

# Acetylene-Substituted Two-Photon Absorbing Molecules With Rigid Elongated $\pi$ -Conjugation: Synthesis, Spectroscopic Properties and Two-Photon Fluorescence Cell Imaging Applications

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**Abstract** Two asymmetrical molecules with substituted acetylene as central rigid elongated conjugation are reported as potential chromophores for two-photon microscopic imaging. These molecules consist of a typical D– $\pi$ –A structure, have different donors (D), the same  $\pi$ -conjugated center ( $\pi$ ) and the same acceptor (A). Structural characterization and spectroscopic properties, including single-photon (linear) absorption, quantum yields, single-photon fluorescence, and two-photon absorption spectra, were studied in solvents with different polarity. These acetylene-substituted molecules were found to have high two-photon absorption cross-sections (for example, 690 GM for molecule 1 in toluene), which were determined by a two-photon induced fluorescence method using a femtosecond Ti: sapphire laser as excitation source. Single- and two-photon cellular imaging experiments demonstrate that the substituted acetylene derivatives could be one kind of promising two-photon fluorescence probes for cellular imaging.

**Keywords** Spectroscopic property · Structure-property relationship · Substituted acetylene · Two-photon absorption · Two-photon microscopic imaging

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## Introduction

Beginning from the last 90s, there is an increasingly interest in the synthesis of organic chromophores with improved two-photon absorption (2PA) properties due to their potential applications in several areas, such as optical limiting, two-photon pumped lasing, two-photon photodynamic therapy, 3D microfabrication, 3D optical data storage and two-photon fluorescence microscopic imaging (2PM) [1–5]. Especially, intensive attentions have been focused on the application of organic chromophores in 2PM, because 2PM has some significant advantages over conventional single-photon fluorescence imaging techniques, including a capacity for a highly confined excitation, intrinsic three-dimensional resolution and the possibility of imaging at an increased penetration depth in tissue with reduced photodamage and background fluorescence [6].

To synthesis more effective 2PA molecules, extensive work has been devoted to investigate the relationship between molecular structures and 2PA properties. It is found that the 2PA cross-section ( $\sigma_{2PA}$ ), a characteristic parameter for the two-photon performance of molecules, depends heavily on the donor/acceptor strength, chain length, molecular symmetry, molecular dimensionality as well as the planarity of  $\pi$ -conjugation [7–9]. Basing on these findings, large amount of organic chromophores with attractive 2PA characteristics have been successfully synthesized and studied. However, these works were mainly focused on hydrophobic molecules [10–14]. Organic molecules with both efficient 2PA ability and appropriate water solubility (compatible or relevant to biological environments, which are necessary for efficient cell or tissue staining), are still rare [15]. On the other hand, covalently linking some hydrophilic groups to the side-chain

of hydrophobic molecules is a well-known way to increase their water solubility and thus increase their compatibility with biological environments. As this kind of structure modification has no or little influence on the extent of the conjugation system, which is critical to the corresponding 2PA performance, these subsequent chromophores with improved biocompatibilities could probably possess similar 2PA characteristics as their hydrophobic counterparts [16]. Furthermore, it would be interesting to look closer into the labeling efficiency and specificity of the molecules with improved water solubility.

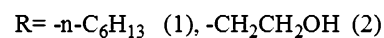
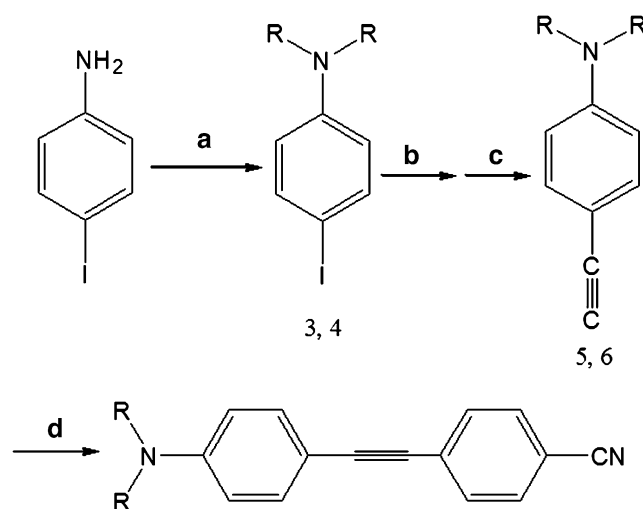
On the other hand, it is worthy to notice that current studies in the field of 2PA material are mainly focused on those chromophores with double-bond (C=C) as  $\pi$ -conjugation. However, little is known about 2PA chromophores containing triple bonds (C≡C). Although the C=C bond is an excellent conjugation bridge for the intramolecular charge transfer (ICT) from donor to acceptor, it readily undergoes *trans* to *cis* photoisomerization, which may hamper the efficiency and lifetime of the materials [17, 18]. Moreover, He et al. pointed out that the alkene compounds have a limited photochemical stability, especially under the exposure to a UV irradiation [19]. However, no photoisomerization (flip–flop) existed when they replaced the vinyl group with an ethynyl group [17]. These findings imply that it would be interesting to study the 2PA ability of the molecules with triple bonds as  $\pi$ -conjugation.

In this paper, two asymmetrical organic molecules 4-[4-*N,N*-(dihexylamino)phenyl]ethynyl benzonitrile (1) and 4-{4-*N,N*-[di(2-hydroxyethyl)amino]phenyl}ethynyl benzonitrile (2) are reported. These molecules consist of a typical D– $\pi$ –A structure, where an *N,N*-dihexylamino or *N,N*-[di(2-hydroxyethyl)amino] group is employed as donor (D), a substituted acetylene as  $\pi$ -conjugated center ( $\pi$ ) and a cyano group as acceptor (A), respectively. Single- and two-photon spectroscopic properties of the molecules are investigated systematically. The potential applications of these chromophores in single- and two-photon fluorescence cell imaging are also evaluated.

## Results and discussion

### Synthesis and characterization

For synthesis of molecules 1 and 2, a multistep approach with coupling procedures was designed (Scheme 1). The key intermediates (terminal acetylenes 5 and 6) were synthesized by Sonogashira's Hagihara reaction with high yields (82–86%). Molecules 1 (or 2) was synthesized by Palladium(II)-catalyzed reaction of molecule 5 (or 6) and 4-bromobenzonitrile in the presence of triethylamine and acetonitrile (76–80% yield). The Palladium(II)-catalyzed



**Scheme 1** Reagents and conditions: (a) (R = -*n*-C<sub>6</sub>H<sub>13</sub>): DMF, K<sub>2</sub>CO<sub>3</sub>, 1-iodohexane 120 °C, 36 h; (R = -CH<sub>2</sub>CH<sub>2</sub>OH): DMF, K<sub>2</sub>CO<sub>3</sub>, chlorohydrin, 80 °C, 10 h; (b) 2-methyl-3-butyn-2-ol, PdCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub>, CuI, triethylamine, reflux, 16 h; (c) KOH, toluene, isopropanol, reflux, 2 h; (d) 4-Bromobenzonitrile, PdCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub>, CuI, triethylamine, CH<sub>3</sub>CN, 60 °C, 10 h

reactions were carried out under dilute hydrogen atmosphere, where the yields can be drastically increased [20]. Standard Schlenk technique was used in the reactions performed under inert gas and anhydrous condition.

### Single-photon spectroscopic properties

The profiles of the single-photon absorption (SPA) or single-photon fluorescence (SPF) spectra of the two molecules are found to be very similar to each other. Only a small shift in the peak wavelength can be observed in the SPA and SPF spectra, which is probably due to the donor electronic density [21]. The single-photon (linear) spectroscopic properties of molecules 1 and 2 in solvents of different polarity are summarized in Table 1. Specifically, in the case of molecule 1, with increasing the solvent polarity (polarity: toluene < CHCl<sub>3</sub> < DMF < PBS) [22], the maximum peaks of the SPA spectra slightly red-shift, while the SPF peaks red-shift significantly. This implies that molecule 1 may possess larger dipole moment in the excited state than that in the ground states [23, 24]. The same phenomena can be seen for molecule 2 in several organic solvents (toluene, CHCl<sub>3</sub> and DMF) except PBS, where a large blue-shift was observed in both SPA and SPF spectra. This abnormal behavior is probably due to the hydrogen bonding interaction between the solvent (PBS) and the solute (molecule 2) [25]. In addition, the molecules have moderate quantum yields ranging from 0.21 to 0.62 and

**Table 1** Spectroscopic data for molecules 1 and 2 in different solvents

Comp	Solvent	$\lambda_{\text{max}}^{\text{SPA}}/\text{nm}^{\text{a}}$	$\lambda_{\text{max}}^{\text{SPF}}/\text{nm}^{\text{b}}$	$\lambda_{\text{max}}^{\text{2PF}}/\text{nm}^{\text{c}}$	$\eta^{\text{d}}$	$\sigma_{\text{2PA}}/\text{GM}^{\text{e}}$
1	In toluene	378	427	494	0.60	690
	In $\text{CHCl}_3$	382	465	506	0.51	338
	In DMF	386	533	546	0.42	121
	In $\text{PBS}^{\text{f}}$	387	536	— <sup>g</sup>	0.25	— <sup>g</sup>
2	In toluene	374	426	498	0.62	658
	In $\text{CHCl}_3$	377	453	500	0.52	283
	In DMF	380	536	545	0.44	182
	In $\text{PBS}^{\text{f}}$	367	458	— <sup>g</sup>	0.21	— <sup>g</sup>

<sup>a</sup> Peak wavelengths of single-photon absorption (SPA) spectra

<sup>b</sup> Single-photon fluorescence (SPF) spectra

<sup>c</sup> Two-photon fluorescence (2PF) spectra

<sup>d</sup> Fluorescence quantum yield relative to fluorescein in 0.1 mol.l<sup>-1</sup> NaOH ( $\eta=0.90$ ) [30]

<sup>e</sup> Two-photon absorption cross-section ( $\sigma_{\text{2PA}}$ ) values given in GM at 780 nm. Fluorescein with the same concentration in NaOH (pH=11) is used as reference standard. 1 GM (Göppert-Mayer)=10<sup>-50</sup> cm<sup>4</sup> s photon<sup>-1</sup>

<sup>f</sup> A mixture of PBS and DMSO (PBS/DMSO=20:1, V/V)

<sup>g</sup> Not available

the quantum yields of molecules decrease with increasing the polarity of solvents.

#### Two-photon spectroscopic properties

Two-photon excited fluorescence (2PF) of the molecules were measured in solvents of varying polarity at a concentration of  $5 \times 10^{-4}$  mol.l<sup>-1</sup> using a mode-locked femtosecond Ti:sapphire laser (Mai Tai, Spectra-Physics Inc., USA) as excitation source. The up-converted fluorescence was recorded in the excitation wavelength range from 720 to 960 nm with a step size of 20 nm. To confirm that the up-converted emission underwent 2PA process under the experimental conditions, the dependence of the total integrated up-converted fluorescence intensity for molecules 1 and 2 in different solvent were measured as a function of the incident intensity (pump power), and a representative dependence curve for the molecule 2 in toluene is shown in Fig. 1. The quadratic dependence indicates that the up-converted fluorescence is induced by 2PA. The profiles of the 2PF spectra are found to be identical to the corresponding SPF spectra, while the differences in emission peaks between SPF and 2PF are probably due to reabsorption effect [21]. Table 1 summarizes the two-photon spectroscopic performances of the molecules in solvents of different polarity. Solvatochromism behavior can be seen, which is similar to that observed in the corresponding single-photon process.

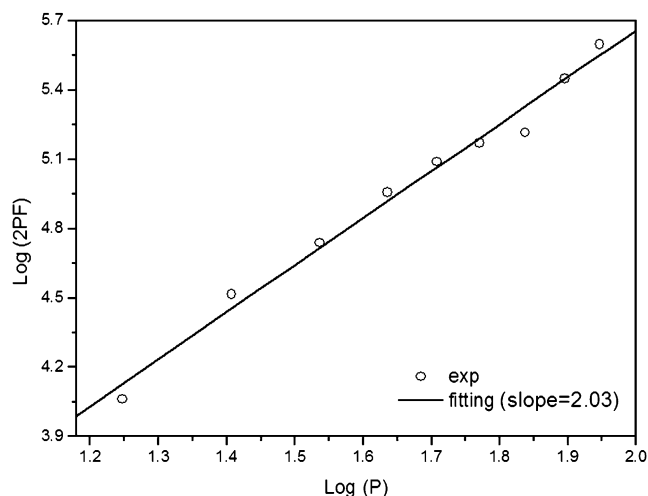
#### Two-photon absorption cross-section

The two-photon absorption cross-section ( $\sigma_{\text{2PA}}$ ) of molecules 1 and 2 were obtained by comparison the 2PF emission of the analyte with a fluorescein calibration standard (both at a concentration of  $5 \times 10^{-4}$  mol l<sup>-1</sup>). The detailed procedures

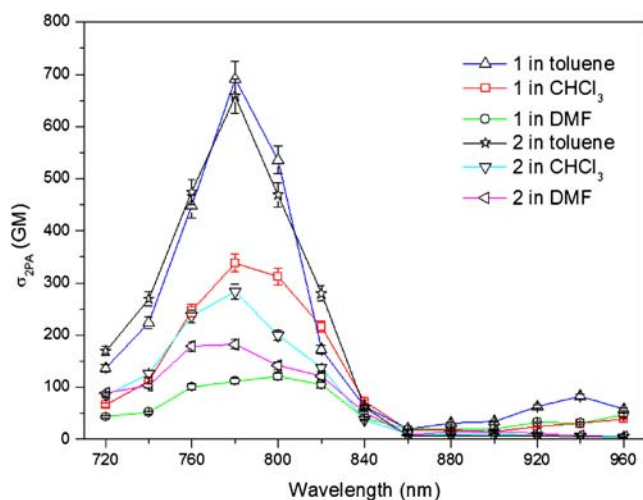
are similar to the reference [26] and had been described in our previous work [13]. Using similar procedures, the  $\sigma_{\text{2PA}}$  values were calculated and shown (see Fig. 2 and Table 1). Interestingly, the  $\sigma_{\text{2PA}}$  values of the two molecules decrease with the increasing polarity (Fig. 2) and both molecules have nearly the same  $\sigma_{\text{2PA}}$  values, indicating that replacing a hydrophobic side group (*N,N*-dihexylamino) with a hydrophilic side group (*N,N*-[di(2-hydroxyethyl)amino]) has little or no changes to the 2PA performance.

#### Single- and two-photon cellular imaging

In order to evaluate the potential applications of molecules 1 and 2 in single- and two-photon cellular imaging, HepG2 cells were incubated with these molecules for 15 min at room temperature. Bright field transmission and single-



**Fig. 1** Input intensity dependent up-converted fluorescence of molecule 2 ( $5 \times 10^{-4}$  mol l<sup>-1</sup>) in toluene excited at 780 nm



**Fig. 2** Two-photon excitation spectra of molecules 1 and 2 in different solvents

photon fluorescence microscopic images (Olympus IX 71, Japan) of the HepG2 cells stained with molecules 1 and 2 were collected with a 20 $\times$  objective (NA=0.4) and are shown in Fig. 3. The control cells showed very weak or no autofluorescence under the same single-photon excitation (images not shown). It is found that the cells could be efficiently stained with both molecules (Fig. 3b and d).

Two-photon fluorescence microscopic images were collected on a commercial laser scanning two-photon microscope (Fluoview 1000, Olympus Inc., Japan). The excitation light was provided by a mode-locked Ti:sapphire

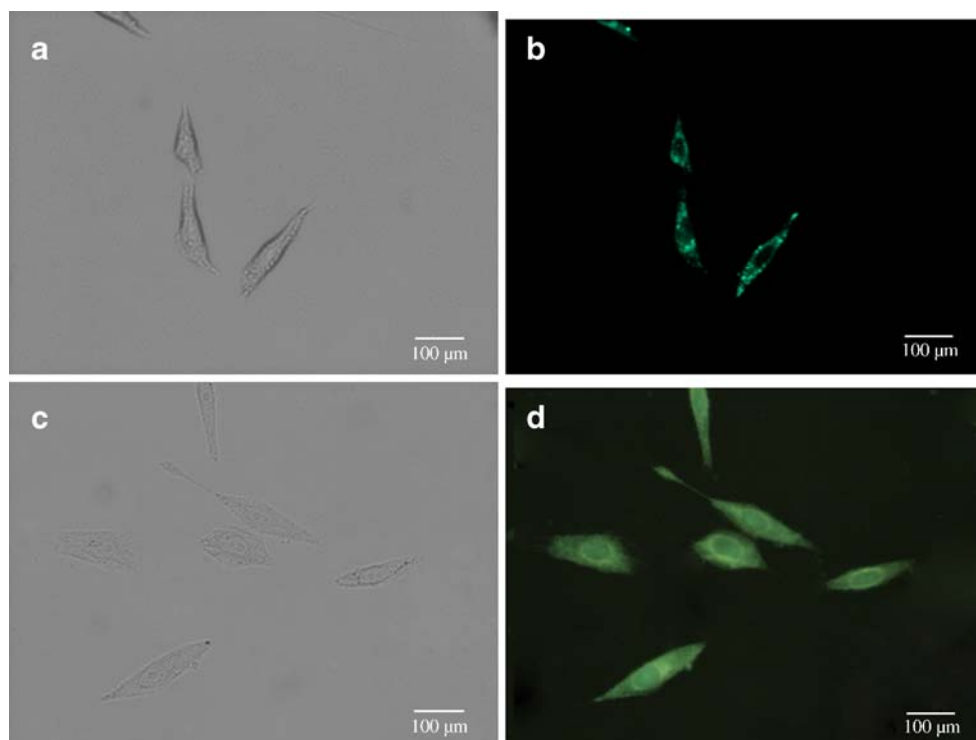
laser (Mai Tai, Spectra-Physics Inc., USA) tuned to 800 nm, operating at 100 fs pulse width and 82 MHz repetition rate. The average incident power at the sample was adjusted to be about 1 mW. A broad band-pass filter (480–600 nm) was used as emission filter. A 60 $\times$  objective oil lens (NA=1.35, Olympus Inc., Japan) was used to collect the 2PF emission from the cells. The control cells did not show any autofluorescence under this kind of excitation. The 2PM images of the cells stained with molecules 1 and 2 are shown in Fig. 4. In addition, it is found that the contrast in 2PM images (Fig. 4) is much higher than that in the single-photon microscopic images (Fig. 3). Further experiments are still ongoing to fully evaluate the potentials of these molecules in two-photon fluorescence imaging.

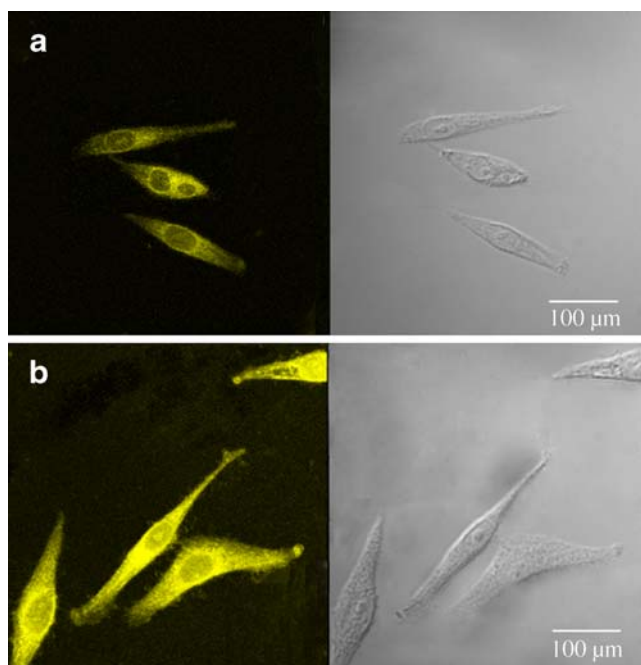
## Experimental

### Materials, methods, and instruments

All the solvent and chemicals were purchased from Shanghai Chemical Reagent Co. Ltd. (China) and were purified by standard procedures. [27] Dichlorobis(triphenylphosphine) palladium(II) ( $\text{PdCl}_2(\text{PPh}_3)_2$ ) (Fluka) was used without further purification. 4-Iodo-*N,N*-dihexylaniline (3) and 4-iodo-*N,N*-di(2-hydroxyethyl) aniline (4) were prepared according to the literature [28, 29]. All melting points are uncorrected.  $^1\text{H}$  NMR spectra were recorded on a Mercury

**Fig. 3** Bright field transmission (left) and single-photon fluorescence microscopic (right) images of HepG2 cells stained with 1 (a and b) and 2 (c and d), respectively





**Fig. 4** Differential interference contrast (*right*) and 2PM (*left*) images of HepG2 cells stained with (a) 1 and (b) 2, respectively

VX-300 (Varian, 300 MHz) spectrometer using tetramethylsilane (TMS) as the internal standard.  $^{13}\text{C}$  NMR spectra were recorded on a Bruker AV400 (Bruker, 400 MHz) spectrometer using the carbon signal of the deuterated solvent as the internal standard. Elemental analyses were performed on a Perkin-Elmer 240C instrument. The SPA spectra were recorded on a UV-2550 UV-visible Spectrophotometer (SHIMADZU, Japan). The SPF spectra and the quantum yields were measured on a LS-55 Luminescence Spectrometer (PerkinElmer, USA). The quantum yields were measured by the optically dilute measurement method using fluorescein in  $0.1 \text{ mol l}^{-1}$  sodium hydroxide as the reference standard ( $\eta=0.90$ ) [30]. The experimental setup for the  $\sigma_{2\text{PA}}$  measurements was similar to the reference [31]. Bright field transmission and single-photon fluorescence microscopic imaging of HepG2 cells, which were stained with the molecules studied, were recorded on the inverted fluorescence microscope (IX71, Olympus Inc., Japan). 2PM images of cells stained with the molecules were collected on a commercial two-photon microscope (Fluo View 1000, Olympus Inc., Japan). The excitation light was provided by a mode-locked Ti:sapphire laser (Mai Tai, Spectra-Physics Inc., USA) tuned 800 nm, operating at a 100 fs pulse width and a 82 MHz repetition rate. The average power at the sample was controlled to be about 1 mW. A broad band-pass filter (480–600 nm) was used as the emission filter. The differential interference contrast (DIC) images were taken using a thermoelectrically cooled charged-couples device (CCD) (Penguin 150CL, Pixera, USA).

## Synthesis

### General procedure for the synthesis of molecules 1 and 2

3 (1.01 g, 2.6 mmol) or 4 (0.80 g, 2.6 mmol) and 2-methyl-3-butyn-2-ol (0.33 g, 3.9 mmol) in a 50 ml Schlenk reaction tube were refluxed in the presence of 20 ml anhydrated triethylamine (previously purged with Ar),  $\text{PdCl}_2(\text{PPh}_3)_2$  (21 mg, 0.03 mmol) and a trace amounts of CuI [32, 33]. The reaction was stirred under argon/hydrogen (7:3) mixture for 16 h. After evaporating, brine was added (30 ml) and the solution was extracted with ether. The Palladium salts were eliminated by passing the ether extract through a flash alumina column (neutral, activity I). The ether was removed and the remaining product was used in the followed reaction without further purification. KOH (0.14 g, 2.6 mmol) and the above raw products were added to the mixture of isopropanol (3 ml) and toluene (25 ml). After a 2 h reflux, the solvent was removed under vacuum, brine (25 ml) was added, and then the solution was extracted with  $\text{CHCl}_3$ . The combined organic phase was dried over  $\text{MgSO}_4$ . The residue was purified by column chromatography (silica gel, light petroleum ether/ethyl acetate, 1:1) to give the product 4-*N,N*-Dihexylaminophenylacetylene (5) or 4-*N,N*-di(2-hydroxyethyl)amino phenylacetylene (6).

4-Bromobenzonitrile (0.25 g, 1.4 mmol),  $\text{PdCl}_2(\text{PPh}_3)_2$  (10 mg, 0.015 mmol), and a trace amounts of CuI were placed in a 50 ml Schlenk reaction tube and degassed. 5 (0.31 g, 1.1 mmol) or 6 (0.22 g, 1.1 mmol) was added the reaction mixture of triethylamine (1 ml) and  $\text{CH}_3\text{CN}$  (20 ml), which was previously purged with Ar. The reaction was stirred under argon/hydrogen (7:3) mixture gas atmosphere at 60 °C for 10 h. The solvent was removed under vacuum. Brine (40 ml) was added, and then the mixture was extracted with ether and dried over  $\text{MgSO}_4$ . The solvent was evaporated and the residue was purified by column chromatography (silica gel, light petroleum ether/ethyl acetate, 1:1) to give 1 or 2.

*4-N,N-Dihexylaminophenylacetylene (5)* Yellow oil. Yield: 86%.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ , ppm)  $\delta$ : 7.29 (d, 2H), 6.50 (d, 2H), 3.25 (t, 4H), 2.95 (s, H), 1.56 (m, 4H), 1.30 (m, 12H), 0.89 (t, 6H). Calculated analyses for  $\text{C}_{20}\text{H}_{31}\text{N}$ : C, 84.15; H, 10.95; N, 4.90. Found: C, 84.15; H, 10.99; N, 5.01%.

*4-[4-N,N-(Dihexylamino)phenyl]ethynyl benzonitrile (1)* Yellow solid. Yield: 80%. Melting point (m.p.) 88–90 °C.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ , ppm)  $\delta$ : 7.57 (d, 2H), 7.51 (d, 2H), 7.34 (d, 2H), 6.56 (d, 2H), 3.28 (t, 4H), 1.56 (m, 4H), 1.32 (m, 12H), 0.90 (t, 6H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ , ppm)  $\delta$ : 148.59, 133.25, 131.90, 131.48, 129.49, 118.84, 111.24, 110.15, 107.44, 96.24, 86.22, 50.97, 31.69, 27.19, 26.78,

22.66, 14.01. Calculated analyses for  $C_{27}H_{34}N_2$ : C, 83.89; H, 8.87; N, 7.24. Found: C, 83.94; H, 8.90; N, 7.30%.

*4-N,N-Di(2-hydroxyethyl)amino phenylacetylene (6)* Yellow solid. Yield: 82%. m.p. 105–107 °C.  $^1H$  NMR (300 MHz,  $CDCl_3$ , ppm)  $\delta$ : 7.32 (d, 2H), 6.58 (d, 2H), 3.85 (t, 4H), 3.59 (t, 4H), 2.97 (m, 1H). Calculated analyses for  $C_{12}H_{15}NO_2$ : C, 70.22; H, 7.36; N, 6.82. Found: C, 70.28; H, 7.41; N, 6.90%.

*4-{4-N,N-[Di(2-hydroxyethyl)amino]phenyl}ethynyl benzonitrile (2)* Yellow solid. Yield: 76%. m.p. 174–176 °C.  $^1H$  NMR (300 MHz,  $CDCl_3$ , ppm)  $\delta$ : 7.55 (d, 2H), 7.49 (d, 2H), 7.22 (d, 2H), 6.63 (d, 2H), 3.88 (t, 4H), 3.63 (t, 4H).  $^{13}C$  NMR (100 MHz, DMSO, ppm)  $\delta$ : 149.28, 133.44, 132.90, 131.87, 128.90, 119.10, 111.86, 110.13, 107.08, 96.44, 86.82, 58.54, 53.54. Calculated analyses for  $C_{19}H_{18}N_2O_2$ : C, 74.49; H, 5.92; N, 9.14. Found: C, 74.52; H, 5.98; N, 9.20%.

#### Cells and staining

HepG2 cells were plated on a slice in a 3.5 cm-dish, and kept in a humidified atmosphere of 5%  $CO_2$  at 37 °C 24 h prior to staining. The cells were in Dulbecco's modification of Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Sigma). At the beginning of staining, cells were washed with PBS (phosphate buffer saline, pH=7.2) for three times to remove growth medium, then 15  $\mu$ l of each molecule (1 or 2,  $5 \times 10^{-5}$  mol.l $^{-1}$ ) dissolved in anhydrous DMSO (Sigma) and 200  $\mu$ l PBS were added into the dish separately. After 15 min incubating at room temperature, the cells were washed with PBS ( $\times 3$ ) to remove unlabeled molecules, and then the cells were ready for imaging.

#### Conclusions

Two substituted acetylene derivatives with a typical D– $\pi$ –A structure have been designed and synthesized. Single- and two-photon spectroscopic studies were performed. The quantum yields and two-photon absorption cross-sections of the molecules in different solvents were found to decrease from nonpolar to polar solvents. However, when replacing the hydrophobic donor with a hydrophilic donor, no significant changes in quantum yields and two-photon absorption cross-sections can be found in this kind of acetylene derivatives. The potential applications of the molecules in single- and two-photon cellular imaging were also evaluated. This work demonstrates that the substituted acetylene derivatives could be one kind of promising two-photon fluorescence probes for two-photon cellular imaging.

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