Coumarin-based fluorescent probe for the rapid detection of peroxynitrite ‘AND’ biological thiols

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1. UV-Vis and fluorescence analysis

Figure S1. Absorption spectra of ROS-AHC (20 μM) only with and without ONOO⁻ (18 μM) wait 5 min/ GSH (26 μM) wait 5 min, and with addition of GSH (26 μM) wait 5 min then addition of ONOO⁻ (18 μM) with 5 min incubation before measurements in PBS buffer solution (10 mM, pH = 7.40).
Figure S2. (a) Fluorescence spectra of ROS-AHC (5 μM) with increasing additions of ONOO− (from 0 to 6 μM) in PBS buffer solution (10 mM, pH = 7.40) after 5 min. (b) Fluorescence intensity changes (based on the intensities at 461 nm) against ONOO− concentration. λ<sub>ex</sub> = 400 nm (bandwidth 8 nm).
Figure S3. Fluorescence intensity changes ($I/I_{\text{ONOO}^-}$) for ROS-AHC (5 µM) with addition of ONOO$^-$ (6 µM), wait 5 min, then additions of GSH (0 – 4.5 µM) with 5 min incubation before measurement in PBS buffer solution (10 mM, pH = 7.40). $\lambda_{\text{ex}} = 400$ nm (bandwidth 8 nm)/$\lambda_{\text{em}} = 461$ nm.
Figure S4. (a) Fluorescence spectra of ROS-AHC (5 μM) with increasing additions of GSH (from 0 to 6 μM) with 5 min incubation before measurement in PBS buffer solution (10 mM, pH = 7.40). (b) Fluorescence intensity changes (based on the intensities at 461 nm) against GSH concentration. λ<sub>ex</sub> = 400 nm (bandwidth 8 nm).
**Figure S5.** Fluorescence intensity changes ($\frac{I}{I_{\text{GSH}}}$) for ROS-AHC (5 μM) with addition of GSH (6 μM), wait 5 min, then additions of ONOO$^-$ (0 – 5.5 μM) with 5 min incubation before measurement in PBS buffer solution (10 mM, pH = 7.40). $\lambda_{\text{ex}} = 400$ nm (bandwidth 8 nm)/$\lambda_{\text{em}} = 461$ nm.

**Figure S6.** Selectivity bar chart of ROS-AHC (5 μM) with addition of ONOO$^-$ (6 μM), wait 5 min, then addition of various amino acids wait 5 min or 60 min before measurement in PBS buffer solution (10 mM, pH = 7.40), 1 – blank; 2 – GSH (Glutathione, 4 μM); 3 – Cys (Cysteine, 4 μM); 4 – Hcy (Homocysteine, 4 μM); 5 – Glu (Glutamic acid, 500 μM); 6 – Phe (Phenylalanine, 500 μM); 7 – Asp (Aspartic acid, 500 μM); 8 – Pro (Proline, 500 μM); 9 – Val
(Valine, 500 μM); 10 – Ser (Serine, 500 μM); 11 – Lys (Lysine, 500 μM); 12 – Iso (Isoleucine, 500 μM); 13 – His (Histidine, 500 μM); 14 – Arg (Arginine, 500 μM). \( \lambda_{ex} = 400 \text{ nm} \) (bandwidth 8 nm)/ \( \lambda_{em} = 461 \text{ nm} \).

**Figure S7.** Selectivity bar chart of ROS-AHC (5 μM) with addition of GSH (6 μM), wait 5 min, then addition of various ROS wait 5 min or 60 min before measurement in PBS buffer solution (10 mM, pH = 7.40). 1 – blank; 2 – ONOO\(^-\) (5 μM); 3 – HOCl (100 μM); 4 – H\(_2\)O\(_2\) (100 μM); 5 – ROO\(^\cdot\) (100 μM); 6 – •OH (100 μM); 7 – O\(_2\)\(^{.-}\) (100 μM); 8 – \(^1\)O\(_2\) (100 μM). \( \lambda_{ex} = 400 \text{ nm} \) (bandwidth 8 nm)/ \( \lambda_{em} = 461 \text{ nm} \).
Figure S8. Fluorescence intensity over time of the addition of ROS-AHC (5 μM) followed by the addition of GSH (4 μM) to ONOO⁻ (6 μM) in PBS buffer solution (10 mM, pH = 7.40). λ_ex = 400 nm (bandwidth 8 nm)/ λ_em = 461 nm.

Experimental for Figure S8: A solution of ONOO⁻ (6 μM) in PBS buffer solution was placed in a Greiner Bio-One microplate (96-well, PS, f-bottom (chimney well), black-walled), and the intensity was measured every 25 s for 200 s. A solution of ROS-AHC (6 μM) was then pumped into this solution, and fluorescence was then measured every 25 s from 228 s to 1303 s. A solution of GSH (4 μM) was then added, and fluorescence was measured every 25 s from 1331 s to 3006 s.
Figure S9. (a) Fluorescence intensity over time of the addition of ROS-AHC (5 μM) with addition of GSH (6 μM) at 200 s in PBS buffer solution (10 mM, pH = 7.40). λ<sub>ex</sub> = 400 nm (bandwidth 8 nm)/ λ<sub>em</sub> = 461 nm. (b) Fluorescence intensity over time of the addition of the addition of a pre-mixed (5 min) solution of ROS-AHC (5 μM) and GSH (6 μM) to ONOO (5 μM) at 200 s in PBS buffer solution. λ<sub>ex</sub> = 400 nm (bandwidth 8 nm)/ λ<sub>em</sub> = 461 nm.

Experimental for Figure S9: (a) A solution of ROS-AHC (5 μM) in PBS buffer solution was placed in a Greiner Bio-One microplate (96-well, PS, f-bottom (chimney well), black-walled), and the intensity was measured every 25 s for 200 s. A solution of GSH (6 μM) was then pumped into this solution, and fluorescence was measured every 25 s from 228 s to 803 s. (b) A solution of ONOO (6 μM) in PBS buffer solution was place in a Greiner Bio-One microplate (96-well, PS, f-bottom (chimney well), black-walled), and the intensity was measured every 25 s for 200 s. A solution of ROS-AHC (5 μM) and GSH (6 μM), premixed for 5 min, was then pumped into this solution, and the fluorescence was measured every 25 s from 228 s to 803 s.
2. Generation of various ROS

**ROO•**
ROO• was generated from 2, 2'-azobis (2-amidinopropane) dihydrochloride. AAPH (2, 2’ azobis (2-amidinopropane) dihydrochloride, 1 M) was added into deionizer water, and then stirred at 37 °C for 30 min.

**O2•−**
Superoxide was generated from KO2. KO2 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 (H2O2, 10 eq) was added to Fe(ClO4)2 in deionised water.

**¹O2**
¹O2 was generated by reacting H2O2 (1 mM) with NaClO (1 mM). The solution of H2O2 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 NaNO2, 0.6 M HCl, 0.7 M H2O2 was added simultaneously to a 3 M NaOH solution at 0 °C. 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).

**H2O2**
The concentration of H2O2 was determined from the absorption at 240 nm (Ɛ = 43.6 M⁻¹ cm⁻¹).
3. Mass spectroscopic analysis

Figure S10. HRMS spectrum of ROS-AHC (45 μM).
Figure S11. LC-MS spectrum of ROS-AHC (45 μM) + ONOO⁻ (1.5 equiv.).
Figure S12. LC-MS spectrum of **ROS-AHC (45 μM)** + **ONOO⁻ (1.5 equiv.) + GSH (3.0 equiv.)**.
4. Synthesis and characterisation of compounds 1-4 and ROS-AHC

![Chemical structure diagram]

Scheme S1. Synthesis of target ROS-AHC.

Synthesis of compounds 1-4

Compounds 1-4 were synthesized using adapted literature procedures.\(^1\)\(^2\)

**Compound 4**

M.p. 241 °C; \(^1^H\) NMR (500 MHz, DMSO-\(d_6\)) \(\delta_H\) 9.75 (s, 1H), 8.62 (s, 1H), 7.74 (d, \(J = 8.5\) Hz, 1H), 7.27 (d, \(J = 2.1\) Hz, 1H), 7.13 (dd, \(J = 8.5, 2.2\) Hz, 1H), 2.30 (s, 3H), 2.17 (s, 3H); \(^{13}\)C NMR (126 MHz, DMSO-\(d_6\)) \(\delta_C\) 170.2 (s), 168.9 (s), 157.3 (s), 150.9 (s), 149.9 (s), 128.5 (s), 124.1 (s), 123.1 (s), 119.0 (s), 117.4 (s), 109.7 (s), 23.9 (s), 20.8 (s). HRMS (ESI\(^+\)): calc. for C\(_{13}\)H\(_{11}\)NO\(_5\) [M+H]\(^+\) 262.0710 m/z, found 262.0711 m/z.

**Compound 3**

M.p. 247 °C; \(^1^H\) NMR (500 MHz, DMSO-\(d_6\)) \(\delta_H\) 9.80 (s, 1H), 7.23 (d, \(J = 8.4\) Hz, 1H), 6.69 – 6.64 (m, 2H), 5.22 (bs, 2H); \(^{13}\)C NMR (126 MHz, DMSO-\(d_6\)) \(\delta_C\) 159.0 (s), 156.1 (s), 149.3 (s), 130.2 (s), 125.7 (s), 113.5 (s), 112.9 (s), 109.8 (s), 101.8 (s). HRMS (ESI\(^+\)): calc. for C\(_9\)H\(_7\)NO\(_3\) [M+H]\(^+\) 178.0499 m/z, found 178.0500 m/z.

**Compound 2**

M.p. 247 °C; \(^1^H\) NMR (500 MHz, DMSO-\(d_6\)) \(\delta_H\) 9.80 (s, 1H), 7.23 (d, \(J = 8.4\) Hz, 1H), 6.69 – 6.64 (m, 2H), 5.22 (bs, 2H); \(^{13}\)C NMR (126 MHz, DMSO-\(d_6\)) \(\delta_C\) 159.0 (s), 156.1 (s), 149.3 (s), 130.2 (s), 125.7 (s), 113.5 (s), 112.9 (s), 109.8 (s), 101.8 (s). HRMS (ESI\(^+\)): calc. for C\(_9\)H\(_7\)NO\(_3\) [M+H]\(^+\) 178.0499 m/z, found 178.0500 m/z.

**Compound 1**

M.p. 189 °C; \(^1^H\) NMR (500 MHz, DMSO-\(d_6\)) \(\delta_H\) 10.45 (bs, 1H), 10.05 (s, 1H), 8.57 (s, 1H), 7.56 (d, \(J = 8.6\) Hz, 1H), 6.81 (dd, \(J = 8.5, 2.3\) Hz, 1H), 6.75 (d, \(J = 2.2\) Hz, 1H), 6.72 (d, \(J =
11.8 Hz, 1H), 6.50 (d, J = 11.8 Hz, 1H), 3.69 (s, 3H); $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta_C$
167.1 (s), 163.1 (s), 159.8 (s), 157.7 (s), 151.7 (s), 130.9 (s), 129.5 (s), 129.2 (s), 126.6 (s),
120.6 (s), 113.7 (s), 111.3 (s), 101.9 (s), 51.6 (s). HRMS (ESI$^+$): calc. for C$_{14}$H$_{11}$NO$_6$
[M+Na]$^+$ 312.0479 m/z; found 312.0485 m/z.

**Synthesis of ROS-AHC**

4-Bromomethylphenylboronic acid pinacol ester (0.22 g, 0.74 mmol) and K$_2$CO$_3$ (0.10 g, 0.74
mmol) were added to a solution of 1 (0.18 g, 0.62 mmol) in dry DMF (6 mL) under a N$_2$
atmosphere. The resulting suspension was stirred at room temperature for 5 h. The reaction
mixture was diluted with EtOAc (90 mL) and washed with brine (90 mL × 3), dried over
anhydrous Na$_2$SO$_4$ and concentrated in vacuo. The crude product obtained was purified by
flash chromatography (SiO$_2$, 30% EtOAc in petroleum ether) to afford the desired product
ROS-AHC as a yellow solid (72 mg, 23% yield). M.p. 177 ºC; $^1$H NMR (500 MHz, CDCl$_3$)
$\delta_H$ 9.95 (s, 1H), 8.76 (s, 1H), 7.84 (d, J = 8.0 Hz, 2H), 7.43 (d, J = 8.0 Hz, 2H), 7.40 (d, J =
8.6 Hz, 1H), 6.95 (dd, J = 8.7, 2.4 Hz, 1H), 6.89 (d, J = 2.2 Hz, 1H), 6.42 (d, J = 12.7 Hz, 1H),
6.27 (d, J = 12.7 Hz, 1H), 5.14 (s, 2H), 3.86 (s, 3H), 1.35 (s, 12H); $^{13}$C NMR (126 MHz,
DMSO-$d_6$) $\delta_C$ 166.3 (s), 163.1 (s), 160.6 (s), 158.8 (s), 151.9 (s), 139.1 (s), 136.3 (s), 135.3 (s),
129.0 (s), 127.1 (s), 126.7 (s), 125.6 (s), 121.8 (s), 114.0 (s), 113.4 (s), 102.0 (s), 84.0 (s), 70.6
(s), 52.9 (s), 25.0 (s). HRMS (ESI$^+$): calc. for C$_{27}$H$_{28}$BNO$_8$ [M+H]$^+$ 506.1986 m/z; found
506.1990 m/z.
5. NMR spectra

Figure S13. $^1$H NMR (500 MHz, DMSO – $d_6$) of compound 4.

Figure S14. $^{13}$C($^1$H) NMR (126 MHz, DMSO – $d_6$) of compound 4.
Figure S15. $^1$H NMR (500 MHz, DMSO – $d_6$) of compound 3.

Figure S16. $^{13}$C{^1}H NMR (126 MHz, DMSO – $d_6$) of compound 3
Figure S17. $^1$H NMR (500 MHz, DMSO – $d_6$) of compound 2.

Figure S18. $^{13}$C{$^1$H} NMR (126 MHz, DMSO – $d_6$) of compound 2.
Figure S19. $^1$H NMR (500 MHz, DMSO – $d_6$) of compound 1.

Figure S20. $^{13}$C($^1$H) NMR (126 MHz, DMSO – $d_6$) of compound 1.
Figure S21. $^1$H NMR (500 MHz, CDCl$_3$) of ROS-AHC.

Figure S22. $^{13}$C($^1$H) NMR (126 MHz, CDCl$_3$) of ROS-AHC.
6. Author contributions
Luling Wu – conceived the idea, synthesized the probe and wrote the manuscript.
Xue Tian – wrote the manuscript with Luling Wu and carried out the optical experiments
Robin R. Groleau – provided advice and reviewed and edited the manuscript
Jie Wang – aided Hai-Hao Han with the cellular experiments
Hai-Hao Han – carried out the cellular experiments
Shaun B. Reeksting – helped with the mass spectroscopic analysis
Adam C. Sedgwick – provided advice and reviewed and edited the manuscript
Xiao-Peng He – supervisor of Hai-Hao Han and Jie Wang
Steven D. Bull – supervisor of Luling Wu, Xue Tian and Robin R. Groleau
Tony D. James – lead supervisor

7. References