Electronic Supplementary Information

A fast responsive, highly selective and light-up fluorescent probe for two-photon imaging of carboxylesterase in living cells

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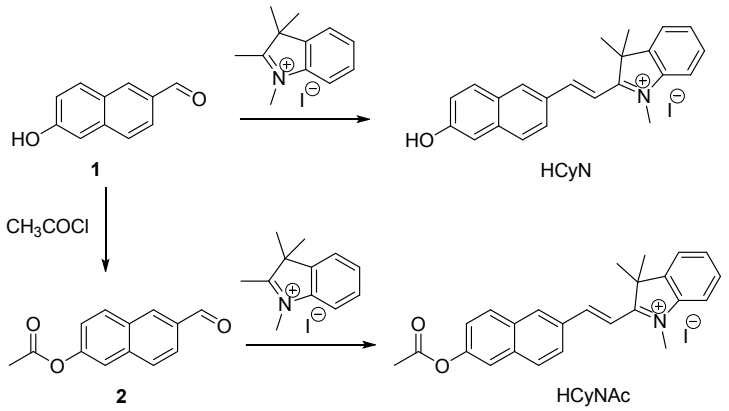
Experimental section

All solvents were purified and dried following standard procedures unless special statements. All other chemical reagents were purchased from Heowns, Sigma Aldrich or Aladdin, unless otherwise stated. ^{1}H NMR spectra were obtained on a Brucker DMX-400MHz spectrophotometer. High resolution mass spectra were obtained on Brucker APEX IV (7.0 T) FT_MS. Fluorescence emission spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer. Confocal fluorescence imaging experiments were performed with an Olympus FV-1000 laser scanning microscopy system, based on an IX81 (Olympus, Japan) inverted microscope. The microscope was equipped with 375 nm (CW) laser lines and UPLSAPO 60x/N.A 1.42 objective. Images were collected and processed with Olympus FV10-ASW Ver.2.1b software.



Scheme S1. The synthetic route of **HCyNAc** and **HCyN**.

Synthesis of 6-formylnaphthalen-2-yl acetate (compound 2)

After the solution of 6-hydroxy-2-naphthaldehyde (compound **1**, 172.2 mg, 1 mmol) and triethylamine (TEA, 121.4 mg, 1.2 mmol) in CH_2Cl_2 was cooled to 0 ℃, acetyl chloride (94.2 mg, 1.2 mmol) was added. The reaction mixture was stirred at room temperature and monitored with TLC until **1** disappeared. After that, the mixture was washed with brine. The organic layer was collected and dried over anhydrous Na_2SO_4. 6-formylnaphthalen-2-yl acetate (compound **2**) was obtained by recrystallization from a mixed solvent of ethyl acetate and petroleum ether. Yield: 92 %. ^{1}H NMR (400 MHz, DMSO) δ 10.15 (s, 1H), 8.61 (s, 1H), 8.22 (d, *J* = 8.9 Hz, 1H), 8.06 (d, *J* = 8.5 Hz, 1H), 7.92 (dd, *J* = 8.5, 1.1 Hz, 1H), 7.81 (d, *J* = 1.9 Hz, 1H), 7.47 (dd, *J* = 8.8, 2.2 Hz, 1H), 2.35 (s, 3H).

Synthesis of compound HCyNAc

A mixture of compound **2** (214.2 mg, 1.0 mmol), 1,2,3,3-tetramethyl-3H-indol-1-ium iodide (compound **3**, 301.2 mg, 1.0 mmol) in ethanol was refluxed under nitrogen atmosphere for 16 h. After cooling to room temperature, ethyl ether was added and compound **HCyNAc** was precipitated and collected as a red solid. Yield: 94 %. ^{1}H NMR (400 MHz, DMSO) δ 8.77 (s, 1H), 8.59 (d, *J* = 16.4 Hz, 1H), 8.39 (d, *J* = 8.7 Hz, 1H), 8.10 (d, *J* = 8.8 Hz, 2H), 7.94 – 7.87 (m, 2H), 7.85 – 7.78 (m, 2H), 7.68 – 7.61 (m, 2H), 7.46 (dd, *J* = 8.8, 2.2 Hz, 1H), 4.20 (s, 3H), 2.36 (s, 3H), 1.84 (s, 6H). ^{13}C NMR (101 MHz, MeOD) δ 181.93, 168.94, 153.18, 150.51, 142.91, 141.20, 135.66, 133.09, 131.24, 130.51, 130.14, 129.03, 128.49, 128.04, 123.61, 121.96, 121.92, 118.06, 114.12, 111.86, 52.00, 33.29, 24.28, 19.01. HRMS (MALDI-TOF) m/z: $[M]^+$ calcd for $C_{25}H_{24}NO_2^+$, 370.1802, found: 370.1817.

Synthesis of HCyN

A mixture of compound **1** (172.2 mg, 1.0 mmol), 1,2,3,3-Tetramethyl-3H-indol-1-ium iodide (compound **3**, 301.2 mg, 1.0 mmol) in ethanol was refluxed under nitrogen atmosphere for 16 h. After cooling to room temperature, ethyl ether was added and compound **HCyN** was precipitated and collected as a red solid. Yield: 95 %. ^{1}H NMR (400 MHz, DMSO) δ 10.41 (s, 1H), 8.61 (s, 1H), 8.53 (d, *J* = 16.3 Hz, 1H), 8.24 (d, *J* = 8.9 Hz, 1H), 7.94 – 7.81 (m, 4H), 7.64 (m, 3H), 7.25 – 7.18 (m, 2H), 4.15 (s, 3H), 1.82 (s, 6H). HRMS (MALDI-TOF) m/z: $[M]^+$ calcd for $C_{23}H_{22}NO^+$, 328.1696, found: 328.1684.

General procedure for detection of CaE.

Unless otherwise noted, all the spectral measurements were performed in 5 mM phosphate buffer (pH 7.4) according to the following procedure. The stock solution (1.0 mM) of probe **HCyNAc** was first prepared in DMSO. Stock solution of pig liver esterase was prepared in PBS. 20 μL of **HCyNAc** stock solution was added to 2 mL PBS followed by addition of different volume of CaE stock solution. The mixture was incubated for certain times at 37 ℃ and then, the reaction solution was transferred to a quartz cell with 1-cm optical length for measurements. In the meantime, the blank solution without CaE was also prepared and measured under the same conditions for comparison.

Determination of the detection limit of HCyNAc toward addition of CaE

Based on the linear fitting in Fig. 1B, the detection limit (*C*) is estimated as follows:

$$C = 3\sigma/B$$

Where σ is the standard deviation obtained from three individual fluorescence measurements (I_{567} nm) of **HCyNAc** (10 μM) without any CaE and B is the slope obtained after linear fitting the titration curves within certain ranges.

Cell culture and cell imaging

HeLa cells were grown on glass-bottom culture dishes (Corning Inc.) using Dulbecco's modified eagle media (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 μg mL^{-1}) and streptomycin (100 μg mL^{-1}) in a humidified 37 ℃, 5% CO_2 incubator. Before use, the

adherent cells were washed three times with FBS-free DMEM. For imaging, the cells were incubated with 10 μM of **HCyNAc** in DMEM at 37 ℃ for 15 min, and then washed three times with PBS (pH 7.4). Further experiments were carried out by pretreatment of cells with BNPP or AEBSF. Briefly, the cells were firstly washed with DMEM (without FBS) for three times, and then incubated with BNPP or AEBSF at 1.0 mM for 30 min. After that, the cells were washed with PBS three times, and then incubated with **HCyNAc**. The cells were viewed with a Nikon multi-photon microscope (A1R MP) equipped with a 60× water-immersion objective lens and living cell workstation. A 457 nm Laser was used as the light source. The two-photon fluorescence imaging were irradiated (80 MHz, ≤140 fs) at 800 nm at the same region of the dish.

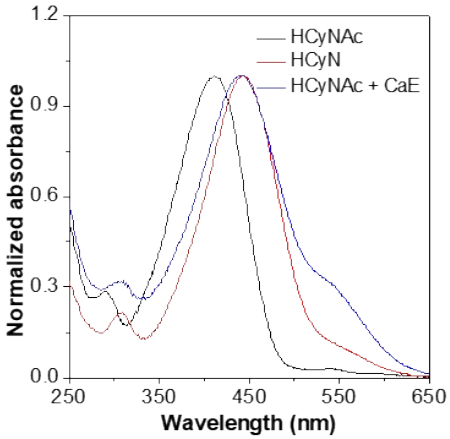


Fig. S1. UV/Vis spectra of **HCyNAc** (10 μM), **HCyN** (10 μM) and **HCyNAc** (10 μM) after incubation with CaE (0.1 U/mL) at 37 ℃ for 7 min in PBS solution.

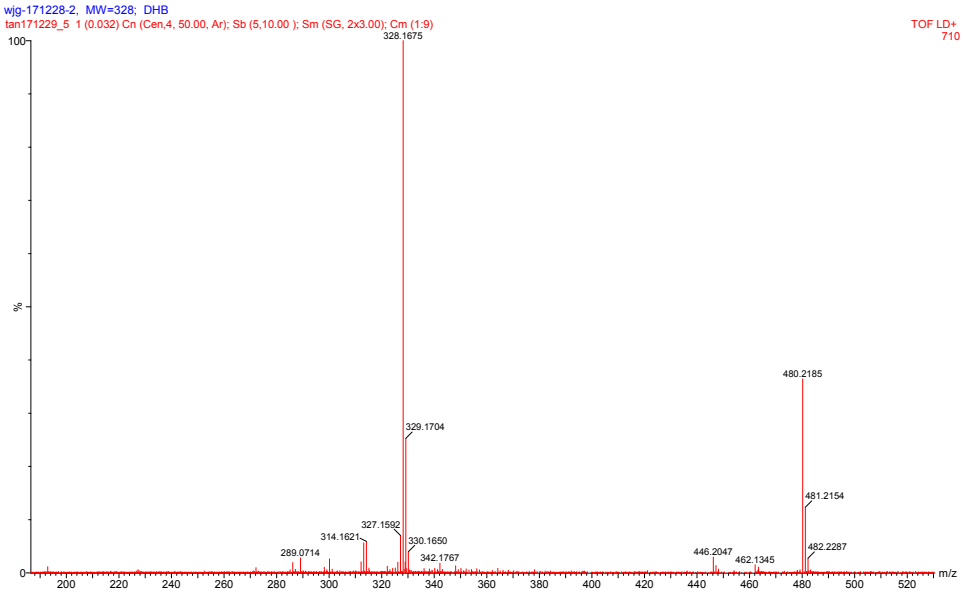


Fig. S2. HRMS spectrum of **HCyNAc** after incubation with CaE for 7 min at 37 ℃.

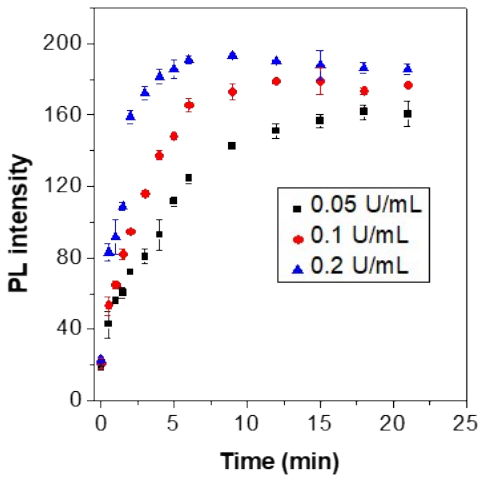


Fig. S3. The fluorescence intensity at 567 nm of **HCyNAc** (10 μM) incubated with different concentrations of CaE as a function of time. (E_{ex} = 443 nm).

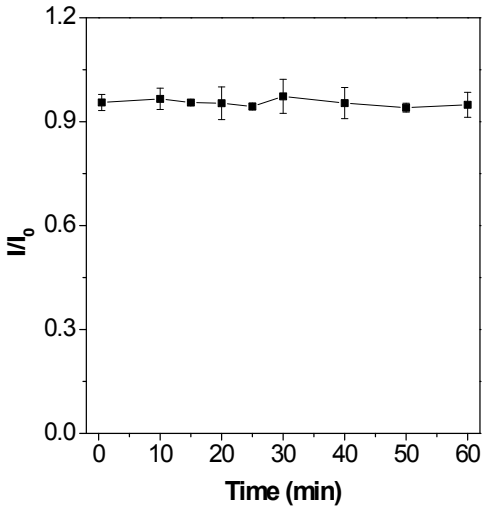


Fig. S4. Stability of **HCyNAc**. I_t/I_0 is the fluorescence intensity ratio at 525 nm after and before t-minute incubation at 37 ℃. (E_{ex} = 443 nm).

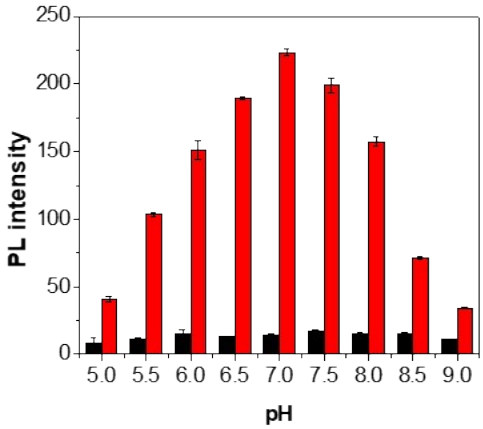


Fig. S5. Fluorescence intensity of **HCyNAc** (black bars) and **HCyNAc** + CaE (red bars) at different pH value in buffered solution. E_{ex}=443 nm.

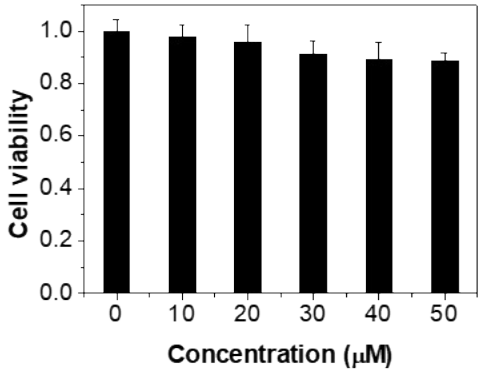


Fig. S6. Cell viability of HeLa cells at varied concentrations of **HCyNAc** using MTT method.

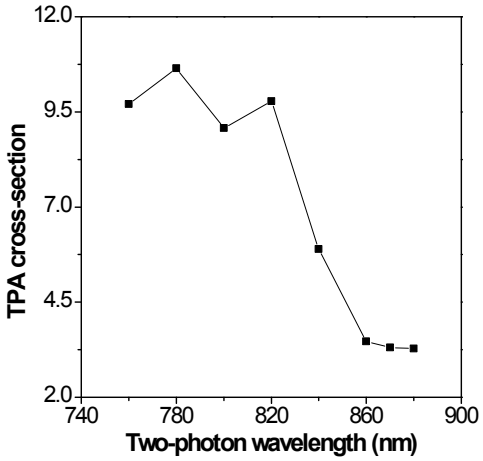


Fig. S7. Two-photon absorption cross-sections of HCyN in the 760–880 nm region.

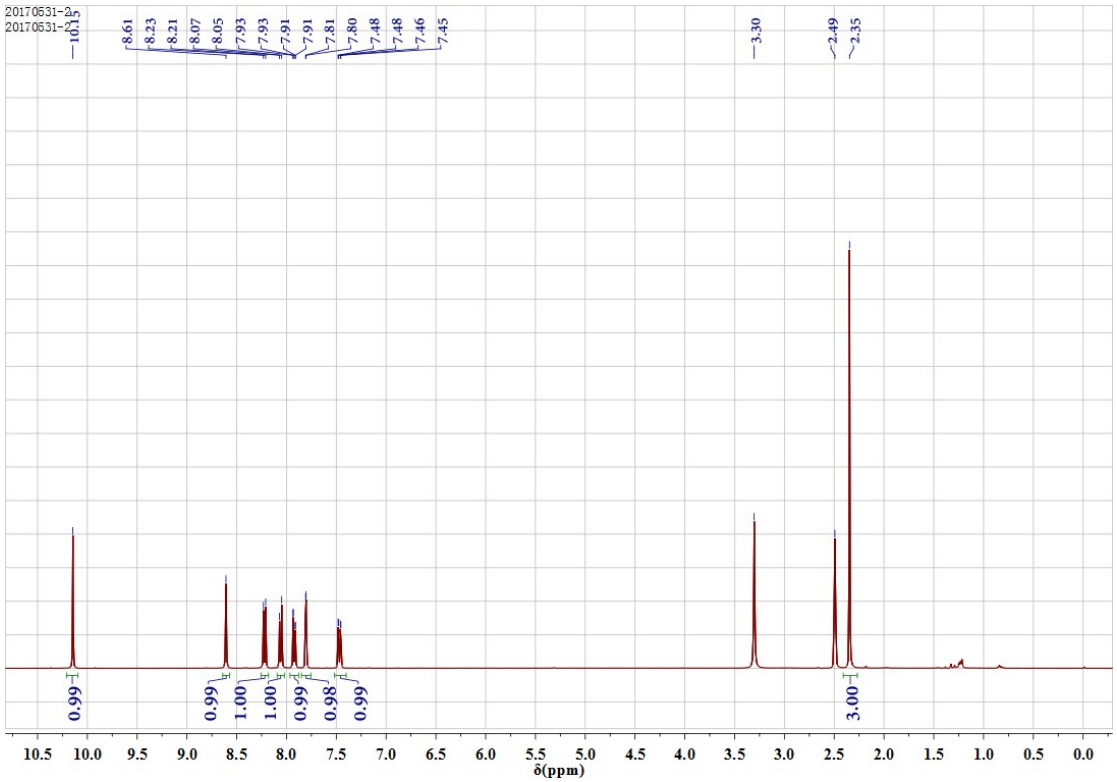


Fig. S8. ^{1}H NMR of 6-formylnaphthalen-2-yl acetate (compound **2**) in d_6-DMSO.

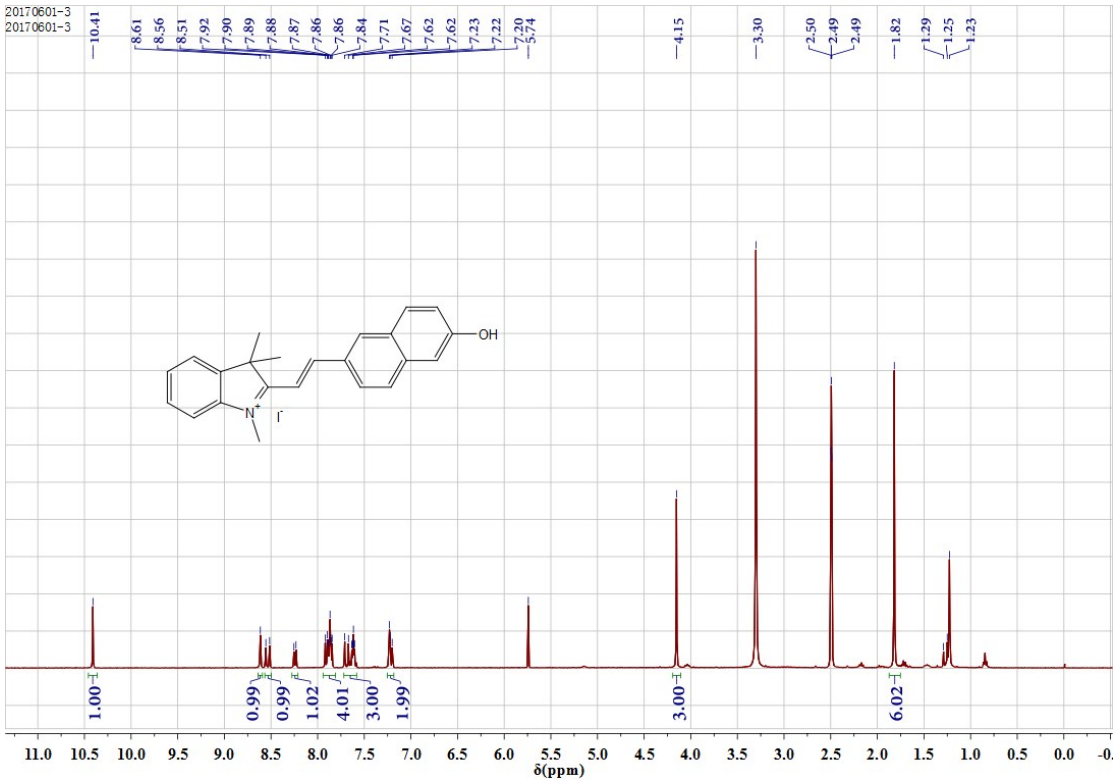


Fig. S9. ^{1}H NMR of **HCyN** in d_6-DMSO.

Fig. S10. HRMS spectrum of **HCyN**.

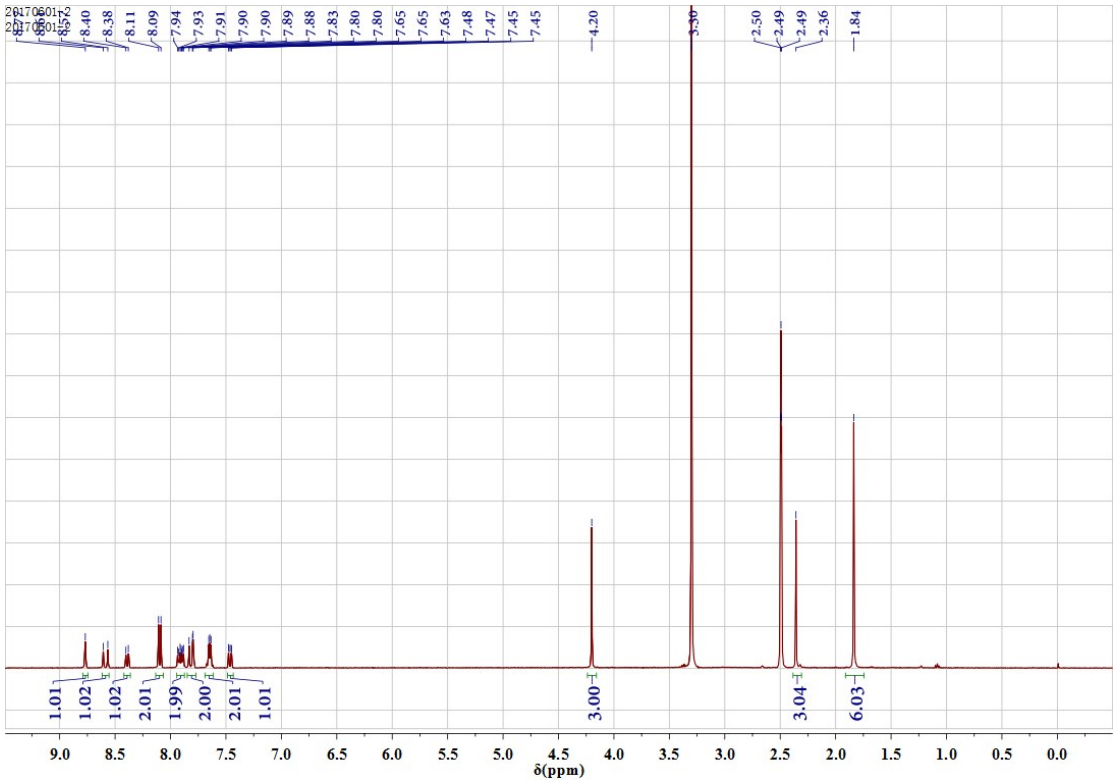


Fig. S11. ^{1}H NMR of **HCyNAc** in d_6-DMSO.

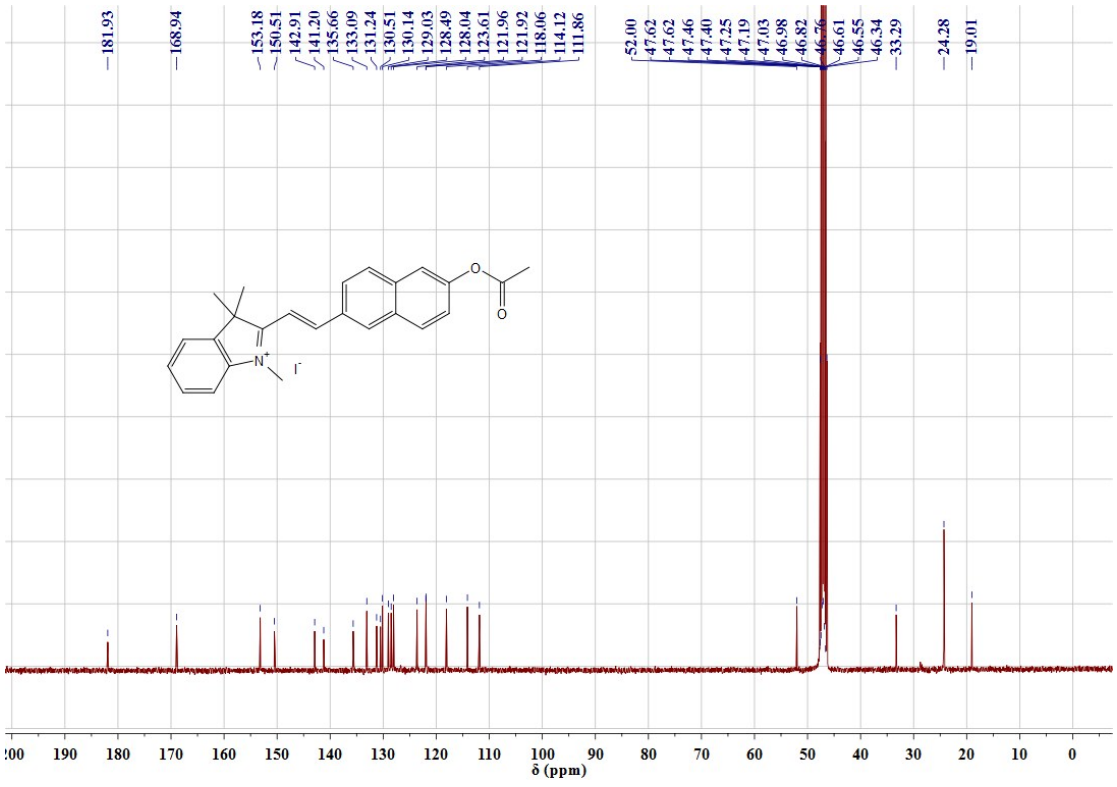


Fig. S12. ^{13}C NMR of **HCyNAc** in d_4-Mehanol.

Fig. S13. HRMS spectrum of **HCyNAc**.

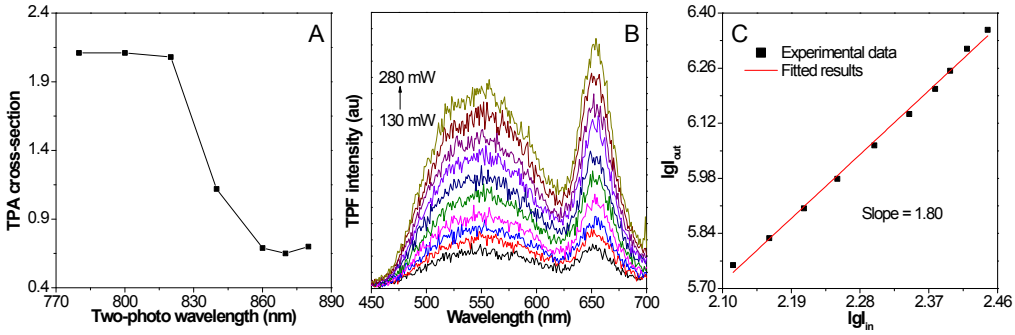


Fig. S14. (A) Two-photon absorption cross-sections of HCyNAc in the 780–880 nm region. (B) The two photon excited fluorescence spectra (λ_{ex} = 820 nm) of HCyNAc in H_2O (contain 1% DMF) with a solution concentration of 1.0 $\times 10^{-4}$ at different input powers. (C) The logarithmic plots of the output fluorescence integral area versus input laser power.

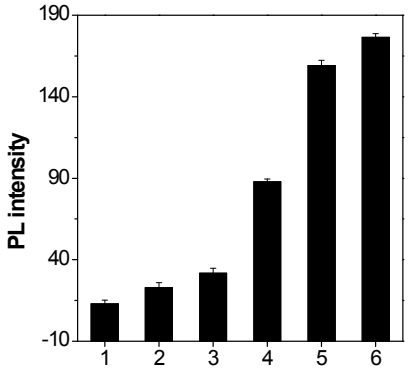


Fig. S15. The dose-dependent inhibitory effect of BNPP. 1: HCyNAc only; 2-6: HCyNAc + 0.1 U/mL CaE + (100 μM, 10 μM, 1 μM, 0.1 μM and 0 μM) BNPP. E_x=443 nm.

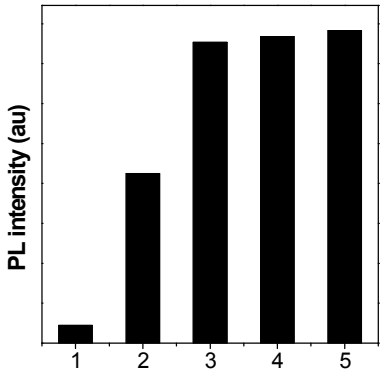


Fig. S16. Fluorescence intensity of HCyNAc in 5-fold diluted serum samples obtained from patients with or without hepatocellular carcinoma. 1: HCyNAc only; 2: HCyNAc + serum sample of patient without hepatocellular carcinoma; 3-5: HCyNAc + serum samples of patients (A, B and C) with hepatocellular carcinoma. E_x=443 nm.

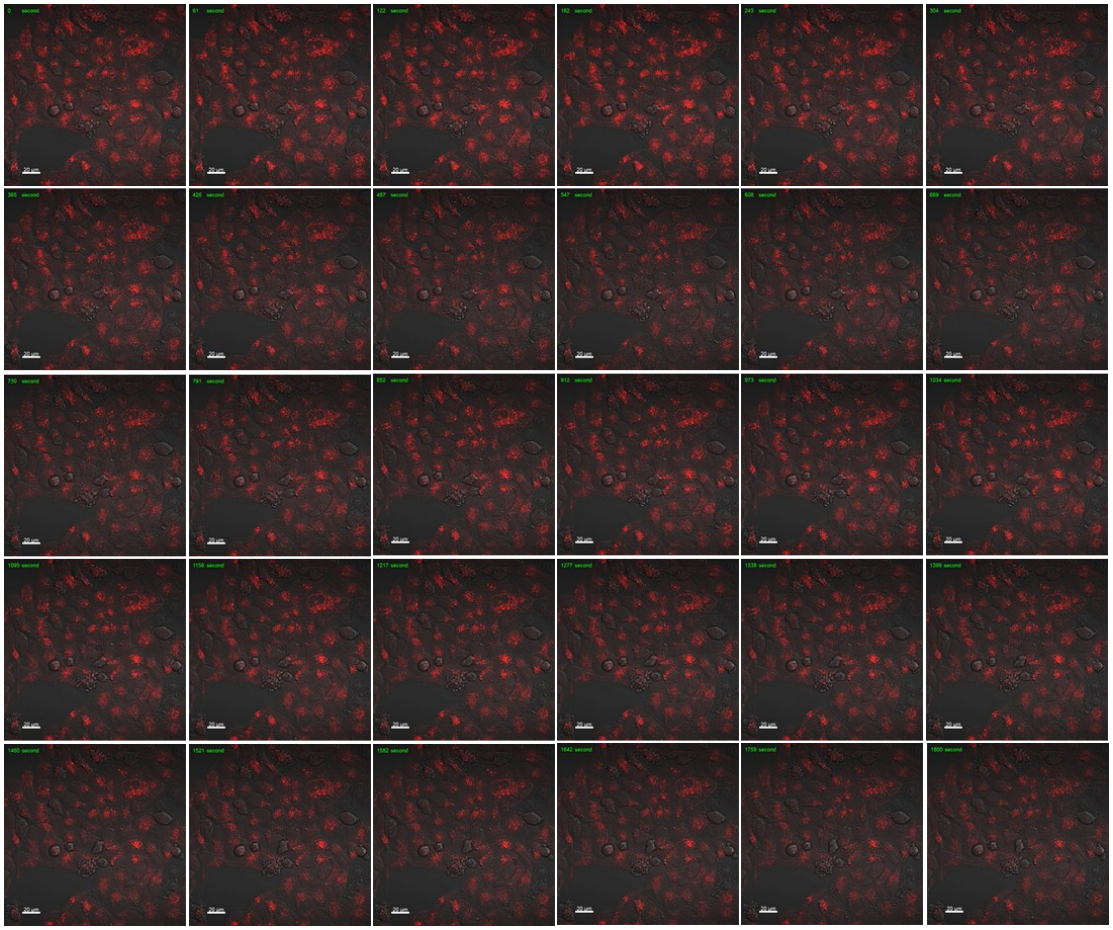


Fig. S17. Photo-stability of HCyNAc in HeLa cells during 30 min period under imaging condition. Excitation wavelength: 405 nm.

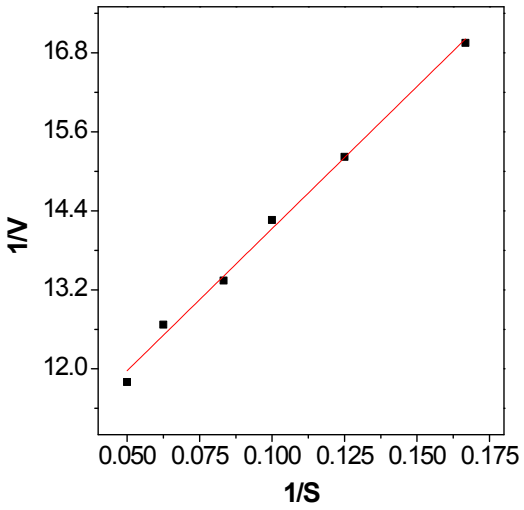
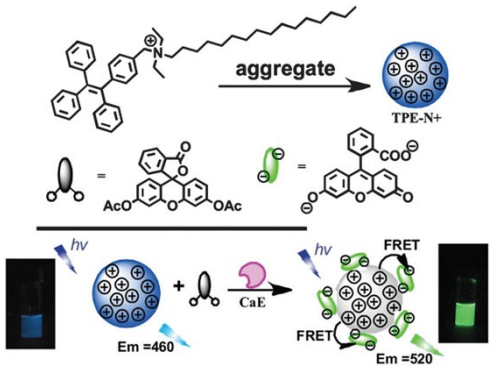
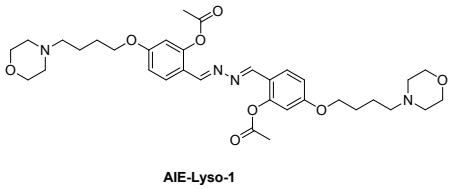
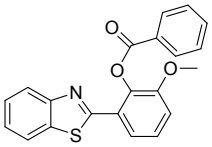
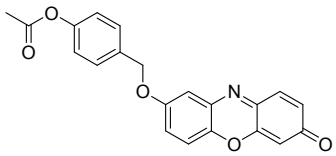
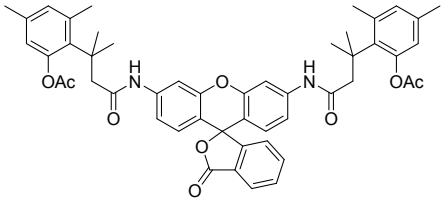
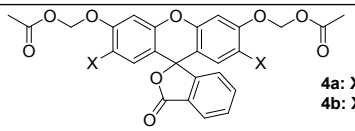


Fig. S18. Lineweaver-Burk plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: $V=V_{max}[S]/(K_m+[S])$, where V is the reaction rate, [S] is the concentration of the probe HCyNAc, and K_m is the Michaelis constant. Points were fitted using a linear regression model (correlation coefficient $R^2 = 0.995$).

Table S1. Comparison of **HCyNAc** with other small-molecule fluorescent probes for CaE.

Structure and Ref. No.	Response time	OPM or TPM	Detection limit	$\lambda_{ex}/\lambda_{em}$ (nm)	Applicaiton
 *Chem. Commun.*, **2015**, 51, 12791	30 min	OPM	0.26 U/L	355/ 520-460	Serum
 AIE-Lyso-1 *J. Mater. Chem. B*, **2014**, 2, 3438	15 min	OPM	2.4 U/L	356/532	Cell imaging
 *Biosen. Bioelectron.*, **2014**, 57, 30	18 min	OPM	Not mentioned	304/ 488-368	Cell imaging
 *Analyst*, **2012**, 137, 716	30 min	OPM	0.086 U/L	550/585	Cell imaging
 *J. Am. Chem. Soc.*, **2005**, 127 1652	4 h	OPM	Not mentioned	492/520	Cell imaging
 *Chem. Sci.*, **2011**, 2, 521	Not mentioned	OPM	Not mentioned	496/520	Cell imaging

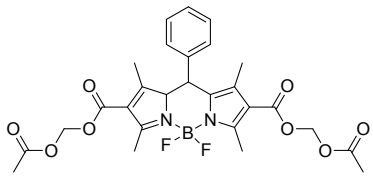
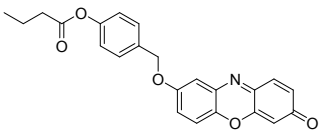
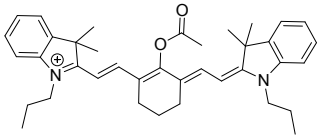
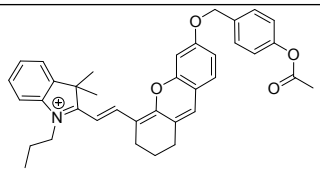
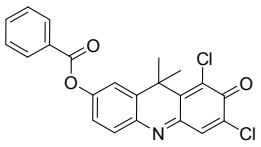
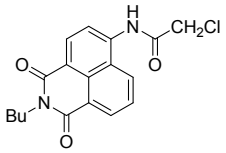
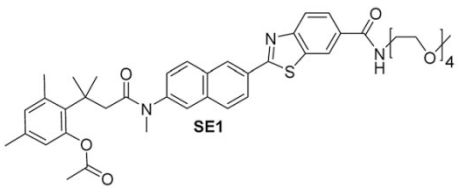
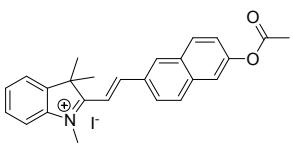
 *Chem. Commun.*, **2009**, 7015	Not mentioned	OPM	Not mentioned	500/ 525-505	Cell imaging
 *Bioorg. Med. Chem. Letters*, **2011**, 21, 3206	> 15 min	OPM	Not mentioned	571/585	Cell imaging
 *Bioorg. Med. Chem. Letters*, **2007**, 17, 5054	30 min	OPM	Not mentioned	535/625	Cell imaging
 *J. Agric. Food Chem.,* **2017**, 65, 4209−4215	15 min	OPM	4.5 U/L	670/705	Cell and zebrafish imaging
 *Biosen. Bioelectron.*, **2016**, 83, 193-199	20 min	OPM	70 ng/mL	600/662	Cell, organ and live mice imaging
 *ACS Appl. Mater. Interfaces*, **2015**, 7, 28474−28481	60 min	TPM	12 ng/mL	800/ 542-452	Cell and tissue imaging
 *Chem. Sci*., **2016**, 7, 3703	1 h	TPM	0.5 nM	740/ 455-540	Cell and tissue imaging
 **HCyNAc** *This work*	7 min	TPM	1.8 U/L	800/567	Cell imaging

Table S2. Photophysical properties of HCyNAc and HCyN.

	λ_{abs} (nm)	λ_{em} (nm)	Φ (%)[a]
HCyNAc	413	550	0.78
HCyN	443	593	4.77

[a]Φ = fluorescence quantum yield measured by using fluorescein as standard in PBS solution.