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Studies on the Fluorescence Characteristics of Fluorescamine Derivatives

TOSHIYUKI SAKANO,* SACHIKO MASUDA, and TAMEYUKI AMANO

Shionogi Research Laboratory, Shionogi and Co., Ltd. Sagisu,
Fukushima-ku, Osaka 553, Japan

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Fluorescamine derivatives of butylamine, benzylamine, *p*-anisidine, *p*-toluidine, aniline, *p*-chloroaniline, sulfanilamide, sulfamethoxazole and N¹-acetylsulfamethoxazole were isolated and various characteristics of the fluorophoric derivatives were investigated, *i.e.*, the effects of solvents, substituent groups, and pH, as well as hydrogen bonding in organic solvents, conversion to non-fluorescent lactones, and thin-layer chromatography behavior.

Keywords—fluorescamine; fluorescamine derivative; fluorescence; solvent effect; hydrogen bonding; reaction yield

Fluorescamine, 4-phenylspiro[furan-2(3*H*), 1'-phthalan]-3,3'-dione (FL), was introduced by Weigele *et al.*¹⁾ as a reagent for fluorometric quantitation of primary amines. FL has been utilized for the analysis of various amino acids,²⁾ proteins,³⁾ catecholamines⁴⁾ and other primary amines.⁵⁻⁷⁾ FL has been also employed in spectroscopic studies of FL derivatives,⁸⁾ studies of the reaction kinetics and hydrolysis of FL⁹⁾ and the quantitation of N-nitrosamine.¹⁰⁾

We have reported the application of FL for the fluorometric determination of residual sulfamethoxazole in animal tissues.¹¹⁾ In this paper, we describe the fluorescence characteristics of various FL derivatives (I—X, Chart 1), *i.e.*, the effects of solvents, substituent groups and pH, as well as hydrogen bonding properties, conversion to non-fluorescent lactones and thin-layer chromatography (TLC) behavior.

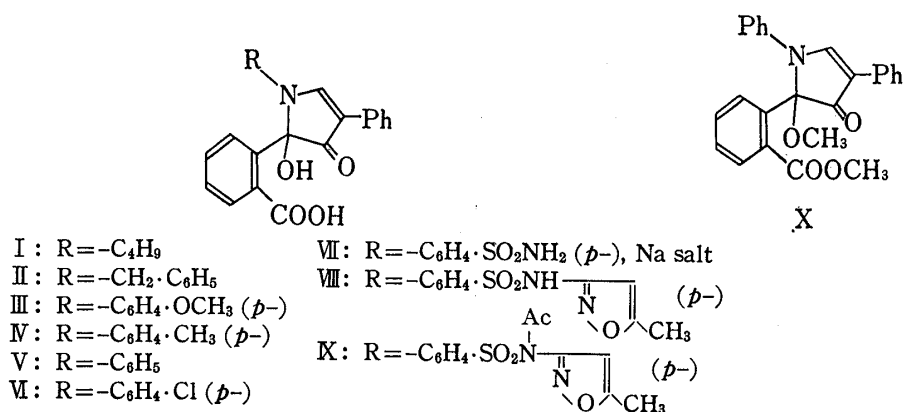


Chart 1

Experimental

Apparatus—Fluorescence excitation and emission spectra were measured with Hitachi MPF-2A and MPF-4 spectrofluorometers. Ultraviolet (UV) spectra were recorded with a Hitachi 323 spectrophotometer and infrared (IR) spectra with a Jasco DS-403G grating spectrometer and a 215 Hitachi grating infrared spectrophotometer. Fluorescence excitation and emission spectra on TLC plates were measured with a Hitachi MPF-2A spectrofluorometer equipped with a Hitachi TLC attachment. All melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected.

Materials—Fluorescamine (Floram, FL) was obtained from Hoffmann-La Roche. Benzene, dichloromethane and methanol were from Wako (Dotite Reagent Luminasol). Acetone, acetonitrile, dioxane, and

ethyl acetate were also from Wako (Dotite Reagent Spectrosol). Ethanol of reagent grade was distilled after dissolving Na metal in it. Dimethyl sulfoxide (DMSO) of reagent grade was distilled at 53–55°C/4 mmHg. Diethyl ether of reagent grade was purified by distillation.

FL Derivatives—1-Butyl-5-(2-carboxyphenyl)-5-hydroxy-3-phenyl-2-pyrrolin-4-one (I), 1-benzyl-5-(2-carboxyphenyl)-5-hydroxy-3-phenyl-2-pyrrolin-4-one (II) and 5-(2-carboxyphenyl)-5-hydroxy-1,3-diphenyl-2-pyrrolin-4-one (V) were prepared by the method of De Bernardo *et al.*⁸⁾

5-(2-Carboxyphenyl)-5-hydroxy-1-(4-methoxyphenyl)-3-phenyl-2-pyrrolin-4-one (III): To 49 mg of *p*-anisidine dissolved in 6 ml of MeCN were added 56 μ l of Et₃N and 113 mg of FL. The mixture was stirred for 30 min in an ice bath. The reaction mixture was then distributed between cold 0.05 N HCl (20 ml) and CH₂Cl₂ (30 ml). The organic extract was washed with half-saturated brine (20 ml) then dried (Na₂SO₄), and the solvent was removed under reduced pressure. The crystalline substance in the residual syrup was recrystallized from CH₂Cl₂ to give III (yield 31.5 mg, 19%). Yellow powder, mp 138–142°C. *Anal.* Calcd for C₂₄H₁₉NO₅: C, 71.81; H, 4.77; N, 3.49. Found: C, 71.74; H, 5.05; N, 3.22. IR $\nu_{\text{max}}^{\text{Nujol}}$ cm⁻¹: 3150, 1697, 1630.

5-(2-Carboxyphenyl)-5-hydroxy-1-(4-methylphenyl)-3-phenyl-2-pyrrolin-4-one (IV) was prepared analogously from *p*-toluidine (9%), mp 145–147°C (from CH₂Cl₂), as a pale yellow powder. *Anal.* Calcd for C₂₄H₁₉NO₄: C, 74.79; H, 4.97; N, 3.63. Found: C, 74.62; H, 4.96; N, 3.65. IR $\nu_{\text{max}}^{\text{Nujol}}$ cm⁻¹: 3160, 1691, 1621.

5-(2-Carboxyphenyl)-1-(4-chlorophenyl)-5-hydroxy-3-phenyl-2-pyrrolin-4-one (VI) was prepared analogously from *p*-chloroaniline (50%), mp 116–118°C (from CH₂Cl₂), as a yellow powder. *Anal.* Calcd for C₂₃H₁₆ClNO₄: C, 68.07; H, 3.97; N, 3.45. Found: C, 68.18; H, 3.75; N, 3.65. IR $\nu_{\text{max}}^{\text{Nujol}}$ cm⁻¹: 3150, 1685, 1635.

5-(2-Carboxyphenyl)-5-hydroxy-3-phenyl-1-(4-sulfamoylphenyl)-2-pyrrolin-4-one Na salt (VII): To 69 mg of sulfanilamide dissolved in 6 ml of MeCN were added 62 μ l Et₃N and 142 mg of FL. The reaction mixture was stirred for 90 min in an ice bath. The mixture was poured into 20 ml of cold water. The aqueous layer was washed with Et₂O (30 ml) and Et₂O–MeCN (5:1, 36 ml), and lyophilized. The resulting yellow powder was dissolved in a small amount of Me₂CO. The solution was chromatographed on a silica gel TLC plate containing 5% NaHCO₃ with MeOH–CH₂Cl₂–Et₃N (50:200:1). The fluorescent fraction of *R_f* value between 0.2 and 0.4 was extracted with MeOH containing 1% Et₃N. After the MeOH had been evaporated off, the residue was dissolved in Me₂CO and filtered. The filtrate was concentrated, and the residue was crystallized from a MeOH–CH₂Cl₂ mixture. Recrystallization from MeCN–Et₂O then from MeCN–MeOH afforded a hygroscopic yellow powder (7.8%), dp 245–246°C. *Anal.* Calcd for C₂₃H₁₇N₂O₆·SNa·H₂O: C, 56.32; H, 3.90; N, 5.71; Na, 4.69. Found: C, 55.97; H, 4.08; N, 5.46; Na, 4.67. IR $\nu_{\text{max}}^{\text{Nujol}}$ cm⁻¹: 3480, 3340, 3250, 3060, 1680.

5-(2-Carboxyphenyl)-5-hydroxy-[4-(5-methylisoxazolylsulfamoyl)phenyl]-3-phenyl-2-pyrrolin-4-one (VIII) was prepared by the reaction of sulfamethoxazole and FL in the manner described for III. The reaction time was 60 min in an ice bath, and the yield was 18%, mp 152–153°C. *Anal.* Calcd for C₂₇H₂₁N₃O₇S: C, 61.01; H, 3.98; N, 7.91. Found: C, 60.84; H, 3.72; N, 7.75. IR $\nu_{\text{max}}^{\text{Nujol}}$ cm⁻¹: 3220, 3180, 3080, 1692, 1642, 1636.

5-(2-Carboxyphenyl)-5-hydroxy-1-[4-(5-methylisoxazolyl-N-acetylsulfamoyl)phenyl]-3-phenyl-2-pyrrolin-4-one (IX): To 118 mg of N¹-acetylsulfamethoxazole dissolved in 6 ml of MeCN were added 56 μ l of Et₃N and 111 mg of FL. The mixture was stirred for 3.5 h in an ice bath. The reaction mixture was then distributed between cold 0.05 N HCl (20 ml) and CH₂Cl₂ (30 ml). The organic extract was washed with half-saturated brine (20 ml) then dried (Na₂SO₄), and the solvent was removed under reduced pressure. Crystallization from Me₂CO–petroleum ether afforded a yellow powder. The powder was dissolved in Me₂CO, and the solution was chromatographed on a silica gel TLC plate containing 5% NaHCO₃ with MeOH–CH₂Cl₂–Et₃N (50:200:1). The fluorescent fraction of *R_f* between 0.35 and 0.45 was extracted with MeOH containing 1% Et₃N. After the MeOH had been evaporated off, the residue was combined with 60 ml of acetate buffer (pH 5) and 15 ml of 0.05 N HCl, then extracted with 80 ml of CH₂Cl₂. The extract was washed with half-saturated brine (30 ml) and dried (Na₂SO₄), and the solvent was removed under reduced pressure. Recrystallization of the residual powder from Et₂O–CH₂Cl₂ afforded 34 mg (15%) of yellow powder, mp 153–155°C. *Anal.* Calcd for C₂₉H₂₃N₃O₈S: C, 60.73; H, 4.04; N, 7.33. Found: C, 60.62; H, 3.81; N, 7.60. IR $\nu_{\text{max}}^{\text{Nujol}}$ cm⁻¹: 3180, 3070, 1695, 1620.

5-Methoxy-5-(2-methoxycarbonylphenyl)-1,3-diphenyl-2-pyrrolin-4-one (X): To 35 mg of V dissolved in 5 ml of MeOH was added 5 ml of Et₂O solution of CH₂N₂ and the reaction was allowed to continue for 1 h at room temperature. After the solvent had been evaporated off, the residue was dissolved in Et₂O and chromatographed on a silica gel TLC plate with Et₂O. The fraction of *R_f* between 0.49 and 0.67 (blue-green fluorescence) was extracted with Et₂O. After the Et₂O had been evaporated off, the residue was recrystallized from Et₂O to give X (yield 14 mg, 37%). Yellow powder, mp 57–58°C. *Anal.* Calcd for C₂₅H₂₁NO₄: C, 75.17; H, 5.30; N, 3.51. Found: C, 75.32; H, 5.54; N, 3.62. IR $\nu_{\text{max}}^{\text{Nujol}}$ cm⁻¹: 1780, 1720, 1685.

Results and Discussion

Fluorescence characteristics of FL derivatives of the pyrrolinone type in various organic solvents are shown in Table I. Excitation and emission maxima of FL derivatives of aromatic

amines (III—VII) in various organic solvents were found to have a blue shift with increasing σ -values (Hammett's substituent constant) (III: p -OCH₃, -0.268 ; IV: p -CH₃, -0.170 ; V: H, 0.000 ; VI: p -Cl, 0.227 ; VII: p -SO₂NH₂, 0.621)¹²⁾ of *para*-substituent groups on the phenyl group bonded to the nitrogen atom of the pyrroling ring and with increasing dielectric constants (ϵ) of solvents. Various correlations among the emission maxima of III—VII, dielectric constants (ϵ) of solvents and Hammett σ -values of *para*-substituent groups on the phenyl group were recognized (Table II). On the other hand, excitation and emission maxima of FL derivatives of aliphatic amines (I, II) were on a range of shorter wavelength than those of III—VII and at wavelengths similar to those of FL derivatives of sulfonamides (VIII, IX). Excitation and emission maxima of X, which is the dimethyl derivative of V, are little affected by aprotic solvents. However, those in ethanol had a red shift and their wavelengths approximated to those of V in ethanol. In Table I, IX in ethyl acetate exhibited the highest fluorescence intensity. In general, FL derivatives (I—IX) seemed to fluoresce strongly in semiprotic solvents and solvents with comparatively low dielectric constants, such as ether, ethyl acetate and acetone, and to fluoresce weakly in benzene, dichromethane, acetonitrile and dimethyl sulfoxide. On the other hand, the fluorescence intensity of X was little affected by solvents. These results suggest that the fluorescence abilities of I—IX are affected by intra- or inter-molecular hydrogen bonding *via* carboxyl, hydroxyl, and carbonyl groups in the molecules.

TABLE II. Multiple Correlation Analysis

σ	ϵ	Em	
		Partial correlation coefficients	Simple correlation coefficients
σ		$-0.6792^{a)}$	$-0.6113^{a)}$
ϵ		$-0.5508^{a)}$	-0.4359
Em	$[0.7510^{a)}]$		

[]: multiple correlation coefficients, $n=40$.

σ : Hammett's σ .

ϵ : Dielectric constant of solvent.

Em: Fluorescence emission maximum.

$a)$ Highly significant ($p < 0.01$).¹⁴⁾

Dichloromethane or benzene solutions of pyrrolinones are unstable. The changes of absorption and fluorescence spectra of V in dichloromethane are shown in Fig. 1. The absorption spectra of V at 6, 60 and 180 min after dissolution in dichloromethane changed with two isosbestic points at 314 and 400 nm, and the absorption maximum wavelength of 420 nm was blue-shifted to 370 nm. In the fluorescence spectra, the wavelengths of excitation and emission maxima were unchanged and the fluorescence intensity gradually decreased with the passage of time. However, this change was reversed on addition of a small amount of methanol, dimethyl sulfoxide or triethylamine. Such a change also occurred in benzene. The change in the infrared spectrum of V in dichloromethane is shown in Fig. 2. The carbonyl absorptions at 1737 and 1654 cm⁻¹ decreased with time, and reappeared at 1779 and 1632 cm⁻¹ (in Nujol: 1680, 1620 cm⁻¹; in acetonitrile: 1738, 1644 cm⁻¹, unchanged with the passage of time). It can therefore be presumed that the fluorescence intensity of V decreases with hydrogen bonding of the pyrrolinone carbonyl and increases with hydrogen bonding of C=O of the carboxyl group.

FL derivatives of the fluorescent pyrrolinone type are converted into a nonfluorescent lactone form by a proton.¹⁾ The half-life periods of fluorescence decay of I and III—IX in 1 *N* HCl—AcONa buffer (pH 1) and acetone mixtures are shown in Table III. The decay curve of pyrrolinones followed good pseudo first-order kinetics. Half-life periods at 0—1°C were

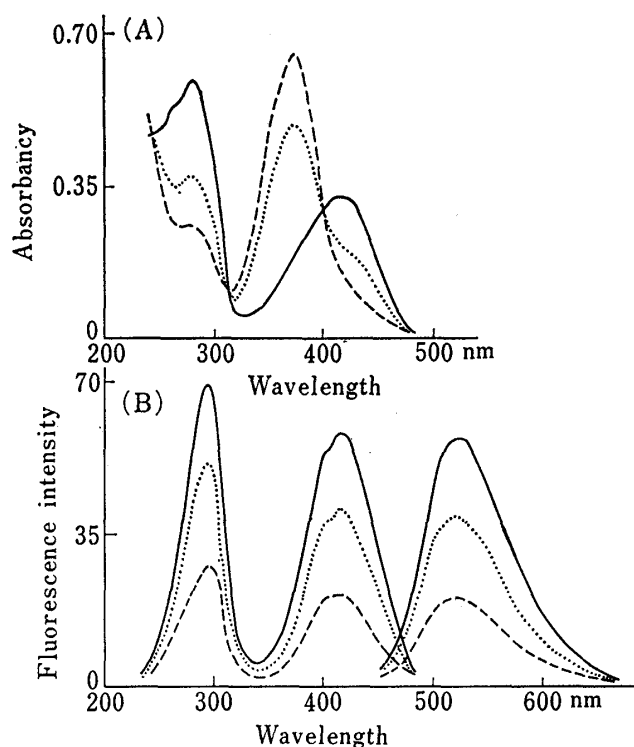


Fig. 1. Changes of Absorption and Fluorescence Spectra of V in Dichloromethane

(A): Absorption spectra, 1×10^{-5} M.
(B): Fluorescence spectra, 1×10^{-6} M.
—: 6 min,: 60 min, ----: 180 min.

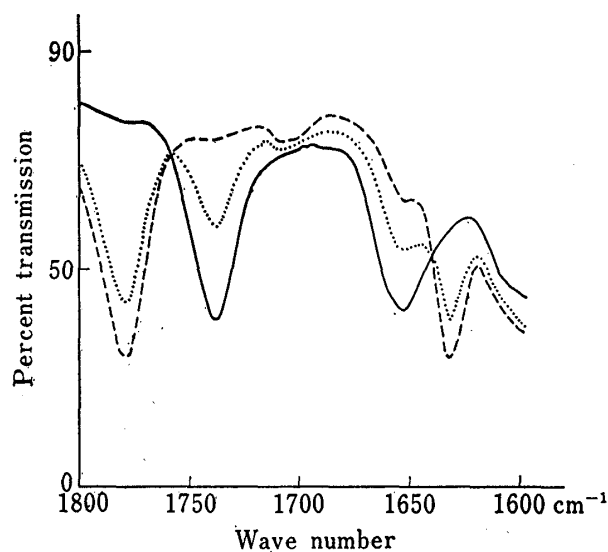


Fig. 2. Changes of Infrared Spectra of V in Dichloromethane

—: 2 min,: 60 min, ----: 180 min.

6—30 times longer than those at room temperature. Increase in the acetone content or addition of FL slowed down the conversion time of pyrrolinones.

The effects of pH on the fluorescence of the pyrrolinones (I, III, V, and VIII) at 2 and 120 min after dissolution are shown in Fig. 3. I and VIII showed maximum fluorescence intensity in the acidic range, V showed the maximum in the neutral range, and the fluorescence intensity of III increased with increasing pH. In all cases, the fluorescence intensities decreased faster in acid media than in alkaline media. These results suggest the need for pH control in fluorometry with FL. The fluorescence intensity change of I with pH (Fig. 3) agreed fairly well with the experimental results of De Bernardo *et al.*⁸⁾

TABLE III. Half-Life Periods of Fluorescent pyrrolinones in 1 N HCl-AcONa Buffer (pH 1) and Acetone Mixtures

Compound ^{a)}	Half-life period, $t_{1/2}$ (min)		
	$25 \pm 1^\circ\text{C}$		$0-1^\circ\text{C}$
	17% Me ₂ CO	29% Me ₂ CO	17% Me ₂ CO
I	24.9	29.5	138
III	10.1		206
IV	11.2	20.1	273
V	9.2	13.7	223
VI	5.1		154
VII	10.2		148
VIII	19.4	31.3	249
K	18.5		176

a) ca. 3×10^{-3} mol/ml

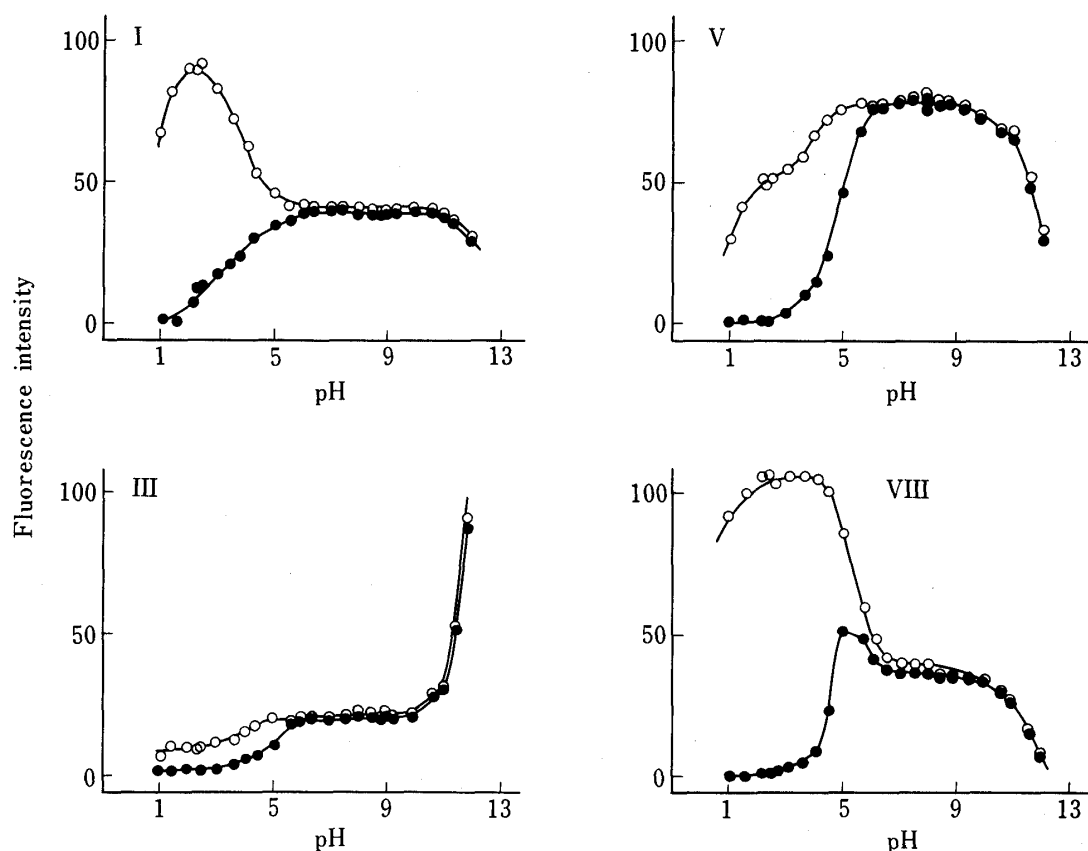


Fig. 3. Effects of pH on the Fluorescence of FL Derivatives of Pyrrolinone Type

—○—: 2 min, —●—: 2 h.

In general, FL reagent is used dissolved in acetone, dioxane or acetonitrile, and the amine sample is used in buffer solution. The chemical yields of the fluorogenic reaction of several amines in buffer solutions of pH 1, 6, and 9 with FL acetone solution were determined by comparing the fluorescence intensities of the reaction mixtures with those of the corresponding pyrrolinones (Table IV). The results suggest that the reaction yield depends on the pH of the reaction solution and the pK_a value of the primary amines. In these cases, the maximum reaction yield could be obtained at the pH corresponding to the pK_a value of the amine. There was a positive correlation between the pK_a value of aniline derivatives and the reaction yield in a medium at pH 9 ($r=0.9070$, $n=5$).

TABLE IV. Reaction Yields of Pyrrolinone Type Derivatives

Amine	pK_a	Reaction yield (%)		
		pH		
		1 ^{a)}	6 ^{b)}	9 ^{c)}
<i>n</i> -Butylamine	10.61	0.0	25.6	45.1
<i>p</i> -Anisidine	5.29		106.1	59.7
<i>p</i> -Toluidine	5.07		103.7	54.8
Aniline	4.58	1.6	100.9	33.2
<i>p</i> -Chloroaniline	3.81		87.0	18.4
Sulfamethoxazole	1.37	43.7	17.5	4.6
N ¹ -Acetyl sulfamethoxazole		30.2		1.4

a) 1N HCl-AcONa.

b) Citric acid- Na_2HPO_4 buffer

c) Borate buffer.

TABLE V. Fluorescence Properties and *R_f* Values of FL Derivatives on Thin Layer Plates

FL derivative ^{a)}	<i>R_f</i> value ^{b)}	Ex. max ^{c)} (nm)		Em. max ^{d)} (nm)
I	0.51	295	386	484
III	0.51	Not measurable ^{e)}		
IV	0.41	294	400	506
V	0.49	290	398	502
VI	0.50	292	398	504
VII	0.27	296	398	491
VIII	0.42	298	398	492
IX	0.41	297	398	495

a) About 1 μg/spot.

b) TLC: Merck silica gel precoated plates developed with methanol-dichloromethane-triethylamine (50:200:1).

c) Fluorescence excitation maximum, uncorrected.

d) Fluorescence emission maximum, uncorrected.

e) Very weak.

FL derivatives fluoresced strongly on TLC plates. Fluorescence excitation and emission spectra of I and III—IX on TLC plates were measured after development with methanol-dichloromethane-triethylamine (50:200:1) mixture. As shown in Table V, the excitation and emission maxima on TLC plates were similar to those in ethanol solution. Detection limits were about 1 ng/spot except for III.

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