Preparation of BODIPY probes for multicolor fluorescence imaging studies of membrane dynamics

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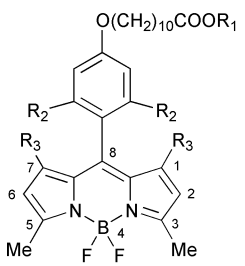
Three different colored fluorescent fatty acids containing BODIPY with extremely high fluorescence quantum yields have been synthesized as probes for investigating the dynamics of membranes. Colored vesicles containing each probe, which were located in the interior of the bilayer membranes, were distinguished from each other by fluorescence microscopy.

Analysis of the dynamics of membranes is important for understanding biological functions in living cells.[1] Fluorescence microscopic analysis is of use for this purpose because the dynamic behavior of membranes can be visualized directly.[2] Nowadays hundreds of fluorescence probes are commercially available and they have been used in various biological experiments.[3] Among fluorophores, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) is lipophilic and exhibits high fluorescence efficiency and excellent photostability.[4] The advantage of BODIPY derivatives is that their absorption and emission maxima can be shifted to longer wavelengths by chemical modification of the π-conjugating system.[5] Accordingly, BODIPY derivatives may be suitable for investigating mixed membranes composed of different membrane components, which are required to be distinguished from each other simultaneously. In this communication, we report the synthesis of three spectroscopically differentiable fluorescent probes **2c**, **4b** and **6b** from readily available chemicals. The problem with BODIPY derivatives carrying aryl groups at the 3- and 5-positions of the *s*-indacene skeleton is the low quantum yield of fluorescence.[5] As for alkyl homologs, the fluorescence quantum yield can be enhanced by introducing methyl groups at the C(1) and C(7) positions as R_3.[5,6] However, introduction of methyl groups at these positions of diaryl derivatives is not without its synthetic difficulties. In this work we report the preparation of highly fluorescent diaryl derivatives of BODIPY carrying methyl groups at the *ortho* positions (R_2) of the phenyl group substituted at C(8) of the *s*-indacene skeleton.

4-Hydroxybenzaldehyde and 2,6-dimethyl-4-hydroxybenzaldehyde, the latter obtained from 3,5-dimethylphenol using the Reimer–Tiemann reaction,[7] were reacted with ethyl 11-bromoundecanoate to afford a fatty acid ester. The formyl group of the product can be converted to the pigment by the following treatment.[8] Condensation of the benzaldehyde derivatives with the corresponding substituted pyrroles, which were synthesized in the same manner as in previous papers,[9,10] followed by an oxidation procedure gave dipyrromethane intermediates, which were treated with $BF_3 \cdot Et_2O$ to produced fluorescent esters **1**, **3** and **5**.[5] Finally, a conventional alkaline hydrolysis gave the desired fatty acid probes **2**, **4** and **6** (Scheme 1).

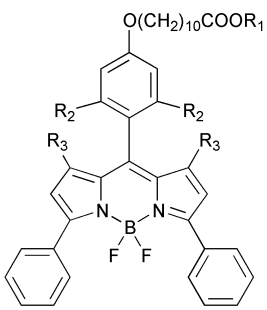
The fluorescence of the probes turned out to be diminished in non-polar solvents. This might be because **2**, **4** and **6** are thought to form a hydrogen-bonded dimer in these solvents and their fluorescence is self-quenched. This interpretation is supported by the fact that the fluorescence intensity was partly restored when a small amount of acetic acid was added to the solution. In order to estimate their photo-physical properties, the absorption and emission spectra of ethyl esters **1**, **3** and **5** were measured in dichloromethane (Fig. 1). The absorption spectra of **1a**–**d**, **3a**,**b** and **5a**,**b** show absorption maxima (λ_{abs}) in the ranges 500–511, 548–553 and 572–578 nm, respectively. These maximum positions are mainly determined by the substituent at the C(3) and C(5) positions, although they vary slightly depending on the nature (Me or H) of the R_2 and/or R_3 substituents. The fluorescence spectra



1 (R_1 = Et), **2** (R_1 = H)

1a: R_2 = H, R_3 = H
1b: R_2 = Me, R_3 = H
1c: R_2 = H, R_3 = Me
1d: R_2 = Me, R_3 = Me

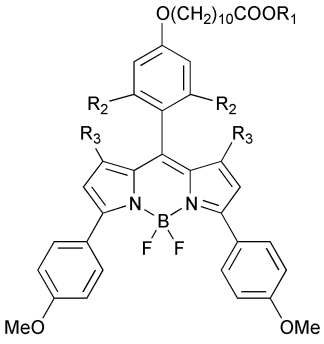
2c: R_2 = H, R_3 = Me



3 (R_1 = Et), **4** (R_1 = H)

3a: R_2 = H, R_3 = H
3b: R_2 = Me, R_3 = H

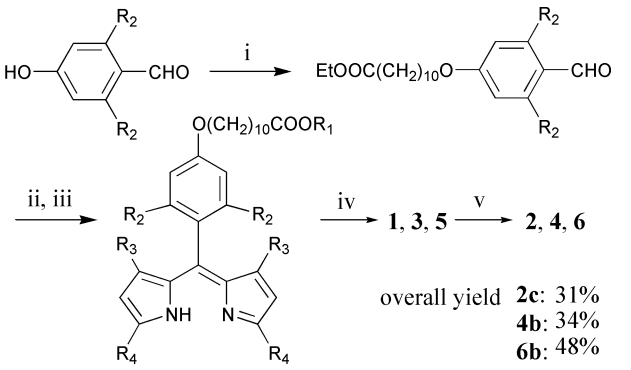
4b: R_2 = Me, R_3 = H



5 (R_1 = Et), **6** (R_1 = H)

5a: R_2 = H, R_3 = H
5b: R_2 = Me, R_3 = H

6b: R_2 = Me, R_3 = H



Scheme 1 (i) Ethyl 11-bromoundecanoate, K_2CO_3, acetone; (ii) 2-substituted pyrrole, CF_3COOH, CH_2Cl_2; (iii) DDQ, CH_2Cl_2; (iv) $BF_3 \cdot Et_2O$, Et_3N, toluene, reflux; (v) KOH, ethanol, reflux.

of **1a–d**, **3a**, **b** and **5a**,**b** were found to show emission maxima (λ_{Em}) in the ranges 512–524, 583–587 and 616–620 nm, respectively, suggesting that their fluorescent colors are determined by the substituent at the C(3) and C(5) positions as well: **1a–d** are green, **3a**,**b** are orange and **5a**,**b** are wine-red. These spectral features facilitate signal identification and analysis when these probes are buried in membranes.

Fluorescence quantum yields for **1**, **3** and **5** were measured using *N*,*N*′-bis(1-hexylheptyl)-3,4:9,10-perylenebis(carboximide) as a reference compound.[11] The quantum yields of the fluorophores having methyl groups at the ortho positions of the phenyl group (R_2) and/or at the C(1) and C(7) positions (R_3), are two or three times larger than those of the derivatives without methyl groups at these positions (Table 1). This trend indicates that the methyl groups suppress non-radiative deactivation by restricting internal rotation of the phenyl ring at the C(8) position. As for the synthesis of **1d**, the synthetic yield of the corresponding dipyrromethane is low due to the steric hindrance induced by the methyl groups at R_2 and R_3. On the other hand, 2-methylpyrrole, which is the starting material for **1b**, is not commercially available. Considering the above situation, **1c** is the most suitable compound for a large scale synthesis.

When fluorescent images of vesicles composed of oleic acid containing several mol% of fatty acid probes **2c**, **4b** and **6b** were recorded, only membranes were found to glisten strongly. Since the pigments are highly lipophilic, they are considered to be buried diffusely in the interior of the bilayer membrane, no appreciable self-quenching being observed.[12] Furthermore, the colored vesicles containing each fluorescent probe turned out to be clearly distinguishable from each other, even when using an identical optical filter (Fig. 2). Accordingly, this technique enables the video recording of the dynamic behavior of membranes.

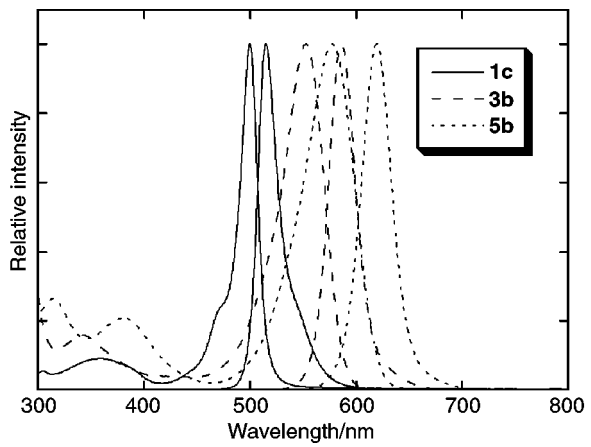


Fig. 1 Normalized absorption and fluorescence spectra of (——) **1c**, (— —) **3b** and (– – –) **5b** in CH_2Cl_2.

Table 1 Photophysical properties of BODIPY compounds in CH_2Cl_2

Compound	λ_{abs}/nm	λ_{Em} [a]/nm	Stokes shift/nm	Φ_f [b]
1a	508	524	16	0.36[c]
1b	511	524	13	0.88
1c	500	516	16	0.71[c]
1d	501	512	13	0.94
3a	548	583	35	0.21[c]
3b	553	587	34	0.68
5a	572	616	44	0.30[c]
5b	578	620	42	0.56

[a] Excited at λ_{abs}. [b] *N*,*N*′-Bis(1-hexylheptyl)-3,4 : 9,10-perylenebis(dicarboximide) was used as a reference ($\Phi_f = 1.00$). [c] Similar fluorophores in which a *p*-iodophenyl group had been introduced at C(8) of the BODIPY derivatives exhibited lower fluorescence quantum yields compared with the current BODIPY derivatives (**1a**, **1c**, **3a** and **5a**). The suppression of the quantum yields may be ascribed to acceleration of intersystem crossing from S_1 to T_1 by the heavy atom effect.

In summary, we have revealed that introduction of methyl groups at the *ortho* positions of aryl-substituted BODIPYs is an efficient way to increase the fluorescence quantum yield.[13] Three fatty acid probes tagged with an individual fluorophore were buried within oleic acid vesicles and these vesicles were identified by multicolor fluorescence microscopy at the same time. Moreover, these fluorescent probes, having a carboxyl group at the terminus, can be attached easily to lysophospholipids. Investigations on the dynamics of mixed membranes composed of multi-amphiphiles and multi-probes are in progress in our laboratories.

Experimental

Syntheses

2,6-Dimethyl-4-hydroxybenzaldehyde. 3,5-Dimethylphenol (92.97 g, 0.76 mol) and KOH (78.00 g, 1.39 mol) were dissolved in water (300 ml). Distilled $CHCl_3$ (121 ml, 0.68 mol) was added over 10 h, keeping the temperature at 60 °C, and the solution was stirred at 60 °C for 8 h. The solution was cooled and then poured into dilute H_2SO_4. The yellow residue was washed with $CHCl_3$ to yield 10.09 g (10%) of grayish white solid. ^{1}H NMR (270 MHz, DMSO-d_6): δ 10.29 (1H, s), 10.28 (1H, br s), 6.50 (2H, s), 3.41 (6H, s). ^{13}C NMR (67.5 MHz, DMSO-d_6): δ 191.1, 161.2, 144.0, 124.1, 116.1, 20.6.

General procedure for the synthesis of 1, 3 and 5. A mixture of 4-hydroxybenzaldehyde derivative (with or without methyl groups at the 2 and 6-positions) and ethyl 11-undecanoate was refluxed in dry acetone overnight in the presence of potassium carbonate. The crude mixture was purified by silica gel column chromatography using $CHCl_3$ to afford an ester-tagged benzaldehyde (56–83%). The obtained benzaldehyde (1.0 mmol) and the corresponding substituted pyrrole (2.0 mmol) were dissolved in CH_2Cl_2 (25 ml) and the solution was degassed by bubbling nitrogen through it (30 min). One drop of CF_3COOH was added and the solution was stirred over-

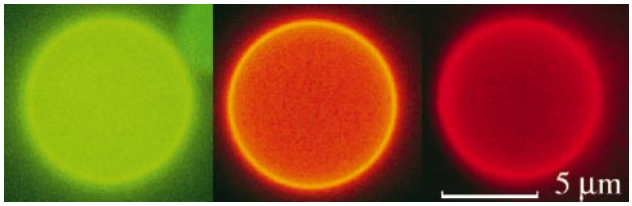


Fig. 2 Fluorescent microscopic images of oleic acid vesicles containing **2c** (left), **4b** (center) and **6b** (right).

night at room temperature under a nitrogen atmosphere. 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (0.25 g, 1.1 mmol) was added, and the mixture was stirred for another 4 h. The reaction mixture was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated.

The crude product was purified by alumina column chromatography using hexane–CH_2Cl_2 to afford the dipyrromethane derivative as a colored oil. The product (0.26–0.41 g, 0.44–0.60 mmol) and $BF_3 \cdot Et_2O$ (0.72 ml, 3.8 mmol) were dissolved with Et_3N (0.50 ml, 6.8 mmol) in toluene (10 ml) and the solution was refluxed for 1 h. The reaction mixture was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated. The fluorescent oil was purified by silica gel column chromatography using hexane–CH_2Cl_2 to afford the desired compound.

1c. Yield 56%. IR (KBr): ν/cm^{-1} 1738 (C=O). ^1H NMR (270 MHz, $CDCl_3$): δ 7.14 (2H, d, $J = 7$), 6.98 (2H, d, $J = 7$), 5.97 (2H, s), 4.12 (2H, quar, $J = 7$), 4.00 (2H, t, $J = 7$), 2.55 (6H, s), 2.29 (2H, t, $J = 7$), 1.82 (2H, quin, $J = 7$), 1.62 (2H, m), 1.49 (2H, m), 1.44 (6H, s), 1.4–1.2 (10H, m), 1.26 (3H, t, $J = 7$ Hz).

3b. Yield 44%. IR (KBr): ν/cm^{-1} 1732 (C=O). ^1H NMR (270 MHz, $CDCl_3$): δ 7.88 (4H, m), 7.46–7.36 (6H, m), 6.70 (2H, s), 6.66 (2H, d, $J = 4$), 6.55 (2H, d, $J = 4$), 4.13 (2H, quar, $J = 7$), 4.01 (2H, t, $J = 7$), 2.30 (2H, t, $J = 7$), 2.21 (6H, s), 1.85 (2H, quin, $J = 7$), 1.63 (2H, m), 1.51 (2H, m), 1.4–1.2 (10H, m), 1.26 (3H, t, $J = 7$ Hz).

5b. Yield 59%. IR (KBr): ν/cm^{-1} 1735 (C=O). ^1H NMR (270 MHz, $CDCl_3$): δ 7.89 (4H, d, $J = 9$), 6.95 (4H, d, $J = 9$), 6.69 (2H, s), 6.60 (2H, d, $J = 4$), 6.53 (2H, d, $J = 4$), 4.12 (2H, quar, $J = 7$), 4.00 (2H, t, $J = 7$), 3.83 (6H, s), 2.30 (2H, t, $J = 7$), 2.20 (6H, s), 1.85 (2H, quin, $J = 7$), 1.63 (2H, m), 1.51 (2H, m), 1.4–1.2 (10H, m), 1.26 (3H, t, $J = 7$ Hz).

General procedure for the synthesis of 2, 4 and 6. The esters **1**, **3** or **5** (0.34 mmol) and KOH (0.80 g, 14 mmol) were dissolved in EtOH (10 ml) and the solution was refluxed for 1 h. After cooling, the mixture was neutralized with dilute HCl, extracted with $CHCl_3$ and dried over Na_2SO_4. The fatty acid probes were used without further purification.

2c. Yield 100%. IR (KBr): ν/cm^{-1} 1708 (C=O). ^1H NMR (270 MHz, THF-d_8–D_2SO_4 19:1): δ 7.17 (2H, d, $J = 7$), 7.06 (2H, d, $J = 7$), 6.00 (2H, s), 4.02 (2H, t, $J = 7$), 2.52 (6H, s), 2.21 (2H, t, $J = 7$ Hz), 1.9–1.3 (22H, m).

4b. Yield 92%. IR (KBr): ν/cm^{-1} 1708 (C=O). ^1H NMR (270 MHz, pyridine-d_5): δ 8.14 (4H, m), 7.53–7.39 (6H, m), 7.03 (2H, d, $J = 4$), 6.98 (2H, s), 6.70 (2H, d, $J = 4$), 4.09 (2H, t, $J = 7$), 2.53 (2H, t, $J = 7$ Hz), 2.28 (6H, s), 1.9–1.7 (4H, m), 1.5–1.2 (12H, m).

6b. Yield 97%. IR (KBr): ν/cm^{-1} 1705 (C=O). ^1H NMR (270 MHz, pyridine-d_5): δ 8.14 (4H, d, $J = 9$), 7.19 (4H, d, $J = 9$), 7.03 (2H, d, $J = 4$), 6.98 (2H, s), 6.69 (2H, d, $J = 4$), 4.09 (2H, t, $J = 7$), 3.70 (6H, s), 2.53 (2H, t, $J = 7$), 2.20 (6H, s), 1.9–1.7 (4H, m), 1.5–1.2 (12H, m).

Recording of images

The fluorescent images were recorded using an Olympus Power BX51 microscope, equipped with a halogen lamp, an Olympus WIB filter set (ex = 460–490 nm; em = >515 nm), and a CCD camera connected to an image recording and processing system.

Acknowledgements

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