Supporting information

Fluorescence turn-on detection of hypochlorous acid *via* HOCl-promoted dihydrofluorescein-ester oxidation and its application *in vivo*

Yi Zhou,[a] Ju-Ying Li,[a] Kai-Hui Chu,[a] Ke Liu,[a] Cheng Yao *[a], Jing-Yun Li[b]

(a) *State Key Laboratory of Materials-Oriented Chemical Engineering and College of Science, Nanjing University of Technology, Nanjing 210009, P. R. China*

(b) *Animal Model Research Center, Nanjing University, Nanjing 210061, P. R. China*

Email: yaochengnjut@126.com Tel.: +86 25 83587433; Fax: +86 25 83587433;

Contents

Reagents and Apparatus

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents were purified and dried using standard procedures. Electrospray ionization mass spectra (*ESI-MS*) were measured on a Micromass LCTTM system. ^{1}H-NMR and ^{13}C-NMR were measured on a BrukerAV-500 or BrukerAV-300 spectrometer with chemical shifts reported in ppm (in $CDCl_3$ or DMSO-d_6; TMS as internal standard). UV-visible spectrums were recorded on a Perkin-Elmer 35 spectrometer. Fluorescence measurements were performed at room temperature on a Perkin-Elmer LS 50B fluorescence spectrophotometer. TLC analysis was performed on silica gel plates. Column chromatography was conducted over silica gel (mesh 200–300), and both were obtained from the Qingdao Ocean Chemicals.

Preparation of ROS and RNS

FCN1 ~ FCN3

A stock solution of **FCN1** ~ **FCN3** (1 mM) was prepared in DMSO and was stored at -20 °C for spectrum and *vivo* investigation.

HOCl

HOCl was prepared from the source of NaOCl at room temperature in HEPES buffer (pH 7.2). The concentration of HOCl was determined by titration with $Na_2S_2O_3$.

NO_2^-

NO_2^- was prepared from the source of $NaNO_2$ at room temperature in HEPES buffer (pH 7.2).

$ONOO^-$

The synthesis of peroxynitrite involved nitrosation of H_2O_2 at pH ≥12.0 by isoamyl nitrite. The peroxynitrite concentration was determined by using an extinction coefficient of 1670 ± 50 $cm^{-1}(mol/L)^{-1}$ at 302 nm.[1]

OH•

Hydroxyl radicals was generated by the addition of Fe^{2+} (100 mM) and H_2O_2 (100 mM) at room temperature in HEPES buffer (pH 7.2) and the mixture was then stirred for 30 min.

$O_2•^-$

Superoxide was prepared from the source of KO_2 at room temperature in HEPES buffer (pH 7.2).

NO

Nitric oxide was prepared from a saturated NO aqueous solution (2 mM) at room temperature.

HNO

Nitroxyl donor (HNO) was generated from sodium trioxodinitrate ($Na_2N_2O_3$, Angeli's salt). Angeli's salt was prepared as described by King and Nagasawa and was stored at -20 °C until needed.[2]

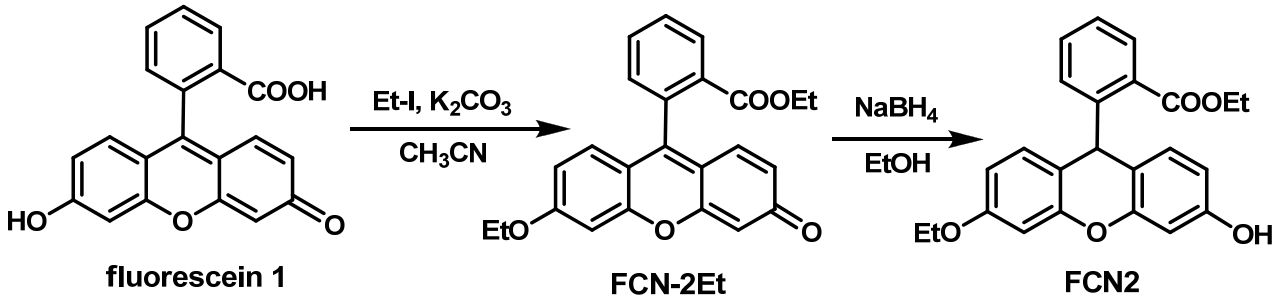
ROO•

ROO• was generated from 2,2'-azobis(2-amidinopropane)dihydrochloride (CAS: 2997-92-4), which was dissolved in deionized water first and then added into probe testing solutions at room temperature in HEPES buffer (pH 7.2) for 30 min.

Synthetic details

Our initial attempt to convert fluorescein as the starting material into dihydrofluorescein by sodium borohydride failed, presumably its carboxyl group partially forms spirocyclic ring to resist the reduction (Fig. S1, ESI†). However, deprotonation of fluorescein afforded fluorescein-ester, which was further installed by the $NaBH_4$-mediated reduction to give **FCN1** and **FCN2** successfully.

2-(3′-Hydroxy-6′-ethoxy-9′*H*-xanthen-9′-yl)-benzoic acid ethyl ester (FCN2)

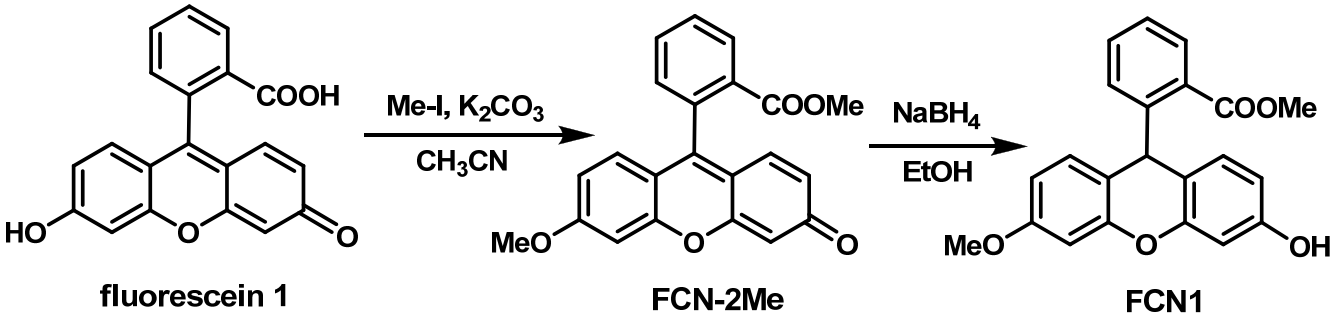


2-(6′-ethoxy-3′-oxo-3′*H*-xantene-9′-yl)-benzoic acid ethyl ester (FCN-2Et): EtI (4.68 g, 30.0 mmol) was added to the mixture of fluorescein (3.32 g, 10.0 mmol) and K_2CO_3 (4.14 g, 30.0 mmol) in 50 ml of CH_3CN. After heating at 60 °C for 24 h, the reaction mixture was concentrated under reduced pressure and diluted with saturated $NaHCO_3$ (40 ml) solution. The resulting mixture was extracted three times with CH_2Cl_2 (40 ml). The combined organic layer was dried over anhydrous Na_2SO_4, and concentrated under vacuum. Compound **FCN-2Et** was isolated using a silica gel chromatographic column eluted with Dichloromethane/MeOH (v/v, 95:5), resulting a light yellow solid (R_f = 0.38, 2.91 g, yield: 75%).^{1}H-NMR (500 MHz, DMSO-d_6): δ = 8.20 (dd, J= 7.9 Hz, J= 1.1 Hz, 1H, Ar-H), 7.86 (t,

J= 7.6 Hz, 1H, Ar-H), 7.79 (t, J= 7.8 Hz, 1H, Ar-H), 7.50 (dd, J= 7.6 Hz, J= 1.0 Hz, 1H, Ar-H), 7.21 (d, J= 2.4 Hz, 1H, Ar-H), 6.89 (dd, J= 8.9 Hz, J= 2.4 Hz, 1H, Ar-H), 6.85 (d, J= 8.9 Hz, 1H, Ar-H), 6.81 (d, J= 9.7 Hz, 1H, Ar-H), 6.39 (dd, J= 9.7 Hz, J= 2.0 Hz, 1H, Ar-H), 6.24 (d, J= 2.0 Hz, 1H, Ar-H), 4.18 (q, J= 7.0 Hz, 2H, -CH_2-), 3.96 (m, 2H, -CH_2-), 1.36 (t, J= 7.0 Hz, 3H, -CH_3), 0.86 (t, J= 7.1 Hz, 3H, -CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$): 183.76, 164.90, 163.12, 158.27, 153.53, 149.83, 133.42, 132.93, 130.62, 130.50, 130.29, 129.99, 129.91, 129.30, 128.86, 116.67, 114.21, 113.76, 104.52, 100.81, 64.38, 60.81, 14.28, 13.27. Anal. Calcd for $C_{24}H_{20}O_5$: C, 74.21; H, 5.19. Found: C, 74.01; H, 5.06. *ESI*-MS: m/z 389.1 $[M+H]^+$.

FCN2: To a solution of **FCN-2Et** (776 mg, 2.0 mmol) in MeOH (30 mL), $NaBH_4$ (152 mg, 4.0 mmol)was added at 0 °C. The resulting mixture was stirred at 0 °C for 10 min, and slowly brought to room temperature for another 2 hour. After removal of the solvent, the residue was diluted with water. The mixture was then extracted three times with CH_2Cl_2 (40 ml), dried over Na_2SO_4, and concentrated in vacuo to yield the crude product. Compound **FCN-2** was purified using a silica gel chromatographic column eluted with Dichloromethane/petroleum ether (v/v, 7:3), resulting a light yellow solid (R_f = 0.52, 710 mg, yield: 91%). ^{1}H-NMR (500 MHz, DMSO-d_6): δ = 7.69 (t, J= 7.6 Hz, 1H, Ar-H), 7.39 (t, J= 6.6 Hz, 1H, Ar-H), 7.24 (t, J= 7.6 Hz, 1H, Ar-H), 6.97 (t, J= 7.6 Hz, 1H, Ar-H), 6.89 (dd, J= 8.6 Hz, J= 2.4 Hz, 1H, Ar-H), 6.81 (dd, J= 8.5 Hz, J= 2.6 Hz, 1H, Ar-H), 6.68 (d, J= 2.4 Hz, 1H, Ar-H), 6.56 (dd, J= 8.6 Hz, J= 2.3 Hz, 1H, Ar-H), 6.53 (d, J= 2.3 Hz, 1H, Ar-H), 6.44 (dd, J= 8.4 Hz, J= 2.1 Hz, 1H, Ar-H), 6.00 (s, 1H, Ar_3-CH), 4.38 (q, J= 7.1 Hz, 2H, -CH_2-), 4.00 (q, J= 7.0 Hz, 2H, -CH_2-), 1.34 (t, J= 7.1 Hz, 3H, -CH_3), 1.30 (t, J= 7.0 Hz, 3H, -CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$): 168.23, 167.81, 158.18, 157.10, 150.88, 147.87, 132.32, 132.19, 130.91, 130.19, 129.43, 129.07, 126.13, 116.35, 114.77, 111.45, 110.69, 102.41, 101.44, 63.22, 61.16, 37.04, 14.55, 14.06. Anal. Calcd for $C_{24}H_{22}O_5$: C, 73.83; H, 5.68. Found: C, 73.69; H, 5.61. *ESI*-MS: (positive ion mode) m/z 413.1 $[M+Na]^+$; (negative ion mode) m/z 389.1 $[M-H]^-$.

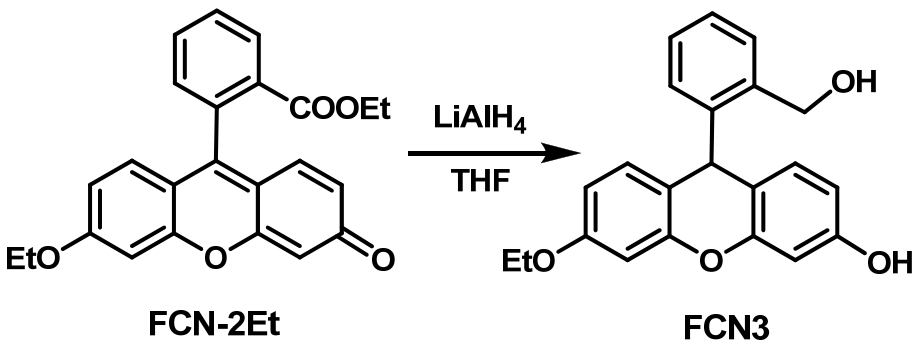
2-(3′-Hydroxy-6′-methoxy-9′*H*-xanthen-9′-yl)-benzoic acid methyl ester (FCN1)



2-(6′-Methoxy-3′-oxo-3′*H*-xantene-9′-yl)-benzoic acid methyl ester (FCN-2Me): This compound was synthesized from fluorescein **1** using a synthetic procedure for **FCN-2Et** (82%, yellow solid). ^{1}H-NMR (300 MHz, DMSO-d_6): 8.18 (dd, J= 7.8 Hz, J= 1.1 Hz, 1H, Ar-H), 7.73~7.81 (m, 2H, Ar-H), 7.49 (dd, J= 7.9 Hz, J= 1.2 Hz, 1H, Ar-H), 7.20 (d, J= 2.5 Hz, 1H, Ar-H), 6.80-6.89 (m, 3H, Ar-H), 6.39 (dd, J= 9.5 Hz, J= 2.2 Hz, 1H, Ar-H), 6.21 (d, J= 2.3 Hz, 1H, Ar-H), 3.87 (s, 3H, -CH_3), 3.79 (s, 3H, -CH_3). *ESI*-MS: m/z 361.1 $[M+H]^+$.

FCN1: FCN1 was synthesized from **FCN-2Me** using a synthetic procedure for **FCN2** (88%, yellow solid). ^{1}H-NMR (500 MHz, $CDCl_3$): 7.76 (d, J= 7.6 Hz, 1H, Ar-H), 7.31 (td, J= 7.5 Hz, J= 1.4 Hz, 1H, Ar-H), 7.21 (t, J= 7.6 Hz, 1H, Ar-H), 7.06 (dd, J= 7.5 Hz, J= 1.5 Hz, 1H, Ar-H), 6.90 (d, J= 8.3 Hz, 1H, Ar-H), 6.85 (d, J= 8.3 Hz, 1H, Ar-H), 6.60 (d, J= 2.5 Hz, 1H, Ar-H), 6.57 (d, J= 2.5 Hz, 1H, Ar-H), 6.48 (dd, J= 8.5 Hz, J= 2.4 Hz, 1H, Ar-H), 6.40 (dd, J= 8.5 Hz, J= 2.4 Hz, 1H, Ar-H), 6.10 (s, 1H, Ar_3-CH), 3.91 (s, 3H, -CH_3), 3.74 (s, 3H, -CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$): 169.23, 159.21, 155.46, 151.57, 151.41, 148.36, 132.57, 131.90, 130.95, 130.18, 129.62, 129.41, 126.28, 116.97, 116.90, 111.15, 110.21, 103.16, 101.17, 55.51, 52.47, 37.86. Anal. Calcd for $C_{22}H_{18}O_5$: C, 72.92; H, 5.01. Found: C, 72.70; H, 4.96. *ESI*-MS: (positive ion mode) m/z 385.1 $[M+Na]^+$; (negative ion mode) m/z 361.1 $[M-H]^-$.

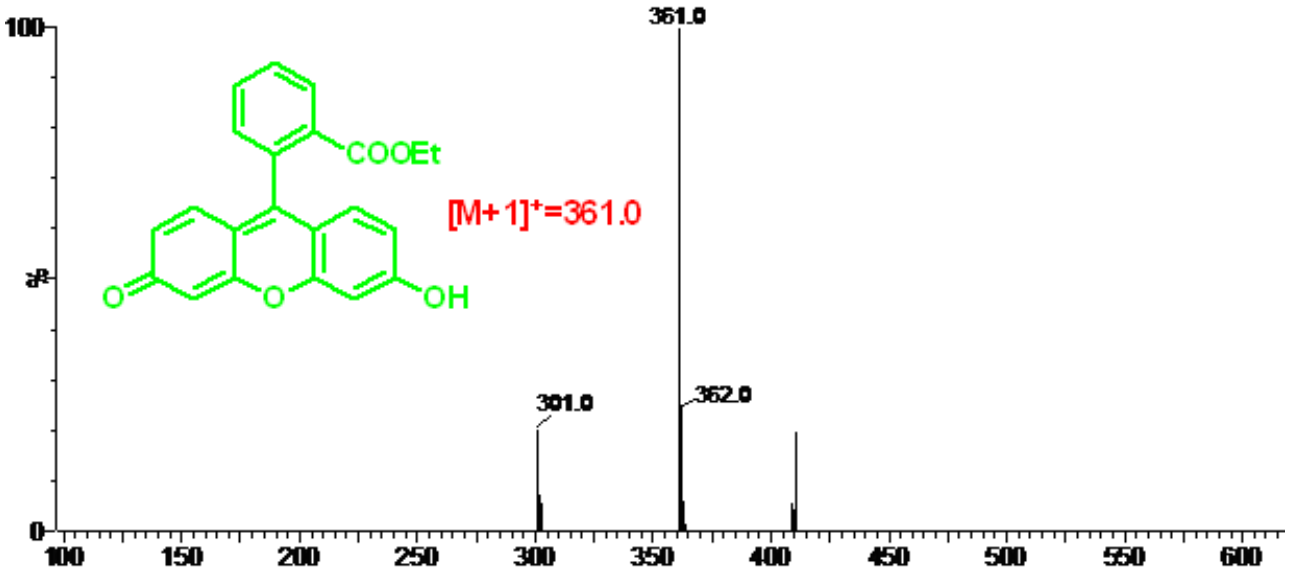
2-(3′-Hydroxy-6′-ethoxy-9′*H*-xanthen-9′-yl)-phenylethyl alcohol (FCN3)



FCN3: To a solution of **FCN-2Et** (388 mg, 1.0 mmol) in anhydrous THF (20 ml, was prepared by refluxing the commercial THF over $LiAlH_4$ under an argon atmosphere for 24 hours followed by distillation), $LiAlH_4$ was added slowly in small portions at 0 °C until the starting materials were consumed (over 30 min). The suspended mixture was stirred at 0 °C with another 2 h and neutralized with 5 M HCl (40 ml) solution. After evaporation of the solvent, the residue was extracted three times with CH_2Cl_2 (20 ml). The combined organic layer was dried over anhydrous Na_2SO_4, and concentrated under vacuum. Compound **FCN-3** was purified using a silica gel chromatographic column eluted with Dichloromethane/petroleum ether (v/v, 7:3), resulting a yellow viscous solid (R_f = 0.31, 219 mg, yield: 63%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.37 (t, J= 4.9 Hz, 1H, Ar-H), 7.14~7.24 (m, 3H, Ar-H), 6.73 (d, J= 8.6 Hz, 1H, Ar-H), 6.67 (d, J= 8.4 Hz, 1H, Ar-H), 6.59 (d, J= 2.6 Hz, 1H, Ar-H), 6.51 (d, J= 2.4 Hz, 1H, Ar-H), 6.45 (dd, J= 8.6 Hz, J= 2.6 Hz,1H, Ar-H), 6.32 (dd, J= 8.4 Hz, J= 2.6 Hz,1H, Ar-H), 5.41 (s, 1H, Ar_3-CH), 4.59 (s, 2H, -CH_2OH), 3.97 (q, J= 7.0 Hz, 2H, -CH_2-), 1.37 (t, J= 7.0 Hz, 3H, -CH_3). ^{1}H-NMR (75 MHz, $CDCl_3$): 158.56, 155.89, 151.13, 151.08, 144.37, 137.81, 131.26, 130.21, 130.01, 129.26, 128.34, 127.03, 116.23, 115.96, 111.01, 110.59, 103.05, 101.67, 67.93, 63.60, 39.69, 14.72. Anal. Calcd for $C_{22}H_{20}O_4$: C, 75.84; H, 5.79. Found: C, 75.59; H, 5.68. *ESI*-MS: (positive ion mode) m/z 471.1 $[M+Na]^+$; (negative ion mode) m/z 347.1 $[M-H]^-$.

Detection of HOCl-promoted FCN2 oxidation (HPLC-MS positive ion mode)

The probe **FCN2** (39.1 mg, 0.1 mmol) was dissolved in 50% CH_3CN/H_2O (10 mL) and then diluted to 10 μM with HEPES buffer at pH 7.2. Then 20 equiv of HClO was injected into the probe. The reaction mixture was stirred at room temperature for 40 min and conversion was checked by analytical HPLC. (4.6 mm x 150 mm 5 μm C18 column; 5 μL injection; 50% CH_3CN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); *ESI*-MS: m/z 361.0 $[M+H]^+$.



Supplementary spectra data and tables

Fluorescence Analysis: Fluorescence emission spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. The probe **FCN2** (DMSO, 1.0 mM) was diluted to 1.5 μM in 20 mM HEPES buffer, and was further added to a 10.0-mL color comparison tube for test. The mixture was equilibrated for 30 min before measurement. The fluorescence intensity was measured simultaneously at $\lambda_{ex}/\lambda_{em}$ = 415/485 nm, respectively. Φ_F was determined with rhodamine B as a standard (Φ_F = 0.69 in ethanol).[3]

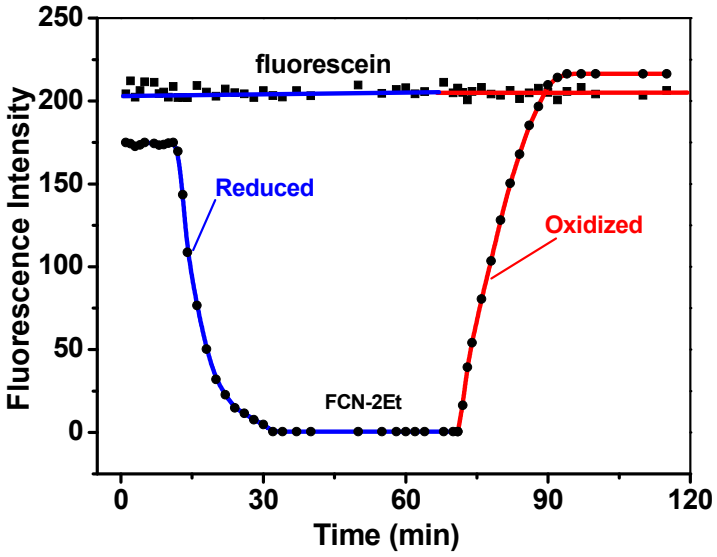


Figure S1: Time course of fluorescein (5.0 μM) and FCN-2Et (5.0 μM) was measured by a spectrofluorometer in EtOH. FCN-2Et was reduced by 1.5 equiv $NaBH_4$ for 70 min (blue line), then further treated with 5 equiv of HClO for another 50 min (red line).

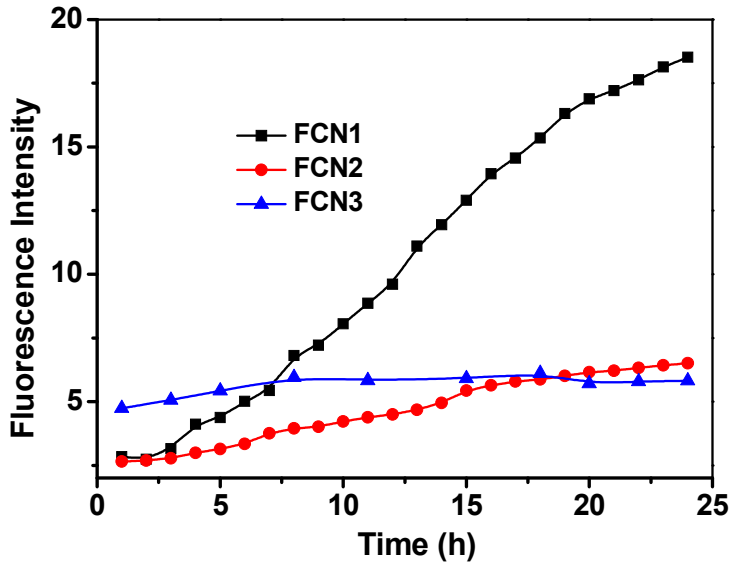


Figure S2: The fluorescence intensity of **FCN1**~**FCN2** showed 6.5-fold and 2.4-fold enhancement upon the exposure to 20 mM HEPES buffer (pH 7.2) for 24 h in air, indicating its auto-oxidation characteristic. However, **FCN3** did not show any auto-oxidation for more than 48 h.

Table S1. Fluorescence intensity (**F.I.**) of **FCN2** to biologically relevant ROS and RNS in 20 mM HEPES buffer.

FCN2	HOCl	H_2O_2	NO_2^-	$ONOO^-$	OH•	$O_2•^-$	NO	HNO	ROO•
F.I.	944.95	0.65	0.47	26.53	6.45	0.56	0.59	2.78	0.94

Detection limit:

The detection limit was calculated based on the fluorescence titration. To determine the S/N ratio, the emission intensity of probe **FCN2** in the absence of HClO was measured. The value of **[DL]** was estimated on the basis of the signal-to-noise ratio: For **FCN2** with HClO: **[DL]**= 6.68 nM (0.71 ppb).

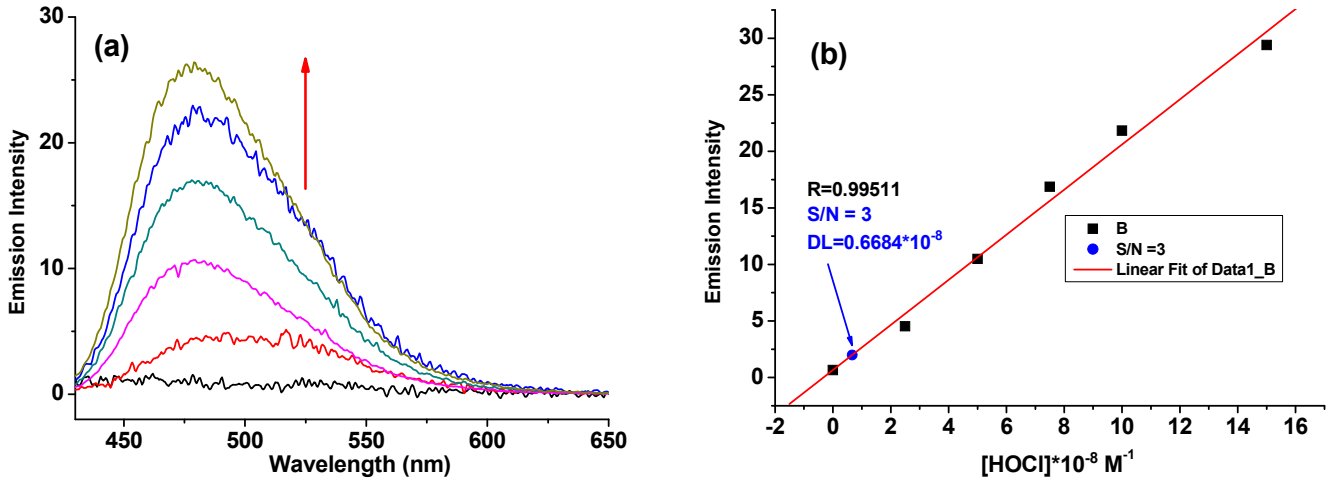


Figure S3: (a) Fluorescence titration of **FCN2** (0.5 μM) upon addition of HClO (by 25 nM ~ 150 nM) in 20 mM HEPES buffer (pH 7.2) with excitation at 415 nm. (b) The fluorescence intensities at 485 nm.

Kinetic Studies:

The kinetic Studies of probe **FCN1~FCN3** (1 μM) with HOCl were determined in HEPES buffer (20 mM, 0.1M KNO_3, pH7.2) at room temperature. The *pseudo*-first-order rate constant value was fitted from the emission intensity data at 485 nm following the modified *pseudo*-first-order equation:

$$\mathbf{Ln\ [(\Delta I_{max} - \Delta I_t)/\Delta I_{max}] = - k't}$$

Here $\Delta I_t = I_t - I_{min}$ and $\Delta I\ max = I_{max} - I_{min}$, where I_{min}, I_t, and I_{max} are the fluorescence intensities of FCN1~FCN3 considered in the absence of HOCl, at an intermediate time t, and at a reaction was complete. k' is the *pseudo*-first-order rate constant. The *pseudo*-first-order plots for the reaction of HOCl with **FCN1~FCN3** are shown in Figures S4-6, respectively. From the plot of Ln ($\Delta I_{max} - \Delta I_t / \Delta I_{max}$) against t for **FCN1~FCN3**, the value of k' was determined by fluorescence time course method for **FCN1~FCN3** with HOCl : $k' = 5.50\times10^{-3}\ s^{-1}$, $2.54\times10^{-3}\ s^{-1}$, and $5.94\times 10^{-4}\ s^{-1}$, respectively.

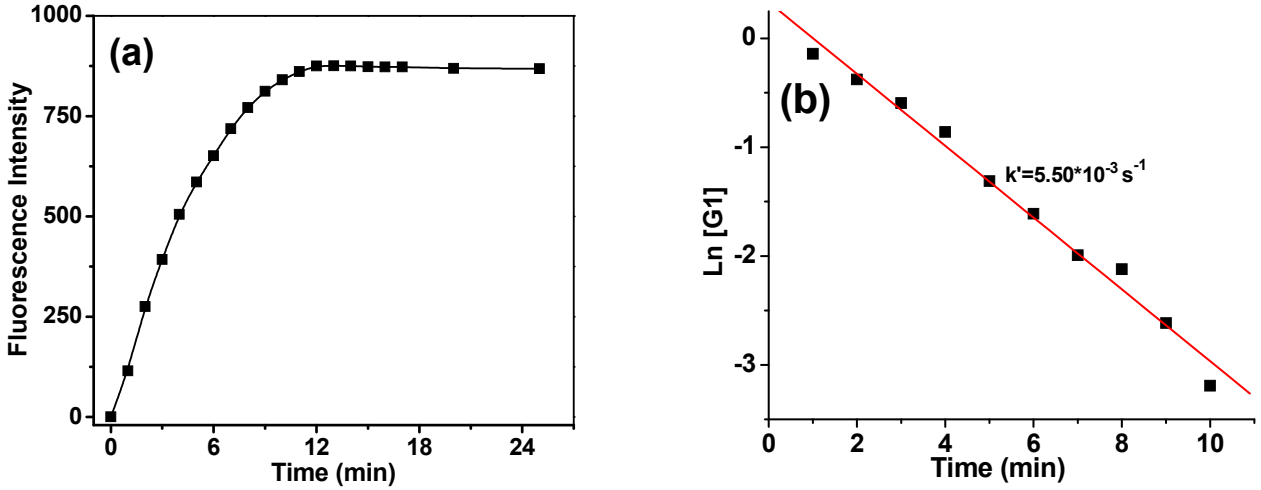


Figure S4: (a) Time course of reaction of **FCN1** (1.5 μM) with HOCl (5 μM) in 20 mM HEPES buffer (pH 7.2) at room temperature for 0-25 min. (b) *Pseudo*-first-order kinetic plot of the reaction of **FCN1** (1.5 μM) with addition of HOCl in 50 mM HEPES buffer (pH 7.2) at room temperature. [G1] = ($\Delta I_{max} - \Delta I_t / \Delta I_{max}$), Slope = 5.50×10^{-3} s^{-1}, R= 0.99281.

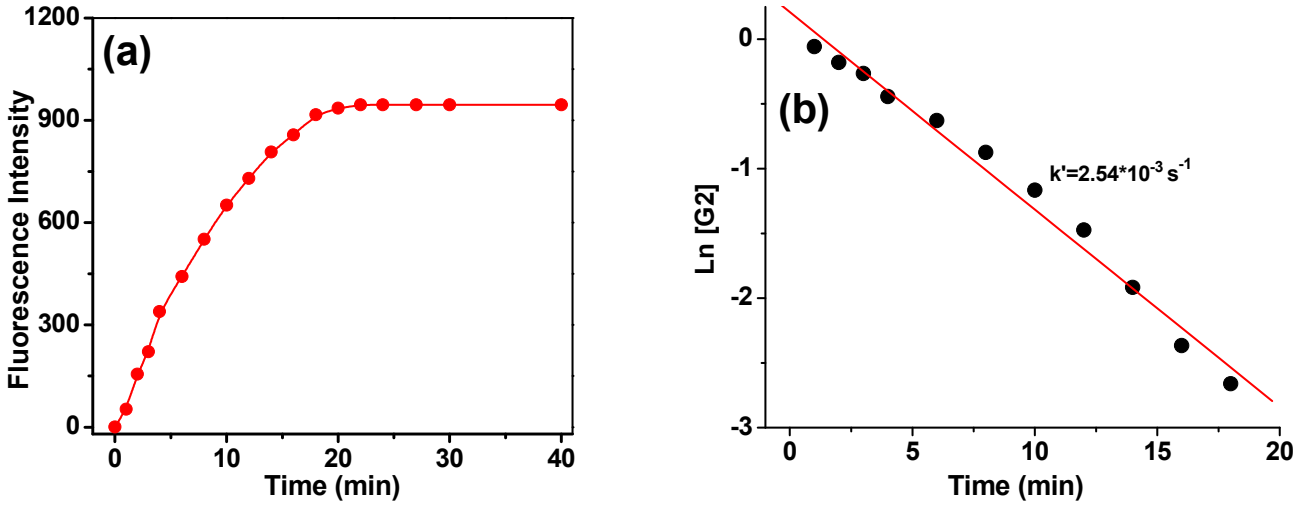


Figure S5: (a) Time course of reaction of **FCN2** (1.5 μM) with HOCl (5 μM) in 20 mM HEPES buffer (pH 7.2) at room temperature for 0-40 min. (b) *Pseudo*-first-order kinetic plot of the reaction of **FCN2** (1.5 μM) with addition of HOCl in 50 mM HEPES buffer (pH 7.2) at room temperature. [G2] = ($\Delta I_{max} - \Delta I_t / \Delta I_{max}$), Slope = 2.54×10^{-3} s^{-1}, R= 0.99234.

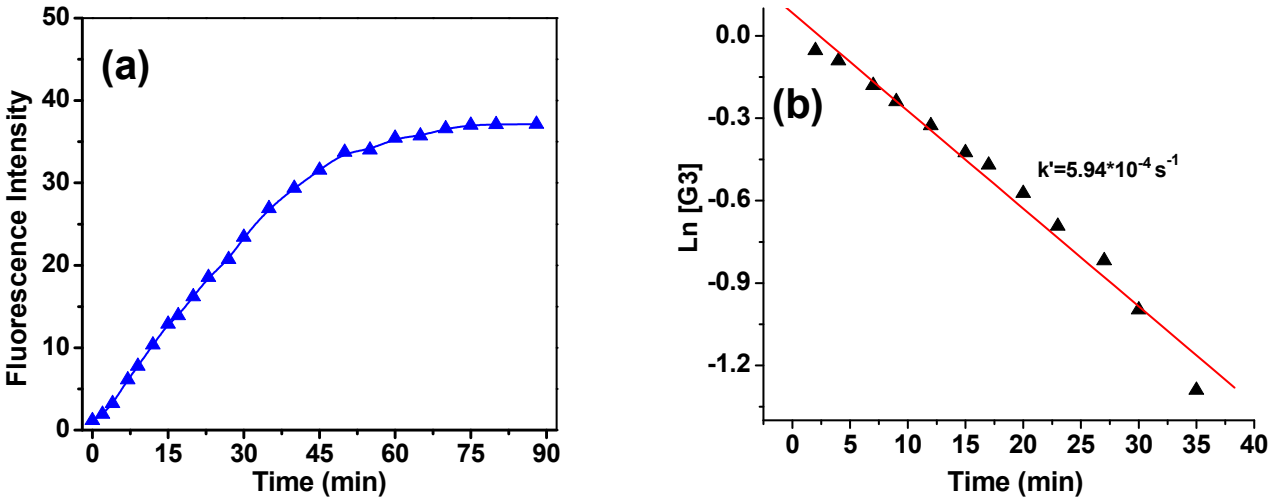
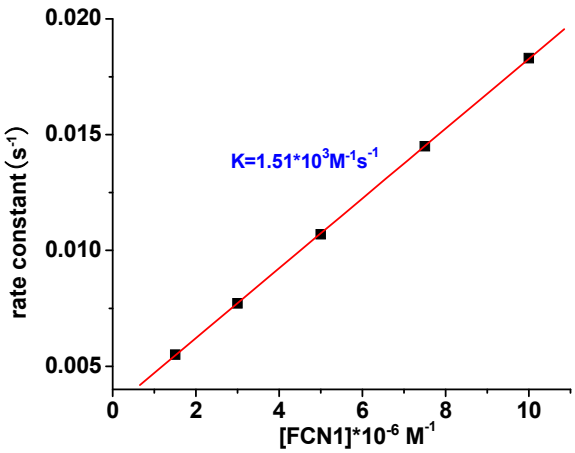


Figure S6: (a) Time course of reaction of **FCN3** (1.5 μM) with HOCl (5 μM) in 20 mM HEPES buffer (pH 7.2) at room temperature for 0-90 min. (b) *Pseudo*-first-order kinetic plot of the reaction of **FCN3** (1.5 μM) with addition of HOCl in 20 mM HEPES buffer (pH 7.2) at room temperature. [G3]= ($\Delta I_{max} - \Delta I_t / \Delta I_{max}$), Slope = 5.94× 10^{-4} s^{-1}, R=0.98924.

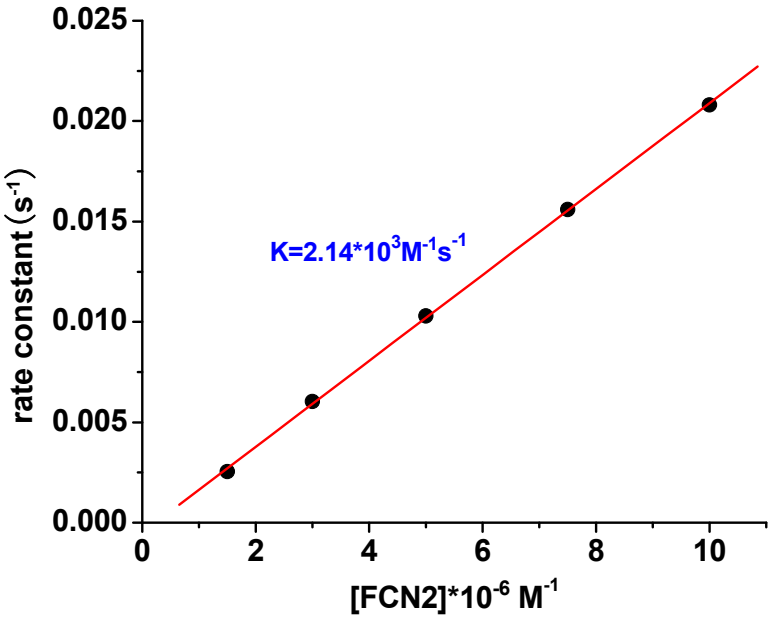
The second-order rate constant value was related to *pseudo*-first-order rate constant following the equation:

$$\mathbf{k' = k\,[M]}$$

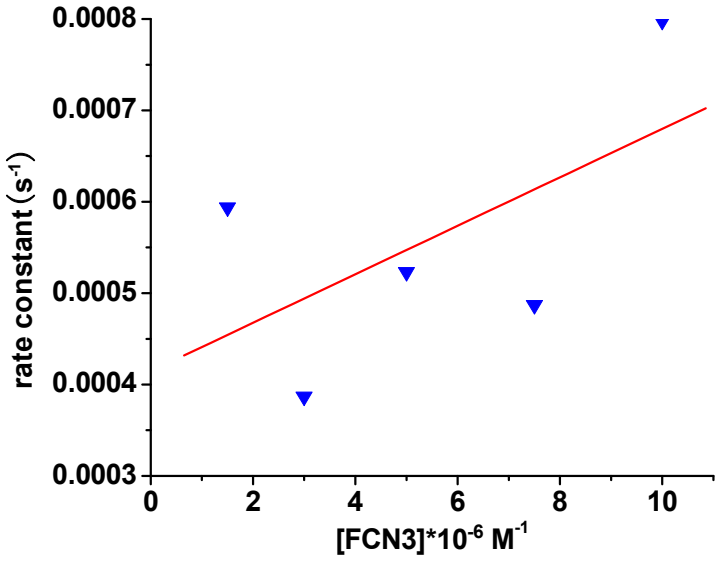
Where [M] is the concentration of HOCl, k is the second-order rate constant, k' is the *pseudo*-first-order rate constant. The second-order plots for the reaction of HOCl with **FCN1**~**FCN3** are shown in Figures S7-9. The second-order rate constant for this reaction is the slope of the linear plot of k' against the concentration of HOCl: $k' = 1.51\times10^{3}$ $M^{-1}s^{-1}$, and 2.14× 10^{-4} s^{-1}, respectively.



Figures S7**:** Dependence of the rate constant of the observed processes on **FCN1** concentration for HOCl. The solid line is the best fit to a straight line. Conditions: 20 mM HEPES buffer (pH 7.2) at room temperature. Slope = 1.51× 10^3 M^{-1} s^{-1}.



Figures S8**:** Dependence of the rate constant of the observed processes on **FCN2** concentration for HOCl. The solid line is the best fit to a straight line. Conditions: 20 mM HEPES buffer (pH 7.2) at room temperature. Slope = 2.14× 10^3 M^{-1} s^{-1}.



Figures S9**:** **FCN3** does not meet the strict definition of the second-order equation.

Table S2. The comparison of the photophysical properties and rate constants of **FCN1~FCN3** with HOCl.

Probe	FCN1	FCN2	FCN3
F_{HOCl}/F_{ap}[a]	1232.8-fold	1643.4-fold	31.6-fold
Φ_{HOCl}	0.63	0.71	~0.02
K'(s^{-1})[b]/(K'$_{FCN2}$/ K')[c]	5.50×10^{-3}(0.46)	2.54×10^{-3}(1.0)	5.94×10^{-4}(4.28)
K($M^{-1}s^{-1}$)[d]	1.51×10^{3}	2.14×10^{3}	__[e]
auto-oxidation[f]	6.5-fold	2.4-fold	stable

[a] The relative emission intensity of **FCN1~FCN3** with HOCl compared to the blank probe. [b] The *pseudo*-first-order rate constant of **FCN1~FCN3** with HOCl. [c] The relative *pseudo*-first-order rate constant. [d] The second-order rate constant of **FCN1** and **FCN2** with HOCl. [e] **FCN3** does not meet the strict definition of the second-order equation. [f] The exposure of probe to 20 mM HEPES buffer for 24 h in air.

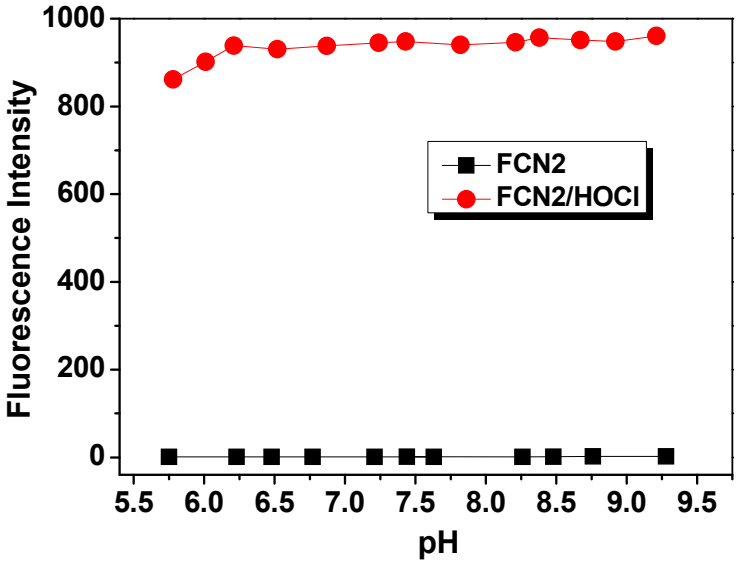
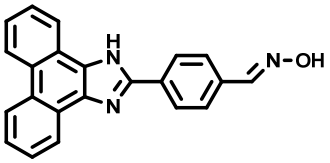
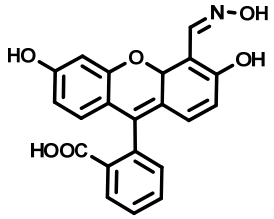
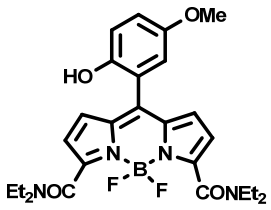
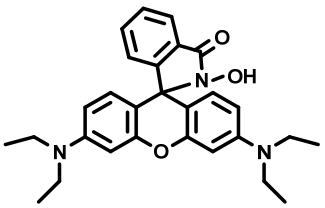
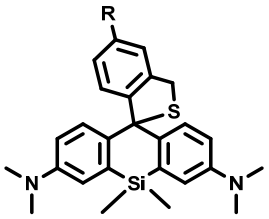
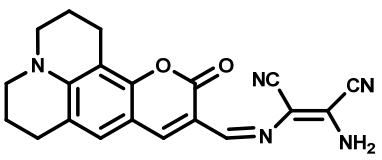
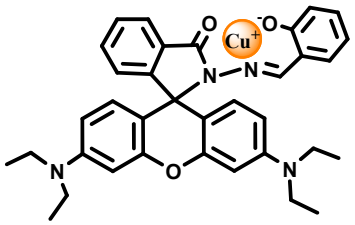
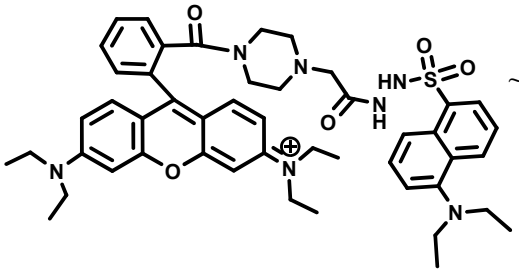
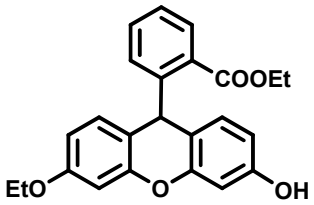


Figure S10: Fluorescence responses of **FCN2** (1.5 μM) with HOCl (5 μM) at different pH.

Table S3 Comparison of the recently reported HOCl fluorescent probes

probe	detection limit	selectivity	Comments
 *Chem. Eur. J.* 2009, **15**, 2305	**no data available**	**good**	**PBS/DMF (v/v, 1:4)** **I_{509}/I_{439}=9.79-fold** **Ratiometric Fluorescent Probe**
 *Chem. Commun.* 2011, **47**, 11978	**no data available**	**good**	**HEPES/DMSO (v/v, 9:1)** **(I_{530})= 61-fold** **Fluorescence enhancement** **Living cell imaging**
 *Org. Lett.* 2008, **10**, 2171	**no data available**	**medium**	**100% aqueous media** **(I_{541})= 1079-fold** **Fluorescence enhancement** **Living cell imaging**
 *Org. Lett.* 2009, **11**, 859	**~ 25 nM**	**good**	**PBS/DMF(0.1%)** **Fluorescence enhancement** **Living cell imaging**
 *J. Am. Chem. Soc.* 2011, **133**, 5680	**no data available**	**good**	**100% aqueous media** **NIR Fluorescence enhancement** **model mouse imaging** **Living cell imaging**
 *Chem. Commun.* 2011, **47**, 12691	**0.2 μ M**	**good**	**PBS/DMF (v/v,2:8)** **I_{505}/I_{585}=235-fold** **Ratiometric Fluorescent Probe** **Living cell imaging**

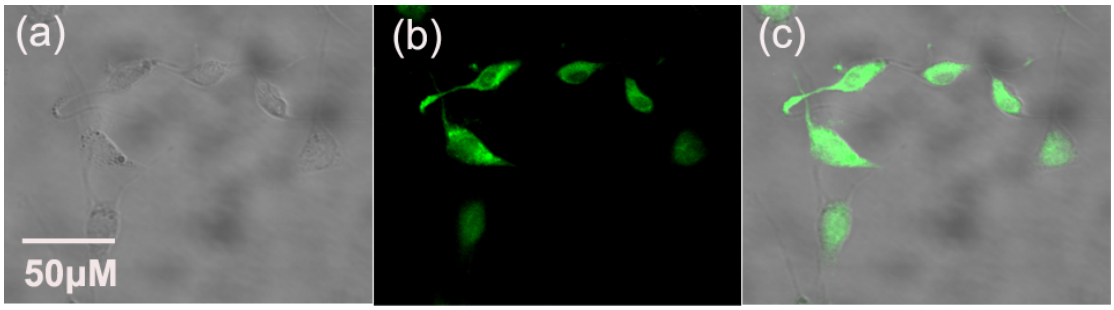
probe	detection limit	selectivity	Comments
 *Chem. Commun.* 2011, **47**, 3189	**0.81 μ M**	**medium**	**Water/CH_3CN (v/v, 1:1)** **A_{555} good sensitive colorimetric probe in tap water**
 *Chinese Sci. Bull.* 2011, **56**, 3266	**~80 nM**	**good**	**Phosphate buffer, pH 7.4** **Ratiometric Fluorescent Probe** **Living cell imaging**
 ***this work* FCN2**	**6.68 nM (0.71 ppb)**	**good**	**100% aqueous media** **(I_{485})= 1643.4-fold Fluorescence enhancement** **Living cell imaging** **model zebrafish imaging-organ level**

Cell Culture and Fluorescence Imaging

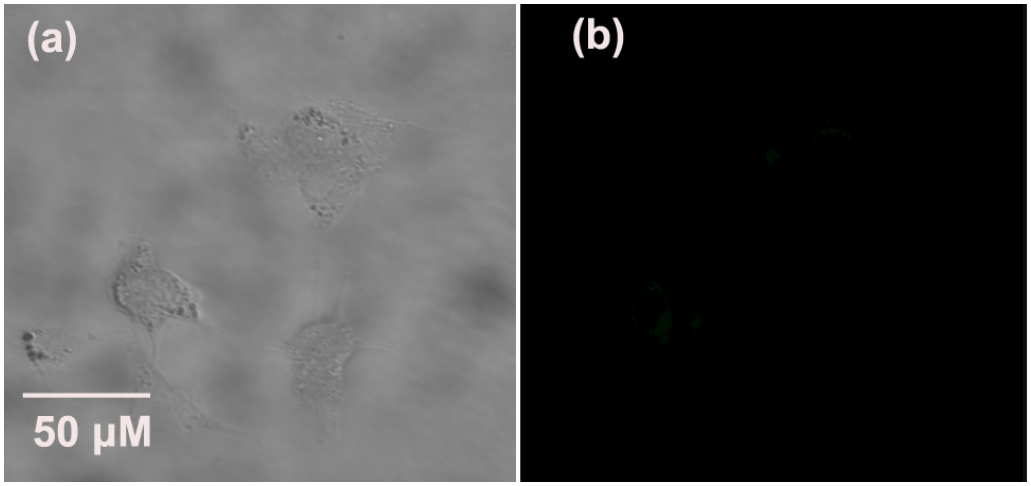
The **FCN2** working solution for cell staining was prepared from a 1 mM stock solution (DMSO, 1.0 mM) of probe by diluting with PBS to a final concentration of 5 μM. Myeloperoxidase (human neutrophils) was purchased from J&K (CAS: 9003-99-0). NIH3T3 cells (mouse embryonic fibroblast cells) were dropped on the poly-D-lysine-coated 35 mm glass bottom dishes (Mat Tek Corp) at a density of 2×10^3 cells per well in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin (100 μg mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37 °C in a humidified amosphere with 5% CO_2 and 95% air for 24 h prior to staining. All cellular fluorescent images were collected on an FV1000-IX81 confocal microscope.

Experiments to detection of HOCl were performed in the PBS media supplemented with MPO (1.5 U/100 mL), NaCl (250 mM) and 5 μM **FCN2** for 30 min. After washing twice with PBS (phosphate

buffered saline, pH=7.2, Gibco) to remove the remaining sensor, then the treated cells were stimulated with 10 μM H_2O_2. Images were collected using IPP software (Olympus) by confocal microscope.



Figures S11: Similar fluorescence enhancements were observed by using **FCN1** under the H_2O_2 stimulated production of HOCl. DIC (a) and confocal fluorescence (b) images of NIH3T3 cells preincubated with NaCl (250 mM) and 5 μM **FCN1** in MPO (1.5 U/100 mL) enzymatic system. (c) overlay image of (a) and (b).



Figures S12: Negligible fluorescent enhancements were observed by using **FCN3** under the H_2O_2 stimulated production of HOCl. DIC (a) and confocal fluorescence (b) images of NIH3T3 cells preincubated with NaCl (250 mM) and 5 μM **FCN3** in MPO (1.5 U/100 mL) enzymatic system.

MTT Assay

To ascertain the cytotoxic effect of **FCN2** treatment over a 24 h, the MTT assay was performed. NIH3T3 cells (5×10^4) were passed and plated to 70% confluence in 96-well plates 24 h before treatment. 5, 25, 50, 75, and 100 μM **FCN2** was added to the cells and incubated at 37°C for 12 h. Cytotoxicity was then determined by the method of Thiazolyl Blue Tetrazolium Bromide (MTT) assay (Cell Proliferation Kit; keygen biological products, Nanjing, China), following the instructions of the kit. Subsequently, the cells were incubated with 5 mg/mL MTT reagent at 37°C for 4 h and the

absorbance of each well was measured by a microplate reader (SPECTRA SLT; Labinstruments, Salzburg, Austria). The excitation wavelength was 492 nm, and the emission was read at 690 nm. Each treatment was done in six wells, and the experiments were repeated three times. Cytotoxicity was calculated relative to the absorbance of the control for each treatment. Data were expressed as means □□SD. The reported percent cell survival values are relative to untreated control cells.

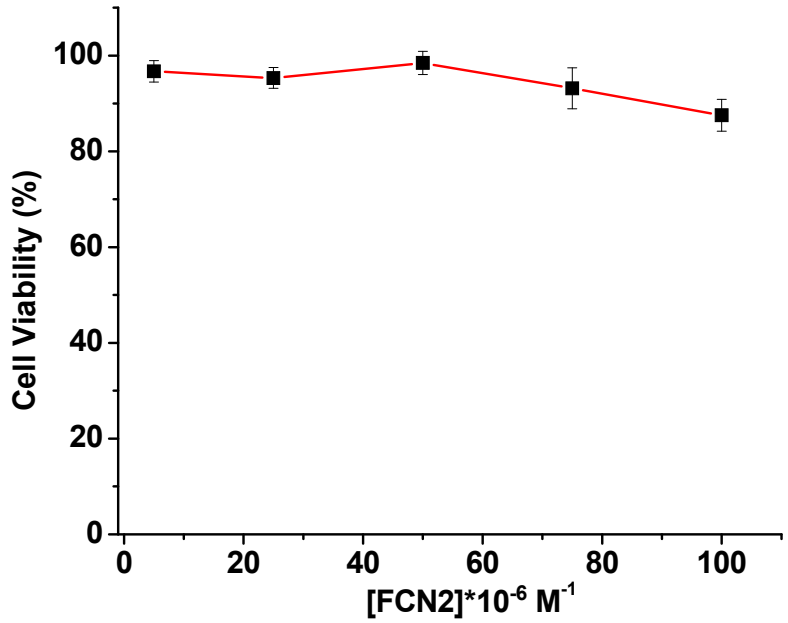


Figure S13: Cell viability was quantified by the MTT assay (NIH3T3 cells, 12h).

Experimental for the fluorescence imaging of zebrafish

Zebrafish were kept at 28.5°C and maintained at optimal breeding conditions. For mating, male and female zebrafish were maintained in one tank at 28.5°C on a 12 h light/12 h dark cycle and then the spawning of eggs were triggered by giving light stimulation in the morning. Almost all the eggs were fertilized immediately. The 19 dpf and 54 dpf old zebrafish was maintained in E3 embryo media (15 mM NaCl, 0.5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM $NaHCO_3$, 10-5% methylene blue; pH 7.5).[4] Experiments to detection of HOCl were performed in E3 embryo media with 5 μM **FCN2** for 30 min. All zebrafish fluorescent images were collected on a fluorescent dissecting microscope (Leica) equipped with a DP70 digital imaging system (Olympus, Tokyo, Japan) with a GFP filter set. Images were collected using IPP software (Olympus).

Ethical statement:

In this paper, all experiments with live animals were performed in compliance with guidelines issued by Ethical Committee of Model Animal Research Center of Nanjing University, and the institutional committee(s) have approved the experiments.

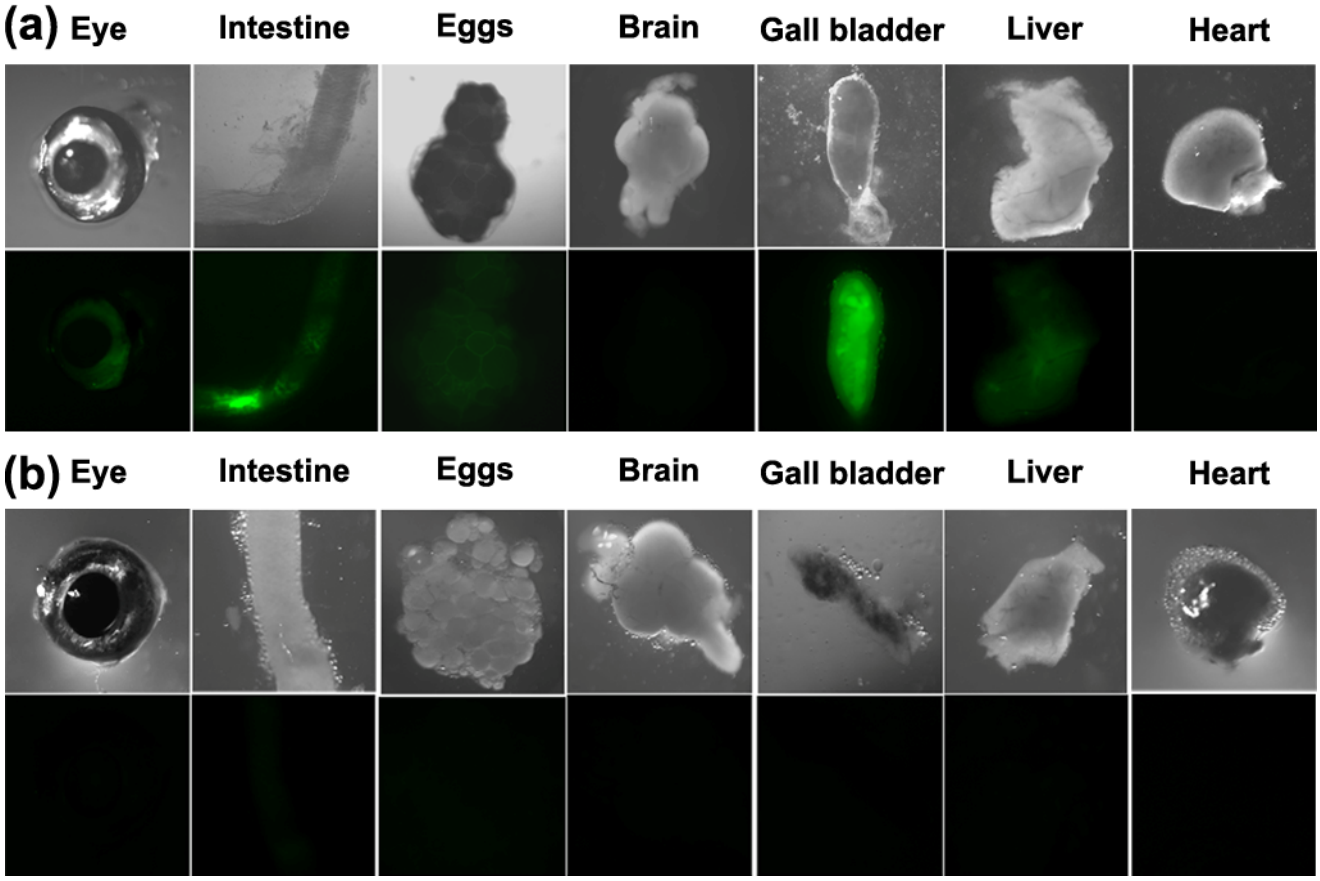


Figure S14: Images of adult zebrafish organs treated with 5 μM **FCN2** in (a) the absence and (b) presence of external HOCl.

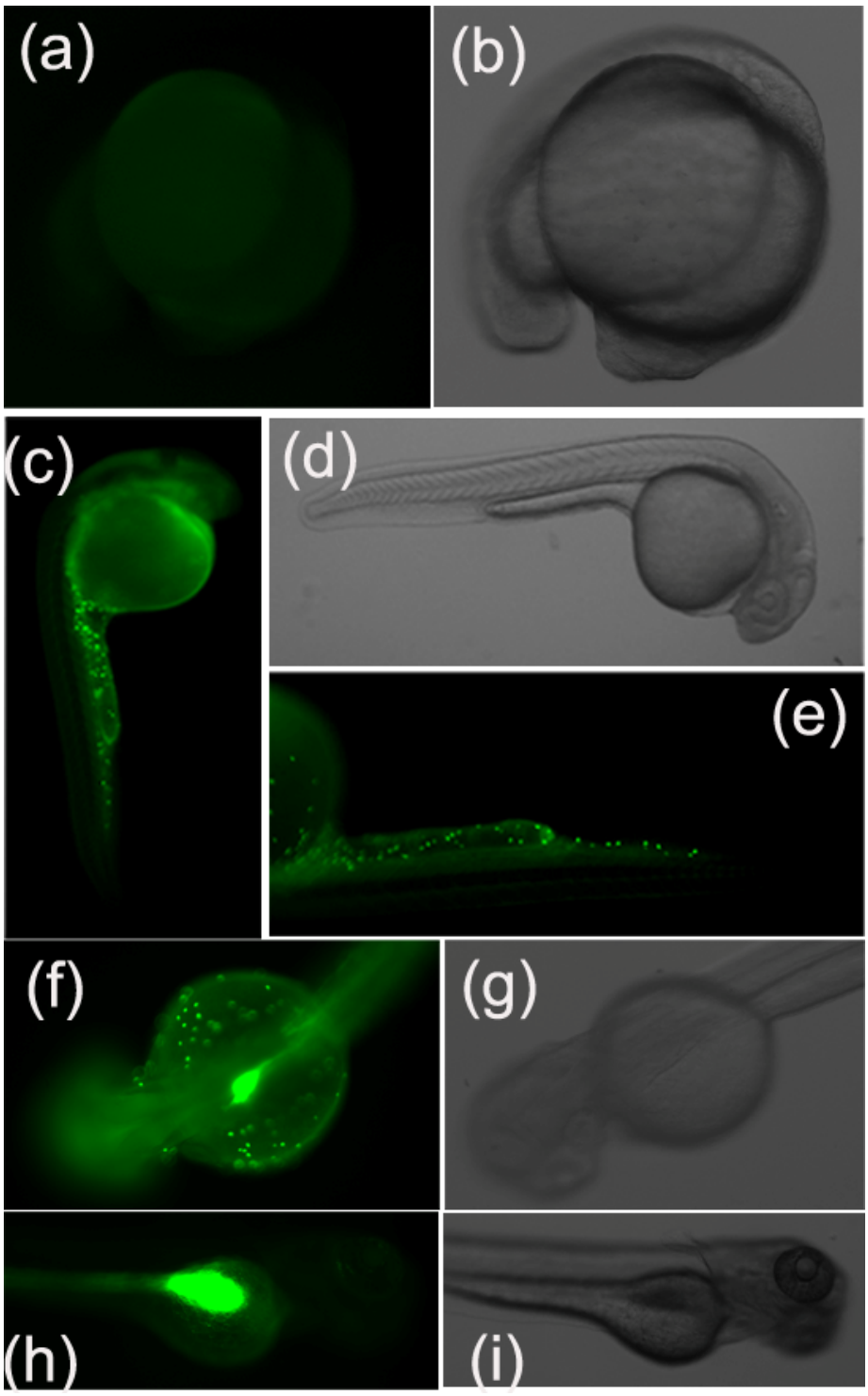


Figure S15: Fluorescence microscopy images of AB/Tubingen larvae zebrafish incubated with 10 μM HOCl (E3 embryo media, 28.5°C) during the development. 1-Phenyl-2-thiourea (PTU, 0.003%) was added to depress the development of pigment after 8 h of incubation. Fluorescence and DIC images of (a, b) 18 h-old, (c, d, e) 28 h-old, (f, g) 54h-old, and (h, i) 78h-old zebrafish were further incubated with 5 μM **FCN2** for 1 h.

References

(1) Hughes, M. N.; Nicklin, H. G. *J. Chem. Soc. (A)* 1968, ***2***, 450.

(2) King, S. B.; Nagasawa, H. T. Chemical Approaches Toward Generation of Nitroxyl. In Methods in Enzymology Vol. 301, Part C. Packer, L., Ed; Academic Press: San Diego, 1999; pp 211-220.

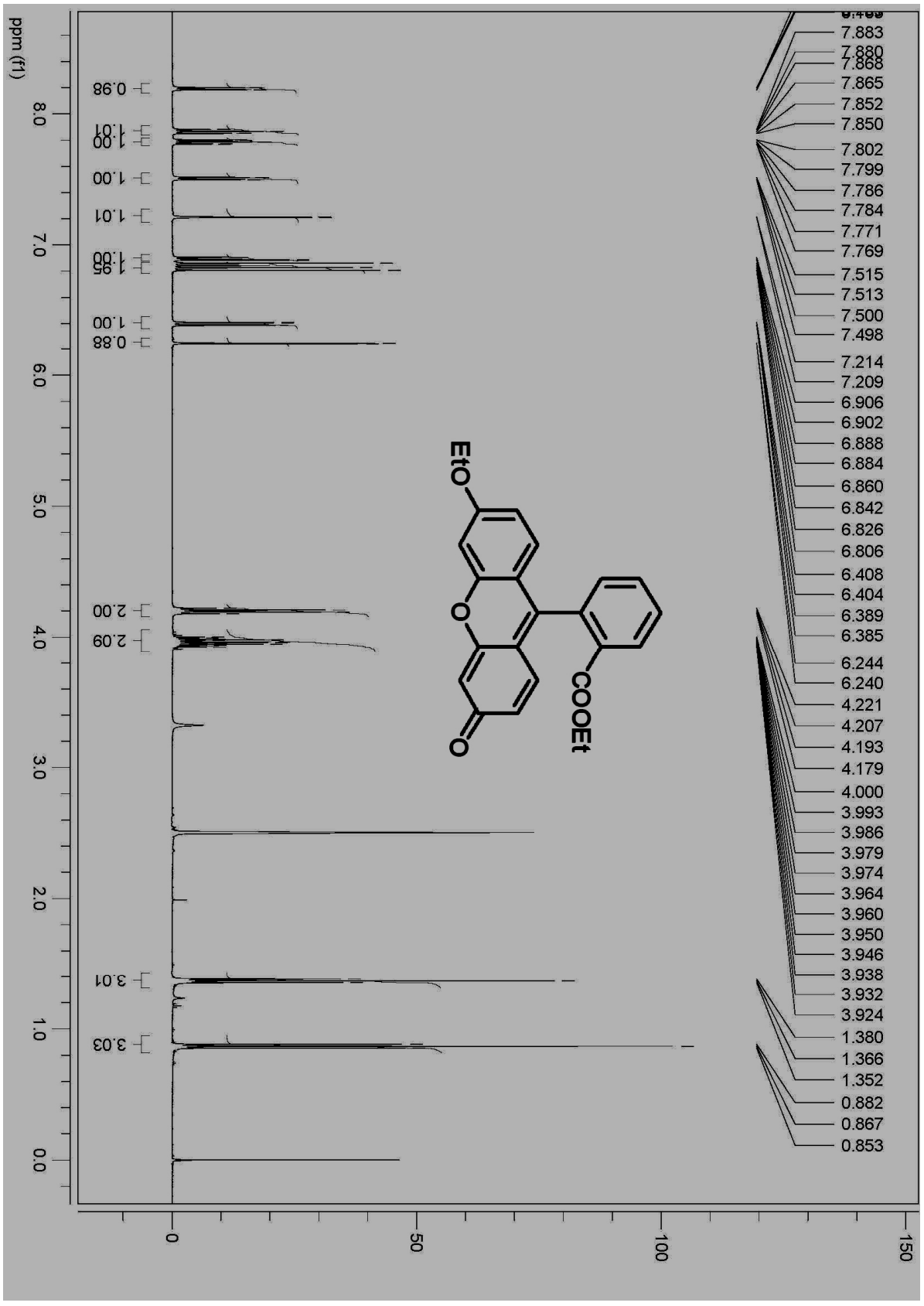
(3) C. A. Parker and W. T. Rees, Analyst, 1960, **85**, 587.

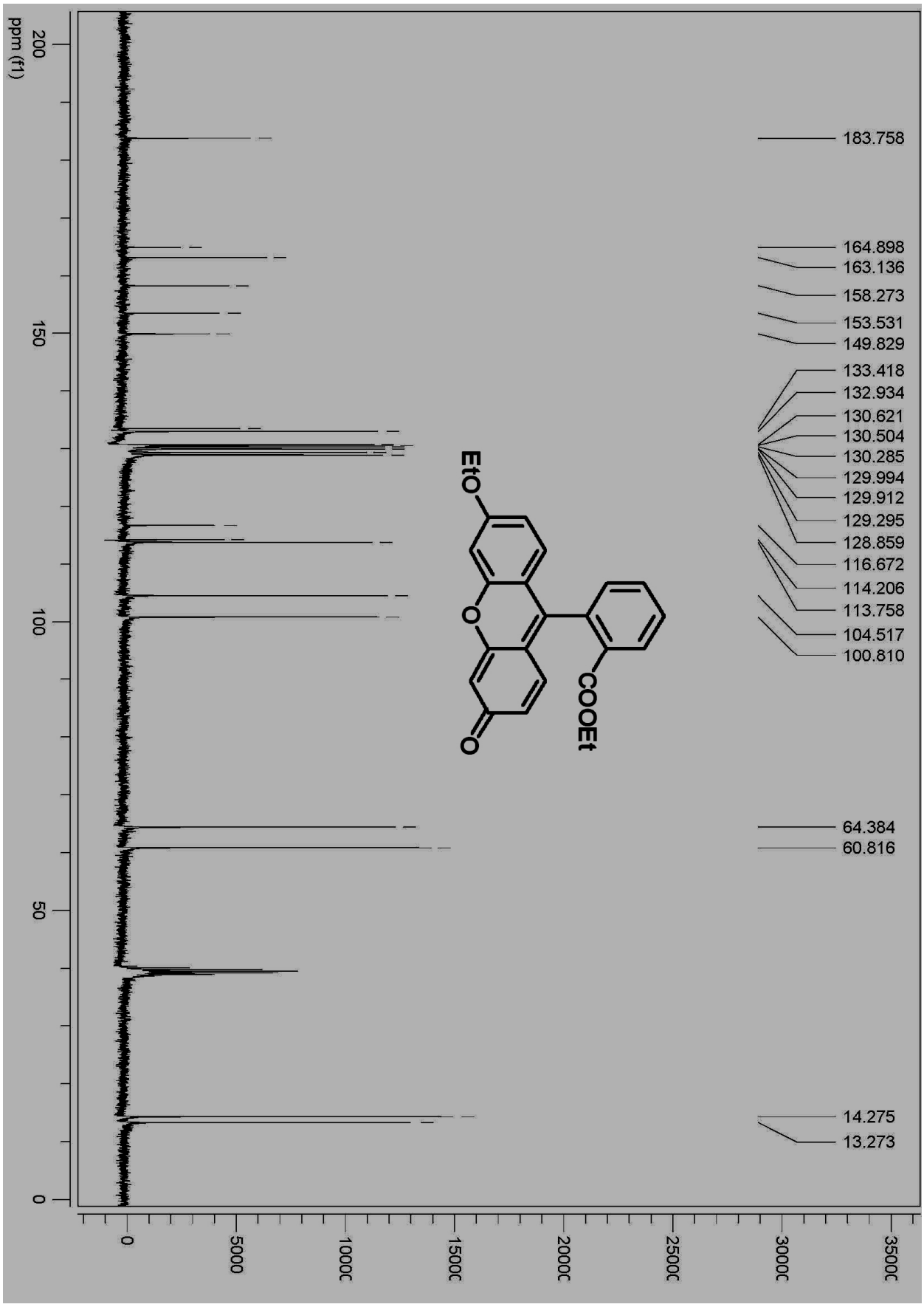
(4) Y. K. Yang, S. K. Ko, I. Shin and J. Tae, *Nat. Protocols* 2007, ***2***, 1740.

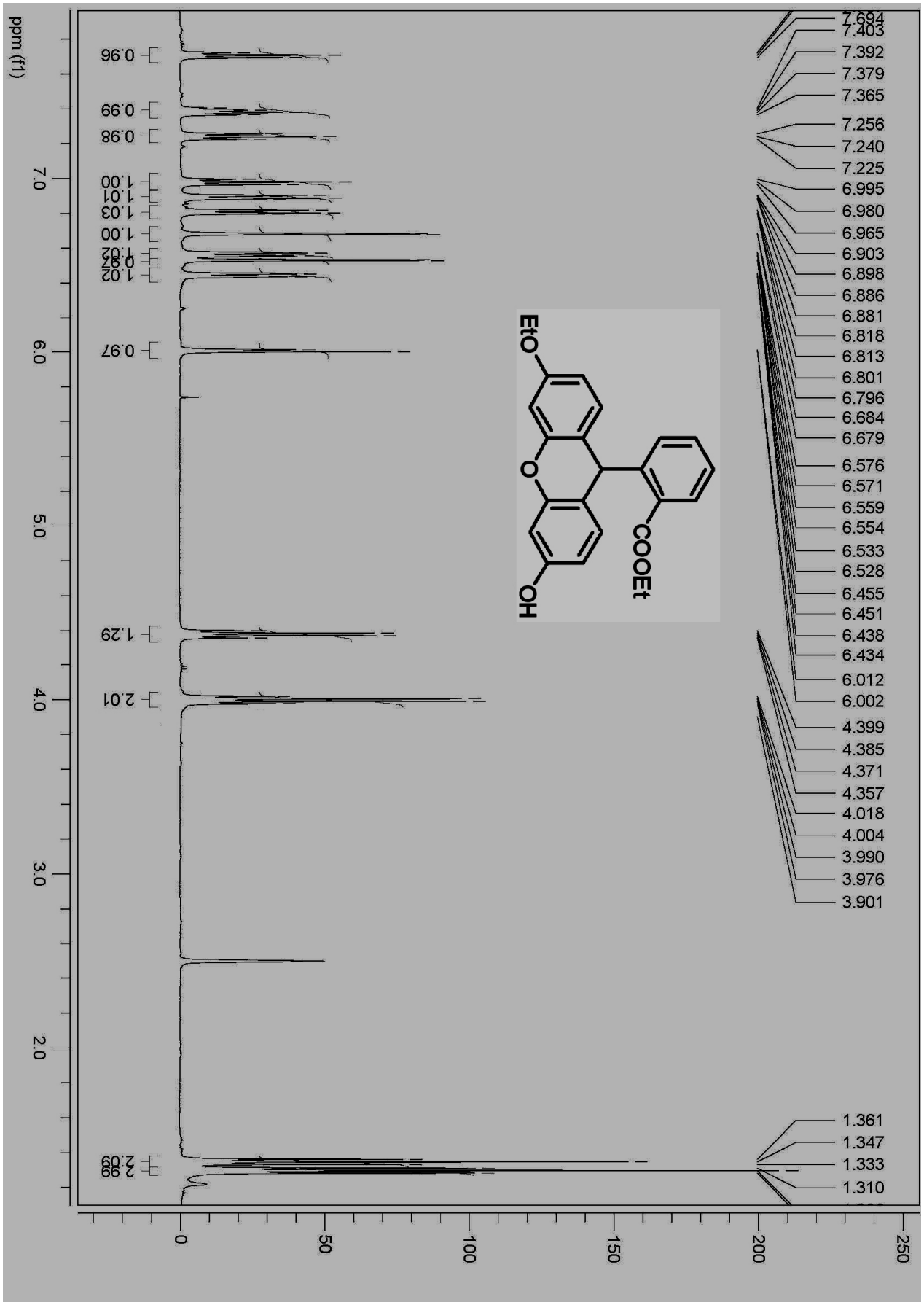
Acknowledgements

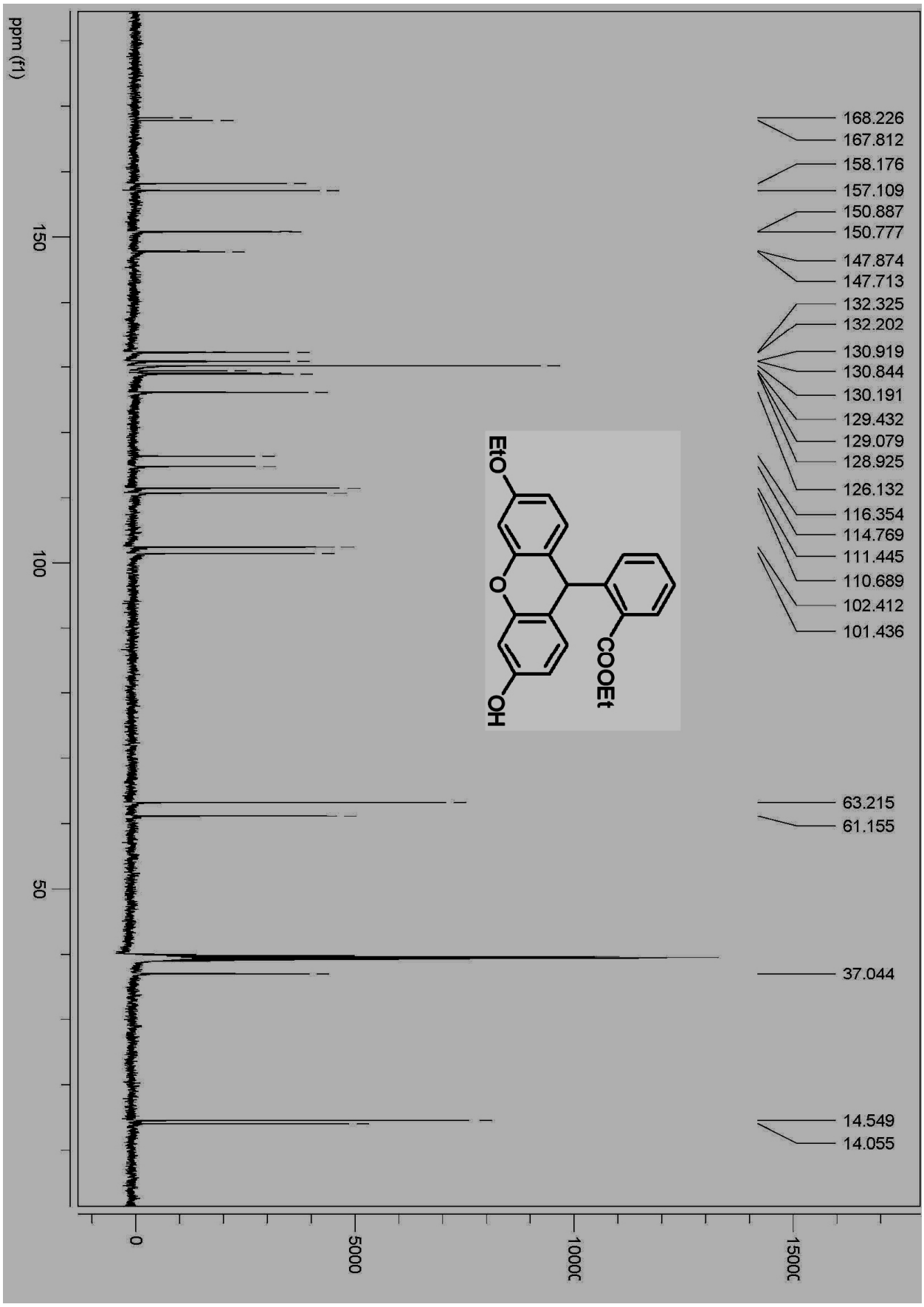
This work is supported by the Open Fund of the State Key Laboratory of Materials-Oriented Chemical Engineering (KL09-9), the postgraduate practice innovation fund of Jiangsu province. We thank Prof. Qin-Shun Zhao (Nanjing University) for expert technical assistance in bioimaging.

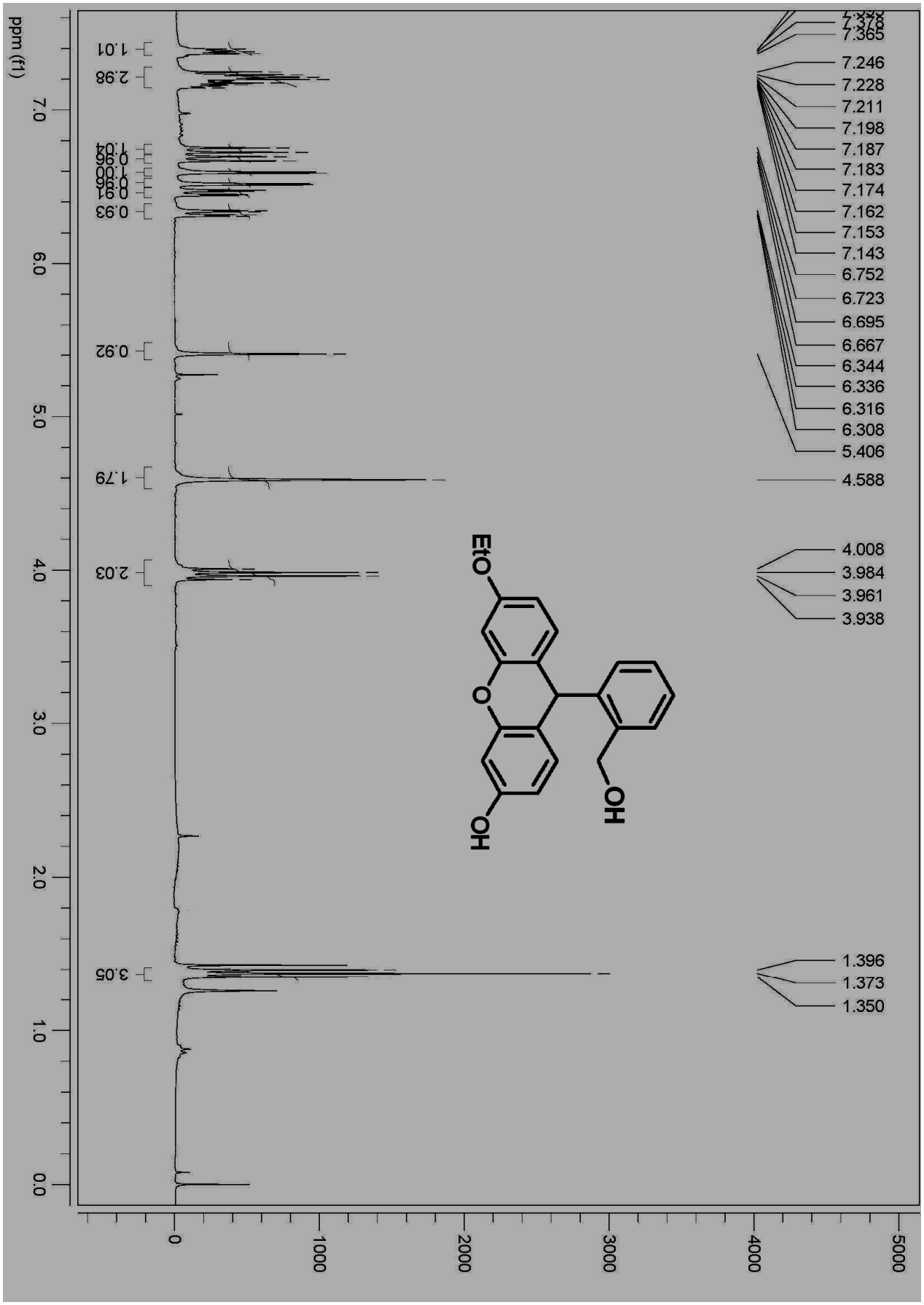
^{1}H NMR, ^{13}C NMR and *ESI*-MS spectra Supplementary spectra data

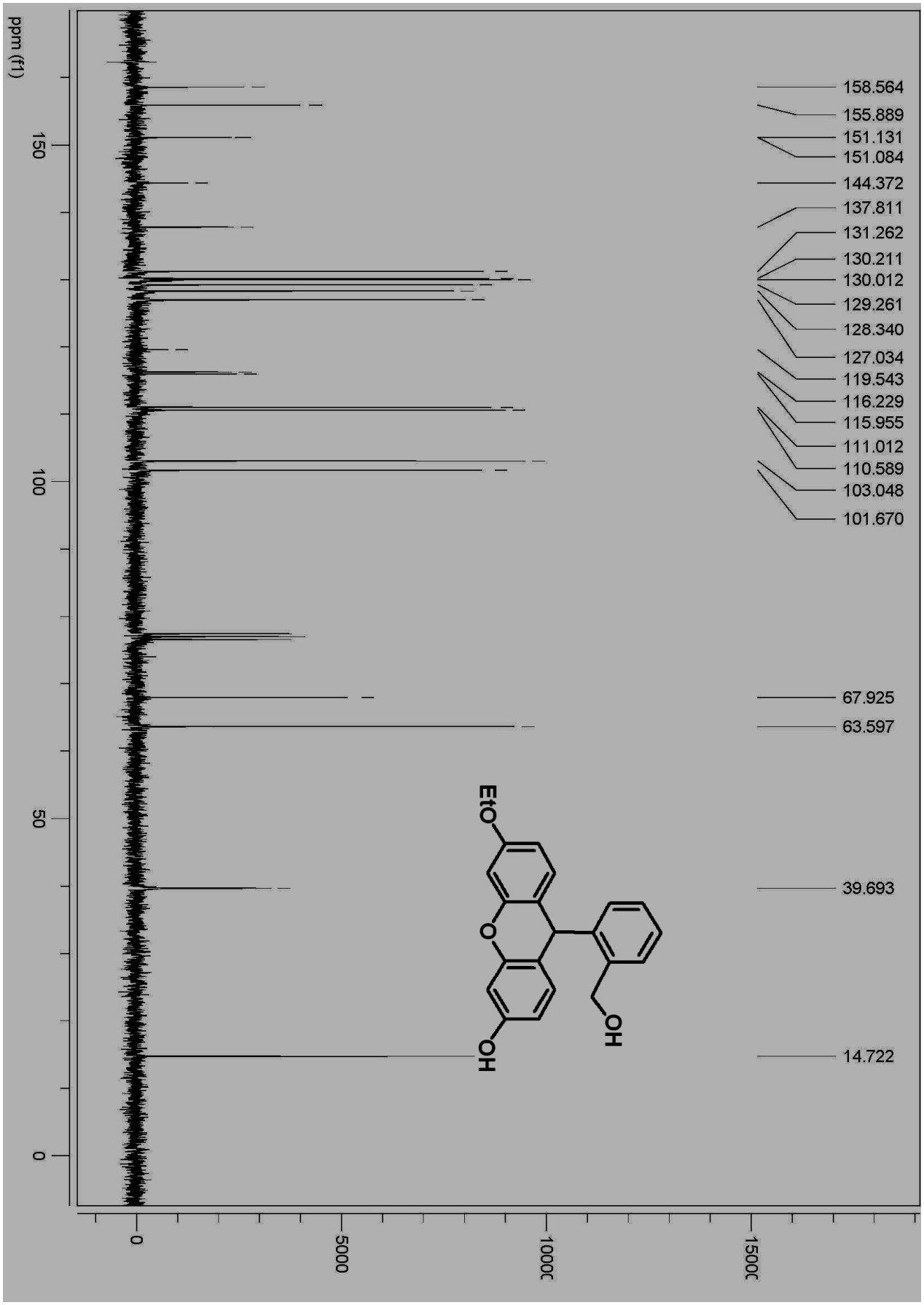



ppm (f1)
200
150
100
50
0
0
5000
10000
15000
20000
25000
30000
35000
183.758
164.898
163.136
158.273
153.531
149.829
133.418
132.934
130.621
130.504
130.285
129.994
129.912
129.295
128.859
116.672
114.206
113.758
104.517
100.810
64.384
60.816
14.275
13.273
EtO
O
O
COOEt


ppm (f1)
7.694
7.403
7.392
7.379
7.365
7.256
7.240
7.225
6.995
6.980
6.965
6.903
6.898
6.886
6.881
6.818
6.813
6.801
6.796
6.684
6.679
6.576
6.571
6.559
6.554
6.533
6.528
6.455
6.451
6.438
6.434
6.012
6.002
4.399
4.385
4.371
4.357
4.018
4.004
3.990
3.976
3.901
1.361
1.347
1.333
1.310
0.96
0.99
0.98
1.00
1.01
1.03
1.00
1.02
0.97
1.02
0.97
1.29
2.01
2.09
2.99
EtO
O
OH
COOEt


ppm (f1)
150
100
50
0
5000
10000
15000
168.226
167.812
158.176
157.109
150.887
150.777
147.874
147.713
132.325
132.202
130.919
130.844
130.191
129.432
129.079
128.925
126.132
116.354
114.769
111.445
110.689
102.412
101.436
63.215
61.155
37.044
14.549
14.055
EtO
O
OH
COOEt


ppm (f1)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1.01
2.98
1.04
0.96
1.00
0.96
0.91
0.93
0.92
1.79
2.03
3.05
7.365
7.246
7.228
7.211
7.198
7.187
7.183
7.174
7.162
7.153
7.143
6.752
6.723
6.695
6.667
6.344
6.336
6.316
6.308
5.406
4.588
4.008
3.984
3.961
3.938
1.396
1.373
1.350
EtO
O
OH
OH
0
1000
2000
3000
4000
5000


ppm (f1)
150
100
50
0
158.564
155.889
151.131
151.084
144.372
137.811
131.262
130.211
130.012
129.261
128.340
127.034
119.543
116.229
115.955
111.012
110.589
103.048
101.670
67.925
63.597
39.693
14.722
EtO
O
OH
OH
0
5000
10000
15000