Notes

12-(Difluoro-1,3,5-triazinyl)-benz[*f*]isoindolo[1,2-*b*][1,3]benzothiazolidine as a Fluorescent Labeling Reagent for Estrogens in High-Performance Liquid Chromatography

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Regarding the application of fluorescent labeling techniques for the trace analysis of biological materials, the ability to excite the labeled fluorescent compounds in the visible region enhances the selectivity of the assay, since there are few, if any, absorbing species in biological samples that complete for the exciting radiation in this region. In a previous study, 3-(difluoro-1,3,5-triazinyl)-1-(ethylthio)-2-*n*-propylbenz[*f*]isoindole (FEPBI)¹ was synthesized as a precolumn fluorescent labeling reagent for estrogens; it was found that the excitation wavelengths of FEPBI and its derivatives were almost the same as that of the product of primary amine with 3-(2-furoyl)quinoline-2-carbaldehyde.² In this paper we describe the preparation of a new highly sensitive labeling reagent, 12-(difluoro-1,3,5-triazinyl)-benz[*f*]isoindolo[1,2-*b*][1,3]benzothiazolidine (FBIBT), which shows longer excitation and emission wavelengths than those of FEPBI as well as the optimum conditions for the derivatization of estrogens in high-performance liquid chromatography (HPLC).

Experimental

Instrumental conditions

Proton nuclear magnetic resonance (¹H-NMR) spectra were recorded on a JNM-GX400 spectrometer (JEOL Ltd., Tokyo) at 400 MHz. The chemical shifts were reported in parts per million relative to tetramethylsilane as an internal standard. For describing the NMR characteristics, the following abbreviations are used: s=singlet, d=doublet and t=triplet. The mass spectra were taken with a JMS-DX303HF mass spectrometer (JEOL Ltd., Tokyo). For measurements of the excitation and emission spectra, a Hitachi 650-60 fluorescence spectrometer with a 1 cm quartz cell was used without any spectral correction. A Hitachi L-6000 pump was used; it was equipped with a Rheodyne 7125 syringe-loading sample injector valve (20 μ l loop) and a Hitachi

F-1000 fluorescence spectrophotometer fitted with a 12 μ l flow-cell operating at 560 nm emission and 540 nm excitation. Separations were performed using a TSKgel ODS-120T column (250 $\times$ 4.6 mm i.d., particle size 5 μ m, TOSOH, Tokyo). The mobile phase consisted of a mixture of acetonitrile-water (50:1, v/v) and was filtered (0.45 μ m) prior to use. The chromatographic separation was performed isocratically at ambient temperature at a flow rate of 1.0 ml/min.

Synthesis of FBIBT

To a stirred solution of 2,3-naphthalenedialdehyde (400 mg) in tetrahydrofuran (THF) (12 ml) was added 2-aminothiophenol (240 μ l). Then, cyanuric fluoride (380 μ l) was immediately poured into the solution. After stirring for 30 min at room temperature the precipitate was filtered off, washed with THF, and recrystallized from acetone to yield FBIBT (30 mg) as red needles (mp 276–280°C). *Anal.* Calcd. for C₂₁H₁₀F₂N₄S: 64.94% C, 2.57% H, 14.43% N, 9.79% F; Found: 64.93% C, 2.65% H, 14.23% N, 9.73% F. *MS* *m/z*: 388 (*M*⁺). ¹H-NMR (*p*-dioxane-*d*₈) δ : 7.39 (1H, t, *J*=7 Hz, 9-H), 7.48 (1H, t, *J*=8 Hz, 8-H), 7.62 (1H, t, *J*=7 Hz, 2-H), 7.73 (1H, t, *J*=7 Hz, 3-H), 7.93 (1H, d, *J*=8 Hz, 1-H), 8.05 (1H, d, *J*=7 Hz, 10-H), 8.13 (1H, d, *J*=8 Hz, 4-H), 8.56 (1H, s, 11-H), 9.42 (1H, s, 6-H), 9.89 (1H, d, *J*=8 Hz, 7-H).

Labeling procedure

To 10 μ l of a standard mixture of estrogens in dimethylsulfoxide (DMSO) (each 500 pmol/ml) was added 50 μ l of 0.4 mM FBIBT in DMSO, followed by the addition of 10 μ l of 20 mM aqueous NaOH. After standing at room temperature for 5 s, 10 μ l of 52 mM aqueous acetic acid was added; the components were mixed thoroughly. Then, 10 μ l of the final solution was injected into the HPLC apparatus.

Results and Discussion

We have studied the FEPBI analogues which were more sensitive than FEPBI. The reaction of 2,3-naphthalenedialdehyde with 2-aminothiophenol and cyanuric fluoride afforded FBIBT. This structure, as shown in Fig. 1, was confirmed by mass, $^1\text{H-NMR}$ spectral and elemental analyses. FBIBT was stable for at least 6 months in the crystalline state in a light-protected desiccator. FBIBT reacts with phenolic hydroxyl groups under alkaline conditions at room temperature. The FBIBT derivative of estrone was purified by preparative silica gel thin-layer chromatography and subjected to mass spectrometry in order to confirm this structure. The mass spectrum showed the molecular ion peak of the estrone derivative at m/z 638. Figure 2 shows the fluorescence excitation and emission spectra of estrone labeled with FBIBT in acetonitrile–water (50:1, v/v) used as the mobile phase. The relative fluorescence intensities of the estrone derivative decreased with increasing water concentration in the medium. Therefore, the HPLC determination of estrogens is optimum at lower concentrations of water in the eluent. Introduction of the benzothiazolidine ring into the benz[*f*]isoindole skeleton afforded red shifts (each *ca.* 40 nm) of both the excitation and emission maximum wavelengths, compared with those of FEPBI. FBIBT readily reacted with each estrogen in the presence of NaOH at room temperature to produce a corresponding single fluorescent chromatographic peak. For analytical applications of this reagent, the reaction conditions for derivatization, such as the choice of solvent, the concentration of FBIBT or NaOH and the reaction time were optimized by the batch method using estrone as a model compound. Attempts have been made to maximize the peak intensities of the estrone labeled with FBIBT on the chromatogram. We first investigated the reaction solvents, using DMSO, THF, dimethylformamide (DMF), acetone and acetonitrile due to their complete miscibility with aqueous NaOH during labeling. However, acetone and acetonitrile were not available, due to the poor solubility of FBIBT; this was because the reaction was slower at low concentrations of FBIBT and no constant peak height was obtained, even after 1 h. Although FBIBT was dissolved at a concentration of less than 0.4 mM in THF or DMF, the derivatization in both THF and DMF offered no peak corresponding to estrone labeled with FBIBT on the chromatogram. Thus, DMSO was employed as a solvent for the labeling reaction. Maximum and constant peak heights were attained at a concentration of 285 μM for FBIBT and 2.85 mM for NaOH in the reaction solution (70 μl) at room temperature for 5 s. After labeling, the reaction mixture was neutralized by the addition of 10 μl of 52 mM aqueous acetic acid to prevent the degradation of column materials. From the above-mentioned results, the optimum conditions were fixed, as mentioned under Experimental. The pattern of the derivatization re-

Fig. 1 Structure of FBIBT.

Fig. 2 Fluorescence excitation and emission spectra of estrone labeled with FBIBT (0.5 μM) in acetonitrile–water (50:1, v/v).

action with FBIBT was almost comparable to that of FEPBI. Figure 3 shows the separation of 5 pmol for each of the standard estrogens labeled with FBIBT. The peak identities are given in the figure legend. Complete resolution of the adducts and reagent was achieved with all of the estrogens tested. No decrease was observed in the peak height of the adducts in the final solution after 6 h from a time-course study. The activity of FBIBT in DMSO showed no loss for at least a week at room temperature in the dark. The calibration graph of estrone, the amount of estrone *versus* the peak height of the fluorescent response, showed excellent linearity in the range 25 – 1200 fmol per injection. The relative standard deviation at a level of 800 fmol of estrone was 4.6% ($n=8$). The detection limit (signal-to-noise ratio of 2) of the authentic derivative obtained from the reaction of estrone with FBIBT was 10 fmol for an injection volume of 10 μl , which is *ca.* 8-times higher than that of FEPBI.

In conclusion, the newly designed reagent, FBIBT, has been easily synthesized from commercially available starting materials by a one-step reaction and is recommended for the determination of estrogens owing to its good reactivity, excellent fluorescence characteristy and high sensitivity in the low femtomole range. The

Fig. 3 Chromatogram of estrogens labeled with FBIBT.
Peaks identity: a, reagent blank; 1, ethinylestradiol; 2, estrone; 3, 17α -estradiol; 4, estriol; 5, 17β -estradiol.

development of FBIBT analogues having reacting groups toward the other functional group is now being conducted; details will be reported elsewhere in the near future.

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References

1. H. Fujino and S. Goya, *Chem. Pharm. Bull.*, **37**, 1939 (1989).
2. S. C. Beale, Y. Z. Hsieh, D. Wiesler and M. Novotny, *J. Chromatogr.*, **499**, 579 (1990).

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