Supporting information

A ratiometric fluorescent probe for fast and sensitive detection of peroxynitrite：boronate ester as the receptor to initiate cascade reaction

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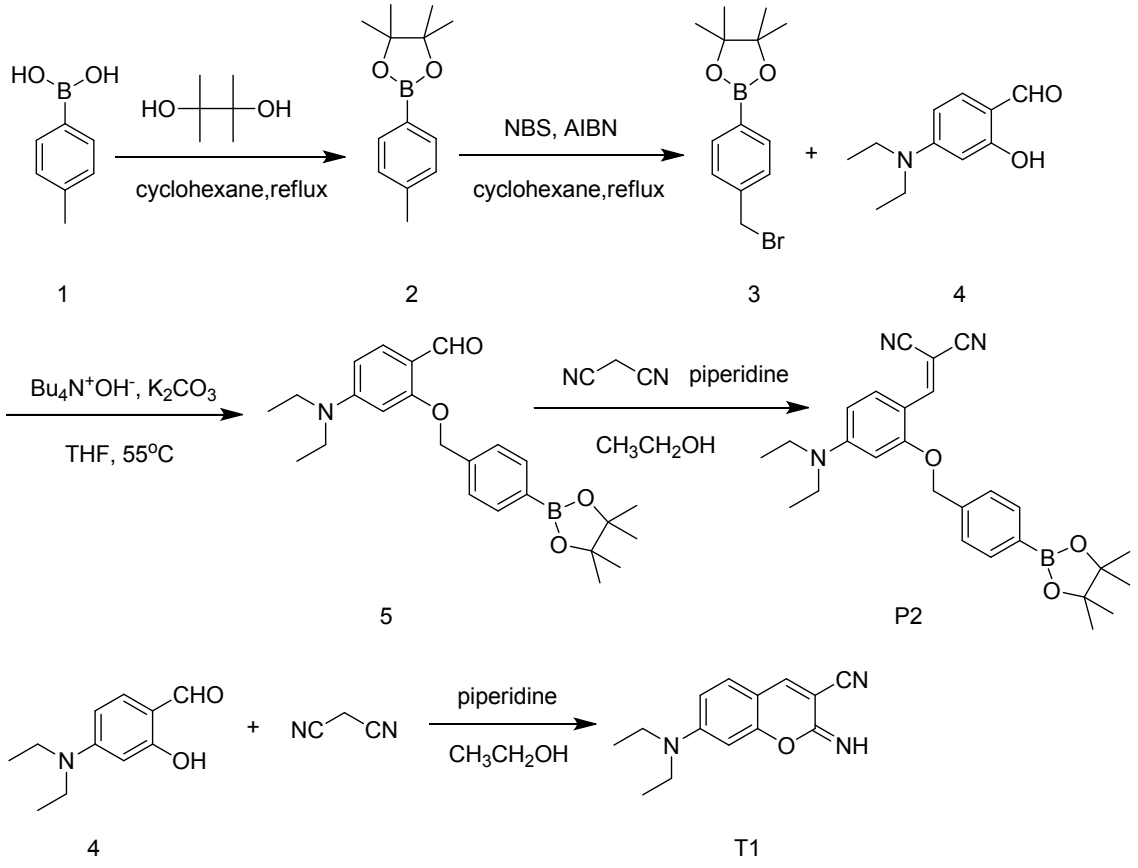
1. Instruments

Instruments: Fluorescence spectra were collected by a Varian Cary Eclipse Fluorescence Spectrometer. Absorption spectra were recorded by a Varian Cary 100 UV-Vis spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were taken in $CDCl_3$ and DMSO-d_6 at 25 ^{o}C on a Bruker AV-400 spectrometer in NMR Facility of East China University of Science and Technology (ECUST). The chemical shifts were reported in ppm (TMS as internal standard). Mass spectra were performed in the Analysis Center of East China University of Science and Technology (ECUST).

2. Synthesis

Synthesis of compounds 2-P2.



Scheme S1 The synthesis of the probe **P2**

Compound 2. To a solution of p-tolylboronic acid (2 g, 14.7 mmol) in cyclohexane (40 mL) was added pinacol (6.26 g, 52.9 mmol). Then the reaction mixture was refluxed for 10 h. The reaction mixture was concentrated in vacuo to get the crude product 2. The residue was purified by silica gel flash chromatography with 20:1 PE / CH_2Cl_2 to give the title compound 2 (2.56 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (d, J = 7.2 Hz, 2H), 7.23 (d, J = 7.2 Hz, 2H), 2.41 (s, 3H), 1.38 (s, 12H).

Compound 3[1]. Compound 2(2 g, 9.17 mmol), NBS (2.14 g, 12 mmol), AIBN (0.083 g) were dissolved in 40 mL cyclohexane and the mixture was refluxed under Ar for 7 h. It was then filtered under reduced pressure, and the filtrate was concentrated. The residue was purified by silica gel flash chromatography with 5:1 PE / CH_2Cl_2 to give the title compound 3 (2.34 g, 86%).

Compound 5[2]. Compound 3 (1.54 g, 5.18 mmol), compound 4 (1 g, 5.18 mmol), $nBu_4N^+OH^-$ (2.68 g, 1.04 mmol) and K_2CO_3 (2.86 g, 21 mmol) were dissolved in 20 mL THF and the mixture was was stirred at 55 ^oC for 24 h. After the reaction was completed, the mixture was concentrated in vacuo, then 60 mL water and CH_2Cl_2 were added. The organic layer was washed with brine, dried over anhydrous Na_2SO4, and concentrated in vacuo. The residue was purified by silica gel flash chromatography with 1:1 PE / CH_2Cl_2 to give the title compound 5 (0.85 g, 40%). ^1H NMR (400 MHz, $CDCl_3$): δ 10.28 (s, 1H), 7.85 (d, J = 7.6 Hz, 2H), 7.76 (d, J = 8.8 Hz, 1H), 7.47 (d, J = 7.6 Hz, 2H), 7.40 (d, J = 7.6 Hz, 1H), 6.34 (d, J = 8.8 Hz, 1H), 5.22 (s, 2H), 3.39 (q, J = 6.8 Hz, 4H), 1.37 (s, 12H), 1.18 (t, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 186.9, 163.2, 153.8, 139.8, 135.1, 130.4, 126.2, 114.4, 104.6, 94.1, 83.9, 77.4, 44.8, 24.9, 12.5. HRMS (TOF-ESI): Calcd for $C_{24}H_{33}BNO_4$ [M+H^+] 410.2503; Found, 410.2492.

Compound P2. Compound 5 (0.4 g, 1 mmol), malononitrile (0.066 g, 1 mmol), piperidine (0.85 g, 10 mmol) and absolute ethanol (15 mL) was stirred under Ar at 25 ^oC for 1.5 h. Then the mixture was filtered. The yellow solid was washed with ethanol and then purified by silica gel flash chromatography to get the title compound **P2** (0.32 g, 70%). ^1H NMR (400 MHz, DMSO-d_6): δ 8.05 (d, J = 9.2 Hz, 1H), 7.97 (s, 1H), 7.71 (d, J = 7.6 Hz, 2H), 7.50 (d, J = 7.6 Hz, 2H), 6.55 (d, J = 9.2 Hz, 1H), 6.23 (d, J = 0.8 Hz, 1H), 5.32 (s, 2H), 3.47 (q, J = 6.8 Hz, 4H), 1.30 (s, 12H), 1.08 (t, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.0, 154.9, 150.7, 140.1, 135.2, 130.4, 127.5, 117.6, 116.6, 109.2, 106.8, 94.8, 84.2, 70.2, 66.3, 44.9, 25.1, 12.9. HRMS (TOF-ESI): Calcd for $C_{27}H_{32}BN_3O_3$ [M+H^+] 458.2615; Found, 458.2610.

Compound T1[3]. Compound 4 (0.193 g, 1 mmol), malononitrile (0.066 g, 1 mmol), piperidine (0.009 g, 0.1 mmol) and absolute ethanol (10 mL) was stirred at 25 ^oC for 15 min. Then the mixture was filtered. The yellow solid was recrystallized with absolute ethanol and then purified by silica gel flash chromatography to get the title compound **T1** (0.145 g, 60%). ^1H NMR (400 MHz, $CDCl_3$): δ 7.55 (s, 1H), 7.13 (d, J = 8.8 Hz, 1H), 6.47 (dd, J_1 = 8.8 Hz, J_2 = 1.6 Hz, 1H), 6.31 (s, 1H), 3.43 (q, J = 7.2 Hz, 4H), 1.23 (t, J = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.6, 152.6, 145.2, 130.2, 116.2, 108.3, 106.4, 97.1, 45.0, 12.5. HRMS (TOF-ESI): Calcd for $C_{14}H_{16}N_3O$ [M+H^+] 242.1293; Found, 242.1293.

3. Methods and data

3.1 The pH titration

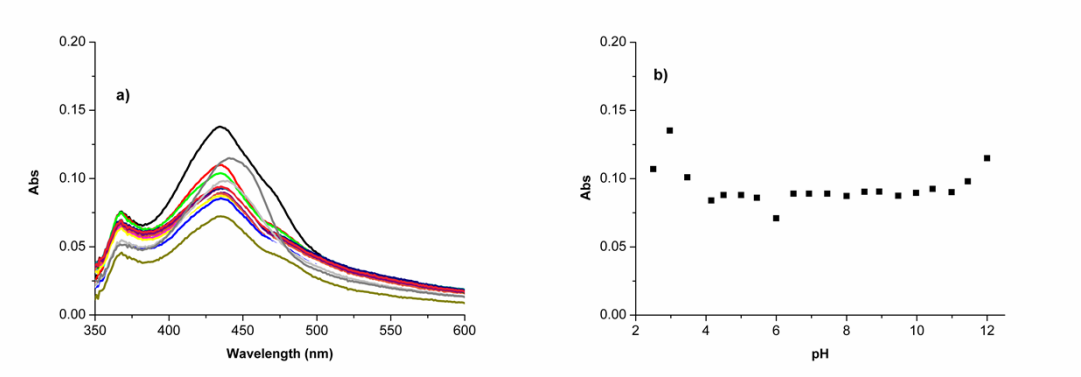


Figure S1. (a) Absorption responses of **P2** (20 μM) to various pH in water with 10% CH_3CN as the co-solvent. (b) The absorption (at 440 nm) versus different pH values in water with 10% CH_3CN as the co-solvent. pH 2-12.

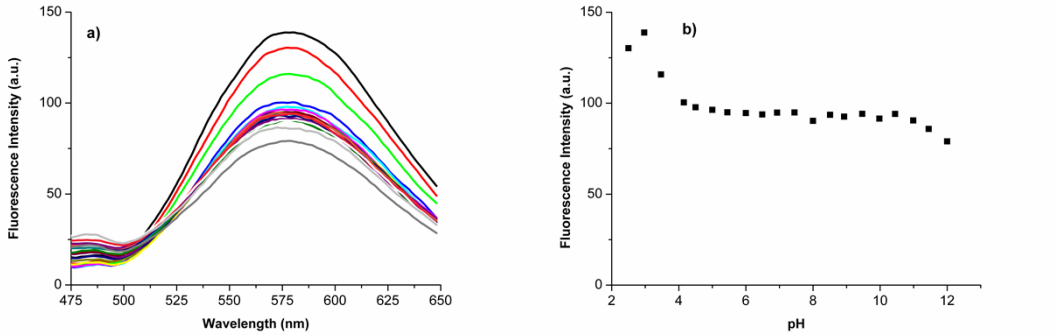


Figure S2. (a) Fluorescence responses of **P2** (20 μM) to various pH in water with 10% CH_3CN as the co-solvent. (b) The fluorescence (at 580 nm) versus different pH values in water with 10% CH_3CN as the co-solvent. pH 2-12, slit: 5 nm, 5 nm.

3.2 The detection limit

The detection limit was calculated according to the previous literature. The fluorescence intensity of **P2** was measured by ten times and the standard deviation was calculated. The fluorescence intensity at 480 nm was plotted as a concentration of $ONOO^-$. By using detection limit 3σ/k, the detection limit was calculated as 0.35 nM. σ is the standard deviation of the fluorescence intensity of **P2**, k is the slope between the fluorescence intensity at 480 nm versus the $ONOO^-$ concentration.

3.3 The kinetic curves of the probe P2 and $ONOO^-$

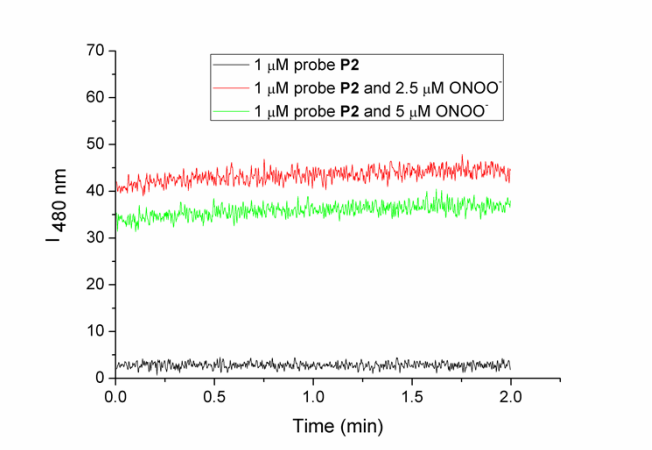


Figure S3. The kinetic curve of **P2** (1 μM) and $ONOO^-$ (2.5 and 5 μM)

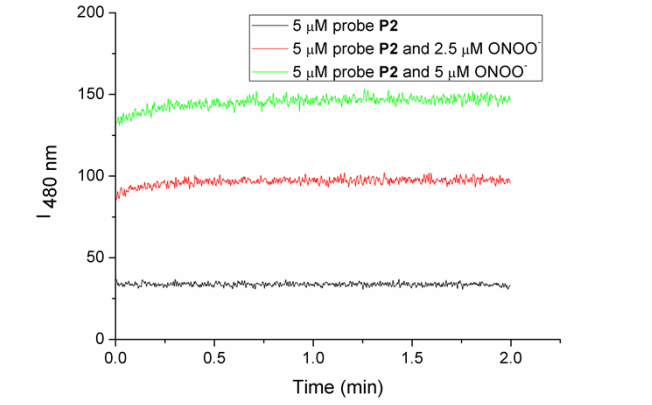


Figure S4. The kinetic curve of **P2** (5 μM) and $ONOO^-$ (2.5 and 5 μM)

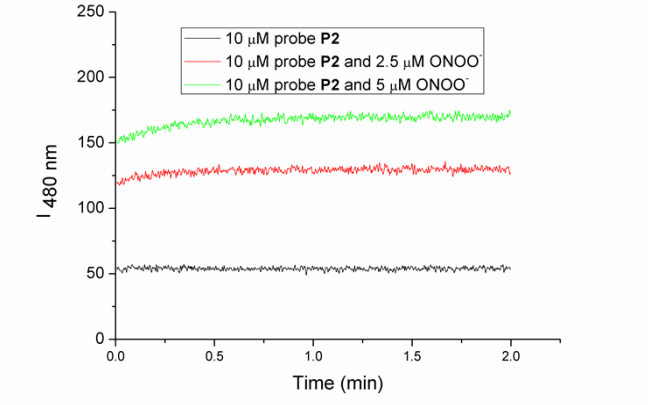


Figure S5. The kinetic curve of **P2** (10 μM) and $ONOO^-$ (2.5 and 5 μM)

From the above figures, we found that even though the concentration of the probe was low, its reaction with $ONOO^-$ was very quick which could be finished mostly within 10 s. This was good for detection cause H_2O_2 would react with the probe much slower than $ONOO^-$ which required at least half an hour.

3.4 The reaction between the probe and H_2O_2

We could observe that H_2O_2 was also able to induce a similar fluorescence enhancement at 480 nm. However the reaction was very slow and it took about at least 140 min for the reaction to complete. In addition, the reaction was not sensitive which required more than 100 μM H_2O_2.

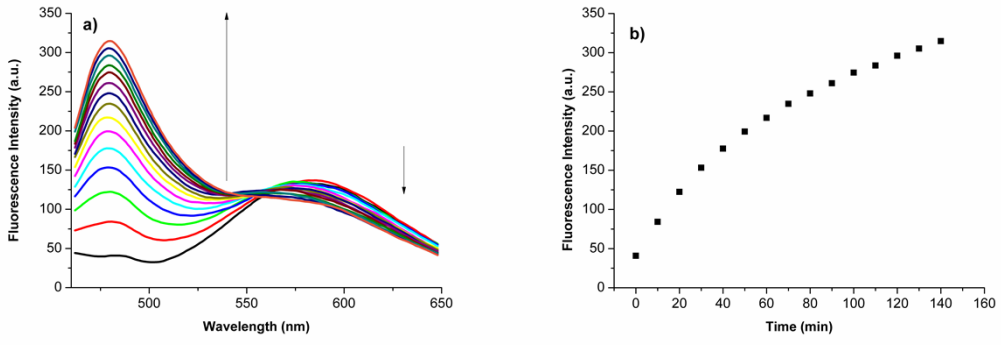


Figure S6. (a) The emission spectra of probe **P2** (20 μM) upon addition of 100 μM H_2O_2 in PBS buffer (0.01M, pH 7.4) with 10% CH_3CN as a co-solvent. The data were recorded every 10 min. (b) Fluorescence responses ($I_{480\ nm}$) of **P2** (20 μM) to H_2O_2 (100 μM). Excitation wavelength was 440 nm. Slit: 5 nm, 5 nm.

3.5 HPLC condition

HPLC was performed on a ZoRBAX RX-C18 column (Analytical 4.6×250mm 5-Micron, Agilent) with a HP 1100 system. The HPLC solvents employed were acetonitrile and buffer (acetic acid and ammonium acetate pH 6.0). HPLC conditions were as follows: solvent A: solvent B = 30:70 (0 min)-100:0 (20 min), flow rate 1 mL/min, detection by UV (430 nm).

3.6 Cell Culture and Imaging

HeLa cells were obtained from American Type Culture collection, and grown in Dulbecco's modification of Eagle's medium Dulbecco (DMEM/high: with 4500 mg/L Glucose, 4.0 mM L-Glutamine, and 110 mg/L Sodium Pyruvate) supplemented with 10% foetal bovine serum (FBS). Cells were incubated in a 5% CO_2 humidified incubator at 37 ^oC and typically passaged with sub-cultivation ratio of 1:4 every two days.

For fluorescence microscopy, HeLa cells were seeded in 24-well culture plate for one night. The cells were first incubated with **P2** (15 μM) for 30 min at 37 ^oC and washed with phosphate buffer (pH 7.4). Then the cells were treated with or without $ONOO^-$ (10 μM) for another 2 min at 37 ^oC and washed with phosphate buffer (pH 7.4) for three times. Fluorescence imaging was performed with Nikon Ti-S with Xenon lamp. Exposure time is 1 s

for blue emission.

3.7 The toxicity of the probe **P2**

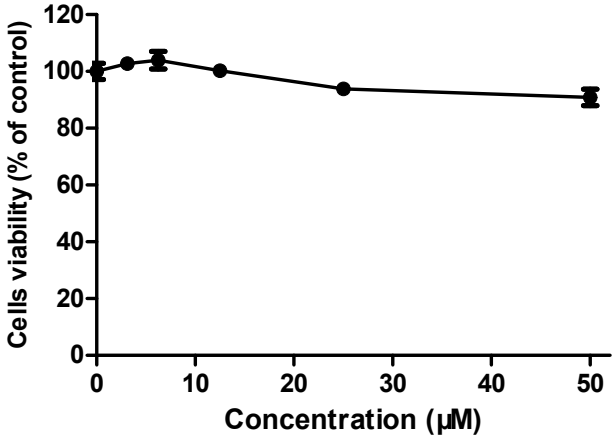


Figure S7. The toxicity of **P2**

The cytotoxicity was carried out by MTT method. Hela cells were placed in 96-well culture plates (1×10^4 cells/well), and allowed to attach for 24 h before the next treatment. The cells were incubated in the absence and presence of **P2** at different concentrations for 24 h at 37 ^oC. Then 20 μL of MTT solution (5 mg/mL) was added and 100 μL of DMSO was replaced after 4 h. Absorbance at 570 nm was measured with EnSpire Multimode Plate Reader (Perkin Elmer, Boston,MA). Cell viability was showed as percentage of untreated control cells.

From the figure, we know that the probe had very little toxicity towards Hela cells.

3.8 The second-order rate constants for the reaction of the probe with $ONOO^-$ and H_2O_2.

First, we detected the kinetic profiles of the reaction under pseudo-first-order conditions with a large excess of $ONOO^-$ and H_2O_2 over probe **P2** (10 μM) in pH 7.4 PBS (containing 10% CH_3CN as cosolvent) at room temperature.

The pseudo-first-order rate constant k' was calculated according to equation[4] (1):

$$\ln[(F_{max}-F_t)/F_{max}] = -k't \quad (1)$$

Where F_t and F_{max} are the fluorescence intensities at 480 nm at time t and the maximum value obtained after the reaction is complete, respectively, and k' is the pseudo-first-order rate constant. In the process, we found that if there was too much $ONOO^-$, then the fluorescence would first increase and later decrease, therefore we chose the fluorescence enhancement as the calculated scope of the equation.

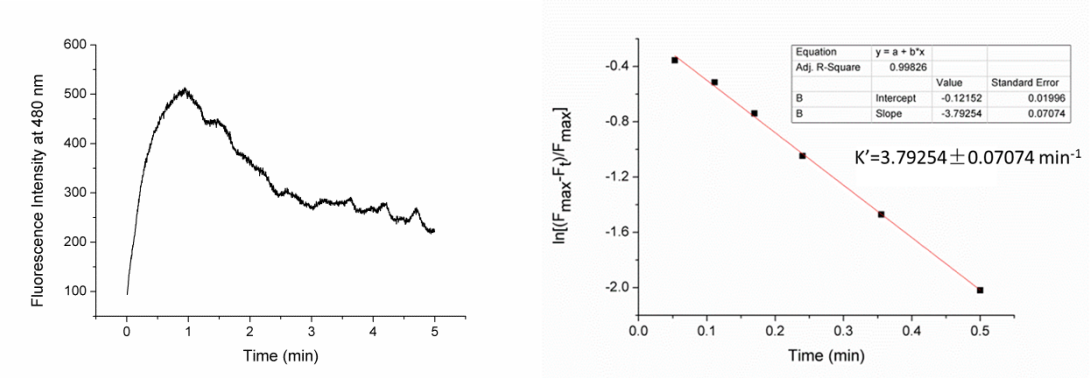


Figure S8. The kinetic curve of the reaction between the probe **P2** (10 μM) and $ONOO^-$ (250 μM) and the pseudo-first-order rate constant k', k' was 3.79254±0.07074 min^{-1}.

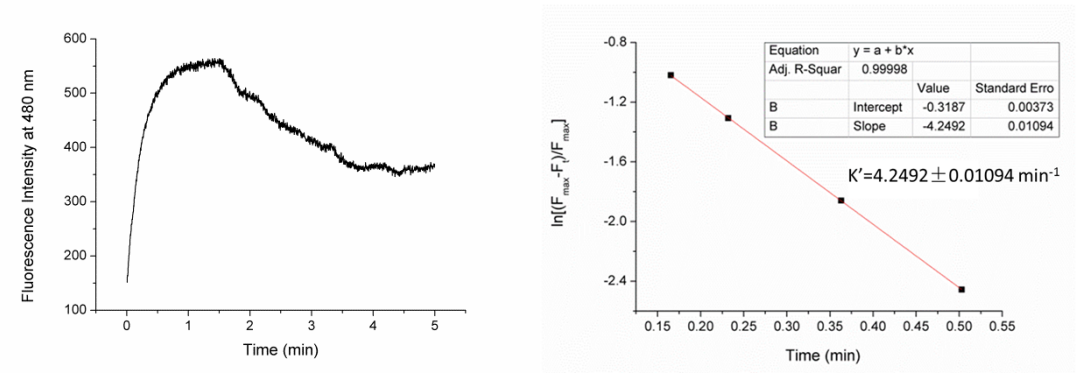


Figure S9. The kinetic curve of the reaction between the probe **P2** (10 μM) and $ONOO^-$ (300 μM) and the pseudo-first-order rate constant k', k' was 4.2492±0.01094 min^{-1}.

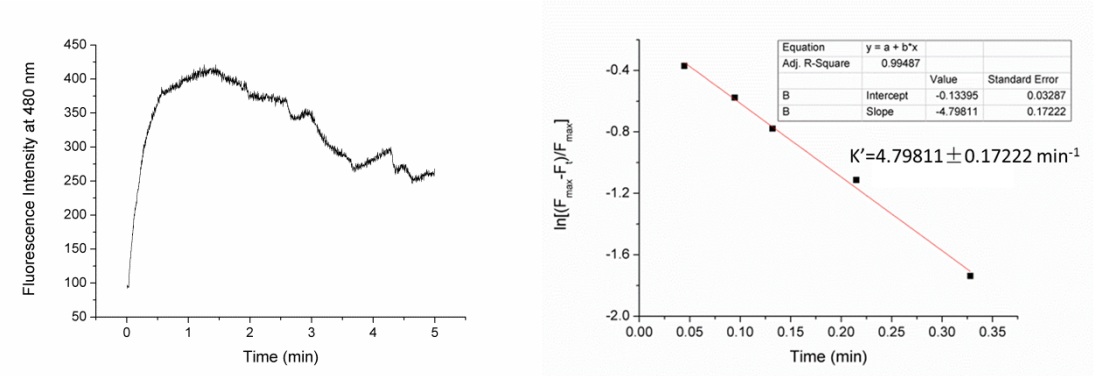


Figure S10. The kinetic curve of the reaction between the probe **P2** (10 μM) and $ONOO^-$ (375 μM) and the pseudo-first-order rate constant k', k' was 4.79811±0.17222 min^{-1}.

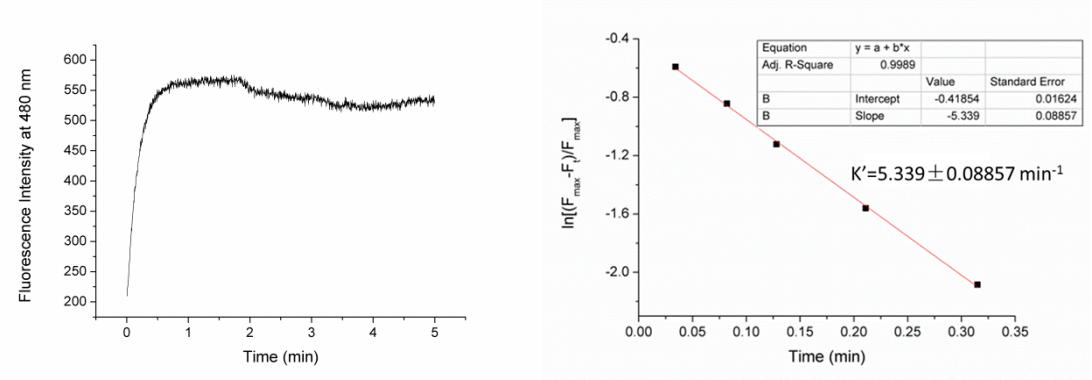


Figure S11. The kinetic curve of the reaction between the probe **P2** (10 μM) and $ONOO^-$ (450 μM) and the pseudo-first-order rate constant k', k' was 5.339±0.08857 min^{-1}.

The second-order rate constant for this reaction is thus the slope of the linear plot of *k'* versus the concentration of $ONOO^-$.

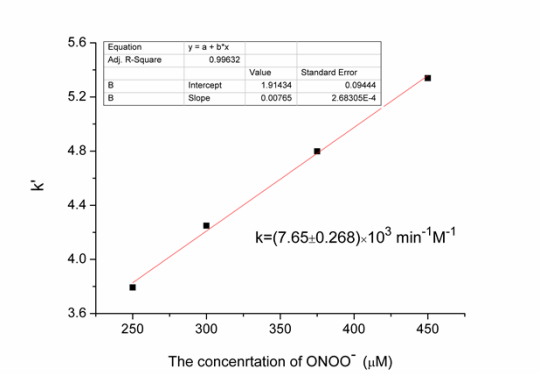


Figure S12. Plot of the apparent rate constant k' versus the concentrations of $ONOO^-$, k was (7.65±0.268)×10^3 $min^{-1}M^{-1}$.

Therefore the rate constant for the reaction between the probe and $ONOO^-$ is (7.65±0.268)×10^3 $min^{-1}M^{-1}$.

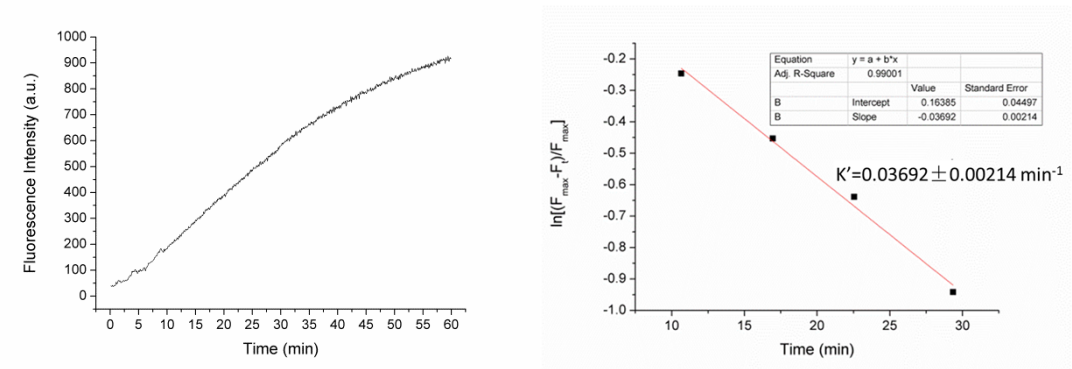


Figure S13. The kinetic curve of the reaction between the probe **P2** (10 μM) and H_2O_2 (500 μM) and the pseudo-first-order rate constant k', k' was 0.03692±0.00214 min^{-1}.

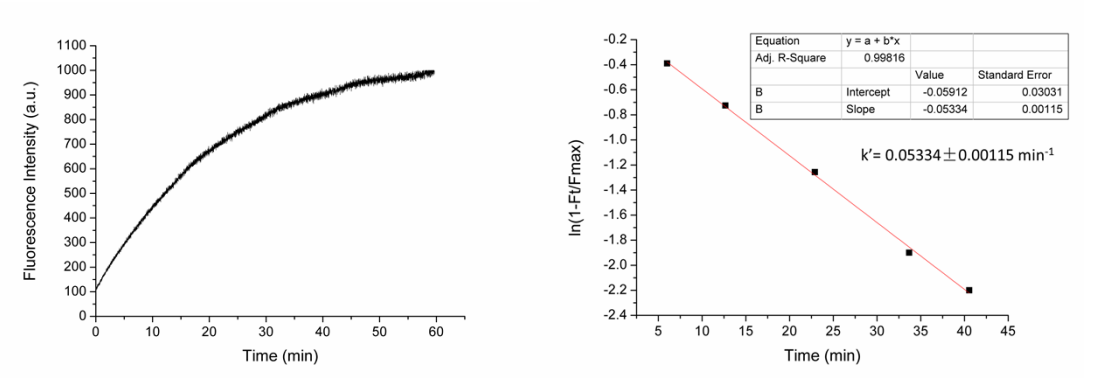


Figure S14. The kinetic curve of the reaction between the probe **P2** (10 μM) and H_2O_2 (680 μM) and the pseudo-first-order rate constant k', k' was 0.05334±0.00115 min^{-1}.

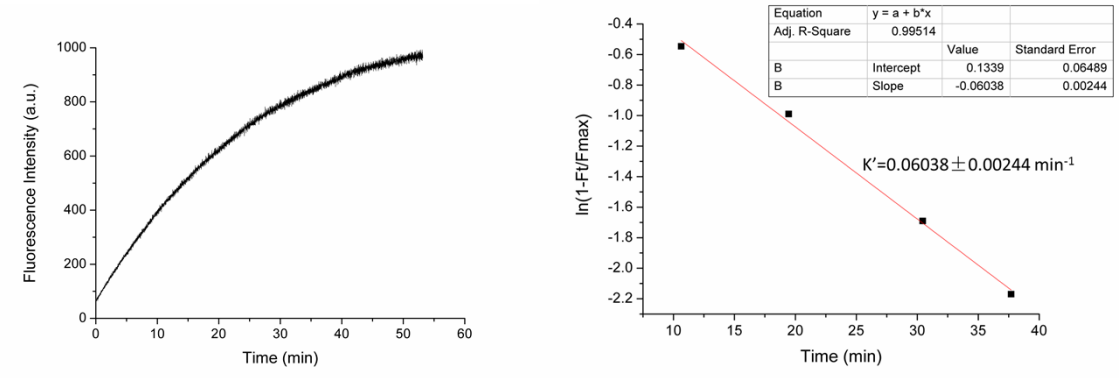


Figure S15. The kinetic curve of the reaction between the probe **P2** (10 μM) and H_2O_2 (750 μM) and the pseudo-first-order rate constant k', k' was 0.06038±0.00244 min^{-1}.

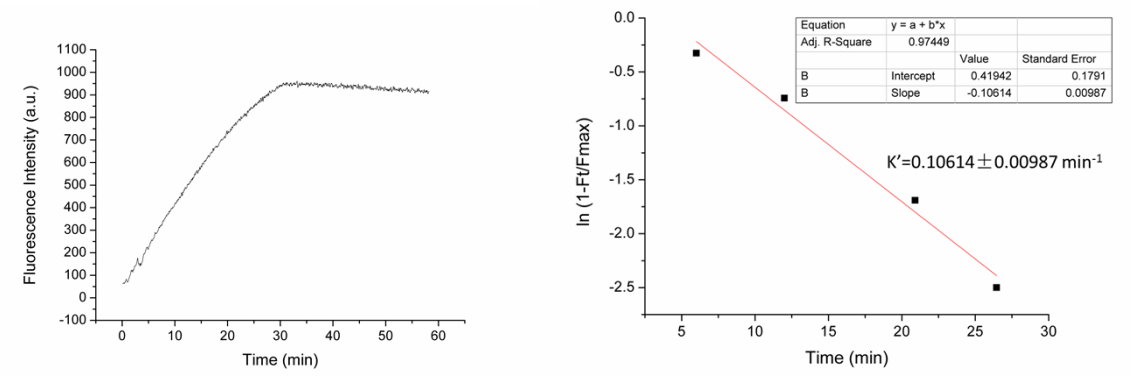


Figure S16. The kinetic curve of the reaction between the probe **P2** (10 μM) and H_2O_2 (1400 μM) and the pseudo-first-order rate constant k', k' was 0.10614±0.00987 min^{-1}.

The second-order rate constant for this reaction is thus the slope of the linear plot of *k'* versus the concentration of H_2O_2.

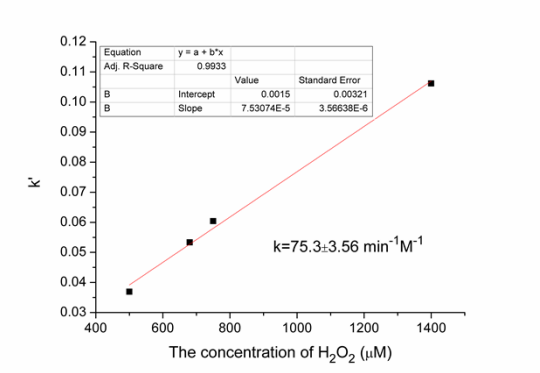
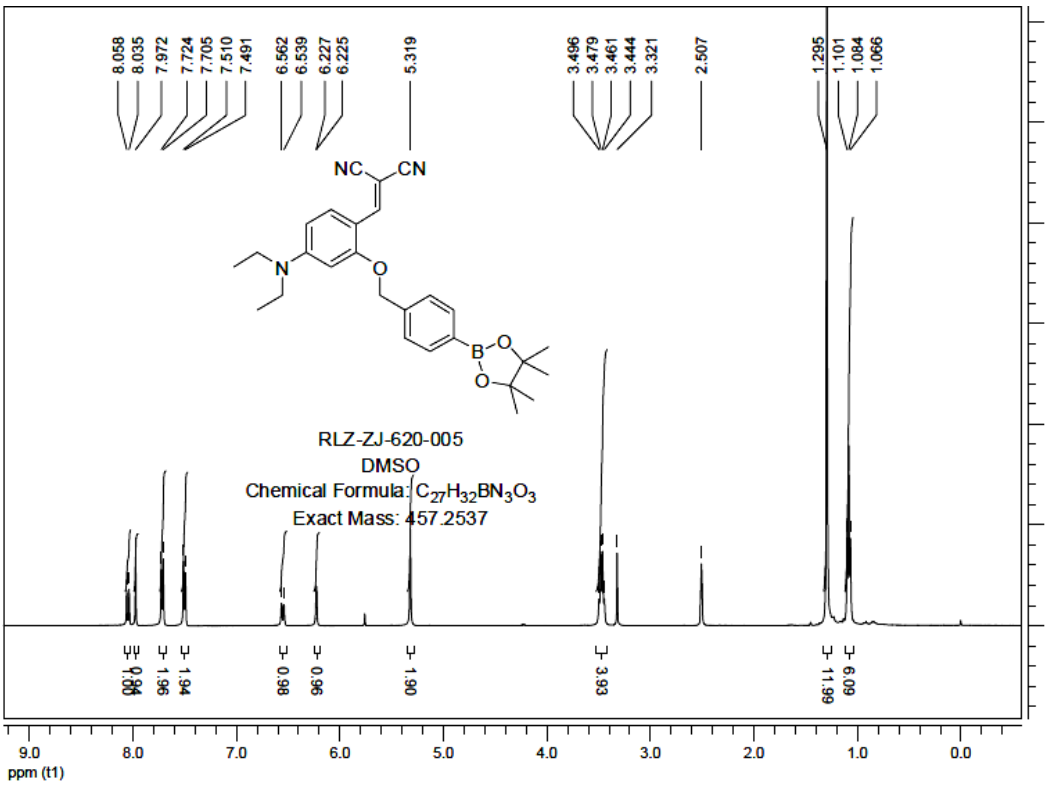
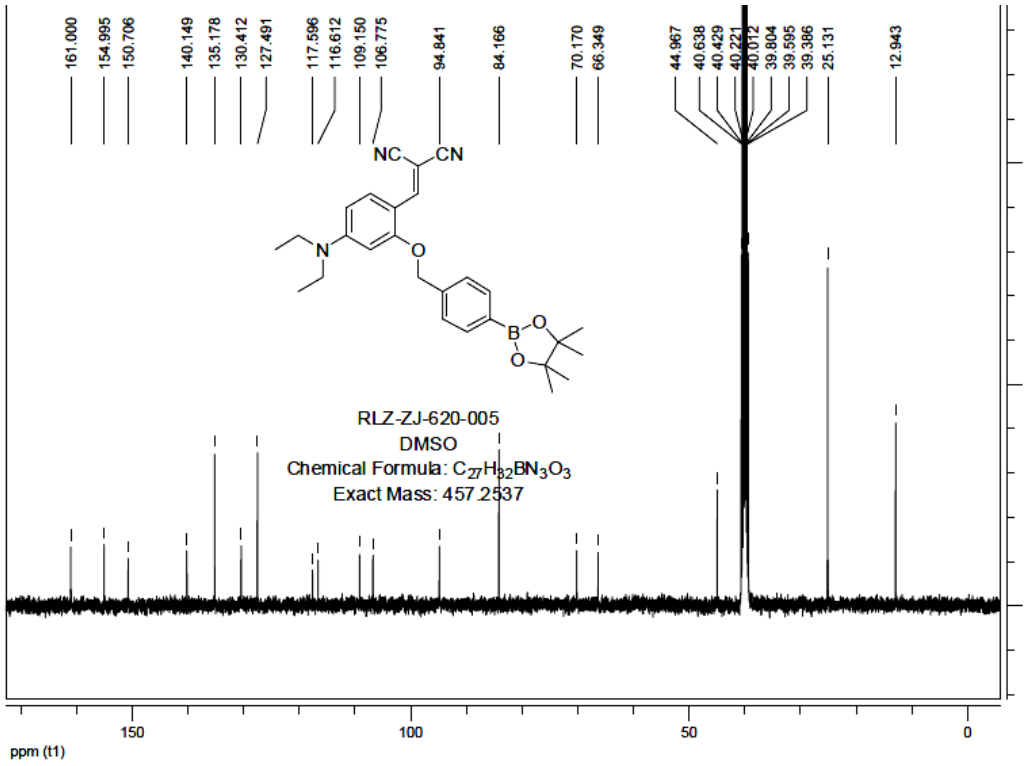


Figure S17. Plot of the apparent rate constant k' versus the concentrations of H_2O_2, k was 75.3±3.56 $min^{-1}M^{-1}$.

Therefore the rate constant for the reaction between the probe and H_2O_2 is 75.3±3.56 $min^{-1}M^{-1}$.

4. **NMR spectra**

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O
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O
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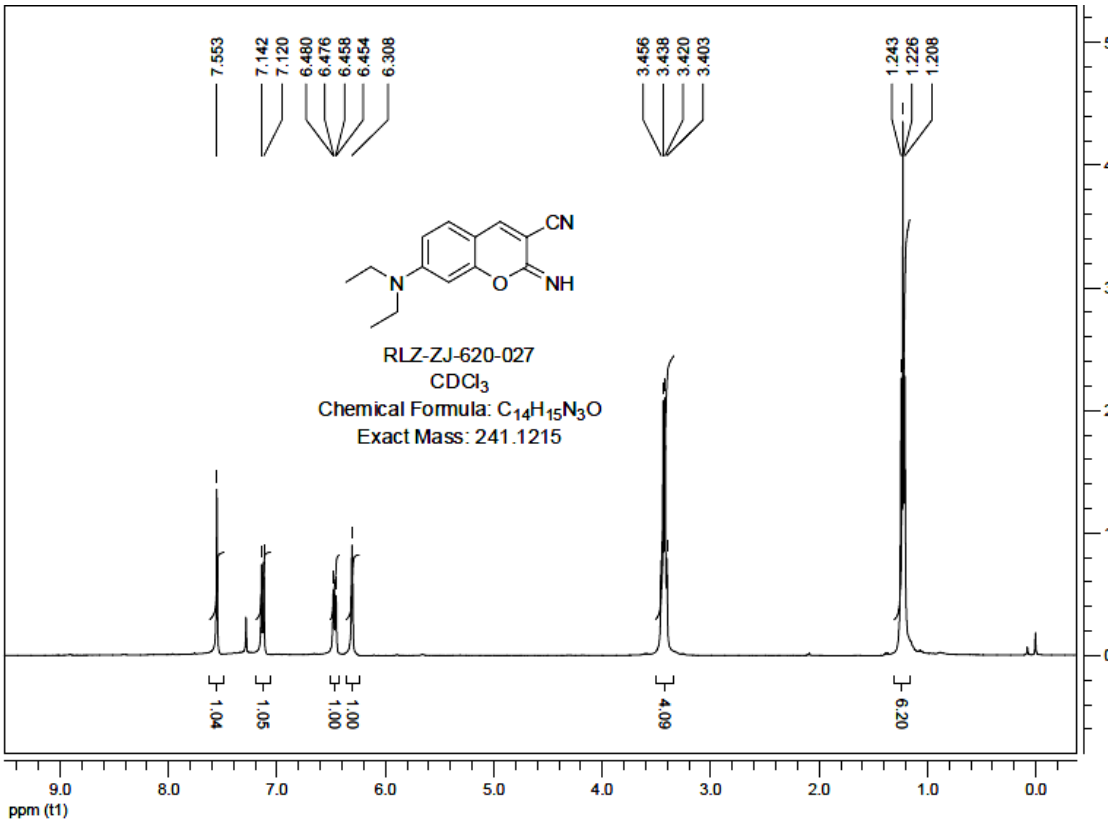
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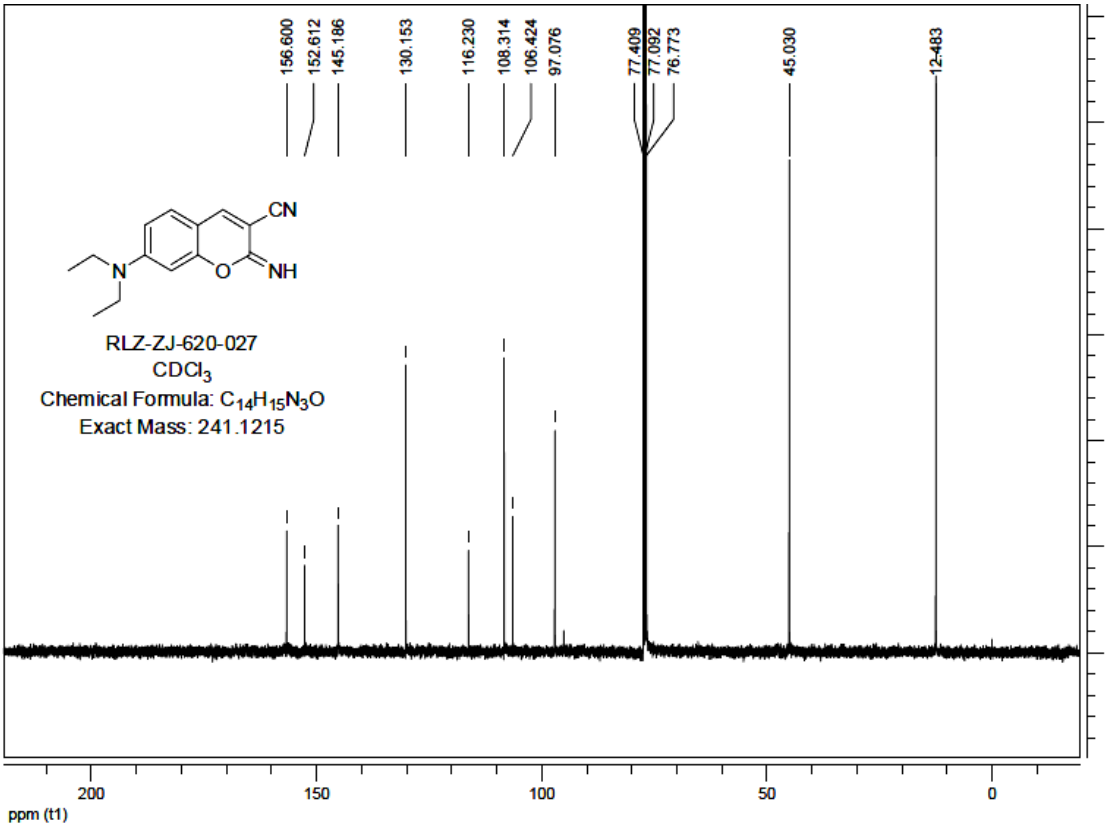
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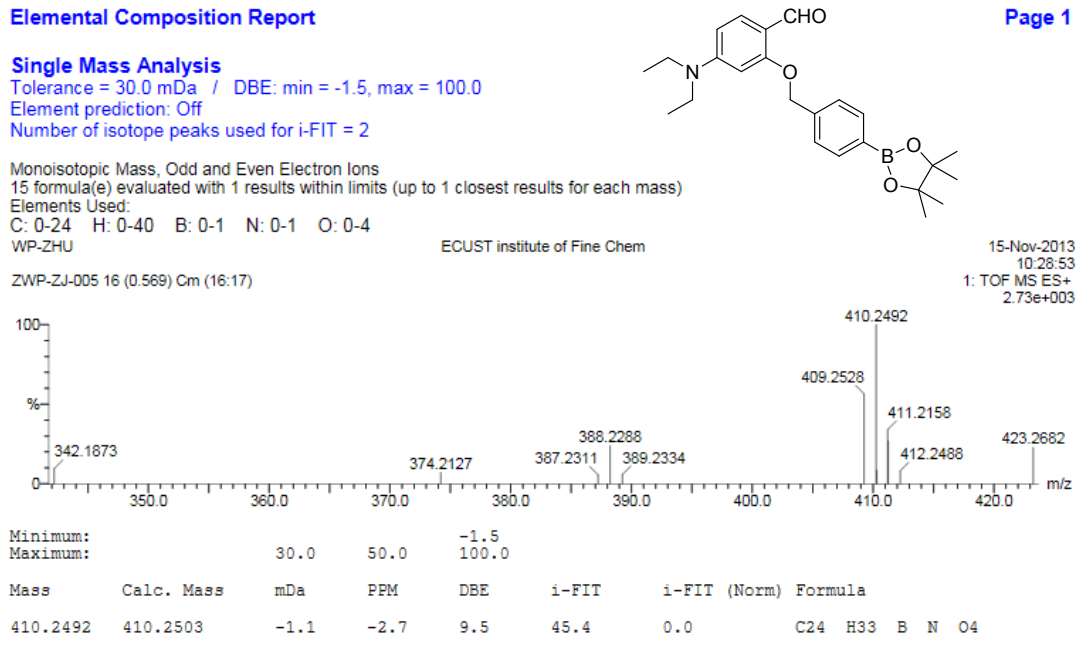
ppm (t1)

5. HRMS data


Elemental Composition Report
Page 1
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Element prediction: Off
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Monoisotopic Mass, Odd and Even Electron Ions
15 formula(e) evaluated with 1 results within limits (up to 1 closest results for each mass)
Elements Used:
C: 0-24 H: 0-40 B: 0-1 N: 0-1 O: 0-4
WP-ZHU
ECUST institute of Fine Chem
15-Nov-2013
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2.73e+003
CHO
N
O
B
O
O
100
%
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389.2334
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m/z
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Maximum:
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i-FIT (Norm)
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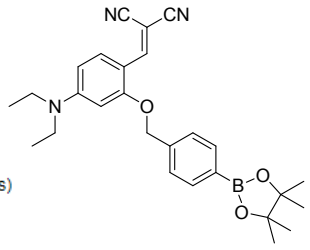
C24 H33 B N O4

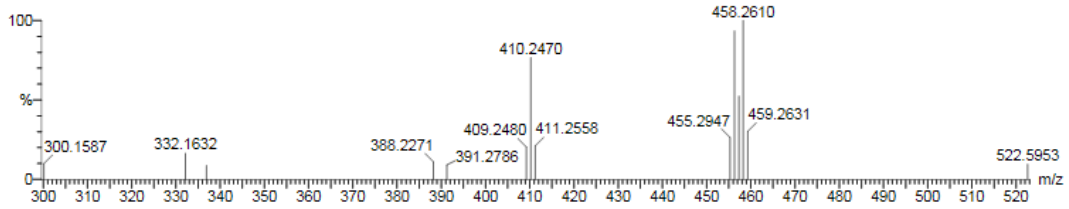
Elemental Composition Report **Page 1**



Single Mass Analysis
Tolerance = 30.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
24 formula(e) evaluated with 1 results within limits (up to 1 closest results for each mass)
Elements Used:
C: 0-27 H: 0-40 11B: 0-1 N: 0-3 O: 0-3

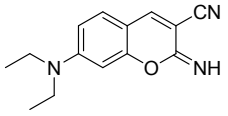
WP-ZHU ECUST institute of Fine Chem 15-Nov-2013 10:40:22
ZWP-ZJ-006 28 (0.937) Cm (28:29) 1: TOF MS ES+ 7.53e+002



Minimum:				-1.5			
Maximum:		30.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
458.2610	458.2615	-0.5	-1.1	13.5	6.9	0.0	C27 H33 11B N3 O3

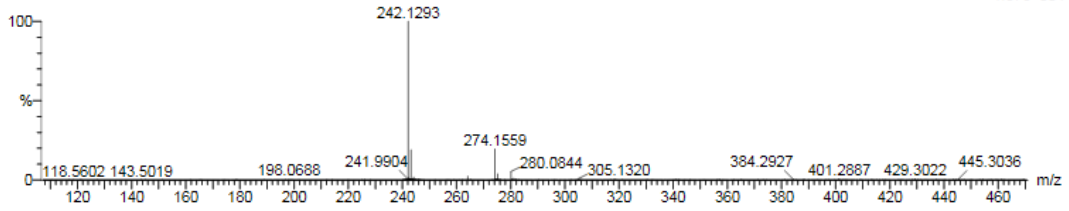
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 30.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2



Monoisotopic Mass, Even Electron Ions
47 formula(e) evaluated with 4 results within limits (up to 1 closest results for each mass)
Elements Used:
C: 0-47 H: 0-80 N: 0-6 O: 0-1

WP-ZHU ECUST institute of Fine Chem 10-Jan-2014 16:52:24
ZWP-ZJ-620-027 125 (0.862) Cm (120:127) 1: TOF MS ES+ 1.57e+004



Minimum:				-1.5			
Maximum:		30.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
242.1293	242.1293	0.0	0.0	8.5	230.7	0.0	C14 H16 N3 O

6. References

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3.H. Turki, S. Abid, Y. Le Bigot, S. Fery‐Forgues and R. El Gharbi, *Synthetic*

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