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Imidazopyridine- and Purine-Thioacetamide Derivatives: Potent Inhibitors of Nucleotide Pyrophosphatase/Phosphodiesterase 1 (NPP1)

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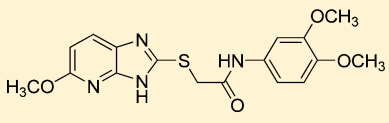
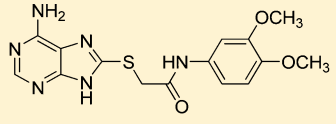
S *Supporting Information*



NPP1 K_i = 0.0296 μM (vs. *p*-Nph-5'-TMP)
NPP1 K_i = 5.34 μM (vs. ATP)



NPP1 K_i = 0.00500 μM (vs. *p*-Nph-5'-TMP)
NPP1 K_i = 18.0 μM (vs. ATP)

ABSTRACT: Nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) belongs to the family of ecto-nucleotidases, which control extracellular nucleotide, nucleoside, and (di)phosphate levels. To study the (patho)physiological roles of NPP1 potent and selective inhibitors with drug-like properties are required. Therefore, a compound library was screened for NPP1 inhibitors using a colorimetric assay with *p*-nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP) as an artificial substrate. This led to the discovery of 2-(3*H*-imidazo[4,5-*b*]pyridin-2-ylthio)-*N*-(3,4-dimethoxyphenyl)acetamide (**5a**) as a hit compound with a K_i value of 217 nM. Subsequent structure−activity relationship studies led to the development of purine and imidazo[4,5-*b*]pyridine analogues with high inhibitory potency (K_i values of 5.00 nM and 29.6 nM, respectively) when assayed with *p*-Nph-5′-TMP as a substrate. Surprisingly, the compounds were significantly less potent when tested versus ATP as a substrate, with K_i values in the low micromolar range. A prototypic inhibitor was investigated for its mechanism of inhibition and found to be competitive versus both substrates.

INTRODUCTION

The enzyme family of human nucleotide pyrophosphatases/phosphodiesterases (NPPs) consists of seven members (NPP1−7). They exist as membrane-bound glycoproteins with an extracellular active site or as soluble proteins in body fluids. These enzymes hydrolyze phosphodiester bonds in a wide variety of substrates. Three members of the NPP family hydrolyze nucleotides, i.e., NPP1 (PC-1), NPP2 (autotaxin), and NPP3. Besides nucleoside di- and triphosphates, they also catalyze the hydrolysis of NAD^+, FAD, UDP-sugars, and dinucleoside polyphosphates. Moreover, artificial phosphoric acid esters, such as *p*-nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP), are accepted by the enzymes as substrates. Ecto-NPPs are able to terminate extracellular nucleotide signaling through the hydrolysis of natural nucleotide agonists of P2X and P2Y receptors. Extracellular nucleotides that are NPP1 substrates, e.g., ATP, UTP, UDP, UDPglucose, and dinucleoside polyphosphates, can activate certain G protein-coupled P2Y receptors and ligand-gated ion channel receptors (P2X receptors). Both receptor families are involved in a wide variety of physiological and pathological processes and conditions. Thus, NPPs modulate purinergic signaling.[1−3]

NPP1 has been implicated in different biological processes. NPP1 is expressed on the extracellular membrane of osteoblasts and chondrocytes and plays an important role in bone mineralization.[4,5] NPP1 has also been reported to affect insulin signaling. Overexpression of NPP1 inhibits insulin receptor tyrosine kinase activity in peripheral tissues that are major targets of insulin action (liver, muscle, and fat), leading to insulin resistance and hyperglycemia in several animal models.[6,7] Therefore, inhibitors of NPP1 hold promise for the treatment of type 2 diabetes. NPP1 expression has been reported to be increased in membranes of rat C6 glioma cells,[8] human astrocytic brain tumors,[9] and human glioblastoma stem-like cells.[10] NPP1 expression was found to be increased according to the grade of the tumors.[9] Therefore, NPP1 inhibitors might be useful for the treatment or prevention of brain cancers. Despite the therapeutic potential of NPP1

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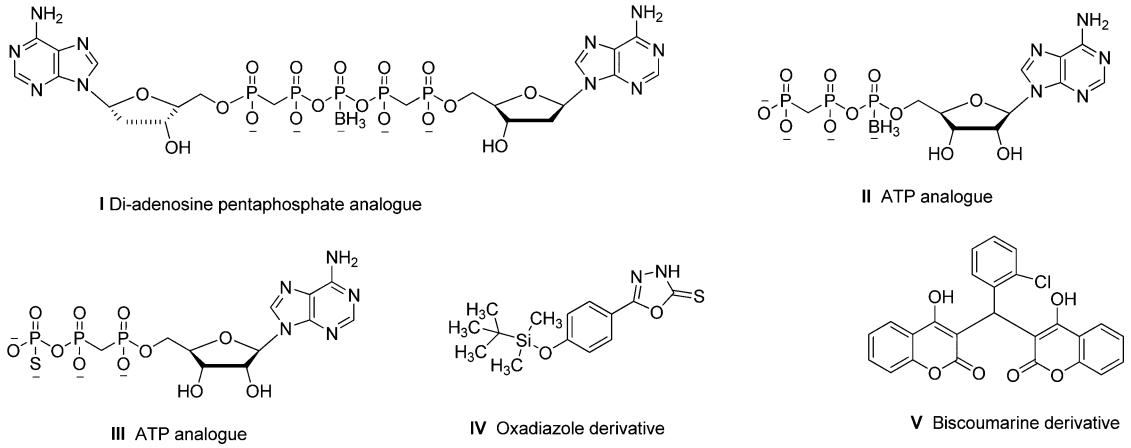

Figure 1. Known NPP1 inhibitors.

inhibitors, only a very limited number of inhibitors are known so far. However, potent and selective NPP1 inhibitors are required as research tools for investigating the (patho)-physiological functions of NPP1.

A series of diadenosine 5′,5″-(borono)polyphosphonate analogues has been prepared, with the most potent congener **I** (Figure 1) displaying a K_i value of 9 μM, using a spectrophotometric assay and *p*-Nph-5′-TMP as a substrate.[11] Recently, a new series of metabolically stable nucleoside diphosphate and triphosphate analogues was designed and prepared. The most promising NPP1 inhibitor of that series (compound **II**, Figure 1) was endowed with a K_i value of 0.5 μM determined with a colorimetric assay using the same artificial substrate.[12] In addition, highly potent and selective NPP1 inhibitors based on an adenosine-5′-methylenetriphosphate scaffold have been prepared.[13] The most potent congener of this series (compound **III**, Figure 1) displayed a K_i value of 20 nM at NPP1 using *p*-Nph-5′-TMP as surrogate substrate. Although these are promising results, the described compounds suffer from laborious preparation and tedious purification. In addition, these nucleotide analogues are negatively charged at physiological pH and it will be difficult to develop them as orally bioavailable drugs. Therefore, our strategy was to focus on the identification of small-molecule heterocyclic compounds as potential inhibitors of NPP1 rather than targeting nucleotide-based inhibitors. So far only very few reports describe SAR studies of heterocyclic structures as inhibitors of NPP1. A series of 1,3,4-oxadiazole-2(3*H*)-thione derivatives was evaluated as NPP1 inhibitors, of which the best compound (**IV**, Figure 1) was endowed with a K_i value of only 360 μM.[14] Among a series of biscoumarine derivatives, the most active member **V** (Figure 1) displayed a K_i value of 50 μM.[15] These marginal inhibitory activities do not allow researchers to use these compounds in cell biological experiments. Recently, quinazolin-4-piperidine-4-ethylsulfamide derivatives were reported as potent NPP1 inhibitors, but they also show high affinity binding to hERG potassium channels, which results in drug-induced QT prolongation and precludes their development as drugs.[16]

To identify novel inhibitors of NPP1, a compound library was screened and the main hit was optimized to obtain highly potent and selective inhibitors of NPP1.

RESULTS AND DISCUSSION

Screening of a Library for NPP1 Inhibitors. A commercial compound library consisting of 1612 compounds based on different bicyclic scaffolds (such as benzimidazoles, indoles, quinazolines, and quinolines) was screened in a high-throughput manner for inhibition of NPP1 using a spectrophotometric method with *p*-Nph-5′-TMP as an artificial substrate.[17] The main hit was 2-(3*H*-imidazo[4,5-*b*]pyridin-2-ylthio)-*N*-(3,4-dimethoxyphenyl)acetamide (**5a**, Figure 2), which was characterized as a potent NPP1 inhibitor with a K_i value of 217 nM.[17]

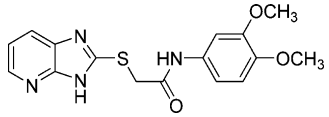

Figure 2. Hit compound **5a** identified as a potent NPP1 inhibitor (K_i = 217 nM).

Because of the drug-like properties of the hit compound, we decided to synthesize a series of derivatives and analogues to study the SARs of this new, non-nucleotidic class of NPP1 inhibitors.

Chemistry. The hit compound **5a** was resynthesized as shown in Scheme 1 in order to confirm its biological activity. A series of derivatives was prepared according to the same procedure. This procedure was also used for the preparation of a number of analogues with a different bicyclic scaffold (purine, benzimidazole, imidazo[4,5-*c*]pyridine, and imidazo[4,5-*b*]-pyrazine) and for the preparation of imidazo[4,5-*b*]pyridine and purine derivatives with simple substituents (e.g., chlorine, bromine, amine, and hydroxyl). The synthesis started from appropriate diamino-substituted building blocks **1a**−**i**. Most of these starting materials were commercially available, with the exception of pyrazine-2,3-diamine **1i**, which was accessible from 3-chloropyrazin-2-amine according to a published procedure.[18] Treatment of **1a**−**i**, with either potassium ethyl xanthate or carbon disulfide as one-carbon equivalents, gave access to a series of novel bicyclic scaffolds **2a**−**i**. Subsequent alkylation of the thio group with alkyl chloride **4** (derived from the reaction of 3,4-dimethoxyaniline **3** and 2-chloroacetyl chloride)[19] yielded compounds **5a**−**i** (Table 1).

A simplified analogue of the parent compound **5a**, in which the pyridinyl moiety is absent, was prepared from 2-

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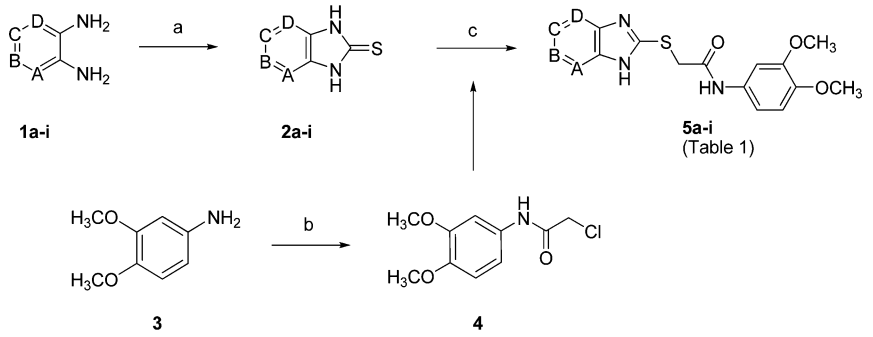
Scheme 1. Synthesis of Bicyclic *N*-(3,4-Dimethoxyphenyl)-2-mercaptoacetamide Analogues[a]



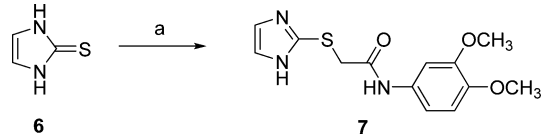
[a]Reagents and conditions: (a) ethyl xanthate, EtOH, H_2O, 80 °C or CS_2, KOH or $NaHCO_3$, EtOH, H_2O, 80 °C; (b) 2-chloroacetyl chloride, Et_3N, CH_2Cl_2, rt; (c) DMF, K_2CO_3, **4**, 85 °C.

Table 1. Overview of Synthesized Bicyclic *N*-(3,4-Dimethoxyphenyl)-2-mercaptoacetamide Analogues

compd	A	B	C	D	target scaffold
1a, 2a, 5a	–N–	–CH–	–CH	–CH–	imidazo[4,5-*b*]pyridine
1b, 2b, 5b	–N–	–CH–	–CCl–	–CH–	imidazo[4,5-*b*]pyridine
1c, 2c, 5c	–N–	–CH–	–CBr–	–CH–	imidazo[4,5-*b*]pyridine
1d, 2d, 5d	–CH–	–CH–	–CH	–CH–	benzimidazole
1e, 2e, 5e	–CH–	–N–	–CH	–CH–	imidazo[4,5-*c*]pyridine
1f, 2f, 5f	–N–	–CH–	–N–	–CH–	purine
1g, 2g, 5g	–N–	–CH–	–N–	$-CNH_2-$	purine
1h, 2h, 5h	–N–	–CH–	–N–	–COH–	purine
1i, 2i, 5i	–N–	–CH–	–CH–	–N–	imidazo[4,5-*b*]pyrazine

thioimidazole (**6**) and alkyl chloride **4**, yielding derivative **7** (Scheme 2).

Scheme 2. Synthesis of Imidazole Derivative 7[a]



[a]Reagents and conditions: (a) DMF, K_2CO_3, **4**, rt.

Some congeners were synthesized from commercially available 3-nitropyridine derivatives **8**–**9** (Scheme 3). Reduction of the nitro group was achieved either chemically (using iron and calcium chloride) or catalytically (using hydrogen gas and Pd/C as a catalyst), affording the 2,3-diaminopyridine derivatives **10** and **11**. Subsequent imidazole ring formation followed by alkylation led to the target compounds **12** and **13**.

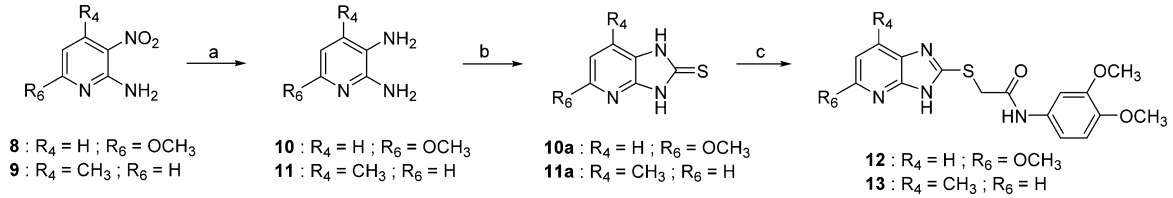
To elongate and branch the linker between the aromatic ring structures, two different alkyl chlorides, **14** and **15**, were prepared from 3,4-dimethoxyaniline (**3**), using an appropriate acyl chloride (Scheme 4). Coupling with 2-thioimidazo[4,5-*b*]pyridine **2a** yielded access to derivatives **17** and **18**, respectively.

The synthesis of a sulfonamide derivative started with the condensation of 3,4-dimethoxyaniline (**3**) with chloromethanesulfonyl chloride, yielding sulfonamide derivative **16**. Coupling with the imidazo[4,5-*b*]pyridine derivative **2a** furnished the final compound **19** (Scheme 4).

For insertion of a methylene linker between the amino group and the dimethoxyphenyl moiety, 3,4-dimethoxybenzylamine (**20**) was selected as starting material (Scheme 5). Acylation yielded the alkyl chloride **21**, which was used to alkylate 2-thioimidazo[4,5-*b*]pyridine (**2a**), leading to the desired derivative **22**.

The synthesis of a series of imidazo[4,5-*b*]pyridines **28a**–**f** is depicted in Scheme 6. Commercially available 2-amino-6-chloro-3-nitropyridine **23** was converted to 6-substituted-2-amino-3-nitropyridine derivatives **24a**–**f** by nucleophilic aromatic substitution with a selection of amines and sodium alkoxides. The thiomethyl derivative **24f** was obtained via the 2-thio-pyridine derivative **25** by methylation with iodomethane.

Scheme 3. Synthesis of Imidazo[4,5-*b*]pyridine Derivatives 12 and 13[a]



[a]Reagents and conditions: (a) for **10**, Fe, $CaCl_2$, EtOH, H_2O, 60 °C, for **11**, H_2, Pd/C, THF, rt; (b) CS_2, KOH, EtOH, H_2O, reflux; (c) DMF, **4**, K_2CO_3, 90 °C.

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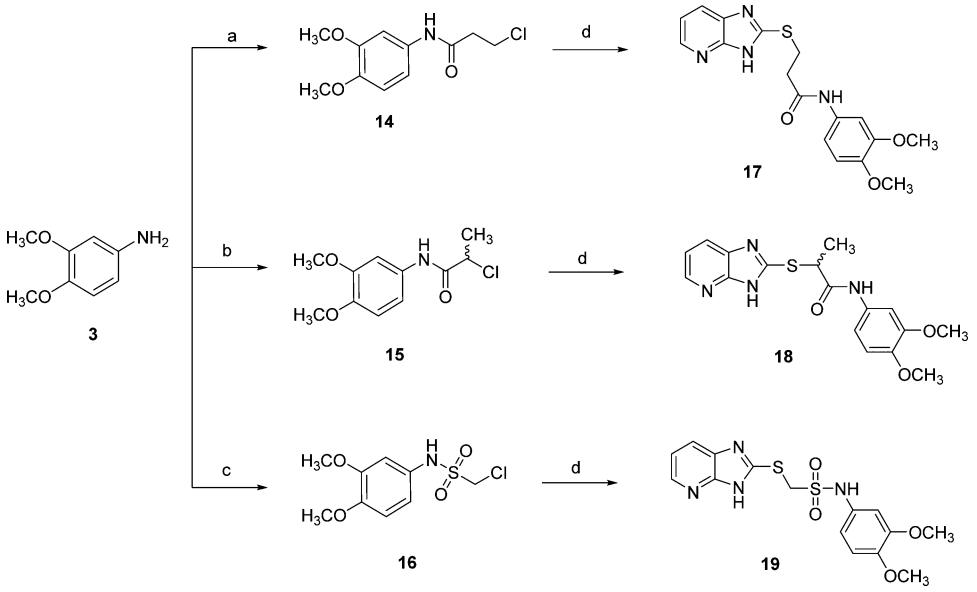
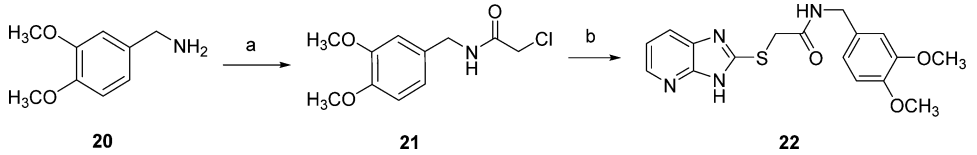
Scheme 4. Synthesis of Imidazo[4,5-*b*]pyridine Derivatives 17−19[a]



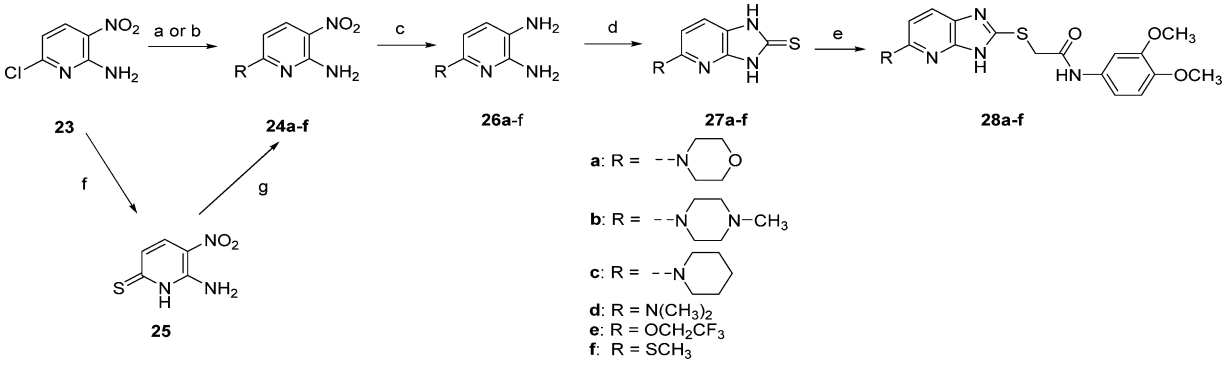
[a]Reagents and conditions: (a) 2-chloropropionyl chloride, Et_3N, CH_2Cl_2, 0 °C to rt; (b) 3-chloropropionyl chloride, Et_3N, CH_2Cl_2, 0 °C to rt; (c) chloromethanesulfonyl chloride, CH_2Cl_2, Et_3N, rt; (d) **2a**, K_2CO_3, DMF, 80 °C.

Scheme 5. Synthesis of Imidazo[4,5-*b*]pyridine Derivative 22[a]



[a]Reagents and conditions: (a) 2-chloroacetyl chloride, CH_2Cl_2, Et_3N, 0 °C to rt; (b) DMF, K_2CO_3, **2a**, 80 °C.

Scheme 6. Synthesis of Imidazo[4,5-*b*]pyridine Derivatives 28a−f[a]



[a]Reactions and conditions: (a) for **24a−d**, RH, K_2CO_3, DMF, 80 °C; (b) for **24e**, CF_3CH_2OH, NaH, THF, rt; (c) for **26a−e**, Pd/C, H_2, MeOH, rt; for **26f**, $Na_2S_2O_4$, H_2O, MeOH, 85 °C; (d) for **27a−e**, CS_2, MeOH, 50 °C; for **27f**, CS_2, $NaHCO_3$, EtOH, H_2O, 55 °C; (e) **4**, NaOH, MeOH/EtOH, rt; (f) NaSH, DMF, 80 °C; (g) MeI, K_2CO_3, DMF, rt.

Catalytic or chemical reduction of the nitro group of pyridines **24a−f**, followed by cyclization by treatment with carbon disulfide, yielded the 2-thio-imidazo[4,5-*b*]pyridines **27a−f**. Alkylation of the thio moiety provided target compounds **28a−f**.

Furthermore, a series of purine analogues was synthesized containing an amino group and electron-donating group at positions 2 and 6 of the purine moiety, respectively (Scheme 7). To obtain building blocks **32a−e**, 2-amino-5-nitrosopyrimidine derivatives **31a−e** were used as starting material (Scheme 7B). The advantage of this class of compounds is that they are chemically stable in free form (not as a salt) and they are either commercially available or easy to synthesize (Scheme 7A). Upon treatment of 5-nitrosopyrimidines **31a−e** with sodium dithionite,[20,21] the crude 2,5,6-triaminopyrimidine derivatives **32a−e** were obtained, which were reacted with CS_2, affording 8-thiopurines **33a−e**. Alkylation with alkyl chloride **4** afforded the final compounds **34a−e**.

In a similar way, 2-methylthio-6-aminopurine **40** was prepared (Scheme 8). 4-Amino-6-chloro-2-methylthiopyrimi-

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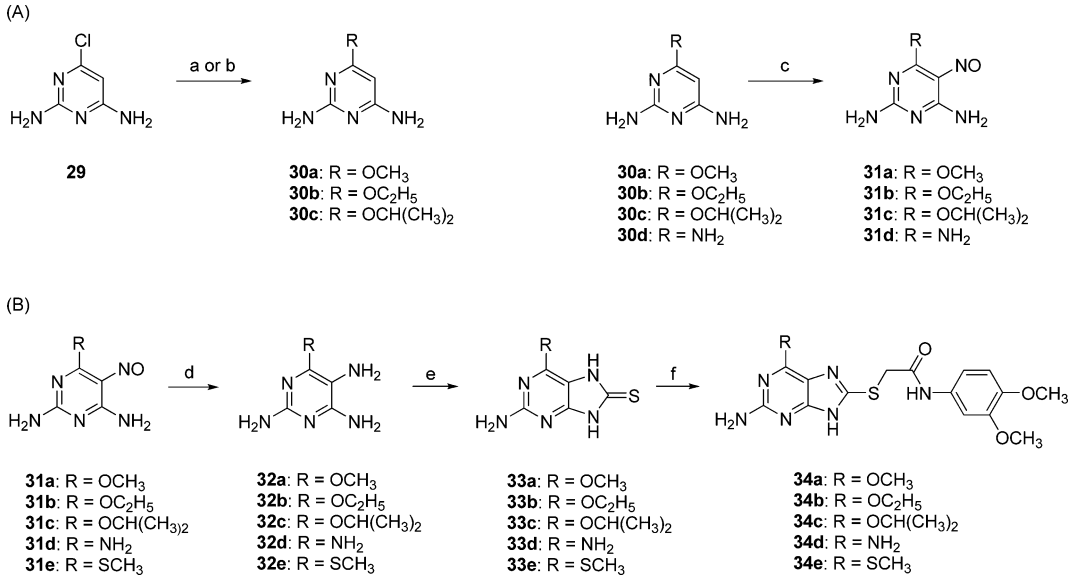
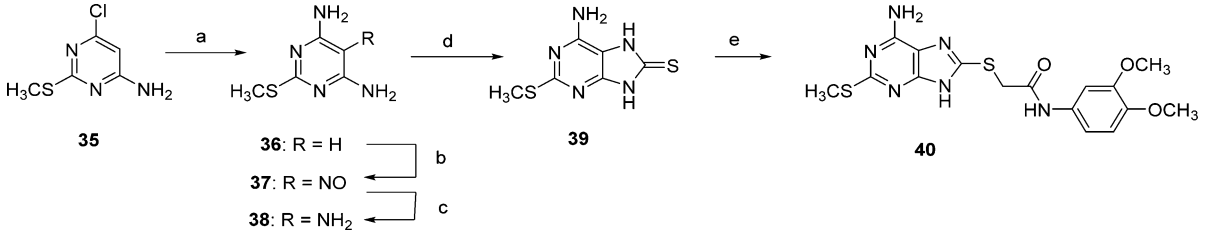
Scheme 7. Synthesis of Purine Derivatives 34a–e[a]



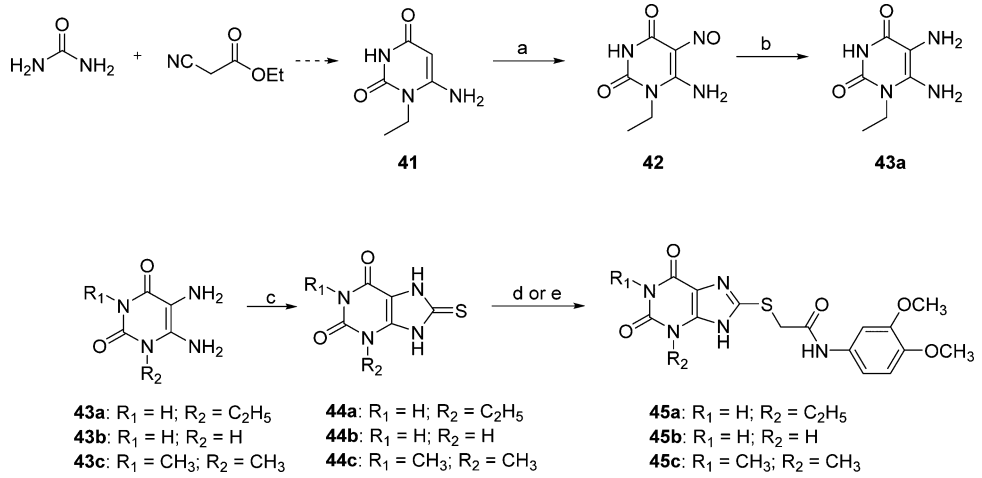
[a]Reagents and conditions: (a) NaOR, ROH, reflux; (b) *i*-PrOH, NaH, reflux; (c) $NaNO_2$, AcOH, H_2O, rt–60 °C; (d) $Na_2S_2O_4$, H_2O, MeOH, 50–70 °C; (e) CS_2, $NaHCO_3$, H_2O, EtOH, reflux; (f) **4**, K_2CO_3, DMF, rt–85 °C.

Scheme 8. Synthesis of Purine Derivative 40[a]



[a]Reagents and conditions: (a) NH_3/MeOH, 120 °C; (b) $NaNO_2$, AcOH, H_2O, rt; (c) $Na_2S_2O_4$, H_2O, MeOH, 50–70 °C; (d) CS_2, $NaHCO_3$, H_2O, EtOH, reflux; (e) **4**, NaOH, H_2O, rt.

Scheme 9. Synthesis of Xanthine Derivatives 45a–c[a]



[a]Reactions and conditions: (a) $NaNO_2$, AcOH, H_2O, EtOH, rt; (b) $Na_2S_2O_4$, H_2O, MeOH, 50–70 °C; (c) CS_2, $NaHCO_3$, H_2O, EtOH, reflux; (d) **4**, K_2CO_3, DMF, rt–85 °C; (e) **4**, NaOH, H_2O, rt.

dine (**35**) was treated with ammonia in methanol to form the diaminopyrimidine derivative **36**, which was then reacted with $NaNO_2$, affording the 5-nitrosopyrimidine **37**. Subsequent reduction followed by cyclization with carbon disulfide gave access to the 8-thiopurine derivative **39**. Reaction of **39** with alkyl chloride **4** afforded product **40** in moderate yield.

On the basis of the chemistry described above, xanthine derivatives **45a–c** were synthesized (Scheme 9). Whereas

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commercially available diaminouracil derivatives **43b–c** were used, 5,6-diamino-1-ethyluracil (**43a**) was prepared from compound **41**.[22,23] Reaction of **41** with $NaNO_2$ under acidic conditions yielded the desired 5-nitrosouracil derivative **42**, which was reduced to the corresponding 5,6-diaminouracil **43a**.[23] Treatment of 5,6-diaminouracil **43a–c** with carbon disulfide, followed by alkylation with alkyl chloride **4**, afforded the xanthine derivatives **45a–c** in moderate to good yields.

Biological Evaluation. All final products were initially investigated at a concentration of 10 μM for their inhibition of soluble human NPP1 versus the artificial substrate *p*-Nph-5′-TMP (400 μM). For compounds that showed at least 70% inhibition at 10 μM, concentration–inhibition curves were determined and K_i values were calculated from the obtained IC_{50} values using the Cheng–Prusoff equation and the K_m value of 222 μM determined for *p*-Nph-5′-TMP.[24] The most potent NPP1 inhibitors were additionally investigated versus the natural substrate ATP (400 μM) using a capillary electrophoresis (CE) assay, concentration–inhibition curves were determined, and K_i values were calculated from the obtained IC_{50} values using the Cheng–Prusoff equation and the K_m value of 8.17 μM determined for ATP. For compound **5a**, the mechanism of NPP1 inhibition was investigated by determining enzyme kinetics in the presence of different concentrations of inhibitor. To investigate the selectivity of the compounds for NPP1 versus other ecto-NPPs, the effects of the most potent compounds on human NPP2 and NPP3 were also determined.

Structure–Activity Relationships. To understand the structure–activity relationships (SARs) of the newly identified class of NPP1 inhibitors, hit compound **5a** was divided into three fragments (Figure 3): part A, the terminal aryl moiety, part B, the linker region, and part C, the bicyclic scaffold.

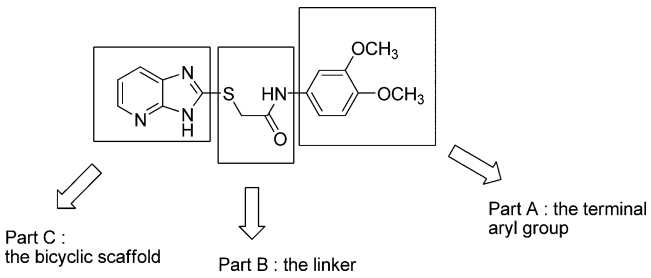

Figure 3. Subdivision of the identified NPP1 inhibitor lead structure into three structural parts.

Modification of the Terminal Aryl Group (part A). To probe the optimal substitution pattern on the phenyl ring, a series of differently substituted derivatives was purchased (Table 2, compounds **C1–C20**). Most of the analogues were devoid of NPP1 inhibitory activity. Only three compounds (**C7**, **C8**, and **C12**) showed significant activity, displaying around 50% inhibition at 10 μM. Substitution in the *p*- and *m*-position was better tolerated than *o*-substitution. However, all of the compounds were less potent than the 3,4-dimethoxyphenyl-substituted lead structure **5a**, indicating that this was already the best substitution pattern.

Modification of the Linker (Part B). As the 3,4-dimethoxyphenyl moiety was optimal for NPP1 inhibition, this structural element was kept constant for future SAR studies. The linker was elongated and modified in different ways (Table 3). Elongation by a methylene unit on either side of the linker (compounds **17** and **22**) virtually abolished NPP1 inhibitory potency. Similarly, branching of the methylene group (giving rise to racemate **18**) led to a loss in activity. Replacement of the amide by a sulfonamide moiety (compound **19**) afforded a compound displaying 50% inhibition of NPP1 activity at a concentration of 10 μM, showing that the sulfonamide acted as a bioisosteric amide replacement although with lower potency.

Modification of the Bicyclic Scaffold (Part C). Since it had become clear that structural variation was neither tolerated at the terminal aryl group nor at the linker, we kept both parts A and B fixed and examined modifications of the bicyclic imidazopyridine structure. In contrast to parts A and B, structural modifications were tolerated at the bicyclic scaffold C (Table 4). The simplified imidazole analogue **7** still showed substantial inhibitory activity (64% inhibition at 10 μM). A number of novel core structures showed similarly high activity as the original imidazo[4,5-*b*]pyridine scaffold **5a**. The imidazo[4,5-*c*]pyridine **5e**, the imidazo[4,5-*b*]pyrazine **5i**, and the purine derivative **5f** were endowed with low K_i values ranging from 0.0442 to 0.281 μM. The shift of an H-bond accepting N atom in the imidazo[4,5-*b*]pyridine core resulted in an increase in potency by a factor of 5 (compare **5a** and **5e**). However, the absence of any H-bond acceptor site in the ring that is fused to the imidazole led to a loss of potency (as observed in benzimidazole analogue **5d**).

Two nitrogen atoms were well tolerated in the 6-membered ring, as exemplified by the purine analogue **5f** and the imidazo[4,5-*b*]pyrazine derivative **5i**. For further SAR studies, the imidazo[4,5-*b*]pyridine and the purine skeletons were selected to investigate the optimal substitution patterns of the scaffolds. The pyridine moiety of the imidazo[4,5-*b*]pyridine scaffold allowed structural variation at different positions (Table 5). Whereas the introduction of electron-withdrawing groups such as halogen atoms at position 6 (**5b** and **5c**) or electron-donating groups like a methyl substituent in position 7 (**13**) resulted in a slight increase in potency (K_i values of 0.137, 0.192, and 0.127 μM, respectively), the substitution with an electron-donating methoxy group at position 5 led to significantly increased potency (K_i value of 0.0296 μM, compound **12**). This result prompted us to synthesize a series of derivatives **28a–f** with structural variation at position 5 of the imidazo[4,5-*b*]pyridine scaffold. Unfortunately, all cyclic amines were devoid of NPP1 inhibition, and only the dimethylamino-substituted compound **28d**, the trifluoroethoxy derivative **28e**, and the thiomethyl derivative **28f** were endowed with NPP1 inhibitory activity, displaying ca. 60% inhibition at 10 μM. These results suggest that only small, aliphatic substituents are tolerated at that position, with a methoxy group being optimal.

Besides the imidazo[4,5-*b*]pyridine scaffold, the SARs of the purine scaffold were investigated in detail (Table 6). Those derivatives appeared to be of particular interest as they are related to adenine, which is the purine base part of ATP, the natural substrate of NPP1. As such, they might act as competitive inhibitors of the natural substrate. The introduction of a hydroxyl (affording hypoxanthine analogue **5h**) or an amino group (yielding adenine derivative **5g**) on the purine scaffold led to a boost in potency, yielding derivatives with K_i values of 0.0200 and 0.00500 μM, respectively. The insertion of an additional hydroxyl group at position 2 yielded xanthine derivative **45b**, which was still endowed with potent inhibition (K_i = 0.0358 μM). On the other hand, the 2,6-diaminopurine derivative **34d** showed an almost 1000-fold loss in potency (K_i

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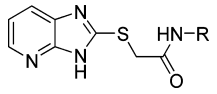
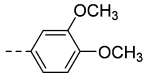
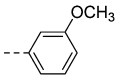
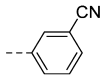
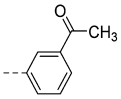
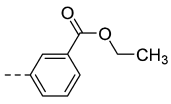
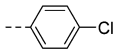
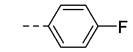
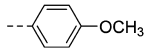
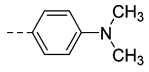
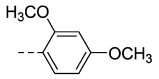
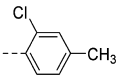
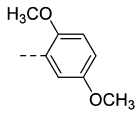
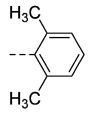
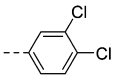
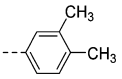
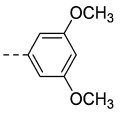
Table 2. NPP1 Inhibitory Potency of Imidazopyridine Derivatives with Differently Substituted Phenyl Substituent (Modification of Part A)



Cmpd.	R	K_i ± SEM (μM) *(or % inhibition ± SD at 10 μM vs. p-Nph-5'-TMP)*[a]
5a (hit)		**0.217** ± 0.052[b]
C1		*(30 ± 1%)*
C2		*(19 ± 2 %)*
C3		*(13 ± 2%)*
C4		*(21 ± 1%)*
C5		*(23 ± 2%)*
C6		*(30 ± 3%)*
C7		*(45 ± 1%)*
C8		*(50 ± 1%)*
C9		*(15 ± 1%)*
C10		*(17 ± 2%)*
C11		*(30 ± 3%)*
C12		*(48 ± 2%)*
C13		*(23 ± 3%)*
C14		*(8 ± 1%)*
C15		*(2 ± 1%)*
C16		*(12 ± 3%)*
C17		*(19 ± 1%)*
C18		*(10 ± 2%)*
C19		*(11 ± 2%)*
C20		*(38 ± 2%)*

[a]Three separate experiments were performed each in duplicate. [b]Results from the ref 17.

= 3.48 μM) when compared to the 6-amino congener **5g**. In contrast, the presence of a thiomethyl group at position 2 of the adenine core led to compound **40**, with a K_i value of 0.152 μM. Mono- and dialkylation of xanthine **45b** afforded compounds **45a** and **45c**. As both derivatives were found to be only weak NPP1 inhibitors, the presence of hydrogen bond donors at these positions appears to be required. Finally, a series of 2-amino-6-substituted purine derivatives **34a–d** was evaluated as NPP1 inhibitors. These compounds were in general much less potent, with the 6-ethoxy derivative having the most promising activity, displaying a K_i value of 1.01 μM. The obtained concentration–inhibition curves for the hit compound **5a** as well as the optimized imidazo[4,5-*b*]pyridine and purine derivatives (**5g**, **5h**, **12**, and **45b**) are presented in Figure 4.

Investigation of Enzyme Inhibition Type. The parent compound **5a** was further investigated to determine its mechanism of inhibition with both the artificial and the natural substrate (*p*-Nph-5′-TMP and ATP, respectively). Thus, enzyme kinetics were determined in the absence and in the presence of various concentrations of inhibitor. The obtained data indicated that inhibitor **5a** displays competitive inhibition of human NPP1, which could be confirmed versus both investigated substrates. Figure 5 shows the Lineweaver–Burk plots for **5a** versus the artificial substrate (A) and versus the

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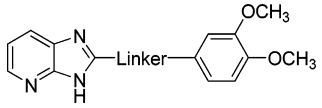
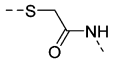
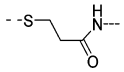
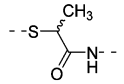
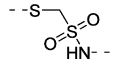
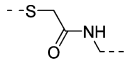
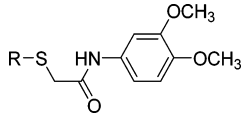
Table 3. NPP1 Inhibitory Potency of Imidazopyridine Derivatives with Different Linkers (Modification of Part B)

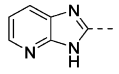
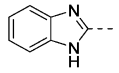
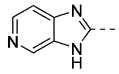
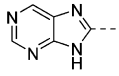
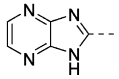


Cmpd.	Linker	K_i ± SEM (μM) *(or % inhibition ± SD at 10 μM vs. p-Nph-5'-TMP)*[a]
5a (hit)		**0.217** ± 0.052[b]
17		*(13 ± 3%)*
18		*(6 ± 3%)*
19		*(50 ± 4%)*
22		*(0 ± 1%)*

[a]Three separate experiments were performed each in duplicate. [b]Results from ref 17.

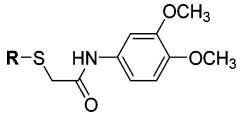
Table 4. NPP1 Inhibitory Potency of Imidazopyridine Derivatives and Analogues with Modification of the Bicyclic Ring Structure (Modification of Part C)

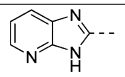
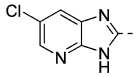
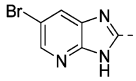
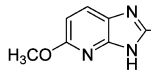
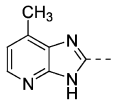
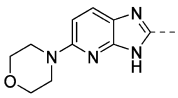
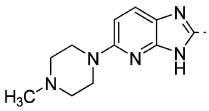
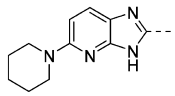
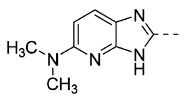
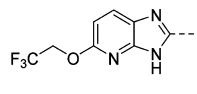
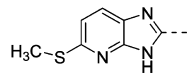


Cmpd.	R	K_i ± SEM (μM) *(or % inhibition ± SD at 10 μM vs. p-Nph-5'-TMP)*[a]
5a (hit)		**0.217** ± 0.052[b]
5d		*(34 ± 1%)*
5e		**0.0442** ± 0.0035
5f		**0.281** ± 0.042
5i		**0.131** ± 0.008
7		*(64 ± 5%)*

[a]Three separate experiments were performed each in duplicate. [b]Results from ref 17.

Table 5. NPP1 Inhibitory Potency of Imidazo[4,5-*b*]pyridines Substituted at the Pyridine Ring



Cmpd.	R	K_i ± SEM (μM) *(or % inhibition ± SD at 10 μM vs. p-Nph-5'-TMP)*[a]
5a (hit)		**0.217** ± 0.052[b]
5b		**0.137** ± 0.043
5c		**0.192** ± 0.003
12		**0.0296** ± 0.0100
13		**0.127** ± 0.036
28a		*(45 ± 1%)*
28b		*(35 ± 1%)*
28c		*(27 ± 1%)*
28d		*(58 ± 1%)*
28e		*(61 ± 3%)*
28f		*(59 ± 2%)*

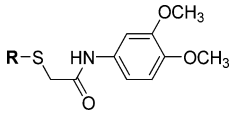
[a]Three separate experiments were performed each in duplicate. [b]Results from ref 17.

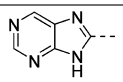
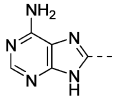
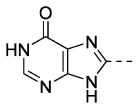
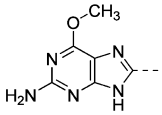
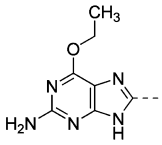
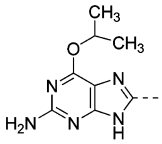
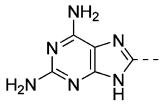
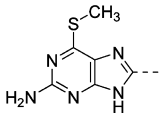
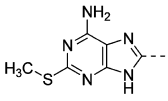
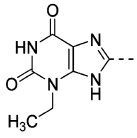
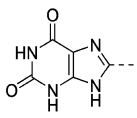
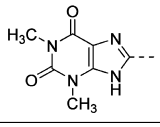
natural substrate ATP (B). The Linewaver–Burk plots visualize the competitive mechanism of inhibition by showing the same *y*-intercept for uninhibited and inhibited enzyme.

Evaluation of Selected Compounds versus the Natural Substrate ATP. Compounds which had shown greater than 70% inhibition of NPP1 at 10 μM concentration in initial screening assays versus the artificial substrate *p*-Nph-5′-TMP were further investigated versus the natural substrate

dx.doi.org/10.1021/jm501434y | J. Med. Chem. XXXX, XXX, XXX–XXX

Table 6. NPP1 Inhibitory Potency of Substituted Purine Derivatives



Cmpd.	R	K_i ± SEM (μM) *(or % inhibition ± SD at 10 μM vs. p-Nph-5'-TMP)* [a]
5f		**0.281** ± 0.042
5g		**0.00500** ± 0.00077
5h		**0.0200** ± 0.0012
34a		**8.04** ± 0.04
34b		**1.01** ± 0.31
34c		**11.0** ± 0.6
34d		**3.48** ± 0.13
34e		**3.65** ± 0.12
40		**0.152** ± 0.025
45a		*(43 ± 2%)*
45b		**0.0358** ± 0.0004
45c		*(23 ± 0%)*

[a]Three separate experiments were performed each in duplicate.

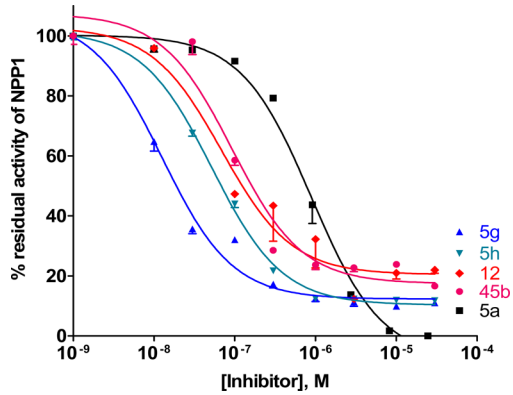


Figure 4. Concentration–inhibition curve of the hit compound **5a** (square) and optimized imidazo[4,5-*b*]pyridine and purine derivatives **5g** (triangle), **5h** (upside down triangle), **12** (tilted square), and **45b** (circle) as inhibitors of NPP1 determined versus the artificial substrate *p*-Nph-5′-TMP. Human enzyme K_m, 222 μM; *p*-Nph-5′-TMP concentration, 400 μM. Data points are from three separate experiments performed in duplicate.

ATP. Table 7 shows the determined K_i values of the presumably competitive inhibitors (see above) versus both substrates. Surprisingly, all investigated compounds displayed significantly lower NPP1 inhibitory potency when investigated versus the natural substrate ATP as compared to the values determined versus the artificial substrate. K_i values were 100–1000-fold higher when determined versus ATP than those obtained versus *p*-Nph-5′-TMP. In a previous study, a similar trend, although less pronounced, had been observed for the standard competitive NPP1 inhibitor 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate (bzATP), which had shown a 5-fold higher K_i value versus the natural substrate ATP as compared to the artificial substrate *p*-Nph-5′-TMP (14.6 μM versus ATP, 2.96 μM *p*-Nph-5′-TMP).[25]

Curiously, the discrepancy between the K_i values was generally higher for purine derivatives than for imidazopyridines. Imidazo[4,5-*b*]pyridines **5b**, **5c**, **12**, and **13** were the most potent inhibitors versus ATP of the present series, with K_i values in the low micromolar range.

Figure 6 shows the correlation of the pK_i values of the 17 most potent NPP1 inhibitors of the present series obtained versus ATP as a substrate and *p*-Nph-5′-TMP as a substrate, respectively. Most of the data were within the 95% confidence band or at its boundary of the best fit line of the linear regression, but the determined correlation coefficient (R^2) between both assays of 0.5046 was fairly low.

A possible explanation for the observed discrepancy might be that, in addition to the orthosteric substrate binding site, there may be an additional allosteric site to which the substrates can bind. Binding of the compounds to that allosteric site may modulate the conformation of the active site depending on the structure of the substrate and thus result in different affinities for the same inhibitor in the different assays. This hypothesis will have to be examined in future studies.

Selectivity. Finally, we investigated the selectivity of the most potent NPP1 inhibitor (**12**) versus the other members of the ecto-NPP family, namely NPP2 and NPP3. This compound was found to be a competitive inhibitor of both NPP subtypes (data not shown). Our study indicated that the selected

dx.doi.org/10.1021/jm501434y | *J. Med. Chem.* XXXX, XXX, XXX–XXX

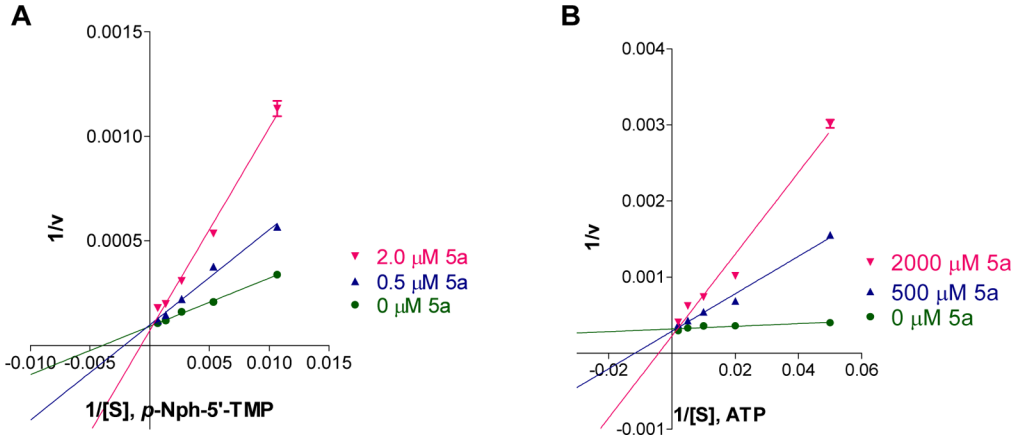


Figure 5. (A) Lineweaver–Burk plot of human NPP1 inhibition by compound **5a**, [S], concentration of substrate *p*-Nph-5′-TMP in μM. Concentration of **5a**: circle, 0 μM; triangle, 0.5 μM; upside down triangle, 2.0 μM. (B) Lineweaver–Burk plot of human NPP1 inhibition by compound **5a**, [S], concentration of substrate ATP in μM. Concentration of **5a**: circle, 0 μM; triangle, 500 μM; upside down triangle, 2000 μM.

compound was 13-fold more potent to block human NPP1 (K_i = **5.34** ± 0.06 μM vs ATP, see Table 7) compared to human NPP2 (K_i = **71.2** ± 1.8 μM determined versus its natural substrate lysophosphatidylcholine (18:1)) and human NPP3 (K_i = **68.1** ± 2.5 μM determined versus ATP). Therefore, it could be characterized as a selective inhibitor of NPP1.

CONCLUSIONS

High-throughput screening utilizing colorimetric assays with the artificial substrate *p*-Nph-5′-TMP led to the identification of 2-((3*H*-imidazo[4,5-*b*]pyridin-2-yl)thio)-*N*-(3,4-dimethoxyphenyl)acetamide (**5a**) as a new, potent inhibitor of human NPP1 (K_i = 0.217 μM). The compound behaved as a competitive NPP1 inhibitor. Initial SAR studies revealed that the 3,4-dimethoxyphenyl group and the thioacetamide linker moiety are essential for NPP1 inhibition. The SARs suggests that different bicyclic scaffolds (imidazo[4,5-*b*]pyridine, imidazo[4,5-*c*]pyridine, imidazo[4,5-*b*]pyrazine, purine) are tolerated by NPP1. Two of these core structures (imidazo-[4,5-*b*]pyridine and purine) were selected for extended SAR studies. In each of these series, potent NPP1 inhibitors were discovered, with a K_i values of 5.00 nM for purine analogue **5g** and a K_i value of 29.6 nM for imidazo[4,5-*b*]pyridine **12**. Surprisingly, these compounds displayed considerably lower NPP1 inhibitory potency when they were tested against the natural substrate ATP instead of the artificial substrate *p*-Nph-5′-TMP. These results suggest that care needs to be taken with the interpretation of results of screening assays using artificial substrates. Further investigations to explain the discrepancy between results with the artificial and natural substrates are in progress. *N*-(3,4-Dimethoxyphenyl)-2-(5-methoxy-3*H*-imidazo-[4,5-*b*]pyridin-2-ylthio)acetamide (**12**), the most potent inhibitor of ATP hydrolysis by NPP1 of the present series (K_i = 5.34 μM), was 13-fold selective versus the ecto-NPP isoenzymes NPP2 and NPP3.

EXPERIMENTAL SECTION

Chemistry. *General.* The compound library contained in total 1612 small-molecule heterocyclic compounds from different suppliers. The 555 analogues were purchased from ChemDiv (San Diego, CA, USA), 360 heterocycles were from InterBioScreen Ltd. (Moscow, RUS), and 697 derivatives were obtained from ChemBridge Corporation (San Diego, CA, USA). All compounds were dissolved in DMSO (at a concentration of 10 mM) prior to screening. For all reactions, analytical grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glassware (135 °C). ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 300 (^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz) or Bruker Avance 500 (^{1}H NMR, 500 MHz; ^{13}C NMR, 125 MHz), using tetramethylsilane as internal standard for ^{1}H NMR spectra, residual solvent peak for DMSO-d_6 (39.52 ppm), or $CDCl_3$ (77.16 ppm) for ^{13}C NMR spectra. Abbreviations used are: s, singlet; d, doublet; t, triplet; q, quartet; sep, septet; m, multiplet; br, broad signal. Coupling constants are expressed in Hz. Mass spectra were obtained with a Finnigan LCQ Advantage Max (ion trap) mass spectrophotometer from Thermo Finnigan, San Jose, CA, USA. High resolution mass spectrometry spectra were acquired on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were infused at 3 μL/min, and spectra were obtained in positive or negative ionization mode with a resolution of 15000 (fwhm) using leucine enkephalin as lock mass. Precoated aluminum sheets (Fluka Silica gel/TLC-cards, 254 nm) were used for TLC. Column chromatography was performed on ICN silica gel 63–200, 60 Å. The purity of the final compounds was determined by RP-HPLC analysis on a XBridge column (C-18, 5 μm, 4.6 mm × 150 mm) in combination with a Waters 600 HPLC system and a Waters 2996 photodiode array detector from Waters, Milford, Massachusetts, USA. Elution was done at flow 1 mL/min using a gradient mixture of H_2O containing 0.2% (vol) of TFA and an organic solvent, either MeOH or CH_3CN as stated in Tables S1–S2 of the Supporting Information. All compounds were at least 95% pure.

Pyrazine-2,3-diamine (1i). To 3-chloropyrazin-2-amine (0.5 g, 3.87 mmol) was added 30% NH_3(aq) solution (8.28 mmol, 9.65 mL). The mixture was stirred overnight at 130 °C in a sealed vessel. The solvent was evaporated, and the crude product was adsorbed on silica and purified by flash chromatography on silica using a mixture of CH_2Cl_2 and MeOH as mobile phase (isocratic 95/5 CH_2Cl_2/MeOH), affording the title compound as a white powder (11%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 7.14 (s, 2H, arom H), 5.84 (s, 4H, NH_2). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 146.14, 130.21.

1*H*-Imidazo[4,5-*b*]pyridine-2(3*H*)-thione (2a). *Procedure A.* To a solution of 2,3-diaminopyridine **1a** (0.5 g, 4.58 mmol) in EtOH (7 mL) and H_2O (1 mL) was added potassium ethylxanthate (6.87 mmol, 1.10 g). The reaction mixture was refluxed overnight at 80 °C. The solvents were evaporated, and the crude residue was purified by flash chromatography on silica, using a mixture of CH_2Cl_2, MeOH, and 30% NH_3(aq) solution as mobile phase (in a ratio gradually ranging from 97.5/2/0.5 CH_2Cl_2/MeOH/NH_3(aq) to 89.5/10/0.5 CH_2Cl_2/MeOH/NH_3(aq).

Procedure B. To a solution of 2,3-diaminopyridine **1a** (1.0 g, 9.16 mmol) in EtOH (44 mL) and H_2O (9 mL) were added CS_2 (9.16 mmol, 0.554 mL) and KOH (9.16 mmol, 515 mg). The reaction

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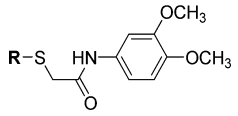
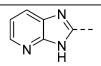
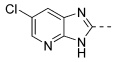
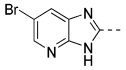
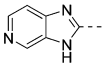
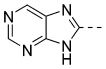
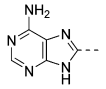
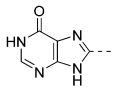
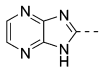
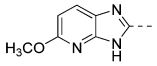
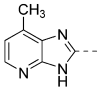
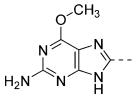
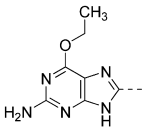
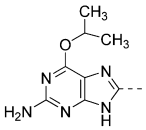
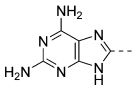
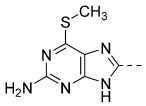
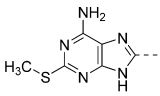
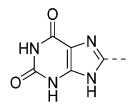
Table 7. Comparison of K_i Values of Selected NPP1 Inhibitors Determined versus the Artificial Substrate p-Nph-5′-TMP and versus the Natural Substrate ATP[a]



Cmpd.	R	K_i ± SEM (μM) vs. *p*-Nph-5'-TMP	K_i ± SEM (μM) vs. ATP
5a (hit)		**0.217** ± 0.052[b]	**22.3** ± 1.3
5b		**0.137** ± 0.043	**9.78** ± 3.68
5c		**0.192** ± 0.003	**10.8** ± 3.7
5e		**0.0442** ± 0.0035	**29.2** ± 5.5
5f		**0.281** ± 0.042	**15.7** ± 0.7
5g		**0.00500** ± 0.00077	**18.0** ± 2.7
5h		**0.0200** ± 0.0012	**14.3** ± 5.9
5i		**0.131** ± 0.008	**30.2** ± 2.1
12		**0.0296** ± 0.0100	**5.34** ± 0.06
13		**0.127** ± 0.036	**7.59** ± 1.04
34a		**8.04** ± 0.04	**42.2** ± 1.8
34b		**1.01** ± 0.31	**33.6** ± 3.1
34c		**11.0** ± 0.6	**89.7** ± 10.1
34d		**3.48** ± 0.13	**78.0** ± 1.2
34e		**3.65** ± 0.12	**67.9** ± 8.8
40		**0.152** ± 0.025	**26.7** ± 6.9
45b		**0.0358** ± 0.0004	**22.5** ± 2.8

[a]Three separate experiments were performed each in duplicate. [b]Results from ref 17.

mixture was refluxed overnight at 80 °C. The solvents were evaporated, and the crude residue was purified by flash chromatography on silica, using a mixture of CH_2Cl_2, MeOH, and 30% NH_3(aq) solution as mobile phase (in a ratio gradually ranging from 97.5/2/0.5 CH_2Cl_2/MeOH/NH_3(aq) to 89.5/10/0.5 CH_2Cl_2/MeOH/NH_3(aq)). Both fractions were combined yielding the pure title compound as a white powder (yield 69%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 7.11−7.16 (dd, 1H, J = 7.9 Hz, J = 5.1 Hz, arom H) 7.47 (d, 1H, J = 7.9 Hz, arom H), 8.10 (dd, 1H, J = 5.1 Hz, J = 1.2 Hz, arom H), 12.71 (s, 1H, NH), 13.12 (s, 1H, SH).

6-Chloro-1*H*-imidazo[4,5-*b*]pyridine-2(3*H*)-thione (2b). This compound was prepared from 5-chloropyridine-2,3-diamine **1b** according to procedure A, as described for the synthesis of **2a**. The crude residue was purified by flash chromatography on silica, using a mixture of CH_2Cl_2 and MeOH as mobile phase (in a ratio gradually ranging from 98.5/1/0.5 CH_2Cl_2/MeOH/NH_3(aq) to 94.5/5/0.5 CH_2Cl_2/MeOH/NH_3(aq)), yielding the title compound (37% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 7.55 (d, 1H, J = 2.0 Hz, arom H), 8.13 (d, 1H, J = 2.2 Hz, arom H), 12.94 (br s, 1H, NH).

6-Bromo-1*H*-imidazo[4,5-*b*]pyridine-2(3*H*)-thione (2c). This compound was prepared from 5-bromopyridine-2,3-diamine **1c** (0.5 g, 2.66 mmol) according to procedure A as described for the synthesis of **2a**. The crude residue was purified by flash chromatography on silica, using a mixture of CH_2Cl_2 and MeOH as mobile phase (in a ratio gradually ranging from 98.5/1/0.5 CH_2Cl_2/MeOH/NH_3(aq) to 92.5/7/0.5 CH_2Cl_2/MeOH/NH_3(aq)), furnishing the title compound (39% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 7.65 (d, 1H, J = 2.0 Hz, arom H), 8.20 (d, 1H, J = 2.0 Hz, arom H), 13.01 (br s, 1H, NH).

1*H*-Benzo[*d*]imidazole-2(3*H*)-thione (2d). This compound was prepared from 2,3-diaminobenzene **1d** (1.0 g, 9.25 mmol) according to procedure A as described for the synthesis of **2a**. The crude residue was purified by flash chromatography on silica, using a mixture of CH_2Cl_2 and MeOH as mobile phase (in a ratio gradually ranging from 1 to 5% of MeOH), yielding the pure title compound as a white powder (65% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 6.43−

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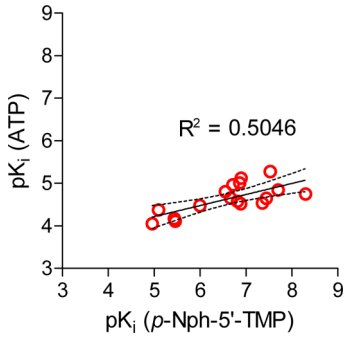

Figure 6. Correlation between results for selected NPP1 inhibitors obtained versus different substrates, ATP and *p*-Nph-5′-TMP, respectively. Correlation coefficient (R^2) were calculated by fitting pK_i values of the *p*-Nph-5′-TMP assay versus those of the ATP assay using Prism 5.0 software; circles, test compounds; solid line, the best fit line of the linear regression; dashed lines, 95% confidence band. The selected compounds for this analysis are listed in Table 7

6.38 (m, 1H, arom H), 6.55−6.51 (m, 1H, arom H), 7.13 (s, 2H, arom H), 12.52 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 109.60 115.11, 117.84 122.43, 132.38 134.63 168.28. ESI-MS (pos): m/z 151.17.

1*H*-Imidazo[4,5-*c*]pyridine-2(3*H*)-thione (2e). This compound was prepared from 3,4-diaminopyridine **1e** (1.0 g, 9.06 mmol) according to procedure A as described for the synthesis of **2a**. The crude residue was purified by flash chromatography on silica, using a mixture of CH_2Cl_2 and MeOH as mobile phase (in a ratio gradually ranging from 1 to 20% of MeOH), affording the title compound as a white powder (81% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 7.18 (d, 1H, J = 5.3 Hz, arom H), 8.25 (d, 1H, J = 5.4 Hz, arom H), 8.38 (s, 1H, arom H), 12.88 (br s, 1H, NH).

7*H*-Purine-8(9*H*)-thione (2f). This compound was prepared from 4,5-diaminopyrimidine **1f** (0.164 g, 2 mmol) according to procedure B as described for the synthesis of **2a**. The crude residue was purified by flash chromatography on silica, using EtOAc, affording the title compound as a white powder (71% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 8.43 (s, 1H, arom H), 8.68 (s, 1H, arom H), 13.01 (s, 1H, SH), 13.53 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz $_6$): δ (ppm) 124.84, 134.77, 151.83, 171.56.

6-Amino-7*H*-purine-8(9*H*)-thione (2g). To a suspension of 4,5,6-triaminopyrimidine sulfate **1g** (100 mg, 0.45 mmol) in a mixture of H_2O and EtOH (2:1 v/v, 2.5 mL) was added $NaHCO_3$ (420 mg, 2.25 mmol) and CS_2 (270 μL). The resulting mixture was refluxed for 2 days. The excess of CS_2 was removed under reduced pressure, and the resulting solution was brought to pH 6 using glacial AcOH. The formed precipitate was filtered off, washed with H_2O, and dried under vacuum, affording the title compound as a solid (42 mg, 56% yield). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 108.11, 147.38, 149.93, 152.61, 166.56.

8-Thioxo-8,9-dihydro-1*H*-purin-6(7*H*)-one (2h). This compound was synthesized from 4,5-diamino-6-hydroxypyrimidine hemisulfate **1h** (500 mg, 2.86 mmol, calcd for $C_4H_6N_4O{\cdot}0.5H_2SO_4$), $NaHCO_3$ (960 mg, 11.43 mmol), and CS_2 (3 mL) according to the method described for the synthesis of compound **2g**. The title compound was isolated as a beige solid (366 mg, 76% yield). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 113.45, 146.22, 148.21, 150.68, 166.27. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_5H_5N_4OS$, 169.0184; found, 169.0186.

1*H*-Imidazo[4,5-*b*]pyrazine-2(3*H*)-thione (2i). Compound **2i** was prepared from 2,3-diaminopyrazine **1i** (0.164 g, 2 mmol) according to procedure B as described for the synthesis of **2a**. The crude residue was purified by flash chromatography on silica, using EtOAc as mobile phase, affording the title compound as a white powder (71% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 8.10 (s, 2H, arom H), 13.49 (s, 2H, SH, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 136.62, 141.04, 172.25.

2-Chloro-*N*-(3,4-dimethoxyphenyl)acetamide (4). To a solution of 3,4-dimethoxyaniline **3** (3 g, 19.58 mmol) in CH_2Cl_2 was added Et_3N (23.50 mmol, 3.267 mL). The reaction mixture was cooled to 0 °C, and 2-chloroacetyl chloride (23.50 mmol, 1.867 mL) was added dropwise. The reaction was allowed to warm to room temperature and was stirred overnight. After reaction completion, the solvents were evaporated and the crude product was washed with ice-cold H_2O. The precipitate was filtered off and washed three times with H_2O, affording the title compound as a white powder (68% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.72 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 4.21 (s, 2H, CH_2), 6.91 (d, 1H, J = 8.7 Hz, arom H), 7.12 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, arom H), 7.27 (d, 1H, J = 2.4 Hz, arom H), 10.15 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 44.66, 56.50, 56.82, 105.65, 112.52, 113.19, 133.10, 146.39, 149.70, 165.25. ESI-MS (pos): m/z 230.09.

2-((3*H*-Imidazo[4,5-*b*]pyridin-2-yl)thio)-*N*-(3,4-dimethoxyphenyl)acetamide (5a). To a solution of 3*H*-imidazo-[4,5-*b*]pyridine-2-thiol **2a** (0.3 g, 1.98 mmol) in DMF (20 mL) was added K_2CO_3 (5.94 mmol, 0.823 g). The mixture was stirred at 85 °C for 30 min. Then 2-chloroacetamide **4** (2.18 mmol, 0.5 g) was added and the reaction mixture was stirred overnight at 85 °C. The solvent was evaporated, and the crude product was purified by flash chromatography on silica using a mixture of CH_2Cl_2 and MeOH as mobile phase (isocratic 98/2 CH_2Cl_2/MeOH), furnishing the title compound as a white powder (79% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.61 (s, 3H, OCH_3), 3.68 (s, 3H, OCH_3), 5.01 (s, 2H, CH_2), 6.69 (d, 1H, J = 8.6 Hz, arom H), 6.76 (d, 1H, J = 4.0 Hz, arom H), 6.79 (s, 1H, arom H), 7.16 (dd, 1H, J = 4.8 Hz, J = 7.9 Hz, arom H), 7.80 (d, 1H, J = 7.9 Hz, arom H), 8.21 (d, 1H, J = 4.6 Hz, arom H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 49.04, 55.30, 55.69, 106.30, 112.15, 113.39, 117.52, 122.42, 130.57, 132.79, 142.62, 146.02, 148.88, 150.35, 152.03. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{16}H_{17}N_4O_3S$, 345.1021; found, 345.1016.

2-((6-Chloro-3*H*-imidazo[4,5-*b*]pyridin-2-yl)thio)-*N*-(3,4-dimethoxyphenyl)acetamide (5b). This compound was prepared from **2b** (0.1 g, 0.539 mmol), according to the procedure for the synthesis of **5a**. The crude product was purified by flash chromatography on silica using a mixture of CH_2Cl_2 and MeOH as mobile phase (gradually ranging from 99/1 CH_2Cl_2/MeOH to 95/5 CH_2Cl_2/MeOH). This was followed by a second flash chromatography using a mixture of cyclohexane and EtOAc (gradually ranging from 60 to 75% of EtOAc), affording the title compound as a white powder (74% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.72 (s, 6H, 2 × OCH_3), 4.30 (s, 2H, CH_2), 6.89 (d, 1H, J = 8.8 Hz, arom H), 7.07 (dd, 1H, J = 6.6 Hz, J = 2.3 Hz, arom H), 7.30 (d, 1H, J = 2.3 Hz, arom H), 7.99 (d, 1H, J = 2.2 Hz, arom H), 8.23 (d, 1H, J = 2.2 Hz, arom H), 10.32 (s, 1H), 13.50 (br s, 1H). HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{16}H_{16}ClN_4O_3S$, 379.0632; found, 379.0620.

2-((6-Bromo-3*H*-imidazo[4,5-*b*]pyridin-2-yl)thio)-*N*-(3,4-dimethoxyphenyl)acetamide (5c). This compound was prepared from **2c** (0.25 g, 1.086 mmol) according to the procedure for the synthesis of **5a**. The crude product was purified by flash chromatography on silica using a mixture of CH_2Cl_2 and MeOH as mobile phase (gradually ranging from 98/2 CH_2Cl_2/MeOH to 97/3 CH_2Cl_2/MeOH), affording the title compound as a white powder (29% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.72 (s, 6H, 2 × OCH_3), 4.31 (s, 2H, CH_2), 6.89 (d, 1H, J = 8.7 Hz, arom H), 7.07 (dd, 1H, J = 8.7 Hz, J = 2.3 Hz, arom H), 7.29 (d, 1H, J = 2.3 Hz, arom H), 8.11 (d, 1H, J = 2.1 Hz, arom H), 8.30 (d, 1H, J = 2.1 Hz, arom H), 10.31 (s, 1H, NH). HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{16}H_{16}BrN_4O_3S$, 423.0121; found, 423.0120.

2-((1*H*-Benzo[*d*]imidazol-2-yl)thio)-*N*-(3,4-dimethoxyphenyl)acetamide (5d). This compound was prepared from **2d** (0.1 g, 0.666 mmol) according to the procedure for the synthesis of **5a**. The crude product was purified by flash chromatography on silica using a mixture of CH_2Cl_2 and MeOH as mobile phase (isocratic 97/3 CH_2Cl_2/MeOH), yielding the title compound as a white powder (34% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.71 (s, 6H, 2 × OCH_3), 4.23 (s, 2H, CH_2), 6.88 (d, 1H, J = 8.7 Hz, arom H), 7.07 (dd, 1H, J = 8.6 Hz, J = 2.3 Hz, arom

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H), 7.13 (dd, 2H, J = 6.0 Hz, J = 3.2 Hz, arom H), 7.29 (d, 1H, J = 2.3 Hz, arom H), 7.46 (dd, 2H, J = 5.9 Hz, J = 3.2 Hz, arom H), 10.41 (s, 1H, NH). HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{17}H_{18}N_3O_3S$, 344.1063; found, 344.1061.

2-((3*H*-Imidazo[4,5-*c*]pyridin-2-yl)thio)-*N*-(3,4-dimethoxyphenyl)acetamide (5e). This compound was prepared from **2e** (0.3 g, 1.98 mmol) according to the procedure for the synthesis of **5a**. The crude product was adsorbed on silica and purified by flash chromatography on silica using a mixture of CH_2Cl_2, MeOH, and NH_3(aq) as mobile phase (gradually ranging from 94.5/5/0.5 CH_2Cl_2/MeOH/NH_3(aq) to 89.5/10/0.5 CH_2Cl_2/MeOH/NH_3(aq)), followed by a second flash chromatography using a mixture of CH_2Cl_2 and acetone as mobile phase (isocratic 50/50 CH_2Cl_2/acetone), yielding the title compound (48% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.71 (s, 6H, 2 × OCH_3), 4.24 (s, 2H, CH_2), 6.89 (d, J = 8.7 Hz, 1H, arom H), 7.07 (dd, 1H, J = 8.6 Hz, J = 2.2 Hz, arom H), 7.29 (d, 1H, J = 2.2 Hz, arom H), 7.49 (d, 1H, J = 5.8 Hz, arom H), 8.17 (d, 1H, J = 5.7 Hz, arom H), 8.75 (s, 1H, arom H), 10.47 (s, 1H, NH). HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{16}H_{17}N_4O_3S$, 345.1021; found, 345.1016.

2-((9*H*-Purin-8-yl)thio)-*N*-(3,4-dimethoxyphenyl)acetamide (5f). To a solution of **2f** (152 mg, 1 mmol) and 2-chloroacetamide **4** (317 mg, 1.38 mmol) in MeOH (15 mL) was added NaOH (240 mg, 6 mmol). The mixture was stirred under N_2 at room temperature overnight. The crude residue was purified using a mixture of CH_2Cl_2 and MeOH (10% of MeOH), affording the title compound as a white powder (59%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.70 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 4.32 (s, 2H, CH_2), 6.88 (d, 1H, J = 8.7 Hz, arom H), 7.08 (dd, 1H, J = 8.7 Hz, J = 2.2, arom H), 7.29 (d, 1H, J = 2.2 Hz, arom H), 8.76 (s, 1H, arom H), 8.87 (s, 1H, arom H), 10.39 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 35.82, 55.39, 55.75, 104.31, 111.10, 112.15, 131.95, 132.46, 141.46, 145.07, 148.62, 150.97, 157.01, 157.38, 165.13. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{15}H_{16}N_5O_3S$, 346.0968; found, 346.0956.

2-(6-Amino-9*H*-purin-8-ylthio)-*N*-(3,4-dimethoxyphenyl)-acetamide (5g). To a solution of **2g** (42 mg, 0.25 mmol) in 1.5 M aq KOH solution (2.2 mL) was added 2-chloroacetamide **4** (66 mg, 0.29 mmol), and the mixture was stirred at room temperature for 1 h. Then the solution was neutralized using glacial AcOH and concentrated to dryness. The crude residue was purified by silicagel flash chromatography, using a mixture of MeOH and CH_2Cl_2 (in a ratio gradually ranging from 2 to 10% MeOH), afforded the pure title compound as a light-brown solid (38 mg, 44%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.70 (s, 6H, 2 × OCH_3), 4.12 (s, 2H, CH_2), 6.88 (d, 1H, J = 8.7 Hz, arom H), 7.02 (br s, 2H, NH_2), 7.10 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, arom H), 7.26 (d, 1H, J = 2.4 Hz, arom H), 8.06 (s, 1H, arom H), 10.28 (s, 1H, NH), 12.99 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 35.88, 55.36, 55.71, 104.30, 111.06, 112.05, 132.38, 145.02, 147.69, 148.55, 151.42, 153.35, 165.74. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{15}H_{17}N_6O_3S$, 361.1077; found, 361.1075.

***N*-(3,4-Dimethoxyphenyl)-2-(6-oxo-6,9-dihydro-1*H*-purin-8-ylthio)acetamide (5h).** A mixture of **2h** (50 mg, 0.30 mmol) and K_2CO_3 (123 mg, 0.89 mmol) in DMF (7 mL) was heated at 85 °C for 15 min. Then 2-chloroacetamide **4** (75 mg, 0.33 mmol) was added, and the resulting mixture was stirred at 85 °C overnight. The volatiles were removed under reduced pressure, and the crude residue was purified by silicagel flash chromatography, using a mixture of MeOH and CH_2Cl_2 as mobile phase (in a ratio gradually ranging from 4 to 10% MeOH), affording the pure title compound as a light-brown solid (90 mg, 83%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.71 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 4.17 (s, 2H, CH_2), 6.89 (d, 1H, J = 8.7 Hz, arom H), 7.09 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, arom H), 7.29 (d, 1H, J = 2.4 Hz, arom H), 7.93 (s, 1H, arom H), 10.44 (s, 1H, NH), 12.23 (br s, 1H, NH), 13.44 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 36.08, 55.35, 55.72, 104.20, 110.96, 112.10, 132.51, 144.49, 144.97, 148.59, 165.49. HRMS (ESI): m/z $[M - H]^-$ calcd for $C_{15}H_{14}N_5O_4S$, 360.0772; found, 360.0776.

2-((1*H*-Imidazo[4,5-*b*]pyrazin-2-yl)thio)-*N*-(3,4-dimethoxyphenyl)acetamide (5i). To a solution of **2i** (152 mg, 1 mmol) and 2-chloroacetamide **4** (317 mg, 1.38 mmol) in MeOH (15 mL) was added NaOH (0.240 mg 6 mmol). The mixture was stirred under N_2 at room temperature overnight. The crude residue was purified using a mixture of CH_2Cl_2 and MeOH (20% of MeOH), affording the title compound in 64% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.71 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 4.37 (s, 2H, CH_2), 6.88 (d, 1H, J = 8.7 Hz, arom H), 7.08 (dd, 1H, J = 8.7 Hz, J = 2.3 Hz, arom H), 7.31 (d, 1H, J = 2.3 Hz, arom H), 8.24 (s, 2H, arom H), 10.34 (s, 1H, NH), 13.67 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 35.66, 55.36, 55.72, 104.31, 111.08, 112.12, 132.46, 137.36, 145.05, 145.96, 148.60, 158.04, 164.98. HRMS (ESI): m/z $[M + Na]^+$ calcd for $C_{15}H_{15}N_5O_3SNa$, 368.0788; found, 368.0787.

2-(1*H*-Imidazol-2-ylthio)-*N*-(3,4-dimethoxyphenyl)-acetamide (7). To a solution of 2-mercaptoimidazole **6** (0.3 g, 3.00 mmol) in DMF (20 mL) was added K_2CO_3 (1.242 g, 9.00 mmol). After stirring for 30 min at 85 °C, 2-chloroacetamide **4** (0.755 g, 3.3 mmol) was added and the reaction mixture was stirred overnight at 85 °C. The solvent was evaporated and the crude product was adsorbed on silica and purified by flash chromatography on silica using a mixture of CH_2Cl_2, MeOH, and 30% NH_3(aq) solution as mobile phase (gradually ranging from 98.5/1/0.5 CH_2Cl_2/MeOH/NH_3(aq) to 94.5/5/0.5 CH_2Cl_2/MeOH/NH_3(aq)), followed by a second flash chromatography using a mixture of CH_2Cl_2 and acetone as mobile phase (isocratic 75/25 CH_2Cl_2/acetone), furnishing the title compound as a white powder (50% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.70 (s, 3H, OCH_3) 3.71 (s, 3H, OCH_3), 3.87 (s, 2H, CH_2), 6.86−6.89 (m, 1H, arom H), 7.02−7.06 (m, 3H, arom H), 7.26 (m, 1H, arom H), 10.26 (br s, 1H, NH), 12.30 (br s, 1H, NH). HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{13}H_{16}N_3O_3S$, 294.0912; found, 294.0908.

6-Methoxypyridine-2,3-diamine (10). To a solution of 6-methoxy-3-nitropyridin-2-amine **8** (300 mg, 1.18 mmol) in EtOH (15 mL) and H_2O (5 mL) was added iron (487 mg, 8.26 mmol) and calcium chloride (100 mg, 0.88 mmol). The reaction was stirred overnight at 60 °C. The solvents were evaporated. Because of the instability of the diamino analogue, the product was immediately used in the next reaction without purification.

5-Methoxy-1*H*-imidazo[4,5-*b*]pyridine-2(3*H*)-thione (10a). To a solution of **10** (0.164 g, 1.18 mmol) in EtOH (9 mL) and H_2O (2 mL) was added KOH (1.18 mmol, 0.066 g) and CS_2 (2.36 mmol, 0.142 mL). The reaction mixture was stirred overnight at 80 °C. The solvents were evaporated, and the crude residue was purified by flash chromatography using a mixture of CH_2Cl_2 and MeOH as mobile phase (isocratic 96/4 CH_2Cl_2/MeOH), affording the title compound as a white powder (84% yield over the two reactions). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.83 (s, 3H, OCH_3), 6.56 (d, 1H, J = 8.5 Hz, arom H) 7.45 (d, 1H, J = 8.5 Hz, arom H), 12.53 (br s, 1H), 12.98 (br s, 1H).

4-Methylpyridine-2,3-diamine (11). To a solution of **9** (0.5 g, 3.26 mmol) in dry THF was added palladium on carbon (10% Pd/C, 0.1 g, 0.94 mmol). The reaction mixture was stirred at room temperature for 5 h under an atmosphere of hydrogen gas. The mixture was filtered over Celite, and the filtrate was collected. The solvent was evaporated to yield the pure title compound (100%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.01 (s, 3H, CH_3), 4.37 (s, 2H, NH_2), 5.25 (s, 2H, NH_2), 6.27 (d, 1H, J = 5.0 Hz, arom H), 7.18 (d, 1H, J = 5.0 Hz, arom H).

7-Methyl-1*H*-imidazo[4,5-*b*]pyridine-2(3*H*)-thione (11a). To a solution of **11** (0.168 g, 1.37 mmol) in a mixture of EtOH (9 mL) and H_2O (2 mL) was added KOH (1.37 mmol, 0.077 g) and CS_2 (2.74 mmol, 0.165 mL). The reaction mixture was stirred overnight. The solvents were evaporated, and the crude residue was adsorbed on silica for purification with flash chromatography using a mixture of CH_2Cl_2 and MeOH as mobile phase (isocratic 95/5 CH_2Cl_2/MeOH), affording the title compound as a white powder (45% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.51 (s, 3H, CH_3), 6.96 (d, 1H, J = 5.2 Hz, arom H), 7.97 (d, 1H, J = 5.2 Hz, arom H), 12.85 (br s, 1H, NH), 13.03 (br s, 1H, SH).

***N*-(3,4-Dimethoxyphenyl)-2-(5-methoxy-3*H*-imidazo[4,5-*b*]-pyridin-2-ylthio)acetamide (12).** To a solution of **10a** (140 g,

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0.843 mmol) in DMF (20 mL) was added K_2CO_3 (350 mg, 2.53 mmol). After stirring for 30 min at 85 °C, 2-chloroacetamide **4** (213 mg, 0.928 mmol) was added and the reaction mixture was stirred overnight at 85 °C. The solvent was evaporated, and the crude product was adsorbed on silica and purified by silicagel flash chromatography, the mobile phase being a mixture of MeOH and CH_2Cl_2 (2% of MeOH), yielding the title compound as a white powder (46% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.72 (s, 6H, 2 × OCH_3), 3.85 (s, 3H, OCH_3), 4.21 (s, 2H, CH_2), 6.60 (d, 1 H, J = 8.5 Hz, arom H), 6.89 (d, 1H, J = 8.7 Hz, arom H), 7.09 (d, 1H, J = 8.9 Hz, arom H), 7.29 (s, 1H, arom H), 7.78 (d, 1H, J = 8.5 Hz, arom H), 10.34 (br s, 1H, NH). HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{17}H_{19}N_4O_4S$, 375.1127; found, 375.1121.

***N*-(3,4-Dimethoxyphenyl)-2-(7-methyl-3*H*-imidazo[4,5-*b*]-pyridin-2-ylthio)acetamide (13).** To a solution of **11a** (0.1 g, 0.61 mmol) in DMF was added K_2CO_3 (1.81 mmol, 0.251 g). The mixture was stirred for 30 min at 90 °C, and 2-chloroacetamide **4** (0.73 mmol, 0.167 g) was added. The mixture was stirred overnight at 90 °C. The solvent was evaporated, and the crude residue was adsorbed on silica for purification by flash chromatography using a mixture of CH_2Cl_2 and MeOH as mobile phase (2% of MeOH), furnishing the pure title compound (37% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.49 (s, 3H, CH_3), 3.72 (s, 6H, 2 × OCH_3), 4.27 (s, 2H, CH_2), 6.90 (d, 1H, J = 8.7 Hz, arom H), 6.99 (d, 1H, J = 4.8 Hz, arom H), 7.08 (d, 1H, J = 8.2 Hz, arom H), 7.31 (s, 1H, arom H), 8.08 (d, 1H, J = 4.9 Hz, arom H), 10.38 (br s, 1H, NH), 13.07 (br s, 1H, NH). HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{17}H_{19}N_4O_3S_1$, 359.1178; found, 359.1177.

3-Chloro-*N*-(3,4-dimethoxyphenyl)propanamide (14). To a solution of 3,4-dimethoxyaniline (**3**, 0.5 g, 3.26 mmol) in CH_2Cl_2 (10 mL) was added Et_3N (3.91 mmol, 0.544 mL). The reaction mixture was cooled to 0 °C on an ice bath, and 3-chloropropanoyl chloride (3.91 mmol, 0.324 mL) was added dropwise. The reaction was allowed to warm to room temperature and was stirred overnight. After reaction completion, the solvent was evaporated and the crude product was washed with ice-cold H_2O. The precipitate was filtered off and washed with H_2O, affording the title compound as a white powder (60% yield). The product was used without additional purification in the next reaction. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.78 (t, 2H, J = 6.2 Hz, CH_2), 3.71 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.87 (t, 2H, J = 6.2 Hz, CH_2), 6.88 (d, 1H, J = 8.6 Hz, arom H), 7.09 (dd, 1H, J = 8.6 Hz, J = 2.3 Hz, arom H), 7.32 (d, 1H, J = 2.2 Hz, arom H), 9.93 (s, 1H, NH).

2-Chloro-*N*-(3,4-dimethoxyphenyl)propanamide (15). To a solution of 3,4-dimethoxyaniline (**3**, 1.5 g, 9.79 mmol) in CH_2Cl_2 (20 mL) was added Et_3N (1.63 mL, 11.75 mmol). The reaction mixture was cooled to 0 °C on an ice bath, and 2-chloropropanoyl chloride (11.75 mmol, 1.16 mL) was added dropwise. The reaction was allowed to warm to room temperature and was stirred overnight. After reaction completion, the solvent was evaporated and the crude product was washed with ice-cold H_2O. The precipitate was filtered off and washed with H_2O, affording the title compound as a white powder (86% yield). The product was used without additional purification in the next reaction. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.60 (d, 3H, J = 6.6 Hz, CH_3), 3.72 (s, 3 H, OCH_3), 3.74 (s, 3 H, OCH_3), 4.64 (q, 1H, J = 6.7 Hz, CH), 6.90 (d, 1H, J = 8.7 Hz, arom H), 7.12 (dd, 1H, J = 8.5 Hz, J = 2.1 Hz, arom H), 7.31 (d, 1H, J = 2.1 Hz, arom H), 10.17 (br s, 1H, NH).

1-Chloro-*N*-(3,4-dimethoxyphenyl)methanesulfonamide (16). To a solution of 3,4-dimethoxyaniline (**3**, 0.3 g, 1.96 mmol) in CH_2Cl_2 was added Et_3N (2.35 mmol, 0.327 mL). The reaction mixture was cooled to 0 °C on an ice bath, and 2-chloromethanesulfonyl chloride (2.35 mmol, 0.214 mL) was added dropwise. The reaction was allowed to warm to room temperature and was stirred overnight. After reaction completion, the solvent was evaporated and the crude product was adsorbed on silica and purified by flash chromatography using a mixture of CH_2Cl_2 and MeOH as mobile phase (1% of MeOH), affording the title compound as a white powder (46% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.73 (s, 6H, 2 × OCH_3), 4.90 (s, 2H, CH_2), 6.77 (dd, 1H, J = 8.5 Hz, J = 2.5 Hz, arom H), 6.84 (d, 1H, J = 2.4 Hz, arom H), 6.93 (d, 1H, J = 8.6 Hz, arom H), 10.06 (s, 1H, NH).

3-(3*H*-Imidazo[4,5-*b*]pyridin-2-ylthio)-*N*-(3,4-dimethoxyphenyl)propanamide (17). To a solution of **2a** (0.100 g, 0.661 mmol) in DMF was added K_2CO_3 (1.98 mmol, 0.274 g). After stirring for 30 min at 85 °C, 3-chloropropanamide **14** (0.727 mmol, 0.177 g) was added and the reaction mixture was stirred overnight at 85 °C. The solvent was evaporated, and the crude product was adsorbed on silica and purified by flash chromatography on silica using a mixture of CH_2Cl_2 and MeOH (isocratic 100% CH_2Cl_2), followed by a second flash chromatography using cyclohexane and EtOAc as the mobile phase (gradually ranging from 55 to 65% of EtOAc), furnishing the title compound as a white powder (30% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.81 (t, 2H, J = 7.0 Hz, CH_2) 3.69 (s, 6H, 2 × OCH_3), 4.51 (t, 2H, J = 7.0 Hz, CH_2), 6.85 (d, 1H, J = 6.8 Hz, arom H), 7.01 (dd, 1H, J = 8.8 Hz, J = 2.1 Hz, arom H), 7.16 (m, 2H, arom H), 7.79 (d, 1H, J = 8.7 Hz, arom H), 8.14 (d, 1H, J = 4.1 Hz, arom H), 9.87 (s, 1H, NH). HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{17}H_{19}N_4O_3S$, 359.1178; found, 359.1176.

2-(3*H*-Imidazo[4,5-*b*]pyridin-2-ylthio)-*N*-(3,4-dimethoxyphenyl)propanamide (18). To a solution of **2a** (0.3 g, 1.98 mmol) in DMF was added K_2CO_3 (5.94 mmol, 0.821 g). After stirring for 30 min at 85 °C, 2-chloropropanamide **15** (0.531 g, 2.18 mmol) was added and the reaction mixture was stirred overnight at 85 °C. The solvent was evaporated, and the crude product was adsorbed on silica and purified by flash chromatography on silica using a mixture of CH_2Cl_2 and MeOH (gradually ranging from 1 to 3% of MeOH), affording the title compound as a white powder (66% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.66 (d, 3H, J = 6.9 Hz, CH_3), 3.72 (s, 6H, 2 × OCH_3), 4.85 (q, 1H, J = 7.0 Hz, CH), 6.90 (d, 1H, J = 8.8 Hz, arom H), 7.09 (dd, 1H, J = 8.7 Hz, J = 2.3 Hz, arom H), 7.18 (dd, 1H, J = 8.0 Hz, J = 4.8 Hz, arom H), 7.31 (d, 1H, J = 2.3 Hz, arom H), 7.87 (d, 1H, J = 7.9 Hz, arom H), 8.24 (d, 1H, J = 4.0 Hz, arom H), 10.45 (br s, 1H, NH). HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{17}H_{19}N_4O_3S$, 359.1178; found, 359.1167.

1-(1*H*-Imidazo[4,5-*b*]pyridin-2-ylthio)-*N*-(3,4-dimethoxyphenyl)methanesulfonamide (19). To a solution of **2a** (0.1 g, 0.66 mmol) in DMF (20 mL) was added K_2CO_3 (1.98 mmol, 0.274 g). The mixture was stirred at 85 °C. After stirring for 30 min, compound **16** (0.73 mmol, 0.192 g) was added and the reaction mixture was stirred overnight at 85 °C. The solvent was evaporated. The crude product was adsorbed on silica and purified by flash chromatography on silica using a mixture of CH_2Cl_2 and MeOH as mobile phase (isocratic 95/5 CH_2Cl_2/MeOH), yielding the title compound as a white powder (57% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.62 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 5.09 (s, 2H, CH_2), 6.71 (m, 3H, arom H), 7.19 (dd, 1H, J = 8.0 Hz, J = 4.9 Hz, arom H), 7.81 (dd, 1H, J = 8.0 Hz, J = 1.3 Hz, arom H), 8.24 (d, 1H, J = 3.7 Hz, arom H). HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{15}H_{17}N4O_4S_2$, 381.0691; found, 381.0688.

2-Chloro-*N*-(3,4-dimethoxybenzyl)acetamide (21). This compound was prepared from 3,4-dimethoxybenzylamine (**20**, 0.5 g, 2.99 mmol, 0.47 mL) and 2-chloroacetyl chloride (3.59 mmol, 0.285 mL) according to the procedure for the synthesis of **4**. The title compound was isolated as a white powder (34% yield). The product was used without additional purification in the next reaction. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.72 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 4.11 (s, 2H, CH_2), 4.23 (d, 2H, J = 5.8 Hz, CH_2), 6.78 (m, 1H, arom H), 6.90 (m, 2H, arom H), 8.66 (s, 1H, NH).

2-(3*H*-Imidazo[4,5-*b*]pyridin-2-ylthio)-*N*-(3,4-dimethoxybenzyl)acetamide (22). This compound was prepared from **2a** (0.139 g, 0.92 mmol) and **21** (0.246 g, 1.01 mmol) according to the procedure for the synthesis of **5a**. The crude residue was purified by flash chromatography on silica using a mixture of CH_2Cl_2 and MeOH as mobile phase (gradually ranging from 1 to 5% of MeOH). This was followed by a second flash chromatography using a mixture of CH_2Cl_2 and acetone (isocratic, 20% of acetone), yielding the title compound as a white powder (29% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.66 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 4.13 (s, 2H, SCH_2), 4.24 (d, 2H, J = 5.8 Hz, NCH_2), 6.81 (m, 3H, arom

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H), 7.16 (dd, 1H, J = 7.9 Hz, J = 4.9 Hz, arom H), 7.81 (dd, 1H, J = 7.9 Hz, J = 1.4 Hz, arom H), 8.22 (dd, 1H, J = 4.8 Hz, J = 1.4 Hz, arom H), 8.73 (t, 1H, J = 5.7 Hz, NH), 13.07 (br s, 1H, NH). HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{17}H_{19}N_4O_3S$, 359.1178; found, 359.1176.

General Procedure for the Synthesis of Compounds 24a−d. To a solution of 2-amino-6-chloro-3-nitropyridine (**23**, 177 mg, 1 mmol) in DMF (10 mL) was added an appropriate amine (2 mmol) and K_2CO_3 (5 mmol). The reaction was stirred overnight at 80 °C. The solvent was evaporated in vacuo and the crude residue was purified by silica gel flash chromatography, yielding the pure title compounds. The following compounds were made according to this procedure.

*6-Morpholino-3-nitropyridin-2-amine (**24a**).* This compound was prepared using morpholine and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 1:1), affording the title compound in 100% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.70 (m, 4H, 2 × NCH_2), 3.76 (m, 4H, 2 × OCH_2), 6.04 (d, 1H, J = 9.4 Hz, arom H), 8.18 (d, 1H, J = 9.4 Hz, arom H).

*6-(4-Methylpiperazin-1-yl)-3-nitropyridin-2-amine (**24b**).* This compound was prepared using *N*-methylpiperazine and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 1:1), affording the title compound in 89% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.20 (s, 3H, CH_3), 2.36 (t, 4H, J = 5.0 Hz, 2 × CH_3NCH_2), 3.70 (br s, 4H, 2 × NCH_2), 6.33 (d, 1H, J = 9.5 Hz, arom H), 7.74 (br s, 2H, NH_2), 8.03 (d, 1H, J = 9.5 Hz, arom H).

*3-Nitro-6-(piperidin-1-yl)pyridin-2-amine (**24c**).* This compound was prepared using piperidine and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 1:1), affording the title compound in 70% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.58 (m, 6H, 3 × CH_2), 3.70 (s, 4H, 2 × NCH_2), 6.32 (d, 1H, J = 9.6 Hz, arom H), 8.00 (d, 1H, J = 9.6 Hz, arom H).

*N^2,N^2-Dimethyl-5-nitropyridine-2,6-diamine (**24d**).* This compound was prepared using dimethylamine and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 1:1), affording the title compound in 100% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.12 (s, 6H, 2 × CH_3), 6.19 (d, 1H, J = 9.5 Hz, arom H), 8.03 (d, 1H, J = 9.5 Hz, arom, H).

3-Nitro-6-(2,2,2-trifluoroethoxy)pyridin-2-amine (24e). To a solution of 2-amino-6-chloro-3-nitropyridine (**23**, 400 mg, 2.3 mmol) in dry THF (10 mL) was added 2,2,2-trifluoroethanol (3 mmol) and NaH (60% in mineral oil, 3 mmol). The reaction was stirred overnight at room temperature. The solvent was evaporated in vacuo, and the crude residue was purified using a mixture of cyclohexane and EtOAc (25% of EtOAc), affording the title compound in 72% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 5.02 (q, 2H, J = 9.0 Hz, CH_2), 6.28 (d, 1H, J = 9.0 Hz, arom H), 8.20 (br s, 2H, NH_2), 8.36 (d, 1H, J = 9.0 Hz, arom H).

6-(Methylthio)-3-nitropyridin-2-amine (24f). A solution of 6-amino-5-nitropyridine-2(1*H*)-thione (**25**) (328 mg, 1.92 mmol) and K_2CO_3 (265 mg, 1.92 mmol) in DMF (2.5 mL) was stirred at 80 °C for 15 min. Then the mixture was cooled in an ice−water bath and iodomethane (120 μL, 1.93 mmol) was added. The cooling bath was removed, and the mixture was stirred for 16 h at room temperature. The solvents were removed under reduced pressure. The crude residue was purified by silicagel flash chromatography, using CH_2Cl_2 as mobile phase, affording compound **24f** as a yellow solid (219 mg, 62%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.55 (s, 3H, CH_3), 6.64 (d, 1H, J = 8.7 Hz, arom H), 8.07 (br s, 2H, NH_2), 8.17 (d, 1H, J = 8.7 Hz, arom H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 12.76, 109.99, 123.24, 134.17, 153.12, 168.22. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_6H_8N_3O_2S$, 186.0337; found, 186.0328.

6-Amino-5-nitropyridine-2(1*H*)-thione (25). To a solution of 2-amino-6-chloro-3-nitro-pyridine **23** (500 mg, 2.88 mmol) in DMF (20 mL) was added NaSH·xH_2O (400 mg) in one portion. The deep dark mixture was stirred at 80 °C for 1.5 h. The solvents were removed under reduced pressure. The crude residue was purified by silicagel flash chromatography, the mobile phase being a mixture of MeOH in CH_2Cl_2 (2% of MeOH), affording compound **25** as an orange solid (336 mg, 68%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 6.50 (d, 1H, J = 9.5 Hz, arom H), 7.58 (br s, 1H, NH_2), 7.92 (d, 1H, J = 9.5 Hz, arom H), 8.67 (br s, 1H, NH_2), 12.61 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 118.79, 119.52, 131.88, 149.44, 183.77, HRMS (ESI): m/z $[M + H]^+$ calcd for $C_5H_6N_3O_2S$, 172.0181; found, 172.0175.

General Procedure for the Synthesis of 3*H*-imidazo[4,5-*b*]pyridine-2-thiones 27a−e. To a solution of appropriate 2-amino-3-nitropyridine **24a−e** (2 mmol) in MeOH (25 mL) was added Pd/C (10%, 0.01 mmol, 10.6 mg). The reaction mixture was stirred overnight under an atmosphere of H_2 at room temperature. The mixture was filtered over Celite. The filtrate was collected and used directly for the next step. CS_2 (5 mL) was added to the filtrate. The reaction mixture was refluxed overnight at 50 °C. The end of the reaction was determined by TLC. The solvents were evaporated, and the crude residue was adsorbed on silica and purified by flash chromatography on silica.

*5-Morpholino-3H-imidazo[4,5-b]pyridine-2-thione (**27a**).* This compound was prepared using 6-morpholino-3-nitropyridin-2-amine (**26a**) and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 1:1), affording the title compound in 71% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.35 (br s, 4H, 2 × NCH_2), 3.69 (t, 4H, J = 4.7 Hz, 2 × OCH_2), 6.57 (d, 1H, J = 8.8 Hz, arom H), 7.32 (d, 1H, J = 8.7 Hz, arom H), 12.17 (s, 1H, SH), 12.51 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 45.89, 65.88, 101.56, 117.85, 118.93, 114.78, 155.94, 167.04.

*5-(4-Methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridine-2-thione (**27b**).* This compound was prepared using 6-(4-methylpiperazin-1-yl)-3-nitropyridin-2-amine (**26b**) and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 1:1), affording the title compound in 68% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.20 (s, 3H, CH_3), 2.39 (t, 4H, J = 4.7 Hz, 2 × CH_3NCH_2), 3.39 (t, 4H, J = 4.6 Hz, 2 × NCH_2), 6.57 (d, 1H, J = 8.8 Hz, arom H), 7.29 (d, 1H, J = 8.7 Hz, arom H), 12.56 (br s, 2H, SH, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 45.53, 45.91, 54.49, 101.80, 117.66, 119.08, 144.98, 156.04, 167.04.

*5-(Piperidin-1-yl)-3H-imidazo[4,5-b]pyridine-2-thione (**27c**).* This compound was prepared using 3-nitro-6-(piperidin-1-yl)pyridin-2-amine (**26c**) and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 1:1), affording the title compound in 76% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.59 (br s, 6H, 3 × CH_2), 3.44 (s, 4H, 2 × NCH_2), 6.52(d, 1H, J = 8.8 Hz, arom H), 7.28 (d, 1H, J = 8.8 Hz, arom H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 25.70, 26.48, 48.13, 103.62, 118.39, 120.50, 146.20, 158.31, 167.25.

*5-(Dimethylamino)-3H-imidazo[4,5-b]pyridine-2-thione (**27d**).* This compound was prepared using N^2,N^2-dimethyl-5-nitropyridine-2,6-diamine (**26d**) and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 7:3), affording the title compound in 66% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.30 (s, 6H, 2 × CH_3), 6.37 (d, 1H, J = 8.7 Hz, arom H), 7.28 (d, 1H, J = 8.7 Hz, arom H), 12.22 (s, 1H, SH), 12.56 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 38.30, 100.32, 116.28, 118.98, 114.95, 156.02, 166.17.

*5-(2,2,2-Trifluoroethoxy)-3H-imidazo[4,5-b]pyridine-2-thione (**27e**).* This compound was prepared using 3-nitro-6-(2,2,2-trifluoroethoxy)pyridin-2-amine (**26e**) and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 7:3), affording the title compound in 50% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 4.82 (q, 2H, J = 8.7 Hz, CH_2), 6.70 (d, 1H, J = 8.5 Hz, arom, H), 7.51 (d, 1H, J = 8.5 Hz, arom H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 62.68, 63.16, 63.63, 64.11, 105.77, 121.93, 122.40, 123.45, 127.12, 159.98, 169.90.

5-(Methylthio)-1*H*-imidazo[4,5-*b*]pyridine-2(3*H*)-thione (27f). A suspension of 3-nitropyridin-2-amine **24f** (287 mg, 1.55 mmol) in a mixture of H_2O and MeOH (1:1 v/v, 10 mL) was treated with $Na_2S_2O_4$ (1.35 g, 7.76 mmol), and the resulting mixture was stirred at 85 °C for 16 h. The solvents were removed in vacuo, and the residue was treated with MeOH. The precipitate was filtered off. The filtrate was concentrated to dryness and used directly in the next step without further purification. The crude pyridine-2,3-diamine **26f** was dissolved in a mixture of H_2O and EtOH (2:1 v/v, 13.5 mL) and $NaHCO_3$ (782 mg, 9.31 mmol) was added, followed by the addition of CS_2 (1.2 mL). The resulting mixture was stirred at 55 °C for 5 h. The

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solvents were removed in vacuo. The solid residue was redissolved in H_2O and the pH was adjusted to 6, using glacial acetic acid. The precipitate was filtered off, washed with H_2O, and dried under vacuum overnight, affording compound **27f** as a solid (183 mg, 0.93 mmol, 60% after 2 steps). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.51 (s, 3H, CH_3, overlapped with residual solvent signal), 7.02 (d, 1H, J = 8.5 Hz, arom H), 7.39 (d, 1H, J = 8.5 Hz, arom H), 12.73 (br s, 1H, NH), 13.06 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 13.40, 115.11, 117.25, 122.52, 146.39, 152.02, 168.83. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_7H_8N_3S_2$, 198.0160; found, 198.0160.

General Procedure for the Synthesis of Compounds 28a−e. To a mixture of appropriate 3*H*-imidazo[4,5-*b*]pyridine-2-thione **27a−e** (1 mmol) and 2-chloroacetamide **4** (317 mg, 1.3 mmol) in MeOH (15 mL) was added NaOH (240 mg, 6 mmol). The mixture was stirred under N_2 at room temperature overnight. The solvents were evaporated, and the crude residue was adsorbed on silica and purified by flash chromatography on silica. The following compounds were made according to this procedure

*N-(3,4-Dimethoxyphenyl)-2-((5-morpholino-3H-imidazo[4,5-b]-pyridin-2-yl)thio)acetamide (**28a**).* This compound was prepared using **27a** and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 6:3), affording the title compound in 23% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.39 (br s, 4H, 2 × NCH_2), 3.7 (br s, 10H, 2 × OCH_3, 2 × OCH_2), 4.18 (s, 2H, CH_2), 6.68 (d, 1H, J = 8.8 Hz, arom H), 6.88 (d, 1H, J = 8.7 Hz, arom H), 7.09 (dd, 1H, J = 8.6 Hz, J = 2.3 Hz, arom H), 7.28 (d, 1H, J = 2.3 Hz, arom H), 7.67 (d, 1H, J = 8.7 Hz, arom H), 10.33 (s, 1H, NH), 12.70 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 35.88, 46.21, 55.32, 55.71, 65.99, 102.49, 104.20, 110.96, 112.12, 125.34, 132.49, 144.98, 147.68, 148.58, 153.94, 155.95, 165.64. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{20}H_{24}N_5O_4S$, 430.1549; found, 430.1543.

*N-(3,4-Dimethoxyphenyl)-2-((5-(4-methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridin-2-yl)thio)acetamide (**28b**).* This compound was prepared using **27b** and was purified using a mixture of MeOH and EtOAc (in a ratio of 7:3), affording the title compound in 23% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.20 (s, 3H, CH_3), 2.40 (s, 4H, 2 × CH_3NCH_2), 3.70 (s, 6H, 2 × CH_3), 4.16 (s, 2H, 2 × NCH_2), 6.67 (d, 1H, J = 8.8 Hz, arom H), 6.88 (d, 1H, J = 8.7 Hz, arom H), 7.08 (dd, 1H, J = 8.6 Hz, J = 2.3, arom H), 7.28 (d, 1H, J = 2.2 Hz, arom H), 7.64 (d, 1H, J = 8.8 Hz, arom H), 10.38 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 35.91, 45.66, 45.79, 54.44, 55.33, 55.71, 102.62, 104.21, 110.98, 112.12, 124.84, 126.56, 132.52, 144.99, 147.87, 148.59, 150.02, 155.88, 165.72. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{21}H_{27}N_6O_3S$, 443.1865; found, 443.1857.

*N-(3,4-Dimethoxyphenyl)-2-((5-(piperidin-1-yl)-3H-imidazo[4,5-b]pyridin-2-yl)thio)acetamide (**28c**).* This compound was prepared using **27c** and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 6:4), affording the title compound in 70% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.56 (s, 6H, 3 × CH_2), 3.46 (s, 4H, 2 × NCH_2), 3.70 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 4.15 (s, 2H, CH_2), 6.66 (d, 1H, J = 8.9 Hz, arom H), 6.88 (d, 1H, J = 8.8 Hz, arom H), 7.08 (dd, J = 8.6 Hz, J = 2.1 Hz, 1H, arom H), 7.28 (d, 1H, J = 2.3 Hz, arom H), 7.62 (d, 1H, J = 8.5 Hz, arom H), 10.32 (br s, 1H, NH), 12.64 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 24.40, 25.06, 36.00, 46.76, 55.41, 55.80, 102.74, 104.27, 111.03, 112.20, 132.58, 145.06, 147.81, 148.66, 155.84, 156.04, 165.30, 165.79. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{21}H_{26}N_5O_3S$, 428.1756; found, 428.1748.

*N-(3,4-Dimethoxyphenyl)-2-((5-(dimethylamino)-3H-imidazo-[4,5-b]pyridin-2-yl)thio)acetamide (**28d**).* This compound was prepared using **27d** and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 1:9), affording the title compound in 43% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.01(s, 6H, 2 × CH_3), 3.70 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 4.15 (s, 2H, CH_2), 6.47 (d, 1H, J = 8.8 Hz, arom H), 6.88 (d, 1H, J = 8.8 Hz, arom H), 7.08 (d, 1H, J = 8.4 Hz, arom H), 7.29 (d, 1H, J = 2.4 Hz, arom H), 7.65 (d, 1H, J = 8.6 Hz, arom H), 10.32 (s, 1H, NH), 12.68 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 35.95, 38.44, 55.34, 55.72, 101.28, 104.22, 110.99, 112.13, 126.53, 127.21, 132.53, 145.01, 145.45, 148.27, 148.62, 155.98, 165.79. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{18}H_{22}N_5O_3S$, 388.1443; found, 388.1438.

*N-(3,4-Dimethoxyphenyl)-2-((5-(2,2,2-trifluoroethoxy)-3H-imidazo[4,5-b]pyridin-2-yl)thio)acetamide (**28e**).* This compound was prepared using **27e** and was purified using a mixture of cyclohexane and EtOAc (in a ratio of 3:7), affording the title compound in 72% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.71 (s, 6H, 2 × OCH_3), 4.24 (s, 2H, CH_2), 4.97 (q, 2H, J = 9.2 Hz, CF_3CH_2), 6.74 (d, 1H, J = 8.5 Hz, arom H), 6.88 (d, 1H, J = 8.6 Hz, arom H), 7.09 (d, 1H, J = 8.6 Hz, arom H), 7.29 (d, 1H, J = 1.4 Hz, arom H), 7.85 (d, 1H, J = 8.4 Hz, arom H), 10.30 (s, 1H, NH), 13.00 (br. s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 36.01, 55.42, 55.80, 60.91, 61.37, 61.83, 62.30, 104.32, 105.13, 111.09, 111.77, 112.03, 112.21, 122.43, 126.11, 132.58, 145.10, 148.68, 157.39, 165.49. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{18}H_{18}F_3N_4O_4S$, 433.1001; found, 433.0995.

***N*-(3,4-Dimethoxyphenyl)-2-(5-(methylthio)-3*H*-imidazo[4,5-*b*]pyridin-2-ylthio)acetamide (28f).** To the compound **27f** (15 mg, 0.08 mmol) in a mixture of 1% aq NaOH solution (304 μL) and EtOH (200 μL), 2-chloroacetamide **4** (18 mg, 0.08 mmol) was added in one portion. The resulting mixture was stirred overnight at room temperature. Then volatiles were removed under reduced pressure and the crude product was purified on preparative TLC (4% of MeOH in CH_2Cl_2), affording compound **28f** as a yellowish solid (20 mg, 0.05 mmol, 63%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.54 (s, 3H, SCH_3), 3.72 (s, 6H, 2 × OCH_3), 4.26 (s, 2H, CH_2), 6.89 (d, 1H, J = 8.7 Hz, arom H), 7.05 (d, 1H, J = 8.4 Hz, arom H), 7.09 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, arom H), 7.30 (d, 1H, J = 2.4 Hz, arom H), 7.32 (d, 1H, J = 8.4 Hz, arom H), 10.36 (s, 1H, NH), 13.09 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 13.20, 35.77, 55.32, 55.70, 104.21, 110.98, 112.10, 115.11, 132.48, 144.98, 148.56, 151.64, 165.36. HRMS (ESI): m/z [M − H]$^-$ calcd for $C_{17}H_{17}N_4O_3S_2$, 389.0742; found, 389.0746.

6-Methoxypyrimidine-2,4-diamine (30a). 6-Chloropyrimidine **29** (1.0 g, 6.92 mmol) was dissolved in anhydrous MeOH (5.8 mL), and a 30% solution of NaOMe in MeOH (2 mL, 10.67 mmol of NaOMe) was added. The resulting reaction mixture was refluxed overnight. After cooling to room temperature, the mixture was neutralized using a 6 N HCl solution. The solvents were removed under reduced pressure, and the crude residue was purified by silicagel flash chromatography, using 5% of MeOH in CH_2Cl_2 as mobile phase, affording compound **30a** as a white solid (780 mg, 5.57 mmol, 80%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.67 (s, 3H, OCH_3), 5.04 (s, 1H, arom H), 5.89 (br s, 2H, NH_2), 6.01 (br s, 2H, NH_2). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 52.41, 75.83, 162.98, 165.94, 170.44.

6-Ethoxypyrimidine-2,4-diamine (30b). This compound was synthesized from 6-chloropyrimidine **29** (1.0 g, 6.92 mmol) using method described for the synthesis of 6-methoxypyrimidine-2,4-diamine. A solution of NaOEt in EtOH (21% w/w, 3.98 mL, 10.67 mmol of NaOEt) and EtOH (5.8 mL) was used, affording the title compound as a white powder (1.03 g, 96%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.22 (t, 3H, J = 7.2 Hz, CH_3), 4.13 (q, 2H, J = 7.2 Hz, CH_2), 5.05 (s, 1H, arom H), 5.99 (br s, 2H, NH_2), 6.11 (br s, 2H, NH_2). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 14.67, 60.44, 76.10, 162.45, 165.50, 169.95.

6-Isopropoxypyrimidine-2,4-diamine (30c). To a solution of 6-chloropyrimidine **29** (500 mg, 3.46 mmol) in *i*-PrOH (15 mL) was added NaH (60% in oil, 277 mg, 6.92 mmol of NaH), and the mixture was refluxed for 60 h. After cooling to room temperature and neutralization using a 6 N HCl solution, the solvents were removed under reduced pressure. The crude residue was purified by silicagel flash chromatography, using a mixture of MeOH and CH_2Cl_2 as mobile phase (in a gradient gradually ranging from 2 to 6% of MeOH), affording compound **30c** as a white solid (571 mg, 98%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.23 (d, 6H, J = 6.0 Hz, 2 × CH_3), 5.05−5.15 (m, 2H, CH and arom H), 6.81 (br s, 2H, NH_2), 6.87 (br s, 2H, NH_2). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 21.89, 68.20, 76.48, 159.10, 162.45, 169.05. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_7H_{13}N_4O$, 169.1089; found, 169.1080.

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6-Methoxy-5-nitrosopyrimidine-2,4-diamine (31a). To a suspension of 6-methoxypyrimidine **30a** (300 mg, 2.14 mmol) in H_2O (1.2 mL) was added $NaNO_2$ (162 mg, 2.36 mmol) in one portion, followed by the dropwise addition of glacial AcOH (192 μL). The resulting mixture was stirred at room temperature for 4 h. The formed purple solid was filtered off, washed with H_2O and ether, and dried at 90 °C under vacuum overnight, affording compound **31a** as a purple solid (344 mg, 95%). The product contained 5% (mol) of starting material. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 4.06 (s, 3H, CH_3), 7.80 (br s, 1H, NH_2), 7.86 (br s, 1H), 8.01 (br s, 1H, NH_2), 10.07 (br s, 1H, NH_2). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 54.12, 139.61, 150.77, 163.45, 171.07. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_5H_8N_5O_2$, 170.0678; found, 170.0675.

6-Ethoxy-5-nitrosopyrimidine-2,4-diamine (31b). The title compound was synthesized from 6-ethoxypyrimidine **30b** (300 mg, 1.95 mmol) according to the procedure described for the synthesis of **31a** (2.1 mL of H_2O was used as a solvent), affording the title compound as a purple solid (324 mg, 91%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.41 (t, 3H, J = 6.9 Hz, CH_3), 4.55 (q, 2H, J = 6.9 Hz, CH_2), 7.77 (s, 1H, NH_2), 7.80 (s, 1H, NH_2), 8.00 (s, 1H, NH_2), 10.12 (s, 1H, NH_2). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 14.37, 62.67, 139.54, 150.85, 163.54, 170.66. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_6H_{10}N_5O_2$, 184.0834; found, 184.0832.

6-Isopropoxy-5-nitrosopyrimidine-2,4-diamine (31c). Title compound was synthesized from 6-isopropoxypyrimidine **30c** (300 mg, 1.78 mmol) according to the procedure described for the synthesis of **31a** (5 mL of H_2O was used as a solvent). After 20 h of stirring at 60 °C, an additional amount of $NaNO_2$ (0.18 mmol) in 100 μL of H_2O and 16 μL of glacial AcOH was added and stirring was continued at room temperature for 5 h. The title compound was obtained as a purple solid (268 mg, 76%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.39 (d, 6H, J = 6.0 Hz, 2 × CH_3), 5.55 (sep, 1H, J = 6.0 Hz, CH), 7.76 (br s, 2H, NH_2), 7.98 (s, 1H, NH_2), 10.12 (s, 1H, NH_2). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 21.81, 69.64, 139.66, 150.95, 163.61, 170.19. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_7H_{12}N_5O_2$, 198.0991; found, 198.0985.

5-Nitrosopyrimidine-2,4,6-triamine (31d). Commercially available 6-aminopyrimidine-2,4-diamine (**30d**) (300 mg, 2.40 mmol) was suspended in H_2O (2.4 mL), and glacial AcOH (220 μL) was added. The mixture was cooled in an ice−water bath, and then a solution of $NaNO_2$ (174 mg, 2.52 mmol) in H_2O (1 mL) was added dropwise. A pink precipitate was formed. The resulting mixture was stirred at room temperature for 15 min. The precipitate was filtered off, washed subsequently with H_2O, acetone, and ether, and dried under vacuum affording compound **31d** as a purple solid (342 mg, 92%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 7.18 (br s, 2H, NH_2), 7.34 (br s, 1H, NH_2), 7.73 (br s, 1H, NH_2), 8.14 (br s, 1H, NH_2), 10.25 (br s, 1H, NH_2). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 137.62, 150.97, 164.85, 166.14. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_4H_7N_6O$, 155.0681; found, 155.0679.

General Procedure for the Synthesis of Purine-8-thiones 33a−e. *Step 1: Synthesis of 4,5-Diaminopyrimidines **32a−e**.* To the 5-nitrosopyrimidine **31a−e** (1 mmol) in a mixture of MeOH and H_2O (1:1, 6 mL) was added $Na_2S_2O_4$ (3 mmol). The resulting mixture was stirred at 50−70 °C until disappearance of the color specific for starting material. Then solvents were removed under reduced pressure, yielding the crude amines **32a−e**, which were used in step 2 without further purification.

Step 2: Ring Closure Using CS_2. The crude amine **32a−e** was dissolved in a mixture of EtOH and H_2O (2:1, 8.5 mL), CS_2 (1.5 mL) was added and the mixture was refluxed overnight. The solvents were removed in vacuo. The crude residue was dissolved in H_2O and neutralized with glacial AcOH. The resulting precipitate was filtered off, washed with H_2O, and dried overnight under vacuum at 90 °C.

*2-Amino-6-methoxy-7H-purine-8(9H)-thione (**33a**).* This compound was synthesized from 6-methoxy-5-nitrosopyrimidine **31a** (150 mg, 0.89 mmol) according to the general procedure, affording the title compound as a yellow solid (131 mg, 75% over 2 steps). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.91 (s, 3H, OCH_3), 6.44 (br s, 2H, NH_2), 12.56 (s, 1H, NH), 12.67 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 53.25, 102.55, 153.13, 153.97, 159.77, 166.44. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_6H_8N_5OS$, 198.0449; found, 198.0439.

*2-Amino-6-ethoxy-7H-purine-8(9H)-thione (**33b**).* This compound was synthesized from 6-ethoxy-5-nitrosopyrimidine **31b** (150 mg, 0.82 mmol) according to the general procedure, affording the title compound as a yellow solid (127 mg, 73% over 2 steps). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.33 (t, 3H, J = 6.6 Hz, CH_3), 4.41 (q, 2H, J = 6.6 Hz, CH_2), 6.39 (s, 2H, NH_2), 12.52 (s, 1H, NH), 12.64 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 14.51, 61.58, 102.56, 152.79, 153.99, 159.73, 166.41. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_7H_{10}N_5OS$, 212.0606; found, 212.0602.

*2-Amino-6-isopropoxy-7H-purine-8(9H)-thione (**33c**).* This compound was synthesized from 6-isopropoxy-5-nitrosopyrimidine **31c** (150 mg, 0.76 mmol) according to the general procedure, affording the title compound as a yellow solid (106 mg, 62% over 2 steps). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.31 (d, 6H, J = 6.0 Hz, 2 × CH_3), 5.36 (sep, 1H, J = 6.0 Hz, CH), 6.36 (s, 2H, NH_2), 12.46 (s, 1H, NH), 12.61 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 21.88, 68.53, 102.77, 152.49, 154.01, 159.74, 166.37. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_8H_{12}N_5OS$, 226.0762; found, 226.0755.

*2,6-Diamino-7H-purine-8(9H)-thione (**33d**).* This compound was synthesized from 5-nitrosopyrimidine **31d** (150 mg, 0.97 mmol) according to the general procedure, affording the title compound as a yellow solid (158 mg, 89% over 2 steps). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 5.89 (s, 2H, NH_2), 6.32 (s, 2H, NH_2), 11.59 (br s, 1H, NH), 12.44 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 101.36, 148.02, 151.95, 160.35, 163.91. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_5H_7N_6S$, 183.0453; found, 183.0441.

*2-Amino-6-(methylthio)-7H-purine-8(9H)-thione (**33e**).* This compound was synthesized from commercially available 5-nitrosopyrimidine-2,4-diamine **31e** (150 mg, 0.81 mmol) according to the general procedure, affording the title compound as a solid (97 mg, 57% over 2 steps). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.54 (s, 3H, CH_3, overlapped with residual solvent signal), 6.44 (s, 2H, NH_2), 12.68 (s, 1H, NH), 12.77 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 10.93, 113.97, 147.91, 151.27, 159.62, 167.50. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_6H_8N_5S_2$, 214.0221; found, 214.0220.

2-(2-Amino-6-methoxy-9*H*-purin-8-ylthio)-*N*-(3,4-dimethoxyphenyl)acetamide (34a). The title compound was synthesized from compound **33a** (110 mg, 0.56 mmol) according to the method described for the synthesis of compound **5g**, using 0.62 mmol of K_2CO_3 and a reaction time of 5 h. The crude residue was purified by silicagel flash chromatography, the mobile phase being a mixture of CH_2Cl_2, MeOH and 7 N methanolic NH_3 (in a ratio of 97:3:2 v/v/v), affording the pure title compound as a white solid (52 mg, 23%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.72 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 4.14 (s, 2H, CH_2), 6.27 (br s, 2H, NH_2), 6.89 (d, 1H, J = 8.7 Hz, arom H), 7.09 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, arom H), 7.28 (d, 1H, J = 2.4 Hz, arom H), 10.38 (s, 1H, NH), 12.66 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 36.03, 53.01, 55.72, 104.14, 110.94, 112.12, 132.50, 144.99, 148.60, 157.51, 158.23, 159.30, 165.61. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{16}H_{19}N_6O_4S$, 391.1188; found, 391.1182.

2-(2-Amino-6-ethoxy-9*H*-purin-8-ylthio)-*N*-(3,4-dimethoxyphenyl)acetamide (34b). The title compound was synthesized from compound **33b** (50 mg, 0.24 mmol) according to the procedure used for the synthesis of compound **5h**, using 0.26 mmol of K_2CO_3 and a reaction time of 16 h. The crude residue was purified by silicagel flash chromatography, the mobile phase being a mixture of CH_2Cl_2, MeOH, and 7 N methanolic NH_3 (98:2:2 v/v/v), affording the pure title compound **34b** as a white solid (37 mg, 39%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.34 (t, 3H, J = 7.2 Hz, CH_2CH_3), 3.71 (s, 6H, 2 × OCH_3), 4.14 (s, 2H, SCH_2), 4.42 (q, 2H, J = 7.2 Hz, OCH_2), 6.21 (s, 2H, NH_2), 6.88 (d, 1H, J = 8.7 Hz, arom H), 7.07 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, arom H), 7.30 (d, 1H, J = 2.4 Hz, arom H), 10.39 (s, 1H, NH), 12.64 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 14.58, 36.08, 55.35, 55.72, 61.31, 104.19, 110.92, 112.11, 132.49, 144.99, 148.58, 159.26, 165.60. HRMS

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(ESI): m/z [M + H]$^+$ calcd for $C_{17}H_{21}N_6O_4S$, 405.1345; found, 405.1331.

2-(2-Amino-6-isopropoxy-9*H*-purin-8-ylthio)-*N*-(3,4-dimethoxyphenyl)acetamide (34c). The title compound was synthesized from compound **33c** (96 mg, 0.43 mmol) according to the method for the preparation of compound **5h**, using 0.47 mmol of K_2CO_3 and a reaction time of 6 h. The crude residue was purified by silicagel flash chromatography, using a mixture of CH_2Cl_2, MeOH, and 7 N methanolic NH_3 (98:2:2 v/v/v) as mobile phase, affording the title compound **34c** as a white powder (96 mg, 53%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.32 (d, 6H, J = 6.0 Hz, CH(CH_3)$_2$), 3.72 (s, 6H, 2 × OCH_3), 4.15 (s, 2H, CH_2), 5.46 (sep, 1H, J = 6.0 Hz, CH), 6.19 (br s, 2H, NH_2), 6.88 (d, 1H, J = 8.7 Hz, arom H), 7.05 (dd, 1H, J = 8.7 Hz, J = 1.8 Hz, arom H), 7.33 (d, 1H, J = 1.8 Hz, arom H), 10.38 (s, 1H, NH), 12.63 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 21.95, 36.12, 55.38, 55.72, 67.92, 104.26, 110.93, 112.11, 132.48, 144.49, 145.01, 148.59, 156.82, 157.85, 159.28, 165.58. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{18}H_{23}N_6O_4S$, 419.1501; found, 419.1485.

2-(2,6-Diamino-9*H*-purin-8-ylthio)-*N*-(3,4-dimethoxyphenyl)acetamide (34d). The title compound was synthesized from compound **33d** (50 mg, 0.27 mmol) according to the method used for the preparation of compound **5h**, using 0.81 mmol of K_2CO_3 and a reaction time of 4.5 h. The crude residue was purified by silicagel flash chromatography, using a mixture of CH_2Cl_2, MeOH, and 7 N methanolic NH_3 (96:4:1 v/v/v) as mobile phase. Fractions containing product were combined and then purified by preparative TLC (CH_2Cl_2/MeOH/7N methanolic NH_3 9:1:0.2 v/v/v), affording the title compound as a solid (18 mg, 18%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.70 (s, 6H, 2 × OCH_3), 4.00 (s, 2H, CH_2), 5.82 (br s, 2H, NH_2), 6.68 (br s, 2H, NH_2), 6.87 (d, 1H, J = 8.7 Hz, arom H), 7.11 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, arom H), 7.24 (d, 1H, J = 2.4 Hz, arom H), 10.26 (s, 1H, NH), 12.34 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 36.22, 55.35, 55.71, 104.27, 111.01, 112.03, 132.40, 144.97, 148.54, 154.05, 159.15, 166.05. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{15}H_{18}N_7O_3S$, 376.1192; found, 376.1187.

2-(2-Amino-6-(methylthio)-9*H*-purin-8-ylthio)-*N*-(3,4-dimethoxyphenyl)acetamide (34e). The mixture of compound **33e** (50 mg, 0.23 mmol), 2-chloroacetamide **4** (54 mg, 0.23 mmol), and K_2CO_3 (33 mg, 0.23 mmol) in DMF (540 μL) was stirred at room temperature for 2 h and then was concentrated to dryness. The solid residue was put on silica gel column. Elution using mixture of MeOH in CH_2Cl_2 (from 2 to 6% of MeOH) afforded the title compound as a powder (19 mg, 22%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.56 (s, 3H, SCH_3), 3.71 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 4.16 (s, 2H, CH_2), 6.33 (br s, 2H, NH_2), 6.89 (d, 1H, J = 8.7 Hz, arom H), 7.09 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, arom H), 7.28 (d, 1H, J = 2.4 Hz, arom H), 10.32 (s, 1H, NH), 12.77 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 10.90, 36.04, 55.36, 55.70, 104.29, 110.19, 111.03, 112.09, 132.39, 145.00, 148.57, 159.14, 165.45. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{16}H_{19}N_6O_3S_2$, 407.0960; found, 407.0947.

2-(Methylthio)pyrimidine-4,6-diamine (36). 6-Chloropyrimidin-4-amine **35** (500 mg, 2.85 mmol) was treated with a 7 N methanolic ammonia solution (7 mL), and the resulting mixture was stirred in sealed vessel at 120 °C for 7 days. After cooling to room temperature, the volatiles were removed in vacuo and the solid residue was purified by silicagel flash chromatography, the mobile phase being a mixture of MeOH and CH_2Cl_2 (isocratic 6% MeOH in CH_2Cl_2), affording the title compound as a yellow solid (130 mg, 29%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.32 (s, 3H, CH_3), 5.13 (s, 1H, arom H), 6.06 (br s, 4H, 2 × NH_2). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 12.87, 78.97, 163.40, 168.77. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_5H_9N_4S$, 157.0548; found, 157.0541.

2-(Methylthio)-5-nitrosopyrimidine-4,6-diamine (37). This compound was synthesized from 2-(methylthio)pyrimidine **36** (120 mg, 0.77 mmol), $NaNO_2$ (0.92 mmol), and glacial AcOH (365 μL) according to the procedure described for the synthesis of **31a** (1.3 mL of H_2O was used as a solvent). After stirring at room temperature for 6 h, an additional amount of $NaNO_2$ (0.62 mmol) was added and stirring was continued at 50 °C for 20 h. Yield: 120 mg (0.65 mmol, 84%) of compound **37** as a blue−green solid (containing 6% of starting material). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.47 (s, 3H, CH_3), 8.01 (br s, 1H, NH_2), 8.40 (br s, 1H, NH_2), 8.99 (br s, 1H, NH_2), 10.18 (br s, 1H, NH_2). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 13.59, 138.97, 145.74, 164.28, 178.61. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_5H_8N_5OS$, 186.0450; found, 186.0444.

6-Amino-2-(methylthio)-7*H*-purine-8(9*H*)-thione (39). This compound was synthesized from 2-(methylthio)-5-nitrosopyrimidine **37** (111 mg, 0.60 mmol) according to the procedure described for the synthesis of compounds **33a−d**, affording the title compound as a solid (91 mg, 71% yield over 2 steps). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.42 (s, 3H, CH_3), 6.82 (br s, 2H, NH_2), 12.65 (br s, 2H, 2 × NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 13.55, 105.53, 147.13, 150.63, 164.14, 165.79. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_6H_8N_5S_2$, 214.0221; found, 214.0213.

2-(6-Amino-2-(methylthio)-9*H*-purin-8-ylthio)-*N*-(3,4-dimethoxyphenyl)acetamide (40). The title compound was synthesized from compound **39** (43 mg, 0.20 mmol) according to the method used for the preparation of compound **28f**. The crude residue was purified by silicagel flash chromatography using mixture of MeOH in CH_2Cl_2 (from 2 to 5% of MeOH), affording the title compound as a white powder (66 mg, 80%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.43 (s, 3H, SCH_3), 3.71 (s, 6H, 2 × OCH_3), 4.10 (s, 2H, CH_2), 6.88 (d, 1H, J = 8.7 Hz, arom H), 7.09 (dd, 1H, J = 8.7 Hz, J = 2.1 Hz, arom H), 7.17 (br s, 2H, NH_2), 7.25 (d, 1H, J = 2.1 Hz, arom H), 10.14 (s, 1H, NH), 13.03 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 13.56, 35.99, 55.35, 55.71, 104.35, 111.10, 112.06, 116.89, 132.31, 144.71, 145.04, 148.55, 152.82, 153.52, 163.21, 165.61. HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{16}H_{19}N_6O_3S_2$, 407.0960; found, 407.0951.

6-Amino-1-ethyl-5-nitrosopyrimidine-2,4(1*H*,3*H*)-dione (42). Compound **41**[22,23] (300 mg, 1.94 mmol) was suspended in mixture of EtOH and H_2O (1:1 v/v, 10 mL). Next, $NaNO_2$ (2.81 mmol) was added, followed by the dropwise addition of glacial AcOH (387 μL). The mixture was stirred at room temperature for 2.5 h. The purple solid was filtered off, washed with H_2O, and dried. The filtrate was concentrated to dryness. A small amount of H_2O was added, and the suspension was cooled in an ice−water bath. The precipitate was filtered off, dried, and combined with the first part of product, affording the title compound in 83% yield (297 mg, 1.61 mmol, 83%) as a purple solid. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.10 (t, 3H, J = 6.9 Hz), 3.82 (q, 2H, J = 6.9 Hz), 9.16 (br s, 1H), 11.50 (s, 1H), 13.30 (s, 1H) . ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 12.00, 35.68, 138.74, 146.49, 148.52, 160.35.

3-Ethyl-8-thioxo-8,9-dihydro-1*H*-purine-2,6(3*H*,7*H*)-dione (44a). This compound was synthesized from 5-nitrosopyrimidine **42** (236 mg, 1.28 mmol) according to the procedure described for the synthesis of **33a−d**. Yield: 178 mg (0.84 mmol, 65%, after 2 steps) of **44a** as yellowish solid. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.14 (t, 3H, J = 6.9 Hz, CH_3), 3.85 (q, 2H, J = 6.9 Hz, CH_2), 11.24 (s, 1H, NH), 12.96 (s, 1H, NH), 13.39 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 13.24, 38.44, 104.21, 139.99, 149.37, 151.91, 163.94. HRMS (ESI): m/z [M − H]$^-$ calcd for $C_7H_7N_4O_2S$, 211.0290; found, 211.0298.

8-Thioxo-8,9-dihydro-1*H*-purine-2,6(3*H*,7*H*)-dione (44b). Title compound was synthesized from commercially available 5,6-diaminouracil sulfate dihydrate (**43b**) (308 mg, 0.74 mmol), $NaHCO_3$ (495 mg, 5.89 mmol), and CS_2 (0.7 mL) according to the method described for the synthesis of compound **2g**. Yield: 271 mg (1.47 mmol, quant.) of **44b** as a solid. ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 103.79, 139.50, 150.32, 152.66, 163.95. HRMS (ESI): m/z [M − H]$^-$ calcd for $C_5H_3N_4O_2S$, 182.9977; found, 182.9978.

1,3-Dimethyl-8-thioxo-8,9-dihydro-1*H*-purine-2,6(3*H*,7*H*)-dione (44c). Title compound was synthesized from commercially available 5,6-diamino-1,3-dimethyluracil (**43c**) (400 mg, 1.94 mmol), $NaHCO_3$ (979 mg, 11.65 mmol), and CS_2 (1.5 mL) according to the method described for the synthesis of compound **2g**. The mixture was refluxed for 7 h. The title compound was obtained as a solid (122 mg, 29%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.18 (s, 3H, CH_3),

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3.36 (s, 3H, CH_3), 12.99 (s, 1H, NH), 13.41 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 27.79, 31.11, 103.57, 139.43, 150.05, 151.51, 163.86. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_7H_9N_4O_2S$, 213.0446; found, 213.0443.

***N*-(3,4-Dimethoxyphenyl)-2-(3-ethyl-2,6-dioxo-2,3,6,9-tetrahydro-1*H*-purin-8-ylthio)acetamide (45a).** The title compound was synthesized from compound **44a** (50 mg, 0.24 mmol) according to the method used for the preparation of compound **34e**. Reaction time: 16 h. The crude residue was purified by silicagel flash chromatography, the mobile phase being a mixture of MeOH and CH_2Cl_2 (5% of MeOH). The fractions containing the desired product were combined and concentrated. The solid residue was boiled in MeOH, cooled down to room temperature, and the precipitate filtered off and dried, affording the pure title compound as a white solid (43 mg, 46%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.13 (t, 3H, J = 6.9 Hz, CH_2CH_3), 3.72 (s, 6H, 2 × OCH_3), 3.91 (q, 2H, J = 6.9 Hz, NCH_2), 4.14 (s, 2H, SCH_2), 6.89 (d, 1H, J = 8.7 Hz, arom H), 7.07 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, arom H), 7.28 (d, 1H, J = 2.4 Hz, arom H), 10.17 (s, 1H, NH), 11.03 (s, 1H, NH), 13.53 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 13.18, 36.47, 37.08, 55.36, 55.70, 104.26, 108.34, 111.06, 112.06, 132.43, 145.01, 148.48, 148.54, 149.38, 150.31, 153.58, 165.26. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{17}H_{20}N_5O_5S$, 406.1185; found, 406.1179.

***N*-(3,4-Dimethoxyphenyl)-2-(2,6-dioxo-2,3,6,9-tetrahydro-1*H*-purin-8-ylthio)acetamide (45b).** The title compound was synthesized from compound **44b** (50 mg, 0.27 mmol) according to the method used for the preparation of compound **34e**. Reaction time: 4.5 h. The crude residue was purified by silicagel flash chromatography, the mobile phase being a mixture of MeOH and CH_2Cl_2 (gradually ranging from 2 to 8% of MeOH), yielding the pure title compound as a solid (41 mg, 41%) ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.72 (s, 6H, 2 × OCH_3), 4.11 (s, 2H, CH_2), 6.89 (d, 1H, J = 8.7 Hz, arom H), 7.08 (dd, 1H, J = 8.7 Hz, J = 2.3 Hz, arom H), 7.27 (d, 1H, J = 2.3 Hz, arom H), 10.25 (s, 1H, NH), 10.78 (s, 1H, NH), 11.56 (br s, 1H, NH), 13.39 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 36.31, 55.38, 55.72, 104.28, 108.34, 111.07, 112.10, 132.39, 145.03, 148.35, 148.58, 149.18, 151.12, 154.48, 165.22. HRMS (ESI): m/z $[M - H]^-$ calcd for $C_{15}H_{14}N_5O_5S$, 376.0716; found, 376.0715.

***N*-(3,4-Dimethoxyphenyl)-2-(1,3-dimethyl-2,6-dioxo-2,3,6,9-tetrahydro-1*H*-purin-8-ylthio)acetamide (45c).** The title compound was synthesized from compound **44c** (50 mg, 0.24 mmol) according to the method used for the preparation of compound **28f**. Reaction time: 4 h. The crude residue was purified by silicagel flash chromatography, the mobile phase being a mixture of MeOH and CH_2Cl_2 (gradually ranging from 2 to 3% of MeOH), yielding the pure title compound as a white powder (69 mg, 71%) ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 3.20 (s, 3H, NCH_3), 3.39 (s, 3H, NCH_3), 3.71 (s, 6H, 2 × OCH_3), 4.17 (s, 2H, CH_2), 6.88 (d, 1H, J = 8.7 Hz, arom H), 7.06 (dd, 1H, J = 8.7 Hz, J = 2.3 Hz, arom H), 7.27 (d, 1H, J = 2.3 Hz, arom H), 10.19 (s, 1H, NH), 13.57 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 27.67, 29.74, 36.52, 55.36, 55.70, 104.30, 107.88, 111.11, 112.06, 132.37, 145.06, 148.19, 148.32, 148.57, 150.91, 153.31, 165.26. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{17}H_{20}N_5O_5S$, 406.1185; found, 406.1179.

Biological Evaluation. *Colorimetric NPP Assay Using p-Nitrophenyl-5′-TMP as a Substrate.* The initial screenings were performed at 37 °C in a total volume of 100 μL in a clear 96-well microplate.[17] The reaction mixture contained 1 mM $CaCl_2$, 200 μM $ZnCl_2$, 50 mM Tris, pH 9.0, 400 μM *p*-Nph-5′-TMP, and 10 μM of each test compounds. The enzyme reactions were started by the addition of 20 ng of human soluble NPP1 (obtained from R&D Systems GmbH, Wiesbaden, Germany), then incubated at 37 °C for 15 min and subsequently terminated by the addition of 20 μL of 1.0 N NaOH. The amounts of released *p*-nitrophenolate were measured at 400 nm. For determination of IC_{50} values, different concentrations of each inhibitor were prepared with 50 mM Tris buffer (pH 9.0) containing 1 mM $CaCl_2$, 200 μM $ZnCl_2$, and 400 μM *p*-Nph-5′-TMP in a final volume of 100 μL. Incubation and operation conditions remained the same as described above. The Cheng–Prusoff equation was used to calculated the K_i values from the IC_{50} values determined by the nonlinear curve fitting program PRISM 5.0 (Graphpad Software, San Diego, CA, USA).[24] The Michaelis–Menten constant (K_m) for *p*-Nph-5′-TMP was 222 μM.

Capillary Electrophoresis-Based NPP Assay with ATP as a Substrate. For the determination of IC_{50} values, the enzyme inhibition assays were performed in 10 mM 2-(*N*-cyclohexylamino)-ethanesulfonic acid (CHES) buffer (pH 9.0) including 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 400 μM of ATP, with different inhibitor concentrations. The reaction mixture was incubated with 20 ng human NPP1 at 37 °C for 30 min in a final volume of 100 μL, and the reactions were stopped by heating at 90 °C for 3 min. Finally, the reaction mixtures were directly measured by capillary electrophoresis (CE). The CE instrumentation and operating conditions were as follows: P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) with a DAD detection system, polyacrylamide-coated capillaries of 40 cm effective length × 50 μm (id) obtained from CS Chromatographie GmbH (Langerwehe, Germany), 50 mM phosphate buffer (pH 6.5) as running buffer, electrokinetic injection (−6 kV, 60 s), separation voltage of −15 kV; the amounts of AMP produced were measured at 260 nm. Data collection and peak area analysis were performed by 32 Karat software obtained from Beckman Coulter (Fullerton, CA, USA). The IC_{50} values of test compounds were calculated by plotting of three independent experiments using the program Prism 5.0, and the K_i values were calculated from the IC_{50} values with the Cheng–Prusoff equation.[24] The Michaelis–Menten constant (K_m) for ATP was 8.17 μM.

Determination of Enzyme Inhibition Mechanisms. The inhibition mechanism of the parent compound **5a** was determined using different concentrations of each substrate (from 10 to 1500 μM), and three different concentrations (0, ∼0.5-fold, and ∼2-fold of IC_{50} values) of **5a**. The operation conditions for those experiments were the same as described for the colorimetric NPP1 assay using p-nitrophenyl-5′-TMP as a substrate and the capillary electrophoresis-based NPP1 assay with ATP as a substrate. Each analysis was performed in three separate experiments. The inhibition type was then evaluated graphically from the Lineweaver–Burk plots using Prism 5.0.

Correlation Analysis between Different Assays. To determine correlation coefficients (R^2) between the results with different substrates, pK_i values (−log K_i) were calculated from the K_i values obtained in *p*-Nph-5′-TMP and ATP assays. Correlation coefficients (R^2) were then obtained by fitting pK_i values between assays using Prism 5.0. The selected compounds for this analysis are listed in Table 7.

Enzyme Inhibition Assays at Human NPP2 and Human NPP3. The inhibitory effects of the compound **12** on lysophospholipase D activity of human NPP2 was assayed in reaction buffer containing 5 mM $MgCl_2$, 5 mM $CaCl_2$, 100 mM Tris, pH 9.0, 400 μM lysophosphatidylcholine (18:1) (LPC (18:1)), and diverse concentrations of the inhibitor. The reaction was initiated by adding 44 μg of human NPP2 and then incubated at 37 °C for 60 min. Subsequently, the released choline was quantified spectrophotometrically at 555 nm after incubation at 37 °C for 10 min with 50 μL of each, the peroxidase reagent (50 mM Tris at pH 9.0, 2 mM 3-(*N*-ethyl-3-methylanilino)-2-hydroxypropanesulfonic acid (TOOS), 5 U/mL peroxidase), and the choline oxidase reagent (50 mM Tris at pH 9.0, 2 mM 4-aminoantipyrine, 5 U/mL choline oxidase). The inhibitory activity of the NPP1 inhibitor (see above) at human NPP3 was performed as described for the capillary electrophoresis-based NPP1 assay with ATP as a substrate (using 43 μg of human NPP3). The expression of human NPP2 and human NPP3 in Sf9 insect cells will be published elsewhere. The IC_{50} values of test compounds at both enzymes were determined by fitting of three independent experiments using the program Prism 5.0, and the K_i values were determined from the IC_{50} values with the Cheng–Prusoff equation.[24] The Michaelis–Menten constants (K_m) was 138 μM for the human NPP2 (LPC (18:1)) and 43.4 μM for the human NPP3, respectively.

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■ ASSOCIATED CONTENT

Supporting Information

HPLC purity data of final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AcOH, acetic acid; ATP, adenosine triphosphate; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EtOAc, ethyl acetate; NPP, nucleotide pyrophosphatase/phosphodiesterase; *p*-Nph-5′-TMP, *p*-nitrophenyl 5′-thymidine monophosphate; rt, room temperature; SAR, structure−activity relationship; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TOOS, 3-(*N*-ethyl-3-methylanilino)-2-hydroxypropanesulfonic acid

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