Development of a Fluorogenic Probe Based on a DNA Staining Dye for Continuous Monitoring of the Histone Deacetylase Reaction

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EXPERIMENTAL SECTION

Synthesis of a BOXTO derivative (compound **8**): Compound **8** was synthesized according to previously reported procedures with minor modifications (Scheme S1).[1,2]

Synthesis of compound 1: A suspension of *o*-aminophenol (2.10 g, 20.0 mol) and *p*-nitro benzoic acid (3.22 g, 20.0 mol) in Eaton's reagent (20 mL) was stirred at 120 °C under reflux for 2 h. After cooling, the reaction mixture was poured into a saturated $NaHCO_3$ aqueous solution and neutralized. The precipitate was collected by filtration, washed with water, and dried to afford compound **1** (4.71 g, 19.6 mmol, 98% yield) as a gray powder. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.45 (d, J = 8.8 Hz, 2H), 8.39 (d, J = 8.8 Hz, 2H), 7.84 (d, J = 8.0 Hz, 1H), 7.64 (d, J = 8.0 Hz, 1H), 7.46-7.40 (m, 2H). MS (ESI+) *m/z*: Calcd for $[M+H]^+$ 241.06, found 241.04.

Synthesis of compound 2: A suspension of compound **1** (1.02 g, 4.24 mmol) and Pd/C (32.5 mg, 10 wt%) in methanol (15 mL) was stirred at room temperature under H_2 atmosphere with balloon pressure. After stirring for 15 h, Pd/C was removed by filtration. The filtered solution was concentrated and dried to afford compound **2** (809 mg, 3.83 mmol, 90%) as a brown solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.86 (d, J = 8.8 Hz, 2H), 7.68-7.65 (m, 2H), 7.33-7.30 (m, *2*H), 6.70 (d, J = 8.8 Hz, 2H), 5.98 (s, 2H). MS (ESI+) *m/z*: Calcd for $[M+H]^+$ 211.09, found 211.08.

Synthesis of compound 3: *N*-bromosuccinimide (202 mg, 1.13 mmol) was added to a solution of compound **2** (215 mg, 1.02 mmol) in DMF on ice in portions over a 1 h period. The reaction mixture was warmed to room temperature and stirred under argon atmosphere. After stirring for 4 h, the reaction mixture was poured into water. The precipitate was collected by filtration, washed with water, and dried to afford compound **3** (294 mg, 0.98 mmol, 96% yield) as a brown powder. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.35 (s, 1H), 8.00 (d, J = 8.4 Hz, 1H), 7.72-7.70 (m, 1H), 7.55-7.53 (m, 1H), 7.33-7.30 (m, 2H), 6.85 (d, J = 8.4 Hz, 1H), 4.50 (s, 2H). MS (ESI+) *m/z*: Calcd for $[M+H]^+$ 313.05, found 313.01.

Synthesis of compound 4: A solution of compound **3** (251 mg, 868 μmol) and potassium ethylxanthate (373 mg, 2.33 mmol) in DMF (15 mL) was stirred at 140 °C under argon atmosphere. After stirring for 5.5 h, the reaction mixture was cooled and concentrated. Addition of 1% aqueous HCl solution gave a brown precipitate. The precipitate was collected by filtration, washed with water, and dried to afford compound **4** (249 mg, 861 μmol, 99% yield) as a brown solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 14.06 (s, 1H), 8.57 (s, 1H), 8.22 (d, J = 8.4 Hz, 1H), 7.82-7.78 (m, 2H), 7.48 (d, J = 8.4 Hz, 1H), 7.45-7.42 (m, 2H). MS (ESI+) *m/z*: Calcd for $[M+H]^+$ 285.02, found 284.99.

Synthesis of compound 5: Iodomethane (90 μL, 1.4 mmol) was added to a solution of compound **4** (206 mg, 724 μmol) and potassium carbonate (205 mg, 1.48 mmol) in DMF (1 mL). The reaction mixture was stirred at room temperature for 1 h. The resultant precipitate was collected by filtration, washed with water, and dried to afford compound **5** (207 mg, 694 μmol, 96% yield) as a brown powder. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.68 (s, 1H), 8.32 (d, J = 8.4 Hz, 1H), 7.98 (d, J = 8.4 Hz, 1H), 7.79-7.77 (m, 1H), 7.61-7.58 (m, 1H), 7.38-7.36 (m, 2H), 2.84 (s, 3H). MS (ESI+) *m/z*: Calcd for $[M+H]^+$ 285.02, found 284.99.

Synthesis of compound 6: A suspension of compound **5** (1.01 mg, 3.42 mmol) and methyl *p*-toluenesulfonate (1.0 mL, 6.6 mmol) in dioxane (3 mL) was stirred at 100 °C under an argon atmos-

phere. After stirring for 24 h, the reaction mixture was cooled and concentrated. Addition of diethyl ether gave a brown precipitate. The precipitate was collected by filtration, washed with ether, and dried to afford compound **6** (673 mg, 1.39 mmol, 40% yield) as a brown powder (as a tosylate salt). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.28 (s, 1H), 8.61 (dd, J = 8.8 Hz, 1.6 Hz, 1H), 8.39 (d, J = 8.8 Hz, 1H), 7.88 (t, J = 8.4 Hz, 2H), 7.53-7.46 (m, 4H), 7.11 (d, J = 8.0 Hz, 2H). 4.16 (s, 3H), 3.18 (s, 3H), 2.28 (s, 3H). MS (ESI+) *m/z*: Calcd for $[M]^+$ 313.05, found 313.01.

Synthesis of compound 7: A solution of 4-methylquinoline (1.77 mg 7.56 mmol) and 6-bromohexanoic acid (1.0 mL, 9.1 mmol) in dioxane (1 mL) was stirred at 100 °C under an argon atmosphere. After stirring for 24 h, the reaction mixture was cooled, and dichloromethane (10 mL) and acetone (10 mL) were added. The precipitate was collected by filtration, washed with ether, and dried to afford compound **7** (1.62 g, 4.76 mmol, 63% yield) as a white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.41 (d, J = 5.6 Hz, 1H), 8.56-8.52 (m, 2H), 8.25 (t, J = 7.6 Hz, 1H), 8.06-8.02 (m, 2H), 4.99 (t, J = 6.8 Hz, 2H), 2.99 (s, 3H), 2.20 (t, J = 7.2 Hz, 2H), 1.93-1.92 (m, 2H), 1.55-1.51(m, 2H), 1.37 (m, 2H). MS (ESI+) *m/z*: Calcd for $[M]^+$ 258.15, found 258.09.

Synthesis of compound 8 (BOXTO-CO_2H): Triethylamine (83 μL, 650 μmol) was added to a suspension of compound **6** (158 mg, 325 μmol) and compound **7** (110 mg, 325 μmol) in ethanol (2 mL). The reaction mixture was stirred at room temperature under an argon atmosphere. After stirring for 10 h, the mixture was concentrated, and diethyl ether was added. The precipitate was collected by filtration, washed with ether, and dried under reduced pressure to afford compound **8** as a red powder (122 mg, 203 μmol, 63% yield). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.83 (s, 1H), 8.79 (d, J = 8.8 Hz, 1H), 8.74 (d, J = 7.6 Hz, 1H), 8.34 (d, J = 8.8 Hz, 1H), 8.17 (d, J = 8.8 Hz, 1H), 8.01 (dd, J = 7.6 Hz, 1H), 7.80-7.77 (m, 3H), 7.45-7.41 (m, 3H), 6.95 (s, 1H), 4.63 (dd, J = 8.0 Hz, 2H), 4.02 (s, 3H), 2.21 (t, J = 7.2 Hz, 2H), 1.87 (t, J = 7.2 Hz, 2H), 1.55 (t, J = 8.0 Hz, 2H), 1.41-1.34 (m, 2H). MS (ESI+) *m/z*: Calcd for $[M+H]^+$ 522.18, found 522.16. HRMS (FAB+) *m/z*: Calcd for $[M+H]^+$ 522.1846, found 522.1844.

Synthesis of BOXTO-GK(Ac)G: BOXTO-GK(Ac)G was synthesized in a stepwise reaction by Fmoc solid-phase peptide synthesis on a 2-chlorotrityl chloride resin (41.5 μmol). In the coupling step, Fmoc-protected amino acids (166 μmol), PyBOP (166 μmol), and *N*,*N*-diisopropylethylamine (DIEA; 166 μmol) in dimethylformamide (DMF; 1 mL) were added to the resin. After mixing by rotation for 3 h, the resin was washed five times with DMF (1 mL). In the deprotection step, 20% piperidine (1 mL) was added to the resin. After mixing by rotation for 10 min, the resin was washed five times with DMF (1 mL). In the coupling of compound **8**, compound **8** (83 μmol), PyBOP (166 μmol), and DIEA (166 μmol) in NMP (2 mL) were added to the resin and mixed by rotation for 16 h. The resin was washed five times with DMF, methanol, and dichloromethane (1 mL), and dried. Then, trifluoroacetic acid (TFA) with triisopylsilane (2.5%) and water (2.5%) were added to the resin and mixed by rotation for 1 h. The resin was removed and the filtrate was concentrated. Addition of chilled diethyl ether gave a red crude product. The crude product was purified using a reversed-phase HPLC with an Inertsil ODS-3 (10.0 × 250 mm) column (20–70% of 0.1% HCOOH/CH_3CN with a linear gradient for 30 min). The detection wavelength was 500 nm. After lyophilization of the collected fraction, BOXTO-GK(Ac)G (7.85 mg, 9.74 μmol, 23% yield) was obtained as a red powder. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.73–8.71 (m, 3H), 8.24 (d, J = 8.4 Hz, 1H), 8.20 (brs, 1H), 8.11 (d, J = 8.4 Hz, 1H), 8.06 (brs, 1H), 7.95 (t, J = 7.6 Hz, 1H), 7.89 (d, J = 8.0 Hz, 1H), 7.82 (d, J = 8.8 Hz, 1H), 7.73-7.66 (m, 4H), 7.36–7.32 (m, 3H), 6.84 (s, 1H), 4.58 (brs, 2H), 4.28-4.26 (m, 1H), 3.97 (s, 3H), 3.74-3.72 (m, 4H), 2.96 (q, J = 6.0 Hz, 2H), 2.16 (t, J = 6.8 Hz, 2H), 1.90–1.85 (m, 2H), 1.76 (s, 3H), 1.67–1.65 (m, 1H), 1.58 (t, J = 6.8 Hz, 2H), 1.52–1.49 (m, 1H), 1.38–1.33 (m, 4H), 1.27–1.23 (m, 2H). MS (ESI+) *m/z*: Calculated for $[M+H]^+$ 806.33, found 806.38. HRMS (FAB+) *m/z*: calculated for $[M+H]^+$ 806.3336, found 806.3325. ε

(MeOH): 81600 (511), 15400 (337), 20700 (278), (5 mM sodium phosphate buffer, pH = 7.0): 61000 (486), 15800 (336), 24000 (282).

Synthesis of BOXTO-GKG: BOXTO-GKG was synthesized as a red powder (13.7 mg, 17.9 μmol, 41% yield) using a procedure similar to that described for the preparation of BOXTO-GK(Ac)G. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.76–8.73 (m, 3H), 8.27 (d, J = 8.4 Hz, 1H), 8.12 (d, J = 8.4 Hz, 1H), 8.07 (brs, 1H), 7.99–7.95 (m, 2H), 7.84 (d, J = 8.8 Hz, 1H), 7.76–7.69 (m, 4H), 7.36–7.34 (m, 3H), 6.86 (s, 1H), 4.59 (br, 2H), 4.30–4.28 (m, 1H), 3.98 (s, 3H), 3.73–3.71 (m, 2H), 3.67–3.65 (m, 1H), 3.55 (brs, 2H), 2.74 (m, 2H), 2.16 (t, J = 6.4 Hz, 2H), 1.89–1.85 (m, 2H), 1.67–1.65 (m, 1H), 1.58–1.50 (m, 6H), 1.39–1.33 (m, 4H). MS (ESI+) *m/z*: Calculated for $[M+H]^+$ 764.32, found 764.39. HRMS (FAB+) *m/z*: calculated for $[M+H]^+$ 764.3230, found 764.3229. ε (MeOH): 81600 (511), 15500 (339), 20200 (278), (5 mM sodium phosphate buffer, pH = 7.0): 62500 (486), 14700 (342), 22900 (282).

Synthesis of BOXTO-K(Ac): BOXTO-K(Ac) was synthesized as a red powder (16.2 mg, 23.4 μmol, 46% yield) using a procedure similar to that described for the preparation of BOXTO-GK(Ac)G. MS (ESI+) *m/z*: Calcd for $[M+H]^+$ 692.29, found 692.36. HRMS (FAB+) *m/z*: calced for $[M+H]^+$ 692.2901, found 692.2906.

CD spectrometry: Compounds were dissolved in DMSO to prepare stock solutions, which were diluted to the required concentrations for measurements. Absorption and CD spectra of BOXTO-GK(Ac)G and BOXTO-GKG (10 μM) were measured in 20 mM Tris·HCl buffer (pH = 8.0) containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and calf thymus DNA (250 μM base pair concentration) at 25 °C. To obtain the CD spectra, the samples were scanned four times.

Fluorescence titration experiment and determination of binding constant: A hairpin shaped oligodeoxynucleotide (ODN) 5′-CGAATTCGCTCTGCTTAAGC-3′ (the hybridizing sequences are underlined) was purchased from Greiner Bio-One Co. Ltd. 2 μM of BOXTO-GKG or BOXTO-GK(Ac)G compounds was mixed with a series of concentrations of the ODN in Tris·HCl buffer (20 mM, pH = 8.0) containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and histone deacetylases (100 nM), pre-incubated at 25 °C. Fluorescence was measured with an excitation at 520 ± 8 nm using an ARVOmx fluorescent microplate reader at 25 °C. The concentration of the complex determined from the fluorescence intensity was plotted with the concentration of ODN. The Equilibrium dissociation constant (K_d) was fitted using the following equation. [L] represents the initial concentration of the ligand, BOXTO-GKG.[3]

$$[\text{complex}] = \frac{(K_d + [ODN] + [L]) - \sqrt{(K_d + [ODN] + [L])^2 - 4[ODN] \times [L]}}{2} \quad \text{(S1)}$$

Detection of p300 histone acetyltransferase (HAT) activity: BOXTO-GKG (3 μM) was reacted with histone p300 acetyltransferase (20 nM) and acetyl-CoA (100 μM) in HAT assay buffer (BPS Biosciences) at 37 °C for 1 h. After addition of 75 μM base pairs ctDNA, the fluorescence was measured using an ARVOmx fluorescent plate reader at 37 °C. The excitation and detection wavelengths were 520 ± 8 and 545 ± 7 nm, respectively.

Conventional fluorescence HDAC activity assay: A Fluor-de-Lys SIRT1 substrate probe (Enzo Lifescience™, 50 μM) in Tris-HCl buffer (20 mM, pH = 8.0) containing 137 mM NaCl, 2.7 mM KCl, 1

mM $MgCl_2$, and NAD^+ (300 μM) in the presence of ctDNA (75 μM base pair concentration) was pre-incubated at 37 °C. SIRT1 deacetylase (100 nM) was added to the sample and incubated at 37 °C for 30 min. Subsequently, the Developer reagent was added and the sample was incubated at 37 °C for 30 min. The fluorescence was measured using an ARVOmx fluorescent plate reader at 37 °C. The excitation and detection wavelengths were 355 ± 40 and 460 ± 40 nm, respectively.

Determination of kinetic parameters: The product concentrations were calculated from the fluorescence using the following equation.

$$[\text{product}] = [S_{\text{total}}] \times \frac{F - F_0}{F_{100} - F_0} \quad (S2)$$

$[S_{\text{total}}]$ represents the total substrate concentration, F_0 the fluorescence in the absence of the enzyme, and F_{100} the fluorescence of BOXTO-GKG, a fully deacetylated product, at the same concentration. The initial reaction rate (v), which was calculated from the linear phase of the progression curve, and the substrate concentration ($[S]$) were plotted, and then fitted to the standard Michaelis-Menten equation:

$$v = \frac{V_{\text{max}}[S]}{K_{\text{m}} + [S]} \quad (S3)$$

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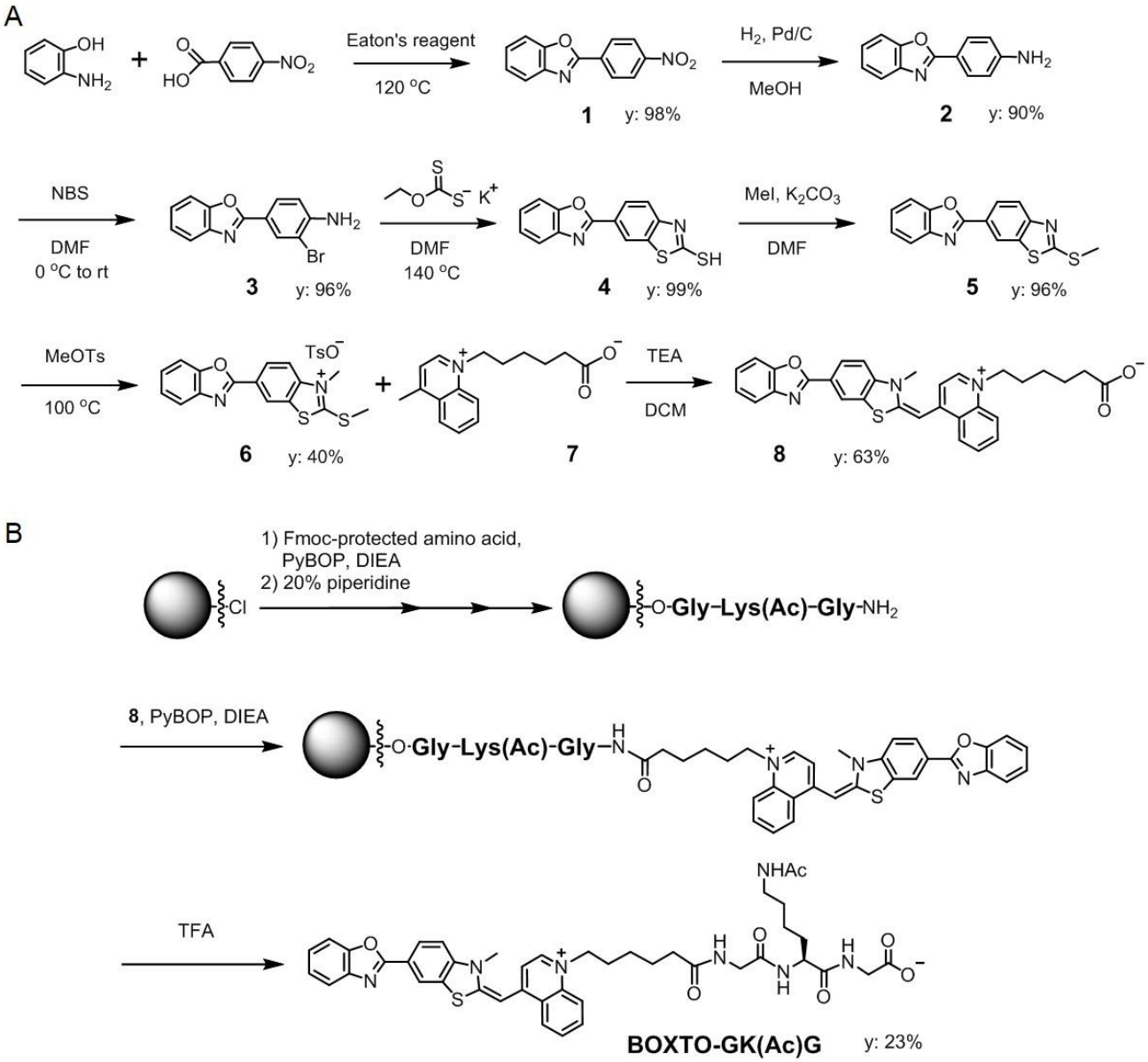
(2) Karlsson, H.; Bergqvist, M.; Lincoln, P.; Westman, G. *Bioorg. Med. Chem.* **2004**, *12*, 2369–2384.
(3) Haq, I.; Ladbury, J. E.; Chowdhry, B. Z.; Jenkins, T. C.; Chaires, J. B. *J. Mol. Biol.* **1997**, *271*, 244–257.



Scheme S1. (A) Synthesis of BOXTO dye derivative **8**. (B) Solid phase synthesis of BOXTO-GK(Ac)G.

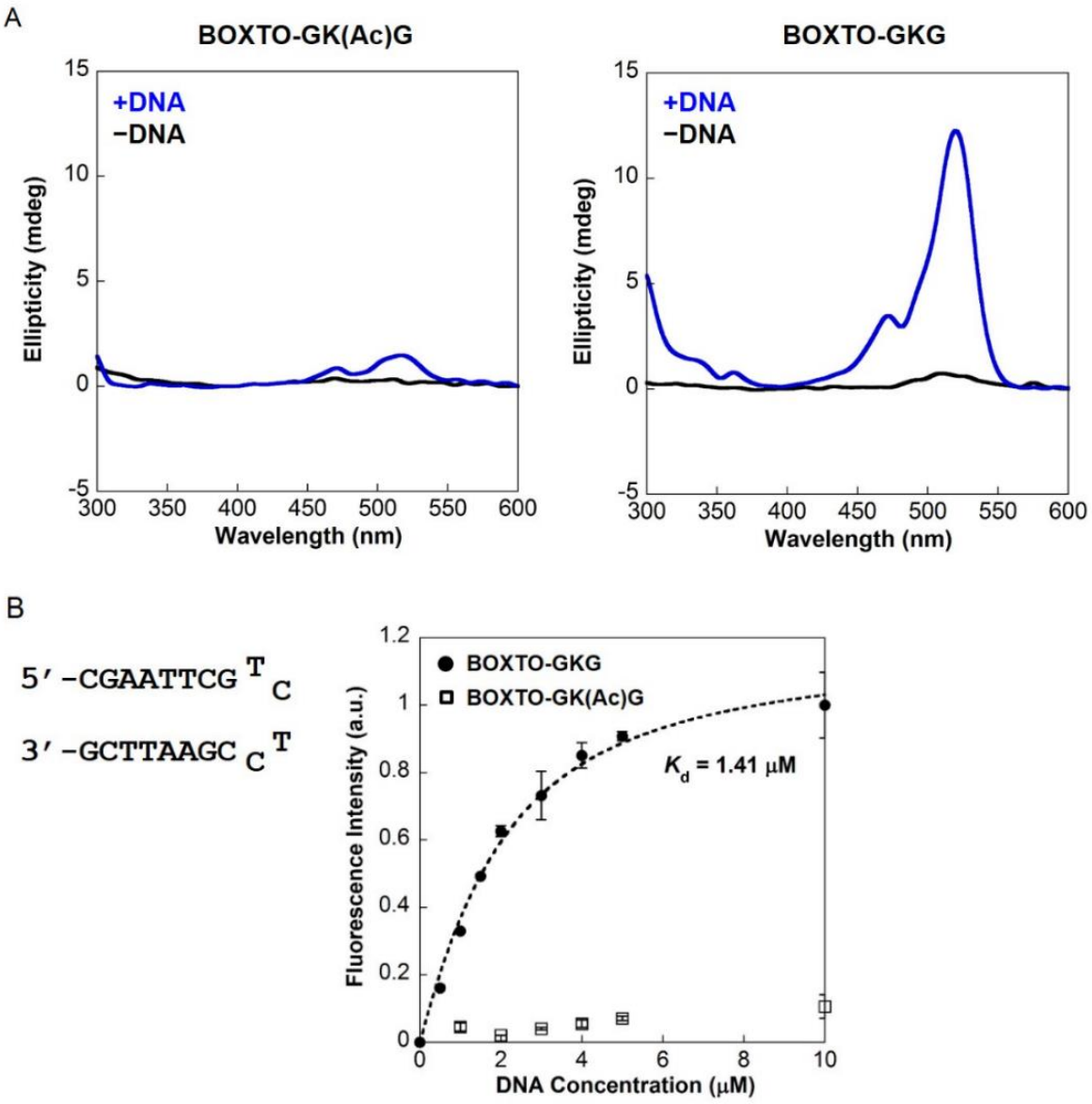


Figure S1. Comparison of the DNA binding abilities of BOXTO-GK(Ac)G and the deacetylated BOXTO-GKG probes. (A) Left: CD spectra of 10 μM BOXTO-GK(Ac)G in the absence (black line) and presence of ctDNA (blue line). Right: CD spectra of 10 μM BOXTO-GKG in the absence (black line) and presence of ctDNA (blue line). (B) Fluorescence titration results of 2 μM BOXTO-GK(Ac)G (filled circles) and BOXTO-GKG (open squares) in the presence of 0–10 μM oligodeoxynucleotide hairpin (shown in left). Error bars represent standard deviation (n = 3).

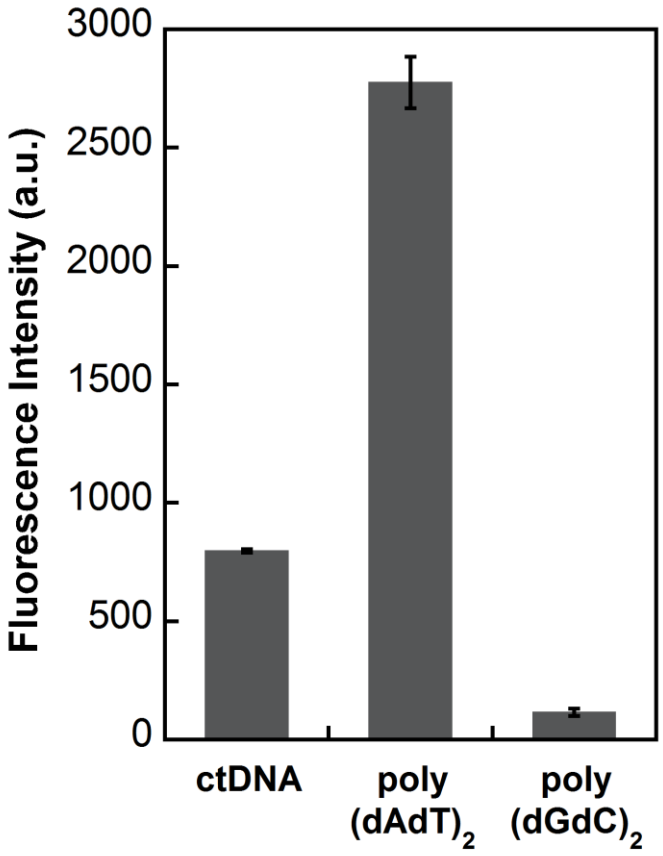


Figure S2. Effects on DNA sequence of the fluorescence of BOXTO-GKG. 3 μM BOXTO-GK(Ac)G in the presence of 75 μM base pairs ctDNA, poly(dA-dT)$_2$ DNA, and poly(dG-dC)$_2$ DNA. The fluorescence was measured using an ARVOmx fluorescent plate reader at 25 °C. The excitation and detection wavelengths were 520 ± 8 and 545 ± 7 nm, respectively. Error bars represent standard deviation (n = 3).

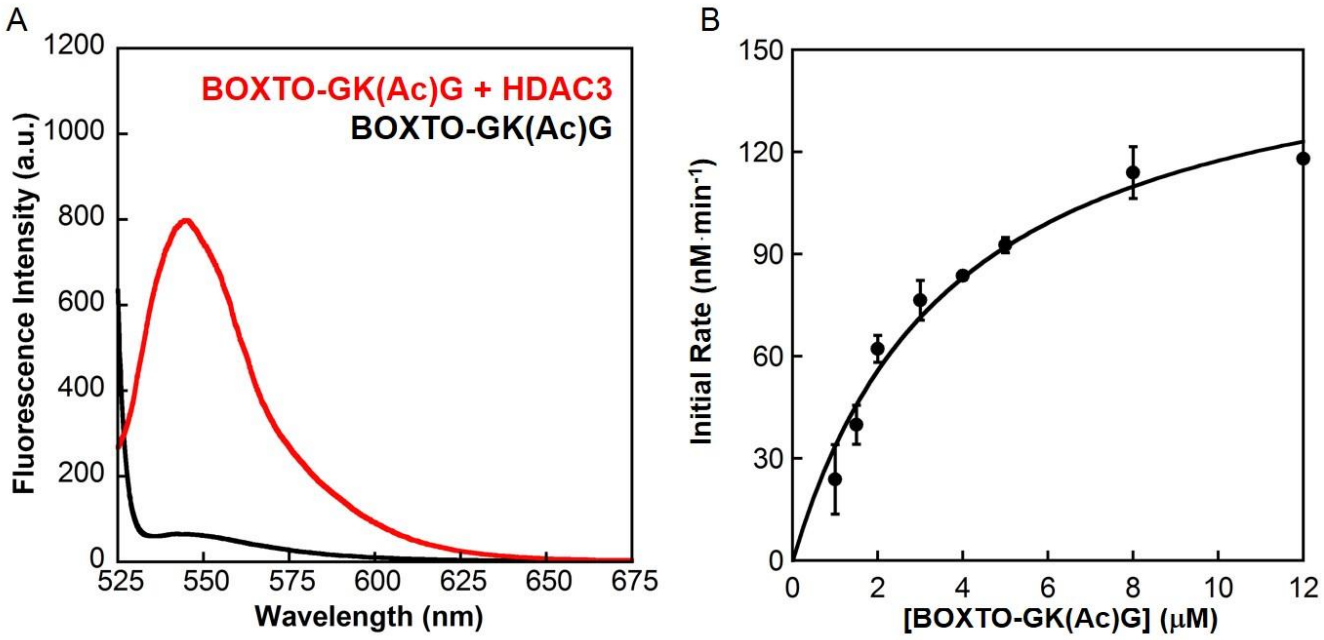


Figure S3. Fluorogenic HDAC3/NCOR1 detection using BOXTO-GK(Ac)G and DNA. (A) Fluorescence spectra of 3 μM BOXTO-GK(Ac)G (black line) and BOXTO-GK(Ac)G reacted with HDAC3/NCOR1 (class I HDAC) for 30 min (red line). After the reaction, the samples were mixed with an excess amount of DNA (75 μM base pair concentration) and their fluorescence spectra were measured. The excitation wavelength was 520 nm. (B) Plot of substrate probe concentration versus initial reaction rate of HDAC3/NCOR1. Error bars represent standard deviation (n = 3).

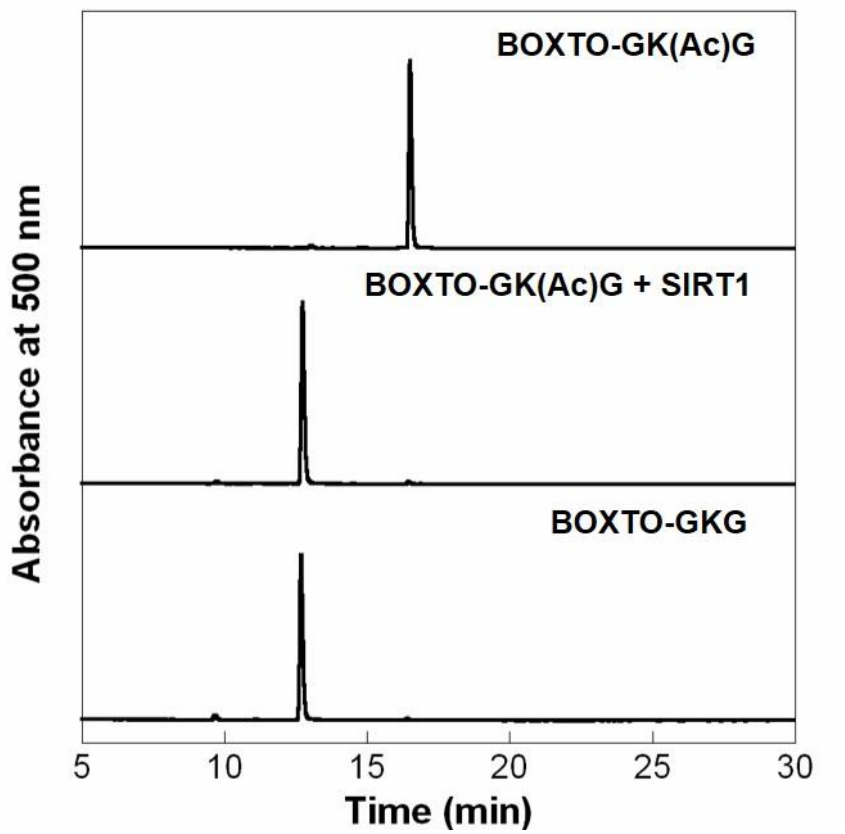


Figure S4. (a) HPLC analysis of BOXTO-GK(Ac)G (top), BOXTO-GK(Ac)G reacted with SIRT1 deacetylase (middle), and BOXTO-GKG (bottom). Analytical conditions: 20-70% CH_3CN in 0.1% formic acid in water with a linear gradient for 30 min; flow rate = 1.0 mL/min; detection wavelength was 500 nm.

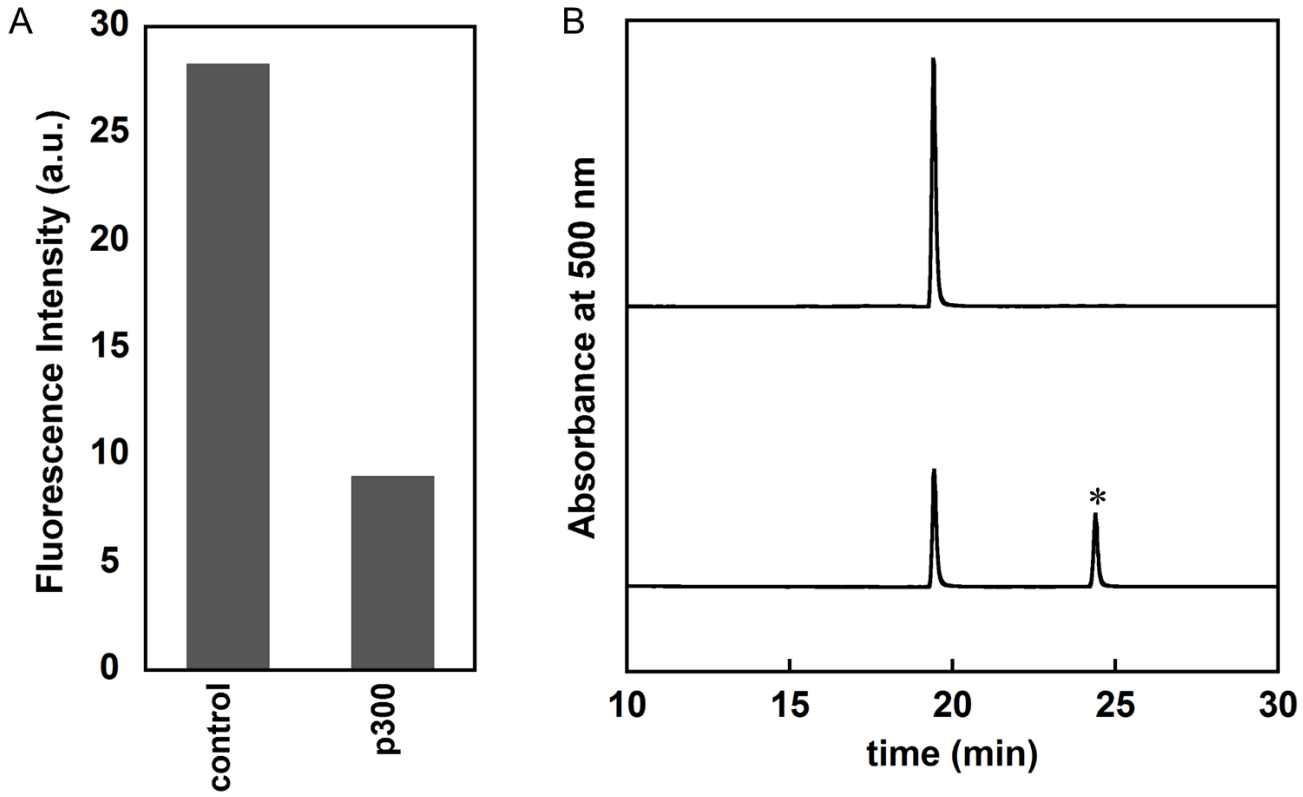


Figure S5. Detection of p300 histone acetyltransferase (HAT) activity. (A) Fluorescence of 3 μM BOXTO-GKG (control) and BOXTO-GKG reacted p300 histone acetyltransferase after addition of 75 μM base pairs ctDNA. (B) HPLC analysis of BOXTO-GKG (top) and BOXTO-GKG reacted with p300 histone acetyltransferase in the presence of 100 μM acetyl-CoA for 1 h (bottom). The peak with an asterisk indicates the acetylated product, BOXTO-GK(Ac)G (MS (ESI+) *m/z*: found 806.38). Analytical conditions: 10–50% CH_3CN in 0.1% formic acid in water with a linear gradient for 30 min; flow rate = 1.0 mL/min; detection wavelength was 500 nm.

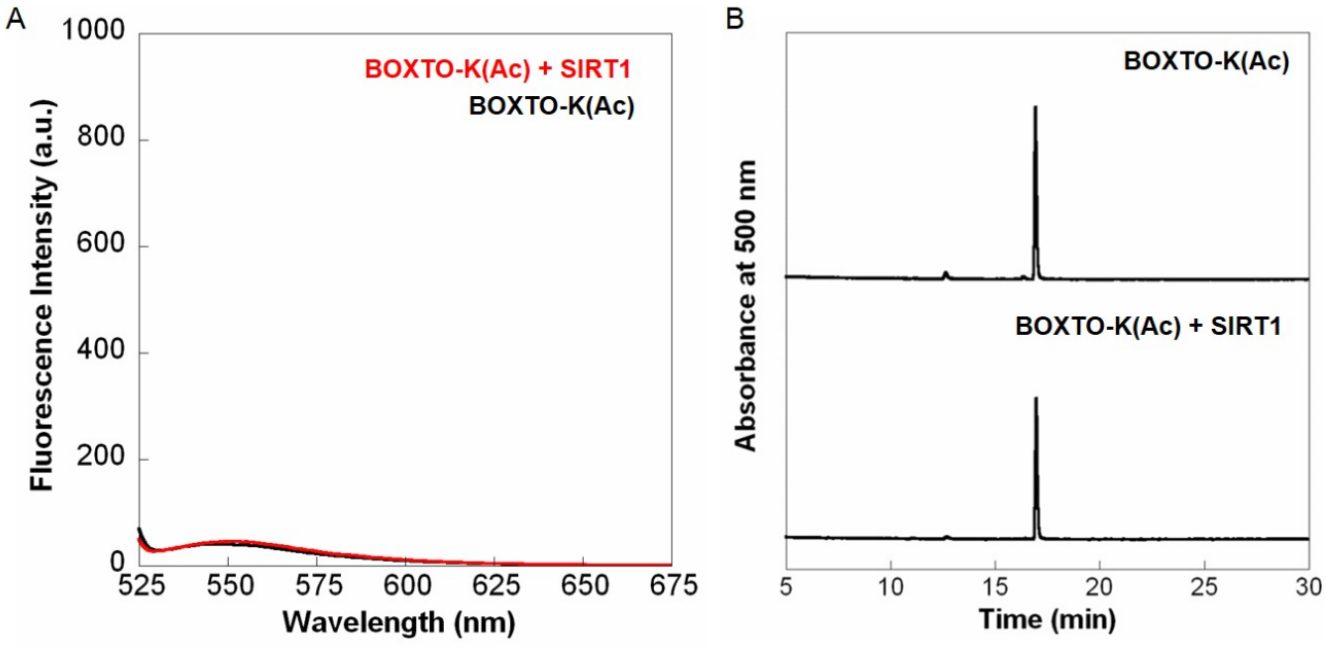


Figure S6. Effects on the flanking amino acids of the probe (A) Fluorescence spectra of 3 μM BOXTO-K(Ac) (black line) and BOXTO-K(Ac) reacted with 100 nM SIRT1 histone deacetylase (red line) for 30 min. (B) HPLC analysis of BOXTO-K(Ac) (top) and BOXTO-K(Ac) reacted with SIRT1 deacetylase (bottom). Analytical conditions: 20–70% CH_3CN in 0.1% formic acid in water with a linear gradient for 30 min; flow rate = 1.0 mL/min; detection wavelength was 500 nm.

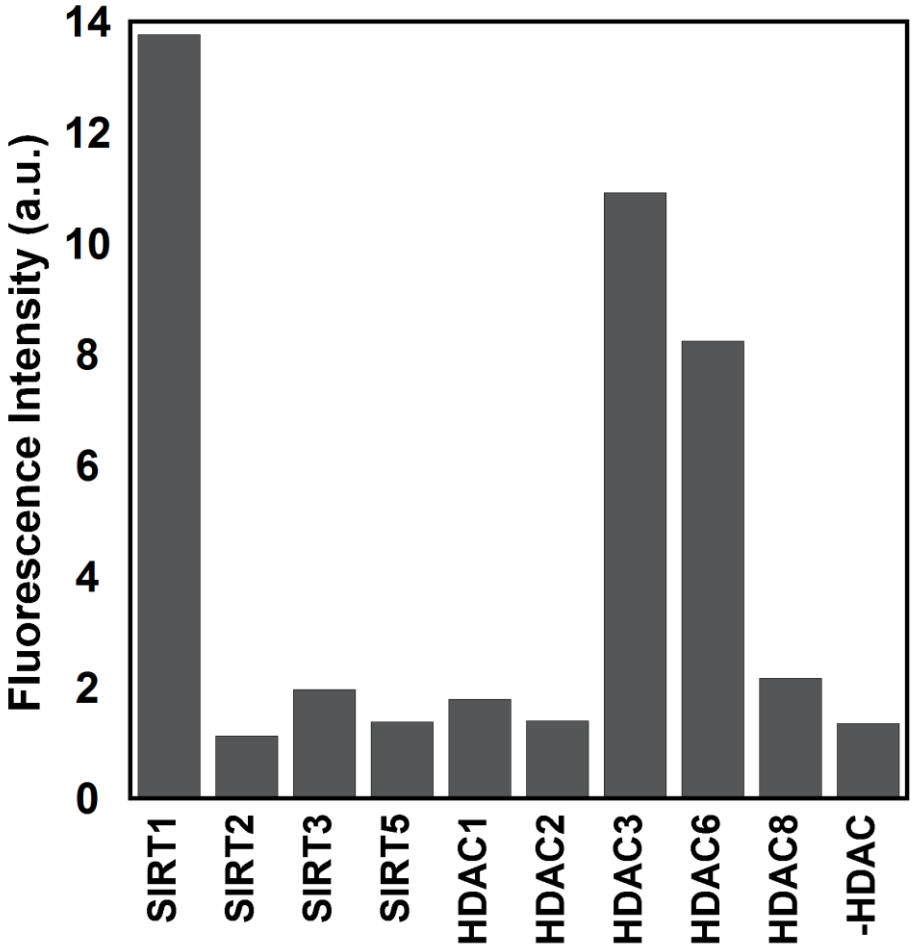


Figure S7. Fluorescence detection of a variety of HDACs using BOXTO-GK(Ac)G and DNA. 3 μM BOXTO-GK(Ac)G was reacted with 100 nM HDACs in the presence of 75 μM base pairs ctDNA for 1 h at 37 °C. The fluorescence was measured using an ARVOmx fluorescent plate reader at 37 °C. The excitation and detection wavelengths were 520 ± 8 and 545 ± 7 nm, respectively. SIRT2-5, and HDAC1, 2, 3, and 6 were purchased from Enzo lifescience™.

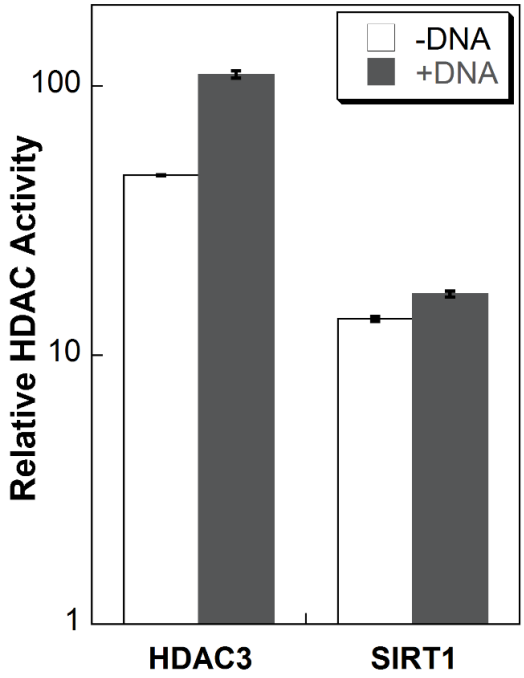


Figure S8. Fluor-de-Lys assay of HDAC3/NCOR1 and SIRT1 deacetylases activity in the absence (white bars) and presence (dark gray bars) of ctDNA (75 μM base pair concentration). Error bars represent standard deviations (n = 3).