Phosphorescent proteins for bio-imaging and site selective bio-conjugation of peptides and proteins with luminescent cyclometalated iridium(III) complexes

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Supporting Information

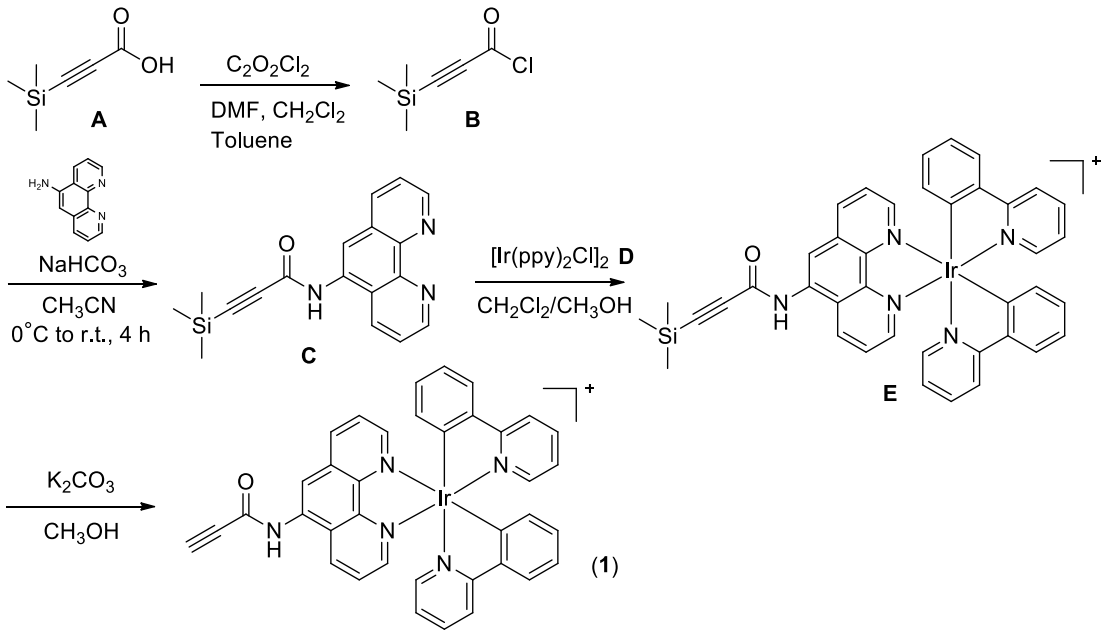
General

All chemicals and solvents (AR grade) were used as received. Acetonitrile for photophysical measurements was distilled over potassium permanganate and calcium hydride. Peptides: STSSSCNLSK (2), AYEMWCFHQK (3), CAYEMWCFHQK (4), CALNN (5), SSCSSCPLSK (6) and HIV-Tat derived peptide GRKKRRQRRRPPQC-amide; proteins: bovine serum albumin (BSA) and human serum albumin (HSA) were obtained from commercial sources and used without further purification. All other solvents were of analytical grade and purified according to conventional methods. TLC analyses were performed on aluminum oxide plates (Fluka, USA) and flash column chromatography was conducted over aluminum oxide (activated, neutral, Brockmann I, Sigma-Aldrich, USA) with methanol/dichloromethane as eluent. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-300 or DPX-400 spectrometer. Chemical shifts (ppm) are referenced to tetramethylsilane (TMS). Mass spectra were measured by EI using Finnigan MAT 95 or by ESI using a hybrid Q-TOF mass spectrometer (QSTAR-XL system, ABI, USA) or by a 4000 QTRAP® mass spectrometer (QTRAP® LC/MS/MS system, ABSCIEX, USA) or by LTQ Orbitrap Velos™ mass spectrometer (LTQ Orbitrap Velos™, Thermo Scientific, USA)

General Spectral Measurements

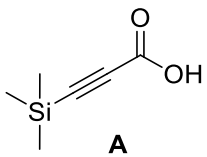
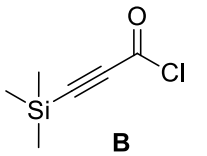
UV/Vis spectra were recorded on a Hewlett Packard 8453 UV/Vis spectrophotometer. Steady-state emission spectra were measured on a SPEX Fluorolog-3 Model FL3–21 spectrofluorometer.

Synthesis of 1



Scheme S1. Synthesis of **1**

Literature Reference of Known Compounds A, B and D

 A	Commercially available;
 B	A. S. Medvedeva, M. V. Andreev, L. P. Safronova, A. V. Afonin, *Russ. J. Org. Chem.* **2005**, *41*, 1463–1466
$[Ir(ppy)_2Cl]_2$ **D**	S. Sprouse, K. A. King, P. J. Spellane, R. J. Watts, *J. Am. Chem. Soc.* **1984**, *106*, 6647–6653 and H.-Y. Shiu, M.-K. Wong, C.-M. Che, *Chem. Commun.* **2011**, *47*, 4367–4369

Preparation and Characterization of B, C, E and 1

B: A mixture of (2.9 g, 20 mmol) of trimethylsilylpropynoic acid **A**, (2.8 g, 22 mmol)

of oxalyl chloride, and 0.04 equiv. of DMF was kept for 30 min. at room temperature. After distillation of oxalyl chloride under reduced pressure, **B** was isolated and used for synthesis without further purification (2.7 g, 80% yield).

C: The in situ generated **B** was added dropwise to a mixture of 5-amino-1,10-phenanthroline (50 mg, 0.26 mmol), sodium bicarbonate (100 mg) and 50 mL acetonitrile at 0 ℃. The reaction was then carried out at room temperature for 4 h. The resulted solution was filtered and washed by acetonitrile. After evaporation of solvent, the residue was purified by flash column chromatography on Al_2O_3 (2% MeOH in CH_2Cl_2) to give product **C** (45.8 mg, 56 % isolated yield).

C: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.10 (s, 1H), 8.93 (dd, *J* = 3.3, 15.7 Hz, 2H), 8.36 (d, *J* = 8.2 Hz, 1H), 8.05 (d, *J* = 8.2 Hz, 1H), 8.02 (s, 1H), 7.49 (dd, *J* = 4.2, 7.9 Hz, 1H), 7.42 (dd, J = 4.2, 8.3 Hz, 1H), 0.19 (s, 9H); EIMS *m/z* 319 ($[M + H]^+$); HRMS (EI) for $C_{18}H_{17}N_3OSi$, calcd 319.1141, found 319.1145.

E: A mixture of $[Ir_2(ppy)_4Cl_2]$ (20 mg, 0.018 mmol) and **C** (11.9 mg, 0.037 mmol) in 30 mL MeOH/CH_2Cl_2 = 1:1 (v/v) was refluxed under an inert atmosphere of N_2 in the dark for 4 h. After evaporation of solvent, the crude mixture **E** was used for synthesis without further purification (13.3 mg, 90% crude yield).

1: **E** (13.3 mg, 0.016 mmol) and potassium carbonate was stirred in 2 mL of methanol at room temperature for 30 min. The solution was then evaporated to dryness and the solid was dissolved in CH_2Cl_2 and purified by column chromatography on Al_2O_3 (8% MeOH in CH_2Cl_2) to give product **1** (11.5 mg, 96% isolated yield).

1: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.57 (d, J = 1.2, 8.5 Hz, 1H), 8.55 (s, 1H), 8.47 (dd, *J* = 1.2, 8.3 Hz, 1H), 8.22 (dd, *J* = 1.2, 5.0 Hz, 1H), 8.18 (dd, *J* = 1.2, 5.0 Hz, 1H), 7.94–7.91 (m, 2H), 7.86 (dd = 5.0, 8.6 Hz, 1H), 7.76–7.67 (m, 5H), 7.33–7.37 (m, 1H), 7.12–

7.07 (m, 2H), 7.01–6.95 (m, 2H), 6.93–6.84 (m, 2H), 6.43–6.37 (m, 2H), 3.28 (s, 1H); ESIMS *m/z* 748 ($[M + H]^+$)

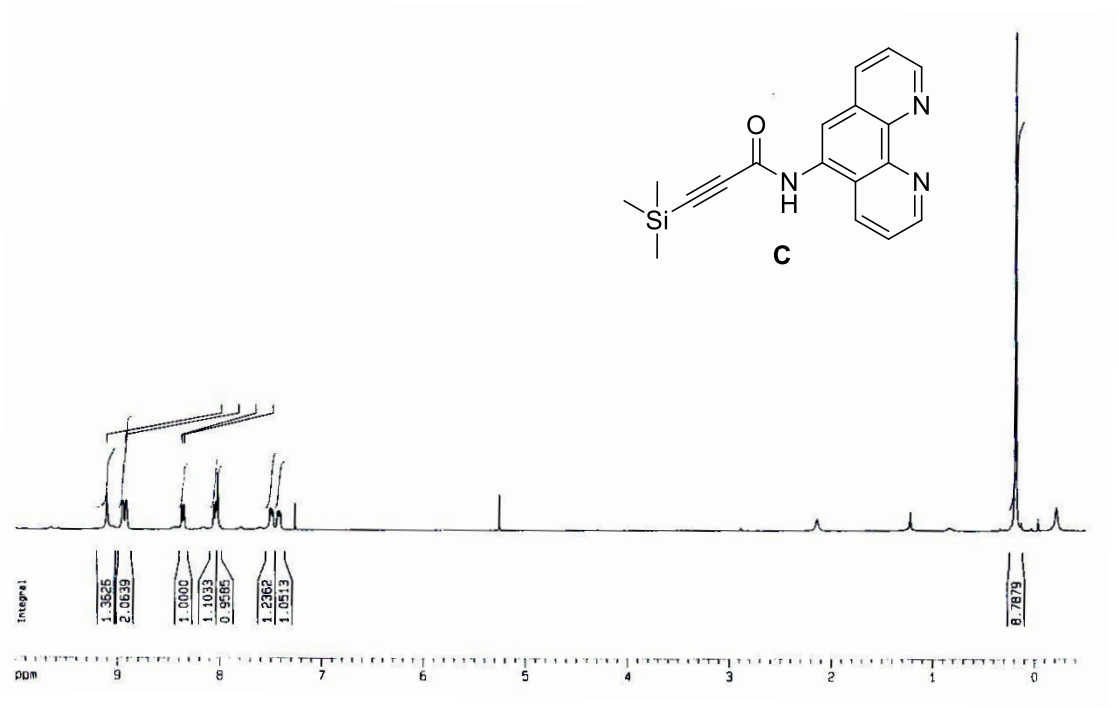


Fig. S1. ^{1}H NMR spectrum of **C**

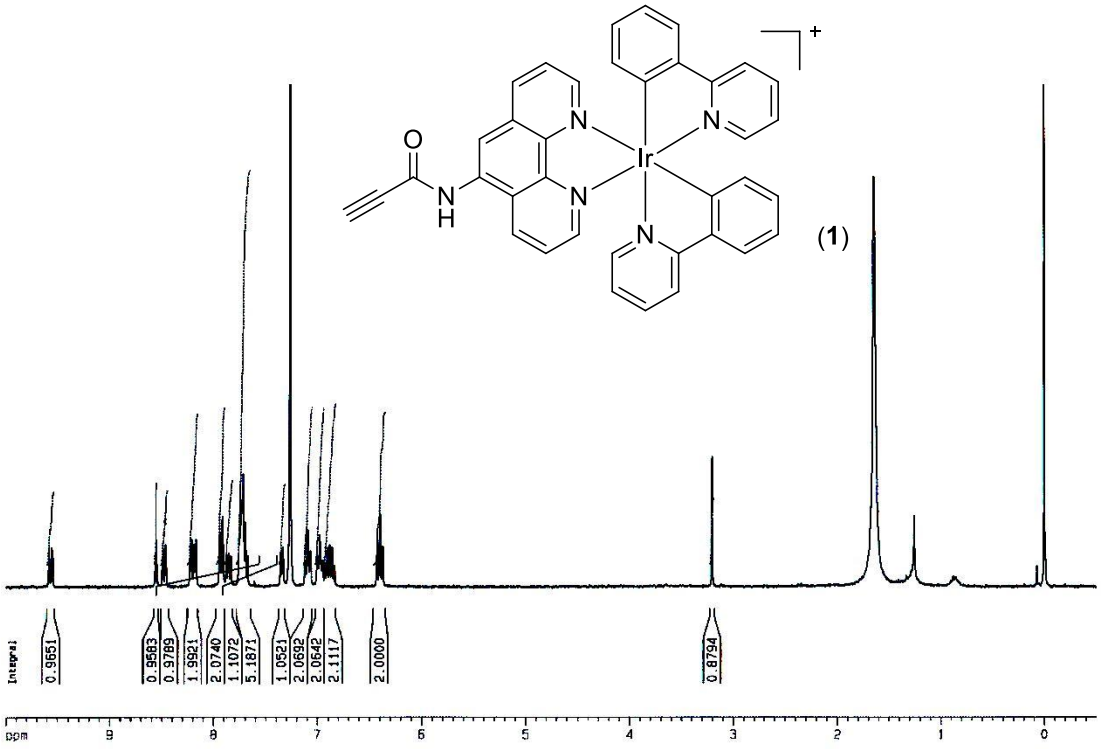


Fig. S2. ^{1}H NMR spectrum of **1**

General procedure for Cysteine Modification of Peptides 2–6 with 1

The peptide (10 μL from 1 mM in H_2O), **1** (1 μL from 10 mM in CH_3CN) [pepetide/iridium(III) complex = 1/1], CH_3CN (9 μL) and pH 8.0 phosphate buffered saline PBS (80 μL) were mixed in a 1.0 mL Eppendorf tube at room temperature for 24 h was examined. The **1**-modified peptides were characterized by LC–MS/MS.

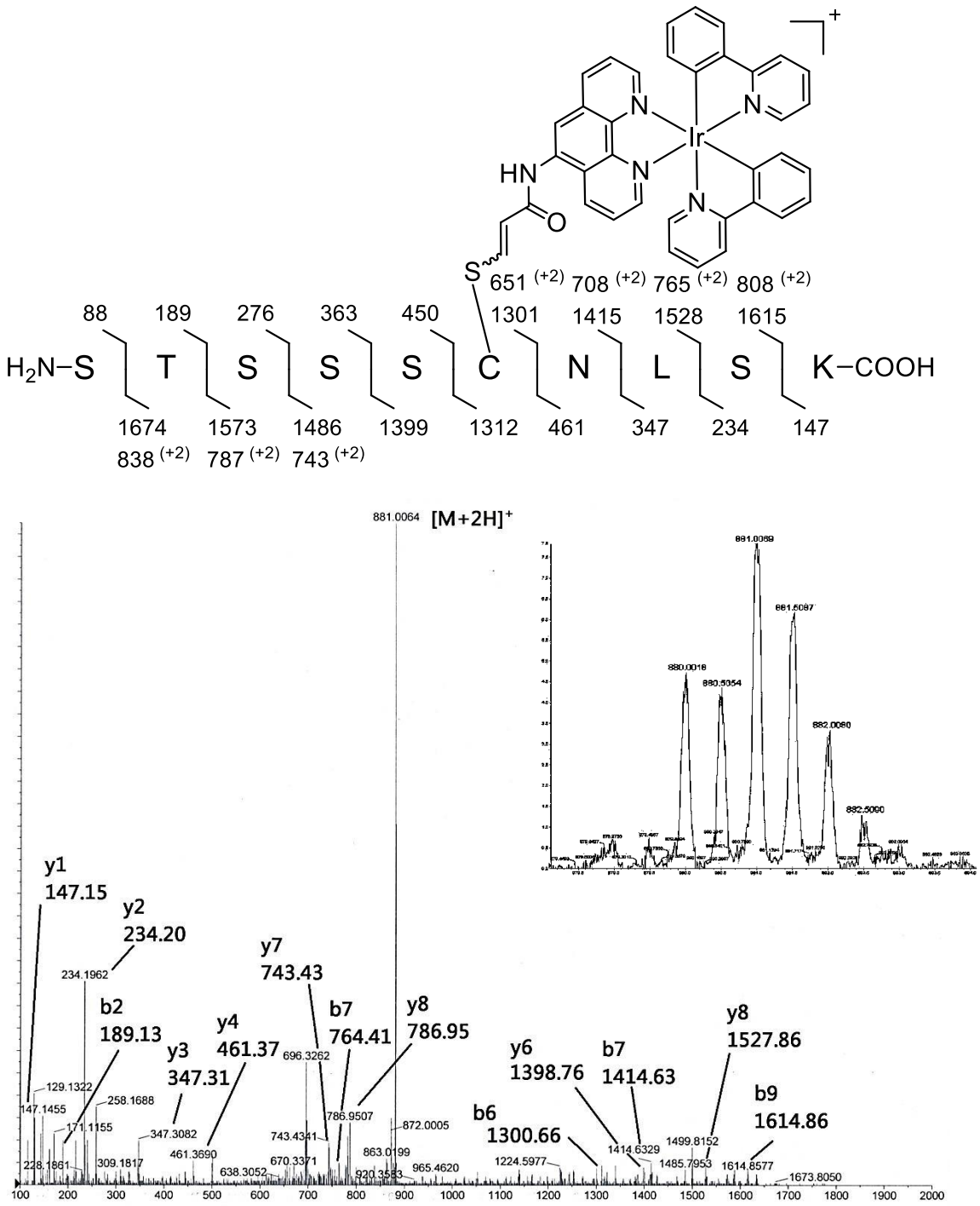


Fig. S3. The QTOF MS/MS of **1**-modified STSSSCNLSK at cysteine and the QTOF MS spectrum of **1**-modified STSSSCNLSK (ESI source, doubly charged ion of *m/z* = 881.0) (inset)

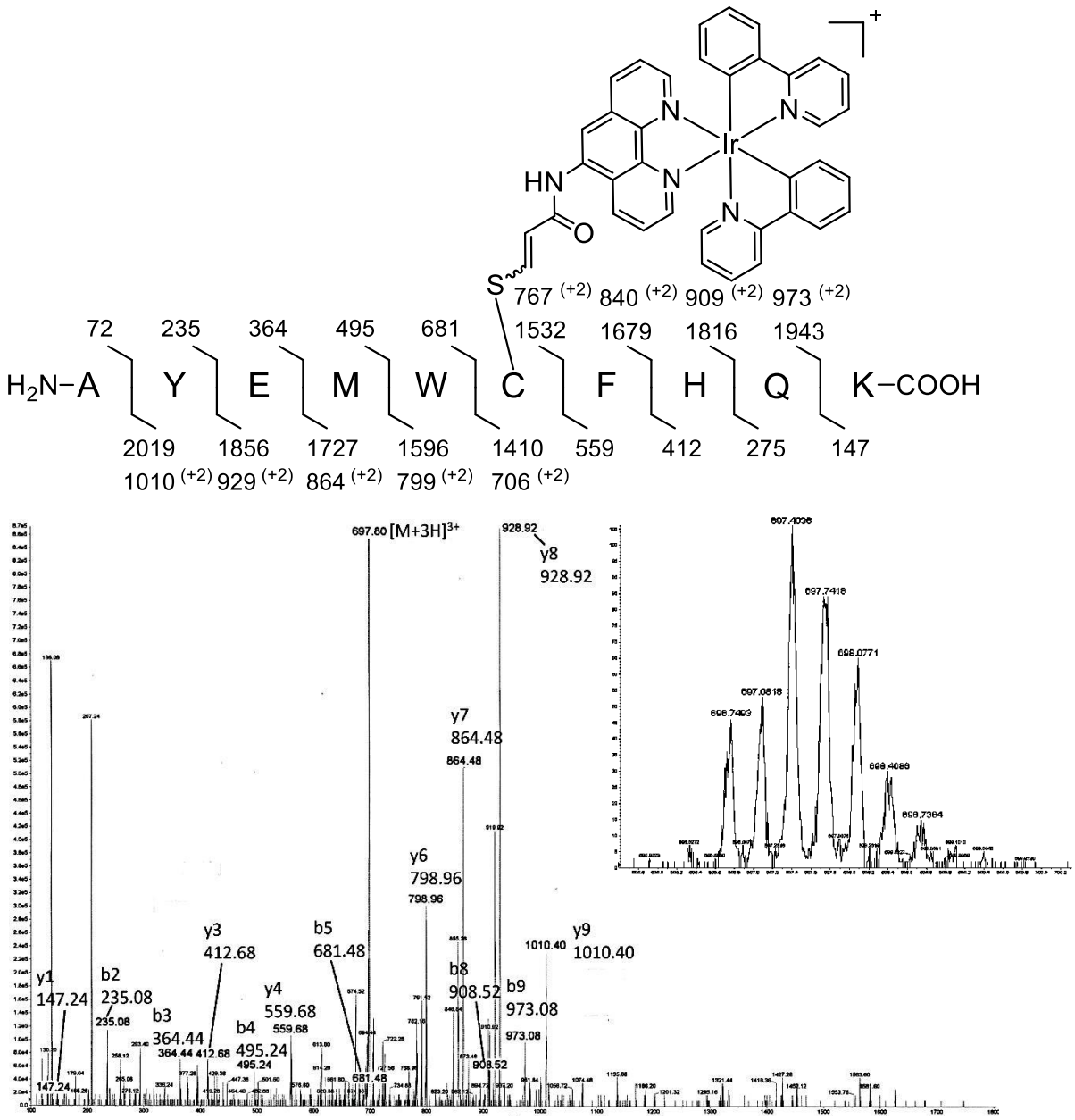


Fig. S4. The QTOF MS/MS of **1**-modified AYEMWCFHQK at cysteine and the QTOF MS spectrum of **1**-modified AYEMWCFHQK (ESI source, triply charged ion of *m/z* = 697.4) (inset)

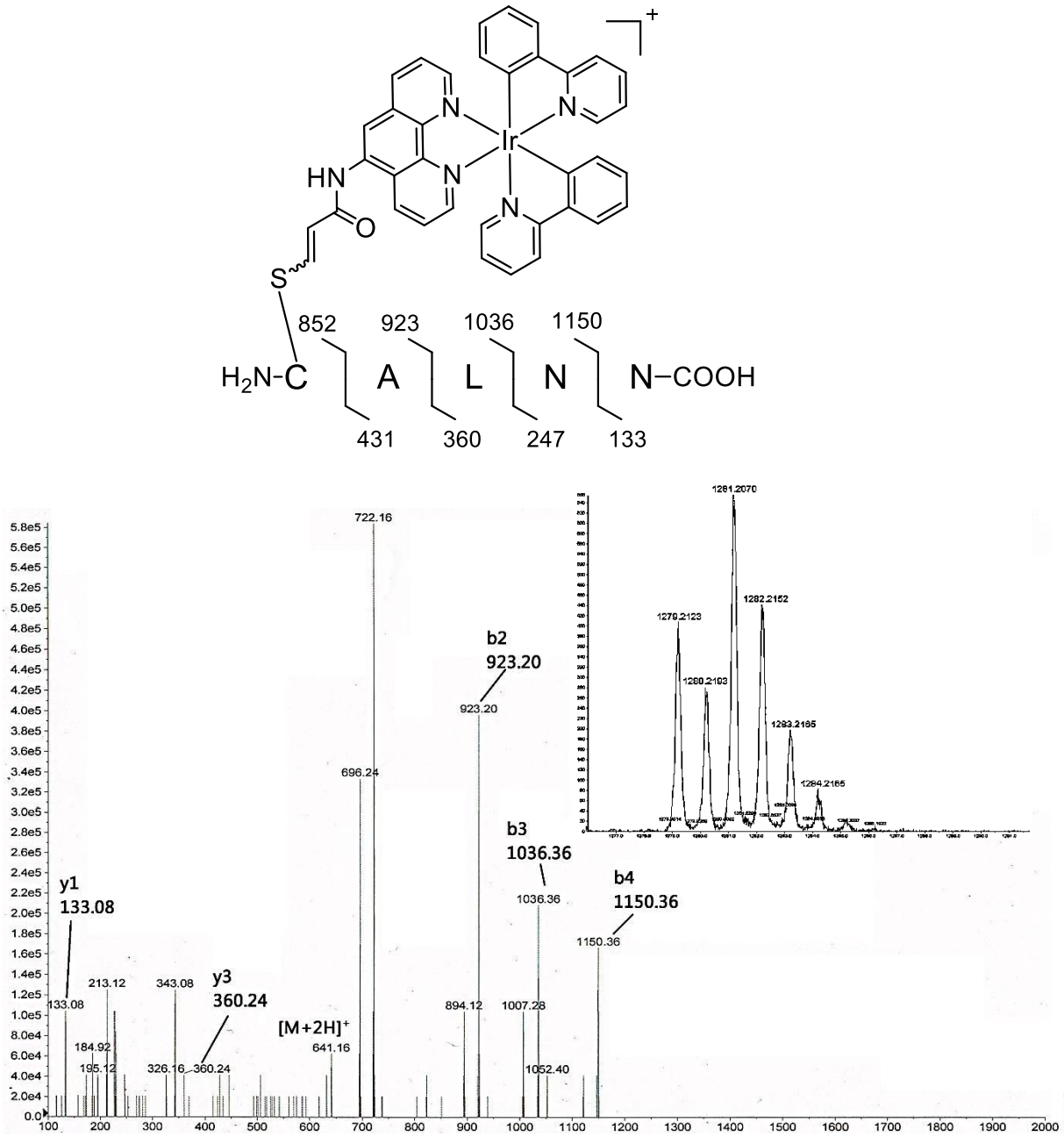


Fig. S5. The QTOF MS/MS of **1**-modified CALNN at cysteine and the QTOF MS spectrum of **1**-modified CALNN (ESI source, singly charged ion of *m/z* = 1281.2) (inset)

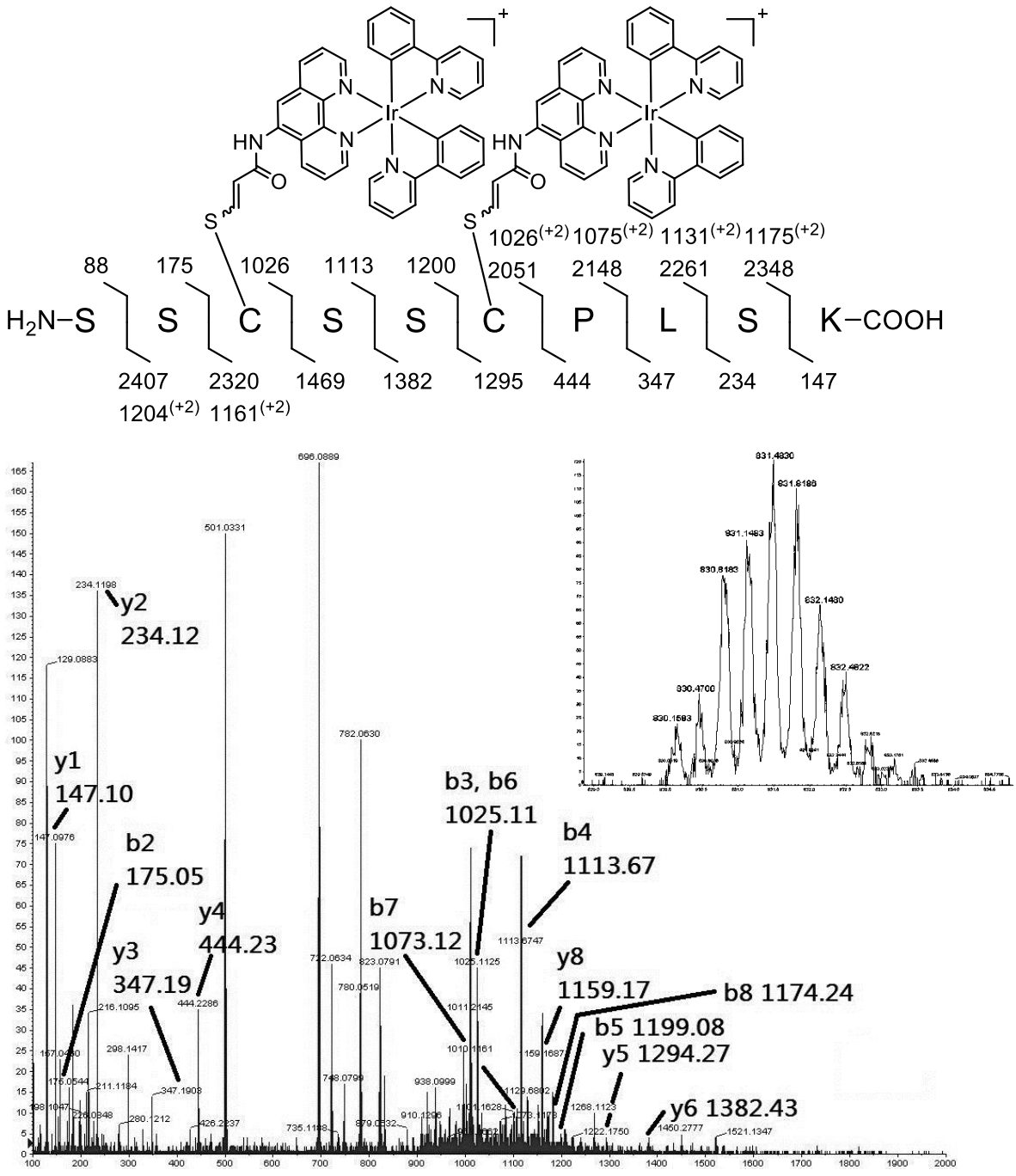


Fig. S6. The QTOF MS/MS of **1**-modified SSCSSCPLSK at cysteine and the QTOF MS spectrum of **1**-modified SSCSSCPLSK (ESI source, triply charged ion of *m/z* = 831.5) (inset)

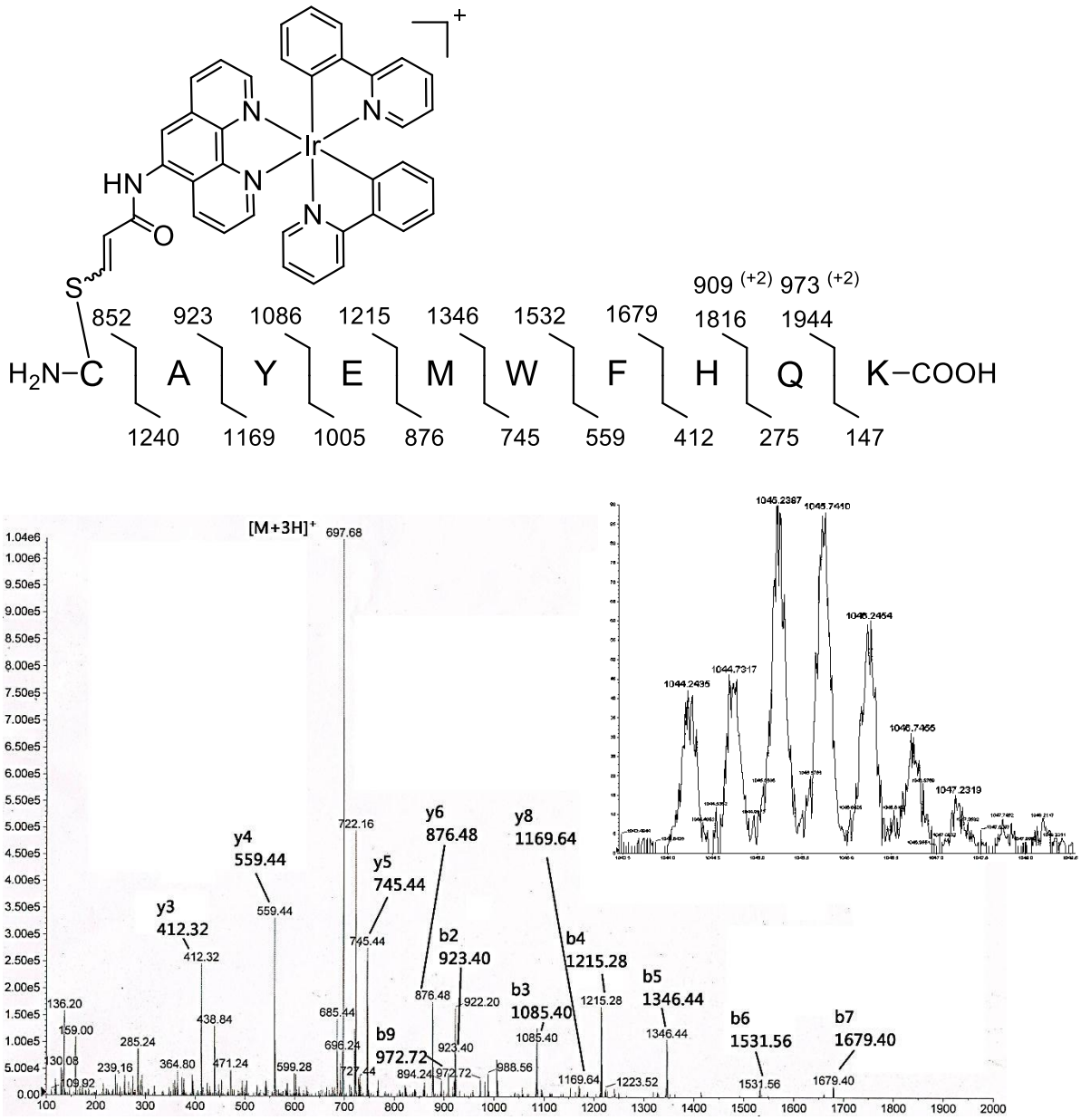


Fig.S7. The QTOF MS/MS spectrum of **1**-modified CAYEMWFHQK at cysteine and the QTOF MS spectrum of **1**-modified CAYEMWFHQK (ESI source, doubly charged ion of *m/z* = 1045.7) (inset)

General procedure for Cysteine Modification of Proteins BSA, HSA and BCArg with 1

The protein (10 μL from 1 mM in H_2O), **1** (10 μL from 10 mM in CH_3CN) [protein/iridium(III) complex = 1/10] and pH 8.0 phosphate buffered saline PBS (80 μL) were mixed in a 1.0 mL Eppendorf tube at room temperature/37 ℃ for 24 h. The **1**-modified protein (50 μL from 0.1 mM in H_2O) were subsequently trypsin digested by sequencing grade modified trypsin (Promega, USA) (protein/trypsin = 50/1) in ammonium bicarbonate (150 μL, 50 mM) at 37 ℃ for 4 h. The reaction mixture was analyzed by LC-MS and LC-MS/MS.

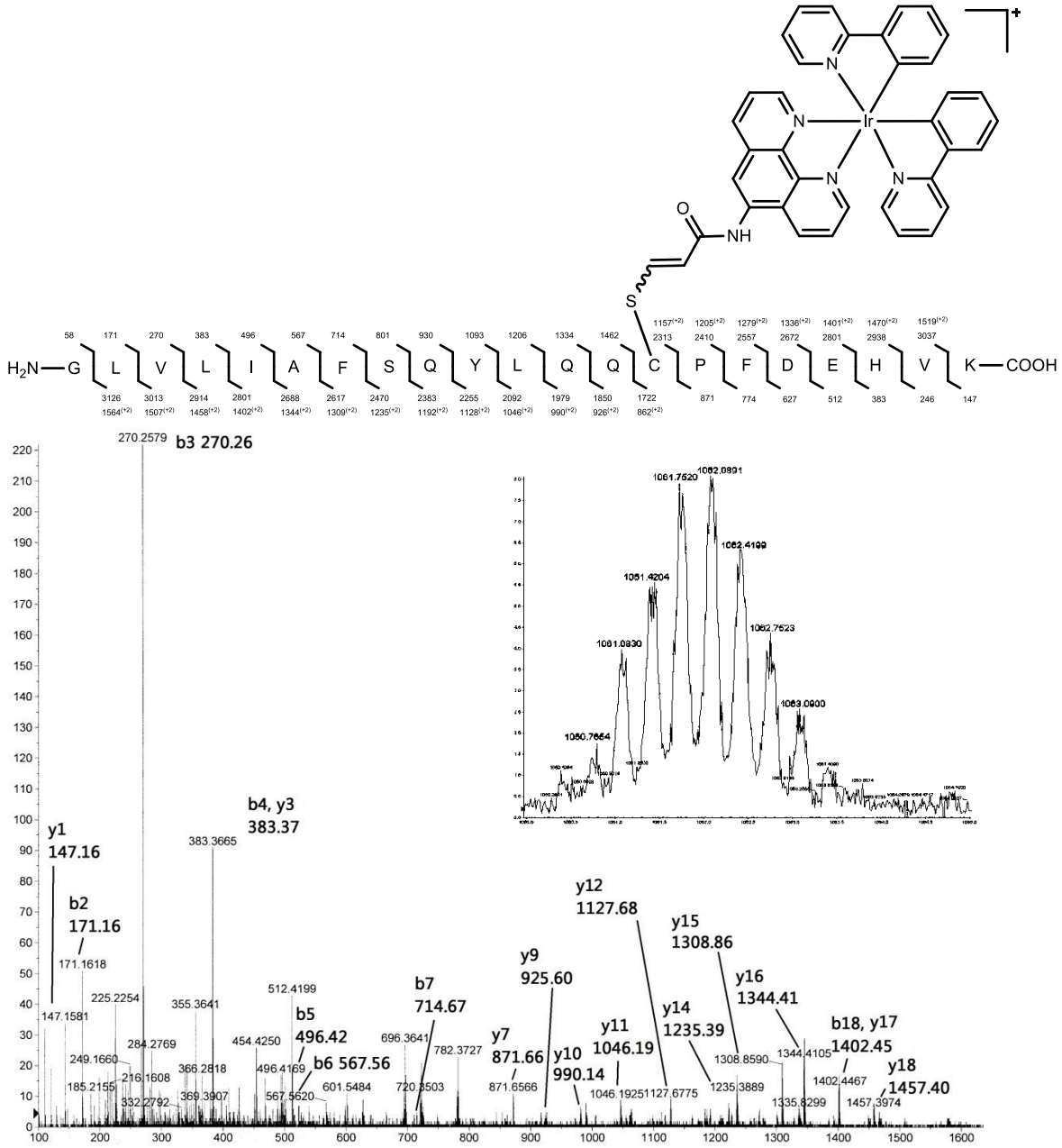


Fig. S8. The QTOF MS/MS spectrum of **1**-modified GLVLIAFSQYLQQCPFDEHVK at cysteine after trypsin digestion of **1**-modified BSA and the QTOF MS spectrum of **1**-modified GLVLIAFSQYLQQCPFDEHVK (ESI source, triply charged ion of *m/z* = 1061.8) (inset)

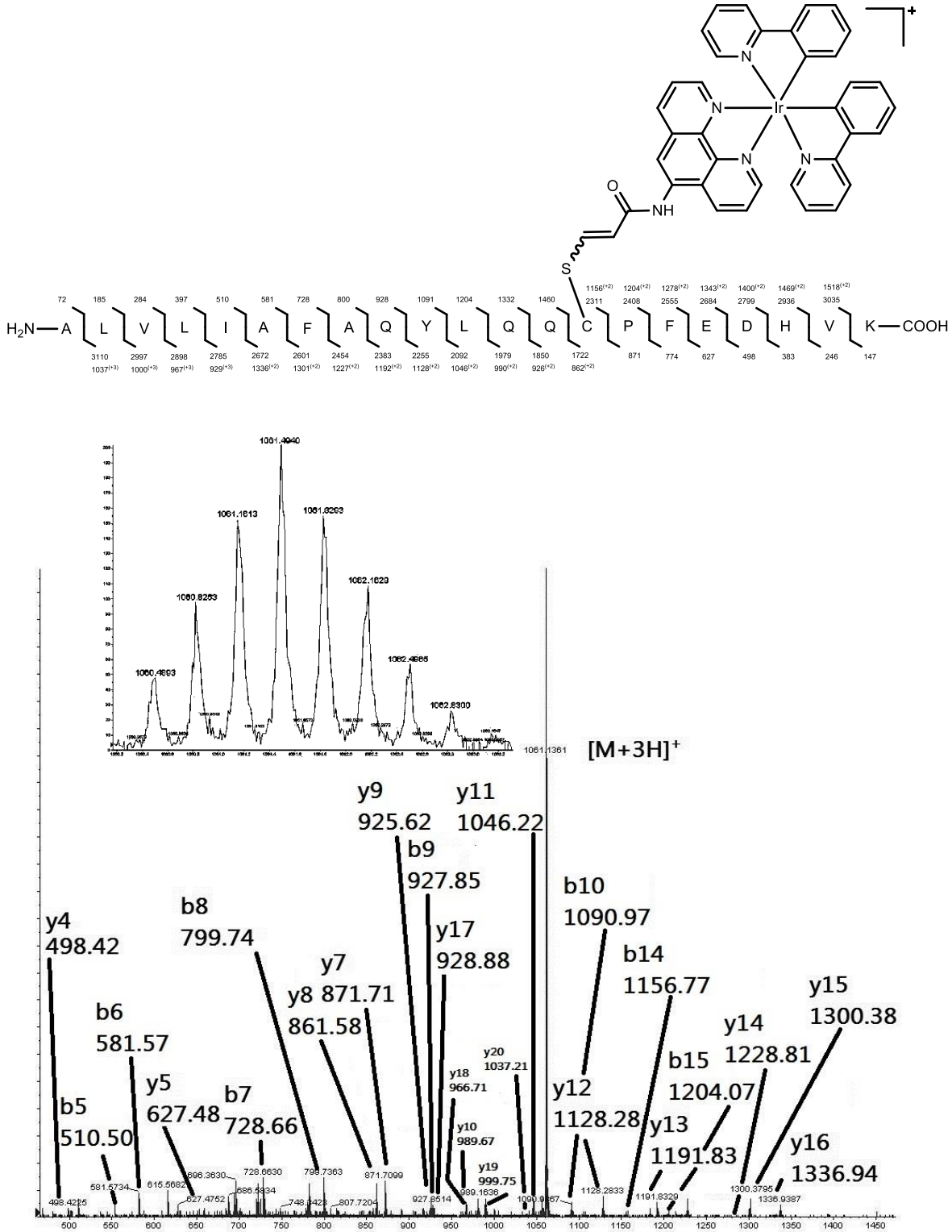


Fig. S9. The QTOF MS/MS spectrum of **1**-modified ALVLIAFAQYLQQCPFEDHVK at cysteine after trypsin digestion of **1**-modified HSA and the QTOF MS spectrum of **1**-modified ALVLIAFAQYLQQCPFEDHVK (ESI source, triply charged ion of *m/z* = 1061.5) (inset)

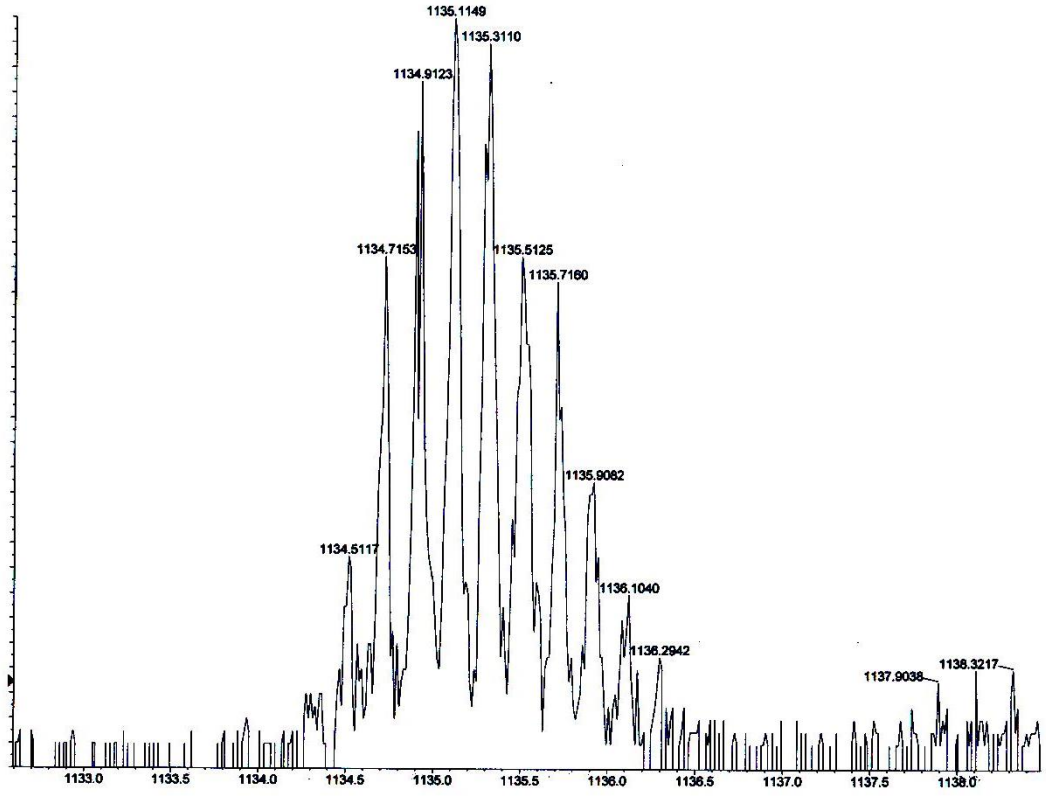


Fig. S10. The QTOF MS spectrum of **1**-modified LGVIWYDAHGDVNTAETSPSGNIHGMPLAASLGFGHPALTQIGGYCPK after trypsin digestion of **1**-modified BCArg (ESI source, quintuply-charged ion of *m/z* =1135.1)

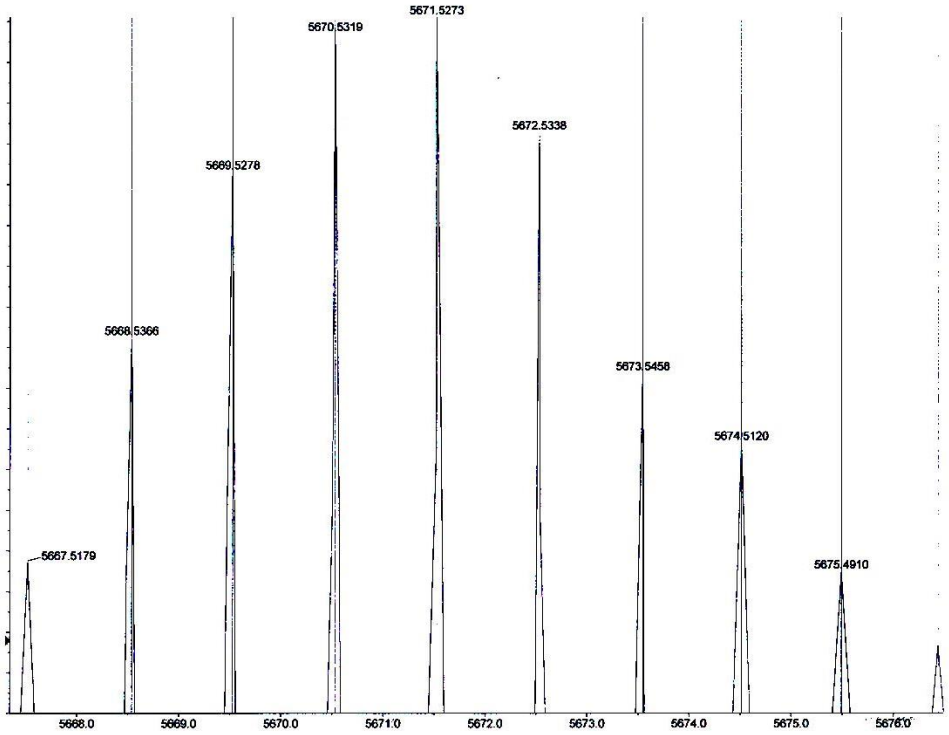


Fig. S11. Mass reconstruction of trypsin-digested peptide fragment from **1**-modified BCArg: LGVIWYDAHGDVNTAETSPSGNIHGMPLAASLGFGHPALTQIGGYCPK incorporated with one molecule of **1**

Absorption and Emission Profile of 1

UV/Vis spectra were recorded on a Hewlett Packard 8453 UV/Vis spectrophotometer. Steady-state emission spectra were recorded on a SPEX Fluorolog-3 Model FL3–21 spectrofluorometer. **1** (10 μM in CH_3CN) were degassed with at least four freeze-pump-thaw cycles. The emission spectra were corrected for monochromator and photomultiplier efficiency and for xenon lamp stability. The excitation wavelength was 380 nm and the excitation and emission slit widths were 3.5 nm. The absorption and emission profiles of 1 were compared in Fig. S12.

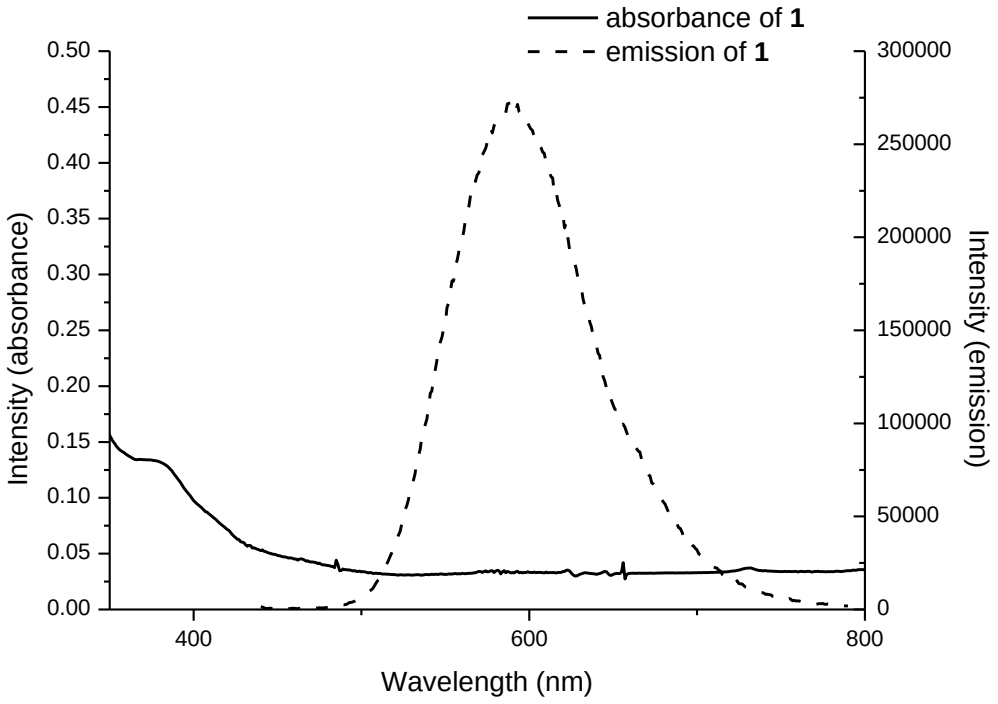


Fig. S12. Absorption and emission profile of **1**

Emission Lifetime Measurement of 1

Emission lifetime measurement of **1** (10 μM in CH_3CN) was performed with a Quanta Ray DCR-3 pulsed Nd:YAG laser system (pulse output 355 nm). Decay times were registered using a FAST Comtec multichannel scaler PCI card with a time resolution of 250 ps.

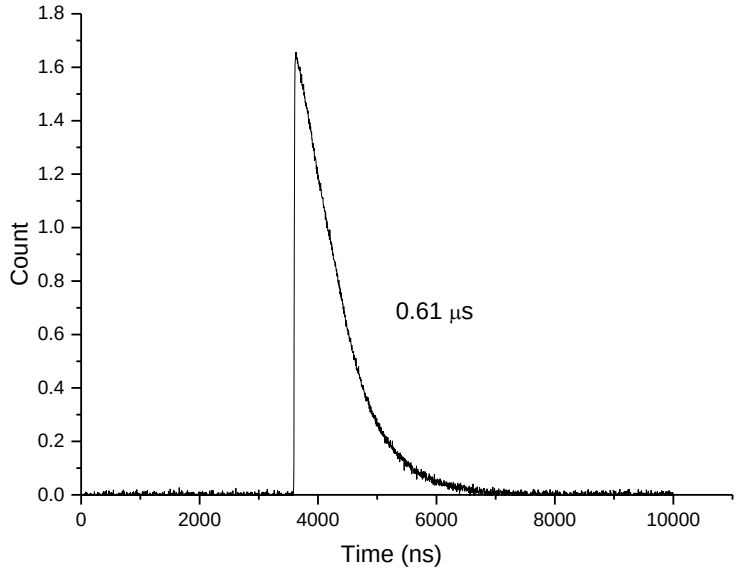


Fig. S13. Emission lifetime spectrum of **1**

Quantum Yield Determination

Luminescence quantum yields were determined by using the method of Demas and Crosby[S1] with $[Ru(bpy)_3]Cl_2$ (bpy=2,2′-bipyridine) in degassed acetonitrile as a standard reference solution (Φ_r=0.062) and calculated according to the following equation: $\Phi_s=\Phi_r(B_r/B_s)(n_s/n_r)^2(D_s/D_r)$ in which the subscripts *s* and *r* refer to sample and reference standard solution respectively, Φ is the luminescence quantum yield, *D* is the area under the corrected emission curve (integrated intensity) and *n* is the refractive index of the solvent. The quantity *B* was calculated by $B=1-10^{-AL}$, in which *A* is the absorbance at the excitation wavelength and *L* is the optical path length. Acetonitrile was used for both the standard and the sample solution; the refractive index ratio was therefore equal to 1.

Here, the quantum yield of **1** was calculated to be 0.12.

S1. J. N. Demas, G. A. Crosby, *J. Phys. Chem.* **1971**, *75*, 991–1024.

Expression of Protein BCArg

The gene encoding *Bacillus caldovelox* arginase (BCArg) is rationally engineered to introduce the cysteine residue and polyhistidine tag. The gene was cloned into the pET3a vector and transformed into the *E.coli* strain BL21(DE3). Over expression of BCArg in the shake flask culture was mediated by isopropyl-beta-D-thiogalactopyranoside (IPTG) induction and 2-step purification through heat treatment and affinity purification was carried out to purify the BCArg to almost homogeneity as examined by SDS-PAGE (Fig. S14).

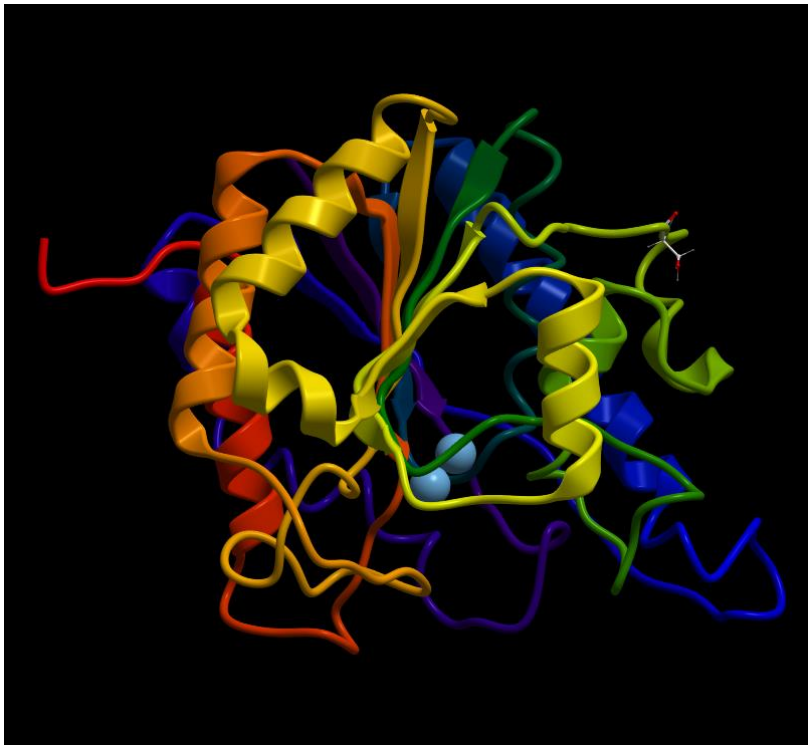


Fig. S14. Crystal structure of BCA (PDB: 2CEV) retrieved from the protein data bank. The ribbon diagram showing the structure of BCArg containing the catalytic dimanganese ions (Blue sphere) and the surface exposed serine residue was showed by the ball and stick representation. This serine residue was replaced by the cysteine residue. The surface exposed C-terminal regions (Red) were sitting opposite to the serine residue.

SDS-PAGE Analysis of BCArg and 1-modified BCArg

In a 1.0 mL eppendorf tube, 50 μL of BCArg solution (0.09 mM in 20 mM phosphate buffer pH 8.0) was mixed with 4.3 μL of **1** (10 mM in CH_3CN). The reaction mixture was kept at room temperature for 24 h. The **1**-modified BCArg was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis in reducing condition.

In a 0.5 mL eppendorf tube, 2 μL of native BCArg or **1**-modified BCArg (0.09 mM) was respectively mixed with 10 μL 2X reducing sample loading buffer and then boiled for 10 min. Samples were analyzed by SDS-PAGE by loading all boiled samples in each lane on an 15% SDS-PAGE and running in a Mini-PROTEAN® Tetra Cell (Bio-Rad, USA) at 150 V at room temperature until the dye front reached the bottom of the gel. After SDS-PAGE separation, the native BCArg and **1**-modified BCArg were visualized with an AlphamagerTM 2200 MultilmageTM Light Cabinet and finally stained with Coomassie blue, as shown in Fig. 4a.

Bioactivity of BCArg and 1-modified BCArg

To prepare **1**-modified BCArg, 50 μL of BCArg solution (0.09 mM in 20 mM phosphate buffer pH 8.0) was mixed with 4.3 μL of 1 (10 mM in CH_3CN). The reaction mixture was kept at room temperature for 24 h.

BCArg or **1**-modified BCArg activity was determined by diacetyl monoxime (DAMO) method[S2], a spectrophotometric assay that can be used to determine the enzymatic product, urea. Briefly, several dilutions of BCArg or **1**-modified BCArg and 720 mM arginine substrate at pH 7.4 were incubated separately at 37 ℃ for 10 minutes. The enzymatic reaction was carried out by adding arginine substrate to the pre-incubated enzyme solutions. The reaction mixture was incubated at 37 ℃ for exactly 5 minutes and quenched by adding 50% trichloroacetic acid (TCA) solution. The urea formed from the enzymatic reaction was determined by DAMO method. One unit of arginase was defined as the amount of enzyme that converts a micormole of arginine to ornithine and urea per minute at 37 ℃, pH 7.4. The respective enzymatic activity of BCArg and **1**-modified BCArg was summarized in Fig. 4b.

Emission Measurement of 1-modified BSA and 1-modified BCArg

Proteins (BSA or BCArg) (10 μL from 1 mM in H_2O), **1** (10 μL from 10 mM in CH_3CN) [protein/iridium(III) complex = 1/10] and pH 8.0 phosphate buffered saline PBS (80 μL) were mixed in a 1.0 mL Eppendorf tube at room temperature for 24 h. The reaction mixture was then purified by microspin column chromatography (MicroSpin G-50 Columns, GE Healthcare, UK) to remove **1** that was added in excess. The eluent (100 μL) was diluted with CH_3CN (30 μL) and pH 8.0 PBS (270 μL) and was shaken well. Steady-state emission spectra were recorded on a SPEX Fluorolog-3 Model FL3–21 spectrofluorometer. The emission spectra were corrected for monochromator and photomultiplier efficiency and for xenon lamp stability. The excitation wavelength was 380 nm and the excitation and emission slit widths were 3.5 nm. The emission profiles of **1** (25 μM in pH 8.0 PBS/CH_3CN = 9/1), **1**-modified BSA and **1**-modified BCArg were compared in Fig. S15.

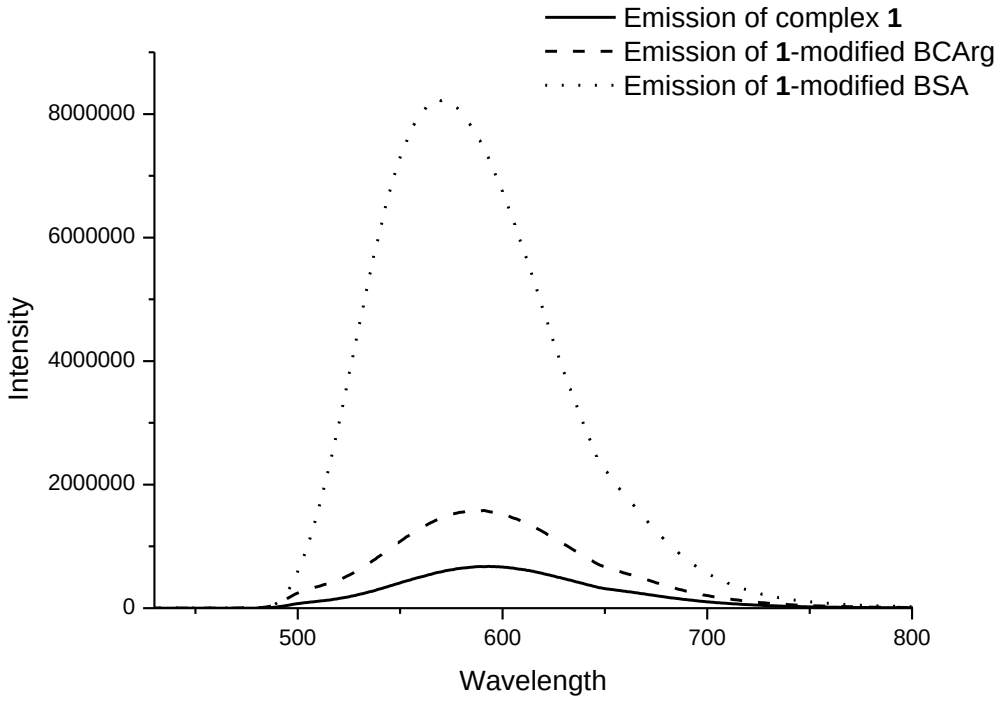


Fig.S15. Emission profiles of **1**, **1**-modified BSA and **1**-modified BCArg

Bioimaging with 1 and 1-modified HTDP

Preparation of 1-modified HTDP

HTDP (10 μL from 1 mM in H_2O), **1** (1 μL from 10 mM in CH_3CN) [peptide/iridium(III) complex = 1/1], CH_3CN (9 μL) and pH 8.0 phosphate buffered saline PBS (80 μL) were mixed in a 1.0 mL Eppendorf tube at room temperature for 24 h was examined. The **1**-modified HTDP were characterized by LC–MS/MS (Fig. S16–22) after desalting (ZipTip®, Merck Millipore, USA).

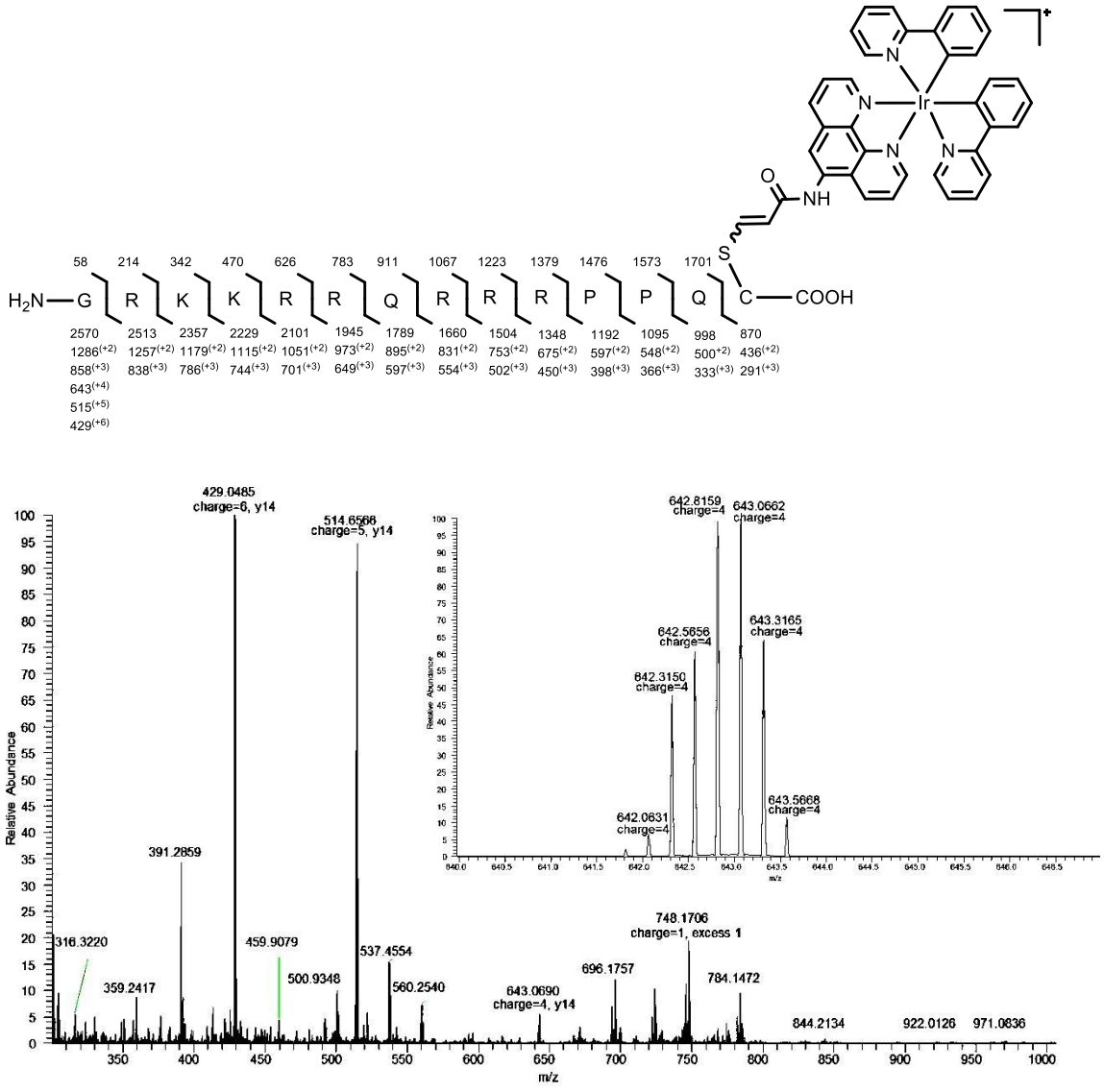


Fig.S16. The Orbitrap MS spectrum of **1**-modified GRKKRRQRRRPPQC (ESI source, quadruply charged ion of *m/z* = 643.1) (inset)

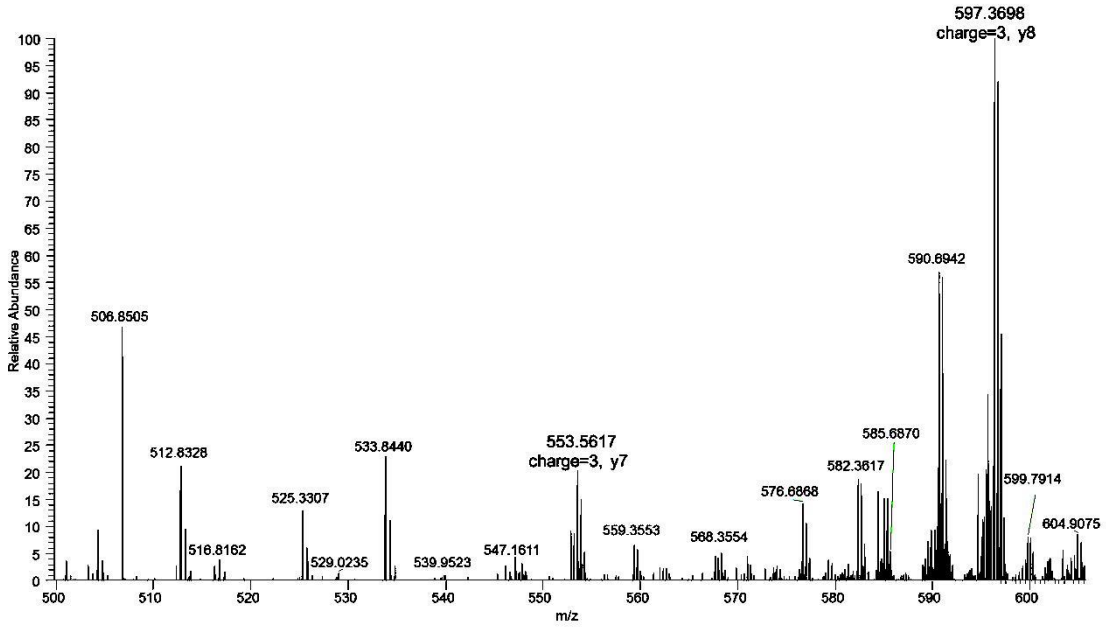


Fig. S17. The Orbitrap MS/MS spectrum of **1**-modified GRKKRRQRRRPPQC at cysteine (ESI source at quadruply charged ion of *m/z* = 643.1, mass range from 500–610)

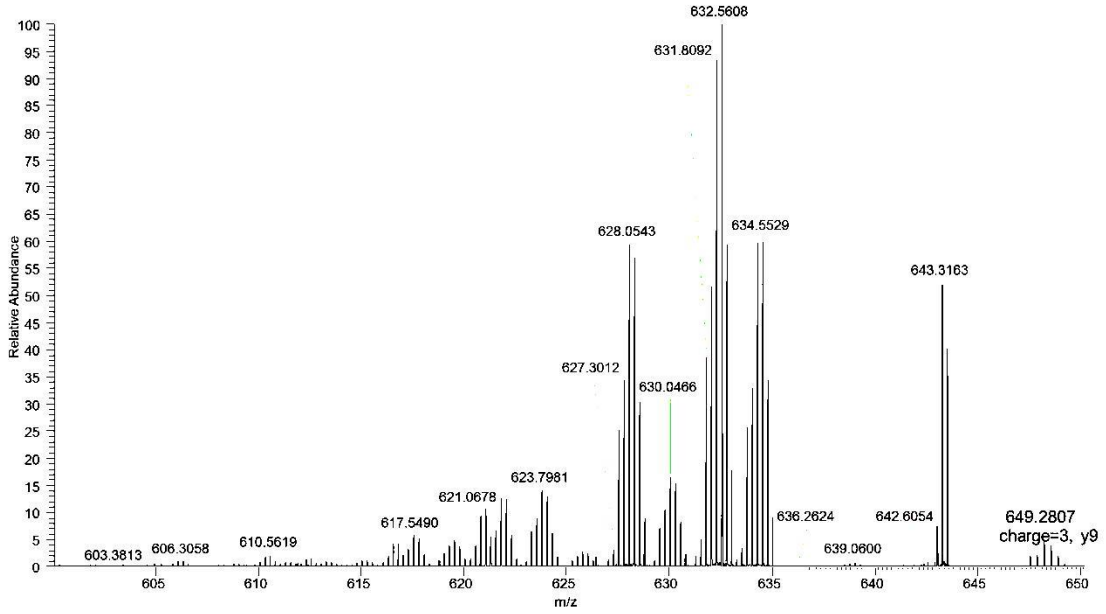


Fig. S18. The Orbitrap MS/MS spectrum of **1**-modified GRKKRRQRRRPPQC at cysteine (ESI source at quadruply charged ion of *m/z* = 643.1, mass range from 600–650)

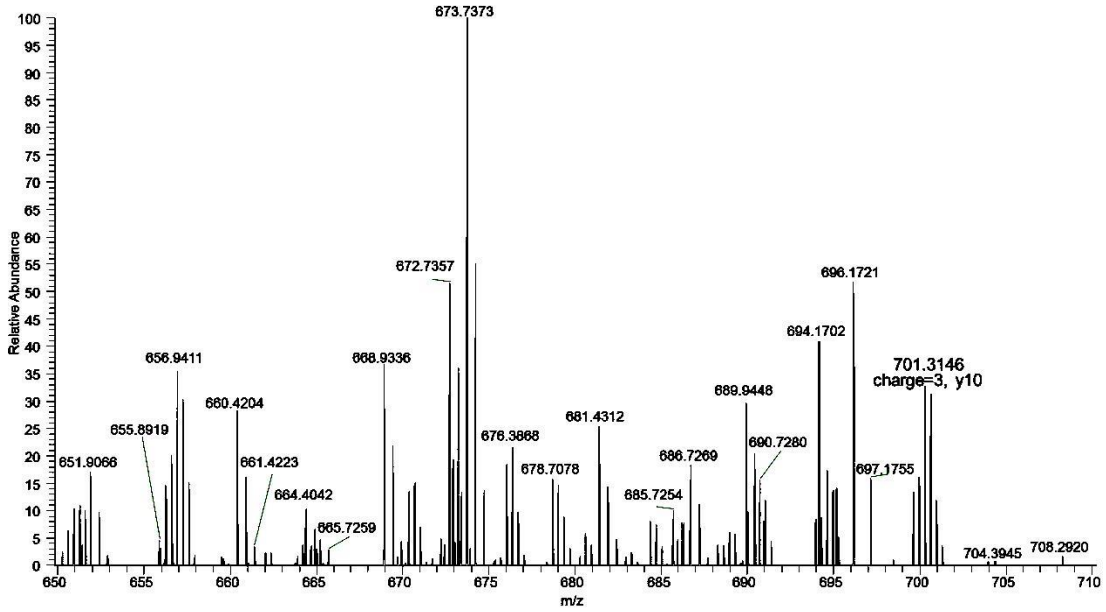


Fig.S19. The Orbitrap MS/MS spectrum of **1**-modified GRKKRRQRRRPPQC at cysteine (ESI source at quadruply charged ion of *m/z* = 643.1, mass range from 650–710)

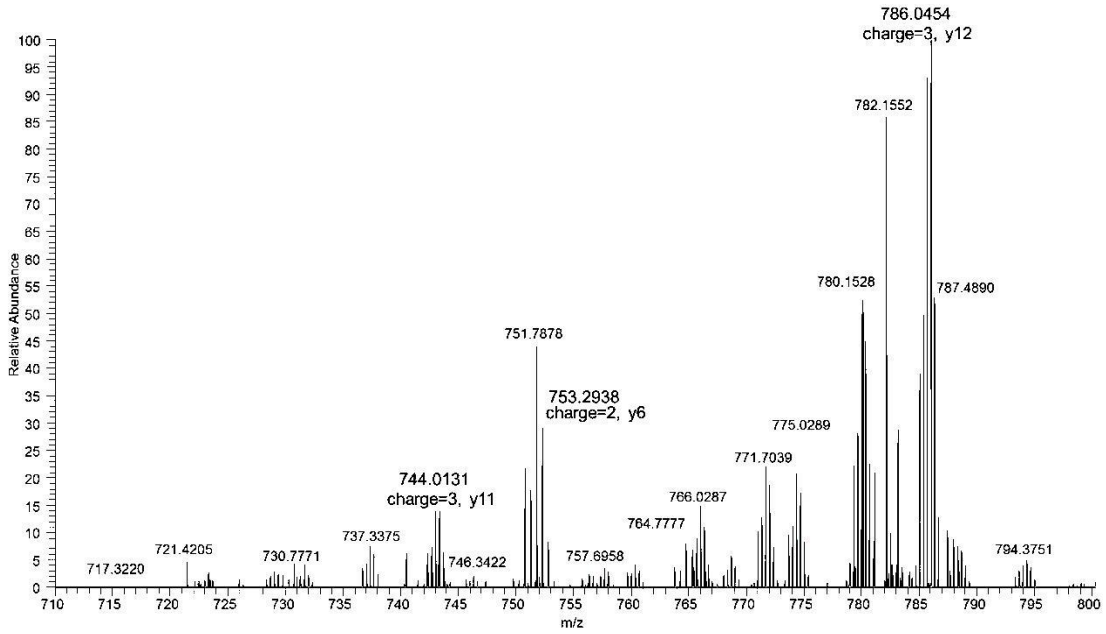


Fig. S20. The Orbitrap MS/MS spectrum of **1**-modified GRKKRRQRRRPPQC at cysteine (ESI source at quadruply charged ion of *m/z* = 643.1, mass range from 710–800)

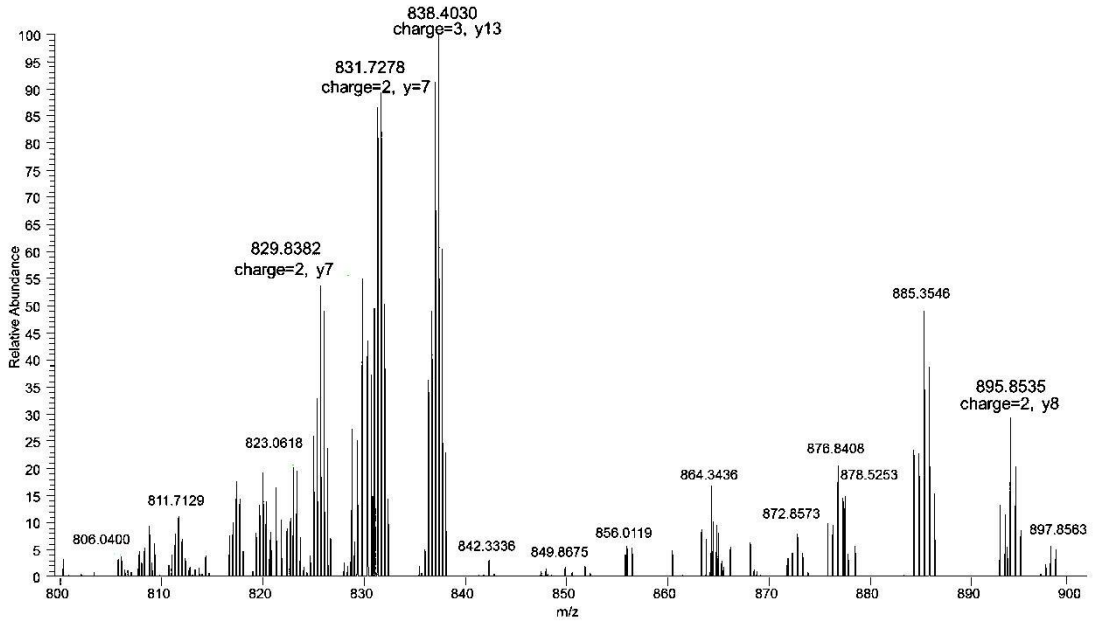


Fig. S21. The Orbitrap MS/MS spectrum of **1**-modified GRKKRRQRRRPPQC at cysteine (ESI source at quadruply charged ion of *m/z* = 643.1, mass range from 800–900)

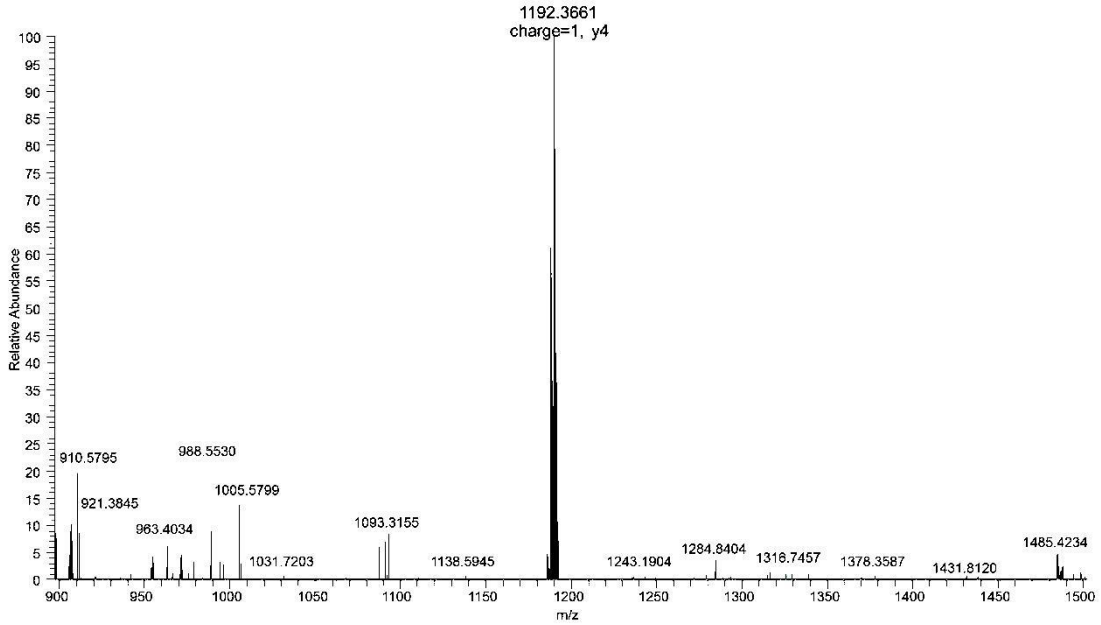


Fig. S22. The Orbitrap MS/MS spectrum of **1**-modified GRKKRRQRRRPPQC at cysteine (ESI source at quadruply charged ion of *m/z* = 643.1, mass range from 900–1500)

Fluorescence Microscopic Studies

HeLa cells (2×10^5 cells) in 2 mL minimal essential medium were seeded in a the 2 cm dish with glass bottom with and were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h. Then the cells were treated with **1** or **1**-modified HTDP with/without Mitotracker® (50 nM) or Lysotracker® (100 nM). After a period of time, the cells were examined by fluorescent imaging. Correlation analysis was conducted using ImageJ according to the reported procedures in C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, **9**, 671-675.

Cytotoxicity to Cancer Cells

HeLa cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C humidified atmosphere with 5% CO_2. The cytotoxicities were determined by MTT assay. In brief, complexes-treated cells were incubated with MTT for 4 h at 37 °C in a humidified atmosphere of 5% CO_2 and were subsequently lysed in solubilizing solution. Cells were then maintained in a dark, humidified chamber overnight. The formation of formazan was measured by using a microtitre plate reader at 580 nm. Cytotoxicity was evaluated by IC_{50} (concentration of a drug causing 50% inhibition of cell growth). Each growth inhibition experiment was repeated at least three times and results were expressed as means ± standard deviation (SD).

Bioimaging with 1

The cellular uptake and intracellular localization of complex **1** by fluorescence microscope was investigated. After incubation of HeLa cells (human cervical cancer) with **1** (2.5 μM, 24 h cytotoxic IC_{50} is 14.3±1.8 μM) for 20 min, bright yellow-red images could be detected in the cytoplasm, with major portion localized in

mitochondria but not in lysosome according to co-localization analysis with commercial markers (Fig. S23). Such localization and the associated cytotoxicities may be partially due to binding interactions with thiol-containing proteins/enzymes (e.g. thioredoxin, glutathione reductase) in mitochondria.

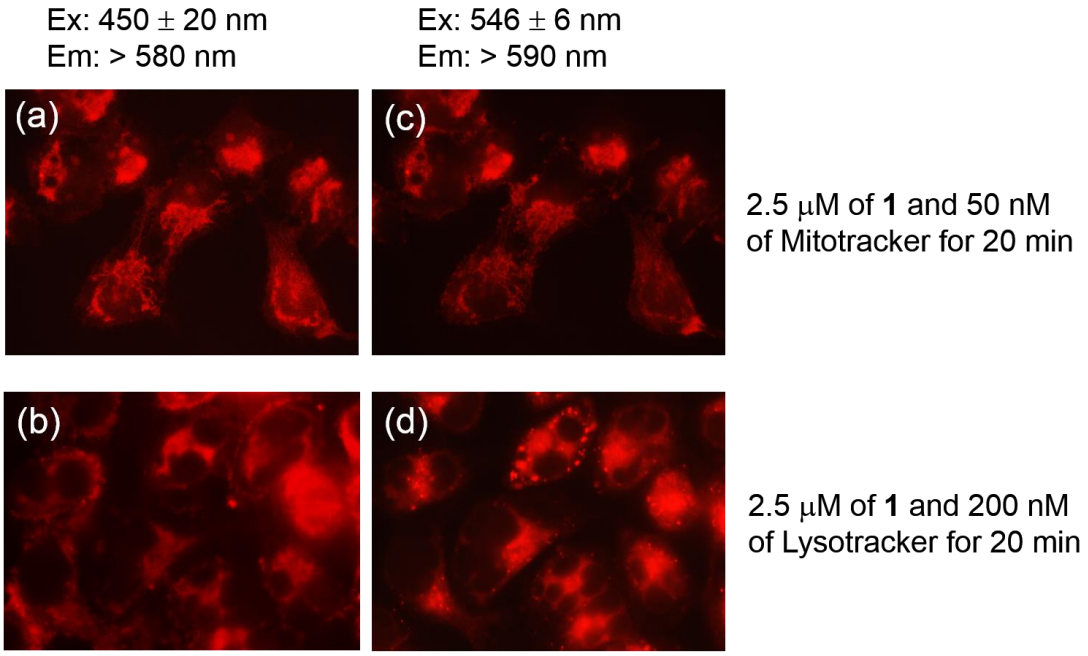


Fig. S23. Fluorescence microscopic analysis of HeLa cells treated with **1** (2.5 μM, panel a and b) and Mitotracker® (50 nM, panel c) or Lysotracker® (200 nM, panel d). The emission and excitation filter are shown.

Bioimaging of 1-modified HTDP

After incubating the **1**-modified HTDP (10 μM, 24 h cytotoxic IC_{50} is 60.1±6.0 μM) with HeLa cells, yellow-red images gradually appeared in cytoplasm after 4 h, indicating the cell penetrating property of the **1**-modified HTDP (Fig. 5).