Electronic Supplementary Information (ESI) for

A sensitive two-photon ratiometric fluorescence probe for γ-glutamyltranspeptidase activities detection and imaging in living cells and cancer tissues

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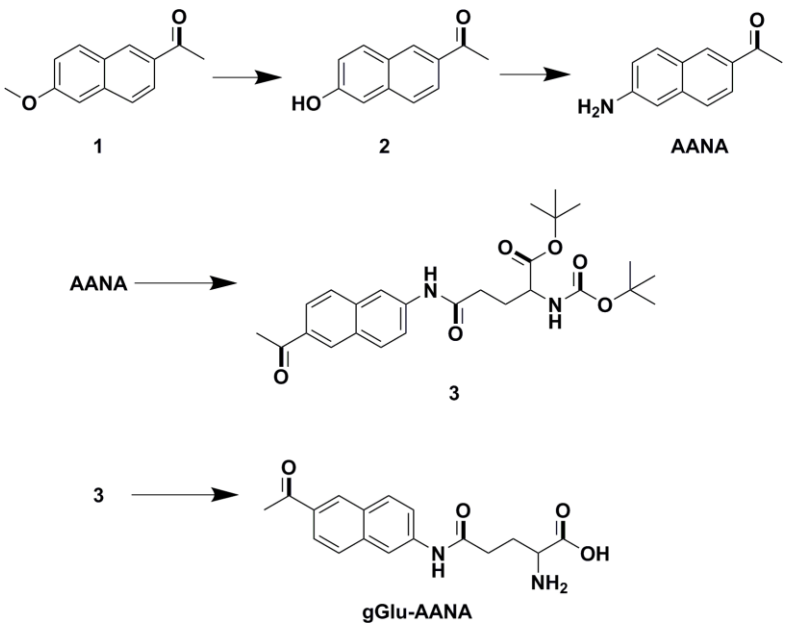
Table of Contents

ADDITIONAL EXPERIMENTAL DETAILS

Materials: Common chemicals were bought from the local commercial suppliers and used without additional purification, unless otherwise noted. Solvents were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China), further purified and dried over 4Å molecular sieves before use. γ-Glutamyltranspeptidase (GGT), GGT inhibitor (6-diazo-5-oxo-L-norleucine, DON) and GGT activities fluorometric assay kit were bought from Sigma-Aldrich (St. Louis, MO, USA). Boc-L-glutamic acid-1-tert-butyl ester (Boc-Glu-OtBu) was obtained from Adamas Reagent Co., Ltd. Trifluoroacetic acid (TFA), 2-(7-aza-1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HATU), and N, N-diisopropylethylamine (DIPEA) were purchased from J&K Chemical Co., Ltd. Thin layer chromatography (TLC) was carried out with silica gel 60 F254, Silica gel (200-300 mesh) was used as the solid phases for column chromatography. Both of them were obtained from Qingdao Ocean Chemicals (Qingdao, China). Compounds were visualized with a 254/365nm, handheld UV lamp (UVP). Water used in all experiments was prepared using ultrapure water which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance >18.25 MΩ. HEK-293T, HepG2, HT-29 and SKOV-3 cells were obtained from the cell bank of Central Laboratory at Xiangya Hospital. The two-photon fluorophore AANA and the probe gGlu-AANA were synthesized following the synthetic methodology shown in Scheme S1.

Instruments: ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker Avance-III 400 instrument. Mass spectrometry (MS) analysis was performed using an LCQ-Advantage ion trap mass spectrometer (Thermo Finnigan). High resolution mass spectrometry (HRMS) was recorded on a LTQ Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). High-performance liquid chromatography (HPLC) analyses were carried out with LC-20 AT solvent delivery unit (Shimadzu, Japan) and Econoshpere C18 column (3.0 μm, 15 cm × 4.6 mm, GL Sciences Inc.). The fluorescence spectra were recorded at room temperature in a quartz cuvette on a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon, Inc., NJ, USA). The UV-vis absorption spectra were plotted using a Shimadzu UV-2450 spectrophotometer. Two-photon excited (TPE) fluorescence spectra were recorded on a DCS200PC single photon counting (Beijing Zolix Instruments Co., Ltd.) which was excited by a mode-locked Ti: sapphire pulsed laser (Chameleon Ultra II, Coherent Inc.). Two-photon fluorescence images of living cells and cancer tissues were acquired on an Olympus FV1000 (TY1318) confocal microscope (Olympus, Japan) with an excitation source of 730 nm, and the emission was collected using blue channel (430-470 nm) and green channel (480-520 nm).

Scheme S1. Synthesis of AANA and gGlu-AANA



Synthesis of 1-(6-hydroxynaphthalen-2-yl)ethanone (2)

Compound 2 was synthesized according to the procedures reported in the literature [S1]. Compound 1 (7.5 g, 25.0 mmol) was dissolved in dichloromethane (DCM, 30 mL), the mixture was added dropwise into 36.0% HCl (500 mL, 4.65 mol) under stirring. Then triethylamine (Et_3N, 5.0 mL, 27.0 mmol) was added dropwise into the solution. The mixture was stirred at 85 °C for 4 .0 h, and the excess acid was neutralized with solid NaOH. The solution was extracted with ethyl acetate and washed with saturated $NaHCO_3$ (aq) and brine. The organic layer was dried with $MgSO_4$ and the solvent was removed in vacuum to gain compound 2 as a pale yellow solid which was pure enough to be used in the next step without further purification.

Synthesis of AANA

AANA was obtained according to the literature method [S2]. In brief, a mixture of compound 2 (6.0 g, 28.0 mmol), $Na_2S_2O_5$ (13.3 g, 70.0 mmol), and NH_4OH (150 mL) in a steel-bomb reactor was stirred at 140 ℃ for 4 days. The product was collected by filtration, washed with water, and then purified by silica gel column chromatography with petroleum ether/ethyl acetate (3/1, v/v) as the eluent to afford AANA as a white solid in 82% yield. ^{1}H-NMR(400 MHz, $CDCl_3$) δ (ppm): 8.32 (s, 1H), 7.94-7.91 (m, 1H), 7.76 (d, *J* = 8.4 Hz, 1H), 7.59 (d, *J* = 8.4 Hz, 1H), 6.98 (d, *J* = 8.4 Hz, 2H), 4.06 (br, 2H), 2.67(s, 3H); ^{13}C-NMR(100 MHz, $CDCl_3$) δ (ppm): 197.80, 146.75, 137.61, 131.45, 131.19, 130.41, 126.50, 125.94, 124.70, 118.71, 107.89, 26.43.

Synthesis of tert-butyl N^5-(6-acetylnaphthalen-2-yl)-N^2-(tert-butoxycarbonyl)glutaminate (compound 3)

A mixture of Boc-Glu-OtBu (304.0 mg, 1.0 mmol), HATU (380.0 mg, 1.0 mmol) and DIPEA (400 μL, 2.5 mmol) were dissolved in 10 mL DCM with stirring at 0 ℃ for 30 minutes, AANA (185.0 mg, 1.0 mmol) in 10 mL DCM was added into the mixture in portions, and the mixture was stirred overnight at room temperature. Then the mixture was concentrated under reduced pressure to give the crude product which was further purified by silica gel column chromatography with petroleum ether/ ethyl acetate (3/, v/v) as the eluent to afford compound 3 as a white solid in 89.9% yield. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 9.43 (s, 1H), 8.39 (s, 2H), 7.99 (d, *J* = 8.8 Hz, 1H), 7.89 (d, *J* = 8.8 Hz, 1H), 7.82 (d, *J* = 8.8 Hz, 1H), 7.64 (d, *J* = 8.8 Hz, 1H), 5.45 (d, *J* = 7.6 Hz, 1H), 4.27 (t, *J* = 7.0 Hz, 1H), 2.71 (s, 3H), 2.53 (t, *J* = 6.4 Hz, 2H), 2.36-2.28 (m, 1H), 1.94-1.89(m, 1H), 1.49 (s, 9H), 1.45 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 198.00, 180.50, 171.14, 156.72, 138.41, 136.41, 133.38, 130.33, 129.80, 129.27, 128.03, 124.45, 120.59, 115.72, 82.83, 80.62, 53.17, 34.27, 30.74, 28.28, 27.91, 26.58. MS (ESI): m/z calcd for $C_{26}H_{34}N_2O_6$ $[M+Na]^+$ 493.2, found 493.2.

Synthesis of gGlu-AANA

Compound 4 (235.0 mg, 0.5 mmol) and trifluoroacetic acid (TFA, 10 mL) in 30 mL DCM were mixed at 0 ℃ and stirred overnight at room temperature. Then the solvent was removed under reduced pressure to afford the crude product, the crude product was washed by ether and DCM, respectively, to obtaining gGlu-AANA as a pale yellow solid in 84.2% yield. ^{1}H-NMR (400 MHz, d_6-DMSO) δ (ppm): 13.97 (s, 1H), 10.43 (s, 1H), 8.58 (s, 1H), 8.38(s, 1H), 8.33 (br, 2H), 8.07 (d, *J* = 9.2 Hz, 1H), 7.95-7.87 (m, 2H), 7.70-7.67 (m, 1H), 4.06-3.97 (m, 1H), 2.68 (s, 3H), 2.65-2.55 (m, 2H), 2.14 (t, *J* = 7.2 Hz, 2H); ^{13}C-NMR (100 MHz, d_6-DMSO) δ (ppm): 198.04, 171.35, 170.89, 139.58, 136.32, 133.36, 130.86, 130.59, 129.13, 128.09, 124.61, 121.09, 115.10, 52.04, 32.11, 27.12, 26.01. MS (ESI): m/z calcd for $C_{17}H_{18}N_2O_4$, $[M+H]^+$ 315.1, found 315.1; HRMS: m/z calcd for 315.1339, found 315.1330.

General procedure for spectra measurement

The stock solution (200 μM) of gGlu-AANA was prepared by dissolving gGlu-AANA in DMSO. The GGT powder was dissolved into ultrapure water to form aqueous solution and was divided into several parts for daily experiments. In order to keep the activity of enzyme, all these enzyme solutions were stored at -20 ℃ in a refrigerator before use according to a reported procedure [S3]. In a test tube, 180 μL PBS buffer (10 mM, pH 7.4) and 10 μL of gGlu-AANA stock solution were mixed, followed by addition of GGT solution or other analysts. The final volume was adjusted to 200 μL. After incubation at 37 ℃ for 30 minutes, 120 μL of the reaction solution was transferred to a quartz cell to record

fluorescence. The excitation wavelength was 355 nm and the emission wavelength was in the range from 380 to 650 nm with both excitation and emission slits of 3.0 nm. At the same time, a solution containing no GGT (control) was prepared and determined for comparison under the same conditions.

Determination of the fluorescence quantum yield and two-photon absorbance cross section

The quantum yield of probe gGlu-AANA and fluorophore AANA was determined using Rhodamine B or quinine sulfate as a reference standard. The quantum yield (Φ) is calculated according to the following formula [S4]:

$$\Phi_x = \Phi_s(D_x/D_s)(A_s/A_x)(n_x/n_s)^2$$

Where Φ is the quantum yield, D is the areas' integral values of the corrected fluorescence spectra, A denotes the absorbance, and n is the refractive index of the solvent used. The subscript x and s stands for the sample and reference standard, respectively. We employed Rhodamine B (Φ = 0.97 in methanol) as the reference standard.

The two-photon absorption (TPA) cross sections (δ) of gGlu-AANA and AANA were determined using two-photon excited (TPE) technique. The TPE fluorescence emission intensity of gGlu-AANA and AANA in DMSO was measured in the emission range of 400-650 nm under excitation at 710-850 nm using Rhodamine B as the reference, whose two-photon properties have been reported [S5]. TPE fluorescence emission intensity of the reference with the same excitation wavelength was recorded. The TPA cross section (δ) was calculated by the following formula [S6]:

$$\delta_x = [(F_x \cdot \Phi_s \cdot C_s \cdot n_x)/(F_s \cdot \Phi_x \cdot C_x \cdot n_s)] \cdot \delta_S$$

Herein F denotes the intensity of TPE fluorescence emission, Φ is the quantum yield, C denotes the concentration, and n is the refractive index of the solvent used. The subscripts x and s denotes the sample and reference standard, respectively.

Cell culture and cytotoxicity assay

SKOV-3 cells were grown in RPMI-1640 medium (Thermo Scientific HyClone) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 U/mL streptomycin. HEK-293T, HepG-2 and HT-29 cells were grown in DMEM (Thermo Scientific HyClone) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 U/mL streptomycin. They were incubated at 37 ℃ in a humidified atmosphere incubator containing 5.0 wt %/vol CO_2. For cytotoxicity assay, the cells were placed in a 96-well plate at 5×10^3 cells per well and grown for 24 h, followed by addition of varying concentrations (0~20 μM) of probe gGlu-AANA. Then the cells were incubated at 37 ℃ in a

humidified atmosphere incubator containing 5.0 wt %/vol CO_2 for 24 h. Thereafter, the cell viability was determined to assess the cytotoxicity of probe gGlu-AANA by the standard MTT assay.

Two-photon fluorescence imaging of GGT in living cells

The normal cells (HEK-293T) and the cancer cells (HepG-2, HT-29, and SKOV-3) were washed with PBS three times, and then incubated in the corresponding medium containing only probe gGlu-AANA (10 μM) at 37 ℃ for 30 min in a humidified atmosphere incubator containing 5.0 wt %/vol CO_2. In the enzyme inhibition experiment, the cancer cells were incubated in the corresponding medium containing the inhibitor DON (1.0 mM) for 1.0 h, and then incubated with this probe (10 μM) for another 30 min in a humidified atmosphere incubator containing 5.0 wt%/vol CO_2. The control experiments were incubated in the corresponding medium. These cells were washed three times with cold PBS before two-photon fluorescence imaging.

Two-photon colon cancer tissues imaging

An 8-week-old mice was firstly injected via skin-pop injection the cancer cells (HT-29 cells). After the colon cancer tissues growth, the mice were killed and anatomized. The colon cancer tissues were cut into 1.0 mm-thick slices. The prepared slices were washed three times with PBS, and then transferred to glass-bottomed dishes. The experiments of the two-photon tissues imaging were divided into two groups: in the first group, the slices were incubated with probe gGlu-AANA (10 μM) in PBS at 37 °C for 1.0 h, and then washed with PBS to remove the residual probe before two-photon fluorescence imaging; in the other group, the slices were first incubated with the inhibitor DON (5.0 mM) for 1.0 h, and followed by incubation with this probe for another 1.0 h in PBS, and then washed three times before imaging. The two-photon fluorescence emission was collected using blue channel (430-470 nm) and green channel (480-520 nm) under excitation at 730 nm.

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ADDITIONAL FIGURES

Figure S1. ^{1}H-NMR spectra of AANA in $CDCl_3$

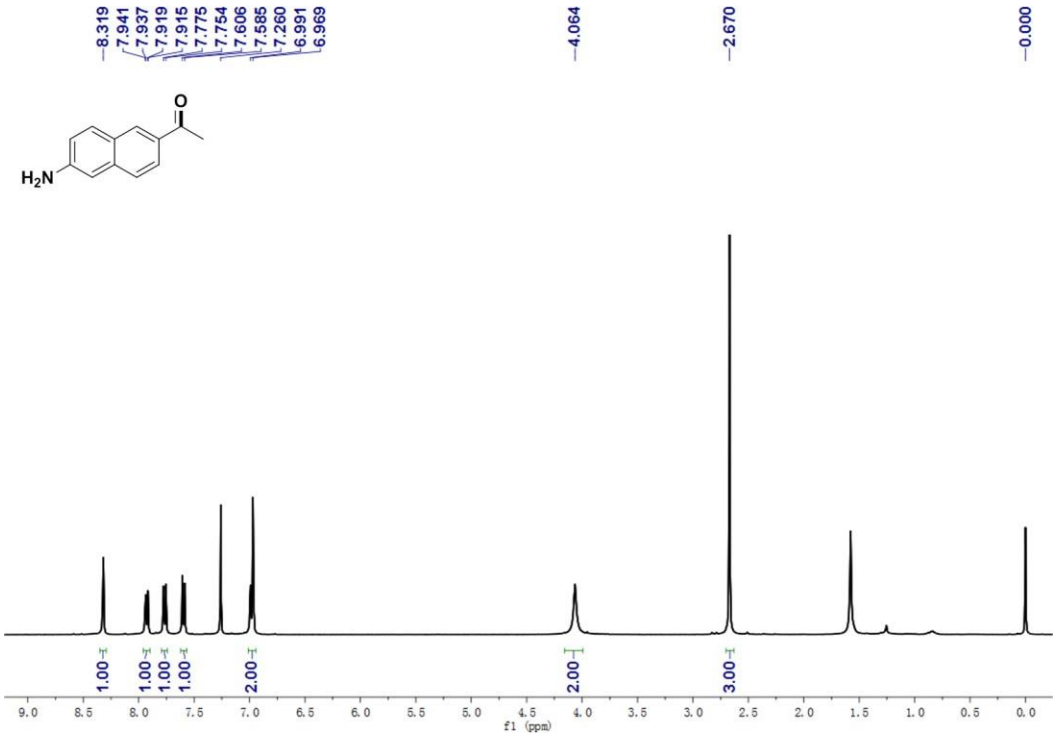

Figure S2. ^{13}C-NMR spectra of AANA in $CDCl_3$

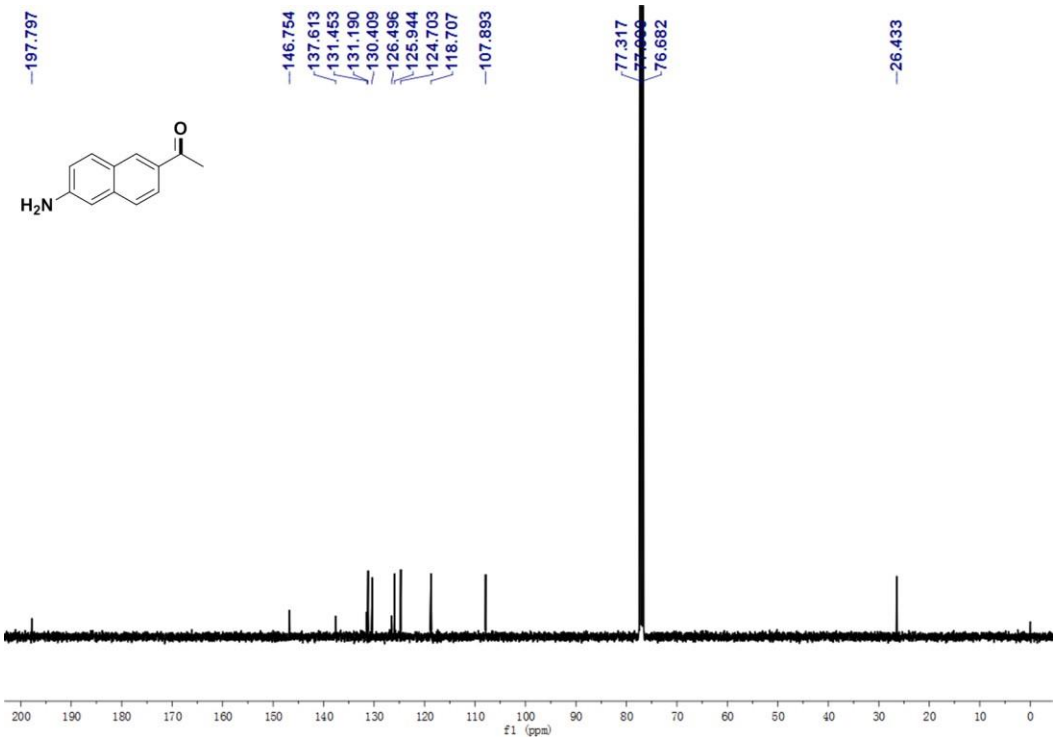

Figure S3. ^{1}H-NMR spectra of compound 3 in $CDCl_3$

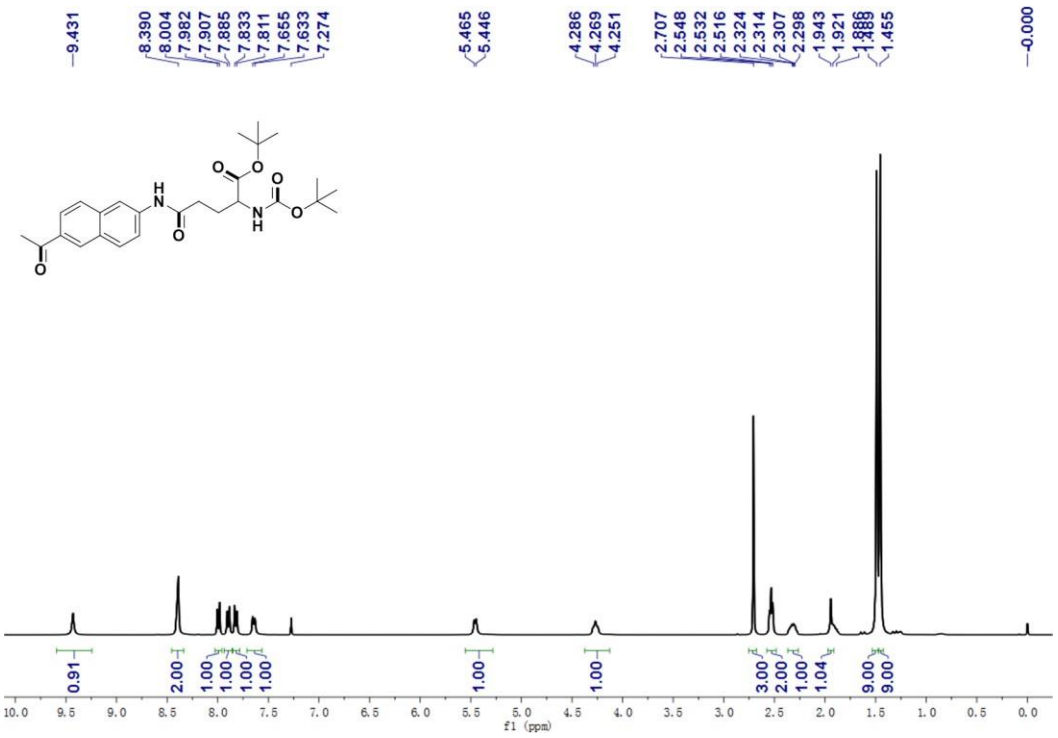

Figure S4. ^{13}C-NMR spectra of compound 3 in $CDCl_3$

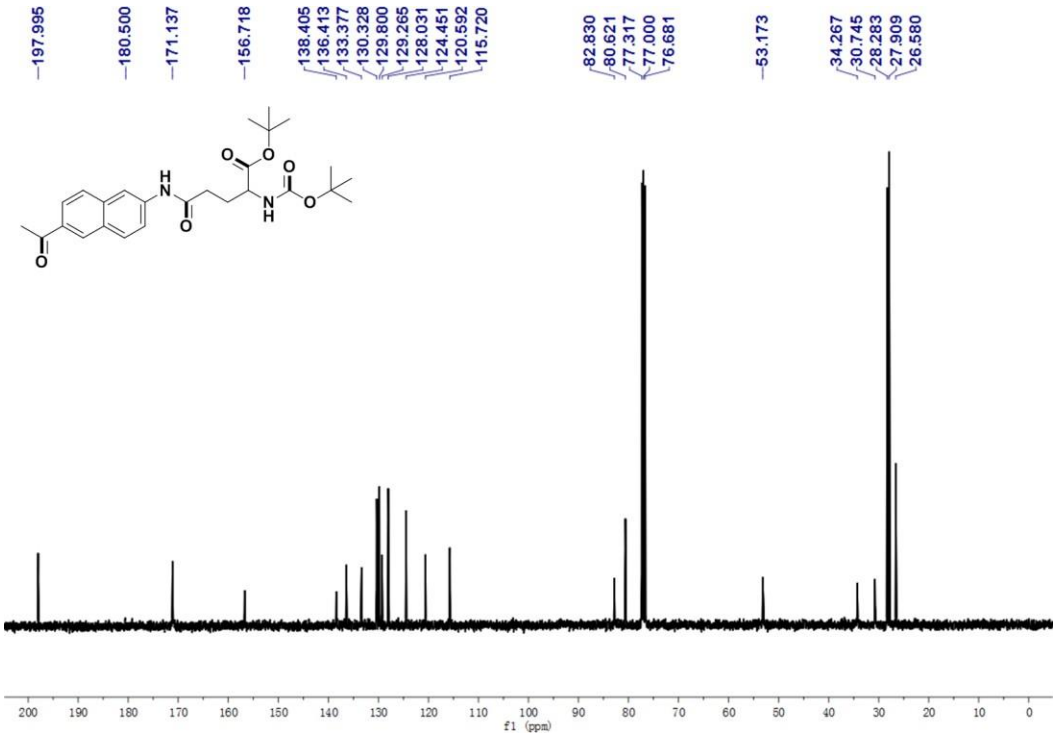

Figure S5. MS of compound 3 in methanol

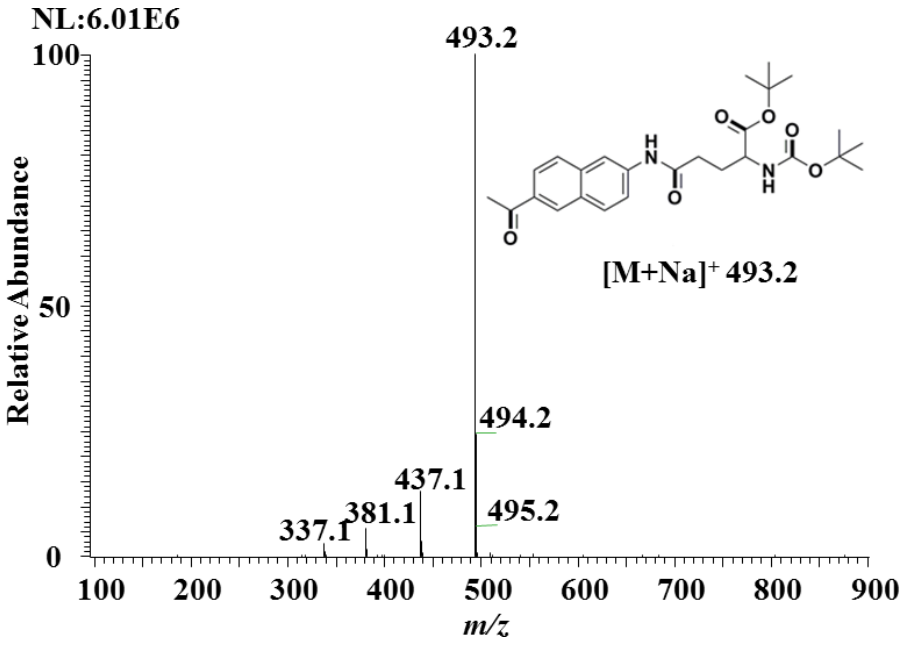

Figure S6. ^{1}H-NMR spectra of gGlu-AANA in d_6-DMSO

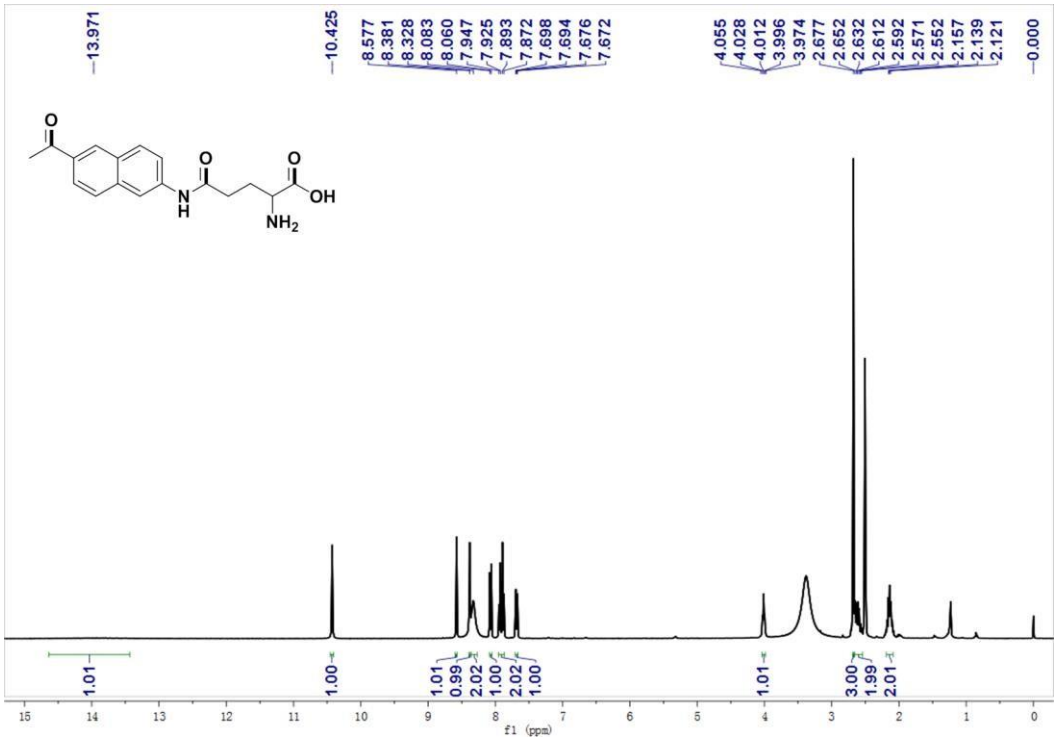

Figure S7. ^{13}C-NMR spectra of gGlu-AANA in d_6-DMSO

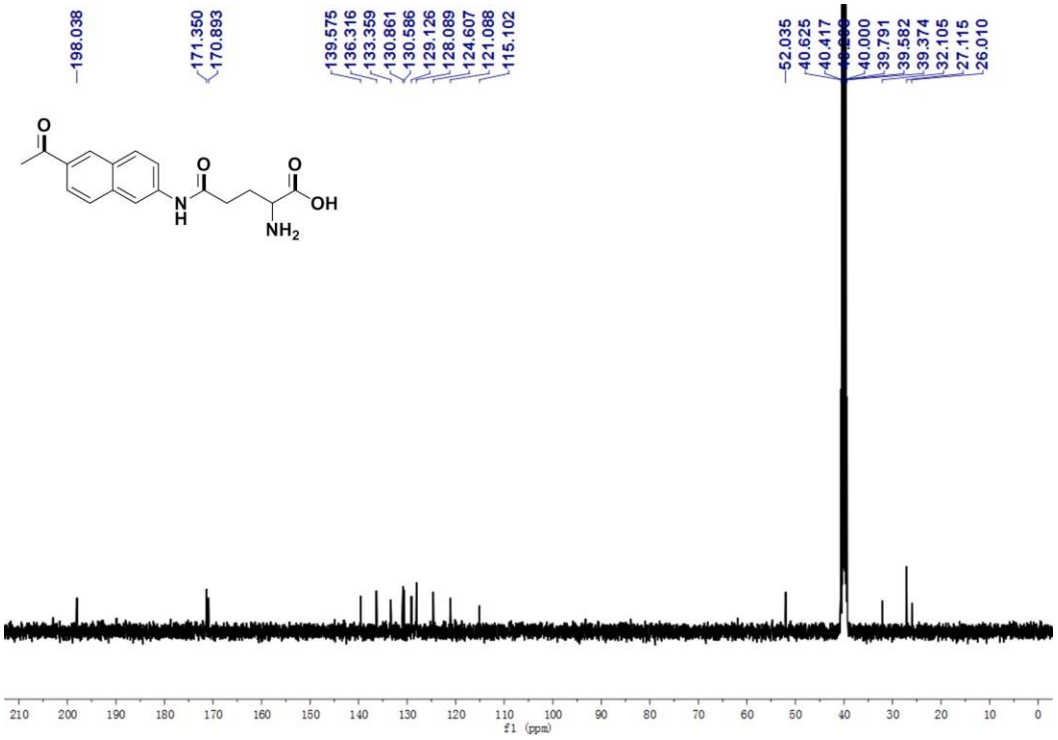

Figure S8. MS of gGlu-AANA in methanol

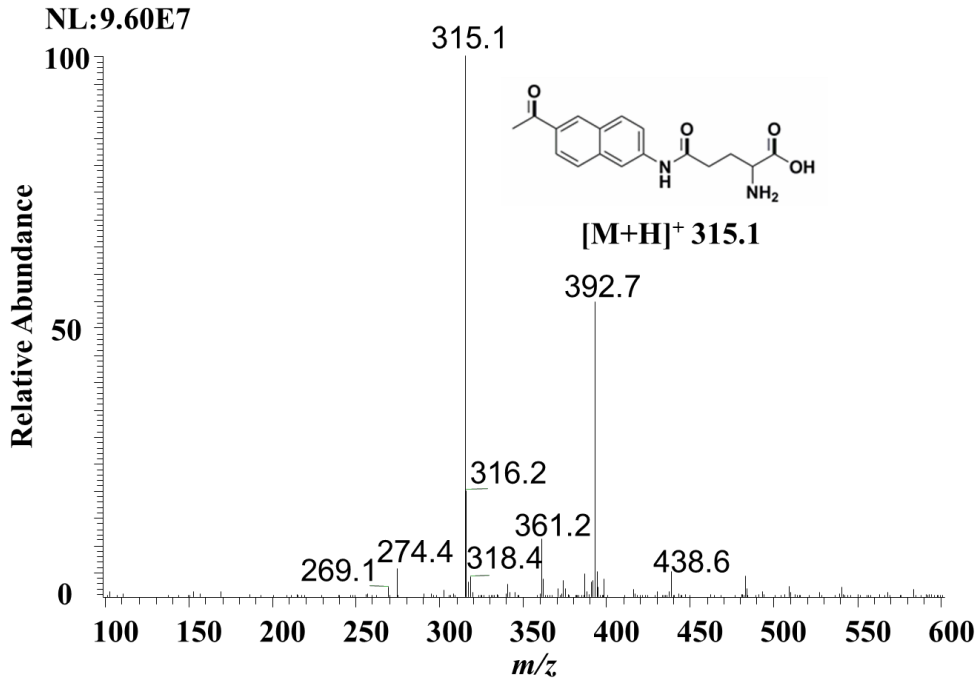

Figure S9. HRMS of gGlu-AANA in methanol

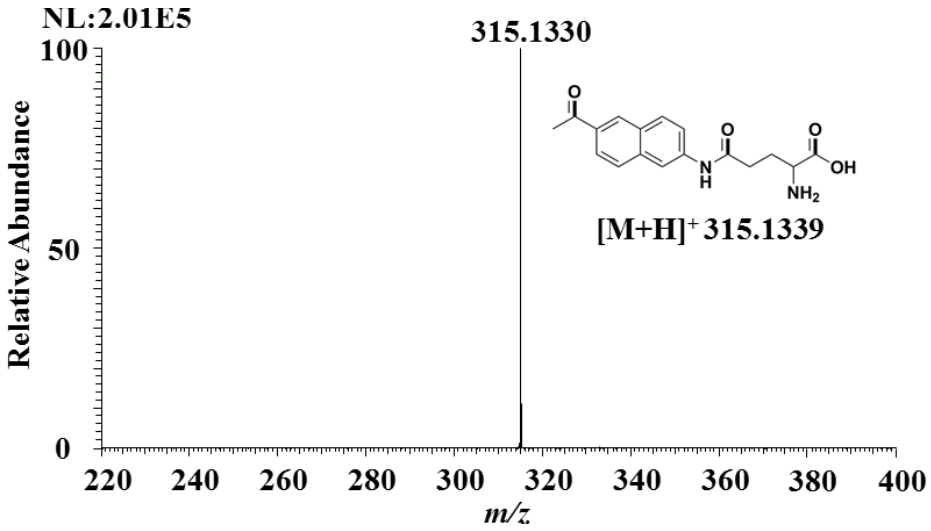

Figure S10. UV-vis absorption spectra of 10 μM probe gGlu-AANA (black line), 10 μM AANA (red line), and the reaction product of probe gGlu-AANA and 50 U/L GGT (blue line) at 37 °C

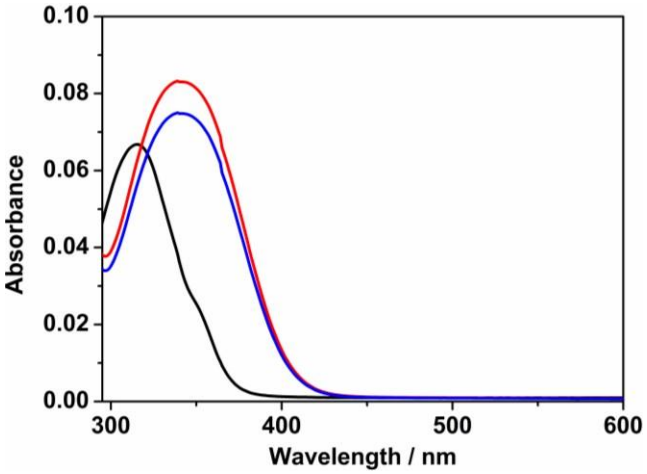

Figure S11. HPLC profiles of different reaction systems: A) 50 μM AANA; B) the reaction product of gGlu-AANA (50 μM) with GGT (100 U/L) for 30 min; C) 50 μM gGlu-AANA. Detection: UV-vis (380 nm) detector. Flow rate: 0.8 mL/min. Temperature: 32 °C. Injecti on volume: 10 μL. Mobile phase: methanol/water = 50/50 (v/v). The assignments of the peaks: 1) 7.164 min, gGlu-AANA; 2) 9.652 min, AANA.

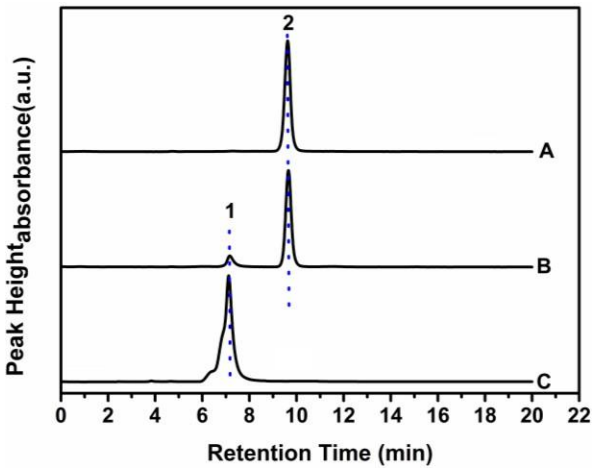

Figure S12. Effects of pH on the fluorescence intensity ratio (I_{500}/I_{445}) of gGlu-AANA (10 μM) with (●) and without (■)GGT (50 U/L) at 37 °C. Reaction at each pH value was repeated three times, and the error bars represent standard deviations. λ_{ex} = 355 nm.

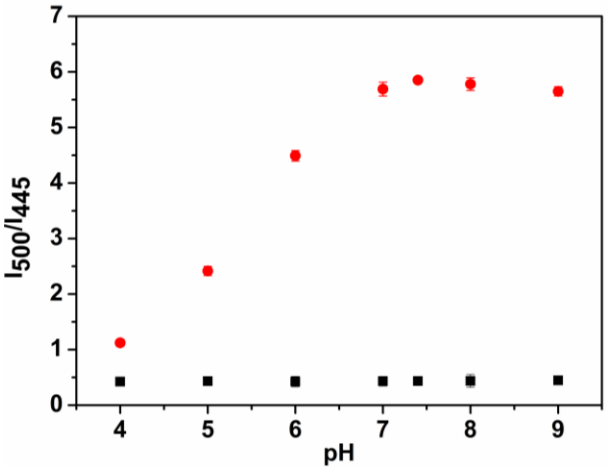

Figure S13. Effects of temperature on the fluorescence intensity ratio (I_{500}/I_{445}) of gGlu-AANA (10 μM) with (●) and without (■)GGT (50 U/L). Reaction at each temperature was repeated three times, and the error bars represent standard deviations. λ_{ex} = 355 nm.

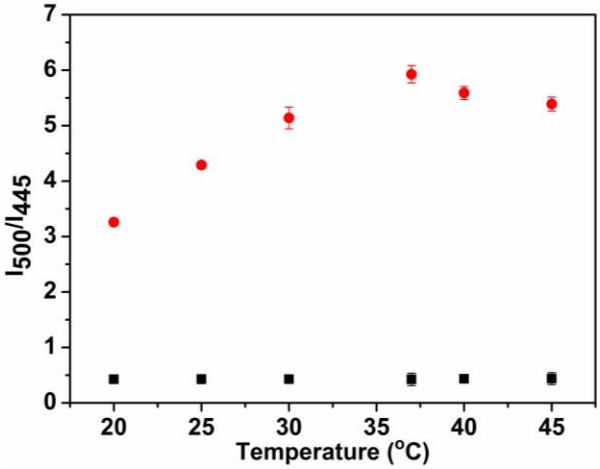

Figure S14. A plot of the fluorescence intensity ratio (I_{500}/I_{445}) versus the reaction time of probe gGlu-AANA (10 μM) with different concentrations of GGT, from bottom to top: 0 U/L (●, control), 5 U/L (●), and 20 U/L (●). All measurements were performed at 37 °C in H_2O/DMSO solution (19/1, v/v, 10 mM PBS, pH 7.4) with λ_{ex} = 355 nm.

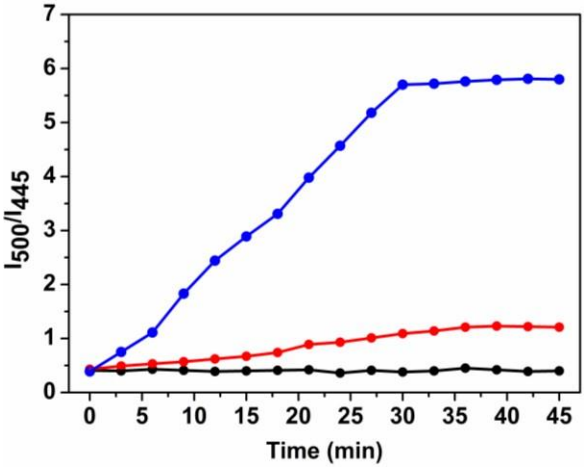

Figure S15. The fluorescence intensity ratio (I_{500}/I_{445}) of gGlu-AANA (10 μM) versus the concentration of GGT, I_{500} and I_{445} were the values of the fluorescence intensity at 500 nm and 445 nm, respectively. The plot of the I_{500}/I_{445} as the function of GGT concentration showed that the fluorescence intensity ratio enhanced gradually with increasing the GGT concentration (0, 1, 2, 5, 10, 20, 50, 75, and 100 U/L), and finally reached a plateau with GGT (20 U/L). All measurements were performed at 37 °C in H_2O/DMSO solution (19/1, v/v, 10 mM PBS, pH 7.4) with λ_{ex} = 355 nm.

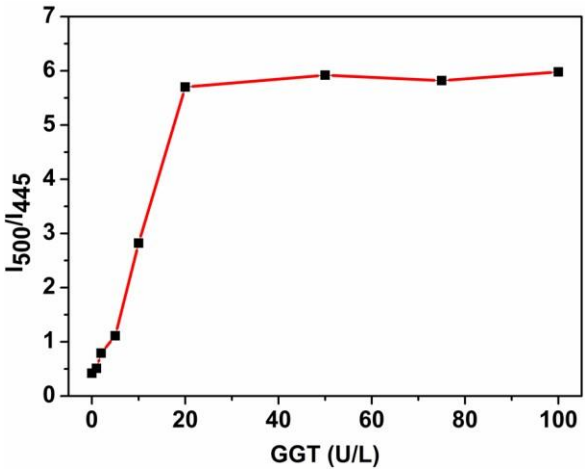

Figure S16. The limit of detection (LOD) of gGlu-AANA (10 μM) toward GGT was derived from the fluorescence titration experiment with lower GGT concentration (0-20 U/L). A good linear relationship (I_{500}/I_{445} = 0.2767×C (U/L) + 0.0897, R^2 = 0.995) was obtained. LOD was calculated by the following equation: LOD = 3σ/*k*, where *k* is the slope of the linear equation obtained from the linear regression of fluorescence intensity ratio (I_{500}/I_{445}) versus the lower GGT concentration, σ is the standard deviation of the eleven times blank measurements. LOD was calculated to be about 0.3 U/L. All measurements were performed at 37 °C in H_2O/DMSO solution (19:1, v/v, 10 mM PBS, pH 7.4) with λ_{ex} = 355 nm.

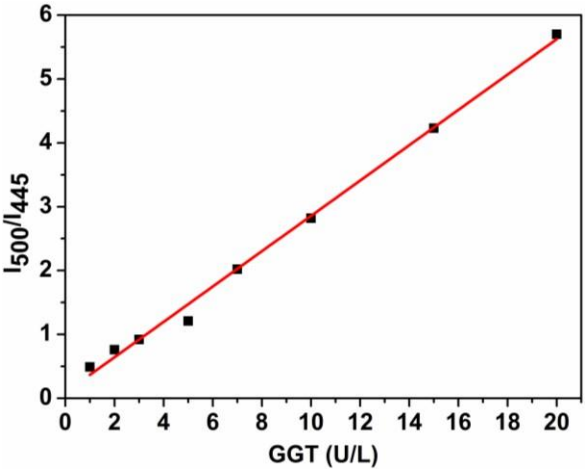

Figure S17. Lineweaver-Burk (or Double-Reciprocal) plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was shown as: $V = V_{max}[\text{probe}]/(K_m+[\text{probe}])$, where V is the reaction rate, [probe] is probe gGlu-AANA concentration (substrate), and K_m is the Michaelis constant. Conditions: 20 U/L GGT, 1-10 μM gGlu-AANA. All measurements were performed at 37 °C, λ_{ex} = 355 nm. Reaction at each probe concentration was repeated three times, and the error bars represent standard deviations. Points were fitted using a linear regression model (correlation coefficient R = 0.997).

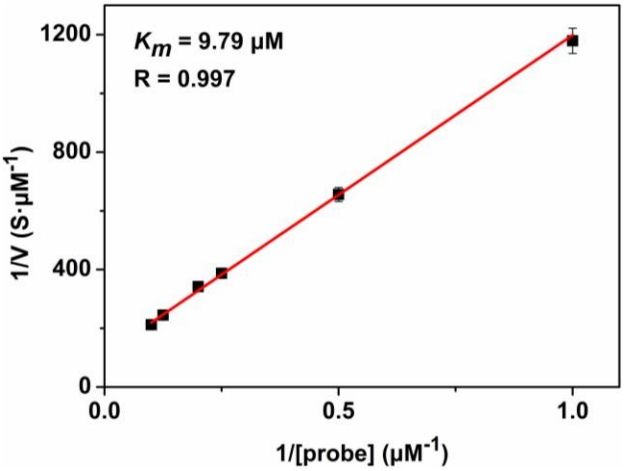

Figure S18. A) Two-photon action (TPA) cross sections of gGlu-AANA (10 μM) and AANA (10 μM) in H_2O/DMSO solution (19:1, v/v, 10 mM PBS, pH 7.4). The relative error of two-photon action cross section values (Φδ) are ± 15%; B) Two-photon excitation spectra of gGlu-AANA (10 μM) reacted with and without GGT (50 U/L), λ_{ex} = 730 nm.

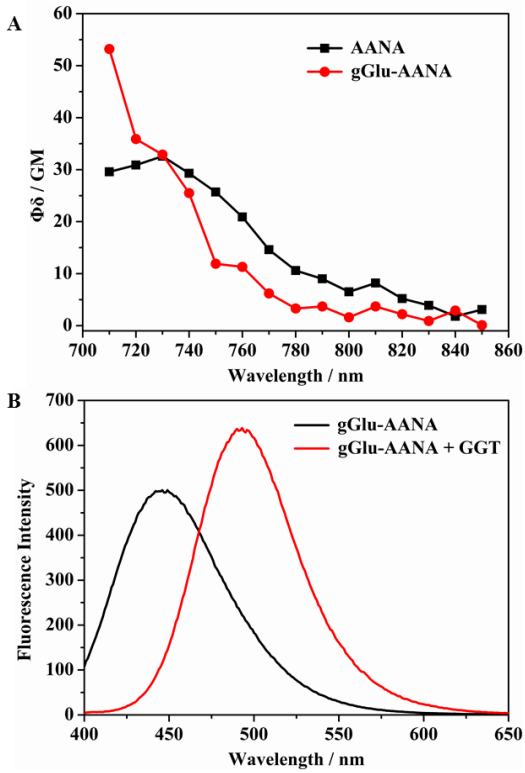

Figure S19. Cytotoxicity assay for the normal cells (HEK-293T) and the cancer cells (HepG-2, HT-29, and SKOV-3). The cells incubated with varying concentrations of gGlu-AANA for 24 h. Data were repeated three times, and the error bars represent standard deviations.

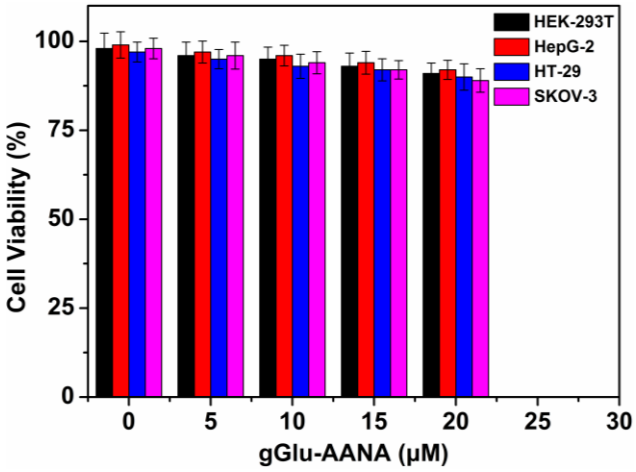

Figure S20. Two-photon fluorescence images of the normal cells (HEK-293T) and the cancer cells (HepG-2, HT-29, and SKOV-3). The cells were incubated with gGlu-AANA (10 μM) for 30 min before imaging. From top to bottom, blue channel: λ_{em} = 430-470 nm, green channel: λ_{em} = 480-520 nm, differential interference contrast (DIC), ratio images generated from blue to green channel. λ_{ex} = 730 nm. Scale bar = 20 μm.

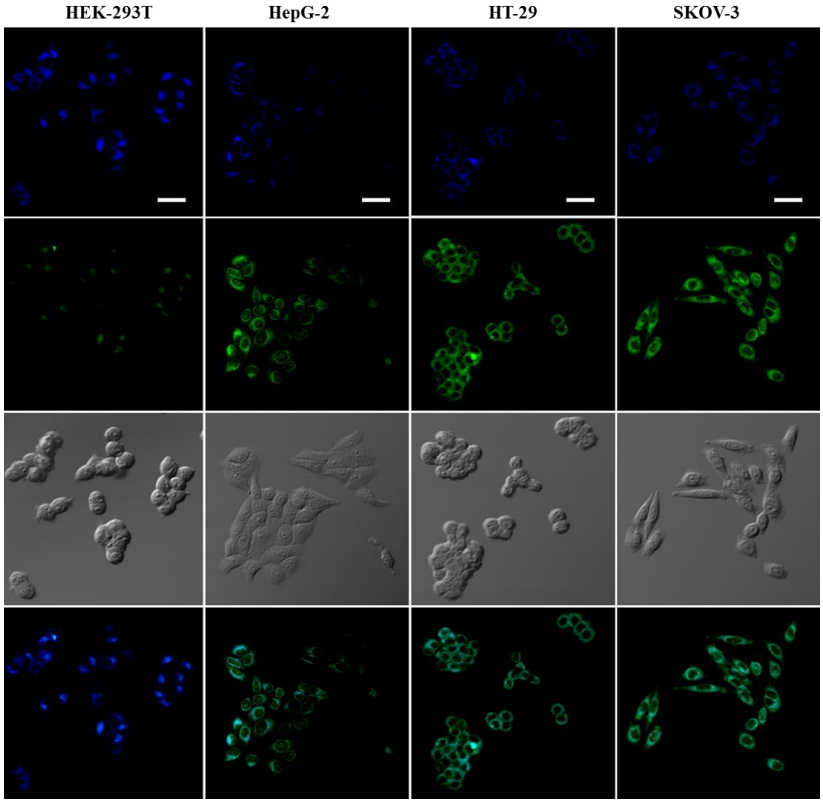

Figure S21. Two-photon fluorescence images of the cancer cells (HepG-2, HT-29, and SKOV-3). The cells were first pretreated with the inhibitor DON (1.0 mM) for 1.0 h, and followed by incubation with gGlu-AANA (10 μM) for another 30 min before imaging. From left to right, blue channel: λ_{em} = 430-470 nm, green channel: λ_{em} = 480-520 nm, differential interference contrast (DIC), ratio images generated from blue to green channel. λ_{ex} = 730 nm. Scale bar = 20 μm.

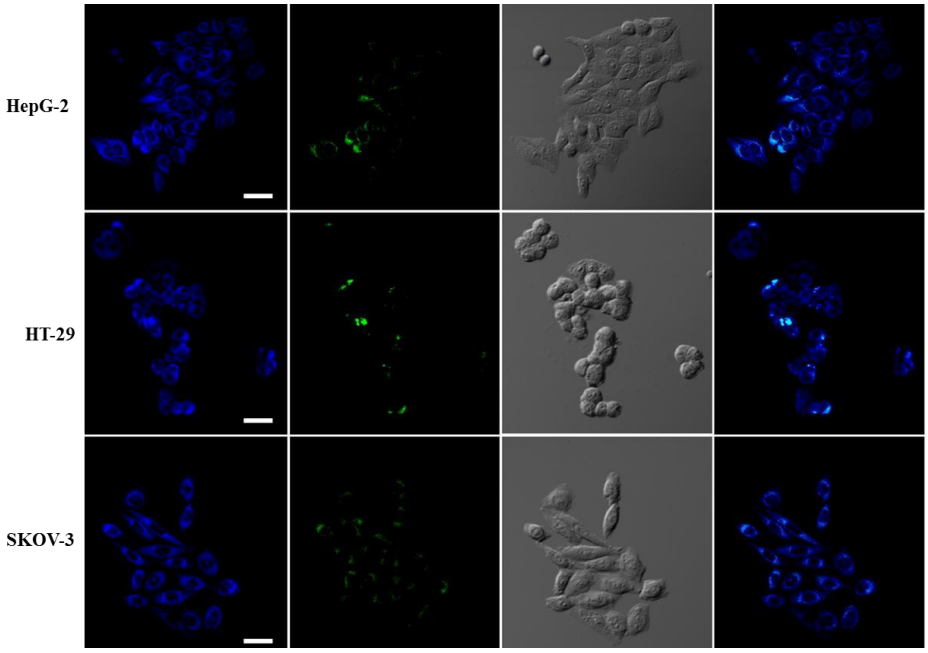

Table S1. Determination of GGT levels in human serum samples using commercial GGT activities fluorometric assay kit and our probe gGlu-AANA.

Methods / Sample No.	GGT level (U/L) by the commercial kit[a]	GGT level (U/L) by our probe[a]
A[b]	62 ± 4	60 ± 2
B[c]	126 ± 10	129 ± 6
C[c]	176 ± 12	172 ± 9
D[c]	150 ± 10	148 ± 5

[a] In both methods, the serum samples were 10-fold diluted for measurement, and the values herein represent GGT levels in the undiluted serum samples;

[b] Serum sample was obtained from healthy people;

[c] Serum samples were obtained from unhealthy people with abnormal GGT levels. The four serum samples were kindly provided by Xiangya Mdical College, Central South University (CSU).