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Isolation and Structural Investigation of the Fluorescent Degradation Products of Ampicillin

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The reaction mechanism of the degradation of ampicillin to fluorescent products has been investigated. High performance liquid chromatography (HPLC) analysis of the degradation products obtained by reaction in the presence of mercuric chloride indicated the formation of a new fluorophore. Isolation of the product followed by spectral investigation showed the structure of the new fluorophore to be 2-hydroxy-3-phenyl-6-penillomethylpyrazine. The degradation in the presence of acetoaldehyde, however, produced another fluorophore which was identified as 2-hydroxy-3-phenyl-6-ethylpyrazine. The reaction mechanism was elucidated in terms of a penilloaldehyde intermediate on the basis of the above results.

Keywords——ampicillin; reaction mechanism; fluorescent degradation products; high performance liquid chromatography; ^{13}C NMR spectra; mass spectra

Introduction

It is known that β-lactam antibiotics having an α-amino group on the side chain (amino-(penicillins and aminocephalosporins) yield a highly fluorescent product when they are degraded under certain conditions. The reaction has been conveniently utilized for the sensitive fluorometric assay of such antibiotics in aqueous solution and in physiological fluids. The reaction so far employed for analytical purposes has been carried out under various conditions of pH, time period, temperature, catalyst, and concentration of reactant.

Jusko,[1] achieving the degradation of ampicillin in a pH 2 solution containing formaldehyde, found that the aminobenzyl group and a cleaved β-lactam ring were necessary for the formation of the fluorophore, because benzylpenicillin which lacks the α-amino group of ampicillin gave no fluorescent product and penicilloic acid obtained by the hydrolysis of ampicillin with penicillinase gave a product with fluorescence as intense as that of the parent penicillin. Jusko also found that formaldehyde accelerated the reaction. Degradation of cephaloglycin (as well as cephalexin) in a weak alkaline solution in the absence of formaldehyde also yielded a strongly fluorescent yellow product (λ_{ex}=433 nm, λ_{em}=355 nm).[2] The fluorophore formed by these reactions had been assumed tentatively to be a substituted 2,5-diketopiperazine derivative without any experimental confirmation.[2]

Although many modified procedures were subsequently applied to other aminopenicillins and aminocephalosporins,[3–12] the excitation and emission wavelengths used for the measurements of fluorescent intensity were essentially the same for most of the antibiotics tested, *i.e.* λ_{ex}=420—440 nm, λ_{em}=340—360 nm. This suggested that the fluorophores derived from different antibiotics might have analogous structures.

On the other hand, it has been reported that a 2,5-diketopiperazine derivative could be formed when cephradine was kept at 5° for 3 weeks in aqueous Na_2CO_3 solution,[13] when solutions of the esters of cephaloglycin and cephalexin in benzene were refluxed for a long time,[14] and when ampicillin was kept at 22° for 20 hrs in an aqueous alkaline solution of glucose.[15] These investigations, however, did not mention whether or not the 2,5-diketopiperazine derivative might be fluorescent.

Thus, the structure of the fluorescent product formed by the degradation of aminopenicillins and aminocephalosporins was uncertain, until Barbhaiya and Lebelle identified it. Bar-

bhaiya[16] showed that the alkaline degradation of various aminopenicillins and aminocephalosporins followed by treatment in citrate buffer solution containing formaldehyde yielded an identical fluorophore whose spectral and chromatographic properties were identical with those of authentic 2-hydroxy-3-phenyl-6-methylpyrazine, which was synthesized from α-phenylglycine amide and methylglyoxal. Lebelle,[17] employing a similar reaction procedure except for the use of formaldehyde, showed that the fluorophore of ampicillin was identical with synthetic 2-hydroxy-3-phenylpyrazine. The proposed reaction mechanism was that penicilloic acid formed by alkaline hydrolysis of ampiciliin is furthre converted into penilloaldehyde through penaldic acid, then penilloaldehyde undergoes intramolecular cyclization to form a Schiff base between amino and aldehyde groups, and subsequent oxidation leads to the pyrazine derivative.

In our studies on the high performance liquid chromatographic (HPLC) analysis of aminopenicillins,[18,19] we found two unknown peaks with appreciable fluorescence intensity appearing on the chromatograms of alkaline degradation products of ampicillin. One of the peaks was found to be due to a new fluorophore obtained when ampicillin was degraded in the presence of acetaldehyde instead of formaldehyde, and the other was to a new fluorophore which was formed by the degradation in the absence of aldehyde. By isolation and spectral investigation of the these fluorescent products, the structure of the former was assigned as 2-hydroxy-3-phenyl-6-ethylpyrazine and that of the latter as 2-hydroxy-3-phenyl-6-penillomethylpyrazine. These findings implied that the previous results could account for only a part of the fluorophore-producing reaction. The present paper describes structural investigation of the new fluorophores and the overall reaction mechanisms.

Experimental

1. **Materials**——Anhydrous ampicillin (AB-PC) and sodium AB-PC were gifts from Takeda Pharm. Ind. Co. Ltd. (Osaka, Japan).

Sodium *n*-heptyl sulfonate used as an ion-pairing agent for HPLC was synthesized by means of the Strecker reaction.[20]

^{12}C-Methanol-d_4 (99.5%), chloroform-*d*, deuterium oxide (D_2O), tetramethylsilane (TMS), and dioxane used for nuclear magnetic resonance (NMR) measurements were purchased from E. Merck, West Germany. Other chemicals used were of analytical reagent grade.

2. **Degradation of Ampicillin**——Procedure 1: Anhydrous AB-PC (10 mg) was dissolved in 10 ml of 0.02 N NaOH and allowed to stand 37° for 45 min. The solution was neutrallized 0.02 N HCl, then 20 ml of aqueous 1 M NaH_2PO_4 solution was added. The mixture was kept at 30° in a thermostated water-bath for 6 hrs.

Procedure 2: Anhydrous AB-PC (500 μg) was dissolved in 1 ml of distilled water. To the solution were added 1 ml of 1/2 M citrate buffer (pH 2) and 0.5 ml of 7% formaldehyde in 0.4 M pH 2 citrate buffer. The solution was then heated at 90° for 2 hrs. This procedure was the same as Jusko's method,[1] but the solvent extraction procedure was omitted.

Procedure 3: Sodium AB-PC (500 mg) in distilled water (50 ml) was treated (10 min; 20°) with 25 ml of 1 N NaOH. To the reaction solution were added 25 ml of 1 N HCl and 150 ml of Sörensen's citrate buffer (pH 4) containing formaldehyde (1.0% v/v). The solution was then heated at 100° for 30 min. This procedure was the same as Barbhaiya's method,[16] but the subsequent solvent extraction procedure was omitted.

Procedure 4: Sodium AB-PC (10 g) was dissolved in distilled water (200 ml), and the solution was maintained at pH 11—12 by addition of 1 N NaOH. After 2 hrs, the solution was adjusted to pH 2 by the addition of 1 N HCl, and heated at 50° for 5 hrs. This procedure was the same as Lebelle's method,[17] but the procedure for solvent extraction of the fluorescent product was omitted.

Procedure 5: Anhydrous AB-PC (1 μg—2 mg) dissolved in distilled water (1 ml) was treated (5 min; room temperature) with 0.5 ml of 1 N NaOH, neutralized with 0.5 ml of 1 N HCl and mixed with pH 2.5 citrate buffer (1 ml) containing $HgCl_2$ (0.04% w/v). After 5 min, 6 ml of phosphate buffer solution (pH 6.0) was added. The solution was then heated at 40° for 20 min. This procedure was the same as Miyazaki's method,[3] but the solvent extraction procedure was omitted. For isolation of the fluorophore, the reaction solution was lyophilized, and the residue was purified by column chromatography with a stationary phase of LiChroprep RP-8 (E. Merck) packed in 310 mm × 25 mm i.d. glass tubing. The column was eluted first with 600 ml of a mixture of methanol/water (3/2, v/v), then with 2000 ml of methanol. The last 1800 ml fraction was collected, and the solvent was removed by evaporation. Lyophilization of the oily residue

dissolved in water gave a reddish-yellow amorphous solid (product (II), dec. 120—125°).

Procedure 6: Sodium AB-PC was treated according to procedure 3 except that acetaldehyde was used instead of formaldehyde. The isolation of the fluorescent product was achieved as follows. The final reaction solution was repeatedly extracted with ethyl acetate. Removal of the solvent from the extract gave a dull orange solid which was passed through a Sep-Pak silica cartridge (Waters Ass. Inc., U.S.A.) using ethyl acetate as an eluent, followed by further purification by TLC [Silica gel 60 precoated plate (20×20 cm) containing no fluorescent indicator; chloroform/acetone=2:1 (v/v)]. The fluorescent zone (*Rf* 0.61) visualized by UV light (245 nm) was scraped off the plate and extracted with ethyl acetate. The residue obtained by evaporating off the solvent was recrystallized from ethyl acetate, yielding pale-yellow needles (product (I), mp 169—170°).

3. **Measurements**——1) HPLC: A high performance liquid chromatograph (Shimadzu LC-3, Shimadzu, Kyoto, Japan) equipped with a variable wavelength UV detector (SPD-2A, Shimadzu) and a spectrofluorophotometric detector (RF-500, Shimadzu) was used in a reverse phase mode with a stationary phase of Nucleosil $10C_{18}$ (M. Nagel, West Germany) packed in 25 cm×4.6 mm i.d. stainless steel tubing, and operated at ambient temperature. A short pre-column (5 cm×1.5 mm i.d.) filled with LiChrosorb RP-2 (E. Merck) was used to guard the main column. A mixture of methanol/water (5/8, v/v) containing 0.011 M sodium *n*-heptyl sulfonate, 0.005 M NaH_2PO_4 and 1.3% (v/v) of 0.5 N HCl (pH 2.7) was used as the mobile phase at a flow rate of 0.8 ml/min. The effluent was monitored by measurements of UV absorption at 218 nm and fluorescence intensity at λ_{ex} 345 nm and λ_{em} 420 nm.

2) Mass Spectra (MS): Electron impact (El) mass spectra were measured with JEOL JMS-01SG2 mass spectrometer (JEOL Ltd., Tokyo, Japan) equipped with a data processing system, operating at an ionizing potential of 25 and 75 eV, and a total emission current of 300 μA. Samples were introduced by means of a direct insertion probe and the spectra of the degradation products were recorded during fractional volatilization on increasing the sample temperature from 50 to 400°.

Field desorption (FD) mass spectrometry was carried out on a JEOL JMS-01SG2 mass spectrometer (JEOL) equipped with a field desorption ion source, model MS-FD01, operating at an accelerating voltage of 10 kV and an emitter heating current of 5—18 mA.

3) NMR Spectra: ^{13}C NMR spectra were measured on a JEOL FX-100 NMR spectrometer (JEOL) using 5 mm spinning tubes at ambient temperature and employing the deuterium field/frequency lock system. Samples were dissolved in ^{12}C-methanol-d_4, chloroform-*d* and D_2O. Tetramethylsilane (δ=0.0 ppm) and dioxane (δ=67.4 ppm) were used as internal references.

The ^1H NMR spectrum was measured on a JEOL FX-100 NMR spectrometer (JEOL). The sample was dissolved in ^{12}C-methanol-d_4. Chemical shifts are given relative to internal TMS.

Results

1. HPLC of Fluorescent Products

Figure 1 shows chromatograms of the degradation products obtained by procedure 1. The two peaks on the chromatogram with UV detection (shown by dotted line) have been assigned as the penicilloic acid (AB–PA) and penamaldic acid (AB–PM) of ampicillin.[18,19] These products, however, were not fluorescent. Fluorometric detection (shown by solid line) indicates that two fluorescent products (peaks (a) and (b)) were formed by this reaction.

Figure 2 shows chromatograms of the fluorescent products obtained by procedures 2—5. It is clear that the reactions according to procedures 2 and 3 produced exactly the same fluorophore (peak (c) in Fig. 2 (1) and (2)), whose structure had been assigned by Barbhaiya[16] as 2-hydroxy-3-phenyl-6-methylpyrazine, and that no other fluorophores were produced in these reactions. The chromatograms of the reaction solutions obtained by procedures 4 and 5 are shown in Fig. 2 (3) and (4); both reactions produced the same two fluorophores (peak (a) and (b)), which coincide with those obtained by procedure 1 (Fig. 1.). The minor peak (a) can be assigned to 2-hydroxy-3-phenylpyrazine in accord with Lebelle's results, and the major peak (b) is due to a new fluorophore whose structure was investigated in the present work. Figure 2 (5) shows the chromatogram of solvent extracts of fluorophore obtained by procedures 4 and 5. The solvents used were 25% methanol in chloroform for procedure 4 and ethyl acetate for procedure 5, in accord with those employed by Lebelle[17] and Miyazaki,[3] respectively. It is evident that the fluorophore of peak (a) (2-hydroxy-3-phenylpyrazine) was extracted into the organic solvent, leaving the new fluorophore in the aqueous layer. The chromatogram of the aqueous layer (not shown) exhibited only the peak (b) whose retention

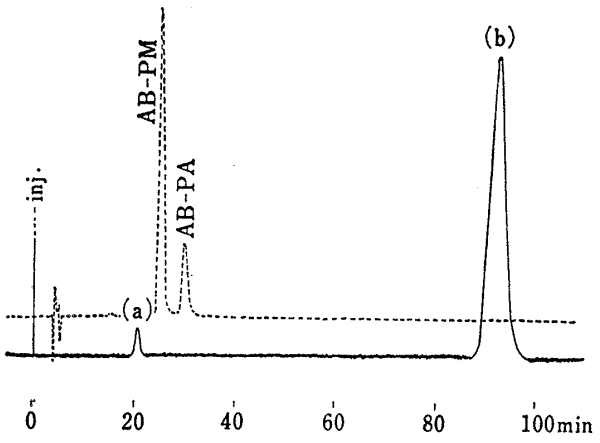


Fig. 1. Chromatograms with UV Detection (---) and Fluorophotometric Detection (—) of Ampicilin which had been allowed to stand at 30° for 6 hrs in Aqueous 1 M NaH_2PO_4 Solution (procedure 1.)

Peak: AB–PA; aminoenicilloic acid,
AB–PM; aminopenamaldic acid.
(a) and (b) fluorescent degradation products of ampicillin.
Conditions: see the text.

time coincided exactly with that of the isolated product (II).

The yield ratio of 2-hydroxy-3-phenylpyrazine and the new fluorophore (II) obtained by procedure 5 was examined upon varying the initial concentration of AB–PC from 100 to 200 μg/ml. The resulting chromatograms shown in Fig. 3 indicate that the increase in the formation of 2-hydroxy-3-phenylpyrazine is accompanied by a simultaneous decrease in that of the new fluorophore (II) as the initial concentration decreases. The same results were obtained when the reaction was carried out according to procedure 4. It is clear, therefore, that the degradation of AB–PC achieved by Lebelle and Miyazaki without the use of formaldehyde produces two fluorophores, whose ratio is a function of the initial concentration. Thus, it follows that the fluorophore, 2-hydroxy-3-phenylpyrazine (peak (a)), isolated by Lebelle is a minor product. It seems that they must have lost the major product (peak (b)) during solvent extraction. Mercuric chloride used in procedure 5 was found to act

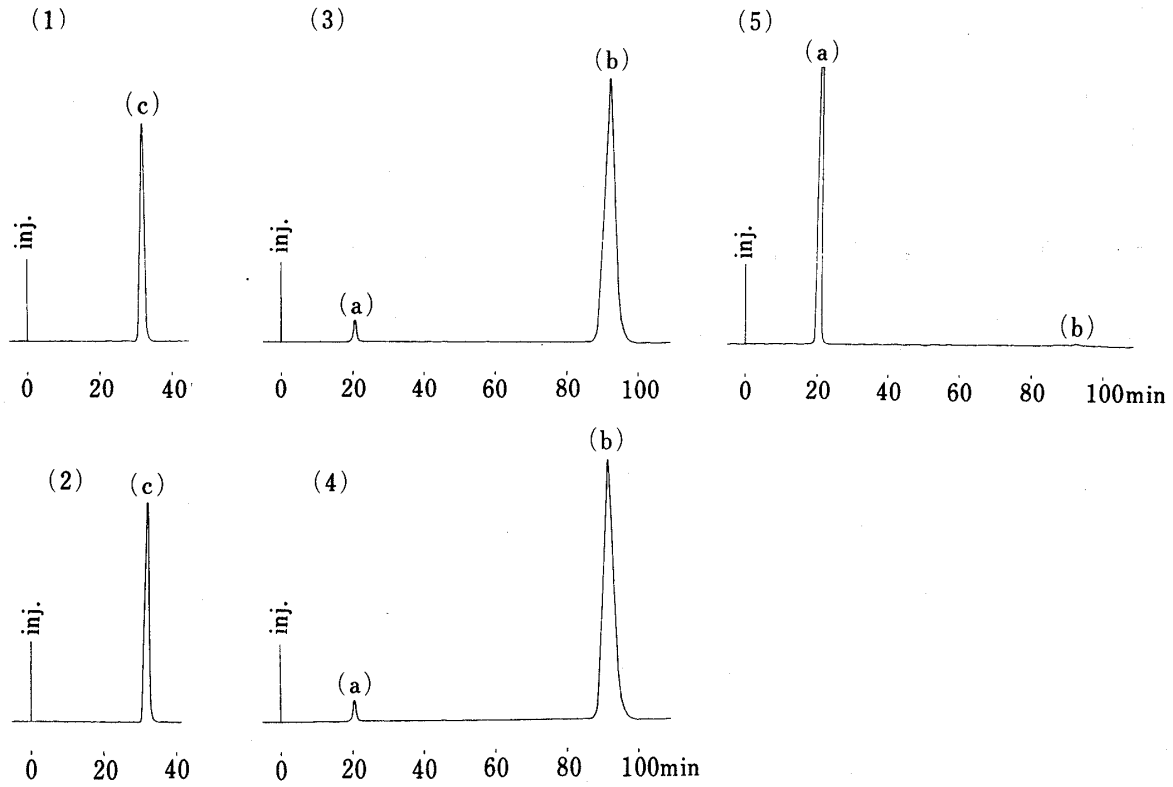


Fig. 2. Chromatograms of the Fuorescent Product of Ampicillin obtained by (1) Procedure 2, (2) Procedure 3, (3) Procedure 4, (4) Procedure 5 and (5) Extract from Procedures 1, 4 and 5 into 25% Methanol in Chloroform or Ethyl Acetate

Peaks (a), (b) and (c): Fluorescent degradation products of ampicillin.
Conditions: See the text.

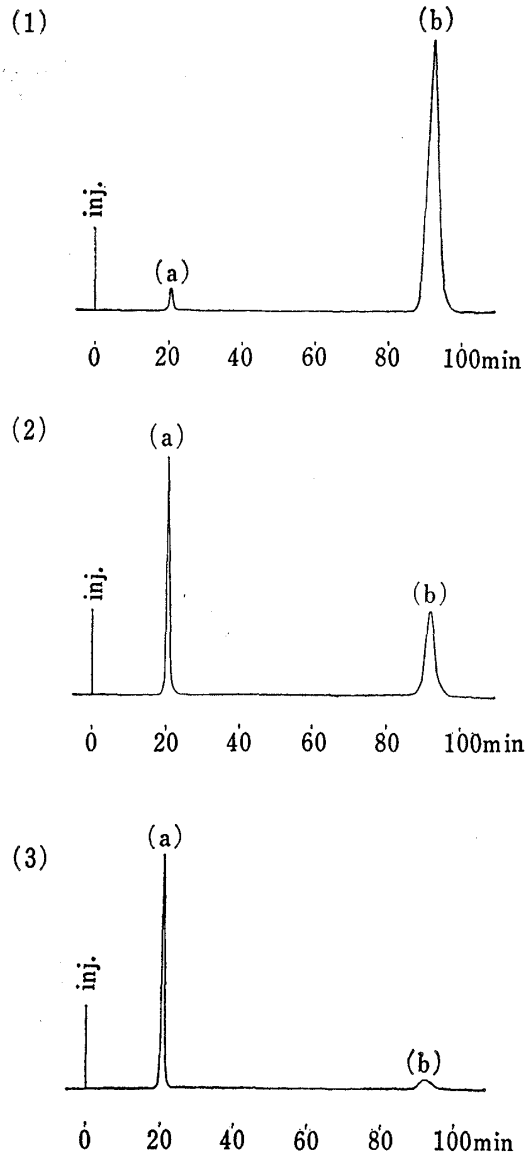


Fig. 3. Chromatograms of the Fluorescent Product of Ampicillin at Initial Concentrations of (1) 200 μg/ml, (2) 150 μg/ml and (3) 100 μg/ml, obtained by Procedure 5

Peaks (a) and (b): Fluorescent degradation products of ampicillin.
Conditions: see the text.

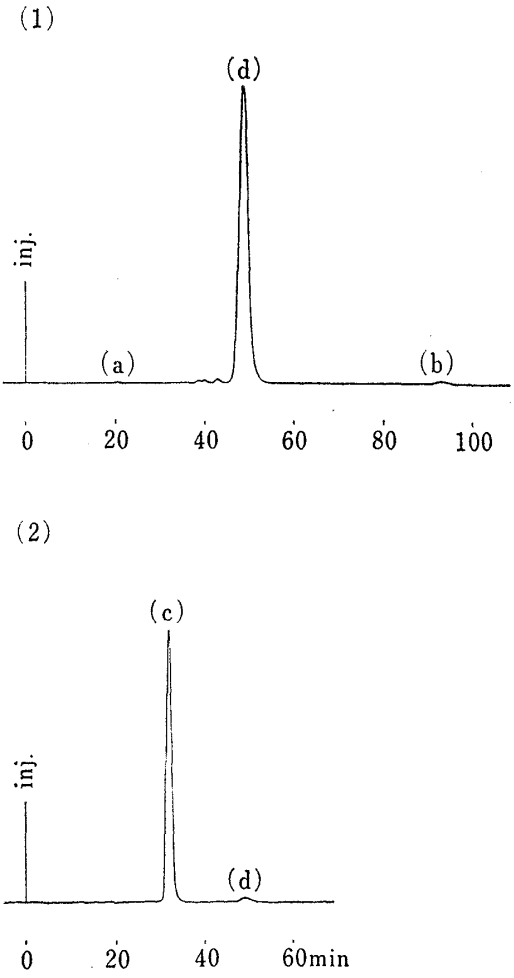


Fig. 4. Chromatograms of the Fluorescent Products obtained by (1) Procedure 6 and (2) Procedure 6 with the Use of Acetaldehyde and Formaldehyde

Peaks (a), (b), (c) and (d): Fluorescent degradation products of ampicillin.
Conditions: see the text.

as a catalyst, because it required about 30 min in its absence at 100° for the reaction to proceed to the same extent as shown in Fig. 3 (1).

The role of formaldehyde used in procedures 3 and 6 was investigated by using acetaldehyde instead. Figure 4 (1) depicts the resulting chromatogram of the reaction solution obtained by procedure 6. The chromatogram of Fig. 4 (2) was obtained from the reaction using both aldehyde at the same time. Figure 4 (1) shows that the use of acetaldehyde produces a different fluorophore (peak (d)) from that obtained by the reaction with formaldehyde, the latter product having been identified as 2-hydroxy-3-phenyl-6-methylpyrazine. The reaction time of peak (d) coincided exactly with that of the isolated fluorophore (I), However, as found from Fig. 4 (2), the production of (I) was depressed when formaldehyde was used together with acetaldehyde, thus resulting in preferential formation of 2-hydroxy-3-phenyl-6-methylpyrazine.

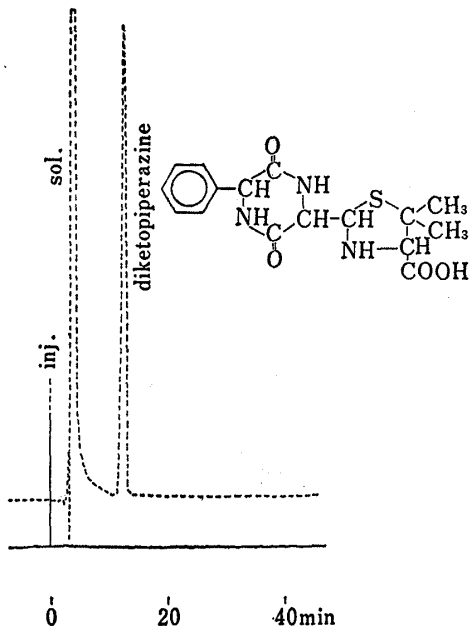


Fig. 5. Chromatograms with UV Detection (---) and Fluorophotometric Detection (——) of 2,5-Diketopiperazine obtained by Bundgarrde's Method

Conditions: see the text.

As mentiond above, it had been considered that the formation of the 2,5-diketopiperazine derivative might be a substantial factor in the development of fluorescence. However, as can be seen in Fig. 5, HPLC of the 2,5-diketopipera zine derivative of AB–PC synthesized according to Bundgaard's method[15] showed no fluorometric response.

2. Mass Spectra

The fluorescent product (I) formed by procedure 6 was isolated, and its EI mass spectrum was measured. The mass spectrum (Fig. 6) shows $M^{+\cdot}$ at *m/z* 200, which is 14 mass units higher than that of 2-hydroxy-3-phenyl-6-methylpyrazine. The precise value of $M^{+\cdot}$ (*m/z* 200.0952) measured by high resolution MS indicated that $C_{12}H_{12}N_2O$ (calc. 200.0950) was the most likely molecular formula. This composition is attained by adding CH_2 to 2-hydroxy-3-phenyl-6-methylpyrazine, and also by adding C_2H_4 to 2-hydroxy-3-phenylpyrazine. The elemental composition of a fragment ion at *m/z* 172.1013 was identified as $C_{11}H_{12}N_2$ (calc. 172.1000), and that of a fragment ion at 157.0779 as $C_{10}H_9N_2$ (calc. 157.0766). These results strongly suggest that (I) may be assignable to 2-hydroxy-3-phenyl-6-ethylpyrazine.[16,17]

Figure 7 shows FD and EI mass spectra of the fluorescent product (II) isolated by procedure 5. Comparison of these spectra indicates that the base peak at *m/z* 349 in FD mass spectrum is assignable to MH^+. The molecular ion with a precise mass number of *m/z* 348.1566 supported the elemental composition of $C_{20}H_{20}N_4O_2$ (calc. 348.1586), which corresponds to $C_{10}H_{13}N_2O$ - substituted 2-hydroxy-3-phenylpyrazine. The fragment ions at 243.0985 and 106.0654 were found to corresoond to $C_{13}H_{12}N_3O_2+H$ (calc. 243.1006) and C_7H_8N (calc. 106.0655), respectively.

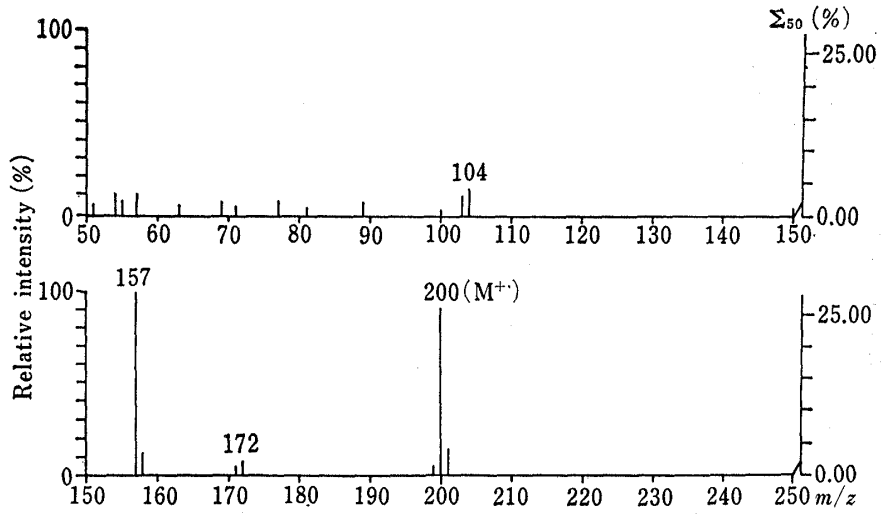


Fig. 6. EI Mass Spectrum of the Fluorescent Product (I) obtained by Procedure 6 (peak (d))

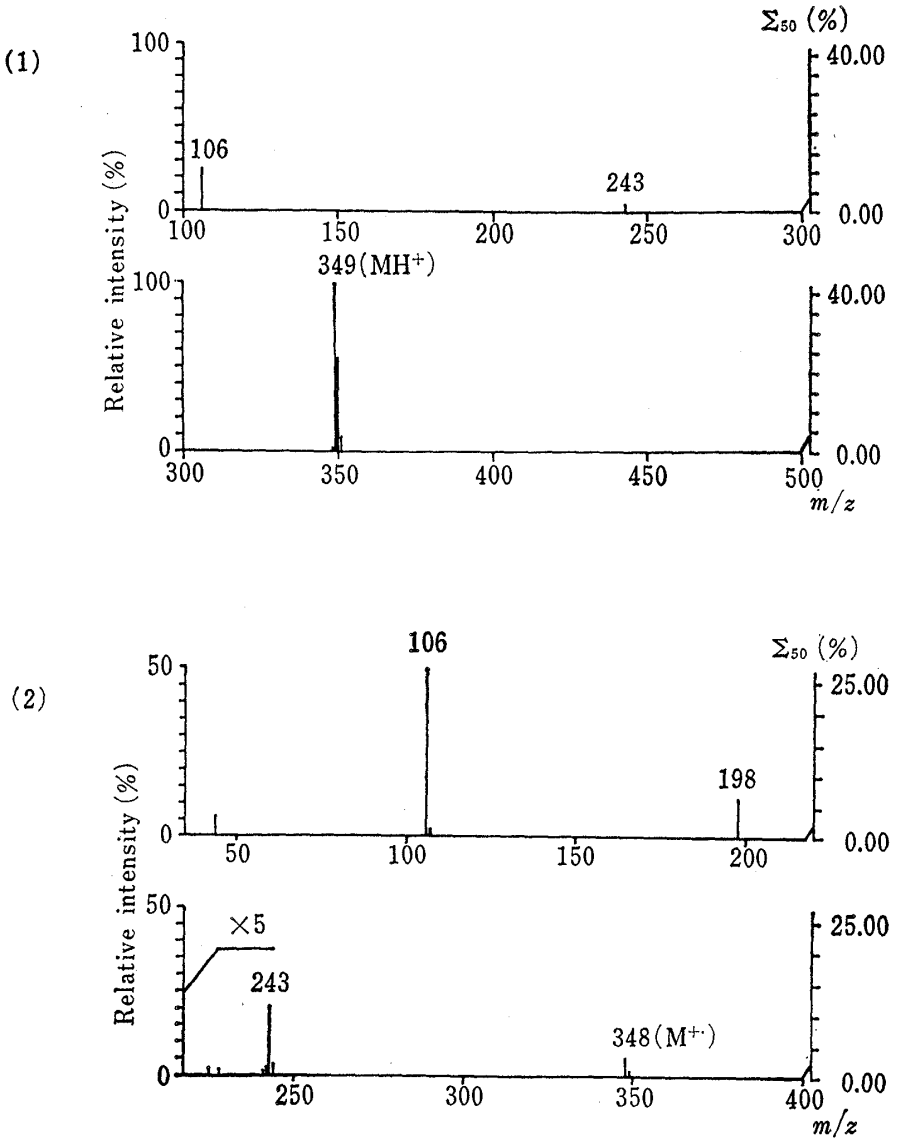


Fig. 7. (1) FD and (2) EI Mass Spectra of the Fluorescent Product (II) obtained by Procedure 5 (peak (b))

3. NMR Spectra

Figure 8 (1) and (2) shows the ^{13}C NMR spectra of AB–PC and 2-hydroxy-3-phenyl-6-methylpyrazine. The assignments were determined by taking into account the results of off-resonance decoupling experiments and the expected chemical shifts.

The ^{13}C and ^{1}H NMR spectra of (I) are shown in Fig. 9. (1) and (2), respectively.

The ^{13}C NMR spectrum of (II) are shown in Fig. 10.

All the observed ^{13}C chemical shifts are summarized in Table I, together with the assignments. The assignments of ^{13}C resonances were made by comparison of the ^{13}C NMR spectra of AB–PC and 2-hydroxy-3-phenyl-6-methylpyrazine.

Discussion

The above results allow us to discuss the structures of (I) and (II). The structures and numbering of the atoms appearing in the following discussion are shown in Fig. 11. The ^{1}H and ^{13}C NMR spectra of (I) are well explained by the structure 2-hydroxy-3-phenyl-6-ethyl-

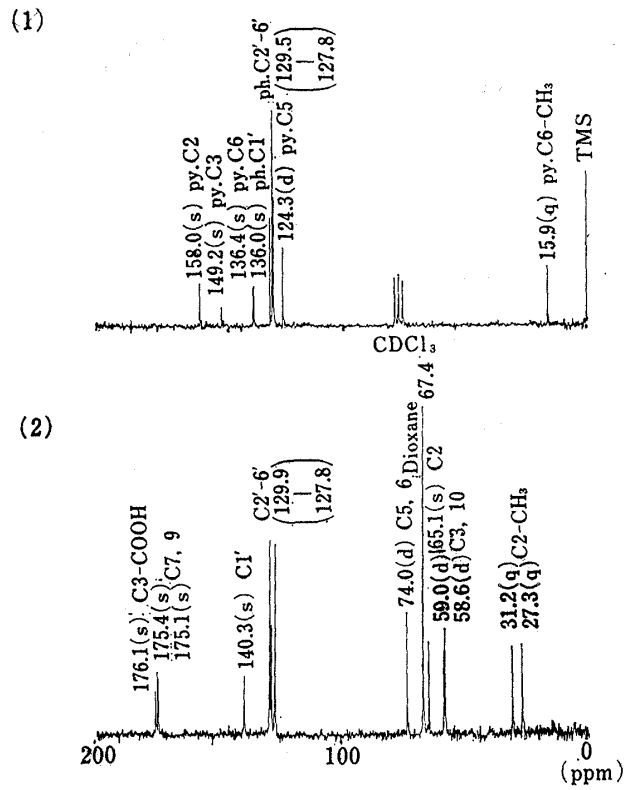


Fig. 8. ^{13}C NMR Spectra of (1) 2-Hydroxy-3-phenyl-6-methylpyrazine and (2) Ampicillin

Chemical shifts and signal multiplicities obtained from the off-resonance decoupled spectrum are given on top of each line.

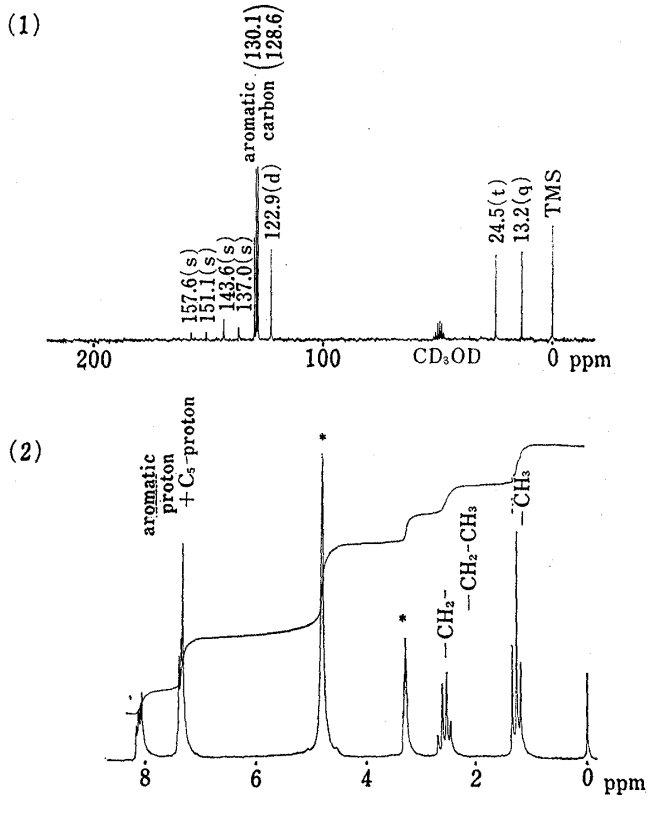


Fig. 9. (1) ^{1}H NMR and (2) ^{13}C NMR Spectra of the Fluorescent Product (I) obtained by Procedure 6 (peak (d))

Chemical shifts and signal multiplicities obtained from the off-resonance decoupled spectrum are given on top of each line. (*) solvent.

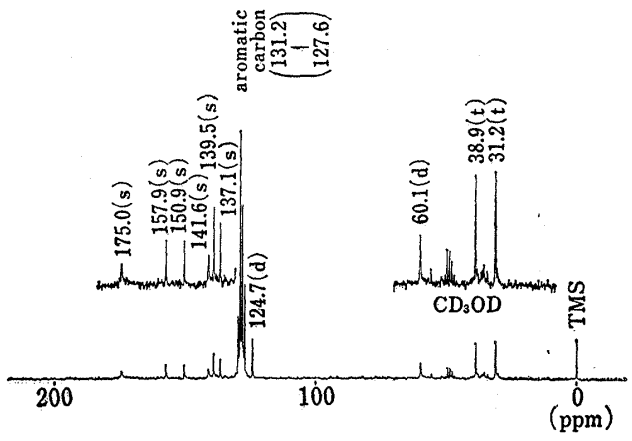


Fig. 10. ^{13}C NMR Spectrum of the Fluorescent Product (II) obtained by Procedure 5 (peak (b))

Chemical shifts and signal multiplicities obtained from the off-resonance decoupled spectrum are given on top of each line.

pyrazine, which was postulated on the basis of the mass spectral results. The existence of an ethyl group is evident from the ^{1}H NMR signals (Fig. 9 (2)) of a triplet at 1.28 ppm and a quartet at 2.60 ppm. The signals at 8.15 and 7.35 ppm indicate that (I) has six olefinic protons, five of which belong to the substituted benzene ring and one of which is located at the C5 position. The ^{13}C NMR results can be well explained by this structure (Table I). The singlet signals of (I) at 157.6, 151.1, 143.6 and 137.0 ppm correlate well with those of 2-hydroxy-3-phenyl-6-methylpyrazine, *i.e.* C2 at 158.0 ppm, C3 at 149.2 ppm, C6 at 136.4 ppm and phenyl C1′ at 136.0 ppm, respectively. The doublet at 122.9 ppm can be correlated with C_5 at 124.3 ppm. The triplet at 24.5 ppm and quartet at 13.2 ppm can be assigned to carbons of $-CH_2-$ and $-CH_3$ groups, respectively. The structure of (I) is thus assigned as 2-hydroxy-3-phenyl-6-ethylpyrazine. Thus, acetaldehyde is transformed into an ethyl group at the C6 position

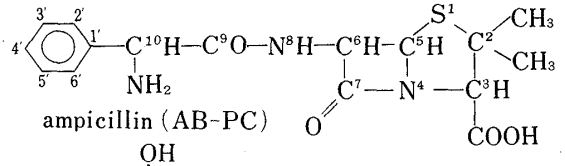
TABLE I. ^{13}C Chemical Shifts and Assignments of Product (II), Product (I), 2-Hydroxy-3-phenyl-6-methylpyrazine and Ampicillin

Product (II) (in CD_3OD)[a]		Product (I) (in CD_3OD)[a]		2-Hydroxy-3-phenyl-6-methylpyrazine (in $CDCl_3$)[a]		Ampicillin (in D_2O)[b]	
Chemical shift ( )[c]	Assignments	Chemical shift ( )[c]	Assignments	Chemical shift ( )[c]	Assignments	Chemical shift ( )[c]	Assignments
175.0 (s)	Aminophenylacetyl Cl					176.1 (s)	C3-COOH
						175.4 (s)	C7,C9
						175.1 (s)	
157.9 (s)	Pyrazine C2	157.6 (s)	Pyrazine C2	158.0 (s)	Pyrazine C2		
150.9 (s)	Pyrazine C3	151.1 (s)	Pyrazine C3	149.2 (s)	Pyrazine C3		
141.6 (s)	Pyrazine C6	143.6 (s)	Pyrazine C6	136.4 (s)	Pyrazine C6		
139.5 (s)	Aminophenylacetyl C3					140.3 (s)	C1′
137.1 (s)	Phenyl C1′	137.0 (s)	Phenyl C1′	136.0 (s)	Phenyl C1′		
131.2	Phenyl C2′-6′	130.1	PhenylC2′-6′	129.5	Phenyl C2′-6′	129.9	C2′-6′
|	Aminophenyl-	|		|		|	
127.6	acetyl C4-8	128.6		127.8		127.8	
124.7 (d)	Pyrazine C5	122.9 (d)	Pyrazine C5	124.3 (d)	Pyrazine C5		
						74.0 (d)	C5,C6
						65.1 (s)	C2
60.1 (d)	Aminophenylacetyl C2					59.0 (d)	C3,C10
						58.6 (d)	
38.9 (t)	Ethyl C1, C2 ($-CH_2-CH_2-$)	24.5 (t)	Ethyl C1 ($-CH_2-$)				
31.2 (t)							
						31.2 (q)	C2-CH_3
						27.3 (q)	
		13.2 (q)	Ethyl C2 ($-CH_3$)	15.9 (q)	Pyrazine C6-CH_3		

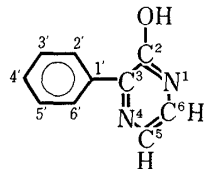
a) Chemical shifts in ppm from internal TMS (δ=0.0 ppm). Accurate $\pm$0.1 ppm.
b) Chemical shifts in ppm from internal dioxane (δ=67.4 ppm). Accurate to $\pm$0.1 ppm.
c) Signal multiplicities obtained from the off-resonance decoupled spectrum. s, singlet; d, doublet; t,triplet;q,quartet.

of the pyrazine skeleton. This result, contrary to what was proposed by Jusko, indicates that the role of aldehyde is not catalytic.

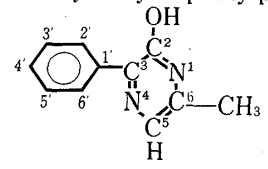
The spectral investigations of (II) lead to the following conclusion. The elemental composition of (II) ($C_{20}H_{20}N_4O_2$) given by high resolution mass spectrometry suggests that $C_{10}H_{13}$-



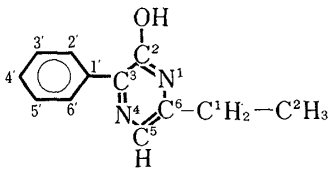
ampicillin (AB-PC)



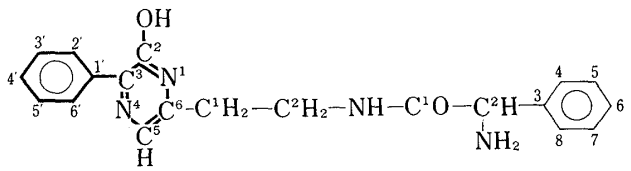
2-hydroxy-3-phenylpyrazine



2-hydroxy-3-phenyl-6-methylpyrazine



2-hydroxy-3-phenyl-6-ethylpyrazine (I)



2-Hydroxy-3-phenyl-6-[2-(aminophenylacetyl)-amino]ethylpyrazine (II) (2-hydroxy-3-phenyl-6-penillomethylpyrazine)

Fig. 11. Structures and Numbering of the Atoms

N_2O may be substituted onto 2-hydroxy -3-phenylpyrazine. The structure of this substituent can be elucidated by addition of one hydrogen atom to penilloaldehyde with simultaneous elimination of one oxygen atom, *i.e.* $C_{10}H_{12}N_2O_2+H-O$. This suggests that the substituent may arise from another penilloaldehyde molecule, namely, a penillomethyl moiety. The ^{13}C NMR results can be well explained by this structure. As shown in Table I, the ^{13}C NMR signals of (II) indicate that the signals at 175.0, 157.9, 150.9, 141.6, 139.5 and 137.1 ppm can be assigned to carbons at the positions of aminophenylacethyl Cl, pyrazine C2, C3 and C6, aminophenylacethyl C3, and phenyl C1′, respectively. These assignments are supported by comparison with those for AB–PC and 2-hydroxy-3-phenyl-6-methylpyrazine. The doublets at 124.7 and 60.1 ppm correspond to carbons at C5 of 2-hydroxy-3-phenyl-6-methylpyrazine and at C10 of AB–PC, respectively, and the triplets at 38.9 and 31.2 ppm can be assigned to two carbons in a $-CH_2-CH_2-$ group. These observations confirm the structure of (II) to be 2-hydroxy-3-phenyl-6-[2-(aminophenylacetyl)amino]ethylpyrazine (2-hydroxy-3-phenyl-6-penillomethylpyrazine).

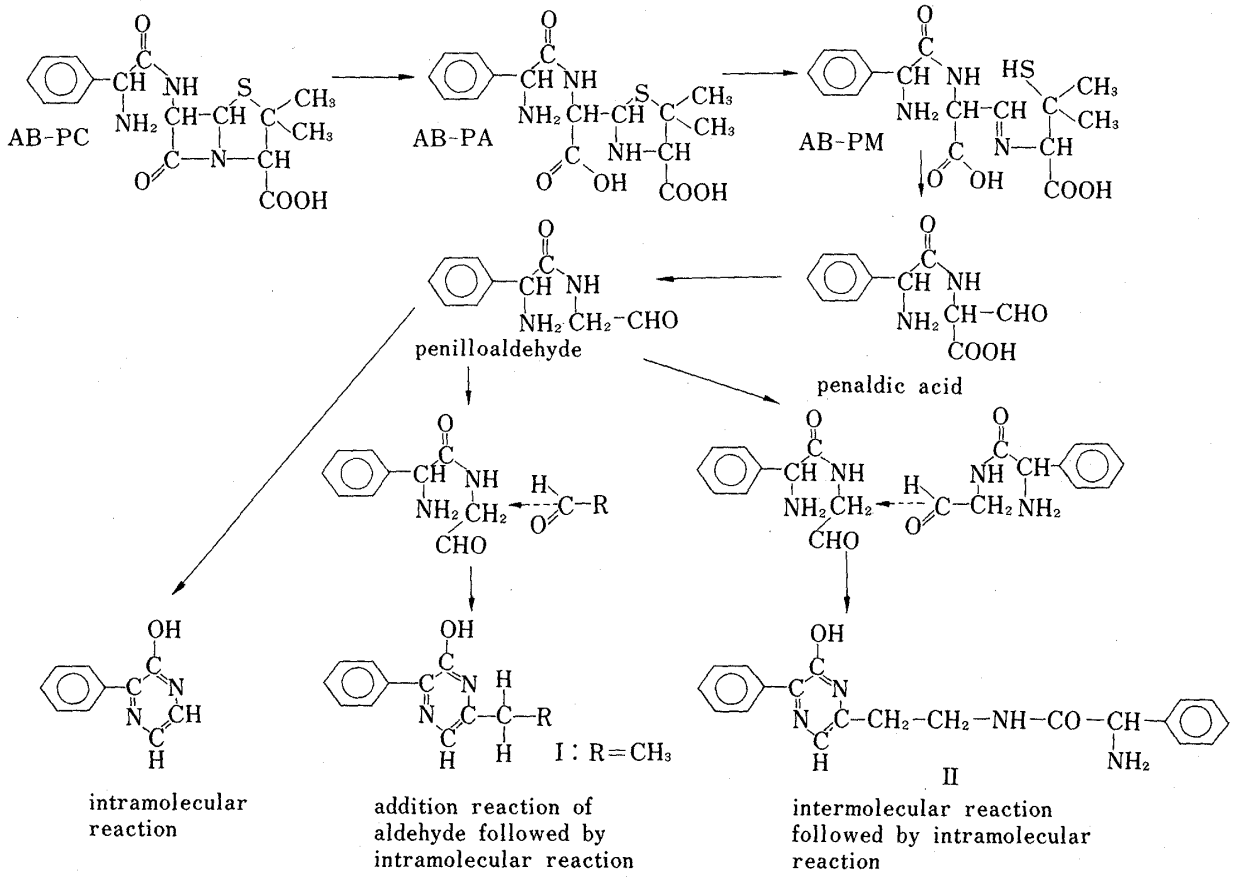


Fig. 12. Reaction Pathway for the Formation of Fluorescent Degradation Products of Ampicillin

The reaction mechanism leading to the formations of (I), (II), and the known fluorophores, 2-hydroxy-3-phenylpyrazine and 2-hydroxy-3-phenyl-6-methylpyrazine, is illustrated in Fig. 12. The first four steps from AB–PC to penilloaldehyde proceed in accordance with a known mechanism.[21] If a certain aldehyde is involved in this reaction, it will attack at the methylene group of penilloaldehyde. The reaction with formaldehyde or acetaldehyde occurs thus, and subsequent intermolecular cyclization to form the Schiff base between aldehyde and amino groups produces a dihydropyrazine skeleton which is easily oxidized to 2-hydroxy-3-phenyl-6-methylpyrazine or (I). If these is no other aldehyde, penilloaldehyde undergoes

either intramolecular cyclization to form 2-hydroxy-3-phenylpyrazine, as found by Lebelle,[17,22,23] or intermolecular reaction with another penilloaldehyde molecule to form (II). The competition between these reactions depends on the initial concentration of AB–PC; a higher concentration (>200 μg/ml) favors the intermolecular reaction and a lower concentration (<100 μg/ml) favors the intramolecular reaction. It follows from the present results that the formation of 2-hydroxy-3-phenyl-6-methylpyrazine or 2-hydroxy-3-phenylpyrazine depends on the presence or absence of formaldehyde. This conclusion differs from that of Barbhaiya, who stated that when the products obtained by the reactions with and without the use formaldehyde[16] were compared chromatographically and spectroscopically, they were found to be the same substance. This discrepancy probably arose from the difference in the method used for the separation of fluorescent products, because re-examination of the silica gel TLC method employed by Barbhaiya showed that it did not separate 2-hydroxy-3-phenyl-6-methylpyrazine and 2-hydroxy-3-phenylpyrazine. The ion-pair reversed phase HPLC used in this study permitted effective separation.

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