

## Studies on Fluorometric Determination of Aromatic Aldehydes with 1,2-Diaminonaphthalene. II.<sup>1)</sup> On the Reaction Products of Benzaldehyde, Cinnamaldehyde, and Pyruvic Acid<sup>2)</sup>

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Fluorescent compounds, I and II, were obtained from the ethanolic and sulfuric acid reaction mixtures of benzaldehyde and of cinnamaldehyde with 1,2-diaminonaphthalene monosulfate, respectively, and proved to be identical fluorescent compounds with those produced under the conditions of the method of aldehyde determination. I and II were characterized as 2-substituted naphth[1,2-*d*]imidazole derivatives shown in Chart 1 and 2.

From the sulfuric acid reaction mixture of pyruvic acid, fluorescent compounds, III and IV, were separated. III was determined as a benzoquinoxalinone derivative shown in Chart 3 and found not to be fluorescent compound produced under the conditions of the method. IV was main fluorescent compound produced from pyruvic acid under the conditions of the method though its structure could not be elucidated. The thin-layer chromatographic and fluorescence spectral data indicated that IV was identical with a fluorescent compound derived from acetaldehyde under the conditions of the method.

In the previous paper,<sup>1)</sup> a new fluorometric method was presented for the determination of aromatic aldehydes, which was based on the finding that 1,2-diaminonaphthalene reacted selectively with aromatic aldehyde in sulfuric acid or neutral solution to give a compound which fluoresced intensely in an alkaline medium. The method was shown to be interfered only by 2-oxo acids such as pyruvic acid which also fluoresced when treated under the conditions of the method. Then the method was effectively applied to the assay of serum monoamine oxidase activity where benzaldehyde produced from the substrate, benzylamine, in the enzyme reaction was determined.<sup>4)</sup> This paper describes the separation and characterization of fluorescent compounds produced in the reactions of benzaldehyde, cinnamaldehyde,<sup>5)</sup> and pyruvic acid with 1,2-diaminonaphthalene.

### Results and Discussion

#### Fluorescent Compound from Benzaldehyde

An aqueous ethanolic solution of benzaldehyde was mixed with 1,2-diaminonaphthalene monosulfate (1,2-DNS) and refluxed for several hours with stirring.<sup>6)</sup> The resulting mixture left almost colorless powder of mp 190° (I-sulfate) in a high yield. The data of elemental analysis of the compound agreed with the formula of  $C_{17}H_{12}N_2 \cdot 1/2H_2SO_4$ , whose elemental composition was consistent with that of phenylnaphthimidazole sulfate. The infrared (IR) spectrum of I-sulfate indicated the existence of sulfate ion in the molecule by the characteristic absorption bands ascribable to the vibrations of S=O and S-O at 1105 and 615  $cm^{-1}$ ,

1) Part I: Y. Ohkura and K. Zaitzu, *Talanta*, **22**, 547 (1974).

2) This paper forms "Organic Analysis XCII," Part XCI: Y. Ohkura, M. Yamaguchi and T. Momose, *Chem. Pharm. Bull.* (Tokyo), **22**, 1414 (1974).

3) Location: *Maidashi, Higashi-ku, Fukuoka*; a) To whom all inquiries should be addressed.

4) Presented partly at the 93rd Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April, 1973.

5) Benzaldehyde and cinnamaldehyde were employed as model compounds of aromatic and unsaturated arylaliphatic aldehydes, respectively, to establish the reaction conditions in the method of determination,<sup>1)</sup> and so they were also selected for the present purpose.

6) The fluorescence reaction was already shown to proceed in aqueous ethanol as in the sulfuric acid solution.<sup>1)</sup>

respectively. The mass spectrum (MS) of I-sulfate showed the strong peak responsible for the molecular ion ( $M^+$ ) of free phenylnaphthimidazole<sup>7)</sup> and the pattern of fragmentation was analogous to that of 2-phenylbenz[1,2-*d*]imidazole.<sup>8)</sup> Therefore, I-sulfate was phenylnaphthimidazole sulfate.

The free imidazole (I) was obtained as colorless powder of mp 217—218° by treating I-sulfate with ammonium hydroxide. I was identified as 2-phenylnaphth[1,2-*d*]imidazole<sup>9)</sup> (Chart 1) by the comparison of its physical properties<sup>10)</sup> with those of the authentic sample.

I has been considered to exist as mesohydric tautomer<sup>11)</sup> (Chart 1).

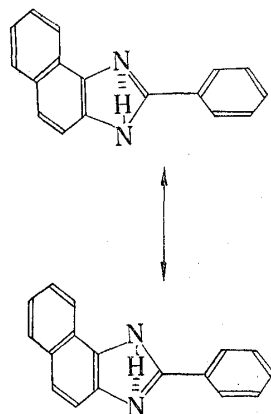


Chart 1. Mesohydric Tautomerism of I

I fluoresced strongly when dissolved in an aqueous sodium hydroxide solution<sup>12)</sup> whose concentration was adjusted to be identical with that of the final reaction mixture in the method for the determination of aldehyde.<sup>1)</sup> The fluorescence excitation and emission spectra (the excitation maxima, 290 and 353 nm; the emission maximum, 385 nm) were identical with those of the final reaction mixture of benzaldehyde as shown in Fig. 1, thus indicating that I was the fluorescent compound in the reaction of benzaldehyde in the method of determination.

I was confirmed to produce also in the reaction in a sulfuric acid solution<sup>13)</sup> used in the method of aldehyde determination by extracting the product from the reaction mixture, then purifying it by alumina column chromatographic technics and finally comparing its *R<sub>f</sub>* value in the thin-layer chromatogram and fluorescence spectra with those of I.

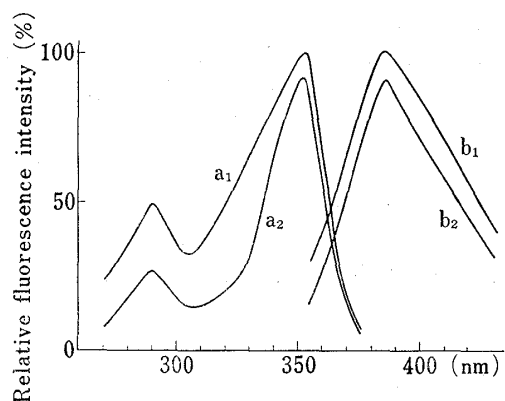


Fig. 1. Excitation and Emission Spectra of I and the Reaction Mixture of Benzaldehyde

a: excitation spectra; b: emission spectra

a<sub>1</sub> and b<sub>1</sub>: I was dissolved in the sodium hydroxide solution<sup>12)</sup> ( $2.5 \times 10^{-2}M$ ).

a<sub>2</sub> and b<sub>2</sub>: 1.0 ml of  $1 \times 10^{-5}M$  benzaldehyde solution was mixed with 2.0 ml of 45  $\mu g/ml$  1,2-DNS solution (dissolved in the sulfuric acid solution<sup>13)</sup>), heated at 100° for 20 min and diluted with 2.0 ml of 2.5*N* sodium hydroxide solution. The fluorescence of the reagent blank was subtracted.

excitation wavelength (nm): a<sub>1</sub> and a<sub>2</sub>, 353  
emission wavelength (nm): b<sub>1</sub> and b<sub>2</sub>, 385

### Fluorescent Compound from Cinnamaldehyde

An ethanolic solution of *trans*-cinnamaldehyde similarly treated with 1,2-DNS as in the reaction of benzaldehyde gave yellow powder of mp 221—223° (II-sulfate). The same compound was also obtained when cinnamaldehyde was treated with 1,2-DNS in the sulfuric acid solution<sup>13)</sup> and the product was purified by column chromatographic technics described in Experimental.

The molecular formula of II-sulfate was established from the data of elemental analysis as  $C_{19}H_{14}N_2 \cdot 1/2H_2SO_4$ , whose composition was consistent with that of styrylnaphthimidazole sulfate.

In the MS of the compound,  $M^+$  of free styrylnaphthimidazole was observed as in the MS of I-sulfate. The IR spectrum indicated the existence of styryl group in the molecule by the bands ascribable to C=C of benzene ring and C-H of *trans*-ethylene group at 1580 and 976  $cm^{-1}$ , respectively,

7) 1,2-DNS also showed  $M^+$  of free 1,2-diaminonaphthalene in the MS.

8) S.O. Lowesson, J.H. Bowie and R.G. Cooks, *Tetrahedron*, **24**, 1875 (1968).

9) Z. Yoshida and R. Oda, *Kogyokagaku Zasshi*, **55**, 447 (1952).

10) NMR, IR and mass spectra.

11) C.F. Kelly and A.R. Day, *J. Am. Chem. Soc.*, **67**, 1074 (1945).

12) 0.4*N* sodium hydroxide solution.

13) Approximately 0.34*N* sulfuric acid solution.

and also indicated the existence of sulfate ion by the bands due to S=O and S-O at 1100 and 618  $\text{cm}^{-1}$ , respectively. The nuclear magnetic resonance (NMR) spectrum showed multiplet with the integral intensity of 15 at  $\delta$  6.88–8.67 ppm. The intensity of the multiplet decreased by 2 on adding heavy water, indicating that the signals due to the imino and iminium protons in II-sulfate were included in the multiplet. From the data described above, II-sulfate was determined as *trans*-2-styrylnaphth[1,2-*d*]imidazole sulfate.

The free base of II-sulfate (II) was obtained as yellow powder of mp 104–106° by treating II-sulfate with ammonium hydroxide. The data of physical properties<sup>10)</sup> and elemental analysis proved that II was *trans*-2-styrylnaphth[1,2-*d*]imidazole. II might exist as mesohydric tautomer in analogy with I (Chart 2).

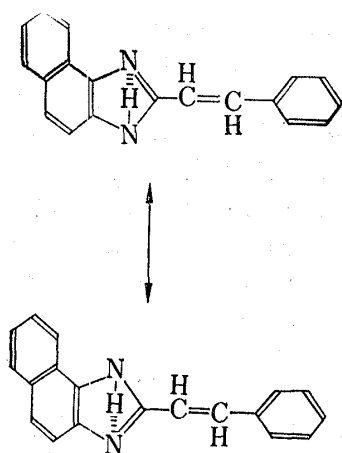


Chart 2. Mesohydric Tautomerism of II

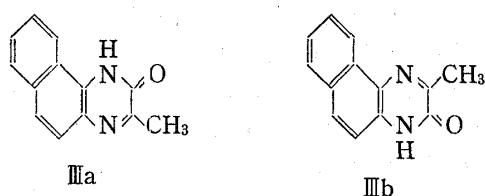


Chart 3. Structure of III

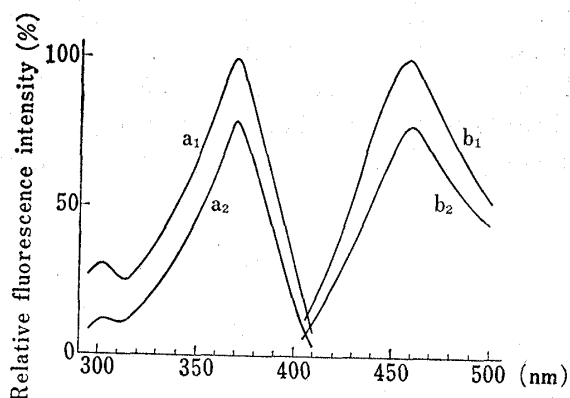


Fig. 2. Excitation and Emission Spectra of II and the Reaction Mixture of Cinnamaldehyde

a: excitation spectra; b: emission spectra  
 a<sub>1</sub> and b<sub>1</sub>: II was dissolved in the sodium hydroxide solution<sup>12)</sup> ( $2.5 \times 10^{-7}\text{M}$ ).  
 a<sub>2</sub> and b<sub>2</sub>: 1.0 ml of  $1 \times 10^{-5}\text{M}$  cinnamaldehyde solution was treated as in a<sub>2</sub> and b<sub>2</sub> in Fig. 1. The fluorescence of the reagent blank was subtracted.  
 excitation wavelength (nm): a<sub>1</sub> and a<sub>2</sub>, 370  
 emission wavelength (nm): b<sub>1</sub> and b<sub>2</sub>, 460

II dissolved in the sodium hydroxide solution<sup>12)</sup> showed the fluorescence excitation spectrum with the maxima at 300 and 370 nm and the emission spectrum with the maximum at 460 nm, which were identical with those of the final reaction mixture of cinnamaldehyde in the method of determination as shown in Fig. 2. Therefore, II was the fluorescent compound produced in the method.<sup>1)</sup>

### Fluorescent Compounds from Pyruvic acid

An aqueous solution of lithium pyruvate was mixed with a suspension of 1,2-DNS in the sulfuric acid solution<sup>13)</sup> and heated at 100° for 2 hours. The mixture was extracted with benzene. The extract was chromatographed on a silica gel column with ethyl acetate as developing solvent. The major fraction was collected, which showed a blue fluorescence in the sodium hydroxide solution,<sup>12)</sup> and gave light yellow needles of mp 242–244° (III). III was determined to have the formula of  $\text{C}_{13}\text{H}_{10}\text{ON}_2$  from the data of elemental analysis and its  $\text{M}^+$  in the MS, whose elemental composition corresponded to that of a possible reaction product, methylbenzoquinoxalinone. The presence of lactam moiety in the molecule was indicated by the characteristic IR absorption bands due to imino and carbonyl groups at

3300 and 1650  $\text{cm}^{-1}$ , respectively. Therefore, III was 3-methylbenzo[1,2-*h*]-2(1*H*)-quinoxalinone (IIIa) or 2-methylbenzo[1,2-*f*]-3(4*H*)-quinoxalinone (IIIb) shown in Chart 3.

The aqueous layer which remained after the benzene extraction of the reaction mixture was neutralized with sodium hydroxide and extracted with ethyl acetate. The concentrated extract was subjected to silica gel thin-layer<sup>14)</sup> chromatography with ethyl acetate. The major fraction on the chromatogram, which showed a blue fluorescence in the sodium hydroxide solution,<sup>12)</sup> was purified by repeating the thin-layer chromatography. A small amount of faint yellow-brownish viscous matter (IV) thus obtained could not be successfully crystallized. IV decomposed gradually to a brownish resin on standing in air and in daylight and its structure could not be elucidated successfully.

The fluorescence excitation and emission spectra of III dissolved in the sodium hydroxide solution<sup>12)</sup> were quite different from those of pyruvic acid treated under the conditions of the method of aldehyde determination<sup>1)</sup> (Fig. 3), thus indicating that III was not the main fluorescent compound in the method. On the other hand, IV dissolved in the sodium hydroxide solution<sup>12)</sup> showed the fluorescence spectra with the excitation maxima at 260 and 333 nm and the emission maximum at 395 nm, which were closely similar to those of the reaction mixture of pyruvic acid as shown in Fig. 3. The fact indicated that IV was the main fluorescent compound derived from pyruvic acid under the conditions of the method.<sup>1)</sup>

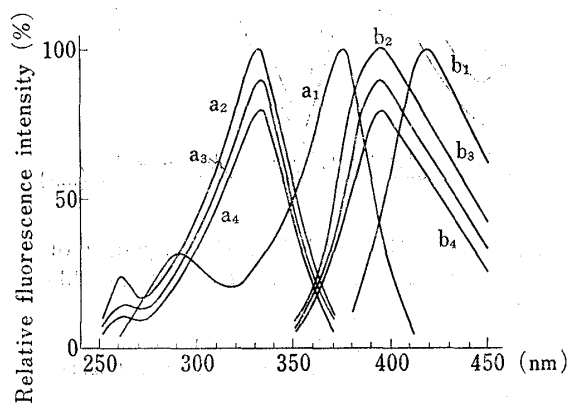


Fig. 3. Excitation and Emission Spectra of III, IV and the Reaction Mixtures of Pyruvic Acid and of Acetaldehyde

a: excitation spectra; b: emission spectra

a<sub>1</sub> and b<sub>1</sub>: III was dissolved in sodium hydroxide solution<sup>12)</sup> ( $2.5 \times 10^{-6} \text{M}$ ).

a<sub>2</sub> and b<sub>2</sub>: 1.0 ml of  $2 \times 10^{-5} \text{M}$  pyruvic acid solution was treated as in a<sub>2</sub> and b<sub>2</sub> in Fig. 1.

The fluorescence of the reagent blank was subtracted.

a<sub>3</sub> and b<sub>3</sub>: 1.0 ml of  $1 \times 10^{-5} \text{M}$  acetaldehyde solution was treated as in a<sub>2</sub> and b<sub>2</sub>. The fluorescence of the reagent blank was subtracted.

a<sub>4</sub> and b<sub>4</sub>: IV was dissolved in the sodium hydroxide solution<sup>12)</sup> ( $2 \mu\text{g/ml}$ ).

excitation wavelength (nm): a<sub>1</sub>, 375; a<sub>2</sub>, 330; a<sub>3</sub> and a<sub>4</sub>, 333  
emission wavelength (nm): b<sub>1</sub>, 420; b<sub>2</sub>, b<sub>3</sub> and b<sub>4</sub>, 395

The fluorescence excitation and emission spectra of the reaction mixture of pyruvic acid and of IV were observed to be identical with those of the reaction mixture of acetaldehyde (Fig. 3). This fact suggested that some part of pyruvic acid decomposed to acetaldehyde under the conditions of the method, which reacted with 1,2-DNS to form IV. The formation of IV from acetaldehyde was then confirmed by the fact that the spot ascribed to IV was observed on the chromatogram in the thin-layer<sup>14)</sup> chromatographic separation of the reaction mixture obtained by treating the aldehyde under the conditions of the method,<sup>1)</sup> though IV could not be obtained successfully as pure form from the reaction mixture for further investigation.

### Effect of Sulfate Ion on the Imidazole Formation

In order to clarify whether the presence of sulfate ion in the reaction was required to form the imidazole, the reaction of benzal-

dehyde was examined with free 1,2-diaminonaphthalene in aqueous and ethanolic media, respectively, and subsequently the products were separated chromatographically on silica gel thin-layers.<sup>14)</sup> A spot corresponding to I was clearly observed in both cases of the reactions in aqueous and ethanolic media. Therefore, the sulfate ion was not requisite for the imidazole formation. A more concentrated sulfuric acid than 0.7*N* in the reaction mixture was already reported to interfere the fluorescence reactions of benzaldehyde and cinnamaldehyde.<sup>1)</sup>

14) 0.25 mm Layer of Wakogel B-5, activated at 105° for 1 hour.

These observations showed that free 1,2-diaminonaphthalene could be used as reagent for the determination of aldehyde, but the free amine was very unstable in air and in daylight. Thus, 1,2-DNS, which was stable enough to use as reagent, and the minimum concentration of sulfuric acid to dissolve the reagent were to be employed in the method of aldehyde determination.<sup>1)</sup>

### Experimental<sup>15)</sup>

**Isolation of I-Sulfate from the Ethanolic Reaction Mixture**—To 2.5 g of freshly distilled benzaldehyde dissolved in 200 ml of 95% EtOH, 1.3 g of 1,2-DNS was added, refluxed for 6 hr with stirring, cooled and left with continuous stirring for several hr to precipitate almost colorless powder, which was filtered and recrystallized from H<sub>2</sub>O to colorless powder (I-sulfate), mp 190°. Yield 0.8 g. *Anal.* Calcd. for C<sub>17</sub>H<sub>12</sub>N<sub>2</sub>·1/2 H<sub>2</sub>SO<sub>4</sub>: C, 69.61; H, 4.47; N, 9.55. Found: C, 70.35; H, 4.47; N, 9.24. IR  $\nu_{\text{max}}^{\text{KBr}}$  cm<sup>-1</sup>: 2600 (N<sup>+</sup>H, broad), 1200 (C=N, skeletal), 1105 (S=O), 615 (S-O). UV  $\lambda_{\text{max}}^{\text{EtOH}}$  nm (log  $\epsilon$ ): 232 (4.53), 279 (4.39), 318 (4.04), 332 (4.27), 348 (4.30). NMR (in Me<sub>2</sub>SO-*d*<sub>6</sub>)  $\delta$ (ppm): 6.76–8.80 (13H, multiplet, the integral intensity decreased by 2 on adding D<sub>2</sub>O). Mass Spectrum  $m/e$ : 244 (M<sup>+</sup> of I, base peak), 140 (M<sup>+</sup> of I-C<sub>7</sub>H<sub>8</sub>N), 114 (C<sub>9</sub>H<sub>6</sub>).

**I from I-Sulfate**—I-Sulfate (0.4 g) was dissolved in 500 ml of boiling H<sub>2</sub>O, made alkaline with 10% NH<sub>4</sub>OH while hot and cooled. The separated precipitate was filtered and washed with cold 10% NH<sub>4</sub>OH. Colorless powder (I), mp 217–218°. Yield 0.15 g.

No depression of mp was observed on admixture with the authentic sample of 2-phenylnaphth[1,2-*d*]-imidazole.<sup>9)</sup> The IR, NMR and mass spectra were entirely identical with those of the authentic sample.

**Identification of I in the Mixture Resulted from the Reaction in the Sulfuric Acid Solution**—To a suspension of 0.5 g of 1,2-DNS in 100 ml of 0.33N H<sub>2</sub>SO<sub>4</sub>, 1.5 g of freshly distilled benzaldehyde was added and heated in a boiling water-bath for 2 hr. After cooling, the mixture was neutralized with 10% Na<sub>2</sub>CO<sub>3</sub> and extracted with AcOEt. The extract was dried over Na<sub>2</sub>SO<sub>4</sub> and passed through a short column packed with about 10 g of acidic Al<sub>2</sub>O<sub>3</sub><sup>16)</sup> to remove resinous matter. The resulting solution was concentrated to dryness *in vacuo*, dissolved in a small amount of C<sub>6</sub>H<sub>6</sub>, poured onto a column packed with about 100 g of Al<sub>2</sub>O<sub>3</sub><sup>16)</sup> and eluted with CHCl<sub>3</sub>. The main fraction, which showed a blue fluorescence, was collected and concentrated to dryness *in vacuo*. The residue, a viscous matter, was dissolved in a small amount of EtOH and subjected to the separation on silica gel thin-layers<sup>14)</sup> with 95% EtOH as developing solvent. The major spot (*R*<sub>f</sub>=0.68), which showed a blue fluorescence under UV light<sup>17)</sup> after spraying 1N NaOH, was identified with I by overlapping method in the development. The spot was scraped and extracted with the NaOH solution<sup>12)</sup> and centrifuged to remove the silica gel. The supernatant clear solution showed identical fluorescence excitation and emission spectra with those of I dissolved in the NaOH solution<sup>12)</sup> shown in Fig. 1.

**Identification of I in the Mixture Resulted from the Reaction with Free 1,2-Diaminonaphthalene in the Absence of Sulfate Ion I. in Aqueous Reaction Mixture**—To 0.32 g of free 1,2-diaminonaphthalene suspended in 100 ml of H<sub>2</sub>O, 1.0 g of freshly distilled benzaldehyde was added and heated in a boiling water-bath for 2 hr. After cooling, the mixture was extracted with AcOEt. Then, the extract was treated in the same way as in Identification of I in the mixture resulted from the reaction in the sulfuric acid solution. The resulting substance was spotted on silica gel thin-layer<sup>14)</sup> and developed with *n*-BuOH–10% NH<sub>4</sub>OH (100:3). The main fluorescent spot located at *R*<sub>f</sub>=0.86 was identified with the spot due to I by overlapping method in the development. The NaOH<sup>12)</sup> extract of the spot showed identical fluorescence excitation and emission spectra with those of I dissolved in the NaOH solution.<sup>12)</sup>

**I in Ethanolic Reaction Mixture**—To 0.32 g of free 1,2-diaminonaphthalene dissolved in 100 ml of 95% EtOH, 1.0 g of freshly distilled benzaldehyde was added and refluxed for 2 hr. The resulting mixture was concentrated to dryness *in vacuo*. The residue was dissolved in AcOEt and then treated in the same way as described in I in Aqueous Reaction Mixture. The existence of I was confirmed by the spot in the thin-layer chromatogram and the fluorescence spectra of its NaOH extract.

**Isolation of II-Sulfate from the Ethanolic Reaction Mixture**—To 2.3 g of *trans*-cinnamaldehyde dissolved in 150 ml of 95% EtOH, 1.3 g of 1,2-DNS was added, refluxed for 6 hr, cooled and left standing for 3 hr. Yellow powder thus precipitated was filtered and recrystallized from HCON(CH<sub>3</sub>)<sub>2</sub> to light yellow crystalline

15) Fluorescence excitation and emission spectra were taken on a Hitachi 203 Spectrofluorometer equipped with a xenon arc-lamp in a quartz cell of 1×1 cm optical path lengths, UV spectra on a Hitachi 124 Spectrophotometer in a cell of 1 cm optical path length, IR spectra on a Nihonbunko 701G Spectrometer in KBr pellets, NMR spectra on a JEOL JNM-PS-100 Spectrometer at 100 MHz with tetramethylsilane as an internal standard, and mass spectra on a Nihondenshi JMS-01-SG Spectrometer. All melting points and fluorescence excitation and emission spectra were uncorrected.

16) Acidic alumina, Merck, Grade I.

17) A commercial UV-lamp (Manasul Light Long Wave 3650Å, Manasul Kogaku Kogyo, Ltd.) was used.

powder (II-sulfate), mp 221—223°. Yield 60 mg. *Anal.* Calcd. for  $C_{19}H_{14}N_2 \cdot 1/2 H_2SO_4$ : C, 71.48; H, 4.73; N, 8.77. Found: C, 71.52; H, 4.70; N, 8.59. IR  $\nu_{\max}^{KBr}$   $cm^{-1}$ : 2560 (N<sup>+</sup>H, broad), 1580 (C=C, benzene skeletal), 1210 (C=N, skeletal), 1100 (S=O), 976 (C-H, *trans*-ethylene), 618 (S-O). NMR (in  $Me_2SO-d_6$ )  $\delta$  (ppm): 6.88—8.64 (15H, multiplet, the integral intensity decreased by 2 on adding  $D_2O$ ). Mass Spectrum  $m/e$ : 270 ( $M^+$  of II), 181 ( $M^+$  of II- $C_7H_5$ ), 168 ( $C_{11}H_8N_2$ ), 140 ( $C_{10}H_6N$ ), 115 ( $C_9H_7$ ).

**Isolation of II-Sulfate from the Mixture Resulted from the Reaction in the Sulfuric Acid Solution**—

To 0.6 g of 1,2-DNS suspended in 200 ml of the  $H_2SO_4$  solution,<sup>13)</sup> 1.0 g *trans*-cinnamaldehyde was added and heated in a boiling water-bath for 3 hr with stirring. The resulting mixture was extracted with AcOEt and the extract was concentrated to almost dryness *in vacuo*. The residue was dissolved in a small amount of  $CHCl_3$ , poured onto a column packed with about 100 g of silica gel<sup>18)</sup> and developed with  $CHCl_3$ . The main fraction, which gave a fluorescence identical with II-sulfate when mixed with the NaOH solution,<sup>12)</sup> was scraped and eluted with AcOEt. The elute was dried over  $Na_2SO_4$  and concentrated. The residue was dissolved in a small amount of AcOEt to which  $(C_2H_5)_2O$  was added to form yellow crystalline powder (II-sulfate), mp 221—223°. Yield 30 mg. No depression of mp was observed on admixture with II-sulfate. Its IR spectrum was identical with that of II-sulfate.

**II from II-Sulfate**—To 0.1 g of II-sulfate dissolved in 1.5 g of 28%  $NH_4OH$ , 3 ml of 95% EtOH was added. To the resulting solution, 50 ml of hot  $H_2O$  was added and left standing overnight in a refrigerator. The crystalline precipitate thus separated was washed with cold 10%  $NH_4OH$  and dried over  $P_2O_5$  at 90° *in vacuo*. Yellow crystalline powder (II), mp 104—106°. Yield 40 mg. *Anal.* Calcd. for  $C_{19}H_{14}N_2$ : C, 84.42; H, 5.22; N, 10.36. Found: C, 84.39; H, 5.15; N, 10.29. IR  $\nu_{\max}^{KBr}$   $cm^{-1}$ : 1210 (C=N, skeletal), 963 (C-H, *trans*-ethylene), 695 (C-H, monosubstituted benzene). UV  $\lambda_{\max}^{EtOH}$  nm (log  $\epsilon$ ): 209 (4.43), 237 (4.50), 289 (4.26), 359 (4.46), 376 (4.33). NMR (in  $Me_2SO-d_6$ )  $\delta$  (ppm): 6.70—8.70 (14H, multiplet, the integral intensity decreased by 1 on adding  $D_2O$ ). Mass Spectrum  $m/e$ : 270 ( $M^+$ , base peak), 181 ( $M^+$ - $C_7H_5$ ), 168 ( $M^+$ - $C_6H_5$ -CH=C), 140 ( $C_{10}H_6N$ ), 115 ( $C_9H_7$ ).

**Isolation of III and IV**—To 1.1 g of Li pyruvate· $H_2O$  dissolved in 100 ml of  $H_2O$ , 2.4 g of 1,2-DNS suspended in 20 ml of 6N  $H_2SO_4$  was added and heated in a boiling water-bath for 2 hr. After cooling, the mixture was extracted with  $C_6H_6$  and the extract was concentrated to dryness. The residue was dissolved in a small amount of AcOEt and subjected to the separation on a column packed with about 50 g of silica gel.<sup>18)</sup> The column was eluted with AcOEt and the largest fraction was collected. The elute was concentrated and the resulting residue was recrystallized from  $CHCl_3$ -EtOH (2:3) to light yellow needles (III), mp 242—244°. Yield 80 mg. *Anal.* Calcd. for  $C_{13}H_{10}ON_2$ : C, 74.27; H, 4.79; N, 13.33. Found: C, 74.05; H, 4.74; N, 13.22. IR  $\nu_{\max}^{KBr}$   $cm^{-1}$ : 3300 (N-H, weak), 1653 (C=O), 1480 and 1370 ( $CH_3$ ). UV  $\lambda_{\max}^{EtOH}$  nm (log  $\epsilon$ ): 231 (4.59), 283 (4.25), 319 (3.63), 362 (3.84). NMR (in  $Me_2SO-d_6$ )  $\delta$  (ppm): 7.28—9.00 (6H, multiplet, arom. H), 12.8 (1H, broad singlet, NH, disappeared on adding  $D_2O$ ). Mass Spectrum  $m/e$ : 210 ( $M^+$ ), 182 ( $M^+$ -CO, base peak), 155 ( $M^+$ -CO-HCN), 141 ( $C_{10}H_7N$ ), 128 ( $M^+$ -CO-2HCN), 115 ( $C_9H_7$ ).

The reaction mixture which remained after the  $C_6H_6$  extraction was washed with AcOEt, neutralized with 1N NaOH and extracted continuously with AcOEt. The extract was concentrated, spotted on the silica gel thin-layers<sup>14)</sup> and developed with AcOEt. After drying the chromatograms in the air, the largest spot located at  $R_f=0.17$  was scraped and extracted with AcOEt. The extract gave a viscous residue on evaporating the solvent. The residue was dissolved in a small amount of AcOEt and rechromatographed in the same way as described above. The procedure was repeated 2 times to give absolutely single spot. The spot was scraped and extracted with AcOEt and the extract gave a small amount of slightly yellow-brownish viscous matter (IV) on evaporating the solvent. IV also gave a single spot at  $R_f=0.53$  when chromatographed on the silica gel thin-layer<sup>14)</sup> with a solvent system of  $C_6H_6$ - $Me_2CO$  (1:1).

**Identification of IV in the Reaction Mixture of Acetaldehyde**—To 10 mg of 1,2-DNS dissolved in 100 ml of 0.5N  $H_2SO_4$ , 13 mg of acetaldehyde dissolved in 50 ml of  $H_2O$  was added and heated in a boiling water-bath for 30 min. After cooling, the mixture was washed with AcOEt, neutralized with 10% NaOH and extracted with  $C_6H_6$ .

The extract was dried over  $Na_2SO_4$  and concentrated to dryness *in vacuo*. The residue was dissolved in a small amount of EtOH, spotted on the silica gel thin-layer<sup>14)</sup> and developed with  $C_6H_6$ -( $CH_3$ )<sub>2</sub>CO (1:1). The spot located at  $R_f=0.53$  showed a blue fluorescence on spraying 1N NaOH and was identified with that ascribed to IV by overlapping method in the development. The spot was scraped and extracted with the NaOH solution.<sup>12)</sup> The extract showed identical fluorescence excitation and emission spectra with those of IV dissolved in the NaOH solution.<sup>12)</sup>

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18) Silicic acid, Mallinckrodt, 100 mesh.