

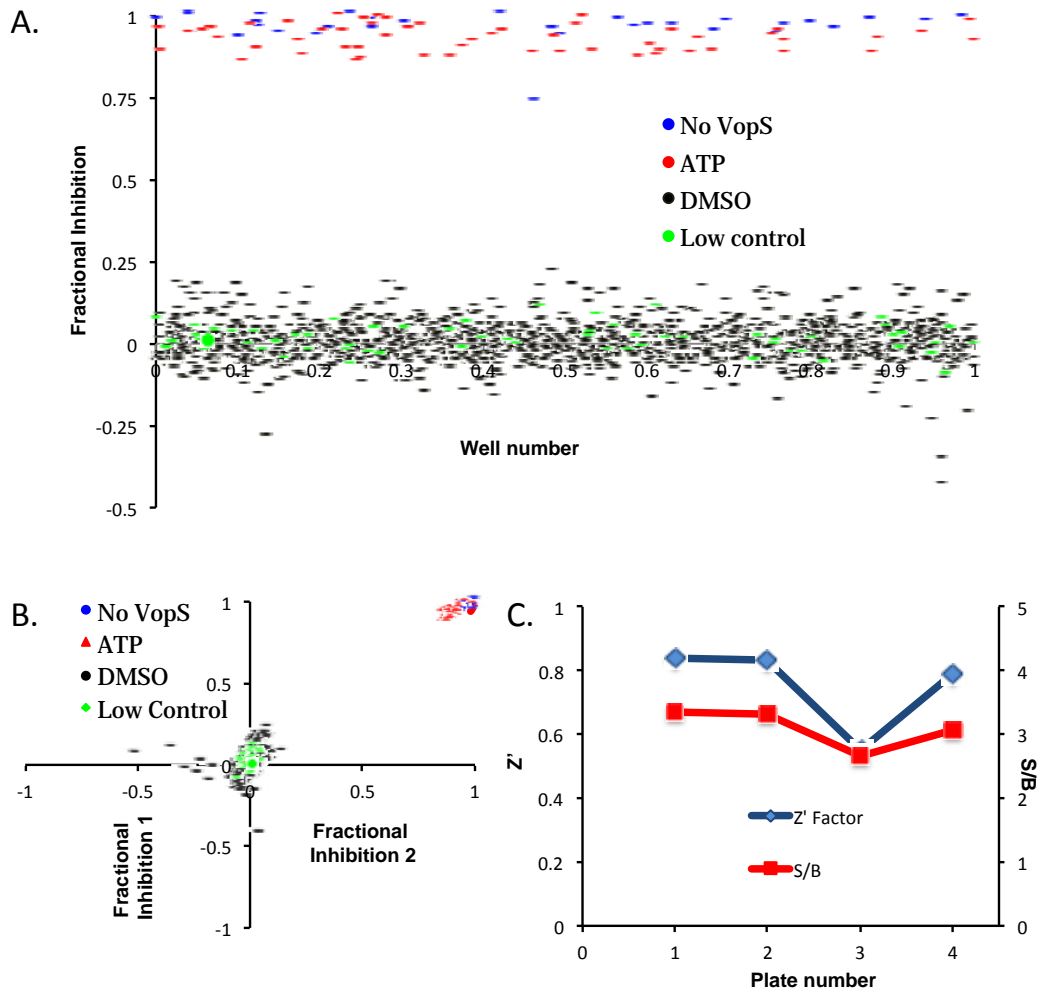
**Supporting Information for:**

**Inhibiting AMPylation: A novel screen to identify the first small molecule  
inhibitors of protein AMPylation<sup>‡</sup>**

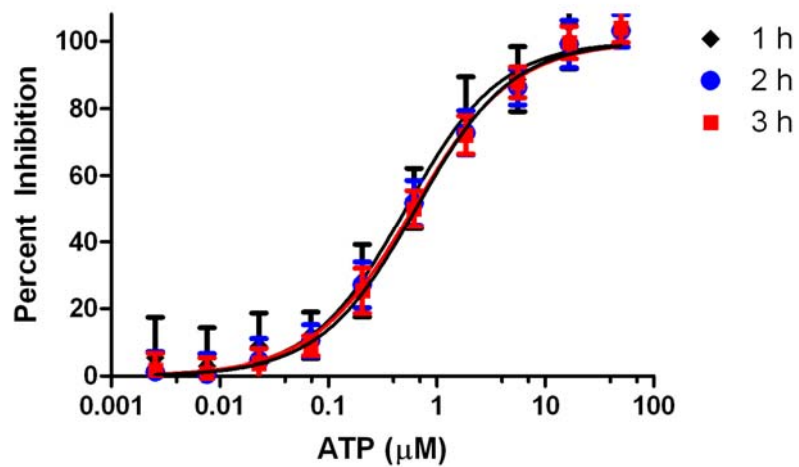
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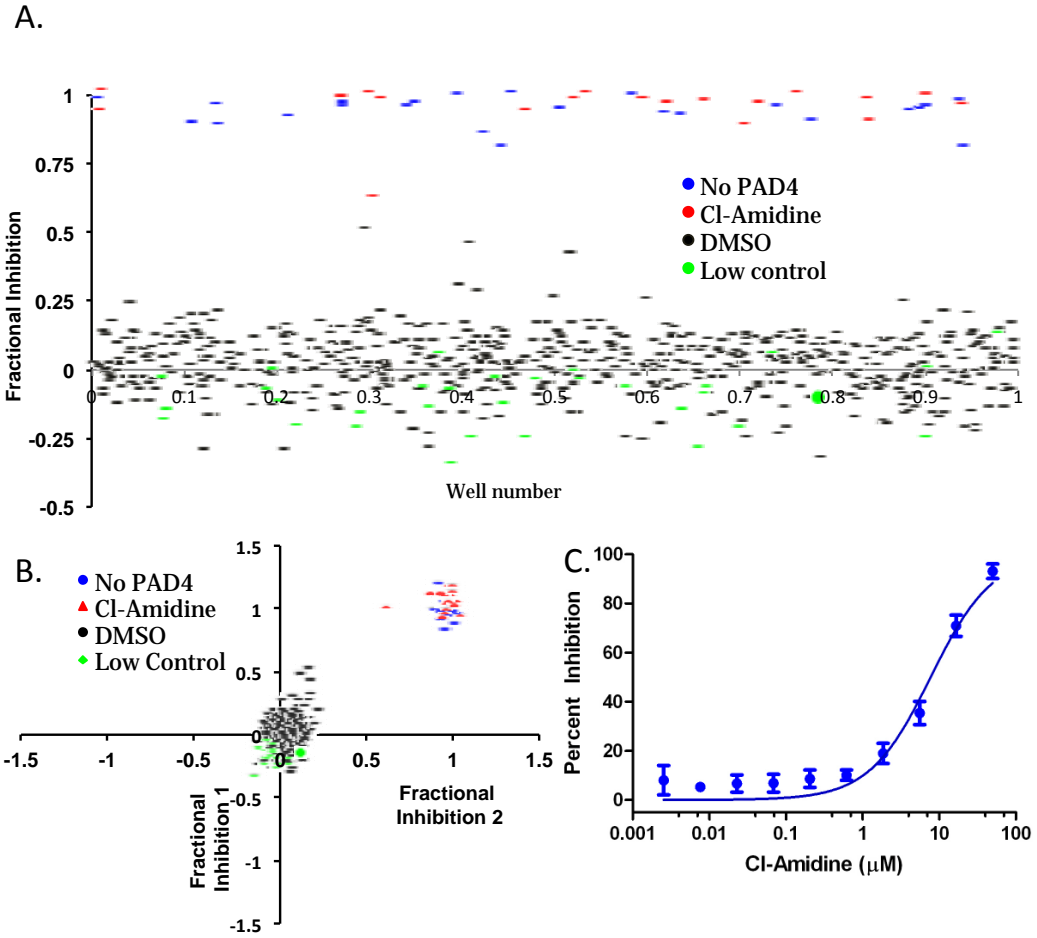
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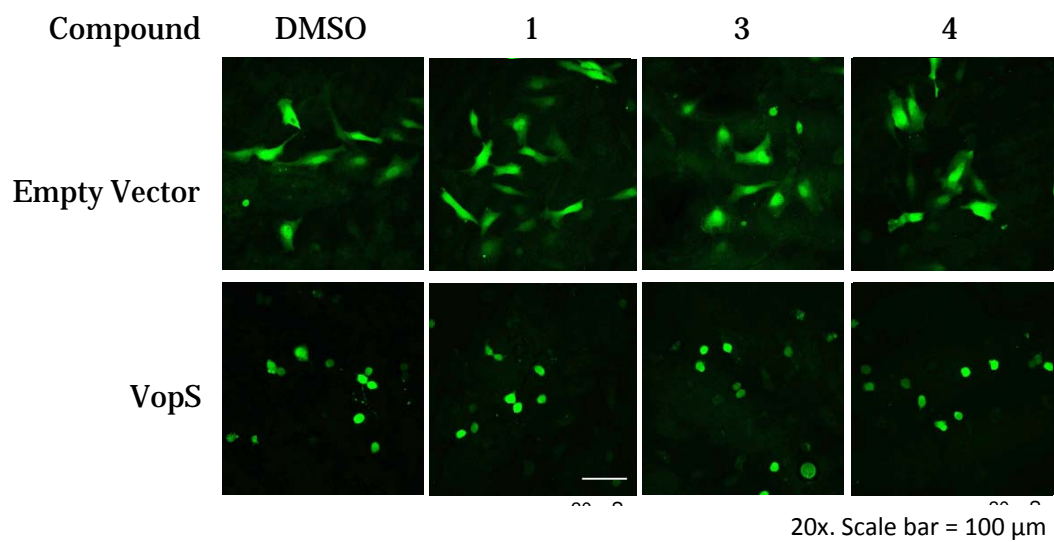
**Figure S1** FI-ATP assay validation. a) Random well scatter of the 3 h normalized FP values. b) Well correlation between 2 of the DMSO plates. c) Z' and S/B plots for each of the 4 DMSO plates.



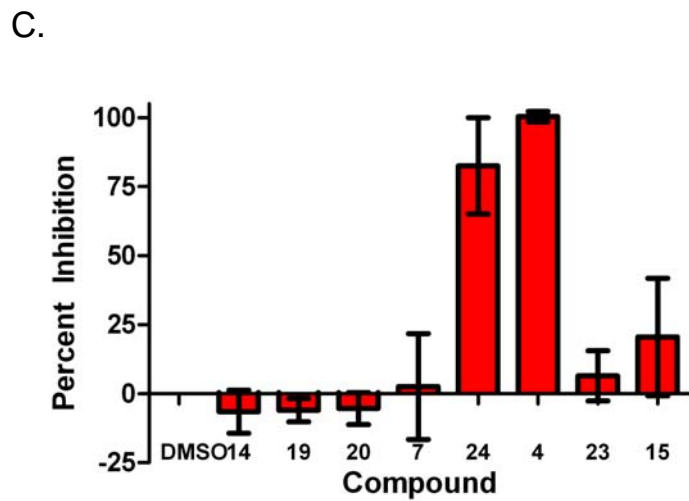
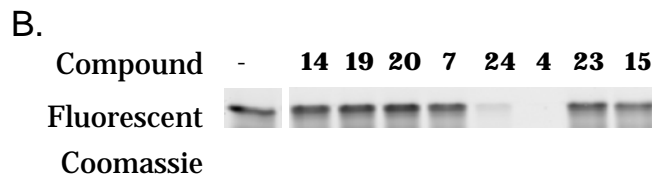
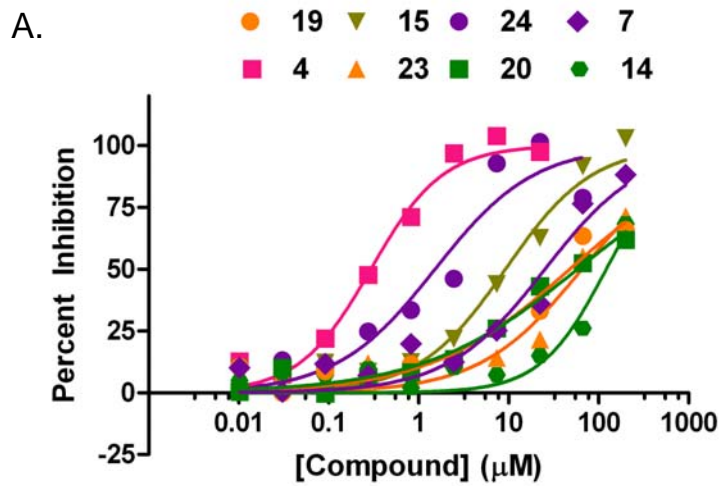
**Figure S2** FI-ATP HTS assay reproducibility. Three different plates of VopS (0.02 μM) were preincubated with seven replicates of 1/3 dilutions of ATP. Substrates (0.25 μM FI-ATP and 5 μM Cdc42) were added and the FP was measured after 1,2 and 3 h.



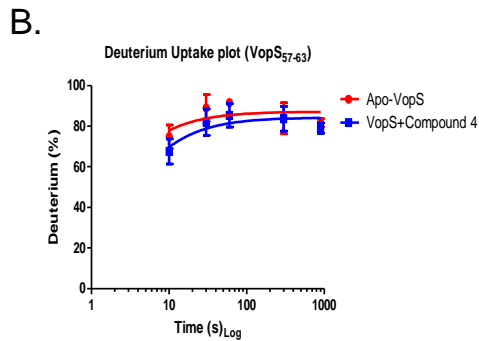
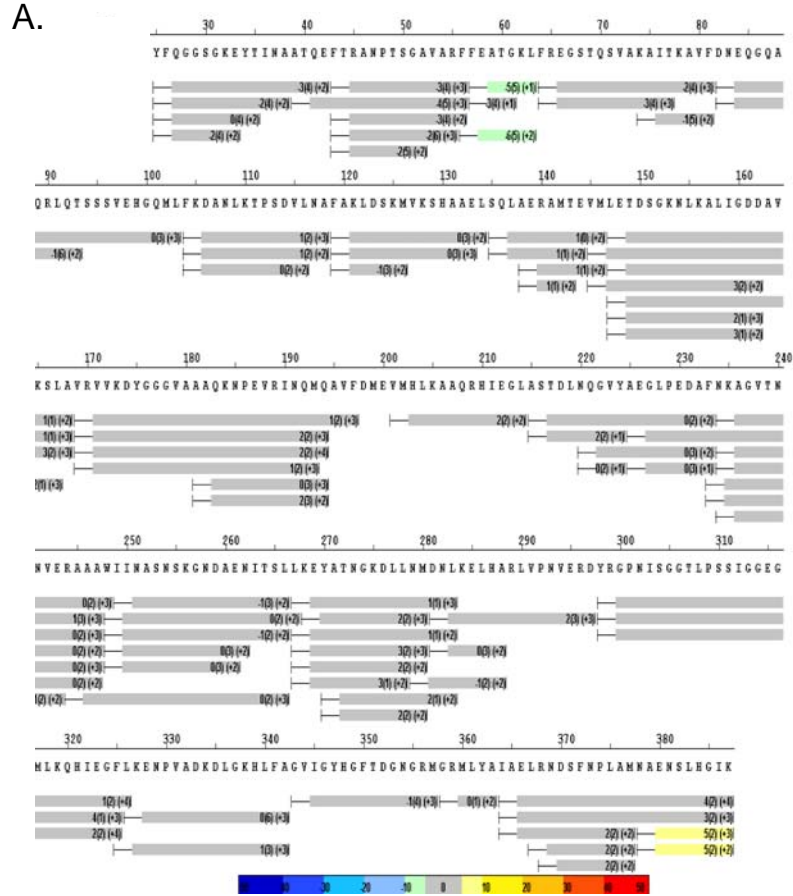
**Figure S3** FP-ABPP HTS assay validation with PAD4 and FFA. a) Random well scatter of the 3 h normalized FP values. b) Well correlation between 2 of the DMSO plates. c) CRC of Cl-amidine with PAD4 at the 3 h time point.



**Figure S4** Cellular characterization of validated hits. Fluorescent microscopy of GFP or VopS/GFP transfected HeLa cells in the presence or absence of **4** (5  $\mu$ M), **3** (10  $\mu$ M) or **1** (10  $\mu$ M). Cells were fixed with paraformaldehyde after incubating with inhibitors for 18 h.



**Figure S5** Calmidazolium derivatives. a) CRCs of the top 12 calmidazolium derivatives as determined by percent inhibition. b) Gel-based inhibition assay using 50  $\mu\text{M}$  inhibitor with VopS and Cdc42. c) Measured percent inhibition values from b).



**Figure S6** Differential HDX of VopS $\Delta$ 30 with **4**. a) Heat map of the change in deuterium uptake of VopS $\Delta$ 30 incubated with 30  $\mu$ M of **4** shows decreased protection in the N-terminal region. b) Deuterium uptake plots for VopS 57-63 shows protection at 30  $\mu$ M **4**.

## **Abbreviations**

AMP, adenosine monophosphate; ATP, adenosine triphosphate; MeCN, acetonitrile; PP<sub>i</sub>, inorganic pyrophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); EDTA, ethylenediamine tetraacetic acid; CRC, compound response curves; MALDI, matrix-assisted laser desorption/ionization; DTT, dithiothreitol; Fl-ATP, N6-(6-amino)hexyl-ATP-5-carboxyl-fluorescein; Fl-AMP N6-(6-amino)hexyl-AMP-5-carboxyl-fluorescein; FFA, fluorescein tagged F-amidine. Reverse Phase High-performance liquid chromatography (RP-HPLC); Dimethylformamide (DMF); Dimethylsulfoxide (DMSO); Trifluoroacetic acid (TFA); Acetonitrile (MeCN); benzyl bromide (BnBr); sodium hydride (NaH); benzyl chloride (BnCl); methanol (MeOH).



## Material and methods:

**Chemicals and proteins.** *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), dithiothreitol (DTT), fluorescein isothiocyanate (FITC), and ethylenediamine tetraacetic acid (EDTA), adenosine triphosphate (ATP), pluronic acid (Cat#P2443), GW7647 (Cat#G6793), MK886 (Cat#M2692), calmidazolium chloride (Cat#C3930), farnesylthiosalicylic acid (Cat#F8175) and protease inhibitor cocktail (Cat#P8465) were purchased from Sigma Aldrich (St. Louis, MO). N6-(6-Amino)-hexyl-ATP-5-carboxyfluorescein (Fl-ATP) was purchased from Jena Bioscience GmbH (Germany).  $\alpha$ -<sup>33</sup>P-ATP was purchased from American Radio Chemicals. The synthesis of fluorescein-tagged F-amidine (FFA) has previously been described.<sup>1</sup> VopS $\Delta$ 30, Cdc42Q61L (1–179) and PAD4 were prepared as previously described.<sup>2, 3</sup> Compounds **6–14**, **18–21** and **24** were obtained from the NCI/DTP Open Chemical Repository.<sup>4</sup> Imidazole (A10221) and benzhydrylbromide (L02211) were purchased from Alfa Aesar. Anhydrous DMF (227056), BnBr (B17905), NaH (452912), BnCl (185558), and 1-(2,4-dichloro-phenyl)-2-imidazol-1-yl-ethanol (cds003858) were purchased from Aldrich. Styrene oxide (132800100) and deuterated MeOH (d4) (325360100) were purchased from Acros. MeCN (AX0149-3) was purchased from EMD. Deuterated DMSO (d6) (DLM-10-10) was purchased from Cambridge Isotope Labs.

**PAD4 counter screen.** 8  $\mu$ L of PAD Screening Buffer (50 mM HEPES pH 7.6, 100 mM NaCl, 1 mM TCEP, 10 mM CaCl<sub>2</sub>, 0.01% pluronic acid) (column 1) or 8  $\mu$ L of PAD4 (2  $\mu$ M final) in PAD Screening Buffer (columns 2–23) were added to a black 384-well microtiter plate (Greiner 784076) using a Beckman Coulter FRD. Controls (columns 1: DMSO (no PAD4, high control); 2: 5 mM Cl-amidine; and 23: DMSO (no inhibitors, low control)) and CRC molecules (columns 3–22) were pinned using

the 100 nL head on a Beckman Coulter BioMek NXP to achieve a final concentration of 2 nM to 50  $\mu$ M. 2  $\mu$ L of Fluorescein-conjugated F-amidine (FFA) (150 nM final in PAD Screening Buffer), a PAD targeted ABPP,<sup>5</sup> was added using the FRD. The plates were read after incubating 3 h at 37 °C using a Perkin-Elmer EnVision plate reader as described above.

**Kinetic assays.** VopS $\Delta$ 30 activity was measured by detecting pyrophosphate formation as previously described.<sup>2</sup> Different concentrations of inhibitor were added to VopS $\Delta$ 30 (20 nM final) in Reaction Buffer (20 mM HEPES pH 8.0, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% BSA, 0.01% pluronic acid and 1 mM DTT) in a 384 well plate and incubated for 20 min before the reaction was initiated by adding Cdc42Q61L, ATP, PNP, pyrophosphatase and 2-Amino-6-mercapto-7-methylpurine riboside (MESG). Absorbance values were measured at 360 nm every 20 s and the initial rates were determined directly from the slopes using a pyrophosphate standard curve. The initial rates were fit by using a non-linear least fit squares approach to equation (2),

$$v = V_{\max}[S]/(K_M+[S]) \quad (2),$$

yielding values for  $k_{cat}$ ,  $K_M$  and  $k_{cat}/K_M$ . Note, all assays were performed at least in duplicate.

In inhibitor assays in which Cdc42Q61L is the varied substrate (0 – 750  $\mu$ M), rates were measured with a constant concentration of ATP (300  $\mu$ M) and several concentrations of inhibitor. Alternatively, in inhibitor assays in which ATP is the varied substrate (0 – 1 mM), a constant concentration of Cdc42Q61L (300  $\mu$ M) and several concentrations of inhibitor were used. The data were globally fit to equations representing competitive (equation (3)), non-competitive (equation (4);  $K_{is} = K_{ii}$ ), and mixed inhibition (equation (4);  $K_{is} \neq K_{ii}$ ),

$$v = V_{\max}[S]/(K_M(1 + [I]/K_{is}) + [S]) \quad (3),$$

$$v = V_{\max}[S]/(K_M(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})) \quad (4),$$

using GraFit version 5.0.1.1, where  $v$  is velocity,  $V_{\max}$  is the maximum rate,  $K_M$  is the Michaelis-Menten constant,  $[S]$  is the concentration of substrate,  $[I]$  is the concentration of inhibitor,  $K_{is}$  is the dissociation constant for the EI complex, and  $K_{ii}$  is the dissociation constant for the ESI complex.

Initial rates in the presence and absence of calmidazolium were determined using a radioactive assay with  $\alpha^{33}\text{P}$ -ATP. Different concentrations of inhibitor were added to VopS $\Delta$ 30 (10 nM final) in Reaction Buffer and incubated for 20 min before the reaction was initiated by adding Cdc42Q61L and ATP. In inhibitor assays in which Cdc42Q61L is the varied substrate (0 – 750  $\mu\text{M}$ ), rates were measured with a constant concentration of ATP (500  $\mu\text{M}$ ). Alternatively, in inhibitor assays in which ATP is the varied substrate (0 – 1 mM), a constant concentration of Cdc42Q61L (300  $\mu\text{M}$ ) was used. After initiation, 2  $\mu\text{L}$  of the reaction mixture was spotted on nitrocellulose after 6, 10, 13 or 20 min. The nitrocellulose was washed 1x with 1 mM NaCl 0.1%  $\text{H}_3\text{PO}_4$  and then 3x with 0.5%  $\text{H}_3\text{PO}_4$ . The blots were dried and exposed to a storage phosphor screen (GE Healthcare) overnight before scanning on a Typhoon imager. Percent incorporation was determined by normalizing the radioactivity to 2  $\mu\text{L}$  of spotted but unwashed reaction mixture. The initial rates and global analysis were done as described above.

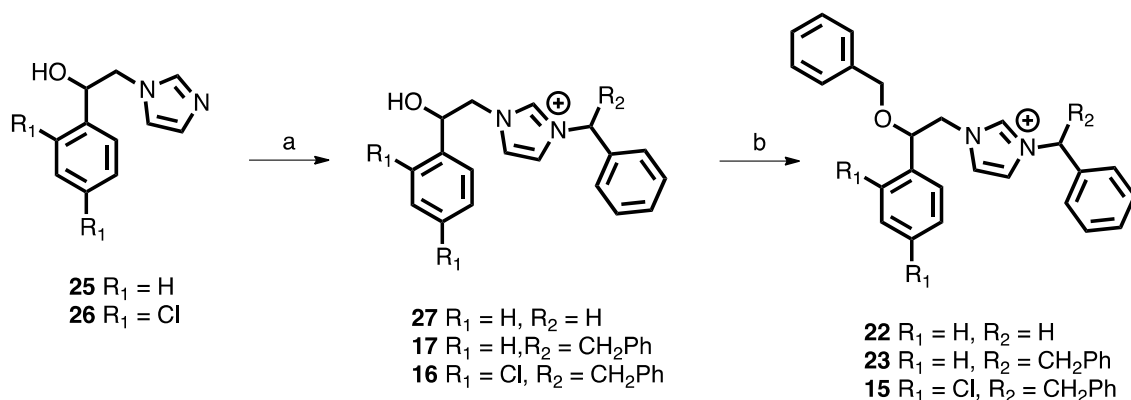
**Differential Hydrogen deuterium exchange (HDX) Mass Spectrometry.** Solution-phase amide HDX experiments were carried out with a fully automated system (CTC HTS PAL, LEAP Technologies, Carrboro, NC; housed inside a 4 °C cabinet) as described previously with slight modifications.<sup>6</sup> VopS $\Delta$ 30 (10  $\mu\text{M}$ ) was mixed with a

1:3 or 1:10 (protein:ligand) molar excess of calmidazolium (**4**), and MK886 (**3**), and incubated for 1 h at 4 °C for complex formation before subjecting to HDX. For the differential HDX experiments, 5 µL of VopSΔ30 with or without compound were mixed with 20 µL of D<sub>2</sub>O-containing HDX buffer (20 mM HEPES pH 8.0, 150 mM NaCl, 1 mM EDTA) and incubated at 4 °C for 0, 10, 30, 60, 90 or 3,600 s. Unwanted forward or back exchange was minimized by the addition of 25 µL of a quench solution (0.1% v/v TFA in 3 M urea and 50 mM TCEP) to denature the protein. Denatured samples were passed through an immobilized pepsin column<sup>7</sup> at 50 µL/min (0.1% v/v TFA, 15 °C) and the resulting peptides were trapped on a C<sub>8</sub> trap column (Hypersil Gold, Thermo Fisher). The bound peptides were gradient-eluted (5-50% CH<sub>3</sub>CN w/v and 0.3% w/v formic acid) across a 1 mm × 50 mm C<sub>18</sub> HPLC column (Hypersil Gold, Thermo Fisher) for 5 min at 4 °C. The eluted peptides were subjected to electrospray ionization directly coupled to a high resolution (60,000) Orbitrap mass spectrometer (LTQ Orbitrap XL with ETD, Thermo Fisher). Each HDX experiment was done in triplicate.

**Peptide Identification and HDX data processing.** Product ion spectra were acquired in a data-dependent mode and the five most abundant ions were selected for product ion analysis. The MS/MS \*.raw data files were converted to \*.mgf files and then submitted to Mascot (Matrix Science, London, UK) for peptide identification. Peptides included in the peptide set used for HDX detection had a MASCOT score of 20 or greater. The MS/MS MASCOT search was also performed against a decoy (reverse) sequence and ambiguous identifications were ruled out. The MS/MS spectra of all the peptide ions from the MASCOT search were further manually inspected and only the unique charged ions with the highest MASCOT score were used

in estimating the sequence coverage. The intensity weighted average  $m/z$  value (centroid) of each peptide isotopic envelope was calculated with the latest version of our in-house developed software, MS Peptide Workbench.<sup>8</sup>

**General methods.** All compounds were purified by RP-HPLC unless otherwise noted using a Varian C18 column on a Varian Prostar semi-prep HPLC. Solvent A was 0.1% TFA in H<sub>2</sub>O and solvent B was 0.1% TFA in MeCN. Fractions containing the correct compound were concentrated *in vacuo* and lyophilized. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on Bruker Avance 400 MHz ULTRASHield spectrometer. Chemical shifts are reported in  $\delta$  (ppm) units using <sup>13</sup>C and residual <sup>1</sup>H signals from deuterated solvents as references. Spectra were analyzed with Mest-Re-Nova (Mestrelab Research). Electrospray ionization spectra were recorded using an Agilent 1220 Infinity LC coupled to a 6120 Quadrupole MS and analyzed using Agilent Chemstation.



**Reagents and conditions:** a. BnCl or benzhydryl bromide, MeCN 50 °C. b. BnBr, NaH, DMF 0 °C -RT.

**General N-alkylation:** Aryl halide (1.1 eq) was added to monosubstituted imidazoles (**25**<sup>9</sup> or **26**) in MeCN. The reaction was heated to 50 °C in an oil bath for 6 h then concentrated *in vacuo*. The crude solid was washed with cold acetone, filtered, dried and purified by RP-HPLC.

**3-methyldiphenyl-1-(2-hydroxy-2-(2,4 dichlorophenylethyl)imidazolium bromide (16),** (yield = 50%).

<sup>1</sup>H NMR (400 MHz, MeOD) δ 8.67 (s, 1H), 7.64 (d, 1H, *J* = 9.6 Hz), 7.48 (m, 5H), 7.37 (m, 3H), 7.30 (m, 3H), 7.22 (m, 3H), 7.02 (s, 1H), 5.41 (t, 1H, *J* = 4.4 Hz), 4.50 (m, 2H). <sup>13</sup>C NMR (100 MHz, MeOD) δ 146.0, 138.3, 138.0, 135.8, 133.6, 133.5, 130.0, 129.4, 128.8, 128.2, 127.8, 125.5, 123.4, 77.0, 68.6, 55.5. LRMS (ESI): *m/z* calcd for [C<sub>24</sub>H<sub>21</sub>Cl<sub>2</sub>N<sub>2</sub>O]<sup>+</sup>: 423.1; found 423.1.

**3-methyldiphenyl-1-(2-hydroxy-2-phenylethyl)imidazolium bromide (17),** (yield = 60%).

<sup>1</sup>H NMR (400 MHz, MeOD) δ 8.52 (t, 1H, *J* = 1.6 Hz), 7.60 (t, 1H, *J* = 1.6 Hz), 7.59, 7.46 (t, 1H, *J* = 2.0 Hz), 7.42-7.39 (m, 6H), 7.29-7.25 (m, 5H), 7.14-7.10 (m, 4H), 6.93 (s, 1H), 5.03 (dd, 1H, *J* = 16.6, 3.6 Hz), 4.44 (dd, 1H, *J* = 12.0, 3.6 Hz), 4.35 (dd, 1H, *J* = 13.8, 6.4 Hz). <sup>13</sup>C NMR (100 MHz, MeOD) δ 141.6, 138.1, 138.0, 130.5, 130.5, 129.7, 129.3, 129.3, 126.9, 125.3, 123.3, 72.4, 68.5, 57.6. LRMS (ESI): *m/z* calcd for [C<sub>24</sub>H<sub>23</sub>N<sub>2</sub>O]<sup>+</sup>: 355.2; found 355.2.

**3-Benzyl-1-(2-hydroxy-2-phenylethyl)imidazolium chloride (27),** (yield = 43%). Spectral values matched those previously reported.<sup>10</sup>

*General O*—alkylation. BnBr (1.1 eq) was added to disubstituted imidazoles **16**, **17** or **27** (1 eq) in anhydrous DMF. The reaction was cooled to 0 °C and NaH (1.2 eq) was added and the reaction stirred for 1 h. The reaction was quenched with MeOH, diluted with H<sub>2</sub>O and MeCN, and purified by RP-HPLC.

**3-methyldiphenyl-1-(2- *O*-benzyl-2-(2,4 dichlorophenylethyl)imidazolium trifluoroacetate, (15)**, (yield = 18%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 8.64 (t, 1H, *J* = 1.6 Hz), 7.59 (t, 1H, *J* = 2.0 Hz), 7.52 (m, 2H), 7.42-7.39 (m, 6H), 7.34 – 7.32 (m, 3H), 7.26 - 7.24 (m, 3H), 7.15 - 7.12 (m, 5H), 6.95 (s, 1H), 5.16 (dd, 1H, *J* = 6.8, 3.6 Hz), 4.52 - 4.46 (m, 3H), 4.29 (d, 1H, *J* = 12.0 Hz); <sup>13</sup>C NMR (100 MHz, MeOD) δ 138.4, 138.1, 137.9, 137.8, 136.5, 135.0, 134.4, 130.9, 130.5, 130.5, 130.2, 129.7, 129.3, 129.2, 129.2, 125.3, 123.7, 75.9, 72.5, 68.6, 54.2. LRMS (ESI): *m/z* calcd for [C<sub>31</sub>H<sub>27</sub>Cl<sub>2</sub>N<sub>2</sub>O]<sup>+</sup>: 513.2; found 513.2.

**3-Benzyl-1-(2- *O*-benzyl-2-phenylethyl)imidazolium trifluoroacetate, (22)** (yield = 12%) <sup>1</sup>H NMR (400 MHz, DMSO) δ 9.22 (t, 1H, *J* = 1.6 Hz), 7.81 (t, 1H, *J* = 2.0 Hz), 7.73 (t, 1H, *J* = 2.0 Hz), 7.45-7.37 (m, 11H), 7.26 (m, 2H), 7.09 (m, 2H), 5.45 (s, 2H), 4.81 (m, 1H), 4.49 (m, 2H), 4.42(d, 1H, *J* = 12.0 Hz), 4.23(d, 1H, *J* = 12.0 Hz); <sup>13</sup>C NMR (100 MHz, DMSO) δ 137.4, 137.3, 136.6, 134.8, 129.0, 128.8, 128.8, 128.6, 128.2, 128.2, 127.6, 127.3, 126.7, 123.5, 122.2, 78.2, 69.9, 54.0, 51.9. LRMS (ESI): *m/z* calcd for [C<sub>25</sub>H<sub>25</sub>N<sub>2</sub>O]<sup>+</sup>: 369.2; found 369.2.

**3-methyldiphenyl-1-(2-*O*-benzyl-2-phenylethyl)imidazolium trifluoroacetate, (23)**, (yield = 15%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 8.61 (t, 1H, *J* = 1.6 Hz), 7.63 (t, 1H, *J* = 1.6 Hz), 7.52 (t, 1H, *J* = 2.0 Hz), 7.41-7.36 (m, 8H), 7.36 - 7.31 (m, 4H), 7.26 - 7.24 (m, 3H), 7.14-7.10 (m, 5H), 6.94 (s, 1H), 4.77 (m, 1H), 4.45 (m, 3H), 4.22 (d, 1H, *J* = 12.0 Hz); <sup>13</sup>C NMR (100 MHz, DMSO) δ 141.0, 137.5, 136.9, 136.8, 129.1, 128.9, 128.8, 128.3, 127.9, 127.6, 127.3, 126.7, 125.8, 123.9, 122.2, 78.2, 70.0, 65.7, 53.9. LRMS (ESI): *m/z* calcd for [C<sub>31</sub>H<sub>29</sub>N<sub>2</sub>O]<sup>+</sup>: 445.2; found 445.2.

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