Electronic Supplementary Material (ESI) for ChemComm.
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Supporting Information for

Efficient two-photon fluorescent probe for monitoring mitochondrial singlet oxygen in tissues during photodynamic therapy

Hong-Wen Liu [†], Shuai Xu [†], Peng Wang, Xiaoxiao Hu, Jing Zhang, Lin Yuan, Xiao-Bing Zhang* and Weihong Tan

Molecular Science and Biomedicine Laboratory, State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China.

* To whom correspondence should be addressed.

E-mail: xbzhang@hnu.edu.cn.

[†] co-first author

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Synthesis and the response mechanism of the probe

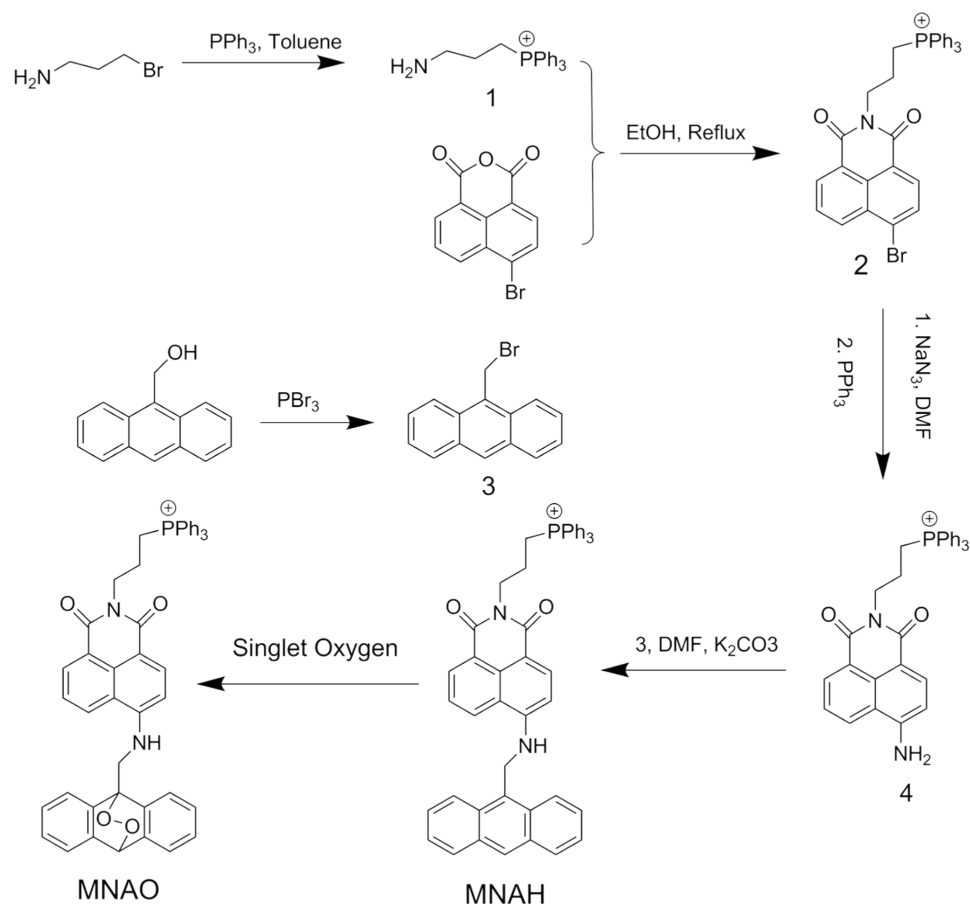


Fig. S1. Structures and synthetic route of **MNAH** and the response mechanism of **MNAH**.

Synthesis of Compound 1

3-Bromopropanamide (3.04 g, 20 mmol) and triphenylphosphine (5.24 g, 20 mmol) were dissolved in toluene and the mixture was stirred at 116 °C for 24 hours and monitored by TLC. After cooling to room temperature, 50 mL of petroleum ether were added and the precipitated solid was filtered. The solid was washed with petroleum ether/ dichloromethane (v/v, 3/1), and vacuum-dried to afford compound **1** as gray solid, which was used without further purification in the preparation of **2**.

Synthesis of compound 2. Compound **1** (1 g, 2.5 mmol) and 4-Bromo-1,8-Naphthalic Anhydride (0.7 g, 2.5 mmol) were dissolved in ethanol (50 mL) and the mixture was stirred at

85 °C for 6 hours and monitored by TLC. After completion of the reaction, the solvent was removed under reduced pressure. The crude solid was washed with H_2O, EtOH/H_2O (v/v, 1/1), and vacuum-dried to afford compound **2** as gray solid, which was used without further purification in the preparation of **4**.

Synthesis of compound 3. Compound **3** was synthesized according to previous work.[1]

Synthesis of compound 4. Compound **2** (0.659 g, 1 mmol) and NaN_3 (0.195 g, 3 mmol) were dissolved in DMF/H_2O (v/v, 10/1, 20mL) and stirred at 90 °C for 5h. After completion of the reaction, the solvent was poured into ice water (10 mL), and the precipitated solid was filtered. The solid was dissolved in THF, and added PPh_3 (3 mmol) and stirred at room temperature for 1h. 10 mL of acetic acid solution were added and stirred for 30min, then poured into ice water (100 mL). The precipitated solid was filtered. The solid was washed with water and petroleum ether/dichloromethane(v/v, 1/1), recrystallization with EtOH/ petroleum ether, and vacuum-dried to afford compound **4** as yellow solid, which was used without further purification in the preparation of probe **MNAH**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): δ 8.67 (d, *J* = 8.66 Hz, 1H), δ 8.41 (d, *J* = 8.40 Hz, 1H), δ 8.18 (d, *J* = 8.17 Hz, 1H), δ 7.91 (t, *J* = 7.89 Hz, 3H), δ 7.80-7.75 (m, *J* = 7.77 Hz, 12H), δ 7.66 (t, *J* = 7.66 Hz, 1H), δ 7.55 (s, 2H), δ 6.87 (d, *J* = 6.86 Hz, 1H), δ 4.20 (t, *J* = 4.19 Hz, 2H), δ 3.79 (t, *J* = 3.75 Hz, 2H), δ 1.98 (m, *J* = 1.95 Hz, 2H). HRMS (ESI): m/z 515.1862 $[M]^+$, calculated for $C_{33}H_{28}N_2O_2P^+$ 515.1885.

Synthesis of probe MNAH.

Compound **4** (0.297 g, 0.5 mmol), Compound **3** (0.135 g, 0.5 mmol) and K_2CO_3 (0.138 g, 1 mmol) were dissolved in DMF (20 mL) and stirred at 90 °C for 1h. The solvent was removed under reduced pressure. The solid was further purified by the silica gel chromatography using

CH_2Cl_2 /CH_3OH (10:1, v/v) as the mobile phase to afford probe **MNAH** as a yellow solid (0.243 g, 62.1%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ(ppm): δ 8.90 (d, *J* = 8.89 Hz, 1H), δ 8.71 (s, 1H), δ 8.37 (d, *J* = 8.37 Hz, 1H), δ 8.22 (d, *J* = 8.21 Hz, 1H), δ 8.08 (d, *J* = 8.07 Hz, 1H), δ 1.80 (t, *J* = 1.79 Hz, 3H), δ 7.79-7.52 (m, *J* = 7.61 Hz, 19H), δ 7.09 (s, 1H), δ 4.91 (s, 2H), δ 3.96 (t, *J* = 3.94 Hz, 2H), δ 3.56 (t, *J* = 3.52 Hz, 2H), δ 1.79 (m, *J* = 1.75 Hz, 2H). HRMS (ESI): m/z 705.2644 $[M]^+$, calculated for $C_{48}H_{38}N_2O_2P^+$ 705.2665.

Materials and apparatus

All chemicals were purchased from commercial suppliers and used without further purification. Water was purified and doubly distilled by a Milli-Q system (Millipore, USA). The one-photon excited fluorescence measurements were conducted at room temperature on a Fluoromax-4 spectrofluorometer (HORIBA JobinYvon, Edison, NJ). NMR spectra were recorded on a Bruker DRX-400 spectrometer using TMS as an internal standard. Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan). Two-photon fluorescence images of HeLa cells and tissues were obtained using an Olympus FV1000-MPE multiphoton laser Scanning confocal microscope (Japan). All living cells, living nude mice and rat experiments were performed in compliance with the relevant laws and institutional guidelines, and also the institutional committee of Hunan University has approved the experiments.

General procedure for the spectra measurement

Both the fluorescence and UV-Vis absorption measurement experiments were carried out in 10 mM phosphate buffered solution containing 5% DMSO as the co-solvent. The fluorescence emission spectra were recorded at an excitation wavelength of 440 nm with emission wavelength ranged from 460 to 650 nm. A 1×10^{-3} M stock solution of probe **MNAH** was prepared by dissolving compound **MNAH** in DMSO. The solutions of various testing species were prepared from H_2O_2, tert-butylhydroperoxide (t-BuOOH), NaClO, $NaNO_2$, NaOH, $FeSO_4$, $MgSO_4$, $ZnCl_2$, $CuSO_4$ and $Fe(NO_3)_3$ in twice-distilled water. Hydroxyl radical (•OH) was generated by reaction of 1 mM Fe^{2+} with 100 μM H_2O_2. Peroxynitrite ($ONOO^-$) was used from stock solution 10 mM in 0.3 M NaOH. Superoxide ($O_2^{\bullet-}$) was delivered from enzymatic reaction of xanthine (X; 1 mM) and xanthine oxidase (XO; 2.5 U/mL). The test solution of the **MNAH** (5μM) in 2 mL of 10 mM PBS buffer DMSO solution (pH 7.4) was

prepared by placing 10 μL of the **MNAH** stock solution (1×10^{-3} M) in 1.99 mL of the various analytes buffer/DMSO solution (by adding DMSO to the resulting solution to keep the ratio of organic phase at 5%). The resulting solutions were kept at ambient temperature for 30 min and then the fluorescence intensities were measured.

Cell cytotoxic assays and two-photon fluorescence imaging

To evaluate the potential cytotoxicity of probe **MNAH**, HeLa cells were seeded at 1×10^5 cells per well in 96-well plates and incubated for 24 h and 48 h respectively. After that different concentration (0-10 μM) of probe **MNAH** was added to the cells and these cells were cultured for an additional 24 h or 48 h. And then the cytotoxic effects of **MNAH** was determined using MTT assays. Following incubation, the HeLa cells were washed three times with Dulbecco's phosphate buffered saline (DPBS). Before images the cells were irradiating with 405 nm laser (at 20% laser power) of Olympus FV1000-MPE multiphoton laser Scanning confocal microscope for different time. The two-photon excitation wavelength of the femtosecond laser was fixed at 820 nm; the emission wavelengths were recorded at 460-540 nm.

Two-photon fluorescence imaging of frozen mouse liver slices

Frozen tissue slices were prepared from the livers of nude mice. The slices were incubated with probe **MNAH** or **MNAH** and Ce6 at 37 °C for 1 h. The slices were washed with DPBS three times. The tissues were irradiated for 6 min with 635 nm laser (at 30% laser power) of Olympus FV1000-MPE multiphoton laser Scanning confocal microscope, and then two-photon fluorescence microscopy images were collected. The excitation wavelength of the femtosecond laser was set at 820 nm, the emission wavelengths were recorded at 460-540 nm.

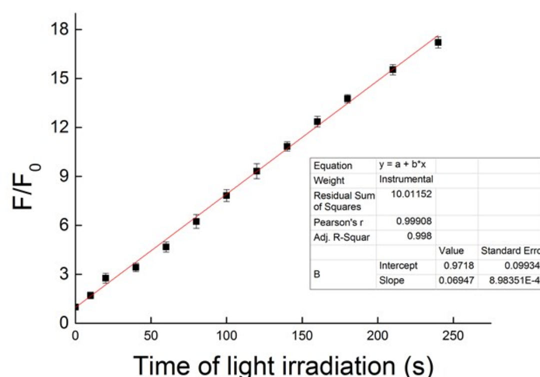


Fig. S2. MNAH shows linear fluorescence increase versus [$^1O_2$] generated by

photoirradiation of Chlorin e6. λ_{ex} = 440 nm.

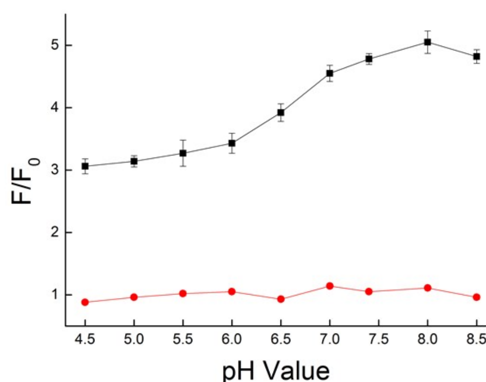


Fig. S3. Effect of pH on the fluorescence intensity of **MNAH** (5 μM) in buffered/DMSO (95/5, v/v, pH= 4.5-8.5, 10 mM). λ_{ex} = 440 nm. Fluorescence responses are shown before (●) and after (■) photoirradiation of codissolved Chlorin e6 for 60 s, respectively.

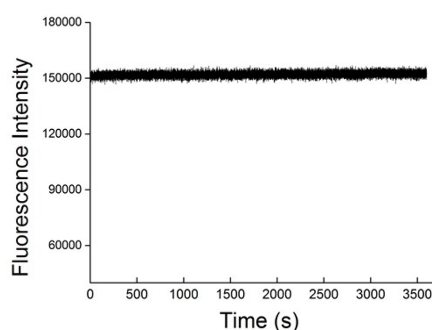
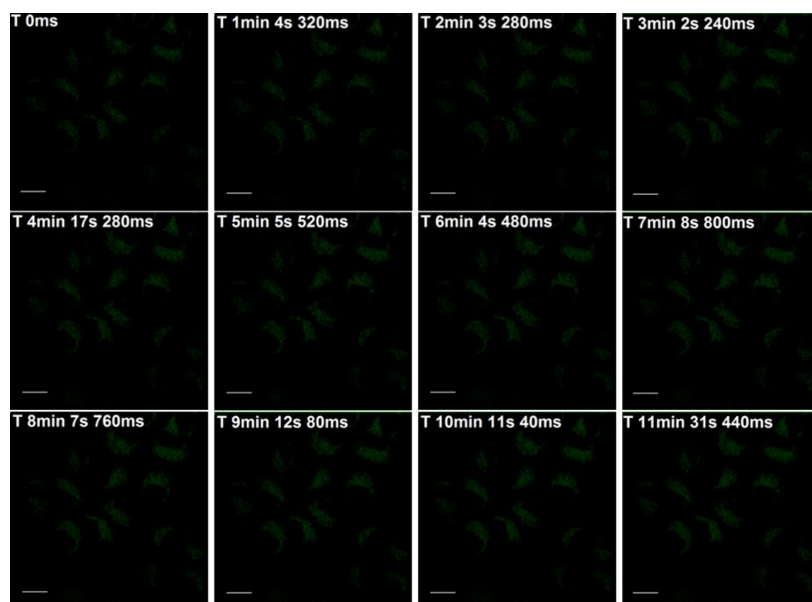


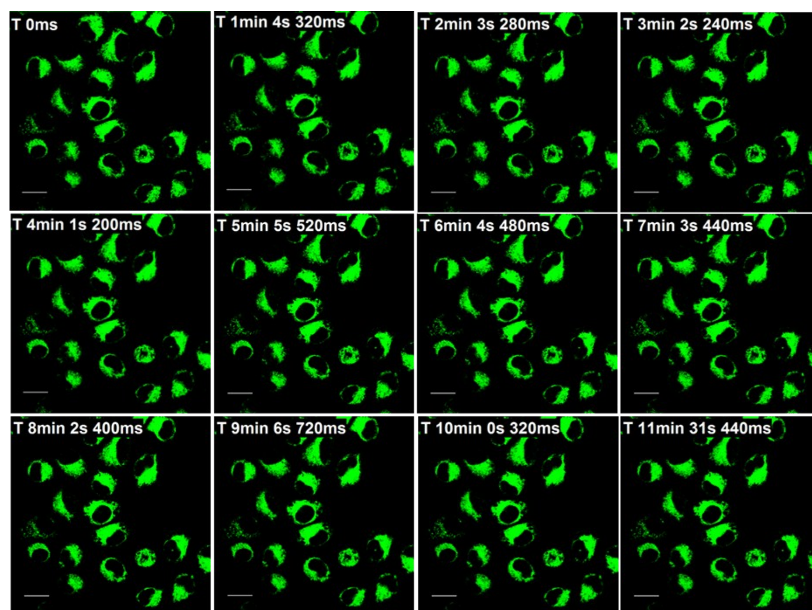
Fig. S4. Photostability experiment of **MNAO** (**MNAH** react with $^1O_2$). In buffered/DMSO (95/5, v/v, pH= 7.4, 10 mM). λ_{ex} = 440 nm. λ_{ex} = 536 nm. The result showed that the MNAO is quite stable under the experiment condition.

Photostability studies of the probe in live HeLa cells

We then examined the photostability of **MNAH** or **MNAO** (**MNAH** react with $^1O_2$) in the live HeLa cells toward continuous irradiation. The cells were pre-incubated with 2.5 μM probe for 30 min. Subsequently, the HeLa cells were irradiated at 7% laser power by a Olympus FV1000-MPE multiphoton laser Scanning confocal microscope equipped with 60× objective lens with the excited wavelength set at 820 nm and the emission wavelengths collected from 460 to 540 nm. As shown in Fig. S7, the results indicate that the probe has relatively good photostability for biological imaging applications.



(a)



(b)

Fig. S5. TP Fluorescent images of the cells incubated with the (a) **MNAH** and (b) **MNAO**. Scale bar: 20 μm. λ_{ex} = 820 nm. λ_{em} = 460-540 nm.

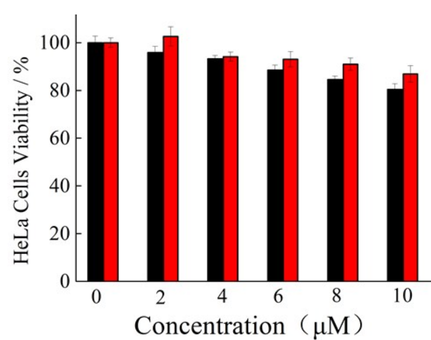


Fig. S6. Cytotoxicity of both **MNAH** against HeLa cells as determined by MTT assay: HeLa cells were treated with **MNAH** (0-10 μM). Black bar and red bar represents incubation for 48 h and 24 h respectively.

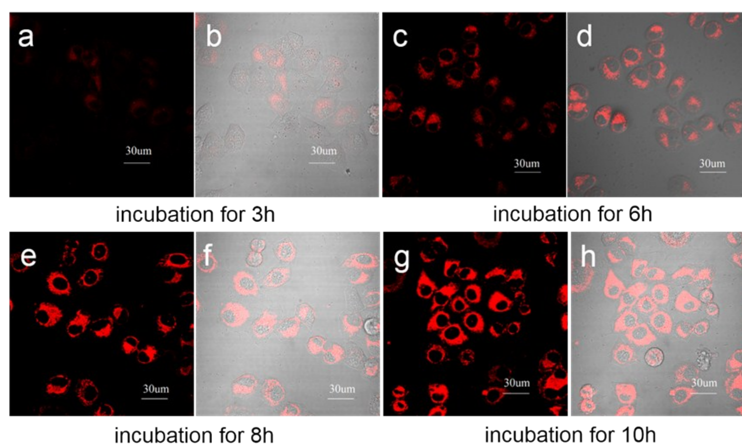


Fig. S7. Fluorescence imaging of endogenously produced PpIX in HeLa cells with different incubation times of [5-ALA] = 100 μM (3 h, 6 h, 8 h and 10h). (a-b) Incubation of [5-ALA] for 3 h. (c-d) Incubation of [5-ALA] for 6 h. (e-f) Incubation of [5-ALA] for 8 h. (g-h) Incubation of [5-ALA] for 10 h. We tested only up to 10 h incubation time since amounts of PpIX is reported to decrease upon prolonged incubation time longer than 10 hours, and diffuse to the cytoplasm.[2] λ_{ex} = 405 nm. λ_{em} = 600-660 nm.

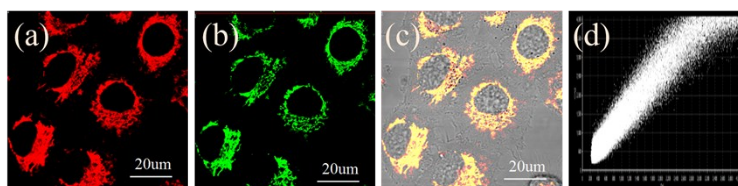


Fig. S8. Intracellular localizations of 5-ALA-derived PpIX. (a) 5-ALA-derived PpIX (100 μM, λ_{ex} = 405 nm, λ_{em} = 600-660 nm). (b) MitoTracker Green (0.5 μM, λ_{ex} = 488 nm, λ_{em} = 495-550 nm). (c) Overlay of (a) and (b). (d) Intensity correlation plot of stain, The Pearson's correlation factor is 0.973. The results demonstrated that after 5-ALA was incubated for 8 h, the biosynthesized PpIX is initially localized in mitochondria.

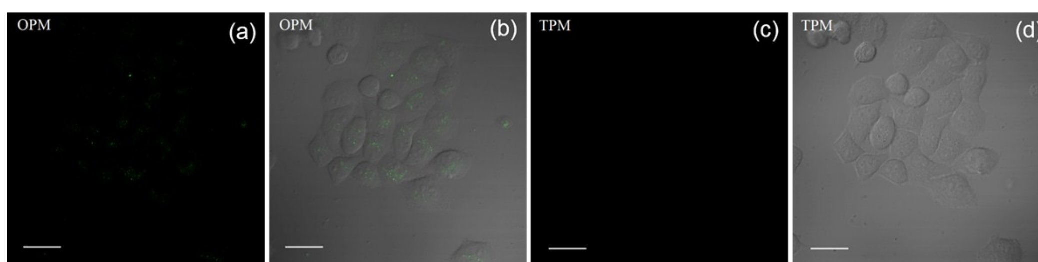


Fig. S9. Confocal images of Hela cells without probe. Confocal fluorescence images of one-photon (a), merge of fluorescence images and DIC (b), and the fluorescence images of two-photon (c), (d) merge of fluorescence images and DIC.

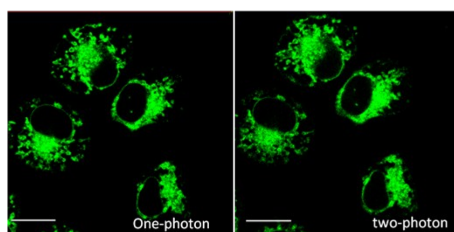


Fig. S10. Confocal images of Hela cells with 5-ALA-derived PpIX and **MNAH**, after 405 nm laser irradiating for 120 s. Confocal fluorescence images of one-photon (a), and the fluorescence images of two-photon (b).

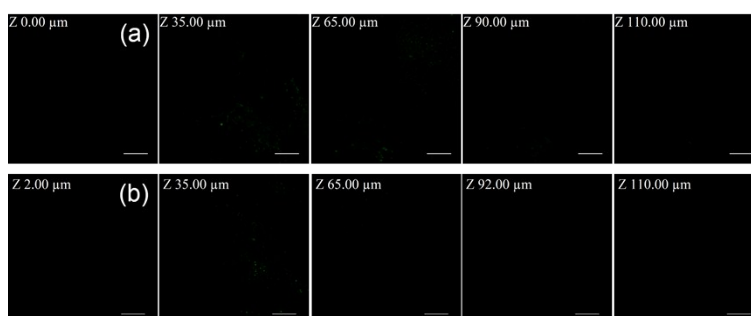


Fig. S11. Depth TP fluorescence images of a rat liver frozen slice. Liver slides stained with **MNAH** (10 µM) for 1 h (a). Liver slides stained with **MNAH** (10 µM) and Chlorin e6 (10 µM) for 1 h without Photoirradiation (b).

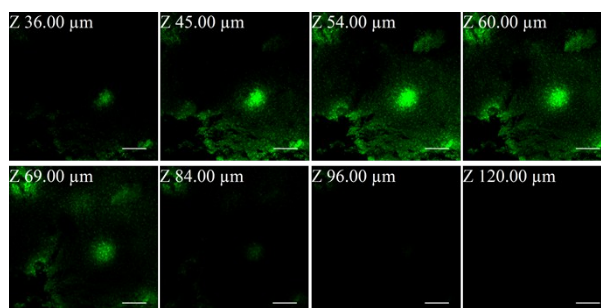


Fig. S12. Depth one-photon confocal fluorescence images of a rat liver frozen slice stained with **MNAH** (10 µM) and Chlorin e6 (10 µM) for 1 h, after 635 nm laser irradiating for 6 min.

References

1. R. C. Wende, A. Seitz, D. Niedek, S. M. Schuler, C. Hofmann, J. Becker and P. R. Schreiner, *Angew. Chem. Int. Ed.*, 2016, **55**, 2719-2723.
2. Q. Peng, K. Berg, J. Moan, M. Kongshaug and J. M. Nesland, *Photochemistry and photobiology*, 1997, **65**, 235-251.

Mass Spectra and ^{1}HNMR

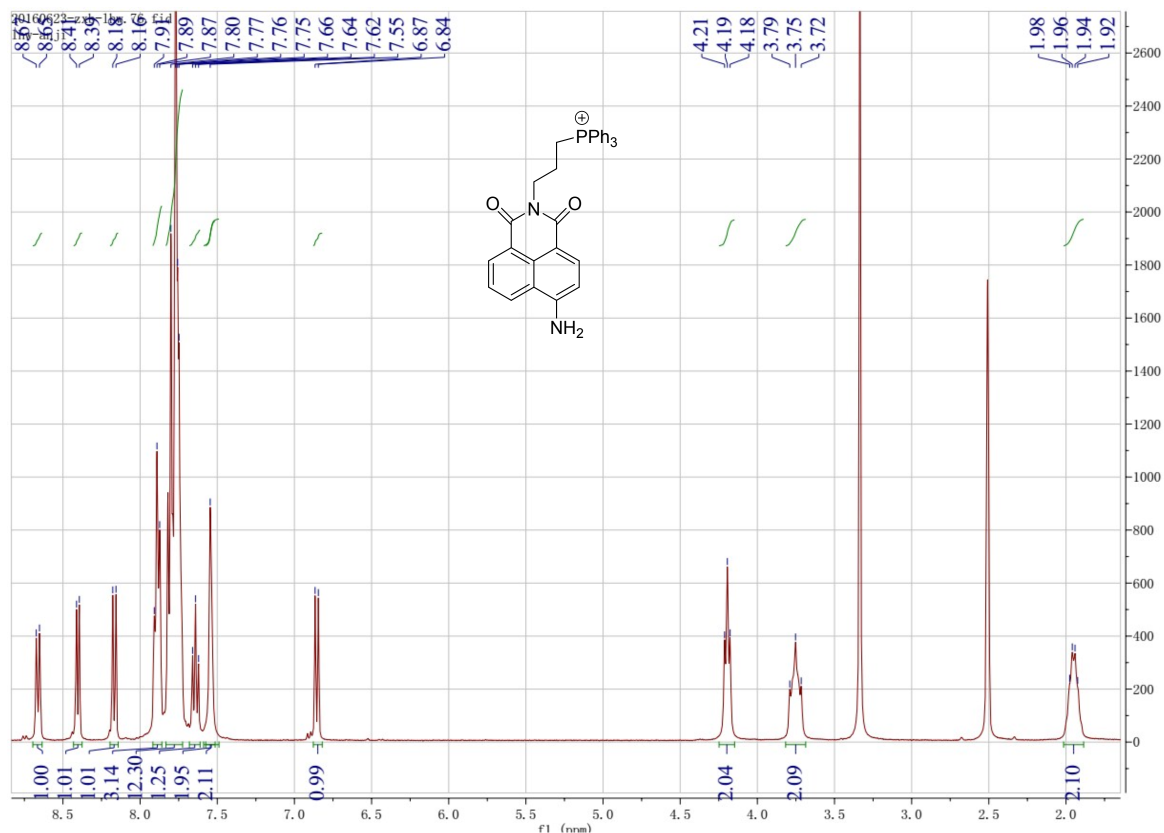
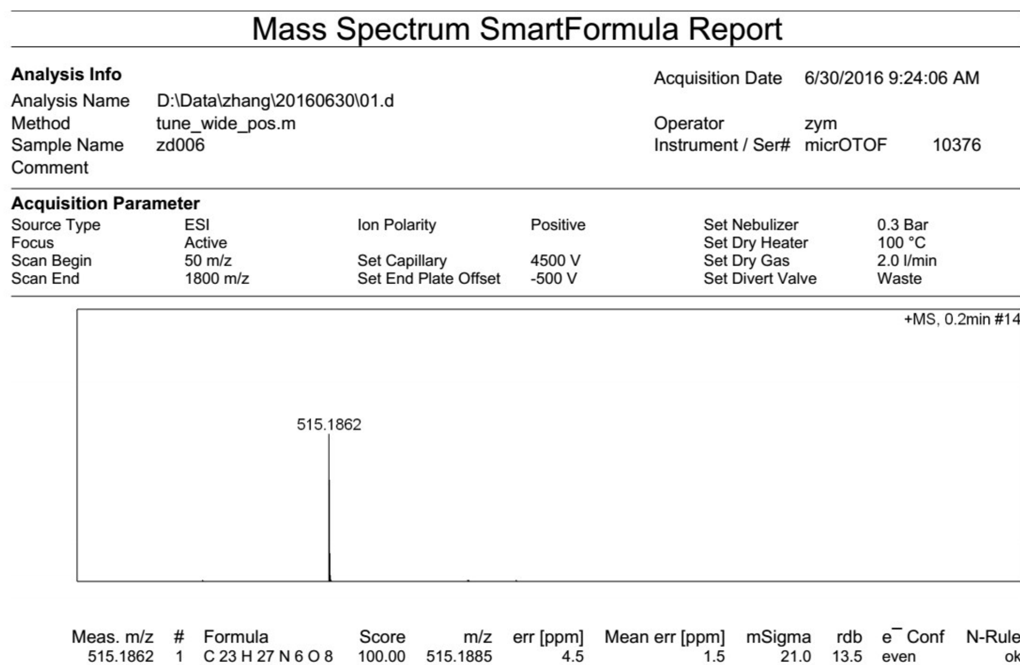


Fig. S13. ^{1}HNMR spectrum of the compound **4**



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Comment			

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Fig. S14. ESI mass spectrum of the compound **4**.

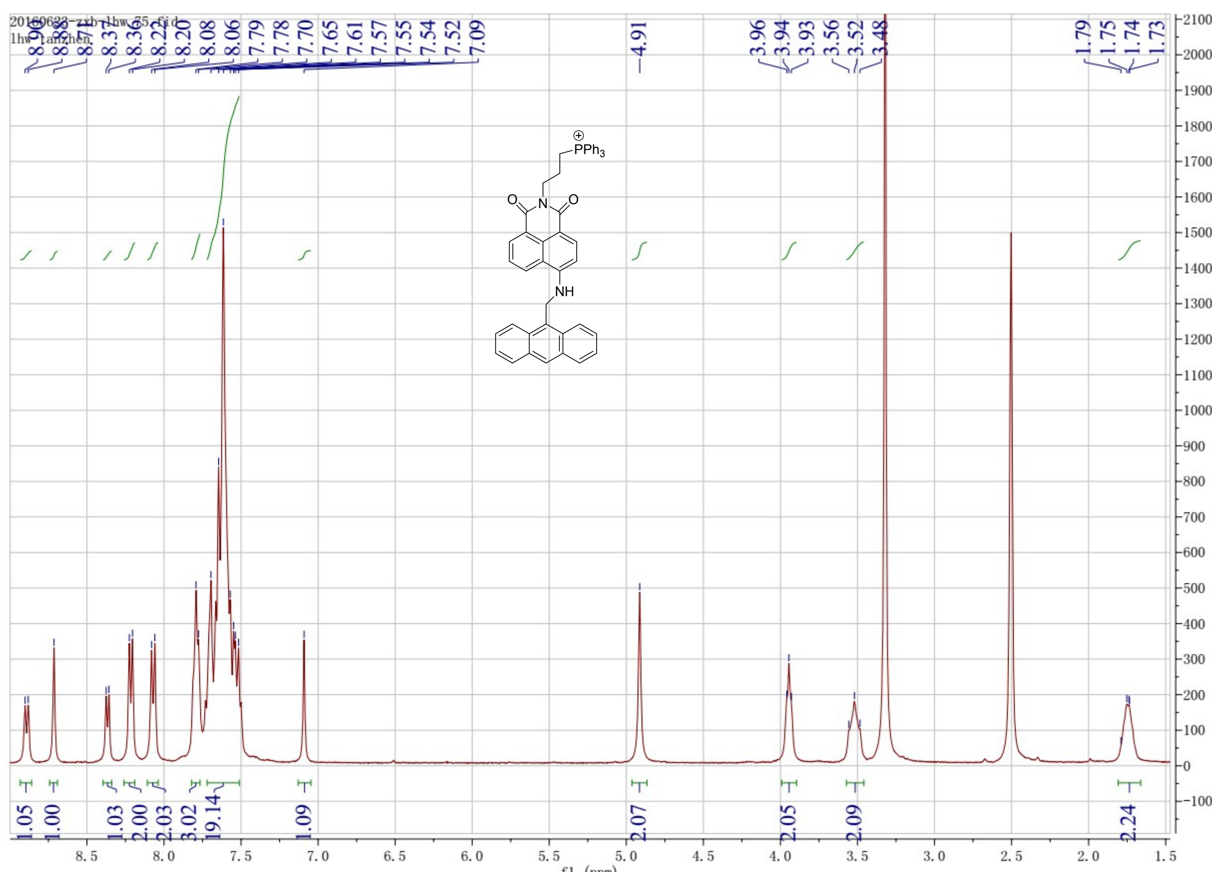
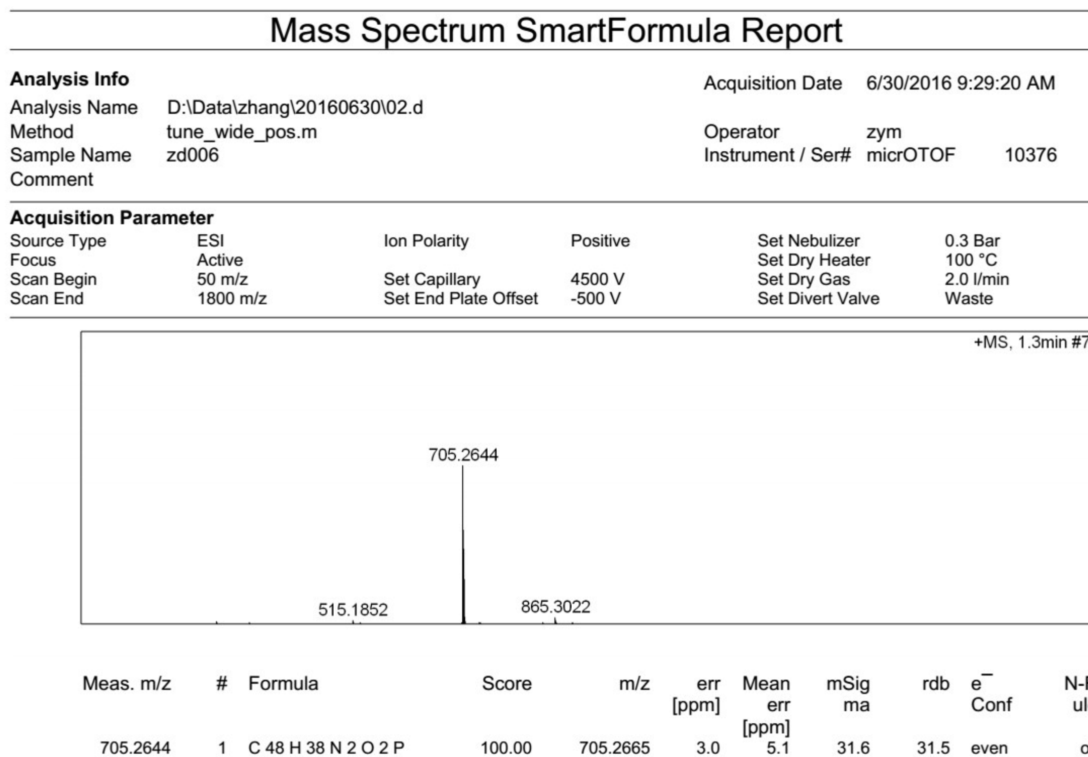


Fig. S15. ^{1}HNMR spectrum of the compound **MNAH**.



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Meas. m/z	#	Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
705.2644	1	C 48 H 38 N 2 O 2 P	100.00	705.2665	3.0	5.1	31.6	31.5	even	ok

Fig. S16. ESI mass spectrum of the compound **MNAH**.

Display Report

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Comment			

Acquisition Parameter

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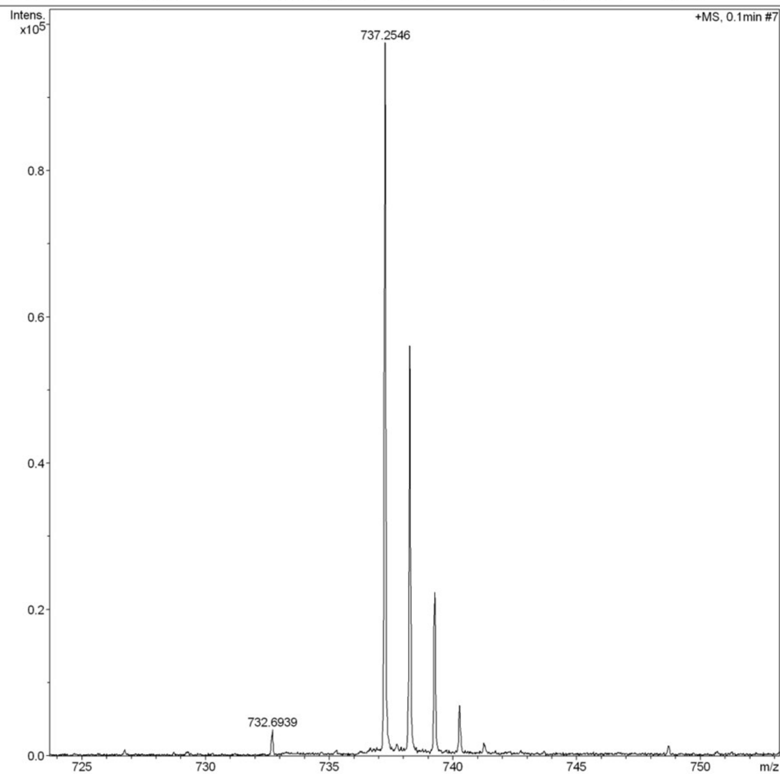

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Mass Spectrum SmartFormula Report

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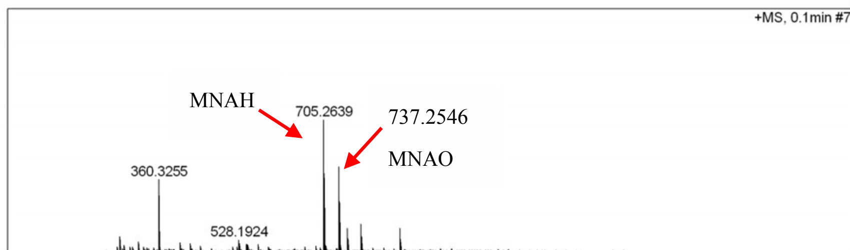

Meas. m/z	#	Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
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Fig. S17. ESI mass spectrum of the compound **MNAO**.