Supporting Information

Naphthalene-based fluorescent probes for glutathione their applications in living cells and patients with sepsis

Jun Li[1, †], Younghee Kwon[2, †], Kyung Soo Chung[3,†], Chang Su Lim[5, †],Dayoung Lee[1], Yongkang Yue[4], Jisoo Yoon[1], Gyoungmi Kim[1], Sang-Jip Nam[1], Youn Wokk Chung,[2] Hwan Myung Kim[5,*], Caixia Yin[4,*], Ji-Hwan Ryu[2,*], Juyoung Yoon[1,*]

1. Department of Chemistry and Nano Science, Ewha Womans University, Seoul 120-750, Korea
2. Severance Biomedical Science Institute, Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, Korea.
3. Division of Pulmonology, Department of Internal Medicine, Institute of Chest Disease, Severance Hospital, Yonsei University College of Medicine, Seoul 120-752, Korea.
4. Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Key Laboratory of Materials for Energy Conversion and Storage of Shanxi Province, Institute of Molecular Science, Shanxi University, Taiyuan 030-006, China.
5. Department of Chemistry and Energy Systems Research, Ajou University, Suwon, Korea.

† These authors contributed equally to this work

*Corresponding authors: Juyoung Yoon, e-mail: jyoon@ewha.ac.kr; Ji-Hwan Ryu, e-mail: yjh@yuhs.ac; Caixia Yin, e-mail: yincx@sxu.edu.cn,Hwan Myung Kim, e-mail: kimhm@ajou.ac.kr.

General experimental procedures Unless otherwise stated, NDA, all starting materials and reagents, such as anhydrous DMF, dichloromethane (DCM), triethylamine (Et_3N), and human serum, were obtained commercially. ^1H and ^{13}C NMR spectra were collected in $CDCl_3$ and DMSO-d_6 solutions using a Bruker AM-300 spectrometer with tetramethylsilane (TMS) as the internal standard. Mass spectra were measured in electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) mode. UV/Vis spectra were obtained using a Scinco 3000 spectrophotometer (1 cm quartz cell) at 25°C. Fluorescence spectra were recorded on an RF-5301/PC (Shimada) fluorescence spectrophotometer (1 cm quartz cell) at 25°C. Deionized water was used to prepare all aqueous solutions. ^1H and 2D NMR spectral data were recorded in DMSO-d_6 solution containing Me_4Si as an internal standard on a Varian Inova 500 MHz spectrometer. ^{13}C NMR spectra were acquired on a Bruker Avance III 300 (75 MHz) spectrometer. Low-resolution LC/MS data were collected using a Hewlett-Packard series 1100 LC/MS system with a reversed-phase C18 column (Phenomenex Luna, 4.6 mm × 100 mm, 5 μm) at a flow rate of 0.7 mL/min. The crude extract (200 mg) was purified by reversed-phase HPLC (Phenomenex Luna C-18(2), 250 × 100 mm, 2.0 mL/min, 5 μm, 100 Å, UV = 254 nm) using a isocratic solvent system (40% CH_3CN [0.01% trifluoroacetic acid (TFA)]) to afford 8.0 mg of oil.The pseudo-first-order rate constant (Kobs) of the reaction between probes and thiols was calculated by fitting the fluorescence intensity data in the following equation: $Ln(F_{max} - F_t) = Ln\ F_{max} - K_{obs}t$.The quantum yield was determined according to the reported reference (Chen et al, *Chem. Commun*., 2017, **53**, 4791)

Study design, patients, and clinical setting

We used the data from a retrospective cohort study of prospectively collected samples from the medical ICU (MICU) of Severance Hospital, a 2000-bed (30-bed MICU) university tertiary referral hospital in Seoul, South Korea. The study included 15 healthy volunteers and 97 consecutive patients aged >19 years who had been admitted to the MICU because of sepsis between March 2015 and August 2015, for a total of 112 participants. According to the 2016 revised sepsis definition (Sepsis-3), sepsis is defined as when patients have suspected infection and a SOFA score ≥ 2 points.[1] All patients presenting with sepsis were treated according to the guidelines of the Surviving Sepsis Campaign.[2] The study protocol was submitted as an ICU cohort and approved by the Institutional Review Board (IRB) of Severance Hospital (IRB number: 4-2013-0585). In addition, this study was performed in compliance with the principles set forth in the Declaration of Helsinki.

1. M. Shankar-Hari, G. S. Phillips, M. L. Levy, C.W. Seymour, V. X. Liu,C. S. Deutschman, D. C. Angus, G. D. Rubenfeld, M. Singer,*JAMA,*2016, **315**, 775-787.

2. W. A. Knaus, E. A. Draper, D. P.Wagner , J. E. Zimmerman, *Crit. Care. Med.,* 1985, **13**, 818-829.

Clinical data and blood sample collection

Data from all of the patients admitted to the MICU were collected from hospital electronic medical records and blood samples. The severity of each patient's condition was classified according to two different scoring systems. The SOFA and APACHE II scores were calculated. Clinical parameters, such as the blood culture positivity, 28-day mortality, and other demographic characteristic data, were evaluated. Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) within 24 hours after admission to the MICU. Simultaneously, we determined the C-reactive protein (CRP), procalcitonin, creatinine, and albumin levels; blood culture results; white blood cell and platelet counts; and APACHE II and SOFA scores. Plasma was prepared by centrifugation for 15 minutes at 800 × g and 4ºC. Supernatants from centrifuged blood were immediately aliquoted and stored at -80ºC until analysis. The levels of cytokines (IL-1β, IL-10, IL-18, IL-6, TNF-α, and interferon gamma induced protein [IP]-10) were measured using a Human Magnetic Luminex® Screening Assay kit (R&D Systems, Inc., Minneapolis, MN, USA) and performed according to the manufacturer's protocols. All samples and standards were assayed in duplicate using a Luminex 200TM System (Merck Millipore, Darmstadt, Germany).

Measurement of the GSH level in blood samples by NDA and FNDA

Collected blood samples were diluted to 10% solutions in HEPES (10 mM, pH = 7.4) buffer. The diluted samples were reacted with 40 μM NDA or 20 μM FNDA at room temperature for 15 minutes, and the fluorescence intensity of each solution was read using a fluorescence spectrometer.

Statistical analysis

Data are described as medians with IQRs. The chi-squared and Fisher's exact tests or the Mann–Whitney U test was used to assess differences between two groups. Pearson correlation analyses were performed to estimate associations between the biomarkers and the APACHE II or SOFA score. AUC analyses of the receiver operating characteristic (ROC) curves were performed to compare the GSH level and APACHE II and SOFA scores. In all cases, a p-value of < 0.05 was considered to be statistically significant. SPSS version 20 (IBM, Armonk, NY, USA) was used for the statistical analysis.

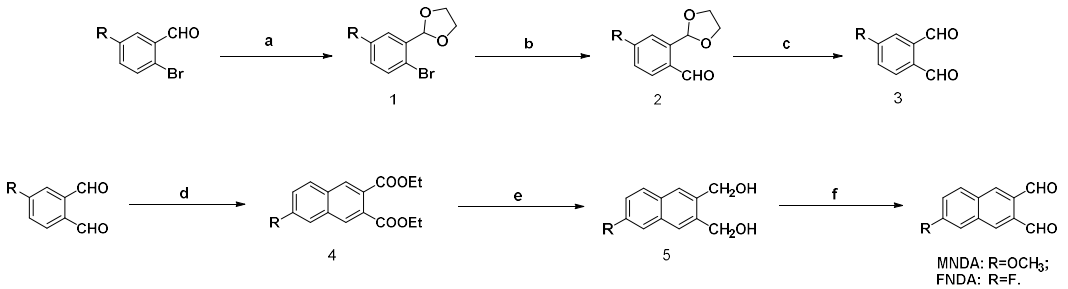
Measurement of two-photon cross section. The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described(S. K. Lee, W. J. Wang, J. J. Choi, C. H. Kim, S. J. Jeon,B. R. Cho, *Org. Lett*. 2005, **7**, 323-326.). MNDAwere dissolved in 30 mM HEPES buffer (pH 7.4) at concentrations of 1.0×10^{-5} M and then the two-photon induced fluorescence intensity was measured at 720−980 nm by using Rhodamine 6G as the reference, whose two-photon property has been well characterized in the literature(N. S. Makarov, M.Drobizhev, A. Rebane, *Opt. Express* 2008, **16**, 4029-4047.). To measure the two-photon cross section (δ) of the reaction product between MNDA (1.0×10^{-5} M) and GSH (100 μM), the reaction mixture was kept at RT for 1hrs before the measurement was conducted. The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were

determined. The TPA cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\varphi_r c_r)/(S_r\Phi_s\varphi_s c_s)$: where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as *S*. Φ is the fluorescence quantum yield. φ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as *c*. δ_r is the TPA cross section of the reference molecule.

Two-photon fluorescence microscopy. Two-photon fluorescence microscopy images of probe-labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP8 MP) with a ×100 (NA = 1.30 OIL) and ×10 (NA = 0.30 DRY) objective lens, respectively. The two-photon fluorescence microscopy images were obtained with a DMI6000BMicroscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Mai Tai HP; Spectra Physics, 80 MHz pulse frequency, 100 fs pulsewidth) set at wavelength 900 nm and output power 3050 mW, which corresponded to approximately 15mW average power in the focal plane. To obtain images at 400-600 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 512 × 512 pixels at 400 Hz scan speed.

Preparation and staining of fresh rat Hippocampal slices. Slices were prepared from the hippocampi of 2-weeks-old rat (SD). Coronal slices were cut into 400 μm-thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; 124 mMNaCl, 3 mMKCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM D-glucose, 2.4 mM $CaCl_2$, and 1.3 mM $MgSO_4$). Slices were incubated with 50 μMMNDA in ACSF bubbled with 95% O_2 and 5% CO_2 for 50min at 37 °C. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (MatTek) and observed in a spectral confocal multiphoton microscope. To assess the effect of NMM, the slices were treated with 200 μM of NMM for 30 min before MNDA was added.

Synthetic route of probe MNDA and FNDA



Reagents and conditions: (a) ethylene glycol, p-toluenesulfonic acid, toluene, reflux; (b) n-BuLi/DMF, THF, -78°C, N_2; (c) $FeCl_3.6H_2O$, acetone or 10% HCl, r.t; (d) PEt_3, dimethylmaleate, DBU, CH_2Cl_2; (e) diisobutylaluminium hydride solution, -50°C; (f) DMSO, $(COCl)_2$, -78°C, N_2.

Compound 3 was synthesized as described previously (M. Gravel, et al. *Org. Lett*., 2010, **12**, 5772.).

General synthetic method for diethyl 6-substituent naphthalene-2,3-dicarboxylate (4)

Triethylphosphine (2.8 mmol, 330 mg) was added dropwise to a CH_2Cl_2 solution of diethylmaleate (2.6 mmol, 447 mg) with stirring at 0 °C under N_2 protection. The mixture was stirred at room temperature for 30 minutes. The above solution was added dropwise to a CH_2Cl_2 solution of phthalaldehyde (2 mmol, 320 mg) with stirring at 0 °C. After completing the above addition, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (0.2 mmol, 31 mg) was added to the mixture. The reaction was stirred for an additional 5 h and then washed with water and saturated NaCl solution. The organic layer was dried by anhydrous magnesium sulfate. The solvent was removed, and the residue was purified by column chromatography to obtain the desired compound as a colourless oil.

For 6-methoxynaphthalene-2,3-dicarboxylate: ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.25 (s, 1H), 8.09 (s, 1H), 7.84 (d, *J* = 9.0 Hz, 1H), 7.30–7.27 (m, 2H), 7.20 (d, *J* = 3.0 Hz, 1H), 4.43 (q, *J* = 4.2 Hz, 4H), 3.96 (s, 3H), 1.40 (t, *J* = 4.2 Hz, 6H.).

For 6-fluoronaphthalene-2,3-dicarboxylate: ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.25 (s, 1H), 8.13 (s, 1H), 7.93-7.89 (m, 1H), 7.53-7.50 (m, 1H), 7.41-7.34 (m, 1H), 4.40 (q, *J* = 7.2 Hz, 4H), 1.39 (t, *J* = 7.2 Hz, 6H).

Synthesis of (6-methoxynaphthalene-2,3-diyl) dimethanol (5)

A diisobutylaluminium hydride solution in 5 mL of toluene was slowly added to a toluene solution of diethyl 6-methoxynaphthalene-2,3-dicarboxylate (1 mmol, 302 mg) at -50°C under N_2 atmosphere. After the completion of the reaction, a saturated solution of sodium potassium tartarate was added, and the reaction mixture was extracted with ethyl acetate three times, followed by drying over anhydrous magnesium sulfate. The solvent was removed under reduced pressure, and the desired product was obtained as a white solid.

For (6-methoxynaphthalene-2,3-diyl) dimethanol: ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.80–7.77 (m, 3H), 7.28 (d, *J* = 3.0 Hz, 1H), 7.10 (dd, *J* = 3.0 Hz, 1H), 5.23–5.14 (m, 2H), 4.69–4.64 (m, 4H), 3.86 (m, 3H).

For (6-fluoronaphthalene-2,3-diyl) dimethanol: ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.99–7.90 (m, 3H), 7.68 (dd, *J*1 = *J*2 = 2.4 Hz, 1H), 7.39–7.32 (m, 1H), 5.32–5.25 (m, 2H), 4.69 (t, *J* = 6.0 Hz, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 161.95, 158.73, 139.93, 137.91, 137.88, 133.27, 133.15, 130.67, 130.55, 129.59, 125.36, 124.66, 124.59, 116.08, 115.75, 110.96, 110.69, 60.90, 60.87.

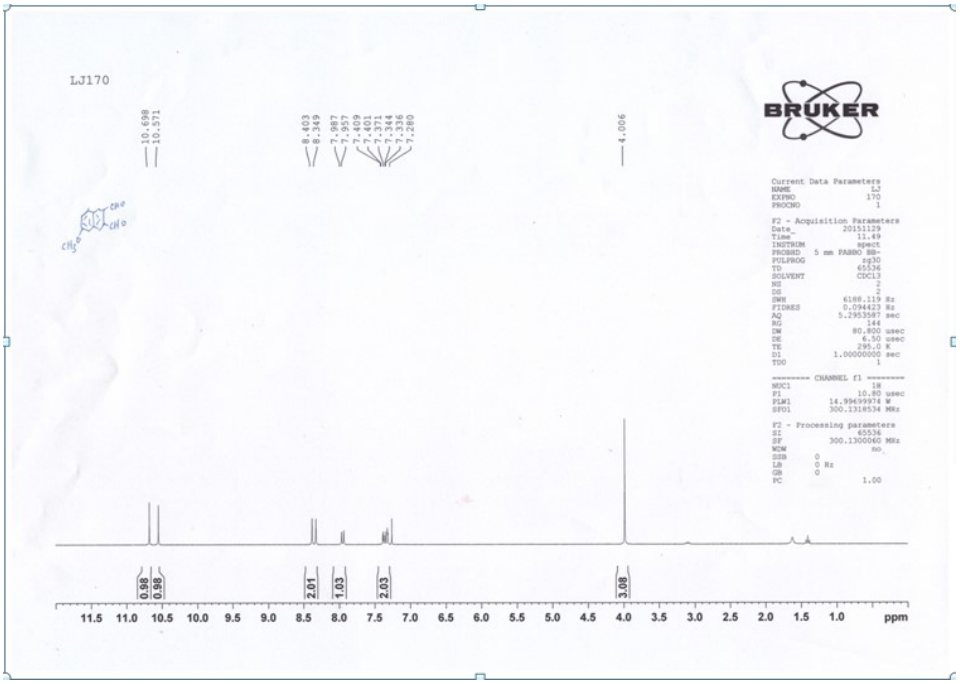
Synthesis of the substituent naphthalene-2,3-dicarbaldehyde

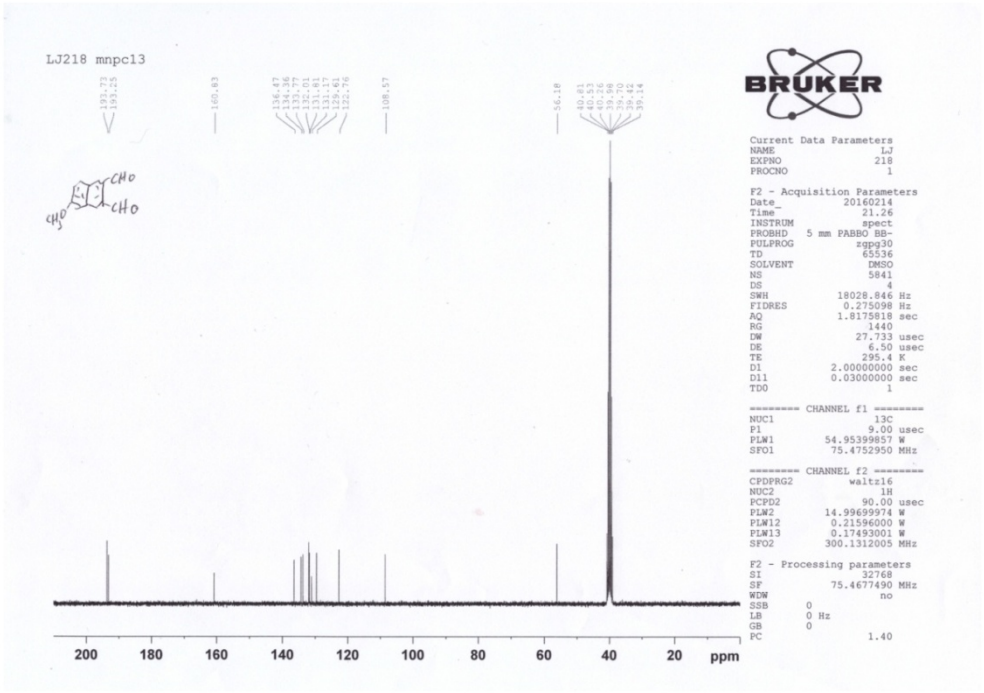
A solution of 5 mL of DMSO and 10 mL of CH_2Cl_2 was added dropwise to a CH_2Cl_2 solution of oxalyl chloride (3.5 mmol, 441 mg) with stirring at -78°C under N_2. The resulting solution was stirred for an additional 30 minutes. Subsequently, a solution of

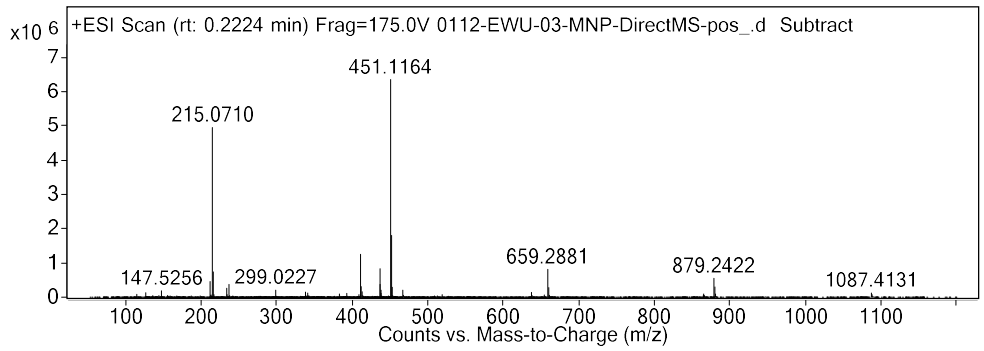
(6-substituent naphthalene-2,3-diyl) dimethanol in the mixture of 5 mL of DMSO and 10 mL of CH_2Cl_2 was added dropwise to the above mixture. Then, the reaction was stirred overnight and quenched with 5 mL of Et_3N. Water was added, and the mixture was extracted with CH_2Cl_2, followed by drying over anhydrous magnesium sulfate. The solvent was removed, and the residue was purified by column chromatography to obtain the desired compound as a white solid.

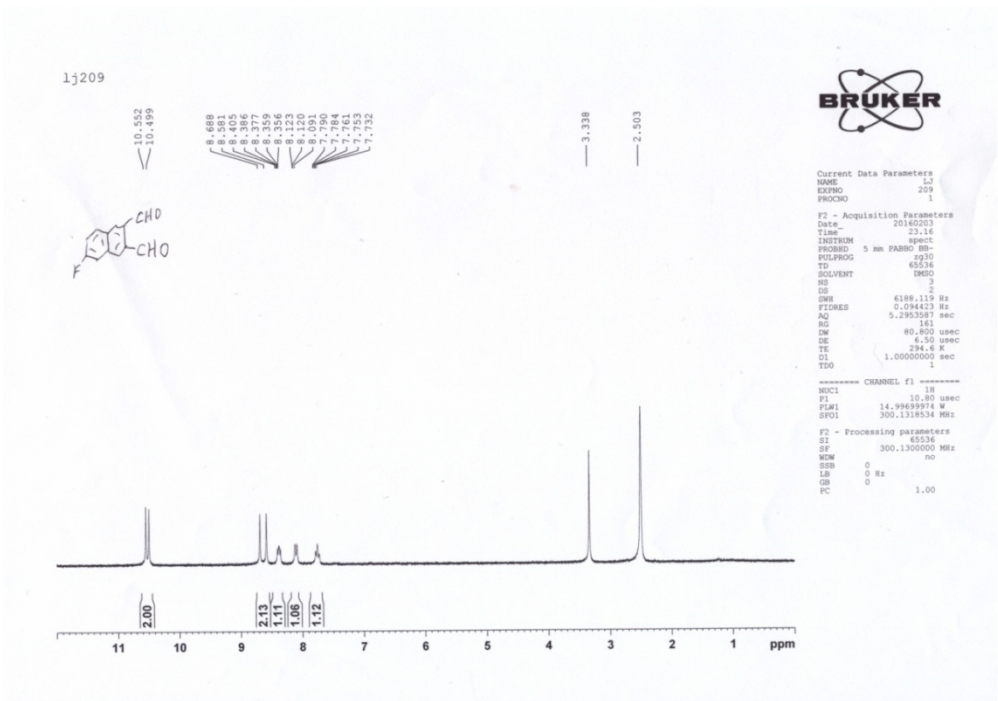
For 6-methoxynaphthalene-2,3-dicarbaldehyde: ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.70 (s, 1H), 10.57 (s, 1H), 8.38 (d, *J* = 16.2 Hz, 2H), 7.97 (d, *J* = 9.0 Hz, 1H), 7.41–7.34 (m, 2H), 4.00 (m, 3H). ^{13}C NMR (75 MHz, DMSO-d_6):δ193.73, 193.25, 160.83, 136.47, 134.36, 133.77, 132.01, 131.81, 131.17, 129.61, 122.76, 108.57, 56.18. HRMS calcd for [M+H$^+$]: 215.0708, found: 215.0710.

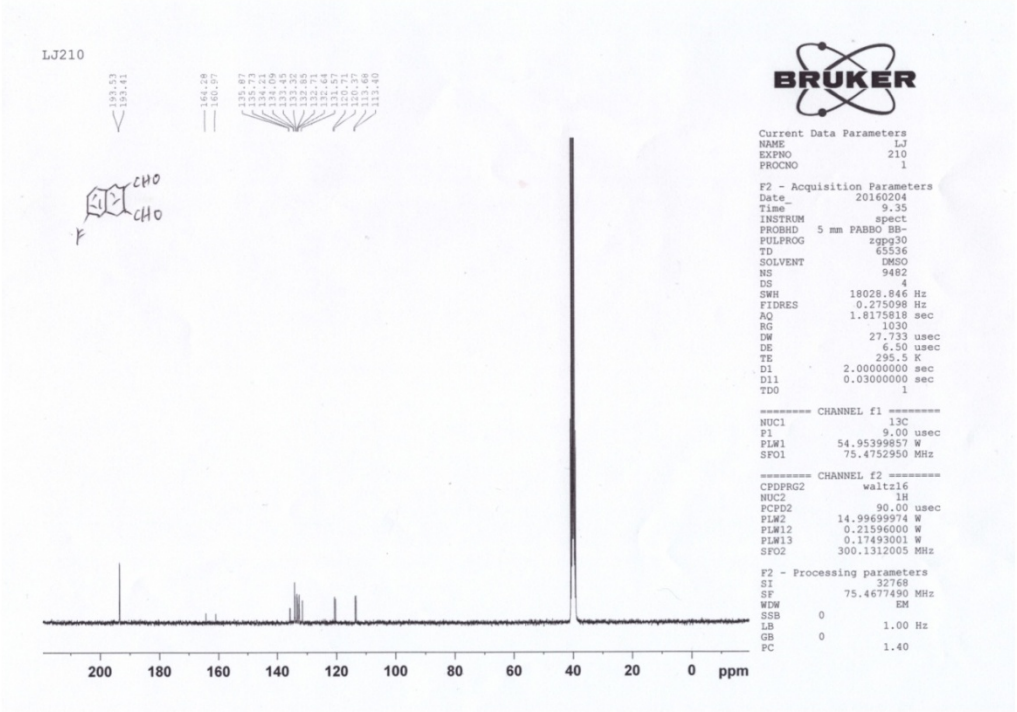
For 6-fluoronaphthalene-2,3-dicarbaldehyde: ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.55 (s, 1H), 10.50 (s, 1H), 8.69 (s, 1H), 8.58 (s, 1H), 8.40–8.36 (m, 1H), 8.12–8.09 (m, 1H), 7.79–7.73 (m, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 193.53, 193.41, 164.28, 160.97, 135.87, 135.73, 134.21, 134.09, 133.45, 133.32, 132.85, 132.71, 132.64, 131.57, 120.71, 120.37, 113.68, 113.40. HRMS calcd for [M+H$^+$]: 203.0508, found: 203.0504.

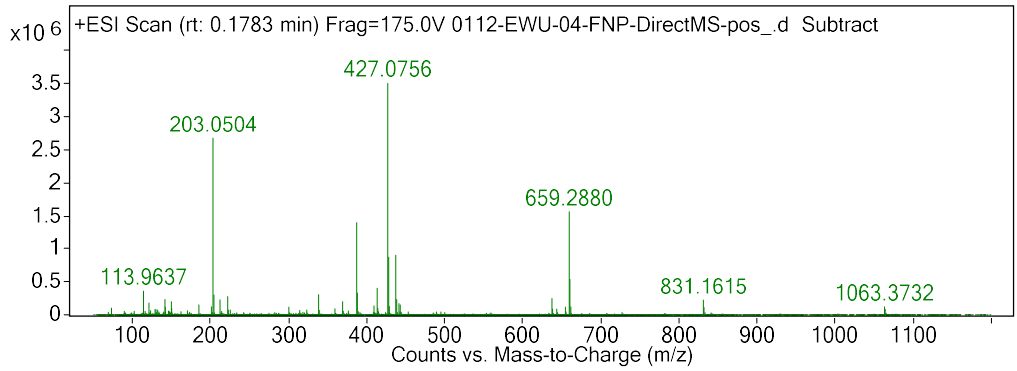



LJ218 mnpc13
BRUKER
Current Data Parameters
NAME LJ
EXPNO 218
PROCNO 1
F2 - Acquisition Parameters
Date_ 20160214
Time 21.26
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 5841
DS 4
SWH 18028.846 Hz
FIDRES 0.275098 Hz
AQ 1.8175818 sec
RG 1440
DW 27.733 usec
DE 6.50 usec
TE 295.4 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
CHANNEL f1
NUC1 13C
P1 9.00 usec
PLW1 54.95399857 W
SFO1 75.4752950 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 90.00 usec
PLW2 14.99699974 W
PLW12 0.21596000 W
PLW13 0.17493001 W
SFO2 300.1312005 MHz
F2 - Processing parameters
SI 32768
SF 75.4677490 MHz
WDW no
SSB 0
LB 0 Hz
GB 0
PC 1.40
ppm


+ESI Scan (rt: 0.2224 min) Frag=175.0V 0112-EWU-03-MNP-DirectMS-pos_.d Subtract
x10 6
451.1164
215.0710
147.5256
299.0227
659.2881
879.2422
1087.4131
Counts vs. Mass-to-Charge (m/z)


lj209
BRUKER
Current Data Parameters
NAME LJ
EXPNO 209
PROCNO 1
F2 - Acquisition Parameters
Date_ 20160203
Time 23.16
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 65536
SOLVENT DMSO
NS 3
DS 2
SWH 6188.119 Hz
FIDRES 0.094423 Hz
AQ 5.2953587 sec
RG 161
DW 80.800 usec
DE 6.50 usec
TE 294.6 K
D1 1.00000000 sec
TD0 1
CHANNEL f1
NUC1 1H
P1 10.80 usec
PLW1 14.99699974 W
SFO1 300.1318534 MHz
F2 - Processing parameters
SI 65536
SF 300.1300000 MHz
WDW no
SSB 0
LB 0 Hz
GB 0
PC 1.00
2.00
2.13
1.11
1.06
1.12
ppm





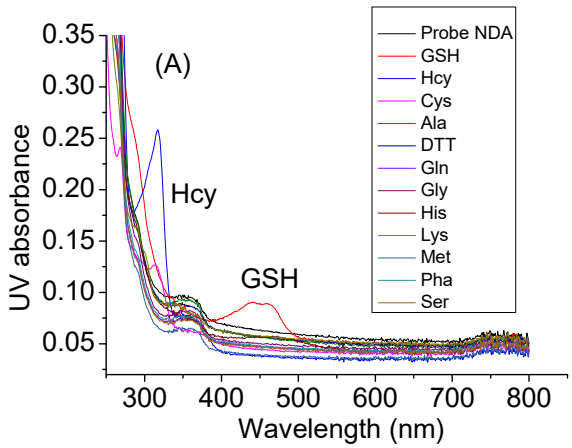


Figure S1. (A) UV absorbance of NDA (10 μM) in the presence of 10 equiv. of amino acid in DMSO: HEPES buffer (1:9, v/v, 0.01 M) at 25 °C after incubation for 30 min.

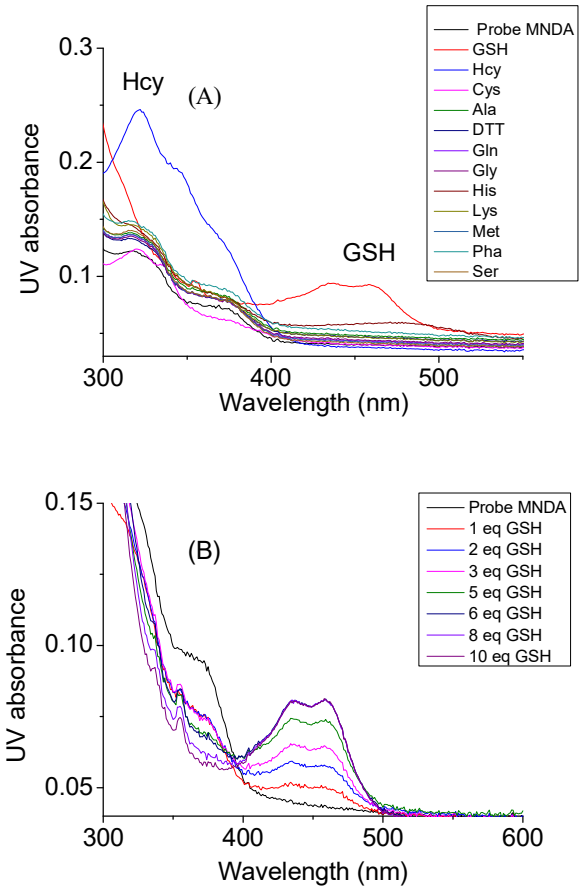
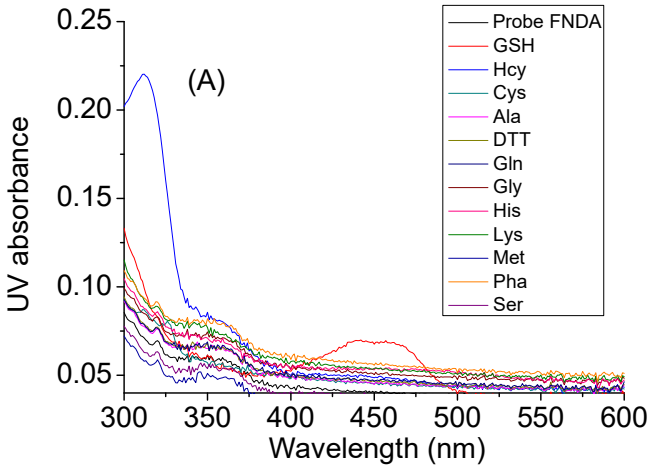


Figure S2. (A) UV absorbance of MNDA (10 μM) in the presence of 10 equiv. of amino acid in DMSO: HEPES buffer (1:9, v/v, 0.01 M) at 25 °C after incubation for 30 min. (B) UV absorbance of MNDA (10 μM) in different concentrations of GSH in DMSO: HEPES buffer (1:9, v/v, 0.01 M) at 25 °C after incubation for 30 min.



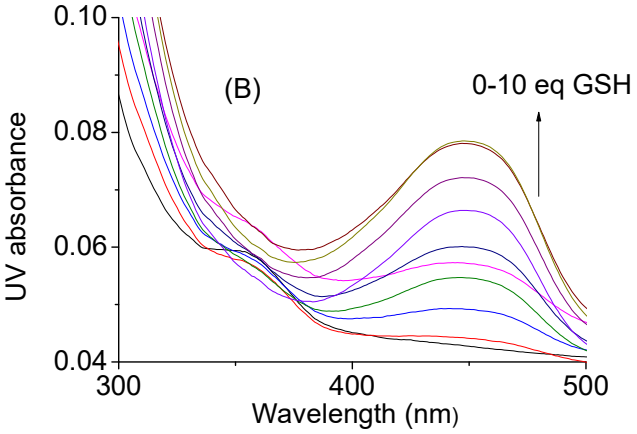


Figure S3. (A) UV absorbance of FNDA (10 μM) in the presence of 10 equiv. of amino acid in DMSO:HEPES buffer (1:9, v/v, 0.01 M) at 25 °C after incubation for 30 min. (B) UV absorbance of FNDA (10 μM) in different concentrations of GSH in DMSO:HEPES buffer (1:9, v/v, 0.01 M) at 25 °C after incubation for 30 min.

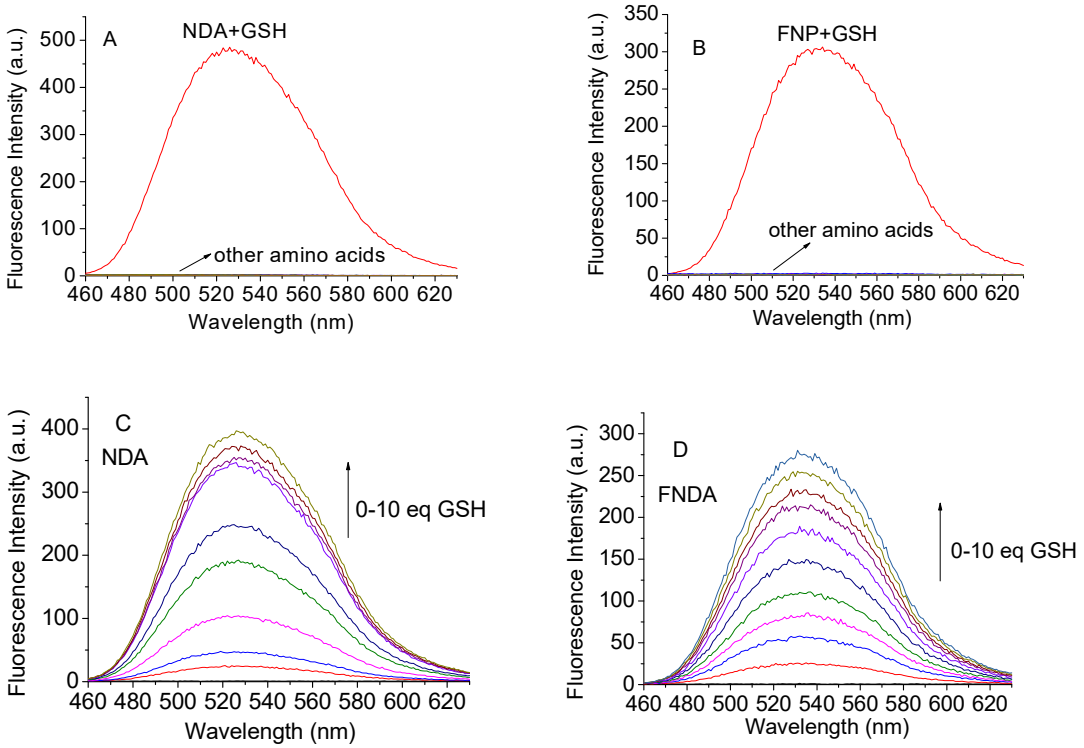


Figure S4. Fluorescence responses of NDA (A) and FNDA (B) (10 μM) to various amino acids (100 μM). Each spectrum was recorded 30 min after addition of the amino acids (including GSH, Cys, Ala, DTT, Gln, Gly, His, Lys, Met, Pha and Ser). Gradient titrations of NDA (C) and FNDA (D) in HEPES buffer solution (0.01 M, containing 10% DMSO, pH = 7.4, excitation: 450 nm, and slit width: 3, 3).

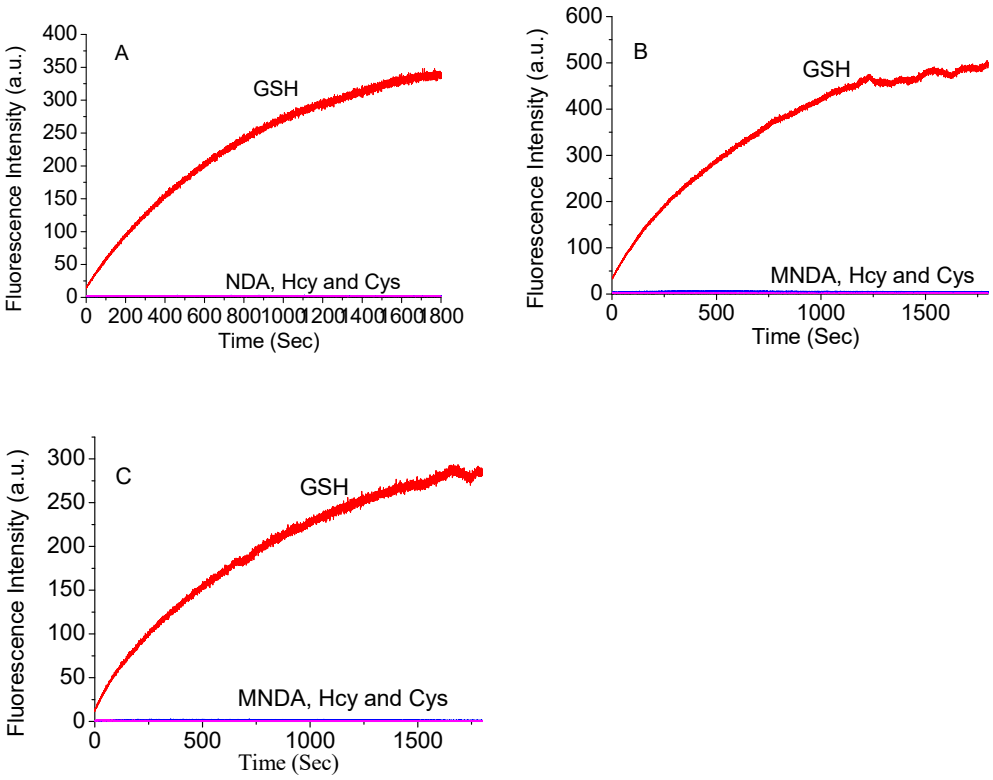


Figure S5. Time dependence of NDA (A), MNDA (B) and FNDA (C) (10 μM) in the presence of 100-μM GSH, Hcy and Cys in HEPES buffer (0.01 M, containing 10% DMSO, pH = 7.4).

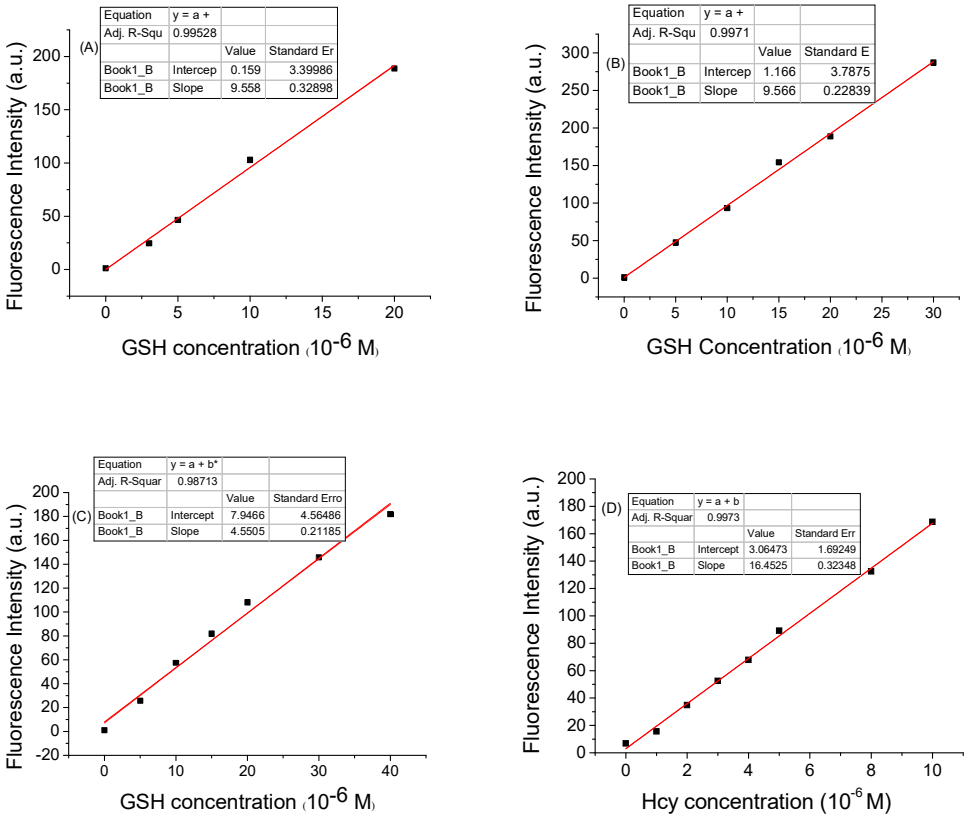


Figure S6. Linear relationships of NDA (A), MNDA (B) and FNDA (C) in the presence of different concentrations of GSH (λ_{ex} = 450 nm, λ_{em} = 531 nm, slit width: 3, 3) and MNDA (D) in the presence of different concentrations of Hcy (λ_{ex} = 350 nm, λ_{em} = 476 nm, slit width: 1.5, 3)

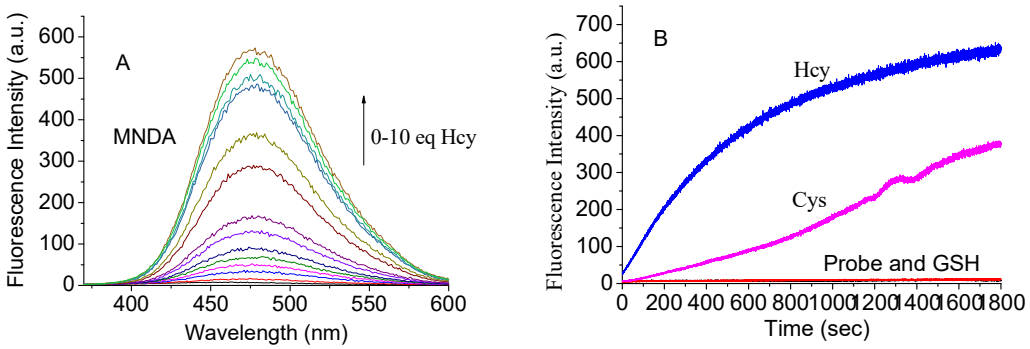
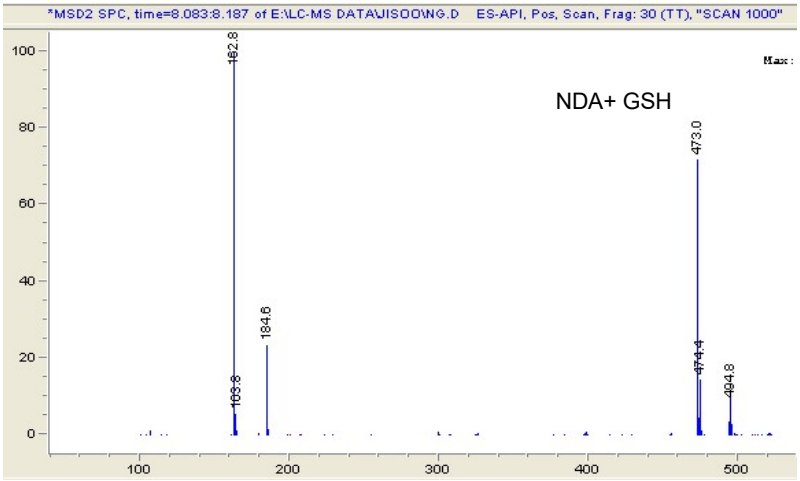
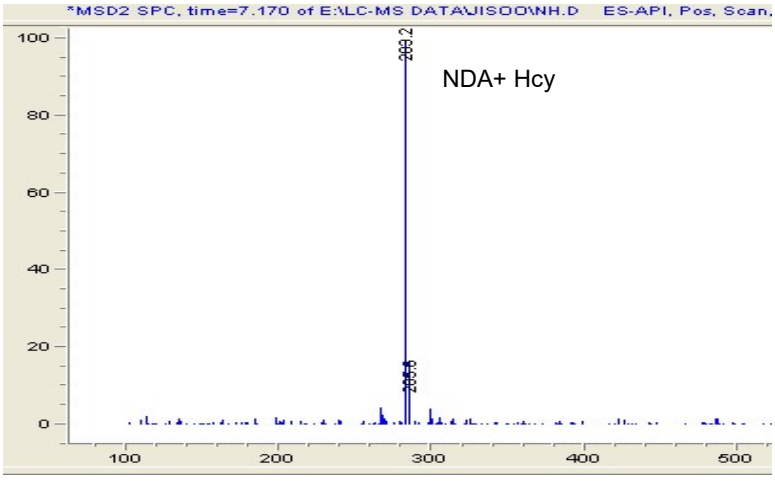
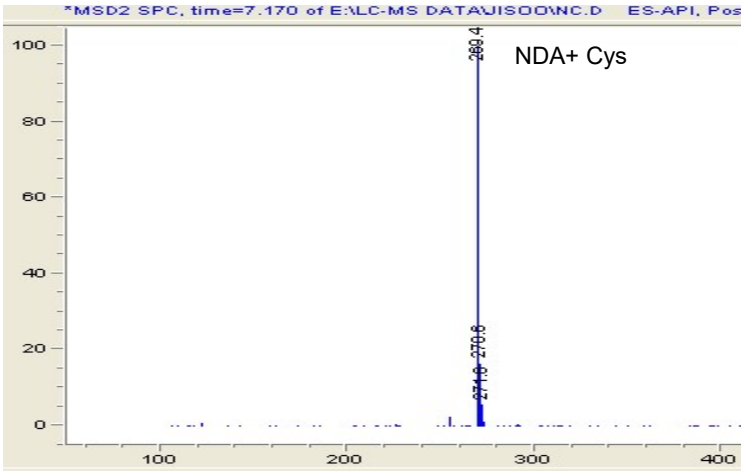
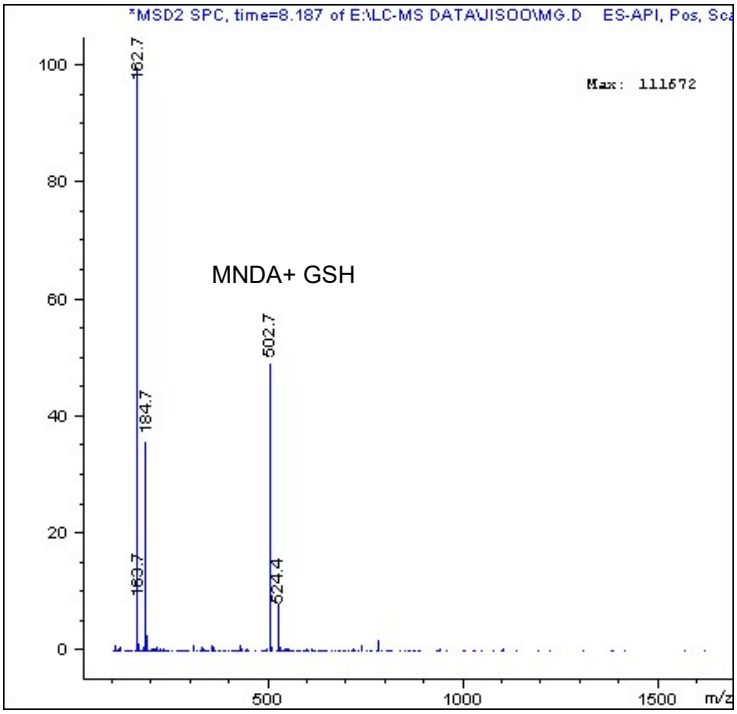


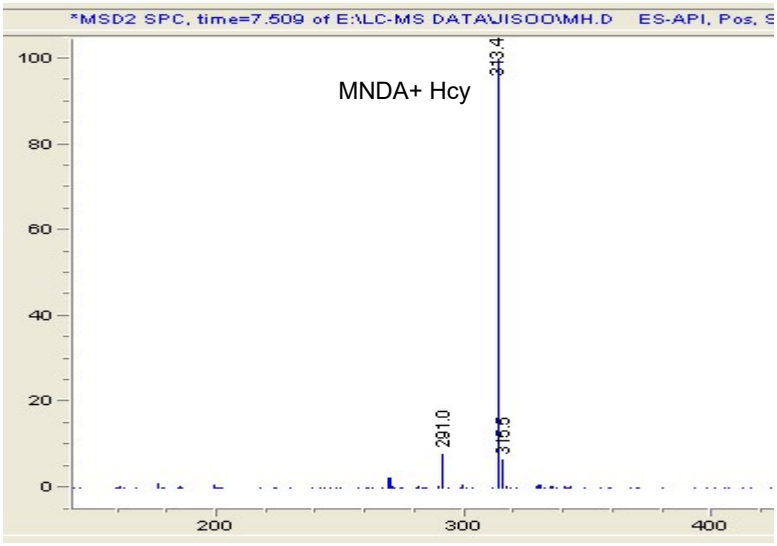
Figure S7. (a) Gradient titration of MNDA in HEPES buffer solution. (b) Time dependence of MNDA(10 μM) in the presence of 100-μM Hcy in HEPES buffer (0.01 M, containing 10% DMSO, pH = 7.4, excitation: 350 nm, and slit width: 1.5, 3).

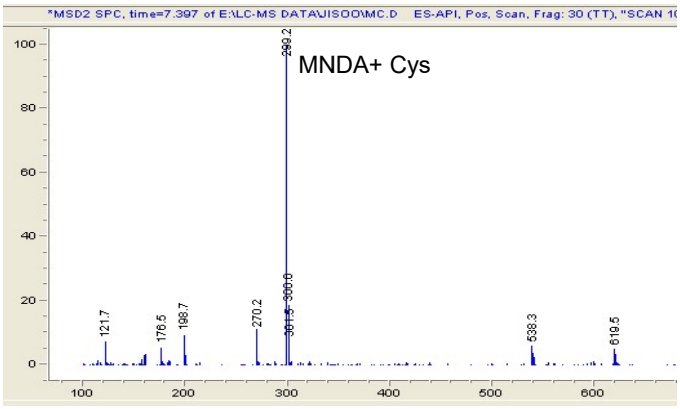


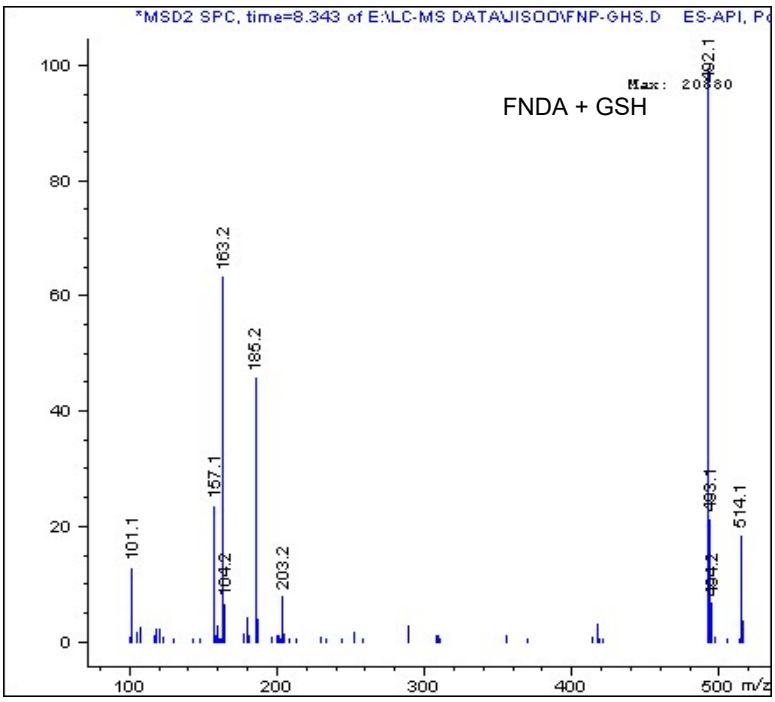


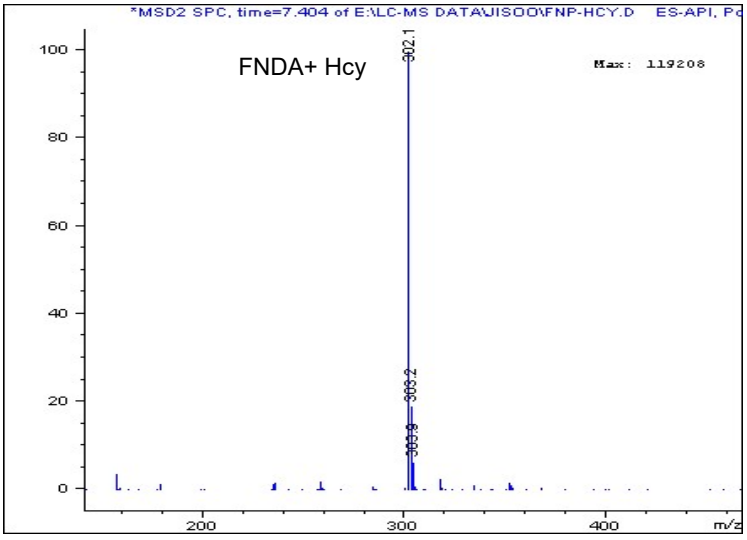

*MSD2 SPC, time=7.170 of E:\LC-MS DATA\JISOO\NC.D ES-API, Pos
NDA+ Cys
269.4
270.8
100
80
60
40
20
0
100
200
300
400


*MSD2 SPC, time=8.187 of E:\LC-MS DATA\JISOO\MG.D ES-API, Pos, Sca
Max: 111572
MNDA+ GSH
162.7
184.7
163.7
502.7
524.4
100
80
60
40
20
0
500
1000
1500
m/z


*MSD2 SPC, time=7.509 of E:\LC-MS DATA\JISOO\MH.D ES-API, Pos, S
MNDA+ Hcy
313.4
291.0
315.5
100
80
60
40
20
0
200
300
400


*MSD2 SPC, time=7.397 of E:\LC-MS DATA\JISOO\MC.D ES-API, Pos, Scan, Frag: 30 (TT), "SCAN 1
MNDA+ Cys
299.2
121.7
176.5
198.7
270.2
301.5
300.6
538.3
619.5


*MSD2 SPC, time=8.343 of E:\LC-MS DATA\JISOO\FNP-GHS.D ES-API, Pc
FNDA + GSH
Max: 20
492.1
163.2
185.2
157.1
101.1
104.2
203.2
493.1
514.1
m/z


*MSD2 SPC, time=7.404 of E:\LC-MS DATA\JISOO\FNP-HCY.D ES-API, Pc
FNDA+ Hcy
Max: 119208
302.1
303.2
m/z

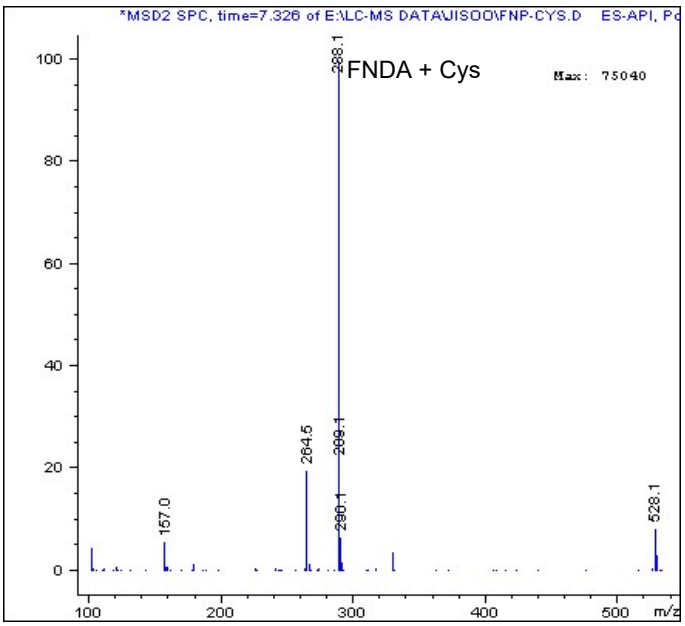
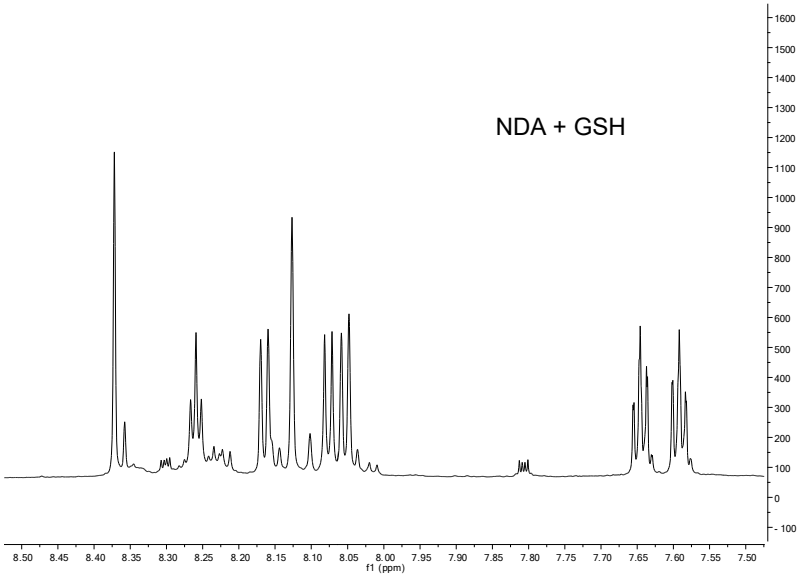
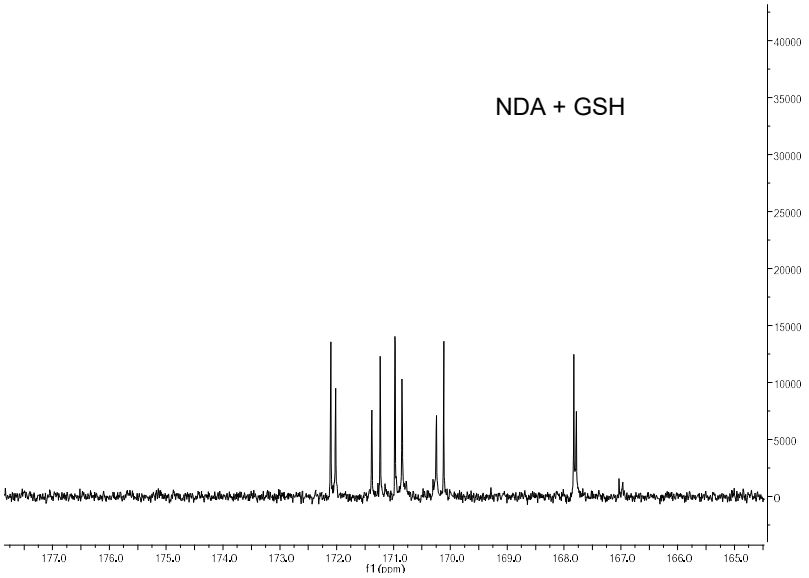
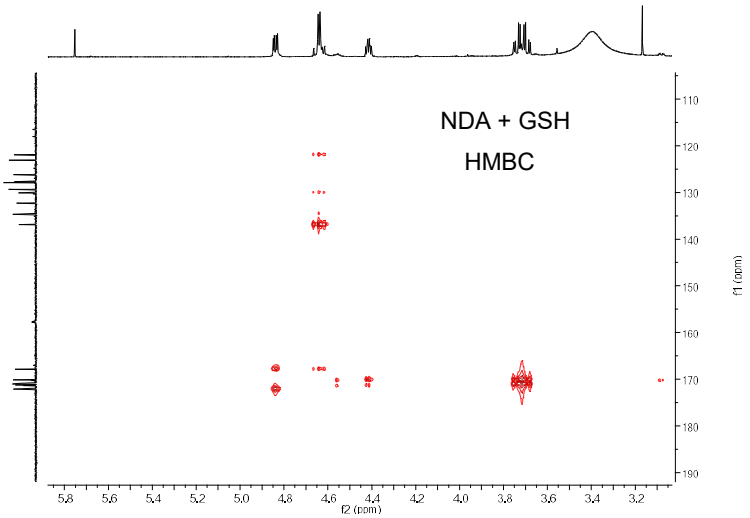


Figure S8. HPLC-MS data of the probes' reactions with GSH, Hcy and Cys.




NDA + GSH
40000
35000
30000
25000
20000
15000
10000
5000
0
177.0
176.0
175.0
174.0
173.0
172.0
171.0
170.0
169.0
168.0
167.0
166.0
165.0
f1 (ppm)


NDA + GSH
HMBC
110
120
130
140
150
160
170
180
190
f1 (ppm)
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
f2 (ppm)

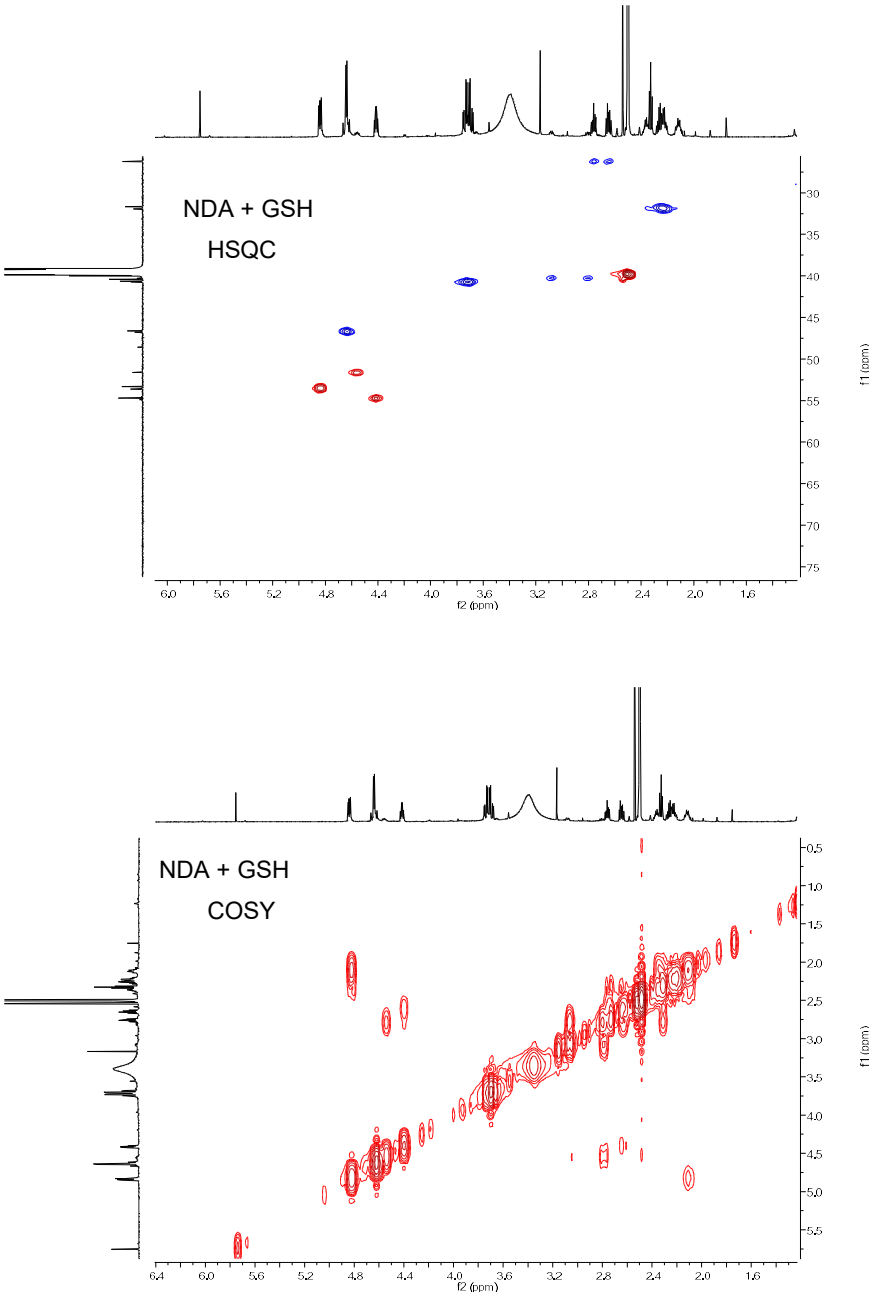
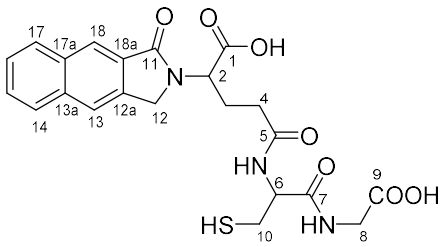


Figure S9. NMR data of the reaction of GSH with NDA.



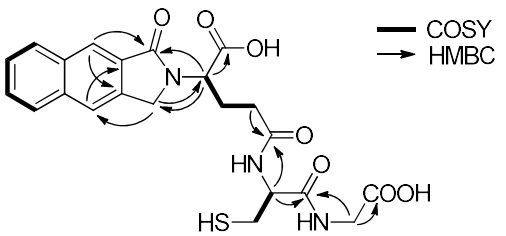


Figure S10. The peak assignments and COSY and key HMBC correlations for the structural elucidation of the product.

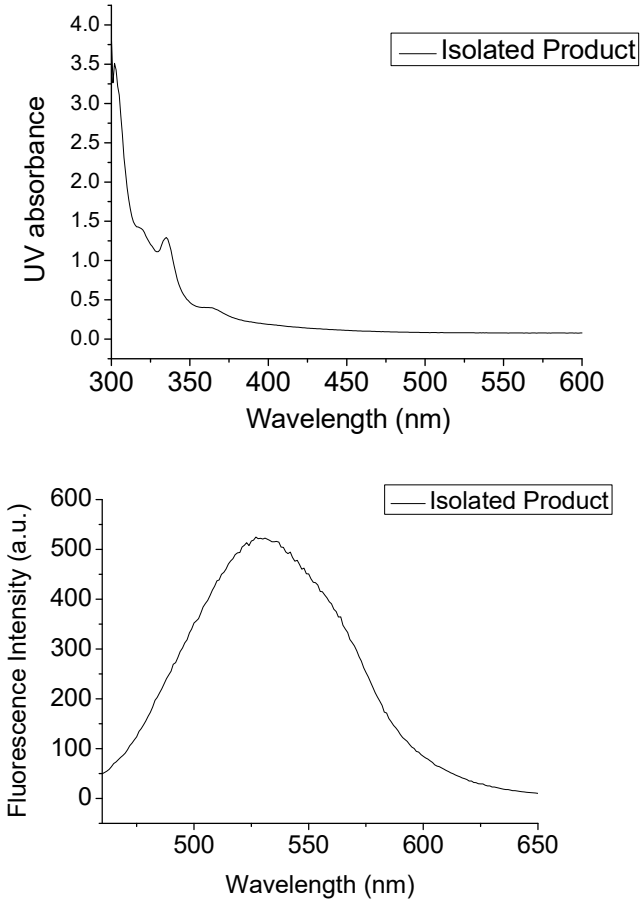


Figure S11. UV absorbance and Fluorescence spectrum of the isolated product of the reaction of GSH with NDA in HEPES buffer. (λ_{ex} = 450 nm, slit width: 5, 5, 1.0 mg of isolated product dissolved in 3 mL of solution [HEPES:DMSO = 1:1]).

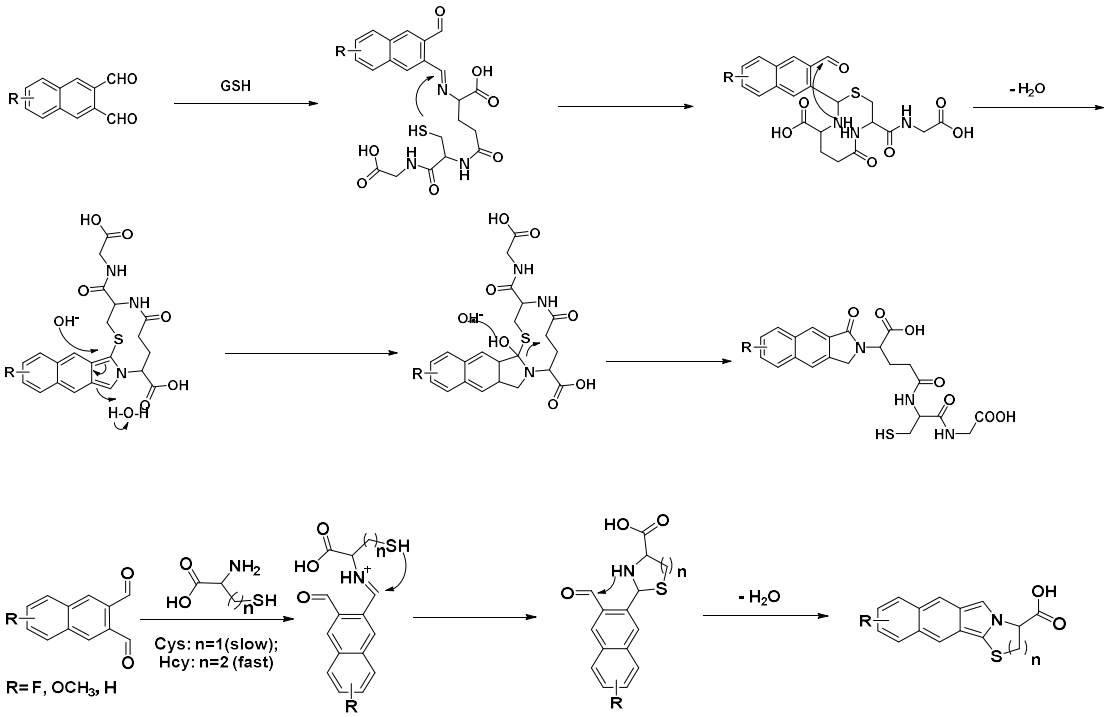


Figure S12. Mechanisms underlying the reactions of the probes with GSH, Hcy/Cys.

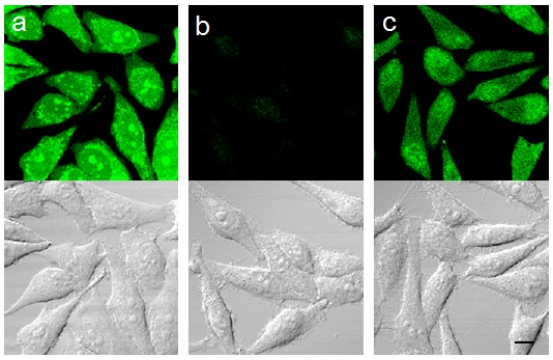


Figure S13. Fluorescence properties in the cells. (a) HeLa cells were incubated with 3 μM NDA for 30 min; (b) pretreated with 1 mM NMM for 30 min and incubated with 3 μM NDA for 30 min; (c) pretreated with 1 mM NMM for 30 min and then treated with 1 mM GSH-MEE for 60 min, followed by incubation with 3 μM NDA. Ex. 473 nm/em. 490-590 nm. Scale bar: 10 μm.

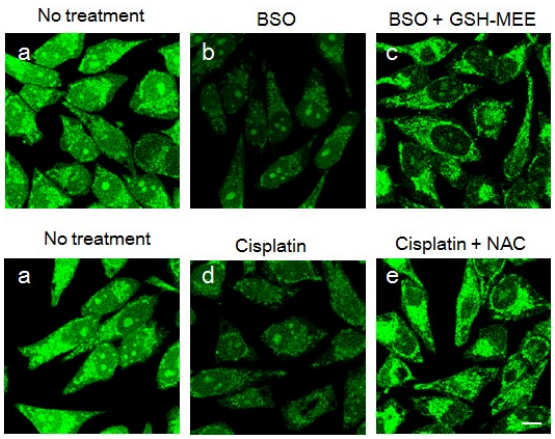


Figure S14. Changes in the intracellular GSH level after drug treatment. (a) HeLa cells were incubated with 3 μM NDA; (b) 100 μM BSO without 1 mM GSH-MEE for 6 hr and stained with 3 μM NDA for 30 min; (c) 100 μM BSO with 1 mM GSH-MEE for 6 hr and stained with 3 μM NDA for 30 min; (d) HeLa cells were incubated with 50 μM cisplatin without 2 mM NAC for 6 hr and stained with 3 μM NDA for 30 min; (e) HeLa cells were incubated with 50 μM cisplatin with 2 mM NAC for 6 hr and stained with 3 μM NDA for 30 min; Ex. 473 nm/em. 490-590 nm. Scale bar: 10 μM.

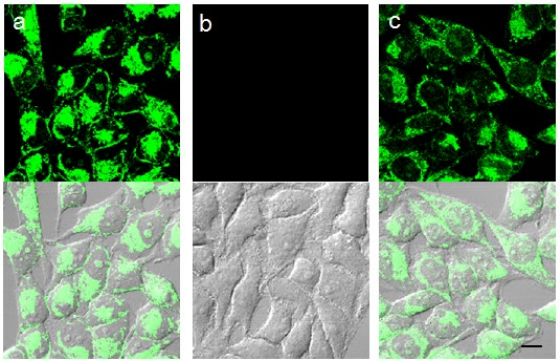


Figure S15. Fluorescence properties of FNDA in the cell. (a) HeLa cells were incubated with 3 μM FNDA for 30 min; (b) pretreated with 1 mM NMM for 30 min and incubated with 3μM FNDA for 30 min; (c) and pretreated with 1 mM NMM for 30 min and then treated with 1 mM GSH-MEE for 60 min, followed by incubation with 3 μM FNDA (c). Ex. 473 nm/em. 490-590 nm. Scale bar: 10 μm.

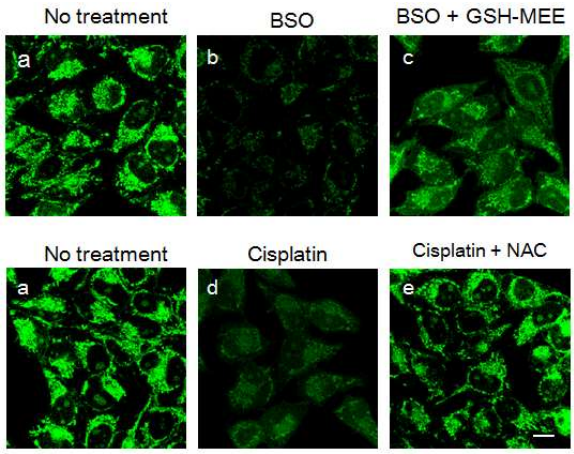


Figure S16. Changes in the intracellular GSH level after drug treatment. (a) HeLa cells were incubated with 3 μM FNDA; (b) 100 μM BSO without 1 mM GSH-MEE for 6 hr and stained with 3 μM FNDA for 30 min; (c) 100 μM BSO with 1 mM GSH-MEE for 6 hr and stained with 3 μM FNDA for 30 min; (d) HeLa cells were incubated with 50 μM cisplatin without 2 mM NAC for 6 hr and stained with 3 μM FNDA for 30 min; (e) HeLa cells were incubated with 50 μM cisplatin with 2 mM NAC for 6 hr and stained with 3 μM FNDA for 30 min; Ex. 473 nm/em. 490-590 nm. Scale bar: 10 μM.

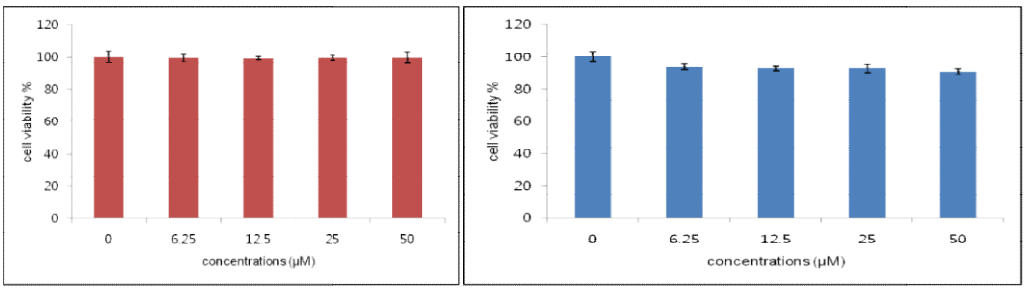
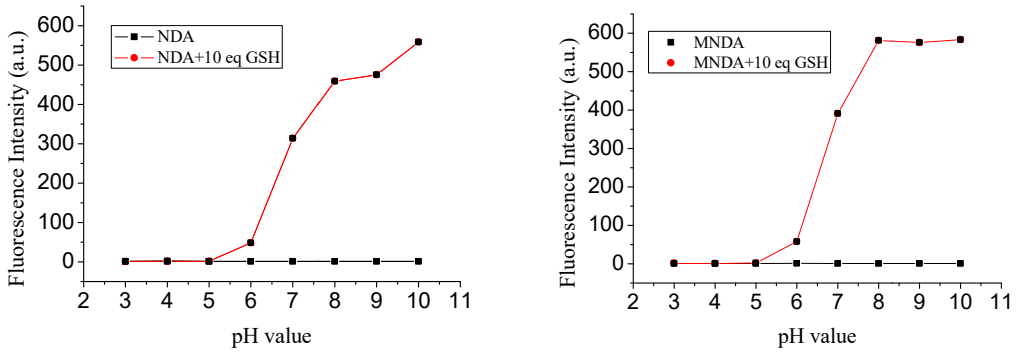


Figure S17. HeLa cells were incubated with each probe concentration (left: MNDA; right: FNDA) for 24 hr.



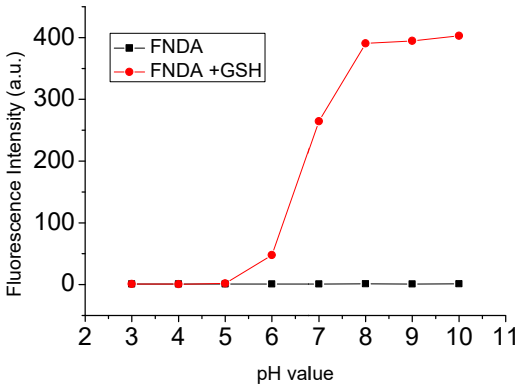


Figure S18. pH dependence of the NDA, MNDA and FNMA with 10 equiv. of GSH in DMSO:HEPES buffer (1:9, v/v, 0.01 M) at 25°C after incubation for 30 min. (λ_{ex} = 450 nm, λ_{em} = 531 nm, slit width: 3, 3)

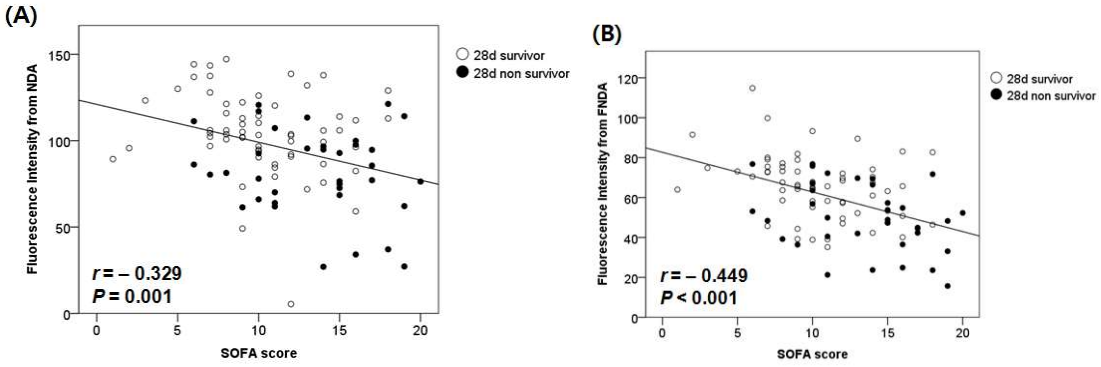
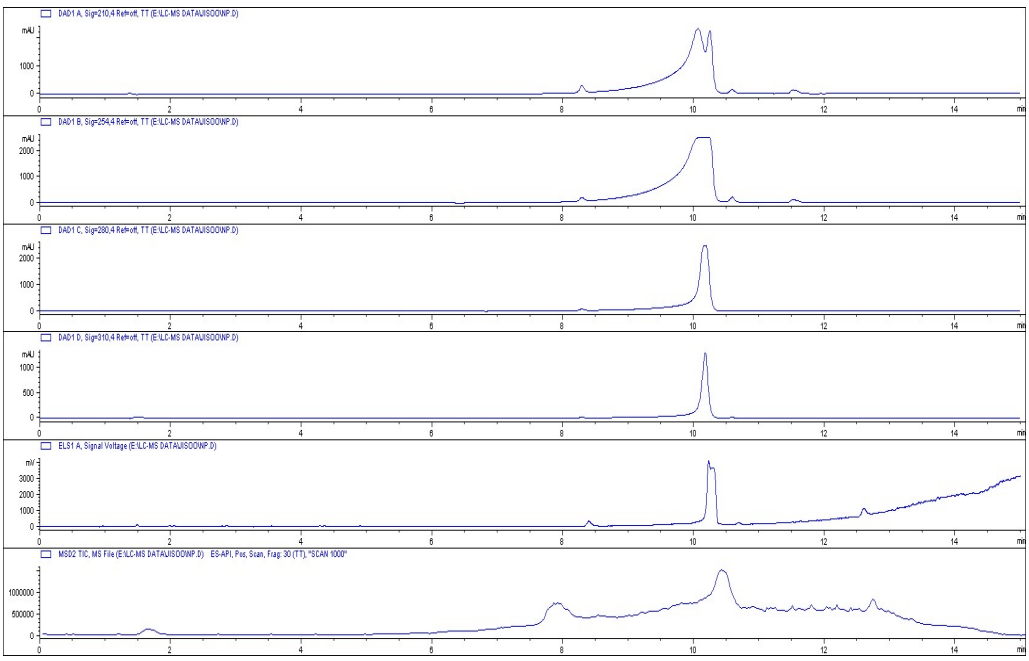


Figure S19. Correlations of the SOFA score with NDA and FNDA. (A) Correlations of the SOFA score with NDA. (B) Correlations of the SOFA score with FNDA.

NDA



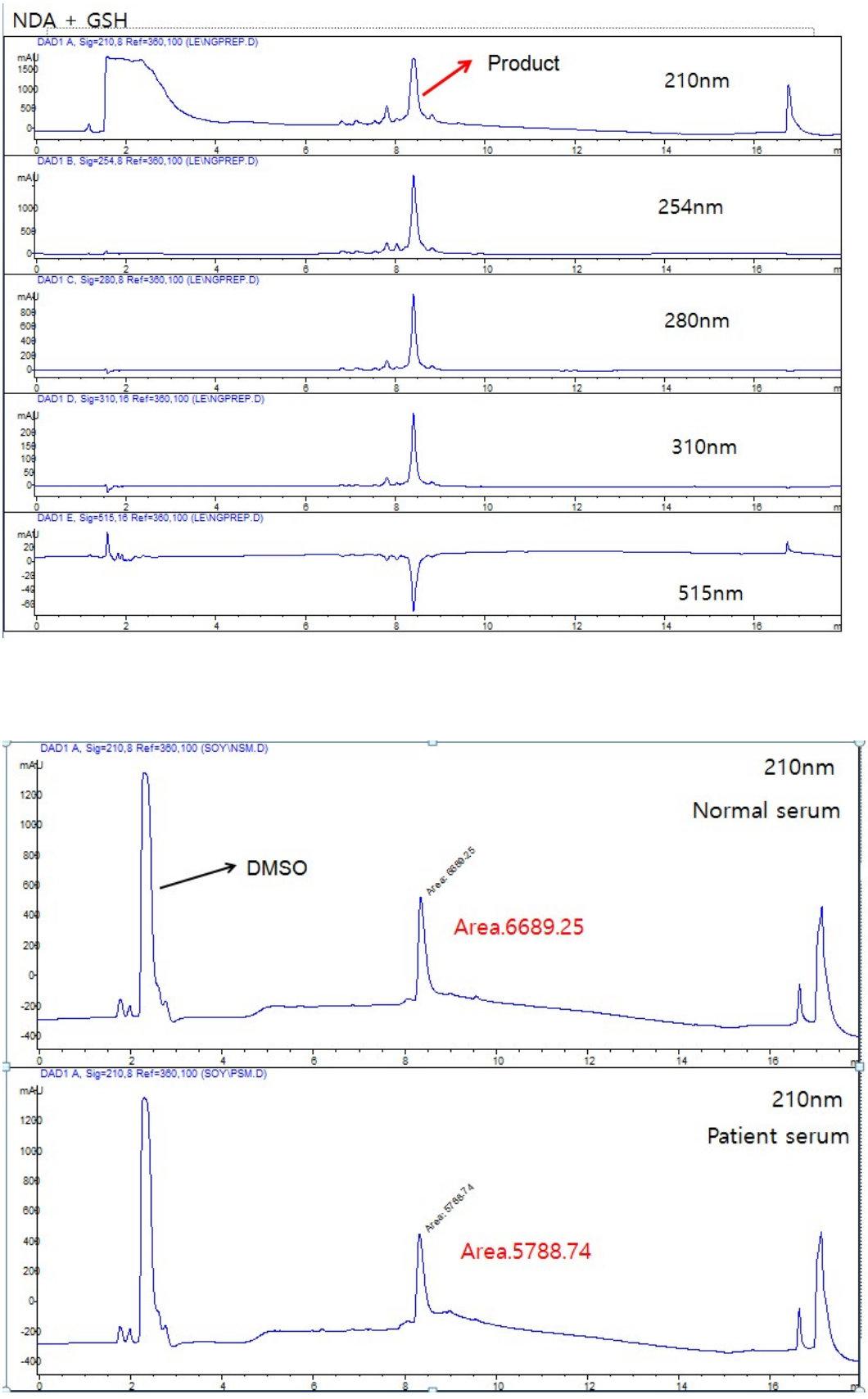


Figure 20. HPLC data of Probe NDA, NDA with GSH in DW solution and probe NDA in serum

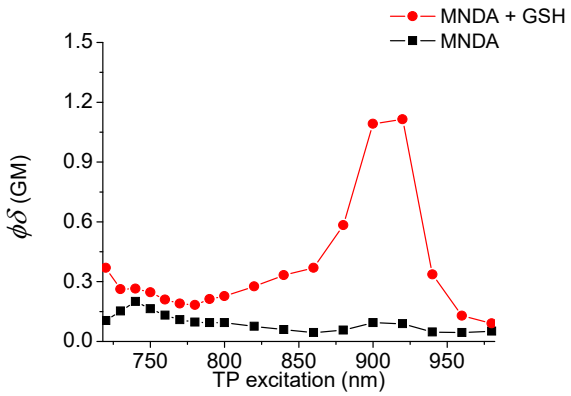


Figure S21. Two-photon excitation spectrafor MNDA in the absence and presence of100 μM GSH inHEPES buffer.

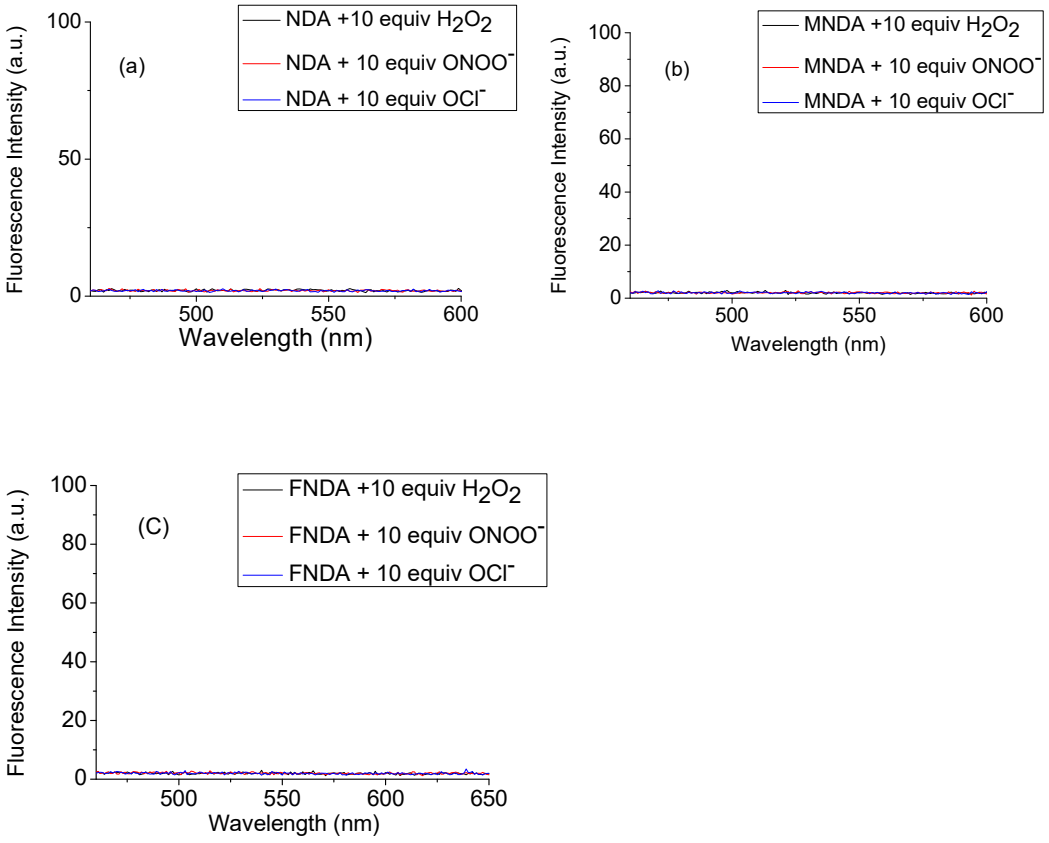


Figure S22. Fluorescence response of probes to 10 equiv.ROS (a: NDA, b: MNDA, C: FNDA; λ_{ex} = 450 nm, slit width: 3, 3).

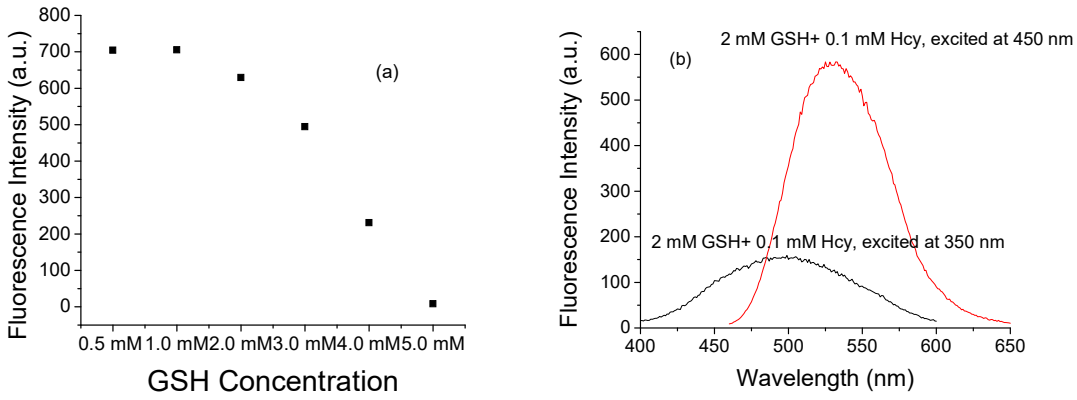


Figure S23. (a) Fluorescence spectrum of MNDA (10 μM) with high concentrations of GSH; excitation: 450 nm, emission: 531 nm; (b) Fluorescence response of MNDA with Hcy in the present of GSH both in blue (excitation: 350 nm) and green channel (excitation: 450 nm).

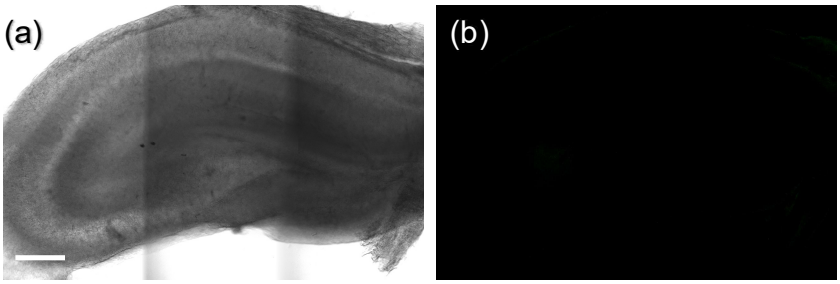
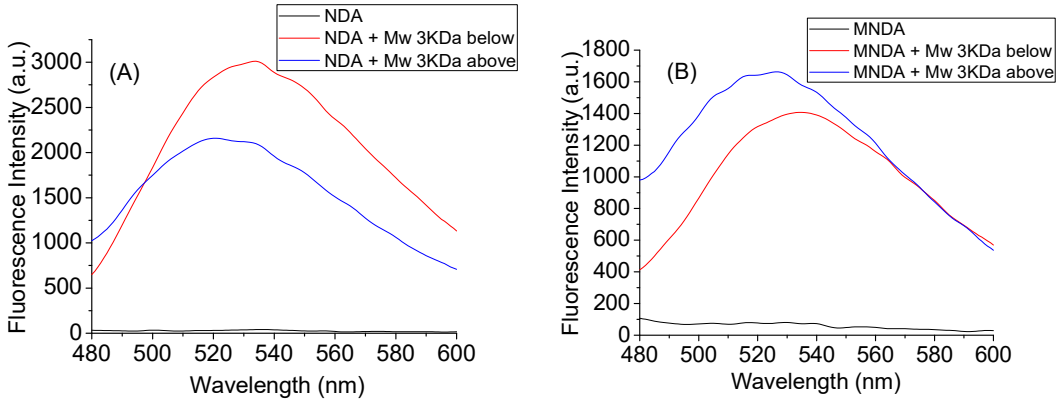


Figure S24. (a) Bright field image and (b) TPM image of a fresh rat hippocampal slice without incubation of the probe with magnification at 10×. The TPEF were collected at 400-600 nm upon excitation at 900 nm with fs pulse. Scale bar, 300 μm.



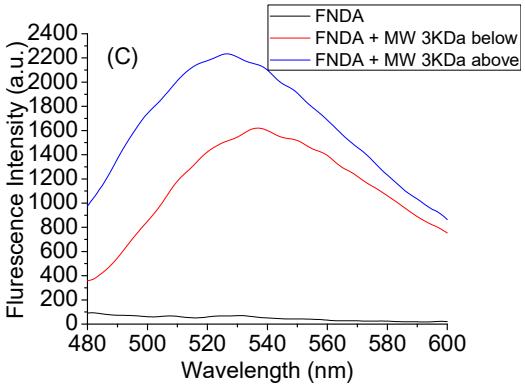


Figure S25. Fluorescence spectrum of probe NDA (A), MNDA (B) and FNDA (C) in the present of cell proteins.

(Table S1). Baseline characteristics of all participants, including the site of infection and laboratory values of sepsis patients and both the cytokine levels and NDA and FNDA fluorescence intensities in healthy controls and sepsis patients. The values are expressed as the median (interquartile range [IQR]) unless otherwise indicated.

		Healthy controls	Sepsis patients	P-value
		(n = 15)	(n = 97)	
Age, year		26.0 (24.0–32.0)	71 (62.0–77.0)	<0.001
Male		9 (60.0)	70 (72.2)	0.369
Site of infection				
	Pulmonary	-	60 (61.9)	
	Hepatobiliary	-	9 (9.3)	
	GI tract	-	13 (13.4)	
	Urogenital	-	8 (8.2)	
	Others	-	7 (7.2)	
Laboratory values				
	WBCs (109/L)	-	11.9 (5.7–20.1)	
	Platelets (109/L)	-	113.0 (52.0–196.0)	
	Bilirubin (mg/mL)	-	0.7 (0.4–1.4)	
	Creatinine (mg/dL)	-	1.4 (0.8–2.6)	
	CRP (mg/L)	-	93.2 (41.3–145.8)	
	PCT (ng/mL)	-	1.9 (0.4–13.4)	
	IL-1beta (pg/mL)	36.0 (12.8–68.5)	16.5 (12.0–32.8)	0.645

	IL-10 (pg/mL)	9.0 (8.0–13.3)	46.5 (20.8–225.5)	0.276
	IL-18 (pg/mL)	206.5 (167.8–247.0)	537.5 (342.6–1061.0)	<0.001
	IL-6 (pg/mL)	44.0 (40.5–47.5)	875.5 (243.6–7297.9)	<0.001
	IP-10 (pg/mL)	158.8 (122.3–174.0)	1044.5(311.8–3077.3)	<0.001
	TNF-alpha (pg/mL)	58.8 (25.5–123.0)	43.8(29.6–87.3)	0.700
NDA		173.9 (153.0–175.5)	97.7 (79.7–113.2)	<0.001
FNDA		88.1 (80.2–118.7)	63.6 (47.2–72.5)	<0.001

(Table S2) Baseline characteristics of 28-d survivors and 28-d non-survivors: fluorescence intensities from NDA and FNDA and APACHE II and SOFA scores in 28-d survivors and 28-d non-survivors. The values are expressed as the median (IQR) unless otherwise indicated.

		Sepsis		
		28d-survivor	28d-non survivor	P-value
		(n = 61)	(n = 36)	
Age, year		71.0 (62.0–77.0)	70.5 (63.0–77.8)	0.778
Male		46 (75.4)	24 (66.7)	0.415
Site of infection				
	Pulmonary	37 (60.7)	23 (63.9)	0.441
	Hepatobiliary	5 (8.2)	4 (11.1)	
	GI tract	9 (14.8)	4 (11.1)	
	Urogenital	7 (11.5)	1 (2.8)	
	Others	3 (4.9)	4 (11.1)	

Positive Blood culture, n (%)		20 (32.8)	10 (28.6)	0.820
NDA		103.8 (93.1–120.8)	80.8 (66.6–97.5)	<0.001
FNDA		66.3 (56.3–74.5)	48.7 (39.5–65.7)	<0.001
APACHE II		24.0 (17.0–30.0)	30.0 (21.0–37.8)	0.012
SOFA score		10.0 (8.0–12.5)	14.0 (10.0–16.8)	<0.001

(Table S3) The AUC, cut-off, sensitivity and specificity values of NDA, FNDA, the APACHEII score and the SOFA score for the prediction of mortality in sepsis patients

	AUC	95% CI	P-value	Cut-off	Sensitivity	Specificity
APACHE II	0.658	0.542-0.774	0.01	28.5	0.721	0.639
SOFA score	0.724	0.617-0.830	<0.001	12.5	0.754	0.611
NDA	0.758	0.657-0.858	<0.001	95.6	0.772	0.721
FNDA	0.744	0.641-0.846	<0.001	57.6	0.772	0.738