Visualizing ozone fluctuations employing a fluorescent probe in stimulated-epilepsy cell models

Jian Zhang, [a,d#] Yu Duan, [a,#] Chang Liu, [a] Mingyue Zhang, [a] Heng Liu, *[,c] Xingchen Ming, [a] Yong Li, [a] Xiaoyun Jiao, [a] Xu Wang, *[,a] Bo Tang *[,a,b]

a College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Provincial Key Laboratory of Clean Production of Fine Chemicals, Shandong Normal University, Jinan 250014, P. R. China.

b Laoshan Laboratory, Qingdao 266237, P. R. China.

c Department of Radiotherapy, The First Affiliated Hospital of Hainan Medical University, Hainan Medical University, Haikou, People's Republic of China.

d State Key Laboratory of Molecular Engineering of Polymers (Fudan University), Shanghai 200438, People's Republic of China.

E-mail: tangb@sdnu.edu.cn, wangxu@sdnu.edu.cn, liuheng11b@muhn.edu.cn

#These authors contributed equally to this work.

1. Materials and Instruments

All chemicals were purchased from Adamas Reagent, Ltd. (China), and analytical grade solvents were used without further purification. All aqueous solutions were prepared using ultrapure water (ultrapure water, 18 MΩ cm^{-1}). CCK-8 was purchased from Sigma Corporation, column chromatography silica gel (200-300 mesh) was purchased from Qingdao Haiyang Reagent Co., Ltd. The RPMI1640 medium, penicillin/streptomycin and fetal calf serum was purchased from Gibco Corporation. PC12 cells were purchased from Procell Life Science & Technology Co., Ltd.

Fluorescence data was measured by a F-4700 fluorescence spectrophotometer (Hitachi) at room temperature (slit: 5.0 nm, 5.0 nm). The absorption spectra were measured on a UV-1700 spectrophotometer (Shimadzu, Japan). The mass spectra were obtained by Maxis MHR-TOF ultra-high resolution quadrupole time of flight mass spectrometer (Bruker Germany). The ^{1}H NMR and ^{13}C NMR spectra were acquired on a nuclear magnetic resonance spectrometer (400 MHz, Bruker Co., Ltd., Germany). The δ value represents the shift of the spectrum relative to TMS ($(CH_3)_4Si$ = 0.00 ppm). The LC-Mass were performed on a high-performance liquid chromatography-mass spectrometer (LC-16, Shimadzu, Japan). Confocal imaging data were obtained on TCS SP8 confocal laser scanning microscope (CLSM, Leica Co., Ltd., Germany). The data of CCK-8 experiment was measured with a microplate reader (TRITURUS).

Preparation of various interference substances. All reagents were used right after they were ready. Cys, Hcy, GSH, Citric acid and vitamin C (Vc) were all used as received. S^{2-} was used as its sodium salt and prepared as the stock solutions.

K^{+}, Na^{+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, $Fe^{2+}$and Zn^{2+} were all used as their chloride salt and prepared as the stock solutions. All these compounds were commercial available with analytical purity and used directly.

We prepared reactive oxygen species (ROS) as follows:

Ozone: O_3 prepared by ozone generator, the high pure oxygen was introduced into the ozone generator, and then the O_3 generated was pumped into PBS (50 mM, pH = 7.4) at 0 ℃. The absorbance of O_3 at 258 nm was measured by UV-visible absorption spectroscopy, and the concentration of O_3 was calculated according to Lambert-Beer law (c = A /εb, A is

the absorbance of O_3 at 258 nm, the molar extinction coefficient of O_3 at 258 nm $\varepsilon = 2900$ $L \cdot mol^{-1} \cdot cm^{-1}$, and b is the width of the absorption cell is 1cm)

Peroxynitrite ($ONOO^-$): hydrochloric acid (0.6 M) was added to the mixture of $NaNO_2$ (0.6 M) and H_2O_2 (0.7 M), then NaOH (1.5 M) was added. The resulted faint yellow solution was split into small aliquots and stored at lower than -20 °C. The concentration of the prepared peroxynitrite was determined by testing the absorption of the solution at 302 nm. The extinction coefficient of $ONOO^-$ solution is 1670 M^{-1} cm^{-1} at 302 nm. $C_{ONOO^-} = Abs_{302nm}/1.67$ (mM).

Hydroxyl radical (·OH) was prepared by the reaction of Fe^{2+} with H_2O_2 (1:6), and the concentration of ·OH is equal to the concentration of Fe^{2+}.

Singlet oxygen ($^1O_2$) was prepared in situ by addition of the H_2O_2 stock solution into a solution containing 10 eq of NaClO.

Superoxide solution ($O_2^{\bullet-}$) was prepared by adding KO_2 to dry dimethylsulfoxide and stirring vigorously for 10 min.

Hydrogen peroxide (H_2O_2) was diluted appropriately in water. The concentration of H_2O_2 was determined by measuring the absorbance at 240 nm with a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

Nitric oxide (NO) was used from a stock solution prepared by sodium nitroprusside.

Hypochlorous acid (HClO) was obtained by diluting commercial aqueous solutions. The concentration was determined by measuring the absorbance at 292 nm with a molar extinction coefficient of 391 M^{-1} cm^{-1}.

2. Fluorescence analysis

All the measurements about BID-Ozo were carried out in DMSO/PBS = 5/95 without special instructions, and PBS buffer solution (50 mM, pH = 7.4, 37 °C).

Fluorescence titration profiles of the probe were constructed by mixing BID-Ozo (5.0 μM, DMSO/PBS = 5/95) with different level of O_3 (0-18.94 μM) in PBS buffer solution (50 mM, pH = 7.4, 37 °C). The measurement was carried out at $\lambda_{ex}/\lambda_{em}$ = 520/573 nm for BID-Ozo. The specificity experiments of BID-Ozo towards O_3 were carried out by incubation of the probe with O_3 and other biorelevant species, including reactive oxygen and nitrogen species (·OH, $^1O_2$, $O_2^{\cdot-}$, H_2O_2, HClO, NO, $ONOO^-$), reactive sulfur species (GSH, Cys, Hcy, Citric acid and S^{2-}), reductive species (HSO_3^-, and Vc), and metal ions (K^+, Na^+, Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}) in PBS buffer solution (50 mM, pH = 7.4, 37 °C). The kinetic studies of fluorescence responses were performed by incubating BID-Ozo with O_3 at $\lambda_{ex}/\lambda_{em}$ = 520/573 nm.

Determination of BID-Ozo fluorescence quantum yield (Φ_F):

The absorbance of BID-Ozo and the corresponding product was adjusted to ca. 0.05. The emission spectra were obtained by exciting with corresponding maximal excitation wavelength and the integrated areas of the fluorescence spectra were calculated. The fluorescence quantum yield was determined by comparing the integrated emission intensity of the test samples with that of a solution of quinine sulfat (the fluorescence standard, Φ_F = 0.54 in 0.1 M H_2SO_4) and calculated by following equation.

$$\Phi_F = \Phi_S \frac{A_S F_X}{A_X F_S}\left(\frac{n_x}{n_s}\right)^2$$

$$\Phi_{F(BID-Ozo} = 0.012$$
$$\Phi_{F(BID-OH} = 0.097$$

where Φ_F is the fluorescence quantum yield, “A” is the absorbance at the excitation wavelength, “F” is the area under the emission curve, and “n” is the refractive index of the solvents used. Subscripts “S” and “X” refer to the standard and the samples to be tested, respectively.

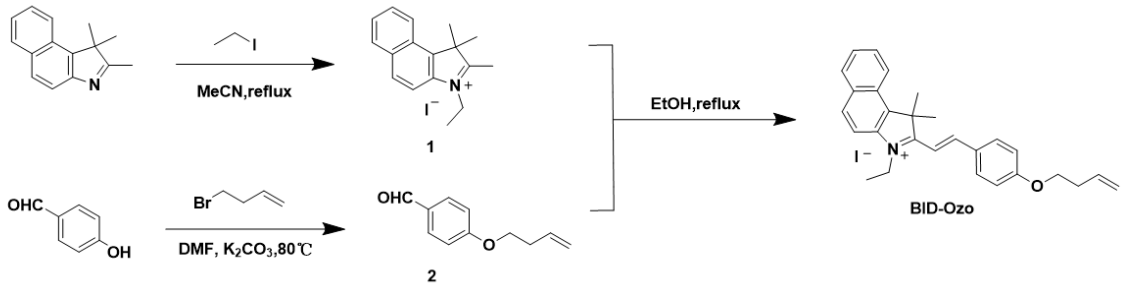
Determination of octanol-water partition coefficient (log P)

N-octanol and purified water were selected and added to the closed container according to the volume ratio (1:1). A certain amount of probe (30 μM) was added, and the total volume (6 mL) was recorded. The total volume was shaken vigorously in the constant temperature shaker (25 ^{o}C, 200 rpm, 24 h) to make the two phases fully mixed and reach the distribution equilibrium. The concentration of the probe in the lipid and aqueous phases was determined by direct measurement of the absorbance respectively, and the partition coefficient was calculated according to the following formula ($C_{lipid\ phase}$ and $C_{aqueous\ phase}$ are the concentrations in the two phases after equilibration).

$$\log P = \log\left(\frac{C_{lipid\ phase}}{C_{aqueous\ phase}}\right)$$

$$\log P = 1.544$$

3. Synthesis and characterization of BID-Ozo



Scheme S1 Synthesis procedure of BID-Ozo

Synthesis of compound 1

1,1,2-Trimethyl-1H-benz[e]indole (2.0 g, 10 mmol, 1 eq) and iodoethane (3.1 g, 20 mmol, 2 eq) were dissolved in acetonitrile (20 mL), and the mixture was stirred at 80 ℃ for 12 h. After the reaction, it was cooled to room temperature, the excess solvent was removed under reduced pressure, the precipitate was washed with petroleum ether. After drying, compound 1 was obtained as a gray white solid (3.4 mg, yield: 92.8 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.37 (d, J = 8.0 Hz, 1H), 8.30 (d, J = 8.8 Hz, 1H), 8.22 (d, J = 7.6 Hz, 1H), 8.15 (d, J = 8.8 Hz, 1H), 7.80 - 7.70 (m, 2H), 4.64 - 4.59 (m, 2H), 3.36 (s, 3H), 1.76 (s, 6H), 1.50 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 196.42, 138.67, 137.50, 133.50, 131.19, 130.19, 128.88, 127.74, 127.71, 123.89, 113.66, 55.94, 43.82, 21.96, 14.15, 13.37. HR-MS(ESI): calcd for $C_{17}H_{20}NI$ $[M-I]^+$:238.1590, found 238.1608.

Synthesis of compound 2

Under the protection of argon, 4-Hydroxybenzaldehyde (122 mg, 1 mmol, 1eq), 4-Bromo-1-butene (300 μL, 3 mmol, 3 eq) and K_2CO_3 (522 mg, 4 mmol, 4 eq) were dissolved in *N*,*N*-Dimethylformamide (4 mL) and heated to 80 ℃ for 12 h. After cooling to room temperature, the solvent was removed under reduced pressure, and then the crude product was purified by column chromatography producing Compound 2 (151 mg, yield: 86.0 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.87 (s, 1H), 7.82 (d, J = 8.4 Hz, 2H), 6.99 (d, J = 8.8 Hz, 2H), 5.94 - 5.84 (m, 1H), 5.20 - 5.11 (m, 2H), 4.09 (t, J = 6.8 Hz, 2H), 2.60 - 2.54 (m, 2H);

^{13}C NMR (101 MHz, $CDCl_3$) δ 190.95, 164.02, 133.86, 132.36, 132.05, 129.86, 117.50, 116.01, 114.80, 67.55, 33.41. HR-MS(ESI): calcd for $C_{11}H_{12}O_2$ $[M+Na]^+$:199.0729, found 199.0698.

Synthesis of BID-Ozo

Under the protection of argon, Compound 1 (81.0 mg, 0.34 mmol, 1 eq) and Compound 2 (60.0 mg, 0.34 mmol, 1 eq) were dissolved in ethanol (4 mL) and heated to 80 ℃ for 12 h. After cooling to room temperature, the solvent was removed under reduced pressure, and then the crude product was purified by column chromatography producing an orange solid BID-Ozo (118 mg, yield: 66.5 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 - 8.28 (m, 3H), 8.20 (d, J = 8.4 Hz, 1H), 8.10 (d, J = 8.8 Hz, 1H), 8.04 (d, J = 8.0 Hz, 1H), 7.79 (s, 1H), 7.75 - 7.71 (m, 2H), 7.64 (t, J = 7.2 Hz, 1H), 7.05 (d, J = 8.8 Hz, 2H), 5.92-5.82 (m, 1H), 5.20-5.10 (m , 4H), 4.10 (t, J = 6.8 Hz, 2H), 2.58 - 2.53 (m, 2H), 2.09 (s, 6H), 1.66 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 181.70, 164.46, 154.06, 137.74, 134.35, 133.77, 133.60, 131.80, 130.36, 128.61, 127.40, 127.35, 126.77, 122.62, 117.55, 115.78, 112.02, 109.26, 67.79, 53.69, 44.19, 33.35, 27.09, 14.48. HR-MS(ESI): calcd for $C_{28}H_{30}NOI$ $[M-I]^+$:396.2321, found 396.2273.

HPLC analysis

HPLC analysis of BID-Ozo after incubation without or with O_3 was performed on an AGILENT 1200 system (AGILENT Co. Ltd., US). The reaction mixture was analyzed with the detection wavelength at 520 nm. Running conditions were as follows: mobile phase composition was MeOH/H_2O: 4/1; GL sciences C18-WR column of 4.6*150mm; and flow rate of 0.6 mL/min.

Cell culture

The PC12 cell lines were grown in Roswell Park Memorial Institute 1640 (RPMI1640) with 1 % 100 U mL^{-1} antibiotics penicillin/streptomycin and 10 % fetal bovine serum (FBS) at 37 °C under a humidified atmosphere containing 5 % CO_2.

Cytotoxicity assay

The cytotoxicity of the probe was tested in PC12 cells using a standard Cell Counting Kit-8 assay. Cells in the logarithmic phase were harvested and plated into 96-well plates (5000 cells/well). After 24 hours, cells were treated with different concentrations (0, 5, 10, 20, 50, 100 μM) of compounds. After incubation of 8 h, cells were washed by PBS and incubated in the 100 μL fresh medium with 10 μL CCK8 dye for more 2 h. The absorbance at 450 nm was determined using a microplate reader; The IC_{50} value was calculated according to GraphPad Prism.

Cell imaging

PC12 cells were employed to perform the imaging.

Colocalization assay:

PC12 cells were washed with PBS three times and then co-incubated with Mito-Tracker Deep Red FM (1.0 μM) and BID-Ozo (10.0 μM) for 15 min at 37 °C. After washing with PBS again, the cells were imaged by confocal microscopy. BID-Ozo: E_x = 514 nm, E_m = 545-650 nm; Mito-Tracker: E_x = 651 nm, E_m = 700-720 nm.

Confocal fluorescent imaging of exogenous O_3 in PC12 cells with BID-Ozo:

The cells were washed with PBS for three times firstly. Group a: PC12 cells were incubated with BID-Ozo (10.0 μM) alone for 15 min, the BID-Ozo solution was washed away and the imaging was carried out. Group b: PC12 cells were pretreated with O_3 (5.0 μM) for 10 min and washed with PBS for three times; After culturing with BID-Ozo (10.0 μM) for 15 min, the BID-Ozo solution was washed away and the imaging was carried out. Group c:

PC12 cells were pretreated with O_3 (10.0 μM) for 10 min and washed with PBS for three times; After culturing with BID-Ozo (10.0 μM) for 15 min, the BID-Ozo solution was washed away and the imaging was carried out. Group d: PC12 cells were pretreated with O_3 (10.0 μM) for 10 min and then cultured with ethyl 4-vinylbenzoate (50.0 μM) for 20 min, and washed with PBS for three times; After culturing with BID-Ozo (10.0 μM) for 15 min, the BID-Ozo solution was washed away and the imaging was carried out. BID-Ozo: E_x = 514 nm, E_m = 545-650 nm.

Confocal fluorescent imaging of endogenous O_3 in PC12 cells with BID-Ozo

The cells were washed with PBS for three times firstly. Group a: PC12 cells were incubated with BID-Ozo (10.0 μM) alone for 15 min, after washing away BID-Ozo solution, PBS was added again to image as control group. Group b: PC12 cells were pretreated with PMA (2 μg/mL) for 20 min and washed with PBS for three times; After culturing with BID-Ozo (10.0 μM) for 15 min, the BID-Ozo solution was washed away and the imaging was carried out. Group c: PC12 cells were pretreated with PMA (2 μg/mL) for 20 min and washed with PBS for three times, then treated with ethyl 4-vinylbenzoate (50.0 μM) for 20 min. After culturing with BID-Ozo (10.0 μM) for 15 min, the BID-Ozo solution was washed away and the imaging was carried out.

Confocal fluorescent imaging of O_3 in PC12 cells with BID-Ozo under oxidative stress

Group a: PC12 cells were incubated with BID-Ozo (10.0 μM) alone for 15 min, after washing away BID-Ozo solution, PBS was added again to image as control group. Group b: The PC12 cells were incubated with Glutamic acid (Glu, 10 mM, 12 h)/ Pentetrazol (PTZ, 10 mM, 1 h)/ Penicillin G (PG, 2.5 mM, 12 h) to induce oxidative stress, then washed with PBS for three times; After culturing with BID-Ozo (10.0 μM) for 15 min, the BID-Ozo solution was washed away and the imaging was carried out. Group c: The PC12 cells were pretreated with Glu (10 mM, 12 h)/ PTZ (10 mM, 1 h)/ PG (2.5 mM, 12 h), then incubated with ethyl 4-vinylbenzoate (50.0 μM) for 20 min and washed with PBS for three times; After culturing with BID-Ozo (10.0 μM) for 15 min, the BID-Ozo solution was washed away and the imaging was carried out.

Confocal fluorescent imaging of O_3 in PC12 cells treated with anti-oxidant drugs by BID-Ozo:

The cells were washed with PBS for three times firstly. Group a: PC12 cells were incubated

with medium containing 5% DMSO for 12 h, washed with PBS for three times, then PC12 cells were incubated with BID-Ozo (10.0 μM) alone for 15 min, after removing BID-Ozo solution, PBS was added again to image as control group. Group b-j: PC12 cells were pretreated with difference antioxidant drugs (20.0 μM) for 12 h and washed with PBS for three times; After culturing with BID-Ozo (10.0 μM) for 15 min, the BID-Ozo solution was removed and the imaging was carried out. Ascorbic acid, Naringin, Curcumin, Glutathione, Coenzyme Q10, Baicalein, Sulfamethoxazole, Caffeic acid and Folic acid were used in groups b-j, respectively.

Confocal fluorescent imaging of O_3 in PC12 cells for signaling pathway studies:

Group a: PC12 cells were incubated with BID-Ozo (10.0 μM) alone for 15 min, after washing away BID-Ozo solution, washed with PBS for three times, and the imaging was carried out. Group b: The PC12 cells were incubated with Glu (10 mM) for 12 h to construct the epilepsy cell model, and then the cells were washed three times with PBS. Group c-e: PC12 cells were pretreated with Glu (10 mM) for 12 h and washed with PBS for three times, then incubated with ascorbic acid (20.0 μM, 12 h) / sulindac (20.0 μM, 12 h) / ethyl 4-vinylbenzoate (50.0 μM, 20 min) and washed with PBS for three times; After culturing with BID-Ozo (10.0 μM) for 15 min, the BID-Ozo solution was washed away and the imaging was carried out.

Western blot:

Group a: control. Group b: PC12 cells were incubated with Glu (10 mM) for 12 h. Group c-e: PC12 cells were incubated with Glu (10 mM) for 12 h, then incubated with ascorbic acid (20.0 μM, 12 h) / sulindac (20.0 μM, 12 h) / ethyl 4-vinylbenzoate (50.0 μM, 20 min). The cells were digested with trypsin and the cell precipitate was collected by centrifugation. The subsequent experiments were completed by BOSTER Co., Ltd.

In Vivo imaging:

All animal experimental protocols complied with the Animal Management Rules of the Ministry of Health of the People's Republic of China and were approved by the Animal Care Committee of Shandong Normal University. Ethics No. AEECSDNU2025045. C57BL/6J mice (female, age 8 weeks) were purchased from Beijing Weishang Lide Biotechnology Co., LTD. C57BL/6J mice were housed in pathogen-free cages and fed under alternating light and dark conditions (given adequate water and food).

Construction of mice model of epilepsy: After anesthesia, the hair on the top of the head was removed, and the mice were fixed on the brain stereotaxic apparatus. Kainic acid was injected into the unilateral hippocampal CA1 region, and penicillin was given to the wound. Then the mice were reheated with a heating pad to wake up, and returned to the cage.

Normal mice were injected with BID-Ozo (0.5mg/Kg) via the tail vein in the control group, kainate (KA) -treated mice were injected with BID-Ozo (0.5mg/Kg) via the tail vein in the epilepsy group and curcumin-treated group. Epileptic mice were given curcumin (60 mg/Kg, 1d) followed by a tail vein injection of BID-Ozo (0.5 mg/Kg). Fluorescence images of mouse brains 60 min after BID-Ozo injection were further determined using an IVIS spectral imaging system with a 520 nm excitation filter and an acquisition wavelength range of 550 to 600 nm.

Statistical Analysis

All experiments were replicated at least three times. Data values were presented as mean ± standard deviation (SD). Statistical significance was assessed using one-way ANOVA. *$p < 0.05$, **$p < 0.01$. ***$p < 0.001$.

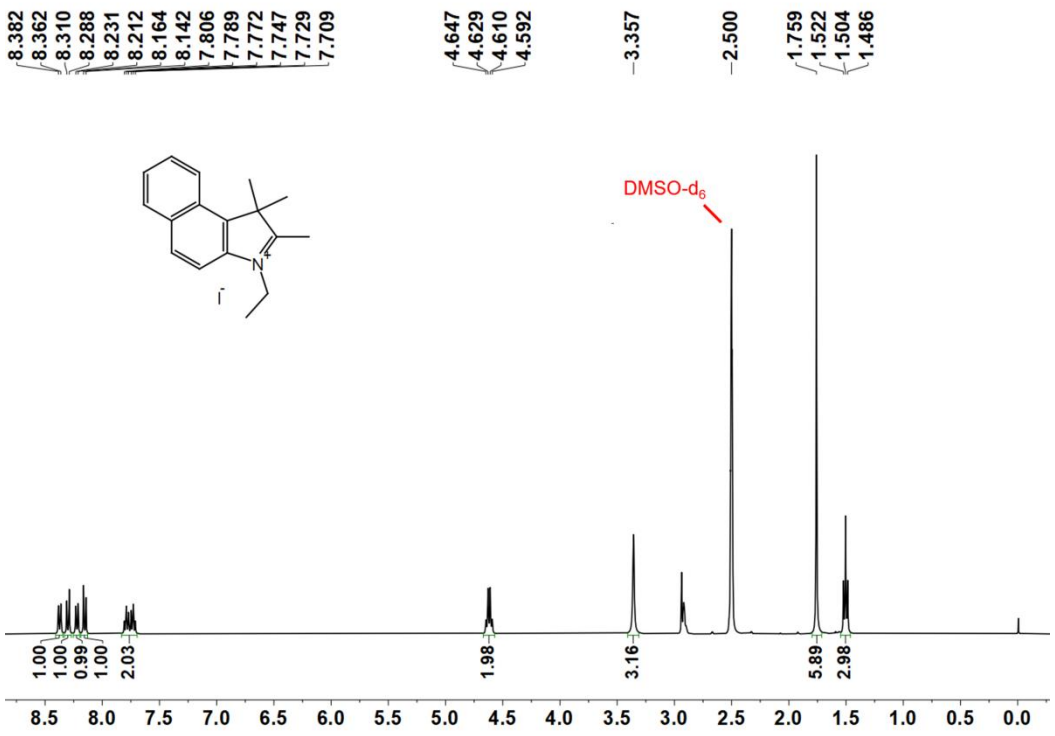


Fig. S1 ^{1}H NMR spectrum of compound 1 in DMSO-d_6.

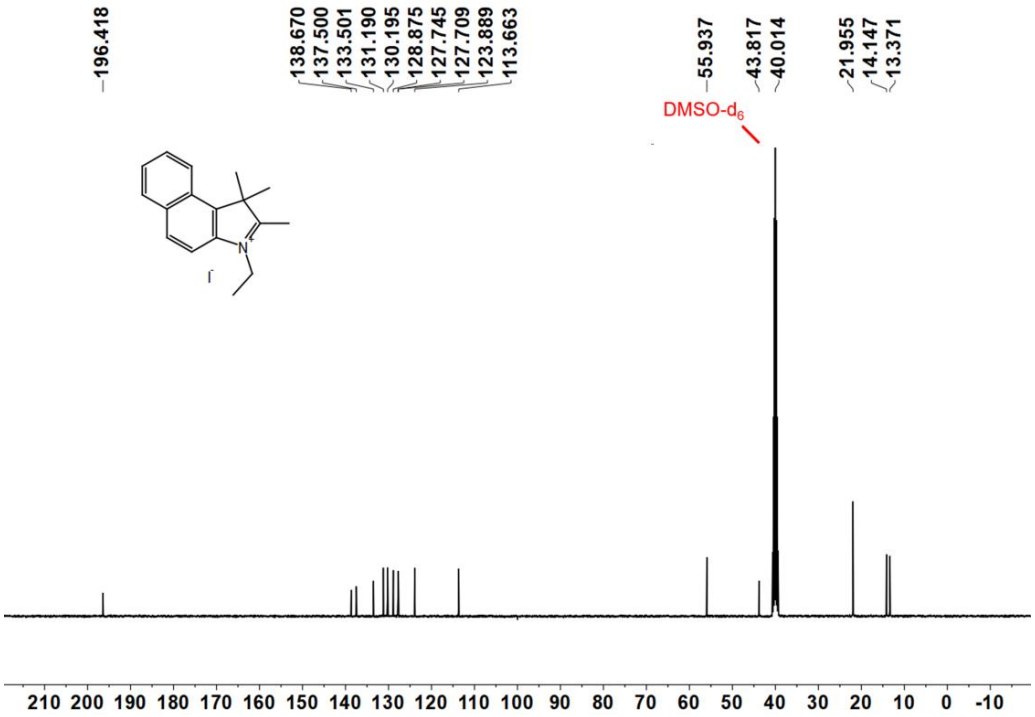


Fig. S2 ^{13}C NMR spectrum of compound 1 in DMSO-d_6

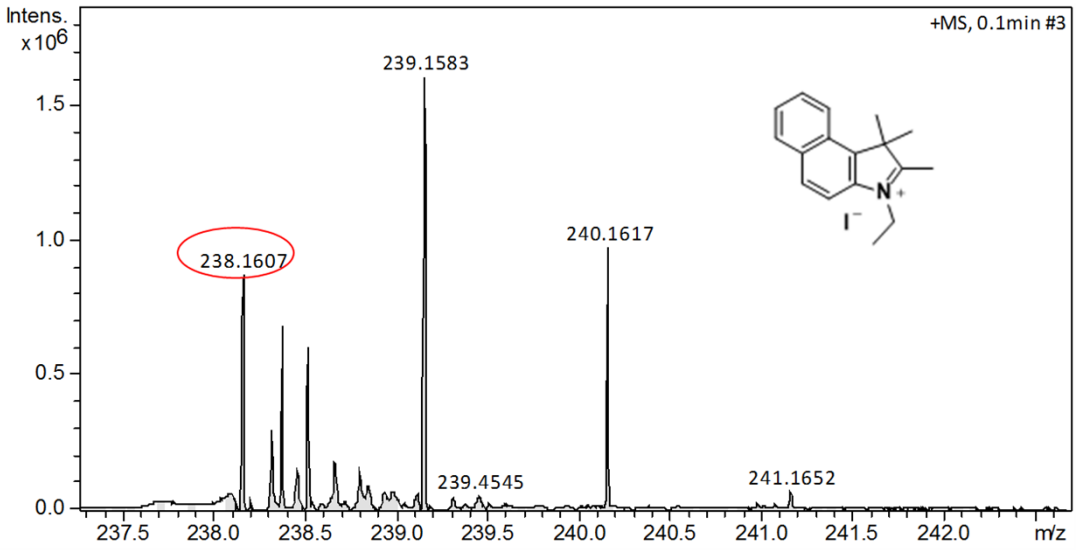


Fig. S3 HRMS of compound 1.

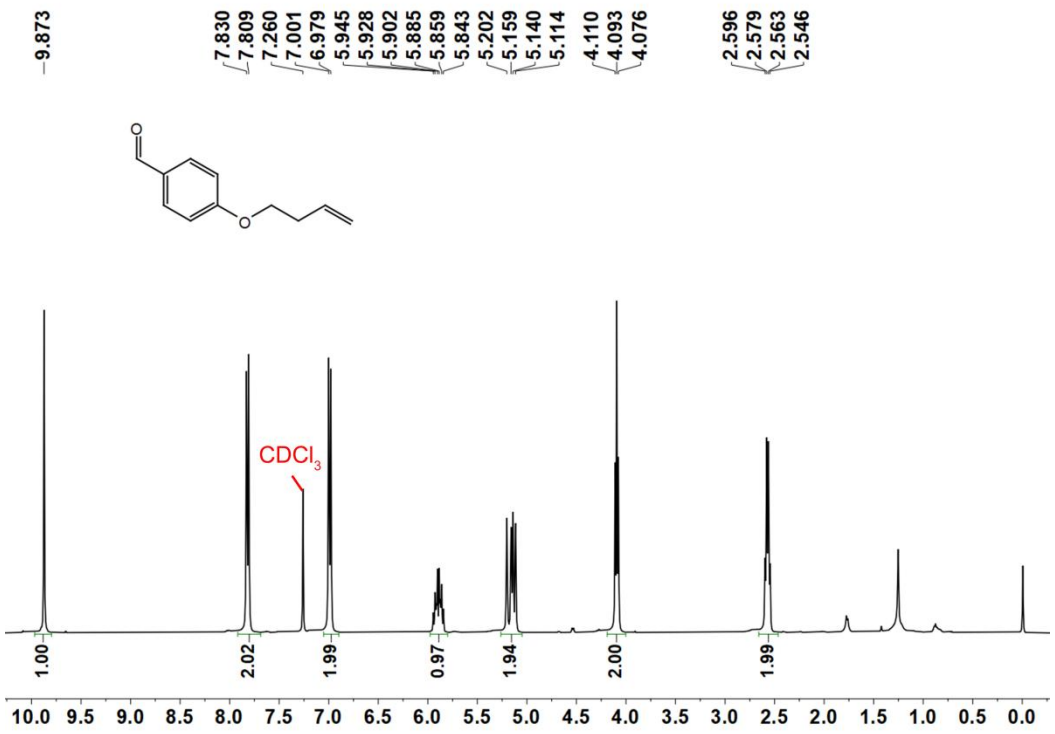


Fig. S4 ^{1}H NMR spectrum of compound 2 in $CDCl_3$.

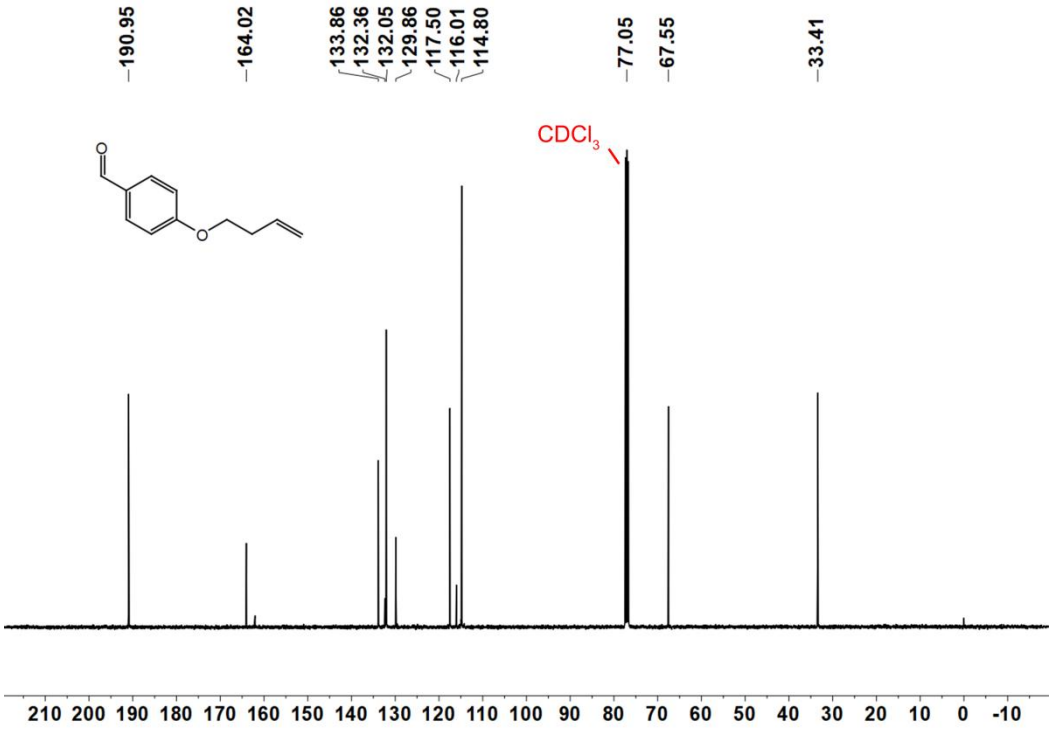


Fig. S5 ^{13}C NMR spectrum of compound 2 in $CDCl_3$.

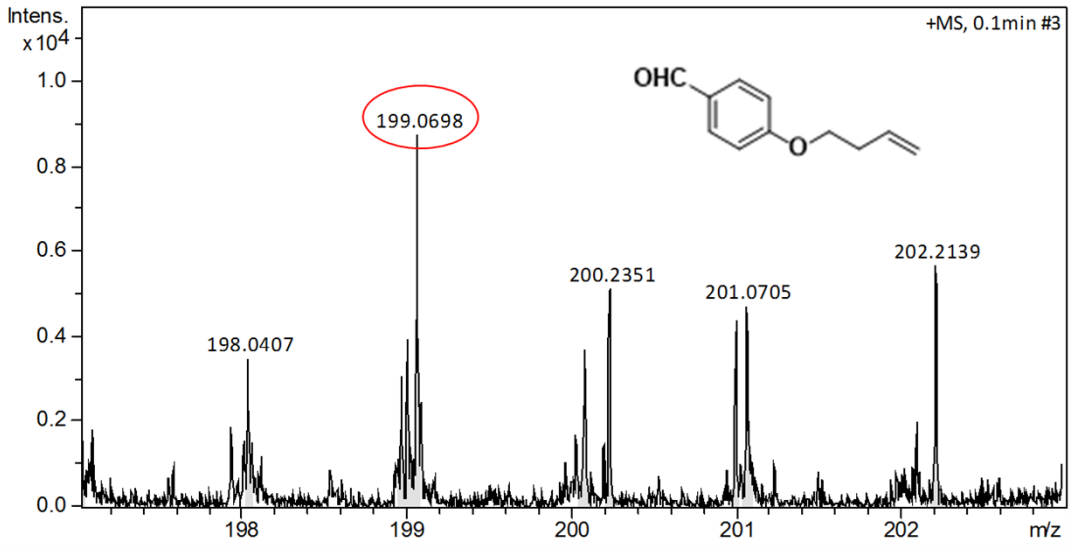


Fig. S6 HRMS of compound 2.

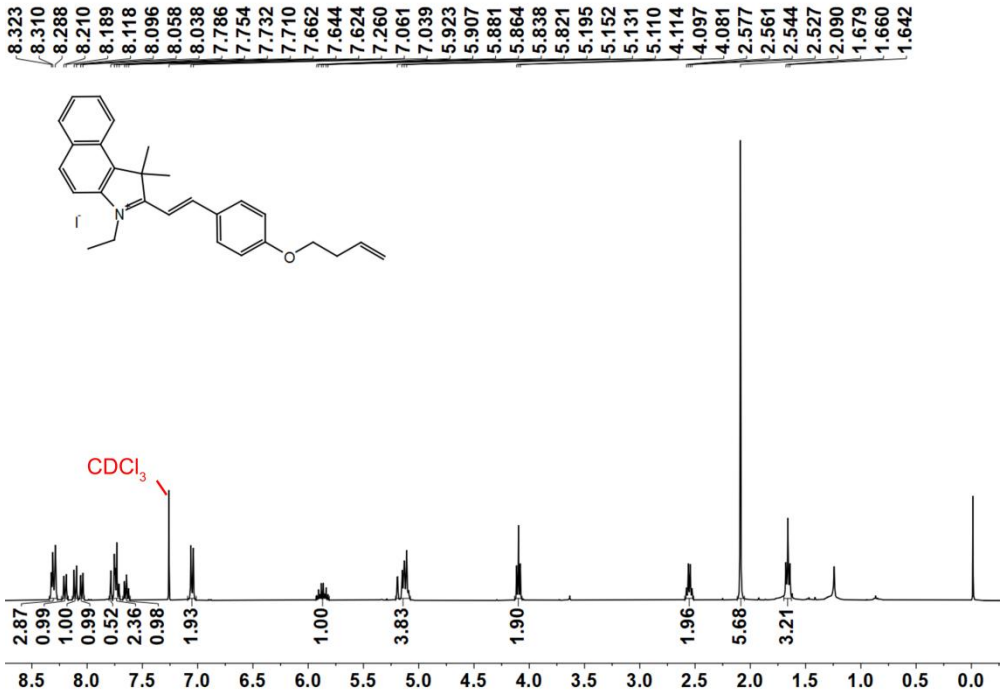


Fig. S7 ^{1}H NMR spectrum of BID-Ozo in $CDCl_3$.

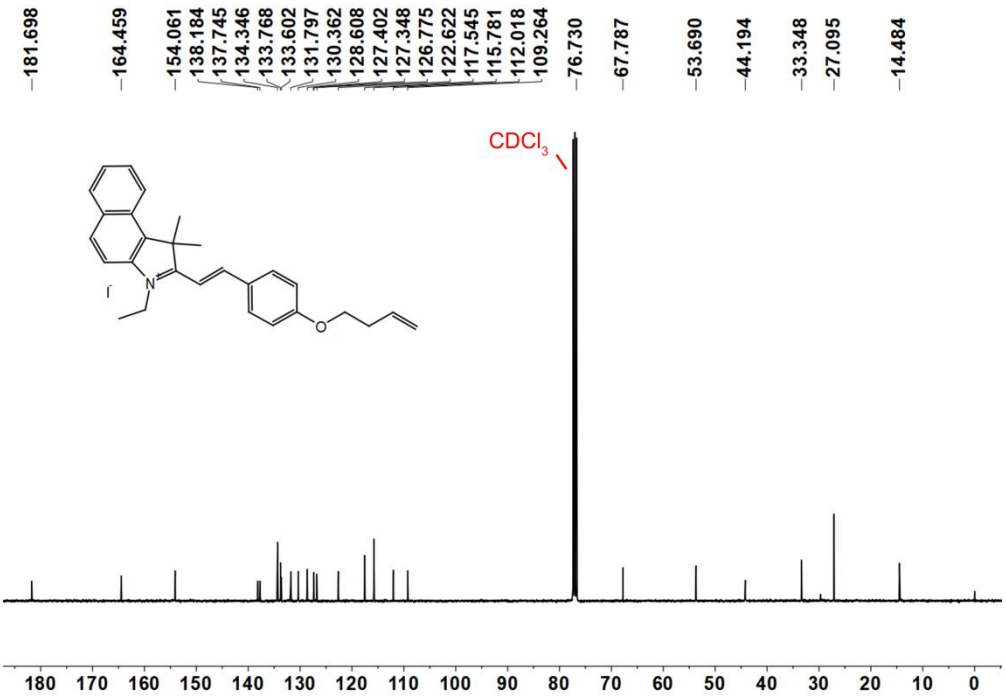


Fig. S8 ^{13}C NMR spectrum of BID-Ozo in $CDCl_3$.

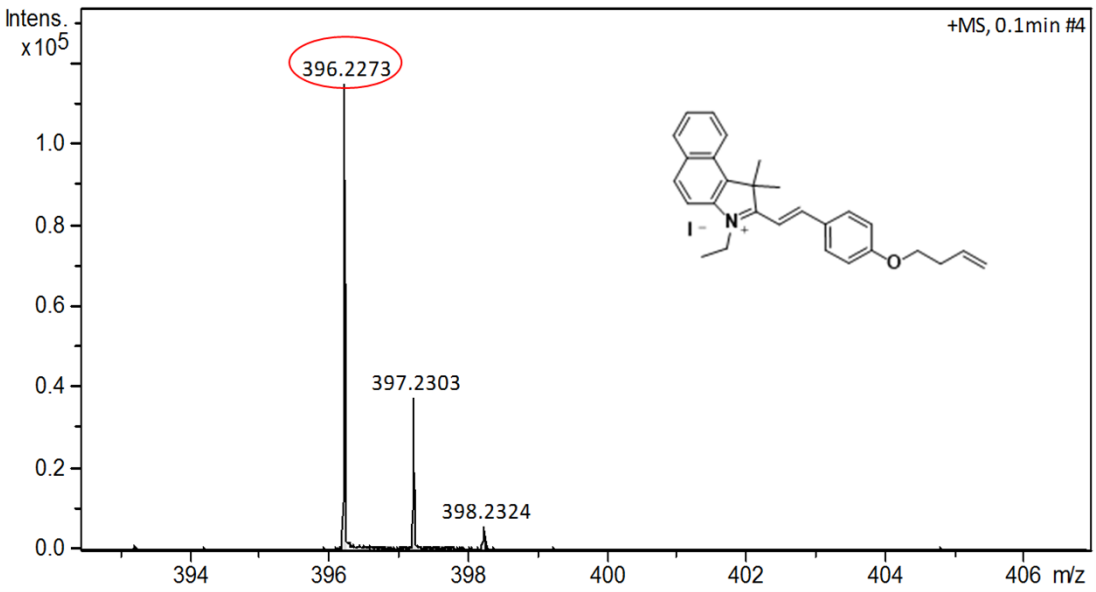


Fig. S9 HRMS of BID-Ozo.

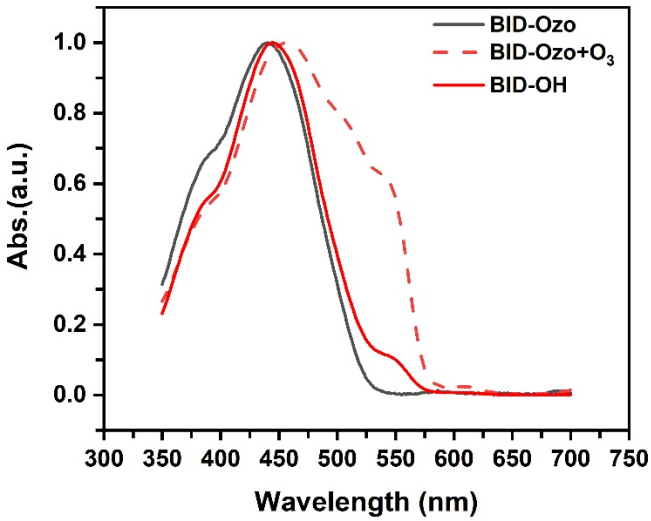


Fig. S10 UV-visible absorption spectra of BID-OH (10 μM, DMSO/PBS = 5/95) and BID-Ozo (10 μM, DMSO/PBS = 5/95) in the absence and presence of O_3. PBS buffer solution (50 mM, pH = 7.4)

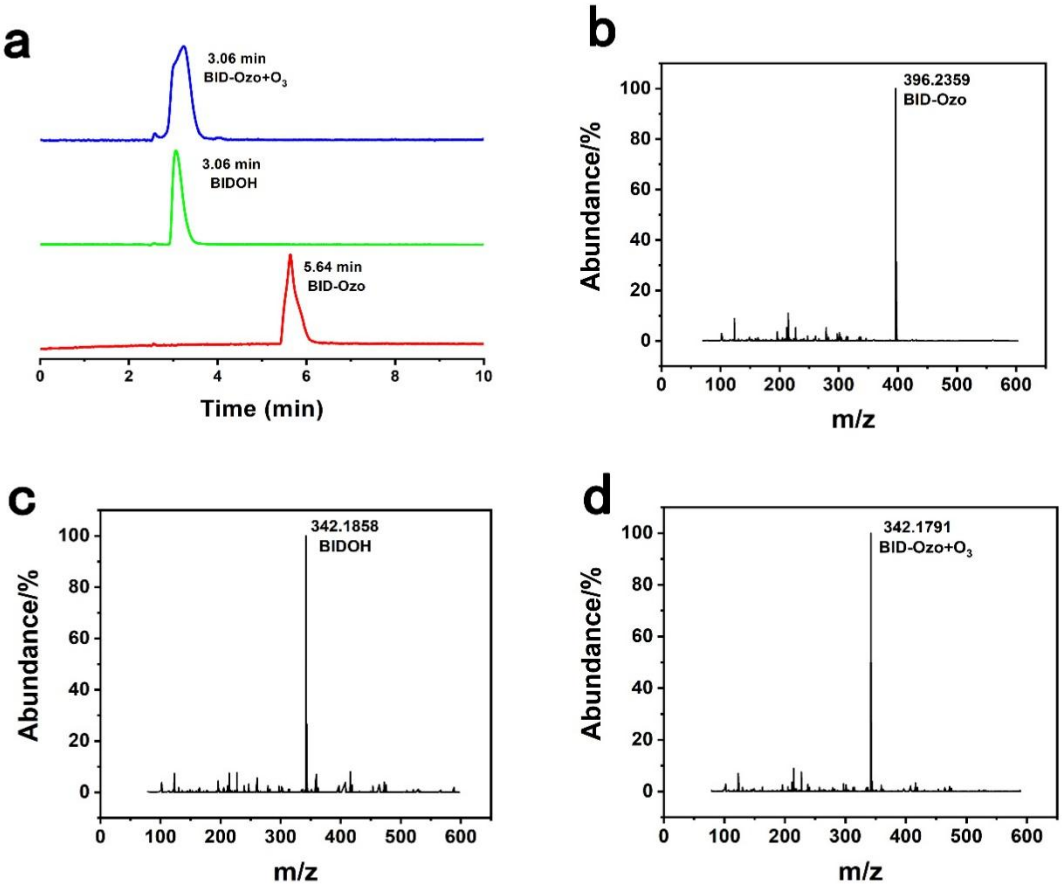


Fig. S11 HPLC-MS analysis of the reaction between BID-Ozo and O_3. (a) HPLC results of BID-Ozo, BIDOH and BID-Ozo with O_3 respectively; MS spectra of (b) BID-Ozo, (c) BIDOH and (d) BID-Ozo and O_3 respectively.

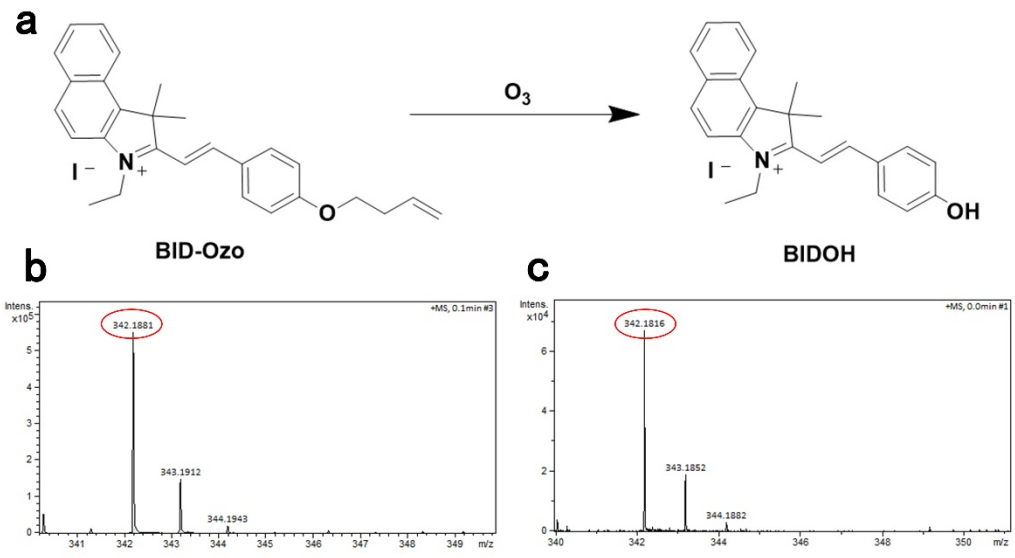


Fig. S12 (a) The reaction between BID-Ozo and O_3; (b) HRMS of the mixture of BID-Ozo and O_3; (c) HRMS of BIDOH.

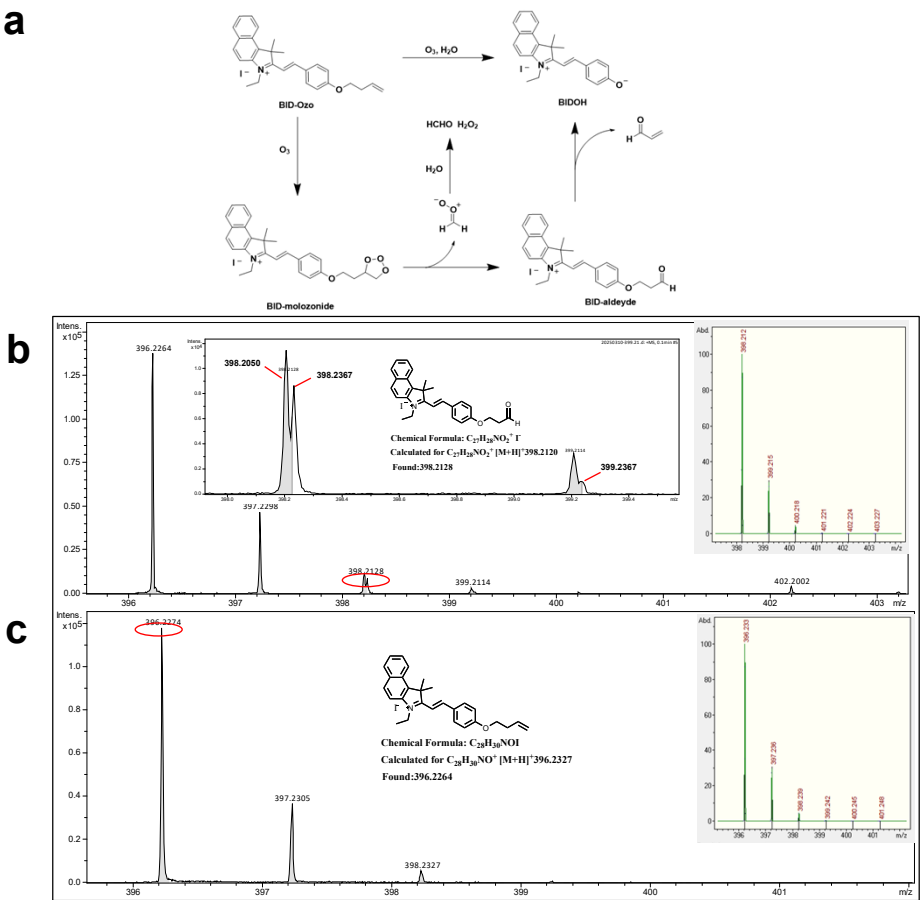


Fig. S13 (a) The reaction mechanism between BID-Ozo and O_3; (b) HRMS of the mixture of BID-Ozo and O_3; (c) HRMS of BID-Ozo.

The isotopic peak cluster of BID-Ozo covers the molecular weight range that includes 398. However, a magnified view (Fig. S13b) reveals that the mass spectrum peak at this position exhibits a double-peak shape, corresponding to BID-Ozo (398.2327) and BID-aldehyde (398.2128), both of which match the theoretical values. This confirms that the mass spectrum signal of BID-aldehyde has been captured. Additionally, the isotopic peak cluster of the reaction mixture extends from 396.2264 to 403.2037. Theoretical predictions indicate that the isotopic peak cluster range of BID-Ozo is 396.2327-401.2484, which aligns with the experimental results (Fig. S13c), while the predicted range for BID-aldehyde is 398.2120-403.2271, also consistent with the observations. Based on these evidence, it can be concluded that BID-aldehyde is indeed an intermediate in the reaction between BID-Ozo and O_3.

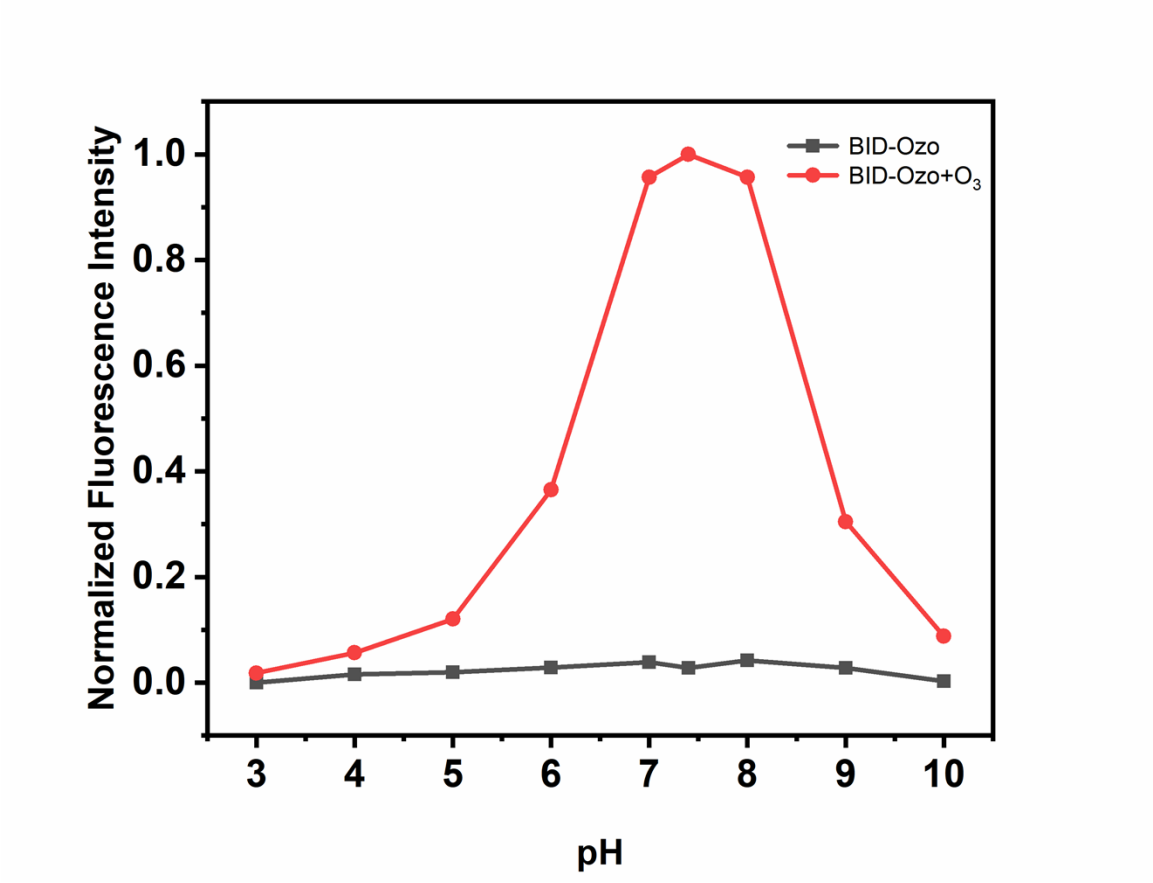


Fig. S14 Effect of pH on the response of BID-Ozo (10 μM, DMSO/PBS = 5/95) towards O_3 (18 μM). ($\lambda_{ex}/\lambda_{em}$ = 520/573 nm) PBS buffer solution (50 mM, pH = 7.4)

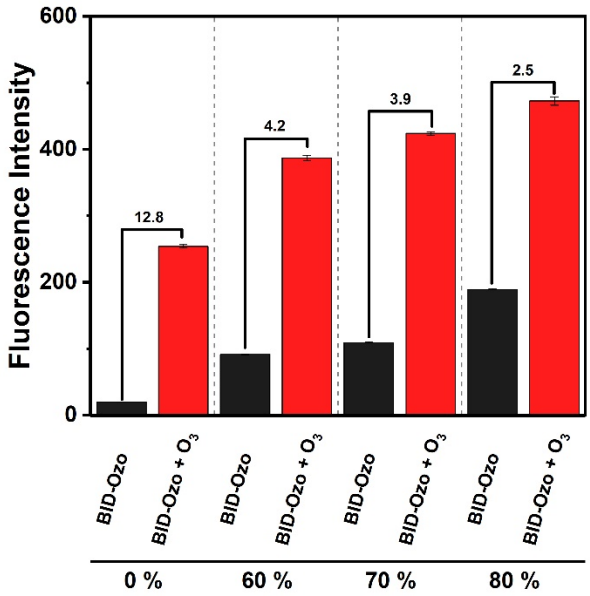


Fig. S15 Effect of different viscosity on the fluorescence intensity of BID-Ozo (5 μM) and BID-Ozo (5 μM) + O_3 (15 μM) at 573 nm. ($\lambda_{ex}/\lambda_{em}$ = 520/573 nm, excitation slit and emission slit: 5 nm)

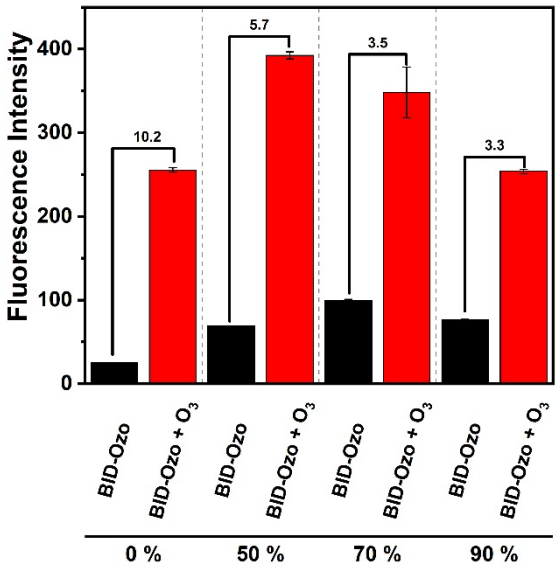


Fig. S16 Effect of different polarity on the fluorescence intensity of BID-Ozo (5 μM) and BID-Ozo (5 μM) + O_3 (15 μM) at 573 nm. ($\lambda_{ex}/\lambda_{em}$ = 520/573 nm, excitation slit and emission slit: 5 nm)

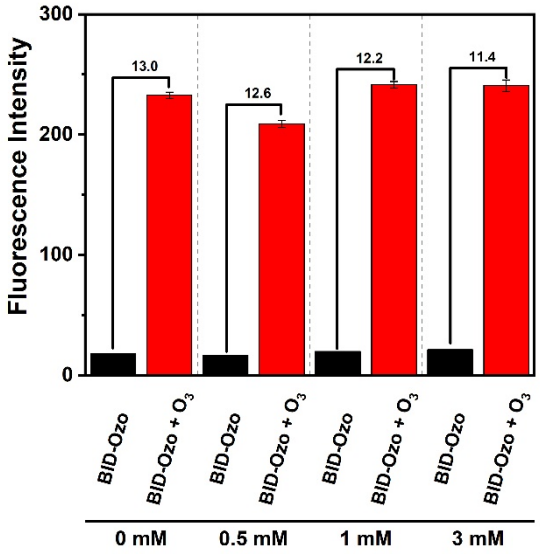


Fig. S17 Effect of different concentrations of BSA on the fluorescence intensity of BID-Ozo (5 μM) and BID-Ozo (5 μM) + O_3 (15 μM) at 573 nm. ($\lambda_{ex}/\lambda_{em}$ = 520/573 nm, excitation slit and emission slit: 5 nm)

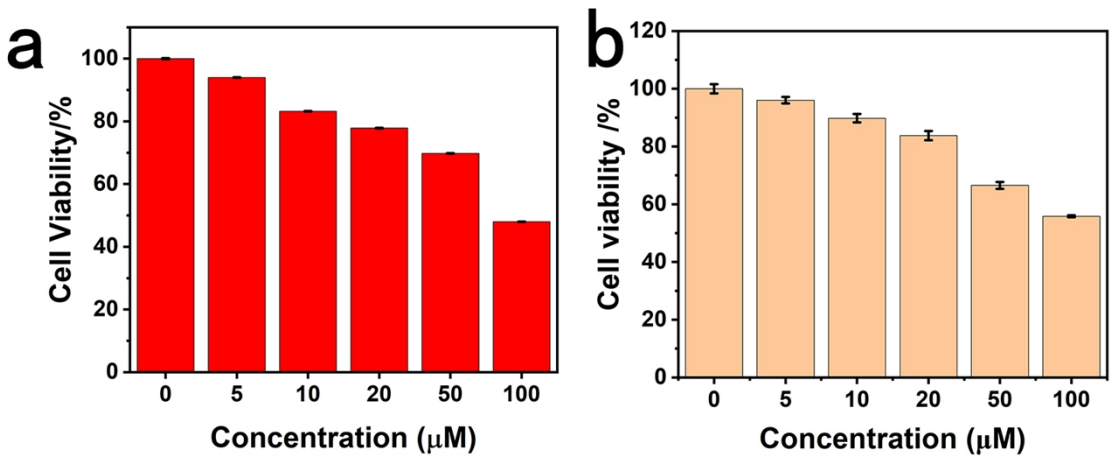


Fig. S18 CCK-8 assay of PC12 cells (a) and SVGP-12 cells (b) with different concentrations of BID-Ozo.

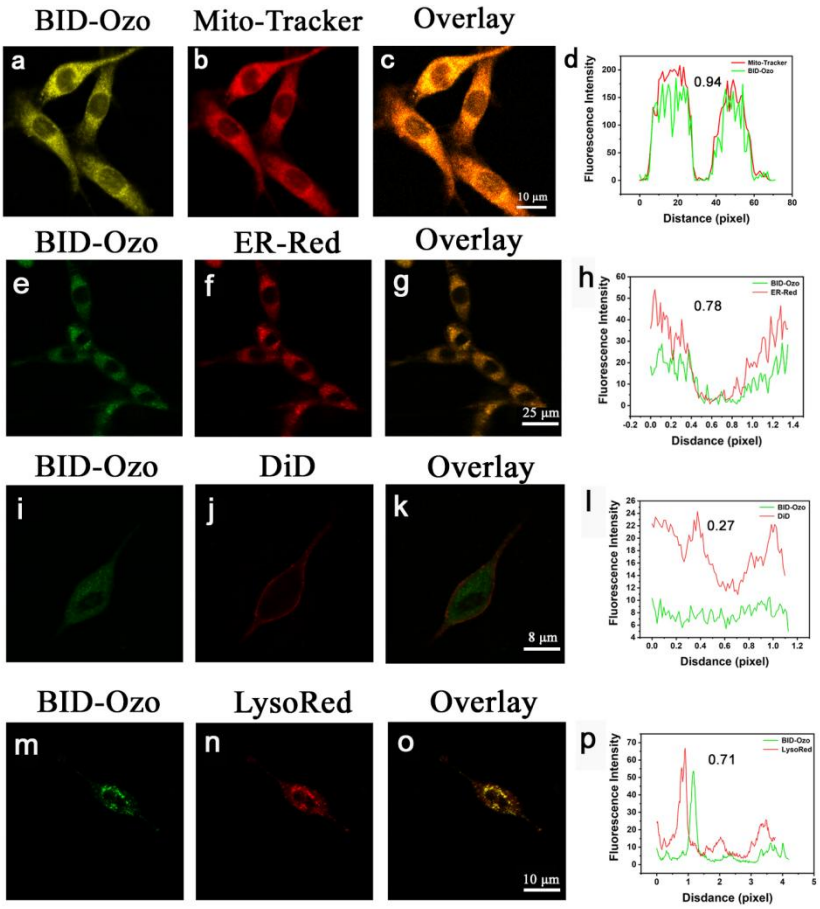


Fig. S19 Confocal imaging plots of PC12 cells after the coincubation of BID-Ozo with Mito-Tracker, ER-Red, DiD and LysoRed. (a) Confocal imaging by BID-Ozo in yellow fluorescent channel; (b) Confocal imaging by Mitto-Tracker in red fluorescent channel; (c) Superposition of panels a and b; (d) Plots of two-channel fluorescence intensity in the underlined part in Fig. c; (BID-Ozo: E_x = 514 nm, E_m = 545-650 nm; Mito-Tracker: E_x = 651 nm, E_m = 700-720 nm; Scale bar: 10 μm). (e) Confocal imaging by BID-Ozo in green fluorescent channel; (f) Confocal imaging by ER-Red in red fluorescent channel; (g) Superposition of panels e and f; (h) Plots of two-channel fluorescence intensity in the underlined part in Fig g. (BID-Ozo: E_x = 488 nm, E_m = 545-650 nm; ER-Red: E_x = 561 nm, E_m = 660-720 nm; Scale bar: 25 μm). (i) Confocal imaging by BID-Ozo in green fluorescent channel; (j) Confocal imaging by DiD in red fluorescent channel; (k) Superposition of panels i and j; (l) Plots of two-channel fluorescence intensity in the underlined part in Fig k. (BID-Ozo: E_x = 488 nm, E_m = 545-650 nm; Mito-Tracker: E_x = 638 nm, E_m = 663-700 nm; Scale bar: 8 μm). (m) Confocal imaging by BID-Ozo in green fluorescent channel; (n) Confocal imaging by DiD in red fluorescent channel; (o) Superposition of panels m and n; (p) Plots of two-channel fluorescence intensity in the underlined part in Fig o. (BID-Ozo: E_x = 488 nm, E_m = 545-650 nm; Mito-Tracker: E_x = 561 nm, E_m = 615-660 nm; Scale bar: 10 μm).

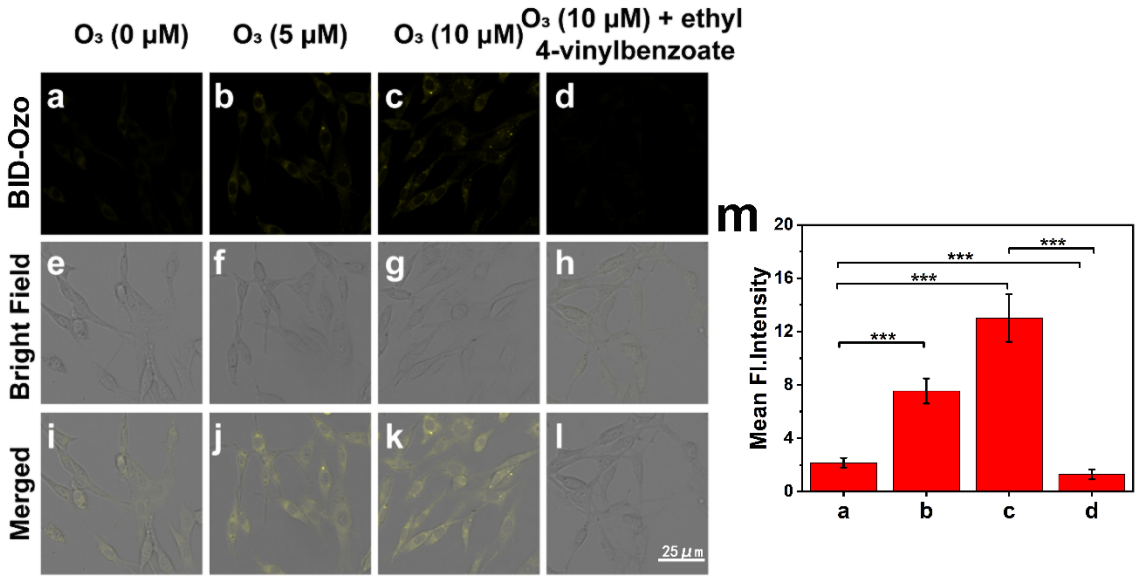


Fig. S20 Confocal imaging of exogenous O_3 in PC12 cells. (a-l) Confocal imaging of PC12 cells treated with different [O_3] (a, 0 μM, b, 5 μM, c, 10 μM), and (d) both O_3 (10 μM) and 4-vinyl ethyl benzoate (50 μM) followed by addition of BID-Ozo (10 μM); (m) Fluorescence intensity quantification in panels a-d. (E_x = 514 nm, E_m = 545-650 nm; The values are the mean ± s.d. for n =3, *p < 0.05, **p < 0.01, ***p < 0.001; scale bar: 25 μm).

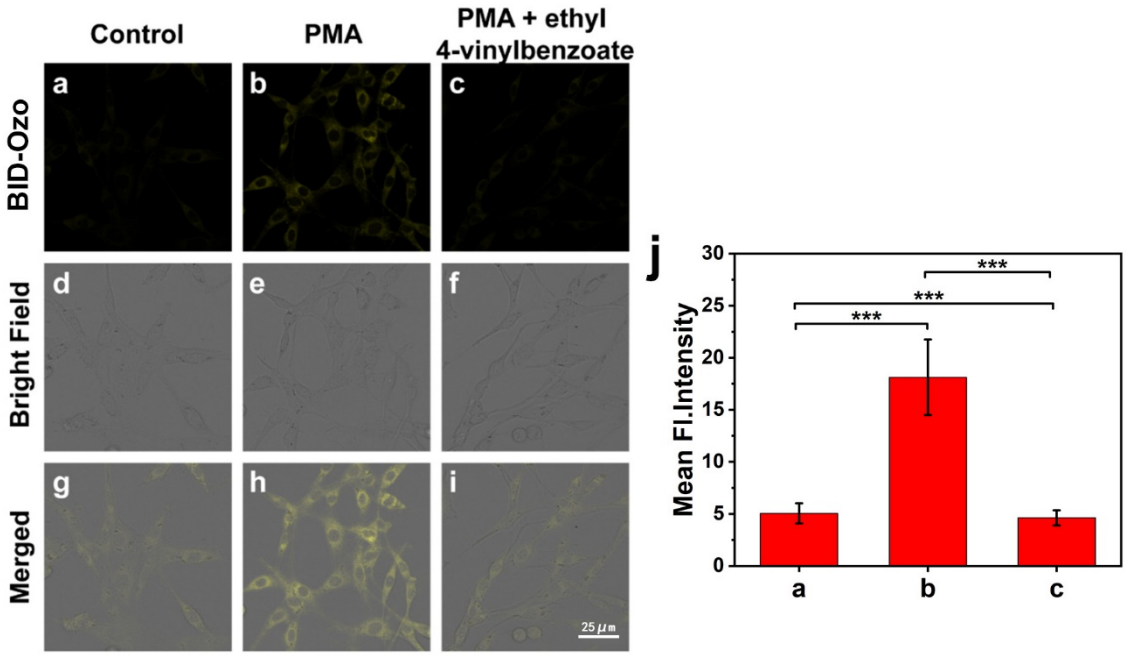


Fig. S21 Confocal imaging of endogenous O_3 in PC12 cells. (a-i) Confocal imaging of PC12 cells treated with different treatments (control, PMA (2.0 μg/ml), PMA (2.0 μg/ml0 + ethyl vinylbenzoate (50 μM)) and incubated with BID-Ozo (10 μM); (j) Quantification of fluorescence intensity in panels a-c. (E_x = 514 nm, E_m = 545-650 nm; The values are the mean ± s.d. for n =3, *p < 0.05, **p < 0.01, ***p < 0.001; scale bar: 25 μm).

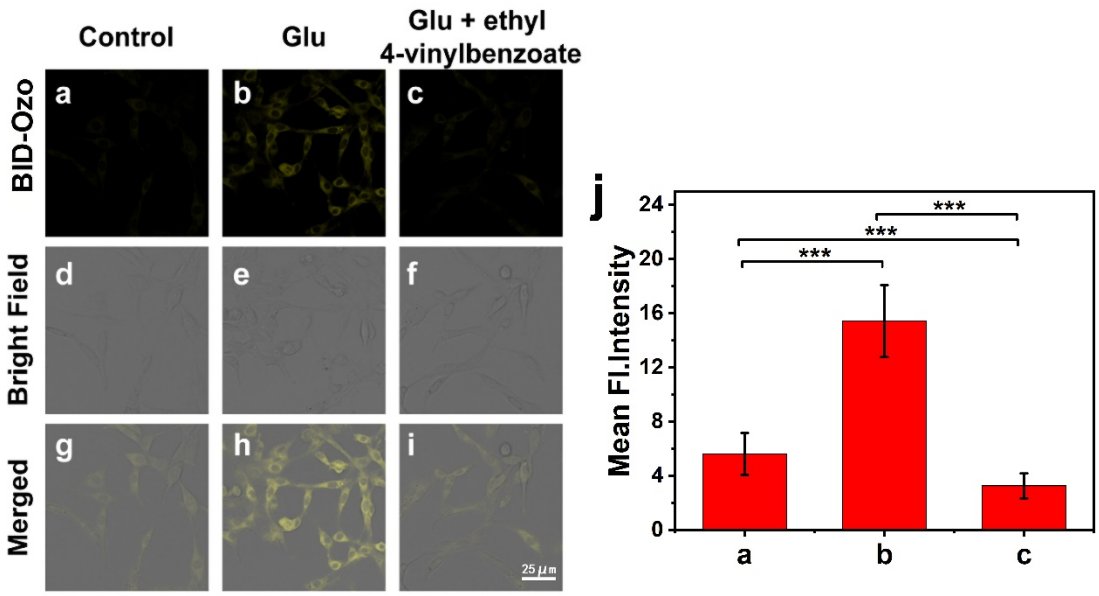


Fig. S22 Fluorescence imaging of PC12 cells under Glu-treated oxidative stress. (a-i) Confocal imaging images of PC12 cells treated with different treatments (control, Glu (10 mM), Glu (10 mM) + ethyl vinyl benzoate (50 μM)) and incubated with BID-Ozo (10 μM); (j) Quantification of fluorescence intensity of the cells shown in panels a-c. (E_x = 514 nm, E_m = 545-650 nm; The values are the mean ± s.d. for n =3, *p < 0.05, **p < 0.01, ***p < 0.001; scale bar: 25 μm).

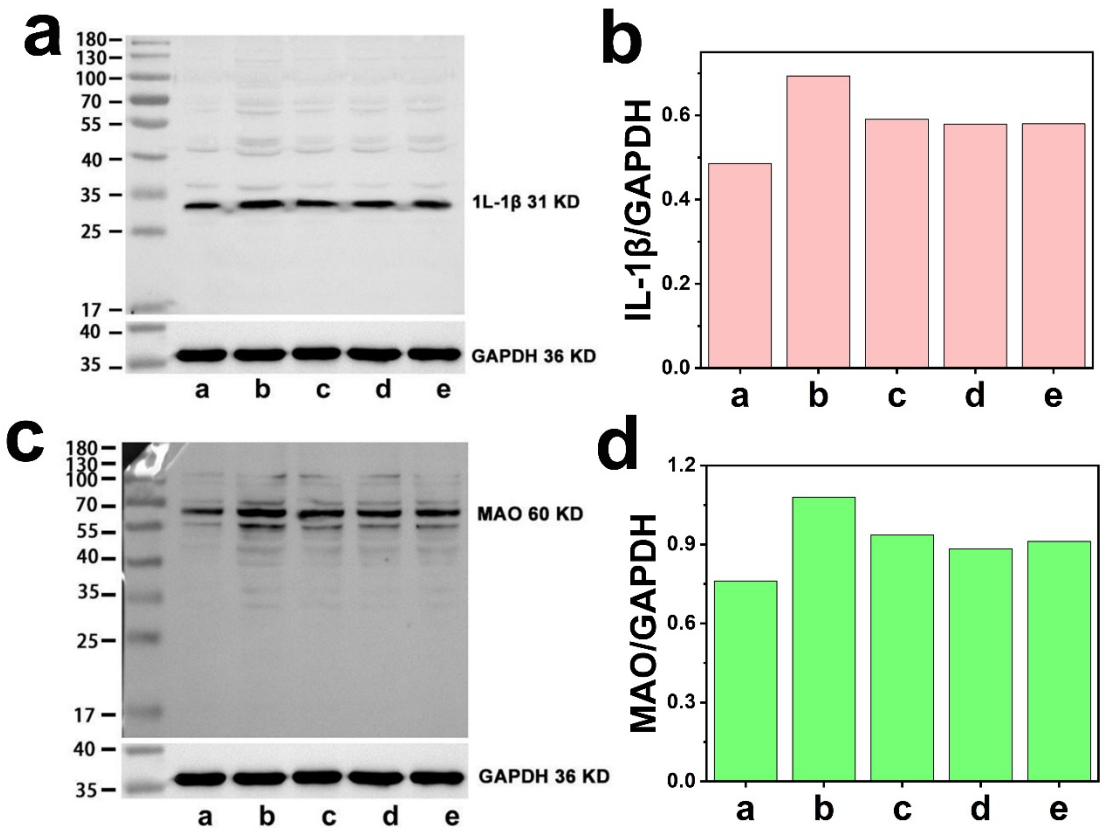


Fig. S23 Western Blot assay of IL-1β (a) and MAO (c) in PC12 cells under different treatments. Quantitative data from Western Blot assays for IL-1β (b) and MAO (d.)

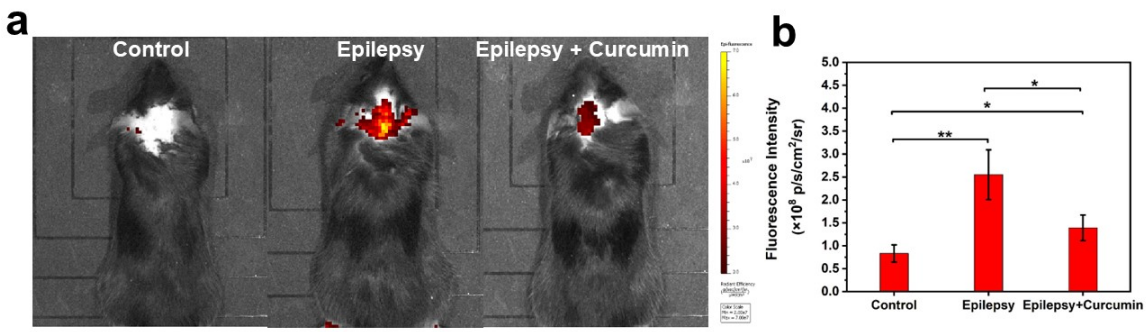


Fig. S24 Fluorescence images of three groups of mice at 60 min after injection with BID-Ozo (0.5mg/Kg). (a) Fluorescence images of mice with different treatment (control, epilepsy, epilepsy + curcumin) (b) Quantification of fluorescence intensity of three groups of mice. (Ex = 520 nm, Em = 550-600 nm; The values are the mean ± s.d. for n =3, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$;)

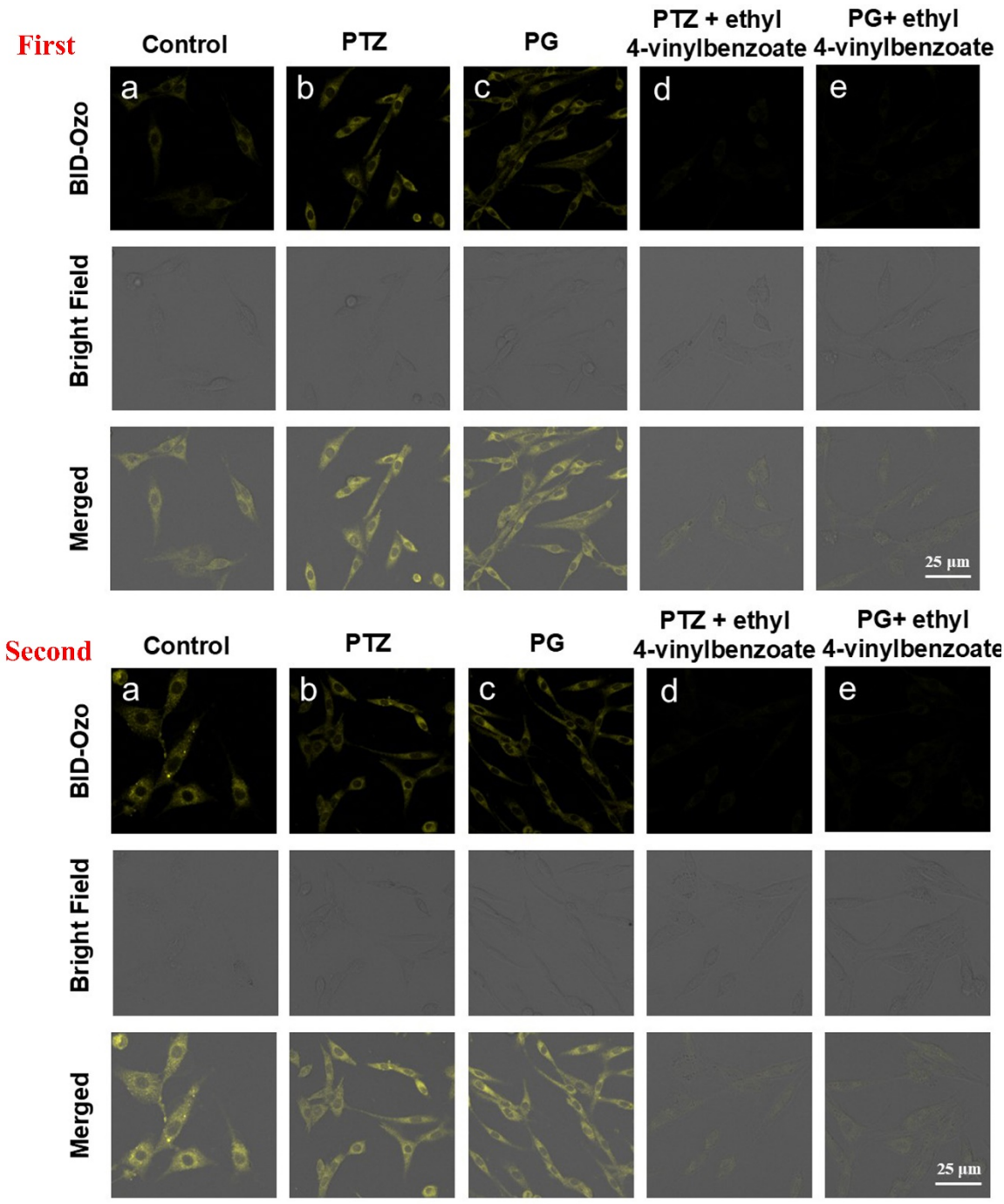


Fig. S25 Another two parallel sets of data in Fig.2.

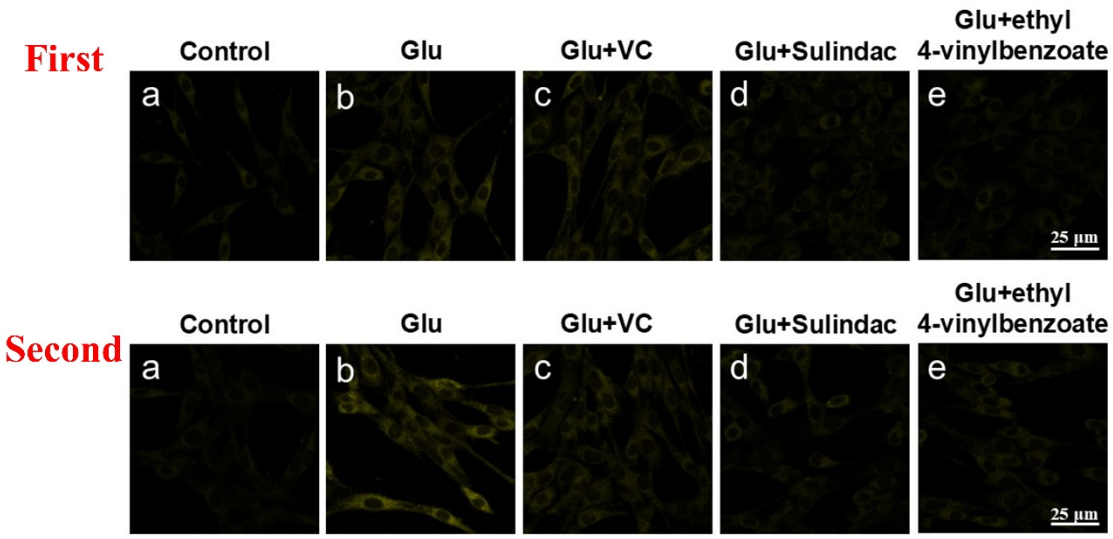


Fig. S26 Another two parallel sets of data in Fig.3.

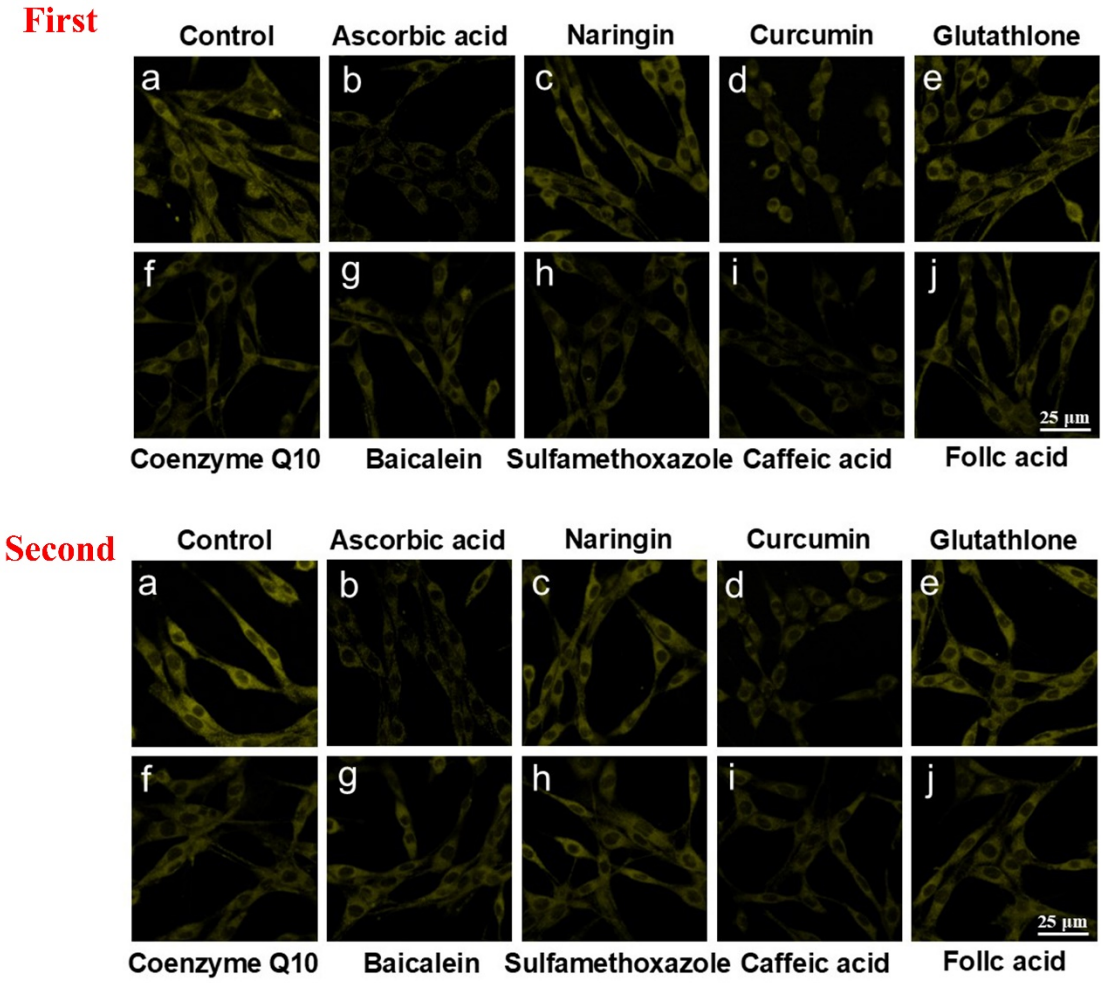
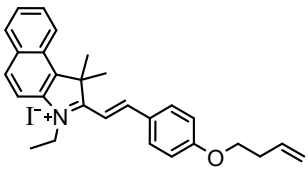
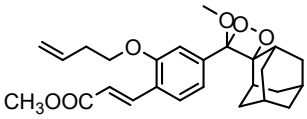
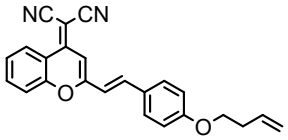
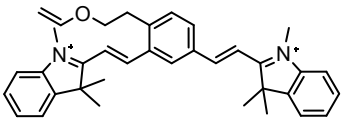
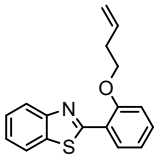
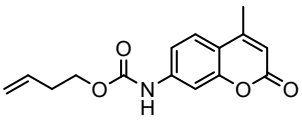
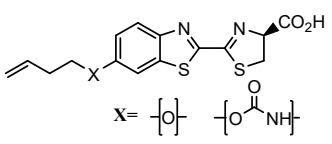
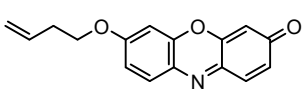
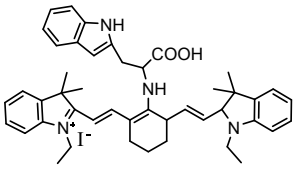
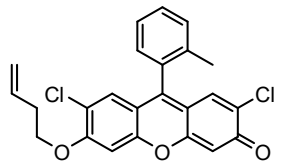


Fig. S27 Another two parallel sets of data in Fig.4.

Table S1 Currently reported O_3 probes.

Name of probe	Probe Structure	Type of probe	Excitation /emission (nm)	Detection limit (nM)	Applications	Reference
BID-Ozo		Fluorescent probe	520/573	12.2	Exogenous and endogenous O_3 in cell and epilepsy mice	This work
CL-O_3		Chemiluminescent probe	None/550	8.5	Selective detection of O_3 in acute inflamed mice	Chem. Commun., **2022** *58*, 4184-4187
DCM-O_3		Fluorescent probe	560 /680	620	Exogenous O_3 in cell	Spectrochim. Acta. A Mol. Biomol. Spectrosc., **2021** *248*, 119192
Acy7		Fluorescent probe	570 /690	10	Exogenous and endogenous O_3 in mice with depression phenotypes	Chem. Sci., **2019** *10*, 2805
HBT-OZO		Fluorescent probe	385/460	34	Paper-based sensor for visualize O_3	Sensor Actuat BChem., **2018** *266*, 717
Cou-Bu		Fluorescent probe	325/395	4.7	Exogenous O_3 in cell and identify the relative ozone index based on colors.	Sensor. Actuat. B-Chem., **2018** *258*, 501-507
		Bioluminescence probe	None/560 or 580	0.01 or 0.11	Environmental sample	Chem. Commun., **2016** *52*, 1128-1130

		Fluorescent probe	550 /580	5.9	Exogenous O_3 in cell	Sci Rep., **2013** *3*, 2830
Trp-Cy		Fluorescent probe	630 /770	17	Exogenous and endogenous O_3 in cell	Chem. Commun., **2012** *48*, 684-686
		Fluorescent probe	497 /523	50	Environmental sample and exogenous O_3 in cell	Nat chem., **2009** *1*, 316