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Clickable Long-Wave "Mega-Stokes" Fluorophores for Orthogonal Chemoselective Labeling of Cells

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Dedication to Prof. Dr. Péter Huszthy on the occasion of his 60th birthday

In vitro and in vivo fluorescence imaging of biological structures that use near-infrared (NIR) fluorophores is becoming more and more important for their high sensitivity, excellent temporal and spatial resolution, and their potential for multichannel imaging.[1] Fluorophores in the spectral region between the far-red and NIR region are particularly suitable for biological (both in vitro and in vivo) labeling as they are less or not at all interfered by biological background luminescence. Therefore there is an increasing demand for water-soluble labels fluorescing in the NIR regime.[2] It is crucial that the covalent ligation of these probes to the biomolecule of interest is fast, high-yielding, and biocompatible and the functional groups do not interfere with other naturally occurring reactive sites. For these techniques the term "bioorthogonality" was introduced. Little effort has been made to develop bioorthogonal labels in this spectral regime.[3] In a bioorthogonal labeling scheme the introduction of reporter tags relies on their selective and efficient reaction under physiological conditions with functional groups available at the biomolecules of interest. (Bio)orthogonal chemical reporters that are "non-native, non-perturbing chemical handles that can be modified in living systems through highly selective reactions with exogenously delivered probes" are particularly suitable for fluorescent labeling of biological species.[4] Among these tagging reactions, the Staudinger ligation,[5c] the inverse electron demand Diels–Alder reaction between tetrazines and strained alkenes,[5d,e] the photoactivable dipolar cycloaddition of tetrazoles and alkenes,[5f] and the so-called click reaction represented by the Cu^{I}-catalyzed azide–alkyne cycloaddition (CuAAC)[5g,h] are the most valuable ones.[5] Their superiority over other methods is due to the inertness of the chemical reporters, the exogenously delivered probes and their selective and efficient reaction with each other with the tolerance of the majority of functional groups. The extreme rareness of azide and alkyne functions in biological systems as well as the easy access of azide/alkyne-modified biological building blocks further increase the importance of tagging by means of copper-catalyzed azide–alkyne cycloaddition. This reaction or its copper-free, strain-promoted alternative (SPAAC) has been shown to be quite versatile in terms of biological applications.[6,7] CuAAC also finds widespread applications in high-throughput screening of libraries.[8] The versatility of the combined use of copper-catalyzed and Cu-free click chemistries in sequential labeling methods was also demonstrated recently.[9] Besides the fundamental criteria, for example, chemical stability, water solubility, bright emission, etc. that a conjugatable dye should meet, a synthetic routine providing fast and good-yielding access to these dyes is also demanding. Herein we report an efficient routine to access azide/alkyne-modified, therefore "clickable", dyes that emit in the far-red/NIR regime and have very large Stokes shifts.

As part of our program on the development of FRET labeled MMP-2 substrates we wished to develop clickable NIR-dyes with large Stokes shifts.[9] The work of Czerney et al.[10] on the development of the so-called "mega-Stokes" dye family has directed our attention to the polymethine scaffolds shown in Scheme 1. In our recent report we have shown the applicability of this dye family in the synthesis of red-emitting clickable dyes.[3b] We expected that installation

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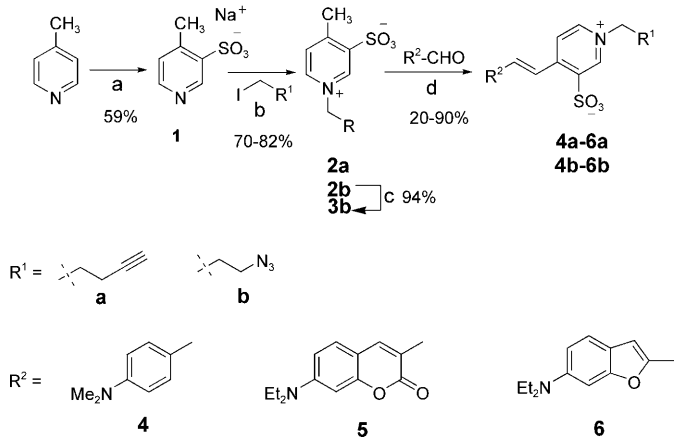

Scheme 1. Synthetic route for preparation of clickable dyes. Reaction conditions: a) 20% oleum, cat. $HgSO_4$; b) 5-iodo-1-pentyne (**2a**) or 1,3-diiodopropane/MeCN, reflux (**2b**); c) NaN_3/MeCN, reflux; d) EtOH, cat. piperidine, reflux.

of a sulfonyl function onto the picoline ring will increase water solubility and shift the spectral properties towards the NIR region of the spectrum relative to derivatives that do not possess a sulfonyl function.[3b]

The synthetic procedure started with the introduction of the sulfonyl function onto the picoline ring. Treatment of 4-picoline with 20% oleum furnished the desired sulfonylpicoline salt **1** in good yield. Alkylation of **1** was then accomplished either by reaction with 5-iodo-1-pentyne or 1,3-diiodopropane to obtain the corresponding sulfonylpicolinium betaines **2**. Compound **2b** was further subjected to halogen–azide exchange, which was effected by treatment with NaN_3. Precursors **2** were then condensed with the appropriate aldehydes in the presence of a catalytic amount of piperidine. This synthetic route afforded each dye molecule in acceptable to good yields. The synthetic routine justifies an easy access to these dyes without the need for special chemicals or difficult reaction steps.

The fluorescence excitation and emission spectra of the dyes showed that each possesses a large Stokes shift (>100 nm). To our delight both excitation and emission spectra were shifted towards the NIR regime and emission maxima were positioned at 625, 674, and 734 nm for **4**, **5**, and **6**, respectively (Table 1). Regardless of the starting aldehyde, the photophysical studies revealed that all dyes have acceptable quantum yields and excellent photostabilities. For example, continuous irradiation of **5** and **6** for 1 h resulted in only 4 and 21% loss of the original intensity, respectively. As expected, all photophysical characteristics of the fluorophores were found to be identical for the **a** and **b** series dyes.

Table 1. Spectral characteristics of clickable dyes.

Dye	Solvent	λ_{max} (exc) [nm]	λ_{max} (em) [nm]	ε ($\times 10^4$) [$M^{-1}cm^{-1}$]	ϕ[b]
4	MeOH	519	625	5.6	0.8.
	PBS[a]	523	630	4.3	n.d.
5	MeOH	538	674 (695)	4.8	15.7
	PBS	544	675 (697)	5.3	1.0
6	MeOH	586	735	4.0	1.0
	PBS	588	744	2.8	n.d.

[a] PBS: phosphate-buffered saline solution (pH 7.4). [b] Using cresyl violet as reference standard. n.d. = not determined.

The excitation spectra of the new dyes (Figure 1) show that each of them is compatible with laser sources (e.g., Ar, He-Ne, diode lasers) widely used in fluorescence instrumentation such as cell sorters and imagers. The large Stokes shifts make these dyes ideal candidates for fluorescence resonance energy transfer (FRET) applications. An often encountered problem in FRET assays using common fluorophores is that there is a direct excitation of acceptor emission by the light used to excite the donor. This problem reduces the dynamic range of assays where most of the acceptor is unbound. Another problem is the overlap between the emission of the donor and the emission band of the acceptor dye. The large Stokes shift is likely to reduce these difficulties.

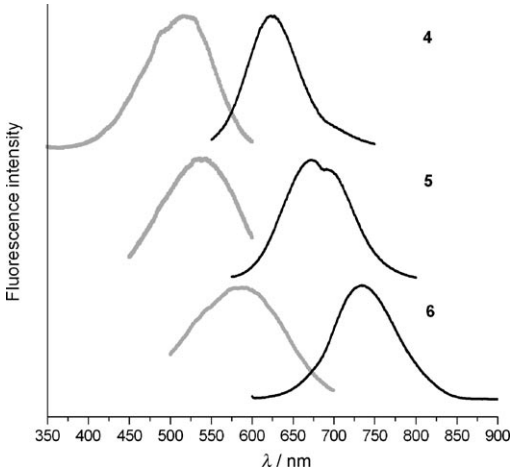

Figure 1. Fluorescence excitation (gray) and emission (black) spectra of labels in methanol.

To test the feasibility of clickable dyes in chemoselective tagging reactions the surface glycoprotein labeling of fixed cells supplied on azido sugar containing nutrients have frequently been used recently.[3a,7a] We have adapted this system to demonstrate the ability of our dyes to undergo orthogonal labeling reactions efficiently. Prior to labeling, Chinese hamster ovary (CHO) cells were incubated with **4a**–**6a** to assess the possible cytostatic effects of these dyes. Based on experimental data, neither dye has a cytostatic effect on CHO cells (see the Supporting Information). Subsequently, CHO cells were treated with azidoacetylmannosamine (ManNAz) for 3 days. The cells, which incorporated the azido sugar metabolically yielding azido sialic acid residues in their surface glycoproteins were then fixed with methanol at −20 °C for 10 min, and acetone for 1 min. Fixed cells were then subsequently treated with **4a**, **5a**, or **6a** (20 μM).

The reaction was facilitated with $CuSO_4$-sodium-ascorbate (100 μM/1 mM) and tris(benzyltriazolylmethyl)amine (100 μM, TBTA[11]) in PBS (pH 7.0) at 37 °C. The results have shown that after 25 min reaction all labels had caused fluorescent tagging of the cells. Variations in the brightness of the images indicated differences probably in the quantum yields of the dyes. In the case of control cells that previously were not supplemented with ManNAz only negligible fluorescence could be observed owing only to unspecific labeling of the cells (Figure 2 A, B, and C). Contrary to this, cells bearing azido sialic acid residues in their surface glycans were labeled efficiently as shown in Figure 2 D, E, and F. We have also studied fluorescent labeling of azido-modified cells on the time scale. The fixed cells were washed three times with PBS and incubated with the dyes for 5, 25, and 60 min (Figure 2 G, H, and I). Results showed that even after 5 min reaction time efficient labeling could be observed. For the confocal microscopy images (Figure 2) conventional laser sources as 514 nm Ar, 543 nm He-Ne, and 633 nm He-Ne lasers were used for **4a**, **5a**, and **6a**, respectively, indicating the suitability of the present dyes for microscopic and/or flow-cytometric applications. Moreover dye **6** was excitable both by green and red lasers.

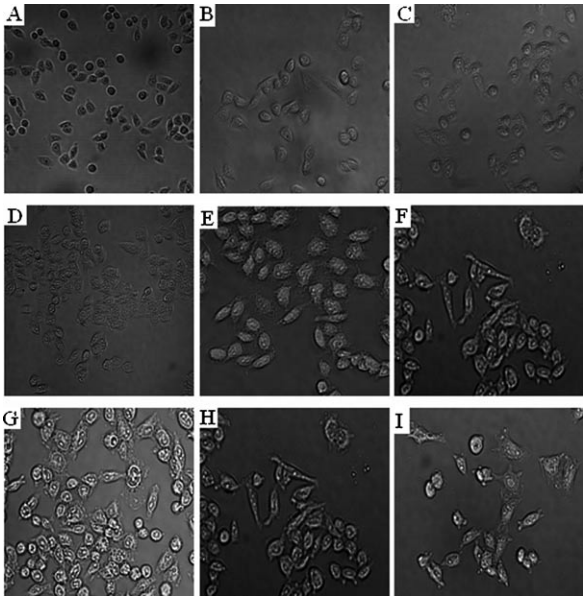

Figure 2. Fluorescent microscopy images of CHO cells. Control experiments with cells not bearing azido sialic acid and treated with **4a**, **5a**, and **6a** for 25 min (A, B, C); cells modified with ManNAz and labeled with **4a**, **5a**, and **6a** for 25 min (D, E, F); time dependency: ManNAz-treated cells labeled with **6a** for 5, 25, and 60 min (G, H, I). For color images, see the Supporting Information.

In conclusion, the set of clickable fluorophores presented here is suitable for fluorescent labeling of biomolecules. The synthetic routine provides efficient and easy access to these dyes. The tagging molecules possess acceptable quantum yields, which is best documented by the contrast of the microscopy images. The easy access to these dyes justifies them to be expected to draw much interest in a large variety of applications in chemoselective and (bio)orthogonal labeling schemes of proteins, sugars, lipids, nucleic acids, or organelles both in vivo and in vitro.

Experimental Section

Unless otherwise indicated, all starting materials were obtained from commercial suppliers (Sigma–Aldrich, Fluka) and used without further purification. Analytical thin-layer chromatography (TLC) was performed on Polygram SIL G/UV 254 precoated plastic TLC plates with 0.25 mm silica gel from Macherey–Nagel + Co. Silica gel column chromatography was carried out with flash silica gel (0.040–0.063 mm) from Merck. The NMR spectra were recorded on a Bruker DRX-250 or Varian Inova 600 MHz spectrometer. Chemical shifts (δ) are given in parts per million (ppm) using solvent signals as the reference. Coupling constants (J) are reported in Hertz (Hz). Splitting patterns are designated as s (singlet), d (doublet), t (triplet), quint (quintuplet), m (multiplet), dd (doublet of a doublet). Cells were imaged using Olympus Fluoview500 software of an Olympus IX81 confocal laser scanning microscope using the 514 argon-ion, 543 argon-helium or 633 argon-helium laser lines to excite dyes.

Sodium 4-methylpyridine-3-sulfonate (**1**)[12]: A reaction vessel was charged with mercury-sulfate catalyst (210 mg) and 20% fuming sulphuric acid (53.0 g). To this mixture was added 4-picoline (10.0 g, 0.107 mol) dropwise at room temperature. After the addition was complete the temperature was raised to 225–235 °C and the reaction mixture was vigorously stirred for 5 h at this temperature. The reaction mixture was neutralized with 50% NaOH to pH 9–10 while it was cooled on an ice-water bath. The solvent was then evaporated and the product was extracted with methanol. The methanolic extract was evaporated and the crude product (12.3 g, 59%) was recrystallized from ethanol to give pure product as an off-white solid. ^{1}H NMR ([D$_6$]DMSO): δ = 2.53 (3H, s), 7.20 (1H, d, J = 4.9 Hz), 8.37 (1H, d, J = 4.9 Hz), 8.79 ppm (1H, s); ^{13}C NMR ([D$_6$]DMSO): δ = 19.4, 125.7, 141.4, 144.9, 146.9, 149.7 ppm. M.p. > 240 °C. IR (neat): $\tilde{\nu}$ = 3052, 1230, 1213 cm^{-1}. HRMS (ESI) calcd for $C_6H_6NO_3S^-$ [M−Na]$^-$: 172.0074; found: 172.0078.

1-(5-Pentyn-1-yl)-3-sulfonato-4-methylpyridinium betaine (**2a**): Sodium 4-methylpyridine-3-sulfonate (1.0 g, 5.12 mmol) and 5-iodo-1-pentyne[13] (2.10 g, 10.8 mmol) were heated at reflux in DMF for 14 h. After cooling to room temperature the solvent was removed in vacuo and the product was purified on silica (dichloromethane-methanol-triethylamine 10:1:0.1) to give 0.86 g (70%) of the desired product as a yellowish solid. ^{1}H ([D$_6$]DMSO): δ = 2.08 (2H, quint., J = 7.1 Hz), 2.21–2.30 (2H, m), 2.79 (3H, s), 2.85 (1H, t, J = 2.7 Hz), 4.63 (2H, t, J = 7.1 Hz), 8.01 (1H, d, J = 6.2 Hz), 8.92 (1H, dd, J = 1.4 Hz, J = 6.2 Hz), 9.09 ppm (1H, d, J = 1.4 Hz); ^{13}C ([D$_6$]DMSO): δ = 14.7, 20.2, 29.2, 59.2, 72.3, 82.4, 129.8, 141.4, 144.0, 145.4, 156.7 ppm. M.p. 175–179 °C. IR (neat): $\tilde{\nu}$ = 3278, 3044, 2935, 1213 cm^{-1}. HRMS (ESI) calcd for $C_{11}H_{14}NO_3S^+$ [M+H]$^+$: 240.0689; found: 240.0685.

1-(3-Iodopropyl)-3-sulfonato-4-methylpyridinium betaine (**2b**): Sodium 4-methylpyridine-3-sulfonate (1.0 g, 5.12 mmol) and 1,3-diiodopropane (6.06 g, 20.5 mmol) were heated at reflux in DMF for 4 h. After cooling to room temperature the solvent was removed in vacuo and the product was purified on silica (dichloromethane-methanol 10:1 to 5:1 v/v%) to give 1.43 g (82%) of the desired product as a yellowish solid. ^{1}H ([D$_6$]DMSO): δ = 2.42 (2H, quint., J = 7.3 Hz), 2.78 (3H, s), 3.21 (2H, t, J = 7.3 Hz), 4.61 (2H, t, J = 7.1 Hz), 8.01 (1H, d, J = 6.3 Hz), 8.92 (1H, dd, J = 1.4 Hz, J = 6.2 Hz), 9.09 ppm (1H, d, J = 1.3 Hz); ^{13}C ([D$_6$]DMSO): δ = 1.3, 20.2, 34.1, 60.5, 129.8, 141.4, 143.9, 145.4, 156.7 ppm. M.p. 136–139 °C. IR (neat): $\tilde{\nu}$ = 3110, 3033, 2952, 1213 cm^{-1}. HRMS (ESI) calcd for $C_9H_{13}INO_3S^+$ [M+H]$^+$: 341.9655; found: 341.9649.

1-(3-Azidopropyl)-3-sulfonato-4-methylpyridinium betaine (**3b**): 1-(3-Iodopropyl)-3-sulfonato-4-methylpyridinium betaine (0.85 g, 2.5 mmol) and NaN_3 (0.49 g, 7.5 mmol) were dissolved in an acetonitrile (40 mL)-DMF (5 mL) mixture and the resulting solution was heated at reflux for 3 h.

After cooling to room temperature the solution was filtered to remove salts and the filtrate was brought to dryness. To the remainder solid was added dichloromethane and the solution was filtered through a plug of silica. Evaporation of the solvent gave 0.6 g (94%) product as a yellow crystalline solid. ^{1}H ([D$_6$]DMSO): δ=2.15 (2H, quint., J=6.6 Hz), 2.78 (3H, s), 3.45 (2H, t, J=6.6 Hz), 4.64 (2H, t, J=7.1 Hz), 8.03 (1H, d, J=6.3 Hz), 8.95 (1H, dd, J=1.4 Hz, J=6.3 Hz), 9.10 ppm (1H, d, J=1.3 Hz); ^{13}C ([D$_6$]DMSO): δ=20.1, 29.7, 47.5, 57.6, 129.7, 141.4, 144.0, 145.3, 156.6 ppm. M.p. 94–97 °C. IR (neat): $\tilde{\nu}$=3023, 2098, 1214 cm^{-1}. HRMS (ESI) calcd for $C_9H_{13}N_4O_3S^+$ $[M+H]^+$: 257.0703; found: 257.0698.

1-(5-Pentyn-1-yl)-4-([E]-2-(4-dimethylaminophenyl)-vinyl]-3-sulfonato pyridinium betaine (**4a**): 1-(5-Pentyn-1-yl)-3-sulfonato-4-methylpyridinium betaine (0.25 g, 1.05 mmol) and 4-(dimethylamino)benzaldehyde (0.156 g, 1.05 mmol) were stirred at 60 °C in ethanol (30 mL) in the presence of catalytic amount of piperidine (six drops) for 14 h. The solvent was removed in vacuo and the product was purified on silica (dichloromethane-methanol 9:1 v/v%) to give 0.30 g (77%) product as a red solid. ^{1}H ([D$_6$]DMSO): δ=2.07 (2H, quint, J=7.0 Hz), 2.25–2.28 (2H, m), 2.87 (1H, t, J=2.9 Hz), 3.03 (6H, s), 4.51 (2H, t, J=7.0 Hz), 6.81 (2H, d, J=8.8 Hz), 7.51 (2H, d, J=8.8 Hz), 7.92 (2H, AB, J=15.8 Hz), 8.36 (1H, d, J=7.0 Hz), 8.70 (1H, d, J=7.0 Hz), 8.97 ppm (1H, d, J=1.8 Hz); ^{13}C ([D$_6$]DMSO): δ=14.7, 29.1, 40.0, 58.2, 72.3, 82.5, 111.9, 116.3, 121.1, 122.8, 130.1, 141.2, 141.9, 142.0, 142.4, 150.8, 151.9 ppm. M.p. >240 °C. IR (neat): $\tilde{\nu}$=3224, 3072, 2990, 1213 cm^{-1}. HRMS (ESI) calcd for $C_{20}H_{23}N_2O_3S^+$ $[M+H]^+$: 371.1424; found: 371.1423.

1-(5-Pentyn-1-yl)-4-([E]-2-(7-diethylamino-2-oxo-2H-chromen-3-yl)-vinyl]-3-sulfonato pyridinium betaine (**5a**): 1-(5-Pentyn-1-yl)-3-sulfonato-4-methylpyridinium betaine (0.20 g, 0.836 mmol) and 7-diethylamino-2-oxo-2H-chromene-3-carbaldehyde[14] (0.21 g, 0.836 mmol) was stirred at 60 °C in ethanol (30 mL) in the presence of a catalytic amount of piperidine (six drops) for 14 h. The solvent was removed in vacuo and the product was purified on silica (dichloromethane-methanol 9:1 v/v%) to give 0.35 g (90%) product as a dark red solid. ^{1}H ([D$_6$]DMSO): δ=1.14 (6H, t, J=7.1 Hz), 2.09 (2H, quint, J=6.6 Hz), 2.22–2.32 (2H, m), 2.88 (1H, t, J=2.7 Hz), 3.48 (4H, q, J=6.6 Hz), 4.57 (2H, t, J=7.1 Hz), 6.61 (1H, d, J=2.2 Hz), 6.77 (1H, dd, J=2.4 Hz, J=9.0 Hz), 7.60 (1H, d, J=9.0 Hz), 7.75 (1H, d, J=16.3 Hz), 8.13 (1H, s), 8.35 (1H, d, J=16.2 Hz), 8.39 (1H, d, J=6.9 Hz), 8.81 (1H, d, J=6.6 Hz), 9.07 ppm (1H, d, J=1.3 Hz); ^{13}C ([D$_6$]DMSO): δ=12.3, 14.7, 29.2, 44.2, 58.6, 72.3, 82.4, 96.2, 108.2, 109.8, 114.3, 121.9, 122.4, 130.7, 136.1, 142.3, 142.4, 143.1, 143.6, 150.8, 151.8, 156.3, 159.5 ppm. M.p. >240 °C. IR (neat): $\tilde{\nu}$=3272, 3073, 2971, 1610, 1185 cm^{-1}. HRMS (ESI) calcd for $C_{25}H_{27}N_2O_5S^+$ $[M+H]^+$: 467.1635; found: 467.1624.

1-(5-Pentyn-1-yl)-4-([E]-2-(6-diethylamino-benzofuran-2-yl)-vinyl]-3-sulfonato pyridinium betaine (**6a**): 1-(5-Pentyn-1-yl)-3-sulfonato-4-methylpyridinium betaine (0.15 g, 0.631 mmol) and 6-diethylamino-benzofuran-2-carbaldehyde[15] (0.16 g , 0.631 mmol) were stirred at 60 °C in ethanol (30 mL) in the presence of a catalytic amount of piperidine (six drops) for 14 h. The solvent was removed in vacuo and the product was purified on silica (dichloromethane-methanol 9:1 v/v%) to give 0.056 g (20%) product as a dark solid. ^{1}H ([D$_6$]DMSO): δ=1.14 (6H, t, J=7.0 Hz), 2.08 (2H, quint, J=7.0 Hz), 2.21–2.34 (2H, m), 2.89 (1H, t, J=2.4 Hz), 3.44 (4H, q, J=7.0 Hz), 4.53 (2H, t, J=7.0 Hz), 6.76 (1H, dd, J=1.9 Hz, J=8.8 Hz), 6.88 (1H, s), 7.17 (1H, s), 7.48 (1H, d, J=8.8 Hz), 7.93 (2H, AB, J=15.9 Hz), 8.38 (1H, d, J=7.0 Hz), 8.76 (1H, d, J=7.0 Hz), 9.03 ppm (1H, s); ^{13}C ([D$_6$]DMSO): δ=12.3, 14.8, 29.1, 44.2, 58.5, 72.3, 82.5, 92.2, 110.2, 114.5, 117.2, 118.4, 121.7, 122.6, 127.7, 141.8, 142.2, 142.8, 148.2, 149.7, 150.7, 158.5 ppm. M.p. >240 °C. IR (neat): $\tilde{\nu}$=3280, 2968, 2362 cm^{-1}. HRMS (ESI) calcd for $C_{24}H_{27}N_2O_4S^+$ $[M+H]^+$: 439.1686; found: 439.1682.

1-(3-Azidopropyl)-4-([E]-2-(4-dimethylaminophenyl)-vinyl]-3-sulfonato pyridinium betaine (**4b**): 1-(3-Azidopropyl)-3-sulfonato-4-methylpyridinium betaine (0.3 g, 1.2 mmol) and 4-(dimethylamino)benzaldehyde (0.175, 1.2 mmol) were stirred at 60 °C in ethanol (30 mL) in the presence of a catalytic amount of piperidine (six drops) for 3 h. The solvent was removed in vacuo and the product was purified on silica (dichloromethane-methanol 9:1 v/v%) to give 0.256 g (56%) product as a deep-red solid. ^{1}H ([D$_6$]DMSO): δ=2.14 (2H, quint, J=6.5 Hz), 3.02 (6H, s), 3.45 (2H, t, J=6.5 Hz), 4.52 (2H, t, J=6.5 Hz), 6.80 (2H, d, J=8.2 Hz), 7.51 (2H, d, J=8.5 Hz), 7.93 (2H, AB, J=15.9 Hz), 8.39 (1H, d, J=6.5 Hz), 8.72 (1H, d, J=6.5 Hz), 8.97 ppm (1H, s); ^{13}C ([D$_6$]DMSO): δ=29.6, 43.7, 47.6, 56.8, 111.9, 116.2, 121.2, 122.8, 125.6, 130.1, 141.1, 142.1, 142.5, 150.7, 151.9 ppm. M.p. 188–191 °C. IR (neat): $\tilde{\nu}$=2905, 2095, 1564, 1524, 1158 cm^{-1}. HRMS (ESI) calcd for $C_{18}H_{22}N_5O_3S^+$ $[M+H]^+$: 388.1438; found: 388.1429.

1-(3-Azidopropyl)-4-([E]-2-(7-diethylamino-2-oxo-2H-chromen-3-yl)-vinyl]-3-sulfonato pyridinium betaine (**5b**): 1-(3-Azidopropyl)-3-sulfonato-4-methylpyridinium betaine (0.21 g, 0.815 mmol) and 7-diethylamino-2-oxo-2H-chromene-3-carbaldehyde (0.20 , 0.815 mmol) were stirred at 60 °C in ethanol (30 mL) in the presence of a catalytic amount of piperidine (six drops) for 14 h. The solvent was removed in vacuo and the product was purified on silica (acetonitrile-acetonitrile/NH_4PF_6) to give 0.120 g (36%) product as a dark solid. ^{1}H ([D$_6$]DMSO): δ=1.15 (6H, t, J=7.1 Hz), 2.17 (2H, quint, J=6.9 Hz), 3.45–3.51 (6H, m), 4.58 (2H, t, J=6.9 Hz), 6.62 (1H, d, J=2.4 Hz), 6.78 (1H, dd, J=2.4 Hz, J=8.9 Hz), 7.61 (1H, d, J=8.9 Hz), 7.76 (1H, d, J=16.3 HZ), 8.14 (1H, s), 8.37 (1H, d, J=16.3 Hz), 8.39 (1H, d, J=6.9 Hz), 8.82 (1H, dd, J=1.2 Hz, J=6.9 Hz), 9.09 ppm (1H, d, J=1.2 Hz); ^{13}C ([D$_6$]DMSO): δ=12.3, 29.6, 44.2, 47.6, 57.1, 96.2, 108.2, 109.8, 121.9, 122.4, 130.7, 136.1, 142.3, 142.4, 143.2, 143.3, 143.6, 150.8, 151.8, 156.3, 159.5 ppm. M.p. >240 °C. IR (neat): $\tilde{\nu}$=3047, 2928, 2086, 1714, 1515 cm^{-1}. HRMS (ESI) calcd for $C_{23}H_{26}N_5O_5S^+$ $[M+H]^+$: 484.1649; found: 484.1644.

1-(3-Azidopropyl)-4-([E]-2-(6-diethylamino-benzofuran-2-yl)-vinyl]-3-sulfonato pyridinium betaine, (**6b**): 1-(3-Azidopropyl)-3-sulfonato-4-methylpyridinium betaine (0.21 g, 0.815 mmol) and 6-diethylamino-benzofuran-2-carbaldehyde (0.20 , 0.815 mmol) were stirred at 60 °C in ethanol (30 mL) in the presence of 1.2 equiv piperidine (six drops) for 14 h. The solvent was removed in vacuo and the product was purified on silica (acetonitrile-acetonitrile/NH_4PF_6) to give 0.102 g (27%) product as a dark solid. ^{1}H ([D$_6$]DMSO): δ=1.14 (6H, t, J=7.0 Hz), 2.16 (2H, quint, J=7.0 Hz), 3.41–3.59 (6H, m), 4.55 (2H, t, J=7.0 Hz), 6.76 (1H, dd, J=2.3 Hz, J=8.8 Hz), 6.88 (1H, s), 7.18 (1H, s), 7.48 (1H, d, J=8.8 Hz), 7.93 (2H, AB, J=15.8 Hz), 8.39 (1H, d, J=7.0 Hz), 8.78 (1H, d, J=7.0 Hz), 9.05 ppm (1H, d, J=1.8 Hz); ^{13}C ([D$_6$]DMSO): δ=12.3, 29.6, 44.2, 47.6, 57.0, 92.2, 110.2, 114.5, 117.2, 118.4, 121.7, 122.7, 127.7, 141.8, 142.3, 142.8, 148.2, 149.7, 150.7, 158.5 ppm. M.p. 163–166 °C. IR (neat): $\tilde{\nu}$=3044, 2100, 1559 cm^{-1}. HRMS (ESI) calcd for $C_{22}H_{26}N_5O_4S^+$ $[M+H]^+$: 456.1700; found: 457.1692.

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