Expedient total synthesis of small to medium-sized membrane proteins via Fmoc chemistry

Ji-Shen Zheng[a], Mu Yu[b], Yun-Kun Qi[a], Shan Tang[a], Fei Shen[a], Zhi-Peng Wang[a], Liang Xiao[b], Long-hua Zhang[b], Chang-Lin Tian[b,*], Lei Liu[a,*]

Email: lliu@mail.tsinghua.edu.cn; cltian@ustc.edu.cn

a. *Tsinghua-Peking Center for Life Sciences, Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology (Ministry of Education), Department of Chemistry, Tsinghua University, Beijing 100084, China*

b. *High Magnetic Field Laboratory, Chinese Academy of Sciences, and School of Life Sciences, University of Science and Technology of China, Hefei 230026, China*

Supplementary Information

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Supplementary Methods

Peptide synthesis

C S Bio Co. automated synthesizer. Use a CS136XT synthesizer running with a scale of 0.1 mmol. A general protocol as follow: 2 × DMF, 30 s; 1 × 20% (vol/vol) piperidine in DMF, 5 min; 1 × 20% (vol/vol) piperidine in DMF, 20 min; 2 × DMF, 30 s; 2 × DCM, 30 s; 3 × DMF, 30 s; amino acids (AA) activation by HBTU or HCTU in the presence of DIEA in DMF for ~0.5–1 min; Add AA solution to resin, couple 0.5–1 h; 2 × DMF, 30 s; 1 × DCM, 30 s.

Manual peptide-synthesis apparatus. Peptide synthesis vessels were purchased from Synthware Glass Co., Ltd.. Fmoc amino acids, DIEA, HOBt, HBTU, HCTU, HATU, HOAt, DIC, Boc-Met-OH, Boc-Cys(Trt)-OH, Rink amide-AM resin and 2-Cl-(Trt)-Cl resin were from GL Biochem (Shanghai) Ltd. NovaPEG Wang resin was used for the synthesis of peptide acid. The Side-chain AAs were Arg(Pbf), Asn(Trt), Asp(O^{t}Bu), Cys(Trt), His(Trt), Glu(O^{t}Bu), Gln(Trt), Lys(Boc), Ser(^{t}Bu), Thr(^{t}Bu), Trp(Boc), and Tyr(^{t}Bu). Choose the proper coupling conditions for each amino acid, including the number of coupling reactions (e.g., single or double coupling), reaction time (e.g., 60–90 min) and the type of coupling reagent. A single coupling reaction using HBTU or HCTU and a 30-45 min reaction is enough for most amino acids. However, a double coupling strategy is needed for sterically hindered amino acids (e.g., Fmoc-Ile-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH), especially when the peptide is assembled beyond 20 amino acids or transmembrane peptide. Double coupling was used when necessary. It is necessary to perform a double or three coupling step for amino acids after Pro or Gly^{RBM0} residue. Piperidine (20% in DMF) was added to the resin for 15-25 min (twice: 5-10 min and 10-15 min) to remove the Fmoc protecting group. The deprotection of Side chain protecting groups and final cleavage from the resin were achieved by TFA cocktails for 3-4 h at room temperature (rt). The cleavage cocktail (85% TFA, 5% thioanisole, 5% EDT, 2.5% phenol and 2.5% H_2O) or (95%TFA, 2.5%

TIPS, 2.5% H_2O) could be used. The combined solutions were concentrated by blowing with N_2. The crude peptides were obtained by precipitation with cold ether and centrifugation. The crude peptides were dissolved in CH_3CN/H_2O and purified by semi- RP-HPLC. Peptide identity was confirmed by MALDI-TOF/MS or ESI-MS.

Reversed-Phase HPLC (RP-HPLC). Analytical and semi-preparative RP-HPLC were performed with a Prominence LC-20AT with SPD-20A UV/Vis detector. A Vydac C4 column (5 μm, 4.6 mm×150 mm, 4.6 mm×250 mm) with a 1 mL/min flow rate was used for analytical RP-HPLC, and a Vydac C4 column (10 μm, 10×250 mm10 or 22 mm×150 mm) with a 3-6mL/min flow rate was used for semi-preparative RP-HPLC with different buffers: buffer A (0.1% TFA in water), buffer B (0.1% TFA in CH_3CN) and buffer C (0.1% TFA in the mixed solvents iPrOH-CH_3CN-water (v/v/v, 6:3:1)). Gradients and temperature for all peptides were detailed below. Data were recorded and analyzed using the software system LC Solution.

Mass Spectrometry (MS). Product containing fractions were identified by ESI-MS (electrospray ionization mass spectrometry) or MALDI-TOF-MS (matrix-assisted laser desorption ionization-time of flight mass spectrometer). ESI-MS was performed on a Agilent 1200/6340 mass spectrometer in Center of Biomedical Analysis or a Bruker Daltonics Inc. APEX IV FT-ICR Mass Spectrometer in Analytical Instrumentation Center (Tsinghua University). The buffers for LC/MS analysis were the same but using formic acid instead of TFA to increase MS ionization of peptides. MALDI-TOF/MS was performed on a Bruker Daltonics Inc. autoflex I mass spectrometer or an Applied Biosystems 4800PLUS mass spectrometer in Center of Biomedical Analysis, Tsinghua University. The matrix used for MALDI-TOF was α-cyano-4-hydroxycinnamic acid.

Solubility of peptides FITC-based fluorophores. Solubility of peptides was determined by quantifying the amount of FITC fluorophore via UV/Vis spectrophotometry. Absorbance measurements of FITC-labeled peptides were recorded on a UV-2100 spectrometer in a quartz cell with 1 mm path length at 25°C.

Circular dichroism (CD) spectroscopy. CD spectra were recorded on a Pistar π-180 spectrometer from 260 nm to 195 nm at 25°C in a quartz cell with 1 mm path length. The final concentration of samples was about 10 μM. The spectra for each peptide was performed in triplicate, averaged, subtracted from blank and smoothed.

Chemical ligation using peptide hydrazides as thioester surrogates. Peptide hydrazides were dissolved using 0.2 M phosphate solution containing 6 M Gn•HCl (pH 3.0-3.1) in a 2-ml Eppendorf reaction tube and then the reaction tube was placed in a -15 ^{o}C ice–salt bath, and gently agitate solution by magnetic stirring. 0.5 M $NaNO_2$ (10-15 equiv) were pipetted and the solution was gently agitated for 15 min at -15 ^{o}C to oxidize the peptide hydrazide to the azide. Subsequently, the phosphate solution containing MPAA (4-mercaptophenylacetic acid, ~100 equiv) and N-terminal Cys-peptide (0.7-1.6 equiv) was added to the reaction mixture to convert the peptide azide to the thioester for native chemical ligation. The tube was removed from the ice-salt bath and warmed to rt. The pH of the ligation reaction at the pH to 6.5-6.8 was monitored using micro pH probe. The ligation was monitored by analytical HPLC and ESI-MS. After ligation completed, 0.1 M neutral TCEP solution (~100 equiv) was added for 20 min to reduce the ligation system.

Reconstituted in Unilamellar Liposome Vesicles. All lipids used were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The chemical synthesized protein samples (Kir5.1[64-179] as an example, ~20 μg) was centrifuged at 14,000 rpm for 10 minutes to recover the sample that sticks to the tube wall. 200 μl reconstitution buffer (0.1% SDS, 20 mM Tris, 200 mM NaCl, pH 8.0) was added into the tube. Sample was completely dissolved and uniformly dispersed using pipettes. The lipids of 1-palmitoyl-2-oleoyl-sn-glycero- 3-phosphocholine (POPC, 1.5 mg/ml) and 1-palmitoyl-2-oleoyl-sn-glycero-3- phosphor-(1'-rac-glycerol) (sodium salt) (POPG, 0.5 mg/ml) were mixed and well dispersed in reconstitution buffer (0.1% SDS, 20 mM Tris, 200 mM NaCl, pH 8.0) through 5 cycles of liquid nitrogen freeze and thawing at rt. The protein solution was added into the liposome solution to a final molar ratio of 1/600 (protein/lipid). The mixture was rotated at 4 °C for 1 hour, and

dialyzed for 3 days at 25 °C in Tris buffer (20 mM Tris, 200 mM NaCl, pH 8.0) to completely remove the detergent. To prepare unilamellar liposome vesicles, samples were extruded using a 400 nm polycarbonate membrane by the Avanti Mini-Extruder (Alabaster, AL). Final concentration of protein sample was ~40 μg/ml.

Single Channel Conductance Measurement in Planar Lipid Bilayer. Channel conductance measurements in planar lipid bilayer were conducted using Ionovation Compact (Osnabrück, Germany). Two polycarbonate compartments in volume of 1.2 ml were separated by a TEFLON foil with 25 μm thickness and 50-100 μm aperture diameter. The pre-mixed liposome (POPC/POPG= 3/1) was employed to paint the aperture. Planar lipid bilayer formation was monitored optically or by capacitance measurements. After successful formation of a stable bilayer in the aperture, proteoliposomes (protein sample in POPC/POPG) were added to the cis chamber next to the bilayer. Fusion of protein and planar lipid bilayer was detected through observation of channel conductance. Working solution (5 mM HEPES, 150 mM KCl, pH 8.0) were present in both cis- and trans- chambers.

Different DC voltages were used to measure channel conductance using an EPC-10 amplifier (HEKA Elektronik). Currents were measured with a 2 kHz low-pass filter at 10 kHz sampling rate. Data was analyzed using the pCLAMP 10.0 software (Axon Instruments). In each measurement at various voltages, current amplitudes were recorded referring to a pre-defined baseline, based on 50% threshold crossing methods. Current-voltage (I-V) relationship was plotted with the measured current amplitudes against different applied voltages. The I-V plot was regressed to a linear line with the slope representing channel conductance of the membrane protein in planar lipid bilayer.

Chemical Syntheses

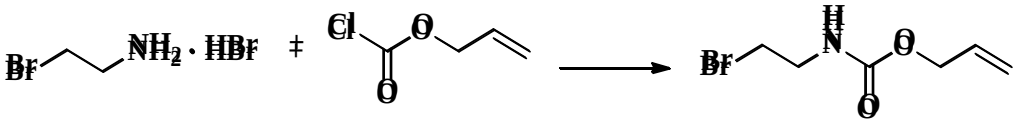
General Methods

All reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd or Alfa Aesar and were purified if necessary. The composition of mixed solvents was given by the volume ratio (v/v). Thin-layer chromatography (TLC) was performed on silica 60F-254 plates. The spots were visualized by UV light, ninhydrin solution, iodine vapors or potassium permanganate staining. Flash column chromatography was carried out on silica gel 60 (300-400 mesh). ^{1}H- and ^{13}C-NMR spectra were recorded on a JOEL 300 or 400 MHz instrument at rt in $CDCl_3$ unless otherwise indicated. Chemical shifts (δ) were reported relative to TMS (^{1}H 0 ppm) or $CDCl_3$ (^{1}H 7.26 ppm) for ^{1}H-NMR and $CDCl_3$ (77.0 ppm) or d6-DMSO (39.5 ppm) for ^{13}C-NMR spectra. *J* were given in Hertz (Hz), and the splitting patterns were designed as follows: s, singlet; s, br, broad singlet; d, doublet; t, triplet; m, multiplet. ESI-MS was carried out with a Bruker Daltonics Inc. APEX II Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer.

Part I: Synthesis of Fmoc-Gly^{RMB0}-OH

To apply the new RBM strategy for SPPS, we synthesized the building block Fmoc-Gly^{RMB0}-OH **S5**. As shown in **Supplementary Scheme 1**, **S5** was obtained through five synthetic steps from commercial 2,4-dihydroxybenzaldehyde. The procedures were detailed below.

Allyl (2-bromoethyl)carbamate

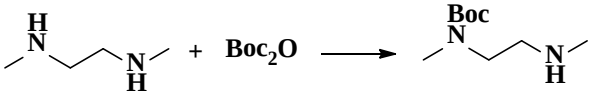


Triethylamine (53.12 g, 525 mmol) was added to the mixture of 2-bromoethylene ammonium bromide (30.73 g, 150 mmol) in dry CH_2Cl_2 (300 mL) at rt and stirred for 30 min. The mixture was cooled to 0 °C. Then a portion of allyl chloroformate (18.08 g, 150 mmol) in dry CH_2Cl_2 (100 mL) was added dropwise over 1.5 h under 0 °C and stirred overnight at rt. Water (120 mL) was added and the organic phase was separated. The organic phase was washed with 1 M HCl (80 mL × 3) and saturated NaCl solution, dried (Na_2SO_4) and the organic solvent was concentrated under reduced pressure. The residue was purified by flash chromatographywith petroleum ether / ethyl acetate (PE/EtOAc=4:1) to afford 17.34 g of the desired compound as a pale yellow oil (yield, 56%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.87 – 5.97 (m, 1 H), 5.28 – 5.34 (dq, J_1 = 17.2 Hz, J_2 = 1.6 Hz, 1 H), 5.21 – 5.24 (dq, J_1 = 10.4 Hz, J_2 = 1.2 Hz, 1 H), 4.58 (d, J = 5.2 Hz, 2 H), 3.58 – 3.61 (q, J = 5.6 Hz, 2 H), 3.46 – 3.49 (t, J = 5.6 Hz, 2 H).

^{13}C-NMR (400 MHz, $CDCl_3$): δ 156.21, 132.75, 117.84, 65.78, 42.80, 32.34.

Tert-butyl methyl(2-(methylamino)ethyl)carbamate



N,*N*'-dimethylethylenediamine (8.01 g, 90.8 mmol) was dissolved in dry CH_2Cl_2 (100 mL) and cooled to 0 °C. A solution of Boc_2O (6.00 g, 27.5 mmol) in dry CH_2Cl_2 (40 mL) was added dropwise under 0 °C. And then the mixture was allowed to warm

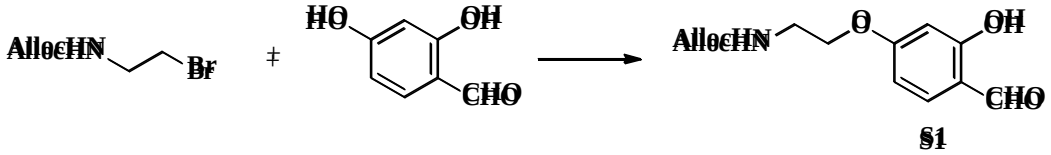
to rt slowly. The mixture was stirred at rt overnight and the solvent was removed under vacuum. The residue was purified by column chromatography eluting with CH_2Cl_2/MeOH to afford yellow oil (4.24 g, yield 82%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.63 (t broad, 2 H), 2.93 (s, 3 H), 2.86(t broad, 2 H), 2.71 (s, 3 H), 1.45 (s, 9 H).

^{13}C-NMR (400 MHz, $CDCl_3$): δ 80.31, 47.08, 45.95, 45.22, 33.14, 28.30, 8.69.

ESI-MS *m/z* calcd for $C_9H_{20}N_2O_2$ 188.2; found ($M+H^+$) 189.2.

Allyl (2-(4-formyl-3-hydroxyphenoxy)ethyl)carbamate **S1**



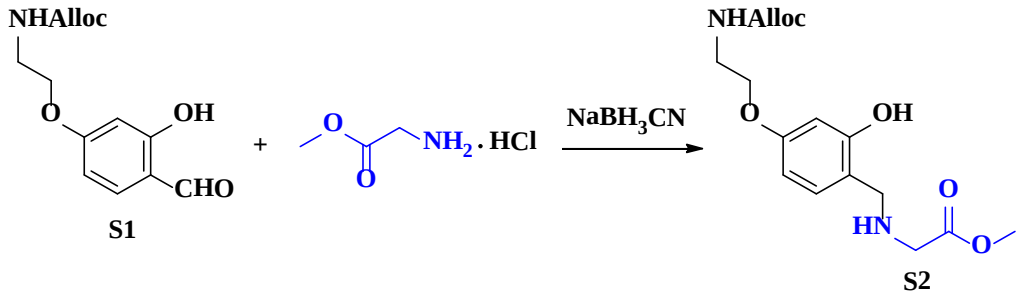
2,4-Dihydroxybenzaldehyde (3.04 g, 22 mmol), $KHCO_3$ (3.00 g, 30 mmol), KI (0.66 g, 4 mmol) and *N*-allyloxycarbonyl-2-bromoethylamine (4.16 g, 20 mmol) were mixed in 250 mL of dry acetone. The mixture was heated under reflux for 26 h, then filtered to remove any insoluble solids. 1 M HCl was added to neutralize the warm solution, which was then extracted with ethyl acetate (80 mL × 3). The combined organic extract was sequentially washed with 1 M HCl, saturated NaCl solution, dried with Na_2SO_4. a pale yellow solid product was afforded after evaporation of the solvents. The solid was purified by column chromatography using a mixture of PE/EtOAc (v / v, 5 / 1) as eluent. Evaporation of the solvent afforded a white solid product S1 (2.44 g, yield 46%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 11.44 (s, 1 H), 9.72 (s, 1 H), 7.43 (d, *J* = 8.4 Hz, 1 H), 6.52 –6.55 (d, *J* = 8.4 Hz, 1 H), 6.41 (d, *J* = 2.0 Hz, 1 H), 5.87 – 5.97 (m, 1 H), 5.29 – 5.33 (d, *J* = 17 Hz, 1 H), 5.21 – 5.23 (d, *J* = 10 Hz, 1 H), 4.58 (d, *J* = 5.2 Hz, 2 H), 4.09 (t, *J* = 5.2 Hz, 2 H), 3.60 – 3.64 (q, *J* = 5.2 Hz, 2 H).

^{13}C-NMR (400 MHz, $CDCl_3$): δ 194.52, 165.68, 164.43, 156.34, 135.47, 132.76, 117.95, 115.51, 108.35, 101.47, 67.48, 65.84, 40.31.

ESI-MS *m/z* calcd for $C_{13}H_{15}NO_5$ 265.1; found ($M+Na^+$) 288.2.

Methyl 2-((4-(2-(((allyloxy)carbonyl)amino)ethoxy)-2-hydroxybenzyl)amino)acetate **S2**



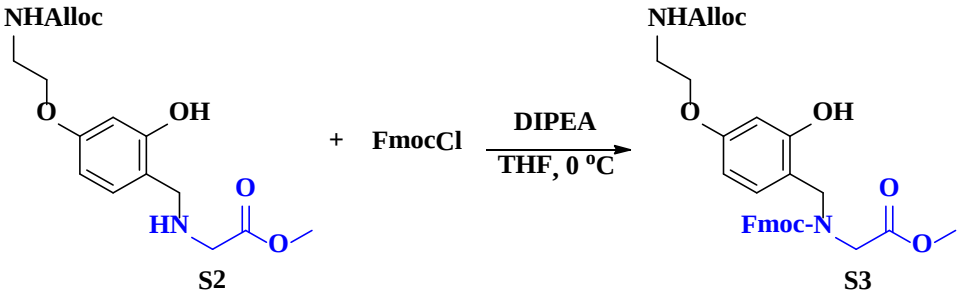
Aldehyde **S1** (2.12 g, 8 mmol) was dissolved in 100 mL dry ethanol and then cooled to 0 °C. The mixture of glycine methyl ester hydrochloride (2.01 g, 16 mmol) and triethylamine (1.66 g, 16.4 mmol) in 45 mL dry ethanol was added portion-wise to the above solution under 0 °C. Then sodium triacetoxyborohyride (5.09 g, 24 mmol) was added portion-wise over 8 minutes under 0 °C. The mixture was then stirred under 0 °C for about 3 h. The reaction was terminated by addition of 150 mL water, then a clear solution was formed. The solution pH was adjusted to 5 with 1 M HCl solution. The bulk of ethanol was removed under reduced pressure. The residue was extracted successively with ethyl acetate (40 mL) and CH_2Cl_2 (40 mL). The pH of the remaining aqueous mixture was adjusted to about 8.5 by addition of saturated aqueous $NaHCO_3$ solution. The above mixture was extracted successively with ethyl acetate (40 mL × 2) and CH_2Cl_2 (40 mL × 2). The combined organic extracts were washed with saturated NaCl solution, dried with Na_2SO_4. The residue was purified by flash chromatography (CH_2Cl_2/MeOH, 20:1) to afford the desired compound **S2** as a colorless oil (2.17 g, yield 80%).

^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.33 – 7.36 (t, J = 5.6 Hz, 1 H), 6.91 – 6.93 (d, J = 8.8 Hz, 1 H), 6.26 – 6.28 (m, 2 H), 5.83 – 5.90 (m, 1 H), 5.21 – 5.25 (dd, J_1 = 17.2 Hz, J_2 = 1.6 Hz, 1 H), 5.11 – 5.14 (dd, J_1 = 10.4 Hz, J_2 = 1.2 Hz, 1 H), 4.43 – 4.44 (d, J = 5.2 Hz, 2 H), 3.84 – 3.87 (t, J = 5.6 Hz, 2 H), 3.65 (s, 2 H), 3.59 (s, 3 H), 3.26 – 3.29 (t, J = 5.6 Hz, 2 H), 2.45 – 2.47 (quintet, J = 1.6 Hz, 1 H), 1.86 (s, 2 H).

^{13}C-NMR (400 MHz, DMSO-d_6): δ 172.61, 159.13, 158.14, 156.61, 134.22, 130.12, 117.46, 117.38, 105.07, 102.50, 66.65, 64.85, 51.92, 49.24, 49.08, 21.61.

ESI-MS *m/z* calcd for $C_{16}H_{22}N_2O_6$ 338.2; found ($M+H^+$) 339.1.

Methyl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)(4-(2-(((allyloxy)carbonyl)amino) ethoxy)-2-hydroxybenzyl)amino)acetate **S3**



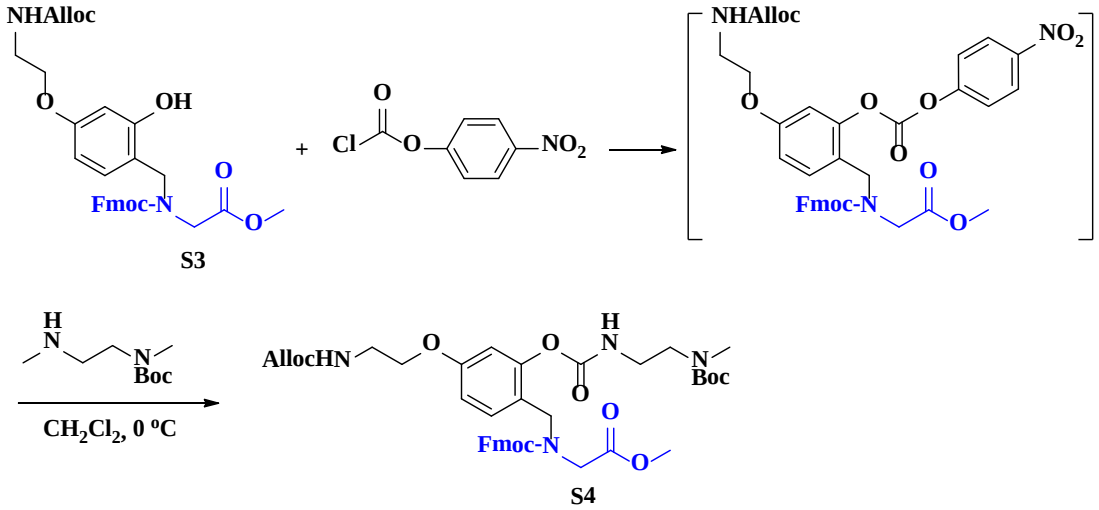
N,*N*-Diisopropylethylamine (1.13 mL, 6.5 mmol) and 9-fluorenylmethyl chloroformate (1.36 g, 5.25 mmol) were sequentially added to an ice-cooled solution of the secondary amine **S2** (1.69 g, 5 mmol) dissolved in dry tetrahydrofuran (45 mL). After 90 min reaction under 0 °C, saturated aqueous ammonium chloride solution (100 mL) was added. The resulting mixture was extracted sequentially with CH_2Cl_2 (50 mL × 2) and ethyl acetate (50 mL × 2). The combined organic extracts were dried with Na_2SO_4. The dried solution was concentrated under vacuum to afford a pale yellow oil. The residue was then purified by flash chromatography (PE/EtOAc, 3:2) to afford the Fmoc-protected objective product **S3** (2.58 g, yield 92%) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.75 (broad s , 1 H), 7.73 – 7.75 (d, J = 7.6 Hz, 2 H), 7.49 – 7.52 (m, 2 H), 7.36 – 7.40 (t, J = 7.4 Hz, 2 H), 7.25 – 7.30 (m, 2 H), 6.90 – 6.92 (d, J = 8.0 Hz, 1 H), 6.48 (s, 1 H), 6.33 – 6.36 (dd, J_1 = 8.0 Hz, J_2 = 2.4 Hz, 1 H), 5.86 – 5.96 (m, 1 H), 5.28 – 5.32 (d, J = 15.6 Hz, 1 H), 5.15 – 5.22 (m, 2 H), 4.56 – 4.57 (d, J = 4.8 Hz, 2 H), 4.48 – 4.50 (d, J = 6.4 Hz, 2 H), 4.37 (s, 2 H), 4.19 – 4.23 (t, J = 6.2 Hz, 1 H), 3.97 – 4.00 (t, J = 4.8 Hz, 2 H), 3.86 (s, 2 H), 3.66 (s, 3 H), 3.54 – 3.58 (q, J = 5.2 Hz, 2H).

^{13}C-NMR (400 MHz, $CDCl_3$): δ 169.42, 160.38, 158.23, 157.25, 156.40, 143.52, 141.36, 132.83, 131.76, 127.84, 127.18, 124.86, 120.06, 117.85, 114.87, 106.14, 103.48, 68.74, 66.96, 65.76, 52.41, 48.54, 47.73, 47.11, 40.54.

ESI-MS *m/z* calcd for $C_{31}H_{32}N_2O_8$ 560.2; found ($M+Na^+$) 583.2.

Methyl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)(4-(2-(((allyloxy)carbonyl)amino)ethoxy)-2-(((2-((tert-butoxycarbonyl)(methyl)amino)ethyl)carbamoyl)oxy)benzyl)amino)acetate **S4**



Triethylamine (520 μL, 3.75 mmol) and 4-nitrophenyl chloroformate (0.53 g, 2.63 mmol) were sequentially added to a solution of the phenol **S3** (1.40 g, 2.5 mmol) dissolved in 60 mL dry CH_2Cl_2 under 0 °C. The reaction was stirred under 0 °C for 2 h. Then triethylamine (416 μL, 3.0 mmol) and the Boc-protected N,N'-dimethylethylenediamine (0.56 g, 3.0 mmol) were added under 0 °C. The reaction was stirred at rt for additional 3 h. The solution was diluted with ethyl acetate (120 mL), washed with 1 M HCl (20 mL × 2) and saturated $NaHCO_3$ solution (10 mL × 2), dried with Na_2SO_4, and then condensed under reduced pressure. The resulting pale yellow oil was purified by column chromatography (PE/EtOAc, 2:1) to afford a white solid product **S4** (1.21 g, yield 62%).

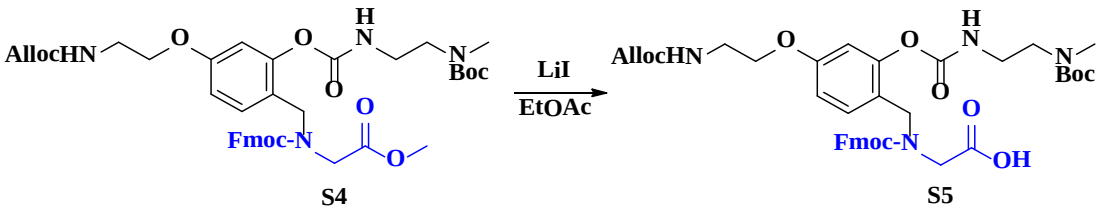
^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.73 – 7.76 (t, *J* = 6.2 Hz, 2 H), 7.52 – 7.54 (d, *J* = 7.6 Hz, 2 H), 7.37 – 7.41 (m, 2 H), 7.27 – 7.32 (m, 2 H), 7.13 – 7.16 (m, 1 H), 6.71 – 6.75 (m, 1 H), 6.64 – 6.68 (m, 1 H), 5.88 – 5.97 (m, 1 H), 5.29 – 5.33 (m, 1 H), 5.20–5.23 (d, *J* = 10.4 Hz, 1 H), 4.43 – 4.58 (m, 6 H), 4.20 – 4.28 (m, 1 H), 4.01 – 4.03 (m,

2 H), 3.93 (s, 1 H), 3.79 (s, 1 H), 3.65 – 3.70 (m, 3 H), 3.37 – 3.59 (m, 6 H), 2.88 – 3.06 (m, 6 H), 1.44 – 1.46 (d, *J* = 8.0 Hz, 9 H).

^{13}C-NMR (400 MHz, $CDCl_3$): δ 170.08, 158.86, 156.17, 156.01, 155.49, 153.90, 150.78, 143.74, 141.20, 132.79, 130.93, 130.73, 129.85, 127.62, 126.99, 124.82, 121.11, 119.91, 117.56, 79.84, 67.73, 67.00, 65.50, 51.90, 47.16, 47.07, 46.56, 45.15, 40.30, 35.04, 28.36.

ESI-MS *m/z* calcd for $C_{41}H_{50}N_4O_{11}$ 774.3; found ($M+Na^+$) 797.9.

2-((((9H-fluoren-9-yl)methoxy)carbonyl)(4-(2-(((allyloxy)carbonyl)amino)ethoxy)-2-(((2-((tert-butoxycarbonyl)(methyl)amino)ethyl)carbamoyl)oxy)benzyl)amino)acetic acid **S5**



Lithium iodide (4.28 g, 32 mmol) was added to a stirred solution of the methyl ester **S4** (3.10 g, 4 mmol) in dry ethyl acetate (90 mL) under nitrogen atmosphere. The mixture was heated under reflux for additional 25 h. Then the reaction was quenched by the addition of 90 mL 1 M HCl. The resulting mixture was extracted with EtOAc (60 mL × 2). The combined organic extracts were sequentially washed with 5% aqueous $Na_2S_2O_3$ solution (8 mL × 3), 1 M HCl (15 mL) and saturated NaCl solution (15 mL × 2), dried with Na_2SO_4, filtered and evaporated in vacuum. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 30:1) to afford the target compound **S5** (white solid, 2.28 g, yield 75%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.66 – 7.68 (d, *J* = 7.6 Hz, 2 H), 7.44 – 7.55 (m, 2 H), 7.27 – 7.31 (m, 2 H), 7.19 – 7.24 (m, 2 H), 7.04 – 7.16 (m, 1 H), 6.50 – 6.72 (m, 2 H), 5.81 – 5.89 (m, 1 H), 5.21 – 5.26 (d, *J* = 15.4 Hz, 1 H), 5.13– 5.16 (d, *J* = 10.0 Hz, 1 H), 4.31 – 4.59 (m, 6 H), 4.12 – 4.23 (m, 1 H), 3.90 – 4.00 (m, 2 H), 3.76 – 3.89 (m, 2 H), 3.29 – 3.55 (m, 6 H), 2.75 – 3.03 (m, 6 H), 1.29 – 1.43 (d, *J* = 12.0 Hz, 9 H).

^{13}C-NMR (400 MHz, $CDCl_3$): δ 172.34, 159.03, 156.49, 156.16, 154.43, 150.84, 143.76, 141.23, 132.75, 131.10, 130.11, 127.73, 126.96, 125.03, 121.13, 119.96, 117.68, 112.52, 109.27, 80.22, 68.08, 67.06, 65.72, 53.49, 47.17, 46.83, 46.62, 40.33, 35.13, 28.27.

ESI-MS *m/z* calcd for $C_{40}H_{48}N_4O_{11}$ 760.3; found ($M+Na^+$) 783.9.

Part II: Synthesis and Deprotection of Gly^{RMB}-Containing Peptides

The resulting Fmoc-Gly^{RMB}-OH amino acids could be coupled onto the resin (e.g., Rink amide AM resin) via customary coupling (**Supplementary Scheme 2**). Through standard Fmoc-SPPS protocols, the peptides were assembled in a stepwise manner according to the predesigned sequence. It was noted that the last amino acids for coupling must be one with Boc-protected amino groups (such as Boc-Cys(Trt)-OH) or acetylation of amino groups. At the end of peptide chain assembly, Alloc was removed by $Pd(PPh_3)_4/PhSi_3$ and then another standard Fmoc-SPPS protocols were performed for solubilizing Arg-Tag. At last, standard TFA cleavage was conducted to remove the side-chain protection groups and release the Gly^{RMB}-containing peptides.

Synthesis of Gly ^{RMB}-Containing Peptides Ac-AQFRG^{RBM}SLA-NH$_2$

The Rink amide-AM Resin (60 mg, 0.02 mmol) was swollen with CH_2Cl_2/DMF (1/1, v/v) for 30 min. The resin washed (3×DMF, 3×CH_2Cl_2, 3×DMF), and followed by deprotection of the Fmoc group with 20% piperidine/DMF (2×5 min). After another washing step, Fmoc-Ala-OH (31 mg, 0.1 mmol) was coupling onto the resin with HCTU (38 mg, 0.09 mmol) and DIEA (35 ul, 0.2 mmol) for 45 min. The peptide-chain assembly was then performed using standard Fmoc-SPPS protocols excepted for Gly^{RMB0} and the Arg following the Gly^{RMB0}. The Gly^{RMB0} amino acid residues was coupling using Fmoc-Gly^{RMB0}-OH (30 mg, 0.04 mmol), HATU (15 mg, 0.04 mmol), HOAt (5 mg, 0.04 mmol) and DIEA (7 ul, 0.08 mmol) for 1.5h, and then capping with Ac_2O/DIEA/DMF (1/1/8, v/v/v). After deprotection of Fmoc, it was performed a double coupling step for the Fmoc-Arg(Pbf)-OH with 1.5 h each and given a Ac_2O capping step. After completing peptides assembly, 10 mg of Pd $(PPh_3)_4/PhSiH_3$ in 5 ml of CH_2Cl_2/DMF (1/1, v/v) was added to removal the Alloc protection group. The deprotection step was repeated once for 30-50 min each time. The Arg-Tag was coupled by standard Fmoc-SPPS protocols. The peptide was cleaved from the resin with TFA/PhOH/TIPS/H_2O cocktails and isolated by semi-RP-HPLC to obtain the target peptide **1** (19 mg, 60% of isolated yield) which

was determined by ESI-MS (**Supplementary Figure 1**). As described previous literature[1], modified peptides were not completely stable under ESI-MS mass spectrometry conditions and the peptides with partial cleavage of the carbamate group were evident as molecular masses of 114 less than expected.

The First Intramolecular conversion of Gly^{RMB}-Containing Peptides

In previous paper[2], Saari and co-workers had been investigated the rate of the intramolecular cyclization reaction for hydroxyanisole derivatives and the half-life time was 36.3 min at pH 7.4, 37 ^{o}C. The cyclization reaction was studied employing the model peptide Ac-AQFRG^{RMB}SLA-NH_2 under ligation conditions (0.2 M phosphate buffer containing 6M Gn•HCl, pH 7.2 at rt). 1.8 mg of model peptide **1** was dissolved in 1 mL of ligation buffer, adjusted to pH 7.2, and then monitored by RP-HPLC (**Supplementary Figure 2**). The detailed test indicated that the parent phenol was generated with the half-life time $t_{1/2}$ = 50 min and the formation of N,N'-dimethylimidazolidinone was accompanied at pH 7.2 and rt. The reaction can be was completed up to 95% after 5 h. What's more, the phenol derivatives were totally stable up to 7 days under ligation conditions. In summary, the carbmates was released rapidly and cleanly under the native chemical ligation conditions, and the released peptide product **2** with phenolic functionality was compatible with standard NCL's conditions.

The Second Deprotection of Gly^{RMB}-Containing Peptides by TFA cocktails

After completing the ligation, the backbone modified group of product should be removed. To this end, the modified peptide was exposed to TFA cocktails. As described by Johnson et al that the reversible protection group of the peptide backbone using the N-(2-hydroxy-4-methoxybenzyl) (Hmb) group could be cleavage by TFA.[3] The deprotection reaction was studied employing the model peptide under TFA/TIPS/H_2O (95/2.5/2.5, v/v/v). 1.6 mg of model peptide **2** was dissolved in 4 mL of TFA cocktails, and monitored by analytical RP-HPLC and MALDI-TOF-MS (**Supplementary Figure 3**). The native peptide can be obtained quantitatively within

3 h with TFA. After finishing the cleavage, cleavage mixture was concentrated, precipitated with Et_2O and then the native product **3** was obtained in 69% isolated yield (0.6 mg) by a chromatographic step. It is noted that, for the aggregation-prone membrane proteins, the proteins could be purified handily by precipitation with Et_2O and then H_2O.

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Part III: Synthesis of Transmembrane Peptides by RBM Strategy

Signal peptide peptidase (SPP) is a novel family of membrane proteases which cleave peptide bonds under intramembrane. This family has been implicated in Alzheimer's disease.[1] Kent et al. employed the N-methyl backbone modification to the fourth transmembrane peptides from SPP. The helix-breaking caused the improved solubility of peptide. But the modification was permanent and could not be used to synthesize integral membrane proteins.[2] Here, the fourth transmembrane peptides from SPP was chosen as an initial target to test the new Arg-Tag containing RBM strategy for solubility of transmembrane peptides. Seven peptides labeled by fluorescein isothiocyanate (FITC) (**SPP4-1** to **SPP4-7**, **Supplementary Scheme 3**) were synthesized by Fmoc-SPPS, characterized by ESI-MS and purified by RP-HPLC except **SPP4-1** (**Supplementary Scheme 5** and **6**). The **SPP4-1** showed insolubility in 1:1 ACN/H_2O + 0.1% TFA and did not elute as well-defined peaks from the C4 reversed-phase column (data not shown). We observed precipitation of the peptides **SPP4-2** within 15–30 min during the preparation of the sample.

Solubility of peptides was determined by quantifying the amount of FITC fluorophore via UV/Vis spectrophotometry. First, we got two standard curves of FITC in different solvent systems (in 50% CH_3CN containing 0.1% TFA (buffer D) at λ=442 nm and 6M Gn•HCl containing 0.2 M phosphate buffer at pH 7.4 (buffer E) at λ=499 nm) as shown in **Supplementary Figure 4**. Subsequently, the peptides **SPP4-1** to **SPP4-7** dissolved in minimized buffer D to obtain saturated solutions. The solutions were centrifuged and the supernatant was did a 20-fold dilution for **SPP4-2** to **SPP-7**. (For measurements in buffer E, peptides **SPP4-1** to **SPP4-7** dissolved in minimized buffer E and then treated under pH 7.4 overnight at rt to obtain the **SPP4-1'** to **SPP4-7'** (**Supplementary Scheme 7**). The solutions were centrifuged and the supernatant was did a 50-fold dilution for **SPP4-2'** to **SPP-7'**.) The absorbance of solutions was measured at λ=442 nm for buffer D and λ=499 nm for buffer E (**Supplementary Table 1**). The data indicated that Arg_4-taged backbone modification was an effective strategy to improve the solubility and therefore, handling properties

of MP peptides.

We speculate the improvement of the solubility by new Arg-tagged RBM method is twofold: (1) N-backbone modification disrupts helix formation[2]; (2) solubilizing Arg-tag increases the solubility of TM peptides[3]. Backbone modification can disrupt the secondary structure helix formation as revealed by our circular dichroism (CD) analysis (**Supplementary Fig. 8**). In this CD experiment, **SPP4-2** showed a very similar secondary structure as **SPP4-1**, whereas **SPP4-3** to **SPP4-7** with a N-backbone modification exhibited dramatically different spectra.

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Part IV: Synthesis and Single Channel Activity of Wide-Type and Ser64-Phosphorylated M2 Channel

The integral membrane protein M2 from influenza A is a proton channel protein containing 97 amino acids and is homotetramer in its native state. The M2 protein is important for the influenza A virus entry and infection. Therefore, it is an idea target for the inhibition of viral fusion by the amantadine class of antiviral drugs.[1] The crystal structure and NMR spectroscopy of the M2 channel indicated a physical occlusion mechanism for antiviral activity.[2-4]

A number of post-translational modifications (such as palmitoylation and phosphorylation) occured to the M2 protein. Previous work indicated that the phosphorylation of a loop in the cytoplasmic tail of M2 does not influence the ion channel function of M2, but maybe promotes tetramer formation. It was puzzling what true role those modifications played in viral infections.[5,6] To determine the role of phosphorylation for the assembly of M2 into its native tetrameric state, the integral membrane protein was synthesized using native chemical ligation of unprotected peptide segments.

The total synthesis of M2 protein was achieved using native chemical ligation by Kochendoerfer et al. This strategy was based on Boc-SPPS for synthesis of unprotected peptides.[7] However, Boc-SPPS strategy cannot obtain peptides with HF-sensitive phosphate groups. Besides, the poor handling properties of M2[1-49] transmembrane peptides resulted in complication of purification and peptides ligation. Here, we employed the new solubilizing strategy for chemical synthesis of M2 protein with phosphorylation at S64.

Total Synthesis of Wide-Type and Ser64-Phosphorylated M2

The membrane spanning M2 segment **5** M2[1-49, 4Arg-Tag]-$NHNH_2$ was synthesized on a 2-Cl-Trt-$NHNH_2$ resin (0.1 mmol scale) using standard Fmoc-SPPS. M2 segment **6** M2[50-97, Ser64] and M2 segment p**6** M2[50-97, pSer64] were synthesized analogously on a Nova-PEG Wang resin (0.1 mmol scale). The use of 0.1M HOBt/20% piperidine solution was recommended to reduce the aspartimide

formation, a common side reaction that occured in peptides containing 'Asp-Ser'. The three M2 segments were purified by preparative RP-HPLC with an acetonitrile-water system containing 0.1% TFA at 56^{o}C. In both cases, fractions containing objective peptide were collected and lyophilized. Finally, the three purified peptides were obtained in final yields of 12%, 20%, 15% respectively (**5**, **6**, and **p6**) and identified by ESI-MS (**Supplementary Figure 9**: M2[1-49,4Arg-Tag]-$NHNH_2$, observed M.W. 6516.6 and calcd M.W. 6517.7(average isotope composition); M2[50-97, pSer64], observed M.W. 5683.3 and calcd M.W. 5684.1(average isotope composition); M2[50-97, Ser64], observed M.W. 5602.5 and calcd M.W. 5604.1(average isotope composition)).

The native chemical ligation of M2[1-49, 4Arg-Tag]-$NHNH_2$ and M2[50-97, pSer64] p**6** was performed using the *in situ* $NaNO_2$ activation/thiolysis strategy. 6.5 mg of M2[1-49, 4-Arg-Tag]-$NHNH_2$ **5** was placed into a 2 mL Eppendorf reaction tube, and 0.4 mL of buffer (0.2 M sodium phosphate buffer containing 6 M Gn•HCl with pH 3.0-3.1) was added to completely dissolve the peptide hydrazide. The reaction tube was taken into the −15 ^{o}C ice-salt bath, and gently agitated by a magnetic stirring. 40 μL of 0.5 M $NaNO_2$ was pipetted into the buffer and buffer solution was gently stirred for 15 min at −15 ^{o}C. During the oxidation step, 8.5 mg of N-terminal Cys-peptide M2[50-97, pSer64] p**6** and 13.6 mg of MPAA was dissolved in 0.4 ml of buffer (0.2 M sodium phosphate buffer containing 6 M Gn•HCl with pH 7.0). The mixture was adjusted to about pH 6.5 with 6 M NaOH. The solution containing M2[50-97, pSer64] p**6** was pipetted to the oxidation buffer. The reaction tube was warmed to rt. The ligation solution was adjusted to pH 6.5-6.8 with 6 M NaOH. The reaction was monitored by RP-HPLC. The ligation mixture was stirred for 3 h at rt and then raised the pH to 7.4-7.5 to accelerate the intramolecular cyclization. The reactions were completed within 5h at pH 7.4. The ligation mixture was subsequently reduced with 0.4 mL of 0.2 M TCEP solution for 30 min. The product was purified by preparative RP-HPLC. Fractions containing ligation products were collected and lyophilized to obtain 4.3 mg of purified products with 36 % isolated yield. The final ligation product M2[1-97, 4Arg-Tag, pSer64] p**7** was

identified by ESI-MS and MALDI-TOF (exact mass: 12048.0 and observed: 12051.5).

It is noted that our new RBM strategies for wild-type and phosphorylated M2 channel employ standard Fmoc-SPPS, standard peptide purification strategy (ACN/H_2O), and standard native chemical ligation condition (0.2 M phosphate buffer containing 6 M Gn•Cl). This backbone modification with Arg-tag facilitates the handling of membrane peptides and ligation product.

The 4Arg-Tag can be removed reversibly by TFA cocktails (TFA/TIPS/H_2O= 95/2.5/2.5). 3.6 mg of M2 protein was dissolved in 2 mL TFA cocktails. The Ser64 phosphorylated M2 protein can be obtained within 5 h with 2 ml of TFA cocktails. TFA was concentrated by N_2 blowing and precipitated with Et_2O. The objective product M2[1-97, pSer64] was identified by MALDI-TOF (exact mass: 11258.5 and observed:11261.9). The final Ser64-phosphorylated M2 (**p8**) was obtained through a further RP-HPLC separation in 17% yield.

CD measurements were made in PiStar-180 spectrometer (Applied Photophysics) at rt. The Ser64 phosphorylated M2 p**7** was dissolved with dodecylphosphocholine (DPC, Avanti Polar Lipids) in TFE. The organic solvent was removed by nitrogen blowing, and the film was kept overnight with high vacuum pump. The solid was dissolved to ~5 mM with 20 mM Tris-HCl buffer containing 50 mM NaCl and 0.5 mM TCEP with pH 7.5, and then vortexed vigorously to clarity for CD spectra.[7]

The native M2[1-97] protein can also be obtained by the same procedures as phosphorylated M2 (**Supplementary Figure 10**). The ligation reaction proceeded smoothly in 39% yield and the final step for wild-type Ser64-phosphorylated M2 (**8**) was obtained through a further RP-HPLC separation in 26% yield.

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Part V: Synthesis and Single-Channel Activity of Inward Rectifier K^+ Channel Protein Kir5.1[64-179]

Inward rectifier potassium channels are important to maintain the membrane excitability, and control the intra- and extracellular potassium ionic homeostasis.[1-3] Among the members of Kir family, Kir5.1 is widely expressed in brain and kidney. This channel protein has been implicated in a number of important physiological and pathological processes such as the function and modulation of brainstem neurons and the SeSAME/EAST syndrome.[4] It has been long believed that the Kir5.1 formed no functional channel when expressed alone in heterogonous expression system. Yet a functional homomeric Kir5.1 was reported in a rare case, when it was expressed in HEK293T cells with the help of PSD-95, a member of the membrane-associated guanylate kinase (MAGUK) family which can help receptors or ion channels to locate on plasma membrane.[5] This largely overlooked observation raised a hypothesis that the no detection of functional homomeric Kir5.1 may due to the lack of ability to locate on plasma membrane. But very little is known about the physiology of Kir5.1 due to the fact that no functional channel of homomeric Kir5.1 was detected when expressed alone. Nevertheless, very few further physiological and structural studies of the Kir5.1 channel were reported. Here, we aimed to functionally reconstitute the chemical synthesized amino acid segment in planar lipid bilayers and to test its single channel activity.

Chemical Total Synthesis of Kir5.1[64-179] Channel

To address the problem that whether or not pure Kir5.1 can form a functional channel by itself, we chemically synthesized the core transmembrane domain of Kir5.1 (64-179, 116 amino acids) containing two transmembrane helices and a membrane-bound P-loop. For this purpose we divided the protein into four segments, Kir5.1[64-89]-$NHNH_2$, Kir5.1[90-116]-$NHNH_2$, Kir5.1[117-137, 4Arg-Tag]-$NHNH_2$, Kir5.1[138-179] and conducted *N*-to-*C* sequential ligation (**Supplementary Scheme**

4). The three segments (i.e. Kir5.1[64-89]-$NHNH_2$, Kir5.1[90-116]-$NHNH_2$, and Kir5.1[138-179]) can be directly synthesized in 15%, 20%, and 12% isolated yields by Fmoc-SPPS.The pseudoproline units Fmoc-Gln(Trt)-Ser[PSI(Me,Me)Pro]-OH, Fmoc-Ala-Thr(Psi(Me,Me)pro)-OH and Fmoc-Gly(Dmb)-OH were used during Fmoc-SPPS (**Supplementary Figure 11**). However, the P-loop segment Kir5.1[127-147]-$NHNH_2$ cannot prepare by general Fmoc protocol as the crude peptide was hardly soluble. Fortunately, the peptide was smoothly obtained after addition of a 4Arg-Tag at Gly133 through automated Fmoc SPPS. The purified peptide was also nicely soluble (**Supplementary Figure 11**).

The first ligation of two peptide segments **9** and **10** underwent in 6 M Gn•HCl with 0.2 M sodium phosphate in the presence of 17 mg/mL dodecylphosphocholine (DPC). 100 mM MPAA was used as a thiolysis mediator. The ligation product **13** was obtained in 57% isolated yields (**Supplementary Figure 12**) Using the similar ligation conditions, the next two rounds of ligation and subsequent purification could be successfully carried out in 29%, and 45% isolated yields (**Supplementary Figure 13** and **Supplementary Figure 14**). Thus we obtained Kir5.1[64-179, 4Arg-Tag] **15** in *ca.* 7.5% overall isolated yield (as calculated from the peptide segments **9**). This Arg-tagged molecule was still isolated by RP-HPLC to high purity (>95%) in CH_3CN-H_2O solvent system and easily characterized by ESI-MS (**Supplementary Figure 14d**).

The last step of the synthesis was to dissolve the purified, backbone-modified Kir5.1[64-179] powder into the TFA/TIS/H_2O cocktail for 5 hours at rt. Then the solution was concentrated by nitrogen blowing and the final target protein was obtained through precipitation from the cold diethyl ether treatment. The final Kir5.1[64-179] protein could not be eluted from a C_4-column on RP-HPLC in our experiments. The protein **16** also could not be characterized by ESI-MS. The SDS-PAGE of Kir5.1[64-179] protein **16** indicated that the De-Arg-tag product was pure (**Supplementary Figure 15a**).

Single Channel Activity of Kir5.1[64-179] in Planar Lipid Bilayer

In this study, the chemically synthesized Kir5.1[64-179] was in high purity, without any source of channel contaminants or functional modulators. Here, the measured channel currents immediately demonstrated the successful functional refolding of chemically synthesized channel proteins in lipid bilayers.

Single channel conductance was measured to analyze purified Kir5.1[64-179] channels' properties in lipid bilayer. First, we underwent functional refolding for a chemically synthesized protein in liposome and consequent channel conductance measurement in planer lipid bilayer. Secondly, channel conductance measurement of Kir5.1[64-179] was performed. Relative high concentration of Kir5.1[64-179] was added to ensure successful fusion of channels to planar lipid bilayer in the aperture in the TEFLON plate, which resulted in more than one channel present in the lipid bilayers and consequent tendency of channel opening in both positive and negative voltage (**Supplementary Figure 15**). With further optimization of the channel fusion to planar lipid bilayer, channel conductance of Kir5.1[64-179] could be measured with unique orientation, only in opening state in positive or negative voltage (**Supplementary Figure 15**). At the same time, we confessed that the reconstituted Kir5.1[64-179] channel current measurement could only sustained in short time, resulting in difficulties in channel conductance estimation and blocking assay using channel blockers (such as Mg^{2+}) (**Supplementary Figure 16**). It is probably due to lack of the cytoplasmic domains of Kir5.1 protein, which was known to play important roles in the modulation of Kir channel gating.[1]

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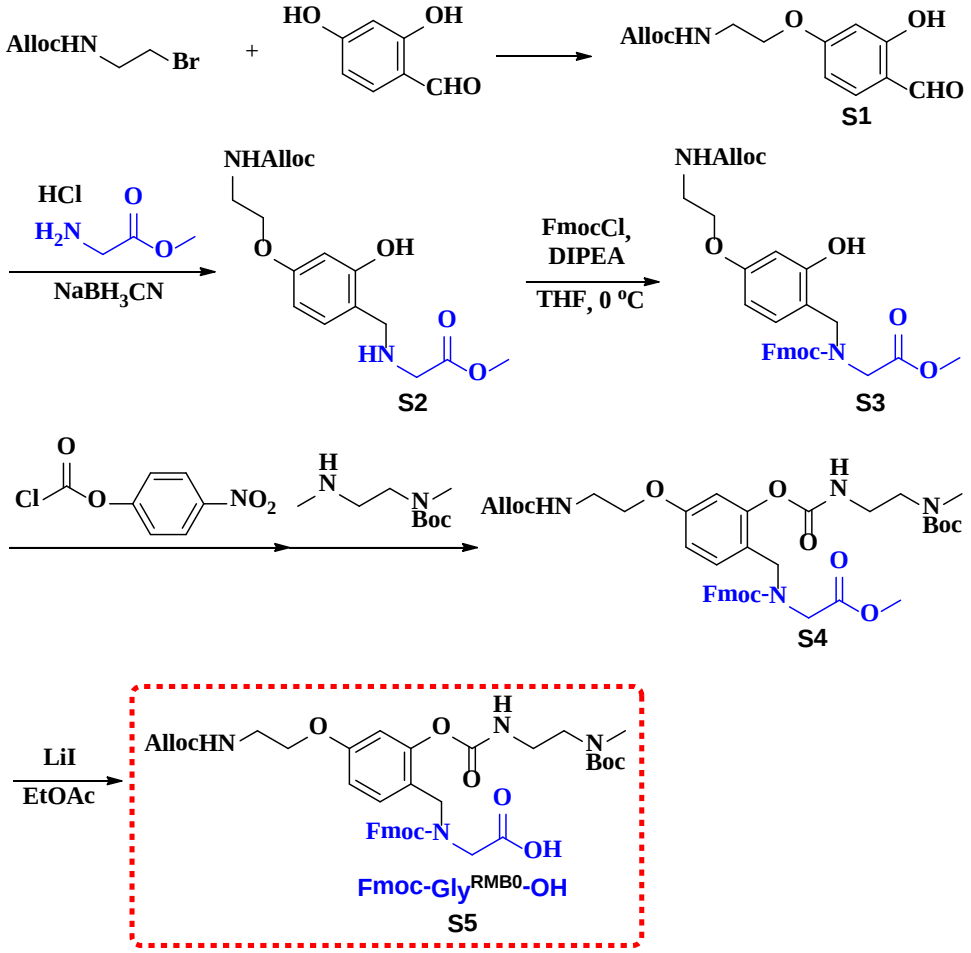
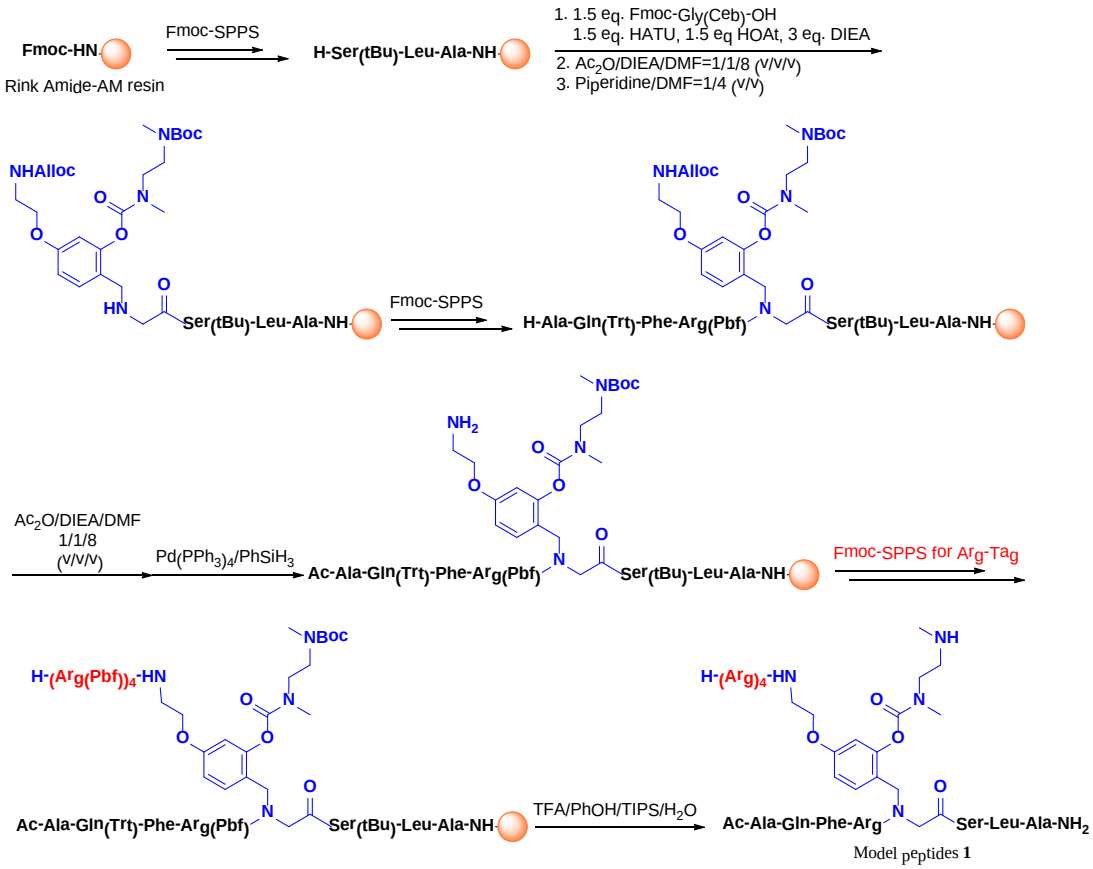
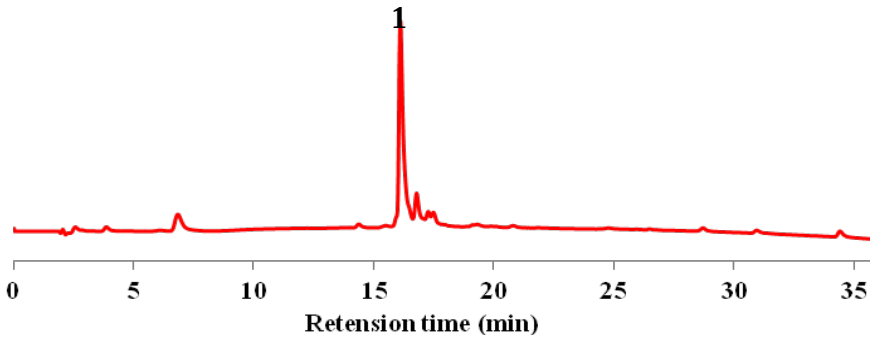


Supplementary Scheme 1. Synthetic route for Fmoc-Gly^{RMB0}-OH **S5**.

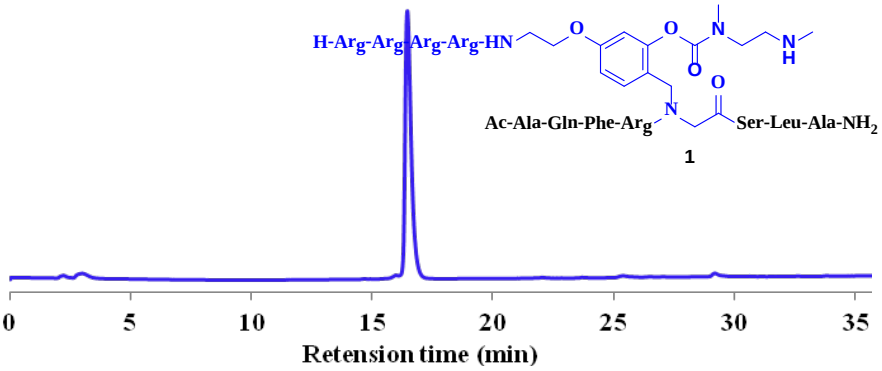


Supplementary Scheme 2. The general synthetic routes for Gly^{RMB}-containing model peptides **1**.

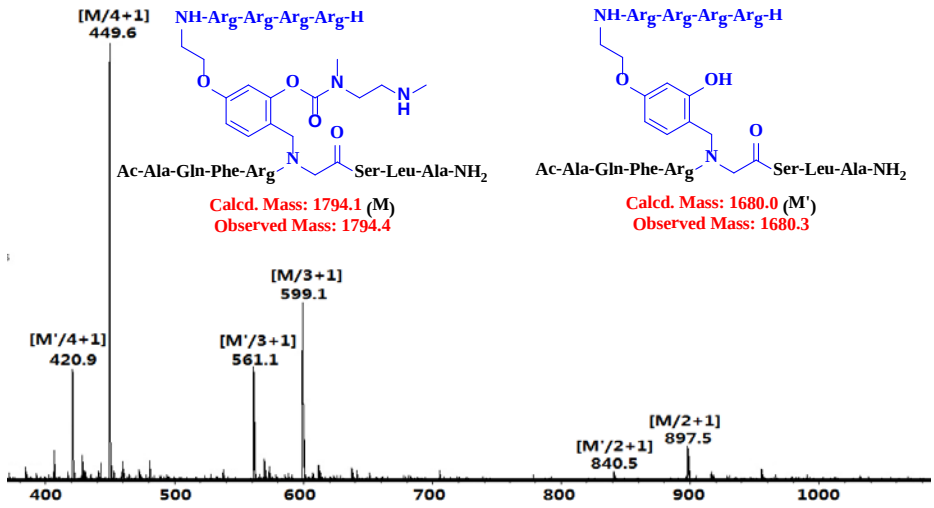
a. HPLC of crude peptides **1**



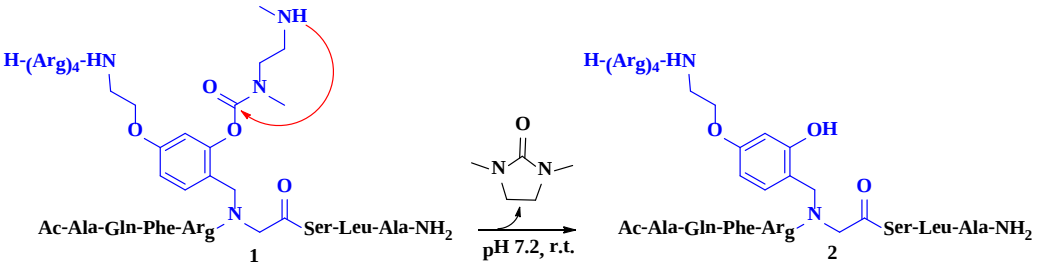
b. HPLC of purified peptides **1**



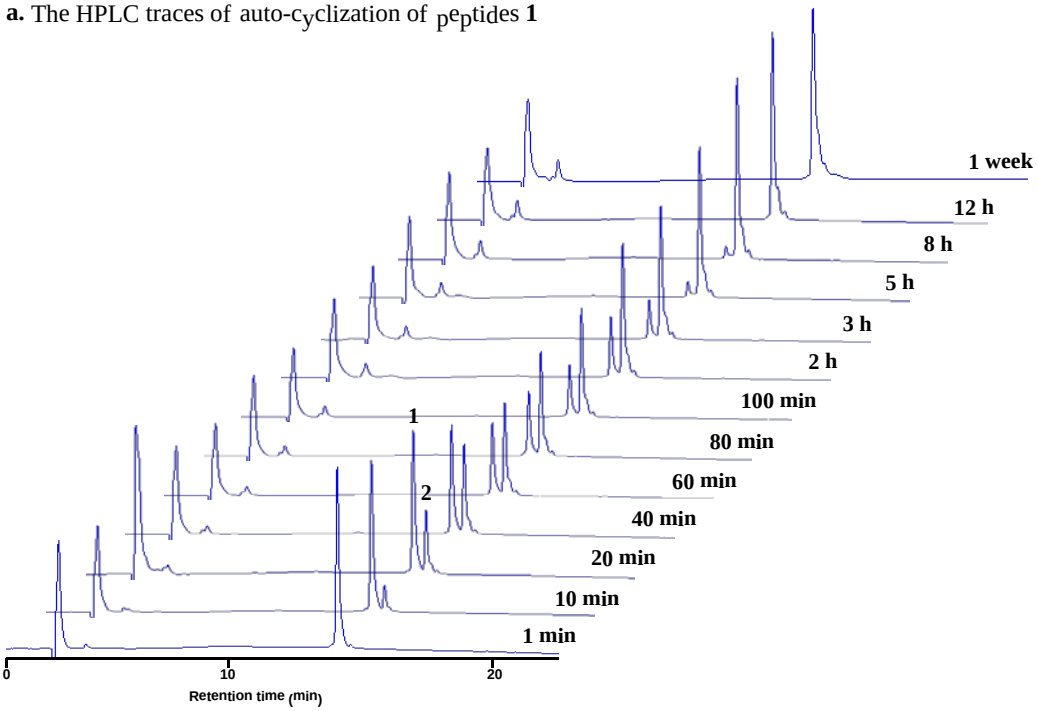
c. ESI-MS of purified peptide **1**



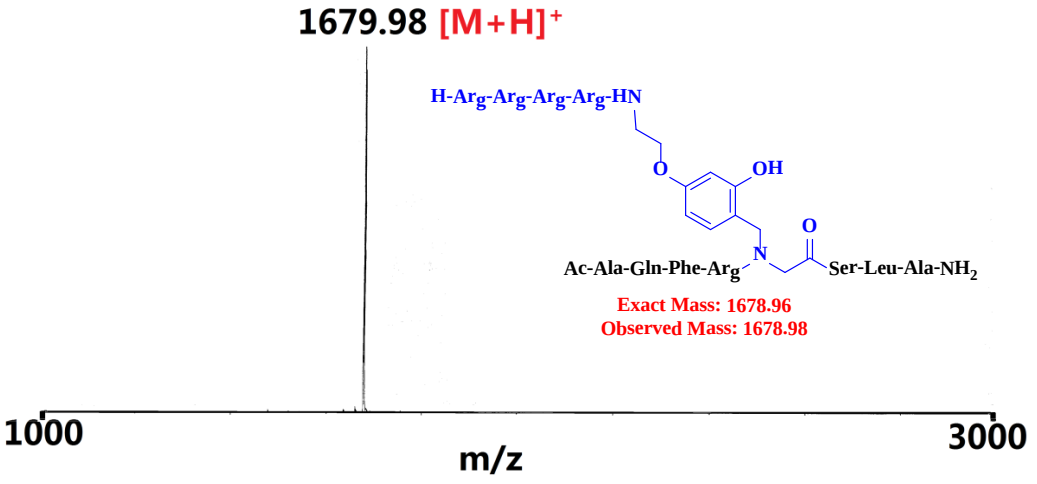
Supplementary Figure 1. Analytical RP-HPLC and ESI-MS characterization of peptides **1**. (a) The HPLC trace of crude peptides **1**. (b) The HPLC trace of purified peptides **1**. (c) ESI-MS of purified peptides **1**. Peptides were eluted from a C4 column (Vydac, 4.6×150 mm, 300Å, 5 μm) using a gradient of 5% buffer B (acetonitrile/0.8% TFA) in buffer A (water/0.1% TFA) to 65% B in A over 30 min at rt.



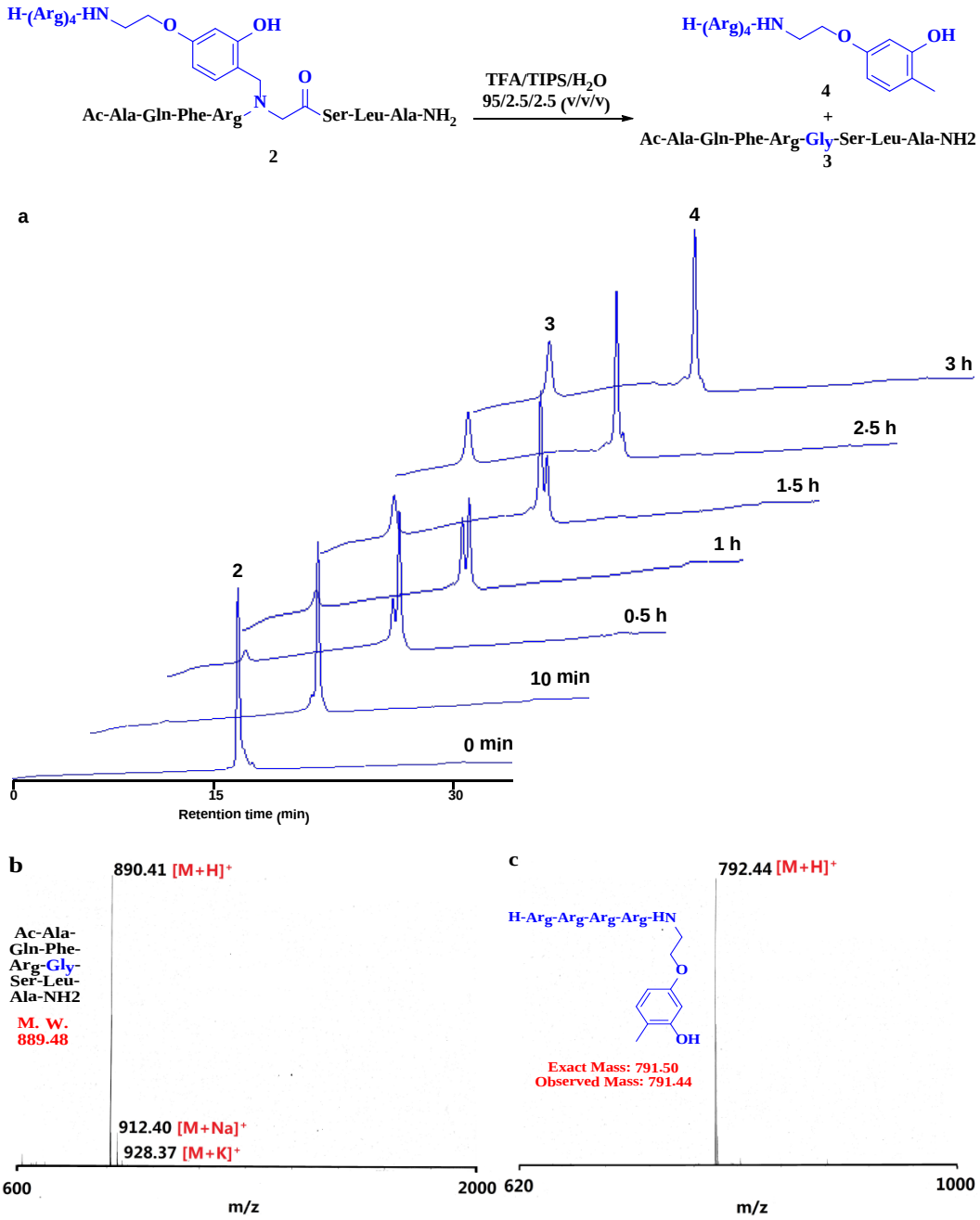
a. The HPLC traces of auto-cyclization of peptides **1**



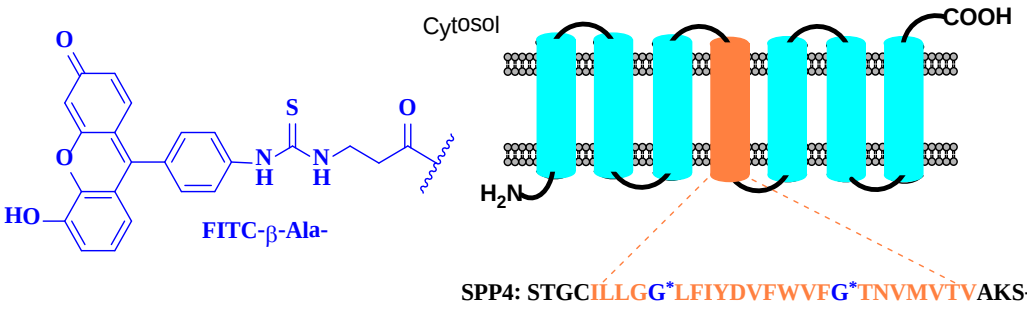
b. MALDI-TOF-MS of purified peptide **2**



Supplementary Figure 2. RP-HPLC and MALDI-TOF-MS characterization of intramolecular cyclization of peptides **1**. (a) The RP-HPLC traces of of the reversible backbone modification peptides **1** under pH 7.2 at rt. (b) The MALDI-TOF-MS of peptide product with phenolic functionality. Peptides were eluted from the C4 column using a gradient of 5% buffer B in buffer A to 65% B in A over 30 min at rt.



Supplementary Figure 3. RP-HPLC and MALDI-TOF-MS characterization of reversible cleavage of N-backbone modification using TFA cocktails. (a) RP-HPLC trace of deprotection of the reversible backbone modification peptides **2** with TFA at rt. (b) The MALDI-TOF-MS of the native peptide product **3**. (c) The MALDI-TOF-MS of solubilizing Arg-tag **4**. Peptides were eluted from the C4 column using a gradient of 5% buffer B in buffer A to 35% B in A over 30 min at rt.



SPP4: STGCILLGG*LFIYDVFWVFG*TNVMVTVAKS-NH_2

SPP4-1: FITC-β-Ala-STGCILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2

SPP4-2: FITC-β-Ala-RRRR-STGCILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2

SPP4-3: Ac-STGCILLGGLFIYDVFWVFG*(FITC-β-Ala-Heb)TNVMVTVAKS-NH_2

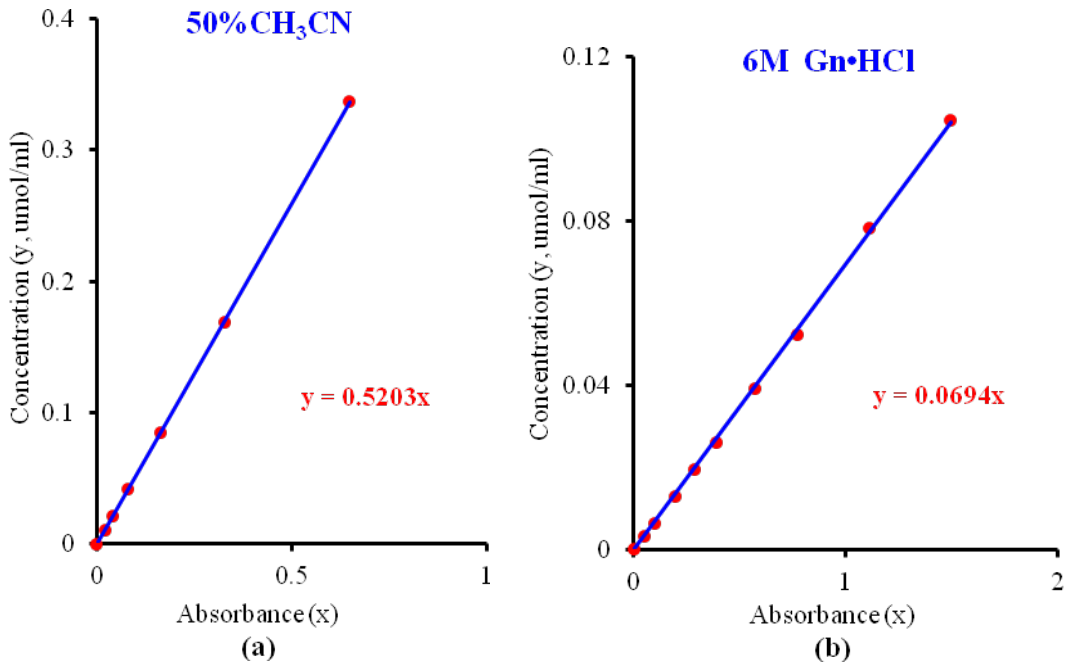
SPP4-4: Ac-STGCILLGGLFIYDVFWVFG*(FITC-β-Ala-RR-Heb)TNVMVTVAKS-NH_2

SPP4-5: Ac-STGCILLGGLFIYDVFWVFG*(FITC-β-Ala-RRRR-Heb)TNVMVTVAKS-NH_2

SPP4-6: Ac-STGCILLGGLFIYDVFWVFG*(FITC-β-Ala-RRRRRR-Heb)TNVMVTVAKS-NH_2

SPP4-7: Ac-STGCILLGG*(FITC-β-Ala-RRRR-Heb)LFIYDVFWVFGTNVMVTVAKS-NH_2

Supplementary Scheme 3. Structures of FITC-labeled model transmembrane peptide SPP4-1 and its annlogues.

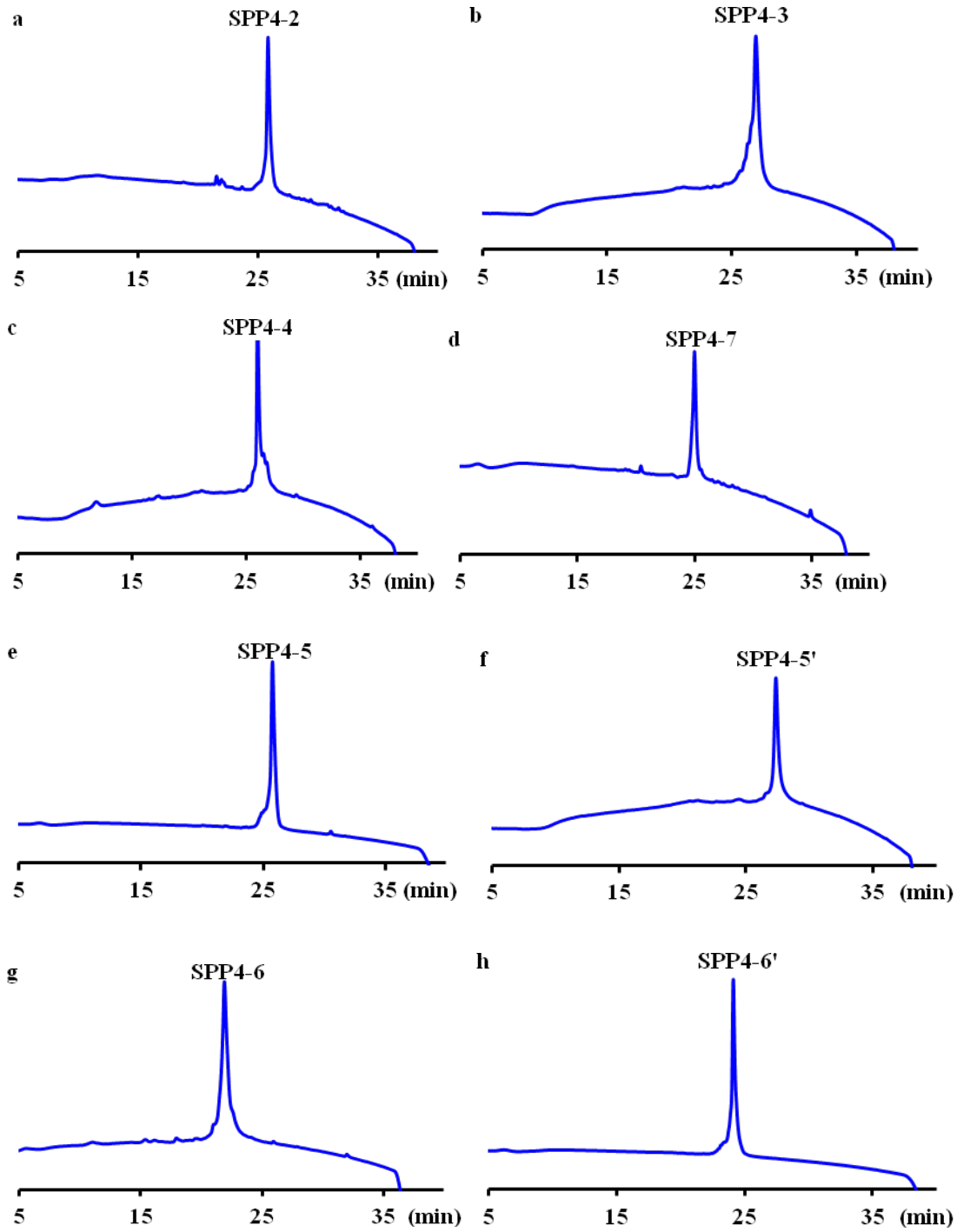


Supplementary Figure 4. Beer-Lambert plots of FITC fluorophor in different solvent systems. (a) Beer-Lambert plot of standard FITC fluorophore in 50%CH_3CN containing 0.1% TFA at λ=442 nm. (b) Beer-Lambert plot of standard FITC fluorophore in 6M Gn•HCl containg 0.2 M phosphate buffer at pH 7.4 at λ=499 nm.

Supplementary Table 1. Solubility of FITC-labeled peptides SPP4-1 and analogues.

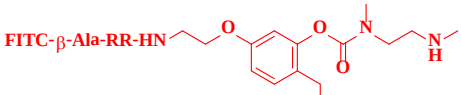
		SPP4-1*	SPP4-2	SPP4-3	SPP4-4	SPP4-5	SPP4-6	SPP4-7
In Buffer D	Abs $_{\lambda=442\ nm}$	0.365	0.061	0.118	0.213	0.656	0.727	0.596
	Solubility/**mM**	**0.19**	**0.63**	**1.23**	**2.22**	**6.83**	**7.57**	**6.20**
	/mg.ml^{-1}	0.7	2.7	4.9	9.5	31.4	37.2	28.5
In Buffer E′	Abs $_{\lambda=499\ nm}$	0.720	0.148	0.268	0.544	1.628	1.756	1.408
	Solubility/**mM**	**0.05**	**0.37**	**0.93**	**1.89**	**5.65**	**6.09**	**4.89**
	/mg.ml^{-1}	0.2	1.6	3.7	8.1	26.0	29.9	22.5

* Crude peptide ′ Peptides were treated under pH 7.4 overnight at rt.



Supplementary Figure 5. Analytical RP-HPLC of FITC-labeled peptides SPP4-1 and its analogues. RP-HPLC running on a C4 column (4.6×150 mm) using a gradient of 15% buffer B in buffer A to 75% B in A over 30 min at 56°C.

a

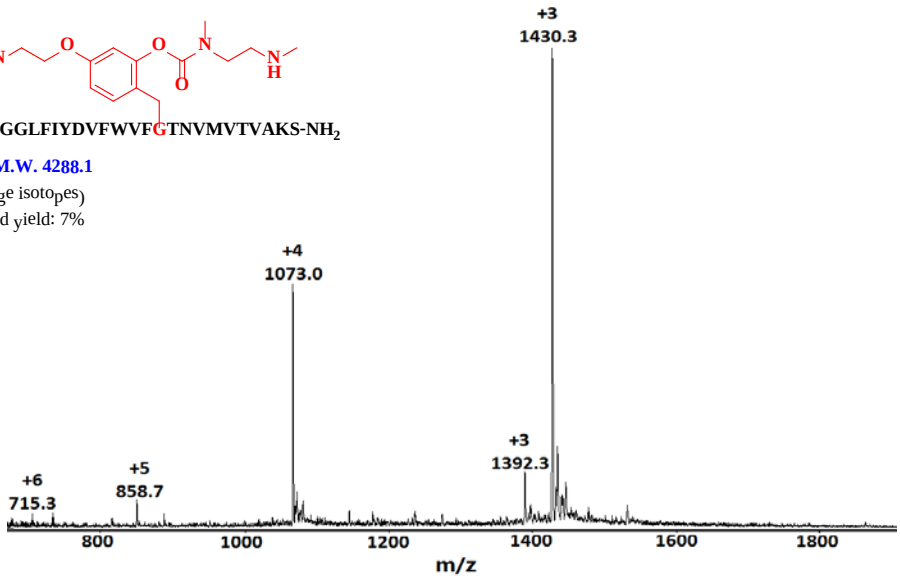


Ac-STGCILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2

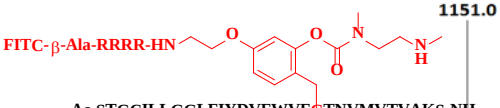
Calcd M.W. 4288.1

(average isotopes)

Isolated yield: 7%



b

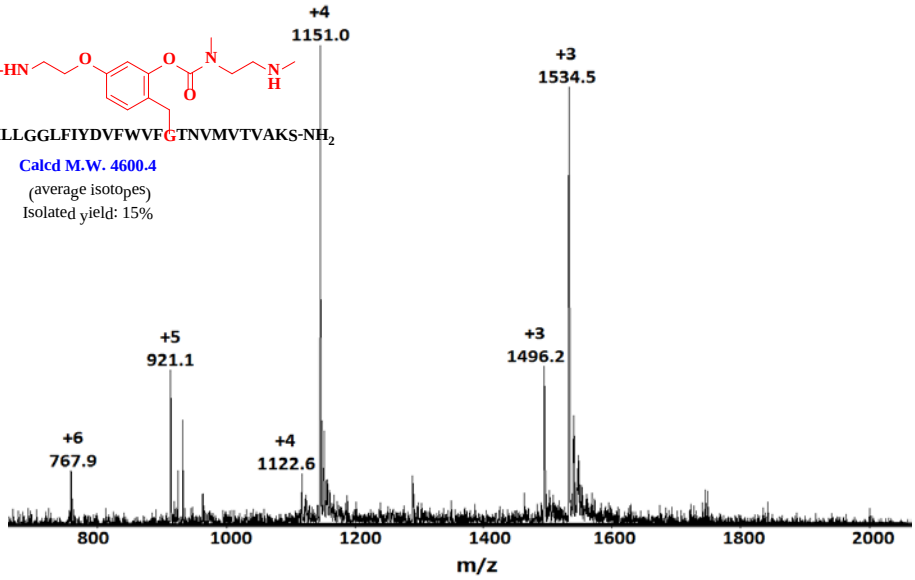


Ac-STGCILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2

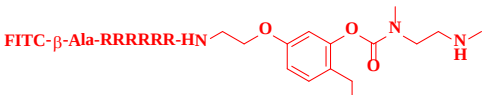
Calcd M.W. 4600.4

(average isotopes)

Isolated yield: 15%



c

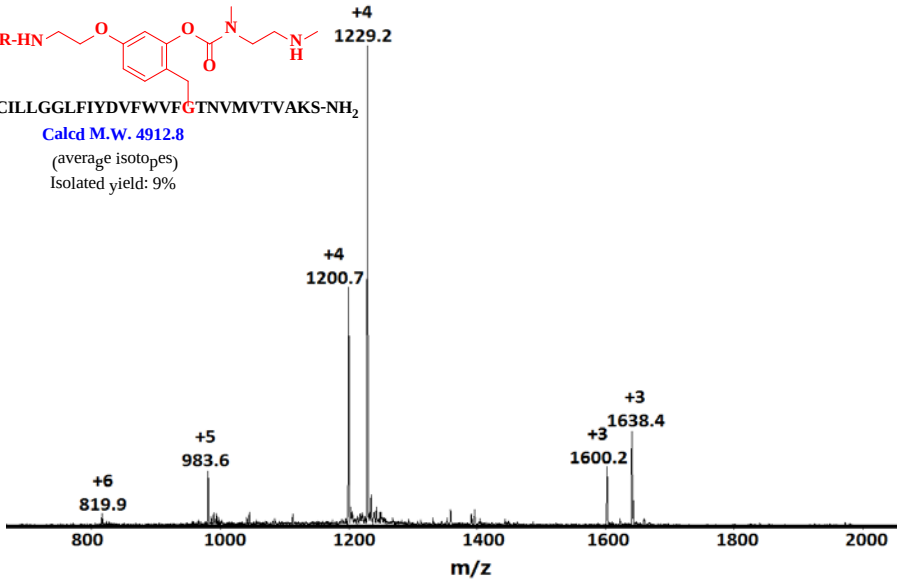


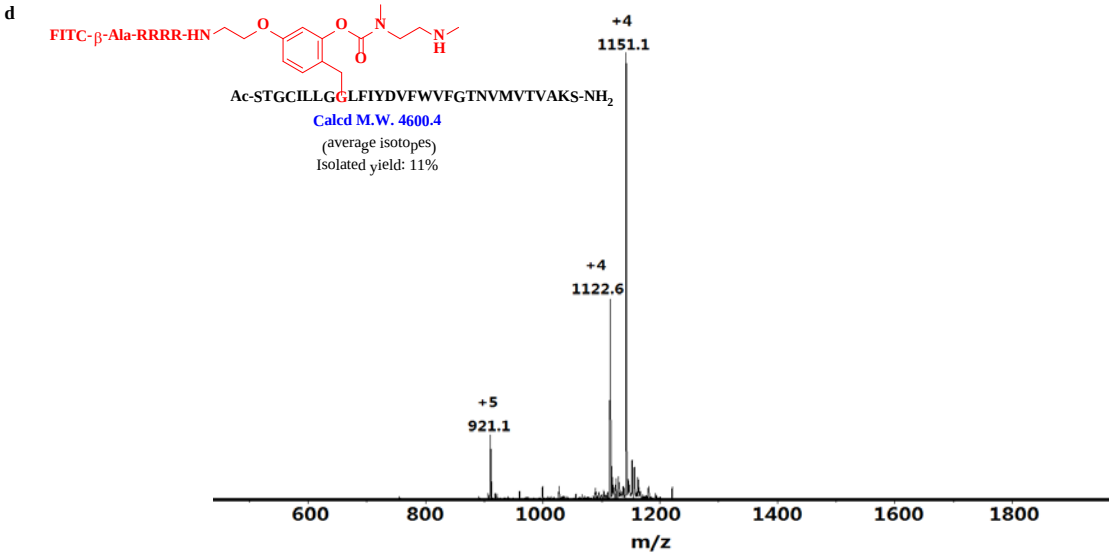
Ac-STGCILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2

Calcd M.W. 4912.8

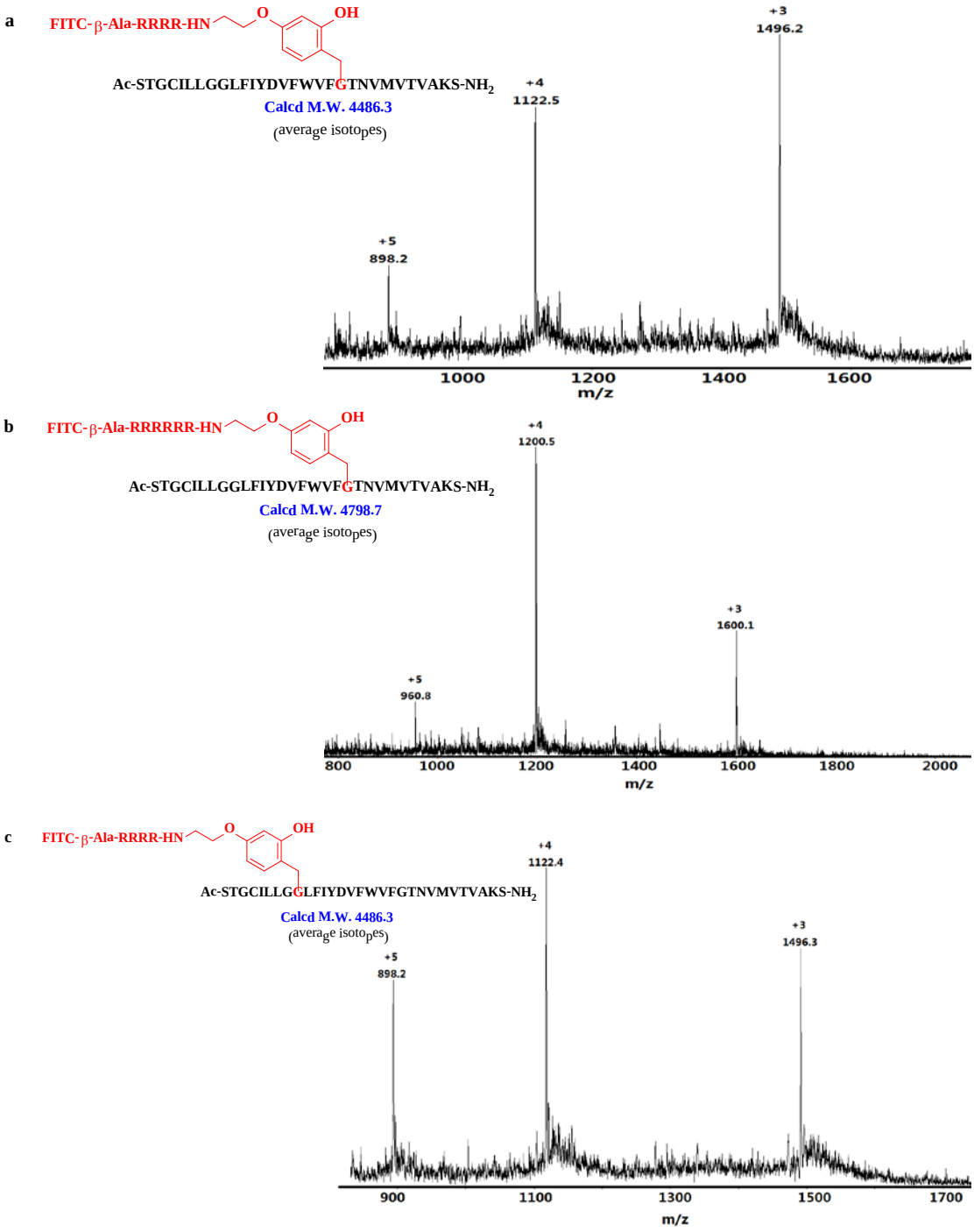
(average isotopes)

Isolated yield: 9%

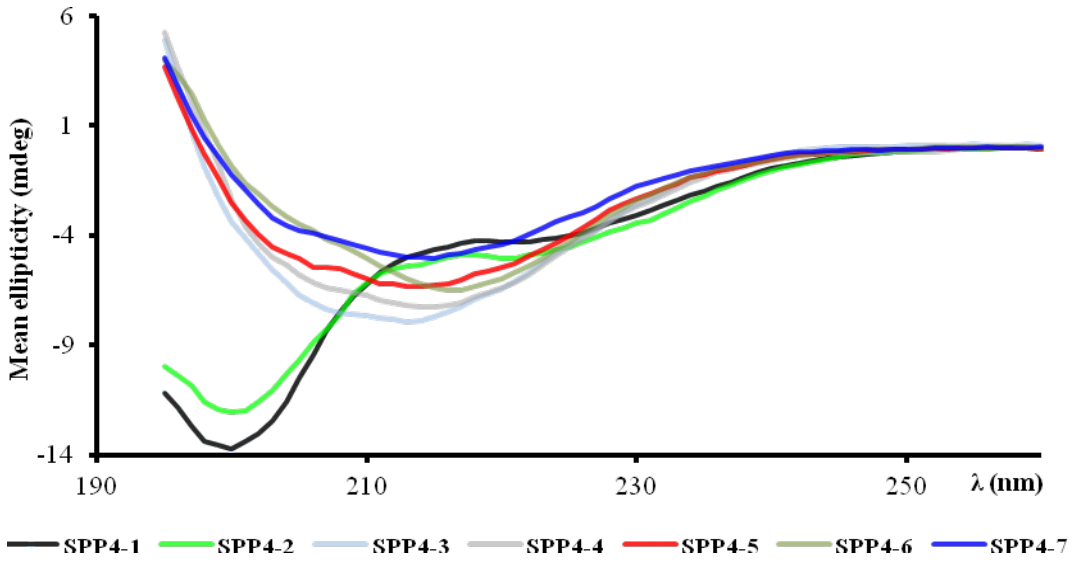




Supplementary Figure 6. ESI-MS data of FITC-labeled peptides analogues. (a) The ESI-MS of SPP4-4. (b) The ESI-MS of SPP4-5. (c) The ESI-MS of SPP4-6. (d) The ESI-MS of SPP4-7.

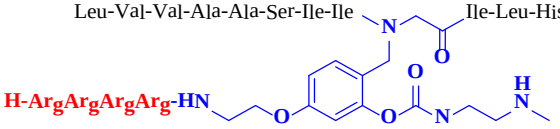


Supplementary Figure 7. ESI-MS data of FITC-labeled peptides analogues. (a) The ESI-MS of SPP4-5'. (b) The ESI-MS of SPP4-6'. (c) The ESI-MS of SPP4-7'.



Supplementary Figure 8. CD spectra of SPP4-1 and its analogues. Peptides were dissolved at about 0.05 mg/mL based on dry weight in 1:1 acetonitrile/H_2O, and CD spectra were recorded on a Pistar π-180 spectrometer from 260 nm to 195 nm using a 1 mm path length quartz cuvette. The spectra for each peptide was performed in triplicate, averaged, subtracted from blank and smoothed.

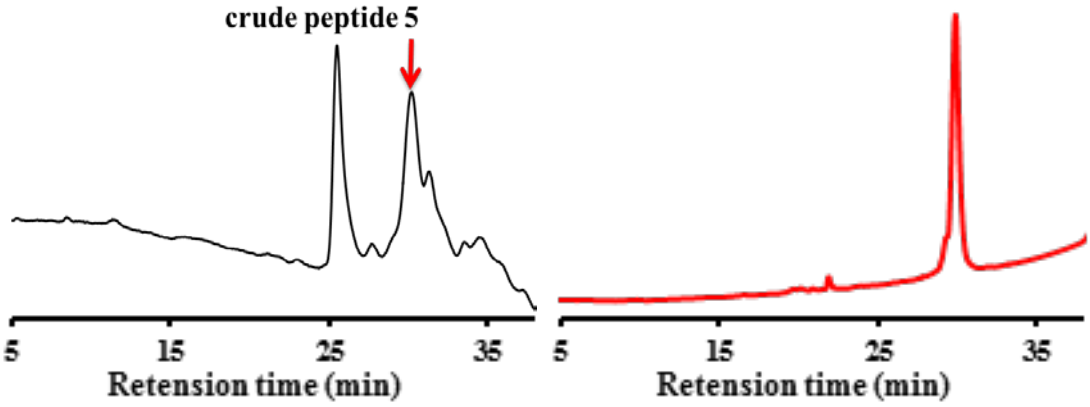
a H-Met-Ser-Leu-Leu-Thr-Glu-Val-Glu-Thr-Pro-Ile-Arg-Asn-Glu-Trp-Gly-Cys-Arg-Cys-Asn-Asp-Ser-Ser-Asp-Pro-Leu-Val-Val-Ala-Ala-Ser-Ile-Ile

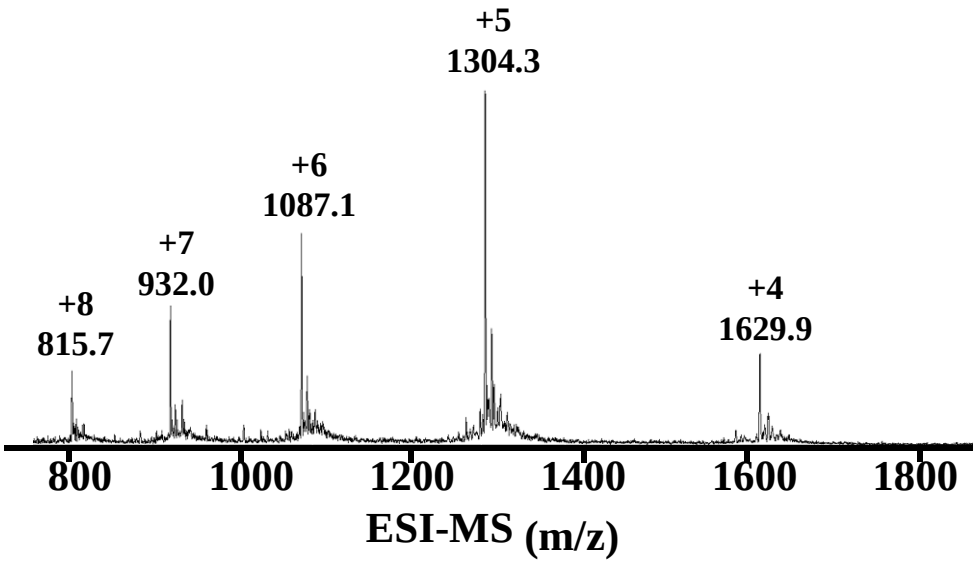


Ile-Leu-His-Leu-Ile-Leu-Trp-Ile-Leu-Asp-Arg-Leu-Phe-Phe-Lys-NHNH2

Calcd M.W. 6517.7 (average isotopes)

(78 mg, Isolated yield: 12%)

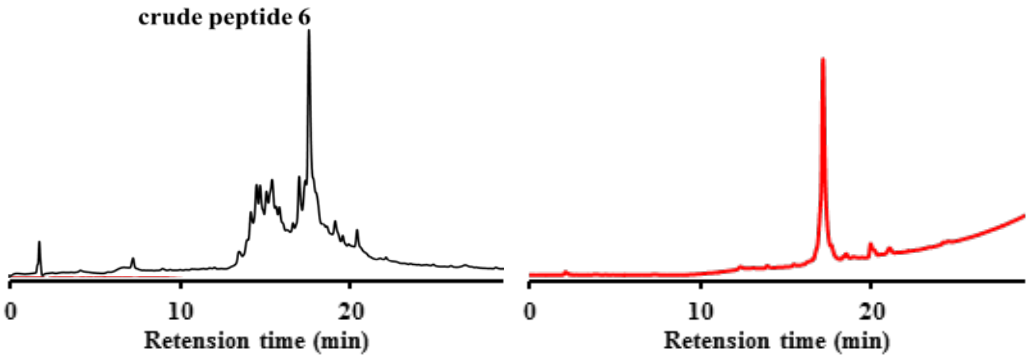


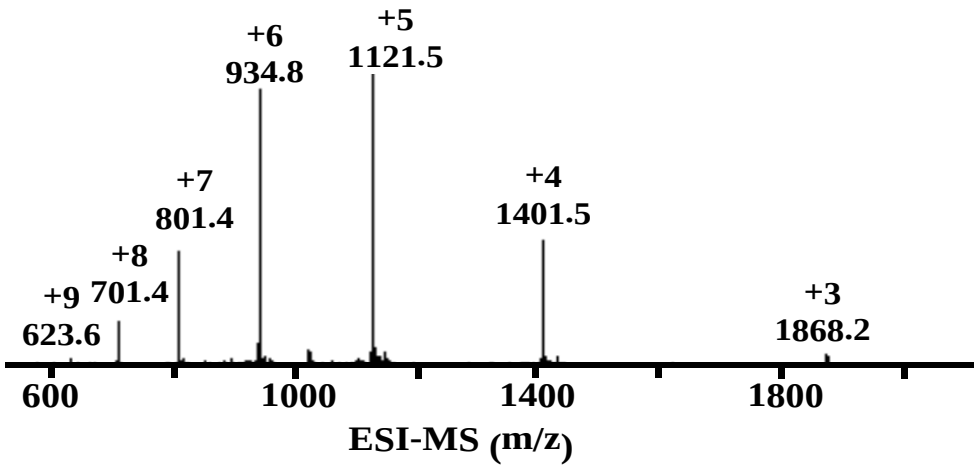


b H-Cys-Ile-Tyr-Arg-Phe-Phe-Glu-His-Gly-Leu-Lys-Arg-Gly-Pro-Ser-Thr-Glu-Gly-Val-Pro-Glu-Ser-Met-Arg-Glu-Glu-Tyr-Arg-Lys-Glu-Gln-Gln-Ser-Ala-Val-Asp-Ala-Asp-Asp-Ser-His-Phe-Val-Ser-Ile-Glu-Leu-Glu-OH

Calcd M.W. 5604.1 (average isotopes)

(112 mg, Isolated yield: 20%)

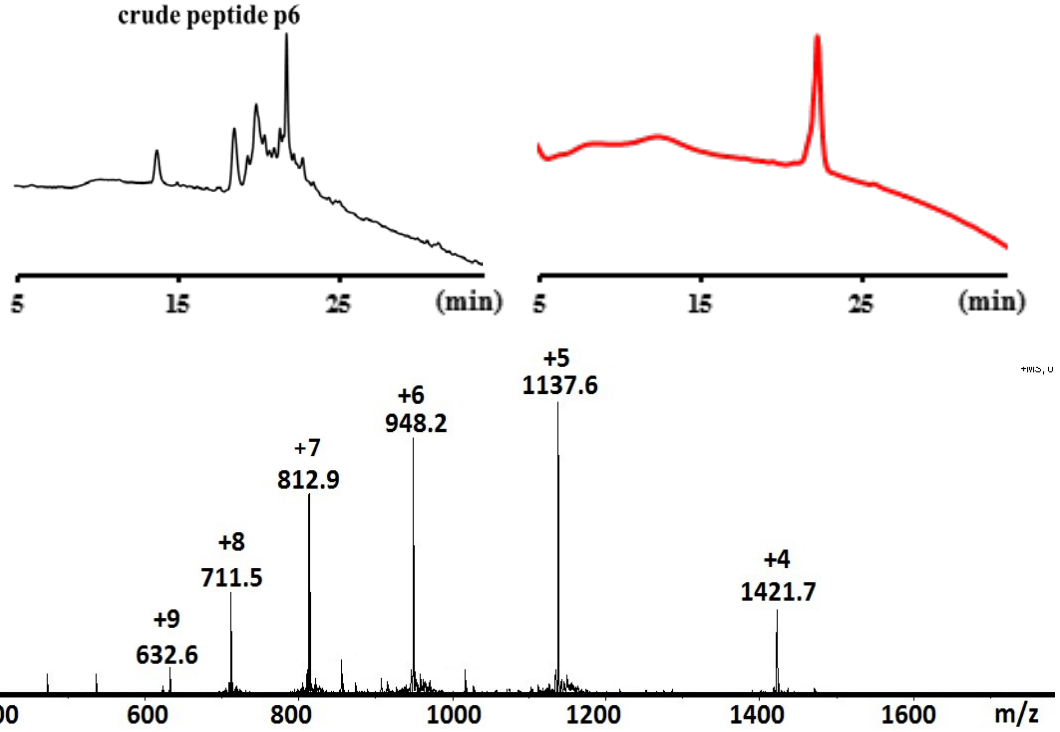




€ H-Cys-Ile-Tyr-Arg-Phe-Phe-Glu-His-Gly-Leu-Lys-Arg-Gly-Pro-pSer-Thr-Glu-Gly-Val-Pro-Glu-Ser-Met-Arg-Glu-Glu-Tyr-Arg-Lys-Glu-Gln-Gln-Ser-Ala-Val-Asp-Ala-Asp-Asp-Ser-His-Phe-Val-Ser-Ile-Glu-Leu-Glu-OH

Calcd M.W. 5683.3 (average isotopes)

(85 mg, Isolated yield: 15%)



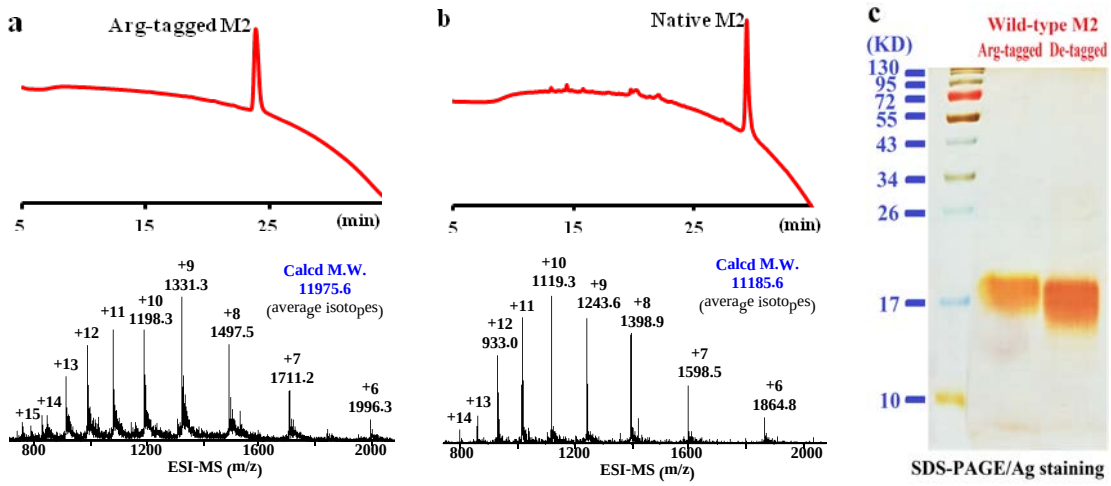
Supplementary Figure 9. Analytical RP-HPLC and ESI-MS characterization of purified peptides **5**, **6**, and **p6** of M2 protein.

(a) Up: RP-HPLC of M2[1-49, 4Arg-Tag]-$NHNH_2$ **5** (Left: crude, Right: purified) using a gradient of 15% buffer B in A to 75% B in A over 30 min on a C4-column (4.6×150 mm) at 56ºC. Down: ESI-MS of **5** showing the [M+4H], [M+5H], [M+6H], [M+7H] and [M+8H] peaks.

(b) Up: RP-HPLC of M2[50-97] **6** (Left: crude, Right: purified) using a gradient of 5% buffer B in A to 95% B in A over 30 min on a C4-column (4.6×150 mm) at 56ºC. Down: ESI-MS of **6** showing the [M+4H], [M+5H], [M+6H], [M+7H], [M+8H] and

[M+9H] peaks.

(c) Up: RP-HPLC of M2[50-97] bearing pSer64 **p6** (Left: crude, Right: purified) using a gradient of 5% buffer B in A to 65% B in A over 30 min on a C4-column (4.6×150 mm) at 56^{o}C. Down: ESI-MS of **p6** showing the [M+4H], [M+5H], [M+6H], [M+7H], [M+8H] and [M+9H] peaks.

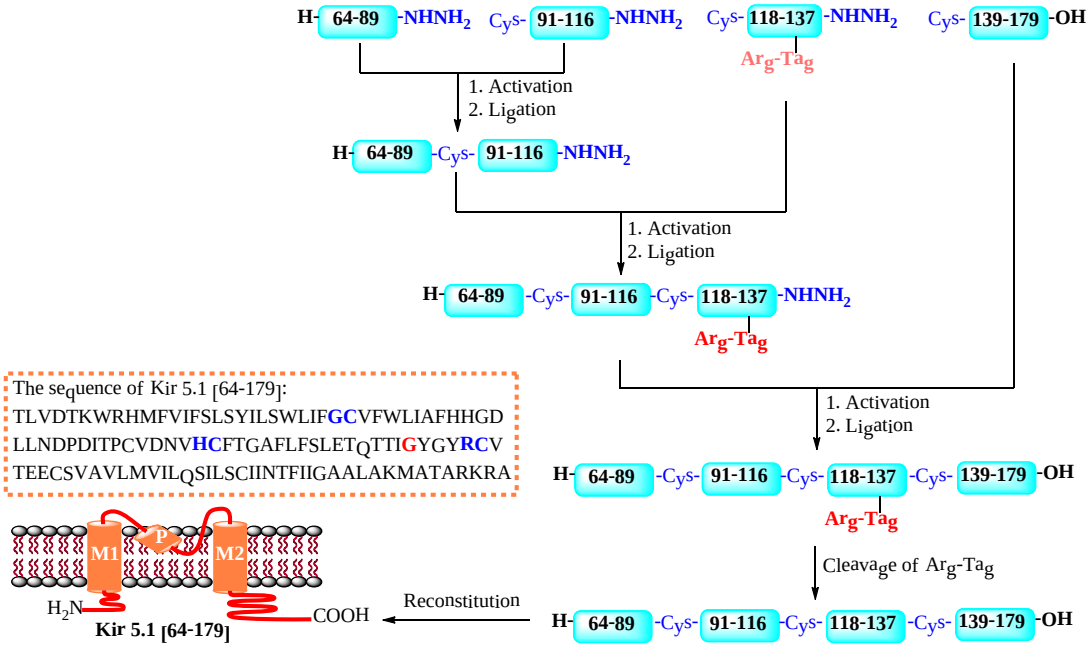


Supplementary Figure 10. Analytical data for synthesis of native M2[1-97] **8**.

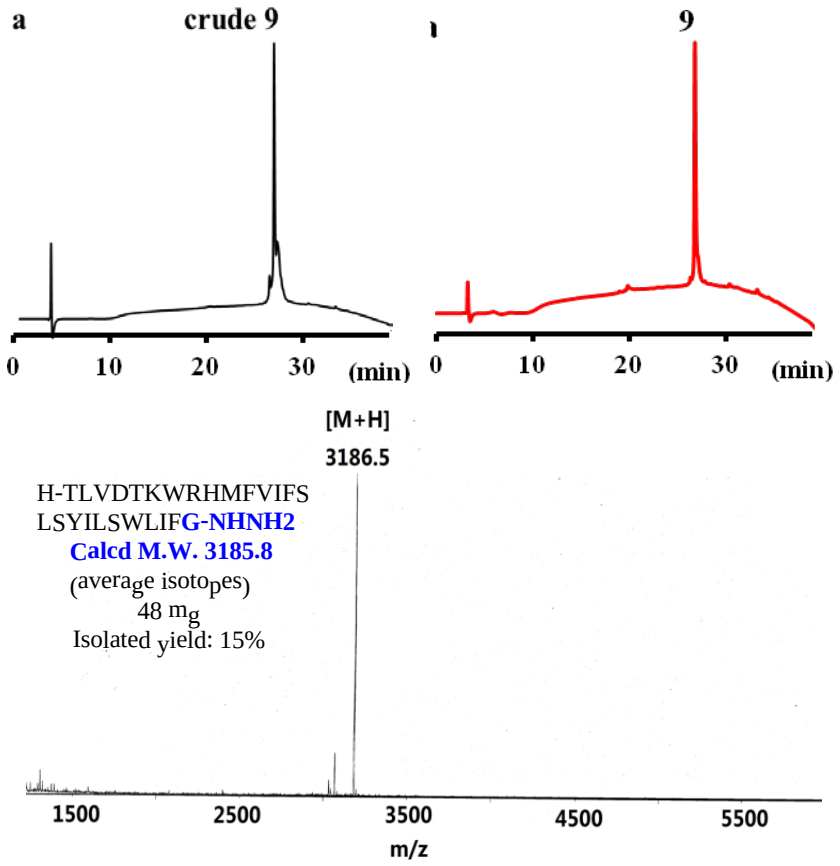
(a) Up: RP-HPLC of purified Arg-Tagged M2[1-97] **7** using a gradient of 5% buffer B in buffer A to 95% B in A over 30 min on a C4-column (4.6×150 mm) at 56°C. Down: ESI-MS data of Arg-Tagged M2[1-97] **7** (1.9 mg, isolated yield: 39%, based on **5**).

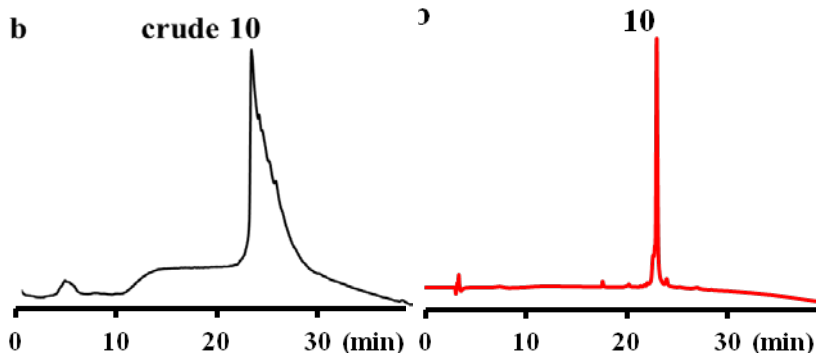
(b) Up: RP-HPLC of purified native M2[1-97] **8** using a gradient of 5% buffer B in buffer A to 95% B in A over 30 min on a C4-column (4.6×150 mm) at 56°C. Down: ESI-MS data of native M2[1-97] **8**.

(c) SDS-PAGE/Ag staining: lane left, the ligation products Arg-tagged M2[1-97] **7**; lane right, de-tagged products native M2[1-97] **8** by TFA cocktails.



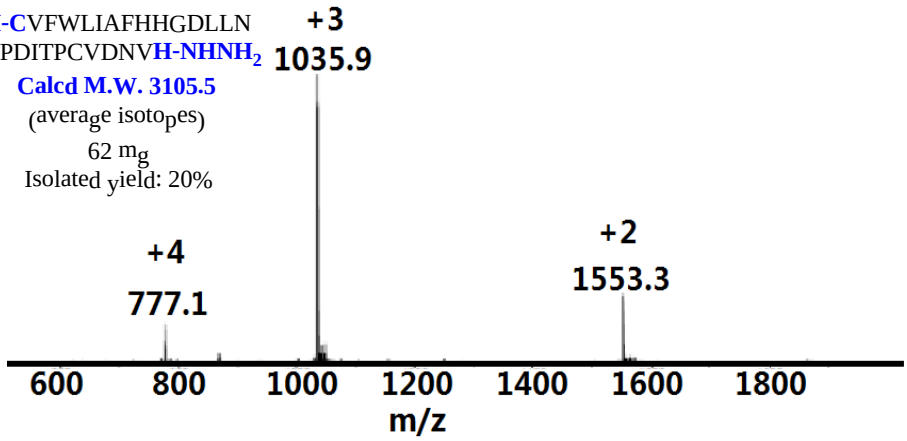
Supplementary Scheme 4. Chemical synthetic strategy of Kir 5.1[64-179] protein.

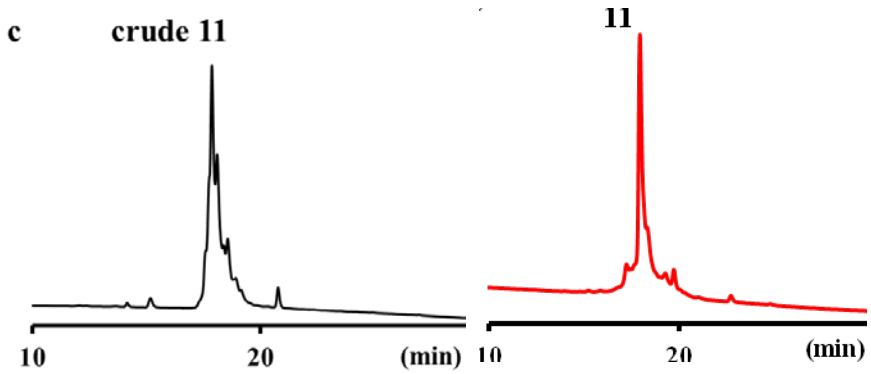


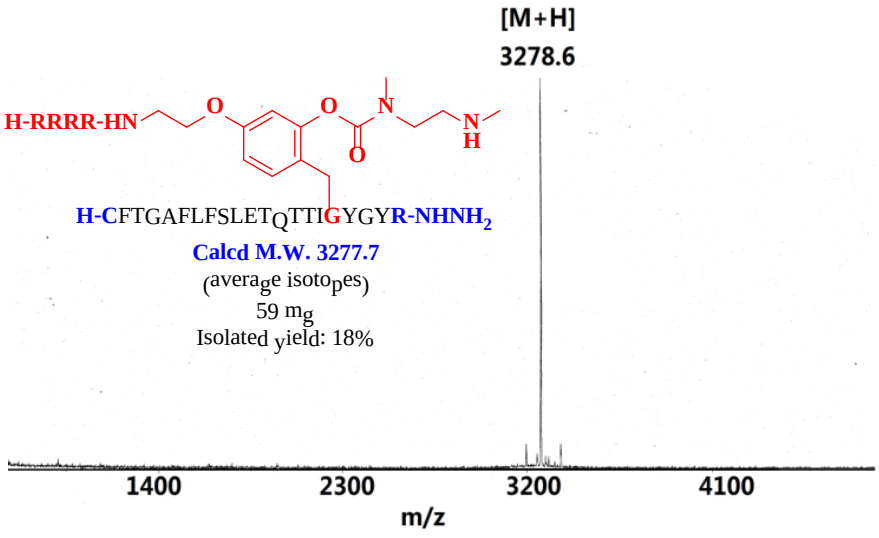


H-CVFWLIAFHHGDLLN
DPDITPCVDNVH-NHNH$_2$

Calcd M.W. 3105.5
(average isotopes)
62 mg
Isolated yield: 20%

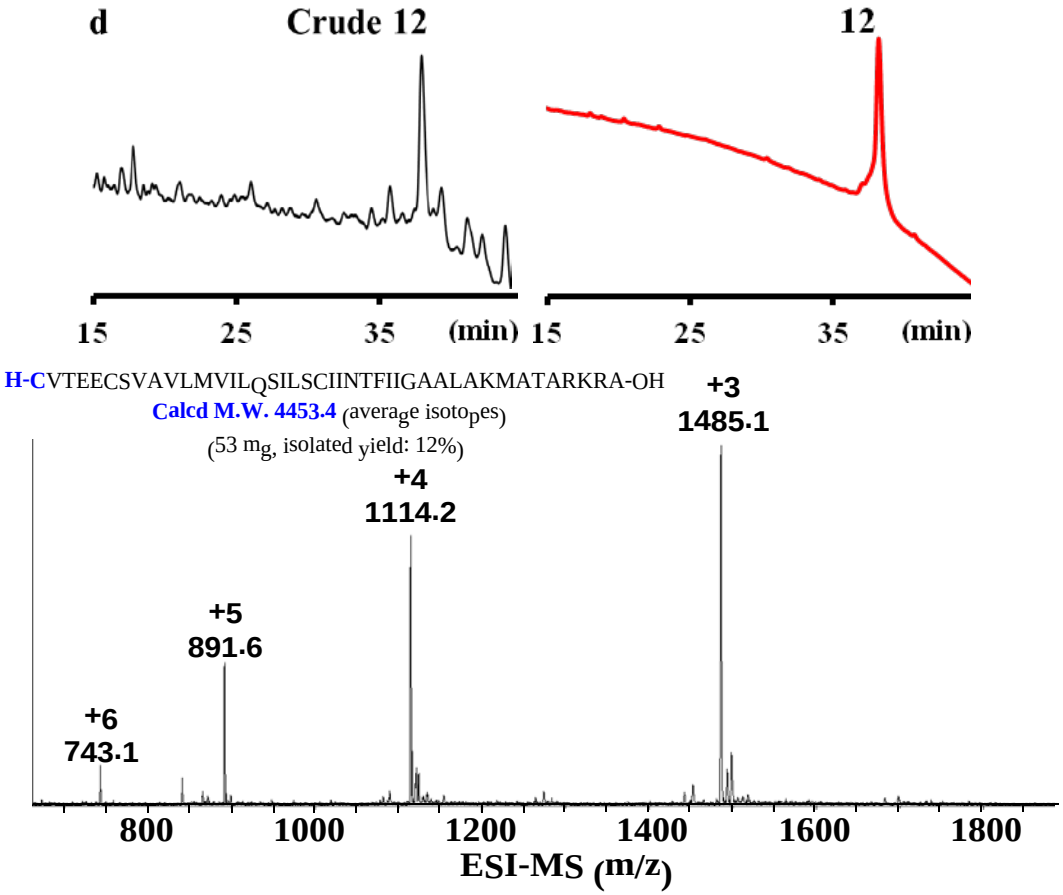






H-CFTGAFLFSLETQTTIGYGYR-NHNH$_2$

Calcd M.W. 3277.7
(average isotopes)
59 mg
Isolated yield: 18%



Supplementary Figure 11. Analytical RP-HPLC and MS characterization of purified peptides **9-12**.

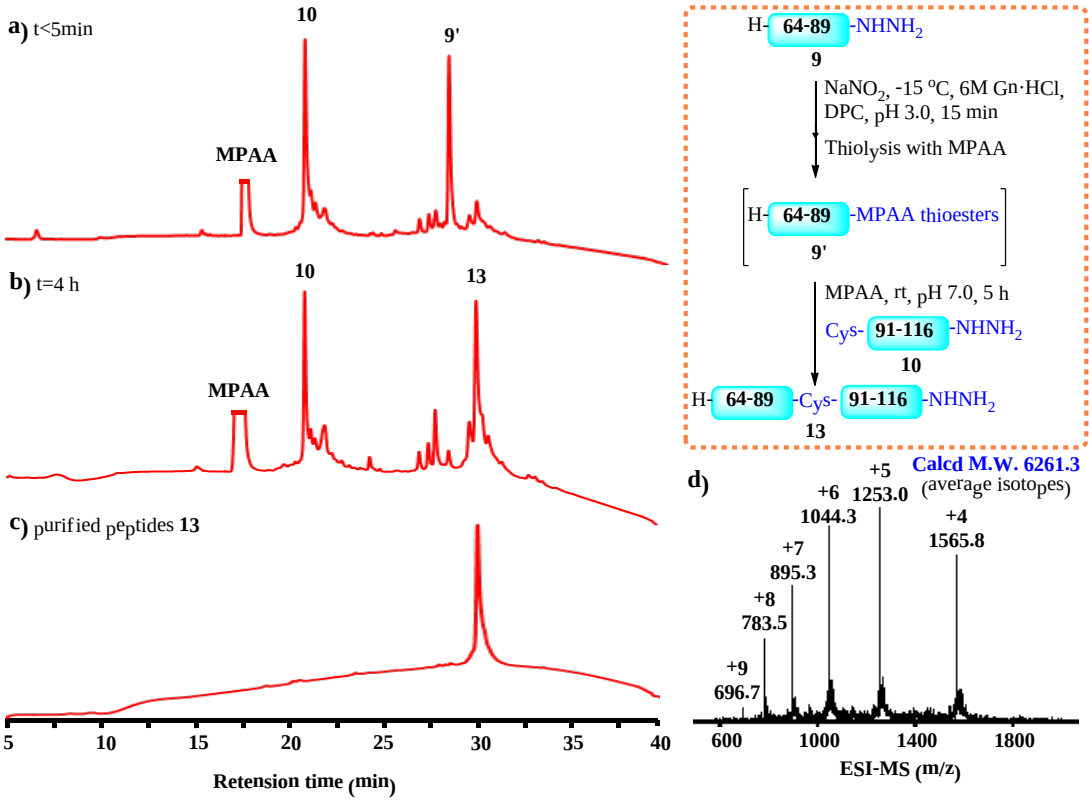
(a) Up: RP-HPLC trace of Kir5.1 [64-89]-$NHNH_2$ **9** (Left: crude, Right: purified) using a gradient of 5% buffer B in buffer A to 95% B in A over 30 min on a C4-column (4.6×250 mm) at rt. Down: MALDI-TOF-MS of **9** showing the [M+H] peak.

(b) Up: RP-HPLC trace of Kir5.1 [90-116]-$NHNH_2$ **10** (Left: crude, Right: purified) using a gradient of 5% buffer B in buffer A to 95% B in A over 30 min on a C4-column (4.6×250 mm) at rt. Down: ESI-MS of **10** showing the [M+2H], [M+3H] and [M+4H] peaks.

(c) Up: RP-HPLC trace of Kir5.1 [117-137, 4Arg-Tag]-$NHNH_2$ **11** (Left: crude, Right: purified) using a gradient of 20% buffer B in buffer A to 50% B in A over 30 min on a C4-column (4.6×150 mm) at rt. Down: MALDI-TOF-MS of **11** showing the [M+H] peak.

(d) Up: RP-HPLC trace of Kir5.1 [138-179]-OH **12** (Left: crude, Right: purified) using a gradient of 45% buffer B in buffer A to 85% B in A over 40 min on a C4-column (4.6×250 mm) at 56°C. Down: ESI-MS of **10** showing the [M+2H], [M+3H] and [M+4H] peaks.

H-TLVDTKWRHMFVIFSLSYILSWLIF**GC**VFWLIAFHHGDLLNDPDITPCVDNV**H-NHNH$_2$**



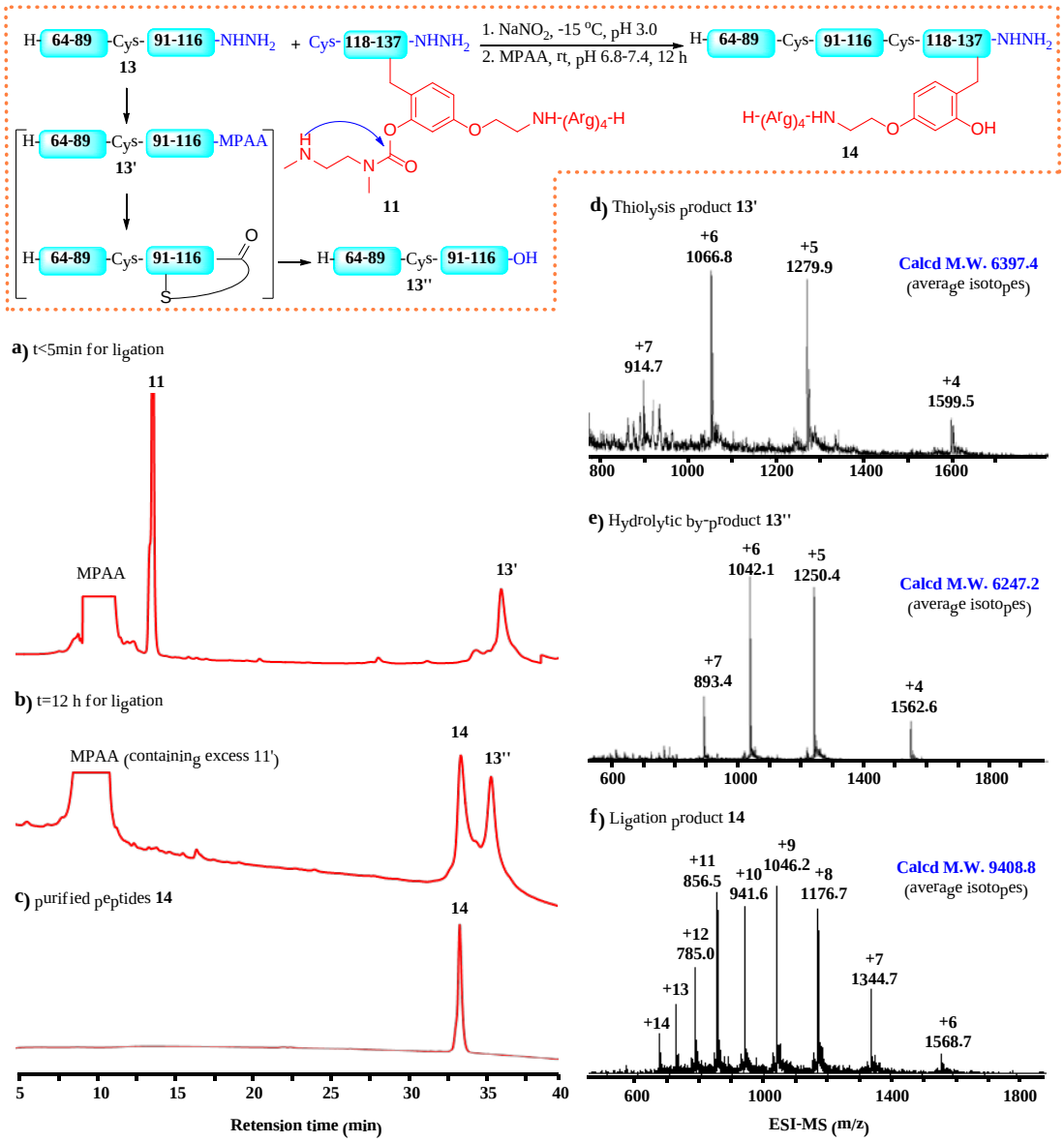
Supplementary Figure 12. Analytical data for chemical ligation of peptide hydrazides **9** and **10**. Inset shows the synthetic route for peptides **13**.

(a) RP-HPLC of ligation after oxidation of peptide hydrazides **9**, thiolysis with MPAA and subsequent ligation within 5 min.

(b) RP-HPLC of ligation for 4 h.

(c) RP-HPLC of purified peptides **13**. RP-HPLC runs on a C4 column (4.6×250 mm) using a gradient of 5% buffer B in buffer A to 95% B in A over 30 min at 56°C.

(d) ESI-MS data of ligaiton prduct **13** (10.7 mg, isolated yield: 57%, based on **9**).



Supplementary Figure 13. Analytical data for chemical ligation of peptide hydrazides **13** and **11**. Inset shows the synthetic route for peptides **14**.

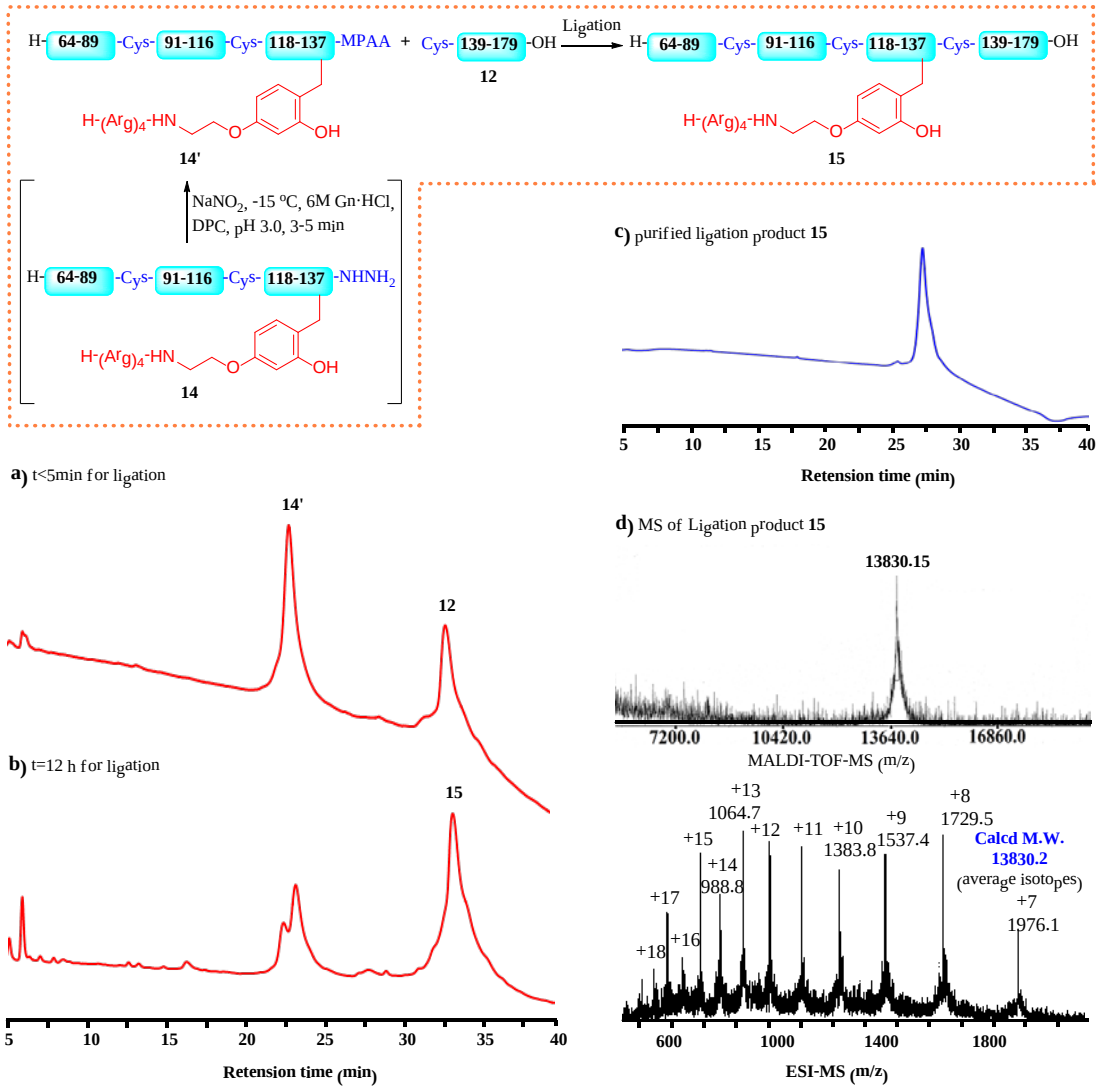
(a) RP-HPLC of ligation after oxidation of peptide hydrazides **13**, thiolysis with MPAA to obtain MPAA-thioesters **13'**, and subsequent ligation within 5 min with pH 6.8 at rt.

(b) RP-HPLC of ligation for 6h with pH 6.8 and subsequent for 6 h with pH 7.4 at rt. The hydrolysis product **13''** of thioesters **13'** was observed in this ligation.

(c) RP-HPLC of purified peptides **14**. RP-HPLC runs on a C4 column (4.6×150 mm) using a gradient of 5% buffer B in buffer A for 2min ,and then 35 to 65% B in A over 30 min at 56°C.

(d) ESI-MS data of the hydrolysis product **13''**.

(e) ESI-MS data of ligation product **14** (4.4 mg, isolated yield: 29%, based on **13**).



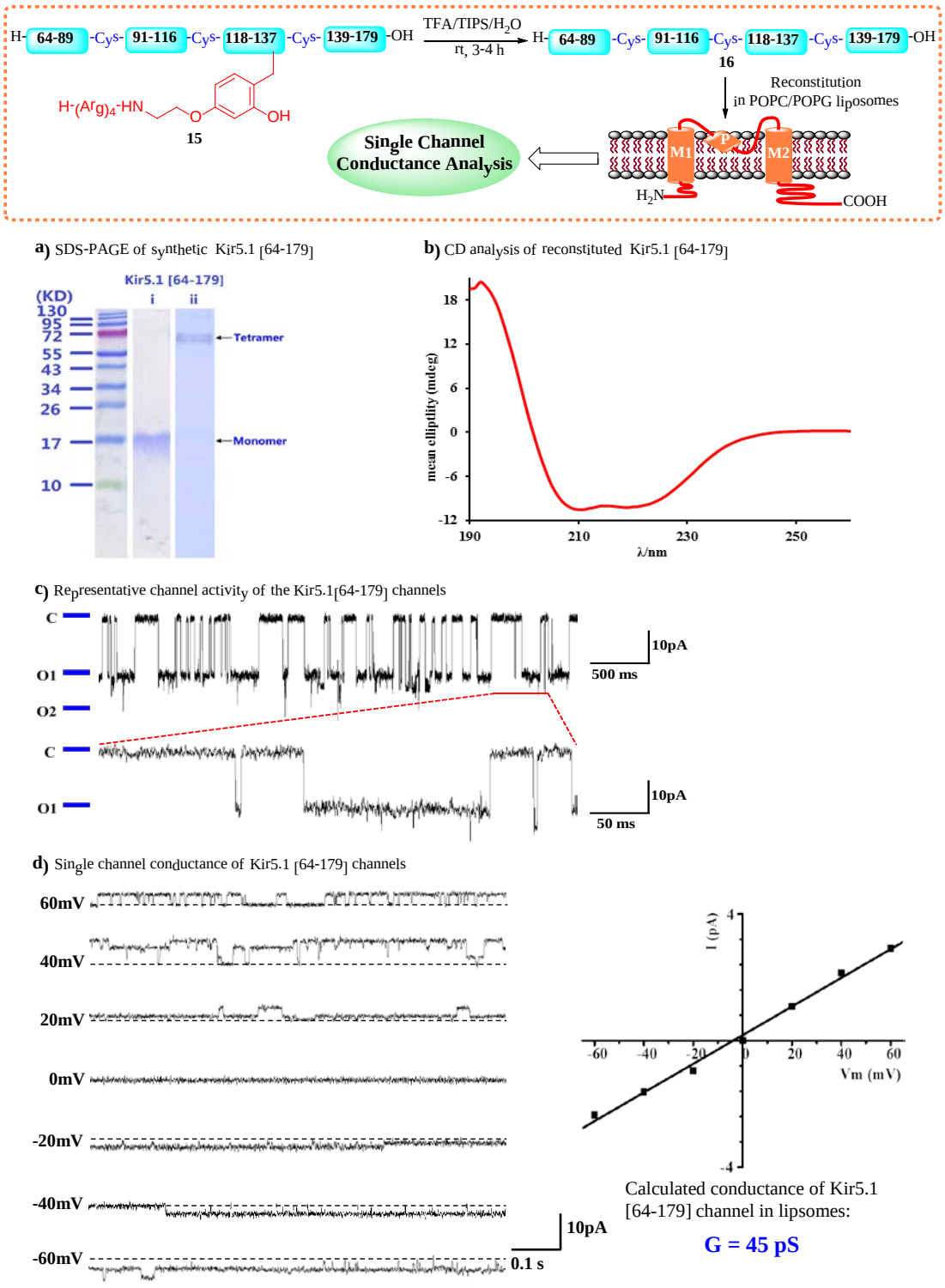
Supplementary Figure 14. Analytical data for chemical ligation of peptide hydrazides **14** and **12**. Inset shows the synthetic route for peptides **14**.

(a) RP-HPLC of ligation after rapid oxidation of peptide hydrazides **14**, thiolysis with MPAA to obtain MPAA-thioesters **13'**, and subsequent ligation within 5 min.

(b) RP-HPLC of ligation for 12 h with pH 7.0 at rt. RP-HPLC runs on a C4 column (4.6×150 mm) using a gradient of 5% buffer B in buffer A for 2min ,and then 35 to 75% B in A over 40 min at 56°C.

(c) RP-HPLC of purified peptides **15** using a C4 column (4.6×150 mm) with a gradient of 5% buffer B in buffer A to 95% B in A over 30 min at 56°C.

(d) MS data of ligation product **15** (1.9 mg, isolated yield: 45%, based on **12**). Up: MALDI-TOF-MS of **15** showing the [M+H] peak. Down: ESI-MS of product **15.**



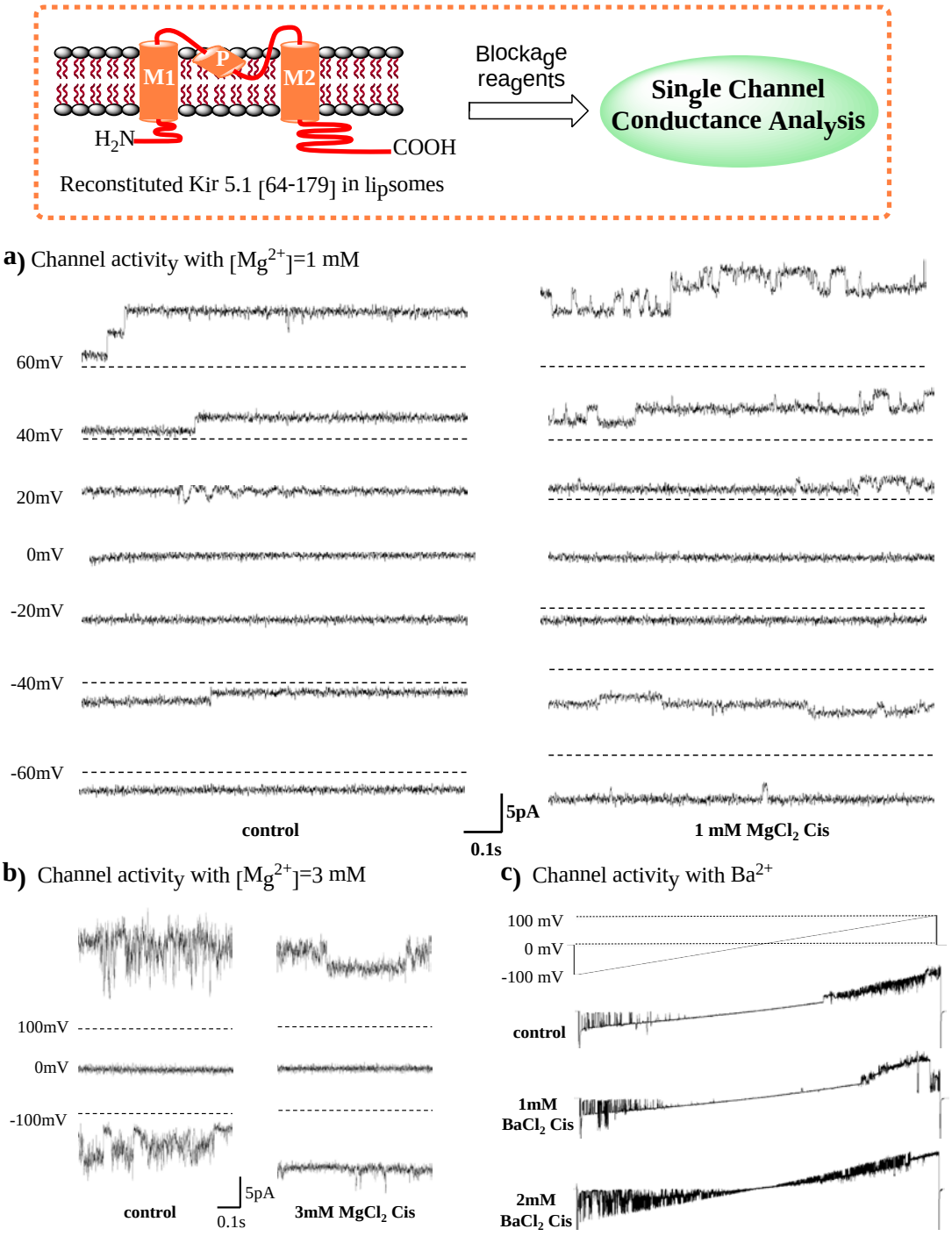
Supplementary Figure 15. Removal of Arg-Tag and biophysical characterization of functional Kir5.1 [64-179] channels by total chemical synthesis.

(a) SDS-PAGE/Ag staining analysis: lane i, the de-tagged products Kir5.1 [64-179] **16** by TFA cocktails; lane ii, reconstituted tetrameric Kir5.1 [64-179] in POPC/POPG liposomes.

(b) CD analysis of functional Kir5.1 [64-179] channels (~10 μM, in a 1 mm quartz cell).

(c) Representative channel activity of the Kir5.1[64-179] channels after reconstituted in POPE/POPG (3:1) lipid vesicles. The traces were recorded at -100 mV in 5mM HEPES/ 150 mM KCl (pH 8.0) both inside and outside. A zoom-in of the gating behavior is shown beneath. C: level of channel closure; O1: level 1 of channel opening; O2: level 2 of channel opening.

(d) Single-channel conductance of Kir5.1 [64-179] in planar lipid bilayers. Left: Single-channel currents were recorded at various membrane potentials. Right: Single-channel conductance was calculated from the current-voltage plot, with the slope conductance of 45 pS. The traces were recorded at -100 mV in 5 mM HEPES/150 mM KCl (pH 8.0) both inside and outside.



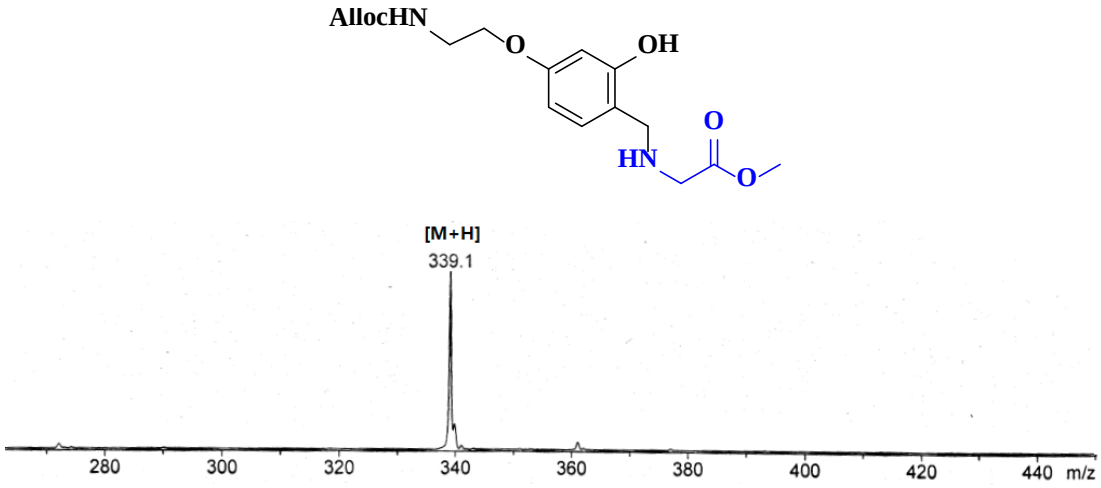
Supplementary Figure 16. Data of conductance measurement with different blockage reagents.

(a) No channel blockage observed upon addition of Mg^{2+} (1 mM $MgCl_2$).

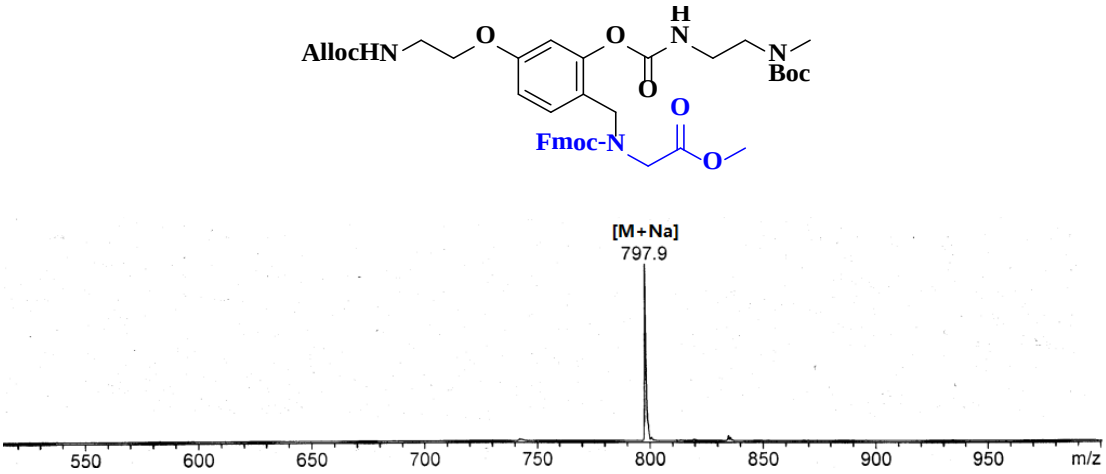
(b) No channel blockage observed at raised concentration of Mg^{2+} (3 mM $MgCl_2$).

(c) No Kir5.1-TMS Channel Blockage Observed upon Addition of Ba^{2+} (Conductance Measurement with Ramp Voltage).

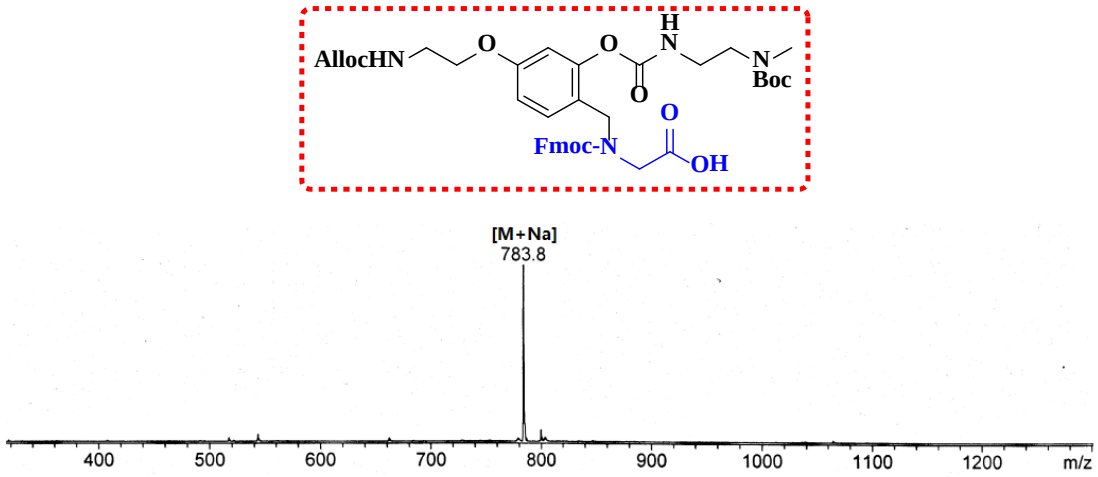
The ESI-MS Date for Organic Compounds



The ESI-MS Spectrum of S2

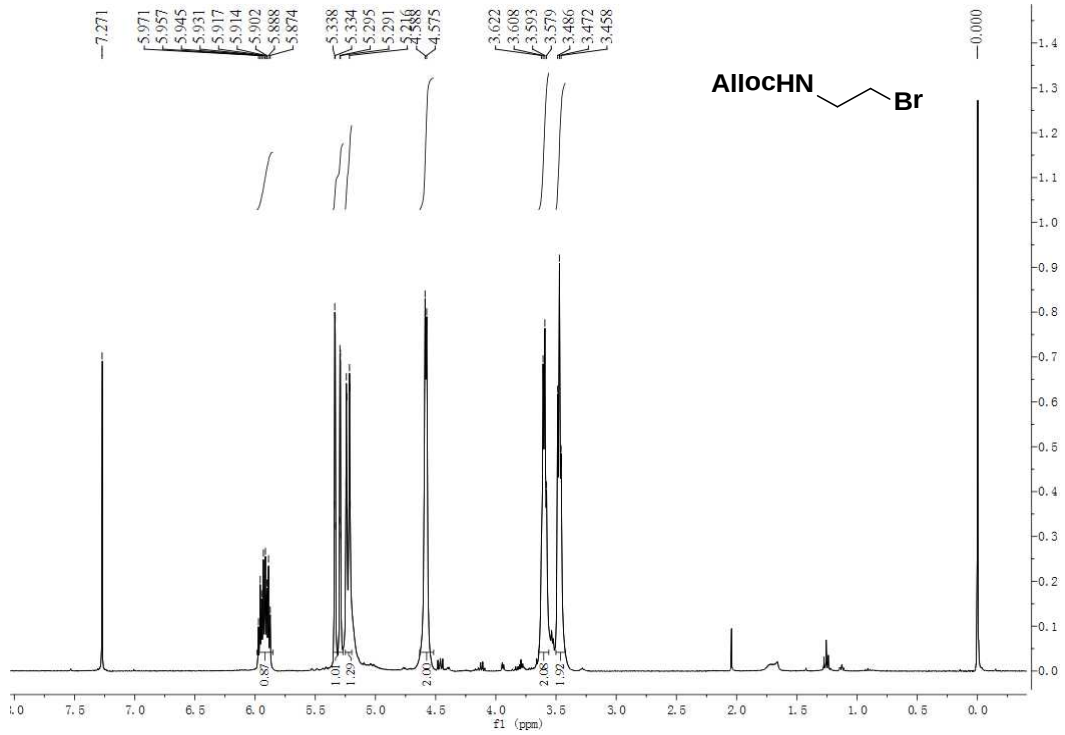


The ESI-MS Spectrum of S4

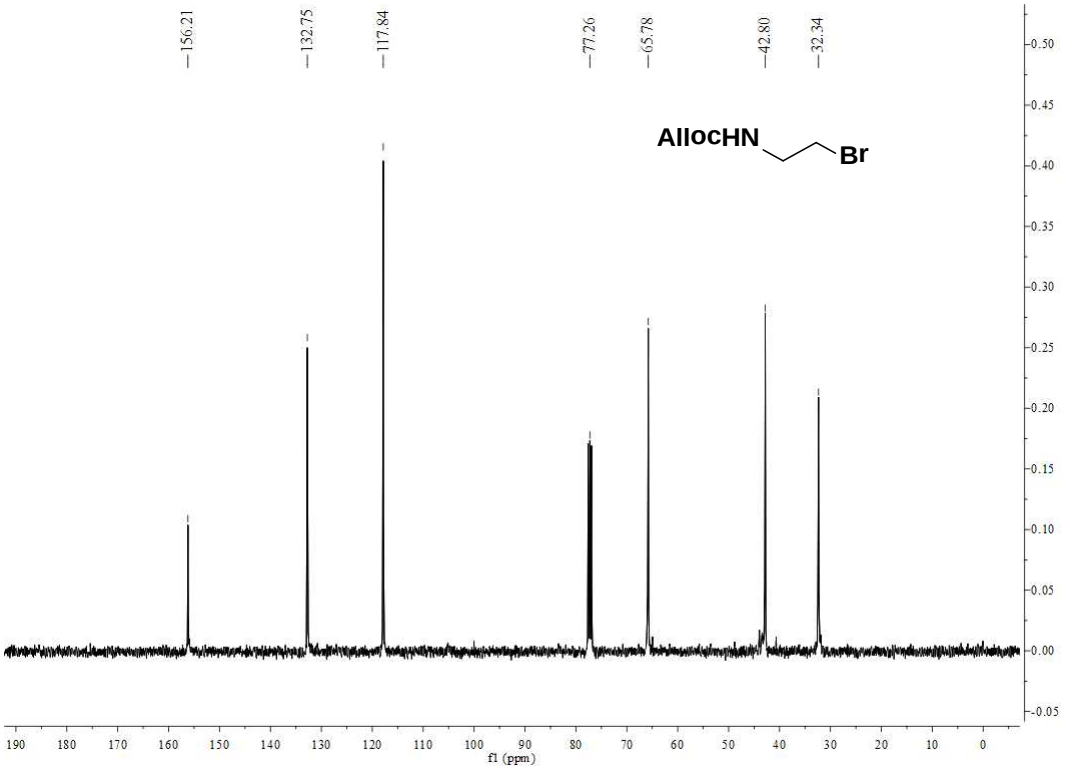


The ESI-MS Spectrum of S5

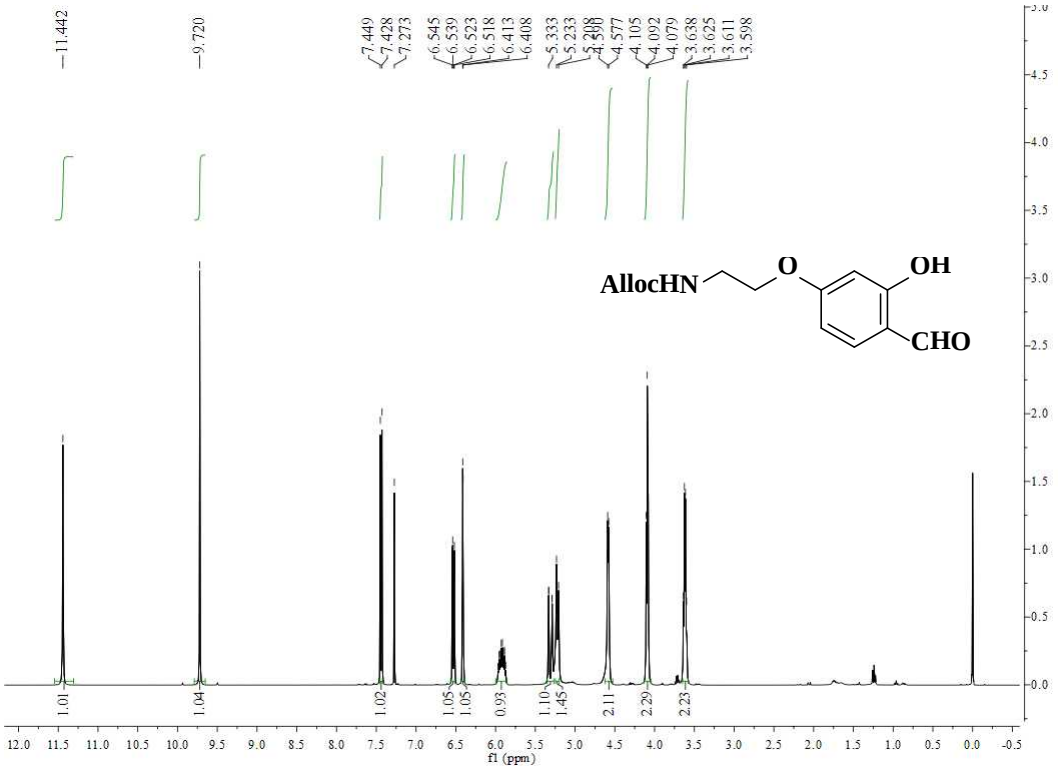
The NMR Date for Organic Compounds



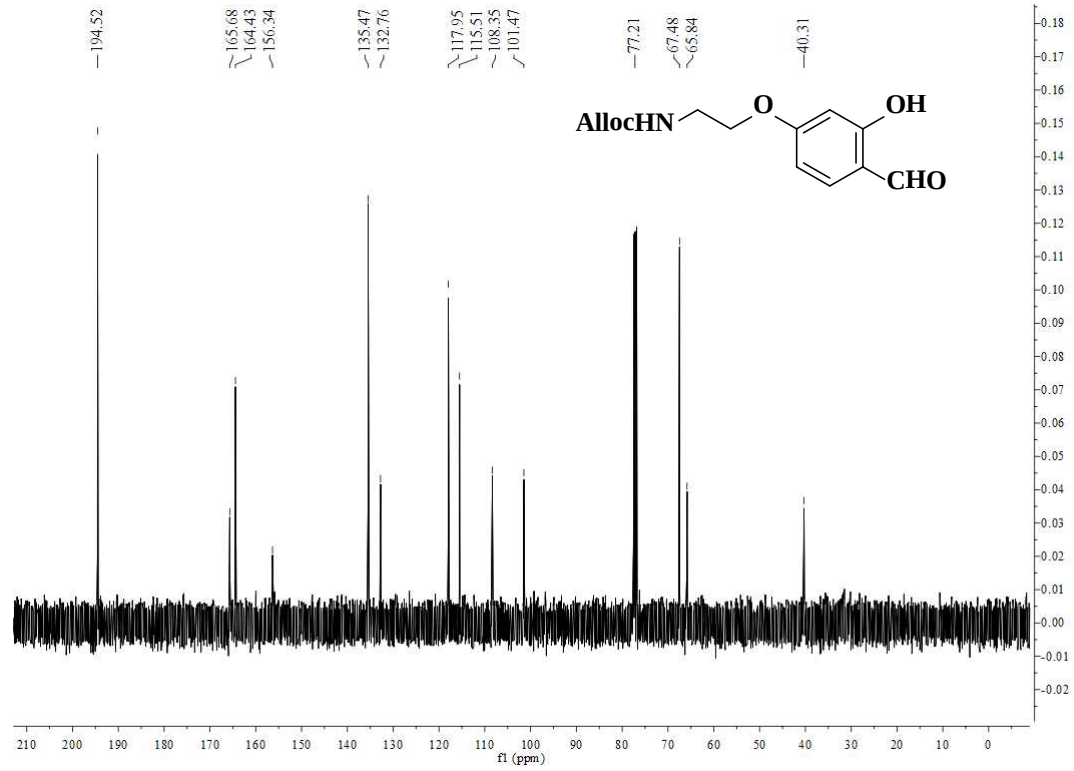
The ^{1}H NMR Spectrum



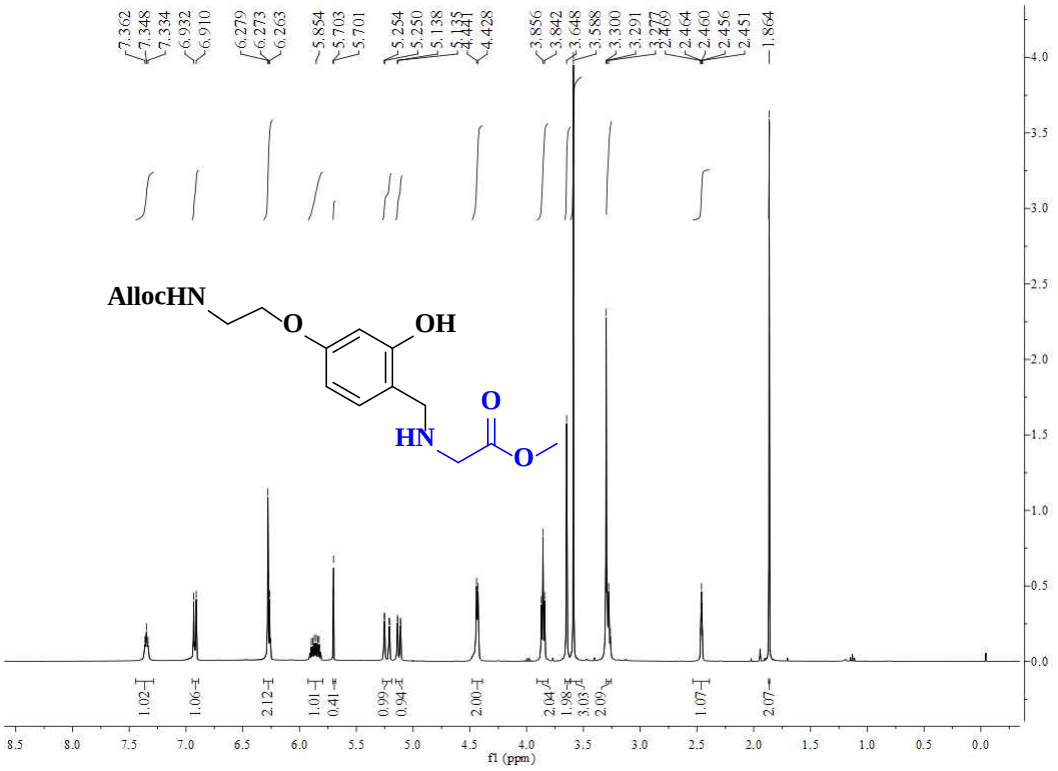
The ^{13}C NMR Spectrum



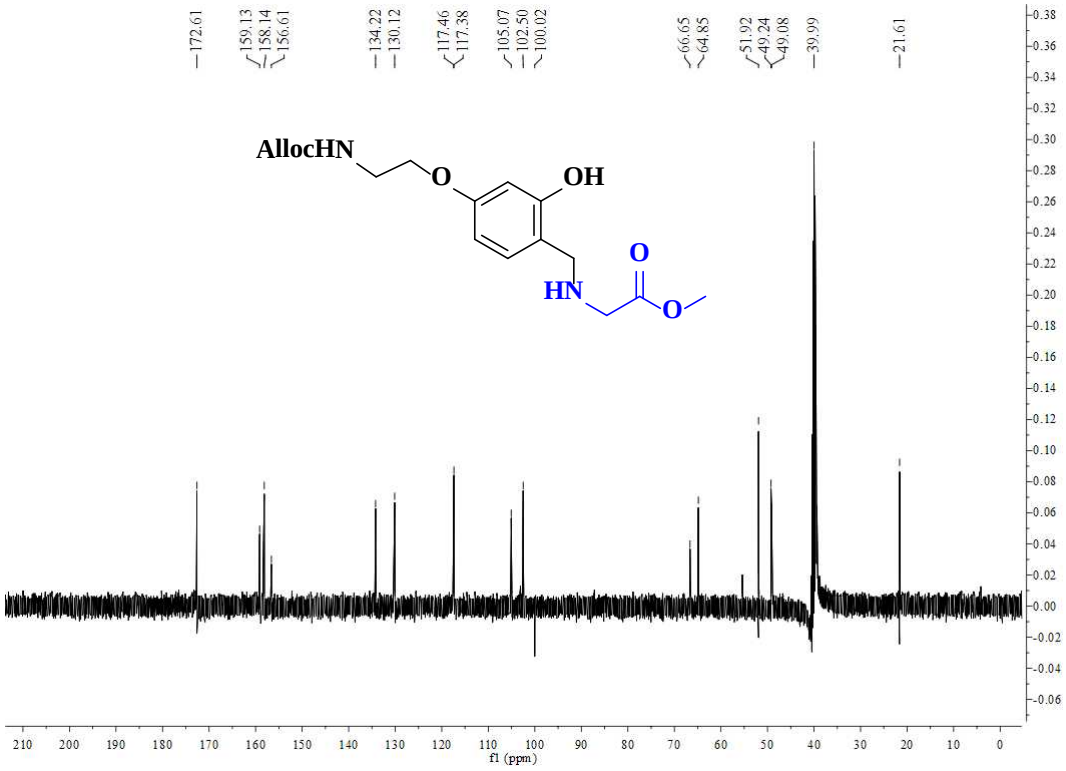
The ^{1}H NMR Spectrum of S1



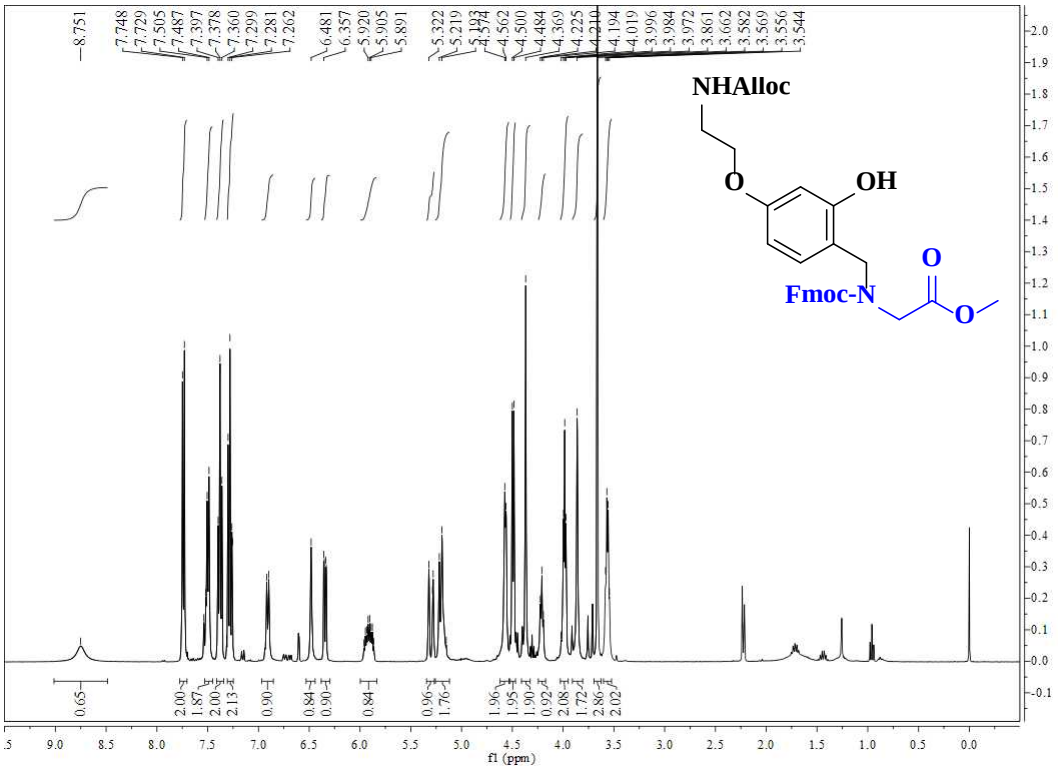
The ^{13}C NMR Spectrum of S1



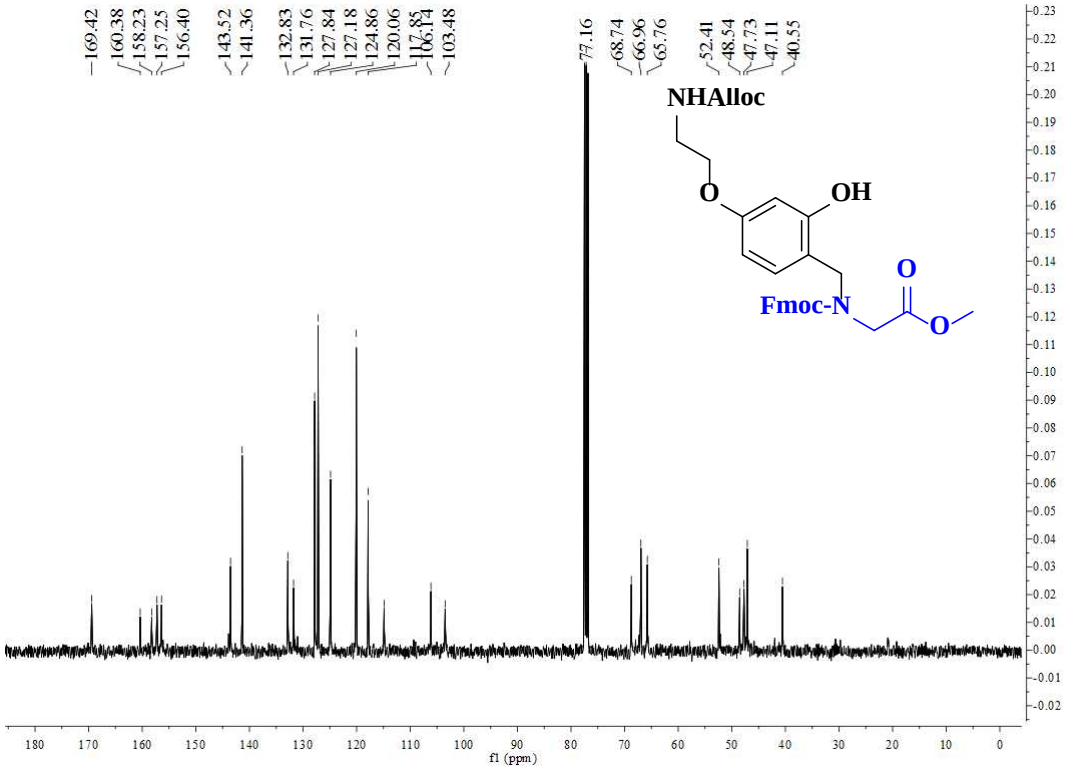
The ^{1}H NMR Spectrum of S2



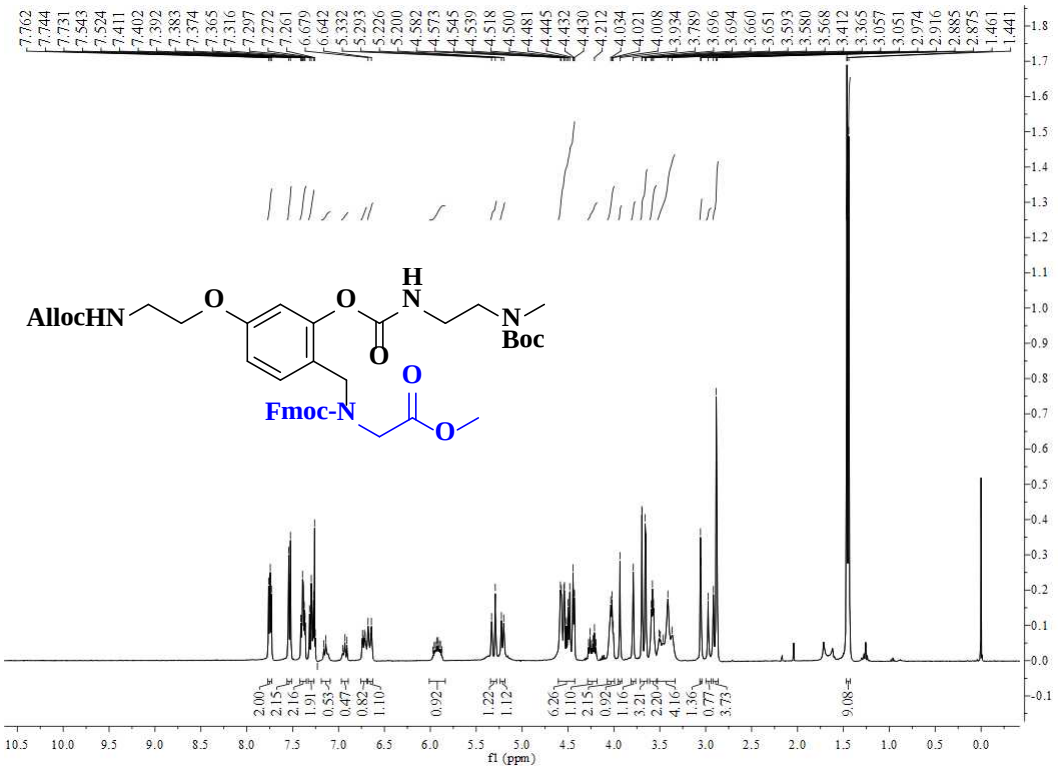
The ^{13}C NMR Spectrum of S2



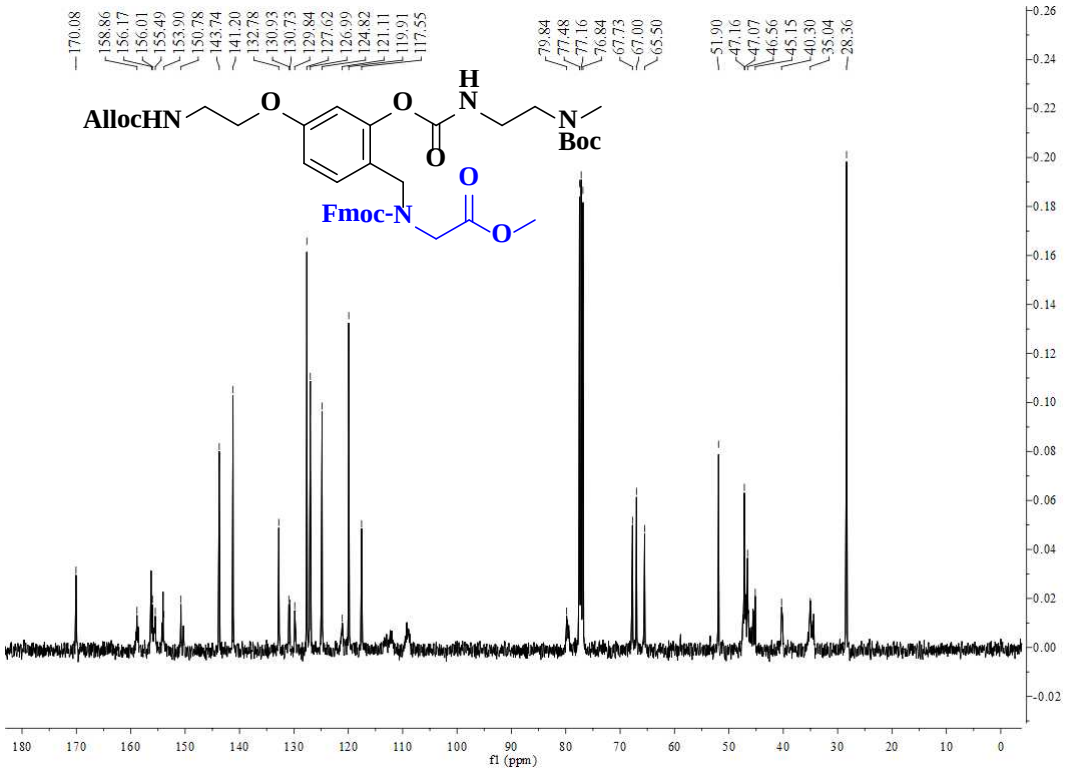
The ^{1}H NMR Spectrum of S3



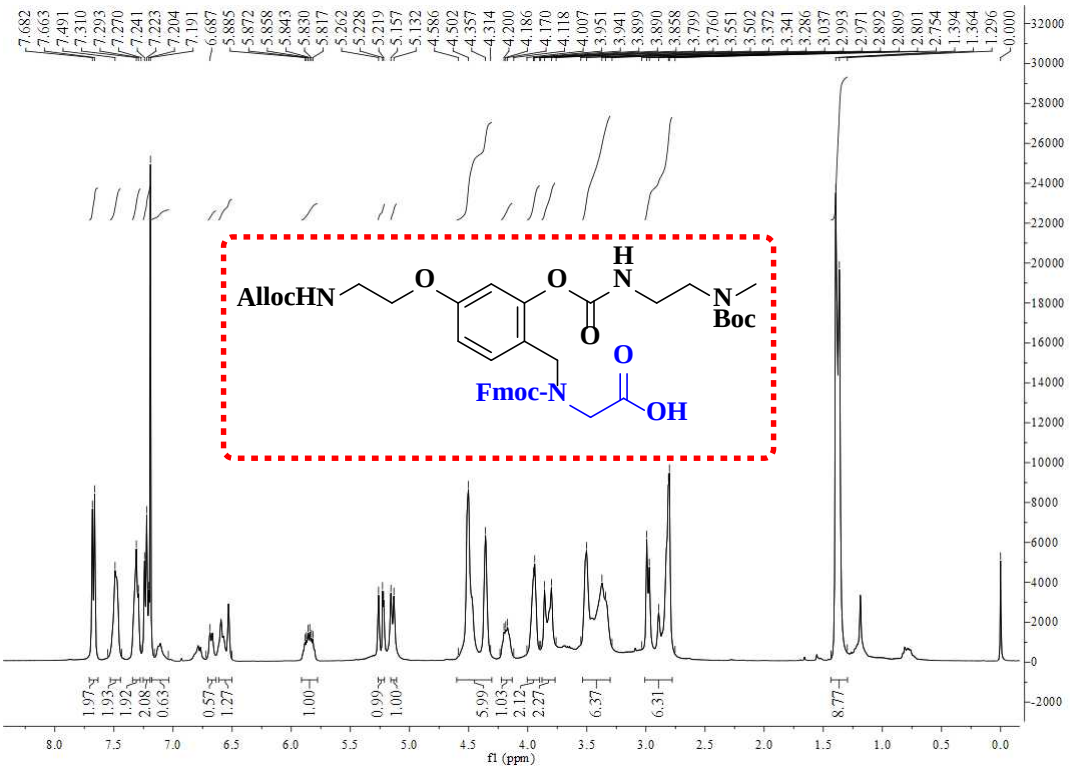
The ^{13}C NMR Spectrum of S3



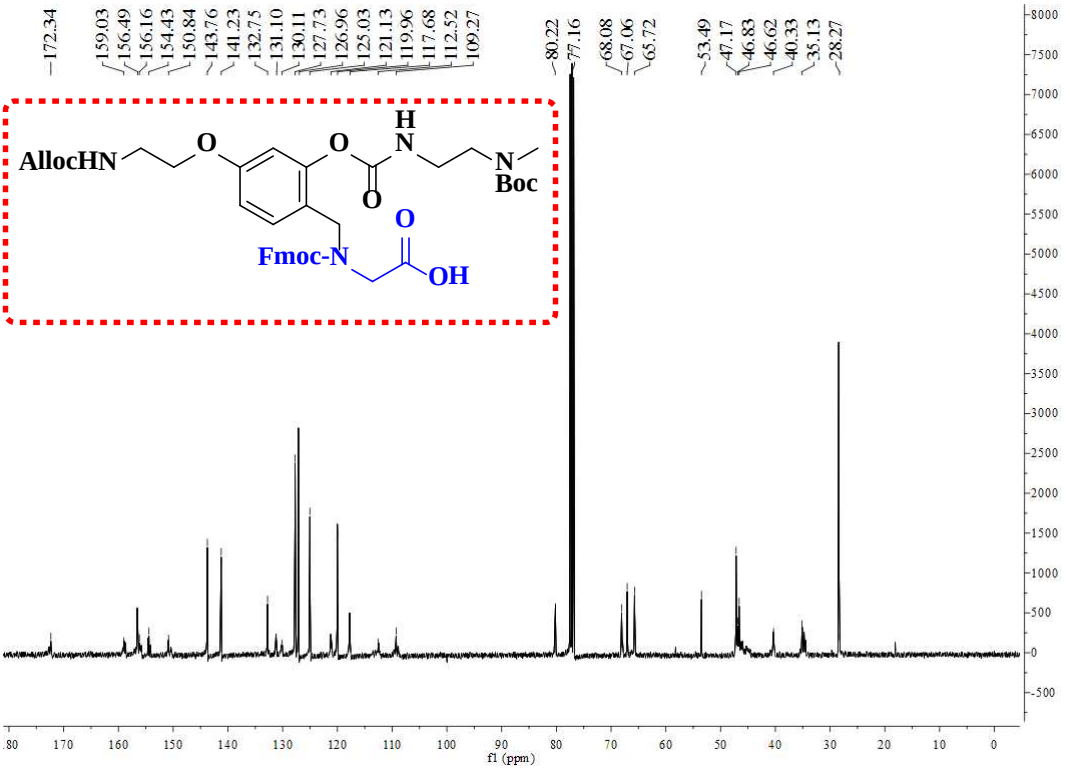
The ^{1}H NMR Spectrum of S4



The ^{13}C NMR Spectrum of S4



The ^{1}H NMR Spectrum of S5



The ^{13}C NMR Spectrum of S5