Discovery of 4,4'-Dipyridylsulfide Analogs as "Switchable Electrophiles" for Covalent Inhibition

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ABSTRACT:

Electrophilic heterocycles offer attractive features as covalent fragments for inhibitor and probe development. A focused library of heterocycles for which protonation can enhance reactivity (called "switchable electrophiles") is screened for inhibition of the proposed drug target dimethylarginine dimethylaminohydrolase (DDAH). Several novel covalent fragments are identified: 4-chloroquinoline, 4-bromopyridazine and 4,4-dipyridylsulfide. Mechanistic studies of DDAH inactivation by 4,4-dipyridylsulfide reveals selective covalent S-pyridinylation of the active-site Cys through catalysis by a neighboring Asp residue. Inactivation (k_{inact}/K_I = 0.33 $M^{-1}s^{-1}$) proceeds with release of 4-thiopyridone (0.78 equiv) and structure-activity relationships reveal that the leaving group pK_a can be modulated to tune reactivity. The use of a "switchable electrophile" strategy helps impart selectivity, even to fragment-sized modifiers. Identification of 4,4-dipyridylsulfide analogs as inactivators offers an easily tunable covalent fragment with multiple derivatization sites on both the leaving and staying groups.

The use of electrophilic fragments (also called covalent fragments[1, 2]) for inhibitor development has broadened to include aromatic fragments that can undergo nucleophilic aromatic substitution (S_NAr) by protein nucleophiles. Aryl halides, electrophilic heterocycles, and 2-sulfonylpyridines are a few notable examples used in fragment libraries and in selective inhibitors.[3-5] Electrophilic aromatic fragments represent promising scaffolds in which reactivity can be predictably tuned by swapping heterocycles, installation of electron withdrawing or donating groups, and substitution of different leaving groups. These fragments often have a nascent protein binding surface and offer multiple sites for derivatization that are distant from the site of nucleophilic attack, providing a potential advantage over other covalent fragments that are more restricted and less extensible.

Most often, covalent fragments are transformed into selective inhibitors through a combination of decreasing the inherent reactivity of the electrophile and increasing non-covalent binding affinity to the target protein through addition of protein binding substituents.[6] However, there are other less-used strategies to increase the selectivity of covalent inhibitors. One such example that we previously described is inactivation by fragment-sized 4-halopyridines of DDAH (dimethylarginine dimethylaminohydrolase), a Cys-dependent hydrolase that degrades N^{ω}-methylated arginine residues and a proposed target for anti-angiogenic, anti-sepsis, and anti-fibrotic drug development.[7-12] Mechanistic analysis revealed that 4-halopyridines act as "switchable electrophiles" that are predominately neutral and unreactive in solution but can be "switched on" to a highly-reactive state by stabilization of the protonated form of the inhibitor when bound to a target protein (Figure 1).[7, 8] Model studies revealed a ~4500-fold increase in reactivity with thiols upon formation of the cationic pyridinium.[13] This strategy can impart selectivity even to fragment-sized covalent modifiers because of the dual requirements of the targeted site to 1) stabilize the protonated form of the inhibitor and 2) provide an appropriately placed nucleophile. The use of protonation as a switching mechanism provides a way to impart additional selectivity to a fragment-sized inhibitor, but is simpler than many mechanism-based

inactivators and might be more easily applied during inhibitor design. Here we broaden the scope of "switchable electrophile" scaffolds through the discovery of 4,4'-dipyridylsulfide and related analogs as covalent protein modifiers.

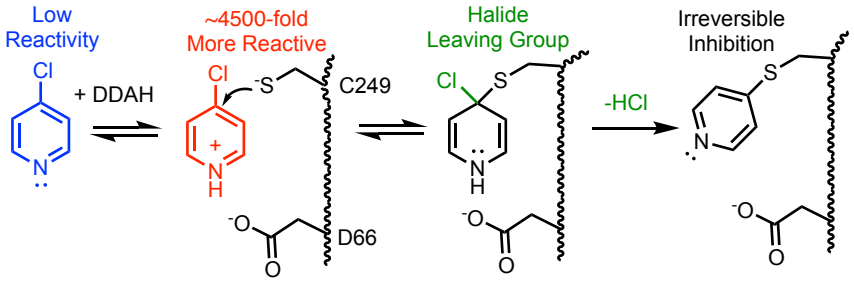


Figure 1. Use of a "Switchable Electrophile" as an enzyme inactivator. Predominately neutral 4-chloropyridine derivatives have low reactivity (blue) with glutathione in solution, but a cationic pyridinium (red) is ~4500-fold more reactive. The Asp66 of *Pa* DDAH stabilizes the pyridinium, "switching on" this electrophile when bound adjacent to Cys249, leading to facile reaction, loss of the halide (green) and irreversible inactivation catalyzed by the targeted protein.

We compiled a focused library of fragment-sized (109 - 314 Da) compounds (**1**-**51**) that we predicted to have enhanced electrophilicity upon protonation of a heterocyclic nitrogen (Figure 2, Table S1). Our initial library consists mostly of pyridines and quinolines that have a pK_a value between -1 and 8.7 and a potential leaving group at the 2- or 4-position. All the listed pK_a values are calculated (Supporting Information). Other potential scaffolds were included that could be activated in a similar manner (e.g. 3-chloromethylquinoline[14]). Other "switchable electrophiles" can be envisioned using the same principles. To gauge reactivity, each compound (4 mM) was incubated (1h) with glutathione (GSH, 300 μM) as a model thiol nucleophile and then assayed to quantify remaining thiol groups. Six different thiol-reactive compounds (**52**-**57**) that were included as positive controls readily reacted with the glutathione thiol group, but all of the other compounds showed only <10 % reaction (Table S1; pH 7.3, 25 °C), consistent with the library compounds being mostly unreactive in their neutral forms.

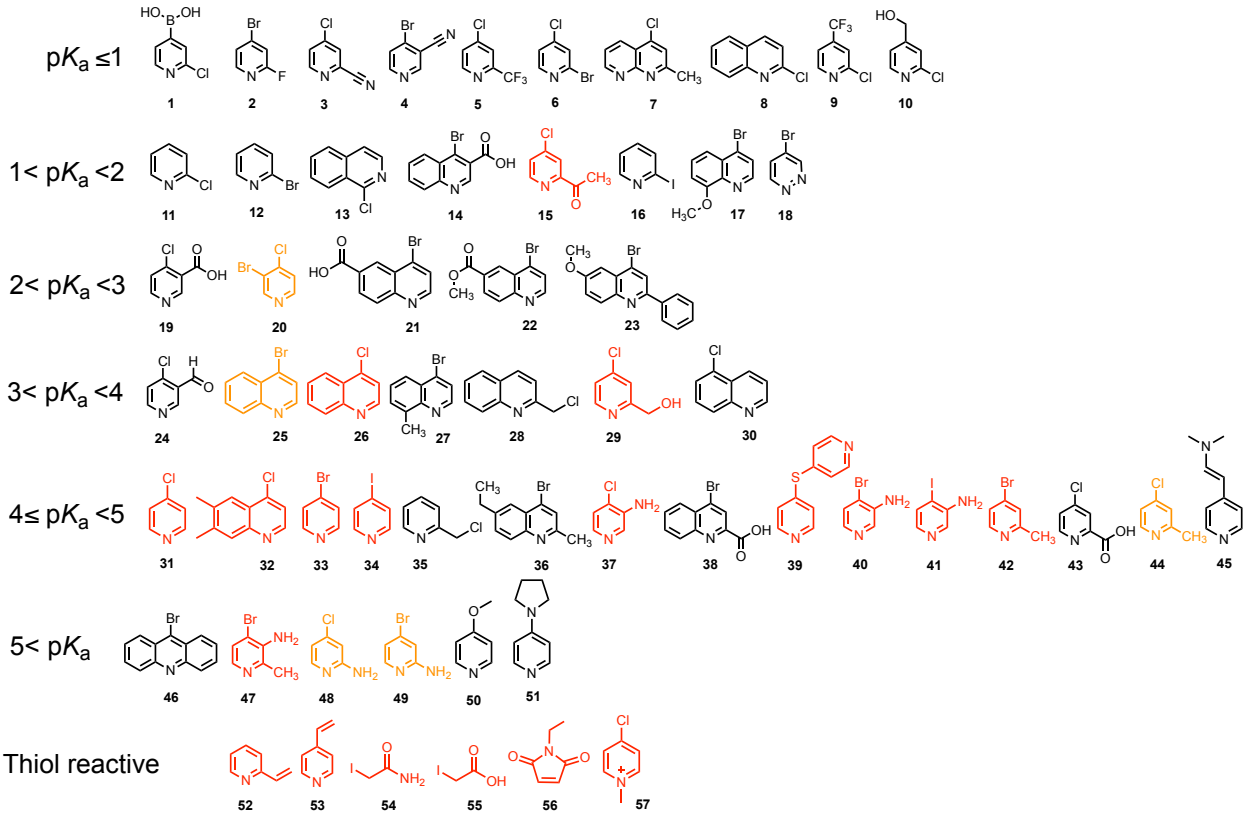


Figure 2. "Switchable Electrophile" Fragment Library. Fragment-sized compounds (**1**-**51**) are proposed to have enhanced electrophilicity upon protonation of the heterocyclic nitrogen. The fragments are depicted in rows of increasing pK_a for the heterocyclic nitrogen. The bottom row consists of compounds (**52**-**57**) that do not require protonation to facilitate reaction with thiols and serve as positive controls. Compounds are color coded to indicate the percent inhibition of *Pa* DDAH after incubation at 4 mM compound for 1 h: Black 0-10 %, Orange 11-85 %, Red 86-100 %.

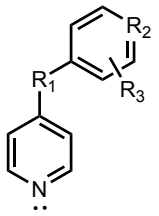
Compounds (4 mM) were then incubated (1h) with either *Pseudomonas aeruginosa* (*Pa*) DDAH or *human* DDAH1 (Figures 2, S1, Table S1) and subsequently diluted into saturating amounts of substrate to detect remaining activity in comparison with an uninhibited control to reveal the extent of irreversible inhibition. A number of inactivators were identified. The high hit rate is likely due to overlap of the library's focus with the pharmacophore of DDAH and not due to non-selective thiol reactivity. Clear structure-activity relationships are observed: leaving groups at the 4-positions, and not the 2-positions, are preferred and carboxylates at the 2-positions are disfavored. These hits likely reflect constraints of the DDAH active site for positioning the nucleophile and the pyridinium-stabilizing amino acid side chain, along with repulsion by nearby

anionic residues (e.g. Asp66 in *Pa* DDAH[15]). Despite 29% amino acid identity (45% similarity), DDAH ortholog-selective inhibitors were found in the library: The larger active-site of *Pa* DDAH is readily inactivated by **32** but *human* DDAH-1 is not inactivated, although the slightly smaller compound **26** was a good inactivator of both isoforms. In general, more hits are found in "switchable electrophiles" with higher pK_a values, reflecting the easier barrier to reach the reactive protonated form. Most notably, several new "switchable electrophile" scaffolds are identified as DDAH inhibitors: 4-chloroquinoline (**26**) inactivates *Pa* DDAH (k_{inact}/K_I = 12.0 $\pm$ 0.3 $M^{-1}s^{-1}$), 4-bromopyridazine (**18**) inactivates human DDAH1, and 4,4'-dipyridylsulfide (**39**) inactivates *Pa* DDAH (k_{inact}/K_I = 0.33 $\pm$ 0.02 $M^{-1}s^{-1}$) (Figures 2, 3, S1, S2, Table S1). An elaborated 4-chloroquinoline has been reported elsewhere as a covalent inhibitor for a different target and is reidentified here as a covalent fragment.[16] In both cases, the 4-chloroquinoline moiety likely acts as a "switchable electrophile." We could not identify any previous use of halopyrazidines as covalent inhibitors, but inactivation by this compound was relatively weak and limited to one DDAH ortholog, so was not further characterized here.

In contrast to the other hits, 4,4'-dipyridylsulfide (**39**) offers an opportunity to vary leaving group attributes much more widely, so we characterized this inactivator in more detail. This compound is reported elsewhere as a rapid (no time-dependence observed) competitive inhibitor of cytochrome CYP2A6 that likely binds to the heme iron through coordination by the pyridine nitrogen.[17, 18] To the best of our knowledge, 4,4'-dipyridylsulfides were not previously known to be covalent protein modifiers and so represent a novel covalent fragment. We determined that inhibition of *Pa* DDAH is irreversible to dialysis (Figure S3). Preincubation of **39** with an equimolar concentration of GSH did not appreciably slow inactivation rates, showing that **39** is not just a non-selective thiol-reactive reagent (Figure S4). The lack of reactivity with thiols in solution can be rationalized by low reactivity of the predominating neutral form

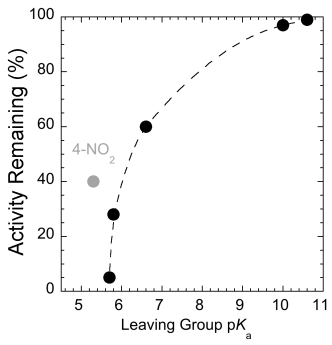
(predicted pK_a values of **39** are 4.4 and 5). To probe structure activity relationships in more depth, we prepared several analogs (Figure 3).

a



Compound	R_1	R_2	R_3	Pyridine pK_a	Leaving Group pK_a	Activity Remaining (%)	k_{inact}/K_I ($M^{-1}s^{-1}$)
39	S	N	H_4	4.4	5.7	5	0.33 ± 0.02
39a	S	CH	H_4	4.7	6.6	60	0.029 ± 0.001
39b	S	CNO_2	H_4	4.7	5.3	40	N.D.
39c	O	N	H_4	5.6	10.6	99	N.D.
39d	O	CH	H_4	6.0	10.0	97	N.D.
39e	O	CF	F_4	5.8	5.8	28	0.072 ± 0.006

b



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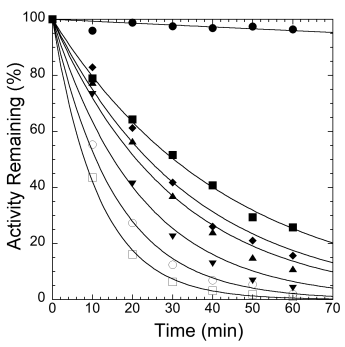

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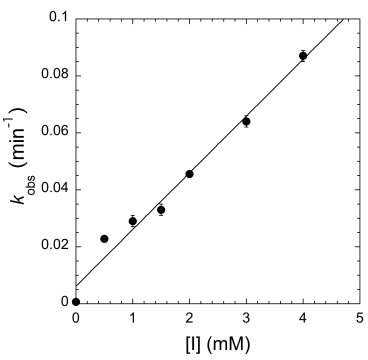
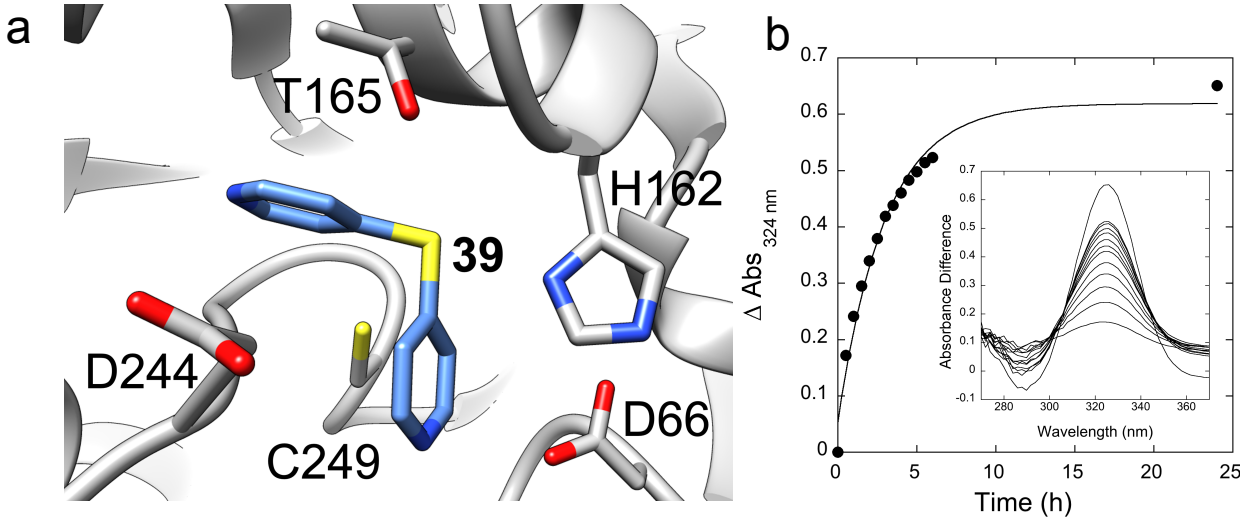


Figure 3. Time- and Concentration-Dependent Inactivation of *Pa* DDAH by **39** and Analogs. a) Analogs of **39** were tested for inhibition (activity remaining errors ± 1 %) as described in Figure 2, and k_{inact}/K_I determined for selected compounds (N.D., not determined), graphs shown in Supporting Information. b) Graph depicting the impact of leaving group pK_a on inhibition, with dashed line interpolating between points to emphasize the trend. Compound **39b** (grey circle) was not included in the trend due to steric differences from the series. c) Time-dependent inhibition of *Pa* DDAH by **39**: 0, 0.5, 1, 1.5, 2, 3 and 4 mM **39** (top to bottom) showing fits for single exponential decay kinetics. d) Replot of k_{obs} at each concentration **39** from panel c, with a linear fit with the slope yielding k_{inact}/K_I (listed in panel a).

One pyridine of the symmetrical **39** can be replaced by a phenyl substituent (**39a**), although inactivation rates are decreased. We reasoned that the increase of leaving group pK_a in **39a** might disfavor inactivation and so installed an electron-withdrawing 4-NO_2 substituent to lower this pK_a. The resulting compound **39b** displayed improved inhibition rates. Replacement of the thioether bridge with an oxygen ether resulted in a loss of almost all ability to inactivate DDAH using the dipyridinyl analog (**39c**) or the phenyl analog (**39d**). However, lowering the pK_a of the phenol leaving group through pentafluoro substitution (**39e**) is sufficient to regain irreversible

inhibition. We interpret these results to indicate that the leaving group pK_a, and not the difference in bond angles between oxygen ethers and thioethers, is the dominant factor. The leaving group pK_a trend is observed for all of the analogs of **39** but deviates for the bulky 4-NO_2 substituent that may impair binding (Figure 3b). Determination of k_{inact}/K_I for selected compounds confirms the same rank order and shows that, as expected for covalent fragments, none of the inactivators display saturation kinetics at the concentrations used (Figures 3a, c, d, S2, S5, S6; with the possible exception of **26**, Figure S2). We previously demonstrated how the nitrogen pK_a of 4-halopyridines can be used to predictably tune k_{inact}/K_I, with higher pK_a values correlating with increased reactivity.[13] Here we show that the leaving group pK_a of 4,4'-dipyridylsulfide analogs can also be used to tune k_{inact}/K_I.

Next, we investigated how *Pa* DDAH helps catalyze its own inactivation (Figure 4). Docking of unreacted **39** to the *Pa* DDAH active site suggests that a close approach of the Cys249 thiol to the C4 of **39** is possible and shows two potential stabilizing residues for the cationic pyridinium group < 3 Å away from the docked ligand: Asp66 and Asp244. Asp66 was previously shown to be the pyridinium stabilizing residue that catalyzes inactivation by 4-halopyridines.[7]



c

Pa DDAH Preparation	Calc'd Mass (Da)	Protein-Only Mass (Da)	Mass of Proteins After Incubation with **39** (Da)		Mass of Proteins After Incubation with **39f** (Da)	
			$Mass_{obsd}$	Difference	$Mass_{obsd}$	Difference
Wild-type	30498	30500	30577	77	30591	91
C249S	30482	30484	30484	0	30484	0
D66N	30497	30498	30498	0	30590	93
D244N	30497	30497	30575	78	30589	92

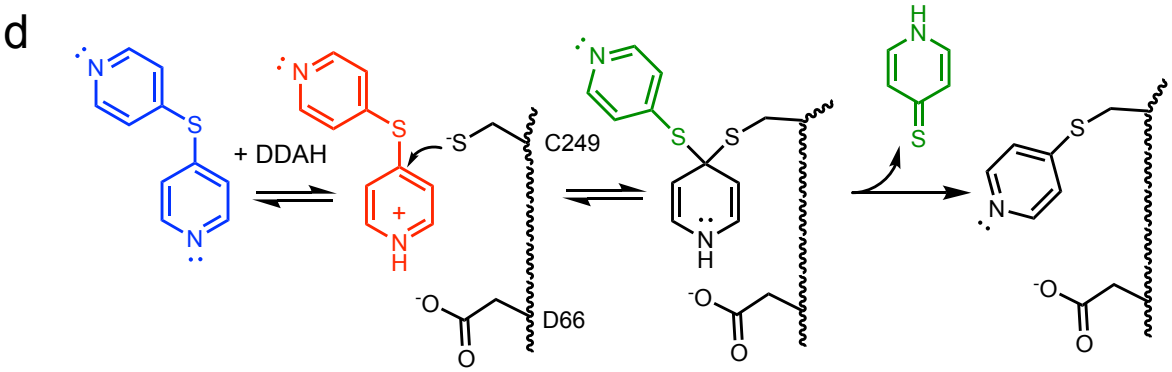


Figure 4. Probing the Mechanism of *Pa* DDAH Inactivation by **39**. a) One docked conformation (AutoDock Vina) of **39** shows proximity to active-site Cys249 and Asp66 of *Pa* DDAH (PDB code 1H70 with a computational S249C reversion to approximate the wild-type structure). The conformation with the closest association of Cys249 and C4 of **39** (~3 Å) is shown, although other lower energy docking conformations are predicted as well (not shown). Side chains < 3 Å distant from **39** are labeled. b) UV-vis absorbance changes upon *Pa* DDAH incubation with **39** at pH 7. Inset shows difference spectra at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, and 24h (bottom to top). Main panel shows time-dependent change of $Abs_{324\ nm}$ fit by a single exponential to give an endpoint of 0.62 ± 0.2 Abs units, k_{obs} = 0.34 ± 0.4 h^{-1}. c) Protein ESI-MS results for four *Pa* DDAH variants, alone or treated with **39** or **39f**. d) Proposed mechanism for *Pa* DDAH inactivation by **39**.

Here, a C249S mutant blocks formation of a 77 Da covalent adduct observed after treating wild-type *Pa* DDAH with **39**, consistent with proposing modification at the active-site Cys. *Pa* DDAH has a total of five Cys residues, although most are buried, and the C249S mutant has been shown to have the same fold as wild type.[15] The D244N mutation does not block covalent adduct formation but D66N does, implicating Asp66 as the residue that stabilizes the protonated ("switched on") form of **39**. A mono-*N*-methyl analog (**39f**, Figure S7) was synthesized as a permanently "switched on" mimic of cationic **39** and recovers the ability to form a covalent adduct with D66N *Pa* DDAH in a chemical rescue experiment, resulting in an adduct 14 Da greater than that observed with **39** due to the pyridine NH replacement by NCH_3. These experiments implicate Cys249 and Asp66 as essential residues for inactivation by **39** and suggests that the role of Asp66 is to stabilize the pyridinium to catalyze inactivation. In catalytic turnover of substrates by wild-type *Pa* DDAH, Asp66 makes binding interactions with the guanidine of N^{ω}-methylarginine but is not directly involved in proton transfer.[9, 15]

We evaluated four different inactivation mechanisms for **39**. First, selective protonation of the leaving group pyridine nitrogen only (Figure S8a) could facilitate product formation. This route is disfavored because inactivation would have to proceed through an unfavorable anionic intermediate and because inactivation by the phenyl derivative **39a** demonstrates that protonation of a leaving group pyridine nitrogen is not essential. Second, hydrolysis of **39** would yield 4-hydroxypyridine and 4-mercaptopyridine (Figure S8b), with predominant tautomers 4-pyridone and 4-thiopyridone, respectively. These products represent possible electrophiles that could lead to inactivation by reaction with an enzyme nucleophile. The mass of the observed adduct (77 Da) differs from those expected from hydrolysis products (96 or 112 Da, Figures 3c, S8b), and multiple turnovers are not required for inactivation (see below), so these products do not likely lead to DDAH inactivation. Third, a 4-mercaptopyridine hydrolysis product might undergo oxidative disulfide bond formation with a DDAH Cys residue, leading to inactivation (Figure S8c). As precedence, disulfide exchange is seen with the disulfide analog of **39**, 4,4'-

dipyridyl**di**sulfide.[19] However, the mass adduct expected from disulfide formation with 4-mercaptopyridine (110 Da) also differs from the observed mass, disfavoring this mechanism. The fourth proposed mechanism is favored (Figure 4d): In solution, **39** is not very reactive because its neutral form predominates. However, in the **39**:DDAH complex, residue Asp66 stabilizes protonated **39**, which is expected to be significantly more electrophilic ("switched on"). Attack of nearby Cys249 results in a neutral tetrahedral intermediate, avoiding the negative charge observed in typical Meisenheimer complexes, and subsequent elimination of the leaving group is facilitated by the low pK_a of the leaving group with the 4-thiopyridone tautomer predominating in solution, leaving behind a pyridine adduct substituted at the 4-position by the thiol of Cys249 (expected mass of 78 Da). Covalent modification of this active-site Cys blocks substrate binding and results in the observed inhibition. Release of the 4-thiopyridone product was verified using UV-vis spectroscopy (Figure 4b), and quantified ($\varepsilon_{324\ nm}$ = 21,000$M^{-1}cm^{-1}$)[19] as producing 0.78 $\pm$ 0.03 equivalents of product per equivalent of *Pa* DDAH, indicating that multiple turnovers are not required for inactivation. This proposed mechanism is well supported by structure activity relationships from analogs of **39**, low-reactivity of **39** with glutathione in solution, computational docking of **39** to *Pa* DDAH, mass spectrometry of wild-type and mutant *Pa* DDAH after treatment with **39**, the chemical rescue experiment with **39f**, and UV-vis spectroscopy and quantitation of the released 4-thiopyridone product. In this proposed mechanism, DDAH catalyzes its own inactivation, but Asp66 is not used for proton transfer during turnover of the normal N^{ω}-methylarginine substrates,[9] so we classify **39** as a quiescent affinity label[6] of *Pa* DDAH and report it here as the first example of covalent inhibition by a 4,4'-dipyridylsulfide.

Substituted pyridines are emerging as useful scaffolds for development of covalent inhibitors. The recent report of 2-sulfonylpyridines as Cys modifiers describes the use of an electron withdrawing group ortho to the leaving group to activate pyridines for S_NAr reactions.[5]

Other ring substitutions are also shown to impact electrophilicity of similar heterocycles.[4] This type of tuning is expected to enhance reactivity in a non-selective manner with all types of thiol nucleophiles. As a contrasting complement to this approach, the "switchable electrophile" strategy is represented here by the first report of 4,4'-dipyridylsulfide as a covalent protein modifier. The pyridine is activated for attack by target-stabilized protonation of the pyridine nitrogen. Because the neutral form of the inhibitor predominates in solution, covalent modification is selectively catalyzed when bound to the protein. This requirement for catalysis represents an alternative strategy to increase selectivity, even in fragment-sized covalent inhibitors, but also narrows the applicability of these probes to targeted sites that can provide both a nucleophile and a group to stabilize the protonated form. The relative arrangement of nucleophilic and activating residues is likely not unique to DDAH, but further studies are required to identify other targets amenable to this inactivator scaffold. The related switchable electrophile 2-chloropyridine is significantly less reactive with proteomic extracts as compared to other aryl halides, consistent with proposing increased selectivity for this type of covalent modifier.[20] The discovery of 4,4'-dipyridylsulfide analog fragments as a new "switchable electrophile" scaffold broadens the potential application of this strategy for inhibitor and probe design by enabling predictable tuning of pK_a values for both the pyridine nitrogen and the leaving group in order to target a wider variety of sites, and by providing multiple derivatization sites for installation of protein binding groups on either the leaving or staying groups.

ASSOCIATED CONTENT

Supporting Information

Supporting Information Available: This material is available free of charge via the Internet.

Supplementary table and figures, detailed experimental procedures (PDF)

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Notes

The authors declare no competing financial interest.

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Graphical Table of Contents Image for:

Discovery of 4,4'-Dipyridylsulfide Analogs as "Switchable Electrophiles" for Covalent Inhibition

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