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Development of water-soluble fluorogenic reagents having a 2,1,3-benzoxadiazole structure and their application to the determination of peptides

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Water-soluble fluorogenic reagents having a 2,1,3-benzoxadiazole (benzofurazan) structure, 2-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)ethanesulfonic acid (ES-ABD-F), 4-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)benzenesulfonic acid (*p*-BS-ABD-F) and 3-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)benzenesulfonic acid (*m*-BS-ABD-F), were synthesized and evaluated for their fluorescence characteristics and reactivities with peptides. Although these reagents showed no fluorescence, their derivatives with valine exhibited fluorescence at maximum wavelengths from 562 to 566 nm with excitation from 424 to 426 nm. The derivatives with ES-ABD-F and *m*-BS-ABD-F showed a similar sensitivity to that of 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F), a previously developed fluorogenic reagent for amino compounds. The fluorescence intensity obtained with the *p*-BS-ABD-F derivative was about one-third of that of the others. Angiotensin II (ANG II), an octapeptide, was successfully derivatized with ES-ABD-F and *m*-BS-ABD-F and determined by using high performance liquid chromatography (HPLC) with fluorescence detection. Good linearity was obtained between the amount of peptide (1–200 pmol) and the peak area of their derivatives (correlation coefficients > 0.999). The detection limits of the ANG II derivatives with ES-ABD-F and *m*-BS-ABD-F were 210 and 270 fmol, respectively. *m*-BS-ABD-F, however, showed some disadvantages in the simultaneous determination of peptides: a possible interference with the separation of derivatized peptides due to some blank peaks, and its complicated and changeable pH-reactivity profile. From these results, ES-ABD-F was recommended for the sensitive and simultaneous determination of peptides. The derivatization reaction with ES-ABD-F commonly reached a plateau at 60 °C and pH > 8.5 for several hours. The peptide derivatives of ES-ABD-F (ANG I, ANG II, ANG III, bradykinin, and substance P) were well separated by reversed phase HPLC. In addition, the amyloid β-peptide (1–40) [Aβ(1–40)] derivative with ES-ABD-F was successfully eluted from a YMC-pack C4 (150×4.6 mm id, 5 μm particle size) under conditions such that underivatized Aβ(1–40) was not eluted, suggesting that the derivatization with ES-ABD-F decreased the adsorption of hydrophobic peptides to the HPLC stationary phase.

Introduction

The presence of various endogenous peptides in living animals has been reported in the last two decades. Accompanied by the development of peptides as therapeutic agents, the biological activities of peptides in neural, hormonal or immunological regulation have been greatly elucidated. Also, there has been growing demand for a sensitive and specific analytical method for peptides.

Bioassay, radioimmunoassay (RIA) and enzyme immunoassay (EIA), because of their high sensitivities, have often been employed to determine trace amounts of peptides. However, their usefulness is limited, as they do not show sufficient specificity so that chromatographic separation is necessary before assay. In addition, they are not able to detect more than one analyte at a time.

On the other hand, as previously reviewed,[1] numerous analytical methods for peptides have been developed using HPLC or capillary electrophoresis (CE) combined with various detection methods such as ultraviolet (UV), mass spectrometric (MS) and fluorescence (FL) techniques.

Among these methods, a pre- or post-column fluorescence derivatization method combined with an HPLC-FL system is one of the most effective approaches to detect femto- to picomole amounts of peptides.[2,3] In this method, peptides are often derivatized with fluorescent reagents at their N-terminal amino group to make them detectable by spectrofluorimetry. For instance, dansyl chloride (Dns-Cl),[4,5] fluorescein-5-isothiocyanate (FITC)[6] and fluorenylmethyloxycarbonyl chloride (FMOC)[7,8] have been used for the pre-column derivatization of peptides. *o*-Phthalaldehyde (OPA),[9–11] naphthalene-2,3-dialdehyde (NDA)[12,13] and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA)[14] have been applied to the post-column derivatization of peptides. Fluorescamine can be used as a post- or pre-column derivatization reagent.[15–17] Although, as described in detail elsewhere,[2] some of these reagents have disadvantages such as instability of derivatives, large blank peaks or no reactivity toward secondary amino groups.

In our laboratory, we have also developed a series of fluorogenic reagents having a 2,1,3-benzoxadiazole (benzofurazan) structure. Among them, 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F)[18] and 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F)[19] have been applied to the determination of primary and secondary amines. Application of these reagents to peptide analysis has already been reported: NBD-F for opioid peptides and bradykinin in rat brain and plasma,[20] DBD-F for calcitonin standard material[21] and for a series of synthesized peptides having an N-terminal proline residue.[22]

However, it is generally understood that adsorption of peptides can be a serious problem in their analysis. A loss of peptides occurs during handling of samples and also in their

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separation because of binding to the stationary phase in the HPLC column or the capillary wall in CE, resulting in a deterioration of precision and accuracy of the assay. Derivatization using fluorescent reagents further exaggerates this tendency because the high hydrophobicity of these reagents results in a decrease in the solubility of the analyte to aqueous media.[23] For example, in the derivatization of calcitonin with DBD-F,[21] adsorption of the calcitonin derivative on the surface of the sample tube was observed, and hence the addition of sodium dodecyl sulfate (SDS) was necessary to solve the problem. We also have experienced a gradual decrease in the peak area of the NBD-F–angiotensin II (ANG II) derivative when it was allowed to stand in a polypropylene tube, which might be due to adsorption but not the degradation of the derivative.

Considering the above background, we aimed to develop water-soluble fluorogenic derivatization reagents for the peptides having a benzofurazan structure. In this paper, we describe the synthesis of three water-soluble reagents, 2-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)ethanesulfonic acid (ES-ABD-F), 4-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)-benzenesulfonic acid (*p*-BS-ABD-F) and 3-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)benzenesulfonic acid (*m*-BS-ABD-F). These reagents were synthesized by introducing a sulfonic acid residue on the chlorosulfonyl group of 4-chrolosulfonyl-7-fluoro-2,1,3-benzoxadiazole (CBD-F), which led to high solubility in an aqueous medium. The fluorescence properties of the derivatives of amino acids and their reactivity towards peptides were investigated. Finally, sensitivity and chromatographic separation were studied by use of an HPLC-FL system.

Experimental

Materials and reagents

DBD-F was purchased from Tokyo Kasei (Tokyo, Japan). 4-(Chlorosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (CBD-F) was synthesized according to the method reported previously.[24] Taurine was obtained from Wako Pure Chemical Industries (Osaka, Japan). 3-Aminobenzenesulfonic acid and 4-amino-benzenesulfonic acid were purchased from Tokyo Kasei. Ethylenediamine tetraacetic acid disodium salt (Na_2EDTA) was supplied by Kanto Chemical (Tokyo, Japan). Amyloid β-peptide (1–40) [Aβ(1–40)] was purchased from Bachem (Bubendorf, Switzerland). All other peptides were obtained from the Peptide Institute (Osaka, Japan). All other chemicals were of analytical or guaranteed reagent grade and were used without further purification.

Unless described otherwise, 0.2 M borate buffers (pH 8.0, 8.5 or 9.0) containing 2 mM Na_2EDTA and 0.2 M phosphate buffers (pH 6.5, 7.0 or 7.5) containing 2 mM Na_2EDTA were used in all the investigations.

Apparatus

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a JEOL (Tokyo, Japan) JMN-LA500 spectrometer with tetramethylsilane as an internal standard in acetonitrile-d_3 (abbreviations used: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad). Mass spectra were obtained on a ThermoQuest (Tokyo, Japan) LCQ mass spectrometer using electrospray ionization (ESI). Fluorescence spectra were measured on a Hitachi (Tokyo, Japan) F-4500 instrument. A Shimadzu (Kyoto, Japan) LC-8A high performance liquid chromatograph system equipped with a Shimadzu SPD-6A UV detector and an L-column ODS (250 × 20 mm id, 5 μm particle size) (Chemicals Inspection and Testing Institute, Tokyo, Japan) was used for the purification of the synthesized products. The high-performance liquid chromatograph used for the analysis of derivatives was constructed from a Hitachi L-6300 pump, a Rheodyne (Cotati, CA, USA) Model 7125 injector, a Hitachi L-5025 column oven, a Hitachi F-1080 fluorescence detector and a Hitachi D-2500 integrator. Unless specially mentioned, the fluorescence detector was set at 426 and 564 nm for the excitation and emission, respectively. An L-column ODS (150 × 4.6 mm id, 5 μm particle size) (Chemicals Inspection and Testing Institute) or a YMC-pack C4 (150 × 4.6 mm id, 5 μm particle size) (YMC, Tokyo, Japan) was used for the analysis. The total flow rate was fixed at 1.0 mL min^{-1}. Unless noted otherwise, a linear gradient elution from 100% eluent A [acetonitrile–water–trifluoroacetic acid (TFA) (250 + 750 + 0.5)] to 30% eluent B [acetonitrile–water–TFA (750 + 250 + 0.5)] over 30 min with an L-column ODS (150 × 4.6 mm id, 5 μm particle size) was adopted for the separation. The reaction temperature was controlled by a Taiyo (Tokyo, Japan) TAL-1 heater.

Synthesis of 2-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)ethanesulfonic acid (ES-ABD-F)

To a solution of CBD-F (0.20 g, 0.85 mmol) in 75% acetonitrile (15 mL) was added taurine (0.11 g, 0.87 mmol) in 0.1 M borate buffer (pH 8.5, 8 mL), and the mixture was stirred for 5 min at ambient temperature. After the addition of 2 M hydrochloric acid to acidify the reaction mixture, it was evaporated under reduced pressure and the residue was chromatographed on silica gel with ethyl acetate–methanol (4 + 1) to afford a crude product. The crude product was purified on an L-column ODS (250 × 20 mm id, 5 μm) using HPLC with acetonitrile–water–TFA (180 + 820 + 0.5) as the eluent. ES-ABD-F was eluted at 7.5 min with 12 mL min^{-1} flow rate. The eluate was collected and evaporated under reduced pressure to afford ES-ABD-F (23 mg, yield 8.3%) as a pale yellow powder. ^{1}H NMR, δ_H 8.00 (1H, dd, J_{ab} = 7.8 Hz, J_{aF} = 4.3 Hz, H_a), 7.24 (1H, dd, J_{ab} = 7.8 Hz, J_{bF} = 9.8 Hz, H_b), 3.35 (2H, m), 3.11 (2H, m). ESI-MS, m/z 324 ($[M - H]^-$).

Synthesis of 4-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)benzenesulfonic acid (*p*-BS-ABD-F)

To a solution of CBD-F (0.10 g, 0.42 mmol) in 60% acetonitrile (5 mL) were added 4-amino-benzenesulfonic acid (0.11 g, 0.64 mmol) and 0.1 mL of pyridine and the mixture was stirred for 5 min at ambient temperature. After the addition of 2 M hydrochloric acid to acidify the reaction mixture, it was evaporated under reduced pressure and the residue was chromatographed on silica gel with ethyl acetate–methanol (4 + 1) to afford a crude product. The crude product was purified on an L-column ODS (250 × 20 mm id, 5 μm) using HPLC with acetonitrile–water–TFA (250 + 750 + 0.5) as the eluent. *m*-BS-ABD-F was eluted at 11 min with 12 mL min^{-1} flow rate. The eluate was collected and evaporated under reduced pressure to afford *p*-BS-ABD-F (67 mg, yield 42%) as a oily yellow powder. ^{1}H NMR, δ_H 8.09 (1H, dd, J_{ab} = 7.8 Hz, J_{aF} = 4.1 Hz, H_a), 7.59 (2H, d, J = 8.6), 7.22–7.18 (3H, m). ESI-MS, *m/z* 372 ($[M - H]^-$).

Synthesis of 3-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)benzenesulfonic acid (*m*-BS-ABD-F)

To a solution of CBD-F (0.10 g, 0.42 mmol) in 60% acetonitrile (5 mL) were added 3-aminobenzenesulfonic acid (0.11 g, 0.64 mmol) and 0.1 mL of pyridine, and the mixture was stirred for 5 min at ambient temperature. After the addition of 2 M hydrochloric acid to acidify the reaction mixture, it was

evaporated under reduced pressure and the residue was chromatographed on silica gel with ethyl acetate–methanol (4 + 1) to afford a crude product. The crude product was purified on an L-column ODS (250 × 20 mm id, 5 μm) using HPLC with acetonitrile–water–TFA (260 + 740 + 0.5) as the eluent. *m*-BS-ABD-F was eluted at 10 min with 12 mL min^{-1} flow rate. The eluate was collected and evaporated under reduced pressure to afford *m*-BS-ABD-F (43 mg, 27%) as a oily yellow powder. ^1H NMR, δ_H 8.02 (1H, dd, J_{ab} = 7.8 Hz, J_{aF} = 4.1 Hz, H_a), 7.51 (1H, s), 7.43 (1H, d, J = 7.3 Hz), 7.27 (1H, m), 7.21 (1H, d, J = 8.2 Hz), 7.18 (1H, dd, J_{ab} = 7.8 Hz, J_{bF} = 9.7 Hz, H_b). ESI-MS, *m/z* 372 ($[M - H]^-$).

Measurement of fluorescence spectrum of valine derivative

A 50 μL portion of each fluorogenic reagent solution, ES-ABD-F, *m*-BS-ABD-F, *p*-BS-ABD-F and DBD-F, in 80% acetonitrile (20 mM), was mixed with the same volume of a valine solution (1 mM) in the borate buffer (pH 9.0). The mixture was allowed to stand for 4 h at 60 °C. After the derivatization reaction, a 50 μL aliquot of the reaction mixture was injected into the L-column ODS (150 × 4.6 mm id, 5 μm). HPLC separation was performed isocratically using acetonitrile–water–TFA (250 + 750 + 0.5) as the eluent. The eluate was collected at the retention time of each valine derivative. The fluorescence spectra of these eluates were measured on a spectrofluorimeter with a 5 nm excitation and emission slit width and a 1200–2400 nm min^{-1} scan speed.

pH profile of derivatization reaction and detection limit of valine derivative

A portion of each fluorogenic reagent solution, ES-ABD-F, *m*-BS-ABD-F and *p*-BS-ABD-F, in 80% acetonitrile (20 mM), was mixed with the same volume of the valine solutions (50 μM) in the buffers in the pH range 6.5–9.0. Each reaction mixture was allowed to stand at 60 °C and a 1 μL aliquot of the reaction mixture was periodically injected onto the HPLC column. The fluorescence detector was set at the maximum excitation and emission wavelengths obtained with the investigation described above. HPLC separation was performed isocratically using acetonitrile–water–TFA (250 + 750 + 0.5) as the eluent with an L-column ODS (150 ×4.6 mm id, 5 μm).

The detection limits were determined under the conditions showing the highest yield of the derivatization reaction: 60 °C/pH 8.5/2 h for ES-ABD-F, 60 °C/pH 7.5/4 h for *p*-BS-ABD-F and 60 °C/pH 7.5/8 h for *m*-BS-ABD-F. To compare the sensitivity with DBD-F, the valine derivative with DBD-F was also prepared at 60 °C/pH 9.3 for 1 h. The reaction conditions for DBD-F were taken from a previous report[19] and the completion of the reaction was confirmed by periodic analysis using the HPLC system. Each detection limit (signal-to-noise ratio = 3) was calculated from the chromatogram obtained with a 1 μL injection of the diluted reaction mixture. HPLC separation was performed isocratically using acetonitrile–water–TFA (250 + 750 + 0.5) as the eluent with an L-column ODS (150 × 4.6 mm id, 5 μm).

Comparison of reactivity to N-terminal amino group of peptides between ES-ABD-F and *m*-BS-ABD-F

ES-ABD-F and *m*-BS-ABD-F were used as the fluorogenic reagent. A 30 μL aliquot of the fluorogenic reagent solution in 80% acetonitrile (20 mM) was mixed with the same volume of ANG II aqueous solution (125 μM) and each buffer (pH 7.0–9.0). The reaction mixture was allowed to stand for 6 h at 50 °C. After the reaction a 10 μL portion of 0.5 M acetic acid was added to the reaction mixture to stop the reaction. A 1 μL aliquot of the reaction mixture was injected onto the L-column ODS (150 × 4.6 mm id, 5 μm).

At the optimum pH obtained, the time course of the derivatization reaction to ANG II was investigated. The derivatization mixtures were prepared in the same way as described above using the borate buffers of pH 8.5 and 8.0 for ES-ABD-F and *m*-BS-ABD-F, respectively. A 1 μL aliquot of each reaction mixture was periodically injected onto the L-column ODS (150 × 4.6 mm id, 5 μm).

Linearity and detection limit of ANG II derivative

ES-ABD-F and *m*-BS-ABD-F were used for this investigation. A 50 μL aliquot of each fluorogenic reagent solution in 80% acetonitrile (20 mM) was mixed with the same volume of ANG II aqueous solution (1–200 μM) and the buffer. The mixture was allowed to stand at 50 °C for 10 h. The borate buffers of pH 8.5 and 8.0 were used for ES-ABD-F and *m*-BS-ABD-F, respectively. After the derivatization, a 3 μL aliquot of each reaction mixture was injected onto the HPLC column. Detection limits (signal-to-noise ratio 3) were calculated from the chromatograms obtained with 1.0 pmol/3 μL.

Reaction conditions and reactivity of ES-ABD-F to peptides

A 50 μL aliquot of ANG II solution (125 μM) was mixed with the same volume of the fluorogenic reagent solution in 80% acetonitrile and borate buffer. The reaction temperature (50–60 °C), pH of the buffer (8.5–9.0) and reagent concentration (20–60 mM) were changed. The time course for these reaction mixtures were investigated by periodic injection onto the HPLC column. The HPLC conditions described in the Apparatus section were adopted for the separation.

Bradykinin and leucine-enkephalin were also derivatized with ES-ABD-F at 60 °C/pH 8.5. A 50 μL aliquot of peptide aqueous solution (125 μM) was mixed with the same volume of the fluorogenic reagent solution in 80% acetonitrile (20 mM) and borate buffer (pH 8.5). The mixtures were allowed to stand at 60 °C and were periodically injected onto the HPLC column. The HPLC conditions described in the Apparatus section was adopted for leucine-enkephalin. For the determination of the bradikinin derivative, a linear gradient elution from 87% eluent A [acetonitrile–water–TFA (100 + 900 + 0.5)] to 22% eluent B [acetonitrile–water–TFA (750 + 250 + 0.5)] from 0 to 20 min was adopted.

Separation and detection of peptide derivatives of ES-ABD-F by HPLC-FL

To a 50 μL portion of a mixture of ANG I, ANG II, ANG III, bradykinin and substance P were added the same volume of ES-ABD-F solution in 80% acetonitrile (20 mM) and borate buffer (pH 8.5). The reaction mixture was allowed to stand for 4 h at 60 °C. The concentration of each peptide in the reaction mixture was 23.8 μM. A 1 μL aliquot of the reaction mixture was injected onto the L-column ODS (150 × 4.6 mm id, 5 μm). Linear gradient elution from 87% eluent A [acetonitrile–water–TFA (100 + 900 + 0.5)] to 22% eluent B [acetonitrile–water–TFA (750 + 250 + 0.5)] over the first 10 min was performed, which was followed by another linear gradient elution from 22 to 32% eluent B over the next 30 min (from 10 to 40 min).

A 50 μL aliquot of Aβ(1–40) solution (50 μM) in the buffer was mixed with the same volume of the fluorogenic reagent solution in 80% acetonitrile (20 mM). The reaction mixtures

were allowed to stand at 50 °C for 10 h and a 1 μL aliquot of the reaction mixture was injected onto a YMC-pack C4 (150 × 4.6 mm id, 5 μm). Isocratic elution with eluent A [acetonitrile–water–TFA (250 + 750 + 0.5)] for the first 5 min was followed by another linear gradient elution from 100% eluent A to 87.5% eluent B [acetonitrile–water–TFA (750 + 250 + 0.5)] over the next 30 min (from 5 to 35 min).

Results and discussion

Synthesis of ES-ABD-F, *m*-BS-ABD-F and *p*-BS-ABD-F

An outline of the synthetic route to water-soluble fluorogenic reagents having a benzofurazan structure is shown in Scheme 1. In this reaction, as reported previously,[25] not only the chlorosulfonyl group of CBD-F but also its 7-fluoro moiety can be replaced with an amino group, resulting in the generation of by-products **1** and **2**. Actually we obtained three main products in the synthesis of each reagent. Although these products could not be separated from each other by silica gel column chromatography, we were able to separate them and obtain final products by using HPLC with an ODS column to purify them. The structures of these final products (Scheme 1) were identified from their mass and NMR spectra. All the final products showed the expected mass number as the negative pseudo-molecular ion ([M − H]$^-$). Although by-product **1** and the final product have the same molecular weight, the NMR spectra of each final product showed the aromatic H–F couplings (J_{aH} = 4.1–4.3 Hz, J_{bH} = 9.7–9.8 Hz). These coupling constants were similar to those of DBD-F in dimethyl-d_6 sulfoxide reported previously (J_{aH} = 4.4 Hz, J_{bH} = 10.0 Hz).[25] Only the H_b–F coupling constant of *p*-BS-ABD-F could not be calculated owing to the overlapping signals of other protons.

Measurement of fluorescence spectrum of valine derivative

Under the derivatization conditions of 60 °C for 4 h, all the reagents, DBD-F, ES-ABD-F, *m*-BS-ABD-F and *p*-BS-ABD-F, gave the peaks for the respective valine derivatives in their HPLC traces and these peaks were well separated from the blank peaks. The fluorescence properties of valine derivatives obtained with the combined HPLC effluent are given in Table 1. The valine derivatives with the newly developed reagents showed excitation and emission wavelengths, 424–426 and 562–566 nm, respectively, which were almost the same as those of DBD-F.

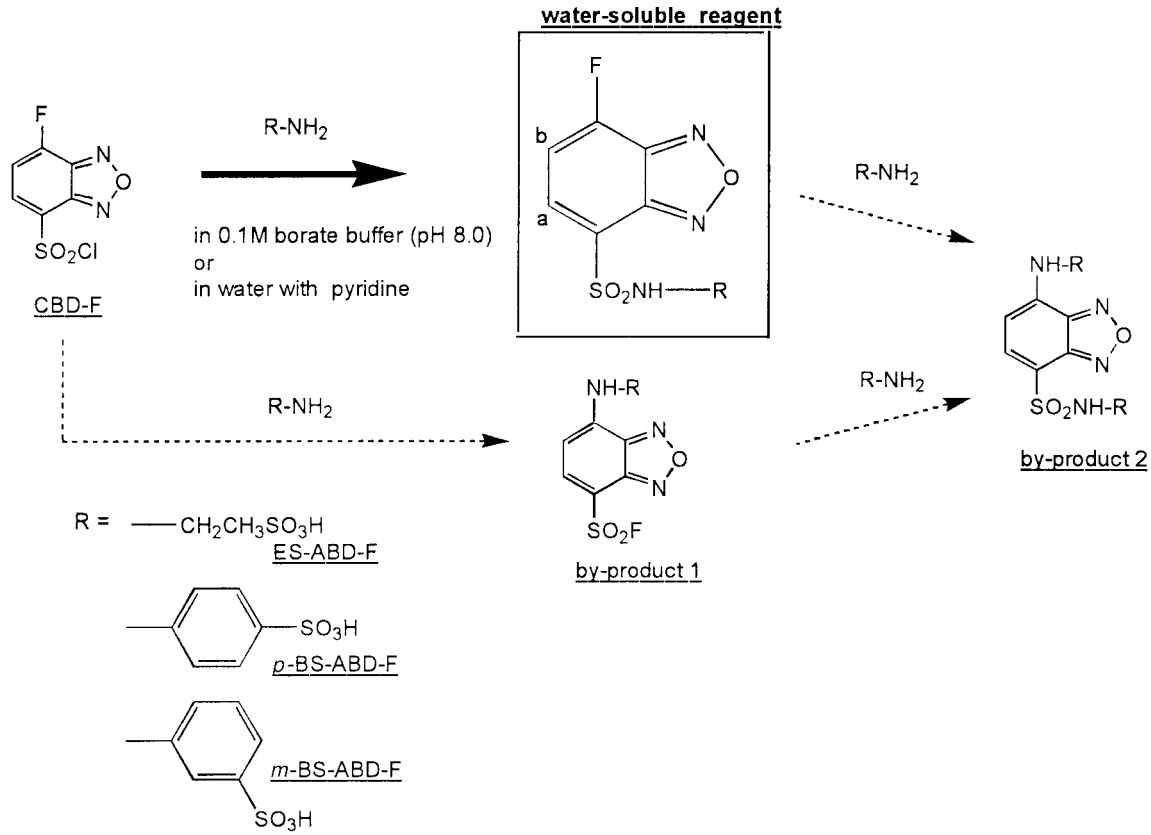
pH profile for derivatization reaction and detection limit of valine derivative

We have previously developed DBD-F as a derivatization reagent for amino compounds having a benzofurazan structure. DBD-F showed a higher reactivity to amino compounds at higher pH[25] owing to the deprotonation of the amino group.

Table 1 Maximum wavelengths and detection limits of valine derivatives of DBD-F, ES-ABD-F, *p*-BS-ABD-F and *m*-BD-ABD-F

Reagent	Wavelength/nm		Detection limit[a]/fmol
	λ_{ex}	λ_{em}	
ES-ABD-F	424	566	190
p-BS-ABD-F	426	562	640
m-BS-ABD-F	426	564	230
DBD-F	424	566	350

[a] Detection limits (signal-to-noise ratio = 3) were calculated from the chromatogram obtained by HPLC using acetonitrile–water–TFA (250 + 750 + 0.5) as the eluent.



Scheme 1 Outline of synthetic route and structures of water-soluble fluorogenic reagents having a benzofurazan structure.

Considering this result, the reactivity–pH profiles of newly developed reagents were investigated.

The time courses of the derivatization reactions at the different pH values are depicted in Scheme 1. ES-ABD-F showed a higher reactivity with valine at pH > 8.5, indicating a similar pH–reactivity profile to that of DBD-F. The derivatization reaction reached a plateau within 2 h at 60 °C/pH 8.5 [Fig. 1(A)], indicating its high reactivity toward the amino group.

In contrast, both *m*-BS-ABD-F and *p*-BS-ABD-F showed the highest reactivity at pH 7.5 [Fig. 1(B) and (C)], but their pH–reactivity profiles were complicated. Although it has never been clearly explained, the strong electron withdrawing effect of the benzenesulfonic acid group may be the cause. The pK_b value of the amino group of the analyte and the pK_a value of the sulfonylamide group of the reagents should affect their reactivity. This resulted in a lower reactivity at pH > 7.5. The highest yields were obtained after 4 and 8 h of reaction at pH 7.5 for *p*-BS-ABD-F and *m*-BS-ABD-F, respectively. The detection limits obtained under the optimized conditions are given in Table 1. ES-ABD-F and *m*-BS-ABD-F showed similar detection limits to that of DBD-F, and were expected to be useful for the highly sensitive detection of amino compounds. The *p*-BS-ABD-F derivative gave significantly weaker fluorescence than the others. From these results, ES-ABD-F and *m*-BS-ABD-F were chosen for further investigations.

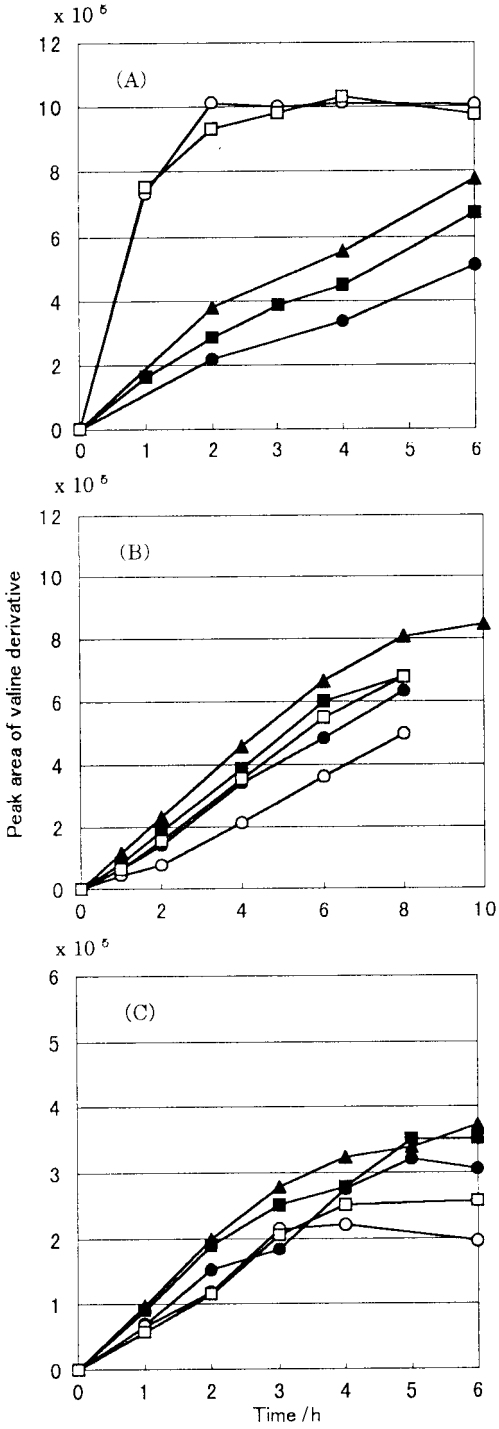


Fig. 1 Time course of derivatization reaction to valine with ES-ABD-F, *p*-ABS-ABD-F and *m*-ABS-ABD-F at various pH values. (A) ES-ABD-F: ●, pH 7.0; ■, pH 7.5; ▲, pH 8.0; ○, pH 8.5; □, pH 9.0. (B) *m*-BS-ABD-F: ●, pH 6.5; ■, pH 7.0; ▲, pH 7.5; ○, pH 8.0; □, pH 9.0. (C) *p*-BS-ABD-F: ●, pH 6.5; ■, pH 7.0; ▲, pH 7.5; ○, pH 8.0; □, pH 9.0. The reaction conditions are described in the text.

Comparison of reactivity to N-terminal amino group of peptides between ES-ABD-F and *m*-BS-ABD-F

The reactivities of ES-ABD-F and *m*-BS-ABD-F to the N-terminal amino group of peptides were studied using ANG II as a model peptide. ANG II, an endogenous octapeptide related to hypertension, has no lysine residue in its amino acid sequence. Our results are shown in Fig. 2. ES-ABD-F showed a similar reactivity–pH profile to that obtained with valine derivatization. The maximum reaction yield was obtained at pH > 8.5. *m*-BS-ABD-F, however, showed a different optimum pH from valine with a complicated pH–reactivity profile.

The time courses of the derivatization reaction toward ANG II at the optimum pH and typical chromatograms are shown in Fig. 3(A) and Fig. 4(A) and (B), respectively. Under these conditions, the derivatization reaction proceeded only moderately rapidly and it took more than 10 h to reach the plateau. Some blank peaks were found at around 15 min in the case of *m*-BS-ABD-F, indicating the possibility of interference with the determination of other peptides.

Linearity and detection limit of ANG II derivative

Both ES-ABD-F and *m*-ABS-ABD-F showed good linearity between peak area and the theoretical amount of ANG II derivatives in the range 1–200 pmol (r > 0.999). The detection limits (signal-to-noise ratio = 3) of the ANG II derivatives of ES-ABD-F and *m*-BS-ABD-F calculated from the chromatograms obtained with 1.0 pmol per injection were 270 and

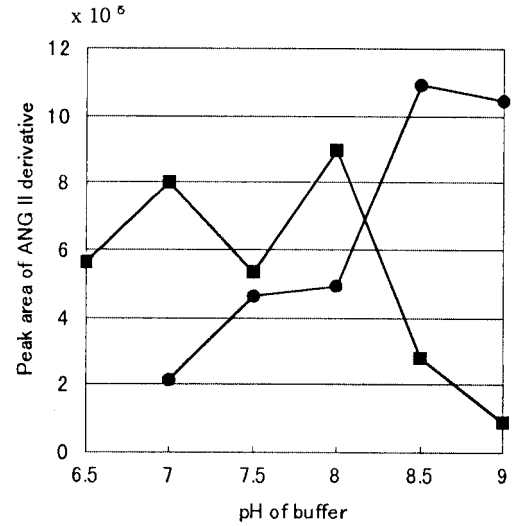


Fig. 2 Effect of pH on the reaction yield of the derivatization reaction to ANG II. ●, ES-ABD-F; ■, *m*-BS-ABD-F. The reaction conditions are described in the text.

210 fmol, respectively. These values were in good agreement with those obtained from valine derivatives, indicating that the derivatization was fully complete without loss of peptides.

Reaction conditions and reactivity of ES-ABD-F to peptides

Considering the disadvantages found with *m*-ABS-ABD-F, that is, its complicated and changeable pH–reactivity profile and the blank peaks found in the chromatograms, ES-ABD-F was recommended for the sensitive and simultaneous determination of peptides.

The effect of the reaction temperature and the reagent concentration were studied using ES-ABD-F [Fig. 3(B)]. An increase in the reaction temperature from 50 to 60 °C greatly accelerated the reaction. Although an increase in the reagent concentration and/or the pH also accelerated the reaction, an increase in blank peaks was observed. In the case of ANG II, a higher pH led to the generation of one more peak due to the decomposition of the peptide.

Under the conditions of 60 °C/pH 8.5 and a 6.7 mM reagent concentration in the reaction mixture, ANG II, bradykinin and leucine-enkephalin, whose N-terminal amino acid residues are aspartic acid, arginine and tyrosine, respectively, were derivatized with ES-ABD-F. The time courses of the reactions are shown in Fig. 3(C). The derivatization reactions of bradykinin and leucine-enkephalin were faster than that of ANG II, being completed in about 3 and 4 h, respectively. The reaction velocity of each peptide seemed to be mainly determined by the pK_a value of the N-terminal amino group. Although the reaction conditions should be optimized for each peptide to obtain the maximum yield, the reagent could be used for the practical assay of peptides.

It was noticed that the leucine-enkephaline derivative showed about a four-fold times lower fluorescence intensity than the others. In a recent study, it was found that the fluorescence of a benzofurazan compound was quenched by an intra-molecule interaction with aromatic compounds including phenol (unpublished data). Therefore, the low fluorescence intensity of the leucine-enkephaline derivative might be due to the fluorescence quenching by its N-terminal tyrosine residue.

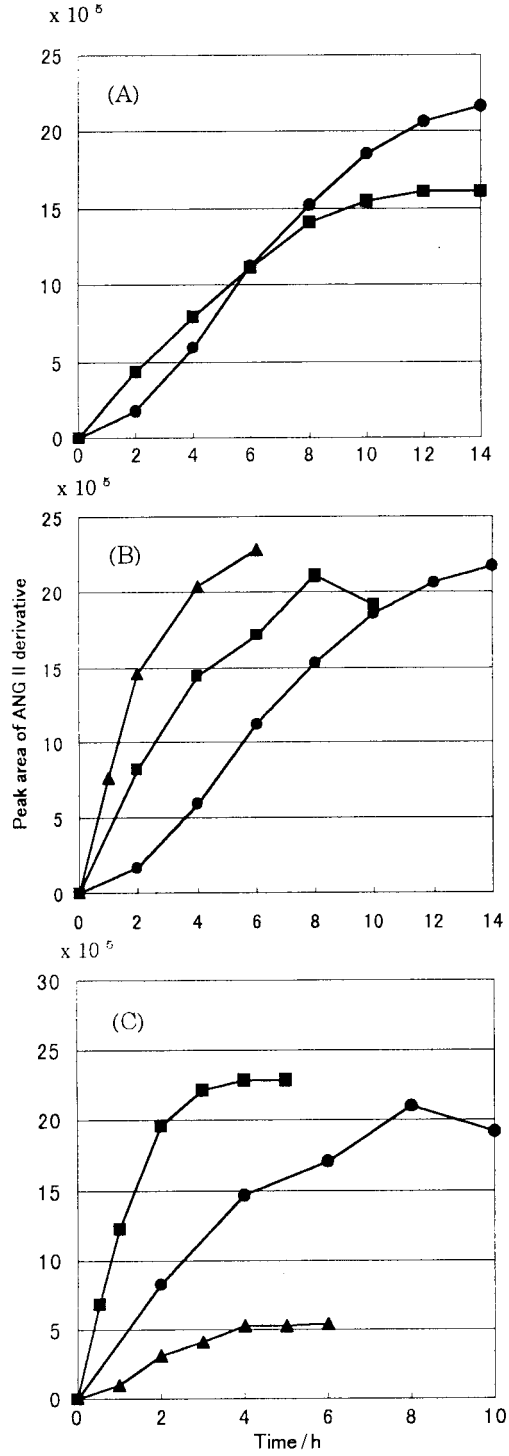


Fig. 3 Time course of derivatization reaction to peptides with the newly developed water-soluble reagents. (A) Derivatization reaction to ANG II: ●, ES-ABD-F; ■, *m*-BS-ABD-F. (B) Derivatization reaction to ANG II with ES-ABD-F under different conditions (temperature/reagent concentration in the reaction mixture/pH of buffer): ●, 50 °C/6.7 mM/pH 8.5; ■, 60 °C/6.7 mM/pH 8.5; ▲, 60 °C/20 mM/pH 9. (C) derivatization reaction to peptides with ES-ABD-F at 60 °C/pH 8.5: ●, ANG II; ■, bradykinin; ▲, leucine-enkephalin. The reaction conditions are described in the text.

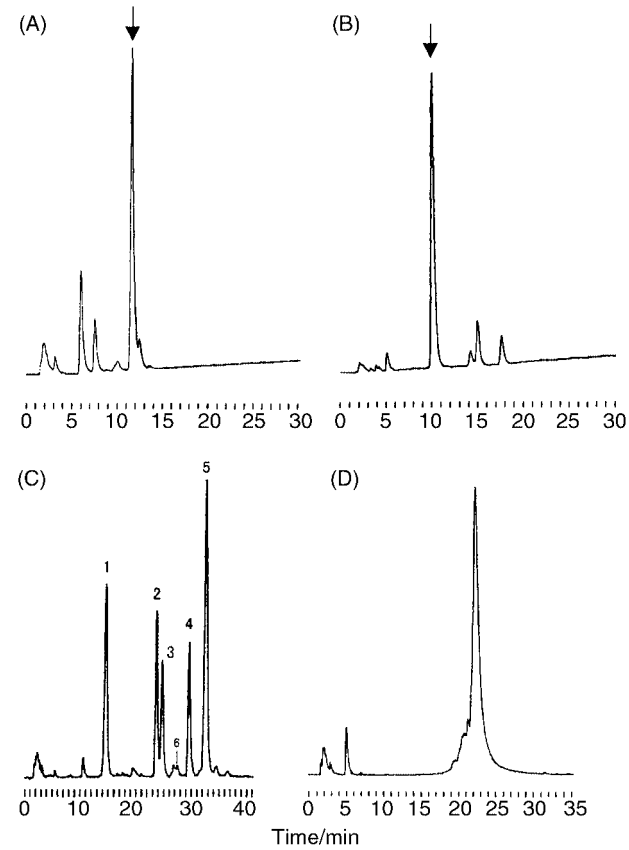


Fig. 4 Chromatograms obtained from the peptides derivatized with the newly developed water-soluble reagents. (A) ANG II–ES-ABD-F (37.5 pmol); (B) ANG II–*m*-BS-ABD-F (37.5 pmol); (C) peptide mixture–ES-ABD-F; 1, bradykinin; 2, ANG II; 3, ANG III; 4, ANG I; 5, substance P (disubstituted); 6, substance P (monosubstituted); (23.8 pmol each); (D) Aβ(1–40)–ES-ABD-F (25 pmol) (peptide/reagent). The reaction conditions and HPLC conditions are described in the text.

Separation of peptide derivatives

The separation of peptide derivatives was performed by HPLC with gradient elution. In spite of the high hydrophilicity of ES-ABD-F, the derivatives of ANG I, ANG II, ANG III, bradykinin and substance P were successfully separated on the ODS column with gradient elution using acetonitrile–water–TFA as the eluent [Fig. 4(C)]. As regards the derivatization of substance P, an increase in peak 5 accompanied by a decrease in peak 6 was observed, which resulted in one main peak (peak 5) after reaction at 60 °C/pH 8.5 for 4 h. This suggests the complete derivatization of substance P to both the N-terminal amino group and ε-amino group of the lysine residue.

ANG I, ANG II, ANG III and bradykinin are well known as endogenous peptides related to the regulation of blood pressure. Recently, it has been suggested that the renal renin–angiotensin and kallikrein–kinin systems are greatly involved in the regulation of blood pressure. In microdialysis studies of the rat kidney, the existence of nanomolar concentrations of ANG II[26] and bradykinin[27] in the renal interstitial fluids was reported. However, only the amount of ANG II was determined and not those of the other angiotensin peptides.[26] In addition, the specificity of the RIA methods used for the determination of bradykinin seemed to be insufficient.[27] Derivatization with ES-ABD-F followed by separation and detection by HPLC-FL could be applied to the simultaneous determination of a series of angiotensin peptides and bradykinin in rat renal interstitial fluids.

Aβ(1–40) was successfully derivatized with ES-ABD-F to give one main peak [Fig. 4(D)]. In a previous study,[28] the addition of ethylene glycol to the mobile phase (acetonitrile–water–TFA) was necessary to elute Aβ(1–40) from a YMC-pack C4 (150 × 4.6 mm id, 5 μm). In this investigation, Aβ(1–40) derivatized with ES-ABD-F could be eluted from this column using acetonitrile–water–TFA as the eluent, suggesting that the derivatization with ES-ABD-F decreased the adsorption of hydrophobic peptide on the stationary phase.

Conclusion

Newly developed water-soluble derivatization reagents having a benzofurazan structure, ES-ABD-F and *m*-BS-ABD-F, were successfully applied to the highly sensitive fluorescence detection of peptides by HPLC-FL. ES-ABD-F was recommended for the sensitive and simultaneous determination of peptides. The derivatization reaction with ES-ABD-F was completed at 60 °C and pH > 8.5 in a few hours. ES-ABD-F derivatives of peptides were well separated with gradient elution for reversed-phase HPLC. Not only the N-terminal amino acid, but also the ε-amino group of the lysine residue was effectively derivatized with ES-ABD-F. In addition, it was demonstrated that derivatization with ES-ABD-F resulted in a decrease in the hydrophobicity of the peptide.

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