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Long-wavelength Fluorogenic Derivatization of Aryl Halides Based on the Formation of Stilbene by Heck Reaction with Vinylbenzenes

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The long-wavelength fluorogenic derivatization method for aryl halides was developed based on stilbene formation by the Heck coupling reaction between aryl halides and vinylbenzenes in the presence of palladium(II) acetate as a catalyst. Fluorescent maximum wavelengths of the derivative obtained by the proposed reaction were 365 – 450 nm, which were 50 – 100 nm longer than those of the biphenyl derivatives formed with our previously developed fluorogenic derivatization method. Also, by the investigation using vinylbenzenes containing electron-donating or -withdrawing functional groups, it was found that an internal charge transfer system could contribute to extend the emission wavelength of the derivative. Furthermore, the proposed reaction was applied to develop a pre-column derivatization HPLC with fluorescence detection method for aryl bromides using 4-vinylanisole. *p*-Substituted aryl bromide derivatives (*i.e.*, *p*-bromobenzonitrile, *p*-bromoanisole, bromobenzene, *p*-bromobenzoic acid ethyl ester, *p*-bromotoluene) were successfully detected within 40 min with the detection limit of 0.007 – 0.264 μM. Despite the short reaction time of 10 min, the reaction yields for *p*-bromoanisole and bromobenzene were good at 101 and 87%, respectively.

Keywords Fluorogenic derivatization, Heck reaction

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Introduction

The fluorescence detection method has been applied to trace analysis in combination with high-performance liquid chromatography (HPLC) because it has high sensitivity and selectivity. However, most compounds are non-fluorescent or weakly fluorescent. Thus, in order to convert non-fluorescent or weakly fluorescent compounds to fluorescent ones, fluorescence derivatization techniques have frequently been utilized. Until now, various fluorescence derivatization reagents for typical functional groups such as amine, phenol, and carbonyl groups have been developed.[1-9] However, fluorescence derivatization can be difficult if the target molecules do not have typical functional groups that can be derivatized. Among such compounds that are difficult to derivatize, our research group focused on aryl halides. We previously developed a fluorescence derivatization method for aryl halides based on the Suzuki coupling reaction, which is a palladium catalyzed cross-coupling reaction between aryl halide and aryl boronic acid.[10] In this method, we employed fluorescent aryl boronic acid, 4-(4,5-diphenyl-1*H*-imidazole-2-yl) phenylboronic acid (DPA), as a derivatization reagent. DPA can react selectively with aryl halides to produce fluorescent derivatives even in the presence of numerous biogenic substances. So far, we successfully applied DPA for the monitoring of various aryl halide drugs, including clofibrate,[10] haloperidol[11] and hydroxyzine,[12] in body fluids. However, DPA itself has strong fluorescence, which can interfere with detection of the target derivative. To overcome this issue, we developed a fluorogenic derivatization reaction for aryl halides based on the formation of biphenyl with non-substituted phenylboronic acid (PBA) as a reagent (Fig. 1).[13] The derivatization with PBA did not cause interfering blank peaks derived from excess derivatization reagent. However, the formed fluorescent biphenyl has a fluorescence emission maximum at the short-wavelength region of 315 – 350 nm, and is susceptible to interference by coexisting materials.

In the present study, we attempted to develop a long-wavelength fluorogenic derivatization reaction for aryl halides based on the formation of stilbene by Heck coupling reaction (Fig. 1). Heck coupling is a palladium-catalyzed cross-coupling reaction between aryl halide and vinylbenzene to form a stilbene derivative.[14,15] It can be expected that longer conjugated systems of stilbene than biphenyl provide long-wavelength fluorescence.[16,17] Therefore, we evaluated vinylbenzenes as a long-wavelength fluorogenic derivatization reagent for aryl halides. Firstly, the fluorescence spectra of the reaction mixture of aryl bromides with vinylbenzene were measured and compared with those of biphenyls. In addition to non-substituted vinylbenzene, 4-vinylanisole (4-VA) containing electron donating group and 4-(trifluoromethyl)vinylbenzene (4-TFV) containing electron withdrawing group were also tested to extend the emission wavelength of the derivative induced by internal charge transfer (ICT).[17-19] Subsequently, we established a HPLC with fluorescence detection method for aryl bromides after pre-column derivatization with 4-VA.

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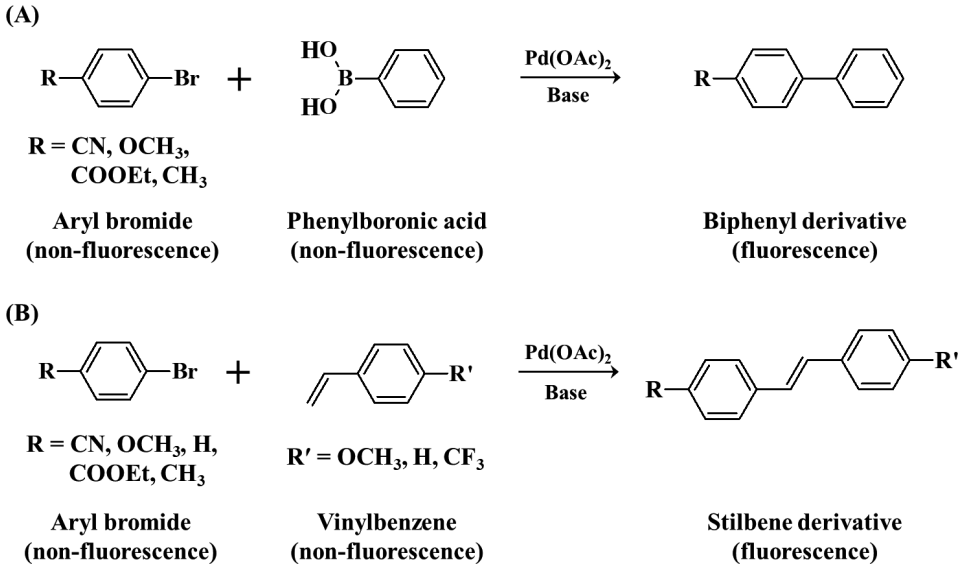

Fig. 1 Scheme of fluorogenic derivatization for aryl halide by (A) the reaction with phenylboronic acid (PBA) based on the Suzuki coupling reaction and (B) the reaction with vinylbenzenes based on the Heck coupling reaction.

Experimental

Materials and reagents

4-VA was purchased from Ark Pharm (Illinois, USA). Vinylbenzene, *p*-bromobenzonitrile (Br-Ph-CN), *p*-bromoanisole (Br-Ph-OCH_3), bromobenzene (Br-Ph-H), *p*-bromobenzoic acid ethyl ester (Br-Ph-COOEt), *p*-bromotoluene (Br-Ph-CH_3), 4-methoxy-*trans*-stilbene and 4,4′-dimethoxy-*trans*-stilbene were from Tokyo Chemical Industries (Tokyo, Japan). 4-TFV were from Oakwood products (South Carolina, USA). Palladium(II) acetate ($Pd(OAc)_2$) and *N*,*N*-dimethylacetamide (DMAc) were from Nacalai Tesque (Osaka, Japan). Triethylamine (TEA) was purchased from Wako (Tokyo, Japan). Acetonitrile was from Kanto Chemical (Tokyo, Japan).

Derivatization procedures

To the DMAc solution including aryl halides (50 µL), 15 mM 4-VA in DMAc (50 µL), 10 mM $Pd(OAc)_2$ in DMAc and aqueous solution of 60 mM TEA (50 µL) were successively added and mixed in an amber-colored screw-capped vial. After purging with N_2 (5.0 mL/s) for 10 s, the reaction mixture was heated at 100°C for 10 min to form a fluorescent derivative.

HPLC system and conditions

The HPLC system consisted of a pump LC-20A (Shimadzu, Kyoto, Japan), a Shimadzu RF-20A fluorescence detector, a 7125 injector with a 20-µL loop (Rheodyne, Cotati, CA), and a Chromato-PRO (RTC, Tokyo) as a recorder. Chromatographic separation was performed on a Cosmosil 5C18AR-II (250 × 4.6 mm, i.d., Nacalai Tesque, Kyoto) *via* isocratic elution with a mixture of acetonitrile and water (60:40, v/v) at a flow rate of 1.0 mL/min. The fluorescence wavelength of the detector was changed according to the time program to detect each derivative at a maximum wavelength (Table S1, Supporting Information). After filtration through Hawach Scientific (Shaanxi, China) 0.45-µm PTFE membrane filter, a 20-µL aliquot of the derivatization reaction mixture was injected into the HPLC system.

Measurement of fluorescence spectra of the derivatives

The fluorescence spectra of the derivative were investigated after the purification in order to minimize the influence of the fluorescence derived from the reagent blank. The formed derivative in the reaction mixture was purified by HPLC-UV system at 254 nm under the separation conditions described above. The fluorescence excitation and emission spectra of the peak fraction were measured with a Shimadzu RF-1500 spectrofluorometer.

Results and Discussion

Comparison of fluorescence spectra of the reaction mixture

Figure 2 shows the fluorescence spectra of the reaction mixture of Br-Ph-COOEt with 4-VA. The fluorescence was observed at excitation and emission maximum wavelength of 320 and 450 nm, respectively. This result indicated that Br-Ph-COOEt was derivatized to fluorescent compound by the reaction with 4-VA. Meanwhile, the intense fluorescence was not observed from the reagent blank. In the reaction solution of the aryl halide and vinylbenzene, there was no significant change in the fluorescence intensity depending on the ratio of water. Table 1 lists the fluorescence excitation and emission maxima of the derivative obtained by the reaction of five aryl bromides with vinylbenzene, 4-VA and 4-TFV. On the other hand, the fluorescence excitation/emission maxima (nm) of the Br-Ph-CN, Br-Ph-OCH_3, Br-Ph-COOEt and Br-Ph-CH_3 after the reaction with PBA were reported at 285/335, 280/330, 290/350 and 275/315, respectively. The derivatives formed with vinylbenzene were found to have a fluorescence maximum wavelength on the long wavelength side of 35 – 45 nm compared with PBA. This longer wavelength emission could be attributed to the difference of conjugated system between stilbene and biphenyl. The derivatives formed with 4-VA and 4-TFV also gave longer wavelength emission than biphenyl derivatives. It should be noted that a further extension of emission wavelength was observed from the derivatives formed from 4-VA and aryl bromides containing electron withdrawing group (Br-Ph-CN

and Br-Ph-COOEt). The similar red shift was also observed from the derivative between 4-TFV and aryl bromides containing electron donating group. These extensions of emission wavelength could be attributed to the ICT between electron donating and withdrawing groups in the formed stilbene derivative. Among tested vinylbenzenes, 4-VA could provide the derivative that emits the longest wavelength fluorescence. Consequently, we selected 4-VA as the derivatization reagent for further studies of the development of a pre-column derivatization HPLC method.

Chromatographic analysis of the formed aryl halides derivatives of 4-VA

Figures 3(A) and 3(B) shows the chromatograms obtained by the injection of the reagent blank (4-VA) and the reaction mixture of aryl bromides with 4-VA into the HPLC system, respectively. Under the HPLC conditions described above, the peaks of the derivatives of Br-Ph-CN, Br-Ph-OCH_3, Br-Ph-H, Br-Ph-COOEt and Br-Ph-CH_3 were detected at 15, 21, 23, 32 and 36 min, respectively. On the other hand, the peaks considered to be reagent blank were hardly detected because 4-VA should be non-fluorescent. In order to confirm the formation of stilbene derivative by the proposed reaction, the authentic solution of 4,4′-dimethoxy-*trans*-stilbene and 4-methoxy-*trans*-stilbene were injected in to the HPLC system. As shown in Fig. 3(C), the retention times of 4,4′-dimethoxy-*trans*-stilbene and 4-methoxy-*trans*-stilbene were identical with those of the derivative of Br-Ph-OCH_3 and Br-Ph-H, respectively, which revealed that aryl bromides were converted to stilbenes by the reaction with 4-VA.

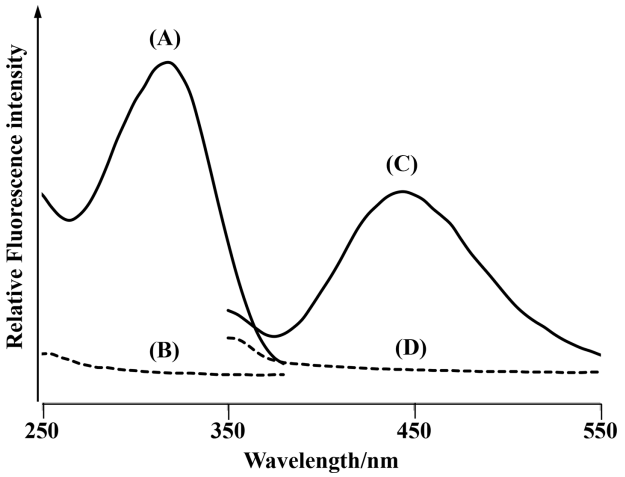

Fig. 2 Fluorescence spectra obtained from the reaction mixture of Br-Ph-COOEt with 4-VA and the reagent blank. Excitation spectra with the emission wavelength at 450 nm of the product (A, solid line) and the reagent blank (B, dashed line). Emission spectra with the excitation wavelength at 320 nm of the product (C, solid line) and the reagent blank (D, dashed line). Solvent: acetonitrile/water = 60/40 (v/v%).

Optimization of the reaction condition between 4-VA and aryl halides

In order to get higher reactivity, the derivatization reaction conditions were optimized using a standard solution of aryl bromides. Different kinds of solvents, including DMAc, 1,4-dioxane (Dioxane), acetonitrile (CH_3CN), ethanol and *N*,*N*-dimethylformamide (DMF), were examined. Among the tested solvents, DMAc yielded the largest peak area of aryl bromide derivatives (Fig. S1, Supporting Information). The concentration of 4-VA was investigated over a range of 1 – 20 mM, and the largest peak area of derivatives was obtained at

Table 1 Fluorescence properties of derivatives obtained by the reaction of aryl bromides with 4-VA, vinylbenzene and 4-TFV

Aryl bromide	4-VA		Vinylbenzene		4-TFV	
	λ_{ex}/ nm	λ_{em}/ nm	λ_{ex}/ nm	λ_{em}/ nm	λ_{ex}/ nm	λ_{em}/ nm
Br-Ph-OCH_3	290	370	290	370	305	400
Br-Ph-CH_3	290	365	290	350	300	370
Br-Ph-H	290	370	290	340	290	350
Br-Ph-COOEt	320	450	310	390	300	360
Br-Ph-CN	320	435	300	380	300	355

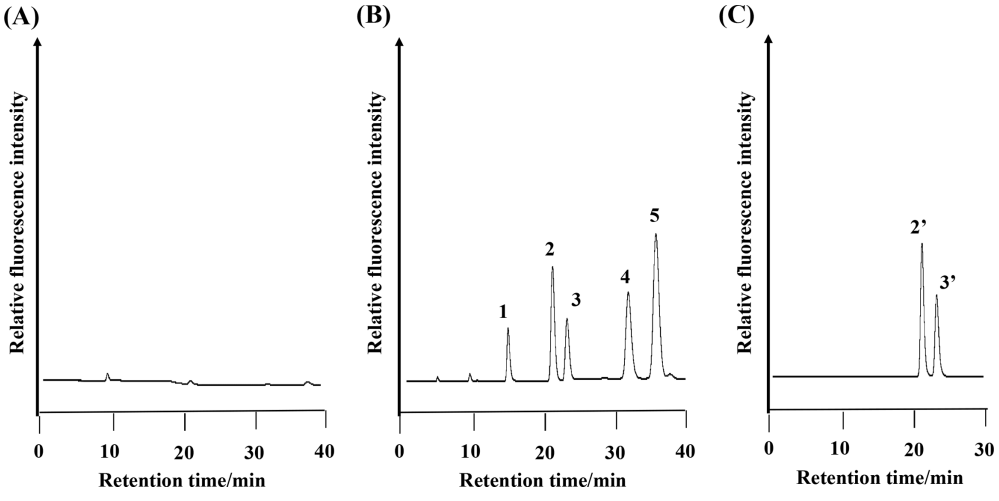

Fig. 3 Chromatograms of (A) reagent blank, (B) reaction mixture and (C) authentic stilbenes. Peaks: 1, Br-Ph-CN derivative; 2, Br-Ph-OCH_3 derivative; 3, Br-Ph-H derivative; 4, Br-Ph-COOEt; 5, Br-Ph-CH_3; 2′, 4,4′-dimethoxy-*trans*-stilbene; 3′, 4-methoxy-*trans*-stilbene. Sample concentration: 100 μM except for Br-Ph-OCH_3 (Br-Ph-OCH_3 10 μM).

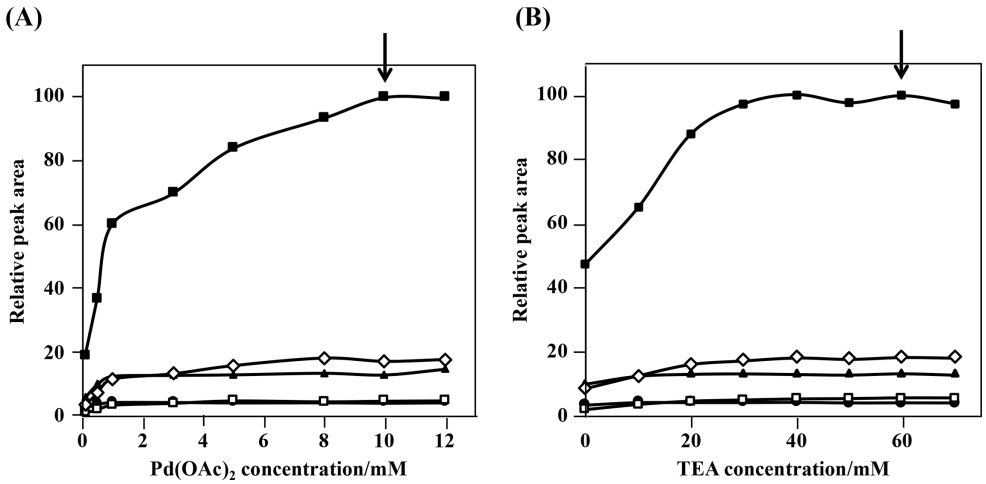

Fig. 4 Effect of the (A) $Pd(OAc)_2$ and (B) TEA concentrations on the peak area of aryl bromides. Compounds: ■, *p*-bromoanisole; ◇, *p*-bromotoluene; ▲, *p*-bromobenzoic acid ethyl ester; □, bromobenzene; ●, *p*-bromobenzonitrile. Sample concentration: 30 μM for all compounds.

Table 2 Calibration curves and detection limits for aryl bromides

Aryl halide	Calibration curve			LOD[b] μM (pmol/injection)
	Range/μM	Equation[a] /mean ± SD (n = 3)	r^2	
Br-Ph-CN	1 - 250	$Y = (3.14 \times 10^2 \pm 36.2)X + (6.38 \times 10^2 \pm 1.29 \times 10^2)$	0.999	0.264 (1.3)
Br-Ph-OCH_3	0.05 - 25	$Y = (7.10 \times 10^3 \pm 8.83 \times 10^2)X + (5.90 \times 10^3 \pm 2.29 \times 10^3)$	0.998	0.007 (0.04)
Br-Ph-H	1 - 250	$Y = (3.81 \times 10^2 \pm 40.3)X + (2.05 \times 10^2 \pm 72.0)$	0.999	0.120 (0.60)
Br-Ph-COOEt	1 - 250	$Y = (4.69 \times 10^2 \pm 63.0)X + (1.14 \times 10^3 \pm 7.62 \times 10^2)$	0.997	0.094 (0.47)
Br-Ph-CH_3	1 - 250	$Y = (9.01 \times 10^2 \pm 1.18 \times 10^2)X + (2.13 \times 10^3 \pm 6.14 \times 10^2)$	0.998	0.073 (0.37)

a. Y = Peak height; X = concentration (μM). b. S/N = 3.

a concentration of 15 mM (Fig. S2, SI). The concentration of $Pd(OAc)_2$ was investigated over a range of 0.1 - 12 mM. The maximum and constant peak area of aryl bromides was obtained at more than 8 mM. For the optimal condition, 10 mM of $Pd(OAc)_2$ was selected (Fig. 4(A)). Different kinds of bases, including TEA, *N*,*N*-diisopropylethylamine (DIPEA), potassium fluoride (KF), and sodium hydrogen phosphate (Na_2HPO_4), were tested. As shown in Fig. S3 (SI), the addition of a base could improve the peak area of the derivatives except for Na_2HPO_4. Among the tested bases, TEA provided the largest peak area of aryl bromide derivatives. Then, the concentration of TEA was investigated over a range of 0 - 70 mM, and the largest or constant peak area of aryl bromides was obtained at 60 mM. Therefore, 60 mM of TEA was selected (Fig. 4(B)). The effects of the reaction temperature and time were investigated, and optimum conditions were achieved with heating at 100°C for 10 min (Figs. S4 and S5, SI).

Under the optimized conditions, the reaction yields of Br-Ph-OCH_3 and Br-Ph-H, were calculated by comparing the peak areas of the reaction products with those of authentic 4,4′-dimethoxy-*trans*-stilbene and 4-methoxy-*trans*-stilbene. The estimated reaction yields of Br-Ph-OCH_3 and Br-Ph-H were 101 and 87%, respectively, and therefore it was confirmed that the proposed derivatization reaction proceeded with excellent yield.

Validation of the proposed method

Calibration curves were prepared with a standard mixture of aryl bromides and good linearities ($r^2 > 0.997$) were obtained between the peak height and the concentration of aryl bromides in the range of 1.0 - 250 μM except for *p*-bromoanisole (0.05 - 25 μM) (Table 2). The detection limits of aryl bromides calculated at a signal-to-noise ratio (S/N) of 3 ranged from 0.007 to 0.264 μM. The optimum reaction conditions and the sensitivities of the proposed method were compared to those of the previously developed methods by our research group (Table 3). The sensitivities of the proposed method were relatively superior to those of the DPA.[10] Although the proposed method was less sensitive than the PBA method[13] except for Br-Ph-OCH_3, the longer wavelength fluorescence emission should be effective to reduce the interfering peaks derived from co-existing components. In addition, the proposed method could provide excellent reaction yields within shorter reaction time. The repeatability of the proposed method was examined using three different concentrations of aryl bromides in the calibration range (25, 150 and 250 μM). The relative standard deviations for within-day (n = 5) and between-day (n = 5) runs were less than 7.7 and 8.6%, respectively (Table 4), and therefore sufficient repeatability of the proposed method could be confirmed.

Conclusion

In this study, we reported a long-wavelength fluorogenic derivatization reaction for aryl halides based on the formation of

Table 3 Comparison of the derivatization conditions and LODs obtained with various fluorescence derivatization reagents for aryl bromides

Reagent		4-VA[a]	DPA[b]	PBA[c]
Derivatization condition		100°C, 10 min	100°C, 45 min	100°C, 30 min
LOD/μM	Br-Ph-CN	0.264	—	0.003
	Br-Ph-OCH_3	0.007	0.050	0.013
	Br-Ph-H	0.120	0.35	—
	Br-Ph-COOEt	0.094	—	0.007
	Br-Ph-CH_3	0.073	0.35	0.030

a. The proposed method.
b. N. Kuroda, S. Sugihara, Y. Sugihara *et al.*, Journal of Chromatography A, 1066 (2005) 119 – 125.
c. N. Kishikawa, K. Kubo, S. F. Hammad *et al.*, Journal of Chromatography A, 1216 (2009) 6873 – 6876.

Table 4 Intra- and inter-day precision of the proposed method

Aryl bromide	Concentration/ μM	Precision (RSD, %)	
		Within-day (n = 5)	Between-day (n = 5)
Br-Ph-CN	25	1.2	1.0
	150	1.0	2.3
	250	2.4	6.1
Br-Ph-OCH_3	2.5	4.9	6.7
	15	5.7	5.1
	25	7.7	2.9
Br-Ph-H	25	5.2	8.6
	150	2.4	7.4
	250	3.7	4.6
Br-Ph-COOEt	25	0.8	2.7
	150	1.9	5.4
	250	1.8	6.7
Br-Ph-CH_3	25	7.3	6.1
	150	5.7	5.5
	250	4.8	4.0

stilbene by Heck coupling reaction. Since the maximum fluorescence wavelengths of the derivatives obtained by the proposed method using 4-VA were 50 – 100 nm longer than those of the derivative obtained by the previous method using PBA, it should be expected to reduce the interfering peaks derived from autofluorescence of samples. Moreover, it should be noteworthy that the proposed derivatization reaction could proceed with high reaction yields (>87%) within a short time (10 min). Hence, by taking advantage of the long wavelength and short reaction time, the proposed method will be a promising tool for the development of post-column derivatization HPLC systems for aryl halides.

Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/. The supporting information includes the time program for the fluorescence detection and the results of optimization studies.

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