Supporting Information

A Versatile Fluorescent Probe for Hydrogen Peroxide in Serotonergic Neurons of Living Mouse Brains with Depression

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Experimental Procedures

Instruments

^{1}H NMR spectra were obtained at 400 MHz using Bruker NMR spectrometers, and ^{13}C NMR spectra were recorded at 100 MHz. The mass spectra were obtained using the Bruker maXis ultra-high-resolution-TOF MS system. Absorption spectra were measured on an Evolution 220 UV-vis spectrophotometer (Thermo Scientific Co., Ltd.) All fluorescence measurements were carried out at room temperature on an FLS-980 Edinburgh fluorescence spectrometer. The co-localized images were recorded on a Leica TCS SP8 microscope with a 63× oil-immersion objective (N/A 1.3). The forced swimming tests and tail suspension tests were analysed by DepressionScan (Clever Sys. Inc.).

Apparatus and Reagents

All chemicals were purchased from Adamas Reagent, Ltd. (China) and Energy Chemical Ltd. (China), and analytical grade solvents were used without further purification. Dicyclohexylcarbodiimide (DCC), paroxetine hydrochloride hemihydrate, 4-bromomethylphenylboronic acid pinacol ester, caesium carbonate, 5(6)-carboxyfluorescein was from Shanghai Macklin Biochemical Co., Ltd. 4-Dimethylaminopyridine was from Shanghai Aladdin Biochemical Technology Co., Ltd. We prepared reactive oxygen species (ROS) as follows. H_2O_2 was diluted from a 30 % aqueous solution. Tertbutyl hydroperoxide (TBHP) was diluted from a 70 % aqueous solution. Hypochlorite (NaOCl) was diluted appropriately in 0.1 M NaOH aq. Superoxide ($O_2^{\bullet-}$) was generated from KO_2 in DMSO solution, and the concentration of $O_2^{\bullet-}$ was determined by the concentration of KO_2. Singlet oxygen ($^1O_2$) was prepared using the ClO^-/H_2O_2 system (1:1), and peroxynitrite was prepared from a stock solution of 10 mM in 0.3 M NaOH. Lipid peroxyl radicals were generated via thermolysis of the azo-initiators 2,2'-azobis(2,4-dimethyl) valeronitrile (AMVN) and 2,2'-azobis(2-methylpropionitrile) (ABIN) in acetonitrile solution at 37 °C for 30 min. Hydroxyl radical (•OH) was produced by the reaction of Fe^{2+} with H_2O_2 (1:6).

Cell culture

HEK293 cells were purchased from Wuhan Procell Life Science&Technology Co., Ltd. HEK293 cells were cultured in DMEM medium supplemented with 10 % fetal bovine serum, 1 % penicillin, and 1% streptomycin at 37 ℃ (w/v) in a 5 % CO_2 / 95 % air MCO-15AC incubator (SANYO, Tokyo, Japan). One day before imaging, the cells were detached and placed in glass-bottomed dishes.

Neurons were isolated from embryonic day 17 mice and plated onto poly-l-lysine-coated petri dishes. The neurons were grown in neuronal media (Neurobasal, 2% B27, 0.5 mM glutamine) and 1% streptomycin at 37 ℃ (w/v) in a 5 % CO_2／95 % air MCO-15AC incubator (SANYO, Tokyo, Japan) for 7 days.

Cytotoxicity assays

The cytotoxicity was measured by Cell Counting Kit-8 (CCK8) assay. HEK293 cells were seeded in a 96-well plate at a concentration of 2000 cells $well^{-1}$ in 100 μL of DMEM medium with 10 % fetal bovine serum, 1 % penicillin, and 1 % streptomycin and maintained at 37 ℃ in a 5 % CO_2 incubator for 12 h. Then, cells were exposed to different concentrations of probe (1, 10, 20, 200, 500 μM) for 24 h. The total volume of 96-well plates is 200 μL well-1. Configure 10 % CCK8 medium solution, add it in the form of liquid change, and cultivate for 4 h in the incubator.

Absorbance was measured at 450 nm in a Triturus microplate reader.

Cell transfection

hsert-pcDNA3 was purchased from AddGene and RFect Plasmid DNA Transfection Reagent was purchased from BIOG, Changzhou Bio-generating Biotechnology Corp. We used 12-well plates for transfection. According to the instructions, plates were spread 24 h before the start of transfection, and then incubated for 48 h before subsequent experiments were performed with 2.5 μL RFect, 1.5 μL plasmid (at a concentration of 1 μg/μL) and 1 mL complete medium (90 % DMEM, 10 % Fetal bovine serum) added to each well.

Mouse models with depression-like behaviours

Adult male C57BL/6J mice (age: 10 weeks; average body weight: 22 ± 2 g) were purchased from the Experimental Animal Center of Shandong University (Jinan, PR China) (approval number AEECSDNU2022099). All animal care and 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. The mice were housed in cages under a controlled 12-h/12-h light-dark cycle (lights on: 7:00 a.m.) and given free access to water and food. The mice were allowed to adapt to stable environmental conditions for 1 week and then randomly divided into 2 groups, control and stress. The stress models were established by feeding mice with water added corticosterone. The control group were given water without corticosterone. After 21 days, mice were tested based on the sucrose preference test, forced swimming test and tail suspension test to prove the stress model was established successfully. For all animal studies, the tester was blind to the group allocation.

Sucrose preference test

Anhedonia is one of the core symptoms of depression.[1] The sucrose preference test (SPT) is a reward-based test, used as in indicator of anhedonia.[2] Mice are born with an interest in sweet foods or solutions. Reduced preference for sucrose in SPT represents anhedonia.[3] Compared with the normal mice, the preference of mice with depression for sucrose water was significantly reduced, indicating the mice with depression were successfully developed.

Sucrose preference test was conducted using a two-bottle choice procedure before and after medication procedure. Before the sucrose preference test (SPT), mice were habituated to drink a 1 % sucrose solution for 24 h with two bottles. Then, the sucrose solution was replaced with water for an additional 24 h. At the start of the test, mice were given access to the two bottles, one filled with sucrose solution and the other with water. The position of the water and sucrose bottles (left or right) was switched every 30 min for 3 h. Then the mice were left undisturbed, and their overnight fluid consumption was measured at the next morning. The volume of sucrose or water of every bottle was recorded. The sucrose preference was defined as the ratio of the volume of sucrose to the total volume of sucrose and water consumed.

Forced swimming test

Forced swimming test (FST) as a 2 days program were carried out following reference.[4,5] In the FST, each mouse was placed in a cylindrical tank (24 cm height × 10 cm diameter) filled to 6 cm with water at a temperature of 24 ± 1 ℃. The mice could swim freely. On the first day, the mice represented an escape-like behaviours and found an immobility posture that they could maintain their head above water easily for conserving energy. After rested for 24 h, mice would stay

immobile quickly. The mice were subjected to 6 min of swimming, but only the last four minutes were considered in the analysis.

Tail suspension test

In the tail suspension test (TST), each mouse was suspended by the tail using adhesive scotch tape from a hook connected to a strain gauge that detected all the movements of the mouse and transmitted them to a central unit, which calculated the total duration of immobility during a 6 min test. However, only the last four minutes were considered in the analysis.[6]

Immunofluorescence assay

Treated cells were fixed for 2 h using methanol, closed for 24 h using PBST solution with 1 % BSA (PBS, 0.1 % Tween 20), incubated for 24 h with primary antibody and 1 h with secondary antibody, and finally incubated for 2 h with PF-H_2O_2 for imaging.

Fluorescence imaging experiments *in vivo*

In the in vivo imaging experiments, mice were first injected intraperitoneally with 0.5 mg/kg PF-H_2O_2. 1 h later, mice were anesthetized by intraperitoneal injection of 400 mg/kg of tribromoethanol. Then, mice brain hair removal followed by fluorescence imaging at 500 nm excitation.

Statistical analysis

All data are expressed as the mean ± SD. The data under each condition were accumulated from at least three independent experiments. For each experiment, unless otherwise noted, n represents the number of individual biological replicates. For each biological replicate and for all in vitro and ex vivo studies, $n \geq 3$. The statistical analyses were performed using Student's t-test. $P < 0.05$ was considered statistically significant.

Data availability

All relevant data that support the findings of this study are available from the corresponding author upon reasonable request.

Synthesis of PF-H_2O_2

5-carboxyfluorescein (0.37632 g, 1 mmol) was dissolved in 6 ml N,N dimethylformamide (DMF), DMAP (0.122 g, 1 mmol) and DCC (0.26 g, 1 mmol) for 30 min. Paroxetine (0.3748 g, 1 mmol) was then added and stirred at room temperature for 24 h. The compound PF (0.42 g, 60% yield) was purified by thin layer chromatography.

0.34357 g (0.5 mM) of PF was dissolved in 5 mL of DMF, 0.296996 g (1 mM) of 4-bromomethylphenylboronic acid pinacol ester, 0.65 g (2 mM) of cesium carbonate at 80 ℃ for 18 h, and purified by TLC to obtain PF-H_2O_2 (0.18 g, 0.18 g), Yield 32%).

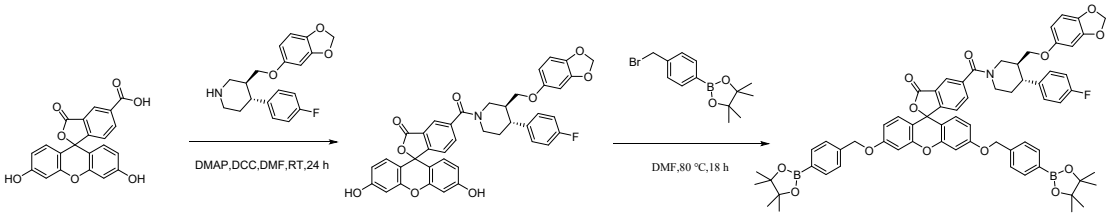

Fig. S1. Synthesis process of PF-H_2O_2.

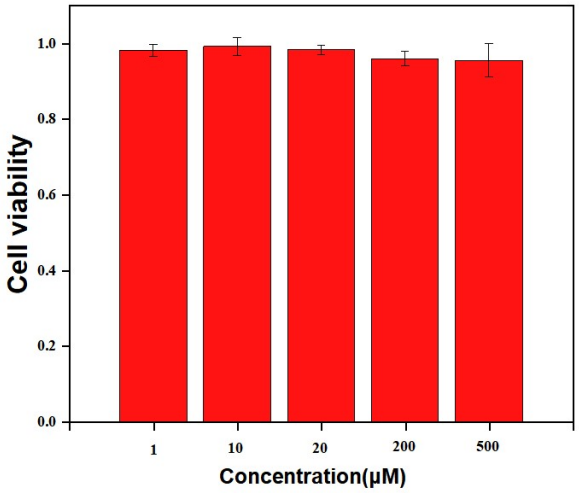

Fig. S2. CCK8 detection of PF-H_2O_2. For the CCK8 assay of HEK293 cells, the IC_{50} of PF-H_2O_2 was 2.913×10^7 μM.

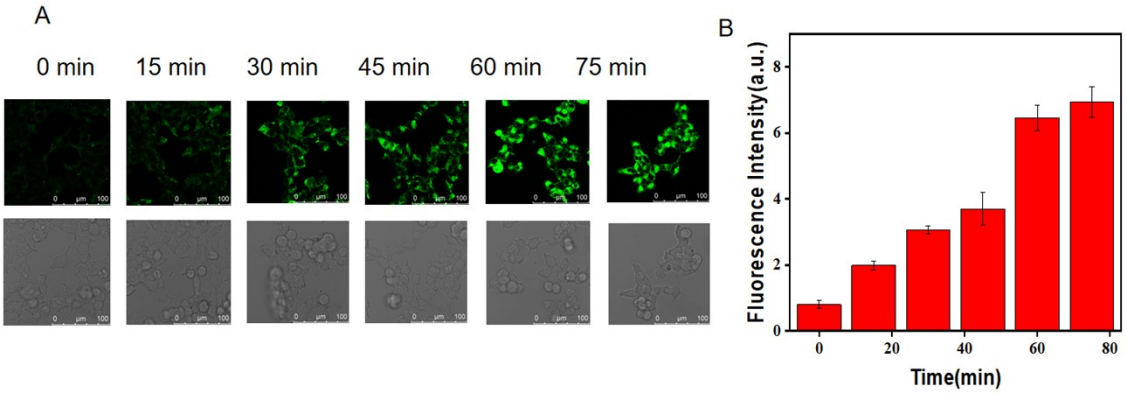

Fig. S3. Time-dependent fluorescence imaging of PF-H_2O_2 in hsert HEK293 cells. hsert-HEK293 cells were incubated with 10 mM glutamic acid for 12 h, then 25 μM PF-H_2O_2 was added, and the imaging was performed at an interval of 15 min. Images acquired by excitation at 480 nm, λex =480 nm. Fluorescence emission windows: λ_{em} = 510-540 nm. Scale bar = 100 μm. The data are expressed as the mean ± SD.

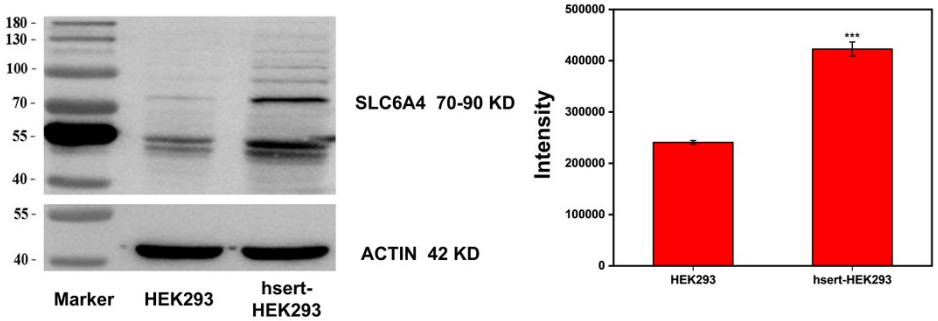

Fig. S4. Expression of SERT protein in transfected/untransfected cells (SERT-SLC6A4, the gatekeeping control protein-ACTIN).

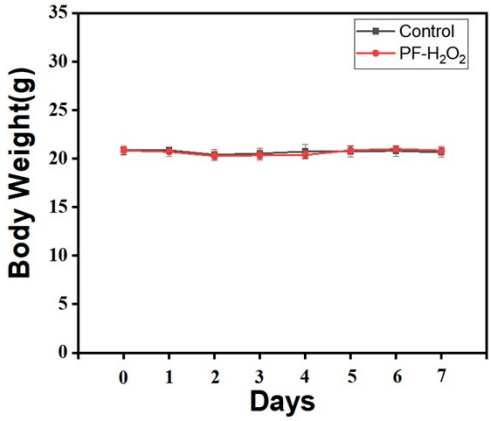

Fig. S5. In vivo toxicity test, the mice were intraperitoneally injected with 0.7 mg/kg PF-H_2O_2 for 7 consecutive days and weighed. It was found that the weight of the three groups of mice did not decrease significantly, which proved that the experimental concentration was less toxic.

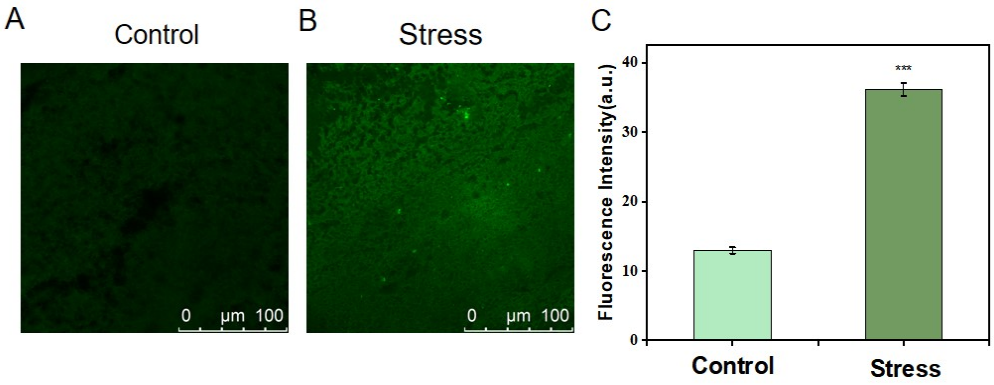

Fig. S6. Mouse brain tissue section imaging. First, mice were intraperitoneally injected with 0.5 mg/kg PF-H_2O_2. One hour later, the mice were anesthetized by intraperitoneal injection of 400 mg/kg tribromoethanol. After that, the decapitated brains of the mice were removed and cut into 50 μM using frozen slices. Finally, the observations were made by confocal microscope. Images acquired by excitation at 480 nm, λex =480 nm. Fluorescence emission windows: λem = 510−540 nm. Scale bar = 100 μm. The data are expressed as the mean ± SD. ***P <0.001 is compared with control group.

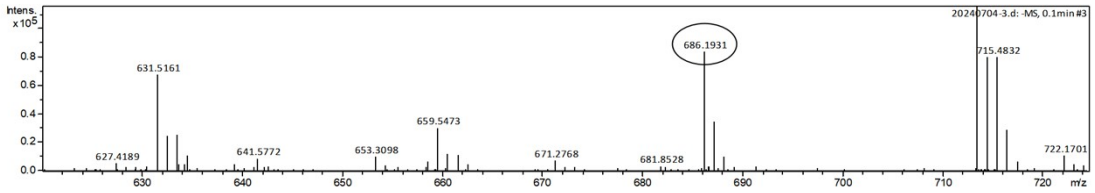

Fig. S7. HRMS of PF

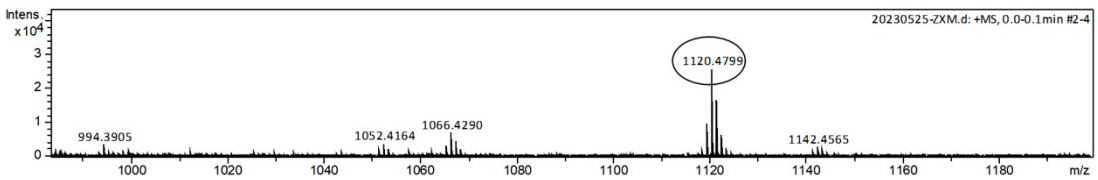

Fig. S8. HRMS of PF-H_2O_2

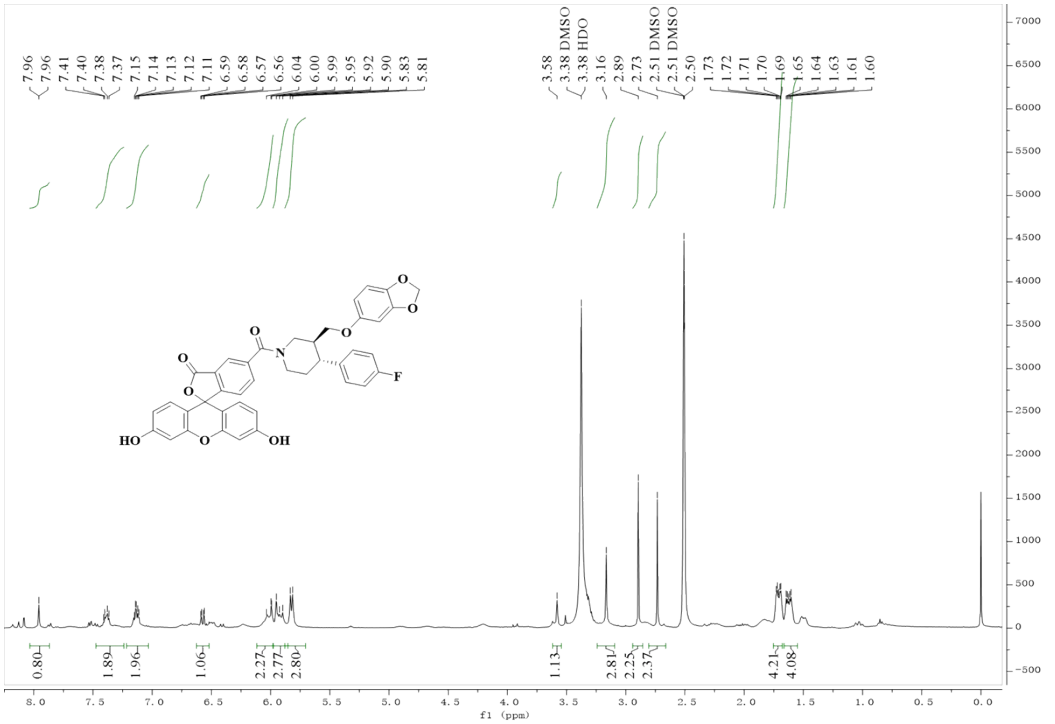

Fig. S9. ^{1}H NMR of PF

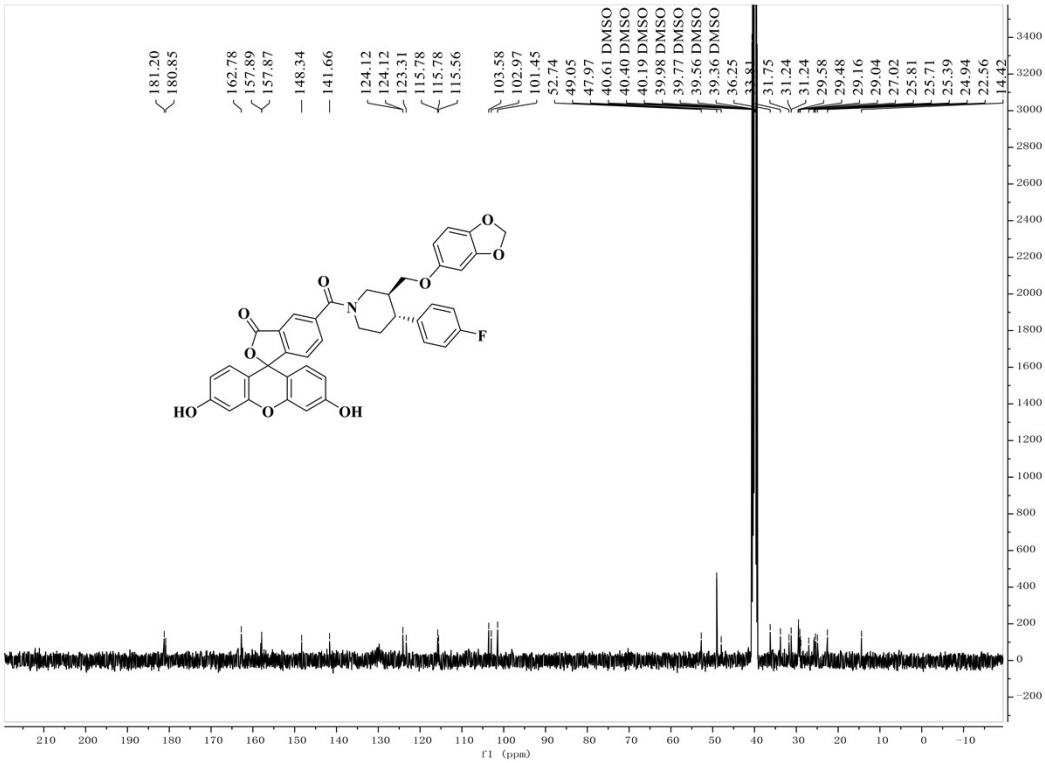

Fig. S10. ^{13}C NMR of PF

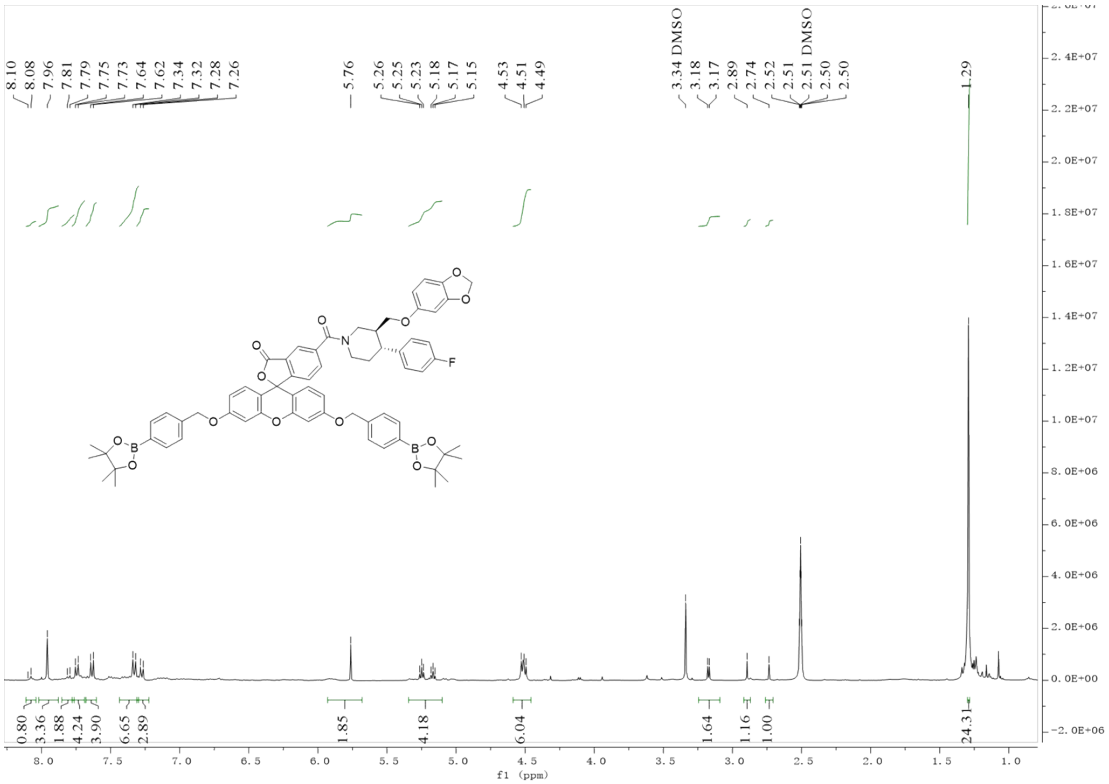

Fig. S11. ^{1}H NMR of PF-H_2O_2

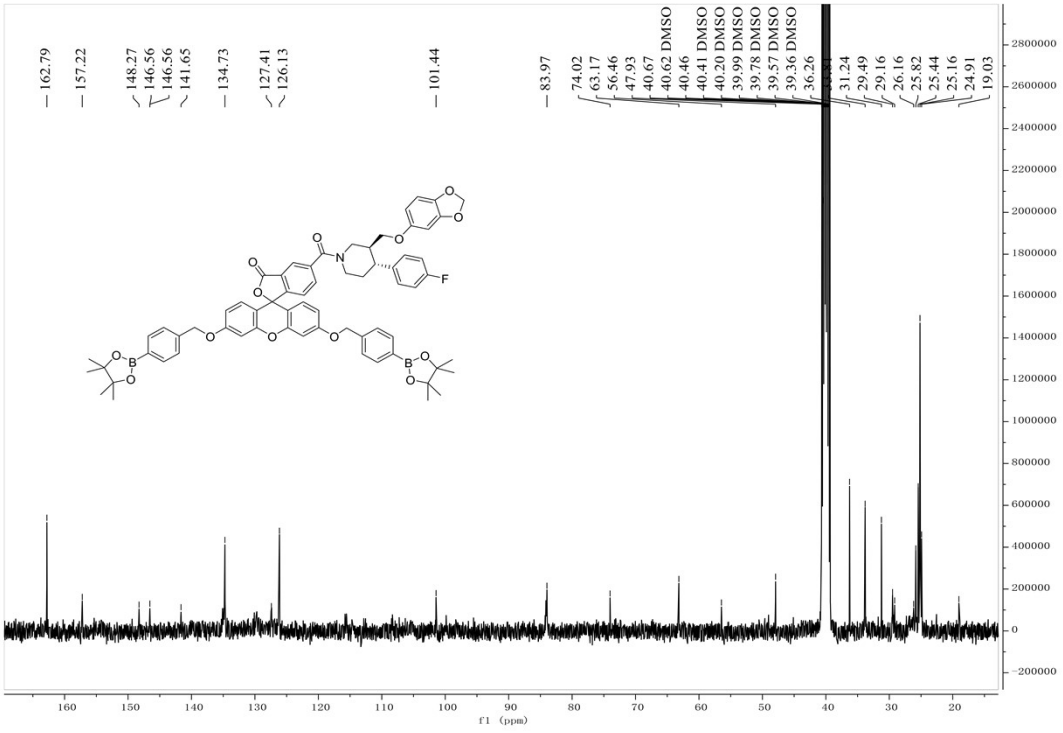

Fig. S12. ^{13}C NMR of PF-H_2O_2

REFERENCES

[1] M.-Y. Liu, C.-Y. Yin, L.-J. Zhu, X.-H. Zhu, C. Xu, C.-X. Luo, H. Chen, D.-Y. Zhu, Q.-G. Zhou, Nature Protocols 13 (2018) 1686-1698.

[2] M. Gross, A. Pinhasov, Behavioural Brain Research 298 (2016) 25-34.

[3] S. Madiha, S. Haider, Metabolic Brain Disease 34 (2019) 297-308.

[4] B. Petit-Demouliere, F. Chenu, M. Bourin, Psychopharmacology 177 (2005) 245-255.

[5] M.J. Detke, J. Johnson, I. Lucki, American Psychological Association, US, 1997, pp. 107-112.

[6] H.-C. Yan, X. Cao, M. Das, X.-H. Zhu, T.-M. Gao, Neuroscience bulletin 26 (2010) 327-337.