

# Mitochondria-Targeted Approach: Remarkably Enhanced Cellular Bioactivities of TPP2a as Selective Inhibitor and Probe toward TrxR

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## **1. Chemistry**

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## 1. Chemistry

**General** Unless otherwise stated, all manipulations were carried out without taking precautions to exclude air and moisture. All chemicals and solvents (HPLC grade or better) were sourced commercially and used as received. Melting points were determined with OptiMelt MPA100. Mass spectra were recorded on Shimadzu LCMS-IT-TOF mass spectrometer. The HPLC analyses were performed on DIONEX Ultimate 3000 HPLC System, C18 column (250 mm 4.6 mm, 5  $\mu$ m). The optimum HPLC separation was achieved on proper mode with a mobile phase composed of methanol, acetonitrile, and 0.1% trifluoroacetic acid at a flow rate of 1 mL min<sup>-1</sup>. The <sup>1</sup>H, <sup>13</sup>C and <sup>31</sup>P NMR spectra were recorded using tetra-methylsilane and phosphoric acid as the internal standard on a Bruker BioSpin GmbH spectrometer at 400, 101 and 162 MHz, respectively; and the coupling constants are reported in hertz. The following abbreviations were used to explain the multiplicities: s, singlet; d, doublet; t, triplet; dd, double doublet; m, multiplet, br, broad. The reactions were followed by thinlayer chromatography (TLC) on glass-packed precoated silica gel plates and visualized in an iodine chamber or with a UV lamp.

**Synthesis of M1** Acetyl acetone (10 g, 100 mmol) and boron trioxide (0.49 g, 7.0 mmol, 0.7 eq.) were mixed and stirred in ethyl acetate at 70 °C for 30 min, then vanillin (1.52 g, 10 mmol) and tributyl borate (2.3 g, 10 mmol) were added and the mixture was stirred for further 30 min. Next, n-butylamine (0.73 g, 10 mmol) dissolved in dry ethyl acetate (3.0 mL) was added dropwisely, the mixture was stirred for 2.5 h at 85 °C. After that, Hydrochloric acid (1.0 mol L<sup>-1</sup>, 60 °C) was added and the organic phase was separated and washed with water, dried with Na<sub>2</sub>SO<sub>4</sub>, and

evaporated in vacuo after filtration. The raw material was purified by flash chromatography on silica gel using the eluent petroleum ether-ethyl acetate to give M1 (1.05 g, 45% yield) as a yellow solid: m.p. 144.0-145.3 °C. <sup>1</sup>H NMR (400 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 15.73 (s, 1H), 9.60 (s, 1H), 7.49 (d, J = 16.0 Hz, 1H), 7.29 (s, 1H), 7.11 (d, J = 8.1 Hz, 1H), 6.81 (d, J = 8.1 Hz, 1H), 6.64 (d, J = 15.9 Hz, 1H), 5.85 (s, 1H), 3.82 (s, 3H), 2.12 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 196.96, 178.02, 147.78, 146.85, 140.10, 127.66, 122.65, 120.31, 114.86, 109.57, 100.68, 55.94, 26.79 ppm. HRMS (ESI) m/z [M + Na]<sup>+</sup> for C<sub>13</sub>H<sub>14</sub>O<sub>4</sub> predicted 257.0784, measured 257.0661; HPLC purity of 96.12%.

**Synthesis of 2a** M1 (0.47 g, 2.0 mmol) and boric anhydride (0.12 g, 1.7 mmol, 0.7eq.) were dissolved in ethyl acetate and stirred at 70 °C for 30 min. Then 5-methyl-2-furaldehyde (0.22 g, 2.0 mmol) and tributyl borate (0.46 g, 2.0 mmol) were added, and the mixture was stirred for 30 min. After that, n-butylamine (0.15 g, 2.0 mmol) dissolved in ethyl acetate was added dropwise. After increasing the temperature to 85 °C, stirring was continued for 2.5 h. The mixture was then hydrolyzed by adding aqueous HCl (1.0 mol L<sup>-1</sup>) and stirring at 60 °C for 30 min. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate three times. The combined organic layers were washed with water until neutral. The solvent was removed in vacuo. The crude products were purified by flash column to give 2a (0.33 g, 50% yield) as an orange red solid: m.p. 123.3-124.7 °C. <sup>1</sup>H NMR (400 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 7.56 (d, J = 15.9 Hz, 1H), 7.34 (dd, J = 16.4, 8.7 Hz, 2H), 7.14 (dd, J = 8.2, 1.7 Hz, 1H), 6.93-6.74 (m, 3H), 6.73 (d, J = 15.8 Hz, 1H), 6.46 (d, J = 15.6

Hz, 1H), 6.30 (d,  $J = 3.2$  Hz, 1H), 6.09 (s, 1H), 3.84 (s, 3H), 2.36 (s, 3H).  $^{13}\text{C}$  NMR (101 MHz,  $(\text{CD}_3)_2\text{SO}$ )  $\delta$  183.79, 181.79, 155.66, 149.77, 149.41, 147.98, 141.04, 126.43, 126.26, 123.20, 121.03, 119.58, 117.63, 115.68, 111.27, 109.63, 101.18, 55.65, 13.62 ppm. HRMS (ESI)  $m/z$   $[\text{M} + \text{Na}]^+$  for  $\text{C}_{19}\text{H}_{18}\text{O}_5$  predicted 349.1046, measured 349.0842; HPLC purity of 97.22%.

**Synthesis of M2** To a solution of 2a (0.33 g, 1 mmol) in acetone (30 mL), anhydrous  $\text{K}_2\text{CO}_3$  (1.10 g, 8.0 mmol, 8 eq.) and 1,10-dibromodecane (2.40 g, 8.0 mmol, 8 eq.) were added, the mixture was stirred overnight at RT. Then  $\text{K}_2\text{CO}_3$  was removed by filtration, and the solvent was evaporated under the vacuum. The crude products were purified by flash chromatography on silica gel using the eluent petroleum ether-ethyl acetate to give M2 (0.38 g, 70% yield) as orange solid: m.p. 89.1-90.3°C.  $^1\text{H}$  NMR (400 MHz,  $(\text{CD}_3)_2\text{SO}$ )  $\delta$  7.58 (d,  $J = 15.8$  Hz, 1H), 7.35 (dd,  $J = 13.7, 8.5$  Hz, 2H), 7.23 (d,  $J = 8.4$  Hz, 1H), 7.00 (d,  $J = 8.4$  Hz, 1H), 6.86 (d,  $J = 3.2$  Hz, 1H), 6.79 (d,  $J = 15.9$  Hz, 1H), 6.47 (d,  $J = 15.6$  Hz, 1H), 6.31 (d,  $J = 2.4$  Hz, 1H), 6.11 (s, 1H), 4.00 (t,  $J = 6.5$  Hz, 2H), 3.82 (s, 3H), 3.52 (t,  $J = 6.7$  Hz, 2H), 2.36 (s, 3H), 1.88 – 1.66 (m, 4H), 1.45-1.17 (m, 12H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  183.51, 182.60, 155.53, 150.71, 150.40, 149.56, 140.41, 127.94, 126.77, 122.64, 122.05, 120.05, 116.66, 112.48, 110.30, 109.16, 101.62, 69.02, 56.05, 34.00, 32.81, 29.39, 29.33, 29.30, 29.04, 28.73, 28.15, 25.90, 13.96 ppm. HRMS (ESI)  $m/z$   $[\text{M} + \text{Na}]^+$  for  $\text{C}_{29}\text{H}_{37}\text{BrO}_5$  predicted 567.1717, measured 567.1732; HPLC purity of 98.44%.

**Synthesis of TPP2a** The mixture of M2 (0.38 g, 0.69 mmol) and  $\text{PPh}_3$  (0.90 g, 3.45 mmol, 5 eq.) in 15 mL acetonitrile was stirred for 2 d at 100°C. Evaporation of the

solvent, the solid was washed with ethyl acetate. The crude products were purified by flash chromatography on silica gel using the eluent ethyl acetate-methanol to afford the desired product TPP2a (0.25 g, 45% yield) as a crimson solid: m.p. 67.5-68.4°C. <sup>1</sup>H NMR (400 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 7.93-7.74 (m, 15H), 7.58 (d, J = 15.8 Hz, 1H), 7.37 (d, J = 15.6 Hz, 1H), 7.34 (d, J = 1.8 Hz, 1H), 7.23 (dd, J = 8.4, 1.7 Hz, 1H), 7.00 (d, J = 8.4 Hz, 1H), 6.86 (d, J = 3.2 Hz, 1H), 6.80 (d, J = 15.9 Hz, 1H), 6.47 (d, J = 15.6 Hz, 1H), 6.31 (m, 1H), 6.12 (s, 1H), 3.99 (t, 2H), 3.81 (s, 3H), 3.55 (t, 2H), 2.36 (s, 3H), 1.70 (m, 2H), 1.53-1.21 (m, 14H). <sup>13</sup>C NMR (101 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 183.39, 182.20, 155.75, 150.39, 149.74, 149.14, 140.52, 134.83(4C), 133.54(6C), 130.19(6C), 127.44, 126.61, 122.98, 121.95, 119.55, 118.98, 118.13, 117.79, 112.61, 110.63, 109.68, 101.32, 68.18, 55.67, 29.81, 29.65, 28.76, 28.59, 28.03, 25.43, 21.68, 20.43, 19.94, 13.65 ppm. <sup>31</sup>P NMR (162 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 24.08. HRMS (ESI) m/z [M]<sup>+</sup> for C<sub>47</sub>H<sub>52</sub>O<sub>5</sub>P<sup>+</sup> predicted 727.3547, measured 727.3548; HPLC purity of 99.07%.

**Synthesis of TPPOH** A solution of 10-bromo-1-decanol (0.24 g, 1 mmol) in dry acetonitrile (50 mL) was treated with triphenylphosphine (1.31 g, 5 mmol). The solution was refluxed with stirring for 48 h. The solvent was removed in a vacuum, and the crude product was subjected to chromatography on silica gel (EtOAc, MeOH) to yield compound TPPOH as a colorless oil (0.30 g, 60%). <sup>1</sup>H NMR (400 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 8.19-7.57 (m, 15H), 3.56 (m, 2H), 3.40-3.35 (m, 2H), 1.57-1.36 (m, 6H), 1.18 (m, 10H). <sup>13</sup>C NMR (101 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 134.83(4C), 133.60(6C), 130.26(6C), 118.99, 118.14, 60.65, 32.46, 29.82, 29.66, 28.86, 28.83, 28.62, 28.05, 25.44, 21.72 ppm. HRMS (ESI) m/z [M]<sup>+</sup> for C<sub>28</sub>H<sub>36</sub>OP<sup>+</sup> predicted 419.2498,

measured 419.2502; HPLC purity of 98.05%.

## **2. Experimental methods**

**Sample preparation** TPP2a, 2a, TPPOH and DNCB were dissolved in DMSO to obtain stock solutions (10 mM). NACs, DTT, metal salts were dissolved in double distilled water to obtain stock solutions (10 mM); TrxR, Trx, HRP, SOD, BSA, trypsin, tubulin are dissolved in PBS buffer to obtain stock solutions (10  $\mu$ M). These stock solutions were diluted with PBS buffer (pH 7.4) to the desired concentration before use, and the DMSO is no more than 1% (v/v) in all experiments. For fluorescence detection, TrxR, Trx are activated by DTT (10  $\mu$ M) at room temperature for 30 min before use; for consistency, other proteins are also added DTT (10  $\mu$ M) at room temperature for 30 min before use.

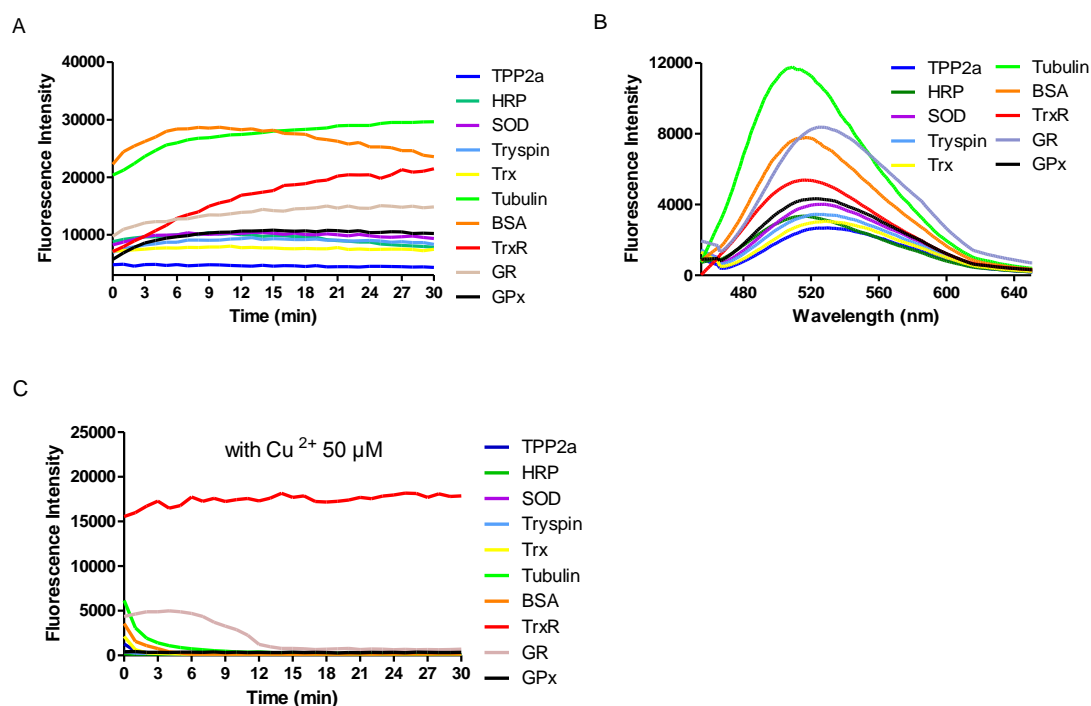
**Molecular docking** The complex model of TrxR with TPP2a was generated by the MOE software. The protein structure was extracted from the PDB file (PDB code 2ZZB) of the human TrxR,<sup>1</sup> and the ligand conformer was generated by MOE using the MMFF94x force field. The ligand was manually covalent to Sec498 residue of TrxR, and the final structure of the ligand with the free C-terminal tail (from residue Ile 492 to Gly 499) of TrxR was generated by conformational search module with the LowModeMD method.<sup>2</sup> The structural figures were drawn in PyMOL.<sup>3</sup>

**RNA interference** Small interfering RNAs (siRNA) that target human TrxR2 was based on nucleotides 1160-1178 (GTCTGAATTTGGAGAAGGC) relative to the translation start site.<sup>4</sup> TrxR2-specific sense (5'-GUCUGAAUUUGGAGAAGGC-3') and antisense (3'-CAGACUAAAACCUCUCCG-5') siRNA oligonucleotides were synthesized by RiboBio. The cells were seeded to 6-well plates. After incubation for 24 h, the cells were transfected with siRNA for 3 d employing Lipofectamine 3000 (Invitrogen). The N-Control non-targeting siRNA (RiboBio) served as a negative control for siRNA activity. After three days, TrxR2 knockdown was determined by Western blotting.

**Real-time RT-PCR analyses** HeLa cells were plated in a 6-well dish and exposed to

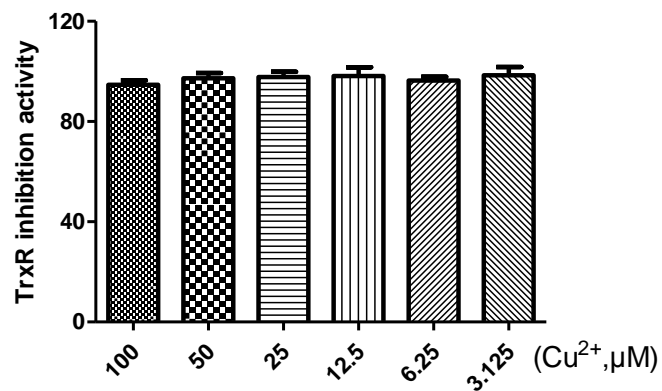
treatment with siRNA for 24 h, the medium was removed and cells were rinsed twice with PBS. Total RNA was isolated and purified from HeLa cells by RNA Trizol extraction (Invitrogen). cDNA was produced using Taqman reverse transcription (Invitrogen) kits. The primers used in each reaction were as follows: TrxR2 forward 5' -GTTCCCACGACCGTCTTCAC-3' and reverse 5' -GTGATAGACCTCAACATGCTCCTG -3' ;<sup>5</sup> GAPDH, forward 5' -GCA CCG TCA AGG CTG AGA AC-3' and reverse 5' -TGG TGA AGA CGC CAG TGG A-3' (Sangon Biotech). Real-time RT-PCR experiments were performed using SYBR1Green Real-time PCR Master Mix (TOYOBO) according to the manufacturer's instructions. Real-time PCR (QRT-PCR) was performed in triplicate on LightCyclerH 480 II (Roche). Primers were designed to be compatible with a single QRT-PCR thermal profile (95 °C for 10 min, and 40 cycles of 95 °C for 30 s and 60 °C for 1 min). After normalized to GAPDH gene, expression levels for each target gene were calculated using the comparative threshold cycle (CT) method.

### 3. Supporting Information Figures mentioned in text

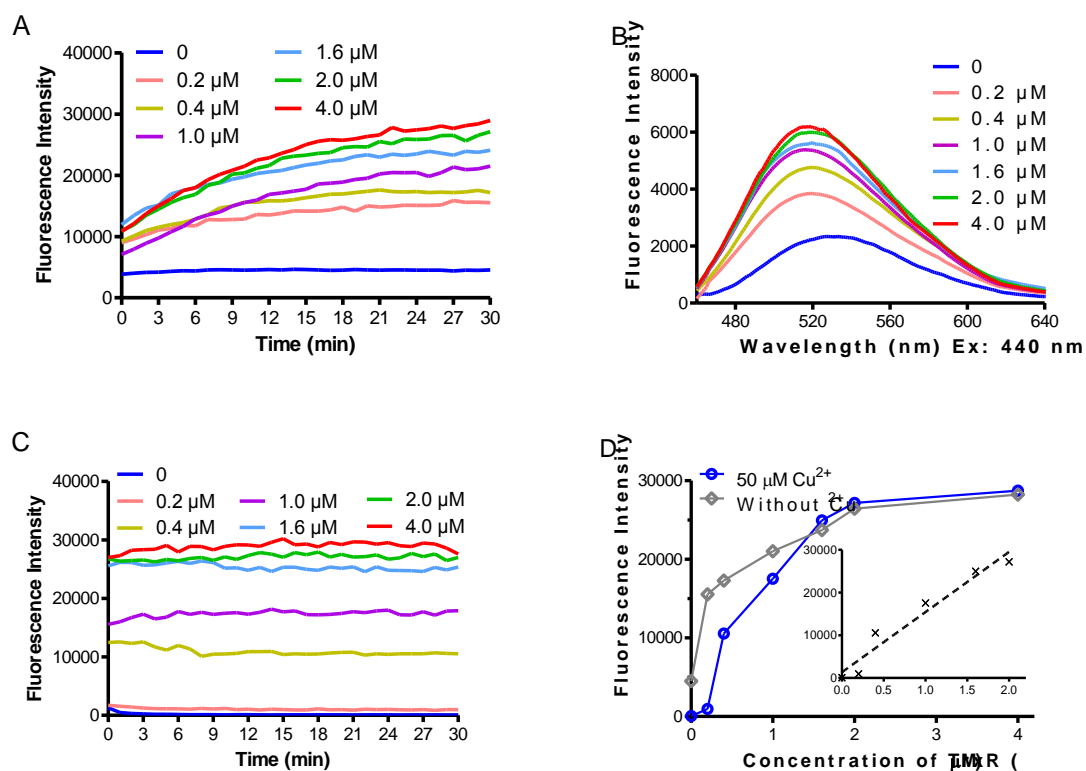


**Figure S1.** Fluorescence responses of TPP2a (2.0 μM) to putative proteins with or without  $\text{Cu}^{2+}$  (50 μM). (A) Time course of the fluorescence changes of TPP2a in the presence of 1.0 μM different proteins. (B) Fluorescent emission spectra of TPP2a to various proteins. (C) The effect of the  $\text{Cu}^{2+}$  to the fluorescence of TPP2a with in different proteins. Fluorescent kinetics is recorded with  $\lambda_{\text{ex}} = 440 \text{ nm}$ ,  $\lambda_{\text{em}} = 520 \text{ nm}$ . Fluorescent emission is recorded with  $\lambda_{\text{ex}} = 440 \text{ nm}$ . All spectra are recorded in PBS solution (pH 7.4) containing 1% (v/v) of DMSO at 37 °C.





**Figure S2.** Effect of cupric acetate on thioredoxin reductase activity. The enzymes were incubated with the indicated concentrations of cupric acetate for 5 min at room temperature, and the TrxR activity was determined by DTNB assay. DTNB reduction was estimated spectrophotometrically at 412 nm.



**Figure S3.** Fluorescence responses of TPP2a (2.0  $\mu\text{M}$ ) to various concentrations of TrxR in the absence and presence of  $\text{Cu}^{2+}$  (50  $\mu\text{M}$ ). The fluorescence kinetics (A) and emission spectra (B) were recorded. After the addition of  $\text{Cu}^{2+}$ , the fluorescence kinetics were recorded again (C), and stable fluorescence intensity (30 min) was compared in the absence and presence of  $\text{Cu}^{2+}$  (D) inserts, linear regression of the dose-dependent response of TPP2a with TrxR in the presence of  $\text{Cu}^{2+}$ .

#### 4. $^1\text{H}$ NMR, $^{13}\text{C}$ NMR, $^{31}\text{P}$ NMR and HRMS Spectra

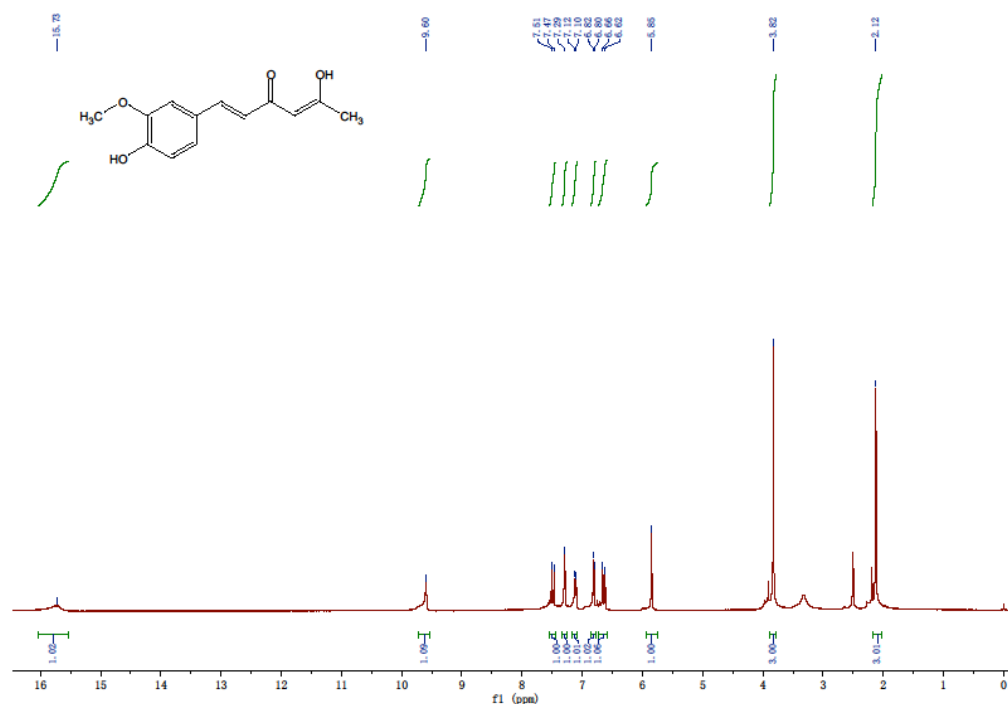


Figure S4.  $^1\text{H}$ -NMR spectrum of M1 in  $(\text{CD}_3)_2\text{SO}$  (400 MHz).

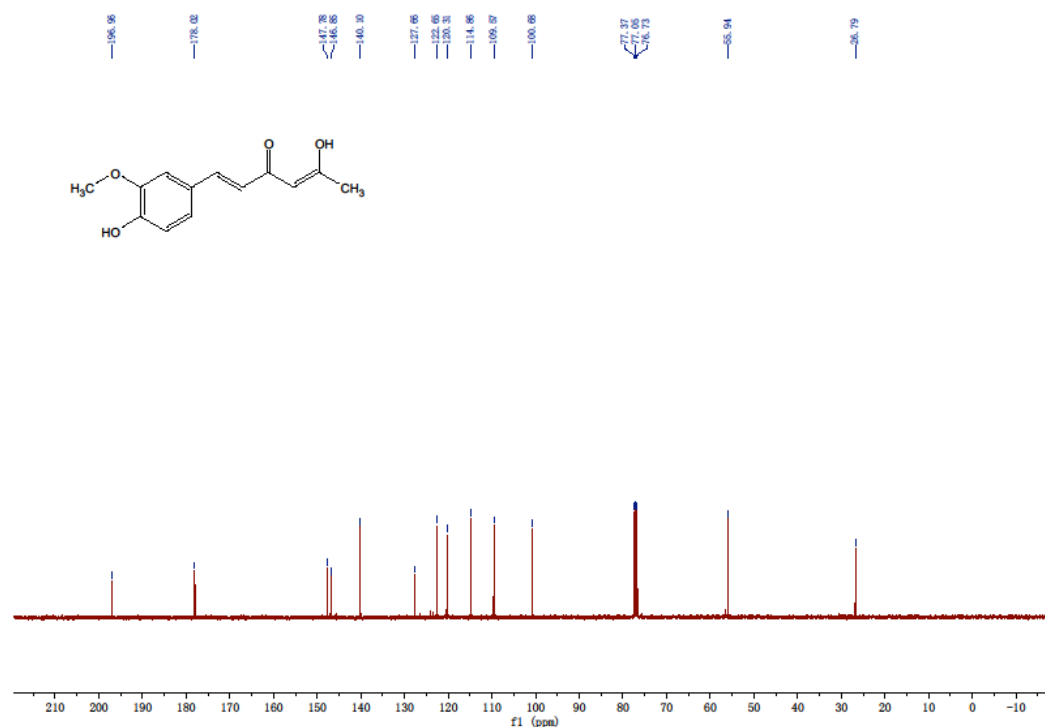
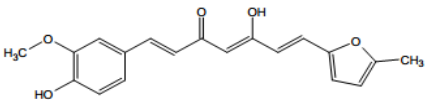
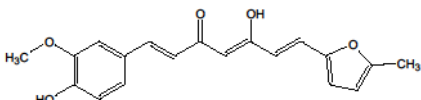


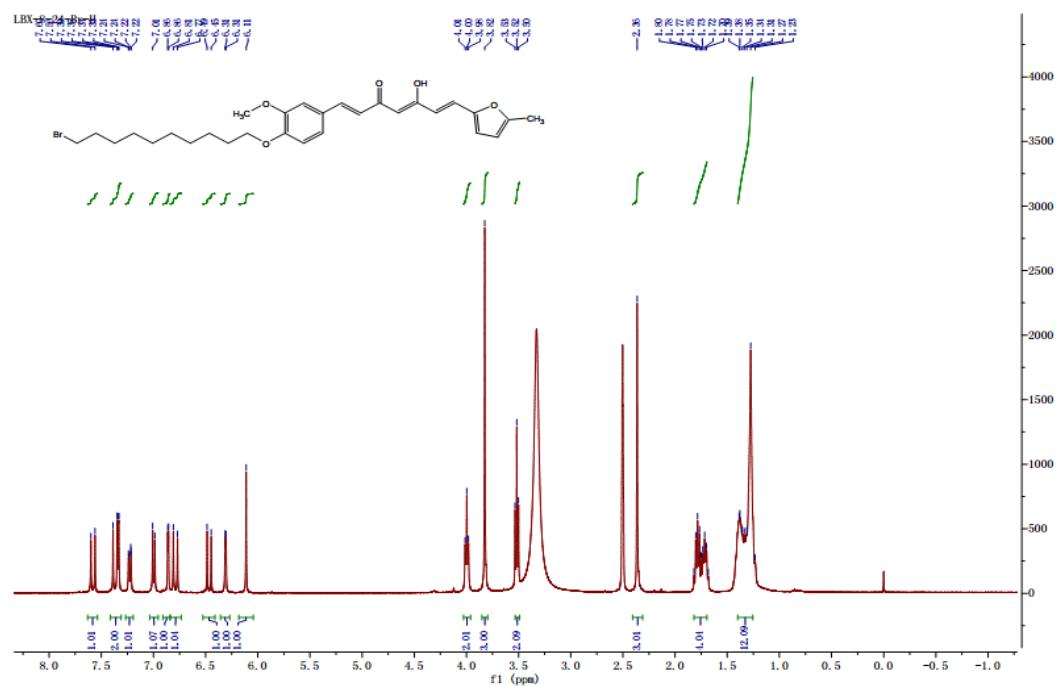
Figure S5.  $^{13}\text{C}$  NMR spectrum of M1 in  $\text{CD}_3\text{Cl}_3$  (101 MHz).



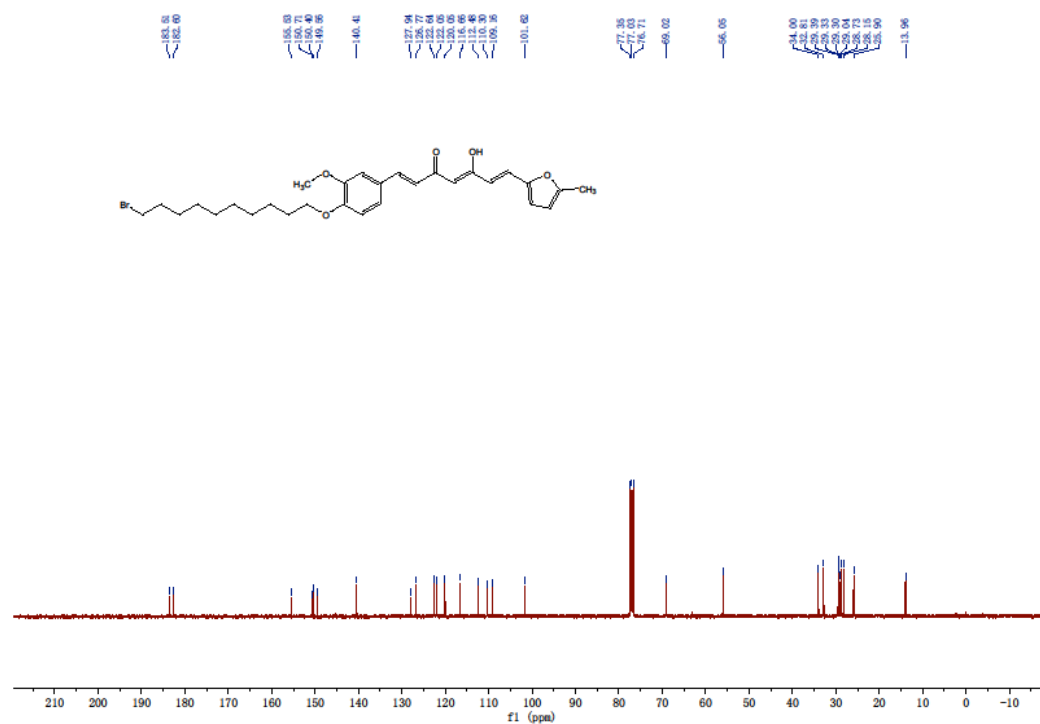
**Figure S6.**  $^1\text{H}$ -NMR spectrum of 2a in  $(\text{CD}_3)_2\text{SO}$  (400 MHz).



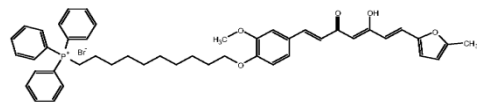
**Figure S7.**  $^{13}\text{C}$  NMR spectrum of 2a in  $(\text{CD}_3)_2\text{SO}$ . (101 MHz).



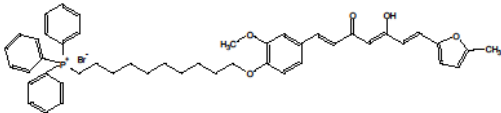
**Figure S8.** <sup>1</sup>H-NMR spectrum of M2 in (CD<sub>3</sub>)<sub>2</sub>SO (400 MHz).



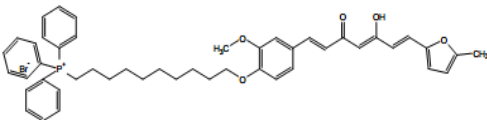
**Figure S9.** <sup>13</sup>C NMR spectrum of M2 in CDCl<sub>3</sub> (101 MHz).



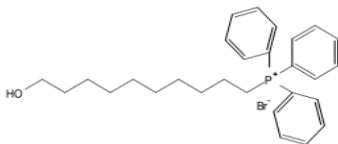
**Figure S10.**  $^1\text{H}$ -NMR spectrum of TPP2a in  $(\text{CD}_3)_2\text{SO}$  (400 MHz).



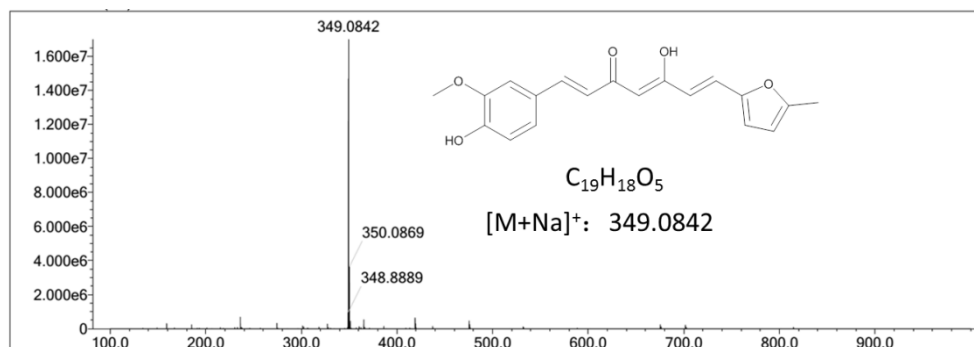
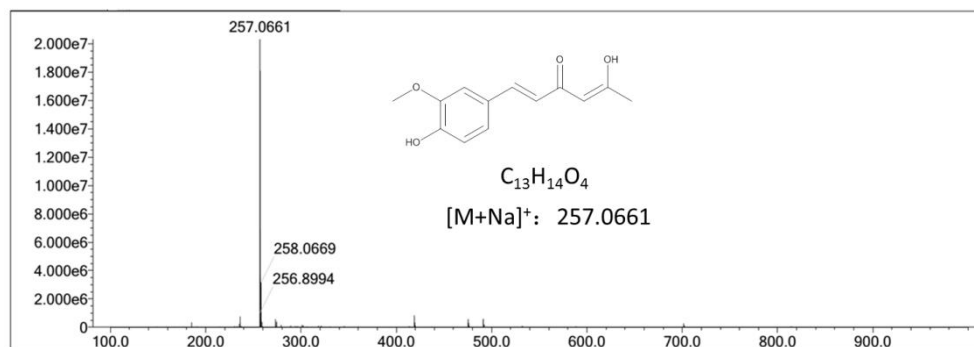
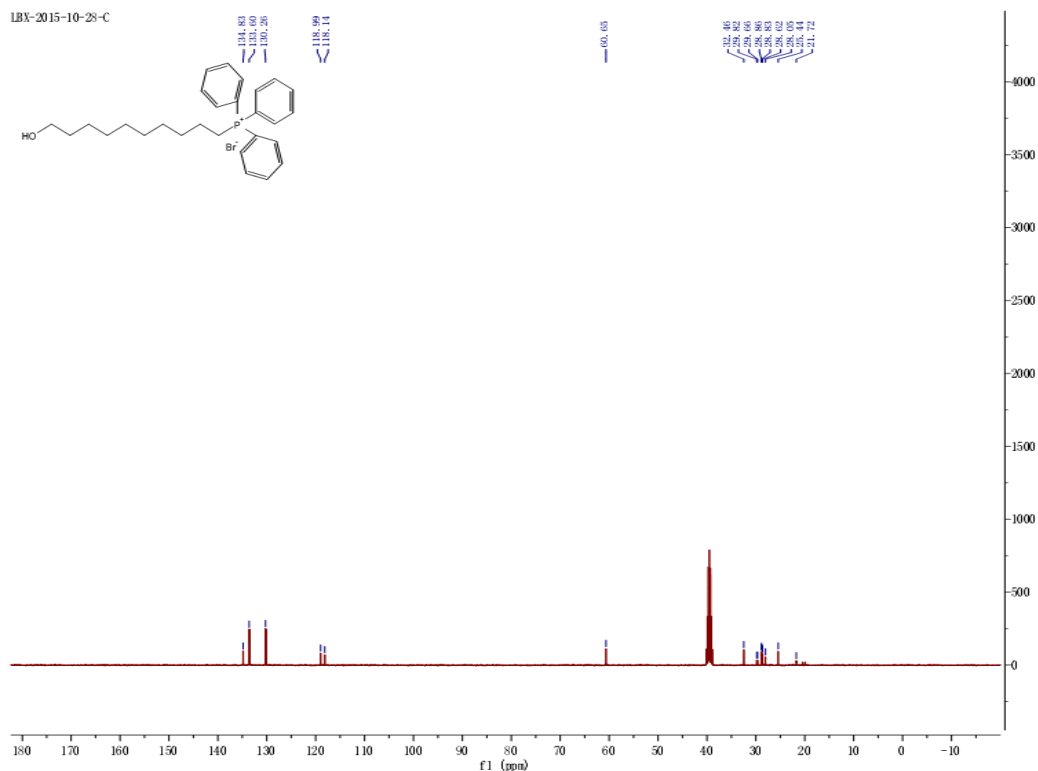
**Figure S11.**  $^{13}\text{C}$  NMR spectrum of TPP-2a in  $(\text{CD}_3)_2\text{SO}$  (101 MHz).



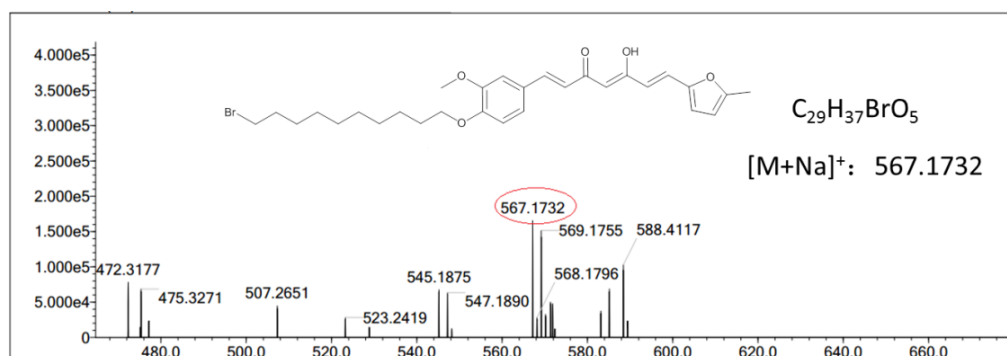
**Figure S12.**  $^{31}\text{P}$  NMR spectrum of TPP2a in  $(\text{CD}_3)_2\text{SO}$  (162 MHz).



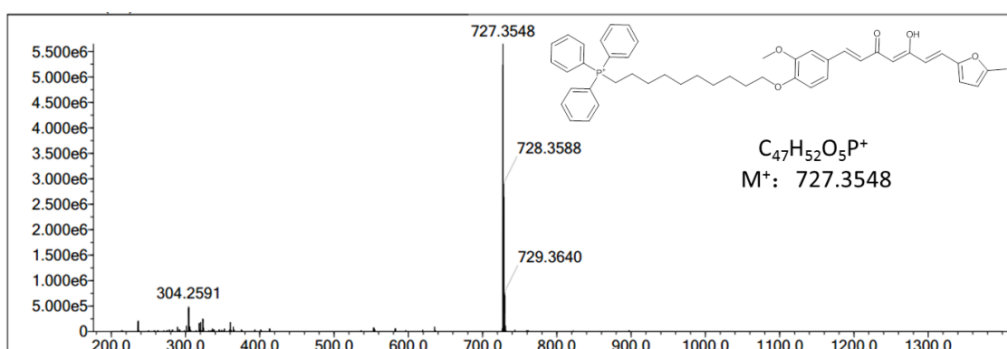
**Figure S13.**  $^1\text{H}$ -NMR spectrum of TPPOH in  $(\text{CD}_3)_2\text{SO}$  (400 MHz).



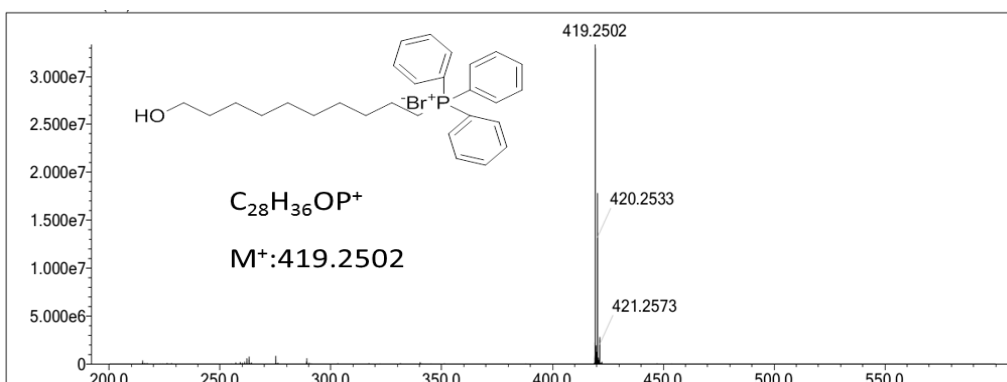




**Figure S17.** HRMS spectrum of M2.



**Figure S18.** HRMS spectrum of TPP2a.



**Figure S19.** HRMS spectrum of TPPOH.

## 5. Supporting Information References

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