

## Supplementary Information

### Mechanism-based Affinity Capture of Sirtuins

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### S1. Expression and purification of sirtuin enzymes

*Archaeoglobus fulgidus* Sir2 (Af2Sir2) was expressed and purified according to previously published protocols.<sup>1</sup> Yeast Sir2p plasmid was a gift from the Guarente laboratory and was expressed and purified as described.<sup>2</sup> The coding sequences for SirT1, SirT2, SirT3(118-399), SirT5(34-302) and SirT6 were cloned into a pSTblue vector (Novagen) by blunt cloning and subsequently cloned into a pET28a vector (Novagen) for expression. The sequences of the cloned genes were verified by nucleotide sequencing and confirmed against the NCBI published sequence. The plasmids containing the SIRT sequences were then transfected into BL21-CodonPlus(DE3)-RIPL competent cells (Stratagene) and protein expression induced by IPTG when the cells reached OD<sub>600</sub> of 0.6~0.7 in LB media. Protein induction was optimized for each sirtuin isoform. Specifically, SirT1 was induced by 100 μM IPTG overnight at 25 °C; SirT2 was induced with 500 μM IPTG for 1 h at 37 °C; SirT3(118-399) was induced with 300 μM IPTG overnight at 25 °C; SirT6 was induced with 500 μM IPTG for 1 h at 37 °C; and SirT5 and yeast Sir2 were induced with 500 μM IPTG for 3 h at 37 °C. Cultures were pelleted at 4000 rpm for 30 minutes, and cells lysed by three freeze-thaw cycles. Lysate was incubated with Ni-NTA resin (GBiosciences) at 4 °C for 2 h and then washed sequentially with Buffer A (20 mM potassium phosphate, 300 mM NaCl, 10 mM imidazole) and Buffer B (20 mM potassium phosphate, 300 mM NaCl, 20 mM imidazole) and protein was eluted with Buffer C (20 mM potassium phosphate, 300 mM NaCl, 200 mM Imidazole). The sirtuin proteins were frozen with 15% glycerol, 2.5 mM DTT and stored at -80 °C. Purity was determined by SDS-PAGE and silver

staining. Protein concentration was determined by Bradford assay. In the case of SirT5 and yeast Sir2 Ni-NTA resin was washed sequentially with Buffer A (50 mM potassium phosphate, 100 mM NaCl, 10 mM imidazole), Buffer B (50 mM potassium phosphate, 100 mM NaCl, 20 mM imidazole) and protein was eluted with Buffer C (50 mM potassium phosphate, 100 mM NaCl, 250 mM imidazole).

## **S2. Sirtuin inhibition studies and pull-down assay**

Peptides utilized as sirtuin substrates are identified as follows: (1) peptide based on p53 sequence (KKGQSTSRHK(AcK)LMFKTEG) is called p53mer; (2) peptide based on p300 sequence (ERSTEL(AcK)TEI(AcK)EEEDQPSTS) is called p300mer; (3) peptide based on histone H3 sequence (ARTKQTAR(AcK)STGG(AcK)APRKQLAS is called H3mer; (4) peptide based on histone H4 sequence (SGRG(AcK)GG(AcK)GLG(AcK)GGA(AcK)RHR) is called H4mer. The peptides were synthesized by the Proteomic Resource Center at Rockefeller University. The non-thioacetylated peptide N<sup>α</sup>-4-nitrobenzoyl-N<sup>ε</sup>-acetyl-lysiny-alaniny-alanine methyl ester abbreviated NBKAcAAOMe was synthesized in our laboratory (refer to synthetic procedures).

**Assay for sirtuin inhibition via thioacetylated peptide 1.** A typical reaction containing NAD<sup>+</sup>, at 300 μM for SirT1, 800 μM for SirT2, 750 μM for SirT3(118-399), 800 μM for Af2Sir2, and 500 μM for yeast Sir2, and enzyme, 8.75 μM SirT1, 5.5 μM SirT2, 2.35 μM SirT3(118-399), 12.08 μM Af2Sir2, and 2.06 μM yeast Sir2, was incubated for 30 min with varying concentrations of inhibitor **1** (0 to 1000 μM) at 37 °C in 50 mM KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.0. Deacetylation activity was initiated with the introduction of substrate, 200 μM p53mer for SirT1, 500 μM p53mer for SirT2, 500 μM H3mer for SirT3, 540 μM NBKAcAAOMe for Af2Sir2, and 500 μM H3mer for yeast

Sir2, to the reaction and incubated at 37 °C for 15 min (Af2Sir2) or 30 min (SirT1, SirT2, SirT3(118-399) and yeast Sir2). Reactions were quenched with 8  $\mu$ L 10% TFA on ice. Quenched samples were subjected to centrifugation and then injected on an HPLC fitted to a Macherey-Nagel Nucleosil C18 column. AADPR, NAD<sup>+</sup> and nicotinamide (NAM) peaks were resolved using a gradient of 0 to 10% methanol in 20 mM ammonium acetate pH 7. Chromatograms were analyzed at 260 nm. Data was plotted in a semi-log fashion as percent remaining enzyme activity as a function of the log[**1**] in nanomolar using Kaleidagraph software. Curve fits were generated using the following equation:  $v(\%) = v(\%)_I - [v(\%)_I(10^x)/(10^x + IC_{50})]$ .  $v(\%)$  represents turnover rate expressed as percent enzyme activity remaining and  $v(\%)_I$  represents the initial turnover rate expressed as an enzyme activity of 100%. The variable x represents the log[**1**] in nanomolar; therefore,  $10^x$  is indicative of the concentration of **1** in nanomolar.  $IC_{50}$  values were calculated from this equation. Data for all enzymes tested are summarized in Table 1 of main text and shown in Fig. S1.

**Compound 1 competitive inhibition of SirT1.** Reactions containing either 0  $\mu$ M, 5  $\mu$ M, or 10  $\mu$ M inhibitor **1** were incubated with 300  $\mu$ M NAD<sup>+</sup>, with varying concentrations of p300mer substrate (25, 50, 75, 125, or 200  $\mu$ M) and 2.02  $\mu$ M SirT1 in 180 mM KH<sub>2</sub>PO<sub>4</sub>/KOAc buffer (pH 7.25) for 60 min at 37 °C. Reactions were quenched with 10  $\mu$ L 10% TFA on ice. Quenched samples were subjected to centrifugation and then injected on an HPLC fitted to a Macherey-Nagel Nucleosil C18 column. AADPR, NAD<sup>+</sup> and nicotinamide (NAM) peaks were resolved using a gradient of 0 to 10% methanol in 20 mM ammonium acetate. Chromatograms were analyzed at 260 nm.

Double reciprocal plots ( $1/\text{rate}$  versus  $1/[\text{p300}]$ ) were generated using Kaleidagraph® and fit to a linear curve representative of the following Lineweaver-Burk relationship:  $1/v = (1/v_{\text{max}}) + [((1 + [I]/K_i)K_m/v_{\text{max}})](1/[\text{p300}])$ . Intersection at the y-axis ( $1/v_{\text{max}}$ ) indicates competitive inhibition. An average value for the  $K_i$  of 611 nM with a standard deviation of 248 nM was obtained for **1** inhibition of SirT1.

**Validation of **2** as a substrate for SirT1.** Reactions containing 500  $\mu\text{M}$  of **2** or  $\text{NAD}^+$  and either: (1) 500  $\mu\text{M}$  of p53mer peptide; (2) 250  $\mu\text{M}$  of inhibitor peptide **1**; or, (3) a combination of 500  $\mu\text{M}$  of p53mer and 250  $\mu\text{M}$  of **1** in 100 mM phosphate buffer at pH 7.5 were initiated by addition of SirT1 to a final concentration of 10.7  $\mu\text{M}$ . The reactions were incubated at 37 °C for 20 min before quenched by addition of 10% TFA to pH 2. After centrifugation at 13,000 g for 2 min to remove precipitates, reactions were injected on an HPLC fitted to a Macherey-Nagel Nucleosil C18 column, **2** and corresponding products were resolved by running a gradient of 0 to 10% methanol in 20 mM ammonium acetate. Chromatograms were analyzed at 260 nm. Deacetylation product for **2** (6-AMX-2', 3'-AADPR) was isolated by HPLC and assayed by MALDI-TOF ( $M+\text{Na} = 743$ , positive ion mode, Figure S3). For comparison,  $\text{NAD}^+$  and p53mer in 100 mM phosphate buffer pH 7.5 were incubated with SirT1 at 37 °C, and this procedure generated 2'- and 3'-AADPR. 2'- and 3'-AADPR were isolated and MALDI confirmed the corresponding mass ( $M+\text{Na} = 624$ , positive ion, Figure S3).

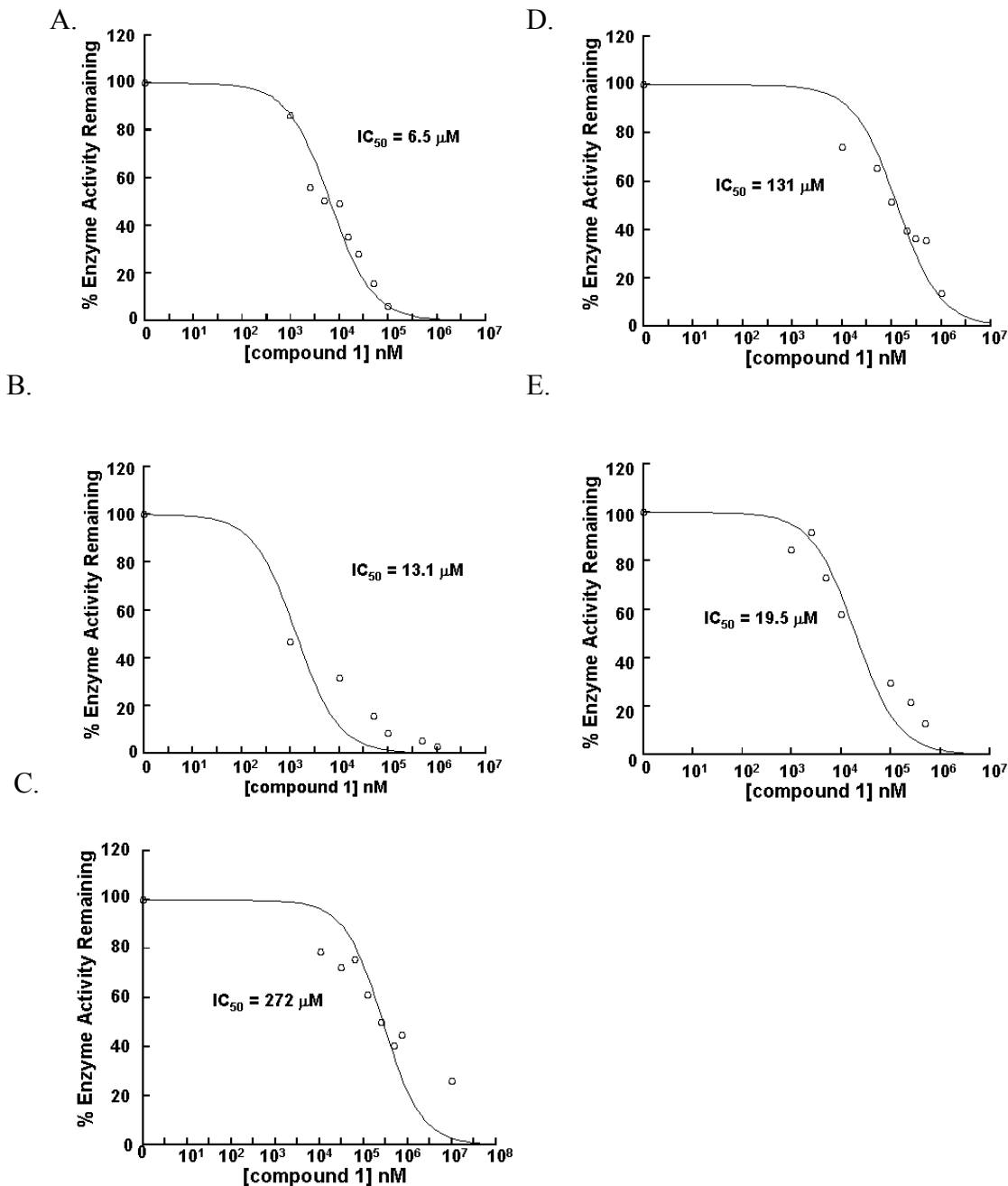
**Steady state substrate parameters for **2**.** Reactions containing 250  $\mu\text{M}$  of p53mer and various concentrations of **2** (0, 10, 20, 50, 75, 100, 125, 150, 200, 400, 500, 800, 1000 and 2000  $\mu\text{M}$ ) in 100 mM phosphate buffer pH 7.5 were initiated with the addition of SirT1 (final concentration 4.04  $\mu\text{M}$ ). The reactions were incubated at 37°C for 1 h and

quenched by addition of 10  $\mu\text{L}$  of 10% TFA. After centrifugation at 13,000 g for 2 min to remove precipitates, reactions were injected on an HPLC fitted to a Macherey-Nagel Nucleosil C18 column, and **2** and corresponding products were resolved by running a gradient of 0 to 10% methanol in 20 mM ammonium acetate and chromatograms were analyzed at 260 nm. Reactions were quantified by integrating areas of peaks corresponding to **2** and deacetylation product. Rates were plotted versus the concentration of **2** and fit to the Michaelis-Menten equation performed by Kaleidagraph®.

**Sirtuin capture assay.** A typical reaction was performed in 300 mM phosphate buffer pH 7.5 in a total volume of 50  $\mu\text{L}$ . Each reaction contained 800  $\mu\text{M}$  of **2** and 400  $\mu\text{M}$  of **1**, three control experiments were run at the same time: the first one contained 800  $\mu\text{M}$  of **2** and 500  $\mu\text{M}$  of peptide substrate, p53mer for SirT1, H3mer for SirT2, H3mer for SirT3, p53mer for SirT5, p53mer for SirT6, p53mer for Af2Sir2, H4mer for yeast Sir2 and p53mer for sirtuin mixture; the second control sample contained 800  $\mu\text{M}$  of **2** only; and the third control had 400  $\mu\text{M}$  of **1** only. Reactions were initiated by addition of enzyme with final concentrations: 16.5  $\mu\text{M}$  for SirT1, 12.5  $\mu\text{M}$  for SirT2, 11.5  $\mu\text{M}$  for SirT3, 13.6  $\mu\text{M}$  for SirT5, 17.1  $\mu\text{M}$  for SirT6, 19.7  $\mu\text{M}$  for Af2Sir2 and 6.2  $\mu\text{M}$  for yeast Sir2. For sirtuin mixture sample, enzyme concentrations were 16.5  $\mu\text{M}$  for SirT1, 12.5  $\mu\text{M}$  for SirT2, 11.5  $\mu\text{M}$  for SirT3, 13.6  $\mu\text{M}$  for SirT5 and 17.1  $\mu\text{M}$  for SirT6. Reactions were incubated at 37 °C for 30 min and then adjusted to pH 6 by addition of 25  $\mu\text{L}$  of 300 mM phosphate buffer pH 4.25. Compound **3** was then added to each sample to a final concentration of 1 mM, and incubations were continued at 25 °C for 1 h. Then 50  $\mu\text{L}$  of avidin agarose beads were added to each sample, and the reactions were allowed to incubate at 25 °C for 1 h. After removal of the supernatant, the beads were washed with 1

mM NAD<sup>+</sup> in 300 mM phosphate buffer (3 x 100  $\mu$ L), the washes were removed and saved for gel analysis. The beads were boiled in SDS-PAGE sample buffer for 10 minutes and subjected to SDS-PAGE for analysis. Gels for SirT1, SirT3, SirT6 and sirtuin mixture are shown in the main text as Figure 3. Representative gels for Af2Sir2, yeast Sir2, SirT5 and SirT2 are shown in Figure S5.

S3. Figure S1.

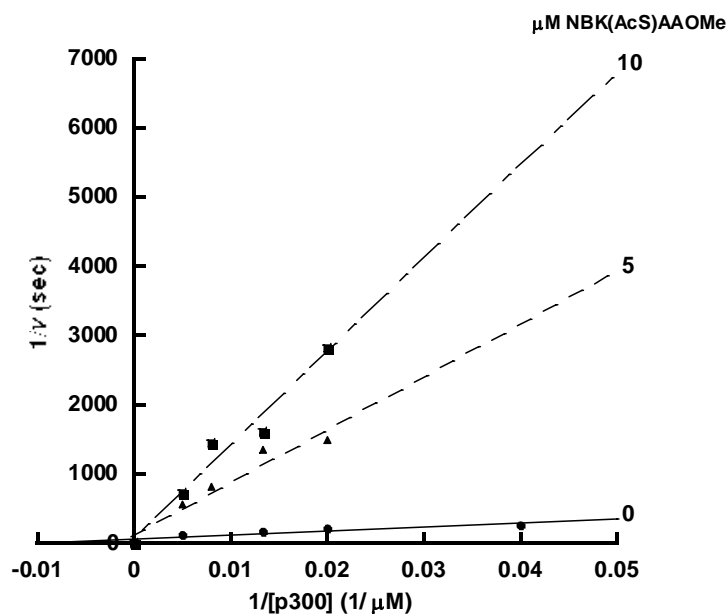


Enzymes were preincubated with varying concentrations of **1** and  $NAD^+$  and initiated with substrate addition. A. 300  $\mu M$   $NAD^+$ , 200  $\mu M$  p53mer, and 8.75  $\mu M$  SirT1. B. 800  $\mu M$   $NAD^+$ , 500  $\mu M$  p53mer, and 5.5  $\mu M$  SirT2. C. 750  $\mu M$   $NAD^+$ , 500  $\mu M$  H3mer, and 2.35  $\mu M$  SirT3. D. 800  $\mu M$   $NAD^+$ , 540  $\mu M$  **1**, and 12.08  $\mu M$  Af2Sir2. E. 500  $\mu M$



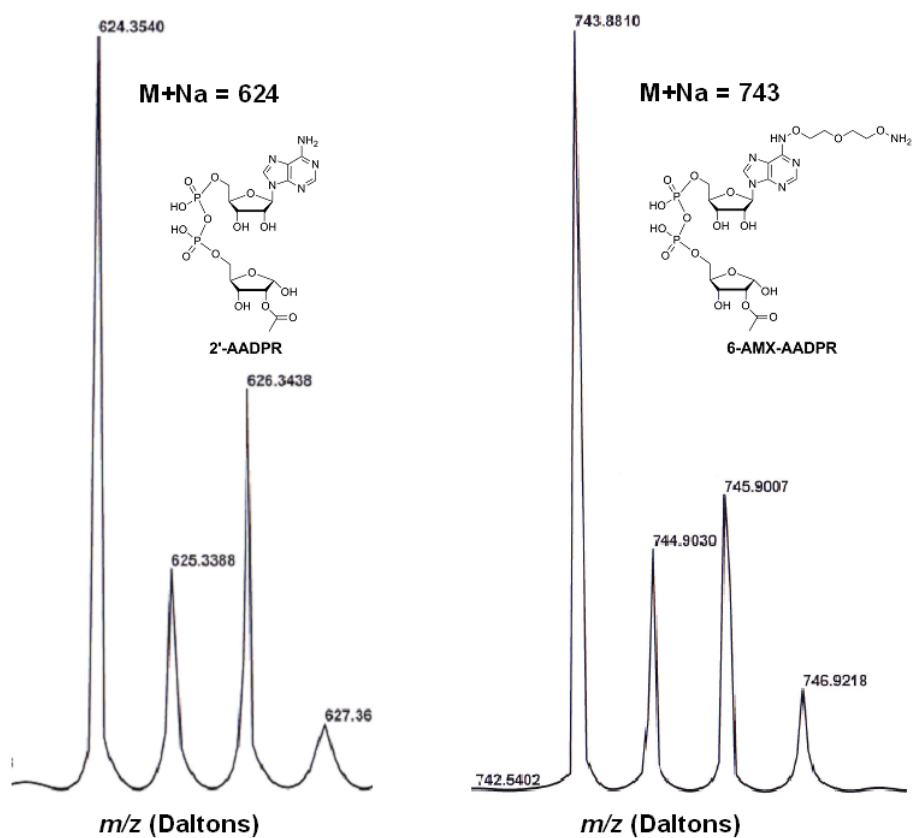
NAD<sup>+</sup>, 500 μM H3mer, and 2.06 μM yeast Sir2. Data is plotted as percent enzyme activity remaining as a function of the log values of [**1**] in nanomolar. The legend for the x-axis is presented as concentration of **1** in nanomolar. Curve fits were generated using the following equation:  $v(\%) = v(\%)_I - [v(\%)_I(10^x)/(10^x + IC_{50})]$ .  $v(\%)$  represents turnover rate expressed as percent enzyme activity remaining and  $v(\%)_I$  represents the initial turnover rate expressed as an enzyme activity of 100%. The variable x represents the log of [**1**] in nanomolar; therefore,  $10^x$  is indicative of the concentration of **1** in nanomolar. IC<sub>50</sub> values were calculated from this equation.

S4. Figure S2.



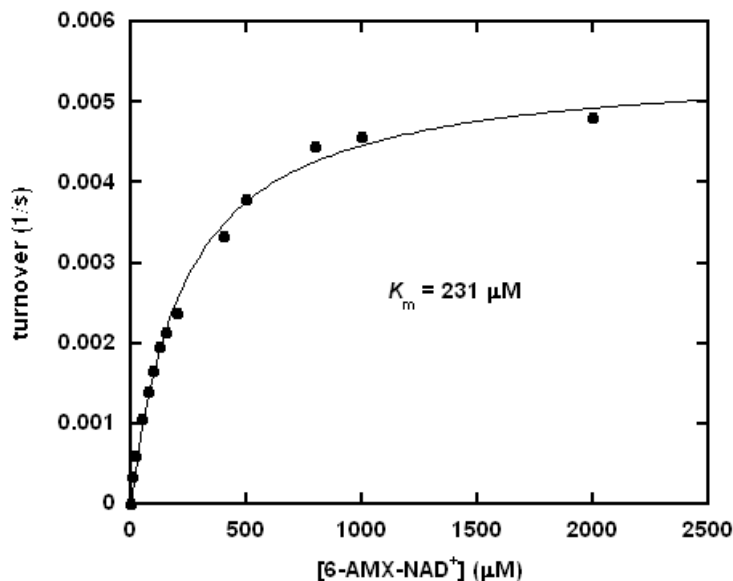
Double-reciprocal sirtuin inhibition plot. Reactions carried out using 0, 5, or 10  $\mu\text{M}$  **1**, 300  $\mu\text{M}$   $\text{NAD}^+$ , varying concentrations of p300mer (25 – 200  $\mu\text{M}$ ) and 2.02  $\mu\text{M}$  SirT1 for 60 min at 37°C. Lines are best fit to the Lineweaver-Burk equation for competitive inhibition.  $K_i = 611 \text{ nM} \pm 248 \text{ nM}$ .

S5. Figure S3.



MALDI-MS data showing the  $m/z$  values for 2'-AADPR ( $M+Na = 624$ ) and 6-AMX-AADPR ( $M+Na = 743$ ).

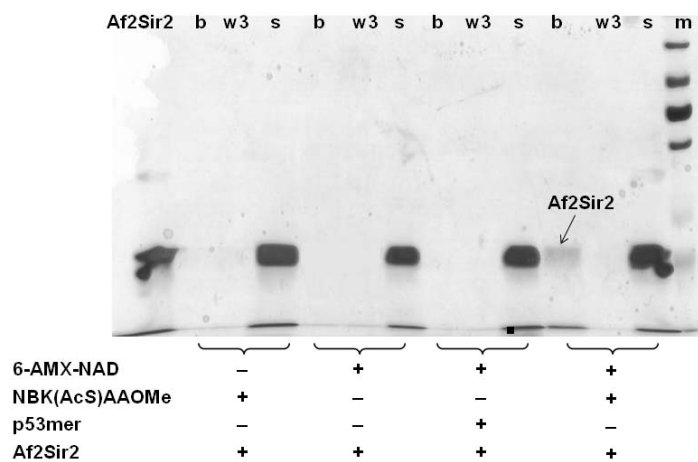
**S6. Figure S4.**



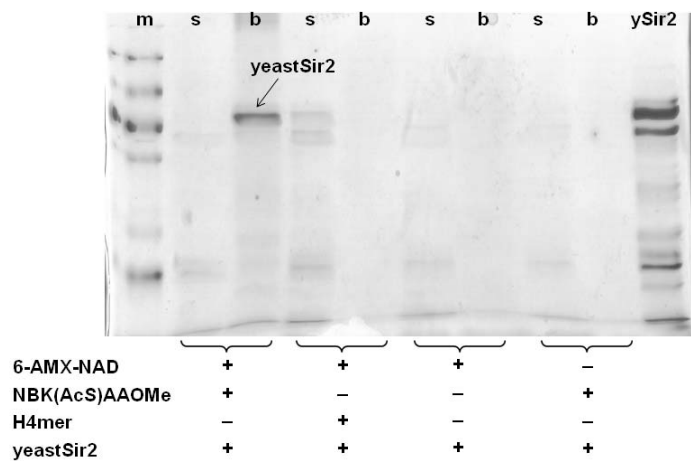
Steady-state parameter curve for reaction of **2** as a substrate on SIRT1. Reactions containing 250 μM of p53mer and various concentrations of **2** (0, 10, 20, 50, 75, 100, 125, 150, 200, 400, 500, 800, 1000 and 2000 μM) in 100 mM phosphate buffer pH 7.5 were initiated with the addition of SirT1 (final concentration 4.04 μM). The reactions were incubated at 37 °C for one hour and quenched by addition of 10 μL of 10% TFA. Rates were determined as described in the Supplementary Information experimental. Rates were plotted versus [**2**] and fit to the Michaelis-Menten equation by Kaleidagraph®.

**S7. Figure S5.**

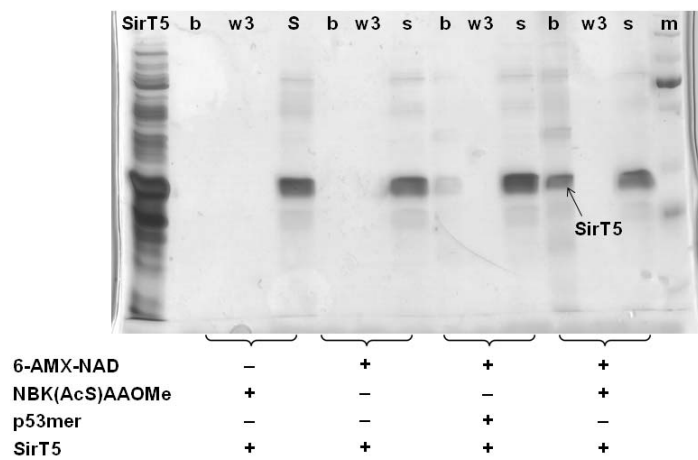
**A.**



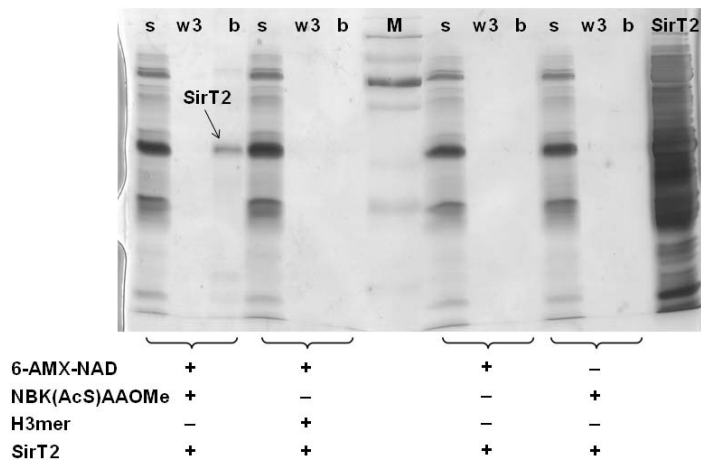
**B.**



**C.**



**D.**

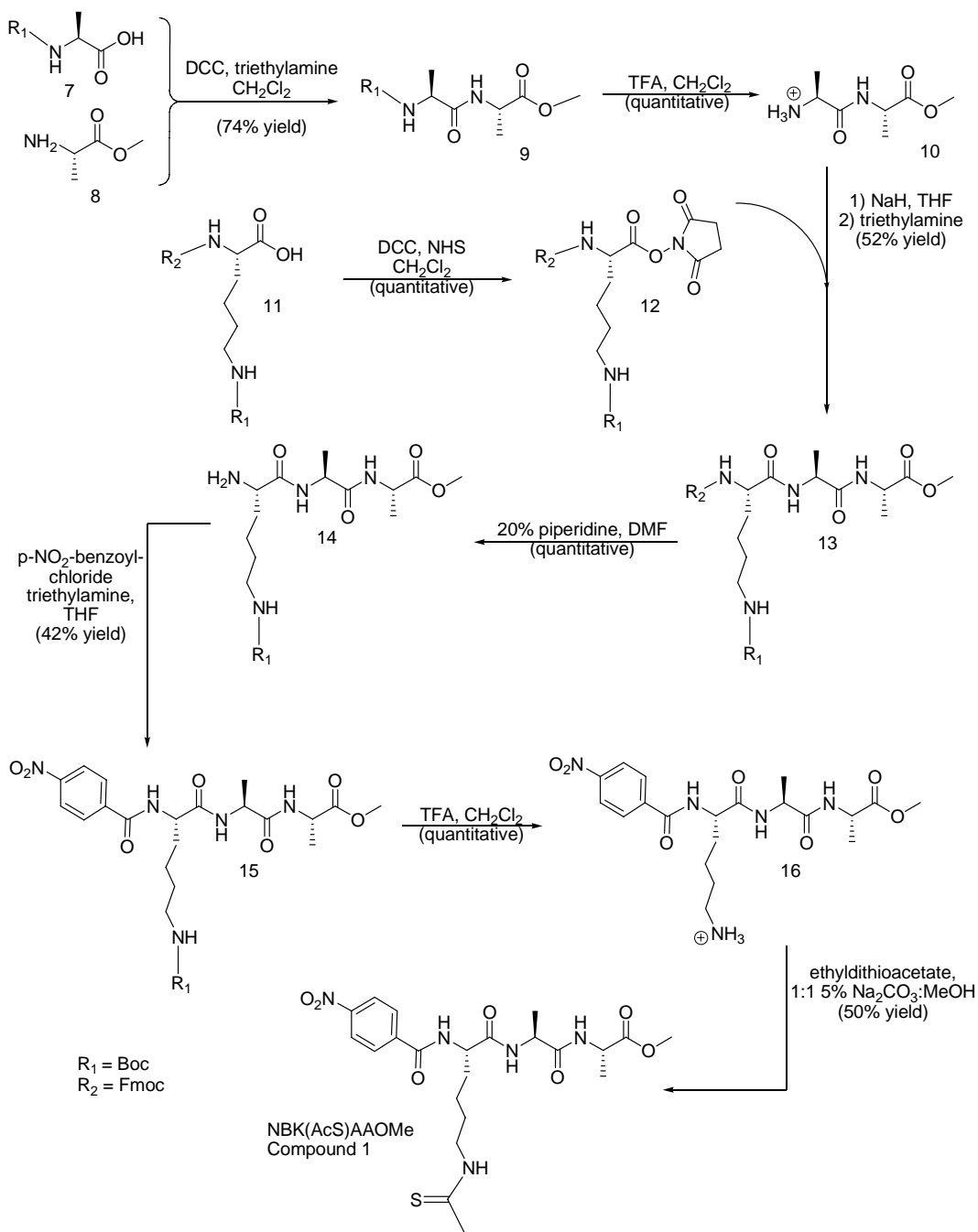


**Figure S5:** SDS-PAGE results for sirtuin capture by **1** and **2**. A) Af2Sir2; B) yeast Sir2; C) SirT5; D) SirT2. Reactions were performed in 300 mM phosphate buffer pH 7.5 in a total volume of 50  $\mu$ L. Each “complete” reaction contained 800  $\mu$ M of **2** and 400  $\mu$ M of **1**. In addition, three control experiments were run at the same time: the first one contained 800  $\mu$ M of **2** and 500  $\mu$ M of peptide substrate, p53mer for Af2Sir2, H4mer for yeast Sir2, p53mer for SirT5 and H3mer for SirT2; the second control sample contained 800  $\mu$ M of **2** only; and the third control had 400  $\mu$ M of **1** only. Reactions were initiated by addition of enzyme, 19.7  $\mu$ M for Af2Sir2, 6.2  $\mu$ M for yeast Sir2, 13.6  $\mu$ M for SirT5 and 12.5  $\mu$ M for SirT2. Reactions were incubated at 37  $^{\circ}$ C for 30 min and then adjusted to pH 6 by addition of 25  $\mu$ L of 300 mM phosphate buffer pH 4.25. Compound **3** was added to each sample to a final concentration of 1 mM and incubations were continued at 25  $^{\circ}$ C for 1h. Then 50  $\mu$ L of avidin agarose beads were added to each sample, and the reactions were allowed to incubate at 25  $^{\circ}$ C for 1 h. After removal of the supernatant, the beads were washed with 1 mM NAD<sup>+</sup> in 300 mM phosphate buffer (3 x 100  $\mu$ L), the washes were removed and saved for gel analysis. The beads were boiled in SDS-PAGE sample buffer for 10 minutes and subjected to SDS-PAGE for analysis. Abbreviations: s=supernatant; w3=wash 3; b=beads; m=molecular weight marker.

## **S8. Synthetic procedures**

### **General information**

*N*<sup>α</sup>-Boc-alanine (**7**), alanine-methyl ester monochloride (**8**), *N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-Boc-lysine (**11**) and *N*<sup>α</sup>-Fmoc-lysine (**17**) were of (L) absolute configuration and were purchased at the highest purity available from Novabiochem and used without further purification. 6-Chloropurine riboside and nicotinamide mononucleotide were purchased from SigmaAldrich. Additional reagents used, unless synthesized according to the provided protocols, were purchased from commercial suppliers at the highest purity available and used without further purification. Unless otherwise specified all synthetic reactions were carried out under argon pressure. NMR spectral data was obtained using a Bruker Avance DMX 500 MHz spectrometer.



**Scheme S1. Synthesis of compound 1 synthetic route 1**

**Synthetic route S1.**

*N*<sup>α</sup>-Boc-alaninyl-alanine-methyl ester **9**. Alanine methyl ester monochloride **8** was treated with a NaOMe/MeOH (1.0 mol equivalent). NaCl precipitate was filtered and



filtrate was evaporated, redissolved in dichloromethane and filtered again. Second filtrate was evaporated to the oil alanine methyl ester **8**. In 5 mL distilled dichloromethane, **8** (996 mg, 9.67 mmol) was added a solution of **7** (2.06 g, 10.63 mmol) in distilled dichloromethane (5 mL). Dicyclohexylcarbodiimide (DCC; 2.19 g, 10.63 mmol) was dissolved in 3 mL distilled dichloromethane and added to the solution. Dicyclohexylurea (DCU) precipitation was noted immediately after DCC addition. Triethylamine (3 mL, 21.52 mmol) and additional distilled dichloromethane (4 mL) were added to the reaction mixture. Reaction proceeded for a total of three hours. Reaction was placed at -20 °C to promote additional DCU precipitation. Precipitate was filtered through a Büchner funnel and rinsed with cold dichloromethane. Combined filtrate and rinses were dried under vacuum and purified by silica-gel chromatography to yield dipeptide **9** (2.03 g, 7.14 mmol, 74%). <sup>1</sup>H-NMR (chloroform-d) δ (ppm): 1.34 (d, *J*= 14.1 Hz, 3H); 1.38 (d, *J*= 14.3 Hz, 3H); 1.40 (s, 9H); 3.73 (s, 3H); 4.14 (s-broad, 1H); 4.55 (quintet, *J*= 14.5 Hz, 1H); 4.97 (s-broad, 1H); 6.59 (s-broad, 1H).

**Alaninyl-alanine methyl ester trifluoroacetate 10.** Trifluoroacetic acid (1.7 mL) and triethylsilane (0.200 mL) were added to a 1.7 mL solution of **9** (964 mg, 3.30 mmol) in distilled dichloromethane. Reaction was allowed to proceed for 30 min and then concentrated to an oil affording the deprotected dipeptide **10** in quantitative yield. <sup>1</sup>H-NMR (D<sub>2</sub>O) δ (ppm): 1.43 (d, *J*= 14.7 Hz, 3H); 1.55 (d, *J*= 14.3 Hz, 3H), 3.76 (s, 3H), 4.09 (q, *J*= 14.2 Hz, 1H); 4.46 (q, *J*= 14.6 Hz, 1H).

***N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-Boc-lysine-*N*-hydroxysuccinimide ester **12**** Peptide **11** (1.57 g, 3.35 mmol) and *N*-hydroxysuccinimide (NHS; 360 mg, 3.21 mmol) was dissolved in distilled dichloromethane (4 mL). DCC (862 mg, 4.18 mmol), dissolved in distilled dichloromethane (4 mL), was added to stirring solution. Precipitation was noted immediately and reaction was permitted to proceed an additional 12 h. DCU precipitation was filtered and rinsed with cold dichloromethane. Filtrate and rinses were concentrated under vacuum to yield the activated ester **12** in quantitative yield. <sup>1</sup>H-NMR (chloroform-*d*) δ (ppm): 1.36 (s, 9H); 1.44 (m, 4H); 1.90 (m, 2H); 2.80 (s, 4H); 3.12 (m, 2H); 4.21 (t, *J*= 13.7 Hz 1H); 4.41 (m, 2H); 4.69 (m-broad, 2H); 5.52, (s-broad, 1H); 7.29 (t, *J*= 14.7 Hz, 2H); 7.37 (t, *J*= 14.9 Hz, 2H); 7.57 (d, *J*= 14.4 Hz 2H); 7.73 (d, *J*= 15.0 Hz 2H).

***N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-Boc-lysiny-*l*-alaniny-*l*-alanine methyl ester **13**** A solution of **10** (780 mg, 2.54 mmol) in distilled THF (10 mL) was treated with NaH (67 mg, 2.79 mmol) on an ice/water bath. Activated ester **12** (1.40 g, 2.49 mmol) dissolved in distilled THF (5 mL) was added to the stirring reaction. Triethylamine (0.531 mL, 3.81 mmol) was added. Reaction proceeded until consumption of dipeptide starting material was evident (1.5 h). Crude reaction mixture was concentrated under vacuum to dryness and purified by silica-gel chromatography to obtain the desired tripeptide **13** (823 mg, 1.32 mmol, 52%). <sup>1</sup>H-NMR (chloroform-*d*) δ (ppm): 1.37 (d, *J*= 13.5 Hz, 6H); 1.41 (s, 9H); 1.48 (m, 2H); 1.67 (m, 2H); 1.81 (m, 2H); 2.68 (m, 2H); 3.72 (s, 3H); 4.15 (s-broad, 1H); 4.18 (t, *J*= 13.9 Hz, 1H); 4.37 (d, *J*= 13.0 Hz 1H); 4.49(m, 2H); 4.72 (s-broad, 1H); 5.61 (s-broad, 1H);

6.68 (d,  $J= 13.4$  Hz, 1H); 7.27 (t,  $J= 14.7$  Hz, 2H); 7.37 (t,  $J=14.8$  Hz, 2H); 7.56 (d,  $J= 14.6$  Hz, 2H); 7.73 (d,  $J= 15.0$  Hz, 2H).

***N*<sup>ε</sup>-Boc-lysiny-alaniny-alanine methyl ester 14** Method 1: Peptide **13** (74 mg, 0.131 mmol) was dissolved in dimethylformamide (DMF, 0.800 mL). Piperidine (0.200 mL) was added. Reaction proceeded for 20 min and then the reaction mixture was pumped to dryness. Residue was resuspended in H<sub>2</sub>O and washed twice with an equal volume of hexanes. Aqueous layer was then lyophilized to yield pure tripeptide **14** in quantitative yield. <sup>1</sup>H-NMR (chloroform-d): 1.34 (d,  $J= 13.8$  Hz, 3H); 1.36 (d,  $J= 14.1$  Hz, 3H); 1.39 (s, 9H); 1.50 (m, 4H); 1.83 (m, 2H); 3.10 (m, 2H); 3.34 (d,d,  $J = 8.7, 16.1$  Hz, 1H); 3.73 (s, 3H); 4.41 (q,  $J= 14.2$  Hz, 1H); 4.52 (q,  $J= 14.5$  Hz, 1H); 4.55 (s-broad, 1H); 6.60 (d,  $J= 13.8$  Hz, 1H); 7.69 (m, 1H).

Method 2: Peptide **11** (466 mg, 0.747 mmol) was dissolved in DMF (1.60 mL). Piperidine (0.300 mL) was then added. Reaction proceeded for 30 min. Solution was pumped to dryness, redissolved in DMF and dried again. The DMF evaporation process was repeated four times. Solid residue was redissolved in a minimal volume of dichloromethane and addition of hexanes to the solution caused precipitation. Additional precipitation was promoted by overnight incubation at -20 °C. After removal of solids, the volume was reduced by evaporation to yield an oily residue that was washed with cold hexanes. Remaining residue was characterized and compared to the <sup>1</sup>H-NMR spectra in procedure 1. Pure product **14** was afforded (250 mg, 0.622, 88% yield).

***N*<sup>α</sup>-4-nitrobenzoyl-*N*<sup>ε</sup>-Boc-lysiny-alaniny-alanine methyl ester 15**

Peptide **14** (89.7 mg, 0.223 mmol) was dissolved in distilled THF (5.0 mL) in a flask chilled by an ice/H<sub>2</sub>O bath. Triethylamine (0.065 mL, 0.279 mmol) was added to the solution. 4-nitrobenzoyl chloride (51 mg, 0.469 mmol) was then added to the stirring reaction. Precipitation was noted immediately. After 30 min, reaction mixture was evaporated to dryness and redissolved in H<sub>2</sub>O. The aqueous suspension was extracted three times with ethyl acetate (EtOAc) and the organic layers collected. Combined organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated under vacuum. Silica-gel chromatography was performed to yield the purified tripeptide **15** (51.3 mg, 0.093 mmol, 42%). <sup>1</sup>H-NMR (chloroform-d) δ (ppm): 1.37 (s, 9H), 1.40 (d, *J*= 15.3 Hz, 3H); 1.43 (m, 4H); 1.50 (m, 2H) 1.69 (m, 1H); 1.80 (m, 1H); 1.92 (m, 1 H); 3.06 (m, 2H); 3.72 (s, 3H); 4.52 (quintet, *J*= 14.4 Hz, 1H); 4.50 (quintet, *J*= 14.4 Hz, 1H); 4.1 (q, *J*= 14.9 Hz, 1H); 4.78 (s-broad, 1H); 6.91 (d, *J*= 14.5 Hz, 1H); 7.08 (d, *J*= 14.9 Hz, 1H); 7.49 (d, *J*= 14.2 Hz, 1H); 8.00 (d, *J*= 16.9 Hz, 2H); 8.24 (d, *J*= 17.4 Hz, 2H).

***N*<sup>α</sup>-4-nitrobenzoyl-lysiny-l-alaniny-l-alanine methyl ester trifluoroacetate 16**

Trifluoroacetic acid (0.500 mL) and triethylsilane (0.100 mL) were added to a solution of **15** (51.3 mg, 0.093 mmol) in distilled dichloromethane (0.500 mL). Reaction was allowed to proceed for forty minutes and then concentrated to an oil affording tripeptide **16** in quantitative yield. <sup>1</sup>H-NMR (MeOD) δ (ppm): 1.40 (t, *J*= 15.3 Hz, 6H); 1.56 (q, *J*= 15.3 Hz, 2H); 1.72 (m, 1H); 1.84 (m, 1H); 1.94 (m, 1H); 2.96 (t, *J*= 14.9 Hz, 2H); 3.71 (s, 3H), 4.39 (q of d, *J*= 4.5, 14.6, 2H); 4.57 (t, *J*= 13.7 Hz 1H); 8.06 (d, *J*= 17.6 Hz 2H); 8.32 (d, *J*= 17.5 Hz, 2H).

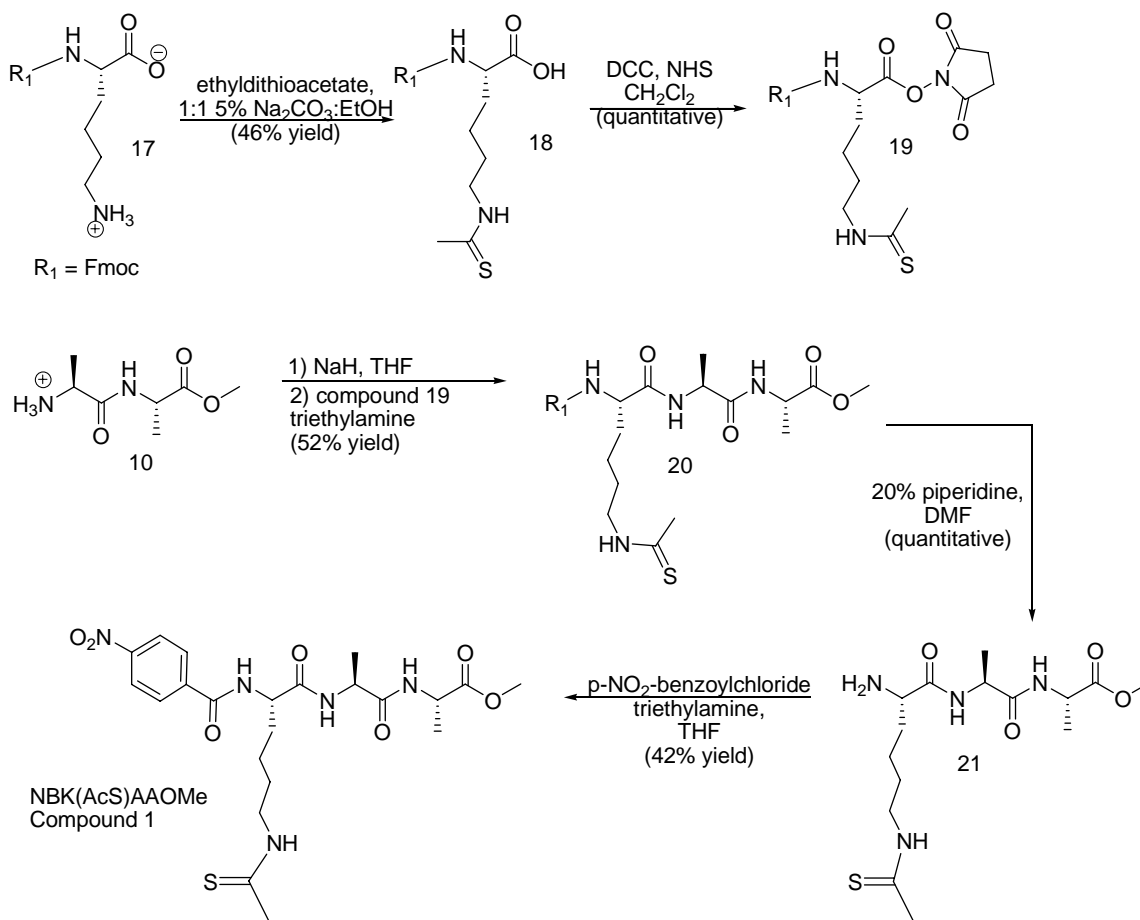
***N*<sup>α</sup>-4-nitrobenzoyl-*N*<sup>ε</sup>-thioacetyl-lysiny-alaniny-alanine methyl ester **1**<sup>3</sup>**

Tripeptide **16** (18 mg, 0.0318 mmol) was dissolved in methanol (abbr. MeOH, 1.0 mL) in a flask chilled on an ice/water bath. Drop wise over several minutes, a 5% Na<sub>2</sub>CO<sub>3</sub> (0.500 mL) solution was added to the stirring solution. Then, ethyl dithioacetate (0.010 mL, 0.105 mmol) was added dropwise to the solution. Reaction proceeded for 50 min whereupon TLC indicated completion of reaction. 1:1 MeOH:H<sub>2</sub>O (1.0 mL) solution was then added to the reaction. MeOH was removed by evaporation under vacuum and the remaining aqueous solution was acidified with 6 N HCl to pH ~1-2, as indicated by pH paper. The aqueous solution was then extracted four times with dichloromethane and the combined organic layer was washed with brine and then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Evaporation concentrated this layer to yield a crude oil. Purification by silica-gel chromatography afforded pure thioacetylated tripeptide **1** (9.8 mg, 0.016 mmol, 50%).  
<sup>1</sup>H-NMR (acetone-d<sub>6</sub>) δ (ppm): 1.31 (d, *J*= 14.4 Hz, 3H); 1.33 (d, *J*= 14.6 Hz, 3H); 1.54 (m, 2H); 1.70 (m, 2H); 1.87 (m, 1H); 1.96 (m, 1H); 2.43 (s, 3H); 3.60 (q, *J*= 13.5 Hz, 2H); 3.67 (s, 1H); 4.40 (q, *J*= 13.7 Hz, 1H); 4.49 (q, *J*= 14.2 Hz, 1H); 4.66 (m, 1H); 7.60 (d, *J*= 14.2 Hz, 1H); 7.70 (d, *J*= 14.5 Hz, 1H); 8.14 (d, *J*= 14.7 Hz, 1H); 8.17 (d, *J*= 17.6 Hz, 2H); 8.32 (d, *J*= 17.6 Hz, 1H); 9.16, s, 1H). See full NMR data in section S9 of the Supplementary Information.

***N*<sup>α</sup>-4-nitrobenzoyl-*N*<sup>ε</sup>-acetyl-lysiny-alaniny-alanine methyl ester NBKAcAAOMe**

To a solution of tripeptide **16** (52.5 mg, 0.093 mmol) in distilled THF (400 μL), acetic anhydride (100 μL, 1.06 mmol) was added. Triethylamine (10 mL, 0.072 mmol) was then added and immediate precipitation was noted. Reaction was quenched after 30min

with saturated  $\text{NaHCO}_3$  and then extracted with ethyl acetate (3x). Combined organic layers were dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated to dryness. Crude residue was then purified by silica-gel chromatography to afford the peptide “**NBKAcAAOMe**” as a white solid (16.8 mg, 0.034 mmol, 37%).  $^1\text{H-NMR}$  (MeOD)  $\delta$  (ppm): 1.38 (t, 4H); 1.50 (m, 2H); 1.55 (m, 2H); 1.83 (m, 1H); 1.91 (2, 3H); 1.93 (m, 1H); 3.18 (t, 2H); 3.71 (s, 3H); 4.40 (m, 2H); 4.5 (t,  $J= 11.5$  Hz, 1H); 8.07 (d,  $J= 17.5$  Hz, 2H); 8.32 (d,  $J= 17.6$  Hz, 1H).



### Scheme S2. Synthesis of compound 1 synthetic route 2

#### Synthetic route S2.

***N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-thioacetyl-lysine 18<sup>3</sup>** Dropwise, 5% Na<sub>2</sub>CO<sub>3</sub> (4.0 mL) was added to a stirring suspension of **17** (750 mg, 2.03 mmol) in ethanol (4.0 mL) in a flask chilled on an ice/water bath. Ethyldithioacetate (0.250 mL, 2.65 mmol) was added dropwise to the suspension. Reaction proceeded for 220 min and then was diluted with 1:1 EtOH:H<sub>2</sub>O (2.0 mL). Ethanol was evaporated using an air stream and the remaining aqueous solution was treated according to the protocol employed above for the synthesis of **1**. Purification by silica-gel chromatography using an EtOAc/MeOH gradient containing 1% acetic acid yielded an oil. The oil was redissolved in dichloromethane and washed with

100 mM HCl. The organic fraction was subsequently dried over Na<sub>2</sub>SO<sub>4</sub> to yield the pure thioacetylated lysine **18** (402 mg, 0.945 mmol, 46%). <sup>1</sup>H-NMR (chloroform-d) δ (ppm): 1.46 (m, 2H); 1.70 (m, 3H); 1.92 (m, 1H); 2.49 (s, 3H); 3.60 (m, 2H); 4.20 (t, *J*= 13.7 Hz, 1H); 4.41 (m, 3H); 5.37 (d, *J*= 15.3 Hz, 1H); 7.29 (t, *J*= 14.9 Hz, 2H); 7.38 (t, *J*= 14.8 Hz, 2H); 7.471 (s-broad, 1H); 7.55 (d-broad, 2H); 7.75 (d, *J*= 15.0 Hz, 2H).

***N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-thioacetyl-lysine-*N*-hydroxysuccinimide ester **19**** *N*-

hydroxysuccinimide (88.5 mg, 0.770 mmol) was added to a stirring solution of **18** (298 mg, 0.699 mmol) in distilled dichloromethane (8.0 mL). DCC (151 mg, 0.734 mmol) dissolved in distilled dichloromethane (2.0 mL) was added to the stirring solution. Precipitation was noted within 10 min of reaction time. Reaction was allowed to proceed 2 h and stored at -20 °C to promote additional DCU precipitation. DCU was filtered and rinsed as described above. Filtrates and rinses were concentrated under vacuum and the resulting crude solid was purified by silica-gel chromatography to yield the desired NHS-ester **19** (131 mg, 0.250 mmol, 36%). <sup>1</sup>H-NMR (chloroform-d) δ (ppm): 1.50 (m, 2H); 1.67 (m, 1H), 1.77 (m, 1H); 1.96 (m, 2H); 2.51 (s, 3H); 2.84 (s, 4H); 3.59 (m, 1H); 3.71 (m, 1H); 4.20 (t, *J*= 13.2 Hz, 1H); 4.43 (d, *J*= 13.4 Hz, 2H); 4.777 (q, *J*= 12.4 Hz, 1H); 5.36 (d, *J*= 16.2 Hz, 1H); 7.29 (t, *J*= 14.8 Hz, 2H); 7.38 (t, *J*= 14.9 Hz, 2H); 7.55 (d, *J*= 14.8 Hz, 2H); 7.74 (d, *J*= 15.1 Hz, 2H); 7.78 (s-broad, 1H).

***N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-thioacetyl-lysiny-alaninyl-alanine methyl ester **20**** Dipeptide **10** (68 mg, 0.222 mmol) was dissolved in distilled THF (3.0 mL). NaH (7.5 mg, 0.3125 mmol) was added to the stirring reaction. Solution was vented to allow for off-gassing. Once



off-gassing was no longer observed, a solution of NHS ester **16** (55 mg, 0.143 mmol) in distilled THF (3.0 mL) was added to the solution. Triethylamine (0.054 mL, 0.387 mmol) was added to the solution after reaction had proceeded for 10 min. Precipitation was noted. Reaction was allowed to proceed for 2.5 after which TLC analysis indicated consumption of the NHS-ester. Reaction was evaporated to dryness and the crude residue was purified by silica-gel chromatography to yield the Fmoc-protected tripeptide **20** (70 mg, 0.120 mmol, 84 %). <sup>1</sup>H-NMR (chloroform-d)  $\delta$  (ppm): 1.35 (d,  $J$ = 20.6 Hz, 6H); 1.70 (m, 6H); 2.49 (s, 3H); 3.59 (q,  $J$ = 12.0 Hz, 2H); 3.70 (s, 3H); 4.17 (t,  $J$ = 13.9 Hz, 1H); 4.23 (q,  $J$ = 14 Hz, 1H); 4.37 (m, 2H); 4.47 (m, 2H); 5.69 (d,  $J$ = 15.6 Hz, 1H); 6.73 (d,  $J$ = 14.4 Hz, 1H); 6.78 (d,  $J$ = 13.4 Hz, 1H); 7.27 (t,  $J$ = 14.9 Hz, 2H); 7.37 (t,  $J$ = 15.1 Hz, 2H); 7.54 (d,  $J$ = 15.0 Hz, 2H); 7.73 (d,  $J$ = 15.1 Hz, 2H); 8.11 (s-broad, 1H).

***N*<sup>ε</sup>-thioacetyl-lysiny-alaniny-alanine methyl ester 21** Piperidine was added (0.200 mL) to a solution of tripeptide **20** (85 mg, 0.146 mmol) in DMF (0.800 mL) and the deprotection reaction allowed to proceed to completion. Once complete, the reaction solution was evaporated to dryness and treated according to procedure 2 employed for the synthesis of tripeptide **14**. Pure product tripeptide **21** was obtained in 90% yield (47 mg, 0.130 mmol). <sup>1</sup>H-NMR (chloroform-d)  $\delta$  (ppm): 1.38 (d,  $J$ = 14.1 Hz, 6H); 1.46 (m, 2H); 1.63 (m; 4H); 2.53 (s, 3H); 3.39 (t-broad, 1H); 4.42 (m, 1H); 4.51 (m, 1H); 6.70, 6.71 (d-broad,  $J$ = 11.8 Hz, 1H); 7.63 (s-broad, 1H); 7.90 (s-broad; 1H).

***N*<sup>α</sup>-4-nitrobenzoyl-*N*<sup>ε</sup>-thioacetyl-lysiny-alaniny-alanine methyl ester 1** Chilled triethylamine (0.055 mL, 0.3948) was added to a chilled solution of tripeptide **21** (68 mg,

0.188 mmol) in anhydrous THF (5.0 mL) in a flask chilled on an ice/water bath. Chilled 4-nitrobenzoylchloride (44 mg, 0.143 mmol) in anhydrous THF (5.0 mL) was added to the reaction. Additional 4-nitrobenzoylchloride (44 mg, 0.143 mmol) was added as a solid after 25 min. Formation of product was evident within several minutes of solid addition. Reaction was quenched with 10 mL H<sub>2</sub>O. Aqueous layer was extracted with ethyl acetate three times. Combined organic fractions were dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated to a crude residue. Silica-gel chromatography yielded pure product **1** (28 mg, 0.055 mmol, 30%). <sup>1</sup>H-NMR (acetone-d<sub>6</sub>) δ (ppm): 1.33 (d, *J*= 14.2 Hz, 3H); 1.35 (d, *J*= 14.6 Hz, 3H); 1.55 (m, 2H); 1.70 (m, 2H); 1.87 (m, 1H); 1.96 (m, 1H); 2.43 (s, 3H); 3.60 (q, *J*= 13.3 Hz, 2H); 3.67 (s, 1H); 4.41 (quintet, *J*= 14.5 Hz, 1H); 4.47 (quintet, *J*= 14.2 Hz, 1H); 4.65 (q, *J*= 15.5 Hz 1H); 7.57 (d, *J*= 13.7 Hz, 1H); 7.66 (d, *J*= 14.3 Hz, 1H); 8.11 (d, *J*= 14.6 Hz, 1H); 8.17 (d, *J*= 17.5 Hz, 2H); 8.32 (d, *J*= 17.5 Hz, 1H); 9.14 (s, 1H). <sup>13</sup>C-NMR (acetone-d<sub>6</sub>) δ (ppm): 17.76 (Ala-βCH<sub>3</sub>); 18.27 (Ala βCH<sub>3</sub>); 23.83 (Lys βCH<sub>2</sub>); 27.99 (Lys δCH<sub>2</sub>); 32.60 (Lys γCH<sub>2</sub>); 32.61 (thioacetyl); 46.33 (Lys εCH<sub>2</sub>); 48.85 (methyl ester); 49.45 (Lys αH); 52.34 (Ala αH); 54.78 (Ala αH); 124.32, 129.73, 140.96, 150.57 (aromatics); 160.05 (*N*-terminal carbonyl); 171.90, 172.90, 173.66 (carbonyls); 200.98 (thioamide carbonyl).

### **Synthesis of nucleoside, nucleotide and dinucleotide.**

Synthetic scheme is shown in the main text as Scheme 3, and compounds are numbered accordingly.

**6-chloropurine riboside 5'-phosphate 4** 6-Chloropurine riboside 5'-phosphate was synthesized as previously described with slight modifications.<sup>4</sup> 6-Chloropurine riboside

(100 mg, 0.35 mmol) was dissolved in 1.75 mL of trimethyl phosphate. Phosphoryl chloride (160 mg, 1.05 mmol) was dissolved in a mixture of 0.17 mL of trimethylphosphate and 6.3  $\mu$ L of water. Both solutions were cooled to 0 °C, mixed and stirred on a magnetic stirrer at 0 °C. After 6 h, ice was added to quench the reaction and the pH was adjusted to 7 by addition of an ammonium hydroxide solution. The crude product was concentrated and subsequently purified on a C18 reverse phase column using water as the mobile phase. The fractions containing desired product were combined and lyophilized to dryness to afford **4** (96 mg, 0.26 mmol, 75%) as a white solid.  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 500 MHz)  $\delta$  (ppm): 4.20 (m, 2H), 4.42 (m, 1H), 4.55 (t,  $J= 4.6$  Hz, 1H), 4.84 (m, 1H), 6.28 (d,  $J= 5.1$  Hz, 1H), 8.79 (s, 1H), 8.86 (s, 1H).

***O*-[2[(2-aminooxy-ethoxy)-ethyl]hydroxyamine 5** This compound was synthesized as described previously.<sup>5</sup> The conjugates made from this linker are described in Scheme 3 in the main text, and this moiety is abbreviated as ‘AMX’.

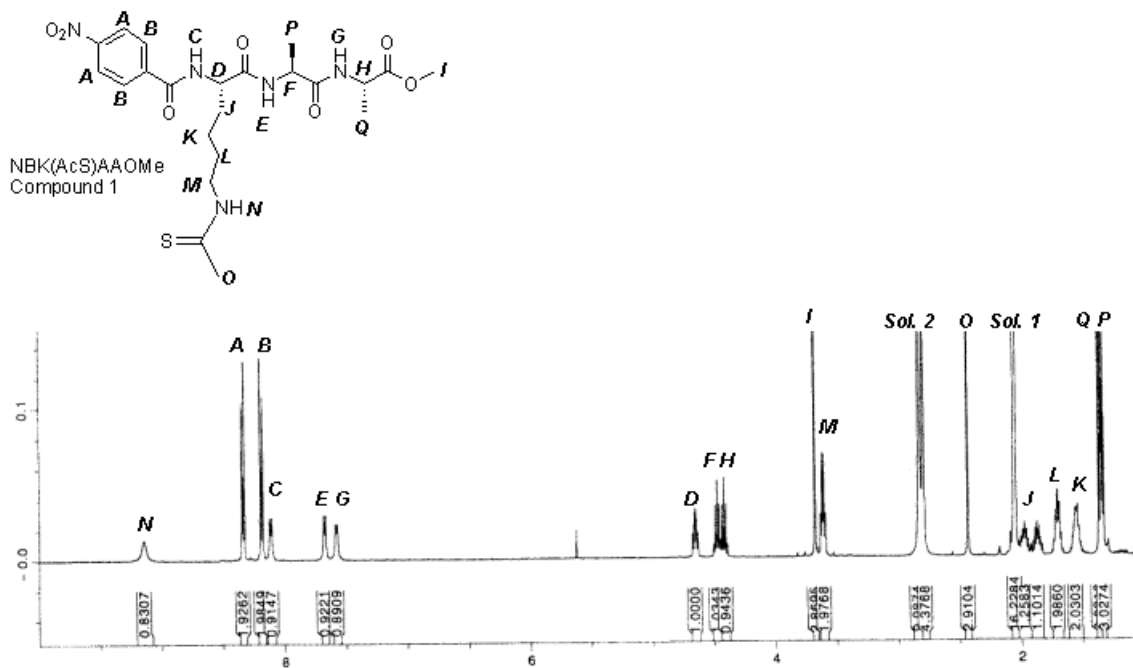
**6-‘AMX’-AMP 6** The solution of **4** (15 mg, 0.041 mmol) in 0.34 mL of water was treated with a solution of **5** (56 mg, 0.41 mmol) and triethylamine (14 mg, 0.14 mmol) in 0.33 mL of water at 25 °C overnight. The reaction mixture was concentrated and purified on C18 reverse phase column using water and methanol as mobile phase. The desired product eluted in 10% methanol-90% water fractions. These fractions were combined and lyophilized to dryness to afford **6** (8.6 mg, 0.018 mmol, 45%) as a off-white solid.  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 500 MHz)  $\delta$  (ppm): 3.90 (dt,  $J= 4.2, 18.9$  Hz, 4H), 4.14 (m, 2H), 4.28 (dt,  $J=$

4.1, 20.6 Hz, 4H), 4.42 (t,  $J= 2.1$  Hz, 1H), 4.52 (t,  $J= 4.6$  Hz, 1H), 4.82 (m, 1H), 6.18 (d,  $J= 5.0$  Hz, 1H), 8.21 (s, 1H), 8.68 (s, 1H).

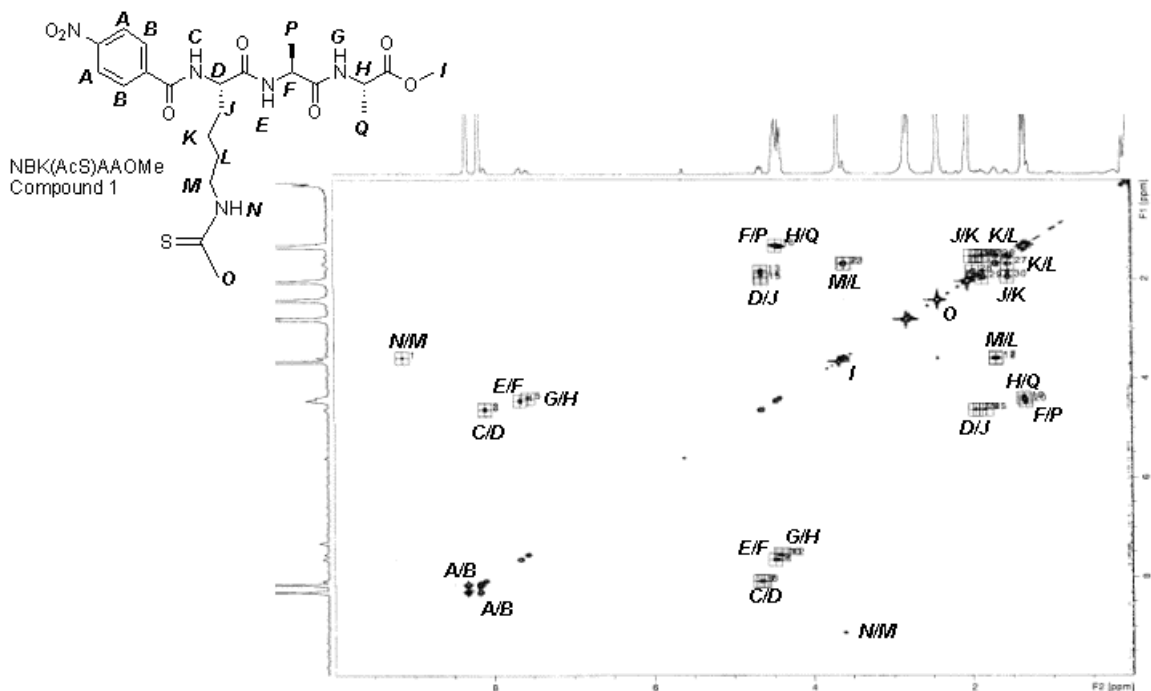
**6-‘AMX’-NAD<sup>+</sup> 2** A solution of **6** (3 mg, 0.0065 mmol), nicotinamide mononucleotide (4.3 mg, 0.013 mmol) and MgCl<sub>2</sub> (14.8 mg, 0.156 mmol) in 1 mL of water was concentrated to dryness, 500  $\mu$ L of 1.5 M HEPES-NaOH and 500  $\mu$ L of 5 M EDCI were added to the residue to initiate the coupling reaction. Incubation overnight at 37 °C was followed by dilution with 1 mL of water. The reaction mixture was purified by HPLC on a Waters XBridge® Prep Shield RP18 column (solvent was 0.1% TFA. Compound was eluted at a flow rate of 2 mL/min) to afford **2** in the main text ( $t_R= 14.5$  min, 3.4 mg, 0.0043 mmol, 67%). <sup>1</sup>H NMR (D<sub>2</sub>O)  $\delta$  (ppm): 3.78 (m, 4H), 4.00 (m, 4H), 4.19 (m, 1H), 4.25 (stack, 3H), 4.38 (stack, 2H), 4.46 (m, 1H), 4.52 (stack, 2H), 4.57 (m, 1H), 6.07 (d,  $J= 5.9$  Hz, 1H), 6.12 (d,  $J= 5.5$  Hz, 1H), 8.21 (s, 1H), 8.23 (dd,  $J= 6.6, 7.8$  Hz, 1H), 8.47 (s, 1H), 8.87 (d,  $J= 8.1$  Hz, 1H), 9.19 (d,  $J= 6.2$  Hz, 1H), 9.37 (s, 1H).

**N-biotinyl-6-aminohexanal 3** *N*-biotinyl-6-aminohexanal, **3**, was synthesized as previously described.<sup>6</sup>

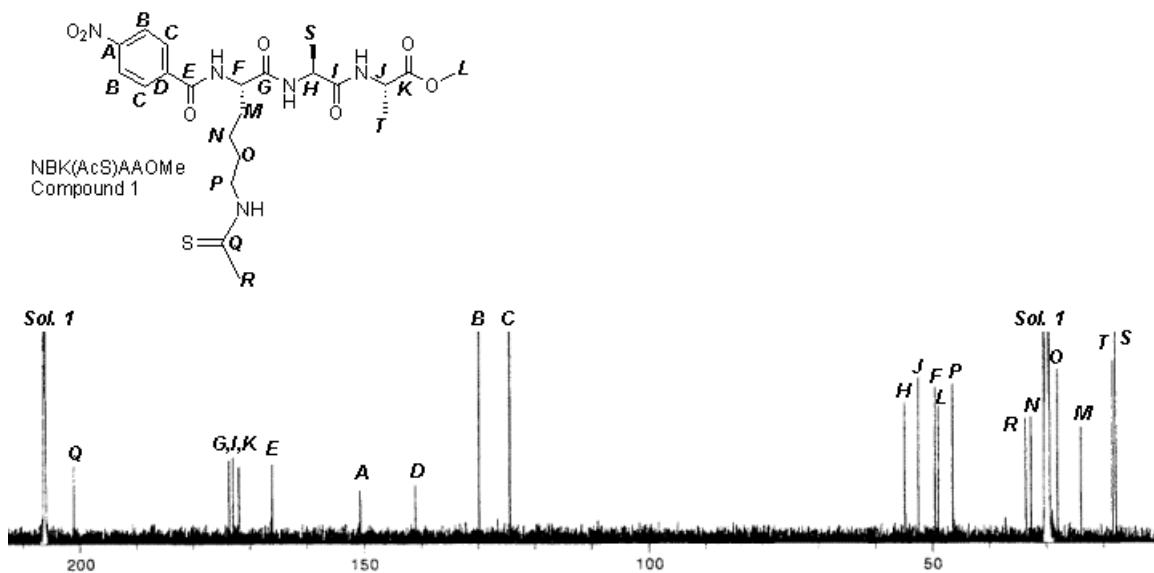
### S9. NMR data of 1.



1D <sup>1</sup>H NMR of **1** in acetone-d<sub>6</sub>. NMR spectrum corresponds to **1** synthesized by methodology 2 described above. Solvent (Sol.) residual signals are indicated. Sol. 1 = acetone; Sol. 2 = water. Letters correspond to labeled protons of compound **1**.

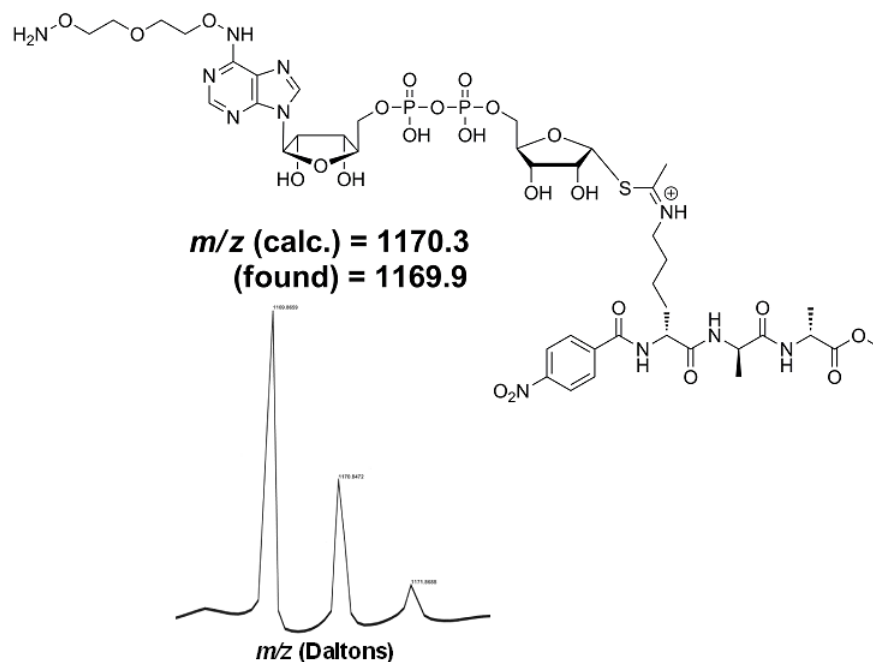


2D-COSY of **1** in acetone- $d_6$ . NMR spectrum corresponds to **1** synthesized by methodology 2 described above. Letters represent cross-coupling of protons corresponding to compound **1**. *I* indicates C-terminal methyl ester, *O* indicates thioacetyl, both of which exhibit no cross-coupling to intramolecular proton signals.



1D <sup>13</sup>C NMR of **1** in acetone-d<sub>6</sub>. NMR spectrum corresponds to **1** synthesized by methodology 2 described above. Sol. 1 = acetone. Letters indicate signals corresponding to the labeled carbon atoms of **1**.

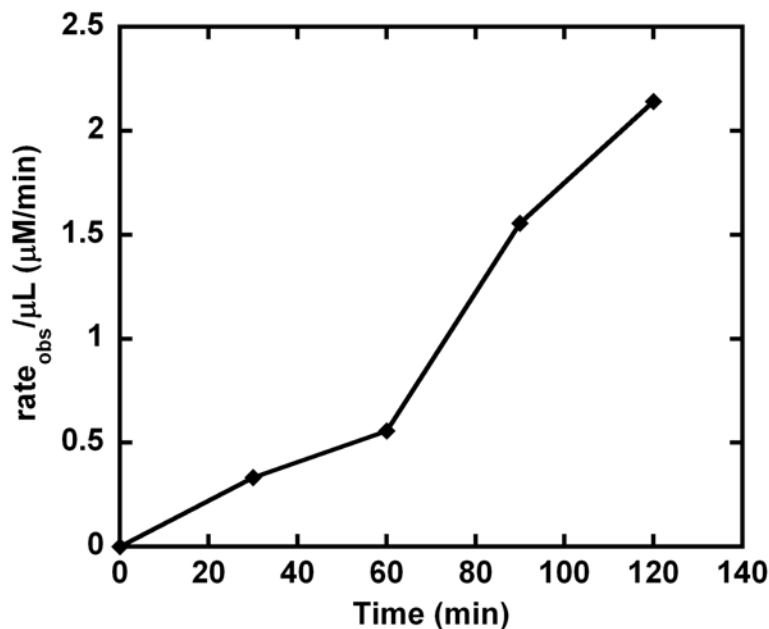
**S10. Figure S6**



MALDI-MS data showing detection of thioimidate ( $M+1 = 1169.9$ ) formation on SIRT1. Reaction containing 800  $\mu\text{M}$  of **2**, 700  $\mu\text{M}$  of **1** and 10  $\mu\text{M}$  of SirT1 in 50 mM ammonium acetate pH 7.0 was incubated at 37  $^\circ\text{C}$  for 30 min. Control experiments in which **2** or **1** or SirT1 was removed from the reaction mixture were carried out in parallel. After incubation, reaction mixtures were stored on ice and subjected to MALDI analysis using a PerSeptive Voyager DE STR MALDI-TOF mass spectrometer in reflective positive mode. The species with  $m/z = 1169.9$  only appeared in the reaction with **1**, **2** and SirT1.



S11. Figure S



**Time-dependent release of Af2Sir2 from avidin beads.** In order to capture Af2Sir2 on avidin beads reactions were performed in 300 mM phosphate buffer pH 7.5 in a final volumes of 50  $\mu\text{L}$ . One reaction contained 800  $\mu\text{M}$  of **2** and 400  $\mu\text{M}$  of **1** (final concentrations), whereas a control experiment contained only 800  $\mu\text{M}$  of **2**. Both reactions were initiated by addition of concentrated Af2Sir2 enzyme (25.5  $\mu\text{M}$  final concentration). Reactions were incubated at 37  $^{\circ}\text{C}$  for 30 min and then adjusted to pH 6 by addition of 25  $\mu\text{L}$  of 300 mM phosphate buffer pH 4.25. Compound **3** was added to each sample to a final concentration of 1 mM, and incubated further at 25  $^{\circ}\text{C}$  for 1 h. 50  $\mu\text{L}$  of avidin agarose beads was then added to each sample, and the reactions incubated at 25  $^{\circ}\text{C}$  for 1 h. Supernatant was then removed from the beads, and beads were washed with 1 mM  $\text{NAD}^{+}$  in 300 mM phosphate buffer pH 7.5 (3 x 100  $\mu\text{L}$ ). To monitor release of Af2Sir2 from these beads 80  $\mu\text{L}$  of 100 mM phosphate buffer pH 7.5 containing 800

$\mu\text{M}$  of  $\text{NAD}^+$  was added to each set of beads. Bead buffer mixtures were incubated at 37 °C, and 15  $\mu\text{L}$  of aliquots were sampled from the buffer (no removal of beads) at 30 min intervals. Enzymatic activity in each aliquot was assayed by HPLC. To the aliquot was added 5  $\mu\text{L}$  of p53mer (final concentration 800  $\mu\text{M}$ ), followed by incubation at 37 °C for 1 h. Reactions were quenched by addition of 5  $\mu\text{L}$  of 10% TFA. After centrifugation at 13,000 g for 2 min to remove precipitates, reactions were injected on a Macherey-Nagel Nucleosil C18 column and  $\text{NAD}^+$  and AADPR resolved by 0.1% TFA as eluant with HPLC chromatograms analyzed at detection wavelength of 260 nm. Reactions were analyzed by quantitation of peak areas corresponding to  $\text{NAD}^+$  and AADPR. Control showed no product formation at any time.  $\text{Rate}_{\text{obs}}$  (see y-axis, Figure S7) at each time point was pmol AADPR formed/min. This was divided by the volume of the aliquot removed from the beads to provide a measure of enzymatic activity per unit volume at each time point.  $\text{Rate}_{\text{obs}}/\mu\text{L}$  was then plotted versus time to provide the graph in Figure S7.

## S12. Supporting References

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