

## Two-Photon Fluorescence Probes for Imaging of Mitochondria and Lysosomes

Wanggui Yang<sup>a</sup>, Pui Shan Chan<sup>b</sup>, Miu Shan Chan<sup>a, d</sup>, King Fai Li<sup>c</sup>, Pik Kwan Lo<sup>d</sup>, Kok Wai Cheah<sup>c</sup>, Nai Ki Mak<sup>b, \*</sup>, and Man Shing Wong<sup>a, \*</sup>

<sup>a</sup> Department of Chemistry and Institute of Advanced Materials, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China. E-mail: mswong@hkbu.edu.hk

<sup>b</sup> Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China. E-mail: nkmak@hkbu.edu.hk

<sup>c</sup> Department of Physics and Institute of Advanced Materials, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China. E-mail: kwcheah@hkbu.edu.hk

<sup>d</sup> Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, SAR China.

---

### Contents:

- (1) Absorption and emission spectra of cyanines
- (2) UV-Vis absorption and emission spectra of cyanines in buffer with different pH values
- (3) The logarithmic plots of the power dependence of relative two-photon induced fluorescence intensity of cyanines
- (4) Fluorescence confocal microscopy images of HK-1 cells stained with cyanines
- (5) Experimental procedures
- (6) <sup>1</sup>H and <sup>13</sup>C NMR and HRMS spectra of cyanines

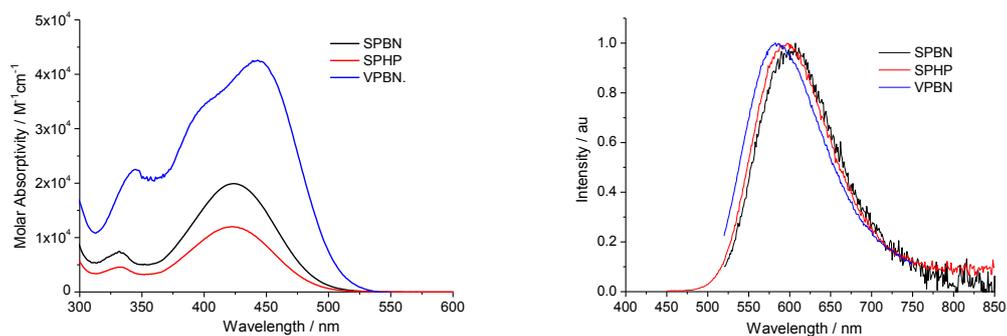
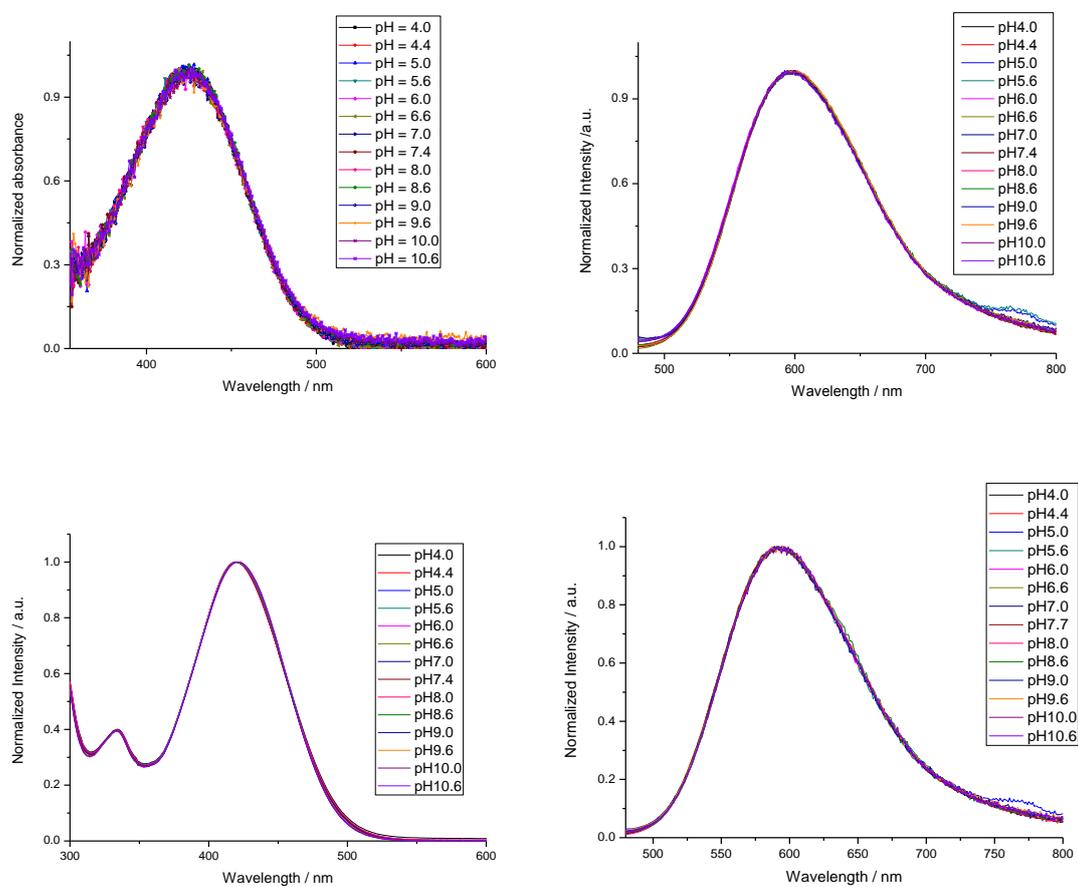
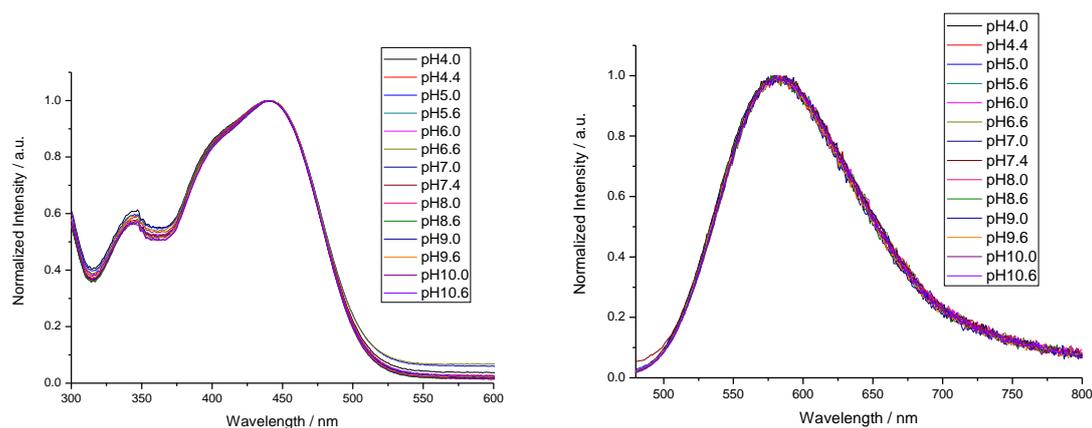
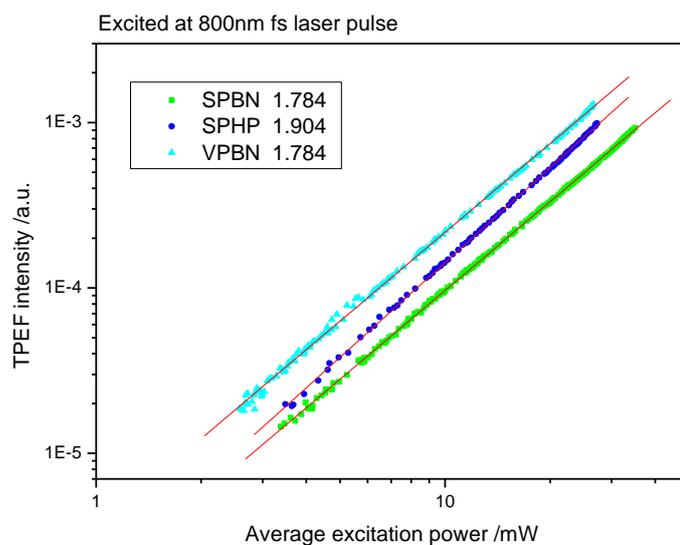


Figure S1. Absorption and emission spectra of cyanines in buffered medium

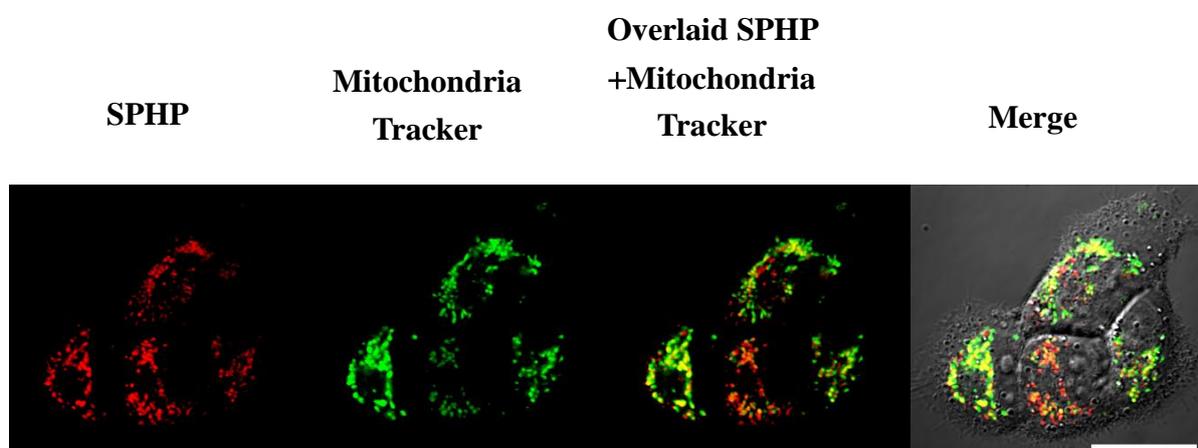
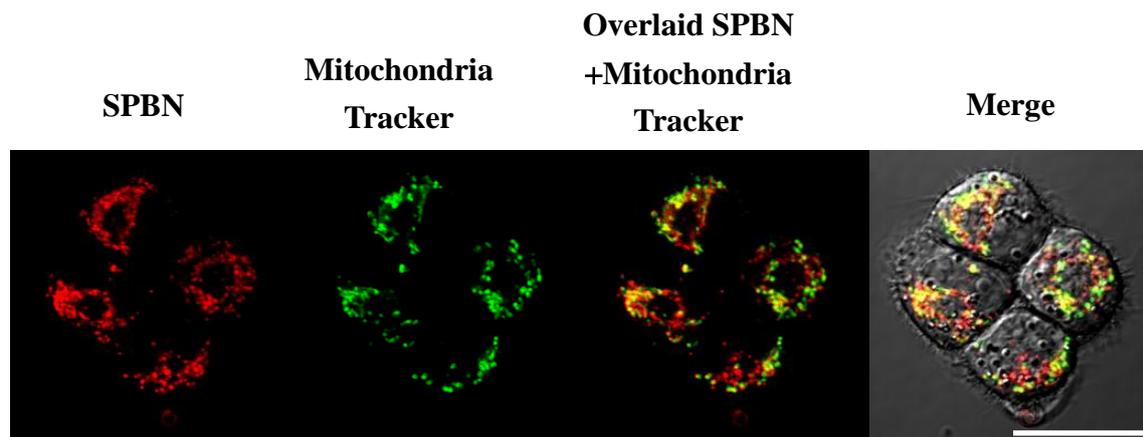




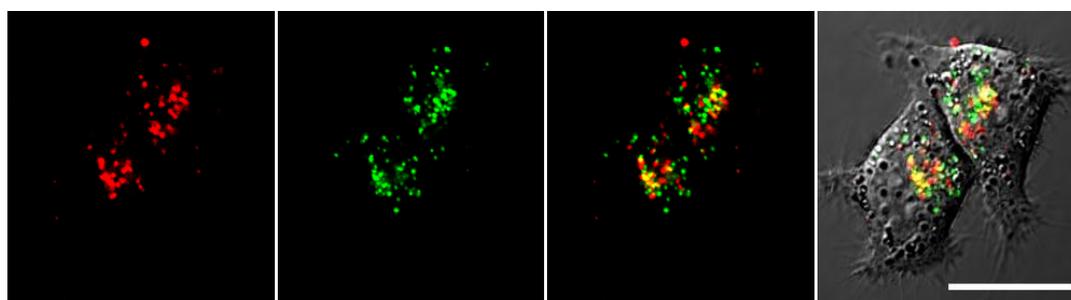
**Figure S2.** UV-Vis absorption and emission spectra of (a) **SPBN** (upper row) (b) **SPHP** (middle row), and (c) **VPBN** (lower) in buffer with different pH values.



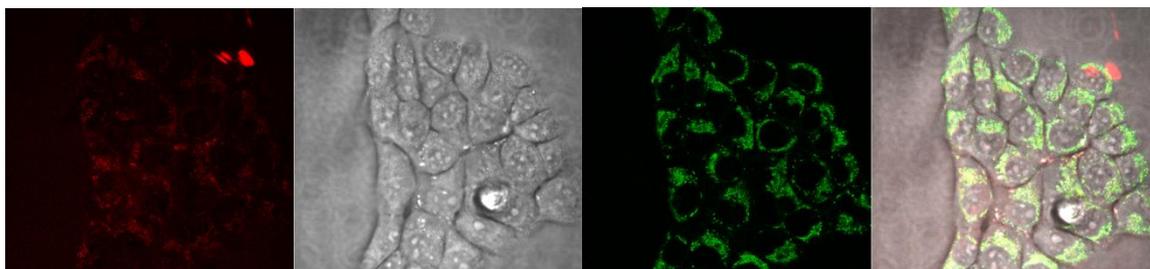
**Figure S3.** The logarithmic plots of the power dependence of relative two-photon induced fluorescence intensity of the cyanines as a function of pump power at an excitation wavelength of 800 nm, respectively. The solid lines are the best-fit straight lines with gradient,  $n = 1.8-1.9$ .



**Figure S4.** Fluorescence confocal microscopy images of HK-1 cells incubated with (a) **SPBN** (upper) and **SPHP** (lower) (5  $\mu$ M, 6 h). (b) images of the same sample after being co-stained with Mito tracker. (c) the overlap of (a) and (b); (d) overlapped images of (c) and the DIC image.



**Figure S5.** Fluorescence confocal microscopy images of HK-1 cells incubated with (a) **VPBN** (5  $\mu$ M, 6 h). (b) images of the same sample after being co-stained with Lyso tracker. (c) the overlap of (a) and (b); (d) overlapped images of (c) and the DIC image.



**Figure S6.** (a) TPEF and (b) Transmission images of HK-1 cells upon incubation with **SPBN** for 24 h at a concentration of 2  $\mu\text{M}$ . (c) one-photon confocal microscopic images of the same sample after being co-stained with Mito tracker (100 nM) for 10-20 minutes in dark (d) overlapped images of (a), (b) and (c).

## Experimental Procedure:

### Two-photon Bio-imaging

HK-1 cells ( $5 \times 10^4$ ) were seeded onto 35 mm glass bottom Petri dish (TatTek) and incubated for overnight. The cells were incubated with the fluorescent dye at a concentration of 2  $\mu\text{M}$  for 24 hours in dark. The cells were then rinsed with cRPMI and the images were examined under a two-photon confocal microscope. The excitation wave length was 850 nm for **SPBN**, 850 nm for **SPHP**, and 910 nm for **VPBN**. A long pass filter of 560 nm is placed before the CCD camera. After capturing of the TPA images, the cells were rinsed with cRPMI and then incubated with Lyso or Mito tracker at a concentration of 100 nM for 10 to 20 minutes in dark. The images of Lyso and Mito tracker-stained cells were examined under the one-photon confocal microscope. The excitation wave length for the trackers was 488 nm and the emission was collected at 500 - 550 nm.

### MTT method:

HK-1 cells ( $2 \times 10^4$  cells /well) were seeded onto 96-well plate and the cells were cultured in cRPMI for overnight. The cells were then treated with various concentrations of the compounds (0.5  $\mu\text{M}$  – 8  $\mu\text{M}$ ) for 24 hours in dark. The cells were then rinsed with PBS and incubated with MTT (250  $\mu\text{g}/\text{ml}$ ) for 3 hours in a  $\text{CO}_2$  incubator. The formazan crystal was dissolved in DMSO. The absorbance of the dissolved crystal was measured at 540 nm and 690 nm using the ELISA plate reader. Results were expressed as the mean  $\pm$  S.D. of three separate trials.

**4-bromo-*N,N,N*-triethylbutan-1-aminium bromide (1).** A solution of triethylamine (0.33 g, 3.3 mmol) and 1,4-dibromobutane (1.4 g, 6.6 mmol) in CH<sub>3</sub>CN (35 mL) was refluxed for 4 h. Then the reaction mixture was cooled to room temperature and the solvent was removed. The residue was dissolved into water and washed with ethyl acetate for 3 times. Water was removed under vacuum and the solid was washed with CH<sub>3</sub>CN and ethyl acetate (CH<sub>3</sub>CN: EA = 1:4) affording **1** (0.48 g) in 46% yield. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 3.65-3.69 (t, *J* = 6.4 Hz, 2H), 3.55-3.60 (q, *J* = 7.2 Hz, 6H), 3.05 (m, 2H), 2.02 (m, 4H), 1.37-1.41 (t, *J* = 7.2 Hz, 9H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>) δ 55.0, 52.1, 34.0, 28.9, 19.7, 7.2.

**(4-methyl-1-(4-(triethylammonio)butyl)pyridinium) dibromide (2).** A solution of **1** (0.48 g, 1.5 mmol) and picoline (0.3 g, 3.2 mmol) in CH<sub>3</sub>CN (25 mL) was refluxed for 8 h. Then the solution was cooled to room temperature and the solvent was removed. The residue was dissolved into water and washed with ethyl acetate for 3 times. Water was removed under vacuum and the solid was washed with CH<sub>3</sub>CN and ethyl acetate (CH<sub>3</sub>CN: EA = 1:3) affording **2** (0.38 g) in 61% yield. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 8.96-8.98 (d, *J* = 6.4 Hz, 2H), 8.02-8.03 (d, *J* = 6.4 Hz, 2H), 4.58-4.62 (t, *J* = 7.2 Hz, 2H), 3.22 (m, 8H), 2.62 (s, 3H), 1.92-1.95 (t, *J* = 7.2 Hz, 2H), 1.62-1.64 (t, *J* = 7.2 Hz, 2H), 1.15-1.18 (t, *J* = 7.2 Hz, 9H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>) δ 158.8, 143.7, 128.3, 59.0, 55.4, 52.2, 27.6, 21.4, 18.1, 7.4.

**(6-bromohexyl)triphenylphosphonium bromide (3).** A solution of triphenylphosphine (0.52 g, 2 mmol) and 1,6-dibromohexane (1.5 g, 6 mmol) in CH<sub>3</sub>CN (35 mL) was refluxed for 4 h. The mixture was cooled to room temperature and the solvent was removed under vacuum. The residue was dissolved into water and extracted with ethyl acetate for 3 times. Water was removed under vacuum and the solid was washed with CH<sub>3</sub>CN and ethyl acetate (CH<sub>3</sub>CN: EA = 1:4) affording **3** (0.41 g) in 41% yield. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 7.75-7.92 (m, 15H), 3.6-3.7 (m, 2H), 3.48 (t, *J* = 6.8 Hz, 2H), 1.69-1.76 (m, 2H), 1.37-1.54 (m, 6H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>) δ 134.8, 133.6, 133.5, 130.3, 130.1, 118.9, 118.1, 34.9, 31.9, 28.8, 26.7, 21.6, 20.4, 19.9.

**4-methyl-1-(6-(triphenylphosphonio)hexyl)pyridinium bromide (4).** A solution of **3** (0.41 g, 0.8 mmol) and picoline (0.25 g, 2 mmol) in CH<sub>3</sub>CN (25 mL) was refluxed for 8 h. Then the solution was cooled to room temperature and the solvent was removed under vacuum. The residue was dissolved into water and washed with ethyl acetate for 3 times. Water was removed under vacuum and the solid was washed with CH<sub>3</sub>CN and ethyl acetate (CH<sub>3</sub>CN: EA = 1:3) affording **4** (0.25 g) in 52% yield. <sup>1</sup>H NMR (400 MHz,

CH<sub>3</sub>CN-d<sub>3</sub>) 9.15 (d, *J* = 6.4 Hz, 2H), 7.63-7.87 (m, 17H), 4.67 (t, *J* = 7.2 Hz, 2H), 3.61-3.68 (m, 2H), 2.52 (s, 3H), 1.78-1.85 (m, 2H), 1.51-1.55 (m, 4H). 1.28-1.35 (m, 2H). <sup>13</sup>C NMR (100 MHz, CH<sub>3</sub>CN-d<sub>3</sub>) δ 159.8, 144.5, 135.6, 134.5, 134.4, 130.9, 129.2, 119.6, 118.7, 118.2, 60.4, 31.1, 29.4, 24.9, 22.2, 21.9, 21.6.

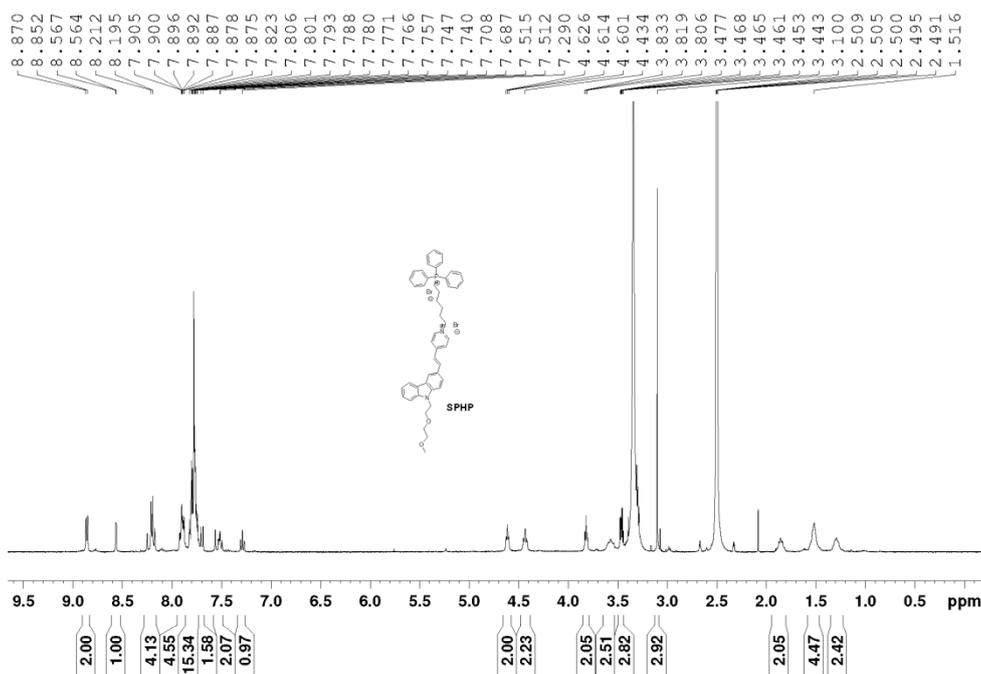
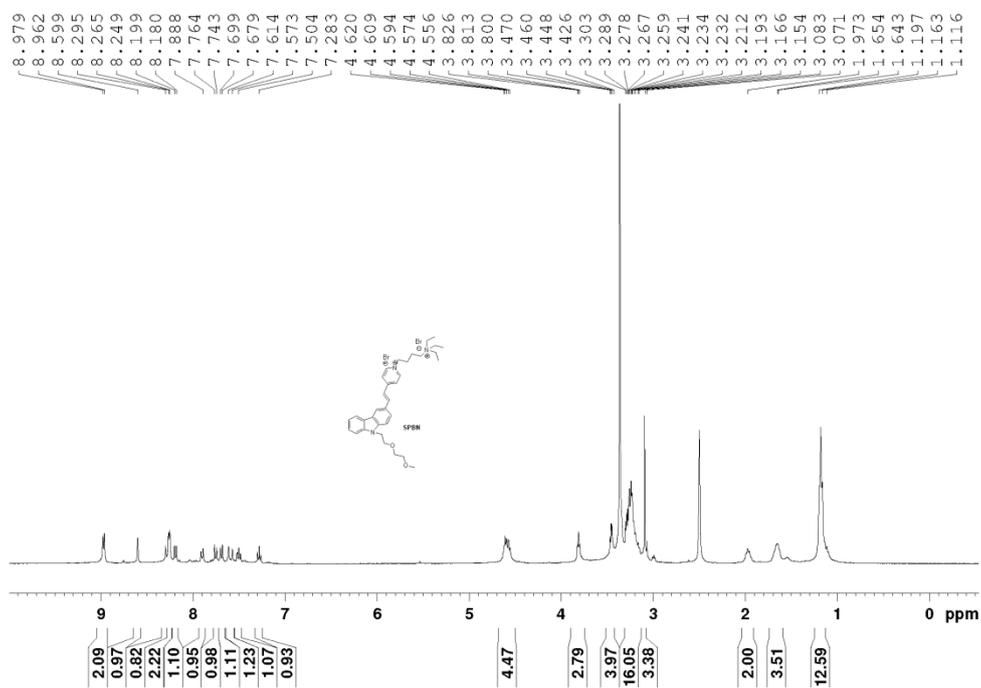
**(E)-4-(2-(9-(2-(2-methoxyethoxy)ethyl)-9H-carbazol-3-yl)vinyl)-1-(4-(triethylammonio)butyl)pyridinium bromide (SPBN).** A solution of **2** (0.38 g, 0.91 mmol), 9-(2-(2-methoxyethoxy)ethyl)-9H-carbazole-3-carbaldehyde (0.22 g, 0.76 mmol) and piperidine (0.2 mL) in ethanol (15 mL) was heated to reflux overnight. After cooling down to room temperature, the organic solvent was removed and the residue was purified by recrystallization using ethanol and methanol to afford **SPBN** (0.22 g, 0.33 mmol) in 44% yield. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.96-9.98 (d, *J* = 6.8 Hz, 2H), 8.60 (s, 1H), 8.25-8.29 (m, 3H), 8.18-8.20 (d, *J* = 7.6 Hz, 1H), 7.89-7.91 (d, *J* = 8.8 Hz, 1H), 7.74-7.76 (d, *J* = 8.4 Hz, 1H), 7.68-7.70 (d, *J* = 8.0 Hz, 1H), 7.57-7.61 (d, *J* = 16.4 Hz, 1H), 7.48-7.52 (t, *J* = 7.6 Hz, 1H), 7.27-7.30 (t, *J* = 7.6 Hz, 1H), 4.60 (m, 4H), 3.80-3.83 (t, *J* = 5.2 Hz, 2H), 3.46 (m, 4H), 3.25 (m, 6H), 3.08 (s, 3H), 1.96-1.99 (t, *J* = 6.8 Hz, 2H), 1.64 (m, 4H), 1.16 (m, 9H). <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>) δ 153.6, 144.0, 142.8, 141.8, 140.9, 126.4, 126.3, 126.2, 123.1, 122.7, 122.1, 121.1, 120.3, 120.2, 120.0, 119.8, 110.6, 110.3, 71.3, 69.8, 68.8, 58.6, 58.1, 55.3, 52.1, 43.7, 42.9, 27.5, 22.3, 21.7, 18.1, 7.3. HRMS (MALDI-TOF) *m/z* Calcd for C<sub>34</sub>H<sub>47</sub>BrN<sub>3</sub>O<sub>2</sub><sup>+</sup> 610.2832 Found 610.2847 [M]<sup>+</sup>.

**(E)-4-(2-(9-(2-(2-methoxyethoxy)ethyl)-9H-carbazol-3-yl)vinyl)-1-(6-(triphenylphosphonio)hexyl)pyridinium bromide (SPHP).** A solution of **4** (0.36 g, 0.6 mmol), 9-(2-(2-methoxyethoxy)ethyl)-9H-carbazole-3-carbaldehyde (0.15 g, 0.5 mmol) and piperidine (0.2 mL) in ethanol (15 mL) was heated to reflux overnight. After cooling down to room temperature, the organic solvent was removed and the residue was purified by recrystallization using ethanol and methanol to afford **SPHP** (0.17 g) in 38% yield. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ 8.86 (d, *J* = 7.2 Hz, 2H), 8.56 (s, 1H), 8.23 (d, *J* = 16.0 Hz, 1H), 8.19 (t, *J* = 7.6 Hz, 1H), 7.87-7.92 (m, 4H), 7.75-7.82 (m, 15H), 7.69 (d, *J* = 8.4 Hz, 1H), 7.54 (d, *J* = 16.0 Hz, 1H), 7.51 (t, *J* = 7.6 Hz, 1H), 7.29 (t, *J* = 8.0 Hz, 1H), 4.61 (t, *J* = 5.6 Hz, 2H), 4.43 (t, *J* = 6.8 Hz, 2H), 3.82 (t, *J* = 5.2 Hz, 2H), 3.42-3.48 (m, 4H), 3.10 (s, 3H), 1.83-1.89 (m, 2H), 1.46-1.51 (m, 4H), 1.23-1.29 (m, 2H). <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>) 153.5, 143.9, 142.7, 141.9, 140.9, 135.0, 133.7, 130.4, 130.3, 126.5, 126.3, 126.2, 123.1, 122.7, 121.2, 120.4, 120.0, 119.9, 119.0, 118.1, 110.6, 110.4, 71.3, 69.8, 68.9, 59.4, 58.1, 54.9, 30.2, 24.8. HRMS (MALDI-TOF) *m/z* Calcd for C<sub>48</sub>H<sub>51</sub>BrN<sub>2</sub>O<sub>2</sub>P<sup>+</sup> 799.2866 Found 799.2859 [M]<sup>+</sup>.

**4,4'-(1E,1'E)-2,2'-(9-(2-(2-methoxyethoxy)ethyl)-9H-carbazole-3,6-diyl)bis(ethene-2,1**

**-diyl)bis(1-(4-(triethylammonio)butyl)pyridinium) bromide (VPBN).** A solution of **2** (0.43 g, 1.03 mmol), 9-(2-(2-methoxyethoxy)ethyl)-9H-carbazole-3,6-dicarbaldehyde (0.15 g, 0.47 mmol) and piperidine (0.3 mL) in ethanol (30 mL) was heated to reflux for 40 h. After cooling down to room temperature, organic solvent was removed and the residue was purified by recrystallization from ethanol and then from methanol to afford **VPBN** (0.2 g) in 39% yield.  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.95-8.97 (d,  $J = 6.4$  Hz, 4H), 8.62 (s, 2H), 8.29 (m, 6H), 7.97-7.99 (d,  $J = 8.0$  Hz, 2H), 7.83-7.85 (d,  $J = 8.8$  Hz, 2H), 7.60-7.64 (d,  $J = 24$  Hz, 2H), 4.67 (m, 2H), 4.55-4.58 (t,  $J = 6.4$  Hz, 4H), 3.83-3.85 (d,  $J = 4.8$  Hz, 2H), 3.47 (m, 6H), 3.28 (m, 12H), 3.09 (s, 3H), 1.95-1.97 (t,  $J = 6.4$  Hz, 4H), 1.66 (m, 6H), 1.17-1.20 (t,  $J = 6.8$  Hz, 18H).  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$  153.4, 144.1, 142.4, 127.2, 126.5, 123.4, 122.7, 121.4, 120.7, 111.3, 71.3, 69.8, 68.9, 58.7, 58.1, 55.3, 52.1, 48.6, 27.5, 18.1, 7.3. HRMS (ESI)  $m/z$  Calcd for  $\text{C}_{51}\text{H}_{75}\text{N}_5\text{O}_2$  197.3974 Found 197.3971 [ $\text{M}^{4+}$ ].

# $^1\text{H}$ NMR spectra





### <sup>13</sup>C NMR spectra

