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## Unique D- $\pi$ -A- $\pi$ -D type fluorescent probes for the two-photon imaging of intracellular viscosity

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Institute of Fluorescent Probes for Biological Imaging, School of Materials Science and Engineering, School of Chemistry and Chemical Engineering, University of Jinan, Jinan, Shandong 250022, P. R. China. \*E-mail: weiyinglin2013@163.com **Materials and instruments.** All solvents and reagents were purchased from J&K, or Sinopharm Chemical Reagent Co., and used without further purification unless for special needs. Doubly distilled water was used in the experiments. Thin-layer chromatography (TLC) analysis was performed on silica gel plates, which was obtained from the Qingdao Ocean Chemicals. <sup>1</sup>H NMR (400 MHz) and <sup>13</sup>C NMR (100 MHz) spectra were recorded on an AVANCE III 400 MHz Digital NMR Spectrometer, using CDCl<sub>3</sub> or DMSO-*d*<sub>6</sub> as a solvent and tetramethylsilane (TMS) as internal reference. High-resolution mass spectra (HRMS) for the characterization of structures were collected using a Bruker apex-Ultra mass spectrometer (Bruker Daltonics Corp., USA) in electrospray ionization (ESI) mode. Viscosity determination was carried out with a NDJ-8S rotational viscometer, and each viscosity value was recorded. The fluorescence spectra and relative fluorescence intensity were measured with a Hitachi F-4600 spectrofluorimeter with a 1-cm quartz cuvette. UV/vis spectra were made with a Shimadzu UV-2700 spectrophotometer. Fluorescence imaging was performed with a Nikon A1R confocal microscope.

Synthesis.



(c) Terephthalaldehyde, NaOH, EtOH, r.t. stir; (d) Benzaldehyde, NaOH, EtOH, r.t. stir.

Scheme S1 Synthesis routes of Ph-cyclo, CHO-cyclo and H-cyclo.

2,5-Bis(4-(dimethylamino)benzylidene)cyclopentanone (**Ph-cyclo**): Cyclopentanone (0.17 g, 2 mmol) and 4-(dimethylamino)benzaldehyde (0.60 g, 4 mmol) were dissolved in ethanol (20 mL) containing 0.1 g of NaOH as catalyst. The mixture was stirred at r.t. for 20 min, and then filtered for yellow residues. The crude product were recrystallized by dry ethanol one time to obtain compound **Ph-cyclo** (yield 90%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.56 (d, *J* = 8.6 Hz, 6H), 6.76 (d, *J* = 8.8 Hz, 4H), 3.10 (s, 4H), 3.06 (s, 12H). HRMS: m/z [M+H]<sup>+</sup> calcd for [C<sub>23</sub>H<sub>27</sub>N<sub>2</sub>O]<sup>+</sup> 347.2123, found 347.2133.

2-(4-(Dimethylamino)benzylidene)cyclopentanone (compound **3**): Cyclopentanone (0.17 g, 2 mmol) and 4-(dimethylamino)benzaldehyde (0.30 g, 2 mmol) were dissolved in ethanol (20 mL) containing 0.05 g of NaOH as catalyst. The mixture was stirred at r.t. for 10 min, and then filtered for yellow residues. The crude compound **3** was used directly for the next step without further purification.

CHO-cyclo and H-cyclo were obtained by the same procedure of Ph-cyclo.

4-(3-(4-(Dimethylamino)benzylidene)-2-

oxocyclopentylidene)methyl)benzaldehyde (CHO-cyclo): red solid, yield 63%. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta = 10.06$  (s, 1H), 8.00 (d, J = 7.5 Hz, 2H), 7.90 (d, J = 7.5 Hz, 2H), 7.58 (d, J = 8.5 Hz, 2H), 7.43 (s, 2H), 6.81 (d, J = 8.5 Hz, 2H), 3.10 (d, J = 31.5 and 7.4 Hz, 4H), 3.03 (s, 6H). HRMS: m/z [M+H]<sup>+</sup> calcd for [C<sub>22</sub>H<sub>22</sub>NO<sub>2</sub>]<sup>+</sup> 332.1651, found 332.1682.

2-Benzylidene-5-(4-dimethylamino-benzylidene)-cyclopentanone(**H-cyclo**): yellow solid, yield 51%. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  = 7.61-7.54 (m, 6H), 7.45-7.34 (m, 3H), 6.74 (d, *J* = 9.0 Hz, 2H), 3.13-3.03 (m, 7H), 1.55 (s, 3H). HRMS: m/z [M+H]<sup>+</sup> calcd for [C<sub>21</sub>H<sub>21</sub>NO]<sup>+</sup> 304.1701, found 304.1738.



Scheme S2 Synthesis routes of Indol-cyclo and Ph-ene-cyclo.

Compounds Indol-cyclo and Ph-ene-cyclo were obtained by the same procedure described above.

2,5-bis(2-(1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclopentanone (Indolcyclo), yellow solid, yield 30%. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta = 7.46$  (d, J = 13.1 Hz, 2H), 7.33 (d, J = 7.3 Hz, 2H), 7.21 (t, J = 7.6 Hz, 2H), 6.94 (dd, J = 16.3, 7.9 Hz, 4H), 5.27 (d, J = 13.2 Hz, 2H), 3.24 (s, 6H), 2.23 (t, J = 7.7 Hz, 4H), 1.53 (s, 12H). <sup>13</sup>C NMR (100 MHz, DMSO- $d_6$ ):  $\delta = 204.64$ , 164.67, 144.59, 139.17, 128.86, 128.34, 127.33, 122.21, 121.24, 107.90, 93.04, 46.41, 38.80, 29.65, 28.57, 26.95, 19.95. HRMS: m/z [M+H]<sup>+</sup> calcd for [C<sub>23</sub>H<sub>27</sub>N<sub>2</sub>O]<sup>+</sup> 347.2123, found 347.2185.

2,5-bis(3-(4-(dimethylamino)phenyl)allylidene)cyclopentanone (**Ph-ene-cyclo**), red solid, yield 60%. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.41 (d, *J* = 8.8 Hz, 4H), 7.23-7.26 (m, 2H), 6.90 (d, *J* = 15.6 Hz, 2H), 6.81-6.77 (m, 2H), 6.75-6.69 (m, 4H), 3.02 (s, 12H), 2.87 (s, 4H). HRMS: m/z [M+H]<sup>+</sup> calcd for [C<sub>27</sub>H<sub>31</sub>N<sub>2</sub>O]<sup>+</sup> 399.2436, found 399.2428. **Theoretical calculations.** All the calculations were implemented with the Gaussian09 program package. The initial geometries of the compounds were generated by the Gauss View software. The ground state structures of the compounds were optimized using time-dependent density functional theory (TD-DFT) using a B3LYP/6-31G\* level of theory. The solvent effects were modeled with the polarizable continuum model (PCM).

Absorption and fluorescence spectroscopy. The solvents were obtained by mixing water-glycerol systems in different proportions. Test solutions of **Ph-ene-cyclo** of different viscosity were prepared by adding the stock solution (DMSO, 1 mM) to 3 mL of solvent mixture (water-glycerol solvent systems) to obtain the final concentration of the dye (10.0  $\mu$ M). These solutions were sonicated for 5 min to mix and eliminate air bubbles. The solutions were measured after standing for 30 min at room temperature.

Fluorescence quantum yield was determined by the relative comparison with fluorescein ( $\Phi_s = 0.79$  in 0.1 N NaOH aqueous solution) as standard for **Ph-ene-cyclo** in water-glycerol mixture solutions, and quinine sulfate ( $\Phi_s = 0.56$  in 0.1 N H<sub>2</sub>SO<sub>4</sub> aqueous solution) as standard for **Ph-ene-cyclo** in different organic compounds, and they were calculated by equation 1.<sup>27</sup>

$$\Phi = \Phi_s(IA_s/I_sA)(\eta^2/\eta_s^2) \tag{1}$$

in which, A is the absorbance, I is the integrated fluorescence intensity, and  $\eta$  is the refractive index of the solvent.

The logarithm of the fluorescence ratio  $(I/I_0)$  has a linear relationship with that of the viscosity ( $\eta$ ) of the solution, which is as expected from the Förster-Hoffmann equation:

$$\log I_f = C + x \log \eta$$

which C is a concentration that is temperature-dependent and x is a dye-dependent constant.

Compounds		DCM	THF	DMF	DMSO	MeOH
Ph-cyclo	$\lambda_{abs,\;max} \; / nm$	465	455	467	475	481
	$\lambda_{em,\;max} \; / nm$	551	538	562	575	611
	Stokes shift /nm	86	83	95	100	130
H-cyclo	$\lambda_{abs,\;max}\;/\!nm$	446	429	447	452	454
	$\lambda_{em,\ max}$ /nm	560	590	567	600	587
	Stokes shift /nm	114	161	120	148	133
CHO-cyclo	$\lambda_{abs,\ max}$ /nm	421	414	420	426	433
	$\lambda_{em,\ max}$ /nm	477	467	477	495	498
	Stokes shift /nm	56	53	57	62	65
Indol-cyclo	$\lambda_{abs,\;max} \; / nm$	464	457	473	471	469
	$\lambda_{em, max} / nm$	622	620	571	582	607
	Stokes shift /nm	158	163	98	111	138
Ph-ene-cyclo	$\lambda_{abs,max}/nm$	504	494	507	517	521
	$\lambda_{em,\;max} \; / nm$	642	627	657	672	720
	Stokes shift /nm	138	133	150	155	199

 Table S1 Photophysical data of four compounds in different solvents.

Table S2 Fluorescence quantum yield of Ph-ene-cyclo in different solvents

solvents	DCM	THF	DMF	DMSO	MeOH	water <sup>a</sup>	50% <sup>b</sup>	95%°
Ф	0.12	0.15	0.041	0.052	0.050	0.003	0.009	0.038

Footnote: The ratio of glycerol-water is 0 (a), 50% (b) and 95% (c).

## Figures of UV-Vis and fluorescence spectra



Fig. S1 Fluorescence spectra of H-cyclo (10  $\mu$ M) in different ratios of glycerol-water mixtures at room temperature ( $\lambda_{ex} = 400$  nm).



Fig. S2 Fluorescence spectra of CHO-cyclo (10  $\mu$ M) in different ratios of glycerolwater mixtures at room temperature ( $\lambda_{ex} = 450$  nm).



Fig. S3 Emission spectra of Ph-ene-cyclo (10  $\mu$ M) in different solvents with corresponding maximum absorption at room temperature.



Fig. S4 Photos of Ph-ene-cyclo in different organic solvents.



Fig. S5 Upconversion emission spectra of Ph-ene-cyclo (10  $\mu$ M) in (a) DCM or (b) DMSO with different excited wavelengths (700-900 nm).



Fig. S6 UV-Vis absorption spectra (a) and emission spectra (b) of Ph-ene-cyclo (10  $\mu$ M) in H<sub>2</sub>O with different values of pH (silit: 5.0/5.0 nm,  $\lambda_{ex}$  = 420 nm, pH = 5.01, 6.05, 7.00, 8.11, 9.00 and 10.06).



Fig. S7 (a) The fluorescence intensity at 630 nm of Ph-ene-cyclo (10  $\mu$ M) vs different viscosity; (b) the linear response between the log (I/I<sub>0</sub>) at 630 nm of Ph-ene-cyclo (10  $\mu$ M) and the log (viscosity) in the water/glycerol solvent ( $\lambda_{ex} = 420$  nm).



Fig. S8 Fluorescence intensity ratio  $(I_{630}/I_{497})$  of Ph-ene-cyclo (10  $\mu$ M) vs different viscosity ( $\lambda_{ex} = 420$  nm).



Fig. S9 Normalized excitation spectra of Ph-ene-cyclo ( $10 \mu$ M) in 90% water-glycerol mixtures with corresponding maximum emission wavelengths at room temperature.



**Fig. S10** Fluorescence spectra of **Ph-ene-cyclo** (10  $\mu$ M) in different ratios of water (W) -glycerol (G) mixtures at room temperature ( $\lambda_{ex} = 860$  nm).



Fig. S11 The linear response between the log (I/I<sub>0</sub>) at 630 nm of Ph-ene-cyclo (10  $\mu$ M) and the log (viscosity) in the water/glycerol solvent ( $\lambda_{ex} = 860$  nm).

Cell culture and fluorescence imaging. HeLa cells were cultured in DMEM medium supplemented with 10% FBS and incubated at 37 °C in air atmosphere (5% CO<sub>2</sub>). The cells were seeded into glass bottom dishes with appropriate density. After 24 h, the cells were incubated with **Ph-ene-cyclo** (10  $\mu$ M) for 30 min at 37 °C. The control group of living cells was preincubated with 10  $\mu$ M Monensin for 30 min, and then **Ph-ene-cyclo** (10  $\mu$ M) was added and incubated for another 30 min. For fluorescence imaging, the cells were washed three times with PBS (pH 7.4) and then underwent imaging measurement by a Nikon A1R confocal microscope with the 488 nm excitation filter and emission channel of 500-550 nm (green channel). Imaging analysis involved use of ImageJ.

**Cytotoxicity assay.** The *in vitro* cytotoxicity of **Ph-ene-cyclo** to HeLa cells was measured by MTT assay. Briefly, HeLa cells were loaded in 96-well culture plates at 7000 cells per well. After culture for 24 h, cells were incubated with fresh cell culture containing 5.0, 10.0 and 20.0  $\mu$ M **Ph-ene-cyclo** for 24 h, respectively. After another 24 h, the cells were added 10  $\mu$ L MTT to each well, and after sufficient reaction with cells, 100  $\mu$ L DMSO was used to dissolve the MTT. Absorbance at 570 nm was measured in a 96-well multiwall-plate reader (TECAN).

## **Fluorescence images**



**Fig. S12** Viability of HeLa cells incubated with different concentration of **Ph-ene-cyclo** (0, 5  $\mu$ M, 10  $\mu$ M and 20  $\mu$ M) for 24 h. Data are mean±SD (bars) (n = 3).



Fig. S13 Fluorescence images of HeLa cells. (a-d) HeLa cells; (e-h) HeLa cells were treated with 10  $\mu$ M Ph-ene-cyclo for 60 min; (i-l) HeLa cells were pre-incubated with 10  $\mu$ M Monensin for 60 min, then with 10  $\mu$ M Ph-ene-cyclo for another 60 min. The images were collected at 500-550 nm, one-photon excitation at 488 nm, two-photon

excitation at 860 nm. Scale bar: 20  $\mu m.$ 

## NMR spectra



Fig. S14 <sup>1</sup>H NMR spectrum of Ph-cyclo (CDCl<sub>3</sub>).



**Fig. S15** <sup>1</sup>H NMR spectrum of **CHO-cyclo** (DMSO-*d*<sub>6</sub>).



Fig. S16 <sup>1</sup>H NMR spectrum of H-cyclo (CDCl<sub>3</sub>).



Fig. S17 <sup>1</sup>H NMR spectrum of Indol-cyclo (DMSO-*d*<sub>6</sub>).



**Fig. S18** <sup>13</sup>C NMR spectrum of **Indol-cyclo** (DMSO-*d*<sub>6</sub>).



**Fig. S19** <sup>1</sup>H NMR spectrum of **Ph-ene-cyclo** (DMSO-*d*<sub>6</sub>).