Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

A Small Molecule Probe Reveals Declined Mitochondrial Thioredoxin Reductase Activity in a Parkinson's Disease Model

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EXPERIMENTAL SECTION

Materials

The recombinant rat TrxR was essentially prepared as described [1] and is a gift from Prof. Arne Holmgren at Karolinska Institute, Sweden. The recombinant U498CTrxR mutant (Sec→Cys) was produced as described.[2] The PC12 and HeLa cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Dulbecco's modified Eagle's medium (DMEM), reduced glutathione (GSH), dimethyl sulfoxide (DMSO), glutathione reductase (GR) were obtained from Sigma-Aldrich (St. Louis, MO, USA). NADPH was obtained from Roche (Mannheim, Germany). Ethylene diaminetetraacetic acid (EDTA) was obtained from J&K Scientific (Beijing, China) Fetal bovine serum (FBS) was obtained from Sijiqing (Hangzhou, China). Penicillin and streptomycin were obtained from Sangon (Shanghai, China). Acylase from Hog Kidney and L-selenocystine were obtained from J&K Scientific (Beijing, China). The Sec (10 μM) was generated in situ by mixing Cys (1 mM) and selenocytine (5 μM). MitoTracker Deep Red was obtained from Invitrogen. The antibody against TrxR2 was purchased from Santa Cruz. All other reagents were of analytical grade and were purchased from commercial supplies. Absorption spectra were recorded on UV−vis spectrometer evolution 200 (Thermo Scientific). Fluorescence studies were carried out using a Cary Eclipse Fluorescence Spectrophotometer (Agilent) or Leica inverted fluorescence microscope. Flow cytometry experiments were carried by a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA). MS spectra were recorded on Trace DSQ GC-MS spectrometer or Bruker Daltonics esquire 6000 mass spectrometer. HRMS was obtained on Orbitrap Elite (Thermo Scientific). The quantum yields (ϕ) of **6** and Mito-TRFS were determined on FLS920 spectrometer (Edinburgh Instruments, U.K.). Melting points (mp) were determined on a Fisher-Johns melting apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Advance 400, and tetramethylsilane (TMS) was used as a reference.

Chemical synthesis

Compounds **1**, **7**, and **8** was prepared by adapting the published procedures.[3]

Synthesis of compound 2

4-Nitronaphthalic-1,8-anhydride (**1**, 1.5 g, 6.17 mmol) and 2-(2-aminoethoxy)ethanol (617 μL, 6.17 mmol) were added to THF (50 mL) in a round-bottom flask. The reaction mixture was refluxed for 3 h and then was cooled to room temperature. The solvent was evaporated and the resulting residue was dissolved in a minimum amount of MeOH and poured into water. The product was filtered, washed with water, and dried to afford compound **2** (1.0 g, 50% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.75 (m, 1H), 8.67 (m, 1H), 8.63 (d, J=8.0 Hz, 1H), 8.35 (d, J=8.0 Hz, 1H), 8.00 (m, 1H), 4.42 (t, J = 5.6 Hz, 2H), 3.85 (t, J = 5.6 Hz, 2H), 3.66 (m, 4H), 2.54 (brs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 163.4, 162.6, 149.5, 132.5, 129.9, 129.8, 129.3, 128.9, 126.6, 123.8, 123.5, 122.6, 72.2, 68.0, 61.7, 39.8; mp: 105.3−106.2 °C; ESI-MS (m/z): $[M + H]^+$ 331.5.

Synthesis of compound 3

To a cold (0 °C) magnetically stirred solution of compound **2** (3.0g, 9.2 mmol) and triethylamine (1.4 mL, 10 mmol) in dry dichloromethane (50 mL) was added methanesulfonyl chloride (1.6 mL，20mmol) dropwisely over 30 min and the reaction was left to stir at room temperature for 6 h. The resulting solution was then poured into aqueous HCl (100 mL, 1 M) and extracted with dichloromethane (DCM, 50 mL x 3). The DCM layer was combined and washed with saturated sodium hydrogen carbonate and brine. The organic layer was dried over anhydrous Na_2SO_4 and purified by silica gel column chromatography (petroleum ether : EtOAc = 1:1, v/v) to afford compound **3** (2.1 g, 55 % yield) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.77 (m, 1H), 8.67 (m, 1H), 8.63 (d, J=8.0 Hz, 1H), 8.36 (d, J=8.0 Hz, 1H), 8.00 (m, 1H), 4.43 (t, J = 5.7 Hz, 2H), 4.30 (m, 2H), 3.86 (t, J = 5.7 Hz, 2H), 2.97 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 163.2, 162.4, 149.5, 132.4, 129.8, 129.3, 128.9, 126.6, 123.8, 123.5, 122.7, 69.0, 68.4, 67.9, 39.3, 37.4; mp: 112.9−114.4 °C; ESI-MS (m/z): $[M + H]^+$ 409.4.

Synthesis of compound 4

The compound **3** (2.0 g，5mmol) was added to NaI (1.5 g, 10 mmol) in dry acetone (50 mL) and the mixture was refluxed for 12 h. The solvent was evaporated and the residue was directly purified by silica gel column chromatography (petroleum ether : EtOAc = 4:1, v/v) to give compound **4** (1.7 g, 80 % yield) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.84 (d, J=8.7 Hz, 1H), 8.74 (d, J=7.3 Hz, 1H), 8.70 (d, J=8.0 Hz, 1H), 8.41 (d, J=8.0 Hz, 1H), 8.00 (m, 2H), 4.47 (t, J =

5.7 Hz, 2H), 3.87 (t, J = 5.7 Hz, 2H), 3.77 (t, J = 6.6 Hz, 2H), 3.21 (t, J = 6.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 163.4, 162.6, 149.6, 132.5, 129.9, 129.4, 129.2, 126.9, 123.9, 123.7, 122.9, 71.2, 67.4, 39.6, 2.9; mp: 113.2−113.5 °C; ESI-MS (m/z): $[M + H]^+$ 440.8.

Synthesis of compound 5

To a stirred cloudy solution of compound 4 (880 mg, 2 mmol) in ethanol (50 mL) was added dropwisely the solution of $SnCl_2{\cdot}2H_2O$ (2.71 g, 12 mmol) in concentrated hydrochloric acid (2 mL) at room temperature. Next, the reaction was refluxed for 6 h. Then, a solution of 5 M NaOH was employed to neutralize the excessive acid, followed by extraction with ethyl acetate. The organic layer was dried with anhydrous Na2SO4 and evaporated to dryness. The crude product was purified by silica gel column chromatography (petroleum ether : EtOAc = 1:1, v/v) to afford the desired product (0.47 g, 58% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.63 (d, J=8.4 Hz, 1H), 8.43 (d, J=7.2 Hz, 1H), 8.20 (d, J=8.4 Hz, 1H), 7.67 (m, 1H), 7.44 (s, 2H), 6.85 (d, J=8.4 Hz, 1H), 4.23 (t, J = 6.5 Hz, 2H), 3.70 (m, 4H), 3.31 (t, J = 6.4Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 163.8, 162.8, 152.7, 133.9, 131.0, 129.7, 129.3, 123.9, 121.7, 119.3, 108.1, 107.4, 70.5, 66.6, 38.2, 5.3; mp: 176.3−177.1 °C; ESI-MS (m/z): $[M + Na]^+$ 433.0.

Synthesis of compound 6

To a solution of the compound **5** (410 mg, 1 mmol) in CH_3CN (10 mL) was added PPh_3 (365 mg, 1.2mmol). The resulting solution was stirred for 14 h under reflux. The solvent was evaporated and the residue was directly purified by silica gel column chromatography (DCM : methanol=10:1, v/v) to give compound **6** (530 mg, 80 % yield) as an orange solid. ^{1}H NMR (400 MHz, CD_3OD) δ: 8.50 (m, 1H), 8.49 (m, 1H), 8.18 (d, J=8.4 Hz, 1H), 7.73 (m, 16H), 6.87 (d, J=8.4 Hz, 1H), 4.15 (t, J = 5.6 Hz, 2H), 3.86 (m, 1H), 3.81 (m, 1H), 3.73 (m, 2H), 3.49 (t, J = 5.6 Hz, 2H), 3.35 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ: 166.1, 165.5, 154.7, 135.9, 135.8, 135.7, 134.9, 134.8, 132.8, 131.6, 131.2, 131.1, 130.4, 125.3, 123.2, 121.1, 120.8, 119.9, 109.7, 109.4, 69.5, 64.7, 64.6, 39.6, 25.6, 25.0; mp: 258.1-258.4°C; ESI-MS (m/z): $[M]^+$ 545.2; HRMS (m/z): $[M]^+$ calcd for 545.1989, found 545.1981.

Synthesis of compound Mito-TRFS

To a mixture of compound 6 (134 mg, 0.2 mmol) and DIPEA (52 mg, 0.4 mmol) in dry DCM (25 mL) was added a solution of diphosgene (28 μL, 0.24 mmol) in DCM, and the reaction was kept on ice for 1 h. The resulting solution was heated to reflux for 3 h. After cooling to room temperature, compound 8 (30 mg, 0.24 mmol) was added, and the solution was stirred at room temperature for an additional 3 h. Then the reaction mixture was concentrated, and the crude product was purified by silica gel column chromatography (DCM : methanol=15:1, v/v) to give mito-TRFS (16 mg, 10% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.56 (d, J=6.9 Hz, 1H), 8.50 (d, J=8.2 Hz, 1H), 8.36 (m, 2H), 7.86 (s, 1H), 7.78 (m, 16H), 4.11 (t, J=6.0 Hz, 2H), 4.05 (m, 2H), 3.97 (m, 1H), 3.91 (m, 1H), 3.49 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 163.9, 163.4, 152.7, 139.4, 134.8, 134.7, 133.9, 133.8, 132.3, 131.4, 130.2, 130.1, 128.8, 127.7, 126.7, 123.2, 122.6, 118.9, 118.1, 117.5, 117.2, 79.7, 68.1, 63.9, 63.9, 44.9, 38.7, 29.7, 25.9, 25.4; ESI-MS (m/z): $[M]^+$ 693.4; HRMS (m/z): $[M]^+$ calcd for 693.1641, found 693.1646.

UV−vis and Fluorescence Spectroscopy. UV−vis spectra were acquired from UV−vis spectrometer evolution 200 (Thermo Scientific). Fluorescence spectroscopic studies were performed with Cary Eclipse Fluorescence Spectrophotometer (Agilent). The slit width was 5 nm for both excitation and emission. For spectra measurements, Mito-TRFS was dissolved in DMSO to obtain a stock solution, which was diluted with TE to the desired concentrations. The organic solvent is no more than 1 % (v/v) in the in vitro assays, and no more than 0.1 % (v/v) in the cell experiments.

Confocal Fluorescence Imaging. HeLa cells were seeded in a 12-well plate and cultured overnight in DMEM supplemented with 10 % FBS, 2 mM glutamine, penicillin (100 units/mL), streptomycin (100 units/mL) at 37 °C in a humidified atmosphere of 5 % CO_2. The cells were treated with vehicle (0.1 % (v/v) DMSO) or Auranofin (0.5, 1, 2 μM) for 4 h followed by further treated with Mito-TRFS (1μM) for 2 h. Then the cells were rinsed with the medium for three times to remove the remaining Mito-TRFS. And the medium was replaced with fresh medium containing 100 nM of MitoTracker Deep Red and incubated for another 15 min at 37 °C. After washing the cells with PBS three times, the fluorescence images were carried out on an Olympus FV1000 laser scanning confocal microscope. The colocalization coefficient is analyzed by the software along with the confocal microscope.

General Fluorescence Imaging. PC12 cells were seeded in a 12-well plate and cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, penicillin (100 units/mL), streptomycin (100 units/mL) at 37 °C in a humidified atmosphere of 5% CO_2 overnight. The cells were treated with 6-OHDA (0, 50, 100, 200 μM) for 10 h or 22 h. Then Mito-TRFS (1 μM) was added and continued incubation for another 2 h. The cells were visualized and photographed under a Leica inverted fluorescence microscope.

Flow Cytometry Experiments. PC12 cells (5×10^5 cells/well) were seeded into 6-well plates and allowed to grow overnight. The cells were treated with 6-OHDA (0, 50, 100, 200 μM) for 10h or 22 h. Then Mito-TRFS (1 μM) was added and continued incubation for another 2 h. The cells were detached with trypsin and resuspended in PBS, and subjected to analysis by a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) using the FITC channel. The data represents ~10,000 cells for each analysis. The relative fluorescence intensity (F. I.) was quantified by the following equation: F. I. % = $(F_{sample}-F_{blank}) / (F_0-F_{blank})$ x 100, where F_{sample} is the F. I. of the cells treated with 6-OHDA and Mito-TRFS, F_{blank} is the F. I. of the cells without any treatment, and F_0 is the F. I. of the cells treated with Mito-TRFS only.

MTT assay. Five thousand PC12 cells or HeLa cells cells were incubated with Mito-TRFS in a 96-well plate for 20h at 37 °C in a final volume of 100 μL. Cells treated with DMSO alone were used as controls. At the end of the treatment, 10 μL MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C. An extraction buffer (100 μL, 10% SDS, 5% isobutanol, 0.1% HCl) was added, and the cells were incubated overnight at 37°C. The absorbance was measured at 570 nm on Multiskan GO (Thermo Scientific).

Trypan blue exclusion assay. As 6-OHDA interfere with the MTT assay, we adopted the trypan blun exclusion assay to determine the cytotoxicity of 6-OHDA to PC12 cells. The cells were seeded at 2×10^4 cells per well in 24-well plates and treated with different concentrations of 6-OHDA (50, 100 or 200 μM) for 12 h or 24h. Cells treated with DMSO alone were used as controls, and the cell viability was determined by the

trypan blue exclusion assay. After treatment, the cells were stained with trypan blue (0.4%, w/v), and the number of viable (non-stained) and dead (stained) cells were counted under microscope.

References

[1] E. S. Arner, H. Sarioglu, F. Lottspeich, A. Holmgren, A. Bock, *J. Mol. Biol.* **1999**, *292*, 1003-1016.

[2] a) Y. Liu, D. Duan, J. Yao, B. Zhang, S. Peng, H. Ma, Y. Song, J. Fang, *J. Med. Chem.* **2014**, *57*, 5203-5211; b) L. Zhong, A. Holmgren, *J. Biol .Chem.* **2000**, *275*, 18121-18128.

[3] L. Zhang, D. Duan, Y. Liu, C. Ge, X. Cui, J. Sun, J. Fang, *J. Am. Chem. Soc.* **2014**, *136*, 226-233.

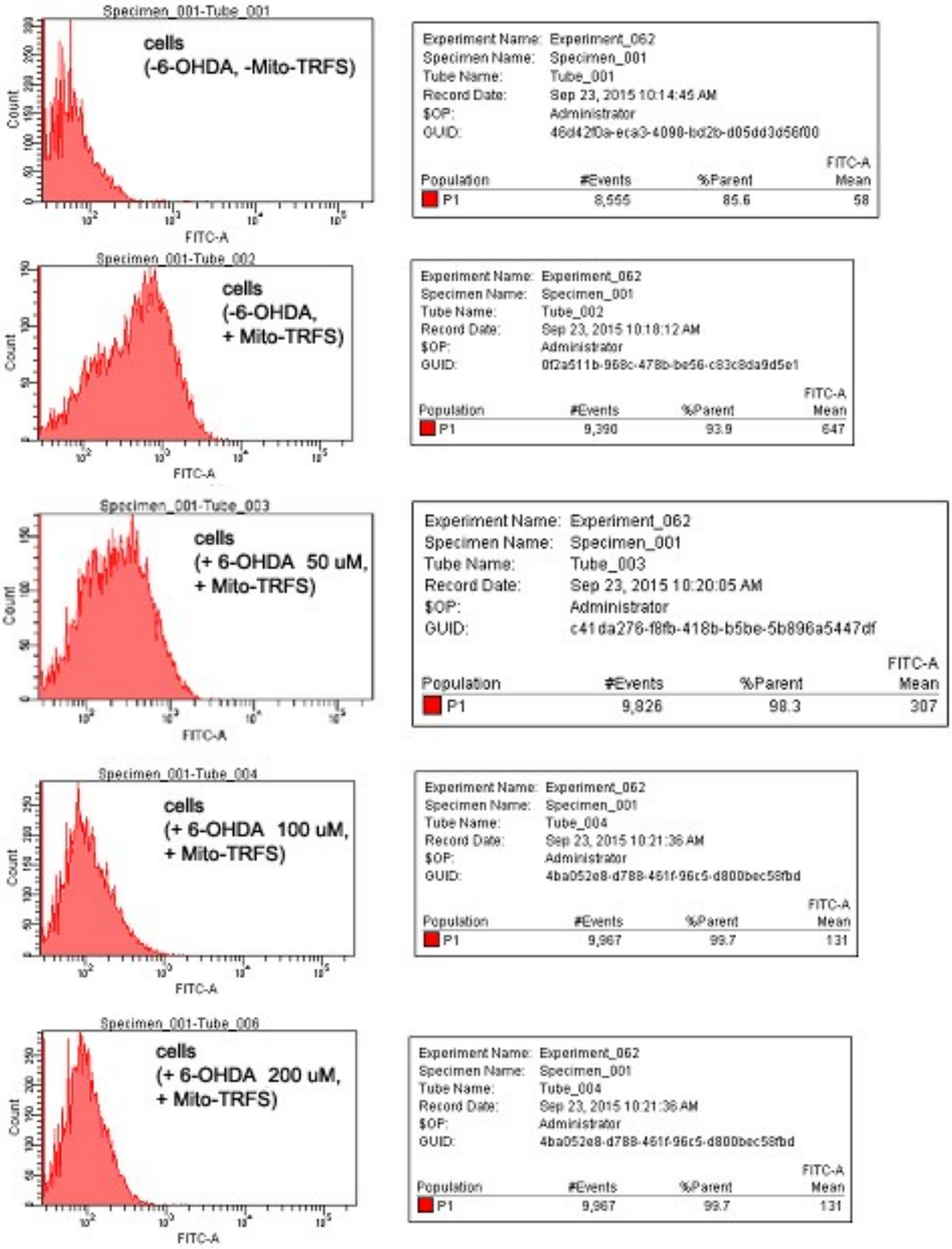

Figure S1. Flow cytometry analysis of the fluorescence intensity in 6-OHDA-treated PC12 cells after Mito-TRFS staining. The cells were treated with 6-OHDA for 10 h, and then stained with Mito-TRFS for 2 h. The cells were detached with trypsin and resuspended in PBS, and subjected to analysis by a FACSCanto flow cytometer.

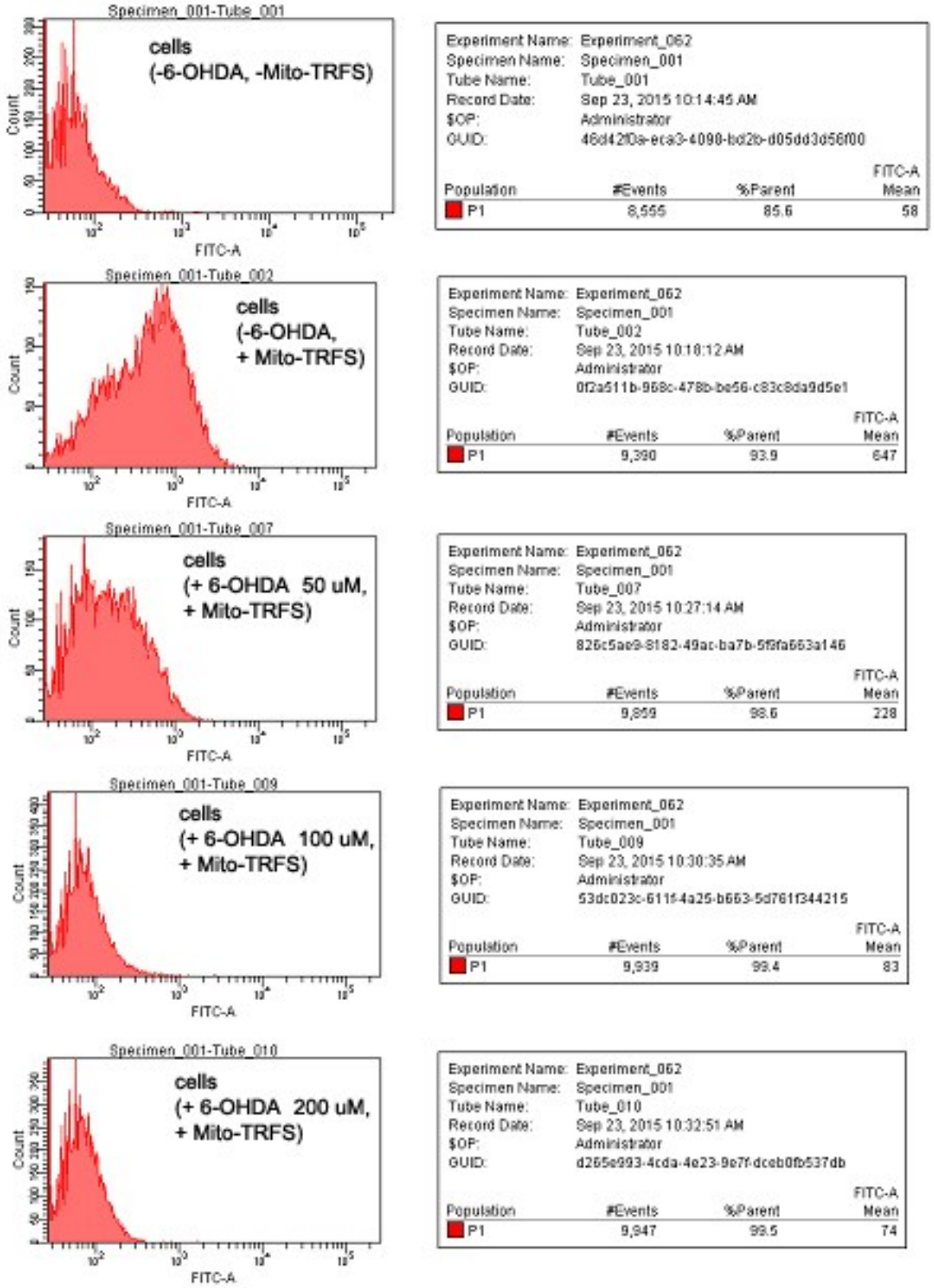

Figure S2. Flow cytometry analysis of the fluorescence intensity in 6-OHDA-treated PC12 cells after Mito-TRFS staining. The cells were treated with 6-OHDA for 22 h, and then stained with Mito-TRFS for 2 h. The cells were detached with trypsin and resuspended in PBS, and subjected to analysis by a FACSCanto flow cytometer.

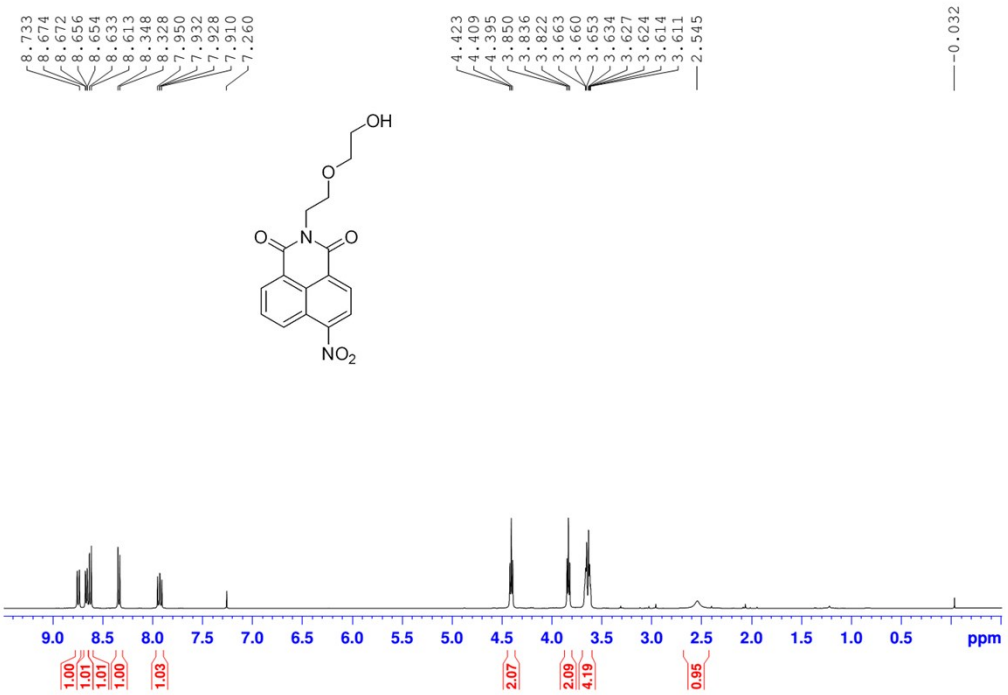

Figure S3. ^{1}H NMR Spectrum of compound **2** in $CDCl_3$ (400 MHz)**.**

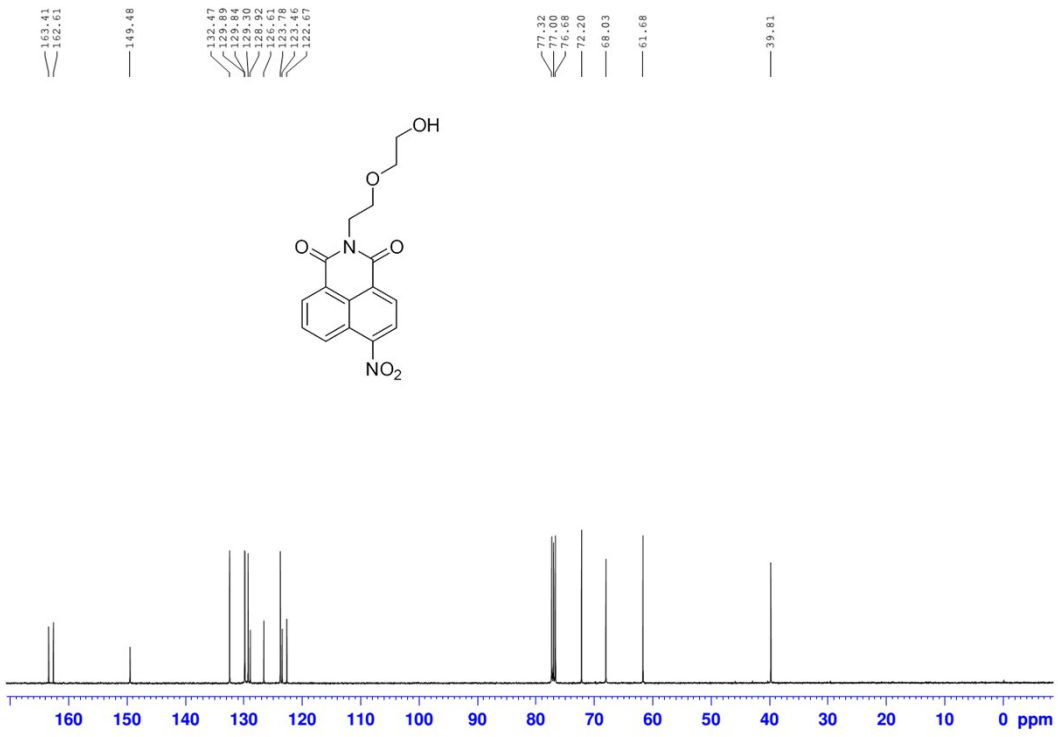

Figure S4. ^{13}C NMR Spectrum of compound **2** in $CDCl_3$ (100 MHz)**.**

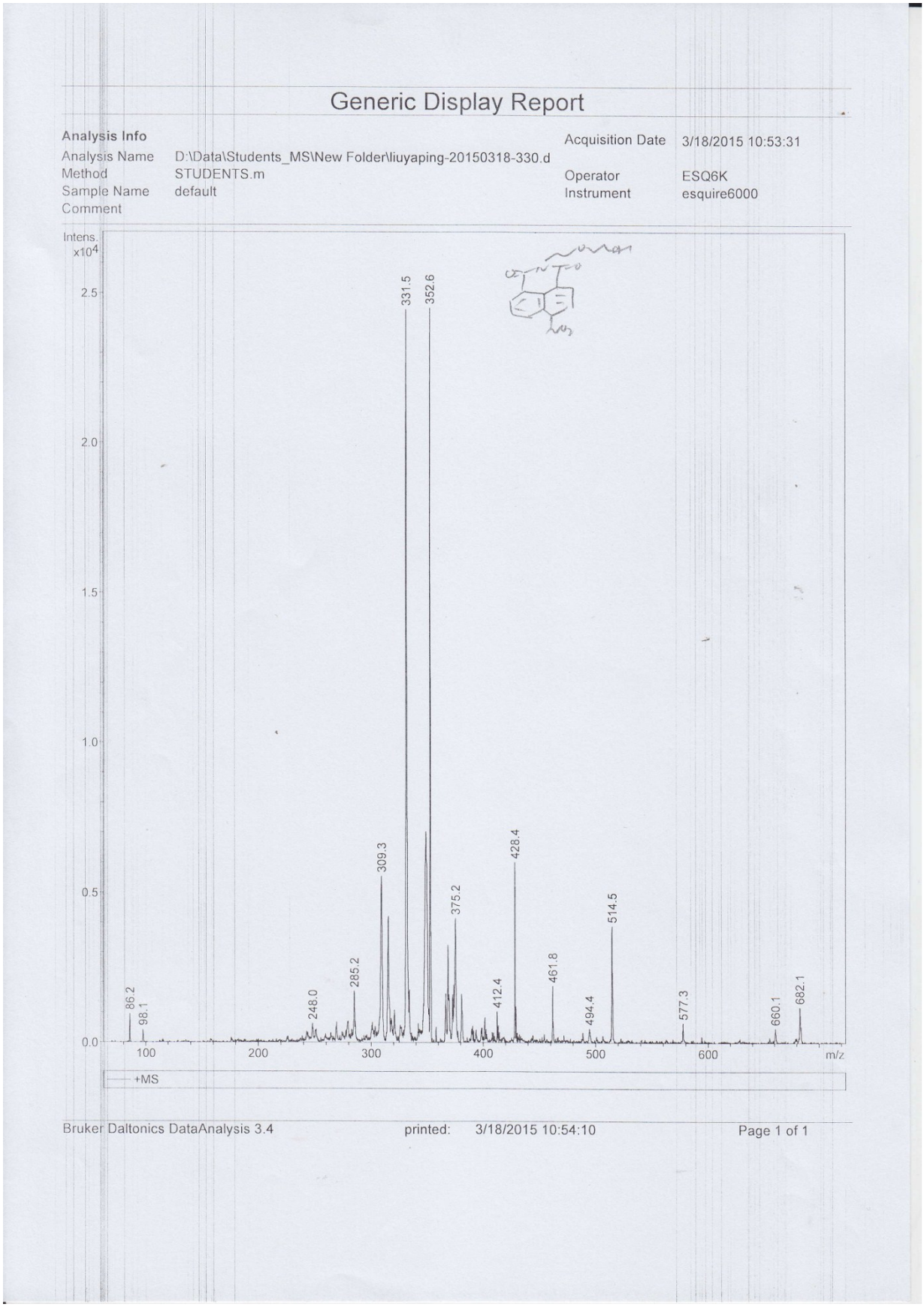

Figure S5. ESI-Mass spectrum of compound **2** (ESI-MS).

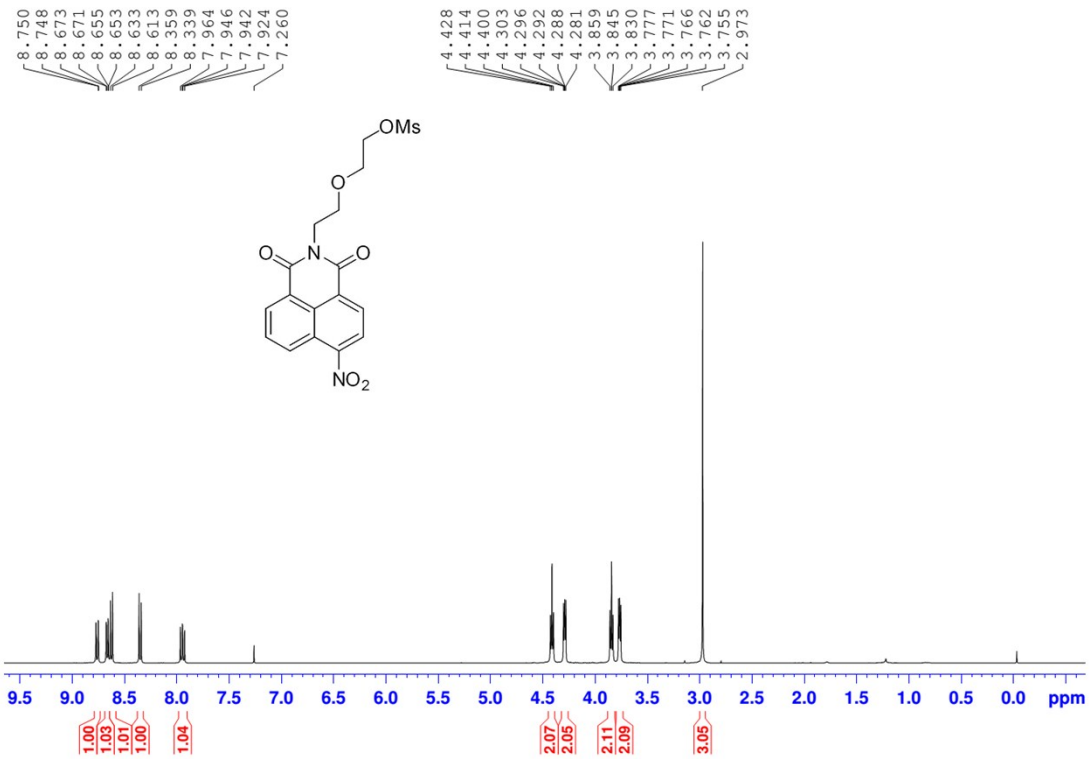

Figure S6. ^{1}H NMR Spectrum of compound **3** in $CDCl_3$ (400 MHz)**.**

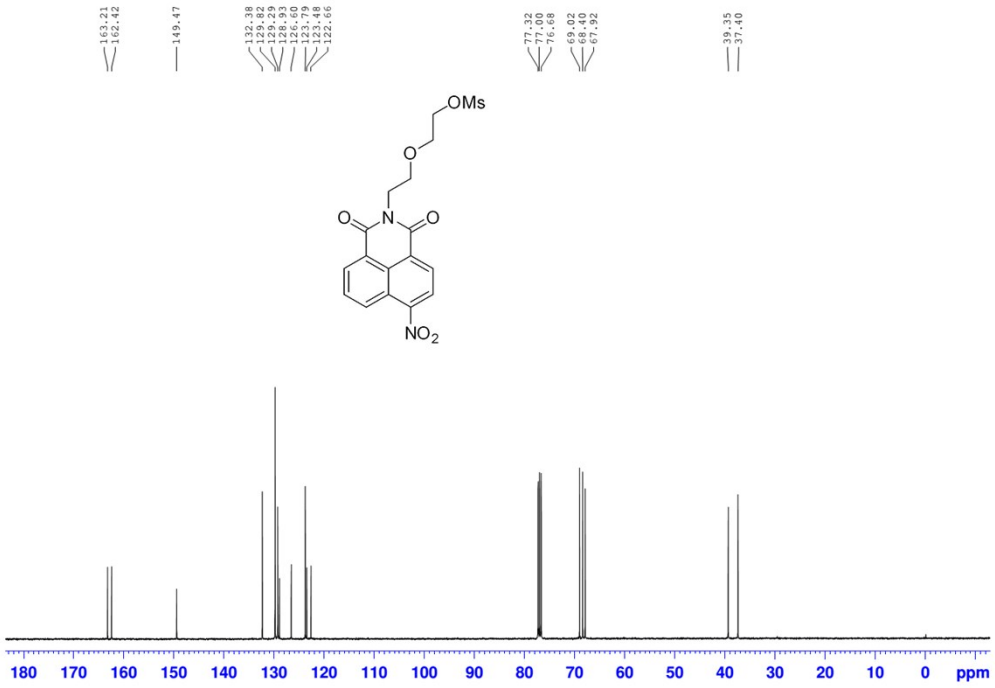

Figure S7. ^{13}C NMR Spectrum of compound **3** in $CDCl_3$ (100 MHz)**.**

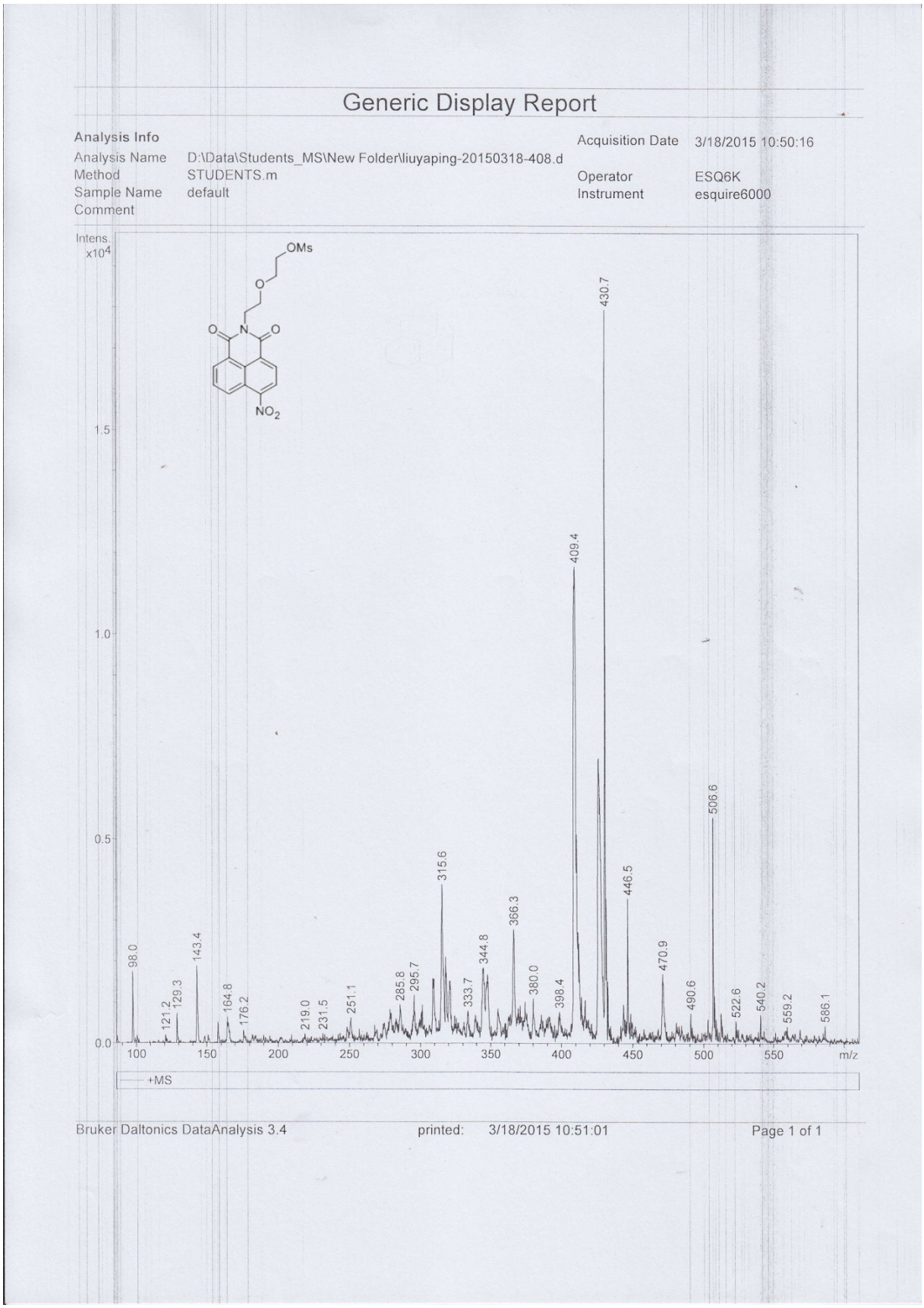

Figure S8. ESI-Mass spectrum of compound **3** (ESI-MS).

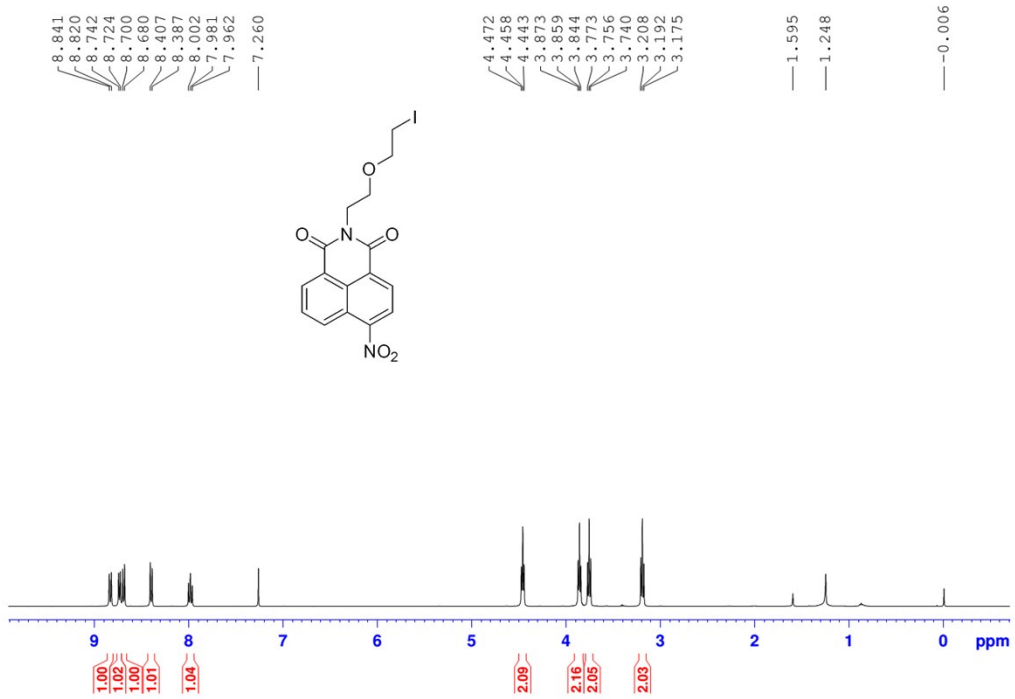

Figure S9. ^{1}H NMR Spectrum of compound **4** in $CDCl_3$ (400 MHz)**.**

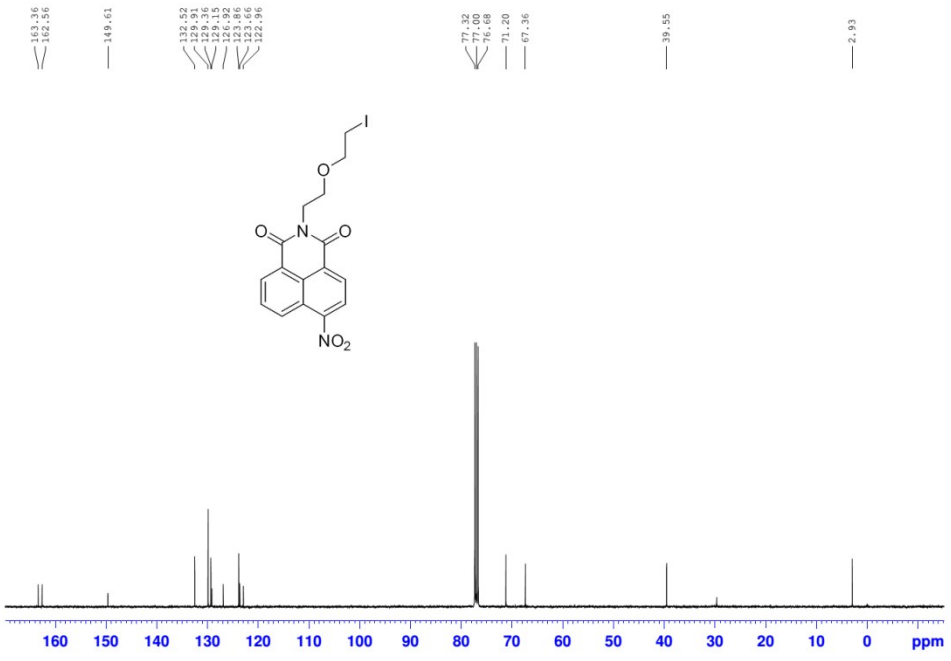

Figure S10. ^{13}C NMR Spectrum of compound **4** in $CDCl_3$ (100 MHz)**.**

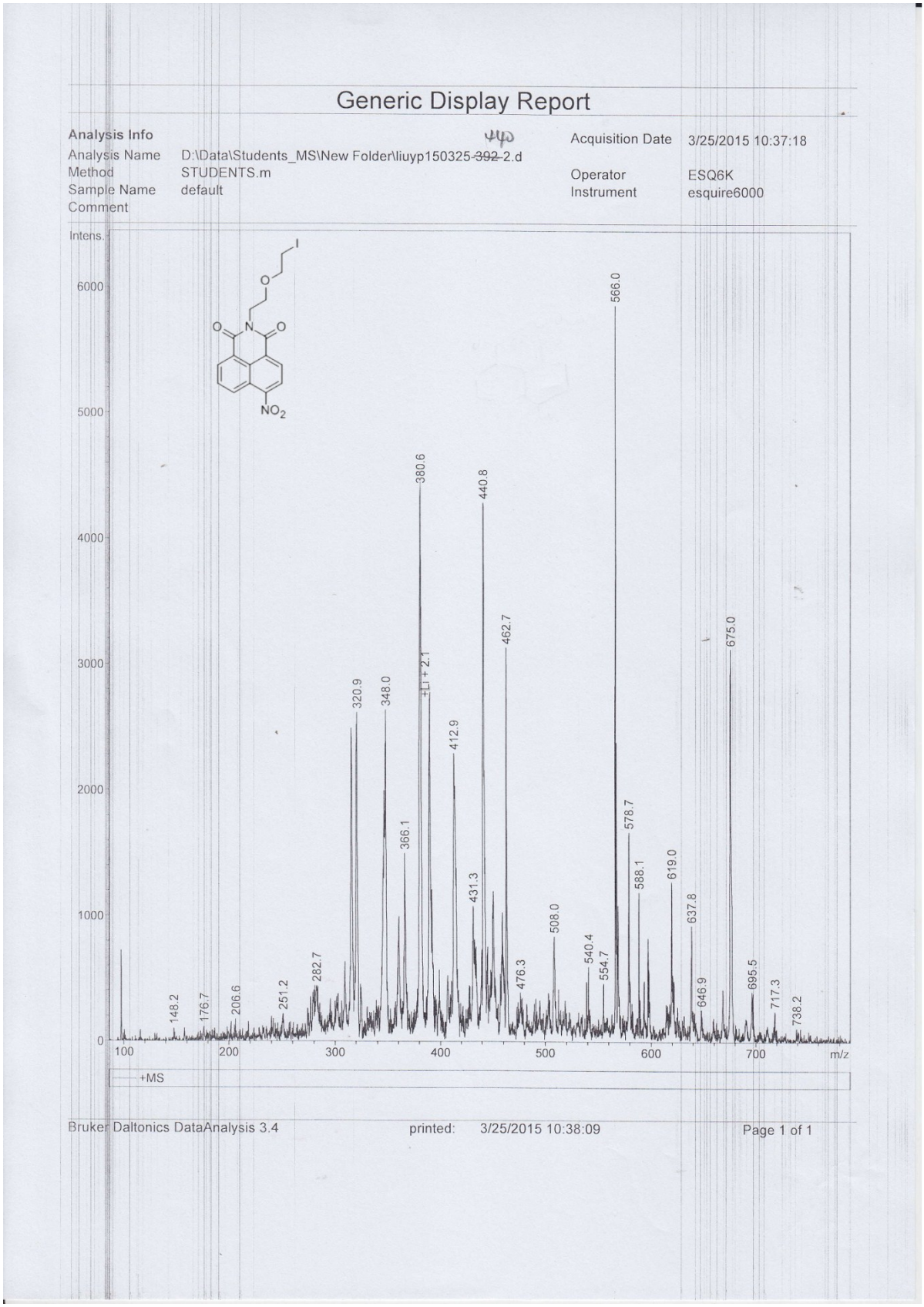

Figure S11. ESI-Mass spectrum of compound **4** (ESI-MS).

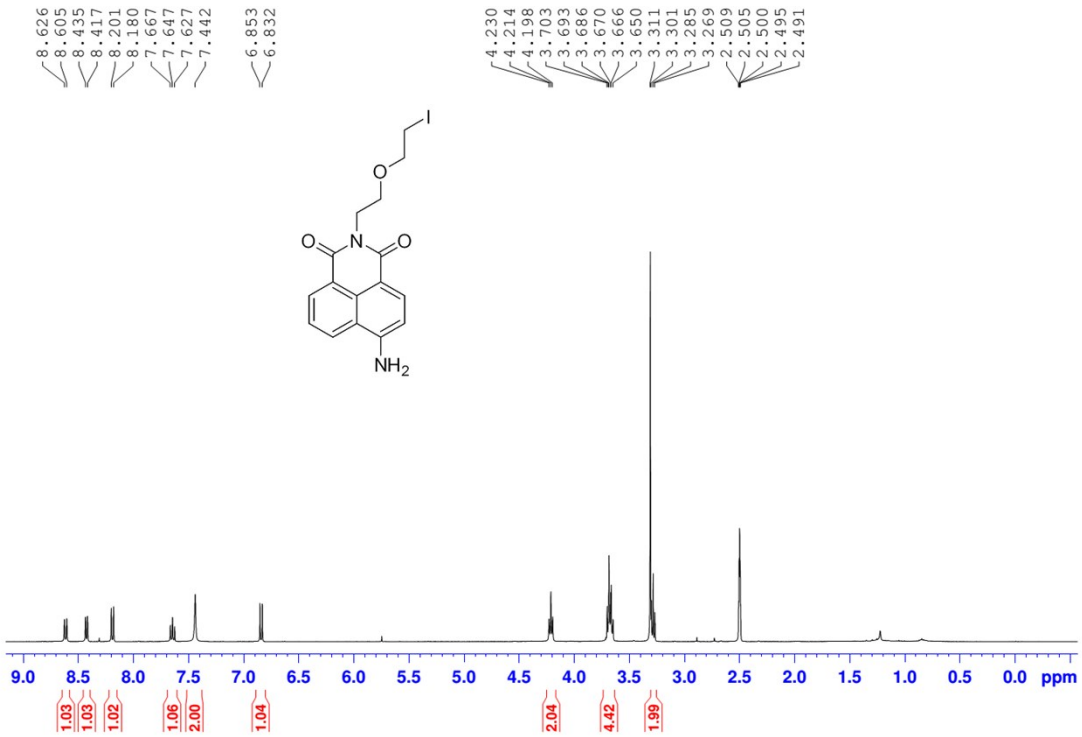

Figure S12. ^{1}H NMR Spectrum of compound **5** in DMSO-d_6 (400 MHz)**.**

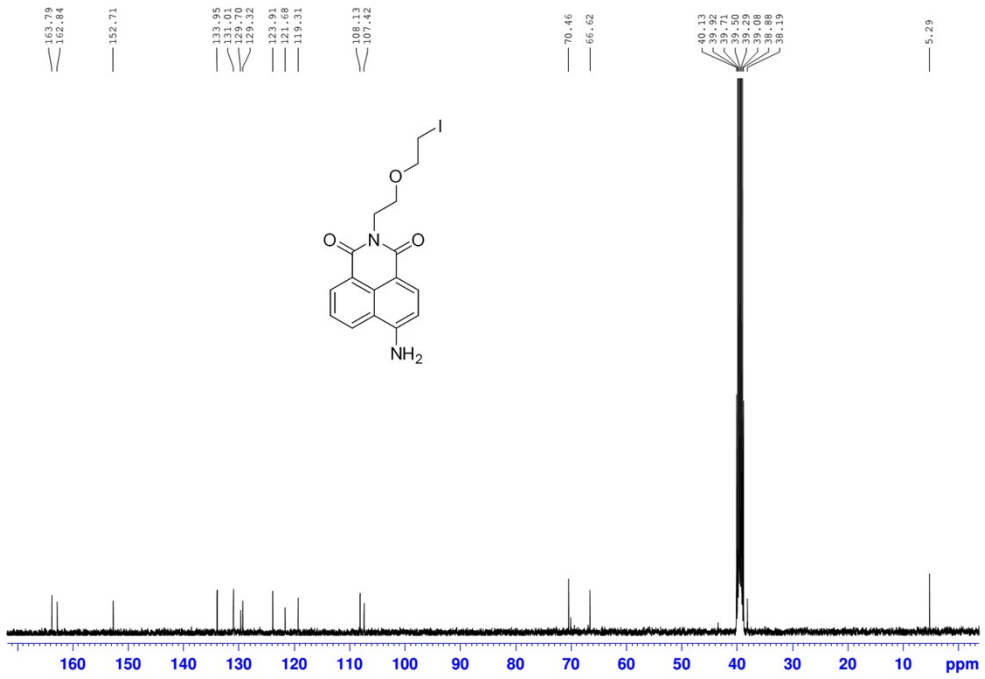

Figure S13. ^{13}C NMR Spectrum of compound **5** in DMSO-d_6 (100 MHz)**.**

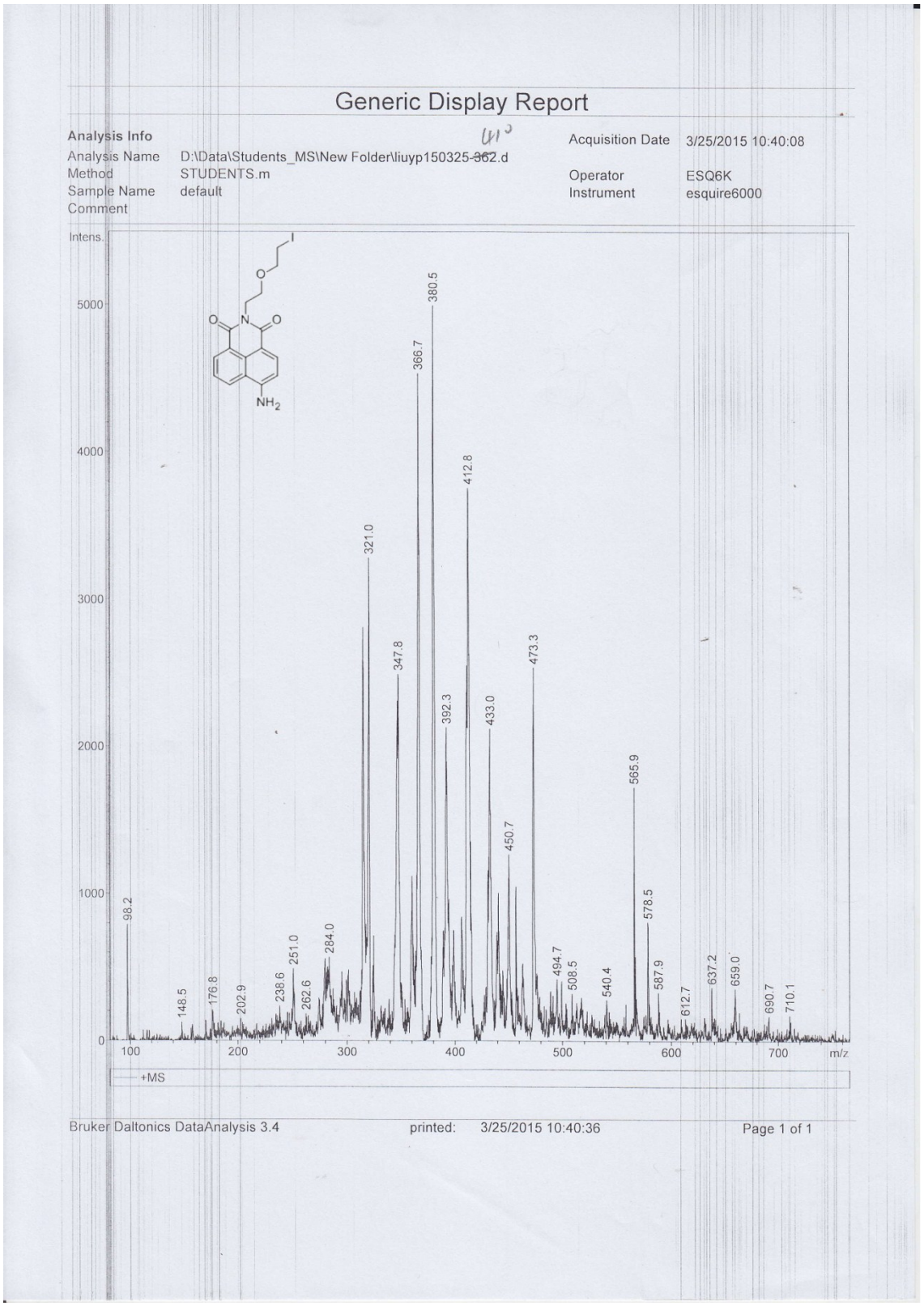

Figure S14. ESI-Mass spectrum of compound **5** (ESI-MS).

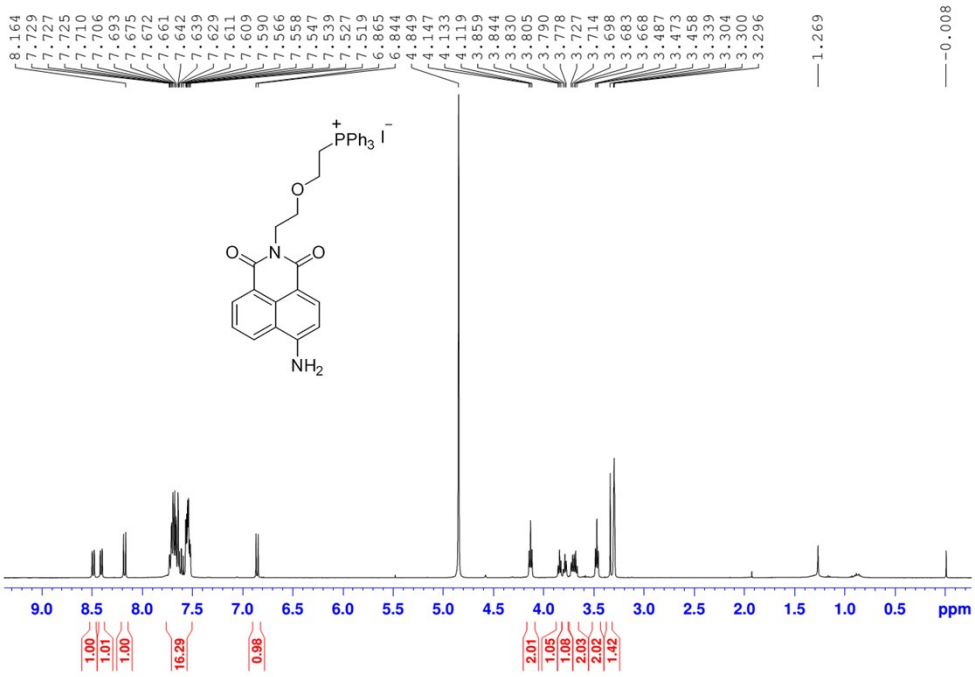

Figure S15. ^{1}H NMR Spectrum of compound **6** in CD_3OD (400 MHz)**.**

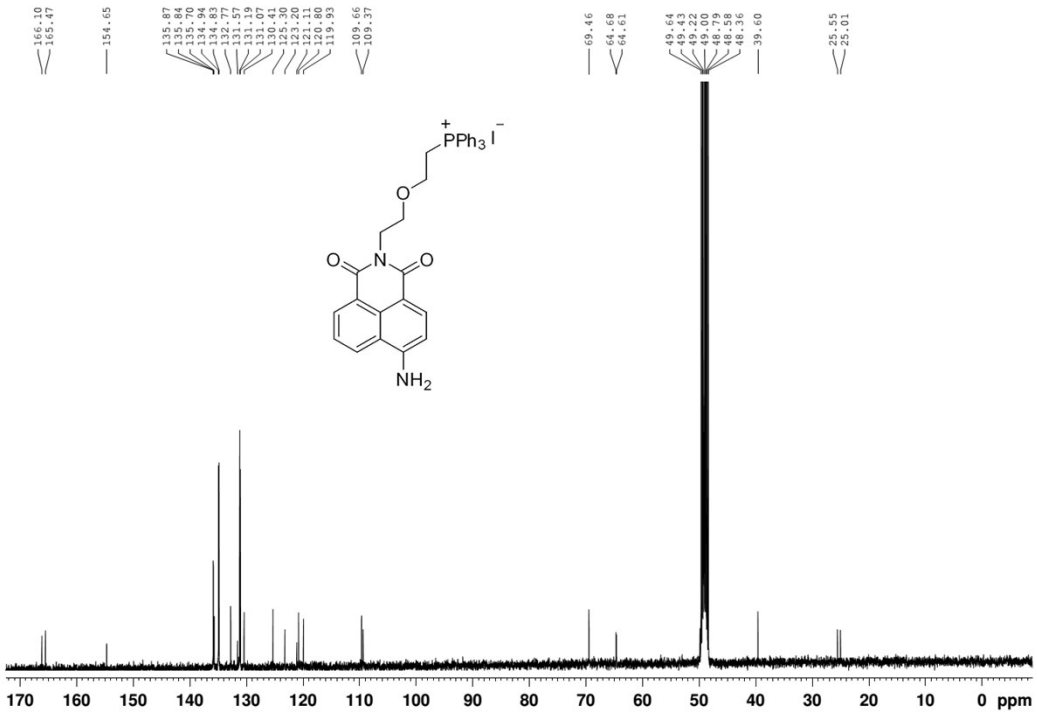

Figure S16. ^{13}C NMR Spectrum of compound **6** in CD_3OD (100 MHz)**.**

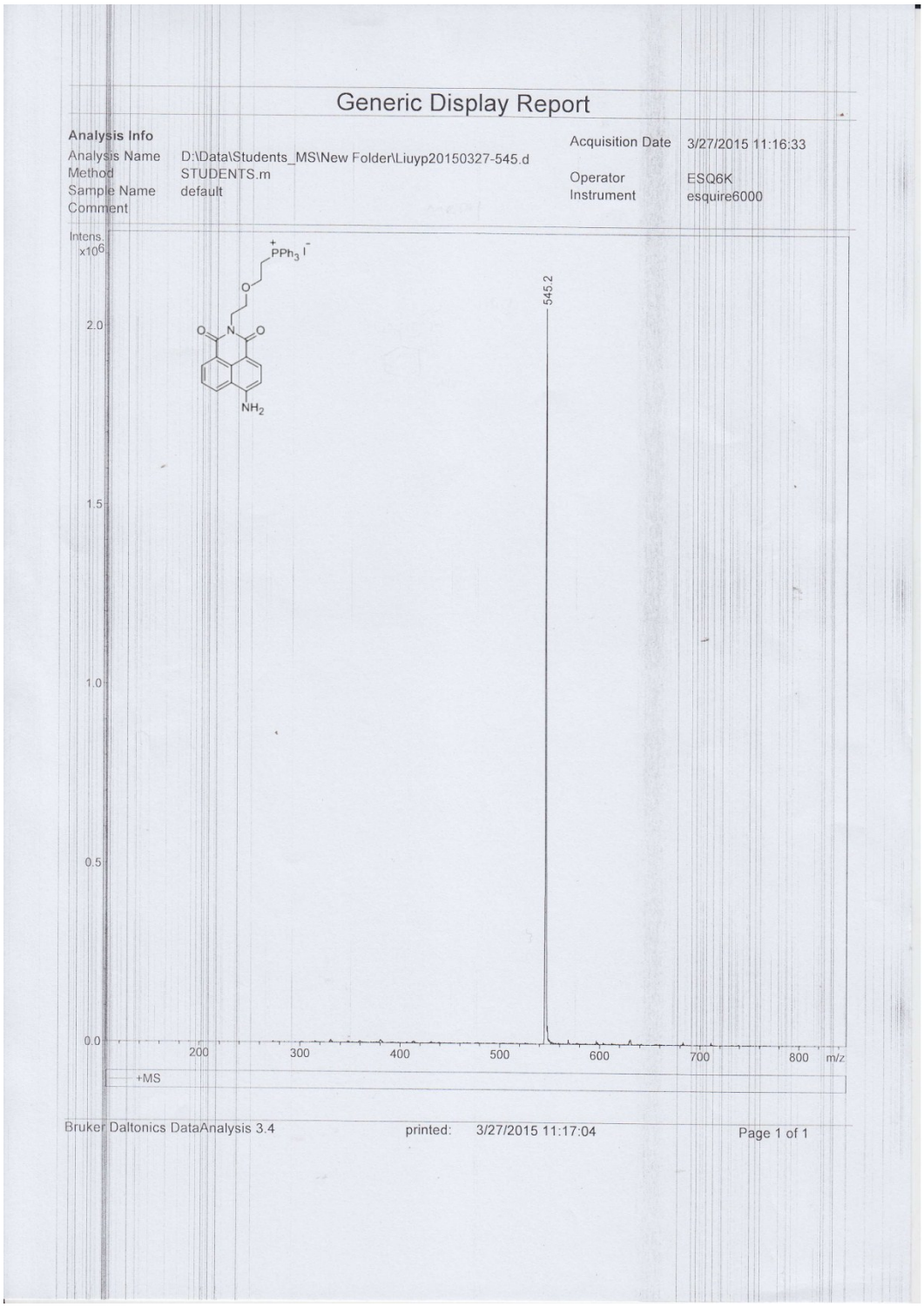

Figure S17. ESI-Mass spectrum of compound **6** (ESI-MS).

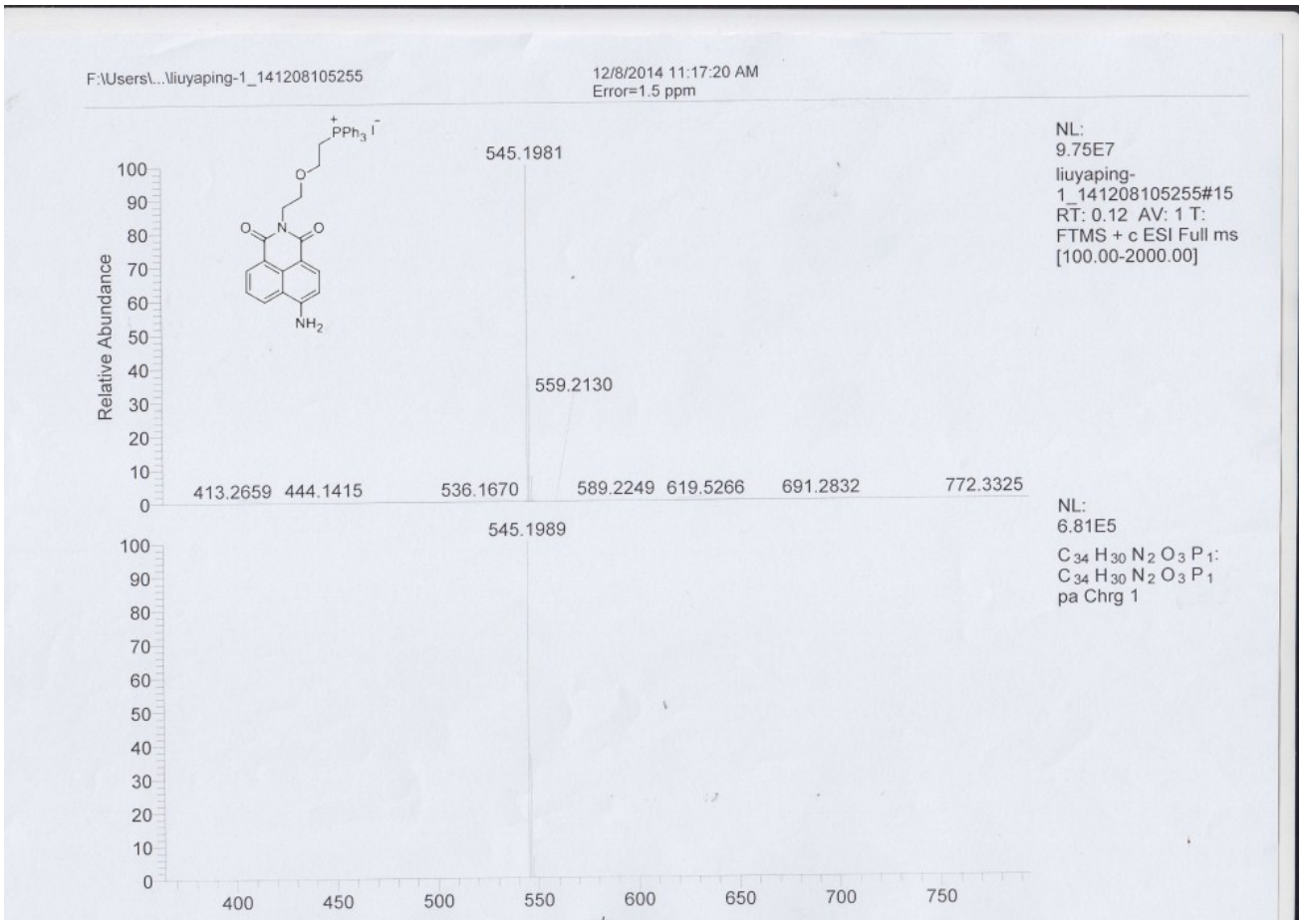

Figure S18. HR-MS of compound **6** (ESI).

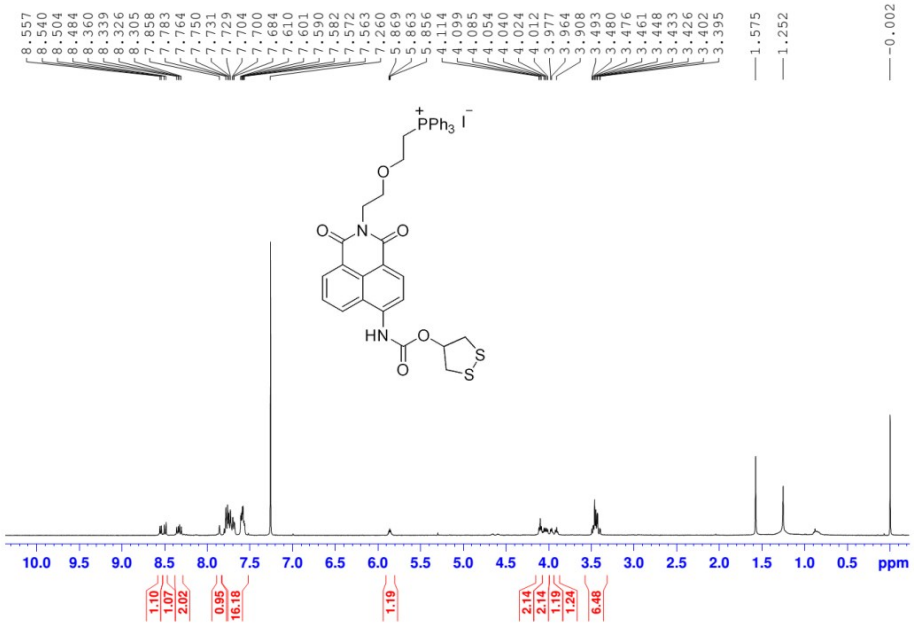

Figure S19. ^{1}H NMR Spectrum of Mito-TRFS in $CDCl_3$ (400 MHz)**.**

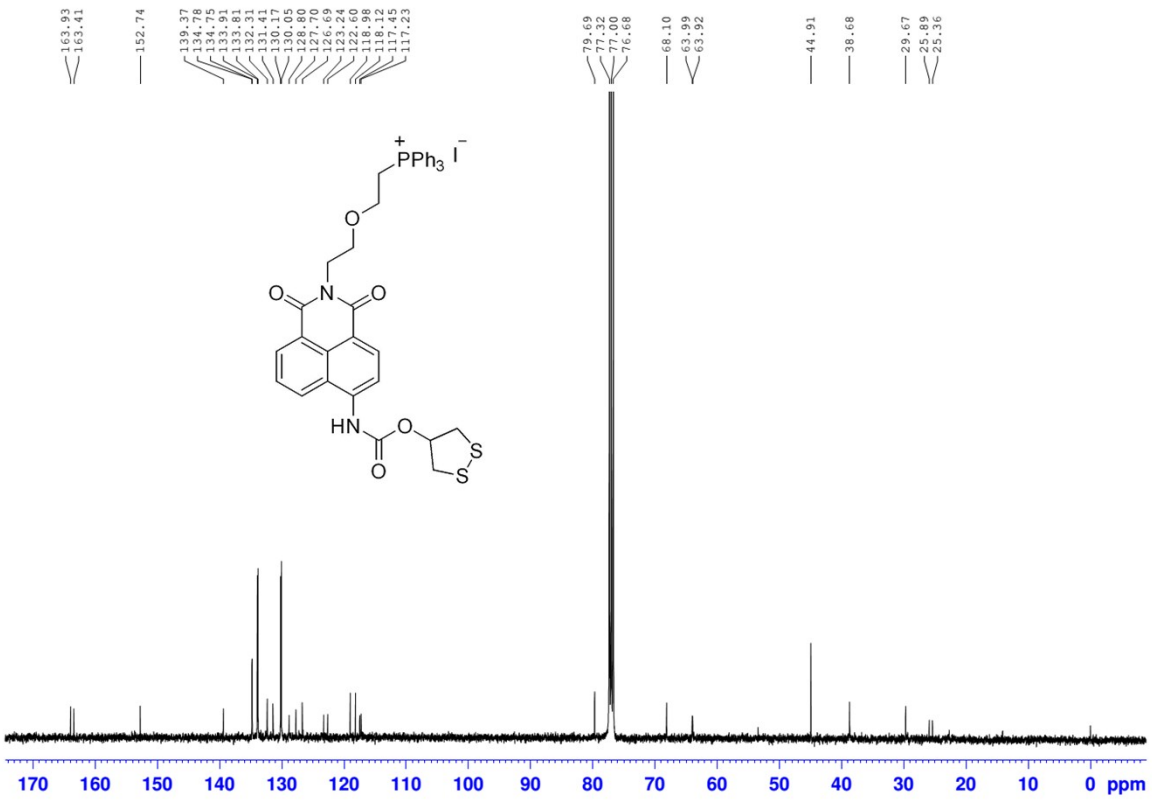

Figure S20. ^{13}C NMR Spectrum of Mito-TRFS in $CDCl_3$ (100 MHz)**.**

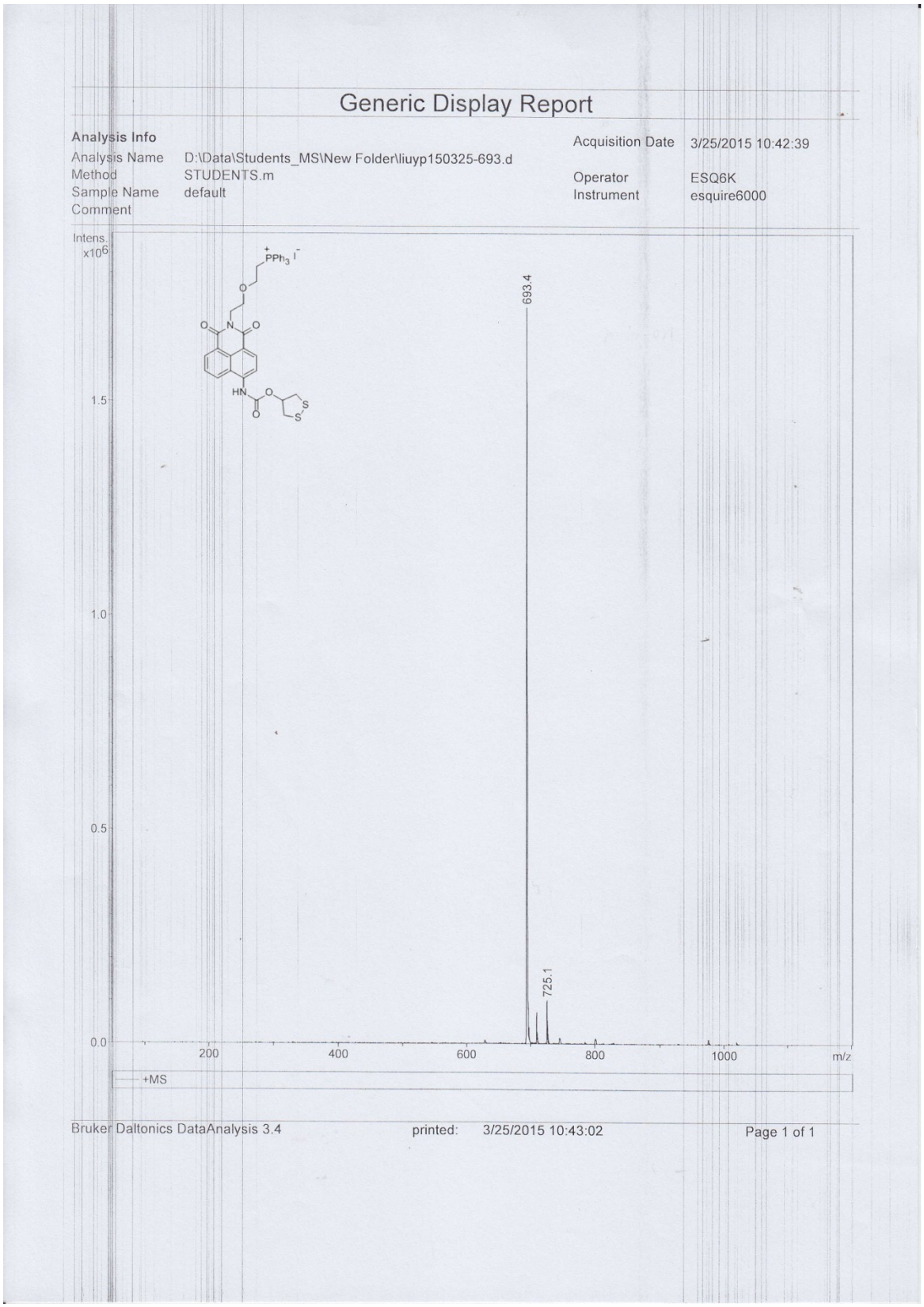

Figure S21. ESI-Mass spectrum of Mito-TRFS (ESI-MS).

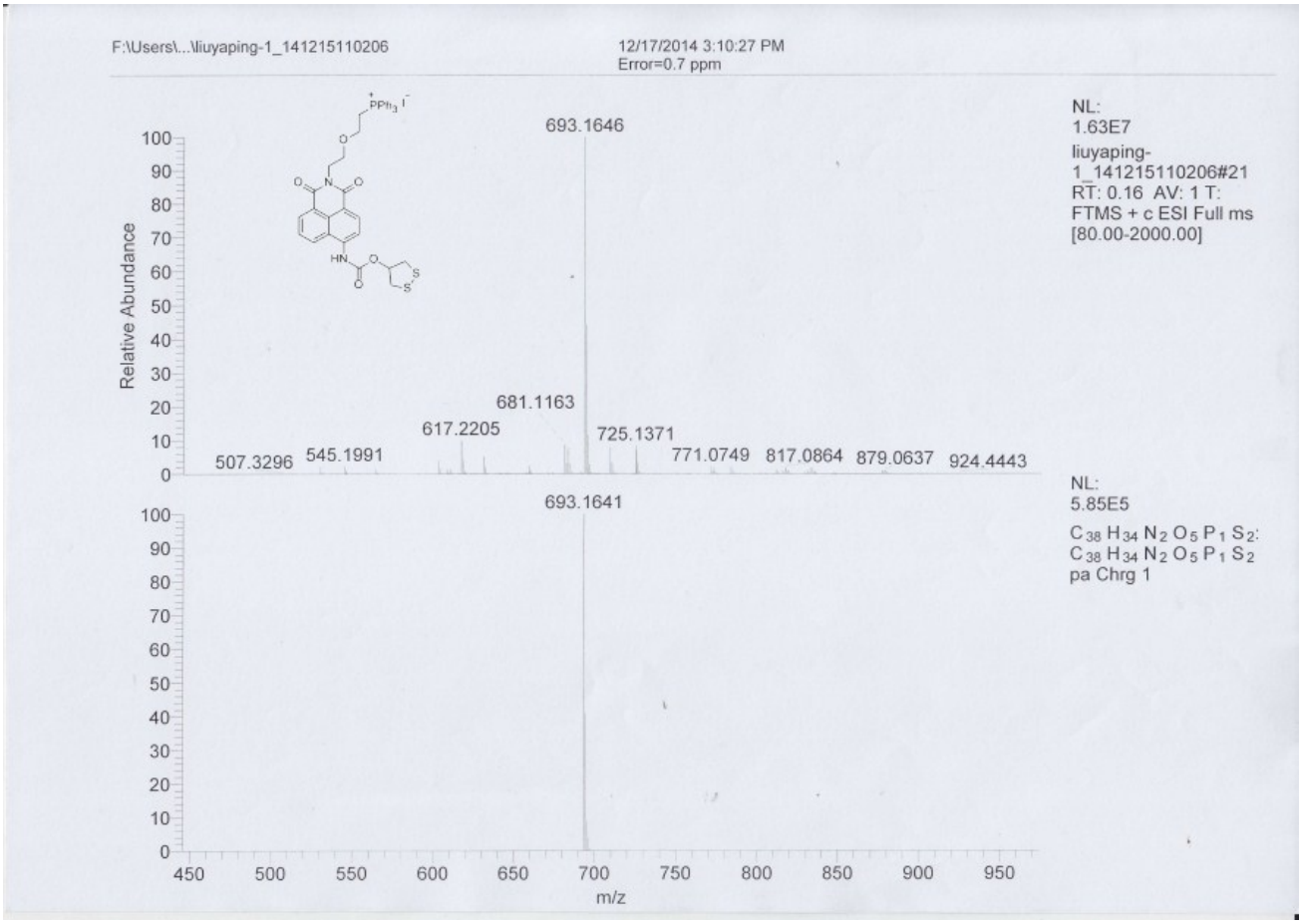

Figure S22. HRMS spectrum of Mito-TRFS (ESI).

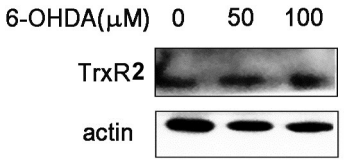

Figure S23. No significant alteration of the protein level of TrxR2 after treatment of the PC12 cells with 6-OHDA for 12 h. The protein expression was determined by the Western blotting, and actin was used as a loading control.