3-(Difluoro-1,3,5-triazinyl)-1-(ethylthio)-2-*n*-propylbenz[*f*]isoindole as a Fluorescence Derivatization Reagent for Estrogens in High-Performance Liquid Chromatography

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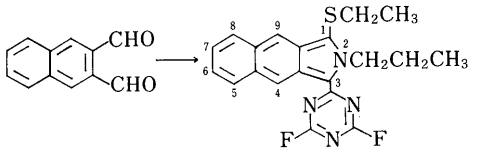
A new fluorogenic reagent, 3-(difluoro-1,3,5-triazinyl)-1-(ethylthio)-2-*n*-propylbenz[*f*]isoindole, which reacts with phenolic hydroxyl groups, has been synthesized. This reagent reacts with estrogens in aqueous alkali–acetone mixtures at room temperature for 5 s to form the corresponding fluorescent derivatives, which can be separated by reversed-phase chromatography on TSK gel ODS-120A using methanol as a mobile phase. The detection limit for estrone was 80 fmol for an injection volume of 10 μl.

Keywords 3-(difluoro-1,3,5-triazinyl)-1-(ethylthio)-2-*n*-propylbenz[*f*]isoindole; phenolic hydroxyl group; estrogen; fluorogenic reagent; high-performance liquid chromatography

The fluorogenic labeling of nonfluorescent or weakly-fluorescent compounds has found a great deal of use in biological and pharmaceutical analyses. Recently we reported that 1-(ethylthio)-3-(difluoro-1,3,5-triazinyl)-2-*n*-propylisoindole (EFPI)[1] was useful as a fluorescent precolumn derivatizing reagent for estrogens in high-performance liquid chromatography (HPLC). In this paper, we describe the synthesis of 3-(difluoro-1,3,5-triazinyl)-1-(ethylthio)-2-*n*-propylbenz[*f*]isoindole (FEPBI) as a more sensitive reagent and a preliminary investigation of fluorescence labeling of estrogens with FEPBI in HPLC.



FEPBI

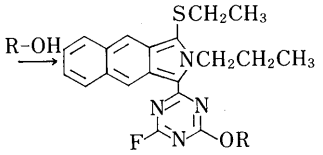


Chart 1

Experimental

Materials All chemicals were of analytical reagent grade. Water was purified with Milli-Q apparatus (Millipore Corp., Bedford, MA) after passage through ion-exchange resin. Organic solvents were purified by distillation prior to use.

Instrumental Conditions Proton and fluorine nuclear magnetic resonance (^{1}H- and ^{19}F-NMR) spectra were recorded on a JNM-GX400 spectrometer at 400 MHz for ^{1}H and at 376 MHz for ^{19}F (JEOL Ltd., Tokyo). Chemical shifts were reported in parts per million relative to tetramethylsilane (δ 0.00) for ^{1}H-NMR and benzotrifluoride (ϕ 67.75) for ^{19}F-NMR as internal standards. Mass spectra (MS) were taken with a JMS-DX303HF spectrometer (JEOL Ltd.). Uncorrected fluorescence spectra were measured with a 650-60 spectrofluorometer (Hitachi Ltd.) in 10 × 10 mm quartz cells; spectral bandwidths of 10 nm were used in both the excitation and emission monochromators. A 655A-11 high-performance liquid chromatograph (Hitachi Ltd.) was used with an F1000 fluorescence detector (Hitachi Ltd.) operating at Ex 490 nm and Em 520 nm. The separation was achieved using a stainless-steel column (250 × 4.6 mm i.d.) packed with TSK gel ODS-120A (particle size 5 μm: Tosoh, Tokyo). Methanol was used as a mobile phase at a flow rate of 1.0 ml/min at room temperature.

Synthesis of FEPBI Ethanethiol (81 μl) and *n*-propylamine (90 μl) were added to a stirred suspension of 2,3-naphthalenedialdehyde (200 mg) in anhydrous ether (20 ml) at room temperature, then the mixture was cooled in ice-water. Cyanuric fluoride (146 μl) was introduced into this mixture *via* a syringe with stirring. Stirring was continued for 1 min, then the solvent was removed under reduced pressure. The red–black oily residue was dissolved in 20 ml of acetone and applied to preparative silica gel thin-layer plates, which were developed with benzene. The band of FEPBI was scraped off and extracted with acetone. The extraction solvent was removed *in vacuo*. The residue was purified by precipitation from a concentrated acetone solution by the dropwise addition of water, followed by drying under reduced pressure to yield 20 mg of dark purple needles (mp 158—160 °C). *Anal.* Calcd for $C_{20}H_{18}F_2N_4S$: C, 62.48; H, 4.72; N, 14.57. Found: C, 62.63; H, 4.78; N, 14.30. MS *m/z*: 384 (M^+, base peak). ^{1}H-NMR ($CDCl_3$) δ: 1.08 (3H, t, J=7 Hz, $NCH_2CH_2\underline{CH_3}$), 1.28 (3H, t, J=7 Hz, $SCH_2\underline{CH_3}$), 1.90 (2H, tq, J=7 Hz, $NCH_2\underline{CH_2}CH_3$), 3.06 (2H, q, J=7 Hz, $S\underline{CH_2}CH_3$), 5.19—5.23 (2H, m, $N\underline{CH_2}CH_2CH_3$), 7.36—7.44 (2H, m, C_6-H, C_7-H), 7.94 (1H, d, J=8 Hz, C_5-H), 8.04 (1H, d, J=8 Hz, C_8-H), 8.44 (1H, s, C_4-H), 9.34 (1H, s, C_9-H). ^{19}F-NMR ($CDCl_3$) ϕ: 92.10—93.25 (m, temperature-dependent).

Derivatization Procedure To an acetone solution of estrogens (each 10 pmol, 20 μl), FEPBI in acetone (1.5 mM, 20 μl), was added, followed by 10 μl of 20 mM aqueous NaOH. The mixture was kept for 5 s at room temperature, then 10 μl of 174 mM aqueous acetic acid was added. The components were mixed thoroughly, then 10 μl of the final solution was injected into the HPLC apparatus.

Results and Discussion

Preparation and Properties of FEPBI and Its Derivatives FEPBI was prepared from 2,3-naphthalenedialdehyde, ethanethiol, and *n*-propylamine with cyanuric fluoride (Chart 1). This reagent was found to be stable for at least several months in the crystalline state and for a week in acetone solution when protected from light at room

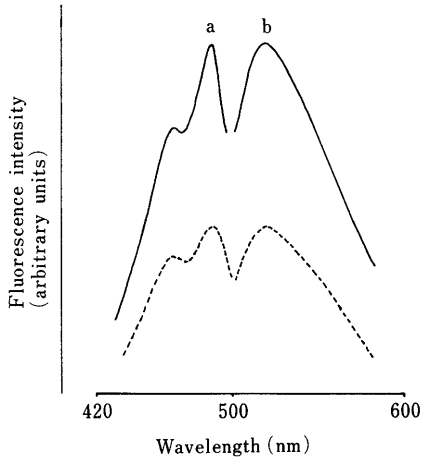


Fig. 1. Fluorescence Excitation and Emission Spectra of FEPBI (----, 2×10^{-7} M) and the Estrone Derivative (——, 2×10^{-7} M) in Methanol

a, excitation spectra; b, emission spectra.

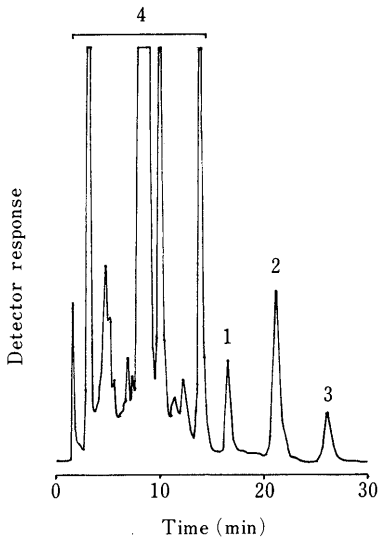


Fig. 2. Chromatogram of the FEPBI Derivatives of Estrogens

A portion (20 μl) of a standard mixture of the estrogens (each 500 pmol/ml) was treated with 20 μl of FEPBI (1.5 μmol/ml) and 10 μl of 20 mM aqueous NaOH. Peaks: 1, ethinylestradiol; 2, estrone; 3, 17β-estradiol; 4, reagent blank.

temperature. FEPBI reacts with phenolic hydroxyl groups under alkaline conditions at room temperature, but not with primary or secondary aliphatic hydroxyl groups. The structure of the derivatives was confirmed by mass spectrometry. The molecular ion peak of the estrone derivative appeared at m/z 634. The fluorescence excitation and emission spectra of FEPBI and the estrone derivative in methanol are shown in Fig. 1. The fluorescence intensity of the estrone derivative was *ca.* twice that of FEPBI.

Derivatization Conditions For analytical application of this reaction, the reaction conditions were optimized with estrone as a model compound. FEPBI gave the most intense peaks at concentrations greater than 0.6 mM in aqueous acetone solution (50 μl). Maximum and constant peak heights were attained at 4 mM NaOH in aqueous acetone solution (50 μl). At room temperature (20 °C), the peak height for estrone was maximal after standing for 5 s. After labeling, the alkaline reaction mixture was neutralized by the addition of 10 μl of 174 mM aqueous acetic acid to protect the column. The FEPBI derivatives in the final solution were stable for at least 6 h at room temperature with protection from light. Figure 2 shows a chromatogram obtained with a derivatized mixture of estrogens. Under the analytical conditions, a linear relationship was observed between the peak height ratios of estrone to 17β-estradiol derivatives (17β-estradiol: 4 pmol) and the amounts of estrone between 0.08 and 4.2 pmol per injection volume (10 μl). The limit of detection of estrone was 80 fmol/injection volume (10 μl) at a signal-to-noise ratio of 2. This method yields *ca.* 3.3-fold higher sensitivity than that of the method with EFPI.[1]

FEPBI as a fluorescence derivatization reagent is more sensitive than EFPI, and further studies on the clinical applications of FEPBI are in progress.

References

1) H. Fujino and S. Goya, *Anal. Sci.*, **5**, 105 (1989).