

# **CHEMISTRY**

---

## **A EUROPEAN JOURNAL**

### Supporting Information

© Copyright Wiley-VCH Verlag GmbH & Co. KGaA, 69451 Weinheim, 2009

#### **Red-Emitting Rhodamine Dyes for Fluorescence Microscopy and Nanoscopy**

**Kirill Kolmakov, Vladimir N. Belov,\* Jakob Bierwagen, Christian Ringemann,  
Veronika Müller, Christian Eggeling, and Stefan W. Hell\*<sup>[a]</sup>**

chem\_200902309\_sm\_miscellaneous\_information.pdf

**Table 1S.** Commercially available fluorescent dyes for the red optical region upon the excitation with He-Ne red laser (633 nm) or 635 nm spectral line of red diode lasers.\*

| Name  | $\lambda_{max}$<br>( <i>abs.</i> )<br>nm | $\lambda_{max}$<br>( <i>fl.</i> )<br>nm | Solvent                  | $\epsilon \times 10^{-5}$<br>$M^{-1}$<br>$cm^{-1}$ | $\Phi_{fl}$<br>%    | $\tau_{fl}$<br>ns                                 | Provider<br>(structure)      | Solubility<br>(polarity)                             |
|---|--|---|--------------------------|--|---------------------|---|------------------------------|--|
| BODIPY <sup>®</sup><br>630/650  | 625                                      | 640                                     | MeOH                     | 1.01   | “good”              | 3.9 (H <sub>2</sub> O)<br>4.4 (EtOH) <sup>1</sup> | Invitrogen<br>(+)            | DMSO, MeCN<br>(non-polar)                            |
| Atto 633 <sup>2</sup>   | 629                                      | 657                                     | H <sub>2</sub> O         | 1.3  | 64                  | 3.2 (H <sub>2</sub> O)                            | Atto-tec<br>(-)              | DMF, DMSO,<br>(H <sub>2</sub> O) MeCN <sup>2</sup>   |
| Alexa <sup>®</sup><br>633 <sup>3</sup>  | 632                                      | 647                                     | MeOH                     | 1.0  | –                   | 3.2   | Invitrogen<br>(+)            | DMSO, H <sub>2</sub> O <sup>3</sup>                  |
| Atto 635  | 635                                      | 659                                     | H <sub>2</sub> O         | 1.2  | 25                  | 1.9   | Atto-tec<br>(+)              | DMF, DMSO,<br>MeCN (H <sub>2</sub> O) <sup>2,4</sup> |
| Atto 637 <sup>5</sup>   | 635                                      | 659                                     | H <sub>2</sub> O         | 1.2  | 25                  | 1.9   | Atto-tec<br>(-)              | H <sub>2</sub> O, DMF,<br>DMSO, MeCN <sup>4,5</sup>  |
| DyLight <sup>®</sup><br>633   | 638                                      | 658                                     | un-<br>known             | 2.0  | –                   | –   | Thermo<br>Fisher Sci.<br>(-) | –  |
| DY-630 <sup>6</sup>   | 636<br>627 <sup>1</sup>                  | 657<br>651 <sup>1</sup>                 | EtOH<br>H <sub>2</sub> O | 2.0  | varies <sup>6</sup> | 0.21<br>(H <sub>2</sub> O) <sup>1,6</sup>         | Dyomics<br>(+)               | MeOH, EtOH,<br>DMF, DMSO                             |
| DY-631, <sup>7</sup><br>632, <sup>7</sup><br>633, <sup>7</sup> 634 <sup>7</sup> | 635-<br>637                              | 657-<br>658                             | EtOH                     | 2.0  | –                   | –   | Dyomics<br>(+)               | H <sub>2</sub> O, MeOH,<br>DMF, DMSO                 |
| DY-635  | 647<br>635 <sup>1</sup>                  | 671<br>669 <sup>1</sup>                 | EtOH<br>H <sub>2</sub> O | 2.0  | –                   | 0.48<br>(H <sub>2</sub> O) <sup>1,6</sup>         | Dyomics<br>(+)               | MeOH, EtOH,<br>DMF, DMSO                             |
| DY-636 <sup>8</sup>   | 645                                      | 671                                     | EtOH                     | 2.0  | –                   | –   | Dyomics<br>(+)               | H <sub>2</sub> O, EtOH, DMF,<br>DMSO                 |
| DY-650 <sup>9</sup>   | 653                                      | 674                                     | EtOH                     | 2.2  | –                   | 0.64 (H <sub>2</sub> O) <sup>1</sup>              | Dyomics                      | MeOH, EtOH,  |

\*  $I_{max}$  (*abs.*),  $I_{max}$  (*fl.*): absorption and fluorescence maxima, respectively;  $\epsilon$ : molar extinction coefficient;  $\tau_{fl}$ : excited state lifetime.

<sup>1</sup> V. Buschmann, K. D. Weston, M. Sauer, *Bioconj. Chem.* **2003**, *14*, 195–204.

<sup>2</sup> According to the definition of the producer, this dye is “moderately hydrophilic”.

<sup>3</sup> B. Agnew, K. R. Gee, T. G. Nyberg (Invitrogen), US Pat. US 2007/0249014

<sup>4</sup> Slowly decomposes at pH>8.5.

<sup>5</sup> Hydrophilic version of Atto 635.

<sup>6</sup> Addition of (bio)polymers (BSA, Tween20) increases the low fluorescence quantum yield of DY fluorophores (ca. 5% in H<sub>2</sub>O) and their fluorescence lifetime: P. Czerney, F. Lehmann, M. Wenzel, V. Buschmann, A. Dietrich, G. J. Mohr, *Biol. Chem.* **2001**, *382*, 495–498

<sup>7</sup> The same fluorophore as in DY-630; the solubility in water is increased due to the presence of up to 4 sulfonic acid residues.

<sup>8</sup> The same fluorophore as in DY-635; the solubility in water is increased due to the presence of two sulfonic acid residues.

|                                |                  |                  |                   |     |                     |                                      |                   |                          |
|--------------------------------|------------------|------------------|-------------------|-----|---------------------|--------------------------------------|-------------------|--------------------------|
|                                | 646 <sup>1</sup> | 670 <sup>1</sup> | H <sub>2</sub> O  |     |                     |                                      | (+)               | DMF, DMSO                |
| Evoblue                        | 647              | 664              | EtOH              | 1.0 |                     | 0.64 (H <sub>2</sub> O) <sup>1</sup> | Fluka             | H <sub>2</sub> O, MeOH,  |
| 30 <sup>9</sup>                | 650 <sup>1</sup> | 667 <sup>1</sup> | H <sub>2</sub> O  |     |                     |                                      | (+)               | DMF, DMSO                |
| Cy <sup>®</sup> 5 <sup>9</sup> | 647              | 663              | H <sub>2</sub> O  | 2.5 | 27                  | 1 (PBS) <sup>11</sup>                | GE                | DMF, DMSO                |
|                                |                  |                  |                   |     | (PBS) <sup>10</sup> | 0.9 (H <sub>2</sub> O) <sup>1</sup>  | Healthcare        | (H <sub>2</sub> O)       |
|                                |                  |                  |                   |     |                     |                                      | (+)               |                          |
| Alexa <sup>®</sup>             | 651              | 672              | MeOH              | 2.7 | 33 <sup>14</sup>    | 1.0 (H <sub>2</sub> O) <sup>1</sup>  | Invitrogen        | H <sub>2</sub> O, DMSO   |
| 647 <sup>9,12,13</sup>         | 649 <sup>1</sup> | 666 <sup>1</sup> | H <sub>2</sub> O  |     |                     |                                      | (+) <sup>15</sup> | hydrophilic              |
| Atto                           | 644              | 669              | PBS <sup>11</sup> | 1.5 | 65                  | 3.4                                  | Atto-tec          | DMF, DMSO                |
| 647N <sup>9,16,17</sup>        |                  |                  |                   |     |                     |                                      | (+) <sup>18</sup> | (non-polar) <sup>2</sup> |
| DyLight <sup>®</sup>           | 646              | 674              | –                 | 2.5 | –                   | –                                    | Thermo            | –                        |
| 649 <sup>9</sup>               |                  |                  |                   |     |                     |                                      | Fisher Sci.       |                          |
|                                |                  |                  |                   |     |                     |                                      | (–)               |                          |

<sup>9</sup> Fluorescence of this dye may be excited by the 647 nm line of the krypton ion laser or with diode laser emitting at 650 nm.

<sup>10</sup> R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis, A. S. Waggoner, *Bioconj. Chem.* **1993**, *4*, 105–111.

<sup>11</sup> [www.iss.com/resources/fluorophores.html](http://www.iss.com/resources/fluorophores.html) (PBS = phosphate buffer saline: 50 mM potassium phosphate, 150 mM NaCl, pH 7.2)

<sup>12</sup> Cy<sup>®</sup> 5 dye was shown to be “brighter” but less photostable than Alexa<sup>®</sup> 647: J. L. Ballard, V. K. Peeva, C. J. de Silva, J. L. Lynch, N. R. Swanson, *Mol. Biotechnol.* **2007**, *36*, 175–183.

<sup>13</sup> Photostability of the fluorescent dyes decreases in the following order: Alexa<sup>®</sup> 647 > Alexa<sup>®</sup> 633 > Cy<sup>®</sup> 5 (R. P. Haugland, *A Guide to Fluorescent Probes and Labelling Technologies*, Invitrogen Corp., Carlsbad, **2005**, p. 38).

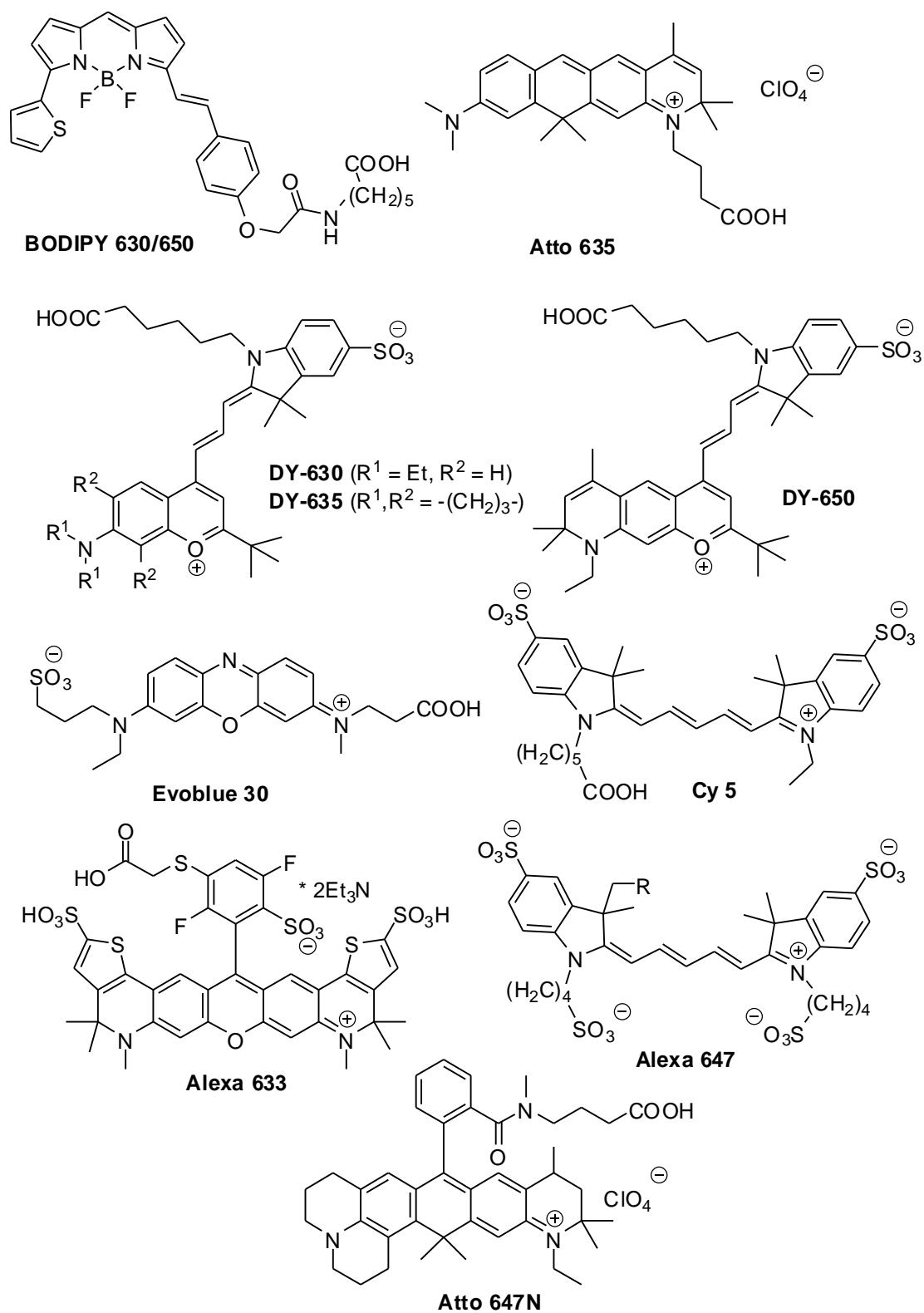
<sup>14</sup> Measured at 22 °C relative to fluorescein in 0.01 M NaOH ( $F_{fl} = 0.92$ ); [www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/tables/Fluorescence-quantum-yields-and-lifetimes-for-Alexa-Fluor-dyes.html](http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/tables/Fluorescence-quantum-yields-and-lifetimes-for-Alexa-Fluor-dyes.html)

<sup>15</sup> For the structure of Alexa 647, see *Supporting Information* of the following report: M. Bates, B. Huang, G. T. Dempsey, X. Zhuang, *Science*, **2007**, *317*, 1749–1753.

<sup>16</sup> According to the data of Atto-tec GmbH, photoresistance of the fluorescent dyes decreases in the following order: Atto 633 > Atto 647 > Cy<sup>®</sup> 5 ([www.atto-tec.de](http://www.atto-tec.de))

<sup>17</sup> Mixture of two diastereomers with practically identical physical properties.

<sup>18</sup> For the structure of Atto 647, see *Supporting Information* of the following report: C. Eggeling, C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, S. Polyakova, V. N. Belov, B. Hein, C. v. Middendorff, A. Schönle, S. W. Hell, *Nature*, **2009**, *457*, 1159–1162.



**Figure 1S.** Structures of the commercially available fluorescent dyes for the red optical region (allow the excitation with He-Ne red laser (633 nm) or 635 nm spectral line of red diode lasers).

## Display Report

### Analysis Info

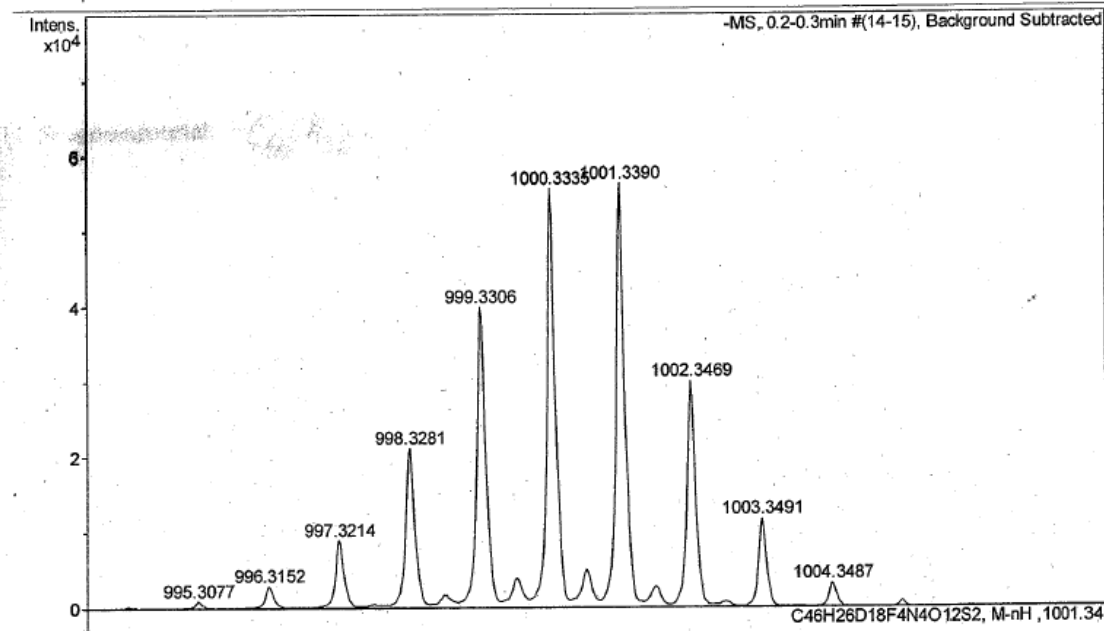
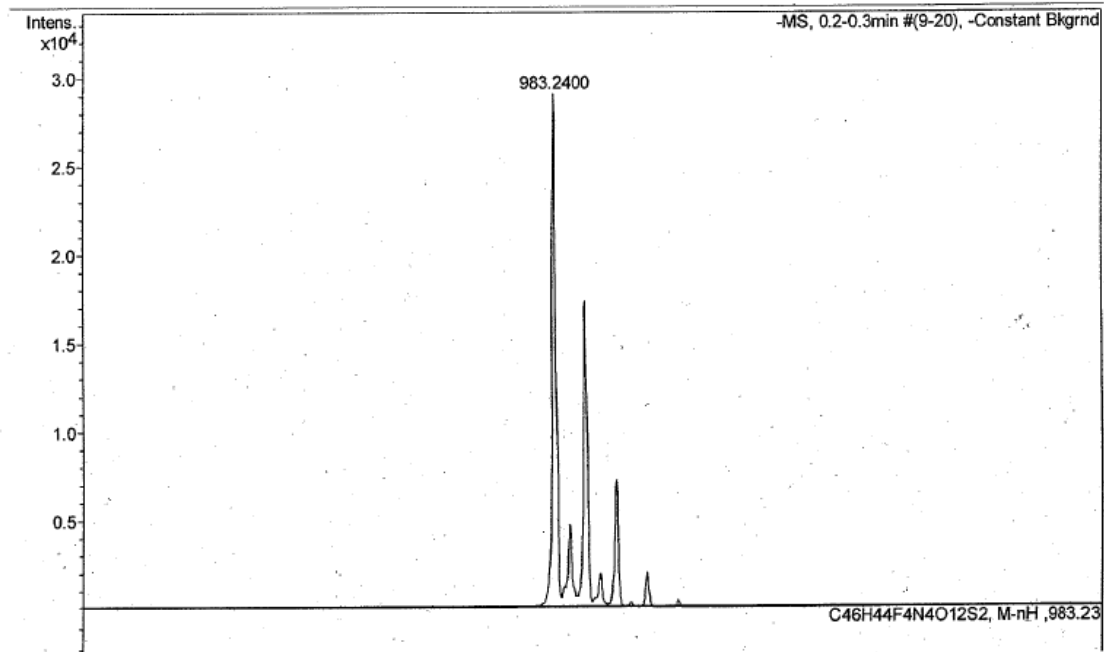
Analysis Name Z:\Data\ms\sam011208\neg\KK114NHS\_19\_01\_2039.d  
Method fiamsauto\_neg.m  
Sample Name KK114NHS  
Comment

Acquisition Date 01.12.2008 12:13:08

Operator BDAL@DE  
Instrument / Ser# micrOTOF 10237

### Acquisition Parameter

|             |            |                      |          |                  |           |
|-------------|------------|----------------------|----------|------------------|-----------|
| Source Type | ESI        | Ion Polarity         | Negative | Set Nebulizer    | 0.4 Bar   |
| Focus       | Not active |                      |          | Set Dry Heater   | 180 °C    |
| Scan Begin  | 50 m/z     | Set Capillary        | 3800 V   | Set Dry Gas      | 4.0 l/min |
| Scan End    | 3000 m/z   | Set End Plate Offset | -500 V   | Set Divert Valve | Source    |



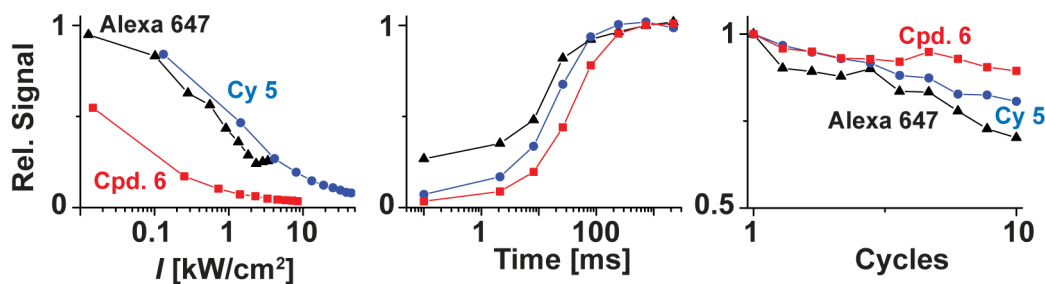
**Figure 2S.** Electro spray ionization spectra (negative mode) of the NHS esters **9** (KK114-NHS; upper panel) and **9D** (lower panel) displaying the natural und artificial molecular mass distributions of the molecular ions, respectively.

## GSDIM imaging

**GSDIM microscope:** The GSDIM setup has been described in detail in ref. [1]. A 633 nm laser (HeNe-Laser R-32413, Newport, Irvien, CA, USA) was used for excitation with intensity  $I = 10 \text{ kW/cm}^2$ . For the GSDIM image, we recorded 11 stacks of 10.240 camera frames at a camera frame rate of 100 Hz. The final reconstruction was realized from 1.046.460 detected single-molecule events as outlined in detail previously.<sup>[1]</sup> Before recording, the sample was pre-irradiated for approximately 10 seconds to transfer most of the dye molecules into the long living dark state. To accelerate single-molecule return at a later stage of recording we added 375 nm UV light ( $2 \text{ kW/cm}^2$ , 375 nm iPulse, Toptica, Munich, Germany) for 1-100  $\mu\text{s}$  during the camera frames. UV-irradiation was started in the fifth stack of camera frames for 1  $\mu\text{s}$  and was increased to 2  $\mu\text{s}$  in the sixth stack, 5  $\mu\text{s}$  in the seventh stack, 10  $\mu\text{s}$  in the eighth stack, 50  $\mu\text{s}$  in the ninth stack and 100  $\mu\text{s}$  in the tenth and eleventh stacks. The UV light accelerates the dark state return by optical deshelling and consequently image recording time.<sup>[1,2]</sup> The GSDIM-Image was reconstructed from 1046460 detected events. The optical resolution was found to be approximately 30 nm.

**Sample preparation:** PVA-coated samples of the labeled  $\beta$ -tubuline strands in PtK2 cells were prepared using sheep-anti-mouse secondary antibodies which had been conjugated with compound **6** (used as NHS-ester **9**) according to a standard protocol.<sup>[1]</sup>

**Pump-probe experiments:** The performance of compound **6** for GSDIM and its comparison to the performance of the cyanine dyes Cy 5 (GE Healthcare) and Alexa 647 (Invitrogen) were done in a pump-probe measurement on a setup described previously.<sup>[1]</sup> The dyes were dissolved in PVA and spin-coated on microscope cover glass (see <sup>[1]</sup> for details). The relative fluorescence signal, i.e., the switch off level of Supplementary Figure 3S (left panel) was determined by comparing the fluorescence observed with an excitation intensity  $I = 100 \text{ W/cm}^2$  for 0.5 ms before and after strong irradiation for 10 ms with the given laser intensity. The fluorescence recovery (Supplementary Figure 3S, middle panel) has been determined by checking the signal level at different time laps after strong irradiation for 10 ms with 5 (Alexa 647), 15 (Cy 5) and  $10 \text{ kW/cm}^2$  (compound **6**). Comparison of photobleaching has been realized by plotting (Supplementary Figure 3S, right panel) the level of fluorescence signal after the given number pump-probe cycles applying a of pump intensity of  $10 \text{ kW/cm}^2$ .



**Figure 3S.** Superior performance of compound **6** for GSDIM: comparison to Cy 5 and Alexa 647. (Left) Laser intensity required to switch off an ensemble of the dye molecules in PVA as determined by the pump-probe experiment with 633 nm light. (Middle) Recovery of fluorescence after dark state shelving. (Right) Level of photobleaching with number of pump-probe cycles.

### (STED-)FCS experiments

**Synthesis of the fluorescent sphingomyelin (SM) derivatives:** Atto647N-labelled *lys*-sphingomyelin (*N*-(Atto647N)-*D*-erythro-sphingosine phosphocholine, *N*-Atto647-sphingomyelin - acyl chain replacement) was purchased from Atto-Tec (Siegen, Germany). SM lipids labeled with compound **6** and **5-H** – substances **12-H** and **12-SO<sub>3</sub>H** – were prepared as as decribed in a later section.

**Triplet parameter determination by FCS:** We determined the rates of intersystem crossing  $k_{ISC}$  and of triplet depopulation  $k_T$  for the compounds **6/6D** in aqueous solution at 22° C (Table 1) according to the common FCS theory.<sup>[2]</sup> The setup and analysis have been decribed in detail before.<sup>[3]</sup> The very same setup was used to determine the fluorescence lifetime by time-correlated single-photon counting.<sup>[2]</sup>

**STED-FCS measurements:** Inclusion of the fluorescent lipid analogues into the plasma membrane of living PtK2 cells and STED-FCS measurements were performed and analyzed as reported.<sup>[3]</sup>

[1] J. Fölling, M. Bossi, H. Bock, R. Medda, C. A. Wurm, B. Hein, S. Jakobs, C. Eggeling, S. W. Hell, *Nat. Methods* **2008**, *5*, 943–945.

[2] a) J. Widengren, U. Mets, R. Rigler, *J. Phys. Chem.* **1995**, *99*, 13369-13379; b) C. Ringemann, A. Schoenle, A. Giske, C. v. Middendorff, S. W. Hell, C. Eggeling J, *ChemPhysChem* **2008**, *9*, 612-624.

[3] C. Eggeling, C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, S. Polyakova, V. N. Belov, B. Hein, C. v. Middendorff, A. Schönle, S. W. Hell, *Nature* **2009**, *457*, 1159–1163.

## Synthesis of the fluorescent dyes

**General remarks:** UV-visible absorption spectra were recorded on a Varian Cary 4000 UV-Vis spectrophotometer, and fluorescence spectra on a Varian Cary Eclipse fluorescence spectrophotometer. Reactions were carried out upon magnetic stirring in Schlenk flasks equipped with septa or reflux condensers with bubble-counters under argon using a standard manifold with vacuum and argon lines. Routine NMR spectra were recorded with a Varian MERCURY-300 spectrometer at 300.5 ( $^1\text{H}$ ), 75.5 ( $^{13}\text{C}$  and APT), and 282.4 MHz ( $^{19}\text{F}$ ).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were also recorded with Varian INOVA 600 (600 MHz) and Varian INOVA 500 (125.7 MHz) instruments, respectively. All NMR spectra are referenced to tetramethylsilane as an internal standard ( $d = 0$  ppm) using the signals of the residual protons of  $\text{CHCl}_3$  (7.26 ppm) in  $\text{CDCl}_3$ ,  $\text{CHD}_2\text{OD}$  (3.31 ppm) in  $\text{CD}_3\text{OD}$ , HOD (4.75 ppm) in  $\text{D}_2\text{O}$ ,  $[\text{D}_5]\text{acetone}$  (2.04 ppm) in  $[\text{D}_6]\text{acetone}$  or  $[\text{D}_5]\text{DMSO}$  (2.50 ppm) in  $[\text{D}_6]\text{DMSO}$ . Multiplicities of signals are described as follows: s = singlet, br. s = broad singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet,  $m_c$  = centrosymmetrical multiplet. Coupling constants ( $J$ ) are given in Hz. Multiplicities in the  $^{13}\text{C}$  NMR spectra were determined by APT (Attached Proton Test) measurements. Low resolution mass spectra (electro spray ionization, ESI) were obtained with LCQ and ESI-TOF mass-spectrometers (MICROTOF (focus), Fa. Bruker). The MICROTOF spectrometer equipped with ESI ion source Apollo and direct injector with LC autosampler Agilent RR 1200 was used for obtaining high resolution mass spectra (ESI-HRMS). ESI-HRMS were obtained also on APEX IV spectrometer (Bruker). HPLC system (Knauer): Smartline pump 1000 (2 $\times$ ), UV detector 2500, column thermostat 4000 (25  $^\circ\text{C}$ ), mixing chamber, injection valve with 20 and 100  $\mu\text{L}$  loop for the analytical and preparative columns, respectively; 6-port-3-channel switching valve; analytical column: Eurospher-100 C18, 5  $\mu\text{m}$ , 250 $\times$ 4 mm, 1.1 mL/min; preparative column: Eurospher-100-5 C18, 5  $\mu\text{m}$ , 250 $\times$ 8 mm, 3.7 mL/min; solvent A: water + 0.1 % v/v trifluoroacetic acid (TFA); solvent B: MeCN + 0.1 % v/v TFA; detection at 636 nm (if not stated otherwise). Normal phase analytical TLC was performed on MERCK ready-to-use plates with silica gel 60 (F<sub>254</sub>) and reversed phase TLC – on RP-18W/UV<sub>254</sub> precoated TLC plates (Macherey-Nagel). Preparative TLC was performed on MERCK HPTLC silica gel 60 plates (10 $\times$ 10 cm, layer thickness 0.2 mm). Column chromatography: MERCK silica gel, grade 60, 0.04–0.063 mm or Polygoprep 60-50 C18 (Macherey-Nagel).



**10-Methoxy-5,5,7-trimethyl-2,3-dihydro-1*H*,5*H*-pyrido[3,2,1-*ij*]quinoline (2-Me).** The alkylation of compound **1** with 1-bromo-3-chloropropane was carried out similarly to the procedure described in US Pat 6,372,907 for its closest analog, the corresponding 10-pivaloyl ester. To a solution of compound **1** (2.71 g, 13 mmol) and 1-bromo-3-chloropropane (8.32 g, 53 mmol) in CH<sub>3</sub>CN (45 mL), the finely ground powders of NaHCO<sub>3</sub> (2.18 g, 26 mmol) and KI (17.3 g, 0.104 mol) were added, and the mixture was refluxed for 25 h with vigorous stirring. The solids were filtered off at room temperature and washed with CH<sub>2</sub>Cl<sub>2</sub> (2×60 mL). The organic solutions were combined, washed with water (2×100 mL), dried and evaporated *in vacuo*. The residue was separated over a column with silica gel (100 g) using a hexane/CH<sub>2</sub>Cl<sub>2</sub> (4:1) mixture as a mobile phase. The main fraction was evaporated *in vacuo* to afford 2.64 g (83%) of compound **2-Me** as colorless crystals with m. p. 87–88 °C. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): *d* = 1.28 (s, 6 H), 1.90 (quint, <sup>3</sup>*J*<sub>H,H</sub> = 6 Hz, 2 H), 1.94 (br. s, 3H), 2.62 (t, <sup>3</sup>*J*<sub>H,H</sub> = 6.5, 2 H), 3.23 (t, <sup>3</sup>*J*<sub>H,H</sub> = 5.6, 2 H), 3.78 (s, 3 H), 5.15 (br. s, 1 H), 6.16 (d, <sup>3</sup>*J*<sub>H,H</sub> = 8.6, 1 H), 6.87 (d, <sup>3</sup>*J*<sub>H,H</sub> = 8.6, 1 H) ppm; <sup>13</sup>C NMR (75.5 MHz, CDCl<sub>3</sub>): *d* 18.7 (Me), 21.4 (CH<sub>2</sub>), 21.5 (CH<sub>2</sub>), 26.7 (Me×2), 41.5 (CH<sub>2</sub>), 55.2 (MeO), 55.8 (C), 97.8 (CH), 109.9 (C), 116.5 (C), 121.5 (CH), 127.4 (CH), 128.1 (C), 142.3 (C), 157.2 (C) ppm; EI-MS: *m/z* = 243 (8) [M<sup>+</sup>], 228 (100) [M–Me]<sup>+</sup>

**1,3,3-Trimethyl-6,7-dihydro-3*H*,5*H*-pyrido[3,2,1-*ij*]quinolin-8-ol (2-H).** Compound **2-Me** (2.43 g, 10.0 mmol) was dissolved in a mixture of glacial AcOH (9 mL) and 48 aq. % HBr (9 mL), the solution was transferred into to a Schlenk flask with a magnetic stirring bar and a reflux condenser, stirred and maintained at reflux for 22 h (oil bath temp. 135–140 °C) under Ar. After cooling, the red solidified material was mixed with 120 mL of CHCl<sub>3</sub>. Upon vigorous stirring and cooling with an external ice-bath, 10% aq. NaOH (36 mL) was added to the mixture at such a rate that the temperature did not exceed 0 °C. The aqueous layer was separated and extracted with CHCl<sub>3</sub> (40 mL); organic solutions were combined and washed with brine (30 mL), saturated NaHCO<sub>3</sub> solution (40 mL), and dried over MgSO<sub>4</sub> under an argon atmosphere. Phenol **2-H** (an air-sensitive compound, especially in solutions) was isolated by column chromatography on SiO<sub>2</sub> (50 g) using a three-component EtOAc/CH<sub>2</sub>Cl<sub>2</sub>/hexane (1:1:10) mixture as a mobile phase. The solvents were evaporated *in vacuo* at the temperatures not exceeding 35 °C to furnish 1.58 g (67%) of the compound **2-H**, whose identity and purity was confirmed by the spectroscopic data given below; pale yellow crystals, m. p. 125–126 °C. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): *d* = 1.29 (s, 6 H), 1.92 (d, <sup>4</sup>*J*<sub>H,H</sub> = 1, 3

H), 1.93 (m, 2 H), 2.61 (t,  $^3J_{\text{H,H}} = 6.5$  Hz, 2 H), 3.24 (m, 2 H), 4.70 (br. s, 1 H, OH), 5.12 (q,  $^4J_{\text{H,H}} = 1$ , 1 H), 6.04 (d,  $^3J_{\text{H,H}} = 8.3$  Hz, 1 H), 6.77 (d,  $^3J_{\text{H,H}} = 8.3$  Hz, 1 H) ppm;  $^{13}\text{C}$  NMR (75.5 MHz,  $\text{CDCl}_3$ ):  $d$  18.8 (Me), 21.2 ( $\text{CH}_2$ ), 21.4 ( $\text{CH}_2$ ), 26.7 (Me $\times$ 2), 41.4 ( $\text{CH}_2$ ), 55.9 (C), 102.4 (CH), 108.1 (C), 116.3 (C), 121.9 (CH), 126.9 (CH), 128.2 (C), 142.6 (C), 153.2 (C) ppm; EI-MS:  $m/z = 229$  (9) [ $\text{M}^+$ ], 214 (100) [ $\text{M}-\text{Me}$ ] $^+$ ; MS (ESI):  $m/z$  (positive mode, %) = 230 (48) [ $\text{M}+\text{H}$ ] $^+$ , 262 (100) [ $\text{M}+\text{Na}$ ] $^+$ ; HRMS ( $\text{C}_{15}\text{H}_{19}\text{NO}$ ): 230.15402 (found  $\text{M}+\text{H}$ ), 230.15394 (calc.).

**2,3,4,5-Tetrafluoro-6-(10-hydroxy-5,5,7-trimethyl-2,3-dihydro-1*H*,5*H*-pyrido[3,2-*i*]quinoline-9-carbonyl)-benzoic acid (3).** Compound 2-H (1.57 g, 6.8 mmol) was refluxed with the tetrafluorophthalic anhydride (1.51 g, 6.9 mmol) in 22 mL of toluene for 5 h under an argon atmosphere. The precipitate was filtered, washed with hexane and dried in air. The crude product was dissolved in  $\text{CHCl}_3$  (300 mL), the solution was stirred for 5 min with 1.5 g of  $\text{SiO}_2$ ; a dark green material was removed by filtration, and the filtrate was diluted with 300 mL of warm (50 °C) hexane. The resulting solution was slowly evaporated to approx. 1/2 of its initial volume; the crystalline powder was filtered off and dried in air to furnish 2.19 g (71%) of the pure compound **3** as a bright yellow crystalline solid, which decomposed at about 280 °C.  $^1\text{H}$  NMR (300 MHz, acetone- $d_6$ ):  $d = 1.37$  (s, 6 H), 1.71 (d,  $^4J_{\text{H,H}} = 1.2$ , 3 H), 1.87 (m, 2 H), 2.61 (t,  $^3J_{\text{H,H}} = 6.5$  Hz, 2 H), 3.24 (m, 2H), 5.27 (q,  $^4J_{\text{H,H}} = 1.2$ , 1 H), 6.75 (d,  $J = 2$  Hz, 1 H), 12.7 (br.s, 1 H) ppm;  $^{13}\text{C}$  NMR (75.5 MHz, acetone- $d_6$ ; highly splitted signals of the fluorinated  $^{13}\text{C}$ -atoms were not registered):  $d$  18.5 (Me), 21.0 ( $\text{CH}_2$ ), 21.3 ( $\text{CH}_2$ ), 28.6 (Me $\times$ 2), 43.0 ( $\text{CH}_2$ ), 58.6 (C), 106.1 (C), 109.9 (C), 115.4 (C), 125.5 (CH), 126.7 (C), 128.5 (CH), 149.9 (C), 162.7 (C), 163.1 (C), 188.7 (C) ppm;  $^{19}\text{F}$  NMR (282.4 MHz, acetone- $d_6$ ):  $d = -155.1$  (m,  $J = 20, 4.5$  and  $1.5$ , 1 F),  $-152.0$  (m,  $J = 21, 7.2$  and  $3.4$ , 1 F),  $-141.5$  (m,  $J = 23, 12, 4.4$  and  $1.8$ , 1 F),  $-138.2$  (m,  $J = 21, 12$  and  $7.5$ , 1 F) ppm; HRMS ( $\text{C}_{23}\text{H}_{19}\text{F}_4\text{NO}_4$ ): 448.1177 (found  $\text{M}-\text{H}$ ), 448.1172 (calc.).

**Rhodamine 4.** With some modifications, the recipe described in US Pat 6,372,907 was followed. In a typical experiment, a suspension of compound **3** (224 mg, 0.5 mmol) in 6 mL of 1,2-dichloroethane was stirred at RT with  $\text{POCl}_3$  (0.19 ml, 2.0 mmol) for 15 min, then phenol 2-H (115 mg, 0.5 mmol) was added in one portion, and the stirring was continued at 63–65 °C (external oil bath) for 3.5 h under an argon atmosphere. Upon cooling to RT, the dark blue solution was vigorously stirred for 1 h with 20 mL of water containing  $\text{NaHCO}_3$  (505 mg, 6.0 mmol). The organic layer was separated, dried over  $\text{Na}_2\text{SO}_4$ , and the solvent was

removed *in vacuo*. The chromatographic separation was performed over a column containing 70 g of SiO<sub>2</sub>. The column was eluted with gradient CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>CN (2/1→1/9), until brown and yellow impurities separated. Then the mobile phase was switched to CH<sub>2</sub>Cl<sub>2</sub>/MeOH (10:1→5:1); the main fraction, containing a blue dye with a red fluorescence, was collected and the solvents evaporated *in vacuo* to give 113 mg (35%) of rhodamine **4**, whose identity and purity was confirmed by the spectroscopic data, TLC and HPLC. Bronze-like crystalline powder, insoluble in water, soluble in most organic solvents giving blue solutions with intense red fluorescence. HPLC:  $t_R = 6.9$  min (A/B 20/80 – 0/100 in 25 min, analytical column, detection at 616 nm). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $d = 1.42/1.43$  (s, 12 H), 1.86 (d, <sup>4</sup> $J_{H,H} = 1.2$ , 6 H), 1.99 (m, 4 H), 2.90 (t, <sup>3</sup> $J_{H,H} = 6.6$  Hz, 4 H), 3.47 (m, 4 H), 5.37 (m, 2 H), 6.88 (s, 2 H) ppm; <sup>13</sup>C NMR (75.5 MHz, CDCl<sub>3</sub>; highly splitted signals of the fluorinated <sup>13</sup>C-atoms were not registered):  $d$  18.7 (Me), 20.1 (CH<sub>2</sub>), 20.7 (CH<sub>2</sub>), 28.7/28.8 (Me×2), 43.1 (CH<sub>2</sub>), 59.3 (C), 104.9 (CH), 113.8 (C), 121.2 (CH), 122.5 (C), 126.5 (C), 130.9 (C), 146.9 (C), 150.0 (C), 153.5 (C), 163.1 (C) ppm; <sup>19</sup>F NMR (282.4 MHz, CDCl<sub>3</sub>):  $d = -159.0$  (br. s, 1 F),  $-152.5$  (t,  $J = 22$ , 1 F),  $-140.1$  (br. m, 1 F),  $-137.7$  (dd,  $J = 23$  and  $13$ , 1 F) ppm. MS (ESI):  $m/z$  (positive mode, %) = 643 (100) [M+H]<sup>+</sup>; HRMS (C<sub>38</sub>H<sub>34</sub>F<sub>4</sub>N<sub>2</sub>O<sub>3</sub>): 643.2571 (found M+H), 643.2588 (calc.).

**Compound 5-*t*Bu.** Rhodamine **4** (224 mg, 0.35 mmol) and POCl<sub>3</sub> (0.27 ml, 2.80 mmol) was refluxed for 2.5 h in 12 mL of 1,2-dichloroethane under an argon atmosphere in a Schlenk flask. The solvent was removed *in vacuo*, the residue kept for an additional hour *in vacuo* (0.4–0.6 mbar) and, after argon had been introduced into the flask, dissolved in CH<sub>3</sub>CN (10 mL). The flask was then cooled to  $-10^{\circ}\text{C}$  (external dry ice bath) and a solution containing *N*-methyl-**b**-alanine *tert*-butyl ester (free base, 64 mg, 0.40 mmol) and Et<sub>3</sub>N (0.16 mL, 1.10 mmol) in 2 mL of CH<sub>3</sub>CN was added in one portion upon stirring. After stirring for 15 min at RT, the solvent was removed *in vacuo* without heating, and the residue shaken with water (10 mL) and CH<sub>2</sub>Cl<sub>2</sub> (40 mL). The organic layer was separated, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and the solvent thoroughly removed *in vacuo* (0.1–0.2 mbar) to afford 260 mg (91%) of the title compound as an amorphous dark blue solid; insoluble in water, soluble in most organic solvents (except alkanes) with intense red fluorescence. HPLC:  $t_R = 8.0$  min (A/B 20/80 – 0/100 in 25 min, analytical column). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, 1:3 mixture of two amide rotamers with diastereotopic methyl and CH groups):  $d = 1.33/1.38$  (s, 9 H, *t*BuO), 1.48/1.51 (br. s, 12 H), 1.80/1.82/1.86/1.88 (br. s, 6 H, MeC=CH), 2.07 (br. m, 4 H), 2.17/2.35 (m, 2 H, CH<sub>2</sub>CO), 2.86/2.87 (br. s, 3 H, NMe), 2.98 (br.m, 4 H), 3.45 (m, 2 H,

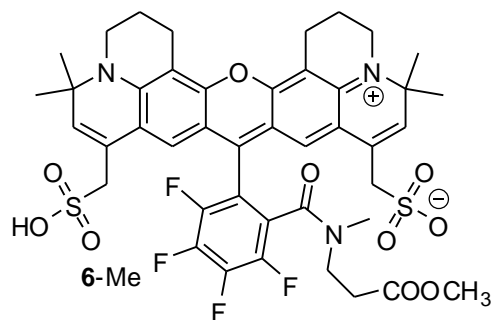
NCH<sub>2</sub>), 3.58 (br. m, 4 H), 5.46/5.48/5.49/5.54 (br. s, 2 H), 6.44/6.56/6.61/6.70 (br. s, 2 H) ppm; <sup>19</sup>F NMR (282.4 MHz, CDCl<sub>3</sub>, 1:3 mixture of two amide rotamers): *d* = -151.3/-150.4 (dt, *J* = 22 and 4, 1 F), -148.7 ÷ -149.0 (m, 1 F), -137.4/-136.9 (ddd, *J* = 23, 12 and 5, 1 F), -135.2/-134.7 (ddd, *J* = 23, 13 and 5, 1 F) ppm. MS (ESI): *m/z* (positive mode, %) = 784 (100) [M<sup>+</sup>]; HRMS (C<sub>46</sub>H<sub>50</sub>F<sub>4</sub>N<sub>3</sub>O<sub>4</sub><sup>+</sup>\*Cl<sup>-</sup>): 784.3732 (found M<sup>+</sup>), 784.3732 (calc.).

**Compound 5-Me.** Rhodamine **4** (76 mg, 0.118 mmol) and POCl<sub>3</sub> (0.27 ml, 0.950 mmol) in 3 mL of 1,2-dichloroethane were heated at 60°C for 3 h under an argon atmosphere in a Schlenk Flask equipped with a magnetic stirrer and a septum. The solvent was removed, the residue kept for an additional hour *in vacuo* (0.4–0.6 mbar) and, after argon had been introduced into the flask, dissolved in CH<sub>3</sub>CN (4 mL). The flask was then cooled to 0°C, and a solution containing methyl 3-(*N*-methylamino)propionate hydrochloride (22 mg, 0.142 mmol) and Et<sub>3</sub>N (0.050 ml, 0.360 mmol) in 1 mL of CH<sub>3</sub>CN was added in one portion with stirring. After stirring for 15 min at RT, the solvent was removed *in vacuo* at 25°C, and the residue shaken with water (10 mL) and CH<sub>2</sub>Cl<sub>2</sub> (20 mL). The organic layer was separated, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and the solvent removed *in vacuo*. The crude compound was purified by flash-chromatography over SiO<sub>2</sub> (9 g) with a MeOH/CH<sub>2</sub>Cl<sub>2</sub> (1:7) mixture as a mobile phase. Evaporation at RT and 0.4–0.6 mbar afforded 75 mg (81%) of the title compound. Amorphous dark blue solid, insoluble in water, soluble in most organic solvents (except alkanes) with intense red fluorescence. HPLC: *t*<sub>R</sub> = 10.4 min (A/B 80/20 – 10/90 in 25 min, analytical column). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, 1:3 mixture of two amide rotamers with diastereotopic methyl and CH groups): *d* = 1.48/1.51 (br. s, 12 H), 1.78/1.81/1.85/1.87 (br. s, 6 H, MeC=CH), 2.06 (br. m, 4 H), 2.23 (m, 2 H, CH<sub>2</sub>CO), 2.78/2.85 (br. s, 3 H, NMe), 2.99 (br.m, 4 H), 3.47 (t, <sup>3</sup>*J*<sub>H,H</sub> = 7, 2 H, NCH<sub>2</sub>), 3.59 (br. m, 4 H), 5.46/5.47/5.51/5.54 (br. s, 2 H), 6.43/6.51/6.61/6.69 (br.s, 2 H) ppm; <sup>19</sup>F NMR (282.4 MHz, CDCl<sub>3</sub>, 1:3 mixture of two amide rotamers): *d* = -151.0/-150.4 (dt, *J* = 22 and 5, 1 F), -148.7 (m, 1 F), -137.3/-137.1 (ddd, *J* = 23, 13 and 5, 1 F), -135.0/-134.7 (ddd, *J* = 22, 12 and 5.5, 1 F) ppm. <sup>13</sup>C NMR (75.5 MHz, CDCl<sub>3</sub>; 1:3 mixture of two amide rotamers with diastereotopic methyl and CH groups; highly splitted signals of the fluorinated <sup>13</sup>C-atoms were not registered): *d* 18.5/18.6, 19.7/19.8, 20.6/20.7, 28.7/28.8, 31.2, 32.5/32.6, 37.20/37.23, 43.6/43.8, 51.7/52.0, 59.99/60.05, 105.7/106.5, 112.8, 114.0, 118.0, 120.8, 121.3, 122.8, 123.0, 124.8, 125.8, 132.2, 133.3, 150.3, 150.9, 153.2, 171.1/171.3 ppm; MS (ESI): *m/z* (positive mode, %) = 742 (100) [M<sup>+</sup>]; HRMS (C<sub>43</sub>H<sub>44</sub>F<sub>4</sub>N<sub>3</sub>O<sub>4</sub><sup>+</sup>\*Cl<sup>-</sup>): 742.3255 (found M<sup>+</sup>), 742.32625 (calc.).

**Rhodamine 6.** *tert*-Butyl ester **5-tBu** (32 mg, 0.04 mmol) was introduced in one portion into a vial containing 1.0 mL of the commercial (97%) sulfuric acid pre-cooled to 0°C. The vial was sealed, the mixture stirred at RT until the solid had completely dissolved, and left for 40 h. The viscous red solution was transferred to a vessel containing a frozen mixture (-10÷-15°C; dry ice bath) of 1,4-dioxane (2 mL) and dry diethyl ether (5 mL). Then a larger amount of diethyl ether (40 mL) was added followed by hexane (15 mL). After standing at 0°C for 1 h, the liquid was decanted from a blue viscous oil which was then thoroughly washed with absolute diethyl ether (4×10 mL). The crude product was dissolved in 5mL of water and subjected to chromatography over a column with Polygoprep 60-50 C18 (30 g). The column was eluted with water (50 mL) to remove the residual sulfuric acid, then with CH<sub>3</sub>CN/H<sub>2</sub>O (1:2) containing 0.05 % (v/v) CF<sub>3</sub>COOH. The first (main) fraction was collected, and the bulk of the solvent evaporated *in vacuo*. Subsequent freeze-drying furnished 26 mg (72%) of the title compound, whose purity (HPLC-area 99%) and identity (structure **6**) was confirmed by the spectroscopic methods. Upon prolonged storage or moderate heating in methanol-containing solutions, particularly in the course of solvent evaporation, compound **6** formed the corresponding methyl ester (**6-Me**; the structure is given below), whose MS and HRMS (ESI) agreed with the proposed structure. Dark blue crystalline powder, perfectly soluble in water and polar organic solvents (with an intense red fluorescence), insoluble in diethyl ether, aliphatic, chlorinated, and aromatic hydrocarbons. HPLC: *t*<sub>R</sub> = 13.0 min (100%, A/B 80/20 – 50/50 in 25 min, analytical column), RP TLC: CH<sub>3</sub>CN/H<sub>2</sub>O (1:1) + 0.1% CF<sub>3</sub>COOH (v/v), *R*<sub>f</sub> ~ 0.2. <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O): *d* = 1.63/1.65/1.69/1.71 (br. s, 12 H), 1.91/1.93 (s, 6H, MeC=CH), 2.02 (br. m, 4 H), 2.55 (m, 2 H, CH<sub>2</sub>CO), 2.83/2.90 (br. s, 3 H, NMe), 2.98 (br. m, 4 H), ~3.5 (br. m, 2 H, NCH<sub>2</sub>), ~3.7 (br. m, 4 H), 3.94 (d, <sup>2</sup>*J*<sub>H,H</sub> = 13, 2 H, CH<sub>A</sub>H<sub>B</sub>), 4.32 (d, <sup>2</sup>*J*<sub>H,H</sub> = 14, 2 H, CH<sub>A</sub>H<sub>B</sub>), 6.14/6.16 (br. s, 2 H), 7.32 (br.s, 2 H) ppm; <sup>19</sup>F NMR (282.4 MHz, D<sub>2</sub>O; ca. 1:9 mixture of the two amide rotamers; signals of the major rotamer are given): *d* = -150.8 (dt, *J* = 21 and 5.5, 1 F), -149.3 (dt, *J* = 22 and 5.5, 1 F), -138.7 (ddd, *J* = 22, 11 and 5.5, 1 F), -136.0 (m, 1 F) ppm. <sup>13</sup>C NMR (75.5 MHz, D<sub>2</sub>O; highly splitted signals of the fluorinated <sup>13</sup>C-atoms were not registered): *d* 16.7/16.8 (CH<sub>2</sub>), 17.4 (CH<sub>2</sub>), 24.6/24.87/24.93/25.1 (Me; disappear in compound **6D**), 28.3 (CH<sub>2</sub>), 35.9 (NMe), 40.9/41.0/41.1 (CH<sub>2</sub>), 50.5/50.9 (CH<sub>2</sub>SO<sub>3</sub>; disappear in compound **6D**), 57.4/57.7 (C), 104.0/104.3 (C), 109.7/110.3 (C), 117.6 (C), 118.1 (CH), 119.4 (C), 119.9 (CH), 135.5/136.2 (CH=[CMe]; disappear in compound **6D**), 137.9 (C), 147.8/148.3 (C), 150.1/150.2 (C), 160.2 (C), 171.8 (CO) ppm; MS (ESI): *m/z* (negative mode, %) = 442.6 (100) [M-2H]<sup>-</sup>, 908 (45) [M-2H+Na]<sup>-</sup>; *m/z* (positive mode, %) = 910 (49) [M+Na]<sup>+</sup>, 932 (45) [M-H+2Na]<sup>+</sup>; HRMS

(C<sub>42</sub>H<sub>41</sub>F<sub>4</sub>N<sub>3</sub>O<sub>10</sub>S<sub>2</sub>): 888.22386 (found M+H), 888.22423 (calc.); 910.20587 (found M+Na), 910.20617 (calc.).

**Methyl ester 6-Me** (formed by esterification of compound **6** in methanolic solutions upon standing) was isolated by preparative TLC on silica plates using CH<sub>3</sub>CN/H<sub>2</sub>O (5:1) as a mobile phase). MS (ESI): *m/z* (positive mode, %) = 902 (100) [M+H]<sup>+</sup>, 924 (27%) [M+Na]<sup>+</sup>; (ESI): *m/z* (negative mode, %) = 900 (100) [M-H]<sup>-</sup>; HRMS (C<sub>43</sub>H<sub>43</sub>F<sub>4</sub>N<sub>3</sub>O<sub>10</sub>S<sub>2</sub>): 902.23994 (found M+H), 902.23988 (calc.).



**Rhodamine 5-H** (cleavage of the *tert*-butyl ester **5-tBu**). Compound **5-tBu** (30 mg, 0.038 mmol) was dissolved in dry dioxane (1 mL) and 4 M HCl solution in dry 1,4-dioxane (1 mL) was added at 0 °C, and then the reaction mixture was left for 2 days at RT. The solution was mixed with CH<sub>2</sub>Cl<sub>2</sub> (40 mL), washed with brine (2×10 mL), dried and evaporated to dryness *in vacuo*. The pure title compound was isolated in 70% yield by flash chromatography over 15 g of SiO<sub>2</sub> with CH<sub>3</sub>CN/H<sub>2</sub>O (1:10? 1:3) mixture as a mobile phase. Dark blue crystalline powder, very slightly soluble in water, moderately soluble in polar organic solvents, also in CHCl<sub>3</sub> and CH<sub>2</sub>Cl<sub>2</sub>. HPLC: *t*<sub>R</sub> = 5.0 min (100%, A/B 20/80 – 0/100 in 25 min, analytical column). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, ca. 1:1 mixture of two amide rotamers with diastereotopic methyl and CH groups): *d* = 1.45/1.49 (br. s, 12 H), 1.88/1.90 (br. s, 6 H, MeC=CH), 2.05 (br. m, 4 H), 2.35 (br. m, 2 H, CH<sub>2</sub>CO), 2.74/2.79 (br. s, 3 H, NMe), 2.95 (br. m, 4 H), 3.15 (m, 2 H, NCH<sub>2</sub>), 3.54 (br. m, 4 H), 5.43/5.44/5.49/5.50 (br. s, 2 H), 6.43/6.62/6.69/6.74 (br. s, 2 H) ppm; <sup>19</sup>F NMR (282.4 MHz, CDCl<sub>3</sub>, 1:1 mixture of two amide rotamers): *d* = –152.2 (m, 1 F), –149.4/–148.4 (m, 1F), –134.5 (m, 2 F) ppm. MS (ESI): *m/z* (positive mode, %) = 728 (100) [M<sup>+</sup>] 750 (60) [M+Na]<sup>+</sup>; HRMS (C<sub>42</sub>H<sub>41</sub>F<sub>4</sub>N<sub>3</sub>O<sub>4</sub>): 728.3100 (found M+H), 728.3106 (calc.); 750.2923 (found M+Na), 728.2925 (calc.).

*Saponification of the ester 5-Me (an alternative procedure for the preparation of the acid 5-H)*. A freshly prepared aqueous 10 wt. % KOH solution (0.045 mL, 0.080 mmol) was diluted with water (1.5 mL) and combined with a solution of methyl ester **5-Me** (14 mg, 0.018 mmol) in THF (1.2 mL) at RT. The resulting homogeneous solution was left overnight in a sealed vial, then acidified with 1 M aq. HCl (0.3 mL, 0.3 mmol), mixed with brine (10 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (2×30 mL). The extract was washed with brine (10 mL), dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated *in vacuo* to afford 10 mg (68%) of a pure (~99%, HPLC) product, which proved to be identical (spectroscopic data, HPLC retention parameters) to the previously obtained acid **5-H**; no additional purification required.

*Sulfonation of the acid 5-H.* Rhodamine **5-H** was sulfonated with H<sub>2</sub>SO<sub>4</sub> under conditions described above for the *tert*-butyl ester **5-tBu** and gave the product identical to the previously obtained rhodamine **6** (HPLC, spectroscopic data) in an 88% yield.

**N-hydroxysuccinimidyl Ester 9.** To rhodamine **6** (5–10 mg, 6–12 μmol), taken as a 2% solution in dry DMF, Et<sub>3</sub>N (60–12 μmol) was added followed by TSTU (24–48 μmol), which was added at RT in small portions over a period of 5–15 h, while the reaction was monitored by HPLC (A/B: 80/20 → 50/50 in 25 min, 1 mL/min, detection at 636 nm, analytical column) The solvent was removed *in vacuo* (< 1 mbar) at the temperatures not exceeding 30 °C, and the ester **9** isolated by means of preparative HPLC: *t*<sub>R</sub> = 16.0 min (A/B 20/80 – 0/100 in 25 min, preparative column) followed by freeze drying of the fractions. Dark blue solid soluble in water (purity check showed 98% HPLC area); decomposes in aqueous solutions at RT, stable in a solid state under Ar at –20°C. MS (ESI): *m/z* (negative mode, %) = 983 (100) [M–H]<sup>–</sup>; HRMS (C<sub>46</sub>H<sub>44</sub>F<sub>4</sub>N<sub>4</sub>O<sub>12</sub>S<sub>2</sub>): 983.2400 (found M–H), 983.2261 (calc.).

**Maleimide 11** was synthesized from the freshly prepared NHS ester **9** (2 mg, 2.2 μmol) and *N*-(2-aminoethyl)maleimide trifluoroacetate (2.8 mg, 11 μmol) which were combined in dry DMF (0.2 mL) in the presence of Et<sub>3</sub>N (3 μl, 21 μmol) at room temperature and left with stirring overnight. The solvent was evaporated *in vacuo* (1 mbar), and the title compound was isolated by preparative HPLC; *t*<sub>R</sub> = 15.4 min (A/B 20/80 – 50/50 in 25 min, analytical column, 636 nm) followed by freeze drying of the fractions. Dark blue solid soluble in water (purity check gave 100% HPLC area); MS (ESI): *m/z* (negative mode, %) = 1008 (100) [M–H]<sup>–</sup>. It decomposes in methanol containing solutions (adding one molecule of MeOH, apparently with opening of the maleimide cycle and forming the compound with M = 1041; [M–H] = 1040 in the negative mode of the electro-spray ionization); stable in a solid state under Ar. The title compound reacts with *N*-acetylcysteine in aq. NaHCO<sub>3</sub> at pH = 7.5 forming the conjugate with *t*<sub>R</sub> = 13.8 min (A/B 20/80 – 50/50 in 25 min, analytical column, 636 nm).



**2,3,5,6-Tetrafluorophenyl Ester 8.** Rhodamine 5-H (6 mg, 7  $\mu$ mol) in 2.5 mL of dry  $\text{CH}_3\text{CN}$  was mixed with 2,3,5,6-tetrafluorophenol (1.68 mg, 10  $\mu$ mol, 1% solutions in  $\text{CH}_2\text{Cl}_2$ ), and the commercial  $\text{Me}_2\text{NCH}_2\text{CH}_2\text{CH}_2\text{N}=\text{C}=\text{NEt}$  (free base, 1.37 mg,  $\mu$ mol, 1% solutions in  $\text{CH}_2\text{Cl}_2$ ) was added over a period of 2 h. The reaction was monitored by TLC on  $\text{SiO}_2$  with  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:10) mixture as a mobile phase. Compound **8** was isolated in 65% yield using HPTLC plates (normal phase  $\text{SiO}_2$ ) with the same eluent followed by freeze drying. HPLC:  $t_R = 18.0$  min (70–80% HPLC area, A/B 20/80 – 0/100 in 25 min, analytical column). For the preparative experiments, the active ester **8** was used without further purification. MS (ESI):  $m/z$  (positive mode, %) = 876 [ $\text{M}^+$ ]; HRMS ( $\text{C}_{48}\text{H}_{42}\text{F}_8\text{N}_3\text{O}_4^+ \cdot \text{OH}^-$ ): 876.3052 (found  $\text{M}^+$ ), 876.3042 (calc.).

**The deuterated analog of compound 1.** The method for the preparation of **1** outlined by L. A. Robinson and M. E. Theoclitou [*Tetrahedron Lett.* **2002**, *43*, 3907–3910] was used utilizing  $[\text{D}_6]$ acetone as a starting material. An additional treatment of the evaporated reaction mixture with MeOD in order to “restore” the methine deuterium atoms in the fragment  $\text{CCD}_3=\text{CD}$  was necessary. They proved to be largely (and unexpectedly) exchanged to H in the course of the synthesis. Presumably, the methine groups coordinate to the ytterbium catalyst and then receive protons from  $\text{H}_2\text{O}$  formed in the course of the reaction or added during the work-up.

Finely powdered ytterbium(III) triflate (the commercially available hydrate, 1.30 g, ~26 mmol) was dried in a Schlenk flask with stirring at  $130^\circ\text{C}$  for 1.5 h. Then the flask was filled up with argon, cooled to  $0^\circ\text{C}$ , and a solution of *m*-anisidine (1.75 g, 14 mmol) in  $[\text{D}_6]$ -acetone (92 mL, 1.14 mol) was added in one portion through a septum. The mixture was stirred overnight at RT, the solvent removed in vacuo at  $25\text{--}30^\circ\text{C}$ , MeOD (10 mL) was added to the residue followed by  $\text{CH}_2\text{Cl}_2$  (200 mL), and it was well shaken with water (30 mL). The organic layer was separated, washed with brine (30 mL), dried over  $\text{Na}_2\text{SO}_4$  and evaporated *in vacuo*. Column chromatography on  $\text{SiO}_2$  (90 g) with  $\text{CH}_2\text{Cl}_2$ /hexane mixture (1:3? 1:1) as an eluent furnished the deuterated compound **1** (1.76 g, 59%). The intensity of the singlet signal at 5.2 ppm (CH group at the double bond) in the  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ ) indicated the degree of deuteration of ca. 50% at this carbon atom, while other aliphatic protons, which originated from acetone- $[\text{D}_6]$ , were

deuterated to a degree of about 90% and more. To further improve the deuterium content, the obtained product (1.76 g, 0.83 mmol) was heated with a reflux condenser at 50°C in 25 mL of CH<sub>3</sub>OD (0.72 mol) with 96% D<sub>2</sub>SO<sub>4</sub> (0.87 g, 15 mmol) for 30 h. The solution was cooled to -35°C (dry ice bath), neutralized with an excess of dry Na<sub>2</sub>CO<sub>3</sub> (1.40 g, 13 mol) upon stirring, and evaporated *in vacuo* at RT. The residue was extracted with CH<sub>2</sub>Cl<sub>2</sub>, evaporated and subjected to column chromatography exactly as described above (using 50 g of SiO<sub>2</sub>), which afforded 1.23 g (41%) of the deuterated compound **1** with the higher degree of deuteration (ca. 75%).

**Compound 6D** was obtained from the deuterated analogue of compound **1** as described above using Na<sub>2</sub>CO<sub>3</sub> (instead of NaHCO<sub>3</sub>) and CD<sub>3</sub>CN in the alkylation step, followed by treatment with 48% DBr in D<sub>2</sub>O (Deutero GmbH) in AcOD for the dealkylation. Further steps of the synthesis were performed exactly as described for the non-deuterated compounds (see above). The sulfonation of the deuterated compound **5-*t*Bu** (65 mg, 0.08 mmol) was performed with 96% D<sub>2</sub>SO<sub>4</sub> in D<sub>2</sub>O (VWR International). It improved the deuterium content in the fragment C(CD<sub>3</sub>)=CD of the title compound. Yield – 30 mg (41%). MS (ESI): *m/z* (negative mode, %) = 451.7 (100) [M-2H]<sup>-</sup>, 904.4 (45) [M-H]<sup>-</sup>; C<sub>42</sub>H<sub>23</sub>D<sub>18</sub>F<sub>4</sub>N<sub>3</sub>O<sub>10</sub>S<sub>2</sub>: 905.4. Molecular mass distribution of the molecular ion peaks was as follows: *m/z* (negative mode, %) = 900 (15), 901 (36), 902 (67), 903 (94), 904 (100), 905 (46%), 906 (18%). Subtracting the natural deuterium content, this molecular mass distribution corresponds to the following amounts of the new deuterium atoms, introduced in the course of the labelling procedures: 905 (D<sub>18</sub>): 5%; 904 (D<sub>17</sub>): 43%; 903 (D<sub>16</sub>): 24%; 902 (D<sub>15</sub>): 13%; 901 (D<sub>14</sub>): 8%.

**NHS Ester 9D** was prepared from acid **6D** (11 mg, 12 μmol) and *N*-hydroxysuccinimide (13 mg, 0.11 mmol) dissolved in dry DMF (0.5 mL). HATU (12 mg, 32 μmol) was added to this solution at room temperature followed by Et<sub>3</sub>N (25 μl, 0.17 mmol), and the reaction mixture was stirred under Ar overnight. The solvent was removed *in vacuo*, and the title compound was isolated by preparative HPLC. Dark blue very light powder; HPLC: *t<sub>R</sub>* = 15.6 min (A/B 20/80 – 0/100 in 25 min, analytical column). MS (ESI): *m/z* (negative mode, %) = 1001.4 (100) [M-H]<sup>-</sup>; C<sub>46</sub>H<sub>26</sub>D<sub>18</sub>F<sub>4</sub>N<sub>4</sub>O<sub>12</sub>S<sub>2</sub>: 1002.4. Molecular

mass distribution of the molecular ion peaks was as follows:  $m/z$  (negative mode, %) = 997 (16), 998 (37), 999 (70), 1000 (97), 1001 (100), 1002 (54), 1003 (21).

**General method of the preparation of sphingomyelin (SM) derivatives labelled with compounds 5-H and 6: synthesis of the fluorescent SM analogs 12-H and 12-SO<sub>3</sub>H.**

Lyso-SM (Avanti Polar Lipids, USA) (1.2 mg) was combined with the freshly prepared active esters **8** or **9** (1.4 mg) in ca. 0.2–0.5 mL of the dry DMF/methanol mixture (1:1), and dry Et<sub>3</sub>N (3 μl) was added. The reaction mixture was stirred at room temperature overnight, the solvents were evaporated in vacuo (ca. 1 mbar) at room temperature, and the title products were isolated from the residue by preparative HPTLC on the commercially available plates coated with SiO<sub>2</sub> (VWR, 10×10 cm, thickness of the layer 0.2 mm). A mixture of CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O (65:35:8, v/v/v) was used as an eluent. The stained stripes with  $R_f$ s lower than these of the starting active esters were scratched out, the products were extracted with the eluent, and the pooled solutions were evaporated by passing the gentle stream of argon above the solution followed by liophilization. The homogeneity of the isolated products was controlled by HPTLC and, if necessary, the purification procedure was repeated.

**Compound 12-H** (which is difficult to extract from the silica gel completely) was isolated as a dark blue waxy solid. MS (ESI):  $m/z$  (positive mode, %) = 1174.6 (100) [M<sup>+</sup>]; HRMS (C<sub>65</sub>H<sub>88</sub>F<sub>4</sub>N<sub>5</sub>O<sub>8</sub>P<sup>+</sup>): 1174.6412 (found M<sup>+</sup>), 1174.6379 (calc.).

**Compound 12-SO<sub>3</sub>H** was isolated as a dark blue solid. MS (ESI):  $m/z$  (negative mode, %) = 1332.6 (100) [M<sup>-</sup>]; HRMS (C<sub>65</sub>H<sub>87</sub>F<sub>4</sub>N<sub>5</sub>O<sub>14</sub>PS<sub>2</sub><sup>-</sup>): 1332.5555 (found M<sup>-</sup>), 1332.5365 (calc.).