Two-photon ESIPT-based fluorescent probe using 4-hydroxyisoindoline-1,3-dione for the detection of peroxynitrite

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Table of contents

1. Materials and instruments

All starting materials and reagents were purchased from Sigma Aldrich, Alfa Aesar, Fluorochem, or Acros Organics, and used as received without any further purification. Unless otherwise stated, all solvents used were reagent grade and were used without distillation. All water was deionized which is generated by a reverse osmosis (Ultra-Purified Type I, 18.2 Megohm water). Thin-layer chromatography was performed using commercially available Fluorochem aluminum-backed plates coated with a layer of silica gel (60 Å) with fluorescent indicator UV254. These plates were visualized using ultraviolet light with a wavelength of either 254 or 365 nm. Silica gel column chromatography was carried out using Sigma Aldrich 60 Å silica gel (200-400 mesh). All NMR spectra were obtained using an Agilent ProPulse 500 with all spectra recorded in chloroform-*d*. LC−MS analyses were performed using an Agilent QTOF 6545 with Jetstream ESI spray source coupled to an Agilent 1260 Infinity II Quat pump HPLC with 1260 autosampler, column oven compartment and variable wavelength detector (VWD).

2. Generation of various ROS/RNS

ROO•

ROO• was generated from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). AAPH was added into deionizer water, and then stirred at 37 °C for 30 min to give 0.1 M AAPH.

$O_2^{\bullet-}$

Superoxide was generated from KO_2. KO_2 and 18-crown-6 ether (2.5 eq) were dissolved in DMSO to afford a 0.25 M solution.

•OH

Hydroxyl radical was generated by the Fenton reaction. To prepare •OH solution, hydrogen peroxide (H_2O_2, 10 equiv.) was added to $Fe(ClO_4)_2$ in deionised water.

$^1O_2$

$^1O_2$ was generated by reacting H_2O_2 (1 mM) with NaClO (1 mM). The solution of H_2O_2 was added in one portion to the aqueous solution of NaClO and stir for 2 minutes, using the prepared solution immediately.

$ONOO^-$

0.6 M $NaNO_2$, 0.6 M HC1, 0.7 M H_2O_2 was added simultaneously to a 3 M NaOH solution at 0 ºC. The concentration of peroxynitrite in a 0.5 M NaOH aqueous solution was determined

from the absorption at 302 nm (Ɛ = 1670 M^{-1} cm^{-1}).

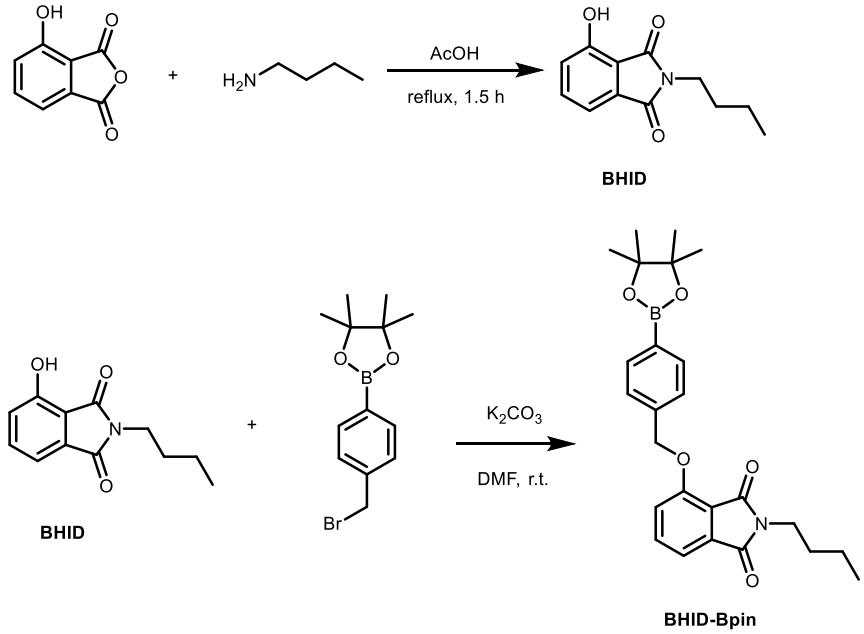
ClO^-

The concentration of ClO^- was determined from the absorption at 292 nm (Ɛ = 350 M^{-1} cm^{-1}).

H_2O_2

The concentration of H_2O_2 was determined from the absorption at 240 nm (Ɛ = 43.6 M^{-1} cm^{-1}).

3. Synthesis of BHID and probe BHID-Bpin



Scheme S1. Synthesis of **BHID** and probe **BHID-Bpin**

BHID

3-Hydroxyphthalic anhydride (0.77 g, 4.69 mmol, 1.0 equiv.) was dissolved in acetic acid (40 mL), then butylamine (0.51 g, 7.04 mmol, 1.5 equiv.) was added. The mixture was further heated under reflux for 1.5 hours, TLC was used to monitor the reaction until the 3-hydroxyphthalic anhydride was consumed. The solvent was removed *in vacuum*, the crude product was purified by silica chromatography using elute solvents (PE/EtOAc = 50/1, v/v). **BHID** was then obtained as a white solid (0.90 g, yield 88%). M.p. 83 - 86 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.66 (s, 1H), 7.56 – 7.53 (m, 1H), 7.35 (d, J = 7.2 Hz, 1H), 7.13 (d, J = 8.4 Hz, 1H), 3.63 (t, J = 7.2 Hz, 2H), 1.66 – 1.61 (m, 2H), 1.39 – 1.31 (m, 2H), 0.93 (t, J = 7.3 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 170.6, 168.1, 154.7, 136.3, 132.3, 122.6, 115.9, 114.8, 37.7, 30.7, 20.2, 13.7. HRMS (ESI^+): calculated $[M+Na]^+$ 242.0788 *m/z*, found 242.0781 *m/z*. M represents $C_{12}H_{13}NO_3$ (chemical formula of compound **BHID**).

BHID-Bpin

BHID (202 mg, 0.92 mmol, 1.0 equiv.), 4-bromomethylphenylboronic acid pinacol ester (327 mg, 1.10 mmol, 1.2 equiv.) and K_2CO_3 (254 mg, 1.84 mmol, 2.0 equiv.) were dissolved in dry DMF (5 mL), The mixture was then stirred for 6 hours at room temperature, TLC was used to monitor the reaction until the **BHID** consumed. The solvent was removed *in vacuum*, the crude product was purified by silica chromatography using elute solvents (PE/EtOAc = 200/1, v/v), then the crude product was washed with n-hexane. **BHID-Bpin** was then obtained as a white solid (245 mg, yield 61%). M.p. 125 – 129 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.82 (d, J = 8.1 Hz, 2H), 7.54 (dd, J = 8.4, 7.3 Hz, 1H), 7.47 (d, J = 8.1 Hz, 2H), 7.41 (d, J = 7.2 Hz, 1H), 7.13 (d, J = 8.4 Hz, 1H), 5.36 (s, 2H), 3.66 (t, J = 7.3 Hz, 2H), 1.69 – 1.63 (m, 2H), 1.41 - 1.36 (m, 2H), 1.34 (s, 12H), 0.94 (t, J = 7.4 Hz, 3H).^{13}C NMR (126 MHz, $CDCl_3$) δ_C 168.3, 167.1, 155.7, 139.1, 135.8, 135.3, 134.6, 126.1, 119.6, 118.3, 115.9, 84.0, 71.1, 37.8, 30.8, 25.0, 20.2, 13.8. HRMS (ESI$^+$): calculated $[M+Na]^+$ 458.2114 *m/z*, found 458.2113 *m/z*. M represents $C_{25}H_{30}BNO_5$ (chemical formula of compound **BHID-Bpin**).

4. UV-Vis and one-photon fluorescence analyses

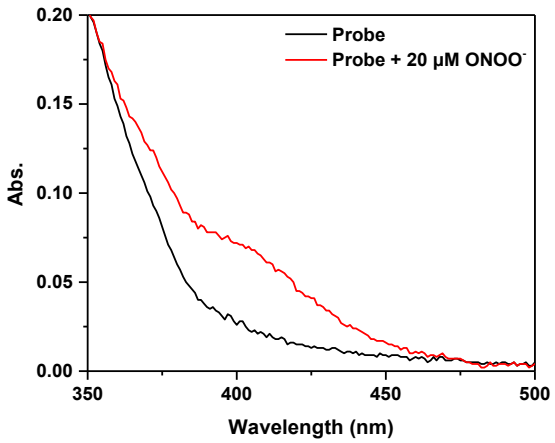


Figure S1. Absorption spectra of probe **BHID-Bpin** (10 μM) with and without $ONOO^-$ (20 μM) in PBS buffer solution (10 mM, pH = 7.40) after 1 min.

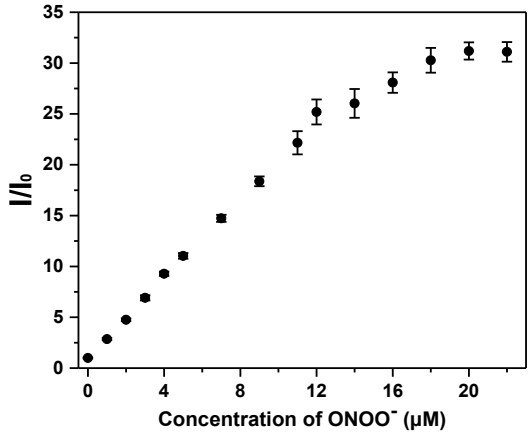


Figure S2. Emission at 515 nm of the probe **BHID-Bpin** (10 μM) with increasing addition of $ONOO^-$ (from 0 to 22 μM) in PBS buffer solution (10 mM, pH = 7.40) after 1 min. λ_{ex} = 400 (bandwidth 8) nm.

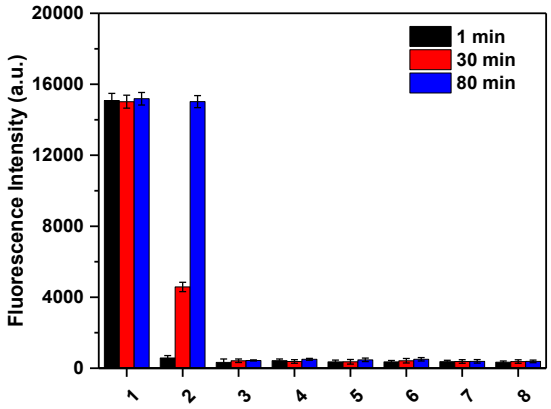


Figure S3. One-photon selectivity bar chart of probe **BHID-Bpin** (10 μM) in PBS buffer solution (10 mM, pH = 7.40) with $ONOO^-$ (20 μM), H_2O_2 (95 μM) and other ROS. 1, $ONOO^-$ (100 μM); 2, H_2O_2; 3, probe **BHID-Bpin** alone; 4, ClO^- (100 μM); 5, $ROO^{\bullet}$ (200 μM); 6, $^{\bullet}OH$ (100 μM); 7, $O_2^{\bullet-}$ (100 μM); 8, $^1O_2$ (100 μM); $\lambda_{ex/em}$= 400 nm /515 nm. Time points were taken at 1 min (black bars), 30 min (red bars) and 80 min (blue bars).

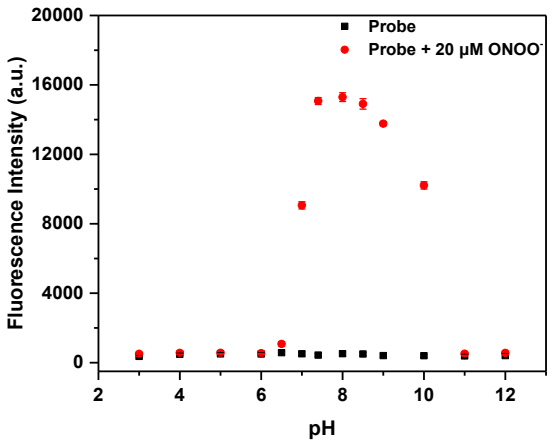


Figure S4. Effects of pH on the fluorescence of the probe **BHID-Bpin** (10 μM) in the absence and presence of $ONOO^-$ (20 μM) after 1 min. $\lambda_{ex/em}$= 400 (bandwidth 8) nm /515 nm.

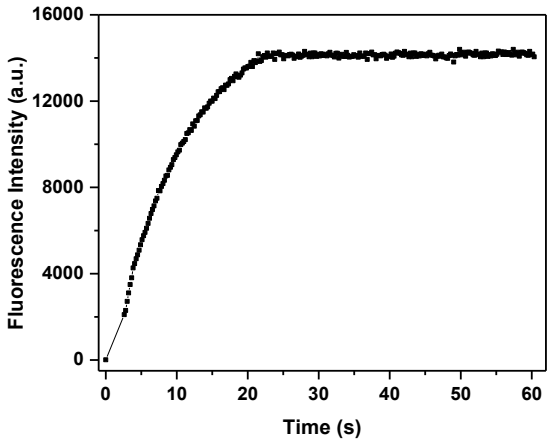


Figure S5. Plots of fluorescence intensity *vs.* the reaction time of the probe **BHID-Bpin** (10 μM) with 20 μM $ONOO^-$ ($\lambda_{ex/em}$= 400 (bandwidth 8) nm /515 nm) in PBS buffer solution (10 mM, pH = 7.40).

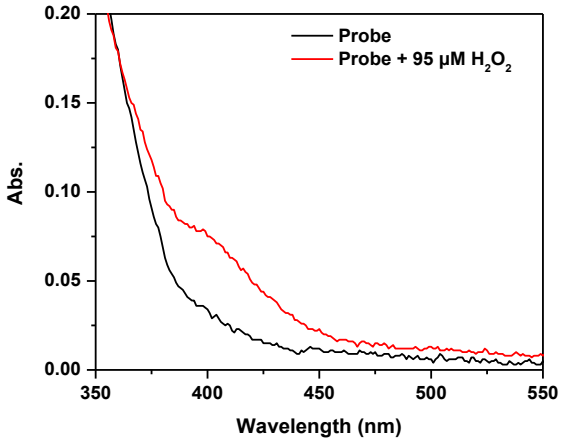


Figure S6. Absorption spectra of probe **BHID-Bpin** (10 μM) with and without H_2O_2 (95 μM) in PBS buffer solution (10 mM, pH = 7.40) after 80 min.

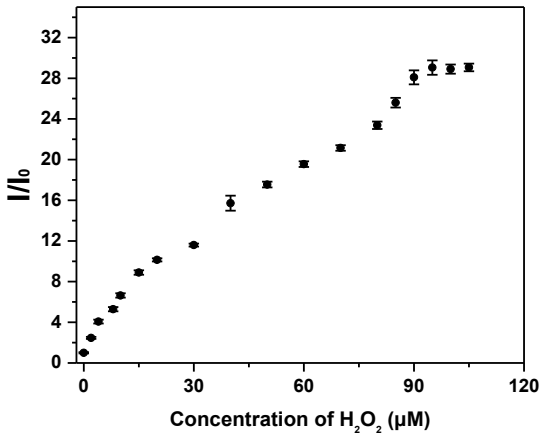


Figure S7. Emission at 515 nm of the probe **BHID-Bpin** (10 μM) with increasing addition of H_2O_2 (from 0 to 105 μM) in PBS buffer solution (10 mM, pH = 7.40) after 80 min. λ_{ex} = 400 (bandwidth 8) nm.

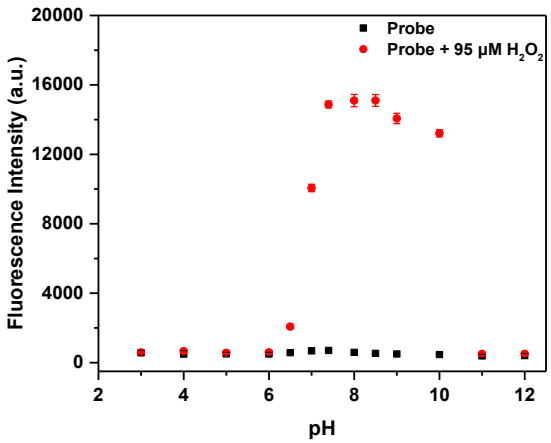


Figure S8. Effects of pH on the fluorescence of the probe **BHID-Bpin** (10 μM) in the absence and presence of 95 μM H_2O_2. $\lambda_{ex/em}$= 400 (bandwidth 8) nm /515 nm after 80 min.

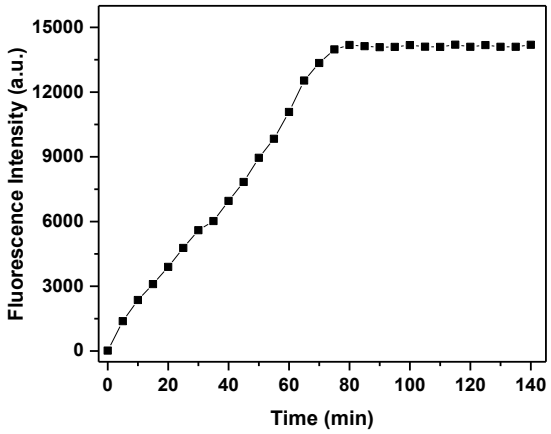
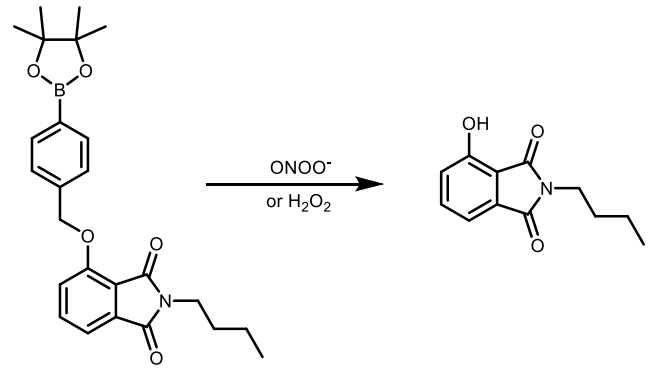


Figure S9. Plots of fluorescence intensity *vs.* the reaction time of the probe **BHID-Bpin** (10 μM) with 95 μM H_2O_2 ($\lambda_{ex/em}$= 400 (bandwidth 8) nm /515 nm) in PBS buffer solution (10 mM, pH = 7.40).

5. Mass spectroscopic analyses



Chemical Formula: $C_{25}H_{30}BNO_5$ — **BHID-Bpin**

Chemical Formula: $C_{12}H_{13}NO_3$ — **BHID**

Scheme S2. Proposed mechanism for the reaction of probe **BHID-Bpin** upon addition of $ONOO^-/H_2O_2$.

Probe **BHID-Bpin** (5 mM, in DMSO) was diluted to 10 μM in H_2O (Figure S11), then $ONOO^-$ (20 μM) was added (Figure S12); H_2O_2 (95 μM) was added to **BHID-Bpin** (10 μM) in H_2O (Figure S13).

Sample Type	Sample	**Position**	P1-A1
Instrument Name	6545 QTof	**User Name**	Xue Tian
Acq Method	Pos_5mins_MS.m	**Acquired Time**	4/11/2021 11:44:33 AM
IRM Calibration Status	Success	**DA Method**	Pos_5mins_MS.m
Comment			

Sample Group		**Info.**	
Walkup Sample Description		**Walkup Method**	Pos_5Mins_C18
Formula	C12H13NO3	**Walkup Method Description**	Positive mode ionization using C18 column chromatography
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.09.00 (B9044.0)

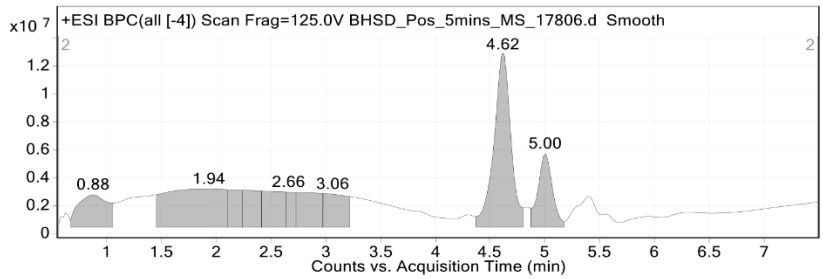


Figure 1: Base peak chromatogram

User Chromatogram Peak List

RT (min)	Area	Area %	Area Sum (%)	Base Peak (m/z)	Width (min)
0.88	43274051	32.37	8.75	224.0902	0.300
1.94	102426229	76.61	20.71	141.9591	0.590
2.15	21394667	16.00	4.32	141.9591	0.120
2.31	28045588	20.98	5.67	141.9591	0.150
2.46	34036458	25.46	6.88	141.9591	0.220
2.66	13383461	10.01	2.71	141.9591	0.080
2.82	36222512	27.09	7.32	141.9590	0.200
3.06	34132994	25.53	6.90	141.9590	0.240
4.62	133698081	100.00	27.03	301.0810	0.160
5.00	48076422	35.96	9.72	279.1607	0.140

Compound Table

Compound Label	RT (min)	Observed mass (m/z)	Neutral observed mass (Da)	Theoretical mass (Da)	Mass error (ppm)	Isotope match score (%)
Cpd 1: C12 H13 N O3	4.59	242.0781	219.0897	219.0895	0.89	83.02

Mass errors of between -5.00 and 5.00 ppm with isotope match scores above 60% are considered confirmation of molecular formulae

Walkup MS Report

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Compound specific information

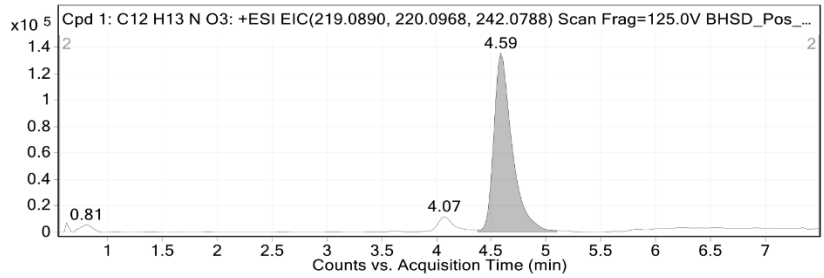

Figure: Extracted ion chromatogram (EIC) of compound.

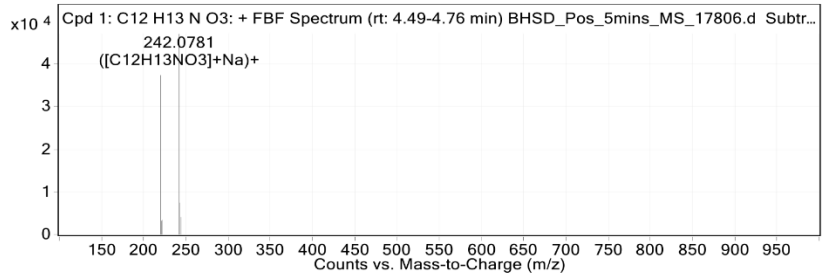

Figure: Full range view of Compound spectra and potential adducts.

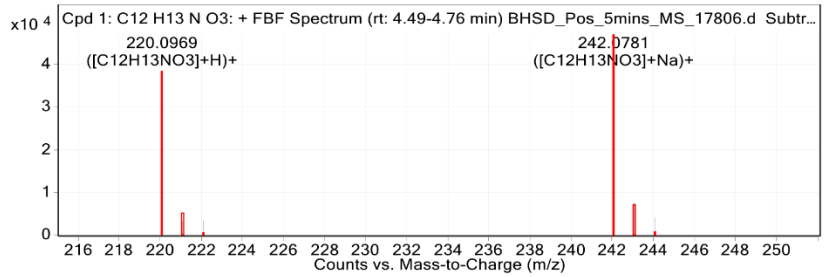

Figure: Zoomed Compound spectra view
(red boxes indicating expected theoretical isotope spacing and abundance)

Compound isotope peak List

m/z	z	Abund	Formula	Ion
220.0969	1	37380.8	C12H13NO3	(M+H)+
221.1014	1	3252.3	C12H13NO3	(M+H)+
222.1139	1	3422.3	C12H13NO3	(M+H)+
242.0781	1	47046.3	C12H13NO3	(M+Na)+
243.0828	1	7480.7	C12H13NO3	(M+Na)+
244.0834	1	4074.1	C12H13NO3	(M+Na)+

--- End Of Report ---

Figure S10. LC-MS of **BHID** (10 μM).

Sample Type	Sample	**Position**	P1-A3
Instrument Name	6545 QTof	**User Name**	Xue Tian
Acq Method	Pos_5mins_MS.m	**Acquired Time**	02/10/2020 10:46:21
IRM Calibration Status	Success	**DA Method**	Pos_5mins_MS.m
Comment			

Sample Group		**Info.**	
Walkup Sample Description		**Walkup Method**	Pos_5Mins_C18
Formula	C25H30BNO5	**Walkup Method Description**	Positive mode ionization using C18 column chromatography
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.09.00 (B9044.0)

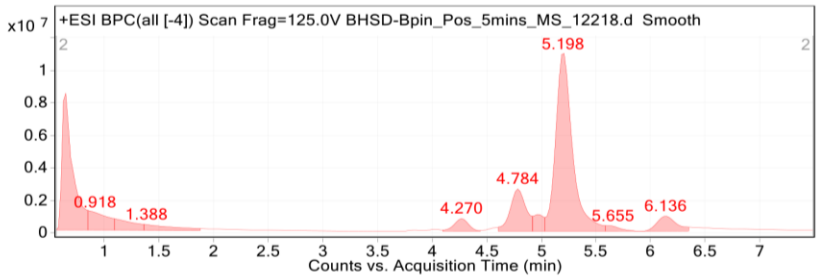

Figure 1: Base peak chromatogram

User Chromatogram Peak List

RT (min)	Area	Area %	Area Sum (%)	Base Peak (m/z)	Width (min)
0.64	58520187	49.30	22.45	101.0031	0.100
0.92	14069821	11.85	5.40	101.0028	0.198
1.16	8365370	7.05	3.21	101.0028	0.199
1.39	6706124	5.65	2.57	922.0015	0.249
4.27	7224754	6.09	2.77	185.1139	0.142
4.78	26163920	22.04	10.04	757.3030	0.151
4.97	6440782	5.43	2.47	301.1393	0.103
5.20	118706009	100.00	45.55	893.4295	0.165
5.66	2996504	2.52	1.15	670.2900	0.144
6.14	11428807	9.63	4.39	125.9859	0.185

Compound Table

Compound Label	RT (min)	Observed mass (m/z)	Neutral observed mass (Da)	Theoretical mass (Da)	Mass error (ppm)	Isotope match score (%)
Cpd 1: C25 H30 B N O5	5.81	458.2113	434.2253	434.2253	-0.12	99.86

Mass errors of between -5.00 and 5.00 ppm with isotope match scores above 60% are considered confirmation of molecular formulae

Walkup MS Report

Compound specific information

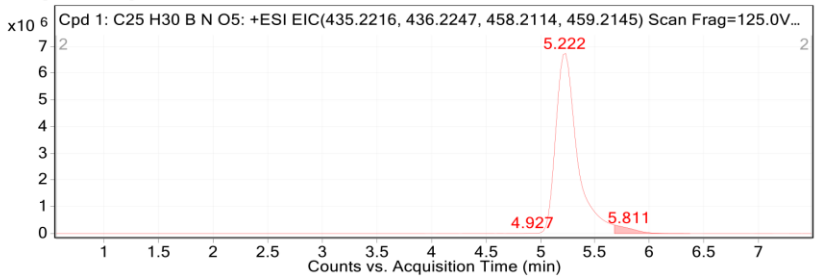

Figure: Extracted ion chromatogram (EIC) of compound.

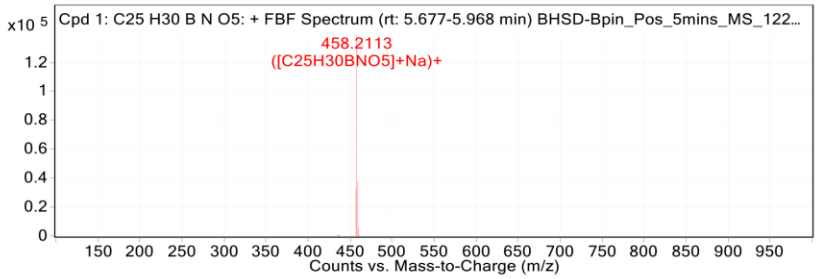

Figure: Full range view of Compound spectra and potential adducts.

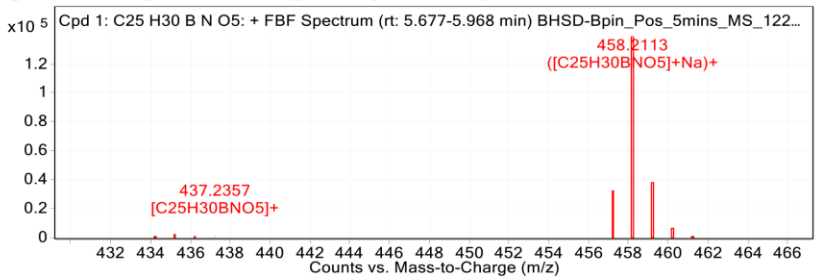

Figure: Zoomed Compound spectra view
(red boxes indicating expected theoretical isotope spacing and abundance)

Compound isotope peak List

m/z	z	Abund	Formula	Ion
434.2481	1	58.7	C25H30BNO5	M+
435.2296	1	924.3	C25H30BNO5	M+
436.2308	1	714.2	C25H30BNO5	M+
437.2357	1	1271.1	C25H30BNO5	M+
457.2142	1	32482.7	C25H30BNO5	(M+Na)+
458.2113	1	139064.6	C25H30BNO5	(M+Na)+
459.2142	1	37723.4	C25H30BNO5	(M+Na)+
460.2171	1	6184.0	C25H30BNO5	(M+Na)+
461.2202	1	959.6	C25H30BNO5	(M+Na)+
462.2160	1	84.0	C25H30BNO5	(M+Na)+

Figure S11. LC-MS of **BHID-Bpin**.

Sample Type	Sample	**Position**	P1-A1
Instrument Name	6545 QTof	**User Name**	Xue Tian
Acq Method	Pos_5mins_MS.m	**Acquired Time**	10/2/2020 10:29:26 AM
IRM Calibration Status	Success	**DA Method**	Pos_5mins_MS.m
Comment			

Sample Group		**Info.**	
Walkup Sample Description		**Walkup Method**	Pos_5Mins_C18
Formula	C12H13NO3	**Walkup Method Description**	Positive mode ionization using C18 column chromatography
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.09.00 (B9044.0)

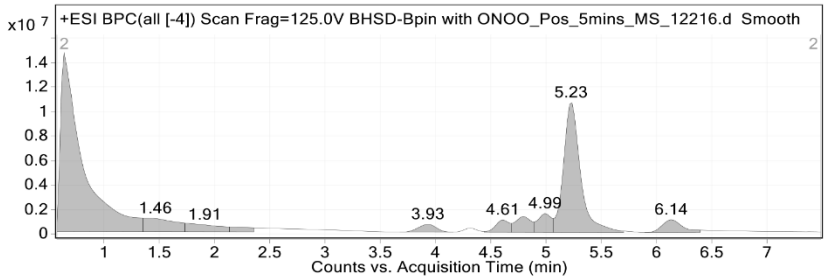

Figure 1: Base peak chromatogram

User Chromatogram Peak List

RT (min)	Area	Area %	Area Sum (%)	Base Peak (m/z)	Width (min)
0.64	204926167	100.00	49.44	101.0034	0.180
1.46	21594166	10.54	5.21	101.0027	0.300
1.91	13108566	6.40	3.16	101.0028	0.310
2.20	4968370	2.42	1.20	101.0028	0.180
3.93	7410797	3.62	1.79	185.1137	0.180
4.61	8660820	4.23	2.09	364.1133	0.130
4.80	12756365	6.22	3.08	757.3026	0.150
4.99	13077941	6.38	3.16	301.1395	0.130
5.23	114576241	55.91	27.65	893.4297	0.170
6.14	13375191	6.53	3.23	125.9858	0.200

Compound Table

Compound Label	RT (min)	Observed mass (m/z)	Neutral observed mass (Da)	Theoretical mass (Da)	Mass error (ppm)	Isotope match score (%)
Cpd 1: C12 H13 N O3	4.74	242.0785	219.0886	219.0895	-4.44	92.71

Mass errors of between -5.00 and 5.00 ppm with isotope match scores above 60% are considered confirmation of molecular formulae

Walkup MS Report

Compound specific information

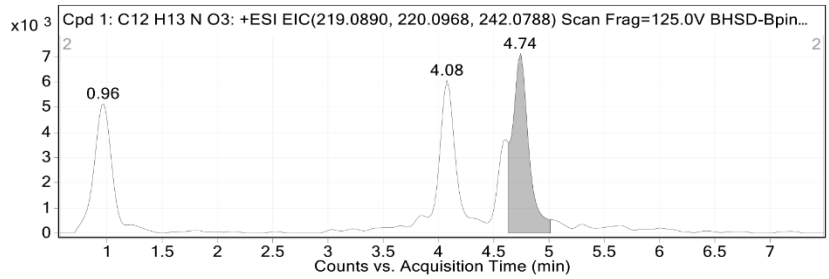

Figure: Extracted ion chromatogram (EIC) of compound.

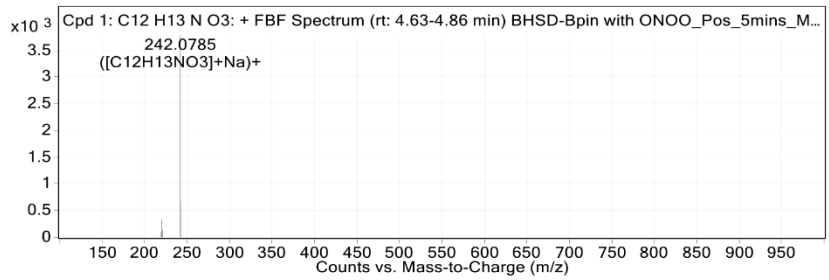

Figure: Full range view of Compound spectra and potential adducts.

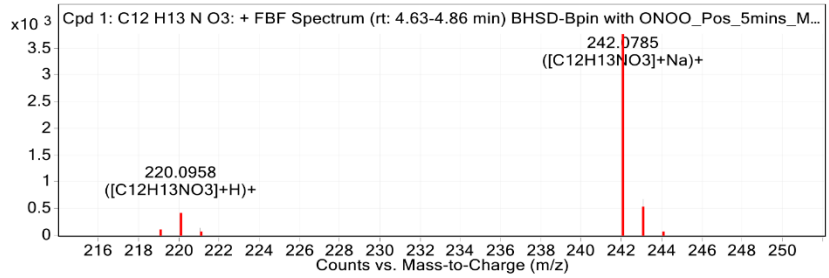

Figure: Zoomed Compound spectra view
(red boxes indicating expected theoretical isotope spacing and abundance)

Compound isotope peak List

m/z	z	Abund	Formula	Ion
219.0837	1	93.8	C12H13NO3	M+
220.0958	1	330.6	C12H13NO3	(M+H)+
221.0777	1	136.0	C12H13NO3	(M+H)+
242.0785	1	3764.4	C12H13NO3	(M+Na)+
243.0826	1	672.4	C12H13NO3	(M+Na)+
244.0714	1	22.5	C12H13NO3	(M+Na)+

Figure S12. LC-MS of **BHID-Bpin** with 2 equiv. $ONOO^-$.

Sample Type	Sample	**Position**	P1-A4
Instrument Name	6545 QTof	**User Name**	Xue Tian
Acq Method	Pos_5mins_MS.m	**Acquired Time**	10/2/2020 12:11:13 PM
IRM Calibration Status	Success	**DA Method**	Pos_5mins_MS.m
Comment			

Sample Group		**Info.**	
Walkup Sample Description		**Walkup Method**	Pos_5Mins_C18
Formula	C12H13NO3	**Walkup Method Description**	Positive mode ionization using C18 column chromatography
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.09.00 (B9044.0)

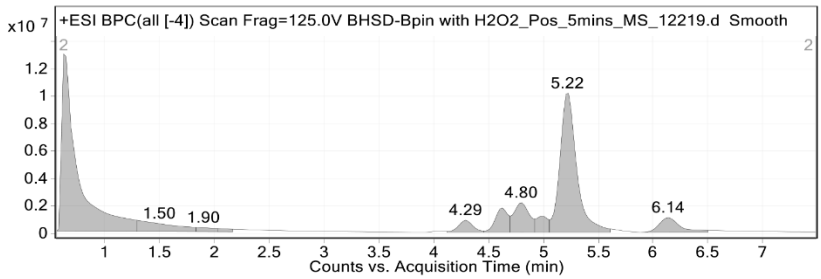

Figure 1: Base peak chromatogram

User Chromatogram Peak List

RT (min)	Area	Area %	Area Sum (%)	Base Peak (m/z)	Width (min)
0.64	133616203	100.00	40.52	101.0034	0.160
1.50	16730884	12.52	5.07	101.0029	0.420
1.90	3257829	2.44	0.99	922.0019	0.150
2.04	1670455	1.25	0.51	922.0020	0.120
4.29	8140960	6.09	2.47	185.1141	0.140
4.62	14230218	10.65	4.32	364.1136	0.120
4.80	21221816	15.88	6.44	757.3035	0.150
4.99	8580639	6.42	2.60	301.1397	0.100
5.22	108110284	80.91	32.78	893.4299	0.160
6.14	14212406	10.64	4.31	125.9861	0.200

Compound Table

Compound Label	RT (min)	Observed mass (m/z)	Neutral observed mass (Da)	Theoretical mass (Da)	Mass error (ppm)	Isotope match score (%)
Cpd 1: C12 H13 N O3	4.74	242.0785	219.0892	219.0895	-1.72	92.02

Mass errors of between -5.00 and 5.00 ppm with isotope match scores above 60% are considered confirmation of molecular formulae

Walkup MS Report

Compound specific information

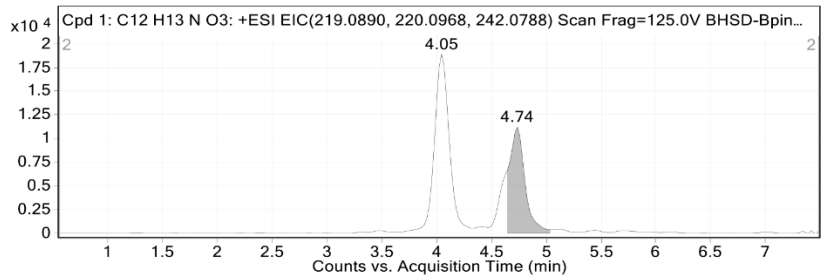

Figure: Extracted ion chromatogram (EIC) of compound.

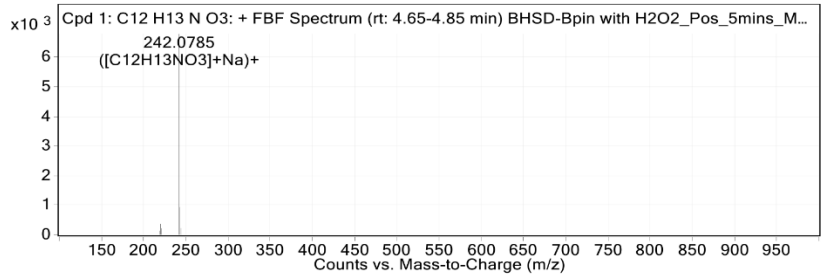

Figure: Full range view of Compound spectra and potential adducts.

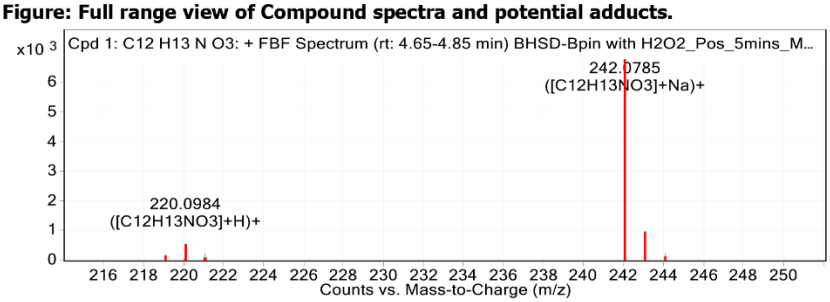

Figure: Zoomed Compound spectra view
(red boxes indicating expected theoretical isotope spacing and abundance)

Compound isotope peak List

m/z	z	Abund	Formula	Ion
219.0858	1	130.2	C12H13NO3	M+
220.0984	1	367.3	C12H13NO3	(M+H)+
221.0810	1	213.9	C12H13NO3	(M+H)+
242.0785	1	6777.5	C12H13NO3	(M+Na)+
243.0835	1	927.7	C12H13NO3	(M+Na)+
244.0900	1	223.3	C12H13NO3	(M+Na)+

--- End Of Report ---

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Figure S13. LC-MS of **BHID-Bpin** with 9.5 equiv. H_2O_2.

6. Measurement of two-photon action cross section (Φδ)

The measurement of the two-photon absorption cross section (δ) was carried out using the femto second (fs) fluorescence measurement technique widely used in previous studies.[1] **BHID** (5 μM) or **BHID-Bpin** (5 μM) were dissolved in PBS buffer (15 mM, pH 7.40) and the fluorescence intensity measured through a two-photon light source was measured at 730−860 nm by using rhodamine 6G (1 μM， dissolved in methanol) as the reference, whose two-photon property has been well characterized in the literature.[2, 3] The integral intensities value of the fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The δ was calculated by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$: where the subscripts *s* and *r* stand for the sample and reference molecules. The integral intensity of the signal collected by a charge coupled device (CCD) detector was denoted as *S*. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as *c*. δ_r is the two-photon absorption cross section of the reference molecule.

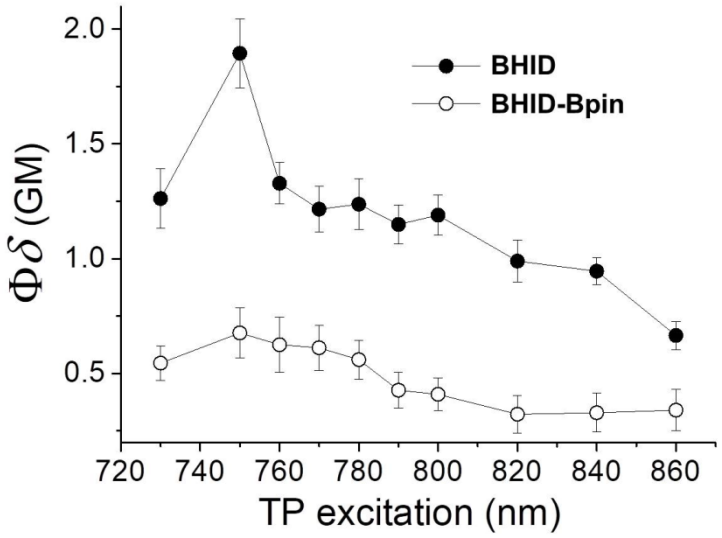

Figure S14. Two-photon action cross section spectra of **BHID** (5 μM) and **BHID-Bpin** (5 μM) in PBS buffer (pH 7.40, 15 mM).

7. Cell viability

The HeLa cells were seeded in a 96-well plate with culture media for 30 h. After overnight culture, cells were incubated with 1 to 20 μM concentration of **BHID-Bpin** for 24 h. To identify cell viability, reagents were removed and 1 mg/mL of CCK-8 kit (Cell Counting Kit-8; Dojindo, Japan) media was added to cells in 96-well plate and incubated for 4 h at

37 °C in a CO_2 incubator, and the produced formazan was dissolved in 0.1 mL DMSO and read with a Spectra Microwell plate reader. Absorption value was analyzed, and the average cell viability was calculated as a percentage of the mean vehicle control. Experiments were performed in 5 independent viability tests.

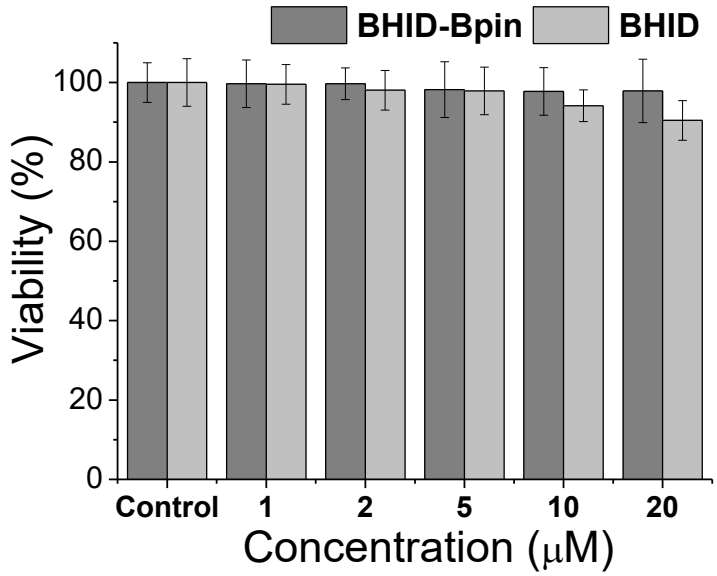

Figure S15. Viability of HeLa cells in the presence of **BHID** and **BHID-Bpin** as measured by using CCK-8 kit. The cells were incubated with 1-20 μM **BHID** and **BHID-Bpin** for 24 h.

8. Two-photon fluorescence microscope imaging

Two-photon fluorescence microscopic images of **BHID-Bpin** labeled cells and tissues were obtained with spectral two-photon microscopes (Leica TCS SP8 MP) with × 40 oil objectives (numerical aperture (NA) = 1.30). The two-photon fluorescence microscopic images were obtained with a DMI6000B Microscope (Leica) by exciting the **BHID-Bpin** with a mode-locked titanium-sapphire laser source (Mai Tai HP; Spectra Physics, 80 MHz pulse frequency, 100 fs pulse width) set at excitation wavelength 750 nm and output power 2670 mW, which corresponded to approximately 1.3 mW average laser power at the imaging focal point. The cell and tissue imaging was performed using live cell incubator systems (Chamlide IC; Live Cell Instrument) for stable environment by maintaining 37 °C in 5% CO_2 condition under the long-time incubation. To obtain two-photon images at 400–600 nm range, internal photomultiplier tubes (PMTs) were used to collect the signals in an 8 bit unsigned 512×512 pixels at 400 Hz scan speed.

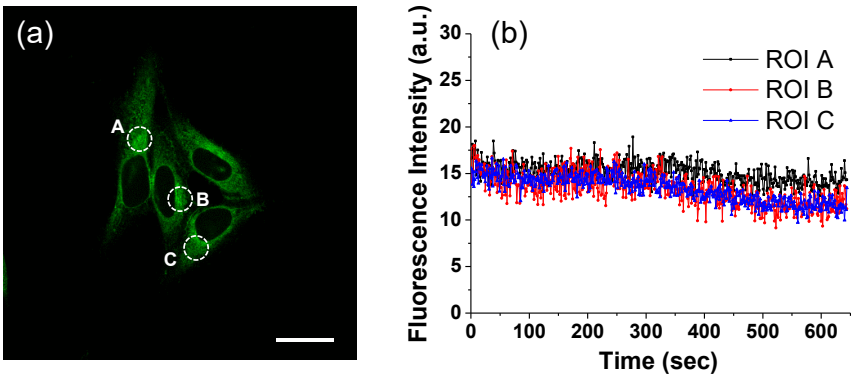

Figure S16. (a) TPM image of **BHID-Bpin** labeled (5 μM) HeLa cells collected at 400-600 nm. (b) The relative TPEF intensity as a function of time. The digitized intensity was recorded with 1.63 sec intervals for the duration of 10 min using xyt mode (λ_{ex} = 750 nm, ~100 fs). Cells shown are representative images from replicate experiments (n = 5). Scale bar: 50 μm.

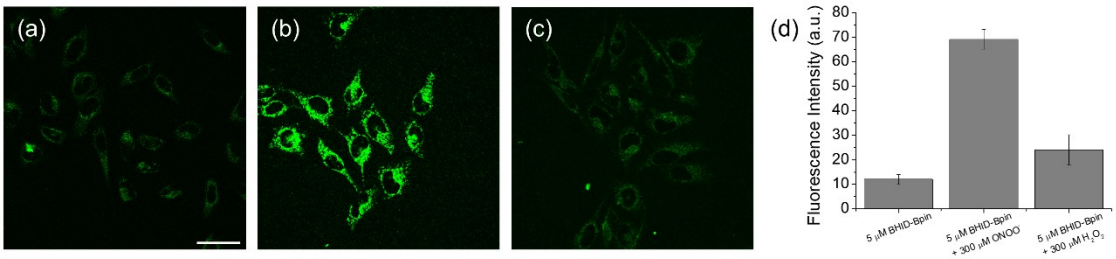

Figure S17. Two-photon excitation microscopy images of exogenous $ONOO^-$ and H_2O_2 in HeLa cells. The cells stained with (a) 10 μM **BHID-Bpin** for 30 min. No treatment, (b) 300 μM $ONOO^-$, (c) 300 μM H_2O_2 for 20 min, (d) TPEF intensity in a–c. The images were obtained by collecting emissions at 400–600 nm upon excitation at 750 nm. Scale bar: 50 μm.

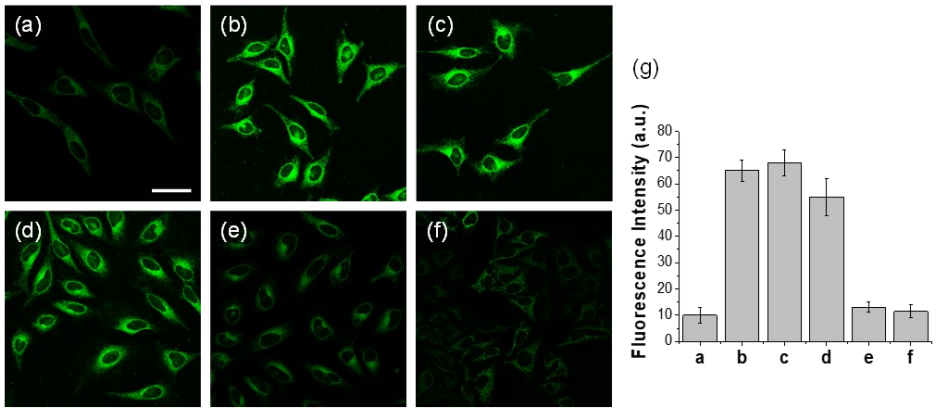

Figure S18. Two-photon excitation microscopy images of exogenous and endogenous $ONOO^-$ in HeLa cells. The cells stained with (a) 5 μM **BHID-Bpin** for 30 min. No treatment, (b) 300 μM $ONOO^-$, (c) 20 μM SIN-1 for 10 min, (d) 50 ng/mL IFN-γ and 500 ng/mL LPS for 4 h, (e) 200 μM aminoguanidine, 20 μM SIN-1 for 20 min, and (f) 100 μM ebselen, 20 μM SIN-1. (g) TPEF intensity in a–f. The images were obtained by collecting emissions at 400–600 nm upon excitation at 750 nm. Scale bar: 50 μm.

9. Cell culture

All cells were grown by processing in 35 mm glass bottom dishes (NEST) at a density of 3×10^5 cells per dish for 3 days prior to imaging experiments. The cells were cultured in a humidified atmosphere of 5/95 (v/v) of CO_2/air at 37 °C. PBS buffer solution (15 mM, pH 7.40): 800 mL of distilled water in a suitable container with 0.2 g KCl, 8 g NaCl, 1.5 g $NaHPO_4$, 0.24 g KH_2PO_4, then add distilled water until volume is 1 L, finally, use an autoclave for sterilization. The culture mediums for each cell are as below. HeLa human cervical carcinoma cells (ATCC, Manassas, VA, USA): MEM (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units/ml), and streptomycin (100 μg/mL).

10. Preparation and staining of fresh rat hippocampal slices

Slices were prepared from the hippocampus of a 2week-old rat (SD). Coronal slices were cut into 400 μm-thick slices by using a vibrating-blade microtome and stored in artificial cerebrospinal fluid (ACSF; 138.6 mM NaCl, 3.5 mM KCl, 21 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 9.9 mM d-glucose, 1 mM $CaCl_2$, and 3 mM $MgCl_2$). Slices were labelled with 20 μm **BHID-Bpin** in ACSF bubbled with 95% O_2 and 5% CO_2, for 30 min at 37°C. Slices were then washed three times with ACSF and transferred to glass-bottomed imaging dishes (MatTek Corp.). The image of tissue slices were observed using a two-photon microscope. All protocol were performed according to the Korean Code of Practice for the Care and Use of Animals and approved by the Laboratory Animal Research center of Ajou University Medical Center, Suwon, Korea.

11. NMR spectra

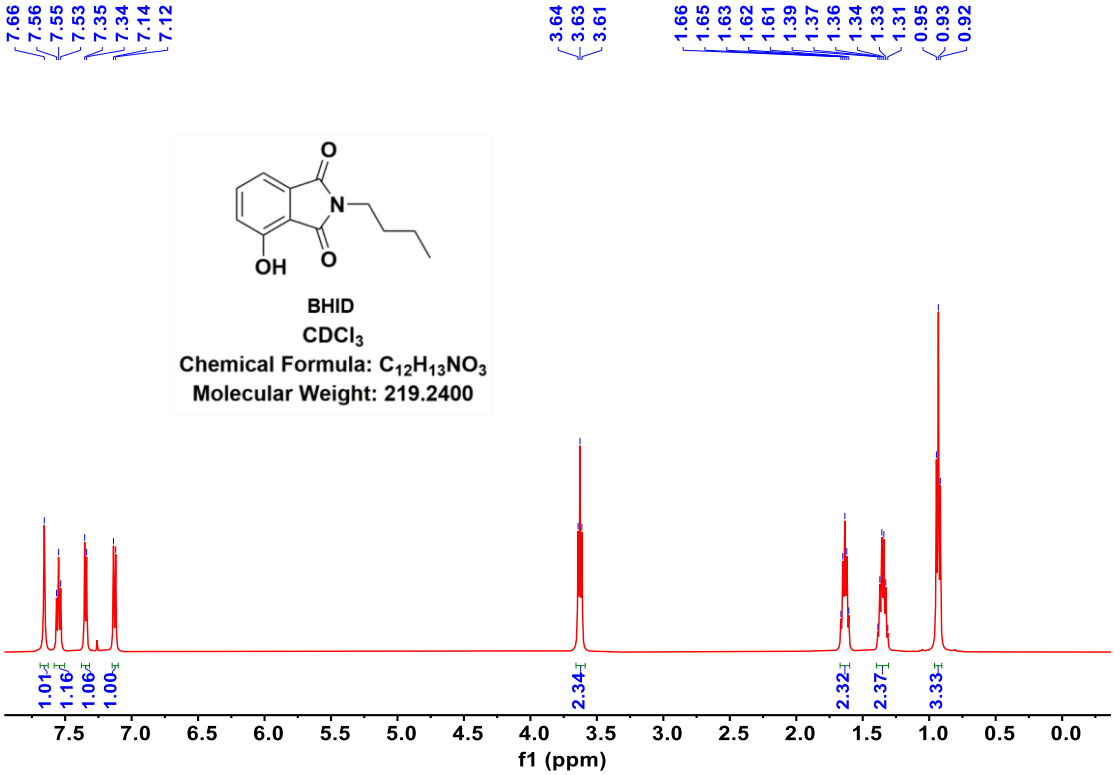

Figure S19. ^{1}H NMR (500 MHz, $CDCl_3$) of **BHID**.

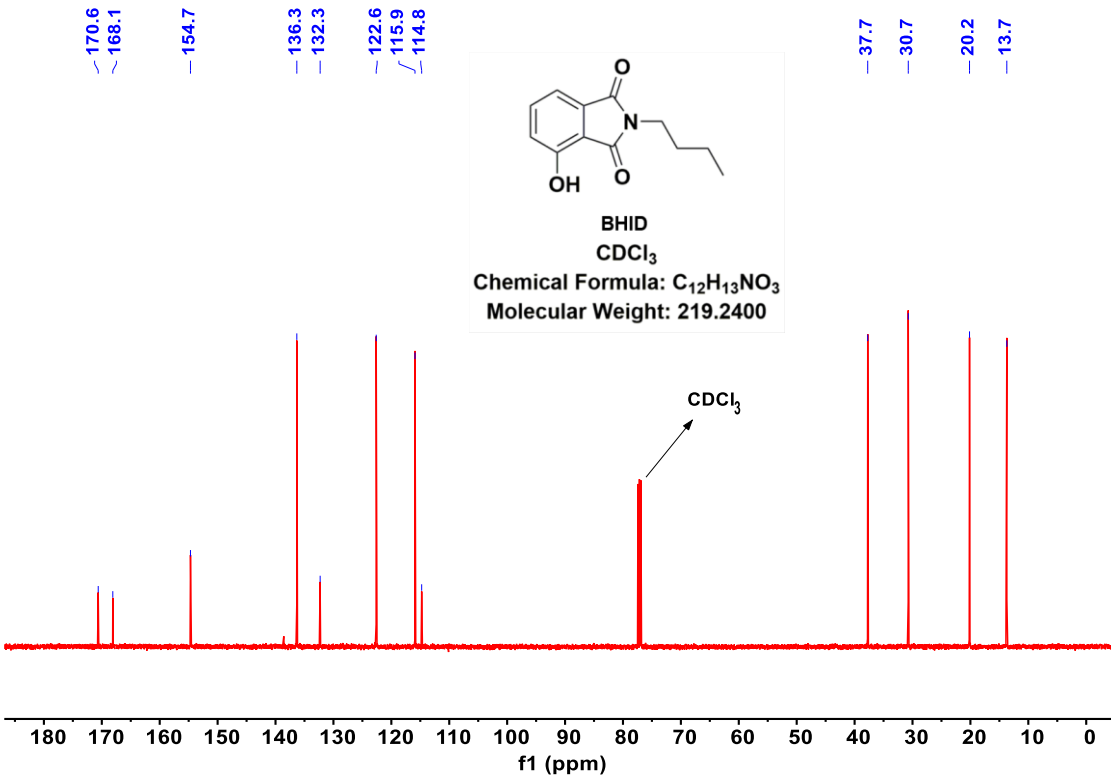

Figure S20. ^{13}C NMR (126 MHz, $CDCl_3$) of **BHID**.

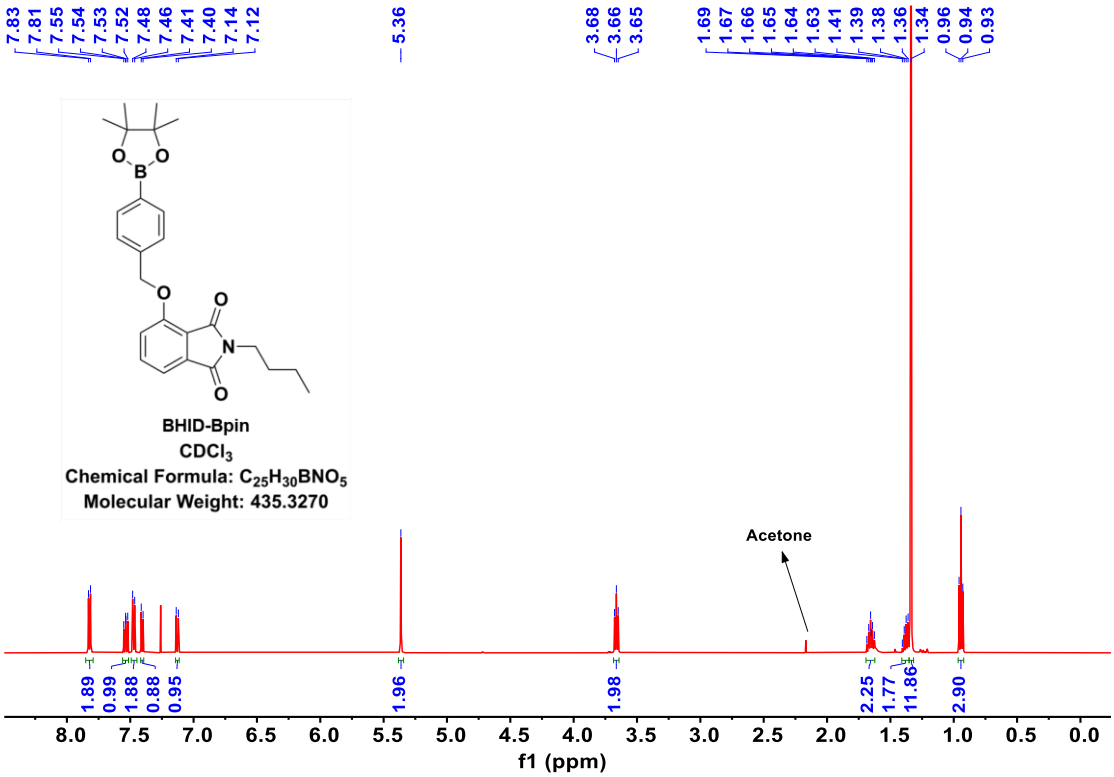

Figure S21. ^{1}H NMR (500 MHz, $CDCl_3$) of **BHID-Bpin**.

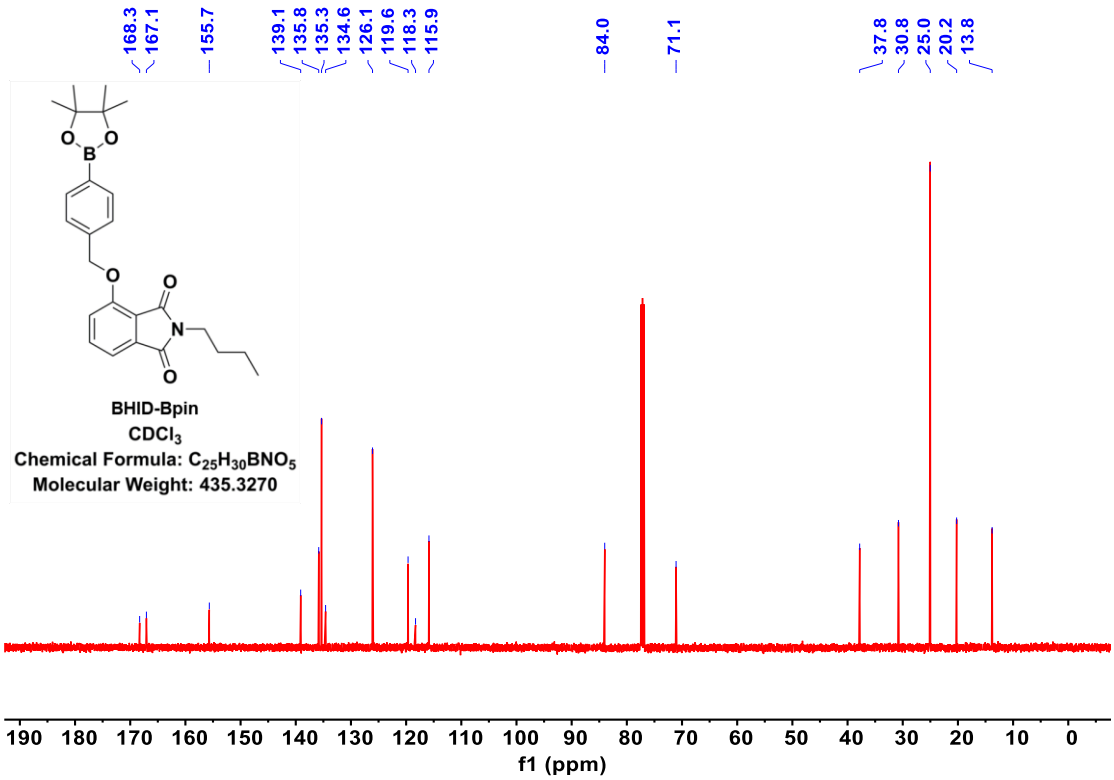

Figure S22. ^{13}C NMR (126 MHz, $CDCl_3$) of **BHID-Bpin**.

12. References

1. S. K. Lee, W. J. Yang, J. J. Choi, C. H. Kim, S.-J. Jeon and B. R. Cho, *Org. Lett.*, 2005, **7**, 323-326.
2. C. Xu and W. W. Webb, *J. Opt. Soc. Am. B*, 1996, **13**, 481-491.
3. N. S. Makarov, M. Drobizhev and A. Rebane, *Opt. Express*, 2008, **16**, 4029-4047.