Published in final edited form as:

Chembiochem. 2017 August 04; 18(15): 1551–1556. doi:10.1002/cbic.201700104.

Selective Covalent Protein Modification by 4-Halopyridines Through Catalysis

Christopher L. Schardon², Alfred Tuley¹, Joyce A. V. Er¹, Jake C. Swartzel¹, and Walter Fast^{1,3,*}

¹Division of Chemical Biology and Medicinal Chemistry, College of Pharmacy, The University of Texas, Austin, Texas 78712, United States

²Biochemistry Graduate Program, The University of Texas, Austin, Texas 78712, United States

³LaMontagne Center for Infectious Disease, The University of Texas, Austin, Texas 78712, United States

Abstract

4-Halopyridines are developed herein as selective, tunable, and “switchable” covalent protein modifiers for use in the development of chemical probes. Non-enzymatic reactivity of 4-chloropyridine with amino acids and thiols is ranked with respect to common covalent protein modifying reagents and found to have reactivity similar to that of acrylamide, but can be switched to a reactivity similar to that of iodoacetamide upon stabilization of the positively charged pyridinium. Diverse fragment-sized 4-halopyridines inactivate human dimethylarginine dimethylaminohydrolase-1 (DDAH1) through covalent modification of the active-site Cys, acting as quiescent affinity labels that require “off pathway” catalysis via a stabilization of the protonated pyridinium by a neighboring Asp residue. A series of 2-fluoromethyl-substituted 4-chloropyridines demonstrate that pK_a and k_{inact}/K_I can be predictably varied over several orders of magnitude. Covalent labeling of proteins in an *E. coli* lysate is shown to require folded proteins, indicating that alternative proteins can be targeted and modification is likely to be catalysis-dependent. 4-Halopyridines, and quiescent affinity labels in general, represent an attractive strategy to develop reagents with “switchable” electrophilicity as selective covalent protein modifiers.

Graphical Abstract

Covalent warheads that become more electrophilic upon protein binding represent a novel strategy in the design of selective covalent probes. Herein is described the non-enzymatic, enzymatic, and proteomic reactivity of 4-halopyridines as covalent protein modifiers with ‘switchable’ electrophilicity that is enhanced upon binding to targeted proteins including human dimethylarginine dimethylaminohydrolase-1.

*Corresponding Author: walt.fast@austin.utexas.edu.

Notes

The authors declare no competing financial interests.

Supporting Information, including figures for halopyridine reactivity with free amino acids and glutathione, structure lists, example k_{inact}/K_I determination, halide dependence, materials, synthetic methods, and NMR spectra.

Keywords

covalent inhibitor; halopyridine; enzyme inactivation; covalent probe

INTRODUCTION

Many biochemical probes and a subset of therapeutic agents use a strategy of selective covalent protein modification.^[1] Those that are categorized as affinity labels typically contain electrophilic groups that rely on non-covalent binding to provide a high effective concentration of the electrophile in proximity to a protein-derived nucleophile, which increases the rate of covalent bond formation. Because of the high effective concentration, the electrophilicity of the reactive group can be drastically decreased to provide a degree of selectivity for labeling one site on a particular protein in competition with non-selective labeling of other proteins or small molecules that contain nucleophiles with similar reactivity. A number of other complementary strategies have been applied to increase the selectivity of affinity labels in proteomic mixtures, cells, or organisms, with three listed here as examples: The proton pump inhibitor omeprazole uses a prodrug approach where the reactive electrophile that acts as an affinity label is only generated near the site of action;^[2] The opposite “soft drug” approach^[3] has been applied to acrylamide-based modifiers by designing them to be rapidly metabolized into unreactive compounds when not bound to their targeted site;^[4] A third strategy increases the reversibility of covalent bond formation in order to decrease off-target labeling for which there is little non-covalent affinity to drive the equilibrium forward.^[5]

A fundamentally different approach to increase the selectivity of covalent protein modification is that of mechanism-based enzyme inactivation (although not all inhibitors in this category use a covalent mechanism).^[6] These inactivators differ from affinity labels in that they start as unreactive compounds, and through the normal catalytic mechanism, are converted into reactive groups (often electrophiles), which can covalently modify the targeted enzyme before they dissociate. For these latent electrophiles, the utilization of the same mechanism as substrate imparts very stringent selectivity to covalent modification. However, this strategy is often perceived as being too complicated to easily implement by design.

A conceptual middle ground between affinity labels and mechanism-based inactivators is represented by a category of covalent modifiers called quiescent affinity labels.^[1c] These compounds are very weak electrophiles that do not readily react with nucleophiles in solution, yet act as covalent protein modifiers through a reaction that is catalyzed by the

target protein. These compounds differ from mechanism-based inactivators, in part because the catalysis required is “off pathway” and not found in the normal turnover mechanism. The mechanisms for catalyzing covalent bond formation by quiescent affinity labels can be potentially quite simple and may represent a promising approach to achieve greater selectivity of covalent modification than classic affinity labels, without all of the constraints found with mechanism-based inactivators. For one example, we discovered that fragment-sized (< 300 Da) 4-halopyridines are quiescent affinity labels of the enzyme dimethylarginine dimethylaminohydrolase (DDAH).^[7] These compounds typically have pK_a values < 5 and are not very reactive to nucleophiles in solution at neutral pH, due to the unfavorable formation of two lone electron pairs on the pyridine nitrogen in the σ -complex (Scheme 1). DDAH catalyzes the modification of its active-site Cys residue simply by stabilizing the more reactive protonated pyridinium through interaction with a neighboring Asp residue. Rate-pH profiles^[8] and computational prediction^[9] do not suggest a shifted pK_a for this Asp, so the halopyridine likely either acquires a proton from solvent after binding, or the enzyme selectively binds the fraction of protonated pyridine already present in solution. Since the electrophilicity of 4-halopyridines is “switchable” by a simple protonation mechanism, we suggest that these moieties may be more broadly useful for selective covalent modification of other protein targets that can stabilize the protonated form. Herein, we compare 4-halopyridine non-enzymatic reactivity to other commonly used electrophiles and quantify how much the “switch” (formation of the charged pyridinium) increases reactivity. We illustrate how pK_a modulation can predictably tune enzyme inactivation kinetics over several orders of magnitude, and demonstrate broader application of 4-halopyridines for labeling different proteins in a proteomic extract in a manner that likely also requires catalysis.

RESULTS & DISCUSSION

First, the non-enzymatic reactivity of 4-chloropyridine was estimated and ranked with respect to well-characterized electrophilic compounds used to modify proteins: *N*-ethylmaleimide, phenyl vinylsulfonate, iodoacetamide, iodoacetate, acrylamide, styrene oxide, and ampicillin. Each electrophile was mixed with an equivalent molar amount of glutathione (GSH), which serves here as a typical thiol nucleophile (GSH thiol pK_a = 9.7).^[10] After an incubation period, the remaining thiol was quantified (Figure 1A). As expected, *N*-ethylmaleimide was the most reactive compound, and acrylamide did not significantly react with GSH during this timeframe. 4-Chloropyridine also did not significantly react with GSH. In contrast, *N*-methyl-4-chloropyridine, which contains a fixed positive charge and serves as a model of the fully protonated pyridinium, reacts quite readily and ranks close to iodoacetamide. This relative ranking can be compared to more extensive lists of electrophiles published elsewhere.^[11] To better quantify the comparison between the commonly used acrylamide moiety and the cationic and neutral forms of 4-chloropyridine, we replaced GSH with the more reactive nucleophile thiophenol (pK_a = 6.5),^[10] increased the ratio of electrophile:thiol, and monitored thiol modification to determine second order rate constants (Figure 1B). The rate constant for 4-chloropyridine was very similar (only 4.8-fold larger) to that of acrylamide. However, *N*-methylation of 4-chloropyridine resulted in an impressive ~4500-fold reaction rate enhancement. Here, only the reaction with thiols

was assessed because Cys was the only free amino acid tested that showed significant reactivity with *N*-methyl-4-chloropyridine at pH 7.5 (Figure S1). In sum, 4-chloropyridine shows a reactivity on par with acrylamide, but can be “switched on” to enhance reactivity by approximately 4500-fold upon formation of the pyridinium, reaching a reactivity similar to iodoacetamide. The full difference between neutral and charged forms of the halopyridine is likely larger than 4500-fold (*vide infra*) since the observed reaction may be due mostly to the small fraction of the pyridine found in the protonated state (~ 1 in 3333 molecules; $pK_a = 4.0$; $pH = 7.5$).

Because charge has such a profound effect on reactivity, we next sought to determine how modulation of the 4-halopyridine pK_a affects enzyme inactivation kinetics of the human enzyme DDAH-1 (DDAH1), a member of the penten superfamily^[12] and a proposed drug target for septic shock and idiopathic pulmonary fibrosis.^[13] To explore which positions of the pyridine might be substituted without adversely impacting interactions with the enzyme, we determined the second order inactivation rate constants (k_{inact}/K_I) for a panel of nineteen structurally diverse fragment-sized 4-halopyridines that showed little or no non-enzymatic reactivity with glutathione (Figure 2A, Figure S2, Figure S3). An example determination with 4-bromopyridine is included in Supporting Information (Figure S4). These 4-halopyridines showed little or no reactivity with glutathione. From this set of compounds, we observed some correlation of k_{inact}/K_I with pK_a , but there were many exceptions to the trend. Small neutral substitutions at the 2-position appeared to be well tolerated, so we determined k_{inact}/K_I for a series of 2-fluoromethyl substituted 4-chloropyridines with pK_a values ranging from 4.7 to 0.4 (Figure 2B, See Supporting Information for synthetic methods). Although increasing the strength of the electron-withdrawing group would be expected to increase pyridine electrophilicity in the neutral state, we observe a clear increasing correlation of k_{inact}/K_I values with increasing electron donation by the 2-substituent. This result indicates that the impact of substituents on pK_a is what dominates the control of 4-halopyridine reactivity with the enzyme. Notably, reactivity and pK_a of these compounds can be predictably tuned over several orders of magnitude. This finding also impacts selectivity; compounds with pK_a values well below that of 4-chloropyridine still serve as effective DDAH1 inactivators but would be less reactive with competing thiols in solution, further increasing selectivity of covalent modification.

The influence of varying halide leaving groups was not as clear to interpret (Figure S5, 2A). The k_{inact}/K_I values for both underivatized and comparably derivatized 4-halopyridines have a rank order of $Br > I > Cl$ for the 4-halide leaving group, which we interpreted preliminarily as an indication that breaking the C-halide bond may be the rate-determining step in the inactivation (similar to 4-chlorobenzoyl-CoA dehalogenase^[14]), assuming the large iodide substitution had adverse steric effects. However, non-enzymatic reaction rates with thiophenol gave the same rank order, instead of the $Cl > Br > I$ ranking that we originally expected due to influence on the electrophilicity of the adjacent carbon. Addition of a radical scavenger did not change the rank order for enzymatic inactivation (data not shown). Varying the 4-halide does not greatly impact pK_a , so leaving group identity represents another variable that can be used to tune reactivity, although more studies will be required to better understand the underlying mechanism. Regardless, in all cases, the 4-chloro substituent experimentally ranked the lowest in reactivity, and would be expected to have the

least non-selective reactivity. So, we chose the 4-chloro substitution to use in the proteomic studies below.

Covalent modification of DDAH1 by 4-halopyridines requires a Cys residue adjacent to a neighboring Asp residue.^[7] This configuration of Cys and Asp/Glu residues is not likely unique to DDAH and different mechanisms to catalyze labeling by 4-chloropyridines may exist. Therefore, we sought to determine whether the naïve soluble proteome of *E. coli* (which lacks DDAH1) contains other protein targets (Figure 3). A 4-chloropyridine probe with an alkyne appended through an ether linkage at the 2-position was synthesized in one step and incubated with soluble *E. coli* lysates. The resulting covalently labeled proteins were visualized by appending a biotin tag to all available alkyne groups, resolving proteins through SDS-PAGE, and Western blotting to detect bands that became linked to biotin. A number of labeled bands are observed in the probe-treated lane and not in the probe-omitted lane; these bands represent alternative protein targets of 4-chloropyridines. Endogenously biotinylated proteins were not observed under these conditions. Heat denaturation of the lysate before addition of the probe resulted in no observed labeling. This result is consistent with the proposed requirement of catalysis for labeling by 4-halopyridines since protein denaturation would unfold the reactive sites. The requirement for catalysis was then removed by synthesis of the *N*-methylated probe to fix the positive charge of the pyridinium. Subsequent incubation with lysates led to labeling of a larger number of proteins than that by the unmethylated probe, consistent with the presence of more total accessible nucleophilic sites than the subset that can also provide catalysis. In sharp contrast to the unmethylated probe, heat denaturation led to a significant increase in the number of proteins labeled by the *N*-methylated probe, likely due to an increase in accessibility to previously buried nucleophiles. The low non-selective reactivity of 4-halopyridines is consistent with a prior study that reported very limited labeling of proteomic extracts by 2-chloro- and 2,4-dichloropyridine probes with respect to more reactive *p*-chloronitrobenzene or chlorotriazine probes.^[15] In our hands, 2-chloropyridines are approximately 70-fold more reactive with thiols than 4-chloropyridine (data not shown). Further studies will determine the identity of proteins labeled by the 4-chloropyridine probe, as well as stoichiometry and mechanism of modification, but the results presented here demonstrate that proteins other than DDAH can be targeted and that a folded modification site and likely some type of catalysis are requirements for facile covalent bond formation.

In conclusion, 4-halopyridines are shown to be selective, tunable, and “switchable” covalent protein modifiers with a “resting” thiol-reactivity similar to that of acrylamide (or less). For 4-chloropyridine, the protonation “switch” imparts a ~4500-fold increase in reactivity. Facile covalent protein modification can be achieved through simple catalysis by stabilizing the protonated pyridinium form, which has a reactivity similar to that of iodoacetamide. Substituents can predictably vary the pK_a of the halopyridine and k_{inact}/K_I values for enzyme inactivation over several orders of magnitude, and the requirement for a folded reaction site, and likely catalysis, is demonstrated for targets other than DDAH1 in a proteomic extract. Both the specific example of 4-halopyridines, and the more general concept of quiescent affinity labels, have an additional dimension (the requirement for catalysis) that can be exploited and optimized in the design of covalent protein modifiers. In

our opinion, 4-halopyridines and other “switchable” electrophiles represent a promising strategy for the development of specific biochemical probes and therapeutic agents.

EXPERIMENTAL SECTION

Materials

Unless noted otherwise, all chemicals are from Sigma- Aldrich Chemical Co. (St. Louis, MO). Human dimethylarginine dimethylaminohydrolase-1 (DDAH1) was purified and assayed as described previously.^[16] 2-Methyl-4-chloropyridine, 3-amino-4-bromopyridine, and 2-amino-4-bromopyridine were from Chem-Impex International, Inc. (Wood Dale, IL). 3-Amino-4-iodopyridine and 2-methyl-4-bromopyridine were from Oakwood Chemical (Estill, SC). 2-Trifluoromethyl-4-chloropyridine was from Ark Pharm Inc. (Libertyville, IL). 3-Carboxy-4-chloropyridine was from Enamine LLC. (Monmouth Jct., NJ). The 2-fluoromethyl and 2-difluoromethyl-4-chloropyridine compounds were synthesized as described below. *N*-Methyl-4-chloropyridine was synthesized as described elsewhere.^[17] The two alkyne-containing 4-chloropyridine probes described in the main text were synthesized as described below. Unless specifically referenced, pK_a values were calculated using MarvinSketch version 15.11.16.0.^[18]

Non-enzymatic Reaction of Electrophiles with Glutathione

Reduced glutathione (GSH) was incubated with selected electrophiles at a 1:1 ratio (1 mM each) in Reaction Buffer (KH_2PO_4 (0.15 M), NaCl (0.1 M), ethylenediaminetetracetic acid (EDTA) (1 mM) at pH 7.5) for 45 min. Reactions were quenched by addition of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB; 1.85 mM final concentration). Reaction Buffer was added to each sample to bring the total volume to 1.25 mL and the absorbance at 412 nm was measured in order to determine the amount of GSH remaining. The concentration of GSH was calculated using $\epsilon_{412\text{ nm}} = 14,150\text{ M}^{-1}\text{cm}^{-1}$.^[19]

Non-enzymatic Reaction of Electrophiles with Thiophenol

Reduced thiophenol (1 mM) was incubated with selected electrophiles (10 mM) in Reaction Buffer. At various time points (0 – 80 min) aliquots (20 μ L) were diluted into a Reaction Buffer solution containing DTNB (1.85 mM final concentration) for a final volume of 1 mL and the amount of unmodified thiophenol determined as described above for GSH. The observed reaction rates (k_{obs}) were determined by fitting the time-dependence of remaining thiol concentration to a single-exponential equation using KaleidaGraph (Synergy Software, Reading, PA). The resulting k_{obs} values were then plotted against compound concentration and linear fitting used to determine the second order rate constant for each compound.

Enzyme Inactivation Assays

Time- and concentration-dependent inactivation assays were carried out with a dilution method as previously described using N^{ω},N^{ω} -dimethyl-L-arginine as a saturating DDAH1 substrate and the COLDER assay to detect product.^[7a, 20] The observed inactivation rates (k_{obs}) were determined by fitting the time-dependence of remaining activity (%) to a single-exponential equation. The resulting k_{obs} values were then plotted against inactivator

concentration to determine the second order rate constant for each compound tested using a linear equation.

Covalent Labeling and Visualization of Soluble Lysates from *Escherichia coli*

Escherichia coli (*E. Coli*) BL21(DE3) were grown in Luria-Bertani (LB) medium (500 mL) at 37 °C with shaking and harvested by centrifugation 1 h after transition into stationary phase ($OD_{600} \approx 1.5$). Cells were resuspended in K_2HPO_4 (100 mM) buffer at pH 8, containing KCl (100 mM) and protease inhibitor cocktail (170 µg/mL phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL pepstatin A, 1 µg/mL leupeptin). Soluble lysates were obtained by sonication (210 s total, 10 s pulse on/45 s pulse off, 70 % amplitude) with a sonic dismembrator (Fisher Scientific Model 500), equipped with a 0.5 inch disruptor horn, followed by centrifugation to pellet cell debris, which was subsequently discarded.

Lysate samples (80 µg total protein as determined by Bradford Assay) were treated as described in Figure 3 using each probe (1 mM) in K_2HPO_4 (100 mM) buffer at pH 8 containing KCl (100 mM) at room temperature for 3 h as described previously. A biotin tag was selectively appended to alkynes found in the reaction mixture by treating samples with *N*-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethyl]hexahydro-2-oxo-(3a*S*,4*S*,6a*R*)-1*H*-thieno[3,4-*d*]imidazole-4-pentanamide (Biotin- PEO_3 -azide, 100 µM, Sigma), tris(2-carboxyethyl)phosphine (TCEP, 1 mM), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA, 100 µM), and $CuSO_4$ (1 mM), making the final reaction volume 50 µL. Samples were gently vortexed and incubated 1 h at room temperature. The azide-alkyne click reaction was quenched by addition of 4 × Laemmli Sample Buffer (16 µL, from BioRad).

Labeled and unlabeled proteins were then visualized as follows. Each sample was resolved by two replica stacking, gradient (4 – 20 %) SDS-PAGE gels (BioRad) run in parallel. One gel was stained for total protein content using GelCode Blue stain (Thermo Scientific) and used as a loading control. The other was used for Western blotting to determine if any bands were covalently labeled by biotin. Two antibodies were used: IgG fraction monoclonal mouse anti-biotin primary antibody (1:200, Jackson ImmunoResearch, West Grove, PA) and IR Dye 800CW goat anti-mouse secondary antibody (1:10000) as obtained in the Odyssey IR Dye Western Blot Kit I (Li-Cor Biosciences, Lincoln, NE). Molecular Weight (MW) markers are Precision Plus Protein Dual Color Standards (BioRad). Images were scanned using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) at the core DNA Facility (University of Texas, Austin).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported in part by National Institutes of Health (NIH) Grant GM069754 and the Robert A. Welch Foundation Grant F-1572. A.T. acknowledges a postdoctoral fellowship from NIH 1K12GM102745.

We thank Haley Kenefick for assistance with the kinetic studies.

References

1. a) Shannon DA, Weerapana E. *Curr Opin Chem Biol.* 2015; 24:18–26. [PubMed: 25461720] b) Singh J, Petter RC, Baillie TA, Whitty A. *Nat Rev Drug Discov.* 2011; 10:307–317. [PubMed: 21455239] c) Krantz A. *Bioorg Med Chem Lett.* 1992; 2:1327–1334. d) Wofsy L, Metzger H, Singer SJ. *Biochemistry.* 1962; 1:1031–1039. [PubMed: 14001461]
2. Bohnenkamp W, Eltze M, Heintze K, Kromer W, Riedel R, Schudt C. *Pharmacol.* 1987; 34:269–278.
3. Bodor N, Buchwald P. *Med Res Rev.* 2000; 20:58–101. [PubMed: 10608921]
4. Zaro BW, Whitby LR, Lum KM, Cravatt BF. *J Am Chem Soc.* 2016; 138:15841–15844. [PubMed: 27960302]
5. Bradshaw JM, McFarland JM, Paavilainen VO, Bisconte A, Tam D, Phan VT, Romanov S, Finkle D, Shu J, Patel V, Ton T, Li X, Loughhead DG, Nunn PA, Karr DE, Gerritsen ME, Funk JO, Owens TD, Verner E, Brameld KA, Hill RJ, Goldstein DM, Taunton J. *Nat Chem Biol.* 2015; 11:525–531. [PubMed: 26006010]
6. Silverman RB. *Method Enzymol.* 1995; 249:240–283.
7. a) Johnson CM, Linsky TW, Yoon DW, Person MD, Fast W. *J Am Chem Soc.* 2011; 133:1553–1562. b) Johnson CM, Monzingo AF, Ke Z, Yoon DW, Linsky TW, Guo H, Robertus JD, Fast W. *J Am Chem Soc.* 2011; 133:10951–10959. [PubMed: 21630706]
8. Hong L, Fast W. *J Biol Chem.* 2007; 282:34684–34692. [PubMed: 17895252]
9. Olsson MH, Sondergaard CR, Rostkowski M, Jensen JH. *J Chem Theor Comp.* 2011; 7:525–537.
10. Jencks, WP., Regenstein, J. *Handbook of Biochemistry.* The Chemical Rubber Co; Cleveland: 1968. p. J150-J189.
11. Schwobel JA, Koleva YK, Enoch SJ, Bajot F, Hewitt M, Madden JC, Roberts DW, Schultz TW, Cronin MT. *Chem Rev.* 2011; 111:2562–2596. [PubMed: 21401043]
12. Linsky T, Fast W. *Biochim Biophys Acta.* 2010; 1804:1943–1953. [PubMed: 20654741]
13. a) Wang Z, Lambden S, Taylor V, Sujkovic E, Nandi M, Tomlinson J, Dyson A, McDonald N, Caddick S, Singer M, Leiper J. *Biochem J.* 2014; 460:309–316. [PubMed: 24611830] b) Pullamsetti SS, Savai R, Dumitrascu R, Dahal BK, Wilhelm J, Konigshoff M, Zakrzewicz D, Ghofrani HA, Weissmann N, Eickelberg O, Guenther A, Leiper J, Seeger W, Grimminger F, Schermuly RT. *Sci Trans Med.* 2011; 3:87ra53.
14. Crooks GP, Copley SD. *J Am Chem Soc.* 1993; 115:6422–6423.
15. Shannon DA, Banerjee R, Webster ER, Bak DW, Wang C, Weerapana E. *J Am Chem Soc.* 2014; 136:3330–3333. [PubMed: 24548313]
16. Wang Y, Monzingo AF, Hu S, Schaller TH, Robertus JD, Fast W. *Biochemistry.* 2009; 48:8624–8635. [PubMed: 19663506]
17. Sprague RH, Brooker LGS. *J Am Chem Soc.* 1937; 59:2697–2699.
18. ChemAxon. 2017. (<http://www.chemaxon.com>) MarvinSketch 17.1.16.0
19. Riddles PW, Blakeley RL, Zerner B. *Method Enzymol.* 1983; 91:49–60.
20. Knipp M, Vasak M. *Anal Biochem.* 2000; 286:257–264. [PubMed: 11067748]

Figure 1.

Non-enzymatic reactivity of selected electrophiles with thiols. A. Reactivity is estimated by incubation of 1:1 glutathione (GSH):electrophile (1 mM each) for 45 min at a pH of 7.5, 25 °C and quantification of remaining thiol. Compound numbers in this figure correspond to those depicted in Figure 1B. B. Structures of electrophiles tested include **1** (4-chloropyridine), **2** (ampicillin), **3** (styrene oxide), **4** (acrylamide), **5** (iodoacetate), **6** (*N*-methyl-4-chloropyridine), **7** (iodoacetamide), **8** (phenyl vinylsulfonate), **9** (*N*-ethylmaleimide). C. The second order rate constants for reaction of thiophenol with acrylamide (○), 4-chloropyridine (□), and *N*-methyl-4-chloropyridine (■) are determined by plotting k_{obs} values versus the concentration of electrophile and linear fitting gives $(2.5 \pm 0.1) \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$, $(1.2 \pm 0.2) \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$, and $54 \pm 7 \text{ M}^{-1}\text{s}^{-1}$, respectively. Error bars for panels A and C are the standard deviation from three replicate experiments.

Figure 2.

Effect of 4-Halopyridine Substituents on k_{inact}/K_I for DDAH1. A. Structurally diverse 4-halopyridines inactivated DDAH1 with varying k_{inact}/K_I values. The structures of the nineteen 4-halopyridine derivatives used here are numbered differently than in Figure 1 and are shown in Supporting Information Figure S2. B. A series of structurally similar 4-halopyridine derivatives with various 2-substituents (Left to Right: CF_3 , CHF_2 , CH_2F , H, CH_3) inactivate DDAH1 with k_{inact}/K_I values that increase with increasing pK_a for the corresponding compound. The relationship of $\log (k_{\text{inact}}/K_I)$ to pK_a is well fit by a linear equation with slope of 0.49 ± 0.04 and y -intercept of -2.9 ± 0.1 . Error bars are the fitting errors from the individual k_{inact}/K_I determinations.

Figure 3.

Selectivity of Halopyridines In Labeling *E. coli* Cell Lysates. A. Western blot with red indicating pre-labeled molecular weight markers and green indicating biotin/probe-labeled proteins. Lanes left to right are: molecular weight markers (bottom to top: 10, 15, 20, 25, 37, 50, 75, 100, 150, (250 only in B) kDa), untreated lysate, lysate heated to 95 °C then cooled and treated with the alkyne-tagged 4-chloropyridine probe shown, unheated lysate treated with the same probe, unheated lysate treated with the alkyne-tagged *N*-methyl-4-chloropyridine probe shown, lysate heated to 95 °C then cooled and treated with the *N*-methylated probe shown above. B. Coomassie-stained SDS-PAGE gel run in parallel with that used to create the Western blot in A, with lanes corresponding to those described above.

Scheme 1.

Nucleophilic aromatic substitution of 4-halopyridines. A. Most 4-halopyridine derivatives tested have pK_a values < 5 , and at neutral pH disfavor stabilization of two electron pairs on the pyridine nitrogen. B. DDAH catalyzes covalent modification of the active-site Cys (C249) by stabilizing the protonated 4-halopyridinium (in red) through interaction with a neighboring Asp (D66, residue numbering corresponds to *Pseudomonas aeruginosa* DDAH).