Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 13, pp. 1479-1482, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00263-6

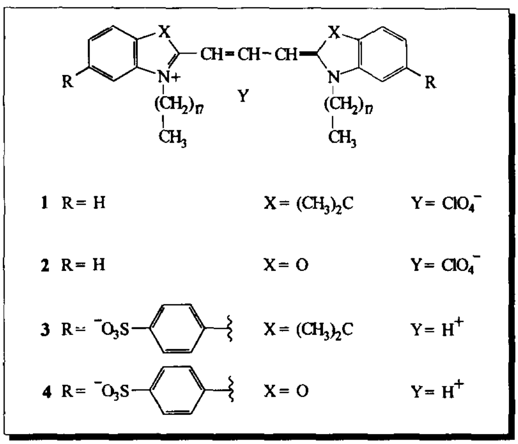
LIPOPHILIC SULFOPHENYLCARBOCYANINE DYES: SYNTHESIS OF A NEW CLASS OF FLUORESCENT CELL MEMBRANE PROBES

Wai-Yee Leung, Fei Mao,* Richard P. Haugland, and Dieter H. Klaubert

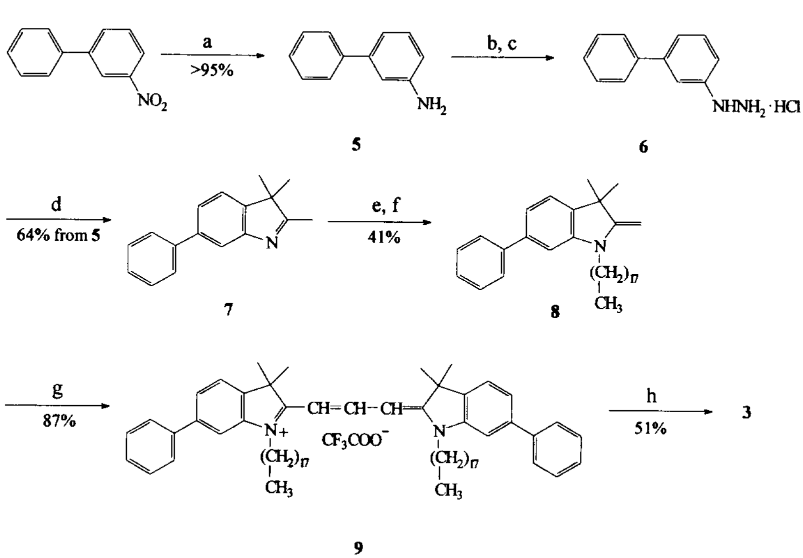
Molecular Probes, Inc.
4849 Pitchford Ave, Eugene, OR 97402

Abstract: Two lipophilic sulfophenylcarbocyanine dyes, **3** and **4**, were synthesized as a new class of fluorescent cell membrane probes. Compared with other commonly used membrane probes, the new dyes have improved fluorescent quantum yield, retention in cell membranes and are easier to use in staining cells.

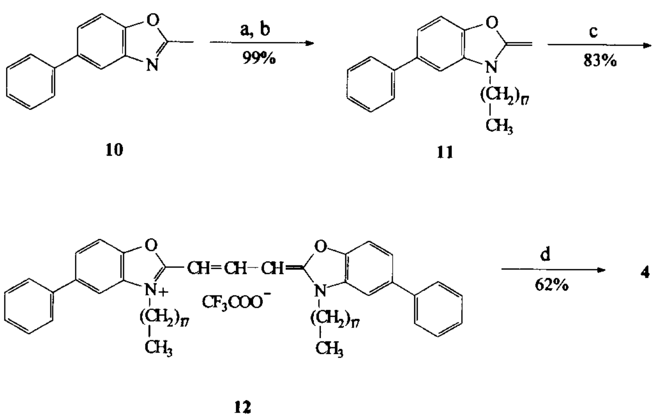
Lipophilic carbocyanine dyes such as $DiIC_{18}(3)$ **1**[1,2] and $DiOC_{18}(3)$ **2**[1,2] have been used extensively as fluorescent cell membrane probes, particularly in neuronal tracing studies.[3] The excellent retention in cell membranes and relatively low toxicity of these dyes make them useful for long term cell tracing studies. However, because of their low solubility in aqueous media, the dyes are difficult to use for labeling cells in suspension. In addition, cells stained with **1** or **2** lose the dye when extracted with acetone, a common procedure in histochemistry to permeabilize cell membranes. Thus, there is a need for fluorescent membrane dyes that have both improved water solubility for easy labeling of cells as well as good retention in cell membranes.



In this paper, we report the syntheses of lipophilic carbocyanine dyes that contain negatively charged sulfophenyl groups on the aromatic nucleus of the fluorophore. These sulfonated dyes, 6,6′-DSP $DiIC_{18}(3)$ **3**[1,2] and 5,5′-DSP $DiOC_{18}(3)$ **4**,[1,2] can be easily loaded into cells without the use of osmolarity-regulating agents, which are required for other carbocyanine dyes.[4] Furthermore, these new dyes are about five times more fluorescent than **1** and **2** in cell membranes.



Scheme 1: (a) 10% Pd/C, H_2; (b) $NaNO_2$, conc HCl, 0 °C; (c) $SnCl_2$; (d) 3-methylbutan-2-one, AcOH, reflux; (e) octadecyl *p*-chlorobenzenesulfonate, neat, 120 °C; (f) saturated $NaHCO_3$; (g) triethyl orthoformate, pyridine, trifluoroacetic acid, reflux; (h) 20% fuming H_2SO_4, 0 °C.



Scheme 2: (a) octadecyl triflate, rt; (b) saturated $NaHCO_3$; (c) triethyl orthoformate, pyridine, trifluoroacetic acid, reflux; (d) 20% fuming H_2SO_4, 0 °C.

The synthesis of **3** is outlined in Scheme 1. Hydrogenation of 3-nitrobiphenyl gave 3-aminobiphenyl **5** in almost quantitative yield. Diazotization of **5** followed by treatment with stannous chloride yielded the biphenylhydrazine **6**, which, without purification, was reacted with 3-methyl-2-butanone in refluxing acetic acid to give a mixture of 6-phenyl-2,3,3-trimethylindolenine **7** and 4-phenyl-2,3,3-trimethylindolenine ($<10\%$), separable by column chromatography. Quarternization of **7** with octadecyl *p*-chlorobenzenesulfonate[5] at 120 °C and then neutralization afforded the free base **8**. Treatment of **8** with triethyl orthoformate (4 equiv) and trifluoroacetic acid (1 equiv) in refluxing pyridine resulted in the formation of carbocyanine dye **9**,[6] which was sulfonated with 20% fuming H_2SO_4 to yield **3**.[6]

The synthesis of **4** started with the commercially available 2-methyl-5-phenylbenzoxazole **10** [Scheme 2]. Unlike **7**, the quarternization of **10** at room temperature required the more reactive octadecyl triflate, generated in situ from 1-octadecanol and triflic anhydride. Subsequent neutralization with $NaHCO_3$ gave **11**, which was converted to the oxacarbocyanine dye **12**.[6] Sulfonation of **12** with 20% fuming H_2SO_4 at 0 °C afforded **4**.[6]

In addition to some bathochromic shift of their absorption and emission wavelengths, both **3** and **4** have higher extinction coefficients than their counterpart dyes **1** and **2** [Table 1]. Moreover, the quantum yields (Φ) of **3** and **4** in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes, an environment that mimics the lipophilic cell membrane, are about five times higher than those of **1** and **2**, respectively. The negatively charged sulfonate groups may contribute to the large fluorescence enhancement by preventing dye aggregation, which is a common and major cause of fluorescence quenching.[7]

Table 1. Spectral Data of the Carbocyanine Dyes

Dye	$\lambda_{abs}{}^{a}$ (nm)	$\lambda_{em}{}^{a}$ (nm)	ϵ (L/mol/cm)	Relative Φ^{b}
1	550	565	148,000	Φ_1
3	557	573	164,000	4.8 Φ_1
2	484	501	154,000	Φ_2
4	497	513	175,000	5.5 Φ_2

[a] Measured in methanol. [b] In DOPC liposomes.

Preliminary studies showed that loading of the dyes **3** and **4** into cell membranes can be carried out directly from their corresponding buffer solutions without the addition of any osmolarity regulating agents. Furthermore, the dyes remain in the membrane following aldehyde fixation and lipid extraction with acetone. The detailed results of this study will be reported elsewhere.

Acknowledgment: We thank Hans Engel for purification of the products and Diane Ryan for the UV and fluorescence data.

References and Notes

1. The full names for **1** to **4** are: **1**, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; **2**, 3,3′-dioctadecyloxacarbocyanine perchlorate; **3**, 1,1′-dioctadecyl-6,6′-di(4-sulfophenyl)-3,3,3′,3′-tetramethylindocarbocyanine and **4**, 3,3′-dioctadecyl-5,5′-di(4-sulfophenyl)oxacarbocyanine.

2. For the generic acronyms $DiIC_{18}(3)$ and $DiOC_{18}(3)$, the subscript designates the number of carbon atoms in each alkyl tail and the bracketed number refers to the number of carbon atoms in the bridge between the indole or benzoxazole ring systems.

3. (a) Wolf, D. E. *Spectroscopic Membrane Probes*; Loew, L. M., Ed.; CRC: 1988; Vol. 1, p 193; (b) Tsien, R.; Waggoner, A. S. *Handbook of Biological Confocal Microscopy*; Pawley, J. B., Ed.; Plenum: 1990; p 169; (c) Waggoner, A. S. *Flow Cytometry and Sorting*, 2nd Edition; Melamed, M. R., Ed.; Wiley-Liss: 1990; p 209; (d) Peters, R. *Naturwissenschaften* **1983**, *70*, 294; (e) Fritzsch, B.; Wilm, C. *Trends in Neuroscience* **1990**, *13*, 14; (f) Rashid, F.; Horobin, R. W.; Williams, M. A. *J. Histochem.* **1991**, *23*, 450; (g) Spikes, J. P. *Photochem. Photobiol.* **1991**, *54*, 1079; (h) Honig, M. G.; Hume, R. I. *Trends in Neuroscience* **1989**, *9*, 333.

4. (a) Horan, P. K.; Slezak, S. E. US Patent 4 762 701, 1988; (b) Horan, P. K.; Jensen, B. D.; Slezak, S. E. US Patent 4 783 401, 1988; (c) Horan, P. K.; Jensen, B. D.; Slezak, S. E. US Patent 4 859 584, 1989.

5. Sondermann, J. *Liebigs Ann. Chem.* **1971**, *183*, 749.

6. **3**: TLC (SiO_2) CH_3OH:$CHCl_3$=3:7, R_f=0.24; HPLC (Microsorb-MV Cyano reverse phase column 4.1 x 150 cm, 0.1 M NH_4OAc:CH_3CN=60:40 to 0:100): 99%; mp: 226-228 °C (dec); ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD=1:1): δ 8.45 (t, J=12 Hz, 1H), 7.97 (d, J=8 Hz, 4H), 7.61 (d, J=8 Hz, 4H), 7.49 (s, 4H), 7.34 (s, 2H), 6.22 (d, J=12 Hz, 2H), 4.12 (m, 4H), 1.92-1.78 (m, 16H), 1.50-1.14 (m, 60H), 0.83 (t, J=7 Hz, 6H); HRMS (FAB) mass calcd for $C_{71}H_{105}N_2O_6S_2$ (M^++H) 1145.7419, found 1145.7400.
4: TLC (SiO_2) CH_3OH:$CHCl_3$=2:8, R_f=0.25; HPLC (Microsorb-MV Cyano reverse phase column 4.1 x 150 cm, 0.1 M NH_4OAc:CH_3CN=60:40 to 0:100): 99%; mp: >300 °C; ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD=1:1): δ 8.50 (t, J=15 Hz, 1H), 7.95 (d, J=10 Hz, 4H), 7.60-7.40 (m, 10H), 6.00 (d, J=15 Hz, 2H), 4.15 (m, 4H), 1.87 (m, 4H), 1.45-1.15 (m, 60H), 0.80 (t, J=7Hz, 6H); HRMS (FAB) mass calcd for $C_{65}H_{93}N_2O_8S_2$ (M^++H) 1093.6377, found 1093.6400.
9: TLC (SiO_2) CH_3OH:$CHCl_3$:EtOAc=2:5:5, R_f=0.24; mp: 80-82 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.45 (t, J=12 Hz, 1H), 7.62-7.25 (m, 18H), 4.32 (m, 4H), 2.00-1.70 (m, 16H), 1.60-1.20 (m, 60H), 0.89 (t, J=7 Hz, 6H); HRMS (FAB) mass calcd for $C_{71}H_{106}N_2$ (M^+-CF_3CO_2+H) 986.8361, found 986.8360.
12: TLC (SiO_2) CH_3OH:$CHCl_3$:EtOAc = 1:4.5:4.5, R_f=0.3; mp: 120-122 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.45 (t, J=15 Hz, 1H), 7.70-7.30 (m, 16H), 6.75 (d, J=15 Hz, 2H), 4.35 (m, 4H), 2.00-1.00 (m, 64H), 0.90 (t, J=7 Hz, 6H); HRMS (FAB) mass calcd for $C_{65}H_{94}N_2O_2$ (M^+-CF_3CO_2+H) 934.7320, found 934.7320.

7. (a) West, W.; Pearce, S. *J. Phys. Chem.* **1965**, *69*, 1894.; (b) Hamer, F. *Cyanine Dyes and Related Compounds*; Interscience: 1964; p 86.

(*Received in USA* 29 *April* 1996; *accepted* 28 *May* 1996)