Electronic supplementary information

A Two-photon Fluorescent Probe for Formaldehyde Detection and Regeneration in Living Cells

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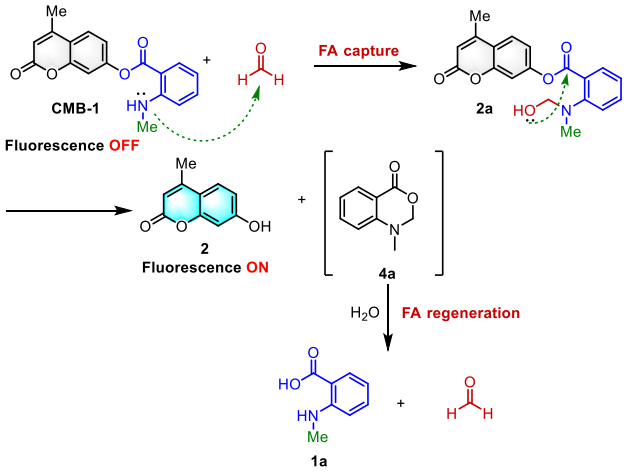
1. General Information

All reagents and solvents were obtained commercially and used without further purification unless otherwise noted. Analytical thin-layer chromatography (TLC) was performed on silica gel plates and analyzed by UV light or by potassium permanganate stains followed by heating. Flash chromatography was carried out utilizing silica gel (200-300 mesh). ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ at room temperature on a Bruker AM-400 spectrometer (400 MHz ^{1}H, 100 MHz ^{13}C). The chemical shifts are reported in ppm relative to either the residual solvent peak (^{13}C) (δ = 77.00 ppm for $CDCl_3$;), (^{1}H) (δ = 7.26 ppm for $CDCl_3$, δ = 7.16 ppm for C_6D_6) or TMS (^{1}H) (δ = 0 ppm) as an internal standard. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, dd = doublet doublet), coupling constants (Hz), integration. Data for ^{13}C NMR are reported as chemical shifts. HRMS were performed on a Bruker Apex II mass instrument (ESI).

All UV − visible spectra and fluorescence spectra were recorded using an Agilent Cary 5000 spectrophotometer and Horiba FluoroMax-4 luminescence spectrometer, respectively. Fluorescence spectra were measured after the addition of FA for 2 h. Fluorescent quantum yields were determined to be 0.01, 0.01, 0.02 for probe **CMB-1**, **CMB-2**, **CMB-3**, and 0.70 for compound **2**, respectively by an absolute method using an integrating sphere on FLS920 of Edinburgh Instrument. Two-photon fluorescence imaging was obtained using an FV1200 (Olympus) multiphoton laser scanning microscope with a Coherent Mira900-D. In two photon experiments, the excitation wavelengths were 800 nm from a Ti : sapphire femtosecond laser source (Coherent Chamelon Ultra) and the incident power on the samples was modified using an attenuator and examined with a Power Monitor (Coherent).

2. Recognition Mechanism Studies



Scheme S1. Proposed mechanism for FA detection and regeneration of **CMB-1**.

HPLC Studies of the Reaction between CMB-1 and FA: The probe **CMB-1** reacting with FA was analyzed by the High-Performance Liquid Chromatography (HPLC) instrument (SHIMADZU LC-20AT) using the following procedure. Firstly, **CMB-1**, compounds **2** and **1a** were dissolved at a concentration of 1 mg/mL in n-hexane/isopropanol (90/10). Secondly, the reaction system of **CMB-1** with FA was prepared with 0.05 mmol of **CMB-1** and 5 mmol of FA in THF/H_2O (3:2) and incubated for 2 h at 37 °C before the measurement. Then, the reaction system is extracted with dichloromethane and the solvent was removed to yield the solid, which was further dissolved in n-hexane/isopropanol (90/10). Finally, 15 μL of each sample was injected into an Inertsil SIL-100A column (250 mm × 4.6 mm; particle size, 5 μm) and chromatographed using a solution of n-hexane/isopropanol (95/5) for 15 min at a flow rate of 1 mL/min.

The Sensing Reaction of CMB-1 with FA at Different pH using Different Methods:

(i) The pH effects of photophysical responses between **CMB-1** and FA were explored to disclose the reactivity between **CMB-1** and FA using time-dependent fluorescence studies. The fluorescence of **CMB-1**(10 μM) in the absence and presence of FA (1.75 mM) was collected at pH 5.0, 7.4, and 9.0 at different time points, respectively. Data were acquired at the time points 0, 20, 30, 45, 60, 90, and 120 min.

(ii) CMB-1 (3.1 mg, 0.01 mmol) was dissolved in 3 mL THF and 2 mL H_2O at pH 5.0, 7.4, and 9.0, respectively, in which 37% FA solution (14.8 μL, 0.2 mmol) was added and stirred at 37 °C for 2 h. The reaction was monitored by TLC assay and the photos of the TLC plate and the solution was taken under a UV lamp (254 nm).

(iii) MS Analysis: Finally, the reaction mixture in THF-PBS at pH 7.4 was submitted for MS analysis. $[M + H]^+$ peaks at 152.0778 and 177.0630 were found and assigned as compounds **1a** and **2**, respectively. HRMS (ESI) m/z: $[M + H]^+$ calcd. for compound **1a** $C_8H_{10}NO_2$, Exact Mass: 152.0712, found 152.0778. HRMS (ESI) m/z: $[M + H]^+$ calcd. for compound **2** $C_{10}H_9O_3$, Exact Mass: 177.0552, found 177.0630.

^{1}H NMR Studies of the Intermediate 4a: CMB-1 (3.1 mg, 0.01 mmol) was dissolved in 3 mL THF and 2 mL PBS at pH of 7.4, in which 37% FA solution (74 μL, 1 mmol) was added and stirred at 37 °C. The reaction was monitored by TLC assay and stopped until the majority of **CDM-1** was diminished. The mixture was immediately extracted with ethyl acetate three times, from which the organic layer was collected and dried over the vacuum to yield a white solid. C_6D_6 was used to dissolve the solid, and the upper clear solution was collected for the measurement of ^{1}H NMR spectra.

Compared with compounds **2** and **1a**, **4a** displayed much better solubility in both ethyl acetate and C_6D_6, which resulted in accumulated **4a** in the upper clear solution for the ^{1}H NMR experiment. As shown in Figure. S6, peaks for **4a** were noticed and assigned as followed: ^{1}H NMR (400 MHz, C_6D_6) δ 8.20 (dd, J = 7.8, 1.5 Hz, 1H), 7.02 – 6.96 (m, 1H), 6.63 – 6.57 (m, 1H), 6.15 (d, J = 8.3 Hz, 1H), 4.16 (s, 2H), 1.99 (s, 3H).

3. Determination of the Detection Limit

The detection limit was calculated based on fluorescence titration. The emission spectrum of **CMB-1** was measured by thirty times and the standard deviation (σ) of this blank measurement was achieved. The slope (k) was derived from the calibration curve for quantitative analysis of FA. The detection limit was determined with the following equation:

Detection limit = $3\sigma/k$. (1.0 μM)

4. Cell Culture and MTT Experiment

HepG2 cells were cultured in DMEM, supplemented with 10% FBS, 1% penicillin, and 1% streptomycin sulfate in a humidified 5% CO_2/95% air incubator at 37 °C. The growth medium was replaced every two days. Cells were routinely detached with a trypsin-EDTA solution and then seeded in a 25 mL cell culture bottle. The cells reached about 80% confluence prior to experiments. The cytotoxicity of **CMB-1** to HepG2 cells was examined by the MTT assay method. HepG2 cells were seeded at a density of 5×10^4 cells/mL in a 96-well micro-assay culture plate. After growth at 37 °C in a 5% CO_2 incubator for 24 h, the culture medium was replaced with the freshly prepared medium containing different concentrations of **CMB-1**. The group with the addition of culture medium only was employed as the control, and the wells containing culture media without cells were used as blanks. After incubation at 37 °C in a 5% CO_2 incubator for 24 h, the cell culture medium was removed, and cells were carefully washed three times with PBS. Then, the MTT solution in PBS (100 μL, 0.5 mg/mL) was added to each well for further incubation for 4 h. The excess MTT solution was then carefully removed from each well, and the formed formazan was dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorbance at 490 nm was measured in an Infinite F50 Microplate Reader.

The results from the five individual experiments were averaged. The following formula was used to calculate the viability of cell growth:

Vialibity (%) = (mean of absorbance value of treatment group-blank)/(mean absorbance value of control-blank) × 100.

5. Fluorescence Imaging of Formaldehyde in Living HepG2 Cells

For fluorescence cell imaging, a stock solution of **CMB-1** (10 mM) was prepared in DMSO. HepG2 cells were typically seeded at a density of 5×10^4 cells/mL in a covered glass-bottomed cell culture dish ($\phi = 20$ mm) for fluorescence microscopic cell imaging.

Exogenous FA Imaging: After 24 h growth, the culture medium was removed, and the cells were further incubated with a freshly prepared medium containing FA (200 μM and 400 μM) at 37 °C in a 5% CO_2/95% air incubator for another 1 h, respectively. The excess FA was discarded. Cells were washed with PBS thrice, to which **CMB-1**

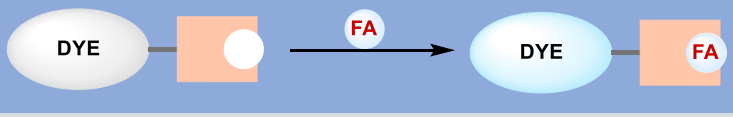
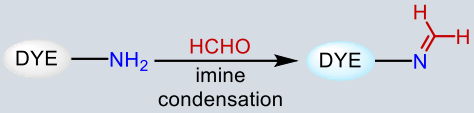
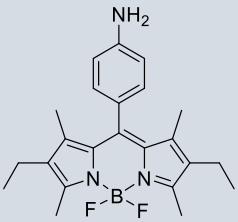
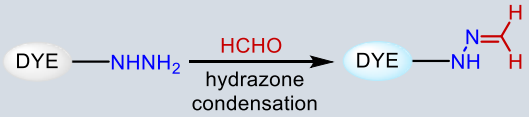
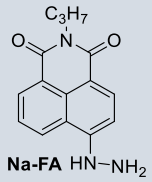
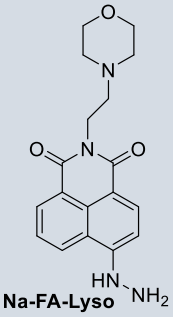
(10 μM, 0.5% DMSO as the co-solvent) was subsequently added to each and incubated for 0.5 h before imaging. For comparison, one group of cells was only treated with **CMB-1** for 0.5 h.

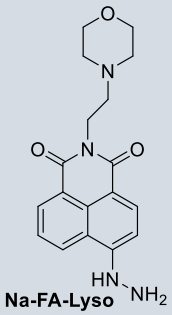
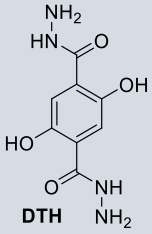
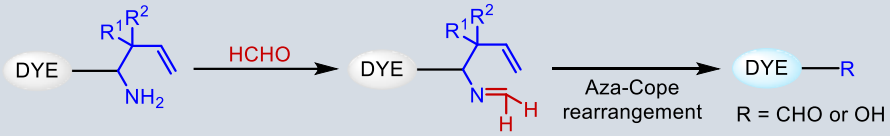
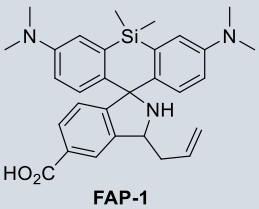
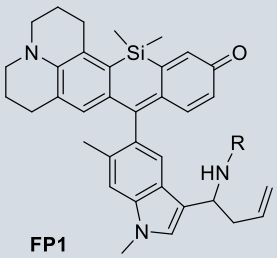
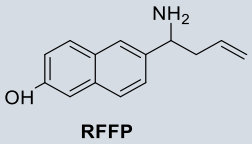
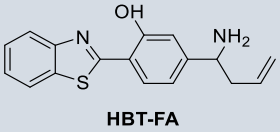
Endogenous FA Imaging: Three groups of cells were performed to image endogenous FA. The cells in Group I were incubated with a freshly prepared medium containing **CMB-1** (10 μM, 0.5% DMSO as the co-solvent) at 37 °C in a 5% CO_2/95% air incubator for 0.5 h before the image. The cells in Group II were firstly incubated with $NaHSO_3$ (200 μM) for 2 h, and then excess $NaHSO_3$ was discarded. Cells were washed with PBS thrice, to which **CMB-1** (10 μM, 0.5% DMSO as the co-solvent) was subsequently added and incubated for 0.5 h before imaging. The cells in Group III were treated with THFA (200 μM) and incubated for 2 h. After removing the excess $NaHSO_3$ and washing with PBS thrice, **CMB-1** (10 μM, 0.5% DMSO as the co-solvent) was subsequently added and incubated for 0.5 h before imaging measurements.

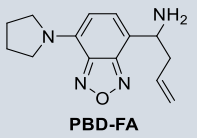
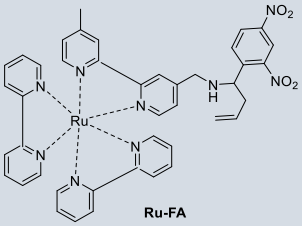
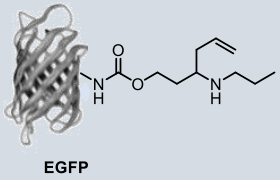
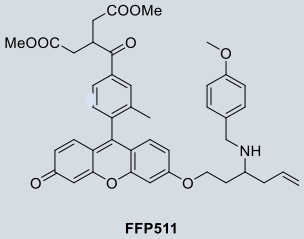
Two-photon Imaging of FA: Four groups of cells were performed. The cells in Group I were incubated with a freshly prepared medium containing **CMB-1** (10 μM, 0.5% DMSO as the co-solvent) at 37 °C in a 5% CO_2/95% air incubator for 0.5 h before the image. The cells in Group II were firstly incubated with $NaHSO_3$ (200 μM) for 2 h, and then excess $NaHSO_3$ was discarded. Cells were washed with PBS thrice, to which **CMB-1** (10 μM, 0.5% DMSO as the co-solvent) was subsequently added and incubated for 0.5 h before imaging. The cells in Group III and IV were treated with 300 μM and 600 μM FA, respectively, and incubated for 2 h. After removing the excess $NaHSO_3$ and washing with PBS thrice, **CMB-1** (10 μM, 0.5% DMSO as the co-solvent) was subsequently added and incubated for 0.5 h before imaging measurements.

6. Supplementary Data, Tables and Figures

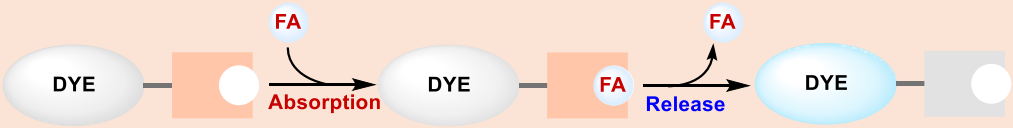
Table S1. Summary of the fluorescent probes for FA

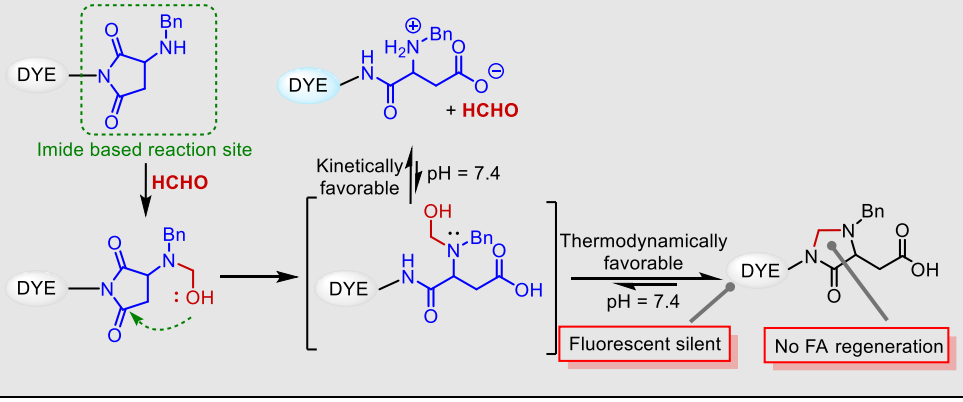
Structure	Response mode	Two-photon	Response time (min)	LOD (μM)	Solution	Ref.
Selected analyte consumed fluorescent probes for FA (many works) 						
						
 **AnB**	Turn on	N.D	N.R.	0.165	CH_3OH (PH 8.0)	*Tetrahedron Lett.*, 2012, **53,** 4913. [1]
 **BOD-NH_2**	Turn off	NO	120	0.05	HEPES buffer (pH 7.4)	*Analyst*, 2018, **143,** 429. [2]
						
 **Na-FA**	Turn on	Yes	30	0.71	PBS buffer (pH 7.4, 1% DMSO)	*Angew. Chem.*, 2016, **128,** 3417. [3]
 **Na-FA-Lyso**	Turn on	NO	30	5.02	PBS buffer (pH 7.4, 1% DMSO)	*Anal. Chem.*, 2016, **88,** 9359. [4]

 Na-FA-Lyso	Turn on	NO	6	0.02	DMF : PBS (1:1, v/v, pH=7.4)	*Sens. Actuators B Chem.*, 2018, **255,** 3292. [5]
 DTH	Ratiometric	NO	13	0.29	PBS buffer (pH = 7.4)	*Sens. Actuators B Chem.*, 2020, **320,** 128354. [6]
						
 FAP-1	Turn On	NO	>120	5	PBS buffer (pH = 7.4)	*J. Am. Chem. Soc.*, 2015, **137,** 10886. [7]
 FP1	Turn On	NO	>180	10	PBS buffer (pH = 7.4)	*J. Am. Chem. Soc.*, 2015, **137,** 10890. [8]
 RFFP	Ratiometric	NO	240	59.6	PBS buffer (1% acetone)	*Chem. Commun.*, 2016, **52,** 4029. [9]
 HBT-FA	Ratiometric	NO	420	410	MeCN/PBS (40/60, pH 7.4)	*Sens. Actuators B Chem.*, 2018, **258,** 156. [10]

 PBD-FA	Turn on	NO	180	0.0435 and 0.0497	MeCN/PBS (20/80, pH 7.4).	*J. Am. Chem. Soc.*, 2018, **140,** 854. [11]
 Ru-FA	Turn on	NO	120	0.0198	HOAc/OAC⁻ buffer (pH 5.0)	*J. Am. Chem. Soc.*, 2019, **141,** 8462. [12]
 EGFP	468/510	NO	60	25	PBS buffer (pH 7.4)	*Angew. Chem.*, 2020, **59,** 16352. [13]
 FFP511	Turn on	NO	120	1	PBS buffer, (pH 7.4)	*Chem. Sci.*, 2021, **12,** 13857. [14]

Analyte regeneration fluorescent probes for FA (only two works reported)





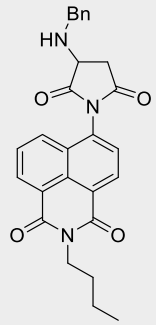
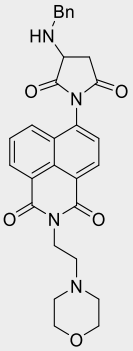
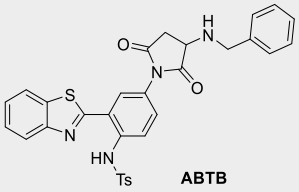
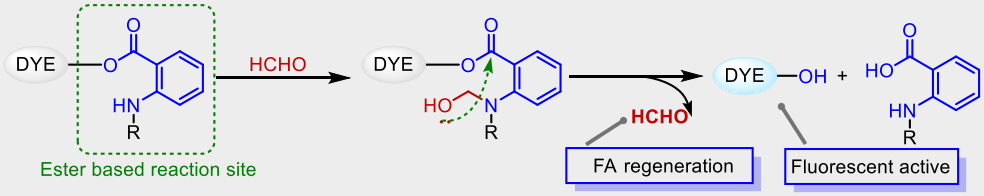
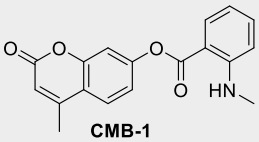
 NAP-FAP-1	Turn on	NO	120	0.48	PBS buffer (pH 5.0)	*J. Am. Chem. Soc.*, 2018, **140,** 16408. [15]
 NAP-FAP-2	Turn on	NO	240	0.145	PBS buffer (pH 5.0)	*J. Am. Chem. Soc.*, 2018, **140,** 16408. [15]
 ABTB	Ratiometric	NO	N.D.	0.432	PBS	*Chem. Commun.*, 2021, **57,** 3496. [16]
						
 CMB-1	Turn On	Yes	60	1	DMSO/PBS (30/70, pH 7.4)	*This work*

Table S2. Photophysical properties of **CMB-1 – CMB-3**.

	λ_{abs} (nm) [a]	λ_{em} (nm) [a]	ε (cm^{-1} M^{-1})	Φ[a]
CMB-1	368	445	7,600	1%
CMB-2	368	445	9,600	1%
CMB-3	375	445	12,500	2%
2	366	445	13,800	70%

[a] Measured in PBS buffer solution (10 mM, pH = 7.4, containing 30% DMSO). Ex = 368 nm.

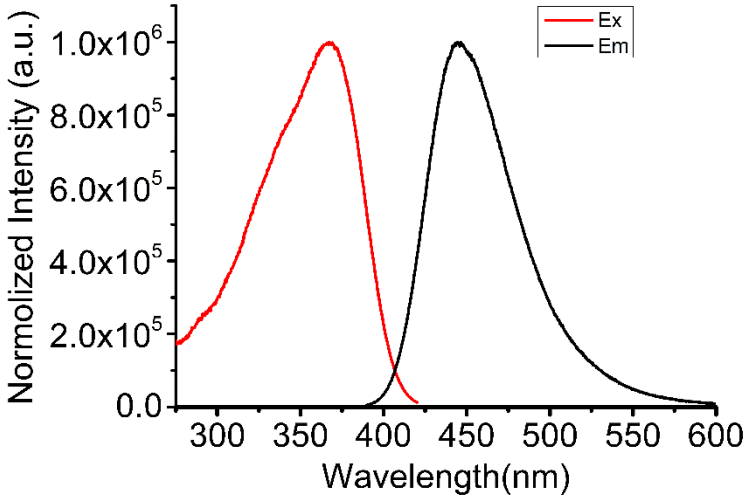


Figure S1. Excitation (red line) and emission (black line) spectra of probe **CMB-1** (10 μM) in PBS buffer solution (10 mM, pH = 7.4, containing 30% DMSO). Ex = 368 nm. Slit: 1.0 nm/1.0 nm.

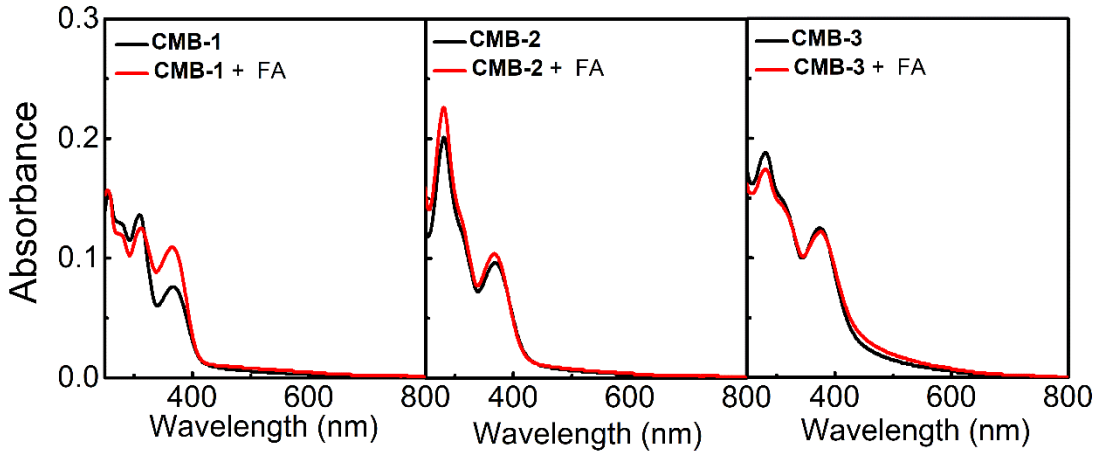


Figure S2. UV-vis absorption spectra of **CMB-1**(10 μM), **CMB-2** (10 μM), and **CMB-3** (10 μM) in the absence and presence of FA (1.75 mM). All spectra were acquired 2 h after FA addition at 37 ℃ in PBS buffer solution (10 mM, pH = 7.4, containing 30% DMSO).

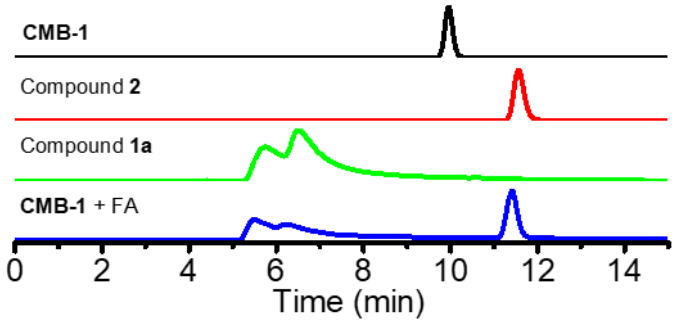


Figure S3. HPLC analysis of the sensing reaction.

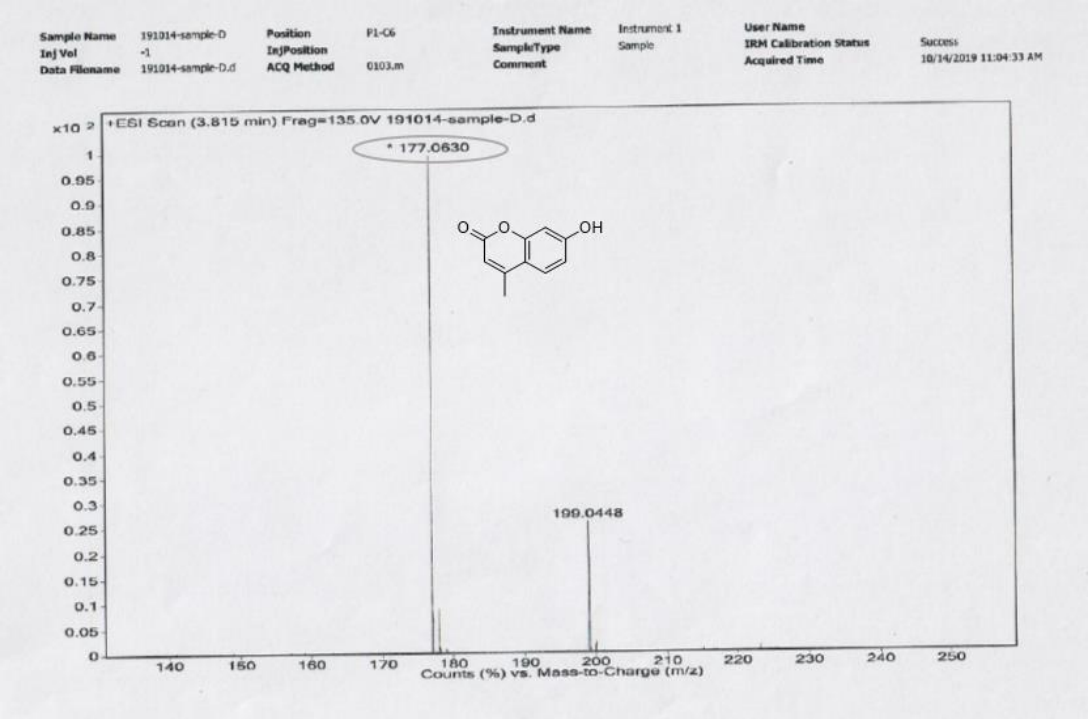


Figure S4. ESI spectra of the reaction mixture from the mechanism reaction.

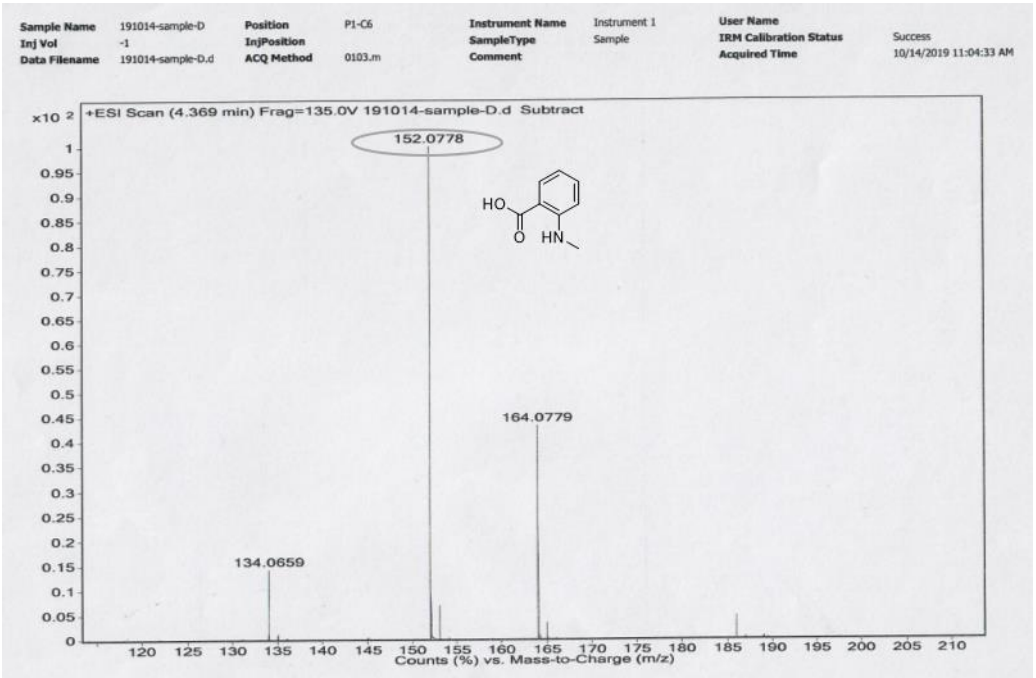


Figure S5. ESI spectra of the reaction mixture from the sensing reaction.

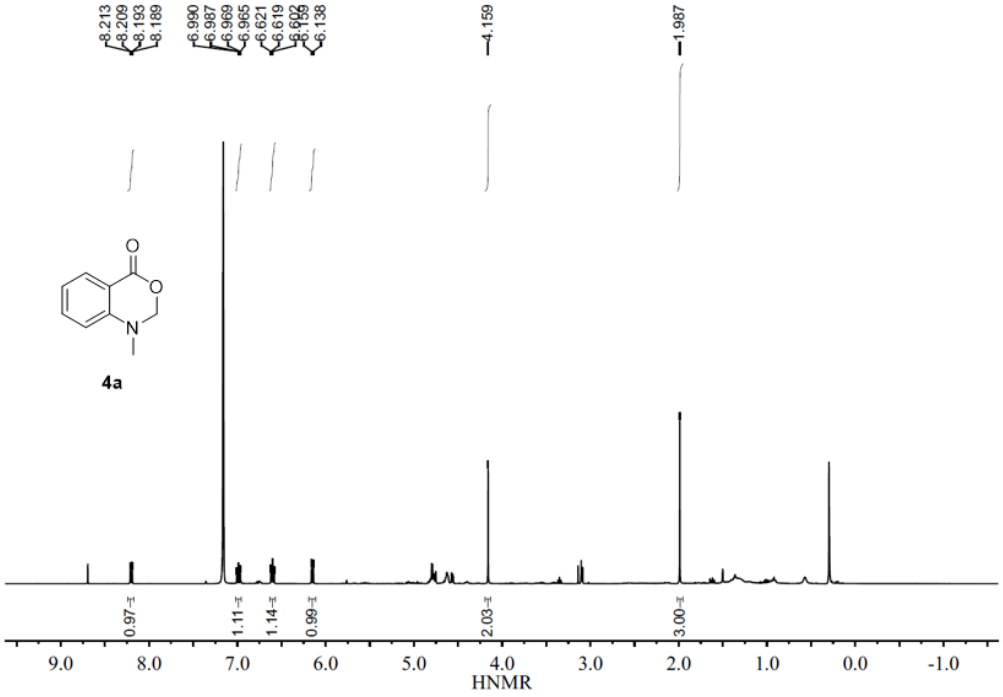


Figure S6. ^{1}H NMR spectra of the reaction mixture in C_6D_6 from the sensing reaction. Selected peaks are assigned to compound **4a**.

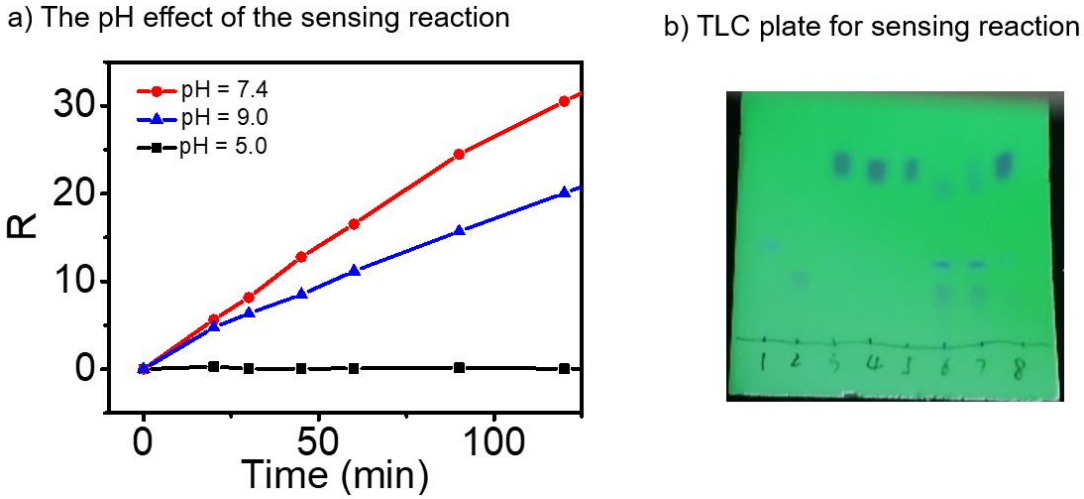


Figure S7. The sensing mechanism studies. (a) Time-dependent relationship between the fluorescent intensity ratio (the changes of the emission intensity at each time point between the **CMB-1**-FA and free **CMB-1** versus emission intensity at time point 0 min) at 445 nm of **CMB-1** at pH 5.0, 7.4, and 9.0 respectively in and absence and in presence of FA (1.75 mM). Ex = 368 nm. Slit: 1.0 nm/1.0 nm. (b) Photo of TLC plate for the sensing reaction under the UV lamp (254 nm). From spot 1 to 8: compound **2** (spot 1), compound 1a (spot 2), **CMB-1** without (spot 3) or with FA (20 eq.) (spot 6) at pH 9.0; **CMB-1** without (spot 4) or with FA (20 eq.) (spot 7) at pH 7.4; **CMB-1** without (spot 5) or with FA (20 eq.) (spot 8) at pH 5.0.

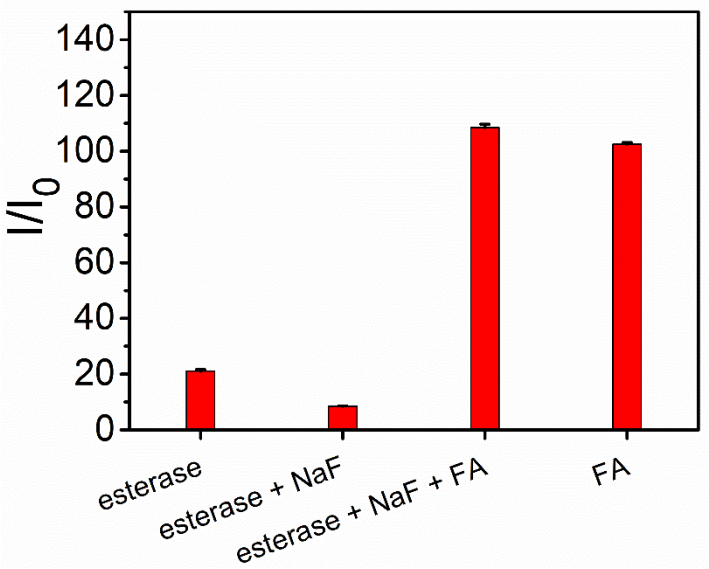


Figure S8. The emission response of **CMB-1** towards the porcine liver esterases. From left to right: **CMB-1** (10 μM) with the addition of esterase (20 U/L); **CMB-1** (10 μM) with the addition of esterase (20 U/L) pretreated with NaF (1 mM); **CMB-1** (10 μM) with the addition of esterase (20 U/L) pretreated with NaF (1 mM) and FA (1000 eq.); **CMB-1** (10 μM) with the addition of FA (1000 eq.). Data were acquired at 37 °C in PBS buffer solution (10 mM, pH = 7.4, containing 30% DMSO) after incubation of 2 h. Ex = 368 nm. Slit: 1.0 nm/1.0 nm.

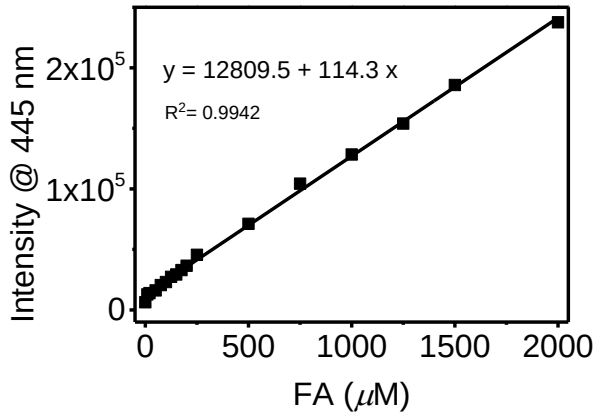


Figure S9. Linear relationship between the fluorescent intensity at 445 nm of **CMB-1** and FA concentration (0 – 2 mM). Data were acquired at 37 °C in PBS buffer solution (10 mM, pH = 7.4, containing 30% DMSO) after incubation of 2 h. Ex = 368 nm. Slit: 1.0 nm/1.0 nm.

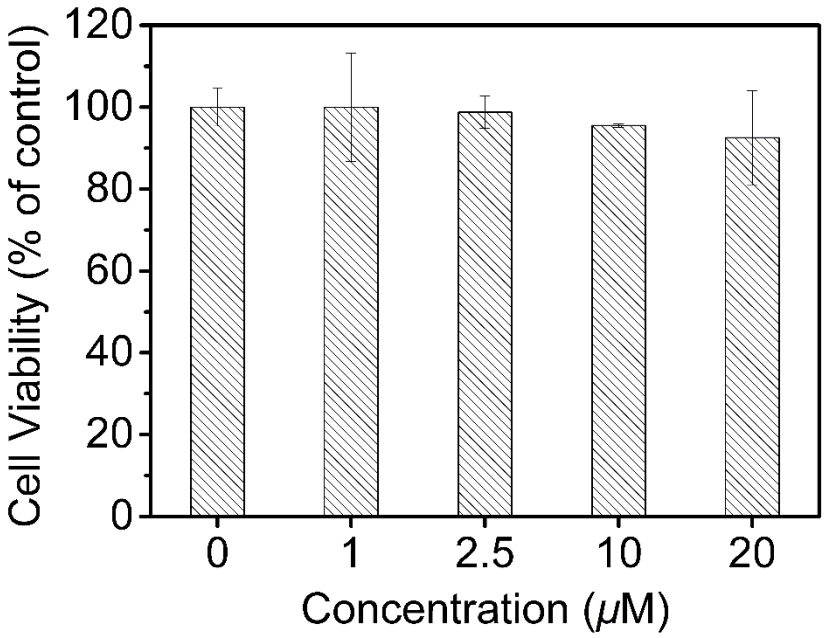


Figure S10. Cell viabilities of **CMB-1** at various concentrations for HepG2 cells after 24 h incubation. Error bars = SD (n = 5).

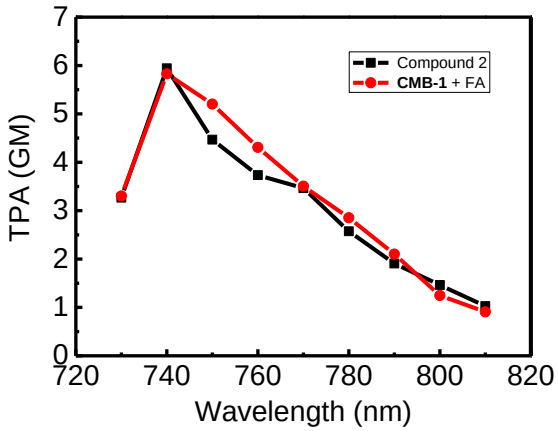


Figure S11. Two-photon action spectra of compound **2** and the probe **CMB-1** after reaction with FA in PBS buffer (10 mM, containing 30% DMSO, pH 7.4).

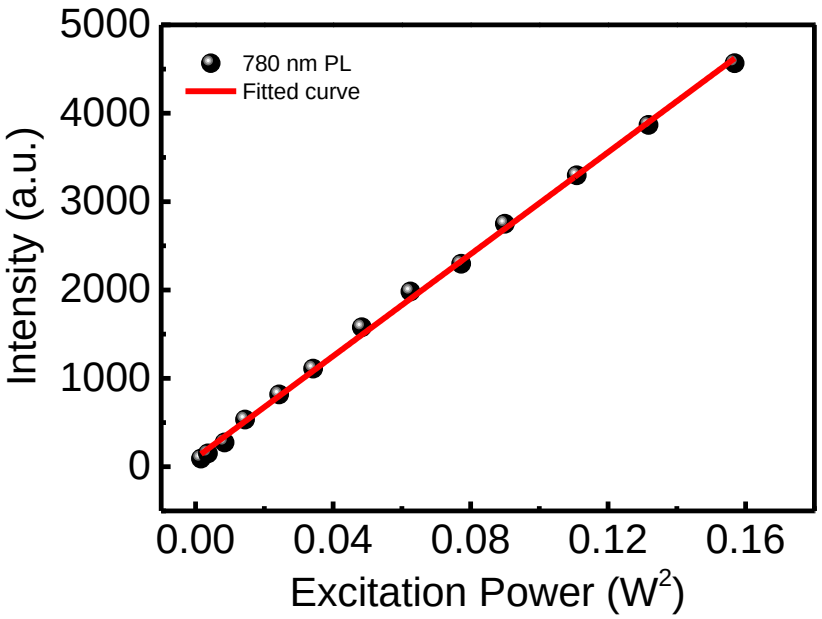


Figure S12. The intensity changes of the system of probe **CMB-1** with FA in PBS buffer (10 mM, containing 30% DMSO, pH 7.4) versus the excitation power. Ex = 780 nm, Em = 445 nm.

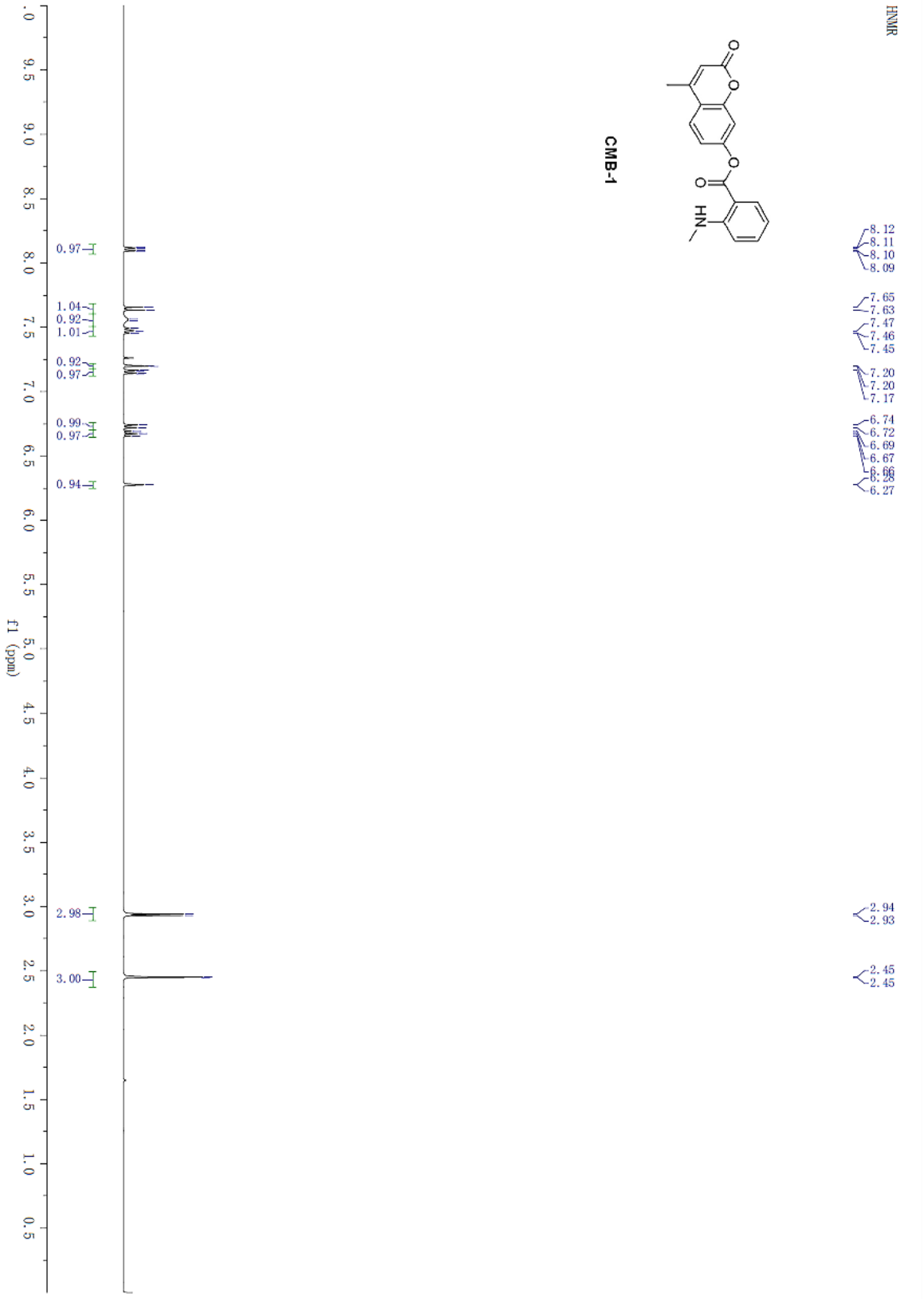


Figure S13. ^{1}H NMR spectra of **CMB-1**.

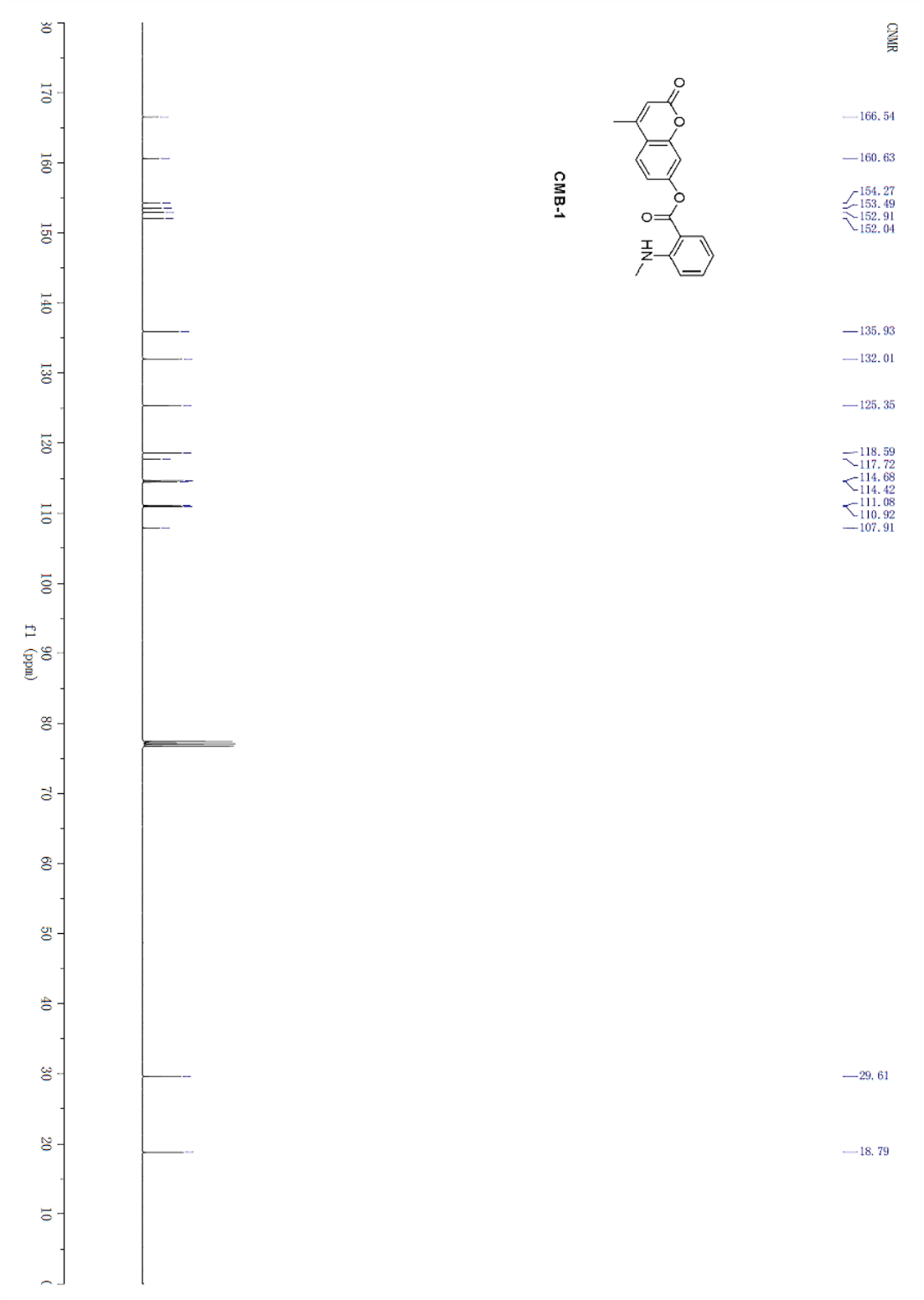


Figure S14. ^{13}C NMR spectra of **CMB-1**.

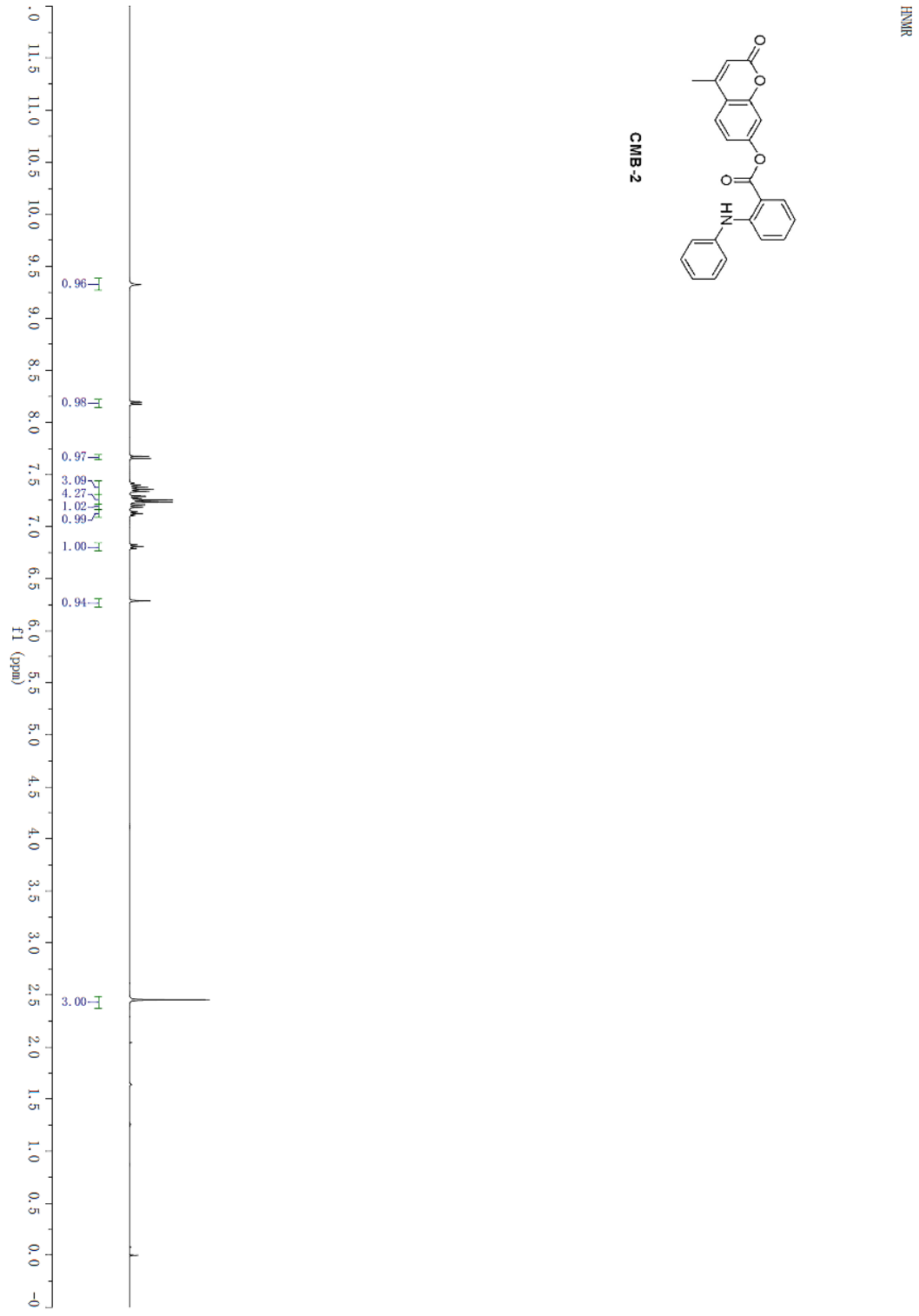


Figure S15. ^{1}H NMR spectra of **CMB-2**.

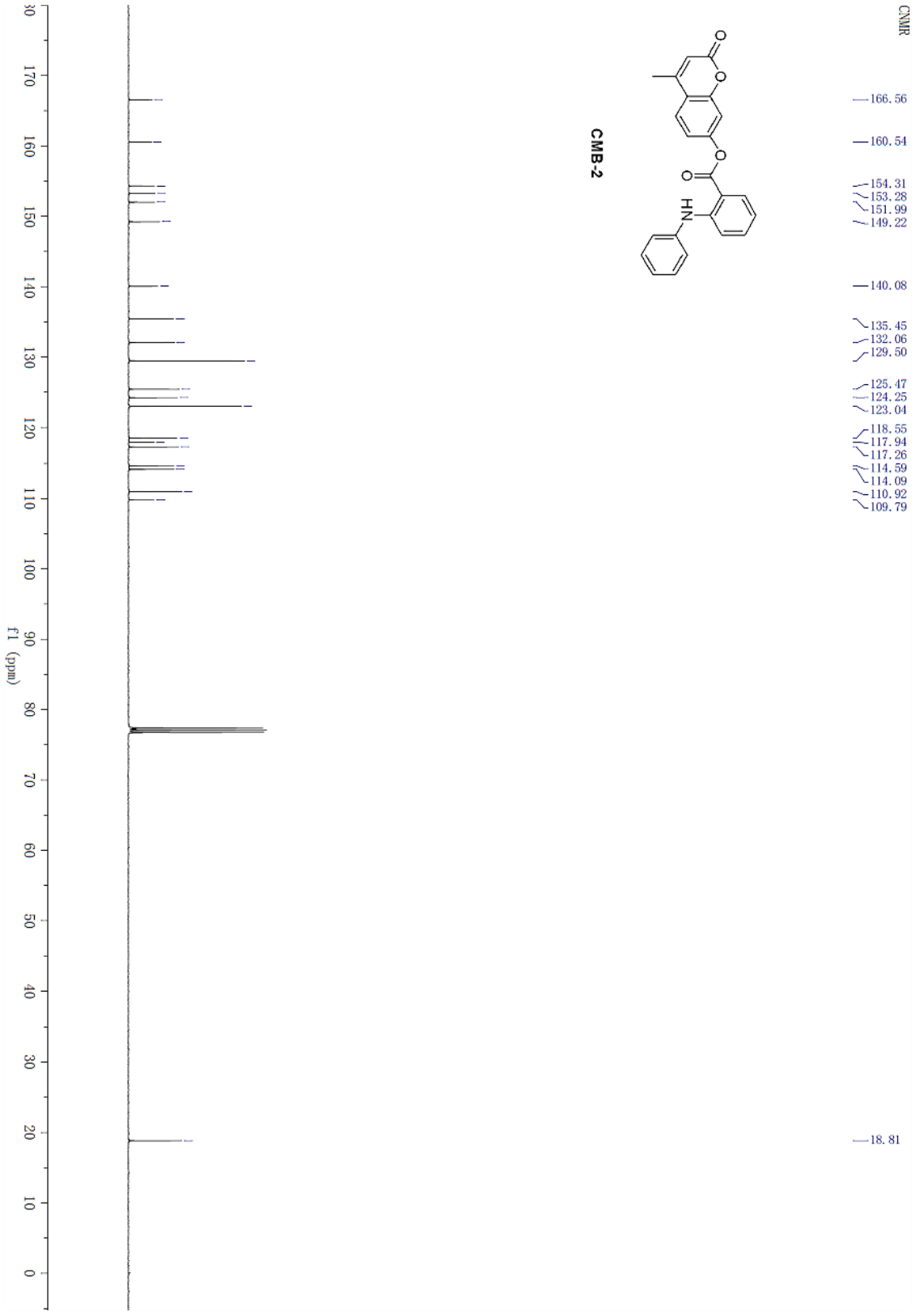


Figure S16. ^{13}C NMR spectra of **CMB-2**.

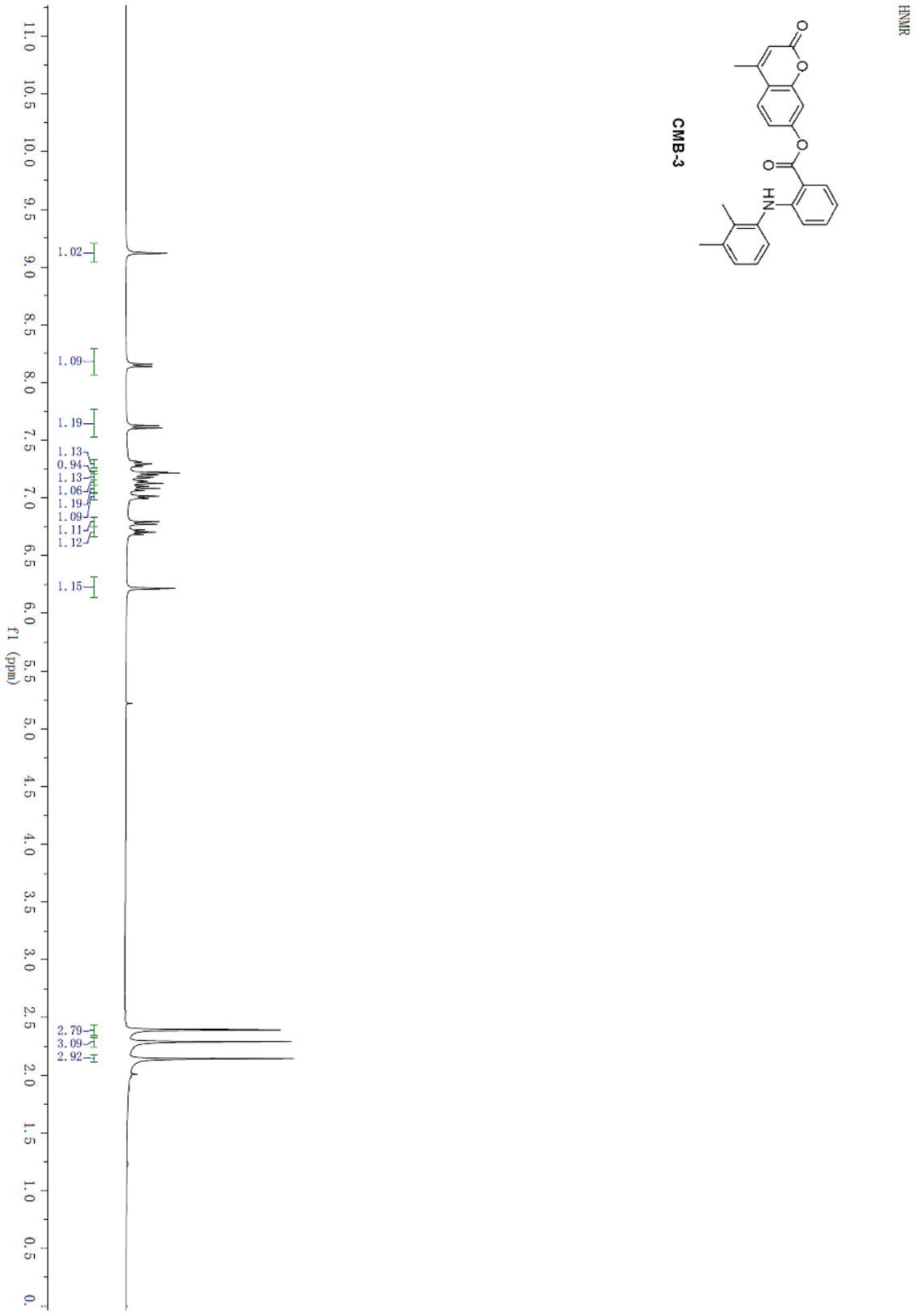


Figure S17. ^{1}H NMR spectra of **CMB-3**.

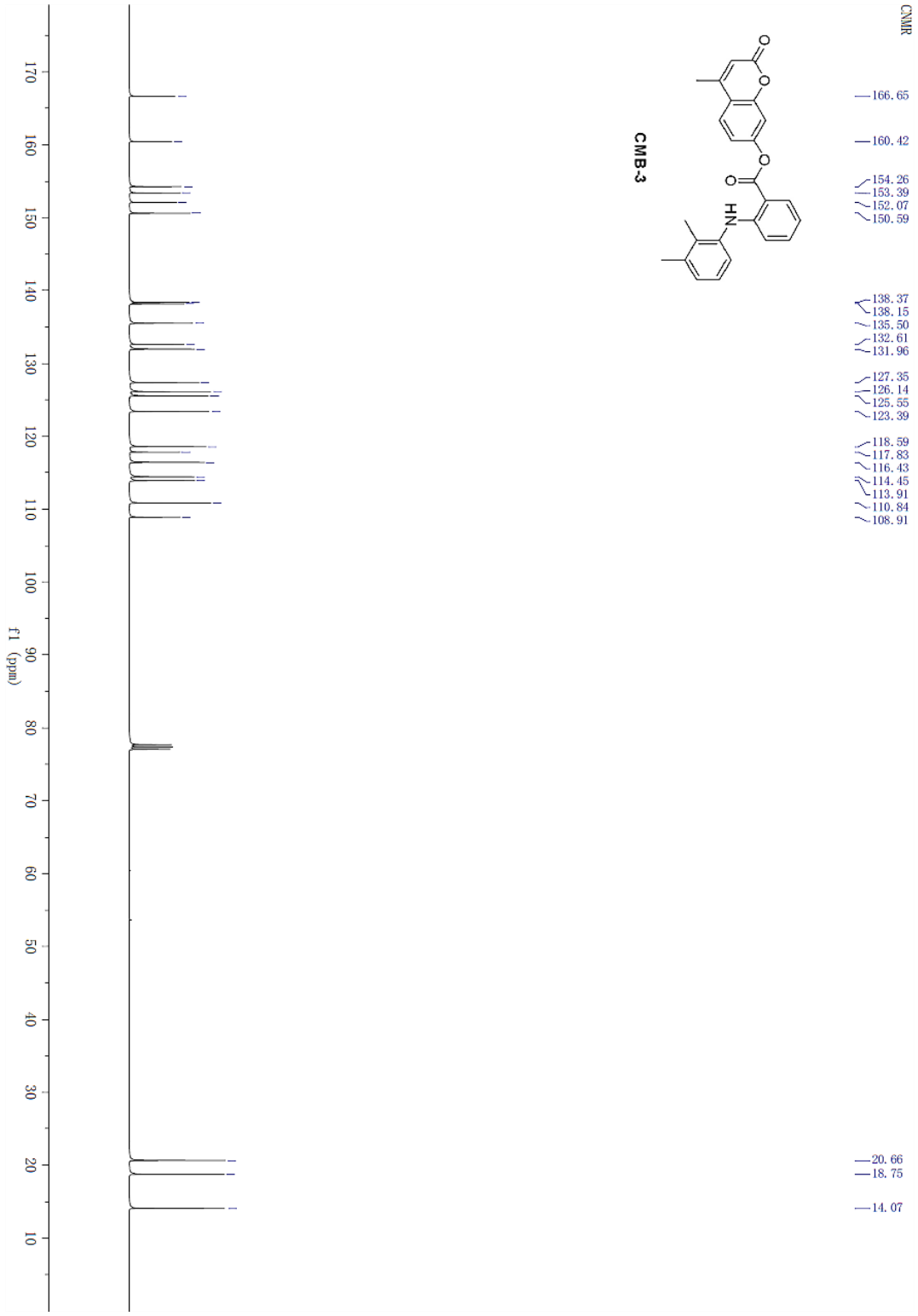


Figure S18. ^{13}C NMR spectra of **CMB-3**.

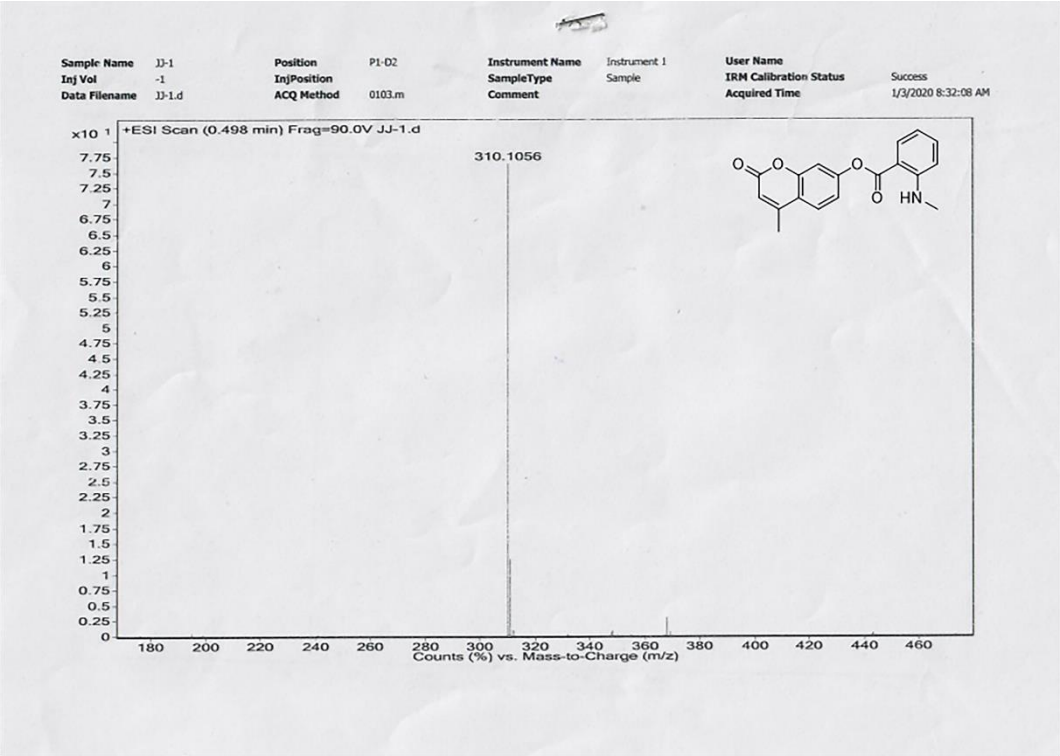


Figure S19. ESI spectra of **CMB-1**.

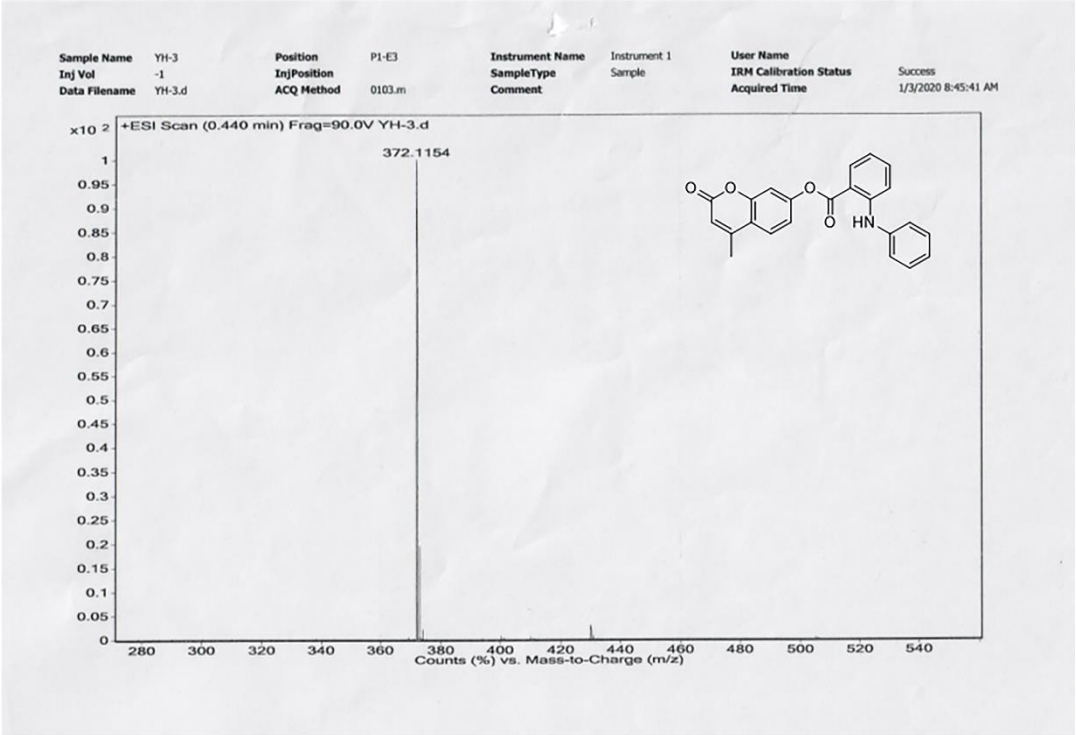


Figure S20. ESI spectra of **CMB-2**.

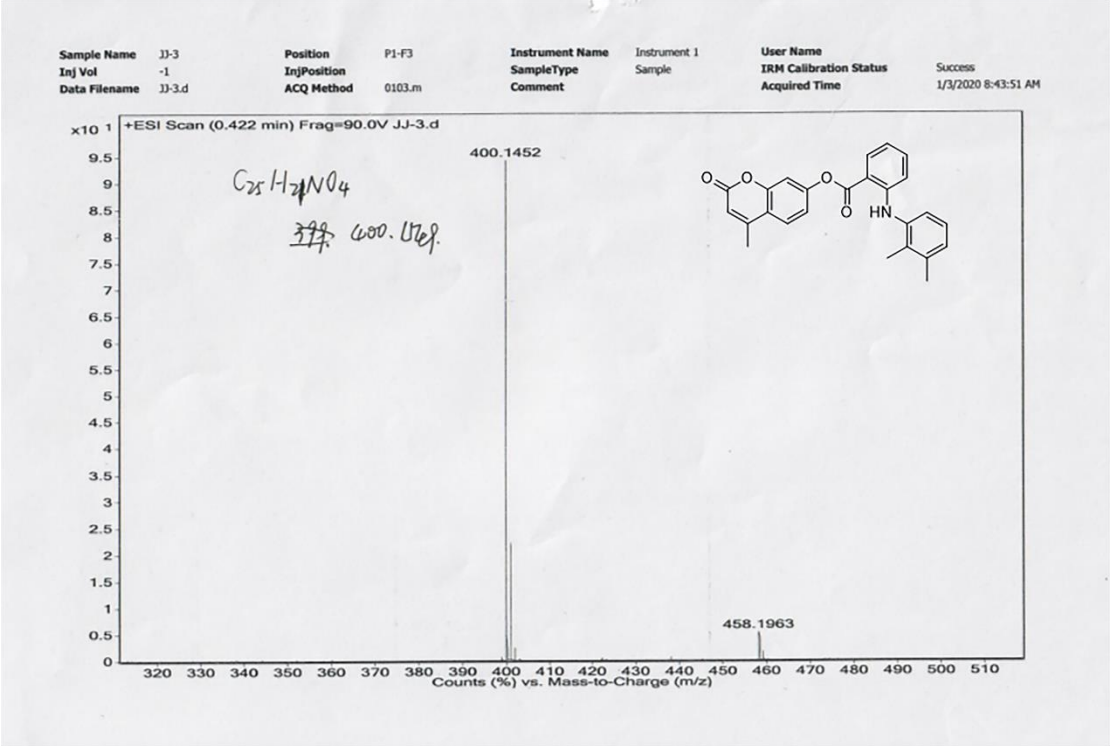


Figure S21. ESI spectra of **CMB-3**.

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