

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

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UPDATED 1st February 2021: The original version of this ESI was first published on 3rd April 2020. This version of the ESI replaces the previous copy in which there was a minor error in the NMR characterization data for Compound 3, and Figure S15 was incorrectly shown.

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1. UV-Vis and fluorescence analysis

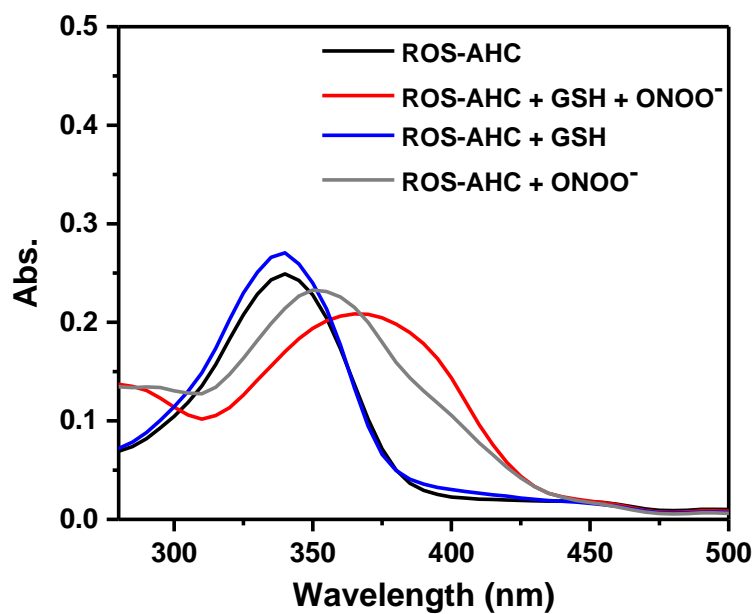


Figure S1. Absorption spectra of **ROS-AHC** (20 μM) only with and without ONOO^- (18 μM) wait 5 min/ GSH (26 μM) wait 5 min, and with addition of GSH (26 μM) wait 5 min then addition of ONOO^- (18 μM) with 5 min incubation before measurements in PBS buffer solution (10 mM, pH = 7.40).

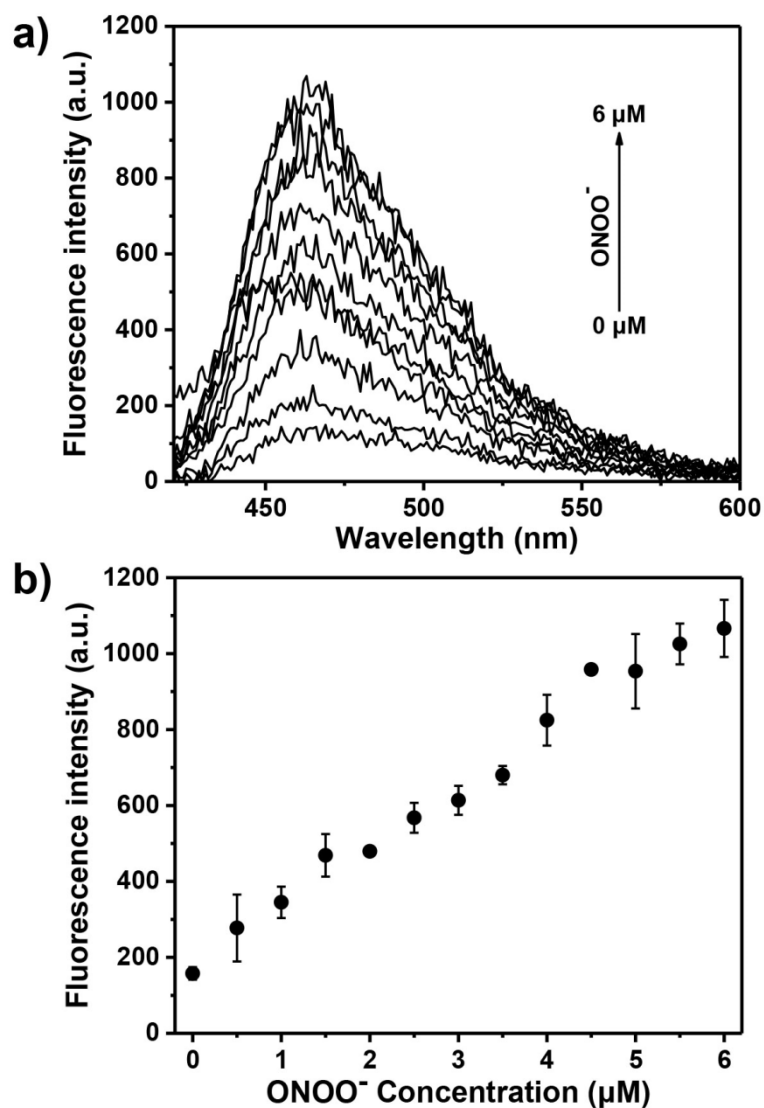


Figure S2. (a) Fluorescence spectra of ROS-AHC (5 μM) with increasing additions of ONOO⁻ (from 0 to 6 μM) in PBS buffer solution (10 mM, pH = 7.40) after 5 min. (b) Fluorescence intensity changes (based on the intensities at 461 nm) against ONOO⁻ concentration. $\lambda_{\text{ex}} = 400$ nm (bandwidth 8 nm).

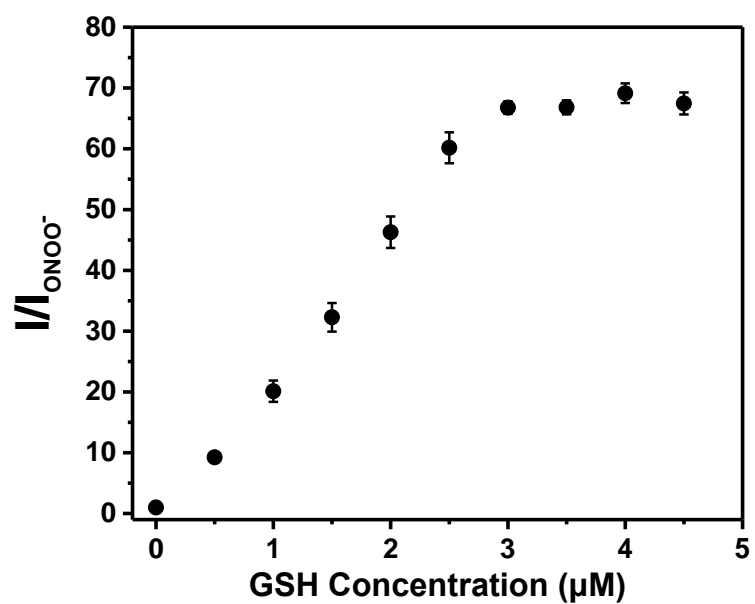


Figure S3. Fluorescence intensity changes (I/I_{ONOO^-}) for **ROS-AHC** (5 μM) with addition of ONOO^- (6 μM), wait 5 min, then additions of GSH (0 – 4.5 μM) with 5 min incubation before measurement in PBS buffer solution (10 mM, pH = 7.40). $\lambda_{\text{ex}} = 400$ nm (bandwidth 8 nm)/ $\lambda_{\text{em}} = 461$ nm.

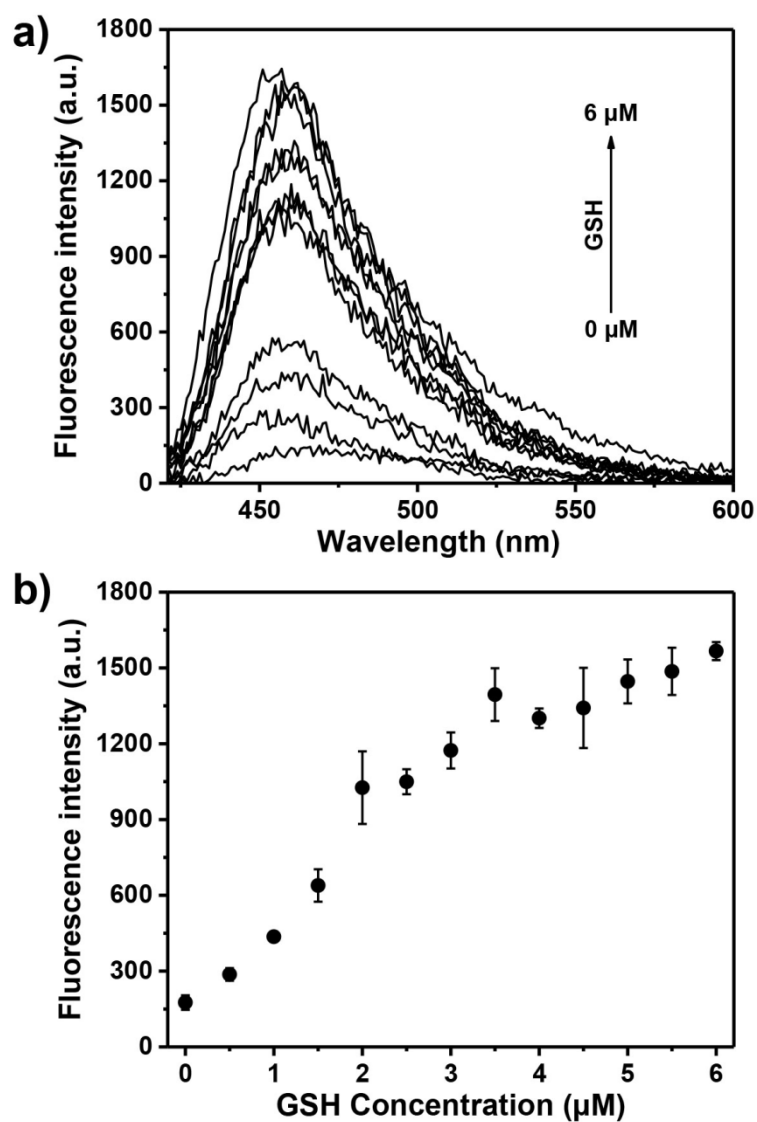


Figure S4. (a) Fluorescence spectra of ROS-AHC (5 μM) with increasing additions of GSH (from 0 to 6 μM) with 5 min incubation before measurement in PBS buffer solution (10 mM, pH = 7.40). (b) Fluorescence intensity changes (based on the intensities at 461 nm) against GSH concentration. $\lambda_{\text{ex}} = 400 \text{ nm}$ (bandwidth 8 nm).

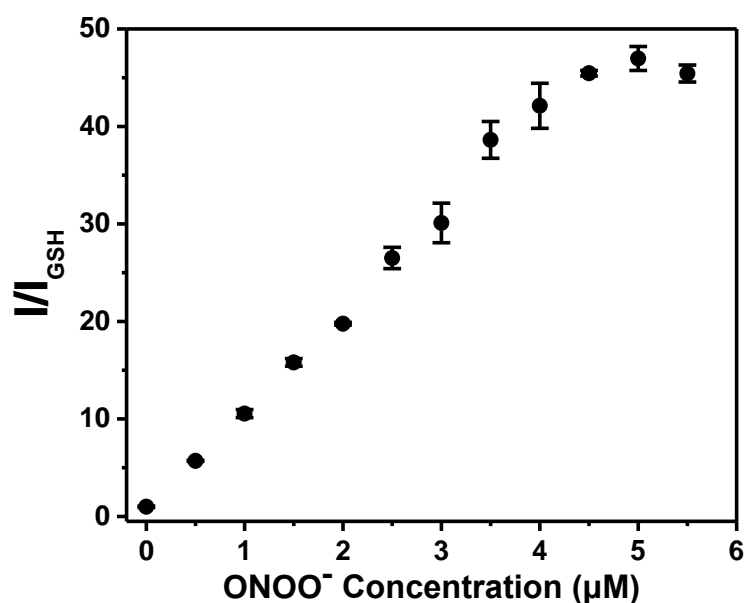


Figure S5. Fluorescence intensity changes (I/I_{GSH}) for **ROS-AHC** ($5 \mu\text{M}$) with addition of **GSH** ($6 \mu\text{M}$), wait 5 min, then additions of ONOO^- ($0 - 5.5 \mu\text{M}$) with 5 min incubation before measurement in PBS buffer solution (10 mM , $\text{pH} = 7.40$). $\lambda_{\text{ex}} = 400 \text{ nm}$ (bandwidth 8 nm)/ $\lambda_{\text{em}} = 461 \text{ nm}$.

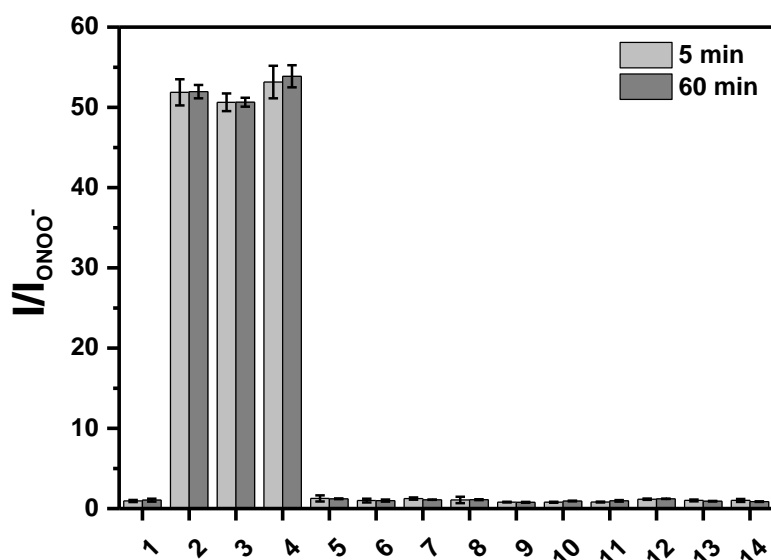


Figure S6. Selectivity bar chart of **ROS-AHC** ($5 \mu\text{M}$) with addition of ONOO^- ($6 \mu\text{M}$), wait 5 min, then addition of various amino acids wait 5 min or 60 min before measurement in PBS buffer solution (10 mM , $\text{pH} = 7.40$), 1 – blank; 2 – **GSH** (Glutathione, $4 \mu\text{M}$); 3 – **Cys** (Cysteine, $4 \mu\text{M}$); 4 – **Hcy** (Homocysteine, $4 \mu\text{M}$); 5 – **Glu** (Glutamic acid, $500 \mu\text{M}$); 6 – **Phe** (Phenylalanine, $500 \mu\text{M}$); 7 – **Asp** (Aspartic acid, $500 \mu\text{M}$); 8 – **Pro** (Proline, $500 \mu\text{M}$); 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). $\lambda_{\text{ex}} = 400 \text{ nm}$ (bandwidth 8 nm)/ $\lambda_{\text{em}} = 461 \text{ nm}$.

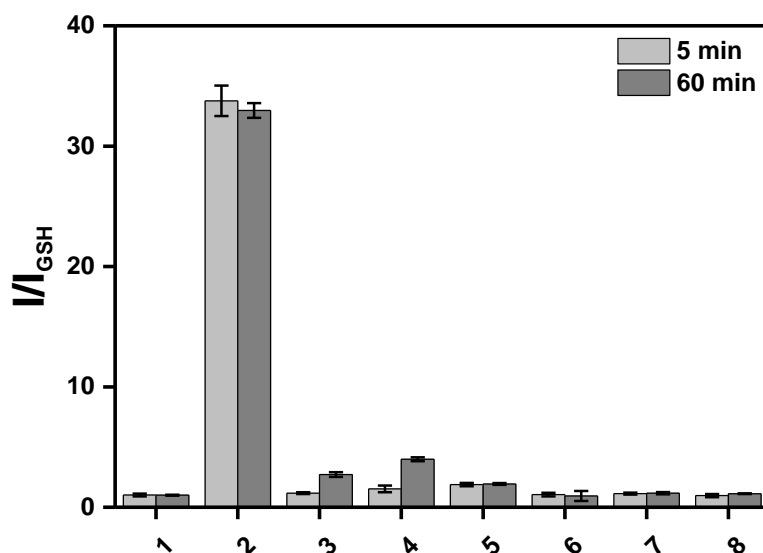


Figure S7. Selectivity bar chart of **ROS-AHC** (5 μM) with addition of GSH (6 μM), wait 5 min, then addition of various ROS wait 5 min or 60 min before measurement in PBS buffer solution (10 mM, pH = 7.40). 1 – blank; 2 – ONOO^- (5 μM); 3 – HOCl (100 μM); 4 – H_2O_2 (100 μM); 5 – $\text{ROO}\cdot$ (100 μM); 6 – $\cdot\text{OH}$ (100 μM); 7 – $\text{O}_2^{\cdot-}$ (100 μM); 8 – $^1\text{O}_2$ (100 μM). $\lambda_{\text{ex}} = 400 \text{ nm}$ (bandwidth 8 nm)/ $\lambda_{\text{em}} = 461 \text{ nm}$.

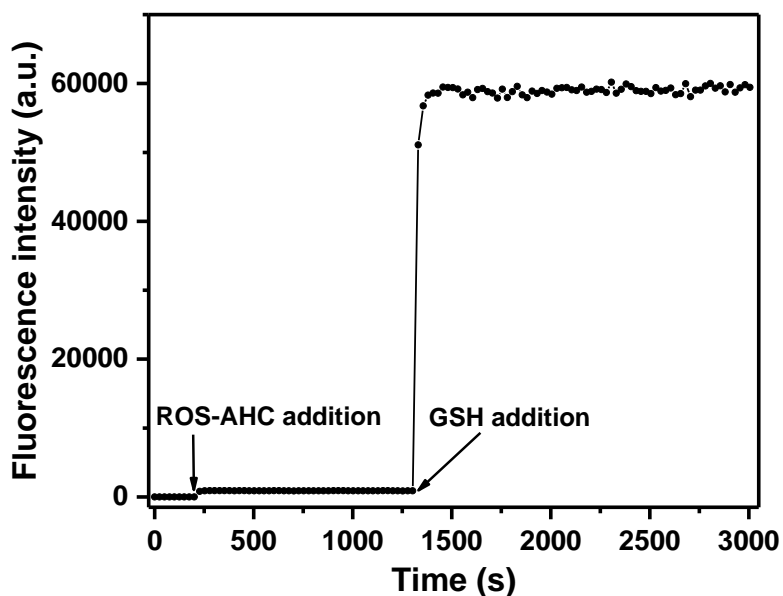


Figure S8. Fluorescence intensity over time of the addition of **ROS-AHC** (5 μM) followed by the addition of **GSH** (4 μM) to ONOO^- (6 μM) in PBS buffer solution (10 mM, pH = 7.40). λ_{ex} = 400 nm (bandwidth 8 nm)/ λ_{em} = 461 nm.

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.

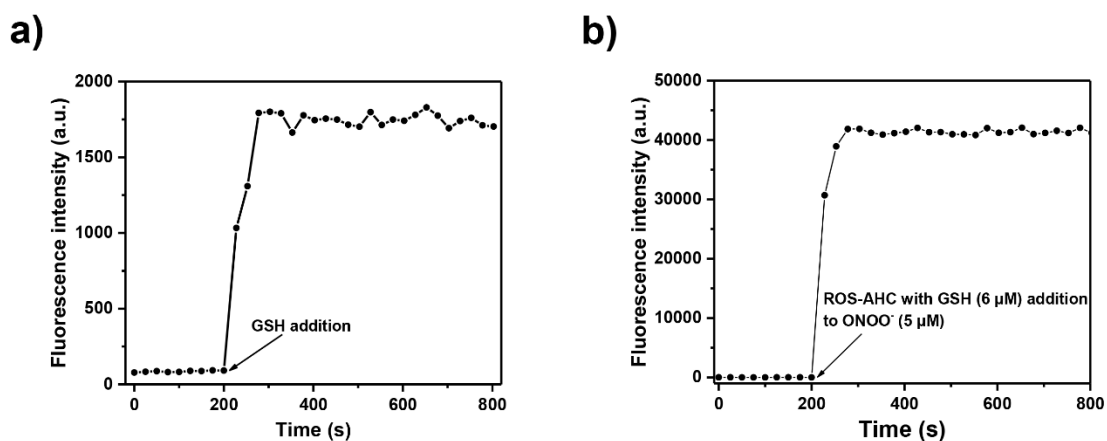


Figure S9. (a) Fluorescence intensity over time of the addition of **ROS-AHC** ($5 \mu\text{M}$) with addition of **GSH** ($6 \mu\text{M}$) at 200 s in PBS buffer solution (10 mM , $\text{pH} = 7.40$). $\lambda_{\text{ex}} = 400 \text{ nm}$ (bandwidth 8 nm)/ $\lambda_{\text{em}} = 461 \text{ nm}$. (b) Fluorescence intensity over time of the addition of the addition of a pre-mixed (5 min) solution of **ROS-AHC** ($5 \mu\text{M}$) and **GSH** ($6 \mu\text{M}$) to ONOO^- ($5 \mu\text{M}$) at 200 s in PBS buffer solution. $\lambda_{\text{ex}} = 400 \text{ nm}$ (bandwidth 8 nm)/ $\lambda_{\text{em}} = 461 \text{ nm}$.

Experimental for Figure S9: (a) A solution of **ROS-AHC** ($5 \mu\text{M}$) 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** ($6 \mu\text{M}$) was then pumped into this solution, and fluorescence was measured every 25 s from 228 s to 803 s. (b) A solution of ONOO^- ($6 \mu\text{M}$) 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** ($5 \mu\text{M}$) and **GSH** ($6 \mu\text{M}$), 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 peroxyntirite in a 0.5 M NaOH aqueous solution was determined from the absorption at 302 nm ($\epsilon = 1670 \text{ M}^{-1} \text{ cm}^{-1}$).

ClO⁻

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

H₂O₂

The concentration of H₂O₂ was determined from the absorption at 240 nm ($\epsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$).

3. Mass spectroscopic analysis

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: C ₂₇ H ₂₈ BNO ₈	0.68	506.1990	504.1948	504.1944	0.78	98.86

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

Figure: Extracted ion chromatogram (EIC) of compound.

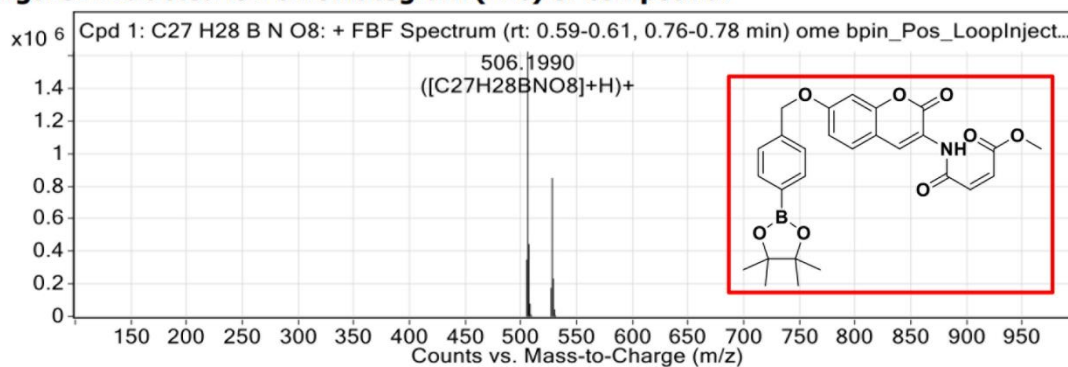


Figure: Full range view of Compound spectra and potential adducts.

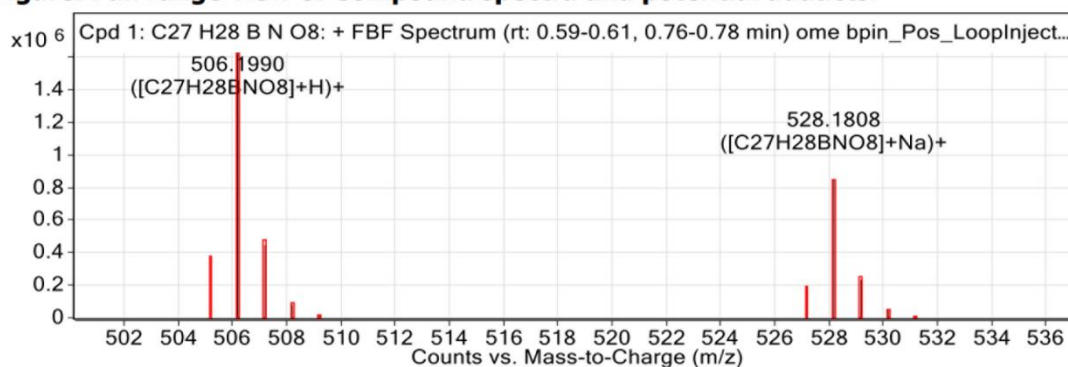


Figure S10. HRMS spectrum of ROS-AHC (45 μ M).

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: C ₁₄ H ₁₁ N O ₆	4.15	290.0657	289.0586	289.0586	-0.25	74.23

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

Figure: Extracted ion chromatogram (EIC) of compound.

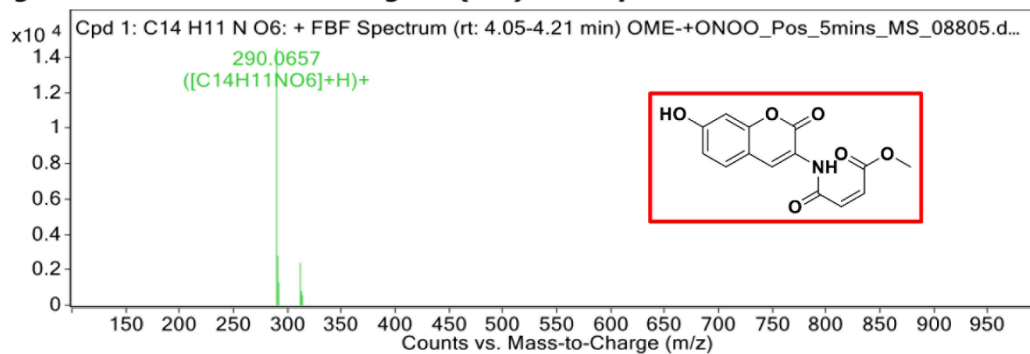


Figure: Full range view of Compound spectra and potential adducts.

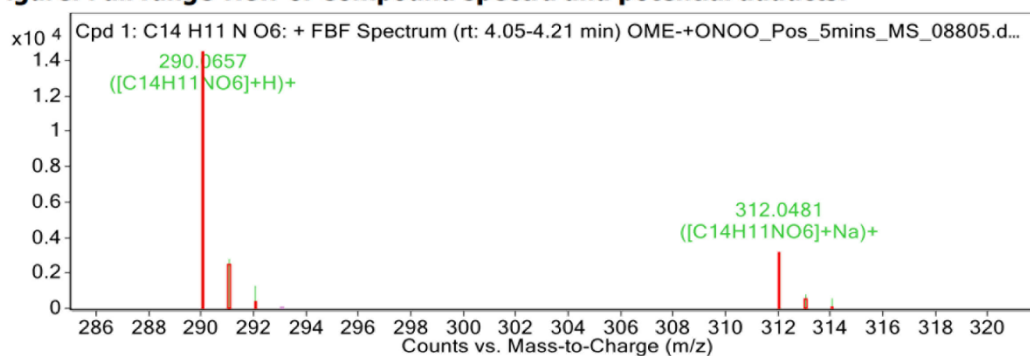


Figure S11. LC-MS spectrum of ROS-AHC (45 μ M) + ONOO⁻ (1.5 equiv.).

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: C ₂₄ H ₂₈ N ₄ O ₁₂ S	0.96	597.1491	596.1404	596.1424	-3.47	72.99

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

Figure: Extracted ion chromatogram (EIC) of compound.

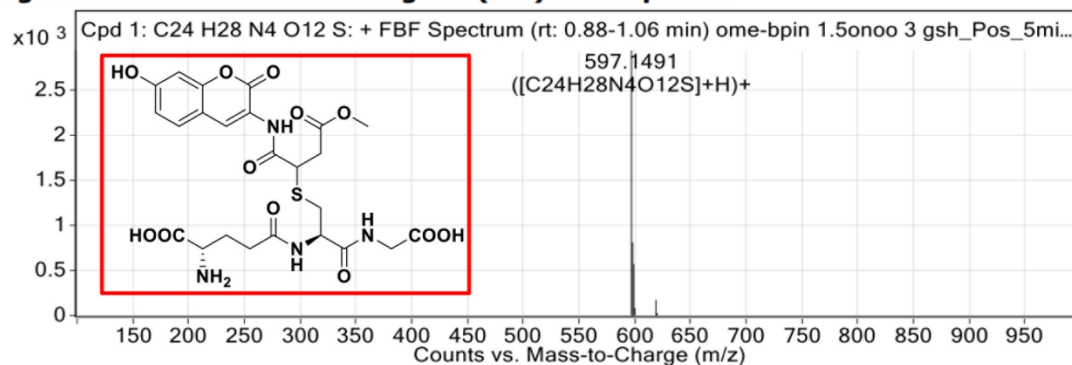


Figure: Full range view of Compound spectra and potential adducts.

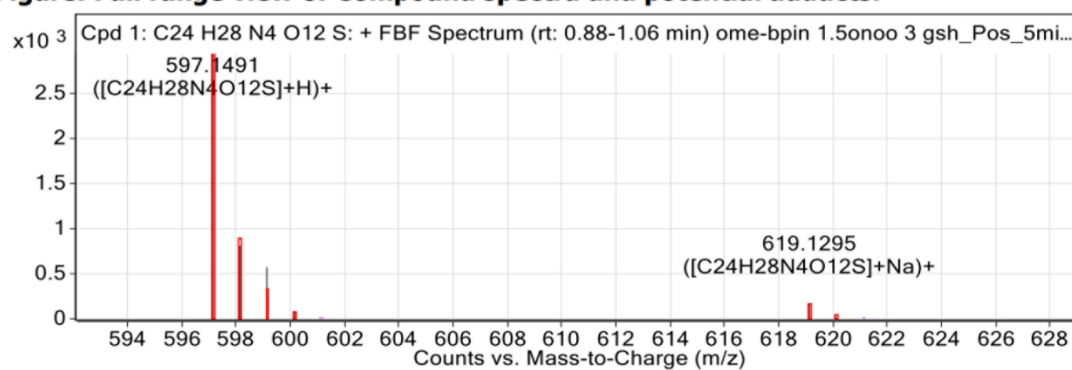
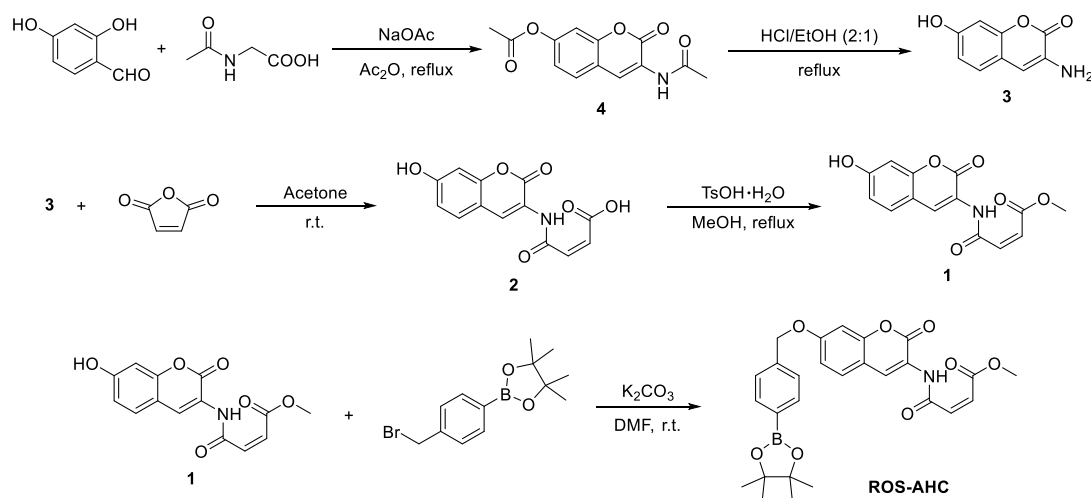


Figure S12. LC-MS spectrum of ROS-AHC (45 μ M) + ONOO⁻ (1.5 equiv.) + GSH (3.0 equiv.).

4. Synthesis and characterisation of compounds 1-4 and ROS-AHC



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; ¹H NMR (500 MHz, DMSO-*d*₆) δ_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); ¹³C NMR (126 MHz, DMSO-*d*₆) δ_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 C₁₃H₁₁NO₅ [M+H]⁺ 262.0710 *m/z*, found 262.0711 *m/z*.

Compound 3

M.p. 247 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ_H 9.80 (s, 1H), 7.23 (d, *J* = 8.4 Hz, 1H), 6.69 – 6.65 (m, 3H), 5.22 (bs, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ_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 C₉H₇NO₃ [M+H]⁺ 178.0499 *m/z*, found 178.0500 *m/z*.

Compound 2

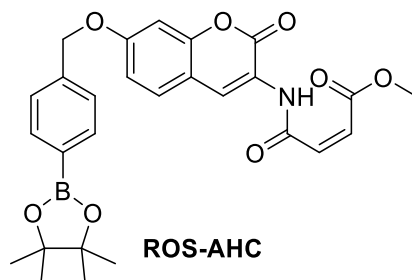
M.p. 247 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ_H 12.96 (bs, 1H), 10.41 (s, 1H), 10.14 (s, 1H), 8.61 (s, 1H), 7.55 (d, *J* = 8.5 Hz, 1H), 6.80 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.74 (d, *J* = 2.2 Hz, 1H), 6.62 (d, *J* = 12.1 Hz, 1H), 6.40 (d, *J* = 12.0 Hz, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ_C 167.6 (s), 163.6 (s), 159.8 (s), 157.7 (s), 151.7 (s), 132.2 (s), 129.2 (s), 128.7 (s), 126.6 (s), 120.6 (s), 113.7 (s), 111.3 (s), 102.0 (s). HRMS (ESI⁺): calc. for C₁₃H₉NO₆ [M+H]⁺ 276.0503 *m/z*, found 276.0502 *m/z*.

Compound 1

M.p. 189 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ_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); ¹³C NMR (126 MHz, DMSO-*d*₆) δ_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 C₁₄H₁₁NO₆ [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 K₂CO₃ (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 N₂ 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 Na₂SO₄ and concentrated *in vacuo*. The crude product obtained was purified by flash chromatography (SiO₂, 30% EtOAc in petroleum ether) to afford the desired product **ROS-AHC** as a yellow solid (72 mg, 23% yield). M.p. 177 °C; ¹H NMR (500 MHz, CDCl₃) δ_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); ¹³C NMR (126 MHz, DMSO-*d*₆) δ_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 C₂₇H₂₈BNO₈ [M+H]⁺ 506.1986 *m/z*, found 506.1990 *m/z*.

5. NMR spectra

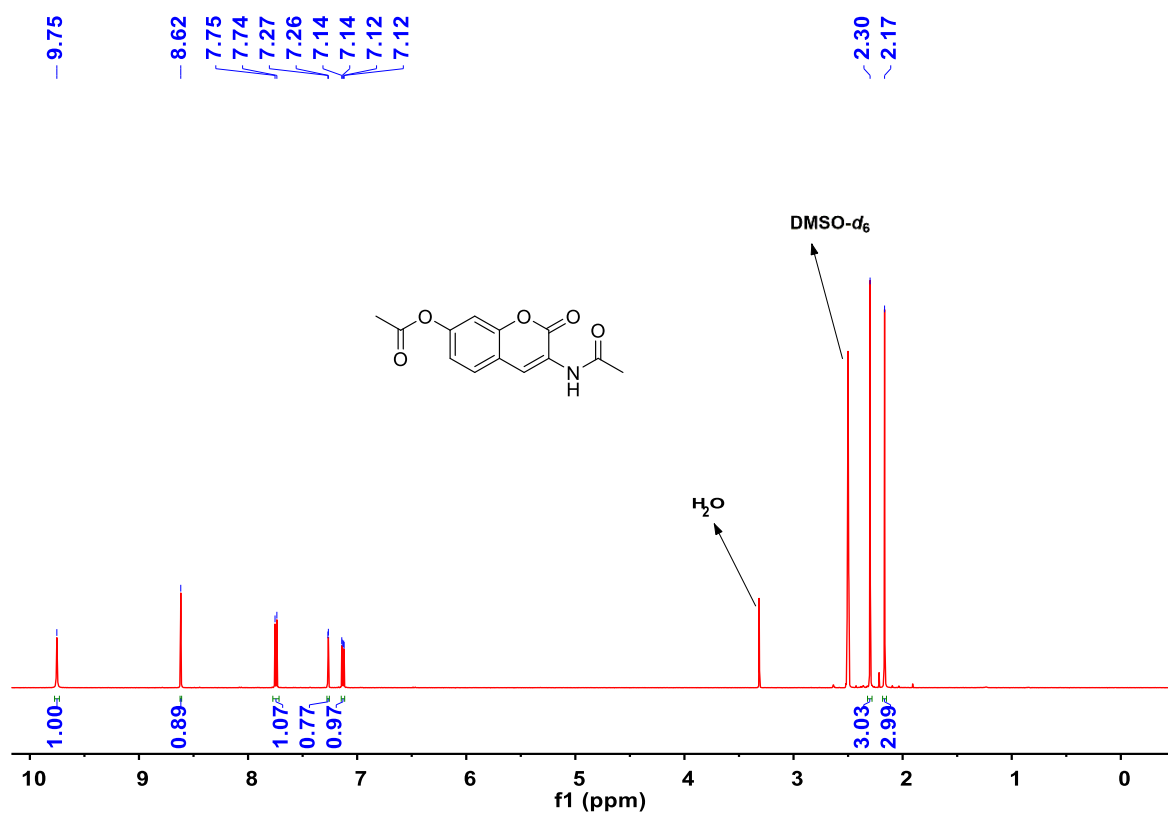


Figure S13. ^1H NMR (500 MHz, $\text{DMSO} - d_6$) of compound 4.

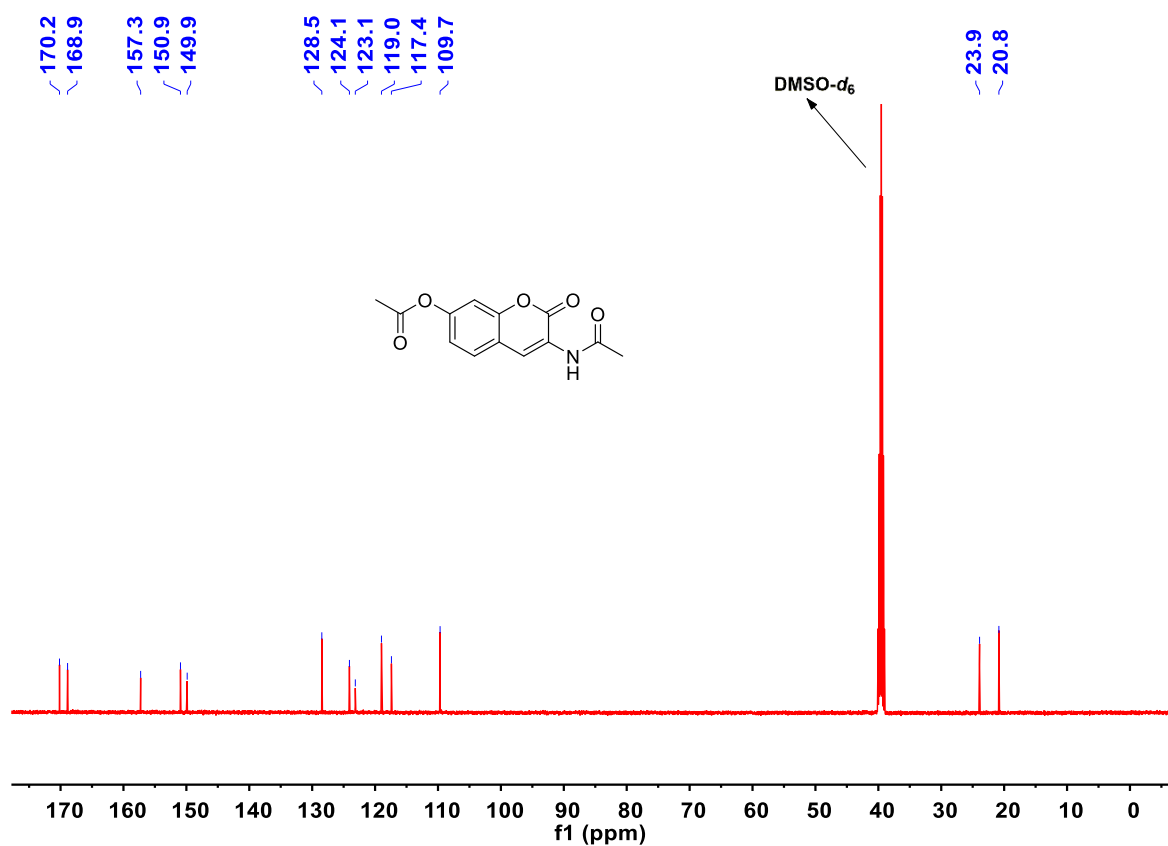


Figure S14. $^{13}\text{C}\{^1\text{H}\}$ NMR (126 MHz, $\text{DMSO} - d_6$) of compound 4.

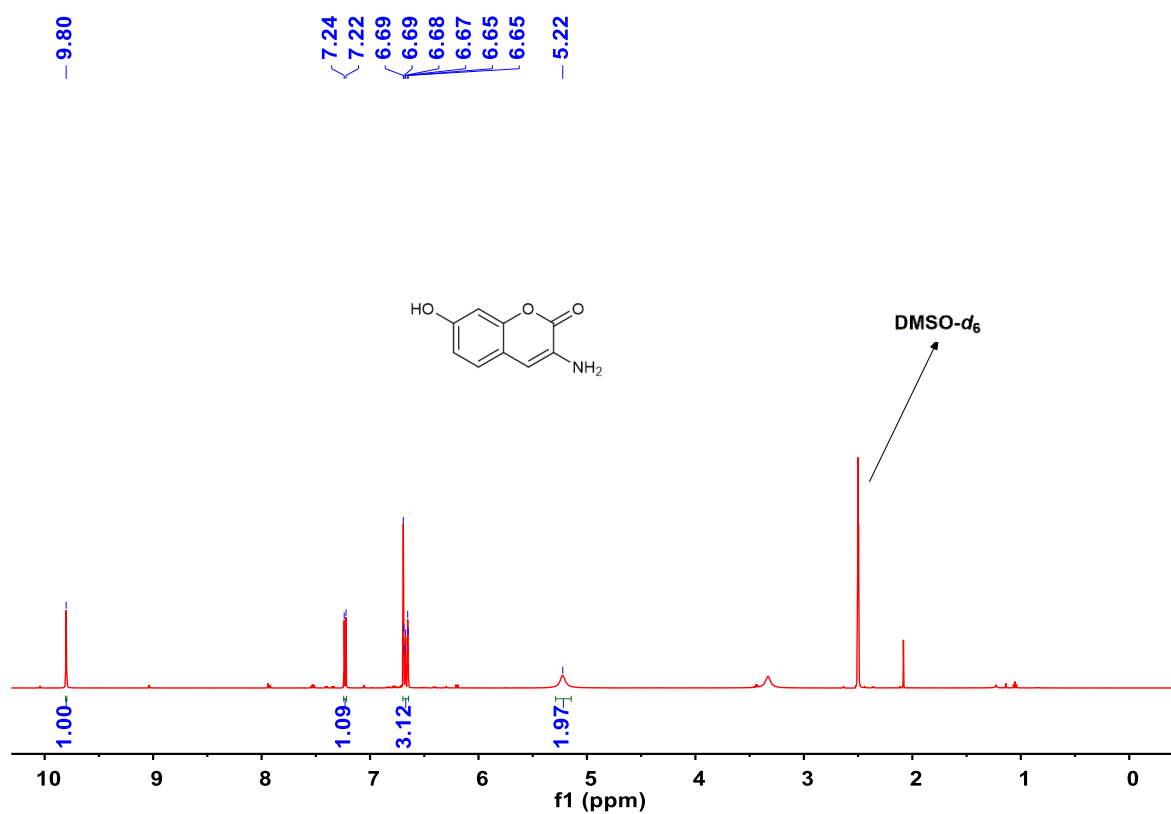


Figure S15. ¹H NMR (500 MHz, DMSO – *d*₆) of compound 3.

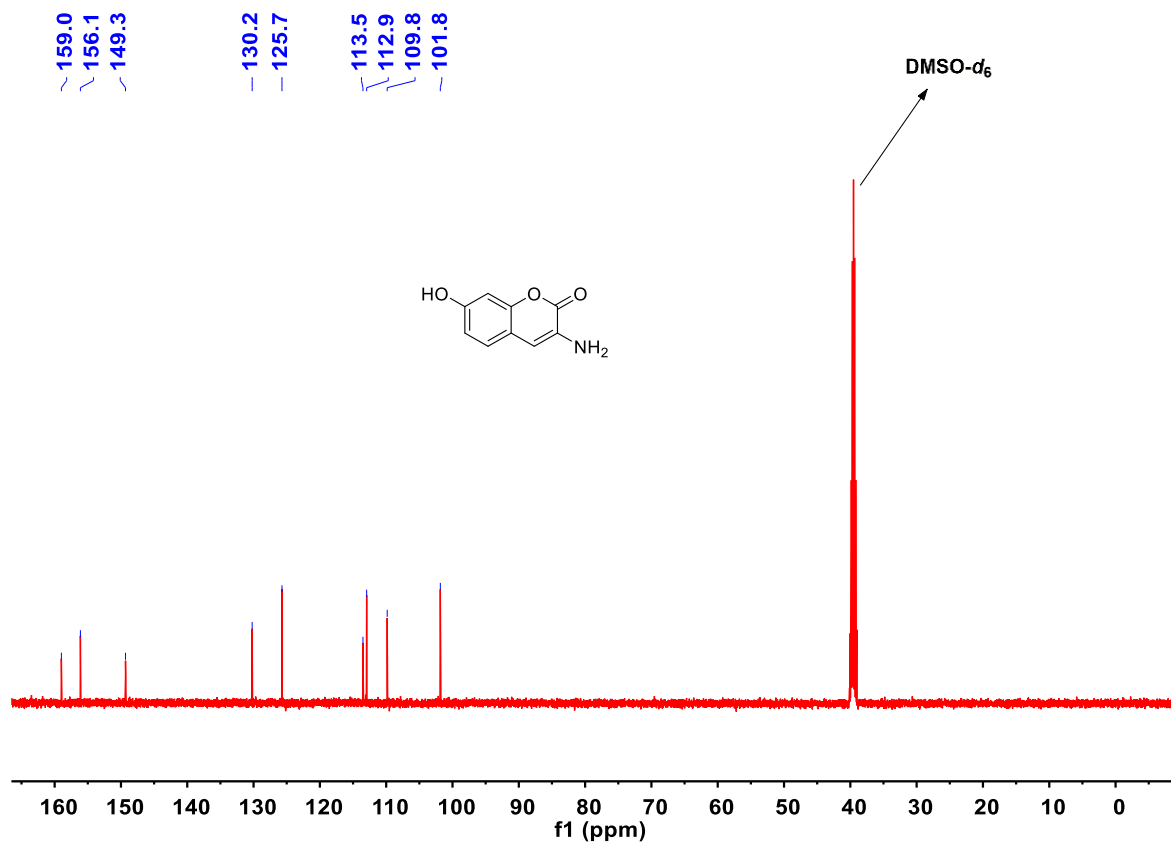


Figure S16. ¹³C{¹H} NMR (126 MHz, DMSO – *d*₆) of compound 3.

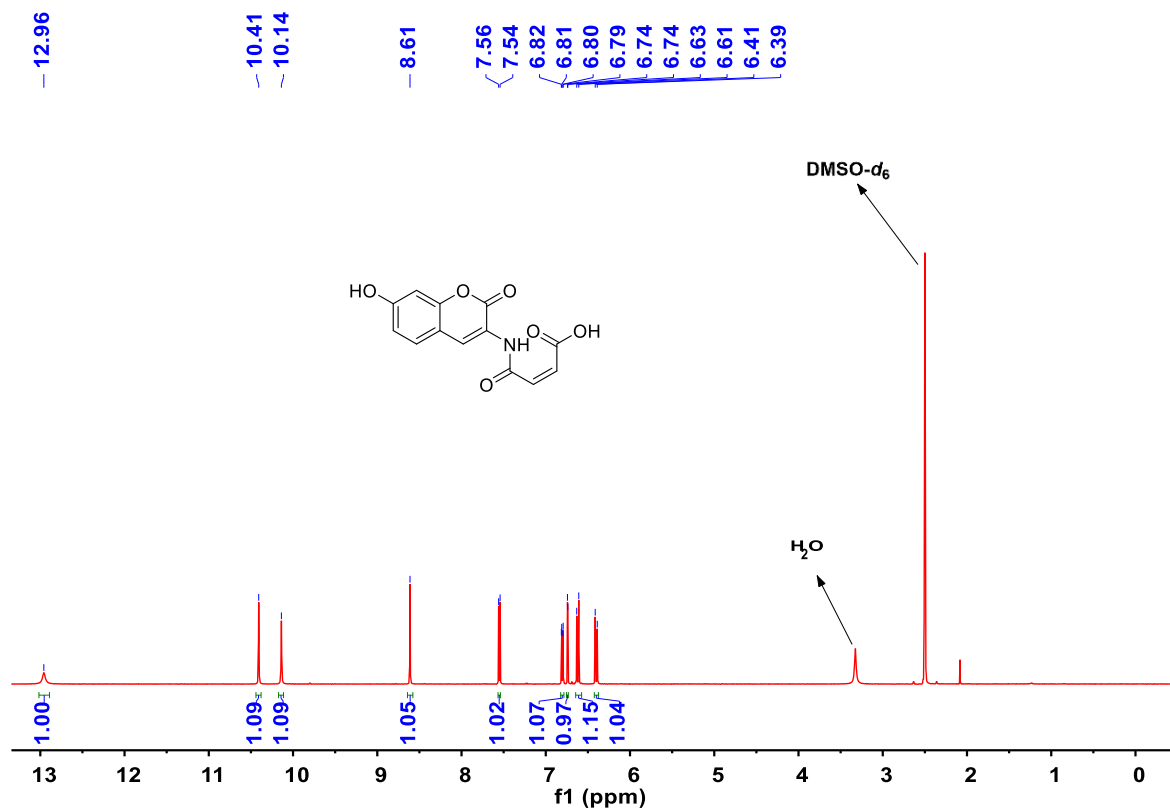


Figure S17. ^1H NMR (500 MHz, $\text{DMSO} - d_6$) of compound 2.

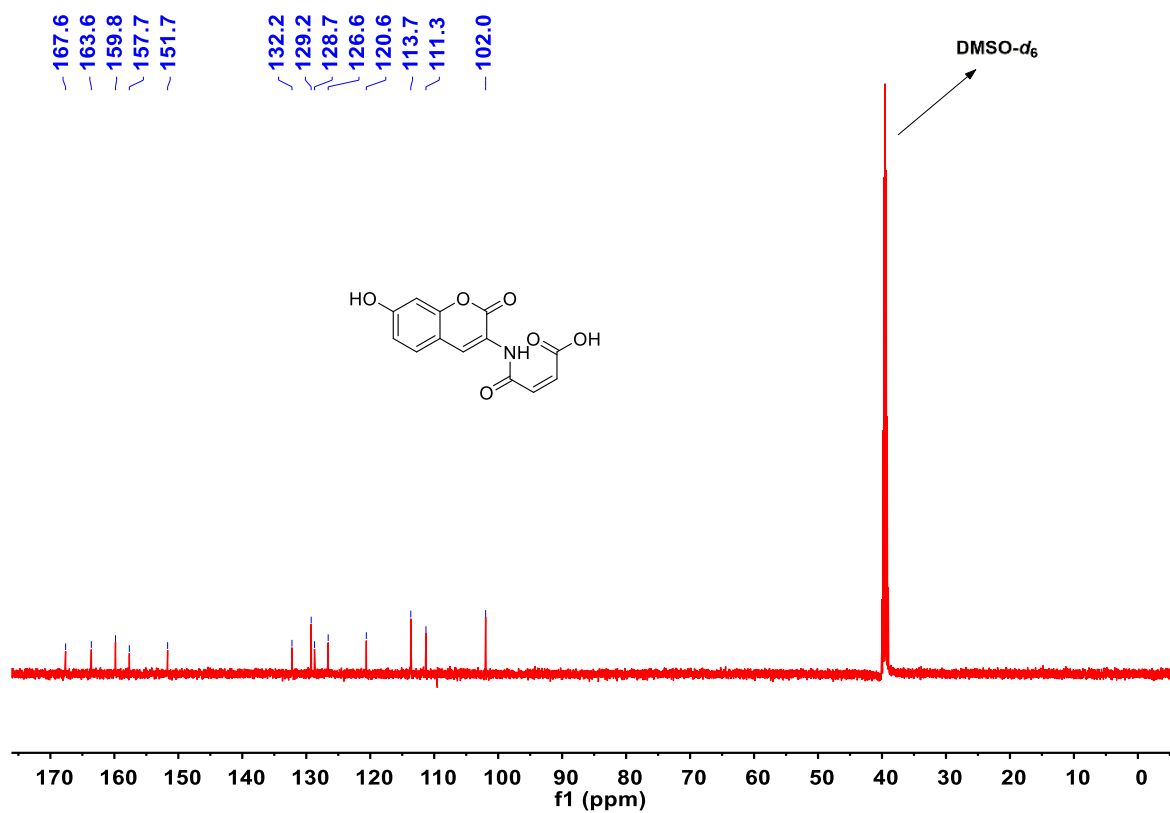


Figure S18. $^{13}\text{C}\{^1\text{H}\}$ NMR (126 MHz, $\text{DMSO} - d_6$) of compound 2.

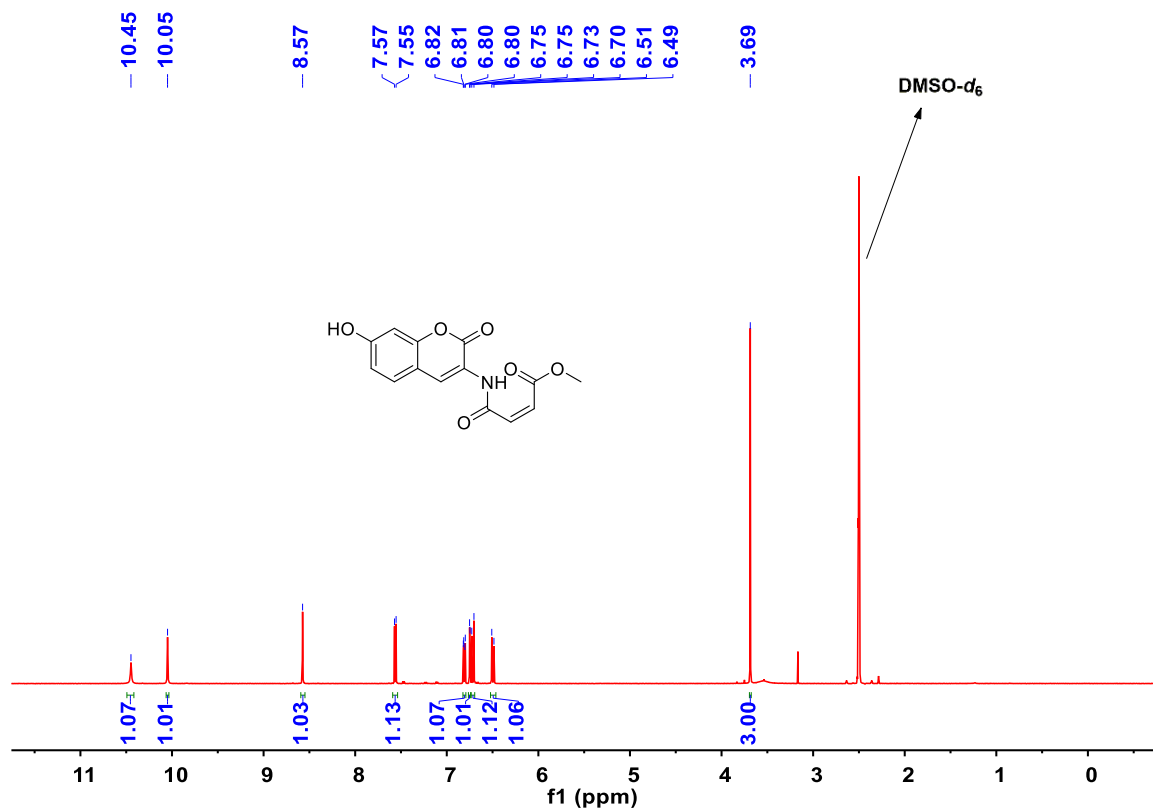


Figure S19. ^1H NMR (500 MHz, $\text{DMSO} - d_6$) of compound 1.

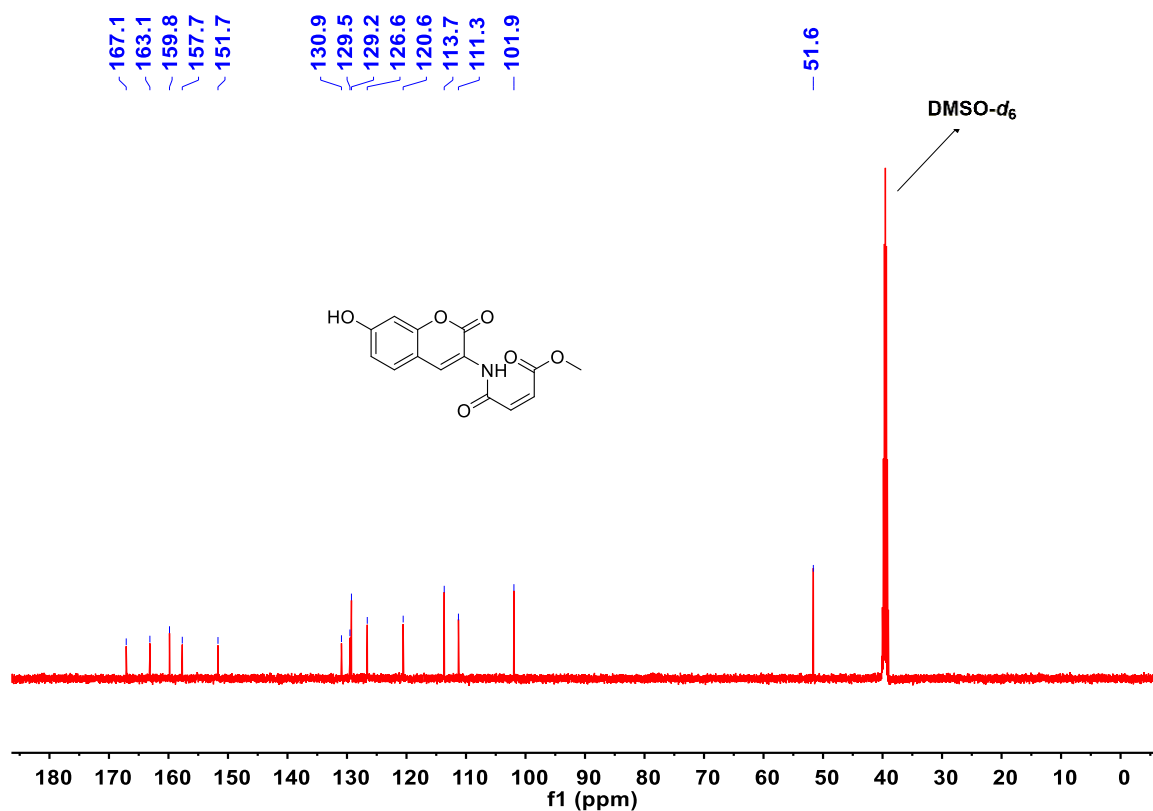


Figure S20. $^{13}\text{C}\{^1\text{H}\}$ NMR (126 MHz, $\text{DMSO} - d_6$) of compound 1.

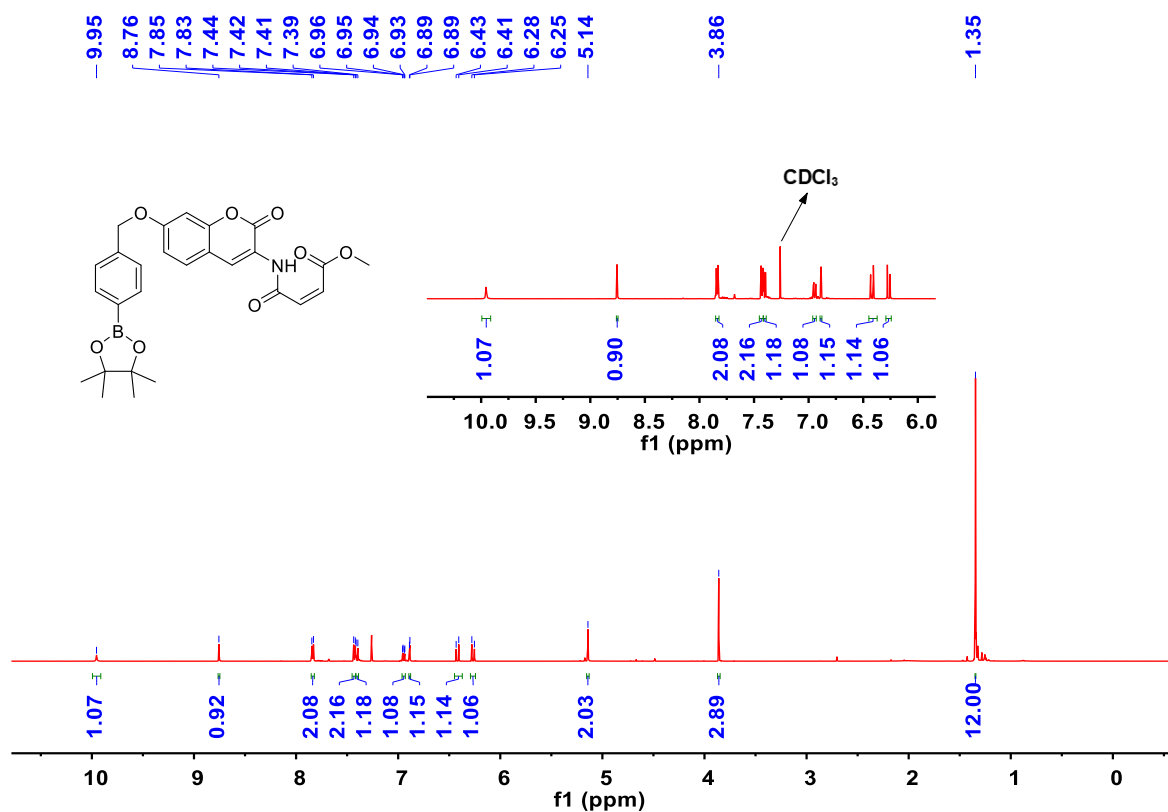


Figure S21. ^1H NMR (500 MHz, CDCl_3) of ROS-AHC.

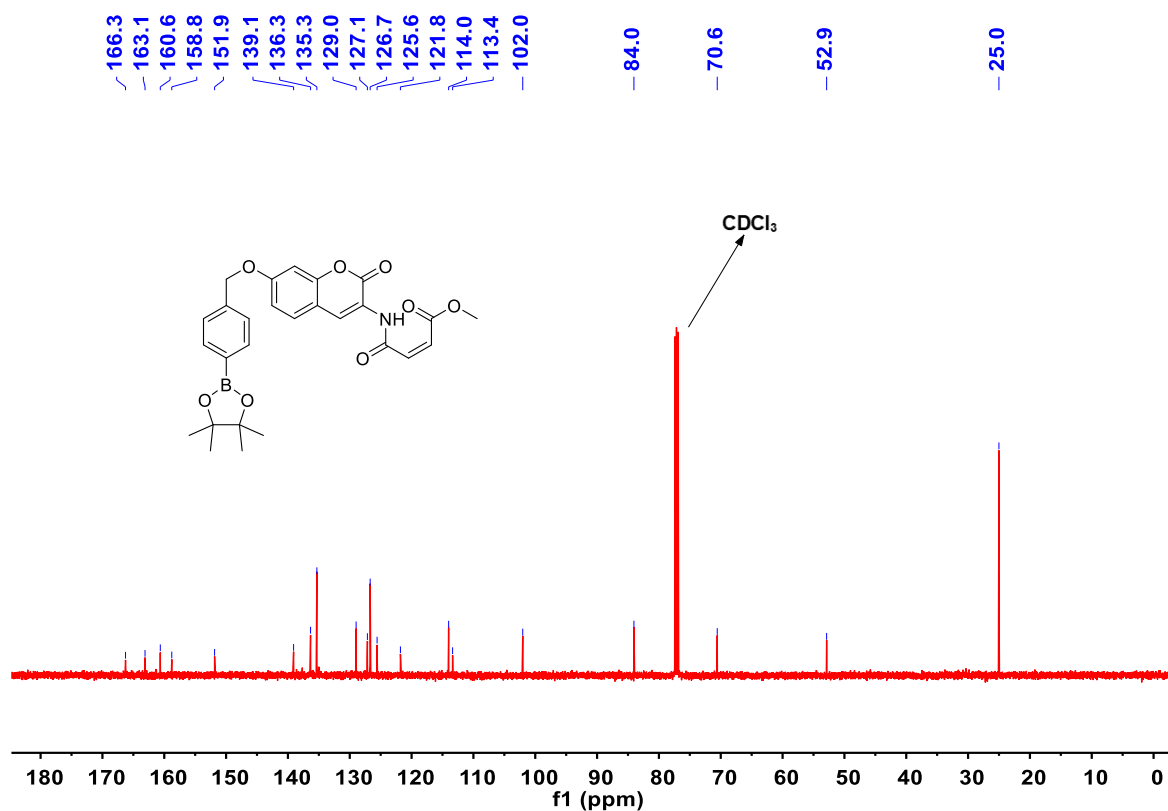


Figure S22. $^{13}\text{C}\{^1\text{H}\}$ NMR (126 MHz, CDCl_3) of ROS-AHC.

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

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