Chiral Fluorescent Labeling Reagent Derived from Rhodamine B for Flurbiprofens

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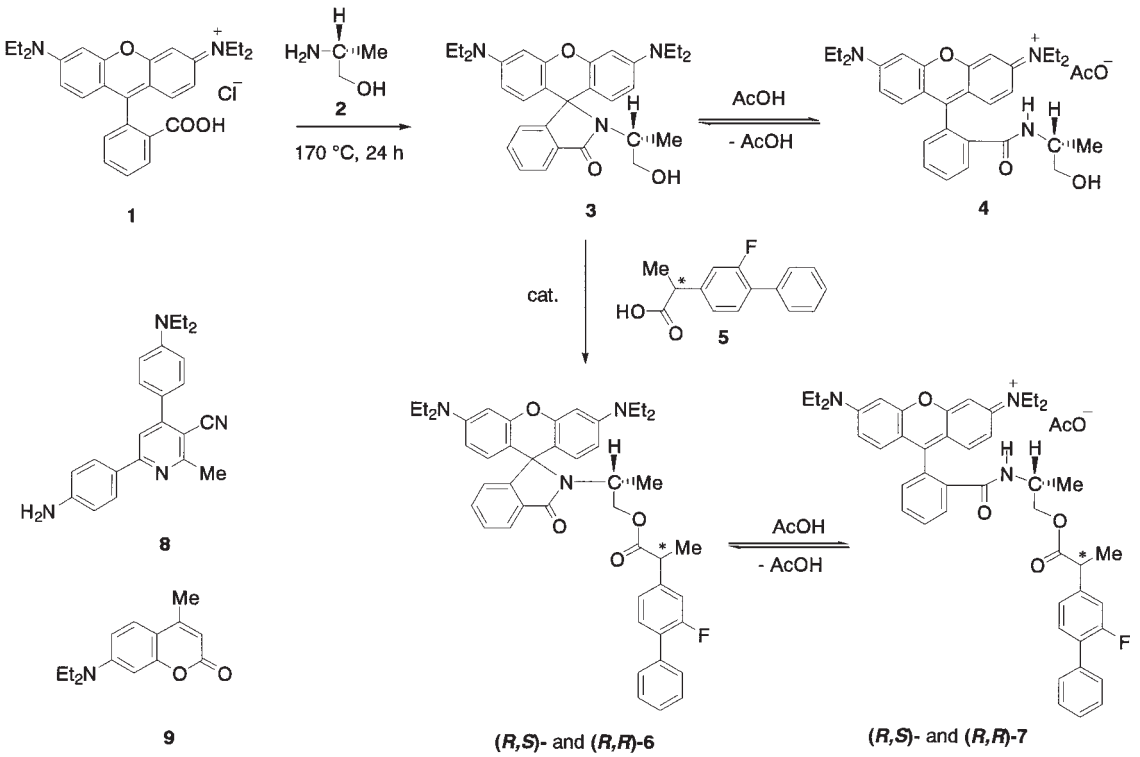
A lactam-type enantiomeric fluorescent labeling reagent was prepared from Rhodamine B. This reagent showed its absorption and emission maxima at λ 557 and 580 nm in an acetonitrile–acetic acid (9:1) mixed solution, respectively. Racemic Flurbiprofens reacted with this reagent to produce diastereomeric esters, which were separated by non-chiral high-performance liquid chromatography (HPLC).

Fluorescent labeling reagents are required to have intense and bathochromic fluorescence.[1,2] Biologically active materials having a chiral center in a molecule can be analyzed in non-chiral columns by forming their diastereomers. HPLC analyses of amino acids and peptides by benzoxadiazoles[3–5] and carboxylic acids by anthracene-2,3-dicarboximide derivatives[6,7] have been reported. Xanthene dyes, such as fluorescein and Rhodamine B, are known to show intense fluorescence. Especially, Rhodamine dyes are interesting and important compounds due to their strong and bathochromic fluorescence. Therefore, they have been used as indicators for calcium ion,[8] labeling reagents for nucleic acids,[9] proteins,[10] muscle fibers,[11] and DNA sequencing.[12] Unfortunately, no chiral fluorescent labeling reagents derived from intensely fluorescent and bathochromic Rhodamine dyes have been reported so far. We report here on the synthesis and properties of a chiral fluorescent labeling reagent derived from Rhodamine B for the analysis of Flurbiprofens.

Results and Discussion

Scheme 1 shows the synthesis of fluorescent labeling reagent **3** derived from Rhodamine B (**1**). Compound **1** reacted with (*R*)-(−)-2-amino-1-propanol (**2**) to produce a lactam **3** in a good yield. The optical purity (ee%) of this compound



Scheme 1.

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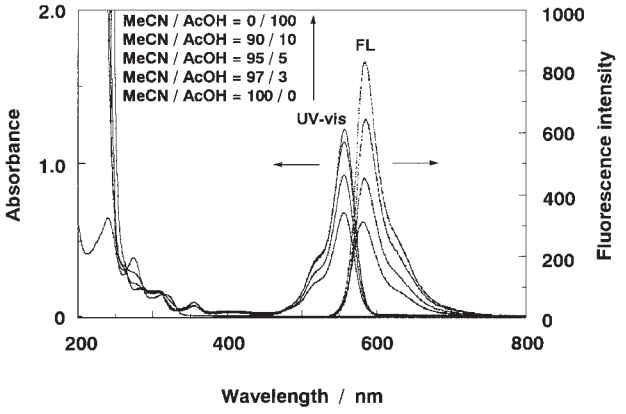

Fig. 1. UV-vis absorption and fluorescence spectra of **3** (**4**) in an acetonitrile–acetic acid mixed solution.

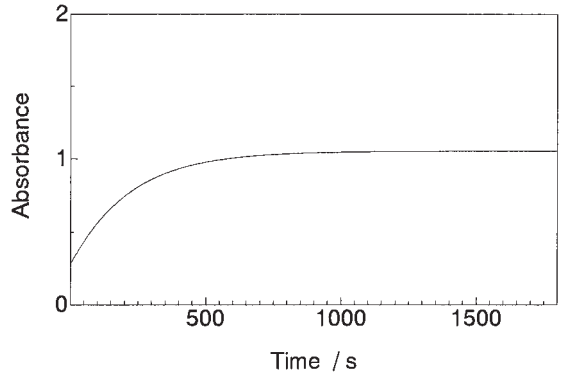

Fig. 2. Relationship between fluorescence intensity of **4** and reaction time. An acetonitrile–acetic acid (9:1) mixed solution of **4** (1×10^{-5} mol dm^{-3}) was allow to stand at 25 °C.

measured by HPLC (CHIROBIOTIC V (4.6 mm × 250 mm), hexane–ethanol (85/15), 0.8 cm^{-3} min^{-1}, detection: 254 nm) was higher than 99%.

Compound **3** was colorless and non-fluorescent in acetonitrile. However, compound **3** turned red and showed fluorescence in an acetonitrile–acetic acid (9:1) mixed solution. The UV-vis absorption and fluorescence spectra are shown in Fig. 1. The absorption (λ_{max}) and emission maxima (λ_{em}) were observed at 557 and 580 nm, respectively. The fluorescence intensity increased up to an acetic acid ratio of 20%. Then, the intensity was almost constant in the range of acetic acid ratios of 20–100%. Isosbestic points were observed at 265, 285, 315, and 335 nm, indicating that the formation of colored and fluorescent form **4** by the addition of acetic acid was in equilibrium with the non-fluorescent form **3**, as shown in Scheme 1.

The relationship between the absorbance and the reaction time in an acetonitrile–acetic acid (9:1) mixed solution is shown in Fig. 2. When compound **3** was kept in the solution at 25 °C, the absorbance gradually increased, and became constant after 10 min.

The relationship among the absorbance, fluorescence intensity, and temperature is shown in Fig. 3. No change in the absorption spectra was observed in the range of 25 to 65 °C. Meanwhile, the fluorescence intensity drastically decreased at

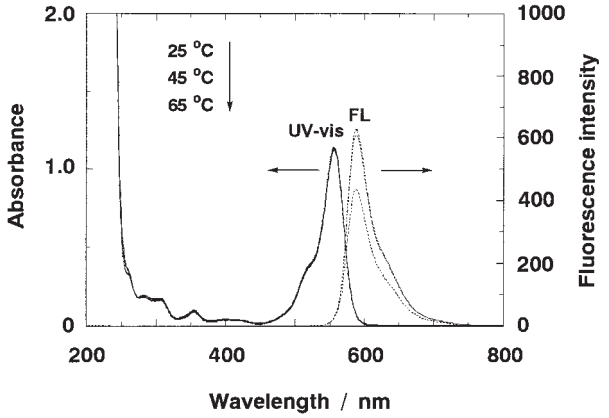

Fig. 3. Relationship among UV-vis absorption spectra, fluorescence spectra, and temperature. An acetonitrile–acetic acid (9:1) mixed solution of **4** (1×10^{-5} mol dm^{-3}) was allow to stand.

Table 1. UV-Vis Absorption and Fluorescence Spectral Data of **4**, **8**, **9**, and Rhodamine B

Compd	λ_{max} (ε)	λ_{ex}	λ_{em}	RFI
	nm (dm^3 mol^{-1} cm^{-1})	nm	nm	
4[a)]	557 (104900)	557	580	643
8[b)]	353 (53100)	353	508	100
9[b)]	369 (27000)	364	435	447
Rhodamine B[c)]	545 (106000)	530	565	819

a) Measured in an acetonitrile–acetic acid (9:1) mixed solution on 1.0×10^{-5} mol dm^{-3} of substrate at 25 °C. b) Measured in acetonitrile on 1.0×10^{-5} mol dm^{-3} of substrate at 25 °C. c) Measured in methanol on 1.0×10^{-5} mol dm^{-3} of substrate at 25 °C.

65 °C. These results indicate that only a small amount of open-form **4** was converted into the lactam-form **3** with increasing temperature. The remarkable decrease in the fluorescence intensity can be attributed to the thermal deactivation of **4** in the excited singlet state.

Table 1 summarizes the UV-vis absorption and fluorescence spectra of **4**, 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)-phenyl]-2-methylpyridine (**8**), 7-diethylamino-4-methylcoumarin (**9**), and Rhodamine B. Compounds **8** and **9** were reference materials throughout our study on the fluorescent labeling reagents.[13–17] The Stokes shift of **4** was calculated to be 23 nm, being very small, as normally observed for Rhodamine dyes. The molar absorption coefficient (ε) of Rhodamine B in methanol was calculated to be 106000 dm^3 mol^{-1} cm^{-1} at λ_{max} 545 nm, suggesting that most of lactam form **3** were converted into the open-form **4** in an acetonitrile–acetic acid mixed (9:1) solution. Both λ_{max} and λ_{em} of **4** were most bathochromic among **4**, **8**, and **9**. Furthermore, the relative fluorescence intensity (RFI) of **4** was most intense among them.

Since the HPLC analysis of water-soluble compounds is usually performed in a reverse phase, the effect of water on the RFI of **4** was examined. The result is shown in Fig. 4. The RFI of **8** and **9** drastically decreased by the addition of water. However, the decrease of RFI in **4** was only 16% even in a water ratio 90%. This result indicates that compound **3** can act

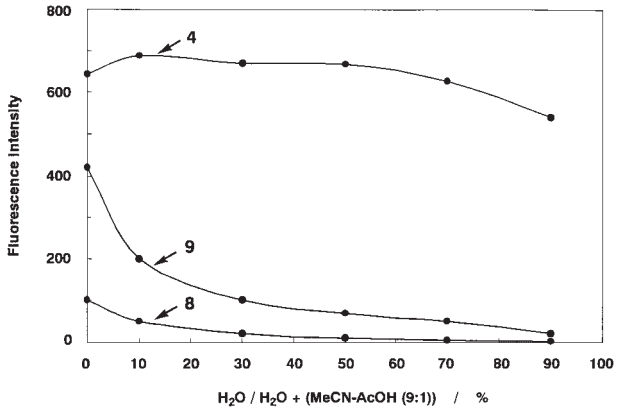

Fig. 4. Effect of water on fluorescence intensity of **4**, **8**, and **9** (1×10^{-5} mol dm^{-3}). In the cases of **8** and **9**, the abscissa indicates the % ratio of (H_2O/(H_2O + MeCN)).

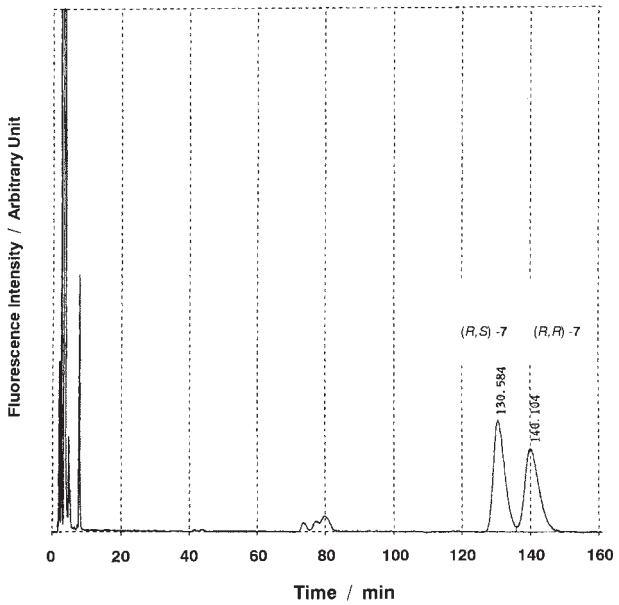

Fig. 5. HPLC analysis of labeled diastereomeric (*R*,*S*)- and (*R*,*R*)-**7**. Column: Mightysil RP-18 GP (4.6 mm × 150 mm); Mobile phase: acetonitrile–tetrahydrofuran–0.5% acetic acid (28:5:67); Flow rate: 0.8 cm^3 min^{-1}; Excitation: 562 nm, Detection: 582 nm.

as a good fluorescent labeling reagent.

Finally, racemic Flurbiprofens (**5**), which acts as inhibitors for cyclooxygenase, reacted with **3** in the presence of 4-(1-pyrrolidinyl)pyridine and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) to produce diastereomeric esters (*R*,*R*)- and (*R*,*S*)-**6**, which reacted with acetic acid to form the corresponding open forms of (*R*,*S*)- and (*R*,*R*)-**7**, as shown in Scheme 1. When an acetonitrile–acetic acid (9:1) mixed solvent was used as a mobile phase, no good separation of the diastereomers in HPLC was observed. Therefore, after the formation of open-forms **7**, the diastereomers were analyzed by HPLC. An acetonitrile–tetrahydrofuran–aqueous 0.5% acetic acid (28:5:67) mixed solvent was used as a mobile phase. The HPLC analysis of racemic Flurbiprofens (**5**) is shown in Fig. 5. The labeled diastereomers derived from (*S*)- and (*R*)-Flurbiprofens, (*R*,*S*)- and (*R*,*R*)-**7**, were observed at retention times of around 130 and 140 min, respectively. The retention time of the (*R*,*S*) derivative was checked by comparing that of the product obtained by the reaction of (*S*)-**5** with **3**. The ratio between (*R*,*S*)- and (*R*,*R*)-**7** was 55.8 to 44.2. Several pmol of racemic Flurbiprofens could be analyzed by this method.

Conclusion

An enantiomeric fluorescent labeling reagent for carboxylic acids was prepared from Rhodamine B. The absorption and emission maxima of this reagent was observed at λ 557 and 580 nm in an acetonitrile–acetic acid (9:1) mixed solution, respectively. Racemic Flurbiprofen smoothly reacted with this reagent in the presence of EDC to produce the corresponding diastereomeric esters, which were separated by non-chiral HPLC.

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. EIMS spectra were recorded on a Shimadzu QP-1000 instrument. UV-vis and fluorescence spectra were measured with Hitachi U-3500 and F-4500 spectrometers, respectively. HPLC was performed with a Jeol Triroter-V instrument.

Materials. Rhodamine B (**1**) was purchased from Tokyo Kasei Co., Ltd. (*R*)-(−)-2-Amino-1-propanol (**2**) was obtained from ACROS Organics. Racemic and (*S*)-Flurbiprofens were purchased from Aldrich Co., Ltd.

Synthesis of 3. To Rhodamine B (**1**) (120 mg, 0.25 mmol) was added (*R*)-(−)-2-amino-1-propanol (**2**) (500 mg, 6.67 mmol). The mixture was heated at 170 °C for 24 h. After the reaction was completed, the mixture was poured into brine. The product was extracted with dichloromethane and recrystallized from ethyl acetate. (106 mg, 79%). Mp 258.5–259.0 °C; ^{1}H NMR ($CDCl_3$) δ 1.10 (d, $J = 7.1$ Hz, 3H), 1.165 (t, $J = 7.1$ Hz, 6H), 1.171 (t, $J = 7.1$ Hz, 6H), 3.21–3.24 (m, 1H), 3.34 (q, $J = 7.1$ Hz, 4H), 3.35 (q, $J = 7.1$ Hz, 4H), 3.48 (ddd, $J = 11.7$, 8.7, and 4.6 Hz, 1H, a doublet peak ($J = 8.7$ Hz) disappears in the presence of D_2O), 3.55 (d, $J = 11.7$ Hz, 1H), 5.03 (d, $J = 8.7$ Hz, 1H, disappeared in the presence of D_2O), 6.27–6.30 (m, 2H), 6.37 (dd, $J = 9.5$ and 2.8 Hz, 2H), 6.47 (dd, $J = 9.5$ and 4.1 Hz, 2H), 7.05–7.08 (m, 1H), 7.44–7.48 (m, 2H), 7.87–7.90 (m, 1H); EIMS (70 eV) m/z (rel intensity) 499 (M^+; 18), 469 (42), 468 (100). $[\alpha]_D^{25}$ ($c = 0.0455$, $CHCl_3$) −52.7°. Anal. Found: C, 74.23; H, 7.66; N, 8.13%. Calcd for $C_{31}H_{37}N_3O_3$: C, 74.52; H, 7.46; N, 8.41%.

HPLC Analysis of Racemic Flurbiprofens (5). To a dichloromethane solution (0.6 cm^3) of racemic Flurbiprofens **5** (1.2 μmol) were added a dichloromethane solution (0.6 cm^3) of 4-(1-pyrrolidinyl)pyridine (10 μmol dm^{-3}, 6 μmol), a dichloromethane solution (0.6 cm^3) of **3** (10 μmol dm^{-3}, 6 μmol), a dichloromethane solution (0.6 cm^3) of EDC (10 μmol dm^{-3}, 6 μmol), and aflatoxin G2 (10 μg dm^{-3}). Aflatoxin G2 was used as a reference compound in the HPLC analysis. The mixture reacted in a vial tube at 20 °C for 7 h. After drying the reaction mixture, 2 cm^3 of an acetonitrile–0.5% acetic acid (55:45) mixed solution was added. The solution was then analyzed by HPLC (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).

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