Targeting Acute Myelogenous Leukemia Using Potent Human Dihydroorotate Dehydrogenase Inhibitors Based on the 2-Hydroxypyrazolo[1,5- a]pyridine Scaffold: SAR of the Biphenyl Moiety

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Targeting Acute Myelogenous Leukemia using potent *human* dihydroorotate dehydrogenase inhibitors based on the 2-hydroxypyrazolo[1,5-*a*]pyridine scaffold: SAR of the biphenyl moiety

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Keywords

Bioisosterism, dihydroorotate dehydrogenase (DHODH) inhibitors, brequinar, BAY-2402234, hydroxylazoles, pyrazolo[1,5-*a*]pyridine, *acute myelogenous leukemia* (AML), myeloid differentiation, apoptosis.

Abstract

Since its recent connection with *acute myelogenous leukemia* (AML), human dihydroorotate dehydrogenase (*h*DHODH), a key enzyme in pyrimidine biosynthesis, has attracted significant interest from Pharma as a possible new AML therapeutic target. In 2018, we discovered

compound **1**, which can be seen to be representative of a novel class of *h*DHODH inhibitors that are based on an unusual carboxylic group bioisostere; 2-hydroxypyrazolo[1,5-a]pyridine. As a potent *h*DHODH inhibitor (IC_{50} 1.2 nM), **1** can induce myeloid differentiation in AML cell lines (THP1) in the low nM range (EC_{50} 32.8 nM), which is an improvement on *brequinar's* Phase I/II clinical trial (EC_{50} 265 nM, NCT03760666). Herein, we investigate the *drug-like* properties of **1** that shows good metabolic stability in rat hepatic liver microsomes and no toxic profile when administered at doses of 10 and 25 mg/Kg every 3 days for 5 weeks in Balb/c mice. Moreover, we report a deep SAR investigation of this class of compounds and provide analogues with better *drug-like* profiles; **17** is characterized by higher potency in inducing myeloid differentiation (EC_{50} 14.5 nM), in the same low nM range of BAY-2402234 (EC_{50} 3.3 nM) a Phase I/II clinical trial *lead*. As a strong proapoptotic agent (EC_{50} 18 nM), **17** is a relatively safe compound with low cytotoxicity toward non-AML cells (EC_{30} (Jurkat) >100 µM), which is superior to BAY-2402234.

INTRODUCTION

Human *dihydroorotate dehydrogenase* (*h*DHODH, EC 1.3.99.11) is a flavin-dependent enzyme that plays a fundamental role in *de-novo* pyrimidine biosynthesis. In humans, class 2 DHODH is anchored at the inner mitochondrial leaflet where it enzymatically catalyzes the oxidation of dihydroorotate to orotate by involving cofactor *flavin mononucleotide* (FMN). In order to regenerate FMN, a second redox reaction occurs with *coenzyme Q* (ubiquinone), which is recruited from the inner mitochondrial membrane and is a key player in the *mitochondrial electron transport chain* (ETC).[1]

*h*DHODH has been validated as a therapeutic target in diseases that involve wide cellular proliferation, such as autoimmune diseases and cancer.[2-4] Small molecules that can interfere with *h*DHODH enzymatic activity by targeting the host's pyrimidine synthesis may also show great potential in reducing viral replication against a broad spectrum of viruses.[5] *h*DHODH was initially included in the list of therapeutic options to be tested against SARS-CoV-2 infected cells.[6] It was then validated as a target for COVID-19,[7, 8] and became one of the most interesting therapeutic options for this disease.[9, 10]

It is quite recent the discovery,[11, 12] that *h*DHODH is also involved in regulating myeloid differentiation in AML; this has opened new scenarios for possible treatments of the disease. As the most common acute leukemia in adults, AML affects the myeloid lineage of white blood cells; if left untreated it is typically fatal within weeks or months, while current chemotherapies give an over-five-year survival rate of only around 25 %. By remaining blocked in an immature form, and so losing the ability to differentiate into adult white blood cells, the leukemic blast accumulates in the bone marrow and interferes with the production of normal blood cells. The mechanism that associates *h*DHODH inhibition with myeloid differentiation had not been fully understood.[2, 13] However, the effect seems to be strictly connected to the depletion of pyrimidine biosynthesis being rescued by the presence of excesses uridine,[14] which bypasses the requirement for *de-novo* pyrimidine synthesis by feeding the *salvage* pathway. This concept was explained well by Sykes *et al*.,[15] who were the first to suggest that AML cells, unlike non-leukemia cells, may be particularly sensitive to "*pyrimidine starvation*", and choose differentiation over self-renewal. This scenario realistically opens the possibility of expanding, to all AML types, the developments in M3 subclass *acute promyelocytic leukemia* (APL), whose clinical management was completely transformed by the introduction of a differentiation therapy that was based on *all-trans*-retinoic acid (ATRA), in association with a pro-apoptotic agent (chemotherapy or arsenic trioxide);[16-18] APL is currently curable in up to 90 % of cases.[19] Looking to the future, using the same powerful treatment strategy for non-APL AML, i.e. forcing differentiation and apoptosis using a *h*DHODH inhibitor, for example, may increase AML-patient survival rates.

Five major companies are currently running Phase I/II AML clinical trials with new generation *h*DHODH inhibitors and *brequinar* (Chart 1).[3]

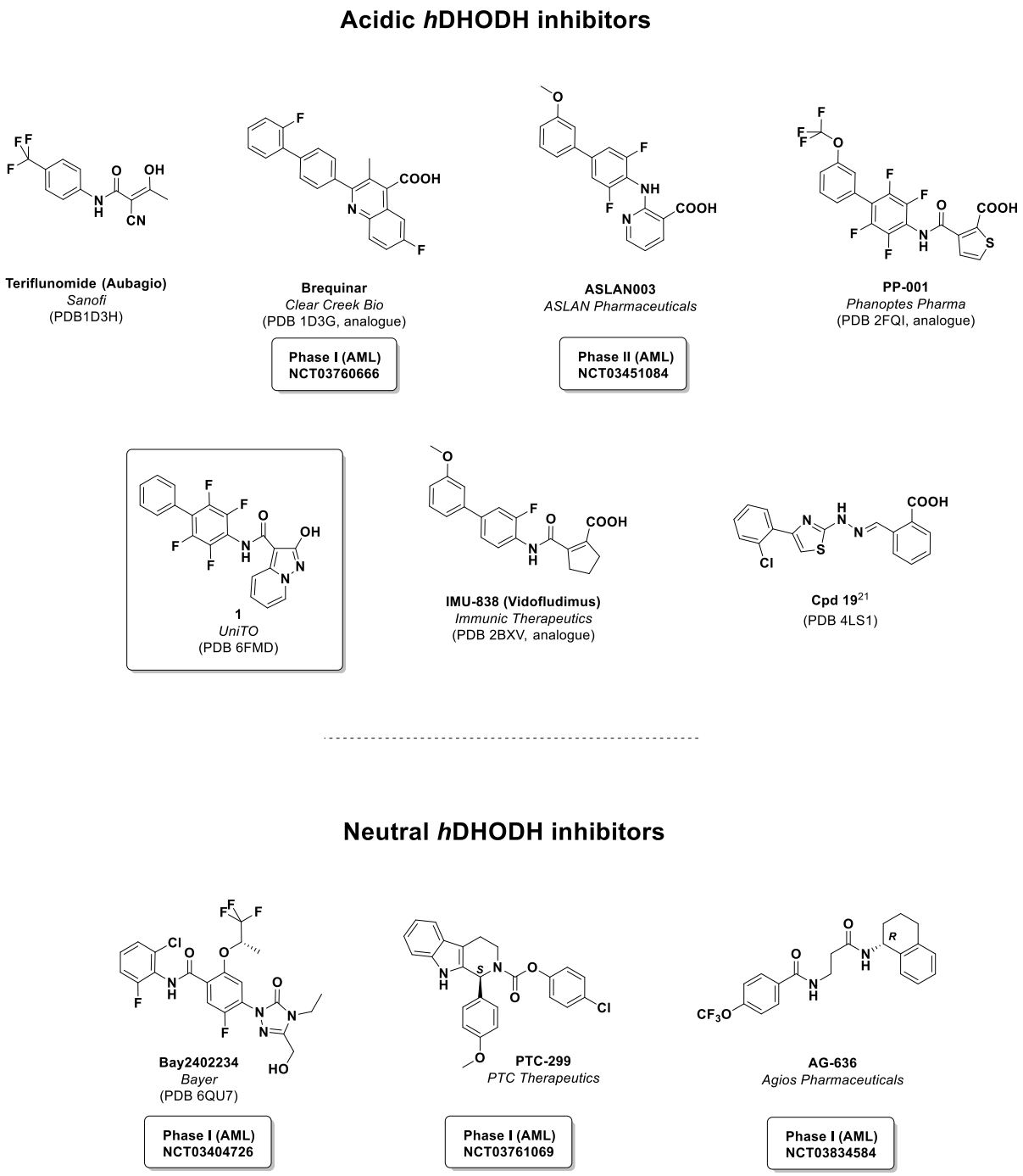


Chart 1. Present landscape of the most potent *h*DHODH inhibitors. Where available, the PDB ID of the inhibitor-*h*DHODH complex in the Protein Data Bank is indicated in brackets as well as the current AML related clinical trials.

Brequinar, designed by *Du Pont* in 1985,[20] is one of the most potent *h*DHODH inhibitors discovered to date. Despite showing clear *in-vitro* anticancer properties, it had never been

tested in AML until November 2018, when *Clear Creek Bio*, who acquired it the previous year from *Bristol Myers Squibb*, obtained an active IND for the study of *brequinar* in relapsed/refractory AML. *Brequinar* is currently in a Phase I/II clinical trial for the treatment of patients with relapsed/refractory AML (NCT03760666). A second acid DHODH inhibitor in a clinical trial, in addition to *brequinar*, is ASLAN003 (ASLAN Pharmaceuticals), which is currently being evaluated in a Phase IIa clinical trial in AML patients (NCT03451084).[21] Li, S. *et al*., have designed, in a *hit-to-lead* process, a series of benzylidenehydrazinyl-substituted thiazoles of which **"cpd 19"** was the most potent.[22] Although still in the preclinical stage, **cpd 19** is comparable to brequinar, at the enzymatic level, in presenting notable anti-arthritic efficacy and acceptable pharmacokinetic profiles *in vivo.* On the other hand, there is a small group of neutral inhibitors in trials: PTC-299 (PTC pharmaceuticals, Phase I since 29 Oct 2018, NCT03761069), AG 636 (Agios pharmaceuticals, Phase I since 18 Feb 2019, NCT03834584) and Bayer's BAY-2402234 (BY, in Phase I clinical trials since January 2018, NCT03404726). Recently, the pharmaco-chemical properties of this latter have been well detailed by Christian, S., *et al*.[23]

In 2018, the authors, developing a modulation of hydroxyazole scaffolds, [24, 25] discovered compound **1**, [14] (Chart 1) and took it as being representative of a novel class of *h*DHODH inhibitors that are based on an unusual carboxylic group bioisostere, 2-hydroxypyrazolo[1,5-a]pyridine, which is effectively able to mimic the interactions of brequinar carboxylate in the ubiquinone binding site.[14, 25] As it demonstrates comparable potency to *brequinar* itself on the enzymatic level (IC_{50} 1.2 nM vs 1.8 nM respectively), **1** was found to induce myeloid differentiation in AML cell lines (THP1) in the low nM range (EC_{50} 29.7 nM), which is around one log digit superior to the Phase I candidate *brequinar* (EC_{50} 270 nM).

In this work, we move forward from that discovery in two directions. On one hand, we have thoroughly explored the Structure-Activity Relationships **(**SAR) of this class of compounds and have attempted to provide analogues with better potency and *drug-like* profiles. On the

other, we continue the investigation into the drug-like properties of **1**, in particular, its *in-vitro* metabolism and *in-vivo* toxicity, in order to evaluate whether it may be a suitable candidate for future *in-vivo* testing. The data obtained during the study, which was supported by *in-silico* design based on the crystallographic poses of **1**, as well as by extensive biochemical and physicochemical characterization, has been compared with that of clinical trial *leads* (*brequinar* and BAY-2402234). Moreover, we have also compared the apoptotic, differentiating and cytotoxic properties of the synthesized compounds in AML cell lines.

RESULTS AND DISCUSSION

Target-compounds design. Since the ligands designed herein must be able to reach the inner mitochondrial leaflet, where *h*DHODH is located,[26] lipophilicity plays a central role in the translation of *h*DHODH enzymatic activity into a substantial effect on cells. While developing **1**, we observed how the LogP and LogD of compounds were correlated to the potency of their differentiation effect in AML cell lines. For instance, two acidic inhibitors with comparable IC_{50} values, such as **1** and brequinar, but with different LogDs **(**2.35 and 1.83 respectively) have different effects; **1** induced myeloid differentiation at a concentration that was 1-log lower than that of brequinar.

As higher lipophilicity is usually associated with reduced solubility and adverse ADME, the further modulation of **1** was performed to optimize the compound's lipophilicity in order to achieve the desired cellular effect, while also retaining high enzymatic inhibition activity. The cocrystal structure of *h*DHODH in complex with **1** (PDB code: 6FMD) provided insight into the ligand-binding mode and was used to support the *in-silico* studies. Similarly to brequinar, **1** effectively binds the so called "*lipophilic patch*",[26] which is the pathway followed by ubiquinone (coenzyme Q) to reach FMN. Acidic hydroxypyrazolo[1,5-a]pyridine forms an ion bridge with the side chain of Arg136 and a hydrogen bond interaction with Gln47. The pyridine

moiety extends toward *subsites 3* and *4*, fitting between Val134 and Val143. The last interaction with *h*DHODH occurs in the lipophilic channel defined as *subsite 1*, within the tetrafluorobiphenilic scaffold, with residues Met43, Leu42, Leu46, Ala59, Phe62, Phe98, Leu68, Leu359 and Pro364.

Figure 1 shows the three different portions of the cpd **1** structure (Rings A, B and C), which were the subject of this SAR study, as well as the designed analogues (compounds **2 - 17**). The SAR of the pyridine moiety (A ring), started in a previous study,[14] showed that position C7 best tolerated the substitution of the hydrogen. Compounds **4** and **5** were designed to complete the investigation of the A ring and the interaction with *subsite 4*. In the tetrafluorophenyl scaffold (B ring), the importance of fluorine atoms was analyzed and detailed as part of the development of compound **1**. The SAR of this substructure inside other hydroxyazole analogues highlighted that the complete saturation of the B ring with fluorine is fundamental to maintaining high binding affinity.[24, 25, 27] On the basis of the SAR-transfer concept,[28] we can assume that analogs with a biphenylic scaffold share similar SAR, since X-ray structures clearly show superimposable binding modes.[25] Thus, it is reasonable to suppose that fluorine has an important role to play in compound **1** and its derivatives, making the B ring difficult to modulate without losing activity.

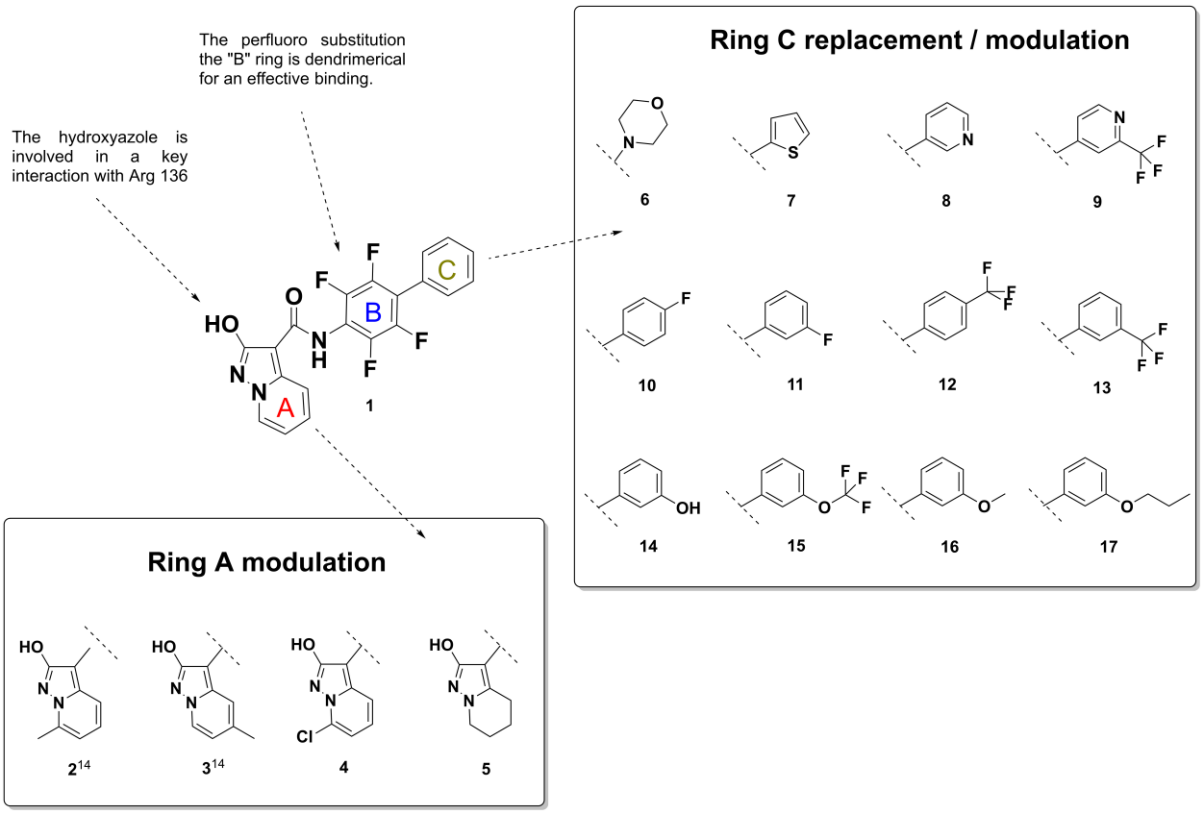


Figure 1. The compounds involved in the SAR exploration.

Moving now to the outer second ring (C ring), previous SAR studies carried out on **1** analogues have identified in this position possible opportunities for modulation.[24] Firstly, four compounds (**6 - 9**) were designed to investigate the possible phenyl bioisosteric replacement of the C ring. As guided by *in-silico* methodologies (Table S1: docking scores), we designed eight compounds (**10 – 17**) to investigate the effect of substituents in the *meta* and *para* positions of the phenyl ring; optimal replacement should not change the optimal *dihedral* angle between rings B and C, as explored in Bonomo *et al.* with *ortho* substituents.[27] The binding mode of **1** and its derivatives places the C ring next to the entrance of the ubiquinone binding pocket, where the phenyl is mainly involved in hydrophobic interactions with Phe62, Pro364, Leu68 and Tyr38 (Figure 2).

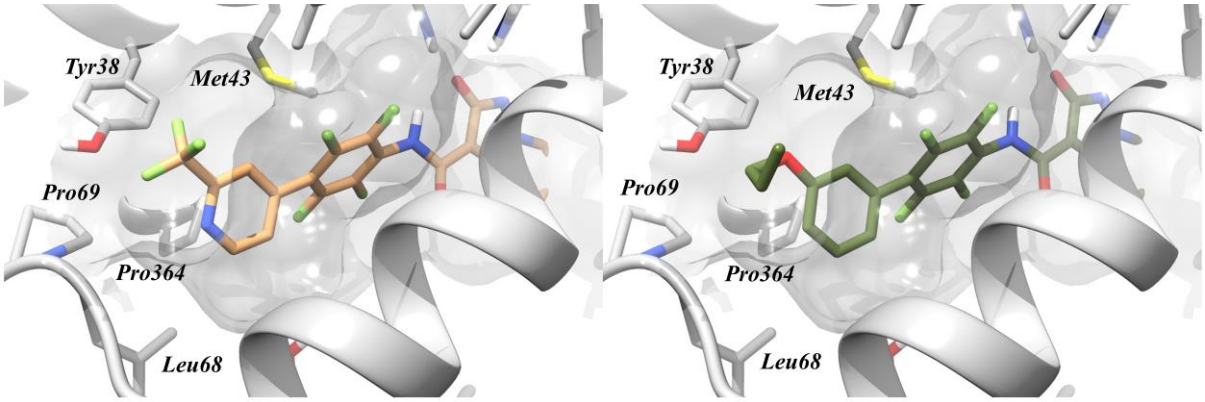


Figure 2. View of the entrance of the ubiquinone binding site, with the predicted binding mode of **9**, on the left, and **17** on the right.

Moreover, as shown in Figure 2, substituents in the *meta* position are located in an empty area on the border between the pocket and the vacuum. The solvent exposure of this modulation might lead to marginal effects on inhibitor activity. In this sense, the *meta* position may be quite strategic for the development of *h*DHODH inhibitors with increased lipophilicity that retain low nM enzymatic activity profiles.

***h*DHODH inhibitory activity and SAR.** We evaluated the recombinant *h*DHODH inhibition activity of compounds **4 - 17** using two clinical-trial candidates (brequinar and BAY-2402234) and **1** as comparisons. While BAY-2402234 was purchased from a commercial source, brequinar was synthesized following known procedures. In order to complete the scenario and prepare the discussion of the following cell-based studies, $LogD^{7.4}$, solubility in PBS and protein binding were also measured for each compound.

SAR analysis of the A ring. As reported in our previous publication,[14] the interaction between **1** and the small lipophilic pocket created by Val134 and Val143 (subsite 4) was explored using Molecular Dynamics (MD) free energy perturbation (FEP),[29] as a possible source of further modulation. Of the four sites on the A ring (positions 4 - 7), *in-silico* analyses suggested that position 7 is the most profitable for hydrogen substitution. Moreover, the study indicated that chlorine derivatives were generally preferred over methyl ones. Moving to experimental work, taking into account the MD/FEP results, a derivative with a chlorine substituent in position 7

(**4**, IC_{50} = 3.4 nM) was synthesized. Compared to the methyl analogue (**2**, IC_{50} = 4.3 nM), the chlorine is better tolerated, leading to an analogue of **1** with comparable activity, but higher $LogD^{7.4}$. We therefore also considered a reduced *4,5,6,7*-tetrahydropyrazolo[1,5-a]pyridine analogue, **5** (IC_{50} = 5.8 nM), which gave a slight decrease in potency compared to **1**.

Compound	*h*DHODH[a] IC_{50} ± SE (nM)	Log $D^{7.4}$ ± SDc	Solubility (µM) in PBS	Protein Binding (% bond)
Brequinar	1.8 ± 0.3	1.83 ±0.02	229	98.83
BAY-2402234	6.0 ± 0.6 (1.2 from lit[23])	2.7[23]	<1[30]	90.1[23]
ASLAN003	35[21]	nd	nd	> 99[21]
1	1.2 ± 0.2	2.35 ±0.02	12	99.10
2	4.3 ± 0.5	2.70 ± 0.02	< LOD	nd
3	35 ± 3	2.47 ± 0.09	< LOD	nd
4	3.4 ± 0.5	2.81 ±0.13	< LOD	nd
5	5.8 ± 0.4	2.36 ± 0.02	< LOD	nd

Table 1: Enzymatic inhibitor activity of compounds **2 - 5**, *brequinar*, Bay2402234, ASLAN003 and **1**, with relative $LogD^{7.4}$, solubility and protein binding. The effect of the compounds is expressed as IC_{50} values. Limit of Detection (LOD): value 6 µM. The "nd" notation indicates that the compound was not tested in that specific assay.

While the A ring modulations did not result in increased inhibitory activity compared to **1**, higher $LogDs^{7.4}$ were observed in all compounds, but were unfortunately all associated with reduced solubility. Solubility in PBS was not measured, as the concentration of the soluble fraction was below the LOD value (6 µM). In terms of protein binding, any significant improvement was observed.

SAR analysis of the C ring. ***phenyl replacement/modulation.*** Moving to the C ring, we assigned the first four compounds (**6 – 9**) to the investigation of its possible isosteric replacement (Table 2).

Compound	*h*DHODH[a] IC_{50} ± SE (nM)	Log $D^{7.4}$ ± SDc	Solubility (µM) in PBS	Protein Binding (% bond)
6	90.9 ± 13.1	0.66 ± 0.08	438	nd
7	1.35 ± 0.45	nd	nd	nd
8	6.23 ± 0.63	0.98 ± 0.03	47.3	99.58
9	150 ± 15	1.84 ± 0.06	20.2	nd
10	17.7 ± 3.30	*Insoluble*	<LOD	nd
11	2.03 ± 0.44	2.09 ± 0.04	<LOD	99.96
12	71.8 ± 9.42	*Insoluble*	<LOD	nd
13	6.34 ± 0.63	2.69 ± 0.03	<LOD	99.94
14	2.78 ± 0.32	1.82 ± 0.09	55.3	nd
15	2.30 ± 0.33	3.27 ± 0.19	8.1	100
16	2.75 ± 0.31	2.46 ± 0.04	74.3	99.95
17	5.09 ± 1.23	3.28 ± 0.12	12.9	100

Table 2: Enzymatic inhibitor activity of compounds **6 - 17** and relative $LogD^{7.4}$, solubility and protein binding. The effect of the compounds is expressed as IC_{50} values. Limit of Detection (LOD): value 6 µM. The "nd" notation indicates that the compound was not tested in that specific assay.

The incorporation of a morpholine substituent (**6**, IC_{50} = 90.9 nM) was not well tolerated, as a phenyl ring, and resulted in around a 50-fold potency decrease compared to **1**. The introduction of heteroatoms that may interact with the lipophilic sub-pocket, composed of Pro69 and Leu68, may induce repulsive interactions, as the potency decrease is also observed for pyridine derivate **9** (Figure 2). However, **6** was the most soluble of the series, showing almost twice the solubility of brequinar. The replacement of the C ring with classical bioisostere thiophen (**7**, IC_{50} = 1.35 nM) retained the inhibitory profile. The optimal $logD^{7.4}$

range for optimal drug absorption, via the phenomena of passive permeability or diffusion, is considered to be in the range between 1 and 3.[31] In the case of *h*DHODH inhibitors, the literature indicates an optimal $logD^{7.4}$ value superior to 2.50 reduce adsorption issue.[30] In terms of activity, the replacement of the phenyl position of **1** with a classic isostere nitrogen, as in **8** and **9**, resulted in losses of activity (IC_{50} = 6.23 nM and 150 nM), as *meta* replacement is better tolerated. To better understand this result, **9** must be compared with **13** (IC_{50} = 6.34 nM) in which the -CF_3 in the *meta* position is still present, but the nitrogen is ideally removed. The two pyridine analogues, **8** and **9** display better solubility than **1**, four- and 1.5-times respectively. In terms of protein binding, any significant improvement was observed.

Moving on, we investigated the positions on the C ring that are suitable for substitution in compounds **10 - 17**. The binding mode of **1** and derivatives places the C ring next to the entrance of the ubiquinone binding pocket (Figure 2), exposing the *meta* position to an empty area of the binding site, on the border between the pocket and the vacuum. With **10 - 13**, we investigated the effect of lipophilic substitutions, such as F and CF_3, in the *para* (**10**, **12**) and *meta* (**11**, **13**) positions of the C ring. Analyzing the results (Table 2), it can be observed how *meta* replacement, **11** and **13** with IC_{50} = 2.03 nM and IC_{50} = 6.34 nM respectively, were better tolerated than the *para* isomers, **10** and **12** with IC_{50} = 17.7 nM and IC_{50} = 71.8 nM. While finding the activity of **11** to be in the same range as **1** is not surprising, as the fluorine is a classical proton bioisosteric replacement, this cannot be said for **13**, for which small lipophilic groups, such as -CF_3, are well accepted. This replacement validated the predicted binding mode of **13**, in which a trifluoromethyl is placed in an empty area of the binding site. These modulations resulted in compounds being more lipophilic, as expected, but unfortunately, this property is associated with insolubility, and these values are largely below the reference limit of 6 μM. Focusing on substitution on the *meta* position, we obtained compound **14** (IC_{50} = 2.78 nM), which is comparable to **1** itself in terms of potency, but characterized by better solubility (around five times), as the oxygen atom is able to form hydrogen bonds with water, and has a

$logD^{7.4}$ comparable with that of brequinar itself. By ideally modulating **14**, we introduced substitution to the phenolic oxygen, giving **15 - 17**. This modulation resulted in an IC_{50} that is comparable to that of **1**, proving the predicted binding mode again, and was associated with an increase in $LogD^{7.4}$ for each compound. The most interesting compound is **17** (IC_{50} = 5.09 nM), which is characterized by the introduction of a propoxy group. This is the most interesting compound in the series here described, as it is comparable to the *lead* **1** in terms of potency although showing similar solubility but higher $LogD^{7.4}$ (above the 2.5 threshold).

Cell-based assays: differentiation, apoptosis and cytotoxicity. Several research groups have observed that *h*DHODH inhibitors can induce differentiation and apoptosis in multiple AML models.[11, 14, 23] An ideal *h*DHODH inhibitor should work at low concentrations in AML cells, but should be non-toxic against non-AML cells, or at least only be toxic at high concentrations. This would guarantee strong specificity against AML, minimizing systemic toxicity. In order to assess the biological activities of the new *h*DHODH inhibitors discussed above, we evaluated their ability to induce differentiation and apoptosis in the THP1-AML cell line, and their cytotoxic effects on non-AML cells. The differentiation process was tracked by analyzing CD14 expression, as this antigen is typically present in mature myeloid cells; the apoptotic rate was assessed with Annexin V, whose expression indicates the beginning of the apoptotic process. We performed a preliminary selection by treating THP1 cells with *h*DHODH inhibitors at 1.0 µM (Figure 3a), and the most promising molecules were then challenged in a new experiment at 0.1 µM (Figure 3b). The best compounds were finally characterized in detail, their EC_{50} for differentiation and apoptosis in THP1 were assessed, their toxicity profiles on non-AML cells were evaluated and their performance was compared to that of brequinar and BAY-2402234 (Table 4).

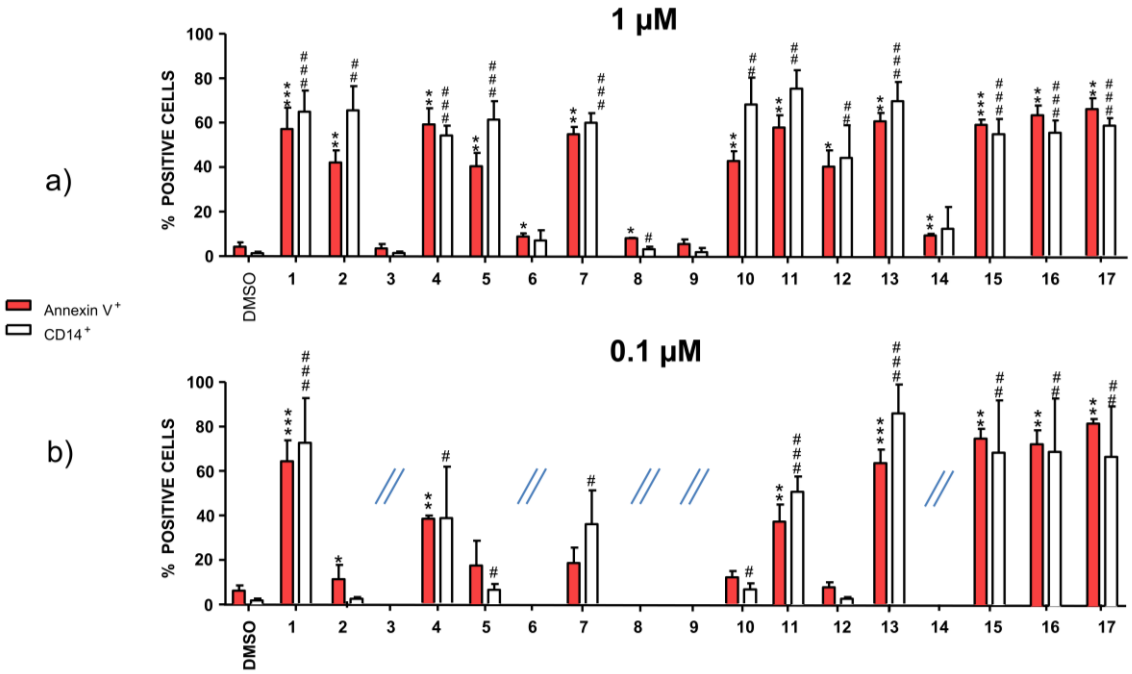


Figure 3: Differentiation (CD14 expression, white histogram) and apoptosis (Annexin V expression, red histogram) as induced by inhibitors **1** - **17** at 1 µM (Figure 3a) and 0.1 µM (Figure 3b) in THP1 cells. DMSO (dimethyl sulfoxide) acts as the negative control group as it was used to solubilize *h*DHODH inhibitors. *, **, *** represent the statistical significance for apoptosis (respectively p < 0,05, < 0,01 and <0,001); #, ## and ### represent the statistical significance for differentiation (respectively p < 0,05, < 0,01 and <0,001). The statistical significance is calculated by comparing the compounds to DMSO.

First of all, we can observe that apoptosis and differentiation are substantially associated, with no compound inducing just one phenomenon or the other. This suggests that they are both the consequence of the same mechanism, i.e. pyrimidine starvation. The performance of these new *h*DHODH inhibitors confirms our preliminary hypothesis. As observed earlier, *h*DHODH inhibitors with comparable IC_{50} at the enzymatic level can have different effects on cells, depending on their LogD. For example, **1** (IC_{50} 1.2 nM, $LogD^{7.4}$ 2.35) can induce differentiation at a concentration that is 1-log lower than possible with brequinar (IC_{50} 1.8 nM, $LogD^{7.4}$ 1.83). The close correlation between cell-based potency and $logD^{7.4}$ in *h*DHODH inhibitors has also been underlined by Gradl *et al.* In particular, they observed that, in a series

of BAY-2402234 analogues, *c*logD values lower than 2.5 led to compounds with low cellular activity, probably due to insufficient lipophilicity.[30]

Compounds **6**, **8**, **9** and **14** confirmed this phenomenon. In fact, despite potently inhibiting *h*DHODH at the enzymatic level, they were basically inactive in cells even at 1 µM. It is possible, in fact, that the low $LogD^{7.4}$ of these compounds prevented them from reaching the target deep inside the second mitochondrial leaflet. We have also previously observed that one-digit nM enzymatic inhibition IC_{50} values are usually needed to observe potent myeloid differentiation.[14] This could explain the inactivity of **3** at 1 µM (Figure 3a).

When challenged at 0.1 µM (Figure 3b), only five compounds (**11**, **13**, **15**, **16** and **17**) besides **1**, were still highly active. Interestingly, they were all characterized by *meta* substitution, confirming again how substituents in *meta* position are favored compared to the *para* analogs.

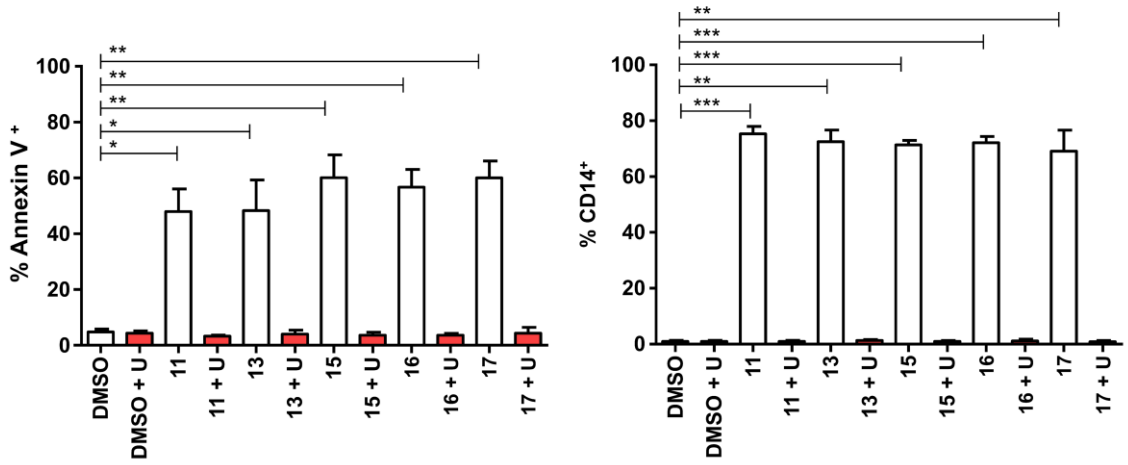


Figure 4: Differentiation (CD14 expression, left panel) and apoptosis (Annexin V expression, right panel) induced by inhibitors **11**, **13**, **15**, **16**, **17** at 1 µM, with and without uridine at 100 µM. *, **, *** represent the statistical significance for apoptosis (respectively $p < 0,05$, $< 0,01$ and $<0,001$). The statistical significance is calculated by comparing the compounds to DMSO.

In order to demonstrate that apoptosis and differentiation were indeed caused by pyrimidine depletion, rather than off-target effects, experiments with the best five compounds in the series were repeated in the presence of uridine. As already mentioned, uridine is a downstream

product of *h*DHODH, and is basically the antidote to *h*DHODH inhibitors. Accordingly, when differentiation and apoptosis experiments were performed in the presence of uridine, the complete rescue of the phenomena was observed (Figure 4).

Table 4 shows that, of the selected compounds, **13, 15 - 17** are at least as effective as the *lead* **1** in terms of myeloid differentiation and pro-apoptotic profile. In **11**, the introduction of the *m*-Fluorine was unable to significantly increase the $LogD^{7.4}$ and hence improve performances. The best results were obtained with **17**, which was characterized by a differentiation EC_{50} of 14.5 nM, which is basically in the same low nM range as Phase I BAY-2402234 (EC_{50} 2.4 nM), and 18-times more potent than Phase I/II brequinar. Similar behavior was observed for apoptosis. Importantly, the toxicity profiles of our compounds, and especially **17**, are extremely favorable, and superior to those of **1**, *brequinar* and BAY-2402234.

Compound	***h*DHODH**[a] **IC_{50} ± SE (nM)**	**Differentiation EC_{50} (µM) (C.L.95%)**	**Apoptosis EC_{50} (µM) (C.L.95%)**	**Cytoxicity (µM) (effect≥30% ±SD)**
Brequinar	1.8 ± 0.3	0.2651 (0.1033 - 0.6802)	0.2992 (0.1550 - 0.5778)	48 ± 1[14]
BAY-2402234	6.0 ± 0.6 (1.2 from lit[23])	0.0024 (0.0010 - 0.0054)	0.0033 (0.0017 - 0.0067)	36 ± 4
1	1.2 ± 0.2	0.0328 (0.0123 - 0.0876)	0.0695 (0.0340 - 0.142)	60 ± 1[14]
11	2.03 ± 0.44	0.0589 (0.0375 - 0.0925)	0.2222 (0.1422 - 0.3470)	39 ± 3
13	6.34 ± 0.63	0.0320 (0.0147 - 0.0700)	0.0429 (0.0225 - 0.0816)	>100 µM
15	2.30 ± 0.33	0.0280 (0.0118 - 0.0662)	0.0326 (0.0160 - 0.0666)	>100 µM
16	2.75 ± 0.31	0.0269 (0.0104 - 0.0698)	0.0296 (0.0077 - 0.1136)	68± 7
17	5.09 ± 1.23	0.0145 (0.0079 - 0.0268)	0.0180 (0.0102 - 0.0319)	>100 µM

Table 4: Analysis of the biological activity (enzymatic-inhibitor activity, differentiation, apoptosis and cytotoxicity) of compounds **1**, **11**, **13**, **15 - 17**, compared to *brequinar*,

BAY2402234 and **1**. The differentiation and apoptotic data are expressed as EC_{50}, and the cytotoxic effect was determined as the concentration that induced cytotoxicity in more than 30 % of the cells. The "nd" notation indicates that the EC_{50} and cytotoxicity of the compound were not determined.

***In-vitro* metabolic profile of 1.** Moving in parallel to the exploration of the SAR of this class of *h*DHODH inhibitors, we continued the investigation into the *drug-like* properties of **1**, the *lead* first-generation that displayed an optimal toxicity profile and was highly selective on-target,[14] in order to evaluate whether it may be a suitable candidate for future *in-vivo* testing. Here we characterize the major metabolic pathways responsible for the metabolism of compound **1** *in-vitro* using rat-liver microsomes, and therefore move the *in-vivo* evaluation forward. The *in-vitro* metabolic profile of **1** was assessed using the following combination of methods: (C) incubation at 37 °C with active rat-liver microsomes and a regenerating system that slowly generated coenzyme units over the incubation time, leading to a better reproduction of *in-vivo* behavior; (C1) incubation at 37 °C with heat-inactivated microsomes (via a 10-minute heating cycle at 90 °C) and a regenerating system;(C2) incubation at 37 °C with microsomes without a regenerating system and, finally, (B) incubation with the blank medium. SyGMa (*Systematic Generation of potential Metabolites*) software, a tool that lists predicted metabolites with associated empirical probability scores, was used to identify putative metabolites, which were then investigated by analyzing samples with liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS). For each series of samples (C, C1 and C2), incubation was stopped after 60 minutes (t 60) and after 120 minutes (t 120). The full-scan MS data acquired for all of the samples were analyzed to find the *m/z* values of the predicted molecular structures. In order to exclude interfering signals, the results obtained were compared to blank samples and common background peaks were not considered. In sample (C), we found peaks whose accurate mass data were in accordance with the mono-hydroxylated, di-hydroxylated and methoxylated metabolites of **1** (Table 5). As expected, we

did not identify the same metabolites in samples C1 and C2, confirming the fundamental role of CYP450 in phase I metabolism.

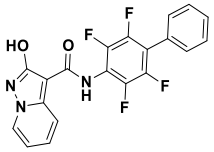
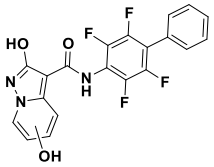
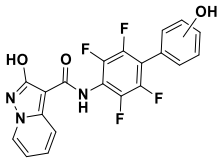
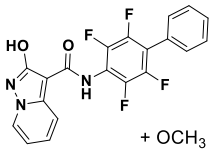
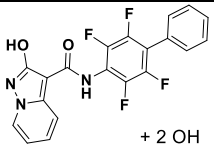
Compound metabolites	RT (min)	Calculated [M-H]- (m/z)	Identified [M-H]- (m/z) and mass error (± ppm)	Hypothesis	Relative Structure
Parent compound	9.49	400.0715	400.072 (± 1.25)	Parent compound	
M1	8.6	416.0663	416.0672 (± 2.16)	Hydroxy-derivate	
M2	7.44	416.0663	416.0672 (± 2.16)	Hydroxy-derivate	
M3	7.7	416.0663	416.0672 (± 2.16)	Hydroxy-derivate	
M4	8.51	430.082	430.0825 (± 1.16)	Methoxy-derivate	
M5	8.77	430.082	430.0825 (± 1.16)	Methoxy-derivate	
M6	8.07	432.0613	432.0619 (± 1.39)	Dihydroxy-derivate	
M7	8.6	432.0613	432.0619 (± 1.39)	Dihydroxy-derivate	

Table 5. List of metabolites of **1**, with chromatographic retention times, calculated accurate masses (m/z M-H-), identified accurate masses (m/z M-H-) in samples, chemical formulas and structures.

In order to confirm the presence and the chemical structures of the metabolites, a second set of experiments, based on the MS2 fragmentation of selected peaks (MS2-DIA analysis), was performed. In this way, we confirmed the structures of **1** and its metabolites that were found in sample C by interpreting the MS and MS2 spectra of each chromatographic run. Following the

criteria proposed by Schymanski *et al.*,[32] the metabolites identified are to be considered as "probable structures" (level 2b) or "tentative candidates" (level 3). Figure 5 reports extracted ion chromatograms for the putative metabolites. The different retention times of the hydroxy and methoxy derivatives of **1** indicate that there may have been modifications to different parts of the molecule.

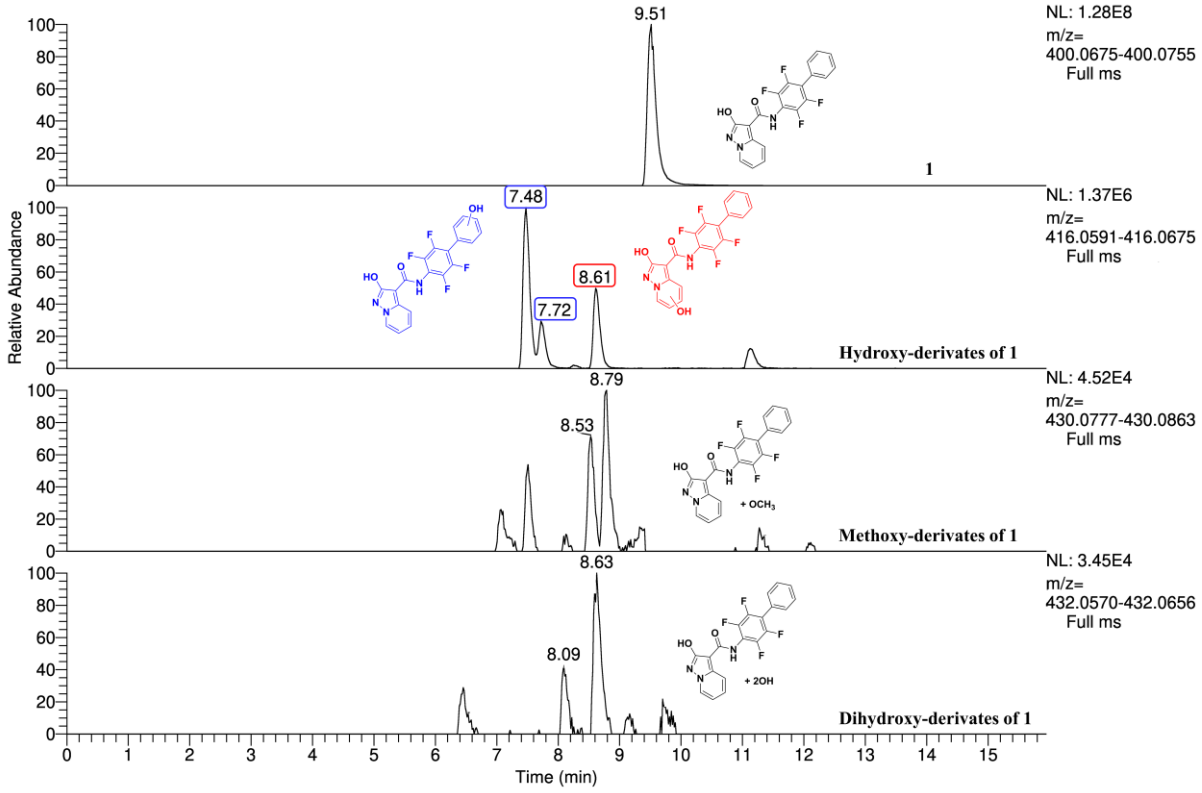


Figure 5. Extracted ion chromatograms of identified metabolites of **1** in sample C after incubation (time-point 2 hours).

The interpretation of the fragmentation spectra allowed the metabolites with hydroxyl substitution on the pyrimidine ring and on the phenyl ring to be distinguished (Figure S1). The high-resolution MS2 spectra revealed mutually exclusive ions and was thus capable of distinguishing metabolites that originated from the same precursor ion. For instance, for hydroxylated metabolites (precursor ion m/z 416.0672), we found the fragment ions at m/z 256.0391 when the -OH group was on the phenyl ring, but at m/z 240.0442 when the hydroxylation was on the pyrimidine ring. However, the MS2 spectra could not provide information on the exact ring position of hydroxylation. An examination of the results for

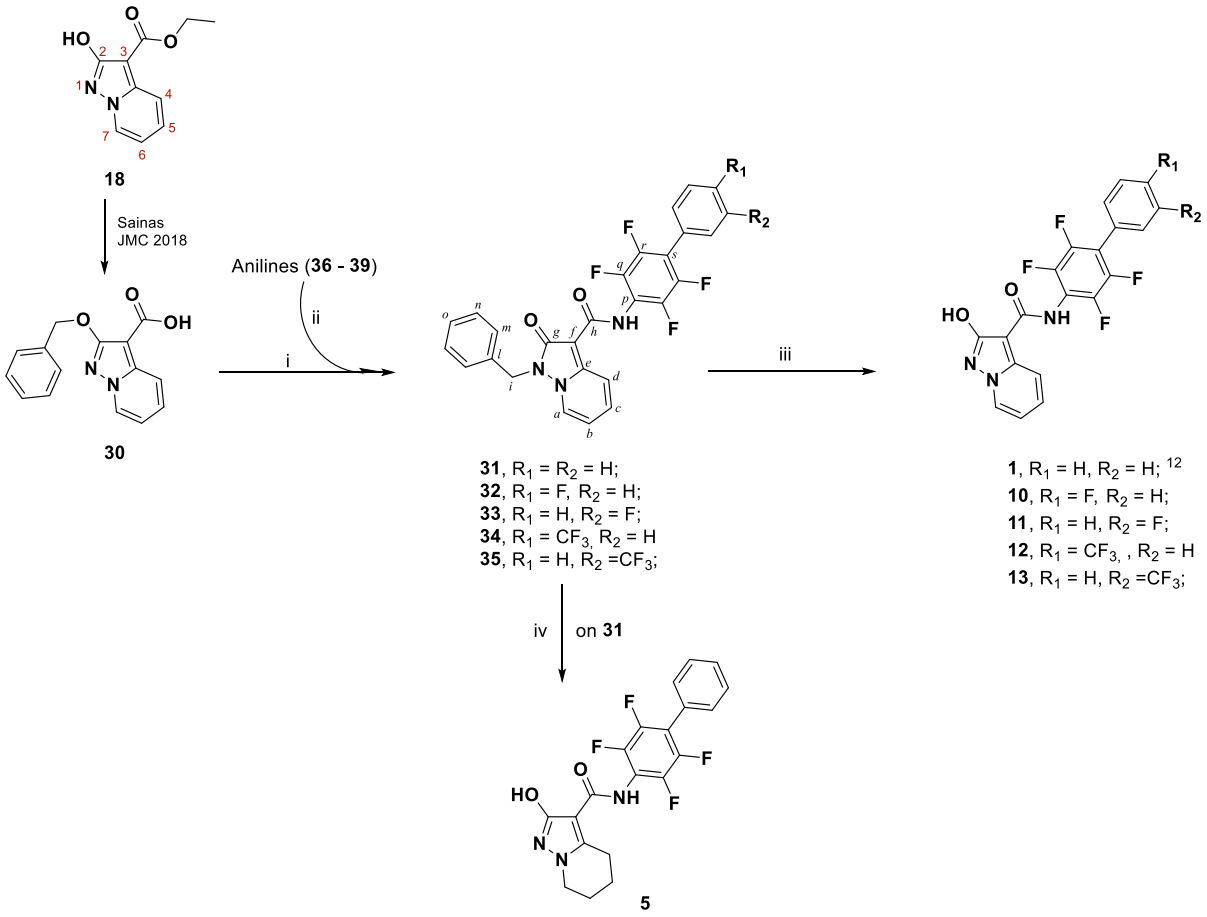
compound **1**, which had undergone P-450-mediated biotransformation for an incubation period of 2 h, highlighted that compound **1** is metabolically stable and 98% of compound **1** was recovered.

***In-vivo* toxicity profile of 1.** As the potency of compound **1** in AML cell lines is 10 times higher than brequinar, we decided to assess its potential toxic effects, and adopted a similar administration schedule as reported for brequinar in Sykes *et al*.;[11] Balb/c mice were treated with 10 and 25 mg/kg of the drug (every 3 days, via intraperitoneal injection i.p) for 35 days. First of all, none of the mice in the various treatment groups died during the trial and all the animals were alive at the end of the experiment. The mice were checked and weighed, before treatment. As described in **Figure S2**, no statistically significant loss of weight was observed over time at both compound **1** concentrations up to the end of the treatment. In addition, food uptake was normal, and no differences were observed in the two treatment groups, compared to controls (**Figure S3**).

Finally, to better test whether compound **1** induced pathological changes in hematological profile and in kidney and liver function, we pooled the blood samples of the mice at the end of the treatments and, in collaboration with the Veterinary Analysis laboratory (Turin, Italy), performed hematological profiling and the biochemical analysis of renal and hepatic function parameters. As indicated in **Table S2**, no differences in blood count, and the kidney and liver tests were observed after 35 days of treatment in the mice at both 10 and 25 mg/kg of compound **1**, compared to controls. Taken together these data demonstrate that compound **1** present a low *in vivo* toxicity profile and could be a good candidate for future *in vivo* efficacy experiment.

CHEMISTRY

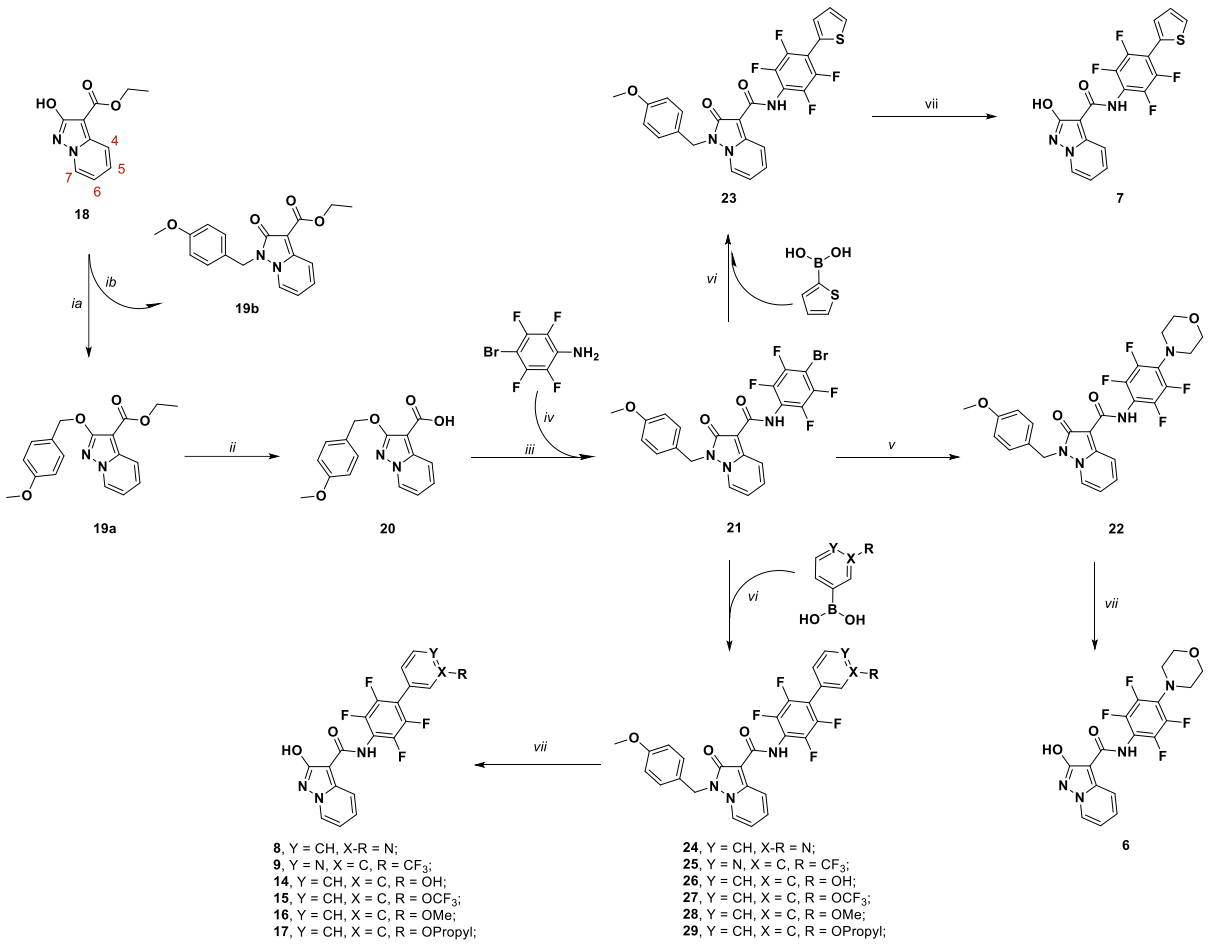
For the syntheses of target compounds **5** and **10** - **13**, a chemical strategy, which had already been tested to obtain *lead* compound **1**, was used (see Scheme 1).[14] The scheme starts from protected 2-hydroxypyrazolo[1,5-a]pyridine building block **30**, which is obtained from **18** in two steps.[14] From **30**, the corresponding acyl chloride was obtained and used directly without further purification. Due to their poor reactivity with acyl chloride, each aniline (**36** – **39**, a detailed description of the synthetic methodologies for the functionalized aniline used in this manuscript has been included in the SI) was converted into the more reactive dimethylaluminum amide, which were reacted with the above acyl chloride to give the desired amides **31** - **35** in a 31 - 40 % yield range. Note how, during the coupling step, the benzylic protecting group transferred from the exocyclic oxygen to the endocyclic N1 nitrogen in the pyrazolo[1,5-a]pyridine system. During the synthesis of **1**,[14] the removal of the benzyloxy moiety from **31** via hydrogenation was always impacted by the presence of a side reaction that lead to traces of reduced compound **5**. On this occasion, **5** was obtained in a 44 % yield by applying stronger catalytic hydrogenation conditions (40 bar) and using SynthWAVE apparatus. On the other hand, to avoid such side reactions, compounds **32** - **35** were converted to the desired target compounds **10** - **13** by applying room-pressure catalytic hydrogenation in the presence of 1.0 eq. of 37 % w/w HCl.



Scheme 1. Synthetic methodologies for the synthesis of targets **1**, **5**, **10**-**13**: *i*) oxalyl chloride, dry DMF, dry THF; *ii*) $AlMe_3$, dry toluene, reflux; *iii*) H_2, Pd/C, 37 % w/w HCl, ethanol; *iv*) H_2, Pd/C, dry THF, 40 bar, 65 °C, SynthWAVE.

For the synthesis of compounds **6** - **9** and **14** - **17**, we designed a more convenient synthetic approach that made use of the late-step Suzuki coupling of compound **21**, as a common intermediate. Once again, the scheme started from 2-hydroxypyrazolo[1,5-a]pyridine **18**, which was protected by a 4-methoxybenzylic group. We exchanged the benzylic protecting group with a 4-methoxybenzylic one as it can be easily removed in acidic conditions, which are also applicable to molecules containing sulphur atoms and pyridine rings, both known to poison metal catalysts during hydrogenation.[33] The reaction with 4-methoxybenzil bromide afforded a mixture of the regio-isomers **19a** and **19b** at a ratio of 61 and 27 %, respectively. The mixture was resolved by flash chromatography and the structure characterization of each isomer was attributed using the benzylic ^{13}C chemical shift (70.7 and 43.2 for $ArCH_2O$ and

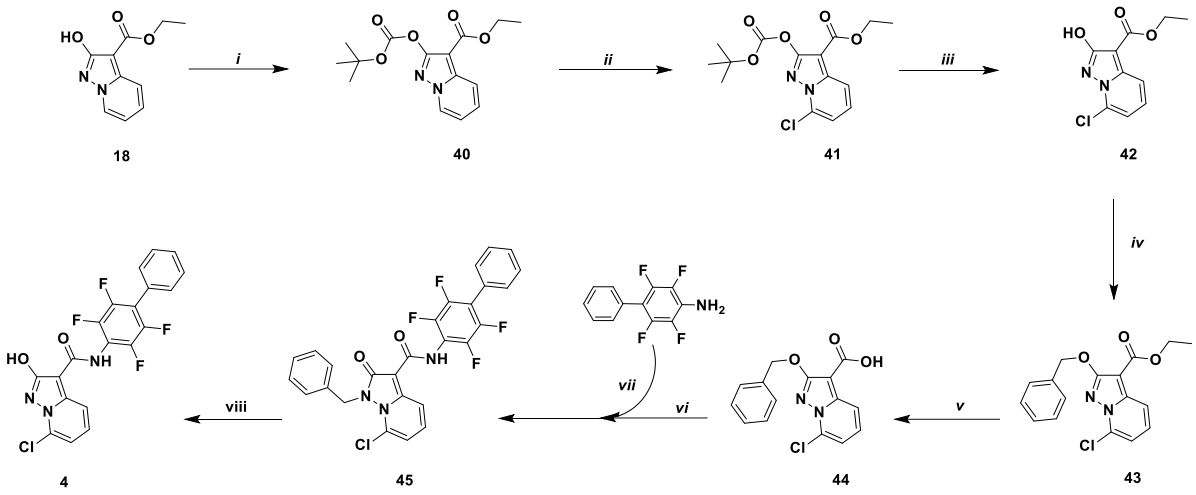
$ArCH_2N$ respectively) according to a previous 2D-NMR spectra analysis reported in Sainas *et al*. 2018[14] and ^{13}C chemical shift analysis of other oxygen vs nitrogen alkylated compounds.[34-41] Ester **19a** was then hydrolyzed under basic conditions to obtain the corresponding acid **20** (quantitative yield), which was then used for the preparation of the common intermediate **21**. Starting from acid **20**, the corresponding acyl chloride was obtained via treatment with oxalyl chloride and was used without any further purification in the reaction with the dimethylaluminum amide of 2,3,5,6-tetrafluoro-4-bromoaniline, giving the desired amide **21** in a 55 % yield. Once again, the benzylic protecting group transferred from the exocyclic oxygen to the endocyclic N1 nitrogen in the pyrazolo[1,5-a]pyridine system. Compound **21** was used as a common building block for desired compounds **22**, **23** - **29**. Firstly, *Buchwald-Hartwig* coupling conditions,[42] with morpholine, were used to obtain **22** (59 % yield), while a *Suzuki* reaction, involving the corresponding boronic acids, gave **23** - **29** (yield range: 70 – 94 %). Compounds **22** – **29** were then converted to the desired targets **6** - **9**, **14** - **17** via treatment with trifluoroacetic acid (TFA) in the presence of thioanisole, which was used as a scavenger of benzylic cation.



Scheme 2. Synthetic methodologies for the synthesis of targets **6 – 9**, **14 -17**: *i*) *a*) Cs_2CO_3, 4-MeOBnBr, dry DMF; *b*) flash chromatography *ii*) *a*) 5M NaOH, ethanol, 75 °C; *b*) HCl 2M, rt; *iii*) nitrogen atmosphere, oxalyl chloride, dry DMF, dry THF; *iv*) $AlMe_3$, dry toluene, reflux; *v*) nitrogen atmosphere, morpholine, Cs_2CO_3, $Pd(OAc)_2$, BINAP, dry toluene, sealed tube, 110°C; *vi*) *a*) $Pd(Ph_3)_4$, K_2CO_3, dioxane/water (9:1 v/v), 1 h r.t, *b*) corresponding boronic acid, reflux; *vii*) thioanisole, trifluoroacetic acid, 70 °C.

A dedicated synthetic scheme was applied for the synthesis of compound **4**; the hydroxyl group of **18** was O-protected with a Boc group to afford **40**.[35] Using lithium hexamethyldisilylazide on **40**, the pyrazolo[1,5-a]pyridine moiety was selectively deprotonated on the 7 position. Subsequently, quenching the lithium salt of **40** with hexachloroethane, which was used as an electrophile source of Cl^+, afforded compound **41** in a good yield.[43] In order to move the reaction scheme forward and prepare the subsequent coupling steps, the Boc group was ideally exchanged for a benzylic group that is more stable in acidic conditions. The Boc group was

quantitatively removed under mild acidic conditions (TFA), giving hydroxyazole **42**, which was reacted with benzyl bromide affording compound **43** (90 % over two steps). It is worth noting that, in this case, the endocyclic N1 benzylated isomer was obtained only in traces because of the presence of a chlorine in position 7. Ester **43** was then hydrolyzed under basic conditions to give the corresponding acid **44** (quantitative yield), which was then used for the preparation of amide **45**, under the conditions described above; 2,3,5,6-tetrafluoro-4-phenylaniline was activated with trimethylaluminum and reacted with the **44** acid chloride to give the desired amide **45** in a 38 % yield. Compound **45** was then converted to the desired target **4** via treatment with TFA in the presence of thioanisole, which was used as a scavenger of the benzylic cation.



Scheme 3. Synthetic methodologies for the synthesis of compound **4**: *i*) Cs_2CO_3, *tert*-butoxycarbonyl anhydride, dry THF, reflux; *ii*) *a*) nitrogen atmosphere, lithium hexamethyldisililazide (LiHMDS, 1.0 M, dry THF), -78°C, 1h, *b*) nitrogen atmosphere, hexachloroethane r.t.; *iii*); trifluoroacetic acid, dry dichloromethane, r.t.; *iv*) benzyl bromide, Cs_2CO_3, dry DMF, r.t.; *v*) *a)* 6 M NaOH, ethanol, 75 °C; *b)* 2M HCl, r.t.; *vi*) nitrogen atmosphere, oxalyl chloride, dry DMF, dry THF; *vii*) $AlMe_3$, dry toluene, reflux; *viii*) thioanisole, trifluoroacetic acid, 70°C.

CONCLUSIONS

In this work, we have investigated the *drug-like* properties and SAR of compound **1**, the *lead* of a novel class of *h*DHODH inhibitors that are based on an unusual carboxylic group bioisostere; 2-hydroxypyrazolo[1,5-a]pyridine. **1** showed good metabolic stability in rat hepatic liver microsomes after incubation for 2h, and showed a non-toxic *in-vivo* profile when administered at doses of 10 and 25 mg/Kg every 3 days for 5 weeks in Balb/c mice. These data demonstrate that compound **1** present a low *in vivo* toxicity profile and could be a good candidate for future *in vivo* efficacy experiment. Starting from **1** and investigating its SAR, we have identified the *meta* position of its C ring as the most favorable for substitution. Of the series produced, comparable enzymatic IC_{50} values resulted in dramatic differences in cellular activity. In particular, all modulations that were intended to improve solubility profiles and result in more polar compounds gave compounds with reduced cellular differentiation and lower apoptotic activity. On the other hand, all substituents with improved $LogD^{7.4}$ resulted in compounds with improved cellular potency. Among the new derivatives, the best result was obtained with **17**, which was able to induce myeloid differentiation with an EC_{50} 14.5 nM, in the same low nM range as Phase I BAY-2402234 (EC_{50} 3.3 nM). Despite being a strong proapoptotic agent (EC_{50} (THP1) 18 nM), **17** seems to be a safe compound as it is characterized by low cytotoxicity towards non-AML cells (EC_{30} (Jurkat) >100 µM), which indicates lower toxicity than **1** and brequinar itself. It is worth noting how the improvement of **17**, in terms of cell activity, was not associated with lower solubility, as the solubility of **17** and **1** are just comparable.

We can conclude that this class of *h*DHODH inhibitors contains candidates, such as **1** and **17**, that are characterized by strong antileukemic activity and an optimal toxicity profile, and whose performance is, at least, comparable to that of other competitors that are already in clinical trials.

EXPERIMENTAL SECTION

Chemistry

General methods. All chemical reagents were obtained from commercial sources (Sigma Aldrich, Alfa Aesar, FluoroChem), and used without further purification. Thin-layer chromatography (TLC) was carried out to monitor reaction progress. Analytical-grade solvents (acetonitrile, diisopropyl ether, diethyl ether, dichloromethane [DCM], dimethylformamide [DMF], ethanol 99.8 % v/v, ethyl acetate [EtOAc], hexane, methanol [MeOH], petroleum ether b.p. 40 - 60°C [*petroleum ether*], toluene), were used without further purification. When needed, solvents were dried over 4 Å molecular sieves. Tetrahydrofuran (THF) was distilled from Na and benzophenone under N_2 immediately prior to use. Thin layer chromatography (TLC) was carried out on silica gel on 5 x 20 cm plates at a 0.25 mm layer thickness. Anhydrous Na_2SO_4 was used as a drying agent for the organic phases. Compound purification was either achieved using flash column chromatography on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM), and the eluents indicated in the procedures for each compound, or using CombiFlash Rf 200 (Teledyne Isco) with 5–200 mL/min, 200 psi (with an automatic injection valve), and RediSep Rf Silica columns (Teledyne Isco), with the eluents indicated in the procedures for each compound. Compounds synthesized in our laboratory generally varied between 90 % and 99 % purity. Biological experiments were performed on compounds with a purity of at least 95 %. Purity was checked using two UHPLC analytical methods. HPLC analyses were performed on an UHPLC chromatographic system (Perkin Elmer, Flexar). The analytical columns were an UHPLC Acquity CSH Fluoro-Phenyl (2.1x100 mm, 1.7 µm particle size, Waters) and a reverse-phase (RP) C18 Phenomenex column (2.1x100 mm, 1.7 µm particle size). Compounds were dissolved in acetonitrile and injected through a 20 µl loop. The mobile phase consisted of acetonitrile / water with 0.1 % trifluoroacetic acid (ratio between 60 / 40 and 40 / 60, depending on the compound's retention factor). UHPLC retention times were obtained at flow

rates of 0.5 mL/min, and the column effluent was monitored at 230, 254 and 262 nm, referenced against a 360 nm wavelength. Melting points (m.p.), were measured on capillary apparatus (Büchi 540). Final m.p. determination was achieved by placing the sample at a temperature that was 10 °C below the m.p. and applying a heating rate of 1° C min^{-1}. All compounds were routinely checked by ^{1}H- and ^{13}C-NMR, and mass spectrometry. The IR spectra of solid compounds were recorded on an FT-IR (PerkinElmer SPECTRUM BXII, KBr dispersions), using the diffuse reflectance apparatus DRIFT ACCY. MS spectra were performed on a Waters Micromass ZQ equipped with an ESCi source for electrospray ionization mass spectra. ^{1}H- and ^{13}C-NMR spectra were performed on a JEOL ECZR600. The following abbreviations are used for coupling patterns: br = broad, s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet. Chemical shifts (δ) are given in parts per million (ppm). In this work, protons and carbons are labeled (*a, b, c, d, e, f, g, h, l, m, n*, *o, p, q, r and s*) according to Scheme 1. Values marked with an asterisk (*, ** and ***) are interchangeable. Detailed ^{13}C spectra of tetrafluorinated biphenyl compounds (final compounds **4** – **17** and protected final compounds) have not been entirely reported due to their especially complicated patterns (attributable to the multiple couplings between fluorine and carbon atoms). For these spectra, only the ^{13}C signals, caused by the heterocyclic substructure and non-aromatic carbons, are assigned. For the final compounds **4 - 17**, HRMS spectra were recorded on an LTQ-Orbitrap XL Plus (Thermo Scientific, Bremen, Germany) mass spectrometer, equipped with an atmospheric pressure interface and an ESI ion source instrument. Compounds **18**, **30** and **31** were prepared according to previously described procedures. [14]

Ethyl 2-((4-methoxybenzyl)oxy)pyrazolo[1,5-a]pyridine-3-carboxylate (**19a**) and *ethyl N-(4-methoxybenzyl)-2-oxo-pyrazolo[1,5-a]pyridine-3-carboxylate* (**19b**). 4-Methoxybenzyl bromide (645 mg, 3.20 mmol, 1.10 eq) was added dropwise to a mixture of **18** (600 mg, 2.91 mmol) and Cs_2CO_3 (2.295 g, 7.04 mmol, 2.4 eq) in dry DMF (15 mL). The reaction mixture was stirred overnight at room temperature, and water (100 mL) was then added. The mixture

was extracted using EtOAc (4 × 70 mL), the combined organic layer was dried under Na_2SO_4 and evaporated under reduced pressure to give a yellow oil. This oil showed two spots on the TLC (eluent: petroleum ether/EtOAc 60/40 v/v) that were ascribed to the two pyrazolo[1,5-a]-pyridine regioisomers. The mixture was separated using flash chromatography (eluent: *petroleum ether*/EtOAc 2/1 v/v, after elution of first isomer dichloromethane/MeOH 95/5 v/v).

19a) First isomer eluted. White solid after a first trituration with hexane, followed by filtration and a second trituration with water (111.3 – 112.5 °C). Yield 61 %. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 1.40 (*t*, J = 7.1 Hz, 3H, -OCH$_2$C*H*$_3$); 3.81 (*s*, 3H, -OC*H*$_3$), 4.36 (*q*, J = 7.1 Hz, 2H, -OC*H*$_2$CH$_3$), 5.43 (*s*, 2H, -OC*H*$_2$Ar), 6.83 (*t*, 1H, J = 6.7 Hz, *H-b*), 6.91 (*d*, 2H, J = 8.6 Hz, *H-n*), 7.35 (*t*, 1H, J = 7.7 Hz, *H-c*), 7.48 (*d*, 2H, J = 8.5 Hz, *H-m*), 8.00 (*d*, 1H, J = 8.8 Hz, *H-d*), 8.29 (*d*, 1H, J = 6.8 Hz, *H-a*), ^{13}C NMR (151 MHz, Chloroform-*d*) δ 14.7 (-OCH$_2$*C*H$_3$), 55.4 (-O*C*H$_3$), 59.7 (-O*C*H$_2$CH$_3$), 70.7 (-O*C*H$_2$Ar), 88.5 (*C-f*), 112.6 (*C-b*), 113.9 (*C-n*), 118.3 (*C-d*), 127.8 (*C-c*)*, 128.9 (*C-a*)*, 129.0 (*C-l*)*, 129.3(*C-m*), 142.9 (*C-e*), 159.5 (*C-o*), 163.4 (*C-g*)*, 165.2 (*C-h*)*. MS (ES+): 327 (M+1).

19b) Second isomer eluted. White solid. (158.3 – 159.2 °C, from diisopropyl ether). Yield 27 %. ^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 1.28 (*t*, 3H, J = 7.1 Hz, -OCH$_2$C*H*$_3$); 3.69 (*s*, 3H, -OC*H*$_3$), 4.21 (*q*, 2H, J = 7.1 Hz, -OC*H*$_2$CH$_3$), 5.35 (*s*, 2H, -NC*H*$_2$Ar), 6.88 (*d*, 2H, J = 8.5 Hz, *H-n*), 6.96 (*t*, 1H, J = 6.8 Hz, *H-b*), 7.19 (*d*, 2H, J = 8.4 Hz, *H-m*), 7.58 (*t*, 1H, J = 8.0 Hz, *H-c*), 7.91 (*d*, 1H, J = 8.8 Hz, *H-d*), 8.45 (*d*, 1H, J = 6.8 Hz, *H-a*); ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 14.6 (-OCH$_2$*C*H$_3$), 43.2 (-N*C*H$_2$Ar), 55.1 (-O*C*H$_3$), 58.5 (-O*C*H$_2$CH$_3$), 83.5 (*C-f*), 112.4 (*C-b*), 114.3 (*C-n*), 116.3 (*C-d*), 125.3 (*C-a*), 125.7 (*C-l*), 128.8 (*C-m*), 132.4 (*C-c*), 142.8 (*C-e*), 159.0 (*C-o*), 160.0 (*C-h*)*, 163.2 (*C-g*)*; MS (ES+): 327 (M+1).

2-((4-Methoxybenzyl)oxy)pyrazolo[1,5-a]pyridine-3-carboxylic acid (**20**). 6 M NaOH (5.0 eq) was added to a solution of compound **19a** (785 mg, 2.40 mmol) in EtOH (20 mL). The mixture was stirred for 4 h at 75 °C, and then neutralized with 6 M HCl, and was concentrated under reduced pressure. The mixture was cooled to 0 °C, then acidified with 2 M HCl until pH 2 was

reached, granting a suspension. This suspension was filtered to give **20** as a white solid (162.8 – 163.9 °C dec., from water). Yield 90 %. ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.76 (*s*, 3H, -OCH_3), 5.34 (*s*, 2H, -OCH_2Ar), 6.96 (*d*, 2H, J = 8.4 Hz, *H-n*), 7.02 (*t*, 1H, J = 6.7 Hz, *H-b*), 7.45 (*d*, 2H, J = 8.3 Hz, *H-m*), 7.51 (*t*, 1H, J = 7.9 Hz, *H-c*), 7.92 (*d*, 1H, J = 8.8 Hz, *H-d*), 8.66 (*d*, 1H, J = 6.7 Hz, *H-a*), 12.07 (*s*, 1H, -COO*H*); ^{13}C NMR (151 MHz, DMSO-d_6) δ 55.1 (-O*C*H$_3$), 70.1 (-O*C*H$_2$Ar), 87.6 (*C-f*), 113.1 (*C-b*), 113.8 (*C-n*), 117.3 (*C-d*), 128.4 (*C-a*)*, 128.5 (*C-l*)*, 129.5 (*C-c*)*, 129.9 (*C-m*), 142.3 (*C-e*), 159.2 (*C-o*), 163.5 (*C-h*)*, 164.4 (*C-g*)*; MS (ES+): 299 (M+1).

N-(4-Bromo-2,3,5,6-tetrafluorophenyl)-2-((4-methoxybenzyl)oxy)pyrazolo[1,5-a]pyridine-3-carboxamide (**21**). Oxalyl chloride (0.54 mL, 6.30 mmol, 3.0 eq) and dry DMF (1 drop) were added to a cooled (0 °C) solution of **20** (630 mg, 2.10 mmol) in dry THF (15 mL) kept under a nitrogen atmosphere. The resulting mixture was stirred for 2 h at room temperature. In parallel, a 2M solution of $AlMe_3$ in toluene (1.8 mL, 3.57 mmol, 1.7 eq) was added to a solution of 4-bromo-2,3,5,6-tetrafluoroaniline (769 mg, 3.15 mmol, 1.5 eq) in dry toluene (10 mL) under a nitrogen atmosphere. The resulting suspension was stirred 3 h at room temperature. The solution of acyl chloride was then concentrated under reduced pressure, and the residue was dissolved in dry THF (10 mL, this step was repeated three times to eliminate all gaseous residues). The acyl chloride was dissolved in dry toluene (15 mL) and the solution was added to the suspension described above. The reaction mixture was stirred at 85 °C overnight, then cooled to room temperature, quenched with methanol and evaporated. The residue was dissolved in EtOAc (80 mL), 0.5 M HCl (50 mL) was then added and the layers were separated. The aqueous phase was extracted twice with EtOAc and the combined organic layers were washed with brine, dried and evaporated under reduced pressure. The crude material was purified using flash chromatography (eluent: petroleum ether/EtOAc/DCM 2/1/1 v/v/v) to afford the title compound as a white solid (177.4 – 178.0 °C, triturated with diisopropyl ether). Yield 55 %. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 3.79 (*s*, 3H, -OCH_3), 5.41 (*s*, 2H, -NCH_2Ar),

6.77 (*t*, 1H, J = 6.9 Hz, *H-b*), 6.90 (*d*, 2H, J = 8.5 Hz, *H-n*), 7.21 (*d*, 2H, J = 8.5 Hz, H-*m*), 7.46 (*t*, 1H, J = 7.9, Hz, *H-c*), 7.75 (*d*, 1H, J = 6.9 Hz, *H-a*), 8.27 (*t*, 1H, J = 8.8 Hz, *H-d*), 9.98 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 45.2 (-N*C*H$_2$Ar), 55.5 (-O*C*H$_3$), 87.1 (*C-f*), 96.4 (*t*, J = 22.6 Hz, *C-s*)*, 112.9 (*C-b*), 115.0 (*C-n*), 117.0 (*t*, J = 14.8 Hz, *C-p*)*, 118.3 (*C-d)*, 123.1 (*C-a*), 124.2 (*C-l*), 128.6 (*C-m*), 131.8 (*C-c*), 142.5 (*C-e*), 142.8 (*dd*, J = 251.6, 14.9 Hz, (*C-r*)**, 145.2 (*dd*, J = 246.4, 14.2 Hz, (*C-q*)**, 160.0 (*C-o*)***, 161.4 (*C-h*)***, 162.2 (*C-g*)***; MS (ES+): 524 / 526 (M+1).

1-(4-Methoxybenzyl)-2-oxo-N-(2,3,5,6-tetrafluoro-4-morpholinophenyl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**22**). Cs_2CO_3 (782 mg, 2.4 mmol, 3.00 eq) was added to a solution of **21** (420 mg, 0.80 mmol, 1.00 eq) and morpholine (209 mg, 2.40 mmol, 3.00 eq) in toluene (30 mL). After degasification with nitrogen for 10 min, $Pd(OAc)_2$ (18 mg, 0.08 mmol, 0.10 eq) and BINAP (100 mg, 0.16 mmol, 0.20 eq) were added, and the mixture was degassed again for 5 min. The resulting suspension was heated at 110 °C in a sealed flask under a nitrogen atmosphere. After 3.5 h, the heating was stopped, the mixture concentrated to reduced pressure and water was added. The resulting suspension was extracted with EtOAc (3 x 50 mL). The combined organic fractions were collected, dried and concentrated under reduced pressure. The crude product was purified by flash chromatography (eluent: petroleum ether/ EtOAc/ DCM 1/1/1 v/v/v) giving a solid that was triturated with diisopropyl ether to give the title compound as a white solid (237.2 –237.5 °C dec). Yield: 59 %. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 3.24 – 3.28 (*m*, 4H, -NC*H*$_2$CH$_2$O-), 3.79 (*s*, 3H, -OC*H*$_3$), 3.81 – 3.85 (*m*, 4H, -NCH$_2$C*H*$_2$O-), 5.40 (*s*, 2H, -NC*H*$_2$Ar), 6.74 (*t*, 1H, J = 6.9 Hz, *H-b*), 6.90 (*d*, 2H, J = 8.5 Hz, *H-n*), 7.21 (*d*, 2H, J = 8.5 Hz, *H-m*), 7.44 (*t*, 1H, J = 7.9 Hz, *H-c*), 7.73 (*d*, 1H, J = 6.9, Hz, *H-a*), 8.28 (*d*, 1H, J = 8.9 Hz, *H-d*), 9.75 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 45.1 (-N*C*H$_2$Ar), 51.5 (-N*C*H$_2$CH$_2$O-), 55.5 (-O*C*H$_3$) , 67.5 (-NCH$_2$*C*H$_2$O-), 87.2 (*C-f*), 111.3 (*t*, *J* = 15.5 Hz, *C-p*)*, 112.6 (*C-b*), 114.9 (*C-n*), 118.3 (*C-d*), 123.1 (*C-a*), 124.3 (*C-l*), 127.8 (*t*, J = 11.0 Hz, *C-s*)*, 128.6 (*C-m*), 131.6 (*C-c*), 142.5 (*C-e*),

143.2 (*d*, J = 248.2 Hz, *C-q*)**, 143.5 (*d*, J = 247.9, Hz, *C-r*)**, 160.0 (*C-o*)***, 162.0 (*C-h*)***, 162.1 (*C-g*)***; MS (ES+): 553 (M+Na).

General Procedure: the Suzuki reaction used for the production of compounds **23 - 29**. $Pd(PPh_3)_4$ (90 mg, 0.08 mmol, 0.20 eq) was added to a solution of **21** (200 mg, 0.38 mmol, 1.00 eq) and K_2CO_3 (158 mg, 1.14 mmol, 3.00 eq) in dioxane/water mixture (9:1 v/v). After stirring the resulting mixture under a nitrogen atmosphere for 1 h at r.t., the corresponding boronic acid (0.760 mmol, 2.0 eq) was added. The reaction mixture was then heated at reflux under a nitrogen atmosphere. After 2 h, an additional amount of boronic acid (0.38 mmol, 1.0 eq) was added, the reaction mixture was heated at reflux for another 2h before it was cooled to room temperature and concentrated under reduced pressure. The crude material was taken-up with water (100 mL) and the mixture was extracted with EtOAc (3 x 60 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography (see the conditions below).

1-(4-Methoxybenzyl)-2-oxo-N-(2,3,5,6-tetrafluoro-4-(thiophen-2-yl)phenyl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**23**). The crude product was purified by flash chromatography (eluent: *petroleum ether*/ EtOAc 1/1 v/v) giving a solid that was recrystallized from acetonitrile (8 mL) to give the title compound as a white solid (197.4 –198.1 °C from acetonitrile). Yield: 72 %. ^{1}H NMR (600 MHz, Chloroform-d) δ 3.79 (*s*, 3H, -OC*H*$_3$), 5.41 (*s*, 2H, -NC*H*$_2$Ar), 6.76 (*t*, 1H, J = 6.6 Hz, *H-b*), 6.90 (*d*, 2H, J = 8.5 Hz, *H-n*), 7.16 – 7.20 (*m*, 1H, *aromatic proton*), 7.21 (*d*, 2H, J = 8.5 Hz, *H-m*), 7.45 (*t*, 1H, J = 7.7 Hz, *H-c*), 7.54 (*d*, 1H, J = 5.0 Hz, *aromatic proton*), 7.59 (*d*, 1H, J = 3.2 Hz, *aromatic proton*), 7.75 (*d*, 1H, J = 6.9 Hz, *H-a*), 8.28 (*d*, 1H, J = 8.8 Hz, *H-d*), 10.00 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-d) δ 45.2 (-NCH$_2$Ar), 55.5 (-OCH$_3$), 87.2 (*C-f*), 112.3 (*C-b*), 115.0 (*C-n*), 115.7 (*t*, J = 16.2 Hz, *C-p*)*, 118.4 (*C-d*), 123.1 (*C-a*), 124.3 (*C-l*), 127.3 (*thiophene carbon*), 127.9 (*thiophene carbon*), 128.1 (*t*, J = 3.3 Hz, *C-s*)*, 128.6 (*C-m*), 128.5 (*thiophene carbon*), 130.0 (*t*, J = 5.3 Hz, *thiophene carbon*), 131.7 (*C-c*), 142.6 (*C-e*), 142.9 (*dd*, J = 248.0, 15.8 Hz, *C-q*)**, 144.0 (*d*,

J = 247.0, Hz, *C-r*)**, 160.0 (*C-o*)***, 161.6 (*C-h*)***, 162.2 (*C-g*)***. MS (ES+): 528.2 (M+1).

1-(4-Methoxybenzyl)-2-oxo-N-(2,3,5,6-tetrafluoro-4-(pyridin-3-yl)phenyl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**24)**. The crude product was purified by flash chromatography (eluent: from petroleum ether/EtOAc 6/4 v/v to 3/7 v/v) giving a solid. This solid was then triturated with diisopropyl ether to give the title compound as a white solid (m.p. 202.6 – 203.8 °C from trituration with diisopropyl ether). Yield: 90 %. ^{1}H NMR (600 MHz, Chloroform-d) δ 3.78 (*s*, 3H, -OC*H*$_3$), 5.41 (*s*, 2H, -NC*H*$_2$Ar), 6.77 (*t*, 1H, J = 6.7 Hz, *H-b*), 6.89 (*d*, 2H, J = 8.5 Hz, *H-n*), 7.21 (*d*, 2H, J = 8.5 Hz, *H-m*), 7.43 – 7.49 (*m*, 2H, *aromatic protons and H-c*), 7.77 (*d*, 1H, J = 6.9 Hz, H-*a*), 7.84 (*d*, 1H, J = 7.8 Hz, *aromatic proton*), 8.27 (*d*, 1H, J = 8.8 Hz, *H-d*), 8.69 (*d*, 1H, J = 2.9 Hz, *aromatic proton*), 8.76 (s, 1H, *aromatic proton*), 10.06 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-d) δ 45.2 (-NCH_2Ar), 55.5 (-OCH_3), 87.1 (*C-f*), 112.9 (*C-b*), 114.0 (*t*, J = 16.9 Hz, *C-p*)*, 115.0 (*C-n*), 117.4 (*t*, J = 15.8 Hz, *C-s*)*, 118.3 (*C-d*), 123.1 (*C-a*), 123.7 (*pyridine carbon*), 124.2 (*C-l*), 124.3 (*pyridine carbon*), 128.6 (*C-m*), 131.8 (*C-c*), 137.9 (*pyridine carbon*), 142.6 (*C-e*), 142.9 (*d*, J = 252.7 Hz, *C-q*)**, 144.2 (*d*, J = 249.7 Hz, *C-r*)**, 149.9 (*pyridine carbon*), 150.5 (*pyridine carbon*), 160.0 (*C-o*)***, 161.6 (*C-h*)***, 162.2 (*C-g*)***; MS (ES+): 523 (M+1).

1-(4-Methoxybenzyl)-2-oxo-N-(2,3,5,6-tetrafluoro-4-(2-(trifluoromethyl)pyridin-4-yl)phenyl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**25**). The crude product was purified by flash chromatography (eluent: from *petroleum ether*/ EtOAc 6/4 v/v to 3/7 v/v) giving a solid. This solid was then triturated with diisopropyl ether, and the title compound was obtained as a pale-yellow solid (m.p 192.7 – 193.9 °C from trituration with diisopropyl ether). Yield: 70 %. ^{1}H NMR (600 MHz, Chloroform-d) δ 3.79 (*s*, 3H, -OC*H*$_3$), 5.42 (*s*, 2H, -NC*H*$_2$Ar), 6.79 (*t*, 1H, *J* = 6.7 Hz, *H-b*), 6.90 (*d*, 2H, J = 8.5 Hz, *H-n*), 7.22 (*d*, 2H, *J* = 8.5 Hz, *H-m*), 7.48 (*t*, 1H, J = 7.9 Hz, *H-c*), 7.64 (*d*, 1H, J = 4.9 Hz, *pyridine proton*), 7.78 (*d*, 1H, *J* = 6.9 Hz, *H-a*), 7.84 (*s*, 1H, *pyridine proton*), 8.29 (*d*, 1H, J = 8.8 Hz, *H-d*), 8.88 (d, 1H, J = 4.9 Hz, *pyridine proton*),

10.15 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-d) δ 45.3 (-N*C*H$_2$Ar), 55.5 (-O*C*H$_3$), 87.1 (*C-f*), 113.0 (*C-b*), 113.2 (*t*, J = 15.6 Hz, *C-p*)*, 115.0 (*C-n*), 118.4 (*C-d*), 118.9 (*t*, J = 14.7 Hz, *C-s*)*, 121.5 (*q*, J = 274.5 Hz, -CF$_3$), 121.7 (*q*, J = 2.1 Hz, *pyridine carbon*), 123.1 (*C-a*), 124.2 (*C-l*), 127.6 (*pyridine carbon*), 128.6 (*C-m*), 131.9 (*C-c*), 137.9 (*pyridine carbon*), 142.6 (*C-e*), 142.8 (*d*, J = 247.0 Hz, *C-q*)**, 144.2 (*d*, J = 245.4 Hz, *C-r*)**, 149.0 (*q*, J = 34.9 Hz, *pyridine carbon*), 150.6 (*pyridine carbon*), 160.1 (*C-o*)***, 161.4 (*C-h*)***, 162.3 (*C-g*)***; MS (ES+): 591.

1-(4-Methoxybenzyl)-2-oxo-N-(2,3,5,6-tetrafluoro-3'-hydroxy-[1,1'-biphenyl]-4-yl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**26**). The crude product was purified by flash chromatography (eluent: *petroleum ether*/ EtOAc 1/2 v/v) giving a solid. This solid was then triturated with diisopropyl ether in order to remove traces of O=PPh$_3$, giving the title compound as a white solid (236.9 –237.4 °C from diisopropyl ether). Yield: 79 %. ^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 3.71 (*s*, 3H, -OC*H*$_3$), 5.49 (*s*, 2H, -NC*H*$_2$Ar), 6.88 – 6.97 (*m*, 5H, *aromatic protons* and *H-n*), 7.07 (*t*, 1H, J = 7.1Hz, *H-b*), 7.28 (*d*, 2H, J = 8.3 Hz, *H-m*), 7.35 (*t*, 1H, J = 8.1 Hz, *aromatic proton*), 7.67 (*t*, 1H, J = 7.9 Hz, *H-c*), 8.03 (*d*, 1H, J = 8.8 Hz, *H-d*), 8.57 (*d*, 1H, J = 6.9 Hz, *H-a*), 9.78 (*br s*, 1H, -O*H*), 10.07 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 43.8 (-N*C*H$_2$Ar), 55.1 (-O*C*H$_3$), 85.5 (*C-f*), 113.2 (*C-b*), 114.4 (*C-n*), 116.2 (*C-d*), 116.3 (*aromatic carbon*), 116.6 (*t*, J = 14.6 Hz, *C-p*)*, 116.9 (*2 carbon, C-s partially overlapped with aromatic carbon*)*, 120.7 (*aromatic carbon*), 125.3 (*C-a*), 125.4 (*C-l*), 127.7 (*aromatic carbon*), 128.8 (*C-m*), 129.9 (*aromatic carbon*), 132.9 (*C-c*), 141.4 (*C-e*), 142.3 (*d*, J = 248.9 Hz, *C-q*)**, 143.3 (*d*, J = 251.3 Hz, *C-r*)**, 157.5 (*aromatic carbon*), 159.1 (*C-o*)***, 160.7 (*C-h*)***, 161.7 (*C-g*)***; MS (ES+): 538 (M+1).

1-(4-Methoxybenzyl)-2-oxo-N-(2,3,5,6-tetrafluoro-3'-(trifluoromethoxy)-[1,1'-biphenyl]-4-yl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**27**). The crude product was purified by flash chromatography (eluent: petroleum ether/ DCM 2 /1 v/v) giving a solid. This solid was then triturated with diisopropyl ether, and the title compound was obtained as a white solid

(162.7 –163.0 °C from diisopropyl ether). Yield: 82 %. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 3.80 (*s*, 3H, -OC*H*$_3$), 5.42 (*s*, 2H, -NC*H*$_2$Ar), 6.77 (*t*, 1H, J = 6.9 Hz, *H-b*), 6.91 (*d*, 2H, J = 8.5 Hz, *H-n*), 7.22 (*d*, 2H, J = 8.5 Hz, *H-m*), 7.32 (*d*, 1H, J = 8.1 Hz, *aromatic proton*), 7.37 (*s*, 1H, *aromatic proton*), 7.43 (*d*, 1H, J = 7.7 Hz, *aromatic proton*), 7.47 (*t*, 1H, J = 7.9 Hz, *H-c*), 7.53 (*t*, 1H, J = 8.0 Hz, *aromatic proton*), 7.76 (*d*, 1H, J = 6.9 Hz, *H-a*), 8.31 (*d*, 1H, J = 8.8 Hz, *H-d*), 10.03 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 45.2 (-N*C*H$_2$Ar), 55.4 (-O*C*H$_3$), 87.1 (*C-f*), 112.8 (*C-b*), 115.0 (*C-n*), 115.9 (*t*, *J* = 16.1 Hz, *C-p*)*, 117.0 (*t*, *J* = 15.6 Hz, *C-s*)*, 118.3 (*C-d*), 120.6 (*q*, *J* = 257.6 Hz, -O*C*F$_3$), 121.5 (*aromatic carbon*), 123.0 (*aromatic carbon*), 123.1 (*C-a*), 124.2 (*C-l*), 128.5 (*C-m*), 128.8 (*aromatic carbon*), 129.4 (*aromatic carbon*), 130.1 (*aromatic carbon*), 131.7 (*C-c*),, 142.5 (*C-e*), 142.8 (*d*, J = 247.5 Hz, *C-q*)**, 144.0 (*d*, J = 249.5 Hz, *C-r*)**, 149.3 (*aromatic carbon*), 160.0 (*C-o*)***, 161.6 (*C-h*)***, 162.2 (*C-g*)***; MS (ES+): 606.6, 628.6.

1-(4-Methoxybenzyl)-2-oxo-N-(2,3,5,6-tetrafluoro-3'-methoxy-[1,1'-biphenyl]-4-yl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**28**). The crude product was purified by flash chromatography (eluent: *petroleum ether*/EtOAc/dichloromethane 1.5/1/2 v/v/v) giving the title compound as a beige solid (172.9 –173.8 °C from diisopropyl ether). Yield: 94 %. ^{1}H-NMR (600 MHz, Chloroform-*d*): δ 3.79 (*s*, 3H, -OC*H*$_3$), 3.85 (*s*, 3H, -OC*H*$_3$), 5.42 (*s*, 2H, -NC*H*$_2$Ar), 6.76 (*t*, 1H, J = 6.9 Hz, *H-b*), 6.90 (*d*, 2H, J = 8.5 Hz, H-*n*), 6.97 – 7.03 (*m*, 2H, *aromatic protons*), 7.06 (*d*, 1H, J = 7.5 Hz, *aromatic proton*), 7.22 (*d*, 2H, J = 8.5 Hz, *H-m*), 7.41 (*t*, 1H, J = 7.9 Hz, *aromatic proton*), 7.45 (*t*, 1H, J = 7.9 Hz, *H-c*), 7.75 (*d*, 1H, J = 6.9 Hz, *H-a*), 8.29 (*d*, 1H, J = 8.8 Hz, *H-d*), 9.99 (*s*, 1H, -N*H*); ^{13}C-NMR (150 MHz, Chloroform-*d*): δ 45.2 (-N*C*H$_2$Ar), 55.5 (2 x -O*C*H$_3$), 87.2 (*C-f*), 112.3 (*C-b*), 114.9 (*aromatic carbon*), 115.0 (*C-n*), 115.8 (*aromatic carbon*), 116.3 (*t*, *J* = 15.0 Hz, *C-p*)*, 117.6 (*t*, *J* = 19.6 Hz, *C-s*)*, 118.4 (*C-d*), 122.7 (*aromatic carbon*), 123.1 (*C-a*), 124.3 (*C-l*), 128.6 (*C-m*), 128.8 (*aromatic carbon*), 129.7 (*aromatic carbon*), 131.7 (*C-c*), 142.6 (*C-e*), 142.9 (*d*, J = 248.6 Hz, *C-q*)**,,

144.2 (*d*, J = 244.2, Hz, *C-r*)**, 159.7 (*aromatic carbon*)***, 160.0 (*C-o*)***161.7 (*C-h*)****, 162.2 (*C-g*)****; MS (ES+): 552.5 (M+1).

1-(4-Methoxybenzyl)-2-oxo-N-(2,3,5,6-tetrafluoro-3'-propoxy-[1,1'-biphenyl]-4-yl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**29**). The crude product was purified by flash chromatography (eluent: petroleum ether/ EtOAc 2/3 v/v) to give the title compound as a beige solid (168.8 –169.9 °C from diisopropyl ether). Yield: 86 %. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 1.06 (*t*, 3H, J = 7.4 Hz, -OCH$_2$CH$_2$C*H*$_3$), 1.84 (*h*, 2H, J = 7.3 Hz, -OCH$_2$C*H*$_2$CH$_3$), 3.80 (*s*, 3H, -OC*H*$_3$), 3.96 (*t*, 2H, J = 6.6 Hz, -OC*H*$_2$CH$_2$CH$_3$), 5.42 (*s*, 2H, -NC*H*$_2$Ar), 6.76 (*t*, 1H, J = 7.0 Hz, *H-b*), 6.91 (*d*, 2H, J = 8.6 Hz, *H-n*), 6.99 (*dd*, 1H, J = 8.4, 2.2 Hz, *aromatic proton*), 7.00 (*s*, 1H, *aromatic proton*), 7.04 (*d*, 1H, J = 7.6 Hz, *aromatic proton*), 7.22 (*d*, 2H, J = 8.6 Hz, *H-m*), 7.39 (*t*, 1H, J = 7.9 Hz, *aromatic proton*), 7.46 (*t*, 1H, J = 7.9 Hz, *H-c*), 7.75 (*d*, 1H, J = 7.0 Hz, *H-a*), 8.31 (*d*, 1H, J = 8.8 Hz, *H-d*), 9.98(*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 10.6 (-OCH$_2$CH$_2$*C*H$_3$), 22.7 (-OCH$_2$*C*H$_2$CH$_3$), 45.2 (-N*C*H$_2$Ar), 55.5 (-O*C*H$_3$), 69.8 (-O*C*H$_2$CH$_2$CH$_3$), 87.2 (*C-f*), 112.8 (*C-b*), 115.0 (*C-n*), 115.5 (*aromatic carbon*), 116.2 (*t*, *J* = 15.6 Hz, *C-p*)*, 116.4 (*aromatic carbon*), 117.7 (*t*, J = 16.8 Hz, *C-s*)*, 118.3 (*C-d*), 122.5 (*aromatic carbon*), 123.1 (*C-a*), 124.3 (*C-l*), 128.6 (*C-m*), 128.7 (*aromatic carbon*), 129.7 (*aromatic carbon*), 131.7 (*C-c*), 142.5 (*C-e*), 142.8 (*dd*, *J* = 248.5, 15.3 Hz, *C-q*)**, 144.2 (*d*, *J* = 248.5 Hz, *C-r*)**, 159.2 (*aromatic carbon*)***, 160.0 (*C-o*)***161.7 (*C-h*)****, 162.2 (*C-g*)****; MS (ES+): 580 (M+1).

*General procedure for the synthesis of pyrazolo[1,5-a]pyridine related amides **32 – 35**.*

Oxalyl chloride (3.0 mmol) and dry DMF (1 drop) were added to a cooled (0 °C) solution of O-protected pyrazolo[1,5-a]pyridine acid (1.0 mmol) **30** in dry THF (20 mL), under a nitrogen atmosphere. The obtained solution was stirred at room temperature for 2 hours. The solution was then concentrated under reduced pressure and the residue dissolved in dry THF (10 mL, this step was repeated three times to eliminate all gaseous residues). The resulting acyl chloride was immediately used without any further purification and was dissolved in 10 mL of dry

toluene and transferred to the solution described below. Trimethylaluminium (2.0 M in hexane, 1.5 mmol), was added to a solution of the appropriate aniline (see supporting info for the synthesis, 1.5 mmol), in dry toluene (15 mL), under a nitrogen atmosphere. The resulting mixture was stirred for 2 hours at room temperature producing a brown suspension, then the solution of the previously described acyl chloride in dry toluene (30 mL) was quantitatively added. The mixture was heated overnight at 90 °C and then cooled to r.t. The reaction was quenched with 1M HCl. The layers were resolved, and the aqueous phase was exhaustively extracted using EtOAc. The combined organic layer was washed with 1M NaOH and brine, dried and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography.

1-Benzyl-2-oxo-N-(2,3,4',5,6-pentafluoro-[1,1'-biphenyl]-4-yl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**32**). *2,3,4',5,6-Pentafluoro-[1,1'-biphenyl]-4-aniline* was used. Flash chromatography (eluent: *petroleum ether* / EtOAc from 80:20 v/v to 50:50 v/v) was performed. Grey solid (m.p. 234.2 – 235.4 °C from trituration with diisopropyl ether). Yield 31%. ^{1}H NMR (600 MHz, Chloroform-*d*): δ 5.48 (*s*, 2H, -NC*H*$_2$Ph), 6.77 (*t*, 1H, J = 6.9 Hz, *H-b*), 7.19 (*t*, 2H, J = 8.6 Hz, *aromatic protons*), 7.28 (*d*, 2H, J = 7.4 Hz, *aromatic protons*), 7.32 -750 (*m*, 6H, *aromatic protons*), 7.73 (*d*, 1H, J = 6.9, *H-a*), 8.30 (*d*, 1H, J = 8.8, *H-d*), 9.98 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-*d*): δ 45.7 (-N*C*H$_2$Ph), 87.2 (*C-f*), 112.9 (*C-b*), 115.9 (*d*, J = 21.9 Hz, *aromatic carbon*), 116.4 (*t*, J = 17.1 Hz, *C-p*)*, 116.7 (*d*, J = 16.7 Hz, *C-s*)*, 118.4 (*C-d*), 123.0 (*C-a*), 123.5 (*aromatic carbon*), 127.1 (*aromatic carbon*), 129.0 (*aromatic carbon*), 129.7 (*aromatic carbon*), 131.8 (*C-c*), 132.8 (*d*, J = 8.4 Hz, *aromatic carbon*), 132.5 (*aromatic carbon*), 142.6 (*C-e*), 142.8 (*d*, J = 244.1 Hz, *C-q*)**, 144.2 (*d*, J = 250.7 Hz, *C-r*)**, 161.7 (*C-g*)***, 162.2 (*C-h*)***, 163.1 (*d*, J = 249.5 Hz, -*C*F); MS (ESI) 510 (M+1).

1-Benzyl-2-oxo-N-(2,3,3',5,6-pentafluoro-[1,1'-biphenyl]-4-yl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**33**). *2,3,3',5,6-Pentafluoro-[1,1'-biphenyl]-4-aniline* was used.

Flash chromatography (eluent: *petroleum ether* / EtOAc from 80:20 v/v to 50:50 v/v) was performed. Pale yellow solid (m.p. 195.4 – 196.3 °C from trituration with diisopropyl ether). Yield 40 %. ^{1}H NMR (600 MHz, DMSO-d_6): δ 5.57 (*s*, 2H, -NC*H*$_2$Ph), 7.07 (*t*, 1H, J = 6.9 Hz, *H-b*), 7.29 – 7.45 (*m*, 7H, *aromatic protons*), 7.50 (*d*, 1H, J = 9.5 Hz, *aromatic proton*), 7.62 (*dd*, 1H, J = 14.3, 7.7 Hz, *aromatic proton*), 7.68 (*t*, 1H, J = 7.9 Hz, *H-c*), 8.05 (*d*, 1H, J = 8.7 Hz, *H-d*), 8.53 (*d*, 1H, J = 7.0 Hz, *H-a*), 10.12 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, DMSO-d_6): δ 44.2 (-N*C*H$_2$Ph), 85.4 (*C-f*), 113.3 (*C-b*), 115.4 (*t*, J = 17.3 Hz, *C-p*)*, 116.2 (*C-d*), 116.4 (*d*, J = 20.8 Hz, *aromatic carbon*), 117.0 (*C-s*)*, 117.2 (*d*, J = 23.1 Hz, *aromatic carbon*), 125.2 (*C-a*), 126.5 (*C-l*), 127.2 (*aromatic carbon*), 128.2 (*C-c*), 128.8 (*d*, J = 9.8 Hz, *aromatic carbon*), 129.1 (*aromatic carbon*), 130.9 (*d*, J = 8.4 Hz, *aromatic carbon*), 132.9 *(aromatic carbon)*, 133.7 *(aromatic carbon)*, 141.4 (*C-e*), 142.2 (*d*, J = 242.5 Hz, *C-q*)**, 143.3 (*d*, J = 245.8 Hz, *C-r*)**, 144.1, 160.6 (*C-g*)***, 161.2 (*C-h*)***, 162.0 (*d*, J = 244.3 Hz, *aromatic carbon*); MS (ESI) 508 (M-1).

1-Benzyl-2-oxo-N-(2,3,5,6-tetrafluoro-4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**34**). 2,3,5,6-Tetrafluoro-4'-(trifluoromethyl)-[1,1'-biphenyl]-4-aniline was used: Flash chromatography eluent: from *petroleum ether*/EtOAc 80:20 v/v to 50:50 v/v. Pale yellow solid (m.p. 262.2 – 263.5 °C from trituration with diisopropyl ether). Yield 41 %. ^{1}H NMR (600 MHz, Chloroform-*d*): δ 5.49 (*s*, 2H, -NC*H*$_2$Ph), 6.78 (*t*, 1H, J = 6.8 Hz, *H-b*), 7.29 (*d*, 2H, J = 7.4 Hz, *aromatic protons*), 7.33 - 7.43 (*m*, 3H, *aromatic protons*), 7.48 (*t*, 1H, J = 7.9 Hz, *H-c*), 7.62 (*d*, 2H, J = 7.9 Hz, *aromatic protons*), 7.73 (*d*, 1H, J = 6.9 Hz, *H-a*), 7.76 (*d*, 2H, J = 8.1 Hz, *aromatic protons*), 8.31 (*d*, 1H, J = 8.8 Hz, *H-d*), 10.04 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-*d*): δ 45.7 (-N*C*H$_2$Ph), 87.2 (*C-f*), 112.9 (*C-b*), 116.1 (*t*, J = 16.5 Hz, *C-p*)*, 117.2 (*t*, J = 15.6 Hz, *C-s*)*, 118.4 (*C-d*), 123.0 (*C-a*), 124.0 (*q*, J = 272.5 Hz, -*C*F$_3$), 125.7 (*q*, J = 3.8 Hz, *aromatic carbon*), 127.1 (*aromatic carbon*), 129.0 (*C-c*), 129.7 (*aromatic carbon*), 130.8 (*aromatic carbon*), 131.1 (*q*, J = 32.8 Hz, *aromatic carbon*), 131.4 (*aromatic carbon*), 131.9 (*aromatic carbon*),

132.5 (*aromatic carbon*), 142.6 (*C-e*), 142.8 (*dd*, J = 251.8, 17.4 Hz, *C-q*)**, 144.1 (*dd*, J = 244.7, 15.4 Hz, *C-r*)**, 161.6 (*C-g*)***, 162.2 (*C-h*)***; MS (ESI) 560 (M+1).

1-Benzyl-2-oxo-N-(2,3,5,6-tetrafluoro-3'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**35**). 2,3,5,6-Tetrafluoro-3'-(trifluoromethyl)-[1,1'-biphenyl]-4-aniline was used. Flash chromatography eluent: from *petroleum ether* / EtOAc 80:20 v/v to 50:50 v/v. White solid (m.p. 190.9 – 191.8 °C from trituration with diisopropyl ether). Yield 40 *%*. ^{1}H NMR (600 MHz, Chloroform-*d*): δ 5.49 (*s*, 2H, -NC*H*$_2$Ph), 6.78 (*t*, 1H, J = 6.8 Hz, *H-b*), 7.29 (*d*, 2H, J = 7.3 Hz, *aromatic protons*), 7.33 – 7.42 (*m*, 3H, *aromatic protons*), 7.48 (*t*, 1H, J = 7.9 Hz, *H-c*), 7.64 (*t*, 1H, J = 7.7 Hz, *aromatic proton*), 7.68 (*d*, 1H, J = 7.5 Hz, *aromatic proton*), 7.72 (*t*, 2H, J = 7.6 Hz, *aromatic protons*), 7.76 (*s*, 1H, *aromatic proton*), 8.31 (*d*, 1H, J = 8.8, *H-d*), 10.04 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-*d*): δ 45.7 (-N*C*H$_2$Ph), 87.2 (*C-f*), 112.9 (*C-b*), 116.4 (*t*, J = 16.2 Hz, *C-p*)*, 117.2 (*t*, J = 15.2 Hz, *C-s*)*, 118.5 (*C-d*), 123.0 (*C-a*), 124.0 (*q*, J = 272.1 Hz, -*C*F$_3$), 125.9 (*q*, J = 3.9 Hz, *aromatic carbon*), 127.1 (*aromatic carbon*), 127.2 (*aromatic carbon*), 128.5 (*aromatic carbon*), 129.0 (*aromatic carbon*), 129.3 (*aromatic carbon*), 129.7 (*aromatic carbon*), 131.3 (*q*, J = 32.4 Hz), 131.8 (*C-c*), 132.5 (*aromatic carbon*), 133.7 (*aromatic carbon*), 142.7 (*C-e*), 142.8 (*d*, J = 252.0 Hz, *C-q*)**, 144.2 (*d*, *J* = 248.2 Hz, *C-r*)**, 161.6 (*C-g*)***, 162.2 (*C-h*)***; MS (ESI) 560 (M+1).

2-Hydroxy-N-(2,3,5,6-tetrafluoro-[1,1'-biphenyl]-4-yl)-4,5,6,7-tetrahydropyrazolo[1,5-a]pyridine-3-carboxamide (**5**). Palladium on carbon (Pd/C, 20% w / w) was added to a solution of compound **31**, (1.0 mmol) in dry THF (10 mL). The resulting mixture was stirred under a hydrogen atmosphere of 40 bar, at a temperature of 65°C for 3 hours using a Microwave SynthWAVE. The suspension was filtered through Celite and the cake was washed with methanol. The filtrate was concentrated under reduced pressure. The obtained solid was further purified by flash chromatography (eluent: dichloromethane / EtOAc / HCOOH 80:20:1 v/v/v). White solid (m.p. 270.9 – 272.9 °C dec, from diisopropyl ether). Yield 40% ^{1}H NMR (600

MHz, DMSO-d_6): δ 1.70-1.80 (*m*, 2H, *H-b*), 1.89-1.98 (*m*, 2H, *H-c*), 2.92 (*t*, 2H, J=6.1 Hz, *H-d*), 3.86 (*t*, 2H, J=5.8 Hz, *H-a*), 7.49 – 7.55 (*m*, 5H, *aromatic protons*), 9.11 (*s*, 1H, -N*H*), 11.94 (v *br s*,1H, -O*H*). Exchangeable proton signals overlapped with the water signal; ^{13}C NMR (151 MHz, DMSO-d_6): δ 18.5 (*C-b*), 22.1 (*C-d*), 22.9 (*C-c*), 46.5 (*C-a*), 95.5 (*C-f*), 116.8 (*t*, J = 14.3 Hz, *C-s*)*, 117.0 (*t*, J = 17.4 Hz, *C-p*)*, 126.7 (*aromatic carbon*), 128.9 (*aromatic carbon*), 129.4 (*aromatic carbon*), 130.1 (*aromatic carbon*), 141.8 (*C-e*) 143.2 (*d*, J = 248.4, 21.8 Hz, *C-q*)**, 144.3 (*d*, J = 244.0 Hz, *C-r*)**, 159.7 (*C-g*)***, 160.7 (*C-h*)***; MS (ES-) 404 (M-1). IR (KBr) *ν* (cm^{-1}): 3338, 2924, 2519, 1685, 1577, 1522, 1437, 1374, 1316, 1283, 1241, 1144, 992. ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{20}H_{16}F_4N_3O_2$, 406.1173; obsd, 406,1170.

General Procedure: removal of the 4-methoxybenzyloxy moiety to give final compounds **6 – 9, 15-17**. Thioanisole (220 µL, 1.87 mmol, from 5.0 eq to 10 equivalent) was added to a solution of the corresponding starting material (200 mg, 0.37 mmol, 1.0 eq) in TFA (3 mL). The mixture was heated at 70 °C for 2h then cooled to r.t.. The mixture was partially concentrated, and the crude product was taken up with water to give a suspension that was filtered, and the solid washed with an additional amount of cold water. The resulting solid was triturated with diisopropyl ether to produce the title compounds, often directly in pure form (see details above).

2-Hydroxy-N-(2,3,5,6-tetrafluoro-4-morpholinophenyl)pyrazolo[1,5-a]pyridine-3-carboxamide (**6**). Compound **22** (200 mg, 0.38 mmol, 1.0 eq) was dissolved in a solution of thioanisole (250 µL, 2.26 mmol, 6.0 eq) in TFA (2 mL). The residue was triturated with hexane and diisopropyl ether, and then purified by flash chromatography (eluent: *petroleum ether*/ DCM/ MeOH 5/4/0.4 v/v/v). The resulting solid was triturated with diisopropyl ether to give the title compound as a white solid (276.5 –277.2 °C dec. from diisopropyl ether). Yield: 42%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.18 – 3.25 (*m*, 4H, -NC*H*$_2$CH$_2$O-), 3.68 – 3.75 (*m*, 4H, -NCH$_2$C*H*$_2$O-), 7.0 (*t*, 1H, J = 6.8 Hz, *H-b*), 7.48 (*t*, 1H, J = 7.8 Hz, *H-c*), 7.94 (*d*, 1H, J = 8.7

Hz, *H-d*), 8.58 (*d*, 1H, J = 6.7 Hz, *H-a*), 8.76 (*s*, 1H, -N*H*), 12.83 (v *br s*, 1H, -O*H*); ^{13}C NMR (151 MHz, DMSO-d_6) δ 51.0 (-NCH_2CH_2O-), 66.7 (-NCH_2CH_2O-), 88.2 (*C-f*), 111.5 (*t, J* = 15.1 Hz, *C-p*)*, 113.1 (*C-b*), 116.8 (*C-d*), 127.5 (*t, J* = 11.2 Hz, *C-s*)*, 128.2 (*C-a*), 129.1 (*C-c*), 141.7 (*C-e*), 142.2 (*dd, J* = 243.5, 6.2 Hz, *C-q*)**, 143.4 (*dd, J* = 244.6, 14.4 Hz, *C-r*)**, 160.8 (*C-h*)***, 162.7 (*C-g*)***. MS (ES-): 409 (M-1). ESI−HRMS (*m*/*z*): [M-H]$^-$ calcd for $C_{18}H_{13}F_4N_4O_3$, 409.0929; obsd, 409.0925.

2-Hydroxy-N-(2,3,5,6-tetrafluoro-4-(thiophen-2-yl)phenyl)pyrazolo[1,5-a]pyridine-3-carboxamide (**7**). The solid was crystallized three times from acetonitrile (20 mL) to give the title compound as a grey solid (278.4 –279.9 °C dec. from acetonitrile). Yield: 23 %. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.03 (*t*, 1H, J = 6.6 Hz, *H-b*), 7.31 (*t*, 1H, J = 4.3 Hz, *aromatic proton*), 7.51 (*t*, 1H, J = 7.8 Hz, *H-c*), 7.64 (*d*, 1H, J = 2.8 Hz, *aromatic proton*), 7.92 (*d*, 1H, J = 5.0 Hz, *aromatic proton*), 7.97 (*d*, 1H, J = 8.8 Hz, *H-d*), 8.61 (*d*, 1H, J = 6.7 Hz, *H-a*), 8.95 (*s*, 1H, -N*H*), 12.90 (*br s*, 1H, -O*H*); ^{13}C NMR (151 MHz, DMSO-d_6) δ 88.2 (*C-f*), 110.9 (*t*, J = 15.6 Hz, *C-p*)*, 113.8 (*C-b*), 17.03 (*t*, J = 16.5 Hz, *C-s*)*, 116.8 (*C-d*), 126.2 (*thiophene carbon*), 127.8 (*thiophene carbon*), 128.4 (*C-a*), 129.2 (*thiophene carbon*), 129.5 (*C-c*), 130.7 (*t*, J = 4.0 Hz, *thiophene carbon*), 141.8 (*C-e*), 143.0 (*dd, J* = 246.5, 14.3 Hz, *C-q* and *C-r*), 160.4 (*C-h*)**, 162.7(*C-g*)**. MS (ES-): 406 (M-1). ESI−HRMS (*m*/*z*): [M-H]$^-$ calcd for $C_{18}H_8F_4N_3O_2S$, 406.0279; obsd, 406.0275.

2-Hydroxy-N-(2,3,5,6-tetrafluoro-4-(pyridin-3-yl)phenyl)pyrazolo[1,5-a]pyridine-3-carboxamide (**8**). Pale yellow solid (m.p. 283.4 – 286.7 °C dec. from trituration with diisopropyl ether). Yield: 76%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.98 (*t*, 1H, J = 6.7 Hz, *H-b*), 7.47 (*t*, 1H, J = 7.9 Hz, *H-c*), 7.61 (*dd*, 1H, J = 7.7, 5.0 Hz, *aromatic proton*), 7.95 (*d*, 1H, J = 8.6 Hz, *H-d*), 8.05 (*d*, 1H, J = 7.4 Hz, *aromatic proton*), 8.57 (*d*, 1H, J = 6.5 Hz, *H-a*), 8.71 (*d*, 1H, J = 4.1 Hz, *aromatic proton*), 8.78 (*s*, 1H, *aromatic proton*), 9.30 (*br s*, 1H, -N*H*); ^{13}C NMR (151 MHz, DMSO-d_6) δ 88.3 (*C-f*), 112.9 (*C-b*), 113.8 (*t*, J = 17.4 Hz, *C-p*)*, 116.6 (*C-d*), 117.9 (*t*, J = 14.7 Hz, *C-s*)*, 123.3 (*pyridine carbon*), 124.0 (*pyridine carbon*), 128.0 (*C-a*),

128.9 (*C-c*), 137.9 (*pyridine carbon*), 141.7 (*C-e*), 142.6 (*dd*, J = 245.2, 15.1 Hz, *C-q*)**, 143.4 (*dd*, J = 245.7, 17.2 Hz, *C-q*)**, 150.2 (*pyridine carbon*), 150.3 (*pyridine carbon*), 160.6 (*C-h*)***, 163.9 (*C-g*)***. MS (ES+): 403 (M+H). ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{19}H_{11}F_4N_4O_2$, 403.0813; obsd, 403.0810.

2-Hydroxy-N-(2,3,5,6-tetrafluoro-4-(2-(trifluoromethyl)pyridin-4-yl)phenyl)pyrazolo[1,5-a]pyridine-3-carboxamide (**9**). Pale yellow solid (m.p. 258.4 – 259.2 °C dec. from trituration with diisopropyl ether). Yield: 55%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.01 (*t*, 1H, J = 6.6 Hz, *H-b*), 7.49 (*t*, 1H, J = 7.7 Hz, *H-c*), 7.96 (*d*, 1H, J = 8.6 Hz, *H-d*), 8.00 (*d*, 1H, J = 3.5 Hz, *aromatic proton*), 8.22 (*s*, 1H, *aromatic proton*), 8.59 (*d*, 1H, J = 6.6 Hz, *H-a*), 8.99 (*d*, J = 4.6 Hz, 1H, *aromatic proton*), 9.22 (*br s*, 1H, -N*H*); ^{13}C NMR (151 MHz, DMSO-d_6) δ 88.2 (*C-f*), 113.1 (*C-p* overlapped with C-b*), 116.7 (*C-d*), 118.9 (*t*, J = 13.5 Hz, *C-s*)*, 121.5 (*q*, J = 274,6 Hz, -CF_3), 121.8 (*pyridine carbon*), 128.2 (*C-a*), 128.5 (*C-c*), 129.1 (*q*, J = 12.5 Hz, *pyridine carbon*), 137.3 (*pyridine carbon*), 141.7 (*C-e*), 142.6 (*d*, J = 246.8 Hz, *C-q*)**, 143.3 (*d*, J = 247.0 Hz, *C-r*)**, 147.1 (*q*, J = 34.1 Hz, *pyridine carbon*), 151.0 (*pyridine carbon*), 160.3 (*C-h*)***, 163.4 (*C-g*)***. MS (ES+): 470 (M+H). ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{20}H_{10}F_7N_4O_2$, 471.0686; obsd, 471.0684.

2-Hydroxy-N-(2,3,5,6-tetrafluoro-3'-hydroxy-[1,1'-biphenyl]-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**14**). white solid (275.9 –276.4 °C dec. from diisopropyl ether). Yield: 58%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.84 – 6.99 (*m*, 3H, *aromatic protons*), 7.03 (*t*, 1H, J = 6.8 Hz, *H-b*), 7.35 (*t*, 1H, J = 8.2 Hz, *aromatic proton*), 7.51 (*t*, 1H, J = 7.8 Hz, *H-c*), 7.98 (*d*, 1H, J = 7.4 Hz, *H-d*), 8.61 (*d*, 1H, J = 4.5 Hz, *H-a*), 8.90 (*s*, 1H, -Ar-O*H*), 9.78 (*s*, 1H, -N*H*), 12.86 (*s*, 1H, -O*H*); ^{13}C NMR (151 MHz, DMSO-d_6) δ 88.2 (*C-f*), 113.3 (*C-b*), 114.7 (*t*, J = 13.5 Hz, *C-p*)*, 116.3 (*C-d*), 116.8 (*aromatic carbon*), 117.4 (*t*, *J* = 16.3 Hz, *C-s*)*, 120.7 (*aromatic carbon*), 127.6 (*aromatic carbon*), 128.4 (*C-a*), 128.3 (*aromatic carbon*), 129.1 (*C-c*), 129.9 (*aromatic carbon*), 141.7 (*C-e*), 142.7 (*d*, *J* = 248.4 Hz, *C-q*)**, 143.2 (*d*, *J* = 244.8 Hz, *C-*

r)**, 157.5 (*aromatic carbon*), 160.4 (*C-h*)***, 162.6 (*C-g*)***; MS (ES-): 416 (M-1). ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{20}H_{12}F_4N_3O_3$, 418.0809; obsd, 418.0807.

2-Hydroxy-N-(2,3,5,6-tetrafluoro-3'-(trifluoromethoxy)-[1,1'-biphenyl]-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**15**). The crude product was partially dissolved in methanol, filtered from the insoluble solid, concentrated and precipitated via the addition of diisopropyl ether. Beige solid (228.1 –229.5 °C dec. from diisopropyl ether). Yield: 26%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.03 (*t*, 1H, J = 6.7 Hz, *H-b*), 7.51 (*t*, 1H, J = 7.8 Hz, *H-c*), 7.55 (*d*, 1H, J = 8.0 Hz, *aromatic proton*), 7.60 – 7.67 (*m*, 2H, *aromatic protons*), 7.71 (*t*, 1H, J = 7.9 Hz, *aromatic proton*), 7.98 (*d*, 1H, J = 8.7 Hz, *H-d*), 8.61 (*d*, 1H, J = 6.7 Hz, *H-a*), 8.97 (*s*, 1H, -N*H*), 12.94 (*br s*, 1H, -O*H*); ^{13}C NMR (151 MHz, DMSO-d_6) δ 88.2 (*C-f*), 113.2 (*C-b*), 115.5 (*t*, J = 17.2 Hz, *C-p*)*, 116.8 (*C-d*), 117.6 (*t*, J = 15.1 Hz, *C-s*)*, 120.1 (*q*, J = 257.0 Hz, -O*C*F_3), 122.1 (*aromatic carbon*), 123.0 (*aromatic carbon*), 128.4 (*C-a*), 128.8 (*aromatic carbon*), 129.2 (*C-c*), 129.5 (*aromatic carbon*), 130.9 (*aromatic carbon*), 141.7 (*C-e*), 142.7 (*dd*, J = 246.2, 13.6 Hz, *C-q*)**, 143.3 (*d*, J = 245.6 Hz, *C-r*)**, 148.4 (*aromatic carbon*), 160.3 (*C-h*)***, 162.7 (*C-g*)***; MS (ES-): 484 (M-1). ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{21}H_{11}F_7N_3O_3$, 486.0683; obsd, 486.0681.

2-Hydroxy-N-(2,3,5,6-tetrafluoro-3'-methoxy-[1,1'-biphenyl]-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**16**). The residue was treated with diethyl ether, the mixture was filtered and the insoluble residue was purified by flash chromatography (eluent: DCM / MeOH 97 / 3 v/v then *petroleum ether*/dichloromethane/methanol 5/4/0.6 v/v/v) to obtain the title compound as a white solid (223.6 – 224.1 °C dec. from diisopropyl ether). Yield: 47 %. ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.81 (*s*, 3H, -OC*H*$_3$), 7.02 (*t*, 1H, J = 6.6 Hz, *H-b*), 7.08 – 7.16 (*m*, 3H, *aromatic protons*), 7.45 – 7.54 (*m*, 2H, *H-c* and *aromatic proton*), 7.98 (*d*, 1H, J = 8.6 Hz, *H-d*), 8.61 (*d*, 1H, J = 6.6 Hz, *H-a*), 8.99 (*br s*, 1H, -N*H*); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 55.4 (-O*C*H$_3$), 88.2 (*C-f*), 113.2 (*C-b*), 115.0 (*C-d*), 115.8 (*aromatic carbon*), 116.8 (*aromatic carbon*), 117.0 (*t*, J = 16.1 Hz, *C-s and C-p*)*, 122.3 (*aromatic carbon*), 127.8 (*C-a*), 128.3 (*aromatic carbon*),

129.1 (*C-c*), 130.0 (*aromatic carbon*), 141.8 (*C-e*), 142.9 (*d*, *J* = 246.3 Hz, *C-q*)**, 143.4 (*d*, *J* = 252.5 Hz, *C-r*)**, 159.4 (*aromatic carbon*), 160.5 (*C-h*)****, 163.0 162.2 (*C-g*)****; MS (ES-): 430 (M-1), MS (ES+) 432 (M+1). ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{21}H_{14}F_4N_3O_3$, 432.0966; obsd, 432.0969.

2-Hydroxy-N-(2,3,5,6-tetrafluoro-3'-propoxy-[1,1'-biphenyl]-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**17**). Beige solid (209.4 – 210.0 °C from diisopropyl ether). Yield: 69 %. ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.98 (*t*, 3H, J = 7.2 Hz, -OCH$_2$CH$_2$C*H*$_3$), 1.68 – 1.81 (*m*, 2H, -OCH$_2$C*H*$_2$CH$_3$), 3.98 (*t*, 2H, J = 6.1 Hz, -OC*H*$_2$CH$_2$CH$_3$), 7.02 (*t*, 1H, J = 6.5 Hz, *H-b*), 7.05 – 7.14 (*m*, 3H, *aromatic protons*), 7.46 (*t*, 1H, J = 7.8 Hz, *aromatic proton* or *H-c*), 7.51 (*t*, 1H, J = 7.6 Hz, *aromatic proton* or *H-c*), 7.99 (*d*, 1H, J = 8.7 Hz, *H-d*), 8.61 (*d*, 1H, J = 6.5 Hz, *H-a*), 8.91 (*s*, 1H, -N*H*), 12.86 (*s*, 1H, -O*H*); ^{13}C NMR (151 MHz, DMSO-d_6) δ 10.4 (-OCH$_2$CH$_2$*C*H$_3$), 22.0 (-OCH$_2$*C*H$_2$CH$_3$), 69.2 (-O*C*H$_2$CH$_2$CH$_3$), 88.2 (*C-f*), 113.3 (*C-b*), 115.5 (*C-d*), 116.2 (*aromatic carbon*), 116.8 (*aromatic carbon*), 116.9 – 117. 7 (*m*, *C-s and C-p*), 122.2 (*aromatic carbon*), 127.8 (*aromatic carbon*), 128.4 (*C-a*), 129.2 (*C-c*), 129.9 (*aromatic carbon*), 141.7 (*C-e*), 142.8 (*dd*, J = 245.5, 21.8 Hz, *C-q*)**, 143.3 (*d*, J = 245.6 Hz, *C-r*)**, 158.8 (*aromatic carbon*)***, 160.4 (*C-h*)****, 162.6 (*C-g*)****; MS (ES-): 458 (M-1), MS (ES+): 460 (M+1). ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{23}H_{18}F_4N_3O_3$, 460.1279; obsd, 460.1280.

General hydrogenation procedure for the production of target compounds **10 - 13**.

Palladium on carbon (Pd/C, 6% w/w) was added to a solution of the appropriate amide (compounds **32 - 35**, 1.0 mmol), in dry THF (15 mL), and 37 % HCl (1.0 mmol). The resulting mixture was vigorously stirred under a hydrogen atmosphere for 6 hours. The suspension was filtered through Celite, and the cake was then washed with methanol. The filtrate was concentrated under reduced pressure. When necessary, the obtained solid was further purified by flash chromatography (see details below).

2-Hydroxy-N-(2,3,4',5,6-pentafluoro-[1,1'-biphenyl]-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**10**). Obtained from **32**, flash chromatography (eluent: dichloromethane / EtOAc / HCOOH 80:20:1 v/v/v). White solid (m.p. 293.4 – 294.5 °C dec. from trituration with diisopropyl ether). Yield 75 *%.* ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.03 (*t*, 1H, J = 6.7 Hz, *H-b*), 7.42 (*t*, 2H, J = 8.7 Hz, *aromatic protons*), 7.51 (*t*, 1H, J = 7.8 Hz, *H-c*), 7.57 – 7.71 (*m*, 2H, *aromatic protons*), 7.98 (*d*, 1H, J = 8.7, *H-d*), 8.62 (*d*, 1H, J = 6.7, *H-a*), 8.93 (*s*, 1H, -N*H*), 12.88 (*br s*, 1H, -O*H*); ^{13}C NMR (151 MHz, DMSO-d_6): δ 88.2 (*C-f*), 113.3 (*C-b*), 116.0 (*d*, J = 22.0 Hz, *aromatic carbon*), 116.3 (*t*, J = 17.4 Hz, *C-p*)*, 116.8 (*C-d*), 117.1 (*t*, J = 14.1 Hz, *C-s*)*, 123.0 (*aromatic carbon*), 128.4 (*C-a*), 129.2 (*C-c*), 132.5 (*d*, J = 8.4 Hz, *aromatic carbon*), 141.7 (*C-e*), 142.8 (*d*, J = 246.4 Hz, *C-q*)**, 143.3 (*d*, J = 248.6 Hz, *C-r*)**, 160.4 (*C-g*)***, 162.6 (*d*, J = 247.1 Hz, -*C*F), 162.7 (*C-h*)***; MS (ESI) 420 (M+1). ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{20}H_{11}F_5N_3O_2$, 420.0766; obsd, 420.0766.

2-Hydroxy-N-(2,3,3',5,6-pentafluoro-[1,1'-biphenyl]-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**11**). Obtained from **33**, flash chromatography eluent: dichloromethane/EtOAc / HCOOH 80:20:1 v/v/v. White solid (m.p. 255.4 – 256.2 °C dec. from trituration with diisopropyl ether). Yield 75 *%.* ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.03 (*t*, 1H, J = 6.8 Hz, *H-b*), 7.33 – 7.56 (*m*, 4H, *aromatic protons*), 7.62 (*dd*, 1H, J = 14.3, 7.7 Hz, *aromatic proton*), 7.99 (*d*, 1H, J = 8.7, *H-d*), 8.62 (*d*, 1H, J = 6.8, *H-a*), 8.94 (*s*, 1H, -N*H*), 12.83 (*br s*, 1H, -O*H*); ^{13}C NMR (151 MHz, $CDCl_3$): δ 88.2 (*C-f*), 113.3 (*C-b*), 115.9 (*t*, J = 17.4 Hz, *C-p*)*, 116.4 (*d*, J = 20.9 Hz, *aromatic carbon*), 116.9 (*C-d*), 117.2 (*d*, J = 23.0 Hz, *aromatic carbon*), 117.4 (*t*, J = 14.8 Hz, *C-s*)*, 126.5 (*aromatic carbon*), 128.4 (*C-a*), 128.7 (*d*, J = 9.6 Hz, *aromatic carbon*), 129.2 (*C-c*), 130.9 (*d*, J = 8.3 Hz, *aromatic carbon*), 141.7 (*C-e*), 142.8 (*d*, J = 243.6 Hz, *C-q*)**, 143.2 (*d*, J = 244.6 Hz, *C-r*)**, 160.4 (*C-g*)***, 162.0 (*d*, J = 244.4 Hz, -*C*F), 162.7 (*C-h*)***; MS (ESI) 418 (M-1). ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{20}H_{11}F_5N_3O_2$, 420.0766; obsd, 420.0763.

2-Hydroxy-N-(2,3,5,6-tetrafluoro-4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**12**). Obtained from **34**, flash chromatography eluent: dichloromethane/EtOAc /HCOOH 80:20:1 v/v/v. White solid (m.p. 286.1 – 286.8 °C dec. from trituration with diisopropyl ether). Yield 95 *%*. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.03 (*t*, 1H, J = 6.7 Hz, *H-b*), 7.51 (*t*, 1H, J = 7.8 Hz, *H-c*), 7.83 (*d*, 2H, J = 7.8 Hz, *aromatic protons*), 7.94 (*d*, 2H, J = 8.0 Hz, *aromatic protons*), 7.98 (*d*, 1H, J = 8.7 Hz, *H-d*), 8.61 (*d*, 1H, J = 6.7 Hz, *H-a*), 9.00 (*s*, 1H, -N*H*), 12.92 (br *s*, 1H, -O*H*); ^{13}C NMR (151 MHz, DMSO-d_6): δ 88.2 (*C-f*), 113.3 (*C-b*), 115.7 (*t*, J = 16.9 Hz, *C-p*)*, 116.8 (*C-d*), 117.8 (*t*, J = 13.1 Hz, *C-s*)*, 124.0 (*q*, J = 272.4 Hz, -*C*F_3), 125.8 (*q*, J = 3.3 Hz, *aromatic carbon*), 128.4 (*C-a*), 129.2 (*C-c*), 129.7 (*q*, J = 32.3 Hz, *aromatic carbon*), 131.0 (*aromatic carbon*), 131.2 (*aromatic carbon*), 141.7 (*C-e*), 142.7 (*dd*, J = 246.0, 14.9 Hz, *C-q*)**, 143.2 (*dd*, J = 244.9, 19.5 Hz, *C-r*)**, 160.3 (*C-g*)***, 162.7 (*C-h*)***; MS (ESI) 468 (M-1). ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{21}H_{11}F_7N_3O_2$, 470.0734; obsd, 470.0731.

2-Hydroxy-N-(2,3,5,6-tetrafluoro-3'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**13**). Obtained from **35**, flash chromatography eluent: dichloromethane/EtOAc/HCOOH 80:20:1 v/v/v. Pale pink solid (m.p. 220.3 – 220.7 °C dec. from trituration with diisopropyl ether). Yield 98 *%*. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.03 (*t*, 1H, J = 6.7 Hz, *H-b*), 7.51 (*t*, 1H, J = 7.8 Hz, *H-c*), 7.82 (*t*, 1H, J = 7.7 Hz, *aromatic proton*), 7.91 (*d*, 2H, J = 7.6 Hz, *aromatic protons*), 7.95 -8.02 (m, 2H, *aromatic proton* and *H-d*), 8.62 (*d*, 1H, J = 6.7, *H-a*), 8.98 (*s*, 1H, -N*H*), 12.87 (*br s*, 1H, -O*H*); ^{13}C NMR (151 MHz, DMSO-d_6): δ 88.2 (*C-f*), 113.3 (*C-b*), 115.6 (*t*, J = 17.0 Hz, *C-p*)*, 116.8 (*C-d*), 117.6 (*t*, J = 15.6 Hz, *C-s*)*, 123.9 (*q*, J = 271.9 Hz, -*C*F_3), 126.2 (*aromatic carbon*), 126.9 (*aromatic carbon*), 127.9 (*aromatic carbon*), 128.4 (*C-a*), 129.2 (*C-c*), 129.7 (*q*, J = 32.4 Hz, *aromatic carbon*), 130.1 (*aromatic carbon*), 134.4 (*aromatic carbon*), 141.7 (*C-e*), 143.8 (*d*, J = 246.0 Hz, *C-q*)**, 143.9 (*d*, J = 246.9 Hz, *C-r*)**, 160.3 (*C-g*)***, 162.7 (*C-h*)***; MS (ESI) 468 (M-1). ESI−HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{21}H_{11}F_7N_3O_2$, 470.0734; obsd, 470.0735.

Ethyl 2-((tert-butoxycarbonyl)oxy)pyrazolo[1,5-a]pyridine-3-carboxylate (**40**). Cs_2CO_3 (2.86 g, 8.74 mmol) and tert-butoxycarbonyl anhydride (0.699 g, 3.2 mmol) were added to a solution of **18** (0.600 g, 2.91 mmol) in dry THF (25 mL). The reaction mixture was stirred under reflux overnight and allowed to reach room temperature. The solvent was concentrated under reduced pressure and the residue was dissolved in water (50 mL) and extracted with diethyl ether (3 x 50 mL). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography (eluent: *petroleum ether*/ethyl acetate 80:20 v/v) to afford the title compound as a white solid (m.p. 95.4 – 96.4 °C from trituration with diisopropyl ether). Yield 93%. ^{1}H NMR (600 MHz, Chloroform-d_3): δ 1.39 (*t*, 3H, J = 7.1 Hz, -OCH$_2$C*H*$_3$), 1.58 (s, 9H, -OC(*CH*$_3$)$_3$), 4.36 (*q*, 2H, J = 7.0 Hz, -OC*H*$_2$CH$_3$), 6.96 (*t*, 1H, J = 6.8 Hz, *H-b*), 7.42 (*t*, 1H, J = 7.9 Hz, *H-c*), 8.08 (*d*, 1H, J = 8.8 Hz, *H-d*), 8.38 (*d*, 1H, J = 6.6 Hz, *H-a*). ^{13}C NMR (151 MHz, Chloroform-d_3): δ 14.6 (-OCH$_2$*C*H$_3$), 27.8 (-C(*C*H$_3$)$_3$), 60.2 (-O*C*H$_2$CH$_3$), 84.8 (*C-f*), 93.4(-*C*(CH$_3$)$_3$), 114.1 (*C-b*), 119.2 (*C-d*), 128.0 (*C-a*), 129.3 (*C-c*), 142.3 (*C-e*), 150.2 (*C-h*), 158.2 (*C*-g)*, 162.1 (*C-i*)*. MS (ESI) 307 (M+1).

Ethyl 2-((tert-butoxycarbonyl)oxy)-7-chloropyrazolo[1,5-a]pyridine-3-carboxylate (**41**). LiHMDS (1.0 M THF solution: 0.980 mL, 0.98 mmol, 1.5 equiv.) was added dropwise to a solution of **40** (0.400 g, 0.654 mmol) in dry THF (10 mL) and cooled to -78°C. The mixture was stirred at -78°C for 1 h, and a solution of hexachloroethane (0.170 g, 0.72 mmol, 1.1 equiv.) in dry THF was then added at -78°C, and the reaction mixture was stirred for 15 minutes at room temperature. Subsequently, the reaction was quenched with an aqueous sutured solution of NH_4Cl (100 mL). The water phase was extracted with dichloromethane (4 x 100 mL). The combined organic phases were dried over Na_2SO4, filtered and evaporated to dryness under vacuum. The crude product was purified by flash chromatography (eluent: *petroleum ether*/ethyl acetate 80:20 v/v) to afford the title compound as a white solid (m.p. 104.6 – 106.0 °C from trituration with diisopropyl ether). Yield 81%. ^{1}H NMR (600 MHz, Chloroform-d_3):

δ 1.40 (*t*, 3H, J = 7.1 Hz, -OCH$_2$C*H$_3$*), 1.58 (s, 9H, -OC(*CH$_3$*)$_3$), 4.37 (*q*, 2H, J = 7.2 Hz, -OC*H$_2$*CH$_3$), 7.11 (*dd*, 1H, J = 7.5, 1.1 Hz, *H-b*), 7.39 (*dd*, 1H, J = 8.9, 7.5 Hz, *H-c*), 8.09 (*dd*, 1H, J = 8.9, 1.2 Hz, *H-d*). ^{13}C NMR (151 MHz, Chloroform-d$_3$): δ 14.6 (-OCH$_2$*C*H$_3$), 27.8 (-C(*C*H$_3$)$_3$), 60.5 (-O*C*H$_2$CH$_3$), 85.0 (*C-f*) $_3$, 95.2(-*C*(CH$_3$)$_3$), 114.4 (*C-b*), 117.5 (*C-d*), 128.2 (*C-c*), 131.0 (*C-a*), 143.9 (*C-e*), 150.0 (*C-h*), 158.0 (*C*-g)*, 161.8 (*C-i*)*. MS (ESI) 241 (M+1, -Boc).

Ethyl 7-chloro-2-hydroxypyrazolo[1,5-a]pyridine-3-carboxylate (**42**). Trifluoroacetic acid (10 mL) was added to a solution of **41** in dry dichloromethane (25 mL) and the reaction mixture was stirred at room temperature for 4 h. The mixture was quenched with water and the layers were separated. The aqueous solution was further extracted with dichloromethane (3 x 25 mL). The combined organic phases were dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure. The crude product was purified by flash chromatography (eluent: dichloromethane/methanol 98:2 v/v) to afford the title compound as a white solid (m.p. 134.0 – 135.8 °C from trituration with diisopropyl ether). Yield 94%. ^{1}H NMR (600 MHz, Chloroform-d$_3$): δ 1.44 (*t*, 3H, J = 7.2 Hz, -OCH$_2$C*H$_3$*), 4.44 (*q*, 2H, J = 7.2 Hz, -OC*H$_2$*CH$_3$), 7.02 (*dd*, 1H, J = 7.5, 1.2 Hz, *H-b*), 7.35 (*dd*, 1H, J = 8.7, 7.5 Hz, *H-c*), 7.72 (*dd*, 1H, J = 8.7, 1.2 Hz, *H-d*), 9.09 (*s*, 1H, -O*H*). ^{13}C NMR (151 MHz, Chloroform-d$_3$): δ 14.6 (-OCH$_2$*C*H$_3$), 60.9 (-O*C*H$_2$CH$_3$), 87.9 (*C-f*), 113.6 (*C-b*), 115.5 (*C-d*), 128.4 (*C-c*), 131.3 (*C-a*), 142.0 (*C-e*), 166.0 (*C-h*)*, 166.9 (*C*-g)*. MS (ESI) 241 (M+1).

Ethyl 2-(benzyloxy)-7-chloropyrazolo[1,5-a]pyridine-3-carboxylate (**43**). Benzyl bromide (645 mg, 3.20 mmol, 1.10 eq) was added dropwise to a mixture of **42** (600 mg, 2.91 mmol) and Cs_2CO_3 (2.295 g, 7.04 mmol, 2.4 eq) in dry DMF (15 mL). The reaction mixture was stirred overnight at room temperature, and water (100 mL) was then added. The mixture was extracted with EtOAc (4 × 70 mL), the combined organic layer was dried under Na_2SO_4 and evaporated under reduced pressure to give a yellow oil. The mixture was separated using flash chromatography (eluent: *petroleum ether*/EtOAc 6/4 v/v) to afford the title compound as a pale

yellow solid (m.p. 98.2 – 99.3 °C from trituration with diisopropyl ether). Yield 85% ^{1}H NMR (600 MHz, Chloroform-d_3): δ 1.41 (*t*, 3H, J = 7.1 Hz, -OCH$_2$C*H$_3$*), 4.38 (*q*, 2H, J = 7.1 Hz, -OC*H$_2$*CH$_3$), 5.58 (*s*, 2H, -OC*H$_2$*Ph), 6.96 (*dd*, 1H, J = 7.4, 1.2 Hz, *H-b*), 7.27 – 7.34 (*m*, 2H, *H-c and aromatic proton*), 7.39 (*t*, 2H, J = 7.5 Hz, *aromatic protons*), 7.59 (*d*, 2H, J = 7.4 Hz, *aromatic protons*), 7.99 (*dd*, 1H, J = 8.8, 1.2 Hz, *H-d*). ^{13}C NMR (151 MHz, Chloroform-d_3): δ 14.6 (-OCH$_2$*C*H$_3$), 60.0 (-O*C*H$_2$CH$_3$), 71.1 (-O*C*H$_2$Ph), 90.1 (*C-f*), 112.8 (*C-b*), 116.5 (*C-d*), 127.8 (*aromatic carbon*), 127.9 (*aromatic carbon*), 128.0 (*C-c*), 128.5 (*aromatic carbon*), 130.6 (*C-a*), 136.7 (*aromatic carbon*), 144.6 (*C-e*), 163.1 (*C-h*)*, 164.8 (*C*-g)*. MS (ESI) 331 (M+1).

2-(Benzyloxy)-7-chloropyrazolo[1,5-a]pyridine-3-carboxylic acid (**44**). 6 M NaOH (5.0 eq) was added to a solution of compound **43** (785 mg, 2.40 mmol) in EtOH abs (20 mL). The mixture was stirred for 4 h at 75 °C, then neutralized with 6 M HCl and concentrated under reduced pressure. The mixture was cooled to 0 °C and then acidified with 2 M HCl until pH 2 was reached, giving a suspension. This suspension was filtered to give the title compound as a white solid (m.p. 178.4 – 179.8 °C dec. with gas developed, from trituration with diisopropyl ether). Yield 84 %. ^{1}H NMR (600 MHz, DMSO-d_6): δ 5.48 (*s*, 2H, -OC*H$_2$*Ph), 7.31 (*dd*, 1H, J = 7.5, 1.1 Hz, *H-b*), 7.35 (*t*, 1H, J = 7.4 Hz, *aromatic proton*), 7.41 (*t*, 2H, J = 7.4 Hz, *aromatic protons*), 7.52 (*dd*, 1H, J = 8.7, 7.6 Hz, *H-c*), 7.55 (*d*, 2H, J = 7.5 Hz, *aromatic protons*), 7.95 (*dd*, 1H, J = 8.9, 1.1 Hz, *H-d*), 12.34 (*br s*, 1H, -COO*H*). ^{13}C NMR (151 MHz, DMSO-d_6): δ 70.5 (-O*C*H$_2$Ph), 89.4 (*C-f*), 113.3 (*C-b*), 116.1 (*C-d*), 127.9 (*C-a*), 128.1 (*aromatic carbon*), 128.4 (*aromatic carbon*), 128.9 (*aromatic carbon*), 129.4 (*C-c*), 136.4 (*aromatic carbon*), 144.1 (*C-e*), 163.3 (*C-h*)*, 164.0 (*C*-g)*. MS (ESI) 301 (M-1).

1-Benzyl-7-chloro-2-oxo-N-(2,3,5,6-tetrafluoro-[1,1'-biphenyl]-4-yl)-1,2-dihydropyrazolo[1,5-a]pyridine-3-carboxamide (**45**). Oxalyl chloride (3.0 mmol) and dry DMF (1 drop) were added to a cooled (0°C) solution of **44** (1.0 mmol) 1-3, in dry THF (20 mL), under a nitrogen atmosphere. The obtained solution was stirred at room temperature for

2 hours. The solution was then concentrated under reduced pressure and the residue dissolved in dry THF (10 mL, this step was repeated three times). The resulting acyl chloride was immediately used without any further purification, and was dissolved in 10 mL of dry toluene and transferred to the solution described below. Trimethylaluminium (2.0 M in hexane, 1.5 mmol), was added to a solution of 4-phenyl-2,3,5,6-tetrafluoroaniline (1.5 mmol) in dry toluene (15 mL), under a nitrogen atmosphere. The resulting mixture was stirred for 2 hours at room temperature, giving a brown suspension, and the solution of the previously described acyl chloride in dry toluene (30 mL) was then quantitatively added. The mixture was heated overnight at 90 °C and then cooled to r.t. The reaction was quenched with 1M HCl, the layers resolved, and the aqueous phase was exhaustively extracted using EtOAc. The combined organic layer was washed with 1M NaOH and brine, dried and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (eluent: from *petroleum ether*/EtOAc 8:2 v/v to *petroleum ether*/EtOAc 4:6 v/v) to afford the title compound as a white solid (m.p. 201.1 – 202.4 °C from trituration with diisopropyl ether). Yield 38 %.^{1}H NMR (600 MHz, Chloroform-d$_3$): δ 5.74 (*s*, 2H, -NC*H*$_2$Ph), 6.73 (*dd*, 1H, J = 7.5, 1.2 Hz, *H-b*), 7.06 (*dd*, 2H, J = 7.4, 1.7 Hz, *aromatic protons*), 7.22- 7.30 (*m*, 3H, *aromatic protons*), 7.35 (*dd*, 1H, J = 8.8, 7.6 Hz, *H-c*), 7.42 – 7.53 (*m*, 5H, *aromatic protons*), 8.28 (*dd*, 1H, J = 8.8, 1.2 Hz, *H-d*), 10.01 (*s*, 1H, -N*H*); ^{13}C NMR (151 MHz, Chloroform-d$_3$): δ 52.6 (-NCH$_2$Ph), 89.2 (*C-f*), 114.6 (*C-b*), 115.9 (*t*, J = 16.3 Hz, *C-p*)*, 116.7 (*C-d*), 118.0 (*t*, J = 17.2 Hz, *C-s*)*, 127.2 (*aromatic carbon*), 127.6 (*C-a*), 128.6 (*aromatic carbon*), 128.7 (*aromatic carbon*), 129.1 (*aromatic carbon*), 129.2 (*aromatic carbon*), 130.4 (*C-c*), 130.6 (*aromatic carbon*), 133.7 (*aromatic carbon*), 133.8 (*aromatic carbon*), 142.8 (*dd*, J = 248.8, 15.1 Hz, *C-q*)**, 144.2 (*d*, J = 248.3 Hz, *C-r*)**, 150.3 (*C-e*), 161.2 (*C-g*)***, 167.5 (*C-h*)***. MS (ESI) 526 (M-1).

7-Chloro-2-hydroxy-N-(2,3,5,6-tetrafluoro-[1,1'-biphenyl]-4-yl)pyrazolo[1,5-a]pyridine-3-carboxamide (**4**). Thioanisole (240 µL, 1.90 mmol, 10.0 eq) was added to a solution of **45** (100 mg, 0.19 mmol, 1.0 eq) in TFA (2 mL). The mixture was heated at 70 °C for 3h then cooled to

r.t.. The mixture was partially concentrated, and the crude product was taken up with water giving a suspension that was filtered. The solid was washed with an additional amount of cold water. The resulting solid was triturated with diisopropyl ether to afford the title compound, in pure form, as a white solid (m.p. 259.3 – 260.4 °C dec. from trituration with diisopropyl ether). Yield 64%. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.32 (*dd*, 1H, J = 7.5, 1.1 Hz, *H-b*), 7.53 (*dd*, 1H, J = 8.7, 7.5 Hz, *H-c*), 7.53 – 7.60 (*m*, 5H, *aromatic protons*), 8.02 (*dd*, 1H, J = 8.8, 1.1 Hz, *H-d*), 8.99 (*s*, 1H, -N*H*), 13.33 (*br s*, 1H, -O*H*). ^{13}C NMR (151 MHz, DMSO-d_6): δ 89.9 (*C-f*), 113.5 (*C-b*), 115.6 (*C-d*), 116.7 (*t*, J = 17.8 Hz, *C-p*)*, 117.4 (*t*, J = 17.7 Hz, *C-s*)*, 126.6 (*C-a*), 128.8 (*aromatic carbon*), 128.9 (*aromatic carbon*), 128.9 (*C-c*), 129.4 (*aromatic carbon*), 130.1 (*aromatic carbon*), 142.9 (*d*, J = 246.6 Hz, *C-q*)**, 143.2 (*d*, J = 241.3 Hz, *C-r*)**, 143.5 (*C-e*), 160.2 (*C-g*)***, 162.6 (*C-h*)***. MS (ESI) 436 (M+1). ESI−HRMS (m/z): [M+H]+ calcd for $C_{20}H_{11}ClF_4N_3O_2$, 436.0470; obsd, 436.0472.

Molecular modeling

Docking. The predicted binding modes shown herein were carried out using the Glide XP Docking Protocol.[44-46] The protein structure of **1** in complex with *h*DHODH (PDB code: 6FMD) [14] was retrieved from the Protein Data Bank[47] and prepared for the docking procedure. The crystal structure of the protein underwent a preparation process that was performed using the Protein Preparation Wizard tool,[48] implemented in Maestro™ GUI [49-51]missing hydrogen atoms were added, missing bonds or atoms were fixed, bond orders were assigned and water molecules were removed. The prediction of protonation states for the protein was accomplished using PROPKA™, with the pH set at 7.4 [52-54]. The docking procedure was validated by performing a self-docking protocol and verifying the correct positing of the co-crystallized ligand. The coordinates of the bound crystallographic ligand were used as the centroid of the grid box.

Enzymatic assays

Protein expression and purification. BL21DE3 PyrD E. Coli cells were transformed using the plasmid construct pFN2A−hDHODH (kindly given by *Department of Drug Science and Technology, University of Turin, Turin*). The vector produces *h*DHODH as an N-terminal GST-fusion protein. Cells were grown at 37 °C in LB medium supplemented with 0.1 mM flavin mononucleotide (Cayman Chemical). After 20 h of growth, cells were induced with 0.8 mM isopropyl-D-thiogalactopyranoside at an OD600 of 0.5−0.7 at 28 °C for an additional 6 h. A cell pellet from 250 mL of culture was lysed in 20 mL of PBS (50 mM Na2HPO4, 50 mM NaH2PO4, 500 mM NaCl), which had been supplemented with 24 mg of lysozyme and 0.2% v/v protease inhibitor cocktail, incubated for 30 min over ice, and disrupted by sonication (total sonication time: 8 minutes with On/Off cycles of 10”/50”). Triton X-100 was added to the lysate, to a final concentration of 1%, before centrifugation at 14000g for 40 min at 4 °C. The clarified supernatant was incubated with DNase I for 30 min at room temperature, supplemented with 2 mM dithiothreitol (DTT), and filtered through a 0.45 μm syringe filter as previously described by Sainas *et al*.[14]. The GST-fused enzyme was purified from the bacterial lysate using affinity chromatography on immobilized glutathione-sepharose columns (GE-HiTrap Protein G HP 1ml). The GST tag was not cleaved for further analysis. All the reagents used in the protein expression and purification were supplied by Merck / Sigma-Aldrich, if not otherwise specified.

hDHODH inhibition assay. The enzymatic inhibition assay was optimized for being performed on a 96 well plate and to achieve higher throughput. For each well of the plate a total volume of 200 μL was used: 5 μL of purified GST-hDHODH; 60 μL of 2,6-dichloroindophenol (DCIP) 500 μM; 20 μL of coenzyme Q10 enzyme 100 μM; 20 μL of dihydroorotate (DHO) 500 μM; Tris-HCl pH8 up to a final volume of 200 μL. Inhibitory activity was assessed by monitoring

the reduction of DCIP, which is associated with the oxidation of dihydroorotate as catalysed by the DHODH enzyme. The enzyme was pre-incubated for 5 min at 37 °C in Tris-HCl pH8 with coenzyme Q10, with DCIP (50 μM) and with the compounds to be tested used at different concentrations (final DMSO concentration 0.1% v/v). The reaction was initiated by the addition of DHO (500 μM), and the absorbance kinetic reduction was monitored at λ = 650 nm using a multi-plate reader (Tecan, M1000Pro). In order to assess the minimum and maximum absorbance values of the enzymatic reaction, a Min control value was obtained by measuring the absorbance without DHO. Similarly, a Max value was obtained by measuring the absorbance with DHO, but none inhibitor. A blank reduction calculation was also performed by measuring the absorbance values using 180 μL of Tris-HCl and 20 μL of coenzyme Q10. The Instrument was set to read the absorbance values every 10s for a total read time of 10 minutes at 37 ^{o}C. The initial rate was measured in the first 5 min (ε = 10 400 M^{-1} cm^{-1}) and an IC_{50} value was calculated, [55]using GraphPad Prism 7 software. Values are means ± SE of three independent experiments.

Cell-based assay

Cell lines and drug treatment. Human cells, THP1 (acute monocytic leukemia) and Jurkat (T cell leukemia), were cultured in complete RPMI 1640 (Invitrogen Life Technologies, Gaithersburg, MD), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (GIBCO, Invitrogen, Milan, Italy). Each compound was solubilized in DMSO (Sigma Sigma-Aldrich, Milan, Italy) at a final concentration of 10 mM, which was used as the stock solution for all experiments. Final dilutions were made in culture medium.

CFSE-based cytotoxic activity assay. Briefly, the Jurkat cell line was incubated with 1 μM carboxyfluorescein diacetate succinimidyl ester dye (CFSE, Vybrant CFDA SE cell tracer kit; Molecular Probes, Invitrogen Carlsbad, CA), at 10^7 / ml for 20 min at 37 °C. At the end of the labeling process, cells were resuspended and washed in RPMI-1640 supplemented with 1 % fetal bovine serum. Cells were then resuspended in RPMI 1640 supplemented with 10 % FBS and incubated for 20 minutes at 37 °C. Cells were centrifuged and plated ($1x10^4$ in 200 μl of medium), with increasing concentrations of the DHODH inhibitors (1 μM to 100 μM), for 3 days. Cells were harvested and 1 μg/ml of propidium iodide was added to assign the ratio of cell death. The percentage of specific lysis was calculated as described and in accordance with the following equation: [dead targets in sample (%) – spontaneously dead targets (%))/(100-spontaneously dead targets (%))] × 100. Spontaneous release was obtained by incubating cell lines in medium supplemented with the corresponding percentage of DMSO used for the dilution of compounds. Values represent the concentration that induces significant cytotoxic effects (≥ 30 %).

Annexin assay. For compound screening, $1x10^4$ THP1 cells were plated in 96-well round-bottom plates and *h*DHODH inhibitors were added, at 0.1 μM or 1.0 μM, in a volume of 200 μl of medium. For the determination of EC50, $1x10^4$ THP1 cells were plated in 96-well round-bottom plates and treated with increasing doses of DHODH inhibitors from 0.001 μM to 10 μM. After three days of culture, the apoptotic assay was performed using the Annexin V-FITC Kit (Miltenyi Biotec, Italy), according to the manufacturer's instructions. The apoptotic cells were acquired on FacsVerse and analyzed using Kaluza software version 1.2 (Beckman Coulter Fullerton, CA). The annexin assay was also performed in the presence of uridine 100 μM (Sigma Sigma-Aldrich, Milan, Italy).

Differentiation assay. For compound screening, $1x10^4$ THP1 cells were plated in 96-well round-bottom plates and the *h*DHODH inhibitors were added, at 0.1 μM or 1.0 μM, in a 200 μl volume of medium. For the determination of EC_{50}, $1x10^4$ THP1 cells were plated in 96-well

round-bottom plates and treated with increasing doses of the *h*DHODH inhibitors from 0.001 μM to 10 μM. After three days of culture, the differentiation pathway was monitored by analyzing the expression of CD14 (FITC-conjugated Beckam Coulter CA, USA) via flow cytometry analysis. Cells were washed and resuspended in staining buffer (phosphate-buffered saline, 2 % bovine serum albumin, 1mM EDTA), and incubated with antibodies at 4 °C for 45 min. Samples were acquired on a FACSVerse (BD-Biosciences San Jose- CA) and dead cells were excluded from the analyses, according to the use of propidium iodide (Sigma-Aldrich, Milan, Italy). Data were processed using Kaluza software version 1.2 (Beckman Coulter Fullerton, CA). The differentiation assay was also performed in the presence of uridine 100 μM.

Statistical analysis. Statistical analyses were performed on Prism software, version 5.0 (GraphPad Software, San Diego, CA). Data are reported as means ± SD. Two-tail paired Student's t tests were calculated to assess the differences between mean values, and $P < 0.05$ was considered significant. For the determination of EC_{50}, a non-linear regression model was applied.

Preliminary ADME and chemophysical profiling

Solubility assay at pH 7.4. Solubility was assayed in Phosphate Buffered Saline (PBS: 12 mM with NaCl 137 mM and KCl 2.7 mM, pH 7.4). Each solid compound (1 mg) was added to 1 mL of PBS. The samples were shaken in an orbital shaker at 25 °C for 24 h. These suspensions were filtered through a PTFE 0.45 μm filter (VWR), and the solutions were chromatographically analyzed using a Perkin Elmer Ultra High Performance Liquid Chromatography (UHPLC) instrument, equipped with a reverse-phase (RP) C18 Phenomenex column (2.1x100 mm, 1.7 μm particle size). Gradient elution: the ratio of eluents A and B (0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile, respectively) changed linearly from 60% A-40% B to 0% A-100% B in 12 minutes, followed by 5 minutes

in isocratic elution at 100% of eluent B and then 4 minutes in equilibration elution to reset the starting conditions. The flow rate was 0.5 mL/min. Standard injection volumes were either 2 or 4 µl for poorly soluble compounds. The detection system was a Perkin Elmer diode-array-detector. The wavelengths that were monitored for each compound were defined according to the compound's own absorption spectrum. Solubility, expressed as µM concentration of the saturated solution, was calculated via interpolation with external calibration curves that were obtained with solutions of each compound in acetonitrile.

Clog P and log D (pH 7.4). ClogP values were calculated using the Bio-Loom program for Windows, Version 1.5 (BioByte). The partition coefficients between n-octanol and PBS at pH 7.4 (log $D^{7.4}$) were obtained using the shake-flask technique at room temperature. In the shake-flask experiments, 50 mM of phosphate buffered saline pH 7.4 was used as the aqueous phase. The organic (n-octanol) and aqueous phases were mutually saturated by shaking for 4 h. The compounds were solubilized in the buffered aqueous phase at the highest concentration compatible with solubility and appropriate amounts of *n*-octanol were added. The two phases were shaken for about 20 min, by which time the partitioning equilibrium of solutes had been reached, and then centrifuged (10000 rpm, 10 min). The concentration of the solutes was measured in the aqueous phase using a UV spectrophotometer (Varian Cary 50BIO); absorbance values (recorded for each compound at the wavelength of maximum absorption), were interpolated in calibration curves obtained using standard solutions of the compounds (r^2 >0.99). Each log D value is an average of at least six measurements.

Protein binding in vitro. This was achieved via ultrafiltration using commercially available membrane systems (Centrifree ultrafiltration devices with ultracel YM-T membrane, Merck). A solution of the selected compound in DMSO was added to human serum (sterile-filtered from human male AB plasma, Sigma-Aldrich), to give the final concentration of 50 µM with 2% of co-solvent. 1 mL of the solution obtained in the sample reservoir of the ultrafiltration device was gently shaken in an orbital shaker at 37 °C for 1 h. The tube was then centrifuged

at 1000 x g for 15 min. The concentrations of the compounds in the ultrafiltrate and filtrate were determined using reverse-phase UHPLC and the chromatographic conditions were those described above with different injection volumes; 20 µL for ultrafiltrate samples and 2 µL for filtrate samples. The quantitation of the compounds in the filtrate and in ultrafiltrate was performed using two different calibration curves of compound standard solutions (linearity determined in concentration ranges of 0.5-25 µM with injection volume of 20 µL for ultrafiltrate and 10-100 µM with injection volume of 2 µL for filtrate ; $r^2 > 0.99$). The recovery of the ultrafiltration process was calculated in order to discover whether any compound was lost during ultrafiltration, in view of the limited solubility of tested compounds.

$$\text{Recovery} = 100 \times [(\text{vol.}_{\text{bound}} \times \text{conc}_{\text{bound}}) + (\text{vol.}_{\text{unbound}} \times \text{conc}_{\text{unbound}})] / \text{vol}_{\text{initial serum}} \times \text{conc}_{\text{initial}}$$

$\text{vol.}_{\text{bound}}$: calculated by dividing the weight of the bound fraction (difference between the weights of the sample reservoir after ultrafiltration and empty), by its density (0.991 g/mL assessed by weighing five replicates of a known volume of the bound fraction).

$\text{vol.}_{\text{unbound}}$: calculated by dividing the weight of the unbound fraction (difference between the weights of the ultrafiltrate cup after and before ultrafiltration), by its density (0.999 g/mL assessed by weighing five replicates of a known volume of the unbound fraction).

$\text{conc}_{\text{bound}}$: calculated using the RP-HPLC method.

$\text{conc}_{\text{unbound}}$: calculated using the RP-HPLC method (calibration with standard additions)

Average recovery was 90% for all tested compounds.

In-vivo toxicity assays. All procedures have been described previously. [56] Briefly, twelve female Balb/c mice (4-5 weeks old, 16 - 22 g of weight) were randomly assigned to 3 groups

(4 mice in each group) and animals were given access to food and water *ad libitum*. The control group was intraperitoneally inoculated with the vehicle (every 3 days, *i.p),* for 35 days). The other two groups of mice received 10 and 25 mg/kg of **1** (every 3 days, *i.p,* for 35 days) and were weighed before treatment. Compound **1** was dissolved in 50 % PBS, 25 % cremophor (Kolliphor EL) and 25 % ethanol (vehicle). At the end point of the study, we examined the following parameters: mortality, clinical signs, body weight, food consumption (every week), hematology and serum biochemistry parameters. The organs (liver, heart, lung, kidney) were collected at sacrifice and fixed in 4% buffered formaldehyde for histological analyses. At the time of sacrifice, 1 mL of blood from each mouse was collected from the heart, using a hypodermic syringe, stored in a tube containing heparin, and hematological and biochemical analyses were performed. All results were compared to standard parameters for normal mouse blood. These analyses were performed at the Veterinary Analysis laboratory (Turin, Italy). All animal procedures were approved by the Ethics Committee at the University of Turin and by the Italian Ministry of Health (MoH), in compliance with international laws and policies (MoH approved project # 337/216-PR).

In-vitro metabolic behavior:

Incubation conditions and sample preparation. Rat-liver microsomes (Sprague-Dawley, male, Sigma Aldrich; 20 mg/mL protein concentration) were incubated with the candidate compound solution (5 µM final concentration, with 1 % DMSO) and TRIS buffer (0.1 M, pH = 7.4). The regenerating system, which slowly generated coenzyme units over the incubation time, leading to a better reproduction of *in-vivo* behavior, was composed of $MgCl_2$ (3.3 mM), $NADP^+$ (1.3 mM), Glu6P (3.5 mM) and Glu6Pdehydrogenase (0.5 U/mL). In addition to the compound sample that was incubated with active microsomes and the regenerating system ("C") and drug-free matrix blank sample (B), two other series of specimens were used to provide more information for the interpretation of experimental results:

- In the “C1” control sample, the tested drug was incubated with heat-inactivated microsomes (inactivation via a 10 minutes heating cycle at 90 °C).

- In the “C2” control sample, there was no regenerating system in the incubation medium.

The incubation time started with the addition of the microsomes suspension. Time point t_0 was immediately collected and the following samples were collected at 60 and 120 min in order to evaluate short-term stability and longer-term stability, respectively.

Metabolic reactions were stopped by adding 200 µL of cooled acetonitrile to the 100 µL sample of the incubation mixture. Samples were centrifuged to provoke protein precipitation, and the supernatants were immediately stocked at -80 °C, until analysis, to prevent the potential degradation of unstable products.

Identification of metabolites using high-resolution mass spectrometry. The products of *in-vitro* metabolism were identified using a high-resolution mass spectrometer (Q-Exactive Orbitrap, Thermo Scientific) coupled to an HPLC instrument (1200 system Agilent). All analytes were separated on an Ascentis C18 column (150 x 2.1 mm, 5 µm particle size) maintained at 35 °C. The elution mixture was composed of solvent A (0.1% formic acid in water for positive ionization mode and 0.05% acetic acid for negative ionization mode) and solvent B (acetonitrile). The elution gradient was from 1 to 99% of solvent B in 24 minutes; held at 99% for 4 minutes and re-equilibration for 6 min at 1% of solvent B. The injection volume and flow rate were 4 µL and 180 µL/min, respectively. Mass spectrometric analyses were performed in positive- and negative-ion mode using a HESI II source under the following conditions: heated capillary temperature 320 °C, spray voltage 3 kV (positive ions) or -2.2 kV (negative ions), auxiliary gas temperature 160 °C, flow rate of 6 (arbitrary units), sheath-gas flow rate 32 (arbitrary units), sweep-gas flow rate 2 (arbitrary units). Accurate mass measurements were obtained using full-scan mass spectra (resolving power R=70000; mass range: m/z 105-850 Da) and f with data dependent MS2 acquisition, in which the four most abundant ions of the previous full-scan spectrum were selected for fragmentation. After a first explorative analysis,

samples were re-analyzed using a data-independent (DIA) method, in which the molecular ions that corresponded to the metabolites that were found were always selected for MS2 fragmentation. This allowed the acquisition of MS2 spectra even for metabolites present in very low concentrations. MS2-DIA scans were acquired at R = 17500 and with variable collision energies, ranging from 30 to 45, depending on the m/z of the parent ion. Results from the DIA analyses allowed the main characteristic fragments for each metabolite to be identified.

ASSOCIATED CONTENT

Supporting Information. A PDF file containing: synthesis of compounds **36 – 39** and characterization**,** MS2 spectra of hydroxylated metabolites of **1,** a table with docking scores of compounds **4 - 17**, *in vivo* toxicity figures and ^{1}H-NMR, ^{13}C-NMR spectra and HRMS of representative compounds.

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Notes

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