Electronic Supplementary Information for

In vivo observation of pH alternation in mitochondria upon various external stimuli

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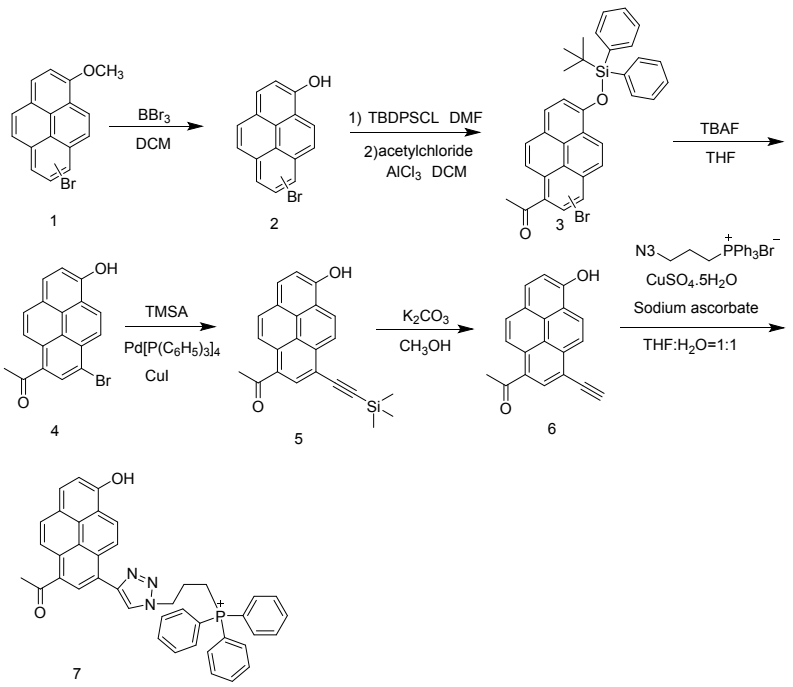
Experimental apparatus

All the reagents were obtained from J&KCHEM and used without further purification. Ultrapure water (18 MΩ/cm) was used for the preparation of all aqueous solutions.

Synthetic scheme

The synthesis of the 1-Bromo-6-methoxypyrene/1-Bromo-8-methoxy pyrene (1) were completed according to the literature.[1]



Scheme S1. Synthesis of the mitochondria -targeting fluorescent pH probe Mito-pH-1.

Synthesis of compound 2.

Under argon, to a 50 mL Schlenk flask were added 1-Bromo-6-methoxypyrene / 1-Bromo-8-methoxy pyrene (12g, 0.038mol) and dry methylene chloride (200 mL).

Then BBr_3 (5.7ml, 0.058mol) was added slowly to the solution at 0°C, and the stirring was continued for the next 4 hours at room temperature. Finally the reaction solution was poured into ice water, the crude extracted with ethyl acetate, and the separated organic phase was dried over $MgSO_4$. The solvent was evaporated to yield a white powder (11.2g, 100%). It was then used directly for next reaction.

Synthesis of compound 3.

1-Bromo-6-hydroxy pyrene / 1-Bromo-8-hydroxy pyrene (7g, 0.023mol) and TBDPSCL(9.1ml, 0.035mol), imidazole (4g, 0.059mol)were dissolved in DMF. After stirring for two hours, the solvent was removed under reduced pressure to yield 1-Bromo-6-OTIPS pyrene / 1-Bromo-8-OTIPS pyrene as a yellow solid (12.3g, 100% yield). It was then used directly for the next reaction. To a solution of 1-bromo-6-OTIPS pyrene / 1-bromo-8-OTIPS pyrene (9g, 0.017mol) in dichloromethane (100 mL) was added dropwise the mixture of acetylchloride (1.08mL, 0.015mol) and Aluminum chloride (4g, 0.031mol) in dichloromethane (20 mL) in 30 minutes at 0^oC.

After the reaction was completed, the mixture was washed with brine (3 × 30 mL) and dried over $MgSO_4$. The filtrate was concentrated and the residue was purified by chromatography on silica gel to afford a mixture of 1,3,6- and 1,3,8- trisubstitutes of pyrene as a yellow solid (7.8 g, 80% yield). HRMS (ESI) m/z $[M+H]^+$calc. 577.11930, found:577.11923, mp : 60^oC-61^oC.

Synthesis of compound 4.

To a stirred solution of the mixture of 1,3,6- and 1,3,8- trisubstitutes of pyrene (3 g, 5.2 mmol) in tetrahydrofuran (30 ml) was added tetrabutyl ammonium fluoride (1.6g, 6 mmol). The reaction stirred at r.t. for 30 minutes, diluted with ethyl acetate (20 ml), extracted with water (3 × 20 ml) and brine (3 × 20 ml), dried with $MgSO_4$ and concentrated in vacuo. Purification by flash column chromatography (methylene chloride) gave the desired compound 4 (1,3,6- trisubstitutes of pyrene, 860 mg, 98% yield). ^1H NMR (400 MHz, DMSO-d6) δ 11.16 (s, 1H), 8.96 – 8.72 (m, 2H), 8.54 (d, J = 9.8 Hz, 1H), 8.39 – 8.30 (m, 2H), 8.16 (d, J = 9.1 Hz, 1H), 7.71 (d, J = 8.3 Hz, 1H), 2.88 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d6) δ 200.94, 154.57, 132.62, 131.97, 131.73, 131.15, 129.43, 129.22, 126.20, 124.88, 124.33, 123.65, 122.90, 121.85, 118.20, 117.09, 114.85, 30.92. HRMS (ESI) m/z $[M-H]^-$calc. 336.98587, found : 336.98572. mp : 185^oC-186^oC.

Synthesis of compound 5.

Under a argon atmosphere, compound 4 (100 mg, 0.29 mmol), copper(I) iodide (6 mg, 0.03 mmol) and tetrakis(triphenylphosphine)-palladium(0) (17 mg, 0.015 mmol) were dissolved in anhydrous triethylamine (10 ml). Then trimethylsilylacetylene (58 mg, 0.59 mmol) was added and the mixture was allowed to stir at 45^oC for a total period of 12 hours. The mixture was concentrated, the remaining residue dissolved in ethyl acetate (20 ml), washed three times with water (50 ml). Evaporation of the solvent under reduced pressure and chromatographic purification on silica gel (ethyl acetate : petroleum ether = 1 : 20) furnished compound 5 (61.9 mg, 60%) as a yellow solid. ^1H NMR (300 MHz, DMSO-d6) δ 11.16 (s, 1H), 8.85 (d, J = 9.5 Hz, 1H), 8.59 (s, 1H), 8.55 (d, J = 9.7 Hz, 1H), 8.40 – 8.21 (m, 3H), 7.69 (d, J = 8.4 Hz, 1H), 2.87 (s,

3H), 0.38 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d6) δ 200.40, 153.56, 134.07, 130.71, 130.50, 129.19, 129.13, 128.51, 124.07, 123.86, 122.71, 121.98, 120.25, 117.21, 113.75, 113.60, 103.06, 99.65, 29.84, -0.50. HRMS (ESI) m/z $[M-H]^-$calc. 355.11488, found : 355.11456. mp: 234^{o}C-235^{o}C.

Synthesis of compound 6.

Compound 5 (150 mg, 0.42 mmol) was dissolved in a mixture of methanol (6 ml) and tetrahydrofuran (4 ml). A solution of potassium carbonate (116 mg) in water (4 ml) was added and the mixture was stirred at ambient temperature for 3 hours. The solution was neutralised with 1 M hydrochloric acid, then treated with ethyl acetate (25 ml), and the aqueous phase was extracted three times with ethyl acetate (15 ml). The combined organic layers were dried with $MgSO_4$ and the solvent was removed under reduced pressure. Compound 6 (118 mg, , 99% yield) was obtained as an dark red solid. ^{1}H NMR (300 MHz, DMSO-d6) δ 11.18 (s, 1H), 8.85 (t, *J* = 7.6 Hz, 1H), 8.62 (d, *J* = 8.2 Hz, 1H), 8.58 – 8.51 (m, 1H), 8.31 (dt, *J* = 13.1, 6.4 Hz, 3H), 7.76 – 7.58 (m, 1H), 4.83 (s, 1H), 2.87 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d6) δ 201.35, 154.54, 135.23, 132.08, 131.43, 130.22, 130.03, 129.42, 125.05, 124.87, 123.73, 122.98, 121.26, 118.24, 114.62, 114.41, 86.69, 82.51, 30.82. HRMS (ESI) m/z $[M-H]^-$ calc. 283.07536, found : 283.07532, mp: 204^{o}C-205^{o}C.

Synthesis of compound 7 (Mito-pH-1).

Under a argon atmosphere, the compound 6 (123 mg, 0.43 mmol), (3-azidopropyl)triphenylphosphonium bromide (185 mg, 0.43 mmol), sodium ascorbate (26 mg, 0.13 mmol), and $CuSO_4 \cdot 5H_2O$ (10 mg, 0.04 mmol) were suspended in a mixture of tetrahydrofuran and water (v/v 10 mL/10 mL) in a 50 mL round bottom flask. The mixture was stirred at room temperature for 24 h. After the materials were consumed completely, the mixture was washed three times with water(20 ml) and extracted with ethyl acetate. The combined organic phase was dried with $MgSO_4$. The solvent was evaporated, and the residue was purified by silica gel column chromatography using eluent (CH_2Cl_2/CH_3OH = 10:1). A yellow solid compound 7 was obtained (153 mg, yield 50%). ^{1}H NMR (400 MHz, DMSO) δ =11.07 (s, 1H), 8.98-8.85 (m, 2H), 8.70 (s, 1H), 8.62 (d, J = 9.2 Hz, 1H), 8.54 (d, J = 9.6 Hz, 1H), 8.26 (dd, J = 16.1, 8.9 Hz, 2H), 7.96-7.90 (m, 3H), 7.89-7.84 (m, 4H), 7.83-7.74 (m, 8H), 7.71 (d, J = 8.3 Hz, 1H), 4.75 (t, J = 6.7 Hz, 2H), 3.81 (t, J = 15.3 Hz, 2H), 2.90 (s, 3H), 2.38-2.27 (m, J = 14.5, 7.7 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d6) δ = 202.03, 154.16, 146.28, 135.56, 135.52, 134.22, 134.09, 131.57, 130.88, 130.78, 130.71, 130.62, 129.68, 129.13, 128.64, 125.79, 125.74, 125.56, 124.32, 123.74, 123.08, 121.44, 119.20, 118.45, 118.06, 114.47, 50.11, 49.84, 31.00, 23.59, 19.19, 18.50. HRMS (ESI) m/z $[M-H]^-$calc. 629.21484, found : 629.21456. mp 189^{o}C-190^{o}C.

Computational details.

Geometry optimizations for the ground state of the luminogens of Mito-pH-1 were carried out by using the GAUSSIAN 09 software package.[2] Becke's three-parameters hybrid method, using the Lee-Yang-Parr correlation functional (B3LYP), was employed here. Structures of the lowest excited state of luminogens were obtained by

TDDFT (time-dependent density functional theory) methods. The geometries were fully optimized without symmetry constraints. On the basis of ground and excited state optimization, TDDFT was applied to investigate the excited state electronic properties. All calculations above employed the 6-31G(d) basis set for carbon, hydrogen, oxygen and nitrogen atoms. The calculated transition dipole moments are 6.3 and 5.9 Debye for neutral and anionic forms of luminogen of Mito-pH-1.

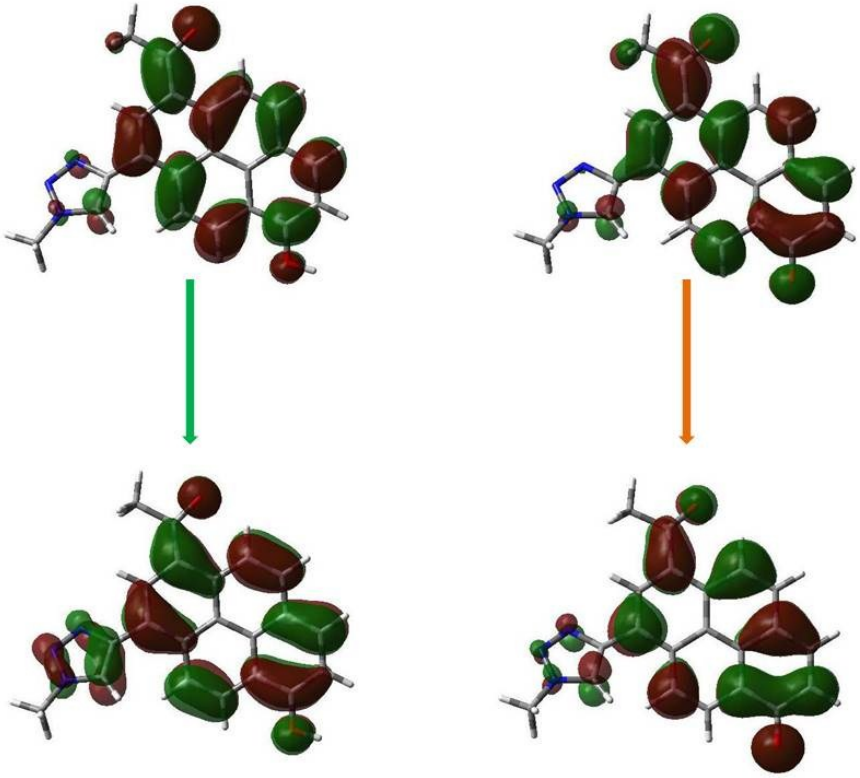


Figure S1 Contours of the frontier orbitals for anionic (left) and neutral (right) forms of luminogen of Mito-pH-1.

Table S1 Optimized excited state coordination of two forms of luminogen of Mito-pH-1.

Neutral form:

C	-1.60500900	1.50806700	-0.05589600
C	-1.53150900	0.14004800	-0.09770600
C	-0.23712600	-0.50747900	-0.13168100
C	0.94502400	0.30213000	-0.00817100
C	0.86524900	1.72853000	0.08114600
C	-0.44948100	2.34970100	-0.00025900
C	-0.08324300	-1.90304100	-0.30928700
C	2.23231400	-0.33037200	0.01599200
C	2.33334400	-1.75022300	-0.12041700
C	1.14993600	-2.51349400	-0.30539100
C	3.61664000	-2.34898900	-0.08687800
C	4.76619800	-1.57507500	0.07124100
C	4.67238900	-0.19442500	0.19582100

C	3.41976500	0.45414400	0.16759200
C	3.29324400	1.86445500	0.28020200
C	2.05927500	2.46903400	0.23386800
H	4.19118000	2.46508800	0.40031900
H	-0.96338700	-2.50934500	-0.48749500
H	-2.59378100	1.95109200	-0.05477800
H	1.23401800	-3.58439300	-0.45102700
H	5.74115500	-2.05991400	0.09441200
O	3.66711700	-3.71218100	-0.21693000
H	4.59501300	-3.99192500	-0.19100800
C	-2.80649700	-0.59889100	-0.08959100
C	-3.16605100	-1.81270400	0.47321900
N	-4.49844000	-1.92403800	0.24235200
H	-2.61375600	-2.55533200	1.02661200
N	-3.94607700	-0.04778700	-0.62563000
N	-4.95736300	-0.84362400	-0.42720300
H	2.00931600	3.55054000	0.33109800
C	-5.40495000	-3.00279200	0.59122900
H	-6.40362100	-2.69489000	0.27968600
H	-5.12760400	-3.92511600	0.07143100
H	-5.39505700	-3.17724600	1.67129800
H	5.57394900	0.40038900	0.31446300
C	-0.61945100	3.73127500	-0.01997900
C	-1.98549900	4.43597700	-0.12131000
H	-2.50213700	4.08153100	-1.01651400
H	-2.57521500	4.20311600	0.76951000
H	-1.85035100	5.51705600	-0.18751300
O	0.29613900	4.63245900	0.03198300

Anionic form:

C	-1.53198000	1.53017700	-0.11190600
C	-1.49164500	0.13425300	-0.15623100
C	-0.23476600	-0.53981300	-0.19038900
C	0.96404800	0.26998600	-0.05123800
C	0.89350000	1.68552000	0.03517700
C	-0.38425300	2.35008200	-0.03840000
C	-0.08633800	-1.94875700	-0.38431900
C	2.23961200	-0.39686500	-0.00072400
C	2.32829500	-1.79682500	-0.13103300
C	1.13748200	-2.56425600	-0.35145800
C	3.61527900	-2.49432700	-0.07167200
C	4.78545000	-1.65552000	0.12714700
C	4.69458300	-0.28892200	0.24481100
C	3.44387700	0.38172600	0.18207700

C	3.33507800	1.79272600	0.29038300
C	2.12325800	2.42686400	0.21771700
H	4.24503000	2.37488200	0.43056700
H	-0.96837600	-2.54019500	-0.60787400
H	-2.51479400	1.98276100	-0.11575200
H	1.25318300	-3.63189400	-0.50359700
H	5.74481300	-2.16582100	0.17119600
O	3.70469900	-3.74261600	-0.18457000
C	-2.78016300	-0.56785400	-0.10783800
C	-3.12195500	-1.81129200	0.40788000
N	-4.46957600	-1.89884800	0.25857500
H	-2.54200500	-2.58414000	0.88519300
N	-3.95004500	0.02081300	-0.53831100
N	-4.95802700	-0.77441600	-0.32217100
H	2.04392200	3.50314800	0.28461800
C	-5.36146200	-2.98370300	0.60388800
H	-6.37317900	-2.65850900	0.35599600
H	-5.11911300	-3.88791400	0.03409300
H	-5.30306100	-3.20989400	1.67428900
H	5.59246900	0.31248400	0.38673500
C	-0.56082300	3.81635800	-0.01769800
C	-1.99087400	4.36562500	-0.02616200
H	-2.54508000	4.07589700	-0.92990400
H	-2.58216600	4.01566100	0.83172200
H	-1.92234200	5.45547200	0.01115100
O	0.36242900	4.65438200	-0.00482600

General procedure for UV/Vis and fluorescence spectroscopy

A series of pH buffers were prepared by mixing 20mM Na_2HPO_4 and 20 mM NaH_2PO_4. All pH values were measured by METTLER-TOLEDO FE20 pH-meter. Absorption spectra were recorded on Hitachi UV-3010, and the fluorescence spectra were recorded on Hitachi F-7000.

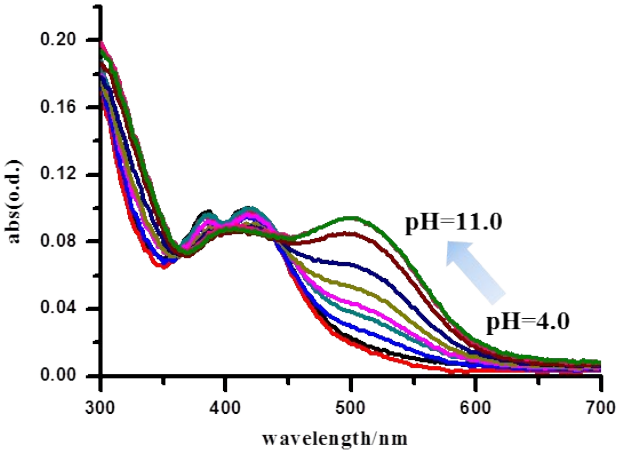


Figure S2 Absorption spectra of Mito-pH-1 (1.0 μM) recorded at different pH values (4.0-11.0).

Fluorescence selectivity of Mito-pH-1

The biologically relevant analytes including Ca^{2+} (10 mM); Cu^{2+} (1 mM); K^+ (10 mM); Mg^{2+} (10 mM); Na^+ (10 mM); Zn^{2+} (1 mM); Fe^{2+} (1 mM); Cys (1 mM); GSH (1 mM); H_2O_2 (0.1 mM); ·OH (0.1 mM); O_2^- (0.1 mM); Histamine (0.1 mM) were prepared and the pH value was fixed at 8.0.

Fluorescence reversibility of Mito-pH-1

The pH of Mito-pH-1 solution (1.0μM) between pH 4.0 and pH 9.0 was adjusted back and forth by 2 M HCl or NaOH, and then measured by pH-meter. The fluorescence spectra were recorded with λ_{ex} = 405 nm.

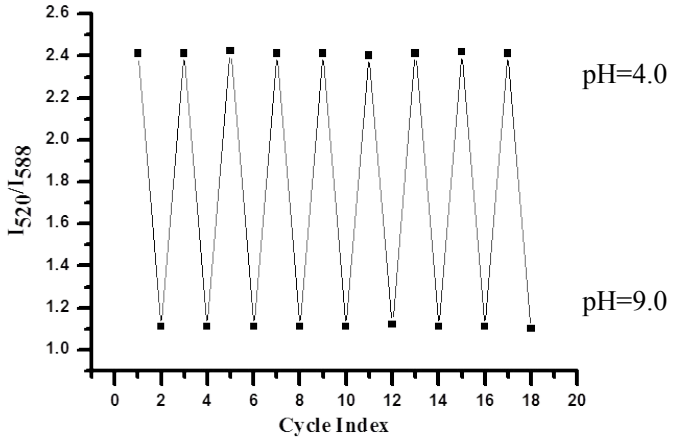


Figure S3 Reversible fluorescence changes of Mito-pH-1 between pH 4.0 and pH 9.0.

Cell culture and fluorescence imaging

HeLa, NIH/3T3 Fibroblasts, HBE and MCF-7 cells were grown at 37°C under 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Gibco; Invitrogen) supplemented with 10% fetal bovine serium. When labeling, the cells were treated with Mito-pH-1 (1.0 μM) at 37 °C under 5% CO_2 for 40 min. The treated cells were washed three times with PBS and then cell images were obtained using a confocal microscope FV-1000-IX81 and analysized with FV10-ASWsoftware.

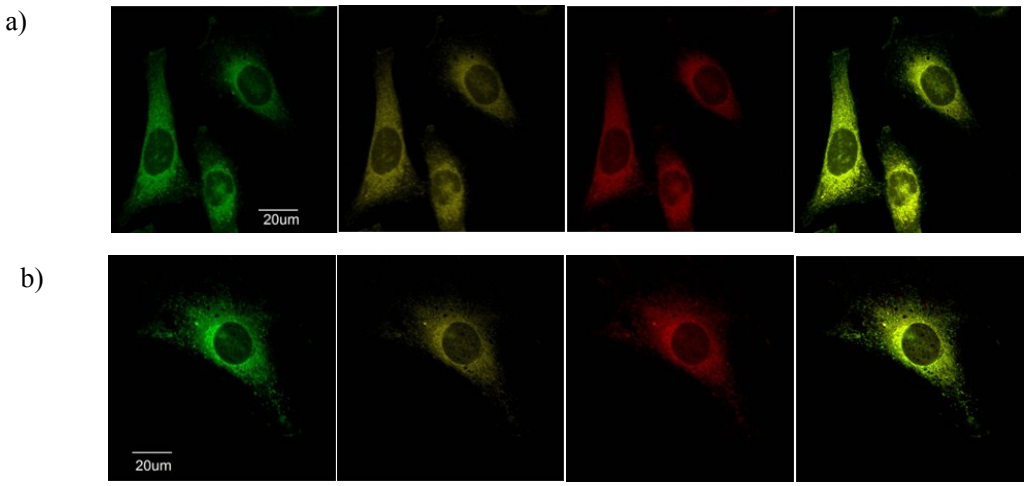
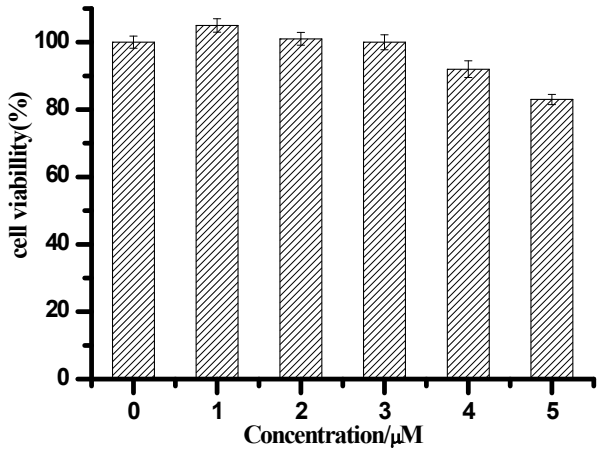


Figure S4 Confocal microscopy images of HeLa cells and NIH/3T3 Fibroblasts cells treated with Mito-pH-1 (1.0 μM) and MTR (1.0 μM). a) Fluorescence image of HeLa cells stained with Mito-pH-1 (green channel, λex=405 nm, λem=460–525 nm, yellow channel, λex=405 nm, λem=570–610 nm); Fluorescence image of HeLa cells stained with MTR (red channel, λex=543 nm, λem=650–710 nm); the merged image of three channels (forth image). b) Fluorescence image of NIH/3T3 Fibroblasts cells stained with Mito-pH-1 (green channel, λex=405 nm, λem=460–525 nm, yellow channel, λex=405 nm, λem=570–610 nm); Fluorescence image of NIH/3T3 Fibroblasts cells stained with MTR (red channel, λex=543 nm, λem=650–710 nm); the merged image of three channels (forth image).

Viability assay

The cytotoxicity of Mito-pH-1 was evaluated by the standard MTT assay. HeLa and NIH/3T3 Fibroblasts cells were seeded in 96-well U-bottom plates at a density of 7000 cells/well and incubated with Mito-pH-1 at varied concentrations (0-5μM) at 37°C for 24 h. Then 10μL of the MTT solution (5 mg/mL in DMEM) was added to each well and followed by incubation at 37°C for another 4 h. The supernatant was removed, and 150 μL of DMSO was added to each well to dissolve the formed formazan. After shaking the plates for 15 min, absorbance values of the wells were read with a microplate reader at 490 nm. The cell viability rate (VR) was calculated according to the equation: $VR = A/A_0 \times 100\%$, where A is the absorbance of the experimental group (i.e., the cells were treated by Mito-pH-1) and A_0 is the absorbance of the control group (i.e., the intact cells).

a)



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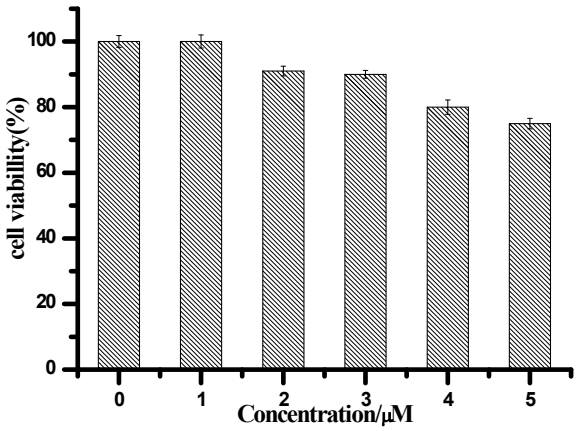


Figure S5 Effects of Mito-pH-1 on the viability of a) HeLa cells and b) NIH/3T3 Fibroblasts cells at varied concentrations.

Intracellular pH calibration

The Mito-pH-1-loaded cells were incubated at 37 °C for 15 min in high K^+ buffer (30 mM NaCl, 120 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1 mM NaH_2PO_4, 5 mM glucose, 20 mM HEPES) with various pH values in the presence of 10 μM nigericin.

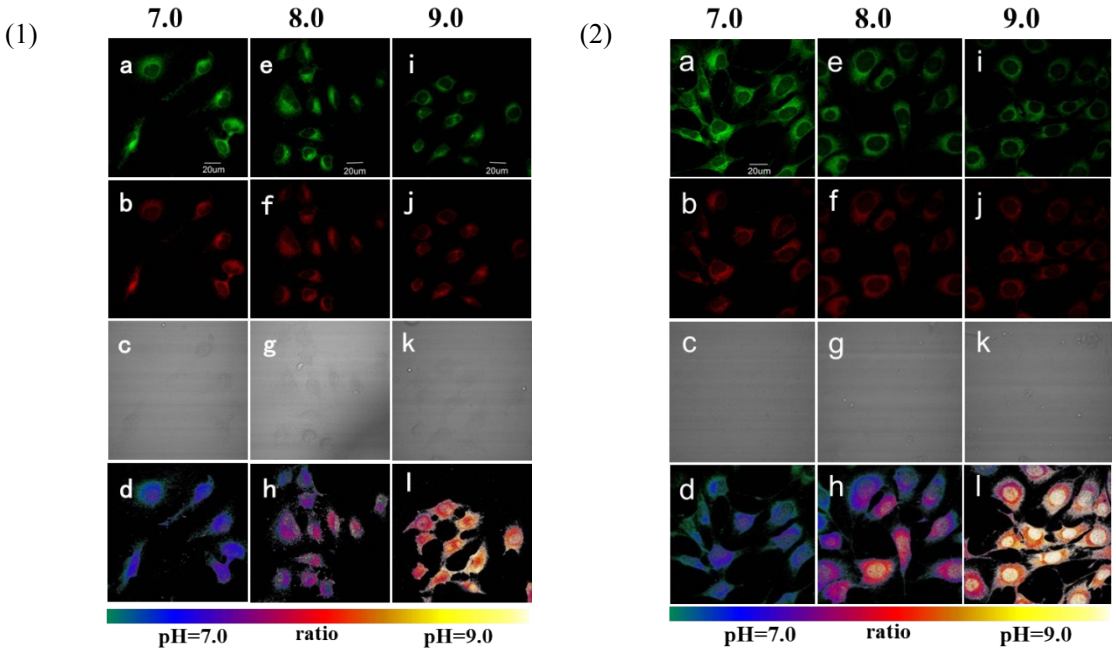
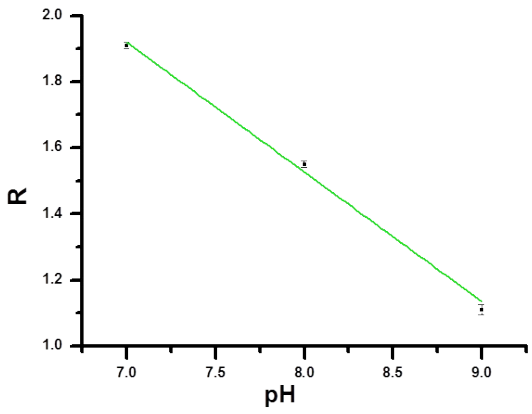


Figure S6 Confocal microscopy images of Mito-pH-1 (1.0 μM) in (1) HeLa cells clamped at pH 7 (a–d), 8 (e–h), 9 (i–l). The images were collected at 425–510 nm (first row) and 530–600 nm (second row). The corresponding contrast images (third row) and ratio images obtained from the green and red channels (fourth row). (2) NIH/3T3 Fibroblasts cells clamped at pH 7 (a–d), 8 (e–h), 9 (i–l) 9. The images were collected at 425–510 nm (first row) and 530–600 nm (second row). The corresponding contrast images (third row) and ratio images obtained from the green and red channels (fourth row). The bottom color strip represents the pseudocolor change with pH. λex=405nm. Scale bar: 20 μm.

a)



b)

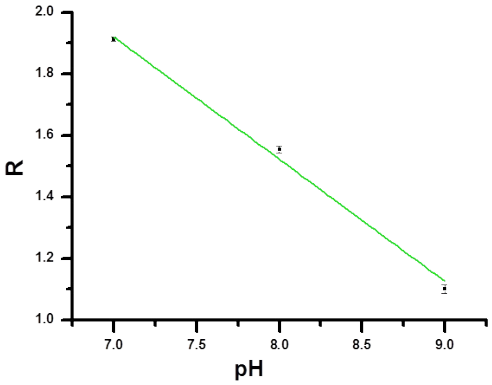


Figure S7 Intracellular pH calibration curves of Mito-pH-1. a) Linear relationship in HeLa cells. b) Linear relationship in NIH/3T3 Fibroblasts cells. R = Igreen/Ired.

Monitor the change of mitochondria pH when treated with H_2O_2 by Mito-pH-1

HeLa or NIH/3T3 Fibroblasts, HBE and MCF-7 cells were incubated with Mito-pH-1 (1.0 μM) at 37 °C for 40 min, and then treated with H_2O_2 and corresponding fluorescence imaging experiments were performed on confocal microscope FV-1000-IX81.

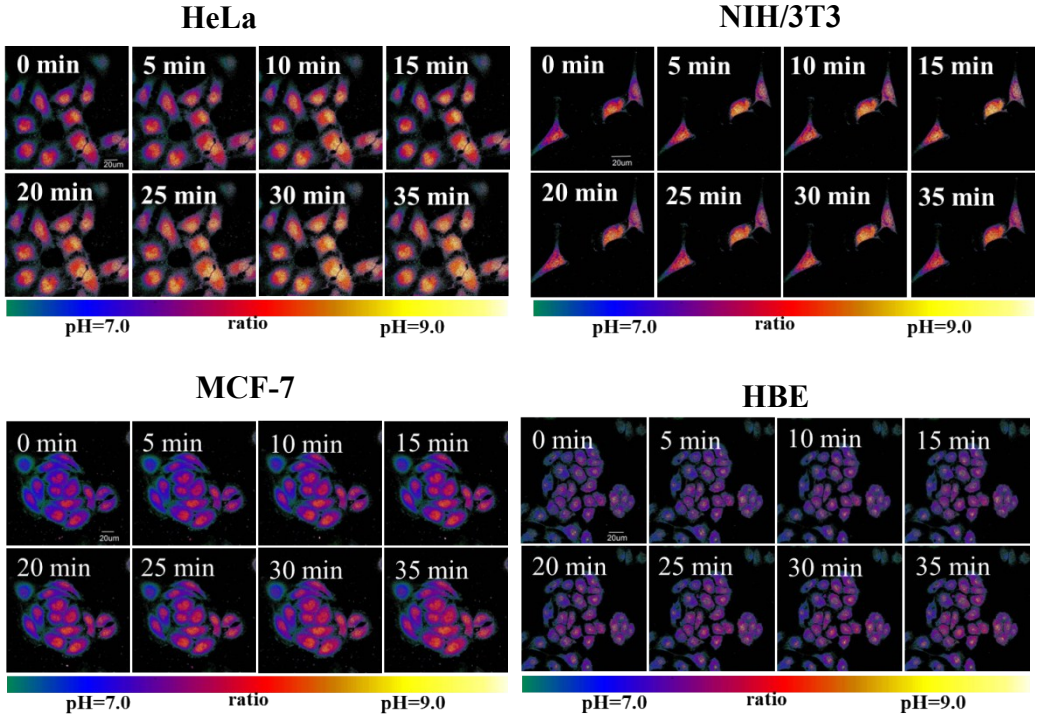
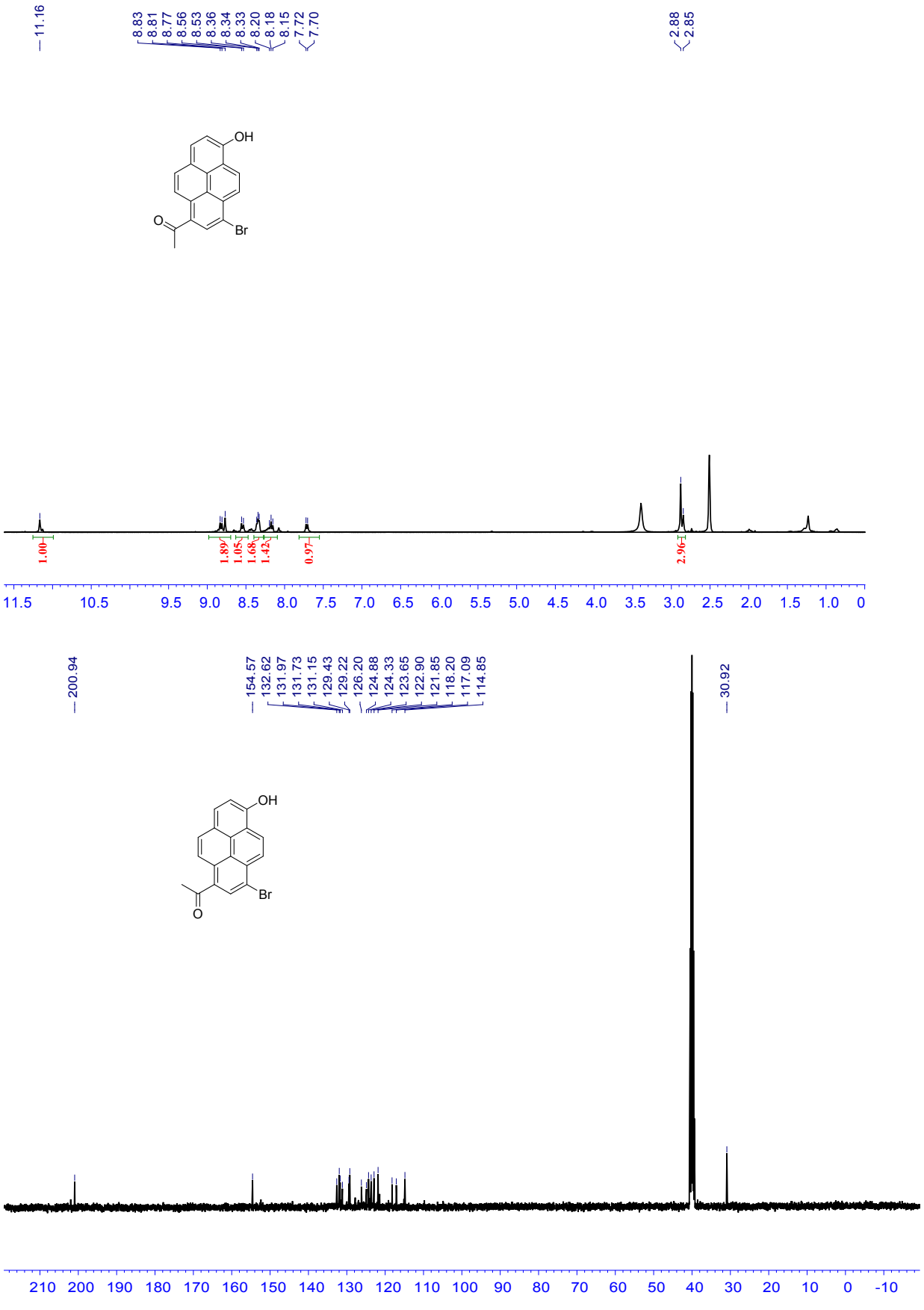
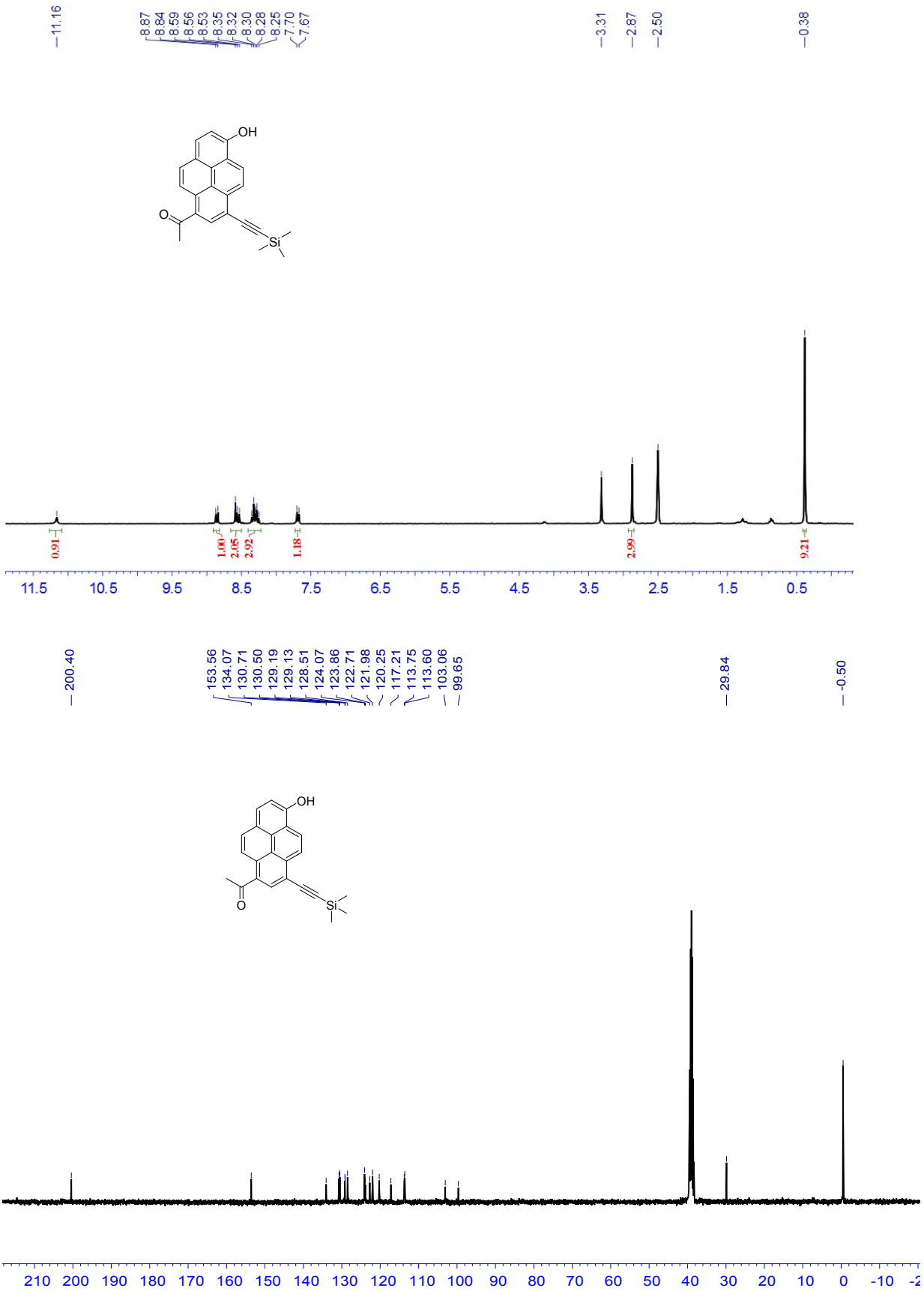


Figure S8 Ratiometric images of HeLa, NIH/3T3, MCF-7 and HBE cells loaded with Mito-pH-1 (1.0 μM) and treated with 100μM H_2O_2 for different times.The bottom color strip represents the pseudocolor change with pH. λex=405nm. Scale bar: 20 μm.

^{1}H NMR, ^{13}C NMR Spectra




11.16
8.87
8.84
8.59
8.56
8.53
8.35
8.32
8.30
8.28
8.25
7.70
7.67
3.31
2.87
2.50
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OH
O
Si
0.91
1.00
2.05
2.92
1.18
2.99
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11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
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130.71
130.50
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128.51
124.07
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113.75
113.60
103.06
99.65
29.84
-0.50
OH
O
Si
210
200
190
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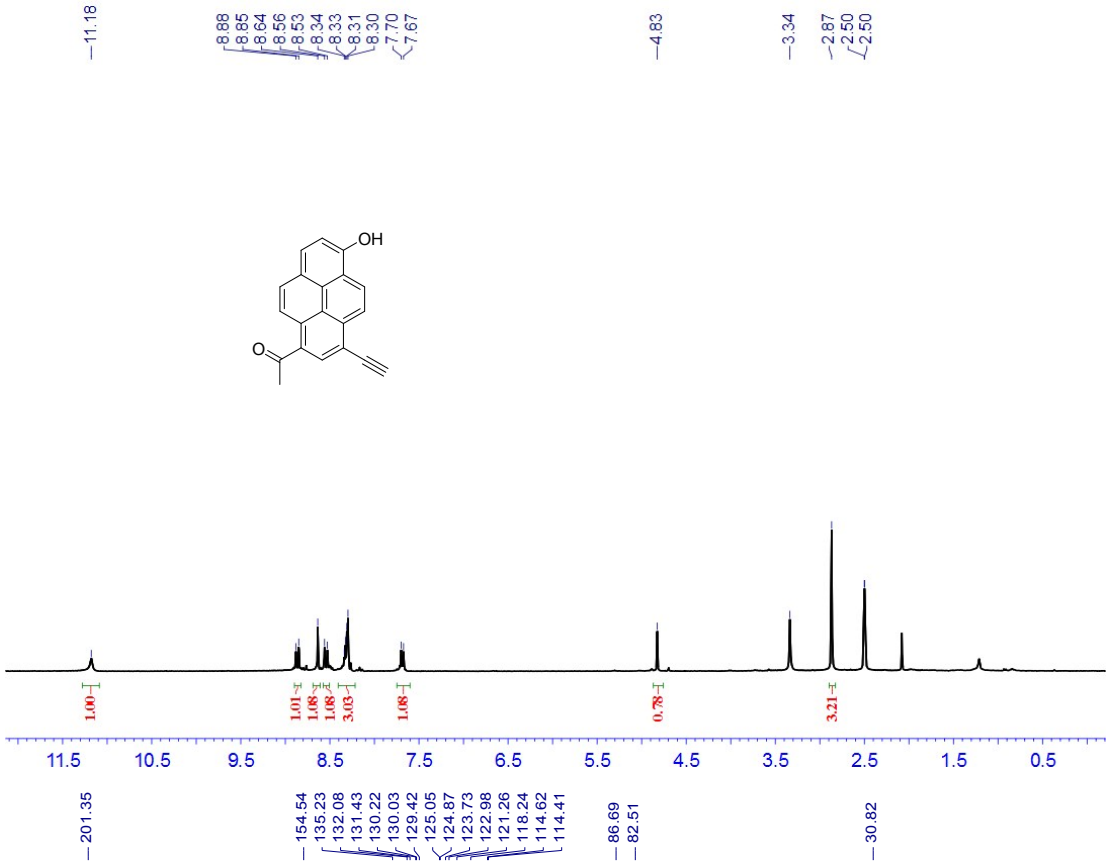
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2.87
2.50
2.50
OH
O
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1.01
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3.03
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114.41
86.69
82.51
30.82
OH
O
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200
190
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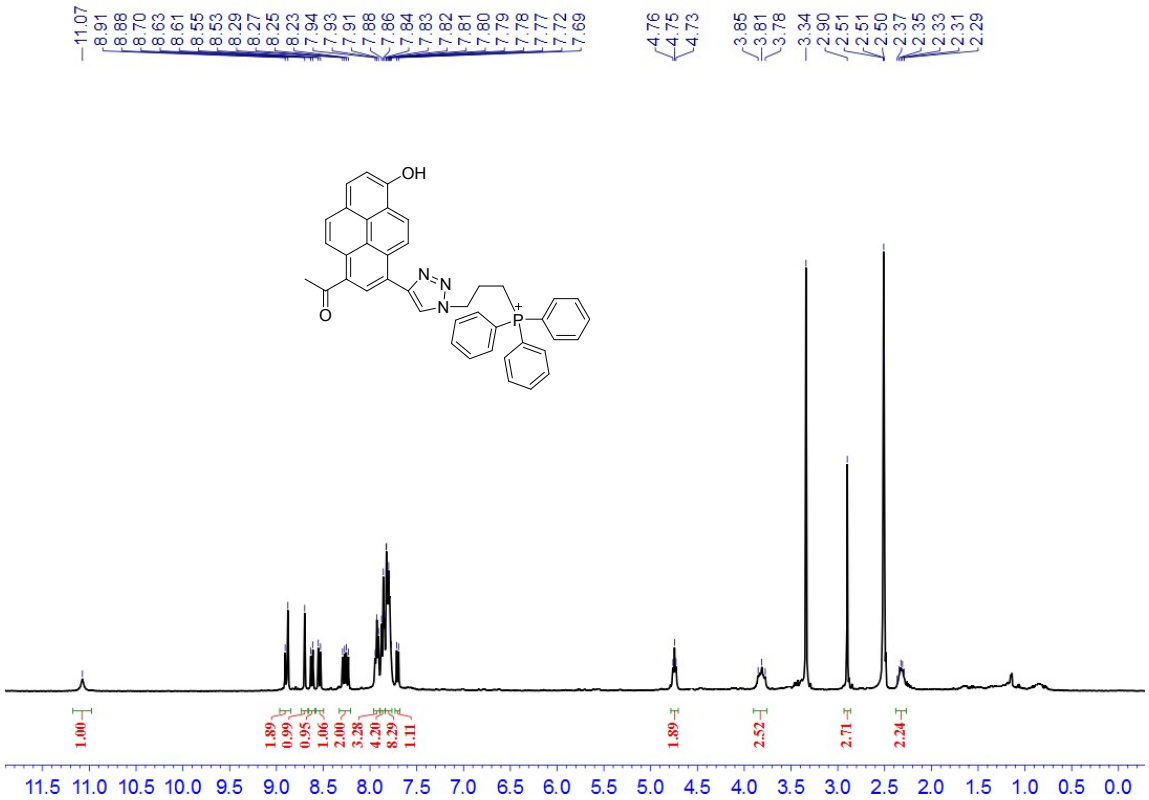

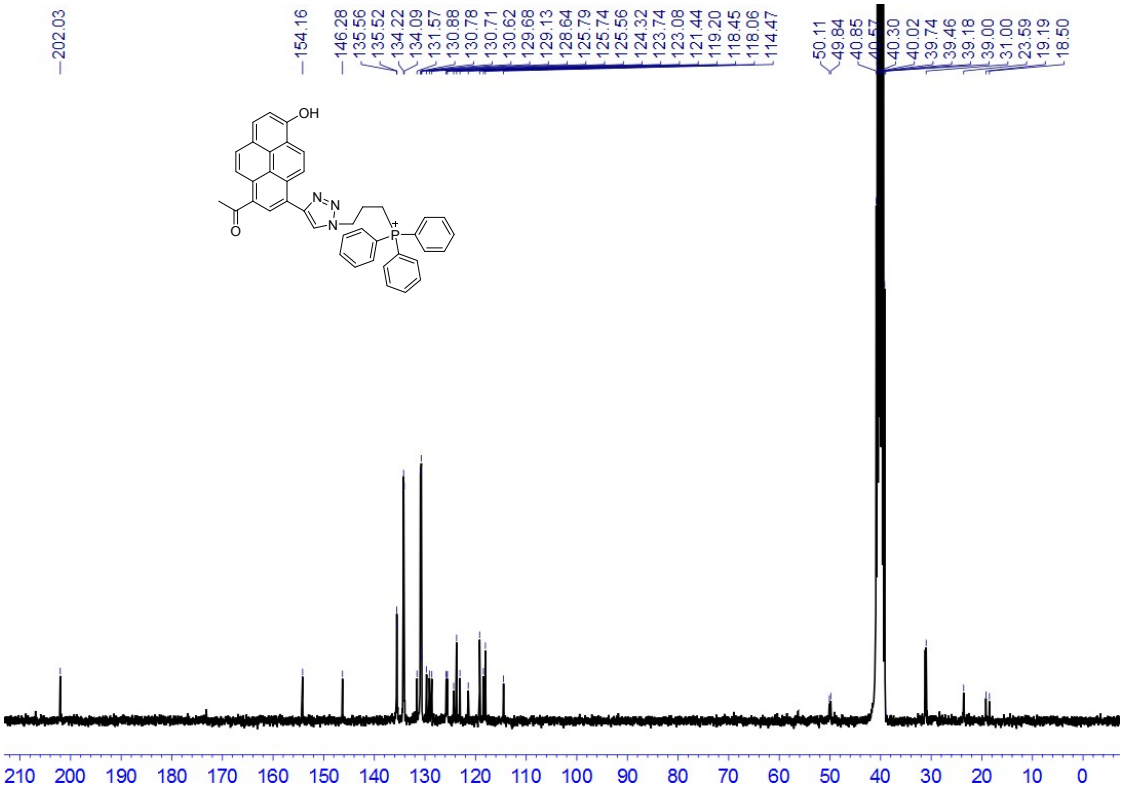

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