Radioiodinated Nitroxide Derivative for the Detection of Lipid Radicals

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1. Synthetic Experimental Procedure

General Methods

Reagents and solvents from commercial suppliers were used without further purification. The reaction was monitored by thin layer chromatography on silica gel *60* plates. The compounds were detected by examination under UV light and by staining with iodine vapor or 5% phosphomolybdic acid EtOH solution. Flash column chromatography was performed with prepacked silica gel column. HRMS were measured on a Thermo Fisher Scientific Exactive spectrometer. NMR data for **2** and **3** were obtained by *in situ* reduction with phenylhydrazine ($PhNHNH_2$) to reduce the broadening effect derived from the paramagnetism. ESR spectra were obtained by X-band ESR spectrometer in DMSO solution at rt. The purity was checked by HPLC measurement. The measurement condition was described in Figure S5 and S6 legend.

4-(4-Iodobenzamido)-2,2,6,6-tetramethylpiperidine-1-oxyl (2). To a solution of **1** (0.50 g, 2.9 mmol) in THF (3 mL), 4-iodobenzoic acid (0.72 g, 2.9 mmol) and DMT-MM (1.22 g, 4.4 mmol) were added and stirred for 1.5 hours at rt. After extraction with EtOAc and drying over Na_2SO_4, the solvent was evaporated under reduced pressure, and purification was performed by flash column chromatography using hexane: EtOAc = 2: 1 (R_f: 0.23) as an elution solvent to afford orange solid **2** (0.92 g, 79%). ^{1}H NMR (300 MHz, $CDCl_3$ w/ $PhNHNH_2$) δ ppm 1.45 (s, 6 H), 1.56 (s, 6 H), 2.02 - 2.14 (m, 2 H), 2.14 - 2.33 (m, 2 H), 4.41 - 4.74 (m, 1 H), 6.43 (d, *J*=7.6 Hz, 1 H), 7.50 (d, *J*=8.4 Hz, 2 H), 7.78 (d, *J*=8.4 Hz, 2 H). ESR (DMSO): g = 2.0060, a_N = 1.549 mT (triplet). HRMS (ESI/Q-TOF) m/z: $[M+Na]^+$ Calcd for $C_{16}H_{22}IN_2NaO_2^{\cdot+}$ 424.0618; Found 424.0622.

2,2,6,6-Tetramethyl-4-(4-(tributylstannyl)benzamido)piperidine-1-oxyl (3). To a solution of **2** (0.20 g, 0.5 mmol) in anhydrous toluene (5 mL), Pd $(PPh_3)_4$ (29 mg, 25 μmol) is added, and bubbling is carried out for 5 minutes while stirring, then $(SnBu_3)_2$ (0.50 mL, 1.0 mmol) was added and refluxed at 100°C in an oil bath for 24 hours. After the reaction, the solution was evaporated under reduced pressure, and purification was performed by flash column chromatography by using hexane: Et_2O = 3: 1 (R_f: 0.38) as an elution solvent to afford orange solid **3** (145 mg, 51%). ^{1}H NMR (300 MHz, $CDCl_3$ w/ $PhNHNH_2$) δ ppm 0.88 (t, *J*=7.2 Hz, 9 H), 1.02 - 1.13 (m, 6 H), 1.27 - 1.39 (m, 6 H), 1.47 - 1.59 (m, 6 H), 1.49 (s, 6 H), 1.63 (s, 6 H), 2.05 - 2.18 (m, 2 H), 2.20 - 2.43 (m, 2 H), 4.50 - 4.69 (m, 1 H), 6.25 (d, *J*=8.1 Hz, 1 H), 7.54 (d, *J*=8.0 Hz, 2 H), 7.67 (d, *J*=8.0 Hz, 2 H). ESR (DMSO): g = 2.0060, a_N = 1.572 mT (triplet). HRMS (ESI/Q-TOF) m/z: $[M+Na]^+$ Calcd for $C_{28}H_{49}N_2NaO_2Sn^{\cdot+}$ 588.2708; Found 588.2711.

Radiolabeling for 4-(4-[^{125}I]Iodobenzamido)-2,2,6,6-tetramethylpiperidine-1-oxyl ([^{125}I]2). Compound **3** and NCS were each dissolved in MeOH: AcOH (100: 1) to 1 mg/mL. The solutions of **3** (50 μl) and NCS (50 μl) were added to Na^{125}I, vortexed and allowed to stand at rt for 15 minutes. The reaction mixture was injected into HPLC (mobile phase: A: H_2O, B: MeCN; 10% B (t = 0 min), 100% B (t = 5 to 30 mins); column: COSMOSIL 5C_{18}-AR-II, 4.6 mm I.D. x 250 mm; flow rate: 1 mL/min), to purify the desired compound. Collected fraction was concentrated using N_2 gas flow.

Reactivity assay. The conditions of each reaction are as follows. (i) Lipid alkyl radical: Arachidonic acid, 0.8 mM; soybean lipoxidase (LOX), 0.05 mg/mL; **2**, 50 μM. (ii) H_2O_2: H_2O_2, 1%; **2**, 50 μM. (iii) Hydroxyl radical (·OH): H_2O_2, 1%; $FeSO_4$, 0.2 mM; **2**, 50 μM; (iv) Superoxide anion radical ($O_2^{\cdot -}$): hypoxanthine, 1.25 mM; xanthine oxidase, 78 mU/mL; **2**, 50 μM. (v) Hypochlorite ion (ClO^-): NaClO, 0.1%; **2**, 50 μM. Each solution was mixed in 10 mM phosphate buffer (pH 7.4) in equal volumes (40 μL), then the mixed solution was transferred to a capillary tube and set into the ESR cavity. The ESR spectra were recorded on an X-band ESR spectrometry. The amounts of the nitroxide were calculated from the double-integrated ESR signal intensity, using Mn^{2+} as an external standard. The conditions for the ESR measurements were as follows: power, 10 mW; frequency, 9.4 GHz; magnetic field, 337 mT; modulation amplitude, about one-third of the line width; time constant, 0.3 s.

Cell culture. HepG2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL) and penicillin (100 U/mL). Cells were maintained at 37 ˚C in 5% CO_2/95% air.

Cellular uptake assay. HepG2 cells were plated at a density of 2 x 10^5 cells per well in a 24-well plate for 1 day prior to the assay. The medium was removed and medium containing each additive, such as 500 μM arachidonic acid and [^{125}I]**2** (0.5 μCi with 1% EtOH and 0.1% Tween 80 as co-solvents), was added to each well and allowed to stand in a CO_2 incubator at 37 °C for 5, 10, 15 minutes. Then the medium was removed, cells were washed with PBS, and dissolved in 0.2 M NaOH. The radioactivity was measured with a γ counter. In addition, the amount of protein was measured by BCA protein assay kit (Thermo Fisher Scientific).

Statistical Analysis. All results are expressed as the mean ± standard deviation of at least three independent experiments. Statistical analyses were performed using Student's t test. The minimum level of significance was set at $p < 0.05$.

2. NMR spectra

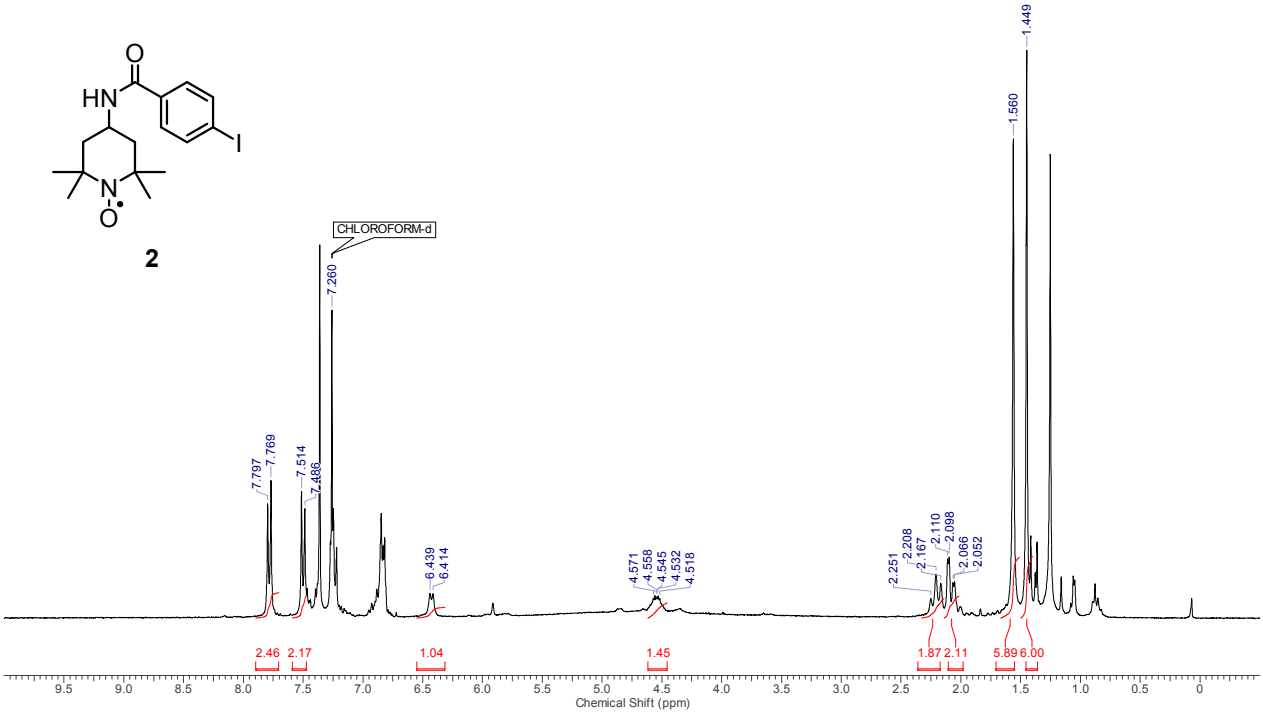

Figure S1. NMR spectrum of **2** w/ $PhNHNH_2$.

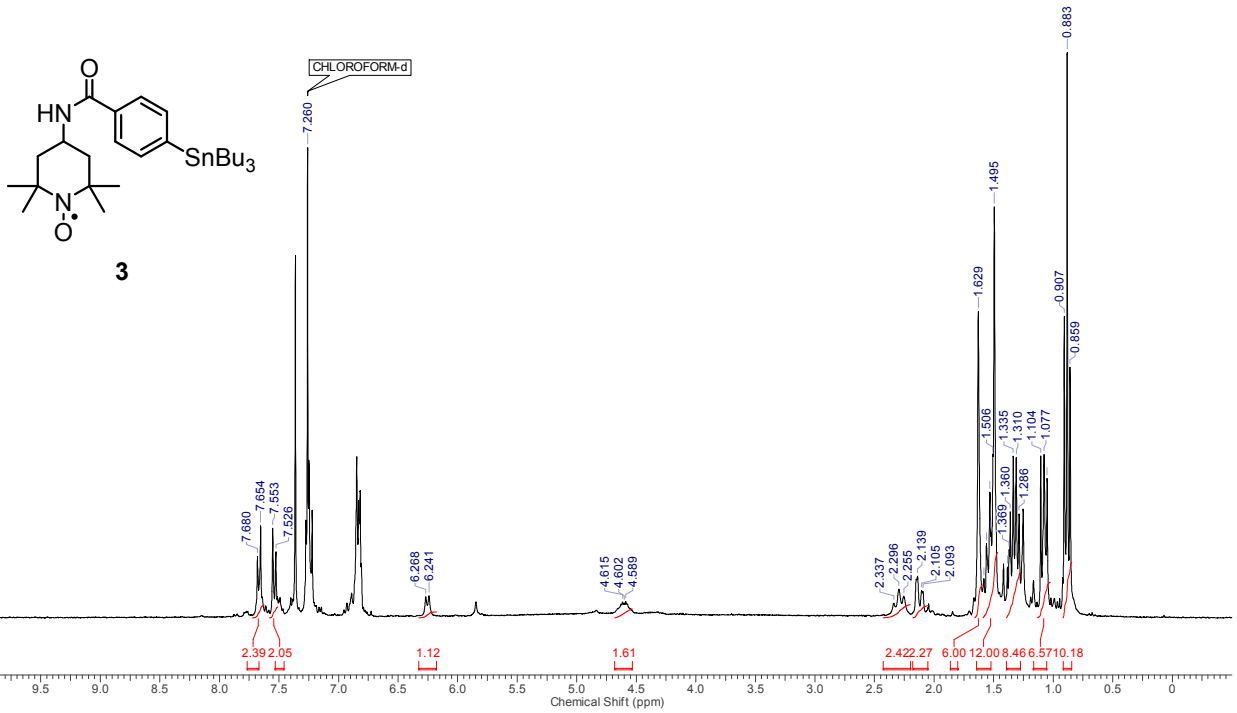

Figure S2. NMR spectrum of **3** w/ $PhNHNH_2$.

3. ESR spectra

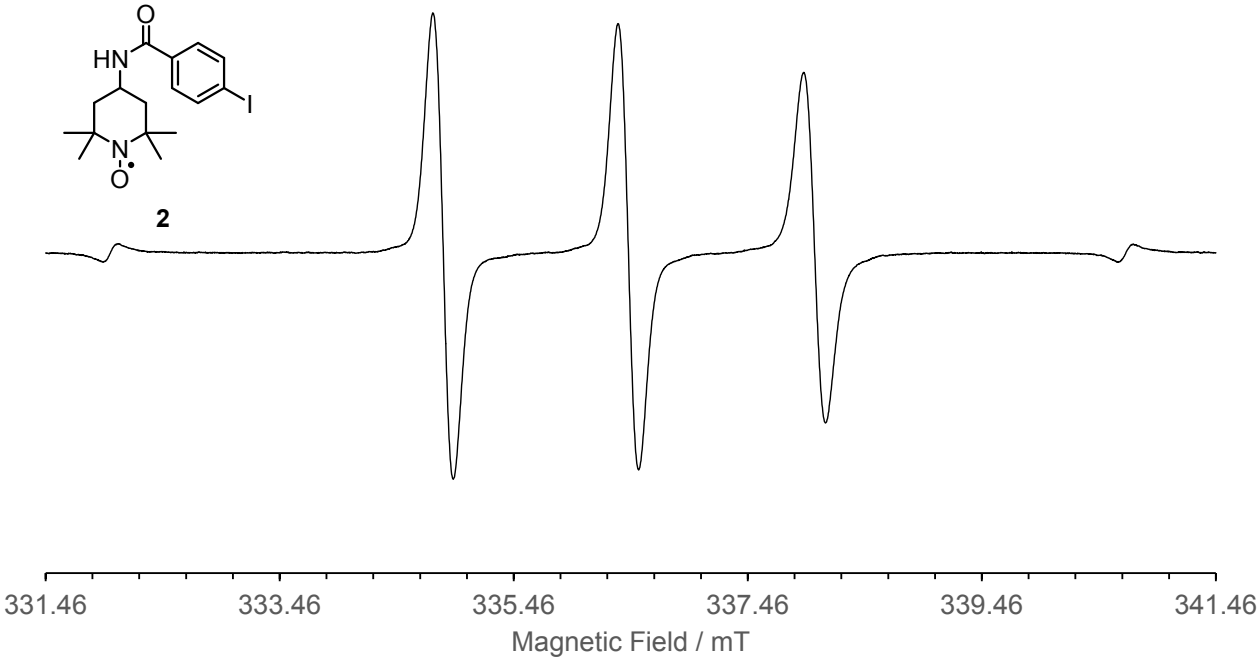

Figure S3. ESR spectrum of **2** in DMSO.

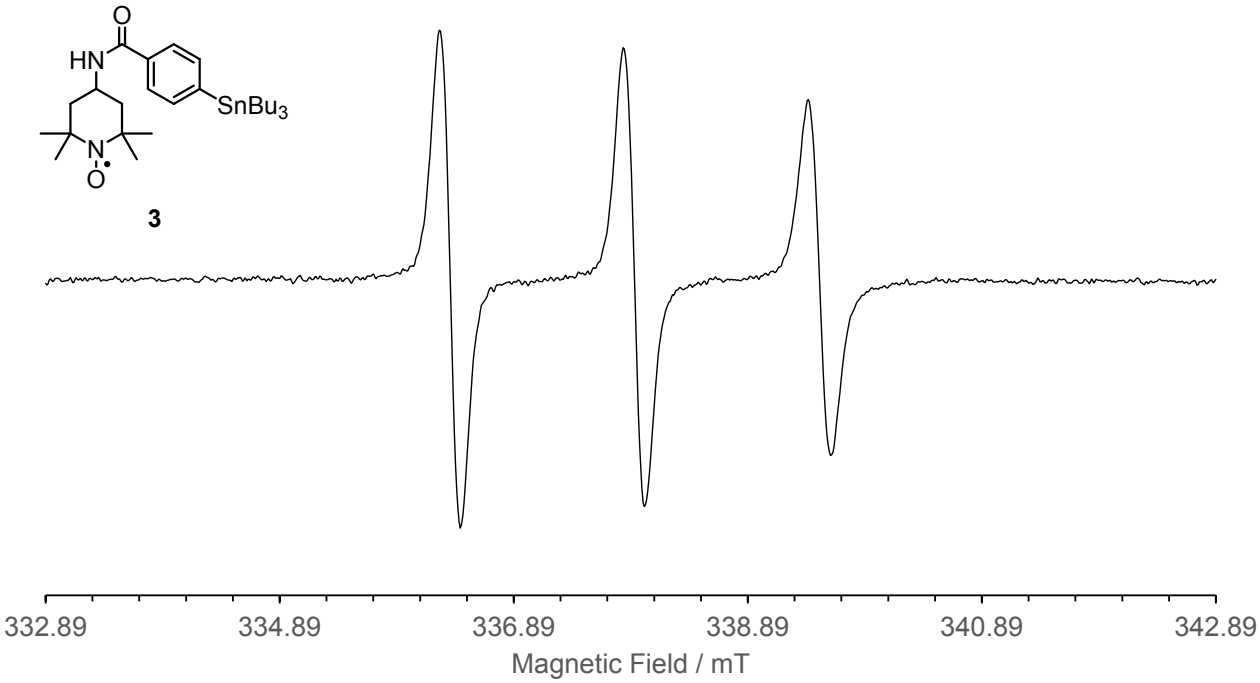

Figure S4. ESR spectrum of **3** in DMSO.

4. HPLC chromatograms

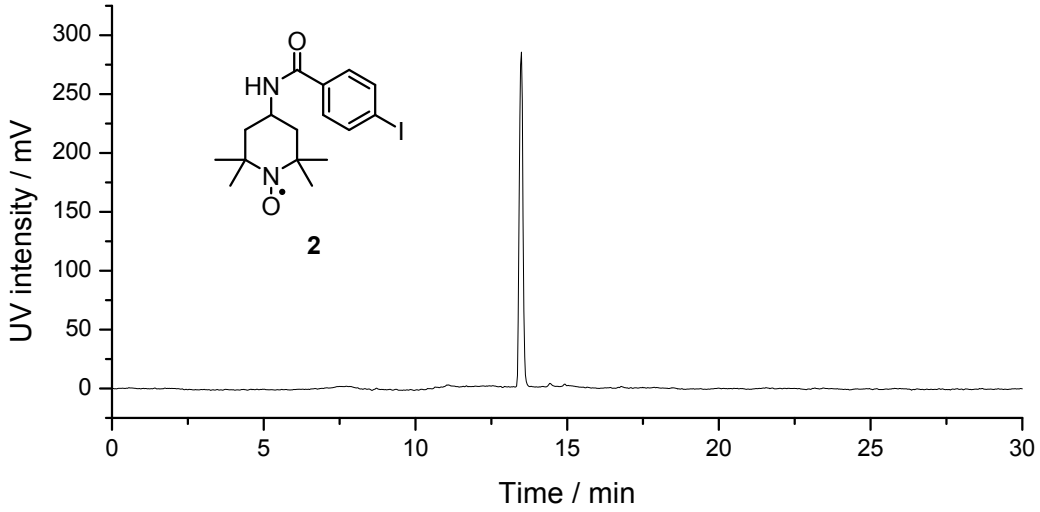

Figure S5. HPLC chromatogram of **2**. Mobile phase: A: H_2O, B: MeCN; 10% B (t = 0 min), 100% B (t = 5 to 30 mins); column: COSMOSIL $5C_{18}$-AR-II, 4.6 mmI.D. x 250 mm; UV: 254 nm; flow rate: 1 mL/min.

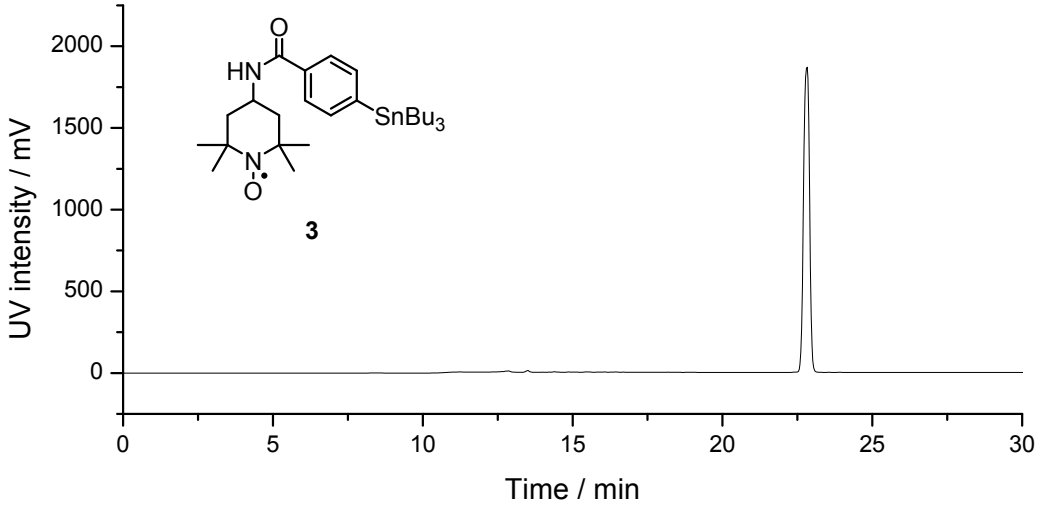

Figure S6. HPLC chromatogram of **3**. Mobile phase: A: H_2O, B: MeCN; 10% B (t = 0 min), 100% B (t = 5 to 30 mins); column: COSMOSIL $5C_{18}$-AR-II, 4.6 mmI.D. x 250 mm; UV: 254 nm; flow rate: 1 mL/min.