Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Supporting Information

A fluorogenic assay for screening Sirt6 modulators

Jing Hu, Bin He, Shiva Bhargava, Hening Lin*

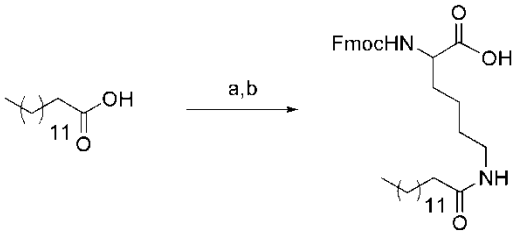
Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853, USA

Contents of Supporting Information

A. Reagents and Methods

General methods and materials. Human Sirt6 was expressed as previously described.[1] Reagents were obtained from Aldrich or Acros in the highest purity available and used as supplied. Sirtinol, AGK-2, Cambinol and Tenovin-1 inhibitors were from Calbiochem (EMD Millipore). BSA was from Themo Scientific and trypsin was from Gibco (0.5% Trypsin-EDTA, 10X). ^{1}H and ^{13}C-NMR were performed on INOVA 400 spectrometers. 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% acetic acid and acetonitrile with 0.1% acetic 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 326 nm. Preparative HPLC purification was carried out using Targa^{TM} Prep C18 10µm 250×20mm reverse phase column with UV detection at 215 nm and 260 nm. Solvents used in Preparative HPLC were water with 0.1% TFA and acetonitrile with 0.1% TFA. Fluorescence assay was recorded by BIO-TEK® Synergy HT plate reader (Optics Position: Top, Sensitivity: 50, Excitation at the wavelength of 360 nm and Emission at the wavelength of 460 nm.

Synthesis of the Fmoc-Lys(Myristoyl)-OH building block for peptide synthesis



1 **2a**

Reagents and conditions: a) N-Hydroxysuccinimide, DCC, room temperature, 2 hrs. b) Fmoc-Lys-OH, ^{i}Pr$_2$NEt, room temperature, overnight.

To a solution of myristic acid **1** (2.28 g, 10 mmol) in anhydrous *N, N'*-dimethylformamide (DMF, 20 mL), N-hydroxysuccinimide (1.15 g, 10 mmol) was added with stirring at room temperature. Then *N,N'*-dicyclohexylcarbodiimide (DCC, 2.06 g, 10 mmol) in anhydrous DMF (20 mL) was added to the reaction. After stirring at room temperature for 2 hrs, the reaction mixture was filtered. The filtrate was added to a solution of Fmoc-Lys-OH (3.68 g, 10 mmol) with N,N-diisopropylethylamine (DIEA, 1.74 mL, 10 mmol) in anhydrous DMF (20.0 mL) at room temperature. The resulting reaction mixture was stirred for another 24 hrs at room temperature. Then the reaction mixture was poured into water and neutralized with 1 M HCl to adjust pH to 2~3. The precipitate was collected by filtration and dried to give the crude. Then the crude compound was purified by silica gel column chromatography using 50:1 CH_2Cl_2:CH_3OH to give compound **2a** as a white solid (5.03 g, 87% yield). ^{1}H NMR (400 MHz, CD_3OD): δ 7.77 (d, 2H, *J* = 4.0 Hz), 7.66 (t, 2H, *J* =4.0), 7.37 (t, 2H, *J* = 8.0 Hz), 7.29 (t, 2H, *J* =8.0 Hz), 4.35-4.30 (m, 2H), 4.20 (t, 1H, *J* = 8.0), 4.15-4.10 (m, 1H), 3.15 (t, 2H, *J* = 4.0 Hz), 2.13 (t, 2H, *J* = 8.0 Hz), 1.90-1.67 (m, 2H), 1.67-1.35 (m, 6H), 1.35-1.18 (m, 20H), 0.87 (t, 3H, *J* =8.0 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ174.9, 174.5, 157.3, 143.9, 143.7,

141.2, 127.3, 126.7, 124.9, 124.8, 119.5, 66.6, 53.8, 38.6, 35.8, 31.6, 30.9, 29.4, 29.3, 29.3, 29.2, 29.0, 28.9, 28.5, 25.7,22.9, 22.3, 13.0. LCMS (ESI) calcd. for $C_{35}H_{50}N_2O_5$ $[M+H]^+$ 579.4, obsd. 579.3.

Synthesis of peptide Ac-Glu-Ala-Leu-Pro-Lys-Lys(Acyl)-(7-amino-4-methyl-coumarin)

Ac-Glu-Ala-Leu-Pro-Lys-Lys(Myristoyl)-(7-amino-4-methyl-coumarin) (4a). A protected peptide AcGlu(^{t}Bu)Ala-Leu-Pro-Lys(Boc)Lys(Myr)OH was synthesized by solid phase peptide synthesis using a 2-chlorotrityl resin and then cleaved from support under mild conditions (AcOH: CF_3CH_2OH: CH_2Cl_2, 1:1:8, v/v/v). To a solution of AcGlu(^{t}Bu)Ala-Leu-Pro-Lys(Boc)Lys(Myr)OH (50 mg, 0.046 mmol) and N-methylmorpholine (NMM, 0.01 mL, 0.091 mmol) in dry dichloromethane (2 mL), isobutylchloroformate (0.009 mL, 0.067 mmol) was added dropwisely at 0°C. The reaction mixture was stirred for 2 hrs at 0°C. 7-amino-4-methyl-coumarin (16mg, 0.091mmol) in DMF (2 mL) was added at 0°C and the reaction mixture was stirred 24 hrs at room temperature. Then the solution was partitioned between CH_2Cl_2 (10ml) and brine (10ml). The aqueous phase was extracted with CH_2Cl_2 (2×15ml). The combined organic phase was dried over anhydrous $MgSO_4$ and then evaporated to dryness. The obtained residue was treated with 1% triisopropylsilane in TFA (1mL). After stirring for 2 hrs, the reaction mixture was evaporated to dryness, and then co-evaporated with toluene and MeCN. The resulting residue was applied to preparative HPLC purification. Peptide **4a** was eluted at 62 mins with a flow rate of 10 ml/min. The gradient was: 0% solvent B for 10 min, 0% to 80% solvent B over 50 mins, then 80% to 95% solvent B for 5 mins. After lyophilization the desired peptide **4a** was obtained as a white solid (**4a,** 12 mg, 23.8%). LCMS (ESI) calcd. for $C_{57}H_{91}N_9O_{12}$ $[M+H]^+$ 1094.7, obsd. 1094.9.

Ac-Glu-Ala-Leu-Pro-Lys-Lys(Ac)-(7-amino-4-methyl-coumarin) (4b) Peptide **4b** was synthesized with the same synthetic method of peptide **4a** and peptide **4b** was eluted at 61 mins with a flow rate of 10 ml/min. The gradient was: 0% solvent B for 10 mins, 0% to 40% solvent B over 50 mins, then 40% to 95% solvent B for 5 mins. After lyophilization the desired peptide 4b was obtained as a white solid (**4b**, 20.5%). LCMS (ESI) calcd. for $C_{45}H_{67}N_9O_{12}$ $[M+H]^+$ 926.5, obsd. 926.7.

Ac-Glu-Ala-Leu-Pro-Lys-Lys-(7-amino-4-methyl-coumarin) (4c) Peptide **4c** was synthesized with the same synthetic method of peptide **4a** and peptide **4c** was eluted at 57 min with a flow rate of 10 ml/min. The gradient was: 0% solvent B for 10 mins, then 0% to 30% solvent B over 50 mins. After lyophilization the desired peptide was obtained as a white solid (**4c**, 21.0%). LCMS (ESI) calcd. for $C_{43}H_{65}N_9O_{11}$ $[M+H]^+$ 884.5, obsd. 884.6.

Monitoring trypsin-catalyzed hydrolysis of AMC-free lysine peptides. Trypsin (5 mg/mL, 60 μL) was added to a 60 μL solution containing the AcEALPKK-AMC peptide (10 μM), NAD (1 mM) and dithiothreitol (DTT, 1 mM) in Tris-HCl buffer (pH 8.0, 20 mM). The reaction mixture was incubated for 0.5, 1, 2, 3, 4, 6 or 8 hrs at 37°C. The mixture (100 μL) was transferred to a 96 well plate and the fluorescence was recorded by a plate reader.

Testing the Sirt6 fluorogenic assay with different concentration of the AMC- myristoyl lysine peptide. Sirt6 (1 μM) was incubated with AcEALPK(MyrK)-AMC peptide (1~20 μM), NAD (1 mM), BSA(1 mg/mL), and dithiothreitol (DTT, 1 mM) in Tris-HCl buffer (pH 8.0, 20 mM) in a 60 μL reaction for 2 hrs at 37°C. Then trypsin (5 mg/mL, 60 μL) containing nicotinamide (8 mM) was added and the reactions were incubated for 2 hrs at 37°C. Then the mixture (100 μL) was transferred to a 96-well plate and the fluorescence was recorded by a plate reader.

Testing the Sirt6 fluorogenic assay with different enzyme concentration.
Sirt6 (0.1~2μM) was incubated with AcEALPK(MyrK)-AMC peptide (10 μM), NAD (1 mM), BSA(1 mg/mL), and dithiothreitol (DTT, 1 mM) in Tris-HCl buffer (pH 8.0, 20 mM) in a 60 μL reaction for 2 hrs at 37°C. Then trypsin (5 mg/mL, 60 μL) containing nicotinamide (8 mM) was added and the reactions were incubated for 2 hrs at 37°C. Then the mixture (100 μL) was transferred to a 96-well plate and the fluorescence was recorded by a plate reader.

Sirt6 fluorogenic assay with Ac-Glu-Ala-Leu-Pro-Lys-Lys(Acyl)-(7-amino-4-methyl-coumarin). Sirt6 (1 μM) was incubated with AcEALPK(AcylK)-AMC peptide (10 μM), NAD (1 mM), BSA(1 mg/mL), and dithiothreitol (DTT, 1 mM) in Tris-HCl buffer (pH 8.0, 20 mM) in a 60 μL reaction for 2 hrs at 37°C. Then trypsin (5 mg/mL, 60 μL) containing nicotinamide (8 mM) was added and the reactions were incubated for 2 hrs at 37°C. Then the mixture (100 μL) was transferred to a 96-well plate and the fluorescence was recorded by a plate reader.

Testing Sirtuin inhibitors with Ac-Glu-Ala-Leu-Pro-Lys-Lys(Myristoyl)-(7-amino -4-methyl-coumarin) for their ability to inhibit Sirt6. Sirt6 (1 μM) was first incubated with sirtuin inhibitors (200 μM from DMSO stock solution), NAD (1 mM), BSA(1 mg/mL) in Tris-HCl buffer (pH 8.0, 20 mM) containing dithiothreitol (DTT, 1 mM) for 0.5 hour at 37°C. Then the substrate AcEALPK(MyrK)-AMC (10 μM) was added to initiate the demyristoylation reaction. The total reaction volume was 60 μL. The reactions were incubated for 2 hrs at 37°C. Then trypsin (5 mg/mL, 60 μL) containing nicotinamide (8 mM) was added and the reactions were incubated for 2 hrs at 37°C. The mixture (100 μL) was transferred to a 96-well plate and the fluorescence was recorded by a plate reader.

Dose-response of nicotinamide with Sirt6 using the fluorogenic assay. Sirt6 (1 μM) was first incubated with nicotinamide (2 μM to 2mM from water stock solution), NAD (1mM), BSA(1mg/mL) in Tris-HCl buffer (pH 8.0, 20 mM) containing dithiothreitol (DTT, 1 mM) for 0.5 hours at 37°C. Then the substrate AcEALPK(MyrK)-AMC (10 μM) was added to initiate the demyristoylation reaction and the reaction (60 μL) was incubated for 2 hrs at 37°C . Then trypsin (5 mg/mL, 60 μL) containing nicotinamide (8 mM) was added and the resulting reaction mixtures were incubated for 2 hrs at 37°C. The reaction mixtures (100 μL) were transferred to a 96-well plate and the fluorescence was recorded by using a plate reader.

Kinetics of the Ac-Glu-Ala-Leu-Pro-Lys-Lys (Myristoyl)-(7-amino-4-methyl-coumarin)

peptide on Sirt6. The AcEALPK(MyrK)-AMC peptide concentration was varied from 1 to 128 μM. The reactions (60 μL with 1 mM NAD, 20 mM Tris, pH 8.0, 1 mM DTT, 1 mg/mL BSA, 0.2 μM Sirt6 and the peptide at different concentrations) were incubated for 60 mins at 37°C. Each reaction was stopped using 60 μL of 0.5N HCl in methanol. The reaction mixtures were spun at 18000g for 10 min and we analyzed on a Kinetex XB-C18 column (100A, 100 mm × 4.60 mm, 2.6 μm, Phenomenex). The gradient of 0 to 40% B in 17 mins and then 40% to 100% for 2 mins at 0.5 mL/min was used. The product and the substrate peaks were quantified using absorbance at 326 nM and converted to initial rates, which were then plotted against the peptide concentrations and fitted using Kaleidagraph.

Reference

1. H. Jiang, S. Khan, Y. Wang, G. Charron, B. He, C. Sebastian, J. Du, R. Kim, E. Ge, R. Mostoslavsky, H. C. Hang, Q. Hao and H. Lin, *Nature*, 2013, **496**, 110-113.

B. Supplementary Figures

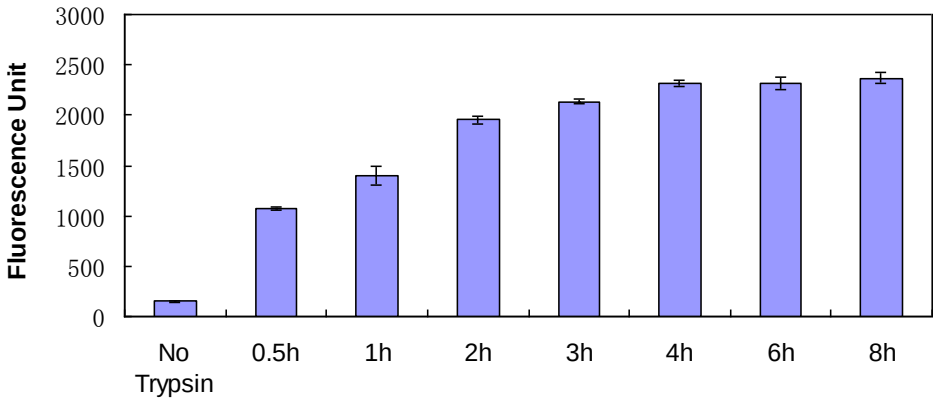


Figure S1. Cleavage of the AcEALPKK-AMC peptide by trypsin with different incubation time (37℃). The experiments were performed in duplicate.

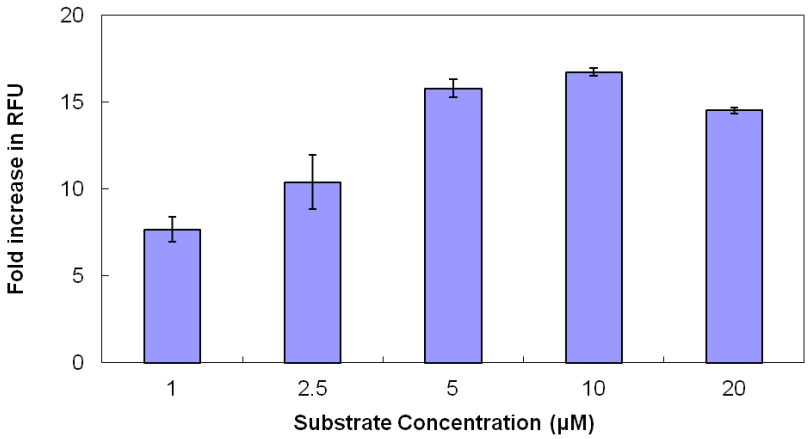


Figure S2. Optimizing the substrate concentration in the Sirt6 fluorogenic assay. Different concentrations of the AcEALPK(MyrK)-AMC peptide was incubated with or without Sirt6 for 2 hrs and then with 2.5 mg/mL trypsin for 2 hrs. The experiments were performed in duplicate.

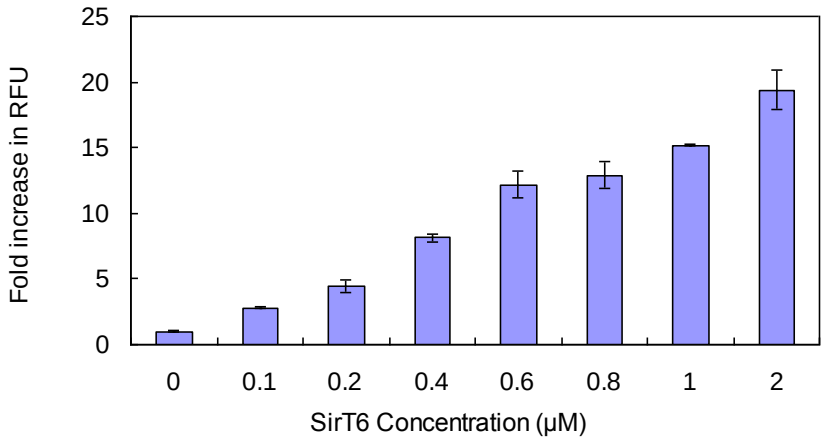


Figure S3. Bar graph for demyristoylation of substrate AcEALPK(MyrK)-AMC. 10 μM of the AcEALPK(MyrK)-AMC peptide was incubated with or without Sirt6 for 2 hrs and then with 2.5 mg/mL trypsin for 2 hrs. Compared with the negative control without Sirt6, the fluorescence was increased 2.8, 4.4, 8.1, 12.2, 12.9, 15.2 and 19.4 -fold with 0.1, 0.2, 0.4, 0.6, 0.8, 1 and 2 μM of SirT6.The data were obtained as end point readings in duplicate.

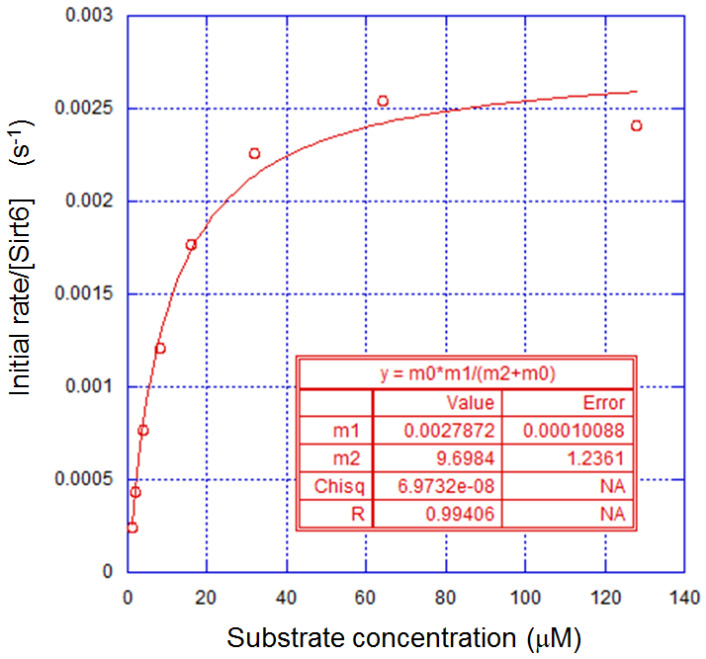


Figure S4. Michaelis-Menten curve of the AcEALPK(MyrK)-AMC peptide with Sirt6. In the reactions, 1 mM NAD, 20 mM Tris pH 8.0, 1 mM DTT and 0.2 μM Sirt6 were used. Peptide concentration used for AcEALPK(MyrK)-AMC was 1, 2, 4, 8, 16, 32, 64, and 128 μM and the incubation time was 60 min. The experiments were performed in duplicate.

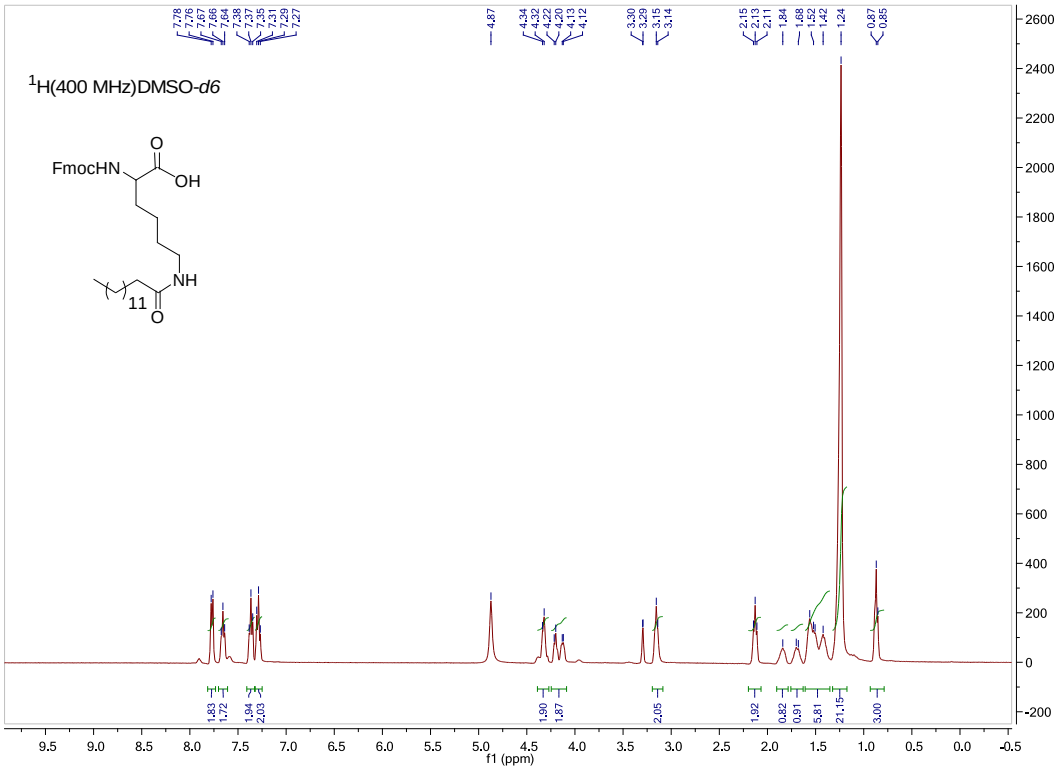


Figure S5. ^{1}H NMR of **Fmoc-Lys(Myr)-OH**

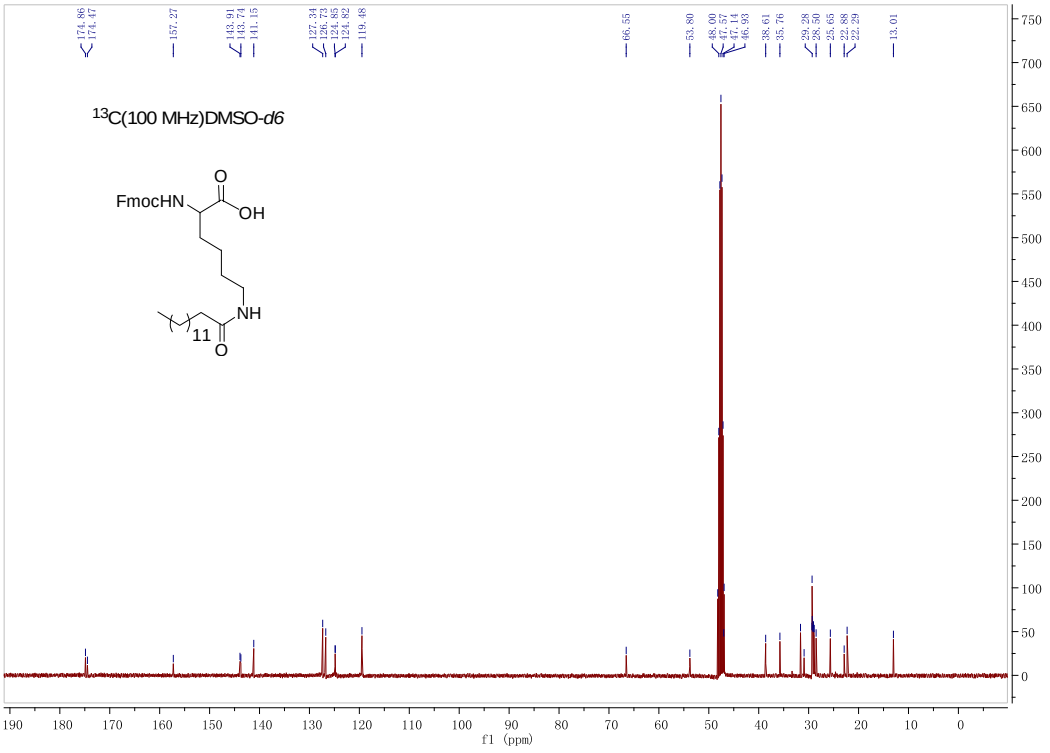


Figure S6. ^{13}C NMR of **Fmoc-Lys(Myr)-OH**

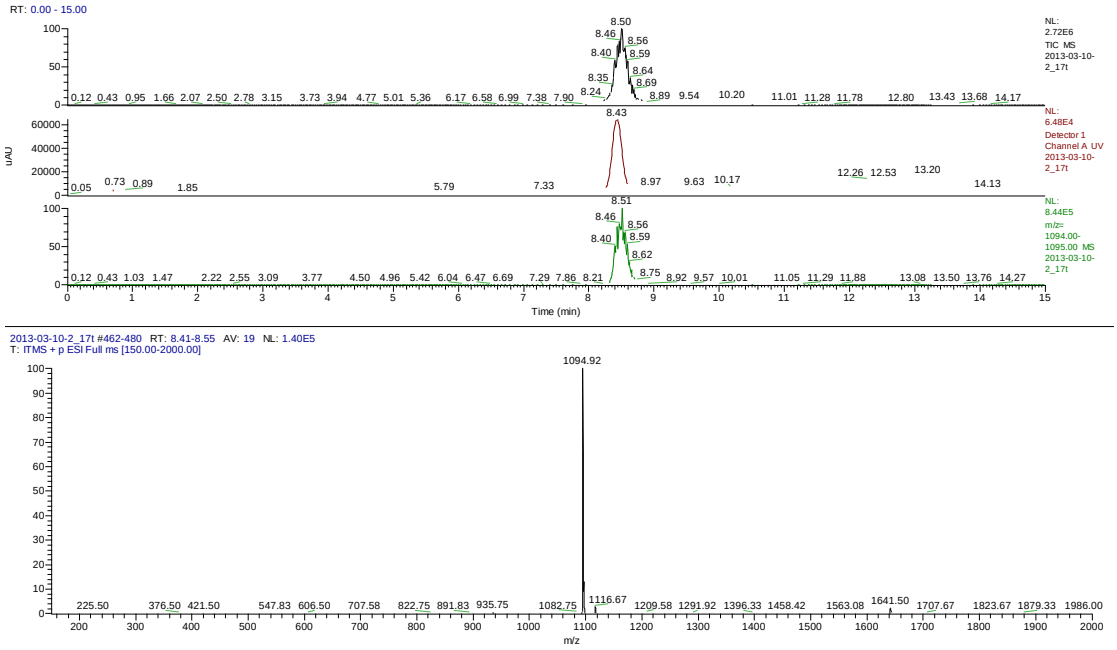


Figure S7. LCMS of **Ac-Glu-Ala-Leu-Pro-Lys-Lys(Myristoyl)-(7-amino-4-methyl-coumarin)**

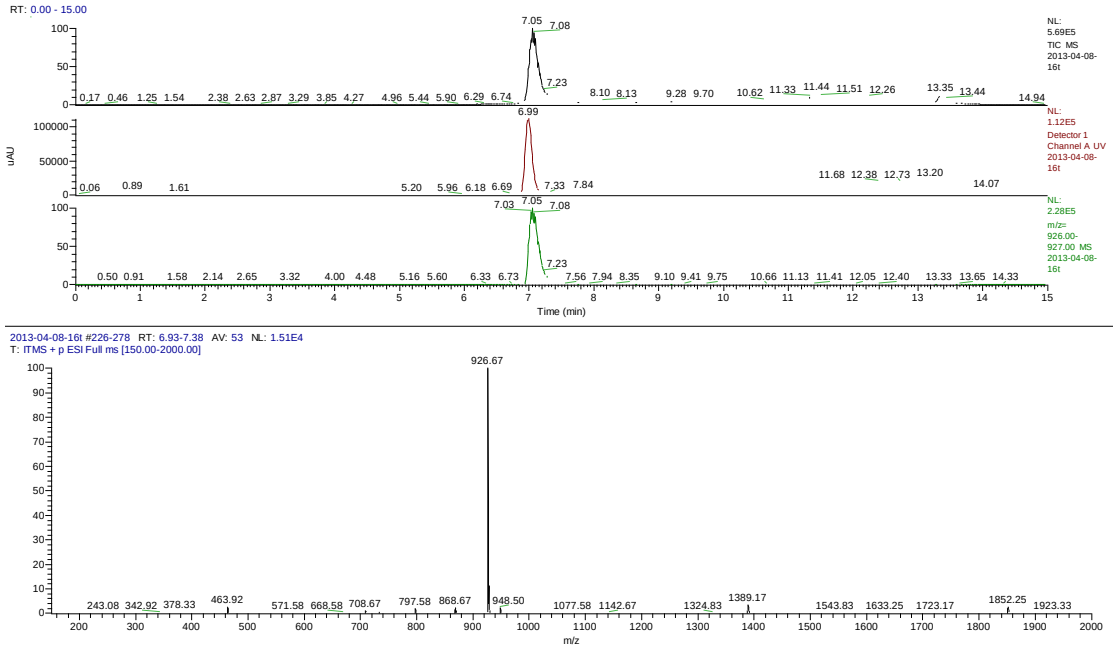


Figure S8. LCMS of **Ac-Glu-Ala-Leu-Pro-Lys-Lys(Ac)-(7-amino-4-methyl-coumarin)**

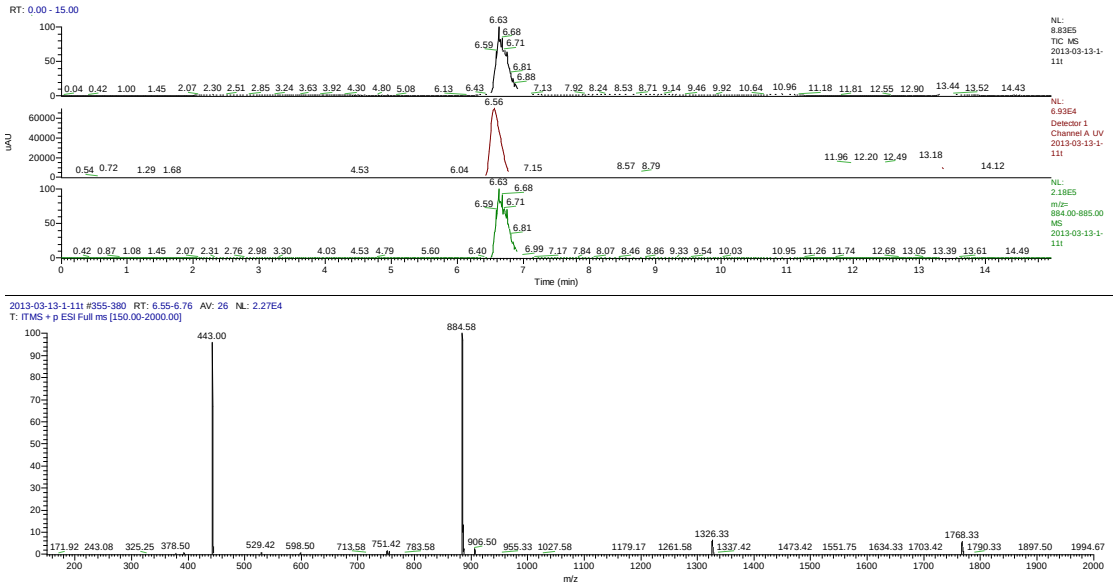


Figure S9. LCMS of **Ac-Glu-Ala-Leu-Pro-Lys-Lys-(7-amino-4-methyl-coumarin)**