Supplementary Material (ESI) for Chemical Communications
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Supporting Information

V-Shape Cationic Dye for Nonlinear Bioimaging

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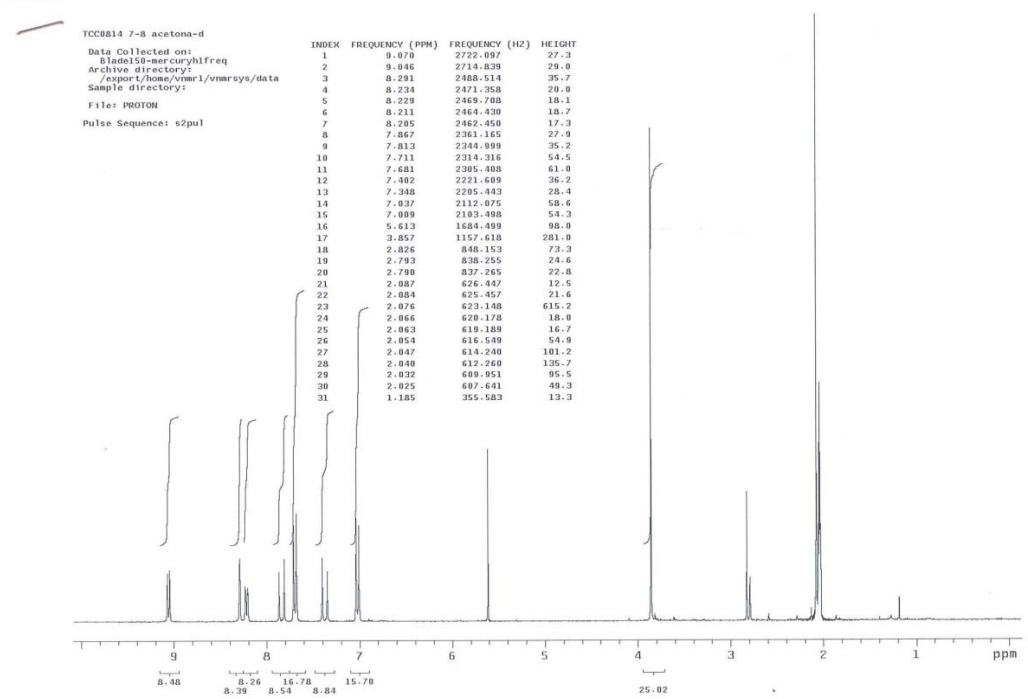
Synthesis

General . Melting points were uncorrected. ^{1}H NMR spectra were recorded at 200 and 300 MHz and ^{13}C NMR at 50 and 75 MHz. Chemical shifts were reported as δ values (ppm). High resolution mass spectra (HRMS) were recorded with *m*-NBA and 35 KeV (Cs Iodide). 4-methoxybenzaldehyde and 4-*N,N*-dimethylaminobenzaldehyde were purchased from Aldrich and were used without further purification. 2,8-dimethylquinolizinium hexafluorophosphate was synthesized as described on blibliography.[1]

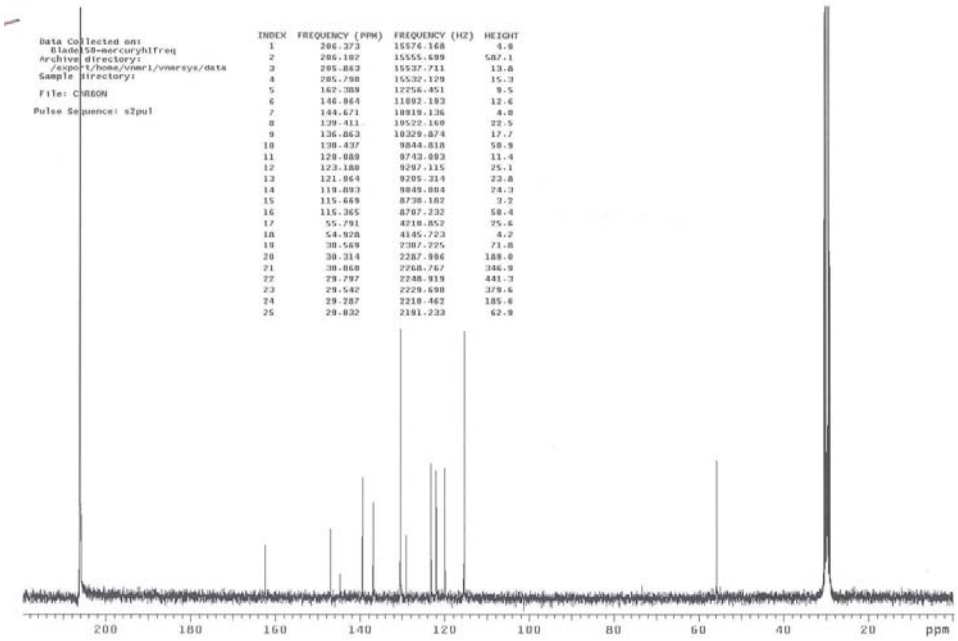
General Procedure. To a refluxing acetonitrile solution (6 ml) of 2,8-dimethylquinolizinium hexafluorophosphate (100 mg, 0.3300 mmol) and arylaldehyde (0.8251 mmol, 2.5 equiv.) was added piperidine (65.3µL, 0.6600 mmol, 2 equiv.). The mixture was refluxed for 2 hours. After cooling of the mixture to room temp., diethyl ether (30 ml) was added. The resulting precipitate was filtered, washed with ether, and dried *in vacuo* to give (*E,E*)-2,8-Bis(arylvinyl)quinolizinium hexafluorophosphates. Anaytically pure sample was obtained by recrystallization from acetonitrile-ethanol.

(*E,E*)-2,8-bis(4-methoxyphenylvinyl)quinolizinium Hexafluorophosphate (V-MOP2). Following the general procedure and using 4-methoxybenzaldehyde (112.3 mg), **V-MOP2** was obtained as yellow solid (166.5 mg, 93%). mp: 207-208 °C. IR, (KBr) $\nu_{máx}$ (cm^{-1}) 3364, 2917, 1651, 1598, 1514, 1455, 1259, 1173, 1027, 846.

^{1}H NMR (300 MHz, CD_3OCD_3) δ 9.06 (d, 2H, *J* = 7.2 Hz), 8.29 (s, 2H), 8.22 (dd, 2H, *J* = 1.6, 6.9 Hz), 7.84 (d, 2H, *J* = 16.2 Hz), 7.69 (d, 4H, *J* = 8.9 Hz), 7.37 (d, 2H, *J* = 16.2 Hz), 7.02 (d, 4H, *J* = 8.6 Hz), 3.85 (s, 6H).



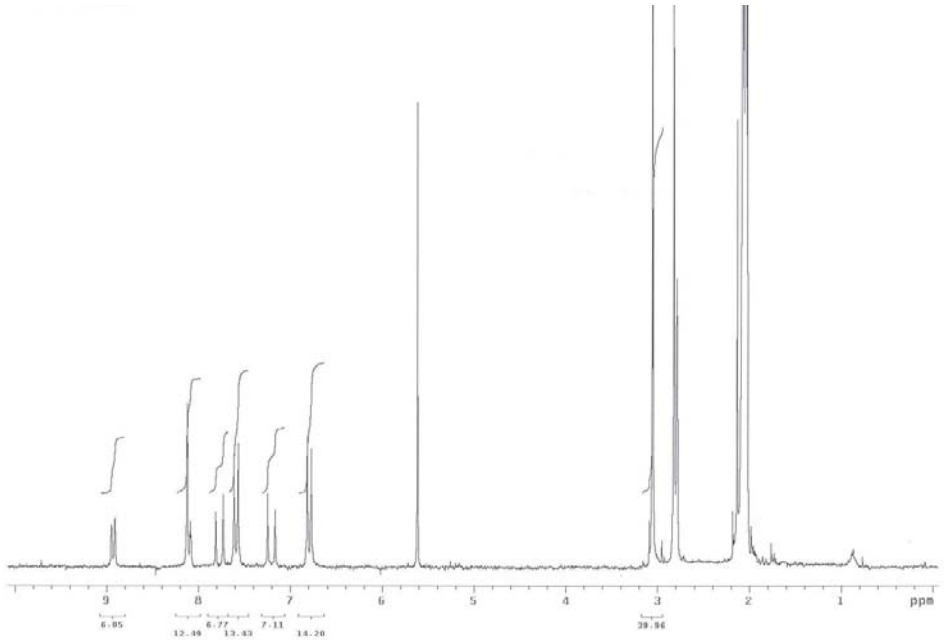
^{13}C-NMR (75 MHz, CD_3OCD_3) δ: 162.4, 146.9, 144.7, 139.4, 136.9, 130.4, 129.1, 123.1, 121.9, 119.9, 115.4, 55.8.



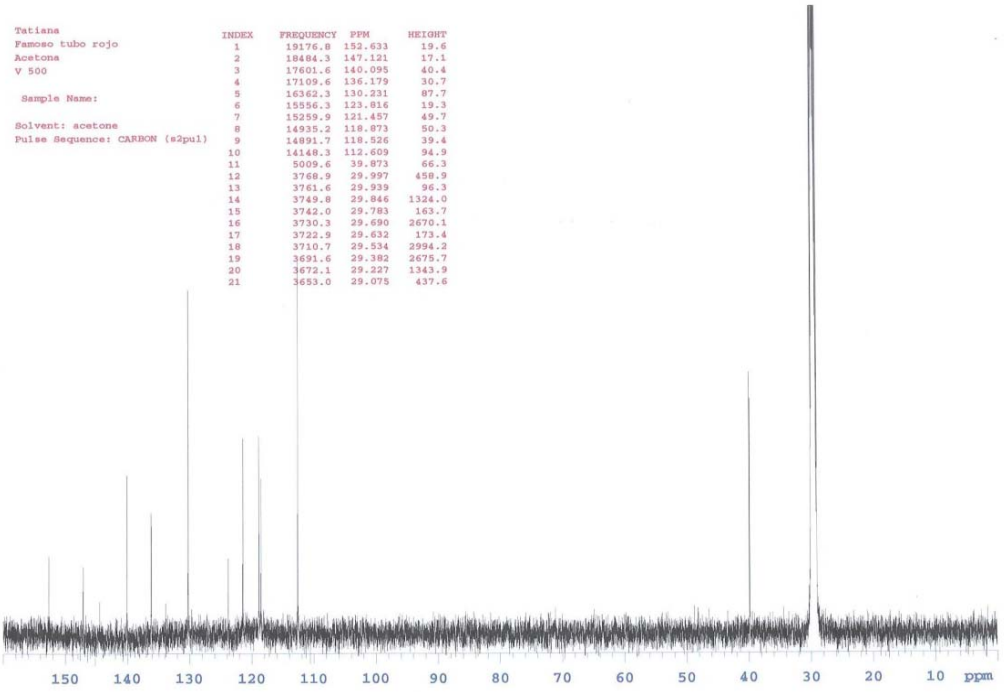
HRMS (ESI-TOF, MeOH) calcd for $C_{27}H_{24}NO_2$ [M]+ 394.1807, found 394.1810.

(*E,E*)-2,8-bis(4-*N,N*-dimethylaminophenylvinyl)quinolizinium Hexafluorophosphate (V-DMA2). Following the general procedure and using 4-*N,N*-dimethylaminobenzaldehyde, **V-DMA2** was obtained as red solid (164.1 mg, 88%). mp 258-259 °C. IR, (KBr) vmáx (cm-1) 3264, 2917, 1651, 1598, 1259, 1173, 1027, 846.

^{1}H NMR (300 MHz, CD3OCD3) δ 8.92 (d, 2H, J = 7.6 Hz), 7.81-7.73 (4H, m), 7.77 (d, 2H, J = 16.2 Hz), 7.59 (d, 4H, J = 8.9 Hz), 7.20 (d, 2H, J = 16.2 Hz), 6.79 (d, 4H, J = 8.9 Hz), 3.01 (s, 12H).



^{13}C-NMR (125 MHz, CD3OCD3) δ: 152.6, 147.1, 144.5, 140.1, 136.2, 130.2, 123.8, 121.4, 118.9, 118.5, 112.6, 39.9.



HRMS (ESI-TOF, MeOH) calcd for C29H30N3 [M]+ 420.2440, found 420.2392.

Spectroscopic Methods

The linear absorption spectra were recorded on a Shimadzu UV-3101PC UV−vis−NIR spectrophotometer, and the fluorescence measurements were obtained on a SLM-Aminco 8110 Series 2 spectrofluorimeter (Spectronics, Rochester, NY) equipped with a 450 W Xe lamp, a double excitation and emission monochromator MC400 and R928P PMT using Rhodamine B quantum counter solution as reference. The spectra were recorded in spectroscopic grade methanol using 5 x 5 mm quartz cells. The fluorescence quantum yields were determined using as standard fluorescein in water at pH=11 (ϕ = 0.92, λ_{em}= 500-600 nm).

The two-photon absorption (TPA) spectra were measured by two-photon excited fluorescence using fluorescein at pH >11 as a standard to account for collection efficiency and pulse characteristics.[2] In the 710-990 nm region a modified setup that follows closely the one described by Xu and Webb was used.[3] In order to select a narrow bandwidth of emission wavelengths an H20Vis Jobin Yvon monochromator was placed at the entrance of a PMC-100-4 photomultiplyer tube (Becker and Hickl GmbH). The excitation source was a Ti:Sapphire laser (Tsunami BB, Spectra-Physics, 710-990 nm, 1.7 W at 800 nm, 100 fs, 82 Mz). Solutions of 4-60 μM concentration in spectroscopic grade methanol were used. The two-photon absorption cross-section was estimated from the relation:

$$\sigma_2 = \left(\frac{F_2}{\phi C n}\right)_q \left(\frac{\phi C n \sigma_2}{F_2}\right)_{ref} \quad (1)$$

where F_2 stands for fluorescence intensity, ϕ is the one-photon excited fluorescence quantum yield, n refers to the refractive index of the solution, C is the concentration, and q and ref are relative to the quinolizinium derivatives and the fluorescein reference, respectively. The emission intensity dependence of the excitation power was checked to be quadratic for all molecules at the excitation maximum.

Cell staining procedure and fluorescence microscopy imaging

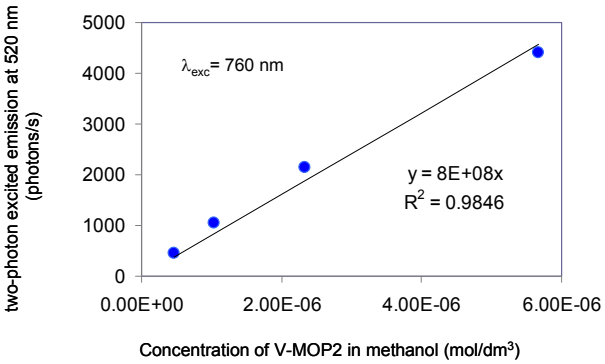
Human embryonic kidney cells (HEK 293) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 g/ml streptomycin in a 5% CO2 incubator at 37 °C. The cells were grown on 1 μ-Slide 8 well uncoated ibidi glass coverslips. Prior to the addition of cells the chambers were coated with poly-L-Lysine for at least 30min, then each well was washed several times with PBS. The cells were subsequently added to the wells and cultured with DMEM without fenol red for two days before each experiment. Cell culture reagents were from Invitrogen Corp. (Carlsbad, CA) and fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Solutions of 10^{-4} M concentration of the V-MOP2 dye in methanol were used as stock solutions. Several staining protocols were attempted by changing the V-MOP2 concentration from 2 to 10 μM, generally by adding a fixed volume of 2μl of stock solutions with different concentration to 200 μL of culture medium. The incubation periods at 37º C with 5% CO_2 ranged from 5-30 min with and without wash. Colocalization experiments using Alexa Fluor 594 wheat germ agglutinin (AF594WGA, plasma membrane stain) were also performed where V-MOP2 (4 μM) was incubated for 30 min and AF594WGA (5μg/l) was incubated for 10 min.

The cells were imaged using a laser scanning microscope (Leica TCS-SP5) equipped with an continuous Ar ion laser (458, 465, 488, 496 and 514 nm) and a Ti:Sapphire (Spectra-Physics Mai Tai BB, 710-990 nm, 100 fs, 80 MHz). Both confocal and multiphoton imaging are possible using either the Ar ion laser or the Ti:Sapphire laser. For fluorescence lifetime imaging (FLIM) a photomultiplyer tube was coupled to the X-port of the microscope and the emitted photons were processed by an SPC board that addresses simultaneously the xy location of the collected photons (Becker and Hickl GmbH, PMC-100-4 and SPC-830). The SPCM acquisition software and the lifetime image processing SPCImage software were used. The emitted light was discriminated from the excitation light by a dichroic beamsplitter (Semrock, FF665) and a short pass dichroic filter (Semrock, FF01-680/SP). A 63x 1.2 N.A. water immersion objective was used. Images were collected with a scan rate of 400 Hz per frame.

Sample preparation for evaluation of the DNA effect on fluorescence emission

Solutions of the symmetric V-shape dyes of μM concentration were prepared in 0.05 M tris-HCl buffer, pH 8.0. About 0.1 mg of Deoxyribonucleic acid sodium salt from salmon test (Sigma-Aldrich) was added to 2 ml of the dye solution making a total DNA concentration of 80 μM b.p.

Concentration dependent emission of V-MOP2 upon excitation at 760 nm



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2. N. S. Makarov, M. Drobizhev and A. Rebane, *Optics Express*, 2008, **16**, 4029-4047.
3. C. Xu and W. W. Webb, *J. Opt. Soc. Am. B*, 1996, **13**, 481-491.