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SUPPORTING INFORMATION

Real-Time Detection of Histone Deacetylase Activity with a Small-Molecule Fluorescent and Spectrophotometric Probe

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1. Supporting figures and tables

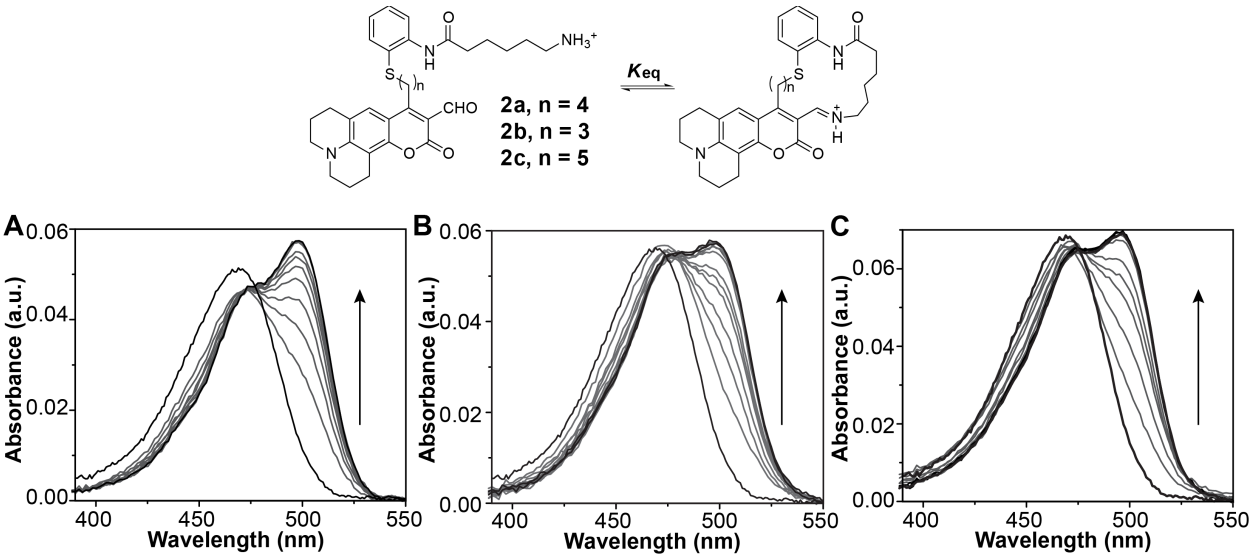


Figure S1. Changes in absorption spectra observed during intramolecular imine formation in 50 mM HEPES, 100 mM KCl buffer at pH 8.0 and 37 °C, starting with 2.1 – 2.7 μM samples of amine **2a** (A), **2b** (B) or **2c** (C). Amines were obtained from quantitative deprotection of Boc-protected compounds **3a**, **3b**, and **3c**, respectively.

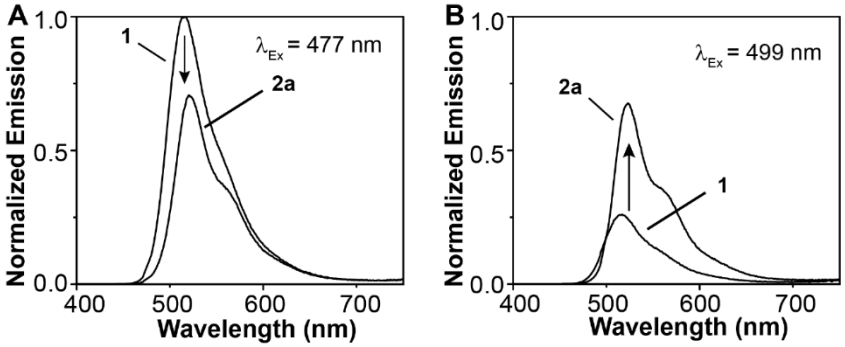


Figure S2. Comparison of fluorescence emission spectra obtained upon excitation at 477 nm (A) and 499 nm (B) of 2.2 μM acetylated probe **1** and chemically-generated amine **2a** after equilibration (10 minutes). Spectra were collected in 50 mM HEPES, 100 mM KCl at pH 8.0 and 37 °C.

Table S1. Photophysical properties of HDAC activity probe **1** and equilibrated mixture of deacetylated product **2a** obtained from deprotection of Boc-protected model compound **3a**.[a,b]

Compound	ε_{471} ($\times 10^3$ $M^{-1}cm^{-1}$)	ε_{477} ($\times 10^3$ $M^{-1}cm^{-1}$)	ε_{499} ($\times 10^3$ $M^{-1}cm^{-1}$)	ε_{510} ($\times 10^3$ $M^{-1}cm^{-1}$)	Φ_{477}[c]	Φ_{499}[c]
Probe **1**	24.4 (7)	22.6 (7)	6.0 (2)	1.9 (2)	0.54 (3)	0.53 (4)
Equilibrated **2a**	22.6 (9)	23.3 (5)	28 (1)	20.7 (8)	0.27 (3)	0.21 (3)

[a]Measurements conducted in 50 mM HEPES, 100 mM KCl, pH 8.0 at 37 °C. [b]Values in parenthesis correspond to the uncertainty in the last significant figure. [c]Fluorescein in 0.1 N NaOH ($\Phi_{492} = 0.95$) was used as a standard.

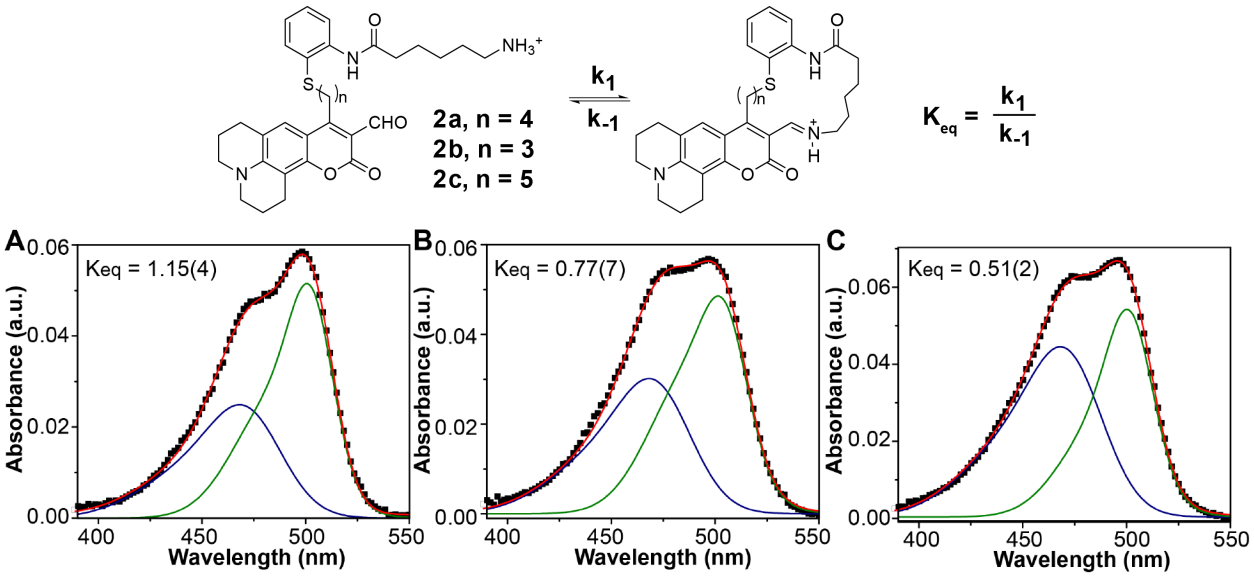


Figure S3. Deconvolution of absorption spectra (black squares) of 2-2.5µM samples of **2a** (A), **2b** (B), and **2c** (C) after equilibration, showing the individual contributions of the free amine (blue line) and the imine (green line) species to the overall absorption (red line). Spectra were collected in 50 mM HEPES, 100 mM KCl at pH 8.0 and 37 °C.

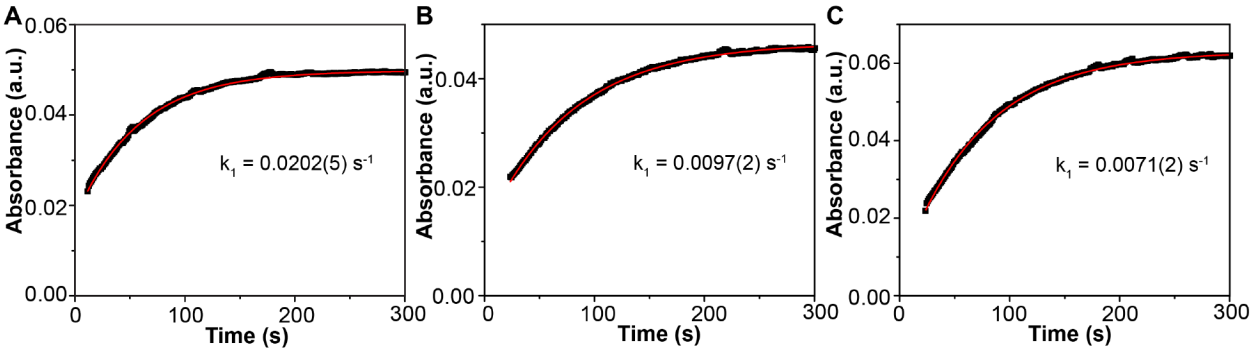


Figure S4. Representative plots of changes in absorption at 499 nm as a function of time for 1.5 -2 µM samples of amines **2a** (A), **2b** (B), and **2c** (C) following a pH jump. Samples were generated by quantitative deprotection of the corresponding *t*-butyl carbamates (**3a-c**) under acidic conditions, followed by dilution in 50 mM HEPES, 100 mM KCl at pH 8.0, 37 °C. Data were fitted to an integrated rate law for a reversible first-order reaction (approach to equilibrium, red line). Values of the kinetic rate constant k_1 for the forward reaction correspond to averages of three replicas.

Table S2. Thermodynamic and kinetic parameters of the intramolecular imine formation step for deacetylated probes with various linker lengths.[a,b]

Alkyl chain length (n)	K_{eq}	k_1 ($\times 10^{-3}$ s^{-1})	k_{-1} ($\times 10^{-3}$ s^{-1})
3	0.77(7)	9.7(2)	12.6(1)
4	1.15(4)	20.2(5)	17.54(4)
5	0.51(2)	7.1(2)	14.06(5)

[a]Samples of pure amines were obtained from quantitative acid cleavage of Boc-protected model compounds **3a-c**. [b]All measurements conducted in 50 mM HEPES, 100 mM KCl aqueous buffer, pH 8.0 at 37 °C.

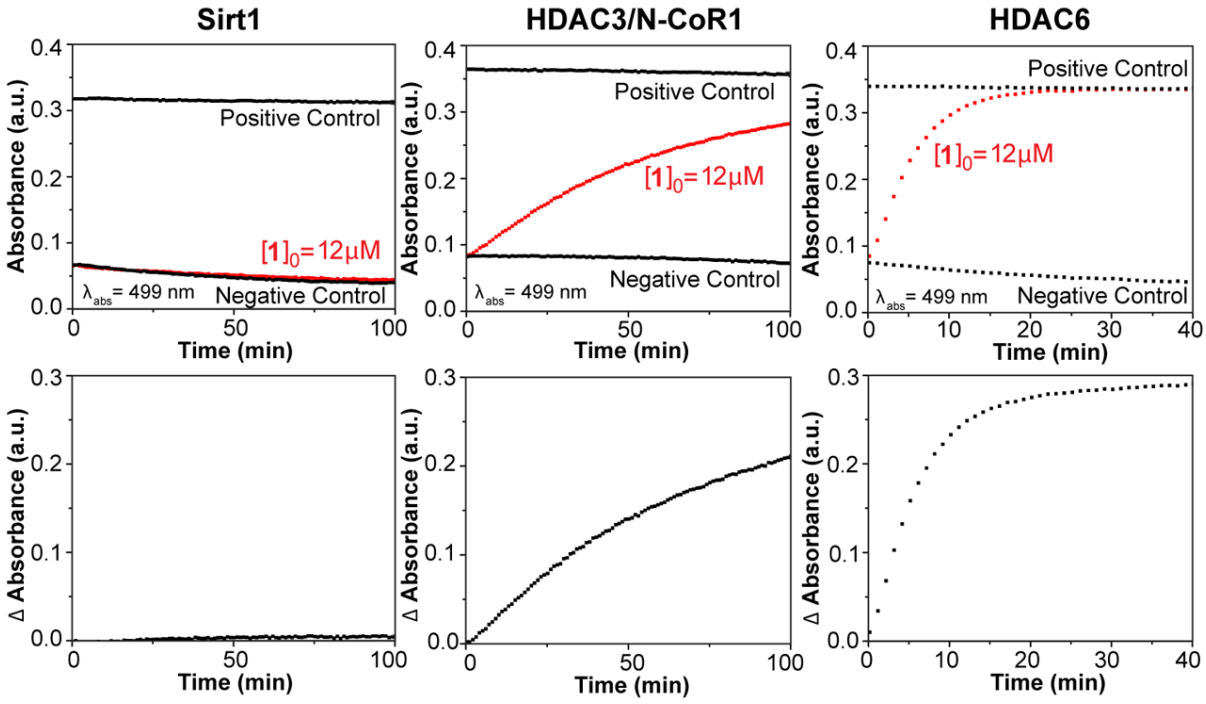


Figure S5. Deacetylation of probe **1** by samples of purified deacetylases at pH 8.0 and 37 °C, monitored by absorption. Top: absorbance (λ_{abs} = 499 nm) as a function of time for probe **1** (red), with corresponding negative and positive controls (black). Bottom: changes in absorption at 499 nm resulting solely from product formation, determined by subtraction of the corresponding negative control.

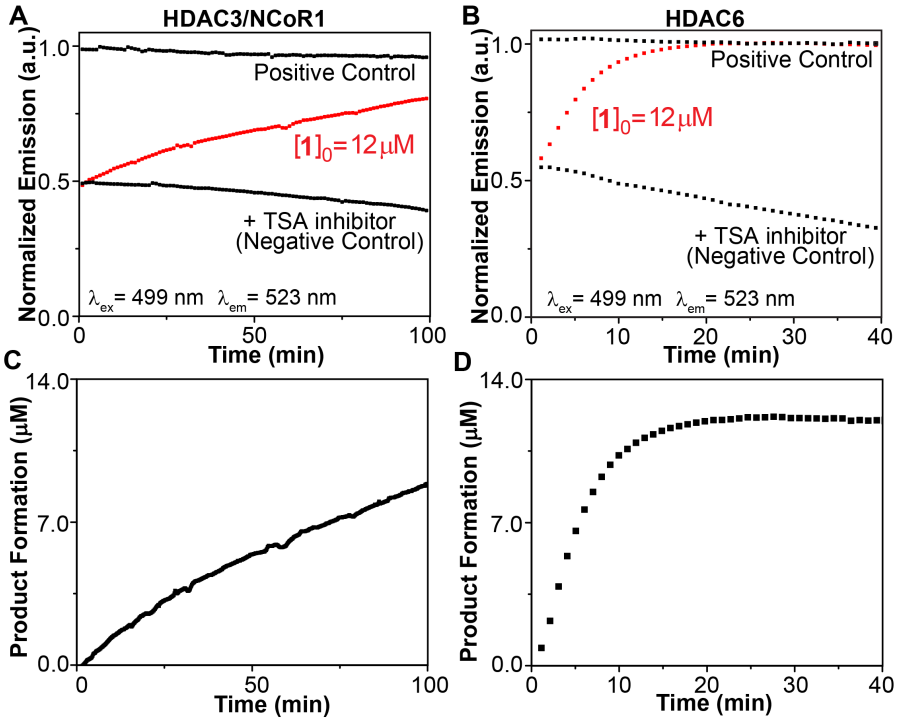


Figure S6. Deacetylation of probe **1** by purified deacetylases at pH 8.0 and 37 °C, monitored by fluorescence. Top: Normalized fluorescence emission (λ_{ex} = 499 nm, λ_{em} = 523 nm) as a function of time for probe **1** (red) and corresponding negative and positive controls (black). Bottom: Product formation as a function of time.

Table S3. Emission fluorescence and absorption Z' factors for assays in diluted HeLa nuclear extract, purified HDAC6, and purified HDAC3/NCOR1.[a,b]

Conditions	Absorption Z'	Fluorescence Z'
HeLa nuclear extract	0.95[c]	0.85[e]
HDAC6	0.93[d]	0.81[e]
HDAC3/NCOR1	0.88[d]	0.82[e]

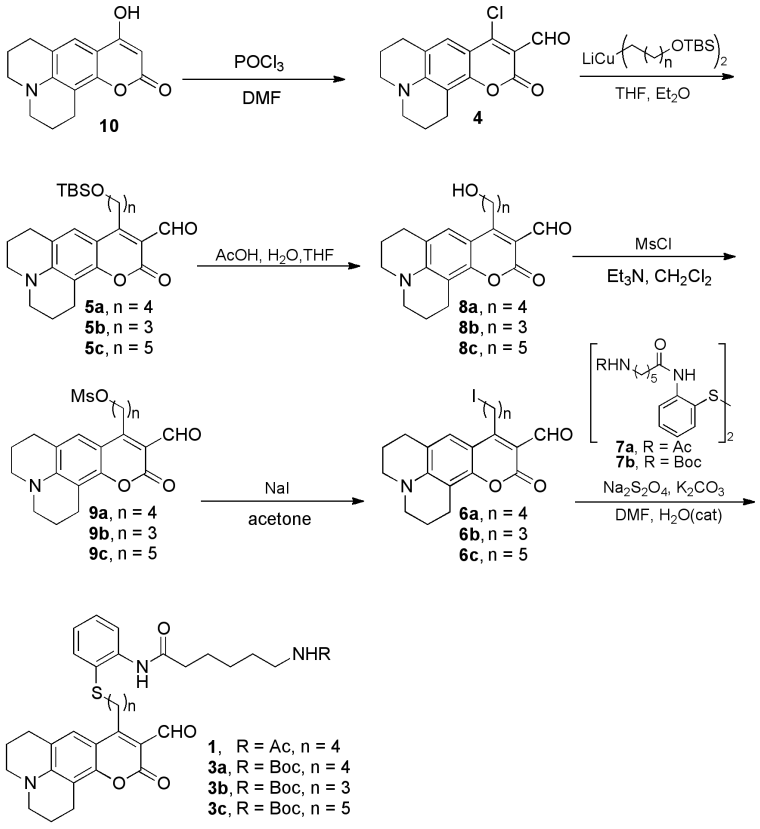
[a]All measurements conducted in 50 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$ aqueous buffer, pH 8.0 at 37 °C. [b]Z' values determined from triplicate measurements. [c]Z' value determined at λ_{abs} = 510 nm. [d]Z' value determined at λ_{abs} = 499 nm. [e]Z' value determined at λ_{ex} = 499 nm and λ_{em} = 523 nm.

2. Experimental Procedures

2.1. Synthetic protocols

General Materials and Synthetic Methods:
Hydroxycoumarin **10**,[1] 1-*tert*-butyldimethylsilyloxy-4-bromobutane,[2] and 1-*tert*-butyldimethylsilyloxy-5-bromopentane[3] were synthesized according to reported procedures. Other reagents were obtained from commercial sources and used as received. NMR spectroscopic data were obtained on Bruker AVANCE-400 NMR or Bruker AVANCE III-600 NMR spectrometers. ^{1}H NMR chemical shifts are reported in ppm relative to $SiMe_4$ ($\delta = 0$) and were referenced internally with respect to residual protio impurity in the solvent (δ 7.26 for $CHCl_3$). ^{13}C NMR chemical shifts are reported in ppm relative to $SiMe_4$ ($\delta = 0$) and were referenced internally with respect to the solvent signal (δ 77.16 for $CDCl_3$). Low-resolution mass spectrometry was conducted on an Agilent 1100 Series LCMSD VL system with single quadrupole detector. High-resolution mass spectra (HRMS) were acquired on an Agilent 6224 Accurate-Mass TOF LC/MS using ES ionization. Chromatograms were obtained on Agilent 1260 Infinity HPLC equipped with diode array and fluorescence detectors.

Scheme S1



Synthesis of compound 4.
A solution of compound **10** (1.99 g, 7.73 mmol) in dry DMF (5.5 mL) was treated with $POCl_3$ (2 mL, 22 mmol) added over 5 minutes under nitrogen atmosphere. After 1 hour at room temperature, the solution was diluted with CH_2Cl_2 (100 mL) and quenched with saturated

aqueous $NaHCO_3$ (100 mL), stirring until gas evolution ceased. The resulting mixture was then transferred to a separatory funnel and the aqueous layer was discarded. The organic layer was washed with saturated aqueous $NaHCO_3$ (2x50 mL), brine (50 mL), and dried over Na_2SO_4. The solvent was removed *in vacuo* to obtain the product formylcoumarin **4** as a red solid (2.18 g, 7.18 mmol, 92%). R_f = 0.3 (1:1 ethyl acetate/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.26 (s, 1H), 7.42 (s, 1H), 3.40-3.35 (m, 4H, -C*H*$_2$-), 2.86 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.79 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.01 – 1.96 (m, 4H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 187.4, 160.4, 153.7, 151.6, 149.8, 125.1, 120.5, 109.8, 107.5, 105.7, 50.6, 50.1, 27.7, 21.1, 20.2, 20.1. ESI-MS (*m/z*): $[M + H]^+$ calcd for $C_{16}H_{14}ClNO_3$ 304.1; found 304.1.

General procedure for the synthesis of compounds 5a-c.
A solution of TBDMS-protected bromoalcohol (2 equiv) in dry ether (0.1M) at -78 °C was treated with *tert*-butyllithium (1.7 M in hexanes, 5 equiv), added slowly under inert atmosphere. The resulting clear solution was stirred at -78 °C for 5 minutes and then allowed to warm up to room temperature over the course of ~2 hours. The organolithium solution was cooled to 0 °C and transferred via cannula to a second flask containing CuI (1.2 equiv) at 0 °C under inert atmosphere. The resulting dark purple solution was stirred at 0 °C for 10 minutes. The reaction was further cooled to -78 °C and dry THF (20 mL/mmol of **4**) was added. After stirring at -78 °C for 30 minutes, the reaction was warmed to -20 °C and compound **4** (1 equiv) was added. The mixture was stirred at -20 °C for 3 hours and allowed to warm to 0 °C for an additional 30 minutes. After this period, the reaction was quenched slowly with aqueous NH_4Cl. The aqueous layer was extracted three times with CH_2Cl_2, and the combined organic layers were dried over Na_2SO_4. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on silica gel (ethyl acetate/hexanes).

Compound 5a. Orange solid (0.55 g, 1.21 mmol, 29%), starting from 1.3 g (4.1 mmol) of compound **4**. R_f = 0.6 (1:2 ethyl acetate/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.33 (s, 1H), 7.19 (s, 1H), 3.66 (t, J = 6 Hz, 2H, -*CH*$_2$OTBS), 3.65 – 3.31 (m, 4H, -C*H*$_2$-), 3.22 (t, J = 8 Hz, 2H, -C*H*$_2$-), 2.86 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.76 (t, J = 6 Hz, 2H, (-C*H*$_2$-)$_2$), 2.01 – 1.94 (m, 4H, -C*H*$_2$-), 1.71 (quin, J = 7 Hz, 2H, -C*H*$_2$-), 1.65 – 1.58 (m, 2H, -C*H*$_2$-), 0.876 (s, 9H, -SiMe$_2$*^{t}Bu*), 0.041 (s, 6H, -Si*Me*$_2$^{t}Bu). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 191.1, 163.8, 163.7, 152.7, 148.6, 124.5, 119.5, 110.8, 108.4, 106.2, 62.9, 50.3, 49.9, 33.2, 27.9, 27.5, 27.2, 26.1, 21.4, 20.4, 20.3, 18.4, -5.1. ESI-MS (*m/z*): $[M + Na]^+$ calcd for $C_{26}H_{37}NO_4Si$ 478.2; found 478.1.

Compound 5b. Orange solid (0.63 g, 1.42 mmol, 40%), starting from 1.1 g (3.7 mmol) of compound **4**. R_f = 0.6 (1:2 ethyl acetate/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.32 (s, 1H), 7.29 (s, 1H), 3.76 (t, J = 6 Hz, 2H, -C*H*$_2$OTBS), 3.35-3.25 (m, 4H, -C*H*$_2$-), 3.27 (t, J = 8 Hz, 2H, -C*H*$_2$-), 2.86 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.75 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.00 – 1.93 (m, 4H, -C*H*$_2$-), 1.79 – 1.72 (m, 2H, -C*H*$_2$-), 0.93 (s, 9H, -SiMe$_2$*^{t}Bu*), 0.084 (s, 6H, -Si*Me*$_2$^{t}Bu). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 191.1, 163.8, 163.7, 152.7, 148.6, 124.8, 119.5, 111.0, 108.5, 106.2, 63.0, 50.4, 49.9, 33.6, 27.9, 26.2, 24.6, 21.4, 20.4, 20.3, 18.5, -5.1. ESI-MS (*m/z*): $[M + Na]^+$ calcd for $C_{25}H_{35}NO_4Si$ 464.2; found 464.2.

Compound 5c. Orange solid (0.4 g, 0.9 mmol, 60%), starting from 0.45 g (1.5 mmol), of compound **4**. R_f = 0.6 (1:2 ethyl acetate/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.33 (s, 1H), 7.18 (s, 1H), 3.62 (t, J = 6 Hz, 2H, -C*H*$_2$OTBS), 3.35-3.31 (m, 4H, -C*H*$_2$-), 3.21 (t, J = 7 Hz, 2H,

-C*H*$_2$-), 2.87 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.78 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.00 – 1.96 (m, 4H, (-C*H*$_2$-), 1.60 – 1.52 (m, 6H, -C*H*$_2$-), 0.88 (s, 9H, -SiMe$_2$*t*-*Bu*), 0.04 (s, 6H, -Si*Me*$_2$*t*-Bu). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 191.1, 163.8 163.7, 152.7, 148.5, 124.5, 119.5, 110.8, 108.4, 106.3, 63.2, 50.3, 49.9, 32.7, 30.6, 28.0, 27.8, 26.5, 26.1, 21.4, 20.4, 20.3, 18.5, -5.1. ESI-MS (*m/z*): [M + Na]$^+$ calcd for $C_{27}H_{39}NO_4Si$ 492.3; found 491.9.

General procedure for the synthesis of alcohols 8a-c.
A solution of compound TBDMS-protected alcohol in THF (0.1 M) was treated with acetic acid (30 mL/mmol **5**) and water (10 mL/mmol **5**) and stirred at room temperature for 6-8 hours. The reaction was quenched slowly by the addition of solid K_2CO_3 until pH 8 was obtained. The aqueous layer was extracted three times with CH_2Cl_2 and the combined organic layers were dried over Na_2SO_4. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on silica gel (ethyl acetate/hexanes).

Compound 8a. Bright orange solid (0.28 g, 0.83 mmol, 95%), starting from 0.4 g (0.9 mmol) of compound **5a**. R_f = 0.2 (3:1 ethyl acetate/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.3 (s, 1H), 7.2 (s, 1H), 3.77 (t, J = 6 Hz, 2H, -*CH*$_2$OH), 3.37 – 3.32 (m, 4H, -C*H*$_2$-), 3.19 (t, J = 8 Hz, 2H, -C*H*$_2$-), 2.86 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.78 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.34 (br s, 1H, OH), 2.03 – 1.93 (m, 4H, -C*H*$_2$-), 1.77 (quin, J = 7 Hz, 2H, -C*H*$_2$-), 1.65 (quin, J = 7 Hz, 2H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 191.4, 163.4, 163.7, 152.8, 148.8, 124.5, 119.7, 110.4, 108.3, 106.3, 61.7, 50.4, 49.9, 32.4, 27.9, 27.1, 26.5, 21.3, 20.3, 20.2. ESI-MS (*m/z*): [M + Na]$^+$ calcd for $C_{20}H_{23}NO_4$ 364.2; found 364.1.

Compound 8b. Bright orange solid (0.1 g, 0.3 mmol, 83%), starting from 0.16 g (0.36 mmol) of compound **5b**. R_f = 0.3 (3:1 ethyl acetate/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.32 (s, 1H), 7.26 (s, 1H), 3.71 (t, J = 6 Hz, 2H, -*CH*$_2$OH), 3.38 – 3.32 (m, 6H, -C*H*$_2$-), 2.88 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.79 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.02 – 1.95 (m, 4H, -C*H*$_2$-), 1.87 (quin, J = 6 Hz, 2H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 191.8, 163.5, 163.1, 152.8, 148.8, 124.7, 119.8, 110.9, 108.3, 106.2, 61.8, 50.4, 49.9, 33.5, 27.9, 24.0, 21.3, 20.3, 20.2. ESI-MS (*m/z*): [M + H]$^+$ calcd for $C_{19}H_{21}NO_4$ 350.1; found 350.1.

Compound 8c. Bright orange solid (0.21 g, 0.63 mmol, 82%), starting from 0.36 g (0.77 mmol) compound **5c**. R_f = 0.2 (2:1 ethyl acetate/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.34 (s, 1H), 7.19 (s, 1H), 3.68 (t, J = 6 Hz, 2H, -*CH*$_2$OH), 3.37 – 3.31 (m, 4H, -C*H*$_2$-), 3.23 (t, J = 7 Hz, 2H, -C*H*$_2$-), 2.87 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.79 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.0 – 1.97 (m, 4H, -C*H*$_2$-), 1.68 – 1.55 (m, 6H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 191.2, 163.7 (2 signals overlapping), 152.7, 148.6, 124.4, 119.5, 110. 8, 108.4, 106.3, 62.9, 50.4, 49.9, 32.5, 30.5, 28.0, 27.8, 26.4, 21.4, 20.4, 20.3. ESI-MS (*m/z*): [M + Na]$^+$ calcd for $C_{21}H_{25}NO_4$ 378.2; found 378.1.

General procedure for the synthesis of mesylated alcohols 9a-c.
A solution of compound alcohol **8a**, **8b**, or **8c** in dry CH_2Cl_2 (0.06 M), under inert atmosphere, was treated with Et_3N (3 equiv) and cooled to 0 °C. Methanesulfonyl chloride (2 equiv) was added and the reaction was stirred for 1.5 – 3 hours at 0 °C. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography on silica gel (ethyl acetate/hexanes).

Compound 9a. Orange solid obtained in quantitative yield from 0.15 g (0.44 mmol) of compound **8a**. R_f = 0.5 (3:1 ethyl acetate/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.32 (s, 1H), 7.20 (s, 1H), 4.33 (t, J = 6 Hz, 2H, -C*H*$_2$OMs), 3.37 – 3.32 (m, 4H, -C*H*$_2$-), 3.25 (t, J = 8 Hz, 2H, -C*H*$_2$-), 3.03 (s, 3H, -OMs), 2.87 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.80 (t, J = 6 Hz, 2H, -C*H*$_2$-), 1.99 – 1.94 (m, 6H, -C*H*$_2$-), 1.72 – 1.64 (m, 2H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 191.2, 163.6, 162.6, 152.8, 148.8, 124.4, 119.8, 110.6, 108.2, 106.3, 69.9, 50.4, 49.9, 37.5, 29.3, 27.8, 26.9, 26.4, 21.3, 20.33, 20.26. ESI-MS (*m/z*): $[M + Na]^+$ calcd for $C_{21}H_{25}NO_6S$ 442.1; found 442.1.

Compound 9b. Orange solid obtained in quantitative yield from 0.13 g (0.40 mmol) of compound **8b**. R_f = 0.5 (3:1 ethyl acetate/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.33 (s, 1H), 7.27 (s, 1H), 4.40 (t, J = 6 Hz, 2H, -C*H*$_2$OMs), 3.39 – 3.32 (m, 6H, -C*H*$_2$-), 3.07 (s, 3H, -OMs), 2.88 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.81 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.08 – 1.95 (m, 6H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 191.2, 163.5, 161.4, 152.79, 149.0, 124.4, 120.0, 110.6, 108.2, 106.3, 70.3, 50.4, 50.0, 37.6, 29.9, 27.8, 24.1, 21.3, 20.3, 20.2. ESI-MS (*m/z*): $[M + Na]^+$ calcd for $C_{20}H_{23}NO_6S$ 428.1; found 428.1.

Compound 9c. Orange solid (0.19 g, 0.44 mmol, 81%) starting from 0.18 g (0.54 mmol) of compound **8c**. R_f = 0.4 (3:1 ethyl acetate/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.34 (s, 1H), 7.18 (s, 1H), 4.27 (t, J = 7 Hz, 2H, -C*H*$_2$OMs), 3.38 – 3.32 (m, 4H, -C*H*$_2$-), 3.23 (t, J = 7 Hz, 2H, -C*H*$_2$-), 3.02 (s, 3H, -OMs), 2.88 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.80 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.04 – 1.95 (m, 4H, -C*H*$_2$-), 1.86 (quin, J = 7 Hz, 2H, -C*H*$_2$-), 1.68 – 1.56 (m, 4H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 191.2, 163.7, 163.2, 152.8, 148.7, 124.3, 119.7, 110.8, 108.3, 106.4, 70.1, 50.4, 49.9, 37.5, 29.9, 28.8, 27.9, 27.6, 25.9, 21.4, 20.4, 20.3. ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{22}H_{27}NO_6S$ 456.2; found 456.1.

General procedure for the synthesis of iodoalkyl coumarins 6a-c.
A solution of compound **9a**, **9b**, or **9c** in acetone (0.077 M) was treated with NaI (3 equiv) and refluxed for 1.5-6.5 hours. The solvent was removed *in vacuo* and the solid was redissolved in CH_2Cl_2 and washed five times with water. The organic layer was dried over Na_2SO_4 and solvent was removed *in vacuo*. The resulting residue was purified by flash chromatography on silica gel (ethyl acetate/hexanes).

Compound 6a. Orange solid (0.19 g, 0.42 mmol, 96%) obtained from 0.19 g (0.44 mmol) of compound **9a**. R_f = 0.4 (1:2 ethyl acetate/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.32 (s, 1H), 7.24 (s, 1H), 3.35 (t, J = 6 Hz, 2H, -C*H*$_2$-), 3.33 (t, J = 6 Hz, 2H, -C*H*$_2$-), 3.27 (t, J = 7 Hz, 2H, -C*H*$_2$-), 3.21 (t, J = 8 Hz, 2H, -C*H*$_2$-) 2.86 (t, J = 7 Hz, 2H, -C*H*$_2$-), 2.80 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.03 (quin, J = 7 Hz, 2H, -C*H*$_2$-), 2.0 – 1.95 (m, 4H, -C*H*$_2$-), 1.65 (quin, J = 7 Hz, 2H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 191.2, 163.6, 162.8, 152.8, 148.7, 124.5, 119.7, 110.7, 108.3, 106.3, 50.4, 49.9, 33.6, 31.2, 27.9, 26.5, 21.3, 20.4, 20.3, 6.7. ESI-MS (*m/z*): $[M + Na]^+$ calcd for $C_{20}H_{22}INO_3$ 474.1; found 474.1.

Compound 6b. Orange solid (0.088 g, 0.201 mmol, 80%) obtained from 0.10 g (0.30 mmol) of compound **9b**. R_f = 0.4 (1:2 ethyl acetate/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.33 (s, 1H), 7.33 (s, 1H), 3.39 – 3.31 (m, 8H, -C*H*$_2$-), 2.88 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.81 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.08 (quin, J = 7 Hz, 2H, -C*H*$_2$-), 2.02 – 1.96 (m, 4H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (151 MHz,

$CDCl_3$) δ 191.2, 163.5, 161.4, 152.8, 148.8, 124.6, 119.8, 110.9, 108.3, 106.3, 50.4, 50.0, 33.8, 28.9, 27.9, 21.3, 20.4, 20.3, 7.2. ESI-MS (*m/z*): $[M + Na]^+$ calcd for $C_{19}H_{20}INO_3$ 460.0; found 460.0.

Compound 6c. Orange solid (0.18 g, 0.38 mmol, 93%) obtained from 0.18 g (0.41 mmol) of compound **9c**. R_f = 0.4 (1:2 ethyl acetate/hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 10.35 (s, 1H), 7.19 (s, 1H), 3.35-3.32 (m, 4H, -C*H*$_2$-), 3.23 – 3.21 (m, 4H, -C*H*$_2$-), 2.88 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.82 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.01 – 1.95 (m, 4H, -C*H*$_2$-), 1.91 (quin, J = 7 Hz, 2H, -C*H*$_2$-), 1.67 – 1.56 (m, 4H, -C*H*$_2$-). $^{13}C\{^1H\}$ NMR (151 MHz, $CDCl_3$) δ 191.2, 163.7, 163.3, 152.8, 148.6, 124.4, 119.6, 110.8, 108.3, 106.4, 50.4, 49.9, 33.1, 31.0, 29.4, 28.0, 27.6, 21.4, 20.4, 20.3, 7.2. ESI-MS (*m/z*): $[M + Na]^+$ calcd for $C_{21}H_{24}INO_3$ 488.1; found 488.1.

Synthesis of probe 1.
A suspension of disulfide **7a** (0.066 g, 0.12 mmol) in dry DMF (0.5M), under inert atmosphere, was treated with K_2CO_3 (0.05g, 0.36 mmol), compound **6a** (0.11 g, 0.24 mmol), $Na_2S_2O_4$ (0.066 g, 0.38 mmol) and one drop of water. The mixture was stirred at room temperature for 6 hours. After this period, the reaction mixture was treated with equal volumes of brine and diethyl ether (50 mL each). The organic phase was collected and the aqueous layer was extracted with diethyl ether (3×50 mL) and with CH_2Cl_2 (2×50 mL). The combined organic layers were dried over Na_2SO_4 and solvent was removed *in vacuo*. The residue was purified by flash chromatography on silica gel (acetone/hexanes) to obtain product compound **1** as an orange solid (0.09 g, 0.14 mmol, 58%). R_f = 0.35 (1:1 acetone/hexanes). ^1H NMR (600 MHz, $CDCl_3$) δ 10.33 (s, 1H), 8.56 (s, 1H), 8.38 (d, J = 8 Hz, 1H, -N*H*CO-), 7.51 (dd, J = 6 Hz, 1 Hz, 1H), 7.32 (td, J = 7 Hz, 1 Hz, 1H), 7.15 (s, 1H), 7.04 (td, J = 7 Hz, 1 Hz, 1H), 5.86 (s, 1H, -N*H*Ac), 3.38 – 3.33 (m, 4H, -C*H*$_2$-), 3.26 (q, J = 7 Hz, 2H, -C*H*$_2$NHAc), 3.21 (t, J = 8 Hz, 2H, -C*H*$_2$S-), 2.88 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.85 (t, J = 7 Hz, 2H, -C*H*$_2$-), 2.78 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.41 (t, J = 7 Hz, 2H, -NHCOC*H*$_2$-), 2.02 – 1.95 (m, 7H, -C*H*$_2$- and -Ac), 1.76 – 1.70 (m, 4H, -C*H*$_2$-), 1.68 – 1.63 (m, 2H, -C*H*$_2$-), 1.56 – 1.52 (m, 2H, -C*H*$_2$-), 1.44 – 1.39 (m, 2H, -C*H*$_2$-). $^{13}C\{^1H\}$ NMR (151 MHz, $CDCl_3$) δ 191.1, 171.4, 170.3, 163.7, 163.0, 152.8, 148.8, 139.7, 135.3, 129.8, 124.3, 124.1, 122.7, 120.4, 119.8, 110.6, 108.2, 106.3, 50.4, 50.0, 39.5, 37.9, 36.0, 29.6, 29.4, 29.3, 27.9, 27.2, 26.5, 25.1, 23.5, 21.3, 20.33, 20.27. HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_{34}H_{41}N_3O_5S$ 626.2659; found 626.2665.

General procedure for the synthesis of Boc-protected compounds 3a-c.
A suspension of disulfide **7b** in dry DMF (0.05 M), under inert atmosphere, was treated with K_2CO_3 (3 equiv), compound **6a-c** (2 equiv), $Na_2S_2O_4$ (3 equiv) and one drop of water. The reaction was stirred at room temperature for 2.5 – 4.5 hours. After this period, the reaction mixture was treated with equal volumes of diethyl ether and brine. The organic layer was collected, and the aqueous layer was further extracted with ether. The combined organic layers were dried over Na_2SO_4 and the solvent was removed *in vacuo*. The residue was taken up in dichloromethane, transferred to a preparative TLC, and eluted with a mixture of ethyl acetate/hexanes.

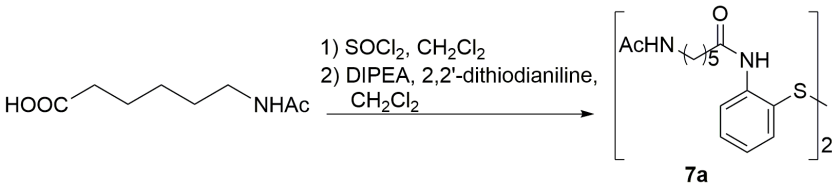
Compound 3a. Orange solid (0.028 g, 0.042 mmol, 53%), obtained from 0.04 g (0.08 mmol) of compound **6a**. R_f = 0.52 (2:1 ethyl acetate/hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 10.34 (s, 1H), 8.54 (s, 1H, -N*H*CO-), 8.40 (d, J = 8 Hz, 1H), 7.50 (dd, J = 8 Hz, 1 Hz, 1H), 7.32 (td, J = 8

Hz, 1 Hz, 1H), 7.15 (s, 1H), 7.04 (td, J = 8 Hz, 1 Hz, 1H), 4.63 (s, 1H, -N*H*Boc), 3.37 – 3.32 (m, 4H, -C*H*$_2$-), 3.20 (t, J = 8 Hz, 2H, -C*H*$_2$NHBoc), 3.15 – 3.10 (m, 2H, -C*H*$_2$-), 2.88 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.83 (t, J = 7 Hz, 2H, -C*H*$_2$-), 2.78 (t , J = 6 Hz, 2H, -C*H*$_2$-), 2.42 (t, J = 7 Hz, 2H, -NHCOC*H*$_2$-), 2.02 – 1.95 (m, 4H, -C*H*$_2$-), 1.79 – 1.72 (m, 4H, -C*H*$_2$-), 1.69 – 1.62 (m, 2H, -C*H*$_2$-), 1.55 – 1.50 (m, 2H, -C*H*$_2$-), 1.43 (s, 9H, -Boc). $^{13}C\{^1H\}$ NMR (151 MHz, $CDCl_3$) δ 191.2, 171.4, 163.6, 162.9, 156.2, 152.8, 148.7, 139.8, 135.4, 129.8, 124.3, 124.0, 122.6, 120.4, 119.7, 110.7, 108.3, 106.4, 50.4, 50.0, 40.5, 38.0, 35.8, 30.0, 29.8, 29.4 (2 signals overlapping), 28.6, 27.9, 27.2, 26.5, 25.3, 21.3, 20.4, 20.3. HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_{37}H_{47}N_3O_6S$ 684.3078; found 684.3075.

Compound 3b. Orange solid (0.04 g, 0.06 mmol, 54%) obtained from 0.05 g (0.12 mmol) of compound **6b**. R_f = 0.73 (2:1 ethyl acetate/hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 10.33 (s, 1H), 8.49 (s, 1H, -N*H*CO-), 8.38 (d, J = 8 Hz, 1H), 7.51 (dd, J = 8 Hz, 1 Hz, 1H), 7.30 (td, J = 8 Hz, 2 Hz, 1H), 7.10 (s, 1H), 7.03 (td, J = 8 Hz, 1 Hz, 1H), 4.62 (s, 1H, -N*H*Boc), 3.37 – 3.29 (m, 6H, -C*H*$_2$-), 3.15 – 3.07 (m, 2H, -C*H*$_2$S-), 2.96 (t, J = 7 Hz, 2H, -C*H*$_2$-), 2.87 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.73 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.44 (t, J = 7, 2H, -NHCOC*H*$_2$-), 2.01 – 1.94 (m, 4H, -C*H*$_2$-), 1.86 – 1.72 (m, 4H, -C*H*$_2$-), 1.62 – 1.49 (m, 4H, -C*H*$_2$-), 1.43 (s, 9H, -Boc). $^{13}C\{^1H\}$ NMR (151 MHz, $CDCl_3$) δ 191.2, 171.4, 163.5, 162.0, 156.1, 152.7, 148.8, 139.4, 134.6, 129.6, 124.2, 124.1, 122.8, 120.7, 119.7, 110.7, 108.1, 106.3, 50.4, 49.9, 40.5, 38.0, 36.2, 30.1, 29.9, 29.8, 28.5, 27.9, 26.8, 26.5, 25.3, 21.3, 20.3, 20.2. HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_{36}H_{45}N_3O_6S$ 670.2921; found 670.2941.

Compound 3c. Orange solid (0.06 g, 0.09 mmol, 41%) obtained from 0.1 g (0.2 mmol) of compound **6c**. R_f = 0.77 (2:1 ethyl acetate/hexanes). ^1H NMR (400 MHz, $CDCl_3$) δ 10.33 (s, 1H), 8.50 (s, 1H, -N*H*COCH$_2$-), 8.37 (d, J = 8 Hz, 1H), 7.49 (d, J = 8 Hz, 1H), 7.29 (t, J = 8 Hz, 1H), 7.13 (s, 1H), 7.02 (t, J = 8 Hz, 1H), 4.63 (s, 1H, -N*H*Boc), 3.36 – 3.31 (m, 4H, -C*H*$_2$-), 3.19 (t, J = 7 Hz, 2H, -C*H*$_2$NHBoc), 3.15 – 3.06 (m, 2H, -C*H*$_2$S-), 2.87 (t, J = 6 Hz, 2H, -C*H*$_2$-), 2.77 (t, J = 6 Hz, 4H, -C*H*$_2$-), 2.43 (t, J = 7 Hz, 2H, -NHCOC*H*$_2$-), 1.99 – 1.95 (m, 4 H, -C*H*$_2$-), 1.78 – 1.45 (m, 12 H, -C*H*$_2$-), 1.42 (s, 9H, -Boc). $^{13}C\{^1H\}$ NMR (151 MHz, $CDCl_3$): 191.3, 171.4, 163.7, 163.4, 156.2, 152.8, 148.7, 139.8, 135.3, 129.7, 124.4, 124.1, 120.4, 119.6, 114.2, 110.9, 108.3, 106.4, 50.4, 50.0, 40.6, 38.1, 36.3, 30.2, 30.1, 29.9, 29.4, 29.2, 28.6, 28.0, 27.6, 26.6, 25.4, 21.4, 20.4, 20.3. HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_{38}H_{49}N_3O_6S$ 698.3234; found 698.3242.

Scheme S2



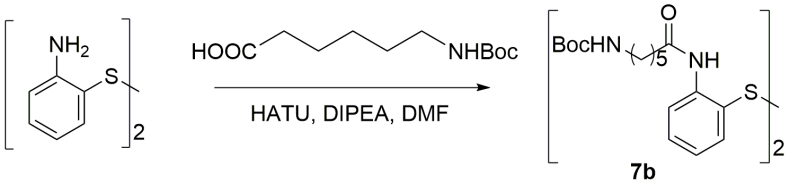
7a

Synthesis of disulfide 7a.

A suspension of 6-acetamidohexanoic acid (1.53 g, 8.83 mmol) in dry CH_2Cl_2 (4 mL) was treated with thionyl chloride (0.7 mL, 9.64 mmol), under inert atmosphere. The resulting clear solution was stirred for 25 min at room temperature and then treated with 2,2'-dithiodianiline (1.00 g, 4.03 mmol) in dry CH_2Cl_2 (4 mL), followed by DIPEA (1.7 mL, 9.76 mmol). The mixture was stirred at room temperature for 6 h. After this period, the yellow solution was

diluted with CH_2Cl_2 (~100 mL), washed with water (4×50 mL), brine (50 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The product was obtained as an off-white solid after recrystallization from hot methanol-water (1.61 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 (d, J = 8 Hz, 1H), 7.97 (s, 1H, -N*H*CO-), 7.44 – 7.38 (m, 2H), 7.02 (td, J = 8, 1.2 Hz, 1H), 5.88 (s, 1H, -N*H*Ac), 3.25 (q, J = 7 Hz, 2H, -C*H*$_2$NHAc), 2.17 (t, J = 7 Hz, 2H, -C*H*$_2$-), 1.97 (s, 9H, -Ac), 1.65 – 1.59 (m, 2H, -C*H*$_2$-), 1.53 (quin, J = 7 Hz, 2H, -C*H*$_2$-), 1.40 – 1.35 (m, 2H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$) δ 171.4, 170.5, 139.8, 136.5, 132.2, 124.6, 124.1, 121.3, 39.5, 37.5, 29.3, 26.5, 25.0, 23.4. ESI-MS (*m/z*): $[M + H]^+$ calcd for $C_{28}H_{38}N_4O_4S_2$ 559.2; found 559.4.

Scheme S3

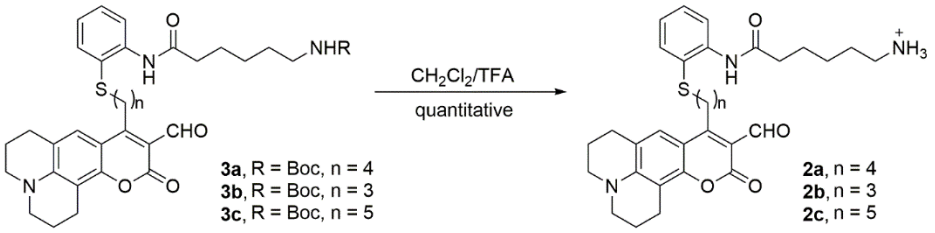


7b

Synthesis of disulfide 7b.

A solution of Boc-protected aminohexanoic acid (0.45 g, 1.9 mmol) and 2,2'-dithiodianiline (0.21 g, 0.82 mmol) in dry DMF (3 mL), under inert atmosphere, was treated with HATU (0.73 g, 1.92 mmol) and DIPEA (0.34 mL, 1.95 mmol) and stirred at room temperature for 46 hours. The solution was diluted with toluene (3 mL) and the solvent was removed *in vacuo*. The residue was redissolved in CH_2Cl_2 (50 mL) and washed sequentially with water (2×50 mL), cold aqueous HCl (1N, 50 mL), saturated aqueous $NaHCO_3$ (50 mL), and brine (50 mL). After drying over Na_2SO_4, the solution was concentrated *in vacuo*. The product was obtained as a white solid (0.27 g, 50%) after purification by flash chromatography (ethyl acetate/hexanes) and trituration with 1:1 ethyl acetate/hexanes. R_f = 0.75 (2:1 ethyl acetate/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.36 (d, J = 7 Hz, 1H), 7.95 (s, 1H, -N*H*CO-), 7.41 – 7.38 (m, 2H), 6.99 (td, J = 8, 1 Hz, 1H), 4.64 (s, 1H, -N*H*Boc), 3.12 (t, J = 7 Hz, 2H, -C*H*$_2$NHBoc), 2.15 (t, J = 7 Hz, 2H, -NHCOC*H*$_2$-), 1.62 (quin, J = 8 Hz, 2H, -C*H*$_2$-), 1.50 (quin, J = 7 Hz, 2H, -C*H*$_2$-), 1.48 (s, 9H, -Boc), 1.43 – 1.33 (m, 2H, -C*H*$_2$-). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 171.3, 156.2, 139.9, 136.6, 132.3, 124.4, 123.8, 121.2, 40.6, 37.6, 29.9, 28.6, 26.5, 25.0. ESI-MS (*m/z*): $[M + Na]^+$ calcd for $C_{34}H_{50}N_4O_6S_2$ 697.3; found 697.2.

Scheme S4



Quantitative deprotection of Boc carbamates 3a-c: synthesis of amines 2a-c.
A sample of compound **3a-c** (2-5 mmol) was dissolved in CH_2Cl_2 (40 μL) and treated with trifluoroacetic acid (40 μL). Upon reaction completion (~4 hours, verified by HPLC analysis of a second reaction, conducted in parallel under the same conditions), the solvent was evaporated and the residue was dried overnight. The residue was dissolved quantitatively in DMSO to obtain a stock solution of the corresponding alkylammonium salt (~1 mM). Aliquots were flash frozen and stored at -20 °C and thawed immediately before use.
A solution prepared in DMSO-d_6 in a similar fashion was employed for ^{1}H NMR spectroscopic analysis to verify the presence of only the aldehyde species, as determined from the integration of the peak at δ 10.12 ppm.

2.2. Spectroscopic methods

General Spectroscopic Methods
Aqueous buffers were prepared with deionized water with a resistivity of ≥ 18 MΩcm^{-1}. Other solvents were purified by standard methods. Absorption data were acquired on a Cary 100 UV-vis Spectrophotometer using Varian Cary Win UV Scan, Kinetics, or Scanning Kinetics software, version 4.10. Fluorescence experiments were conducted on a QuantaMaster 40 Photon Technology International spectrofluorometer equipped with a Xenon lamp source, emission and excitation monochromators, excitation correction unit, and PMT detector. All spectroscopic experiments were conducted at 37.0 °C ± 0.1 °C using a Quantum Northwest cuvette temperature controller. Linear and nonlinear fits were performed in OriginPro9 software.

Determination of extinction coefficients and fluorescence quantum yields
Volumetric samples of probe **1** (0.7 – 2.5 μM) and the corresponding deacetylated compounds obtained from acid deprotection of carbamate **3b** (0.6 – 3.3 μM) were prepared from 1.1 mM DMSO stock solutions diluted in aqueous 50 mM HEPES, 100 mM KCl, pH 8.0. Samples were equilibrated for 10 minutes at 37 °C in the cuvette holder before acquiring absorption or fluorescence data. Absorption spectra were collected from 350 – 600 nm. Emission spectrum (λ_{ex} = 499 nm) at each concentration was integrated from 350 – 700 nm. Solutions of fluorescein in 0.1 N NaOH, with a reported quantum yield of 0.95 (excitation at 492 nm), were used as standards for quantum yield determination.

Determination of equilibrium constant of reversible imine formation
Aliquots of compounds **2a-c** in DMSO were diluted in 50 mM HEPES, 100 mM KCl at pH 8.0 and 37 °C for a final concentration of 2.1 – 2.7 μM. Absorption spectra were collected in the range 390 nm – 550 nm over 10 min, until complete equilibration. The series of spectra were deconvoluted (global analysis) as a sum of Gaussian functions representing the contributions of the aldehyde and imine products to the overall absorption spectrum. The molar absorptivity of the aldehyde component at each wavelength was assumed to be identical to that of the acetylated probe **1**, determined independently. The equilibrium constants for the formation of the imine (K_{eq}) were determined from the relative concentrations of aldehyde and imine in the mixture, calculated from the deconvolution of the final absorption spectrum of the equilibrated sample. Each experiment was conducted in triplicate.

Kinetics of imine formation

An aliquot of amine **2a-c** in DMSO was diluted in aqueous buffer at 37 °C for a final concentration of 1.5 – 2 μM. The solution was mixed by repeatedly inverting the cuvette for 10-20 seconds and placed in cuvette holder set to 37 °C. Absorbance at 499 nm was collected over time. The forward kinetic rate constant (k_1) was obtained from a nonlinear fit of the absorbance as a function of time, using an integrated rate law for a reversible first-order reaction:

$$A(t) = A_e + (A_0 + A_e)e^{-k_1\left(\frac{1}{K_{eq}}+1\right)t}$$

where A_0 is the initial absorbance, A_e is the absorbance at equilibrium, and K_{eq} is the equilibrium constant determined previously from spectral deconvolution. Experiments were conducted in triplicate.

Determination of intermolecular imine association constant

A solution of probe **1** (4 μM) in aqueous buffer (50 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, pH 8.0) was treated with increasing amounts of 6-aminohexanoic acid at 37 °C. Absorption spectra were collected after 10 min of equilibration time. The apparent binding affinity was obtained from a nonlinear fit of the change in absorption at 467 nm as a function of 6-aminohexanoic acid concentration, using a model with 1:1 binding stoichiometry.

2.3. Study of enzymatic deacetylation

General materials and methods

HeLa nuclear extract, HDAC3/NCOR1 (0.48 mg/mL stock at ≥ 90% purity), Trichostatin A (TSA) in DMSO (0.2 mM), stock solution of NAD^+ (50 mM in 50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$), stock solution of nicotinamide (50 mM in 50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$), and valproic acid (VPA) sodium salt were obtained from Enzo Lifesciences. HDAC6 (0.29 mg/mL stock at ≥ 80% purity) and Sirt1 (0.32 mg/mL stock at 91.4% purity) were obtained from Sigma Aldrich. All experiments were conducted in 50 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$ (assay buffer) pH 8.0 at 37.0 °C ± 0.1 °C. Aqueous buffers were prepared with deionized water with a resistivity of ≥ 18 $M\Omega cm^{-1}$. Other solvents were purified by standard methods. Absorption, fluorescence and HPLC data were acquired with the same instrumentation described above. A microcuvette with a path length of 10 mm (Starna Cells) was employed for absorption and fluorescence data. Absorption data for VPA inhibition experiments were acquired with Bio-tek Synergy HT using KC4 (version 3.14, rev. 12) software. Emission fluorescence data for VPA inhibition experiments were acquired with SpectraMax GeminiXS using SoftMax Pro (version 4.7). Corning black 96-well, half area plates with clear bottom were used in plate reader experiments.

Enzymatic deacetylation with purified enzymes studied by absorption spectroscopy

An aliquot of purified enzyme was diluted in HEPES assay buffer (250 μL total volume) to a final concentration as listed in Tables S4 – S6, and allowed to equilibrate at 37 °C for 10 minutes. For experiments with Sirt1, the assay buffer contained NAD+ at a concentration as specified in Table S4. An aliquot of probe **1** (from 0.6 mM DMSO stock solution) was added for a total concentration of 12 μM in the cuvette. The solution was mixed rapidly and absorption data at 499 nm were collected over 40 minutes (HDAC6) or 2 hours (HDAC3/NCOR1 and SIRT1) at 37 °C.

Positive control: an experiment was conducted in a similar fashion using chemically-generated amine product **2a** instead of **1**.
Negative control: the enzyme solution was treated with inhibitor (TSA or Nicotinamide, see Tables S4-S6) and allowed to equilibrate at 37 °C for 10 minutes before addition of probe **1**.
The signal of the negative and positive controls were used to calibrate the absorption signal to 0 and 100% product, respectively, and to calculate the product formation over the course of the reaction. An aliquot was diluted in an equal volume of acetonitrile at the end of the experiment followed by sonication for 30 s and centrifugation for 10 minutes to remove the precipitated protein. The supernatant was analyzed using the HPLC method described below (Chromatographic analysis of enzymatic deacetylation: HeLa nuclear extract) to confirm the presence or absence of product.

Enzymatic deacetylation with purified enzymes studied by fluorescence emission
Sample preparation followed a similar protocol described for experiments studied by absorption spectroscopy. The sample was excited at 499 nm and emission was collected at 523 nm over the course of 40 minutes or 2 hours at 37 °C. HPLC analysis of the reaction mixture was conducted at the end of the run, as described above, to confirm product formation.

Table S4. Experimental conditions for deacetylation of probe **1** by purified SIRT1.

	Experiment	Negative control	Positive control
[SIRT1] (nM)	100	100	100
[NAD^+] (mM)	0.3	0	0.3
[nicotinamide] (mM)	0	0.3	0

Table S5. Experimental conditions for deacetylation of probe **1** by purified HDAC3/NCOR1.

	Experiment	Negative control	Positive control
[HDAC3/NCOR1] (nM)	100	100	100
[TSA] (μM)	0	1.2	0
DMSO vehicle (μL)	1.5	0	1.5

Table S6. Experimental conditions for deacetylation of probe **1** by purified HDAC6.

	Experiment	Negative control	Positive control
[HDAC6] (nM)	25	25	25
[TSA] (μM)	0	1.2	0
DMSO vehicle (μL)	1.5	0	1.5

Enzymatic deacetylation with HeLa nuclear extract studied by absorption spectroscopy
A dilution of HeLa nuclear extract (91.4 μg of protein, from 9.14 mg/mL stock with an activity of 38.6 U/μL) in HEPES assay buffer (234 μL) was treated with Trichostatin A inhibitor (for a final concentration of 1.2 μM, from 0.2 mM DMSO stock solution) or DMSO vehicle and allowed to equilibrate at 37 °C for 10 minutes. An aliquot of probe **1** was added for a total concentration of 4.0 – 11.2 μM in the cuvette (from 0.20 – 0.56 mM DMSO stock solutions). The solution was mixed rapidly and absorption data at 510 nm were collected over 4 hours at 37 °C. As a positive control, an experiment was conducted in a similar fashion using chemically-

generated amine product instead of **1**. The signals of the negative (TSA-inhibited) and positive controls were used to calibrate the absorption signal to 0 and 100% product, respectively, and calculate the product formation over the course of the reaction. An aliquot was diluted in an equal volume of acetonitrile at the end of the experiment followed by sonication for 30 s and centrifugation for 10 minutes to remove the precipitated protein. The supernatant was analyzed using the HPLC method described below (Chromatographic analysis of enzymatic deacetylation: HeLa nuclear extract) to confirm the presence or absence of product.

Enzymatic deacetylation with HeLa nuclear extract studied by fluorescence emission
Sample preparation followed a similar protocol described for experiments studied by absorption spectroscopy. A different lot of HeLa nuclear extract (9.0 mg/mL stock with an activity of 40 U/μL) was used, and the dilution of extract was adjusted accordingly. The sample was excited at 499 nm and emission was collected at 523 nm over the course of 4 h at 37 °C. HPLC analysis of the reaction mixture was conducted at the end of the run, as described above, to confirm product formation.

Chromatographic analysis of enzymatic deacetylation: HeLa nuclear extract
An aliquot of HeLa nuclear extract (91.4 μg of protein, from 9.14 mg/mL stock) in aqueous buffer (234 μL) was treated with Trichostatin A inhibitor (for a final concentration of 1.2 μM, from 0.2 mM DMSO stock solution) or DMSO vehicle and allowed to equilibrate at 37 °C for 10 minutes. The mixture was treated with an aliquot of probe **1** for a total concentration of 11.2 μM (from 0.56 mM DMSO stock containing coumarin 6H as internal standard) and the reaction was kept at 37 °C for 4 hours. Aliquots removed at various time points (0, 10, 20, 30, 60, 120, 150, 180, 210 and 240 minutes) were diluted in aqueous buffer and quenched with an equal volume of acetonitrile. The samples were sonicated for 30 s and centrifuged for 10 minutes to remove the precipitated protein. The supernatant was analyzed on an Agilent Zorbax Extended C18 column (4.6×50 mm, 1.8 μm) at 25 °C using an isocratic method (40% acetonitrile in water, 0.1% trifluoroacetic acid) over 15 minutes. Calibration curves for probe **1** and reaction product **2a** were obtained from standards μprepared in a similar solvent combination as the samples, using coumarin 6H as internal standard.

Calculations of Z' factors:
The Z' statistical parameter[4] for the assay in HeLa nuclear extract, purified HDAC6, and purified HDAC3/NCOR1 was determined for absorption and fluorescence using equation (1):

$$Z' = 1 - \frac{\left(3\sigma_p + 3\sigma_n\right)}{\left|\mu_p - \mu_n\right|} \qquad (1)$$

where μ_p and μ_n are the means of the triplicate signal (absorption at 499 nm for purified enzyme and 510 nm for HeLa nuclear extract or fluorescence excitation at 499 nm and emission at 523 nm) and σ_p and σ_n are the corresponding standard deviations for the positive control using chemically-generated amine product **2a** and the negative control (TSA-inhibited), respectively.

Valproic acid (VPA) inhibition of HDAC3/NCOR1
An aliquot (45 μL) of HDAC3/NCOR1 (61 nM stock in HEPES assay buffer) was mixed with HEPES assay buffer (25 μL) containing the desired concentration of VPA sodium salt (for a total concentration of 0, 0.01 mM, 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM, 1 mM, 2.5 mM, 5 mM, or

10 mM in the final sample volume, 75 μL). The solutions were incubated at 37 °C for 10 minutes. The mixture was treated with an aliquot (5 μL) of warmed probe **1** for a total concentration of 12 μM (from a 0.6 mM DMSO stock) and reactions were kept at 37 °C for 3.5 hours. Samples were heat-shocked at 95 °C for 5 minutes and centrifuged for 5 minutes. The supernatant (50 μL) was transferred to a 96-well plate. Each sample was quantified by fluorescence emission (λ_{ex} = 499 nm, λ_{em} = 523 nm) and absorption (λ_{abs} = 499 nm) at 25 °C. As positive controls, samples were prepared in a similar fashion using chemically-generated amine product **2a** instead of **1**. The half maximum effective concentration (EC_{50}) of VPA was derived from a nonlinear fit of signal as a function of log VPA concentration, using a dose response model:

$$y = A_1 + \frac{\left(A_2 - A_1\right)}{1 + 10^{[p(\log x0 - x)]}},\ EC_{50} = 10^{\log x0}$$

where logx0 is the center, p is the hill slope, and A_1 and A_2 are the bottom and top asymptotes, respectively.

3. NMR spectroscopy and chromatographic characterization data

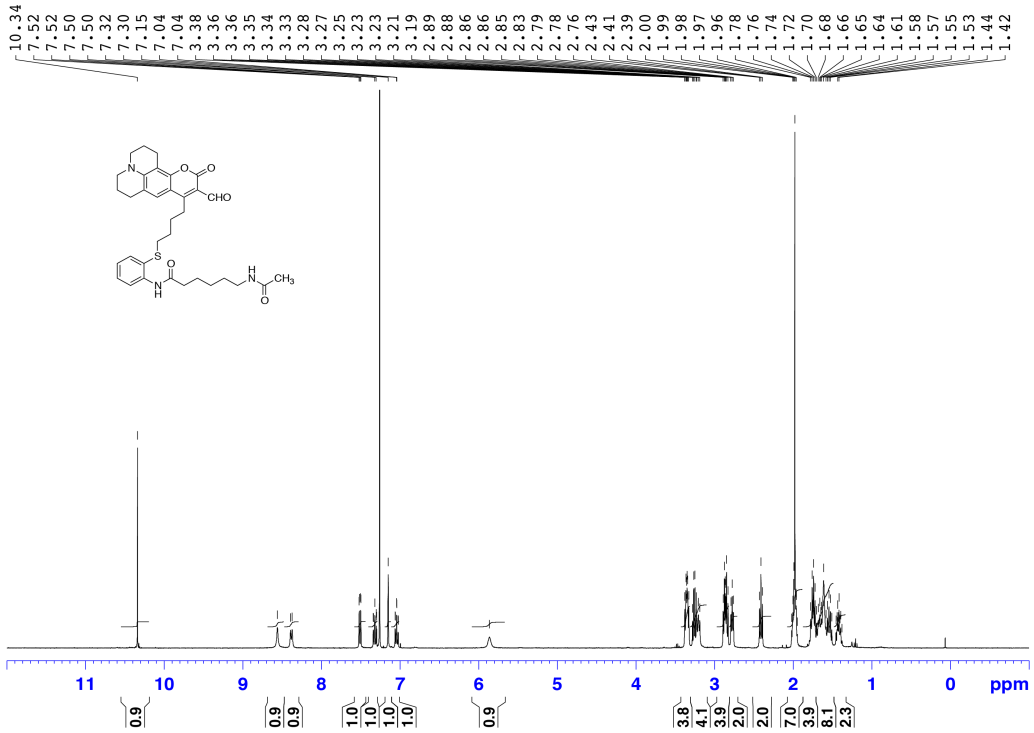


Figure S7. ^{1}H NMR spectrum of acetylated probe **1** in $CDCl_3$.

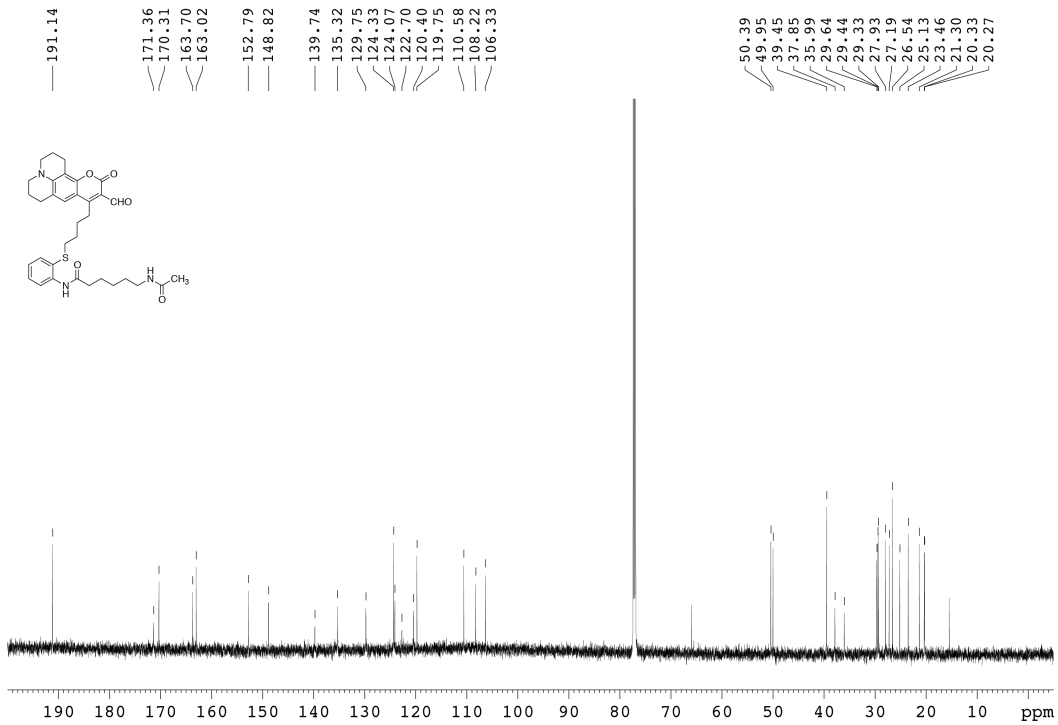


Figure S8. $^{13}C\{^{1}H\}$ NMR spectrum of probe **1** in $CDCl_3$.

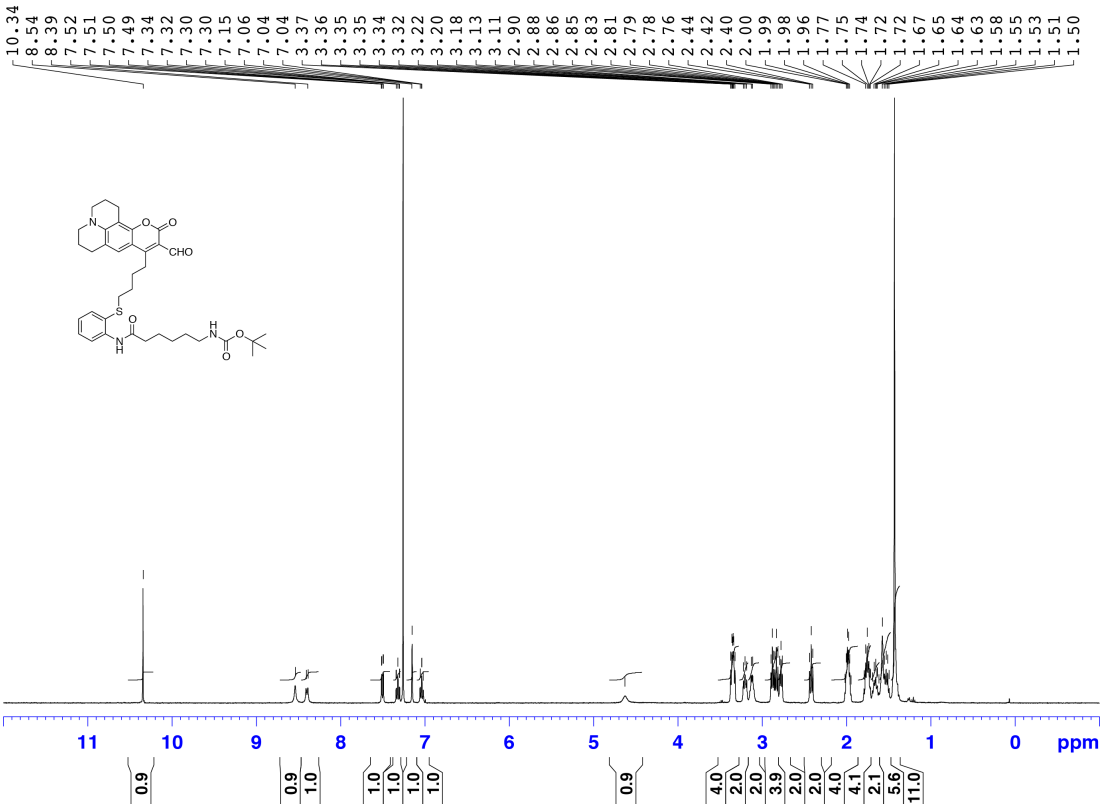


Figure S9. ^{1}H NMR spectrum of Boc-protected model compound **3a** in $CDCl_3$.

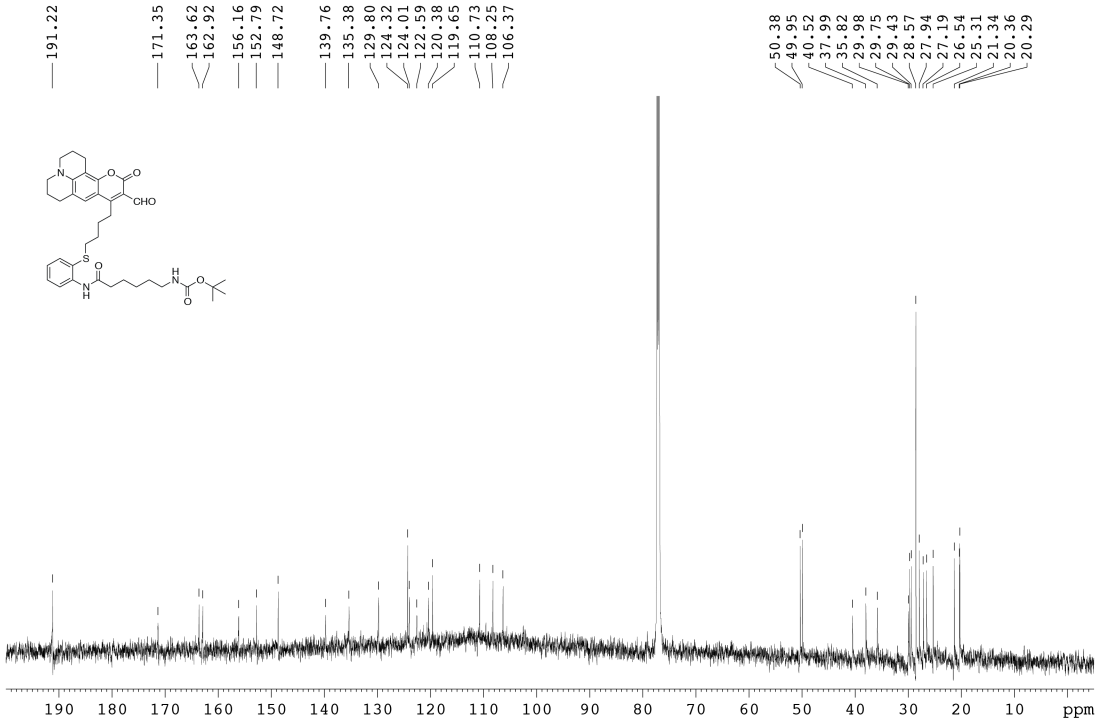


Figure S10. $^{13}C\{^{1}H\}$ NMR spectrum of Boc-protected model compound **3a** in $CDCl_3$.

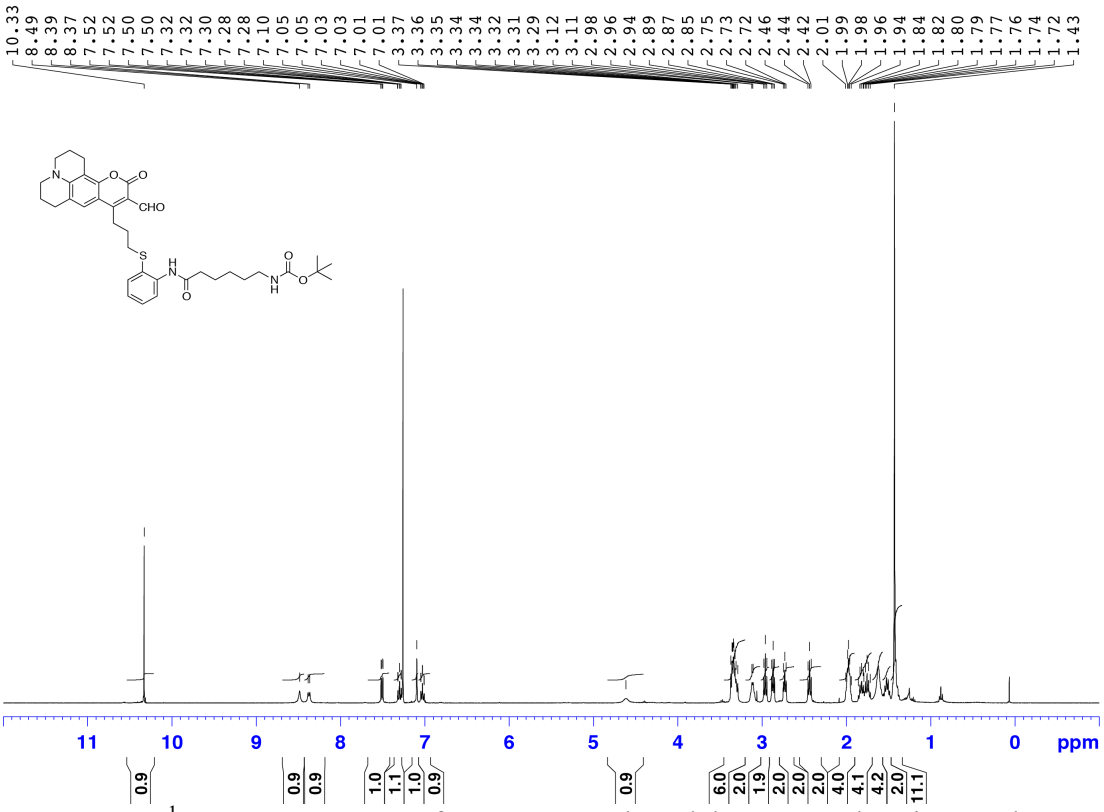


Figure S11. ^{1}H NMR spectrum of Boc-protected model compound **3b** in $CDCl_3$.

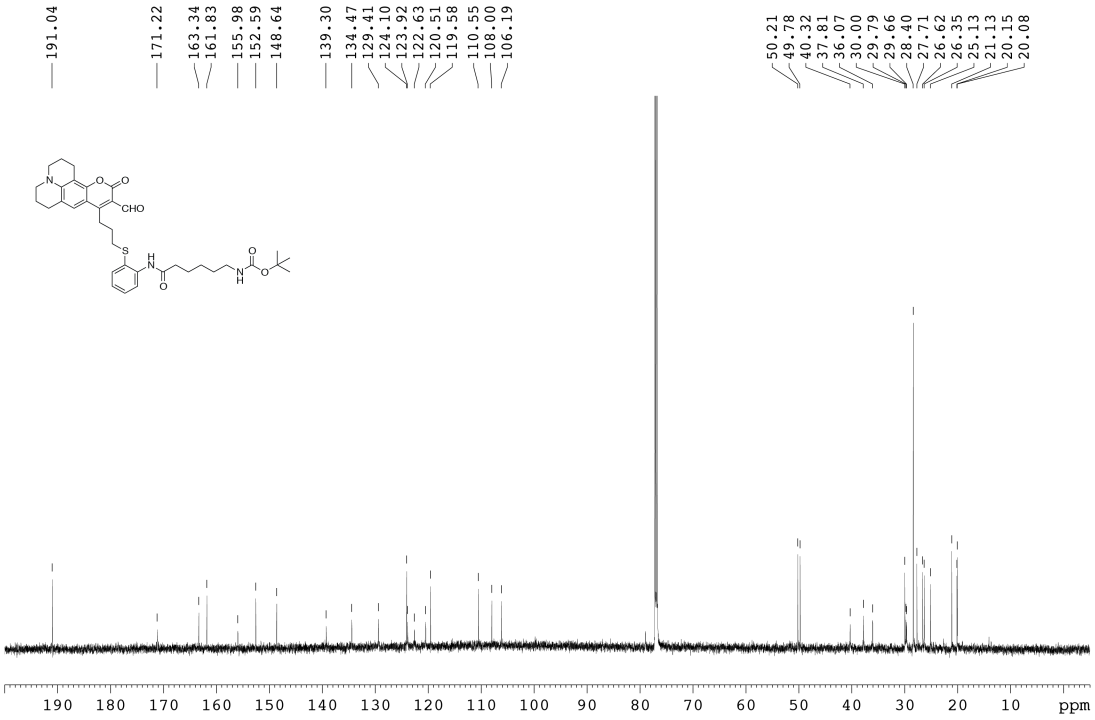


Figure S12. $^{13}C\{^{1}H\}$ NMR spectrum of Boc-protected model compound **3b** in $CDCl_3$.

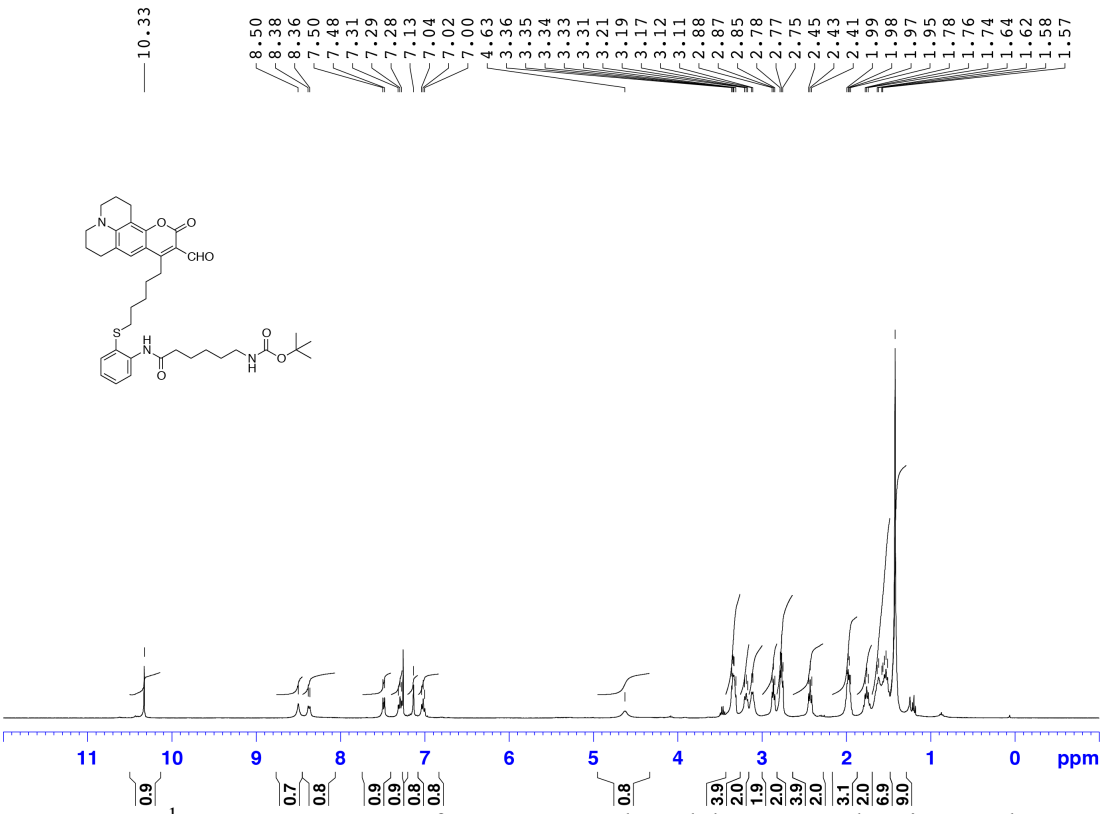


Figure S13. ^{1}H NMR spectrum of Boc-protected model compound **3c** in $CDCl_3$.

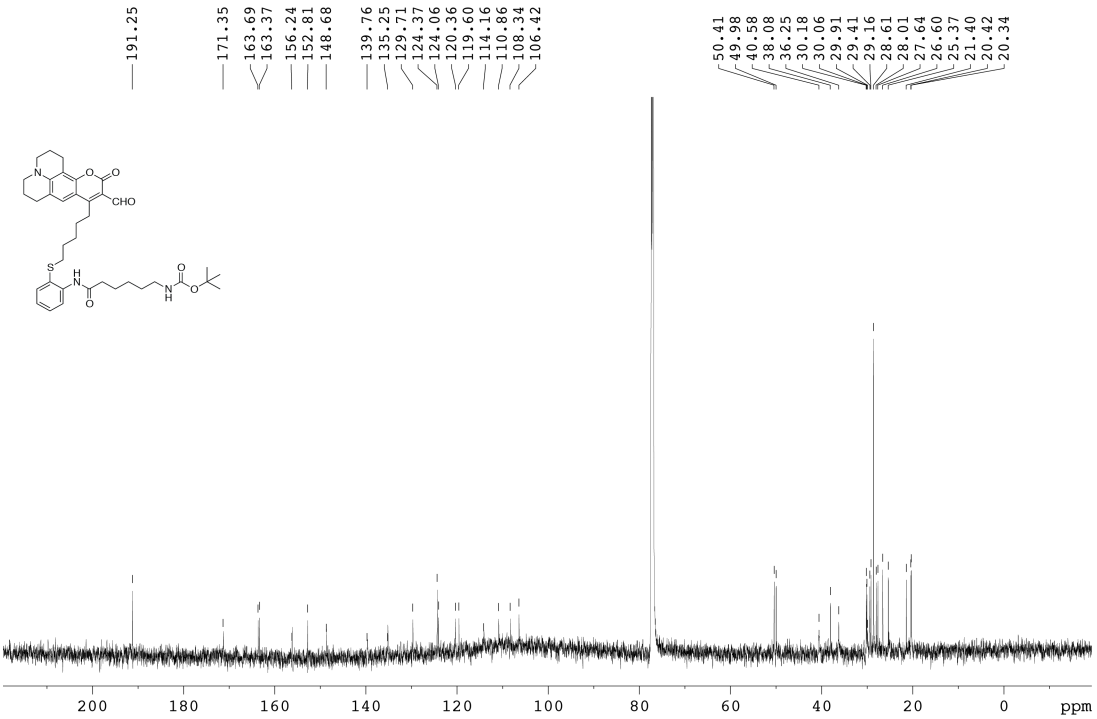


Figure S14. $^{13}C\{^{1}H\}$ NMR spectrum of Boc-protected model compound **3c** in $CDCl_3$.

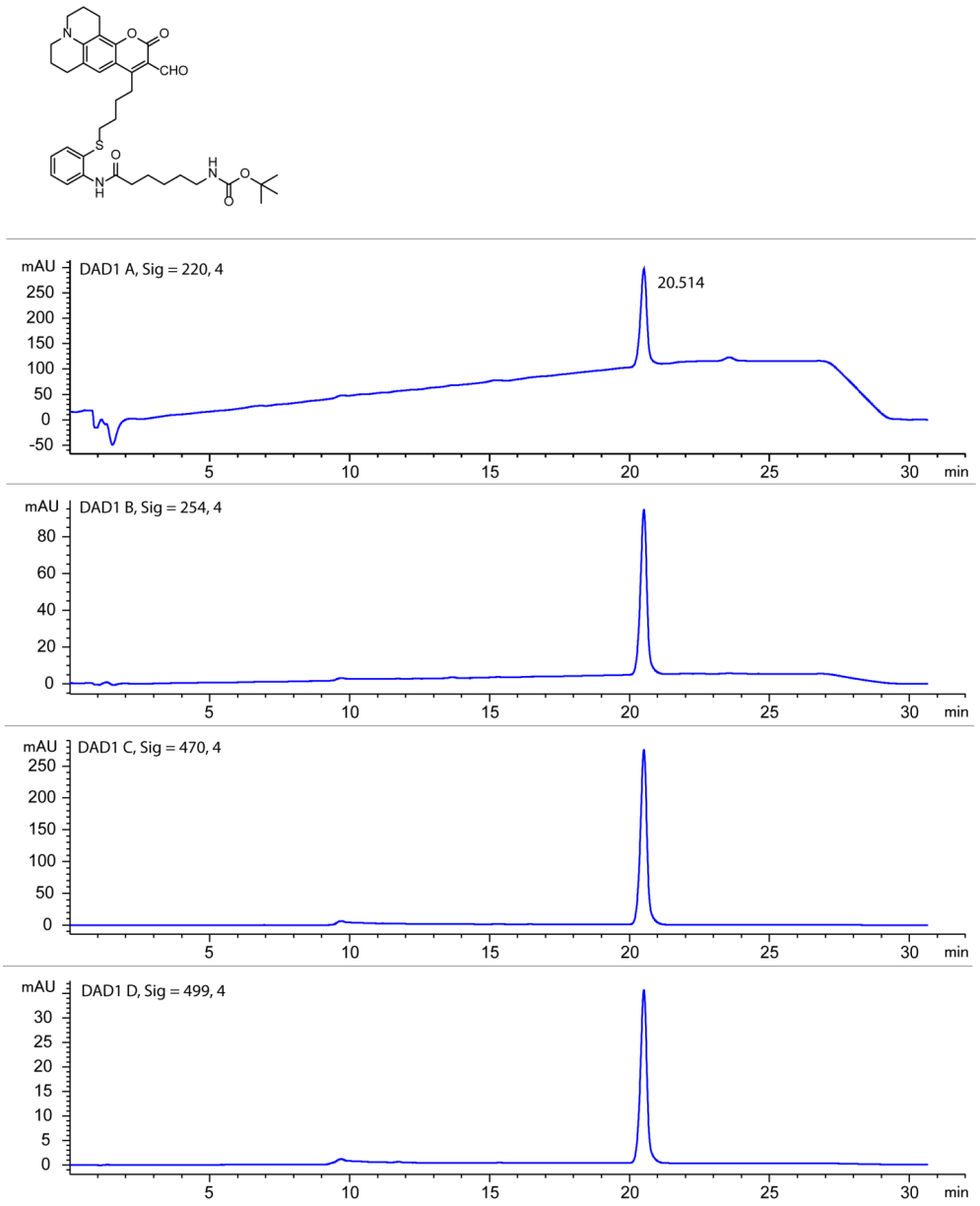


Figure S15. Reverse-phase chromatogram of Boc-protected model compound **3a** eluted with a gradient of acetonitrile/water (+ 0.1% trifluoroacetic acid).

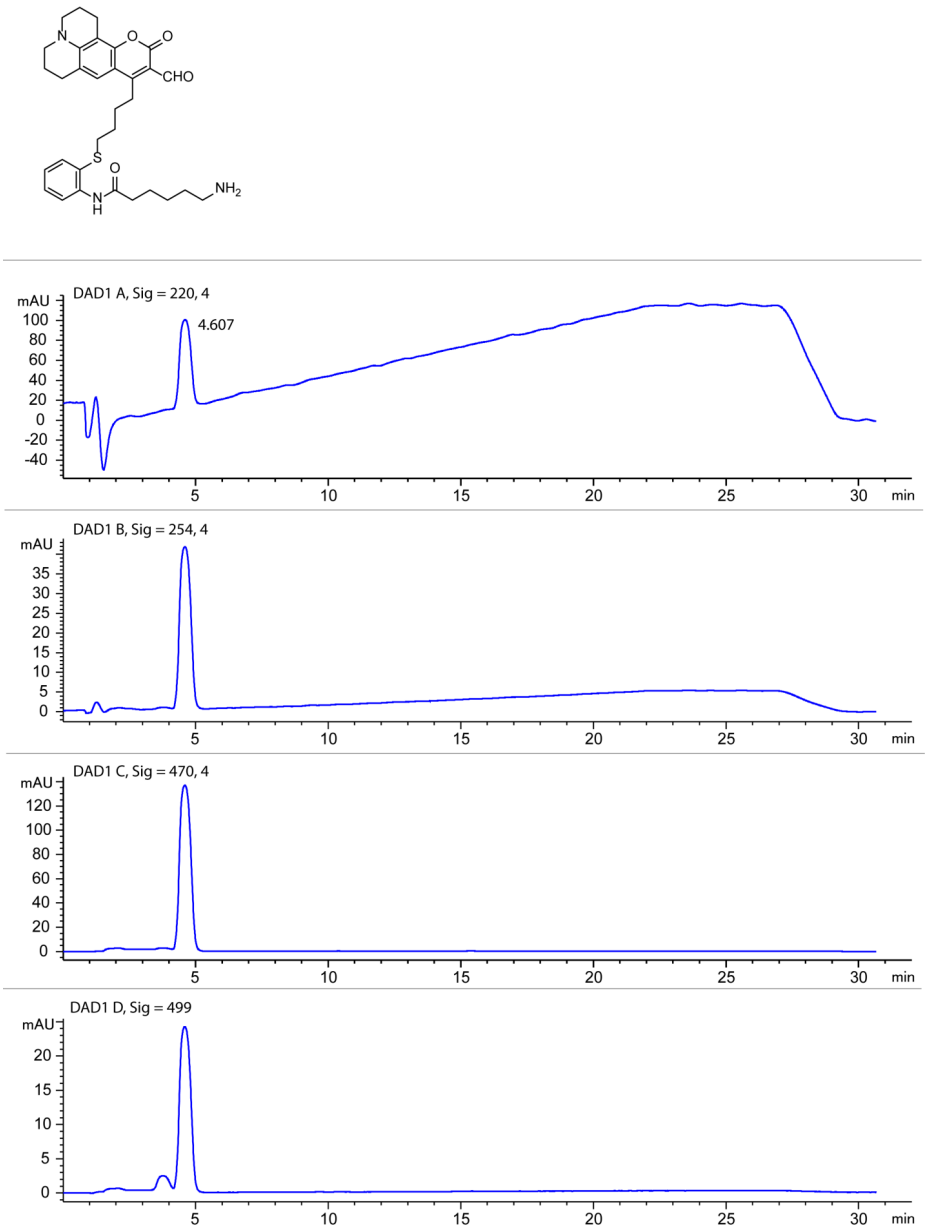


Figure S16. Reverse-phase chromatogram of an aliquot from quantitative deprotection of compound **3a** under acidic conditions eluted with a gradient of acetonitrile/water (+ 0.1% trifluoroacetic acid).

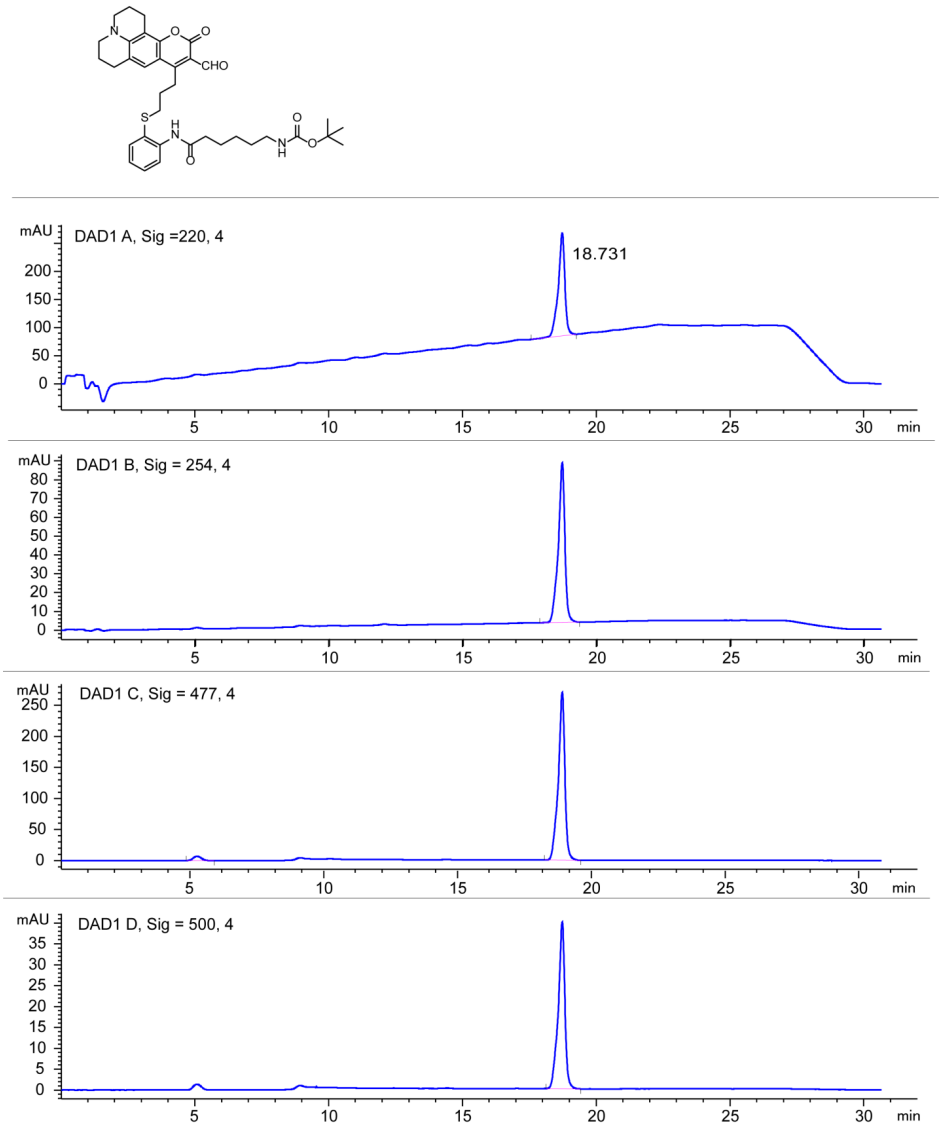


Figure S17. Reverse-phase chromatogram of Boc-protected model compound **3b** eluted with a gradient of acetonitrile/water (+ 0.1% trifluoroacetic acid).

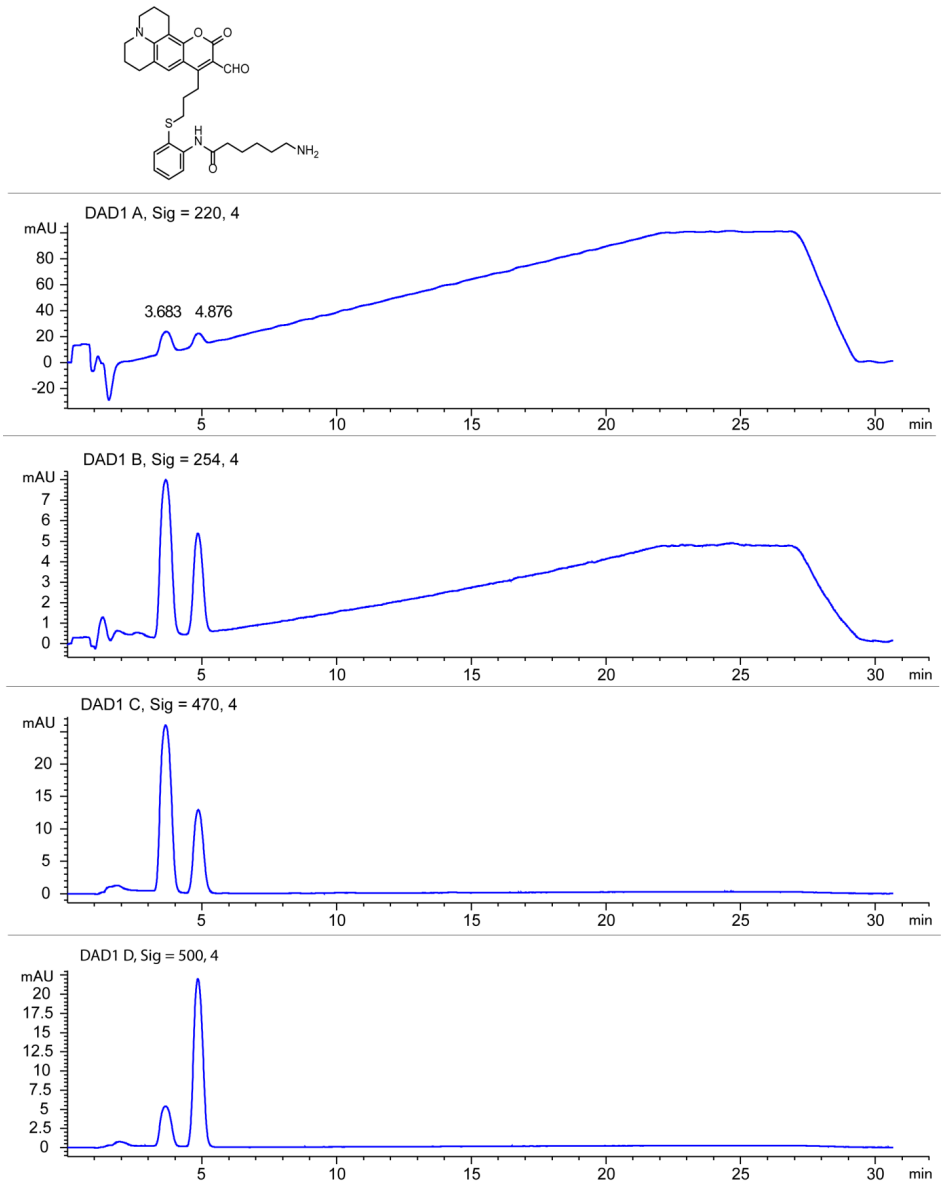


Figure S18. Reverse-phase chromatogram of an aliquot from quantitative deprotection of compound **3b** under acidic conditions eluted with a gradient of acetonitrile/water (+ 0.1% trifluoroacetic acid).

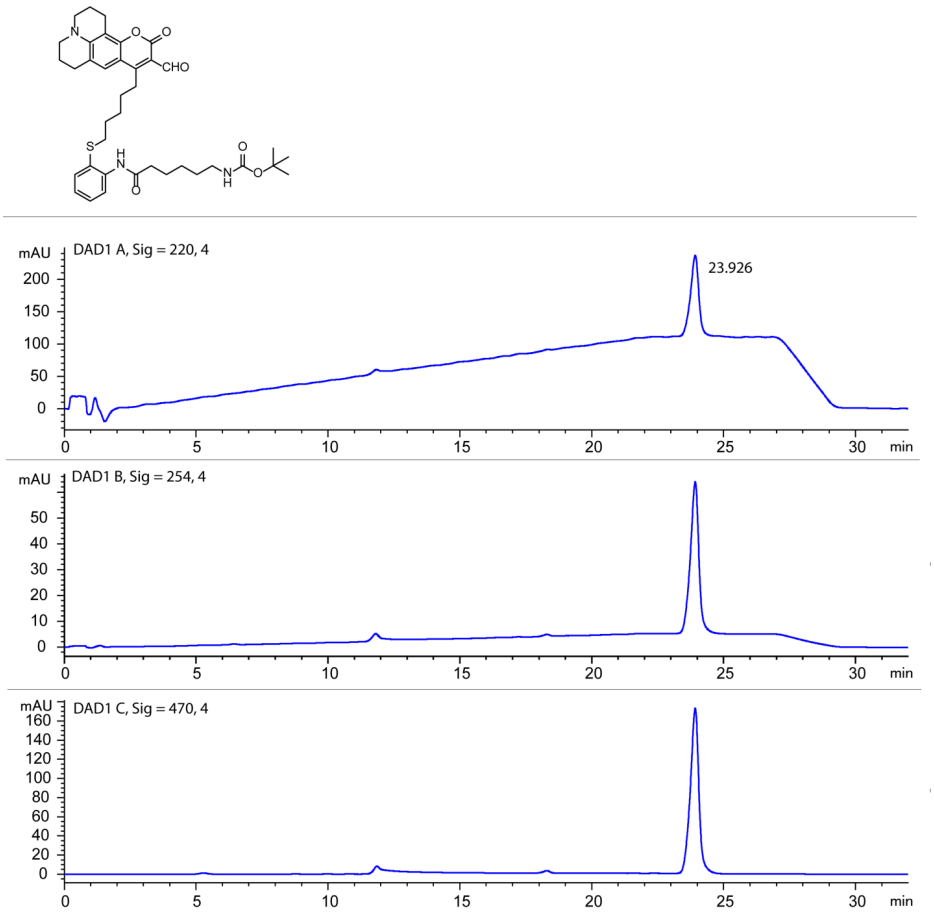


Figure S19. Reverse-phase chromatogram of Boc-protected model compound **3c** eluted with a gradient of acetonitrile/water (+ 0.1% trifluoroacetic acid).

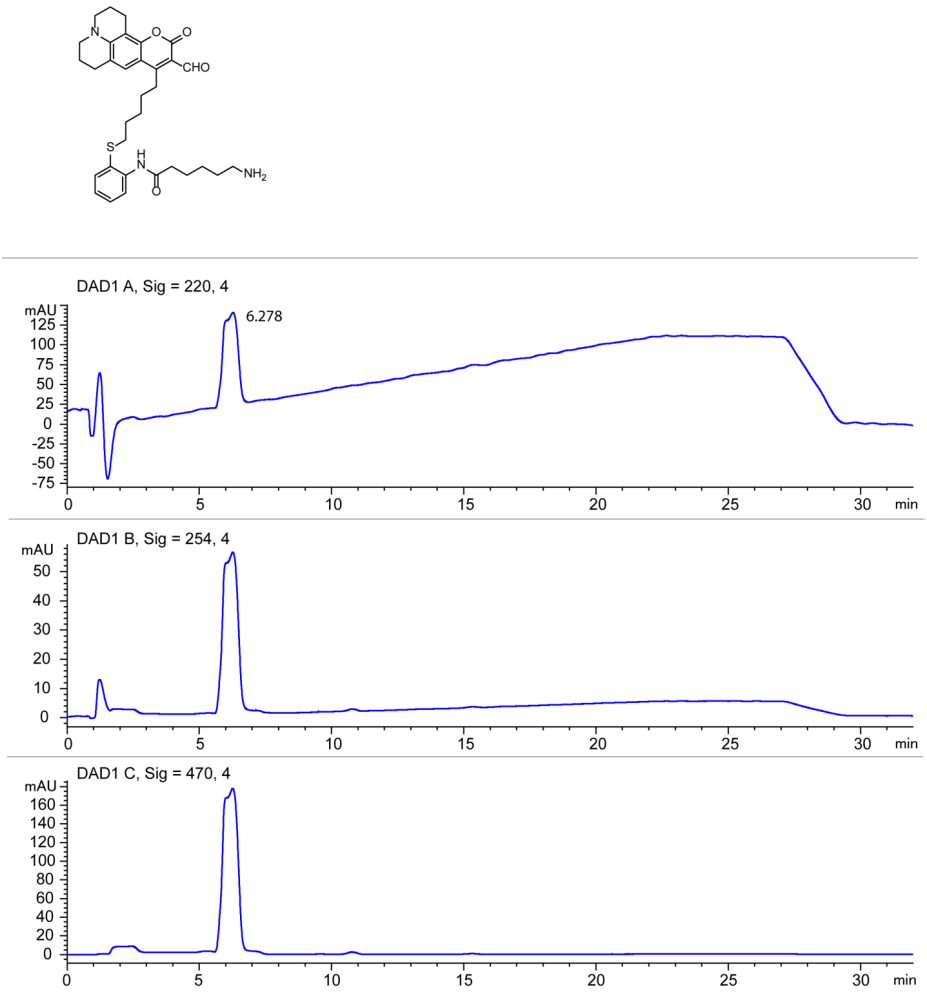


Figure S20. Reverse-phase chromatogram of an aliquot from quantitative deprotection of compound **3c** under acidic conditions eluted with a gradient of acetonitrile/water (+ 0.1% trifluoroacetic acid).

4. References

1) R. S. Coleman and M. L. Madaras, *J. Org. Chem.*, 1998, 63, 5700-5703.
2) B. D. Kelly and T. H. Lambert, *Org. Lett.*, 2011, 13, 740-743.
3) F. Kaiser, L. Schwink, J. Velder and H.-G. Schmalz, *J. Org. Chem.*, 2002, 67, 9248-9256.
4) J.-H. Zhang, T. D. Y. Chung and K. R. Oldenburg, *J. Biomol. Screen.*, 1999, 4, 67-73.