RSC Advances

SUPPLEMENTARY DATA

Development of a mitochondrial sirtuin4 FRET assay based on its activity for removing 3-hydroxy-3-methylglutaryl (HMG) modification

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Contents:

1. General methods.

Human SIRT1, SIRT4 and SIRT5 were expressed as previously described.[1] Reagents were obtained from Aldrich or Acros in the highest purity available and used as supplied. All amino acids for peptides synthesis were purchased from Suzhou Tianma Group, Co., LTD. China. Trypsin was from Gibco (0.5% Trypsin-EDTA, 10X). LCMS was carried out on a SHIMADZU LC and Thermo LCQ FLEET MS with a Sprite TARGA C18 column (40 × 2.1 mm, 5 μm, Higgins Analytical, Inc.) monitoring at 215 and 260 nm. Solvents used in LCMS were water with 0.1% formic acid and acetonitrile with 0.1% formic acid. Analytic HPLC analysis was carried out using Kinetex XB-C18 100A, 100 mm × 4.60 mm, 2.6 μm reverse phase column with UV detection at 215 nm and 336 nm. Preparative HPLC purification was carried out using Targa™ Prep C18 10μm 250×20mm reverse phase column with UV detection at 215 nm and 260 nm. Fluorescence assay was recorded by BIO-TEK® Synergy H4 plate reader (Optics Position: Top, Sensitivity: 50, Excitation at the wavelength of 336 nm and Emission at the wavelength of 490 nm).

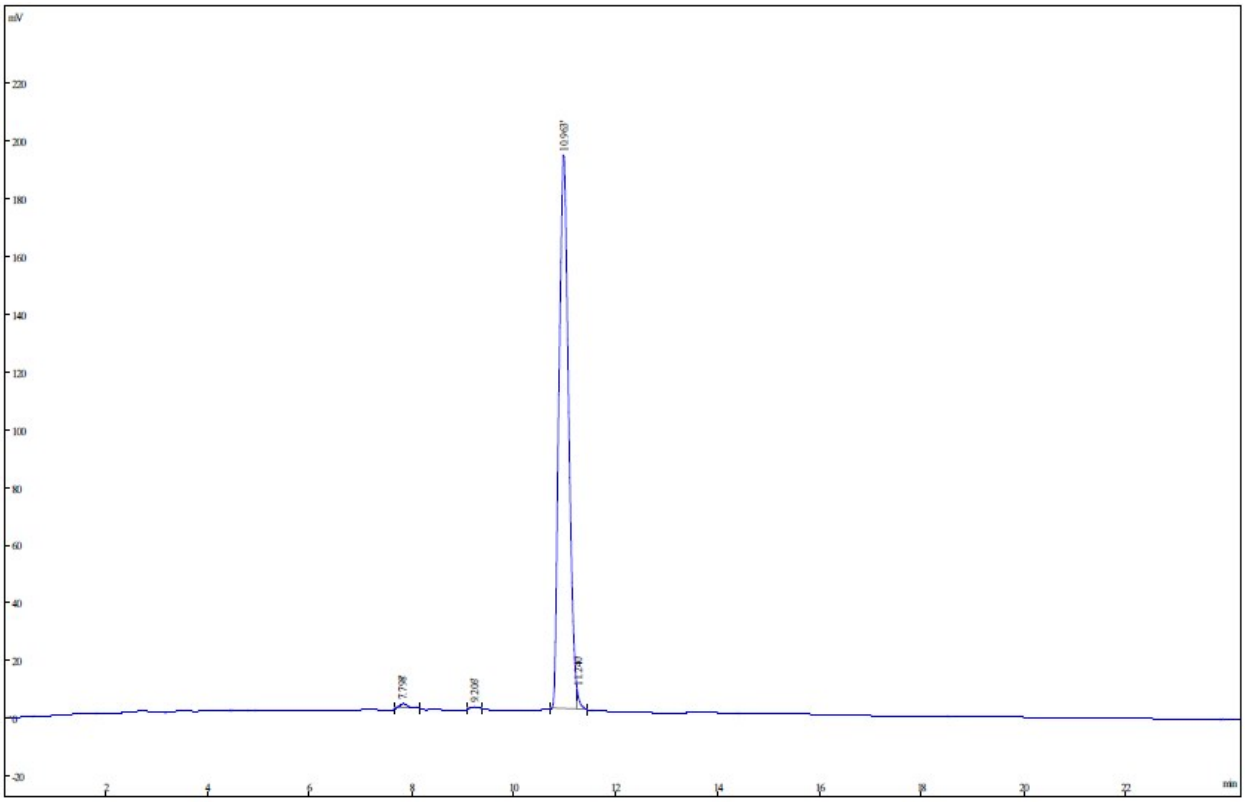
2. Peptide synthesis.

2.1 Synthesis of (DABCYL)GVLK(HMG) EYGVE(EDANS)G peptide

Starting from Fmoc-Gly-Wang Resin (200 mg, 0.1mmol), the following peptide synthesis was based on the standard Fmoc/tBu chemistry O-benzotriazol-N,N,N',N'-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazol (HBTU/HOBt) protocol.[2] The quencher dye, EDANS was introduced into the peptide by using the amino acid of Fmoc-Glu(EDANS)-OH; while the donor dye, DABCYL was coupled by using DABCYL acid directly. The HMGylation of lysine residue was done by using Fmoc-Lys(Dde)-OH coupled into the peptide followed by hydrolysis of 2% hydrazine hydrate and coupling with 3-Hydroxy-3-methylglutaric acid at the last. For cleavage from the resin, resin was suspended in a 1:1:8 by volume mixture of acetic acid/TFE/DCM for 30 mins at RT. Filter the resin and the filtrate was concentrated in vaccum. For removal of protecting groups, the above residue was treated with TFA (2 mL) for 4 hours. The crude peptides were purified by reverse phase HPLC on BECKMAN COULTER System Gold 125P solvent module & 168 Detector with a TARGA C18 column (250 × 20 mm, 10 μm, Higgins Analytical, Inc., Mountain View, CA) monitoring at 215 nm. Mobile phases used were 0.1% aqueous TFA (solvent A) and 0.1% TFA in acetonitrile (solvent B). Peptides were eluted with a flow rate of 10 mL/min with the following gradient: 0% solvent B for 5 min, then 0 % to 25% solvent B over 25 min. The identity and purity of the peptides were verified by LCMS.

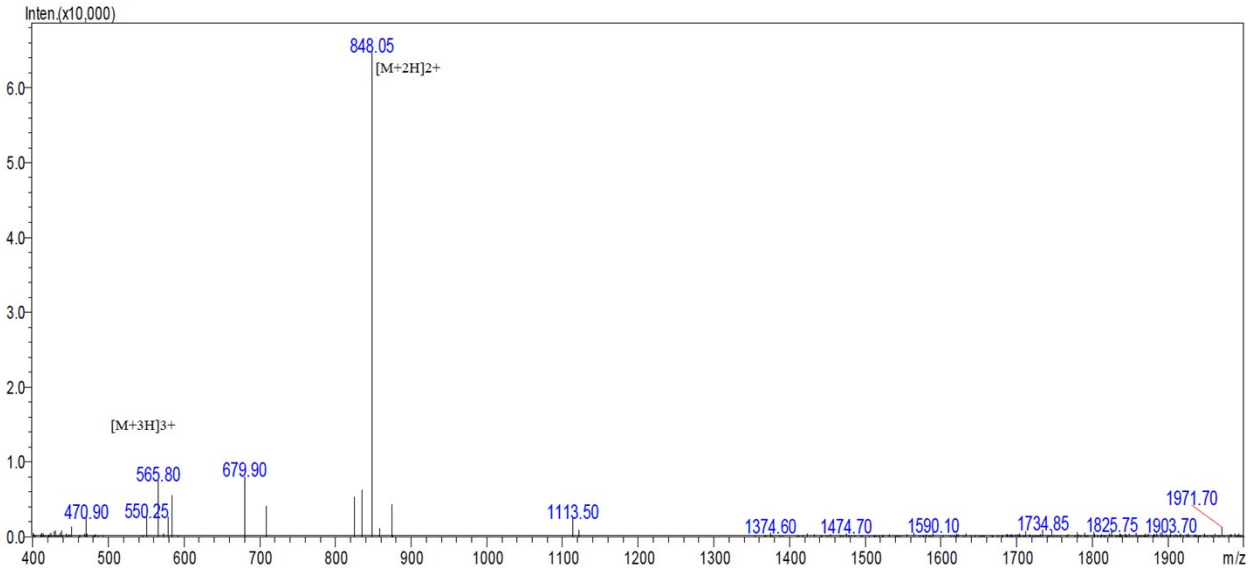
(DABCYL)GVLK(HMG)EYGVE(EDANS)G peptide in a yield of 10% with the purity of 98.2%, LCMS (ESI) calcd. for $C_{80}H_{110}N_{16}O_{23}S$ [M+2H$^+$] 847.38, obsd. 848.05 and calcd. for $C_{80}H_{111}N_{16}O_{23}S$ [M+3H$^+$] 565.26, obsd. 565.80.

HPLC trace for the purity of (DABCYL)GVLK(HMG) EYGVE(EDANS)G peptide



Rank	Time	Conc.	Area	Height
1	7.798	0.9182	23624	1501
2	9.206	0.1910	4914	521
3	10.963	98.2293	2527226	194075
4	11.240	0.6615	17020	5248
Total		100	2572784	201345

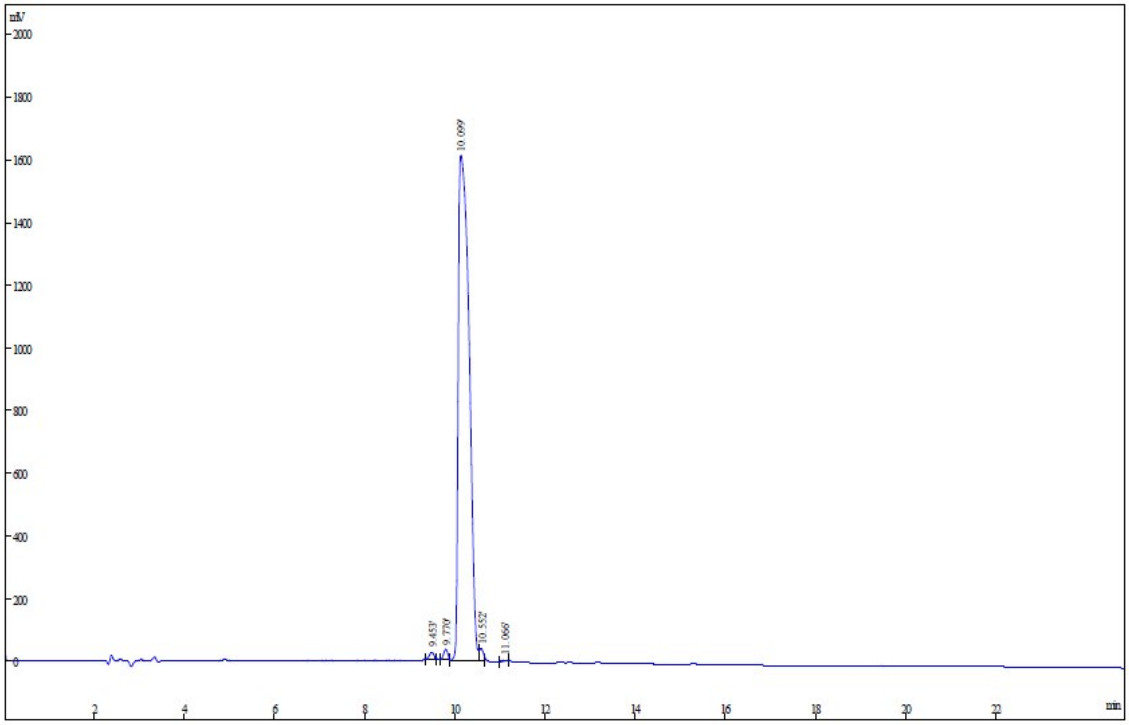
Mass spectrum for the identity of (DABCYL)GVLK(HMG) EYGVE(EDANS)G peptide



2.2 Synthesis of GVLK(HMG)EYGVW peptide

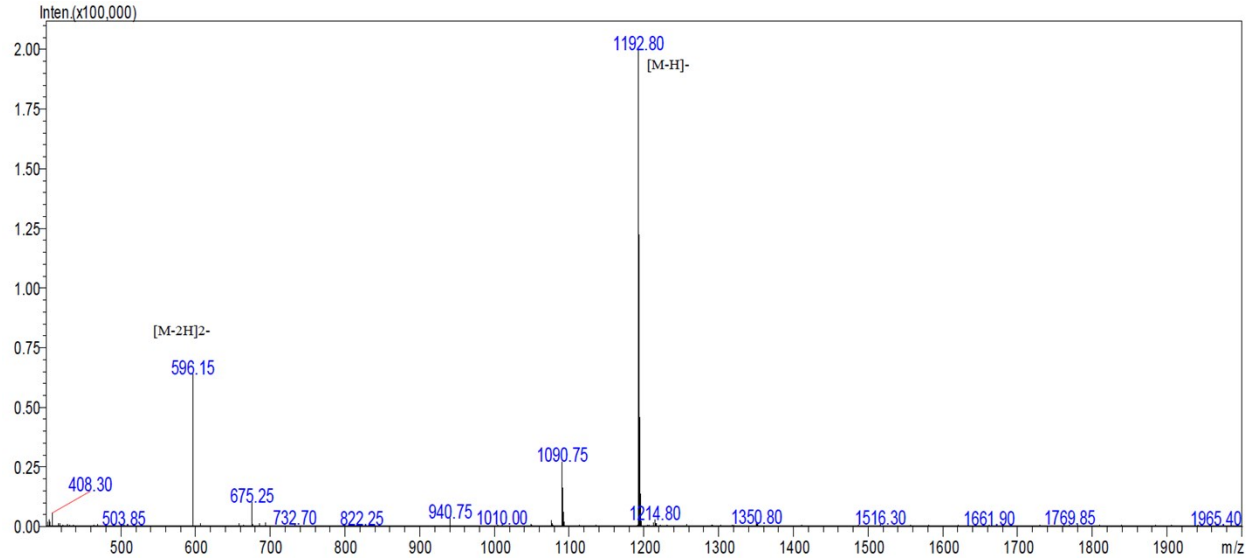
Starting from Fmoc-Trp-Wang Resin (200 mg, 0.1mmol) instead of Fmoc-Gly-Wang Resin, the following peptide synthesis was similar with the previous protocol as the description of **2.1**. GVLK(HMG) EYGVW peptide in a yield of 15% with the purity of 98.0%, LCMS (ESI) calcd. for C57H82N11O17 [M-H$^-$] 1192.59, obsd. 1192.80 and calcd. for C57H81N11O17 [M-2H$^-$] 595.79, obsd. 596.15.

HPLC trace for the purity of GVLK(HMG)EYGVW peptide



Rank	Time	Conc.	Area	Height
1	9.453	0.5386	147006	22046
2	9.770	0.6459	176277	30749
3	10.099	98.03	26755830	1618197
4	10.552	0.6324	172603	34329
5	11.066	0.147	40124	5520
Total		100	27291840	1710841

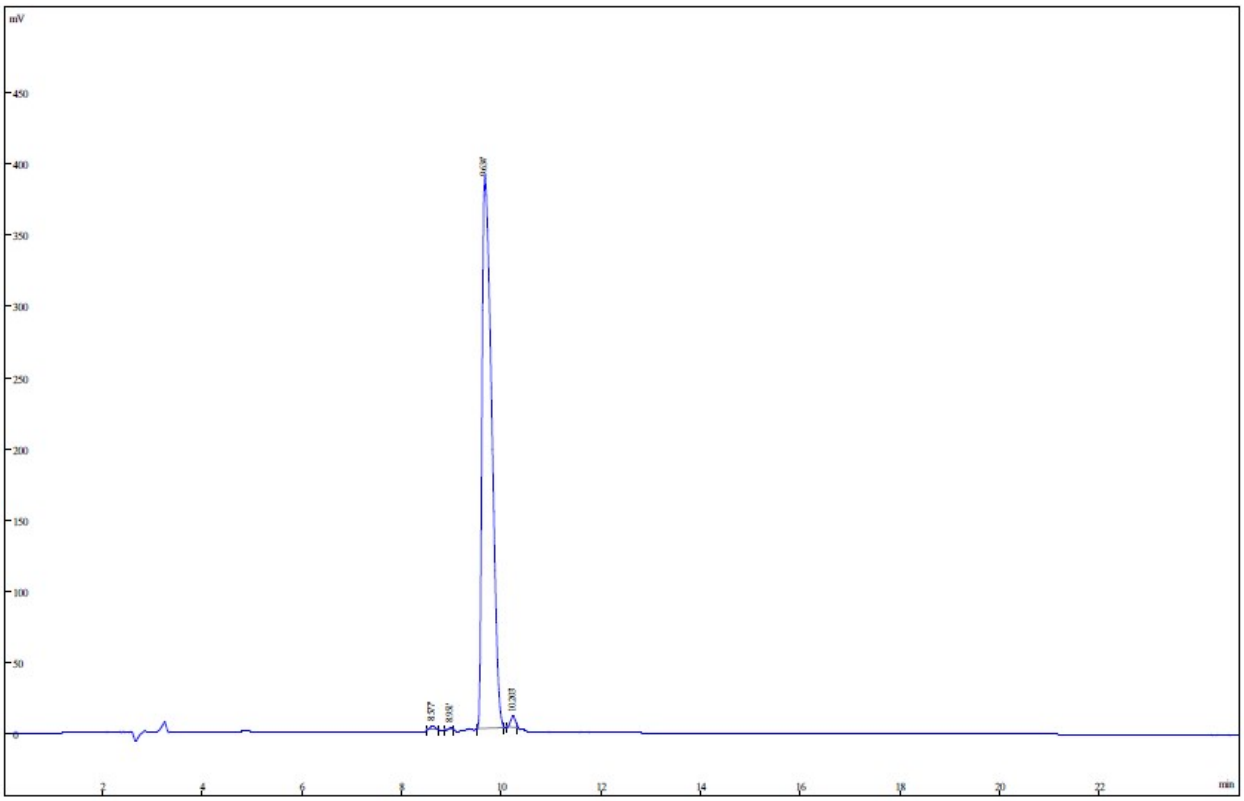
Mass spectrum for the identity of GVLK(HMG)EYGVW peptide



2.3 Synthesis of GVLKEYGVW peptide

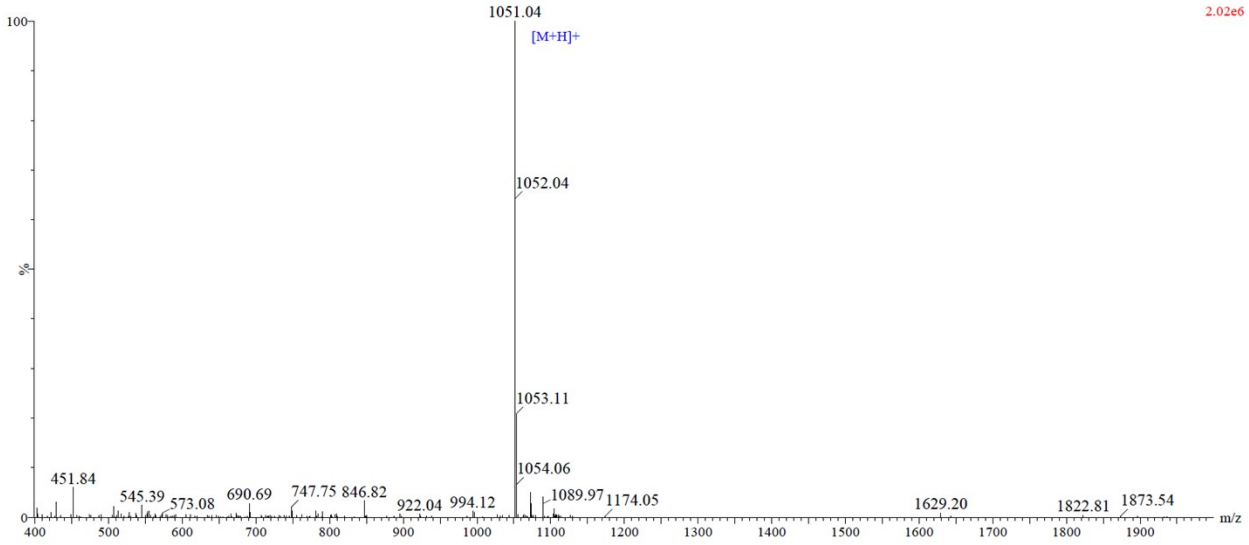
Fmoc-Lys(Boc)-OH (94 mg, 0.2mmol) was used instead of Fmoc-Lys(Dde)-OH during the peptide synthesis. Other than this, the procedure of the peptide synthesis was similar with the description of **2.2**. GVLKEYGVW peptide in a yield of 20% with the purity of 98.0%, LCMS (ESI) calcd. for C51H76N11O13 [M+H$^+$] 1050.56, obsd. 1051.04.

HPLC trace for the purity of GVLKEYGVW peptide



Rank	Time	Conc.	Area	Height
1	8.577	0.5040	26688	3155
2	8.951	0.2820	14932	2326
3	9.634	98.0176	5189764	390244
4	10.203	1.1964	63349	8576
Total		100	5294733	404301

Mass spectrum for the identity of GVLKEYGVW peptide



3. Enzymatic Assays.

3.1 Testing Kinetics of the GVLK(HMG)EYGVW and (DABCYL)GVLK(HMG)EYGVE(EDANS)G peptides on SIRT4.

For the Kinetics of GVLK(HMG)EYGVW peptide on SIRT4:

The GVLK(HMG)EYGVW peptide concentration was varied from 2~256 uM. The reactions (60 uL with 1 mM NAD, 20 mM Tris pH 7.4, 1 mM DTT, 1 uM SIRT4 and the peptide at different concentrations) were incubated for 12 mins at 37°C. Each reaction was stopped using 60 uL of 200 mM HCl and 320 mM acetic acid. The reactions mixtures were spun at 12000g for 10mins and then ananlyzed on a Kinetex XB-C18 column (100A, 100 mm × 4.60 mm, 2.6 um, Phenomenex). The product and the substrate peaks were quantified using absorbance at 336 nM and converted to initial rates, which were then plotted against the peptide concentrations and fitted using Kaleidagraph. All reactions were duplicated.

For the Kinetics of (DABCYL)GVLK(HMG)EYGVE(EDANS)G peptide on SIRT4:

The (DABCYL)GVLK(HMG)EYGVE(EDANS)G peptide concentration was varied from 5~500 uM. The reactions (60 uL with 1 mM NAD, 20 mM Tris pH 7.4, 1 mM DTT, 1 uM SIRT4 and the peptide at different concentrations) were incubated for 40 mins at 37°C. Each reaction was stopped using 60 uL of 200 mM HCl and 320 mM acetic acid. The reactions mixtures were spun at 12000g for 10mins and then ananlyzed on a Kinetex XB-C18 column (100A, 100 mm × 4.60 mm, 2.6 um, Phenomenex). The product and the substrate peaks were quantified using

absorbance at 336 nM and converted to initial rates, which were then plotted against the peptide concentrations and fitted using Kaleidagraph. All reactions were duplicated.

3.2 Testing (DABCYL)GVLK(HMG) EYGVE(EDANS)G peptide without or with different concentrations of SIRT4.

First step, SIRT4 (0.5~3 uM) or no sirtuin was incubated with (DABCYL)GVLK(HMG) EYGVE(EDANS)G (10 uM), NAD (1 mM) in Tris-HCl buffer (pH 7.4, 20 mM) containing dithiothreitol (DTT, 1 mM) in a 60 uL reaction for 1 hour at 37°C. Second step, the above reaction was incubated with trypsin (6.25U) and nicotinamide (10 mM) for 1 hour at 37°C. The mixture was transferred to 96-well plate and the fluorescence was recorded by BIO-TEK® Synergy H4 plate reader (Optics Position: Top, Sensitivity: 50, Excitation at the wavelength of 336 nm and Emission at the wavelength of 490 nm). All reactions were duplicated.

3.3 Screening for SIRT4 inhibitors with (DABCYL)GVLK(HMG)EYGVE(EDANS)G in the FRET-based assay.

First step, SIRT4 (1 uM) was incubated with (DABCYL)GVLK(HMG)EYGVE(EDANS)G peptide (10 uM), indicated inhibitor (300 uM) and NAD (1 mM) in Tris-HCl buffer (pH 7.4, 20 mM) containing dithiothreitol (DTT, 1 mM) in a 60 uL reaction for 1 hour at 37°C. Second step, the above reaction was incubated with trypsin (6.25U) containing nicotinamide (10 mM) was added and the reactions were incubated for 1 hour at 37°C. Then the mixture was transferred to 96-well plate and the fluorescence was recorded by by BIO-TEK® Synergy H4 plate reader (Optics Position: Top, Sensitivity: 50, Excitation at the wavelength of 336 nm and Emission at the wavelength of 490 nm). All reactions were duplicated.

3.4 Secondary screening for filtering non-specific SIRT4 inhibitors using SIRT1 and (DABCYL)ISGASEK(Ac)DIVHSE(EDANS)G in the FRET-based assay.

First step, SIRT1 (1 uM) was incubated with (DABCYL)ISGASEK(Ac)DIVHSE(EDANS)G peptide peptide (10 uM), nicotinamide (300 uM) and NAD (1 mM) in Tris-HCl buffer (pH 7.4, 20 mM) containing dithiothreitol (DTT, 1 mM) in a 60 uL reaction for 1 hour at 37°C. Second step, the above reaction was incubated with trypsin (6.25U) containing nicotinamide (10 mM) was

added and the reactions were incubated for 1 hour at 37°C. Then the mixture was transferred to 96-well plate and the fluorescence was recorded by by BIO-TEK® Synergy H4 plate reader (Optics Position: Top, Sensitivity: 50, Excitation at the wavelength of 336 nm and Emission at the wavelength of 490 nm). All reactions were duplicated.

3.5 Screening for filtering non-specific SIRT4 inhibitors using SIRT5 (DABCYL)ISGASEK(Su)DIVHSE(EDANS)G in the FRET-based assay.

First step, SIRT5 (1 uM) was incubated with (DABCYL)ISGASEK(Su)DIVHSE(EDANS)G peptide (10 uM), indicated inhibitor (30 uM) and NAD (1 mM) in Tris-HCl buffer (pH 7.4, 20 mM) containing dithiothreitol (DTT, 1 mM) in a 60 uL reaction for 1 hour at 37°C. Second step, the above reaction was incubated with trypsin (6.25U) containing nicotinamide (10 mM) was added and the reactions were incubated for 1 hour at 37°C. Then the mixture was transferred to 96-well plate and the fluorescence was recorded by by BIO-TEK® Synergy H4 plate reader (Optics Position: Top, Sensitivity: 50, Excitation at the wavelength of 336 nm and Emission at the wavelength of 490 nm). All reactions were duplicated.

3.6 Dose-response of nicotinamide and suramin with SIRT4 using (DABCYL)GVLK(HMG)EYGVE(EDANS)G in the FRET-based assay.

In presence of different concentration of nicotinamide (50~2000 uM) or suramin (0.5~100 uM), 1 uM SIRT4 was incubated with (DABCYL)GVLK(HMG)EYGVE(EDANS)G peptide (10 uM) and NAD (1 mM) in Tris-HCl buffer (pH 7.4, 20 mM) containing dithiothreitol (DTT, 1 mM) in a 60 uL reaction for 1 hour at 37°C. Second step, the above reaction was incubated with trypsin (6.25U) containing nicotinamide (10 mM) was added and the reactions were incubated for 1 hour at 37°C. Then the mixture was transferred to 96-well plate and the fluorescence was recorded by by BIO-TEK® Synergy H4 plate reader (Optics Position: Top, Sensitivity: 50, Excitation at the wavelength of 336 nm and Emission at the wavelength of 490 nm). All reactions were duplicated.

3.7 The proposed method for high-throughput screening

Firstly, an initial solution of 20 uL in Tris-HCl buffer (pH 7.4, 20 mM) containing DTT (1 mM), NAD (100 uM), BSA (0.5 ug/uL) and SIRT4 (0.2uM) was transferred to each well in 384-well plate at room temperature. Then, a suramin solution (1 mM) of 10 uL as positive control or a candidate compound solution (1 mM) of 10 uL was added into the assay plate, respectively. The reaction was

carried on by addition of a (DABCYL)GVLK(HMG)EYGVE(EDANS)G peptide solution (40uM) of 10uL at room temperature for 1 hour. Following the addition of a trypsin solution (6.25U) of 10uL containing nicotinamide (10 mM), the fluorescence was finally recorded from 384-well plate by BIO-TEK® Synergy H4 plate reader (Optics Position: Top, Sensitivity: 50, Excitation at the wavelength of 336 nm and Emission at the wavelength of 490 nm). All reactions were duplicated.

4. Supplementary Figures

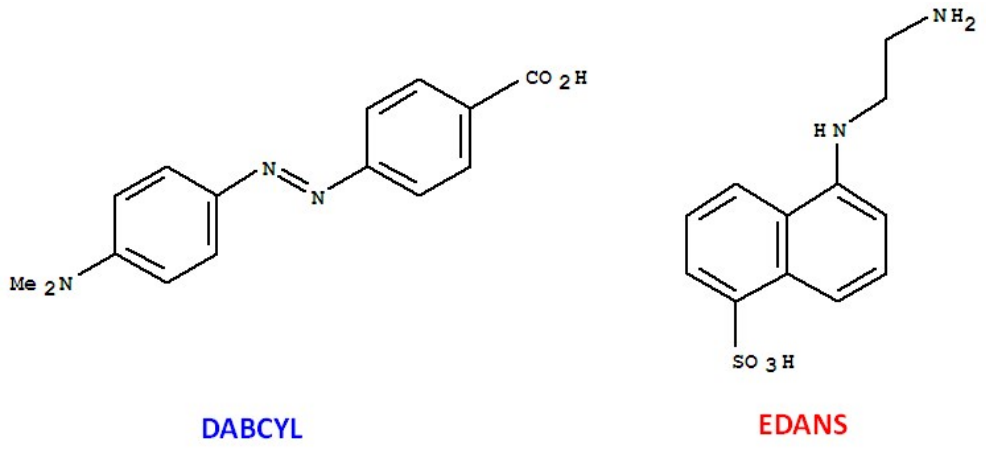


Figure S1. Structures of a pair of FRET dye of DABCYL and EDANS.

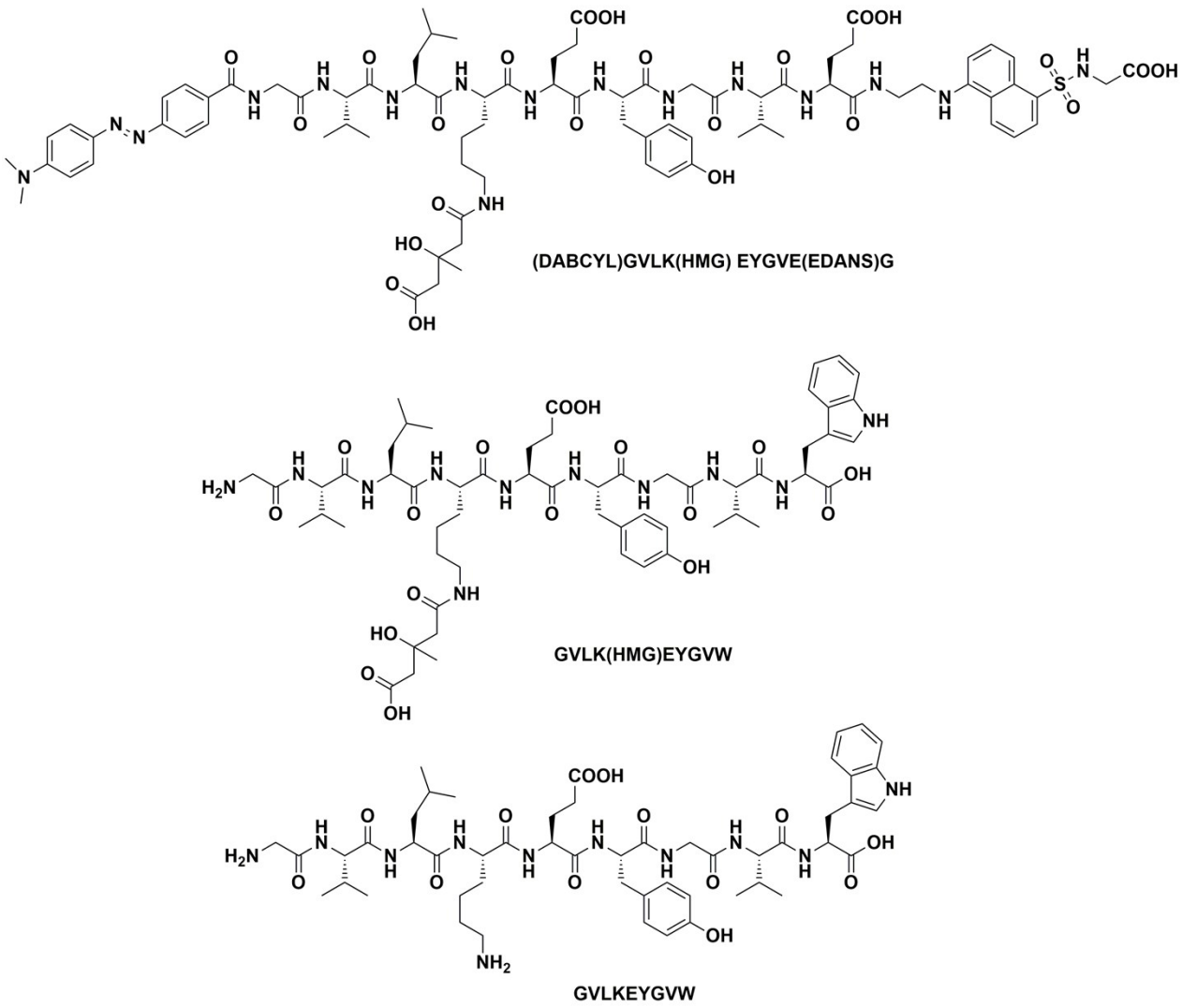


Figure S2. Structures of synthesized peptides for SIRT4 study.

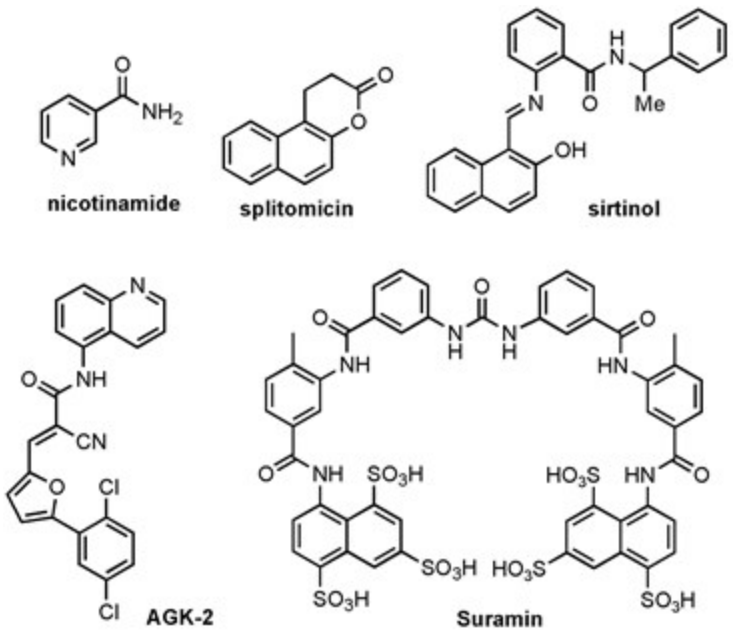


Figure S3. Structures of several reported sirtuin inhibitors used in this study.

5. References

1. Du, J.; Jiang, H.; Lin, H. *Biochemistry* **2009**, *48*, 2878.

2. He, B.; Du, J.; Lin, H. *J. Am. Chem. Soc.* **2012**, *134*, 1922.