Supplementary Information for:

Novel sirtuin inhibitory warheads derived from the N^{ε}-acetyl-lysine analog L-2-amino-7-carboxamidoheptanoic acid

Yanhua He, Lingling Yan,[a] Wenwen Zang,[a] Weiping Zheng*

School of Pharmacy, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, Jiangsu Province, P. R. China

[a]These two authors are the equal second authors.

Contents:

Experimental

General. The following materials were obtained from commercial sources for the compound preparation, and were used as received without further treatment. Sigma-Aldrich China: N-methylmorpholine (NMM), trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF), Rink Amide resin, triethylamine, methylamine in MeOH (2.0 M), ethylamine in MeOH (2.0 M), isopropylamine, methyl 4-aminobutyrate hydrochloride, 4-dimethylaminopyridine (DMAP), NaOH, EtSH, dodecylamine; Alfa Aesar China: 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBt), dimethylamine in MeOH (2.0 M), dicyclohexylcarbodiimide (DCC), N,N'-diisopropyl-carbodiimide (DIC), tert-butyl 2-bromoacetate, LiOH, anhydrous Na_2SO_4; Honeywell China: dichloromethane (DCM), acetonitrile, MeOH, EtOH, ethyl acetate; TCI Shanghai: aniline, glycine methyl ester hydrochloride, β-alanine methyl ester hydrochloride, potassium thioacetate, 1-dodecanol, 1-dodecanethiol; Sinopharm Chemical Reagent Co., Ltd.: piperidine, acetic anhydride, diethyl ether. With the exception of Fmoc-Lys(Mtt)-OH (purchased from GL Biochem (Shanghai) Ltd.) and Fmoc-(S)-Asu(OtBu)-OH (purchased from OKeanos Tech. Co., Ltd.;
Beijing), all the other N^{α}-Fmoc-protected amino acids were purchased from Sigma-Aldrich China, Alfa Aesar China, or TCI Shanghai.

Routine unit-resolution mass spectrometry was performed on a Thermo LXQ LC-ion trap mass spectrometer at Jiangsu University. High-resolution mass spectrometry (HRMS) was performed on an AB 5600+ Q TOF high-resolution mass spectrometer at the Pharmacy School of Fudan University.

The following materials were obtained from commercial sources for the sirtuin inhibition assay and were used as received without further treatment. Sigma-Aldrich China: the active human recombinant His_6-SIRT1, Trizma, Hepes, β-NAD^+, DMSO (molecular-biology grade), a 1.0 M solution of $MgCl_2$ (molecular-biology grade); Cayman Chemical: the active human recombinant GST-SIRT1, the active human recombinant His_6-SIRT2, the active human recombinant His_6-SIRT3, the active human recombinant GST-SIRT5, the active human recombinant His_6-SIRT6; TCI Shanghai: DL-Dithiothreitol (DTT); Alfa Aesar China: NaCl, KCl.

The peptide substrates prepared and used in the sirtuin inhibition assay were: the SIRT1/2/3 substrate H_2N-HK-[N^{ε}-acetyl-lysine]-LM-COOH corresponding to amino acids 380-384 of the human p53 protein acetylated at K^{382}; the SIRT5 substrate CH_3CONH-AR-[N^{ε}-succinyl-lysine]-ST-$CONH_2$ corresponding to amino acids 7-11 of the human histone H3 protein succinylated at K^9; the SIRT6 substrate H_2N-EALPK-[N^{ε}-myristoyl-lysine]-TGGPQ-$CONH_2$ corresponding to amino acids 15-25 of the human tumor necrosis factor α (TNFα) myristoylated at K^{20}.

Compound synthesis. The following describe the solid phase synthesis of the peptidic intermediates **11** and **12**, as well as the solution phase synthesis of the final compounds **2**-**10**, **4a**, **4b**, **7a**, **7b**, **8a**, **8b** from **11** or **12**, according to **Schemes 1-3**. All the compounds were purified by reversed-phase high pressure liquid chromatography (RP-HPLC) on a semi-preparative C18 column (1 x 25 cm, 5 μm). The column was eluted with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 4.5mL/min and monitored at 214 nm. The pooled HPLC fractions were concentrated *in vacuo* to remove acetonitrile and the remaining aqueous solution was lyophilized to give all the compounds as puffy white solids. The purities of the purified compounds were >95% based on RP-HPLC analysis on an analytical C18 column (0.46 x 25 cm, 5 μm). While the exact masses of the purified intermediates **11** and **12** were

confirmed by unit-resolution electrospray ionization-mass spectrometry (ESI-MS) analysis (see below), those of the final compounds **2-10**, **4a**, **4b**, **7a**, **7b**, **8a**, **8b** were confirmed by high-resolution mass spectrometry (HRMS) analysis (see **Table 1**).

Synthesis of 11 (Scheme 1). This intermediate was prepared by the Fmoc chemistry-based manual solid phase peptide synthesis (SPPS) on the Rink amide resin. For each amino acid coupling reaction, 4 equivalents of a N^{α}-Fmoc-protected amino acid, 3.8 equivalents of the coupling reagent HBTU and the additive HOBt were used in the presence of 0.4 M NMM/DMF, and the coupling reaction was allowed to proceed at room temperature for 1 h. A 20% (v/v) piperidine/DMF solution was used for Fmoc removal. The N-terminal α-amino group was acetylated on resin with acetic anhydride in the presence of 0.4 M NMM/DMF. The side chain Mtt protecting group from two Lys(Mtt) residues were selectively removed with a 1% (v/v) TFA/DMF solution before the two exposed free amino groups were acetylated with acetic anhydride on resin. The final treatment with a TFA-containing solution (85% (v/v) TFA, 5% (v/v) ddH_2O, 10% (v/v) DCM) cleaved the crude intermediate **11** from the resin and removed the side chain tert-butyl protecting group from the central (S)-Asu(OtBu) residue as well. Technically, following the TFA treatment at room temperature for 4h, the TFA cleavage filtrate was concentrated *in vacuo* to remove any volatiles before precipitation of the crude intermediate **11** in cold diethyl ether. The crude **11** was then purified by semi-preparative RP-HPLC as above described. The exact mass of the purified **11** was confirmed by a unit-resolution electrospray ionization-mass spectrometry (ESI-MS) analysis: Calcd. m/z for $C_{26}H_{47}N_6O_8$ ($[M+H]^+$) 571.3; found: 571.5.

Synthesis of 2-6 (Scheme 1). A mixture of the purified intermediate **11** (0.025 mmol), HATU (9.5 mg, 0.025mmol), and an amine (methylamine in MeOH (2.0 M), dimethylamine in

MeOH (2.0 M), ethylamine in MeOH (2.0 M), isopropylamine, or aniline) (0.04 mmol, or 0.055 mmol for aniline) in the presence of 0.4M NMM/DMF (500 μL) was stirred at room temperature for 2.5h (or 3h for the reaction with aniline), followed by precipitation of the crude **2**-**6** in cold diethyl ether. The crude compounds **2**-**6** were then purified by semi-preparative RP-HPLC as above described.

Synthesis of 4a (Scheme 1). To a stirred mixture of the purified intermediate **11** (0.0125 mmol) and DMAP (1.25 mg, 0.01 mmol) in EtOH (500 μL) was added a solution of DCC (2.9 mg, 0.014 mmol) in EtOH (200 μL), and the reaction mixture was then stirred at room temperature for 48h. The crude **4a** was precipitated in cold diethyl ether and purified by semi-preparative RP-HPLC as above described.

Synthesis of 4b (Scheme 1). A solution of the purified intermediate **11** (0.0125 mmol) in DMF (500 μL) was neutralized with triethylamine, followed by the addition of DIC (40 μL, 0.25 mmol) and EtSH (18 μL, 0.25 mmol). The reaction mixture was then stirred at room temperature for 48h. The crude **4b** was precipitated in cold diethyl ether and purified by semi-preparative RP-HPLC as above described.

Synthesis of 12 (Scheme 2). This intermediate was also prepared by the Fmoc chemistry-based manual solid phase peptide synthesis (SPPS) on the Rink amide resin. Its N-terminal α-amino group was also acetylated on resin with acetic anhydride in the presence of 0.4 M NMM/DMF. The procedural details of its peptide chain assembly and cleavage from the resin were the same as those described above for intermediate **11**. The crude intermediate **12** precipitated in cold diethyl ether was also purified by semi-preparative RP-HPLC as above described. The exact mass of the purified **12** was also confirmed by a unit-resolution ESI-MS analysis: Calcd. m/z for $C_{26}H_{48}N_9O_{10}$ ($[M+H]^+$) 646.4; found: 646.8.

Synthesis of 7 (Scheme 2). A mixture of the purified intermediate **12** (0.025 mmol), HATU (9.5 mg, 0.025mmol), and dodecylamine (18.6 mg, 0.1 mmol) in the presence of 0.4M NMM/DMF (500 μL) was stirred at room temperature for 3h, followed by precipitation of the crude **7** in cold diethyl ether. The crude compound **7** was then purified by semi-preparative RP-HPLC as above described.

Synthesis of 7a (Scheme 2). To a stirred mixture of the purified intermediate **12** (0.025 mmol), DMAP (2.5 mg, 0.02 mmol), and 1-dodecanol (14 mg, 0.075 mmol) in DMF (1 mL) was added a solution of DCC (5.8 mg, 0.028 mmol) in DMF (200 μL), and the reaction mixture was then stirred at room temperature for 24h. The crude **7a** was precipitated in cold diethyl ether and purified by semi-preparative RP-HPLC as above described.

Synthesis of 7b (Scheme 2). A solution of the purified intermediate **12** (0.0125 mmol) in DMF (500 μL) was neutralized with triethylamine, followed by the addition of DIC (40 μL, 0.25 mmol) and 1-dodecanethiol (60 μL, 0.25 mmol). The reaction mixture was then stirred at room temperature for 48h. The crude **7b** was precipitated in cold diethyl ether and purified by semi-preparative RP-HPLC as above described.

Synthesis of 8-10 (Scheme 3). (a) A mixture of the purified intermediate **11** (0.025 mmol), HATU (9.5 mg, 0.025mmol), and an amine (glycine methyl ester hydrochloride, β-alanine methyl ester hydrochloride, or methyl 4-aminobutyrate hydrochloride) (0.04 mmol) in the presence of 0.4M NMM/DMF (700 μL) was stirred at room temperature for 2.5h, followed by precipitation of the reaction product (i.e. the methyl ester intermediate) in cold diethyl ether. The obtained crude methyl ester intermediate was then purified by semi-preparative RP-HPLC as above described. **(b)** The material from the above reaction was treated with LiOH (4.2 mg, 0.175 mmol) in MeOH/ddH_2O(3/1, v/v) (334 μL), and the reaction mixture was then stirred overnight

at 4 °C, followed by acidification at 0 °C with 1N HCl to pH ~1 and concentration under reduced pressure. The methyl ester hydrolysis products (i.e. **8**-**10**) were then each isolated from the corresponding reaction mixture by semi-preparative RP-HPLC as above described.

Synthesis of 8a (Scheme 3). (a) To a stirred mixture of the purified intermediate **11** (0.025 mmol), triethylamine (8 μL, 0.058 mmol) in DMF (800 μL) was added tert-butyl 2-bromoacetate (5 μL, 0.03 mmol), and the reaction mixture was then stirred at room temperature for 48h. The crude reaction product (i.e. the tert-butyl ester intermediate) was precipitated in cold diethyl ether and then purified by semi-preparative RP-HPLC as above described. **(b)** The material from the above reaction was treated with 85% (v/v) TFA/DCM (1 mL) at 4 °C for 30 min before concentration under reduced pressure. The crude **8a** was then precipitated in cold diethyl ether, and purified by semi-preparative RP-HPLC as above described.

Synthesis of 8b (Scheme 3). (a) Synthesis of tert-butyl 2-mercaptoacetate: A mixture of tert-butyl 2-bromoacetate (176.4 μL, 1.05 mmol), potassium thioacetate (183.0 mg, 1.6 mmol) in EtOH (3 mL) was stirred at room temperature for 24h, followed by centrifugation to discard the pellet. The supernatant in the reaction vessel (a BD Falcon conical centrifuge tube) was then treated with 2M NaOH (2 mL) at room temperature for 10 min before the reaction content was poured into ddH_2O (5 mL). The reaction product was extracted once with ethyl acetate (10 mL); the organic layer was then washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The obtained material was used for the next reaction without further purification. **(b)** A solution of the purified intermediate **11** (0.0125 mmol) in DMF (2 mL) was neutralized with triethylamine, and mixed with DIC (40 μL, 0.25 mmol) and all the material from the above reaction. The reaction mixture was then stirred at room temperature for 48h. The crude reaction product was precipitated in cold diethyl ether and used for the next

reaction without further purification. **(c)** The material from the above reaction was treated with 85% (v/v) TFA/DCM (1 mL) at 4 °C for 30 min before concentration under reduced pressure. The crude **8b** was then precipitated in cold diethyl ether, and purified by semi-preparative RP-HPLC as above described.

***In vitro* sirtuin inhibition assay.** The HPLC-based sirtuin inhibition assay that our laboratory has been using over past several years was employed in the current study and was performed as described previously.[1] An assay solution (50 μL) contained the following components: 50 mM Hepes (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM DTT (omitted for the assay with thioester compounds), β-NAD^+ (0.5 mM for the SIRT1 and SIRT2 assays, 3.5 mM for the SIRT3 assay, 0.8 mM for the SIRT5 assay, and 0.2 mM for the SIRT6 assay), the peptide substrate (0.3 mM of the above-mentioned SIRT1/2/3 substrate for the SIRT1 assay, 0.39 mM of the above-mentioned SIRT1/2/3 substrate for the SIRT2 assay, 0.105 mM of the above-mentioned SIRT1/2/3 substrate for the SIRT3 assay, 0.88 mM of the above-mentioned SIRT5 substrate, 0.02 mM of the above-mentioned SIRT6 substrate), one test compound (**2**-**10**, **4a**, **4b**, **7a**, **7b**, **8a**, or **8b**) with varied concentrations including 0, and a sirtuin (His_6-SIRT1 or GST-SIRT1 (also used for thioester **4b** due to the absence of DTT in this SIRT1 preparation), 320 nM; His_6-SIRT2, 309 nM; His_6-SIRT3, 320 nM; GST-SIRT5 (also used for thioester **8b** due to the absence of DTT in this SIRT5 preparation), 370 nM; or His_6-SIRT6 (also used for thioester **7b** due to the absence of DTT in this SIRT6 preparation), 313 nM). Of note, the same [S]/Km ratios for both substrates (~3.2 for the peptide substrates and ~5.6 for β-NAD^+) were used for the inhibition assays with all the five human sirtuins (SIRT1/2/3/5/6) employed in the current study. An enzymatic reaction was initiated by the addition of a sirtuin at 37 °C and was allowed to be incubated at 37 °C for 10 min (for the SIRT1 assay), 12 min (for the SIRT2 assay), 10 min (for

the SIRT3 assay), 5 min (for the SIRT5 assay), or 12 min (for the SIRT6 assay) until quenched with the following stop solution: 100 mM HCl and 0.16 M acetic acid. The quenched assay solutions were directly injected into a reversed-phase C18 column (0.46 x 25 cm, 5 μm), eluting with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min, and UV monitoring at 214 nm. Turnover of the limiting substrate was maintained at ≤10%. Stock solutions of the test compounds were prepared in either ddH_2O or DMSO. IC_{50} values were estimated from the Dixon plots $(1/v_0$ *vs.*[inhibitor]$)^2$ as an indication of the inhibitory potency.

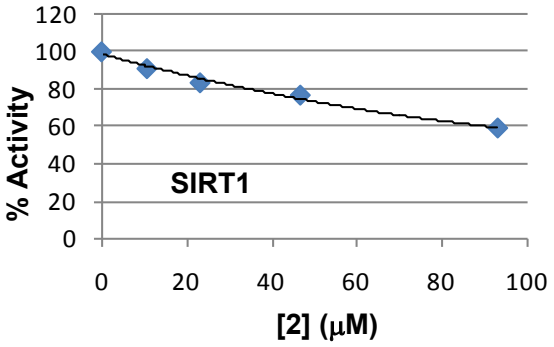
References:

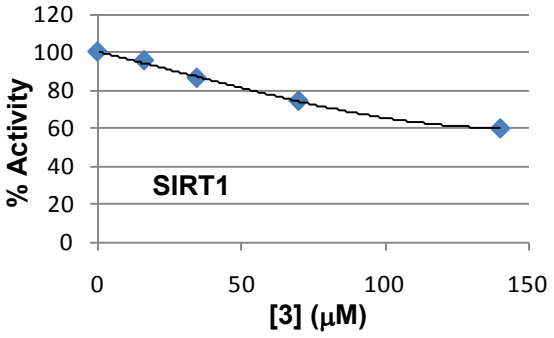
1. Hirsch, B. M.; Gallo, C. A.; Du, Z.; Wang, Z.; Zheng, W. *MedChemComm* **2010**, *1*, 233.

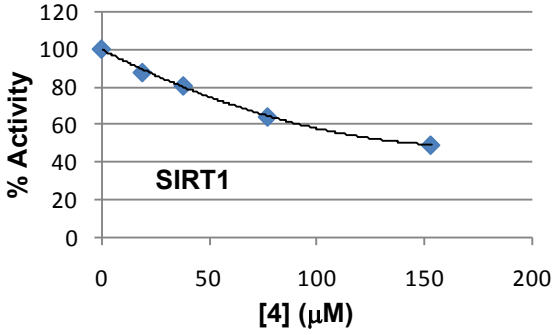
2. Dixon, M. *Biochem. J*. **1953**, *55*, 170.

The following are the representative dose-response curves and Dixon plots generated for all the compounds (**2-10**, **4a**, **4b**, **7a**, **7b**, **8a**, and **8b**) evaluated in the *in vitro* sirtuin inhibition assay in the current study.

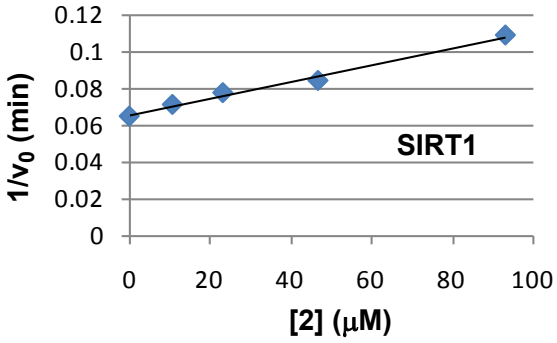
Dose-response curves

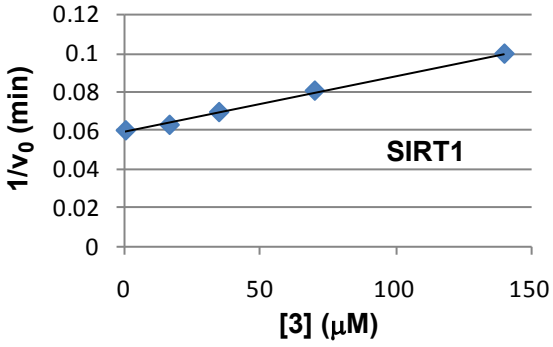


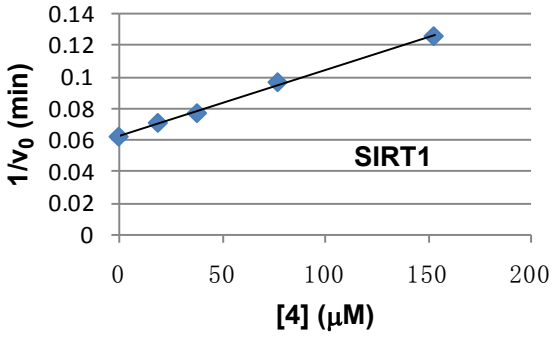


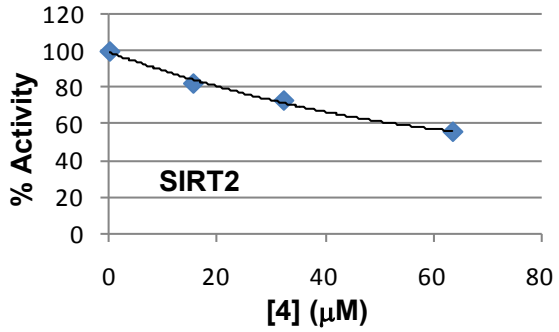


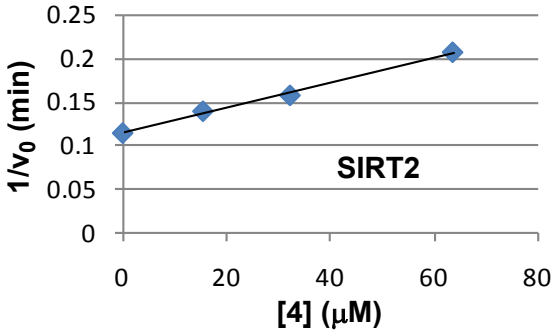
Dixon plots

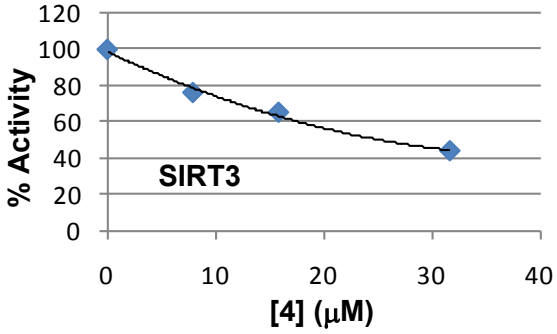


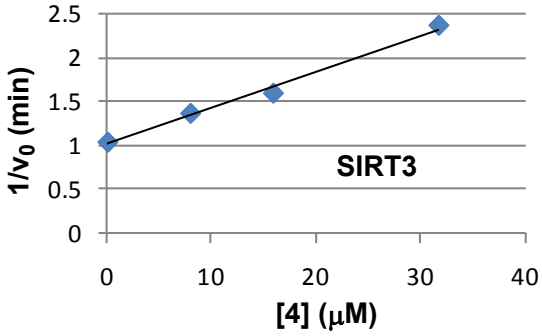


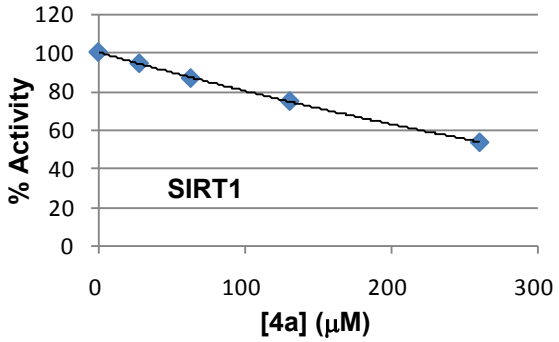


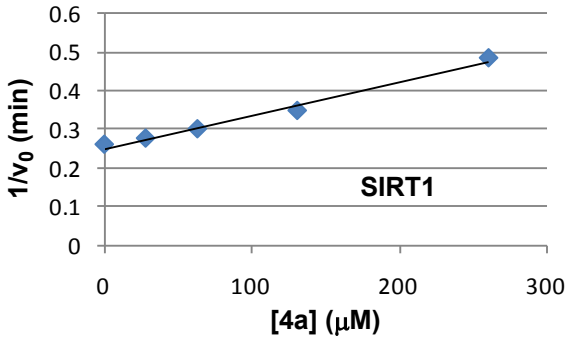

% Activity
SIRT2
[4] (μM)

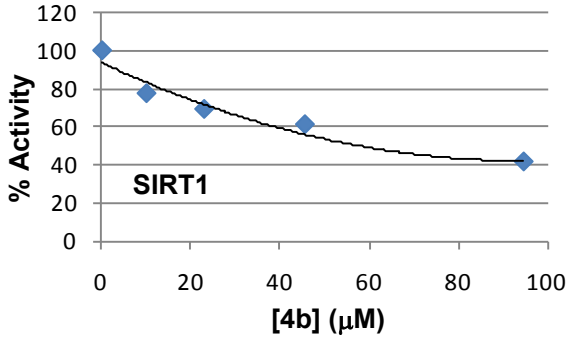

1/v0 (min)
SIRT2
[4] (μM)

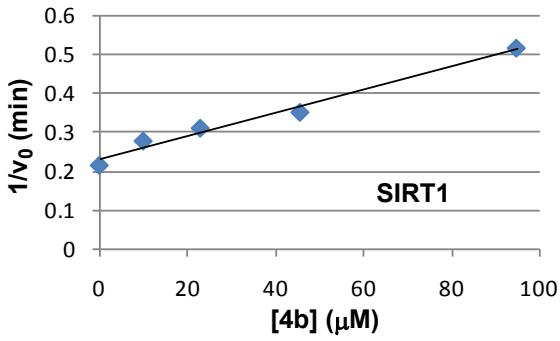

% Activity
SIRT3
[4] (μM)

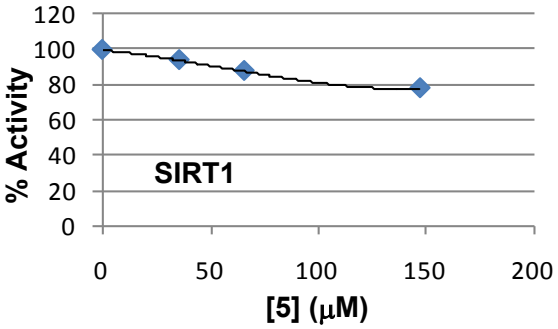

1/v0 (min)
SIRT3
[4] (μM)

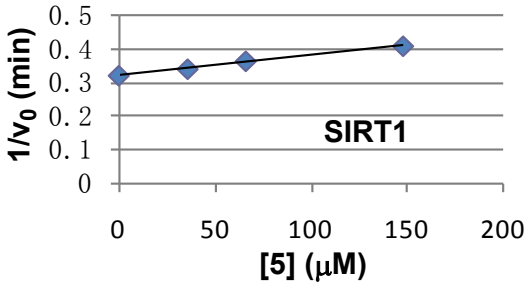

% Activity
SIRT1
[4a] (μM)

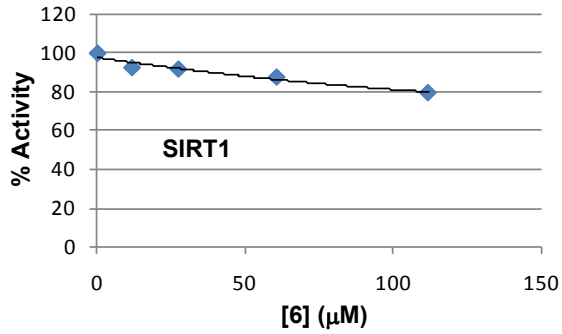

1/v0 (min)
SIRT1
[4a] (μM)

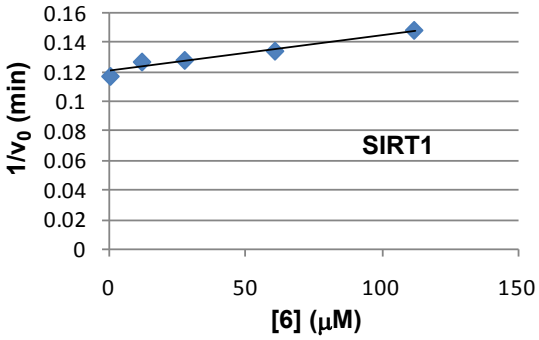

% Activity
SIRT1
[4b] (μM)

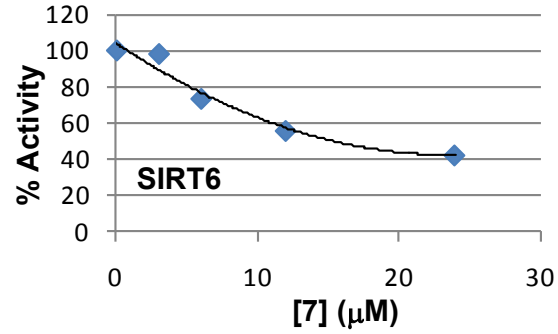

1/v0 (min)
SIRT1
[4b] (μM)

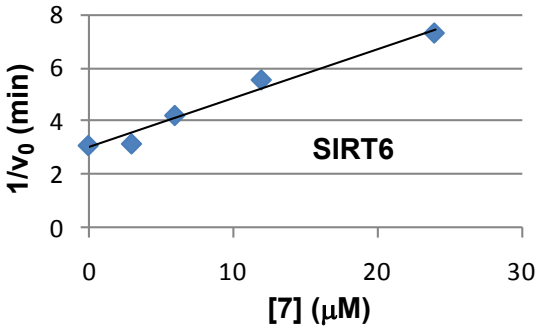

% Activity
SIRT1
[5] (μM)

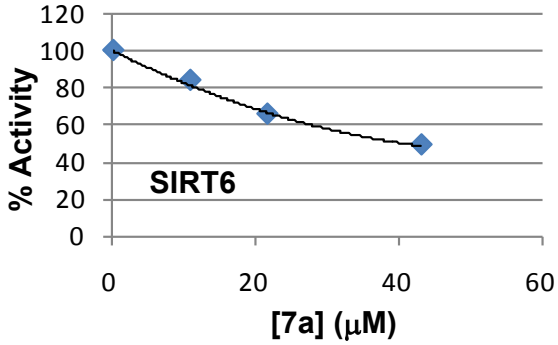

1/v0 (min)
SIRT1
[5] (μM)

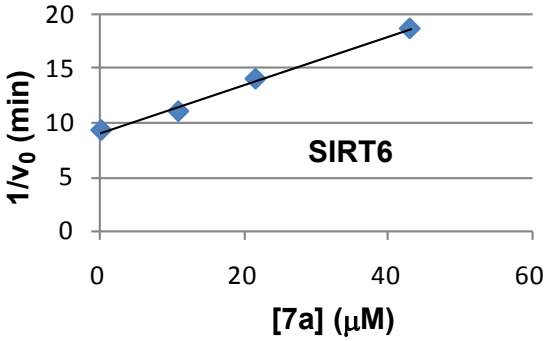

% Activity
SIRT1
[6] (μM)

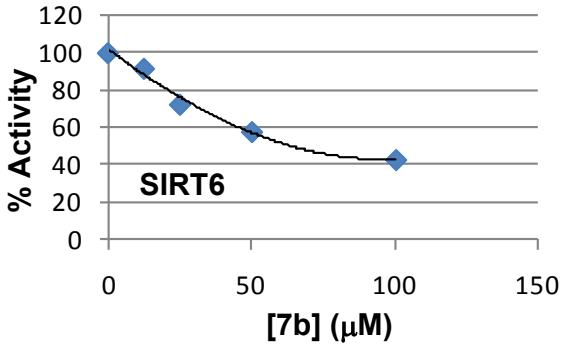

1/v0 (min)
SIRT1
[6] (μM)

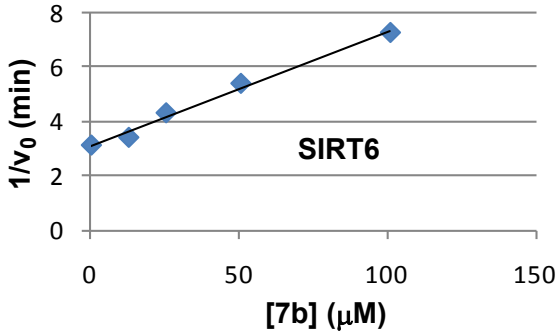

% Activity
SIRT6
[7] (μM)

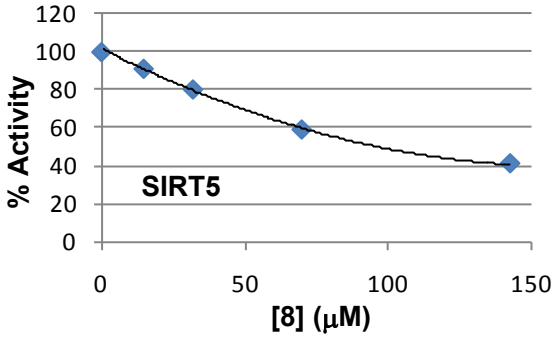

1/v0 (min)
SIRT6
[7] (μM)

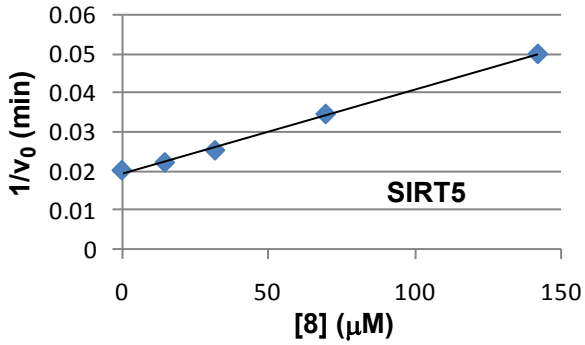

% Activity
SIRT6
[7a] (μM)

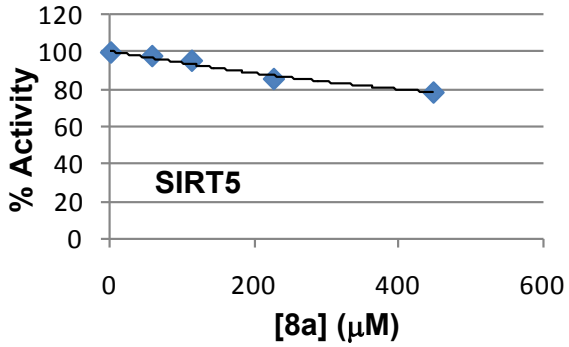

1/v0 (min)
SIRT6
[7a] (μM)

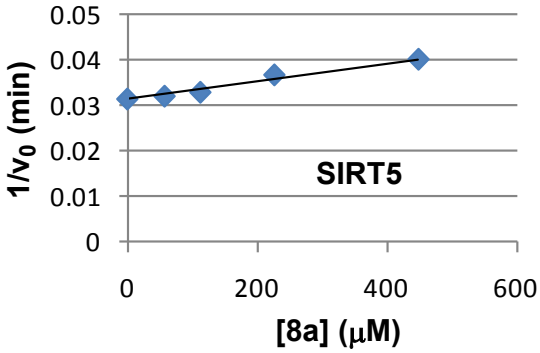

% Activity
SIRT6
[7b] (μM)

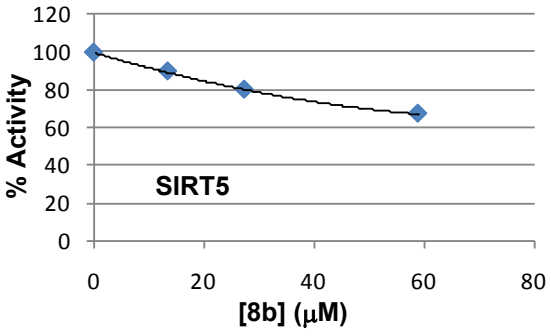

1/v0 (min)
SIRT6
[7b] (μM)

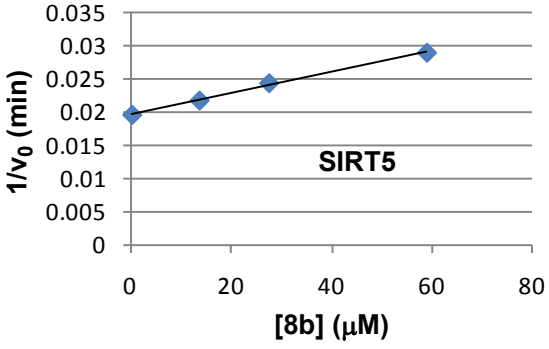

% Activity
SIRT5
[8] (μM)

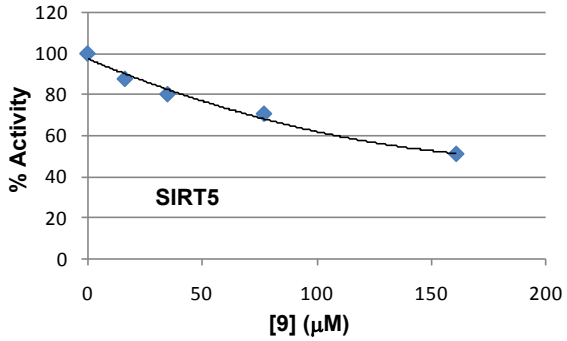

1/v0 (min)
SIRT5
[8] (μM)

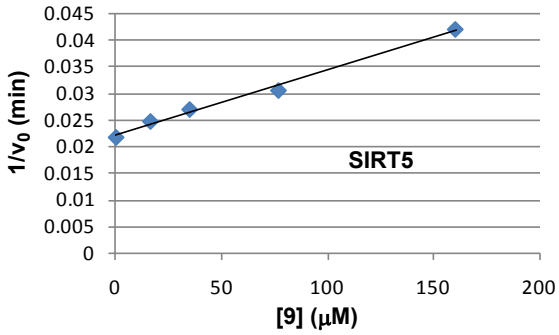

% Activity
SIRT5
[8a] (μM)

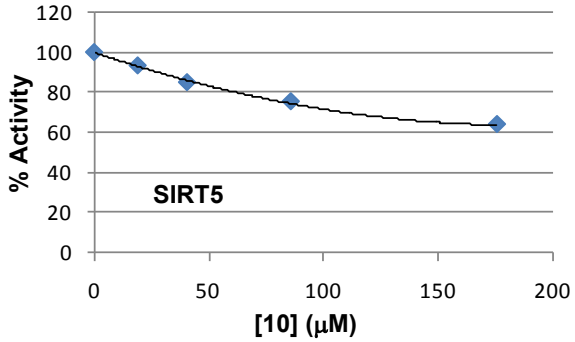

1/v0 (min)
SIRT5
[8a] (μM)

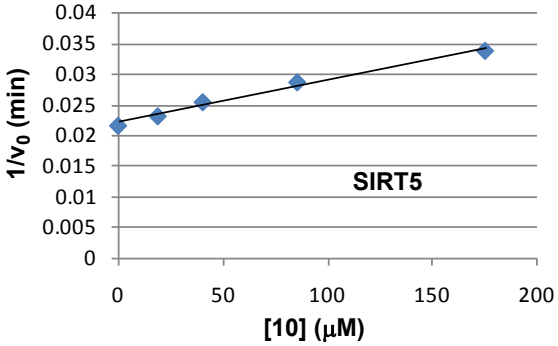

% Activity
SIRT5
[8b] (μM)


1/v0 (min)
SIRT5
[8b] (μM)


120
100
80
60
40
20
0
% Activity
SIRT5
0
50
100
150
200
[9] (μM)


0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
1/v0 (min)
SIRT5
0
50
100
150
200
[9] (μM)


120
100
80
60
40
20
0
% Activity
SIRT5
0
50
100
150
200
[10] (μM)


0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
1/v0 (min)
SIRT5
0
50
100
150
200
[10] (μM)