

Supplementary Information
For
**A Bioorthogonal ‘turn-on’ Fluorescent Probe for Tracking
Mitochondrial Nitroxyl Formation**

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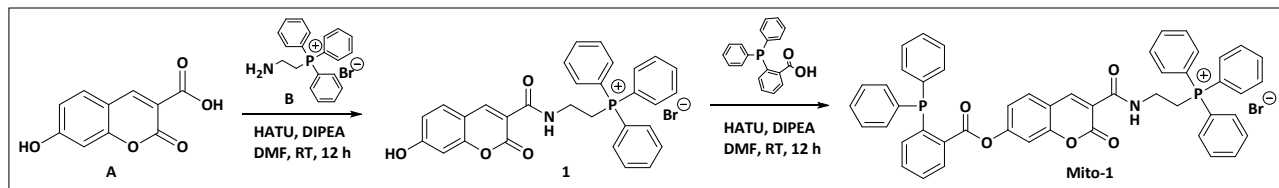
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Materials, methods and instrumentations

2,4-dihydroxybenzaldehyde (TCI), diethylmalonate (Avra, India), 2-(diphenylphosphino) benzoic acid (Alfa-aesar), HATU (Alfa-aesar), DIPEA (Alfa-aesar), THF (Merck), methanol (Merck), DMF (Aldrich), diethyl ether (Loba chem., India), Sodium hydroxide (Loba chem., India) and DCM (Loba chem. India) were purchased commercially and used without further purification. All the materials for UV/Vis and Fluorescence spectroscopy, Angeli's salt(Cayman Chemical), L-cysteine(Acros), potassium superoxide(Aldrich), sodium sulfide (Aldrich), sodium hypochlorite (Alfa Aesar), L-glutathione (Aldrich), hydrogen peroxide (Samchun), homocysteine (Aldrich), sodium nitrite (Aldrich), sodium nitrate (Aldrich), DEA NONOate(Cayman Chemical), 2,2'-Azobis(2-methylpropionamide) dihydrochloride (Aldrich), DMSO (J.T.Baker), sodium hydroxide(Samchun) were purchased from commercial suppliers and were used without further purification. Flash column chromatography was performed using Silica gel (100-200 mesh) and Analytical thin layer chromatography was performed using silica gel 60 (pre coated sheets with 0.25 mm thickness). Mass spectra were recorded on anion SpecHiResESI mass spectrometer. NMR spectra were collected on a 400MHz spectrometer (Bruker, Germany).

Reaction Scheme:



Synthesis of A

Synthesized according to reported literature¹.

Synthesis of B

Synthesized according to reported literature².

Synthesis of **1**

To a solution of **A** (1 g, 4.855 mmol) in DMF (10 mL) at 0°C DIPEA (5.22 mL, 29.13 mmol), **B** (2.73 g, 5.825 mmol) and HATU (2.77 g, 7.282 mmol) were added. The reaction was continued to stir for 12h at RT. The reaction was monitored by TLC. After completion of reaction, the reaction mixture was poured into water (50 mL) and extracted with ethyl acetate (3 x 25 mL). The organic layer was washed with water (2 x 50 mL), brine solution (1 x 50 mL), dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (100-200 mesh) using methanol in DCM (1:9) as eluent to obtain **1** as pale yellow solid (1.8 g; 64.47%). ¹H-NMR (400 MHz, DMSO-d₆): δ 11.10 (s, 1H); 8.96 (s, 1H); 7.89-7.74 (m, 11H); 7.73 (d, *J* = 2.80 Hz, 2H); 6.90 (q, 1H); 6.81 (d, *J* = 1.60 Hz, 1H); 3.87 (m, 2H); 3.71 (d, *J* = 2.4 Hz, 2H); ¹³C-NMR (100 MHz, DMSO-d₆): 164.35, 163.40, 160.09, 157.10, 149.09, 135.01, 134.10, 133.99, 130.25, 119.51, 115.20, 113.09, 111.25, 101.99; ESI- HRMS *m/z* (M+H): calcd. 494.15157, found 494.15048.

Synthesis of Mito-1

To a solution of 2-(diphenylphosphino) benzoic acid (375 mg, 1.23 mmol) in DMF (10 mL) at 0°C DIPEA (0.4 mL, 2.234 mmol), **1** (640 mg, 1.117 mmol) and HATU (640 mg, 1.675 mmol) were added. The reaction was continued to stir for 12 h at RT and the reaction was monitored by TLC. After completion of reaction, reaction mixture was diluted with water and stirred for 15 min. The precipitated solid was filtered, washed with water and dried in vacuo. The crude product was purified by column chromatography over silica gel (100-200 mesh) using Methanol in DCM (0.5: 9.5) as eluent to afford probe, **1** as pale yellow solid (320 mg; 33.20%). ¹H-NMR (400 MHz, CDCl₃): δ 9.05 (d, *J* = 8.0 Hz, 1H); 8.80 (s, 1H); 8.29 (d, *J* = 2.0 Hz, 1H); 8.02 (t, *J* = 2.04 Hz, 1H); 7.99-7.62 (m, 17H); 7.40-7.35 (m, 7H); 7.32-7.06 (m, 5H); 6.92 (d, *J* = 3.0 Hz, 1H); 3.87 (t, 2H); 3.72 (t, *J* = 3.2 Hz, 2H); ¹³C-NMR (100 MHz, DMSO-d₆): 165.02, 162.15, 159.05, 154.05, 148.05, 141.02, 137.20, 135.10, 134.00, 132.50, 130.99, 129.60, 119.99, 118.50, 118.25, 117.59, 116.20, 110.79; ESI-MS *m/z* (M+H): calcd. 782.22, found 783.00; HRMS (M + Oxygen adduct): 798.21234; HPLC ~96.04%.

UV/Vis and Fluorescence Spectroscopy

1×10^{-3} M Stock solution of the probe was prepared by dissolving probe in DMSO. Absorption spectra were recorded on an S-3100 (Scinco) spectrophotometer, and fluorescence spectra were recorded using an RF-5301 PC spectrofluorometer (Shimadzu) fitted with a xenon lamp. The fluorescence emission spectra were recorded at excitation wavelength of 398 nm and emission was monitored over wavelength the range of 400–600 nm ($\lambda_{em} = 452$ nm). The stock solutions of Hcy, GSH, Cys, KO_2 , $NaNO_2$, $NaNO_3$, $NaClO$, H_2O_2 (1×10^{-3} M) in distilled water; DEA NONOate, Na_2S , Angeli's salt (1×10^{-3} M) in $NaOH$ (1×10^{-2} M); KO_2 (1×10^{-3} M) in DMSO were also prepared. Quartz cuvettes were used for absorption and emission measurements (4 mL volume) of samples. All spectroscopic measurements were performed under physiological conditions of pH (PBS buffer, pH 7.4).

Cell culture

Cell culture and data analysis a human cervical cancer cells (HeLa) were purchased from Korean Cell Line Bank (Seoul, Republic of Korea). Cells were cultured in Dulbecco's modified Eagle's medium including high glucose (GE Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany), and 1% penicillin/streptomycin (Hyclone) at $37^\circ C$ in 5% CO_2 .

Two-photon fluorescence microscopy

1×10^5 cells were seeded on Delta T Dishes (Bioprotechs). Cells with 70–85% confluency were washed with PBS and incubated with the probe **Mito-1** ($10 \mu M$ in PBS) and 5% CO_2 at $37^\circ C$ for 5 min. Then, cells were treated with $50 \mu M$ AS in PBS for 30 min and washed with PBS. Two-photon fluorescence microscopy images of probe labeled HeLa cells were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with a $\times 100$ (NA = 1.30 OIL) objective lens. The two-photon fluorescence microscopy images were obtained with a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 740 nm. To obtain images at 420–520 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 512×512 pixels at 400 Hz scan speed.

One-photon fluorescence microscopy

Fluorescence images were taken using a confocal laser scanning microscope (Carl-Zeiss LSM 700 Exciter, Oberko, Germany) with same condition. Fluorescence channel was excited at 405 nm with a Si laser and emission was collected by a 470-500 nm band pass filter. Mito-Track Red (Invitrogen, Carlsbad, CA, USA), Lyso-Tracker Red (Invitrogen), and ER-Tracker Red (Invitrogen) were used for tracing the probe location. Fluorescence images were obtained by using confocal laser scanning microscope (Cal Zeiss)

Endogenous HNO sensing condition: 1×10^5 cells were seeded on a 35 mm-confocal dish (SPL Life Sciences, Seoul, Republic of Korea) containing DMEM medium and incubated in 5% CO₂ at 37 °C for 10 h. Then the cells were treated with 200 µM DEA NONOate for 20 h. The cells were washed with PBS and treated with 0.03 mM NEM in DMEM medium and incubated in 5% CO₂ at 37 °C for 10 min. After pre-treated NEM, the cells were washed with PBS, and treated with Probe **Mito-1** (5 µM) and incubated for 5 min. Cell morphology was taken by using a LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany). For NaAsc treated group, after 5 min probe **Mito-1** treatment, the cells are washed with PBS and treated 1.5 mM NaAsc and incubated in 5% CO₂ at 37 °C for 20 minutes. Then the cells were washed and analyzed by using a fluorescent microscope.

Exogenous HNO sensing condition: 1×10^5 cells were seeded on a 35 mm-confocal dish (SPL Life Science). Cells with 70~85% confluency were washed with PBS and treated with 0.03 mM NEM in DMEM medium for 10 min. Then, the cells were treated with probe **Mito-1** (5 µM) in PBS containing 5% FBS for 5 min. After probe treatment, the cells were washed with PBS and 300 µM Angeli's Salt in PBS was added for 30 min for fluorescent images using a microscope.

Cell cytotoxicity test

Approximately 1×10^4 cells were seeded on 96 well microplate (SPL Life Science, Gyeonggi-do, Korea) and incubated for 24 hr. After incubation, the cells were treated with DMSO and probe for 5 min as various conditions. To analyze cytotoxicity of the probe on cells, we performed cytotoxicity assay using SensoLyte® Cell Cytotoxicity Assay Kit (AnaSpec, Fremont, California). Fluorescence level was analyzed by using SPECTRA MAX GEMINI EM microplate reader (Molecular Devices, Sunnyvale, California). The excitation and emission wavelength were set at 544 nm and 590 nm, respectively.

The Linear Range and Detection Limit

The Fluorescence titration was used to calculate the detection limit. The fluorescence emission spectrum of probe **Mito-1** (2.0×10^{-6} M) was measured ten times and the standard deviation of blank measurement was achieved. The fluorescence intensity at 452 nm was plotted as a concentration of HNO. The detection limit was calculated by using following equation.

Detection Limit $3\sigma/k$:

Where σ is the standard deviation of blank measurement, k is the slope between the fluorescence intensity versus HNO concentration.

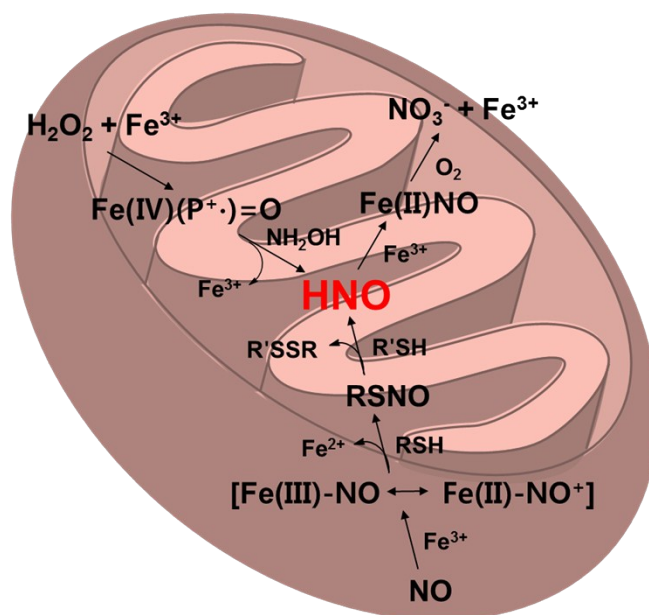


Fig. S1 Schematic presentation of biochemical pathways of nitroxyl formation in cellular system³

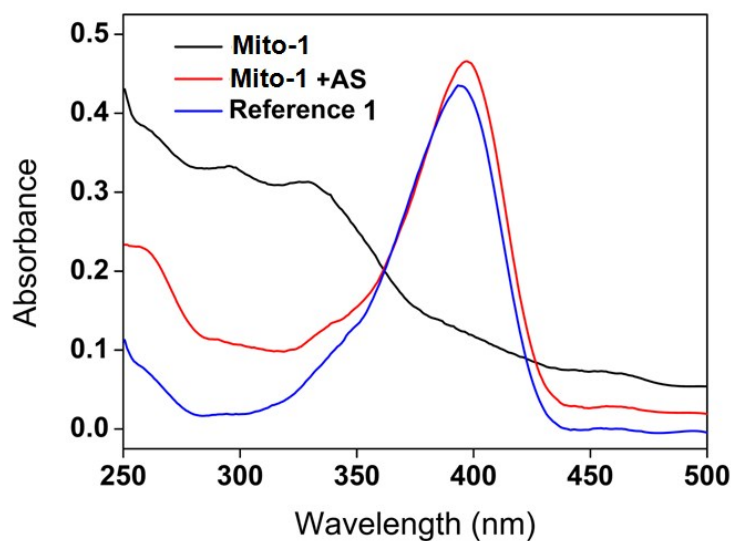


Fig.S2 UV-Vis spectra of the reference **1** (10 μ M) and **Mito-1** (10 μ M) in the absence and presence of AS (30 μ M) in pH 7.4 PBS buffer. Data were obtained after incubation at 25 $^{\circ}$ C for 30 min..

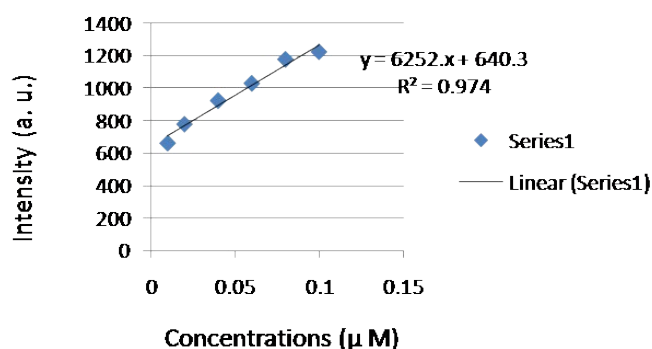


Fig. S3 The changes in the fluorescence intensity of **Mito-1** ($2\mu\text{M}$) at 450 nm against varied concentrations of HNO from 0 to $0.15\mu\text{M}$ in DMSO–PBS buffer (0.01 M , pH 7.4) (V/V= 2:98) with the slit width 5/5 nm.

Previous reported work	Detection limit
C. Liu, H. Wu, Z. Wang, C. Shao, B. Zhu and X. Zhang, <i>Chem. Commun.</i> , 2014, 50 , 6013	0.5 μM
G. J. Mao, X. B. Zhang, X. L. Shi, H. W. Liu, Y. X. Liu, H. Y. Zhou, W. Tan and R. Q. Yu, <i>Chem. Commun.</i> , 2014, 50 , 5790	0.02 μM
Z. Miao, J. A. Reisz, S. M. Mitroka, J. Pan, M. Xian and S. B. King, <i>Bioorg. Med. Chem. Lett.</i> , 2015, 25 , 16	Not reported
K. Zheng, W. Lin, D. Cheng, H. Chen, Y. Liu and K. Liu, <i>Chem. Commun.</i> , 2015, 51 , 5754	0.59 μM
K. N. Bobba, Y. Zhou, L. E. Guo, T. N. Zang, J. F. Zhang, S. Bhuniya, <i>RSC Adv.</i> , 2015, 5 , 84543	0.02 μM
X. Zhu, M. Xiong, H.-W. Liu, G.-J. Mao, L. Zhou, J. Zhang, X. -B. Zhang and W. Tan, <i>Chem. Commun.</i> , 2016, 52 , 733	0.59 μM

Fig. S4 Nitroxyl detection limit of previous reported work (Ref. 12).

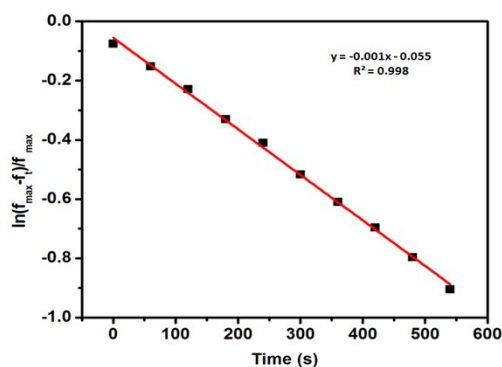


Fig. S5 The estimation of reaction rate constant between **1** and nitroxyl (HNO) by applying regression equation

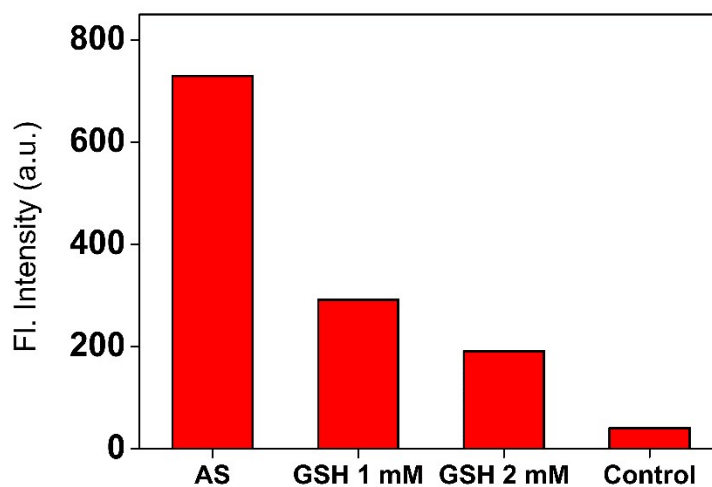


Fig. S6 The fluorescence responses of the probe (5.0 μM) with AS (30 μM /6eq) and high concentration of GSH (1 mM/200eq, 2 mM/400eq respectively) in pH 7.4 PBS buffer. Emission at 451 nm.

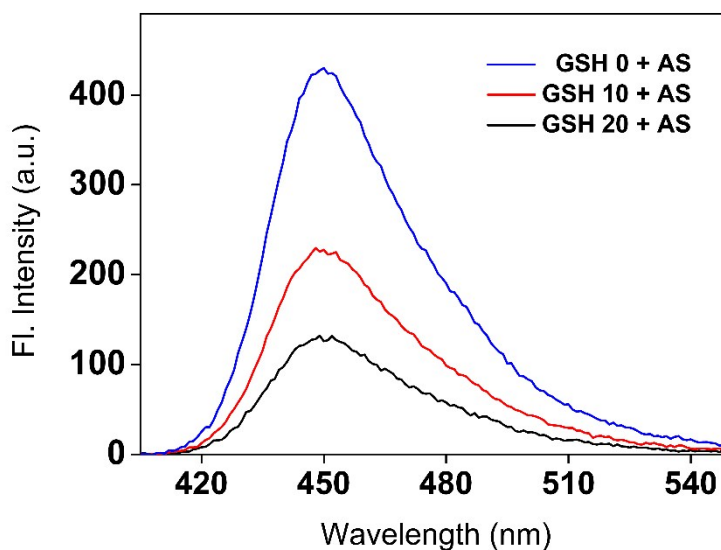


Fig. S7 The GSH dependent fluorescence change of **Mito-1**. Fluorescence spectra of the probe (5.0 μM) in pH 7.4 PBS buffer (0.5% DMSO) with AS (30 μM) and various concentration of GSH (0 μM , 10 μM , 20 μM) were recorded after incubation of the probe with AS and GSH for 20 min.

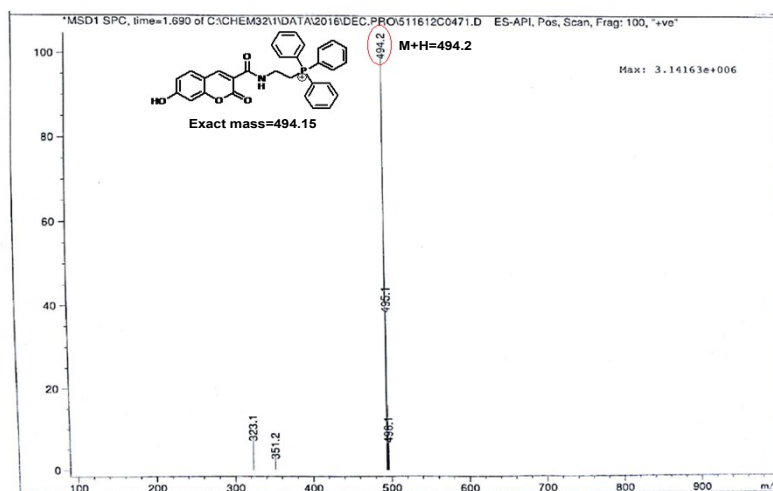
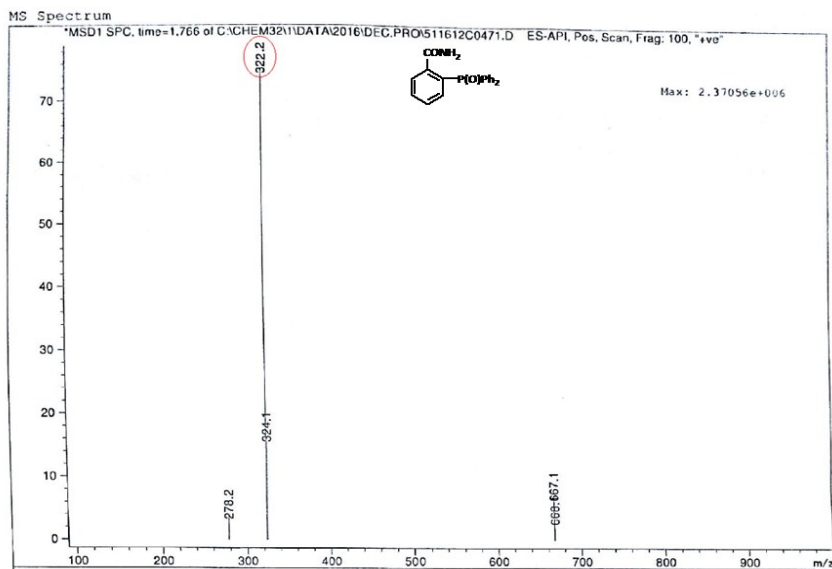
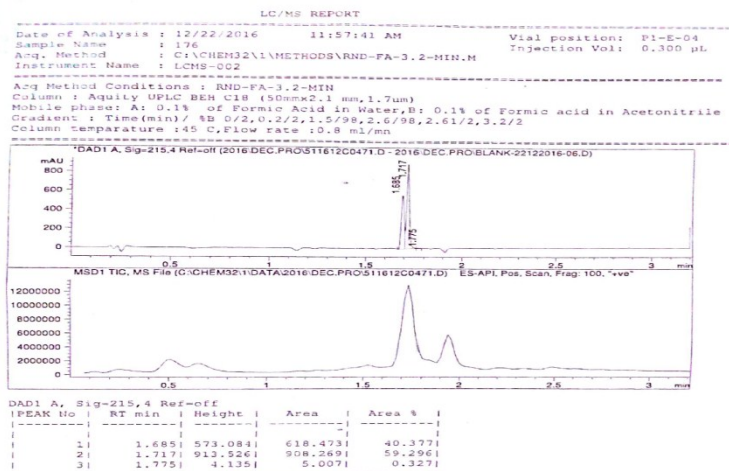


Fig S8. LC-MS data of Mito-1 treated with HNO (AS)

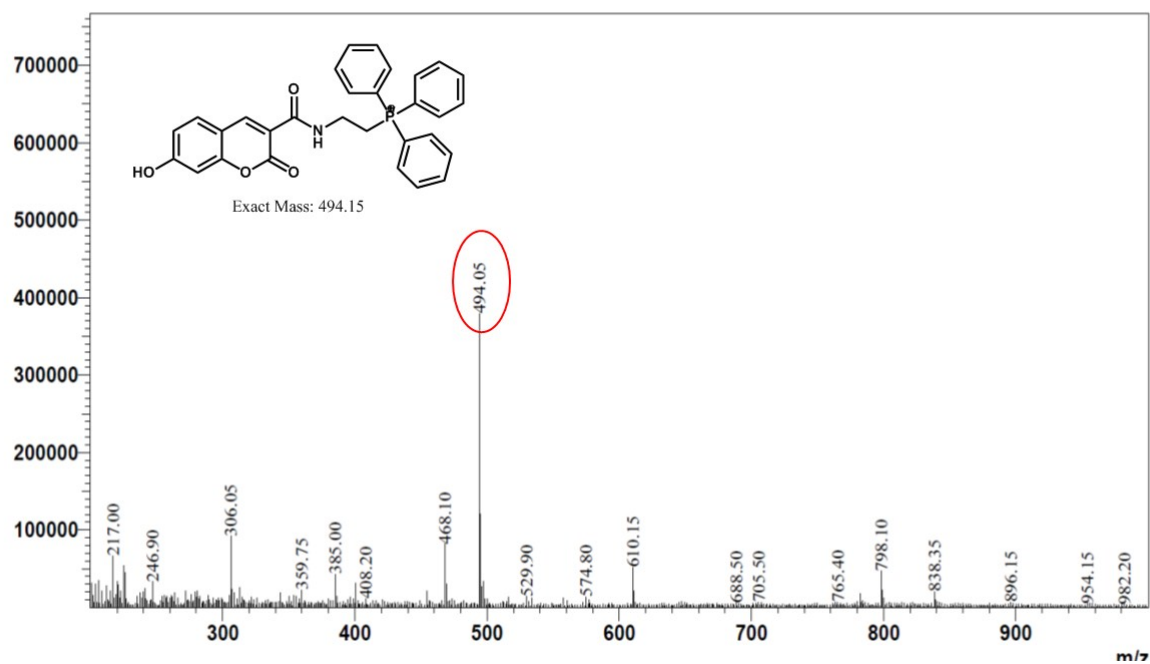


Fig. S9 The High resolution Mass spectrum of probe Mito-1 with AS after 30 min.HRMS (ESI): calcd for $C_{30}H_{24}NO_4P=494.15$, found $m/z=494.050$

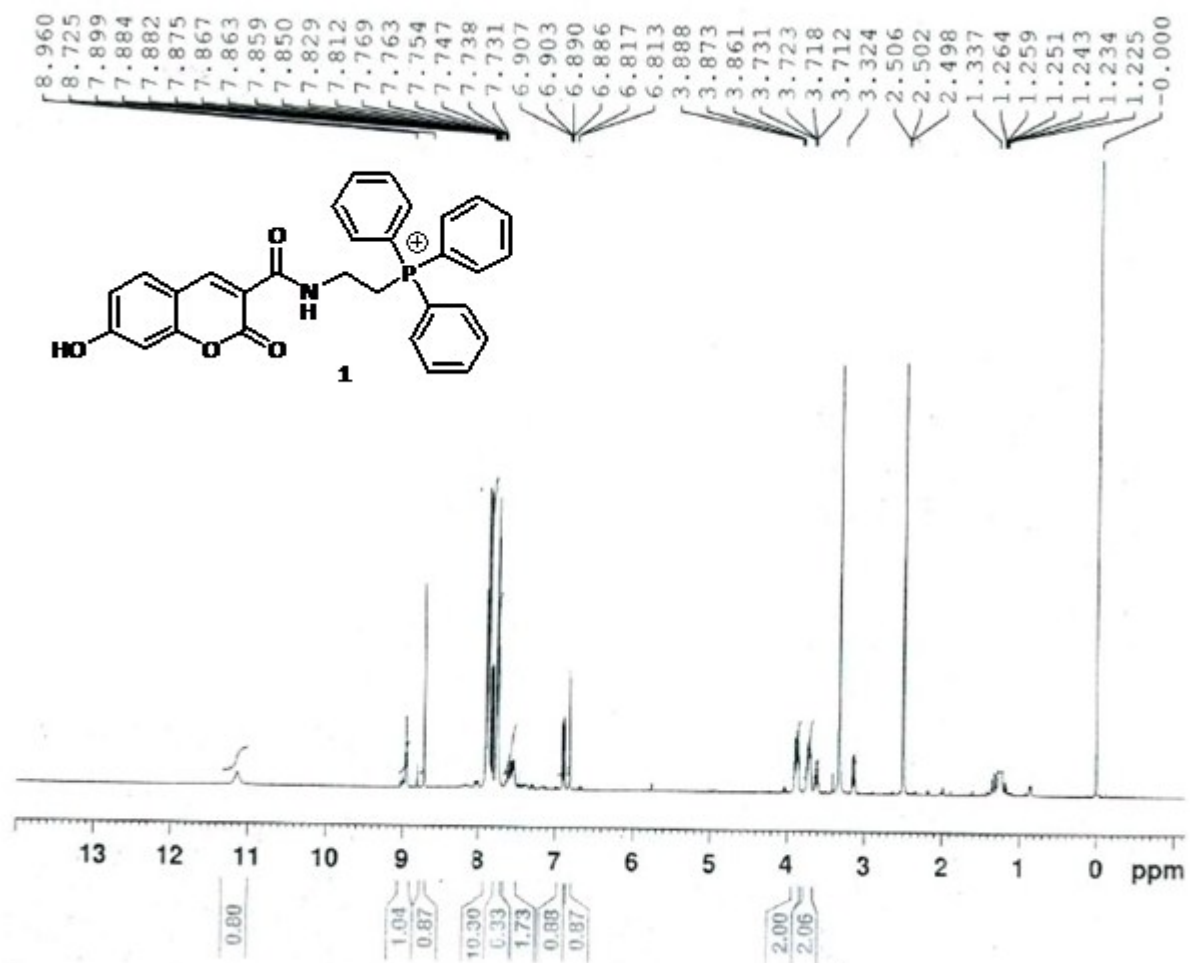


Fig.S10 ^1H -NMR of **1** in DMSO-d_6

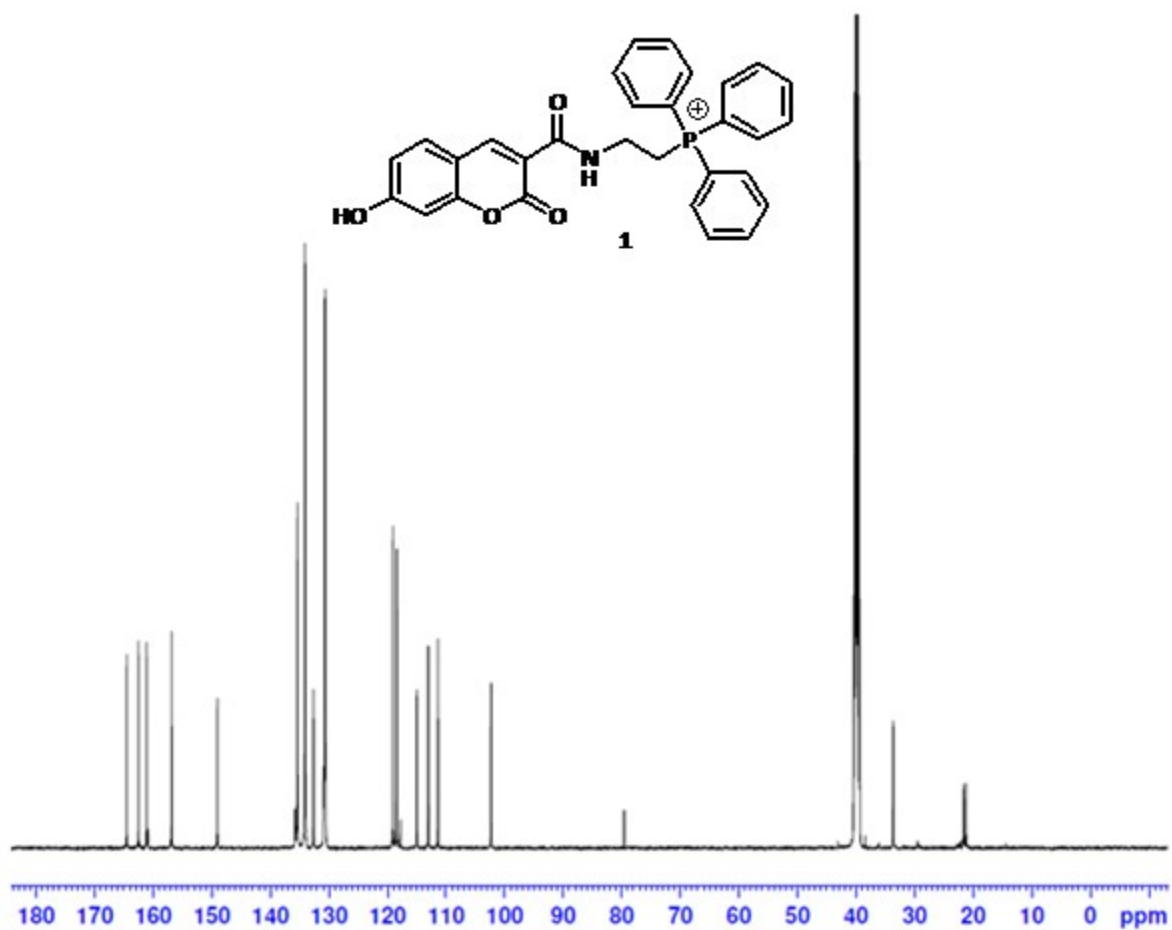


Fig. S11 ^{13}C -NMR of **1** in DMSO-d_6

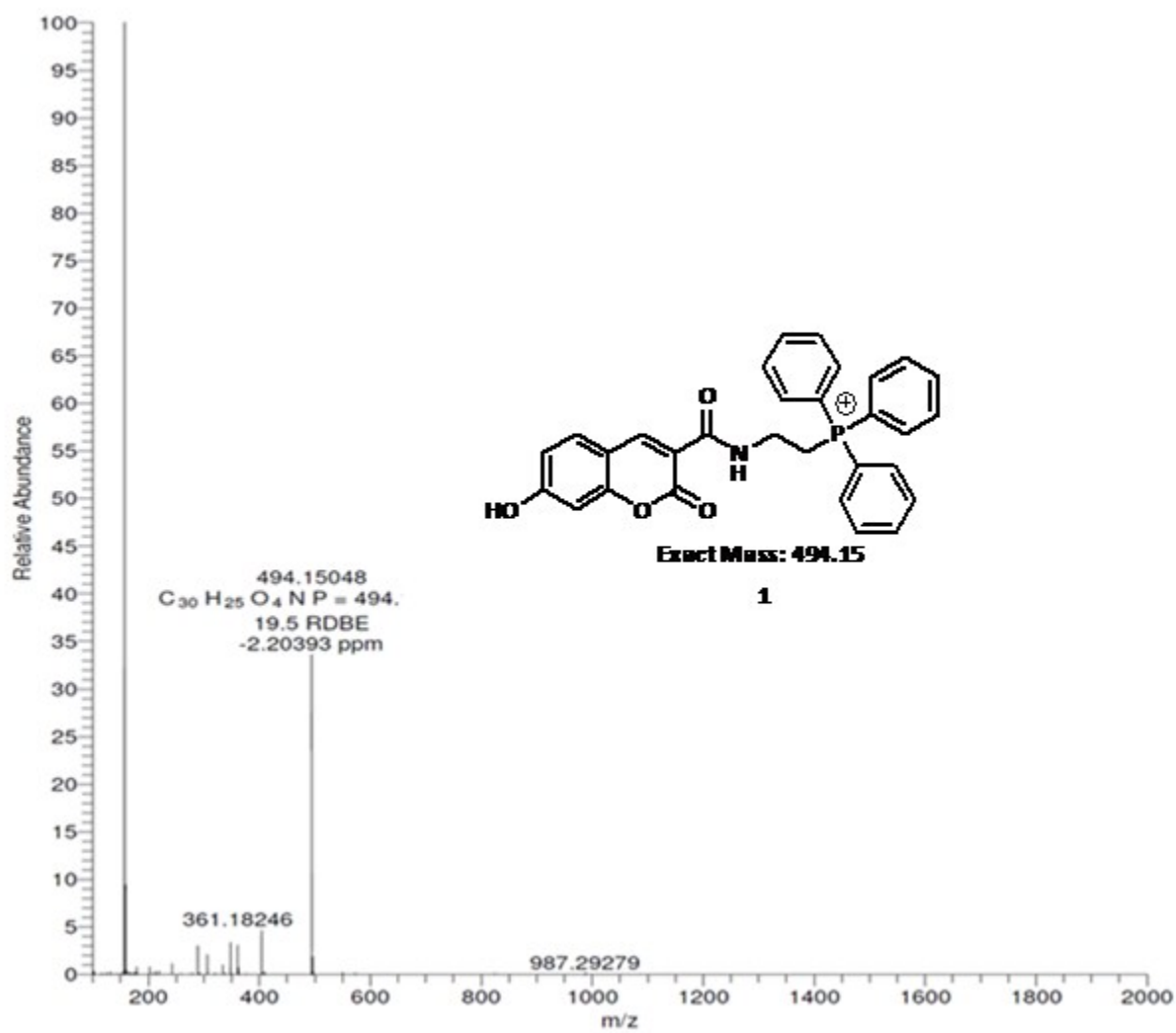


Fig.S12 HRMS of 1

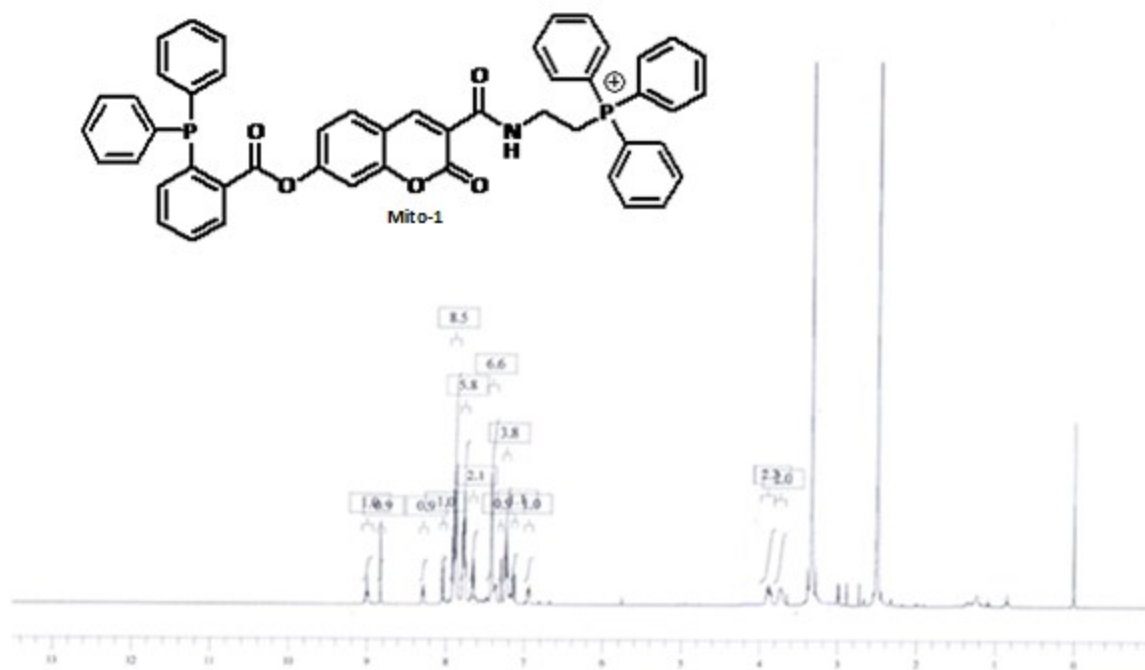


Fig.S13 ^1H -NMR of **Mito-1** in CDCl_3

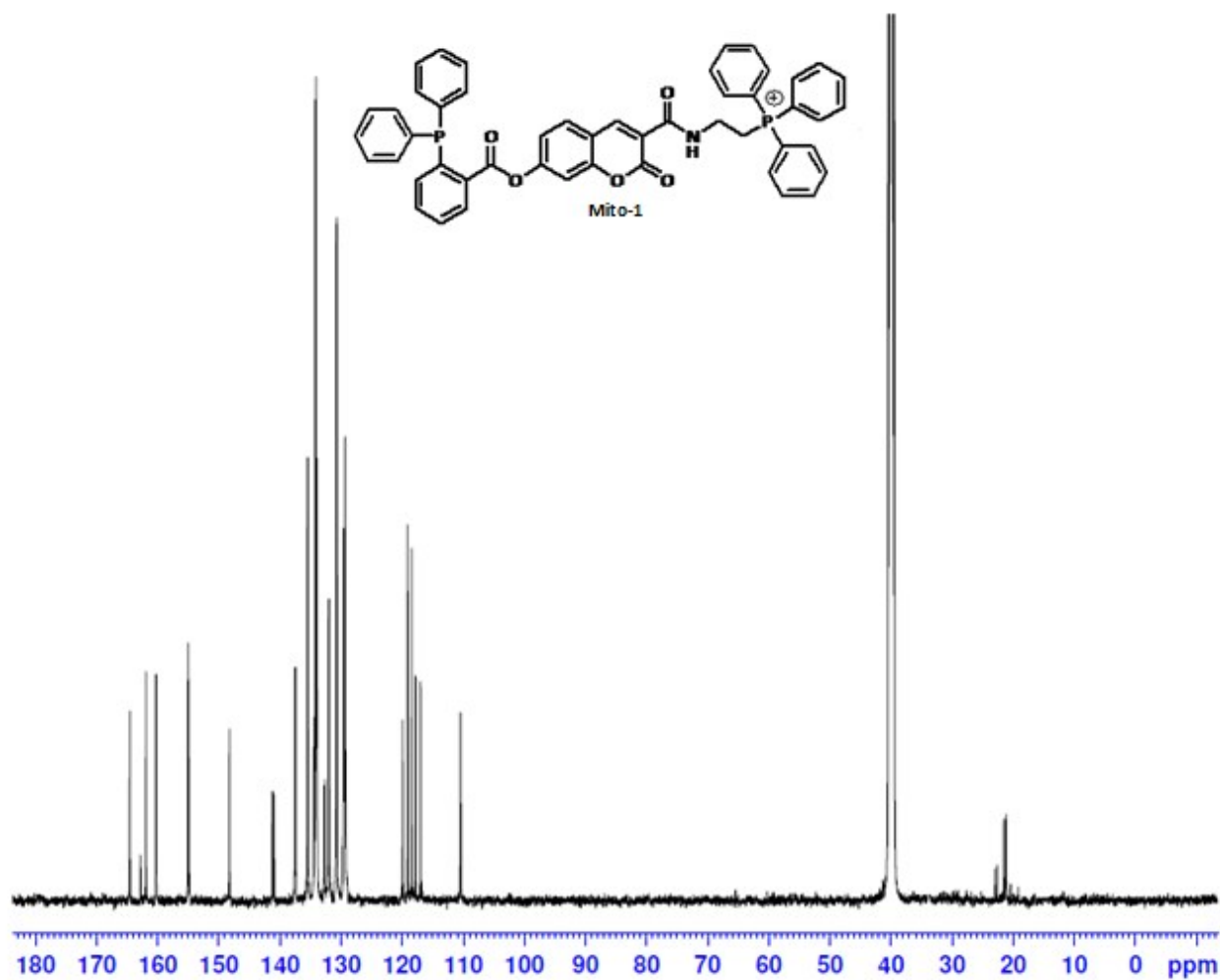


Fig.S14 ^{13}C -NMR of **Mito-1** in DMSO-d_6

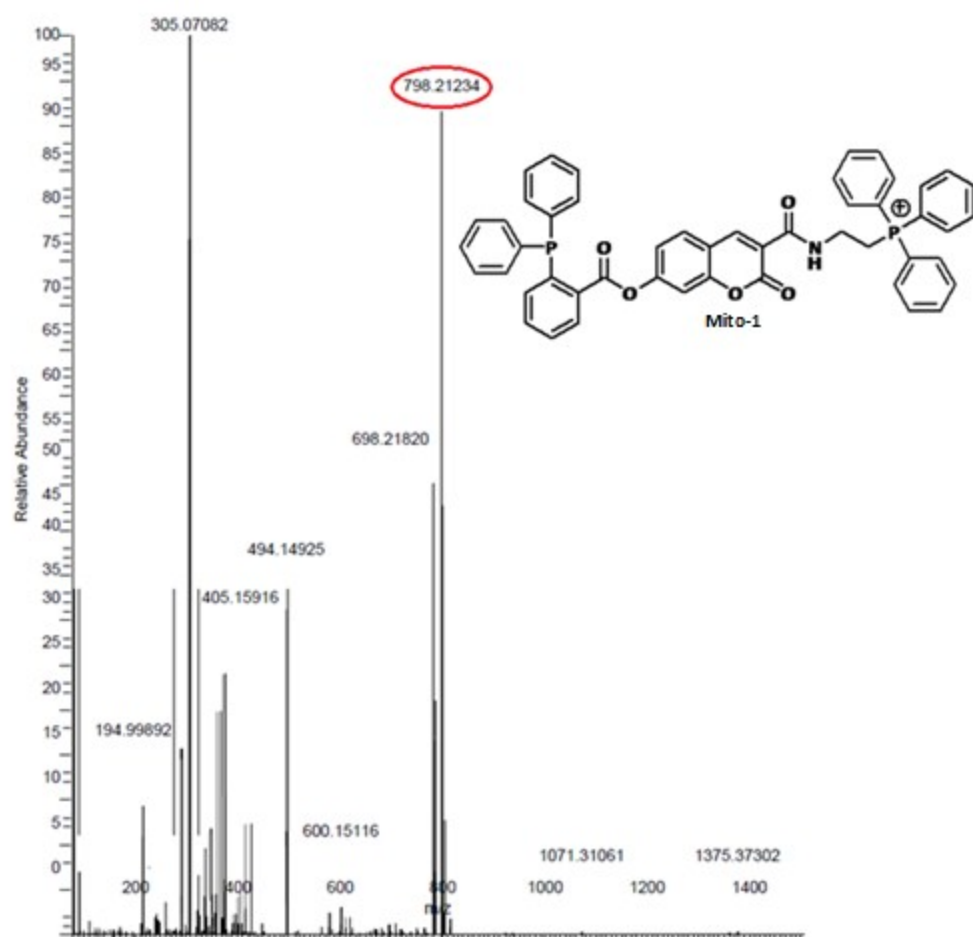


Fig.S15 HRMS of Mito-1

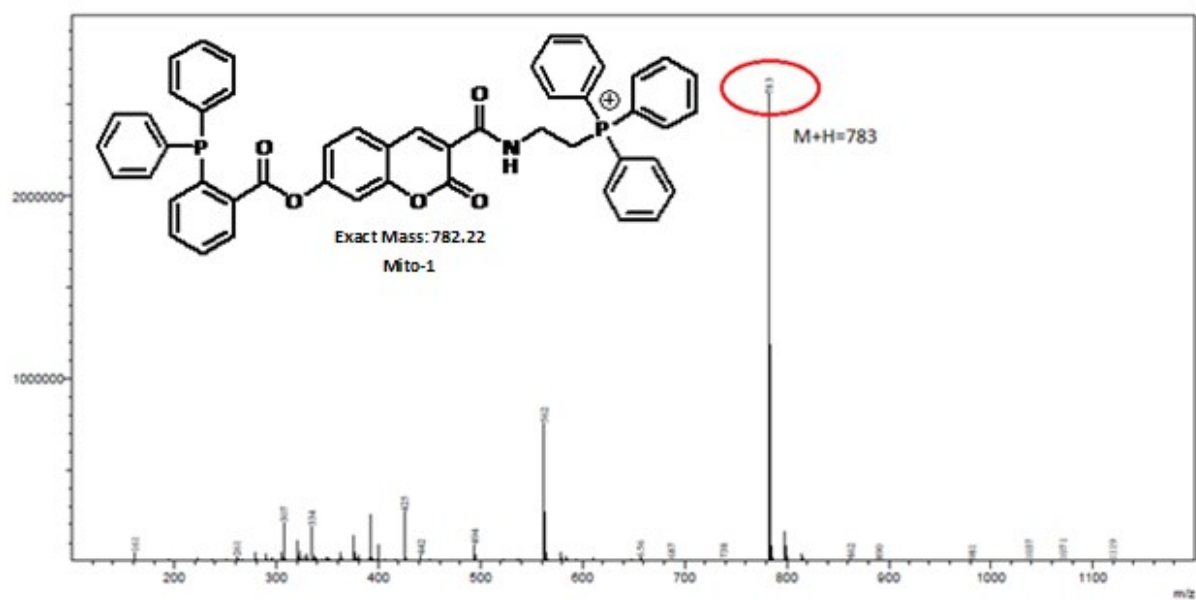
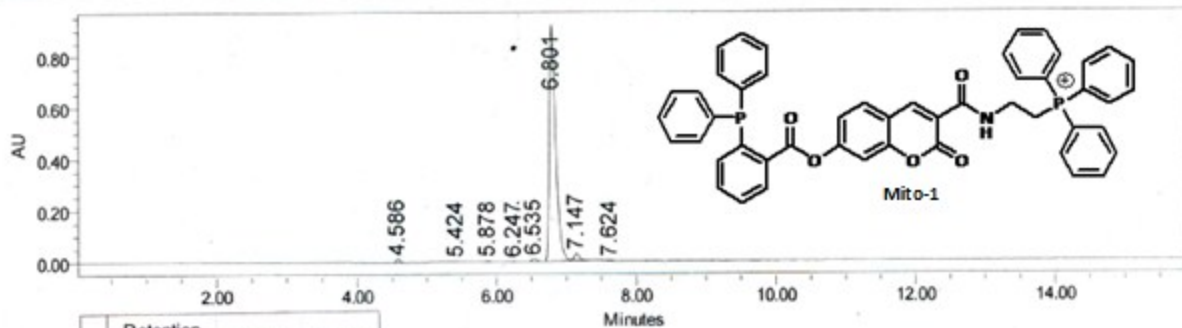


Fig.S16 ESI-MS of **Mito-1**

HPLC REPORT

Column : X-BRIDGE C18 (4.6X150mm) 3.5 μ m
Mobile Phase : A: 10mM Ammonium acetate (aq), B: Acetonitrile
Gradient Time %B : 0/5, 1.2/5, 3/55, 5/70, 7/95, 10/95, 12/100, 14/5, 16/5
Column Temp : Ambient
Flow Rate : 1 ml/min
Diluent : (ACN : WATER)



	Retention Time (min)	Area	% Area
1	4.586	47044	0.78
2	5.424	4135	0.07
3	5.878	10903	0.18
4	6.247	4609	0.08
5	6.535	37726	0.63
6	6.801	5773805	96.04
7	7.147	128702	2.14
8	7.624	4687	0.08

Fig.S17 HPLC of Mito-1

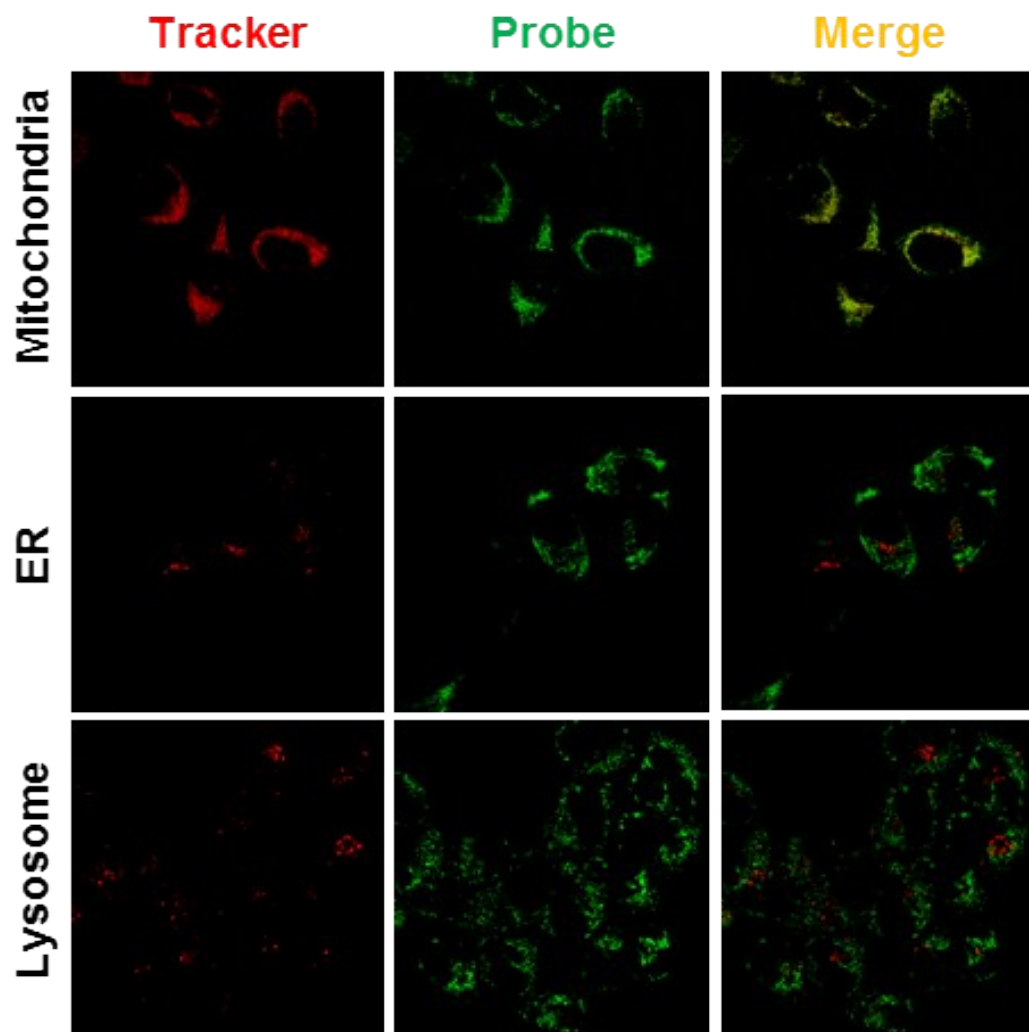


Fig. S18 Confocal microscope images of endogenous HNO with probe and various organelle trackers. Confocal microscope co-localization images of the probe with organelle specific trackers (mitochondria, endoplasmic reticulum and lysosome)

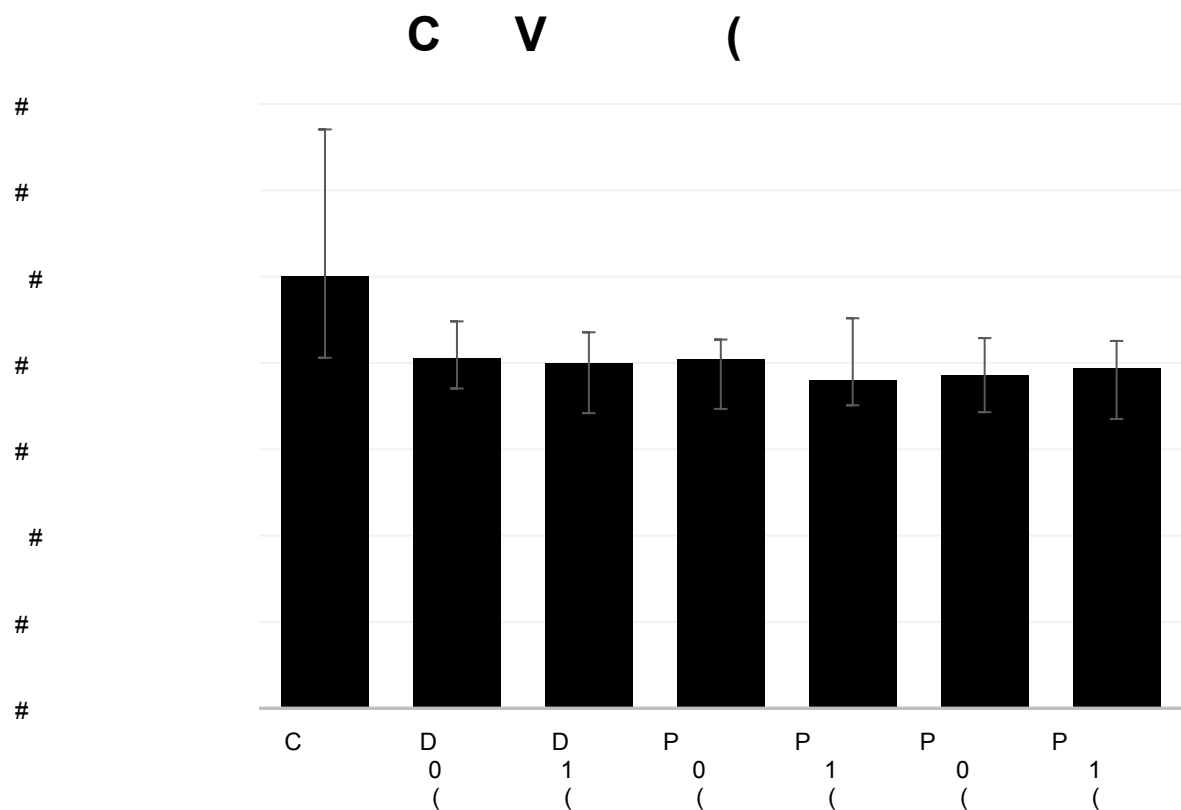


Fig. S19 Cell viability test. 1×10^4 cells were seeded on 96 well microplate (SPL Life Science, Gyeonggi-do, Korea) and incubated for 24 h. After incubation, the cells were treated with DMSO and probe for 5 min as various conditions. To analyze cytotoxicity of the probe on cells, we performed cytotoxicity assay using SensoLyte® Cell Cytotoxicity Assay Kit (AnaSpec, Fremont, California). Fluorescence level was analyzed by using SPECTRA MAX GEMINI EM microplate reader (Molecular Devices, Sunnyvale, California). The excitation and emission wavelength were set at 544 nm and 590 nm, respectively.

References

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2. B. E. Maryanoff, A. B. Reitz and B. A. Duhl-Emswiler, *J. Am. Chem. Soc.*, 1985, **107**, 217.
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