

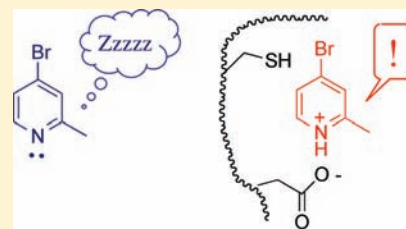
Discovery of Halopyridines as Quiescent Affinity Labels: Inactivation of Dimethylarginine Dimethylaminohydrolase

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S Supporting Information

ABSTRACT: In an effort to develop novel covalent modifiers of dimethylarginine dimethylaminohydrolase (DDAH) that are useful for biological applications, a set of “fragment”-sized inhibitors that were identified using a high-throughput screen are tested for time-dependent inhibition. One structural class of inactivators, 4-halopyridines, show time- and concentration-dependent inactivation of DDAH, and the inactivation mechanism of one example, 4-bromo-2-methylpyridine (**1**), is characterized in detail. The neutral form of halopyridines is not very reactive with excess glutathione. However, **1** readily reacts, with loss of its halide, in a selective, covalent, and irreversible manner with the active-site Cys249 of DDAH. This active-site Cys is not particularly reactive (pK_a ca. 8.8), and **1** does not inactivate papain (Cys pK_a ca. ≤ 4), suggesting that, unlike many reagents, Cys nucleophilicity is not a predominating factor in selectivity. Rather, binding and stabilization of the more reactive pyridinium form of the inactivator by a second moiety, Asp66, is required for facile reaction. This constraint imparts a unique selectivity profile to these inactivators. To our knowledge, halopyridines have not previously been reported as protein modifiers, and therefore they represent a first-in-class example of a novel type of quiescent affinity label.



INTRODUCTION

Human isoforms of dimethylarginine dimethylaminohydrolase (DDAH; EC 3.5.3.18) regulate nitric oxide production by catabolizing N^{ω},N^{ω} -dimethyl-L-arginine, an endogenous inhibitor of nitric oxide synthases.¹ Development of activity-based probes for this enzyme and other putative drug targets in the pentain superfamily of guanidine-modifying enzymes^{2,3} is of interest for both inhibitor design and biological studies because the activity levels of these enzymes do not always correspond to their cellular abundance.⁴ Prior activity-based probes for this superfamily have relied on an α -haloacetamide core^{5,6} that resembles the well-known α -halo ketone motif of many cysteine protease inactivators in structure and mechanism.⁷ The similarities between the amidine inactivators and the guanidine substrates contribute to the target selectivity of these probes but may limit their broader application in profiling the reactivity of proteomic mixtures. In general, many well-studied affinity labels have been used as the reactive groups of activity-based protein profiling reagents.⁸ However, as the methodology for reactivity-based proteomic screening evolves, it will become increasingly important to develop novel reactive groups with different selectivity profiles that will allow access to different subsets of the proteome. To address these issues, we explored “fragment”-sized inhibitors⁹ of DDAH, derived from a high-throughput screen, as a source for potentially novel covalent modifiers. Herein we describe the discovery of 4-halopyridines as quiescent affinity labels of DDAH. The mechanism of

4-bromo-2-methylpyridine is characterized as a paradigm case. To our knowledge, halopyridines have not previously been reported as covalent protein modifiers, and therefore they represent a novel class.

RESULTS AND DISCUSSION

4-Halopyridines Are Time-Dependent, Irreversible Inhibitors.

In an effort to discover structurally diverse inhibitors of DDAH, we performed a high-throughput screen of 4000 “fragment”-sized compounds from a commercial library (Chembridge, San Diego, CA; full description to be published elsewhere). A number of structurally divergent DDAH inhibitors were identified. Among these hits, we sought to distinguish the rapidly reversible inhibitors from the time-dependent inhibitors. A selection of compounds with apparent IC_{50} values $<120 \mu M$ were assayed for time-dependent inhibition of DDAH that could not be readily reversed upon dilution into excess substrate. Three compounds were found to inhibit DDAH in a time-dependent manner: 4-bromo-2-methylpyridine (**1**), 4-chloro-2-hydroxymethylpyridine (**2**), and 4-(2-quinolinyl)-3-buten-2-ol (**3**). Notably, each of these compounds contains a pyridine moiety, and a quinoline-5,8-dione-containing compound has recently been reported

Received: October 25, 2010

Published: January 11, 2011

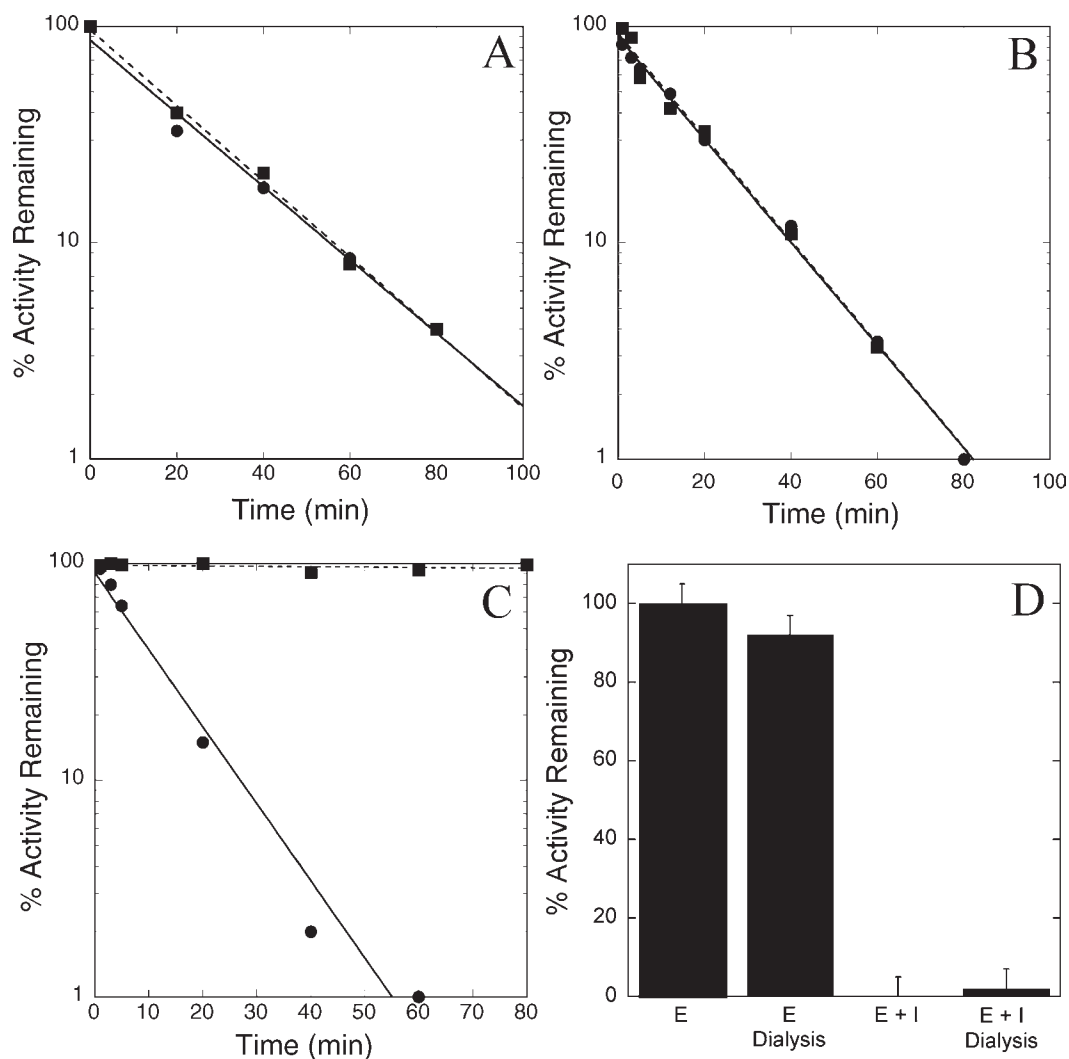
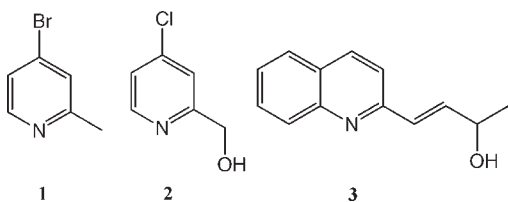


Figure 1. (A–C) Time-dependent inhibition of DDAH by inactivators 1–3. Time-dependent loss in DDAH ($29 \mu\text{M}$) activity (●, solid line) is observed after incubation with 1 ($300 \mu\text{M}$) (A), 2 (1.2 mM) (B), or 3 (1 mM) (C), each in K_2HPO_4 buffer (100 mM), KCl (100 mM), and EDTA (0.05 mM) at pH 7.5, 25°C . Inactivation experiments are also repeated when preceded by an incubation (1 h) of each inactivator with excess glutathione (5 mM) (■, dashed lines). (D) Enzyme inactivated by 1 was dialyzed overnight and assayed for recovery of activity (“E” is an enzyme-only control; “E dialysis” is enzyme-only after dialysis; “E+I” is inactivated enzyme; “E+I dialysis” is inactivated enzyme after dialysis).

as an inactivator of the related enzyme protein arginine deiminase-4.¹⁰



To determine if these inactivators are just reactive species that indiscriminately modify biological nucleophiles, we preincubated each with an excess of glutathione and then retested for time-dependent inhibition of DDAH (Figure 1A–C). Subsequent to its preincubation with glutathione, 3 lost all ability to inactivate DDAH. This compound was not considered further because reactivity with glutathione would likely preclude its use in many biological applications. In contrast, the remaining two compounds, both 4-halopyridines, did not show any reduction in

their ability to inactivate DDAH after preincubation with glutathione, indicating that they are not “reactive” compounds as defined by these experimental conditions.

To further characterize DDAH inactivation by 4-halopyridines, we focused our mechanistic studies on 1. To determine whether the observed time-dependent inhibition is irreversible or just slowly reversible, DDAH was inactivated by 1 and then subjected to extensive dialysis. Control reactions that omit the inactivator retained >90% of the initial activity, but inactivated samples recovered <3% of their initial activity (Figure 1D). These results are consistent with an irreversible inactivation mechanism.

4-Halopyridines Act at the Active Site. The concentration dependence of DDAH inactivation by 1 was measured to determine whether a reversible enzyme–inhibitor (EI) complex forms preceding inactivation. Inactivation rates do not display saturation kinetics but instead show a linear dependence on inhibitor concentration that can be fitted by a second-order inactivation rate constant of $4.8 \pm 0.3 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 2).

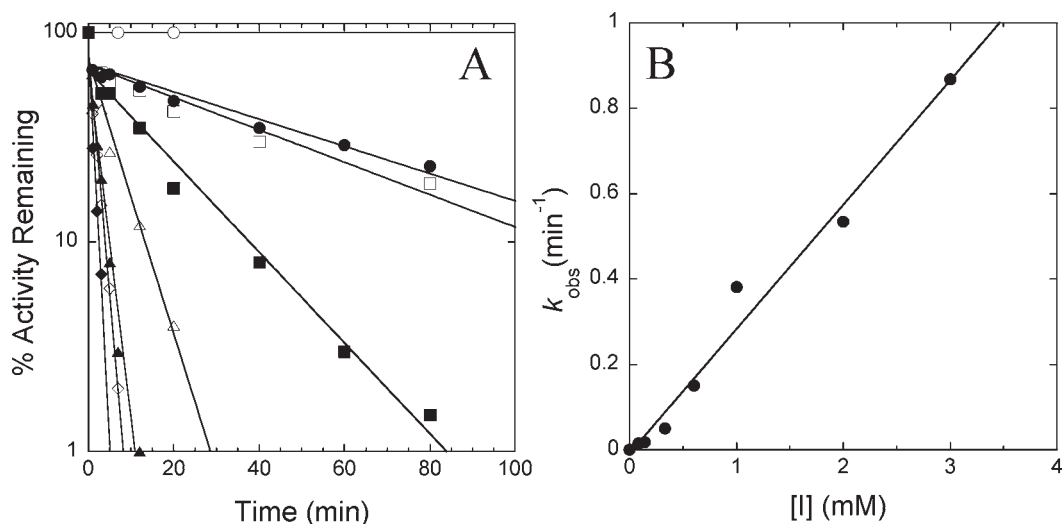


Figure 2. Time- and concentration-dependent inactivation of DDAH by **1** at pH 7.5. (A) Exponential fits to the observed inactivation of DDAH at pH 7.5, 25 °C by different concentrations of inhibitor: 0 (○), 83 μM (●), 140 μM (□), 330 μM (■), 600 μM (△), 1 mM (▲), 2 mM (◇), and 3 mM (◆). (B) Concentration dependence of the pseudo-first-order k_{obs} values, giving a second-order rate constant of $4.8 \pm 0.3 \text{ M}^{-1} \text{ s}^{-1}$ for inactivation.

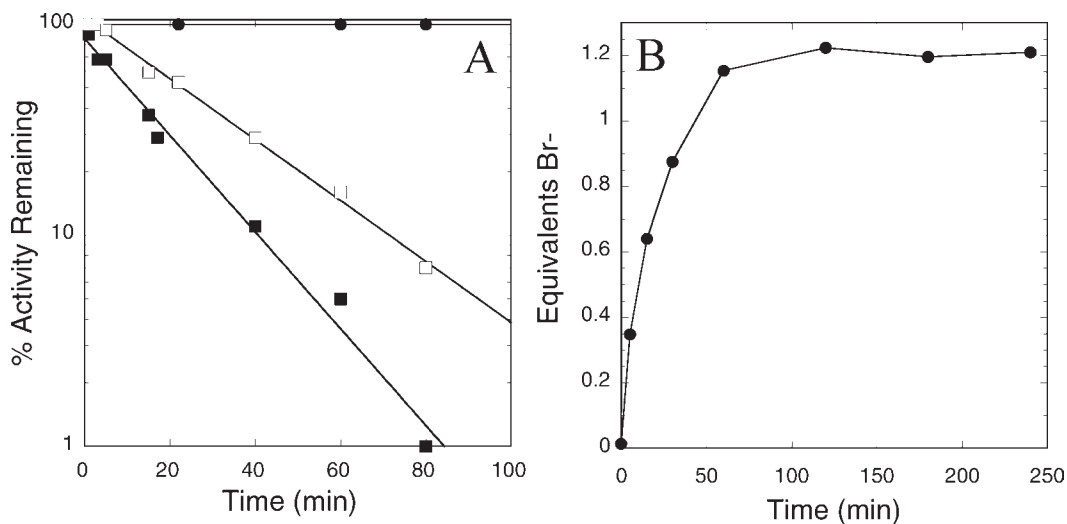


Figure 3. Substrate protection and stoichiometry of bromide ion release. (A) Enzyme preincubated with substrate (*N*⁹,*N*⁹-dimethyl-L-arginine, 3 mM, ●), **1** (300 μM, ■) and both substrate and **1** (□). (B) The molar equivalents ($[\text{Br}^-]/[\text{enzyme}]$, ●) of bromide ion release were monitored at various time points during enzyme inactivation, resulting in total release of approximately 1.2 equiv of Br^- ion.

The lack of observed saturation kinetics suggests several possibilities: that **1** can react with DDAH purely by diffusion encounters, that an EI complex does form but the dissociation rate is much slower than the inactivation rate, or, most likely, that the concentration used here is much less than the inactivator's K_1 value. In the absence of kinetic evidence for the formation of an EI complex, we sought to determine whether the addition of a DDAH substrate could compete for binding at the active site and slow the inactivation rate. Inactivation by **1** is slowed by the presence of excess substrate (Figure 3A), which is consistent with active-site binding. This hypothesis is further supported by additional experiments described below.

Inactivation Proceeds by Covalent Bond Formation and Loss of Bromide. We performed two experiments to characterize the stoichiometry and covalent nature of inactivation. First,

ESI-MS was used to compare uninhibited and fully inactivated DDAH. Treatment of DDAH with **1** leads to a mass addition of $91 \pm 10 \text{ Da}$ (Table 1). The calculated monoisotopic mass is 170.97 Da for **1** and 92.05 Da for **1** without the bromide. Therefore, the observed mass increase upon inactivation is consistent with that calculated for covalent attachment of one methylpyridine adduct to each DDAH monomer, loss of the bromide ion from the inactivator, and loss of one proton from the enzyme (91.04 Da). Second, ion chromatography was used to quantify bromide ion release that occurs during the inactivation process. As inactivation of DDAH progressed to completion, 1.2 ± 0.1 equiv of bromide was released, consistent with a turnover number of approximately one inactivator per inactivation event (Figure 3B). These results support a mechanism in which one inactivator molecule forms a 1:1 covalent adduct with DDAH, with elimination of its bromide ion.

Table 1. Summary of Deconvoluted Protein Masses Observed in ESI-MS Spectra of Control and Inactivated DDAH

DDAH preparation	theoretical calcd mass (Da)	control incubations with no inactivator, obsd mass (Da) ^a	incubations with 1		incubations with 11	
			obsd mass (Da) ^a	mass difference (Da)	obsd mass (Da)	mass difference (Da)
wild-type	30 503	30 498	30 588	91	30 604	106
denatured ^b	30 503	ND ^c	30 497	1	ND	ND
C249S	30 487	30 481	30 481	0	ND	ND
H162G	30 423	30 418	30 508	90	ND	ND
D66N	30 502	30 496	30 496 ^d	0	30 604	108
			30 586 ^d	90		
E65Q	30 502	30 496	30 498	2	30 496	0
D244N	30 502	30 497	30 588	91	30 603	106

^a The masses obtained from deconvoluted ESI-MS spectra are reported with errors of ± 10 Da. ^b Heat-denatured wild-type DDAH. ^c ND = not determined. ^d The modified peak occurs at an approximate 1:2 peak intensity ratio with the unmodified peak.

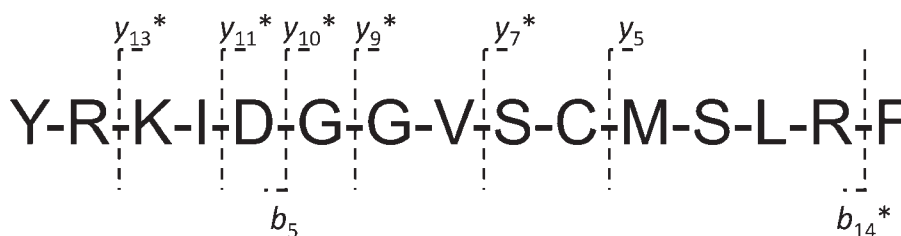


Figure 4. Summary of MALDI-TOF/TOF MS fragmentation pattern obtained for modified peptide 240–254. The 91 Da mass addition is mapped to residue Ser248 or Cys249.

Inactivation Modifies the Active-Site Cys Residue. The point of attachment of the inactivator was mapped, and a more precise mass of the adduct was obtained by digestion of uninhibited and fully inactivated DDAH with V8 endoprotease and characterization of the resulting peptide fragments in each digest by MALDI-TOF/TOF MS. Twenty-six peptides were identified by comparison with masses calculated for a theoretical digest (Table 2). These peptides cover approximately 53% of the primary sequence and include the active-site Cys249 and His162 residues. Two ions with m/z of 2353.14 and 1821.92 do not correspond to predicted peptides but do match the calculated masses for the 235–254 and 240–254 peptides, with each bearing an extra 91.06 ± 0.05 Da mass addition. A third signal with m/z at 1837.91 matches that of the 240–254 peptide bearing a 91.05 Da adduct plus an additional oxidation (16 Da), totaling 107.05 ± 0.05 Da. The control digest of uninhibited DDAH does not contain any signal at these m/z values but instead contains the unmodified 240–254 peptide at m/z 1730.90. Thus, three peptide–adduct species are detected in MALDI-MS of inactivated DDAH digests. Their masses are consistent with addition of a methylpyridine adduct, which gives a theoretical mass addition of 91.04 Da, and with the whole protein ESI-MS mass measurement, which yielded a mass addition of 91 ± 10 Da after inactivation.

Ions from the MALDI-TOF/TOF MS fragmentation spectra of the smallest modified peptide identified above, 240–254, reveal a more precise location of the covalent adduct (Table 3). The b_5 and y_5 daughter ions match those expected from an unmodified peptide. However, the b_{14}^* , y_7^* , y_9^* , y_{10}^* , y_{11}^* , and y_{13}^* ions all have been shifted to higher mass by the addition of

the adduct (Figure 4). These results localize the inactivator attachment site to either of two residues: Ser248 or Cys249. Notably, Cys249 is the active-site nucleophile in the normal catalytic mechanism of DDAH.¹¹

The adduct site was also mapped using site-directed mutagenesis and ESI-MS. Previously, two active-site residues, Cys249 and His162, were shown to be susceptible to covalent modification.^{5,12} Although a peptide containing unmodified His162 was observed (Table 2), this position was still included in our mutational analysis. We purified the catalytically inactive C249S and H162G variants of DDAH and subjected each to incubation with 1, followed by ESI-MS analysis. As expected, the H162G DDAH variant still shows a 90 ± 10 Da mass addition upon treatment with the inactivator (Table 1). However, the C249S variant did not show any significant mass difference after incubation, even though this DDAH variant still contains four other Cys residues. Taken together, these data support a mechanism in which 1 reacts covalently with the active-site Cys249 of DDAH.

Inactivation Requires the Halogen Substituent and Does Not Occur through a Metabolically Activated Species. Because the observed adduct lacks bromide, we tested whether the 4-bromo substituent was required for inactivation. Incubation of DDAH with 2-methylpyridine does not result in time-dependent loss of activity, indicating that the halogen is required for inactivation (Supporting Information, Figure S1). We also considered the possibility that 1 is metabolized by DDAH to produce a reactive product that is released from the active site and subsequently leads to DDAH inactivation. To rule out this possibility, DDAH was fully inactivated, and a fresh aliquot of enzyme was then added to the same

Table 2. Summary of Proteolytic Cleavage of Inactivated DDAH with Endoprotease V8^a

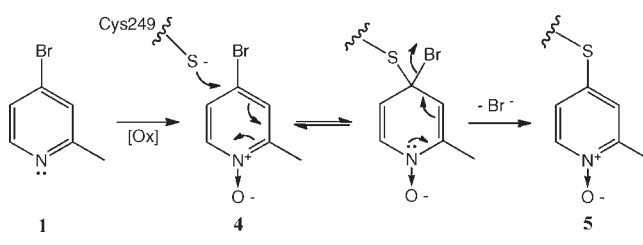
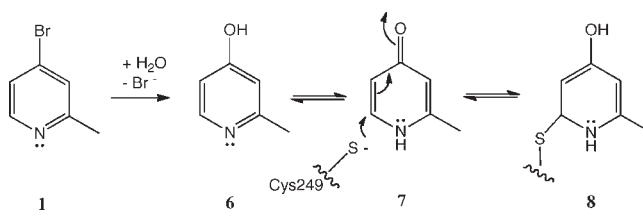
DDAH sequence positions plus leader (-18 to 0)	peptide mass (Da)	
	calcd	obsd ^b
95-105	1322.70	1322.72
95-114	2275.20	2275.22
95-129	3910.90	3910.92
106-114	970.51	970.53
106-129	2606.21	2606.24
115-129	1653.72	1653.72
115-129 + Ox ^c	1669.71	1669.72
115-129 + 2Ox	1685.71	1685.71
130-146	1829.94	1829.96
130-146 + Ox	1845.94	1845.95
137-146	1100.60	1100.61
137-146 + Ox	1116.60	1116.60
147-158	1280.72	1280.74
147-171	2746.59	2746.60
159-171	1483.88	1483.88
159-180	2403.32	2403.35
159-186	3090.69	3090.72
172-186	1624.82	1624.83
211-223	1544.82	1544.84
211-223 + Ox	1560.82	1560.82
211-234	2843.61	2843.63
224-234	1316.79	1316.81
224-239	1848.01	1847.98
235-254 + Me-Pyr ^d	2353.12	2353.14
240-254 + Me-Pyr	1821.90	1821.92
240-254 + Me-Pyr + Ox	1837.90	1837.91
240-254 ^e	1730.86	1730.90

^a Comparison of average calculated peptide masses for up to two missed cleavage sites and two oxidations. Amino acid numbering is assigned to match that of Protein Data Bank entry 1H70. ^b Masses are reported with errors of ± 0.05 Da. ^c Ox stands for addition of +16 Da for one calculated oxidation. ^d Me-Pyr stands for the 2-methylpyridine adduct (**10**) shown in Scheme 3. ^e Peptide mass is taken from a digest done in parallel using an uninhibited DDAH sample.

Table 3. Summary of Major Ions Observed in MALDI-TOF/TOF Fragmentation Spectra from the *m/z* 1821 Parent Ion (YRKIDGGVCSMSLR + Me-Pyr)^a

ion	<i>m/z</i>	
	calcd	obsd ^b
<i>y</i> ₅	653.3	653.3
<i>y</i> ₇ [*] + Me-Pyr	934.4	934.4
<i>y</i> ₉ [*] + Me-Pyr	1090.5	1090.5
<i>y</i> ₁₀ [*] + Me-Pyr	1147.5	1147.6
<i>y</i> ₁₁ [*] + Me-Pyr	1262.6	1262.6
<i>y</i> ₁₃ [*] + Me-Pyr	1503.7	1503.8
<i>b</i> ₅	676.4	676.4
<i>b</i> ₁₄ [*] + Me-Pyr	1657.8	1657.8

^a Me-Pyr stands for the 2-methylpyridine adduct (**10**) shown in Scheme 3. ^b The masses are reported with errors of ± 0.2 Da.

Scheme 1. Proposed Inactivation Mechanism by *N*-Oxidized Inactivator**Scheme 2. Proposed Inactivation Mechanism by Hydrolyzed Inactivator**

preincubation mixture. The observed inactivation rate for the second aliquot of enzyme is not faster than the initial inactivation rate, indicating that **1** is not a metabolically activated inhibitor (Supporting Information, Figure S2). This conclusion is also consistent with the low inactivation turnover number and the observation that excess glutathione, which would react with any released reactive metabolites, does not prevent inactivation.

Proposed Inactivation Mechanism. We examined three possible mechanisms leading to covalent bond formation with DDAH. First we considered the possibility that a minor portion of the stock solution of **1** may have become oxidized to the *N*-oxide (**4**) during storage and handling (Scheme 1). Pyridine *N*-oxides are more reactive than their parent pyridine compounds¹³ and could possibly serve as the inactivating species. The resulting enzyme adduct (**5**) should reflect a 107.04 Da mass increase. This value is close to but slightly above that seen in ESI-MS characterization of the undigested inactivated enzyme (91 ± 10 Da). More accurate masses were determined in the MALDI-TOF/TOF MS analysis of peptide digests. One peptide (*m/z* 1837.91) contains mass addition(s) that total the predicted 107 Da mass, but there are two other peptides that contain mass additions that are 16 Da less than this value. These smaller masses are inconsistent with the mechanism in Scheme 1. These results indicate that *N*-oxidation of the inactivator is not obligatory for covalent bond formation. The observed oxidation of the *m/z* 1837.91 peptide may occur on Met250 rather than on the inactivator and likely explains the observed 16 Da mass addition in the larger peptides.

The second mechanism that we considered was hydrolysis (possibly DDAH-catalyzed) of **1** and tautomerization of the product (**6**) to a pyridone species (**7**) that could react with a nucleophile on the enzyme (Scheme 2). However, the resulting adduct (**8**) should reflect a 108.04 Da mass increase, which is not observed in any of the peptides bearing adducts. Also, significant background hydrolysis was not observed in the bromide

Scheme 3. Proposed Inactivation Mechanism by the Pyridinium Form of the Inactivator

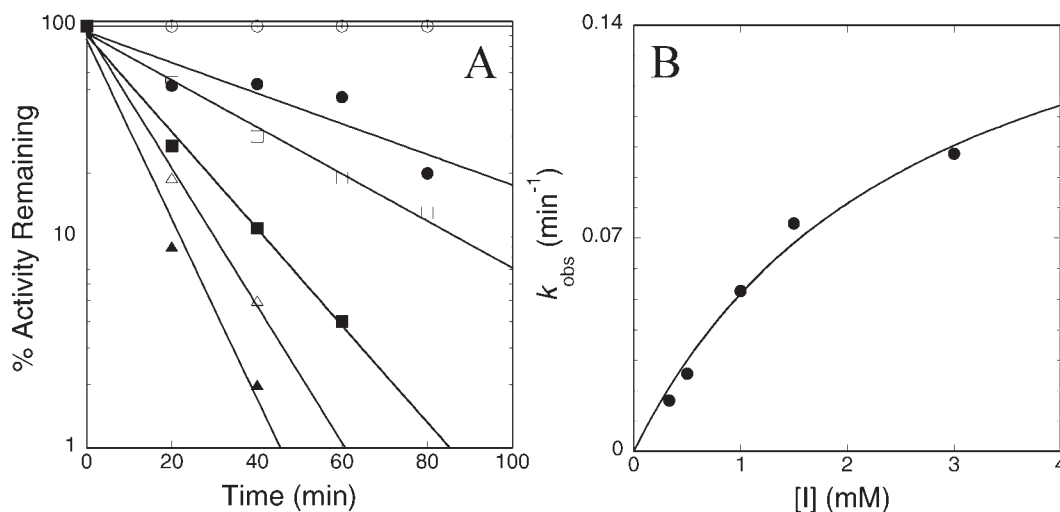
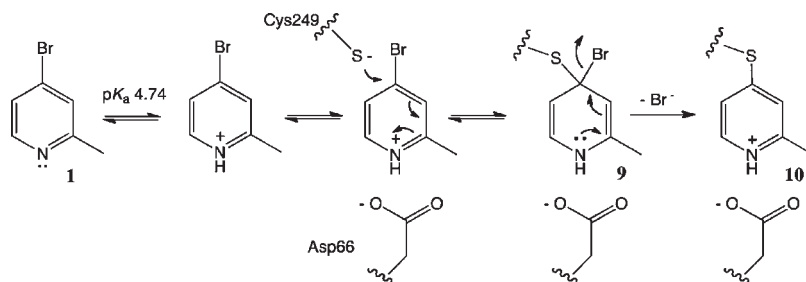


Figure 5. Time- and concentration-dependent inactivation of DDAH by **1** at pH 5.0. (A) Exponential fits to the observed inactivation of DDAH (29 μM) by different concentrations of inhibitor: 0 (\circ), 330 μM (\bullet), 500 μM (\square), 1 mM (\blacksquare), 1.5 mM (\triangle), or 3 mM (\blacktriangle), each in MES buffer (100 mM), KCl (100 mM), and EDTA (0.05 mM) at pH 5.0, 25 $^{\circ}\text{C}$. (B) Concentration dependence of the pseudo-first-order k_{obs} values shows saturation kinetics and is fitted by K_{I} (2.6 ± 0.8 mM) and k_{inact} (0.18 ± 0.03 min^{-1}) values for inactivation.

quantification experiment (Figure 3B), and incubations of DDAH with **6** do not result in enzyme inactivation (Supporting Information, Figure S3). These observations do not support the mechanism shown in Scheme 2.

The third inactivation mechanism that we considered is attack of the active-site Cys249 on the pyridinium form of **1**, formation of a σ -complex (**9**), followed by elimination of bromide to result in a stable 2-methylpyridine-Cys249 adduct (**10**, Scheme 3). This mechanism is most consistent with the experimental results described herein. This mechanism is dependent on enzyme stabilization of the pyridinium form of **1** to enhance its reactivity.

The Pyridinium Form Is Likely the Reactive Species. Typically, 4-halopyridines are not very sensitive to nucleophilic aromatic substitution while in their neutral forms. However, formation of the positive pyridinium ion, often by *N*-oxidation or *N*-methylation, greatly increases reaction rates because of the increased ability of the positive charge to stabilize σ -complex formation upon attack by a nucleophile. For example, methanolysis of 4-chloropyridine by methoxide/methanol at 50 $^{\circ}\text{C}$ shows dramatic rate enhancements of 1.1×10^3 and 5.7×10^9 when the nitrogen is derivatized to the *N*-oxide or *N*-methyl, respectively.¹³ Additionally, nucleophilic aromatic substitution of halopyridines has been shown to be acid-catalyzed

through protonation of the heterocyclic nitrogen.¹⁴ However, the calculated¹⁵ $\text{p}K_{\text{a}}$ of **1** is 4.74, indicating that only a small amount of the more reactive pyridinium form (e.g., 1.7 μM in a 1 mM solution) is present at the incubation pH value of 7.5. Enzyme inactivation by this minor form may explain why saturating inactivation kinetics was not observed at pH 7.5 (Figure 2). We repeated these experiments at pH 5.0 to increase the protonated fraction of **1**. Saturating kinetics were now observed at high inhibitor concentrations and could be fit to give K_{I} (2.6 ± 0.8 mM) and k_{inact} (0.18 ± 0.03 min^{-1}) values (Figure 5). This result is consistent with a mechanism in which the positively charged pyridinium ion is the inhibitory species that binds and inactivates DDAH. Although the K_{I} value is large, its magnitude is not unexpected due to the small “fragment” size of the inactivator. These values compare quite favorably with other “warheads” that have been derivatized into potent biologically useful tools. For example, the protein arginine deiminase-4 inactivator fragment 2-chloroacetamide ($K_{\text{I}} = 20$ mM, $k_{\text{inact}} = 0.7$ min^{-1})³ is greatly enhanced by incorporation into a peptidyl arginine analogue scaffold ($K_{\text{I}} = 180$ μM , $k_{\text{inact}} = 2.4$ min^{-1}).¹⁶ Derivatives of **1** will likely increase potency as well and are a subject of current work.

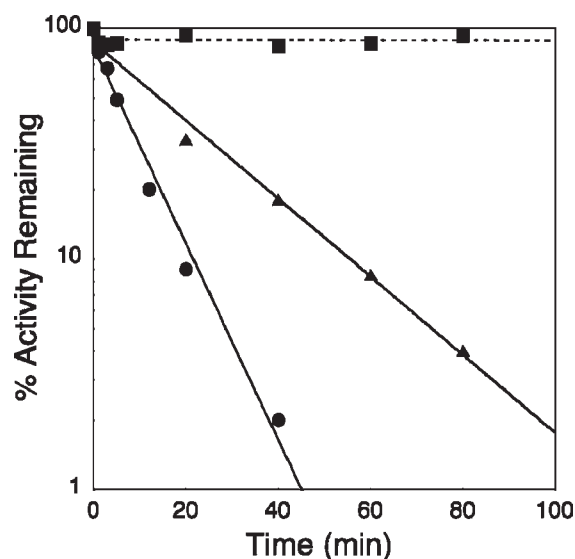
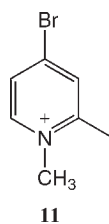


Figure 6. Time-dependent inactivation of DDAH by **11** and protection by glutathione. The observed DDAH inactivation rate by **11** ($300\ \mu\text{M}$, ●) is faster than that of **1** ($300\ \mu\text{M}$, ▲, taken from Figure 1). However, preincubation of **11** with glutathione ($5\ \text{mM}$) prevents any time-dependent inhibition of DDAH (■, dashed line).

To maximize the fraction of the inactivator's pyridinium form without adjusting the pH of the reaction solution, we synthesized 1,2-dimethyl-4-bromopyridinium (**11**).



This compound readily causes time-dependent inactivation of DDAH that cannot be reversed upon dilution into excess substrate. The observed inactivation rate of **11** is faster than that of a similar concentration of **1** (Figure 6). However, preincubation of **11** with excess glutathione completely blocks its ability to inactivate DDAH (Figure 6). Apparently, installing a permanent charge on the inactivator increases its reactivity to an extent that the inhibitor becomes nonselectively reactive, thereby losing much of its biological utility. These results also suggest that the enzyme may play an analogous role in inactivation.

The Active-Site Environment Promotes Inactivation. Two ways that DDAH might participate in the proposed inactivation mechanism are (1) by providing a good active-site nucleophile with a low pK_a and (2) by stabilizing the protonated, more reactive form of the inactivator. However, DDAH (as well as other enzymes in the penten superfamily)² is unusual in that the active-site Cys249 has a high pK_a (ca. 8.8)¹⁷ in the enzyme's resting state. This value is less than the pK_a value of glutathione's thiol (ca. 9.2)¹⁸ but would still be predominantly protonated and presumably less reactive at most physiological pH values (e.g., >95% protonated at pH 7.5). Therefore, it appears that the inactivator's selective reaction with the Cys249 of DDAH is not because the enzyme provides an exceptionally good nucleophile. To further illustrate this point, we tested inactivation of a different

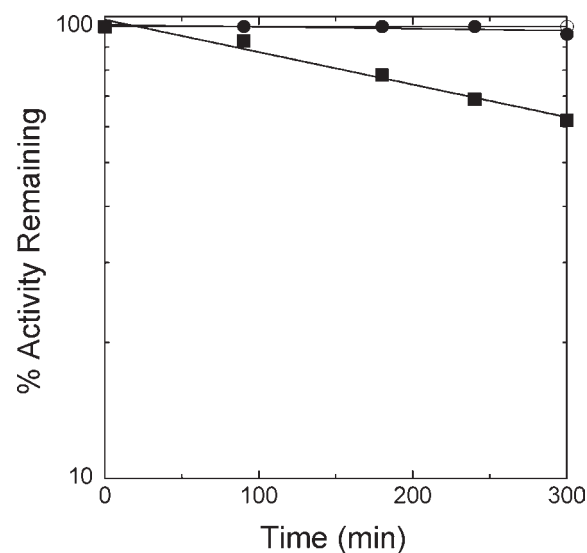


Figure 7. Time-dependent inactivation of papain by **1** and **11**. Time-dependent inactivation by **11** ($1\ \text{mM}$, ■) is observed. No inactivation by **1** ($1\ \text{mM}$, ●) is observed relative to a no-inactivator control (○). Prior to the assays, papain is activated by incubation with reduced L-cysteine ($500\ \mu\text{M}$). Papain samples that are subsequently depleted of free L-cysteine do not lose activity over the experimental time frame (data not shown).

hydrolase, papain. Papain has a very nucleophilic active-site Cys ($pK_a \leq 4$)¹⁹ that can readily react with thiol-modifying reagents. However, preincubation of papain with **1** does not result in any time-dependent inactivation (Figure 7). In contrast, the chemically activated *N*-methylated inactivator **11** does cause inactivation. These results illustrate that a pyridinium-stabilizing group is needed in the target enzyme and that the selectivity of this halopyridine is not solely due to its reaction with good nucleophiles.

Inspection of the structures of DDAH substrates and the 4-halopyridines suggest some similarities. Both contain flat moieties that bear positive charges (guanidinium and pyridinium ions, respectively). The positive charge of the substrate binds to the carboxylate-rich active site of DDAH. Therefore, we considered the possibility that one or more of these carboxylates might also help to bind and stabilize the pyridinium ion of the inactivator, thereby facilitating inactivation. We prepared site-directed mutants of three carboxylates that are located within $8\ \text{\AA}$ of Cys249: Glu65, Asp66, and Asp244. E65Q and D66N are catalytically inactive. D244N retains catalytic activity toward *N*^ω,*N*^ω-dimethyl-L-arginine ($k_{\text{cat}} = 1.32 \pm 0.06\ \text{s}^{-1}$; $K_M = 700 \pm 100\ \mu\text{M}$ at pH 7.5, $25\ ^\circ\text{C}$), with steady-state rate constants similar to those of wild-type enzyme ($k_{\text{cat}} = 1.27\ \text{s}^{-1}$; $K_M = 39\ \mu\text{M}$).¹⁷ These DDAH variants were each incubated with **1** under the same conditions that led to complete inactivation of wild-type DDAH (Table 1). Incubation with the D244N mutant behaves the same as wild-type DDAH, modifying all of the enzyme with a 91 Da mass addition. Incubation with the D66N mutant, however, does not fully modify the enzyme but rather results in mass addition (90 Da) to only a fraction of the enzyme, with a modified:unmodified peak ratio of 1:2. [Comparison of peak intensities assumes similar ionization efficiencies for the unmodified enzyme and enzyme–inactivator adducts.] This is consistent with a mechanism in which Asp66 promotes formation of the covalent adduct. Treatment of the D66N mutant with the

N-methylated inactivator **11** results in complete modification. Therefore, chemical activation of the pyridine is able to “rescue” the impaired D66N mutant with respect to covalent modification. The inactive E65Q mutant completely blocks all covalent adduct formation but also cannot be modified by **11**, suggesting that this mutation may block inactivation by deformation of the active site rather than by destabilizing pyridinium binding. In addition to these three mutants, we also found that incubation of the inactivator with heat-denatured wild-type DDAH does not result in any covalent adduct formation. Taken together, these results demonstrate that the active-site carboxylate Asp66 promotes covalent inactivation, likely by facilitating the binding and stabilization of the reactive pyridinium form of the inactivator.

Inhibitor Classification and Precedence. In classifying the 4-halopyridine inhibitors, we first established that they are not rapidly reversible inhibitors and that their time-dependent irreversible inhibition leads to covalent bond formation at the active site. This inactivation mechanism also does not include release of a reactive species, ruling out metabolic activation.²⁰ Because the 4-halopyridines do not readily react with glutathione, they are not considered to be intrinsically reactive compounds and so cannot be classified as classic affinity labels.²¹ Taking into account the proposed inactivation mechanism (Scheme 3), the 4-halopyridines are also not classified as mechanism-based inactivators because the enzyme does not use a step of the normal catalytic mechanism to unmask a latent reactivity.²⁰ Therefore, considering all of the evidence to date, the most fitting classification for 4-halopyridine inactivators is that of quiescent affinity labels.²² This term was first introduced by Krantz and co-workers to describe acyloxymethylketone inhibitors of cysteine proteases.^{23,24} This type of inhibitor readily reacts when bound in an appropriate enzyme site but is “quiescent” in the presence of other biologically relevant nucleophiles that do not present a suitable complementary surface.

In general, there are some similarities in our proposed inactivation mechanism with other known enzyme inactivators. For example, some inactivation mechanisms use nucleophilic aromatic substitution,²⁵ and others use nucleophilic attack on a dihydropyridinium metabolite.²⁶ There are also similarities in catalytic mechanisms. Some pyridoxal phosphate-dependent enzymes use a carboxylate to stabilize the pyridinium form of the cofactor to facilitate reaction.²⁷ However, to our knowledge, there have been no prior reports of halopyridines used as covalent protein modifiers. One possible exception is a mention of preliminary results showing irreversible inhibition of glucose transport in human erythrocytes by two highly derivatized halopyridines.²⁸ However, no experimental details were given, and it is unclear if the observed inhibition is covalent in nature.

CONCLUSIONS

4-Bromo-2-methylpyridine (**1**) is a quiescent affinity label of DDAH that specifically and covalently modifies the active-site Cys residue. To our knowledge, 4-halopyridine (or the similarly reactive 2-halopyridine) moieties have not previously been identified as covalent modifiers of DDAH or any other proteins. Although the affinity of these reagents for DDAH is weak, they can likely be used as the “warheads” of more elaborate inhibitors with increased potency and selectivity. Selective protein modification by the halopyridines appears to require a binding site with a nucleophilic residue placed at a suitable distance and orientation from a second moiety that binds and stabilizes

the more reactive pyridinium form. These constraints confer a unique selectivity profile to this novel class of quiescent affinity label.

EXPERIMENTAL SECTION

Materials. Unless noted otherwise, all chemicals are from Sigma-Aldrich Chemical Co. (St. Louis, MO). All DDAH enzymes, including wild-type *Pseudomonas aeruginosa* DDAH and the mutants C249S and H162G, were purified and assayed as described previously.¹¹ Synthetic DNA primers are from Sigma-Genosys (The Woodlands, TX) and Invitrogen (Carlsbad, CA). Rapid plasmid isolation kits are from Invitrogen. V8 (Glu-C) endoprotease is from Roche (Indianapolis, IN). 2-Methyl-4-bromopyridine·HBr is from ChemBridge (San Diego, CA), and 2-methyl-4-bromopyridine (the free base form) is from Sigma-Aldrich. *N*^α-Benzoyl-L-arginine *p*-nitroanilide·HCl is from Bachem Americas (Torrance, CA). 4-Hydroxy-2-methylpyridine is from Ark Pharm (Libertyville, IL).

Construction of Vector for E65Q, D66N, and D244N DDAH, Protein Expression, and Purification. Two complementary mutagenic oligonucleotides, (forward) 5′-TCGGTGTTCGTcagGACCCGGTGC-3′ and (reverse) 5′-GCACCGGGTcctgGACGAACACCGA-3′, were designed to introduce a mutant codon (lowercase) encoding an E65Q mutation in the DDAH sequence by Quikchange mutagenesis. Two complementary mutagenic oligonucleotides, (forward) 5′-GGTGTTCGTcGAGaacCCGGTGTCTG-3′ and (reverse) 5′-CAGAGCACCGGgttCTCGACGAACACC-3′, were designed to introduce a mutant codon (lowercase) encoding a D66N mutation in the DDAH sequence by Quikchange mutagenesis. Two complementary mutagenic oligonucleotides, (forward) 5′-CCGAATATCGCAAGATCaacGGCGGGCGTCA-3′ and (reverse) 5′-TGACGCCgttGATGAACGCTATTTCCGG-3′, were designed to introduce a mutant codon (lowercase) encoding a D244N mutation in the DDAH sequence by Quikchange mutagenesis. Briefly, a PCR mixture using a pET28a vector containing *P. aeruginosa* DDAH as a template, the mutagenic primers, a dNTP mixture, and *Pfu* turbo DNA polymerase in the manufacturer's buffer (Stratagene, La Jolla, CA) was run using a temperature program of 95 °C for 1 min, followed by 25 cycles of 95 °C for 30 s, 45–60 °C for 1 min, and 68 °C for 12 min. *DpnI* was then added to the cooled reaction mixture to digest the methylated parent plasmid. After incubation at 37 °C for 1 h, the mixture was transformed into electrocompetent DH5α *Escherichia coli* cells and selected on LB agar plates supplemented with kanamycin (30 μg/mL). The final plasmid was purified from an overnight culture, and the gene insert was fully sequenced (DNA Facility, University of Texas at Austin) to verify the desired sequence. Expression and purification of mutant D66N, E65Q, and D244N DDAH was completed as described previously.¹¹

Time-Dependent Inactivation of DDAH. Purified recombinant DDAH (29 μM) was incubated with KCl (100 mM) and **1** (0.150–3 mM) in K₂HPO₄ buffer (100 mM) and EDTA (0.05 mM) at pH 7.5, 25 °C. Some experiments also included an additional incubation (1 h) of **1** with excess glutathione (5 mM) before addition of enzyme to initiate the inactivation. Substrate protection experiments included excess *N*^ω,*N*^ω-dimethyl-L-arginine (3 mM) in the preincubation mixture. Aliquots were removed from these incubations at various time points (0–80 min) and diluted into an assay solution containing excess (1 mM) substrate *N*^ω,*N*^ω-dimethyl-L-arginine. Remaining enzyme activity was assayed as described previously, using a color-developing reagent (COLDER) to derivatize the urea group of the L-citrulline product.^{11,29} Briefly, COLDER (200 μL) was added to each 100 μL reaction in a clear polystyrene 96-well plate (Nunc, Rochester, NY) and incubated for 15 min at 95 °C. Samples were cooled for 20 min at 25 °C, and the absorbance at 540 nm was determined for each well

using a Perkin-Elmer (Waltham, MA) VICTOR³V 1420 multilabel counter plate reader. The observed inactivation rates (k_{obs}) were determined by fitting the percent remaining activity over time to a single-exponential equation. The resulting k_{obs} values were then plotted against inactivator concentration and fitted to $k_{\text{obs}} = (k_{\text{inact}}[I]) / (K_I + [I])$ to obtain K_I and k_{inact} values.²⁰ All fits were calculated using Kaleida-Graph (Synergy Software, Reading, PA). To further study irreversibility, fully inhibited DDAH was dialyzed overnight at 4 °C against 1000 volumes of phosphate buffer (10 mM) and KCl (100 mM) at pH 7.5, and the resulting activity was assayed as described above. Control reactions were conducted in parallel to ensure that uninhibited DDAH retained activity under these conditions.

Mass Spectral Analysis of Inactivated DDAH and DDAH Mutants. To characterize covalent adducts formed during enzyme inactivation, 90 min incubations of **1** (300 μM) with DDAH were carried out at 25 °C under the same conditions used in the preincubations described above and quenched with trifluoroacetic acid (6 N). Similar incubations were carried out using the C249S, H162G, D66N, D244N, and E65Q DDAH enzymes, along with wild-type DDAH that had previously been heat-denatured. Samples were analyzed by ESI-MS on a ThermoFinnigan LCQ (San Jose, CA) ion trap mass spectrometer as described previously.¹¹

Bromide Quantification by Ion Chromatography. To further investigate the mechanism of inactivation of DDAH, 110 μM enzyme and 300 μM **1** (free base form) were incubated at 25 °C and acid-quenched at varied time points. The enzyme was removed from solution using a Microcon centrifugal filter device with a 3000 Da molecular weight cutoff (MWCO). Samples were centrifuged for 45 min at 11750g at 25 °C. The flow-through was then diluted 1:20 in sodium bicarbonate buffer (20 mM) at pH 7. Inactivated products were analyzed using a Dionex Ion Chromatography System (ICS-1500, Dionex Corp.) equipped with an AG12A precolumn (4 \times 40 mm), an IonPac AS12A column (4 \times 200 mm), an anion self-regenerating suppressor (ASRS ULTRA II, 4 mm), and an AS40 autosampler. Anion detection was performed by suppressed electrical conductivity using an electrochemical detector with an applied current of 25 mA. A solution of 2.7 mM $\text{Na}_2\text{CO}_3/0.3$ mM NaHCO_3 at pH 9.6 was used as the eluent at a flow rate of 1.5 mL/min. Bromide was quantified by use of a calibration curve made with serial dilutions of a NaBr bromide standard (Bromide IC Standard Solution, 1 g/L, 1000 ppm bromide ion, Fluka) in 20 mM NaHCO_3 buffer, pH 9.0. Calibration curve solutions included bromide concentrations of 10, 2.5, 1.25, 0.625, 0.3125, 0.0156, and 0 ppm, all in 20 mM NaHCO_3 buffer at pH 9.0.

Identification of a Covalently Modified DDAH Peptide. To determine the amino acid that is modified by inactivator, fully inactivated DDAH was exchanged into 50 mM ammonium acetate buffer (pH 4) by overnight dialysis. Samples were then digested with V8 endoprotease, and the products were analyzed using MALDI-TOF/TOF MS on an Applied Biosystems 4700 Proteomics Analyzer.

Activity and Inhibition Assays for Papain. Papain from papaya latex was from Sigma-Aldrich. The activity assay for papain was as previously reported,³⁰ using N^α -benzoyl-L-arginine *p*-nitroanilide as the substrate. Briefly, papain was activated by incubating (1 h) with reduced L-cysteine (500 μM) in Na_3PO_4 (2 mM) and EDTA (0.1 mM). Activity was measured by following the release of *p*-nitroanilide at 410 nm ($\epsilon_{410} = 8800 \text{ M}^{-1} \text{ cm}^{-1}$) using a Cary 50 UV-vis spectrophotometer (Varian, Inc., Walnut Creek, CA). As a control, activated papain was subsequently depleted of free L-cysteine (depletion was achieved by multiple washes in a 3000 Da MWCO centrifugal filter) in order to demonstrate that any loss of activity in the experimental assays was not due to a loss of free L-cysteine in the reaction mixture (through reaction with the inactivators).

Synthesis of 1,2-Dimethyl-4-bromopyridine (11). A solution of **1** (0.206 g, 1.19 mmol) in 3.0 mL of CH_2Cl_2 was treated with

CH_3I (0.498 g, 3.50 mmol), and the reaction mixture was stirred overnight at 25 °C. The resulting white precipitate was collected by vacuum filtration, washed with CH_2Cl_2 (30 mL), and dried under high vacuum to yield **11** (0.302 g, 0.962 mmol, 81%). ¹H NMR (400 MHz, $\text{DMSO-}d_6$): δ 8.87 (d, $J = 6.7$ Hz, 1H, Ar-H), 8.46 (d, $J = 2.3$ Hz, 1H, Ar-H), 8.29 (dd, $J = 6.7$ Hz, $J = 2.3$ Hz, 1H, Ar-H), 4.15 (s, 3H, N-CH₃), 2.74 (s, 3H, CH₃). ¹³C NMR (100 MHz, $\text{DMSO-}d_6$): δ 156.80, 146.44, 141.20, 131.89, 128.33, 45.14, 19.75. HRMS (CI) (m/z): M^+ calcd for $\text{C}_7\text{H}_9\text{BrN}^+$, 185.99129; found, 185.99129.

■ ASSOCIATED CONTENT

S Supporting Information. Three figures showing assays for time-dependent DDAH inhibition by 2-methylpyridine, **1** after pretreatment with enzyme, and **6**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ACKNOWLEDGMENT

We thank Gottfried Schroeder (University of Texas, Austin) for assistance with Figure 3B. This work was supported in part by grants from the National Institutes of Health (GM069754), the Robert A. Welch Foundation (F-1572), and a seed grant from the Texas Institute for Drug and Diagnostic Development (Welch Foundation Grant H-F-0032).

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