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*

Fluorescamine derivatives of butylamine, benzylamine, *p*-anisidine, *p*-toluidine, aniline, *p*-chloroaniline, sulfanilamide, sulfamethoxazole and N^1-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(3H), 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] catecholamines[4] 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 FL[9] 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.
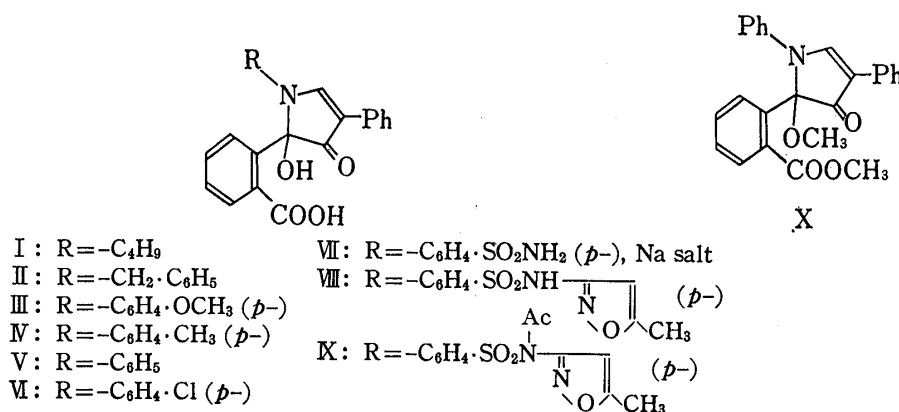

I : R=-C₄H₉
II : R=-CH₂·C₆H₅
III : R=-C₆H₄·OCH₃ (*p*-)
IV : R=-C₆H₄·CH₃ (*p*-)
V : R=-C₆H₅
VI : R=-C₆H₄·Cl (*p*-)

VII : R=-C₆H₄·SO₂NH₂ (*p*-), Na salt
VIII : R=-C₆H₄·SO₂NH— (*p*-)
IX : R=-C₆H₄·SO₂N— (*p*-)

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 (Fluram, 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 μl of Et_3N 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 CH_2Cl_2 (30 ml). The organic extract was washed with half-saturated brine (20 ml) then dried (Na_2SO_4), and the solvent was removed under reduced pressure. The crystalline substance in the residual syrup was recrystallized from CH_2Cl_2 to give III (yield 31.5 mg, 19%). Yellow powder, mp 138—142°C. Anal. Calcd for $C_{24}H_{19}NO_5$: C, 71.81; H, 4.77; N, 3.49. Found: C, 71.74; H, 5.05; N, 3.22. IR ν_{max}^{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 CH_2Cl_2), as a pale yellow powder. Anal. Calcd for $C_{24}H_{19}NO_4$: C, 74.79; H, 4.97; N, 3.63. Found: C, 74.62; H, 4.96; N, 3.65. IR ν_{max}^{Nujol} cm^{-1}: 3160, 1691, 1621.

5-(2-Carboxyphenyl)-1-(4-chlorophenyl)-5-hydroxy-3-phenyl-2-pyrroline-4-one (VI) was prepared analogously from p-chloroaniline (50%), mp 116—118°C (from CH_2Cl_2), as a yellow powder. Anal. Calcd for $C_{23}H_{16}ClNO_4$: C, 68.07; H, 3.97; N, 3.45. Found: C, 68.18; H, 3.75; N, 3.65. IR ν_{max}^{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 μl Et_3N 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 Et_2O (30 ml) and Et_2O–MeCN (5:1, 36 ml), and lyophilized. The resulting yellow powder was dissolved in a small amount of Me_2CO. The solution was chromatographed on a silica gel TLC plate containing 5% $NaHCO_3$ with MeOH–CH_2Cl_2–Et_3N (50:200:1). The fluorescent fraction of Rf value between 0.2 and 0.4 was extracted with MeOH containing 1% Et_3N. After the MeOH had been evaporated off, the residue was dissolved in Me_2CO and filtered. The filtrate was concentrated, and the residue was crystallized from a MeOH–CH_2Cl_2 mixture. Recrystallization from MeCN–Et_2O then from MeCN–MeOH afforded a hygroscopic yellow powder (7.8%), dp 245—246°C. Anal. Calcd for $C_{23}H_{17}N_2O_6$-SNa·H_2O: 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 ν_{max}^{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 $C_{27}H_{21}N_3O_7S$: C, 61.01; H, 3.98; N, 7.91. Found: C, 60.84; H, 3.72; N, 7.75. IR ν_{max}^{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 N^1-acetylsulfamethoxazole dissolved in 6 ml of MeCN were added 56 μl of Et_3N 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 CH_2Cl_2 (30 ml). The organic extract was washed with half-saturated brine (20 ml) then dried (Na_2SO_4), and the solvent was removed under reduced pressure. Crystallization from Me_2CO–petroleum ether afforded a yellow powder. The powder was dissolved in Me_2CO, and the solution was chromatogrophed on a silica gel TLC plate containing 5% $NaHCO_3$ with MeOH–CH_2Cl_2–Et_3N (50:200:1). The fluorescent fraction of Rf between 0.35 and 0.45 was extracted with MeOH containing 1% Et_3N. After the MeOH had been evoporated 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 CH_2Cl_2. The extract was washed with half-saturated brine (30 ml) and dried (Na_2SO_4), and the solvent was removed under reduced pressure. Recrystallization of the residual powder from Et_2O–CH_2Cl_2 afforded 34 mg (15%) of yellow powder, mp 153—155°C. Anal. Calcd for $C_{29}H_{23}N_3O_8S$: C, 60.73; H, 4.04; N, 7.33. Found: C, 60.62; H, 3.81; N, 7.60. IR ν_{max}^{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 Et_2O solution of CH_2N_2 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 Et_2O and chromatographed on a silica gel TLC plate with Et_2O. The fraction of Rf between 0.49 and 0.67 (blue-green fluorescence) was extracted with Et_2O. After the Et_2O had been evaporated off, the residue was recrystallized from Et_2O to give X (yield 14 mg, 37%). Yellow powder, mp 57—58°C. Anal. Calcd for $C_{25}H_{21}NO_4$: C, 75.17; H, 5.30; N, 3.51. Found: C, 75.32; H, 5.54; N, 3.62. IR ν_{max}^{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

TABLE I. Fluorescence Properties of FL Derivatives of Pyrrolinone Type

FL derivative of primary amine[a]

In each compound cell the excitation column lists the secondary (short‑wavelength) excitation maximum followed by the main excitation maximum; for Me₂CO no secondary excitation maximum is given. (Ex^c), Em^d) in nm; RFI^e))

Solvent	ε[b]	I Ex	I Em	I RFI	II Ex	II Em	II RFI	III Ex	III Em	III RFI	IV Ex	IV Em	IV RFI	V Ex	V Em	V RFI	VI Ex	VI Em	VI RFI	VII Ex	VII Em	VII RFI	VIII Ex	VIII Em	VIII RFI	IX Ex	IX Em	IX RFI	X Ex	X Em	X RFI
Benzene	2.275	292, 400	483	25	291, 398	482	45	298, 426	528	9	296, 420	514	76	300, 420	505	109	298, 418	504	257	302, 413	490	107	305, 416	490	99	304, 417	491	300	293, 398	480	411
CHCl₂I₂	8.98	287, 400	490	76	284, 397	490	51	302, 414	496	13	292, 418	524	35	296, 420	510	91	290, 417	512	105	300, 413	494	118	302, 415	495	170	302, 413	494	181	288, 398	475	540
Et₂O	4.335	290, 398	478	108	285, 394	476	31	294, 406	504	38	294, 406	494	194	293, 401	490	340	293, 400	487	263	302, 400	478	339	302, 400	478	339	302, 400	476	639	288, 397	476	510
AcOEt	6.02	292, 397	479	108	288, 396	477	75	296, 414	518	15	295, 416	503	143	296, 415	497	375	294, 411	494	331	300, 407	486	179	303, 408	482	525	303, 400	481	1076	289, 397	486	446
Me₂CO	20.70	397	479	190	397	478	69	409	520	11	414	506	119	413	498	338	408	496	267	406	494	29	403	481	445	403	482	934	397	478	385
EtOH	24.55	290, 394	475	70	285, 388	474	25	298, 410	520	14	296, 405	502	103	295, 403	495	95	294, 400	494	105	300, 399	482	102	303, 399	481	100	304, 398	476	130	288, 400	498	319
MeCN	37.50	292, 388	474	25	287, 395	482	66	302, 407	504	14	294, 409	504	14	294, 410	500	16	294, 403	495	7	300, 406	489	87	302, 403	480	4	302, 404	479	8	288, 397	483	514
DMSO	46.68	291, 370	468	14	291, 370	468	14	309, 412	500	23	304, 410	480	9	304, 404	472	5	300, 405	468	5	306, 402	462	6	302, 400	470	16	307, 404	471	13	294, 398	482	514

a) 2×10^{-9} mol/ml.
b) Dielectric constant.[19]
c) Fluorescence excitation maximum, uncorrected.
d) Fluorescence emission maximum, uncorrected.
e) Relative fluorescence intensity. The fluorescence intensity of VIII in EtOH solution was taken as 100.

amines (III—VII) in various organic solvents were found to have a blue shift with increasing σ-values (Hammett's substituent constant) (III: p-OCH$_3$, −0.268; IV: p-CH$_3$, −0.170; V: H, 0.000; VI: p-Cl, 0.227; VII: p-SO$_2$NH$_2$, 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	Em 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
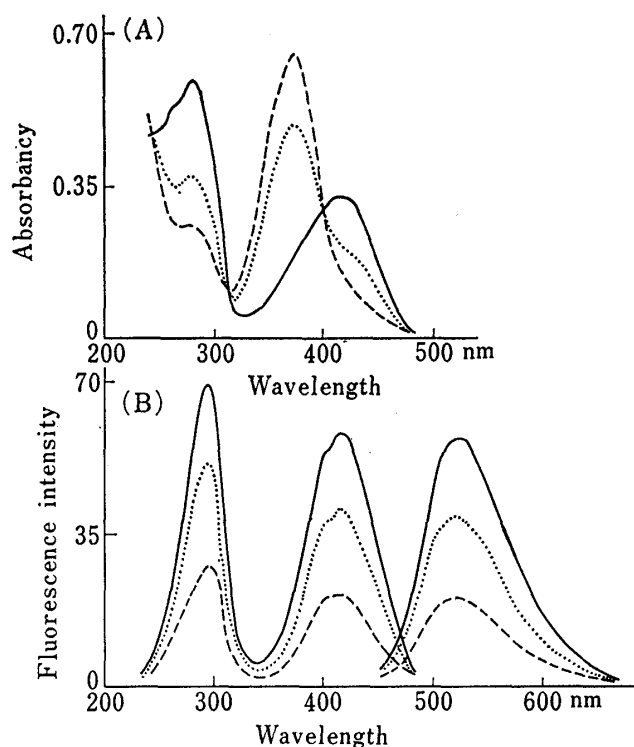
Fig. 1.   Changes of Absorption and Fluorescence
Spectra of V in Dichloromethane

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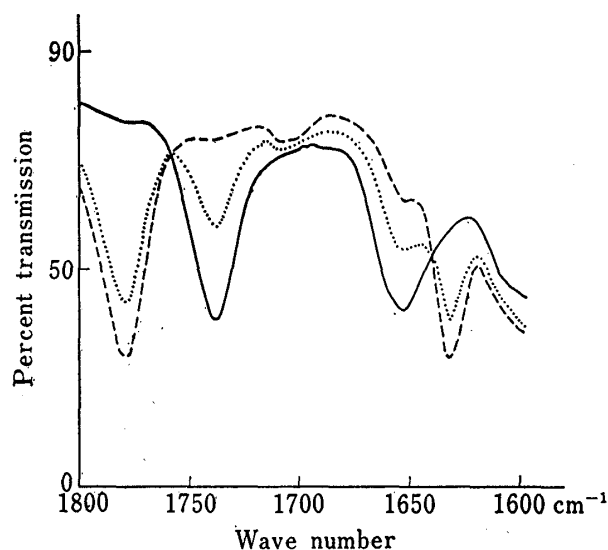——: 6 min, ……: 60 min, ———: 180 min.

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 addi-
tion 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 de-
creased 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)		
	$25 \pm 1°C$		$0—1°C$
	17% Me_2CO	29% Me_2CO	17% Me_2CO
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
IX	18.5		176

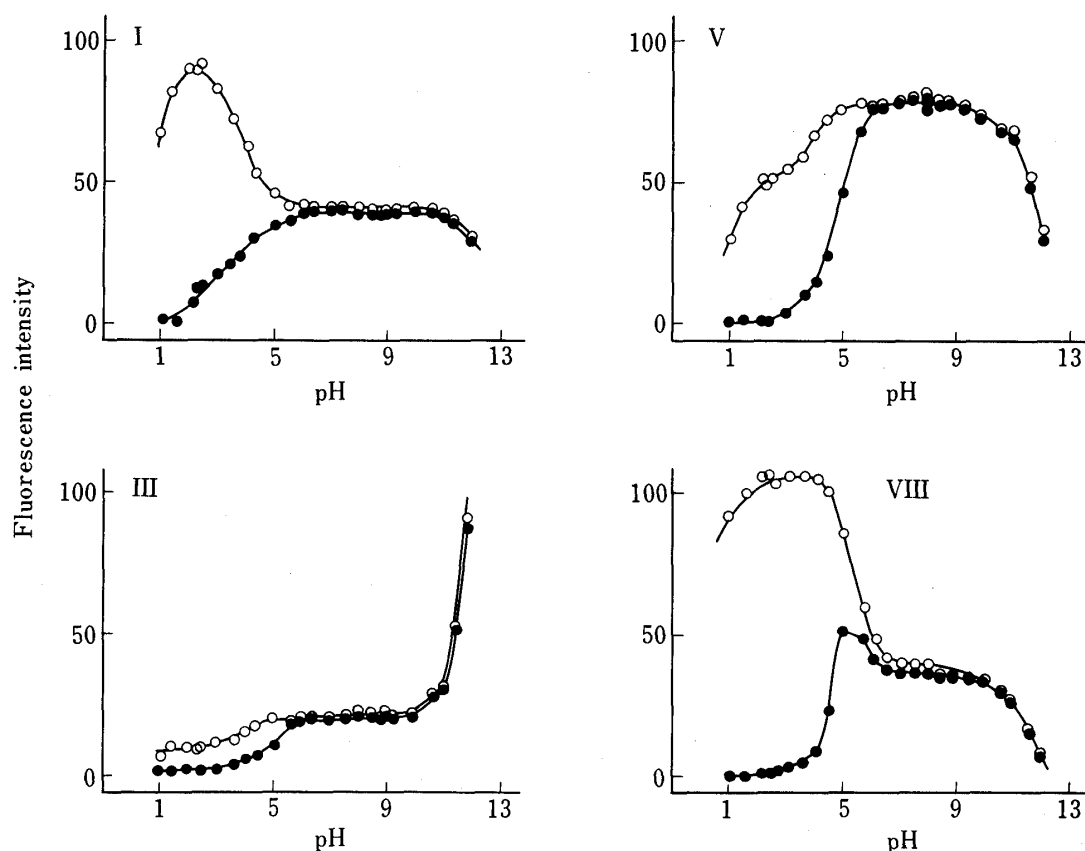a)   ca. 3×10^{-9} mol/ml

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)
n-Butylamine	10.61	0.0	25.6	45.1
p-Anisidine	5.29		106.1	59.7
p-Toluidine	5.07		103.7	54.8
Aniline	4.58	1.6	100.9	33.2
p-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) 1 N HCl–AcONa.
b) Citric acid-Na$_2$HPO$_4$ buffer
c) Borate buffer.

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

FL derivative[a]	Rf 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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