Notes

N-[4-(5,6-Dimethoxy-2-phthalimidyl)phenyl]maleimide as Precolumn Fluorescence Derivatization Reagent for Thiols

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Some nonfluorescent *N*-substituted maleimides react selectively with thiol compounds to form fluorescent derivatives.¹⁻⁶ We previously reported that *N*-[4-(2-phthalimidyl)phenyl]maleimide (PPM) having a phenylphthalimidine moiety as a potential fluorophore is a preferable fluorescent thiol reagent.⁶ Recently, we found that the fluorescent intensity of the dimethoxyphthalimidyl group is higher than that of the unsubstituted phthalimidyl group.

This paper concerns the preparation of *N*-[4-(5,6-dimethoxy-2-phthalimidyl)phenyl]maleimide (DPM) and its applicability to the fluorescence labeling of some thiols.

Experimental

Chemicals

All chemicals were of analytical-reagent grade. 4,5-Dimethoxyphthalaldehyde was obtained from 3,4-dimethoxybenzoic acid via *m*-meconin and 4,5-dimethoxyphthalyl alcohol according to the method of Bhattacharjee *et al.*⁷

Synthesis of *N*-[4-(5,6-dimethoxy-2-phthalimidyl)phenyl]maleimide (DPM)

A mixture of *p*-aminoacetanilide (1.40 g) and 4,5-dimethoxyphthalaldehyde (1.94 g) in 1,4-dioxane (200 ml) was stirred at room temperature for 3 d to yield 4-(5,6-dimethoxy-2-phthalimidyl)acetanilide (yield 980 mg, pale brown leaves from ethanol, m.p. 269–270°C). 4-(5,6-Dimethoxy-2-phthalimidyl)aniline was obtained by

refluxing with 2 M sodium hydroxide in 50% ethanol (100 ml) for 24 h (yield 548 mg, colorless needles, m.p. (dec.) 222–223°C). The aniline derivative was reacted with maleic anhydride (306 mg) in chloroform (60 ml) at 5°C to give the corresponding maleamic acid, which was reacted with excess acetic anhydride in the presence of sodium acetate. The thus-obtained DPM was recrystallized from acetonitrile–ethanol (2:1) (yield 170 mg, pale yellow needles, m.p. 250–253°C). Anal. Calcd. for C₂₀H₁₆N₂O₅: C, 65.93; H, 4.43; N, 7.69%. Found: C, 65.86; H, 4.41; N, 7.71%. Mass spectrum (MS) (*m/z*, M⁺): 364. ¹H-Nuclear magnetic resonance (¹H-NMR) (δ , ppm, in CDCl₃): 3.97 and 3.99 (3H each, s each, CH₃ each), 4.79 (2H, s, CH₂ of phthalimidine), 6.86 (2H, s, H of maleimide), 6.98 and 7.37 (1H each, s each, aromatic H of phthalimidine), 7.49 and 7.97 (2H each, d each, H of benzene ring).

Instrumental conditions

The fluorescence spectra were measured with a Hitachi 650-10S spectrofluorimeter (Hitachi Ltd., Tokyo) using a quartz cell (optical path length 10 mm) at 15°C controlled with circulating water. A Bip-1 high-performance liquid chromatograph system (JASCO, Tokyo), equipped with a Rheodyne 7161 injector (20- μ l loop) and a F-1000 fluorescence detector (Hitachi Ltd., Tokyo), was used. The fluorescence intensity was monitored at Ex 312 nm and Em 422 nm. An ERC-ODS-1161 column (particle size 3 μ m, 100 $\times$ 6 mm i.d., Erma Ltd., Tokyo) was employed at ambient temperature. A mixture of acetonitrile and citrate buffer (1.2 mM, pH 3) (60:40) was used as a mobile phase at a flow rate of 0.3 ml/min.

Derivatization procedure

To a mixture of phosphate buffer (0.01 M, pH 8.0, 700 μ l) and acetonitrile (1200 μ l) were successively added DPM in acetonitrile (0.5 mM, 50 μ l) and an aqueous solution of thiols (20 μ M, 50 μ l). The resulting mixture was allowed to stand at room temperature for 15 min

before measuring the fluorescence at 15°C.

Preparation of DPM-derivative of 2-Mercaptoethanol

2-Mercaptoethanol (60 µl) was reacted with DPM (200 mg) for 2 h according to the derivatization procedure. The reaction product was extracted with chloroform (100 ml, 5 times). The organic layer was evaporated to dryness under reduced pressure. The residue was recrystallized from dichloromethane by adding hexane, to give DPM-derivative of 2-mercaptoethanol as colorless leaves (yield 134 mg, m.p. 190–191°C). Anal. Calcd. for $C_{22}H_{22}N_2O_6S$: C, 59.72; H, 5.01; N, 6.33; S, 7.25%. Found: C, 59.48; H, 4.99; N, 6.31; S, 7.17%. MS (m/z , M^+): 442. 1H -NMR (δ , ppm, in $CDCl_3$): 2.56–4.20 (7H, m, CH_2CH and SCH_2CH_2), other signals appeared at the same ppm as those of dimethoxyphthalimidine-H of DPM described above.

Results and Discussion

DPM was easily obtained from 4-(5,6-dimethoxy-2-phthalimidy)aniline via the maleamic acid according to the conventional method. DPM was soluble in acetonitrile, acetone, dimethylformamide, dimethylsulfoxide and dioxane; the solutions showed little fluorescence. The reagent reacted with thiols to give highly fluorescent derivatives. The reactivity of DPM was investigated using 2-mercaptoethanol as a model compound. The reaction mixture of 2-mercaptoethanol with DPM showed the same fluorescence spectra with excitation and emission wavelengths of Ex 312 nm and Em 422 nm, respectively, as did those of the authentic DPM-derivative of 2-mercaptoethanol. Although the labeling reaction was not affected by any temperature (5–45°C), the fluorescence intensities of the reaction mixture and the solution of the DPM-derivative depended on the temperature. The fluorescence intensities gradually decreased with increasing temperature. Therefore, the fluorescence intensity was measured at 15°C.

Although the pH (5.5–9.0) of the phosphate buffer solution did not influence the fluorescence intensity of the DPM-derivative, the reaction rate was affected by the pH of the buffer solution in the reaction mixture. As shown in Fig. 1, the labeling reaction proceeded slowly at pH values lower than 7.0, and came to completion in 5 min at a pH higher than 8.0.

The extent of conversion of 2-mercaptoethanol into the fluorescent derivatives was 97.5%, which was obtained by comparing the fluorescence intensity of the reaction mixture with that of the DPM-derivative of 2-mercaptoethanol. The analytical sensitivity of DPM was about 3-times higher than that of PPM reported previously.

The reactions of DPM were examined with several thiol compounds under the conditions described above. Glutathione, cysteine, *N*-acetylcysteine, *o*-aminothiophenol and captopril reacted with DPM, showing a

Fig. 1 Time courses of the reaction of 2-mercaptoethanol with DPM at various pHs.

fluorescence spectra with Ex 312–315 nm and Em 420–425 nm. However, the reaction mixtures of 6-mercaptapurine and 2-mercaptothiazoline, which may be present in thiocarbonyl form, did not show any fluorescence.

DPM was also used as a precolumn labeling reagent in HPLC. When each reaction mixture of *N*-acetylcysteine, captopril, 2-mercaptoethanol, cysteine, glutathione and *o*-aminothiophenol with DPM was analyzed by HPLC, the peaks corresponding to these thiols appeared at 7.3, 8.0, 8.3, 11.1, 11.5 and 13.5 min, respectively. The peak heights were correlated to the concentrations of thiols up to 500 pmol per injection (20 µl). The detection limits (signal/noise=2 in 20 µl injection) of thiols labeled with DPM were between 12 fmol (2-mercaptoethanol) and 110 fmol (*o*-aminothiophenol).

DPM will be useful for the determination of physiologically important thiols in biological fluids or therapeutic drug monitoring.

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