SUPPLEMENTARY DATA

Development of a single-step fluorogenic sirtuin assay and its applications for high-throughput screening

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1. Experimental section

1.1 Materials and instruments

Starting materials were obtained from commercial vendors and used directly. The anhydrous solvents used were purchased from Aldrich or Bide Pharmatech Ltd or produced as common procedures. To monitor synthesis reaction, thin layer chromatography (TLC) analysis by UV. Compound purification was performed by flash column chromatography using silica gel (Merck Kieselgel 60, No. 9385, 200-300 mesh ASTM). Nuclear magnetic resonance spectra were obtained with JEOL ECS 400 (operating at 400MHz), with chemical shift in parts per million (ppm, d) downfield from TMS as an internal standard. High-resolution mass spectra (HRMS) were measured with a JEOL (JMS-700) electron impact (EI) mass spectrometer.

pH value was measured with a INESA (PHSJ-3F) pH meter. Analytic HPLC analysis was carried out using SHIMADZU LC16 with InertSustainTM AQ-C18, (250 mm × 4.6 mm, 5 μm) reverse phase column with UV detection at 280 nm and 320 nm. Water with 0.1% trifluoroacetate and acetonitrile with 0.1% trifluoroacetate were used as eluents with the flow rate of 1 mL/ min. LC-MS was analyzed by Thermo Q Exactive Plus with a Hypersil Gold C18 column (50 × 2.1 mm, 1.7 μm, Thermo Fisher). Fluorescence assay was recorded by Thermo Varioskan LUX (Optics Position: Top, Sensitivity: 50, Excitation at the wavelength of 360 nm and Emission at the wavelength of 460 nm).

The expression and purification of recombinant human SIRT1 (aa 193-747), SIRT2 (aa 34-352), SIRT3 (aa 102-399), SIRT5 (aa 34-302) and SIRT6 (full-length) were determined based on the previous studies. *E. coli* expressing pET28a (+)/SIRT1-SIRT3, pDEST XF1/SIRT5 and pET28a (+)/SIRT6 were cultured with shaking at 37°C until OD600 ≈ 0.6, 0.5 mmol/L of isopropyl-D-thiogalactopyranoside was added and cells were cultured at 16°C for 20 h. For recombinant proteins purification, nickel column for His-tag proteins (GE Healthcare, USA) were used to bind these extracellular-expressed proteins in binding buffer (20 mmol/L sodium phosphate, 500 mmol/L NaCl, 5 mmol/L imidazole, pH 8.0). The unspecific proteins were eliminated using a washing buffer (20 mmol/L sodium phosphate, 500 mmol/L NaCl, 40-80 mmol/L imidazole, pH 8.0). Finally, the proteins of interest were eluted by eluting buffer (20 mmol/L sodium phosphate, 500 mmol/L NaCl, 500 mmol/L imidazole, pH 8.0). After concentrating and dialyzing, the required proteins were obtained and stored in 20% glycerol at -80°C. The purity and molecular weight of these proteins were analyzed by SDS-PAGE. The amount of target proteins for assay development were calculated based on the purity. Protein concentration was analyzed using the BCA protein assay kit (Solaibior, China).

1.2 Enzymatic reaction

The probe **4a-d** was incubated with SIRTs and cofactor NAD^+ and DTT for specified time at 37˚C in 20 mM Tris-HCl buffer (pH = 8.0).

1.2.1 Enzymatic reaction monitoring with HPLC and LC-MS using probe **4a-d**

A total reaction volume of 100 μL was used for enzymatic reaction. After specific reaction time, the enzymatic reactions were quenched by addition of methanol (400 μL), vortexed and spun down. Supernatant was collected and applied for reversed HPLC analysis and LC-MS analysis. Water with 0.1% trifluoroacetate and acetonitrile with 0.1% trifluoroacetate were used as the mobile phase with a flow rate (1 mL/ min) for HPLC. Water with 0.1% formic acid and acetonitrile with 0.1% t formic acid were used as the mobile phase with a flow rate (0.25 mL/ min) for LC-MS.

1.2.2 Measurement of fluorescence spectra of probes **4a-d** and **8**

A total reaction volume of 100 μL was set for the reaction. When the reaction was complete, fluorescence spectra were measured. The fluorescence spectra were collected between 400 nm and 550 nm with excitation wavelength of 360 nm and slit width of 1 nm.

1.2.3 Measurement of fluorescence of probes **4a-d** react with enzymes

A total reaction volume of 100 μL was set for the reaction. When the reaction was complete, then the mixture was transferred to 96-well plate and the fluorescence was recorded by Thermo Varioskan LUX (Excitation at the wavelength of 360 nm and Emission at the wavelength of 460 nm).

1.2.4 Kinetic study of probe **4a-d**

A total reaction volume of 100 μL was used for the enzymatic reaction. First measured the fluorescence of **8** (0.5 μM ~ 200 μM) and then made a standard curve by GraphPad Prism 9. Then measured the fluorescence of different **4a-d** (5 μM ~ 200 μM) concentrations with enzyme reactions. Fluorescence was recorded by Thermo Varioskan LUX (Excitation at the wavelength of 360 nm and Emission at the wavelength of 460 nm). The Michaelis equation was fitted by KaleidaGraph 3.5.

1.3 Chemistry

1.3.1 Synthesis of 7-hydroxycoumarin derivatives (**I-2**)

tert-butyl 2-(7-hydroxy-2-oxo-2*H*-chromen-4-yl) acetate (2.76 g, 10 mmol) was dissolved in anhydrous N, N-dimethylformamide (DMF, 50 mL), and sodium hydride (NaH, 288 mg, 12 mmol) was added, then butyl (2,5-dioxopyrrolidin-1-yl) carbonate (3.3 g, 15 mmol) dissolved in anhydrous N, N-dimethylformamide (DMF, 20 mL) was added, and the mixture was stirred at room temperature for 0.5h under an argon atmosphere. *n*-BuOH (10 mL) was added to the reaction mixture on ice and stirred for 20min to stop further reaction with excess NaH. After removing the solvent, the residue was dissolved in 150 mL of AcOEt, washed with 0.1 N HCl aq. (150 mL * 3) and brine (150 mL * 3), and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the crude mixture was purified by silica gel flash column chromatography (AcOEt/*n*-hexane=1/9) to yield *tert*-butyl 2-(7-((butoxycarbonyl) oxy)-2-oxo-2*H*-chromen-4-yl) acetate (colorless powder, 2.3 g, 61.2 % yield). Then *tert*-butyl 2-(7-((butoxycarbonyl) oxy)-2-oxo-2*H*-chromen-4-yl) acetate (2.3 g, 6.3 mmol) was dissolved in dichloromethane (DCM) / trifluoroacetic (TFA) (1/1, 20 mL), and the resulting yellow solution was stirred at room temperature for 2h. The solvent was removed under vacuum to yield 2-(7-((butoxycarbonyl) oxy)-2-oxo-2*H*-chromen-4-yl) acetic acid (colorless powder, 1.9 g, 92% yield). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 7.70 (t, *J* = 8.7 Hz, 1H), 7.21 (d, *J* = 2.6 Hz, 1H), 7.17 (dd, *J* = 8.6, 2.5 Hz, 1H), 6.33 (s, 1H), 4.26 (t, *J* = 6.2 Hz, 2H), 3.85 (s, 2H), 1.71-1.67 (m, 2H), 1.43-1.40 (m, 2H), 0.95 (t, *J* = 7.8 Hz, 3H). HRMS (ESI) for $C_{16}H_{16}O_7$ (M + Na^+): calcd, 343.07882., found, 343.07862.

1.3.2 Synthesis of 7-hydroxycoumarin derivatives (**4a**)

tert-butyl (*S*)-(8,16-dioxo-10-(prop-2-yn-1-ylcarbamoyl)-3,6-dioxa-9,15-diazaheptadecyl) carbamate (200mg, 0.4 mmol) (**3a**) was dissolved in dichloromethane (DCM) / trifluoroacetic (TFA) (1/1, 10 mL), and the resulting yellow solution was stirred at room temperature for 2h. The solvent was removed under vacuum to yield (*S*)-6-acetamido-2-(2-(2-(2-aminoethoxy) ethoxy) acetamido)-N-(prop-2-yn-1-yl) hexanamide (yellow powder, 135 mg, 90% yield). 2-(7-((butoxycarbonyl)oxy)-2-oxo-2H-chromen-4-yl) acetic acid (130 mg, 0.4 mmol) was dissolved in anhydrous tetrahydrofuran (THF, 5 mL), and O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU, 227 mg, 0.6 mmol) was added, then 4-Methylmorpholine (NMM, 170 mg, 1.6 mmol) was added, and the mixture was stirred at room temperature for 10 min. (*S*)-6-acetamido-2-(2-(2-(2-aminoethoxy) ethoxy) acetamido)-N-(prop-2-yn-1-yl) hexanamide (130 mg, 0.4 mmol) was dissolved in anhydrous tetrahydrofuran (THF, 2 mL) was added, then the mixture was stirred at room temperature for 4h. After removing the solvent, the residue was dissolved in 30 mL of DCM, washed with 0.1 N HCl aq. (30 mL * 3) and brine (30 mL * 3), and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the crude mixture was purified by silica gel flash column chromatography (DCM/MeOH=30/1) to yield (*S*)-butyl (2-oxo-4-(2,11,19-trioxo-13-(prop-2-yn-1-ylcarbamoyl)-6,9-dioxa-3,12,18-triazaicosyl)-2H-chromen-7-yl) carbonate (yellow powder, 80 mg, 30 % yield). ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm): 8.57 (t, J = 5.5 Hz, 1H), 8.41 (t, *J* = 5.6 Hz, 1H), 7.83 (d, *J* = 8.8 Hz, 2H), 7.66 (d, *J* = 8.6 Hz, 1H), 7.45 (d, *J* = 2.3 Hz, 1H), 7.31 (dd, *J* = 8.7, 5.6 Hz, 1H), 6.46 (s, 1H), 4.32 (tt, *J* = 11.7, 5.8 Hz, 1H), 4.25 (t, *J* = 6.6 Hz, 2H), 3.94 (d, *J* = 6.5 Hz, 2H), 3.87 (dd, *J* = 5.4, 2.4 Hz, 2H), 3.79 (s, 2H), 3.64 – 3.60 (m, 2H), 3.57 (d, *J* = 3.9 Hz, 2H), 3.47 (t, *J* = 5.6 Hz, 2H), 3.31 – 3.25 (m, 2H), 3.13 (t, *J* = 2.4 Hz, 1H), 2.98 (dd, *J* = 12.7, 6.7 Hz, 2H), 1.78 (s, 3H), 1.72 – 1.63 (m, 2H), 1.41 (dt, *J* = 15.0, 7.4 Hz, 4H), 1.30 – 1.19 (m, 2H), 0.94 (t, *J* = 7.4 Hz, 3H).^{13}C NMR (100 MHz, DMSO-d6) δ 171.75, 169.56, 169.48, 168.15, 160.06, 154.14, 153.38, 152.96, 151.10, 127.09, 118.37, 117.75, 116.11, 110.40, 81.37, 73.67, 70.73, 70.15, 69.89, 69.51, 69.23, 52.07, 39.17, 38.84, 32.58, 30.49, 29.26, 23.16, 23.10, 18.94, 14.06. HRMS (ESI) for $C_{33}H_{44}N_4O_{11}$ (M+H^+): calcd, 673.30793., found, 673.30627.

1.3.3 Synthesis of 7-hydroxycoumarin derivatives (**4b**)

The title compound **4b** was synthe- sized in 32% yield from compound **3b** in a manner similar to that described for the synthesis of compound **4a**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.59 (t, *J* = 5.5 Hz, 1H), 8.46 (t, *J* = 5.6 Hz, 1H), 8.41 (t, *J* = 5.6 Hz, 1H), 7.85 – 7.81 (m, 1H), 7.68 (d, *J* = 8.5 Hz, 1H), 7.54 – 7.48 (m, 1H), 7.45 (dd, *J* = 8.9, 5.0 Hz, 3H), 7.31 (dd, *J* = 8.7, 2.3 Hz, 1H), 6.46 (s, 1H), 4.36 (dd, *J* = 15.3, 7.0 Hz, 1H), 4.25 (t, *J* = 6.6 Hz, 2H), 3.94 (d, *J* = 4.0 Hz, 2H), 3.87 (dd, *J* = 5.5, 2.5 Hz, 2H), 3.79 (s, 2H), 3.58 (d, *J* = 4.3 Hz, 2H), 3.53 (t, *J* = 4.4 Hz, 2H), 3.46 (t, *J* = 5.6 Hz, 2H), 3.26 (dt, *J* = 12.6, 6.7 Hz, 4H), 3.11 (t, *J* = 2.5 Hz, 1H), 1.74 – 1.63 (m, 2H), 1.55 – 1.47 (m, 2H), 1.39 (dt, *J* = 14.6, 7.4 Hz, 2H), 1.32 – 1.25 (m, 2H), 0.93 (t, *J* = 7.4 Hz, 3H).^{13}C NMR (100 MHz, DMSO-d_6) δ 171.83, 169.50, 168.11, 166.66, 160.03, 154.18, 153.40, 152.91, 150.98, 135.10, 131.49, 128.78, 127.59, 127.09, 118.35, 117.74, 116.10, 110.45, 81.36, 73.63, 70.73, 70.15, 69.87, 69.51, 69.18, 52.11, 39.38, 39.18, 32.60, 30.48, 29.27, 28.45, 23.21, 18.93, 14.04. HRMS (ESI) for $C_{38}H_{46}N_4O_{11}$ (M+Na^+): calcd, 735.32358., found, 735.32153.

1.3.4 Synthesis of 7-hydroxycoumarin derivatives (**4c**)

The title compound **4c** was synthe- sized in 24% yield from compound **3c** in a manner similar to that described for the synthesis of compound **4a**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.44 (t, *J* = 5.6 Hz, 1H), 7.84 (d, *J* = 8.8 Hz, 1H), 7.45 – 7.40 (m, 2H), 7.32 (d, *J* = 2.3 Hz, 1H), 7.30 (d, *J* = 2.3 Hz, 1H), 6.46 (s, 1H), 4.26 (td, 1H), 4.24 (t, *J* = 6.6 Hz, 2H), 4.14 (s, 2H), 3.79-3.76 (m, 4H), 3.59 – 3.57 (m, 2H), 3.56 (s, 2H), 3.54 (dd, *J* = 5.0, 2.9 Hz, 2H), 3.46 (t, *J* = 5.6 Hz, 1H), 3.30 – 3.25 (m, 2H), 3.23 (dd, *J* = 7.1, 3.6 Hz, 1H), 2.69 (d, *J* = 1.5 Hz, 2H), 2.52 (dt, *J* = 3.6, 1.8 Hz, 2H), 1.71 – 1.62 (m, 2H), 1.45 – 1.34 (m, 2H), 1.09 (t, *J* = 7.1 Hz, 3H), 1.01 (dd, *J* = 9.0, 5.2 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H).^{13}C NMR (100 MHz, DMSO-d_6) δ 168.28, 168.09, 160.02, 159.14, 158.73, 154.14, 153.38, 152.94, 151.12, 127.09, 118.32, 117.71, 116.04, 110.32, 70.29, 69.93, 69.56, 69.36, 69.18, 40.98, 40.54, 39.74, 39.42, 39.27, 39.17, 38.70, 30.48, 18.89, 14.51, 13.94, 13.33. HRMS (ESI) for $C_{35}H_{46}N_4O_{13}$ ($M+H^+$): calcd, 731.31341., found, 731.31165.

1.3.5 Synthesis of 7-hydroxycoumarin derivatives (**4d**)

The title compound **4d** was synthe- sized in 38% yield from compound **3d** in a manner similar to that described for the synthesis of compound **4a**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57 (dd, *J* = 11.7, 6.2 Hz, 1H), 8.42 (t, *J* = 5.5 Hz, 1H), 7.83 (d, *J* = 8.8 Hz, 1H), 7.74 (t, *J* = 5.4 Hz, 1H), 7.65 (s, 1H), 7.44 (d, *J* = 2.3 Hz, 1H), 7.30 (dd, *J* = 8.7, 2.3 Hz, 1H), 6.46 (s, 1H), 4.33 (td, *J* = 8.4, 5.6 Hz, 1H), 4.25 (t, *J* = 6.6 Hz, 2H), 3.94 (dd, *J* = 8.9, 3.7 Hz, 2H), 3.87 (dd, *J* = 5.4, 2.3 Hz, 2H), 3.79 (s, 2H), 3.65 – 3.59 (m, 2H), 3.57 (d, *J* = 3.6 Hz, 2H), 3.47 (t, *J* = 5.6 Hz, 2H), 3.30 – 3.26 (m, 2H), 3.13 (dd, *J* = 4.8, 2.3 Hz, 1H), 2.99 (dd, *J* = 12.5, 6.6 Hz, 2H), 2.02 (t, *J* = 7.4 Hz, 2H), 1.72 – 1.36 (m, 10H), 1.25 (d, *J* = 13.7 Hz, 22H), 0.94 (t, *J* = 7.4 Hz,31H), 0.86 (t, *J* = 6.8 Hz, 3H).^{13}C NMR (100 MHz, DMSO-d_6) δ 172.41, 171.74, 169.60, 168.08, 160.04, 154.10, 153.40, 152.97, 151.04, 127.07, 118.32, 117.75, 116.09, 110.33, 81.26, 73.65, 70.74, 70.16, 69.85, 69.54, 69.17, 52.06, 39.17, 38.70, 35.93, 31.83, 30.49, 29.61, 29.57, 29.56, 29.48, 29.33, 29.26, 29.22, 28.42, 25.84, 23.11, 22.64, 18.94, 14.48, 14.05. HRMS (ESI) for $C_{45}H_{68}N_4O_{11}$ ($M+H^+$): calcd, 841,49574., found, 841.49463.

1.3.6 Synthesis of 7-hydroxycoumarin derivatives (**8**)

Butyl (*S*)-(2,2-dimethyl-4,13-dioxo-15-(prop-2-yn-1-ylcarbamoyl)-3,8,11-trioxa-5,14-diazanonadecan-19-yl) carbamate (260 mg, 0.5 mmol) (**7**) was dissolved in dichloromethane (DCM) / trifluoroacetic (TFA) (1/1, 10 mL), and the resulting yellow solution was stirred at room temperature for 2h. The solvent was removed under vacuum to yield butyl (S)-(5-(2-(2-(2-aminoethoxy) ethoxy) acetamido)-6-oxo-6-(prop-2-yn-1-ylamino) hexyl) carbamate (yellow powder, 200 mg, 93% yield). 2-(7-hydroxy-2-oxo-2H-chromen-4-yl) acetic acid (110 mg, 0.5 mmol) was dissolved in anhydrous N, N-dimethylformamide (DMF, 10 mL), and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI, 115 mg, 0.6 mmol) was added, then 1-Hydroxybenzotriazole (HOBt, 81 mg, 0.6 mmol) was added, and the mixture was stirred at room temperature for 2 h. butyl (S)-(5-(2-(2-(2-aminoethoxy) ethoxy) acetamido)-6-oxo-6-(prop-2-yn-1-ylamino) hexyl) carbamate (110 mg, 0.5 mmol) was dissolved in anhydrous N, N-dimethylformamide (DMF, 2 mL) was added, then the mixture was stirred at room temperature for 12h. After removing the solvent, the residue was dissolved in 50 mL of AcOEt, washed with 0.1 N HCl aq. (50 mL * 3) and brine (50 mL * 3), and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the crude mixture was purified by silica gel flash column chromatography (DCM/MeOH=20/1) to yield butyl (*S*)-(1-(7-hydroxy-2-oxo-2*H*-chromen-4-yl)-2,11-dioxo-13-(prop-2-yn-1-ylcarbamoyl)-6,9-dioxa-3,12-diazaheptadecan-17-yl) carbamate (yellow powder, 90 mg, 28 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): δ 8.58 (t, *J* = 5.5 Hz, 1H), 8.38 (t, *J* = 5.6 Hz, 1H), 7.68 (d, *J* = 8.5 Hz, 1H), 7.58 (t, *J* = 7.1 Hz, 1H), 7.49 (d, *J* = 8.7 Hz, 1H), 7.06 (t, *J* = 5.6 Hz, 1H), 6.77 (dd, *J* = 8.7, 2.3 Hz, 1H), 6.69 (d, *J* = 2.3 Hz, 1H), 6.14 (s, 1H), 4.32 (td, *J* = 8.5, 5.6 Hz, 1H), 3.92 (dd, *J* = 13.6, 7.0 Hz, 4H), 3.87 (dd, *J* = 5.5, 2.4 Hz, 2H), 3.67 (s, 2H), 3.64 – 3.59 (m, 2H), 3.59 – 3.55 (m, 2H), 3.46 (t, *J* = 5.6 Hz, 2H), 3.32 – 3.23 (m, 2H), 3.12 (t, *J* = 2.5 Hz, 1H), 2.92 (dd, *J* = 12.9, 6.8 Hz, 2H), 1.69 – 1.46 (m, 4H), 1.42 – 1.18 (m, 6H), 0.88 (t, *J* = 7.4 Hz, 3H).^{13}C NMR (100 MHz, DMSO-d_6) δ 171.77, 169.60, 168.35, 160.92, 156.85, 155.63, 151.87, 127.08, 113.73, 111.64, 111.48, 102.83, 81.36, 73.65, 70.73, 70.15, 69.87, 69.48, 63.75, 52.12, 40.51, 39.24, 32.55, 31.29, 29.55, 28.40, 22.98, 19.13, 14.14. HRMS (ESI) for $C_{31}H_{42}N_4O_{10}$ ($M+H^+$): calcd, 631.29737., found, 631.29608.

1.3.6 Synthesis of 7-hydroxycoumarin derivatives (**9**)

The title compound **9** was synthe- sized in 42% yield from compound **3a** in a manner similar to that described for the synthesis of compound **4a**. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): δ 8.57 (dd, *J* = 11.5, 6.0 Hz, 1H), 8.41 (t, *J* = 5.6 Hz, 1H), 7.82 (t, *J* = 8.1 Hz, 2H), 7.65 (t, *J* = 11.7 Hz, 1H), 7.45 (s, 1H), 7.29 (dd, *J* = 5.7, 2.6 Hz, 1H), 6.46 (s, 1H), 4.40 – 4.30 (m, 1H), 3.94 (d, *J* = 6.5 Hz, 2H), 3.89 – 3.84 (m, 2H), 3.79 (s, 2H), 3.57 (d, *J* = 3.9 Hz, 2H), 3.47 (t, *J* = 5.6 Hz, 2H), 3.31 – 3.25 (m, 2H), 3.13 (t, *J* = 3.6 Hz, 1H), 2.98 (dd, *J* = 12.7, 6.7 Hz, 2H), 1.78 (s, 3H), 1.68 (t, *J* = 10.8 Hz, 2H), 1.41 (dt, *J* = 15.0, 7.4 Hz, 2H), 1.24 (t, *J* = 10.9 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.76, 169.56, 169.48, 168.15, 154.12, 153.35, 152.98, 151.10, 127.04, 118.40, 117.75, 110.48, 81.35, 70.79, 70.15, 69.87, 69.23, 52.07, 39.17, 38.84, 32.58, 30.49, 28.44, 23.16, 23.10. HRMS (ESI) for $C_{28}H_{36}N_4O_9$ ($M+H^+$): calcd, 573.25551., found, 573.25508.

2. Supplementary Figures

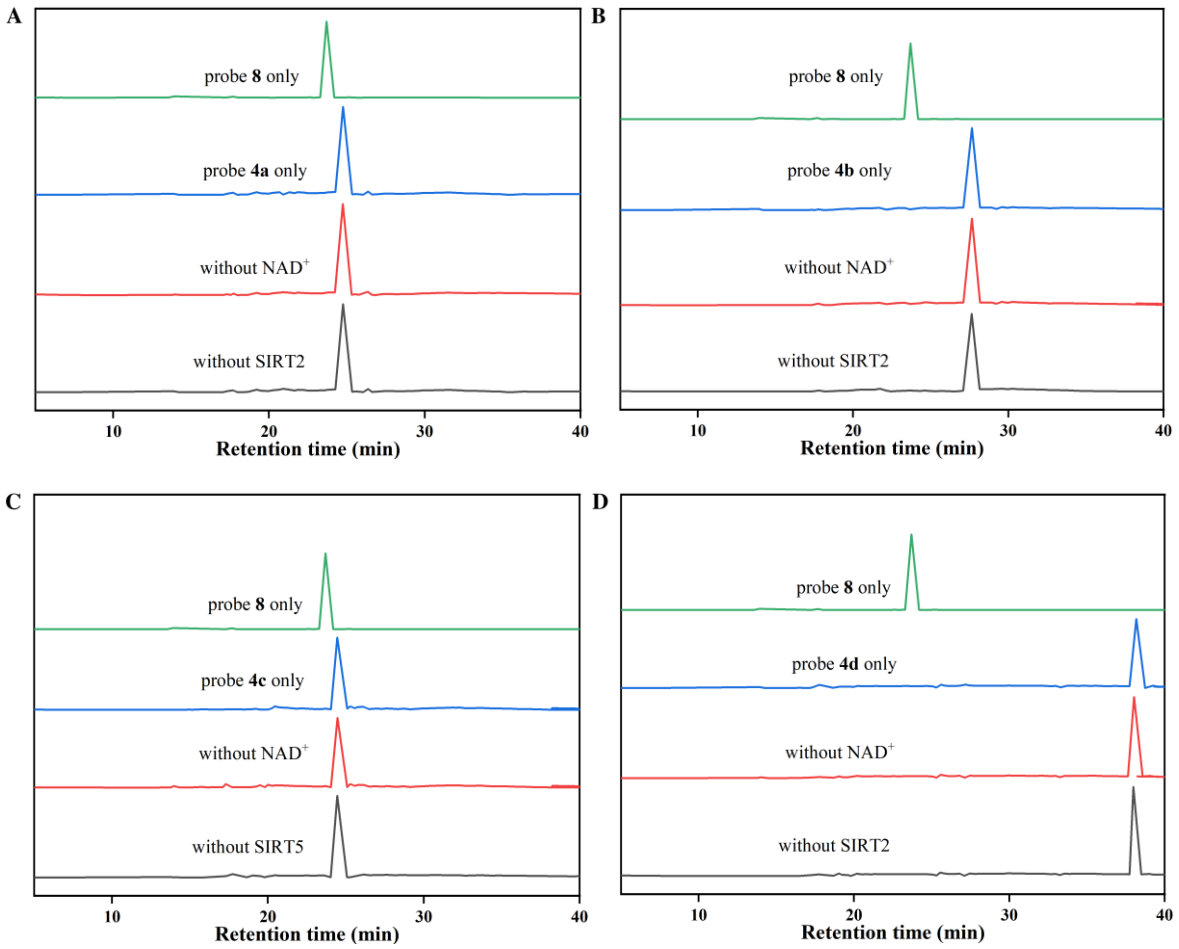
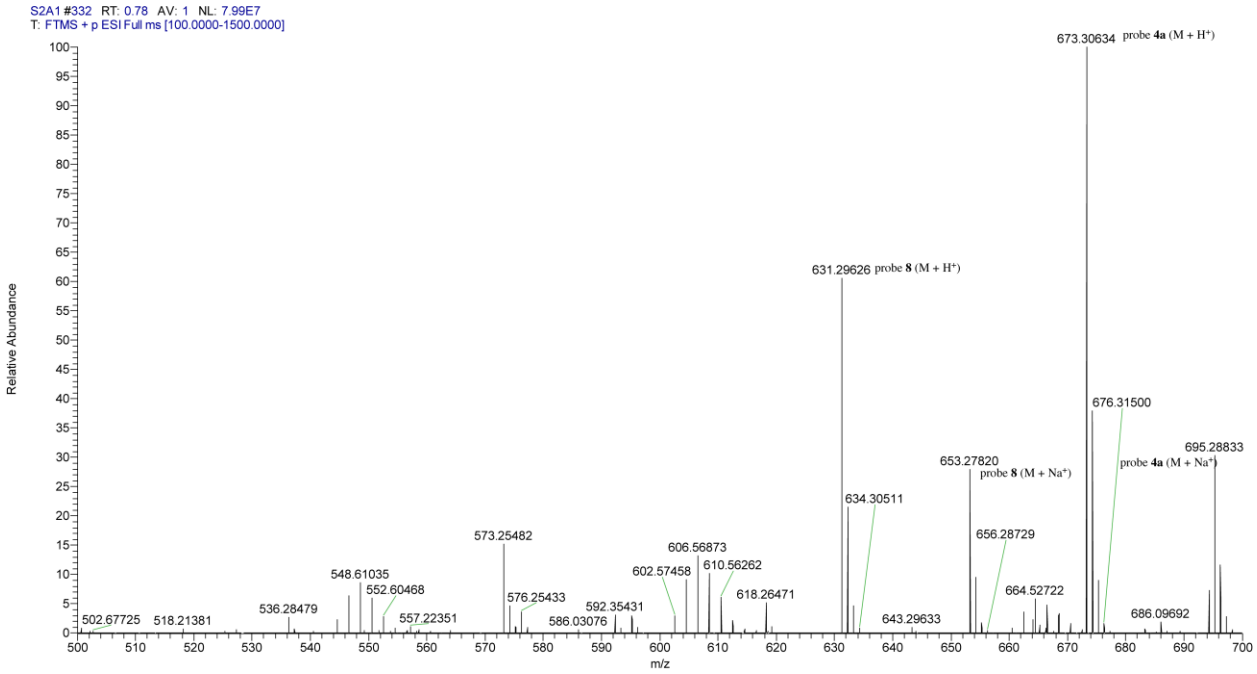
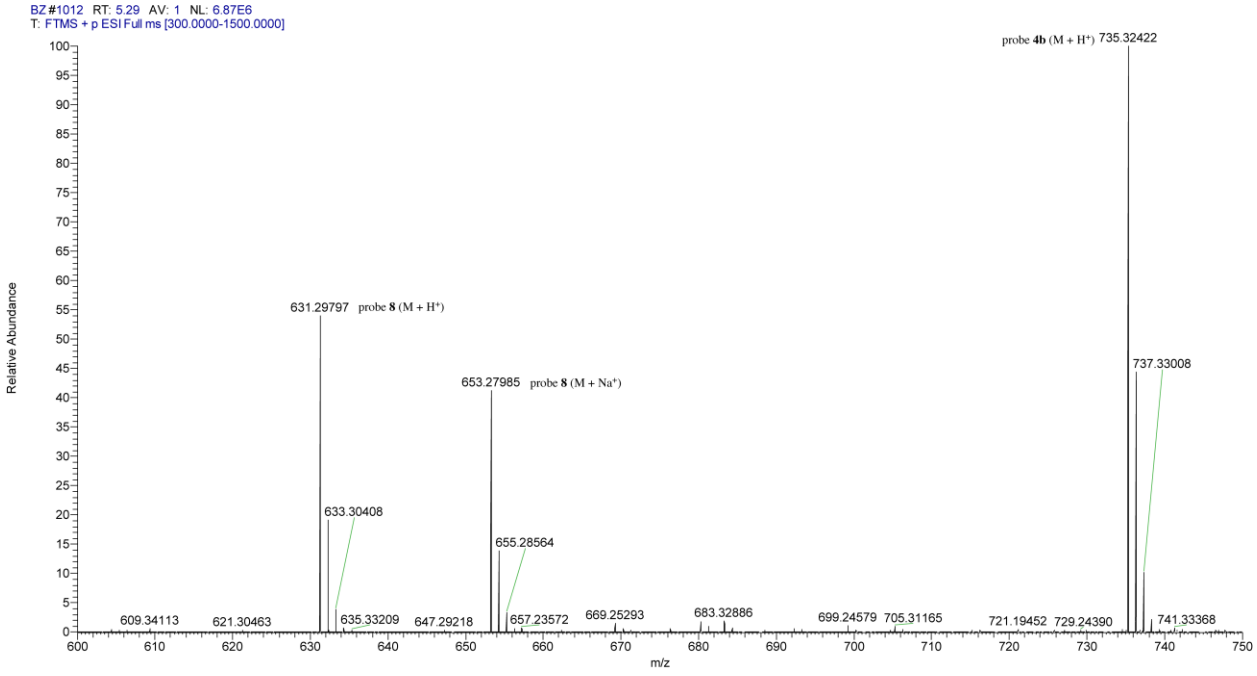


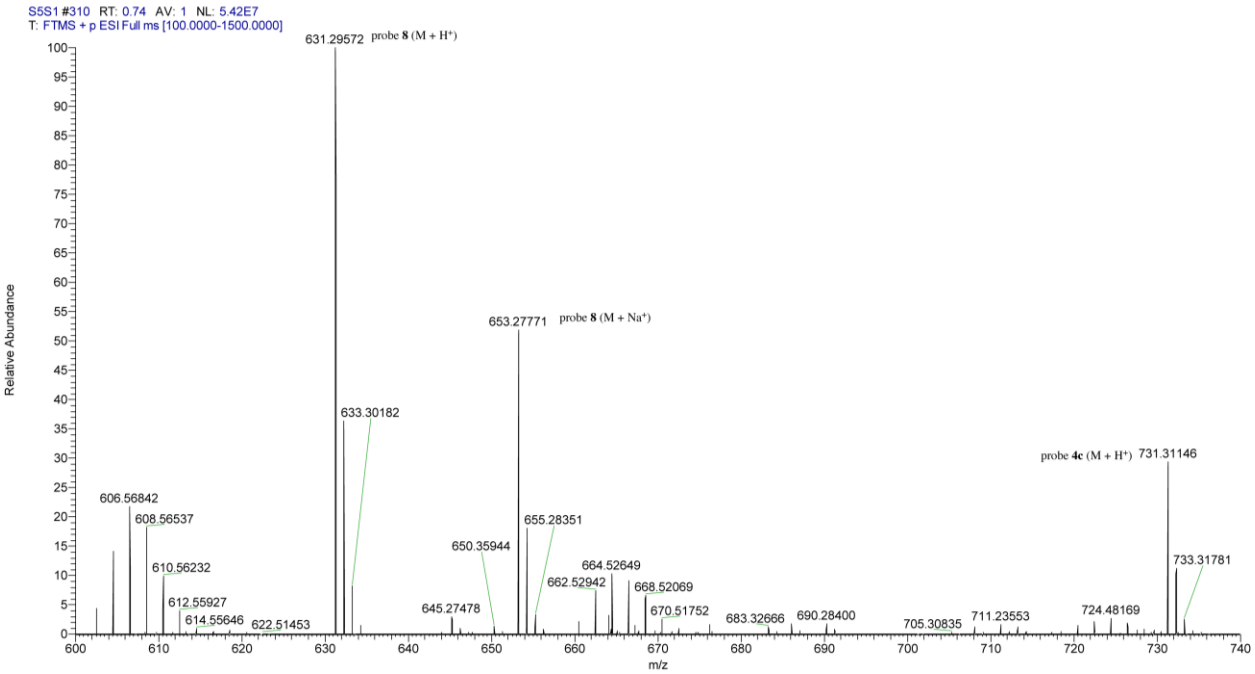
Fig. S1. HPLC analysis of control experiments of the enzymatic reaction of probe **4a-d** with SIRTs: (A) **4a** (100 μM) with or without NAD^+ (1mM) and SIRT2 (5 μM); (B) **4b** (100 μM) with or without NAD^+ (1mM) and SIRT2 (10 μM); (C) **4c** (100 μM) with or without NAD^+ (1mM) and SIRT5 (5 μM); (D) **4d** (100 μM) with or without NAD^+ (1mM) and SIRT2 (5 μM). UV channel: 320 nm.



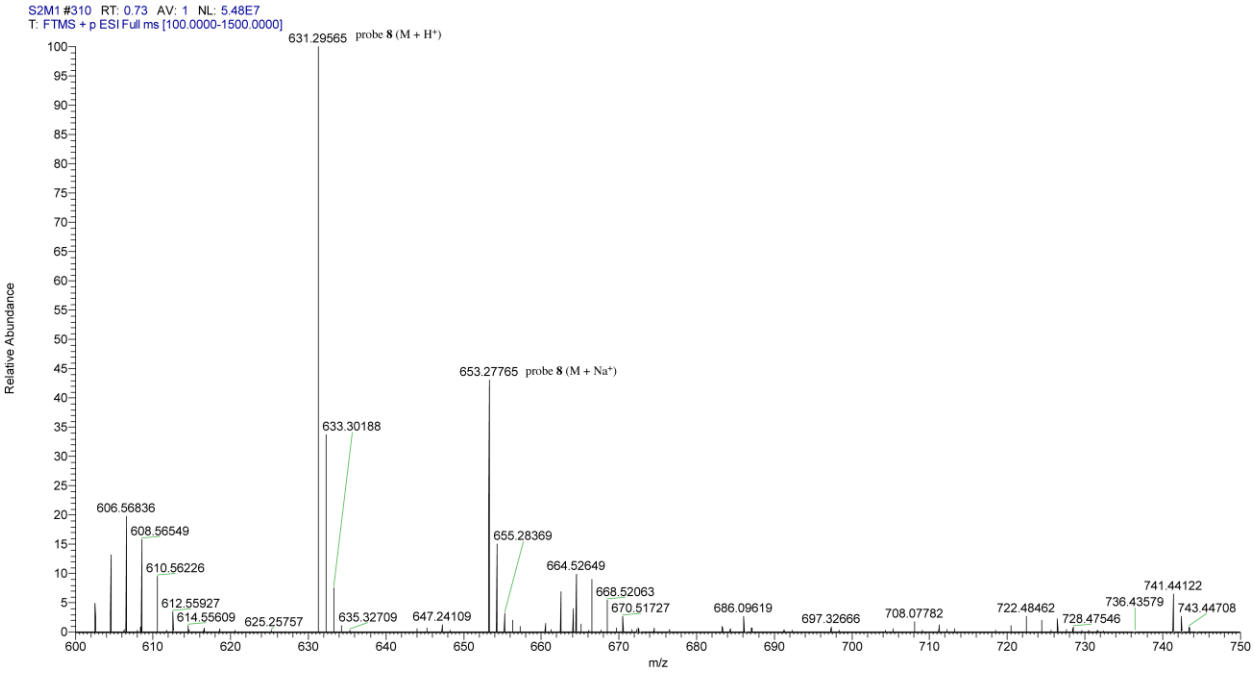
(A) probe **4a** (20 μM) react with SIRT2 (1 μM)



(B) probe **4b** (20 μM) react with SIRT2 (2 μM)



(C) probe **4c** (20 μM) react with SIRT5 (1 μM)



(D) probe **4d** (20 μM) react with SIRT2 (1 μM)

Fig. S2. LC-MS analysis of the enzymatic reaction of probe **4a-d** with SIRTs. (A) probe **4a** (20 μM) react with SIRT2 (1 μM). (B) probe **4b** (20 μM) react with SIRT2 (2 μM). (C) probe **4c** (20 μM) react with SIRT5 (1 μM). (D) probe **4d** (20 μM) react with SIRT2 (1 μM)

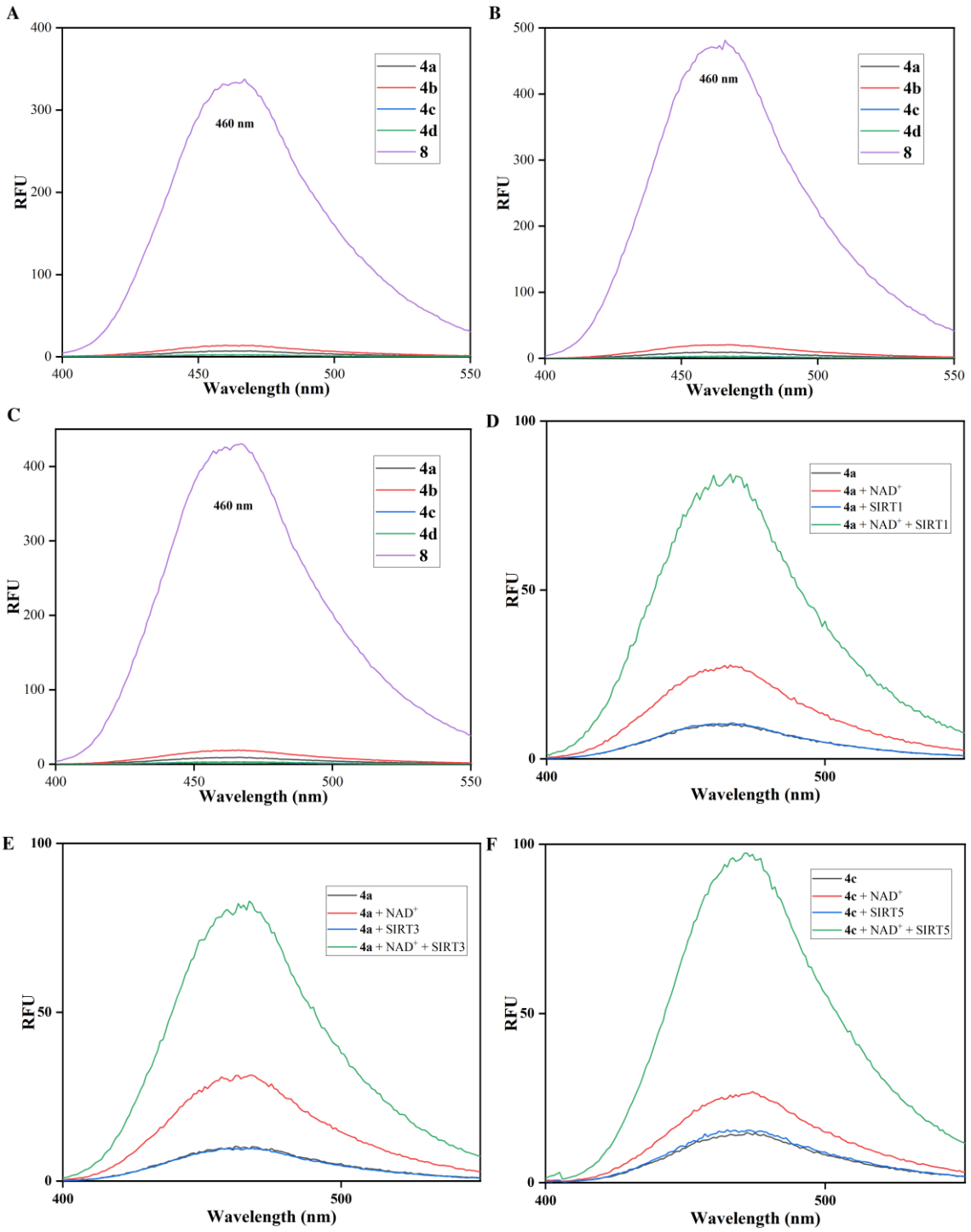


Fig. S3. Fluorescence spectra of **4a-d** and **8** (20 µM, prepared from 2 mM stock in DMSO): (A) Excitation wavelength 340 nm; (B) Excitation wavelength 360 nm; (C) Excitation wavelength 380 nm. Fluorescence spectra of **4a**, **4d** (20 µM,

prepared from 2 mM stock in DMSO): (D) **4a** with SIRT1 (1 µM); (E) **4a** with SIRT3 (1 µM); (F) **4c** with SIRT5 (1 µM). For incubation 60 min, λ_{ex} = 360 nm.

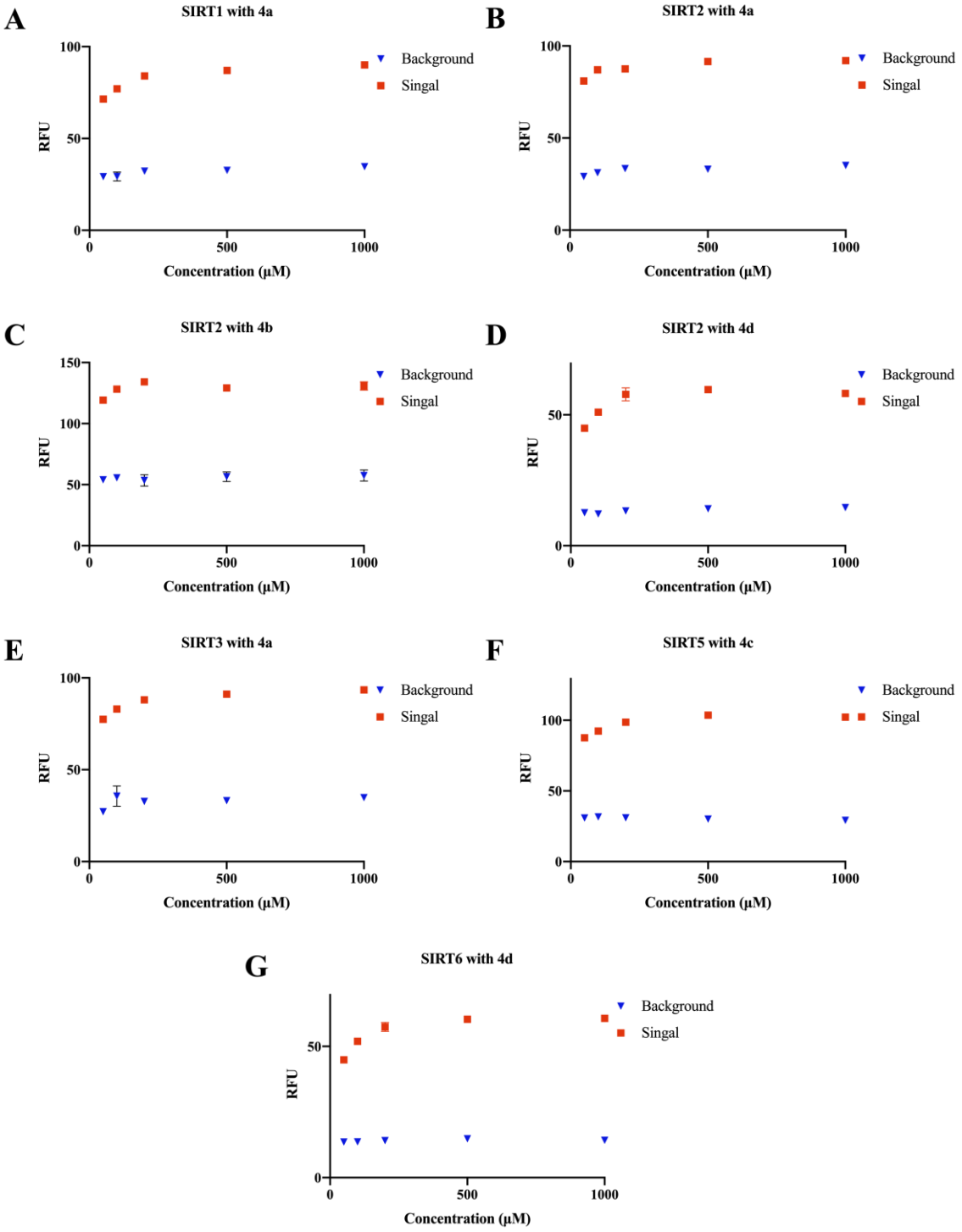


Fig. S4. NAD^+ concentration-dependent experiments of **4a-d** (20 µM) with SIRTs (1 µM or 2 µM SIRT2 for **4b**). For incubation 60 min, detected at 360 nm/ 460 nm.

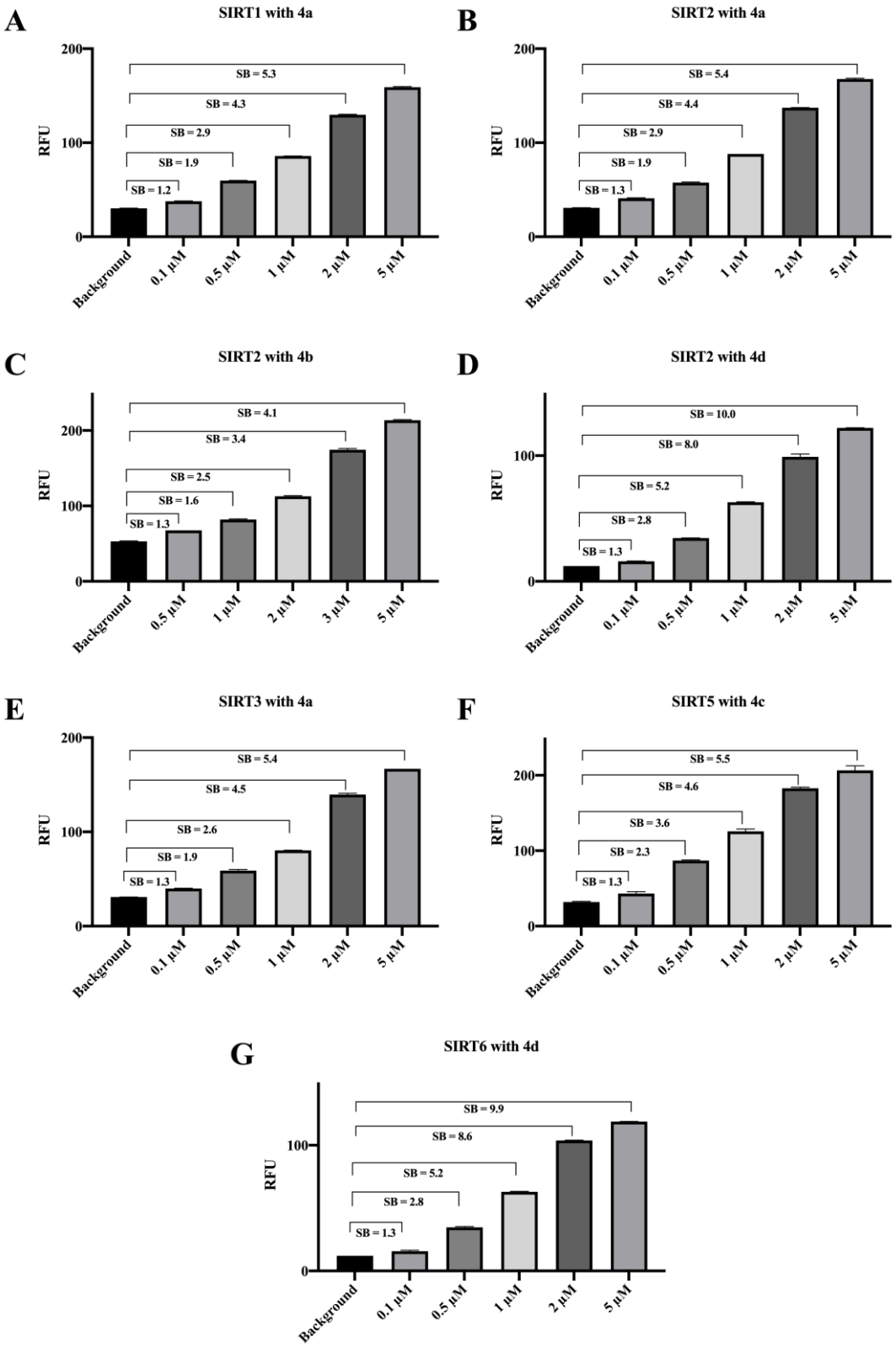


Fig. S5. Enzyme concentration-dependent experiments of **4a-d** (20 µM) with SIRTs. For incubation 60 min, detected at 360 nm/ 460 nm.

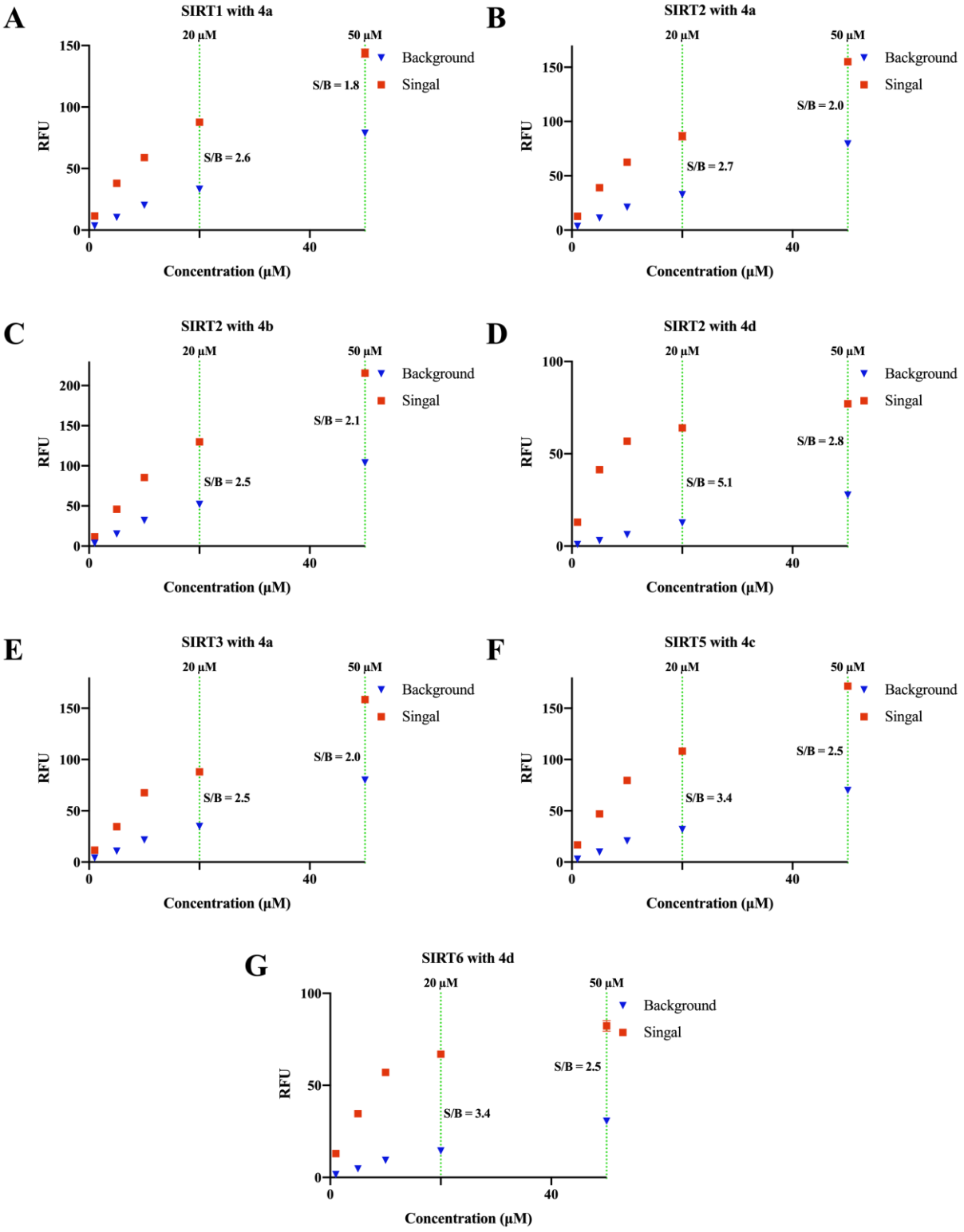


Fig. S6. Substrate concentration-dependent experiments of **4a-d** with SIRTs (1 μM or 2 μM SIRT2 for **4b**). For incubation 60 min, detected at 360 nm/ 460 nm.

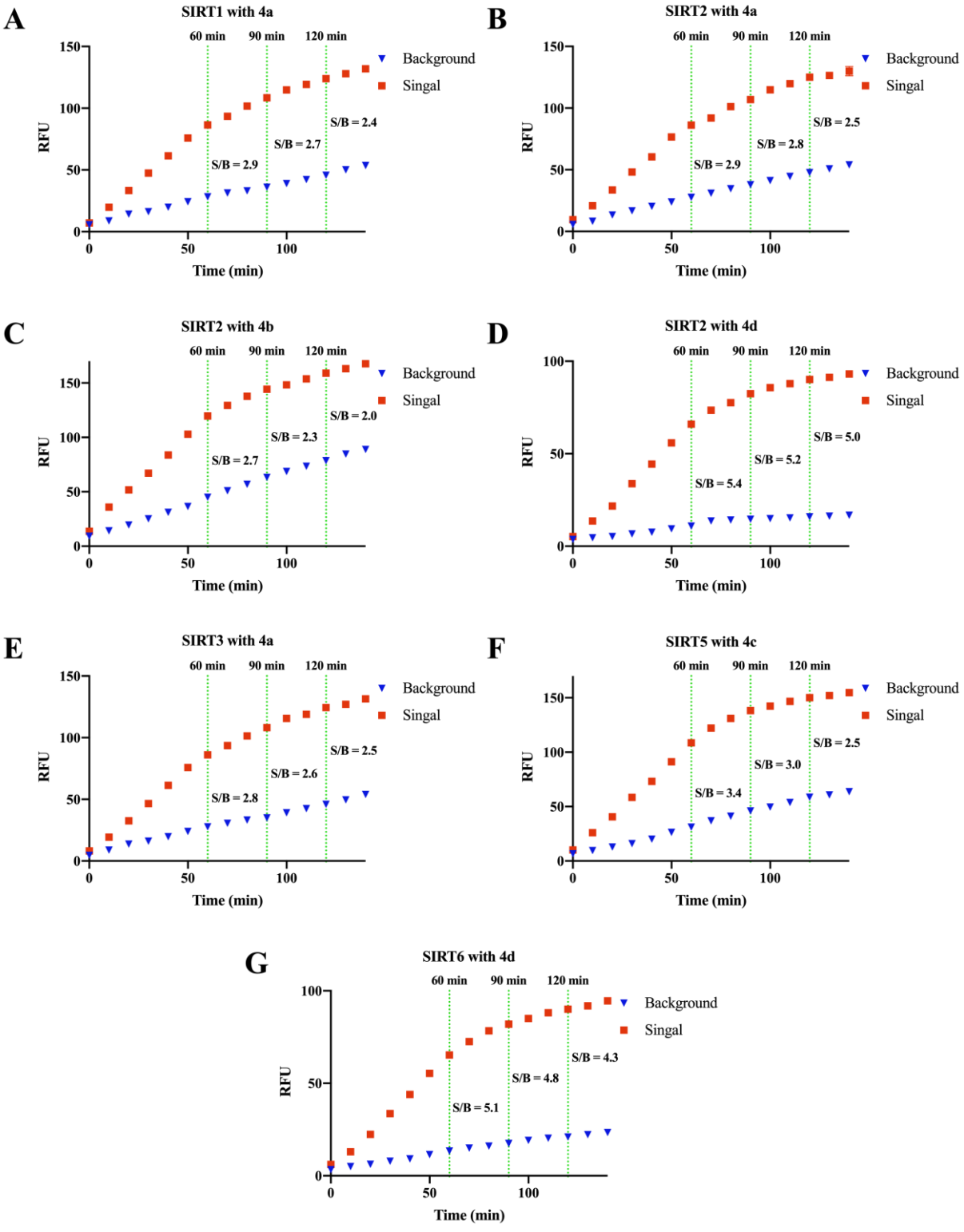


Fig. S7. Time-dependent experiments of **4a-d** (20 μM) with SIRTs (1 μM or 2μM).

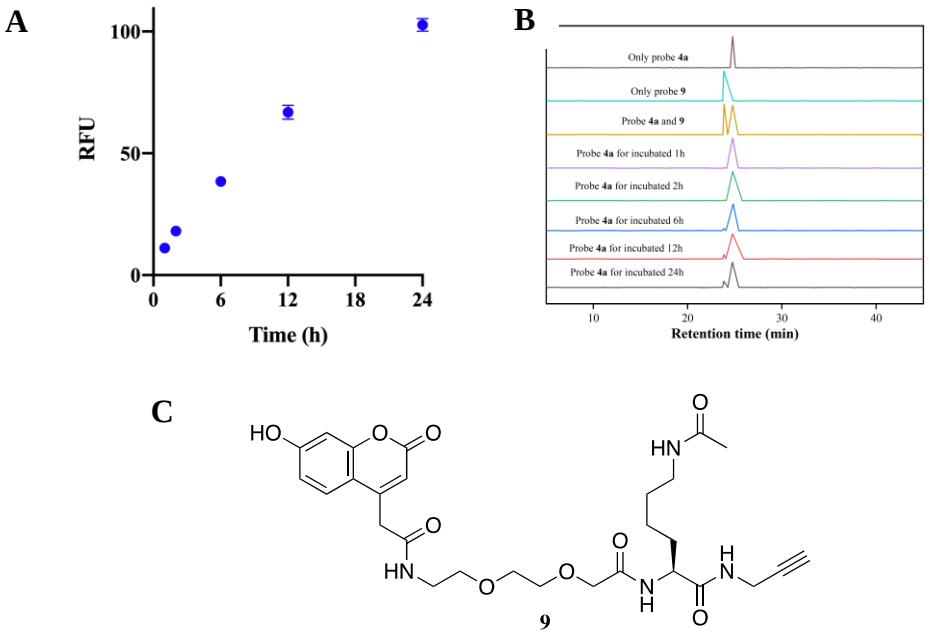
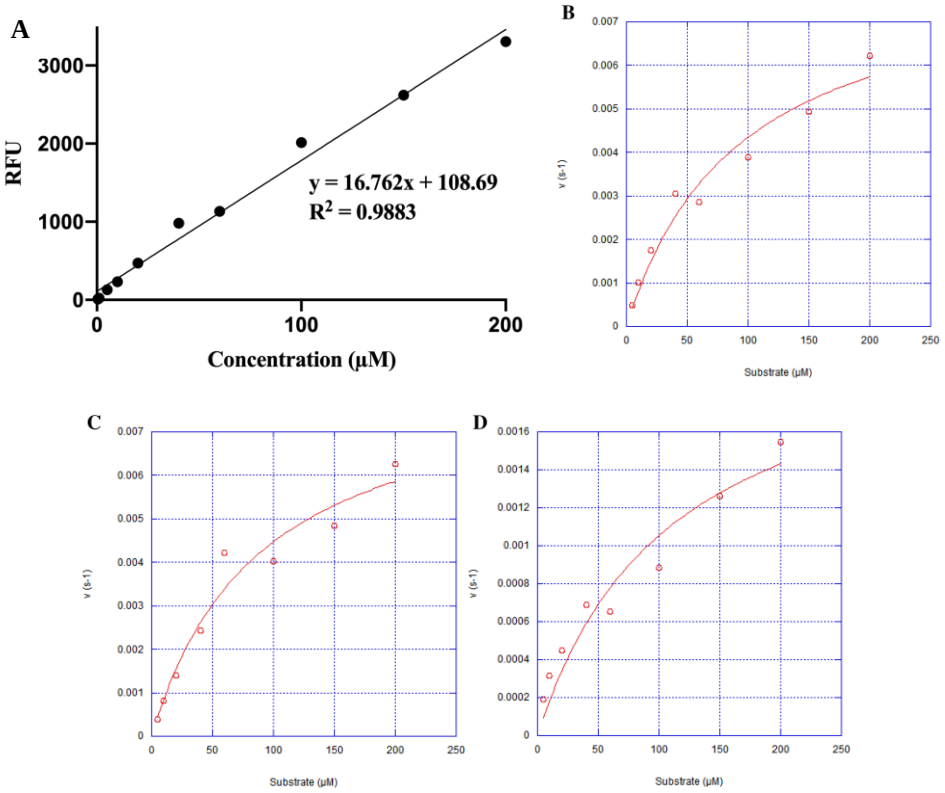


Fig. S8. The stability of **4a** (20 μM) with SIRTs and NAD^+. (A) The stability of **4a** within 24 hours by fluorogenic assay. (B) The stability of **4a** within 24 hours by HPLC assay. (C) The structure of probe **9.**



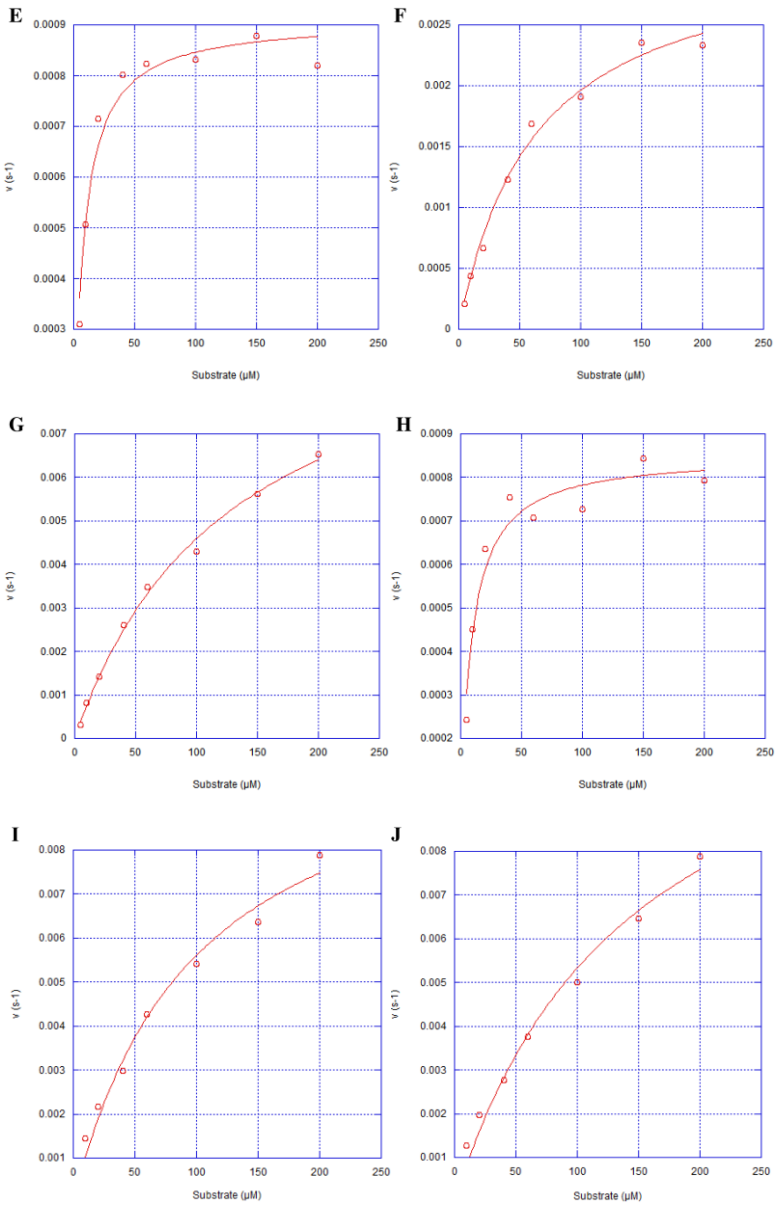
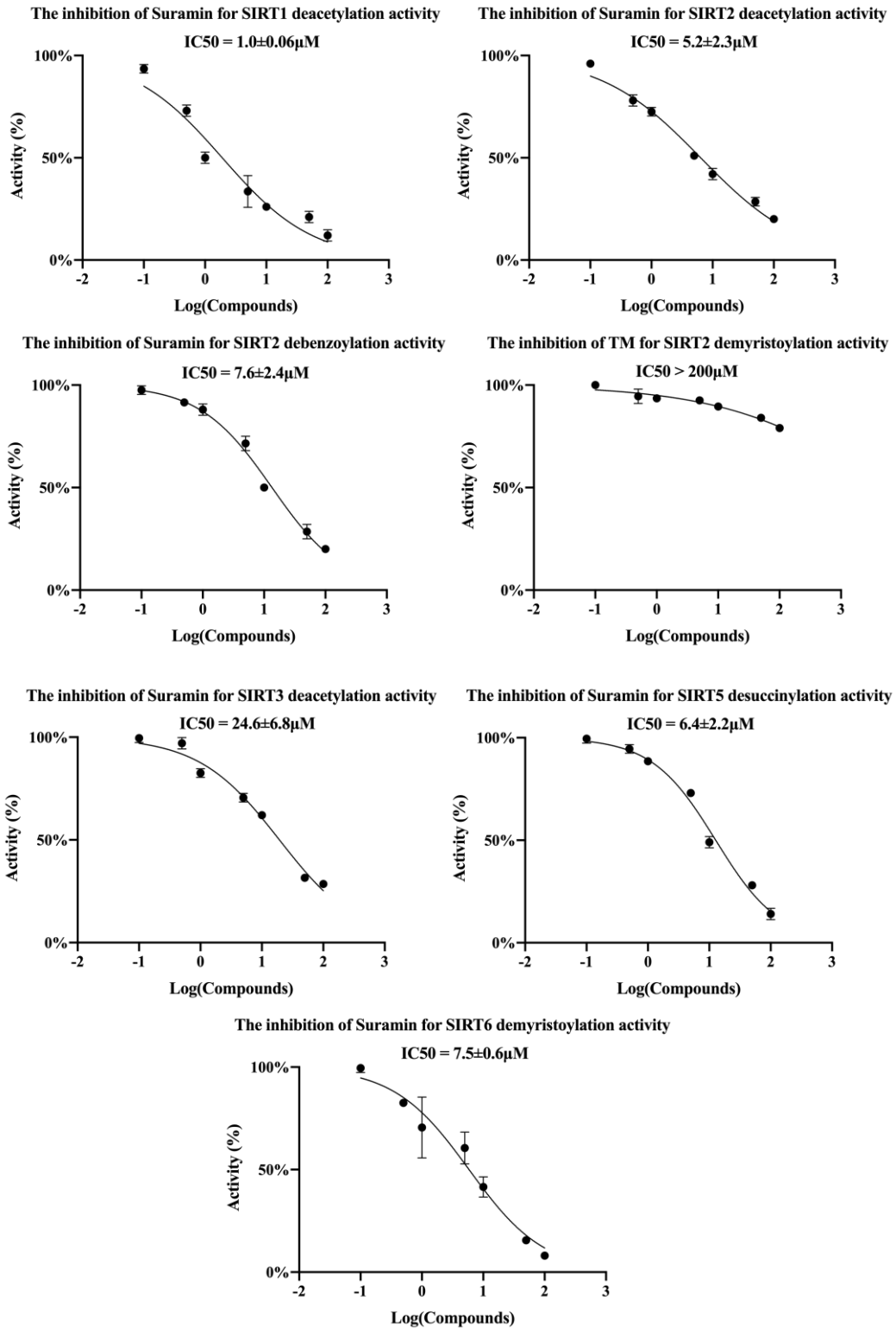
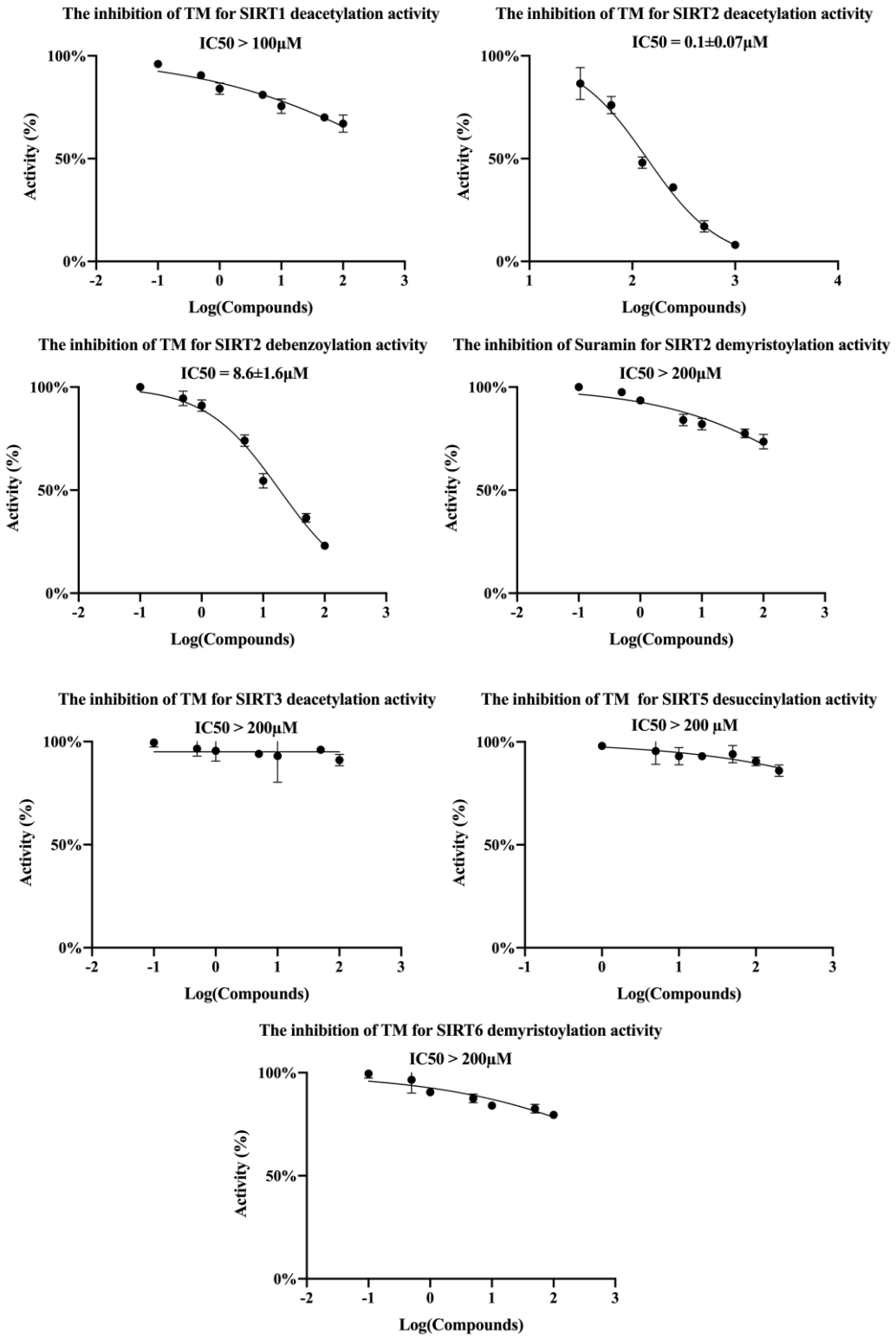


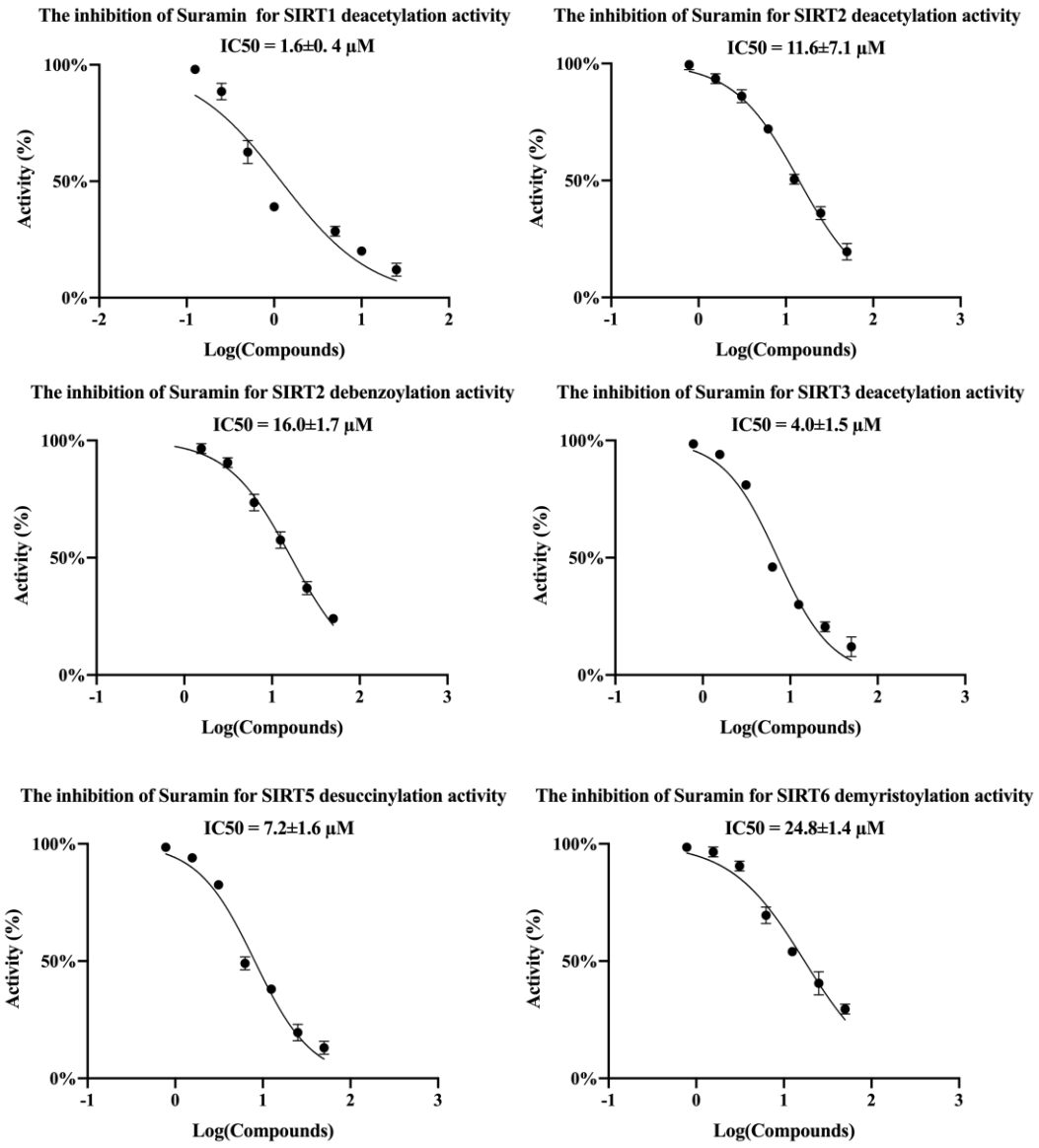
Fig. S9. The kinetic curve of **4a-d**. (A) Standard curve of probe **8**; (B) probe **4a** with SIRT1; (C) probe **4a** with SIRT2; (D) probe **4b** with SIRT2; (E) probe **4d** with SIRT2; (F) probe **4a** with SIRT3; (G) probe **4c** with SIRT5; (H) probe **4d** with SIRT6. (I) probe **4a** with SIRT1 by HPLC assay, UV channel: 320 nm. (J) probe **9** with SIRT1 by HPLC assay. For incubation 60 min, detected at 360 nm/ 460 nm for fluorogenic assay.



(A) Using **4a-d** to determine the inhibitory activity of Suramin for SIRTs by fluorogenic assay



(B) Using **4a-d** to determine the inhibitory activity of TM for SIRTs by fluorogenic assay



(C) Detected the inhibitory activity of Suramin for SIRTs by HPLC assay

Fig. S10. Determination of the inhibitory activity of SIRT2 selective inhibitor TM and pan-SIRTs inhibitor Suramin by fluorogenic assay and HPLC assay. (A) Using **4a-d** to determine the inhibitory activity of Suramin for SIRTs by fluorogenic assay. (B) Using **4a-d** to determine the inhibitory activity of TM for SIRTs by fluorogenic assay. (C) Detected the inhibitory activity of Suramin for SIRTs by HPLC assay

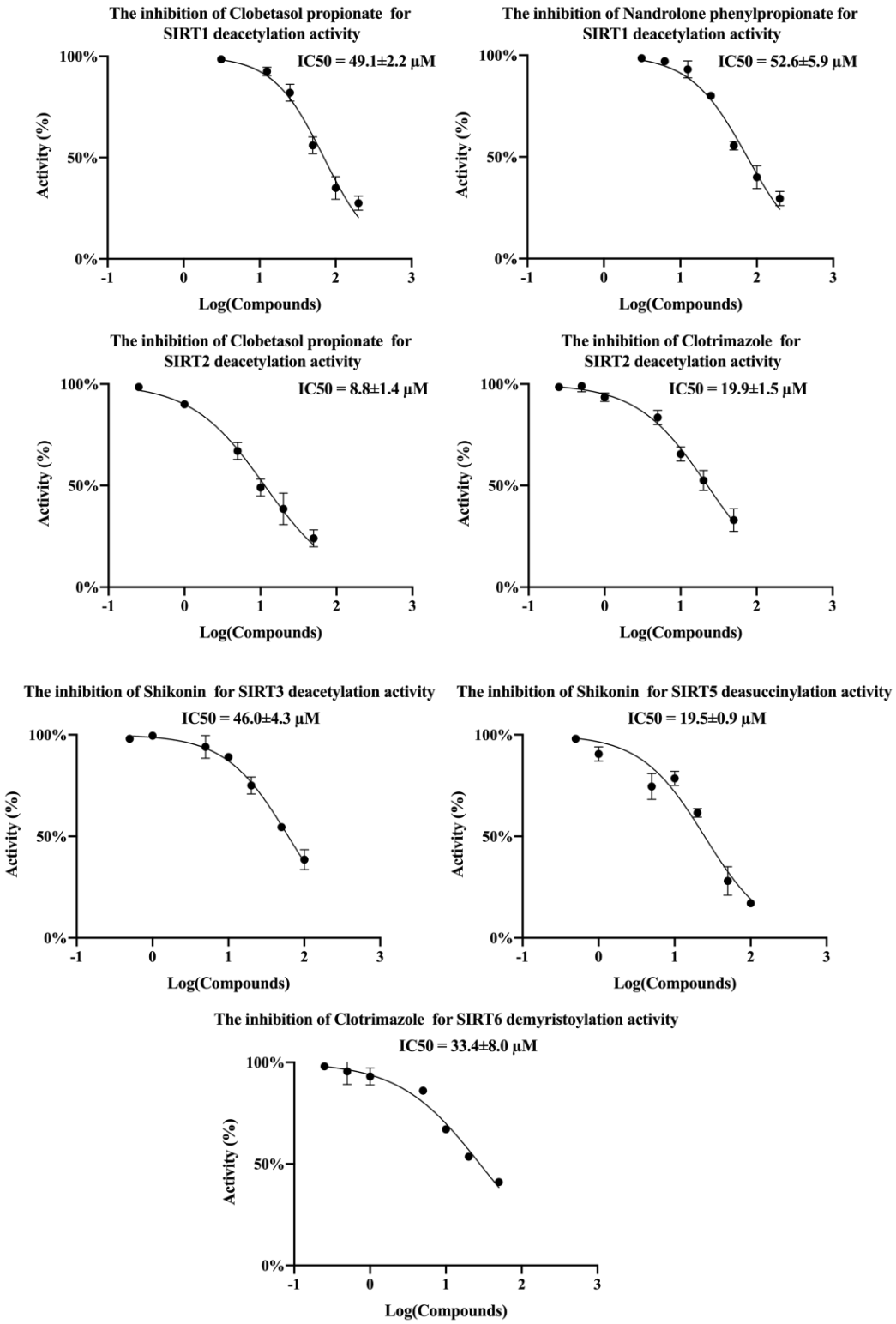


Fig. S11. Determination of the inhibitory activity of shikonin, nandrolone phenylpropionate, clobetasol propionate and clotrimazole against SIRTs by fluorogenic assay.

Table S1. The Kinetic parameters of **4a-d** and **9** with SIRTs

Sirtuin	Substrate	Activity	Km (μM)	kcat (s^{-1})	kcat/Km ($s^{-1}M^{-1}$)
SIRT1	**4a**	Deaceylation	94.0 ± 29.3	0.008 ± 0.001	85.1
	4a	Deaceylation [a]	100.2 ± 20.2	0.01 ± 0.001	99.8
	9	Deaceylation [a]	109.4 ± 32.0	0.01 ± 0.002	91.4
SIRT2	**4a**	Deaceylation	90.3 ± 30.2	0.009 ± 0.001	95.2
	4b	Debenzoylation	111.5 ± 46.7	0.002 ± 0.0004	17.9
	4d	Defatty-acylation	7.6 ± 1.2	0.0009 ± 0.00003	118.4
SIRT3	**4a**	Deaceylation	62.6 ± 8.8	0.003 ± 0.0002	47.9
SIRT5	**4c**	Desuccinylation	129.8 ± 15.2	0.01 ± 0.0006	77.0
SIRT6	**4d**	Defatty-acylation	9.1 ± 1.8	0.0008 ± 0.00005	87.9

a: Detected by HPLC.

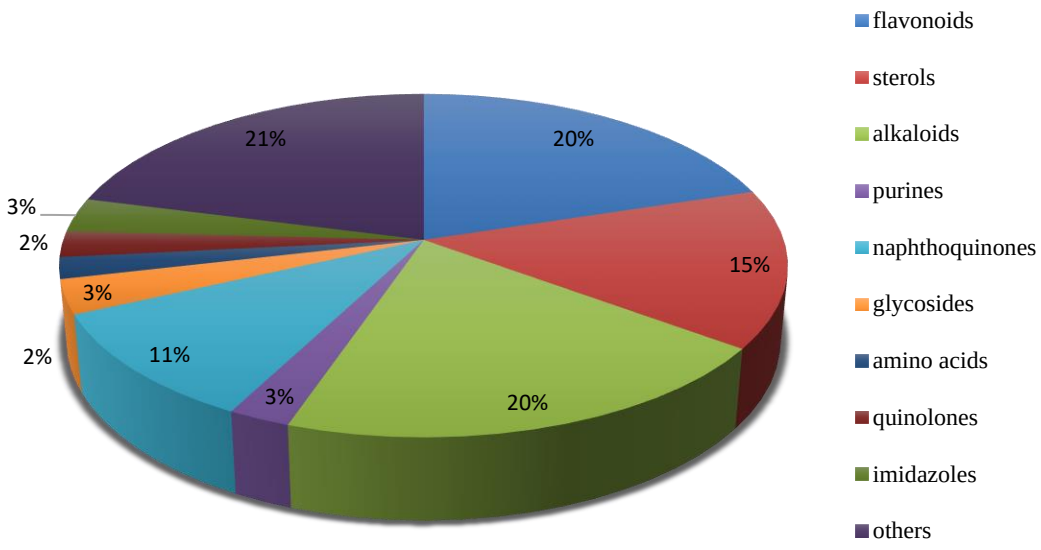
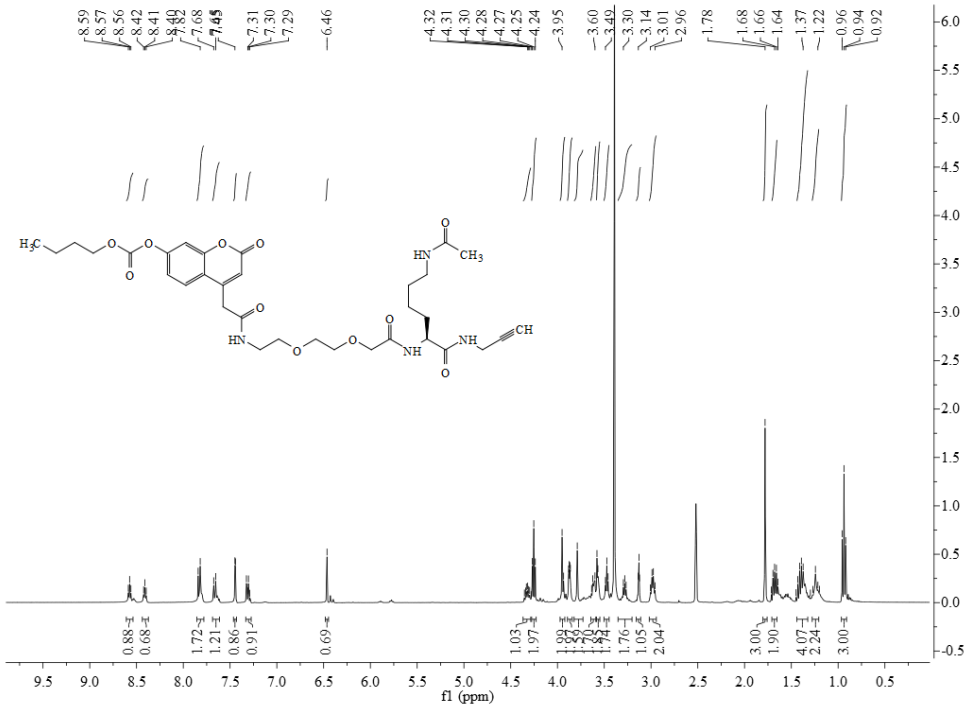


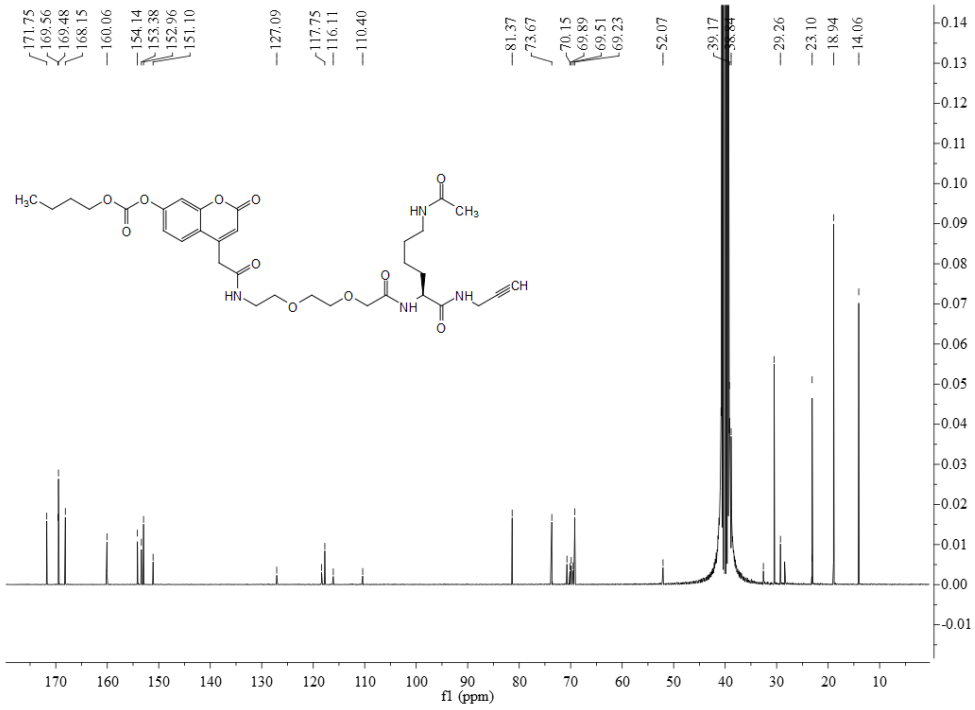
Fig. S12. Small molecule compound library of high-throughput screening. Among them, flavonoids had 50, sterols had 37, alkaloids had 50, purines had 6, naphthoquinones had 26, glycosides had 8, amino acids had 5, quinolone antibiotics had 6, and imidazole antifungal drugs had 8, others had 52.

2. NMR and Mass spectroscopy

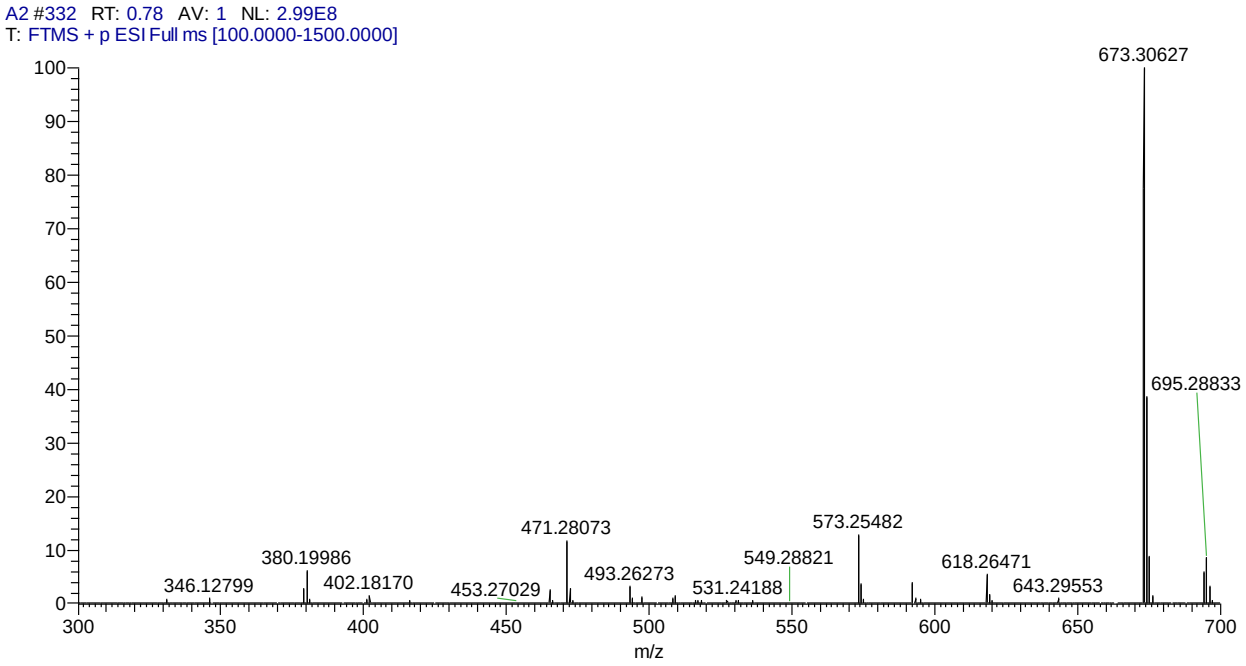
^{1}H NMR of probe **4a**



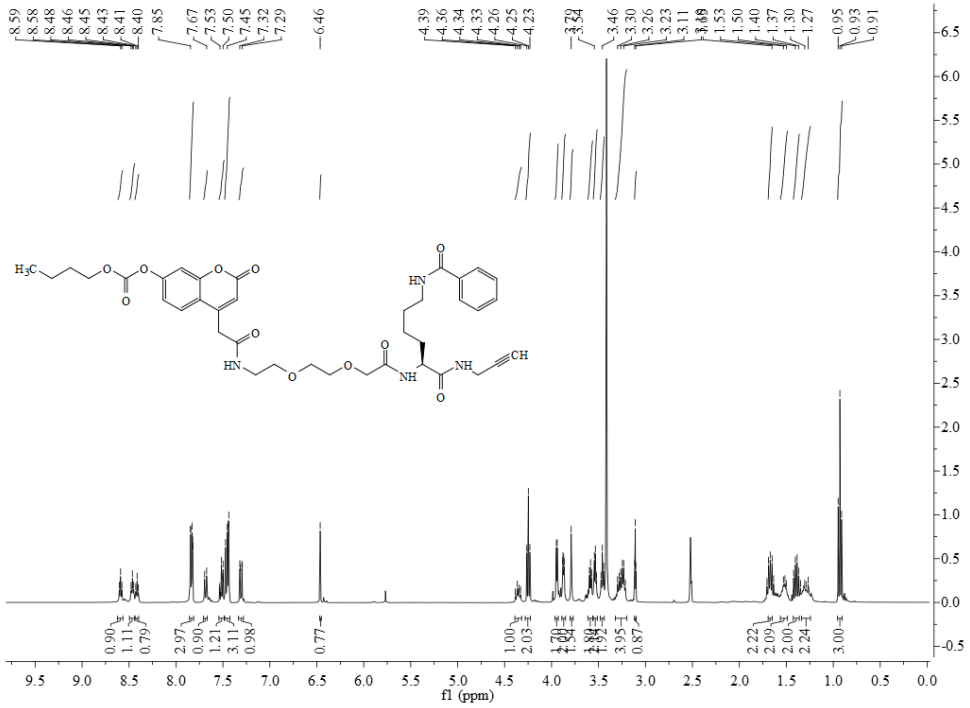
^{13}C NMR of probe **4a**



HRMS of probe **4a**



^{1}H NMR of probe **4b**



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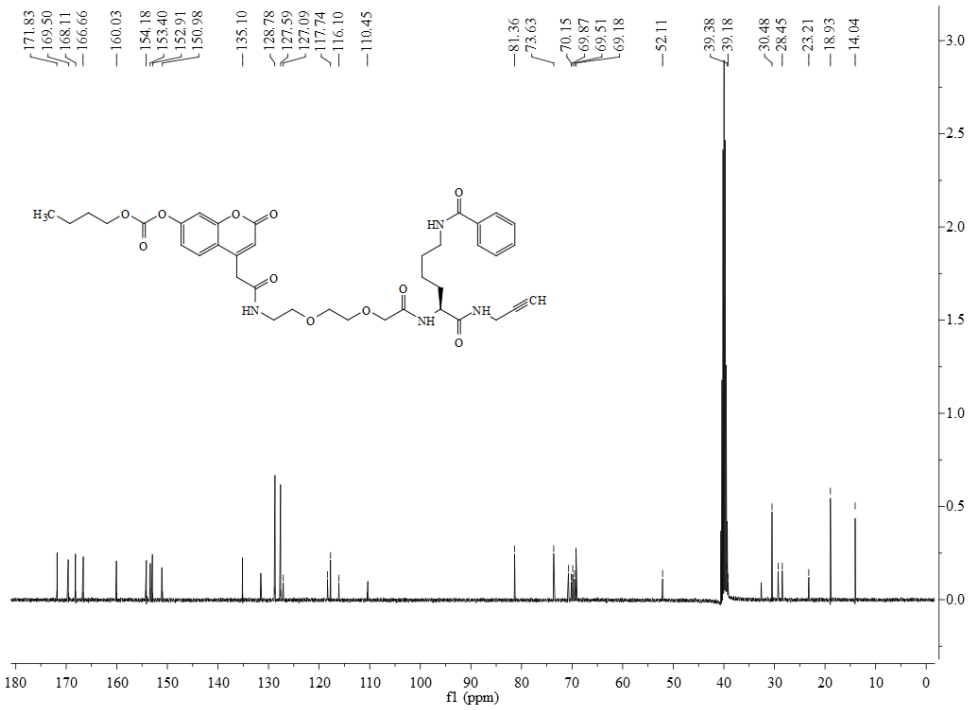
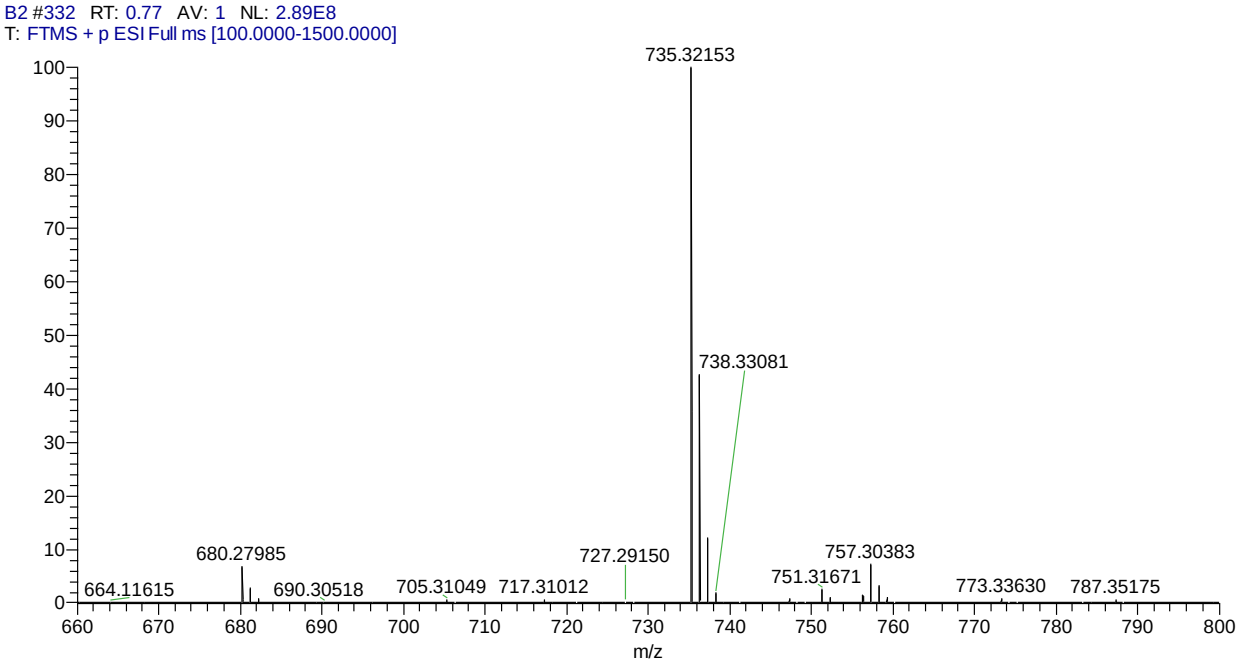


HRMS of probe **4b**



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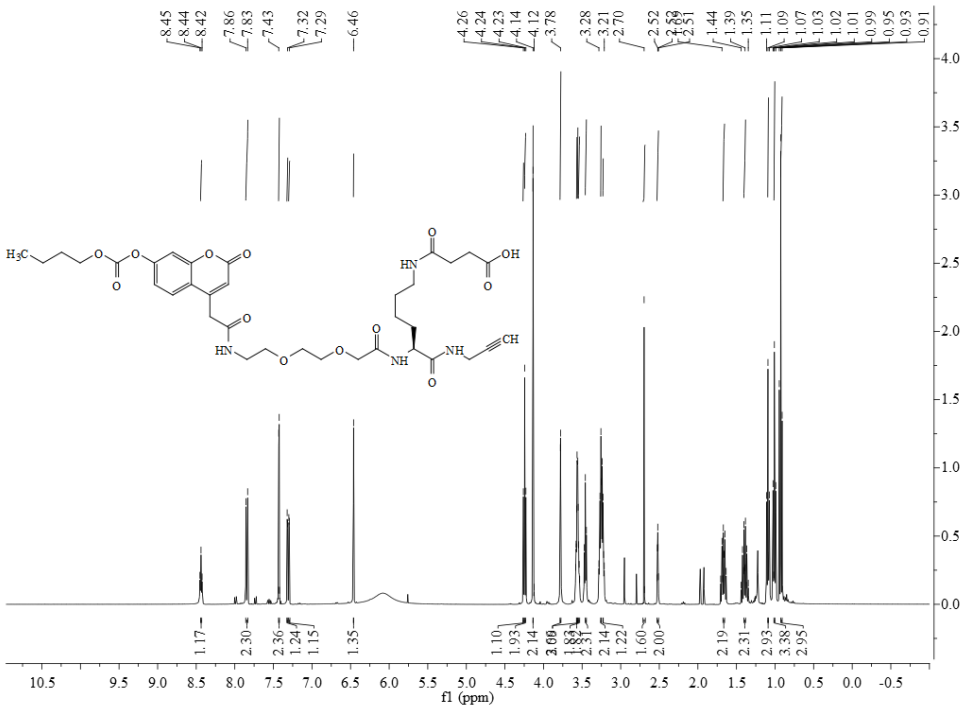
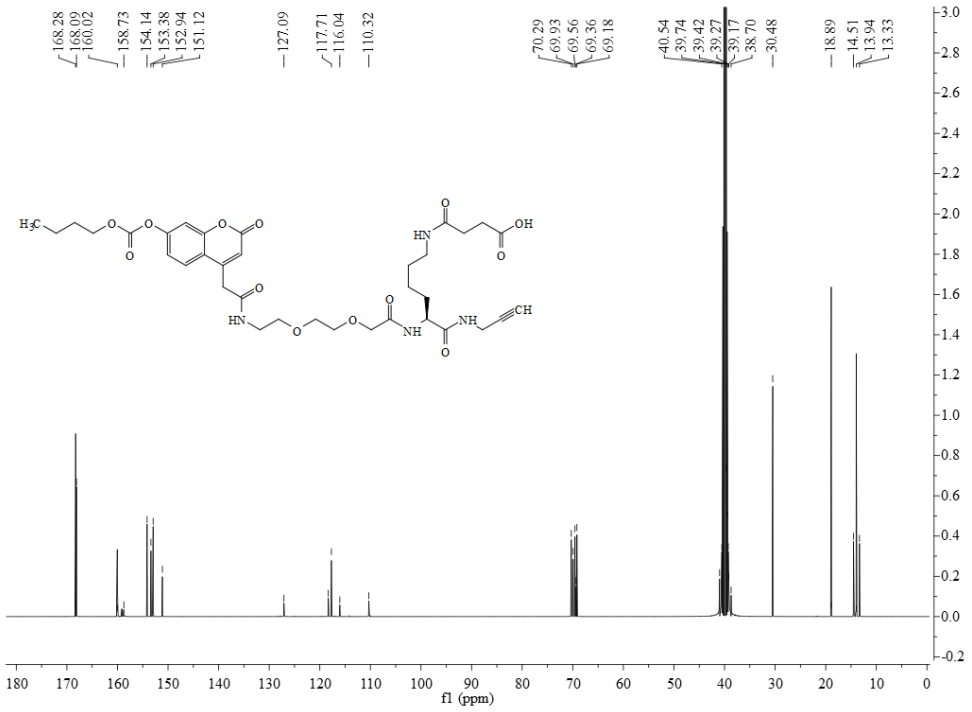
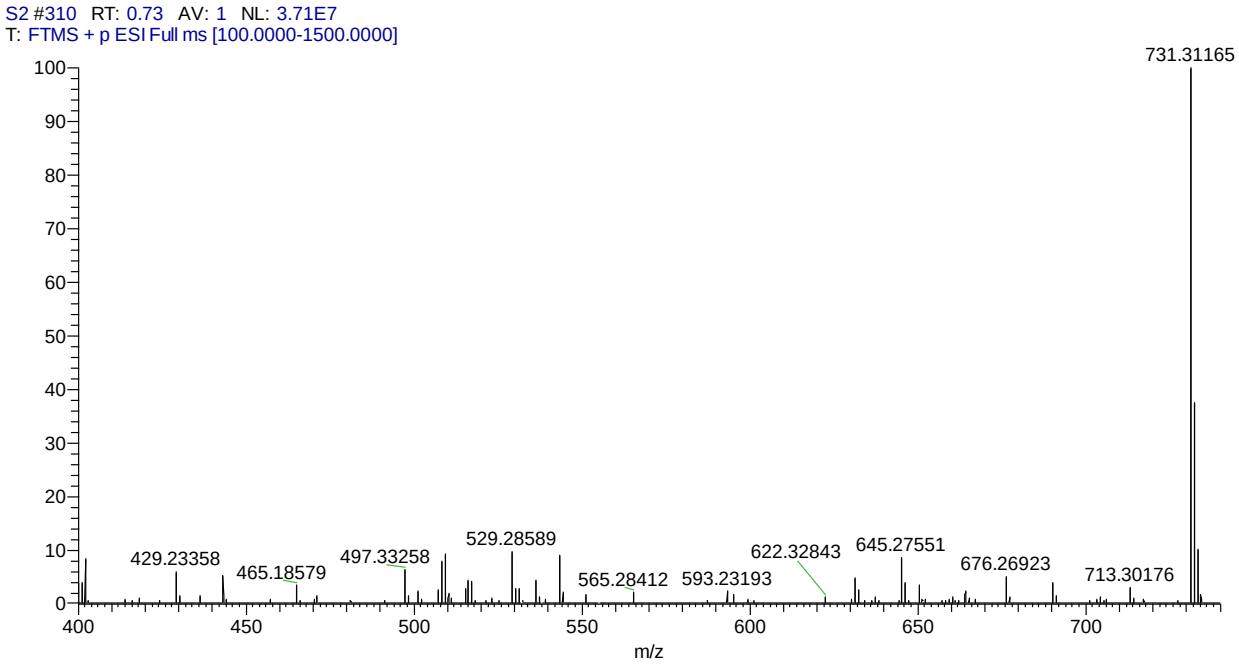


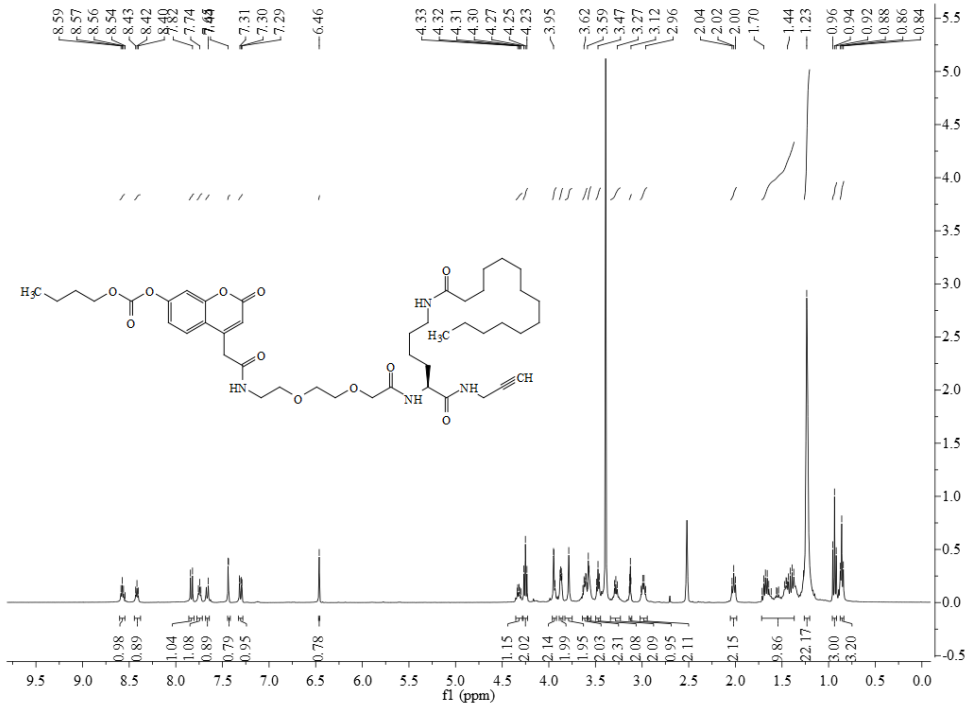
^{13}C NMR of probe **4c**



HRMS of probe **4c**



^{1}H NMR of probe **4d**



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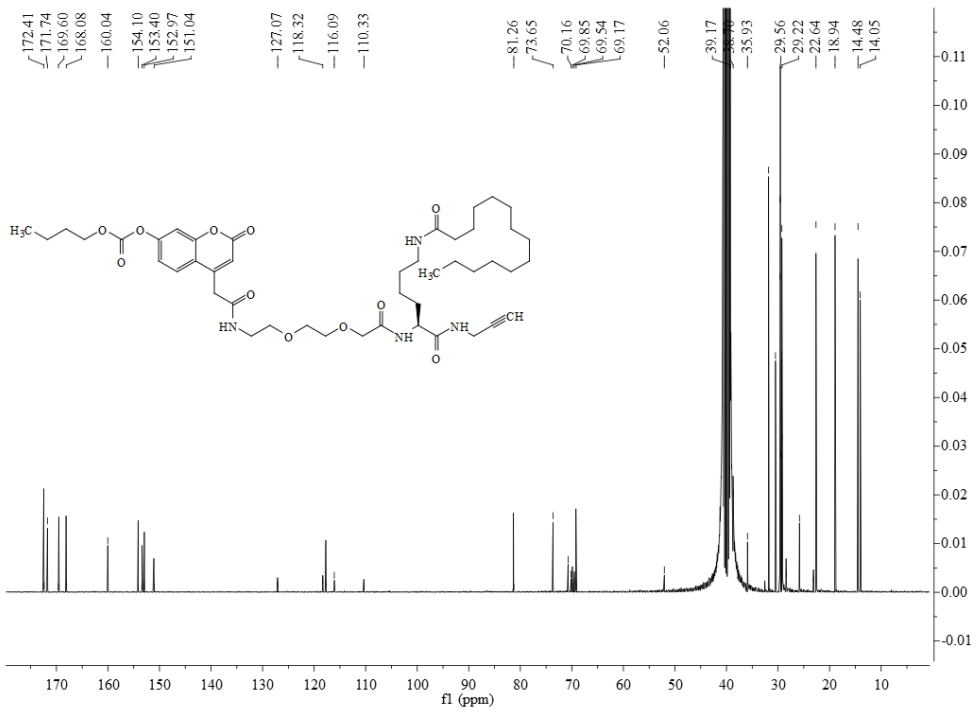
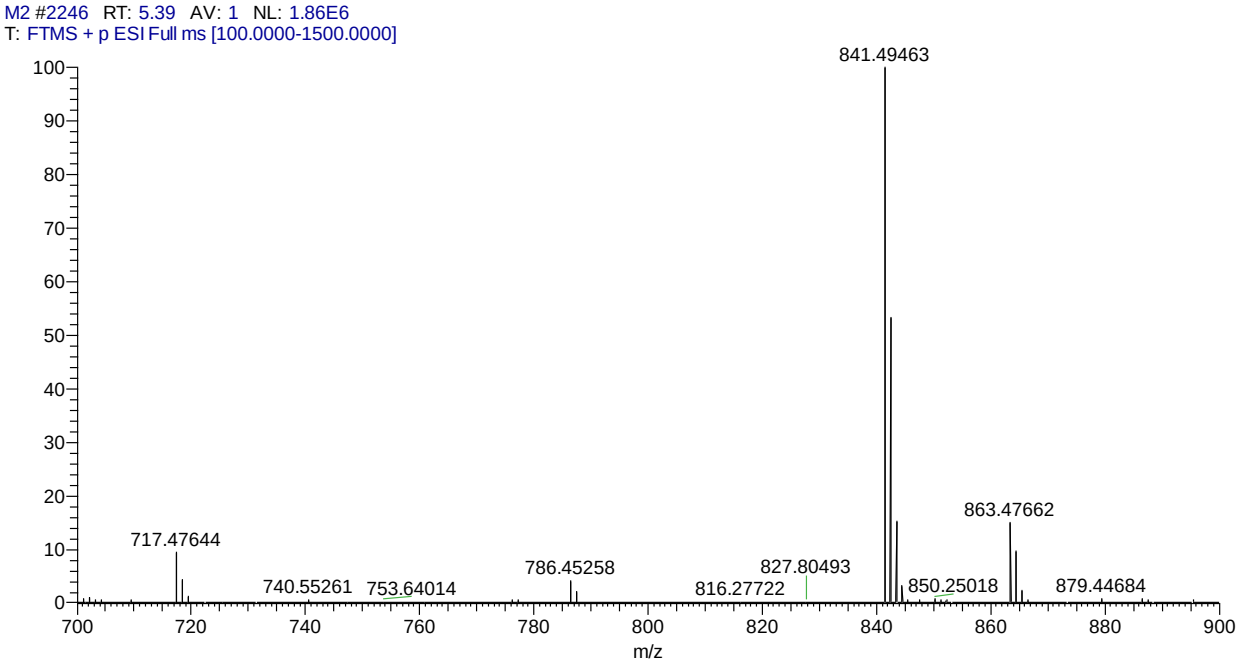


HRMS of probe **4d**



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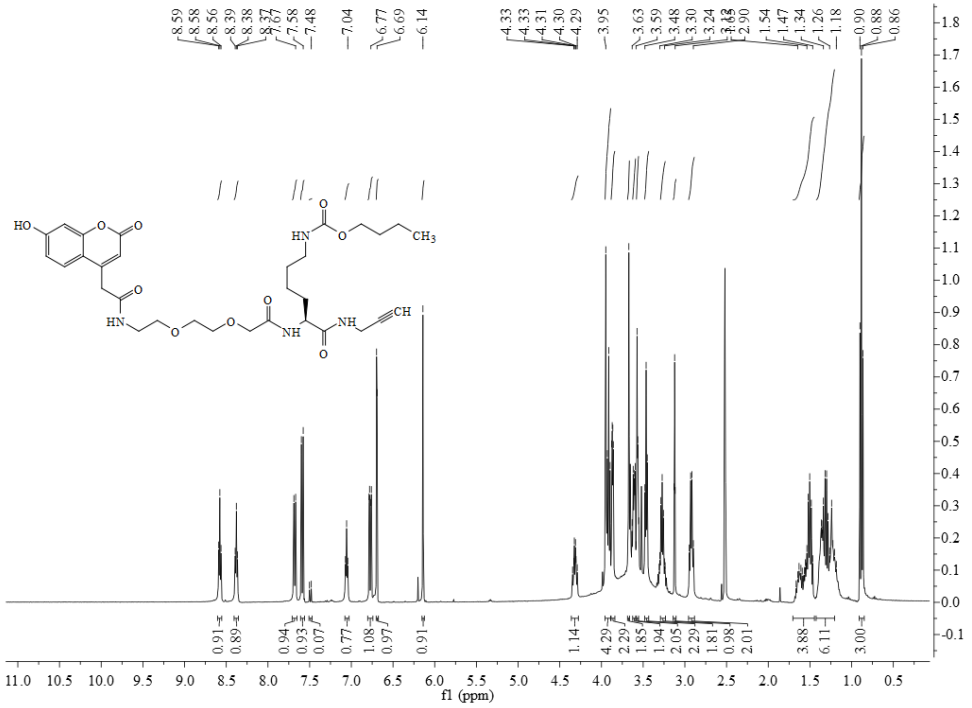

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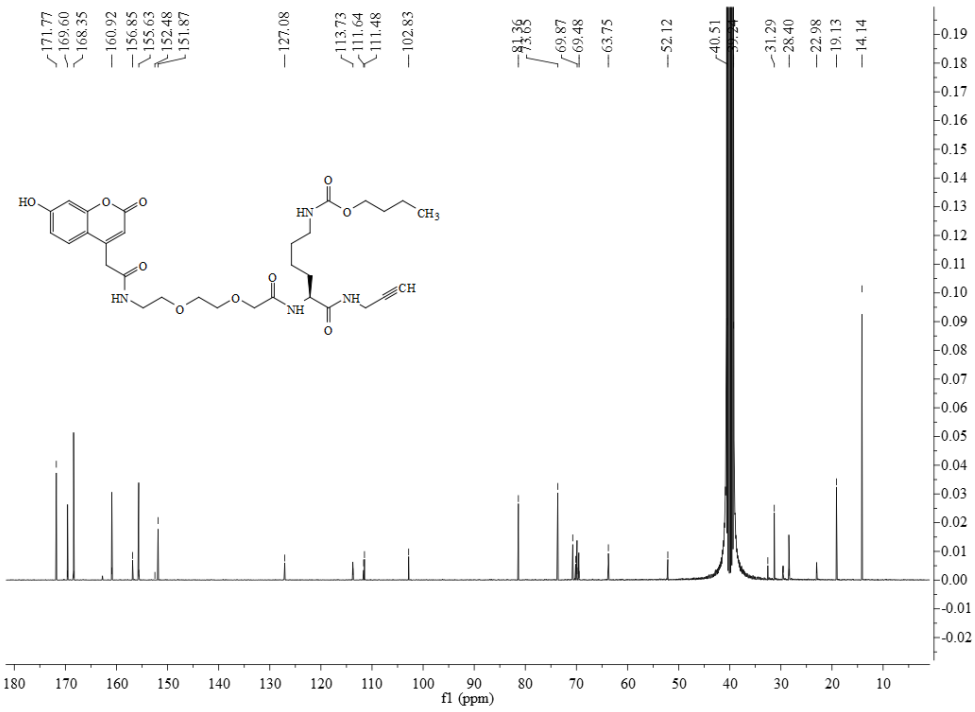
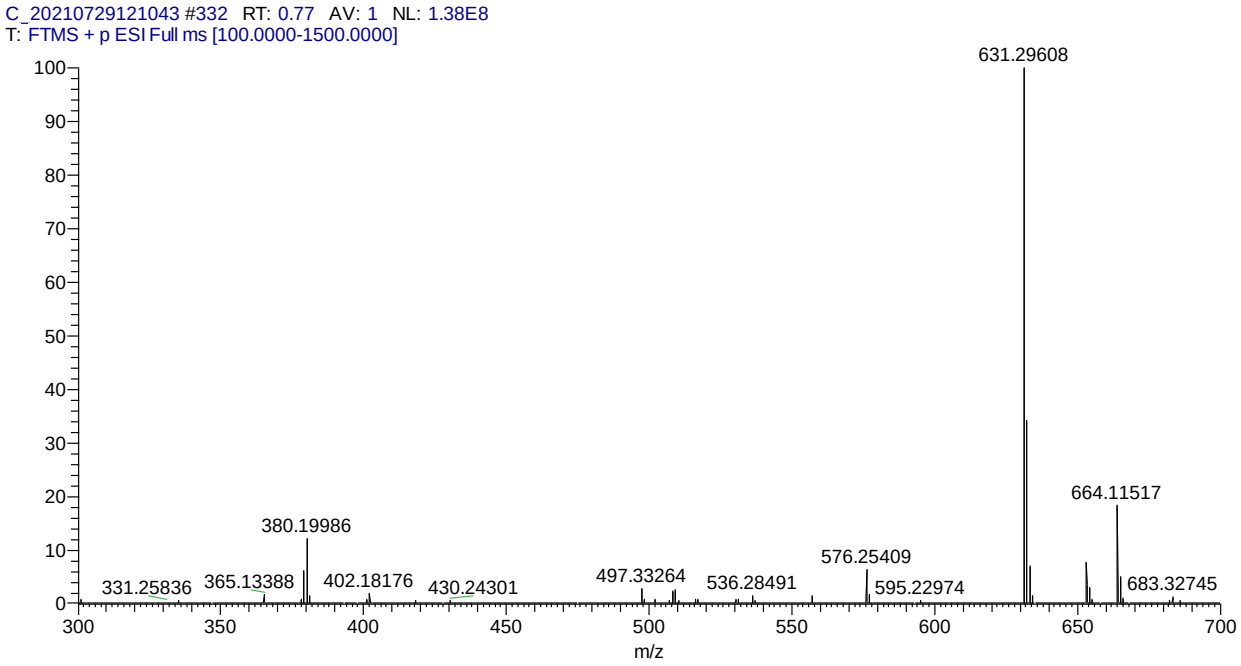
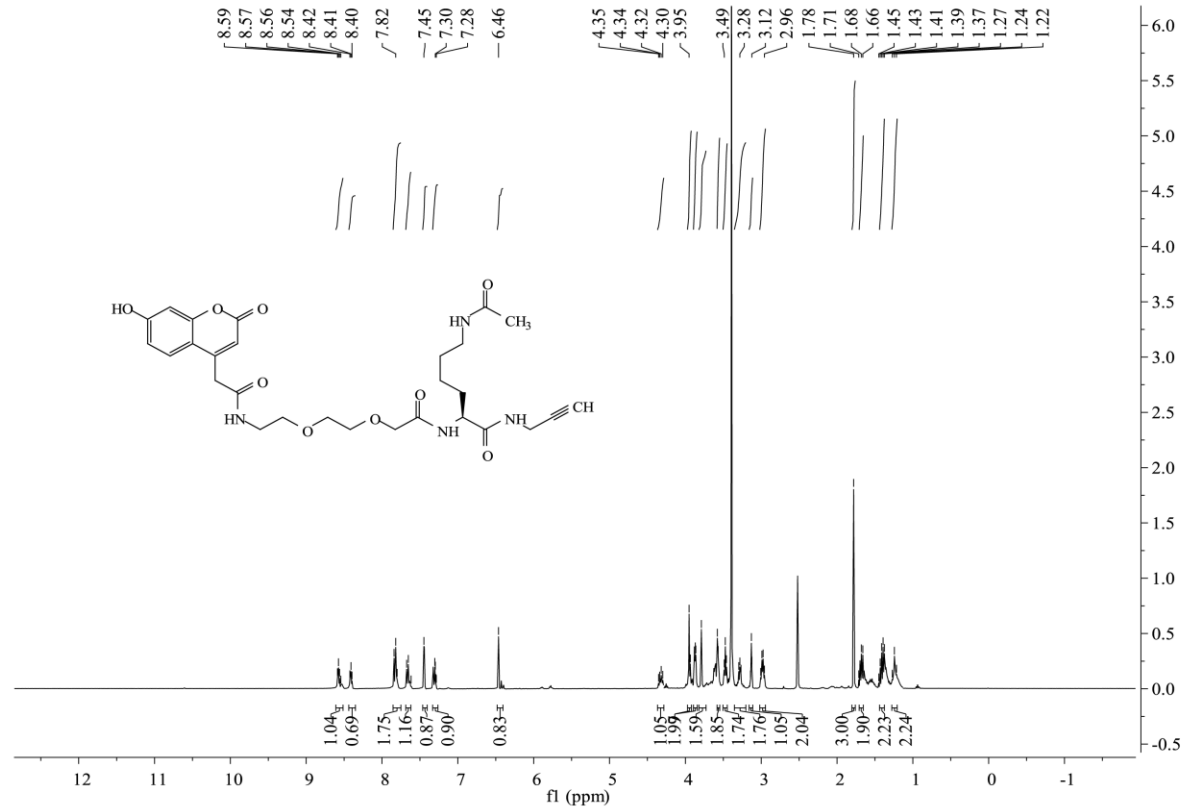


HRMS of probe **8**



^{1}H NMR of probe **9**



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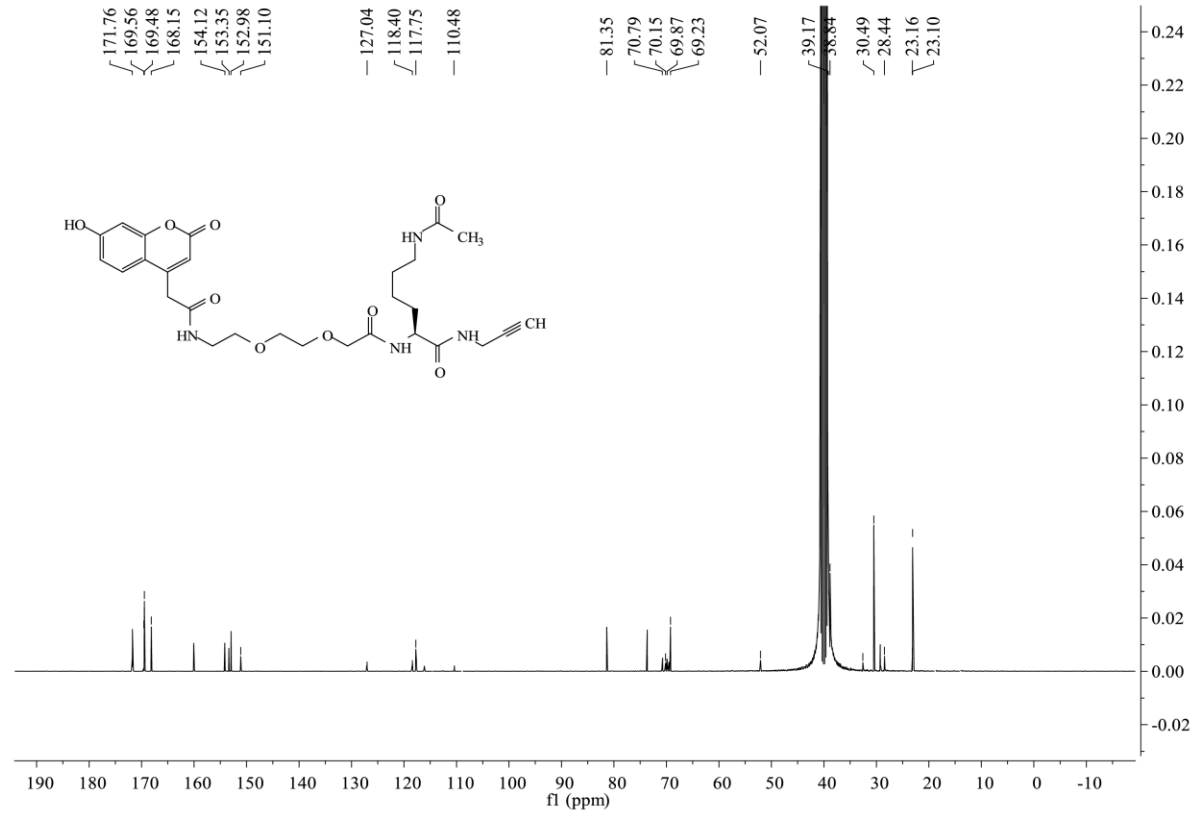

HRMS of probe **9**