Supporting Information

Thiomyristoyl Peptides as Cell Permeable Sirt6 Inhibitors ***

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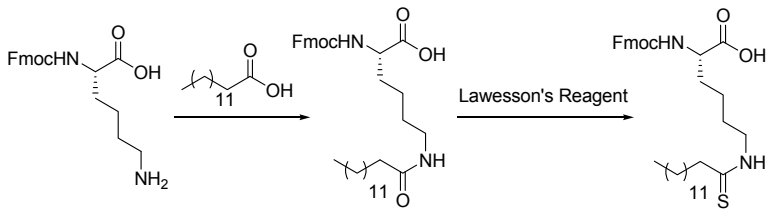
Table of Contents

Materials and methods

Reagents were obtained from Aldrich or Acros in the highest purity available and used as supplied. ^{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 280 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. Anti-Flag M2 affinity gel, anti-Flag M2 antibody conjugated with horseradish peroxidase, and Brefeldin A (BFA) were from Sigma. FuGene 6 transfection reagent was from Promega. Protease inhibitor cocktail was from Sigma. ECL plus western blotting detection reagent was from GE Healthcare. For N-terminal Flag-tagged TNFα, human full-length TNFα cDNA was inserted into pCMV-Tag 4A vector at EcoR V and Xho I sites.

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



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The synthesis of Fmoc-Lys(myristoyl)-OH (**2**) followed the previous published method[1] and a white solid was obtained as product (5.0 g, 85%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.77 (d, 2H, J = 8.0 Hz), 7.66 (t, 2H, J = 8.0 Hz), 7.33 (dt, 4H, J = 32 and 8.0 Hz), 4.35-4.30 (m, 2H), 4.21 (d, 1H, J = 8.0 Hz), 4.17-4.09 (m, 1H), 3.15 (t, 2H, J = 8.0 Hz), 2.13 (t, 2H, J = 8.0 Hz), 1.90-1.80 (m, 1H), 1.76-1.63 (m, 1H), 1.61-1.35 (m, 6H), 1.32-1.17 (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, 29.2, 29.0, 28.9, 28.5, 25.7, 22.9, 22.3, 13.0. LCMS (ESI) calcd. for C35H51N2O5 ($[M+H]^+$) 579.4, obsd. 579.3.

To a solution of Fmoc-Lys(myristoyl)-OH (**2**) (3.0 g, 5.18 mmol) in THF (100 ml) was added Lawesson's reagent (1.9 g, 4.66 mmol) and the resulting mixture was stirred at room temperature overnight. After removing THF using a rotary evaporator, the residue was purified using silica gel column chromatography with 50:1 CH_2Cl_2:CH_3OH to give compound **3** as a white solid (2.0 g, 64.8% yield). ^{1}H NMR (400 MHz, CD_3OD): δ 7.74 (d, 2H, J = 8.0 Hz), 7.63 (t, 2H, J =8.0 Hz), 7.31 (dt, 4H, J = 28 and 8.0 Hz), 4.31 (d, 2H, J = 8.0 Hz), 4.22-4.10 (m, 2H), 3.56 (t, 2H, J = 8.0), 2.55 (t, 2H, J =8.0), 1.95-1.80 (m, 1H), 1.73-1.60 (m, 5H), 1.49-1.35 (m, 2H), 1.29-1.19 (m, 20H), 0.86 (t, 3H, J = 8.0 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 205.0, 174.5, 157.2, 143.9, 143.7,

141.1, 127.4, 126.8, 124.9, 124.8, 119.6, 119.5, 66.6, 53.8, 45.7, 45.2, 31.7, 31.0, 29.4, 29.3, 29.1, 28.6, 26.8, 23.0, 22.4, 13.1. LCMS (ESI) calcd. for C35H51N2O4S $[M+H]^+$ 595.4, obsd. 595.4.

Solid-phase synthesis of thiomyristoyl peptides

Thiomyristoyl peptide was synthesized using standard Fmoc/tBu chemistry O-benzotriazol-N,N,N',N'-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazol (HBTU/HOBt) protocol. BHJH-TM1, BHJH-TM2, BHJH-TM3 and BH-TM4 were synthesized with Wang resin. JH-TM5 was synthesized with Rink resin. Modified lysine was incorporated using *L*-Fmoc-Lys(thiomyrsitoyl)-OH (**3**). Cleavage from the resin and removal of all protecting groups were done by incubating the resin with trifluoroacetic acid (TFA) containing phenol (5%), thioanisole (5%), ethanedithiol (2.5%), and water (5%) for 2 h. The crude peptide was 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). Flow rate was 10 ml/min. BHJH-TM1, BHJH-TM2, BHJH-TM3 and BH-TM4 were using the following gradient: 0% solvent B for 10 min, 0% to 50% solvent B over 50 min, then 50% to 95% solvent B for 5 min. JH-TM5 was purified using the following gradient: 0% solvent B for 10 min, 0% to 80% solvent B over 50 min, then 80% to 95% solvent B for 5 min. The identity and purity of the peptides were verified by LCMS.

BHJH-TM1 (NH_2-PKK(TMy)TG-OH) was eluted at 55 min. LCMS (ESI) calcd. for C37H70N7O7S $[M+H]^+$ 756.51, obsd. 756.58.

BHJH-TM2 (NH_2-PK(TMy)KTG-OH) was eluted at 55 min. LCMS (ESI) calcd. for C37H70N7O7S $[M+H]^+$ 756.51, obsd. 756.50.

BHJH-TM3 (NH_2-LPK(TMy)KT-OH) was eluted at 56 min. LCMS (ESI) calcd. for C41H78N7O7S $[M+H]^+$ 812.57, obsd. 812.58.

BH-TM4 (NH_2-ARK(TMy)ST-OH) was eluted at 56 min. LCMS (ESI) calcd. for C36H70N9O8S $[M+H]^+$ 788.51, obsd. 788.58.

JH-TM5 (Ac-GGK(TMy)G-NH_2) was eluted at 66 min. LCMS (ESI) calcd. for C28H53N6O5S $[M+H]^+$ 585.38, obsd. 585.50.

Cloning, expression and purification of human Sirtuins

Human Sirt6, Sirt1and Sirt3 were expressed as previously described[2, 3]. Human Sirt2 (38-356) was cloned and inserted into pET28a vector for the expression of N-terminal His6-SUMO fusion protein. Then Sirt2 expression vector was introduced into an *E. coli* BL21. Successful transformation were selected by plating the cells on kanamycin (50 μg mL^{-1}) and chloramphenicol (20 μg mL^{-1}) luria broth (LB) plates. Single colonies were selected and grown in LB with kanamycin (50 μg mL^{-1}) and chloramphenicol (20 μg mL^{-1}) overnight at 37 ℃. On the following day the cells were subcultured (1:1000 dilution) into 2 L of LB with kanamycin (50 μg

mL^{-1}) and chloramphenicol (20 μg mL^{-1}). The cells were induced with 20 μM of isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD_{600} of 0.6 and grown overnight at 15 ℃, 200 rpm. The cells were harvested by centrifugation at 8000 rpm for 5 min at 4 ℃ (Beckman Coulter refrigerated floor centrifuge) and passed through an EmulsiFlex-C3 cell disruptor (AVESTIN, Inc.) 3 times. Cellular debris was removed by centrifuging at 20,000 rpm for 30 min at 4 ℃ (Beckman Coulter). The supernatant was loaded onto a nickel column (Histrap, Ge Healthcare) pre-equilibrated with 20mM Tris-HCl pH 8.0 with 500mM NaCl. The protein was eluted with a linear gradient of imidazole (0-500mM). The desired fractions were pooled, concentrated and buffer exchanged. The His 6-SUMO tag of Sirt2 was removed by overnight incubation at 4 ℃ with ULP1, followed by Ni-affinity column purification to remove any undigested SirT2. The tag-free Sirt2 was further purified on a Superdex 75 column (Biorad). The protein was eluted with 20mM Tris-HCl, pH 8.0, 500mM NaCl. After concentration, the target protein was frozen at -80℃.

Inhibition assay for Sirt6

Sirt6 demyristoylation. When using the myristoyl H3K9 peptide as the substrate, different concentrations (0, 1.56, 3.13, 6.25, 12.5, 25, 50 μM) of thiomyristoyl peptides (BHJH-TM1, BHJH-TM2, BHJH-TM3, BH-TM4 and JH-TM5) or different concentration (0, 9.4, 18.8, 37.5, 75, 150, 300 μM) of commercial sirtuin inhibitors, were added to reaction mixtures containing 50 μM KQTAR(MyK)STGGWW, 0.5 mM NAD, 1 mM dithiothreitol (DTT), and 20 mM Tris-HCl pH 7.4. When using the myristoyl-K20 TNFα peptide as the substrate, different concentrations (0, 3.13, 6.25, 12.5, 25, 50 μM) of thiomyristoyl peptides (BHJH-TM1, BHJH-TM2, BHJH-TM3 and BH-TM4) were added to reaction mixtures containing 50 μM EALPK(MyK)TGGPQWW, 0.5 mM NAD, 1 mM DTT, and 20 mM Tris-HCl pH 7.4. Sirt6 (1 μM) was added to the reaction mixture to start the reactions at 37°C. The total reaction volumes were 30 μL and the reactions were allowed to proceed for 15 min at 37°C. The reactions were stopped by adding 90 μL of 200 mM HCl and 320 mM acetic acid in methanol. After centrifugation to remove precipitated proteins, the supernatant was analyzed by HPLC on a reverse phase C18 column (250 × 4.6 mm, 90 A, 10 μm, GraceVydac, Southborough, MA) with a linear gradient of 0% to 20% B for 10 min, 20% to 100% B for 5 min and then 100% for 5 min (0.5 mL/min). Product quantification was based on the area of absorption monitored at 280 nm; assuming hydrolysis of the acyl group does not affect the absorption. All reactions were duplicated.

Sirt6 deacetylation. Different concentrations (0, 1, 2.5, 5, 10, 20 and 40 μM) of thiomyristoyl peptide BH-TM4 and different concentrations (0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μM) of thioacetyl peptide ARK(TAc)ST or different concentration (0, 9.4, 18.8, 37.5, 75, 150, 300 μM) of AGK2, were added to reaction mixtures containing 0.05 mM KQTAR(AcK)STGGWW, 1 mM NAD, 1 mM DTT, and 20 mM Tris-HCl pH 7.4. Sirt6 (10 μM) was added to the reaction mixture to start the reactions at 37°C. The total reaction volumes were 30 μL and the reactions were allowed to proceed for 1 hr at 37°C. The reactions were stopped by adding

90 μL of 200 mM HCl and 320 mM acetic acid in methanol. After centrifugation to remove precipitated proteins, the supernatant was analyzed by HPLC on a reverse phase C18 column (250 × 4.6 mm, 90 A, 10 μm, GraceVydac, Southborough, MA) with a linear gradient of 0% to 20% B for 10 min (0.5 mL/min). Product quantification was based on the area of absorption monitored at 280 nm; assuming hydrolysis of the acyl group does not affect the absorption. All reactions were duplicated.

Inhibition Assay for Sirt1, Sirt2, and Sirt3.

The assay conditions were similar to that described above for Sirt6 with the following differences: KQTAR(AcK)STGGWW was used as the substrate, reaction time was 5 min (Sirt1 and Sirt2) or 10 min (Sirt3).

Sirt6 inhibition assay in mammalian cells.

HEK293T cells were cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum. The pCMV-Tag 4A vector containing TNFα gene was transfected into cells using FuGene 6 transfection reagent. Negative control was transfected with empty pCMV-Tag 4A vector. After transfection of TNFα into 293T cells for 12 h, cells were cultured in fresh medium containing 200 μM of BHJH-TM1, BHJH-TM2, BHJH-TM3, or BH-TM4 for 6 h. Then 50 μM of Alk14 (final concentration) and 4 μg/mL of bredeldin A (final concentration) were added into medium and the cells were cultured for another 6 h. For the dose response experiment, after transfection of TNFα into 293T cells for 12 h, different concentrations of BHJH-TM3 (5, 10, 50, 100 and 200 μM) were incubated with cells for 6 h, followed by incubation of Alk14 (50 μM) and bredeldin A (4 μg/mL) for another 6 h. Cells were collected and lysed using lysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol and 1 % Nonidet P-40) containing protease inhibitor cocktail (1:200 dilution). After centrifugation at 14,000 g for 10 min, the supernatant was collected for immunoprecipitation. 500 μg of total proteins were used and incubated with 20 μL suspension of anti-Flag M2 affinity gel at 4 ˚C for 2 h. The affinity gel was then washed three times with immunoprecipitation washing buffer (25 mM Tris pH 7.4, 150 mM NaCl, 0.2% Nonidet P-40). The gel was re-suspended in 18 μL of immunoprecipitation washing buffer.

Rhodamine-N3 (1 μL of 4.4 mM solution in DMF, final concentration was 200 μM), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (1 μL of 13.2 mM solution in DMF, final concentration was 600 μM), $CuSO_4$ (1 μL of 44 mM solution in H_2O, final concentration was 2 mM) and Tris(2-carboxyethyl)phosphine (1 μL of 44 mM solution in H_2O, final concentration was 2 mM) were added into the re-suspended mixture. The click chemistry reaction was allowed to proceed at room temperature for 1 h. Then protein loading buffer was added and heated at 95 ˚C for 10 min. After centrifugation at 14,000 g for 3 min, the supernatant was collected, treated with hydroxylamine (1 μL of 4.6 mM solution, pH 7.4, final concentration was 200 μM), and heated at 95 ˚C for 5 min.

For Western blot, 2 μL of the supernatant was used. Proteins were resolved by 12% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 5% BSA in TPBS (PBS solution containing

0.1% Tween-20). The anti-Flag M2 antibody was diluted with fresh 5% BSA in TPBS (a dilution of 1:5000) and then incubated with membrane for 1 h at room temperature. After washing three times by TPBS, the membrane was incubated with ECL plus western blotting detection reagents and then the chemiluminescence signal was recorded using Storm 860 Imager (Amersham Biosciences).

For in-gel fluorescence to detect the fatty acylation level, 20 μL of the supernatant was used. The proteins were resolved by 12% SDS-PAGE. Rhodamine fluorescence signal was recorded by Typhoon 9400 Variable Mode Imager (GE Healthcare Life Sciences) with PMT 500 V and normal sensitivity.

Reference:

1. J. Hu, B. He, S. Bhargava and H. Lin, *Org. Biomo. Chem.* 2013, **11**, 5213-5216.
2. J. Du, H. Jiang and H. Lin, *Biochemistry*, 2009, **48**, 2878-2890.
3. 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.

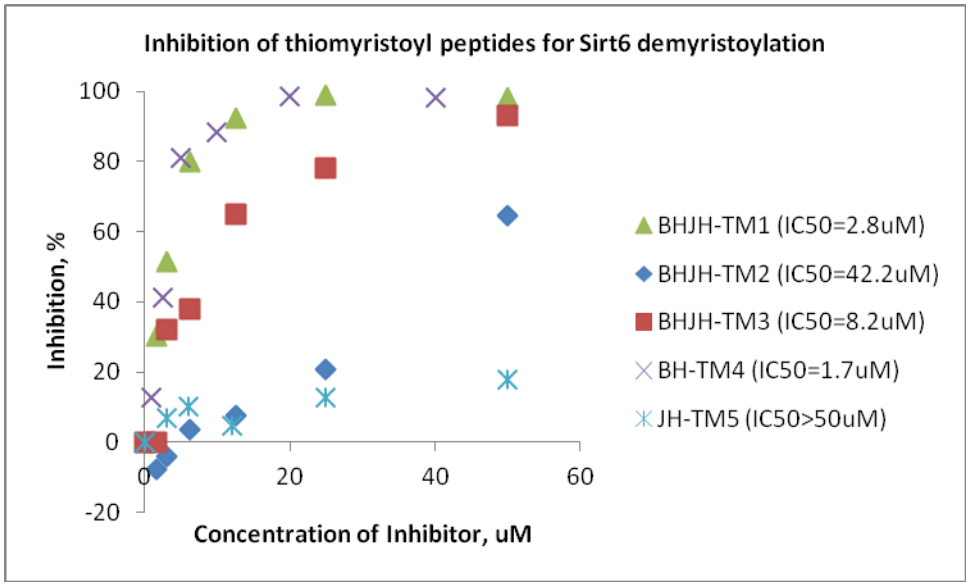

Figure S1. Inhibition curves of thiomyristoyl peptides on Sirt6's demyristoylase activity using Myristoyl H3K9 peptide as substrate.

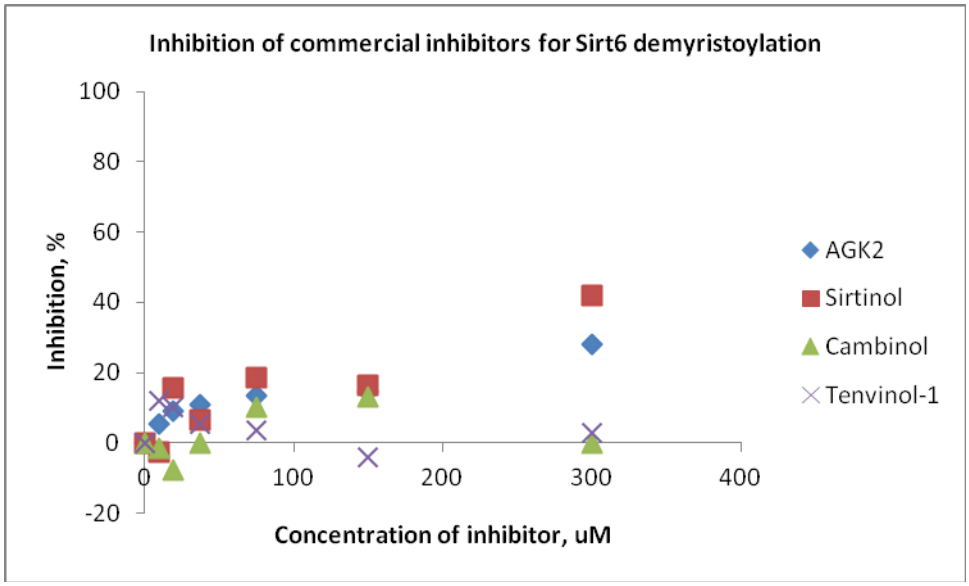

Figure S2. Inhibition curves of commercial inhibitors on Sirt6's demyristoylase activity.

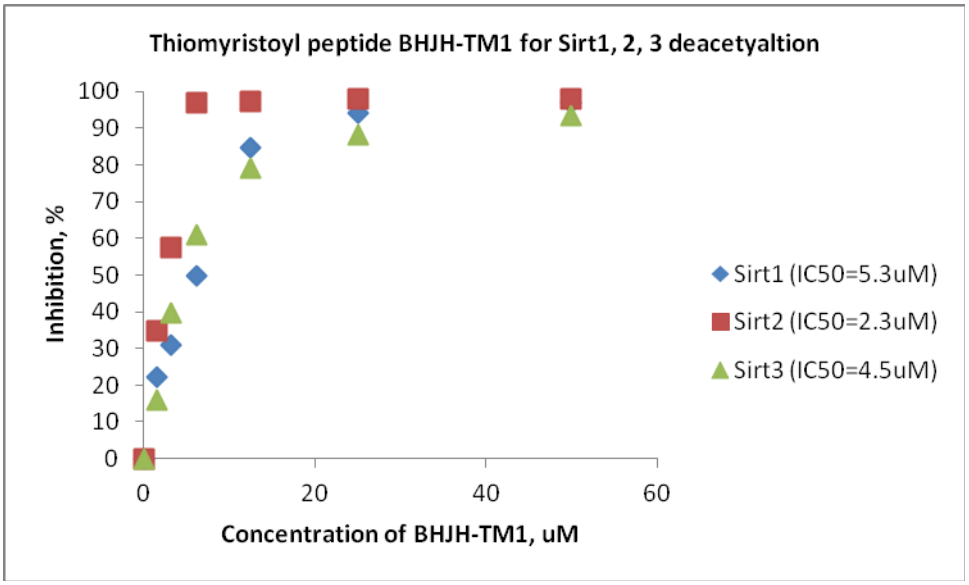

Figure S3. Inhibition curves of BHJH-TM1 on the deacetylase activities of Sirt1, Sirt2, and Sirt3.

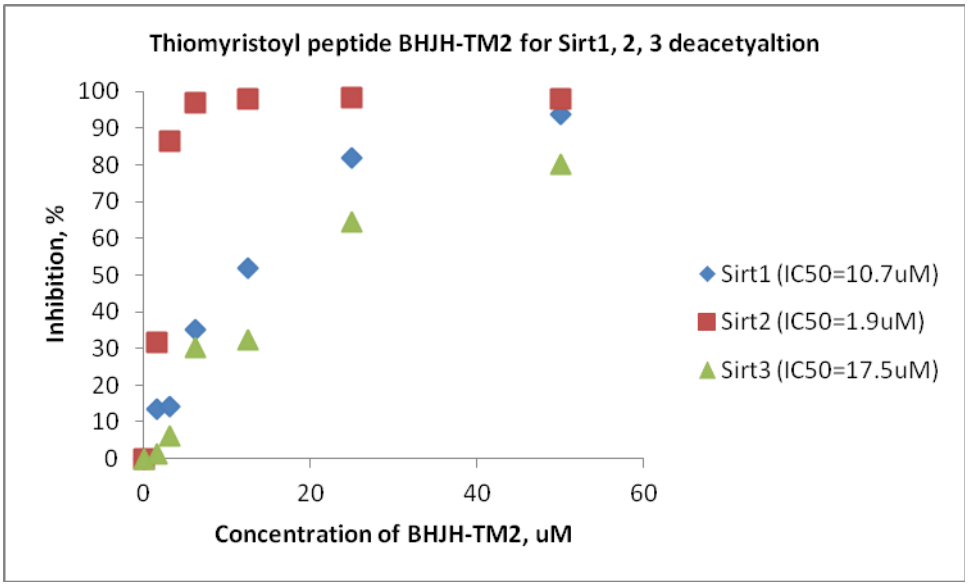

Figure S4. Inhibition curves of BHJH-TM2 on the deacetylase activities of Sirt1, Sirt2, and Sirt3.

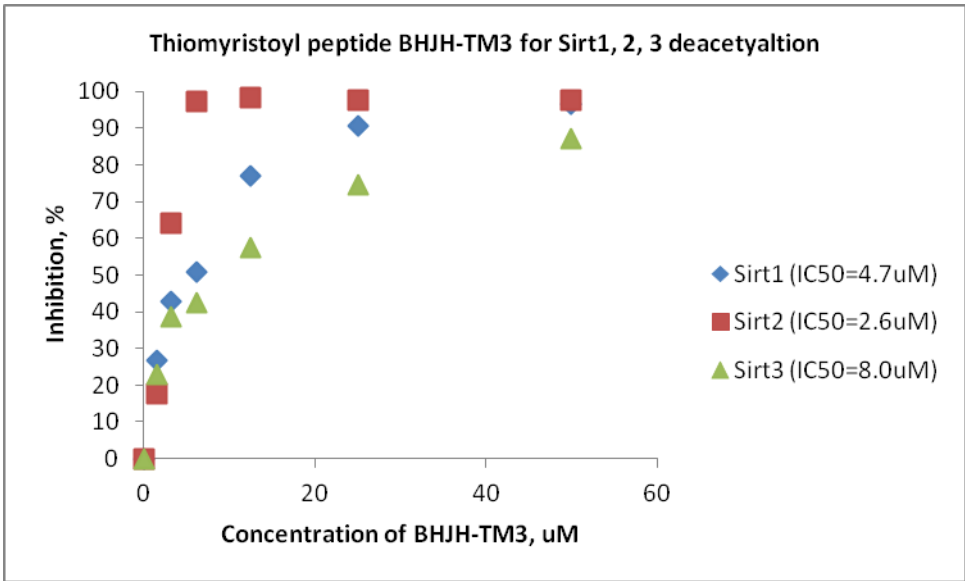

Figure S5. Inhibition curves of BHJH-TM3 on the deacetylase activities of Sirt1, Sirt2, and Sirt3.

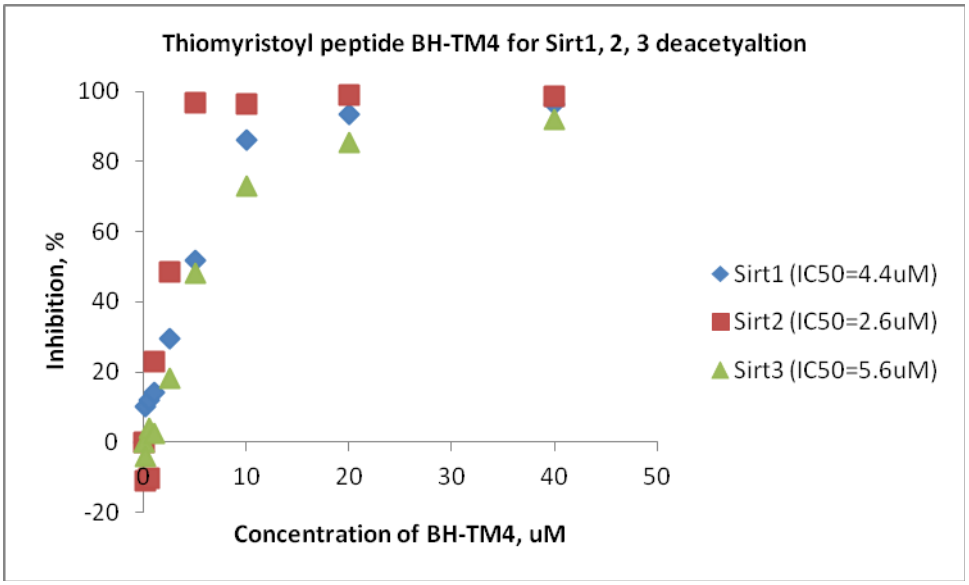

Figure S6. Inhibition curves of BH-TM4 on the deacetylase activities of Sirt1, Sirt2, and Sirt3.

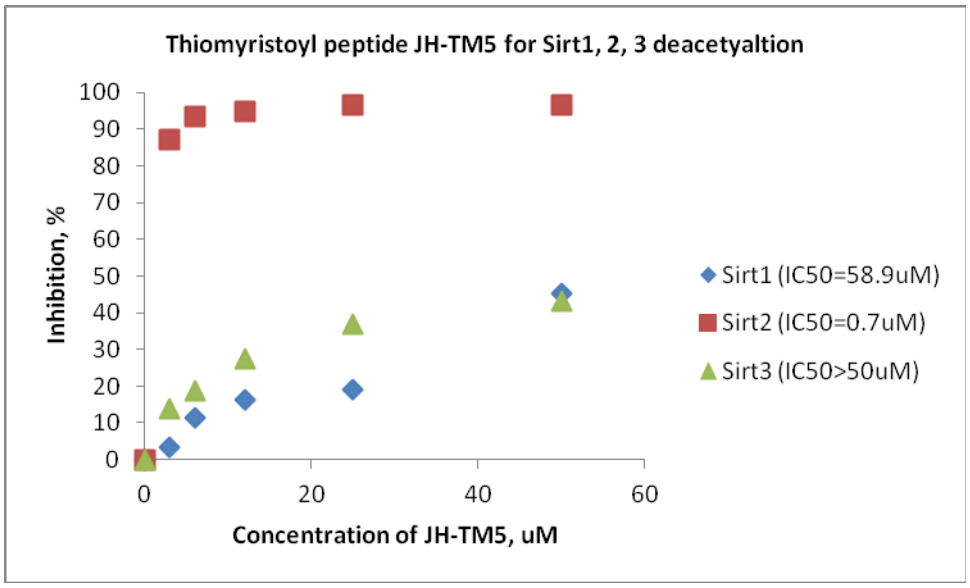

Figure S7. Inhibition curves of JH-TM5 on the deacetylase activities of Sirt1, Sirt2, and Sirt3.

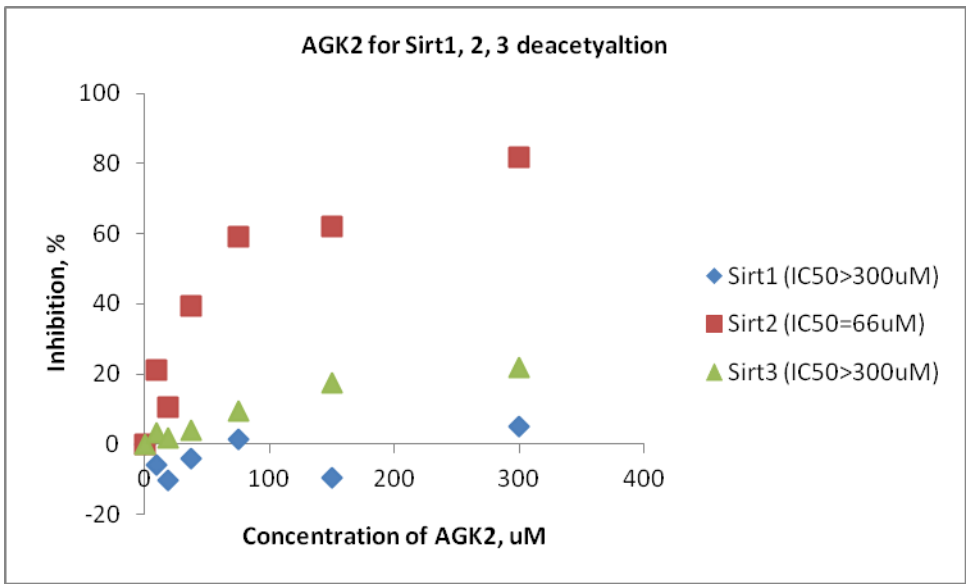

Figure S8. Inhibition curves of AGK2 on the deacetylase activities of Sirt1, Sirt2, and Sirt3.

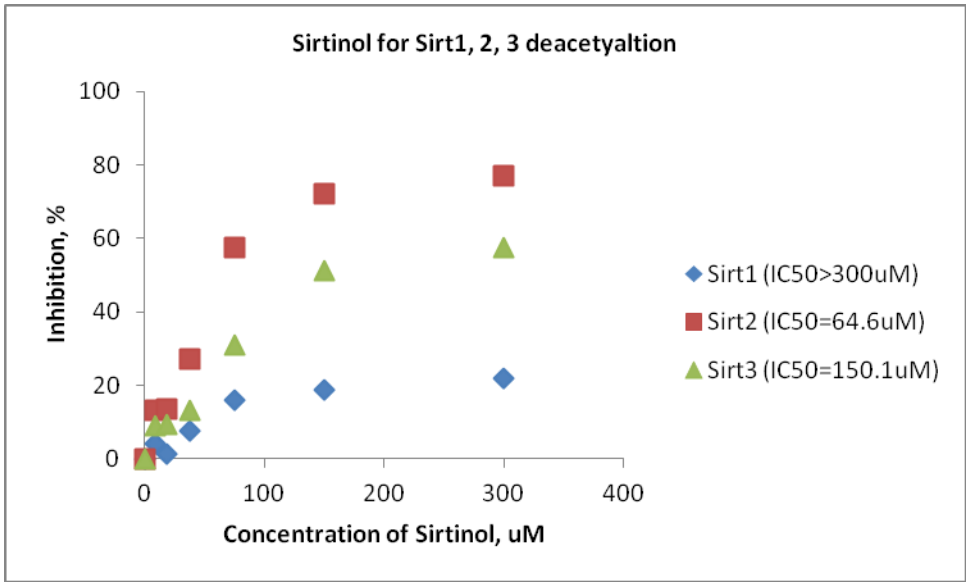

Figure S9. Inhibition curves of Sirtinol on the deacetylase activities of Sirt1, Sirt2, and Sirt3.

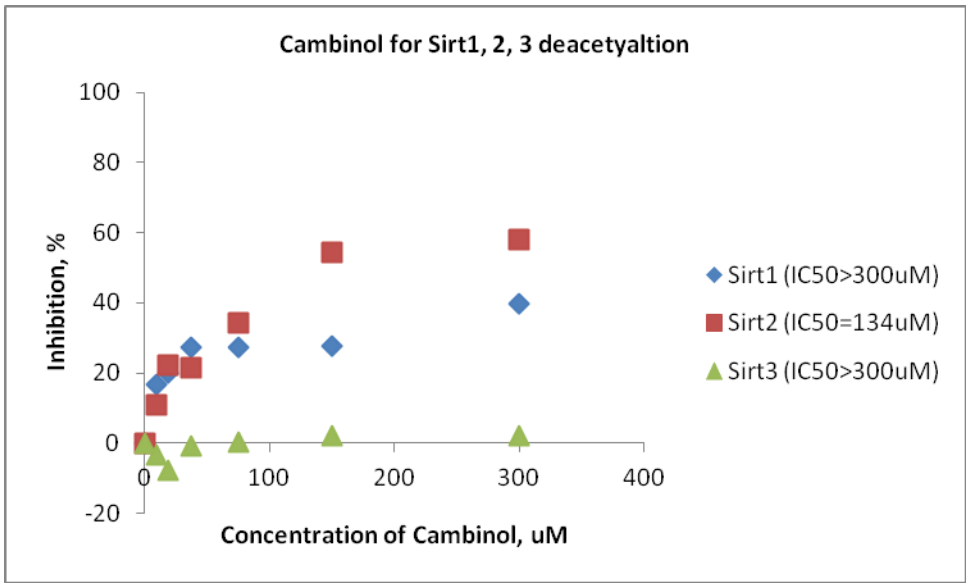

Figure S10. Inhibition curves of Cambinol on the deacetylase activities of Sirt1, Sirt2, and Sirt3.

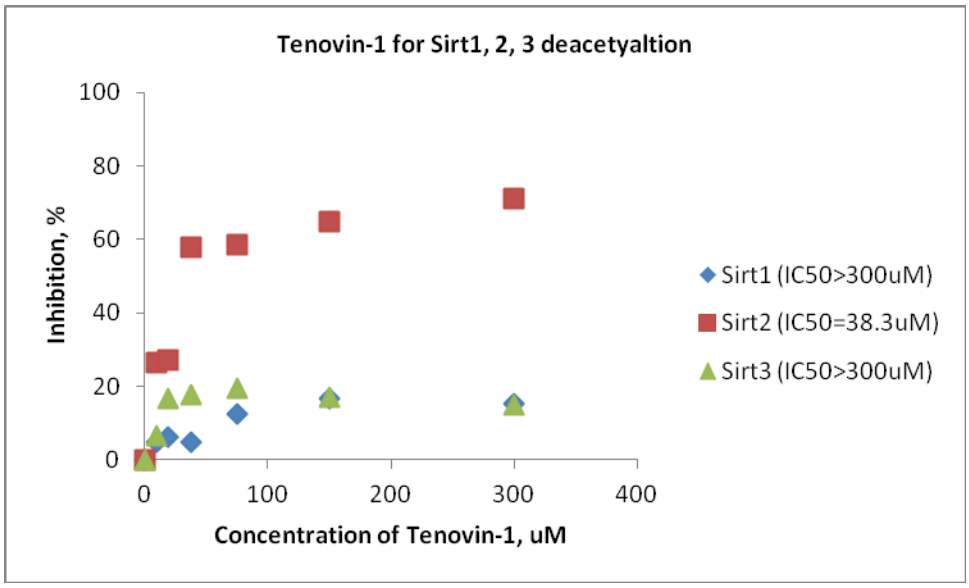

Figure S11. Inhibition curves of Tenovin-1 on the deacetylase activities of Sirt1, Sirt2, and Sirt3

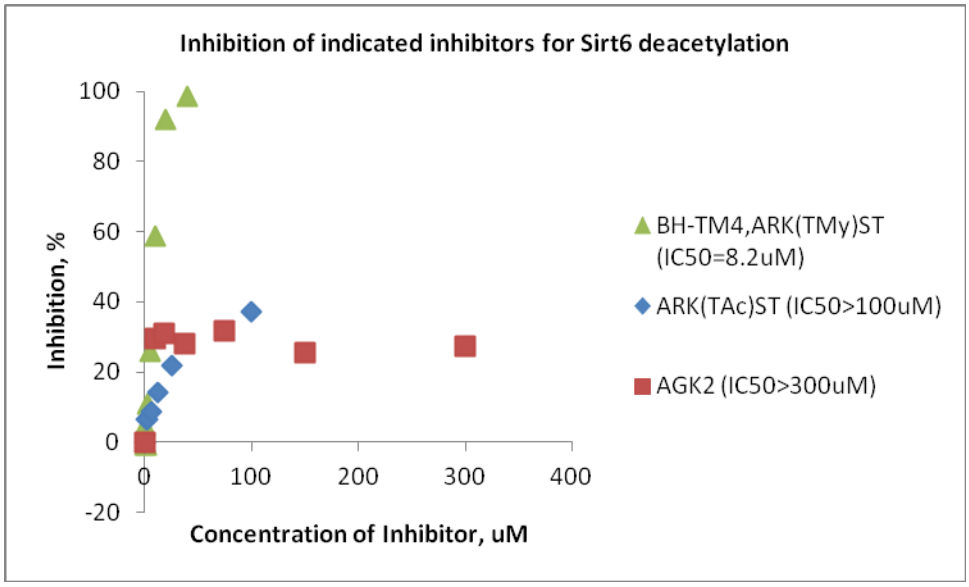

Figure S12. Inhibition curves of BH-TM4, ARK(TMy)ST, and AGK2 on the deacetylase activity of Sirt6., BH-TM4 concentrations used in this assay were 0, 1, 2.5, 5, 10, 20 and 40 μM. ARK(TAc)ST concentrations used were 0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μM. AGK2 concentrations used were 0, 9.4, 18.8, 37.5, 75, 150 and 300 μM. The experiments were performed in duplicate.

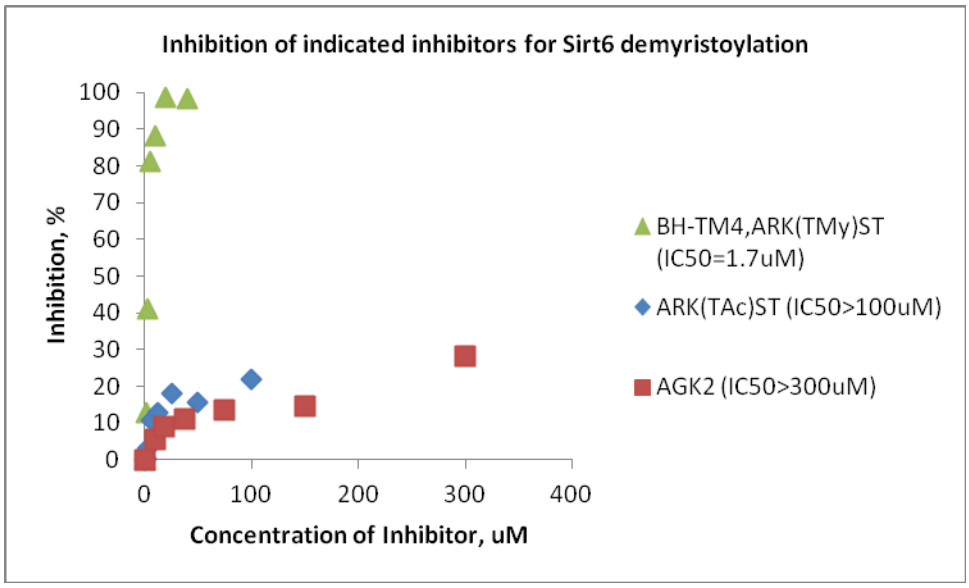

Figure S13. Inhibition curves of BH-TM4, ARK(TAc)ST, and AGK2 on the demyristoylase activity of Sirt6. BH-TM4 concentrations used in this assay were 0, 1, 2.5, 5, 10, 20 and 40 μM. ARK(TAc)ST concentrations used were 0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μM. AGK2 concentrations used were 0, 9.4, 18.8, 37.5, 75, 150 and 300 μM. The experiments were performed in duplicate.

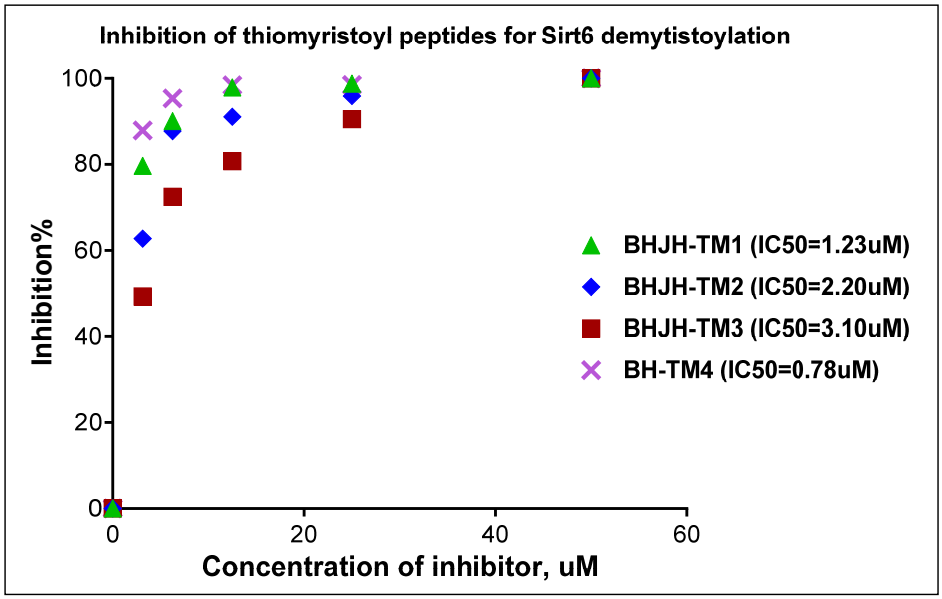

Figure S14. Inhibition curves of thiomyristoyl peptides on Sirt6's demyristoylase activity using Myristoyl-K20 TNFα peptide as substrate.

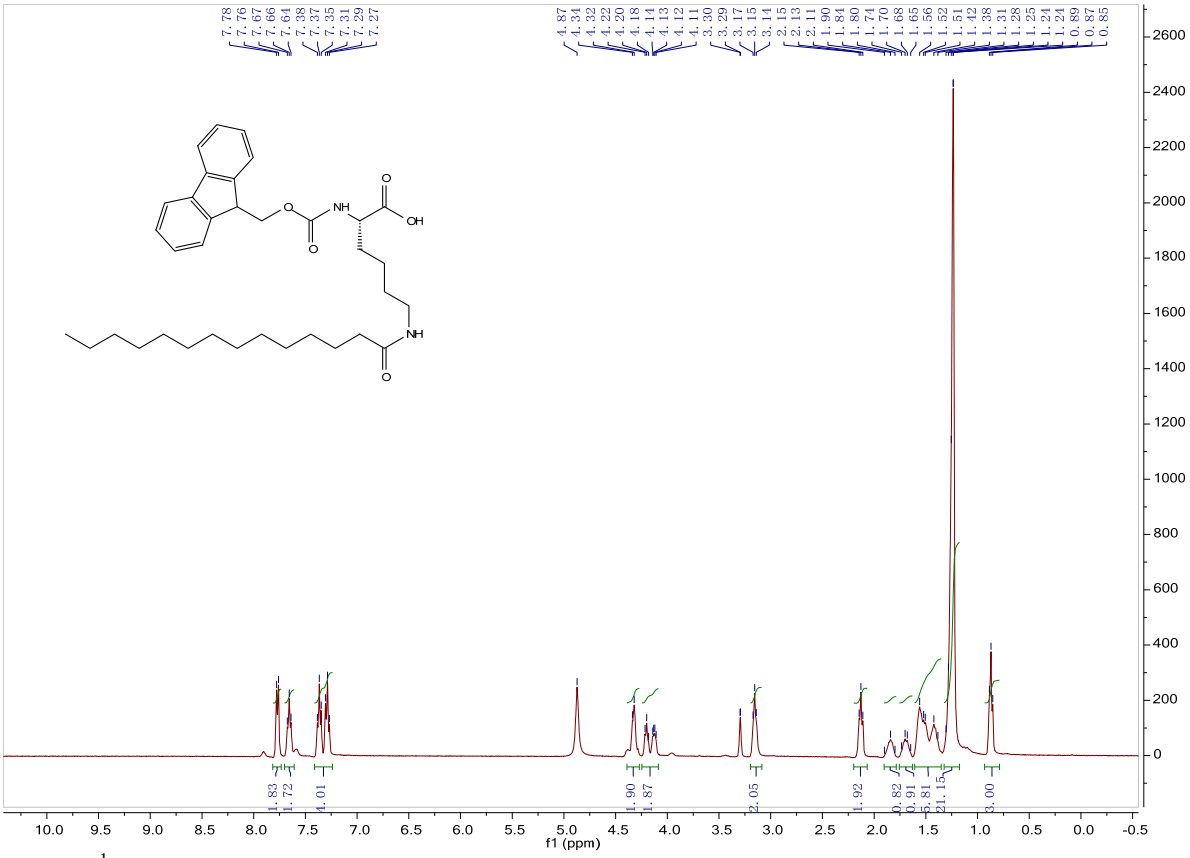

Figure S15. ^{1}H NMR spectrum of Fmoc-Lys(myristoyl)-OH (**2**)

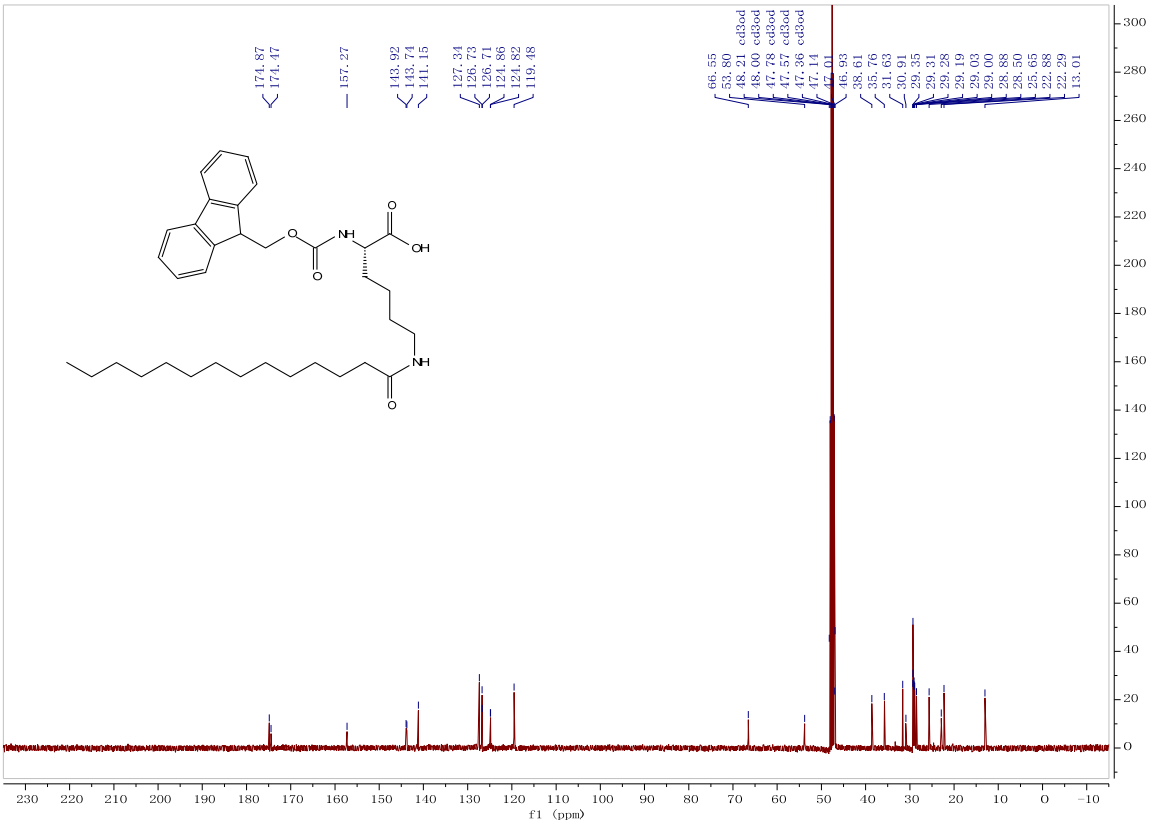

Figure S16. ^{13}C NMR spectrum of Fmoc-Lys(myristoyl)-OH (**2**)

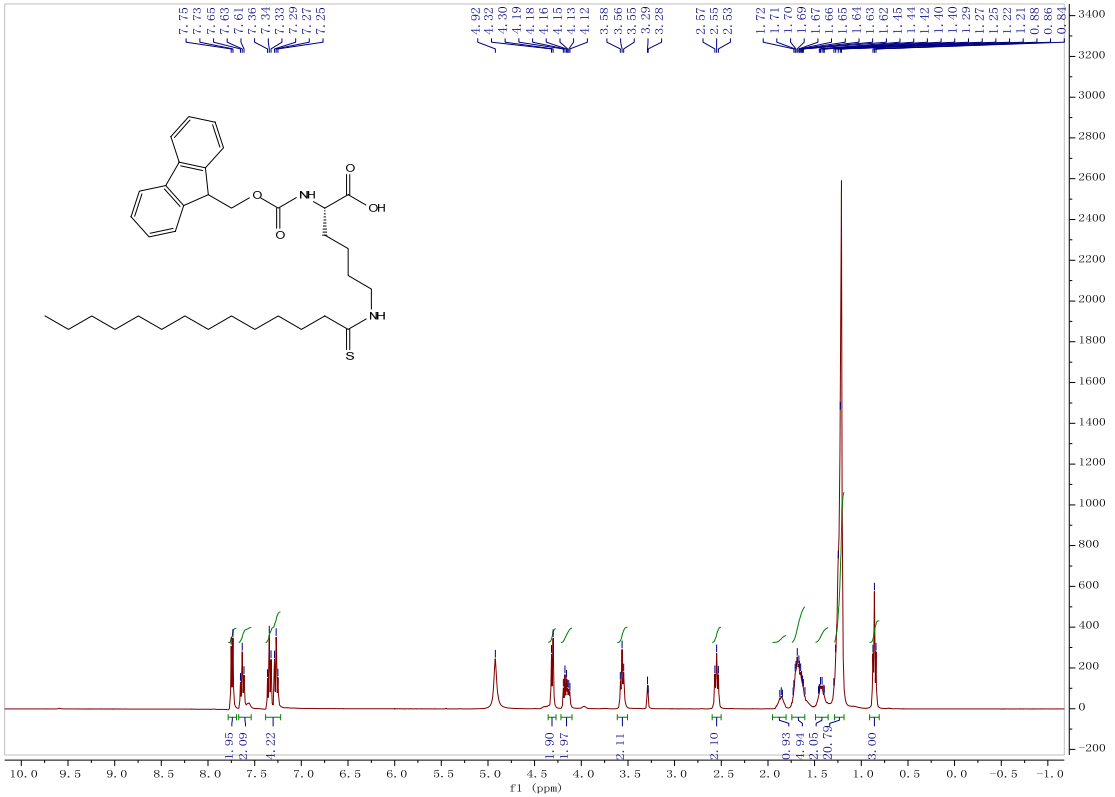

Figure S17. ^{1}H NMR spectrum of Fmoc-Lys(thiomyristoyl)-OH (**3**)

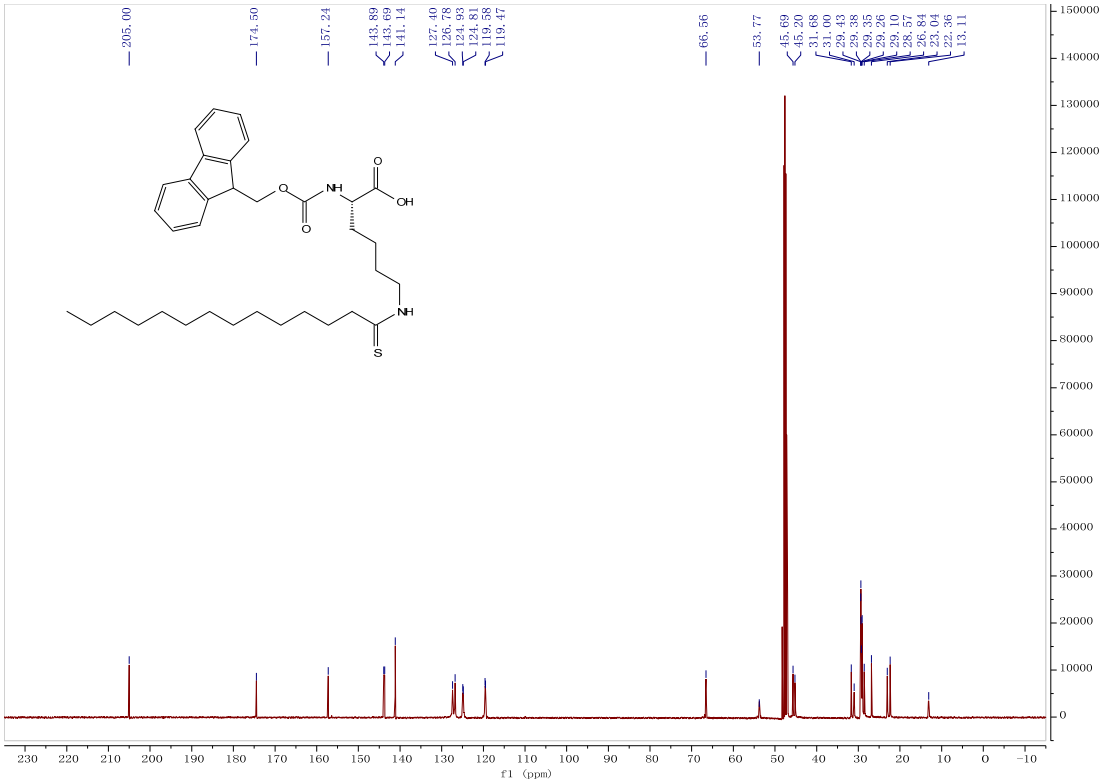

Figure S18. ^{13}C NMR spectrum of Fmoc-Lys(thiomyristoyl)-OH (**3**)

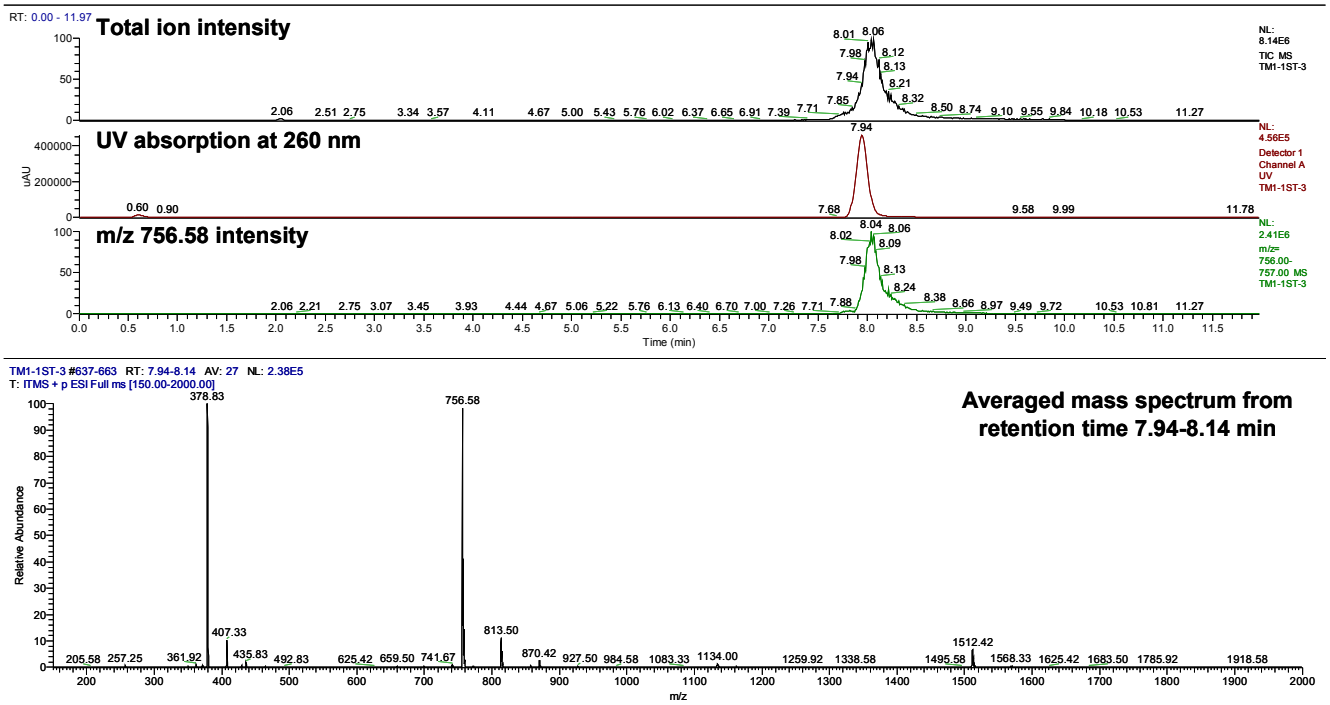

Figure S19. LC-MS of BHJH-TM1, NH_2-PKK(TMy)TG-OH peptide. *m/z* calcd. for $C_{37}H_{70}N_7O_7S$ $[M+H]^+$ 756.51, obsd. 756.58.

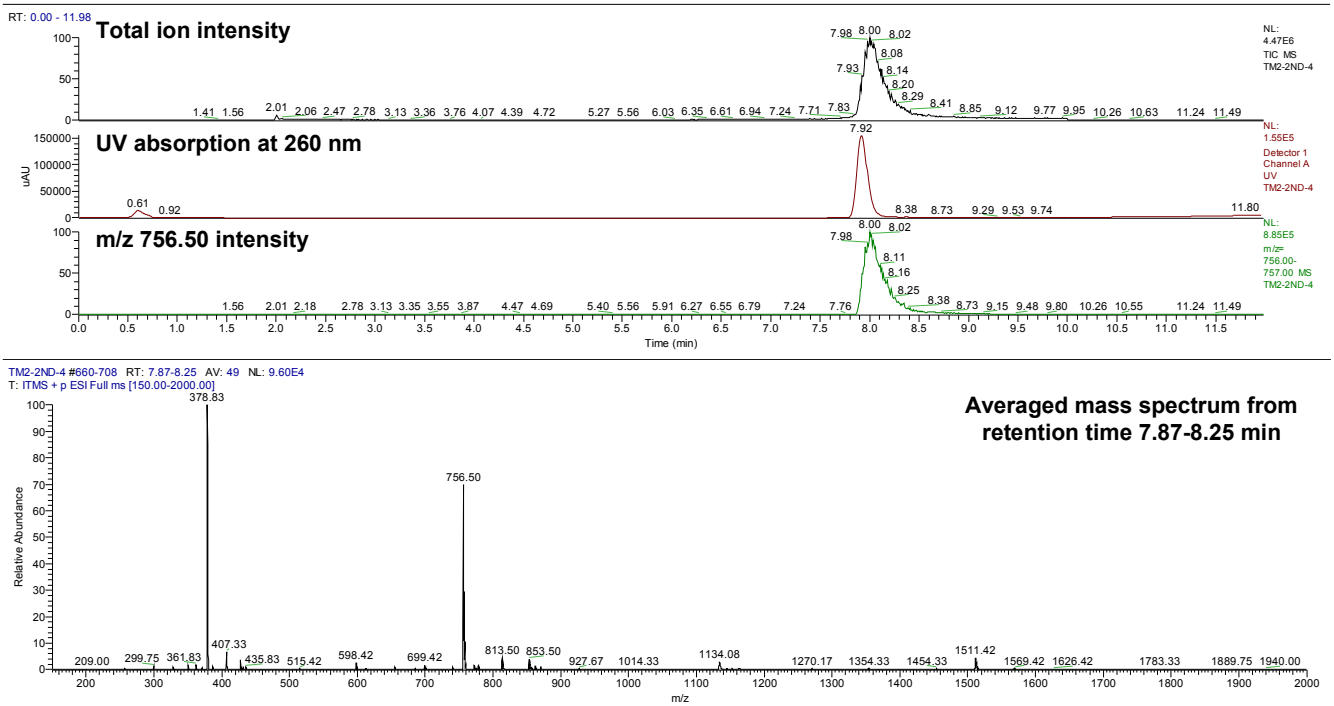

Figure S20. LCMS of BHJH-TM2, NH_2-PK(TMy)KTG-OH. *m/z* calcd. for $C_{37}H_{70}N_7O_7S$ $[M+H]^+$ 756.51, obsd. 756.50.

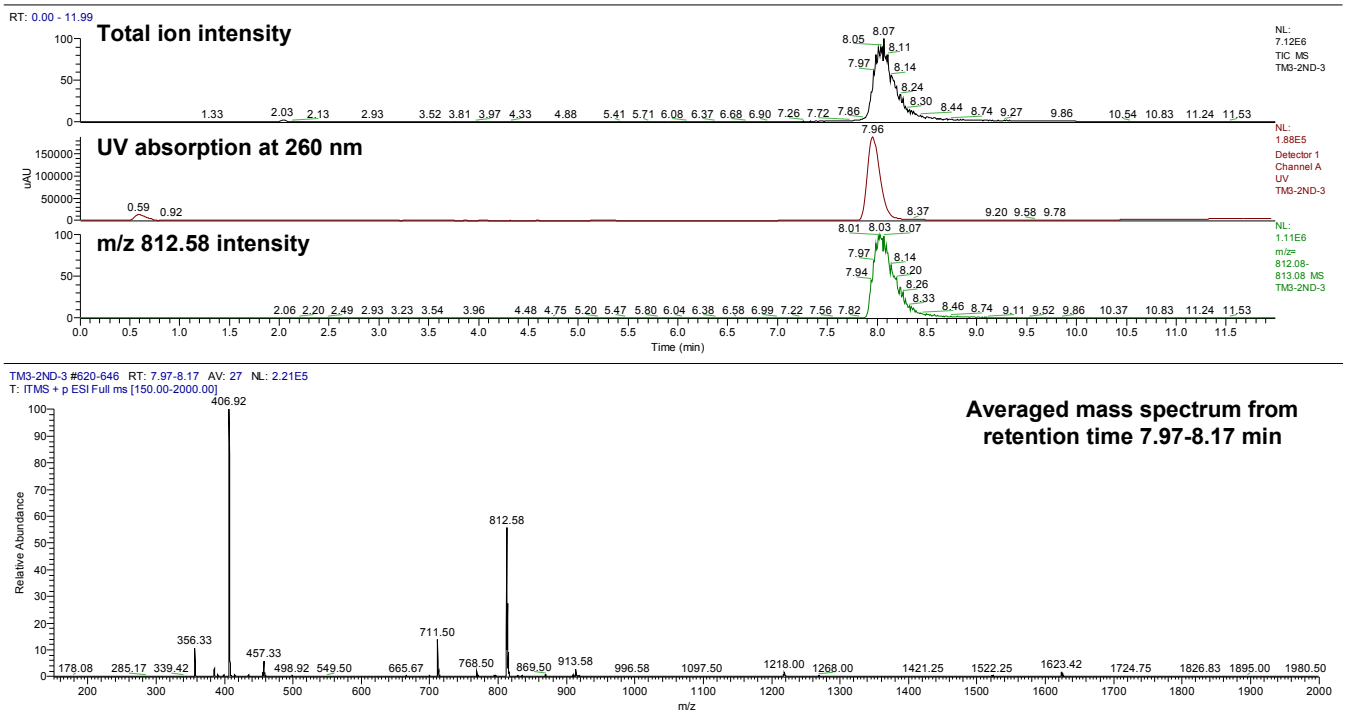

Figure S21. LCMS of BHJH-TM3, NH_2-LPK(TMy)KT-OH. *m/z* calcd. for $C_{41}H_{78}N_7O_7S$ $[M+H]^+$ 812.57, obsd. 812.58.

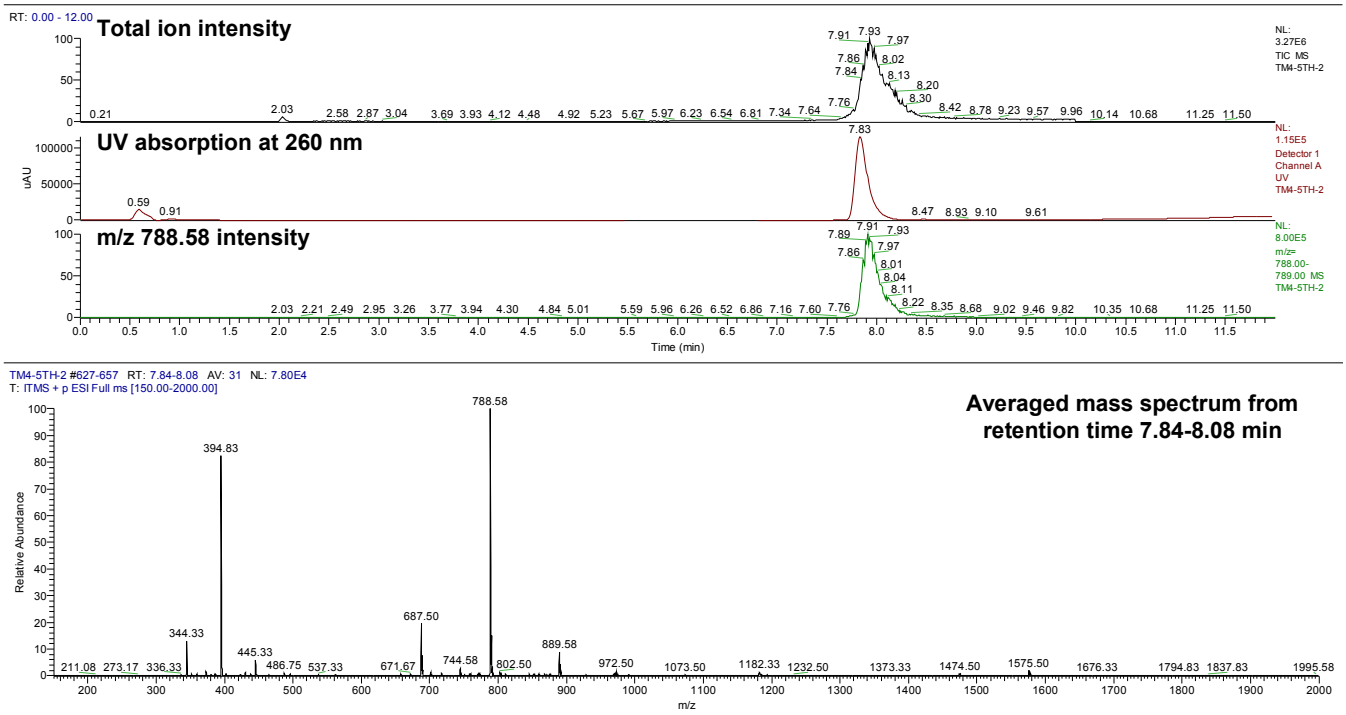

Figure S22. LCMS of BH-TM4, NH_2-ARK(TMy)ST-OH. *m/z* calcd. for $C_{36}H_{70}N_9O_8S$ $[M+H]^+$ 788.51, obsd. 788.58.

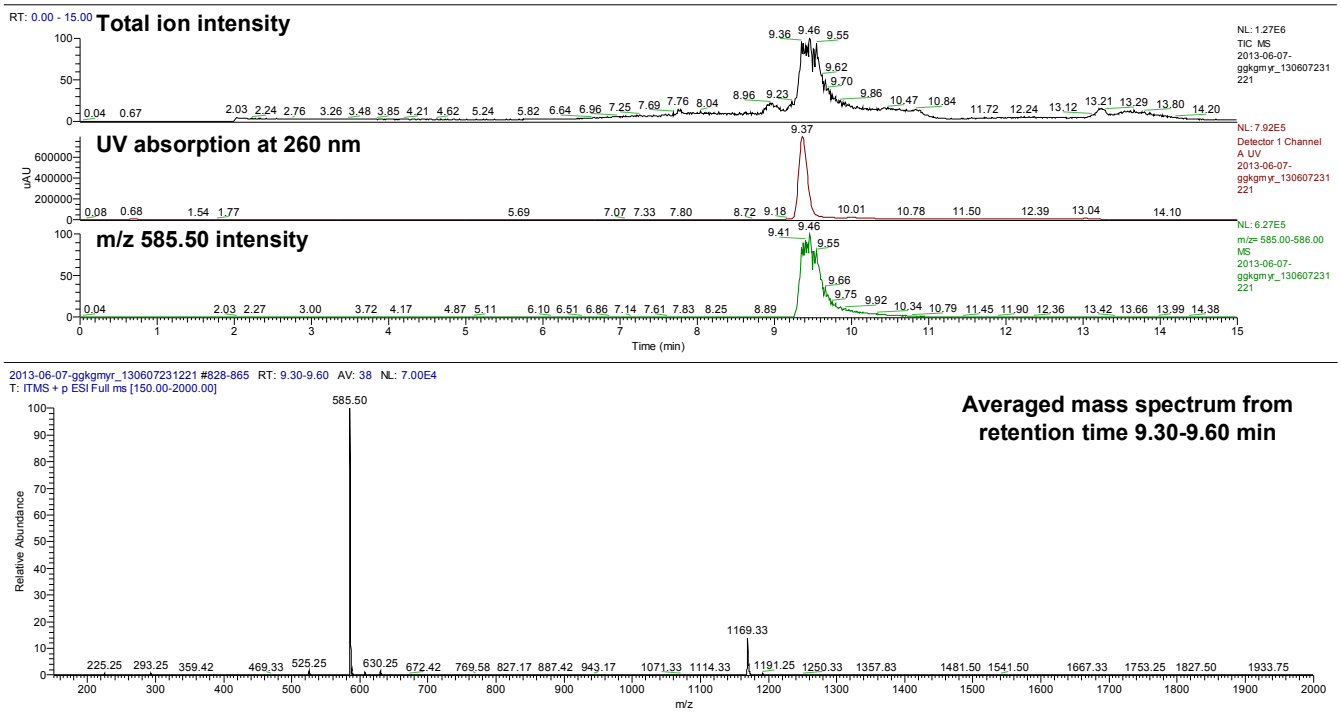

Figure S23. LCMS of JH-TM5, Ac-GGK(TMy)G-NH_2. *m/z* calcd. for $C_{28}H_{53}N_6O_5S$ $[M+H]^+$ 585.38, obsd. 585.50.