Supplementary Data

A chemical strategy for the cell-based detection of HDAC activity

Himashinie V. K. Diyabalanage, Genevieve C. Van de Bittner, Emily L. Ricq and Jacob M. Hooker

Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, United States

Experimental Methods

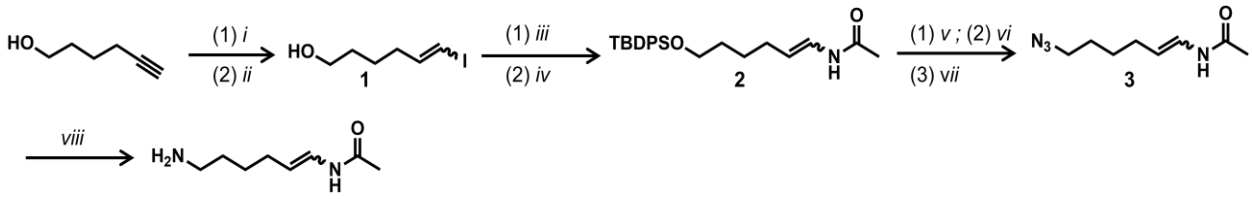
General materials and methods

All chemical reagents were of ACS grade purity or higher, purchased from commercial sources, and used as received without further purification. Reactions were performed using standard techniques, including inert atmosphere of nitrogen with standard Schlenk technique, when necessary. Glassware was oven-dried at 150 ºC overnight. Analytical thin layer chromatography (TLC) was performed on SiliCycle TLC silica Gel 60-F254 plates with visualization by ultraviolet (UV) irradiation at 254 nm. Purifications were performed using HP silica chromatography column by Teledyne Isco. The elution system for each purification was determined by TLC analysis. Chromatography solvents were purchased from commercial sources and used without distillation. NMR spectra were recorded at 22 ºC on a Varian 500 MHz spectrometer. Proton chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-d (δ 7.27, singlet), methanol-d4 (δ 3.31, pentet), or dimethylsulfoxide-d6 (δ 2.50, pentet). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets), dq (doublet of quartet) or m (multiplet). Coupling constants are reported as a *J* value in Hertz (Hz). ^{13}C NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-d (δ 77.1, triplet), methanol-d4 (δ 49.0, septet), dimethyl sulfoxide-d6 (δ 39.5 septet), or acetonitrile-d3 (δ 1.3, singlet; 118.3 septet). HPLC-analysis of organic synthetic reactions was conducted on an Agilent 1100 series HPLC fitted with a diode-array detector, quaternary pump, vacuum degasser, and autosampler. Mass spectrometry data were recorded on an Agilent 6310 ion trap mass spectrometer (ESI source) connected to an Agilent 1200 series HPLC with quaternary pump, vacuum degasser, diode-array detector, and autosampler. Analytical separation by HPLC was achieved by a gradient of acetonitrile with

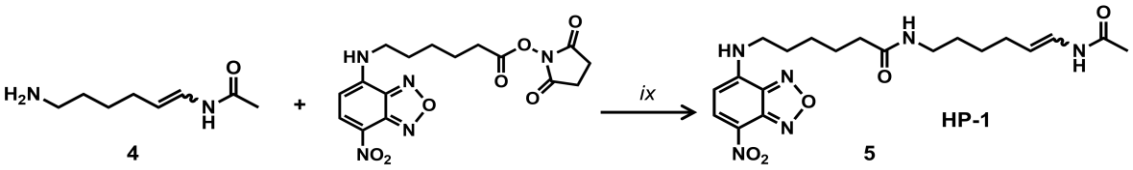
0.01% ammonium formate (10% for 0-3 minutes, 10%-95% for 3-13 minutes, 95% for 13-15 minutes; percentages are % acetonitrile, v/v).

Synthesis procedures and characterization data for HP-1

(a)



(b)



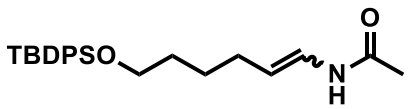
Scheme S1. Synthesis of HP-1*. (a) Building block synthesis for HP-1.[1,2] (b) Attachment to the NBD fluorophore. *Reagents and conditions (*i*) AIBN, Bu_3SnH, THF, 90 ˚C, 12 h, 90% (*ii*) I_2, CH_2Cl_2, 30 min, RT, 61% (*iii*) *t*-Bu(Cl)Ph_2Si, Im, CH_2Cl_2, overnight, 0 ˚C, 73% *(iv)* CuI, CH_3CONH_2, Cs_2CO_3, DMEDA,THF, 20 h, 65 ˚C, 72% (*v*) TBAF, THF, overnight, RT, 80% (*vi*) Pyridine, TsCl, DCM, overnight, RT, 64% (*vii*) NaN_3, DMF, 3h, 80 ˚C, 81% (*viii*) NH_4Cl, Zn, EtOH, H_2O, 3h, RT, 80% (*ix*) Et_3N, CH_2Cl_2, RT, overnight, 75%.

Compound 1:

i & ii. To an oven-dried, round-bottom flask under N_2 was added 5-hexyn-1-ol (3.0 g, 30.57 mmol, 1.0 equiv.) and degassed anhydrous THF (80 mL). Bu_3SnH (8.10 mL, 30.57 mmol, 1.0 equiv.) was added *via* syringe, and the solution was heated to 80 °C. AIBN (1.0 g, 6.11 mmol, 0.2 equiv.) was then added in one portion under a stream of N_2. After 5 minutes, the temperature was raised to 90 °C, and the solution was stirred for 14 hours before cooling to

room temperature. The solvent was removed under vacuum and the intermediate (pale yellow oil) was dissolved in 15 mL of anhydrous CH_2Cl_2. Separately, I_2 (9.31 g, 36.68 mmol, 1.2 equiv.) was dissolved in 20 mL of CH_2Cl_2 and added drop-wise to the vinyl tin solution until a purple color persisted. The reaction mixture was stirred for an additional 30 minutes, then saturated Na_2SO_3 (aq) was added to quench the reaction. The organic phase was extracted, washed with brine, dried over Na_2SO_4, and concentrated under vacuum. The residue was purified *via* flash column chromatography (Rf = 0.5 in 1:1 hexanes/EtOAc) to give a mixture (*trans:cis* = 2.6:1) of vinyl iodide **1** (4.21 g, 18.6 mmol, 61% yield) as a pale yellow oil. *Trans* ^{1}H NMR (500 MHz, $CDCl_3$):1.50 (m, 2H), 1.59 (m, 2H), 2.10 (dq, 2H, *J* = 14.5, 1.1 Hz), 3.66 (m, 2H), 6.02 (br d, 1H, *J* = 14.3 Hz), 6.51 (dt, 1H, *J* = 14.3, 7.1 Hz). *Trans* ^{13}C NMR (125 MHz, $CDCl_3$): 24.52, 31.87, 35.70, 62.53, 74.76, 146.19. *Cis* ^{1}H NMR (500 MHz, $CDCl_3$): 6.20 (m, 2H), 3.66 (m, 2H), 2.19 (q, 2H, *J* = 6.9 Hz), 1.59 (m, 2H), 1.50 (m, 2H). *Cis* ^{13}C NMR (125 MHz, $CDCl_3$): 24.13, 32.03, 34.34, 62.62, 82.67, 140.92.

Compound 2:

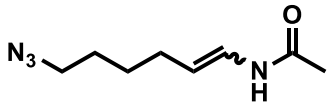


iii. To an oven-dried, round-bottom flask under N_2 was added anhydrous CH_2Cl_2 (30 mL), vinyl iodide (5.31 g, 24.5 mmol, 1.0 equiv.), and imidazole (2.88 g, 42.3 mmol, 1.8 equiv.). The solution was stirred for 5 minutes at room temperature, then chilled to 0 ºC. *t*-Bu(Cl)Ph_2Si (6.01 mL, 24.5 mmol, 1.0 equiv) was added dropwise *via* syringe and a white precipitate formed immediately. The reaction mixture was warmed to room temperature, then stirred for 1 hour. The solution was diluted with CH_2Cl_2 and washed with NH_4Cl (2 x 25 mL), followed by brine (1 x 25 mL). The organic phase was dried over Na_2SO_4, concentrated under vacuum, and purified *via* flash column chromatography (Rf = 0.8 in 8:2 hexanes/EtOAc) to afford a mixture (*trans:cis* = 1.57:1) of *t*-BDPS protected vinyl iodide (8.34 g, 17.95 mmol, 73% yield) as a colorless oil. *Trans* ^{1}H NMR (500 MHz, $CDCl_3$): 1.05 (s, 9H), 1.47 (m, 2H), 1.60 (m, 2H), 2.04 (q, 2H, *J* = 7.3 Hz), 3.66 (m, 2H), 5.95 (d, 1H, *J* = 14.3 Hz), 6.48 (dt, 1H, *J* = 14.3, 7.1 Hz), 7.40 (m, 6H), 7.66 (m, 4H). *Trans* ^{13}C NMR (500 MHz, $CDCl_3$): 19.21, 24.60, 26.85, 31.71, 35.68, 63.56, 74.53, 127.60, 129.55, 133.96, 135.55, 146.40. *Cis* ^{1}H NMR (500 MHz, $CDCl_3$): 1.05 (s, 9H), 1.47 (m, 2H), 1.85 (m, 2H), 2.14 (q, 2H, *J* = 7.0 Hz), 3.75 (m, 2H), 6.16 (m, 2H), 7.40 (m, 6H), 7.66 (m,

4H). *Cis* ^{13}C NMR (125 MHz, $CDCl_3$): 19.21, 24.26, 26.85, 31.95, 34.39, 63.56, 82.36, 127.59, 129.51,134.02,135.56, 141.33.

iv. To an oven-dried, round-bottom flask under N_2 was added CuI (108 mg, 0.56 mmol, 0.1 equiv.), acetamide (662 mg, 11.2 mmol, 2.0 equiv.), and Cs_2CO_3 (2.737 g, 8.4 mmol, 1.5 equiv.). The solids were suspended in anhydrous THF (6 mL), and *N,N*-dimethylethylenediamine (122 µL, 1.12 mmol, 0.2 equiv.) was added dropwise. Separately, the *t*BDPS-protected vinyl iodide was dissolved in anhydrous THF (3 mL) and added dropwise to the acetamide solution *via* syringe. The reaction vessel was flushed with N_2, sealed, and heated to 55 ºC overnight. The reaction mixture was cooled to room temperature, diluted with EtOAc (80 mL), and filtered over a pad of silica gel. After thorough washing, the combined organic solvent was concentrated under vacuum and the mixture (*trans:cis* = 4.6:1) was purified by flash column chromatography (c*is* Rf = 0.45, *trans* Rf = 0.40 in 1:1 hexanes/EtOAc). The major *trans* isomer of the *t*BDPS-protected enamide (**2**, 1.60 g, 4.02 mmol, 72% yield) was obtained as a colorless oil. *Trans* ^{1}H NMR (500 MHz, $CDCl_3$): 1.04 (s, 9H), 1.43 (m, 2H), 1.55 (m, 2H), 1.99 (m, 2H), 2.02 (s, 3H), 3.65 (t, 2H, *J* = 6.2 Hz), 5.07 Hz (dt, 1H, *J* = 14.2, 7.1 Hz), 6.71 (dd, 1H, *J* = 14.2, 10.2 Hz), 6.85 (br d, 1H, *J* = 10.2 Hz), 7.39 (m, 6H), 7.66 (dd, 4H, *J* = 7.7, 1.3 Hz). *Trans* ^{13}C NMR (500 MHz, $CDCl_3$): 19.17, 23.11, 26.08, 26.83, 26.83, 26.83, 29.37, 63.64, 112.71, 122.20, 127.53, 129.46, 134.00, 135.45, 167.10.

Compound 3:



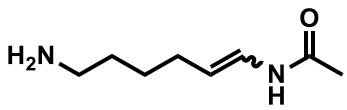
v. To an oven-dried, round-bottom flask under N_2 was added the *trans t*BDPS-protected enamide (2.26 g, 5.68 mmol, 1.0 equiv.) and THF (12 mL). TBAF (8.52 mL, 1.0 M in THF, 1.5 equiv.) was added dropwise *via* syringe under N_2, and the reaction was stirred at room temperature overnight. The solution was then filtered over a pad of silica gel that was pre-washed with 94:5:1 CH_2Cl_2/MeOH/Et_3N. After washing thoroughly, the combined organic solvent was concentrated under vacuum and purified by flash column chromatography (Rf = 0.25 in 94:5:1 CH_2Cl_2/MeOH/Et_3N). The *trans* hydroxy enamide (0.72 g, 4.55 mmol, 80% yield) was obtained as a white solid. *Trans* ^{1}H NMR (500 MHz, $CDCl_3$): 1.20 (br t, 1H, *J* = 5.1Hz), 1.37 (m, 2H), 1.50 (m, 2H), 1.51 (s, 3H), 1.95 (s, 3H), 1.99 (m, 2H), 3.58 (dt, 2H, *J* = 6.2, 5.1 Hz), 5.04

(dt, 1H, *J* = 14.3, 7.1 Hz), 6.68 (dd, 1H, *J* = 14.3, 10.6 Hz), 6.85 (br s, 1H). *Trans* ^{13}C NMR (500 MHz, $CDCl_3$): 23.10, 25.93, 29.36, 32.01, 62.56, 112.67, 122.73, 167.37.

vi. To an oven-dried, round-bottom flask under N_2 was added the *trans* hydroxy enamide (71 mg, 0.45 mmol, 1.0 equiv.) and anhydrous CH_2Cl_2 (5 mL). The solution was chilled to 0 ºC, and TosCl (260 mg, 1.36 mmol, 3.0 equiv.) and anhydrous pyridine (0.22 mL, 2.72 mmol, 6.0 equiv.) were added under N_2. The reaction mixture was warmed to room temperature, and stirred for 1 hour. The solvent was removed under vacuum, and the resulting yellow oil was taken up in 5 mL of CH_2Cl_2 and re-concentrated twice. The residue was purified by flash column chromatography (Rf = 0.45 in hexanes) to obtain the *trans* Tos-protected enamide (87 mg, 0.28 mmol, 64% yield) as a white solid. *Trans* ^{1}H NMR (500 MHz, $CDCl_3$): 1.39 (p, 2H, *J* = 7.6 Hz), 1.64 (m, 2H), 1.97 (dt, 2H, *J* = 10.9, 7.4 Hz), 2.01 (s, 3H), 2.45 (s, 3H), 4.01 (t, 2H, *J* = 6.3 Hz), 5.04 (dt, 1H, *J* = 14.2, 7.4 Hz), 6.68 (dd, 1H, *J* = 14.2, 10.2 Hz), 6.97 (br d, 1H, *J* = 10.2 Hz), 7.35 (d, 2H, *J* = 8.1 Hz), 7.78 (d, 2H, *J* = 8.2 Hz). *Trans* ^{13}C NMR (125 MHz, $CDCl_3$): 21.48, 22.88, 25.42, 27.99, 28.84, 70.38, 111.67, 123.03, 127.65, 132.72, 144.77, 167.47.

vii. To an oven-dried, round-bottom flask under N_2 was added the *trans* Tos-protected enamide (883 mg, 2.83 mmol, 1.0 equiv.) and DMF (10 mL). NaN_3 (386 mg, 5.67 mmol, 2.0 equiv.) was added under a stream of N_2, and the reaction vessel was sealed and heated to 80 ºC for 2 hours. The solution was cooled to room temperature and concentrated under vacuum. The resulting residue was taken up in 10 mL of CH_2Cl_2 and re-concentrated twice. The residue was then diluted in 10 mL of CH_2Cl_2 and washed with brine (3 x 50 mL), dried over Na_2SO_4, and concentrated under vacuum overnight to afford the *trans* azido enamide (**3**, 420 mg, 2.30 mmol, 81% yield) as a pale yellow oil. *Trans* ^{1}H NMR (500 MHz, $CDCl_3$): 1.45 (m, 2H), 1.60 (m,2H), 2.02 (s, 3H), 2.04 (m, 2H), 3.26 (t, 2H, *J* = 7.6 Hz), 5.09 (dt, 1H, *J* = 14.2, 7.0 Hz), 6.75 (dd, 1H, *J* = 14.2, 10.6 Hz), 6.99 (br s, 1H). *Trans* ^{13}C NMR (125 MHz, $CDCl_3$): 23.09, 26.85, 28.14, 29.11, 51.21, 111.93, 122.99, 167.24.

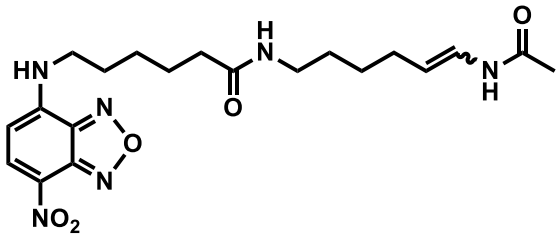
Compound 4:



viii. Zinc powder (8.11 mg, 0.12 mmol, 1.3 equiv.) was added to a solution of compound **3** (17.0 mg, 0.09 mmol, 1.0 equiv.) and ammonium chloride (11.6 mg, 0.22 mmol, 2.4 equiv.) in ethyl alcohol (248 µL) and water (84 µL), and the mixture was stirred vigorously at room temperature.

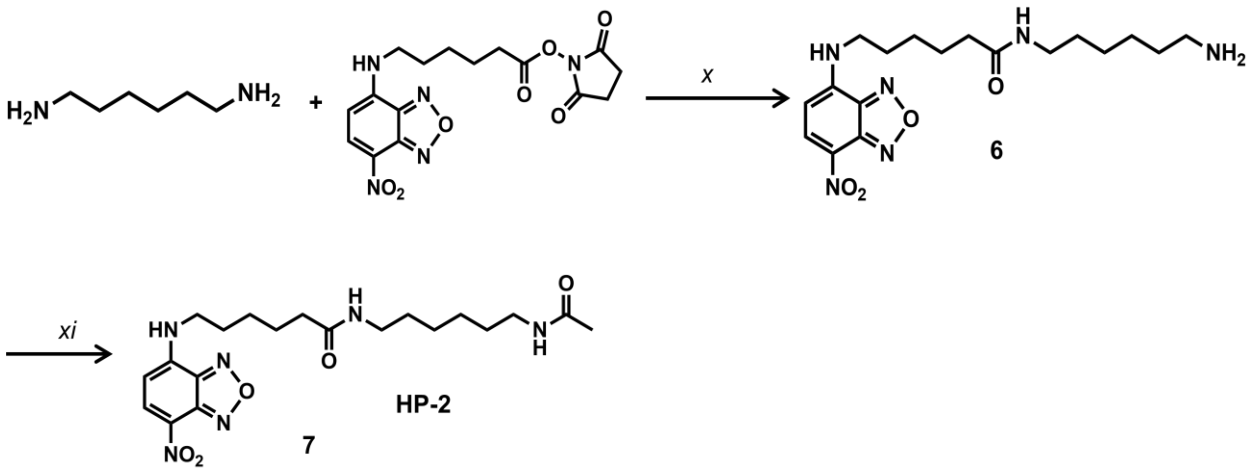
After completion of the reaction (2 hours, monitored by TLC), ethyl acetate (200 µL) and aqueous ammonia (10 µL) were added. The mixture was filtered, and the filtrate was washed with brine and dried over anhydrous Na_2SO_4. After removal of solvent under reduced pressure, the residue was purified by recrystallization with methylene chloride to give the corresponding amine, **4** (a mixture of *trans* and *cis* isomers in 1.5:1 ratio). *Trans* ^{1}H NMR (500 MHz, $CDCl_3$): 1.28 (m, 2H), 1.45 (m, 2H), 1.99 (s, 3H), 2.02 (m, 2H), 2.65 (t, 2H, *J* = 6.7 Hz), 5.08 (dt, 1H, *J* = 14.3, 7.1Hz), 6.70 (m, 1H), 7.11 (br s, 1H). *Trans* ^{13}C NMR (125 MHz, $CDCl_3$): 23.1, 26.7, 28.14, 29.0, 50.2, 110.0, 120.7, 167.24. *Cis* ^{1}H NMR (500 MHz, $CDCl_3$): 1.28 (m, 2H), 1.45 (m, 2H), 2.03 (s, 3H), 2.05 (m, 2H), 2.73 (t, 2H, *J* = 6.2 Hz), 4.72 (dt, 1H, *J* = 16.2, 7.8 Hz), 6.70 (m, 1H), 7.65 (br s, 1H). *Trans* ^{13}C NMR (125 MHz, $CDCl_3$): 23.6, 25.8, 27.1, 31.0, 51.6, 112.0, 123.7, 166.2.

Compound 5 (HP-1):



ix. NBD-SE (Succinimidyl 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoate) (25.0 mg, 0.064 mmol, 1.0 equiv.) and compound **4** (10 mg, 0.064 mmol, 1.0 equiv) were mixed with triethyl amine (28.0 µL, 0.2 mmol, 3.1 equiv.) in methylene chloride (3.0 mL). The mixture was stirred at room temperature overnight. Solvents were removed under vacuum, and the crude mixture was separated by flash column chromatography to obtain compound **5** (20.74 mg, 0.048 mmol, 75%) as a bright orange solid. *Trans* ^{1}H NMR (500 MHz, $CDCl_3$): 1.29 (m, 2H), 1.30 (m, 2H), 1.40 (m, 2H), 1.50 (m, 2H), 1.60 (m, 2H), 1.87 (s, CH_3), 2.10 (m, 2H), 2.22 (m, 2H), 3.00 (m, 2H), 3.4 (m, 2H), 5.10 (dt, 1H, *J* = 14.3, 7.1Hz), 6.80 (m, 1H), 7.10 (br s, 1H), 7.13 (m, 1H), 8.10 (m, 1H). *Cis* ^{1}H NMR (500 MHz, $CDCl_3$): 1.29 (m, 2H), 1.29 (m, 2H), 1.39 (m, 2H), 1.52 (m, 2H), 1.63 (m, 2H), 1.87 (s, CH_3), 2.13 (m, 2H), 2.20 (m, 2H), 3.02 (m, 2H), 3.4 (m, 2H), 4.98 (dt, 1H, *J* = 16.2, 7.8Hz), 7.05 (m, 1H), 7.11 (br s, 1H), 7.12 (m, 1H), 8.12 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): 23.5, 24.1, 25.0, 25.3, 27.0, 31.3, 33.4, 36.7, 38.8, 50.3, 97.8, 110.5, 122.2, 126.3, 132.1, 137.4, 139.6, 144.1, 167.7, 171.1. LCMS (m/z): 433.2 $(M+H)^+$

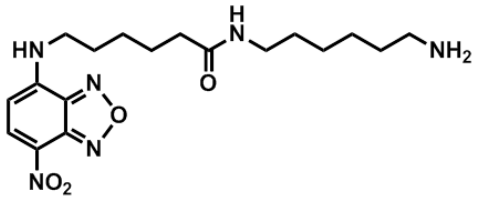
Synthetic procedures and characterization data for HP-2



Scheme S2: Synthesis of HP-2*.

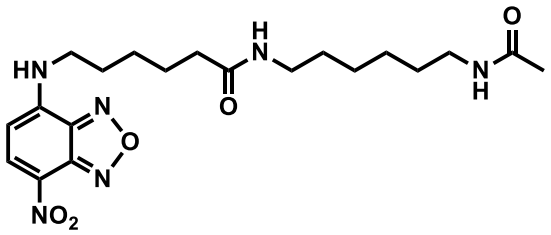
*Reagents and conditions: (*x*) CH_2Cl_2, RT, 3 h, 65% (*xi*) 1M NaOH, acetic anhydride, RT, overnight, 80%.

Compound 6 (DHP-2) :



x. Hexamethylenediamine (58.1 mg, 0.6 mmol, 1.0 equiv.) was dissolved in CH_2Cl_2 (2 mL) and cooled to 0 ºC. NBD-SE (25.0 mg, 0.06 mmol, 1.0 equiv.) was then dissolved in the hexamethylenediamine mixture, and the mixture was stirred at room temperature for 2 hours. Solvents were removed, and the crude mixture was purified by flash column chromatography to obtain compound **6** (15.29 mg, 0.039 mmol, 65%). ^{1}H NMR (500 MHz, $CDCl_3$): 1.29 (m, 2H), 1.29 (m, 2H), 1.30 (m, 2H), 1.30 (m, 2H), 1.51 (m, 2H), 1.52 (m, 2H), 1.60 (m, 2H), 2.11 (m, 2H), 2.23 (m, 2H), 3.00 (m, 2H), 3.4 (m, 2H), 7.13 (m, 1H), 8.10 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): 24.5, 26.1, 26.4, 29.1, 31.5, 32.7, 35.5, 43.0, 50.2, 97.8, 123.4, 132.4, 136.4, 139.5, 146.0, 172.5. LCMS (m/z): 393.2 $(M+H)^+$.

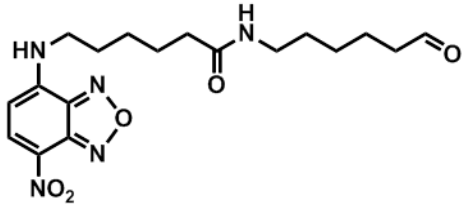
Compound 7 (HP-2):



xi. A mixture of compound **6** (30.0 mg, 0.08 mmol, 1.0 equiv.) in CH_2Cl_2 was made basic by adding 1 drop of 1 M NaOH. Acetic anhydride (72 µL, 0.80 mmol, 10 equiv.) was added, and the mixture was stirred at room temperature overnight. Solvents were removed and the residue was separated by flash column chromatography to obtain compound **7** (27.78 mg, 0.064 mmol, 80%) as a bright orange solid. ^{1}H NMR (500 MHz, $CDCl_3$): 1.29 (m, 2H), 1.29 (m, 2H), 1.30 (m, 2H), 1.30 (m, 2H), 1.51 (m, 2H), 1.52 (m, 2H), 1.60 (m, 2H), 1.80 (s, 3H), 2.11 (m, 2H), 2.23 (m, 2H), 3.00 (m, 2H), 3.4 (m, 2H), 7.13 (m, 1H), 8.10 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): 23.0, 24.5, 26.1, 26.4, 29.1, 31.5, 32.7, 35.5, 43.0, 50.2, 97.8, 123.4, 132.4, 136.4, 139.5, 146.0, 170.0, 172.5. LCMS (m/z): 435.2 $(M+H)^+$.

Synthesis of deacetylated HP-1 (DHP-1)

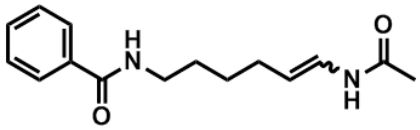
Compound 8:



Compound **5** (20.0 mg, 0.05 mmol) was dissolved in 2 mL of methanol and treated with 1M HCl (5 µL) and stirred at room temperature for 1 hour. The mixture was neutralized with 1 M NaOH, and the solvents were removed under vacuum. The obtained crude product was purified by column chromatography to obtain compound **8** (18.57 mg, 0.045 mmol, 95 %) as a bright yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): 1.29 (m, 2H), 1.30 (m, 2H), 1.40 (m, 2H), 1.50 (m, 2H), 1.60 (m, 2H), 1.87 (s, CH_3), 2.10 (m, 2H), 2.22 (m, 2H), 3.00 (m, 2H), 3.4 (m, 2H), 7.10 (br s, 1H), 7.13 (m, 1H), 8.10 (m, 1H), 9. 71 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): 23.5, 24.1, 25.0, 25.3, 27.0, 31.3, 33.4, 36.7, 38.8, 45.3, 97.8, 110.5, 122.2, 126.3, 132.1, 137.4, 139.6, 144.1, 167.7, 171.1, 201.1. LCMS (m/z): 392.2 $(M+H)^+$.

Synthesis of non-fluorescent benzoyl analogue of HP-1

Compound 9:



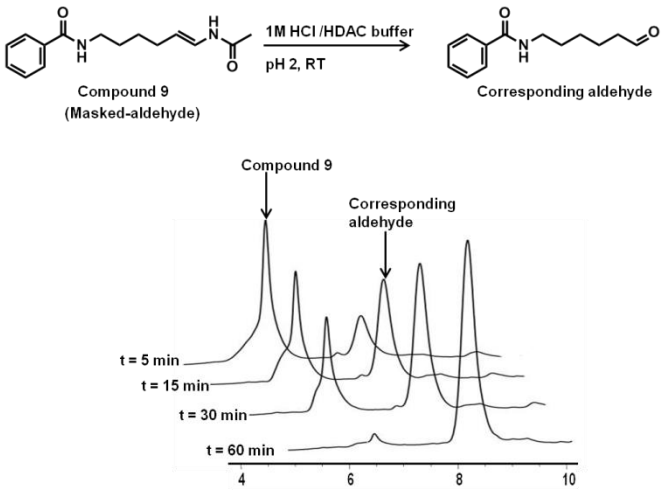
Benzoyl chloride (15.0 µL, 0.128 mmol, 1.0 equiv.) and compound **4** (20 mg, 0.128 mmol, 1.0 equiv.) were mixed with triethyl amine (14.0 µL, 0.1 mmol, 0.78 equiv.) in methylene chloride (3.0 mL). The mixture was stirred at room temperature overnight. Solvents were removed under vacuum and the resultant crude mixture was separated by flash column chromatography to obtain compound **9** (21.33 mg, 0.082, 64%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$): 1.38 (m, 2H), 1.63 (m, 2H), 1.91 (s, 3H), 2.13 (m, 2H), 3.45 (m, 2H), 5.10 (dt, 1H, *J* = 15, 7.5Hz), 7.01 (m, 1H), 7.45 (m, 2H), 7.56 (m, 1H), 7.75 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): 23.4, 24.5, 26.7, 31.0, 38.9, 110.5, 126, 127.2, 128.3, 129.0, 130.1, 132.4, 168.0, 170.1. LCMS (m/z): 261.2 $(M+H)^+$.

HDAC buffer preparation for biological assays

100mL 1M KCl, 50mL 1M HEPES, pH 7.4 (Gibco, 15630-114), 5mL 10% BSA (Invitrogen, P2489), and 20uL 50% Tween-20 (Zymed, 00-3005) were added to Milli-Q water to a final volume of 1L, and the pH was adjusted to 7.4 (final concentrations: HEPES = 50 mM, KCl = 100 mM, BSA = 0.05%, and Tween-20 = 0.001%). The buffer was stored in 45 mL aliquots at -80◦C.

Evaluation of the stability of the enamide (compound 9)

(a) Effect of time on deacetylation at pH 2



(b) Stability at pH 4-12

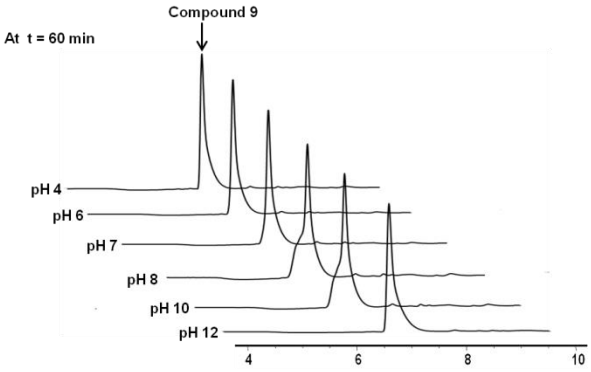
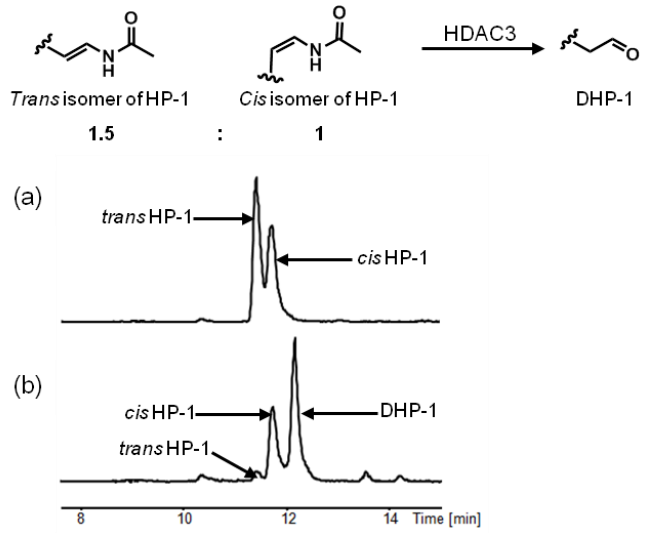


Figure S1. (a) HPLC analysis of acid hydrolysis of **9** over time at pH 2. (b) HPLC analysis of **9** after 60 min at pH 4, 6, 7, 8, 10 and 12.

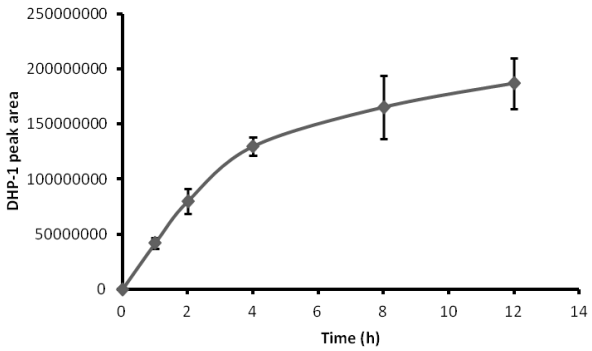
HDAC buffer was adjusted to pH 2, 4, 6, 7, 8, 10 or 12 with 1 M HCl. Compound **9** was dissolved in HDAC buffer at each pH to obtain 200 µL of a 20 µM solution. The solutions were kept at room temperature and 10 µL aliquots were analyzed at selected time points (t = 0, 5, 10, 15, 30 and 60 minutes). Experiments were carried out in duplicate and the hydrolysis was monitored by HPLC and LCMS. HPLC measurements were made using a gradient elution system composed of water and acetonitrile with 0.01% ammonium formate at a flow rate of 1

mL/min on an Agilent Eclipse XDB-C18 chromatographic column (4.6 mm x l50 mm). LCMS was performed with a gradient elution system composed of water and acetonitrile with 0.1% ammonium formate at a flow rate of 0.1 mL/min using an Agilent Eclipse XDB-C8 chromatographic column (5 µm particle size, 2.1mm x l50 mm).

LCMS characterization of HDAC enzymatic action on HP-1 and HP-2



(c) Deacetylation of HP-1 to DHP-1 over time



(d) Calculation of k_{obs}

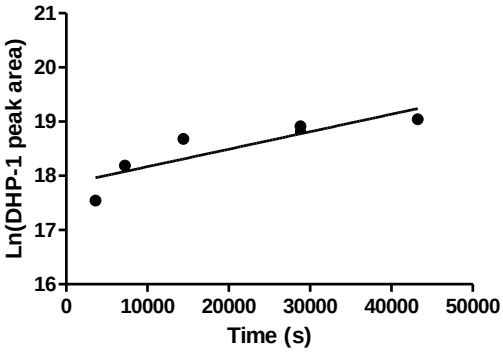


Figure S2. (a) LCMS analysis of 1.5:1 *trans* and *cis* isomer mixture of HP-1. (b) LCMS analysis of HP-1 isomer mixture deacetylation by HDAC3 enzyme. (c) Graph of the production of DHP-1 following HP-1 deacetylation by HDAC3 over 12 hours. (d) Graph of the natural log of DHP-1 formation versus time for calculation of the observed rate constant and the life time ($T_{1/2}$) of the enzyme-catalized reaction.

The enzymatic cleavage of HP-1 and HP-2 was analyzed by performing LCMS assays with HeLa nuclear extract (AnaSpec), HeLa whole cell lysate (Santa Cruz Inc.), and the following purified HDAC isoforms: 1, 2, 3, 6 and 8 (HDAC1, 3 and 8 from Cayman Chemicals and HDAC2 and 6 provided by Dr. Stephen Haggarty). Solutions containing (i) vehicle with DMSO in HDAC buffer, (ii) 5 µM probe in HDAC buffer, (iii) 5 µM probe and HDAC enzyme/HeLa nuclear extract/HeLa whole cell lysate in HDAC buffer, or (iv) 5 µM probe and HDAC enzyme/HeLa nuclear extract/HeLa whole cell lysate pre-incubated with 10 µM SAHA (Provided by Dr. Stephen Haggarty) in HDAC buffer were incubated for 12 hours at 37 ºC. Following incubation, a 20 µL aliquot of supernatant from each sample was analyzed by LCMS. Deacetylation of HP-1 and 2 was confirmed by detection of the $(M+H)^+$.ion following positive electrospray ionization. For HP-1, the $(M+H)^+$ ion for the corresponding aldehyde (**8**, DHP-1) was detected, which formed following reaction of the initial imine deacetylation product with water. For HP-2, the $(M+H)^+$.ion for the corresponding amine (**6**, DHP-2) was identified. The peak area for each detected compound was measured to determine the % deacetylation of each probe. All assay samples were analyzed in triplicate. LCMS measurements were performed with a gradient elution system (5-95%) composed of water and acetonitrile with 0.1% ammonium formate at a flow rate of 0.1 mL/min using an Agilent Eclipse XDB-C8 chromatographic column (5 µm particle size, 2.1mm x l50 mm).

LCMS analysis of HP-1 cleavage by HDAC3 enzyme over time and determination of the observed rate constant (k_{obs}) and the half life ($T_{1/2}$)

The rate of cleavage of HP-1 by purified HDAC3 was analyzed by performing a LCMS assay. Solutions containing 5 µM HP-1 and HDAC3 in HDAC buffer were incubated for 12 hours at 37 ºC. Following incubation, a 20 µL aliquot of supernatant from each sample was analyzed by LCMS at t = 0, 1, 2, 4, 8, and 12h. Deacetylation of HP-1 was confirmed by detection of the $(M+H)^+$ ion following positive electrospray ionization. For HP-1, the $(M+H)^+$ ion for the corresponding aldehyde (**8**, DHP-1) was detected, which formed following reaction of the initial imine deacetylation product with water. The peak area for each detected compound was measured to determine the % deacetylation of each probe. The observed rate constant was determined using Graphpad by plotting Ln(DHP-1 peak area) versus time. Assuming that this is a first order reaction, $T_{1/2}$ (where, $T_{1/2}= \ln 2/k_{obs}$) was estimated. All assay samples were analyzed in triplicate. LCMS measurements were performed with a gradient elution system (5-95%) composed of water and acetonitrile with 0.1% ammonium formate at a flow rate of 0.1 mL/min

using an Agilent Eclipse XDB-C8 chromatographic column (5 µm particle size, 2.1mm x l50 mm).

Characterization of sirtuin (HDAC Class III) and protease enzymatic action on HP-1

The enzymatic cleavage of HP-1 was analyzed by performing LCMS assays with sirtuin 1 and 3 (Cayman Chemicals) and the proteases chymotrypsin (Sigma-Aldrich), cathepsin (EMD Millipore), and pepsin* (Sigma-Aldrich). Solutions containing (i) vehicle with DMSO in HDAC buffer, (ii) 5 µM HP-1 in HDAC buffer, and (iii) 5 µM HP-1 and the enzyme in HDAC buffer were incubated for 12 hours at 37 ºC. Following incubation, a 20 µL aliquot of supernatant from each sample was analyzed by LCMS. Deacetylation of HP-1 was investigated by screening for the DHP-1 $(M+H)^+$ ion following positive electrospray ionization. All assay samples were analyzed in triplicate. LCMS measurements were performed with a gradient elution system (5-95%) composed of water and acetonitrile with 0.1% ammonium formate at a flow rate of 0.1 mL/min using an Agilent Eclipse XDB-C8 chromatographic column (5 µm particle size, 2.1mm x l50 mm).

*(Analysis for pepsin enzyme with HP-1 was carried out in HDAC buffer at pH 4).

Results: HP-1 was not altered by sirtuins 1 and 3 or the proteases chymotrypsin, cathepsin and pepsin, as determined by LCMS.

Positive controls were completed to verify that sirtuin 1 and 3, chymotrypsin, pepsin, and cathepsin B were active in the conditions used for the HP-1 cleavage assays. For sirtuins 1 and 3, the SIRTainty kit (EMD Millipore, Billerica, MA) was used, and the Pierce Protease Assay kit (Thermo Scientific, Rockford, IL) was used for chymotrypsin. For Cathepsin B, the cleavage of the enzyme substrate, z-RR-pNA (Santa Cruz Biotechnology, Dallas, TX) was monitored using a Wallac EnVision 2103 Multilabel fluorescence plate reader (PerkinElmer, Waltham, MA) with a 405 nm excitation filter, a 406 nm emission filter, and a gain of 150. The cleavage of the pepsin substrate, Ac-Phe-Tyr-OH (Chem-Impex Inc.), was monitored by LCMS at pH 4.

IC_{50} measurements

HP-1 and HP-2 IC_{50} values for HDAC1 were determined using the Trypsin–coupled assay as well as the Caliper endpoint assay. HP-1 and HP-2 IC_{50} values for HDAC2, HDAC3, HDAC6 and HDAC8 were determined with the Caliper endpoint assay.

Trypsin–coupled HDAC1 Inhibition assay:

In vitro HDAC1 end point enzymatic assays were performed in optimized 96-well format as previously described[3,4] with the following modifications. Reactions were performed in volume of 120 uL with 30 ng of full-length, recombinant HDAC1 (BPS Biosciences). TCEP was excluded from the assay buffer. HDAC1 was pre-incubated with varying concentrations of HP-1 or HP-2, or DMSO vehicle for 30 minutes. Fluorophore-conjugated acetyl-lysine tripeptide substrate was added at a concentration equivalent to the substrate K_m, 11 uM, and the deacetylation reaction was allowed to run for 45 minutes at RT. Reactions were terminated by 10 μM pan-HDAC inhibitor LBH-589 (Panobinostat) and 150 nM Trypsin (Worthington Biochemical). Fluorescence intensity of the aminomethylcoumarin liberated by deacetylase and trypsin enzymatic activity was monitored at 460 nm using a multilabel plate reader (EnVision, Perkin-Elmer) every 5 minutes until stable, about 25 minutes. Dose response curves were fitted from this end point signal in GraphPad Prism 5 (GraphPad Software Inc.) The background fluorescence intensity of HP-1 or HP-2 was found to be negligible relative to that of aminomethylcoumarin.

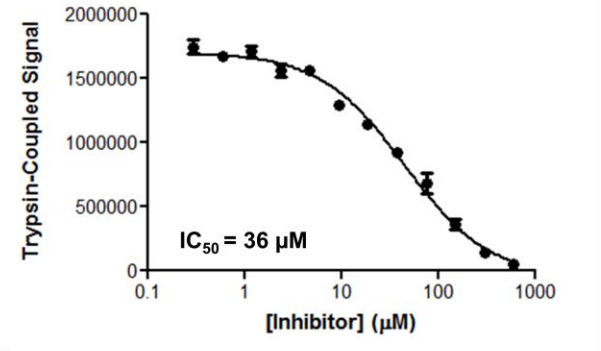
Caliper microfluidic endpoint IC_{50} assay

The Caliper microfluidic assay was performed at the Broad Institute (www.broadinstitute.org) exactly as previously described.[5]

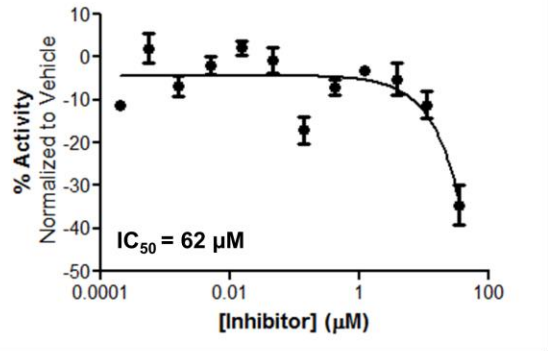
Table S1. Percent cleavage of HP-2 *in vitro* by HDAC enzymes as determined by LCMS.

Enzyme	Maximum % HP-2 to DHP-2	
	- SAHA	+ SAHA
HDAC1	6	0
HDAC2*	0	0
HDAC3	87	0
HDAC6	0	0
HDAC8	0	0

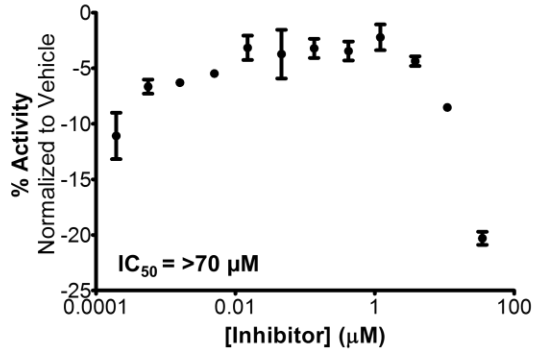
*HP-2 and DHP-2 were not detected



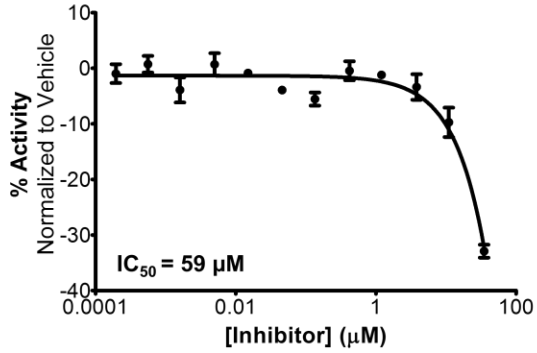
(a) HP-1 IC_{50} curve for HDAC1 by Trypsin-Coupled Assay



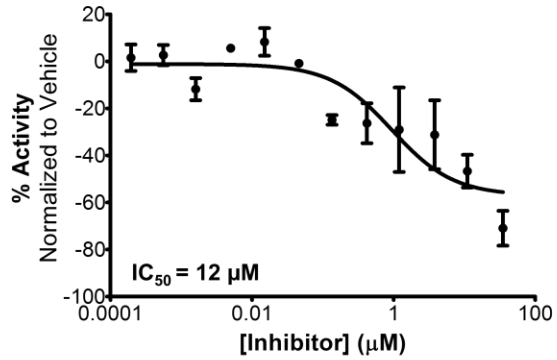
(b) HP-1 IC_{50} curve for HDAC1 by Caliper Assay



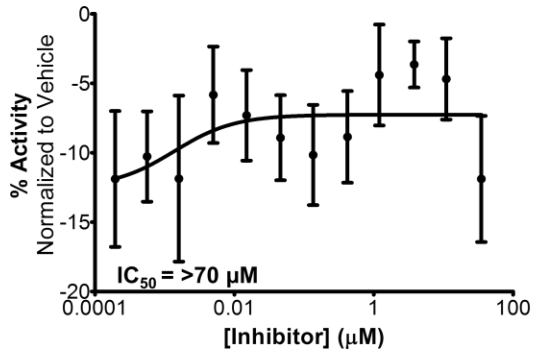
(c) HP-1 IC_{50} curve for HDAC2 by Caliper Assay



(d) HP-1 IC_{50} curve for HDAC3 by Caliper Assay

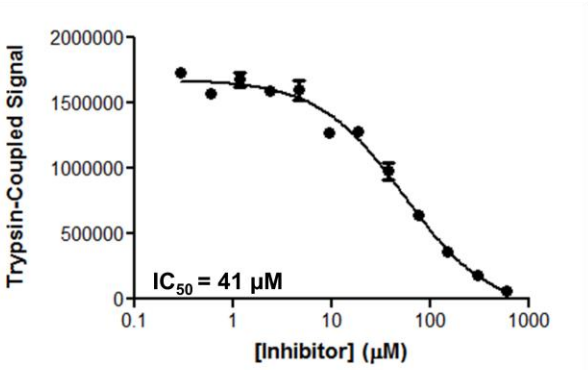


(e) HP-1 IC_{50} curve for HDAC6 by Caliper Assay

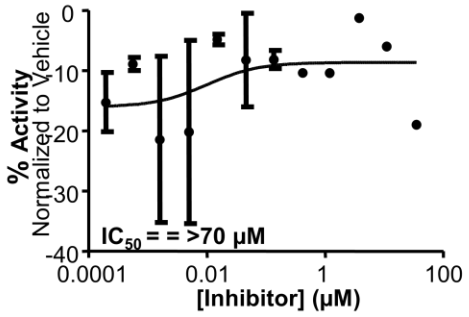


(f) HP-1 IC_{50} curve for HDAC8 by Caliper Assay

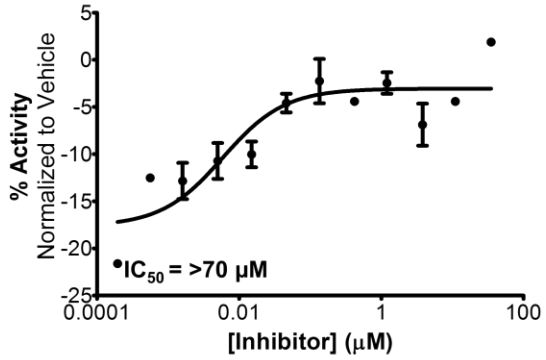
Figure S3. Representative IC_{50} curves for HP-1 and HDAC enzymes using Trypsin-coupled assay and Caliper assay.



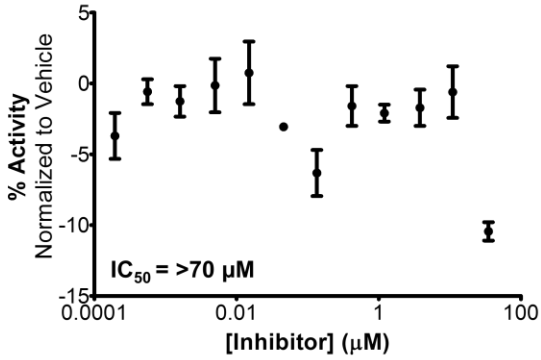
(a) HP-2 IC_{50} curve for HDAC1 by Trypsin-Coupled Assay



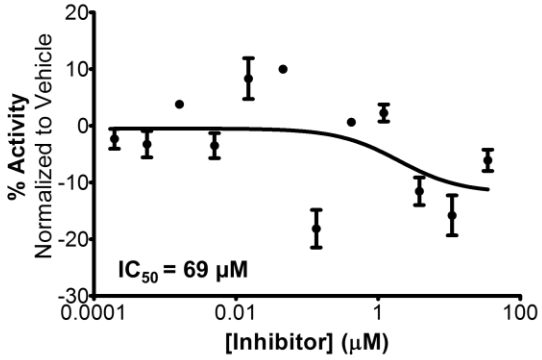
(b) HP-2 IC_{50} curve for HDAC1 by Caliper Assay



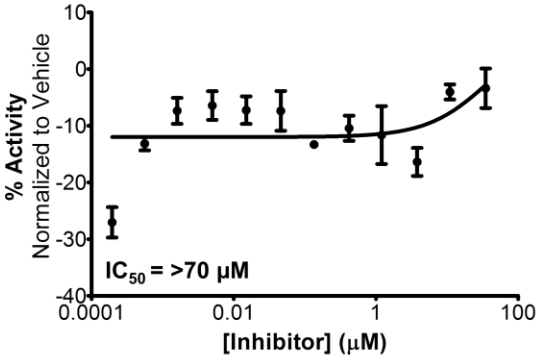
(c) HP-2 IC_{50} curve for HDAC2 by Caliper Assay



(d) HP-2 IC_{50} curve for HDAC3 by Caliper Assay



(e) HP-2 IC_{50} curve for HDAC6 by Caliper Assay



(f) HP-2 IC_{50} curve for HDAC8 by Caliper Assay

Figure S4. Representative IC_{50} curves for HP-2 and HDAC enzymes using Trypsin-coupled assay and Caliper assay.

HP-1 deacetylation and protein binding assay

Solutions containing HP-1 (20 µM) in 30 µL HDAC buffer with 5% DMSO were incubated at 37 ºC for four hours in the presence or absence of HDAC3 (3.6 µM). After incubation with HDAC3, $NaCNBH_3$ (1.4 mM) or vehicle (H_2O) and BSA (6 mg/mL) or vehicle (HDAC buffer), were added to the solutions prior to an additional incubation at 37 ºC for two hours (all reactions were run in triplicate). Following the second incubation, the final solutions (50 µL) were added to G-25 columns (GE Healthcare, Buckinghamshire, UK) pre-equilibrated with milliQ water. The columns were then centrifuged at 700 × g for 60 seconds before addition of milliQ water (50 µL). The columns were again centrifuged at 700 × g for 60 seconds, and the eluent was combined with the eluent from the first spin to make fraction 1. Following this, 11 samples of water (100 µL each) were added to the columns, the columns were centrifuged as above, and the eluent was collected separately to obtain fractions 2-12. 75 µL of each fraction was transferred to a well in a 96-well, black, clear-bottom plate (Corning Incorporated, Corning, NY) and the fluorescence was detected using an IVIS Spectrum (Caliper, Hopkinton, MA). To obtain the fluorescence signals, the 465 nm excitation filter, 530 nm emission filter, and a 5 second exposure were used. For analysis, the total photon flux over the area of each well was determined.

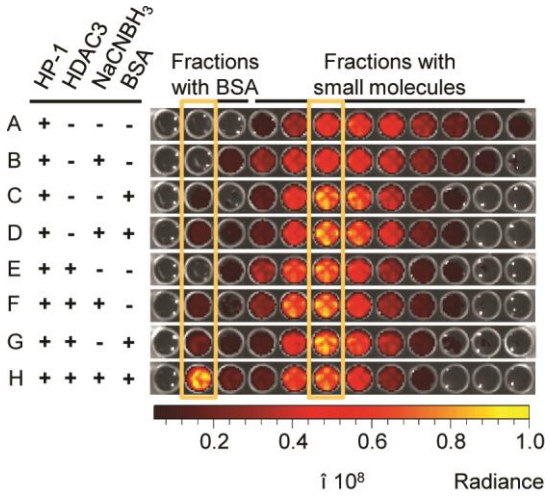


Figure S5. Representative images of fluorescence from fractions collected following gel filtration chromatography of reactions A-H (A: HP-1; B: HP-1 and NaCNBH3; C: HP-1and BSA; D: HP-1, NaCNBH3, and BSA; E: HP-1 and HDAC3; F: HP-1, HDAC3, and NaCNBH3; G: HP-1, HDAC3, and BSA; H: HP-1, HDAC3, NaCNBH3, and BSA). Columns with fractions 2 and 6, which were used to calculate the F2/F6 ratio for Figure 2, are outlined in yellow.

Imaging HDAC activity in HeLa cells with HP-1

HeLa cell culture and treatment with HP-1 and HP-2

HeLa cells (ATCC) were grown as a monolayer in Eagles Minimum Essential Medium (EMEM, GIBCO, BRL, Gaithersburg, MD) with 10% Fetal Bovine Serum (FBS, GIBCO, BRL, Gaithersburg, MD) and 1% penicillin/streptomycin (100mg/mL). All cell culture dishes were maintained in a humidified atmosphere with 5% CO_2 at 37 ºC.

Determination of HDAC activity in HeLa cells by LC-MS

HDAC activity in HeLa cells was analyzed by performing an LCMS assay. Solutions of HP-1 and HP-2 (200 mM each) were prepared in DMSO and diluted in HDAC buffer to a final concentration of 100 µM. SAHA solutions (100 mM) were prepared in DMSO and diluted in HDAC buffer to make a 100 µM solution. All solutions were prepared immediately prior to application to the cells. HeLa cells plated in 600 mL cell culture flasks, were treated with HP-1 or 2 with ± SAHA so that the final concentrations of HP-1 and HP-2 were 5 µM in HDAC buffer (with 0.01% DMSO) ± 10 µM SAHA. Cells were then incubated at 37 °C for t = 1, 2, 4, 8, 12 and 24 h. Following incubation, the medium was removed and the cells were washed three times with DPBS buffer. Cells were scraped off of the flask, lysed in milipore water (90 µL) using a mechanical homogenizer, centrifuged (at 14,000xg for 2 min) and 20 µL aliquot of supernatant from each sample was analyzed by LCMS. Cleavage and accumulation was confirmed by detection of the $(M+H)^+$ ion following positive electrospray ionization. The peak area for each detected compound was measured to determine the % conversion. For HP-1, the corresponding $(M+H)^+$ ion was detected. For HP-2, the $(M+H)^+$ ions for HP-2 and its corresponding amine (**6**, DHP-2) were identified. The peak area for each detected compound was measured to determine the % conversion of each probe. LCMS measurements were performed with a gradient elution system (5-95%) composed of water and acetonitrile with 0.1% ammonium formate at a flow rate of 0.1 mL/min using an Agilent Eclipse XDB-C8 chromatographic column (5 µm particle size, 2.1mm x l50 mm).

Determination of the fraction of protein-bound probe in HeLa cells by fluorescence

HDAC activity in HeLa cells was analyzed by performing IVIS analysis. Solutions of HP-1 and HP-2 (200 mM each) were prepared in DMSO and diluted in HDAC buffer to a final concentration of 100 µM. SAHA solutions (100 mM) were prepared in DMSO and diluted in

HDAC buffer to make a 100 µM solution. All solutions were prepared immediately prior to application to the cells. HeLa cells plated in 600 mL cell culture flasks were treated with HP-1 with ± SAHA so that the final concentration of HP-1 was 5 µM in HDAC buffer (with 0.01% DMSO) ± 10 µM SAHA. The cells were incubated at 37 °C for 24 h. Following incubation, the medium was removed and the cells were washed three times with DPBS buffer. Cells were scraped off of the flask and lysed in milipore water (90 µL) using a mechanical homogenizer. 90 µL from each lysed sample was added to a Micron centrifugal 30K filter device (Millipore Ireland Ltd. Tullagree Carrigtwohill Co. Cork, Ireland). Each device wascentrifuged at 14000 × g for 10 minutes. 75 µL aliquots of each concentrate fraction and corresponding original sample (before concentrating) were transferred to a 96-well, black, clear-bottom plate (Corning Incorporated, Corning, NY) and the fluorescence was detected using an IVIS Spectrum (Caliper, Hopkinton, MA). To obtain the fluorescence signals, the 465 nm excitation filter, 530 nm emission filter, and a 1 second exposure were used. For analysis, the total photon flux over the area of each well was determined.

Imaging HDAC activity in HeLa cells with HP-1 and 2

An acid-washed, poly-lysine-treated sterile glass cover slip was added to each well of 6-well plate, and HeLa cells were plated at a seeding density of ~2.5 × 10^5 cells/mL in 2 mL of growth medium. After 24 hours, the cells reached 80-85% confluence. For all experiments, solutions of HP-1 and HP-2 (200 mM each) were prepared in DMSO and diluted in HDAC buffer to a final concentration of 100 µM. SAHA solutions (100 mM) were prepared in DMSO and diluted in HDAC buffer to make a 100 µM solution. All solutions were prepared immediately prior to application to the cells. Cells were washed with HDAC buffer and treated with HP-1, HP-1 with SAHA, HP-2 or HP-2 with SAHA so that the final concentrations of HP-1 and HP-2 were 5 µM in HDAC buffer (with 0.01% DMSO). Cells were treated with SAHA (10 µM in HDAC buffer with 0.01 %DMSO) or vehicle (HDAC buffer with 0.01%DMSO) 15 min prior to treatment with HP-1 or HP-2. After HP-1 or HP-2 was added, the cells were incubated at 37 °C for 2 hours. Following incubation, the medium was removed and the cells were washed three times with 2 mL HDAC buffer per well. The buffer was removed and 2 mL 4% paraformaldehyde in PBS was added to each well and incubated for 20 minutes at 4 ºC to fix the cells. The fixative was removed and cells were gently washed twice with 2 mL DPBS and twice with 2 mL deionized water. A drop of Gel Mount (anti-fade with DAPI nuclear stain) wasadded to microscope slides, and the cover glasses containing HeLa cells were carefully transferred to the microscope slides. After the slides dried overnight in a dark drawer, they were imaged as described below.

Confocal fluorescence image acquisition and analysis

Confocal fluorescence imaging was performed with a Zeiss laser scanning microscope 710 with a 63× objective lens and Zen 2009 software (Carl Zeiss). HP-1 and HP-2 were excited using a 488 nm Ar laser, and emission was collected using a META detector between 500 and 650 nm. DAPI was excited with a 405 nm diode laser, and emission was collected using a META detector between 450 and 500 nm. One representative image from each coverslip was collected. Each experimental condition was run in triplicate in each of three independent experiments, for a total n of 9 per treatment. The mean fluorescence intensity of 10 cells per coverslip was measured using ImageJ software (http://rsbweb.nih.gov/ij/). Cells were defined using a free-form selection tool using the brightfield image as a guide. The mean background signal was also measured and subtracted from the mean fluorescence signal within the cells. Mean fluorescence intensities and standard deviations were plotted in Microsoft Excel.

Table S2. Hela cell nuclear and whole cell lysate-induced HP-1 and HP-2 deacetylation

Lysate	HP-1 to DHP-1		HP-2 to DHP-2	
	- SAHA	+ SAHA	- SAHA	+ SAHA
HeLa nuclear extract	+	-	+	-
HeLa whole cell	+	-	+	-

References

1. Huang, X.; Shao, N.; Palani, A.; Aslanian, R., Oxidative entry to α-oxy< i> N</i>-acyl aminals and hemiaminals: efficient formation of 2-(< i> N</i>-acylaminal) substituted tetrahydropyrans. *Tetrahedron letters* 2007, *48* (11), 1967-1971.
2. Jiang, L.; Job, G. E.; Klapars, A.; Buchwald, S. L., Copper-catalyzed coupling of amides and carbamates with vinyl halides. *Organic letters* 2003, *5* (20), 3667-3669.
3. Bradner, J. E.; West, N.; Grachan, M. L.; Greenberg, E. F.; Haggarty, S. J.; Warnow, T.; Mazitschek, R., Chemical phylogenetics of histone deacetylases. *Nature chemical biology* 2010, *6* (3), 238-243.
4. Fass, D. M.; Shah, R.; Ghosh, B.; Hennig, K.; Norton, S.; Zhao, W.-N.; Reis, S. A.; Klein, P. S.; Mazitschek, R.; Maglathlin, R. L., Short-chain HDAC inhibitors differentially affect vertebrate development and neuronal chromatin. *ACS Medicinal Chemistry Letters* 2010, *2* (1), 39-42.
5. Wagner, F. F.; Olson, D. E.; Gale, J. P.; Kaya, T.; Weïwer, M.; Aidoud, N.; Thomas, M.; Davoine, E. L.; Lemercier, B. C.; Zhang, Y.-L., Potent and selective inhibition of Histone Deacetylase 6 (HDAC6) does not require a surface-binding motif. *Journal of medicinal chemistry* 2013.