Electronic Supplementary Material (ESI) for Chemical Science.

In Situ Visualization of Ozone in the Brains of Mice with Depression Phenotypes by a New Near-Infrared Fluorescence Probe

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Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Other sources of medicine were as follows. 4-Hydroxyisophthalaldehyde, 4-Bromo-1-butene, 1,2,3,3-Tetramethyl-3H-indol-1-ium iodide, 2,3,3-Trimethylindolenine and 1,3-propanesulfonate were purchased from Shanghai Civi Chemical Technology Co., Ltd. Ozone was produced by an ozone generator (BEYOK ozone, Zhejiang, China) and its solution was prepared in the phosphate buffer of pH 7.0. The concentration of O_3 in the solution was determined by UV absorption spectrometry (λmax = 258 nm; ε = 2900 L mol^{-1} cm^{-1}) and iodometric titration.[1-3] Phorbol 12-myristate 13-acetate (PMA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide(MTT), lipopolysaccharide (LPS) and glutathione (GSH) were supplied by Sigma-Aldrich (Beijing, China). Ethyl 4-vinylbenzoate was from Nanjing Jingrui Jiu'an Biotechnology Co., Ltd. The probe (ACy7) was dissolved in dimethyl sulfoxide (DMSO) to produce 1 mM stock solutions. All the cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The C57 mice were purchased from Shandong University Laboratory Animal Center. Mouse Interleukin-8 (IL-8) ELISA Kit was purchased from USA ImmunoClone Biosciences Co., Ltd (I&C). The preparation of reactive oxygen species (ROS) were as follows. Hydrogen peroxide (H_2O_2), sodium hypochlorite (NaClO) and tert-Butyl hydroperoxide (TBHP) were derived from solutions with a content of 30%, 10% and 70%, respectively. The superoxide anion radical ($O_2^{\cdot-}$) was prepared from potassium superoxide (KO_2) and dimethyl sulfoxide (DMSO). Singlet oxygen ($^1O_2$) was prepared from NaClO and H_2O_2 in amount of substance ratio of 1:1. The hydroxyl radical (·OH) was prepared from Fe(II)EDTA:H_2O_2 in amount of substance ratio of 1:6. Sodium nitroprusside was purchased from Shanghai Dingmiao Chemical Technology Co., Ltd.

NMR spectroscopic characterization was taken on a Bruker Advance 400 MHz spectrometer. HRMS spectra were obtained by a Bruker MaXis UHR-TOF instrument. Absorption spectra were recorded on a UV-Vis spectrophotometer (Evolution 220, Thermo Scientific), and fluorescence measurements were performed using a F-4600 fluorospectrometer (Japan, HITACHI). Fluorescence imaging in cells was carried out on a Leica TCS SP8 confocal laser scanning microscope. The animal behavioral tests were analysed by DepressionScan (Clever Sys. Inc.). The fluorescence imaging of mouse living was obtained using a Caliper IVIS Lumina Series III or IVIS Spectrum small animal *in vivo* imager.

Cells culture

RAW264.7 macrophages and PC12 cells were cultured in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 °C in a 5% CO_2 /95% air incubator MCO-15AC (SANYO, Tokyo, Japan). One day before imaging, the cells were detached and replanted on glass-bottomed dishes.

Construction of a mouse model of depression

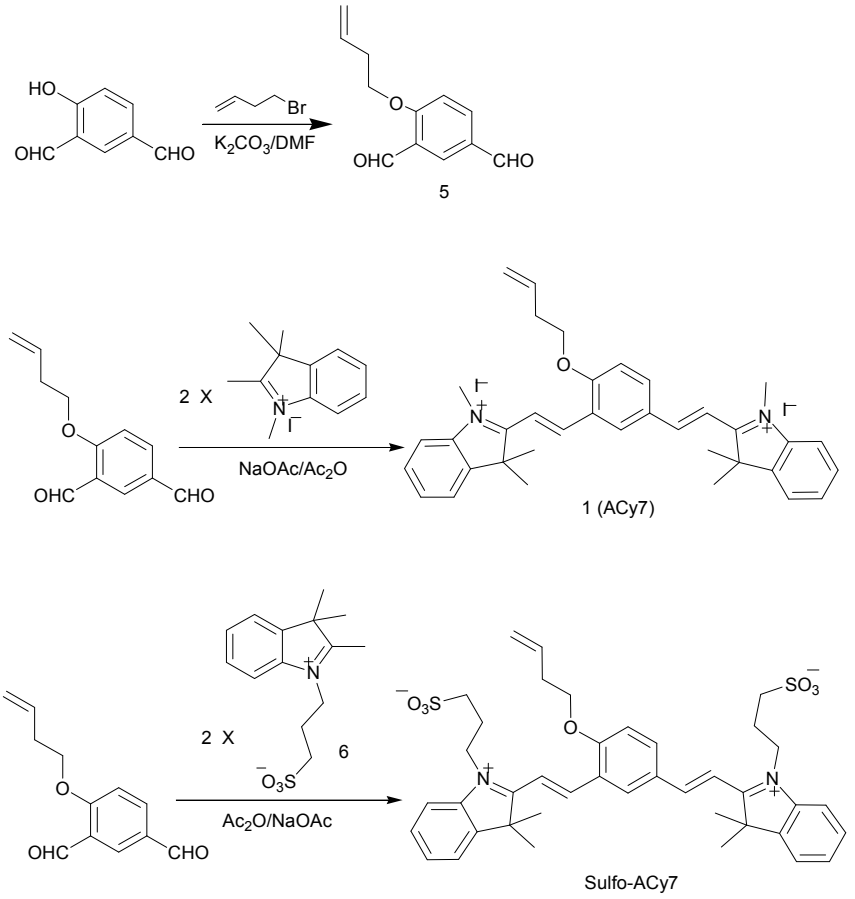
Male wild-type 8-week-old C57 mice were purchased from The Animal Living Center of Shandong University. Mice were housed in a standardized animal room (lights on 7:00 a.m.-7:00 p.m; room temperature 22 ± 2 °C), with food and water provided ad libitum. Prior to modeling, the purchased mice were kept in the rat room for one week to acclimate to the environment.

Chronic unpredictable mild stress protocol (CUMS)

The chronic unpredictable mild stress protocol (CUMS) used in the experiment was constructed as follows. In brief, it was a randomized 4-week schedule which contained various of stressors. Firstly, the mice were divided into a control group and a CUMS group, and the mice in the CUMS group were single-bred alone. Then, we performed unpredictable stressor on mice in the CUMS group. stressor stimuli include: water and food deprivation for 24 h, noise for 30 min, cage shaking for 5 min, 50 °C heat stress for 5 min, clip tail for 5 min, low temperature (0 °C) for 5 min, upside down the day and night, and wet bedding for 24 h. Give mice one stressor every day. Continue to stimulate for 4 weeks. In addition, no single stressor was conducted for two consecutive days. The modeling were evaluated by sucrose preference tests, tail suspension tests and forced swimming tests. The tail suspension tests and forced swimming tests were analysed by DepressionScan (Clever Sys. Inc.).

Acute inflammation‑induced mouse model of depression

Two batches of mouse models were established in parallel for brain fluorescence imaging and IL-8 detection in mice. The mice were randomly divided into three groups: a control group (Control; n=5), an acute inflammation‑induced depression group (LPS; n=5), and a LPS+Lim group (n=5). First, mice in the LPS+Lim group were given by continuously gavage for 7 days with limonene (a natural ozone scavenger) at a dose of 50 mg/kg. The other two groups were intragastrically administered with equal amounts of PBS (0.01 M). On the eighth day of the experiment, LPS (0.83 mg/kg) was administered by intraperitoneal injection to the LPS group and the LPS+Lim group to induce depression-like behaviors of the mice. The control group mice were intraperitoneally injected with an equal volume of PBS. After 24 hours of intraperitoneal injection, the behavioral analysis was completed within 4 h, including sucrose preference tests, tail suspension tests, forced swimming tests. Subsequently, fluorescence imaging of O_3 and detection of IL-8 in the mouse brain were performed immediately.



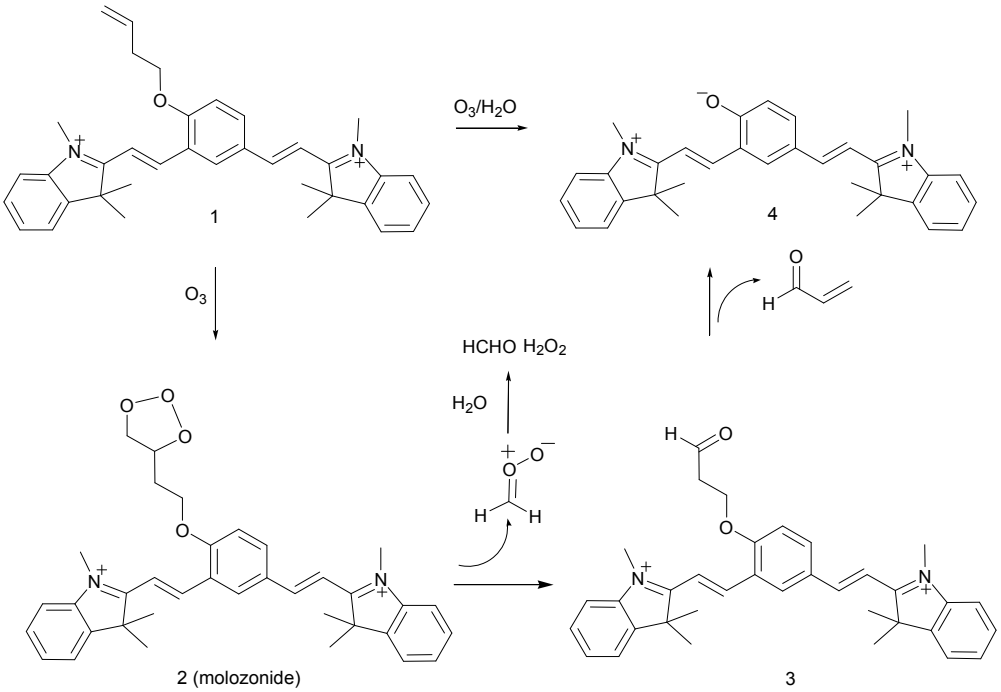
Scheme S1. Synthetic route of ACy7 and Sulfo-ACy7.

Compound 5:4-Bromo-1-butene (168 μL, 1.7 mmol) and K_2CO_3 (0.2630 g, 1.9 mmol) were added to a solution of 4-Hydroxyisophthalaldehyde (0.0720 g, 0.47 mmol) in DMF (2 mL), and the resulting mixture was heated at 80 °C under Ar atmosphere for 36 h. After completion, the reaction flask was neutralized by the addition of saturated NH_4Cl, and CH_2Cl_2 was added to dissolve the solid. The reaction mixture was then poured to saturated NaCl solution and extracted with CH_2Cl_2. The CH_2Cl_2 layer was evaporated to give a crude product as light yellow solid (0.0701 g, 73% yield). ^{1}H NMR (400 MHz, $CDCl_3$) 10.49(s, 1H), 9.94(s, 1H), 8.33(s, 1H), 8.11(d, *J*=8.7 HZ, 1H), 7.13(d, *J*=8.7 HZ, 1H), 5.85-5.95(m, 1H), 5.15-5.24(m, 2H), 4.25(t, *J*=6.4 HZ, 2H), 2.63-2.68(m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) 190.40, 188.85, 165.51, 135.80, 133.54, 132.09, 129.87, 125.18, 118.35, 113.34, 68.60, 33.56. HRMS (ESI) date, m/z calcd for $C_{12}H_{12}O_3$ $[M-H]^-$: 203.0703, found 203.0746.

ACy7: A mixture of Compound 5 (0.0674g, 0.33 mmol), NaOAc (0.0839 g, 1.02 mmol), and commercially available 1,2,3,3-tetramethyl-3H-indolium iodide (0.2106 g, 0.70 mmol) were dissolved in 2 mL Ac_2O. The reaction mixture

was stirred for 30 min at 80 °C under an Ar atmosphere. After completion, the solvent was evaporated under reduced pressure, and the crude product was dissolved in CH_2Cl_2, filtered and concentrated to give a red solid. Then, purification was carried out by thin layer chromatography and column chromatography (eluent: CH_2Cl_2/CH_3OH=15:1) to obtain a relatively pure product ACy7 (0.1424 g, 56% yield). ^{1}H NMR (400 MHz, $CDCl_3$) 9.80(s, 1H), 9.15(d, *J*=8.9 HZ, 1H), 8.88(d, *J*=16.2 HZ, 1H), 8.68(d, *J*=16.4 HZ, 1H), 8.36(d, *J*=16.4 HZ, 1H), 8.15(d, *J*=15.8 HZ, 1H), 8.08(d, *J*=6.9 HZ, 1H), 7.59(t, *J*=4.7 HZ, 4H), 7.49-7.56(m, 6H), 5.88-5.99(m, 1H), 4.58(s, 3H), 4.52(s, 3H), 4.34(t, *J*=6.0 HZ, 2H), 2.68-2.72(m, 2H), 2.02(s, 6H), 1.83(s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) 182.46, 181.79, 161.70, 153.36, 147.23, 142.87, 142.20, 140.33, 138.70, 134.15, 133.09, 129.09, 128.99, 128.90, 128.87, 128.79, 128.72, 128.32, 127.31, 122.19, 121.80, 121.67, 116.88, 113.53, 113.13, 112.93, 67.43, 51.92, 51.35, 49.77, 36.78, 36.47, 32.52, 30.91, 30.89, 28.68. HRMS (ESI) date, m/z calcd for $C_{36}H_{40}N_2O^{2+}$ $[M]^{2+}$: 258.1565, found 258.1581.

Sulfo-ACy7: A mixture of Compound 5 (0.0674g, 0.33 mmol), NaOAc (0.0550 g, 0.67 mmol), and Compound 6[4] (0.1885 g, 0.67 mmol) were dissolved in 2 mL Ac_2O. The reaction mixture was stirred for 30 min at 80 °C under an Ar atmosphere. After completion, the solvent was evaporated under reduced pressure, and the crude product was dissolved in CH_2Cl_2, filtered and concentrated to give an orange solid. Then, purification was carried out by thin layer chromatography and column chromatography (eluent: CH_2Cl_2/CH_3OH=5:1) to obtain a relatively pure product Sulfo-ACy7 (0.1568 g, 65% yield). ^{1}H NMR (400 MHz, DMSO-d6) 9.25(s, 1H), 8.65(d, *J*=8.6 HZ, 1H), 8.52-8.59(m, 2H), 8.19(d, *J*=16.5 HZ,1H), 8.00-8.10(m, 3H), 7.85-7.92(m, 2H), 7.59-7.68(m, 4H), 7.45(d, *J*=9.0 HZ, 1H), 5.94-6.04(m,1H), 5.18-5.32(m, 2H), 4.93-5.02(m, 4H), 4.42(t, *J*=5.8 HZ, 2H), 2.64-2.76(m, 6H), 2.20-2.31(m, 4H), 1.85(s, 6H), 1.79(s, 6H). ^{13}C NMR (101 MHz, DMSO-d6) 182.44, 182.14, 162.27, 153.54, 146.28, 144.43, 144.28, 141.37, 141.28, 138.00, 135.47, 133.14, 130.09, 129.74, 129.72, 129.53, 128.51, 123.94, 123.60, 123.50, 117.99, 115.84, 115.55, 114.58, 114.48, 112.40, 68.65, 52.71, 52.57, 47.57, 46.20, 45.92, 43.80, 33.59, 29.50, 29.30, 29.18, 29.04, 26.66, 26.37. HRMS (ESI) date, m/z calcd for $C_{40}H_{46}N_2O_7S_2$ $[M-H]^-$: 729.2663, found 729.2535.



Scheme S2. Reaction mechanism of ACy7 with O_3.

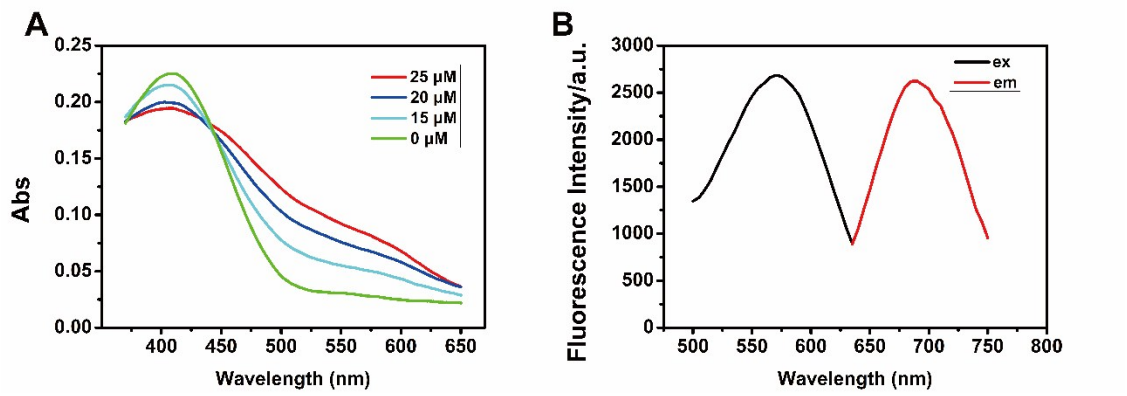


Fig. S1 The spectral properties of the probe ACy7. (A) Absorption of ACy7 before and after reaction with different concentrations of O_3. (B) Excitation and emission spectra of ACy7 after reaction with 26 μM of O_3. (ACy7 (10 μM), PBS (pH=7.4, 20 mM), incubation at 37 °C for 40 min.)

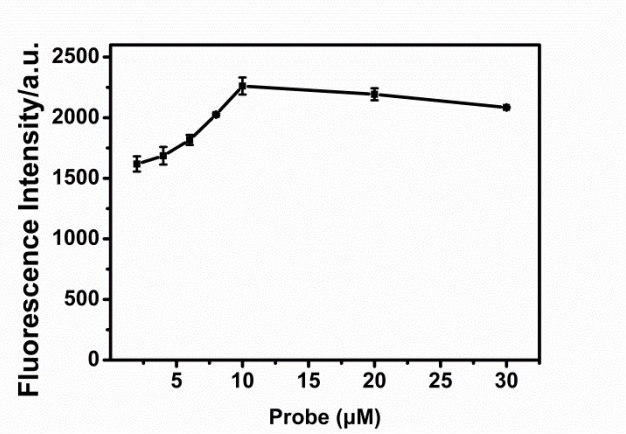


Fig. S2 Effects of probe ACy7 concentration on the fluorescence of ACy7 reacting with O_3. (O_3 (26 μM), PBS (pH=7.4, 20 mM), incubation at 37 °C for 40 min, $\lambda_{ex}/\lambda_{em}$ =570/690 nm.)

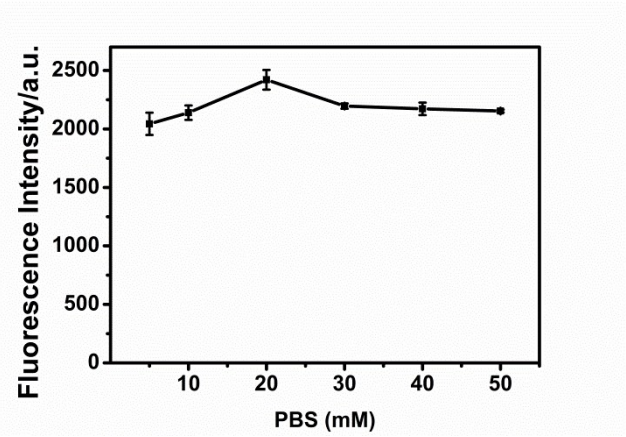


Fig. S3 Effects of the phosphate buffer concentration on the fluorescence of ACy7 reacting with O_3. (ACy7 (10 μM), O_3 (26 μM), PBS (pH=7.4), incubation at 37 °C for 40 min，$\lambda_{ex}/\lambda_{em}$ = 570/690 nm)

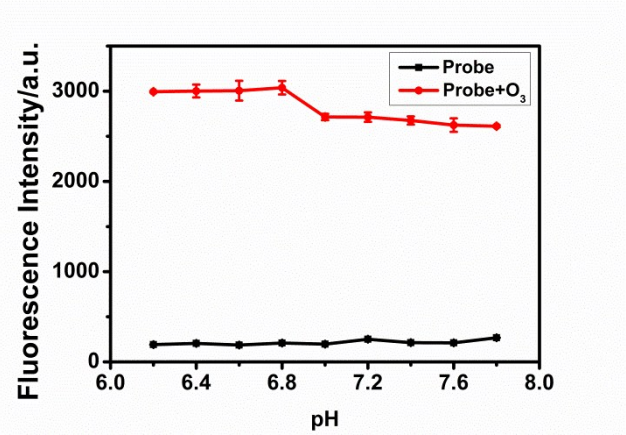


Fig. S4 Effects of the pH on the fluorescence of ACy7 reacting with O_3. (ACy7 (10 μM), O_3 (26 μM), PBS (20 mM), incubation at 37 °C for 40 min，$\lambda_{ex}/\lambda_{em}$ = 570/690 nm)

As can be seen from the figure, ACy7 has a strong fluorescence emission intensity under slightly acidic conditions. Because the ozone decomposition in aqueous media can be catalyzed by hydroxide ions and the acidic environment enhances ozonolysis of probe ACy7 in the first step of Scheme S2.[5] The pH value was 7.2-7.4 under physiological conditions, so we chose pH=7.4 for subsequent studies.

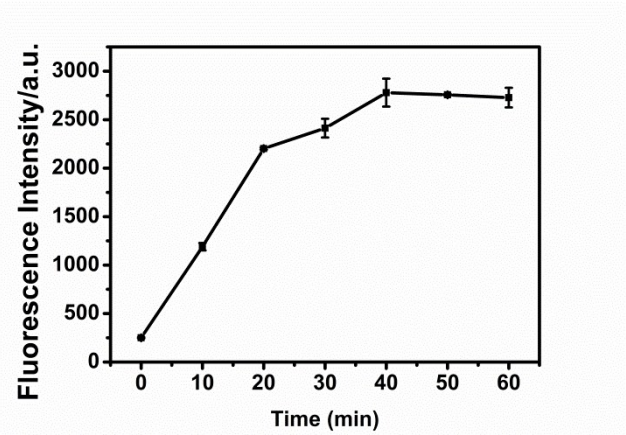


Fig. S5 Effects of the reaction time on the fluorescence of ACy7 reacting with O_3. (ACy7 (10 μM), O_3 (26 μM), PBS (pH=7.4, 20 mM), incubation at 37 °C for 40 min，$\lambda_{ex}/\lambda_{em}$ = 570/690 nm)

As can be seen from Fig.S5 above, it takes 40 min for the probe ACy7 to complete the reaction with O_3. It is speculated that β-elimination is a rate determining step because the formation and cleavage of molecular ozonide is very rapid.[6] Surprisingly, we found that ACy7 could react with O_3 with no more than 15 min in cells (date not shown). Although the exact mechanism of this enhancement is not known, catalysis of β-elimination by action of bovine serum albumin has been previously reported.[7, 8]

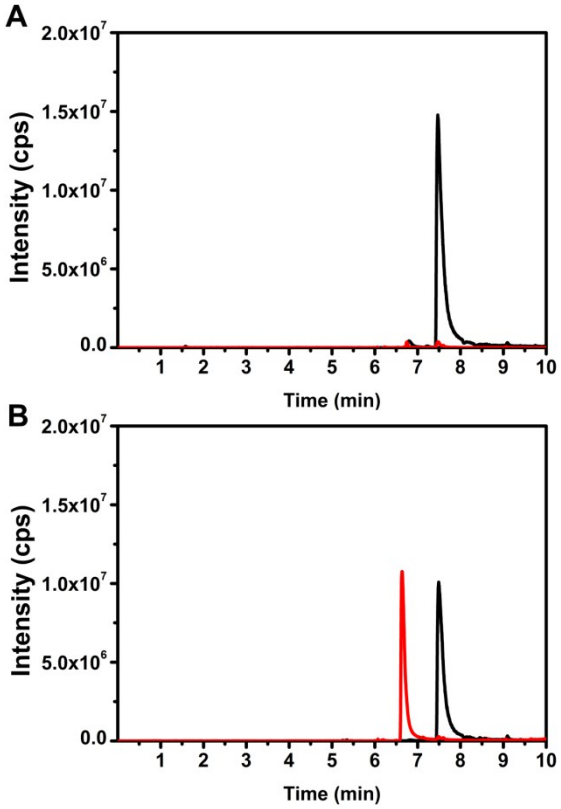


Fig. S6 HPLC-MS chromatograms of 25 μM ACy7 solution (A) and 25 μM ACy7 solution after reaction with O_3 (B). HPLC-MS analyses were performed on a C18 column (Thermo Fisher, 100 mm length × 2.1 mm i.d., 2.6 μm) using an Ultimate 3000 HPLC system (Dionex, Sunnyvale, USA) combined with AB QTRAP 5500 MS system using the characteristic ions m/z 258 for ACy7 (black line) and m/z 461 for QCy7 (red line). Acetonitrile and water containing 0.1% formic acid were used as mobile phases at the flow rate of 0.4 mL/min. The gradient condition was as follows: 0-2 min, 10% acetonitrile; 2-10 min, 10%-66% acetonitrile. The retention time for ACy7 and QCy7 was 7.49 min and 6.65 min, respectively.

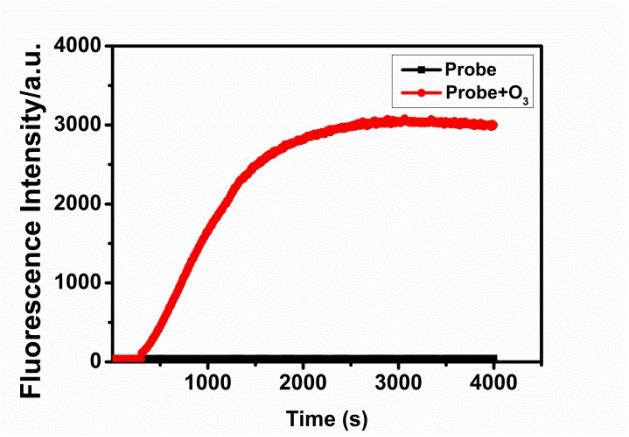


Fig. S7 The reaction kinetics and photostability experiments of 10 μM ACy7 reacting with O_3 (0 μM and 26 μM). (pH=7.4, 20 mM; $\lambda_{ex}/\lambda_{em}$ = 570/690 nm)

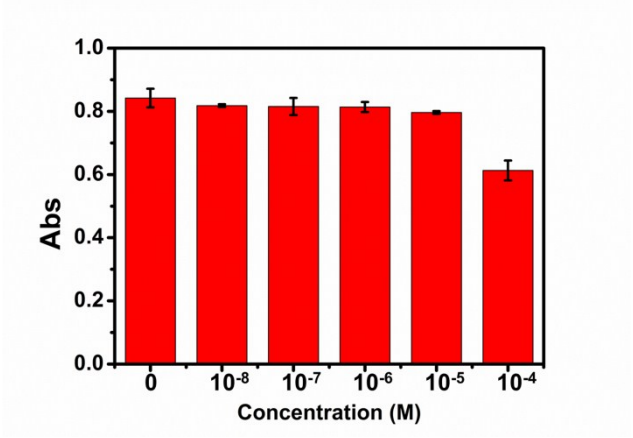


Fig. S8 MTT assay of PC12 cells in the presence of different concentrations of ACy7.

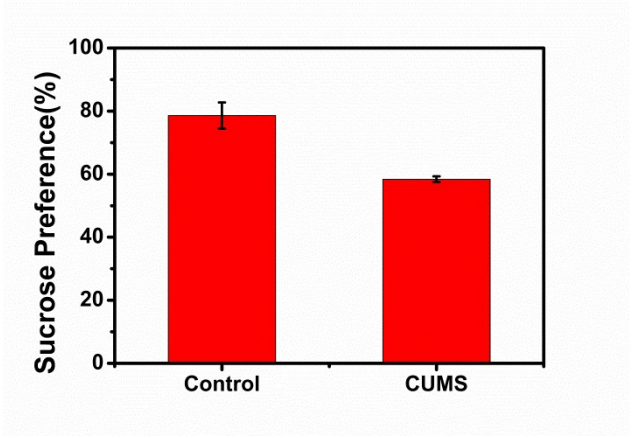


Fig. S9 The sucrose preference test (SPT) performed in the control and CUMS group. (n =3 mice/group)

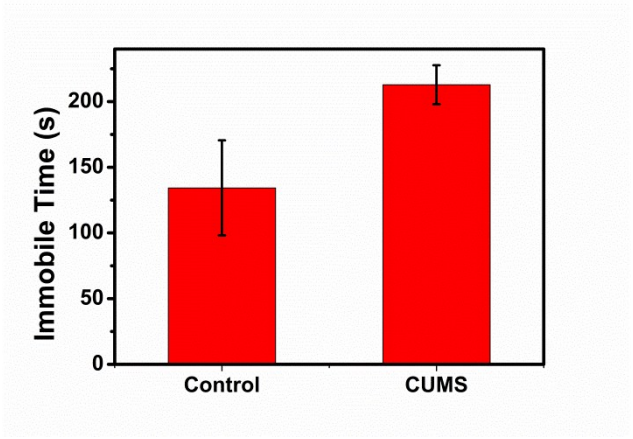


Fig. S10 Immobility times in the tail suspension test (TST) between the control and CUMS group. (n =3 mice/group)

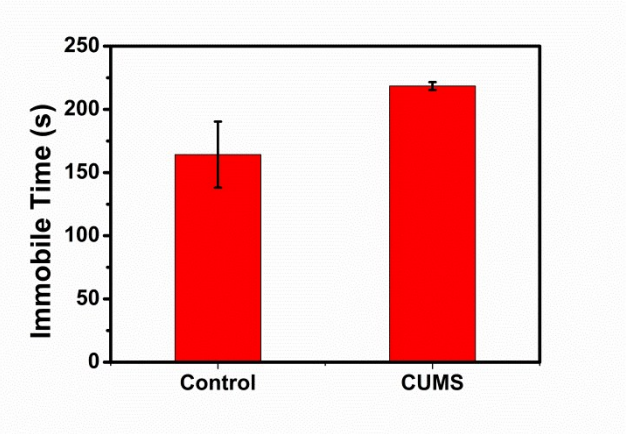


Fig. S11 Immobility times in the forced swimming test (FST) between the control and CUMS group. (n=3 mice/group)

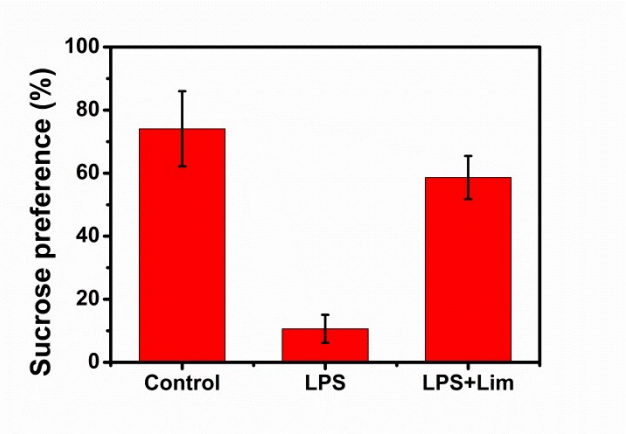


Fig. S12 The sucrose preference test (SPT) performed in the control, LPS and LPS+Lim group. (n = 5 mice/group)

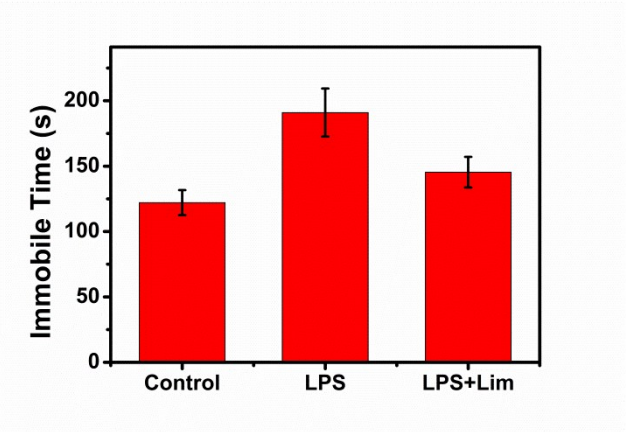


Fig. S13 The tail suspension test (TST) performed in the control, LPS and LPS+Lim group. (n = 5 mice/group)

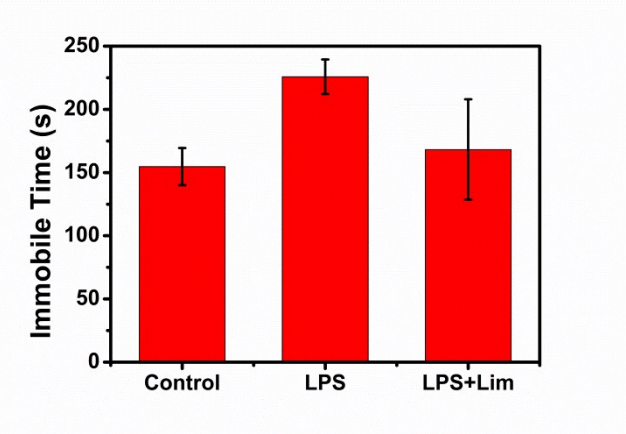


Fig. S14 The forced-swim test (FST) performed in the control, LPS and LPS+Lim group. (n = 5 mice/group)

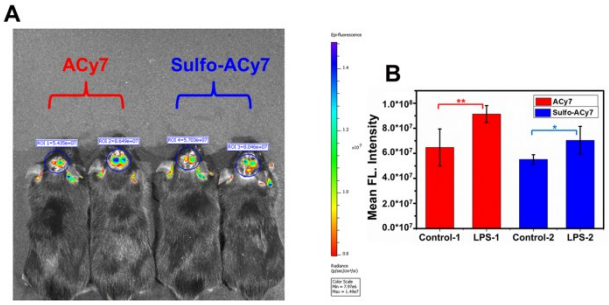


Fig. S15 Visual imaging of O_3 in the brains of mice. (A) Fluorescence imaging (pseudocolor) of 0.15mg/kg ACy7 or Sulfo-ACy7 in control mice (left) and mice with LPS-induced depression phenotypes (right) using IVIS Spectrum. (B) The output of the fluorescence intensity in image (A). The excitation wavelength filter is 570 nm, and the emission wavelength filter is 700 nm. The values are the mean ± s.d. for n =5, *p <0.05 **p <0.01.

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