Pergamon

SCIENCE DIRECT®

Tetrahedron Letters 44 (2003) 4355–4359

TETRAHEDRON LETTERS

Rational design and synthesis of a novel class of highly fluorescent rhodamine dyes that have strong absorption at long wavelengths

Jixiang Liu,[a,*] Zhenjun Diwu,[b] Wai-Yee Leung,[a] Yixin Lu,[a] Brian Patch[a] and Richard P. Haugland[a]

[a]*Molecular Probes, Inc., 29851 Willow Creek Road, Eugene, OR 97402, USA*
[b]*Molecular Devices, Inc., 1311 Orleans Drive, Sunnyvale, CA 94089, USA*

Received 4 February 2003; revised 10 April 2003; accepted 11 April 2003

Abstract—A novel class of strongly fluorescent rhodamine dyes were designed and synthesized by extending the π conjugation of chromophore with limited flexibility. These dyes were shown to have longer absorption in the range of 581 to 631 nm with quantum yields between 0.64 and 0.89.

Rhodamine dyes are highly fluorescent and have great photostability. Additionally, their fluorescence spectra are pH-independent from pH 4 to 10. Over the years a variety of rhodamine dyes have been prepared and used both as laser dyes[1] and as fluorescent detection reagents. For example, carboxyrhodamine 110 succinimidyl ester, carboxyrhodamine 6G succinimidyl ester, carboxytetramethylrhodamine succinimidyl ester, tetramethylrhodamine isothiocyanate, Lissamine rhodamine B sulfonyl chloride and sulforhodamine 101 sulfonyl chloride (Texas Red® sulfonyl chloride) are among the most widely used fluorescent reagents for labeling proteins and oligonucleotides. In particular, researchers commonly use these amine-reactive rhodamines to prepare fluorescent antibodies and avidin conjugates for immunochemistry applications.[2]

Because these rhodamine dyes have absorption maxima that are well below 600 nm, their fluorescence detection sensitivity is severely compromised by background signals caused by biological autofluorescence (e.g. from serum, proteins and other macromolecular compounds). The development of sensitive detectors, such as CCD cameras, and of inexpensive lasers with long-wavelength emission (e.g. He/Ne lasers, 633 nm), as well as technological advances in multicolor labeling and capturing images in the visible–near IR region, has prompted researchers to invent long-wavelength rhodamine dyes with absorption beyond 600 nm. For example, Drexhage, Arden-Jacob and their co-workers developed a new class of long-wavelength pentacyclic rhodamine dyes called 'JA' dyes.[3,4] And recently, Benson et al. reported the synthesis of dibenzorhodamine derivatives with absorption beyond 600 nm.[5]

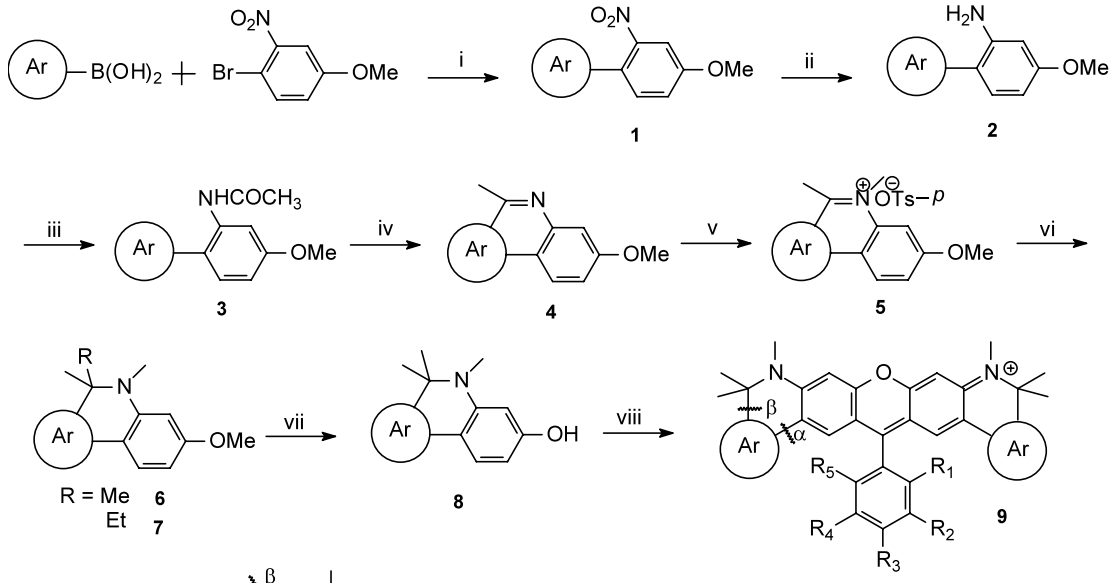
The additional conjugation of the π system, as well as the formation of rigid rings, in organic dyes can shift their absorption and emission maxima to longer wavelengths. Furthermore, the lack of molecular rotation[6] restricts or diminishes the nonradiative deactivation processes, which in turn increases the rigidity of the molecular system and increases the fluorescence quantum yield of the dye. Therefore, we predicted that rhodamine derivatives with different aryl or heteroaryl substituents on the 2′ and 7′ positions (made rigid by dimethylmethylene bridges to the nitrogen atoms of rhodamine) might exhibit the desired characteristics of long-wavelength absorption and emission with high fluorescence quantum yield. Based on this hypothesis, we designed and synthesized a novel class of highly fluorescent rhodamine derivatives with absorption beyond 600 nm.

These new rhodamine derivatives were prepared from commercially available 4-bromo-3-nitroanisole and five different arylboronic acids, as shown in Scheme 1. The improved Suzuki cross-coupling reaction in water gave a high yield of pure product **1**.[7] Catalytic hydrogenation of compound **1** gave the aniline derivative **2**, which was converted to the amide **3** with acetic anhydride. Thiophene or benzothiophene derivatives can poison the Pd/C catalyst, so zinc dust/concentrated hydrochlo-

* Corresponding author. Fax: (+1) 541-344-6504; e-mail: jixiang.liu@probes.com

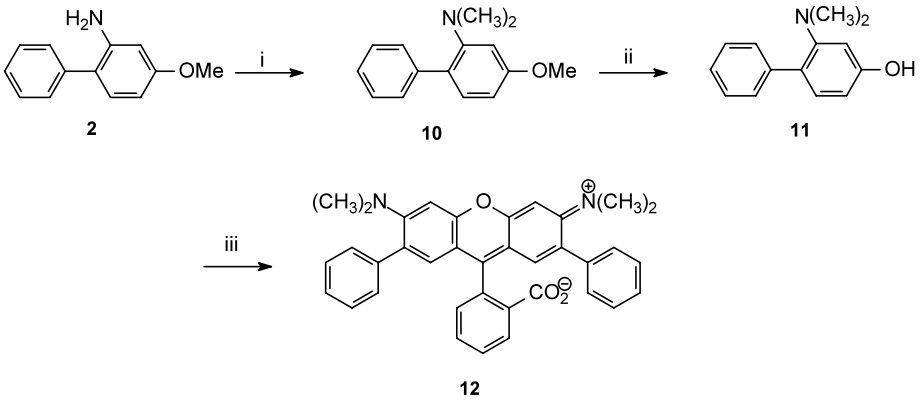
0040-4039/03/$ - see front matter
doi:10.1016/S0040-4039(03)00938-9

ric acid (instead of hydrogenation) was used to reduce compound **1** containing thiophene or benzothiophene moieties. Bischler–Napieralski cyclization of amide **3** with $POCl_3$ afforded compound **4**. Compound **4** was quaternized with *p*-TsOMe to give compound **5**. Compound **5** was reacted with the MeMgCl Grignard reagent to afford the aniline derivative **6**.



	Ar	R_1	R_2	R_3	R_4	R_5
9a		$CO_2^{\ominus}$	H	H	H	H
9b		$CO_2^{\ominus}$	H	H	H	H
9c		$CO_2^{\ominus}$	H	CO_2H (or H)	H (or CO_2H)	H
9d		$CO_2^{\ominus}$	Cl	Cl	Cl	Cl
9e		$CO_2^{\ominus}$	F	F	F	F
9f		$CO_2^{\ominus}$	Cl	Cl	Cl	Cl
9g		$CO_2^{\ominus}$	H	CO_2H (or H)	H (or CO_2H)	H
9h		$SO_3^{\ominus}$	H	SO_3H	H	H
9i		$SO_3^{\ominus}$	H	SO_3H	H	H

Scheme 1. Synthesis of the rhodamine derivatives **9a–i** and their intermediates. *Reaction conditions*: (i) $Pd(OAc)_2$, K_2CO_3, *n*-$BuN^{\oplus}Br^{T}$, H_2O, 80°C, 1–2 h, under N_2, yield 85–91%; (ii) H_2, 10% Pd(C), MeOH, 4 h, yield 100%; or Zn/con. HCl, THF/MeOH, rt, 1 h, yield 82–90%; (iii) Ac_2O, Py, THF, reflux, 1–2 h, yield 91–95%; (iv) $POCl_3$, 80°C, 30 min, yield 62–84%; (v) *p*-TsOMe, chlorobenzene, reflux, 2 days, yield 70–79%; (vi) MeMgCl (large excessive), THF, under N_2, in the dark, yield 65–80%; (vii) BBr_3, CH_2Cl_2, 0°C→rt, 1 h, in the dark, yield 85–93%; (viii) a. phthalic anhydride, cat. *p*-$TsOH{\cdot}H_2O$, $EtCO_2H$, reflux, 6–12 h, in the dark; b. 4-formyl-1,3-benzenedisulfonic acid, disodium salt, TFA, DMF, rt overnight and then 110–120°C, 7–10 h, in the dark.



Scheme 2. Synthesis of rhodamine derivative **12**. *Reaction conditions*: (i) MeI, K_2CO_3, THF, reflux, 1 day, yield 90%; (ii) BBr_3, CH_2Cl_2, 0°C→rt, 1 h, in the dark, yield 91%; (iii) phthalic anhydride, cat. *p*-TsOH·H_2O, $EtCO_2H$, reflux, 6–12 h, in the dark, yield 15%.

Note that the reaction of compound **5** with the MeMgCl Grignard reagent should be carried out in the dark. Otherwise, product **6** continues to react with excess MeMgCl, and a significant amount of by-product **7** is formed, most likely via a free radical reaction.

Demethylation of compound **6** with BBr_3 generated the desired key aminophenol derivative **8**. Finally, the novel rhodamine derivatives **9a**–**i** were obtained via condensation of the aminophenol derivative **8** with different phthalic anhydrides or 4-formyl-1,3-benzenesulfonic acid, disodium salt. Interestingly, only the rhodamine dyes were obtained in the condensation of the aminophenol derivative **8** with 4-formyl-1,3-benzenesulfonic acid, disodium salt, which indicated that the dihydrorhodamine intermediates were readily oxidized by air.

For comparison, the rhodamine derivative **12** was also synthesized from compound **2** as shown in Scheme 2. The alkylation of compound **2** with MeI/K_2CO_3 gave compound **10**, which was demethylated with BBr_3 to afford the aminophenol derivative **11**. The condensation of compound **11** with phthalic anhydride gave the rhodamine derivative **12**. All compounds were confirmed with their MS and ^1H NMR spectra, their R_f values were also measured on silica gel TLC.[8]

Absorption maxima (Abs), fluorescence maxima (Em), fluorescence quantum yields (Φ_f) and extinction coefficient (ϵ) for all of the rhodamine derivatives (compounds **12** and **9a**–**i**) are summarized in Table 1. In general, all of the new rhodamine derivatives have absorption and emission band shapes similar to that of tetramethylrhodamine with a large bathochromic shift. The absorption and emission spectra of compound **9d** was compared with those of tetramethylrhodamine in Figure 1. As expected, the fluorescence spectra of all the new rhodamine derivatives have approximate mirror symmetry with their absorption spectra.

Compared with compound **12**, compound **9a** has absorption and emission maxima that are remarkably

Table 1. The photophysical characteristics of the new rhodamine derivatives (compounds **12** and **9a**–**i**) dissolved in MeOH

Compound	$\lambda_{max.\ abs}$[a] (nm)	$\lambda_{max.\ em}$[a,b] (nm)	Φ_f[c]	ϵ_{max}[d] (M^{-1} cm^{-1})
12	548	571	0.25	82,000
9a	582	604	0.75	125,000
9b	581	605	0.85	130,000
9c	596	620	0.71	126,000
9d	631	648	0.64	141,000
9e	628	649	0.66	140,000
9f	627	645	0.76	124,000
9g	600	627	0.66	132,000
9h	610	629	0.85	130,000
9i	616	633	0.89	143,000

[a] Uncertainty is ±1 cm.

[b] Emission spectra measured on the fluorescence spectrometer Aminco SPF-500C are corrected.

[c] The fluorescence quantum yields of the new dyes are determined by using the reference standards [rhodamine B (Φ_f=0.50 in ethanol)[9] for **12**; rhodamine 101 (Φ_f=0.96 in ethanol)[9] for **9a**–**c**, **9g**–**i** and JA 22 (Φ_f=0.90 in ethanol)[3] for **9c**–**e** as standards]. Uncertainty is $\leq$6%.

[d] Uncertainty is $\leq$5%.

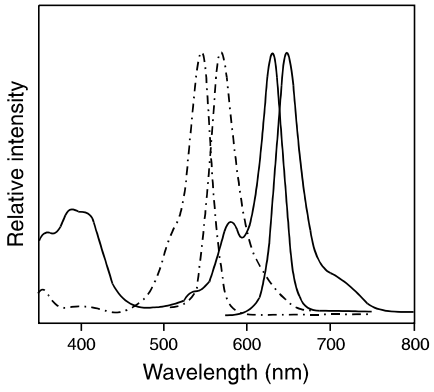


Figure 1. Normalized absorption and emission spectra of compound **9d** (—) and tetramethylrhodamine (-·-) in MeOH.

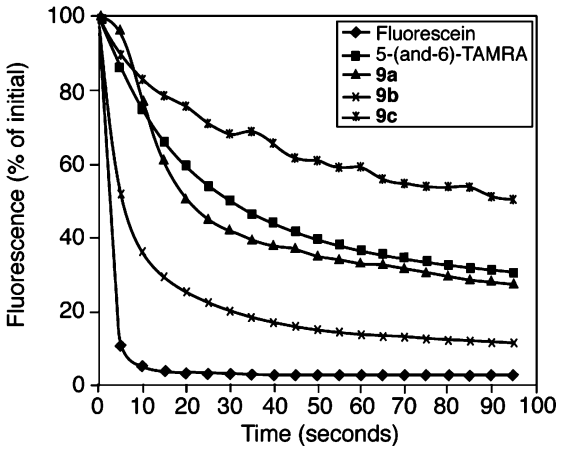


Figure 2. Comparison of photostability of representative dyes.

red-shifted by 34 nm; the fluorescence quantum yield of compound **9a** is also three times higher than that of compound **12**. These differences can be attributed to the dimethylmethylene bridges, which provide a rigidity that reduces rotation of the phenyl groups and nitrogen atoms, thereby preventing the nonradiative process. Some interesting observations were made by the comparison of compounds **9a**,**b** and **9c**. Compounds **9a** and **9b** have almost identical absorption and emission maxima and there is little difference between their fluorescence quantum yields; however, Compound **9c** exhibits much longer absorption and emission maxima. Compounds **9b** and **9c** have thiophenyl substituents at positions 2′ and 7′, whereas compound **9a** has a simple phenyl substituent. Compounds **9b** and **9c** differ only in the position of the sulfur atoms, suggesting that the sulfur atom in compound **9c** also acts as an additional polar substituent incorporated into the π conjugation system.

Compounds **9d** and **9e** have essentially identical photophysical properties, and their absorption and emission maxima are about 30–35 nm longer than those of compound **9c**. This observation supports the hypothesis that the substitution of protons (on the bottom carboxyphenyl moiety) by electron-accepting atoms such as Cl or F can decrease the excitation energy of the rhodamine dyes. As predicted, the additional 'benzo' groups in compounds **9f** and **9g** shift the maxima of absorption and emission to longer wavelengths. Analogous to the effect of sulfonate groups on Lissamine rhodamine B and sulforhodamine 101,[2] the sulfonate groups of the newly reported rhodamines **9h** and **9i** also cause a bathochromic shift in emission wavelength and an increase in fluorescence quantum yields compared with the corresponding carboxylated analog compounds **9c** and **9g**. As expected, some of the new rhodamine dyes display absorption beyond 600 nm with strong fluorescence as shown in Table 1. These dyes also possess high extinction coefficients. For example, the extinction coefficients of compounds **9d** and **9i** were determined to be 141,000 and 143,000 M^{-1} cm^{-1}, respectively, in MeOH.

The photostabilities of representative compounds **9a**,**b** and **9c**, as well as fluorescein and 5-(and-6)-carboxytetramethylrhodamine (5-(and-6)-TAMRA), were tested using glass capillary tubes filled with dyes in MeOH under 100 W mercury arc lamp of the fluorescence microscope and shown in Figure 2. As expected, compounds **9a**,**b** and **9c** have much higher photostabilities than fluorescein, and have similar photostabilities to 5-(and-6)-TAMRA.

In summary, we have reported the rational design and synthesis of novel class of highly fluorescent rhodamine dyes with long-wavelength absorption and emission. These new rhodamine dyes can be readily converted to reactive derivatives and used as fluorescent tags to prepare various bioconjugates for biological applications.[10] Both the methodology used to synthesize these rhodamine dyes and the intermediate aminophenol derivatives involved in that synthesis are significant developments that should open new routes for preparing other fluorescent dyes and heterocyclic compounds.

References

1. Drexhage, K. H. *Top. Appl. Phys.* **1973**, *1*, 144–274.
2. For a sourcebook and comprehensive references, see: Haugland, R. P. *Handbook of Fluorescent Probes and Research Products*, 9th ed.; Molecular Probes, Inc.: Eugene, OR, USA, 2002.
3. Sauer, M.; Han, K.-T.; Muller, R.; Nord, S.; Schulz, A.; Seeger, S.; Wolfrum, J.; Arden-Jacob, J.; Deltau, G.; Marx, N. J.; Zander, C.; Drexhage, K. H. *J. Fluoresc.* **1995**, *5*, 247–261.
4. Herrmann, R.; Josel, H. P.; Drexhage, K. H.; Arden-Jacob, J. US Patent 5,750,409, 1998.
5. Benson, S. C.; Lam, J. Y. L.; Menchen, S. M. US Patent 5,936,087, 1999.
6. Forster, T. *Fluoreszenz Organischer Verbindungen*; Vandenhoeck and Ruprecht: Gottingen, 1951.
7. Badone, D.; Baroni, M.; Cardamone, R.; Ielmini, A.; Guzzi, U. *J. Org. Chem.* **1997**, *62*, 7170–7173.
8. **9a**: blue solid, R_f=0.29 $CHCl_3$/MeOH (v/v 10:1), yield 68%; mp 118–120°C; ^{1}H NMR (400 MHz, [D_4]MeOH): δ=8.24 (d, H; aromatic H), 7.80 (t, 1H; aromatic H), 7.32 (t, 1H; aromatic H), 7.65 (s, 2H; aromatic H), 7.50 (d, 2H; aromatic H), 7.45 (d, 2H; aromatic H), 7.35 (m, 3H; aromatic H), 7.28 (t, 2H; aromatic H), 6.94 (s, 2H; aromatic H), 3.10 (s, 6H; CH_3), 1.60 (s, 6H; CH_3), 1.56 (s, 6H; CH_3); MS (EI): *m*/*z* (%): 591 (100) [M^++H]. **9b**: blue solid, R_f=0.26 $CHCl_3$/MeOH (v/v 10:1), yield 70%; mp 177–180°C; ^{1}H NMR (400 MHz, [D_4]MeOH): δ=8.22 (d, 1H; aromatic H), 7.78 (t, 1H; aromatic H), 7.72 (t, 1H; aromatic H), 7.39 (d, 2H; heteroaromatic H), 7.34 (d, 1H; aromatic H), 7.31 (s, 2H; aromatic H), 7.06 (d, 2H; heteroaromatic H), 6.93 (s, 2H; aromatic H), 3.12 (s, 6H; CH_3), 1.73 (s, 6H; CH_3), 1.69 (s, 3H; CH_3); MS (EI): *m*/*z* (%): 603 (100) [M^++H]. **9c**: blue solid, R_f=0.14/0.34 $CHCl_3$/MeOH (v/v 5:1), yield 67%; mp >300°C; ^{1}H NMR (400 MHz, [D_4]MeOH): δ=8.93 (s, 5-isomer; aromatic H), 8.37 (m, 5- and 6-isomer; aromatic H), 8.32 (d; 6-isomer; aromatic H), 8.00 (s, 6-isomer; aromatic H), 7.39 (m, 2H; heteroaromatic H), 7.08 (m, 2H; heteroaro-

matic H), 6.99 (m, 4H; aromatic H), 3.29, 3.28 (2×s, 6H; CH_3), 1.76, 1.75, 1.74 (3×s, 12H; CH_3); MS (EI): m/z (%) 645 (100) [M^+–H]. **9d**: blue solid, R_f=0.22 $CHCl_3$/MeOH (v/v 10:1), yield 26%; mp 240°C (dec.); ^{1}H NMR (400 MHz, $CDCl_3$) δ=7.10 (d, 2H; heteroaromatic H), 6.85 (d, 2H; heteroaromatic H), 6.65 (s, 2H; aromatic H), 6.40 (s, 2H; aromatic H), 2.97 (s, 6H; CH_3), 1.60 (s, 12H; CH_3); MS (EI): m/z (%) 740 (100) [M^+]. **9e**: blue solid, R_f=0.10 $CHCl_3$/MeOH (v/v 10:1), yield 18%; mp 250°C (dec.); ^{1}H NMR (400 MHz, $CDCl_3$) δ=7.10 (d, 2H; heteroaromatic H), 6.85 (d, 2H; heteroaromatic H), 6.70 (s, 2H; aromatic H), 6.45 (s, 2H; aromatic H), 2.98 (s, 6H; CH_3), 1.60 (s, 12H; CH_3); MS (EI): m/z (%) 675 (100) [M^++H]. **9f**: blue solid, R_f=0.21 $CHCl_3$/MeOH (v/v 10:1), yield 30%; mp 275°C (dec.); ^{1}H NMR (400 MHz, $CDCl_3$) δ=8.14 (d, 2H; aromatic H), 7.72 (d, 2H; aromatic H), 7.65 (d, 2H; aromatic H), 7.52 (m, 2H; aromatic H), 7.40 (m, 4H; aromatic H), 7.01 (s, 2H; aromatic H), 6.41 (s, 2H; aromatic H), 3.02 (s, 6H; CH_3), 2.00 (s, 12H; CH_3); MS (EI): m/z (%) 834 (100) [M^+]. **9g**: blue solid, R_f=0.14/0.41 $CHCl_3$/MeOH (v/v 5:1), yield 61%; mp >300°C; ^{1}H NMR (400 MHz, [D_4]MeOH): δ=8.90 (s, 5-isomer; aromatic H), 8.38 (d, 6-isomer, aromatic H), 8.28 (m, 5- and 6-isomer; aromatic H), 8.09 (s, 6-isomer; aromatic H), 7.93 (d, 5-isomer; aromatic H), 7.89 (d, 2H; aromatic H), 7.78 (d, 2H; aromatic H), 7.34 (m, 4H; aromatic H), 7.07, 7.04 (2×s, 2H; aromatic H), 6.90, 6.85 (2×s, 2H; aromatic H), 3.37, 3.27 (2×s, 6H; CH_3), 2.00, 1.99, 1.96, 1.90 (4×s, 12H; CH_3); MS (EI): m/z (%) 745 (100) [M^+–H]. **9h**: blue solid, R_f=0.09 $CHCl_3$/MeOH (v/v 5:1), yield 85%; mp >300°C; ^{1}H NMR (400 MHz, [D_6]DMSO): δ=8.34 (s, 1H; aromatic H), 7.79 (d, 1H; aromatic H), 7.52 (d, 2H; heteroaromatic H), 7.24 (m, 3H; aromatic and heteroaromatic H), 6.94 (s, 2H; aromatic H), 6.82 (s, 2H; aromatic H), 3.25 (s, 6H; CH_3), 1.72 (s, 6H; CH_3), 1.70 (s, 6H; CH_3); MS (EI): m/z (%): 719 (100) [M^++H]. **9i**: purple–blue solid, R_f=0.21 $CHCl_3$/MeOH (v/v 5:1), yield 88%; mp >300°C; ^{1}H NMR (400 MHz, [D_6]DMSO): δ=8.37 (s, 1H; aromatic H), 8.03 (m, 4H; aromatic H), 7.83 (d, 1H; aromatic H), 7.38 (m, 4H; aromatic H), 7.28 (d, 1H; aromatic H), 6.94 (s, 2H; aromatic H), 6.89 (s, 2H; aromatic), 3.27 (s, 6H; CH_3), 2.00 (s, 6H; CH_3), 1.98 (s, 6H; CH_3); MS (EI): m/z (%): 818 (100) [M^+].

9. Arden, J.; Deltau, G.; Huth, V.; Kringel, U.; Peros, D.; Drexhage, K. H. *J. Luminesc.* **1991**, *48* and *49*, 352–358.
10. For bioconjugates and applications, see: Diwu, Z.; Liu, J.; Haugland, R. P.; Gee, K. R. PCT Int. Appl. WO 02 12,195, 2002.