Electronic Supplementary Information

Intracellular self-assembly of nanoparticles for enhancing cell uptake

Qingqing Miao,[a] Xiaoyu Bai,[a] Yingying Shen,[b] Bin Mei,[a] Jinhao Gao,[c] Li Li[b] and Gaolin Liang*[a,c]

[a]CAS Key Laboratory of Soft Matter Chemistry, Department of Chemistry, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230026, China
[b]Molecular State Key Laboratory of Oncology in Southern China, Imaging Diagnostic and Interventional Center, Cancer Center, Sun Yet-sen University, 651 Dongfeng Road East, Guangzhou 510060, China
[c]The Key Laboratory for Chemical Biology of Fujian Province, Xiamen University, Xiamen 361005, China

***Corresponding author:**

Gaolin Liang, Ph. D.,

Department of Chemistry

University of Science and Technology of China

96 Jinzhai Road, Hefei, Anhui 230026

P. R. China

Tel.: +86-551-3607935

Fax: +86-551-3600730

E-mail: gliang@ustc.edu.cn

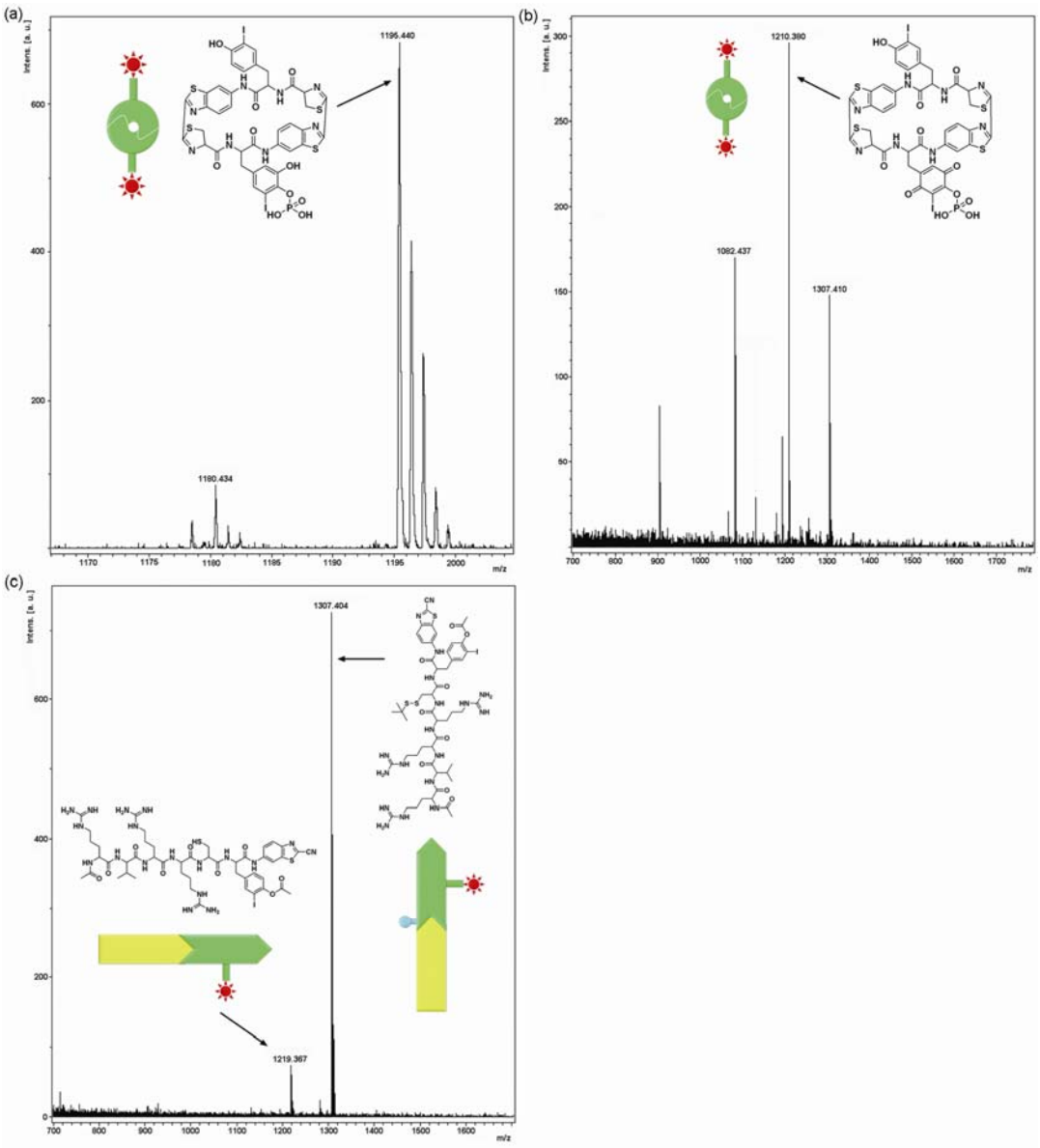


Figure S1. MALDI mass spectra of HPLC peaks at 44.2 min (a), 47-49 min (b), and 49.7 min (c) in figure 2a respectively.

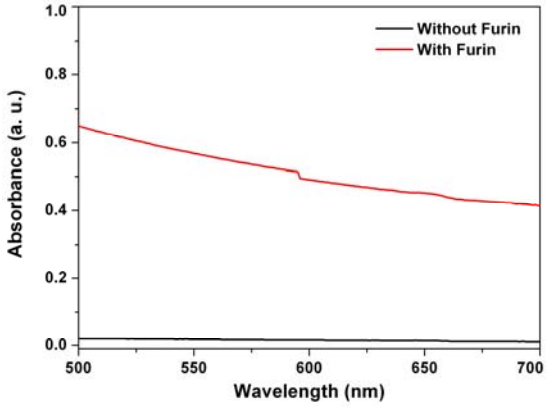


Figure S2. Absorption spectra (500-700 nm due to the light scattering) of **1-Cold** at 100 μM without furin (black) or incubated with furin at 0.5 nmol/U, pH 7.4, and 30 °C for 8 h (red).

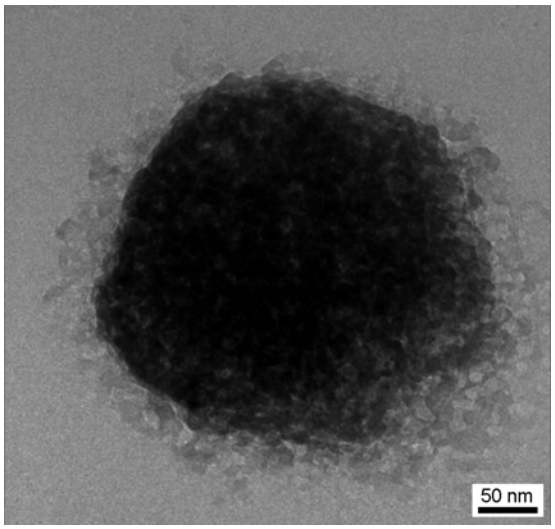


Figure S3. High magnification TEM images of the nanoparticles of **1-Cold** after furin cleavage for 8 h at 0.5 nmol/U and 30 °C.

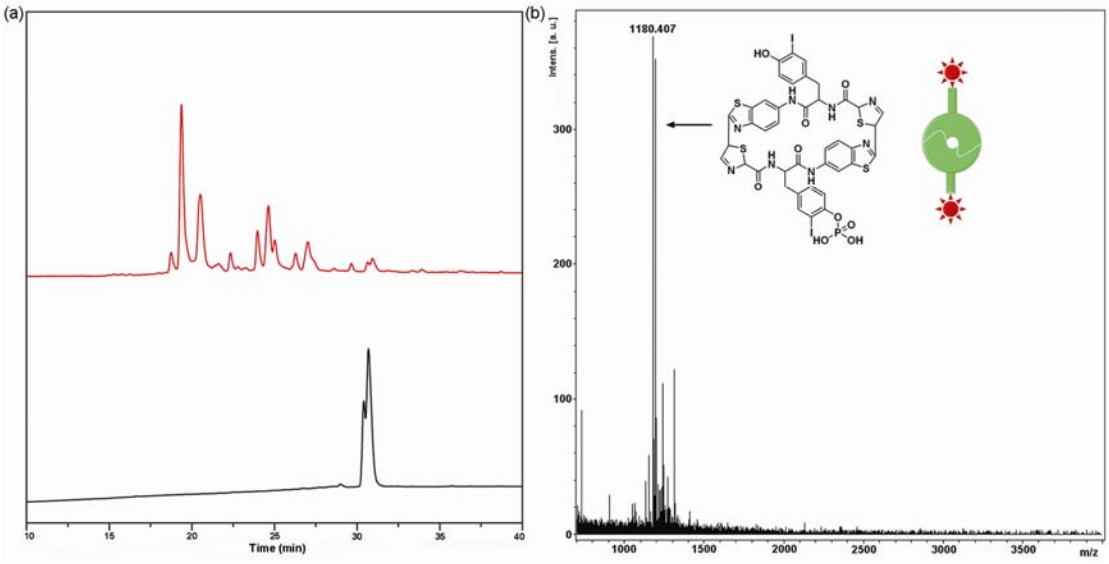


Figure S4. (a) HPLC trace of the incubation mixture of 100 μM of **1-Cold** after 8 h incubation with 0.5 nmol/U of furin at 30 ^{o}C (Upper), and HPLC trace of **1-Cold** in water (Lower). (b) MALDI mass spectrum of peaks at 19-20 min of the upper HPLC trace in a.

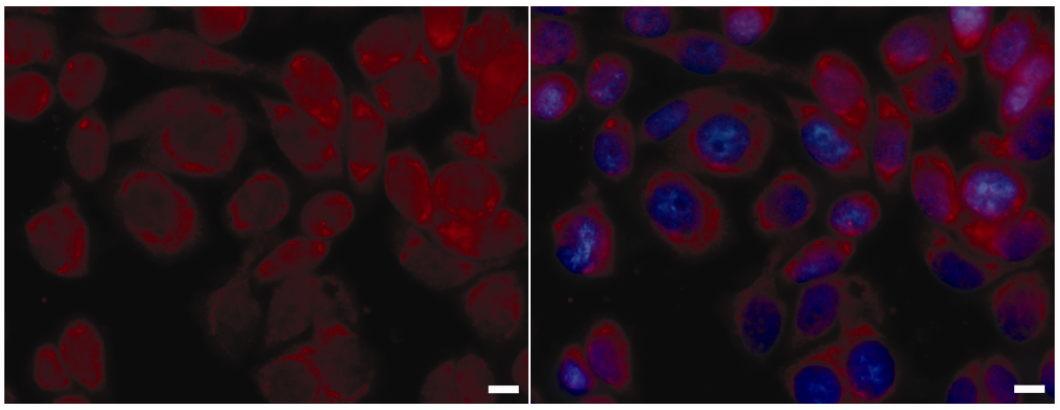


Figure S5. Immunofluorescence staining of MDA-MB-468 cells with rhdomine-labeled antibody against furin: left, DsRed channel (red, furin); right, merged image with DAPI (blue, nucleus). Scale bar: 10 μm.

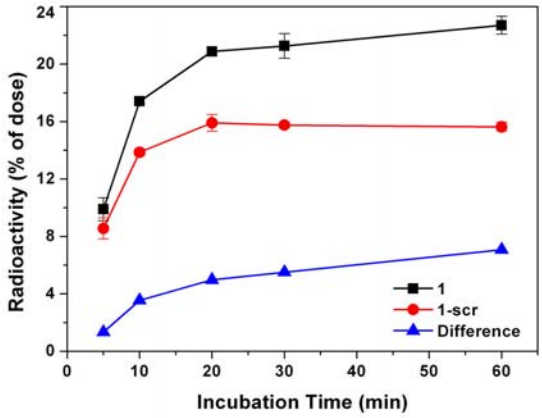


Figure S6. Time course of cellular uptake of **1** and **1-Scr** on MDA-MB-468 cells. 1 million cells were incubated with 1 μCi of **1** or **1-Scr**. At each time point, cells were harvested to count the radioactivity using a γ-counter.

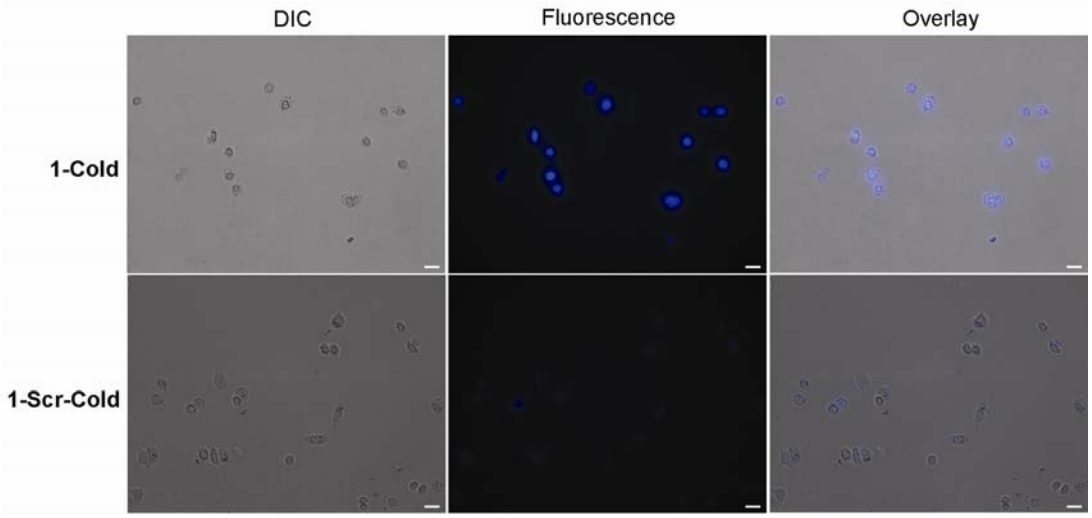


Figure S7. Left: differential interference contrast (DIC) images of MDA-MB-468 cells after incubation with 100 μM of **1-Cold** or **1-Scr-Cold** at 37 °C for 0.5 h. Middle: corresponding fluorescence images (DAPI channel) of the cells. Right: overlay of differential interference contrast (DIC) and fluorescence images. Scale bar: 20 μm.

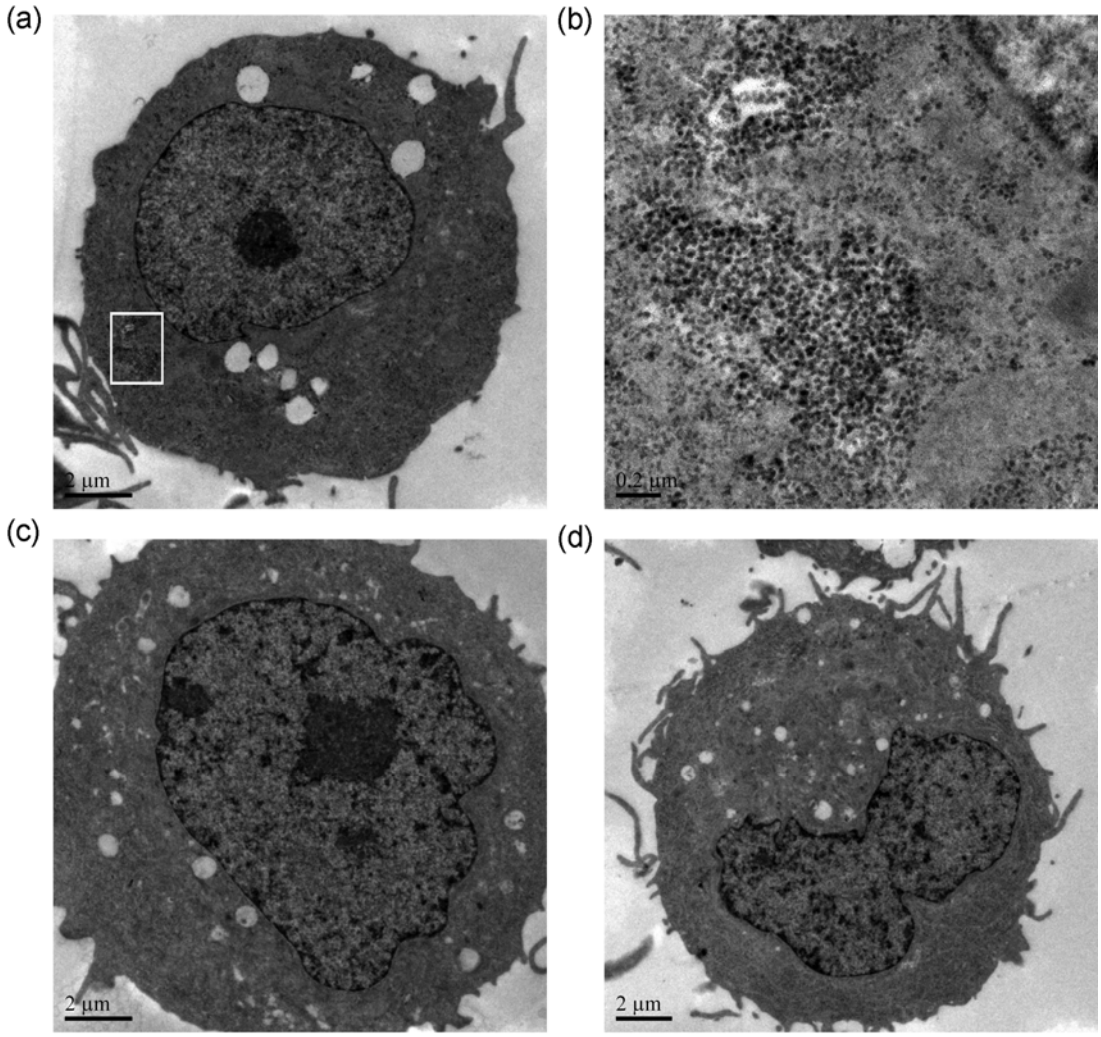


Figure S8. (a) Electron microscopic image of MDA-MB-468 cells after incubation with **1-Cold** at 100 µM for 8 h. (b) High magnification Electron microscopic image of the rectangle area in a. Large area of clustered **I-NPs** of **1-Cold** were found at/near Golgi bodies. (c) Electron microscopic image of MDA-MB-468 cells after incubation with **1-Scr-Cold** at 100 µM for 8 h. (d) Electron microscopic image of MDA-MB-468 cells.

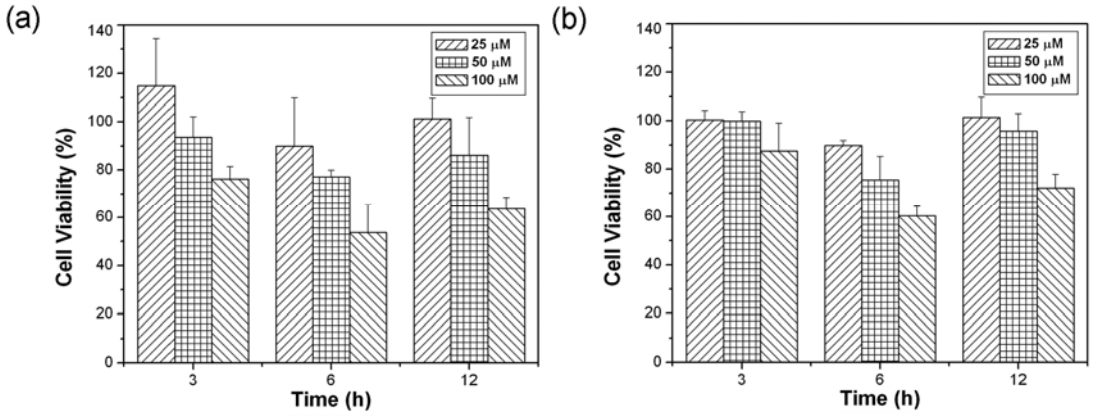


Figure S9. MTT assays of **1-Cold** (a) and **1-Scr-Cold** (b) on MDA-MB-468 cells.

Supplementary Table 1. HPLC condition for the purification of compound **C, 1-Cold, H, and 1-Scr-Cold**.

Time (minute)	Flow (ml/min.)	H_2O %	CH_3OH %
0	7.0	30	70
3	7.0	30	70
35	7.0	0	100
37	7.0	0	100
38	7.0	30	70
40	7.0	30	70

Supplementary Table 2. HPLC condition for the analysis and purification of the enzymatic products of **1-Cold** incubated with furin.

Time (minute)	Flow (ml/min.)	H_2O %	CH_3OH %
0	1.0	90	10
3	1.0	90	10
55	1.0	30	70
57	1.0	30	70
58	1.0	90	10
60	1.0	90	10

Supplementary Methods

General methods. All the starting materials were obtained from Adamas or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All other chemicals were reagent grade or better. Fmoc-3-iodo-D-tyrosine was purchased from Chem-Impex International. Furin was purchased from Biolabs (2,000 U ml^{-1}, one unit (U) corresponds to the amount of furin that releases 1 pmol of methylcoumarinamide (MCA) from the fluorogenic peptide Boc-RVRR-MCA (Bachem) in one minute at 30 °C). ^{1}HNMR spectra were obtained on a 300 MHz Bruker AV 300. MALDI-TOF/TOF mass spectra were obtained on a time-of-flight Ultrflex Ⅱ mass spectrometer (Bruker Daltonics), HPLC analyses were performed on an Agilent 1200 HPLC system equipped with a G1322A pump and in-line diode array UV detector using a YMC-Pack ODS-AM column with CH_3OH (0.1% of TFA) and water (0.1% of TFA) as the eluent. Dynamic light scattering (DLS) was measured on a Zeta Sizer Nano Series (Malvern Instruments). Cell images were obtained on a Zeiss Axio Imager Z1 fluorescence microscope. Transmission electron micrograph (TEM) images were obtained on a JEOL 2010 electron microscope, operating at 200 kV. The cryo-dried samples were prepared as following: a copper grid coated with carbon was dipped into the suspension solvent and placed into a vial, which was plunged into liquid nitrogen until no bubbles were apparent. Then water was removed from the frozen specimen by a freeze-drier.

Cell culture. MDA-MB-468 human breast adenocarcinoma epithelial cells were cultured in Dulbecco's modified eagle medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO). The cells were expanded in tissue culture dishes and kept in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed every other day.

Furin immunofluorescence staining. Cells were fixed with 4% paraformaldehyde for 20 min. Subsequently, the cells were washed with DPBS for three times and permeabilized with 1% Triton-100 in PBS for 20 min. Cells were then blocked with 1% non-fat milk solution in PBS for 20 min at room temperature. This solution was as used as antibody diluents. After blocking, the cells were incubated with primary antibody (1:100, anti-furin rabbit polyclonal antibody, Biomol International) for 1 h at 37 °C. After washing with PBS (3x5 min) and with 1% milk (1x5 min), cells were incubated with secondary antibody (1:100, goat polyclonal anti-rabbit IgG rhodamine-conjugate) for 30 min at 37 °C followed by washing with PBS (3x) and distilled water (1x) before mounted on a glass slide with DAPI-containing mounting media. Images were acquired using a Zeiss fluorescence microscope equipped with a 100x objective. The exposure time was 150 ms for DAPI, 600 ms for FITC, and 600 ms for rhodamine-conjugated IgG, respectively.

Cellular uptake. MDA-MB-468 cells were seeded in 6-well plates at a number of 1 million for each well and for 6 h. After that, the medium was replaced with fresh medium containing 1 μCi of **1** or **1-Scr** for each well. At 5, 10, 20, 30, and 60 min, cells were harvested for counting the radioactivity in a γ-counter.

Cellular efflux titration. MDA-MB-468 cells in 6-well plates at a number of 1 million for each well were incubated with 1 μCi of **1** and **1-Cold** at 0, 25, 50, 100 μM respectively. After 30 minutes' incubation, the medium was replaced with fresh medium and the efflux starts as time 0. At 5, 10, 20, 40, 80, and 160 min, the medium was changed and the radioactivity of the old medium was counted. At 160 min, the cells were also harvested for counting the radioactivity and calculating the total radioactivity at time 0.

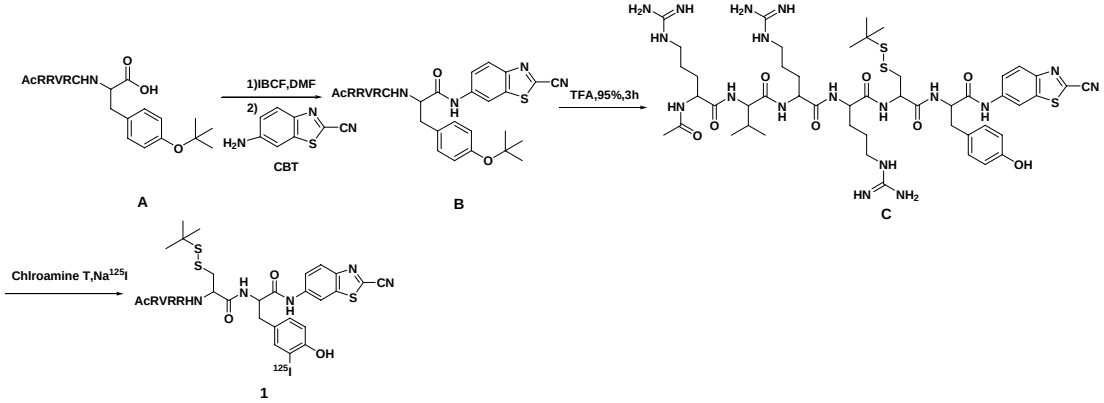
Cellular efflux. MDA-MB-468 cells in 6-well plates at a number of 1 million for each well were incubated with 1 μCi of **1** (or **1-Scr**) and **1-Cold** (**1-Scr-Cold**) at 0, 100 μM respectively. After 30 minutes' incubation, the medium was replaced with fresh medium and the efflux starts as time 0. At 5, 10, 20, 40, 80, and 160 min, the medium was changed and the radioactivity of the old medium was counted. At 160 min, the cells were also harvested for counting the radioactivity and calculating the total radioactivity at time 0.

Chemical synthesis and characterization of compound 1, 1-Cold, 1-Scr, and 1-Scr-Cold

The preparations of compound **1, 1-Cold, 1-Scr, 1-Scr-Cold** were described as below; 2-cyano-6-aminobenzothiazole (CBT) was synthesized following the literature method (White, E. H., Worther, H., Seliger, H. H., McElroy, W. D. Amino analogs of firefly luciferin and biological activity thereof. *J. Am. Chem. Soc.* 1966, **88**, 2015-2019).

*Preparation of Acetyl-Arg-Val-Arg-Arg-Cys(StBu)-Tyr(I-125)-CBT (**1**).*

Scheme S1. Synthetic route for compound **1**.



*Synthesis of **1***: Peptide YCRRVRAc (**A**, 90 mg, 0.05 mmol) was prepared by solid phase peptide synthesis (SPPS). The isobutyl chloroformate (7 mg, 0.05 mmol) was added to a mixture of **A** (90 mg, 0.05mmol) and MMP (4-methylmorpholine, 9.4 mg,

0.1 mmol) in THF (1.5 mL) at 0 °C under N_2 and the reaction mixture was stirred for 20 min. The solution of 2-cyano-6-aminobenzothiazole (9 mg, 0.05 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C then overnight at room temperature. The pure product **B** (34 mg, 35%) was obtained after HPLC purification. The Boc and Pbf protecting groups were cleaved with 95% TFA in CH_2Cl_2 for 3 hrs in the presence of 1% triisopropylsilane. The pure product **C** (10 mg, 50%) was obtained after HPLC purification. **C** was iodinated by the chloramine-T method. The reactions were performed in 50 mM sodium phosphate buffer, pH 7.4. Injecting 50 µL (5 mg/mL) compound **C** in a closed flask, than 74 MBq ^{125}I-NaI was added. 15 µL chloramine-T (2 mg/mL) solution was added to start the iodination reaction. After 1 min reaction at room temperature, the reaction was stopped by the addition of 20 µL (2 mg/mL) sodium metabisulfite. Compound **1** was purified by a HPLC machine equipped with a γ-detector. ^{1}HNMR of compound **C** (d_4-CD_3OD, 300 MHz, Fig. *S*10): 8.63 (s，1 H), 8.13 (d，J=9.0 Hz，1 H)，7.70 (d，J=9.0 Hz，1 H), 7.10 (d, J = 9.0 Hz，2 H), 6.69 (d, J = 9.0 Hz，2 H), 4.68 (t，J = 7.6 Hz, 1 H), 4.58 (q，J = 5 Hz，1 H), 4.34 (m，3 H), 4.16 (d，J = 7.3 Hz，1 H), 3.14-3.25 (m，8 H), 3.10 (d, J = 5.4 Hz, 1 H), 2.96-3.06 (m，2 H), 2.04 (s, 3 H), 1.58-1.95 (br, 14 H), 1.32 (s, 20 H), 0.98 (d, J = 6.7 Hz，6 H). MS of **C**: calculated for $C_{49}H_{74}N_{18}O_8S_3$, $[(M+H)^+]$: 1139.51; obsvd. ESI-MS: m/z 1139.3.

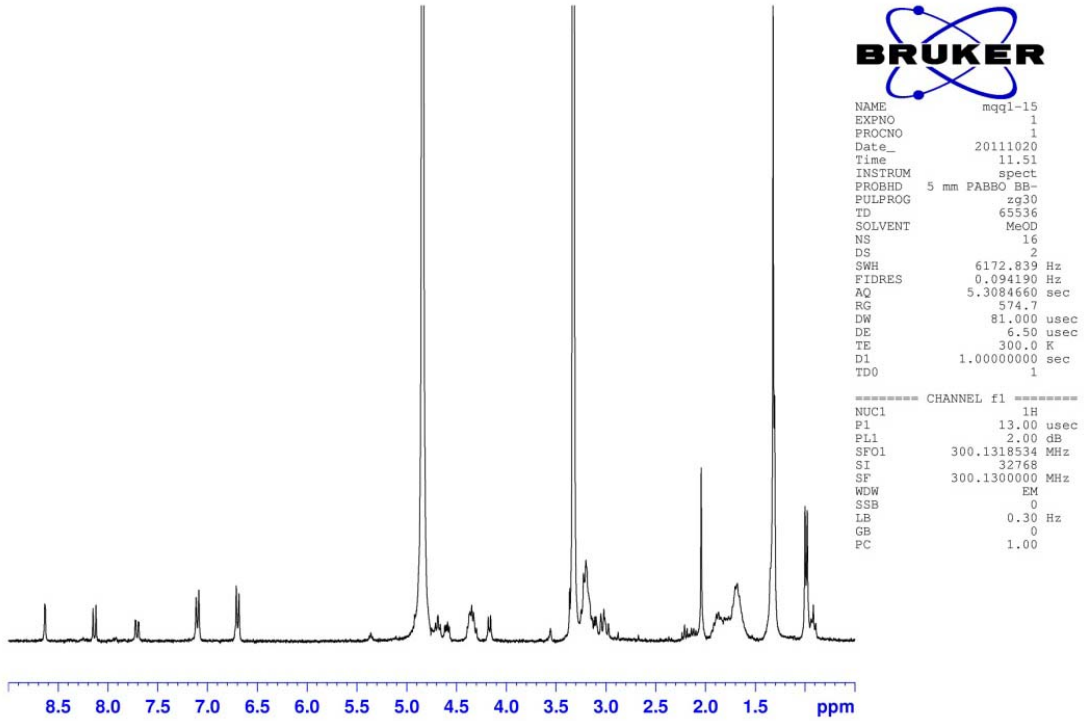
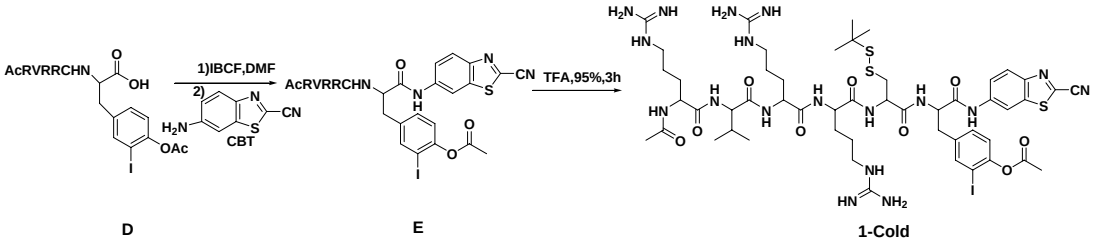


Figure S10. ^{1}HNMR spectrum of compound **C**.

Preparation of Acetyl-Arg-Val-Arg-Arg-Cys(StBu)-Tyr(Ac)(I)-CBT (**1-Cold**).

Scheme S2. Synthetic route for compound **1-Cold**



Synthesis of **1-cold**: Peptide Y(Ac)(I)CRRVRAc (**D**, 95 mg, 0.05 mmol) was prepared by solid phase peptide synthesis (SPPS). The isobutyl chloroformate (7 mg, 0.05 mmol) was added to a mixture of **D** (95 mg, 0.05 mmol) and MMP (4-methylmorpholine, 9.4 mg, 0.1mmol) in THF (1.5 mL) at 0 °C under N_2 and the reaction mixture was stirred for 20 mins. The solution of 2-cyano-6-aminobenzothiazole (9 mg, 0.05 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C then overnight at room temperature. The pure product **E** (36.4 mg, 35%) was obtained after HPLC purification. The Boc and Pbf protecting groups were cleaved with 95% TFA in CH_2Cl_2 for 3 hrs in the presence of 1% triisopropylsilane. The pure product **1-Cold** (10 mg, 50%) was obtained after HPLC purification. ^{1}HNMR of compound **1-Cold** (d_4-CD_3OD, 300 MHz, Fig. S11): 8.62 (d, J = 5 Hz, 1 H), 8.13 (d, J = 9 Hz, 1 H), 7.75 (dd, J_1 = 9 Hz, J_2 = 2 Hz, 2 H), 7.28 (dd，J_1 = 8.5 Hz, J_2 = 2.2 Hz, 1 H)，7.02 (d, J = 8.2 Hz, 1 H), 4.51-4.79 (m, 2 H), 4.11-4.42 (m, 4 H), 2.73-3.27 (m, 11 H), 2.33 (s, 3 H), 2.02 (s, 3 H), 1.50-1.92 (m, 12 H), 1.29-1.36 (m, 12 H), 0.90-0.98 (m, 6 H). MS of **1-Cold**: calculated for $C_{51}H_{76}N_{18}O_9S_3$, $[(M+H)^+]$: 1307.42; obsvd. ESI-MS: m/z 1307.2.

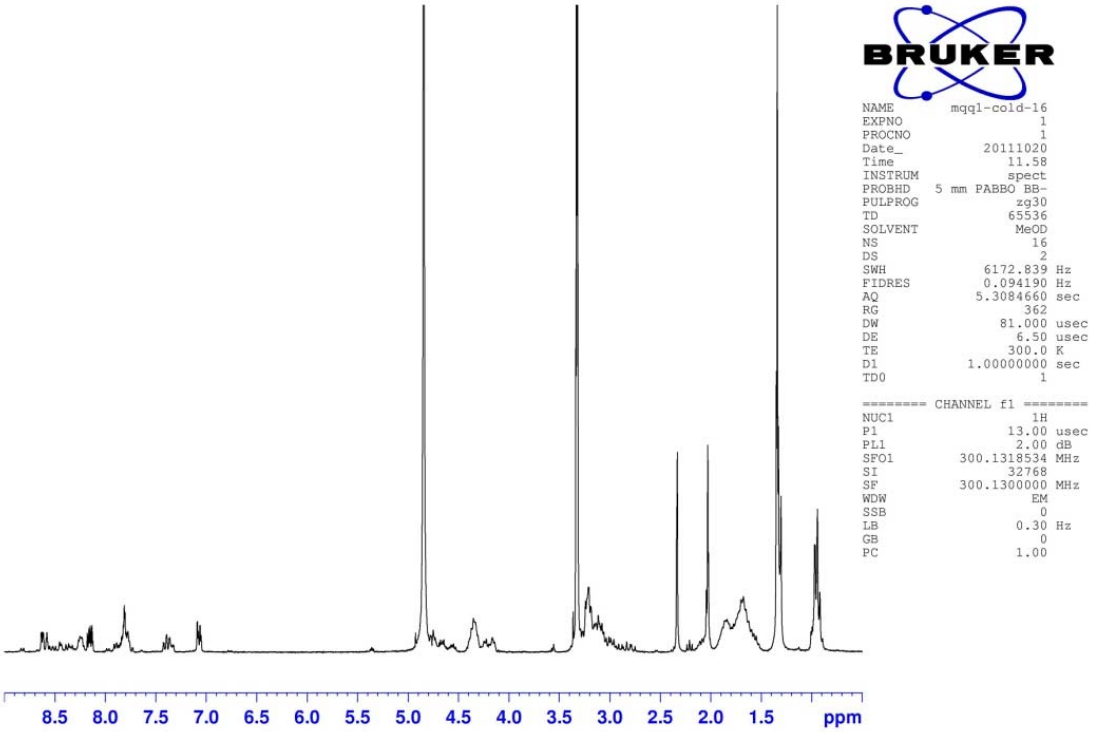
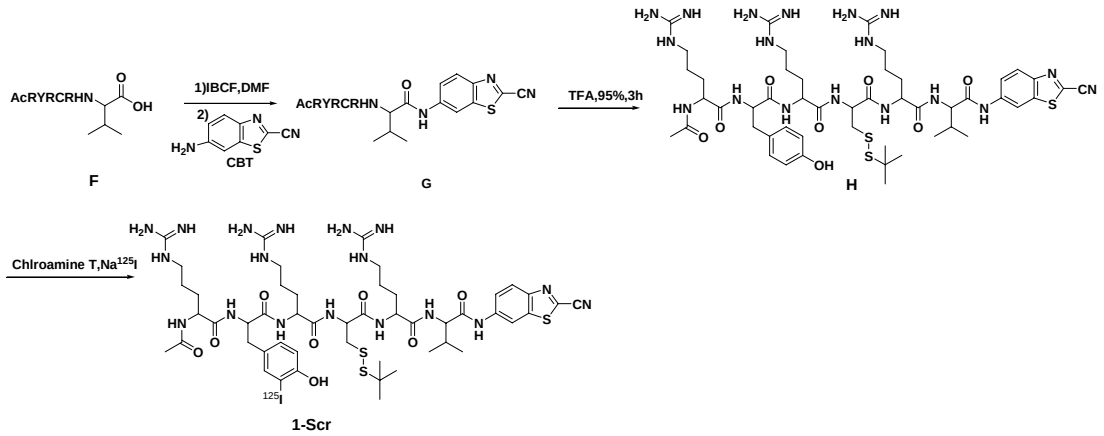


Figure S11. ^{1}HNMR spectrum of compound **1-Cold**.

Preparation of Acetyl-Arg-Tyr(I-125)-Arg-Cys(StBu)-Arg-Val-CBT (**1-Scr**).
Scheme S3. Synthetic route for compound **1-Scr**.



*Synthesis of **1-scr***: Peptide VRCRYRAc (**F**, 90 mg, 0.05 mmol) was prepared by solid phase peptide synthesis (SPPS). The isobutyl chloroformate (7 mg, 0.05 mmol) was added to a mixture of **F** (90 mg, 0.05 mmol) and MMP (4-methylmorpholine, 9.4 mg, 0.1 mmol) in THF (1.5 mL) at 0 °C under N_2 and the reaction mixture was stirred for 20 min. The solution of 2-cyano-6-aminobenzothiazole (9 mg, 0.05 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C then overnight at room temperature. The pure product **G** (34 mg, 35%) was obtained after HPLC purification. The Boc and Pbf protecting groups were cleaved with 95% TFA in CH_2Cl_2 for 3 hrs in the presence of 1% triisopropylsilane. The pure product **H** (10 mg, 50%) was obtained after HPLC purification. **H** was iodinated by the chloramine-T method. The reactions were performed in 50mM sodium phosphate buffer, pH 7.4. Injecting 50 µL (5 mg/mL) compound **H** in a closed flask, than 74 MBq ^{125}I-NaI was added. 15 µL chloramine-T (2 mg/mL) solution was added to start the iodination reaction. After 1 min reaction at room temperature, the reaction was stopped by the addition of 20 µL (2 mg/mL) sodium metabisulfite. Compound **1-Scr** was purified by a HPLC machine equipped with a γ-detector. ^{1}HNMR of compound **H** (d_4-CD_3OD, 300 MHz, Fig. S12): 8.67 (s, 1 H), 8.14 (d, J = 9 Hz, 1 H), 7.76 (d, J = 9 Hz, 1 H), 7.04 (d, J = 9 Hz, 2 H), 6.71 (d, J = 9 Hz, 2 H), 4.14-4.64 (m, 8 H), 3.05-3.25 (br, 9 H), 2.92 (s, 1 H), 2.15-2.27 (br, 1 H), 2.0 (s, 3 H), 1.44-1.89 (br, 13 H), 1.34 (s, 9 H), 1.05 (d, J = 6.4 Hz, 6 H). MS of **H**: calculated for $C_{49}H_{74}N_{18}O_8S_3$, $[(M+H)^+]$:1139.51; obsvd. ESI-MS: m/z 1139.2.

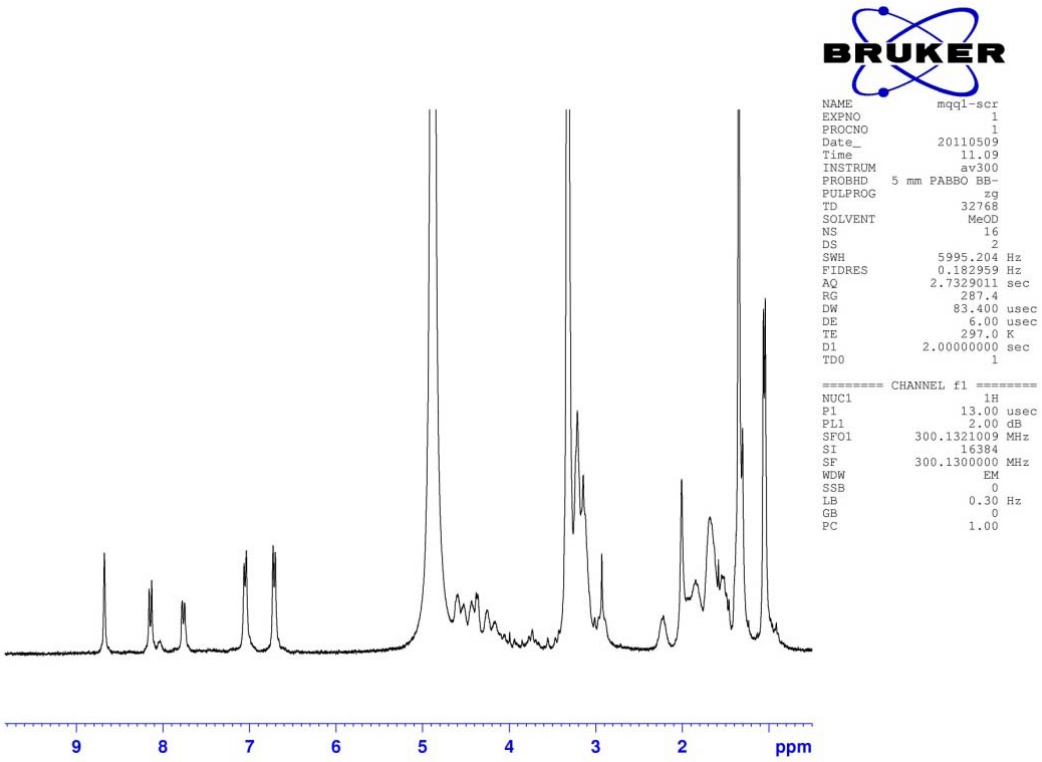
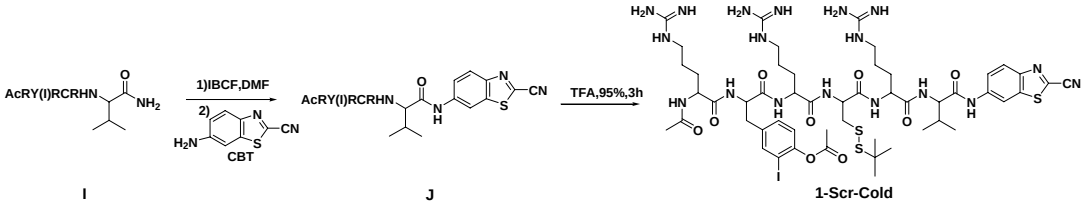


Figure S12. [1]HNMR spectrum of compound **H**.

Preparation of *Acetyl-Arg-Tyr(Ac)(I)-Arg-Cys(StBu)-Arg-Val-CBT* (**1-Scr-Cold**).

Scheme S4. Synthetic route for compound **1-Scr-Cold**



Synthesis of ***1-Scr-Cold***: Peptide VRCRY(Ac)(I)RAc (**I**, 95 mg, 0.05 mmol) was prepared by solid phase peptide synthesis (SPPS). The isobutyl chloroformate (7 mg, 0.05 mmol) was added to a mixture of **I** (95 mg, 0.05 mmol) and MMP (4-methylmorpholine, 9.4 mg, 0.1 mmol) in THF (1.5 mL) at 0 °C under N_2 and the reaction mixture was stirred for 20 mins. The solution of 2-cyano-6-aminobenzothiazole (9 mg, 0.05 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C then overnight at room temperature. The pure product **J** (36.4 mg, 35%) was obtained after HPLC purification. The Boc and Pbf protecting groups were cleaved with 95% TFA in CH_2Cl_2 for 3 hrs in the presence of 1% triisopropylsilane. The pure product **1-Scr-Cold** (10 mg, 50%) was obtained after HPLC purification. [1]HNMR of compound **1-Scr-Cold** (d_4-CD_3OD,300 MHz, Fig. *S*13): 8.65 (d, J = 2 Hz, 1 H), 8.13 (d, J = 9 Hz, 1 H), 7.75 (dd, J_1 = 9 Hz, J_2 = 2 Hz, 2 H), 7.28 (dd，J_1 = 8.5 Hz, J_2 = 2.2 Hz, 1 H)，7.02 (d, J = 8.2 Hz, 1 H)，4.54-4.68 (br，3 H), 4.40-4.48 (m，1 H), 4.37 (d，J = 7.1 Hz，1 H), 4.23-4.32 (m，1 H), 4.11-4.19 (m，1 H), 3.53-3.60 (br，1 H), 2.98-3.26 (br，15 H), 2.33 (s，3 H), 2.03 (s，3 H), 1.48-1.77

(br，13 H), 1.24-1.43 (m，33 H), 1.05 (d，J = 6.8 Hz，7 H). MS of **1-Scr-Cold**: calculated for $C_{51}H_{76}IN_{18}O_9S_3$, $[(M+H)^+]$:1307.42; obsvd. ESI-MS: m/z 1307.2.

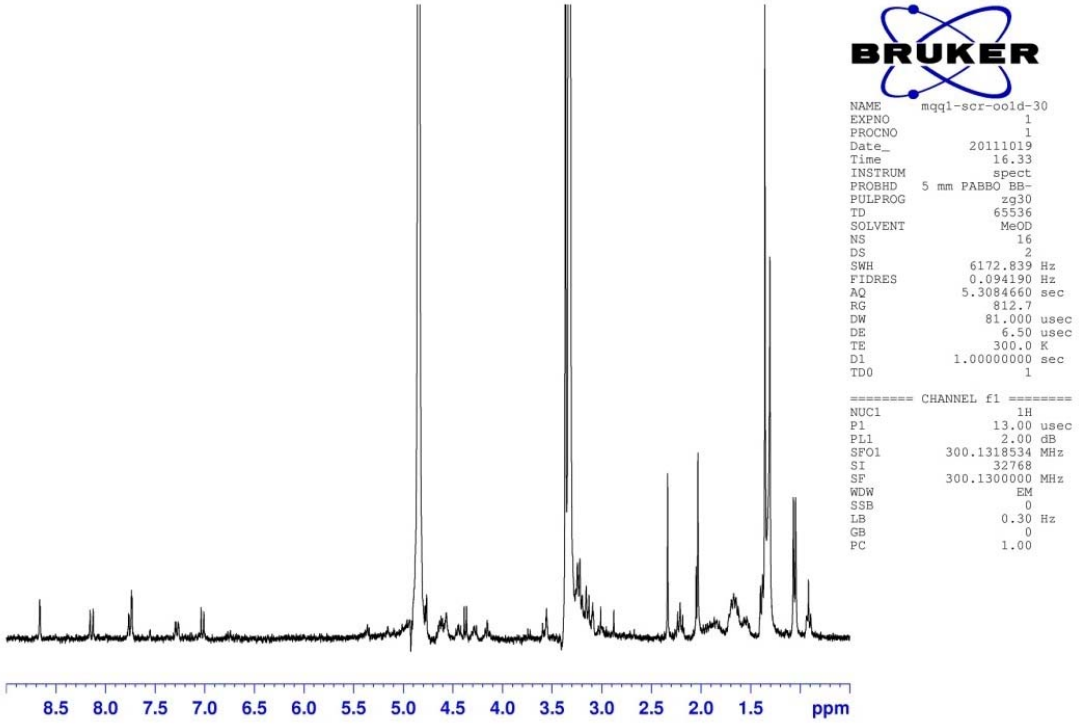


Figure S13. ^{1}HNMR spectrum of compound **1-Scr-Cold**.