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Electronic Supplementary Information

Real-Time Monitoring of Intracellular Nitric Oxide Using a Long-Wavelength-Emitting Probe *via* One-Photon or Two-Photon Excitation

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Experimental section

Materials and instruments. L-arginine (L-Arg), interferon-γ (IFN-γ), lipopolysaccharide (LPS) and 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5) were purchased from Sigma Aldrich Inc. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), HeLa cells and RAW 264.7 cells were purchased from Nanjing KeyGen Biotech. Co., Ltd. Other common reagents were obtained from J&K Chemical Ltd. Compound **1** was prepared according to a previous literature.[S1] NO solution (1.9 mM) was prepared by bubbling distilled water with N_2 gas for 20 min, and then with NO gas for 30 min at room temperature. Reactive species (H_2O_2, $ONOO^-$, ClO^-, NO_2^-, $^{\cdot}OH$, NO_3^-) were prepared according to the literature.[S2] Phosphate buffered saline solution (PBS, pH = 7.4) was consisted of NaCl (0.135 M), KCl (4.7 mM), Na_2HPO_4 (10 mM), and NaH_2PO_4 (2 mM). Mass spectrometry (MS) data were obtained from an UHR TOF LC/MS Mass Spectrometer. ^{1}H NMR and ^{13}C NMR spectra were acquired from a Bruker AVANCE III HD nuclear magnetic resonance spectrometer. Absorption spectra were recorded by a Lambda950 UV-vis spectrophotometer, and fluorescence spectra were obtained from a Varian Fluorescence spectrophotometer. Cresyl

violet (ϕ = 0.54, in methanol) was used as a reference to determine the fluorescence quantum yield. Two-photon absorption spectra were measured by using femtosecond laser pulses (120 fs, 800–1000 nm) generated from a Spectra-physics Ti-sapphire laser system. One-photon excitation imaging was performed on a Lecia SP8 confocal microscope. Two-photon excitation imaging was carried out with an Olympus IX83 inverted microscope, which was based on an upright FVMPE-RS multiphoton microscopy system and a Ti-sapphire laser (Mai Tai DeepSee, Spectra-physics, USA).

Cell culture and fluorescence imaging. Cervical cancer cells (HeLa cells) and Macrophages cells (RAW 264.7 cells) were incubated in DMEM (Dulbecco's modified Eagle's medium) containing 1% (v/v) penicillin/streptomycin (10000 U/mL penicillin, 10000 μg/mL streptomycin) and 10% (v/v) FBS (fetal bovine serum), and maintained at 37 °C in a humidified air atmosphere with 5% CO_2. For exogenous NO detection, HeLa cells were incubated in 35 mm glass-bottomed dishes (Φ=20 mm) for 1 day. When reached 70-90% confluency, the cells were washed twice with DMEM, and then incubated with 2 mL of DMEM containing 25 μM NOC-5 for 60 min. **TTNO** (5 μM) was then added, and the cells were observed under a microscope. For endogenous NO study, RAW 264.7 cells were incubated for 1 day to reach 50-70% confluency, then washed twice with DMEM and treated with 3 mg/mL L-Arg, 200 U/mL INF-r and 20 μg/mL LPS for 8 h. After **TTNO** (5 μM) was added, the images were captured with a microscope.

Cytotoxicity assay. The cytotoxicity was tested using HeLa cells as a model by an MTT assay. HeLa cells were incubated in DMEM with 96-well plates for 12 h. Then the DMEM was replaced with fresh DMEM containing different concentrations of TTNO (0–40 μM). After 24 h, the cells were washed twice with PBS, and 0.5 mg/mL of MTT was added into each well. Formazan crystals were dissolved in DMSO after the cells incubated for further 4 h at 37 °C. Optical densities (O.D.) at 490 nm were tested with a microplate reader. Cell viability was determined according to the equation: viability = (mean O.D. of treated wells/mean O.D. of control wells) × 100%.

Tissue imaging. Tissue slices were prepared from rat liver frozen slices. The tissue was cut into slices with a depth of ~150 μm. The slices were incubated with 10 μM of TTNO for 1 h at

37 °C, and then cultured with 100 μM of NOC-5 for another 1 h. After being washed three times with PBS, the slices were imaged with a two-photon microscope.

Theoretical calculation. The calculations were implemented by using Gaussian 09 program package. The geometrical structures for **TTNO** and **TTNO-P** in the ground state were first optimized by density functional theory (DFT) method at the B3LYP/6-311G (d, p) level with Grimme's D3 empirical dispersion correction.[S3] Then, to determine the vertical excitation properties, 6 singlet excited-states based on the optimized structures in the ground state were calculated by time-dependent density functional theory (TD-DFT) with the same function used in the optimization process, respectively.[S4] Visualization of the frontier molecular orbitals was performed by GaussView.

Synthesis of compound 2. Compound **1** (334 mg, 1.0 mmol), 4-fluoro-1,2-dinitrobenzene (372 g, 2.0 mmol) and K_2CO_3 (276 mg, 2.0 mmol) in 30 mL of MeCN were heated to reflux for 6 h. The solvent was removed by a rotary evaporator, then the residue was purified by silica gel chromatography using petroleum ether/ethyl acetate (10/1, v/v) as eluent to afford compound **2** as a dark green solid (470 mg, 94%). ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C, TMS): δ = 8.34–8.32 (m, 1H), 8.28–8.25 (m, 2H), 7.98 (d, J = 2.8 Hz, 1H), 7.63–7.58 (m, 1H), 7.53 (dd, J_1 = 9.2 Hz, J_2 = 2.4 Hz, 1H), 6.87 (dd, J_1 = 9.2 Hz, J_2 = 2.4 Hz, 1H), 6.72 (d, J = 2.8 Hz, 1H), 6.33 (s, 1H), 3.53 (q, J = 7.2 Hz, 4H), 1.17 (t, J = 7.2 Hz, 6H); ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, TMS): δ = 181.49, 161.77, 156.94, 152.75, 151.78, 147.24, 145.31, 137.62, 134.67, 131.74, 128.99, 124.82, 121.58, 114.92, 114.53, 111.12, 104.82, 100.00, 96.54, 45.07, 12.99. MS (ESI, *m/z*): calcd for $C_{26}H_{20}N_4O_7$ [M + H], 501.1; found, 501.1.

Synthesis of TTNO. Compound **2** (300 mg, 0.6 mmol) was stirred in 20 mL of THF, and 50 mg of 10% Pd/C was added into the reaction flask after the mixture was flushed with H_2 for 15 min. Then the mixture was stirred for 12 h under H_2 atmosphere at room temperature. After solvent was removed by evaporation in *vacuo*, the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH = 15/1 (v/v)) to obtain **TTNO** as a dark red solid (187 mg, 71%). ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C, TMS): δ = 8.08 (d, J = 8.8 Hz, 1H), 7.88 (d, J = 2.8 Hz, 1H), 7.57 (d, J = 8.8 Hz, 1H), 7.25 (dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, 1H), 6.78 (dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, 1H), 6.65 (d, J = 2.4 Hz, 1H), 6.57 (d, J = 8.4 Hz, 1H), 6.35 (d, J = 2.4 Hz, 1H), 6.23 (dd, J_1 = 8.4 Hz, J_2 = 2.4 Hz, 2H), 4.75 (s, 2H), 4.46 (s, 2H), 3.50 (q, J = 7.2 Hz, 4H), 1.16 (t, J = 7.2 Hz,

6H); ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, TMS): δ = 181.81, 162.32, 152.39, 151.36, 146.78, 138.58, 137.29, 132.64, 131.55, 128.07, 126.13, 124.44, 119.65, 115.32, 110.56, 109.40, 108.81, 106.83, 104.66, 96.47, 44.97, 12.98. HR-MS (ESI, *m/z*): calcd for $C_{26}H_{24}N_4O_3$ [M + H], 441.1921; found, 441.1918.

Conversion of TTNO to TTNO-P. NO was bubbled into a solution of **TTNO** (88 mg, 0.2 mmol) in 20 mL of CH_2Cl_2 for 30 min with stirring at room temperature. After the mixture was concentrated in *vacuo*, the residue was purified by silica gel chromatography with petroleum ether/ethyl acetate (10/1, v/v) to produce **TTNO-P** as a dark red solid (73 mg, 81%). ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C, TMS): δ = 8.18 (d, *J* = 8.8 Hz, 1H), 8.11–8.08 (m, 1H), 8.01 (s, 1H), 7.68 (s, 1H), 7.52 (d, *J* = 9.2 Hz, 1H), 7.42 (d, *J* = 8.8 Hz, 1H), 7.36–7.34 (m, 1H), 7.27–7.24 (m, 1H), 6.78 (d, *J* = 9.2 Hz, 1H), 6.67 (s, 1H), 6.27 (s, 1H), 3.50 (q, *J* = 7.2 Hz, 4H), 1.19 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C, TMS): δ = 181.56, 152.42, 151.44, 146.98, 138.01, 134.20, 131.53, 128.38, 124.47, 110.64, 104.64, 96.39, 44.92, 12.90. HR-MS (ESI, *m/z*): calcd for $C_{26}H_{21}N_5O_3$ [M + H], 452.1717; found, 452.1713.

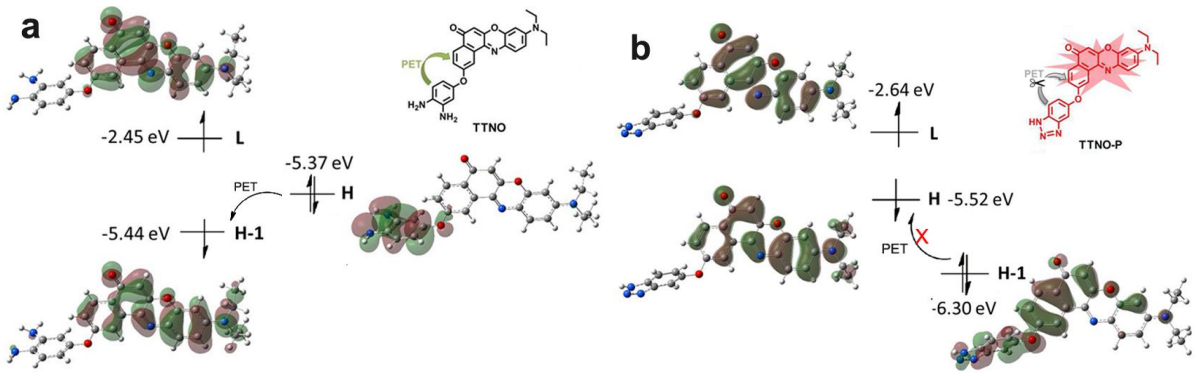


Fig. S1 Geometries and orbits of (a) **TTNO** and (b) the product **TTNO-P** in the excited-states obtained from DFT calculations.

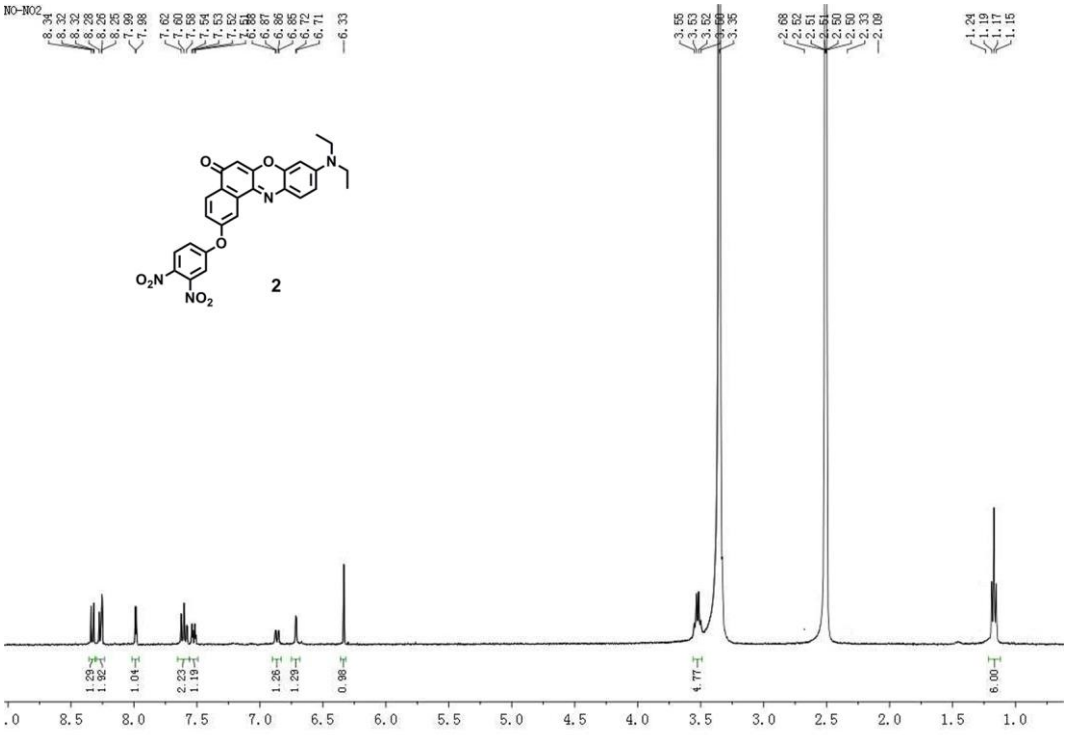


Fig. S2 ^{1}H NMR spectrum of compound **2**.

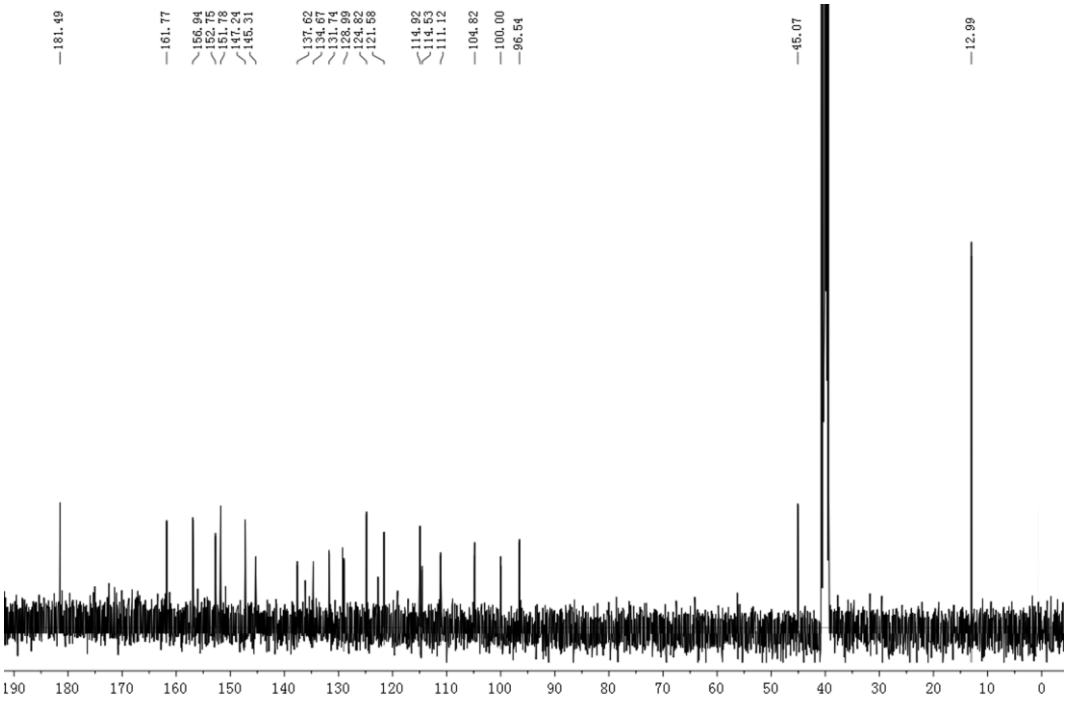


Fig. S3 ^{13}C NMR spectrum of compound **2**.

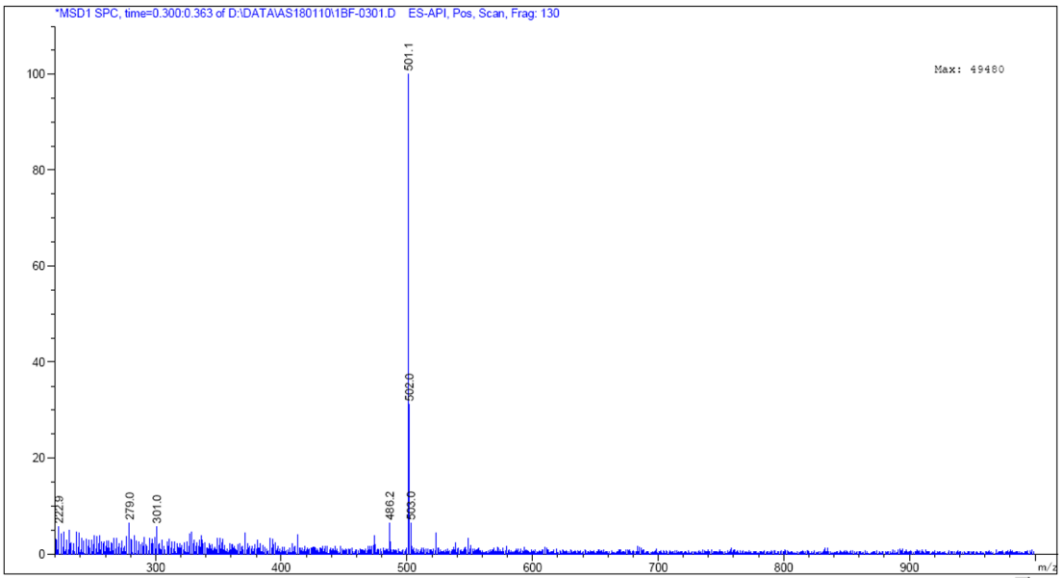


Fig. S4 EI-MS spectrum of compound **2**.

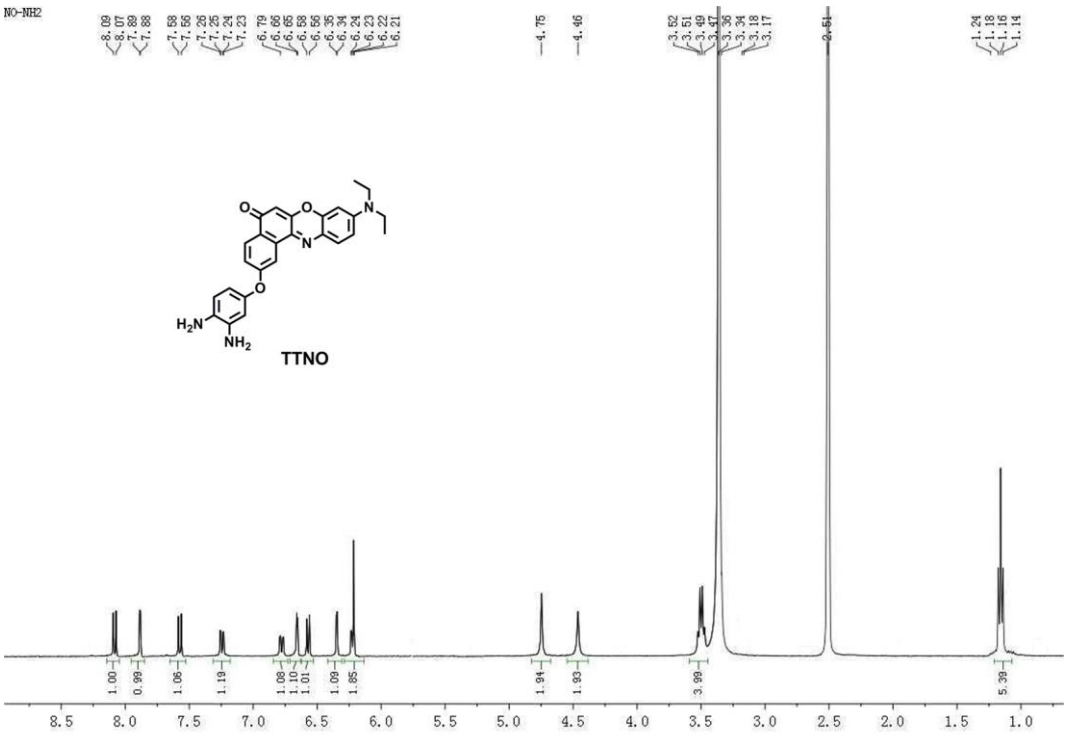


Fig. S5 ^{1}H NMR spectrum of **TTNO**.

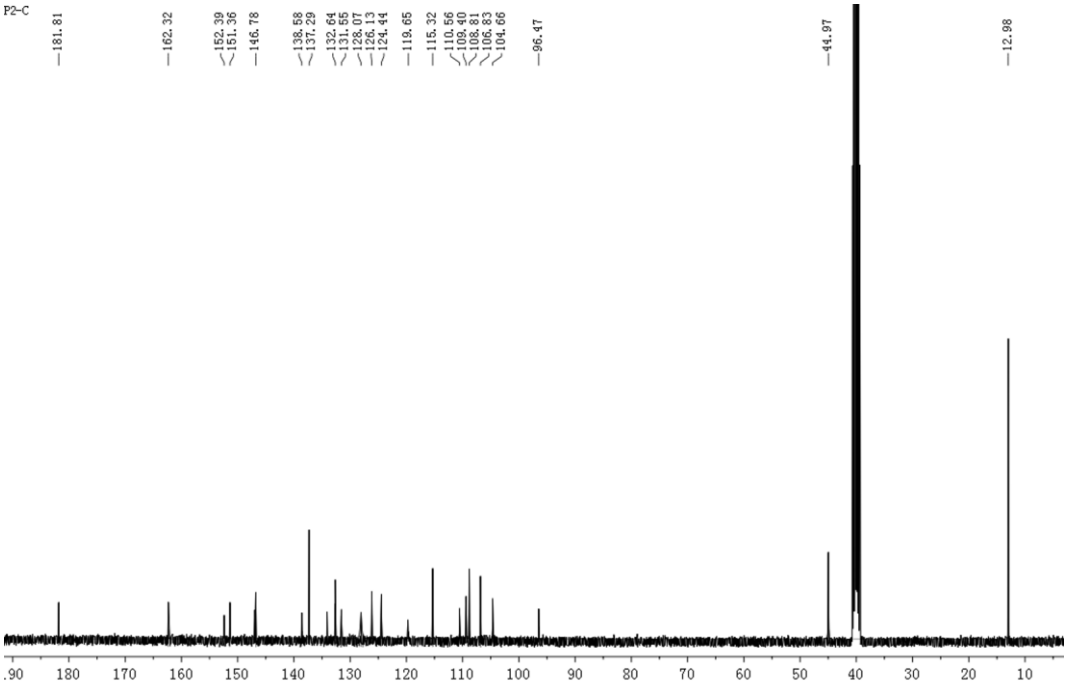
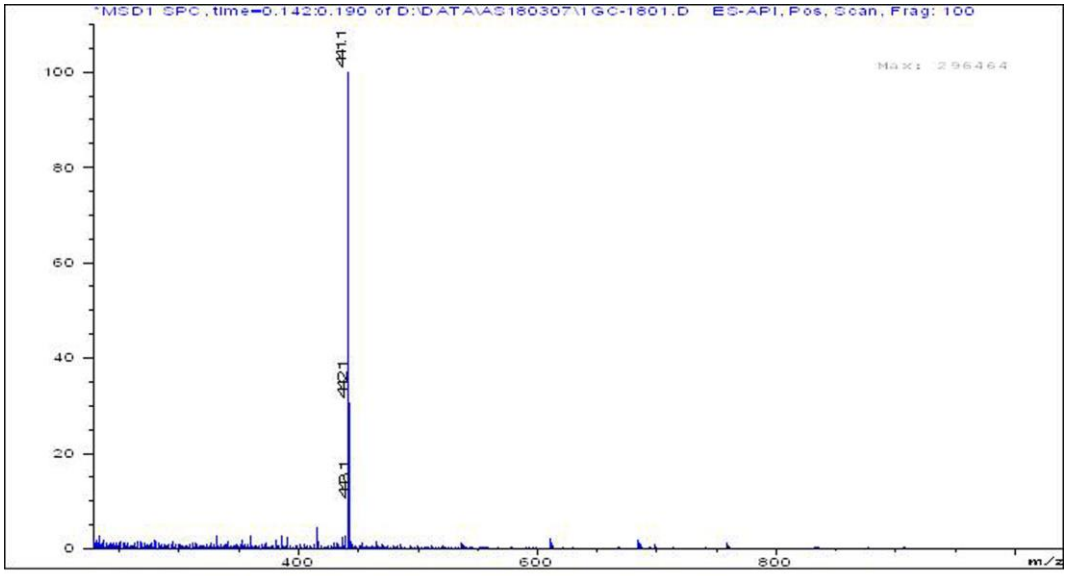


Fig. S6 ^{13}C NMR spectrum of **TTNO**.



Elemental composition search on mass 441.19

m/z= 436.19-446.19

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
441.1918	441.1921	-0.74	16.5	$C_{26}H_{25}O_3N_4$
	441.1908	2.29	11.5	$C_{25}H_{29}O_7$
	441.1935	-3.78	16.0	$C_{28}H_{27}O_4N$

Fig. S7 HR-MS spectrum of **TTNO**.

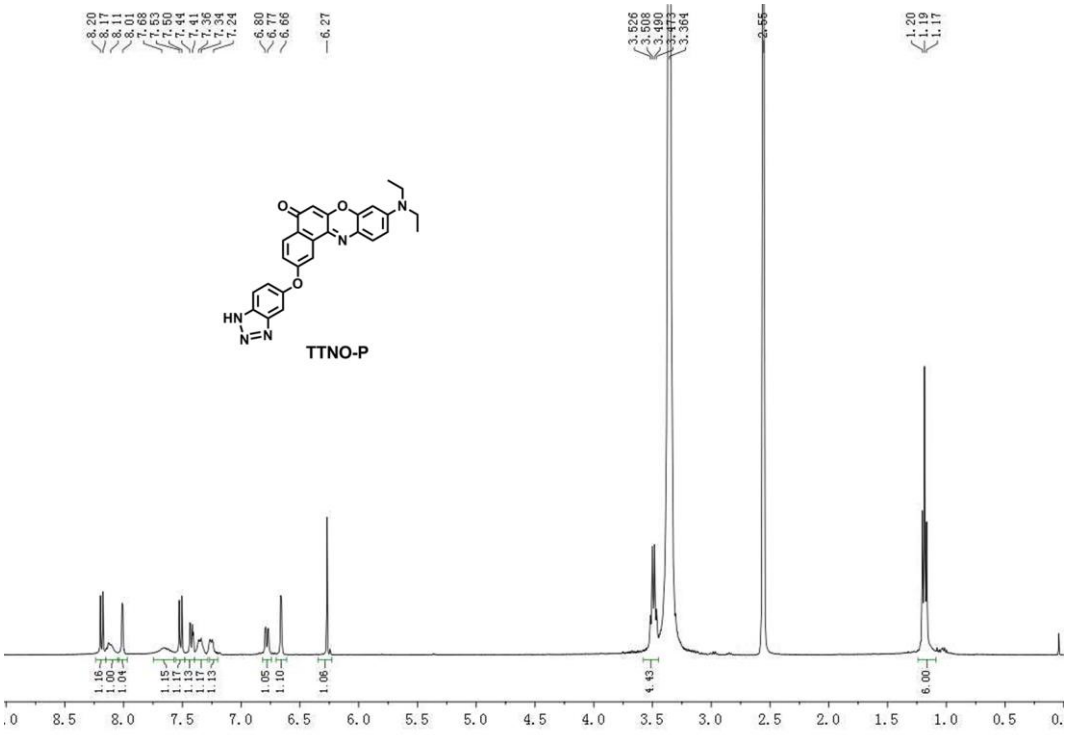


Fig. S8 ^{1}H NMR spectrum of **TTNO-P**.

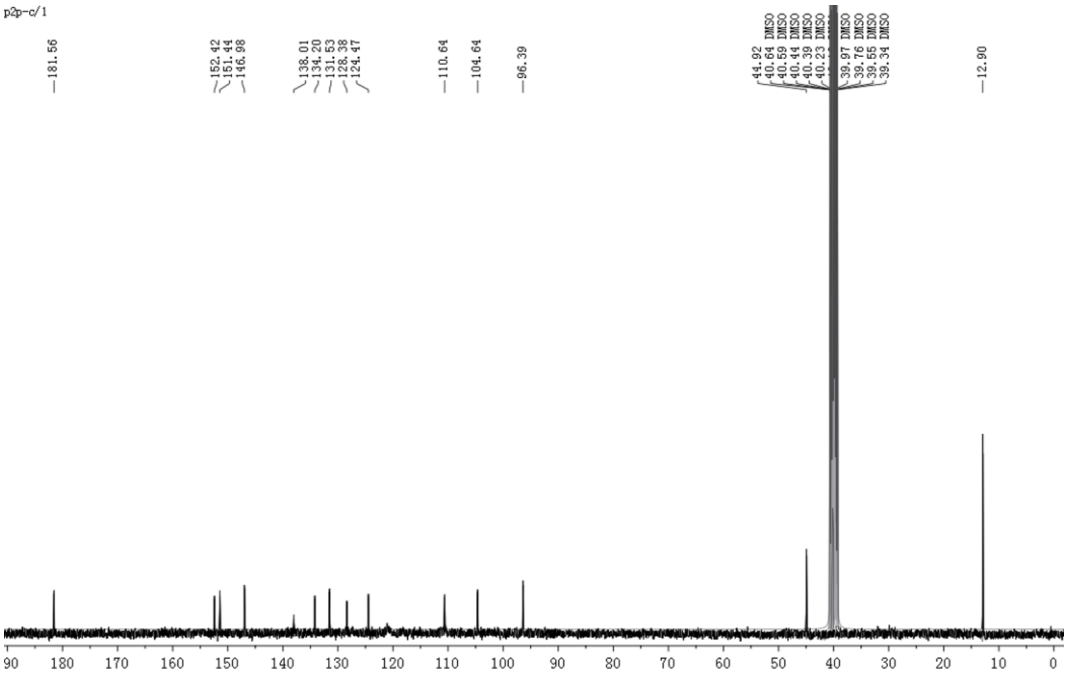
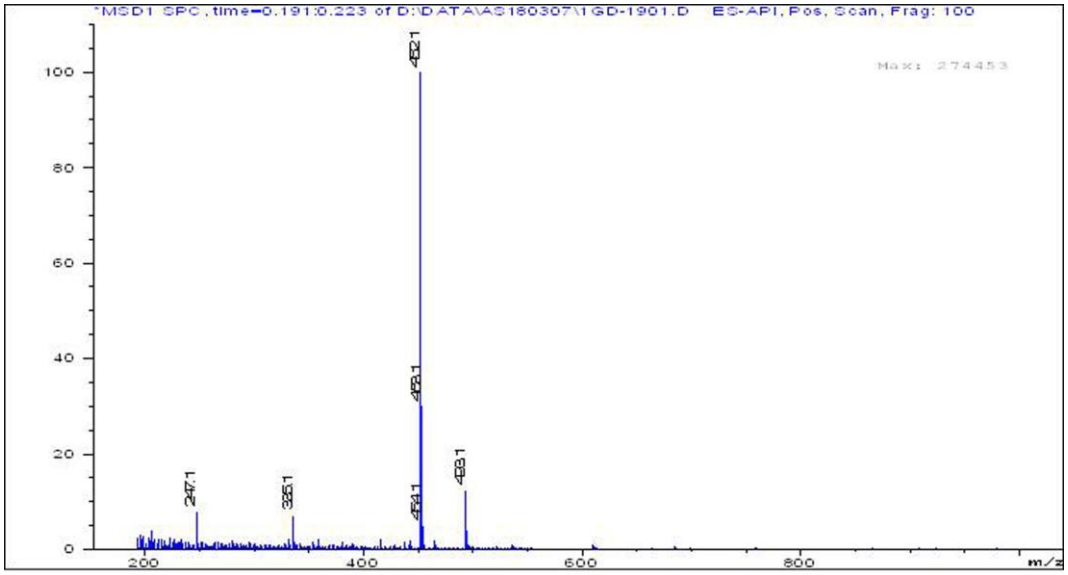


Fig. S9 ^{13}C NMR spectrum of **TTNO-P**.



Elemental composition search on mass 452.17

m/z= 447.17-457.17

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
452.1713	452.1717	-0.85	18.5	$C_{26}H_{22}O_3N_5$
	452.1704	2.10	13.5	$C_{25}H_{26}O_7N$
	452.1731	-3.82	18.0	$C_{28}H_{24}O_4N_2$

Fig. S10 HR-MS spectrum of **TTNO-P**.

Table S1. Sensing data for two-photon NO probes.

Probes[a]	λ_{em}^{max} (nm)[b]	DL (nM)[c]	RT in vitro (s)[d]	RT in cells (min)[e]	Refs
ANO1	502	5	NA	NA	S5
Lyso-NINO	530	5	NA	~15	S6
QNO	535	84	~300	NA	S7
Mem-NO	538	74	NA	~40	S8
NO-QA5	540	15	~25	NA	S9
ADNO	546	NA	~300	>30	S10
Ir-Mito-NO	576	NA	~10	~20	S11
Mito-N	595	21×10^3	~1200	NA	S12
NCNO	613	37	~120	NA	S13
NRNO	650	46	~180	NA	S14
TTNO	658	9	~40	~8	This work
SiRNO	672	14	~90	NA	S15

a) Two-photon NO probes that have been reported in the literatures. b) Emission peak wavelength. c) Detection limit. d) Response-time in vitro. e) Response-time in cells.

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