contrast to **1a** (Figure 3F).

Table 4. Inhibitory activities and cytotoxic activity of bicyclic compounds from series 3. Values are means of at least three independent experiments \pm SD (recombinant CD73) or \pm SEM (cell-based assays).

Compound	Structure	CD73 inhibition [%]	Cell survival [100 μ M]
Series 3		Recombinant CD73 (5 μ M)	Cell-based assay (100 μ M)
8a		53 \pm 4	34 \pm 7
8b		16 \pm 5	120 \pm 13
8c		57 \pm 3	45 \pm 2
8d		50 \pm 8	ND ^[a]
9a		24 \pm 7	3 \pm 6
9b		42 \pm 5	ND ^[a]
9c		25 \pm 4	34 \pm 4
9d		-8 \pm 8	48 \pm 6

[a] Not determined due to low solubility.

Inhibitory function in immune cell-based assay

For the compounds showing reasonable inhibition of CD73 *in vitro* (i.e. % inhibition of hCD73 superior to 65% at 5 μ M and/or inhibition of CD73 activity in cell-based assay superior to 37% at 100 μ M, Tables 2–4), we expanded the evaluation including their ability to reverse the adenosine-mediated blockade of human T cells proliferation, assessed either by the analysis of the pellet size (Figure 4A) or the MTS assay (Figure 4B). In these conditions, AOPCP (used as reference compound) was able to reverse the inhibitory impact of exogenous ATP on T cell proliferation at high concentration (100 μ M) but its efficacy was lost below 10 μ M.^[7d] Thus, compounds **1a–b**, **2a–b**, **2d**, **2aa**, **8c** and **9d** were challenged,

but due to solubility issues or precipitation in biological media the test results for compounds **1a–b**, **2aa** and **8c** were not usable.

In contrast to results obtained on the recombinant enzyme, a higher blockade efficiency was observed in the immune cell-based assay for compound **2d** (10 μ M) than compound **2b** (100 μ M) whereas compound **2a** did not have any activity (Figure 4). It has to be noticed that, at 100 μ M, these three compounds strongly altered the viability of MDA-MB-231 cell line whereas only **2d** was found toxic in the immune cell-based assay. The formation of cyclic derivatives strongly decreased the cellular toxicity at high concentration (100 μ M) on both MDA-MB231 cell line (Table 4) and immune cells. However, the cyclisation was also associated with a reduced functional

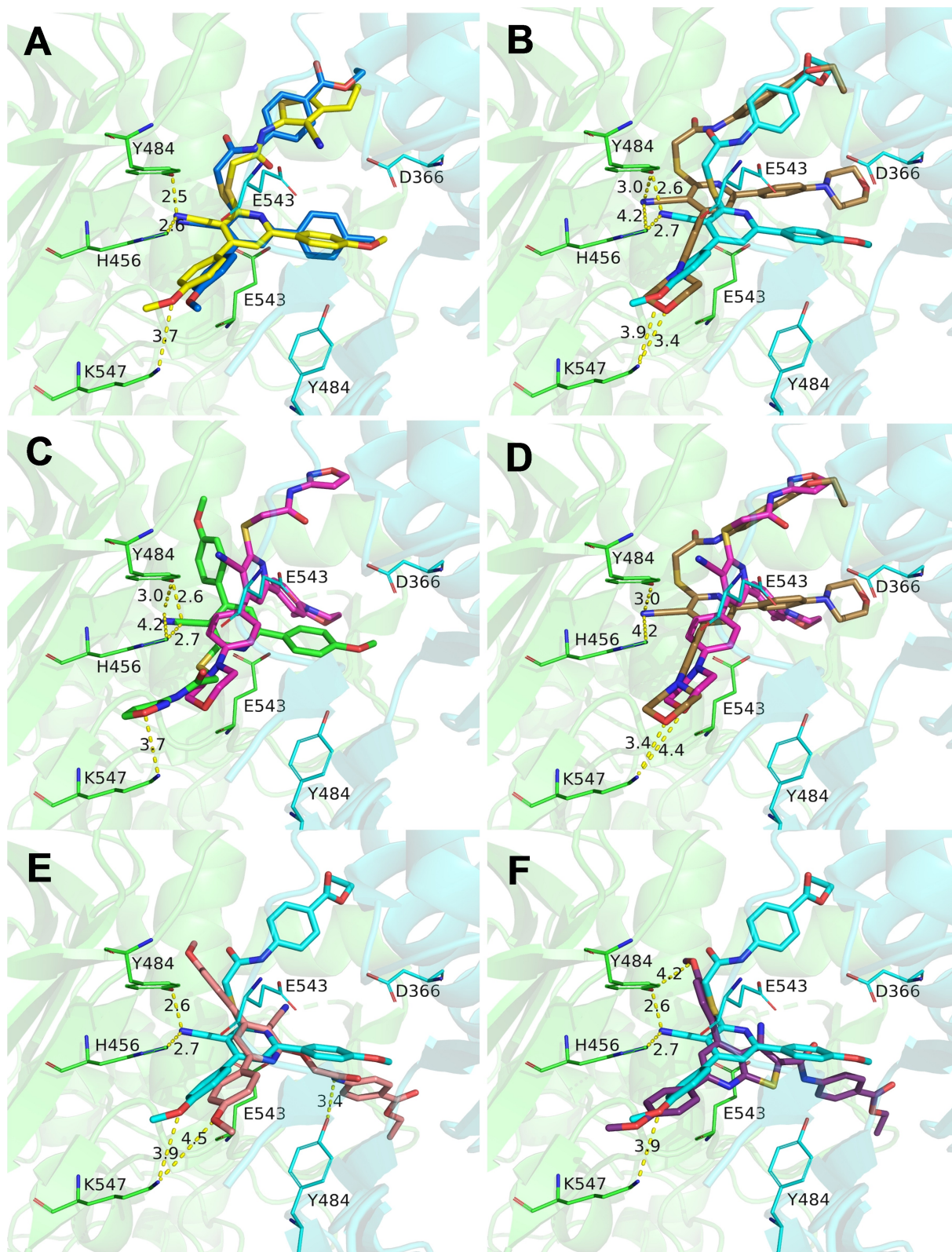


Figure 3. A) Overlay of binding poses obtained by docking at the dimer interface of CD73 for RR4 (yellow) and RR6 (blue). B) Change observed by docking for derivatives 1a and morpholino derivative 2a (depicted in cyan and brown sticks, respectively). C) Comparison of docking poses obtained for compounds 1b (green) and 2b (pink). D) Comparison of derivatives 2a (brown) and 2b (pink). E) Change in binding modes between 1a (cyan) and 1a (salmon). F) Change in binding modes between 1a (cyan) and the corresponding cyclic derivative 8a (purple).

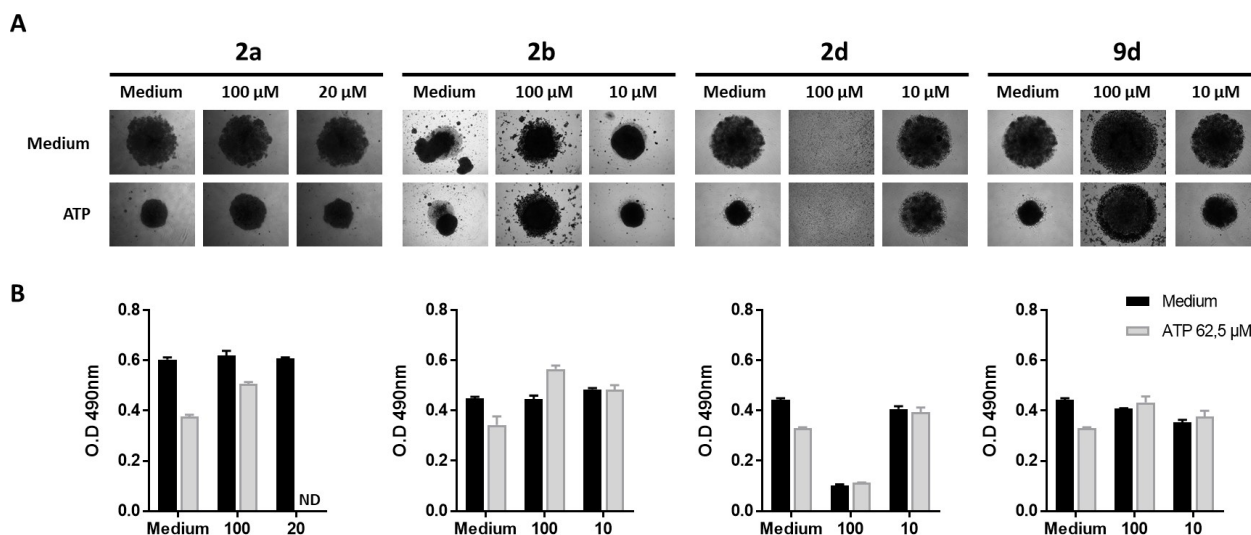


Figure 4. Evaluation of the efficacy of compounds **2a–b**, **2d** and **9d** in a functional assay on immune cells. The capacity of selected CD73 inhibitors to antagonize the blockade of T cells proliferation after TCR triggering induced by CD73 activity was assessed after 3 days by microscopy (A) and MTS assay (B). In (B), data are represented as mean values of optical density at 490 nm (O.D.) + standard deviation (SD) from technical triplicates. These results are representative of two independent experiments.

activity. Indeed, compound **2d** remains efficient at 10 μ M, whereas **9d** was only active at 100 μ M. For this last compound, a discrepancy was noticed between proliferation result (Figure 4A) and MTS assay (Figure 4B) most likely due to the yellow color of the compound that interferes with the colorimetric assay.

Conclusion

The development of PPI inhibitors is often a challenging task as the interacting surface is more hydrophobic and less accessible for hydrophilic/soluble inhibitors. Inhibitory activity depends mainly on reaching its target enzyme and therefore requires both high affinity for CD73 and reasonable aqueous solubility. With this aim we have designed, synthesized and studied three series of 4,6-biaryl-2-thiopyridine derivatives targeting an allosteric pocket previously identified. Two synthetic routes were envisaged and the one involving 4,6-disubstituted 3-cyano-2-chloro-pyridines as intermediates appeared more efficient and convenient to access complex molecules in a few steps.

New structures (such as **2b**, **2d** and **9d**) including morpholino substituents on the 4,6-biaryl-2-thiopyridine core showed improved solubility in aqueous media in comparison to previously identified allosteric CD73-inhibitors,^[10] thus allowing their evaluation in cell culture experiments. These compounds associated both the ability to inhibit CD73 *in vitro* on cancer cell line and to restore T cell proliferation at 100 μ M. In particular, compound **2d** was shown to be in the same range of efficacy as AOPCP in the blockage of the hydrolysis of extracellular ATP into adenosine through the inhibition of the CD73 enzymatic activity.

Experimental Section

Enzyme inhibition assays

Inhibition of the 5'-nucleotidase activity was assessed by steady-state kinetics and quantification of the adenosine produced from AMP hydrolysis by CD73 using the recombinant purified enzyme (as previously described in Rahimova *et al.*^[10]). Briefly, reactions were carried out in a thermostatically controlled beaker under magnetic stirring at 37 °C in a buffer containing Tris-HCl 50 mM pH 7, NaCl 100 mM, MgCl₂ 1 mM, CaCl₂ 1 mM and hCD73 at a final concentration of 5 nM in the absence or in the presence of each inhibitor at 5 μ M. Each reaction was started by addition of the AMP substrate (Sigma-Aldrich) and stopped by addition of 10% of perchloric acid every 5 s. Reaction products (AMP and adenosine) were quantified by HPLC chromatography (Waters Alliance) using a Partisphere 5-SAX anion exchange column (AIT France) and 10 mM ammonium phosphate buffer pH 5.5 as mobile phase. Inhibition percentages were calculated from steady-state rate constants (k_{ss}) and normalized with k_{ss} value obtained in the absence of inhibitor.

Cell-based CD73 assay and cell survival assays

Procedures have been reported previously in Ghotemai *et al.*^[7d,e] Briefly, for CD73 inhibition, CD73-expressing MDA-MB-231 cells were seeded (25,000 cells per well in 96 well plates) and adhered overnight, before washes and incubation (30 min at 37 °C) in a phosphate-free buffer containing or not 200 μ M AMP and compounds. Free inorganic phosphate was quantified by the Green Malachite Phosphate Assay Kit (Gentaur) and spectrophotometry. CD73 inhibition was calculated by $(1 - OD_{inhibitor} / OD_{control}) \times 100 \%$.

Cell survival was assessed by MTT assay after 3 days incubation of MDA-MB-231 cells (seeding 3,000 cells per well in 96 well plates) exposed to 100 μ M of compounds.

Immune cells functional assay

The most interesting compounds highlighted in other assays were tested on a biological immuno-assay previously described^[7d] that is based on the principle that adenosine generated from AMP dephosphorylation by CD73 blocks T cell proliferation after TCR triggering.^[17] Briefly, B cells and T cells were purified from healthy donors' blood by sequential Ficoll ($d = 1.077$, Eurobio) and Percoll ($d = 1.0\text{--}1.3$, 51%, GE Healthcare) density gradients. B and T cells (50,000 per well) were preincubated for 1 hour at 37 °C under 5% CO₂ in triplicate, with chemical compounds selected as CD73 inhibitors (10 or 100 μM) or AOPCP (50 μM , Sigma-Aldrich) as internal control in 96-round-bottom well plates (Falcon) in complete RPMI medium (cRPMI) supplemented with antibiotics, L-glutamine (Life Technologies) and 5% of human serum AB⁺ (EFS). The proliferation was assessed after 3 days culture in the presence of TCR signal (anti-CD3/anti-CD28 beads, ratio 1 bead to 4 cells, Life Technologies) with or without addition of exogenous ATP (62.5 μM , Sigma-Aldrich) by capture of photomicrographs for each condition and the addition of MTS substrate (Promega) for two hours at 37 °C under 5% CO₂. The enzymatic activity of cells that is correlated with living cells number, allowed the degradation of MTS substrate into formazan that was quantified by optical density at 490 nm on a Tecan (ThermoScientific). A negative control of proliferation was obtained by culture of cells without the Expand beads.

Chemistry

¹H NMR and ¹³C NMR spectra were recorded with proton decoupling at ambient temperature on the following spectrometers: Bruker Avance III (600, 500 or 400 MHz). Chemical shifts (δ) are quoted in parts per million (ppm) referenced to the appropriate residual solvent peak: CDCl₃ at 7.26 ppm and 77.2 ppm, DMSO-*d*₆ at 2.50 ppm and 39.5 ppm relative to TMS. When required, COSY experiments were performed in order to confirm proton assignments as well as 2D ¹H–¹³C heteronuclear COSY for the attribution of ¹³C signals. Coupling constants, *J*, are given in Hertz (Hz). Multiplicity are indicated by: s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet), br (broad), dd (doublet of doublet). Thin layer chromatography was performed on pre-coated aluminum sheets of Silica Gel 60 F254 (Merck), and visualization of products was accomplished by UV absorbance followed by spraying with Hanes molybdate reagent. Purifications were carried out on Silica Gel 60 (Merck). Mass spectra were recorded on a Micromass Q-TOF mass spectrometer using electrospray ionization. HRMS were obtained with a Waters Synapt G2S (Waters, SN: UEB205) spectrometer equipped with positive electrospray source ionization (ESI). The capillary voltage was set to 2 kV and the sampling cone voltage was set to 30 V. All moisture sensitive reactions were carried out in anhydrous conditions under argon atmosphere using oven-dried glassware. Solvents were dried and distilled prior to use. Solids were dried over P₂O₅ under reduced pressure at room temperature.

Potassium 3-cyano-4,6-bis(4-methoxyphenyl)pyridine-2-thiolate (3a): To a solution of the chalcone **7a** (200 mg, 0.75 mmol), in *t*-butyl alcohol (2 mL) was added the commercially available 2-cyanothioacetamide (82.6 mg, 1.1 eq.) and *t*BuOK (334.5 mg, 4 eq.) in one portion. The resulting mixture was stirred at 35 °C for 24 hours. The precipitate was filtered and successively washed with cold 2-propanol (30 mL), Petroleum ether (30 mL) and Et₂O (30 mL) to provide the desired compound as a yellow solid (290.6 mg, quantitative yield). *R*_f=(Petroleum ether/EtOAc, 1/1) 0.15. ¹H NMR (600 MHz, DMSO-*d*₆): δ = 8.01 (d, *J* = 8.9 Hz, 2H), 7.52 (d, *J* = 8.7 Hz, 2H), 7.03 (d, *J* = 8.8 Hz, 2H), 6.99 (d, *J* = 8.9 Hz, 2H), 6.91 (s, 1H), 3.81 (2 s, 6H). ¹³C NMR (151 MHz, DMSO-*d*₆): δ = 184.6, 160.1, 159.6, 155.1, 151.5, 131.6, 130.9, 129.6 (C-9), 128.4, 120.9, 113.7, 113.6,

108.2, 106.9, 55.2. Q-TOF MS E⁺: *m/z* 349.1 [M + H]⁺. HRMS Q-TOF MS E⁺: calculated for C₂₀H₁₆N₂O₂S: 349.1005 [M + H]⁺, found 349.1017.

4,6-Bis(4-morpholinophenyl)-2-thioxo-1,2-dihydropyridine-3-carbonitrile (3b): To a solution of the stirred chalcone **7b** (800.4 mg, 2.1 mmol), in EtOH (10 mL) were added the commercially available 2-cyanothioacetamide (424 mg, 2 eq.) and piperidine (210 μL , 1 eq.). The resulting mixture was heated to reflux overnight. Water (20 mL) was added and the mixture was acidified to pH 1 using HCl (1 M) aqueous solution. The resulting precipitate was filtered and dried to provide the desired compound as a yellow solid (502.8 mg, 52%). *R*_f=(CH₂Cl₂) 0.50. ¹H NMR (600 MHz, DMSO-*d*₆): δ = 7.81 (d, *J* = 9.0 Hz, 2H), 7.68 (d, *J* = 8.9 Hz, 2H), 7.08 (d, *J* = 9.0 Hz, 2H), 7.05–7.03 (m, 3H), 3.76–3.73 (m, 8H), 3.30–3.27 (m, 8H). ¹³C NMR (151 MHz, DMSO-*d*₆): δ = 179.4, 155.5, 153.0, 152.5, 151.3, 129.8, 129.6, 124.8, 120.0, 117.8, 113.9, 113.7, 111.0, 109.3, 65.9, 46.9. Q-TOF MS E⁺: *m/z* 459.19 [M + H]⁺. HRMS Q-TOF MS E⁺: calculated for C₂₆H₂₇N₄O₂S: 459.1855 [M + H]⁺, found 459.1858.

General Procedure A: Synthesis of 4,6-Diaryl-3-cyano-2-pyridones 3c and 3d

To a stirred solution of the chalcone **7a** or **7b** (1 eq.), in ethanol (1 mL/mmol) was added the commercially available 2-cyanoacetamide (1.1 eq.). The resulting mixture was cooled to 0 °C and *t*BuOK (4 eq.) was added in one portion. After 15 min, the cooling bath was removed and the mixture was stirred at room temperature for 3 days. The resulting yellow precipitate was filtered and successively washed with cold 2-propanol (30 mL), petroleum ether (30 mL) and diethyl ether (30 mL) to provide the desired compound.

Potassium 3-cyano-4,6-bis(4-methoxyphenyl)pyridin-2-olate (3c): The title compound was obtained as a yellow solid (5.40 g, 78%) in 3 days from compound **7a** (5 g, 18.6 mmol) using general procedure A. *R*_f=(Petroleum ether/EtOAc, 2/8) 0.37. ¹H NMR (600 MHz, DMSO-*d*₆): δ = 7.92 (d, *J* = 8.9 Hz, 2H), 7.52 (d, *J* = 8.8 Hz, 2H), 7.01 (d, *J* = 8.8 Hz, 2H), 6.94 (d, *J* = 9.0 Hz, 2H), 6.45 (s, 1H), 3.81 (s, 3H), 3.79 (s, 3H). ¹³C NMR (151 MHz, DMSO-*d*₆): δ = 172.2, 159.7, 159.4, 158.4, 154.0, 132.8, 131.6, 129.2, 128.1, 121.9, 113.7, 113.5, 102.0, 90.3, 55.2, 55.1. Q-TOF MS E⁺: *m/z* 333.12 [M + H]⁺. HRMS Q-TOF MS E⁺: calculated for C₂₀H₁₇N₂O₃: 333.1239 [M + H]⁺, found 333.1246.

Potassium 3-cyano-4,6-bis(4-morpholinophenyl)pyridin-2-olate (3d): The title compound was obtained as a yellow solid (1.74 g, 92%) in 3 days from compound **7b** (1.61 g, 4.62 mmol) using general procedure A. *R*_f=(EtOAc) 0.40. ¹H NMR (600 MHz, DMSO-*d*₆): δ = 7.81 (d, *J* = 8.9 Hz, 2H), 7.66 (d, *J* = 9.0 Hz, 2H), 7.08 (d, *J* = 9.0 Hz, 2H), 7.03 (d, *J* = 9.1 Hz, 2H), 6.69 (s, 1H), 3.76–3.73 (m, 8H), 3.28–3.25 (m, 8H). ¹³C NMR (151 MHz, DMSO-*d*₆): δ = 162.4, 159.1, 152.8, 152.4, 129.5, 128.7, 125.6, 117.6, 114.0, 113.9, 103.6, 65.9, 47.3, 47.0. Q-TOF MS E⁺: *m/z* 443.21 [M + H]⁺. HRMS Q-TOF MS E⁺: calculated for C₂₆H₂₇N₄O₃: 443.2083 [M + H]⁺, found 443.2090.

General Procedure B: Synthesis of 2-chloro-4,6-Diaryl nicotinonitrile 5a–b

The crude compound **9a** or **9b** (1 eq.) was dissolved under an argon atmosphere with freshly distilled Phosphorus (V) oxychloride (POCl₃, 4 mL/mmol) and the resulting mixture was heated to reflux for 5 days. The crude was poured into a cold saturated solution of K₂CO₃ under stirring for 15 min. The resulting precipitate was filtered and washed with water (3 × 30 mL) and petroleum ether (30 mL) to provide the desired compound.

2-Chloro-4,6-bis(4-methoxyphenyl)nicotinonitrile (5a): The title compound was obtained as a white solid (1.56 g, 83%) in 3 days from compound **9a** (2 g, 5.4 mmol) using general procedure B. R_f = (Petroleum ether/EtOAc, 7/3) 0.75. ^1H NMR (600 MHz, CDCl_3): δ = 8.06 (d, J = 8.7 Hz, 2H), 7.67 (s, 1H), 7.62 (d, J = 8.6 Hz, 2H), 7.07 (d, J = 8.6 Hz, 2H), 7.01 (d, J = 8.7 Hz, 2H), 3.89 (br s, 6H). ^{13}C NMR (151 MHz, CDCl_3): δ = 162.4, 161.6, 159.6, 156.1, 154.1, 130.1, 129.4, 128.8, 128.0, 117.7, 115.9, 114.8, 114.6, 105.6, 55.6. Q-TOF MS E^+ : m/z 351.09 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{20}\text{H}_{16}\text{N}_2\text{O}_2\text{Cl}$: 351.0900 $[\text{M} + \text{H}]^+$, found 351.0903.

2-Chloro-4,6-bis(4-morpholinophenyl)nicotinonitrile (5b): The title compound was obtained as a brown solid (1.09 g, 76%) 3 days from compound **9b** (2.31 g, 4.97 mmol) using general procedure B. R_f = (Petroleum ether/EtOAc, 4/6) 0.57. ^1H NMR (600 MHz, CDCl_3): δ = 8.02 (d, J = 8.7 Hz, 2H), 7.63 (s, 1H), 7.61 (d, J = 8.7 Hz, 2H), 7.01 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 9.1 Hz, 2H), 3.89–3.87 (m, 8H), 3.31–3.38 (m, 8H). ^{13}C NMR (151 MHz, CDCl_3): δ = 159.4, 155.8, 154.2, 153.2, 152.7, 129.8, 129.0, 126.9, 126.2, 116.9, 116.3, 115.0, 114.7, 104.6, 66.8, 48.3, 48.1. Q-TOF MS E^+ : m/z 461.18 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{26}\text{H}_{26}\text{N}_4\text{O}_2\text{Cl}$: 461.1744 $[\text{M} + \text{H}]^+$, found 461.1753.

Ethyl 4-(2-chloroacetamido)benzoate (4a):^[14a] Compound **4a** was obtained according to previously published procedure^[14a] in quantitative yield using commercially available Ethyl-4-aminobenzoate as starting material. Characterization (^1H NMR) agreed with the literature. R_f = (Petroleum ether/EtOAc, 7/3) 0.57. ^1H NMR (400 MHz, CDCl_3): δ = 8.36 (s, 1H), 8.05 (d, J = 8.8 Hz, 2H), 7.65 (d, J = 8.8 Hz, 2H), 4.37 (q, J = 7.1 Hz, 2H), 4.21 (s, 2H), 1.39 (t, J = 7.1 Hz, 3H).

2-Chloro-N-(isoxazol-3-yl)acetamide (4b):^[18] Compound **4b** was obtained according to previously published procedure^[18] in 93% yield using commercially available 5-aminoisoxazole as starting material. Characterization (^1H NMR) agreed with the literature. R_f = (Petroleum ether/EtOAc, 7/3) 0.37. ^1H NMR (400 MHz, CDCl_3): δ = 9.42 (s, 1H), 8.33 (d, J = 1.5 Hz, 1H), 7.08 (d, J = 1.6 Hz, 1H), 4.22 (s, 2H).

General Procedure C: Synthesis of compounds 6a–6c

To a solution of the appropriate compound **4a–c** in anhydrous DMF (4 mL) was added, potassium thioacetate (1.05 eq.). The resulting mixture was stirred at room temperature. After 1 h NaOEt (1.3 eq.) and absolute ethanol (4 mL) were added and the mixture was stirred at room temperature. 6 h later, a saturated aqueous solution of NH_4Cl was added and the resulting mixture was partially concentrated in vacuo and extracted with CH_2Cl_2 (3 \times 20 mL). The combined organic layers were dried over MgSO_4 , filtered and concentrated to dryness to give the crude desired thiol that was used as such for the next step without further purification.

Ethyl 4-(2-mercaptoacetamido)benzoate (6a): The title compound was obtained as a white solid (410 mg, 85%) from compound **4a** (958 mg, 3.96 mmol) using general procedure C. R_f = (Petroleum ether/EtOAc, 6/4) 0.11.

N-(Isoxazol-3-yl)-2-mercaptoacetamide (6b): The title compound was obtained as a brown solid (381 mg, 77%) from compound **4b** (500 mg, 3.11 mmol) using general procedure C. R_f = (Petroleum ether/EtOAc, 6/4) 0.18.

2-Mercapto-N-(thiazol-2-yl)acetamide (6c): The title compound was obtained as a white solid (395 mg, 100%) from compound **4c** (401 mg, 2.27 mmol) using general procedure C. R_f = (Petroleum ether/EtOAc, 6/4) 0.17.

General Procedure D: Synthesis of compounds 1aa and 2aa

To a solution of the compound **3c** or **3d** in dry acetone (8 mL/mmol), were added **4a** (1.2 eq.) and K_2CO_3 (4 eq.). The resulting mixture was heated to reflux overnight. Water (30 mL) was then added and the aqueous layer extracted with CH_2Cl_2 (3 \times 30 mL). The combined organic layers were dried over MgSO_4 , filtered and concentrated to dryness. Purification of the crude was performed on silica gel (petroleum ether/EtOAc) to provide the desired compound.

Ethyl 4-(2-((3-cyano-4,6-bis(4-methoxyphenyl)pyridin-2-yl)thio)acetamido)benzoate (1aa): The title compound was obtained as a yellow powder (187.5 mg, 59%) from compound **3c** (196.2 mg, 0.59 mmol) using general procedure D. R_f = (Petroleum ether/EtOAc, 7/3) 0.44. ^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ = 10.75 (s, 1H), 8.13 (d, J = 9.0 Hz, 2H), 7.94 (d, J = 8.8 Hz, 2H), 7.78–7.74 (m, 5H), 7.16 (d, J = 8.8 Hz, 2H), 6.90 (d, J = 9.0 Hz, 2H), 5.18 (s, 2H), 4.28 (q, J = 7.1 Hz, 2H), 3.86 (s, 3H), 3.78 (s, 3H), 1.30 (t, J = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$): δ = 166.8, 165.3, 163.3, 161.4, 160.8, 156.5, 156.0, 143.0, 130.3, 130.2, 129.1, 128.7, 127.9, 124.5, 118.7, 115.6, 114.3, 114.1, 113.0, 90.6, 65.6, 60.4, 55.4, 55.3, 14.2. Q-TOF MS E^+ : m/z 538.2 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{31}\text{H}_{28}\text{N}_3\text{O}_6\text{S}$: 538.1973 $[\text{M} + \text{H}]^+$, found 538.1970.

Ethyl 4-(2-((3-cyano-4,6-bis(4-morpholinophenyl)pyridin-2-yl)oxy)acetamido)benzoate (2aa): The title compound was obtained as a yellow powder (121.1 mg, 44%) from compound **3d** (189.6 mg, 0.43 mmol) using general procedure D. R_f = (Petroleum ether/EtOAc, 1/1) 0.25. ^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ = 10.72 (s, 1H), 8.04 (d, J = 9.1 Hz, 2H), 7.93 (d, J = 8.8 Hz, 2H), 7.77 (d, J = 8.8 Hz, 2H), 7.67 (d, J = 8.9 Hz, 2H), 7.64 (s, 1H), 7.12 (d, J = 9.0 Hz, 2H), 6.86 (d, J = 9.1 Hz, 2H), 5.16 (s, 2H), 4.28 (q, J = 7.1 Hz, 2H), 3.77 (t, J = 4.9 Hz, 4H), 3.71 (t, J = 4.9 Hz, 4H), 3.26 (t, J = 4.9 Hz, 4H), 3.18 (t, J = 4.9 Hz, 4H), 1.30 (t, J = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$): δ = 166.9, 165.3, 163.4, 156.7, 155.7, 152.5, 152.1, 143.0, 130.3, 129.6, 128.6, 126.2, 125.5, 124.5, 118.8, 116.1, 114.3, 113.9, 112.0, 89.3, 66.0, 65.9, 65.4, 60.4, 47.4, 47.2, 14.2. Q-TOF MS E^+ : m/z 648.28 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{37}\text{H}_{38}\text{N}_5\text{O}_6$: 648.2822 $[\text{M} + \text{H}]^+$, found 648.2823.

General Procedure E: Synthesis of compounds 1a–d and 2a–d

To a solution of compound **5a** or **5b** (1 eq.) in DMF (14 mL/mmol) were added the appropriate thiol **6a–d** (3.6 eq.) and K_2CO_3 (1.6 eq.). The resulting mixture was stirred at room temperature until TLC showed completion. Water (20 mL) was added and the aqueous layer extracted with CH_2Cl_2 (3 \times 20 mL). The combined organic layers were dried over MgSO_4 , filtered and concentrated to dryness. Purification of the crude was performed on silica gel (Petroleum ether/EtOAc) to provide the desired compound.

Ethyl 4-(2-((3-cyano-4,6-bis(4-methoxyphenyl)pyridin-2-yl)thio)acetamido)benzoate (1a): The title compound was obtained as a yellow powder (104.8 mg, 73%) from compound **5a** (90.5 mg, 0.26 mmol) and **6a** (224 mg, 0.94 mmol) in 1 h using general procedure E. R_f = (Petroleum ether/EtOAc, 7/3) 0.37. ^1H NMR (600 MHz, CDCl_3): δ = 9.44 (s, 1H), 8.04 (d, J = 8.9 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H), 7.61 (d, J = 8.9 Hz, 2H), 7.55 (s, 1H), 7.23 (d, J = 8.8 Hz, 2H), 7.07–7.03 (m, 4H), 4.31 (q, J = 7.1 Hz, 2H), 4.13 (s, 2H), 3.89 (s, 3H), 3.88 (s, 3H) 1.35 (t, J = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, CDCl_3): δ = 167.2, 166.2, 162.4, 162.2, 161.6, 159.0, 155.0, 142.0, 130.8, 130.1, 129.4, 129.2, 128.0, 126.0, 118.8, 116.4, 115.7, 115.0, 114.8, 103.1, 61.0, 55.7, 55.6, 35.3, 14.5. Q-TOF MS E^+ : m/z 554.2 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{31}\text{H}_{28}\text{N}_3\text{O}_5\text{S}$: 554.1744 $[\text{M} + \text{H}]^+$, found 554.1734.

2-((3-Cyano-4,6-bis(4-methoxyphenyl)pyridin-2-yl)thio)-N-(isoxazol-3-yl)acetamide (1b): The title compound was obtained as a yellow powder (85.3 mg, 61%) from compound **5a** (103.1 mg, 0.29 mmol) and **6b** (165.1 mg, 1 mmol) in 2 h using general procedure E at 50 °C. R_f =(Petroleum ether/EtOAc, 6/4) 0.60. ^1H NMR (600 MHz, CDCl_3): δ =9.53 (s, 1H), 8.23 (s, 1H), 8.00 (d, J =8.7 Hz, 2H), 7.61 (d, J =8.6 Hz, 2H), 7.53 (s, 1H), 7.06 (d, J =8.6 Hz, 2H), 7.04 (s, 1H), 6.99 (d, J =8.7 Hz, 2H), 4.14 (s, 2H), 3.89 (s, 3H), 3.87 (s, 3H). ^{13}C NMR (151 MHz, CDCl_3): δ =166.9, 162.2, 161.6, 161.2, 159.2, 159.1, 157.2, 154.8, 130.1, 129.3, 129.2, 128.1, 116.3, 115.8, 114.8, 114.7, 103.0, 99.3, 55.6, 34.80. Q-TOF MS E^+ : m/z 473.1 $[\text{M}+\text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{25}\text{H}_{21}\text{N}_4\text{O}_4\text{S}$: 473.1278 $[\text{M}+\text{H}]^+$, found 473.1280.

2-((3-Cyano-4,6-bis(4-methoxyphenyl)pyridin-2-yl)thio)-N-(thiazol-2-yl)acetamide (1c): The title compound was obtained as a yellow powder (27.8 mg, 91%) from compound **10a** (22.6 mg, 0.06 mmol) and **12c** (38.3 mg, 0.22 mmol) in 1 h using general procedure E. R_f =(Petroleum ether/EtOAc, 1/1) 0.30. ^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ =12.59 (s, 1H), 8.10 (d, J =9.0 Hz, 2H), 7.77 (s, 1H), 7.72 (d, J =8.8 Hz, 2H), 7.54 (d, J =3.6 Hz, 1H), 7.22 (d, J =3.6 Hz, 1H), 7.14 (d, J =8.9 Hz, 2H), 6.79 (d, J =9.0 Hz, 2H), 4.39 (s, 2H), 3.85 (s, 3H), 3.77 (s, 3H). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$): δ =166.2, 161.7, 161.3, 160.9, 158.1, 157.9, 153.6, 137.8, 130.3, 129.4, 129.0, 127.8, 116.1, 115.3, 114.4, 114.0, 113.7, 101.2, 55.5, 55.3, 34.0. Q-TOF MS E^+ : m/z 489.1 $[\text{M}+\text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{25}\text{H}_{21}\text{N}_4\text{O}_3\text{S}_2$: 489.1050 $[\text{M}+\text{H}]^+$, found 489.1044.

Methyl 2-((3-cyano-4,6-bis(4-methoxyphenyl)pyridin-2-yl)thio)acetate (1d): The title compound was obtained as a yellow powder (20.5 mg, 61%) from compound **5a** (29.5 mg, 0.08 mmol) and **6d** (30.5 mg, 0.29 mmol) in 1 h using general procedure E. R_f =(Petroleum ether/EtOAc, 7/3) 0.44. ^1H NMR (600 MHz, CDCl_3): δ =8.04 (d, J =8.9 Hz, 2H), 7.59 (d, J =8.7 Hz, 2H), 7.47 (s, 1H), 7.05 (d, J =8.7 Hz, 2H), 7.01 (d, J =8.9 Hz, 2H), 4.10 (s, 2H), 3.89 (1 s, 3H), 3.88 (1 s, 3H), 3.75 (s, 3H). ^{13}C NMR (151 MHz, CDCl_3): δ =169.7, 162.0, 161.9, 161.3, 158.3, 154.2, 130.0, 129.8, 129.1, 128.6, 116.1, 115.2, 114.7, 114.4, 102.5, 55.6, 52.9, 33.0. Q-TOF MS E^+ : m/z 421.1 $[\text{M}+\text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{23}\text{H}_{21}\text{N}_2\text{O}_4\text{S}$: 421.1217 $[\text{M}+\text{H}]^+$, found 421.1207.

Ethyl 4-(2-((3-cyano-4,6-bis(4-morpholinophenyl)pyridin-2-yl)thio)acetamido)benzoate (2a): The title compound was obtained as an orange powder (35.9 mg, 80%) from compound **5b** (31.1 mg, 0.06 mmol) and **6a** (51.7 mg, 0.22 mmol) in 1 h using general procedure E. R_f =(Petroleum ether/EtOAc, 4/6) 0.34. ^1H NMR (600 MHz, CDCl_3): δ =9.59 (s, 1H), 8.01 (d, J =8.9 Hz, 2H), 7.82 (d, J =8.7 Hz, 2H), 7.61 (d, J =8.8 Hz, 2H), 7.53 (s, 1H), 7.23 (d, J =8.7 Hz, 2H), 7.01 (d, J =8.8 Hz, 2H), 6.98 (d, J =8.9 Hz, 2H), 4.31 (q, J =7.1 Hz, 2H), 4.12 (s, 2H), 3.89–3.87 (m, 8H), 3.30–3.28 (m, 8H), 1.35 (t, J =7.1 Hz, 3H). ^{13}C NMR (151 MHz, CDCl_3): δ =167.5, 166.3, 162.2, 158.9, 154.8, 153.2, 152.7, 142.1, 130.7, 129.8, 128.8, 127.2, 126.2, 125.9, 118.8, 116.1, 115.7, 115.1, 102.1, 66.8, 66.7, 61.0, 48.2, 48.0, 35.3, 14.5. Q-TOF MS E^+ : m/z 664.3 $[\text{M}+\text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{37}\text{H}_{38}\text{N}_5\text{O}_5\text{S}$: 664.2588 $[\text{M}+\text{H}]^+$, found 664.2581.

2-((3-Cyano-4,6-bis(4-morpholinophenyl)pyridin-2-yl)thio)-N-(isoxazol-3-yl)acetamide (2b): The title compound was obtained as a yellow powder (32.5 mg, 25%) from compound **5b** (100 mg, 0.22 mmol) and **6b** (125.3 mg, 0.79 mmol) in 24 h using general procedure E. R_f =(Petroleum ether EtOAc, 4/6) 0.28. ^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ =11.53 (s, 1H), 8.78 (d, J =1.6 Hz, 1H), 8.08 (d, J =9.0 Hz, 2H), 7.70 (s, 1H), 7.65 (d, J =8.9 Hz, 2H), 7.11 (d, J =9.0 Hz, 2H), 6.87–6.85 (m, 3H), 4.32 (s, 2H), 3.77–3.74 (m, 8H), 3.27–3.25 (m, 4H), 3.21–3.19 (m, 4H). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$): δ =166.4, 161.7, 160.2, 157.9, 157.6, 153.4, 152.5, 152.2, 129.6, 128.8, 126.5, 125.3, 116.5, 114.3, 114.2, 114.0, 99.9, 99.0, 66.0, 65.9, 47.4,

47.2, 34.5. Q-TOF MS E^+ : m/z 583.2 $[\text{M}+\text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{31}\text{H}_{31}\text{N}_6\text{O}_4\text{S}$: 583.2122 $[\text{M}+\text{H}]^+$, found 583.2110.

2-((3-Cyano-4,6-bis(4-morpholinophenyl)pyridin-2-yl)thio)-N-(thiazol-2-yl)acetamide (2c): The title compound was obtained as a yellow powder (58 mg, 44%) from compound **5b** (100 mg, 0.22 mmol) and **6c** (138 mg, 0.79 mmol) in 1 h using general procedure E. R_f =(Petroleum ether/EtOAc, 4/6) 0.34. ^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ =8.02 (d, J =8.9 Hz, 2H), 7.69 (s, 1H), 7.65 (d, J =8.8 Hz, 2H), 7.54 (d, J =3.5 Hz, 1H), 7.22 (d, J =3.5 Hz, 1H), 7.11 (d, J =8.8 Hz, 2H), 6.77 (d, J =9.0 Hz, 2H), 4.37 (s, 2H), 3.77–3.73 (m, 8H), 3.27–3.25 (m, 4H), 3.18–3.16 (m, 4H). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$): δ =166.3, 161.6, 158.1, 157.9, 153.4, 152.4, 152.2, 137.7, 129.6, 128.8, 126.4, 125.3, 116.5, 114.3, 114.2, 113.9, 113.7, 99.9, 65.9, 65.8, 47.4, 47.2, 33.9. Q-TOF MS E^+ : m/z 599.2 $[\text{M}+\text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{31}\text{H}_{31}\text{N}_6\text{O}_3\text{S}_2$: 599.1894 $[\text{M}+\text{H}]^+$, found 599.1880.

Methyl 2-((3-cyano-4,6-bis(4-morpholinophenyl)pyridin-2-yl)thio)acetate (2d): The title compound was obtained as a yellow powder (22 mg, 54%) from compound **5b** (34.7 mg, 0.07 mmol) and **6d** (26.7 mg, 0.25 mmol) in 1 h using general procedure E. R_f =(Petroleum ether/EtOAc, 4/6) 0.42. ^1H NMR (400 MHz, CDCl_3): δ 8.01 (d, J =8.8 Hz, 2H), 7.58 (d, J =8.7 Hz, 2H), 7.45 (s, 1H), 6.98 (m, 4H), 4.09 (s, 2H), 3.89–3.87 (m, 8H), 3.75 (s, 3H), 3.30–3.26 (m, 8H). Q-TOF MS E^+ : m/z 531.2 $[\text{M}+\text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{29}\text{H}_{31}\text{N}_4\text{O}_4\text{S}$: 531.2061 $[\text{M}+\text{H}]^+$, found 531.2046.

General Procedure F: Synthesis of compounds 8a–d and 9a–d

To a solution of pyridine derivative in DMF (14 mL/mmol) was added K_2CO_3 (6 eq.) and the mixture was stirred at room temperature under an argon atmosphere until TLC showed completion. Water (20 mL) was added and the aqueous layer extracted (3 \times 20 mL). The combined organic layers were dried over MgSO_4 , filtered and concentrated to dryness to provide the desired compound.

Ethyl 4-(3-amino-4,6-bis(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxamido)benzoate (8a): The title compound was obtained as a yellow powder (30.6 mg, 100%) from compound **1a** (30.6 mg, 0.55 mmol) in 16 h using general procedure F. R_f =(Petroleum ether/EtOAc, 7/3) 0.55. ^1H NMR (600 MHz, CDCl_3): δ =8.08 (d, J =8.8 Hz, 2H), 8.04 (d, J =8.7 Hz, 2H), 7.66 (d, J =8.7 Hz, 2H), 7.49 (s, 1H), 7.44 (d, J =8.6 Hz, 2H), 7.29 (s, 1H), 7.07 (d, J =8.7 Hz, 2H), 7.01 (d, J =8.9 Hz, 2H), 6.09 (s, 2H), 4.37 (q, J =7.1 Hz, 2H), 3.91 (s, 3H), 3.88 (s, 3H), 1.40 (t, J =7.1 Hz, 3H). ^{13}C NMR (151 MHz, CDCl_3): δ =166.3, 164.1, 161.4, 160.6, 160.4, 157.3, 148.3, 147.8, 142.4, 130.9, 130.1, 130.1, 129.1, 129.0, 125.9, 121.2, 119.3, 118.6, 114.5, 114.5, 97.0, 61.0, 55.6, 14.5. Q-TOF MS E^+ : m/z 554.2 $[\text{M}+\text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{31}\text{H}_{28}\text{N}_3\text{O}_5\text{S}$: 554.1744 $[\text{M}+\text{H}]^+$, found 554.1733.

3-Amino-N-(isoxazol-3-yl)-4,6-bis(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxamide (8b): The title compound was obtained as a yellow powder (31.1 mg, 100%) from compound **1b** (31.1 mg, 0.06 mmol) in 16 h using general procedure F at 100 °C. R_f =(Petroleum ether/EtOAc, 6/4) 0.80. ^1H NMR (600 MHz, CDCl_3): δ =8.30 (d, J =1.6 Hz, 1H), 8.09 (d, J =8.8 Hz, 2H), 7.89 (s, 1H), 7.50 (s, 1H), 7.43 (d, J =8.6 Hz, 2H), 7.10 (d, J =1.6 Hz, 1H), 7.08 (d, J =8.6 Hz, 2H), 7.02 (d, J =8.8 Hz, 2H), 6.12 (s, 2H), 3.92 (s, 3H), 3.88 (s, 3H). ^{13}C NMR (151 MHz, CDCl_3): δ =163.2, 161.4, 160.8, 160.6, 158.9, 157.7, 149.1, 147.9, 130.8, 130.1, 129.1, 129.0, 120.8, 118.5, 114.6, 114.5, 99.5, 96.2, 55.6. ^{13}C NMR (151 MHz, CDCl_3): δ =163.2, 161.4, 160.8, 160.6, 158.9, 157.7, 149.1, 147.9, 130.8, 130.1, 129.1, 129.0, 120.8, 118.5, 114.6, 114.5, 99.5, 96.2, 55.6. Q-TOF MS E^+ : m/z 473.1 $[\text{M}+\text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{25}\text{H}_{21}\text{N}_4\text{O}_4\text{S}$: 473.1278 $[\text{M}+\text{H}]^+$, found 473.1280.

3-Amino-4,6-bis(4-methoxyphenyl)-N-(thiazol-2-yl)thieno[2,3-b]pyridine-2-carboxamide (8c): The title compound was obtained as a yellow powder (52.2 mg, 100%) from compound **1c** (52.2 mg, 0.1 mmol) in 16 h using general procedure F at 100 °C. R_f = (Petroleum ether/EtOAc, 3/7) 0.70. ^1H NMR (600 MHz, DMSO- d_6): δ = 8.17 (d, J = 8.9 Hz, 2H), 7.60 (s, 1H), 7.53 (d, J = 8.7 Hz, 2H), 7.31 (d, J = 3.9 Hz, 1H), 7.15 (d, J = 8.7 Hz, 2H), 7.05 (d, J = 9.0 Hz, 2H), 6.81 (d, J = 3.6 Hz, 1H), 3.87 (s, 3H), 3.83 (s, 3H). ^{13}C NMR (151 MHz, DMSO- d_6): δ = 168.2, 160.5, 160.5, 159.7, 154.1, 146.5, 130.4, 130.1, 129.2, 128.4, 121.4, 117.1, 114.2, 114.1, 109.9, 55.3. Q-TOF MS E^+ : m/z 489.1 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{25}\text{H}_{21}\text{N}_4\text{O}_3\text{S}_2$: 489.1050 $[\text{M} + \text{H}]^+$, found 489.1050.

Methyl 3-amino-4,6-bis(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxylate (8d): The title compound was obtained as a yellow powder (28.7 mg, 100%) from compound **1d** (28.7 mg, 0.07 mmol) and LiOH (2 mg, 1.2 eq.) in 16 h using general procedure F. R_f = (Petroleum ether/EtOAc, 7/3) 0.60. ^1H NMR (600 MHz, CDCl_3): δ = 8.08 (d, J = 8.9 Hz, 2H), 7.45 (s, 1H), 7.42 (d, J = 8.6 Hz, 2H), 7.07 (d, J = 8.6 Hz, 2H), 7.00 (d, J = 8.9 Hz, 2H), 5.76 (s, 2H), 3.91 (s, 3H), 3.88 (s, 3H), 3.88 (s, 3H). ^{13}C NMR (151 MHz, CDCl_3): δ = 176.5, 166.2, 162.5, 161.3, 160.5, 157.2, 148.2, 147.6, 131.1, 130.1, 129.4, 129.0, 120.4, 117.9, 114.5, 114.4, 55.6, 55.5, 51.7. Q-TOF MS E^+ : m/z 421.1 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{23}\text{H}_{21}\text{N}_2\text{O}_4\text{S}$: 421.1230 $[\text{M} + \text{H}]^+$, found 421.1223.

Ethyl 4-(3-amino-4,6-bis(4-morpholinophenyl)thieno[2,3-b]pyridine-2-carboxamido)benzoate (9a): The title compound was obtained as a yellow powder (25.2 mg, 100%) from compound **2a** (25.2 mg, 0.04 mmol) in 16 h using general procedure F. R_f = (Petroleum ether/EtOAc, 7/3) 0.60. ^1H NMR (400 MHz, CDCl_3): δ = 8.07–8.02 (m, 4H), 7.66 (d, J = 8.7 Hz, 2H), 7.48 (s, 1H), 7.42 (d, J = 8.6 Hz, 2H), 7.29 (s, 1H), 7.05 (d, J = 8.7 Hz, 2H), 6.99 (d, J = 8.9 Hz, 2H), 6.15 (s, 1H), 4.37 (q, J = 7.1 Hz, 2H), 3.93–3.87 (m, 8H), 3.30–3.02 (m, 8H), 1.40 (t, J = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, CDCl_3): δ = 166.4, 164.1, 160.5, 157.4, 152.5, 151.9, 148.5, 147.9, 142.4, 130.9, 129.8, 129.3, 128.6, 127.7, 125.8, 120.9, 119.3, 118.80, 118.3, 115.3, 115.2, 96.6, 66.9, 66.9, 61.0, 48.7, 48.6, 14.5. Q-TOF MS E^+ : m/z 664.3 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{37}\text{H}_{38}\text{N}_5\text{O}_5\text{S}$: 664.2588 $[\text{M} + \text{H}]^+$, found 664.2578.

3-Amino-N-(isoxazol-3-yl)-4,6-bis(4-morpholinophenyl)thieno[2,3-b]pyridine-2-carboxamide (9b): The title compound was obtained as a yellow powder (31 mg, 100%) from compound **2b** (31 mg, 0.05 mmol) in 16 h using general procedure F at 50 °C. R_f = (Petroleum ether/EtOAc, 4/6) 0.60. ^1H NMR (600 MHz, DMSO- d_6): δ = 10.65 (s, 1H), 8.79 (d, J = 1.7 Hz, 1H), 8.11 (d, J = 9.0 Hz, 2H), 7.62 (s, 1H), 7.45 (d, J = 8.8 Hz, 2H), 7.14 (d, J = 8.8 Hz, 2H), 7.04 (d, J = 9.1 Hz, 2H), 6.94 (d, J = 1.7 Hz, 1H), 6.28 (s, 2H), 3.79–3.75 (m, 8H), 3.25–3.23 (m, 8H). ^{13}C NMR (151 MHz, DMSO- d_6): δ = 163.6, 160.8, 159.6, 158.0, 156.4, 152.1, 151.4, 148.6, 147.8, 129.5, 128.1, 127.4, 126.4, 119.5, 117.3, 114.6, 114.4, 100.2, 95.4, 66.0, 66.0, 47.7, 47.5. Q-TOF MS E^+ : m/z 583.2 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{31}\text{H}_{31}\text{N}_6\text{O}_4\text{S}$: 583.2122 $[\text{M} + \text{H}]^+$, found 583.2112.

3-Amino-4,6-bis(4-morpholinophenyl)-N-(thiazol-2-yl)thieno[2,3-b]pyridine-2-carboxamide (9c): The title compound was obtained as a yellow powder (51 mg, 100%) from compound **2c** (51 mg, 0.08 mmol) in 16 h using general procedure F. R_f = (Petroleum ether/EtOAc, 4/6) 0.30. ^1H NMR (600 MHz, DMSO- d_6): δ = 8.10 (d, J = 9.0 Hz, 2H), 7.58 (s, 1H), 7.45 (d, J = 8.7 Hz, 2H), 7.42 (d, J = 3.9 Hz, 1H), 7.14 (d, J = 8.8 Hz, 2H), 7.04 (d, J = 9.0 Hz, 3H), 6.21 (br s, 2H), 3.79–3.75 (m, 8H), 3.25–3.22 (m, 8H). ^{13}C NMR (151 MHz, DMSO- d_6): δ = 161.1, 160.0, 155.6, 152.0, 151.3, 147.5, 129.6, 128.0, 127.71, 126.7, 117.0, 114.6, 114.4, 66.0, 66.0, 47.7, 47.5. Q-TOF MS E^+ : m/z 599.2 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{31}\text{H}_{31}\text{N}_6\text{O}_3\text{S}_2$: 599.1894 $[\text{M} + \text{H}]^+$, found 599.1880.

Methyl 3-amino-4,6-bis(4-morpholinophenyl)thieno[2,3-b]pyridine-2-carboxylate (9d): The title compound was obtained as a yellow powder (53.5 mg, 93%) from compound **2d** (57.6 mg, 0.11 mmol) in 16 h using general procedure F at 50 °C. R_f = (Petroleum ether/EtOAc, 4/6) 0.44. ^1H NMR (600 MHz, CDCl_3): δ = 8.05 (d, J = 8.9 Hz, 2H), 7.44 (s, 1H), 7.40 (d, J = 8.6 Hz, 2H), 7.04 (d, J = 8.7 Hz, 2H), 6.98 (d, J = 8.9 Hz, 2H), 5.82 (br s, 2H), 3.92–3.90 (m, 4H), 3.89–3.87 (m, 7H), 3.29–3.26 (m, 8H). ^{13}C NMR (151 MHz, CDCl_3): δ = 166.2, 162.6, 157.3, 152.4, 151.8, 148.4, 147.7, 129.8, 129.5, 128.6, 128.0, 120.2, 117.7, 115.3, 115.2, 66.9, 51.6, 48.7, 48.6. Q-TOF MS E^+ : m/z 531.2 $[\text{M} + \text{H}]^+$. HRMS Q-TOF MS E^+ : calculated for $\text{C}_{29}\text{H}_{31}\text{N}_4\text{O}_4\text{S}$: 531.2061 $[\text{M} + \text{H}]^+$, found 531.2046.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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