Application of Chiral Pyrromethene–BF_2 Complex Dye as a Fluorescent Labeling Reagent

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A chiral pyrromethene–BF_2 complex dye, showing absorption (λ_{max}) and emission maxima (λ_{em}) at 499 and 518 nm in acetonitrile, respectively, was synthesized. Racemic mexiletine smoothly reacted with this reagent in the presence of EDC to preferentially produce the corresponding diastereomeric amides, which were nicely separated by a non-chiral reverse phase column. The detection limit ($S/N > 3$) of mexiletine was ca. 0.3 pmol.

Since not all chiral compounds can be directly separated by chiral HPLC, it is a good method to separate fluorescently labeled diastereomers derived from chiral compounds by non-chiral HPLC. It is important for fluorescent labeling reagents to show intense and bathochromic excitation and emission maxima.[1,2] In our continuing study on the synthesis and properties of chiral fluorescent labeling reagents,[3–7] a bathochromic and intensely fluorescent reagent derived from rhodamine B has been reported to act as a fluorescent labeling reagent for racemic frulbiprofen.[8] However, the use of acetic acid was essential to analyze the products by this reagent, resulting in difficulty to find optimized analysis conditions in the chromatography, due to the strong polarity of acetic acid. Meanwhile, pyrromethene–BF_2 complex dyes are intensely fluorescent compounds and do not require a polar medium to show fluorescence. The potential applications of non-chiral pyrromethene–BF_2 complex dyes as labeling reagents,[9–11] laser dyes,[12] sensors,[13] and EL emitters[14] have been reported. We report herein on the application of a new chiral pyrromethene–BF_2 complex dye as a fluorescent labeling reagent. Racemic mixiletine was used as a model compound of a chiral amine.

Results and Discussion

A chiral pyrromethene–BF_2 complex dye **4** having a carboxylic group was synthesized, as shown in Scheme 1. (*R*)-(+)-3-Methylglutarate reacted with oxalyl chloride to afford the acid chloride **2**, which was used without further purification. 2,4-Dimethylpyrrole (**1**) reacted with **2** to give **3**, followed by deprotection to provide **4**. The structure of previously reported fluorescent labeling reagent **7**, derived from rhodamine B, is also shown in Scheme 1.

The UV–vis absorption and fluorescence spectra of **4** and **7** are shown in Fig. 1. The λ_{max} and λ_{em} of **4** were observed at 499 and 518 nm in acetonitrile, respectively. The molar absorption coefficient (ε) of **4** was calculated to be 72400 $dm^3\,mol^{-1}\,cm^{-1}$. Meanwhile, the λ_{max} and λ_{em} of **7** were observed at 557 and 580 nm, respectively, being more bathochromic than **4**. However, compound **4** showed more intense fluorescence than **7**.

Since the HPLC analysis of amino acids is usually performed using a reverse phase column, the effect of water on the fluorescence intensity of **4** was examined. The result is shown in Fig. 2. The fluorescence intensity of **4** was scarcely affected by the addition of water, suggesting that **4** can act as a good fluorescent labeling reagent. The solubility of **4** into an aqueous acetonitrile was sufficiently high as a labeling reagent.

The reaction of mexiletine (**5**), which acts as *anti*-arrthythmics, with **4** to form the amide **6**, as shown in Scheme 1, was examined. The results are indicated in Table 1. The reaction did not proceed in the absence of an additive. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) showed a better performance than dicyclohexylcarbodiimide (DCC) to give the product (runs 1–4). The reaction proceeded faster at 60 °C than at 25 °C (runs 1–4). The reaction was completed in the presence of EDC at 60 °C in 3 h (runs 4–7).

The HPLC analyses of (*R*,*S*)- and (*R*,*R*)-**6**, obtained by the reaction of (*S*)-**5** and (*R*)-**5** with **4**, are shown in Figs. 3a and 3b, respectively. The retention time of (*R*,*S*)-**6** and (*R*,*R*)-**6** were observed at 22.7 and 25.2 min, respectively. No epimerization was observed during the reaction, preferentially producing the corresponding diastereomers **6**.

Racemic **5** reacted with **4** to give the corresponding diastereomers **6**, whose HPLC analysis is shown in Fig. 3c. The diastereomers (*R*,*S*)-**6** and (*R*,*R*)-**6** were observed at retention times of 22.8 and 25.2 min, respectively. The integration ratio of (*R*,*S*)-**6** to (*R*,*R*)-**6** was calculated to be 49 to 51. The detection limit ($S/N > 3$) of mexiletine was ca. 0.3 pmol.

In our previous work, racemic flurbiprofen (**8**) was analyzed by forming diastereomers with rohdamine B derivative **7** (column, Mightysil RP18 GP (4.6 mm × 150 mm); mobile phase, acetonitrile–tetrahydrofuran–0.5% aqueous acetic acid (28:5:67); flow rate, 0.8 $cm^3\,min^{-1}$; excitation, 562 nm; detection, 582 nm).[8] However, a survey of mobile phase was very difficult, because the addition of a polar acid, which considerably affects the resolution of the diastereomers, is essential to convert the non-fluorescent derivatives to fluorescent ones. Finally, the acetic acid-containing mobile phase could separate

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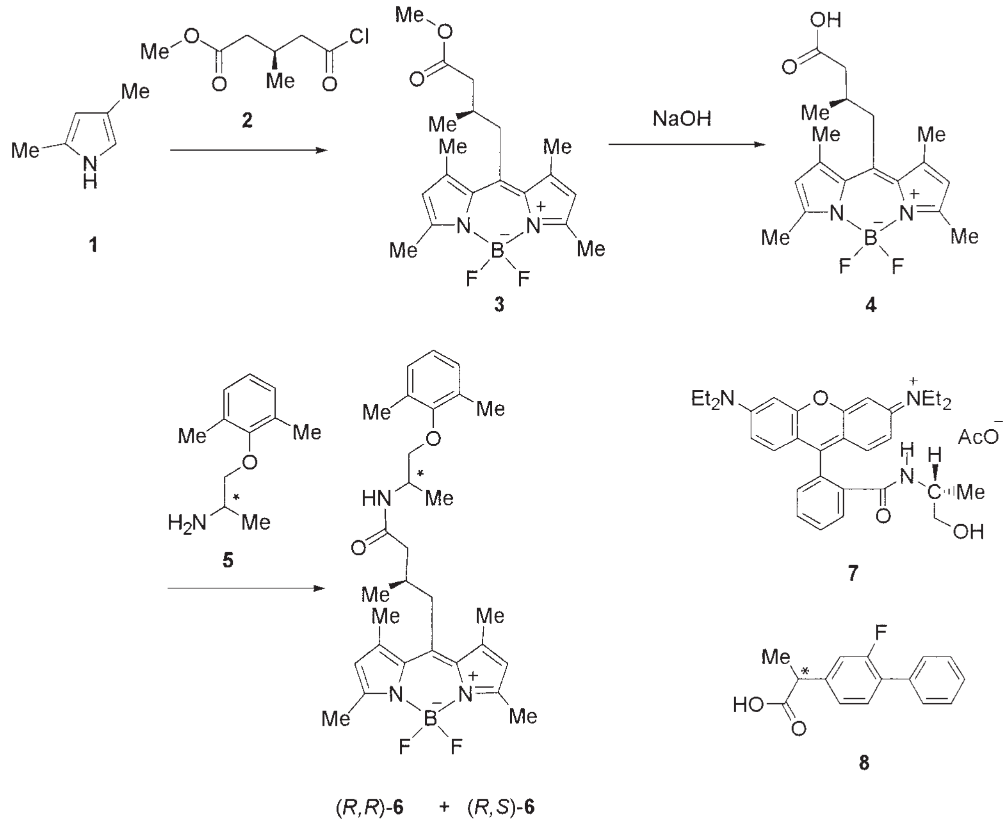

Scheme 1.

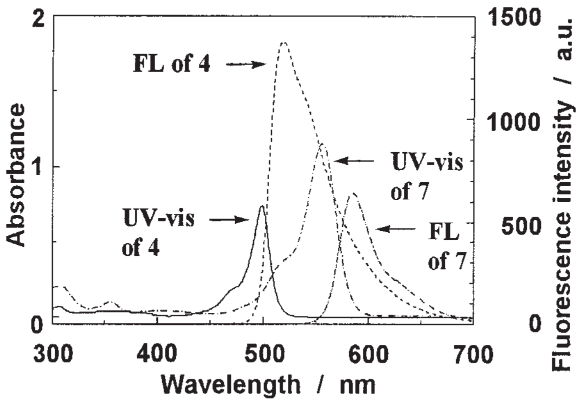

Fig. 1. UV–vis absorption and fluorescence spectra of **4** and **7**. The UV–vis absorption and fluorescence spectra of **4** were measured in acetonitrile at 1×10^{-5} mol dm^{-3} of substrate at 25 °C. The UV–vis absorption and fluorescence spectra of **7** were measured in an acetonitrile–acetic acid mixed solution (9:1) at 1.0×10^{-5} mol dm^{-3} of substrate at 25 °C.

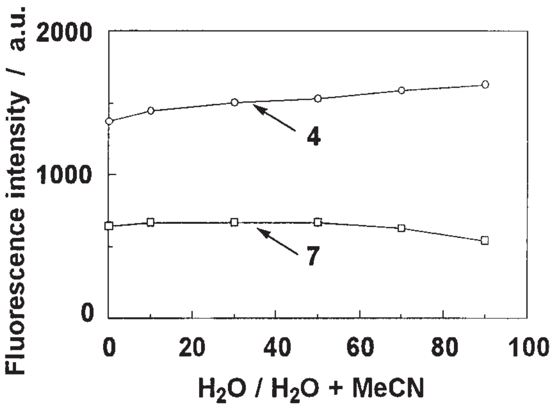

Fig. 2. Effect of water on fluorescence intensity. Measured at 1×10^{-5} mol dm^{-3} of substrate at 25 °C. In the case of **7**, 10% acetic acid is contained in acetonitrile.

diastereomers with broad peaks at a retention time of ca. 140 min. Meanwhile, the mobile phase in this work was very simple, an acetonitrile–water mixed (60:40) solvent; the retention time was short, being ca. 25 min. Furthermore, since the shape of the peaks in this work was sharp and the fluorescence of **4** was more intense than that of **7**, the detection limit was ca. 10-times higher than the previous result. Thus, the pyrromethene–BF$_2$ complex dye **4** could show a better performance than the rohdamine B derivative **7** as a chiral fluorescent labeling reagent.

Conclusion

A novel chiral fluorescent labeling pyrromethene–BF$_2$ complex dye, whose $\lambda_{\max}$ and λ_{em} were observed at 499 and 518 nm in acetonitrile respectively, was synthesized. Racemic mexiletine smoothly reacted with this reagent in the presence of EDC at 60 °C to produce the corresponding diastereomeric amides without epimerization. The labeled diastereomers were nicely separated by a non-chiral reverse-phase column. The detection limit (S/N > 3) of mexiletine was ca. 0.3 pmol. This

Table 1. Reaction of Mexiletine **5** with Pyrromethene–BF$_2$ Complex Dye **4**

Run	Reaction conditions			Conversion of **5**	Relative yield
	Additive	Temp/°C	Time/h	%	%
1	DCC	25	0.5	64	54
2	EDC	25	0.5	67	54
3	DCC	60	0.5	89	77
4	EDC	60	0.5	94	93
5	EDC	60	3	100	99
6	EDC	60	5	100	99
7	EDC	60	20	100	100[a]

a) Standard value.

reagent realized a more convenient HPLC analysis of chiral amines with a higher detection limit and a shorter analysis time compared with the previously reported rhodamine derivative.

Experimental

Apparatus. Melting points were measured with a Yanagimoto MP-S2 micro-melting-point apparatus. NMR spectra were taken on a Jeol α-400 spectrometer using tetramethylsilane as an internal standard. Mass spectra were recorded on a Shimadzu QP-1000 instrument. Elemental analysis was performed with a Yanaco MT-6 CHN corder. UV–vis absorption and fluorescence spectra were measured with Hitachi U-3500 and F-4500 spectrometers, respectively. The HPLC analysis was performed with a Shimadzu LC-10AD instrument.

Materials. Methyl (*R*)-(+)-3-methylglutarate, 2,4-dimethylpyrrole (**1**), and diethyl ether–boron trifluoride (1/1) were purchased from Tokyo Kasei Co., Ltd. Racemic mexiletine (**5**) was obtained from Sigma-Aldrich Co., Ltd. (*R*)- and (*S*)-Mexiletine were prepared as described in the literature.[15]

Synthesis of Protected Pyrromethene–BF$_2$ Complex Dye 3. To a dichloromethane solution (40 mL) of methyl (*R*)-(+)-3-methylglutarate (640 mg, 4 mmol) was added a drop of DMF at 0 °C. To the mixture was added oxalyl chloride (2.5 g, 20 mmol), which was stirred at 0 °C for 15 min and then at room temperature for 2 h. After the reaction was complete, benzene (10 mL) was added to the mixture, which was stirred for 5 min. After the evaporation of benzene, a yellow viscous product was obtained. This crude acid chloride **2** was used without further purification.

To a dichloromethane solution (5 mL) of 2,4-dimethylpyrrole (**1**, 190 mg, 2 mmol) was slowly added a dichloromethane solution (5 mL) of the acid chloride **2**, prepared as mentioned above. After stirring for 10 min, the mixture was refluxed for 2 h. After the reaction was completed, the mixture was poured into hexane (200 mL). The supernatant solution was removed. The resulting precipitate was dissolved in toluene (200 mL). To the solution was added triethylamine (424 mg, 4.2 mmol), which was stirred for 20 min at room temperature. Then, to the mixture was slowly added diethyl ether–boron trifluoride (1/1) (1.69 g, 12 mmol). The mixture was stirred for 20 min at room temperature and refluxed for 2 h. After the reaction was completed, the organic layer was washed with water three times and dried over anhydrous sodium sulfate. The product was isolated by column chromatography (SiO$_2$, hexane/ethyl acetate = 1/1) and recrystallized from a toluene–hexane mixed solvent. Yield 471 mg (33%); mp 137–139 °C; ^{1}H NMR (CDCl$_3$) δ 1.06 (d, $J = 6.4$ Hz, 3H), 2.25–2.35 (m, 2H), 2.43 (s, 3H), 2.44 (s, 3H), 2.46–2.52 (m, 1H), 2.52 (s, 6H), 2.99–3.07 (m, 2H), 3.64 (s, 3H), 6.06 (s, 2H); EIMS (70 eV) *m*/*z* (rel intensity) 362 (M$^+$; 100%), 262 (15).

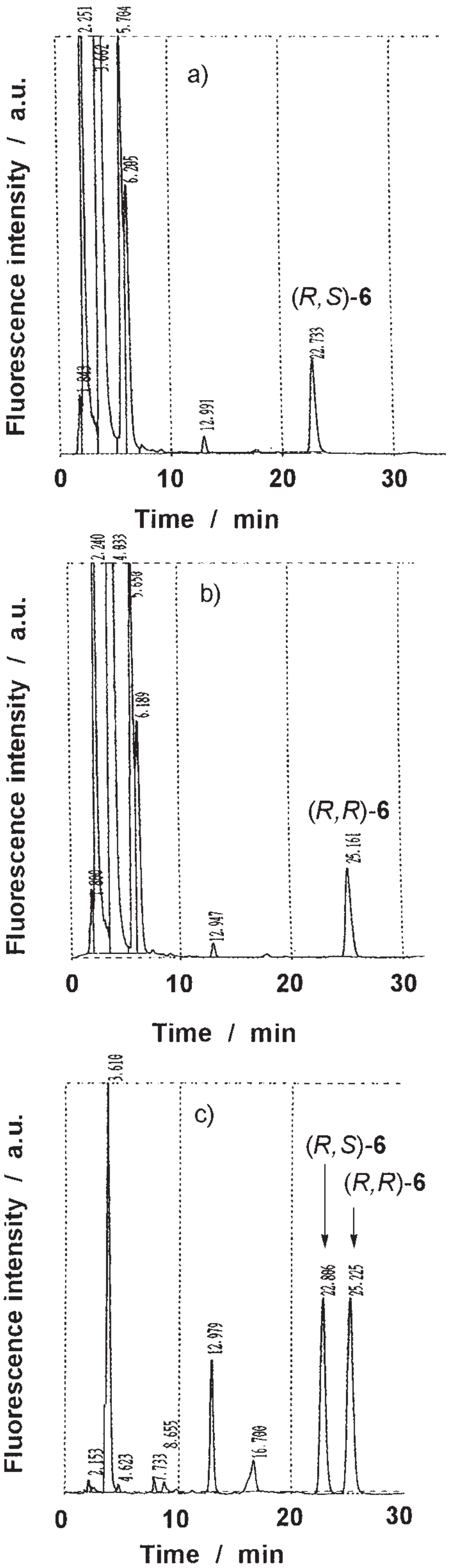

Fig. 3. HPLC analysis of labeled **5**: a) (*S*)-**5**, b) (*R*)-**5**, and c) racemic **5**. Column: Mightysil RP18 GP (4.6 mm × 250 mm); Mobile phase: MeCN/H$_2$O = 60/40, 0.8 mL min^{-1}; Excitation: 499 nm; Detection: 518 nm.

Synthesis of Pyrromethene–BF$_2$ Complex Dye 4 (Deprotection of 3). To a THF solution (10 mL) of the methyl ester **3** (217 mg, 0.6 mmol) was added an aqueous sodium hydroxide solution (1 mol dm^{-3}, 1.8 mL). The mixture was stirred at 50 °C for 4 h. The reaction was monitored by TLC. After the reaction was completed, the mixture was cooled. To the mixture was added ethyl acetate (20 mL). The organic layer was washed with a 10% aqueous citric acid solution (20 mL × 2). The product was isolated by column chromatography (SiO_2, ethyl acetate) and recrystallized from a toluene–hexane 1:1 mixed solvent. Yield 40 mg (19%); mp 149–151 °C; ^{1}H NMR ($CDCl_3$) δ 1.08 (d, $J = 6.4$ Hz, 3H), 2.24–2.34 (m, 3H), 2.42 (s, 3H), 2.43 (s, 3H), 2.51 (s, 6H), 2.99–3.07 (m, 2H), 6.06 (s, 2H); EIMS (70 eV) m/z (rel intensity) 348 (M^+; 100%), 313 (18), 261 (51), 247 (45). Anal. Found: C, 62.22; H, 6.66; N, 8.13%. Calcd for $C_{18}H_{23}BF_2N_2O_2$: C, 62.09; H, 6.66; N, 8.05%.

Reactivity, Checking of Epimerization, and HPLC Analysis of Mexiletine. To a dichloromethane solution (100 µL) of racemic mexiletine **5** (2 µmol dm^{-3}, 0.2 µmol) were added a dichloromethane solution (50 µL) of 4-(1-pyrrolidinyl)pyridine (10 µmol dm^{-3}, 0.5 µmol), a dichloromethane solution (500 µL) of pyrromethene–BF_2 complex dye **4** (1 µmol dm^{-3}, 0.5 µmol), a dichloromethane solution (50 µL) of EDC (10 µmol dm^{-3}, 0.5 µmol) or DCC (10 µmol dm^{-3}, 0.5 µmol), and a dichloromethane solution (100 µL) of Aflatoxin G2 (10 µg dm^{-3}). Aflatoxin G2 was used as a reference compound in the HPLC analysis. The mixture was reacted in a vial tube. Then, the reaction mixture was analyzed by HPLC (column, Mightysil RP18 GP (4.6 mm × 250 mm); mobile phase, $MeCN/H_2O = 60/40$, 0.8 mL min^{-1}; excitation, 499 nm; detection, 518 nm).

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