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Electronic Supplementary Information

Rhodamine Cyclic Hydrazide as Fluorescent Probe for the Detection of Hydroxyl Radical

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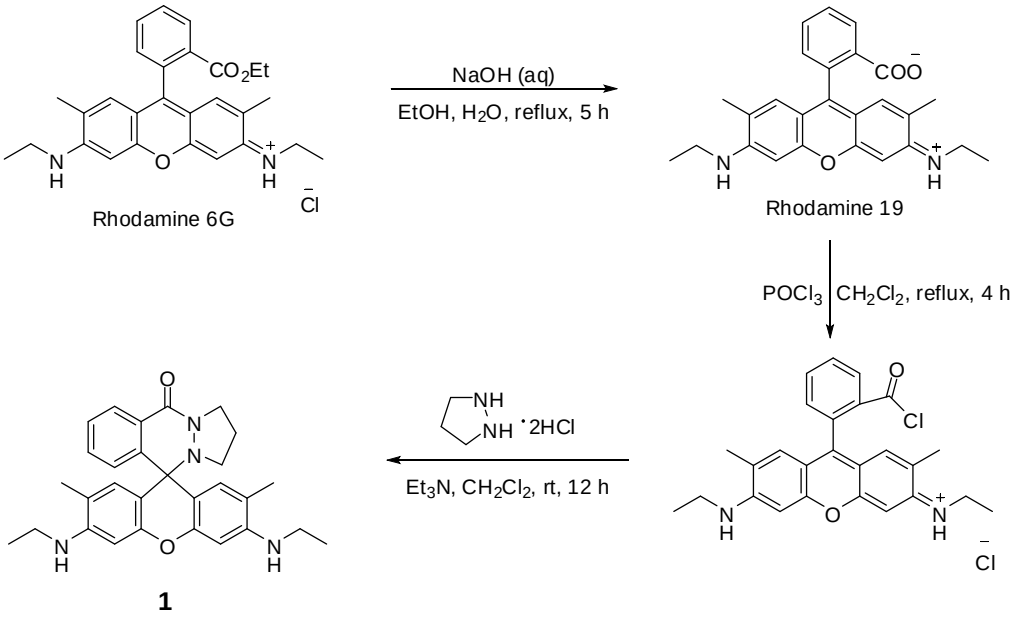
1. General Methods

General Synthetic Materials and Methods: Silica gel 60 (230-400 mesh, Merck) was used for column chromatography. Analytical thin layer chromatography was performed using Merck 60 F254 silica gel (precoated sheets, 0.25 mm thick). All reagents and solvents for reactions were used as received with the following exceptions. 1, 2-Dichloromethane (CH_2Cl_2) and triethylamine (Et_3N) were distilled from calcium hydride (CaH_2). All other chemicals used were purchased from Sigma-Aldrich and were used as received.

Spectroscopic Materials and Methods: Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ unless otherwise stated, with tetramethylsilane (TMS) as internal reference at ambient temperature, mainly on a Bruker Avance II-400 Fourier Transform Spectrometer operating at 400 MHz for ^{1}H and at 100.6 MHz for ^{13}C. Mass spectra were recorded on a ZQ-4000 LC-MS and QUATTRO LC Triple Quadrupole Tandem mass spectrometer for both low resolution and high resolution mass spectra. The pH was recorded by HI-8014 instrument (HANNA). Melting points were measured on a Z289078 (Sigma-Aldrich) microscope. Infrared absorption spectra were recorded as a solution in CH_2Cl_2 on a Avatar 360 FT-IR spectrophotometer. HPLC spectra were recorded using a Agilent Technology 1260 infinity. Fluorescence emission spectra were obtained using a Hitachi F-4500 spectrofluorimeter linked to a Pentium PC running SpectraCalc software package. The slit width was 5.0 nm for both excitation and emission. The photon multiplier voltage was 400 V. A circulating H_2O/DMF bath was used during all experiments to regulate the temperature at 25±1 ℃. Samples were contained in 10.0 nm path length quartz cuvettes (3.5 mL volume). Upon excitation at 500 nm, the emission spectra were integrated over the range 510-630 nm. All measurements were conducted at least in triplicate.

2. Synthesis of 1 from Rhodamine 6G.



Rhodamine 19 was prepared according to the known procedure.[1]

1, 2-Pyrazolidine dihydrochloride was prepared according to the known procedure.[2]

To a rhodamine 6G (12.2 g, 25.5 mmol) in EtOH (300 mL) was added sodium hydroxide (3.04 g, 76.4 mmol) in H_2O (45 mL) and the reaction mixture was stirred for 5 h under reflux conditions. After the solution was cooled to room temperature, distilled H_2O (50 mL) was added to the solution. The solution was cooled to 0 ℃. The resulting precipitates were filtered and dried at 70 ℃ for 30 min to give the 7.0 g (66%) of rhodamine 19.

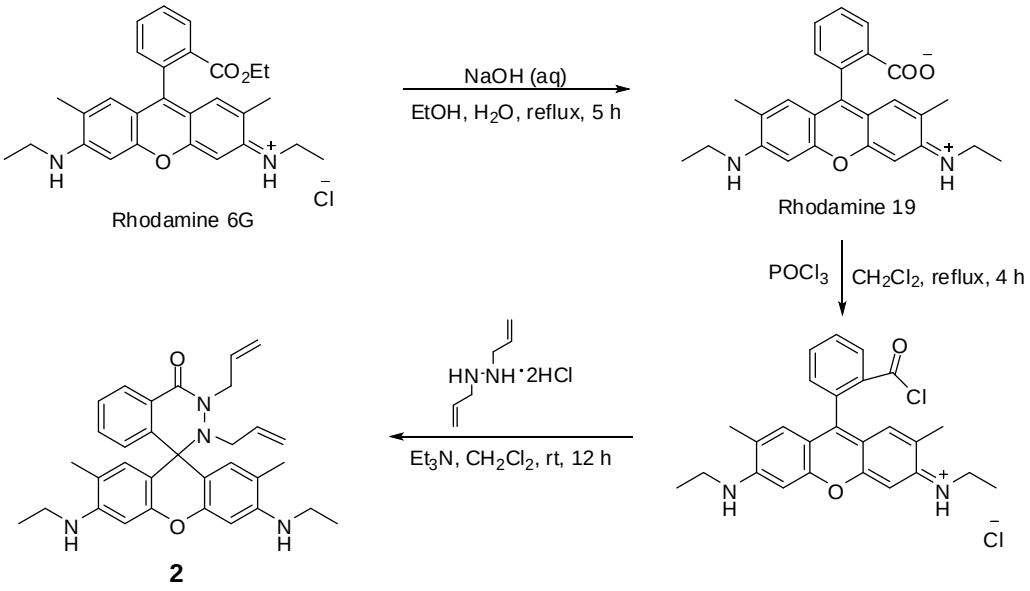
To a solution of rhodamine 19 (973 mg, 2.35 mmol) in dichloromethane (20 mL) was added phosphorus oxychloride (0.65 mL, 7.04 mmol) over 2 min. The solution was refluxed for 4 h. The reaction mixture was cooled and evaporated in vacuum to give rhodamine acid chloride, which was used for next step without purification.

To 1,2-pyrazolidine dihydrochloride (270 mg, 1.86 mmol) in dichloromethane (8 mL) were added slowly triethylamine (1.10 mL, 7.80 mmol) and then the crude rhodamine acid chloride in dichloromethane (10 mL). And the reaction mixture was stirred for 12 h at room temperature. The mixture was concentrated under vacuum and the crude product was purified by column chromatography (hexanes/EtOAc = 2/1 to 1/1) to give 330 mg of compound **1** as a pale red solid (46%

from rhodamine 19).

Compound 1 : R_f = 0.25 (silica gel, hexanes/EtOAc = 1/2); mp 253-255 ℃; ^{1}H NMR (400 MHz, $CDCl_3$) = 8.21-8.19 (m, 1H), 7.40-7.26 (m, 2H), 6.97-6.95 (m, 1H), 6.69 (s, 2H), 6.33 (s, 2H), 3.74 (t, *J* = 4.5 Hz, 2H), 3.53 (bs, 2H), 3.21 (q, *J* = 4.5 Hz, 4H), 2.60 (t, *J* = 4.0 Hz, 2H), 1.97 (s, 6H), 1.86-1.80 (m, 2H), 1.32 (t, *J* = 4.5 Hz, 6H); ^{13}C NMR (100.6 MHz, $CDCl_3$) = 160.8, 151.9, 147.4, 144.8, 132.3, 131.2, 129.0, 128.3, 127.2, 126.7, 117.4, 108.2, 96.0, 63.8, 48.1, 44.8, 38.5, 22.5, 17.1, 15.0; IR (film, cm^{-1}) 3432, 3367, 2973, 2927, 2869, 1634, 1571, 1516, 1468, 1417, 1345, 1263, 1215, 1154, 1094, 1014, 914, 875, 812, 747; HRMS (FAB) *m/z* calcd for $C_{29}H_{33}N_4O_2$ [$(M+H)^+$] 469.2525; found 469.2522.

3. Synthesis of 2 from Rhodamine 6G.

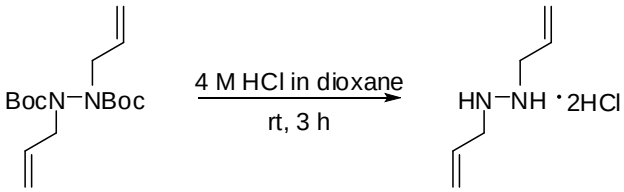


To the 1, 2-diallylhydrazine dihydrochloride (380 mg, 2.10 mmol) dissolved in dichloromethane (10 mL) were added triethylamine (1.2 mL, 8.8 mmol) slowly and then crude rhodamine acid chloride in dichloromethane (10 mL). And the reaction mixture was stirred for 12 h at room temperature. The mixture was concentrated under vacuum and the crude product was purified by column chromatography (hexanes/EtOAc = 5:1 to 3:1) to give 430 mg of compound **2** as pink-red solid (36% from Rhodamine 19).

Compound 2 : R_f = 0.20 (silica gel, hexanes/EtOAc = 2/1); mp 185-187 ℃; ^{1}H NMR (400 MHz, $CDCl_3$) = 8.28-8.26 (m, 1H), 7.58-7.50 (m, 2H), 7.32-7.30 (m, 1H), 6.49 (s, 4H), 5.53-5.43 (m, 1H), 4.93-4.66 (m, 5H), 3.81 (bs, 2H), 3.49 (s, 2H), 3.22 (q, *J* = 4.3 Hz, 6H), 1.96 (s, 6H), 1.33-1.30 (t, *J* =

4.4 Hz, 6H); ^{13}C NMR (100.6 MHz, $CDCl_3$) = 164.2, 153.3, 147.0, 138.6, 134.8, 132.3, 132.1, 130.2, 128.7, 127.8, 127.7, 117.8, 116.9, 115.8, 97.8, 64.3, 56.5, 52.7, 38.5, 17.0, 14.8; IR (film, cm^{-1}) 3434, 3384, 3074, 2973, 2869, 1635, 1574, 1512, 1417, 1342, 1281, 1213, 1155, 1001, 920, 816, 753; HRMS (FAB) *m/z* calcd for $C_{32}H_{37}N_4O_2$ [$(M+H)^+$] 509.2838; found 509.2851

4. Synthesis of 1,2-diallylhydrazine dihydrochloride.

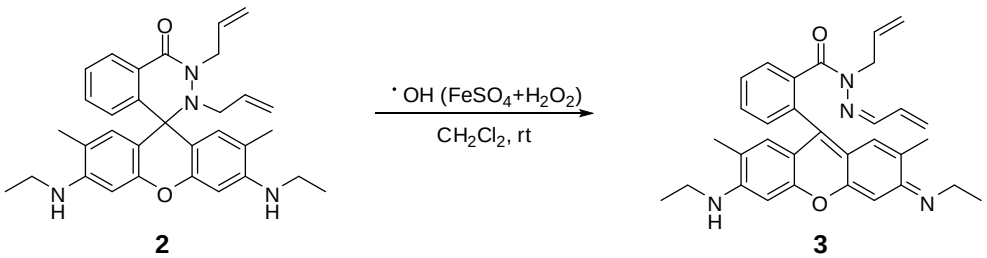


Di-*tert*-butyl 1,2-diallylhydrazine-1, 2-dicarboxylate was prepared according to the known procedure.[3]

Di-*tert*-butyl 1,2-diallylhydrazine-1, 2-dicarboxylate (5.3 g, 16.9 mmol) was dissolved in 4 M hydrogen chloride in 1,4-dioxane (20 mL). And the reaction mixture was stirred for 3 h at room temperature. Diethyl ether (10 mL) was added to the mixture and the mixture was stirred for 10 min. The resulting precipitates were filtered and dried at room temperature for 1 h to give the 2.5 g (89%) of 1, 2-diallylhydrazine dihydrochloride.

1, 2-Diallylhydrazine dihydrochloride: R_f = 0.15 (silica gel, DCM/MeOH = 20/1); mp 163-165 ℃; ^{1}H NMR (400 MHz, MeOD) = 9.62 (bs, 2H), 5.91-5.86 (m, 2H), 5.34-5.29 (m, 2H), 5.23 (dd, *J* = 1.0, 6.5 Hz, 2H), 3.59-3.57 (m, 4H); ^{13}C NMR (100.6 MHz, MeOD) = 131.1, 120.9, 50.5; IR (film, cm^{-1}) 3414, 2259, 2199; HRMS (FAB) *m/z* calcd for $C_6H_{13}N_2$ [$(M+H)^+$] 113.0000; found 113.1004.

5. Synthesis of 3 from the reaction of 2 and •OH.



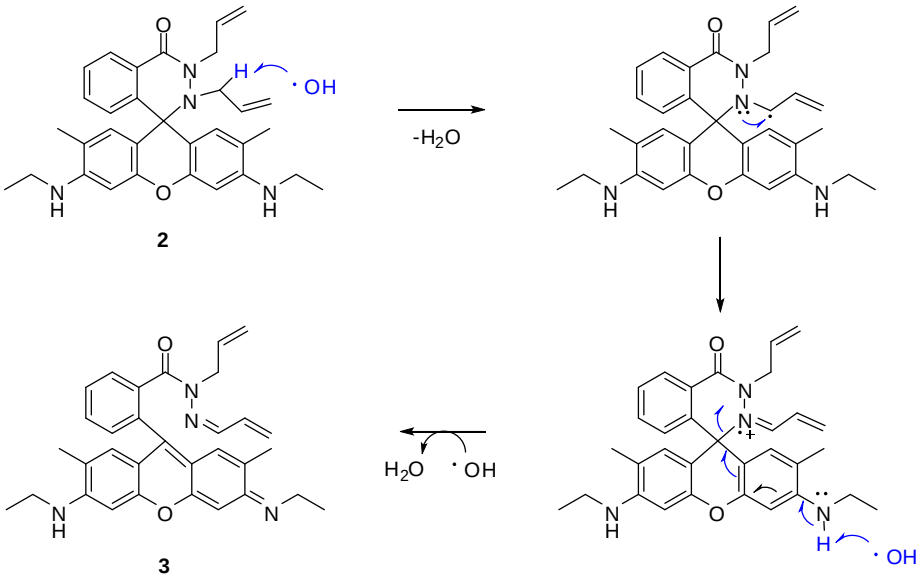
2 **3**

To a solution of **2** (100 mg, 0.19 mmol) in dichloromethane (10 mL) was added hydroxyl

radical (20 eq). The reaction mixture was stirred at room temperature for 4 h. The organic layer was separated and washed with water (20 mL×3) and saturated NaCl solution (20 mL×3) and evaporated under reduced pressure. The crude residue was purified by column chromatography on silica gel (DCM/MeOH = 20/1 to 10/1) to give the 10 mg of **3** (12%) as a red solid.

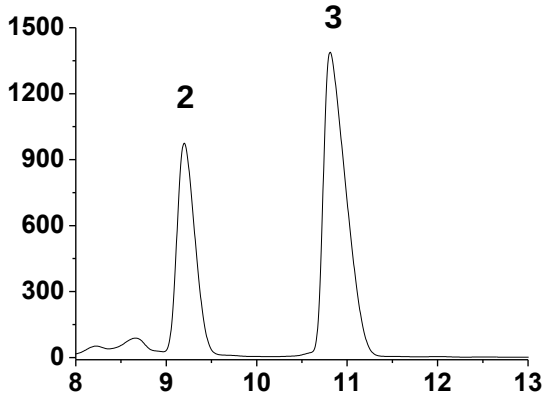
Compound 3: R_f = 0.25 (silica gel, DCM/MeOH = 8/1); mp 190-192 ℃; ^{1}H NMR (400 MHz, $CDCl_3$) = 7.64-7.57 (m, 3H), 7.56-7.53 (bs, 2H), 7.32-7.29 (m, 1H), 7.26 (d, *J* = 5.5 Hz, 1H), 7.01 (s, 2H), 6.52 (s, 2H), 6.30-6.20 (m, 1H), 5.93-5.50 (m, 2H), 5.38-5.28 (m, 1H), 4.91 (dd, *J* = 2.5, 6.5 Hz, 1H), 4.61 (d, *J* = 10.8 Hz, 1H), 4.34 (d, *J* = 3.0 Hz, 2H), 3.55-3.48 (m, 5H), 2.24 (s, 6H), 1.35 (t, *J* = 4.5 Hz, 6H); ^{13}C NMR (100.6 MHz, $CDCl_3$) = 169.5, 157.1, 156.0, 154.5, 143.6, 136.8, 133.6, 131.2, 129.5, 129.4, 128.8, 126.0, 125.3, 117.3, 113.5, 93.4, 43.3, 38.4, 18.5, 13.9; IR (film, cm^{-1}) 3214, 2975, 2926, 1648, 1606, 1499, 1443, 1315, 1185, 1024; LRMS (FAB) *m/z* calcd for $C_{32}H_{35}N_4O_2$ [(M+H)$^+$] 506; found 506.

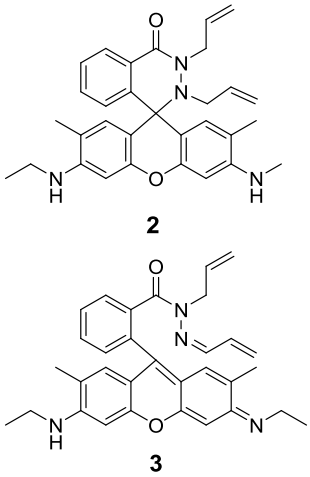
6. Proposed mechanism of reaction between 2 and •OH.

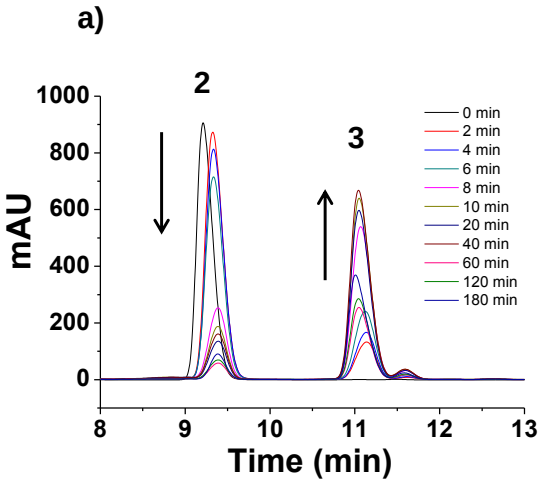


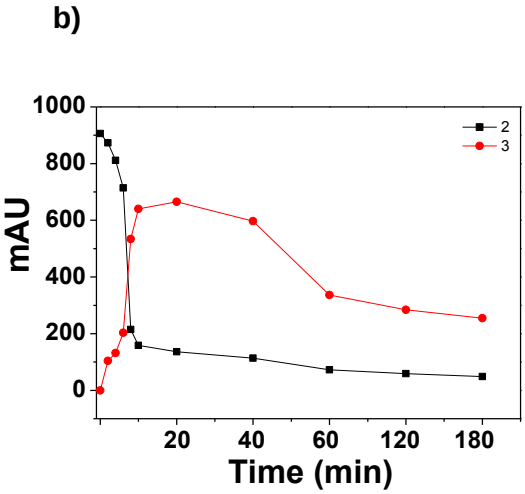
7. HPLC analysis after reaction of 2 with •OH

HPLC analysis: at 520 nm, ACN/H_2O= 60 : 40, flow rate 1.0 mL/min, retention time: 9.2 min (**2**) and 10.8 min (**3**)





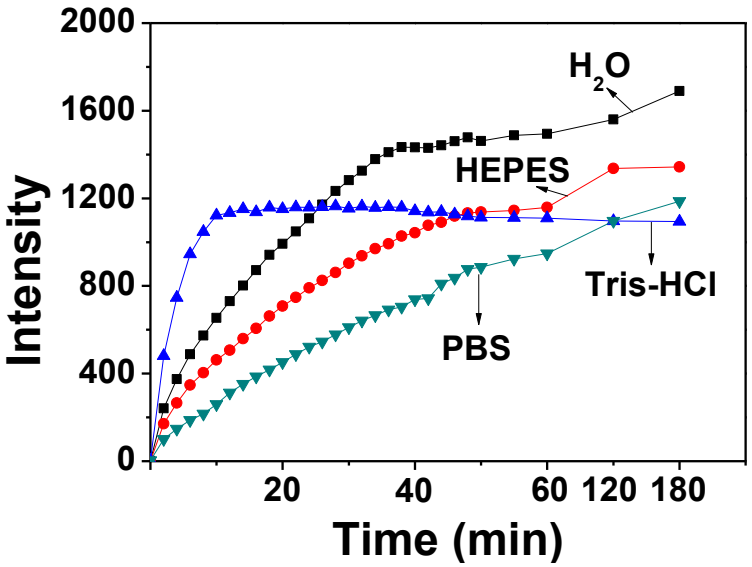


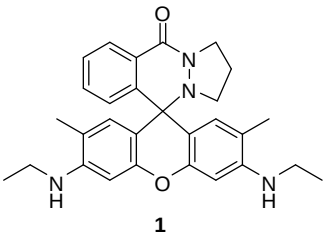


a) Hydroxyl radical (•OH, 1.0 equiv) was added to a solution of **2** (50 μM) in Tris-HCl buffer (DMF 1% v/v) at pH 7.4 (25 ℃). After given time periods, the solutions were quenched with excess Tempol to remove remaining •OH and extract with CH_2Cl_2. The CH_2Cl_2 layers were analyzed by HPLC.
b) Changes of HPLC peaks of **2** and **3** versus time after reaction of **2** with •OH.

(**Note**: The HPLC intensities of **3** are decreasing after initial formation as shown in Figures a) and b). The reaction of **2** and hydroxyl radical generates several spots on TLC analysis, which implies that the product(s) and reactive radical intermediates further react with hydroxyl radical to generate complex mixture.)

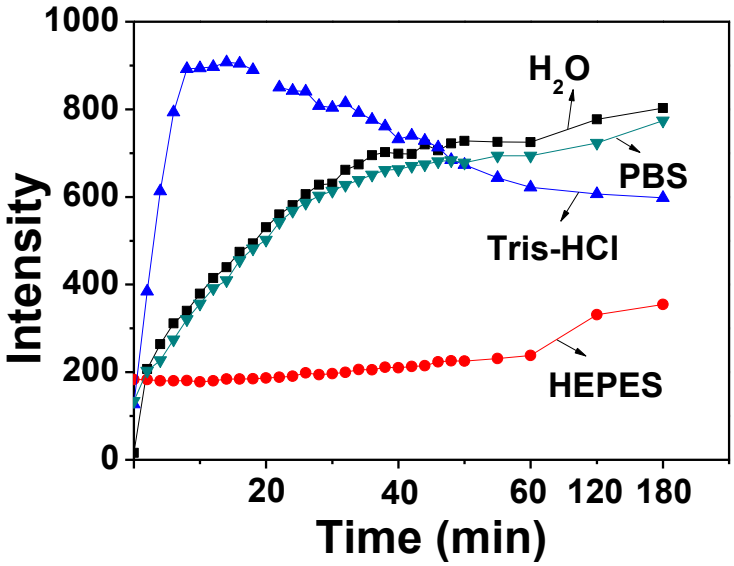
8. Time dependent fluorescence intensity changes of 1, 2 (10 μM) with •OH (1.0 equiv) in various buffer.

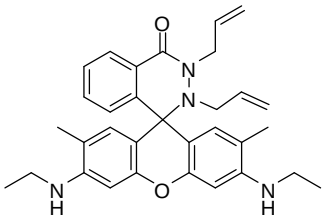




1

Hydroxyl radical (•OH, 1.0 equiv) was added to a solution of **1** (10 μM) in Tris-HCl buffer, HEPES buffer, PBS buffer, H_2O (DMF 1% v/v) at pH 7.4 (25 ℃) (Ex. 500 nm; Em. 550 nm).



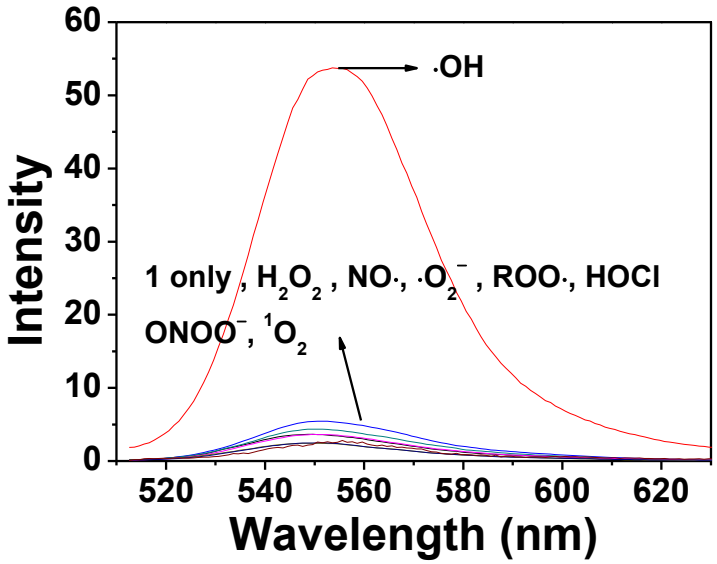


2

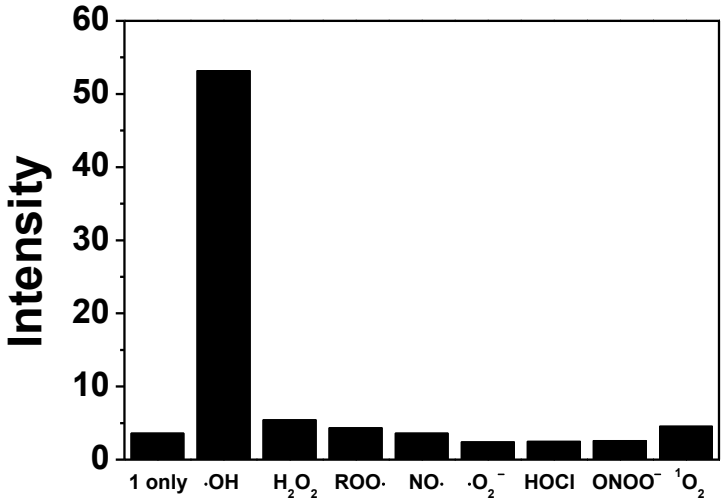
Hydroxyl radical (•OH, 1.0 equiv) was added to a solution of **2** (10 μM) in Tris-HCl buffer, HEPES buffer, PBS buffer, H_2O (DMF 1% v/v) at pH 7.4 (25 ℃) (Ex. 500 nm; Em. 551 nm). To make hydroxyl radical (•OH), Fenton reagents [$CuCl_2$ and H_2O_2] was respectively added in various buffers containing **1, 2** (10 μM).

(Note: It is not clear why probe **1** displays quite different responses to hydroxyl radical in different buffer solutions.)

9. Relative fluorescence intensity of 1 (10 μM) in the presensce of various reactive oxygen species (ROS) and reactive nitrogen species (RNS).



Hydroxyl radical (•OH, 1.0 equiv), hydrogen peroxide (H_2O_2), alkylperoxyl radical (ROO•), nitric oxide (NO•), superoxide ($\bullet O_2^-$), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), peroxy nitrite ($ONOO^-$) (5.0 equiv respectively) was added to a solution of **1** (10 μM) in Tris-HCl buffer (DMF 1% v/v) at pH 7.4 (25 ℃).



Relative fluorescence intensities (at 550 nm) of **1** (10 μM) in the presence of ROS/RNS.

To make hydroxyl radical (•OH), Fenton reagents [$FeSO_4$ and H_2O_2] was respectively added in Tris-HCl buffer containing **1** (10 μM) and mixture was incubated for 30 min. (Ex. 500 nm; Em. 550 nm)

Generation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

Various ROS and RNS including •OH, H_2O_2, ROO•, NO•,$•O_2^-$, HOCl, $^1O_2$ and $ONOO^-$ were prepared according to the following methods.

1) Preparation of •OH (hydroxyl radical) (final 10 μM) [4,5,6]

(a) $CuCl_2$ (copper(II) chloride) was dissolved in H_2O and H_2O_2 was diluted in H_2O respectively. $CuCl_2$ solution (2 mM) and H_2O_2 solution (20 mM) was added to make 10 μM •OH in Tris-HCl buffer (DMF 1% v/v) containing **1** at room temperature and the spectrum was measured 30 min later.

(b) $FeSO_4 \cdot 7H_2O$ (ferrous sulfate hydrate) was dissolved in H_2O and H_2O_2 was diluted in H_2O respectively. $FeSO_4 \cdot 7H_2O$ solution (2 mM) and H_2O_2 solution (20 mM) was added to make 10 μM •OH in Tris-HCl buffer (DMF 1% v/v) containing **1** at room temperature and the spectrum was measured 30 min later. The molar ratio of $FeSO_4$ to H_2O_2 is 1:10

2) Preparation of H_2O_2 (hydrogen peroxide) (10 mM) [7]

H_2O_2 was diluted in H_2O and stirring for 20 min. Then H_2O_2 solution was added in Tris-HCl buffer (DMF 1% v/v) containing **1** at room temperature and the spectrum was measured 30 min later.

3) Preparation of ROO· (alkyl peroxide) (10 mM) [8]

2, 2-azobis (2-amindinopropane) dihydrochloride was diluted in H_2O and stirring for 20 min. Then ROO• solution was added in Tris-HCl buffer (DMF 1% v/v) containing **1** at room temperature and the spectrum was measured 30 min later.

4) Preparation of NO· (Nitric oxide) (10 mM) [8]

SNP (sodium nitroferricyanide(III) dihydrate) was diluted in H_2O and stirring for 20 min. Then NO• solution was added in Tris-HCl buffer (DMF 1% v/v) containing **1** at room temperature and the spectrum was measured 30 min later.

5) Preparation of $•O_2^-$ (superoxide radical) (10 mM) [9]

KO_2 (potassium superoxide) was diluted in DMSO and stirring for 20 min. Then **$•O_2^-$** solution was added in Tris-HCl buffer (DMF 1% v/v) containing **1** at room temperature and the spectrum was measured 30 min later.

6) Preparation of HOCl (hypochlorous acid) (10 mM) [7]

NaOCl was dissolved in 0.1 M NaOH (aq) and stirring for 20 min. Then HOCl solution was added in

Tris-HCl buffer (DMF 1% v/v) containing **1** at room temperature and the spectrum was measured 30 min later.

7) Preparation of $^1O_2$ (singlet oxygen) (10 mM) [10]

NaOCl was dissolved in 1 mM of H_2O_2 to make 10 mM solution and stirring for 20 min. Then $^1O_2$ solution was added in Tris-HCl buffer (DMF 1% v/v) containing **1** at room temperature and the spectrum was measured 30 min later.

8) Preparation of $ONOO^-$ (peroxynitrite)[8]

NaOH (aq) was added in a mixture of $NaNO_2$ (sodium nitrite) 0.6 M, H_2O_2 (hydrogen peroxide) 0.7 M and hydrochloric acid 0.6 M to make 1.5 M alkaline. The excess H_2O_2 was removed by passing the solution through a short column of manganese dioxide. The resulting solution was stored at lower than -18 ℃. The solution was thawed immediately before use. The concentration of peroxynitrite was determined by measuring the absorption of the solution at 302 nm. The extinction coefficient of peroxynitrite solution in 0.1 M NaOH (aq) is 1670 $M^{-1}cm^{-1}$ at 302 nm.

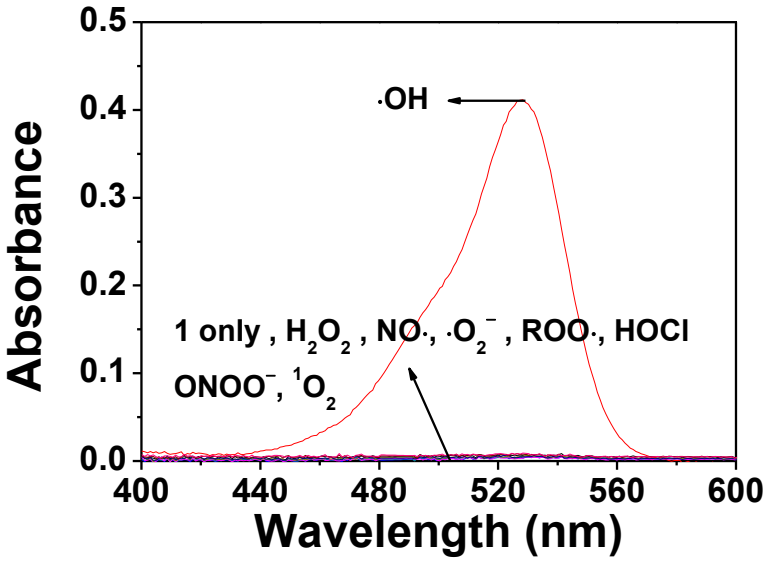
10. Color changes of 1 (20 μM) with •OH and other ROS & RNS.

1only ·OH H_2O_2 ROO· NO· $\cdot O_2^-$ HOCl $ONOO^-$ $^1O_2$

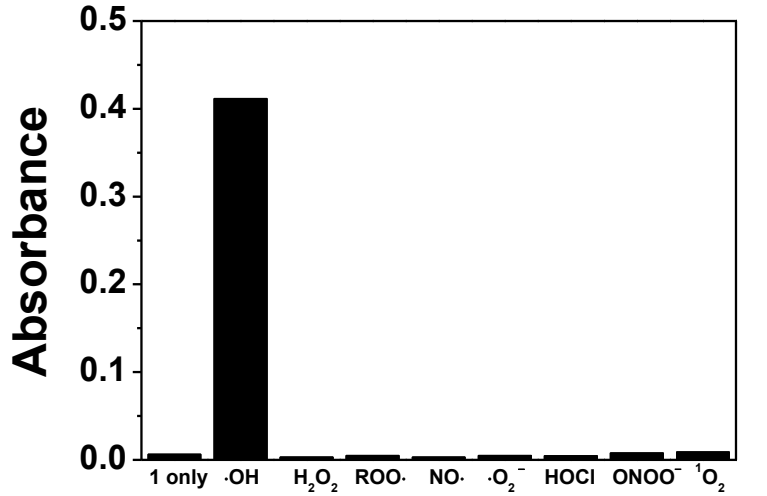
•OH, H_2O_2, ROO•, NO•, $\bullet O_2^-$, HOCl, $^1O_2$, $ONOO^-$ (5.0 equiv respectively) was added to a solution of **1** (20 μM) in Tris-HCl buffer (DMF 1% v/v) at pH 7.4 (25 ℃).

Color changes were taken pictures after incubation for 30 min.

11. UV-Vis absorption of 1 (10 μM) in the presensce of various reactive oxygen species (ROS) and reactive nitrogen species (RNS).

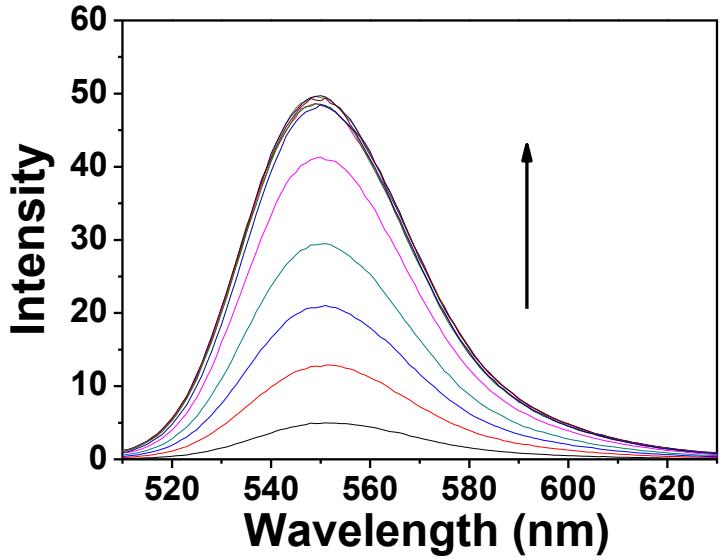


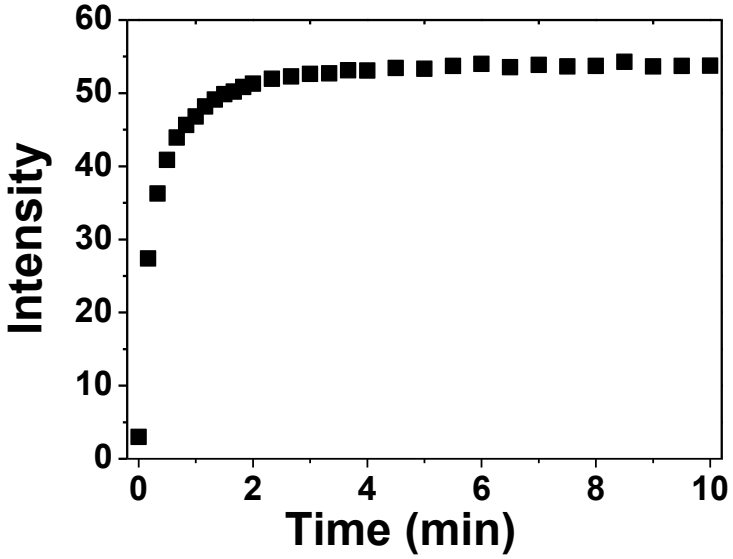
Hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), alkylperoxyl radical (ROO•), nitric oxide (NO•), superoxide ($•O_2^-$), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), peroxy nitrite ($ONOO^-$) (5.0 equiv respectivly) was added to a solution of **1** (10 μM) in Tris-HCl buffer (DMF 1% v/v) at pH 7.4.



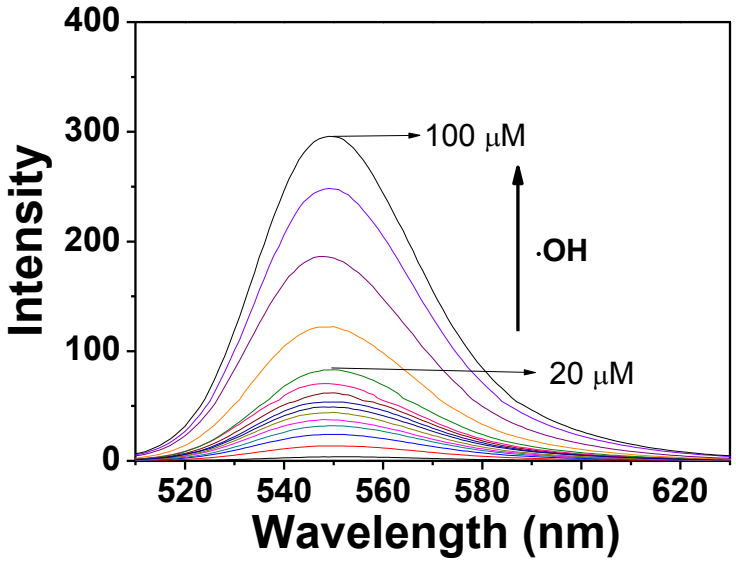
Relative UV-Vis absorptions of **1** (10 μM) in presence of ROS/RNS.

12. Time dependent fluorescence intensity changes of 1 (10 μM) with •OH (1 equiv) in Tris-HCl buffer (DMF 1% v/v) at pH 7.4 (25 ℃). (Ex. 500 nm; Em. 550 nm)



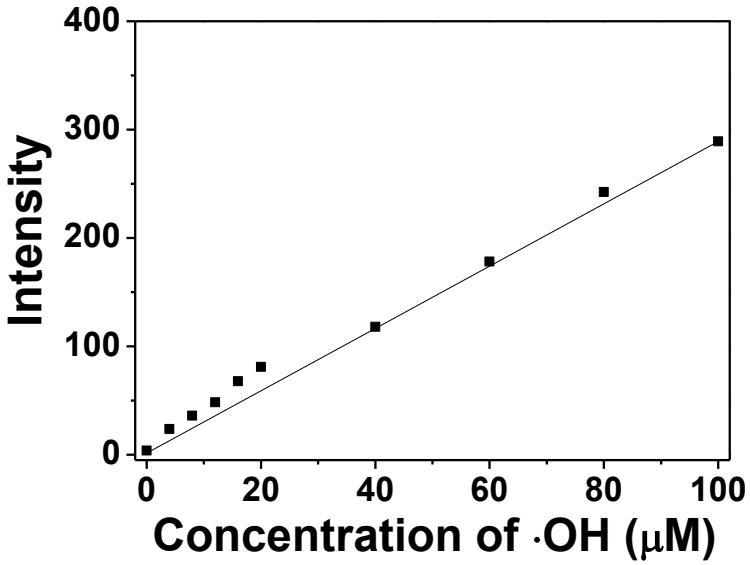


13. Fluorescence titration of 1 (10 μM) with •OH (various concentration) in Tris-HCl buffer (DMF 1% v/v) at pH 7.4 (25 °C). (Ex. 500 nm; Em. 550 nm)[4]



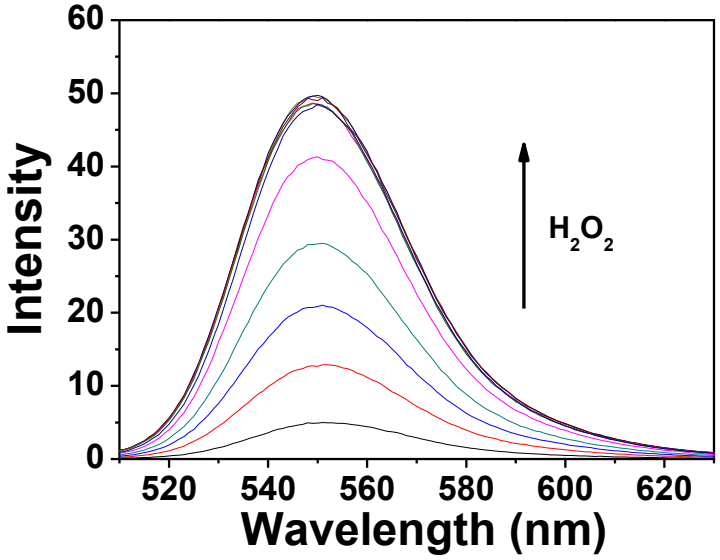
Fluorescence titration (Ex. 550 nm) of **1** (10 μM) in presence of •OH (micro molar concentration = 4, 8, 12, 16, 20, 40, 60, 80, 100 μM).

A solution of **1** (2.0 mL) was placed in a quartz cell and the fluorescence spectrum was recorded. Fluorescence intensity changes (Ex. 550 nm) were recorded after incubation for 30 min at pH 7.4 (25 ℃).

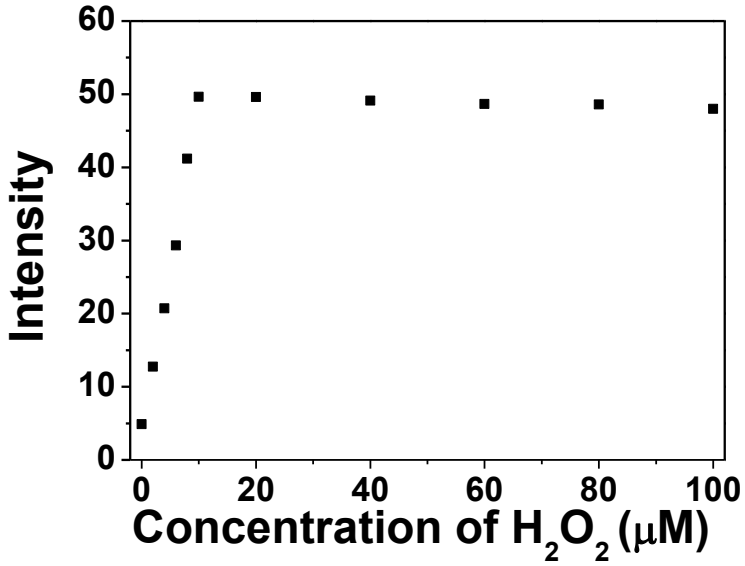


Fluorescence intensity changes (Ex. 550 nm) v.s. micro molar concentration of •OH.

14. H_2O_2 concentration dependent fluorescence intensity changes.

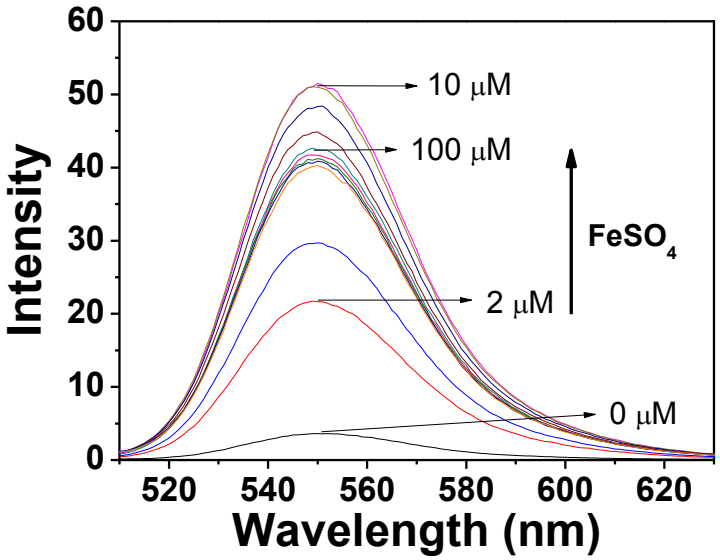


H_2O_2 was added to a solution of **1** (10 μM) in Tris-HCl buffer containing 10 μM $FeSO_4$ (DMF 1% v/v) and mixture was incubated for 30 min at pH 7.4 (25 ℃). (Ex. 500 nm; Em. 550 nm)

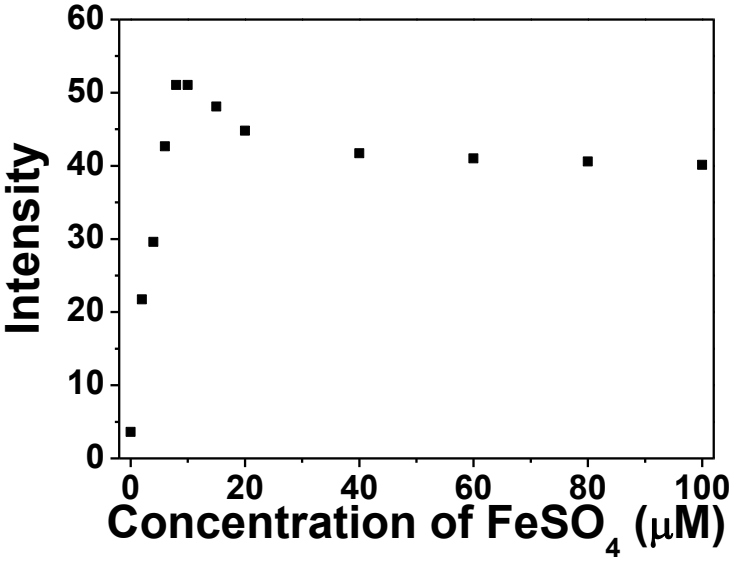


Fluorescence intensity changes (Ex. 550 nm) v.s. micro molar concentration of H_2O_2.

15. $FeSO_4$ concentration dependent fluorescence intensity changes.[11,12,13]

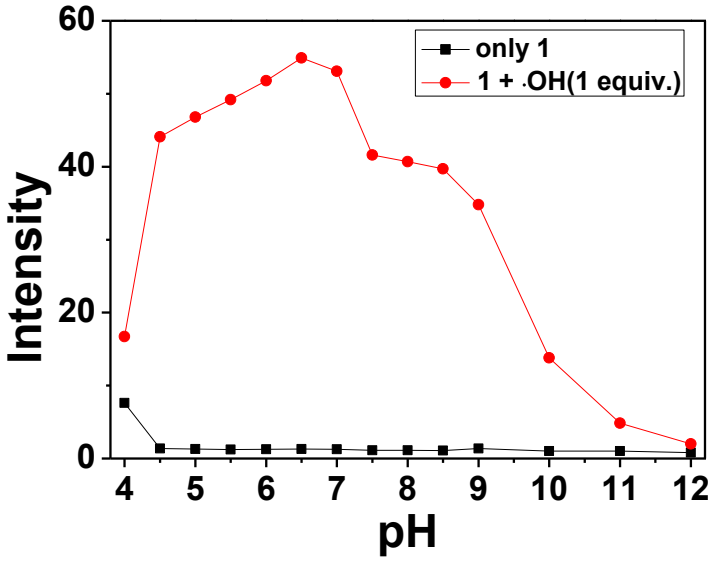


$FeSO_4$ was added to a solution of **1** (10 μM) in Tris-HCl buffer containing 10 μM H_2O_2 (DMF 1% v/v) and mixture was incubated for 30 min at pH 7.4 (25 ℃). (Ex. 500 nm; Em. 550 nm)

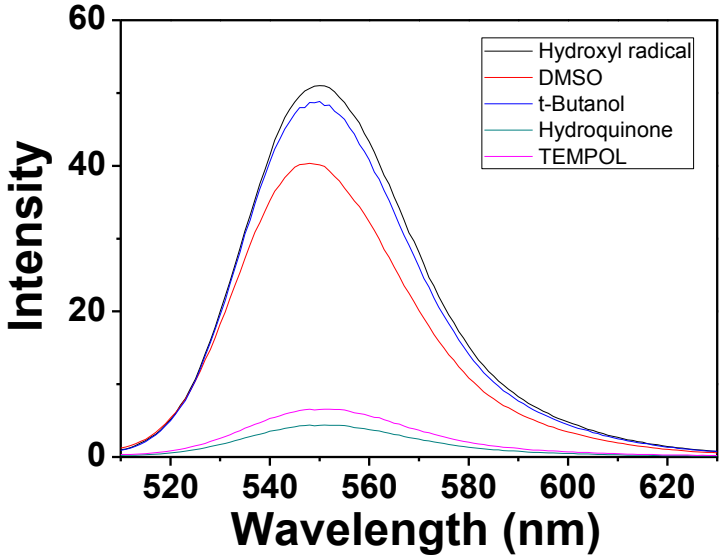


Fluorescence intensity changes (Ex. 550 nm) v.s. micro molar concentration of $FeSO_4$.

16. Fluorescence spectra of 1 (10 μM) with •OH (1 equiv) in H_2O (DMF 1% v/v) at various pH values. (Ex. 500 nm; Em. 550 nm)



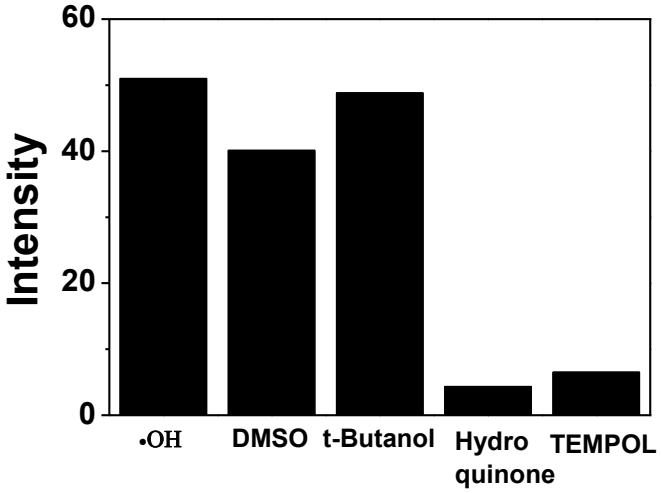
17. Effect of of scavengers (DMSO[6], t-butanol, hydroquinone[14], TEMPOL[15] (0.1 mM)) on flourescence intensity of 1 (10 μM) containing •OH (10 μM) in Tris-HCl buffer (DMF 1% v/v, 25 °C) (Ex. 500 nm; Em. 550 nm).



Scavengers (0.1 mM)* and hydroxyl radical (10 μM) was simultaneously added to a solution of **1** (10 μM) in Tris-HCl buffer (DMF 1% v/v) and mixture was incubated for 30 min at pH 7.4 (25 ℃). (Ex. 500 nm; Em. 550 nm)

* Scavengers : DMSO, t-butanol, hydroquinone, TEMPOL (0.1 mM)

It found that hydroquinone and TEMPOL can be used for scavenger of •OH.



18. Fluorescence imaging of cells with probe 1.[16]

Fluorescent detection of intracellular hydroxyl radicals generated by Fenton's reagent. A549 (human lung adenocarcinoma epithelial cells) and RAW264.7 cells (murine macrophage-like cells) obtained from American Type Culture Collection (Manassas, VA) were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), 50 units/mL of penicillin and 50 μg/mL of streptomycin. A549 and RAW264.7 cells were seeded in a 24-well plate at a density of 1×10^4 cells per well in culture media. After 24 h, the cells were treated with 20 μM probe in culture media containing 0.1% (v/v) DMF for 1 h at 37 ^{o}C. After washing with PBS to remove the remaining probe, the cells were incubated with 20 μM of Fenton's reagent ($FeSO_4$: H_2O_2, 1:10) for 1 h at 37 ^{o}C. Separately, the probe-treated cells were incubated with 5 mM TEMPOL in 0.1% (v/v) DMSO for 1 h and then treated with 20 μM of Fenton's reagent for 1 h. After being washed with PBS, the treated cells were analyzed by using confocal microscopy (LSM 510 META, Carl Zeiss, Germany) (λ_{ex} = 543 nm).

Fluorescent detection of intracellular hydroxyl radicals produced by PMA stimulation. A549 and RAW264.7 cells were treated with 20 μM probe for 1 h at 37 ^{o}C. After being washed with PBS to remove the remaining probe, the cells were incubated with 10 ng/mL PMA for 1 h to generate hydroxyl radicals. Separately, the probe-treated cells were incubated with 5 mM TEMPOL in 0.1% (v/v) DMSO for 1 h and then stimulated by treatment with 10 ng/mL PMA for 1 h. After being washed with PBS, the treated cells were imaged by using confocal microscopy (λ_{ex} = 543 nm).

19. Reference

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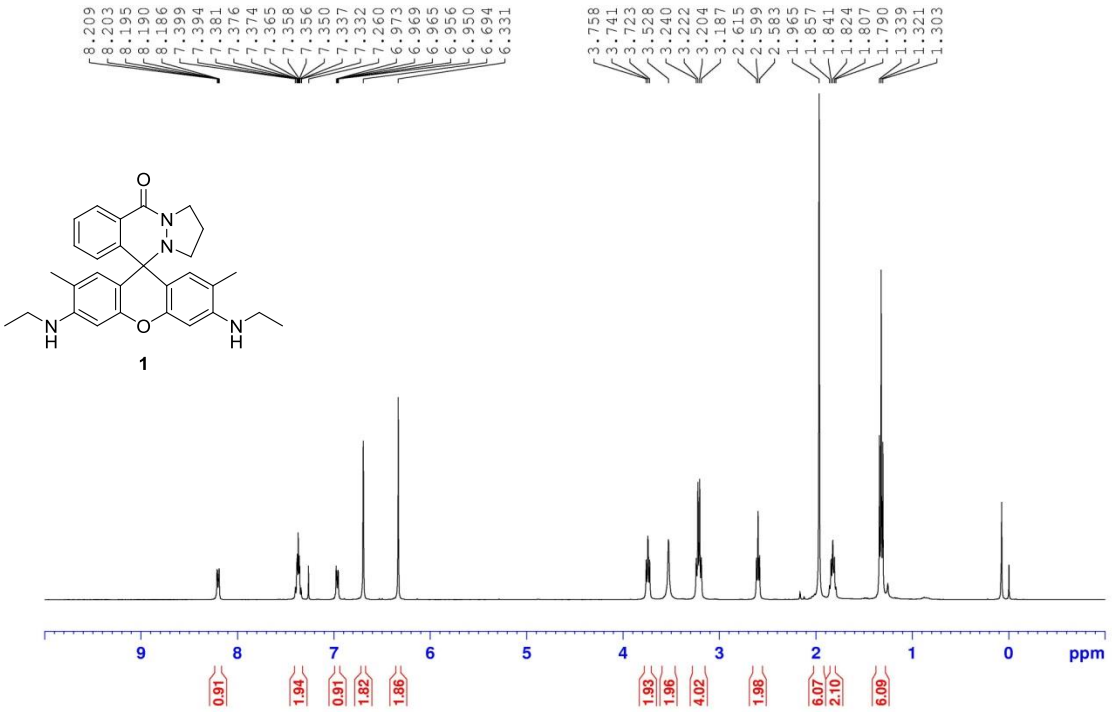
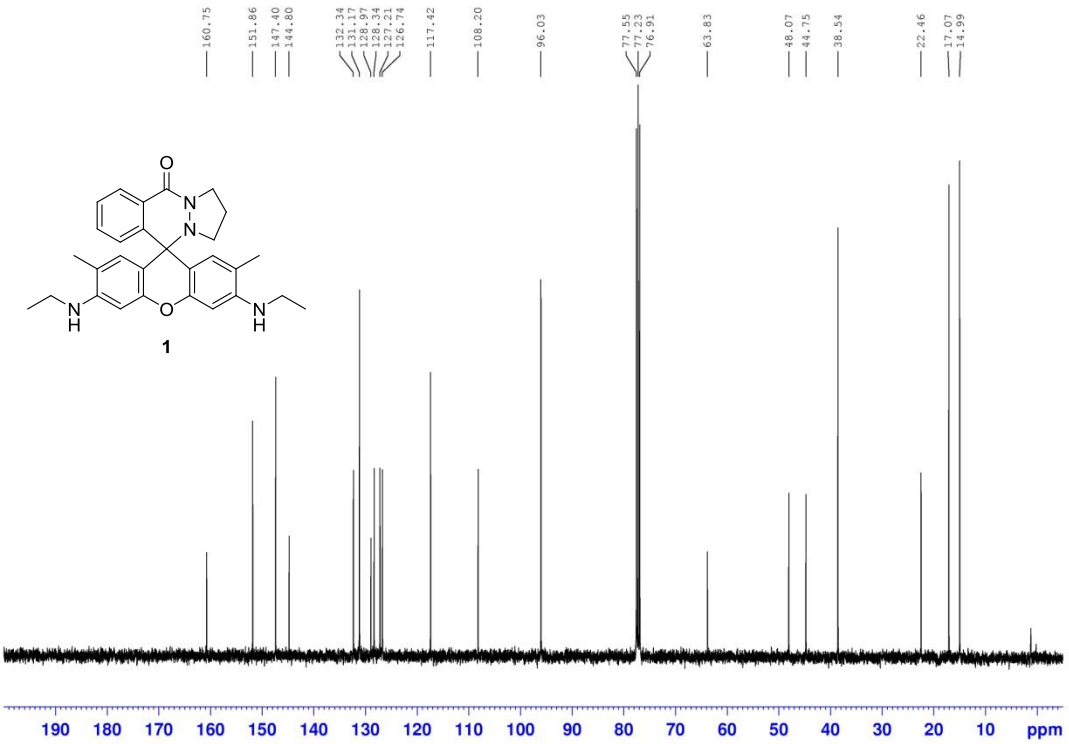
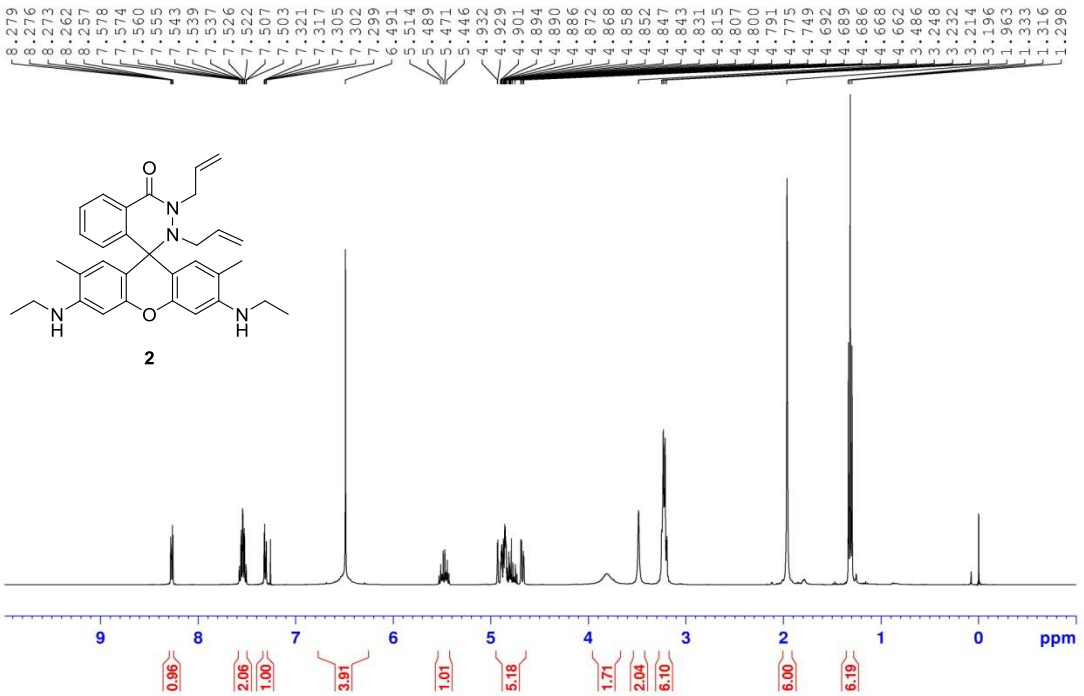
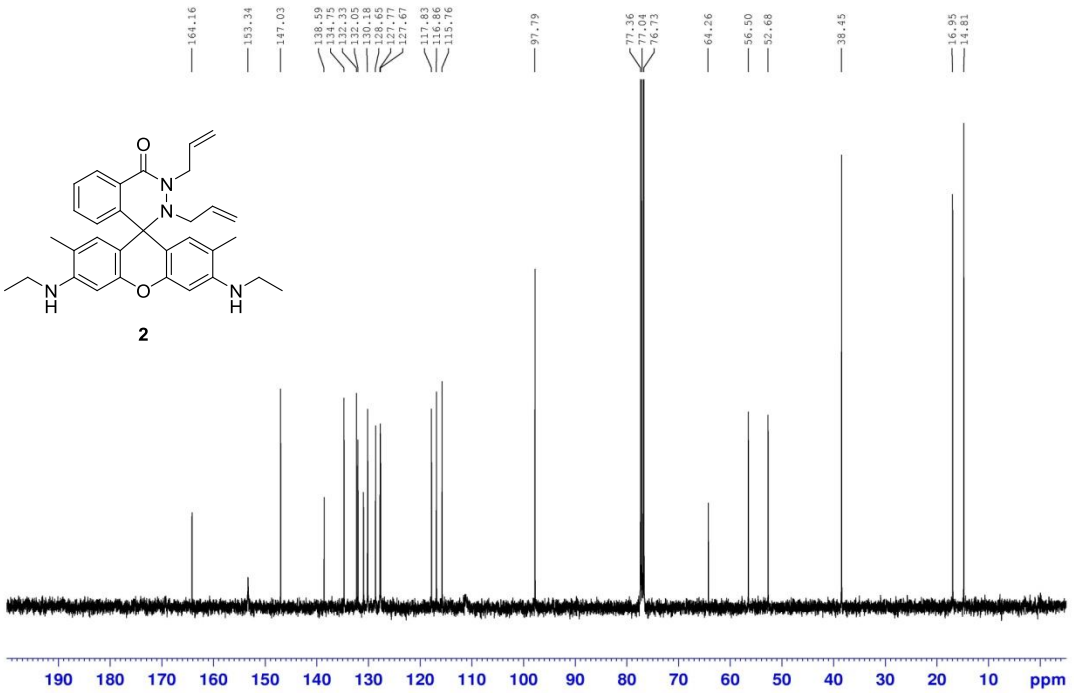
20. ^{1}H NMR and ^{13}C NMR copies of 1.



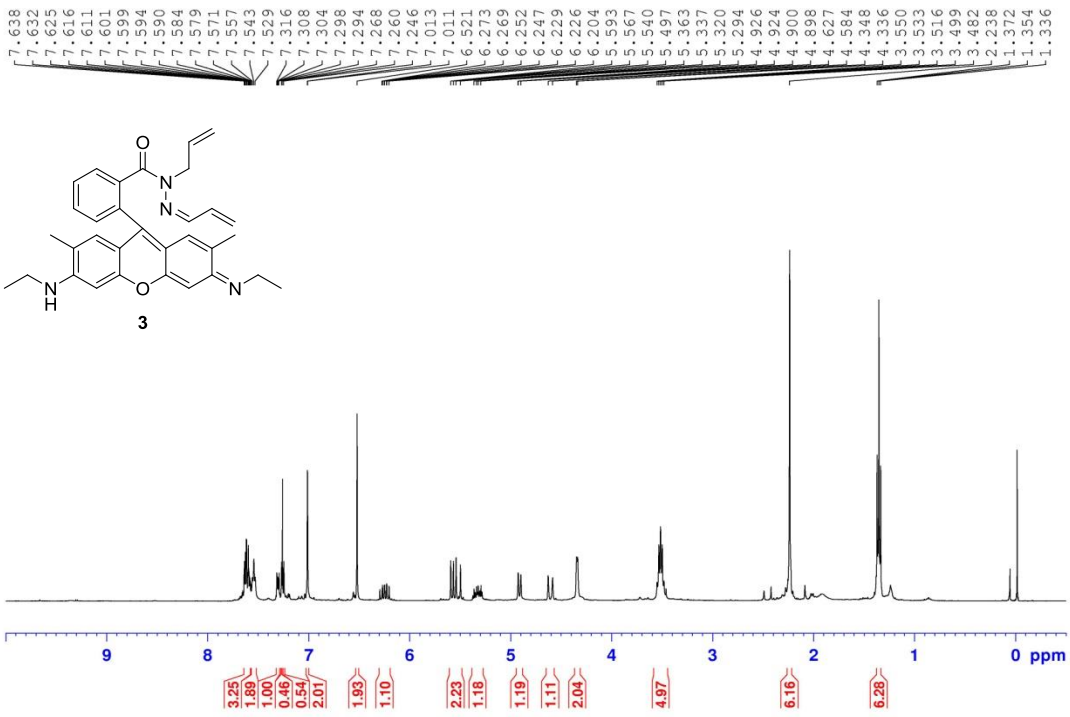


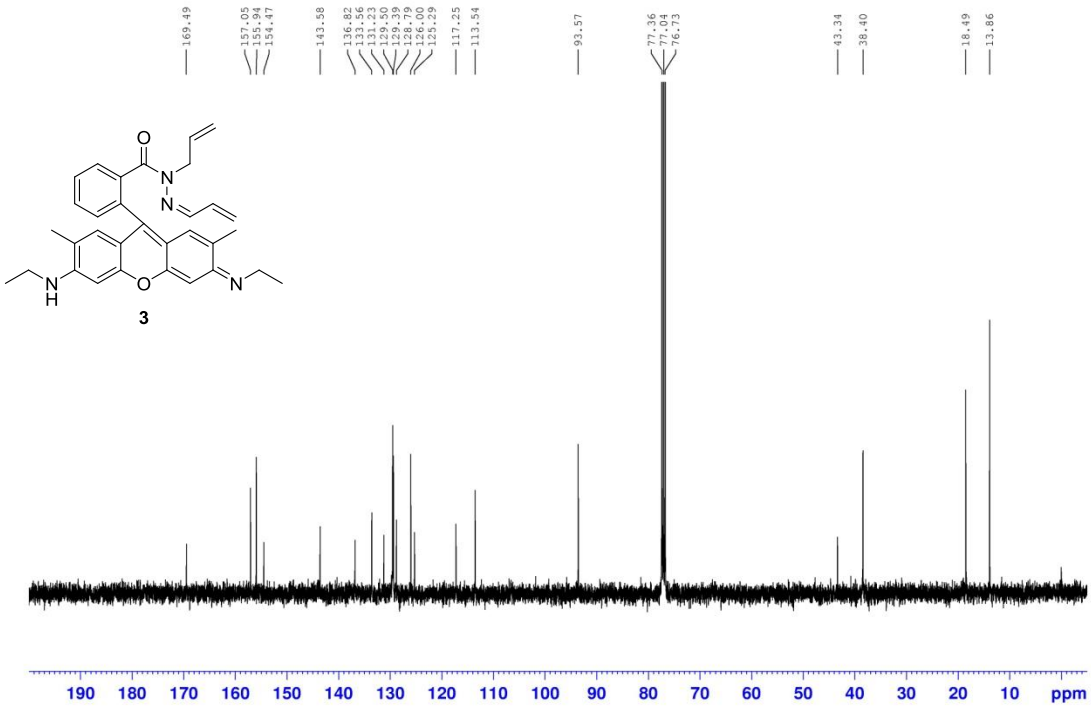
21. ^{1}H NMR and ^{13}C NMR copies of 2.





22. ^{1}H NMR and ^{13}C NMR copies of 3.





23. ^{1}H NMR and ^{13}C NMR copies of 1,2-diallylhydrazine dihydrochloride.