Syntheses and Characterizations of 4-(3,17 β-Dihydroxyestra-1,3,5(10)-trien-6α- and 6β-yl)amino-7-nitro-2,1,3-benzoxadiazoles as Fluorescent Probes

Tomoyuki OE,† Miki IKUTA, and Toshimasa TOYO'OKA

Department of Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

4-(3,17β-Dihydroxyestra-1,3,5(10)-trien-6α- and 6β-yl)amino-7-nitro-2,1,3-benzoxadiazoles have been synthesized and characterized as fluorescent probes for use in a receptor assay and/or a homogeneous immunoassay for estradiol. The fluorescence intensities are strongly dependent upon the solvent polarity used. The intensities in water were reduced to less than 1% of those in ethyl acetate, and a blue shift was also observed in polar solvents. The quenched fluorescence in aqueous solution was recovered by adding bovine serum albumin or an anti-estradiol antibody. Adding intact estradiol inhibited the fluorescence recovered by the antibody.

(Received July 12, 2001; Accepted October 12, 2001)

Introduction

Fluorescence probes are very useful analytical tools for the visualization of trace amounts of biological compounds. The combination of an appropriate fluorescence probe and time-resolved fluorescence imaging or confocal fluorescence imaging microscopies can make it possible to clarify many biological functions using living cells and tissues.[1,2] Many probes have been reported for this purpose, such as Fura[2] for the detection of cytosolic calcium ions[3] and PKH dyes for cell labeling.[4] Generally, a trial-and-error method is needed to develop probes, because several factors must be considered, including water solubility, permeability to the cell membrane, remarkable spectral changes and selectivity for the target compounds. Our ultimate goal is to systematically develop various probes not only for metal ions, but also for bio-molecules, such as hormones.

Our approach for this purpose has been inspired by the hydrophobic probes 4-*N*-(4′-methoxybenzyl)amino-7-nitro-2,1,3-benzoxadiazole (MBD) and 4-*N*-benzylamino-7-nitro-2,1,3-benzoxadiazole (BBD), reported by Kenner and Aboderin.[5] These fluorescence intensities strongly depend upon the solvent polarity. Although the quenching mechanism was presumed by the authors to result from conformational changes between the solvents, the process has not yet been entirely clarified. Our interest is in whether or not the characteristic fluorescence changes can be manipulated by any other interactions besides solvent polarity, such as steric hindrance.

As our first attempt to prove this idea, we have already reported on 4-(4′-methylbenzo-15-crown-5-4′-yl)amino-7-nitro-2,1,3-benzoxadiazole as an alkali metal-ion probe, which showed a relatively larger fluorescence enhancement compared with that of the fluorescence crown compounds that have a fluorophore attached directly to crown moiety.[6] Our data have therefore shown that it is possible to manipulate the quenched fluorescence of 4-*N*-benzylamino-7-nitro-2,1,3-benzoxadiazole derivatives (BzN-NBD derivatives) using various kinds of interactions. We hope that this finding will help us to systematically design other fluorescent probes for bio molecules.

Our approach utilizing the structure of BzN-NBD derivatives has several advantages, as follows (Fig. 1): i) the characteristic fluorescence of benzofurazane derivatives is expected to avoid background fluorescence and to utilize a laser-induced fluorometry,[7] ii) the water solubility and/or the permeability to the cell membrane might be controlled by choosing the 7-substituent on the benzofurazane moiety from nitro to sulfonic acid, sulfonamide or dimethylsulfonamide; each synthetic intermediate is commercially available as a fluorescence reagent for high-performance liquid chromatography (HPLC),[8] iii) systematic development might be accomplished with a simple synthetic approach, which is done by coupling commercially available halogenated benzofurazane and a drug mimetic or recognition site, through their amino group, iv) a free proton located on the 4-nitrogen is easy to introduce to an active site for covalent binding toward biomolecules, such as for photo-affinity labeling using an aziridine, and v) smaller fluorophores, like NBD, have the advantage of avoiding steric effects.

As a next consideration in clarifying the usefulness of BzN-NBD derivatives, we wanted to know whether the characteristic fluorescence can be manipulated selectively with the receptor or the antibody, and also can be utilized for the analytical probe. Many drugs and biological compounds possess aromatic moieties. When we utilize the benzyl moiety as a component of the BzN-NBD derivative, the interaction towards the compound would cause the fluorescence changing. In this paper, 4-(3,17β-dihydroxyestra-1,3,5(10)-trien-6α- and 6β-yl)amino-7-nitro-

† To whom correspondence should be addressed.
Present address: Center for Cancer Pharmacology, University of Pennsylvania, 1253 BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6160, USA.
E-mail: tomo@spirit.gcrc.upenn.edu

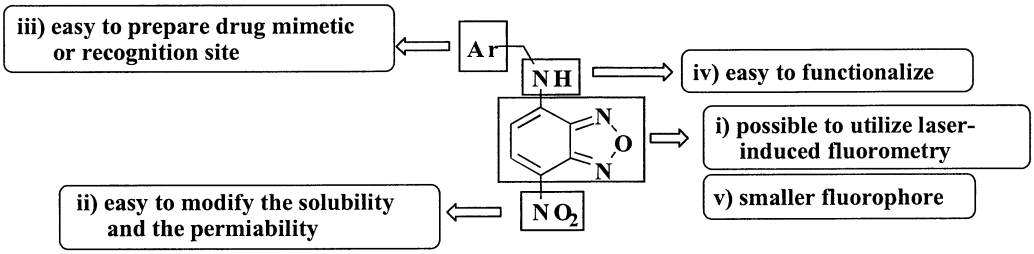

Fig. 1 Design of the BzN-NBD probe family.

2,1,3-benzoxadiazoles (6α- and 6β-E_2-NBD, Fig. 2) have been synthesized and characterized as fluorescent probes in consideration of their application in estrogen receptor assays and/or homogeneous immunoassays for estradiol.

Experimental

Reagents

6-Oxoestra-1,3,5(10)-triene-3,17β-diol (6-oxo-E_2) was purchased from Sigma (Milwaukee, WI, USA). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was purchased from Tokyo Kasei Co. (Tokyo, Japan). β-Estradiol (estra-1,3,5(10)-triene-3,17β-diol; E_2), zinc powder, and bovine serum albumin (BSA, globulin free) were purchased from Wako Pure Chemical Co. (Osaka, Japan). 4-*N*-(4′-Methoxybenzyl)amino-7-nitro-2,1,3-benzoxadiazole (MBD) was purchased from Dojindo Lab. Co. (Kumamoto, Japan). Hydroxylamine hydrochloride was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Lyophilized rabbit antiserum, which was obtained as the radioimmunoassay kit from 17β-E_2-6-(*O*-carboxymethyl)-oxime-BSA, was purchased from Seikagaku Co. (Tokyo, Japan). Deuterated solvents and tetramethylsilane (TMS) for proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy were purchased from Merck (Darmstadt, Germany). All other organic solvents were purchased from Kanto Chemical Co., Inc. and were of spectral or HPLC grade. Water used was purified by distillation and then deionized by ion-exchange columns. All other general reagents were of analytical grade.

Apparatus

^{1}H-NMR spectra were obtained with a JEOL (Tokyo, Japan) GSX-270 (270 MHz) or a GSX-500 (500 MHz) in a 5-mm o.d. tube at room temperature using TMS as an internal standard. The abbreviations used are s = singlet, brs = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, and m = multiplet. The coupling constants and half band widths are shown as Hz values. Thin-layer chromatography (TLC) for purification was carried out on Merck (Darmstadt, Germany) silica gel plates (60 F_{254}, d 0.25 mm, 20 cm × 20 cm) without activation. The melting points (m.p.) were measured between two cover glasses on a micro hot-stage apparatus (Yanagimoto, Kyoto, Japan) and were uncorrected. Liquid chromatography–mass spectrometry (LC-MS) was performed using a Hewlett Packard (Wilmington, DE, USA) HPLC HP 1100 system interfaced with a Finnigan Mat (San Jose, CA, USA) LCQ ion trap mass spectrometer through an electrospray (ESI) ion source. HPLC system consisted of a binary pump, an autosampler, a column oven, and a photodiode array detector. The HPLC elution was done using a GL Science (Tokyo, Japan) ODS 80-A (C18, 5 μm) column (4.6 mm i.d. × 150 mm) eluting at 0.6 mL/min with a linear gradient mode with 5 mM ammonium acetate–1% acetic acid in water: 5 mM ammonium acetate–1% acetic acid in methanol from 65:35 to 5:95 over 30 min. For the ion-trap mass spectrometer, nitrogen was used as the sheath (80 psi) and auxiliary (20 psi) gas to assist with nebulization. A potential of 4 kV was applied to the ESI needle. The metal capillary was maintained at 270°C. The CID energy was set at 30%. Ultraviolet/visible (UV/Vis) spectrophotometry was performed with a Shimadzu (Kyoto, Japan) UV-2100 (scan time, 200 nm/min; slit width, 2 nm) in a quartz cell (10 mm × 10 mm × 45 mm). Fluorescence spectrophotometry was performed with a Hitachi (Tokyo, Japan) F-3010 (scan time, 600 nm/min; slit width, 5 nm) in a quartz cell (10 mm × 10 mm × 45 mm). For an immunological experiment, the fluorescence intensities were measured as accumulative values for 30 s with a slit width of 20 nm. The Shimadzu HPLC system for the purity check consisted of two LC-10AD pumps, an SCL-10A system controller, an SIL-10_{XL} autoinjector, a DGU-12A degasser, and an SPD-10AV UV/Vis spectrophotometer. The HPLC separation was performed using a GL Sciences (Tokyo, Japan) ODS 80-A (C18, 5 μm) column (4.6 mm i.d. × 150 mm) and a Daicel Chemical Co. (Osaka, Japan) CHIRALPAK AS (silica gel coated with α-phenethylcarbamoyl amylose) column (4.6 mm i.d. × 250 mm) eluting at 0.6 mL/min with a linear gradient mode (water–acetonitrile, from 65:35 to 5:95 over 30 min) and at 0.5 mL/min with an isocratic mode (hexane–isopropanol = 50:50), respectively. The chiral HPLC mentioned above was also used to separate each isomer on the 6-position.

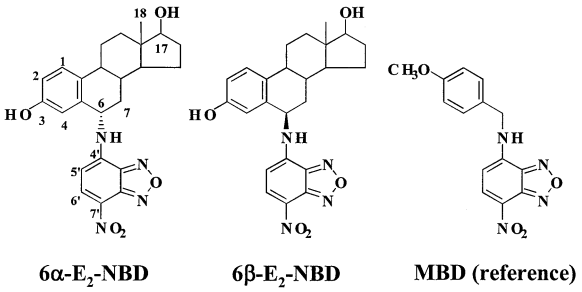

Fig. 2 Structures of 6α- and 6β-E_2-NBD, and MBD (reference).

Synthesis

6α- and 6β-aminoestra-1,3,5(10)-triene-3,17β-diol (6α- and 6β-amino-E_2) were synthesized from 6-oxoestra-1,3,5(10)-triene-3,17β-diol (6-oxo-E_2) through a zinc reduction of 6-hydroximeestra-1,3,5(10)-triene-3,17β-diol (6-oxime-E_2). First, 27.98 mg of 6-oxo-E_2, 34.40 mg of hydroxylamine hydrochloride and 37.82 mg of ammonium acetate in 5 mL of ethanol were refluxed for 3 h. After evaporating to a residue, the precipitate was washed with 3 mL of water three times by centrifugation. The 6-oxime-E_2 was confirmed by ^{1}H-NMR; it

was pure enough to apply the following zinc reduction.

6-Oxime-E_2: Yield (quantitatively). White amorphous. ^{1}H-NMR (270 MHz, $CDCl_3$-CD_3OD = 7:1) δ: 0.75 (3H, s, 18-CH_3), 3.14 (2H, dd, 18.1, 4.4 Hz, 7-H), 3.70 (1H, t, 8.5 Hz, 17α-H), 6.84 (1H, dd, 8.5, 2.4 Hz, 2-H), 7.18 (1H, d, 8.8 Hz, 1-H), 7.28 (1H, d, 2.4 Hz, 4-H).

Next, 30 mg of the oxime, 195.5 mg of zinc powder, and 91.14 mg of ammonium acetate were refluxed in 3 mL of ethanol and 1.5 mL of aqueous ammonium for 7 h. After cooling, excess zinc was removed by filtration. The desired amines were back-extracted using chloroform. The crude amines were applied on a Wakogel LP-60 C18 (4 g), washed with water, and eluted with methanol. The reduction was monitored by Ninhydrin reagent on TLC.

The resulting 6α- and 6β-amino-E_2 mixture (*ca*. 14.2 mg, crude) was subjected to the next reaction with 10 mg of NBD-F, which is a derivatization reagent for amines, in 1 mL of acetonitril–triethylamine (100:1) at room temperature in the usual manner.[9] A considerable amount of by-products resulted from a reaction that was modified not only on the 6-amino position, but also on the phenolic 3-hydroxyl position. Selective removal of NBD from the phenolic position was successful by an exchange reaction using cysteine. The desired 6α- and 6β-E_2-NBD were purified by silica-gel preparative TLC using two solvent systems (chloroform–methanol = 50:1, Rf[4] = 0.15 – 0.21, benzene–ethylacetate = 2:1, Rf[2] = 0.4 – 0.5) as an isomeric mixture (3.2 mg). Each isomer was separated using a chiral HPLC system as described in the apparatus section. The purities were confirmed as being at least > 99.5% by HPLC-UV using a wavelength of 330 nm, corresponding to the benzofurazan moiety; t_R = 26 min (as an isomeric mixture) on a C18 column, t_R = 15 min (6β-E_2-NBD) and 20 min (6α-E_2-NBD) on a chiral HPLC column.

6α-E_2-NBD: Yield (1.1 mg). Orange amorphous (from acetonitrile). m.p.: 187 – 190˚C. ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.78 (3H, s, 18-CH_3), 3.66 (1H, t, J = 8.7 Hz, 17α-H), 5.05 (1H, brs, $h_{1/2}$ = 24 Hz, 6β-H), 6.51 (1H, brs, 5′-H), 6.75 (1H, dd, 8.3, 2.8 Hz, 2-H), 6.69 (1H, d, 2.8 Hz, 4-H), 7.26 (1H, d, 8.7 Hz, 1-H), 8.58 (1H, brs, 6′-H). UV/Vis (ethanol): 224, 279, 333, 471 nm. ESI/MS *m/z*: (positive) 451(M + H)$^+$, (MS/MS from 451) 271(M + H – NBD – NH)$^+$, (negative) 449(M – H)$^-$.

6β-E_2-NBD: Yield (2.1 mg). Orange needles (from acetonitrile–benzene). m.p.: 238 – 242˚C. ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.83 (3H, s, 18-CH_3), 3.68 (1H, t, 8.5 Hz, 17α-H), 5.27 (1H, brs, $h_{1/2}$ = 12 Hz, 6α-H), 6.48 (1H, d, 8.7 Hz, 5′-H), 6.68 (1H, dd, 8.7, 2.8 Hz, 2-H), 6.71 (1H, brs, 4-H), 7.22 (1H, d, 8.7 Hz, 1-H), 8.52 (1H, d, 9.2 Hz, 6′-H). UV/Vis (ethanol): 224, 280, 335, 471 nm. ESI/MS *m/z*: (positive) 451(M + H)$^+$, (MS/MS from 451) 271(M + H – NBD – NH)$^+$, (negative) 449(M – H)$^-$. Each configuration on the 6-position was assigned by reference to the NMR data reported by Adamczyk *et al*.[10,11]

Fluorescence spectrum measurements

After 6α- or 6β-E_2-NBD was dissolved in ethanol, the concentration was confirmed by determining the ε value of MBD (ε = 8000 at 335 nm, benzofurazan moiety) using a UV/Vis spectrophotometer. The ε value of the absorption maximum around 330 nm is not affected by the solvent polarity or the substituents on the benzyl amino moiety.[5] A stock solution was prepared as a 100 μM solution, 30 μL of which was transferred into a tube and diluted up to 3 mL using the desired solvent (final concentration, 1 μM). The pH effect was demonstrated in 50 mM sodium–phosphate buffer. MBD was always measured together under the same conditions as a reference. The BSA concentration was calculated as MW 67000.

Immunological experiment

We performed an immunological experiment following a method reported by Lindner *et al*. with a slight modification.[12] Tris–HCl buffer (0.05 M; pH 8.0) containing 0.1 M NaCl and 0.05% gelatin was used as an assay buffer. Lyophilized antiserum was reconstituted with 5.0 mL of the assay buffer. Fifty microliters of aqueous E_2 solution and 650 μL of the assay buffer were added to assay tubes. After 200 μL of the antibody solution was added to the above tubes and incubated at room temperature for 30 min, 100 μL of aqueous 0.2 μM 6β-E_2-NBD solution (final concentration 20 nM) was added to all tubes, and incubated at 37˚C for 60 min. After cooling, each solution was measured by the method described above.

Results and Discussion

Syntheses of 6α- and 6β-amino-E_2-NBD

The 6-position in estrogens is very desirable as a labeling site for probes and/or an introduction site for the bridge of hapten, because it is apart from both the steroidal A ring and D ring, where estrogens are usually metabolized. In particular, 6α-isomer is preferred because it avoids any steric effect from the 18-angularmethyl group and the 17β-hydroxyl group. NBD-F is known to be a fluorescence derivatization reagent of amines for HPLC-FL. However, it can also react with phenols and thiols under strong basic conditions. The selective replacement of NBD from the phenolic position to a thiol position is an interesting phenomenon, which can be considered for a geographical study of a biopolymer surface. A further investigation on this matter is being done; the data will be reported elsewhere. Each epimer on the 6-position was successfully separated from the others using a chiral column. Exhaustive purification was necessary for this purpose, because interference from the impurities, which might have stronger fluorescence than the desired compound in aqueous solution, should be avoided.

Effect of solvents on the fluorescence intensity

The fluorescence spectra of 6α- and 6β-E_2-NBD in various solvents were measured. Each fluorescence intensity under the optimal wavelength is summarized in Fig. 3. The fluorescence intensities of both 6α- and 6β-E_2-NBD tended to reflect the hydrophobicity of the solvent as well as that of MBD, which was used as a reference compound. The intensities in chloroform (6α-E_2-NBD, 22.0; 6β-E_2-NBD, 18.3) and benzene (6α-E_2-NBD, 39.8; 6β-E_2-NBD, 38.1), which are more hydrophobic, were less than that in ethyl acetate (6α-E_2-NBD, 155; 6β-E_2-NBD, 168). Other interactions, such as charge transfer against the solvent, may result in lower intensities. No remarkable difference was observed between 6α- and 6β-E_2-NBD in any of the solvents examined. A slightly different pattern was observed in two of them compared with MBD (fluorescence intensities: MBD in propanol > MBD in acetonitril, E_2-NBD in propanol < E_2-NBD in acetonitril *etc*.), which seemed to result from the ionic character of the phenolic position on the estradiol moiety, probably due to hydrogen-bond formation. We also observed a blue shift in the hydrophobic solvents. In each solvent, the fluorescences of 6α-E_2-NBD and 6β-E_2-NBD were slightly more quenched than that of MBD. We supposed that the richer π-electron on the benzyl moiety of 6α- and 6β-E_2-NBD caused this difference. A similar

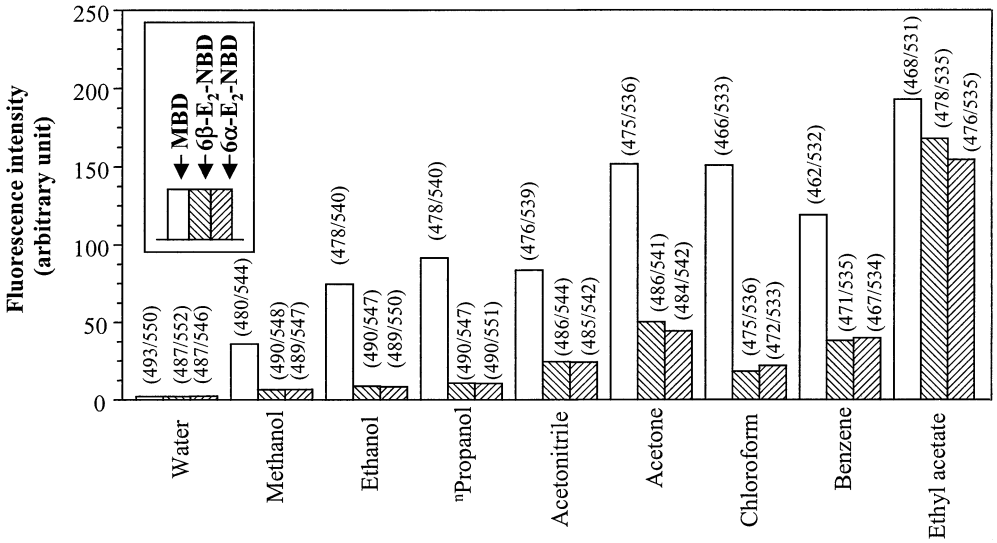

Fig. 3 Effect of solvents on the fluorescence intensities of 6α- and 6β-E_2-NBD. Conditions: concentration, 1 μM; scan time, 600 nm/min; bandwidth, 5 nm each. The optimal wavelengths of the excitation and emission are shown in parentheses.

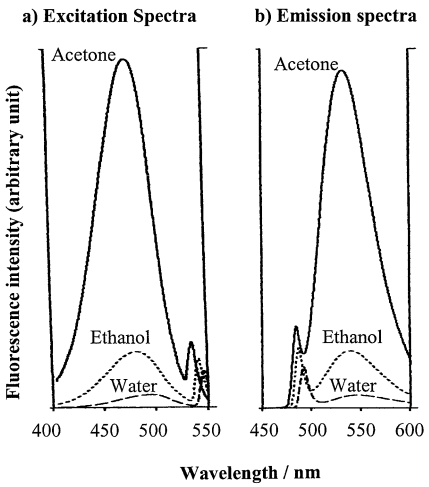

Fig. 4 Typical excitation and emission spectra of 6β-E_2-NBD in water, ethanol, or acetone. a) Excitation spectra, b) emission spectra. Conditions: concentration, 1 μM; scan time, 600 nm/min; bandwidth, 5 nm each.

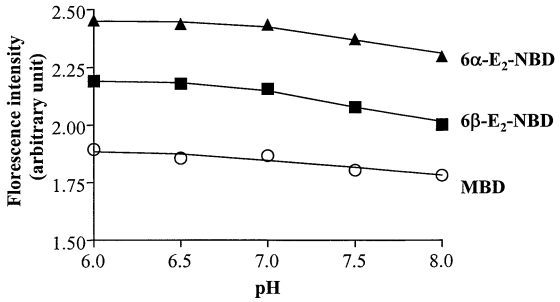

Fig. 5 Effect of the pH value on the fluorescence intensities of 6α- and 6β-E_2-NBD. Conditions: concentration, 1 μM in 50 mM sodium phosphate buffer; scan time, 600 nm/min; wavelength, Ex. 483 nm, Em. 540 nm for E_2-NBD, Ex. 485 nm, Em. 535 nm for MBD; bandwidth, 5 nm each.

difference was reported between MBD and BBD,[5] which has no electron-donating group on the benzyl moiety. The typical excitation and emission spectra using 6β-E_2-NBD are shown in Fig. 4.

Effect of pH on the fluorescence intensity

The pH effect on the fluorescence intensity was examined next. No remarkable change in the pH was observed in the case of MBD at any level of the pH examined, although a slight decrease in pH was observed on both 6α- and 6β-E_2-NBD in alkali solution, as shown in Fig. 5. This decrease can be explained as having resulted from an increased electron density due to dissociation of the phenolic proton.

Effect of BSA on the fluorescence intensity

We also examined the effect of BSA on the fluorescence intensity, as shown in Fig. 6. In this case, 6α- and 6β-E_2-NBD showed different patterns from each other. 6β-E_2-NBD showed the same hydrophobic probe ability as MBD; however, it was more sensitive at the lower concentration of BSA (fluorescence intensities: MBD < 6β-E_2-NBD in 0.01 mM BSA, MBD > 6β-E_2-NBD in 0.03 mM BSA) and plateaued at around 0.05 mM. On the other hand, a smaller change in fluorescence was observed in the case of 6α-E_2-NBD. This phenomenon might be due to a hydrophobic interaction between the probes and BSA. It is known that there are several binding sites on the BSA surface for drugs. These two probes therefore might be binding at different sites and/or with different affinities on the BSA surface. A similar observation was reported when *N*-alkyl-NBDs were used as hydrophobic probes for a binding study of BSA by Matsushita *et al.*[13] This finding is being investigated.

Effect of anti 17β-E_2 antiserum on the fluorescence intensity

We next made a preliminary examination of the change in fluorescence caused by anti 17β-E_2 antiserum. A fluorescence enhancement with anti 17β-E_2 antiserum was observed in the case of 6α- and 6β-E_2-NBD, particularly for 6α-E_2-NBD, even though the concentration was extremely low. This fluorescence

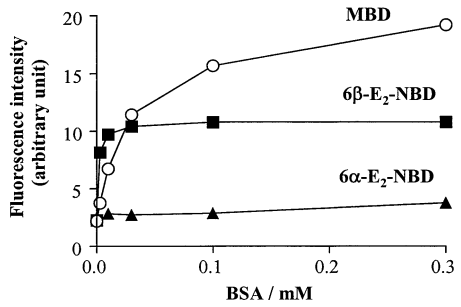

Fig. 6 Effect of BSA on the fluorescence intensities of 6α- and 6β-E_2-NBD. Conditions: concentration, 1 μM; scan time, 600 nm/min; wavelength, Ex. 483 nm, Em. 540 nm for E_2-NBD, Ex. 485 nm, Em. 535 nm for MBD; bandwidth, 5 nm each. The BSA concentration was calculated as M.W. 67000.

enhancement of 6α-E_2-NBD was inhibited (20.4%) by adding an E_2 (13.5 ng) solution which resulted from the competition. However, the reproducibility of this reaction and the sensitivity were still unsatisfying under the current conditions. Since the immunological reaction could be monitored by this system, we are still investigating the possibility of performing a homogeneous immunoassay.

Conclusion

In conclusion, novel fluorescent probes, 6α- and 6β-E_2-NBD, which are estradiol mimic BzN-NBD derivatives utilizing the 6-position as the benzyl moiety, were prepared and characterized. The fluorescence of these compounds was quenched under aqueous solution, and could then be recovered under hydrophobic circumstances, such as in organic solvents, or by adding BSA or the anti E_2 antibody. Although the two isomers showed the same behaviors in the organic solvents examined, they showed the different responses against BSA and the anti E_2 antibody. The phenomenon can therefore be explained as having resulted in the interaction towards the estradiol moiety. The data clearly indicate the possibility of controlling the fluorescence of drug or hormone mimic BzN-NBD derivatives using either their antibodies or their receptors, which can recognize the structures. We are currently investigating homogeneous fluorescence immunoassays and a cytosolic study of estrogen receptors *via* confocal fluorescence microscopy to determine further possible applications of these probes.

Acknowledgements

We thank Dr. Shinichi Miyairi of the Department of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, for valuable discussion.

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